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Mitogen-activated Protein Kinase (MAPK)-regulated Interactions between Osterix and Runx2 Are Critical for the Transcriptional Osteogenic Program*

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Background: *Osterix* and *Runx2* are master genes that transcriptionally promote osteoblast differentiation.
Results: Osterix and Runx2 cooperate to induce osteogenic genes by binding to promoters and interacting with each other.
Conclusion: Osterix and Runx2 exhibit cooperation, subject to further regulation by MAPK signals, during osteogenesis.
Significance: A network of interactions between transcription factors provides a circuit that drives the osteoblast differentiation program.

The transcription factors Runx2 and Osx (Osterix) are required for osteoblast differentiation and bone formation. *Runx2* expression occurs at early stages of osteochondroprogenitor determination, followed by *Osx* induction during osteoblast maturation. We demonstrate that coexpression of Osx and Runx2 leads to cooperative induction of expression of the osteogenic genes *Col1a1*, *Fmod*, and *Ibsp*. Functional interaction of Osx and Runx2 in the regulation of these promoters is mediated by enhancer regions with adjacent Sp1 and Runx2 DNA-binding sites. These enhancers allow formation of a cooperative transcriptional complex, mediated by the binding of Osx and Runx2 to their specific DNA promoter sequences and by the protein-protein interactions between them. We also identified the domains involved in the interaction between Osx and Runx2. These regions contain the amino acids in Osx and Runx2 known to be phosphorylated by p38 and ERK MAPKs. Inhibition of p38 and ERK kinase activities or mutation of their known phosphorylation sites in Osx or Runx2 strongly disrupts their physical interaction and cooperative transcriptional effects. Altogether, our results provide a molecular description of a mechanism for Osx and Runx2 transcriptional cooperation that is subject to further regulation by MAPK-activating signals during osteogenesis.

Bone development and remodeling depend on the activity of the osteoblasts that derive from condensations of mesenchymal stem cells. It is well known that osteochondroprogenitor maturation and the later conversion of preosteoblasts to mature osteoblasts are controlled by a complex network of transcription factors activated by specific osteogenic signals. Among these transcription factors, Runx2 and Osx (Osterix) play a critical role in osteogenesis (1–3). They are considered master osteogenic factors because their null mice do not form mature osteoblasts (4, 5). In *Osx*-null mice, bone calcification is prevented, even though *Runx2* is expressed, suggesting that *Osx* acts downstream of *Runx2* during bone development (5). Moreover, in adult organisms, osteoblast action is still required because the mammalian skeleton undergoes continuous turnover throughout the lifetime. *In vivo* studies have demonstrated that Runx2 and Osx are mandatory for osteoblast maturation as well as bone formation during the adult stage (4, 6, 7). In addition, several *Runx2* and *Osx* mutations or SNPs are related to bone illnesses such as osteoporosis, osteogenesis imperfecta, and cleidocranial dysplasia (8–12).

Several studies have highlighted the role of Runx2 and Osx in osteoblast function at the molecular level. It has been demonstrated that expression of *Osx in vivo* requires Runx2, although osteogenic signals are still able to stimulate *Osx* expression in *Runx2*-deficient cells (13–17). Runx2 regulates the expression of numerous osteoblastic genes such as *Osx*, *Alpl* (alkaline phosphatase), *Col1a1* (collagen type I), *Spp1* (osteopontin), *Ibsp* (bone sialoprotein), *Mmp13* (matrix metalloproteinase 13), and *Bglap* (osteocalcin) (4, 18, 19). Most of these gene promoters are also regulated by Osx (5, 19–22), and in fact, Osx is able to direct its own expression (17). Promoters of several osteoblast-specific genes contain both Runx2-binding sites (TGTGGT) and Sp1 boxes (which are bound by Osx). Thus, it is plausible that Runx2 and Osx work in a collaborative manner to activate the osteoblast genetic program and produce a bone-specific matrix. This hypothesis is supported by the described interaction between Runx2 and Osx in the transcriptional regulation of *Mmp13* and *Col1a1* genes (19, 21). Conversely, in the regulation of *Nell-1* expression, Osx and Runx2 seem to play opposite roles, as the former represses the expression of this gene, and the latter activates it (23).

In the control of downstream events, the master function of Runx2 has also been shown to be tightly regulated by interaction with cofactors. For instance, interaction with Stat1 inhibits its nuclear localization, and interaction with Twist1, Nrf2, or COUP-TFII (chicken ovalbumin upstream promoter transcrip-

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tion factor II) blocks Runx2 DNA-binding ability (20, 24, 25). Positive coactivation of Runx2 has been described for Smad, TAZ, Dlx5, or Gli family members (26–30). In turn, Osx also collaborates with other transcription factors and cofactors such as Sp1, NFATc1, and NO66, which regulate its activity (1, 22, 31). NFATc1 forms a complex with Osx and activates *Col1a1* promoter activity, but it does not activate Runx2-dependent transcription (31). These studies evidence a complex cross-talk between these transcription factors and the transcriptional machinery but also highlight that our knowledge of their regulatory mechanisms is limited.

Recent work has expanded our understanding of the role of p38 and ERK MAPKs in the control of osteogenesis and, in particular, their regulation of Runx2 and Osx transcriptional activity (32). Induction of *Osx* expression requires the activation of Dlx5 through p38-mediated phosphorylation (16). Furthermore, it has been shown that Osx itself is a substrate for p38 (33) and ERK MAPK (34, 35), which increases recruitment of transcriptional coactivators (33). Similarly, Runx2 is strongly regulated through direct p38 and ERK MAPK phosphorylation. Phosphorylation of Runx2 at multiple sites leads to increased transcriptional activity. Thus, a regulatory network exists in which p38 and ERK MAPK phosphorylation is involved in the induction and control of Runx2 and Osx transcriptional activity.

Here, we report functional cooperation between Osx and Runx2 modulating the expression of osteoblast genes *Col1a1*, *Fmod* (fibromodulin), and *Ibsp*, which are involved in the formation of a mature bone matrix. Induction of these genes is mediated through enhancer regions encompassing nearby Sp1 sites and Runx2 DNA-binding sites. Formation of a cooperative complex is mediated through DNA binding of Runx2 and Osx to their cognate sequences as well as protein-protein interactions between them. Moreover, we demonstrate that their phosphorylation by p38 and/or ERK MAPK at specific sites is required for efficient interaction and cooperation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—C2C12, Saos-2, and HEK-293T cell lines (American Type Culture Collection, Manassas, VA) were maintained in DMEM supplemented with 10% FBS, 0.2 mM glutamine, 0.1 mM pyruvate, and 100 units/ml penicillin/streptomycin. MC3T3-E1 preosteoblasts (American Type Culture Collection) were maintained in α-minimal essential medium supplemented with 10% FBS, 0.2 mM glutamine, 0.1 mM pyruvate, and 100 units/ml penicillin/streptomycin. Primary osteoblasts were seeded in culture after collagenase digestion of calvariae from P1–P4 mouse pups. Bones were dissected from euthanized pups, and their sutures and soft tissue were discarded. 8–12 calvariae were pooled and serially digested in a trypsin (0.025%)/collagenase II (1 mg/ml) solution. The product of the first 5 min of digestion was discarded, and the product of a double 40-min digestion was centrifuged (400 × *g*, 5 min) and seeded on 60-mm culture plates. Mouse primary osteoblasts were maintained in α-minimal essential medium supplemented with 10% FBS, 0.2 mM glutamine, 0.1 mM pyruvate, 100 units/ml penicillin/streptomycin, 50 μM ascorbic acid, and 5 mM β-glycerophosphate. C2C12, MC3T3-E1, and Saos-2 cells were transiently transfected using Lipofectamine LTX (Invitrogen). HEK-293T cells were transiently transfected using polyethylenimine.

