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Subnuclear Targeting of Runx1 Is Required for Synergistic Activation of the Myeloid Specific M-CSF Receptor Promoter by PU.1

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Abstract Many types of acute myelogenous leukemia involve chromosomal translocations that target the C-terminus of Runx1/AML1 transcription factor, a master regulator of hematopoiesis. The C-terminus of Runx1/AML1 that includes the nuclear matrix targeting signal (NMTS) is essential for embryonic development, hematopoiesis, and target gene regulation. During the onset and normal progression of hematopoiesis, several lineage-specific factors such as C/EBPα and PU.1 interact with Runx1 to regulate transcription combinatorially. Here we addressed the functional interplay between subnuclear targeting of Runx1 and gene activation during hematopoiesis. Point mutations were generated in the NMTS of the human Runx1 protein and tested for their effect on transcriptional cooperativity with C/EBPα and PU.1 at myeloid-specific promoters. We characterized five mutants that do not alter nuclear import, DNA binding or C/EBPα-dependent synergistic activation of the target gene promoters. However a critical tyrosine in the NMTS is required for subnuclear targeting and activation of the granulocyte-macrophage colony stimulating factor (GM-CSF) promoter. Furthermore, this point mutation is defective for transcriptional synergism with PU.1 on the macrophage colony stimulating factor (MCSF) receptor c-FMS promoter. Our results indicate that the NMTS region of Runx1 is required for functional interactions with PU.1. Taken together, our findings establish that subnuclear targeting of Runx1 is a critical component of myeloid-specific transcriptional control. J. Cell. Biochem. 96: 795–809, 2005.

Key words: AML1; hematopoiesis; transcriptional regulation; nuclear matrix; leukemia

Transcriptional control of myeloid differentiation involves the combinatorial activities of a limited number of key transcription factors including Runx1, Cbfβ, C/EBP, and PU.1 [Speck and Gilliland, 2002; Dahl and Simon, 2003; Friedman et al., 2003; Tenen, 2003]. Genetic evidence indicates that these factors contribute to specific stages of hematopoietic differentiation in vivo. For example, both Runx1 and Cbfβ are essential for definitive hematopoiesis and mice nullizygous for either gene die in utero at 10.5 dpc [Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996a,b; Niki et al., 1997].

The CCAAT enhancer binding proteins (C/EBP), a family of leucine zipper DNA binding transcription factors, play a critical role in development of both the monocytic and myeloid cell lineages [Scott et al., 1992; Katz et al., 1993]. C/EBPα null mice die shortly after birth because of hypoglycemia [Wang et al., 1995]. C/EBPα is critical for expression of many myeloid genes [Rosmarin et al., 2005]. PU.1 is an Ets-family transcription factor that also controls many genes critical for development of the hematopoietic system [Moreau-Gachelin et al., 1988; Scott et al., 1994; McKercher et al., 1996; Hromas et al., 1997]. Targeted disruption of the PU.1 gene leads to loss of myeloid and B-cell

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development and a blockage in differentiation of hematopoietic stem cells [Anderson et al., 1998; Akashi et al., 2000; Dahl et al., 2003]. Furthermore there is evidence for functional interplay between PU.1, C/EBPα, and Runx1 in hematopoiesis [Zhang et al., 1994; Hohaus et al., 1995; Smith et al., 1996]. Thus the genetic requirement of PU.1, Runx1, Cbfβ, and C/EBPα in hematopoiesis and the ability of these factors to interact biochemically suggest that they are also functionally coupled.

Acute myelogenous leukemia is a prevalent hematopoietic failure, characterized by abnormal proliferation and differentiation of myeloid progenitor cells [Bloomfield et al., 1998; Grimwade et al., 1998; Downing, 1999]. Runx1/AML1 and Cbfβ, which form a heteromeric transcription factor complex, are the most frequently deregulated genes in lymphoid and myeloid leukemias [Miyoshi et al., 1991; Erickson et al., 1992; Nisson et al., 1992]. These leukemias involve translocations, point mutations and amplifications that lead to the formation of chimeric gene products. The resulting fusion proteins display a variety of abnormal functions, including a positive effect on the growth of hematopoietic progenitor cells [Mulloy et al., 2005]. These altered functions also represent a gain or loss of molecular interactions with key hematopoietic transcription factors and physiologic co-regulators [Speck and Gilliland, 2002].

Runx1 (AML1/Cbfa2) is a member of the runt related transcription factor family, which also includes Runx2 (AML3/Cbfa1) and Runx3 (AML2/Cbfa3). The Runx1 protein contains multiple modules that are structurally and functionally conserved among the three family members. These include the N-terminal Cbfβ heterodimerization and DNA-binding runt homology domain (RHD), the nuclear localization signal, and the C-terminal nuclear matrix targeting signal (NMTS) along with a context-dependent transcriptional activation/repression domain [Zeng et al., 1997, 1998; Zaidi et al., 2001]. A large number of co-regulatory proteins associate physically with specific domains of Runx1 and are functionally important for regulation of target genes [Wotton et al., 1994; Giese et al., 1995; Hiebert et al., 1996; Rhoades et al., 1996; Petrovick et al., 1998; Rubnitz and Look, 1998; Osato et al., 1999]. Many leukemia related translocations involve a loss or substitution of the Runx1 C-terminal domain. Consistent with these observations, the biological activity of the Runx1 protein requires the presence of an intact carboxy terminus. Knockin mouse models in which the Runx1 C-terminus is deleted or replaced with the ETO protein (AML1-ETO) fail to establish definitive hematopoiesis and die in utero [Castilla et al., 1996; North et al., 1999]. Thus the carboxyl end of Runx1 is essential for embryonic development and hematopoiesis.

Runx proteins are spatially organized in distinct transcriptionally active subnuclear domains that are associated with the nuclear matrix, a major component of nuclear structure [Berezney et al., 1996; Stein et al., 1999]. Our previous work has identified a unique intranuclear trafficking sequence in the Runx1 C-terminus, referred to as the NMTS that targets Runx proteins to these subnuclear domains [Zeng et al., 1997, 1998; Zaidi et al., 2001]. Furthermore, molecular alterations that cause misrouting of Runx1 result in aberrant gene expression and development of disease [Jackson, 1997; Stein et al., 2000a,b]. We postulate that targeting to correct subnuclear locations is critical for Runx1 mediated gene regulatory mechanisms that are physiologically relevant for normal cell growth and differentiation during hematopoiesis.

Here we investigated the role of the Runx1 NMTS in regulating expression of myeloid specific genes. Based on the crystal structure of the Runx1 NMTS, several conserved residues in the interacting surface of the protein were mutated to alanine. These mutations do not affect protein expression, stability, nuclear import or DNA binding. However, we identified a tyrosine residue critical for Runx1 association with the nuclear matrix and for functional synergism with other hematopoietic factors to control myeloid specific gene expression.

MATERIALS AND METHODS

Cell Culture and Transient Transfections

SaOS-2 cells were grown and maintained in McCoy 5A Medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% FBS. For in situ studies, cells were plated on 0.5% gelatin-coated coverslips (Fisher Chemicals, Fairlawn, NJ) in 6-well tissue culture trays at a density of 0.3×10^5 cells per well. Transient transfections were performed 20 h after plating with 0.5 μg/well of wild-type or mutant Runx1 expression vectors using Lipofectamine Plus reagent (Invitrogen

Corp.). Cells were processed 20 h later for in situ immunofluorescence analysis. For biochemical fractionation, SaOS-2 cells were plated at a density of 1.0×10^6 cells per 10 cm dish and transfected 20 h later with 4 μg of wild-type or mutant Runx1 expression vectors as described above. HeLa cells were cultured in DMEM with 10% FBS, while CV-1 cells were grown in α-MEM supplemented with 10% FBS. For promoter-reporter assays, HeLa and CV-1 cells were transfected with 10 μg of reporter and 1 μg of expression plasmid using Calcium Phosphate Kit (Invitrogen Corp.). Timing of the transfection period and concentration of plasmids were optimized to achieve low but detectable expression levels. Cells were harvested 20 h (HeLa) or 40 h (CV-1) post-transfection for luciferase or CAT assays.

