Chapter 2

Identification of Serine 472 as the Essential Residue for Runx2 Degradation

Abstract

Transcription factor Runx2 is the master regulator for cartilage maturation and bone formation. Runx2 is required for late stage chondrocyte maturation and osteoblast commitment and differentiation. The function of Runx2 is regulated at transcriptional and post-translational levels. The functional significance of the post-translational modification of Runx2 has not been fully defined. In this study, through manipulating cyclin D1 expression and monitoring tagged Runx2 protein level and modification in COS cells, we demonstrated that cyclin D1/CDK4 induces Runx2 rapid protein turnover via an ubiquitination-proteasome dependent manner. Serine-472 at the C-terminal of Runx2, a consensus CDK site, is further identified as the critical residue for regulating the protein stability of Runx2. The targeted Runx2 degradation by cyclin D1 identifies a novel mechanism through which Runx2 activity is regulated tightly with the cell cycle machinery in skeletal tissues.
Introduction

Runx2 is a bone- and cartilage-specific transcription factor that belongs to the Runx family (Karsenty et al., 2000). The DNA-binding sites for Runx2 have been identified in the promoter regions of many osteoblast- and chondrocyte-specific genes (Ducy et al., 1995; Geoffroy et al., 1997; Ducy et al., 1997; Javed et al., 2001). Runx2 binds to specific response elements in the promoters of Runx2 target genes and regulates the transcription of these genes. Runx2−/− mice die right after birth and lack bone formation due to the absence of mature osteoblasts (Komori et al., 1997; Otto et al., 1997). Heterozygous mutant mice have skeletal abnormalities, similar to those seen in a human mutation called cleidocranial dysplasia syndrome (Mundlos et al., 1995; Mundlos et al., 1997) and have delayed development of intramembranous bones (Komori et al., 1997; Otto et al., 1997).

Additional studies demonstrated delayed chondrocyte maturation in Runx2−/− mice (Inada et al., 1999; Kim et al., 1999), suggesting that Runx2 also promotes chondrocyte differentiation. Dominant-negative Runx2 inhibits chondrocyte maturation (Ueta et al., 2001; Stricker et al., 2002), whereas overexpression of Runx2 in chondrocytes restores chondrocyte maturation in Runx2−/− mice (Takeda et al., 2001). Recent studies demonstrate that chondrocyte maturation was completely absent in Runx2 and Runx3 double knock-out mice (Yoshida et al., 2004), demonstrating that Runx2 and Runx3 are functionally redundant and play an essential role in chondrocyte maturation.
The Runx2 protein primarily consists of a conserved RUNT domain (DNA binding domain), a C-terminal proline-serine-threonine rich (PST) domain and N-terminal glutamine-alanine rich (QA) domain. The PST domain is thought to be a transcriptional activation domain (Thirunavukkarasu et al., 1998). CBF-β interacts with Runt domain and forms a dimer with Runx2. This complex increases the DNA binding capacity of Runx2 (Thirunavukkarasu et al., 1998; Yoshida et al., 2002).

Zhao and colleagues reported that HECT domain E3 ligase Smurf1 (Smad Ubiquitin Regulatory Factor 1) induces the ubiquitination and degradation of Runx2 in vitro (Zhao et al., 2003). Smurf1 recognizes and interacts with the PY motif (PPXY) of its substrate proteins through its WW domain. A conserved PY motif has been identified in Runx family members, including Runx1, Runx2 and Runx3. In vitro experiments have demonstrated that Smurf1 indeed induces the degradation of Runx2 and Runx3 through the ubiquitin-proteasome pathway. Deletion of the PY motif makes Runx2 and Runx3 proteins are partially resistant to Smurf1-induced degradation (Zhao et al., 2003; Jin et al., 2004; Shen et al., 2006). Since Smurf1 is an important negative regulator of BMP (bone morphogenetic protein) signaling through inducing the proteolytic destruction of Smad1 (Zhu et al., 1999), it is interesting to see Smurf1 also plays a role in mediating Runx2 degradation.
Post-translational modifications modulate the activity of most eukaryotic proteins. Previous works have demonstrated that Runx2 protein may be modified by ubiquitination through cAMP pathway, which then induces rapid turn-over of Runx2; by phosphorylation (Wee et al., 2002) through MAPK signaling (Xiao et al, 2000) and fibroblast growth factor signaling (Xiao et al, 2002).