Plasmids and Reagents—Osx expression vectors were kindly provided by Dr. B. de Crombrugghe. The Ibsp-plux and −2483pCol1a1-lux reporters were generated by PCR, and −2483pCol1a1Δ1 and −2483pCol1a1Δ2 were generated by restriction of −2483pCol1a1-lux (21). OC-p147-lux was a gift from Dr. G. Karsenty. The Sp1-plux reporter was a gift from Dr. C. Caelles. Point mutations in the Runx2 and Sp1 sites of Ibsp-plux and deletions in the OC-p147-lux reporter were generated by PCR. The *Ibsp* enhancer was amplified by PCR with the mutant-specific primers 5′-agaaagagcaattctggcttctcttgggt to mutate the distal Runx2 site, 5′-agaaagagcaattctggcttctcttcgct to mutate the distal Runx2 and Sp1 sites, and 5′-accgcgacgcttgatgtaaaacgagacga to mutate the proximal Runx2 and Sp1 sites. Amplified products were subcloned into pGL2-fos, containing the minimal c-*fos* promoter. Deletions and point mutations of OC-p147-lux were also generated by PCR with the common reverse primer 5′-ttgtcagatctgcaccctccagca, and primers 5′-tgagcacggtacccccaattagtcctggc for OC mut-1, 5′-agcacggtacccactattcgtcctggcagcatcacatgctactcc for OC mut-2, and 5′-agagatcggagagtagccgatataaatgctac for OC mut-3. Amplified products were subcloned into pGL-2Basic. GST-Runx2, GST-Runx2Δ361, and GST-Runx2Δ230 were kindly provided by Dr. M. Montecino. GST-OsxΔ346 and GST-OsxΔ140 were generated by digestion with SmaI and double digestion with NotI and StyI, respectively, from Osx subcloned into the pGEX vector (33). The *Osx*(*S73A/S77A*) mutant expression vector was generated as described previously (33). Myc-*Runx2* and the Myc-*Runx2*(*S43A/S282A/S319A*) mutant were kindly provided by Dr. L. H. Glimcher. SB203580 and U0126 (Calbiochem) were used at final concentrations of 10 and 5 μM, respectively. Antibodies against Osx (Abcam), Runx2 (MBL International Corp.), and α-tubulin (Sigma) were used at a 1:1000 dilution.

Luciferase Reporter Assays—Saos-2 or C2C12 cells were cultured in 6-well plates and transfected for 8 h with Lipofectamine LTX with the indicated plasmids. The transfection efficiency was assessed by GFP expression. Luciferase activities were quantified at 48 h using the Luciferase assay system (Promega) and normalized using the β-Galactosidase Detection Kit II (Clontech).

GST Pulldown Assays—The fusion proteins GST-Osx and GST-Runx2 and their derivatives were produced in *Escherichia coli* BL21 and purified by binding to glutathione-Sepharose beads. For *in vitro* binding assays, cells expressing Osx and/or Runx2 were washed twice with cold PBS and lysed with 0.3% CHAPS, 50 mM Tris (pH 7.5), 150 mM NaCl, and 10% glycerol supplemented with protease and phosphatase inhibitors at 4 °C for 15 min. Lysates were collected and centrifuged at 22,000 × *g* for 5 min to eliminate cellular debris. Supernatants were incubated with the appropriate chimeric protein bound to glutathione-Sepharose beads overnight at 4 °C with rotation. The beads were then collected by centrifugation at 300 × *g* for 1 min and washed five times with wash buffer (0.1% CHAPS, 50 mM Tris (pH 8.0), and 150 mM NaCl). Finally, proteins bound to the beads were subjected to immunoblotting.

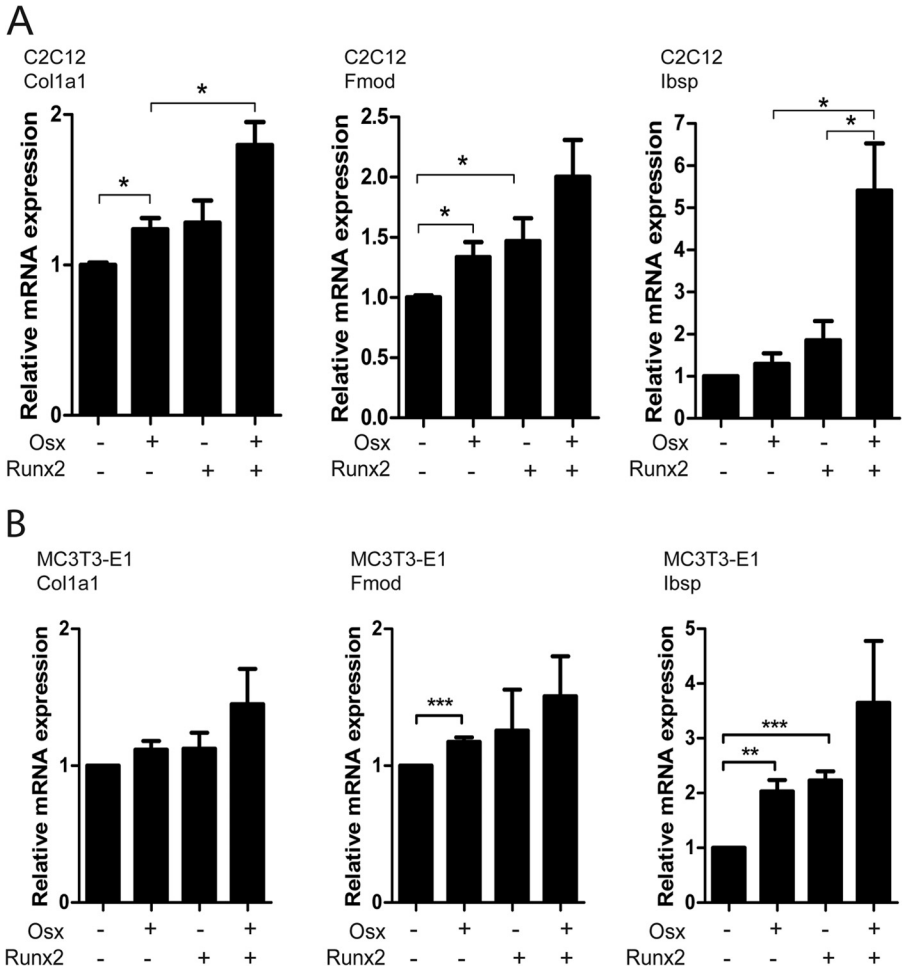

FIGURE 1. **Additive effect of Osx and Runx2.** C2C12 (*A*) or MC3T3-E1 (*B*) cells were cotransfected with Osx and/or Runx2 expression vectors. *Col1a1*, *Fmod*, and *Ibsp* mRNAs from C2C12 or MC3T3-E1 cells were measured by quantitative RT-PCR and normalized to *Gapdh*, and relative expression is presented as the mean ± S.E. of nine independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ using Student's *t* test.

Immunoprecipitation—Primary osteoblasts or transiently transfected HEK-293 cells were lysed as described above. The supernatant fraction was incubated overnight with 1 μg of anti-Osx or anti-Runx2 antibody, followed by incubation with 20 μl of Protein A/G-Sepharose (GE Healthcare) for 1 h. Bound proteins were washed four times with lysis buffer and detected by immunoblotting.

Western Blot Assay—To detect the presence of proteins in the cell extracts or pulldowns, we performed immunoblotting with anti-Osx, anti-Runx2, or anti-α-tubulin antibody diluted at 1:1000. Immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibodies using an ECL kit (Biological Industries).

ChIP—Saos-2 and MC3T3-E1 cells were cultured until confluence and fixed with 1% formaldehyde for 10 min, and the reaction was stopped with 0.01 M glycine for 5 min. Cells were lysed and sonicated to obtain 200–1000-bp fragments. ChIP was carried out using 1 μg of the indicated antibody (anti-Osx, anti-Runx2, anti-RNA polymerase II (Upstate), or anti-IgG (Upstate) as a control) and purified with 20 μl of Magna ChIP Protein A+G magnetic beads (Millipore). The complexes were washed once with four different wash buffers and eluted with a solution containing SDS and $NaHCO_3$. Reversion of cross-linking was carried out by overnight incubation with 0.2 M NaCl at 65 °C, followed by treatment with proteinase K and RNase A. The DNA fragments were purified using the QIAquick gel extraction kit (Qiagen) and analyzed by PCR.