Plasmid Constructs

The full-length human pCMV5-HA-AML1B construct has been reported previously [Zeng et al., 1997]. The HA-tagged deletion mutants of AML1B were generated by PCR-based, site directed mutagenesis. The two deletion mutants with and without the NMTS domain HA-AML1(385 and HA-AML1Δ350, respectively, were PCR amplified using a common forward primer containing an EcoRI site: 5′-ACGCTGAATTCTCTAGAGACG-3′ and reverse primers with a ClaI site: 5′-AAGAATCGATTCAGTAGGGCGGCGGCAGGATGGT-3′ for Δ385 and 5′-AAGAATCGATTCATGGATAGTGCATGCGGGGGT-3′ for Δ350. PCR products were double digested with EcoRI/ClaI and ligated into similarly digested pCMV5-HA vector. To confirm the in frame ligation the positive clones were sequenced from either end using T7 and F353A T3 primers, respectively. Point mutants F350A, Y377A, H378A, Y380A, and Y380C were generated with a two step PCR approach. In the first step, two independent but overlapping PCR products were generated using full-length cDNA as template. For PCR product 1, the common forward primer contained an EcoRI site: 5′-ACGCTGAATTCTCTAGAGACG-3′ while the mutant specific reverse primers were 5′-GAGTAGGAGGCGGCGCCTGGA-3′ for F353A, 5′-GGTGTGGGCGCGCGTGGCCGA-3′ for Y377A, 5′-CAGGTAGGTGGCGTAGCGCGT-3′, for H378A, 5′-CGGCGGCAGGGCGGTGTGGTA-3′ for Y380A, and 5′-CGGCGGCAGGCTGGTGTGGTA-3′ for Y380C. For PCR product 2, the mutant specific forward primers were 5′-TCCAGGCGCCGCCACCTACTC-3′ for F353A, 5′-TCGGCCACGCGCGCCCACACC-3′ for Y377A, 5′-ACGCGCTACGCCACCTACCTG-3′ for H378A, 5′-TACCACACCGCCCTGCCGCCG-3′ for Y380A, and 5′-TACCACACCTGCCTGCCGCCG-3′ for Y380C with the common reverse primer containing a ClaI site and stop codon 5′-AAGAATCGATTCAGTAGGGCCTCCACAACAC-3′. Both PCR products for each mutant plasmid were purified and combined as template to generate full-length PCR product containing the respective mutation. For the full-length PCR reactions, the forward primer with EcoRI site and the reverse primer with ClaI site are the same as above. The final products carrying the respective mutations were double digested with EcoRI/ClaI and ligated into the similarly digested pCMV5-HA vector. Presence of mutated sequences was confirmed by automated sequencing using an internal primer.

Human granulocyte-macrophage colony stimulating factor (GM-CSF) promoter (−75 to +28) was cloned by PCR amplification of genomic DNA from T98G cells. PCR reactions were carried out using XhoI containing forward primer 5′-CCGCTCGAGCCGCCTCCCTGGCAT-3′ and HindIII containing reverse primer 5′-CCCAAGCTTAGAACTTTAGCCTTT-3′. PCR products were digested with XhoI-HindIII and ligated to similarly digested pGL2 basic vector carrying the luciferase reporter gene (Invitrogen, Inc.). Sequences of the cloned promoter segment were confirmed by automated sequencing. The human M-CSF receptor c-FMS promoter fragment (−416 to +71 bp) fused to the luciferase gene was a kind gift of D.G. Tenen, Harvard Institutes of Medicine [Hohaus et al., 1995]. The expression construct for murine PU.1 was obtained from Gerd A. Blobel, University of Pennsylvania School of Medicine [Hong et al., 2002].

In vitro Transcription/Translation of Runx1 Proteins and Preparation of Nuclear Extracts

Wild-type and NMTS mutant Runx1 proteins were synthesized in vitro using the TNT® Quick Coupled Transcription/Translation System (Promega Corp., Madison, WI). Briefly, 1 μg of template DNA and 1 mM methionine were mixed with 47 μl of TNT T7 quick master mix as per the manufacturer's instructions. Reactions were incubated at 30°C for 1.5 h and stored immediately at −70°C until further use.

Nuclear extracts from transfected HeLa cells were prepared essentially as described [Javed et al., 2001]. Briefly HeLa cells were transiently transfected with 10 μg of expression vector using SuperFect transfection reagent (Qiagen, Inc., Valencia, CA). Cells were harvested 24 h post-transfection for isolation of nuclear proteins. Protein quantities were estimated by Bradford assay.

Electromobility Shift Analysis (EMSA)

Runx consensus oligo 5′-CGAGTATTGTG-GTTAATACG-3′ was end labeled essentially as described previously [Javed et al., 2005]. Nuclear proteins isolated from transiently transfected HeLa cells (10 μg) or equal volumes of IVTT reaction (5 μl) were used for the DNA binding reaction. Reaction mixtures were prepared using 50 fmol of probe, 50 mM KCl, 12 mM HEPES, 1 mM EDTA, 1 mM DTT, 12% glycerol, 2 μg of poly(dI-dC).poly(dI-dC), and DNA binding reactions were carried out at 25°C for 20 min. Aliquots were separated in a 4% nondenaturing polyacryl amide gel for 1.5 h at 200 V. The gel was dried and subject to autoradiography.

Immunofluorescence Microscopy

Transfected SaOS2 cells were processed for whole cell (WC) or nuclear matrix intermediate filament (NMIF) preparations, as described [Barseguian et al., 2002]. Briefly cells were washed with ice cold PBS 20–24 h post-transfection and fixed with WC fixative (4% formaldehyde in PBS) for 10 min and permeabilized for 20 min with 0.25% Triton X-100. For the cytoskeletal (CSK) preparation, cells were washed with ice-cold PBS and extracted twice for 15 min each with CSK buffer on ice. Cells were then fixed with CSK fixative (4% formaldehyde in CSK buffer). For NMIF preparations cells were extracted with CSK buffer as above and then extracted twice with digestion buffer containing 40 U/ml of DNaseI at 28°C for 20 min each. Cells were subsequently extracted in 250 mM ammonium sulphate for 10 min and fixed with NMIF fixative (4% formaldehyde in digestion buffer). Fixed cells were incubated with 1:3,000 dilution of mouse monoclonal HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 37°C for 1 h, washed 4× with PBSA (0.5% BSA in PBS) and incubated at 37°C for 1 h with 1:1,000 dilution of anti-mouse Alexa 488 secondary antibody (Molecular Probes). After four washes with PBSA, cells were stained with DAPI (0.02 mg/ml) for 5 min on ice. Cells were then washed once with PBSAT (0.1% Triton in PBSA) and twice with PBS, and then mounted with vectashield mounting media (Vector laboratories, Inc., CA). Digital imaging of cells was performed with a Zeiss Axioplan 2 microscope equipped with fluorescence filters and a charge-coupled device camera (Zeiss, Inc., Hamamatsu, Middlesex, NJ) interfaced with the MetaMorph Imaging System (Universal Imaging Corporation, West Chester, PA).

