



Role of the Transcription Factor Zinc Finger Protein 521 on Runx2 Acetylation

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Role of the Transcription Factor Zinc Finger Protein 521 on Runx2 Acetylation

A Thesis Presented by

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To

The Faculty of the Medicine

In partial Fulfillment of the Requirement

For the Degree of

Doctor of Medical Science

In the Subject of Oral Biology

Research Mentor: Roland Baron, DDS, PhD

Research Supervisor: Francesca Gori, PhD

Harvard School of Dental Medicine

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- Thesis Supervisor: Dr. Francesca Gori
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 - Dr. Giuseppe Intini
- Dr. Baron' lab members

Dedication:

The recognition of this work was only feasible by incredible support of Professor Roland Baron, the primary investigator of this research and Dr. Francesca Gori, research supervisor, which desire to express my ultimate gratefulness. I would like to extend my appreciation to lab members for incredible encouragements and supports.

My enthusiasm for bone biology and research in this area has guided me to begin conducting intriguing research project involving the study of role of zinc finger protein 521 on Runx2 acetylation at Harvard School of Dental Medicine (HSDM). My research experiences in bone biology have solidified my profound interest in this field. I deem my research endeavors have broadened my horizon and provided me a greater sense of gratitude toward this field.

I have had incredible inspiring and rewarding educational experiences in my research year; subsequently it has led me to inscribe my thesis as a partial requirement of my Doctor of Medical Science, DMSc at Harvard School Dental Medicine. Ultimately, my science lectures and research experiences in the area of bone biology have enriched my understanding of science. I am enthusiastic that my academic and personal experiences will allow me to make a positive contribution to the Orthodontics and oral biology.

Ultimately, I dedicate the thesis to my parents for their infinite love, support, and encouragements.

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Abstract

Runx2 is a transcription factor that has a crucial role in the development of bone; haploinsufficiency of Runx2 leads to the autosomal-dominant disorder, cleidocranial dysplasia (CCD) characterized by various skeletal abnormalities. Zinc finger protein 521 (Zfp521) is a transcription factor that is expressed in several cell types including bone. Recent studies demonstrated that Zfp521 interacts with Runx2 and regulates osteoblast maturation at least in part by repressing the transcriptional activity of Runx2; furthermore, it was demonstrated that the repression of Runx2 by Zfp521 involves the recruitment of HDAC3. The interaction of Runx2 with HDAC3 is strongly enhanced by Zfp521. Zfp521 may regulate osteoblast commitment and differentiation by modulating the Runx2 transcriptional activity by decreasing the levels of Runx2 acetylation.

Objective: Runx2 is a key regulator of osteoblast differentiation; Zfp521 may regulate osteoblast commitment and differentiation, at least in part by decreasing the levels of Runx2 acetylation. We investigated 1) Effect of Zfp521 on Runx2 acetylation in HEK293 cells 2) The endogenous Runx2 acetylation levels in MC3T3-E1 cells during osteoblast differentiation.

Results: These studies demonstrated that Runx2 acetylation is decreased when Zfp521 is stable expressed in HEK293 cells. Runx2 acetylation levels were detected using immunoprecipitation analyses. Sodium butyrate (NaB) prevents protein deacetylation by inhibiting HDACs. The treatment of cells with NaB increased global protein

acetylation levels. Importantly, stable expression of Zfp521 did not change global protein acetylation. Therefore, this study suggested that Zfp521 specifically influences Runx2 acetylation. In addition, our findings suggest that Zfp521 impairs Runx2 acetylation by HDACs. Preliminary results show that transient transfection of P300 in HEK293 increased Runx2 acetylation levels. However, stable expression of Zfp521 can still partially decrease Runx2 acetylation levels. This study suggests that Zfp521 function can be linked to P300.

Runx2 acetylation levels were then assessed in MC3T3-E1 cells during osteoblast differentiation. While Runx2 protein levels increase by 7 days in culture, and gradually decreases by days 14, Runx2 acetylation was undetectable.

Conclusion: Runx2 is the transcription factor that has essential role in osteoblast commitment and differentiation. Zfp521 represses the transcriptional activity of Runx2 by recruiting HDAC3. These studies suggest that Zfp521 modulates Runx2 activity by decreasing Runx2 acetylation level. These studies have extended our knowledge of the mechanisms by which Zfp521 regulates osteoblast differentiation and bone formation, which could have important implications for on the development of future osteo-anabolic treatments.

Introduction

Zfp521, a 30 zinc-finger containing protein, is expressed in several cell types including bone. During endochondral bone development, Zfp521 is highly expressed in mesenchymal condensations, in prehypertrophic chondrocytes in the growth plate as well as in osteoblasts and osteocytes. Dr. Baron's group demonstrated that Zfp521 plays a critical role in osteoblast biology by demonstrating that overexpressing Zfp521 in mature osteoblasts *in vivo* increases bone formation and bone mass, whereas deletion of Zfp521 leads to impaired osteoblast maturation and decreased bone matrix formation (Hesse *et al.*, 2010). These studies also suggest that Zfp521 interacts with Runx2 and regulates osteoblast maturation at least in part by repressing the transcriptional activity of this osteoblast transcription factor. Interestingly, these studies demonstrate that the repression of Runx2 by Zfp521 involves the recruitment of HDAC3, a known Runx2 co-repressor (Hesse *et al.*, 2010).

The goals of these studies are to gain further insight into the molecular mechanisms by which Zfp521 modulates Runx2 activity. Several investigations have shown that Runx2 protein levels and activity are regulated independently of mRNA expression and are modulated through post-translational modifications, including acetylation (Jensen *et al.*, 2007; Lian *et al.*, 2004). Acetylation of the ϵ -amino group of lysine is a reversible post-translational modification, catalyzed by acetyltransferases (HATs) and deacetylases (HDACs), which neutralize the positive charge of this amino

acid, thereby changing protein function (Polevoda *et al.*, 2002). The interaction of Runx2 with HDAC3 is strongly enhanced by Zfp521; and that knockdown of HDAC3 by shRNA leads to the loss of Zfp521-induced repression (Hesse *et al.*, 2010). These findings suggest that Zfp521 may modulate Runx2 protein acetylation during osteoblast differentiation

We hypothesized that Zfp521 decreases acetylation of Runx2 protein, thereby affecting its function. Because Runx2 is a key regulator of osteoblast differentiation, Zfp521 may regulate osteoblast commitment and differentiation, at least in part by decreasing the levels of Runx2 acetylation.

Aim I: Investigate whether Zfp521 affects Runx2 acetylation:

- i. Effect of Zfp521 on Runx2 acetylation levels in HEK293 cells stably expressing Zfp521
- ii. Effect of Zfp521 on Runx2 acetylation levels in HEK293T cells transiently transfected with Runx2 and Zfp521
- iii. Effect of Zfp521 on global protein acetylation in HEK293 cells
- iv. Effect of Zfp521 on Runx2 acetylation levels in presence of HDACs inhibitors
- v. Effect of Zfp521 on Runx2 acetylation levels in the presence of P300

Aim II: Investigate whether Runx2 acetylation levels are affected during osteoblast differentiation of MC3T3-E1.

The studies will elucidate our understanding of the mechanisms by which Runx2 and Zfp521 regulate osteoblast differentiation and bone formation, which could have significant implications on improvement of future therapies for skeletal dysfunctions.

Review of the Literature

Bone formation occurs via two major processes, intramembranous and endochondral ossification. Both intramembranous and endochondral ossification begin with condensation of mesenchymal cells that form a template for the skeleton and end with formation of a mineralized skeleton (Karsenty *et al.*, 2009; Goldring *et al.*, 2006). However, while intramembranous ossification occurs by direct differentiation of mesenchymal cells into osteoblasts, the endochondral bone formation is a multistep process where chondrocytes form a template in which vascular invasion occurs followed by formation of an ossification center containing type I collagen-expressing cells. In both processes, osteoblasts play crucial role in directing the deposition and calcification of the bone matrix. This bone matrix provides a reservoir of minerals and growth factors that affect skeletal homeostasis. Mesenchymal stem cells have the potential to differentiate into chondrocytes, adipocytes and myoblasts. Chondrocytes and osteoblasts along with osteoclasts are the major cell types involved in skeletal homeostasis. Proliferation, commitment, and acquisition of molecular and phenotypic markers, as well as their ability to form unique tissues (cartilage and bone) are regulated by tissue-specific transcription factors as well as paracrine and autocrine mechanisms (Karsenty *et al.*, 2009).

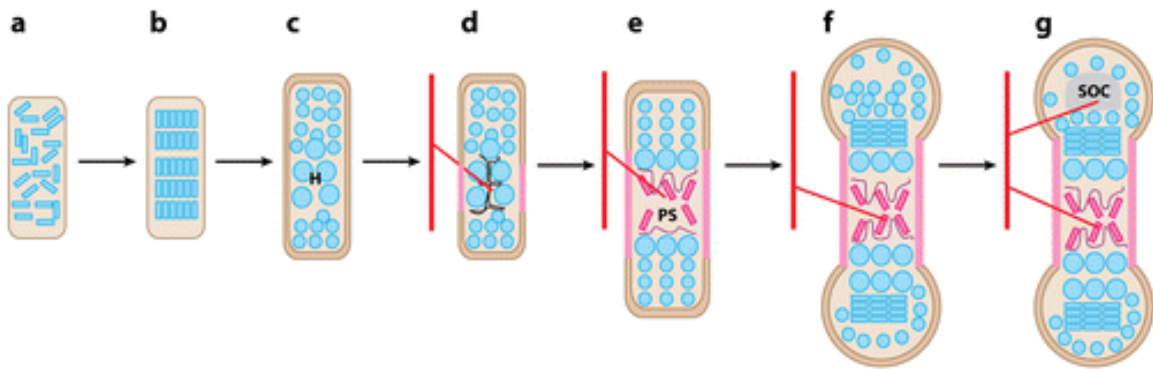


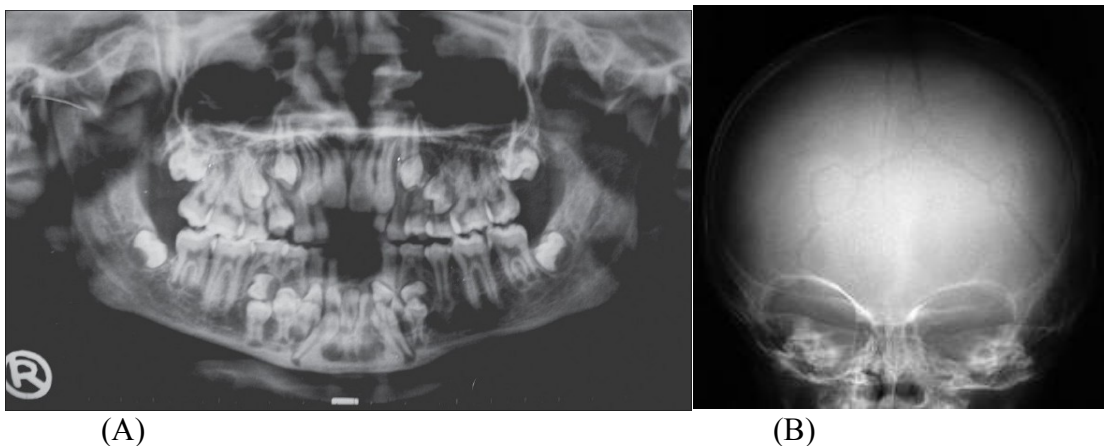
Figure 1 Schematic representation of endochondral bone formation. (a) Mesenchymal cells (blue) condense in the location of the future skeletal element. (b) Cells of condensations differentiate in chondrocytes and start to proliferate. (c) Hypertrophic chondrocyte differentiation (H). (d) Perichondrial cells differentiate in osteoblasts, forming bone collar (pink). Hypertrophic chondrocyte apoptosis favors matrix mineralization and blood vessel invasion (red). (e) Osteoblasts accompany vascular invasion, forming the primary spongiosa (PS). (f) Chondrocytes continue to proliferate, lengthening the bone. Osteoblasts of primary spongiosa form trabecular bone, while at the bone collar osteoblasts form cortical bone. (g) The secondary ossification center (SOC) forms through cycles of chondrocyte hypertrophy, vascular invasion, and osteoblast activity (Karsenty *et al.*, 2009).

