

Review

Runx2 and dental development

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The *Runx2* gene is a master transcription factor of bone and plays a role in all stages of bone formation. It is essential for the initial commitment of mesenchymal cells to the osteoblastic lineage and also controls the proliferation, differentiation, and maintenance of these cells. Control is complex, with involvement of a multitude of factors, thereby regulating the expression and activity of this gene both temporally and spatially. The use of multiple promoters and alternative splicing of exons further extends its diversity of actions. RUNX2 is also essential for the later stages of tooth formation, is intimately involved in the development of calcified tooth tissue, and exerts an influence on proliferation of the dental lamina. Furthermore, RUNX2 regulates the alveolar remodelling process essential for tooth eruption and may play a role in the maintenance of the periodontal ligament. In this article, the structure of *Runx2* is described. The control and function of the gene and its product are discussed, with special reference to developing tooth tissues, in an attempt to elucidate the role of this gene in the development of the teeth and supporting structures.

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Mutations of *Runx2* [also known as Core-binding factor $\alpha 1$ (Cbfa1), PEBP2A1, and AML3] have been identified as being responsible for cleidocranial dysplasia (CCD) (Fig. 1) (1). This is an autosomal-dominant inherited disorder characterized by patent fontanelles, wide cranial sutures, frontal bossing, hypoplasia of the clavicles, short stature, ectopic and delayed eruption of teeth, supernumerary teeth, and other skeletal anomalies. Gene knockout experiments have produced similar skeletal phenotypes in mice (2).

RUNX2 is an osteoblast-specific transcription factor necessary for the differentiation of pluripotent mesenchymal cells to osteoblasts (3). The presence of RUNX2 in fully differentiated cells supports the concept that RUNX2 is also required in maintaining fully functional cells, at least in bone (3–5).

RUNX2 has also been identified as essential for tooth formation (4, 6). Dental abnormalities seen in CCD patients (7, 8) may be a direct result of RUNX2 dysfunction in tooth-forming cells. The dental abnormalities in CCD suggest an important role for RUNX2 during formation of the dentition.

The *Runx2* gene is 220 kb long (9) and contains eight exons (5, 10, 11). It belongs to the runt domain (RUNX) family of genes. These genes, named *Runx1*, -2 and -3, exhibit a high amino acid homology. Their protein products form a heterodimer with core-binding factor β (CBF β) (12).

CBF β is required for the efficient function of RUNX2 in skeletal development (13), allosterically enhancing DNA binding by RUNX proteins at the runt homology domain (RHD) (14). Furthermore, it plays an important

role in stabilizing RUNX proteins against proteolytic degradation by the ubiquitin–proteasome system (15).

Runx2 and bone

Bone tissue consists of hydroxyapatite crystals and various kinds of extracellular matrix (ECM) proteins, including type I collagen, osteocalcin, osteonectin, osteopontin, bone sialoprotein (BSP), and proteoglycans (16, 17). These bone matrix proteins are secreted and deposited by polarized mature osteoblasts, which are aligned on the bone surface (18). The precise roles of matrix proteins in the formation of bone are not fully elucidated (19, 20). The formation of hydroxyapatite crystals is also regulated by osteoblasts.

The establishment of *Runx2* null mice clearly demonstrated that this transcription factor is essential for osteoblast differentiation, as these mice had no bone tissue, osteoblasts or osteoclasts, despite normal cartilaginous skeletal patterning. Chondrocyte maturation, however, is perturbed as a consequence (2, 21).

Runx2 is also active in mature osteoblasts. Mature mice, in whom active RUNX2 levels have been reduced, exhibit decreased expression of the genes encoding the main bone matrix proteins, BSP, osteocalcin, osteopontin and collagen type I (4). These genes are regulated via the RUNX2-binding sites in the proximal promoter segments of the respective genes (14).

RUNX2 has thus been shown to be essential for normal bone formation, with perturbation of bone formation if the levels are insufficient. Overproduction will also

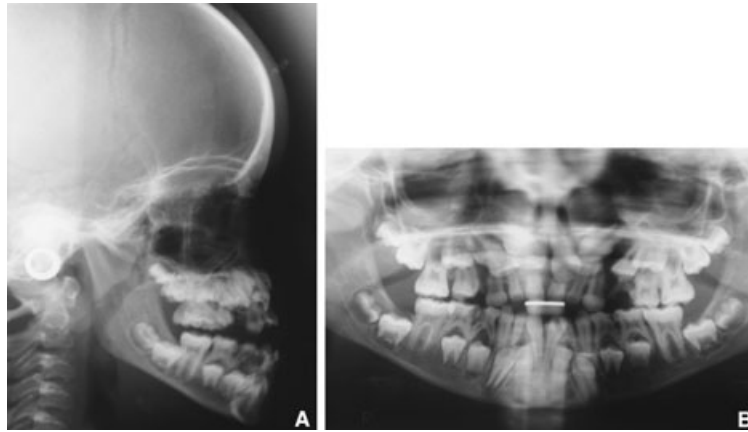


Fig. 1. A 12-yr-old female patient with cleidocranial dysplasia. (A) Lateral skull radiograph showing a wormian bone in the temporal suture region and a hypoplastic maxilla. (B) Panoramic radiograph showing supernumerary and unerupted teeth, some of which are ectopic. Typically, the lower permanent central incisors and all four first permanent molars have erupted.

