Transcription factors RUNX1/AML1 and RUNX2/Cbfa1 dynamically associate with stationary subnuclear domains

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Summary

The runt-related transcription factors (RUNX/Cbfa/AML) are essential for cellular differentiation and fetal development. C-terminal truncations of RUNX factors that eliminate the targeting of these factors to subnuclear foci result in lethal hematopoietic and skeletal phenotypes. Here we demonstrate that in living cells the RUNX C-terminus is necessary for the dynamic association of RUNX into stable subnuclear domains. Time-lapse fluorescence microscopy shows that RUNX1 and RUNX2 localize to punctate foci that remain stationary in the nuclear space. By fluorescence recovery after photobleaching assays, both proteins are shown to dynamically associate at these subnuclear foci, with a 10 second half-time of recovery. A truncation of RUNX2, removing its intranuclear targeting signal (NMTS), increases its mobility by an order of magnitude, resulting in a half-time of recovery equivalent to that of EGFP alone. We propose that the dynamic shuttling of RUNX factors in living cells to positionally stabilized foci, which is dependent on the C-terminus, is a component of the mechanism for gene regulation in vivo.

Movies available on-line

Key words: Intranuclear targeting, Runt homology factors, Green fluorescent protein, Fluorescence recovery after photobleaching, Nuclear matrix

Introduction

Regulatory factors involved in cellular processes including gene transcription, DNA replication and RNA processing are localized to distinct domains in the nucleus (Nickerson et al., 1995; Stein et al., 2000b; Lamond and Earnshaw, 1998; Cook, 1999; Berezney et al., 2000; Misteli and Spector, 1998). One example is the family of runt-related transcription factors (RUNX); these proteins localize within the nucleus to punctate foci that are involved in transcriptional control and associate with the subnuclear scaffold designated as the nuclear matrix (Zeng et al., 1997; Zeng et al., 1998; McNeil et al., 1999; Tang et al., 1999; Stein et al., 2000b). RUNX1 and RUNX2 each contain a C-terminal nuclear-matrix-targeting signal (NMTS) that is necessary for directing these proteins to intranuclear foci (Stein et al., 2000b; Bidwell et al., 1994; Zeng et al., 1997; Zaidi et al., 2001). The C-terminus of RUNX factors is also a functional domain for transcriptional modulation by several co-regulatory proteins (Lutterbach and Hiebert, 2000). RUNX proteins are required for tissue-specific gene expression in hematopoiesis (RUNX1) and skeletogenesis (RUNX2) (Okuda et al., 1996; Wang et al., 1996; Stewart et al., 1997; Komori et al., 1997; Otto et al., 1997; Choi et al., 2001). Recently, gene replacement strategies were used to demonstrate that the C-terminal domain of either RUNX1 or RUNX2, which contains the NMTS, is essential for hematopoiesis and bone formation, respectively, in mice (North et al., 1999; Choi et al., 2001). Thus, intranuclear trafficking of RUNX factors appears to be physiologically relevant for transcriptional regulation.

The dynamics by which proteins traverse and localize within the nucleus may be critical for their biological activity. One important question relates to the relative mobility and compartmentalization of RUNX transcription factors in subnuclear foci in living cells. Previous studies using fluorescence recovery after photobleaching (FRAP) assays have shown that green fluorescent protein (GFP) fused to the splicing factor ASF rapidly associates with splicing compartments and is less mobile than GFP alone (Phair and Misteli, 2000). Other nuclear proteins, such as GFP-histone H2B in chromatin (Kanda et al., 1998; Phair and Misteli, 2000) and GFP-lamin B receptor in the nuclear envelope (Ellenberg et al., 1997), are even more immobile. Hence, an emerging concept is that the mobility of proteins is directly coupled to their function and whether or not they are architecturally linked to specific subnuclear compartments.

To understand the relative mobility of RUNX transcription factors in the nucleus and the dynamics of their association with subnuclear sites, we used time-lapse microscopy and FRAP analysis. Our key result is that RUNX1 and RUNX2 transcription factors are targeted to and dynamically associate with common subnuclear foci that remain stationary within the nuclear space. Furthermore, we show that a C-terminal

truncation of RUNX2 that removes the subnuclear targeting signal increases the mobility of the protein to that of EGFP alone. These findings suggest that the dynamic association of RUNX proteins in stationary foci provides a mechanism for formation of regulatory complexes that are essential for RUNX-dependent cell differentiation and embryonic development.

