Table 1: Published and known phosphorylation sites of human RUNX2. The compilation of RUNX2 phosphorylation sites is based on cited references as well as the open, web-based bioinformatics database of protein post-translational modifications, PhosphoSitePlus (www.phosphosite.org) (Hornbeck et al., 2012). The amino acid residue numbering is according to human type II RUNX2 isoform with the N-terminus 'MASNSL' (521 amino acids, 56.648 kDa), and phosphorylation sites identified in species other than humans are listed in the renumbered form to correspond to the human amino acid numbering for the sake of consistency.

Amino acid residue	Effect of phosphorylation	References
S28	Stimulatory	(Selvamurugan *et al*., 2009;Zou *et al*., 2011)
S43	Stimulatory	(Ge *et al*., 2009)
S118	Inhibitory	(Huang *et al*., 2012;Phillips *et al*., 2006;Wee *et al*., 2002)
S196	Stimulatory	(Pande *et al*., 2013)
T198	Stimulatory	(Pande *et al*., 2013)
T200	Stimulatory	(Pande *et al*., 2013)
S237	Stimulatory	(Zou *et al*., 2011)
S240	Stimulatory	(Kim *et al*., 2006)
S275	Stimulatory	(Zou *et al*., 2011)
S294	Stimulatory	(Zou *et al*., 2011;Ge *et al*., 2009;Sierra and Towler, 2010;Li *et al*., 2012;Park *et al*., 2010)
S312	Stimulatory	(Zou *et al*., 2011;Ge *et al*., 2009;Ge *et al*., 2012;Li *et al*., 2012)
T319	Stimulatory	(Sierra and Towler, 2010)
S347	Stimulatory	(Selvamurugan et al., 2009)
S465	Inhibitory, Stimulatory	(Pierce *et al*., 2012;Zou *et al*., 2011;Qiao *et al*., 2006;Wee *et al*., 2002)
S503	Stimulatory	(Ge *et al*., 2009)

belong to a large group of enzymes generally referred to as lysine acetyltransferases, which are categorised into several protein families (for reviews, see: Kouzarides (2000), Sterner and Berger (2000) and Yang (2004)).

HATs, lysine acetyltransferases in general, as well as HDACs have been documented to interact with and even to acetylate RUNX2. The general conclusion is that acetylation results in a stimulatory effect on RUNX2 stability and transactivation capability.

The p300 protein, also referred to as E1A-associated 300 kDa protein, which functions as a transcriptional co-activator possessing intrinsic HAT activity, is able to acetylate several non-histone proteins (Kouzarides, 2000). Jeon and colleagues reported that p300 mediates RUNX2 acetylation upon BMP-2 signalling, thereby increasing RUNX2 transactivation activity as well as stability (Jeon *et al*., 2006). Furthermore, inhibition of HDAC4 and -5 which deacetylate RUNX2, enforced BMP-2 stimulated *in vitro* osteogenic differentiation and bone formation *in vivo* (Jeon *et al*., 2006). RUNX2 acetylation and stabilisation induced by BMP-2 were shown to depend on MAPK signalling (Jun *et al*., 2010). Upon PTH treatment, RUNX2 has been reported to recruit p300 to the MMP-13 promoter, both of which are required for acetylation of histones H3 and H4, and led to transcriptional activation of the target gene *MMP-13* in rat osteoblastic UMR 106-01 cells (Boumah *et al*., 2009).

Regulation of RUNX2 by ubiquitination

Protein ubiquitination plays a crucial role in protein degradation by the proteasome (for review, see Hershko and Ciechanover (1998)). This degradation pathway takes place in a cascade-like manner governed by E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases (for review, see Pickart (2001)). E3 ubiquitin ligases account for the specificity of protein ubiquitination, and proteins polyubiquitinated by these enzymes are targeted to degradation by the proteasome (for review, see Hershko and Ciechanover (1998)).

It has been shown that RUNX2 is degraded through an ubiquitination-dependent pathway by the proteasome (Tintut *et al*., 1999). An E3 ubiquitin ligase responsible for targeting RUNX2 to proteasomal degradation has been revealed to be Smad ubiquitin regulatory factor 1 (Smurf1) (Zhao *et al*., 2003). Consistently, the suppressing role of Smurf1 in osteoblast differentiation *in vitro* and *in vivo* bone formation has been reported, whereby *Smurf1* overexpression had inhibitory effects, whereas *Smurf1*-deficient mice exhibited increased bone formation through control of proteasomal degradation of MEKK2, also known as MAPK kinase kinase 2, a major upstream kinase of the MAPK pathway (Zhao *et al*., 2004; Yamashita *et al*., 2005).