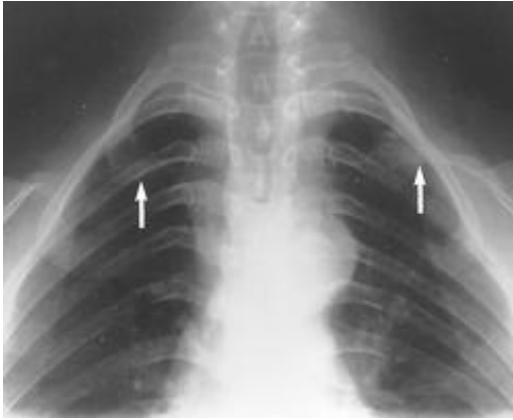
Transcription factors including Runx2 and Osterix are master regulators of osteoblast differentiation, while Sox9 has a crucial role for chondrocyte differentiation and C/EBP and PPAR γ regulate adipocytes differentiation (Komori *et al.*, 1997; Otto *et*

al., 1997, Ducy *et al.*, 1997; Nakashima *et al.*, 2002; Tong *et al.*, 2005). Many signaling pathways regulate these transcriptional programs, including transforming growth factor- β (TGF- β), bone morphogenetic proteins (BMPs), WNT, fibroblast growth factor (FGF) and parathyroid hormone (PTH) among others (Bandyopadhyay *et al.*, 2006, Maruyama *et al.*, 2010, Ono *et al.*, 2011).

Transcription Factor Runx2:

Runx2 is a transcription factor, which has a crucial role in the development and maintenance of mammalian bone and teeth (Li *et al.*, 2011; Liu *et al.*, 2001). In humans, two functional copies of Runx2 are required for proper skeletal and dental development. Haploinsufficiency of Runx2 leads to the autosomal-dominant disorder cleidocranial dysplasia (CCD) characterized by hypoplastic clavicle, large open space between the frontal and parietal bones of the skull, supernumerary teeth, and other skeletal abnormalities (Komori *et al.*, 1997; Otto *et al.*, 1997, Ducy *et al.*, 1997) (Figure 2).





(C)

Figure 2: Characteristic of cleidocranial dysplasia (CCD): A: Panoramic X-ray of the patient with Cleidocranial dysplasia (CCD) presents with numerous supernumerary teeth embedded inside the jaw. B: This image indicating the open space between frontal and parietal skull bone as the result of fontanelle are failed to close in cleidocranial dysplasia patient. C: The arrows indicate the hypoplastic clavicles in CCD patient, which leads to hypermobility of shoulders. Patients with CCD are capable of touching shoulders together in front of chest due to hypoplastic or lack of formation of clavicles.

Although other transcription factors, essential for osteoblast differentiation have been identified, Runx2 was the first osteoblast-specific transcription factor to be discovered (Banerjee *et al.*, 1997, Komori *et al.*, 1997, Stamp *et al.*, 1997; Otto *et al.*, 1997, Ducy *et al.*, 1997, Selvamurugan *et al.*, 1998) and today it is still the earliest and most specific transcription factors expressed in osteoblasts.

There are 3 Runx in mammals: Runx1, Runx2 and Runx3. Runx1 is important for hematopoiesis, Runx2 is essential for regulation of osteoblast differentiation and bone formation, and Runx3 has function in neurogenesis and together with Runx2, regulates endochondral bone formation (Ducy *et al.*, 1997; Knezevic *et al.*, 2011; Greenblatt *et al.*, 2010; Woolf *et al.*, 2006). Through mouse models and cell-culture studies, it is now established that Runx2 is necessary for osteoblast commitment and differentiation as well as for chondrocyte hypertrophy. It has been shown that Runx2 deficient mice lack both intramembranous and endochondral ossification due to absence of osteoblasts and display a delay or absence of chondrocyte hypertrophy (Komori *et al.*, 1997; Otto *et al.*, 1997; Jonason *et al.*, 2009; Bae *et al.*, 2006; Lee *et al.*, 2002; Ducy *et al.*, 1997). On the other hand, overexpression of Runx2 in mature osteoblasts *in vivo* results in delayed osteoblast differentiation and osteopenia and prevents the anabolic effects of PTH (Liu *et al.*, 2009; Merciris *et al.*, 2007), suggesting that Runx2 negatively regulates osteoblast maturation (Maruyama *et al.*, 2007). Thus, Runx2 needs to be tightly regulated in order to ensure proper osteoblast function. At the molecular level, Runx2 interacts with other proteins and regulatory complexes that either activate or repress transcription. Regulation of Runx2 occurs on multiple levels through multiple signaling pathways including BMPs, TGFb, FGF, and the canonical Wnt signaling pathway among others (Shu *et al.*, 2011; Sabbieti *et al.*, 2009; Rodríguez *et al.*, 2011; Ohyama *et al.*, 2011).

As detailed below, Runx2 activity is regulated not only at mRNA level, but also at protein levels through post-translational modifications that likely affects protein-protein

interactions between Runx2 and other members of the Runx2 transcriptional regulatory complexes (Jensen et al., 2007; Lian et al., 2004)

The Zinc finger protein 521:

The Zinc finger protein 521 (Zfp521) gene consists of 30 Kruppel-like zinc finger motifs grouped in five to seven zinc fingers and scattered throughout the protein (Bond *et al.*, 2008). High levels of Zfp521 are found in B-cell lymphomas and in several human acute myeloid leukemias, suggesting that Zfp521 may contribute to these human hematological malignancies (Bond *et al.*, 2008). Zfp521 is thought to have an inhibitory function in hematopoietic stem cell differentiation. Overexpression of Zfp521 in hematopoietic progenitors favors their expansion while blocking their differentiation (Mega *et al.*, 2011; Bond *et al.*, 2004). In addition, it has been recently demonstrated that in the absence of Zfp521 embryonic stem (ES) cells do not undergo neural conversion, suggesting that this transcription factor plays an important role in the differentiation of ES cells into neural progenitors (Kamiya *et al.*, 2011) (Figure 3).

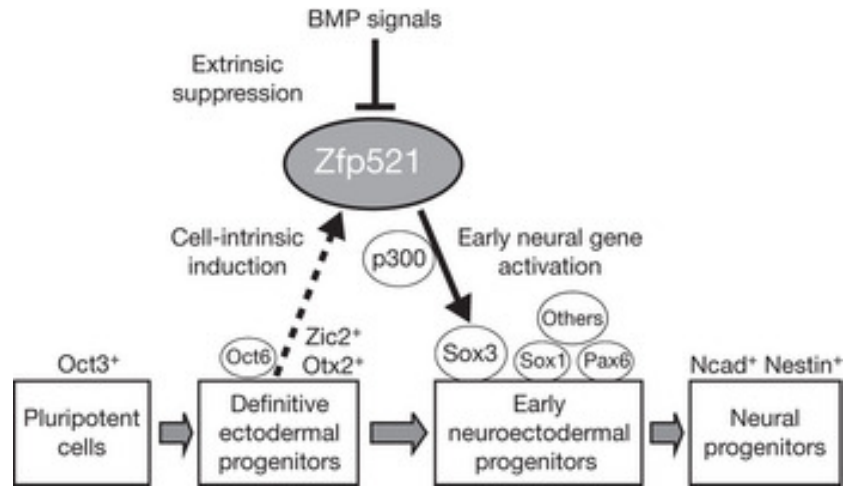
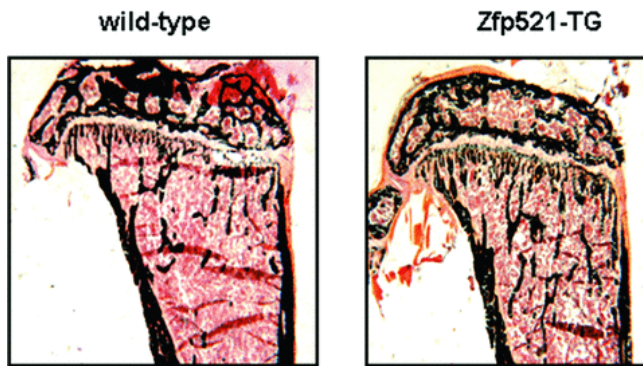


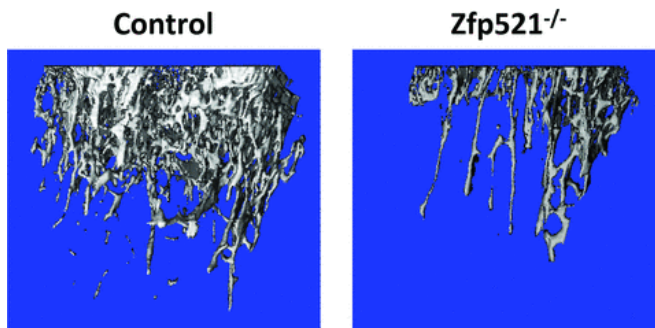
Figure 3: Effect of Zfp521 on neural differentiation: Zfp521 is crucial for differentiation of the Neural cells. The function of zfp521 is linked to histone acetylates transferase (HAT) P300. Zfp521 is associated with a co-activator P300 in inducing the differentiation of embryonic stem cells to neural progenitors.

Zfp521 is expressed in several cell types including bone. During endochondral bone development, Zfp521 is highly expressed in mesenchymal condensations and prehypertrophic chondrocytes in the growth plate as well as in osteoblasts and osteocytes (Hesse *et al.*, 2010; Wu *et al.*, 2008). The expression of Zfp521 increases during osteoblast differentiation *in vitro* and its expression is regulated by PTHrP and BMPs (Correa *et al.*, 2010, Wu *et al.*, 2009). Gain or loss of function studies in mice has demonstrated that Zfp521 plays an important role in osteoblast commitment and differentiation. Overexpression of Zfp521 in mature osteoblasts, using the osteocalcin (OG2) promoter was demonstrated to increase osteoblast numbers and bone mass (Wu *et*

al., 2009; Hesse *et al.*, 2010). In contrast, *in vitro* studies in osteoblasts isolated from OG2-Zfp521 transgenic mice showed that Zfp521 antagonizes osteoblast differentiation, decreasing osteoblast maturation, and bone nodule formation while increasing CFU-F (Hesse *et al.*, 2010)



(A)



(B)

Figure 4: Effect of Zinc Finger protein 521 on bone formation: (A) Histology of osteoblast-targeted gain (Zfp521-TG) demonstrated to increase the bone mass. (B) Germ-line loss (Zfp521^{-/-}) of Zfp521 function mouse models is demonstrated the decreasing bone formation and characterize the osteopenia as shown my Micro-CT

Thus, while Zfp521 regresses the rate of transition of osteoprogenitor into the committed stage, this transcription factor favors the progression of late stage of differentiation (Hesse *et al.*, 2010). By reducing osteoblast commitment, Zfp521 maintains the required pool of precursors. In addition, by increasing the rate of late stage of differentiation, Zfp521 allows matrix production and thereby bone formation (Hesse *et al.*, 2010). Consistent with these data, global and osteoblast-specific deletion of Zfp521 results in decreased bone formation, increased osteoblast numbers, and decreased late osteoblast specific genes. Interestingly, this dual action of Zfp521 on osteoblast function mirrors that of Runx2. In fact, while Runx2 is required for the commitment of mesenchymal cells to the osteoblast lineage, it acts as an antagonist of osteoblast maturation (Hesse *et al.*, 2010).

Dr. Baron's group has demonstrated that Zfp521 and Runx2 physically interact and that Zfp521 represses Runx2 transcriptional activity. Site-directed mutagenesis studies have demonstrated that Zfp521 has two Runx2 binding sites, one in the region spanning ZFs 6 to 10 and one ZFs 26 to 30. Confirming the important role of these ZFs, Zfp521's ability to bind to and repress Runx2 transcriptional activity is compromised when ZFs 6 and 26 are deleted (Hesse *et al.*, 2010). In addition, enforced expression of Runx2 rescues the Zfp521-induced repression of early osteoblast differentiation and Zfp521 antagonizes Runx2 *in vivo*, worsening the CCD phenotype and rescuing the Runx2 overexpression phenotype (Hesse *et al.*, 2010). Zfp521 indeed acts to oppose

Runx2-induced osteoblast differentiation and bone mineralization under physiological conditions in vivo (Hesse *et al.*, 2010) (figure 5).