Materials and Methods

Cell culture

Human osteosarcoma SaOS-2 cells (American Type Culture Collection (ATCC), Manassas, VA) were maintained in McCoy's 5A media supplemented with 15% (v/v) fetal bovine serum (FBS), and human cervical carcinoma HeLa cells (ATCC) were maintained in DMEM media with 10% FBS at 37°C. Both media contained 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine.

Plasmids

cDNA for enhanced green fluorescent protein (EGFP; Clontech, Palo Alto, CA) was cloned into pcDNA3 (Invitrogen, Carlsbad, CA) and fused to full-length RUNX2 (amino acids 1-513), RUNX2Δ361 (amino acids 1-361) and RUNX1 (amino acids 27-480) cDNAs. EGFP-RUNX2 and EGFP-RUNX2Δ361 were generated by inserting a PCR-amplified EGFP cDNA into pcDNA3 and then cloning either RUNX2 or RUNX2Δ361 cDNAs. Briefly, the pEGFP-C3 vector (Clontech) was used as a template for EGFP cDNA PCR amplification using the forward primer 5′-TCTAGAGGTACCATGGTGAGCA-AGGGC-3′, which contains a *Kpn*I restriction site and the reverse primer 5′-ATAGAATTCGGATCCCTTGTACAGCTCGTC-3′ with engineered *Bam*HI and *Eco*RI sites. The *Kpn*I and *Eco*RI sites were used to insert the amplified EGFP cDNA fragment into pcDNA3. RUNX2 and RUNX2Δ361 cDNAs (Javed et al., 2001) were subsequently subcloned into this plasmid at the 3′ end of EGFP using the *Bam*HI site and either *Xba*I or *Xho*I sites, respectively. Similarly, the EGFP-RUNX1 plasmid was generated by inserting a PCR-amplified EGFP cDNA product into pcDNA3 and then adding the RUNX1 cDNA. EGFP was amplified by PCR using the oligonucleotides 5′-GGATCCGGTACCATGGTGAGCAAGGGCG-AGGAG-3′ as the forward primer and 5′-GAATT-CTCTAGACTT-GTACAGCTCGTCCATGCC-3′ as the reverse primer. The PCR product was digested with *Kpn*I and *Xba*I and then ligated to a similarly digested pcDNA3 vector to generate pcDNA3-EGFP. The *Xba*I/*Xba*I fragment of RUNX1 (amino acids 27-480) was then inserted into this plasmid. All clones were then manually sequenced using Sequenase version 2.0 kit (Amersham Pharmacia, Piscataway, NJ).

Transcription assays

HeLa cells were plated in six-well plates at a density of 0.6×10^6 cells per plate and transiently transfected at 50% confluency using, in each well, 5 μl of Superfect reagent (Qiagen, Valencia, CA), 500 ng of each expression vector (as shown in Fig. 1D), 50 ng of the minimal osteocalcin (OC) promoter –83-OC-Luciferase (Towler et al., 1994), which was used as a control for transfection efficiency, and 2.5 μg of the rat –1.1 kb OC promoter-CAT reporter gene (Schepmoes et al., 1991). Reporter activities were determined 36-40 hours following transfection. Cells were lysed with 250 μl of 1× Reporter lysis buffer (Promega, Madison, WI). CAT activities were determined in 50 μl of cell lysate and normalized to luciferase values. The significance of these results was assessed using the analysis of variance (ANOVA) test, and the error bars are shown as the standard error of the mean (s.e.m.).

Western blot analysis

HeLa cells were plated at a density of 0.7×10^6 in 100 mm plates and transfected with 10 μg of expression plasmid and 40 μl of Superfect reagent (Qiagen) following the manufacturer's recommendations. Cell pellets were collected 20 hours after transfection and lysed in 300 μl of lysis buffer containing 8 M urea, 0.1 M NaH_2PO_4, 0.1 M Tris-HCl, pH 8.0 and a cocktail of protease inhibitors including, 1.2 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 10 μg/ml trypsin inhibitor, 2 μg/ml TPCK, 40 μg/ml bestatin, 17 μg/ml calpain inhibitor I, and 1 μg/ml E64 (Roche, Indianapolis, IN). For each sample 20 μg of total protein was separated on a 10% SDS-PAGE gel. EGFP proteins were detected using a GFP monoclonal antibody (Clontech; 1:10,000 dilution). RUNX proteins were detected with polyclonal antibodies against either RUNX2 (1:10,000 dilution) or RUNX1 (1:3,000 dilution) (Meyers et al., 1996). Appropriate HRP-conjugated secondary antibodies (1:10,000 dilution) were detected using the Renaissance chemiluminescence kit (NEN, Boston, MA). Cdk2 protein was detected using an α-cdk2 antibody (1:5,000 dilution) as a control for protein loading. The HRP-conjugated secondary antibodies and the polyclonal cdk2 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

