MINT, the Msx2 Interacting Nuclear Matrix Target, Enhances Runx2-dependent Activation of the Osteocalcin Fibroblast Growth Factor Response Element*

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Msx2 promotes osteogenic lineage allocation from mesenchymal progenitors but inhibits terminal differentiation demarcated by osteocalcin (OC) gene expression. Msx2 inhibits OC expression by targeting the fibroblast growth factor responsive element (OCFRE), a 42-bp DNA domain in the OC gene bound by the Msx2 interacting nuclear target protein (MINT) and Runx2/Cbfa1. To better understand Msx2 regulation of the OCFRE, we have studied functional interactions between MINT and Runx2, a master regulator of osteoblast differentiation. In MC3T3E1 osteoblasts (with endogenous Runx2 and FGFR2), MINT augments transcription driven by the OCFRE that is further enhanced by FGF2 treatment. OCFRE regulation can be reconstituted in the naive CV1 fibroblast cell background. In CV1 cells, MINT synergizes with Runx2 to enhance OCFRE activity in the presence of activated FGFR2. The RNA recognition motif domain of MINT (which binds the OCFRE) is required. Runx2 structural studies reveal that synergy with MINT uniquely requires Runx2 activation domain 3. In confocal immunofluorescence microscopy, MINT adopts a reticular nuclear matrix distribution that overlaps transcriptionally active osteoblast chromatin, extensively co-localizing with the phosphorylated RNA polymerase II meshwork. MINT only partially co-localizes with Runx2; however, co-localization is enhanced 2.5-fold by FGF2 stimulation. Msx2 abrogates Runx2-MINT OCFRE activation, and MINT-directed RNA interference reduces endogenous OC expression. In chromatin immunoprecipitation assays, Msx2 selectively inhibits Runx2 binding to OC chromatin. Thus, MINT enhances Runx2 activation of multiprotein complexes assembled by the OCFRE. Msx2 targets this complex as a mechanism of transcriptional inhibition. In osteoblasts, MINT may serve as a nuclear matrix platform that organizes and integrates osteogenic transcriptional responses.

Bone formation arises via two overlapping yet distinct mechanisms (1). Endochondral ossification occurs via the calcification and vascularization of an initial cartilaginous template, best exemplified by long bone development and fracture repair. Non-endochondral ossification occurs during development in the flat bones of the skull, in teeth, and in the lateral portion of the clavicles. Direct bone deposition occurs in type I collagen-based extracellular matrix; no cartilage template precedes bone deposition. Both in vivo and in cell culture models, a characteristic gene expression program is elaborated as osteoblasts differentiate and mature from osteoprogenitors (2–4). Although transcription factors crucial to bone development (Runx2/Cbfa1, CBF-β, Msx2, Msx1, Dlx5, Akr4, Osx, and Sox9) and metabolic/endocrine regulation (multiple nuclear receptors, Runx2/Cbfa1) have been identified, our understanding of the molecular details of stage-specific osteoblast gene expression is rudimentary (5). “Cross-talk” between these factors occurs in the osteoblast nucleus; an integrative model is lacking and sorely needed to better address unmet clinical needs in metabolic bone disease, craniofacial dysmorphias, and heterotopic ossification (6, 7).

The osteoblast homeodomain protein Msx2 plays an important role in bone formation and the temporospatial timing of osteoblast differentiation (8). Maas and colleagues have disrupted the mouse Msx2 gene by homologous recombination and identified severe deficiencies in both intramembranous and endochondral bone formation (9). Indeed, in the simultaneous absence of Msx2 and Msx1, a highly related and partially redundant Msx gene family member, craniofacial skeletal development is completely abrogated (9). In humans, Wilkie and colleagues demonstrated that haploinsufficiency for Msx2 function results in substantial calvarial mineralization defects; this manifests either as hereditary parietal foramina (10) or as part of a cleidocranial dysplasia syndrome (11). Conversely, Msx2(P148H), a gain-of-function variant of Msx2, causes craniosynostosis, characterized by the precocious mineralization and differentiation of calvarial osteoblasts in cranial sutures. Recent studies in our lab have shown that Msx2 can promote the osteogenic differentiation of vascular progenitors, promoting osteoblast lineage allocation while inhibiting adipogenic potential (12). In this setting, the craniosynostosis variant Msx2(P148H) is much more active than wild-type Msx2 in promoting osteogenic differentiation in synergy with bone morphogenetic protein 2 (12). However, Msx2 also clearly functions to delay the timing of osteoblast terminal differentiation. This is best exemplified by Msx2-dependent suppression of the os-