Additional E3 ubiquitin ligases reported to promote RUNX2 ubiquitination and proteasomal degradation as well as to negatively regulate osteoblast differentiation are C terminus of Hsc70-interacting protein (CHIP) as well as WW domain-containing E3 ubiquitin protein ligase 1 (WWP1) together with the adaptor protein Schnurri-3 (Shn3) (Jones *et al*., 2006; Li *et al*., 2008).

In addition to E3 ubiquitin ligase-induced RUNX2 ubiquitination and degradation, another mechanism leading to ubiquitination and subsequent proteasomal degradation has been reported to be induced by cyclin D1/CDK4 and acts phosphorylation-dependently (Shen *et al*., 2006).

In summary, although the different post-translational regulation mechanisms of RUNX2 have been individually

investigated, they are not unconnected by any means, which is exemplified by the following three studies.

Jeon and colleagues have found that acetylation protects RUNX2 from Smurf1-mediated degradation, clearly suggesting a molecular link between acetylation and ubiquitination-mediated proteasomal degradation (Jeon *et al*., 2006).

Furthermore, it is worth mentioning that although many phosphorylation sites and kinases involved have been investigated, it is still poorly understood how RUNX2 phosphorylation is linked to enhanced transcriptional activity and protein stability.

Recently, Park and colleagues concluded that serine phosphorylation, exemplified with one particular serine residue (S294), triggers RUNX2 acetylation, which in turn accounts for RUNX2 transcriptional activity as well as stabilisation by inhibiting ubiquitin-dependent degradation (Park *et al*., 2010). This study indicates an additional link of the different post-translational regulation mechanisms.

Thirdly, cyclin D1/CDK4 has been reported to phosphorylate RUNX2 at S472 (Shen *et al*., 2006). However, cyclin D1/CDK4 induced not only RUNX2 phosphorylation, but also triggered subsequent ubiquitination and proteasomal degradation (Shen *et al*., 2006). Thus, this study suggests a phosphorylation-dependent proteasomal degradation of RUNX2, another link between different post-translational regulation mechanisms.

Interaction partners of RUNX2

Activity of RUNX2 is modulated by the interactions with a variety of regulatory proteins. The best-known interacting partner of RUNX2 is the non-DNA binding β subunit CBFβ. It interacts with RUNX2 by binding to the Runt domain (Kagoshima *et al*., 1993; Ogawa *et al*., 1993b; Golling *et al*., 1996). The association of RUNX2 with CBFβ both enhances the DNA binding affinity of Runt domain proteins and stabilises the interaction between RUNX2, the α subunit, and the DNA (Ogawa *et al*., 1993a; Golling *et al*., 1996). In *Drosophila*, it could be shown that the interaction between Runt domain proteins and CBFβ additionally impacts the transactivation potential of Runt domain proteins (Li and Gergen, 1999).

Next, TLE proteins (the mammalian homologues of *Drosophila* Groucho) interact with the VWRPY motif at the C-terminus of RUNX2 and in this way act as transcriptional co-repressors (Thirunavukkarasu *et al*., 1998; Javed *et al*., 2000). *Osteocalcin* is an example of a RUNX2 target gene whose activation by is repressed by TLE proteins (Javed *et al*., 2000).

Further interacting partners encompass the basic helix-loop-helix protein Hairy and Enhancer of split 1 (HES-1) which is expressed in rat osteoblastic osteosarcoma ROS17/2.8 cells (Matsue *et al*., 1997). HES-1 was shown to physically interact with RUNX2 and in this way modulates RUNX2 transactivation function (McLarren *et al*., 2000). Yes-associated protein (YAP) acts as a transcriptional co-activator of RUNX2 (Yagi *et al*., 1999), and Smads (Hanai *et al*., 1999; Zhang *et al*., 2000; Lee *et al*., 2000).

In addition, CCAAT/enhancer-binding Proteins (C/EBP) were revealed to physically interact with RUNX2 and to synergistically activate *osteocalcin* gene expression (Gutierrez *et al*., 2002). Interaction of the homeobox protein Msx2 with RUNX2 leads to the repression of transcriptional activity of RUNX2 (Shirakabe *et al*., 2001). The repressive activity of Msx2 gets counteracted by another homeobox protein Dlx5 (Shirakabe *et al*., 2001). Furthermore, c-Fos and c-Jun, the protein subunits making up the heterodimeric activator protein (AP-1), were identified as interaction partners of RUNX2 through the Runt domain, and this interaction was demonstrated to be required to activate rat collagenase 3 promoter (D'Alonzo *et al*., 2002).