In situ immunofluorescence

SaOS-2 cells were grown on 0.5% (w/v) gelatin-coated coverslips and cultured to 70% confluency. Cells were transiently transfected with 0.5 μg of expression plasmid, 3 μl Plus reagent and 2.5 μl Lipofectamine reagent (Life Technologies, Grand Island, NY) following the manufacturer's protocol. Cells were harvested 18-20 hours post-transfection. Whole cell (WC) and nuclear matrix-intermediate filament (NMIF) preparations were performed as previously described (Javed et al., 2000). Briefly, cells were fixed using formaldehyde (3.7%), then permeablized with 0.5% Triton X-100 for whole cell preparations. Nuclear-matrix-intermediate filament preparations were extracted twice for 15 minutes each with CSK buffer [100 mM NaCl, 0.3 M sucrose, 10 mM pipes, pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 2 mM Vanadyl Ribonucleoside Complex (VRC), 0.8 mM 4-(2-Aminoethyl)benzenesulfonyl floride (AEBSF)] and digested twice for 30 minutes each with 400-600 Units/ml of RNase-free DNase I (Roche, Indianapolis, IN) in digestion buffer (CSK buffer with 50 mM NaCl). Cells were extracted with 0.25 M ammonium sulfate in digestion buffer for 10 minutes. Xpress-tagged RUNX2 proteins were detected using a monoclonal α-mouse Xpress antibody (Invitrogen; 1:800 dilution) and a Texas-red-conjugated donkey α-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:200 dilution). Cells were mounted in Vectashield antifade mounting media (Vector Laboratories, Burlingame, CA). Fluorescence and transmitted light images were captured using a Zeiss Axioplan 2 microscope with a 63× Zeiss Plan-Apochromat objective (1.4 N.A.), a 100 W Hg lamp and a Hamamatsu digital charged-couple device (CCD) camera interfaced with the MetaMorph Imaging System (Universal Imaging Corp., Downingtown, PA).

BrUTP labeling

SAOS-2 cells were transfected with 0.25 μg EGFP-RUNX2 as described above and labeled for BrUTP incorporation 18-20 hours following transfection. Cells were incubated for 3 minutes with glycerol buffer (20 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA, 25% glycerol) and then for 3 minutes with glycerol buffer supplemented with 0.05% Triton X-100 and 4 mM AEBSF. Nascent transcripts were labeled with BrUTP for 30 minutes at room temperature in transcription buffer [2× Synthesis buffer (100 mM Tris-HCl pH 7.4, 20 mM $MgCl_2$, 1 mM EGTA, 200 mM KCl, 50% glycerol), 25 μM SAM, 500 μM each of ATP, CTP and GTP (Roche), 750 μM BrUTP (Sigma, St. Louis, MO), 4 mM AEBSF

