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Transcriptional Co-Repressors of Runx2

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Abstract Runx2 is an essential transcription factor for skeletal mineralization because it stimulates osteoblast differentiation of mesenchymal stem cells, promotes chondrocyte hypertrophy, and contributes to endothelial cell migration and vascular invasion of developing bones. Runx2 is also expressed during mouse embryo development in nascent mammary gland epithelium. Recent evidence implicates deregulation of Runx2 as a contributing factor in breast cancer-induced osteolysis and invasion, as well as in ectopic vascular calcification. Like other Runt domain proteins, Runx2 is a context-dependent transcriptional activator and repressor of genes that regulate cellular proliferation and differentiation. Proteins that temporally and spatially associate with Runx2 dictate these opposing transcriptional activities. Recent studies have identified several co-repressor proteins that bind to Runx2 to regulate gene expression. These co-factors include histone deacetylases (HDACs), transducin-like enhancer of split (TLE) proteins, mSin3a, and yes-associated protein (YAP). These proteins do not bind DNA themselves and appear to act by preventing Runx2 from binding DNA, altering chromatin structure, and/or by possibly blocking co-activator complexes. The nuclear localization of several of these factors is regulated by extracellular signaling events. Understanding the mechanisms whereby co-repressor proteins affect Runx2 activity during normal cellular development and tumor progression will identify new therapeutic targets for skeletal disorders such as osteoporosis and for bone metastatic cancers. J. Cell. Biochem. 98: 54–64, 2006.

Key words: HDAC; TLE; mSin3A; YAP; bone; metastasis

Runx2 is one of three mammalian members of the Runt-related transcription factor family. These proteins bind to specific DNA sequences to positively or negatively regulate the expression of genes that contribute to cellular proliferation, tissue differentiation, and the development of numerous pathologies, including tumor progression and metastasis [Ito, 2004; Blyth et al., 2005]. Formerly referred to as Cbfa1, PEBP2αA1, or AML-3, Runx2 is required for osteoblast development and maturation, chondrocyte hypertrophy, and vascular invasion of the developing bone [Komori et al., 1997; Otto et al., 1997]. Consequently, mice lacking both copies of *Runx2* fail to mineralize their skeletons and die shortly after birth. Mutations that alter the structure and/or function of Runx2 cause the rare human skeletal disorder, cleidocranial dysplasia (CCD) [Mundlos et al., 1997], which is a phenotype that is recapitulated in heterozygous null Runx2 animals [Komori et al., 1997; Otto et al., 1997]. In addition to its essential roles in skeletal development and bone mineralization, recent evidence suggests that elevated levels of Runx2 in breast cancer cells contributes to tumor-induced bone destruction and invasion [Barnes et al., 2004; Javed et al., 2005; Pratap et al., 2005]. Enhanced expression of Runx2 may also contribute to the osteomimic properties of metastatic prostate tumors and to vascular calcification [Lin et al., 2001; Brubaker et al., 2003; Tyson et al., 2003]. The transcriptional activity of Runx2 is regulated by numerous signals and co-factors that either affect Runx2 location and function or alter chromatin structure [Schroeder et al., 2005]. In this review, I discuss the co-repressors that are currently known to bind to Runx2 and negatively regulate its activity. The identification of these factors contributes to our understanding of how Runx2 regulates gene expression and cellular phenotypes.

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RUNT DOMAIN TRANSCRIPTIONAL FACTORS ARE A FAMILY OF CONDITIONAL REPRESSORS

Mammalian Runx proteins are more commonly thought of as activators of gene expression rather than repressors because early functional studies identified them as positive regulators of hematopoietic and osteoblastic genes and because leukemia-associated Runx1 fusion proteins that are generated by chromosomal translocations repressed the activity of wild-type Runx proteins in a dominant negative fashion [Geoffroy et al., 1995; Merriman et al., 1995; Meyers et al., 1995]. The roles of mammalian Runx factors as repressors began to be appreciated several years later when TLE/Groucho proteins were found to bind to their carboxy-termini [Aronson et al., 1997]. This discovery affirmed that an early function ascribed to Runt, a *Drosophila* pair-rule gene product and the founding member of the family, as a transcriptional repressor is conserved [Tsai and Gergen, 1994]. Runt is now appreciated to be a context-dependent repressor that is involved in both the establishment and maintenance of repression [Wheeler et al., 2002]. A genetic screen designed to identify factors that contribute to Runt-mediated repression of *engrailed* uncovered four co-repressors: Tramtrack (Ttk), Groucho, dCtBP, and Rpd3 [Wheeler et al., 2002]. Interestingly, the establishment of *engrailed* repression by Runt is independent of DNA binding and is facilitated by Ttk, another DNA binding protein [Wheeler et al., 2002]. These results suggest that Runt proteins can be co-repressors for other transcription factors. In contrast, DNA binding is required for Runt-dependent repression of other genes and for the maintenance of repression [Wheeler et al., 2002]. The histone deacetylase, Rpd3, and co-repressors, Groucho and dCtBP, co-operate with Runt in the maintenance of *engrailed* repression [Wheeler et al., 2002]. These data indicate that Runt recruits chromatin-modifying proteins to facilitate and maintain transcriptional repression of its target genes.