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teocalcin (OC) gene in teeth and calvarial osteoblasts (13–16). During odontogenesis, where stage-specific gene expression programs are spatially resolved, Msx2 and OC are reciprocally expressed (14). Consistent with this, stable expression of Msx2 in MC3T3E1 calvarial osteoblasts suppresses OC, but not osteopontin (OPN), gene expression (14). Unlike recent observations demonstrating that activation of early osteogenic differentiation (e.g., alkaline phosphate expression) is proportional to Msx2 DNA binding activity (12), suppression of the late phenotypic marker OC is independent of intrinsic Msx2 DNA binding (15). Inhibitory protein-protein interactions occur in part between Msx2 and DNA-protein complex assembled by the OC promoter region −154 to −113 (16–18). Because this element also mediates transcriptional responses to activated FGFR1/FGFR2 receptor tyrosine kinase, we called this region the OCFRE (18–20). The OCFRE is a 42-bp DNA domain that encompasses recognition cognates for Runx2 and a novel Kv-antigen transcription factor complex (18). It is noteworthy that this GT-rich duplex DNA is also recognized by MINT, a large ~400-kDa nuclear matrix protein that we first identified by expression cloning using radiolabeled Msx2 (21). MINT is a nuclear DNA- and RNA- binding protein highly expressed in central nervous system, lymphoid tissue, cardiac tissue, and calvarial osteoblasts (21, 23). GT-rich cognate recognition is conferred by the three N-terminal RRM domains of MINT (21). MINT is highly homologous to Drosoiphilan spen, a transcriptional co-regulatory protein genetically identified as regulating fly head development in concert with Hox homeodomain proteins (24) and receptor tyrosine kinase signaling cascades (25). Thus, biochemical and genetic studies place MINT in regulatory pathways under the control of homeodomain transcription factors.

Given the above, we sought to characterize the biochemical relationships between MINT, Msx2, and FGFR2-responsive components of the OCFRE. MINT enhances transcription driven by FGFR2 from the OCFRE in MC3T3E1 osteoblasts that possess endogenous Runx2. We identify that Runx2 possesses the FGFR2-activated transactivation function (TAF) in this complex. In the naive CV-1 cell background, we reconstitute FGFR2 and Runx2-dependent activation of the OCFRE. MINT and Runx2 act synergistically to enhance OCFRE activity, but only in the presence of activated FGFR2. This activation is dependent upon Runx2 activation domain 3 (AD3) and the MINT RRM domains. By contrast, Msx2 suppresses OCFRE activation by Runx2 and MINT, and chromatin immunoprecipitation assays (ChIP) assays reveal that Msx2 displaces Runx2 from OC, but not OPN, chromatin. In confocal microscopy experiments, MINT localizes with phosphorylated RNA Pol II (RNAP IIo), indicating that MINT associates with transcriptionally active chromatin complexes. The limited overlap between MINT and Runx2 is enhanced 2.5-fold by FGFR2 stimulation, providing additional evidence that FGFR signaling regulates Runx2-dependent transcription. Thus, MINT enhances Runx2 and FGFR2 activation of multiprotein complexes assembled by the OCFRE. Msx2 targets this complex as a mechanism of selective transcriptional inhibition. In osteoblasts, MINT may serve as a nuclear matrix platform that organizes and integrates osteogenic transcriptional responses.

**EXPERIMENTAL PROCEDURES**

**Plasmid and Eukaryotic Expression Constructs—**One-hybrid experiments were carried out using pFA-CMV to generate fusion proteins with the yeast Gal4 DNA binding domain and pFR-LUC as the Gal4-responsive reporter (Strategene, La Jolla, CA). Fragments for murine Runx2 (isoform II), murine Ku70 (GenBank accession number P12956), murine Ku80 (GenBank accession number P13010), and human Tbdn100 (GenBank accession number AY112670) were generated and subcloned into Broad Genomics pFA-CMV vector constructs open reading frames downstream of the Gal4 DNA binding domain in pFA-CMV. All eukaryotic Runx2 and Msx2 expression plasmids were prepared in pcDNA3 using techniques described previously (16). Isoform II of murine Runx2 (528 aa, MAS N...; GenBank accession number AF005926) was used to reconstitute Runx2 signaling in naive CV-1 cells. The following C-terminal truncation variants were prepared from Runx2 isoform II: Runx2 (1–142 aa) to remove the Runx2 C-terminal WVRPY repression domain; Runx2 (1–392) to remove the nuclear matrix targeting signal (NMTS); Runx2 (1–312) to remove Activation Domain 3 (AD3) and distal C-terminal sequences, and an internal deletion lacking AD3 in the context of full length Runx2–Runx2 (1–392) [Genebank accession number H11002]. To probe for the cell autonomous FGFR2 stimulation, a plasmid coding for constitutively active FGFR2 Receptor FGFR2-ROS (26) was used in co-transfection experiments. The construction and characterization of OCFRE-LUC has been detailed previously (18).

The full-length mouse MINT cDNA was cloned by long PCR of reverse-transcribed mRNA from MC3T3E1 calvarial osteoblasts cells (21). In brief, 2 μg of total RNA isolated from logarithmically growing MC3T3E1 cells was used in reverse transcription with Oligo(dT) primer and 200 units of Superscript II RT (Invitrogen). The reaction was carried out at 42 °C for 50 min, then diluted 5-fold with nuclease-free water. For PCR, 5 μL of the diluted RT reactions was used for PCR cloning of full-length MINT transcripts. The amplification primer set 5′-CAT GGA GAA GAT GAC AAA ATT AAG AAT CCA TGG GAG ACC GAG ACC TCC GCA-3 and 5′-GCA TGC GGC CGC TCA CAC CGA GGC AAT GAC AAT CAT-3′ PCR reactions were carried out in 50 μL containing 3.5 units of Expand high fidelity Roche polymerase (Roche Diagnostics). DNA was denatured for 3 min at 94 °C, then biphasic PCR carried out for 25 cycles (15 s at 94 °C, 1 min at 68 °C) to obtain a ~11-kb amplicon that was subcloned into pGEM (Promega, Madison, WI). DNA sequencing and in vitro transcription-translation (TNT; Promega) of the subcloned fragment was used to verify expression of full-length MINT protein. The pGEM-MINT plasmid was digested with Sall and NotI, and the full-length MINT cDNA was ligated into pCMV-hemagglutinin tag vector (BD Biosciences Clontech) to make CMV-MINT(1–3576), denoted CMV-MINT or CMV-MINT(1–3576). A similar strategy was used to make the variant CMV-MINT(734–3576), denoted CMV-MINT(ARM) or MINT-ARM. This variant construct retains the MINT mid region and C-terminal sequence downstream of an initiator Met, retains a subset of the nuclear localization cognates, and localizes to the nuclearus (data not shown) but completely lacks intact N-terminal RRM domains (21). For fluorescence imaging of Runx2 and Msx2, full-length cDNAs were cloned in a CMV-driven plasmid downstream of the EGFP coding sequence (Living Colors EGFP-C1 plasmid; BD Biosciences Clontech) to create EGFP-Runx2 and EGFP-Msx2, respectively.

