Journal of Cellular Biochemistry 103:1438–1451 (2008)

p68 (Ddx5) Interacts With Runx2 and Regulates Osteoblast Differentiation

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Abstract Runx2 is an essential transcription factor for osteoblast development from mesenchymal progenitors. Runx2 regulates gene expression by interacting with numerous transcription factors and co-activators to integrate signaling events within the nucleus. In this study we used affinity purification and proteomic techniques to identify novel Runx2 interacting proteins. One of these proteins is the DEAD box RNA helicase, p68 (Ddx5). p68 regulates many aspects of RNA expression, including transcription and splicing. p68 co-localized with Runx2 in punctate foci within the nucleus. In transcription assays, p68 functioned as a co-activator of Runx2, but its helicase activity was not essential for co-activation. In accordance, Runx2 transcriptional activity was muted in p68-suppressed cells. Surprisingly, osteoblast differentiation of the multipotent progenitor C2C12 cell line was accelerated by p68 suppression and Runx2 suppressed p68 expression in calvarial progenitor cells. Together these data demonstrate that p68 is a novel co-activator for Runx2, but it inhibits osteogenic differentiation of progenitor cells. Moreover Runx2 has an active role in regulating p68 levels in osteoblast precursors. Thus, crosstalk between Runx2 and p68 controls osteoblast specification and maturation at multiple levels. J. Cell. Biochem. 103: 1438–1451, 2008.

Key words: CBFa1; DEAD box; Ddx5; osteocalcin; mesodermal cell differentiation

Mesenchymal stem cells give rise to multiple cell lineages including osteoblasts, chondrocytes, skeletal myocytes, fibroblasts and adipocytes. Transcription factors direct lineage specification by binding to specific DNA sequences within tissue-specific genes. Other proteins, collectively referred to as co-factors, are broadly expressed and interact with many transcription factors but do not directly contact DNA. They contribute to chromatin reorganization, transcription initiation, elongation and termination, splicing and other events associated with gene expression [Auboeuf et al., 2005; Rosenfeld et al., 2006].

Runx2 is an essential transcription factor for osteoblast development from mesenchymal progenitor cells and regulates gene expression during all stages of osteoblast maturation. It is also required for mesenchymal condensation, chondrocyte maturation and vascular invasion of the developing skeleton [Komori et al., 1997; Otto et al., 1997]. Runx2 is a context-dependent

Eric D. Jensen and Lingling Niu contributed equally to this work.

Grant sponsor: National Institutes of Health; Grant numbers: AR048147, AR050074, AR050938, AR49069, AR48818; Grant sponsor: The Intramural Research Program of the National Institute of Arthritis, Musculoskeletal, and Skin Diseases of the NIH; Grant sponsor: The Association for International Cancer Research; Grant sponsor: Tenovus, Scotland.

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Received 6 April 2007; Accepted 9 July 2007

DOI 10.1002/jcb.21526

regulator of transcription and although necessary for expression of target genes, it is not sufficient for transcription. It associates with co-activators and co-repressors, as well as other transcription factors and the nuclear matrix to integrate a variety signals at gene response elements [Schroeder et al., 2005; Westendorf, 2006].

p68 (Ddx5) is a multifunctional DEAD box protein that regulates gene expression by affecting RNA processing and transcriptional initiation [Fuller-Pace, 2006]. p68 shares 90% identity at the amino acid level across a central conserved core with another DEAD box protein, p72 (Ddx17), which is derived from a distinct gene. p68 and p72 have divergent amino- and carboxy-termini but can form heterodimers in cells [Lamm et al., 1996; Ogilvie et al., 2003]. p68 and p72 interact with many transcription factors, including p53, estrogen receptor-alpha, SMAD3 and MyoD [Endoh et al., 1999; Warner et al., 2004; Bates et al., 2005; Caretti et al., 2006]. They also associate with RNA coactivator SRA [Caretti et al., 2006] and several transcriptional co-activators and corepressors, including p300, CREB-binding protein (CBP), RNA polymerase II and Hdac1 [Rossow and Janknecht, 2003; Wilson et al., 2004]. Suppression of p68/p72 prevents proper muscle cell differentiation of a multipotent cell line, C2C12 [Caretti et al., 2006]. The role of p68 in osteoblast gene expression and differentiation has not been described.

The goal of this study was to identify and characterize novel Runx2-interacting proteins. By affinity purification and mass spectrometry, we identified p68 as a Runx2-binding protein. We verified the interaction by co-immunoprecipitation and demonstrated that p68 associated with the Runx2 target gene, osteocalcin. p68 modestly augmented Runx2-dependent transcription; however, suppression of p68/p72 by RNA interference accelerated BMP-2 dependent osteoblast differentiation of the myo/osteoprogenitor C2C12 cell line. These cell lines were previously shown to be defective in myocyte differentiation [Caretti et al., 2006]. Finally, Runx2 suppressed p68 expression in calvarial cells. Together these data suggest that p68 contributes to the myogenic commitment of progenitor cells and inhibits osteoblast differentiation, but once the cell is committed to the osteoblast lineage, p68 acts as a co-activator for Runx2.

MATERIALS AND METHODS

Cell Culture and Transient Transfections

UMR-106, COS and C2C12 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 200 mM L-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin. C3H10T1/2 and ROS 17/2.8 cells were cultured in minimal essential medium (MEM) containing the supplements listed above. Transient transfections were accomplished with Lipofectamine (Invitrogen) according to the manufacturer's instructions. Immortalized *Runx2* null mouse calvarial cells were generated from *Runx2* knock-out mice 17.5 dpc by mTERT stable integration as described previously [Bae et al., 2007]. Cells were maintained in αMEM supplemented with 10% FBS, 30 mM penicillin–streptomycin and 100 mM L-glutamine at 37°C and 5% CO_2 humidified atmosphere. Wild type and *Runx2* null primary calvaria cells were obtained from *Runx2* wild-type and null homozygous mice as previously described [Choi et al., 2001]. Briefly, calvaria were isolated from 17.5 dpc embryos, chopped and digested three times with collagenase/trypsin and plated in 6-well plates, 100,000 cells/ well in αMEM supplemented with 10% FBS, 30 mM penicillin–streptomycin and 100 mM L-glutamine. Cells were re-plated upon 75% confluency and expanded for three passages before harvesting.

Generation of Stable Cell Lines

High titer infectious retrovirus stocks were generated by transfecting 293T cells with pZOME-1-N-TAP-Runx2 or pZOME-1-N-TAP (provided by CELLZOME, Frankfurt, Germany), gagPol, and pMDG-VSVG using a calcium chloride precipitation method. Retroviral-containing supernatants were collected, mixed with 8 μg/ml polybrene, and added to UMR-106 cultures twice over 3 days. UMR-106 cells stably expressing TAP or TAP-Runx2 were then selected for a minimum of 3 days in DMEM containing 1.5 μg/ml puromycin. UMR-106 cells were chosen because they proliferate rapidly and express Runx2; thus, large quantities of protein can be obtained quickly and TAP-Runx2 would be expected to bind relevant proteins. C2C12 cells stably expressing shRNAs were previously described [Caretti et al., 2006].

