Direct Interactions of Runx2 and Canonical Wnt Signaling Induce FGF18*[^s]

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Canonical Wnt signaling is clearly required for skeletal development and bone formation. However, the targets of Wnt signaling that convert this signal into bone are unclear. Identification of these targets will yield insight into normal bone physiology and suggest new therapeutics for treatment of bone disease. Here we show that an essential regulator of bone development, FGF18, is a direct target of canonical Wnt signaling. A single DNA binding site for the Wnt-dependent transcription factors TCF/Lef accounted for the stimulation of the fgf18 promoter in response to Wnt signaling. Additionally, targeted disruption of βcat blocked fgf18 expression in vivo. Partially overlapping the TCF/Lef binding site is a Runx2 binding site and experiments showed that Runx2 and TCF/Lef work cooperatively to induce fgf18 expression. RNA interference knockdown of Runx2 inhibited and Runx2 forced expression augmented the induction of fgf18 by canonical Wnt signaling. Significantly, Runx2 formed a complex with Lef1 or TCF4 and this complex bound the composite binding site in the fgf18 promoter. These results demonstrate that two transcription pathways that are essential for bone, physically and functionally converge at the fgf18 promoter.

The signaling cascade initiated by the Wnt family of polypeptides controls normal and abnormal development in a variety of tissues (1–3). Wnt signaling is initiated by the binding of the extracellular ligand (Wnt) to a seven-transmembrane spanning receptor and coreceptor. The receptor complex is comprised of frizzled, a multiple membrane spanning protein and a low density lipoprotein-related coreceptor, LR5 or LR6. The receptor–ligand complex initiates a complex cascade of events. Signals downstream of the Wnt receptor complex are separated into canonical and noncanonical pathways (4). Canonical Wnt (cWnt)[^2] signaling regulates the protein levels of β-catenin (βcat). Within the cytosol βcat resides in a multisubunit complex that includes the proteins axin, APC, glycogen-synthase kinase 3 (GSK3) as well as others (5). In the absence of Wnt, GSK3 phosphorylates specific residues near the amino terminus of βcat, leading to its recognition and degradation by a βTrCP-dependent proteosome pathway. When Wnt engages its receptor-coreceptor complex, the protein disheveled is recruited to the membrane, phosphorylated, and directed to the APC-AXIN-GSK-βcat complex where it represses GSK3 activity. Inhibition of GSK3 leads to accumulation of βcat and migration to the nucleus. Within the nucleus βcat interacts with specific DNA binding transcription factors, most notably the TCF/Lef family of transcription factors (6). When associated with TCF/Lef, βcat recruits other transcription cofactors and stimulates the expression of Wnt target genes.

The TCF/Lef transcription factors are a family of 4 proteins that bind to DNA through a conserved high mobility group domain (7). TCF/Lef proteins transduce cWnt signals into changes in gene expression. In the absence of Wnt, βcat levels are low and TCF/Lef proteins act as transcription repressors by recruiting the general transcription inhibitors Groucho/TLE and histone deacetylases (8, 9). Accumulation of βcat in the nucleus leads to direct displacement of Groucho/TLE through the competitive binding of βcat to TCF/Lef (10). βcat activates gene expression by recruiting coactivators like p300/CBP (11) and the Mediator complex (12).

cWnt signaling has profound effects on normal cell and tissue development and directly contributes to pathological conditions like neoplasia. During bone formation and remodeling, Wnt signaling is indispensable. Studies of genetically modified mice have contributed much to our understanding of the many functions of Wnt signaling in the skeleton. Gene knockouts of individual Wnts have shown requirements for Wnt10b (13), Wnt5a, -5b (14), and others during normal skeletal development. To study cWnt signaling during bone growth and remodeling, βcat has been conditionally inactivated in several studies. These studies have proven that Wnt signaling is required at several steps during osteoblast differentiation. Inactivation of βcat at early steps of osteoblast formation using cre recombinase expressed from the Dermo1, type I collagen, and Prx1 promoters caused aborted osteoblast differentiation (15–17). Early osteoblasts expressing the osteoblast transcription factor Runx2 were present, but mature osteoblasts and synthesis of bone matrix was absent. Other studies showed βcat requirements at later stages of osteoblastogenesis (18, 19); mature osteoblasts failed to develop when βcat was deleted at a stage when the transcription factor osterix is expressed, indicating a requirement for cWnt downstream of or coinciding with
ostrich. In addition, cessation of cWnt signaling is required for osteoblast maturation (18). Finally, cWnt signaling also regulates bone osteoclast differentiation and bone resorption by regulating osteoprotegerin expression (20).