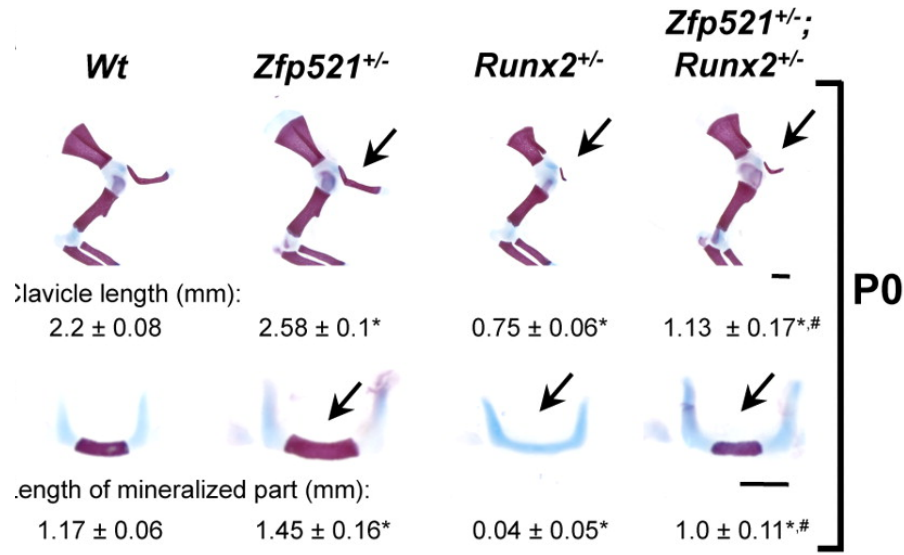


Figure 5: Zfp521 is a physiological antagonist of Runx2 in vivo: Runx2^{+/-} *mice exhibit characters of the cleidocranial dysplasia with hypoplastic clavicles, a hypoplastic hyoid bone with a delay in mineralization due to significant reduction of Runx2 gene dose. However, the length of both the clavicle and the mineralized mid-portion of the hyoid bone were significantly increased in Zfp521*^{+/-} *mice compared with Wt. Mice were generated with a heterozygous germ-line deletion of Zfp521 (Zfp521*^{+/-}*) and crossed these animals with Runx2*^{+/-} *mice to generate Zfp521*^{+/-}*; Runx2*^{+/-} *double heterozygous mice. As the result, the hypoplasia of the clavicle and the delayed mineralization of the mid-portion of the hyoid bone of the Runx2*^{+/-} *mice were partially normalized in the Zfp521*^{+/-}*; Runx2*^{+/-} *mice.*

Most interestingly and with the scope of these studies, repression of Runx2 by Zfp521 involves the recruitment of HDAC3, a histone deacetylase and a known Runx2 co-repressor (Vega *et al.*, 2004; Schroeder *et al.*, 2004; Haberland *et al.*, 2009). The interaction of Runx2 with HDAC3 is strongly enhanced by Zfp521 and inhibition of HDAC3 by HDAC3 shRNA leads to the loss of Zfp521-induced repression, suggesting that Zfp521 acts as an adaptor bridging Runx2 and HDAC3 to repress Runx2 (Hesse *et al.*, 2010). Thus, Runx2 and Zfp521 are important in balancing osteoblast formation and maturation (Hesse *et al.*, 2010) (Figure 6). By interacting with Runx2 and by antagonizing its activity, Zfp521 regulates the rate of osteoblast differentiation (Wu *et al.*, 2009; Hesse *et al.*, 2010).

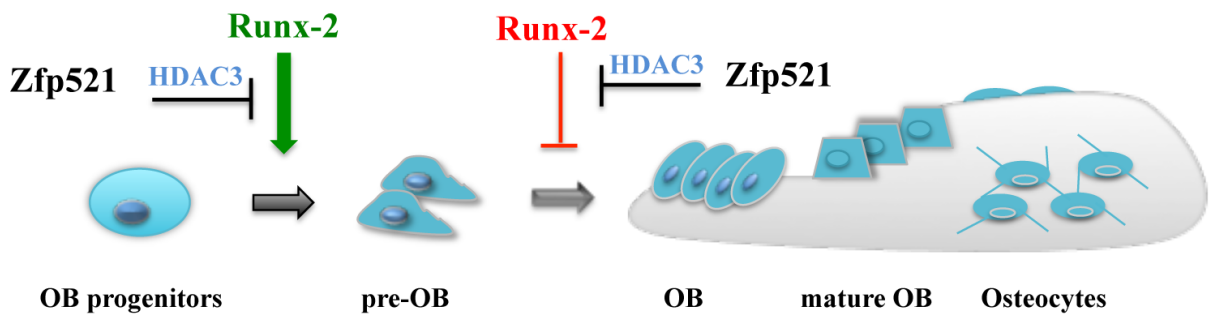


Figure 6. Zfp521 and osteoblast differentiation: Runx2 is required for commitment of progenitor cells to the osteoblast lineage. Zfp521 antagonizes this early positive effect of Runx2, leading to decreased osteoblast differentiation and an increased number of progenitors. Runx2 antagonizes osteoblast differentiation, blocking osteoblast maturation.

Zfp521 antagonizes this negative effect of Runx2, thereby promoting osteoblast maturation. Zfp521 form a complex with HDAC3 and Runx2, stabilizing the association of these two factors.

Protein acetylation:

Gene transcription is a highly regulated process, and acetylation plays a major role in this regulation (Figure 7). Acetylation of the ϵ -amino group of lysine residues is a reversible post-translational modification, which neutralizes the positive charge of this amino acid, thus changing protein function (Polevoda *et al.*, 2002).

Acetylation is a dynamic process that is catalyzed by acetyltransferases (HATs) and deacetylases (HDACs) (Peng *et al.*, 2011). Acetylation can modify the DNA recognition sites, modulate the stability of proteins and alter protein-protein interactions. Acetylation within histone protein tails reduces their affinity for DNA, thereby relaxing the chromatin structure and increasing the accessibility of DNA to transcription factors. Therefore, at the molecular level, this modification can be essential in regulating gene expression (Peng *et al.*, 2011).

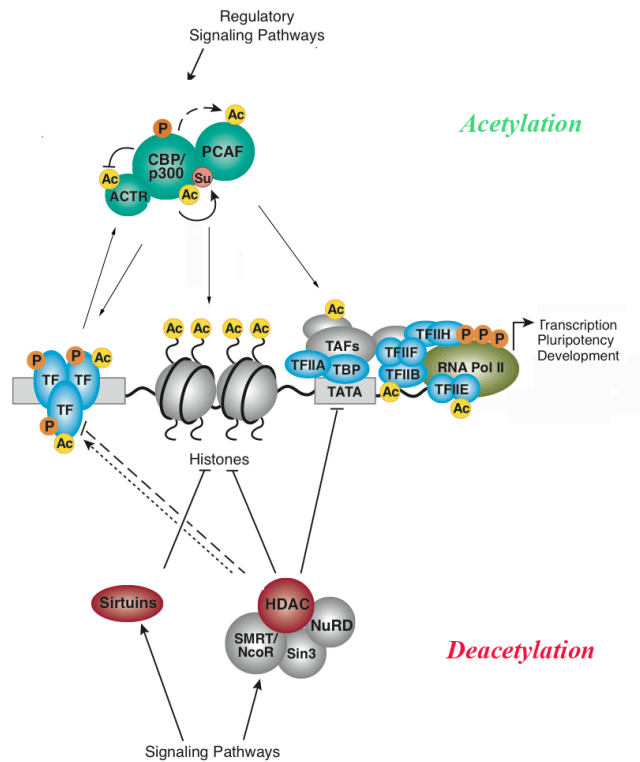


Figure 7. Protein acetylation and deacetylation: Protein acetylation plays a crucial role in regulating chromatin structure and transcriptional activity. Acetylation complexes, including CBP/p300 and PCAF or deacetylation complexes including, HDACs and NuRD are recruited to DNA-bound transcription factors in response to signaling pathways. (Modified from *Cell Signaling*)

HATs and HDACs modulate protein acetylation (Kuo *et al.*, 1998). HATs can effectively catalyze the acetyl group transfer by using oacetyl-CoA (Sternier *et al.*, 2000). The P300/CBP is one of a main group of HATs. P300/CBP and PCAF are transcriptional

co-activators that acetylate histones and non-histone proteins including p53, NF-KB, and Samd group (Kim *et al.*, 2011; Simonsson *et al.*, 2006, Siminsson *et al.*, 2005; Liu *et al.*, 1999; Yao *et al.*, 2001). In addition, HAT activity can be increased by process of auto-acetylation of PCAF and P300 (Kim *et al.*, 2011).

HDACs are grouped into four classes: Class I (HDAC 1, 2, 3, and 8); class II (HDAC 4, 5, 6, 7, 9 and 10); class III or sirtuins (SIRT1, 2, 3, 4, 5, 6, and 7); and Class IV (HDAC II) (Di Marcotullio *et al.*, 2011). The HDAC families prevent the acetylation of histone and non-histone proteins. Transcription-related factors are probably among the most extensively studied proteins regulated by lysine acetylation. Therefore, acetylation controls a variety of cellular processes, such as transcription, proliferation, apoptosis, and differentiation.

Lysine residues are not exclusively acetylated but can also be modified by other posttranslational modifications such as methylation, ubiquitination, and sumoylation. Moreover, posttranslational modifications of amino acids adjacent to a lysine residue, such as phosphorylation of serine or threonine, can regulate the acetylation state of the lysine residue (Bae *et al.*, 2006).

Several investigations have shown that Runx2 protein levels and activity are regulated independently of mRNA expression and are modulated through post-translational modifications, including phosphorylation, ubiquitination, and acetylation (Jonason *et al.*, 2009). For instance, IGF-1 and FGF-2, growth factors essential for cell proliferation and differentiation have been shown to phosphorylate Runx2 protein during

osteoblasts differentiation (Fujita *et al.*, 2004; Lima *et al.*, 2009). Therefore, both IGF and FGF-2 are great examples of induction of post-transcriptional modifications on Runx2. Runx2 transcriptional activity is also negatively regulated by phosphorylation of its serine residues, S104 and S451 (Wee *et al.*, 2002). This phosphorylation abolishes the interaction of Runx2 with its hetero-dimerization partner CBF β , thereby decreasing Runx2 protein stability (Wee *et al.*, 2002).

It is well established that histone acetylation is crucial for chromatin remodeling and thereby for chromatin function. However, it has been demonstrated that acetylation of non-histone proteins plays a key role in several cellular function, including cytoskeleton dynamics, energy metabolism, gene expression, and cell signaling. An example to illustrate the importance of protein acetylation is the effect of acetylation on P53 functions and interactions. P53 is a tumor suppressor gene involved in several cell functions. It has been demonstrated that p53 acetylation promotes its stabilization and consequently its translocation into nucleus. Once in the nucleus, P53 interacts with sequence-specific DNA binding sites of its target genes thereby promoting gene expression (Brooks *et al.*, 2011, Bode, *et al.*, 2004) (figure 8).

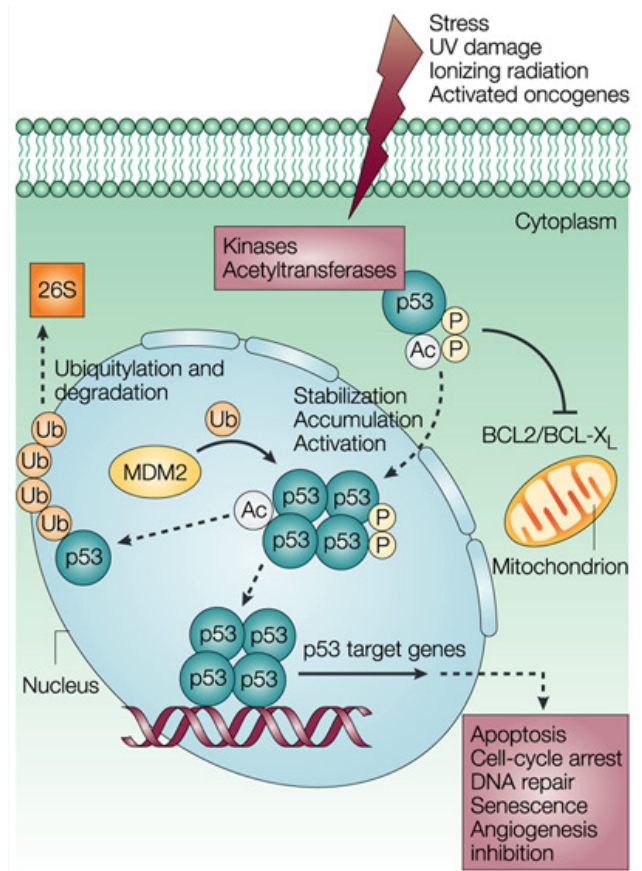


Figure 8: p53 tumor suppressor acetylation by histone acetyl transferases: The post-transcription modification of the P53 protein leads to stabilization, activation, and consequently translocation of the P53 into nucleus to interact with sequence-specific DNA binding sites of its target genes. Therefore, the transcriptional activity of P53 lead to activate the down stream target genes. Lack of acetylation of the P53, it is targeted by ubiquitination and subsequently degradation of P53 (Bode, *et al.*, 2004).