The realization that the product of *AML1* gene is mutated by numerous chromosomal translocations in acute leukemias and contains a stretch of amino acids that are over 70% identical to a region of Runt, led to cloning and characterization of the Runx1 (AML1) translocation fusion genes and other mammalian Runx factors [Ito, 2004; Blyth et al., 2005]. Mammalian Runx factors were simultaneously and independently being purified and cloned as factors that bound to the core binding sequence in enhancers of polyomavirus and murine leukemia viruses [Ito, 2004]. When the Runx1–ETO fusion protein produced by the *t(8;21)* translocation in acute myeloid leukemias was shown to interfere with the ability of Runx1 to activate transcription [Meyers et al., 1995], studies were initiated to identify the mechanism of transcription repression. ETO was eventually found to be a potent co-repressor that bound tightly to histone deacetylases (HDACs) and other co-repressors, namely mSin3A, mSin3B, N/CoR, and SMRT [Lutterbach et al., 1998]. Additional mutagenesis and control experiments showed that wild-type Runx1, as well as Runx2 and Runx3, were transcriptional repressors that associated with mSin3A, but had much weaker and probably indirect interactions with N/CoR [Fenrick et al., 1999; Lutterbach et al., 2000]. Co-transfection experiments with Runx1 and epitope-tagged HDACs demonstrated that Runx1 interacts strongly with HDACs 1, 3, and 9, but weakly with HDACs 2, 5, and 6 [Durst et al., 2003]. Runx1 also interacts with the transducin-like enhancer of split (TLE) proteins that are homologs of Groucho [Aronson et al., 1997]. This interaction was not necessary for repression of Runx1 target gene, $p21^{CIP1/WAF1}$; however, the interaction with mSin3A was required for repression [Lutterbach et al., 2000]. The association between Runx1 and mSin3A was lost upon ERK-dependent phosphorylation of Runx1 [Imai et al., 2004]. Together these studies suggest that Runx1 is a conditional transcriptional repressor that interacts with co-repressors in a different manner than leukemic fusion proteins.

IDENTIFICATION OF REPRESSION DOMAINS IN Runx2

Runx2 shares significant structural similarity with Runx1 and in most cases similar functions. However, a Runx1–Runx2 chimeric protein was not capable of rescuing hematopoietic deficiencies in *Runx1*-deficient embryos [Fukushima-Nakase et al., 2005]. These results strongly suggest that Runx2 interacts with different co-factors and/or is subject to different regulatory mechanisms than Runx1 and Runx3

because of divergent sequences carboxy-terminal to the Runt domain. To begin to understand how Runx2 regulates gene expression, several laboratories used a mutagenesis approach to identify regions of Runx2 that are sufficient to promote transcriptional activation or repression [Thirunavukkarasu et al., 1998; Westendorf et al., 2002; Schroeder et al., 2004]. These experiments typically involve fusing portions of Runx2 to the GAL4–DNA binding domain and then testing the transcriptional activity of the artificial proteins on a heterologous reporter gene driven by a promoter containing multiple GAL4 binding elements. Thirunavukkarasu et al. [1998] showed that progressive deletion of the carboxy-terminus increased the transcriptional activity of a GAL–Runx2 fusion protein. These assays were performed with a luciferase reporter containing five copies of the GAL4 binding sequence (UAS_G) and the simian virus 40 minimal promoter. Deletion of the last five amino acids, VWPRY, which are conserved in all Runt proteins and are sufficient to bind to TLE/Groucho proteins [Aronson et al., 1997], led to a significant increase in transcriptional activity in Cos-1 and NIH3T3 cells [Thirunavukkarasu et al., 1998]. Further truncation of the carboxy-terminus enhanced this repression several fold. These data indicated that a second repression domain was present in the carboxy-terminus that inhibited the adjacent activation domain (Fig. 1). Similar data with Runx1 led to the proposal that an inhibitory domain is present in this region of the carboxy-terminus and prevents activation by intramolecular mechanisms [Kanno et al., 1998].