affect 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 (22). However, adult transgenic mice overexpressing *Runx2* showed osteopenia with a decrease in bone mineral density. This was attributed to reduced osteoblast maturation, but also to enhanced receptor activator of nuclear factor kappa β ligand (RANKL) and matrix metalloproteinase-13 (MMP-13) production with enhanced osteoclastogenesis (23). Neonatal transgenic mice showed maturational blockage of osteoblasts, but did not show enhanced osteoclastogenesis, possibly because of the different ages of the experimental mice (24). The results of these experiments are consistent with evidence that *Runx2* is regulated, in part, 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 (25).

Runx2 expression decreases with age. This may be one possible explanation for impaired osteoblast function and reduced bone formation with aging (26).

RUNX2 is affected by a diversity 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 transcription. These post-translational changes act via the mitogen-activated protein kinase (MAPK) and protein kinase A and C (PKA, PKC) pathways, activating RUNX2 by phosphorylation (27–29) (Fig. 2). Further control of the RUNX2 molecule occurs through the counterplay of acetylation and ubiquitination (29–31) (Fig. 3). The *Runx2* gene, in fact, plays a central role in co-ordinating multiple signaling pathways affecting osteoblast differentiation.

A species-specific difference in action may, however, exist. Osteoblast differentiation in rodents is associated with an increase in RUNX2 production. However, in human bone marrow stem cells, enhanced transactivation activity occurs with no change in *Runx2* mRNA or

protein levels. This activity has been ascribed to post-translational phosphorylation of key residues in the RUNX2 protein (32).

RUNX2 domains

RUNX2 binds to the core binding factor site, also known as the osteoblast-specific *cis*-acting element 2 (OSE2) (33), 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. Together with other factors, such as activator protein 1 (AP-1) and mothers against decapentaplegic homolog (SMADs), it controls their expression (34, 35). The RHD is responsible for the DNA-binding properties of RUNX2 (Fig. 4).

Three transactivation domains and one major repression domain have been identified in the RUNX2 protein (36). 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. In the latter, transactivation depends on a stretch of 29 glutamine residues. Deletion of the alanine stretch does not affect transactivation; however, expansion has a repressive function (1), and expansion may also play a role in nuclear localization (37).

The third activation domain is present in the N-terminal portion of the proline/serine/threonine (PST)-rich domain. A mutation in this region has been shown to cause a failure to interact with SMADs, reducing the response of osteoblasts to the Transforming growth factor- β /Bone morphogenetic protein (TGF- β /BMP) signaling pathway (38). This region has also been shown to interact with the co-activator molecule, p300, affecting expression of the osteocalcin gene (39). This action is independent of the acetyltransferase activity of p300, which protects RUNX2 from degradation by SMAD ubiquitin regulatory factor (SMURF)-mediated ubiquitination and also increases the transactivation potential of RUNX2 (31).

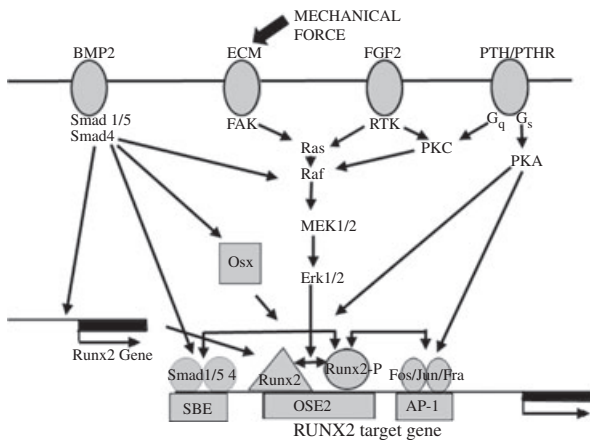


Fig. 2. Multiple signaling pathways converge on RUNX2 to initiate osteoblast differentiation. BMP2, Bone Morphogenic Protein 2; ECM, Extracellular Matrix; FGF2, Fibroblast Growth Factor 2; AP-1, Activator Protein 1; ERK, Extracellular signal related kinase; FAK, Focal adhesion kinase; Fos, Oncogene Fos; Fra, Fos related antigen; G, G alpha protein; Jun, Oncogene Jun; MEK1/2, Mitogen activated protein kinase/Extracellular signal related kinase1/2; OSE2, Osteoblast Specific Element 2; Osx, Osterix; PTH, Parathyroid hormone; PTHrP, Parathyroid hormone related protein; Raf, Oncogene Raf; Ras, Retrovirus associated DNA sequences; RTK, Receptor Tyrosine Kinase; SBE, SMAD binding element; Smad, Mothers against decapentaplegic protein. Modified after Franceschi RT, Xiao G. J. Cell Biochem. Vol.88, 3 Pages: 446-454. Copyright © 2003 Wiley-Liss, Inc., A Wiley Company. Reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