Naturally occurring mutations in humans have proven the relevance of Wnt signaling during osteoblast development and for controlling bone mass. A syndromic form of osteoprosis is caused by loss of function mutations in the Wnt co-receptor LPR5 (21), and a high bone mass phenotype results from mutations in LRP5 that diminish its affinity for Dkk1, an antagonist of Wnt (22, 23). Thus, in accord with the animal studies, Wnt signals are anabolic for bone and decreased Wnt signaling result in clinically significant bone deficiencies.

These findings have proven the significance of cWnt signaling during osteoblast differentiation. Moreover, these studies suggest that careful stimulation of cWnt signaling or antagonism of βcat decay will promote bone formation. Identification of the Wnt target genes that are required for osteoblasts formation in response to cWnt signaling will also signify important pathways that can be modulated for therapeutic benefit of bone disease. In this report we show that the fibroblast growth factor 18 gene (fgf18) is a downstream target of cWnt signaling. FGF18 is expressed in a tissue domain that coincides with (i) sites of osteoblast development and (ii) regions where βcat levels (and therefore Wnt signaling) are increased. This implied that fgf18 is a candidate for a Wnt target gene that translates cWnt signaling into osteoblast differentiation. We find that fgf18 is directly induced by cWnt signaling. TCF/LeF proteins bind to a consensus target sequence of the fgf18 promoter and when stimulated by βcat induce fgf18 expression. Remarkably, the expression of fgf18 is directly coupled to another essential osteoblast transcription factor, Runx2. Partially overlapping the TCF/LeF site of the fgf18 promoter is a recognition motif for Runx2. Experiments showed that Runx2 interacts with TCF/LeF and forms a ternary complex at the FGF18 promoter. RNA interference experiments showed that Runx2 is necessary for stimulation of FGF18 expression by Wnt, demonstrating that Wnt signaling and Runx2 are both physically and functionally linked. These findings reveal exquisite control of fgf18 expression by two essential osteoblast transcription factors that combine to determine tissue specificity and stimulus dependence. These findings suggest that FGF18 may function as an essential component of the Wnt-dependent steps of osteoblastogenesis that are genetically downstream of Runx2.

**MATERIALS AND METHODS**

**Reagents**—SB216763 was from Tocris (Cookson, MO). Wnt3a conditioned medium was prepared from Wnt3a expressing L-cells according to the suppliers instructions (ATCC, Manassas, VA). Conditioned medium without Wnt3a was made using L-cells.

**Plasmids**—To create the −3.3-kb fgf18 reporter plasmid a 3.4-kb MscI fragment of BAC clone RPI 23323L8 was blunt-end cloned into the SmaI restriction site of pGL3basic (Promega, Madison, WI). For the −1.1-kb reporter plasmid a PvuII/KpnI fragment was excised from the −3.3-kb plasmid and blunt-end ligated into the SmaI site of pGL3basic. The −0.8-kb reporter was prepared by SacI digestion of the −3.3-kb reporter that was cloned into pGL2basic rather than pGL3basic. The released fragment was gel purified and cloned into the SacI site of pGL3basic. MluI digestion of the −3.3-kb pGL2basic reporter resulted in a DNA fragment that was cloned into the MluI site of pGL3basic. To produce the trimeric repeats, gel purified oligonucleotides or 415-bp fragments having BamHI or BglII sites at opposite ends were kinased, ligated, and fractionated by agarose gel electrophoresis. The primer was excised from the gel and cloned into the BglII site of pGL3 TATA, a TATA box containing luciferase reporter. pGL3 TATA was created by cloning the oligonucleotide AGATCTGGGTATATAAG- GATCCGGTAAAGCCT and the reverse complement into the BglII/HindIII sites of pGL3basic. LeF1 was cloned into the Stul site of pCS2+ following MluI/HindIII digestion and Klenow fill-in of the image clone 6054813. Runx2 (form II) was PCR amplified from a mouse cDNA library using the primers CCGGTACCATCATGCTTCATTCGCTCA- CAAA and TTCAGATCAATATGGCCGCCAAACAGAC- TCATCCATTCTGGCCGCTAAGATTCA and cloned into pcDNA3.1 (Invitrogen).

