

**Identification and Characterization of MAPK  
Phosphorylation Sites in Runx2**

By

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## DEDICATION

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# CHAPTER I



## 1. BACKGROUND AND SIGNIFICANCE

Runx2/Cbfa1 is a bone-related transcription factor homologous to the *Drosophila* protein, *Runt* (Ducy, 2000). Runx2 protein is essential for the differentiation of osteoblasts from mesenchymal precursors, and bone formation, as homozygous *Cbfa1*<sup>-/-</sup> mice show a complete lack of functional osteoblasts and are devoid of mineralized bone or hypertrophic cartilage (Otto et al., 1997). Mutations in Runx2 on the short arm of Chromosome 6 in humans cause cleidocranial dysplasia (CCD), an autosomal dominant disease characterized by the absence of clavicles, open fontanelles, supernumerary teeth and short stature (Mundlos et al., 1997); (Bergwitz et al., 2001). Runx2 can directly stimulate transcription of osteoblast-related genes such as those encoding osteocalcin (OCN), bone sialoprotein (BSP), type I collagen, osteopontin (OPN) and collagenase 3 by binding to specific enhancer regions containing the core sequence, PuCCPuCA (Ducy et al., 1997).

Runx2/Cbfa1 is present in tooth germs (odontoblasts, ameloblasts, cementoblasts), and some stages of cartilage and osteoblasts (Wise et al., 2002; Yamashiro et al., 2002; Pan et al., 2005). It is essential for differentiation of mesenchymal cells into bone-specific cell types or tooth and mineralization *in vivo* (Chen et al., 2005b). Runx2 gene knockout mice are unable to mineralize their skeletons and exhibit an arrest of tooth development at the cap stage (Aberg et al., 2004a). Thus, Runx2 plays fundamental roles in the development of bones and teeth.

The transition from preosteoblasts to osteoblasts involves the secretion of a complex extracellular matrix (ECM) containing bone morphogenic proteins (BMP),

growth factors and structural components that interact with cells to induce osteoblast-specific gene expression (Xiao et al., 2002c). The mitogen-activated protein kinase (MAPK) pathway is critical for the responsiveness of osteoblasts to ECM, hormonal, growth factor and mechanical stimuli (Xiao et al., 2002a; Xiao et al., 2002c; Franceschi et al., 2003; Jiang et al., 2004). Differentiation is controlled in part by binding of type I collagen to  $\alpha2\beta1$  integrins that signal to the nucleus via the ERK/MAPK pathway to phosphorylate and activate the osteoblast-related transcription factor, RUNX2 (Xiao et al., 1998a). Other growth factors such as FGF2 and IGF, known to signal through MAPK, also stimulate RUNX2 phosphorylation (Xiao et al., 2002c). The significance of MAPK signaling in osteoblast differentiation was established by showing that perturbation of this pathway through the use of specific inhibitors or expression of constitutively active or dominant negative MAPK intermediates, respectively, increased or decreased osteoblast differentiation in cell culture and in transgenic mice. My proposal will focus on identifying and characterizing domains in Runx2 that are phosphorylated by MAPK and necessary for transcriptional activation. The functional activity of specific domains will be established by mutagenesis and testing in bone cells from Runx2-deficient mice. Phosphorylation of Runx2 will also be examined. In this way, we will be able to show how a specific modification of the Runx2 transcription factor can alter its transcriptional activity. This pathway may also be active in odontoblasts since this cell type is also known to be dependent on ECM synthesis for differentiation.

The phosphorylation sites identified in this study will serve as the basis for future work to assess the importance of Runx2 phosphorylation *in vivo*. This will be accomplished through the development of a knock-in model in which inactive mutations will be introduced into the endogenous Runx2 gene. These animals can be used to assess the role of Runx2 phosphorylation in bone formation and remodeling, mechanical loading of bone, and tooth development and movement.

## **2. PURPOSE AND HYPOTHESES**

### **2.1 PURPOSE**

The purpose of this research is to establish the importance of MAPK phosphorylation sites to the biological activity of RUNX2 in cell culture by identifying and characterizing MAPK phosphorylation sites in RUNX2.

### **2.2 HYPOTHESES**

MAPK phosphorylation sites in RUNX2 are critical for responsiveness to several extracellular matrix (ECM) and hormonal signals, and are necessary for Runx2-dependent gene expression.

### **2.3 SPECIFIC AIM**

*Identify and establish the importance of MAPK phosphorylation sites to the biological activity of RUNX2.* Studies in this aim will identify domains of Runx2 necessary for MAPK responsiveness and evaluate the role of MAPK phosphorylation sites in the response of osteoblasts to a known osteoblast-related inducer of MAPK activity, FGF2, in cell culture. Studies with FGF2 will use a reconstituted system in COS7 cells (Runx2-negative) containing an OCN promoter-Luc reporter and Runx2 deletions/mutations and measure FGF2-induced luciferase activity.

### 3. REVIEW OF LITERATURE

The runt box (Runx) is a highly conserved DNA binding and protein-protein interaction domain that defines a family of heterodimeric transcription factors with essential roles in multicellular animal development.

#### 3.1 Runx family

The first member of Runx family to be identified was the *Drosophila* regulatory gene *runt*, which was named by virtue of its function in establishing segmentation patterns during embryogenesis, and subsequently discovered to have additional functions in sex determination and neurogenesis. A second *Drosophila* Runx gene, *lozenge*, is required for cell patterning in the eye and for hematopoiesis. The genome project has revealed the existence of two additional *Drosophila* Runx genes, which have not been fully characterized. Other invertebrate species with well-characterized Runx transcription factors include the nematode, *Caenorhabditis elegans*, and the sea urchin, *Strongylocentrotus purpuratus*, each of which apparently contains only a single Runx gene (Coffman, 2003).

The Runx family of transcription factors has a variety of functions in normal development and neoplasia. In mammals, the Runx gene family consists of RUNX1/AML1, RUNX2 and RUNX3 (van Wijnen et al., 2004). Runx1 is required for hematopoiesis and is genetically altered in leukemia. Indeed, it is the most frequent target of chromosomal translocations associated with human leukemias (Look, 1997; Speck and Gilliland, 2002). Moreover, haploinsufficiency of RUNX1 due to heterozygous loss-of-

function mutations is associated with familial platelet disorder with a predisposition to acute myeloid leukemia (FPD-AML) (Song et al., 1999). Sporadic heterozygous mutations of RUNX1 are also leukemogenic (Osato et al., 1999). In addition, RUNX1 is associated with several autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis and psoriasis (Prokunina et al., 2002; Tokuhiro et al., 2003).

Runx2, also known as Core-binding factor  $\alpha 1$  (Cbfa1), PEBP2A1, AML3, is a bone-related *runt* domain transcription factor (Ducy, 2000). The Runx2 gene encodes a master transcription factor of bone and plays a role in all stages of bone formation (Karsenty, 2001; Lian and Stein, 2003). It also plays an important role in the development of dentition (D'Souza et al., 1999; Gaikwad et al., 2001; Wise et al., 2002; Aberg et al., 2004a; Aberg et al., 2004b; James et al., 2006; Ryoo and Wang, 2006).

RUNX3 is required for the development of CD8-lineage T cells (Taniuchi et al., 2002) and TrkC-dependent dorsal root ganglion neurons (Inoue et al., 2002). It also functions as a tumor suppressor (Bae and Choi, 2004). It is associated not only with gastric cancer, but also with various other cancers of the lung, colon, pancreas, liver, prostate, bile duct, breast, larynx, esophagus, endometrium, uterine cervix and testicular yolk sac (Bae and Lee, 2006).

During development, Runx1, Runx2, and Runx3 are all expressed in mesenchymal condensates. Runx1 is expressed in several cartilage primordia earlier than Runx3, and Runx2 is intensely expressed in all mesenchymal condensations of bones and teeth. Only Runx1 is expressed in epithelia, and in tooth germs transcripts where it was detected in outer dental epithelium and midline epithelium of palatal shelves. In early

tooth morphogenesis, Runx3 is coexpressed with Runx2 in a thin layer of mesenchymal cells underlying dental epithelium. Runx3 is expressed in odontoblasts. However, Runx3 mutant mice do not show an obvious tooth phenotype or deviations of Runx1 and Runx2 expression patterns in the tooth (Yamashiro et al., 2002).

### **3.2 Runx2 and bone**

Bone tissue consists of hydroxyapatite crystals and various kinds of extracellular matrix (ECM) proteins, including type I collagen, osteocalcin (OCN), bone sialoprotein (BSP), osteonectin, osteopontin (OPN), and proteoglycans (Young et al., 1992). These bone matrix proteins are secreted and deposited by polarized mature osteoblasts, which are aligned on the bone surface. The precise roles of matrix proteins in the formation of bone have not been fully elucidated. The formation of hydroxyapatite crystals is also regulated by osteoblasts (Horowitz, 2003; Young, 2003). The Runx2 gene is 220kb long (Levanon et al., 1994; Yamashiro et al., 2002) and contains eight exons (Geoffroy et al., 1998; Xiao et al., 1998b; Quack et al., 1999). Runx2 is essential for osteoblast development and proper bone formation. Runx2 binds specific DNA sequences to regulate transcription of numerous genes and thereby control osteoblast development. Runx2 is essential for the initial commitment of mesenchymal cells to the osteoblastic lineage, and also controls the proliferation, differentiation, and maintenance of these cells. Runx2 expression persists throughout bone development beginning at embryonic day 9.5 (E9.5) in mouse where it peaks at E12.5, well before any mineralization has occurred. After very early expression of Runx2 in skeletal development, it continues during the later stages of bone development and persists in regions of active bone remodeling throughout life (Karsenty, 2001). Although necessary for gene transcription

and skeletal development, Runx2 is not sufficient for optimal gene expression or bone formation. The subsequent development of the osteoblast lineage requires cooperation between Runx2 and several other proteins, including transcription factors and cofactors such as Ostrix, which is essential for subsequent progression of the osteoblast lineage, and ATF4, which regulates osteoblast activity, particularly in postnatal animals (Nakashima et al., 2002; Yang et al., 2004). Runx2 is posttranslationally modified, and associates with the nuclear matrix to integrate a variety of signals and organize crucial events during osteoblast development and maturation. Consistent with its role as a master organizer, alterations in Runx2 expression levels are associated with skeletal diseases (Schroeder et al., 2005).

*In vivo* studies of homozygous Runx2<sup>-/-</sup> mice show no bone tissue, osteoblasts or osteoclasts, despite normal cartilaginous skeletal patterning. However, chondrocyte maturation to the hypertrophic stage is also blocked (Komori et al., 1997; Otto et al., 1997). Mice lacking Runx2 show a complete absence of osteoblasts and bone and succumb at birth due to respiratory distress caused by a defective rib cage (Ducy et al., 1997).

Runx2 overexpression is common in many bone-metastatic cancers (Pratap et al., 2005; Schroeder et al., 2005), and adult transgenic mice overexpressing Runx2 showed osteopenia with a decrease in bone mineral density (Geoffroy et al., 2002). Also, over expression of Runx2 in lymphocytes induces T-cell Leukemia ((Vaillant et al., 2002)).