**Cell Lines and Transfection—**CV-1 fibroblasts and MC3T3E1 calvarial osteoblasts were grown as described previously (19). Transfection by lipofection and luciferase reporter assays were carried out as detailed previously (18), but using 24-well cluster dishes in lieu of 12-well cluster dishes. When indicated, treatment with 10 ng/ml FGF2 (human basic FGF2; BD Biosciences) was initiated 24 h before harvesting cells for luciferase activity. For confocal imaging, MC3T3E1 cells were plated in sterile four-chamber slides (Lab-Tek II; Nalge Nunc, Naperville, IL) and transfected the next day with 0.2 μg/well EGFP-Runx2 and 0.8 μg/well CMV-MINT plasmids using Superfect Reagent (Qiagen, Valencia, CA). After 3 h, transfection mix was removed, cells were rinsed with Dulbecco’s modified Eagle’s medium, and added fresh 1% fetal calf serum in Dulbecco’s modified Eagle’s medium. After 24 h, medium was changed again and half of the wells were treated with 10 ng/ml recombinant human RANKL (100 ng/ml) or vehicle control. All cell culture serum in Dulbecco’s modified Eagle’s medium and incubated for 18 h. Immunostaining—Cells were fixed in 4% paraformaldehyde for 30 min and treated with ammonium chloride to neutralize unspecific cross-linking. Subsequently, cells were permeabilized with 0.1% Triton X-100 in
PBS for 20 min and blocked with 1% bovine serum albumin in PBS for an additional 30 min. The rabbit anti-MINT “R16” primary antibody against the central Msx2 interacting domain (21) was then added at a 1:100 dilution in 1% bovine serum albumin-PBS for 1-4 h at room temperature, mixing in an orbital shaker. Cells were washed three times for 15 min each with 0.1% Triton X-100 in PBS. Secondary Cy3-conjugated or Alexa 647-conjugated goat anti-rabbit antibody (1:400) was then added in 1% bovine serum albumin-PBS and incubated for at least 1 h at room temperature in an orbital shaker. Cells were washed three times for 15 min each with 0.1% Triton X-100 in PBS before adding mounting media, placing a 0.15-mm thick coverslip, and sealing with nail polish. Slides were kept in the dark at 4°C until analyzed.

**Confocal Microscopy and Deconvolution Analyses**—Double-stained cells were imaged using a Bio-Rad Radiance 2000 confocal inverted microscope using 60× oil lens (numerical aperture, 1.4); an optimal pinhole aperture was used at all times, and at least three z-stack sections (every 0.48 μm) were acquired per cell. To avoid bleed-through, images were acquired using line-by-line strobe scanning and with lasers whose excitation/emission spectra do not overlap: argon (488 nm) for EGFP and red diode (647 nm) for MINT. For Cy3 imaging, green HeNe laser was used (543 nm). All images were deconvolved using the 'single image constrained iter deconvolution algorithm' at four iterations, with brightness set at 1.5 (Microtome 7.0; VayTek Software, Fairfield, IA). Images were acquired in grayscale, converted to pseudo-color, and merged using Image Pro-Plus Software (Media Cybernetics, Carlsbad, CA). MINT exhibits a reticular pattern, whereas Runx2 exhibits a partially overlapping punctuate pattern. To determine the proportion of Runx2 punctate foci that had also had the signal for MINT, six high quality, deconvolved images were evaluated characterizing several adjacent cells in the field of view. The total numbers of Runx2 foci were digitally counted (n = 491 in cells treated with vehicle; n = 558 in cells treated with FGF2), and two independent observers counted the number of Runx2 foci stained for MINT (scored only if >50% overlap). Data were averaged and presented as the mean ± S.D. percentage of MINT-positive Runx2 foci. The non-parametric Kruskal-Wallis test on raw data sets was used to test statistical significance for differences in the number of Runx2 foci with MINT staining as a function of FGF2 treatment.