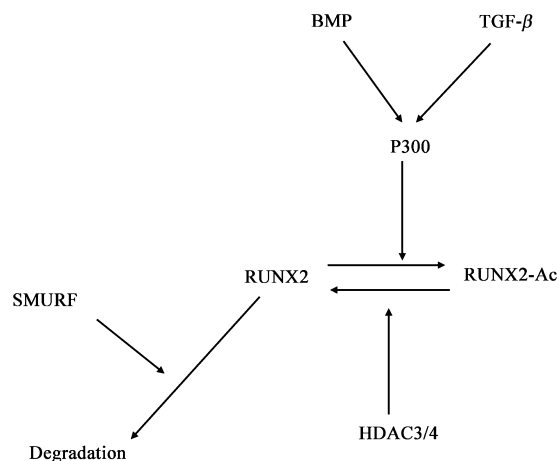


Fig. 3. Bone morphogenetic proteins (BMP) and transforming growth factor- β (TGF- β) act on P300 to acetylate (Ac) RUNX2, protecting it from degradation via the ubiquitin pathway. Histone deacetylases (HDACs) deacetylate RUNX2, accelerating its degradation. SMURF, SMAD ubiquitin regulatory factor.

The C-terminal part of the PST domain is a repression domain (36, 40). The terminal five amino acids (the VWRPY motif) are highly conserved and may bind the corepressor proteins of the transducin-like enhancer of split (TLE) or Groucho-related genes (Grg) family (41).

The down-regulation of TLE/Grg expression during osteoblast differentiation is a potential mechanism for relief of Runx2 repression during cell differentiation (42). Other parts of the molecule have been shown to react with other corepressors, such as histone deacetylases (HDAC) (40), SIN3 (43), and yes-associated proteins (YAP) (44) (Table 1 and Fig. 4). A review of RUNX2 corepression was made by WESTENDORF (45).

RUNX2 is imported to the nucleus after transcription and must bind to specific regions of the nuclear matrix to effect transcriptional control (53), colocalizing with co-activators such as SMADs (35) and RNA polymerases, at nuclear sites that support RNA synthesis (54).

This function is effected by the nuclear matrix targeting signal (NMTS) region, a 38 amino acid segment situated between the RHD and PST domains (55). 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 osteolytic activity (38, 56).

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.

RUNX2 isoforms

RUNX2 has two major N-terminal isoforms, whose transcription from the *Runx2* gene is separately regulated by either a proximal promoter or a distal promoter (Fig. 5) (10). The product of the proximal promoter starts with the amino acid sequence, MRIPVD, and is known as Type I, PEBP2A or P56. That of the distal promoter product starts with the amino acid sequence, MASNSL, and is known as Type II, Til-1 or P57. OSF2 (Type III) is a splice variant of Type II, present only in the mouse. It starts with the sequence MLHSPH, and its function is similar to the Type II isoform. It is not essential for optimal transcriptional activation (57), and it may play a species-specific role in the regulation of transcription (10).

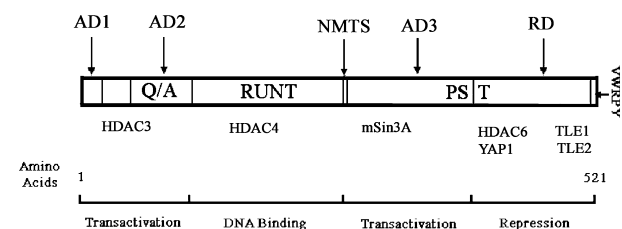


Fig. 4. Distribution of the different domains of the RUNX2 protein. The sites of interaction with various corepressor molecules are also shown. AD, transactivation domain; HDAC, histone deacetylase; SIN3A, SIN3A protein; NMTS, nuclear matrix targeting signal; PST, proline/serine/threonine rich domain; Q/A, glutamine/alanine-rich domain; RD, repression domain; RUNT, Runt homology domain; TLE, transducin-like enhancer of split; VWRPY, conserved repression signal; YAP, yes-associated protein.

Table 1

Known co-activators and corepressors of RUNX2. CBFB, core binding factor subunit B; GR65, Groucho related gene 5; HDAC3, histone deacetylase 3; HDAC5, histone deacetylase 5; HDAC6, histone deacetylase 6; MORF, monocytic leukemia zinc finger protein-related factor; pRb, retinoblastoma 1; Sin3A, Sin3 protein; TAZ, tafazzin; TLE/GROUCHO, transducin-like enhancer of split/Groucho related genes; TWIST, Twist protein; YAP1, yes associated protein 1