Subcellular Fractionation and Western Blotting

SaOS2 cells transfected with 4 μg of expression vectors in 100 mm plates were harvested 24 h post-transfection for biochemical fractionation as described previously [Zeng et al., 1997; Javed et al., 2005]. For WC lysates, 400 μl of direct lysis buffer [2% SDS, 2M urea, 10% glycerol, 10 mM Tris-HCl (pH 6.8), 0.002% bromophenol blue, 10 mM DTT, and 1× Complete^{TM} protease inhibitors (Roche, Indianapolis, IN) was added to the plate. Cell lysate was collected and samples were immediately boiled for 5 min and stored at −70°C until used. For subcellular fractions, cells were collected in ice-cold PBS containing 1× Complete^{TM} protease inhibitors. Cell pellets were then resuspended in 400 μl of CSK buffer (100 mM NaCl, 0.3% sucrose, 10 mM Pipes, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, pH 6.8) and extracted for 10 min on ice. Samples were centrifuged at 4,000 rpm for 5 min at 4°C and the supernatant (CSK fraction) was transferred to another tube, boiled for 5 min with 6× sample loading buffer, and stored at −70°C. Nuclear pellets were resuspended in 350 μl digestion buffer (50 mM NaCl, 0.3M sucrose, 10 mM Pipes, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, pH 6.8, 160 U DNaseI) and extracted for 30 min at 22°C. Samples were then extracted with 54 μl of 2M ammonium sulfate for 10 min on ice and centrifuged to separate soluble nuclear proteins (chromatin fraction) and the NMIF fraction. The pellet (NMIF fraction) was dissolved in 400 μl of direct lysis buffer. Equal volume (10%) of each fraction was separated on 10% SDS–PAGE, transferred to Immobilon membrane (Millipore Corp., MA), and processed for Western blotting. Blots were probed with 1:3,000 dilution of HA antibody to detect Runx1 proteins or 1:2,000 dilution of mouse monoclonal lamin B antibody (Santa Cruz Biotechnology)

and then with HRP-conjugated donkey anti-mouse antibody. Immunoreactive bands were visualized using the enhanced chemiluminescence kit (Amersham Pharmacia). Densitometric quantification of the autoradiograms was carried with Alpha imager software (Alpha Innotech Corp., San Leandro, CA).

Promoter Reporter Assays

HeLa and CV-1 cells were plated at a density of 1×10^6 cells in 100 mm dishes and transiently transfected at 50% confluency using the calcium phosphate transfection method (Invitrogen, Inc., CA). All transfections were performed with 10 μg of M-CSF receptor c-FMS promoter-luciferase and 1 μg of expression plasmid for Runx1, CBFβ, C/EBPα, and PU.1. The total amount of DNA (20 μg) for each plate was maintained by addition of sheared salmon sperm DNA. Cells were harvested 24 h post-transfection, washed twice with 1× PBS buffer and lysed with 500 μl of reporter lysis buffer (Promega, Inc., WI). Luciferase assays were carried out with 20 μl of cell lysate and luciferase reporter assay reagent (Promega Corp.), and luminometric units were determined by using the Monolight 2010 luminometer (Analytical Luminescence Lab., San Diego, CA).

RESULTS

Molecular Determinant for Intranuclear Trafficking of the Myeloid-Related Runx1 Protein

The C-terminus of Runx1, which contains the NMTS, is critical for its biological activity [Zeng et al., 1997; Chen et al., 1998; North et al., 1999]. Here we addressed the molecular contribution of the NMTS in controlling the gene regulatory function of human Runx1 by mutagenesis. Five different amino-acids within the NMTS were selected for substitution mutation based on their conservation among Runx proteins and their location within putative protein interacting surfaces predicted by the NMTS crystal structure [Tang et al., 1999] (Fig. 1A). We first tested if any of the mutations affected protein expression, stability or subcellular distribution using both biochemical and in situ immunofluorescence approaches. For comparison, we assayed Runx1 deletion mutants containing (Δ385) or lacking (Δ350) the NMTS region. All mutant proteins are expressed at equivalent levels when compared with wild-type Runx1 protein (Fig. 1B). Furthermore all proteins show a punctate nuclear distribution typical of Runx factors in both SaOS2 and HeLa cells (data not shown). We also evaluated the DNA binding activities of the Runx1 mutant proteins translated in an in vitro system or expressed in HeLa cells. Gel mobility shift assays indicate that the wild-type and mutant proteins have comparable DNA binding activities (Fig. 1C). Taken together these results demonstrate that the NMTS mutant proteins retain the cellular distribution, expression, and DNA binding properties of wild-type Runx1.

A key feature of the Runx proteins is their tight association with the nuclear matrix, which is mediated through the NMTS [Zeng et al., 1997; Javed et al., 2000; Choi et al., 2001; Zaidi et al., 2001]. We next determined using biochemical fractionation if these mutations of the NMTS disrupt nuclear matrix association. SaOS2 cells were transfected with equal amounts of the wild-type or mutant expression plasmids and harvested 24 h later. Cell pellets were extracted with low salt and detergent to collect the CSK fraction and were further treated with high salt and nuclease digestion to obtain the soluble chromatin (Chrom) fraction. The residual material is the nuclear-matrix-intermediate filament (NMIF) fraction. For comparison a parallel plate of cells was lysed directly and designated as the WC sample. Consistent with previous observations [Zeng et al., 1997], wild-type and the Δ385 mutant Runx1 are present mainly in the NMIF fraction (Fig. 2A,B). However, deletion of the NMTS region in the Δ350 protein results in its extraction into the CSK fraction, primarily.

We next examined the nuclear matrix retention of the five Runx1 NMTS mutant proteins. Mutation of histidine 378 to alanine (H378A) did not alter nuclear matrix association (Fig. 2C,D). F353A and Y377A mutations resulted in partial extraction of Runx1 protein into either CSK or chromatin fractions, although a significant portion of each was retained in the NMIF pellet. Notably, mutation of tyrosine 380 to either alanine or cysteine caused a substantial loss of NM association (Fig. 2C,D). When protein levels were compared to the NMIF marker Lamin B, we observed a 60%–70% loss of nuclear matrix retention for these NMTS mutants. These results indicate that tyrosine 380 is a critical residue for Runx1 association with the nuclear scaffold.

Using in situ immunofluorescence microscopy, we further examined the consequences of mutating the NMTS for subnuclear localization (Fig. 3). All of the NMTS mutants exhibit a punctate nuclear pattern typical of wild-type Runx1 protein in WC preparations (Fig. 3 and data not shown). Thus the NMTS mutations do not affect the nuclear import or cellular distribution of Runx1 protein. However when the cells were extracted to reveal nuclear matrices, we observed a significant loss of NMIF association only for the tyrosine 380 mutant (Y380A) (Fig. 3A and data not shown). This impaired association is similar to that of the Δ350 mutant in which the entire NMTS is deleted. To quantify our results, we counted the total number of Runx1 expressing cells for both WC and NMIF preparations from two independent experiments (Fig. 4B). While the numbers of positive cells were similar for the wild-type and Y380A mutant protein in the WC samples, in the NMIF preparations there was a significant (70%) decrease in the number of cells positive for the Y380A mutant. We conclude that tyrosine 380 in the NMTS of the human Runx1 protein is a critical molecular determinant for subnuclear distribution.