**Osteocalcin Immunoprecipitation Assays**—ChIP assays were carried out essentially as described previously (18), implementing a commercially available polyclonal antibody against the invariant C terminus of Runx2 (C-19; Santa Cruz Biotechnology, Santa Cruz, CA). MC3T3E1 cells, which express endogenous Runx2, were stably transfected with either pcDNA3 or pcDNA3-Msx2 as previously detailed. After transfection, cells selected with G418 for 3 days and then processed for chromatin immunoprecipitation essentially as described previously (18). PCR primers used for OC ChIP were 5′-TAT GAG AGT TGG AGG CCA GTT TAT C-3′ and 5′-CCC TAG AGA CAA GAA CCC TAT GCA T-3′ directed to the mouse OC (OG2) proximal promoter (GenBank accession number U56849). As a control, the osteopontin promoter gene was analyzed, because this gene is not affected by Msx2 (14). Osteopontin genomic PCR primers were 5′-CAC AAA ACC AGA AGA GGA AGT GT T-3′ and 5′-GCT CCA GAC AGT CTG CAC A-3′ directed against the murine OPN proximal promoter (GenBank™ accession number AY220127.1). PCR amplifications were observed in ethidium-stained polyacrylamide gels, captured digitally, and displayed as light-dark inverted images. In completely independent experimental sets, MC3T3E1 cells were transduced either with SFG-LacZ (control) or SFG-Msx2 retroviral expression vectors, generated and characterized as reported previously (12). In this experiment, subconfluent cells were cultured for 1 week in 10% minimal essential medium with 50 g/ml ascorbic acid. OC ChIP assays were performed and quantified by real-time fluorescence PCR with a GeneAmp 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) as detailed previously (18). Twenty nanograms of processed genomic DNA was used in each PCR reaction. The signal arising from 1 pg of mouse genomic DNA was used as a standard. Data were expressed as the mean picograms ± S.D. of OC genomic DNA equivalents present in the Runx2 immunoprecipitate from four independent cultures/precipitations for both SFG-LacZ and SFG-Msx2 cultures.

**RNA Interference and Fluorescence RT-PCR Analysis of OC mRNA**—The siRNA duplexes against MINT were designed following Tuschl’s principles (27). To screen for silencing duplexes, we constructed a reporter MINT fusion protein, MINT-EGFP, by ligating the EGFP cDNA (Invitrogen) by PCR with a pCMV6-Ad5 vector (BD Biosciences Clontech) in a contiguous open reading frame using the unique BglII site in the MINT cDNA. Thus, the 36 C-terminal amino acids of MINT are replaced by EGFP in the MINT-EGFP fusion protein. To screen for active MINT siRNAs, synthetic 21-bp duplexes were cotransfected along MINT-EGFP into MC3T3-E1 cells using Mirrus TransIT-TKO reagent (Mirrus Co., Madison, WI) and evaluated by fluorescence microscopy 24 h later. In parallel, GFP-targeted siRNA was used as a positive control, and luciferase-directed siRNA was used as a negative control. Of the nine MINT-directed siRNA sequences designed and tested, an siRNA duplex against the MINT target sequence AAG CAG CAC GAG reduced luciferase signal comparable with cells transfected with the GFP-targeted siRNA (positive control). This MINT-targeted siRNA duplex was chosen to evaluate the role of MINT on osteocalcin gene expression. In independent duplicate cultures, MC3T3-E1 cells were transfected with either 100 nM MINT-targeted

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**Fig. 1.** A major FGF2-dependent transactivation function resides in Runx2 C terminus. A, one-hybrid assays were performed to identify FGF2-regulated transactivation functions in the OCFRE-binding constituents (18), using the Gal 4 reporter pPR-LUC. As indicated Runx2, Tbdn100, Ku70, and Ku80 were expressed as N-terminal fusion proteins with the Gal4 DNA-binding domain (G4). Wild-type Runx2 lacking the Gal 4 DNA binding domain was used as a negative control. Transfected MC3T3-E1 calvarial osteoblasts were treated with either vehicle or 10 ng/ml FGF2 as indicated, and luciferase assays were carried out as previously detailed (18). Note that most of the basal activity (p < 0.05, G4-Runx2 versus Runx2) and FGF2-activated TAF (p < 0.01, FGF2 versus vehicle) is provided by the Runx2 constituent. By contrast, G4-Tbdn100, G4-Ku70, and G4-Ku80 do not possess significant FGF2-regulated TAF in this assay. B, the OCFRE-LUC reporter plasmid was transiently co-transfected (MC3T3-E1 cells) with either empty expression vector (pcDNA3) or the indicated pcDNA3-Runx2 expression constructs. Constitutively active FGF2-RORS was included as described to activate cell-autonomous FGF2-RORS signaling, and total DNA content was kept constant with empty expression vectors. Data are presented as the mean ± S.D. luciferase reporter activity, normalized to basal OCFRE-LUC activity. Note that Runx2 (1—412), a variant that lacks part of the NMTS and C-terminal VWRPY domain but retains AD3, responds well to FGF2-RORS. Furthermore, note that Runx2 (1—392), a variant that also retains AD3, still activates basal OCFRE-LUC and responds to FGF2-RORS. By contrast, Runx2 (1—312) lacking AD3 is inactive. See text for details. ***, p < 0.01, FGF2-RORS versus corresponding vector control.
siRNA or luciferase-targeted siRNA (negative control). The next day, MC3T3E1 cells were switched to osteogenic differentiation media (10% fetal calf serum in α-minimal essential medium, supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerol phosphate) and cultured for an additional 10 days. RNA was subsequently extracted, contaminating DNA was removed withDNaseI, and OC, OPN, and an additional 10 days. RNA was subsequently extracted, contaminating DNA was removed with DNaseI, and OC, OPN, and β-tubulin mRNA accumulation expression evaluated by fluorescent RT-PCR as described previously (18, 28). The experiment was repeated twice.