The primers used for Saos-2 cells for PCR analysis were as follows: HsFmod F, 5′-ggacccagagctcccaatgtttcaggga; HsFmod R, 5′-cgtccctcgagctggcctccttgggttg; HsIbsp F, 5′-cttctttctcatgtggccaacactcg; HsIbsp R, 5′-tggcatcaggagatgtcctctctt; HsColBE F, 5′-tgctccagagctgcaaacgtggaagc; HsColBE R, 5′-tccattgctgtctccagtcctgcct; HsColProx F, 5′-ggttagcccacgccattctgagga; and HsColProx R, 5′-ggaaggggtcggtgtttctcagatg. Those used for MC3T3-E1 cells were as follows: MmFmod F, 5′-ttcaggctgccaatgctccagggaa; MmFmod R, 5′-tgaccacgtccttctgtctggtct; MmIbsp F, 5′-ccagttttcaaacatccaaatccatagg; MmIbsp R, 5′-ttggcactgggagatgtcctccctt; MmColBE F, 5′-gaaggcactggggtctccttgcaggg; MmColBE R, 5′-tccattgctgtctccagctctgctt; MmColProx F, 5′-tgggtggactcctttccccttccttt; and MmColProx R, 5′-ttagcttcggctcatgcgtggcctca.

Immunofluorescence—Saos-2 and transfected C2C12 cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100, and blocked with normal goat serum for 1 h. Cells were stained with anti-Osx antibody at 1:150 dilution and anti-Runx2 antibody at 1:100 dilution, followed by

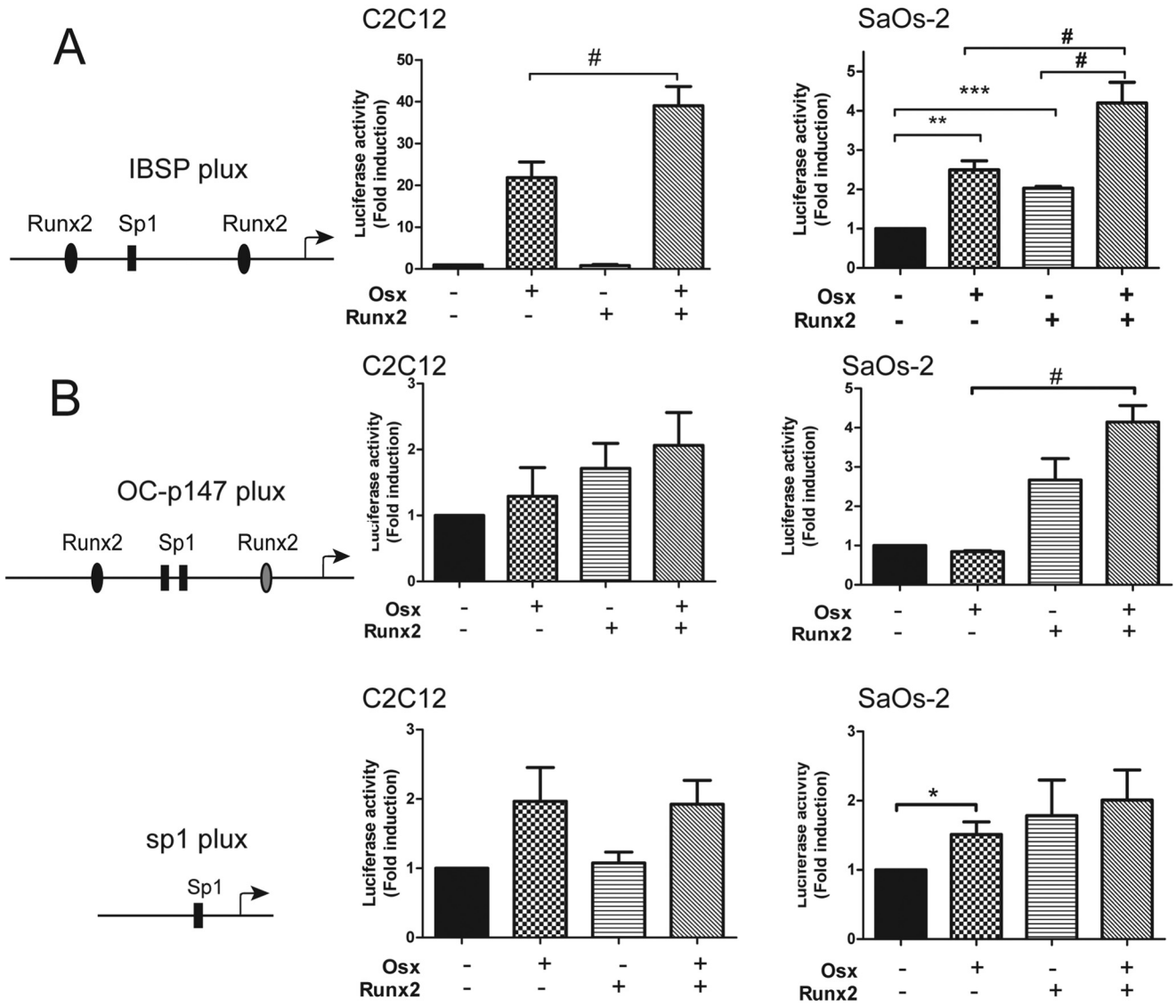

FIGURE 2. **Cooperative transcriptional activation by Runx2 and Osx DNA binding.** C2C12 or Saos-2 cells were cotransfected with Osx and/or Runx2 constructs and the indicated reporters. Schemes of Ibsp-plux (*A*), OC-p147-lux (*Bglap* reporter) (*B*, *upper panel*), and Sp1-plux (*B*, *lower panel*) reporter vectors are shown. Luciferase activity was measured and normalized against β-galactosidase activity. * and #, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

goat anti-rabbit IgG conjugated with Alexa Fluor 555 at 1:500 dilution or anti-mouse IgG conjugated with Alexa Fluor 488. Nuclei were stained using a 1:1000 dilution of DRAQ5. Labeling was detected using a Leica TCS SL inverted laser scanning confocal microscope.

Quantitative RT-PCR Analysis—Total RNA was isolated from C2C12 and MC3T3-E1 cells using TRIsure reagent (Bioline). 5 μg of total RNA was reverse-transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCRs were carried out using the ABI Prism 7900 HT Fast real-time PCR system and a TaqMan 5′-nuclease probe method (Applied Biosystems). All transcripts were normalized to *Gapdh*, and transfection efficiency was assessed by GFP expression. Designed TaqMan assays (Applied Biosystems) were used to quantify gene expression of mouse *Col1a1*, *Fmod*, *Ibsp*, *Gapdh,* and osteocalcin.

Statistical Analysis—Statistical analysis was performed using Student's *t* test. Quantitative data are presented as means ± S.E. Differences were considered significant at $p < 0.05$.

RESULTS

Coexpression of Osx and Runx2 Enhances Transcription of Osteogenic Genes—The expression of many osteogenic markers is modulated by Osx and/or Runx2. To determine their relative relevance, we transfected C2C12 and MC3T3-E1 cell lines with Osx and/or Runx2 expression vectors. As reported previously (21, 33), quantitative RT-PCR assays demonstrated that overexpression of Osx or Runx2 in C2C12 cells can up-regulate the endogenous expression of collagen type 1 (*Col1a1*), fibromodulin (*Fmod*), and bone sialoprotein (*Ibsp*) (Fig. 1*A*). This effect was also observed in MC3T3-E1 preosteoblasts, where *Col1a1*, *Fmod*, and *Ibsp* expression was enhanced when Osx was overexpressed (Fig. 1*B*). More importantly, in both cell lines, coexpression of Osx and Runx2 had a strong additive effect in the expression of these osteogenic genes. To further analyze the mechanism of this cooperation, we focused on the presence of Runx2- or Osx-binding sites in the promoter sequences of these genes. Homology analysis of *Ibsp*, *Fmod*, and *Col1a1* gene pro-