The mechanism through which Runx2 induces osteoblast and chondrocyte differentiation involves both the withdrawal from cell cycle and the activation of osteoblast and chondrocyte-specific genes. Runx2 is expressed in proliferating osteoblasts and chondrocytes and causes efficient withdrawal from the cell cycle prior to the differentiation process. Recent data demonstrate a direct link between Runx2 and cell cycle regulation (Galindo et al., 2005).

Cell proliferation is governed primarily by the cyclin dependent kinases which contain a catalytic subunit, CDK, and a regulatory subunit, cyclin. CDKs drive cell cycle progression by phosphorylating a wide array of substrates. CDKs phosphorylate cyclins and destabilize cyclins at certain time point as a negative feedback of this regulatory mechanism. Hypophosphorylated Rb represses the transcriptional activity of E2F, which activates expression of genes that favor cell cycle progression. CDKs phosphorylate Rb, and abolish its repressive activity on E2F, which then activates gene expression and promotes cell cycle progression.

Acetylation has also been reported to modify Runx3, another member of Runx family, in response to TGF-β treatment, and so prevent the degradation induced by E3 ligase Smurf1 (Jin et al., 2004).

Cell cycle regulation is controlled by a combination of cyclins, CDKs, and CDK inhibitors, which together with the tumor suppressor retinoblastoma (Rb), are involved in the tight control of cell cycle machinery. CDKs, in association with their regulatory partner cyclins, are key regulators of cell cycle progression. Cyclin D1, D2, D3-CDK4/CDK6 and cyclin A/E-CDK2 are involved in regulation of the G1/S transition (Nurse, 1994; Sher, 1994). Cyclin D/CDK4/CDK6 promotes the phosphorylation of Rb. The hypophosphorylated Rb proteins are known to inhibit the function of the E2F proteins, which promote transcription of factors essential for DNA synthesis (Sher, 1996). Thus, phosphorylation of Rb by CDKs, in association with their regulatory partner cyclins, is a key regulator of cell cycle progression. CDK inhibitors (CKI) are important regulators of the activity of CDKs and their substrates.
Rb protein was reported to be essential for osteoblast differentiation in cooperation with Runx2 (Thomas et al., 2001). Loss of Rb but not p107 or p130 blocks late osteoblast differentiation. Rb physically interacts with Runx2 and activates osteoblast specific maker genes expression (Thomas et al., 2001). Although it is not reported that whether this Rb is hypophosphorylated or hyperphosphorylated, it is conceivable that Runx2 interacts with hypophosphorylated Rb. Runx2 not only activates expression of osteoblast specific genes, ectopic expression of Runx2 induces p27 expression in MC3T3-E1 cells (mouse carvarial derived osteoblast-like cells) (Thomas et al., 2004). p27 thereby inhibits the activity of S-phase Cyclin/CDK complexes. This will form a positive loop favoring cell cycle arrest, and promote cell differentiation.

The decision of cell differentiation is often made in the G1 phase of the cell cycle (Zhu and Skoultchi, 2001), and so a higher protein level of differentiation factor in this phase may be necessary. Runx2 protein is accumulated in G1 phase when cell cycle is arrested by different stress and diminishes when cells are exposed to stress favoring cell cycle progression. This observation supports the notion that the tight regulation of Runx2 protein and inversed relationship between proliferation and differentiation (Galindo et al., 2005).

Recently, more observations implicate that in addition to their function as cell cycle...
regulators, the cyclin D proteins also serve as regulators for transcription factors and signaling proteins (Hirai and Sherr, 1996; Inoue and Sherr, 1998; Matsuura et al., 2004). In this report, we show that cyclin D1/CDK4 induces Runx2 phosphorylation, ubiquitination, and proteasome degradation and thereby inhibits differentiation while stimulating proliferation.

**Materials and Methods**

**Cell Culture and Transfection**

COS and C3H10T1/2 cells were cultured in Dulbecco's modified Eagle's medium and supplemented with 10% fetal calf serum at 37 °C under 5% CO2 condition. DNA plasmids were transiently transfected into COS or C3H10T1/2 cells in 6-cm culture dishes using the Lipofectamine 2000 reagents (Invitrogen). Empty vector was used to keep the total amount of DNA transfected constant. FLAG-EGFP plasmid was co-transfected as an internal control for transfection efficiency. Western blot and immunoprecipitation (IP) assays were performed 24 h after transfection.