RESULTS

Runx2 Provides a Major FGF2-Regulated Transactivation Function in the OCFRE Promoter Binding Complex—In the calvarial osteoblast, the OCFRE assembles a multiprotein complex containing Runx2 and a novel Ku-antigen transcription factor complex (18). This complex is the target of both FGFR2-dependent activation and Msx2 suppression. To clarify which of these proteins may provide major transactivation functions versus structural function, we synthesized recombinant TAF in Ga14 one-hybrid analyses. As shown in Fig. 1A, among Ku70, Ku80, Tbdn100, and Runx2, only Runx2 exhibited major basal TAF activity when fused to the Ga14 DNA binding domain when using the Ga14 responsive luciferase reporter pFR-LUC. Importantly, the Runx2 TAF is regulated by FGFR2 signaling. Co-expression of the constitutively active FGFR2, FGFR2-ROS, enhances Runx2 TAF both in one-hybrid assays (Fig. 1A) and on the OCFRE (Fig. 1B).

Because Runx2 provided the major basal and FGF-dependent transactivation function of the OCFRE binding factors, we sought to determine the part of Runx2 that was critical for FGF-dependent activation of the OCFRE. Using a series of C-terminally truncated Runx2 variants and the OCFRE-LUC reporter, we determined that the TAF domain regulated by FGFR2-ROS resides outside of the N-terminal Runt domain and maps between residues 312 and 392 in the C terminus. Runx2 C-terminal deletions caused a stepwise loss of Runx2 activity and FGF responsiveness (Fig. 1B). Runx2 (1–412) lacking the C-terminal VWRPY repressor domain and half of the Runx2 NMTS retains basal and FGF2-stimulated OCFRE-LUC activation resembling that of full-length Runx2. Runx2 (1–392) Runx2 (1–312) removes the additional amino acids 393–528; this completely removes the NMTS (amino acids 397–434) (29) and results in an 80% decrement in basal Runx2 activity (Fig. 1B), indicating that critical TAF domains for Runx2 activity lie between amino acids 312 and 392, a region encompassing the AD3 initially identified by Karsenty and colleagues (30). Thus, Runx2 provides the major transactivation function in the osteoblast nuclear protein complexes assembled by the OCFRE.

MINT Enhances Runx2-dependent Transcription of the OCFRE—In vitro, the RRM domains of MINT bind the GT-rich repeat of the OCFRE, overlapping and adjacent to the cognate for Runx2 recognition (21). To better understand the relationship between Runx2 and OCFRE by MINT, we transiently co-expressed full-length MINT with OCFRE-LUC in MC3T3E1 calvarial osteoblasts, a cell background that has endogenous Runx2 and FGFR expression. MINT significantly enhances both basal and FGF2-induced OCFRE activity by 3-fold (Fig. 2A). To verify and extend these results, we reconstituted FGFR2, MINT, and Runx2 regulation of OCFRE-LUC in CV1 fibroblasts, a naïve cellular background that does not express Runx2, FGFR2, Msx2, or other osteoblast transcription factors. In CV1 cells, expression of MINT alone does not increase OCFRE activity; rather, it weakly suppressed basal OCFRE-LUC activity (Fig. 2B), suggesting that MINT required additional factors to enhance OCFRE activity. Co-expression of a constitutively active FGFR2-ROS demonstrates that MINT alone
OCFRE Regulation by MINT, Runx2, and Msx2

Fig. 3. MINT requires Runx2 Activation Domain 3 (AD3) to enhance FGF-dependent transactivation of the OCFRE. A, the Runx2 isoform II C-terminal deletion mutants generated for mapping FGF2- and MINT-regulated OCFRE activation functions (see “Experimental Procedures”). Activation domains (AD) and Runt domain are depicted according to Karsenty (30). B, using methods detailed previously (18), fibroblastic CV-1 cells (lack endogenous Runx2 and FGFR2) were transiently transfected with the OCFRE-LUC reporter plasmid and the Runx2 and MINT expression constructs in the combinations indicated. Cell-autonomous FGFR2 signaling was provided by co-transfection of a constitutively active FGFR2-ROS as indicated. Data are presented as the mean ± S.D. luciferase reporter activity normalized to basal OCFRE-LUC activity, obtained from three independent transfections. Note that although MINT augments OCFRE activity driven by full-length Runx2 (1–528) or Runx2 (1–392), MINT cannot stimulate transcription driven by Runx2 (1–312) or Runx2 (1–528; Δ308–375), Runx2 variants that lack AD3. It is noteworthy that OCFRE activation by Runx2 does not require AD3, because Runx2 (1–528; Δ308–375) responds to FGFR2-ROS but not to MINT. See text for details.