Another example of the importance of protein acetylation in regulating major cellular functions is the acetylation of the Forkhead box 'Other' (FoxO) proteins (figure 9). FoxO is a subgroup of the Forkhead transcription factor family. Acetylation modulates FoxO function and control nuclear-cytoplasmic shuttling, DNA binding and protein-protein interactions. FoxO transcription factors regulate the expression of genes involved in metabolism, proliferation, and cell renewal by HDAC class II. It has been shown that deacetylation of FoxO activates the transcription of enzymes required for gluconeogenesis (Barthel *et al.* 2005, Mihaylova *et al.*, 2011).

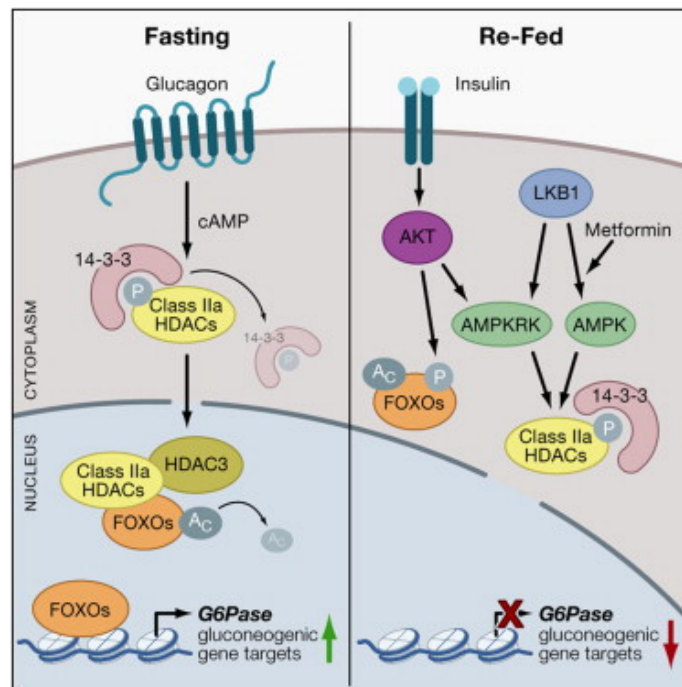


Figure 9: Posttranscriptional modification of the FoxO: Class IIa histone deacetylases (HDACs) dephosphorylated and translocate to the nucleus in respond to fasting hormone glucagon. HDACs deactylates and activates FoxO transcription factor, which activates down stream target genes of gluconeogenic enzymes such as G6Pase (Mihaylova *et al.*, 2011).

Both HATs and HDACs are known to interact with Runx2 and regulate its stability and activity. It has been shown that BMP-2 induces Runx2 acetylation, and that Smad1 and Smad5 facilitate the interaction between Runx2 and p300. In addition, the activation domain of Runx2 physically interacts with p300 in osteoblast cells (Sierra *et al.*, 2003). Runx2 also associates with P/CAF (p300/CBP-associated factor), which cooperates with p300 in activation of the osteocalcin gene promoter (Sierra *et al.*, 2003).

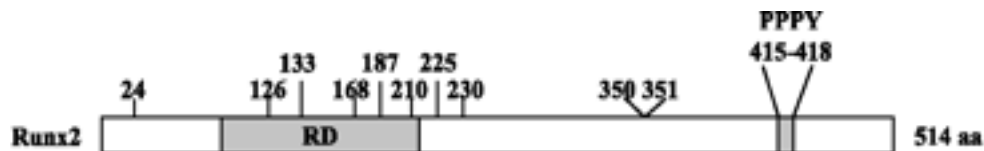


Figure 10: Lysine residues in Runx2 protein: Runx2 has 10 lysine residues that they are known to interact HATs and HDACs. Sites 225, 230, 350, and 351 are specifically acetylated by HAT p300 (Jeon *et al.* 2005).

Several HDACs are crucial in skeletal development (Montgomery *et al.*, 2007, Haberland *et al.*, 2009). HDAC3, HDAC4, and HDAC6 interact with Runx2 to repress

the expression of osteoblast-specific genes (Vega *et al.*, 2004; Schroeder *et al.*, 2004; Lamour *et al.*, 2007; Westendorf *et al.*, 2002). Furthermore, *in vitro* knockdown of HDAC3 leads to the increased expression of Runx2 target genes and osteoblast-targeted deletion of HDAC3 leads to the skeletal disorder and abnormal intramembranous and endochondral bone formation (Razidlo *et al.*, 2010; Hesse *et al.*, 2010). It is believed that overall these modifications affect protein-protein interactions between Runx2 and other members of Runx2 transcriptional regulatory complexes, ultimately determining whether these complexes will activate or repress gene transcription. Alternatively, phosphorylation of a specific residue may provide a recognition sequence for ubiquitination of Runx2 and subsequent degradation (Jonason *et al.*, 2000; Huang *et al.*, 2001). Acetylation, however, may block the availability of a lysine residue for ubiquitination, thereby increasing protein stability (Jin *et al.*, 2004).

Although great advances have been made in understanding how post-translational modifications may affect Runx2 activity, much work is yet to be done to further understand how post-translation modifications affect Runx2 function during osteoblast commitment and differentiation. As mentioned above, work from Dr. Baron's group has demonstrated that Zfp521 enhances the interaction of HDAC3 and Runx2 and that deletion of Zfp521 significantly reduces the interaction between Runx2 and HDAC3 (Hesse *et al.*, 2010).

The proposed studies are designed to address whether Zfp521, a 30 zinc-finger containing protein, regulates acetylation of Runx2 protein and thereby its function during osteoblast differentiation.

Significance:

Runx2 has a crucial role in both bone development and maintenance. Consequently, changes affecting Runx2 lead to several severe skeletal disorders. In addition, Zfp521 has an essential role in osteoblast differentiation. Therefore, the studies proposed would enlighten our understanding of the mechanisms by which Runx2 and Zfp521 regulate osteoblast differentiation and bone formation, which could have significant implications on improvement of future therapies for skeletal dysfunctions.

Hypothesis

We hypothesize that Zfp521 decreases acetylation of Runx2 protein, thereby affecting its function. Because Runx2 is a key regulator of osteoblast differentiation, Zfp521 may regulate osteoblast commitment and differentiation, at least in part by decreasing the levels of Runx2 acetylation.

Specific Aims

Specific Aim I:

Investigate whether Zfp521 affects Runx2 acetylation: This aim is subdivided to following sub-aims:

IA: Effect of Zfp521 on Runx2 acetylation levels in HEK293 cells stably expressing Zfp521:

- A. Transient transfection of the stable cell lines HEK293-EV and HEK293-521 with empty vector and Runx2 expression vectors
- B. Immunoprecipitation (IP) analyses to assess Runx2 acetylation levels

IB: Effect of Zfp521 on Runx2 acetylation levels in HEK293T cells transiently transfected with Runx2 and Zfp521:

- A. Transient transfection of HEK293T cells with empty vector or Zfp521-FLAG and Runx2 expression vectors
- B. Immunoprecipitation (IP) analyses to assess Runx2 acetylation levels

IC: Effect of Zfp521 on global protein acetylation:

- A. Treatment of stable cell lines HEK293-EV and HEK293-521 with NaB for 16 hours
- B. Western analyses to examine global protein acetylation

ID: Effect of Zfp521 on Runx2 acetylation levels in present of HDACs inhibitors:

- A. Transient transfecting the stable cell lines HEK293-EV and HEK293-521 with empty vector and Runx2 plasmids
- B. Treatment of cell with NaB and harvest the cells 16 hours post-treatment
- C. Immunoprecipitation (IP) analyses to assess Runx2 acetylation levels

IE: Effect of Zfp521 on Runx2 acetylation level in present of P300:

- A. Transient transfection of HEK293-EV and HEK293-521 cells with empty vector and Runx2 expression vector or Empty vector and P300-FLAG expression vector
- B. Immunoprecipitation (IP) analyses to assess Runx2 acetylation levels

Specific Aim II:

Investigate whether Runx2 acetylation levels are affected during osteoblast differentiation of MC3T3-E1.

- A. Investigating the Runx2 acetylation during osteoblast differentiation using the osteoblast cell line MC3T3-E1 cells.
- B. Detecting Runx2 acetylation through immunoprecipitation (IP) and immunoblot (IB) experiments

Material and Methods:

Approach for Specific Aim 1:

Investigate whether Zfp521 affects Runx2 acetylation

HEK (Human Embryonic Kidney) 293 cells are relatively easy to work with in terms of culturing and transfection; thus they can be use in experiments encompassing transfection of gene of interest, and investigation of the expressed protein. In the studies proposed in Aim I, HEK293 cells stable expressing Zfp521 and control cells were used to assess whether Zfp521 effects Runx2 protein acetylation.

Cell culture:

HEK293 cells were cultured in in Dulbecco/Vogt modified Eagle's (Harry Eagle) minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) and 1% of penicillin: streptomycin (PS) (Invitrogen).

Plasmid preparation:

QAIGEN Maxi prep technique was used to purify the plasmid DNA from bacteria and amplify the plasmids. This procedure is multistep including; growing bacterial culture, harvesting and lysis of the bacteria, and purification of plasmid DNA. In this experiments, empty vector and Runx2 vector were amplified using the QAIGEN Maxi

prep protocol. The protein concentration was measured using NanoDrop software.

Transfection:

HEK293 cells express a low level of Runx2, thus for this studies, we transiently transfected a Runx2-FLAG expression vector or an empty control vector (EV) or P300-FLAG expression vector (for Aim IE) into HEK293-EV and HEK293-521 cells. Also for Aim IB, empty vector or Zfp521-FLAG expression vector were transiently transfected into HEK293T. The day before transfection, cells were plated at density of 1.5×10^6 cells/plate. 80-90% confluent cells will be transfected using Lipofectamine following the manufacturer's protocol (Invitrogen). Briefly, 8.0 μ g of plasmid DNA was mixed with 20 μ l LipofectamineTM 2000 and incubated at room temperature. After 20 minutes, the DNA- Lipofectamine mix was added directly to each plate. The medium containing the lipofectamine was replaced with fresh DMEM with 10% FBS and 1% PS after 6 hours and cells were treated with NaB (in Aim IC and ID). Immunoprecipitation (IP) experiments were performed 24 hours post-transfection.

Immunoprecipitation:

Immunoprecipitation is a technique used to immunoprecipitate proteins of interest. We performed the following set of IP experiments to detect the Runx2 protein acetylation by Zfp521. The whole cell lysate was immunoprecipitated with α -acetyl

lysine (Cell Signaling). Using the α -acetyl lysine antibody allowed us to immunoprecipitate all acetylated proteins. Subsequently, Runx2 acetylation levels were detected by immunoblot with Runx2 primary antibody.

The immunoprecipitation was performed 24 hours post-transfection. Cells were rinsed with Phosphate Buffered Saline (PBS). Cells were harvested with PBS containing Dithiothreitol (DTT) 1mM and proteinase inhibitors. Sodium butyrate (NaB), a histone deacetylase inhibitor, was added to all buffers to prevent protein deacetylation. Samples were centrifuged for 5 minutes at 2000 rpm, and supernatants were removed. Cells were lysate and homogenized by TNE buffer [Tris-HCl (40mM) pH 7.8, NaCl (0.15M), EDTA (1mM), 1% Nonidet- 40(NP-40)] containing DTT (1mM), proteinase inhibitors, and NaB (90mM). Lysate cells were incubated for 30 minutes at 4°C on a rotator. Cell lysates were then centrifuged at 12000 rpm for 5 minutes at 4°C and the supernatant transferred into new tubes. Input samples were taken out before adding the resin. Cell lysate was incubated with protein-A anti-rabbit IgG beads for 30 minutes at 4°C on rotator. The samples were then centrifuged at 2500g for 2 minutes and supernatants were transferred in to new tube. Cell lysate was then incubated with α -acetyl lysine antibody for one hour 4°C on rotator. Protein A anti-rabbit IgG beads were added and sample were incubated for another one hour. IP protein were eluted from the protein-A anti-rabbit IgG beads and input samples were eluted by adding 2x Laemmli buffer containing 5mM DTT (reducing agent) and boiling for 10 minutes. Samples were centrifuged and subjected to

immunoblot analysis.