**Immunoprecipitation (IP) Assays**

COS cells were transiently transfected with expression plasmids using Lipofectamine 2000 reagents (Invitrogen) and incubated for 24 h before analysis. After transfection cells
were washed once with phosphate-buffered saline, lysed for 30 min in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40) containing protease inhibitors (10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 10 µg/ml aprotinin) and phosphatase inhibitors (1 mM NaF and 1 mM Na3VO4), and clarified by centrifugation at 4 °C for 15 min. Supernatants were pre-cleared with EZ View Red protein G-Sepharose (Sigma) for 1 h at 4 °C. Then 5 µg of antibodies specific for each target protein were added in each sample. Immune complexes were precipitated by EZ View red protein G-Sepharose overnight at 4 °C, washed 5 times with the lysis buffer. The immune complexes were boiled for 10 min in SDS sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromphenol blue, 20% glycerol, 4% SDS) containing 10 mM dithiothreitol and analyzed by 10% SDS-PAGE. Western blot analysis was performed after immunoprecipitation.

**Western Blot Analysis**

The epitope-tagged Runx2, mRunx2(-PY), Runx3, mRunx3(-PY), Smurf1, mSmurf1, Smurf2, WWP1, and Smads 1, 5, 6, and 7 expression plasmids were transfected into COS cells. Cells were lysed on ice for 30 min in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, and 0.1% SDS supplemented with protease inhibitors (10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 10 µg/ml aprotinin) and phosphatase inhibitors (1 mM NaF and 1 mM Na3VO4). Proteins were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected using the following antibodies: anti-FLAG M2, anti-Myc (clone 9E10), and anti-HA (clone HA-7) monoclonal antibodies.
(Sigma). Immunostaining was detected using an enhanced chemiluminescence (ECL) system (Amersham Biosciences).

**PCR-based Site-directed Mutagenesis**

FLAG-tagged mouse Runx2 cDNA (MASN isoform, NCBI accession number: NM_009820 [GenBank]) was amplified by PCR, sequenced, and cloned into pcDNA3 and pCMV-Tag2 expression vectors (Stratagene, La Jolla, CA). Mutant Runx2 constructs (SA-Runx2 and SE-Runx2) were generated using Stratagene QuikChange site-directed mutagenesis kit and cloned into pcDNA3 and pCMV-Tag2 vector (Stratagene).

**In Vivo Protein Decay Assay**

Cells were seeded in 15-cm culture dishes, and equal amounts of FLAG-Runx2, FLAG-SA-Runx2, and FLAG-SE-Runx2 were transfected, respectively. 24 h after transfection, cells were trypsinized and split into five 10-cm dishes. 12 h after recovery, each sample was cultured in regular medium with 80 µg/ml cycloheximide (Calbiochem), and samples from time 0, 20, 60, 120, and 300 min were harvested. Western blot was performed to detect the decay of wild-type (WT) and mutant Runx2 proteins.

**In Vitro Runx2 Phosphorylation by Reconstituted Cyclin D1-Cdk4**

GST-Runx2 and GST-SA-Runx2 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 and purified from Escherichia coli. The induction of protein expression was detected by Coomassie Blue staining. To isolate soluble proteins, 100 ml of bacterial cultures are spun down, and pellets are 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 min. The lysate was sonicated until no longer viscous and centrifuged at 18,000 x g for 30 min at 4 °C. The supernatant is subjected to 100 µl of GST beads pulldown. After columns have been washed three times with sample buffer, the recombinant proteins were washed off with 5 mM reduced glutathione (Sigma) in 50 mM Tris-HCl, pH 8, 50 mM NaCl, and 1 mM dithiothreitol. Active cyclinD1-Cdk4 was obtained from Upstate Cell Signaling Solutions (Charlottesville, VA). The cyclinD1-Cdk4 enzyme mix was used to phosphorylate recombinant substrates for 40 min in 20-µl reactions containing 35 mM HEPES, pH 7.4, 10 mM MgCl2, 1 mM EGTA, 0.1% Tween 20, 1 mM dithiothreitol, 15 µM ATP at 30 °C. After phosphorylation, the recombinant Runx2 or control proteins were directly denatured by sample buffer and subjected to SDS-PAGE gel analysis. After protein samples have been analyzed by SDS-PAGE gel, the whole gel was subjected to Pro-Q Diamond phosphoprotein staining (Invitrogen) following the manufacturer's protocol.

Luciferase Assay

The 6xOSE2-Luc reporter construct was co-transfected with Runx2 expression plasmid.
and different amounts of cyclin D1 expression plasmid into C3H10T1/2 cells. Cell lysates were extracted 24 h after transfection, and luciferase activity was measured using a Promega Dual Luciferase reporter assay kit (Promega, Madison, WI).