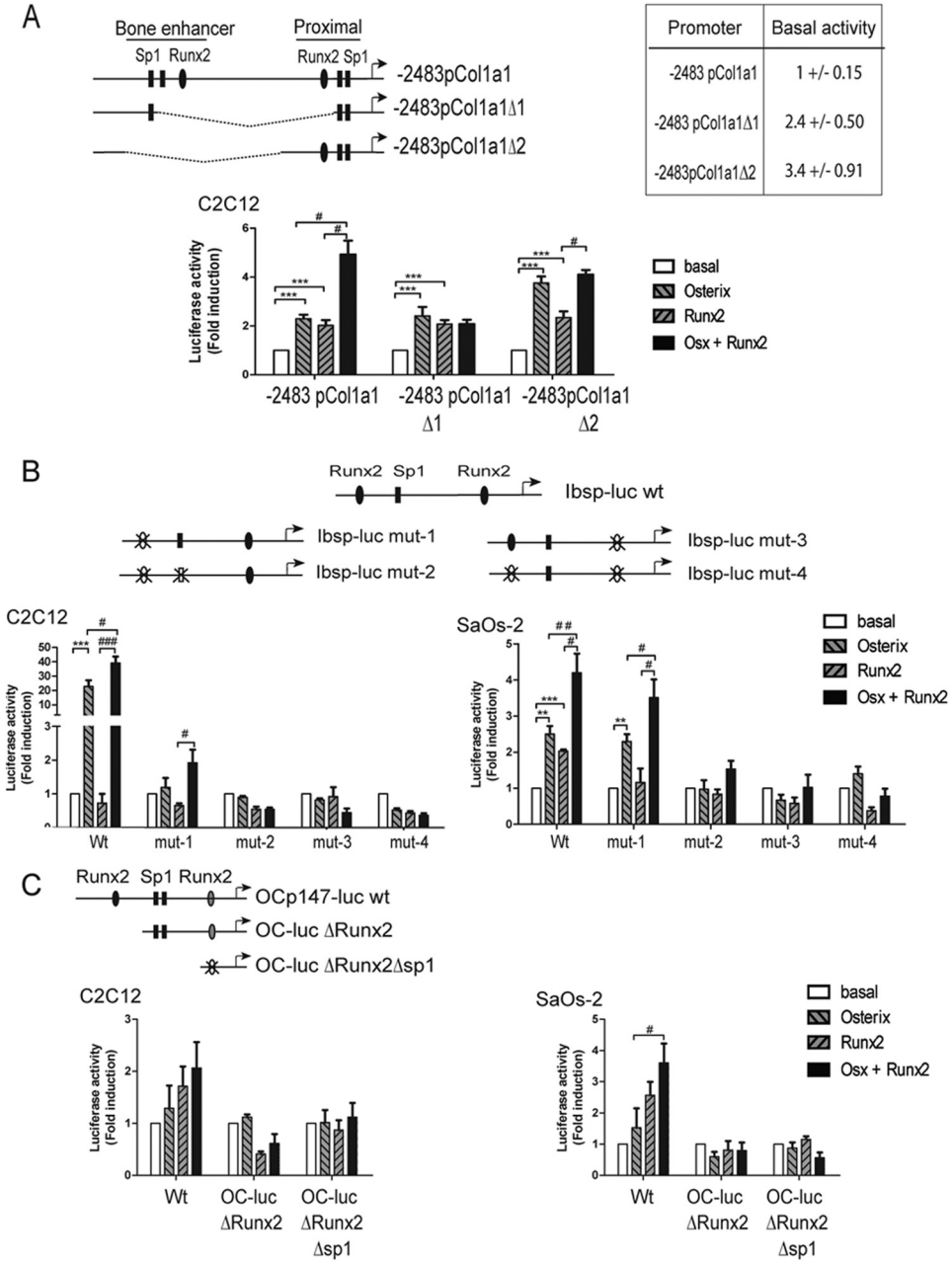

FIGURE 3. **Requirement for Runx2- and Sp1-binding sites in cooperative activation.** *A*, C2C12 cells were cotransfected overnight with Osx and/or Runx2 constructs and the indicated *Col1a1* reporters. A scheme is shown in the *lower panel*. Basal activities refer to those of the −2483pCol1a1-lux reporter. Luciferase activity was measured and normalized against β-galactosidase activity. *B*, C2C12 or Saos-2 cells were cotransfected with Osx and/or Runx2 constructs and the indicated Ibsp-lux reporters. Schemes of the mutated sites introduced in the Ibsp-lux reporter vector are shown. Luciferase activity was measured and normalized against β-galactosidase activity. *C*, C2C12 or Saos-2 cells were cotransfected with Osx and/or Runx2 constructs and the indicated OC-p147-lux reporters. Schemes of the deleted and mutated sites in the OC-p147-lux reporter vector are shown. Luciferase activity was measured and normalized against β-galactosidase activity. Relative luciferase activities are expressed as the mean ± S.E. in triplicate of four independent experiments. #, $p < 0.05$; ** and ##, $p < 0.01$; *** and ###, $p < 0.005$ using Student's *t* test.

moters revealed regions with a high degree of similarity among orthologs, which include one or more Runx2 sites in close proximity to Sp1 sites (Fig. 2). For instance, the study of a distal enhancer of the *Ibsp* gene revealed the presence of two Runx2-binding sites close to an Sp1 site bound by Osx (Fig. 2*A*) (33). We evaluated the *Ibsp* promoter activity in C2C12 cells and in the osteosarcoma cell line Saos-2 using a luciferase reporter driven by the *Ibsp* enhancer (33). Although expression of Runx2 had minor effects on promoter activity in C2C12 cells, we observed a 20-fold induction of Ibsp-plux activity in response to Osx and >40-fold induction when both Osx and Runx2 were coexpressed (Fig. 2*A*). This cooperative induction of the *Ibsp* reporter was similar when analyzed in Saos-2 cells. Thus, these results indicate that Osx and Runx2 have cooperative effects on specific gene expression.

Next, we assessed the importance of the specific *cis*-responsive sequences in the cooperative effects between Runx2 and Osx. The OC-p147-lux reporter is driven by the proximal *Bglap* promoter and contains two Runx2-binding sites and two Sp1 sites (Fig. 2*B*, *left panel*). As described previously (36), overex-