Western Blot Analysis:

Samples were loaded on a 4-15% SDS-Page gel. Gels will be transferred onto a nitrocellulose membrane using the Bio-Rad Mini-Gel Box Electrotransfer. After transfer the membranes were blocked in blocking solution [TBST: Tris-HCl (20mM) pH 7.5, NaCl (150mM), 0.05% Tween] containing 5% non-fat milk for one hour at room temperature. The membranes immunoprecipitated with α -acetyl lysine antibody was incubated with α -Runx2 1:500 diluted in TBST containing 5% nonfat milk. Incubation is for one hour on rotator at room temperature. Membranes were washed with TBST, 3X for 5 minutes and incubated with a secondary antibody HRP conjugated. Membranes were then washed 3X for 5 minutes with TBST, and subjected to the enhanced chemiluminescence (ECL) system (GE Healthcare), and exposed to film (Kodak).

Aim IA: Effect of Zfp521 on Runx2 acetylation levels in HEK293 cells stably expressing Zfp521:

For this aim, HEK239-EV and HEK293-521 cell lines were transiently transfected with Empty vector used as control and Runx2 expression vector. Cells were harvested 24-hours post transfection and subjected to the IP. Whole cell lysates were immunoprecipitated with α -acetyl lysine. Runx2 acetylation levels were then detected by immunoblot with α -Runx2 primary antibody (figure 11).

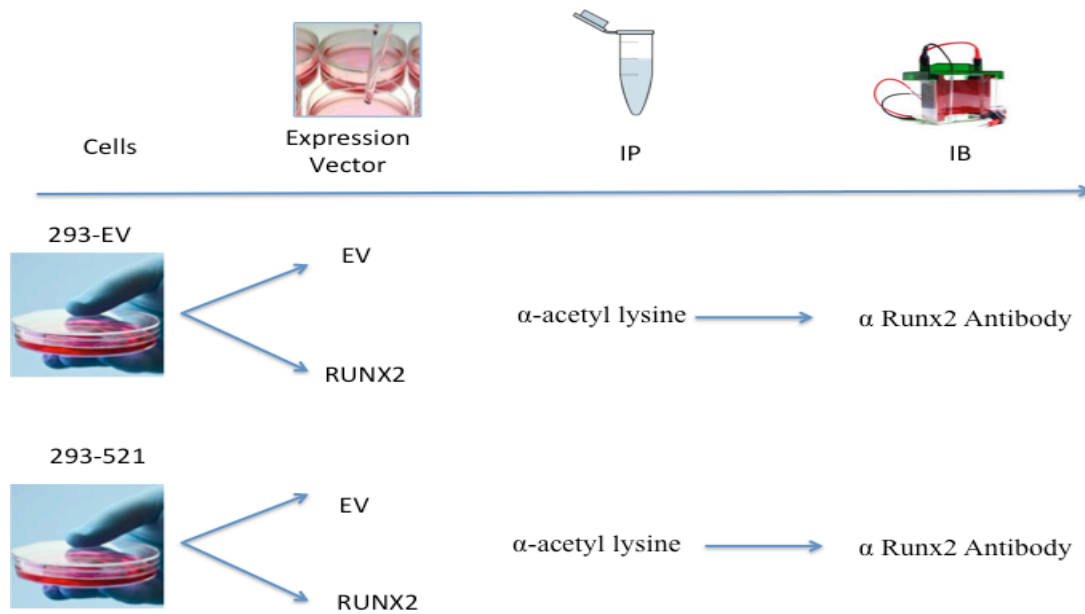


Figure 11: Summery of methods and materials of the Aim IA.

Aim IB: Effect of Zfp521 on Runx2 acetylation levels in HEK293T cells transiently transfected with Runx2 and Zfp521:

In this aim, we used HEK293T. We transiently transfected Runx2-FLAG or 521-FLAG expression vector or an empty control vector into HEK293T cells. Cells were harvested 24 hours post transfection. Whole cell lysates were immunoprecipitated with α -acetyl lysine. Runx2 acetylation levels were detected by immunoblot with α -Runx2 (figure 12).

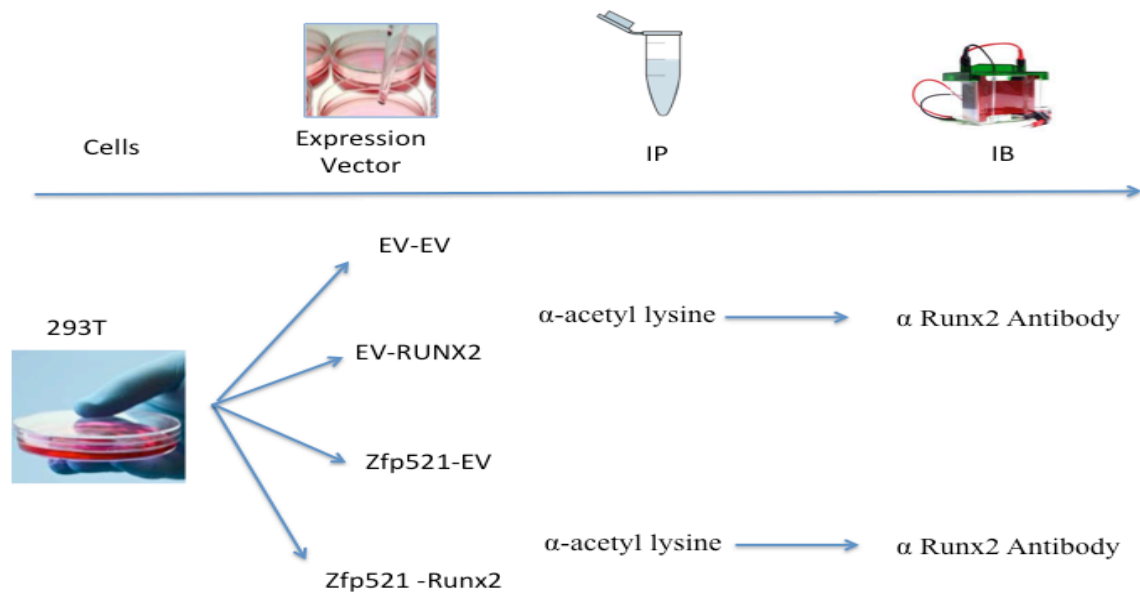


Figure 12: Summery of methods and materials of the Aim IB.

Aim IC: Effect of Zfp521 on global protein acetylation:

In this aim, we examined the role of Zfp521 on global protein acetylation. Sodium butyrate was used to block protein deacetylation. HEK293-EV and HEK293-521 cells

were treated with 5 mM NaB and harvested 16 hours post-treatment. The whole cell lysates were immublot with α -acetyl lysine (Figure 13).

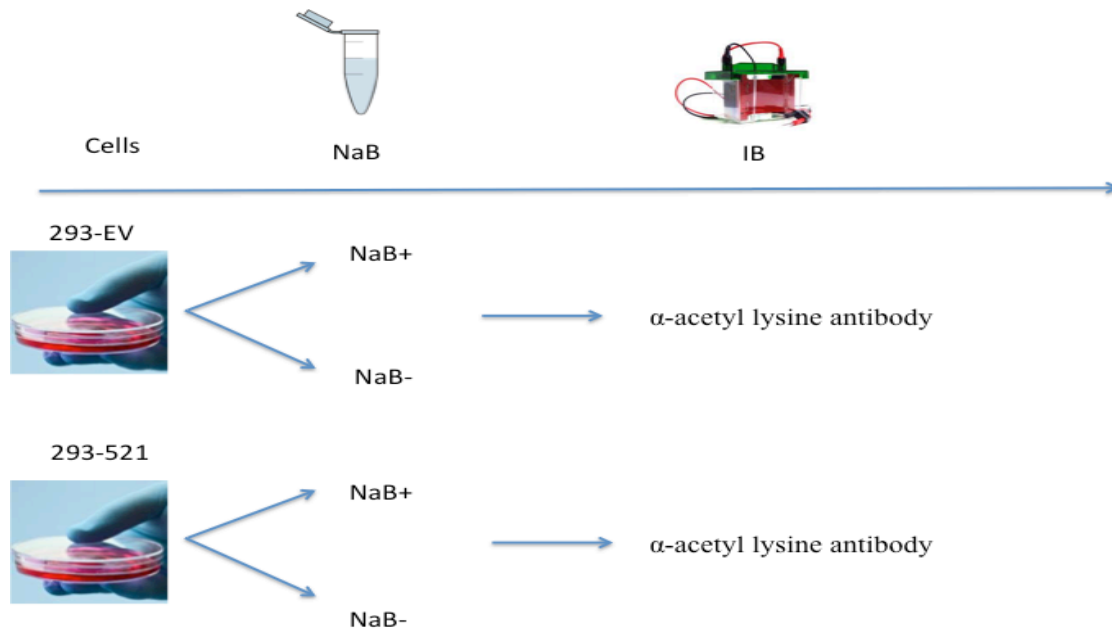


Figure 13: Summery of methods and materials of the Aim 1C.

Aim 1D: Effect of Zfp521 on Runx2 acetylation levels in present of HDACs inhibitors:

We investigated whether treatment with NaB would modulate the effect of Zfp521 on the levels of Runx2 acetylation. We transiently transfected the Runx2-FLAG expression vector or an empty control vector into HEK239-EV and HEK293-521 cells. Six hours after transfection, mediums were changed as detailed above and cells were treated with 5 mM NaB for 16 hours. IP was performed as described above. Runx2

protein acetylation levels were assessed in NaB treated cells and was compared to untreated cells (Figure 14).

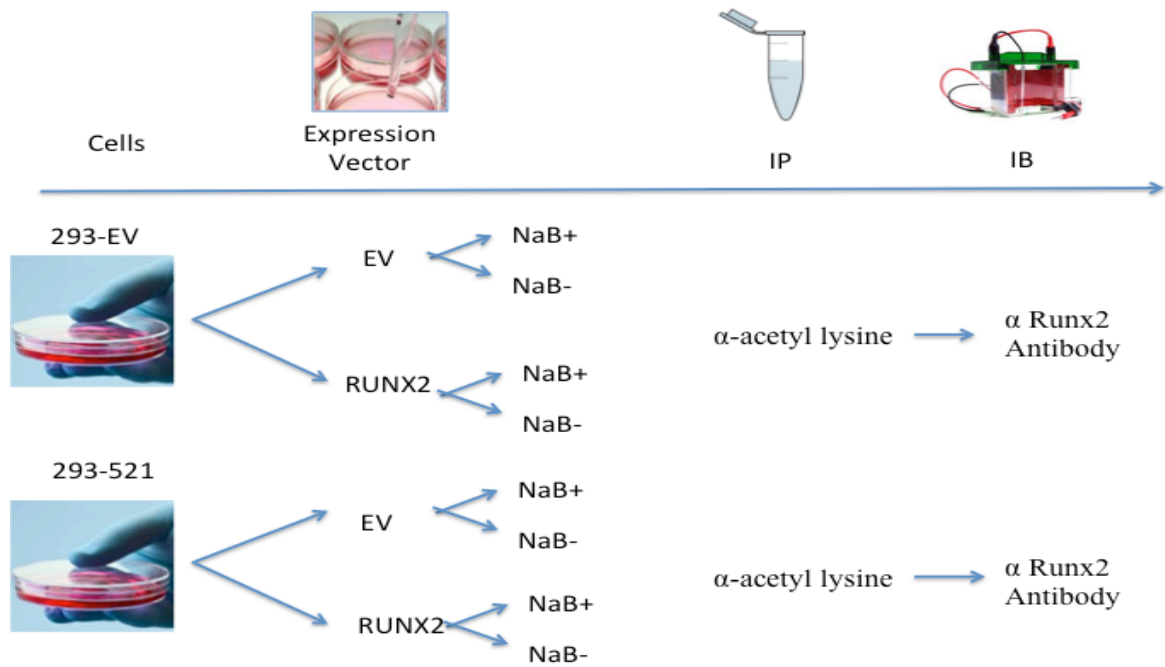


Figure 14: Summary of methods and materials of the Aim ID.

IE: Effect of Zfp521 on Runx2 acetylation level in present of P300:

In this aim, we have investigated whether Zfp521 affect Runx2 protein acetylation levels through P300. HEK239-EV and HEK293-521 cells were transiently transfected with Runx2-FLAG expression vector or P300-FLAG expression vector. Immunoprecipitation with α-acetyl lysine antibody, and immunoblot with α-Runx2 were accomplished (figure 15).