Subnuclear Targeting of Runx1 Is Required for Transcriptional Activation of the GM-CSF Gene Promoter

We investigated the functional consequences of NMTS-mediated intranuclear targeting of Runx1 and compared the ability of NMTS mutant proteins to regulate multiple Runx target gene promoters. Activation of the TCRβ-promoter reporter by the Y380A mutant Runx1 protein is similar to the wild-type (Fig. 4A). However deletion of the C-terminus of Runx1 results in a small decrease (40%) of T-cell

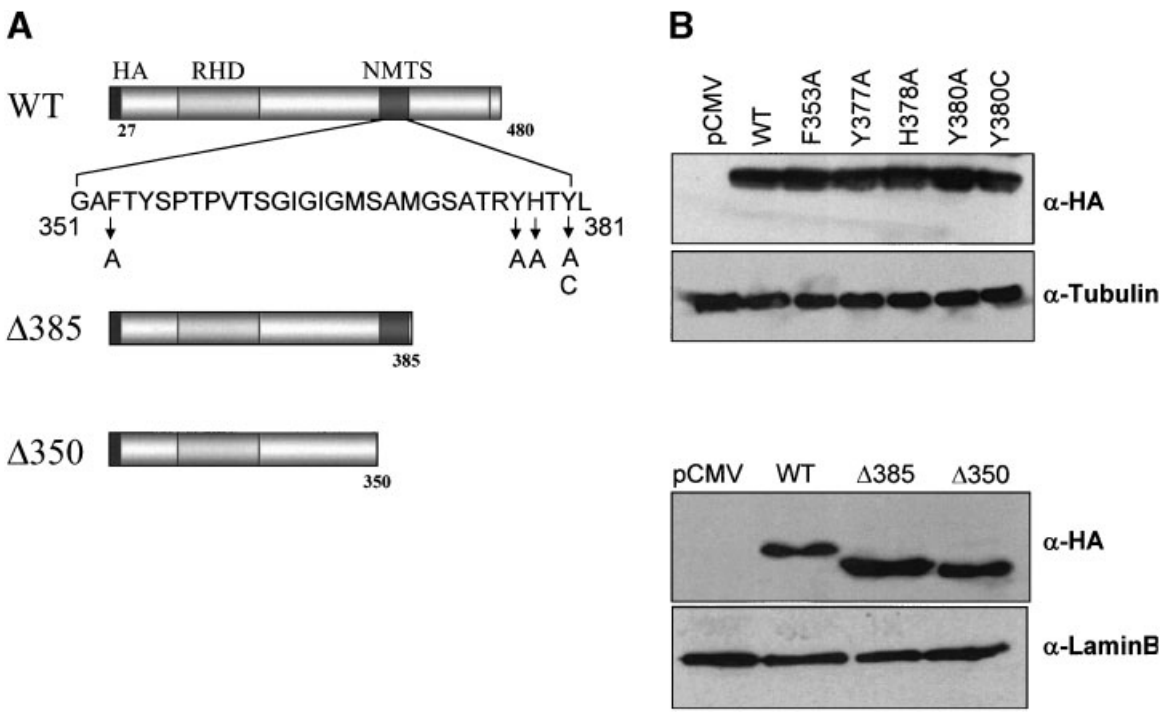


Fig. 1. Mutations in the NMTS domain of the human Runx1 protein do not affect expression or DNA binding. **A**: Diagrammatic description of the wild-type human Runx1 protein. Thirty-one amino-acids that constitute the NMTS domain are shown along with the alanine substitution mutations. The deletion mutants are also shown (Δ385, Δ350). All proteins contain an N-terminal HA epitope; RHD, DNA binding Runt homology domain; NMTS, nuclear matrix targeting signal. **B**: SaOS2 cells plated in 100 mm dishes were transiently transfected with 4 μg of expression plasmid as indicated and lysed directly in plates 24 h later. Proteins were resolved in 10% SDS–PAGE and Western blots were probed with monoclonal HA antibody, stripped and reprobed with Tubulin or Lamin B antibody for loading control. **C**: Electro mobility shift analyses were carried out using 5 μl of in vitro transcribed and translated protein (**left panel**) or 10 μg nuclear extract from transiently transfected HeLa cells (**right panel**). DNA binding reactions were performed in 120 mM KCl for 20 min at 20°C. DNA–protein complexes were resolved in 4% non-denaturing acrylamide gels. Dried gels were exposed to film overnight. Runx1-specific complexes were formed in all lanes. EV, empty vector; NS, non-specific complex.

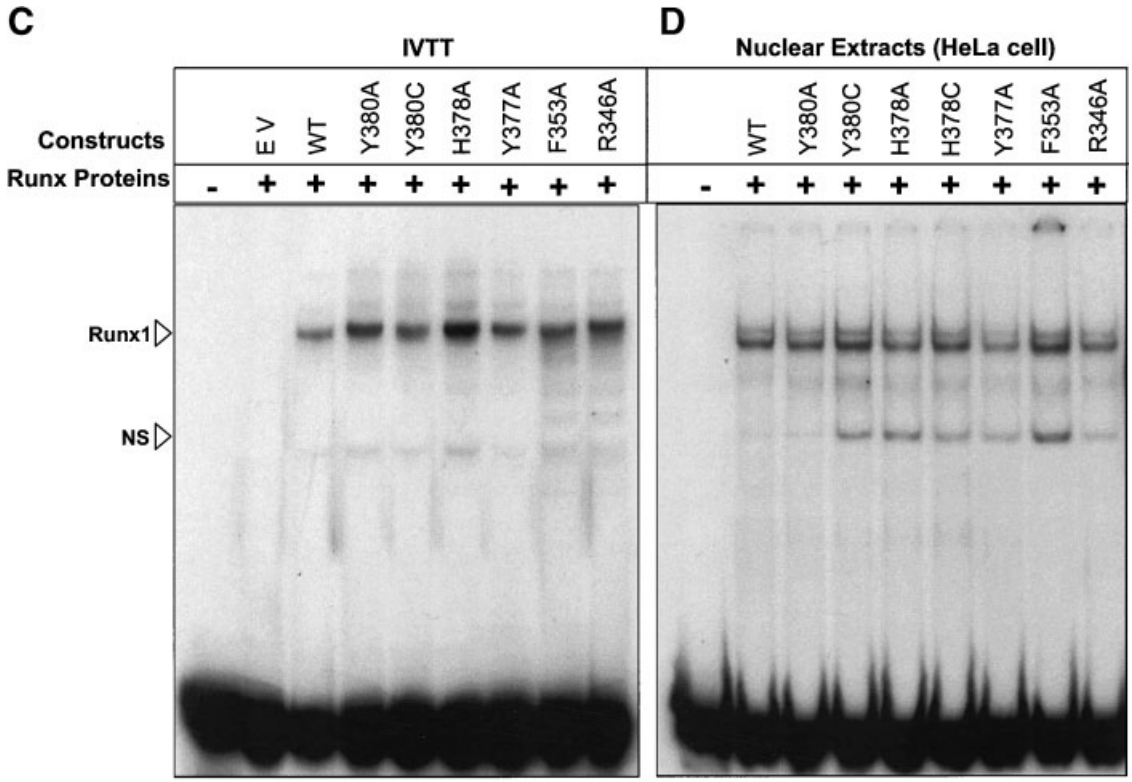


Fig. 1. (*Continued*)

receptor beta-promoter (TCRβ) activity, consistent with the presence of a known activation domain in this region (Fig. 4A). We also find no differences in transcriptional activation of human macrophage colony stimulating factor (MCSF) receptor c-FMS promoter by wild-type and the mutant Runx1 proteins (data not shown). Strikingly, when the GM-CSF promoter was tested, we observed a fourfold transcriptional activation for wild-type Runx1 protein that is completely lost by mutation of tyrosine 380 (Fig. 4B). These data indicate that promoter context may dictate NMTS dependent or independent transcriptional regulation.