Adenoviral Transductions

The adenoviral expression vector Adeno-Vator™ was purchased from Qbiogene. The full-length cDNA encoding the bone-specific P1/MASNS isoform of Runx2 and a carboxy-terminally truncated Runx2 protein (aa 1-361) were each cloned into the vector. Viruses were generated according to the manufacturer's protocol and purified with an adenovirus purification kit (Promega). For infections, *Runx2* null cells were plated in 6-well plates, 0.75×10^6 cells/plate. One day after plating, cells were infected with 100 MOI of each virus in αMEM complemented with 1% FBS for 12 h. Infection efficiencies were monitored by examining GFP expression through IRES mediated bicistronic expression. Runx2 expression was analyzed by western blotting and real-time qPCR analysis.

Immunoblotting

Transiently transfected COS cells were lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA) containing protease inhibitors. Lysates were cleared by centrifugation, resolved by SDS–PAGE and immunoblotted with a polyclonal antibody against Runx2 (kindly provided by Scott Hiebert) or a monoclonal antibody to p68 (Pab204, Upstate Biotech). For western blot analysis of calvarial cells, pellets from each well were resuspended in 150 μl of direct lysis buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% Glycerol, 12% Urea, 25 μM MG132, 100 mM DTT, and 1X Complete protease inhibitors from Roche), boiled 5 min, separated by 10% SDS–PAGE and semi-wet transferred to nitrocellulose membrane. Membranes were blocked in PBS/0.1% Tween with 5% milk for 1 h. Immunoblots were done in PBS/ 0.1% Tween with 1% milk. Mouse monoclonal antibodies to p68 (clone pAb204) were used at a 1:1,000 dilution overnight at +4°C. Runx2-specific mouse monoclonal antibodies were a generous gift of Dr. Y. Ito (Oncology Research Institute, National University of Singapore) and used in 1:2,000 dilution for 1 h at room temperature. CDK2 rabbit polyclonal antibodies and all secondary antibodies HRP-conjugated were used from Santa Cruz Biotechnology in 1:2,000 dilution for 1 h at room temperature. Signal was detected with ECL (Perkin Elmer Western Lighting Chemiluminescence Reagent Plus). Protein levels were quantified using ImageJ. Short exposures of immunoblots obtained with p68 and CDK2 antibodies were each sampled three times. For each densitometric value, local background was subtracted and the p68 blot was normalized to the average CDK2 values for each treatment group. (The error bars reflect sampling error calculated as standard error of the mean.)

Isolation of Nuclei for IgG-Affinity Purification

UMR-106 cells stably expressing either pZOME-N-TAP or pZOME-N-TAP-Runx2 were grown to 80% confluence (about $\times 10^9$ cells). In total, 100 plates (150 mm) of each cell line were collected over several weeks and pooled. For each collection, cells were washed twice with Dulbecco's phosphate-buffered saline (D-PBS). The cell pellets were resuspended in 25 ml of 10 mM HEPES, pH 7.9, 40 mM KCl, 3 mM $MgCl_2$, 5% glycerol, 0.2% NP-40, 0.1 mM DTT, and incubated for 20 min at 4°C. Nuclei were collected by microcentrifugation at 12,000 rpm for 30 s at 4°C, and were stored at −70°C.

IgG Purification

Nuclei were pooled and lysed in 25 ml of TAP lysis buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% NP-40, 0.5 mM DTT, 0.5 mM EDTA, 20 mM NaF, 10 mM β-glycerolphosphate) supplemented with protease inhibitors (Roche). Samples were sonicated (2 s/pulse for 10 pulses). Lysates were cleared by centrifugation at 12,000*g* for 20 min at 4°C. The supernatants were collected and analyzed for protein content. The total yield was 45–50 mg total nuclear protein. TAP-tagged Runx2 and interacting partners were collected with 100 ml of IgG-Sepharose 6 Fast Flow resin (Amersham Biosciences). Lysates were incubated with resin for several hours or overnight at 4°C. Flow-thru was collected by centrifugation. The beads were then washed three times with 20 ml of TAP buffer. Proteins bound to the IgG beads were eluted by incubating the resins in 60 ml of 0.5 M HOAC pH 3.4 for 5 min and neutralized in 2 M Tris while monitoring with litmus paper.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS)

Proteins eluted from the IgG resins were separated by SDS/PAGE electrophoresis on a 4–15% gradient gel, which was then silver stained (Invitrogen). Gel slices were excised and destained according to the manufacturer's

protocol (Invitrogen). After destaining, gels slices were dried by using 100% acetonitrile. Proteins in the gels were subjected to cysteine reduction and alkylation by rehydration in 10 mM DTT/100 mM NH_4HCO_3 at 56°C for 56 min. Samples were removed from heat and cooled. The excess solution in the gel was removed. The gel slices were then incubated in 55 mM iodoacetamide/100 mM NH_4HCO_3 for 30 min in the dark. Gel slices were washed with 100 ml of NH_4HCO_3 for 5 min and dried with 100% acetonitrile. Proteins were in-gel-digested by using sequence-graded trypsin (Promega) in 50 mM NH_4HCO_3 at 37°C overnight. The next day, supernatants were combined with gel extractions by incubating the gel three times with 50% acetonitrile/2% formic acid. Samples were dried to 20 ml by a speed-vac. Digests were analyzed by nanoflow liquid chromatography-tandem MS. Peptides sequences were submitted to the MASCOT program for identification.

Electrophoretic Mobility Shift Assay (EMSA)

COS cells transiently transfected with pCMV5-Runx2 or pCMV5-TAP-Runx2 were lysed in microextraction buffer [Westendorf et al., 2002] and incubated with ^{32}P-labeled double-stranded oligonucleotides containing a Runx binding element as previously described [Meyers et al., 1993]. Competition and supershift assays were also previously described [Kahler and Westendorf, 2003].

Luciferase Assays

C2C12 cells were transfected with Lipofectamine (Invitrogen) in 12-well plates with the indicated amounts of reporter plasmids (p6OSE2-luc and pRL-null) and pCMV-Runx2 or -p68 expression plasmids as indicated. pcDNA3.1 was added to transfections to maintain a uniform amount of total DNA per transfection. Luciferase activity was measured 24 or 48 h after transfection using the dual-luciferase assay system (Promega). Each transfection was performed in triplicate, and normalized to Renilla luciferase activity.

