



Minireview

Post-Translational Regulations of Transcriptional Activity of RUNX2

Hyun-Jung Kim, Woo-Jin Kim, and Hyun-Mo Ryoo*

Department of Molecular Genetics & Dental Pharmacology, School of Dentistry and Dental Research Institute, Seoul National University, Seoul 08826, Korea

*Correspondence: hmryoo@snu.ac.kr

<https://doi.org/10.14348/molcells.2019.0247>

www.molcells.org

Runt-related transcription factor 2 (RUNX2) is a key transcription factor for bone formation and osteoblast differentiation. Various signaling pathways and mechanisms that regulate the expression and transcriptional activity of RUNX2 have been thoroughly investigated since the involvement of RUNX2 was first reported in bone formation. As the regulation of Runx2 expression by extracellular signals has recently been reviewed, this review focuses on the regulation of post-translational RUNX2 activity. Transcriptional activity of RUNX2 is regulated at the post-translational level by various enzymes including kinases, acetyl transferases, deacetylases, ubiquitin E3 ligases, and prolyl isomerases. We describe a sequential and linear causality between post-translational modifications of RUNX2 by these enzymes. RUNX2 is one of the most important osteogenic transcription factors; however, it is not a suitable drug target. Here, we suggest enzymes that directly regulate the stability and/or transcriptional activity of RUNX2 at a post-translational level as effective drug targets for treating bone diseases.

Keywords: osteoblast differentiation, phosphorylation-directed Isomerization, post-translational modification, RUNX2, transcriptional activity

INTRODUCTION

In the field of bone research, 1997 was a landmark year because Runt-related transcription factor 2 (RUNX2) was first described as an essential regulator of skeletal development. Targeted disruption of *Runx2* in mice resulted in a complete lack of bone formation due to impaired osteoblast differentiation, indicating that Runx2 is an essential transcription factor for osteoblast differentiation in both intramembranous and endochondral ossification (Komori et al., 1997; Otto et al., 1997). In humans, haploinsufficiency of the RUNX2 gene is the main cause of cleidocranial dysplasia characterized by hypoplastic clavicles, patent fontanelles, supernumerary and unerupted permanent teeth and other changes in skeletal patterning and growth (Mundlos et al., 1997). Conversely, gain-of-function mutations of the fibroblast growth factor receptors (FGFRs) cause craniosynostosis characterized by the premature closure of craniofacial sutures (Moloney et al., 1997; Su et al., 2014) due to the acceleration of osteoblast proliferation and differentiation by fibroblast growth factor (FGF)/FGFR signal-induced increases in RUNX2 expression and transactivation activity (Kim et al., 2003). These results indicate that the dysregulation of RUNX2 is intrinsically involved in the pathogenesis of bone diseases and understanding the regulatory mechanisms of RUNX2 is required for treating RUNX2-related bone diseases. Various signaling pathways, including FGF/FGFR and bone morphogenic protein (BMP)/BMP receptor (BMPR) signaling pathways are involved in

Received 29 October, 2019; accepted 4 December, 2019; published online 27 December, 2019

eISSN: 0219-1032

©The Korean Society for Molecular and Cellular Biology. All rights reserved.

©This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/3.0/>.

regulating RUNX2 level and transactivation function during osteoblast differentiation. In this process, RUNX2 is strictly regulated at transcriptional, translational, and post-translational levels. Here, we review the post-translational regulations affecting the stability and activity of RUNX2.

RUNX FAMILY AND RUNX2 STRUCTURE

RUNX2 belongs to the RUNX family that contains three members (RUNX1, 2, and 3) with distinct functions. RUNX1 is essential for hematopoiesis and has a crucial role in blood vessel formation (Wang et al., 1996). RUNX3 plays an important role in neurogenesis (Inoue et al., 2002) and also tumor suppression (Li et al., 2002). RUNX2 plays an essential role in osteogenesis (Komori et al., 1997; Otto et al., 1997).

The RUNX2 gene consists of eight exons in both humans and mice, and gene expression is transcriptionally regulated by two promoters, the distal promoter P1 and the proximal promoter P2. The distal promoter P1 regulates transcription of a RUNX2-II transcript that is translated as a protein starting with MASNS, which encodes 521 and 528 amino acids (exons 1-8) in humans and rodents, respectively. This RUNX2-II isoform is predominantly expressed in osteoblasts (Stewart et al., 1997). Conversely, the proximal promoter P2 regulates transcription of the RUNX2-I transcript that is translated into a protein of 513 amino acids starting with MRIPVD (exons 2-8). It is mainly expressed in T cells and osteoblasts (Ogawa et al., 1993b). The differential expression patterns of these two isoforms in bone tissues during development were clearly demonstrated previously (Choi et al., 2002; Park et al., 2001). RUNX2 proteins have several functional domains including a glutamine/alanine-rich domain (QA), Runt homology domain (RHD), nuclear localization signal (NLS), proline/serine/threonine-rich domain (PST), nuclear matrix targeting signal (NMTS), repression domain (RD), and VWRPY domain (Fig. 1) (Levanon and Groner, 2004; Ziros et al., 2008). The QA domain is composed of 23 glutamine and 17 alanine repeats at the N-terminus and acts as a transactivation domain (Thirunavukkarasu et al., 1998). The length of the QA repeat influences the RUNX2 transactivation activity (Morrison et al., 2012). Near the N-terminus of the RUNX protein is a common RHD responsible for DNA binding and heterodimerization with the transcriptional co-activator, core binding

factor- β (CBF- β) (Ogawa et al., 1993a). The PST domain has transactivation as well as transcription repression functions (Bae et al., 1994). Furthermore, this region has several sites important for post-translational modifications by extracellular signal-regulated kinase 1 and 2 (Erk1/2), protein kinase A (PKA), and Akt kinases (Pande et al., 2013; Selvamurugan et al., 2009; Xiao et al., 2002). The NLS of RUNX2 consists of nine amino acids (PRRHRQKLD) and its deletion leads the loss of RUNX2 transactivation function because RUNX2 no longer translocates to the nucleus (Thirunavukkarasu et al., 1998). The NMTS mediates the targeting of RUNX2 to distinct sub-nuclear locations associated with the nuclear matrix and is essential for proper RUNX2 activity (Choi et al., 2001; Zaidi et al., 2001). They also have a common 5 amino acid VWRPY domain at the C-terminus, which interacts with transcriptional corepressor transducing-like enhancer of split 2 (TLE2) (Aronson et al., 1997).