Discussion

MINT Co-Localizes with Transcriptionally Active RNAP IIo and Partially Co-localizes with Runx2—We wished to provide further evidence that MINT participates in transcriptional activation responses, because we initially identified MINT as interacting with Msx2, a homeoprotein suppressor (21). The hyperphosphorylated C-terminal domain of RNA polymerase II, RNAP IIo, demarcates RNA synthesis in transcriptionally active chromatin (31) and can be specifically immunolocalized by the phospho-PolII monoclonal antibody H5 (32). Therefore, we evaluated the subnuclear localization of MINT with respect to RNAP IIo, using dual-color confocal immunofluorescence microscopy (see “Experimental Procedures”). As shown in Fig 4A, RNAP IIo exhibits a reticular pattern in MC3T3E1 osteoblasts and extensively co-localizes with MINT, consistent with a role in transcriptional activation responses. We next examined the subnuclear localization of MINT with Runx2, the major transactivation function recognizing the OCFRE. To do this, we constructed an expression vector for EGFP-Runx2 fusion protein and transiently expressed this in the MC3T3E1 cellular background. As shown in Fig 4B, EGFP-Runx2 exhibits a punctuate pattern (“Runx foci”) that only partially co-localizes with MINT under basal conditions. However, after stimulation with FGF2, the extent of Runx2-MINT co-localization increased ~2.5-fold (Fig. 4, B and C). In the absence of FGF2, 8.5% of the Runx2 foci overlap with MINT. This overlap significantly increases to 20% with 10 ng/ml FGF2 treatment (p < 0.05) (Fig. 4, B and C), consistent with our observations that FGFR2 signaling augments Runx2-MINT activation of the OCFRE (Figs. 2 and 3). Thus, the nuclear matrix protein MINT can be found in subnuclear compartments containing active
transcriptional complexes, indicated by co-localization with RNAP IIo and the major OCFRE transactivator Runx2.

Msx2 Co-localizes with MINT and Displaces Runx2 from OC Chromatin—We demonstrated previously that the OCFRE is a specific target for Msx2-dependent suppression of the OC gene via antagonistic protein-protein interactions (16). Consistent with this observation, activation of the OCFRE by Runx2 and MINT is abrogated by Msx2, either in the presence or absence of activated FGFR2 signaling in CV1 cells (Fig. 5A). Because we initially cloned MINT via its protein-protein interactions with Msx2, we evaluated the subnuclear local of EGFP-Msx2 with respect to MINT. As shown in Fig. 5B, co-expression of Msx2 and MINT in CV1 cells reveals extensive nuclear overlap, revealed by confocal immunofluorescence microscopy. Immunolocalization of MINT again revealed a uniform reticular pattern similar to that found in MC3T3-E1 (Fig. 4B), coincident with EGFP-Msx2 accumulation (Fig. 5B), suggesting that transcriptional inhibition by Msx2 targets nuclear matrix complexes. Because Runx2 possesses the major TAF recognizing and regulating the OCFRE, we employed ChIP (18) to assess the effects of Msx2 on Runx2 localization. The Runx2 localization with endogenous OC chromatin was examined by ChIP

Fig. 4. MINT co-localizes extensively with transcriptionally active chromatin but only partially with Runx2. A, transcriptionally active chromatin (green, upper left) was detected with monoclonal antibody H5 against RNAP IIo, the active, hyperphosphorylated large subunit of RNA polymerase II (32). MINT (red, upper right) was stained with R1 antibody against the Msx2-interacting domain (21). Note the extensive overlap between MINT and RNAP IIo (yellow in merged image). Scale bar, 10 μm. B, EGFP-Runx2 and MINT plasmids were transfected to MC3T3-E1 cells. MINT was immunolocalized with rabbit anti-MINT R1 antibody (21) and Cy3-conjugated anti-rabbit IgG secondary antibody. EGFP-Runx2 (a, d) exhibits a nuclear punctuate distribution (green, left), whereas MINT (b, c) (red, middle) shows a reticular scaffold-like distribution sparing the nucleoli. Co-localizing MINT-Runx2 (yellow areas, arrows; c, f) are more abundantly observed in cells treated with FGF2 (d–f) (see also C). Scale bar, 10 μm. C, the extent of MINT overlap with Runx2 foci increases 2.5-fold with FGF2 treatment (*, p < 0.05). All images were acquired by confocal microscopy and deconvolved. See text for details.
and comparison made with the OPN gene, a genomic target of Runx2 (33) that is not inhibited by Msx2 (14). In MC3T3E1 calvarial osteoblasts stably transfected with pcDNA3 vector control, Runx2 associates with both endogenous OC and OPN chromatin (Fig. 6A, lane 3). However, in cells transfected with pcDNA3-Msx2, Runx2 was displaced from OC, but not OPN, genomic chromatin, consistent with effects on mRNA accumulation. Using a retroviral SFG vector to express Msx2 and fluorescence PCR to quantify ChIP, similar results are obtained; expression of Msx2 significantly decreases the association of OC chromatin by ~75% (Fig. 6B).

We wished to confirm the importance of MINT and Msx2 in control of OC expression in the endogenous chromatin context; therefore, we implemented RNAi, evaluating the effects of MINT-directed siRNA on OC gene expression. Using a MINT-EGFP fusion protein, we screened eight MINT-directed siRNAs and identified one that could greatly reduce MINT protein in MC3T3E1 cells (Fig. 7A and B). Compared with control siRNA (luciferase-directed), lipofection with MINT siRNA significantly reduced endogenous OC gene expression ($p = 0.03$, Fig. 7C; mRNA quantified by fluorescence RT-PCR). By contrast, the accumulation of OPN and tubulin mRNAs was not suppressed by MINT siRNA. Thus, MINT regulates OC gene expression in the endogenous chromatin context of MC3T3E1 calvarial osteoblasts. Msx2 inhibits MINT- and Runx2-dependent activation of the OCFRE. Msx2 accumulates in nuclear compartments occupied by MINT, and nuclear accumulation of Msx2 selectively displaces Runx2 from OC, but not OPN, genomic chromatin complexes (see “Discussion”).