We next tested if the compromised matrix association of the Y380A mutant Runx1 protein alters the co-regulatory protein interactions that mediate transcriptional regulation of target gene promoters. For these studies, we used the MCSF receptor c-FMS promoter (Fig. 5), which has previously been shown to be synergistically activated by Runx1, C/EBPα, and CBFβ in CV-1 cells [Petrovick et al., 1998]. We find a similar pattern of synergism among the three factors in both CV-1 (data not shown) and HeLa cells (Fig. 5B). Each factor alone resulted in a modest activation (∼twofold), but when these proteins were expressed in pairs, a variable enhanced activation was observed (fivefold for CBFβ and Runx1, ninefold for Runx1 and C/EBPα, and twofold for CBFβ and C/EBPα). However, co-expression of C/EBPα with Runx1 and CBFβ resulted in a ∼fivefold synergy (∼21-fold over basal). We then examined if mutation of the Runx1 NMTS affects this functional synergism (Fig. 5C). We observed a significant loss of synergistic activation upon deletion of Runx1 amino-acids 386 to 480 (8.9-fold synergy for wild-type and 5.3-fold for Δ385). Deletion of the NMTS domain however showed no further change (compare 5.3-fold for Δ385 to 5.7-fold for Δ350). Moreover, mutation of the NMTS (Y380A) within the context of the full-length Runx1 protein had no affect on this synergism (compare 8.9-fold for wild-type to 8.6-fold for Y380A). A similar pattern of c-FMS promoter activity was observed in CV-1 cells (data not shown). These data demonstrate the requirement of sequences C-terminal to the NMTS domain of Runx1 protein for synergistic activation with C/EBPα.

Synergistic Activation of the MCSF Receptor c-FMS Promoter by PU.1 Requires Runx1 Subnuclear Targeting

Runx1 has been shown to associate physically and interact functionally with PU.1, a known regulator of MCSF receptor c-FMS transcription and hematopoiesis [Petrovick et al., 1998; Follows et al., 2005]. As with C/EBPα, the maximal transcriptional synergy on the c-FMS promoter is achieved together with Cbfβ. We co-transfected Runx1, CBFβ, and PU.1 in both HeLa and CV-1 cells but find synergistic activation of c-FMS occurs only in HeLa cells (Fig. 6A). When the deletion mutants of Runx1 were co-expressed with PU.1 in HeLa cells, we observe a significant loss of synergism upon deletion of the 31 amino-acid NMTS region (compare 6.1-fold synergy for Δ385 to threefold synergy for Δ350). Importantly, we find a similar loss of synergistic activation of the c-FMS promoter with tyrosine 380 mutant Runx1 (compare 6.7-fold synergy for wild-type to 2.8 for Y380A) (Fig. 6B). Taken together, our findings suggest that the NMTS region of Runx1 not only is required for nuclear matrix association but also contributes to transcriptional regulation and functional interactions with co-regulatory proteins.

DISCUSSION

We performed alanine-substitution mutagenesis of the NMTS domain of Runx1 and identified one specific residue that affects both subnuclear targeting and transcriptional regulation of target genes. Using both biochemical and in situ immunofluorescence approaches, we show that mutation of tyrosine 380 does not alter Runx1 expression, cellular distribution, DNA binding or nuclear import, but blocks its subnuclear targeting. We also demonstrate that the GM-CSF gene is a downstream target of Runx1 and that impaired Runx1 subnuclear association results in loss of transcriptional induction of the GM-CSF promoter. Our data further confirm that Runx1, Cbfβ, C/EBPα, and PU.1 functionally cooperate for positive

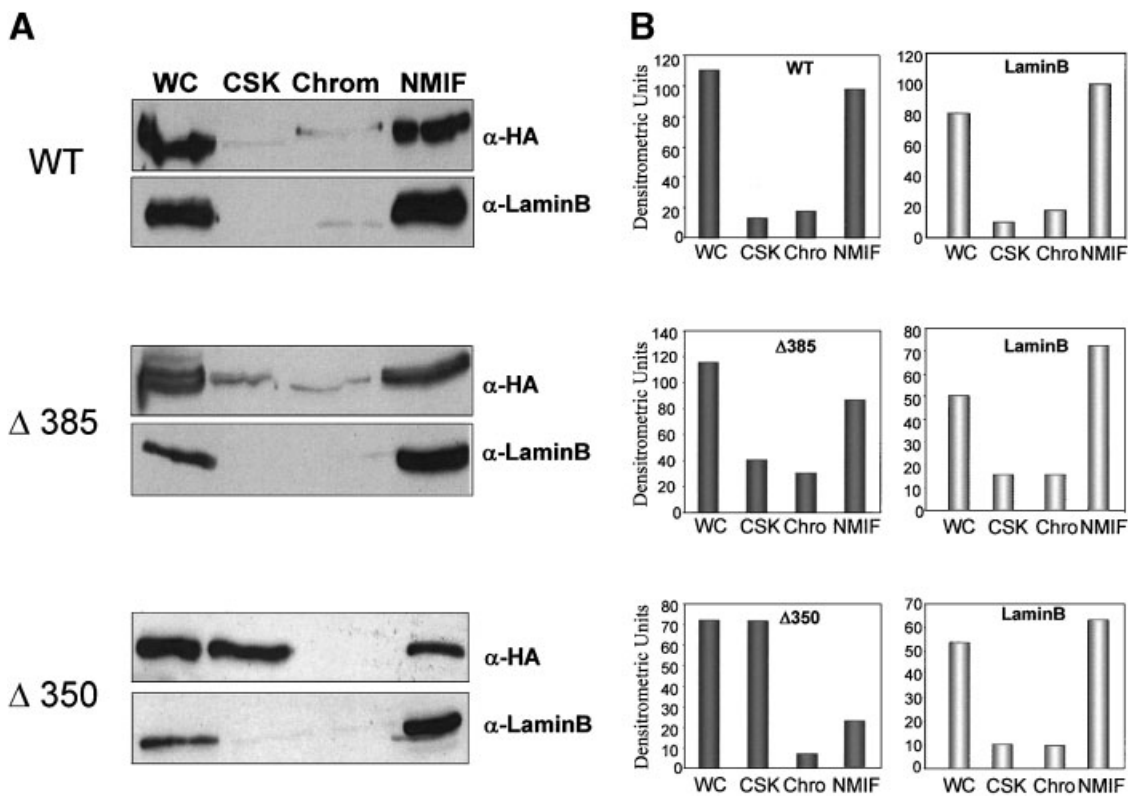


Fig. 2. Mutation of tyrosine 380 in human Runx1 protein results in altered subnuclear distribution. **A, C**: SaOS2 cells cultured in 100 mm dishes were transiently transfected with 4 μg of the indicated expression plasmid. Cells were subjected 24 h later to biochemical extraction as described in Methods. An equal volume (10%) of each fraction was resolved in 10% SDS–PAGE and probed with monoclonal HA antibody. **B, D**: Signals of Western blots were quantified by Alpha imager to determine denstrometric units and absolute values are shown in the bar graphs.