Protein	Reference	Class	Function
CBFB	YOSHIDA <i>et al.</i> (13)	Co-activator	Enhances DNA binding of RUNX2
GRG5	WANG <i>et al.</i> (46)	Co-activator	Dominant-negative inhibitor of larger TLE proteins
HDAC3	SCHROEDER <i>et al.</i> (47)	Corepressor	Blocks RUNX2 dependent transcription
HDAC4	VEGA <i>et al.</i> (48)	Corepressor	Prevents DNA binding
HDAC6	WESTENDORF <i>et al.</i> (40)	Corepressor	Deacetylation of histones
MORF	PELLETIER <i>et al.</i> (49)	Co-activator	Potentiates RUNX2 dependent transcription
MOZ	PELLETIER <i>et al.</i> (49)	Co-activator	Potentiates RUNX2 dependent transcription
SIN3A	FENRICK <i>et al.</i> (43)	Corepressor	Alters subnuclear localization
p-300	JEON <i>et al.</i> (31), SIERRA <i>et al.</i> (39)	Co-activator	Acetylation of RUNX2/potentiates Vitamin D actions
pRb	THOMAS <i>et al.</i> (50)	Co-activator	Potentiates RUNX2 dependent transactivation
TAZ	CUI <i>et al.</i> (51)	Co-activator	Potentiates RUNX2 dependent transactivation
TLE/GROUCHO	McLARRIN <i>et al.</i> (41) JAVED <i>et al.</i> (42)	Corepressor	Colocalizes at subnuclear level
TWIST	BIALEK <i>et al.</i> (52)	Corepressor	Prevents DNA binding of RUNX2
YAP1	ZAIDI <i>et al.</i> (44)	Corepressor	Colocalizes at subnuclear level

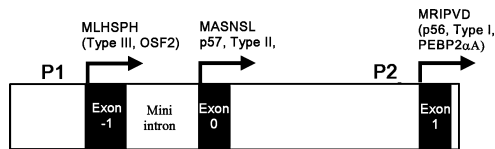


Fig. 5. Diagram of the *Runx2* promoter region. The P2 promoter drives expression of the Type I isoform and is associated with cell proliferation. The P1 promoter drives expression of the Type II variant, associated with commitment to a calcified tissue line. A mini-intron separates Exon 0 from exon -1. Transcription from this site produces the Type III variant, which has similar properties to Type II but is found only in mice. A, arginine; D, aspartic acid; H, histidine; I, isoleucine; L, leucine; M, methionine; N, asparagine; P, proline; R, arginine; S, serine; V, valine.

The presence of multiple isoforms of the *Runx2* gene product is consistent with other members of the *Runx* transcription family, including *Runx1* and *Runx3*, that exist as multiple isoforms with different transactivation potentials (58).

Type I and Type II isoforms differ functionally, but both are crucial in bone development. Type I is expressed ubiquitously in both non-osseous mesenchymal tissues and on osteoblast progenitor cells. It plays an important role in a wide range of cellular differentiation events and its expression is not affected by the differentiation status of the cell. In terms of intramembraneous bone formation, it probably is involved in the initial commitment steps and continues to exert its effects to the final differentiation of osteoblasts.

Type II expression is increased during osteoblast differentiation and is induced as a response to BMP2, suggesting that it is necessary for the maintenance of the osteoblast phenotype and that Type I may have a regulatory role (Fig. 6) (59).

In contrast to the findings of DUCY *et al.* (3) and XIAO *et al.* (10), HARADA *et al.* (60) found all three isoforms to

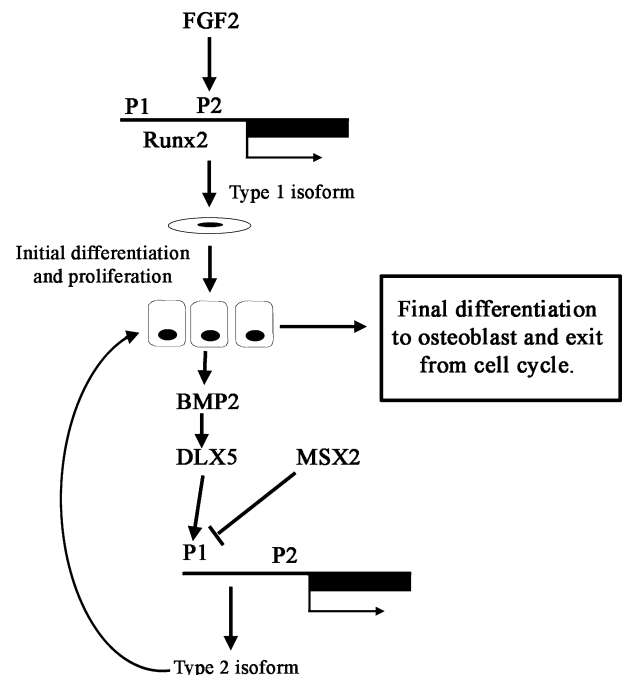


Fig. 6. Fibroblast growth factor 2 (FGF2) stimulates production of the Type I isoform from the P2 promoter to initiate cell commitment and proliferation. Bone morphogenetic protein 2 (BMP2), produced by these cells, acts via distal-less homeobox 5 (DLX5) on the distal P1 promoter to produce the Type 2 isoform, which encourages maturation of the osteoblast and exit from the cell cycle. The action of DLX5 is antagonized by muscle segment homeobox 2 (MSX2). Information derived from BANNERJEE *et al.* (59), CHOI *et al.* (64) and LEE *et al.* (65).

be present in the bones of adult mice and concluded that all three are involved in stimulating osteoblast differentiation, exerting different functions in the process of differentiation depending on the cell type and the

downstream gene promoter. Because production of the Type II isoform is restricted to later events of cell differentiation, being found only in pre-osteoblasts and osteoblasts, its contribution is probably more specific in the final step into osteoblast differentiation and is necessary for osteoblast maturation and skeletogenesis (61, 62). However, the expression pattern and function of both isoforms is not very different in the later stages of osteoblast differentiation (63).