In conclusion, the presence of so many co-regulators that govern RUNX2-mediated transcription indicates a complex regulation of gene expression that RUNX2 holds as a master transcription factor of osteogenesis.

Target genes of RUNX2

RUNX2 is essential for osteoblast differentiation (Banerjee *et al*., 1997; Ducy *et al*., 1997; Komori *et al*., 1997; Otto *et al*., 1997). RUNX2 regulates expression of several genes related or specific to osteoblast differentiation. For RUNX2 to be able to regulate the expression of a particular gene, the target genes require binding sites for RUNX2 in their promoter region and regulatory elements, respectively. OSE2, which was originally identified as a cis-acting element present in the mouse *osteocalcin* promoter accounting for its osteoblast-specific expression (Ducy and Karsenty, 1995), is found in the promoters of many RUNX2 target genes, is recognised by RUNX2 and serves as a RUNX2 binding site (Geoffroy *et al*., 1995). Originally, OSE2 was reported to comprise the sequence ACCACA (Geoffroy *et al*., 1995). Nucleotide sequence comparison between human, rat, mouse, rabbit collagenase 3 promoter regions and human, rat, mouse osteocalcin promoter regions showed sequence identity in the sequence AACCACA, which is generally considered as the consensus RUNX2 binding site (Jimenez *et al*., 1999). Strictly speaking, the term 'OSE2' is designated for the corresponding RUNX2 binding site in mice (Ducy and Karsenty, 1995).

Initially, RUNX2 was reported to transactivate the expression of *osteocalcin* (Ducy and Karsenty, 1995; Geoffroy *et al*., 1995; Merriman *et al*., 1995). Since then *osteocalcin* as a target gene of RUNX2 has been addressed and documented in more detail by many studies (Banerjee *et al*., 1997; Ducy *et al*., 1997; Frendo *et al*., 1998; Javed *et al*., 1999).

Furthermore, RUNX2 was found to both regulate the expression of several osteoblast marker genes in osteoblasts and induce expression of several osteoblast marker genes in non-osteoblastic cells in addition to osteocalcin: Col1α1, BSP, and osteopontin (Ducy *et al*., 1997). As regards BSP as RUNX2 target gene, conflicting results have been reported (Javed *et al*., 2001). Javed and colleagues reported that the *Gallus* BSP promoter, which contains seven functional RUNX2 binding sites, is repressed by RUNX2 both in rat and *Gallus* osteoblasts (Javed *et al*., 2001). They proposed that the repression takes place by a mechanism different from the known transcriptional repression mechanism involving TLE proteins and their

interaction with the VWRPY domain at the C-terminus of RUNX2 (Aronson *et al*., 1997; Thirunavukkarasu *et al*., 1998).

Collagenase 3, also referred to as matrix metalloproteinase 13 (MMP-13), was revealed as another target of RUNX2, as evidenced by both *in vitro* and *in vivo* experiments (Jimenez *et al*., 1999). Furthermore, the TGFβ type I receptor was revealed as another RUNX2 target gene. At least six RUNX2 binding sites were identified in the TGFβ type I receptor promoter and were shown to regulate expression of TGFβ type I receptor, by physically associating with RUNX2 (Ji *et al*., 1998). Moreover, in accordance with (Ducy *et al*., 1997), the ability of RUNX2 to directly regulate the transcriptional activation of *osteopontin* gene was substantiated by another study (Sato *et al*., 1998). Transactivation was revealed to be dependent on OSE2; any change in its nucleotide sequence AACCACA abolished its ability for RUNX2 binding (Sato *et al*., 1998). In short, most of the identified target genes of RUNX2 are regulated in a positive fashion by RUNX2 and are coding for bone ECM proteins.

Another ECM protein RUNX2 target gene is ameloblastin (Dhamija and Krebsbach, 2001). Transcription of the ameloblastin gene, which encodes a tooth-specific ECM protein, has been shown to be regulated in a positive fashion by RUNX2 (Dhamija and Krebsbach, 2001). The ameloblastin promoter region contains RUNX2 binding sites, mediating their physical interaction with RUNX2 (Dhamija and Krebsbach, 2001).