Immunofluorescence

C2C12 cells were transiently transfected with pCMV-p68-GAL and pCMV-Runx2 expression plasmids and grown on glass coverslips. For immunofluorescence experiments, ROS 17/2.8 cells or transfected C2C12 were rinsed in PBS, fixed in 4% paraformaldehyde for 30 min, permeabilized with PBS containing 0.3% Triton X-100 for 5 min and blocked for 30 min in immunofluorescence buffer (3% BSA, 20 mM $MgCl_2$, 0.3% Tween-20 in PBS). Transfected C2C12 cells were incubated with rabbit anti-GAL (Santa Cruz, SC-577) and goat anti-Runx2 (Santa-Cruz, S-19) primary antibodies in immunofluorescence buffer. Cells were washed three times with PBS containing 0.1% Triton X-100, incubated 30 min with donkey anti-goat Alexa546 for 20 min, washed twice, then with goat-anti-mouse Alexa488 for 20 min. ROS 17/2.8 cells were incubated with mouse anti-p68 (pAb204; Upstate Biotech) and rabbit anti-Runx2 (AML3 antibody from Scott Hiebert) primary antibodies in immunofluorescence buffer. Cells were washed three times with PBS containing 0.1% Triton X-100, incubated 30 min with Alexa-conjugated secondary antibodies (anti-mouse-Alexa555, and anti-rabbit-Alexa488) at 1:800 (Invitrogen), washed and mounted in 90% glycerol/0.4% N-propyl-gallate. Images were obtained using an Olympus Fluoview 500 confocal microscope and processed using Adobe Photoshop.

GST Pulldowns

^{35}S-trans-labeled proteins were synthesized from Runx2 and p68-GAL expression plasmids using the T7-TnT System (Promega). GST-Runx2 fusion proteins (1-383 or 383-513 [Westendorf et al., 2002]) and GST-p68 fusion proteins [Wilson et al., 2004] were isolated by lysing cultures of *E. coli* by sonication and purified by binding overnight to glutathione-s-transferase (GST)-sepharose followed by three washes of PBS. Yield and integrity of GST-fusion proteins were verified by SDS–PAGE and Coomassie staining. TnT proteins and GST-fusion proteins were pre-blocked separately in binding buffer containing 50 mM Tris, pH 7.6, 500 mM NaCl, 1 mM EDTA, 0.5% NP-40, 5 mM DTT, and 0.5% BSA for 60 min at 4°, and then co-incubated overnight. The bound complexes were washed three times with binding buffer. Bound complexes were resolved on SDS–PAGE, fixed, incubated with autoradiographic enhancer (Amplify buffer, G.E. Health Systems), dried, and visualized by autoradiography.

Chromatin Immunoprecipitation Assays

ChIP analyses were performed on lysates from ROS17/2.8 cells as previously described

[Schroeder et al., 2004] using antibodies against p68, Runx2 (Santa Cruz, M-70) or normal rabbit IgG (Santa Cruz). Real-time PCR was performed on an iCycler thermocycler (BioRad) using the Quantitect Sybr Green PCR kit (Qiagen, Valenica, CA).

Alkaline Phosphatase Assays

Control and p68-suppressed C2C12 cells were differentiated in growth medium supplemented with 300 ng/ml BMP2, 50 μg/ml ascorbic acid and 10 mM β-glycerol-phosphate. Alkaline phosphatase activity was measured after 3 and 6 days in this differentiation medium as previous described [Schroeder et al., 2004; Schroeder and Westendorf, 2005].

Real-Time Reverse-Transcriptase PCR (RT-PCR)

Control and p68-suppressed C2C12 cells were differentiated in growth medium supplemented with 300 ng/ml BMP2, 50 μg/ml ascorbic acid and 10 mM β-glycerol-phosphate. RNA was harvested with Trizol (Invitrogen) 6, 13, and 18 days later. Real-time RT-PCR was performed with primers for actin and osteocalcin as previously described [Schroeder et al., 2004; Schroeder and Westendorf, 2005] using the comparative threshold method for quantitation. For each timepoint, osteocalcin expression was normalized to actin and is shown relative to the level in control shRNA C2C12 cells at day 6.

Calvarial cells were collected in 1 ml of Trizol reagent. Total RNA was isolated and treated with DNAseI for 15 min and purified with columns (Zymogen). RNA (1 μg) was reverse transcribed with random primers. Resulting cDNA was diluted 75 times, and 5 μl was used in 25 μl qPCR reaction with Power SYBR Green PCR Master Mix (Applied Biosystems) and 0.5 pmol/μl of each of the primers: for mouse Runx2, forward: CGA CAG TCC CAA CTT CCT GT, reverse: CGG TAA CCA CAG TCC CAT CT; for mouse alkaline phosphatase, forward: TTG TGC GAG AGA AAG AGA GAG A, reverse: GTT TCA GGG CAT TTT TCA AGG T; mouse Ddx5, forward: CCT CCA GAG GGC TAG ATG TG, reverse: GCG AGC AGT TCT TCC AAT TC. Data were normalized to rodent GAPDH levels. Initial denaturation was made at 95°C for 10 min followed by 40 cycles of 2-step PCR: 95°C denaturation for 15 s and 60°C for 1 min synthesis.

RESULTS

Identification of Runx2-Interaction Proteins by Affinity Purification

Runx2 is an essential protein for osteoblast development and interacts with numerous nuclear proteins to regulate gene expression (reviewed in Schroeder et al. [2005]). In an effort to identify novel binding partners and regulators of Runx2, we embarked on an affinity purification experiment, using LC-MS to identify Runx2-interacting proteins. Runx2 (MRIPV isoform) was fused to a tandem affinity protein (TAP) consisting of protein A and calmodulin binding peptide (CBP; Fig. 1A). The TAP-Runx2 protein was stable and migrated at a predictably slower rate than wild-type Runx2 (Fig. 1B), bound a Runx binding element in double-stranded DNA (Fig. 1C), and activated transcription of a Runx-responsive reporter, p6OSE2, in a concentration-dependent manner (Fig. 1D). Thus, TAP-Runx2 has the same functional properties as non-tagged Runx2.

To purify Runx2 protein complexes, TAP-Runx2 was stably expressed in a rat osteosarcoma cell line, UMR-106. These cells were chosen because they have a short-doubling time and express Runx2; thus, large quantities of protein could be obtained quickly and TAP-Runx2 would be expected to bind important regulatory proteins in a relevant context. TAP-Runx2 was expressed at a higher level than endogenous Runx2 in the selected stable UMR-106 cells (Fig. 2A, bottom panel), giving it a presumed competitive advantage for binding partners, and was specifically collected by IgG-bound sepharose beads (Fig. 2A, top panel). UMR-106 cells stably expressing TAP or TAP-Runx2 were expanded and nuclear lysates were applied to an IgG column to purify TAP proteins (via the Protein A moieties) and any complexes associated with them. Eluates and remaining beads were resolved by SDS–PAGE. Several silver-stained protein bands were present only in the lysates of the TAP-Runx2 cells (Fig. 2B). Proteins identified exclusively in the TAP-Runx2 elute by LC-MS included TAP-Runx2, as expected. Gelsolin, Ski2w, EPLIN, alpha-actinin, and Hsp70 sequences were also identified. Interestingly, one band unique band was present in the TAP-Runx2 sample that was not eluted from the beads by acetic acid presumably because the interaction was strong enough to

