BMP-2 Induces Osterix Expression through Up-regulation of Dlx5 and Its Phosphorylation by p38*[s]

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Osterix, a zinc-finger transcription factor, is specifically expressed in osteoblasts and osteocytes of all developing bones. Because no bone formation occurs in Osterix null mice, Osterix is thought to be an essential regulator of osteoblast differentiation. We report that bone morphogenetic protein-2 (BMP-2) induces an increase in Osterix expression, which is mediated through a homeodomain sequence located in the proximal region of the Osterix promoter. Our results demonstrate that induction of Dlx5 by BMP-2 mediates Osterix transcriptional activation. First, BMP-2 induction of Dlx5 precedes the induction of Osterix. Second, Dlx5 binds to the BMP-responsive homeodomain sequences both *in vitro* and *in vivo*. Third, Dlx5 overexpression and knock-down assays demonstrate its role in activating Osterix expression in response to BMP-2. Furthermore, we show that Dlx5 is a novel substrate for p38 MAPK *in vitro* and *in vivo* and that Ser-34 and Ser-217 are the sites phosphorylated by p38. Phosphorylation at Ser-34/217 increases the transactivation potential of Dlx5. Thus, we propose that BMP activates expression of Osterix through the induction of Dlx5 and its further transcriptional activation by p38-mediated phosphorylation.

Bone is a dynamic tissue that is constantly remodeled throughout life. Bone remodeling activity is dependent on a strict coupling mechanism of osteoclast resorption and new matrix deposition by osteoblasts, and an imbalance between these two activities leads to pathological states such as osteoporosis and osteosclerosis. Commitment to the osteoblast phenotype is ultimately controlled by a specific set of transcription factors activated by signals and regulatory pathways. Among them, bone morphogenetic protein (BMP)[5] signaling has been

shown to be involved in bone regeneration and osteoblast differentiation *in vitro* and *in vivo* (1–3).

BMP target genes include a growing number of tissue-determining transcription factors that promote differentiation of mesenchymal precursors toward the osseous cell phenotypes. For instance, osteogenic induction of bone marrow mesenchymal stem cells or premyogenic C2C12 cells has identified several types of transcription factors such as Id1, several homeodomain proteins, ATF4, the runt homology domain factor Runx2 (Cbfa1) and Osterix (Osx) (for review, see Refs. 4–6). Runx2 and Osx have been widely accepted as master osteogenic factors since neither Cbfa1 nor Osx null mice form mature osteoblasts (7, 8). Nakashima *et al.* (8) identified Osx as a zinc-finger SP1 family member induced by BMP-2 in C2C12 cells that is specifically expressed in osteoblasts and osteocytes of all developing bones. In Osx-null mice, no bone formation takes place, although Runx2 is expressed, suggesting that Osx acts downstream of Runx2 during bone development (8). It has been suggested that Runx2 would function from the commitment step to the point where osteochondroprogenitors appear, whereas Osx would have a role in the segregation of osteoblasts from osteochondroprogenitors (5). Supporting these differential regulatory pathways for Runx2 and Osx, it has been suggested that Osx promotes proliferation of progenitor cells, whereas Runx2 has an antiproliferative effect (9, 10). In addition, NFATc1 forms a complex with Osx and activates Osx-dependent collagen 1 promoter activity, whereas it does not activate Runx2-dependent transcription (11).

Different homeodomain-containing transcription factors have been implicated in chondrogenesis and osteogenesis. For instance, two isoforms of Prx1, called Prx1A and -B, are critical for cartilage, bone, and tooth development (12–15). Dlx3, -5, and -6 are transcription factors that belong to the distal-less, homeodomain-containing family of transcription factors. Their genes are arranged in tightly linked bigene pairs in the genome and are co-expressed in developing bones (16). Dlx5-null mice have craniofacial abnormalities with a delayed cranial ossification and abnormal osteogenesis (17, 18). Inactivation of both Dlx5 and Dlx6 results in skeletal defects that are considerably more severe, suggesting that they have redundant functions and are involved in endochondral ossification as well (19). Forced expression of Dlx5 or Dlx3 in cell culture leads to increased expression of osteogenic markers such as osteocal-

cin, Runx2, and alkaline phosphatase (20–24). These homeodomain transcription factors exhibit distinct temporal expression profiles during osteoblast differentiation, with Dlx3 expressed in osteoprogenitors and Dlx5 expressed in more mature osteoblasts.

Runx2 and Osx transcription is stimulated by BMP treatment *in vitro* (8, 25). Pretreatment with cycloheximide blocks these inductions, suggesting that either Runx2 or Osx are not direct targets of the BMP signaling cascade but require the expression of newly synthesized intermediates (26, 27). In contrast, BMP-2-induced Dlx5 transcription is unaffected by cycloheximide pretreatment (26). Interestingly, although expression of Osx *in vivo* requires Runx2, BMP-2 is still able to stimulate alkaline phosphatase activity and Osx expression in Runx2 deficient cells, whereas they were completely abrogated by antisense against Dlx5 (27).

Although these data suggest that Dlx5 is involved in BMP-induced Osx expression, little is known about the mechanism for activation of Osx expression in response to BMP signaling. In this study, we identified a homeodomain regulatory sequence as the BMP-2-responsive region in the Osx promoter. The Dlx5 temporal expression pattern and binding to the responsive region as well as overexpression and knock-down of Dlx5 demonstrates its role in activating Osx expression in response to BMP-2. Furthermore, we show that Dlx5 is a novel substrate for p38 MAPK *in vitro* and *in vivo* and that Ser-34 and Ser-217 are the regulatory sites phosphorylated by p38. The transactivation potential of Dlx5 was increased by p38 phosphorylation, resulting in an increased expression of Osx. We propose a regulatory network where BMP activates expression of Osx through the induction of the immediate early gene Dlx5 and its further transcriptional activation by p38-mediated phosphorylation.

EXPERIMENTAL PROCEDURES

Plasmids, Reagents, and Antibodies—The murine Osterix promoter from −1812 to +93 was obtained by PCR and cloned in the pGL2basic vector. Mutants were generated by cutting with SpeI, SmaI, SacI, or Kpn21, respectively. Annealed oligonucleotides encoding from either the −114 to −51 wild type sequence or the indicated mutations were cloned in the minimal promoter of c-fos promoter (28). cDNA encoding Dlx5 was provided by Dr. Watanabe (National Institutes for Longevity Sciences, Japan) and subcloned into pGEX and pcDNA3.1His expression vectors. HisDlx5 was used as a parental plasmid to generate the phosphorylation mutants using PCR approaches. Dlx3, Prx1A, and Prx1B expression vectors were provided by Drs. M. Morasso (NIH, Bethesda, MD) and M. Kern (Medical University of South Carolina), respectively. Activated MKK6 expression vector and recombinant p38 were provided by Dra. P. Muñoz-Canoves (CRG, Barcelona, Spain). Expression vectors encoding p300 and histone acetyltransferase (ΔHAT) mutant were provided by Dr. M. Martinez-Balbas (CID, Barcelona, Spain). BMP-2 was a generous gift from Wyeth (Cambridge, MA). SB203580 (Calbiochem) was used at a final concentration of 10 μM. Antibodies against FLAG (M2 antibody F3165) (Sigma), His (αHis antibody 27–4710) (Amersham Biosciences), α-tubulin (Sigma), and phospho-p38 (Cell Signaling,

Beverly, MA) were used at 1:1000. Antibodies against Dlx5 C-20 (sc-18152) and Y-20 (sc-18151) (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:500.

Cell Culture and Transfection—C2C12 and HEK-293 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, glutamine, pyruvate, and antibiotics. To induce differentiation, the medium was replaced by Dulbecco's modified Eagle's medium without serum and BMP-2 at a final concentration of 2 nM. C2C12 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche Applied Science). HEK-293 cells were transiently transfected using FuGENE 6.