**Site-directed Mutagenesis**—Mutations of the TCF binding site were introduced into the −1.1-kb and (415)3 reporter plasmids via QuiChang site-directed mutagenesis following the manufacturer’s instructions (Stratagene, La Jolla, CA). The mutations were verified by DNA sequencing. The sequence of the mutagenesis primers is available on request.

**Metatarsal Cultures**—Metatarsal were dissected from E15.5 day C57Bl/6 embryos and cultured in serum-free medium as described previously (24). When treated with Wnt3a, the medium was exchanged with conditioned medium (with or without Wnt3a) and the samples were harvested after 24 h.

**In Situ Hybridization**—4-μm sections of paraffin-embedded tissues were processed and hybridized with riboprobes as described previously (25). The FGF18 riboprobe plasmid was a gift from D. Ornitz (St. Louis, MO).

**Immunofluorescence**—4-μm paraffin sections were prepared as previously described (24). Primary antibodies were used at a 1:200 dilution (β-catenin, Santa Cruz sc7963; and FGF18, Santa Cruz sc16830).

**Cell Culture and Transfections**—MC3T3E1 (clone 14; Ref. 47) and HEK293 were obtained from the ATCC and maintained subconfluent in α-minimal essential medium or Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (HyClone), 2 mM glutamine, penicillin G (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified 5% CO2 incubator. Cells were transfected in triplicate using a modified calcium phosphate precipitation and assayed for luciferase and β-galactosidase activity (26, 27). Luciferase activity was normalized to β-galactosidase to control for transfection efficiency.

**Real Time Quantitative-PCR**—Total RNA was prepared from cells using RNeasy according to the manufacturer’s instructions (Qiagen). One μg of total RNA was reverse transcribed as described (28). Real time PCR was done using a SYBR Green PCR mixture (Applied Biosystems) in an ABI 7000 sequence detection system (Applied Biosystems). The sequence of the primers were as reported (28). Ubiquitin was used as the normalizer.
RNA Interference—The small interfering RNA oligonucleotide against mouse Runx2 was targeted to GTCCTATGAC-CAGTCTTAC. A negative control shRNA oligonucleotide directed against TTCTCCGAACGTGTCACGT, unrelated to Runx2, was used. The BD Knock-out RNAi System (BD Bioscience) was used for generating small interfering RNAs. Briefly, a double-stranded DNA oligonucleotide containing the shRNA and BamHI and EcoRI overhangs was cloned into the corresponding sites of RNAi-Ready pSIREN-Shuttle vector. The construct was verified by sequencing. MC3T3E1 cells were transiently transfected with shRNA plasmids using Nucleofection (Amaxa Inc., Gaithersburg, MD). 10^6 cells were resuspended in 100 μl of Nucleofector solution T and transfected with 5 μg of DNA using Program T-20. Transfection efficiency using these conditions was shown to be >80%. RNA was prepared 24 h post-transfection from cells using an RNasy (Qiagen) kit according to the manufacturer’s instructions.

Chromatin Immunoprecipitation—MC3T3 cells at about 80% confluence were stimulated for 4 h with 20 μM SB216763. Thereafter, protein-DNA complexes were cross-linked with 1% formaldehyde for 10 min at room temperature and cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. Cells were washed with phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride, pelleted at 1200 rpm, and solubilized in swelling buffer (5 mM Pipes, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, protease inhibitor mixture), incubated on ice for 20 min, and then Dounce homogenized. The nuclei were collected by microcentrifugation and then resuspended in sonication buffer (1% SDS, 0.1% sodium deoxycholate, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10 mM Tris, pH 8) and precleared for 2 h at 4 °C with 2 mg/ml salmon sperm DNA, 10 mg/ml bovine serum albumin, and 30 μl of Protein G-Sepharose. Prior to use, Protein G-Sepharose was blocked with 0.2 mg/ml of sheared salmon sperm DNA and 1 mg/ml of bovine serum albumin for at least 4 h at 4 °C. Precleared chromatin was incubated with antibody
and rotated at 4 °C for ~12–16 h. Antibodies used included anti-β-catenin (sc-7963, Santa Cruz), anti-Runx C19 (sc-8566, Santa Cruz), and anti-HA (H6908, Sigma). Chromatin-antibody complexes were recovered using 45 μl of Protein G-Sepharose slurry. The bound complexes were washed with 1) RIPA lysis buffer (3 times), 2) RIPA lysis buffer plus 0.5 M NaCl (3 times); 3) LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris (pH 8) (2 times); and 4) TE buffer (2 times). The samples were then eluted with 1% SDS, 0.1 M NaHCO₃ and cross-links were reversed at 65 °C for 4 hours to overnight. The samples were then treated for 1 h at 45 °C in 200 mM NaCl, 10 mM EDTA, 40 mM Tris, pH 6.8 and 30 μg/ml Proteinase K. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. PCR was performed using FGF18 gene primers: 5'-CATTCTTTGGGAACCCCGCCT-3' and 5'-GGCTGGGTGCGCCGCCCACATC-3' for 34 cycles (94 °C for 45 s; 69 °C for 45 s; 72 °C for 45 s).