Using a different reporter construct wherein four copies of the UAS_G sequence are positioned upstream of a minimal thymidine kinase promoter and luciferase gene, my laboratory identified several autonomous repression domains in Runx2 (Fig. 1) [Westendorf et al., 2002; Schroeder et al., 2004]. A GAL4–Runx2 fusion protein repressed the basal activity of this construct by approximately four-fold [Westendorf et al., 2002], which is equivalent to 80% suppression (Fig. 1B). Deletion of the last 15 amino acids reduced the repression to three-fold

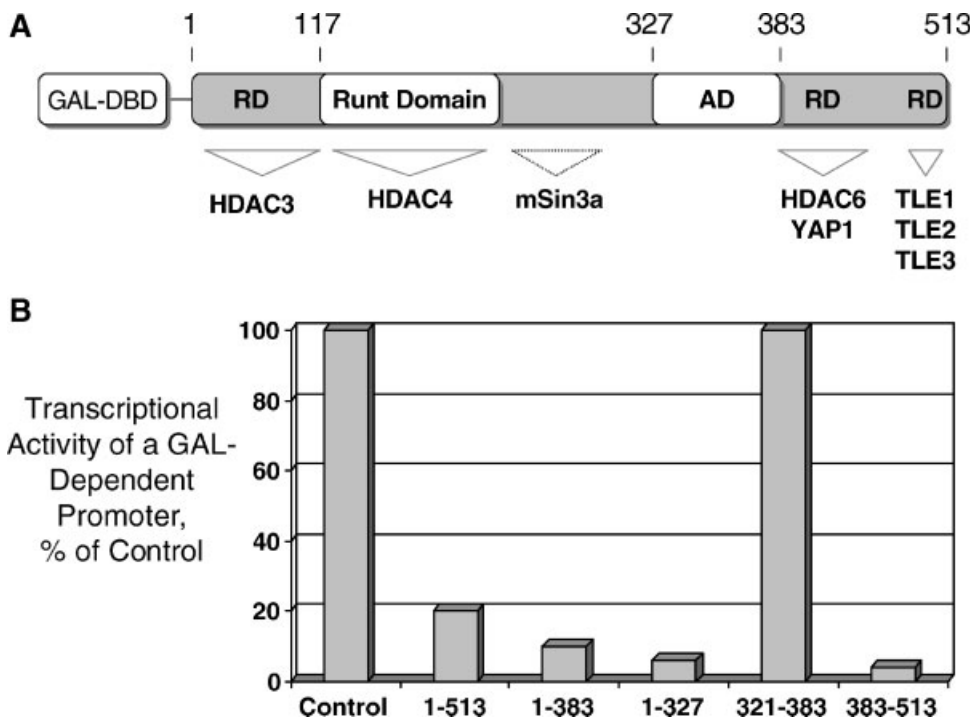

Fig. 1. Runx2 contains multiple repression domains. **A**. This diagram denotes the relative positions of repression domains (RD) in Runx2. The transcriptional activation domain (AD) is also indicated. Distinct regions of Runx2 are sufficient to interact with many co-repressors (below the solid triangles). The dotted triangle above mSin3A indicates that this region contributes to interactions between Runx2 and mSin3A, but another contact site for mSin3A is probable. Fusing Runx2 or parts of it to the GAL4–DNA binding domain (DBD) allowed for the identification of repression domains. **B**. When fused to the GAL–DBD, early all regions of Runx2 repress transcription of a GAL–TK-luciferase reporter in NIH-3T3 cells. The exception is the activation domain.

[Westendorf et al., 2002], demonstrating that the TLE binding contributes to maximal repression. We then tested various regions of Runx2 to identify additional repression domains. We located autonomous repression domains in both the amino- and carboxy-termini [Westendorf et al., 2002; Schroeder et al., 2004]. These truncated proteins were more potent than the full-length protein, likely because they were expressed at higher levels in the cells. The only region of Runx2 that did not have repressive activity contained amino acids 321–383 and encompassed the region required for activation (Fig. 1B). Given the emerging role of HDACs in regulating Runt-domain proteins, we tested whether HDAC activity was responsible for repression by the newly identified repression domains. Trichostatin A (TSA) is a small molecule inhibitor of HDACs. It reversed the activity of GAL–Runx2 fusion proteins to different degrees. Importantly, TSA did not completely reverse the repression of most GAL–Runx2 proteins [Westendorf et al., 2002; Schroeder et al., 2004]. These data indicated that HDAC-dependent and -independent mechanisms contribute to Runx2-mediated repression. They also suggested that Runx2 could recruit cellular co-factors, which are defined as proteins that cannot bind DNA but are directly or indirectly recruited to gene regulatory regions by transcriptions factors. The co-factors currently known to interact with Runx2 are TLEs, mSin3A, HDACs, and yes-associated protein (YAP). Their associations with Runx2 are described in more detail below.