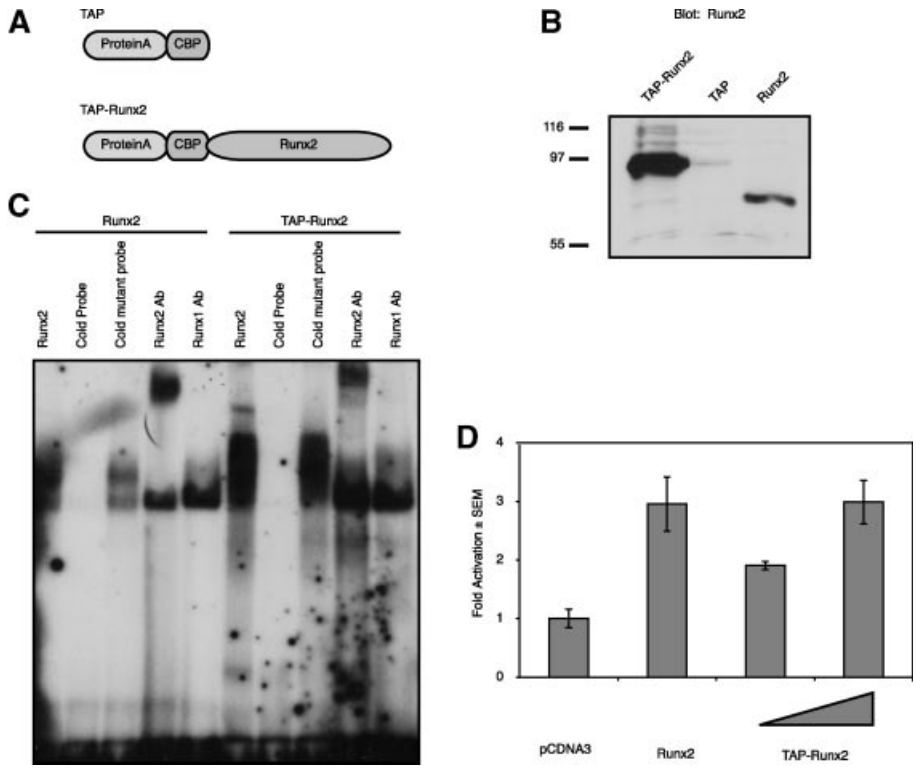

Fig. 1. TAP-Runx2 retains the functional activities of Runx2. **A**: Depiction of the TAP-Runx2 fusion protein. **B**: Protein lysates from UMR-106 cells stably expressing either wild-type Runx2, TAP or TAP-Runx2 were immunoblotted with Runx2 antibodies. **C**: TAP-Runx2 binds to Runx binding sites. Nuclear lysates from COS cells transfected with Runx2 or TAP-Runx2 expression plasmids were incubated with a ^{32}P-labeled Runx2 DNA probe. For competition experiments, 10-fold excess unlabeled wild-type probe (''cold probe'') or mutant probe (''cold mutant probe'') were preincubated with the COS lysates prior to addition of the labeled probe. For supershift experiments, polyclonal antisera against either Runx2 or Runx1 were added to the reaction. **D**: TAP-Runx2 activates transcription similarly to wild-type Runx2. C3H10T1/2 cells were transiently transfected with 200 ng of the p6OSE2-luc reporter, 10 ng of pRL-TK, and either pCMV-Runx2 (500 ng) or pCMV-TAP-Runx2 (500 ng or 1 μg). pcDNA3.1 was added to maintain uniform amount of DNA per transfection. Luciferase activities were measured 48-h after transfection.

resist elution (Fig. 2C). This band was identified as p68 (Ddx5).

p68 Interacts With Runx2

We attempted to verify the interactions by a number of methods. In this report we describe the assays for p68. We confirmed the LC-MS identification of p68 as a Runx2 interacting protein by immunoblotting TAP-Runx2 affinity purified complexes with an antibody that recognizes p68, but not other Ddx proteins (Fig. 3A). p68 is a RNA helicase and co-activator for many proteins; however, its interaction with Runx2 was not dependent on either RNA or DNA (Fig. 3B). Immunofluorescence experiments revealed that p68 and Runx2 co-localize in subnuclear foci. In ROS17/2.8 osteosarcoma cells, a fraction of endogenous Runx2 foci also contained p68 (Fig. 3C). When Runx2 and p68 are overexpressed in cells, nearly all Runx2 foci colocalized with p68 foci (Fig. 3D). GST pull-down assays using in vitro transcribed and translated proteins and *E. coli* purified proteins demonstrated direct interactions between the amino-terminus of Runx2 and the carboxy-terminus of p68 (Fig. 3E,F). These data indicate that p68 physically binds to and colocalizes with Runx2.

p68 is a Runx2 Co-Activator

To determine if p68 might modify Runx2's transcriptional activity, we performed chromatin immunoprecipitation assays and found that p68 bound to a region of the osteocalcin promoter that contains an essential Runx binding element (Fig. 4A). HA and FLAG-tagged p68 proteins did not activate a Runx2 responsive promoter (p6OSE2) on their own, but synergistically augmented Runx2-dependent activation (Fig. 4B,C). A p68 mutant, NEAD, in which

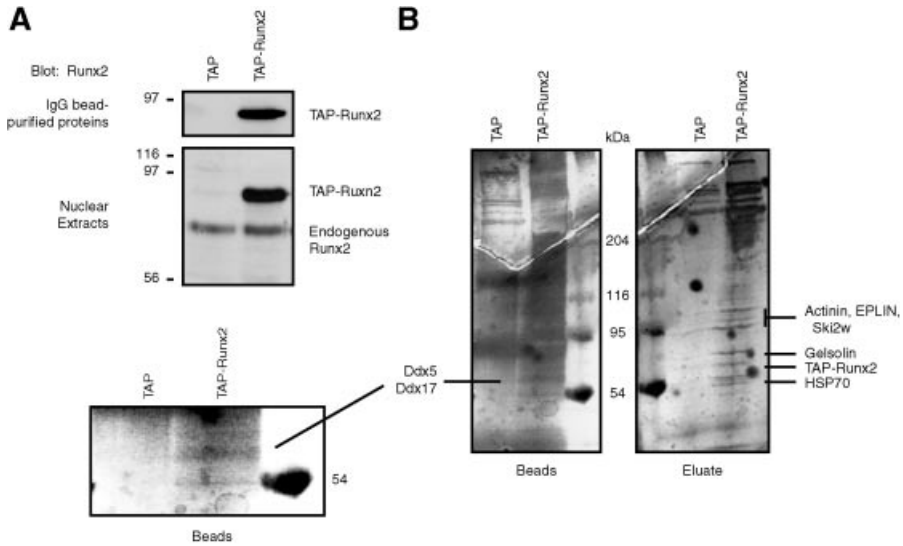

Fig. 2. Affinity purification of Runx2 interacting proteins. **A**: Immunoblots of TAP-Runx2 in nuclear extracts from UMR-106 stably expressing TAP or TAP-Runx2 (**bottom panel**) and in complexes collected by IgG beads from the same cells (**top panel**). **B**: A gel containing proteins from UMR-TAP or -TAP-Runx2 cells that bound tightly to IgG beads (**left panel**) or that were present in eluted fractions (**right panel**) was silver-stained. The indicated bands were excised and subjected to LC/MS. The predicted identities of the proteins are indicated. **C**: An expanded image of the area in the left panel of part (B) containing the band identified as p68.