**Immunoprecipitation**—HEK293 cells were transfected with expression plasmids using a modified calcium phosphate precipitation method (26, 27). 48 h after transfection the cells were lysed with 1% deoxycholate and 1% Triton X-100, 50 mM Tris, pH 8.0, 0.15 M NaCl, 2 mM EDTA, and 50 μg/ml phenylmethylsulfonyl fluoride (Sigma), 0.25 mM orthovanadate (Sigma), and protease inhibitor mixture (Sigma). Clarified lysates were precleared with Protein G-Sepharose (Amersham Biosciences), and then immunoprecipitated with anti-Lef1 (Santa Cruz; sc8591), anti-β-catenin (Santa Cruz; sc7199), or anti-hemagglutinin (Sigma; H6908) absorbed to protein G-Sepharose (Amersham Biosciences). Proteins were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Milli-
Results

We examined by indirect immunofluorescence the presence of FGF18 and βcat in metatarsal bone rudiments isolated from E15.5 mouse embryos. The rudiments were cultured in serum-free medium and differentiated spontaneously. Interestingly, FGF18 protein was found at sites in the perichondrium that coincide with sites of osteoblast differentiation and increased levels of βcat (Fig. 1, A and B, and color micrographs in supplementary data Fig. S1). We previously determined that FGF18 expression is induced following inhibition of GSK3 (24). We therefore reasoned that FGF18 is induced by cWnt signaling. In accord with this, fgf18 expression was induced in the perichondrium of metatarsals cultured in medium containing Wnt3a (Fig. 1, C and D) and in cultured cells (Fig. 1E). To further characterize the regulation of fgf18 by canonical Wnt signaling, we examined fgf18 expression in bones devoid of βcat. βcat was conditionally inactivated in the developing bones by crossing mice with floxed alleles of βcat (30) to the cre-deleter strain Dermo1-cre (29). Consistent with the view that canonical Wnt signaling directly induces FGF18 expression, FGF18 transcripts were absent in the perichondrium of bones lacking βcat (Fig. 1, F and G).