Runx2 INTERACTIONS WITH TLE CO-REPRESSORS

TLEs are the human homologs of Groucho proteins in *Drosophila* and Grg proteins in mice. The longest isoforms are broadly expressed co-repressors and are recruited to promoters/enhancers by numerous transcription factors [Gasperowicz and Otto, 2005]. Runt domain proteins were hypothesized to bind TLEs because their last four amino acids, WRPY, are perfectly conserved and similar to a tetrapeptide sequence, WRPW, in the carboxy-termini of *Drosophila* Hairy and mammalian HES (Hairy, enhancer of split) proteins. The WRPW sequence is required for interactions between HES/Hairy and TLE/Groucho proteins [Fisher et al., 1996; Aronson et al., 1997]. When fused to the GAL4–DNA binding domain, TLE/Groucho proteins are potent transcriptional repressors [Fisher et al., 1996]. Likewise, the extreme carboxy-terminus of Runx2 is a potent repressor in similar assays [Thirunavukkarasu et al., 1998]. The Runx2 carboxy-terminus binds to TLE1, TLE2, TLE3, and Grg3 in yeast-two-hybrid, pull-down and co-localization assays [Javed et al., 2000; McLarren et al., 2000; Wang et al., 2004]. TLEs also repress Runx2-dependent expression of the p6OSE2 and osteocalcin promoters [Thirunavukkarasu et al., 1998; Javed et al., 2000]. The WRPY motif of Runx2 is necessary for TLEs to repress transcriptional activation and for the physical interactions between the two proteins [Thirunavukkarasu et al., 1998; Javed et al., 2000; McLarren et al., 2000]. Another TLE/Groucho protein, Grg5, was also identified in yeast-two-hybrid assays as a Runx2 binding protein [Wang et al., 2004]. In contrast to other family members, it activates Runx2 activity and does not require the WRPY sequence for interactions. Grg5 is a truncated family member and appears to be a dominant-negative inhibitor of larger TLE proteins.

The mechanisms whereby TLE/Groucho proteins repress transcription are poorly defined. They can interact with HDACs and histone H3 [Gasperowicz and Otto, 2005]; thus they may regulate chromatin structure. Hyperphosphorylation of TLEs appears to regulate their ability to repress gene expression. The constitutive protein kinase, CKII, phosphorylates TLEs [Nuthall et al., 2002]. This phosphorylation is directly associated with strong subnuclear localization and transcriptional repression. Binding to transcription factors, such as Hes1 and Runx1, increases TLE hyperphosphorylation [Nuthall et al., 2002]. The downregulation of pan TLE expression during osteoblast differentiation reveals one potential mechanism of relieving repression of Runx2 target genes during lineage progression [Javed et al., 2000].

Runx2 INTERACTIONS WITH THE CO-REPRESSOR, mSin3A

Aronson et al. [1997] demonstrated that Runt-domain proteins repress transcription in TLE/Groucho dependent and -independent manners. These results suggested that other co-repressors of Runx2 must exist. mSin3A is a global transcriptional co-repressor and a stabilizing component of HDAC1/2 complexes

[Silverstein and Ekwall, 2005]. It lacks DNA binding and enzymatic activities, but interacts with numerous proteins and transcription factors to regulate gene expression [Silverstein and Ekwall, 2005]. The identification of mSin3A as a Runx2-interacting protein occurred during studies exploring the mechanisms whereby oncogenic Runx1 fusion proteins repress transcription in acute leukemias. The *t(8;21)* and *t(12;21)* translocation fusion products, Runx1–ETO and TEL–Runx1, respectively, interacted with mSin3A [Fenrick et al., 1999; Lutterbach et al., 2000]. Control and mutagenesis studies showed that metabolically labeled wild-type Runx1, Runx2, and Runx3 also co-immunoprecipitated mSin3A [Fenrick et al., 1999; Lutterbach et al., 2000]. A region of Runx1 just carboxy-terminal to the Runt domain was necessary for its interactions with mSin3A. This domain was also necessary for Runx1 to repress of the p21 promoter [Lutterbach et al., 2000]. The mSin3A binding domain in Runx2 has not been definitely mapped yet. This will be necessary to pursue the mechanisms whereby Runx2 is regulated by mSin3a and associated HDAC complexes. A potential mechanism regulating the interaction between Runx2 and mSin3A is derived from studies with the hematopoietic factor, Runx1. mSin3A protects Runx1 from proteosome-mediated degradation, but it also represses the expression of Runx1-target genes [Imai et al., 2004]. ERK-induced phosphorylation of Runx1, releases mSin3A, changes the subnuclear localization of Runx1, and activates Runx1 transcriptional activities before Runx1 is degraded in a time-dependent manner [Imai et al., 2004]. The ERK-phosphorylation sites are conserved in Runx2. Mutation of these residues partially, but not completely, eliminates interactions of Runx2 with mSin3A (JJW, unpublished data). These data suggest that multiple regions of Runx2 may interact with mSin3A and that Runx2–mSin3A complexes may be regulated by ERK signaling.