**Alkaline Phosphatase Assay and Real-time Reverse transcription-PCR Analysis**

The alkaline phosphatase (ALP) and p27 mRNA expression was examined by real-time reverse transcription-PCR assay (Kaneki et al., 2006), and ALP activity measurements were performed as described previously (Zhao et al., 2004).

**Results**

**Cyclin D1 Induces Runx2 Degradation**

To determine whether cell cycle proteins regulate Runx2 function, we first examined the effect of cyclin proteins on Runx2 degradation. Flag-Runx2 expression plasmid was co-transfected with expression plasmids of cyclin A and cyclin D1 into COS cells, and changes in Runx2 protein levels were detected by Western blot. Transfection of cyclin D1 significantly reduced Runx2 protein level in a dose-dependent manner. In contrast, cyclin A did not alter Runx2 protein levels (Fig. 1a). These results suggest that cyclin D1 induces Runx2 degradation. Cyclin D1 normally forms a complex with CDK4, which
induces phosphorylation of substrate proteins. To determine whether CDK4 interacts with Runx2, we performed an IP assay. Flag-Runx2 and cyclin D1 expression plasmids were transfected into COS cells, and IP was performed using an anti-CDK4 antibody. The interaction of Runx2 with CDK4 was detected by Western blot using an anti-Flag antibody. The interaction of cyclin D1 with CDK4 was also detected by Western blot as a positive control (Fig. 1b). These results demonstrate that the cyclin D1-CDK4 complex directly interacts with Runx2 and induces Runx2 degradation.

**Cyclin D1 Targets the C-terminal Domain of Runx2**

A putative SP motif was identified in the C terminus of the murine Runx2 protein at amino acids 472–475 (MASN isoform, amino acid 1–528). To determine whether the C-terminal region of Runx2 is important for the protein stability, a truncated Runx2 construct with a C-terminal 79-amino acid deletion was generated (mRunx2, 1–449). We found that the mRunx2-(1–449) was more stable than WT-Runx2 in COS cells (Fig. 1c). When cyclin D1 was co-transfected with Runx2 expression plasmids, cyclin D1 induced the degradation of WT but not mutant Runx2 containing a C-terminal 79 amino acid deletion (Fig. 1d). These results suggest that the C-terminal region of Runx2 is important for Runx2 protein stability.

**Serine-472 Is Required for Runx2 Degradation**

To determine the importance of the serine-472 residue for Runx2 stability, site-directed
mutagenesis was used to replace serine-472 with alanine (SA-Runx2) or glutamic acid (SE-Runx2). Prior work has established that replacement of serine with alanine abolishes phosphorylation, whereas replacement with glutamic acid mimics constitutive phosphorylation of cyclin-CDK substrate proteins (Matsuura et al., 2004; Wee et al., 2002). As expected, the Western blot analysis revealed that the SA-Runx2 was more stable than WT-Runx2 and that the SE-Runx2 was very unstable in COS cells and mesenchymal pluripotent C3H10T1/2 cells (Fig. 2a). The results were further confirmed by the protein decay assay. Runx2 protein levels were significantly reduced at 300 min after protein synthesis was stopped by the treatment of cells with a protein synthesis inhibitor, cycloheximide. The SA-Runx2 was stable during the entire 300-min period after cells were treated with cycloheximide. In contrast, the SE-Runx2 was significantly degraded at the 60-120 min period after cells were treated with cycloheximide (Fig. 2b). To determine whether the serine-472 residue of Runx2 plays a critical role in cyclin D1-induced Runx2 degradation, cyclin D1 expression plasmid was co-transfected with either WT- or SA-Runx2 plasmid into COS cells. Cyclin D1 induced WT-Runx2 degradation in a dose-dependent manner (Fig. 2c). In contrast, cyclin D1 had no effect on SA-Runx2 degradation (Fig. 2d). These results indicate that serine-472 is critical for Runx2 stability.

Cyclin D1 Induces Runx2 Phosphorylation

We then examined the role of cyclin D1 in Runx2 phosphorylation. Cyclin D1 plasmid
was co-transfected with Flag-Runx2 or Flag-SA-Runx2 plasmids into COS cells, and IP was performed with the anti-Flag antibody followed by the Western blot using the anti-phosphoserine antibody. Although WT-Runx2 was phosphorylated, minimal phosphorylation of SA-Runx2 was observed (Fig. 3a). To confirm this finding, GST-Runx2 and GST-SA-Runx2 fusion proteins were generated, and the \textit{in vitro} phosphorylation assay was performed. The GST-Rb\textsubscript{379–928} was used as a positive control, and GST-Smad4 was used as a negative control (Matsuura et al. 2004). The \textit{in vitro} phosphorylation was induced by the incubation of recombinant substrate proteins with purified cyclin D1/CDK4 enzymes. Although strong phosphorylation was detected for WT-Runx2, a weak phosphorylation was detected for SA-Runx2. As reported previously, Rb protein was strongly phosphorylated by cyclin D1-CDK4 in this assay, but Smad4 protein was not phosphorylated (Fig. 3b). The results demonstrate serine-472 as a target for cyclin D1-CDK4-induced Runx2 phosphorylation.