The expression of Type I Runx2 is stimulated by FGF2. This commits precursor cells to the osteoblast lineage and permits cellular proliferation. Runx2 Type I stimulates production of BMP2 and this, in turn, affects Runx2, its effect being mediated by distal-less homeobox 5 (DLX5) (64). LEE *et al.* (65) found that DLX5 specifically transactivates the Runx2 distal (P1) promoter in committed osteoblasts and that its action is antagonized by MSX2 (Fig. 6).

The pattern of isoform expression may be different in tooth tissue, with a high expression of Type I and weak expression of Types II and III in the ameloblast and odontoblast layers of neonatal mouse incisors (66). However, Types II/III have been shown to be indispensable for tooth germ development past the bud stage and they play a role in the differentiation of ameloblasts and odontoblasts, which is necessary for transcription of dentine matrix protein 1 (DMP1), dentine sialoprotein (DSPP), amelogenin (AMGN), and ameloblastin (AMBN) in cultured mouse mandibles (67).

Dual translational control of Runx2, via both the internal ribosomal entry site (IRES) and cap-dependent mechanisms, exists for the P1 and P2 promoters. This provides another level of control of RUNX2 isoforms and may be a means to fine-tune Runx2 expression across a wide range of cellular conditions where the amount of Runx2 gene products may be an important determinant of their biological effects (68).

Further isoforms of RUNX2 exist as a result of alternative splicing. This also is consistent with findings for other RUNX factors and may explain the different spatiotemporal patterns of expression of the gene (10, 58, 69, 70). The timing of expression of different RUNX2 isoforms, in conjunction with other transcription factors and depending on the level of differentiation of the cell, may well serve as a method of control of bone and tooth formation.

Runx2 and osteoclastogenesis

Overexpression of Runx2 increases osteoblast number but inhibits their terminal maturation, resulting in accumulation of less mature osteoblasts and consequent osteopenia (24). Osteoclastogenesis is increased, possibly by the increased production of RANKL and MMP-13 (two factors involved in bone formation–resorption coupling) by the immature osteoblasts (23).

Osteoprogenitor cells have greater potential to support osteoclast development than more differentiated cells (71). Osteoclastogenesis is strongly induced by undifferentiated stromal marrow cells, which produce high

RANKL levels. As maturation proceeds, RANKL levels drop and those of its antagonist, osteoprotegerin (OPG), rise (72). The stimulus to osteoclastogenesis is reduced accordingly and the differentiated osteoblasts proceed to the formative phase of bone formation.

The precise role of RUNX2 in osteoclast regulation is controversial. Opg is strongly expressed in Runx2^{−/−} calvarial cell lines, whereas Rankl is not (73, 74). However, RUNX2-binding elements are present in the promoter region of the Opg gene. RUNX2 increases the activity of the Opg promoter, suggesting that RUNX2 regulates osteoclastogenesis by inducing the expression of Opg (75). TGF- β inhibits bone resorption by induction of Opg and its effects are mediated by RUNX2 and SMADs (76).

A RUNX2-binding site is present on the mouse and human Rankl promoter (77), but RUNX2 failed to stimulate the transcriptional activity of the promoter region of the Rankl gene, showing that that RUNX2 possibly does not regulate Rankl in the same manner as other known targets (78). However, RUNX2 induced Rankl expression and suppressed Opg expression in the presence of 1,25(OH)₂D₃ (74). On the other hand, forced production of soluble RANKL was found to be insufficient for the complete rescue of osteoclast differentiation in Runx2^{−/−} mice, suggesting the presence of another requirement for osteoclast differentiation. Also, treatment of RUNX2-deficient calvarial cells with 1,25(OH)₂D₃ affected both Rankl and Opg expression and induced osteoclastogenesis, showing that expression of Rankl and Opg, and initiation of osteoclastogenesis, may be induced via alternative pathways (79).

Colony-stimulating factor 1 (CSF-1) and RANKL are widespread; however, osteoclasts are confined to calcified tissue. The addition of CSF-1 and RANKL to serum-free cell cultures produced no osteoclasts (80). This suggests the existence of other factors or ECM proteins, possibly induced by RUNX2 in osteoblasts, which are required for osteoclast differentiation.

RUNX2 in odontogenesis

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

The expression of Runx2 in both the dental papilla and dental sac suggests a potential involvement of this gene in the differentiation of odontoblasts and osteoblasts lining bone in the periodontal space (6, 81). Mice deficient in RUNX2 exhibit an arrest of molar tooth development at the early cap stage, suggesting a requirement for RUNX2 in the progression of tooth development from the cap stage to the bell stage (6).

In mouse embryonic mandibular first molar tooth germs, *Runx2* expression is initiated by FGF produced by the odontogenic epithelium, shortly after commencement of 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 histo- and morpho-differentiation of the enamel organ (6, 82, 83).

Runx2^{-/-} mouse molars show arrested development at the bud stage, whereas incisors, which develop earlier, progress to the bell stage and show dentine formation, although odontoblasts are abnormal and no enamel is formed (6, 84).