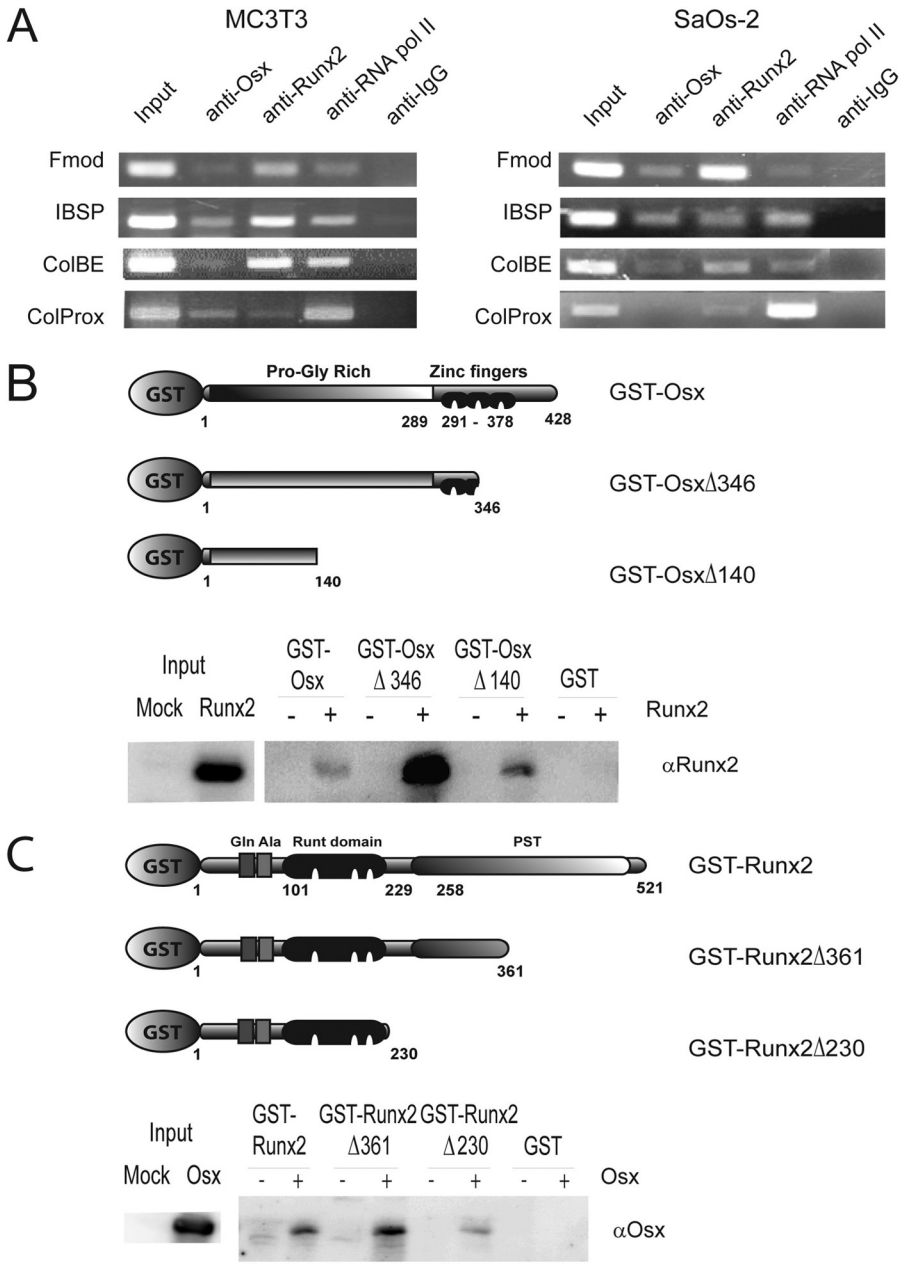

FIGURE 4. **Osx and Runx2 physically interact and associate with the same promoter region.** *A*, ChIP analysis was performed in MC3T3-E1 and Saos-2 cells by incubation of DNA-protein complexes with antibodies against Osx, Runx2, RNA polymerase (*pol*) II, and IgG as a negative control. Primers specific for the *Ibsp* and *Fmod* enhancers, the *Col1a1* bone enhancer (*ColBE*), and the *Col1a1* proximal promoter (*ColProx*) were used for PCR analysis. Extracts of HEK-293T cells expressing Runx2 (*B*) or Osx (*C*) were incubated overnight with the indicated chimeric proteins bound to glutathione-Sepharose beads. Interacting proteins were identified by immunoblotting using anti-Runx2 or anti-Osx antibody. *PST*, Pro/Ser/Thr-rich.

pression of Runx2 induced OC-p147-lux reporter activity (Fig. 2*B*). It has also been reported that although Osx binds to these Sp1 sites, it is unable to induce significant transcriptional activation (22). Our data showed that Osx expression conferred additive effects on Runx2 activation, which were more evident in the Saos-2 osteoblastic cells than in the C2C12 mesenchymal cell line (Fig. 2*B*). To further test the relevance of specific binding sites, we analyzed the activity of the Sp1-plux reporter, an artificial promoter containing a unique Sp1 site (Fig. 2*B*, *left panel*). The reporter was activated 2-fold by Osx expression. However, coexpression of Runx2 failed to induce significant additive transcriptional effects (Fig. 2*B*).

We also analyzed a pCol1a1-lux reporter, which contains functional Runx2-binding boxes and Sp1 sites (Fig. 3*A*, *lower panel*) (18, 37). The −2483pCol1a1-lux reporter was activated by Osx and Runx2, and the coexpression of both factors also notably increased its induction. Furthermore, the −2483pCol1a1Δ1-lux reporter, devoid of Runx2-binding sites, lost cooperativity between the two factors, as shown above with the Sp1-plux reporter. In the −2483pCol1a1Δ2-lux reporter, the proximal region is intact, and although it still maintains a single Runx2 binding site, we did not observe the additive effects, suggesting that this proximal Runx2 site may be less important for these effects. This result is in agreement with previous reports that

Runx2 bound only weakly and did not transactivate the *Col1a1* promoter from this −372 proximal Runx2 site (18). Moreover, we generated a set of Ibsp and OC-p147 reporter constructs with mutations at specific Runx2 and Sp1 sites. Mutation of the distal Runx2 site in the *Ibsp* enhancer did not abolish the additive effects of Runx2 and Osx. However, mutation of either the Sp1 or proximal Runx2 sites suppressed activation by Runx2 and/or Osx (Fig. 3*B*). Similarly, deletion of the most distal Runx2 site in the osteocalcin promoter completely eliminated transcriptional activation by Runx2 or Osx (Fig. 3*C*). Altogether, these results suggest that gene promoters activated cooperatively by Osx and Runx2 require the presence of adjacent Runx2- and Osx-binding sites.

To confirm that functional interaction between Osx and Runx2 occurs *in vivo*, ChIP was performed in Saos-2 and MC3T3-E1 cells. As shown in Fig. 4*A*, both Osx and Runx2 bound to the responsive regions of the osteogenic genes *Fmod*, *Ibsp*, and *Col1a1*. Binding of these factors also correlated with recruitment of RNA polymerase II.

Osx and Runx2 Physically Interact—The presence of Runx2 sites near Osx sites within the same promoter and the functional interdependence between them raised the possibility that both factors might associate through physical interaction. To evaluate this hypothesis, we carried out GST pulldown analyses. HEK-293T cells were transiently transfected with Osx or Runx2 expression vectors and processed with different lysis buffers. Extracts lysed with isotonic buffers containing 0.5% Triton X-100, 0.5% Nonidet P-40, or 0.3% CHAPS were tested. We analyzed the ability of Runx2 or Osx to interact with full-length recombinant GST-Osx or GST-Runx2 and truncated forms *in vitro*. High affinity interaction was maximally retained with the 0.3% CHAPS lysis buffer (data not shown). Using the same approach, we determined which domains of Osx and Runx2 were involved. After Osx pulldown, we found that Runx2 was able to interact with the truncated forms of Osx. OsxΔ346 precipitated higher amounts of Runx2 (Fig. 4*B*), so we concluded that Osx interacted mainly through its N-terminal transactivation domain and that the zinc fingers were not involved. In contrast, whereas Runx2 with a carboxyl-terminal deletion to amino acid 361 still bound Osx, recombinant Runx2 in which amino acids 230–521 had been deleted lost most of its capacity to interact with Osx (Fig. 4*C*). region 230–361 did not encompass the Runt DNA-binding domain of Runx2 and has also been demonstrated to be involved in interaction with other proteins such as the vitamin D receptor and with histone acetyltransferases MORF and MOZ (38, 39). Interaction between Osx and Runx2 was also observed in intact cells. Immunoprecipitation of Osx from transiently transfected C2C12 cell extracts also coprecipitated Runx2 (Fig. 5*A*), further suggesting interaction between these transcription factors *in vivo*.

Regions involved in mutual interaction include the known nuclear localization signals for both Osx and Runx2. Moreover, because it has been demonstrated previously that some Runx2 interactors modify the nuclear or subnuclear localization of Runx2 (40, 41), we analyzed the localization of these two transcription factors by immunofluorescence (Fig. 5*B*). Expression of either Runx2 or Osx alone displayed a constitutive nuclear localization for both. Coexpression of both factors did not alter