**Cyclin D1-induced Runx2 Degradation Is Ubiquitin-Proteasome-dependent**

To determine the role of cyclin D1 in Runx2 ubiquitination, Flag-Runx2 plasmid was co-transfected with cyclin D1 in the presence or absence of proteasome inhibitor 1 (PS1). The IP was performed using the anti-Flag antibody, and Runx2 ubiquitination was detected by Western blot using an anti-HA antibody. Cyclin D1 significantly induced Runx2 ubiquitination. In the presence of PS1, cyclin D1-induced Runx2 ubiquitination was increased (Fig. 3c). To determine the role of serine-472 in Runx2 ubiquitination, the
expression plasmids of WT-, SA-, and SE-Runx2 were co-transfected with HA-ubiquitin into COS cells in the presence of PS1. The ubiquitinated protein ladder of WT-Runx2 was detected. When the SE-Runx2 was immunoprecipitated, a stronger Runx2 ubiquitination band was observed. In contrast, no ubiquitination was detected when SA-Runx2 was immunoprecipitated (Fig. 3d). To determine whether cyclin D1-induced Runx2 degradation is proteasome-dependent, cells were treated with PS1 after they were co-transfected with Runx2 and different amounts of cyclin D1 plasmid. Cyclin D1 induced a dose-dependent degradation of Runx2, which was completely reversed by the addition of PS1 (Fig. 3e). To further determine whether the serine-472 site is critical for Runx2 degradation, COS cells were transfected with WT- and SA-Runx2 and then treated with PS1. The results showed that treatment with PS1 increased the protein level of WT-Runx2 but not SA-Runx2 (Fig. 3f). Taken together, these results further demonstrate that cyclin D1 induces Runx2 ubiquitination and proteasome-dependent degradation and serine-472 is critical for cyclin D1-induced Runx2 degradation.

The Biological Significance of Cyclin D1-induced Runx2 Degradation

To determine whether the cyclin D1-induced Runx2 degradation alters Runx2 transcriptional activity, Runx2 expression plasmid was co-transfected with different amounts of cyclin D1 plasmid and the Runx2 reporter, 6xOSE2-Luc, into C3H10T1/2 cells. Although Runx2 significantly increased reporter activity (6-fold), co-transfection with cyclin D1 dose-dependently inhibited Runx2-induced reporter activity (Fig. 4a).
determine the effect of serine-472 on Runx2 transcriptional activity, WT-, SA-, and SE-Runx2 expression plasmids were co-transfected with 6xOSE2-Luc reporter into C3H10T1/2 cells. The SA-Runx2 had higher activity, and SE-Runx2 had lower activity on activation of the 6xOSE2-Luc reporter when compared with WT-Runx2 (Fig. 4b). The results demonstrate that cyclin D1 induces Runx2 degradation, leading to the changes in Runx2 transcriptional activity. Runx2 plays a key role in osteoblast differentiation and regulates the expression of osteoblast marker genes such as ALP. To further determine the biological significance of serine-472 of Runx2, WT-, SA-, and SE-Runx2 plasmids were transfected into C3H10T1/2 cells, and changes in ALP mRNA expression and ALP activity were examined. We found that transfection of WT-Runx2 significantly stimulated ALP mRNA expression (2.83-fold increase) and ALP activity (3.56-fold increase) in C3H10T1/2 cells. SA-Runx2 had much higher stimulatory effects on ALP mRNA expression (8.1-fold increase) and ALP activity (11.63-fold increase). In contrast, the effects of SE-Runx2 on ALP mRNA expression and activity were 65 and 72% lower, respectively, compared with the WT-Runx2 transfection group (Fig. 4c). Runx2 has also been shown to inhibit the activity of S-phase cyclin complex through inducing the expression of p27KIP1 leading to the dephosphorylation of the retinoblastoma tumor suppressor protein (pRb) and a G1 cell cycle arrest (Thomas et al., 2004). In the present studies, we found that transfection of WT-Runx2 stimulated p27 mRNA expression (2.4-fold increase) in C3H10T1/2 cells. Transfection of SA-Runx2 caused a 6.6-fold increase in p27 expression. In contrast, the effect of SE-Runx2 on p27 expression is 68%
lower than WT-Runx2 (Fig. 4d). These results demonstrate that Ser-472 of Runx2 plays an important role in Runx2-induced osteoblast differentiation, p27 expression, and cell cycle regulation.