RT-qPCR Analysis—Total RNA was isolated from C2C12 cells using the Ultraspec RNA Isolation System (Biotecx, Houston, TX). 5 μg of total RNA were reverse-transcribed using a Ready-to-Go First Strand kit (Amersham Biosciences) and oligo-dT as a primer. Quantitative PCRs were carried out using ABI Prism 7900 HT Fast Real-Time PCR System and a Taqman 5′-nuclease probe method (Applied Biosystems, Foster City, CA). All transcripts were normalized to GAPDH, and transfection efficiency was assessed by green fluorescent protein expression.

Reporter Assays—C2C12 cells were transfected overnight. The next day cells were split before reaching confluence and treated with BMP-2 for 16–20 h. Luciferase activities were quantified using the luciferase assay system (Promega, Madison, WI). Luciferase values were normalized using β-galactosidase activity measured with the luminescent β-galactosidase detection kit II (Clontech, Palo Alto, CA).

Electrophoretic Mobility Shift Assays—100 ng of purified GST-Dlx5 fusion proteins and 1 μl of ^{32}P-labeled probes were incubated in 40 mM Tris-HCl, pH 7.5, 10% glycerol, 10% Sacarose, 5 mM MgCl$_2$, 70 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 1 μg of poly(dI/dC) for 20 min at room temperature. Protein-DNA complexes were resolved on a 5% nondenaturating polyacrylamide gel.

Biotinylated Oligonucleotide Precipitation Assays—HEK-293 cells were transfected and 48 h later were lysed as described previously (28). Biotinylated oligonucleotides encoding the −114 to −51 fragment of the Osterix promoter were incubated overnight with cell extracts in presence of 2 μg of poly(dI/dC) and collected with streptavidin-agarose beads (Pierce).

RNA-mediated Interference Assays—To knock down Dlx5 expression, two siRNA oligonucleotides against Dlx5 mRNA were purchased from Dharmacon (Lafayette, CO): ON-TARGETplus J-041405-09 (J9) and J-041405-10 (J10). As a control, the non-targeting oligonucleotide ON-TARGETplus siCONTROL non-targeting siRNA D-001810-01 was transfected under the same conditions. C2C12 cells at 95% of confluence were transfected by using Lipofectamine 2000 (Invitrogen). After 16 h new medium with 15% fetal bovine serum was added to recover cells, and 8 h later cells were depleted overnight and then treated with or without BMP-2.

Immunofluorescence Assay—24 h after transfection, C2C12 cells were fixed in 3% paraformaldehyde for 30 min, permeabilized in 0.2% Triton X-100, and blocked with 3% bovine serum albumin in Tris-buffered saline. Cells were stained with mouse
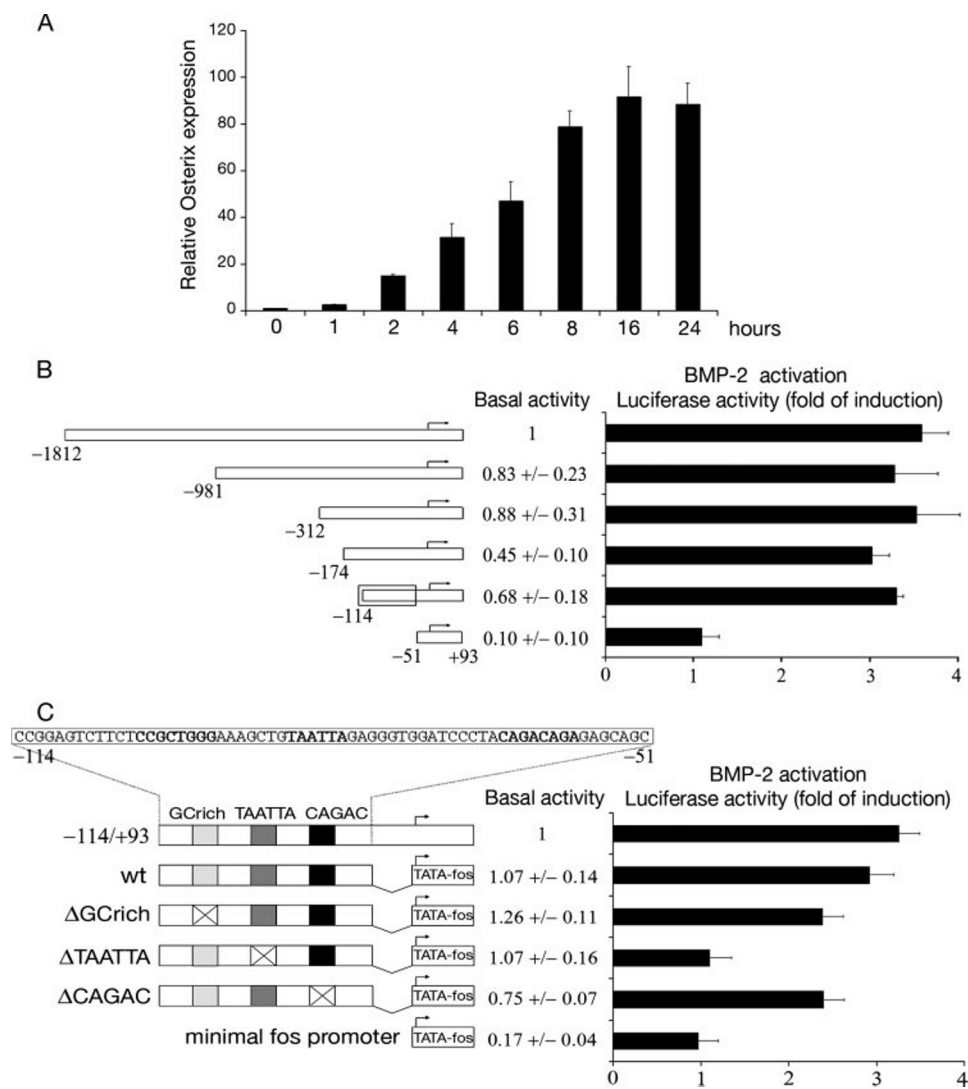
FIGURE 1. **Transcriptional activation of Osx expression by BMP-2.** *A*, confluent C2C12 cells were depleted of serum overnight and treated with BMP-2 2 nM for the indicated times. Osx mRNA was measured by RT-qPCR, normalized to GAPDH, and plotted as relative expression to time 0 ± S.E. of six independent experiments. *B* and *C*, when confluent, transfected C2C12 cells were treated with BMP-2 2 nM in serum-depleted media. 16–20 h later, luciferase activity was measured and normalized against β-galactosidase activity. Basal activities are shown as relative units compared with the activity of the −1812/+93 reporter (*B*) or the −114/+93 reporter construct (*C*). BMP-2 induction of luciferase activities are expressed as -fold induction ± S.E. of five to seven independent experiments. *wt*, wild type.

Chromatin Immunoprecipitation—C2C12 cells were transfected by FuGENE 6 with the indicated constructs. Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions but repeating all the washes twice. Antibodies used were anti-FLAG M2 (Sigma), anti-p300 (N-15, Santa Cruz Biotechnology), anti-acetylhistone H3, anti-RNA polymerase II, and control IgG (Upstate).

RESULTS

Osterix Expression Is Stimulated by BMP-2 in C2C12 Cells—Previous studies had shown that Osx mRNA levels increase after BMP-2 addition in mesenchymal cell types, including primary osteoblasts, mesenchymal stem cells, and C2C12 cells (8, 27, 30, 31). To assess the temporal pattern of this response, we evaluated Osx mRNA levels in C2C12 cells at different time points after BMP-2 addition. Osx was not significantly expressed in undifferentiated C2C12 cells; however, upon BMP-2 addition, Osx mRNA levels were strongly increased (Fig. 1*A*). Osx expression was initially detected at 2 h and reached a maximum expression 8–16 h after BMP addition. This late induction of Osx mRNA suggests that Osx is an indirect target of BMP signaling, which is in agreement with previous data which had indicated that induction of Osx required new protein synthesis (27).