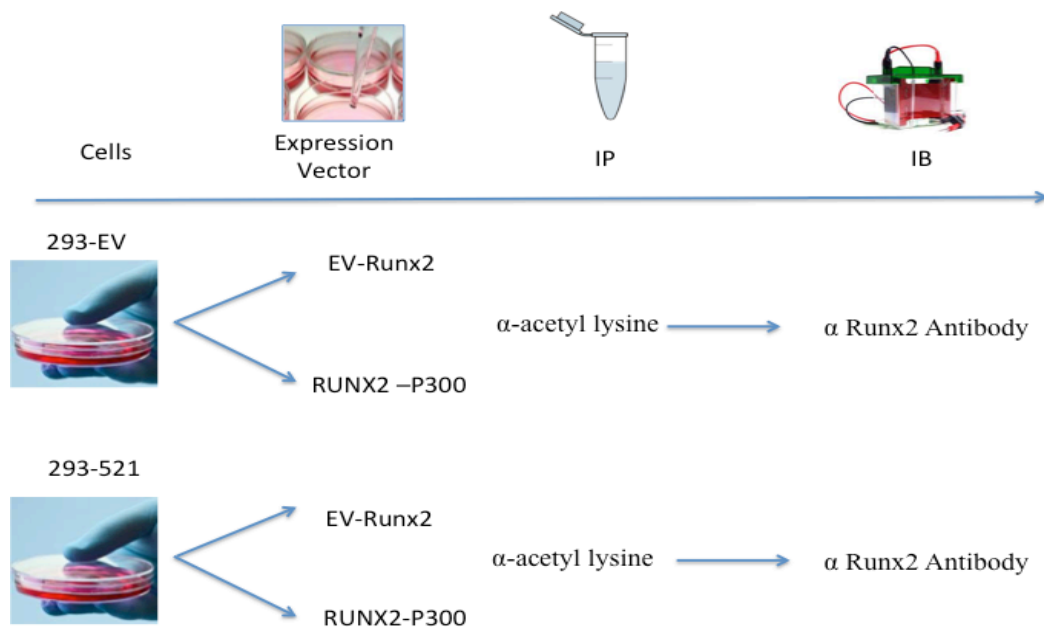


Figure 15: Summery of methods and materials of the Aim IE

Approach for Specific Aim 2:

Investigate whether Runx2 acetylation levels are affected during osteoblast differentiation of MC3T3-E1

In the first aim, we investigated the effect of Zfp521 on Runx2 protein acetylation in HEK293 cells. In this aim, we examined Runx2 acetylation levels during osteoblast differentiation. The cell we have chosen for these investigations is the MC3T3-E1 pre-osteoblast cells. This cell line has been extensively used as an *in vitro* model to study osteoblast biology. These cells display a time-dependent and sequential expression of osteoblast genes analogous to *in vivo* bone formation.

Cell Culture:

MC3T3-E1 cells were cultured in GIBCO™ Minimum Essential Medium (MEM) Alpha Medium (1X) liquid (α -MEM) with 10% FBS and 1% PS. To induce osteoblast differentiation, 10mM β -glycerophosphate, and 5 μ g/ml ascorbic acid were added to the culture media. This medium is called thereafter osteoblastic medium (OBM). Ascorbic acid induces the osteoblast differentiation. It is well established that ascorbic acid is essential for formation of a collagen-containing extracellular matrix for expression of the osteoblastic phenotype (Otsuka et al., 1999).

Transfection:

MC3T3-E1 cells express Runx2 endogenously, thus for this study, we did not transiently transfect the Runx2 expression vector.

Immunoprecipitation:

In Aim II, we investigated the endogenous Runx2 acetylation levels in MC3T3-E1 cells during osteoblast differentiation on day 0, 3, 7, 14 and 21. Cells were cultured in OBM medium. A set of IP was accomplished as follow:

- A. The MC3T3-E1 whole cells lysate were immunoprecipitated with α -acetyl lysine, and subsequently, subjected to immunoblot with α -Runx2. Immunoprecipitation and immunoblot experiments were performed as detailed in specific Aim I.

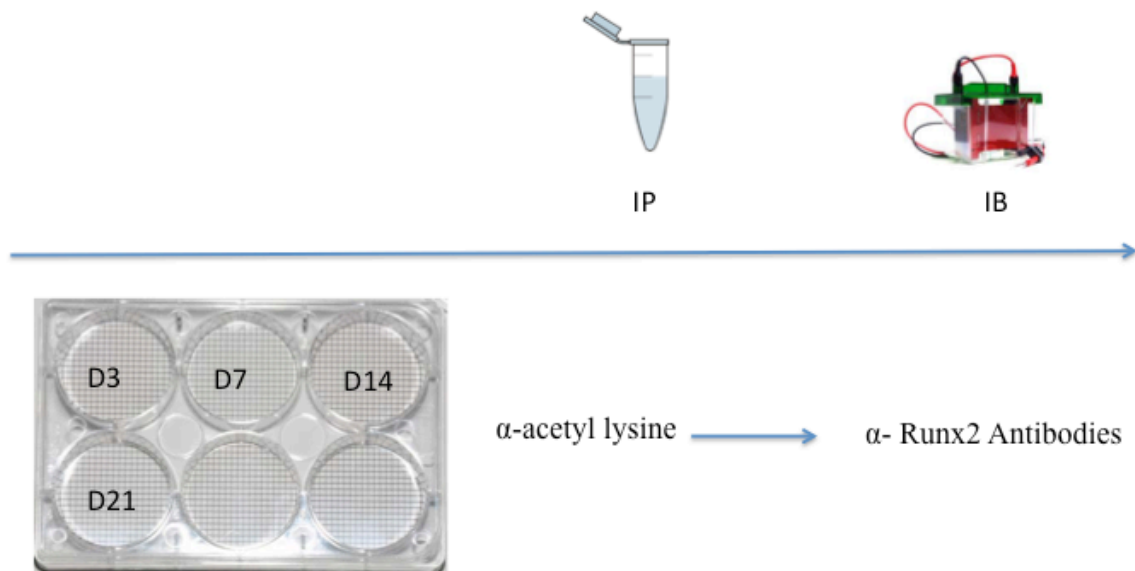


Figure 16: Summary of methods and materials of Aim II: In this aim we investigated the Runx2 acetylation in osteoblast cell line (MC3T3-E1). To induce osteoblast differentiation, cells were cultured in the presence of 10mM β -glycerophosphate, and 5 μ g/ml ascorbic acid . Cells were harvested at on day 3, 7, 14 and 21. The MC3T3-E1 whole cells lysate were immunoprecipitated with α -acetyl lysine, and subsequently, subjected to immunoblot with α -Runx2 1:500 diluted in TBST containing 5% nonfat milk.

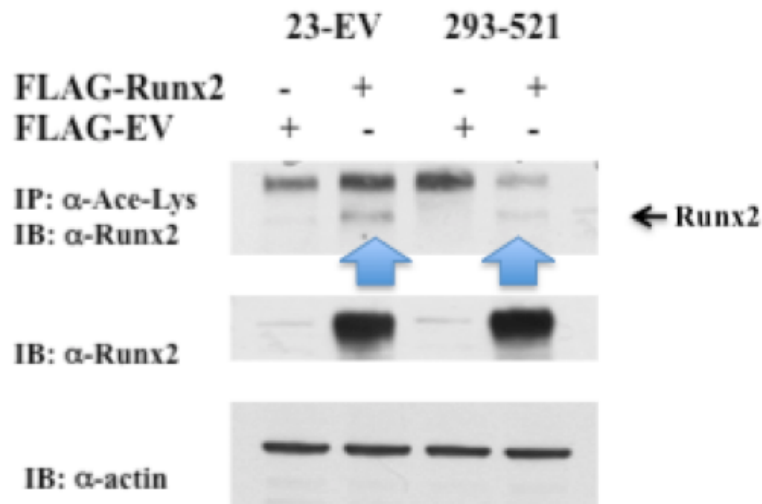
Results

Approach for Specific Aim 1:

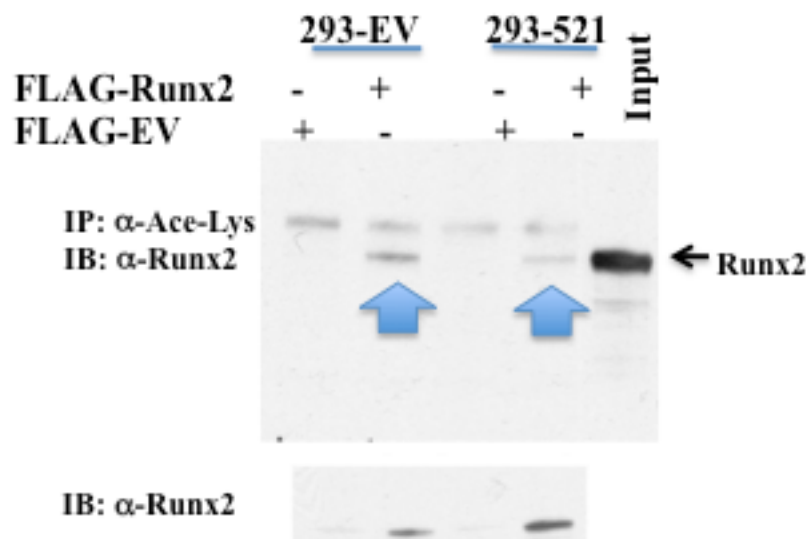
Investigate whether Zfp521 affects Runx2 acetylation

IA: Effect of Zfp521 on Runx2 acetylation levels in HEK293 cells stably expressing Zfp521:

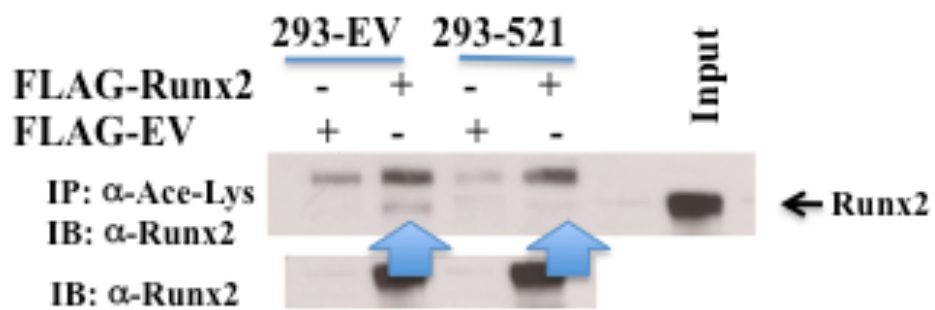
HEK239-EV and HEK293-521 cells were transiently transfected with Runx2-FLAG expression vector or an empty control vector. The result demonstrated that the overexpression of Zfp521 has significantly decreases the Runx2 acetylation in HEK293 cells stably expressing Zfp521. These experiments have been repeated at least 6 times (Figure 17: A, B, C, and D).



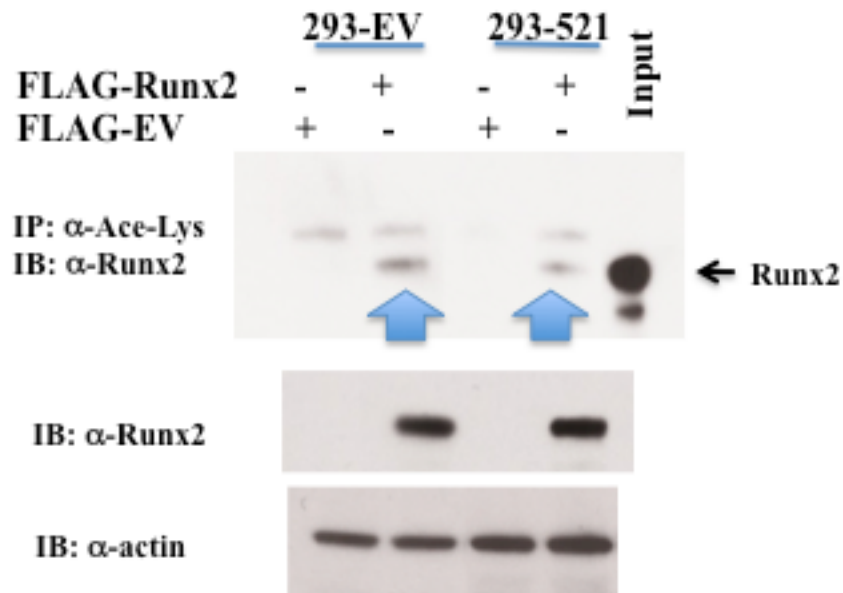
(A)



(B)



(C)



(D)

Figure 17: Effect of Zfp521 on Runx2 protein acetylation levels in HEK 293 cells:

Western analyses demonstrating reduction of Runx2 acetylation protein levels in HEK293-521 cells compared to HEK293-EV cells. Immunodetection with Runx2 is presented to confirm Runx2 expression in transfected cells. An actin antibody is presented to evaluate total protein loaded. The black arrow indicates Runx2. As it shown in blue arrows, the overexpression of Zfp521 has significantly decreases the Runx2 acetylation. Data shown are representative of that obtained with four independent experiments.