To identify the specific targets of Wnt signaling in the fgf18 gene we cloned various portions of the fgf18 promoter into a luciferase reporter vector. Transient transfection of these reporter constructs together with a proteosome-resistant form of βcat or following stimulation of the transfected cells with Wnt3a demonstrated that a 415-base pair motif between −0.8 and −0.5 kb is stimulated by βcat or Wnt3a (Fig. 2, A and B). This 415-bp sequence was sufficient to respond to βcat when cloned into a TATA box containing minimal reporter (Fig. 2C). Stimulation of this sequence by βcat was enhanced when the Wnt-dependent transcription factor, TCF4, was cotransfected (Fig. 2C). These data indicate that the 415-bp motif is stimulated by cWnt signaling; therefore, we examined this sequence for putative TCF/Lef recognition sites (Fig. 2D). Three potential binding sites (A, B, and C) were identified. To further characterize these sites and determine if 1) the DNA sequence associated with the DNA binding domain with putative binding sites in the fgf18 promoter. Competition (comp) was done with a 100-fold molar excess of unlabeled oligonucleotide. B, luciferase assays of trimeric repeats of the indicated oligonucleotides cloned into a TATA box containing minimal reporter. Cotransfections were done in HEK293 cells with βcat and/or a dominant negative form of TCF4 (ΔTCF4). Assays were performed in triplicate and the error bars represent ± S.E. D, PCR results for fgf18 following chromatin immunoprecipitation of βcat (top panel) from MC3T3E1 cells stimulated for 4 h with (+) or without (−) 20 μM SB216763. Input represents amplification following immunoprecipitation using purified rabbit immunoglobulin. The figures show data representative of two or three independent experiments.
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We identified a sequence that regulates the expression of fgf18 in response to Wnt. However, Wnts are widely expressed, yet fgf18 expression is much more restricted. This suggested that additional transcription regulators contribute to the expression of fgf18, and that these regulators may functionally cooperate with βcat and TCF/LeF to induce fgf18 expression. Significantly, fgf18 expression is greatest in the perichondrium. We then questioned if transactivators expressed in perichondrial cells also regulate fgf18 expression. We searched for clues concerning fgf18 expression by examining the DNA sequence surrounding the TCF/LeF site. Interestingly, a putative Runx2 binding site (TGTTG) partially overlaps the TCF/LeF site (Fig. 2E). Notably, Runx2 is expressed in the perichondrium, and therefore is a good candidate for a bone cell-specific transactivator that may restrict fgf18 expression to the perichondrium. To examine effects of Runx2 on fgf18 expression during Wnt activation, we transfected MC3T3E1 osteoblasts with Runx2 and simulated cWnt signaling by inhibiting GSK3 with the pharmacological antagonist SB216763. We chose a concentration of SB216763 that produced submaximal induction of fgf18. Significantly, the combination of Runx2 and SB216763 induced fgf18 to a greater degree (9-fold) than Runx2 alone in the absence of SB216763 (2.8-fold; Fig. 4A). Similar results were obtained with Wnt3a (Fig. 4A, right). Fig. 4B shows that Runx2 did not stimulate the expression of Axin2, a gene that is strongly induced by canonical Wnt signaling (31). These data therefore suggest a cooperative relationship between Runx2 and TCF/LeF at the FGF18 promoter. This cooperation does not, however, extend to all Wnt-regulated genes, as evidenced by the absence of an effect on Axin2. In fact as shown in Fig. 4B, Runx2 may repress Wnt-dependent induction of Axin2. To further analyze the interaction of Wnt-dependent transcription and Runx2, we diminished Runx2 protein levels by RNA inter-
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ference knockdown. Knockdown of Runx2 suppressed induction of FGF18 by the GSK3 antagonist and Wnt3a (Fig. 4C). In contrast, the induction of Axin1 was only modestly effected (Fig. 4D). These data support a functional interdependence of Runx2 and Wnt-dependent transcription at certain target loci like fgf18. The proximity of the TCF/Lef and Runx2 sites of the fgf18 promoter suggests that these transcription factors may interact directly. To examine this more closely we asked if Runx2 stimulates the FGF18 luciferase reporter plasmids. Fig. 4E shows that Runx2 alone did not stimulate the 3.3-kb FGF18 luciferase reporter (nor fragments thereof; data not shown). However, expression of Runx2 together with βcat led to dose-dependent stimulation of the 3.3-kb fgf18 promoter (Fig. 4F). At submaximal levels of βcat the 3.3-kb FGF18 reporter was stimulated 5.3-fold, whereas βcat plus Runx2 stimulated the promoter to 16.6-fold. The 1.1-kb FGF18 reporter was similarly induced by the combination of βcat and Runx2 (Fig. 4G). The costimulation by βcat and Runx2 required an intact TCF/Lef site. Mutation of this site abolished stimulation by the combination of Runx2 and βcat (Fig. 4H). The trimer of the A oligonucleotide and the 417-bp motif (Fig. 5A and data not shown) were also coactivated by βcat and Runx2. Fig. 5A shows that whereas Runx2 alone did not stimulate the reporter, Runx2 combined with βcat caused a 40-fold activation. This compares with a 9.8-fold stimulation by the indicated amount of βcat. Significantly, a TCF/Lef binding site was insufficient for coactivation by Runx2 and βcat. In fact, the Topflash reporter, which contains repeats of an optimum TCF/Lef binding site was inhibited rather than activated by the combination of βcat and Runx2 (Fig. 5B). These data predict that Runx2 interacts specifically with the sequence of fgf18. To examine this, we constructed a reporter wherein the potential Runx2 binding site was mutated (A RUNxmut, see Fig. 2E). Supporting a direct interaction with the Runx2 site, the mutated reporter was not stimulated by Runx2, yet remained inducible by βcat, albeit less so (Fig. 5C). Chromatin immunoprecipitation yields additional evidence that Runx2 interacts with fgf18. Fig. 5D shows that Runx2 is immunoprecipitated with the endogenous fgf18 promoter at the expected site. These data (Runx2 overexpression, Runx2 shRNA, cotransfection of Runx2 and βcat) demonstrate a close functional relationship of Runx2 and the TCF/Lef transcription factors on fgf18. Because the TCF/Lef and Runx2 sites partially overlap, the respective transcription factors may physically interact to regulate the expression of FGF18. To examine this we performed co-immunoprecipitation experiments. First, we examined if βcat interacts with Runx2. If βcat binds to Runx2, this could explain our finding that βcat is required for stimulation of the fgf18 reporters by Runx2. HEK293 cells were transfected with Runx2 or Runx2 and βcat. βcat was immunoprecipitated and immunoblots for Runx2 were done. The results did not reveal interactions of βcat and Runx2 (Fig. 6A). However, Runx2 binds to both Lef1 and TCF4. Fig. 6, B and C, shows that Runx2 was co-immunoprecipitated with either Lef1 or HA-tagged TCF4. These results demonstrated association of TCF4 or Lef1 with RUNx2 in the absence of DNA binding. We next asked whether the proteins also assemble on DNA from the fgf18 gene. EMSA experiments were done using oligonucleo-