*In vitro* studies showed that growth factors with known osteoinductive abilities, like the BMPs, regulate Runx2 expression in osteoblasts (Ducy et al., 1997; Xiao et al., 2002a). Runx2 also binds to and regulates the expression of multiple extracellular matrix



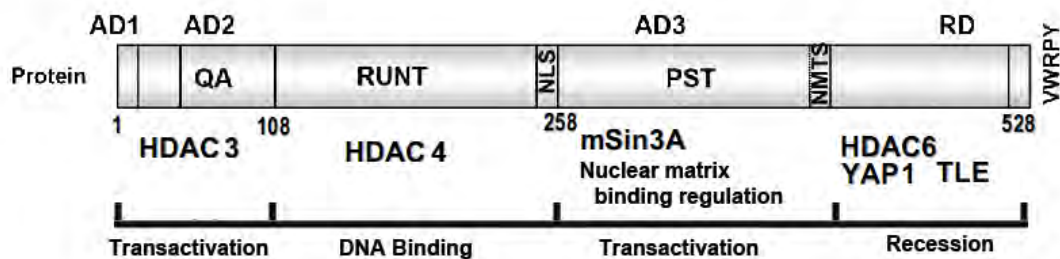
genes in osteoblasts, and its overexpression can induce osteoblast-specific gene expression in fibroblasts and myoblasts (Ducy et al., 1997). These results firmly establish the role of Runx2 as a key transcriptional regulator of osteoblast differentiation during bone formation. *In vivo* and *in vitro* studies indicate that Runx2 is a master gene of osteoblast differentiation whose function is not redundant with that of other genes *in vivo*.

### 3.2.1 Runx2 structure and function

RUNX2 binds to the osteoblast-specific *cis*-acting element 2 (OSE2), the core binding factor site, (Ducy and Karsenty, 1995), which is found in the promoter regions of all the major osteoblast-characteristic genes, such as osteocalcin, type I collagen, BSP, osteopontin, MMP-13, and alkaline phosphatase (ALK). Together with other factors, it controls their expression. The runt homology domain (RHD) is responsible for the DNA-binding of Runx2. Three transactivation domains and one major repression domain have been identified in the RUNX2 protein (Fig.1). The first transactivation domain is located in the N-terminal 19 amino acids of the protein, while the second is located in the glutamine/alanine (Q/A) domain. The transactivation depends on glutamine residues. Deletion of the alanine does not affect transactivation; however, expansion has a repressive function, and expansion may also play a role in nuclear localization (Albrecht et al., 2004). The third activation domain is present in the N-terminal portion of the proline/serine/threonine (PST)-rich domain. A mutation in this region blocked interactions with SMADs, reducing the response of osteoblasts to the Transforming growth factor- $\beta$ /Bone morphogenetic protein (TGF- $\beta$ /BMP) signaling pathway (Zhang et al., 2000).

The C-terminal part of the PST domain is a repression domain (Thirunavukkarasu et al., 1998). Studies identified several co-repressor proteins that bind to Runx2 to regulate gene expression, such as histone deacetylases (HDACs), transducin-like enhancer of split (TLE) proteins, mSin3a, and yes-associated protein (YAP). These proteins do not bind DNA themselves and appear to act by preventing Runx2 from binding DNA, altering chromatin structure, and/or by possibly blocking co-activator complexes. The nuclear localization of several of these factors is regulated by extracellular signaling events (Westendorf, 2006).

RUNX2 is imported to the nucleus after transcription and must bind to specific regions of the nuclear matrix to effect transcriptional control, colocalizing with co-activators such as SMADs and RNA polymerases, at nuclear sites that support RNA synthesis. This function is affected by the nuclear matrix targeting signal (NMTS) region, which is a 38 amino acid segment situated between the RHD and PST domains. Point mutations in the NMTS region have been shown to affect the intranuclear localization of RUNX2, possibly affecting its interaction with target genes that are involved in osteogenic activity (Zhang et al., 2000). The interaction of transcription factors with cellular signal transducers at particular points in the nuclear matrix may partly explain the tissue-specific action of the Runx proteins and of transcription factors in general.

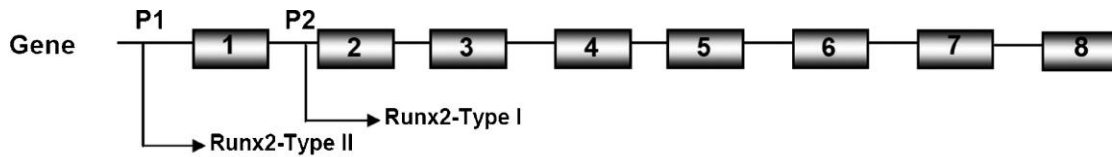


*Fig. 1. Runx2 structure. The Runx2 proteins contain multiple functional domains: AD1-3, transactivation domains; QA, glutamine/alanine rich domain; RUNT, runt homology domain; NLS, nuclear localization signal; PST, proline/serine/threonine rich domain; NMTS, nuclear matrix targeting signal; RD, repression domain; VWRPY, conserved repression “domain” of runt proteins. HDAC, histone deacetylase; mSin3A, SIN3A protein; TLE, transducin-like enhancer of split; YAP, yes-associated protein.*

*(Edit from Ziros et al., 2008)*

### 3.2.2 Runx2 isoforms and expression

Runx2 has two major N-terminal isoforms that are separately transcribed from a proximal promoter or a distal promoter (Fig.2). The P2 promoter drives expression of the Type I isoform, which is associated with cell proliferation. The P1 promoter drives expression of the type II isoform, which appears to be the bone-specific Runx2 isoform. The product of the proximal promoter starts with a different amino acid sequence (Xiao et al., 1998b). The presence of multiple isoforms of Runx2 gene product is similar to the situation seen with other members of the Runx transcription family.



*Fig 2. Runx2 isoforms. The runx2 gene contains eight exons and its transcription is controlled by two promoters, P1 and P2, which give rise to two major transcripts/protein isoforms, type II and type I, respectively. Runx2-Type I, associates with cell proliferation; Runx2-Type II, commitment to a calcified tissue line.*

*(Edit from Ziros et al., 2008)*

Runx2 can directly stimulate transcription of osteoblast-related genes such as those encoding osteocalcin (OCN), type I collagen, bone sialoprotein (BSP), osteopontin (OPN) and collagenase 3 by binding to specific enhancer regions containing the core sequence, PuCCPuCA (Ducy et al., 1997). Over expression of a dominant-negative Runx2 fragment was shown to suppress bone formation in post natal animals thereby proving its role in bone remodeling as well as formation (Ducy et al., 1999).

RUNX2 is essential for normal bone formation, with perturbation of bone formation if the levels are insufficient. Overproduction also affects bone formation. Osteoblasts taken from non-syndromic synostosed sutures in children exhibited an increase in Runx2 expression, possibly explaining the enhanced proliferation and bone-forming ability of these cells (Shevde et al., 2001). However, osteopenia with a decrease in bone mineral density were observed in adult transgenic mice which overexpress Runx2. This was attributed to reduce osteoblast maturation, and also to enhanced receptor activator of nuclear factor kappa  $\beta$  ligand (RANKL) and matrix metalloproteinase-13

(MMP-13) production with enhanced osteoclastogenesis (Geoffroy et al., 2002). Maturation blockage of osteoblasts was observed in neonatal transgenic mice, but no enhanced osteoclastogenesis shown (Liu et al., 2001). Runx2 is regulated partially through a negative feedback loop by activity of the RUNX2 protein on its own promoter, to control variations in gene expression and function during osteogenesis (Drissi et al., 2000). Runx2 expression decreases with age, which may be one possible explanation for impaired osteoblast function and reduced bone formation with aging (Christiansen et al., 2000).

RUNX2 is regulated by a variety of signaling pathways. Binding of ECM proteins to cell-surface integrins, mechanical loading, fibroblast growth factor 2 (FGF2), parathyroid hormone (PTH), and bone morphogenetic proteins (BMP), all influence RUNX2-dependent transcriptional activity. In many cases, Runx2 activity rather than protein levels are regulated *via* post transcriptional modification by the mitogen-activated protein kinase (MAPK) and protein kinase A and C (PKA, PKC) pathways. These pathways alter the phosphorylation state of Runx2. (Xiao et al., 1997; Xiao et al., 1998a; Xiao et al., 2000; Xiao et al., 2002a; Xiao et al., 2002c; Jiang et al., 2004). Further control of the RUNX2 molecule occurs through the counterplay of acetylation and ubiquitination (Bae and Lee, 2006). The Runx2 gene, in fact, plays a central role in coordinating multiple signaling pathways affecting osteoblast differentiation.

### 3.2.3 Bone and Osteocalcin (OCN)

The osteocalcin (OCN), an osteoblast-derived hormone (Karsenty, 2008) used as a specific bone marker, is an excellent tool since it is expressed *in vivo* in osteoblasts and their tooth counterparts, the odontoblasts, and in no other extracellular matrix-producing cells. In mouse, there are two osteocalcin-encoding genes expressed in osteoblasts and odontoblasts only, mOG1 and mOG2 (Desbois et al., 1994). They encode the same protein and display the same expression pattern, and their proximal promoter regions are 93% identical over the first 1.3 kb (Desbois et al., 1994). To understand the mechanisms leading to cell-specific OCN gene expression, studies were conducted on the promoter element of this gene in different laboratories. The results showed that a 200-bp fragment of the rat promoter and a 160-bp fragment of the mouse OG2 promoter were necessary and sufficient to confer osteoblast-specific expression to a reporter gene. Two osteoblast-specific *cis*-acting elements were characterized in this mOG2 promoter fragment (termed OSE1 and OSE2) that were able to confer osteoblast-specific activity to a heterologous promoter (Frendo et al., 1998), while OSE2 is the binding site for Runx2 (Ducy et al., 1997).

### **3.3 Runx2 and Tooth**

Runx2 expression has been reported in pre-osteogenic mesenchyme and active osteogenesis sites, as well as in developing tooth organs in the mouse (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997; D'Souza et al., 1999).

In mammalian tooth development, the cranial neural crest-derived dental mesenchyme consists of the dental papilla and dental sac, which give rise to dental pulp and odontoblasts, as well as the periodontium, including the osteoblasts that contribute to the alveolar process. The alveolar process is a specialized intramembranously formed bone, which provides the primary support structure for the dentition.

In both the dental papilla and dental sac, *Runx2* expression suggests a potential involvement of this gene in the differentiation of odontoblasts and osteoblasts lining bone in the periodontal space. *RUNX2* deficient mice exhibit an arrest of molar tooth development at the early bud/cap stage, which suggests a requirement for *RUNX2* in the progression of tooth development from the cap stage to the bell stage (D'Souza et al., 1999).

*Runx2*<sup>■/■</sup> mouse molars show arrested development at the bud/cap stage. However, the incisors, which develop earlier, progress to the bell stage and show dentine formation, although odontoblasts are abnormal and no enamel is formed (D'Souza et al., 1999; Aberg et al., 2004a; Aberg et al., 2004b). Enamel knot marker genes, such as cyclin-dependent kinase inhibitor 1A (*p21*), *Fgf4*, ectodysplasin A receptor (*Edar*) and *Bmp4*, are down-regulated in *Runx2*<sup>■/■</sup> lower molars, while expressed normally in the upper molars. Sonic Hedgehog (*Shh*) is completely absent in *Runx2*<sup>■/■</sup> lower molars, while weak signals remain at the tip of the tooth bud in the upper molars (Aberg et al., 2004b). Lower molars are more severely affected than upper molars in *Runx2*<sup>■/■</sup> mice, and incisors are less affected than molars. Therefore, *RUNX2* may have different downstream target genes in different regions of the jaw. The different origins of the

neural crest cells give rise to maxillary and mandibular structures with different characteristics and origin. Although both odontoblasts and osteoblasts are derived from mesenchyme, their developmental profiles of Runx2 expression are different, suggesting that the gene may also be differentially regulated in these cells (D'Souza et al., 1999).