FUNCTIONS OF RUNX2 IN SKELETOGENESIS AND OSTEOBLAST DIFFERENTIATION

In intramembranous ossification, the process of the direct differentiation of mesenchymal cells to osteoblasts, RUNX2 directs the differentiation of mesenchymal stem cells to pre-osteoblasts and further differentiation to immature osteoblasts. RUNX2 is expressed in uncommitted mesenchymal cells and its expression is increased in pre-osteoblasts, reaches the maximal level in immature osteoblasts, and is then decreased in mature osteoblasts (Maruyama et al., 2007). Osteoblasts produce osteoblast-specific proteins including type 1 collagen (COL1), alkaline phosphatase (ALP), osteopontin (OP), and osteocalcin (OC), which are differentially expressed during osteoblast differentiation. RUNX2 is responsible for the activation of these osteoblast differentiation marker genes. The expression of ALP, OP, and OC genes is absent in *Runx2*^{-/-} mice (Komori et al., 1997). RUNX2 upregulates the expression of COL1, OP, and OC genes via binding to the consensus sequences, 5'-(Pu/T)ACCPuCPu-3' or 5'-PyGPYGGT(Py/A)-3', referred as osteoblast-specific *cis*-acting element 2 (OSE2) (Ducy and Karsenty, 1995).

During endochondral bone formation where growing cartilage is systemically replaced by bone to form the growing skeleton, hypertrophy of chondrocytes is positively regulated

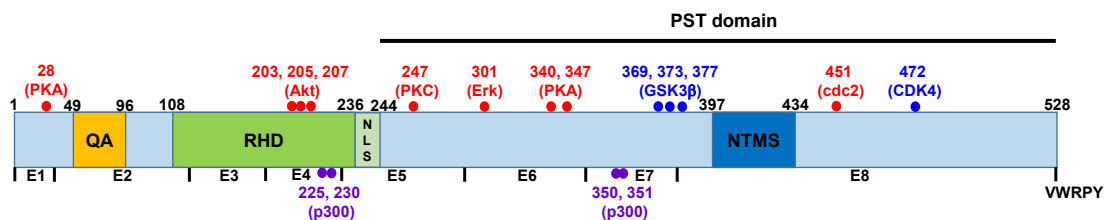


Fig. 1. RUNX2 structure. The structural and functional domains of mouse RUNX2-II (bone specific isoform) and its post-translational modification sites. Mouse RUNX2-II encodes a 528 amino acid proteins (exons 1-8) with functional domains including a glutamine/alanine-rich domain (QA), a Runt homology domain (RHD), a nuclear localization signal (NLS), a proline/serine/threonine-rich domain (PST), a nuclear matrix targeting signal (NMTS) and a C-terminal VWRPY domain. Red and blue circles over the protein indicate phosphorylation sites that activate and inhibit RUNX2, respectively. Acetylation sites are depicted as purple circles under the protein in the figure. E1-E8 indicate Exon1-Exon8.

by RUNX2, which activates the expression of type X collagen (Zheng et al., 2003). RUNX2 regulates endochondral bone formation tightly with key factors of chondrocyte differentiation including Indian hedgehog (IHH) and parathyroid hormone-related protein (PTHrP) in a feedback-loop (Vortkamp et al., 1996). RUNX2 regulates chondrocyte proliferation by regulating IHH expression. Subsequently, IHH upregulates the expression of PTHrP, which inhibits RUNX2 and chondrocyte maturation.

REGULATIONS OF RUNX2 AT POST-TRANSLATIONAL LEVELS

Post-translational regulation modulates the activity, stability and function of many proteins including transcription factors. Phosphorylation, acetylation, and ubiquitination are the most representative post-translational modifications and also phosphorylation-directed conformational changes by enzymes play key roles in regulating protein activity; therefore, we will mainly describe the regulation of RUNX2 activity by these modifications.

Phosphorylation of RUNX2

Protein phosphorylation is one of the most common, yet key post-translational modifications that induce conformational changes in proteins (Pawson and Scott, 2005). The addition of a phosphate group (PO_4) to the polar group R of various amino acids modifies the protein from hydrophobic apolar to hydrophilic polar, allowing proteins to change conformation and consequently affect their functional activities. How phosphorylation of RUNX2 regulates RUNX2 function and how RUNX2 can be further regulated post-phosphorylation has been previously studied.