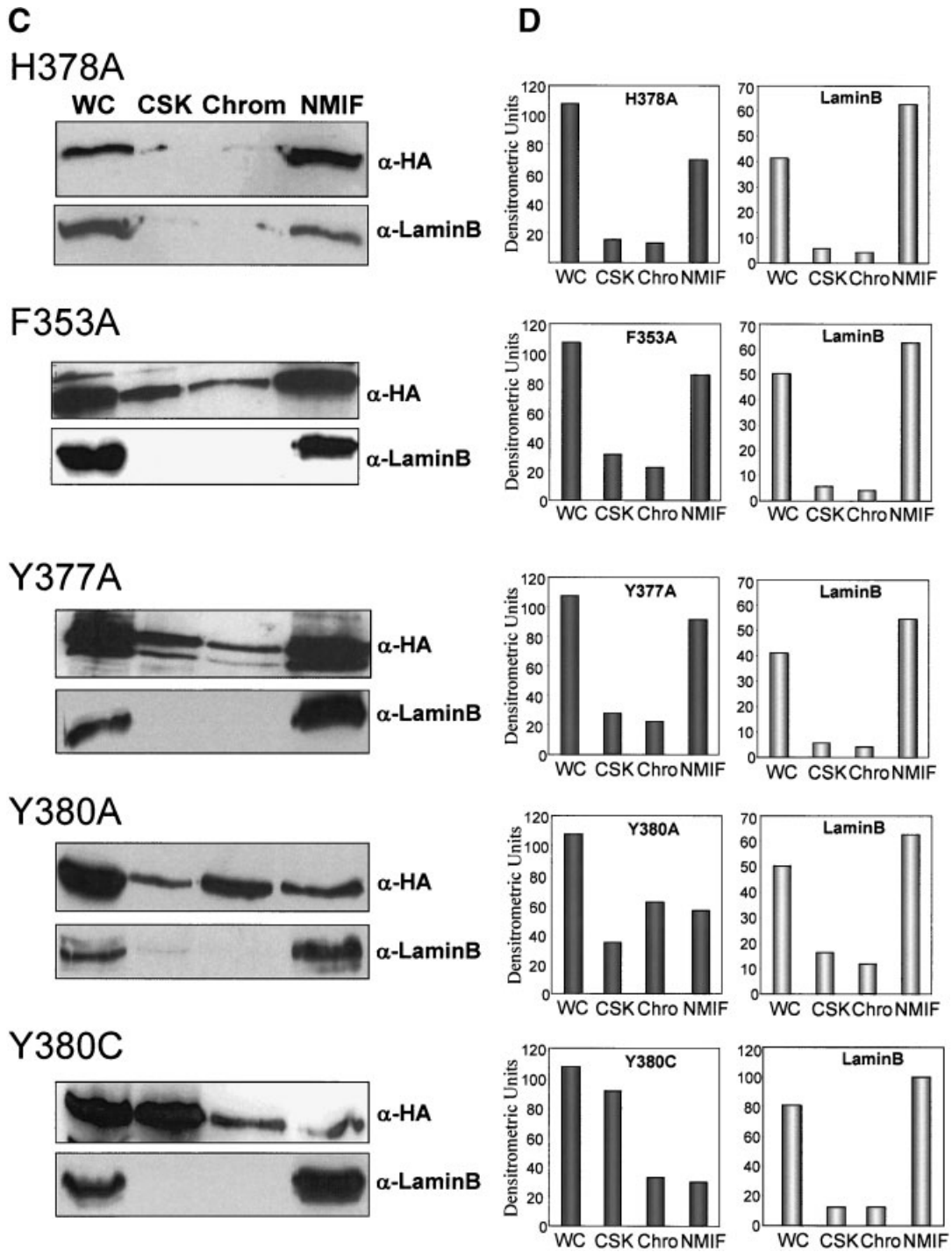
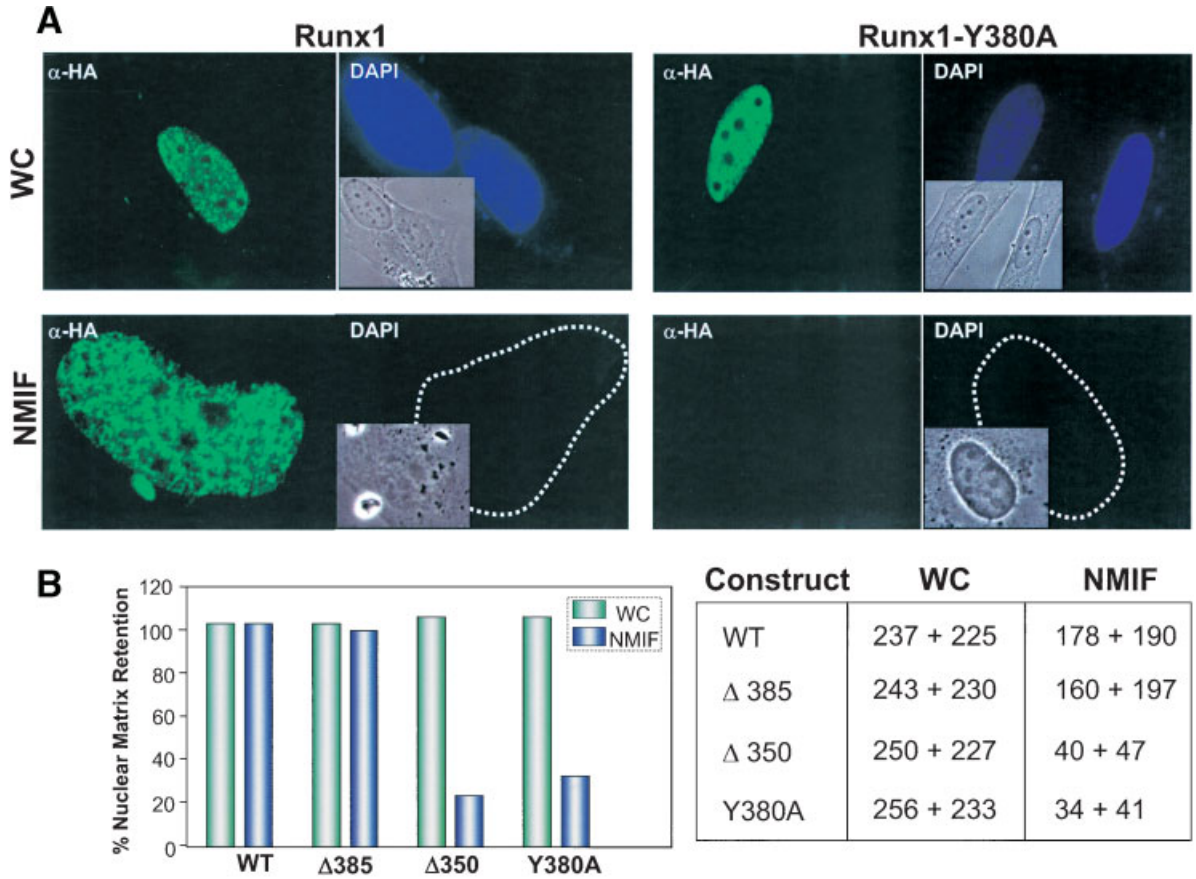


Fig. 2. (*Continued*)

regulation of the MCSF receptor c-FMS promoter and establish that synergistic transactivation depends on the Runx1 NMTS domain. Our results indicate that tyrosine 380 is an essential mediator of functional synergism between Runx1 and PU.1 at the c-FMS promoter.

Runx proteins localize within the nucleus as punctate foci that are involved in transcriptional control and are associated with the subnuclear scaffold, which coordinates the spatial organization of genes and regulatory proteins within the nucleus [Stein et al., 2000a].



Construct	WC	NMIF
WT	237 + 225	178 + 190
Δ 385	243 + 230	160 + 197
Δ 350	250 + 227	40 + 47
Y380A	256 + 233	34 + 41

Fig. 3. Loss of in situ nuclear matrix association of Runx1-Y380A. **A**: SaOS-2 cells grown on gelation-coated coverslips and transiently transfected with 0.5 μg of WT and Y380A mutant Runx1 DNA per well, were processed for whole cell (WC) or nuclear matrix-intermediated filament (NMIF) preparations as described in Methods. Cells were stained with mouse monoclonal HA antibody (1:1,000) to detect tagged Runx1 proteins. Alexa 488 goat anti-mouse secondary antibody was used at a dilution of 1:1,000. Distribution of protein at WC level (**top panel**; 63×) and after extraction with high salt to reveal nuclear matrix (**bottom panels**; 100×). DAPI staining reveals nuclei and is absent in NMIF preparation as chromatin has been removed. Insets show bright field microscopy of the same images. **B**: Positive cells from two coverslips each of WC and NMIF preparation were counted and are plotted as percent nuclear matrix retention.