Before crown development, Runx2 is strongly expressed in dental papilla mesenchyme, which gives rise to the pulpal cells and odontoblasts. Runx2 is markedly down-regulated at the bell stage in the dental papilla after morphogenesis is complete. The expression continues, although at a lower level, in the cells of the dental papilla, particularly near the apical portion, as well as in the odontoblasts lining the pulp chamber. Expression is evident throughout the further development of the tooth and at all stages of root formation, including formation of the periodontium. Cementoblasts, cementocytes, periosteal tissue, osteoblasts, and osteocytes all showed expression of Runx2. No Runx2 expression was found in osteoclasts. The forming periodontium contains a decreasing gradient of transcripts and Runx2 immunostaining from crown to the root tip (Bronckers et al., 2001).

Runx2 expression is initiated by fibroblast growth factor (FGF) produced by the odontogenic epithelium in mouse embryonic mandibular first molar tooth germs, shortly after epithelial thickening, and is followed by expression of RANKL in the early alveolar bone ossification centers and that of its receptor, receptor activator of nuclear factor kappa B (RANK) and OPG in tooth bud epithelium and mesenchyme. Thus, *Runx2* is not involved in the initiation of tooth formation, but is intimately involved in regulating the



expression of mesenchymal molecules that act reciprocally on the epithelium to control the differentiation of the enamel organ (D'Souza et al., 1999; Aberg et al., 2004b).

*Runx2* is thus essential for tooth development up to the bell stage, being necessary for the formation of the enamel knot, which controls growth and folding of the enamel organ epithelium. *Runx2* is a mesenchymally expressed gene that regulates epithelial morphogenesis. It plays an important role in the differentiation of mineralized tooth matrix-producing cells, and its expression in dental mesenchyme is stimulated by epithelial-mesenchymal interactions and by FGFs. Whether *Runx2* is essential for the later stages of tooth development is still unknown, as *Runx2*<sup>■/■</sup> mice do not survive beyond birth.

### 3.3.1 *Runx2* and Ameloblasts

The development of the tooth germ requires complicated molecular interactions that are influenced by the expression of various genes. The expression of *Runx2* is related to the development and differentiation of the tooth germ and controls downstream factors acting on the development of the enamel organ epithelium. Pre-ameloblasts show no *Runx2* mRNA expression, however, it is strongly up-regulated in maturational phase cells. *RUNX2* is present in late secretion stage and maturation stage of ameloblasts. The importance of *Runx2* in amelogenesis is evidenced by the lack of enamel in the incisor tooth germs of *Runx2*<sup>■/■</sup> mice. (D'Souza et al., 1999).

Ameloblastin (AMBN), an extracellular matrix protein, may play a role in enamel crystal formation during tooth development. AMBN is used as an ameloblast-specific gene marker. Interestingly, there are two *Runx2*-binding sites in the murine *Ambn*

promoter. Runx2 can interact with the *Ambn* promoter at these functionally important regions, and mutation of the Runx2-binding sites in *Ambn* promoter resulted in diminishing promoter activity. This indicates that Runx2 has an important function in transcription of the *Ambn* gene. The type II isoform of Runx2 mRNA remains strongly expressed in both immature and mature ameloblast, suggesting that Runx2 is involved in the early stages of enamel organ formation, and tooth morphogenesis. It may also play a direct role in formation of tooth enamel (D'Souza et al., 1999). Histologically, the study of extracted permanent teeth in CCD show evidence of hypoplasia (Fukuta et al., 2001).

### 3.3.2 Runx2 and Odontoblasts

Odontoblasts and Osteoblasts share several similarities, such as the expression of similar genes. The main non-collagenous components of the odontoblastic extracellular matrix dentin sialophosphoprotein or dentin matrix protein 1 (DSPP or DMP1) are also present in other tissues, such as osteoblasts and periodontium, although at much lower levels. The regulation of *Dspp* gene expression is dependent on the state of differentiation of the target cell (Chen et al., 2005a).

RUNX2 is also involved in the regulation of DMP1 in osteoblasts, although it is not essential for DMP1 expression in odontoblasts, which indicates the involvement of other unidentified odontoblast-specific transcription factors or co-activators (Narayanan et al., 2001).

Proliferation and differentiation in *Runx2*<sup>+/-</sup> human primary pulp cells are different to wild-type cells as a result of variations in gene expression patterns and signaling. Thus, haploinsufficiency may well influence the differentiation of odontoblasts

from these cells. Runx2 is up-regulated in early odontoblasts, showing that levels of RUNX2 are necessary at this stage. However, unlike in osteoblasts, Runx2 expression is significantly reduced or undetectable in differentiated odontoblasts (Bronckers et al., 2001). This down-regulation of expression in newly differentiated and functional odontoblasts suggests that *Runx2* plays an essential and stage-specific role in the lineage determination and terminal differentiation of odontoblasts from dental papilla mesenchyme. It also elucidates the different effects of this gene in different tissues.

### 3.3.3 Runx2 and Cementoblasts

Cementoblasts share many characteristics with osteoblasts. It is unclear whether osteoblasts and cementoblasts arise from a common precursor cell (Bosshardt, 2005). Cementoblasts express Runx2 mRNA and protein at different levels. Recently, immunolocalization patterns of Runx2 were examined during rat molar tooth formation. Colocalization of Runx2 was detected in cementoblasts, which penetrated the ruptured Hertwig's epithelial root sheath and attached to root dentin. Moreover, osteopontin was observed in Runx2-positive cementoblasts facing the root surface. However, the cells adjacent to cementoblasts showed only Runx2 reactivity. No Runx2 was seen in cementocytes. These results suggest that Runx2 is important for differentiation into cementoblasts (Hirata et al., 2009).

Variations in cementum formation have been observed in CCD. Both acellular and cellular cementum formation is defective in permanent teeth in CCD, but not in deciduous teeth. Runx2<sup>+/-</sup> showed inconsistent result as well (Camilleri and McDonald, 2006).

### 3.3.4 Runx2 and Periodontal Ligament

Runx2 is also expressed in periodontal ligament (PDL) fibroblasts, although BSP, a marker of osteoblast differentiation is not and biomineralization is not seen. Periodontal ligament cells and osteoblasts located on the alveolar bone surface showed immunoreactivity for Runx2 (Hirata et al., 2009). RUNX2 is suppressed by a mechanism designed to maintain PDL width by S100A4, a member of the S100 calcium-binding protein family. S100A4 is synthesized and secreted by PDL cells, and may act as an inhibitor of mineralization (Kato et al., 2005). However, the PDL cells retained the potential to differentiate to osteoblasts under certain conditions, such as mechanical stress. Deformation of PDL osteoblasts increases Runx2 expression, protein levels, and also its DNA-binding activity. These changes may be initiated by deformation-dependent activation of the ERK (extracellular signal related kinase) MAPK pathway. However, the PDL response to stress did not show difference between heterozygous and wild-type mice (Ziros et al., 2002).

### 3.3.5 Runx2 and Tooth Maturation and Eruption

Dental follicle is a loose connective tissue sac that surrounds the unerupted tooth. It differentiates into the periodontal ligament, and may be the precursor of other cells of periodontium, including osteoblasts and cementoblasts, and plays different roles at different times in the life of a tooth (Yao et al., 2008). It initiates eruption by regulating alveolar bone resorption and formation. Dental follicle is required for eruption (Cahill and Marks, 1980; Marks and Cahill, 1984). During alveolar and root growth, the microenvironment is altered within the superior aspects of the crypt. Consequently, the

activation of transcription factors, cytokines, and growth factors leads to the recruitment and activation of osteoclasts by the dental follicle. The presence of osteoblasts is required for the activation of osteoclasts via the RANKL/OPG pathway. Evidence supports a role for Runx2 in molecular events that regulate tooth eruption (Wise et al., 2002).

Different teeth appear to be regulated by different molecules and mechanisms. Epidermal growth factor (EGF) accelerates rodent incisor eruption, but has little effect on the molar (Lin et al., 1992), whereas colony-stimulating factor-1 (CSF-1) accelerates rat molar eruption, but not incisor eruption (Cielinski et al., 1995; Wise et al., 2005). Dexamethasone accelerates incisor eruption, but not molar eruption (Wise et al., 2001).

In recent years, considerable progress has been made in understanding the biology of tooth eruption. Studies have shown that interactions among osteoblasts, osteoclasts, and dental follicle involve a complex interplay of regulatory genes that encode various transcription factors, proto-oncogenes, and growth factors. For the clinician faced with treating both simple and complex dental complications that arise from abnormal tooth eruption, as seen in numerous genetic and acquired disorders, knowledge about the basic molecular mechanisms involved is essential.

### 3.3.6 Runx2 and Cleidocranial Dysplasia (CCD)

Humans with mutations in RUNX2 present with cleidocranial dysplasia (CCD), an autosomal dominant disorder defined by several skeletal and dental defects. The Runx2 gene is located on the short arm of chromosome 6 in human. Linkage studies and mutational analyses have identified loss-of-function mutations in one allele of Runx2 as responsible for the defects seen in human CCD (Mundlos et al., 1997). Other individuals

affected with CCD are heterozygous for the deletion, frameshift mutation, nonsense mutation, missense mutation, splicing mutation and substitution mutation in the coding region of Runx2 (Yoshida et al., 2003). Thus, the lack of expression of one allele of Runx2 is the basis for the CCD in humans and mice.

Heterozygous mutations in Runx2 in mice are associated with skeletal defects, supernumerary teeth, and delayed eruption (Aberg et al., 2004a). Other dental defects include: irregular and compressed permanent tooth crowns, hypoplastic and hypomineralization defects in enamel and dentin, an excess of epithelial root remnants, the absence of cellular cementum, and abnormally shaped roots (Jensen and Kreiborg, 1993). This phenotypic change is not evident in every adult Runx2<sup>+/-</sup> mouse studied and suggests an effect of incomplete penetrance.

Runx2 controls the maturation of both osteoblasts and odontoblasts. Therefore, a delay in tooth maturation is expected in RUNX2-deficient tissues. This is reflected in the clinical situation, where the dental maturation of CCD subjects is retarded when compared with unaffected subjects (Jensen and Kreiborg, 1993).

### **3.4 Runx2 and mitogen-activated protein kinase (MAPK)**

The mitogen-activated protein kinase (MAPK) pathway plays an important role in osteoblast growth and differentiation by regulation of the transcription factor, Runx2 (Xiao et al., 2000). ERK/MAPK pathway can also regulate the activity of several other tissue/lineage-specific transcription factors, such as MyoD (muscle; (Zetser et al., 2001)), Sox9 (cartilage; (Murakami et al., 2000)), as well as PPAR $\gamma$  (adipose tissue; (Adams et al., 1997a)). In bone, the ERK/MAPK pathway is a major pathway for signaling

transduction from the extracellular environment to the nucleus, and has been implicated in the response to a variety of signals, including hormone/growth factor stimulation (Xiao et al., 2002c; Jiang et al., 2004), extracellular matrix–integrin binding (Xiao et al., 1997; Xiao et al., 2002a), mechanical loading and ECM stiffness (You et al., 2001; Engler et al., 2006).

Osteoblast differentiation requires a type I collagen-containing extracellular matrix (ECM), which is secreted by osteoblast precursors, to form mineralized tissue. Production of ECM is followed by induction of osteoblast-related genes, such as OCN, BSP, alkaline phosphatase (ALK), parathyroid hormone (PTH)/parathyroid hormone-related protein receptor, and ultimately, matrix mineralization (Franceschi and Iyer, 1992; Franceschi et al., 1994; Franceschi, 1999; Xiao et al., 2002c). Differentiation is controlled partially by binding of type I collagen to  $\alpha 2\beta 1$  integrins that transduce a signal to the nucleus *via* the ERK/MAPK pathway to phosphorylate and activate RUNX2. Blocking antibodies or peptides that disrupt the integrin signaling pathway can block ECM-dependent osteoblast differentiation completely (Xiao et al., 1998a).