HDAC-DEPENDENT REPRESSION OF Runx2

HDACs remove acetyl groups from lysine residues on many proteins, including histones. The elimination of the acetyl group alters chromatin structure by removing a mark needed to recruit co-activating proteins and by facilitating chromatin condensation to promote transcriptional repression [Peterson and Laniel, 2004]. Eighteen HDACs have been cloned from mammalian cells and are classified into four phylogenetic groups [Gray and Ekstrom, 2001; Gregoretti et al., 2004]. Class I HDACs (HDAC 1, 2, 3, and 8) are homologous to the *Saccharomyces cerevisiae* (*S. cerevisiae*) Rpd3 protein and are found in the nuclei of most mammalian cells [Gray and Ekstrom, 2001]. HDAC11 shares many characteristics of class I HDACs and was previously part of this group, but it was recently separated into a new class IV because of its early divergence during prokaryotic evolution [Gregoretti et al., 2004]. Class II HDACs (HDAC 4, 5, 6, 7, 9, and 10) are homologous to the *S. cerevisiae* protein Hda1, shuttle between nuclear and cytoplasmic compartments in response to extracellular signals, and exhibit tissue-specific expression patterns [Yang and Gregoire, 2005]. Class III HDACs (SIRT1–7) are homologous the *S. cerevisiae* Sir2 protein and require NAD^{+} for deacetylase activity [Gray and Ekstrom, 2001]. In accordance with their specific mechanism of action, the general enzymatic activities of class III HDACs are sensitive nicotinomide, but insensitive to small molecule inhibitors that generally target the Zn^{+2}-dependent HDACs in classes I, II, and IV [Avalos et al., 2005].

Three lines of evidence pointed to HDACs as co-repressors of Runx2. First, deletion of the last five amino acids (e.g., the TLE interaction domain) of Runx1 or Runx2 did not prevent repression of all promoters and thereby suggested that other co-repressors must exist [Aronson et al., 1997; Thirunavukkarasu et al., 1998; Javed et al., 2000; Westendorf et al., 2002]. Second, TLE and mSin3A are components of HDAC complexes [Gasperowicz and Otto, 2005; Silverstein and Ekwall, 2005]. Third, small molecule inhibitors of class I and II HDACs partially reversed Runx2-mediated repression [Westendorf et al., 2002; Schroeder et al., 2004], but inhibitors of class III HDACs have no effect (JJW, unpublished data). These data prompted us to use a candidate approach to identify the class I and II HDACs that associate with various repression domains of Runx2. Thus far, three HDACs (HDAC3, HDAC4, and HDAC6) are known to interact with Runx2 [Westendorf et al., 2002; Schroeder et al., 2004; Vega et al., 2004].

HDAC6 was identified as a Runx2 binding protein in co-immunoprecipitation experiments designed to identify co-repressors that bind to

the potent carboxy-terminal repression domain of Runx2 [Westendorf et al., 2002]. HDAC6 co-precipitates with endogenous Runx2 in ROS 17/2.8 cells and its exclusive association with the carboxy-terminus of Runx2 does not require the last five amino acids (i.e., the TLE binding domain) [Westendorf et al., 2002]. The majority of HDAC6 is found in the cytoplasm, but over-expression of Runx2 recruits it to chromatin fractions [Westendorf et al., 2002]. Moreover, an inhibitor of the nuclear export traps HDAC6 in the nucleus where it co-localizes with Runx2 at subnuclear foci [Westendorf et al., 2002]. The HDAC6 binding domain of Runx2 is necessary for repressing the p21 promoter. This carboxy-terminal domain also acts as an autonomous repressor when fused to the GAL4–DNA binding domain. This repressive activity is sensitive to the potent and non-selective HDAC inhibitor, TSA, but insensitive to another inhibitor, trapoxin B, which is 300- to 400-fold less effective on HDAC6 than on other HDACs [Westendorf et al., 2002]. These data indicate that the deacetylase activity of HDAC6 is required to repress Runx2 target genes. However, it has not been definitely proven that acetylated histones are the substrates of HDAC6 when Runx2 recruits it to promoters. HDAC6 may alternatively, or additionally, alter the confirmation of Runx2, deacetylate Runx2, and/or prevent the binding of co-activators to Runx2. The mechanisms (i.e., signaling pathways) regulating HDAC6 nuclear localization are not known; however, one likely mechanism regulating HDAC6 interactions with Runx2 is its temporal expression pattern in cells. In the osteoblast lineage, HDAC6 is expressed at high levels in mature cells but weakly detectable in immature cells. Thus, multiple temporal and spatial events regulate the interaction of HDAC6 with the Runx2 carboxy-terminus.