RUNX2 has been documented to regulate the expression of the osteoprotegerin gene whose promoter has been revealed to contain 12 OSE2 elements (Thirunavukkarasu *et al*., 2000). These findings indicate a molecular connection between osteoblastogenesis and osteoclastogenesis, in which RUNX2, in addition to its role in osteoblast differentiation, inhibits osteoclast formation by positively regulating osteoprotegerin, which in turn inhibits osteoclast differentiation (Thirunavukkarasu *et al*., 2000).

Another gene involved in osteoclastogenesis was identified as a RUNX2 target gene, namely receptor activator of NF-κB ligand (RANKL) (Geoffroy *et al*., 2002). This was underlined by the fact that the RANKL promoter exhibits a putative RUNX2 binding site (Kitazawa *et al*., 1999). These findings offer an explanatory approach for the elevated bone resorption rate that exceeds bone formation observed in transgenic mice overexpressing *Runx2* (Geoffroy *et al*., 2002).

During endochondral ossification, hypertrophy of chondrocytes in the cartilaginous template is followed by invasion of blood vessels into cartilage. As a result, osteoblast as well as chondro-/osteoclasts are brought into the cartilaginous template, ultimately remodelling the cartilaginous template into bone. In hypertrophic chondrocytes, RUNX2 was reported to increase the activity of a BMP-responsive region of the promoter of collagen type X (Leboy *et al*., 2001). Together with the fact that the BMP-responsive region of the promoter of collagen type X contains a RUNX2 consensus binding site (Leboy *et al*., 2001), RUNX2 was found to directly regulate the expression of the commonly known hypertrophic chondrocyte marker collagen type X through interaction with its cis-enhancer (Li *et al*., 2011). Moreover, invasion of blood vessels into the cartilage comes along with VEGF upregulation in hypertrophic chondrocytes (Haigh *et al*., 2000). *Vegf* was revealed as another gene, the expression of which gets upregulated upon RUNX2 in hypertrophic chondrocytes (Zelzer *et al*., 2001).

Identification of further putative RUNX2 target genes was approached by searching for genes differentially expressed in C3H10T1/2 mesenchymal precursor cells overexpressing *Runx2* compared to wild type cells, using a differential hybridisation technique and cDNA microarray analysis (Stock *et al*., 2004). The candidate target gene with the strongest difference in expression between *Runx2*-overexpressing and wild type cells was pituitary tumour-transforming 1 interacting protein (*Pttg1ip*) (Stock *et al*., 2004). Furthermore, *Pttg1ip* was not only shown to be expressed in osteoblast-like MC3T3-E1 cells and in primary mouse calvarial cells, but RUNX2 also binds to the 5' flanking region of murine *Pttg1ip* and directly transactivates expression of *Pttg1ip* (Stock *et al*., 2004). These findings provided the presumption that *PTTG1IP* is under transcriptional control of RUNX2 (Stock *et al*., 2004). However, human PTTG1IP has been reported to be ubiquitously expressed in human adult tissues, and its exact function remains blurred (Chien and Pei, 2000). The *Pttg1ip* expression patterns both in different murine cell lines, as well as in mouse embryos, revealed that *Pttg1ip* expression is regulated by RUNX2 in a temporal and tissue-specific manner, but also indicated that other transcription factors must be involved in the transcriptional regulation of *Pttg1ip*. Additionally, RUNX2 has been reported to regulate the transcription of galectin-3, whose promoter contains RUNX2 binding sites (Stock *et al*., 2003). The expression pattern of galectin-3 includes several tissues and developmental stages. Amongst others, galectin-3 had been attributed a role in chondrocyte maturation (Colnot *et al*., 2001). This finding is in line with the fact that RUNX2 functions as a positive regulator on galectin-3 transcription, since RUNX2 is expressed in growth plate chondrocytes. However, RUNX1 and RUNX3 exhibit overlapping expression patterns with galectin-3 expression expressed in growth plate cartilage as well and bind to same consensus sequences like RUNX2. Therefore, galectin-3 expression, both at skeletal and extra-skeletal sites, might not be regulated exclusively by RUNX2, but rather galectin-3 represents a common target of the different RUNXs (Stock *et al*., 2003). In addition, galectin-3 has been implicated in tumourigenesis, tumour progression and metastasis formation (Takenaka *et al*., 2004; Liu and Rabinovich, 2005). More recently, RUNX2 has been revealed to be expressed in human glioma cells and RUNX2-mediated galectin-3 expression was suggested to functionally contribute to glial tumour malignancy (Vladimirova *et al*., 2008).