Induction of MC3T3-E1 preosteoblast cell differentiation by growth in ascorbic acid, results the secretion of a collagenous ECM. Over time, this ECM production dramatically increases both OCN and BSP gene expression. This matrix response requires Runx2 and its DNA binding site in the OCN promoter, osteoblast-specific element 2 (OSE2) (Xiao et al., 1997). Interestingly, this Runx2-dependent increase in transcriptional activity is not accompanied by a significant change in Runx2 mRNA or protein levels although *in vitro* remarked increases are seen in binding of Runx2 to OSE2 DNA as measured by gel retardation assays (Xiao et al., 1998a). U0126, a specific

ERK1/2 phosphorylation inhibitor, rapidly and specifically inhibits both ERK phosphorylation and ECM-dependent induction of the OCN gene expression. Similarly, the ability of BMP to stimulate OCN and BSP mRNAs or OCN promoter activity was synergistically increased in osteoblasts, and these responses also require matrix signals and can be blocked by U0126 (Xiao et al., 2002a).

Overexpression of a constitutively active MEK (MEK(SP)) increases both endogenous OCN mRNA and promoter activity levels, and these responses require Runx2 and an intact OSE2 sequence. Runx2 phosphorylation increased after transfection of cells with MEK(SP), the kinase immediately before ERK1/2 in the MAPK pathway (Xiao et al., 2000). This demonstrates that a MAPK-dependent phosphorylation cascade regulates Runx2 activity.

A transgenic approach was applied to investigate the role of the MAPK pathway in bone. Transgenic mice were generated using a 647-bp mouse osteocalcin (*Ocn*) gene 2 (mOG2) promoter to drive osteoblast-specific expression of constitutively active (MEK-SP) or dominant-negative (MEK-DN) forms of the MAPK intermediate MEK. MAPK stimulation induced by selective expression of MEK(SP) in osteoblasts accelerated *in vitro* differentiation of calvarial cells, as well as *in vivo* bone development; whereas MEK(DN) was inhibitory. Transgenic mice over-expressing MEK(SP) or MEK(DN) were studied in greater detail: (a) Runx2 phosphorylation and transcriptional activity were increased in calvarial osteoblasts from mice over-expressing MEK(SP) and decreased in cells from MEK(DN) over-expressing mice, and (b) the CCD phenotype (hypomorphic clavicles and undemineralized calvaria) of Runx2<sup>+/-</sup> mice was partially rescued by crossing these animals with mice over-expressing MEK(SP); in contrast, when



MEK(DN) animals were crossed with *Runx2*<sup>+/-</sup> mice, a more severe skeletal phenotype were seen. This work demonstrates that the ERK/MAPK pathway has an important *in vivo* function in bone that involves stimulation of RUNX2 phosphorylation and transcriptional activity (Ge et al., 2007a), and provides a clinically relevant *in vitro* and *in vivo* model for the analysis of potential therapeutic modalities involving manipulation of MAPK signaling for the treatment of CCD and other bone or dental diseases.

Runx2 is expressed exclusively in mineralized tissues and their precursors. However, in many cases there is a poor correlation between actual Runx2 mRNA or protein levels and the expression of osteoblast-related genes. During development, Runx2 expression precedes osteoblast differentiation and OCN expression by several days (Ducy et al., 1997). Also, in several osteoblast cell culture systems, Runx2 protein levels are not well correlated with expression of its target genes. For example, cultures of primary osteoblasts or MC3T3-E1 preosteoblast cells do not exhibit major changes in Runx2 protein during *in vitro* differentiation even though expression of osteoblast marker genes like OCN, BSP and ALK is dramatically increased (Xiao et al., 1998a). In addition, although both TGF- $\beta$  and BMP2 dramatically increase Runx2 expression in C2C12 myoblasts, only BMP treatment can induce osteoblast-specific gene expression (Lee et al., 2000). Therefore, these studies indicate that Runx2-dependent transcription is not simply regulated by the level of Runx2 protein. These studies imply that Runx2 is regulated either by post-translational modification, especially phosphorylation, or by protein-protein interactions.

The Franceschi group further showed that the specific amino acid residues in Runx2 necessary for MAPK responsiveness and phosphorylation are located in a 270

amino acid proline/serine/threonine-rich region (P/S/T domain) in the C-terminal portion of the Runx2 molecule(Xiao et al., 2000; Franceschi et al., 2003).

### **3.5 Runx2 and fibroblast growth factor-2 (FGF2)**

Fibroblast growth factor-2 (FGF2) also stimulates Runx2 phosphorylation through the MAPK pathway (Xiao et al., 2002c). FGF2 is an important *in vivo* regulator of skeletal development and growth. Intermittent administration of FGF2 can restore bone mass in the ovariectomized rat, a well-established model for postmenopausal bone loss (Liang et al., 1999). Overexpression of FGF2 in transgenic mice causes premature mineralization, achondroplasia and shortening of long bones, whereas disruption of the FGF2 gene leads to decreased bone mass and bone formation (Coffin et al., 1995; Montero et al., 2000). Furthermore, activating mutations in FGF receptors are associated with a series of craniosynostosis syndromes characterized by accelerated intramembranous bone formation in calvarial sutures. Interestingly, activating mutations in FGFR1 increase Runx2 gene expression and enhance differentiation of calvarial osteoblasts (Zhou et al., 2000). FGF2 can also stimulate OCN gene expression in MC3T3-E1 preosteoblast cells (Boudreaux and Towler, 1996).

A major pathway for FGF receptor signaling involves activation of the MEK/ERK branch of the MAPK pathway. A series of studies were carried out to determine whether FGF2 induction of the OCN gene required MAPK activity and Runx2 phosphorylation (Xiao et al., 2002c). Initial studies demonstrated that FGF2 rapidly induced ERK phosphorylation and stimulated OCN mRNA expression in MC3T3-E1 cells and in bone marrow stromal cells. FGF2 also stimulated the activity of a 1.3 kb

OCN promoter-luciferase reporter gene, whereas this stimulation could be blocked by the MEK/ERK inhibitor, U0126. This stimulation was only seen in cells containing wild type Runx2 and also required an intact Runx2 binding site in the OCN promoter. A P/S/T domain deletion Runx2 mutant did not respond to FGF2. Furthermore, metabolic labeling with [<sup>32</sup>P]-orthophosphate showed that the Runx2 phosphorylation level was increased with FGF2 treatment and that this response could also be blocked by U0126.

Taken together, since Runx2 is involved in bone development, tooth development, as well as tooth movement (Baumert et al., 2004; Chung et al., 2004; Kawarizadeh et al., 2005; Wise and King, 2008), it is important to understand the mechanisms that regulate its activity.

## CHAPTER II

## 1. ABSTRACT

The Mitogen-activated protein kinase (MAPK) pathway plays an important role in osteoblast growth and differentiation. Short-term inhibition of MAPK activity in cell culture using specific inhibitors or MEK(DN), a dominant negative form of MEK, inhibited osteoblast-specific gene expression and responsiveness to BMPs. Activation of the MAPK pathway with MEK(SP), a constitutively active form of MEK, stimulated phosphorylation and transcriptional activity of Runx2. To identify and characterize MAPK-activating domain(s) in Runx2, Runx2 deletion mutants were made and tested for ability to be activated by MAPK in COS-7 cells by transfection. *In vitro* phosphorylation of Runx2 was also examined. The results show deletion of Runx2 from aa528 to aa330 gradually reduced MAPK-stimulated activity. Runx2 (1-330), which contains the first N-terminal 330 amino acid residues, could still be dramatically activated by MAPK. Further deletion from aa330 to aa258 completely abolished MAPK activation indicating that a critical MAPK activating domain is located in this region. Searching the Runx2 protein sequences in this 72-aa region identified four consensus MAPK phosphorylation sites (SP or TP) located at 282(S), 301(S), 319(S), and 326(T), respectively. To determine if these sites can be directly phosphorylated by MAPK, five peptides (Table 1 & Fig. 10A) covering the entire aa330-258 region were synthesized and tested for ability to be directly phosphorylated by purified activated ERK1 *in vitro* in the presence of  $\gamma$ -<sup>32</sup>P-ATP. ERK1 strongly phosphorylated peptides 2, 3, and 4, each of which contains at least one consensus MAPK phosphorylation site, but not peptide 1, which does not contain a consensus site. ERK1 was unable to phosphorylate the same peptides (2, 3, and 4) containing a mutation (from S to A) in the MAPK phosphorylation site. Functional

analysis established the involvement of these three sites in MAPK activation of Runx2. Conclusion: Sites 282(S), 301(S), 319(S) of the Runx2 molecule are required for MAPK activation of Runx2.

## 2. INTRODUCTION

Runx2, a transcription factor related to the *Drosophila* transcription factor, *runt*, directly stimulates transcription of osteocalcin and other osteoblast-related genes in osteoblasts, tooth germs (cells such as odontoblasts, ameloblasts, cementoblasts), and some stages of cartilage via binding to OSE2 core sequences (PuCCPuCA/T). Runx2 is essential for differentiation of mesenchymal cells into osteoblasts and for mineralization *in vivo*. Heterozygous Runx2 deletions or mutations result in Cleidocranial Dysplasia (defects in skeletal and tooth development, hypoplastic clavicles, enlarged fontanelles). Runx2 is a major regulator of osteoblast-specific gene expression, and is subject to post-transcriptional controls including selective proteolysis and phosphorylation. The Mitogen-activated protein kinase (MAPK) pathway plays an important role in osteoblast growth and differentiation. Short-term inhibition of MAPK activity in cell culture using specific inhibitors or MEK(DN), a dominant negative form of MEK, inhibited osteoblast-specific gene expression and responsiveness to BMPs. Activation of the MAPK pathway with MEK(SP), a constitutively active form of MEK, stimulated phosphorylation and transcriptional activity of Runx2. Thus, Runx2 is phosphorylated and activated by the ERK/MAPK pathway (Xiao, Jiang *et al.* 2000, 2002). FGF2 bind to FGFRs to induce receptor dimerization and subsequent transphosphorylation, which initiates intracellular signaling cascades, including activation of MAPK and PKC pathways. A major route for

FGF signaling is through the MAPK pathway. This pathway is important for activation and phosphorylation of Runx2.

ERK/MAPK signaling is also important for *in vivo* bone development. Transgenic over-expression of constitutively active or dominant-negative MEK1 in mouse osteoblasts, respectively, stimulates or inhibits Runx2 phosphorylation and skeletal maturation. Furthermore, the cleidocranial dysplasia phenotype of Runx2 heterozygous null mice can be partially rescued by crossing these animals with mice expressing constitutively-active MEK1, consistent with the *in vivo* actions of the ERK/MAPK pathway being at least in part mediated by Runx2 (Ge, Xiao,2007).

To further understand how the ERK/MAPK pathway regulates Runx2 transcriptional activity, in the present study we identify amino acid residues in Runx2 that are phosphorylated in a ERK/MAPK-dependent manner and show that these sites are necessary for osteoblast-specific gene expression and differentiation.

### 3. MATERIALS AND METHODS

#### A. Reagents

The reagents used in the experiments were obtained from the following sources: Tissue culture media Minimum Essential Medium (Alpha-MEM), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, LipofectAMINE from Invitrogen (Grand Island, NY); U0126 from Promega (Madison, WI); Mouse anti-Runx2 antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and MBL International Corporation (Woburn, MA). Horseradish peroxidase-conjugated goat anti-rabbit IgG from Invitrogen, and FGF2 from Upstate Biotechnology (Lake Placid, NY).  $\gamma$ -[<sup>32</sup>P]-ATP was purchased from Amersham Bioscience (Arlington Heights, IL). Runx2 peptides were synthesized by the core facility of the University of Michigan. [<sup>35</sup>S]-methionine/cysteine translabel from Amersham Bioscience (Arlington Heights, IL).

All other chemicals were analytical grade.