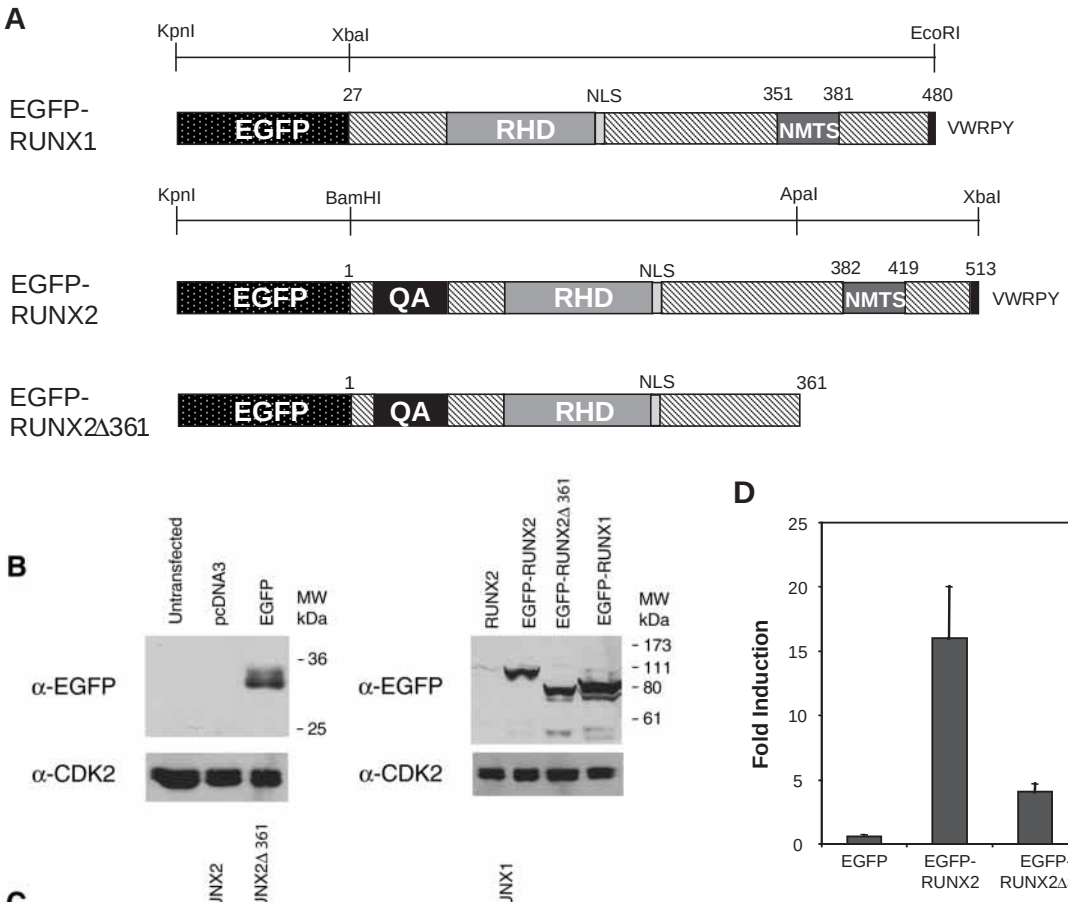

Fig. 1. Structure and expression of functionally active EGFP-RUNX fusion proteins. (A) A schematic of EGFP-RUNX expression constructs. EGFP proteins were fused to the N-termini of RUNX1, RUNX2 and RUNX2Δ361. These constructs were generated using the restriction sites listed above each diagram as described in the Materials and Methods. EGFP-RUNX2Δ361 lacks the C-terminal 152 amino acids of RUNX2 including the NMTS, but retains the RHD and NLS. Conserved functional domains of the fusion proteins are labeled as follows: EGFP: enhanced green fluorescent protein; QA: glutamine-alanine amino-acid stretch, specific to RUNX2; RHD: Runt homology domain; NLS: nuclear localization signal; NMTS: nuclear matrix targeting signal; VWRPY: conserved interacting sequence for TLE/Groucho, a repression protein. (B,C) Western blot analyses are shown of HeLa cell extracts after transfection with either EGFP-RUNX1, EGFP-RUNX2, EGFP-RUNX2Δ361 or EGFP constructs. pcDNA3 empty vector, CMV-RUNX1 and CMV-RUNX2 expression vectors were used as controls. Proteins were detected using either a monoclonal EGFP antibody (B, top), a polyclonal RUNX1 antibody (C, right) or a polyclonal RUNX2 antibody (C, left). Cdk-2 was used as a control for equal protein loading (B and C, bottom panels). Positions of molecular weight markers are indicated on the right side of each blot. (D) CAT activity was assessed from HeLa cell extracts co-transfected with each reporter construct (OC promoter-CAT reporter and –83-OC-LUC) and each expression vector (EGFP, EGFP-RUNX2, EGFP-RUNX2Δ361, RUNX2, EGFP-RUNX1 or RUNX1) as indicated. CAT values were normalized to the luciferase values and fold induction was calculated relative to the empty vectors. The results are means of 15 to 21 samples±s.e.m. $P<0.0001$.

and 40 Units/ml RNase Inhibitor (Roche)]. NMIF extractions were performed on cells as described above. A rat monoclonal α-BrdU antibody (Accurate Chemical and Scientific Corp., Westbury, NY; 1:20 dilution) and an Alexa 568 nm α-rat secondary antibody (Molecular Probes, Eugene, OR; 1:500 dilution) were utilized to detect BrUTP labeling. A Leica SP1 laser scanning confocal microscope interfaced with Scanware software and a Leica 100× Plan Apo 1.4 N.A. objective were used to capture confocal images. Images were taken using an average of four to six sections per cell and 0.365 μm per section. The line scan function in MetaMorph was used to show points of colocalization in a particular area of the nucleus.