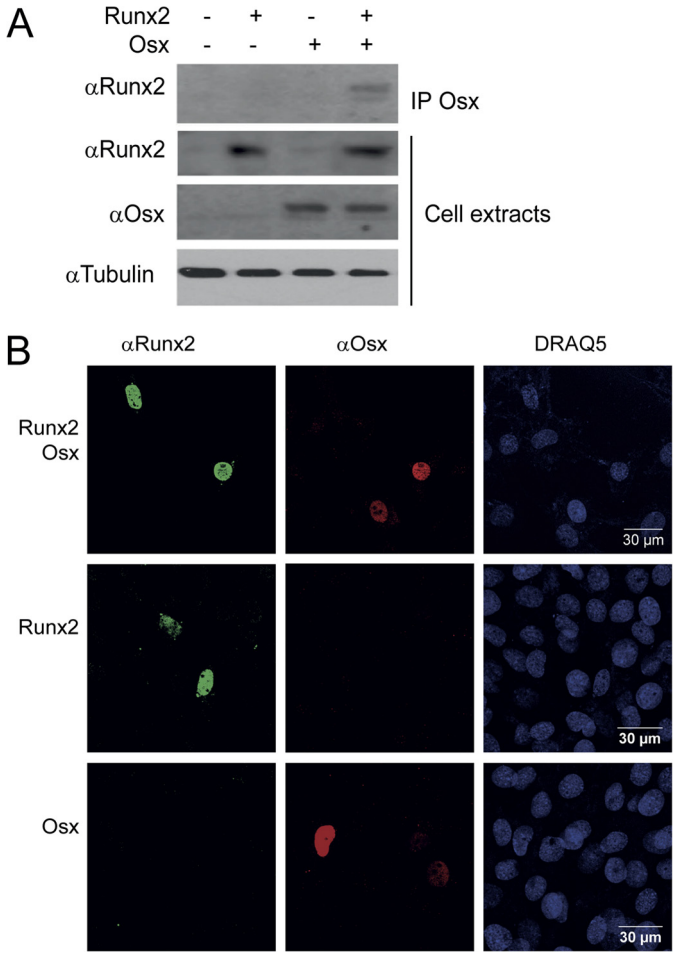

FIGURE 5. ***In vivo* interaction of Osx and Runx2 is restricted to the nucleus.** *A*, C2C12 cells were transfected with Runx2 and/or Osx expression vectors. Expression of the constructs and anti-Osx-co-immunoprecipitated proteins was analyzed by immunoblotting. *B*, the subcellular localization of Runx2 and Osx was analyzed by immunofluorescence with anti-Runx2 or anti-Osx antibody in C2C12 cells transiently transfected with Runx2 and/or Osx expression vectors. *IP*, immunoprecipitated.

their nuclear pattern of localization, suggesting that changes in their nuclear shuttling are not the mechanism involved in their functional interaction.

p38 and ERK MAPK Activities Are Necessary for Functional and Physical Interaction between Runx2 and Osx—The activities of ERK and p38 MAPKs have been shown to phosphorylate and increase the transcriptional activities of Runx2 and Osx (6, 32–34, 42) Moreover, the MAPK phosphorylation sites in Osx and Runx2 identified so far are localized within the regions described above as being involved in their physical interaction (33, 42, 43). Therefore, we tested the effect of the phosphorylation state of Osx and Runx2 on their transcriptional cooperation. We coexpressed Osx and Runx2 in C2C12 cells and treated them with the p38α/β inhibitor SB203580 or the ERK1/2 inhibitor U0126. These inhibitors are known to block phosphorylation of either Runx2 or Osx (33, 43). mRNA expression analysis of *Col1a1*, *Fmod*, *Ibsp*, and *Bglap* demonstrated that inhibition of p38 or ERK signaling resulted in complete abrogation of Osx and Runx2 additive effects in all genes studied (Fig. 6*A*). We also performed similar studies using the −2483pCol1a1-lux, Ibsp-plux, and OC-p147-lux gene report-

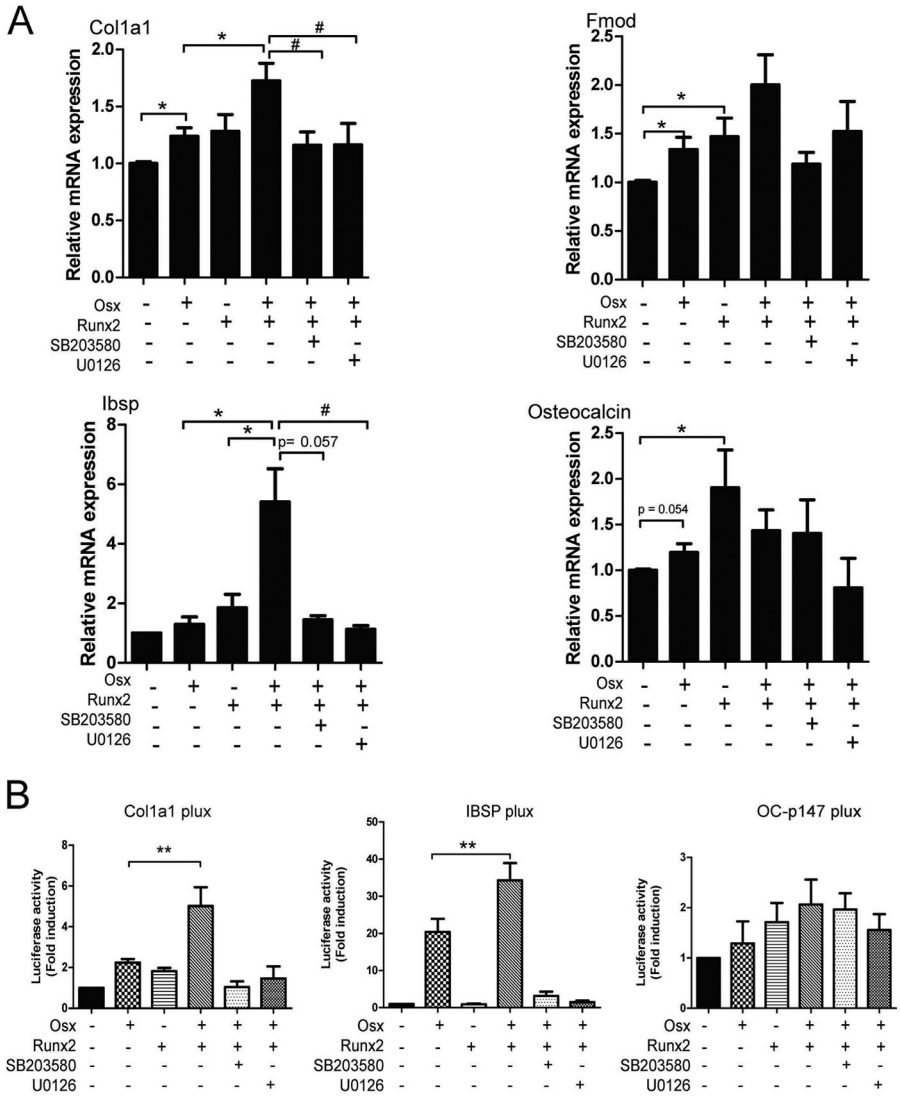

FIGURE 6. **Runx2 and Osx phosphorylation effects on cooperative transcriptional activity.** *A*, C2C12 cells were cotransfected with Osx and/or Runx2 expression vectors and treated with SB203580 or U0126 for 24 h. *Col1a1*, *Fmod*, osteocalcin (*Bglap*), and *Ibsp* mRNAs were measured by quantitative RT-PCR and normalized to *Gapdh*, and relative expression is presented as the mean ± S.E. of four independent experiments. *B*, C2C12 cells were mock-transfected or cotransfected with Osx and/or Runx2 constructs and the indicated reporters. Cells were treated with SB203580 or U0126 for 24 h. Luciferase activity was measured and normalized against β-galactosidase activity. Relative luciferase activities are expressed as the mean ± S.E. in triplicate of at least three independent experiments. * and #, $p < 0.05$; **, $p < 0.01$ using Student's *t* test).

ers. Luciferase assays showed strong and consistent inhibition of reporter activity upon addition of inhibitors (Fig. 6*B*).

The results indicate that functional cooperation between the two transcription factors may be compromised because their phosphorylation is necessary for complete activity. However, these results did not discern whether phosphorylation hampers the interaction between the transcription factors or whether it is required only for the independent recruitment and function of transcriptional coactivators for each one. To further examine whether protein-protein interaction ability depends on the phosphorylated state, we carried out a pulldown assay using full-length GST-Osx and GST-Runx2. Assays performed with GST-Osx and lysates from C2C12 cells expressing Runx2 demonstrated the importance of the Runx2 phosphorylation state. The levels of Runx2 bound to Osx were lower in extracts from cells treated with MAPK inhibitors, despite similar levels of expression (Fig. 7*A*, *upper panel*). A complementary analysis for the requirement of Osx phosphorylation was also performed. As shown in Fig. 7*A* (*lower panel*), the interaction was also lower when extracts from cells treated with MAPK inhibitors were assayed. These data suggest that MAPK phosphorylation of both transcription factors is involved in protein interaction. We investigated whether inhibition of p38 and ERK MAPKs affects localization of endogenous Osx and Runx2 in Saos-2 cells. The addition of SB203580 and U0126 to Saos-2 cells decreased the protein expression levels of both transcription factors. However, they did not impair the nuclear co-localization of endogenous Osx or Runx2 (Fig. 7*B*).