A Homeobox Element Present in the Proximal Promoter Region Is the BMP-2-responsive Element—Previous work had shown that both the human and mouse Osx mRNA have two isoforms arising from two alternative promoters. These include a highly abundant form arising from the most proximal transcription start site and a minor form that is poorly transcribed (32–34). Our analysis indicates that BMP-2 activates the expression of the Osx mRNA form arising from this proximal transcription start site (Fig. 1*A*). This proximal TATA-less promoter contains a consensus Inr sequence at the transcription start site PyPyA(+1)NT/APyPy (35) and a cytosine-rich region between the −51 and −25 nucleotide. To define the BMP-2 responsive elements in the Osx promoter, we isolated this upstream regulatory sequence of the murine Osx gene. Using a reporter construct in C2C12 cells, we observed 3-fold activation with 2 nM BMP-2 (Fig. 1*B*).

anti-FLAG (Sigma) at 1:400 dilution followed by goat anti-mouse IgG conjugated with Alexa 488 at 1:2000. Labeling was detected using a Leica TCS SL inverted laser scanning confocal microscope.

Metabolic Labeling—Labeling with [^{35}S]methionine was performed as previously described (29) using HEK-293 cells expressing the indicated constructs followed by immunoprecipitation of labeled protein with anti-Dlx5 (Y-20 plus C-20, Santa Cruz Biotechnology).

In Vitro Phosphorylation by p38 MAPK—1 μg of GST-p38 MAPK was activated by incubation with MBP-MKK6-DD (5:1 ratio) at 30 °C for 20 min in a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 2 mM dithiothreitol, and 200 μM ATP. Activated p38 (50 ng) was incubated with 1 μg of recombinant Dlx5 in the same buffer supplemented with 1 μM ATP and 3 pmol of [γ-^{32}P]ATP at 30 °C for 20 min.
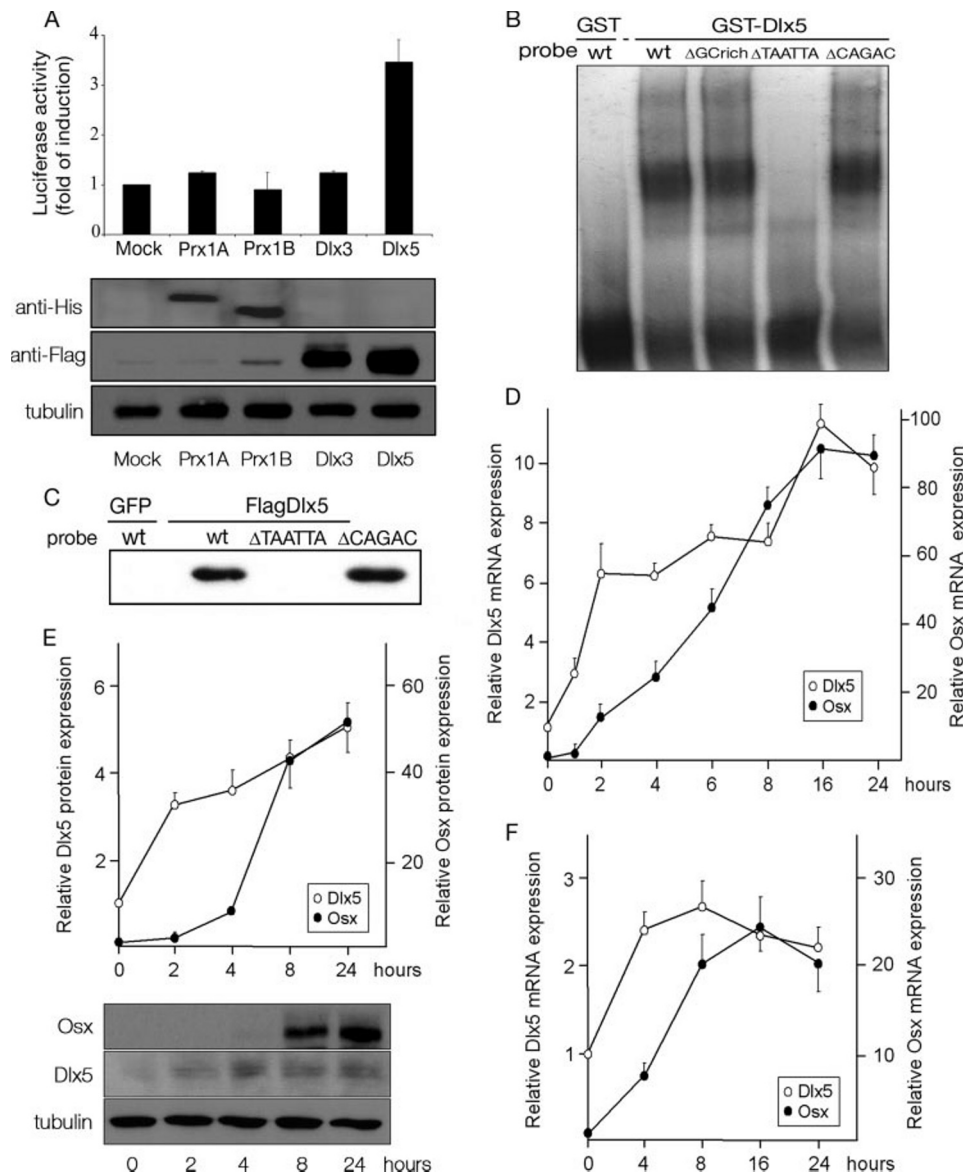
FIGURE 2. **Dlx5 binds and activates the Osx promoter.** *A*, the indicated homeobox transcription factors were co-transfected with the minimal Osx promoter reporter. Luciferase activity was measured and normalized as above. The values, relative to cells transfected with the reporter alone, are expressed as the mean ± S.E. of four independent experiments. Expression levels of the different transcription factors were measured by immunoblotting. Tubulin immunodetection is also shown as a protein loading control. *B*, electrophoretic mobility shift assays were performed using the indicated ^{32}P-labeled probes containing either the −114/−51 wt sequence or the indicated (GC-rich, TAATTA, or CAGAC) mutations. *wt*, wild type. *C*, the wild type, ΔTAATTA, and ΔCAGAC double-stranded biotinylated oligonucleotides were incubated with equal aliquots of extracts from cells expressing FLAG-tagged Dlx5 or green fluorescent protein as control. DNA-protein complexes purified with streptavidin-agarose were analyzed by immunoblotting using anti-FLAG antibody. *D* and *E*, confluent C2C12 cells were depleted of serum overnight and treated with 2 nM BMP-2 for the indicated times. *D*, Osx and Dlx5 mRNAs were measured by RT-qPCR, normalized to GAPDH expression, and plotted as relative expression to time 0 ± S.E. of four independent experiments. *E*, Osx and Dlx5 protein levels were measured by immunoblotting. Quantification was performed using the Bio-Rad Molecular Imager software and normalized to tubulin expression. The values are plotted as relative expression to time zero ± S.E. of four independent experiments. *F*, confluent MC3T3-E1 cells were depleted of serum overnight and treated with 2 nM BMP-2 for the indicated times. Osx and Dlx5 mRNAs were measured by RT-qPCR, normalized to GAPDH expression, and plotted as relative expression to time 0 ± S.E. of three independent experiments.

ability to render responsiveness to BMP-2, we assayed the responses of a construct containing this 63-bp region (comprising from −114 to −51) upstream of a heterologous c-fos minimal promoter. Whereas the minimal c-fos promoter showed no response at all to BMP-2, BMP-2 activated the construct bearing the enhancer (indicated as *wt* in Fig. 1*C*).

Homology analysis showed that the −114/−51 region is almost totally conserved among vertebrate species available in databases. Hence, this 63-bp region was analyzed with Matinspector™ (Genomatix), and three putative transcription factor binding sites were detected; a GC-rich region similar to a BMP responsive Smad binding element (28, 36), a palindromic homeobox binding site (TAATTA), and a CAGAC box. Mutation of the individual enhancer elements in the reporter construct followed by luciferase analysis showed that the homeobox mutant lost BMP-2 responsiveness, whereas no significant effects were observed for the mutated GC-rich construct, and only a slight decrease in the basal levels but not in the induction was observed in the CAGAC box mutation (Fig. 1*C*).