Summary and Discussion

In the present studies, we demonstrate for the first time that cell cycle proteins regulate the key transcription factor Runx2 by inducing its phosphorylation and degradation through the ubiquitin-proteasome pathway. The serine-472 residue plays a critical role in cyclin D1-induced Runx2 phosphorylation and subsequent ubiquitination and degradation. Furthermore, cyclin D1/CDK4 controls Runx2 transcriptional activity and physiological function in osteogenic progenitor C3H10T1/2 cells.

Although regulation by cell cycle proteins has not been characterized for Runx2 in bone and cartilage, regulation of differentiation-associated transcription factors by cell cycle proteins has been well established in other cell types, such as myoblasts. MyoD is a key transcription factor controlling the myoblast differentiation. Its phosphorylation and degradation is controlled and regulated by cyclin B/CDK1/2. Cyclin B induces phosphorylation of serine-200 of MyoD protein and triggers MyoD ubiquitination and rapid degradation (Song et al., 1998; Kitzmann et al., 1999).
An inverse relationship between proliferation and differentiation has been reported in many developmental processes, such as myogenesis and neural development. Previous findings suggest that this inverse relationship is regulated at different levels. For example, cyclin-CDK complexes phosphorylate MyoD and induce its degradation (Kitzmann et al., 1999). Forced expression of MyoD induces p21 expression in myoblasts (Rao et al., 1994, Halevy et al., 1995). In addition, hypophosphorylated Rb acts as a co-repressor for E2F-induced gene expression of cell cycle proteins, and it also serves as a co-activator for Runx2-induced osteoblast differentiation (Thomas et al., 2001). In the present studies, we report that cyclin D1/CDK4 directly phosphorylate Runx2 and induce Runx2 ubiquitination and proteasome degradation, suggesting that coordinated relationship between proliferation and differentiation also exists during skeletal development (Fig. 4e). The existence of these mutual inhibitions provides the handles for regulation of cellular functions by different signaling pathways.

Previously, we have shown that the E3 ubiquitin ligase Smurf1 induces Runx2 degradation (Zhao et al., 2003) and that Smad6 enhances Smurf1-induced Runx2 degradation (Shen et al., 2006). In the present studies, we have analyzed the effect of Smurf1 on the degradation of WT- and SA-Runx2 and found that Smurf1 induced WT as well as SA mutant Runx2 in a similar efficiency, suggesting that Smurf1 is not involved in the cyclin D1-induced, phosphorylation-dependent degradation of Runx2 (Fig. 5).
Further investigation is required to identify the novel E3 ligase that is involved in the cyclin D1/CDK4-induced Runx2 ubiquitination and degradation.

Proper development of bone tissues requires precise spatial and temporal control of cell proliferation and differentiation. Although Runx2 is expressed during early skeletal development (embryonic days 11.5–14.5), the formation of hypertrophic chondrocytes and mature osteoblasts does not occur in most bones until embryonic days 14.5–15.5 (Kim et al., 1999). These observations suggest that Runx2 function must be suppressed during early bone development to allow chondrocyte and osteoblast pools to proliferate and expand. One possible mechanism for the suppression of Runx2 function during early bone development is through cyclin D1/CDK4-induced degradation. More detailed in vivo studies need to be conducted to further investigate this hypothesis.

In summary, our findings demonstrate that the cyclin D1/CDK4 complex phosphorylates Runx2 and induces Runx2 degradation in an ubiquitin-proteasome dependent manner. This study reveals a tightly regulated mechanism between bone cell growth and differentiation.
Figure 2.1. Cyclin D1 induces Runx2 protein degradation.

(a) Runx2 expression plasmid was co-transfected with different amounts of cyclin A and cyclin D1 expression plasmid (0.2, 0.6, and 1.8 µg/dish, 6-cm culture dish) into COS cells. Western blot was performed 48 h after transfection. Cyclin D1 induced Runx2 degradation, whereas cyclin A had no effect on Runx2 degradation. F, Flag.