The mitogen-activated protein kinase (MAPK) was the first reported kinase to phosphorylate and activate RUNX2 (Xiao et al., 2000). It was subsequently reported that FGF2-stimulated phosphorylation of RUNX2 and its transcriptional activity were mediated by Erk1/2 MAPK (Xiao et al., 2002). Our group revealed that the Ser302 of murine RUNX2-II was responsible for the phosphorylation by FGF2-induced Erk MAPK, leading to the transactivation and stabilization of RUNX2 (Park et al., 2010). The Erk MAPK pathway positively modulates RUNX2 at the post-translational level, but it is not involved in the regulation of FGF/FGFR signaling-induced Runx2 mRNA expression (Kim et al., 2003). Conversely, the protein kinase C (PKC) pathway, especially, PKC δ mediates both FGF/FGFR-stimulated transactivation and transcription of RUNX2 (Kim et al., 2003). Upon FGF2 treatment, PKC δ phosphorylates the Ser247 of RUNX2 through a direct interaction with RUNX2 (Kim et al., 2006). Parathyroid hormone (PTH), a bone resorbing hormone, induces PKA-mediated RUNX2 transactivation for matrix metalloproteinase-13 (MMP-13; collagenase-3) gene expression in osteoblasts (Selvamurugan et al., 2000). In the presence of 8-bromo cyclic adenosine mono-phosphate (8-Br-cAMP), a cell permeable form of cAMP, which activates PKA, RUNX2 stimulates MMP-13 promoter activity and the phosphorylation of three sites (Ser28, Ser347, and Thr340) of RUNX2 is essential in this regulation (Selvamurugan et al., 2009). Akt also increas-

es the DNA binding and transcriptional activity of RUNX2 by direct phosphorylation at Ser203, Thr205, and Thr207 within RHD without inhibiting the CBF- β interaction (Pande et al., 2013). The Ser451 phosphorylation of RUNX2 by cell division cycle protein 2 (cdc2) facilitates cell cycle progression possibly through the regulation of G2/M phases, thus, promoting endothelial cell proliferation (Qiao et al., 2006).

Together with the stimulatory effect of phosphorylation on RUNX2 activity, phosphorylation at different sites negatively regulates RUNX2 transcriptional activity. The phosphorylation of RUNX2 at Ser369, Ser373, and Ser377 sites by glycogen synthase kinase 3 beta (GSK3 β) inactivates RUNX2, eventually inhibiting bone formation (Kugimiya et al., 2007). Cyclin D1/CDK4 mediates the phosphorylation of Ser472 of RUNX2, leading to subsequent ubiquitination and proteosomal degradation of RUNX2 (Shen et al., 2006).

It has not been well clarified why RUNX2 activity is differently regulated depending on RUNX2 phosphorylation sites. However, our recent studies provided evidence that sequence-specific phosphorylation alters the RUNX2 structure by recruiting an isomerase, thereby affecting the stability and activity of RUNX2 with subsequent post-translational modifications (this will be described in detail later). Considering these results, it is thought that the sequence-specific phosphorylation of RUNX2 regulates its activity positively or negatively through structural changes, interactions with specific proteins to recognize phosphorylation, and subsequent post-translational modifications. Therefore, integrative studies including protein structure biology are needed to investigate the relationship between RUNX2 phosphorylation and RUNX2 activity.

Acetylation of RUNX2

Post-translational acetylation of lysine is reversible and has emerged as a significant post-translational regulation mechanism. In general, acetylation of histone proteins has been well documented to lead to a reduction in their DNA affinity within the chromatin structure. Furthermore, lysine acetylation of non-histone proteins including transcription factors is an important post-translational modification for regulating function and stability of these proteins. This process is accomplished by histone acetyltransferases (HATs). Conversely, histone deacetylases (HDACs) reverse this process by removing the acetyl group. Both HATs and HDACs are reported to interact with RUNX2 and modulate RUNX2 stability and activity during osteoblast differentiation. BMP2 induces RUNX2 acetylation by the HAT p300, and the acetylation protects RUNX2 from E3 ligase Smad ubiquitin regulatory factor (Smurf)-mediated degradation (Jeon et al., 2006). In this regulation, HDAC4 and HDAC5 deacetylate RUNX2, allowing it to undergo Smurf-mediated degradation. Consistently, HDAC inhibitors including Trichostatin A increase RUNX2 acetylation and potentiate BMP2-stimulated osteoblast differentiation and bone formation (Jeon et al., 2006). FGF2 also stimulated RUNX2 acetylation and the sequential phosphorylation and isomerization of RUNX2 by Erk MAPK and Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) isomerase, respectively, are a prerequisite for FGF2-stimulated RUNX2 acetylation, leading to an increase in RUNX2 stability and

activity (Park et al., 2010; Yoon et al., 2014). HDAC1, 3, and 6 also associate with RUNX2 and function as corepressors of RUNX2 in a deacetylation-dependent manner. HDAC1 interacts with RUNX2 to negatively regulate OP gene expression (Zhang et al., 2012). HDAC3 acts as a corepressor of RUNX2 in regulating the OC and bone-sialoprotein gene expression (Lamour et al., 2007; Schroeder et al., 2004). HDAC6 interacts with the C-terminus of RUNX2, a region that is essential for bone formation, and acts as a corepressor of RUNX2 for p21 gene expression in differentiating osteoblasts (Westendorf et al., 2002). HDACs suppress the stability and activity of RUNX2 through RUNX2 deacetylation; therefore, HDAC inhibitors could be applied to the treatment of RUNX2-related bone diseases. Our group recently described one possibility in an *in vivo* mouse model; the HDAC inhibitor MS275 partially prevented the delayed cranial suture closure in heterozygous RUNX2 null mice by enhancing the stability and transcriptional activity of RUNX2 via the acetylation of RUNX2 (Bae et al., 2017). Suberoylanilide hydroxamic acid (SAHA) also increases RUNX2 acetylation, leading to enhanced stability and transcriptional activity of RUNX2 and effectively reverses soluble receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoporotic bone loss (Lee et al., 2015). Taken together, these results demonstrate that HDACs repress RUNX2 post-translationally and the inhibition of HDACs with small molecule inhibitors or RNAi-mediated suppression accelerates osteoblast differentiation *in vitro* and *in vivo*, suggesting that HDACs modulating RUNX2 stability and activity at the post-translational level could be clinically relevant targets for bone anabolic therapies.