**DISCUSSION**

The molecular mechanisms whereby the osteoblast nucleus integrates transcriptional responses to morphogenetic, metabolic, and mechanical cues are poorly understood. Msx2, Runx2, and Osx have emerged as key regulators of osteoblast- and odontoblast-dependent bone formation (5, 6). Runx2/Cbfa1 and Osx are necessary for proper intramembranous as well as endochondral bone formation. Msx2, by contrast, seems to function primarily to regulate intramembranous ossification and tooth development during craniofacial skeletogenesis (9). Our data indicate that “cross-talk” occurs between Msx2 and Runx2 at the OC promoter. Msx2 inhibits Runx2-dependent
transcription of the OC gene, in part by inhibiting the stable association of Runx2 with OC chromatin. Others have shown that Runx2 can physically interact with Runx2 (34) and thus inhibit Runx2-dependent gene expression. However, in the calvarial osteoblast, Msx2 selectively suppresses the OC but not the OPN gene (14), even though both promoters are supported by Runx2-dependent transcription (33, 35). Our ChIP analyses indicated that Msx2 selectively displaces Runx2 from OC but not from OPN chromatin. Therefore, the Shirakabe model proposes that Msx2 selectively displaces Runx2 from OC but not OPN chromatin (lane 4), even though equivalent amounts of genomic DNA targets were subjected to ChIP (lanes 1–2). B, quantitative fluorescence PCR of OC promoter chromatin immunoprecipitated with anti-Runx2 antibody from MC3T3E1 cells transduced with a control retrovirus (SFG-LacZ) or a virus expressing Msx2 (SFG-Msx2). Data are presented as the mean (± S.D.) amount of OC chromatin precipitated in four independent ChIP assays, normalized to the OC promoter signal obtained from 1 pg of mouse genomic DNA. Runx2 association with OC promoter chromatin was decreased by ~75% in MC3T3E1 cells expressing Msx2.

We initially cloned MINT, the Msx2 interacting nuclear target, using the interaction cloning strategy (36) to identify proteins that directly bind to Msx2 (21). In our initial studies, we noted that expression of the MINT N-terminal RRM domain suppressed FGFR2-activated OC promoter activity in transient transfection. Others subsequently demonstrated that human MINT (a.k.a. SHARP) can function as a suppressor of nuclear receptor signaling (22) or Notch1-regulated transcriptional activation (37) transactivation. As occurs with the large CBP/p300 or p160 adapter/platform proteins that serve structural roles (and acetylase chromatin (38)), transcriptional squelching is commonly observed when only subdomains are expressed or if co-regulator stoichiometry is poorly matched (39). In the present work, we show that full-length MINT enhances Runx2-dependent transcription, mediated via the autonomous Runx2 AD3. Consistent with a role in the activation of gene transcription, we demonstrate that MINT extensively co-localizes with RNAP IIo, the transcriptionally active and phosphorylated form of RNA polymerase (40).

The Ku antigen (heterodimer of Ku70 and Ku80) is a key component of the DNA-protein complex that recognizes and regulates the OCFRE (18). Ku70 and Ku80 bind to the OCFRE, interact with Runx2, and enhance FGFR2 signaling from the element. Although capable of expressing OPN, Ku70+/− calvarial osteoblasts have a profound deficiency in OC gene expression (18). Dynan and colleagues have clearly established a crucial role for Ku antigen in transcriptional activity (32), separate from its DNA-dependent protein kinase catalytic subunit-regulated role in DNA repair. Ku supports multiround transcriptional re-initiation processes (41) and selectively associates with RNAP IIo, the actively phosphorylated and processive form of RNA polymerase II, but not with general transcription factors associated with the pre-initiation complex (32). DNA-dependent protein kinase catalytic subunit is not associated with Ku and RNAP IIo (32), confirming unique localizing and roles for the Ku-RNAP IIo complexes in transcription (32) and the Ku-DNA-dependent protein kinase catalytic subunit complexes in DNA repair (42). Because our novel Ku antigen complex binds Runx2 and the OCFRE (18), it is tempting to speculate that these interactions may provide a mechanism for ensuring the efficient association of Ku with RNAP IIo on the OC gene after promoter clearance of the pre-initiation complex (32, 41).

Incorporating these recent observations with our current data, we propose the following working model for OCFRE regulation (Fig. 8). Runx2 maintains OCFRE activity in concert with Ku antigen and activated FGFR2 signaling. Although Runx2 provides major FGFR2-regulated transactivation function, recruitment of Ku would ensure multiple cycles of transcription via efficient re-initiation. MINT plays a structural role in the osteoblast nuclear matrix, organizing and integrating Runx2- and Msx2-dependent transcription with chromatin remodeling and efficient re-initiation. By interacting with MINT and Ku, Msx2 selectively prevents the stable association and cyclic re-association of Runx2 with the OC chromatin as necessary for support of OC gene expression. We are currently extending the use of RNAi to study the consequences of MINT and Ku depletion on Runx2 sub-cellular localization, chromatin association, and the regulated co-localization of MINT and Runx2 in the osteoblast nucleus; this will permit further evaluation of those features of the model that have yet to be unambiguously established.