(b) FLAG-Runx2 and cyclin D1 expression plasmids were transfected into COS cells. The IP was performed using the anti-CDK4 antibody followed by the Western blot (WB) using the anti-Flag or anti-cyclin D1 antibodies. CDK4 interacts with Runx2 in COS cells. As a positive control, CDK4 also interacts with cyclin D1.

(c) WT and mutant Runx2-(1–449) were transfected into COS cells. Western blot was performed 24 h after transfection. The truncated Runx2 with C-terminal 79 amino acid deletion was more stable than WT-Runx2 protein.

(d) WT and mutant Runx2-(1-449) expression plasmids were co-transfected with different amounts of cyclin D1 expression plasmid (0.2, 0.6 and 1.8 µg/dish) into COS cells. Western blot was performed 24 h after transfection. Cyclin D1 induced a dose-dependent degradation of WT-Runx2 but had no effect on the mutant Runx2.
Figure 2.2. Serine-472 of Runx2 is critical for Runx2 stability.

(a) WT, SA, or SE mutant Runx2 expression plasmids were transfected into COS and C3H10T1/2 cells. Western blot was performed 24 h after transfection. The SA-Runx2 was more stable than WT-Runx2. The SE-Runx2 was quickly degraded after transfection.

(b) Protein decay assay. COS cells were transfected with Runx2, SA-Runx2, or SE-Runx2 plasmids with an equal amount, respectively. Western blot was then performed. Runx2 protein levels are significantly reduced at 300 min after protein synthesis was stopped by cycloheximide. The SA-Runx2 was stable during the entire 300-min period after cells were treated with cycloheximide. In contrast, the SE-Runx2 was significantly degraded at the 60-120-min period after cells were treated with cycloheximide.

(c and d) Wild-type and SA mutant Runx2 expression plasmids were co-transfected with different amounts of cyclin D1 plasmid (0.2, 0.6, and 1.8 µg/dish) into COS cells. Cell lysates were extracted 24 h after transfection, and Western blot was performed. Transfection of cyclin D1 induced the degradation of wild-type Runx2 in a dose-dependent manner (c). In contrast, cyclin D1 had no effect on SA mutant Runx2 (d).
Figure 2.3. Cyclin D1 induces Runx2 phosphorylation, ubiquitination (Ub), and proteasome degradation.

(a) Equal amounts of Flag-Runx2 and SA-Runx2 plasmids were transfected into COS cells and then treated with PS1 (10 µM, 4-hour incubation). Cell lysates were extracted 24 h after transfection. Phosphorylation of Runx2 was detected by Western blot (WB) with the anti-phospho-serine antibody after IP with the anti-Flag antibody. Mutation of serine-472 into alanine dramatically decreases the phosphorylation of Runx2 protein. P-Runx2, phosphorylated Runx2; F, Flag.

(b) Recombinant GST-Rb-(379–928), GST-Runx2, GST-SA-Runx2, and GST-Smad4 were mixed with equal amounts of cyclin D1-CDK4 enzyme and incubated for 40 min at 30 °C, and then the reactions were stopped by sample buffer and subjected to SDS-PAGE gel analysis. The gels were either stained for Coomassie Blue for protein loading or stained for phosphorylated protein using the Pro-Q Diamond phosphoprotein staining kit. P-Protein, phosphorylated protein.

(c) Flag-Runx2 and HA-ubiquitin plasmids were co-transfected with different amounts of cyclin D1 expression plasmid (0.2 and 0.6 µg/dish, 6-cm culture dish) into COS cells in the presence or absence of PS1 (10 µM, 4-h incubation). Cyclin D1 induced Runx2 ubiquitination in a dose-dependent manner. In the presence of PS1, cyclin D1-induced Runx2 ubiquitination is further enhanced.

(d) Flag-tagged WT-, SA-, or SE-Runx2 was co-transfected with HA-ubiquitin plasmid into COS cells in the presence of PS1 (10 µM, 4-h incubation). Runx2 ubiquitination was detected in the WT- and SE-Runx2 groups but not in the SA-Runx2 group.

(e) Flag-Runx2 expression plasmid was co-transfected 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 h after transfection. Transfection of cyclin D1 induced a dose-dependent degradation of Runx2, and treatment with PS1 completely reversed cyclin D1-induced Runx2 degradation.

(f) WT- and SA-Runx2 expression plasmids were transfected into COS cells. Cells were treated with PS1 (10 µM) for 4 h after transfection. The addition of PS1 increased the protein level of WT-Runx2 but not SA-Runx2.
Figure 2.4. Biological significance of cyclin D1-induced Runx2 degradation.