Ubiquitination of RUNX2

The ubiquitination process is a sequential enzymatic reaction that mediates the marking of target proteins by an ubiquitin label, thereby directing them for degradation through the proteasome pathway. The ubiquitination cascade requires the successive action of three enzymes. The first enzyme (E1) involved in this cascade is the ubiquitin-activating enzyme that recruits ubiquitin. The second enzyme (E2) is the ubiquitin-conjugating enzyme that transfers the ubiquitin to the targeted protein. The third enzyme (E3) is the ubiquitin ligase that acts as a scaffold protein, interacts with the E2 enzyme and transfers ubiquitin to the target protein (Dikic et al., 2009). In some cases, the ubiquitination is mutually exclusive and competitive with the acetylation for the same lysine residues of proteins.

RUNX2 protein levels are regulated through a ubiquitin-proteasomal degradation mechanism at the post-translational level. The HECT domain E3 ligase, Smurf1 is the first identified E3 ligase for RUNX2 ubiquitination and degradation (Zhao et al., 2003). Smurf1 interacts directly with RUNX2 and mediates RUNX2 degradation in an ubiquitin- and proteasome-dependent manner and the overexpression of Smurf1 in 2.3Col1-Smurf1 transgenic mice inhibits post-natal bone formation, negatively regulating osteoblast differentiation (Zhao et al., 2003; 2004). Lysine acetylation and ubiquitination occur competitively at the same lysine residues; therefore, p300-dependent acetylation of RUNX2 by BMP2 is resistant to Smurf1-mediated ubiquitin-proteasomal degradation.

Conversely, the deacetylation of RUNX2 by HDAC4 and 5 makes RUNX2 vulnerable to Smurf1-mediated degradation (Jeon et al., 2006). Tumor necrosis factor-induced RUNX2 degradation is also mediated by the upregulation of Smurf1 expression levels in osteoblast precursor cells (Kaneki et al., 2006). Akt enhances RUNX2 protein stability by suppressing Smurf2 function (Choi et al., 2014). Schnurri-3 (Shn3), a zinc finger adapter protein, controls RUNX2 protein levels by promoting its degradation through recruitment of the HECT domain E3 ligase WWP1 to RUNX2, and consistently, RUNX2 protein level, osteoblast function and bone mass are significantly increased in *Shn3* knockout mice (Jones et al., 2006). C-terminus of Hsc70-interacting protein (CHIP), a U-box E3 ligase, also interacts with RUNX2 and enhances RUNX2 ubiquitination and degradation (Li et al., 2008). Overexpression of CHIP in MC3T3-E1 cells leads to RUNX2 degradation and the inhibition of osteoblast differentiation. In contrast, the depletion of CHIP in these cells enhances osteoblast differentiation. S-phase kinase associated protein 2 (Skp2) constituting the SCF^{Skp2} E3 ligase complex promotes ubiquitin-mediated proteasomal degradation of RUNX2 and negatively affects its functions, resulting in inhibited osteoblast differentiation (Thacker et al., 2016). While these E3 ligases accelerate poly-ubiquitination-mediated RUNX2 degradation, WWP2 E3 ligase facilitates RUNX2 transactivation in a mono-ubiquitination manner during osteoblast differentiation and BMPR1A enhances WWP2-modulated RUNX2 ubiquitination and transcriptional activity (Zhu et al., 2017). E3 ligases modulating RUNX2 stability and activity are summarized in Table 1.

In contrast to FGF2-induced phosphorylation of RUNX2, Shen et al. (2006) reported that the phosphorylation of RUNX2 by cyclin D1/CDK4 promotes E3 ubiquitin ligase-induced RUNX2 ubiquitination and degradation, indicating that there are important determinants that decide the post-translational ubiquitination or acetylation of RUNX2 depending on phosphorylation of a specific site.

Phosphorylation-dependent isomerization of RUNX2

Phosphorylation at serine and threonine residues accounts for about 96% of all protein phosphorylation in the cell (Olsen et al., 2006). Although phosphorylation induces conformational changes *per se* (Jiang and McKnight, 2006), the enzymatic conversion of peptide bonds between *cis* and *trans* conformation depending on the phosphorylation state can introduce significant changes in the total protein structure, eventually regulating functional activity of its client proteins (Polonio-Vallon et al., 2014).

Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) is a peptidyl-prolyl isomerase that specifically binds phosphorylated Ser/Thr-Pro motifs and catalyzes the Pro *cis-trans* isomerization of its substrate proteins, thereby altering the conformations of the target proteins (Lu and Zhou, 2007). Pin1-mediated Pro isomerization provides more stringent control of target proteins after phosphorylation-mediated post-translational modification. It was reported that Pin1 has a central role in transducing phosphorylation of tumor suppressor p53 into conformational changes that affect p53 stability and activity upon genotoxic stresses via enhancing p300-mediated acetylation of p53 and p53 dis-

Table 1. E3 ubiquitin ligases regulating RUNX2

Class of E3 ligases	E3 ligases	Regulators	Status	Action to RUNX2	References
HECT E3	Smurf1	Inhibition by BMP2 Activation by TNF α	Poly-Ub	Negative	(Jeon et al., 2006) (Kaneki et al., 2006)
	Smurf2	Inhibition by Akt	Poly-Ub	Negative	(Choi et al., 2014)
	WWP1	Activation by Schnurri-3	Poly-Ub	Negative	(Jones et al., 2006)
	WWP2	Activation by BMPR1A	Mono-Ub	Positive	(Zhu et al., 2017)
RING E3	SCF ^{skp2}	Not determined	Poly-Ub	Negative	(Thacker et al., 2016)
U-BOX E3	CHIP	Not determined	Poly-Ub	Negative	(Li et al., 2008)