We identified HDAC3 and HDAC4 as a Runx2 binding proteins in co-immunoprecipitation experiments designed to identify co-repressors that bind to amino-terminal repression domain(s) of Runx2 [Schroeder et al., 2004]. Vega et al. [2004] simultaneously and independently identified HDAC4 as a Runx2-interacting protein after discovering that *HDAC4*-deficient mice have chondrogenic phenotypes resembling tissue-specific Runx2 transgenic animals and conversely that HDAC4-transgenic animals resemble *Runx2*-null animals. HDAC4 binds to the Runt domain of Runx2 and inhibits Runx2 transcriptional activity by preventing Runx2 from binding to DNA [Vega et al., 2004]. HDAC4 is expressed at high levels in pre-hypertrophic chondrocytes, but it is not detected in primary osteoblasts or most osseous cell lines [Schroeder et al., 2004; Vega et al., 2004]. These data indicate that HDAC4 may repress Runx2 activity in specific tissues. HDAC4 moves out of muscle cell nuclei in a phosphorylation-dependent manner [McKinsey et al., 2000]. The extracellular stimuli that regulate HDAC4 nuclear export in chondrocytes are not known, but understanding these mechanisms will increase our understanding of how it represses Runx2 transcriptional activity.

HDAC3 is a broadly expressed nuclear protein. It binds to the extreme amino-terminus of Runx2, but does not associate with the Runt domain or residues carboxy-terminal to the Runt domain [Schroeder et al., 2004]. HDAC3 interacts with the osteocalcin promoter in a region containing a Runx2-binding site and blocks Runx2-dependent activation of the osteocalcin promoter [Schroeder et al., 2004]. HDAC inhibitors and HDAC3-specific short-hairpin RNAs prevent HDAC3 from repressing Runx2 transcriptional activity [Schroeder et al., 2004]. These data suggest that HDAC3 and its associated deacetylase activity are required to repress Runx2 target genes. However, as with HDAC6, it is not known that acetylated histones are the substrates of HDAC3 when Runx2 recruits it to promoters. HDAC3 may alternatively alter the conformation of Runx2, deacetylate lysine residues in other proteins, including Runx2, and/or prevent the binding of Runx2 to co-activators.

YAP1-MEDIATED REPRESSION OF Runx2

YAP1 (also known as YAP65 or YAP) is a 65 kDa intracellular factor originally isolated as a protein that interacted with the Src family kinase and proto-oncogene product, Yes [Sudol, 1994]. YAP1 shuttles between nuclear and cytoplasmic compartments to transmit signals generated by extracellular ligands and amplified by kinases, notably Akt and Src-family members, to regulate gene expression and cell phenotype. These signals generally promote cellular growth, survival, motility, metabolism, and differentiation and are attractive targets for cancer drug discovery [Cheng et al., 2005; Chong et al., 2005]. YAP1 is ubiquitously

expressed and characterized by WW domains, which are protein–protein interaction motifs that associate with proline-rich motifs in a variety of proteins [Sudol et al., 1995].

YAP1 was identified as a binding partner of mammalian Runx proteins in a yeast-two-hybrid screen wherein the proline-rich activation domain of Runx1 was used as the bait [Yagi et al., 1999]. YAP1 was subsequently shown to interact with full-length Runx2 in osseous cells via co-immunoprecipitation of endogenous proteins and co-immunofluorescence [Zaidi et al., 2004]. Runx2 recruits YAP1 to subnuclear foci and to the osteocalcin gene promoter, but does not affect its nucleo-cytoplasmic shuffling [Zaidi et al., 2004]. The Y residue in the PPPYP motif of Runx2 is essential for interactions with YAP1 [Zaidi et al., 2004]. There is no indication that YAP1 can bind to DNA, but when fused to the GAL4–DNA binding domain, YAP1 acts as a transcriptional co-activator of a heterologous GAL-dependent reporter [Yagi et al., 1999; Zaidi et al., 2004]. Accordingly, YAP doubled the Runx2 (PEBP2aA1)-dependent activation of the IgC alpha promoter in p19 cells and a dominant-negative YAP1 construct blocked Runx2-dependent activation of the osteocalcin promoter in NIH3T3 cells; however, a full-length version of YAP1 was not tested on the osteocalcin promoter in this study [Yagi et al., 1999]. Zaidi et al. [2004] later showed that YAP1 repressed Runx2-dependent activation of the osteocalcin promoter in NIH3T3 cells and four other cells lines. Thus, YAP1-mediated repression of Runx2 activity on the osteocalcin promoter is cell-type independent. YAP1-mediated repression of Runx2 instead seems to be dependent on promoter context. YAP1 blocked Runx2-dependent activation of the TGFßR1 promoter and enhanced Runx2-dependent repression of its own promoter; however it did not affect Runx2's transcriptional effects on the p6OSE2 or p21 promoters [Zaidi et al., 2004]. These data indicate that Runx2 can recruit YAP1 to promoter regions, but the effects of YAP1 on the expression of Runx2 target genes is dependent on the cohort of other DNA binding proteins and co-factors brought to the gene by specific DNA sequences and protein–protein interactions.