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tionally, in the indicated samples we included an anti-Runx2 antibody that produced slower migrating complexes that were more evident on the nondenaturing gels. Fig. 7C shows that oligo A competed for the binding of Runx2 to OSE; both super-shifted and nonsupershifted species were disrupted.

**DISCUSSION**

We showed that the expression of fgf18 is regulated by the combined actions of Wnt-dependent transcription factors TCF/Lef and Runx2. This reflects functional and physical interactions of TCF/Lef and Runx2 at a composite binding site for both transcription factors in the FGF18 promoter. We found that Runx2 and TCF4 or Lef1 form a complex in the absence of DNA and that this complex promotes the binding of Runx2 to its recognition motif in the FGF18 promoter. It is likely that TCF/Lef induces a conformational change in Runx2 that allosterically activates Runx2 DNA binding activity. This is analogous to the interactions of Runx1 and core binding factor β (CBFβ). Binding studies of the runt domain of Runx1 and CBFβ showed a 5-fold decrease in the Runx1-DNA dissociation constant when Runx1 is bound to CBFβ. NMR studies demonstrated changes in the amide bond backbone of Runx1 upon binding to CBFβ and the crystal structure show that Runx-CBFβ has stabilized loop conformations at the site of DNA contact. CBFβ interacts with the runt domain of Runx1 principally through two surface domains that are opposite the DNA binding interface. This same surface may interact with non-DNA binding domains of TCF4 or Lef1. The DNA bending induced by Lef1 and TCF4 at its recognition site may permit simultaneous protein-protein and DNA-protein interactions, and enhanced affinity of Runx2 for DNA. Whether the high mobility group domain of TCF/Lef and the runt domain of Runx2 are sufficient for this complex is unclear. The amino-terminal glutamine/alanine-rich region of Runx2 appears to prevent interactions with the Runx1 binding partner, CBFβ (33). Perhaps in addition to altering affinity for CBFβ, this domain may selectively contribute to binding with TCF/Lef proteins.

After a Runx2-TCF/Lef complex is bound to DNA how does this complex stimulate the expression of FGF18? Clearly, βcat is a key coactivator. The interactions of βcat with TCF/Lef are well recognized and in this way histone acetyltransferases like p300 (11) are recruited to the promoter. If βcat binds to Runx2, then the activator properties of Runx2 at the FGF18 promoter could be explained by the additional binding energy for βcat that is contributed by Runx2. However, βcat did not interact with Runx2 by coimmunoprecipitation and gel-shift experiments did not demonstrate disproportionate binding of βcat to Lef1-Runx2 complexes. Therefore, we speculate that Runx2 activates the FGF18 promoter through mechanisms distinct from the recruitment of βcat. Runx2 may activate expression...
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through actions such as recruitment of histone acetyltransferases like MOZ and MORF (34) or binding to other transcription factors like AP-1 or Smad (35, 36). Alternatively, Runx2 may stimulate \( \text{fgf18} \) expression through pathways not yet described.