#### B. Cell Culture

COS7 cells were cultured in DMEM, 10% FBS, 1% penicillin/Streptomycin. Cells were cultured in a humidified CO<sub>2</sub> incubator at 37°C. Cell culture medium was changed every other day. When cells were confluent, they were detached from 75 cm<sup>2</sup> flasks with Trypsin (Invitrogen), centrifuged, and passed at a proportion of 1:10.

#### C. DNA constructions and plasmid expression vectors

To generate a luciferase reporter plasmid, multimers (six copies) of OSE2 (Osteoblast-specific *cis*-acting element) oligonucleotide were cloned into p4luc



promoterless luciferase expression vector (6OSE2-Luc) described previously (Ducy and Karsenty, 1995; Frendo et al., 1998; Xiao et al., 2002c). pCMVRunx2 expression plasmids (wild type, and C-terminal deletions aa1-410, aa1-330, aa1-286, and aa1-258) containing cDNAs encoding either wild type Runx2 or deletions under CMV promoter control were constructed by ligating the full-length or deletion Runx2 cDNAs into pCMC5 described previously (Xiao et al., 2005). Serine mutants of Runx2 or Runx2 deletions were generated using a Quick Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All sequences were verified by automatic DNA sequencing. pCMV5MEK(SP) expression plasmid encoding a constitutively-active mutant of MAPK/extracellular signal-regulated kinase (MEK1) was previously described (Zheng and Guan, 1993; Ducy and Karsenty, 1995; Xiao et al., 2000). The pCMV5b-gal expression plasmid, used as a negative control for Runx2 overexpression experiments, was constructed by ligating a full-length bacterial  $\beta$ -galactosidase cDNA from plasmid pRSVb-gal into the *HindIII/BamHI* sites of the pCMV5 vector.

#### D. Transfections

COS7 cells were plated at a density of  $2.5 \times 10^5$  cells/cm<sup>2</sup> on 35-mm dishes. After 24 hours, cells were transfected using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. Each transfection contained 0.5 $\mu$ g of the indicated plasmid and 0.05  $\mu$ g of pRL-SV40 containing a cDNA for *Renilla reformis* luciferase as an internal control for transfection efficiency. Cells were then cultured in growth medium for 24 hours and then switched to medium containing 0.1% FBS to minimize the impact of serum growth factors on MAPK signaling. After an additional 24 hours, cells were

harvested and assayed using a Dual Luciferase Assay Kit (Promega, Madison, WI) on a Monolight 2010 luminometer (BD Biosciences Pharmingen, San Diego, CA). Firefly luciferase activity was normalized to *Renilla* luciferase activity. For FGF2 experiments, after transfection, cells were cultured in growth medium for 24 hours and then switched to medium containing 0.1% FBS in the absence or presence of FGF2 at concentration of 25 ng/ml for another 12 hours, then harvested and assayed by the same procedure described above.

#### E. Western Blot Analysis

Whole cell extracts were prepared by rinsing cells layers in phosphate-buffered saline containing 100 ul of Sigma protease inhibitor cocktail / $10^7$  cells. Inhibitor cocktail contained 4-(2-amino-ethyl)-benzenesulfonyl fluoride, pepstatin A, trans-epoxysuccinyl-L-leu-cylamido (4-guanidino) butane, bestatin, leupeptin, and aprotinin and 1.0 mM phenylmethylsulfonyl fluoride. Cells were then harvested in 1 X SDS – polyacrylamide gel electrophoresis loading buffer (2% SDS, 2M urea, 10mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, 10% glycerol, 10 mM Tris-HCl, 0.002% Bromphenol blue). Failure to use a combination of protease inhibitors led to the generation of proteolytic fragments of Runx2. Samples were separated on 4-12% SDS-PAGE gel, and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuel, Keene, NH). Primary Runx2 antibodies were used at a dilution of 1:1,000. Secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) was used at a dilution of 1:10,000. Immunoreactivity was detected using the ECL chemiluminescence reagents (GE Healthcare, piscataway, NJ).

## F. Metabolic Labeling and Immunoprecipitation of Runx2

COS7 cells were transfected with Runx2 expression plasmids, cultured for 30 h and preincubated in phosphate-free DMEM, 0.1% FBS for 12 h. Labeling was conducted for 4 h in phosphate free DMEM containing 200  $\mu\text{Ci/mL}$  [ $^{32}\text{P}$ ] orthophosphate (Phosphorus-32, Amersham Bioscience) or [ $^{35}\text{S}$ ]-methionine/cysteine translabel respectively. Nuclear extracts were prepared as previously described (Xiao et al., 1997) and precleaned twice with 50  $\mu\text{l}$  of protein A/G agarose beads. Appropriate antibodies were added and incubated for 2 h at 4  $^{\circ}\text{C}$  with gentle rocking. Immune complexes were then collected by the addition of 30  $\mu\text{L}$  of protein A/G agarose beads and incubation for 1 h at 4  $^{\circ}\text{C}$  followed by centrifugation. Precipitates were washed five times with 1 x washing buffer (20 mM HEPES, pH7.6, 50 mM KCl, 1 Mm dithiothreitol, 0.25% Nonidet P40, 5 mM NaF, 1 mM EGTA, 5 mM  $\text{MgCl}_2$ , 0.25 mM phenylmethylsulfonyl fluoride). The immunoprecipitated complexes were suspended in SDS sample buffer and analyzed by SDS-PAGE and autoradiography or Western blot analysis using the indicated antibodies.  $^{32}\text{P}$  incorporation was measured using a Packard A2024 InstantImager.

## G. *In vitro* peptide phosphorylation

Synthetic peptides were prepared by the University of Michigan Protein Structure Facility. Each peptide (1  $\mu\text{g}$ ) was incubated with 20  $\mu\text{Ci}$   $\gamma$ -[ $^{32}\text{P}$ ] ATP and 0.5  $\mu\text{l}$  (300 units) of recombinant activated MAPK (Calbiochem) in a buffer containing 25 mM Tris-HCl, pH 7.4, 10 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, 40  $\mu\text{M}$  ATP and 0.5 mM EGTA in a

final volume of 25  $\mu$ l. Samples were incubated at 25°C for 30 minutes. Reactions were terminated by addition of 25  $\mu$ l 2X SDS sample buffer and boiling samples, and analyzed by electrophoresis on 15% SDS gels.

#### H. Statistical Analysis

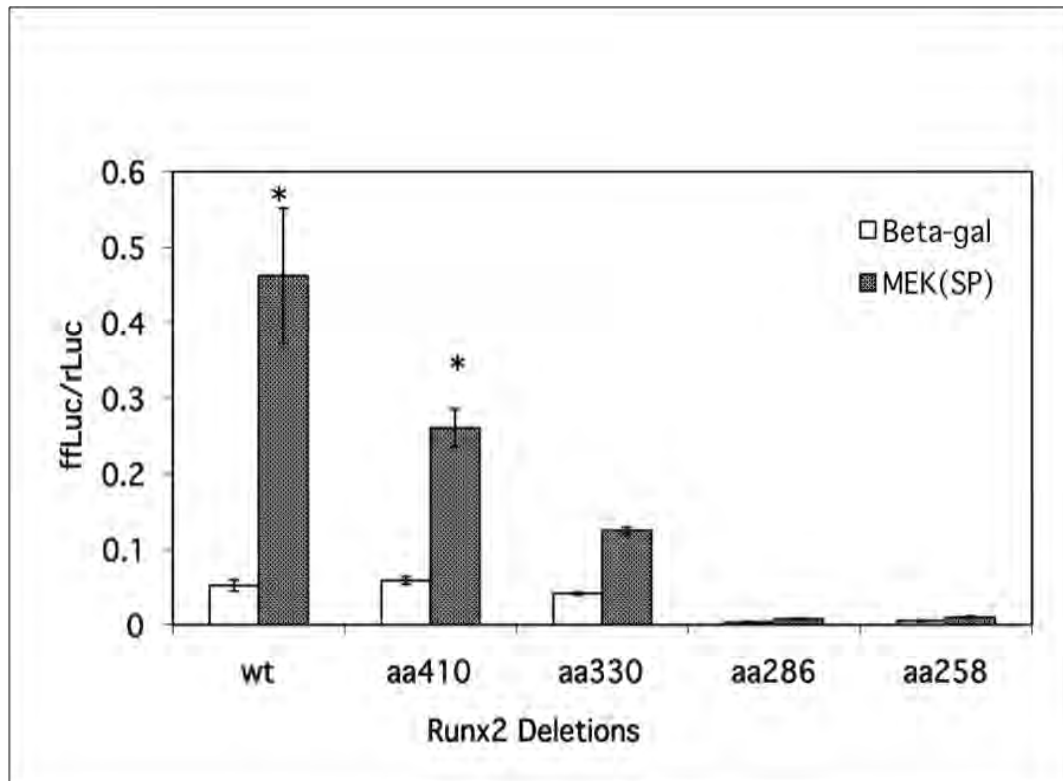
All transfection data are reported as mean  $\pm$  SD based on triplicate independent cell cultures from a representative experiment. All experiments were repeated at least twice, and qualitatively identical results were obtained. Statistical analyses were performed using InStat 3.0 software (GraphPad, Inc., San Diego, CA). A one-way ANOVA analysis was performed. Student's *t* test was used to test for differences between two groups of data. Turkey-Kramer multiple comparisons test was also used to assess statistical significance between samples. Differences with a  $P < 0.05$  were considered as statistically significant.

## 4. RESULTS

*A. ERK/MAPK-dependent phosphorylation and activation of Runx2 requires a specific region of the C-terminal P/S/T domain.* Activation of the ERK/MAPK pathway by overexpression of a constitutively active form of MEK1 or by treatment with FGF2 was previously shown to stimulate *Ocn* mRNA expression and promoter activity via a mechanism requiring Runx2 (Xiao et al., 2000; Xiao et al., 2002b). An initial deletion analysis of the Runx2 coding sequence showed that removal of the entire C-terminal proline/serine/threonine-rich (P/S/T) domain (amino acid residues 258-528 in the mouse sequence) rendered Runx2 completely resistant to MAPK regulation and phosphorylation. In contrast, deletion of the N-terminal Q/A-rich region (amino acids 1-108) lowered basal transcriptional activity without affecting MAPK-dependent activation (Xiao et al., 2000). Similarly, deletion of the P/S/T domain also rendered Runx2 unresponsive to activation by FGF2 (Xiao et al.).

To more precisely define regions of Runx2 necessary for MAPK responsiveness, we carried out a more detailed deletion analysis of the P/S/T domain (Fig. 3, 4, 5, and 6). Wild type Runx2 or several C-terminal deletions (to residues 410, 330, 286 and 258) were transfected into COS7 cells in the presence or absence of constitutively active MEK1 (MEK(SP)) and a p6OSE2-luc Runx2 reporter gene. Samples were then assayed for luciferase activity (Fig. 3). C-terminal Runx2 deletions gradually reduced MAPK activation of Runx2 transcriptional activity from approximately 9-fold with wild type Runx2 to approximately 4.4-fold with the amino acid 410 deletion, 3-fold with the amino acid 330 deletion, and 2.3-fold with the amino acid 286 deletion. This stimulation

gradually decreased with the 410, 330 and 286 deletions and was completely lost after deletion to residue 258 (Fig.3).