Transcriptional repression of Runx2 by YAP1 is dependent on Src-induced activation and phosphorylation of YAP1 [Zaidi et al., 2004]. Dominant-negative Src and YAP1 proteins, as well as Src kinase inhibitors, increased Runx2 transcriptional activation of the osteocalcin promoter in ROS 17/2.8 cells [Zaidi et al., 2004]. Tyrosine phosphorylation of YAP1 is required for its subnuclear co-localization with Runx2 but not for its nucleo-cytoplasmic transport [Zaidi et al., 2004]. Thus, YAP1 is a signal-responsive and context-dependent regulator of Runx2 activity that may facilitate gene expression in response to extracellular signals or oncogene activation.

FUTURE PERSPECTIVES

Runx2 regulates the expression of genes that contribute to normal skeletal development and tumor metastasis [Blyth et al., 2005]. It is commonly believed that Runx2 controls the transcriptional initiation of genes by interacting with other DNA binding factors, co-activators, and co-repressors; thereby organizing an appropriate regulatory complex around a Runx binding element [Schroeder et al., 2005]. Cooperating transcription factors and co-activators are generally thought to open chromatin structure and facilitate the assembly of a complex that recruits and activates RNA polymerase II to initiate gene expression. Conversely, co-repressors would block the proper assembly of this complex by condensing chromatin, recruiting other chromatin modifying proteins and preventing the recruitment of activating complexes. The identification of histone acetyltransferases and HDACs as Runx2-interacting proteins provides support for this model. Although this review focuses on transcriptional co-repressors of Runx2, it is important to also consider that Runx2 may repress gene expression via other mechanisms. For example, Runt is a co-repressor of Tramtrack in *Drosophila* [Wheeler et al., 2002]. In addition, a recent study showed that Runx1 inhibits transcriptional elongation by RNA polymerase II [Jiang et al., 2005]. Thus, Runx factors may repress gene expression via multiple mechanisms and at multiple times during the transcription process.

More than 30 Runx2-interacting proteins have been described [Schroeder et al., 2005]. Of these, 8 proteins can be classified as co-repressors based on their inability to bind DNA directly. Six of these factors (TLE1, TLE2, TLE3, mSin3A, HDAC3, and HDAC6) are components of multi-protein chromatin-modifying complexes

that repress the expression of Runx2 target genes (Fig. 2). HDAC3 and HDAC6 possess deacetylase activities, and therefore may directly affect chromatin structure. In contrast, mSin3A and the TLE proteins do not have any intrinsic enzymatic activities but may recruit HDACs to affect chromatin structure. HDAC4 also represses Runx2 trans-activity but its HDAC activity may not be important because it directly inhibits Runx2 by binding to its DNA binding domain and preventing it from recognizing its target sequence (Fig. 2). With all of the HDACs or HDAC-associated co-repressors, it is important to consider that lysine residues in histones may not be the only or the primary targets of the deacetylases. Runx2 or other proteins may also be post-translationally modified when these enzymes interact with Runx2.