the conserved helicase motif, DEAD, is inactivated also cooperated with Runx2 (Fig. 4C). These data indicate that p68 is a co-activator for Runx2 and that the helicase activity is dispensable for this transcriptional effect. Runx2 did not appear to modify the helicase activities of p68 (data not shown). To determine if p68 is required for Runx2 activity, we assayed Runx2's transcriptional abilities in C2C12 cells expressing shRNAs that target a common region in p68 and p72. These cells are not devoid of p68 and p72, but contain only about half the normal levels (Caretti et al. [2006] and data not shown). Runx2 activated the p6OSE2 reporter in a concentration-dependent manner in both the control shRNA- and p68/p72 shRNA-expressing cells; however, Runx2 activity was approximately 15–40% less in the p68/p72-suppressed cells (Fig. 4D).

p68 Suppression Accelerates Osteoblast Maturation

Because Runx2 is essential for osteoblast development and plays crucial roles in osteoblast differentiation, the effects of p68 suppression on osteoblast maturation were determined. C2C12 cells are myocyte and osteoblast progenitors. In the presence of low serum, they differentiate into myocytes, but shRNAs targeting p68 and p72 attenuates this response [Caretti et al., 2006]. In the presence of BMP2, C2C12 cells acquire osteoblast properties but fail to express myogenic genes [Katagiri et al.,

Fig. 3. Verification of the physical association between Runx2 and p68. **A**: UMR 106 cells stably expressing TAP or TAP-Runx2 were lysed, incubated overnight with IgG-sepharose beads. After three washes, the bound proteins (TAP-purified, **upper panel**) and a saved fraction of the whole cell lysate (**lower panel**) were resolved by SDS–PAGE and immunoblotted with p68 antibodies (PAb204). **B**: The interaction between Runx2 and p68 does not depend on RNA or DNA. UMR-106 cells expressing TAP or TAP-Runx2 were lysed, treated with RNase A or DNase I for 30 min as indicated and affinity-purified by binding to IgG-sepharose beads. Bound materials were resolved by SDS–PAGE and immunoblotted with p68 antibodies. **C**,**D**: p68 and Runx2 co-localize in the nucleus. C: The localization of Runx2 and p68 in ROS17/2.8 cells were examined by immunofluorescence using antibodies against endogenous p68 (red, center) and Runx2 (green, right). Foci of colocalization (yellow, left) were detected in the nucleus. D: C2C12 cells were transiently transfected with p68-GAL4 and Runx2 expression plasmids and incubated with antibodies for GAL4 (green, center) and Runx2 (red, right). Foci of co-localization (yellow, left) were detected in the nucleus. **E**: Binding of p68-GAL fusion proteins to GST-Runx2. ^{35}S-labeled in vitro synthesized p68 proteins (full-length, top; 1-477, middle; or 478-614, bottom) were incubated with GST-Runx2 fusion proteins encoding amino acids 1–383 or 383–513 of Runx2. **F**: Binding of Runx2 to GST-p68 proteins. Assays were performed as in (A) using in vitro synthesized Runx2 (MRIPV isoform) and GST-p68 fusion proteins.

1994]. Under this osteogenic condition, shRNA-mediated suppression of p68 and p72 augmented and accelerated the production of osteoblast markers, alkaline phosphatase (Fig. 5A) and osteocalcin (Fig. 5B). These data suggest that p68 and p72 contribute to the myogenic commitment of these progenitor cells and inhibit their osteoblast differentiation.

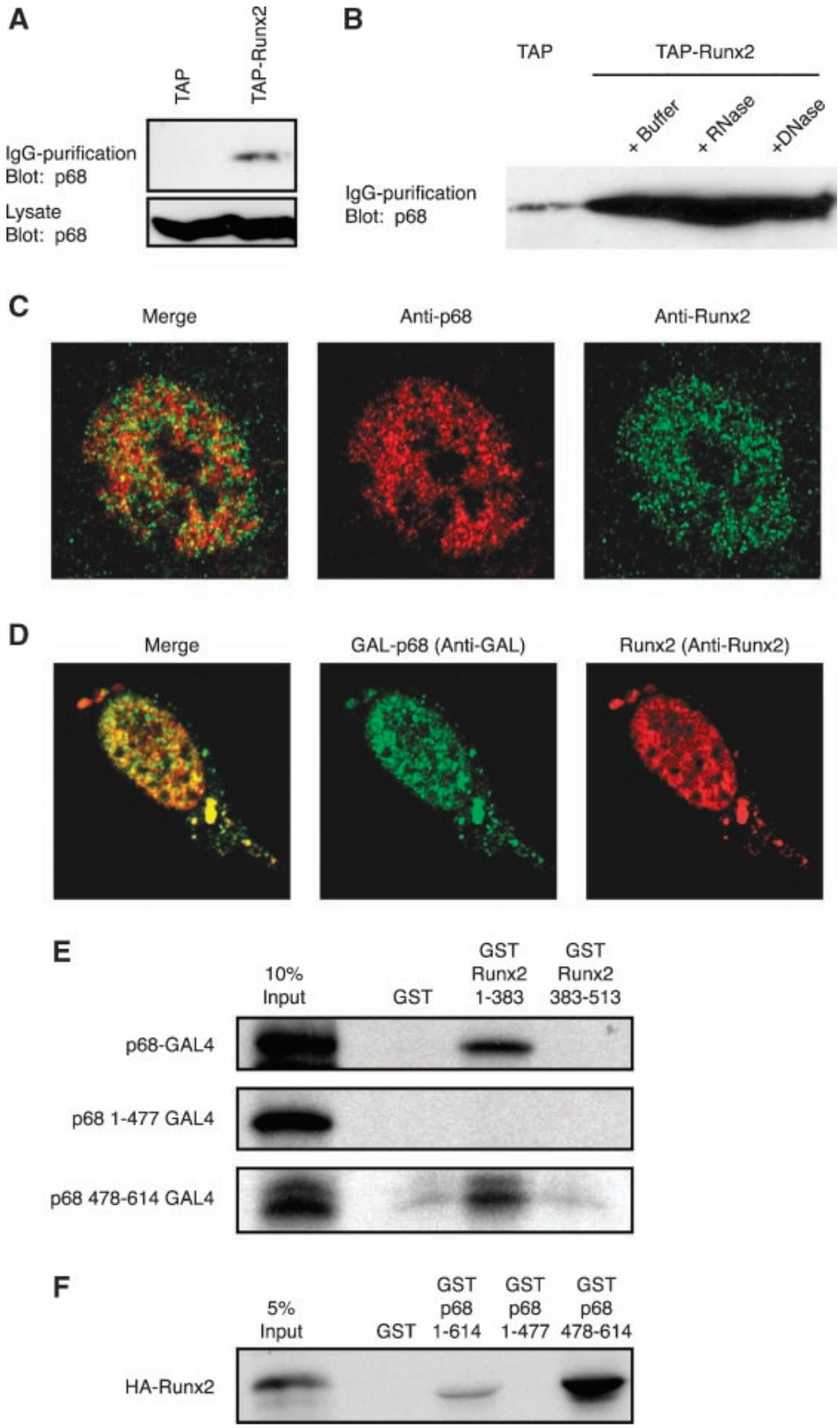

Fig. 3.