Given the biochemical localization of MINT to the nuclear matrix (21), the nuclear pattern generated by immunolocalized MINT caught our attention. Because of its generalized nuclear distribution and considerable thickness, this scaffold should belong to or decorate a major structure of the interphase nucleus. We measured the thickness of these threads because their length seems more a function of the plane observed as a confocal section. The approximate width was 370 ± 130 nm (mean ± S.D.; range, 240–500 nm). These dimensions are in the range of what Verschure et al. (43) found as a substructure of 300–450 nm when staining for chromosomes with specific probes or fluorescent histone H2B. Their model depicts sub-
structures made up of several rounds of 30–100-nm compacted chromatin fibers near transcriptionally active chromatin. Based on bromo-UTP incorporation localizing to diffuse areas between the 300–450-nm substructures, active open chromatin would have to loop out from compacted chromatin. Loops of open chromatin anchor through matrix attachment regions, sequences of DNA associated with the nuclear matrix that help organize nuclear architecture and tissue-specific gene expression (44–47). It remains unknown whether MINT binds matrix attachment region sequences \textit{in situ}. However, in gel shift assays, the recombinant purified MINT RRM domain selectively binds homopolymeric oligo(dG) and oligo(dT) ssDNA, as well as the GT-rich dsDNA of the proximal OC promoter (21).

Applying iterative cyclic amplification and selection of target sequences (CASTing) (48) with recombinant purified MINT RRM domains, we have confirmed that iterative GT-rich dsDNA sequences, like those of the proximal OC promoter, represent highly favored cognates for recognition by the MINT RRM (data not shown). As observed with special AT-rich binding protein 1 (44), MINT interacts with components of the nucleosome remodeling and deacetylase complex (22). Under the converging model of the osteoblast nucleus, (49–51), we propose that MINT binds the GT-rich DNA of the proximal OC promoter via the RRM domains that are necessary for enhancement of Runx2-dependent transcription, providing a structural role that targets the OC gene to the nuclear matrix. It is noteworthy that Shi et al. (22) described how the human homologue of MINT, SHARP, binds RNA molecules that can functional as transcriptional co-adapters. Future studies will examine whether MINT binds matrix attachment region sequences \textit{in situ}, whether the proximal OC promoter becomes tightly associated with the nuclear matrix during osteoblast differentiation and whether Runx2 uses an RNA co-adapter.

Our present work emphasizes studies in MC3T3E1 calvarial osteoblasts, a physiologically relevant cell background for studies of Msx2, Runx2, and MINT gene regulation. It is noteworthy that key features of OCFRE regulation could be recapitulated in the naive CV-1 fibroblastic cell background. However, it remains to be determined whether the key features of MINT-regulated gene expression identified in neural crest-derived craniofacial osteoblasts can be generalized to all osteoblast lineages, including those of the appendicular or axial skeleton. Moreover, cell culture models cannot fully recapitulate all of the morphogenetic, metabolic, and mechanical influences so

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**FIG. 7.** MINT siRNA selectively down-regulates OC mRNA accumulation in MC3T3E1 calvarial osteoblasts. RNA interference (RNAi) was used to manipulate MINT protein expression as described in the text, and the effects on endogenous OC, OPN, and tubulin gene expression quantified by fluorescence RT-PCR. The negative control siRNA was directed toward the firefly luciferase gene (not endogenously expressed in MC3T3E1 osteoblasts). A, MINT-EGFP fusion protein constructed to screen for siRNAs that could down-regulate MINT protein expression in MC3T3E1 cells. B, note that although lipofection with 100 nM control siRNA (luciferase-directed) has no effect on MINT protein accumulation (upper), the siRNA directed to the C-terminal domain of MINT (lower) was able to abrogate protein accumulation (lower set). Similar results were obtained in two separate experiments using either epifluorescence (experiment 1) or confocal (experiment 2) fluorescence microscopy to visualize MINT-EGFP protein. Scale bar, 100 μm. C, endogenous OC, OPN, and tubulin gene expression quantified by fluorescence RT-PCR. Data are presented as the mean (±S.D., n = 4) percentage change in gene expression in response to MINT-targeted siRNA lipofection versus negative control siRNA. Note that cells transfected with 100 nM MINT siRNA significantly down-regulate expression of the endogenous OC gene (p = 0.03). By contrast, OPN and β-tubulin genes are not significantly suppressed. See text for details.
crucial to normal osteoblast physiology. Thus, future studies will necessarily encompass use of vertebrate animal models to confirm and extend our current observations. Because of the extensive co-localization of MINT with RNAP II, it is intriguing to speculate that loss of MINT function in the osteoblast will result in global alterations in integrative transcriptional responses necessary for osteoblast terminal differentiation. The MINT knockout mouse phenotype is embryo-lethal at embryonic day 12 because of cardiovascular defects, consistent with an important role in tissue-specific differentiation (23). It is noteworthy that osteoblast terminal differentiation does not begin until after embryonic day 14.5 in the developing mouse (14); thus, effects of MINT deficiency on osteoblast differentiation could not be assessed in these genetically lesioned embryos. The RNAi strategy we used greatly reduced but did not completely eliminate MINT protein accumulation in transgenic mouse osteoblasts. The RNAi strategy we used greatly reduced but did not completely eliminate MINT protein accumulation in transgenic mouse osteoblasts. The RNAi strategy we used greatly reduced but did not completely eliminate MINT protein accumulation in transgenic mouse osteoblasts. The RNAi strategy we used greatly reduced but did not completely eliminate MINT protein accumulation in transgenic mouse osteoblasts.

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