Dlx5 Interacts Directly with the Homeobox Region in the Osterix Promoter and Mediates BMP-2 induced Osx Expression—Because the TAATTA sequence is a canonical homeodomain binding site, we tested different homeobox transcription factors involved in osteogenesis with the ability to bind the TAATTA sequence as putative activators of the Osx promoter. Neither Prx1A, Prx1B, nor Dlx3 showed significant effects, whereas Dlx5 showed more than a 3-fold induction of the promoter (Fig. 2*A*). Dlx5 failed to induce transcriptional activation of the ΔTAATTA reporter construct (data not shown). Thus, we analyzed whether Dlx5 bound to the BMP-responsive elements of Osx promoter using two complementary approaches. Electrophoretic mobility shift assays were carried out incubating GST-Dlx5 protein with probes corresponding to the wild type or the mutated minimal responsive region. GST-Dlx5 was able to bind all the probes

Then we used reporter assays to analyze serial deletions of the 1.9-kilobase promoter. The deletion of sequences from −1812 to −114 produced only slight decreases in BMP-2 reporter induction. However, further removal of a short region (−114 to −51) resulted in a complete loss of BMP-2 responsiveness (Fig. 1*B*). To test whether this region alone has the
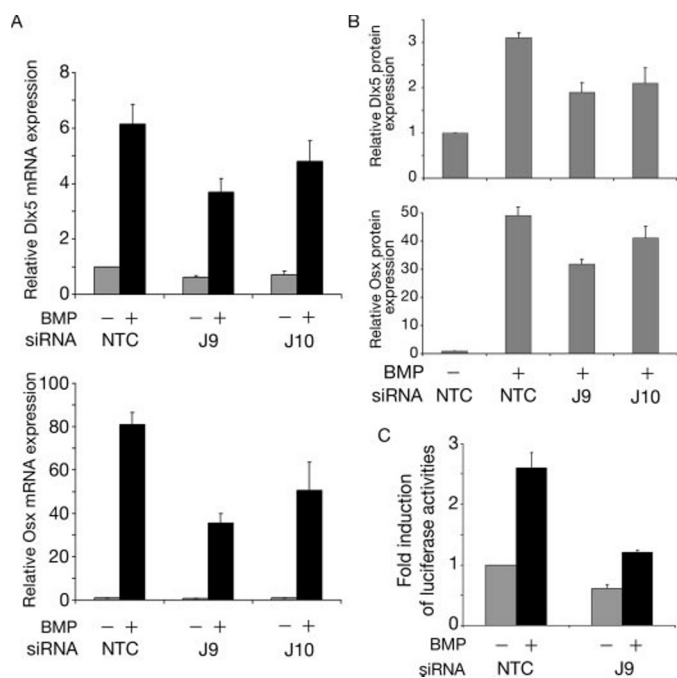
FIGURE 3. **Dlx5 is necessary for BMP-2 induction of Osx.** *A* and *B*, C2C12 cells were transfected with 200 pmol of siRNAs (*J9* and *J10*) targeted against Dlx5 or a scrambled siRNA (non-target control (*NTC*)). 24 h after transfection cells were depleted of serum overnight and treated with BMP-2 2 nM for 8 h. *A*, Dlx5 and Osx mRNA were measured by RT-qPCR, normalized to GAPDH, and expressed as relative expression ± S.E. of five independent experiments. *B*, Osx and Dlx5 protein levels were measured by immunoblotting. Quantification was performed using the Bio-Rad Molecular Imager software and normalized to tubulin expression. The values are plotted as relative expression ± S.E. of four independent experiments. *C*, C2C12 cells were transfected with the minimal Osx promoter reporter construct together with 200 pmol of a siRNA targeted against Dlx5 (*J9*) or an scrambled siRNA (*NTC*). 24 h later cells were serum-deprived and treated with BMP-2. Luciferase activity was measured as above. Values represent the average of four independent experiments ± S.E. after normalization by β-galactosidase activity.

except that which contained a mutated homeobox (Fig. 2*B*). The appearance of the shifted band was abolished by incubation with antibodies against Dlx5, further confirming that the shifted band was indeed Dlx5 (supplemental Fig. 1). Similarly, oligonucleotide pulldown assays of HEK-293 cells overexpressing Dlx5 showed binding of Dlx5 to the homeobox DNA but not when the homeobox sequence was mutated (Fig. 2*C*).

Dlx5 has been shown to be induced in C2C12 cells in response to BMP-2 through the activation of the Smad signaling pathway (6, 26). We analyzed the temporal pattern of expression of both genes to assess whether Dlx5 could act upstream of Osterix. Dlx5 mRNA was induced 1–2 h after BMP-2 addition and increased steadily thereafter. Osterix mRNA levels were also increased showing a delayed profile of induction compared with that of Dlx5 mRNA (Fig. 2*D*). Similar expression profiles were obtained when the protein levels of Dlx5 and Osx were analyzed by Western blot analysis (Fig. 2*E*). We further analyzed the pattern of expression of both Dlx5 and Osx in the osteoblast cell line MC3T3-E1. Similarly to mesenchymal C2C12 cells, BMP-2 induces both Dlx5 and Osx mRNA expression, and the Dlx5 mRNA induction also preceded that of Osx (Fig. 2*F*). Altogether, these data show that BMP-2 induces both Dlx5 and Osx expression in different mesenchymal cell lines and indicates that the induction of Dlx5 by BMP-2 precedes that of Osx.
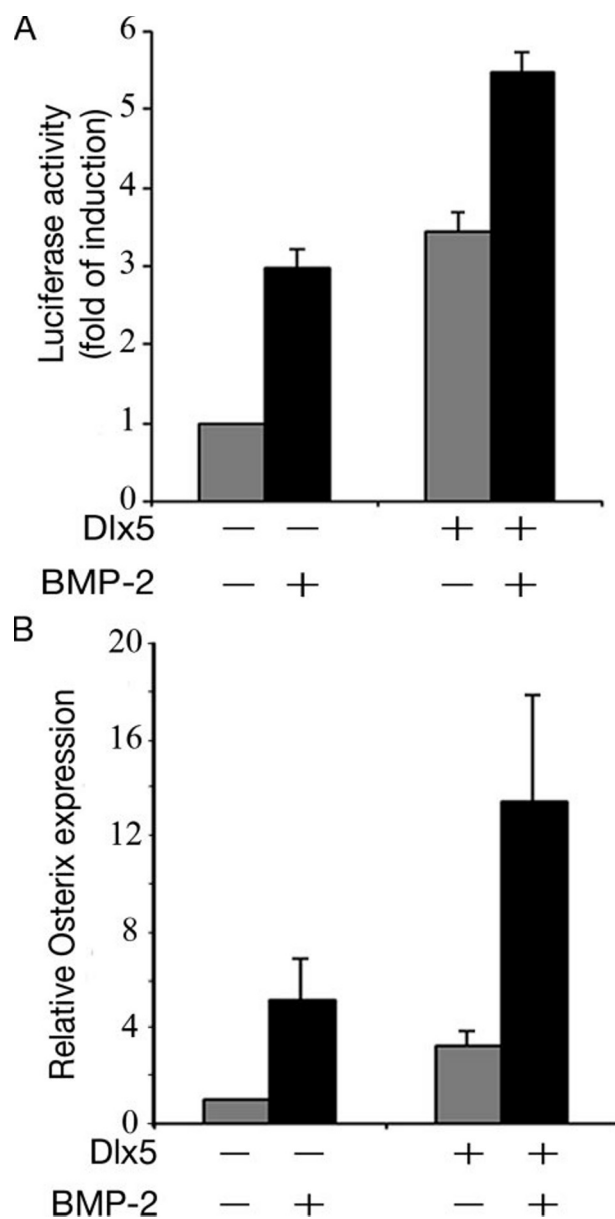

FIGURE 4. **Dlx5 activates Osx expression.** *A*, transfected C2C12 cells were grown to confluence, serum-starved, and treated with BMP-2 for 16–20h. Luciferase activities are expressed as the mean ± S.E. for triplicates from six independent experiments. *B*, transfected C2C12 cells were grown to confluence, serum-starved overnight, and treated with BMP-2 for 8 h. Relative values of Osx mRNA expression were obtained by RT-qPCR and expressed as the mean ± S.E. from four independent experiments after normalization by GAPDH expression.