Enamel knot marker genes, including cyclin-dependent kinase inhibitor 1A (*p21*), *Fgf4*, ectodysplasin A receptor (*Edar*) and *Bmp4*, are down-regulated in *Runx2*^{-/-} lower molars, but are expressed normally in the upper molars. Sonic Hedgehog (*Shh*) is completely absent in *Runx2*^{-/-} lower molars, while weak signals remain at the tip of the tooth bud in the upper molars (83).

Lower molars are more severely affected than upper molars in *Runx2*^{-/-} mice, and incisors are less affected than molars. Hence, RUNX2 may have different downstream target genes in different regions of the jaws. Similar regional differences in molecular regulation is evident in relation to other genes, notably *Dlx* (85) and *ActivinβA* (86). The different origins of the neural crest cells populating the maxillary and mandibular primordia may explain their different behavior (87). However, the developmental profiles of *Runx2* expression in odontoblasts and osteoblasts, both derived from mesenchyme, is also different (6), suggesting that the gene may also be differentially regulated in these cells.

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. Whether *Runx2* is essential for the later stages of tooth development is still unknown, as *Runx2*^{-/-} mice do not survive beyond birth.

Prior to crown development, Type II *Runx2* is strongly expressed in dental papilla mesenchyme, which gives rise to the pulpal cells and odontoblasts. Type II *Runx2* is markedly down-regulated at the bell stage in the dental papilla, after morphogenesis is complete. Expression continues, albeit 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 Type II *Runx2*. No expression was found in osteoclasts. The forming periodontium contains a decreasing gradient of transcripts and immunostaining from crown to the root tip (88) (Figs 7, 8).

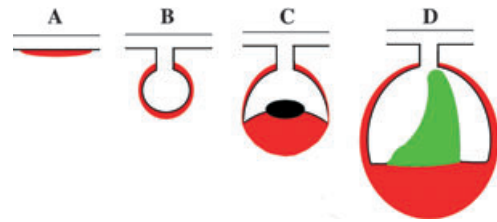


Fig. 7. Patterns of *Runx2* expression. (A) Initiation stage. Expression is induced in the mesenchyme by the odontogenic epithelium. (B) Bud stage. Expression is maintained around the ingrowing dental epithelium. (C) Cap stage. Expression is sustained in the mesenchyme and the dental follicle. RUNX2 is necessary for Sonic Hedgehog (*Shh*) expression and for formation of the enamel knot. (D) Bell stage. In this stage, expression is down-regulated in the dental papilla but is maintained in the dental follicle and surrounding mesenchyme.

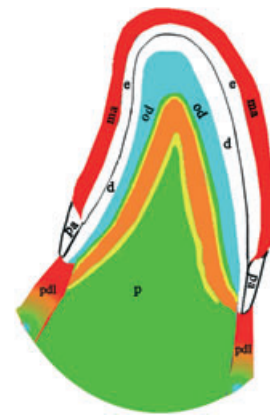


Fig. 8. *Runx2* mRNA expression in the secretory stage. Pre-ameloblasts show no expression; however, this is strongly up-regulated in maturation phase cells. There is diffuse expression throughout the dental papilla, with up-regulation in pre-odontoblasts. Differentiated odontoblasts, however, show no expression. The periodontal ligament shows strong expression as do cementoblasts and cementocytes. d, dentine; e, enamel; od, odontoblasts; ma, maturation-stage ameloblasts; p, dental papilla; pa, pre-ameloblasts; pdl, periodontal ligament.

Ameloblasts

Runx2 expression controls downstream factors acting on the development of the enamel organ epithelium. The importance of *Runx2* in amelogenesis is evidenced by the lack of enamel in the incisor tooth germs of *Runx2*^{-/-} mice. RUNX2 is also present in late secretory- and maturation-stage ameloblasts (6).

AMBN is an extracellular matrix protein that may play a role in enamel crystal formation in the developing dentition. It is used as an ameloblast-specific gene marker. The murine *Ambn* promoter contains two RUNX2-binding sites. RUNX2 interacts with functionally important regions of the *Ambn* promoter, and mutations of the *Ambn* promoter's RUNX2-binding sites diminish promoter activity. This indicates that RUNX2 possesses an important function in transcription of the *Ambn* gene (89). *Runx2* Type II mRNA remains strongly expressed in both immature and mature ameloblasts (81). Thus,

Runx2 is involved in the early stages of enamel organ formation, and tooth morphogenesis and may also play a direct role in formation of tooth enamel. Histological investigation of extracted permanent teeth in CCD show evidence of hypoplasia (90, 91). This does not appear to be the case for deciduous teeth (92).

Odontoblasts

Osteoblasts and odontoblasts share several similarities, including the expression of similar genes. Indeed, the main non-collagenous components of the odontoblastic extracellular matrix (DSPP or DMP1) are also present in other tissues, such as osteoblasts (93) and periodontium (94), albeit at much lower levels.

Multiple RUNX2-binding sites have been identified in the regulatory elements of the mouse *Dspp* gene (66). RUNX2 increased *Dspp* expression in immature odontoblasts, but down-regulated expression in more mature cells, showing that the effect of RUNX2 is dependent on the state of differentiation of the target cell (95).