Aim IB: Effect of Zfp521 on Runx2 acetylation levels in HEK293T cells transiently transfected with Runx2 and Zfp521.

HEK293T cells were transiently transfected with Runx2-FLAG expression vector and 521-FLAG expression vector and an empty control vector: EV-EV, EV-Runx2, 521-EV, and 521-Runx2. A western analysis demonstrates reduction of Runx2 acetylation protein levels in HEK293T-521-Runx2 cells compared to HEK293T-EV-Runx2 cells. The overexpression of Zfp521 significantly decreases the Runx2 acetylation (Figure 18).

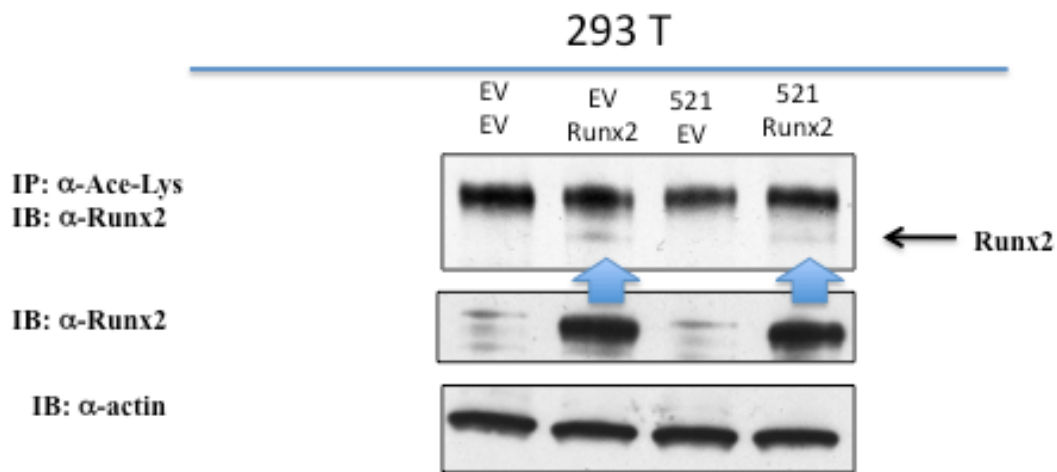


Figure 18: Effect of Zfp521 on Runx2 protein acetylation levels in transient transfection of HEK293T cells: Western analysis is demonstrating the reduction of Runx2 acetylation protein levels in HEK293T-521-Runx2 cells compared to HEK293-EV-Runx2 cells. Immunodetection with Runx2 is presented to confirm Runx2 expression in transfected cells. An actin antibody is presented to evaluate total protein loaded. The black arrow

indicates Runx2. As it shown in blue arrows, the overexpression of Zfp521 has significantly decreased the Runx2 acetylation.

Aim IC: Effect of Zfp521 on global protein acetylation:

HEK293-EV and HEK293-521 cells were cultured with and without 5 mM NaB to investigate global protein acetylation levels and whether stable expression of Zfp521 would effect global protein acetylation. As expected, global protein acetylation levels were significantly increased in presence of NaB (due to inhibition of the HDACs). However, stable expression of the Zfp521 did not affect global protein acetylation, suggesting that Zfp521 specifically modulates Runx2 acetylation without affecting global acetylation levels (Figure 19).

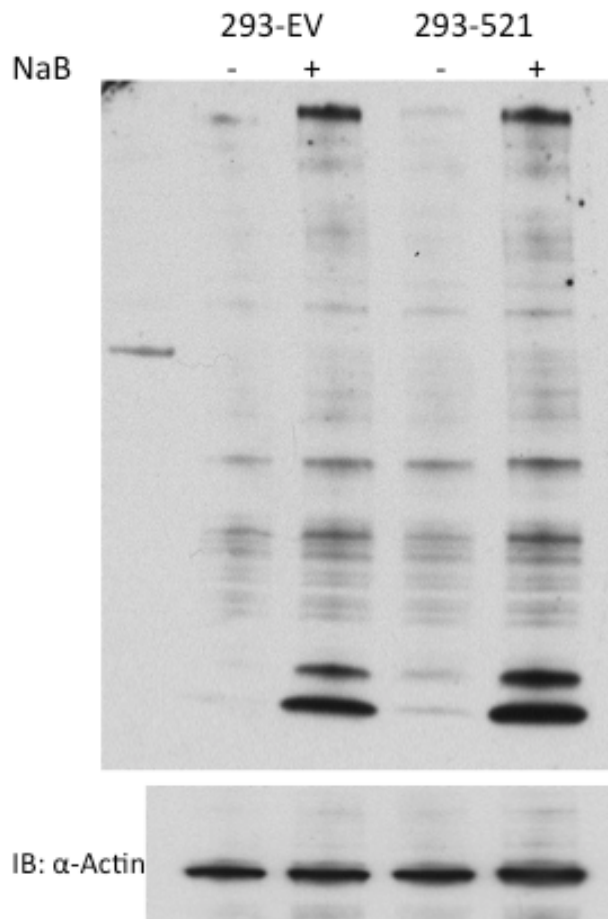


Figure 19: Effect of Zfp521 on global protein acetylation levels in HEK239 cells: The global protein acetylation had been significantly increased in present of the NaB. Western analyses demonstrate no significant changes of global protein acetylation levels in HEK293-521 cells compared to HEK293-EV-Runx2 cells. An actin antibody is presented to evaluate total protein loaded.

Aim ID: Effect of Zfp521 on Runx2 acetylation levels in present of HDACs inhibitors:

HEK239-EV and HEK293-521 cells were transiently transfected with Runx2-FLAG expression vector or an empty vector (Figure 20). Cells were treated with 5 mM NaB for 16 hours. These studies demonstrate that NaB treatment has increased the acetylation by decreasing the deacetylation. NaB increased the acetylation level by inhibiting HDACs in both HEK239-EV and HEK293-521 cells. In addition, the stable expression of Zfp521 significantly decreased Runx2 acetylation levels. This study suggests that Zfp521 impairs the Runx2 acetylation level with HDACs. Despite of present of the NaB, the overexpression of Zfp521 has significantly modulated the Runx2 acetylation by lower the Runx2 acetylation (Figure 20).

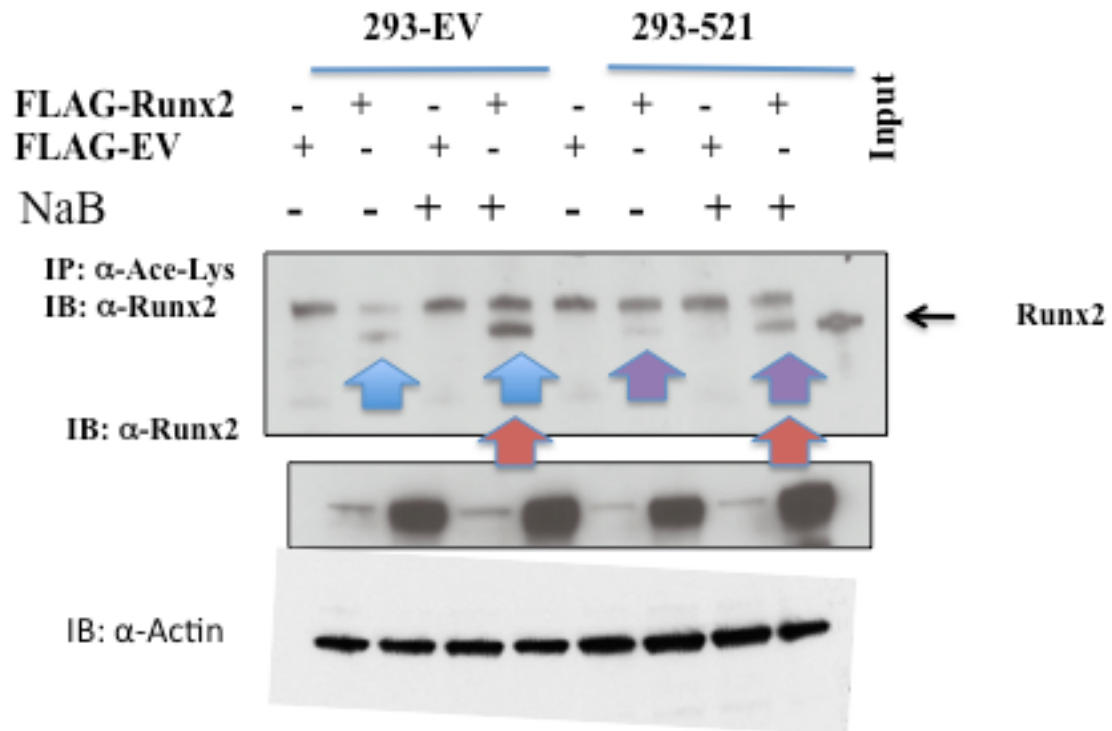


Figure 20: Effect of Zfp521 on Runx2 protein acetylation levels in HEK293 cells with Sodium butyrate (NaB) treatment: The blue arrow is showing that Runx2 acetylation in HEK293-EV cell is increased by NaB treatment due to inhibition of HDACs. The same situation, purple arrow shows the Runx2 acetylation in HEK293-521 cell is increased by NaB treatment. The black arrow indicates Runx2. The overexpression of Zfp521 has significantly decreased the Runx2 acetylation. Western analyses demonstrate reduction of Runx2 acetylation protein levels in HEK293-521 cells compared to HEK293-EV cells, as it shown by red arrow. Immunodetection with Runx2 is presented to confirm Runx2

expression in transfected cells. An actin antibody is presented to evaluate total protein loaded.

IE: Effect of Zfp521 on Runx2 acetylation levels in the presence of P300:

HEK239-EV and HEK293-521 cells were transiently transfected with Runx2-FLAG expression vector or an empty vector or P300-FLAG expression (Figure 21). Preliminary result shows that P300 increases Runx2 acetylation, as it was expected. The stable expression of Zfp521 significantly decreased Runx2 acetylation levels. This study suggests that Zfp521 impairs the Runx2 acetylation level. Despite of present of the P300, the overexpression of Zfp521 has significantly modulated the Runx2 acetylation by lower the Runx2 acetylation (figure 21).

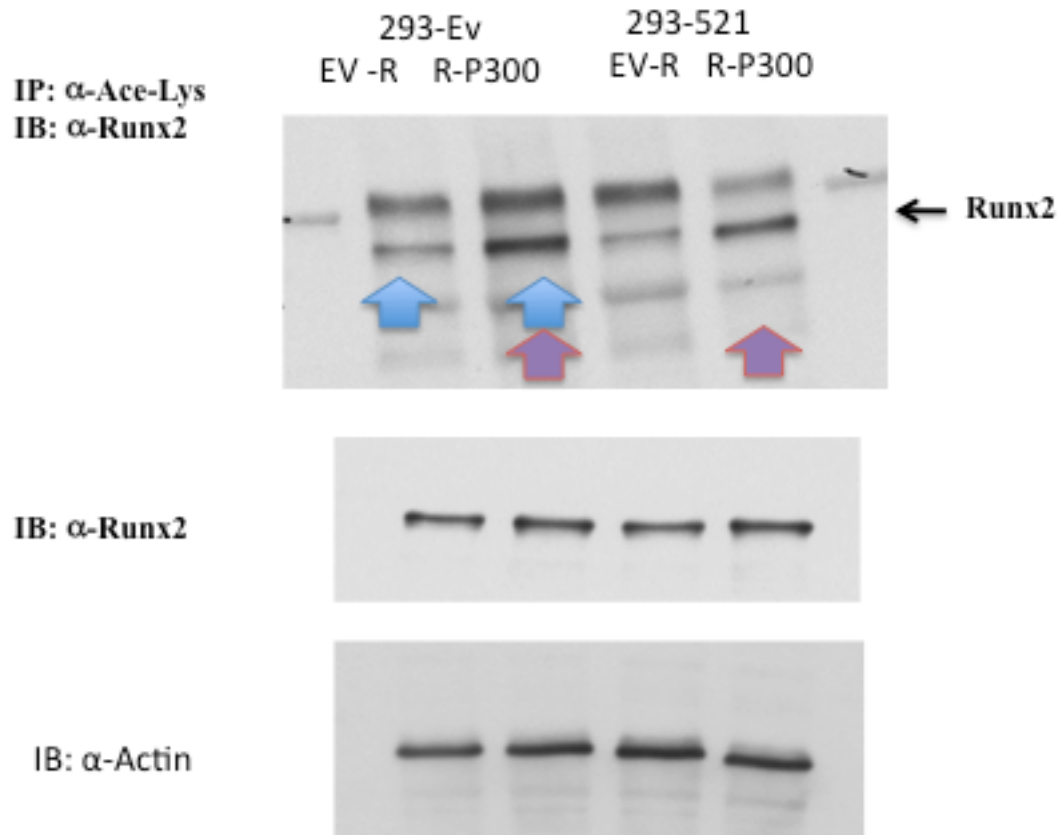


Figure 21: Effect of Zfp521 on Runx2 protein acetylation levels with P300: Western analyses demonstrate reduction of Runx2 acetylation protein levels in HEK293-521 cells compared to HEK293-EV cells. As it shown in blue arrows, the P300 increases Runx2 acetylation. The purple arrow indicates the stable expression of Zfp521 significantly decreased Runx2 acetylation levels. Zfp521 impairs the Runx2 acetylation level. Despite of present of P300, the overexpression of Zfp521 has significantly modulated the Runx2 acetylation by lower the Runx2 acetylation. Immunodetection with Runx2 is presented to

confirm Runx2 expression in transfected cells. An actin antibody is presented to evaluate total protein loaded. The black arrow indicates Runx2.