In summary, the opposing regulation of osteoblast marker genes highlights the importance of the promoter context of RUNX2 binding sites, making up the transcriptional control of the RUNX2 target genes.

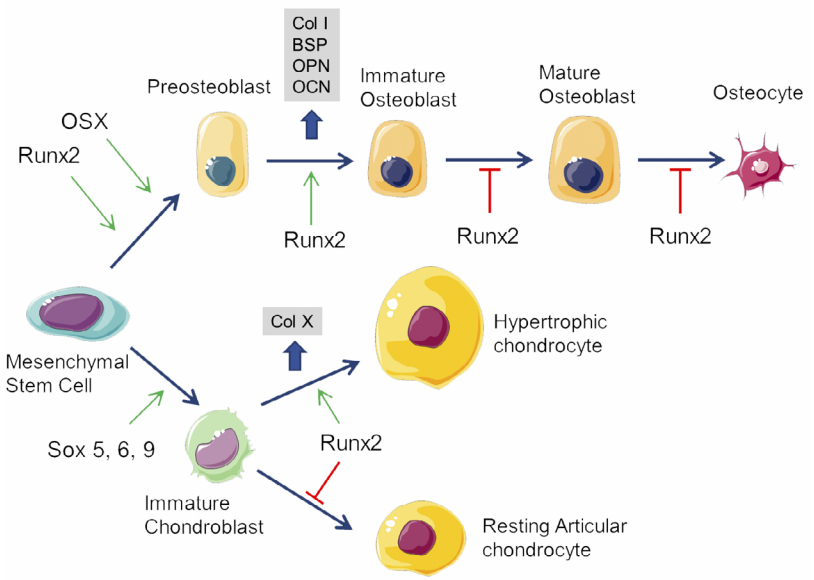

Fig. 2. Regulation of osteoblast and chondrocyte differentiation by Runx2. During the process of osteoblast differentiation, Runx2 is crucial for the commitment of mesenchymal stem cells to the osteoblast lineage and positively influences early stages of osteoblast differentiation. Osterix (OSX) starts playing an important role in osteoblast differentiation following Runx2-mediated mesenchymal condensation. During the process of osteoblast differentiation, Runx2 is involved in the expression of bone matrix genes Col1, osteopontin (OPN), BSP, and osteocalcin (OCN) and maintains the expression of OPN and BSP. For further bone maturation, Runx2 expression has to be downregulated. During the process of chondrocyte differentiation initiated by Sox9-mediated mesenchymal condensation, Runx2 is crucial for chondrocyte maturation from immature to terminal hypertrophic chondrocytes, and inhibits immature chondrocytes from adopting the phenotype of permanent cartilage. Runx2 induces expression of ColX in hypertrophic chondrocytes and is involved in the matrix production of terminal hypertrophic chondrocytes.

Biological functions

RUNX2 is best known as the master regulator of osteoblast differentiation and osteoblast marker gene expression as well as osteoblast function. In fact, the osteogenic activity of bone marrow stromal cells was reported to be enhanced upon *Runx2* overexpression, both *in vitro* and *in vivo* (Zhao *et al*., 2005). Primary murine MSCs transduced with RUNX2-producing AdRunx2 formed more ectopic bone *in vivo* than cells transduced with control virus. However, one drawback arose to be the formation of osteosarcoma (Zhao *et al*., 2005).

A variety of additional biological functions of RUNX2 have been demonstrated, which include:

- antiproliferative role in (pre)osteoblasts (Pratap *et al*., 2003; Galindo *et al*., 2005)
- tooth development (D'Souza *et al*., 1999)
- chondrocyte maturation and hypertrophy (Takeda *et al*., 2001; Yoshida *et al*., 2004), as evidenced by the induction of collagen type X (Col10a), a marker specific for hypertrophic chondrocytes (Enomoto *et al*., 2000)
- tumour metastasis to bone (Pratap *et al*., 2006)
- inhibition of rRNA transcription (Young *et al*., 2007)
- endothelial cell biology as well as angiogenesis (Namba *et al*., 2000; Sun *et al*., 2001; Sun *et al*., 2004).