RUNX2 is also involved in the regulation of DMP1 in osteoblasts, although it is not essential for DMP1 expression in odontoblasts, indicating the involvement of other unidentified odontoblast-specific transcription factors or co-activators (96, 97). JIANG *et al.* (81) found Type III Runx2 expression in immature odontoblasts at all stages, including cells in the dental papilla, confirming their potential to differentiate to odontoblasts.

Growth and differentiation in *Runx2*^{+/-} human primary pulp cells are different to *Runx2*^{+/+} cells as a result of variations in gene expression patterns and signaling (98). Therefore, the effects of haploinsufficiency may well influence the differentiation of odontoblasts from these cells. Runx2 is up-regulated in early odontoblasts (99), showing that levels of RUNX2 are necessary at this stage. However, unlike in osteoblasts, Runx2 expression is remarkably low or undetectable in differentiated odontoblasts (88, 100).

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 (101). It also highlights the different effects of this gene in different tissues.

Cementum

The origins of cementum-producing cells, and whether they share common precursors with osteoblasts, are unclear (102). Most cells embedded in the cellular cementum express Runx2 mRNA and RUNX2 protein to various degrees (88). As cementoblasts are mineralizing cells, this is not a surprising finding.

The result of *Runx2* haploinsufficiency on cemental tissue seems to vary between species. Both acellular and cellular cementum formation is defective in permanent teeth in CCD subjects. This does not seem to

be the case in the deciduous teeth of CCD subjects (7, 103).

Reports on cementum formation in *Runx2*^{+/-} mice are inconsistent (104, 105). CHUNG *et al.* (105) explained the similarity between mutant and wild-type mice in the study of ZOU *et al.* (104) by the mice being too young to have developed their full root length. However, cementum formation in primary teeth in subjects with CCD seems to be normal (92). If the mouse dentition does represent the human primary dentition, gross abnormalities are not expected to be found in heterozygous mice.

Reports of root morphology in CCD literature varies from excessive root length (106), deformation (8,107), and 'spiky' (108).

Periodontal ligament

Type II Runx2 is also expressed in periodontal ligament (PDL) fibroblasts, although BSP, a marker of osteoblast differentiation and biomineralization, is not (81). The action of RUNX2 seems to be suppressed by a mechanism designed to maintain PDL width (109). The factor S100A4 has been implicated (110).

However, these cells maintain the potential to differentiate to osteoblasts under certain conditions, such as mechanical stress (111). Deformation of PDL osteoblasts increases Runx2 expression, protein levels, and also its DNA-binding activity, the latter possibly being caused by activation of the ERK (extracellular signal related kinase) MAPK pathway (34), although the bone response to stress is similar in both heterozygous and wild-type mice. Orthodontic tooth movement is not affected in *Runx2*^{+/-} mice (105); one functioning gene seems to be sufficient to produce an adequate bone response.

Tooth maturation and eruption

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 by as much as 4 years when compared with unaffected subjects (8, 106, 112).

ZOU *et al.* (104) found no difference in dental development or eruption timing between heterozygous knockout and wild-type mice. One functioning allele seems to be sufficient for normal dental development in the mouse. YODA *et al.* (113) reached a similar conclusion as regards tooth development, but found a significant difference in tooth eruption times. This was explained as being caused by a time-specific lack of osteoclastic response, suggesting that heterozygous mice cannot recruit sufficient osteoclasts for active alveolar bone resorption. This is essential for the prompt timing of tooth eruption. The results also suggest the possibility that insufficient alveolar bone resorption is one of the cellular mechanisms of the delayed tooth eruption in CCD patients.

The methods used in these studies are different and may explain the disparity in the conclusions. Furthermore, it must be pointed out that in CCD the primary dentition is rarely affected. It may be assumed that the dentition in mice represents the human primary dentition, where the effects of haploinsufficiency on the eruption and formation of the human dentition may be too subtle to detect in the clinical situation. One major shortcoming of the murine model in this respect is that no secondary dentition develops.

Primary teeth erupt on time in CCD patients. Similarly, the permanent lower incisors and first molars generally erupt on time. However, the subsequent permanent teeth exhibit a delay in eruption, presumably as a result of defective eruption pathway formation (Fig. 1). There does not seem to be a close correlation between the number and positioning of supernumerary teeth and the delay in eruption (114, 115).

Similarly, birth length in CCD children is normal, but height drops below or around the second centile between 4 and 8 yr of age (116, 117). This pattern of development is reflected in the facial morphology, the characteristic frontal bossing and maxillary retrusion often not becoming evident until the later stages of childhood (118). Thus, the effect of haploinsufficiency on the craniofacial complex manifests late in a large number of cases. Whether this is caused by variation in *RUNX2* isoform levels, or simply by a greater requirement for the gene product at that age, is unclear.

As the skeletal symptoms of CCD are not usually a social or physical handicap, and develop late, one of the factors leading to a diagnosis of CCD cases may be the observed anomalies in tooth eruption leading the patient to present for treatment (8).