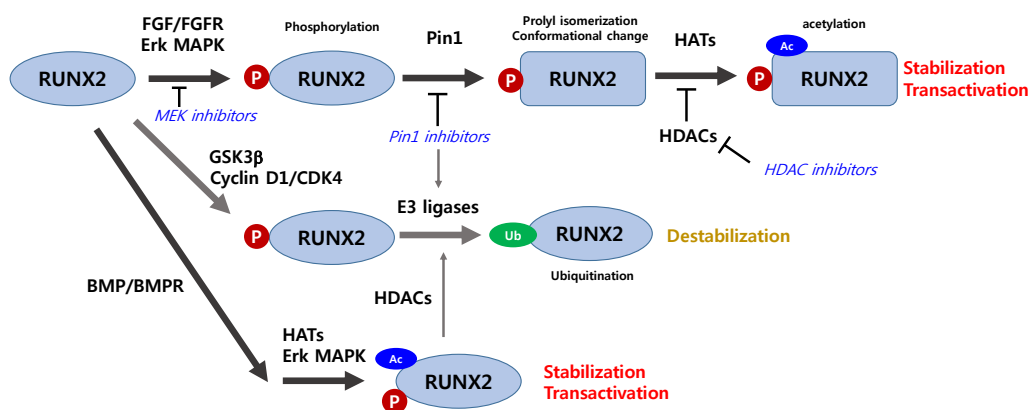


Fig. 2. Regulation of RUNX2 stability and transcriptional activity at post-translational levels. Upon the activation of FGF/FGFR-Erk MAPK signaling pathway, RUNX2 is stabilized and transactivated by multiple post-translational modification cascades: phosphorylation by Erk MAPK, prolyl isomerization by Pin1, acetylation by HAT. Phosphorylation of RUNX2 by GSK3 β and cyclin D1/CDK4 induces ubiquitin-proteasomal degradation of RUNX2. BMP/BMPR stimulates p300-mediated RUNX2 acetylation by protecting RUNX2 from Smurf1-mediated degradation.

sociation from the apoptosis inhibitor iASPP, promoting cell death (Mantovani et al., 2007). Our group reported that Pin1 directly interacts with RUNX2 protein in a phosphorylation-dependent manner and subsequently stabilizes RUNX2 proteins by acetylation (Yoon et al., 2013). In the absence of Pin1, RUNX2 is rapidly degraded by the ubiquitin-proteasome pathway. Conversely, Pin1 overexpression strongly attenuates ubiquitin-dependent RUNX2 degradation. Furthermore, we revealed that Pin1-dependent isomerization of RUNX2 is the critical step required for FGF2-enhanced RUNX2 transcriptional activation and stabilization (Yoon et al., 2014). Phosphorylation and the subsequent Pin1-mediated conformational change of RUNX2 are prerequisites for FGF2-enhanced stability and transcriptional activation of RUNX2. These results also indicate that Pin1-mediated isomerization of RUNX2 is an indispensable step connecting phosphorylation and acetylation for FGF/FGFR-induced RUNX2 stabilization and transcriptional activation. Based on these studies, our group also proved the therapeutic potential of Pin1 inhibition, which prevents premature fusion of cranial sutures through destabilization of RUNX2 by decreasing acetylation using the Apert syndrome mouse model and *in vitro* cell systems (Shin et al., 2018). BMP2-activated ERK MAPK also enhances RUNX2 stability and activity through an increase in p300 levels and its HAT activity (Jun et al., 2010).

Although the study did not mention any structural changes of RUNX2 after phosphorylation, the activation of ERK MAPK precedes RUNX2 acetylation upon BMP2 treatment, suggesting that Pin1 could be involved in the sequential regulation between RUNX2 phosphorylation and acetylation as in the regulation of RUNX2 by FGF2. However, considering that the phosphorylation of Ser-Pro motifs of RUNX2 by cyclin D1/CDK4 promotes RUNX2 degradation unlike the phosphorylation by Erk MAPK, RUNX2 may be susceptible or resistant to degradation due to discriminatory structural changes caused by Pin1 isomerization.

CONCLUSION

RUNX2 plays an essential role in bone development and maintenance. With various intra- and extra-cellular environmental stimuli, RUNX2 is regulated by sequential post-translational modifications accompanied by structural changes, eventually affecting RUNX2 function positively or negatively. In particular, FGF/FGFR-enhanced RUNX2 stabilization and transactivation are controlled by multiple post-translational modification cascades that occur in the following sequence: phosphorylation, prolyl isomerization, and acetylation. However, phosphorylation-coupled proteasomal degradation of RUNX2 by other kinases such as GSK3 β and cyclin D1/

CDK4 might have other links between different post-translational regulation mechanisms. In summary, the stability and transcriptional activity of RUNX2 is stringently controlled by various post-translational modifications (Fig. 2). Although the different post-translational regulation mechanisms of RUNX2 have been individually investigated, they could be cooperatively linked for the regulation of RUNX2 function, thus, further studies are needed to reveal the links between post-translational modifications. Furthermore, RUNX2 modifying enzymes including HATs, HDACs, Pin1, GSK3 β and cyclin D1/CDK4 are interesting therapeutic targets for RUNX2-related bone diseases.

Disclosure

The authors have no potential conflicts of interest to disclose.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea Grants NRF-2017R1A4A1014584 and NRF-2017R1A2B3011778 (to H.M. Ryoo), and NRF-2019R1C1C1003669 (to W.J. Kim).

We thank S.J. Win, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

ORCID

Hyun-Jung Kim <https://orcid.org/0000-0002-5360-1809>
Woo-Jin Kim <https://orcid.org/0000-0002-3471-0006>
Hyun-Mo Ryoo <https://orcid.org/0000-0001-6769-8341>

REFERENCES

Aronson, B.D., Fisher, A.L., Blechman, K., Caudy, M., and Gergen, J.P. (1997). Groucho-dependent and -independent repression activities of Runt domain proteins. *Mol. Cell. Biol.* *17*, 5581-5587.