*Fig. 3. Identification of Regions in Runx2 necessary for ERK/MAPK-dependent transcriptional activation and phosphorylation. COS7 cells were transfected with wild type Runx2 or the indicated C-terminal deletions in the presence of control  $\beta$ -gal or MEK(SP) expression vectors and a 6OSE2-luc reporter as described in Methods. Firefly luciferase activity was normalized for transfection efficiency using renilla reformis luciferase plasmid. For each group, fold-stimulation in MEK(SP) samples was determined. \*, Ratio (MEK(SP)/Beta-gal) is significantly different at  $P < 0.001$ .*

Wild type Runx2 or C-terminal deletions (to residues 410, 330, 286 and 258) were transfected into COS7 cells and a p6OSE2-luc Runx2 reporter gene, then treated in the presence or absence of FGF2, a natural MAPK activator. Similar to the result obtained with MEK(SP) stimulation, C-terminal Runx2 deletions gradually reduced MAPK activation of Runx2 transcriptional activity from approximately 6.74-fold with wild type Runx2 and the amino acid 410 deletion, 3-fold with the amino acid 330 deletion, and 1.7-fold with the amino acid 286 deletion. This stimulation gradually decreased in the 410, 330 and 286 deletions and was completely lost after deletion to residue 258 (Fig. 4).

In addition, wild type Runx2 and increasing amounts of P/S/T domain deletion Runx2 were transfected into COS7 cells in the presence or absence of constitutively active MEK1 (MEK(SP)) and a p6OSE2-luc Runx2 reporter gene. Samples were then assayed for luciferase activity. The P/S/T domain deleted Runx2 inhibited MAPK activation of wild type Runx2 in a dominant negative manner (Fig. 5).

Furthermore, wild type Runx2 and P/S/T domain deletion Runx2 transfected COS7 cells in the presence or absence of constitutively active MEK1 (MEK(SP)) were metabolically labeled with [<sup>32</sup>P]-orthophosphate or [<sup>35</sup>S]-methionine/cysteine translabel and assayed for Runx2 phosphorylation by immunoprecipitation and autoradiography (Fig. 6). The results showed 3.5-fold increase of <sup>32</sup>P incorporation by MEK(SP) stimulation in wild type Runx2, whereas no stimulation by MEK(SP) was seen in P/S/T domain deletion Runx2. There was no significant change in total <sup>35</sup>S incorporation, which indicated the protein level was consistent.

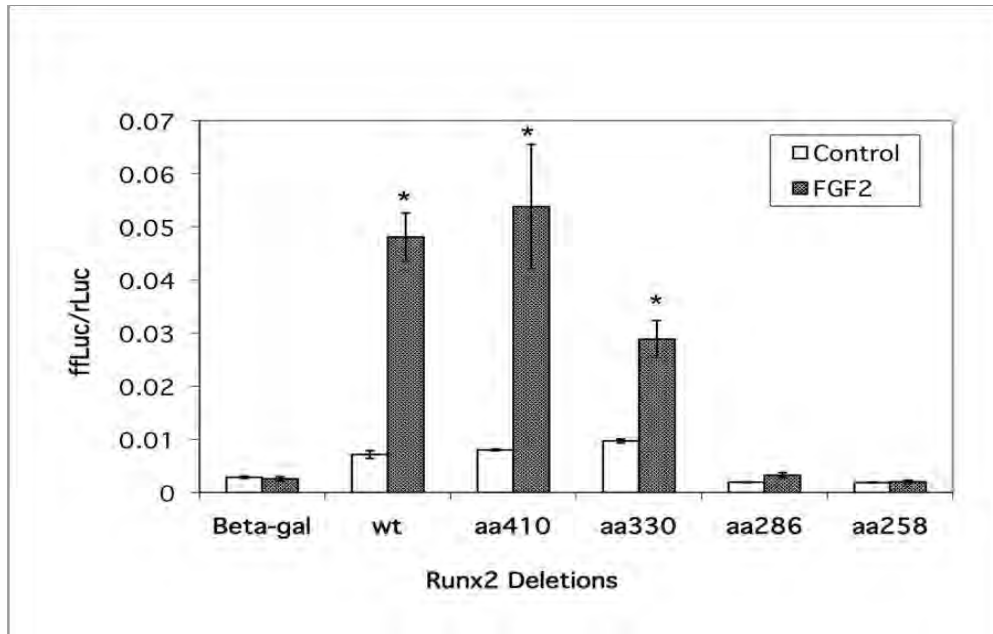
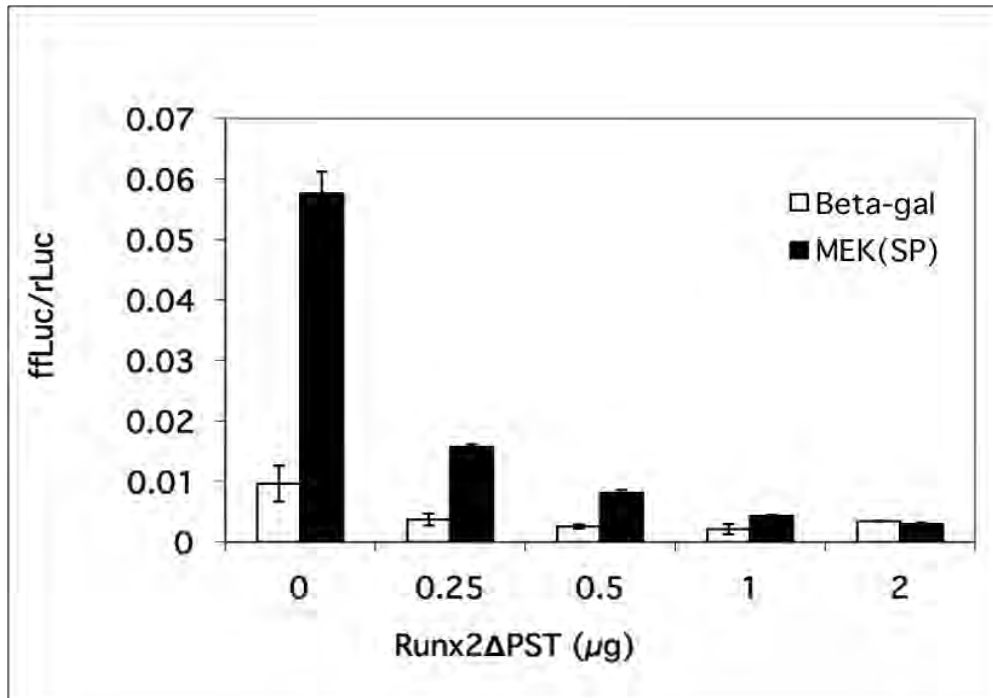


Fig. 4. Localization of an ERK/FGF2 responsive region. COS7 were transfected with the indicated C-terminal Runx2 deletion mutants and 6OSE2-luc. After transfection, cells were cultured in growth medium for 24 hours and then switched to medium containing 0.1% FBS in the absent or presence of FGF2 at a concentration of 25 ng/ml for another 12 hours, then harvested and assayed. Firefly luciferase activity was normalized for transfection efficiency using renilla reformis luciferase plasmid. For each group fold-stimulation in FGF2 treated samples was determined. \*, Ratio (FGF2/Control) is significantly different at  $P < 0.001$ .





*Fig. 5. The P/S/T domain of Runx2 is required for MAPK activation and phosphorylation. The P/S/T domain deleted Runx2 inhibits MAPK activation of wild type Runx2 in a dominant negative manner.*

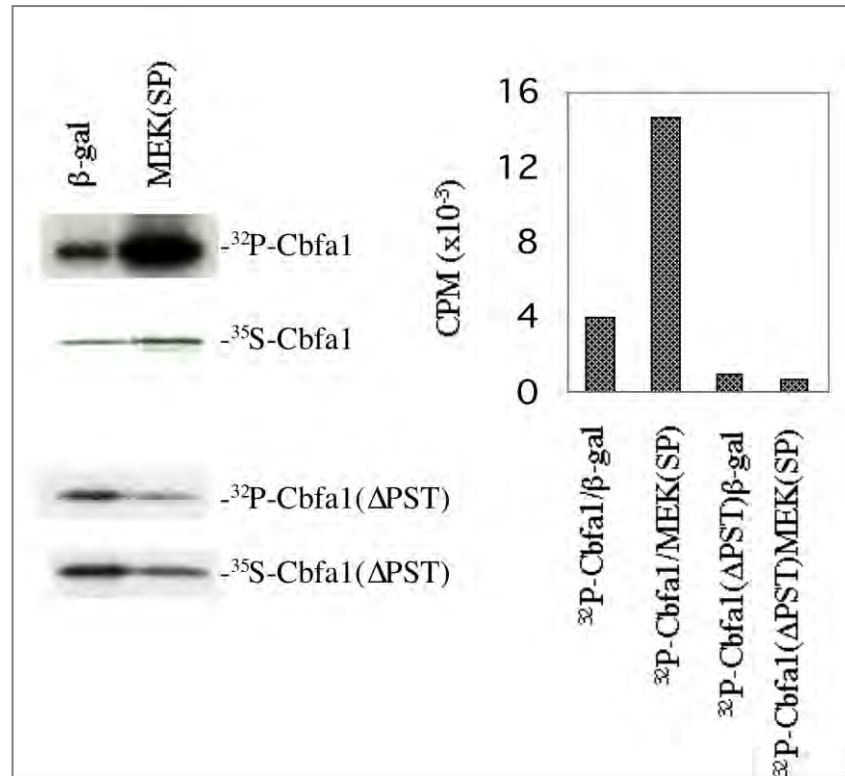


Fig. 6. The P/S/T domain of Runx2 is required for MAPK activation and phosphorylation. Cos7 cells were transfected with Runx2 expression plasmids, cultured for 30 h and preincubated in phosphate-free DMEM, 0.1% FBS for 12 h. Labeling was conducted for 4 h in phosphate free DMEM or sulfate free DMEM containing 200  $\mu\text{Ci/mL}$  [ $^{32}\text{P}$ ] orthophosphate (Phosphorus-32, Amersham Bioscience) or [ $^{35}\text{S}$ ]-methionine/cysteine translabel, respectively. Nuclear extracts were prepared. The immunoprecipitated complexes were suspended in SDS sample buffer and analyzed by SDS-PAGE and autoradiography or Western blot analysis using indicated antibodies.  $^{32}\text{P}$  incorporation was measured using a Packard A2024 InstantImager.

Taken together, these results indicate that the minimal region for MAPK phosphorylation and activation of Runx2 is between amino acids 258 and 330. Subsequent analysis was restricted to this region although it is possible that more C-terminal sites may also participate in this regulation.

*B. Identification and evaluation of Runx2 phosphorylation sites.* Inspection of the peptide sequence of the 72 amino acid region in the Runx2 molecule between 258 to 330 region (Fig. 10A) identified three putative proline-directed serine phosphorylation sites at residues 282, 301 and 319. A similar proline-directed threonine site was also seen (T326). To further characterize these consensus MAPK phosphorylation sites, Runx2 mutants were generated by introducing single point mutations with substitution of Serine to Alanine at S282, 301, or 319 sites (S282A, S301A, S319A), and double-point mutation at S301 and 319 sites (S301/319A), and triple-point mutation at all three of these sites (S282/301/319A) into full-length of Runx2. Wild type Runx2 or Runx2 point mutation expression plasmids were transfected into COS7 cells in the presence or absence of (MEK(SP)) and a p6OSE2-luc Runx2 reporter gene. Samples were then assayed for luciferase activity. As shown in Fig. 7, the ability of MEK(SP) to stimulate transcriptional activity of single point mutation in individual S282A, or S301A, or S319 mutants was not significantly changed compared with wild type Runx2. However, the ability of MEK(SP) to stimulate transcriptional activity in double-point mutation (S310/319A) decreased dramatically, and was completely lost in triple-point mutation (S282/310/319A) Runx2.