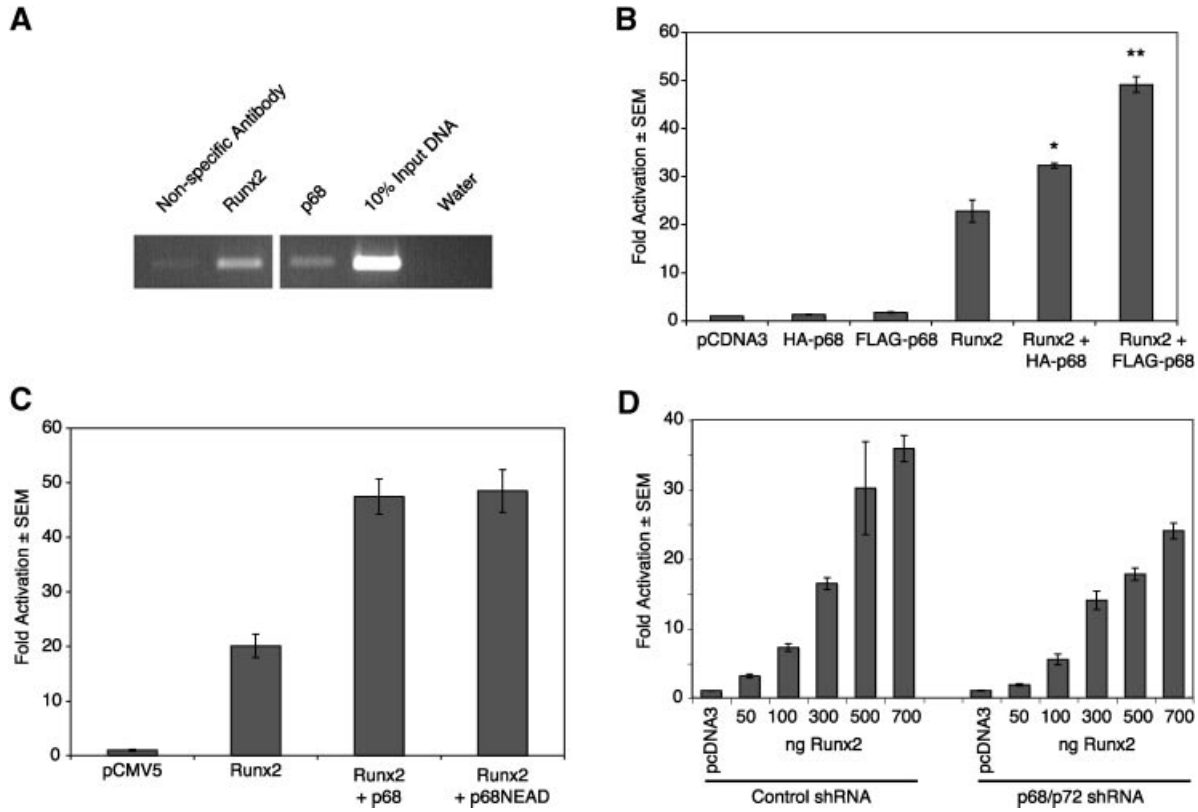

Fig. 4. p68 regulates Runx2 transcriptional activity. **A**: p68 associated with a region of the osteocalcin promoter that contains a Runx binding element in a chromatin immunoprecipitation assay. **B**: p68 is a Runx2 co-activator. C2C12 cells were transfected with the p6OSE2-luc and pRL-null reporters, 150 ng of Runx2 and 450 ng of p68 expression plasmids and pcDNA3.1 to maintain uniform amount of plasmid in each transfection. Each transfection was performed in triplicate. Luciferase readings were taken at 24 h post-transfection and are shown as fold-activation relative to pCDNA3 alone. $*P \leq 0.02$; $**P \leq 0.01$. **C**: The p68-helicase activity is not required for activation of Runx2. C2C12 cells were transfected with p6OSE2-luc and pRL-TK reporters and 500 ng expression plasmids for Runx2, p68 and p68NEAD. Luciferase readings were taken 48 h after transfection as described in (B). **D**: Runx2 transcriptional activation is reduced in p68/p72 suppressed cells. C2C12 cells expressing shRNA against p68/p72 or a control shRNA were transfected as in (B) with varying amounts of Runx2 expression plasmid.

p68 expression is inhibited by Runx2 in calvarial osteoblasts. Because p68/p72 inhibits osteogenic differentiation of progenitor cells and Runx2 promotes osteoblast maturation, we investigated whether Runx2 can modulate p68 expression during differentiation. p68 mRNA levels in primary mouse calvarial cells derived from wild-type mice or mice with a homozygous *Runx2* null mutation were examined. Quantitative PCR analysis revealed that p68 expression was significantly higher in primary calvarial cells from *Runx2* null mice (from 17.5 dpc embryos) compared to cells from the corresponding wild-type mice (Fig. 6A). For comparison, levels of GAPDH were similar, while the levels of alkaline phosphatase were fourfold lower in primary Runx2 null cells. Thus, *Runx2* deficiency enhanced p68 expression. We then performed complementation assays with immortalized *Runx2* null calvarial cells that were infected with adenovirus vector expressing wild type Runx2 protein. Infected cells were cultured up to 4 days in proliferation or differentiation medium (i.e., containing ascorbic acid and β-glycerophosphate) with or without BMP2 (Fig. 6B). As expected, Runx2 or BMP2 strongly enhanced alkaline phosphatase levels under all conditions (Fig. 6B, lower panels). In contrast, re-introduction of Runx2 into *Runx2*-deficient calvarial cells under proliferating conditions transiently suppressed p68 mRNA levels by approximately 40% on day 1 (Fig. 6B, top left). We observed a similar 1.8-fold reduction in p68 mRNA levels in response to Runx2 expression with Affymetrix gene micro-arrays, but p72 (Ddx17) levels were not significantly altered (Nadiya Teplyuk, Gary S. Stein, and Andre van Wijnen, unpublished