Bae, H.S., Yoon, W.J., Cho, Y.D., Islam, R., Shin, H.R., Kim, B.S., Lim, J.M., Seo, M.S., Cho, S.A., Choi, K.Y., et al. (2017). An HDAC inhibitor, entinostat/MS-275, partially prevents delayed cranial suture closure in heterozygous Runx2 null mice. *J. Bone Miner. Res.* *32*, 951-961.

Bae, S.C., Ogawa, E., Maruyama, M., Oka, H., Satake, M., Shigesada, K., Jenkins, N.A., Gilbert, D.J., Copeland, N.G., and Ito, Y. (1994). PEBP2 alpha B/mouse AML1 consists of multiple isoforms that possess differential transactivation potentials. *Mol. Cell. Biol.* *14*, 3242-3252.

Choi, J.Y., Pratap, J., Javed, A., Zaidi, S.K., Xing, L., Balint, E., Dalamangas, S., Boyce, B., van Wijnen, A.J., Lian, J.B., et al. (2001). Subnuclear targeting of Runx/Cbfa/AML factors is essential for tissue-specific differentiation during embryonic development. *Proc. Natl. Acad. Sci. U. S. A.* *98*, 8650-8655.

Choi, K.Y., Lee, S.W., Park, M.H., Bae, Y.C., Shin, H.I., Nam, S., Kim, Y.J., Kim, H.J., and Ryoo, H.M. (2002). Spatio-temporal expression patterns of Runx2 isoforms in early skeletogenesis. *Exp. Mol. Med.* *34*, 426-433.

Choi, Y.H., Kim, Y.J., Jeong, H.M., Jin, Y.H., Yeo, C.Y., and Lee, K.Y. (2014). Akt enhances Runx2 protein stability by regulating Smurf2 function during osteoblast differentiation. *FEBS J.* *281*, 3656-3666.

Dikic, I., Wakatsuki, S., and Walters, K.J. (2009). Ubiquitin-binding domains - from structures to functions. *Nat. Rev. Mol. Cell Biol.* *10*, 659-671.

Ducy, P. and Karsenty, G. (1995). Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Mol. Cell. Biol.* *15*, 1858-1869.

Inoue, K., Ozaki, S., Shiga, T., Ito, K., Masuda, T., Okado, N., Iseda, T., Kawaguchi, S., Ogawa, M., Bae, S.C., et al. (2002). Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons. *Nat.*

Neurosci. *5*, 946-954.

Jeon, E.J., Lee, K.Y., Choi, N.S., Lee, M.H., Kim, H.N., Jin, Y.H., Ryoo, H.M., Choi, J.Y., Yoshida, M., Nishino, N., et al. (2006). Bone morphogenetic protein-2 stimulates Runx2 acetylation. *J. Biol. Chem.* *281*, 16502-16511.

Jiang, Z.G. and McKnight, C.J. (2006). A phosphorylation-induced conformation change in dematin headpiece. *Structure* *14*, 379-387.

Jones, D.C., Wein, M.N., Oukka, M., Hofstaetter, J.G., Glimcher, M.J., and Glimcher, L.H. (2006). Regulation of adult bone mass by the zinc finger adapter protein Schnurri-3. *Science* *312*, 1223-1227.

Jun, J.H., Yoon, W.J., Seo, S.B., Woo, K.M., Kim, G.S., Ryoo, H.M., and Baek, J.H. (2010). BMP2-activated Erk/MAP kinase stabilizes Runx2 by increasing p300 levels and histone acetyltransferase activity. *J. Biol. Chem.* *285*, 36410-36419.

Kaneki, H., Guo, R., Chen, D., Yao, Z., Schwarz, E.M., Zhang, Y.E., Boyce, B.F., and Xing, L. (2006). Tumor necrosis factor promotes Runx2 degradation through up-regulation of Smurf1 and Smurf2 in osteoblasts. *J. Biol. Chem.* *281*, 4326-4333.

Kim, B.G., Kim, H.J., Park, H.J., Kim, Y.J., Yoon, W.J., Lee, S.J., Ryoo, H.M., and Cho, J.Y. (2006). Runx2 phosphorylation induced by fibroblast growth factor-2/protein kinase C pathways. *Proteomics* *6*, 1166-1174.

Kim, H.J., Kim, J.H., Bae, S.C., Choi, J.Y., Kim, H.J., and Ryoo, H.M. (2003). The protein kinase C pathway plays a central role in the fibroblast growth factor-stimulated expression and transactivation activity of Runx2. *J. Biol. Chem.* *278*, 319-326.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M., et al. (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* *89*, 755-764.

Kugimiya, F., Kawaguchi, H., Ohba, S., Kawamura, N., Hirata, M., Chikuda, H., Azuma, Y., Woodgett, J.R., Nakamura, K., and Chung, U.I. (2007). GSK-3beta controls osteogenesis through regulating Runx2 activity. *PLoS One* *2*, e837.

Lamour, V., Detry, C., Sanchez, C., Henrotin, Y., Castronovo, V., and Bellahcene, A. (2007). Runx2- and histone deacetylase 3-mediated repression is relieved in differentiating human osteoblast cells to allow high bone sialoprotein expression. *J. Biol. Chem.* *282*, 36240-36249.

Lee, Z.H., Kim, H.J., and Ryoo, H.M. (2015). A novel osteogenic activity of suberoylanilide hydroxamic acid is synergized by BMP-2. *J. Bone Metab.* *22*, 51-56.

Levanon, D. and Groner, Y. (2004). Structure and regulated expression of mammalian RUNX genes. *Oncogene* *23*, 4211-4219.

Li, Q.L., Ito, K., Sakakura, C., Fukamachi, H., Inoue, K., Chi, X.Z., Lee, K.Y., Nomura, S., Lee, C.W., Han, S.B., et al. (2002). Causal relationship between the loss of RUNX3 expression and gastric cancer. *Cell* *109*, 113-124.