The subnuclear trafficking of Runx1 and Runx2 depends on their C-terminal NMTS domain [Zeng et al., 1997; Javed et al., 2000; Zaidi et al., 2001]. The C-termini of Runx1 and Runx2 are essential for hematopoiesis and bone formation respectively in mouse models [North et al., 1999; Choi et al., 2001]. We previously found that a 31 amino acid segment in the C-terminus of Runx1 (aa351–381) is necessary and sufficient for retention of Runx1 in subnuclear foci [Zeng et al., 1997, 1998]. To define the contribution of subnuclear targeting to Runx1 mediated control of gene expression, we mutated five amino-acids located on either end of the NMTS domain to the neutral and small alanine residue. In the intact cell all of the mutant Runx1 proteins were focally organized within the nucleus and exhibited DNA binding similar to the wild-type. However, only mutation of tyrosine 380 resulted in a major loss of Runx1 association with the nuclear matrix. The observation that four amino-acids of five do not affect nuclear matrix association suggests that there is only a limited number of principal contact points within the 31 amino-acid NMTS region that mediate interaction with components of nuclear architecture. Thus the subnuclear trafficking function is distinct from other nuclear properties of the Runx1 protein.

We have previously reported that other Runx family members (Runx2 and Runx3) also possess NMTS domains [Tang et al., 1999; Stein et al., 2000a]. Interestingly, the point mutation in Runx2 (Y428A) that is analogous to Runx1 Y380A severely impairs subnuclear targeting [Zaidi et al., 2002]. The Y428A mutant protein

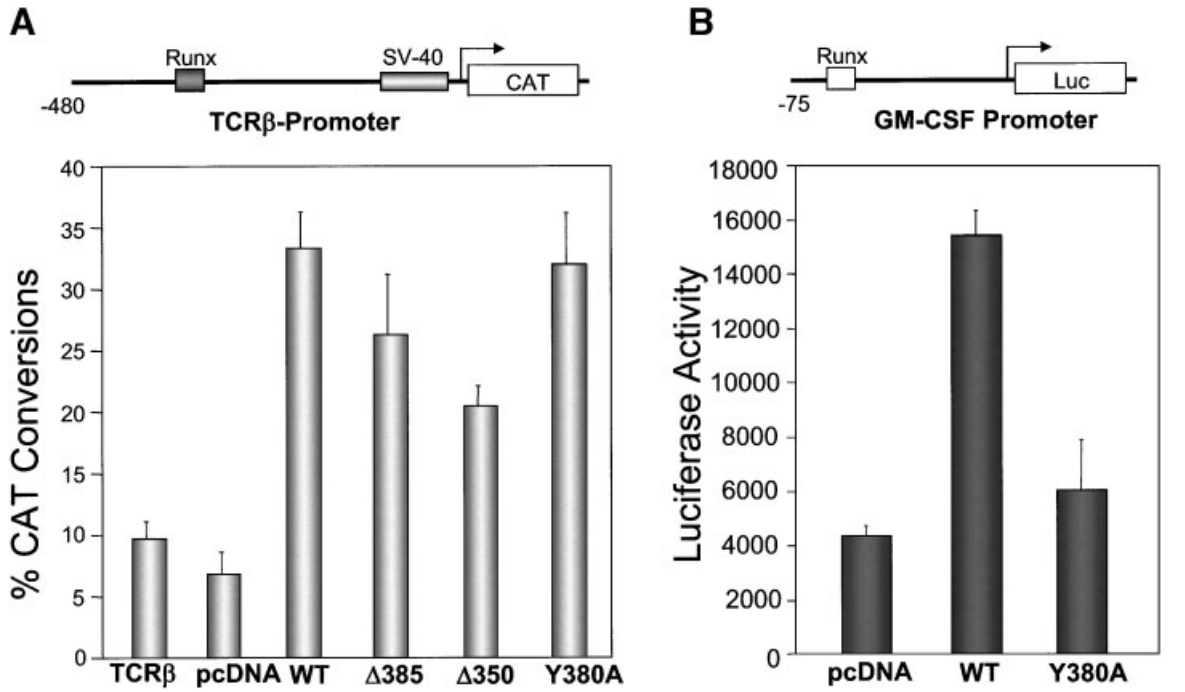


Fig. 4. NMTS-dependent and -independent transcriptional regulation of Runx target gene promoters. **A**: Schematic illustration of the TCRβ-CAT reporter gene (**top panel**). HeLa cells were transiently co-transfected with 2 μg of TCRβ-CAT reporter, 1 μg of expression vector (pcDNA control or wild-type or mutant Runx1) and 50 ng of RSV-Luciferase plasmid as an internal control using SuperFect reagent (Qiagen, Inc.). Cell lysates were prepared 24 h after transfection and used for CAT assays. Samples were resolved on TLC plates and CAT activity was determined by Storm PhosphorImager (Molecular Dynamics, Piscataway, NJ). The graph represents data pooled from three independent determinations with six replicates and normalized to internal control for transfection efficiency. **B**: Position of the Runx motif in the GM-CSF basal promoter is shown diagrammatically (**top panel**). HeLa cells were transiently transfected with 1 μg of GM-CSF-luciferase reporter and 1 μg of wild-type or mutant Runx1 expression vectors. Cells were harvested 24 h later and luciferase activity was determined as described in Methods.

maintains Runx2–Smad5 physical interaction but is defective in the targeting and co-localization of Smad to Runx2 subnuclear foci. This point mutation also abrogates Runx2/Smad co-activation of a reporter gene [Zaidi et al., 2002]. These data suggest that the tyrosine at the C-terminal end of the NMTS domain of the Runx family is a critical residue with key conserved functions related to integration of various signaling pathways.

Specific mechanisms coordinate the spatial organization of genes, transcripts, and regulatory proteins within the nucleus [Stein et al., 2000a]. Consequently, analysis of targeting signals that direct regulatory factors to nuclear matrix associated sites may provide useful information about assembly of the regulatory machinery. In addition to the Runx factors, other nuclear regulatory proteins such as PML, Pit1, YY1, androgen, and glucocorticoid receptors have distinct nuclear matrix targeting determinants [Bushmeyer and Atchison, 1998; Zeng et al., 1998; Mancini et al., 1999; DeFranco and Guerrero, 2000; Stein et al., 2000b; Zaidi et al., 2001]. Thus the nuclear matrix association of multiple factors through their specific targeting signals supports the dynamic organization of spatially distinct and functionally specialized foci within the nucleus.