In osteoblast biology, RUNX2 regulates the process of osteoblast differentiation at different stages. Regulation by RUNX2 takes place in a positive manner at early stages of differentiation, while RUNX2 inhibits the process at later stages (Fig. 2). The whole process from an undifferentiated MSC to an osteoblast occurs in different phases, and each of these phases is characterised by a particular pattern of expressed osteoblast marker genes. RUNX2 controls expression of osteoblast marker genes by binding to OSE2, the RUNX2 binding site, found in the promoter region of all major osteoblast marker genes. The functions of RUNX2 in osteoblast and chondrocyte differentiation are depicted in Fig. 2.

Regulation of osteoblast differentiation by RUNX2 overall demonstrates a stage-dependent shift of Runx2 from a positive to negative regulator of osteoblastic differentiation. In addition, the different RUNX2 isoforms have been assigned the regulation of distinctive stages of osteoblast differentiation. In mice, the two major RUNX2 isoforms, type I and II, have been revealed to possess distinct sub-functions within osteoblast biology. First, as regards the regulation of different stages of osteoblast differentiation, expression of both RUNX2 type I and II isoform have been detected in osteoblasts. However, RUNX2 type I isoform also existed in osteoprogenitor cells and preosteoblasts (Choi *et al*., 2002). Thus, RUNX2 type I has been found to have an exclusive role in early osteoblastogenesis, while RUNX2 type II is necessary for terminal stages of osteoblastic maturation (Choi *et al*., 2002; Xiao *et al*., 2004). Second, while it has been demonstrated that type I isoform is sufficient for

intramembranous ossification, both intramembranous and endochondral ossification have been revealed to be affected in selective deficiency of type II *Runx2* (Xiao *et al*., 2004).

Conclusion

Taken together, it is clear that RUNX2 is a tightly regulated factor and the specific context in which an analysis is performed needs to be considered when using RUNX2 as a marker for *in vitro* studies. Particularly when detecting mRNA message, the particular isoforms need to be considered.

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Discussion with Reviewers

R. Porter: You have provided many examples of how Runx2 activity is regulated both physiologically and pathologically, ranging from transcriptional control to post-translational modification. Does the existing literature point to one or more particular points of regulation that can be exploited for pro-osteogenic applications, such as bone tissue engineering using mesenchymal stem cells?
Authors: There are a number of points of regulation that have been proposed. The main issue is due to the fact that Runx2 expression has differing effects depending on the developmental stage of the cell. In addition, its interaction with other factors, such as Sox9, means that targeting one specific factor may not be sufficient to induce a stable change in phenotype.

R. Porter: Conversely, what about cartilage tissue engineering applications, when Runx2 activity in stem cells may be detrimental to the production of hyaline cartilage? Is there evidence that Runx2 inhibition can prevent the hypertrophic maturation of MSCs *in vitro*, or is the interaction of Runx2 with other transcription factors, namely Sox9, too complex for completely ablating its activity within MSCs?
Authors: Surprisingly little has been published on chondrogenic induction. Inhibiting Runx2 expression does reduce hypertrophy, but as most methods do not completely ablate Runx2 it is not clear whether Sox9 becoming more dominant is sufficient or if Runx2 still plays a role in maintenance of the chondrocyte phenotype. It is unlikely that downregulation of Runx2 in itself will act as a trigger for chondrogenesis. We have demonstrated that knock-down of Sox9 mildly enhances osteogenesis but only when an osteogenic signal is present (Loebel *et al*., 2014, additional reference). It has also been shown that chondrocytes isolated from rib cartilage of Runx2 null mice have an increased tendency to undergo *in vitro* adipogenesis in a process related to IL-11 (Enomoto *et al*., 2004, additional reference). This would suggest that the interplay may involve more than just two transcription factors.

Reviewer IV: Most cited references are from the period around 2000. Why are there so few recent references?
Authors: The reason why most of the references are late 1990s and early 2000s is that this was the time when most of the seminal breakthroughs were made. Runx2 research still proceeds but with fewer more recent breakthroughs.

Reviewer IV: Could you provide a reference for the thesis by the first author to which you refer?
Authors: The reference is Bruderer (2014) (additional references).

Additional References

Bruderer M (2014) Transcription factor-specific reporter constructs – basis for the functional identification and isolation of subpopulations of human mesenchymal stem cells and tool for live cell approaches. Ph.D. Thesis, Swiss Federal Institute of Technology (ETH), Zürich (http://e-collection.library.ethz.ch/view/eth:8760).

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