The MAPK requirement for Osx and Runx2 interaction was confirmed *in vivo* by immunoprecipitation of C2C12 cell extracts expressing Runx2 and Osx and treatment with MAPK inhibitors. Western blot analyses showed a strong decrease in their interaction when p38 or ERK1/2 MAPK activities were restrained. Interestingly, although ERK inhibition alone com-

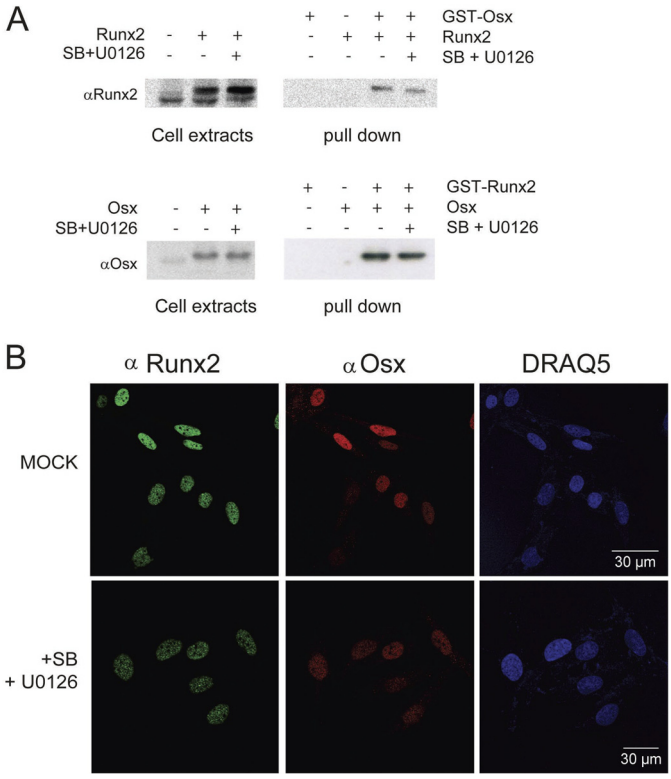

FIGURE 7. **Runx2 and Osx MAPK phosphorylation is involved in protein interaction.** *A*, C2C12 cells were transiently transfected with Runx2 or Osx expression vectors and treated with SB203580 (*SB*) and U0126 for 24 h. Extracts were incubated with the indicated chimeric proteins bound to glutathione-Sepharose beads. Expression of the constructs and interacting proteins was identified by immunoblotting using anti-Runx2 or anti-Osx antibody. *B*, Saos-2 cells were incubated in medium without serum and treated with SB203580 and U0126 for 24 h. The subcellular localization of endogenous Runx2 and Osx was analyzed by immunofluorescence with anti-Runx2 or anti-Osx antibody.

pletely blocked interaction, cells in which p38 MAPK activity was blocked still retained some interaction (Fig. 8*A*). However, because inhibition of one MAPK usually results in activation of the other, it is too early to make definitive conclusions about differences in p38 and ERK MAPK requirements.

We further characterized the interaction between endogenous Runx2 and Osx in primary cultures of mouse calvarial osteoblasts treated with SB203580, U0126, or both inhibitors. As reported previously (16, 21, 42, 44), Runx2 and Osx protein levels were decreased after MAPK inhibition by either SB203580 or U0126 treatment. More importantly, the amount of Runx2 that coprecipitated bound to Osx was significantly decreased by the simultaneous inhibition of both MAPKs (Fig. 8*B*). Together, these results indicate that both MAPK activities are required for a proper physical and functional interaction between these two transcription factors.

MAPK Phosphorylation Sites Are Involved in the Osx-Runx2 Interaction—Previous studies have shown that ERK interacts through a D-domain-docking site and phosphorylates Runx2 at four sites (Ser-43, Ser-301, Ser-319, and Ser-510) (6, 32, 44, 45). Among them, Ser-301 and Ser-319 both contribute to Runx2 function because Ser-to-Ala mutations at these sites greatly reduce its transcriptional activity at specific osteogenic promoters (6). Interestingly, these two sites have also been shown to be phosphorylated by the p38 pathway and are located in the Osx-Runx2 interaction region (Fig. 4) (42). In contrast, Osx is also phosphorylated by p38 at Ser-73 and Ser-77, located in the transactivation domain, which also has a positive effect on its osteogenic activity (21, 33). We then sought to ascertain the importance of the specific phosphorylation sites of these proteins in functional cooperation. We analyzed the interaction *in vivo* by expressing combinations of wild-type Osx and mutant S73A/S77A with wild-type Runx2 and mutant S43A/S282A/S319A. Immunoprecipitation analysis demonstrated that combinations expressing a phosphorylation-deficient mutant form (either Osx(S73A/S77A) or Runx(S43A/S282A/S319A)) showed impaired interaction (Fig. 8*C*). These results prove that the phosphorylation sites targeted by p38 and ERK MAPKs in both Osx and Runx2 are the ones involved in the Osx-Runx2 interaction.

DISCUSSION

It is physiologically and clinically important to understand the mechanisms of the transcriptional network that drives osteoblastogenesis. In this study, we have shown that the key osteogenic transcription factors Runx2 and Osx cooperate in the induction of genes involved in bone matrix formation. Transcriptional activation of these promoters is mediated through enhancer regions encompassing nearby Sp1 and Runx2 DNA-binding sites. Our study shows that both Runx2 and Osx bind to their responsive regions in DNA and interact with each other through the Osx N-terminal transactivation region and the Runx2 Pro/Ser/Thr-rich activation domain. Therefore, the two transcription factors form a complex at specific promoters that increases expression of osteogenic genes such as *Bglap*, *Col1a1*, *Fmod*, and *Ibsp*. In addition, we demonstrated that Runx2 and Osx phosphorylation by p38 and/or ERK MAPK at specific sites located on their interaction surfaces is required for an efficient interaction between them.

Runx2 is expressed as early as embryonic day 10 in developing mouse embryos, and *Osx* appears at embryonic day 18.5 (4, 5). The *Runx2*-expressing osteochondroprecursors, prior to *Osx* expression, remain in the chondrogenic lineage and express high levels of *Sox9* (5). Later, cells already expressing Runx2 and Osx differentiate into mature osteoblasts in which *Sox9* is no longer expressed (1). Thus, it may be suggested that their sequential expression constitutes a mechanism of osteoblast maturation in which, once expressed, Osx controls further transcription independently of Runx2. For instance, in *Osx*-null embryos, there is a strong reduction of *Col1a1* expression and an almost complete lack of late osteogenic markers, including osteonectin, osteopontin, *Ibsp*, and *Bglap*, despite normal expression of Runx2 (1, 5, 6). It may also be suggested that Runx2 and Osx regulate distinct subsets of osteogenic genes or, alternatively, act as allies to cooperatively promote maximal levels of osteogenic gene expression. Our data point to the latter hypothesis, in which Runx2 and Osx are cofactors in the same complex, up-regulating specific osteogenic target genes when they co-occupy their promoters.