Subnuclear targeting to specific foci has been implicated in fidelity of tissue specific transcription. The regulatory consequences of NMTS-mediated subnuclear targeting are directly indicated by a significant contribution to activation or repression of hematopoiesis-specific regulatory genes. Our results show that Runx1 responsive GM-CSF promoter activity requires fidelity of Runx1 subnuclear trafficking, as the Y380A mutant Runx1 protein, which possesses intact DNA binding, fails to activate the GM-CSF promoter. More importantly, the subnuclear targeting of Runx1 appears to be an integral component of multi-factor interaction for control of tissue specific gene expression. Our

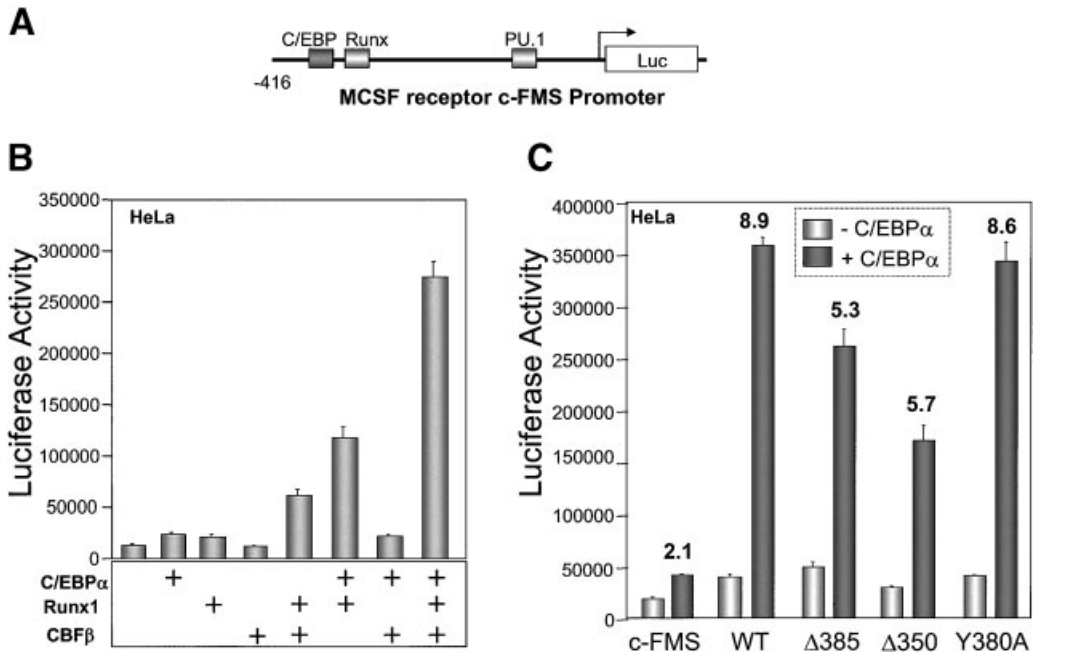


Fig. 5. Runx1, Cbfβ, and C/EBPα are required for NMTS-independent synergistic induction of the MCSF receptor c-FMS promoter. **A**: **Top panel** shows proximal MCSF receptor c-FMS promoter-luciferase gene with C/EBP, Runx, and PU.1 responsive elements. **B**: HeLa cells were transiently transfected with 10 μg of c-FMS-Luc reporter, 1 μg expression plasmid for Runx1, C/EBPα, and Cbfβ or pcDNA empty vector by the calcium phosphate method. Cells were harvested 20 h post-transfection and luciferase activity was determined using the Promega luciferase kit. Data presented are the pooled result of three independent experiments. **C**: HeLa cells were transiently co-transfected with 10 μg of c-FMS-Luc reporter, 1 μg of expression vectors (pcDNA, wild-type or mutant Runx1, C/EBPα, and Cbfβ) by calcium phosphate as described earlier. Cells were harvested 24 h post-transfection and luciferase activity was determined. Fold of synergy for each group is shown above. Each bar represents pooled data of three independent experiments.

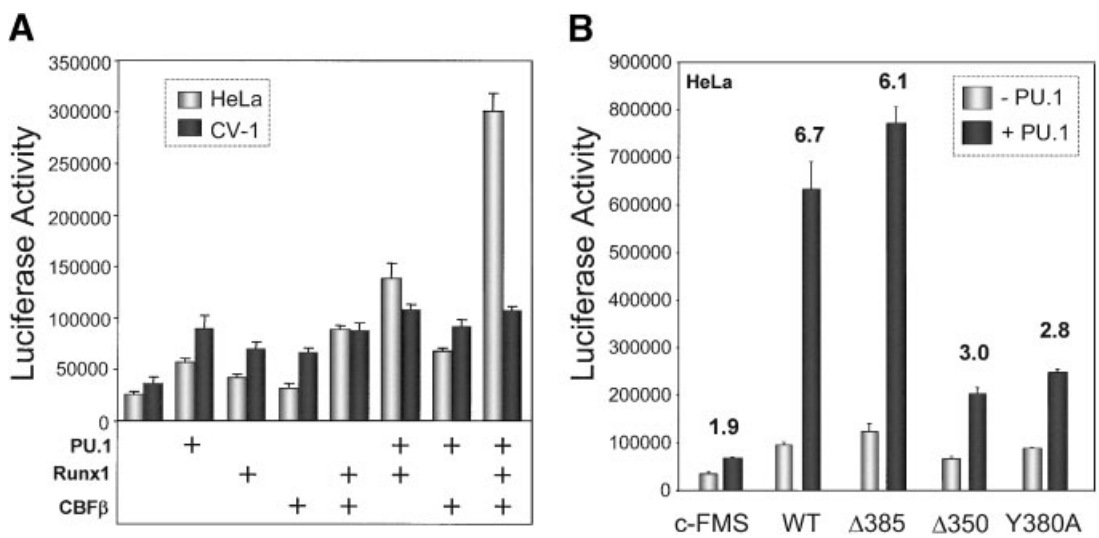


Fig. 6. Synergistic induction of MCSF receptor c-FMS promoter by Runx1, Cbfβ, and PU.1 occurs only in HeLa cells and requires subnuclear targeting of Runx1. **A**: CV-1 or HeLa cells were transiently co-transfected with 10 μg of c-FMS-Luc reporter and 1 μg of expression vector (pcDNA, Runx1, Cbfβ, and PU.1) by the calcium phosphate method. Cells were harvested 20 (HeLa) or 40 h (CV-1) post-transfection and luciferase activity determined. Data represent the pooled results of three independent experiments. Synergistic activation is seen only in HeLa cells. **B**: HeLa cells were transiently co-transfected with 10 μg of c-FMS-Luc reporter and 1 μg of expression vector (pcDNA, wild-type or mutant Runx1, Cbfβ, and PU.1) as above. Cells were harvested 24 h later and luciferase activity was determined. Fold of synergy for each group is shown from pooled results of three independent experiments.

results indicate that functional cooperation of Runx1 with the key hematopoietic factor PU.1 at the c-FMS promoter is completely lost when NMTS residue 380 is mutated. This loss may reflect a lack of physical association between the two factors, as Petrovick et al. [1998] have previously reported that the C-terminus is involved in this interaction. Our observations are in agreement with the concept that regulation of the proximity of genes and cognate transcription factors may be mediated by specific intranuclear targeting signals. Thus molecular alterations that cause misrouting of transcription factors result in compromised gene expression and development of disease.

Functional perturbations of Runx1 are hallmarks of many leukemias and frequently involve alteration of the carboxy terminus. For example, the (8;21) translocation results in formation of an AML1/ETO chimeric protein. Like Runx1/AML1, both AML1/ETO and ETO are nuclear matrix associated [McNeil et al., 1999; Barseguian et al., 2002]. Our previous work has demonstrated that the subnuclear distribution of AML1/ETO is the same as that of ETO but different from that of Runx1/AML1 [McNeil et al., 1999]. Thus AML1/ETO is redirected to different nuclear matrix associated foci and presumably becomes a component of different transcriptional regulatory complexes. This misrouting may play a critical role in the transforming ability of the AML1/ETO oncoprotein and contribute to abnormal gene regulation. Our current data indicate that the tyrosine 380 residue of Runx1 can influence its subnuclear targeting and potentially its participation in gene regulatory complexes [Steffen et al., 2003]. Taken together, our findings suggest that mechanisms for spatial targeting and subnuclear organization of regulatory factors are aberrant in leukemia.

In summary, we have identified a crucial residue within the NMTS domain of Runx1 that is very important for subnuclear distribution and transcriptional regulation of Runx1 target genes. Future experiments will investigate the role of this critical residue in controlling Runx1 function in vivo in mouse models.

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