Li, X., Huang, M., Zheng, H., Wang, Y., Ren, F., Shang, Y., Zhai, Y., Irwin, D.M., Shi, Y., Chen, D., et al. (2008). CHIP promotes Runx2 degradation and negatively regulates osteoblast differentiation. *J. Cell Biol.* *181*, 959-972.

Lu, K.P. and Zhou, X.Z. (2007). The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease. *Nat. Rev. Mol. Cell Biol.* *8*, 904-916.

Mantovani, F., Tocco, F., Girardini, J., Smith, P., Gasco, M., Lu, X., Crook, T., and Del Sal, G. (2007). The prolyl isomerase Pin1 orchestrates p53 acetylation and dissociation from the apoptosis inhibitor iASPP. *Nat. Struct. Mol. Biol.* *14*, 912-920.

Maruyama, Z., Yoshida, C.A., Furuichi, T., Amizuka, N., Ito, M., Fukuyama, R., Miyazaki, T., Kitaura, H., Nakamura, K., Fujita, T., et al. (2007). Runx2 determines bone maturity and turnover rate in postnatal bone development and is involved in bone loss in estrogen deficiency. *Dev. Dyn.* *236*, 1876-1890.

Moloney, D.M., Wall, S.A., Ashworth, G.J., Oldridge, M., Glass, I.A., Francomano, C.A., Muenke, M., and Wilkie, A.O. (1997). Prevalence of