To directly address a functional role of Dlx5 in Osx expression regulation, we tested two different siRNAs against Dlx5 (J9 and J10) in C2C12 cells. BMP-2-induced Dlx5 mRNA and protein expression were decreased compared with cells transfected with a scrambled siRNA (non-target control (*NTC*)) (Fig. 3, *A* and *B*). BMP-2-induced Osx expression was decreased by the two siRNAs (55% by J9 and 35% by J10 at the mRNA level and 35% by J9 and 20% by J10 at the protein level). Moreover, we also co-transfected C2C12 cells with J9 siRNA against Dlx5 together with the minimal responsive Osx reporter. J9 siRNA decreased both basal and BMP-2 mediated responses (Fig. 3*C*). These data are also in agreement with previous data using antisense con-
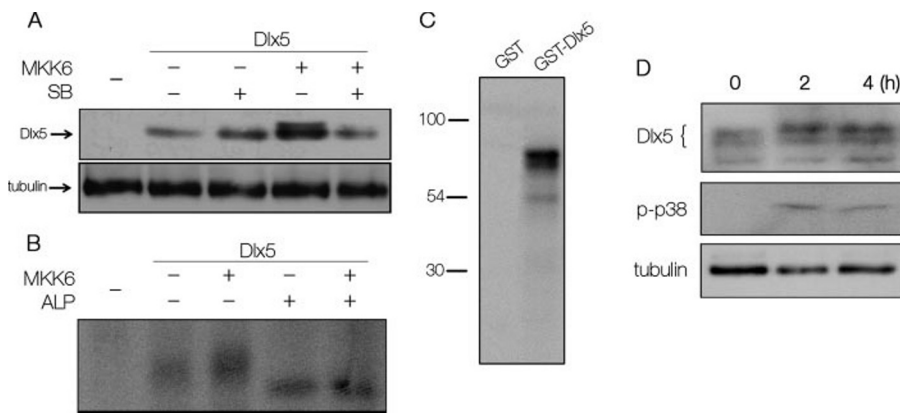
FIGURE 5. **Dlx5 is phosphorylated by p38.** *A*, HEK-293 cells were transfected with the indicated constructs. After 24 h of transfection, cells were treated with SB203580 (*SB*) for 3 h. Dlx5 and tubulin were detected by immunoblotting. *B*, HEK-293 cells transfected with the indicated constructs were labeled with [^{35}S]methionine for 3 h as described under "Experimental Procedures." Aliquots of immunoprecipitated Dlx5 were incubated with alkaline phosphatase (*ALP*) for 1 h and visualized by SDS-PAGE and autoradiography. *C*, GST-Dlx5 fusion protein was incubated with activated p38 MAPK in the presence of γ-[^{32}P]ATP and visualized by SDS-PAGE and autoradiography. *D*, cell extracts from C2C12 cells treated with BMP-2 for 0, 2, and 4 h were analyzed by immunoblotting with the indicated antibodies. *p-*, phosphorylated.

structs against Dlx5 (27). Then we also analyzed the functional effects of up-regulation of Dlx5 expression on the minimal Osx reporter activity assays and endogenous Osx gene expression. Dlx5 overexpression up-regulated transcription of the minimal Osx reporter construct as well as expression of the endogenous Osx gene analyzed by RT-qPCR (Fig. 4). We consistently observed that in cells subjected to transfection procedures, BMP-2 showed a lower ability to stimulate Osx expression. More interestingly, in both experimental assays BMP-2 promoted an additive effect to Dlx5-overexpressing C2C12 cells. These data indicate that, in addition to the induction of Dlx5 expression, BMP-2 activates additional pathways that result in further Osx transcriptional activation.

Dlx5 Is Phosphorylated by p38—The fact that a CAGAC site is also present in the minimal BMP-responsive promoter led us to investigate whether direct binding of Smads was also involved in Osx transcription. However, neither deletion of the CAGAC sequence (Fig. 1*C*) nor overexpression of Smad1 or Smad4 (data not shown) significantly modify BMP-2 responsiveness of the minimal Osx reporter. Alternatively, it has been shown that BMP-2 activates Smad-independent pathways (37). For instance, activation of p38 MAPK by BMP-2 has been shown to be relevant in the osteogenic effects of this cytokine (38–40). Furthermore, the inhibition of the p38 pathway strongly inhibits Osx expression induced by BMP-2, insulin-like growth factor-I, or mechanical stress (41–43). Thus, we analyzed whether Dlx5 could be a target of the p38 pathway. With this objective we used a constitutive active form of MKK6 that phosphorylates and activates p38 and analyzed cell extracts from C2C12 cells expressing Dlx5. Dlx5 appeared as two bands in the presence of MKK6 compared with the single band of the extracts from cells expressing Dlx5 alone (Fig. 5*A*). Moreover, pretreatment of C2C12 cells with SB203580, a potent and selective inhibitor of p38 MAPK, abrogated the retarded band, suggesting that this band is due to p38 phosphorylation. To confirm these results, immunoprecipitates of ^{35}S-labeled Dlx5 from cells co-transfected with Dlx5 and MKK6 were incubated with alkaline phosphatase. Phosphatase treatment converted

the major upper forms of Dlx5 into the lower ones, indicating that the retarded band arose from phosphorylation events (Fig. 5*B*). Then we examined whether Dlx5 was a direct substrate of p38 by *in vitro* phosphorylation assays using recombinant proteins. Dlx5 was phosphorylated by p38 (Fig. 5*C*). Next, we examined whether the activation of p38 induced by BMP-2 was sufficient for the phosphorylation of endogenous Dlx5. In agreement with previous reports (40), treatment of C2C12 cells with BMP-2 induced activation of p38 (Fig. 5*D*). Endogenous Dlx5 appeared as two bands, and BMP-2 treatment increased both the total amount of Dlx5 (in agreement with the RT-qPCR data) and the ratio between retarded *versus* higher mobility Dlx5 bands (from a ratio of 0.95 ± 0.10 at 0 h to a ratio of 1.4 ± 0.2 at 2 h). Taken together, these results demonstrate that p38 is able to directly phosphorylate Dlx5 *in vivo* and *in vitro*.

p38 Phosphorylation Enhances Dlx5 Transcriptional Activity— To investigate whether this phosphorylation has a functional effect on Dlx5 transcriptional activity, C2C12 cells were co-transfected with the Osx minimal promoter construct with Dlx5 and MKK6 in the presence or absence of BMP-2 (Fig. 6*A*). Reporter activity was increased in cells co-transfected with MKK6 alone in a SB-dependent manner (up to 2-fold induction compared with control cells) similar to the induction of cells treated with BMP-2. Furthermore, expression of MKK6 was able to further increase the reporter responsiveness to Dlx5 in a SB-dependent manner (up to 2-fold increase), suggesting a functional role for p38-induced Dlx5 phosphorylation. To confirm the functional effect of phosphorylation by p38, we also analyzed whether MKK6-activated Dlx5 may enhance the endogenous Osx expression by RT-qPCR. Endogenous Osx expression increased when C2C12 cells were transfected with MKK6 (up to 4-fold with respect to control cells) in a SB-dependent manner (Fig. 6*B*). Furthermore, expression of MKK6 was able to cooperate with Dlx5, further increasing the level of Osx expression, whereas SB203580 treatment abolished this effect. These data further support the hypothesis of an enhanced Dlx5 transcriptional activity induced by p38 phosphorylation.