(a) Runx2 expression plasmid was co-transfected with different amounts of cyclin D1 plasmid (0, 0.25, 0.5, 1, and 2 µg/well) and the Runx2 reporter, 6xOSE2-Luc (1 µg/well), into C3H10T1/2 cells. Cell lysates were collected 24 h after transfection, and a luciferase assay was performed. Runx2 significantly increased the luciferase activity of the Runx2 reporter (6-fold). Co-transfection of cyclin D1 inhibited Runx2-induced reporter activity in a dose-dependent manner.*, \( p < 0.05 \) when compared with Runx2 alone, one-way analysis of variance followed by Dunnett's test.

(b) WT-, SA-, and SE-Runx2 were co-transfected with 6xOSE2-Luc into C3H10T1/2 cells. Cell lysates were collected 24 h after transfection, and a luciferase assay was performed. The SA-Runx2 had higher activity and SE-Runx2 had lower activity on activation of the 6xOSE2-Luc reporter when compared with WT-Runx2. *, \( p < 0.05 \) when compared with the WT-Runx2 transfection group, unpaired Student's t test.

(c) and (d) WT-, SA-, and SE-Runx2 were transfected into C3H10T1/2 cells. mRNA was extracted, and cell lysates were collected 24 h after transfection. ALP and p27 mRNA expression was examined by real-time reverse transcription-PCR, and ALP activity was measured using ALP assay kit. The SA-Runx2 had higher activity and SE-Runx2 had lower activity on stimulation of ALP (c) and p27 (d) mRNA expression and ALP activity (c) when compared with the WT-Runx2 transfection group. *, \( p < 0.05 \) when compared with the WT-Runx2 transfection groups for ALP (c) and p27 (d) mRNA expression assay, unpaired Student's t test. **, \( p < 0.05 \), when compared with WT-Runx2 transfection group for ALP activity measurements (c), unpaired Student's t test.

(e) Schematic demonstration of coordinative regulation of proliferation and differentiation in bone cells by cell cycle proteins and Runx2.
Figure 2.5. Smurf1 is not involved in the cyclin D1-induced, phosphorylation-dependent degradation of Runx2

(a) Schematic representation of Runx2 degron, PY, and Serine 472.
(b) Equal amounts of FLAG-Runx2 and SA-Runx2 plasmids were transfected with increasing dosage of Smurf1 into COS cells. Western blot was performed 24 h after transfection. Smurf1 induced protein degradation of both FLAG-Runx2 and SA-Runx2 in similarly efficient manner.
(c) Equal amounts of PY-Runx2 plasmids were transfected with increasing dosage of cyclinD1 into COS cells. Cyclin D1 efficiently induced the degradation of PY-Runx2.
Acetylation has also been reported to modify Runx3, another member of Runx family, in response to TGF-β treatment, and so prevent the degradation induced by E3 ligase Smurf1 (Jin et al., 2004).

Cell cycle regulation is controlled by a combination of cyclins, CDKs, and CDK inhibitors, which together with the tumor suppressor retinoblastoma (Rb), are involved in the tight control of cell cycle machinery. CDKs, in association with their regulatory partner cyclins, are key regulators of cell cycle progression. Cyclin D (D1, D2, D3)-CDK4/CDK6 and cyclin A/E-CDK2 are involved in regulation of the G1/S transition (Nurse, 1994; Sher, 1994). Cyclin D/CDK4/CDK6 promotes the phosphorylation of Rb. The hypophosphorylated Rb proteins are known to inhibit the function of the E2F proteins, which promote transcription of factors essential for DNA synthesis (Sherr, 1996). Thus, phosphorylation of Rb by the cyclin D-CDK complexes relieves inhibition of Rb on the E2F function, promoting the entry of cells into S phase.

Cyclin/CDK inhibitor (CKI) are important regulators of the activities of Cyclin/CDK. They play important roles in regulating both cell cycle progressions and tissue specific differentiation.

In vitro experiments demonstrated that, in MC3T3-E1 cells, there is a protein accumulation of Runx2 when cells arrested in G1/G0 phase through cell culture confluency or serum deprivation (Pratap et al., 2003). Further studies on MC3T3-E1 cells show that Runx2 protein level oscillates during the cell cycle. The

Cyclin/CDK-regulated Runx2 stability is implicated to play a very important role in this case, although the possibility of regulated expression of Runx2 can not be ruled out during this process. If this is true, then the Cyclin/CDK activities promote a cell cycle proceeding and
non-differentiation state. (The last 2 sentences are not very clear to me)