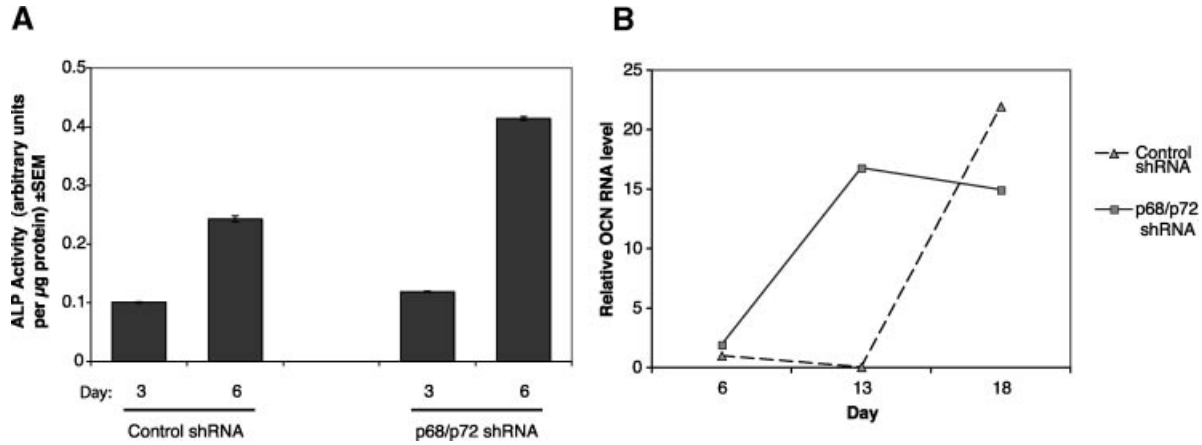

Fig. 5. Suppression of p68 accelerates osteoblast maturation. **A**: Alkaline phosphatase activity is enhanced in p68-suppressed cells. Control and p68-suppressed C2C12 cells were differentiated in growth medium supplemented with 300 ng/ml BMP2, ascorbic acid and β-glycerol-phosphate. Alkaline phosphatase activity was measured after three and 6 days. **B**: Osteocalcin expression is accelerated in p68-suppressed C2C12 cells. Cells were grown in differentiation media as in (A). RNA was harvested by Trizol at the indicated timepoints. Real-time reverse-transcriptase PCR was performed with primers for actin and osteocalcin. For each timepoint, osteocalcin expression was normalized to actin with the comparative threshold method and is shown relative to the level in control shRNA C2C12 cells at day 6.

work). Consistent with these findings, p68 expression was inhibited (~25% reduction) by Runx2 under differentiation conditions on day 4, and this inhibition was more pronounced (~40% reduction) upon treatment with BMP2 (Fig. 6B, top right). Hence, expression of Runx2 is inhibitory for p68, independent of whether cells differentiate.

To confirm that these changes in p68 mRNA levels are reflected in changes in p68 protein levels, we performed immunoblot analysis with protein isolated 1 day after Runx2 re-introduction and under proliferating conditions when we observe the most pronounced effects on p68 mRNA levels. The results showed that expression of wild type Runx2 reduced p68 protein levels approximately twofold (Fig. 6C). Runx2's effects on p68 protein (Fig. 6C) were quantitatively similar to the observed changes in p68 mRNA levels (Fig. 6A,B). Inhibition of p68 protein levels was not seen in cells expressing GFP from the empty adenovirus vector, or in cells expressing a carboxy-terminally truncated Runx2 protein that is defective in suppressing osteoblast proliferation [Pratap et al., 2003; Galindo et al., 2005] and in promoting osteoblast differentiation [Choi et al., 2001].

DISCUSSION

Runx2 is essential for osteoblast development from multipotent mesenchymal progenitors and it contributes to the expression of osteoblast genes throughout lineage maturation. In this study, we identified a novel Runx2-interacting protein, p68, which enhances Runx2 transcriptional activity on a Runx2 responsive reporter. Interestingly, modest suppression of p68 and its closely related protein, p72, accelerated osteoblast differentiation of a myo-osteoprogenitor cell line. The promotion of osteoblast maturation was not anticipated after finding that p68 augments Runx2 activity. However, these results are consistent with the finding that p68/p72 suppression blocks myocyte differentiation [Caretti et al., 2006]. These data support the notion that p68 has an important role in mesenchymal progenitor cell specification into myocytes, perhaps by interacting with MyoD and the noncoding RNA SRA [Caretti et al., 2006], but that it is less essential for osteoblast specification. Runx2 regulation of p68 levels in primary calvarial cells supports this hypothesis. This is not inconsistent with p68's ability to augment Runx2 transcriptional activity, particularly during the maturation of committed osteoblasts or when Runx2 is overexpressed in progenitors (as it is in these studies). The Runx2 and p68 complex might also be important in tumor progression because both factors are reported to be expressed at elevated levels in human cancers [Causevic et al., 2001; Brubaker et al., 2003; Shore, 2005; Dijkman et al., 2006; Lau et al., 2006; Nagaraja et al., 2006]. p68 might also affect other transcription factors