Approach for Specific Aim 2:

Investigate whether Runx2 acetylation levels are affected during osteoblast differentiation of MC3T3-E1

IIA: Runx2 acetylation levels in MC3T3-E1 cells during osteoblast differentiation on day 3, 7, 14, and 21:

We investigated endogenous Runx2 acetylation levels in MC3T3-E1 cells during osteoblast differentiation on day 3, 7, 14, and 21 (Figure 22). MC3T3-E1 cells were differentiated with 10mM β -glycerophosphate, and 5 μ g/ml ascorbic acid. Cells were harvested on days 3, 7, 14, and 21. As shown in Figure 22, we did not detect Runx2 acetylation during osteoblast differentiation. While Runx2 protein levels increase by 7 days in culture, and gradually decreases by days 14, Runx2 acetylation was undetectable (figure 22).

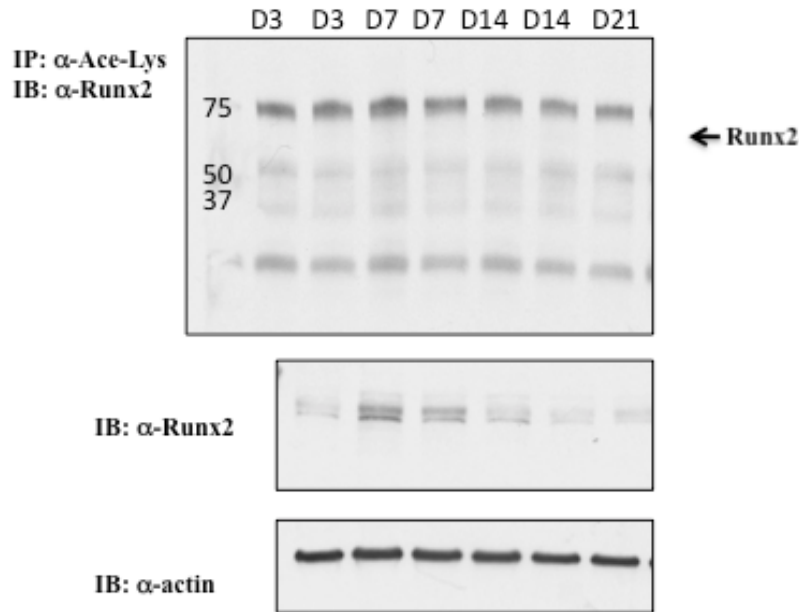


Figure 22: Runx2 acetylation levels in MC3T3-E1 cells during osteoblast differentiation: Western analyses demonstrate endogenous Runx2 acetylation levels in MC3T3-E1 cells during osteoblast differentiation on day 3, 7, 14 and 21. Immunodetection with Runx2 shows Runx2 protein levels increase by 7 days in culture, and gradually decreases by days 14. The black arrow indicates Runx2 acetylation level. The Runx2 acetylation level was undetectable during osteoblast differentiation. An actin antibody is presented to evaluate total protein loaded

IIB: Runx2 acetylation levels in MC3T3-E1 cells during osteoblast differentiation on day 0, 3 and 7:

Since we did not detect Runx2 acetylation during osteoblast differentiation between days 3 to day 21, we examined Runx2 acetylation levels at earlier time points. We assessed Runx2 acetylation on days 0, 3, and 7 (figure 23). Runx2 acetylation was undetectable during osteoblast differentiation. These data suggest that while Runx2 protein levels increase by 7 days in culture; however, Runx2 acetylation was undetectable in earlier time point too.

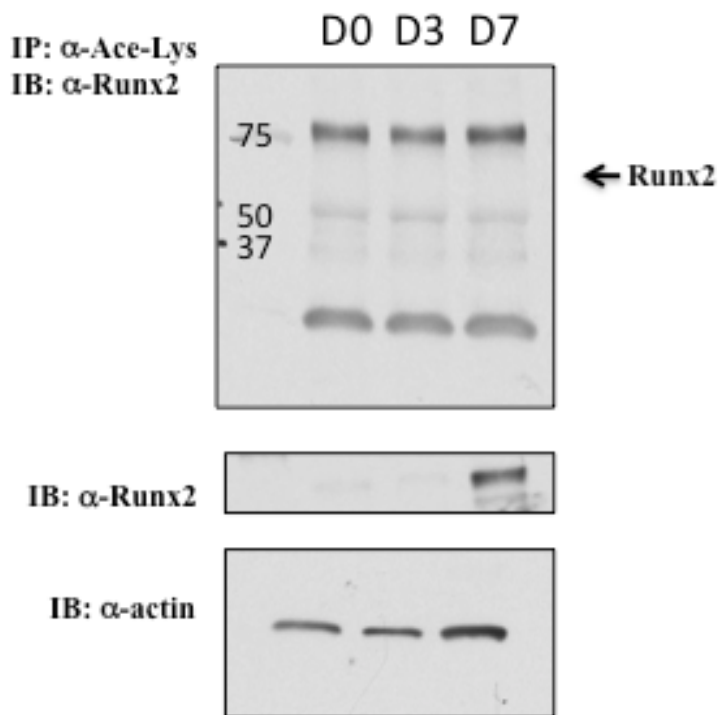


Figure 23: Runx2 acetylation levels in MC3T3-E1 cells during osteoblast differentiation on day 0, 3 and 7: Western analyses demonstrate endogenous Runx2 acetylation levels in

MC3T3-E1 cells during osteoblast differentiation on day 0, 3, and 7. Immunodetection with Runx2 shows Runx2 protein levels gradually increase on day 3 and pick on 7 days in culture. The black arrow indicates Runx2 acetylation level. The Runx2 acetylation level was undetectable during osteoblast differentiation. An actin antibody is presented to evaluate total protein loaded.

Discussion and Conclusion

Runx2 has an essential role in the development of bone and teeth. The haploinsufficiency of Runx2 leads to the autosomal-dominant disorder, cleidocranial dysplasia, characterized by various dental and skeletal abnormalities. Zinc finger protein 521, a 30 zinc-finger containing protein, is a transcription factor that is expressed in several tissue types, including bone. Zfp521 is highly expressed in mesenchymal condensations, in prehypertrophic chondrocytes in the growth plate as well as in osteoblasts and osteocytes. Zfp521 plays a critical role in osteoblast biology as demonstrated by the findings that overexpressing Zfp521 in mature osteoblasts *in vivo* increases bone formation and bone mass, whereas deletion of Zfp521 leads to impaired osteoblast maturation and decreased bone matrix formation (Hesse *et al.*, 2010). Dr. Baron's group has demonstrated that Zfp521 regulates osteoblast differentiation at least in part by interacting with and repressing Runx2 activity by HDAC3 (Hesse *et al.*, 2010).

Runx2 protein can be regulated through post-translational modifications (Polevoda *et al.*, 2002). The interaction of Runx2 with HDAC3 is strongly enhanced by Zfp521, and knockdown of HDAC3 by shRNA leads to the loss of Zfp521-induced repression (Hesse *et al.*, 2010). Thus these findings suggest that Zfp521 perhaps modulate Runx2 protein acetylation during osteoblast differentiation.

In these studies, we investigated whether Zfp521 affects Runx2 acetylation levels. Our findings demonstrated that Zfp521 decreases Runx2 acetylation levels in HEK293

cells stably expressing Zfp521. It has been shown that Runx2 acetylation levels are important for its function (Hesse *et al.*, 2010), thus our findings suggest that Zfp521 might impair Runx2 function by decreasing its acetylation.

In order to prevent changes in the cellular activity by using cells stably expressing Zfp521, transient transfection experiments were performed. Our data demonstrated the reduction of Runx2 acetylation protein levels in HEK293T-521 cells compared to HEK293T-EV cells. The overexpression of Zfp521 has significantly decreases the Runx2 acetylation; therefore we have obtained the same result as stably expressing Zfp521.

We have previously shown that Zfp521 regulates the rate of osteoblast differentiation by interacting with Runx2 and by antagonizing its activity, (Wu *et al.*, 2009; Hesse *et al.*, 2010). The repression of Runx2 by Zfp521 involves the recruitment of HDAC3, a histone deacetylases and a known Runx2 co-repressor (Vega *et al.*, 2004; Schroeder *et al.*, 2004; Haberland *et al.*, 2009). Zfp521 significantly improves the interaction of Runx2 with HDAC3. These findings suggest that Zfp521 interacts with both Runx2 and HDAC3 to repress transcriptional activity of Runx2 by decreasing the Runx2 acetylation. Runx2 and Zfp521 are important in balancing osteoblast formation and maturation (Hesse *et al.*, 2010).

Sodium butyrate (NaB) prevents protein deacetylation by inhibiting the HDACs; therefore it increases the acetylation of global protein. The treatment of HEK293 cells with sodium butyrate increases the acetylation of global protein, however, stable expression of Zfp521 did not change the global protein acetylation. Therefore, Zfp521

specifically modulates Runx2 acetylation by lower the Runx2 acetylation without affecting global protein acetylation levels.

We also investigated the effect of Zfp521 on Runx2 protein acetylation levels in HEK293 cells in response to sodium butyrate treatment. Our findings suggest that not only the stable expression of Zfp521 decreases Runx2 acetylation levels; also Zfp521 impairs the Runx2 acetylation by HDACs. These data confirm the previous finding the repression of Runx2 by Zfp521 involves the recruitment of HDAC3.

P300 is one of a main group of Histone Acetylase Transferases (HATs) that increases acetylation by using acetyl-CoA and it has been recently suggested that during neural cells differentiation, the function of Zfp521 is associated with P300 (Kamiya *et al.*, 2011). We investigated the effect of Zfp521 on Runx2 acetylation in the presence of P300. Our preliminary result shows that although P300 increases Runx2 acetylation levels; Runx2 acetylation is impaired in Zfp521 cells even in the presence of P300. This study suggests that Zfp521 function can be linked to P300 since the Runx2 acetylation was partially halted in stably expressing HEK293-521 in present of P300. Future studies are required to further investigate the mechanism in which Zfp521 interacts with P300.

We assessed whether Runx2 acetylation levels are affected during osteoblast differentiation in MC3T3-E1 cells. While as expected Runx2 protein levels increase by day 7 in culture, Runx2 acetylation was undetectable. The Runx2 acetylation was undetectable perhaps due to either low protein level of the Zfp521 during the osteoblast differentiation in MC3T3-E1 cells or the Runx2 protein level is not high enough to detect

the Runx2 acetylation with immunodetect technique. Future studies are required to further investigate the Runx2 acetylation in osteoblast cells.

These studies demonstrated that Zfp521 modulates the Runx2 acetylation level in HEK293 cell and function of Zfp521 is potentially linked to HDACs and P300. Future studies are required to further investigating the mechanism of actions of Zfp521 in regulating of P300 and HDACs activities. Also future studies are recommended to investigate the Zfp521 on Runx2 acetylation during osteoblast differentiation.

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