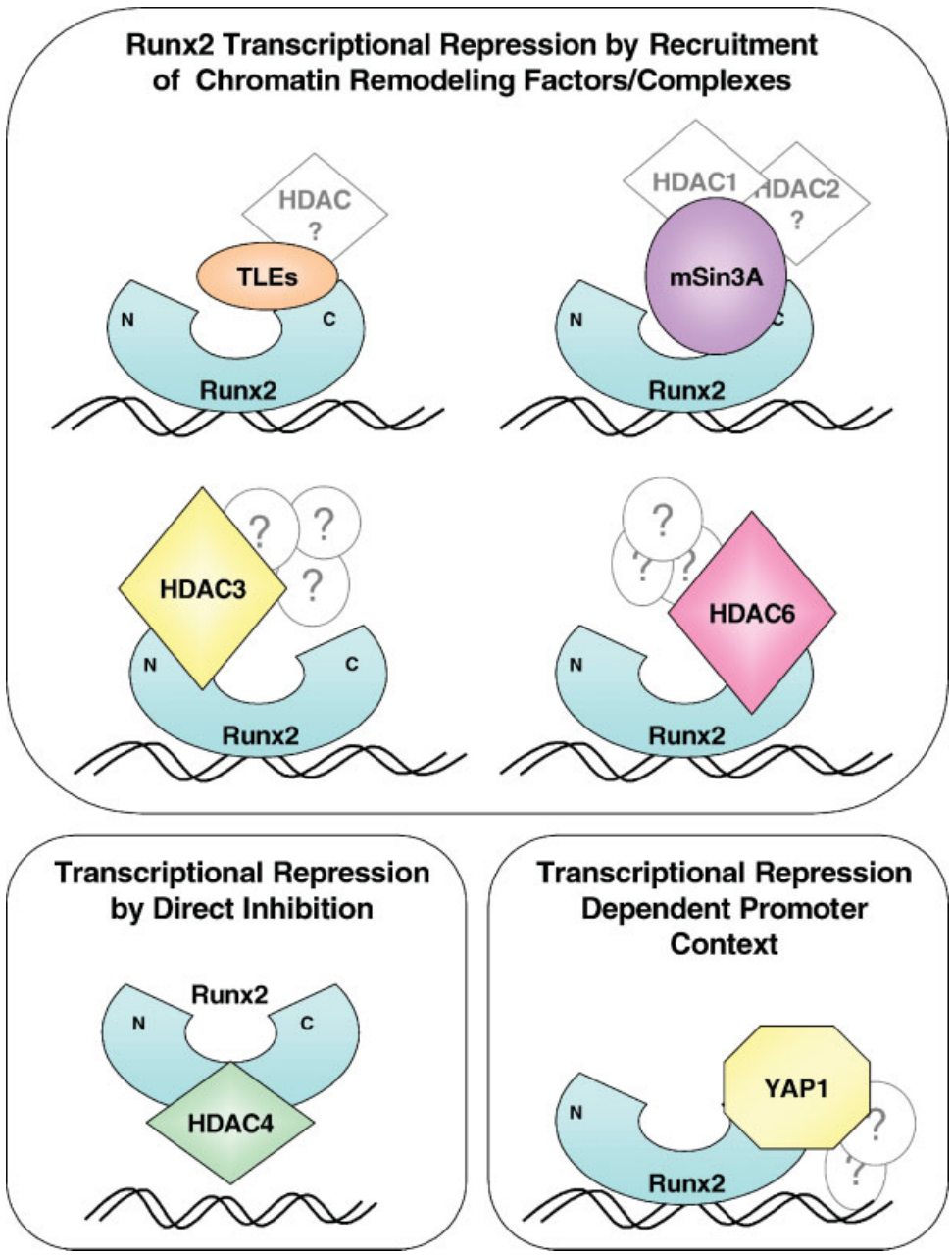

Fig. 2. Co-repressors block Runx2 transcriptional activity via several mechanisms. N and C denote the amino and carboxy-termini of Runx2, respectively. Question marks represent unknown proteins that may be directly or indirectly recruited to promoters as components of multiprotein complexes.

The final co-repressor described within, YAP1, does not have any intrinsic enzymatic activities and in some cases, acts as a co-activator rather a co-repressor. Its co-repressive activity towards Runx2 is dependent on promoter context (Fig. 2).

The identification of Runx2 co-repressors is a necessary step towards understanding the mechanisms regulating Runx2 transcriptional activity. The next challenge is to define the optimal temporal and spatial contexts wherein co-repressor complexes interact with Runx2 and regulate the expression of specific Runx2 target genes. To accomplish this goal, several variables must be considered. These issues include the cell type one is studying, the maturation or differentiation status of the cell, and the structure and sequence of the target gene of interest. The initial studies with HDAC4 demonstrate the importance of cell-type specificity. Although Runx2 is expressed in both osteoblasts and chondrocytes, HDAC4 is more readily detected in chondrocytes [Schroeder et al., 2004; Vega et al., 2004]. Thus, it may be a tissue-specific regulator of Runx2. There is also evidence that some of the co-factors are differentially regulated as osteoblasts differentiate. HDAC6 levels increase during osteoblast differentiation, while TLE levels decrease [Javed et al., 2000; Westendorf et al., 2002]. Changes in the number and type of co-repressors may play a role in regulating the expression of specific genes. Time-dependent chromatin immunoprecipitation assays will address this possibility. Such assays will also contribute to detailed promoter analyses that need to be performed on each Runx2 target gene. The correlation of Runx2 and co-factor binding to nucleosome positioning and structure will ultimately reveal how Runx2 target genes are regulated.

Another issue that must be considered when striving to understand the temporal and spatial regulation of Runx2 transcriptional activity is the involvement of extracellular signals that lead to the post-translational modifications or altered subcellular distributions of Runx2 and/or its associating co-factors. For example, phosphosphorylated TLE proteins interact with Runx2 and have a different subnuclear staining pattern than unphosphorylated TLEs [Nuthall et al., 2002]. Class II HDACs (e.g., HDAC4 and HDAC6) provide a more striking example because they are actively shuttled across the nuclear membrane. Phosphorylation favors their nuclear export and some of the responsible kinases have been identified [Yang and Gregoire, 2005]. It will be necessary to determine whether these kinases target HDACs and Runx2-co-repressor complexes, as well as to identify the extracellular stimuli that activate the kinases. The answers from studies done with osteoblasts may be different than those derived from studies on chondrocytes, endothelial cells, or invasive breast cancer cells. Deciphering these details will bring us closer to the ultimate goal of enhancing skeletal health and eliminating bone metastases.

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