- Pro250Arg mutation of fibroblast growth factor receptor 3 in coronal craniosynostosis. *Lancet* 349, 1059-1062.
- Morrison, N.A., Stephens, A.A., Osato, M., Polly, P., Tan, T.C., Yamashita, N., Doecke, J.D., Pasco, J., Fozzard, N., Jones, G., et al. (2012). Glutamine repeat variants in human RUNX2 associated with decreased femoral neck BMD, broadband ultrasound attenuation and target gene transactivation. *PLoS One* 7, e42617.
- Mundlos, S., Otto, F., Mundlos, C., Mulliken, J.B., Aylsworth, A.S., Albright, S., Lindhout, D., Cole, W.G., Henn, W., Knoll, J.H., et al. (1997). Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell* 89, 773-779.
- Ogawa, E., Inuzuka, M., Maruyama, M., Satake, M., Naito-Fujimoto, M., Ito, Y., and Shigesada, K. (1993a). Molecular cloning and characterization of PEBP2 beta, the heterodimeric partner of a novel Drosophila runt-related DNA binding protein PEBP2 alpha. *Virology* 194, 314-331.
- Ogawa, E., Maruyama, M., Kagoshima, H., Inuzuka, M., Lu, J., Satake, M., Shigesada, K., and Ito, Y. (1993b). PEBP2/PEA2 represents a family of transcription factors homologous to the products of the Drosophila runt gene and the human AML1 gene. *Proc. Natl. Acad. Sci. U. S. A.* 90, 6859-6863.
- Olsen, J.V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006). Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* 127, 635-648.
- Otto, F., Thornell, A.P., Crompton, T., Denzel, A., Gilmour, K.C., Rosewell, I.R., Stamp, G.W., Beddington, R.S., Mundlos, S., Olsen, B.R., et al. (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* 89, 765-771.
- Pande, S., Browne, G., Padmanabhan, S., Zaidi, S.K., Lian, J.B., van Wijnen, A.J., Stein, J.L., and Stein, G.S. (2013). Oncogenic cooperation between PI3K/Akt signaling and transcription factor Runx2 promotes the invasive properties of metastatic breast cancer cells. *J. Cell. Physiol.* 228, 1784-1792.
- Park, M.H., Shin, H.I., Choi, J.Y., Nam, S.H., Kim, Y.J., Kim, H.J., and Ryoo, H.M. (2001). Differential expression patterns of Runx2 isoforms in cranial suture morphogenesis. *J. Bone Miner. Res.* 16, 885-892.
- Park, O.J., Kim, H.J., Woo, K.M., Baek, J.H., and Ryoo, H.M. (2010). FGF2-activated ERK mitogen-activated protein kinase enhances Runx2 acetylation and stabilization. *J. Biol. Chem.* 285, 3568-3574.
- Pawson, T. and Scott, J.D. (2005). Protein phosphorylation in signaling--50 years and counting. *Trends Biochem. Sci.* 30, 286-290.
- Polonio-Vallon, T., Kruger, D., and Hofmann, T.G. (2014). ShaPING cell fate upon DNA damage: role of Pin1 isomerase in DNA damage-induced cell death and repair. *Front. Oncol.* 4, 148.
- Qiao, M., Shapiro, P., Fosbrink, M., Rus, H., Kumar, R., and Passaniti, A. (2006). Cell cycle-dependent phosphorylation of the RUNX2 transcription factor by cdc2 regulates endothelial cell proliferation. *J. Biol. Chem.* 281, 7118-7128.
- Schroeder, T.M., Kahler, R.A., Li, X., and Westendorf, J.J. (2004). Histone deacetylase 3 interacts with runx2 to repress the osteocalcin promoter and regulate osteoblast differentiation. *J. Biol. Chem.* 279, 41998-42007.
- Selvamurugan, N., Pulumati, M.R., Tyson, D.R., and Partridge, N.C. (2000). Parathyroid hormone regulation of the rat collagenase-3 promoter by protein kinase A-dependent transactivation of core binding factor alpha1. *J. Biol. Chem.* 275, 5037-5042.
- Selvamurugan, N., Shimizu, E., Lee, M., Liu, T., Li, H., and Partridge, N.C. (2009). Identification and characterization of Runx2 phosphorylation sites involved in matrix metalloproteinase-13 promoter activation. *FEBS Lett.* 583, 1141-1146.
- Shen, R., Wang, X., Drissi, H., Liu, F., O'Keefe, R.J., and Chen, D. (2006). Cyclin D1-cdk4 induce runx2 ubiquitination and degradation. *J. Biol. Chem.* 281, 16347-16353.
- Shin, H.R., Bae, H.S., Kim, B.S., Yoon, H.I., Cho, Y.D., Kim, W.J., Choi, K.Y., Lee, Y.S., Woo, K.M., Baek, J.H., et al. (2018). PIN1 is a new therapeutic target of craniosynostosis. *Hum. Mol. Genet.* 27, 3827-3839.
- Stewart, M., Terry, A., Hu, M., O'Hara, M., Blyth, K., Baxter, E., Cameron, E., Onions, D.E., and Neil, J.C. (1997). Proviral insertions induce the expression of bone-specific isoforms of PEBP2alphaA (CBFA1): evidence for a new myc collaborating oncogene. *Proc. Natl. Acad. Sci. U. S. A.* 94, 8646-8651.
- Su, N., Jin, M., and Chen, L. (2014). Role of FGF/FGFR signaling in skeletal development and homeostasis: learning from mouse models. *Bone Res.* 2, 14003.
- Thacker, G., Kumar, Y., Khan, M.P., Shukla, N., Kapoor, I., Kanaujiya, J.K., Lochab, S., Ahmed, S., Sanyal, S., Chattopadhyay, N., et al. (2016). Skp2 inhibits osteogenesis by promoting ubiquitin-proteasome degradation of Runx2. *Biochim. Biophys. Acta* 1863, 510-519.
- Thirunavukkarasu, K., Mahajan, M., McLaren, K.W., Stifani, S., and Karsenty, G. (1998). Two domains unique to osteoblast-specific transcription factor Osf2/Cbfa1 contribute to its transactivation function and its inability to heterodimerize with Cbfbeta. *Mol. Cell. Biol.* 18, 4197-4208.
- Vortkamp, A., Lee, K., Lanske, B., Segre, G.V., Kronenberg, H.M., and Tabin, C.J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* 273, 613-622.
- Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A.H., and Speck, N.A. (1996). Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc. Natl. Acad. Sci. U. S. A.* 93, 3444-3449.
- Westendorf, J.J., Zaidi, S.K., Cascino, J.E., Kahler, R., van Wijnen, A.J., Lian, J.B., Yoshida, M., Stein, G.S., and Li, X. (2002). Runx2 (Cbfa1, AML-3) interacts with histone deacetylase 6 and represses the p21(CIP1/WAF1) promoter. *Mol. Cell. Biol.* 22, 7982-7992.
- Xiao, G., Jiang, D., Gopalakrishnan, R., and Franceschi, R.T. (2002). Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx2. *J. Biol. Chem.* 277, 36181-36187.
- Xiao, G., Jiang, D., Thomas, P., Benson, M.D., Guan, K., Karsenty, G., and Franceschi, R.T. (2000). MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. *J. Biol. Chem.* 275, 4453-4459.
- Yoon, W.J., Cho, Y.D., Kim, W.J., Bae, H.S., Islam, R., Woo, K.M., Baek, J.H., Bae, S.C., and Ryoo, H.M. (2014). Prolyl isomerase Pin1-mediated conformational change and subnuclear focal accumulation of Runx2 are crucial for fibroblast growth factor 2 (FGF2)-induced osteoblast differentiation. *J. Biol. Chem.* 289, 8828-8838.
- Yoon, W.J., Islam, R., Cho, Y.D., Woo, K.M., Baek, J.H., Uchida, T., Komori, T., van Wijnen, A., Stein, J.L., Lian, J.B., et al. (2013). Pin1-mediated Runx2 modification is critical for skeletal development. *J. Cell Physiol.* 228, 2377-2385.
- Zaidi, S.K., Javed, A., Choi, J.Y., van Wijnen, A.J., Stein, J.L., Lian, J.B., and Stein, G.S. (2001). A specific targeting signal directs Runx2/Cbfa1 to subnuclear domains and contributes to transactivation of the osteocalcin gene. *J. Cell Sci.* 114, 3093-3102.
- Zhang, Z., Deepak, V., Meng, L., Wang, L., Li, Y., Jiang, Q., Zeng, X., and Liu, W. (2012). Analysis of HDAC1-mediated regulation of Runx2-induced osteopontin gene expression in C3H10t1/2 cells. *Biotechnol. Lett.* 34, 197-203.
- Zhao, M., Qiao, M., Harris, S.E., Oyajobi, B.O., Mundy, G.R., and Chen, D. (2004). Smurf1 inhibits osteoblast differentiation and bone formation in vitro and in vivo. *J. Biol. Chem.* 279, 12854-12859.
- Zhao, M., Qiao, M., Oyajobi, B.O., Mundy, G.R., and Chen, D. (2003). E3 ubiquitin ligase Smurf1 mediates core-binding factor alpha1/Runx2 degradation and plays a specific role in osteoblast differentiation. *J. Biol. Chem.* 278, 27939-27944.
- Zheng, Q., Zhou, G., Morello, R., Chen, Y., Garcia-Rojas, X., and Lee, B.

(2003). Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte-specific expression in vivo. *J. Cell Biol.* *162*, 833-842.

Zhu, W., He, X., Hua, Y., Li, Q., Wang, J., and Gan, X. (2017). The E3 ubiquitin ligase WWP2 facilitates RUNX2 protein transactivation in a mono-

ubiquitination manner during osteogenic differentiation. *J. Biol. Chem.* *292*, 11178-11188.

Ziros, P.G., Basdra, E.K., and Papavassiliou, A.G. (2008). Runx2: of bone and stretch. *Int. J. Biochem. Cell Biol.* *40*, 1659-1663.