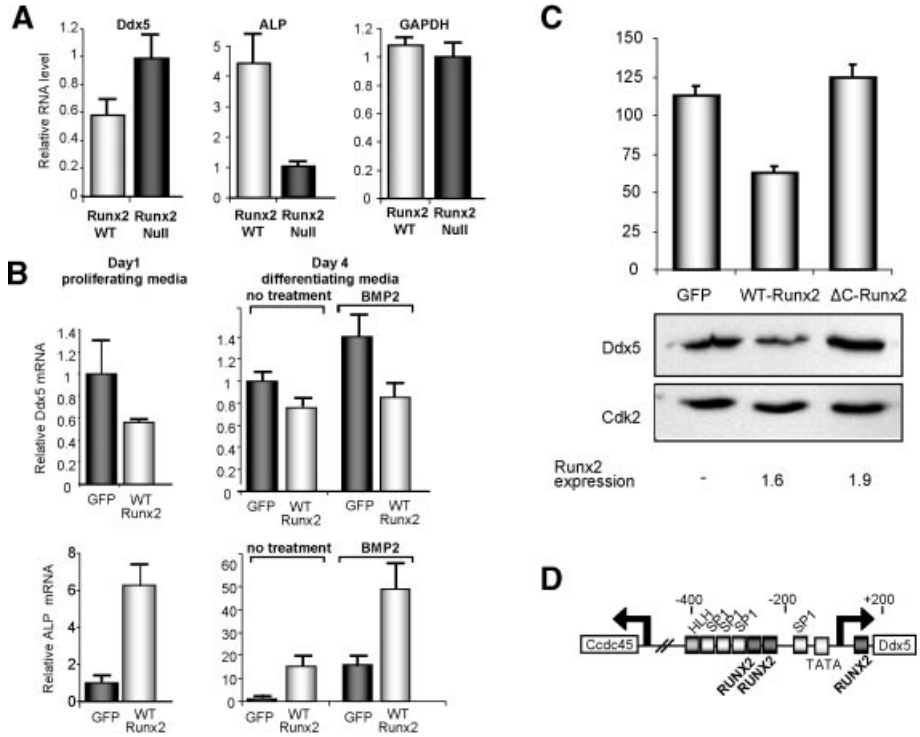

Fig. 6. Runx2 downregulates p68 (Ddx5) expression in osteoblast progenitors. **A**: RNA was isolated from primary calvaria cells (passage #3) from wild-type and *Runx2* null 17.5 dpc mouse embryos. p68 (Ddx5), GAPDH and alkaline phosphatase RNA levels were analyzed by qPCR. **B**: *Runx2* null mouse calvaria cells were infected with adenovirus expressing Runx2 protein or GFP alone (empty adenovirus vector). Cells were maintained in proliferating or osteogenic medium. Fresh medium was added 24 h after infection. Cells were collected 1 or 4 days after infection as indicated. p68, Runx2 and alkaline phosphatase RNA levels were analyzed by qPCR. **C**: Cells were infected with Runx2 and collected 24 h after infection in proliferating conditions. p68 and CDK2 protein levels were analyzed immunoblotting (**bottom**) and signals were quantified using ImageJ. Relative exogenous expression of wild-type and mutant Runx2 protein is numerically shown at the bottom of the figure. **D**: In silico analysis of the Ddx5 promoter reveals three Runx2 motifs near elements previously described for this gene (e.g., TATA box, Myc/HLH site, SP1 sites) [Rossler et al., 2000].

(e.g., estrogen receptors) that contribute to optimal osteoblast function.

In silico promoter analysis indicates that there are three Runx2 motifs near the TATA box of the p68 gene, one of which is a putative high affinity 5′ TGCGGTT element located in the 5′UTR (Fig. 6D). The p68 5′ regulatory region is part of an approximately 1 kb divergently transcribed promoter shared with the CCDC45 gene and this region contains a TATA box, as well as at least one Myc/HLH site and four SP1 sites [Rossler et al., 2000]. The location of the Runx2 motifs in the p68 promoter permits postulation of a suppressive transcriptional mechanism that would not perturb expression of the upstream CCDC45 gene. Taken together, our data show that expression of p68 mRNA and protein is inversely related to modulations in Runx2 levels in osteoblast progenitors, is independent of Runx2 effects on cell proliferation or differentiation, and may be transcriptionally mediated through one or more Runx2 motifs in the p68 promoter.

The identification of p68 as a novel Runx2-interacting protein and co-activator increases our understanding of how Runx2 integrates co-factor complexes within the nuclear matrix. Both Runx2 and p68 are nuclear matrix-associated proteins [Zeng et al., 1998; Akileswaran et al., 2001]. Nuclear matrix association of Runx2 is essential for osteoblast maturation [Choi et al., 2001]. The nuclear matrix-targeting signal of Runx2 is located in its carboxy-terminus and is not required for interactions with p68 (Fig. 3E). Runx2 and p68 colocalize in distinct subnuclear foci (Fig. 3C,D). As would be expected, more co-localization is detected when the proteins are overexpressed (Fig. 3D); however, there is discernable co-localization of the endogenous proteins as well (Fig. 3C). Thus, Runx2 and p68 interact in vivo, but they are not obligate partners. Runx2 transcriptional

activity on a transiently transfected reporter is enhanced by p68 and p68 associates with chromatin containing the osteocalcin promoter in vivo (Fig. 4A), but the effects of p68 on Runx2 activity and vice-versa on gene expression within the nuclear matrix requires further investigation. Preliminary experiments indicate that Runx2 does not affect the helicase activity of p68 in vitro (FVF, data not shown). Both Runx2 and p68 interact with the transcriptional co-activators, CBP and p300 [Rossow and Janknecht, 2003; Sierra et al., 2003; Warner et al., 2004]. It is possible that these three proteins are components of a co-activator complex that maximizes Runx2 activity on certain promoters.

The mechanism whereby p68 enhances Runx2 activity is not known. The observation that the DEAD box is dispensable for p68 cooperation with Runx2 indicates that the helicase activity is not involved in promoting Runx2-dependent gene expression; however, we cannot exclude the possibility that p68-NEAD forms a complex with endogenous p68 or p72, whose helicase activity may contribute to transcription regulation. Similar observations were noted for p53 [Bates et al., 2005]. p68 has many molecular roles in RNA processing and transcription. It is cyclically recruited to estrogen responsive genes following ligand induction [Metivier et al., 2003] and directs assembly of the transcriptome and chromatin remodeling complexes in muscle [Caretti et al., 2006]. p68 interacts with a variety of transcription factors, co-repressors and co-activators [Fuller-Pace, 2006]. The inclusion of Runx2 in this list of p68 binding factors in hindsight is not surprising given the important roles that both protein play in regulating gene expression.

The other proteins identified in this screen for Runx2-interacting factors are under further investigation to verify their associations and to determine their effects on Runx2 transcriptional activity. Ski2w was of initial interest because, like p68, it is a putative helicase [Dangel et al., 1995]; however, little has been done to study it role in mammalian cells. Its association with Runx2 could not be independently verified because overexpressed Ski2w proteins localized only in the cytoplasm. It is possible that alternative isoforms interact with Runx2 in vivo. Thus a limitation of this proteomic analysis is that it cannot discriminate between all protein isoforms. Gelsolin and alpha-actinin are best known for their roles in regulation cytoskeletal organization; although they are also present at comparatively low levels in the nucleus and as part of transcriptional complexes [Archer et al., 2005; Chakraborty et al., 2006; Jasavala et al., 2007]. In preliminary experiments, gelsolin modestly augmented Runx2 transcriptional activity (data not shown). The identification of these proteins in this screen might reflect their relative abundance in the cell and indicates their contributions to organizing Runx2 complexes within the nucleus. We did not identify any known Runx2 interacting proteins, such as transcription factors or histone deacetylases. This is likely due to the transience and context-dependence of those interactions.

In summary, a proteomic screen was undertaken to identify novel Runx2 interaction proteins. We uncovered several abundant proteins that have transcriptional and organization roles in cell nuclei. A known transcriptional activator, p68, was determined to be a novel co-activator for Runx2. Interestingly, p68 suppression accelerated BMP-2 induced differentiation of osteo-myocyte progenitors into the osseous lineage and Runx2 repressed p68 in calvarial cells. These studies indicate that p68 has multiple roles in osteoblasts and regulates lineage commitment.

ACKNOWLEDGMENTS

The authors thank Xiaodong Li for technical assistance, Dr. Ralf Janknecht for providing p68 constructs and Kaleem Zaidi, Yang Lou, Janet Stein and Jane Lian for stimulating discussions and/or reagents. This work was supported by RO1 grants AR48147 (JJW), AR050074 (JJW), AR49069 (AJvW) and AR48818 (GS) from the National Institutes of Health (NIH), an NIH institutional training grant AR050938 in Musculoskeletal Research (to support EDJ), the Intramural Research Program of the National Institute of Arthritis, Musculoskeletal, and Skin Diseases of the NIH (GC and VS), and the Association for International Cancer Research and Tenovus, Scotland (SMN, FVF-P).

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