Significantly, a previous report showed that the human FGF18 promoter is stimulated by a \( \beta \)-cat/TCF complex (37) and that FGF18 contributed to colon cancer cell growth. The sequence of the mouse and human promoters at the TCF/Lef binding site is 100% conserved supporting the significance of this site for FGF18 gene expression. This implies that FGF18 expression is normally targeted to sites where cWnt signaling and Runx2 (and perhaps other members of the Runx family) coincide. We have shown that \( \beta \)-cat is required for the expression of \( \text{fgf18} \) in the perichondrium of long bones during skeletal development. This suggests that both Runx2 and cWnt signaling are required for \( \text{fgf18} \) expression under normal physiological states; the absence of either may result in the loss of expression. That is, the physical coupling of TCF/Lef and Runx2 and the juxtaposition of the DNA binding sites specify a cooperative partnership for \( \text{fgf18} \) expression. Accordingly, recent data show that Runx2 is essential for \( \text{fgf18} \) expression and that Runx2 directly regulates the FGF18 promoter (38). During pathological conditions like colon cancer, \( \text{fgf18} \) may be expressed abnormally and independently of Runx2 as a consequence of excess levels of \( \beta \)-cat.

The interactions of Runx2 and TCF/Lef do not augment the expression of all Wnt target genes. For example, \( \text{Axin2} \) is strongly induced by canonical Wnt signaling, yet its expression is not stimulated by Runx2 (Fig. 4B). Also, the prototypic Wnt-dependent reporter plasmid, Topflash, is repressed rather than stimulated by the combination of Runx2 and \( \beta \)-cat (Fig. 5B).

Kahler and Westendorf (39) also showed that Runx2 binds to Lef1 and this association represses the osteocalcin promoter. Therefore, it is likely that a set of genes are repressed as a consequence of Runx2-TCF/Lef interactions and a different set of genes including \( \text{fgf18} \) are induced by the combination. The outcome will be determined by protein-nucleic acid interactions that are promoter-specific. Given the pivotal importance of Runx2 and Wnt-dependent transcription during osteoblast differentiation, the decision to repress or induce gene expression in response to their combined actions will be paramount for temporal and spatial regulation of osteoblast differentiation. For example, recent data show that cessation of Wnt/\( \beta \)-cat activity is required for full maturation of osteoblasts into osteocalcin expressing cells (18). Coactivation of FGF18 expression by Runx2 and Wnt signaling may support early osteoblast differentiation, but suppress later events. In support of this, we have shown that FGF18 can suppress osteoblast differentiation in cultured metatarsals (24). Alternatively, strong Wnt signaling may induce TCF1 and/or Lef1 expression that lead to dimerization with Runx2 and dissociation from Runx2-dependent promoters like osteocalcin (39). In either case, these possibilities imply that a Runx2-TCF/Lef complex will act decisively at specific points during osteoblast differentiation.

We showed that \( \text{fgf18} \) is induced via an osteoblast-specific, Wnt-dependent pathway. Thus, \( \text{fgf18} \) is a Wnt-target gene that may be essential for translating Wnt signaling into osteoblast development. It is notable that normal osteogenesis is disrupted in \( \text{fgf18} \) null embryos (40, 41). If \( \text{fgf18} \) is responsible for the action of Wnt in bone, then based on our understanding of Wnt signaling in bone \( \text{fgf18} \) must both stimulate and suppress osteoblast differentiation at different stages of development. How this is achieved will require further investigation; however, expansion of an early osteoblast population through stimulation of cell proliferation will almost certainly be a key attribute of FGF18.

What other genes are induced by the Runx2-TCF/Lef combination? These genes are likely to have both TCF/Lef sites and Runx2 within the promoter; however, given the substantial DNA binding that is induced by the high mobility group domain of Lef (42), we predict that these binding sites need not be directly adjacent or overlapping. The dramatic change in DNA conformation caused by Lef1 can bring a distant Runx2 site into close spatial proximity. We predict that certain of these genes have expression patterns that match closely that of FGF18. Interestingly, Dkk1, tcf1, and Runx2 are induced by Wnt signaling (43–45) and are expressed in the perichondrium similar to FGF18. Moreover, Dkk1 and tcf1 expression is lost in Runx2 null embryos at sites where Runx2 and Dkk1 or tcf1 are normally coexpressed (46). Whether these and other genes are coactivated by Runx2 and Wnt-dependent transcription demands further investigation. These genes will surely be vital during osteoblastogenesis and bone formation given that they lay at the convergence of stimulus-dependent and osteoblast-specific transcription pathways that are anabolic for bone.

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