Tooth eruption is controlled by precise osteoclast-osteoblast interaction. Osteoclastogenesis in the alveolar bone, which is essential for the accommodation of normal tooth development and eruption, is mediated by RANK-RANKL signaling (119). The spatiotemporal pattern and relative abundance of CSF-1, RANKL, and OPG during tooth eruption are key determinants of site-specific osteoclast activity in bone surrounding the tooth (120).

Runx2 is expressed in the alveolar bone at all stages of development and during tooth eruption (4,81). Evidence points to *RUNX2* acting, either directly or indirectly, on the OPG/RANK/RANKL system to influence bone remodeling. Communication takes place between tooth germs and bone-forming/resorbing cells, synchronizing the two processes, perhaps to ensure correct spatial positioning of teeth in the jaws (82,121). The delayed and ectopic eruption of teeth seen in subjects with CCD may be caused by loss of function of *RUNX2*, both in respect of reduced CSF-1 production and disruption of the OPG/RANK/RANKL pathway.

Bone healing in CCD patients is not influenced. Normal healing has been reported after maxillofacial surgery (122), and osseointegration seems to be unaffected (123). Deciduous teeth are extracted, and bone is removed over unerupted teeth in order to encourage their eruption (124). It is possible that the inflamma-

tory response to surgery may induce tooth eruption by re-activation of monocyte recruitment and osteoclast formation.

Supernumerary teeth

Supernumerary teeth are considered to be a diagnostic feature of CCD. However, the number of supernumerary teeth is variable, and several reports exist where no supernumeraries exist or hypodontia is reported (125–127).

Minor mutations in the highly conserved RHD are, in general, more likely to produce the classic CCD phenotype than the more robust flanking Q/A and PST domains. The phenotype in mutations involving the RHD is dependent on the residual transactivation potential of the protein (126, 128).

A dose-related effect seems to be present, as the milder cases of CCD, and those exhibiting primary dental anomalies, are associated with mutations that reduce, but do not abolish, protein stability, DNA binding, and transactivation. However, attempts to correlate the number of supernumerary teeth with the severity of skeletal symptoms are inconclusive. Furthermore, identical mutations produce different numbers of supernumerary teeth (126–130). All CCD mutations, including those which primarily feature dental anomalies, have highly variable phenotypic expression. This may indicate the overlying influence of other factors.

Runx2^{-/-} and *Runx2*^{+/-} mice were both found to exhibit lingual buds in front of the upper molars, and these were much more prominent than in wild-type mice (84,87). Furthermore, *Shh* signaling was not inhibited in the lingual buds of the knockout mice (87). *Shh* is necessary for tooth formation, both in the bud and cap stages (131), and its expression is spatially controlled to limit it to regions of tooth development (132).

These buds presumably represent the mouse secondary dentition, and it is likely that *RUNX2* acts to inhibit formation of these buds. It may appear contradictory that the inhibition of *RUNX2* function may arrest primary tooth development but stimulates the formation of secondary teeth. However, it is not unusual, during embryogenesis, for the same gene to have different effects at different developmental stages (87).

RUNX2 may normally function as a cell growth inhibitor in immature osteoblasts. It acts by supporting exit from the cell cycle, thus promoting increased expression of the osteoblast phenotype (133). Human *Runx2*^{+/-} pulp cells proliferate at a far greater rate than their normal counterparts (98). This lends support to the theory that *Runx2* controls the proliferation of cells and may exert specific control on the dental lamina and the formation of successive dentitions.

It is easy to see how loss of function of this gene would encourage proliferation of the dental lamina. It is also easy to see how tooth eruption may be affected. However, the lack of correlation between the loss of *RUNX2* function and the number of supernumerary teeth confuses matters. It is interesting to note that a mutation

affecting just the terminal VWRPY repressor motif produced a phenotype with only mildly hypoplastic clavicles and supernumerary teeth (5).

Concluding remarks

Runx2 is temporally and spatially regulated. The several signaling pathways that converge on this gene, and the existence of numerous splice variants with different N and C termini, substantiate its diverse actions on bone and tooth tissues.

One effect of a mutation may be to alter the proportions of the splice variants of the gene which will, in turn, affect its downstream pathways by altering the levels of interacting products. This would have different effects on different tissues. The inconclusive results of attempts to correlate the number of supernumerary teeth with eruption timing or skeletal effects would support this, as the action of RUNX2 on the dental lamina would be different to that on osteoblast function.

The dosage sensitivity exhibited helps to explain the large intrafamilial variability. Genetic or epigenetic modifiers may influence the phenotype, as may the transcriptional status of the unmutated allele (126).

There exist several cases of clinically diagnosed CCD where no mutation is detectable. Locus heterogeneity is a possible explanation for this; however, all CCD families tested map to the 6p21 locus (134–137).

Another explanation may be mutations within as-yet poorly characterized intronic or regulatory sequences. Variations in the promoter sequence point to a possible alternative mechanism for disruption of normal RUNX2 function (138, 139). Hypermethylation of the P2 promoter of the *Runx3* gene severely affects its function (140, 141). Given the high homology between these genes, the possibility of epigenetic effects on the *Runx2* regulatory regions should not be discounted.

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