The presence of Osx and its association could prevent Runx2 repression by liberating it from factors that prevent Runx2 binding to the DNA. For instance, Twist1, Stat1, or Nrf2 inhib-

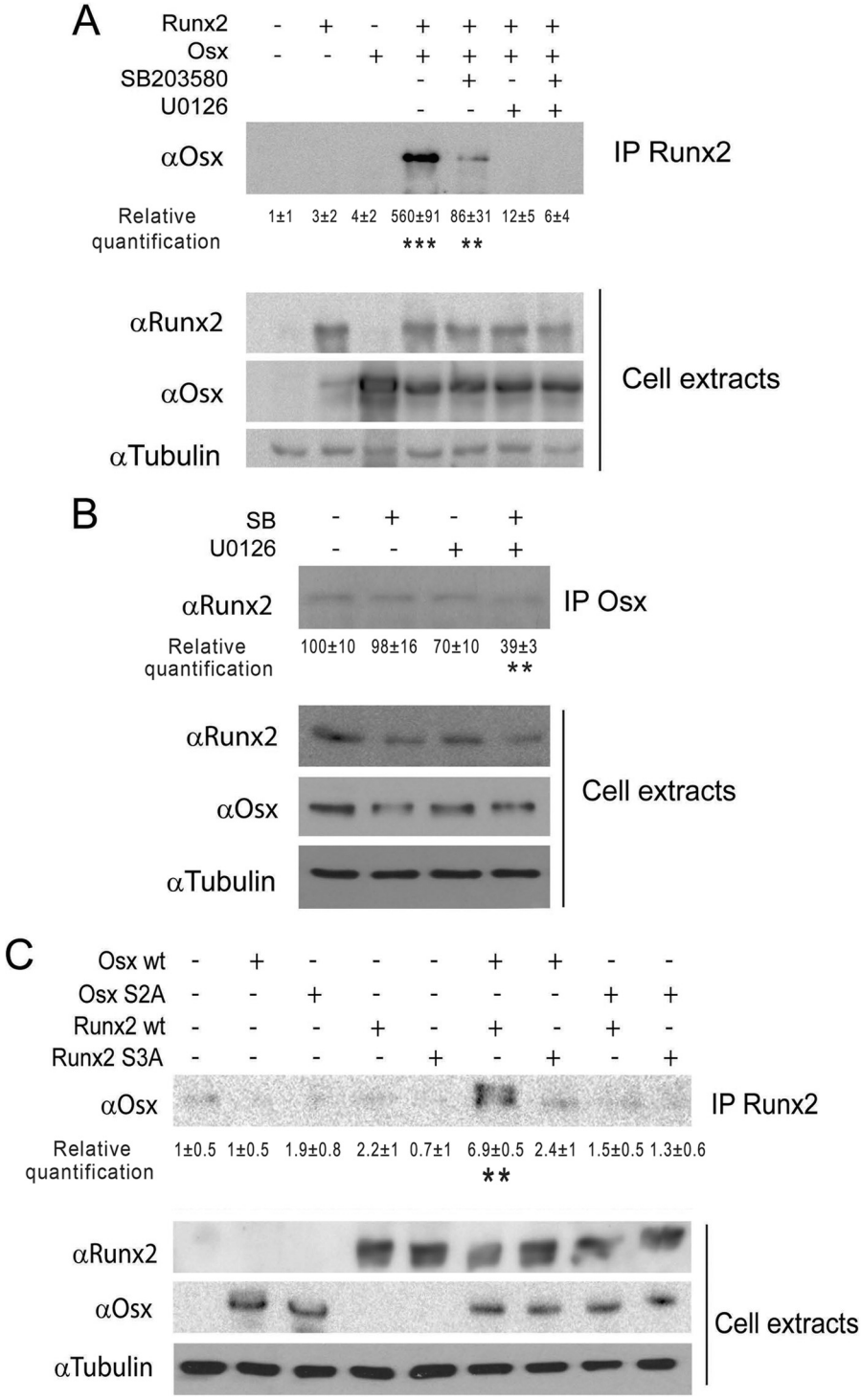

FIGURE 8. **MAPK activities are required for a proper physical interaction.** *A*, C2C12 cells were transiently transfected with Runx2 or Osx expression vectors and treated with SB203580 and/or U0126 for 24 h. Expression of the constructs and anti-Runx2-co-immunoprecipitated proteins was analyzed by immunoblotting. *B*, primary cultures of mouse calvarial osteoblasts were treated SB203580, U0126, or both for 24 h. Endogenous levels of Runx2, Osx, and anti-Osx-co-immunoprecipitated proteins were analyzed by immunoblotting. *C*, C2C12 cells were transiently transfected with the indicated expression vectors and immunoprecipitated (*IP*) with anti-Runx2 antibody. Expression of the constructs and co-immunoprecipitated proteins was analyzed by immunoblotting. The relative levels of immunoprecipitated Osx for each condition are expressed below the panel as the mean ± S.E. of at least three independent experiments. **, $p < 0.01$; ***, $p < 0.005$ using Student's *t* test. *S2A*, S73A/S77A; *S3A*, S43A/S282A/S319A.

its Runx2 transcriptional activity by docking to the Runt DNA-binding domain of Runx2 (24, 25, 41). In osteochondroprogenitors, it has been proven that expression of Sox9 also down-regulates Runx2 transcriptional activity (46, 47). Conversely, some transcription factors such as Dlx5, Gli, and Smad also interact with Runx2 but increase its transcriptional activity (27–30). In these cases, interactions involve domains other than the Runt DNA-binding domain. Although much less is known about Osx, it has been found that additional factors such as Sp1 and NFATc1 are required for functional activity on the *Bglap* or

Col1a1 promoter (22, 31). It has been documented that Runx2 changes its promoter-binding patterns during osteoblastogenesis (24). This study demonstrates that whereas the recruitment of Runx2 to a cluster of genes involved in general cell functions does not change throughout osteoblast maturation, its binding to osteoblast-specific gene promoters increases as osteoblast differentiation progresses. The *Mmp13*, *Fmod*, *Ibsp*, and *Col1a1* gene promoters include one or more Runx2 sites in close proximity (100–200 bp) to Sp1 sites (18, 21, 22, 24, 33, 36). Thus, it is likely that osteogenic genes containing adjacent Runx2 and Osx sites are regulated in a similar fashion. These results are consistent with data reported by Lee and co-workers (34) showing cooperation of Osx and Runx2 in the regulation of osteogenic marker genes during differentiation of adipose stem cells into osteoblasts.

Our results show that the region of Runx2 required for Osx interaction is amino acids 230–361. This region does not involve the Runt DNA-binding domain and partially overlaps with the Pro/Ser/Thr-rich activation domain, which is a transactivation domain in Runt-related proteins (48) targeted by proteins such as MOZ, MORF, and the vitamin D receptor (38, 39). The Runt domain is responsible for Runx2 binding to chromatin and, as mentioned above, is targeted by many Runx2 repressors, including Twist1, Elf1, COUP-TFII, and Stat1, which prevent Runx2 from binding to DNA (20, 24, 41, 49). Our results show that Osx associates with Runx2 through its N-terminal region and that the zinc fingers are not required. Therefore, in the Runx2 and Osx cooperative mechanism, both Runx2 and Osx are able to bind to their responsive sequences on the promoters and interact with each other via regulatory regions that lead to stabilization of the transcriptional complex. This physical interaction between Runx2 and Osx was previously suggested in the regulation of *Mmp13* (19). Runx2 transcription involves interaction with coactivators such p300, MOZ, and MORF (39, 50). Osx also associates with other transcription factors and cofactors such as Brg1, p300, and NO66, which regulate its activity (1, 22, 31). Binding and interaction of both Runx2 and Osx may then also potentiate the recruitment of these coactivators and the function of the transcriptional machinery.

ERK and p38 MAPKs are known to be induced by various stimuli in osteoblasts and play an important role in several steps of osteoblast lineage progression *in vitro* and *in vivo* (6, 42, 51–53). Their effects have been attributed in part to their ability to phosphorylate Osx and Runx2 (6, 33, 42, 43). MAPK phosphorylation of Osx at Ser-73 and Ser-77 does not change its affinity for binding to the Sp1 sequences analyzed but increases its ability to recruit coactivators (33). Runx2 is also a substrate of phosphorylation by ERK and p38 MAPKs, leading to enhanced transcription and recruitment of transcriptional activators (6, 32, 42, 43). In addition, Runx2 contains a consensus MAPK-docking D-site, which allows competitive binding of ERK and p38 MAPKs (43). Binding to this D-site permits phosphorylation of Runx2 by MAPK and is probably also involved in the phosphorylation of Osx when bound together. More importantly, the phosphorylation sites of these two kinases correspond to the amino acids located in the interaction surfaces of both Runx2 and Osx (Ser-301 and Ser-319 for Runx2 and Ser-73 and Ser-77 for Osx). Accordingly, we demonstrated that the phosphorylation by p38 and/or ERK MAPK at these specific sites is required for an efficient interaction and cooperation. Therefore, in addition to their effects on each transcription factor alone, MAPK phosphorylations may modify the osteogenic activity of Runx2 and Osx, enhancing their ability to interact with each other. Phosphorylation of Runx2 and Osx by p38 and ERK signaling would then constitute an integration point at which extracellular stimuli lead to strong modulation of their transcriptional activity and control the osteoblastic phenotype.

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