To examine the mechanisms by which phosphorylation of Dlx5 enhances its transcriptional activity, we first analyzed Dlx5 subcellular localization. Immunofluorescence analysis in C2C12 cells transfected with Dlx5 showed that Dlx5 has nuclear localization (Fig. 6*C*). Next, we analyzed whether MKK6 changed the Dlx5 protein turnover. C2C12 cells were treated with the protein synthesis inhibitor cycloheximide for different times. The degradation pattern of Dlx5 was mostly similar either in the presence or absence of MKK6 (Fig. 6*D*). Electrophoretic mobility shift assays indicated that the ability of Dlx5 to bind to its responsive probe in Osx promoter was not
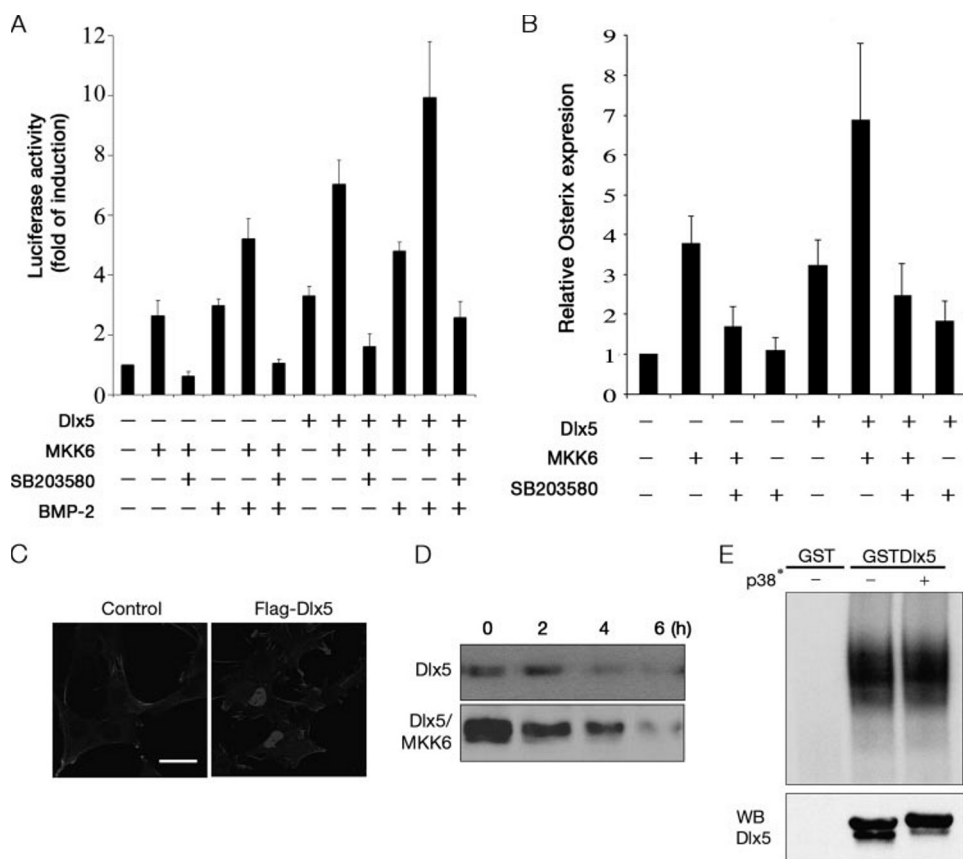
FIGURE 6. **p38 phosphorylation enhances Dlx5 transcriptional activity.** *A*, C2C12 cells were co-transfected with the minimal Osx reporter construct and combinations of Dlx5 and activated MKK6 expression vectors. Cells were grown to confluence and treated with SB203580 and/or BMP-2 for 20 h. Luciferase activities are expressed as the mean ± S.E. of triplicates from four independent experiments. *B*, C2C12 cells were transfected with the indicated constructs. Cells were grown to confluence and treated with SB203580 for 8 h. Relative values of Osx mRNA expression were obtained by RT-qPCR analysis and are expressed as the mean ± S.E. from six independent experiments after normalization by GAPDH expression. *C*, subcellular localization of Dlx5 in C2C12 cells was analyzed by immunofluorescence with anti-FLAG antibody in cells transiently transfected with FLAG-tagged Dlx5 expression vector. *Bar*, 20 μm. *D*, HEK-293 cells were transfected with the indicated constructs. After 24 h cells were treated with cycloheximide for the indicated times. Dlx5 was detected by immunoblotting. *E*, GST-Dlx5 was incubated with activated p38 MAPK. Aliquots were subjected to electrophoretic mobility shift assay with the minimal Osx promoter probe or immunoblotted (*WB*) against Dlx5 antibody.

phorylated bands when co-transfected with either wild type or the mutant forms S206A and S220A (supplemental Fig. 2). MKK6, when co-transfected with the S34A or the S217A forms, only induced partially retarded bands, whereas the double mutant S34A/S217A lost p38-dependent phosphorylation and was detected exclusively as a high-migrating band irrespective of expression of MKK6 (Fig. 8*A*). Then we tested whether activated p38 MAPK could phosphorylate Dlx5(S34A/S217A) *in vitro*. As expected, only the wild type form of Dlx5, but not Dlx5(S34A/S217A), was ^{32}P-labeled (Fig. 8*B*). These results confirm phosphorylation of Dlx5 by p38 at Ser-34 and Ser-217 *in vivo* and *in vitro*.

Then we assessed the relevance of Ser-34 and Ser-217 phosphorylation on transcriptional activity of Dlx5 in the Osx minimal reporter construct. Expression of the different constructs showed no significant differences with respect to the wild type when expressed alone. However, S34A and S34A/S217A were partially refractory to the cooperative transcriptional effects induced by co-transfection of MKK6 compared with either the wild type or the S217A mutant (Fig. 8*C*). The different mutants were also analyzed for activation by BMP-2 (Fig. 8*D*). Whereas induction of the reporter activity by mutant forms of Dlx5 in the absence of BMP-2 was similar to the wild type, induction of luciferase activity by BMP-2 in S34A- and S34A/S217A-transfected cells was significantly reduced. Finally, we analyzed the functional effects of expression of the Dlx5 Ser-34/Ser-217 mutant on expression of endogenous Osx gene. Cells overexpressing the Ser-34/Ser-217 mutant were partially refractory to the stimulatory effects of BMP-2 (Fig. 8*E*). Thus, phosphorylation of Ser-34 and Ser-217 seems to be crucial for the regulation of Dlx5 transcriptional activity by p38.

DISCUSSION

Osx expression is limited to some types of cells of the mesenchymal lineage such as osteoblasts, chondrocytes, and osteosarcoma cell lines (33). Furthermore, Osx expression is not detected before E13 in mice and is more restricted to osteoblasts than Runx2 (8). Analysis of the promoter sequence identified binding sites for transcription factors involved in osteoblast differentiation such as Runx2, vitamin D receptor homeobox family, or NF-κB. For instance, the Runx2 and NF-κB sites have been shown to be functional in regulating the expression

modified by phosphorylation *in vitro* with activated p38 (Fig. 6*E*). To investigate Dlx5 binding in the Osterix promoter *in vivo* we performed chromatin immunoprecipitation assays in C2C12 cells transfected with Dlx5 with or without MKK6. As shown in Fig. 7*A*, Dlx5 bound Osterix promoter *in vivo*. Binding of Dlx5 to the promoter is enough to recruit p300, RNA polymerase II, and acetylated histone H3. To further confirm the involvement of p300 in activation of Osx expression, we analyzed the effects of p300 in the activation of the minimal Osx reporter construct. p300 expression was able to cooperate with Dlx5 in the transcriptional activation of the Osx reporter, whereas a mutated form of p300 devoid of histone acetyltransferase activity was unable to further increase the Dlx5 activation (Fig. 7*B*).