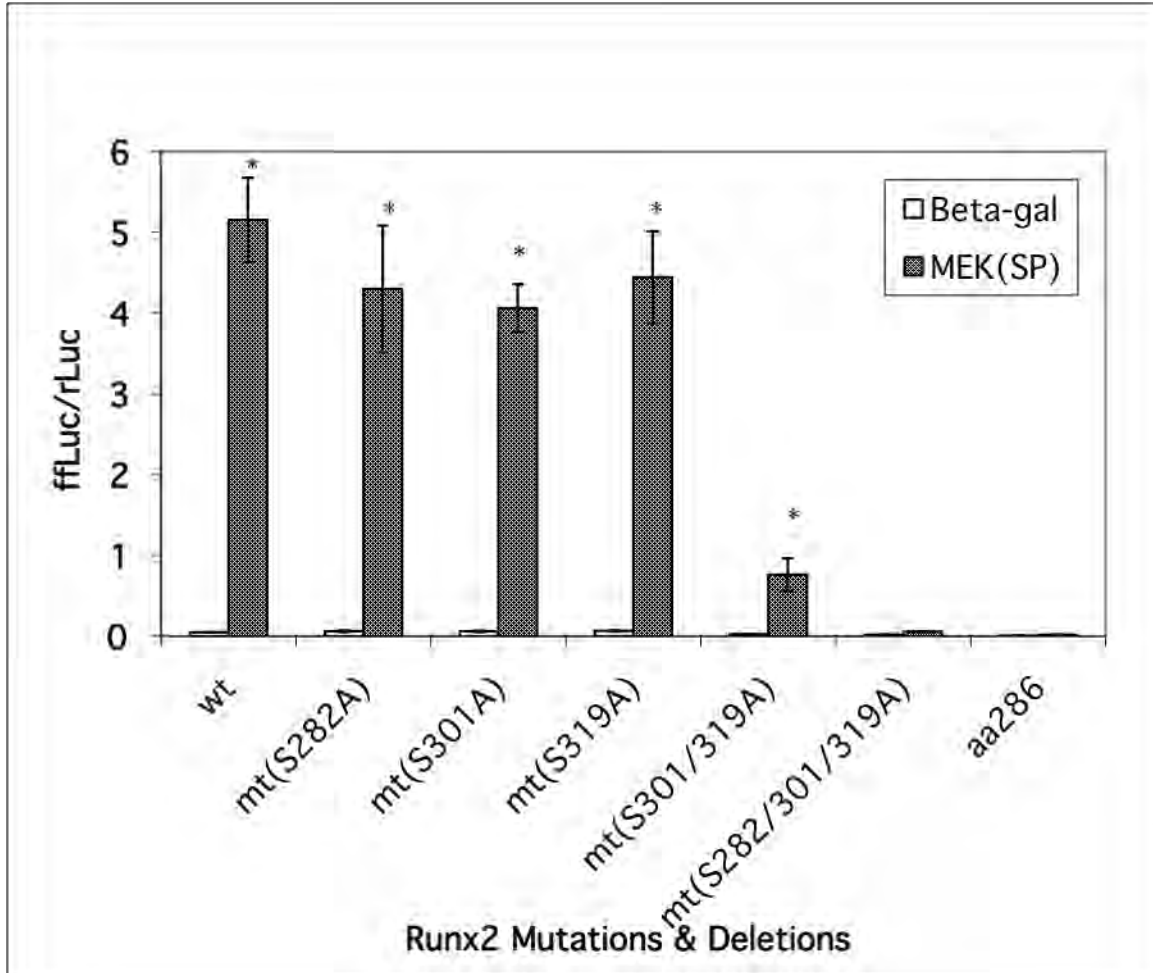
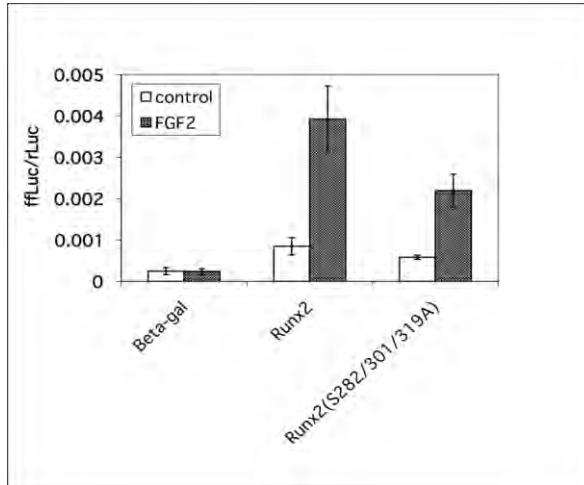


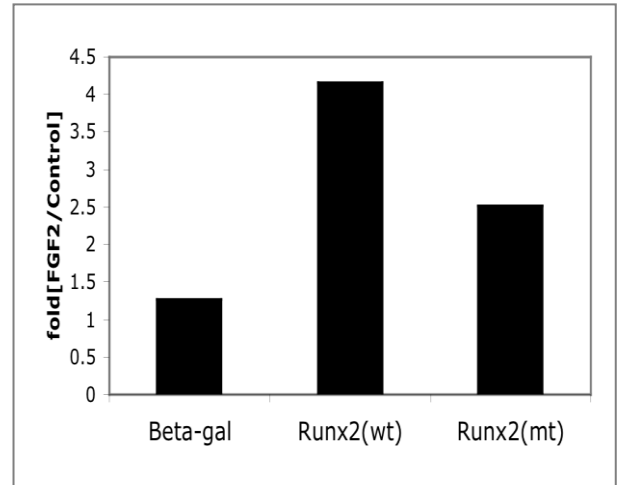
Fig. 7. Mutational analysis of Runx2. COS7 cells were transfected with wild type Runx2 or the indicated point mutations or deletions in the presence of control Beta-gal or MEK(SP) expression vectors and a 6OSE2-luc reporter as described in Methods. Firefly luciferase activity was normalized for transfection efficiency using renilla reformis luciferase plasmid. For each group fold-stimulation in MEK(SP) samples was determined. \*, Ratio (MEK(SP)/Beta-gal) is significantly different at  $P < 0.001$ .

We also assessed the functionality of the above Runx2 phosphorylation sites in mediating the response to FGF2. S/A mutation was generated at all three consensus MAPK phosphorylation sites. Wild type or mutated expression plasmids of Runx2 were transfected into COS7 cells with 6OSE2 $\pm$ FGF2. As shown in Fig. 8A & 8C, the ability of FGF2 to stimulate transcriptional activity in the S/A mutant was partially decreased. Runx2 protein levels tested by Western blot were not significantly changed (Fig. 8B). This indicates that other pathways maybe involved. Transfection of wild type or S/A mutation of Runx2 1-330 and 6OSE2-luc  $\pm$  MEK(SP) plasmids into COS7 cells was used to show that the ability of MEK(SP) to stimulate transcriptional activity was completely lost in triple-point mutation (S282/310/319A) Runx2 1-330 (Fig. 9). These results indicate that all three consensus MAPK phosphorylation sites are functionally responsible for MAPK activation.

8A.



8C.



8B.

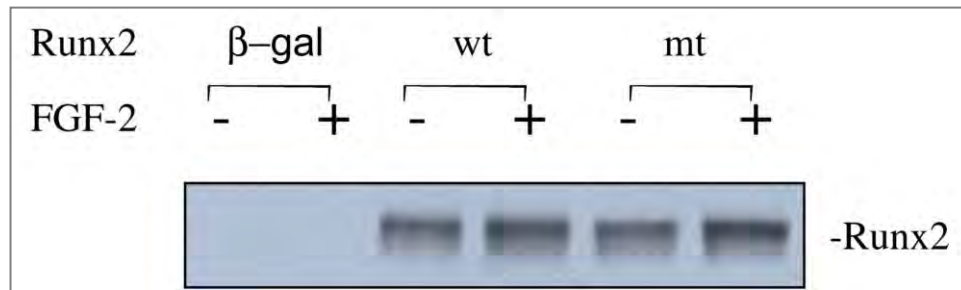


Fig. 8. Mutational analysis of full-length Runx2. A. Wild type or mutated expression plasmids of full-length Runx2 were transfected into COS7 cells with 6OSE2-luc. After transfection, cells were cultured in growth medium for 24 hours and then switched to medium containing 0.1% FBS in the absent or presence of FGF2 at a concentration of 25 ng/ml for another 12 hours, then harvested and assayed. B. Runx2 protein levels tested by Western blot. C. Fold stimulation of Runx2 wild type and S282/301/319A mutant.

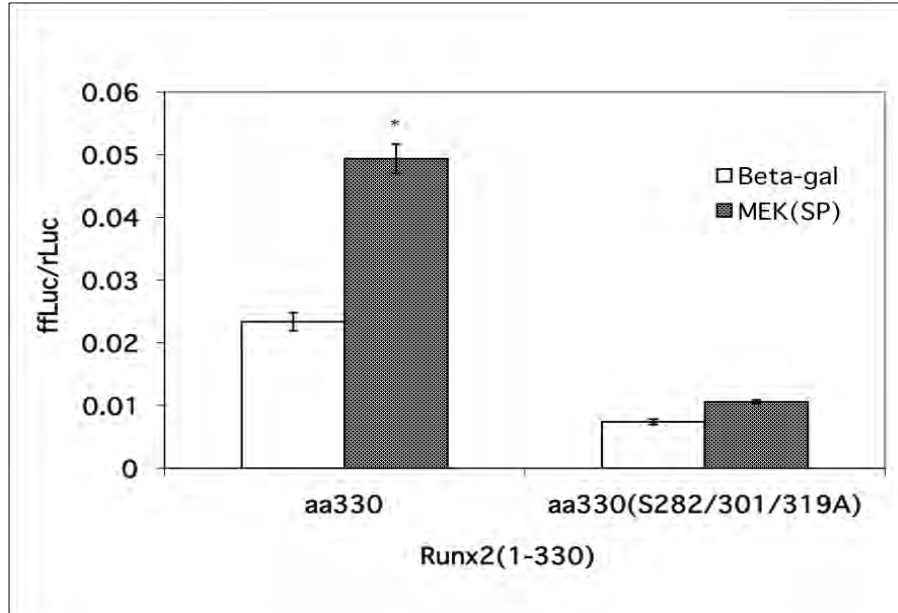


Fig. 9. Mutational analysis of Runx3 1-330 deletion. Mutations of three consensus MAPK phosphorylation sites (282, 301, 319) from Ser to Ala of Runx2 1-330 deletion transfected with 6OSE2-luc in COS7 cells reduced MEK(SP) stimulation of Runx2 1-330 deletion. For each group fold-stimulation in MEK(SP) samples was determined. \*, Ratio (MEK(SP)/Beta-gal) is significantly different at  $P < 0.001$ .

To determine whether MAPK can directly phosphorylate Runx2 peptides *in vitro*, eight synthesized Runx2 peptides (see Table 1 & Fig. 10A), which contain one consensus MAPK phosphorylation site or the same peptide containing a mutation at the MAPK phosphorylation site or none of those sites were examined by *in vitro* phosphorylation experiments. Incubation of peptides spanning the 260 to 333 region with active Erk1 and  $\gamma$ - $^{32}\text{P}$ -ATP revealed that S282, 301 and 319 were all phosphorylated *in vitro* while S/A substitution at each site prevented phosphorylation (Fig 10B). In contrast, T326 was not phosphorylated under these conditions (i.e. Introduction of an S319A mutation in the

316-333 peptide blocked phosphorylation). Peptides containing residues 260-279 and 511-528 were also not phosphorylated. This result indicated that MAPK can directly phosphorylate Runx2 peptides *in vitro*.