Dlx5 Is Phosphorylated at Ser-34 and Ser-217 by p38 MAPK—Sequence analysis of Dlx5 revealed four distinct serines corresponding to consensus sites for MAPK phosphorylation conserved in Dlx5 orthologs. We generated site-directed mutants corresponding to these sites where Ser was changed to Ala. Expression of MKK6 induced the appearance of retarded phos-
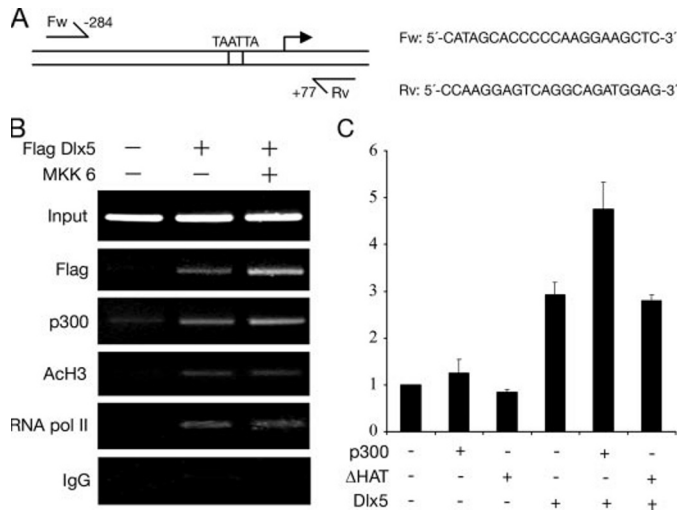
FIGURE 7. **Role of p300 in Dlx5 activation of Osx promoter.** *A*, diagram showing the location of the primers used to amplify Osx promoter (-284 to $+77$) in the chromatin immunoprecipitation analysis. *B*, C2C12 cells were transfected with the indicated constructs. Chromatin immunoprecipitation analysis was performed by incubating DNA-protein complexes with antibodies against FLAG, p300, acetylhistone 3 (*AcH3*), RNA polymerase II (*pol II*), and IgG as a negative control. *C*, either p300 or a ΔHAT (histone acetyltransferase) mutant, which loses the histone acetyltransferase domain, were co-transfected with Dlx5 and the minimal Osx reporter. Luciferase experiments were performed as above.

FIGURE 8. **p38 phosphorylates Ser-34 and -217 of Dlx5.** *A*, HEK-293 cells transfected with the indicated constructs were labeled with [^{35}S]methionine for 3 h as described under "Experimental Procedures." Immunoprecipitated Dlx5s were visualized by SDS-PAGE and autoradiography. *wt*, wild type. *B*, wild type and mutated GST-Dlx5 fusion proteins were incubated with activated p38 MAPK in presence of γ-[^{32}P]ATP and visualized by either SDS-PAGE and autoradiography or immunoblotting (*WB*) against Dlx5. *C* and *D*, C2C12 cells were cotransfected with the indicated constructs. Cells were grown to confluence and either serum-starved (*C*) or serum-starved and treated with BMP-2 for 16–20 h (*D*). Luciferase activities are expressed as the mean ± S.E. for triplicates from five independent experiments after normalization with β-galactosidase activity. *E*, C2C12 cells transfected with the indicated constructs were grown to confluence, serum-starved overnight, and treated with BMP-2 for 8 h. Relative values of Osx mRNA expression were obtained by RT-qPCR and expressed as the mean ± S.E. from four independent experiments after normalization by GAPDH expression.

of Osx, further confirming the genetic evidence that Runx2 expression is required for proper Osx expression (32, 34). Our deletion analysis also indicated that in C2C12 cells, the deletion of the NF-κB binding site decreased basal reporter activity. However, BMP responsiveness, restricted to the region between -114 and -51, does not contain these sites. Mutational analysis of this region identified a homeodomain binding site as being particularly important for BMP-2 responses.

Although we cannot rule out the requirement of additional transcription factors, several evidences strongly suggest that Dlx5 binding activity play an essential role in mediating these effects. First, the induction of Osx mRNA by BMP-2 requires 6–8 h, whereas the induction of Id1, a known direct Smad-responsive gene, only requires 1 h (28). Second, the temporal expression pattern after activation by BMP-2 (Fig. 3) or in other models of differentiation of osteoblast precursors indicate that the expression of Dlx5 always precedes that of Osx and other markers of terminal differentiation such as osteocalcin or alkaline phosphatase (21). Third, chromatin immunoprecipitation assays indicate Dlx5 binding to the Osx proximal promoter region *in vivo*, and recombinant Dlx5 binds specifically to that site *in vitro*. Finally, the overexpression of Dlx5 enhances, whereas siRNAs against Dlx5 reduce, both basal and BMP-induced transcriptional responses of either reporter or endogenous Osx mRNA expression. These data are in agreement with previous results indicating that Dlx5 is an immediate early BMP-responsive gene insensitive to the addition of cycloheximide, whereas the induction of Osx is absolutely sensitive to this protein synthesis inhibitor (26, 27, 44). BMP-2 is still able to stimulate Osx expression in cells with targeted deletion of Runx2, whereas Osx expression is reduced in cells treated with antisense or siRNAs against Dlx5 (Ref. 27 and present work). Furthermore, overexpres-
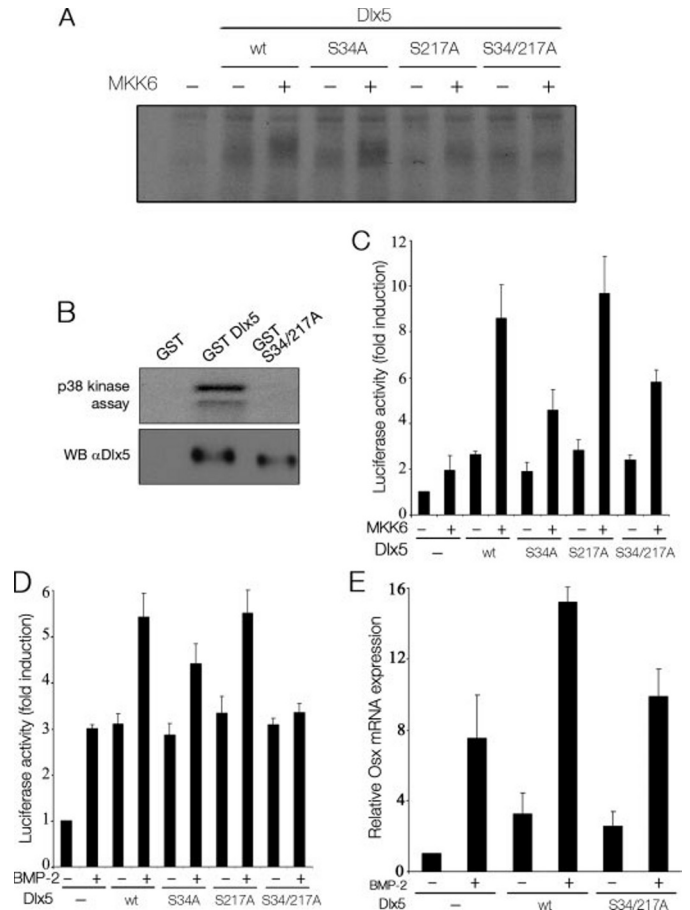
sion of dominant negative Runx2 constructs did not block BMP-2-stimulated Osx mRNA expression (27, 30). Altogether, our data further support the idea that BMP-dependent induction of Osx expression depends on Dlx5 and can occur independently of the changes of Runx2 expression.

Two major Runx2 isoforms are expressed from alternative promoter usage. Expression of Runx2-II, a bone-specific isoform, co-localizes with BMP-2 and Dlx5 during calvarial bone development. Expression of this isoform is specifically regulated by BMP-2 through regulation of Dlx5 function (21, 23). Thus, as suggested in other developmental and differentiation processes, a feed-forward transcriptional mechanism seems to operate in the BMP-induced osteoblast differentiation. Dlx5, acting as an early gene induced by BMP-2, induces direct transcriptional activation of at least two other osteoblast master
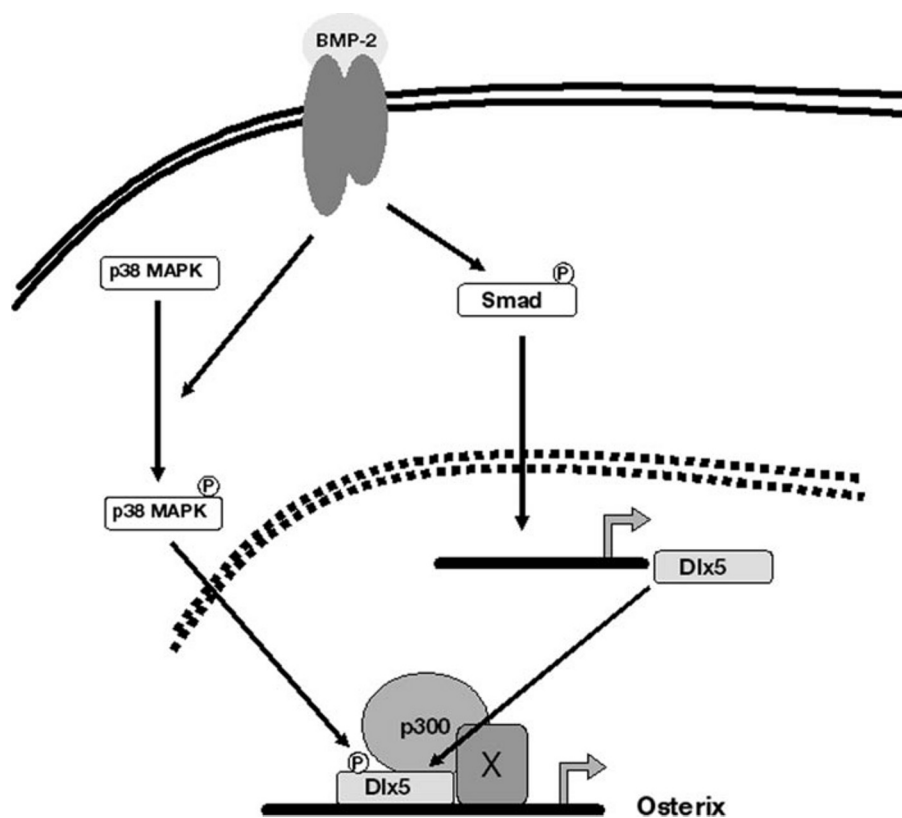
FIGURE 9. **Model of a novel activation mechanism of Dlx5 by BMP-2.** Activation of the BMP signaling pathway leads to increased levels of Dlx5 through the activation of its promoter. In addition activation of p38 by BMP-2 leads to enhanced transcriptional activity of Dlx5 through increased interaction with p300 or additional transcriptional regulators (*X*).

p38 MAPK is known to play an important role in stress response and cell differentiation (47). The phosphorylation and activation of p38 occurs soon after BMP stimulation through an Smad-independent pathway (for review, see Ref. 37). Previous studies have shown that p38 activation is clearly involved in several steps of the osteoblast lineage progression, necessary but not sufficient for the BMP-induced acquisition of the osteoblast phenotype (38). It has been suggested that the p38 signaling may initially upregulate early markers such as alkaline phosphatase but directly or indirectly suppress late markers such as osteocalcin (30, 40, 48). Several lines of evidence also indicate that p38 activity modulates Osx expression induced by BMP in calvarial as well as bone marrow-derived mesenchymal stem cells (30, 41, 43). Here we provide a mechanism by which p38 could activate Osx expression through direct phosphorylation of Dlx5 in Ser-34 and Ser-217. First, activated recombinant p38 phosphorylates Dlx5 *in vitro*, whereas the Dlx5(S34A/S217A) mutant shows a much lower *in vitro* and *in vivo* phosphorylation. Second, phosphorylation of Dlx5 is induced by the addition of BMP-2 or the expression of constitutive active MKK6, whereas it is blocked by the pharmacological inhibition of p38. Moreover, p38-mediated phosphorylation of Dlx5 appears to act primarily at the level of its transactivation function. Indeed, a constitutive active MKK6 increases endogenous Osx expression and activates Dlx5-driven Osx promoter activity through a homeobox binding site. Additionally, a constitutively active MKK6 enhances the transcriptional ability of wild type but not of Dlx5(S34A/S217A). Thus, we propose a regulatory network where BMP activates the expression of Osx through the Dlx5 induction and its further transcriptional activation by p38-mediated phosphorylation (Fig. 9). Our results do not exclude the possibility that other transcription factors involved in Osx expression may also serve as p38 substrates. In fact, the phosphorylation sites found in Dlx5 are well conserved in other Dlx members such as Dlx3 or Dlx6.

genes, Runx2-II and Osx. Then, Dlx5 acting together with either Runx2-II and/or Osx, would ensure an efficient activation of late osteogenic gene products. This functional cooperativity has been demonstrated in the promoter of bone sialoprotein where Runx2 and Dlx5 bind to adjacent sites and physically interact to drive full promoter activation (45). Similarly, it has been shown that both Dlx5 and Osx are required for osteoblast-specific expression of type I collagen and osteocalcin (8, 11, 20, 46), although the precise mechanisms of such cooperativity are still unknown.

BMPs up-regulate the expression of several homeodomain transcription factors critical for osteoblast differentiation such as Dlx5, Dlx3, or Msx2 (4, 21). These homeodomain transcription factors exhibit distinct temporal expression profiles during osteoblast differentiation: Msx2 expressed in the proliferative state, Dlx3 expressed in osteoprogenitors, and Dlx5 expressed in more mature osteoblasts. This fact and the selective association of these factors at different promoters lead to the hypothesis that a hierarchy of multiple homeodomain transcription factors constitutes a network that control the progression of osteoblast differentiation and maturation (20, 21, 23). Here we show that Dlx3, also up-regulated by BMP-2, does not elicit a strong transcriptional activation of Osx reporter construct (Fig. 3). Thus, our data fit with this scenario where Dlx5, which is induced after osteogenic commitment in C2C12 as well as primary calvarial osteoblasts (21), is the main activator of the late osteogenic master gene Osx.

Acetylation of histones in chromatin is commonly associated with transcriptionally active genes, and p300 and histone deacetylases (HDACs) have been shown to bind and regulate the acetylation of the core histones H3 and H4 in several osteogenic gene promoters. For instance, p300 is recruited to the osteocalcin promoter in response to osteogenic signals (46, 49), or JunB recruits p300 to activate dentin matrix protein 1 (50), whereas HDAC4 inhibits Runx2 activity and HDAC4 null mice exhibit strong skeletal defects (51). BMPs have been shown to

switch recruitment from HDAC1 to p300 in osteocalcin or Osx gene promoters and to promote hypomethylation of CpG sites in Dlx5 and Osx promoter regions (46, 52). Similarly, BMPs have been shown to increase the expression and function of the ATP-dependent chromatin-remodeling complex SWI/SNF, which has been shown to be absolutely required for osteogenesis (53, 54). Our study has demonstrated that p300 is recruited by Dlx5 to the Osx proximal promoter and p300 histone acetyltransferase activity is involved in Dlx5-induced activation of Osx transcription. This evidence would be in agreement with data showing that p53 inhibits osteogenesis and Osx expression by impeding p300 from binding to the proximal region of the Osx promoter where Dlx5 binds (31) and the minor requirement of p38 activity to BMP-induced activation of Osx expression in p53-deficient osteoblasts (43). Thus, it could be hypothesized that phosphorylation by p38 increases the ability of Dlx5 to recruit other DNA binding transcription factors or coactivators such as p300 and/or SWI/SNF as has been shown for p38-dependent JunB recruitment of p300 on dentin matrix protein 1 promoter or p38-dependent activation of MyoD and MEF2 in muscle-specific genes (47, 50).

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