Table 1. Peptide sequence

aa260-279 (peptide 1)	PHPSMRVGVPPQNPRPSLNS
aa277-296 (peptide 2)	LNSAPSPFN PQGQS QITDPR
aa277-296 (S282A)	LNSAPAPFN PQGQS QITDPR
aa297-316 (peptide 3)	QAQSSPPWSYDQSYPSYLSQ
aa297-316 (S301A)	QAQSAAPPWSYDQSYPSYLSQ
aa316-333 (peptide 4)	QMTSPSIHSTTPLSSTRG
aa316-333 (S319A)	QMTAPSIHSTTPLSSTRG
aa511-528 (peptide 5)	PTVLNSSGRMDES VWRPY

Taken together, studies with full-length Runx2, Runx2 1-330 and *in vitro* phosphorylation indicate that S282, 301 and S319 phosphorylation sites are critical for MAPK responsiveness although more distal sites outside the 1-330 region may also be needed for full transcriptional activation.

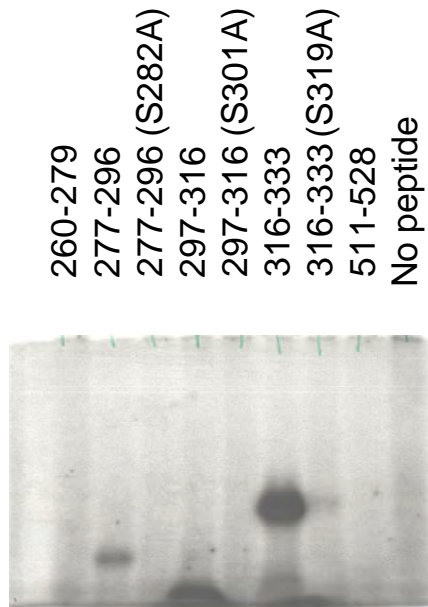


10A.

**Runx2 (aa259-337)**



10B.



*Fig. 10. MAPK directly phosphorylates synthesized Runx2 peptides in vitro. Runx2 peptides were incubated with activated ERK1 and  $\gamma$ -[ $^{32}$ P] ATP. ERK1 strongly phosphorylated the peptide, which contains at least one consensus MAPK phosphorylation site, but not the ones without a consensus site. ERK1 was unable to phosphorylate the mutant peptide at consensus MAPK phosphorylation site.*

## 5. DISCUSSION

In this study, we identified and characterized the MAPK phosphorylation sites in the Runx2 molecule that are necessary for transcriptional activity. Our studies show that: 1) The P/S/T domain of Runx2 is required for MAPK-dependent phosphorylation; 2) The amino acid 258 to 330 region in Runx2 molecule is necessary for MAPK responsiveness/phosphorylation. 3) Three MAPK phosphorylation sites, S282, S301, S319, in Runx2 are required for MAPK-dependent activation of Runx2 transcriptional activity and osteoblast differentiation. These sites are directly phosphorylated by activated ERK1 *in vitro* and functionally responsible for MAPK activation. Recent results of our group also show that these three sites can be phosphorylated in intact cells. Furthermore, inactivating S to A mutations greatly reduced the ability of Runx2 to stimulate expression of *Ocn* and *Bsp* mRNAs in Runx2<sup>-/-</sup> calvarial cells and blocked Runx2-dependent induction of osteoblast gene expression and differentiation in a mesenchymal cell line. In other experiments not show here, S to E (glutamic acid) mutations, which mimic the charge density of phosphorylated amino acids, activated Runx2-dependent transcription (Ge *et al.* in preparation). Taken together, these studies demonstrate that MAPK-dependent phosphorylation plays an important role in controlling Runx2 transcriptional activity in bone.

Runx2 proteins are subject to post-translational modifications that affect their activity (see reviews by Franceschi and Xiao, 2003; Bae and Lee, 2006; Franceschi et al., 2007). As one of the major signal transduction pathways in bone, the ERK/MAPK pathway is able to integrate stimuli from growth and differentiation factor binding to receptor tyrosine kinases (Cobb et al., 1991), ECM-integrin binding and focal adhesion

kinase activation (FAK) (Franceschi and Xiao, 2003), certain non-genomic actions of estrogens (Kousteni et al., 2003) and mechanical stimulation mediated by FAK activation (Moalli et al., 2001) and connexin 43 up-regulation (Lecanda et al., 2000). It also has important functions in the differentiation of post-mitotic mesenchymal and neuronal cells (Kao et al., 2001; Yao et al., 2003) and regulates the activity of several tissue-specific transcription factors including MyoD (muscle(Zetser et al., 2001)), Sox9 (cartilage(Murakami et al., 2000)) and PPAR $\gamma$  (adipose(Adams et al., 1997b)). Furthermore, as previously shown by this laboratory, *in vivo* transgenic stimulation of ERK/MAPK signaling in osteoblasts accelerates bone development and is able to partially rescue the cleidocranial dysplasia phenotype of Runx2 haploinsufficient mice(Ge et al., 2007b). Based on this work, it is likely that the phosphorylation sites we identified are critical for regulating Runx2 activity *in vivo* and may function to integrate the osteogenic response to hormonal, mechanical and environmental stimuli.

Although the three phosphorylation sites identified in this study are clearly important for Runx2 transcriptional activity, it is possible that other sites also participate in the response to ERK/MAPK stimulation. Our deletion/mutation analysis showed that the C-terminal 330 amino acid residues of Runx2 retained the ability to be activated by MAPK and that this activity could be explained by phosphorylation at S282, S301 and S319. Furthermore, combined S/A mutations in the context of full-length Runx2 greatly attenuated the ability of Runx2 to induce expression of *Ocn*, *Bsp* and alkaline phosphatase. However, there are a total of 13 proline-directed serine consensus phosphorylation sites in the Runx2 sequence, some of which may also participate in the MAPK response. In fact, deletion analysis showed a gradual loss of MAPK-dependent

activation of Runx2 with progressively larger C-terminal deletions. It is, therefore, possible that phosphorylation sites in the region between residues 330 and 528 may also participate in the MAPK response, possibly functioning as secondary sites after priming phosphorylations occur at S282, S301 and 319. In addition, S282 /301/319A mutations only partially blocked activation of the 6OSE2-luc reporter by FGF2 (Fig.8A). In this regard, a recent study by Kim and coworkers reported that FGF2 also activates Runx2 via phosphorylation by PKC $\gamma$  at S247 (Kim et al., 2006). Thus, it is possible that FGF2 activates Runx2 transcriptional activity by phosphorylating both ERK/MAPK and PKC sites.

Runx family members exhibit a high degree of amino acid sequence homology, particularly in the DNA binding or Runt domain. Although the C-terminal P/S/T domain of Runx proteins is not highly conserved, the three phosphorylation sites we identified in Runx2 are also present in Runx1 (but not in Runx3). Interestingly, EGF-dependent activation of the ERK/MAPK pathway can stimulate Runx1 transcriptional activity via phosphorylation on these sites (Tanaka et al., 1996). Similarly, stimulation of Runx1 by phorbol esters, which is also largely dependent on ERK/MAPK, requires these same two sites together with an additional conserved site equivalent to T326 in Runx2 (Zhang et al., 2004). Runx1 is essential for hematopoietic cell differentiation (Tanaka et al., 1995; Tanaka et al., 1996) and is also a frequent site for chromosomal translocations in acute myelogenous leukemia. Consistent with this oncogenic activity, transfection of Runx1 into fibroblasts stimulates anchorage-independent growth and transformation. Interestingly, S/A mutations at S249 and S266 in Runx1 (equivalent to S301,319 in Runx2) were shown to inhibit the growth of NIH 3T3 fibroblasts in soft agar, a common

assay for cell transformation (Tanaka et al., 1996). Runx2 can also function as an oncogene under certain conditions and has been associated with cell proliferation and migration of breast cancer cells (Barnes et al., 2004; Pratap et al., 2006). It is, therefore, possible that ERK/MAPK dependent phosphorylation of Runx2 at S301, 319 could also be associated with this metastasis-related behavior.

It is not presently understood how phosphorylation of Runx2 stimulates transcription. Runx2 is also known to serve as a docking site for many other nuclear factors that can form active or inactive transcription complexes on chromatin (Lian et al., 2004). These complexes subsequently recruit additional factors including histone acetyltransferases like p300/CBP to modify chromatin structure thereby allowing the initiation of transcription. Interestingly, the ERK/MAPK-dependent phosphorylation of Runx1 discussed above is associated with the dissociation of the histone deacetylase co-factor, mSin3a, from Runx1, thereby allowing subsequent increases in histone acetylation (Imai et al., 2004). Since Runx1 phosphorylation sites are conserved in Runx2, this observation provides a plausible mechanism for how ERK/MAPK phosphorylation could alter Runx2-dependent transcription.

In addition to the ERK/MAPK-dependent regulation of Runx2 described herein, several other types of post-translational modifications have been described for this molecule. PTH/PKA-mediated phosphorylation of a C-terminal Runx2 site was correlated with induction of MMP13 (Selvamurugan et al., 2000). More recently, Cdk4-mediated phosphorylation at S472 was shown to target Runx2 for ubiquitination and proteosomal degradation during the cell cycle (Shen et al., 2006) while cdc2 phosphorylation at S451 was shown to be necessary for cell cycle progression of

endothelial cells(Qiao et al., 2006). Also, GSK3 $\beta$ -dependent phosphorylation of Runx2 at S369-S373-S377 was shown to reduce transcriptional activity (Kugimiya et al., 2007). Most recently, three phosphorylation sites (S28, S347 and T340) of Runx2 were shown to be critical for PTH activity via protein kinase A (PKA), which phosphorylates and stimulates Runx2-dependent activation of the MMP-13 promoter (Selvamurugan et al., 2009). Lastly, Runx2 can be acetylated on critical lysine residues by p300 acetyltransferase. This modification, which is stimulated by BMP2, increases transcription and stabilizes Runx2 against proteosomal degradation (Jeon et al., 2006). Thus, posttranslational modification appears to be a common mechanism for regulating Runx2 activity and stability.

In summary, phosphorylation of Runx2 at S282, S301 and S319 clearly has an important regulatory role in Runx2-dependent transcription since mutation of these sites in the context of the intact Runx2 molecule severely attenuated the ability of Runx2 to stimulate osteoblast-specific gene expression during differentiation. Ongoing *in vivo* studies will be necessary to assess the full impact of Runx2 phosphorylation to the overall activity of this molecule during skeletal development and remodeling.

Recent studies from our group also show that fluid flow shear stress, a kind of mechanical loading, induces osteoblast-specific gene expression through a MAPK-mediated process (Yan Li, personal communication). The results of this study provide the foundation for future therapeutic strategies to promote bone formation for implant healing, osteointegration, as well as tooth movement in orthodontics. Based on our mutational analysis, future therapeutic means can be developed using knock-in models to manipulate the phosphorylation sites in Runx2 to accelerate or delay the bone formation

for different therapeutic purposes. Furthermore, our transgenic model of MEK(SP), and MEK(DN) also provides the means to cure or rescue CCD patients using Runx2 mutation/deletion. A chemotherapeutic approach could develop drugs to activate or inhibit MEK activity.

Runx2 plays an important role in dental development. Runx2 is required for the differentiation of odontoblasts and osteoblasts lining bone in the periodontal space (D'Souza et al., 1999). The defects seen in the dentition of patients affected with CCD are thought to arise from a disruption in the bone remodeling process. In addition to increased density in the maxilla and mandible, multiple supernumerary teeth are present that show a marked delay or arrest in eruption (Jensen and Kreiborg, 1993). However, the mechanism used by Runx2 to control in dental development is poorly understood. It will be of great value to evaluate the role of Runx2 in dental development.

## **6. CONCLUSIONS**

The P/S/T domain of Runx2 is required for MAPK-dependent phosphorylation. The region from amino acid 258 to 330 in the Runx2 molecule is necessary for MAPK responsiveness/phosphorylation. Three MAPK phosphorylation sites, S282, S301, S319, were identified in Runx2 and shown to be directly phosphorylated by MAPK and functionally responsible for MAPK activation.



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