Time-lapse imaging

SaOS-2 cells were plated at a cell density of 2×10^6 in 100 mm plates containing gelatin-coated 40 mm coverslips (Bioptechs, Butler, PA). Cells were then transiently transfected using 4 μg of either EGFP, EGFP-RUNX1, EGFP-RUNX2, or EGFP-RUNX2Δ361 expression plasmids, 10 μl Lipofectamine reagent and 20 μl Plus reagent (Life Technologies). Mitochondria were stained 15-18 hours following transfection with 100 nM of Mitotracker Red CM-H2XRos dye (Molecular Probes, Eugene, OR) in pre-warmed completed McCoy's 5A media for 30 minutes at 37°C. The Mitotracker Red dye was used as a marker for viability before and after capturing images. Coverslips were then assembled into the FCS-2 closed cell chamber (Bioptechs,

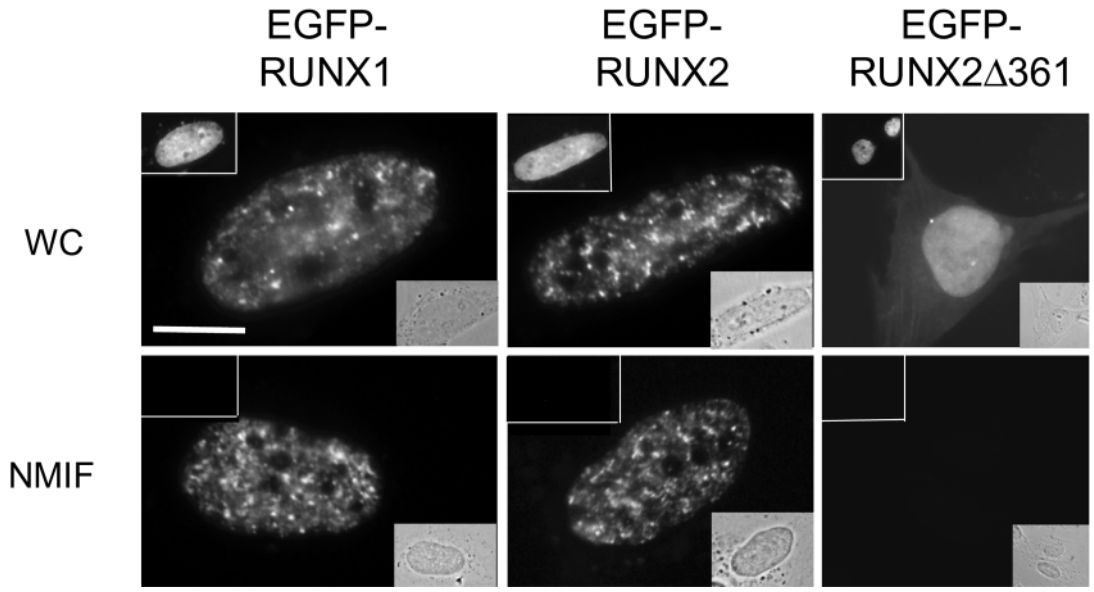

Fig. 2. Absence of subnuclear organization of a mutant RUNX protein in fixed cells. SaOS-2 cells were transfected with either the wild-type EGFP-RUNX1, EGFP-RUNX2 or the mutant EGFP-RUNX2Δ361 expression vectors. Both whole cell (WC) and nuclear-matrix–intermediate-filament (NMIF) preparations were performed as described in the Materials and Methods and show punctate foci for RUNX1 and RUNX2. The green fluorescence of EGFP was captured with a FITC filter (center images). Inserts show DAPI-stained nuclei (top left corners) and transmitted light photographs (lower right corners) of each cell. The scale bar equals 10 μm.

Butler, PA) in which a peristaltic pump (Instech Laboratories Inc, Plymouth Meeting, PA) was used to perfuse complete L-15 media without phenol red (Life Technologies) and 10 nM Mitotracker Red dye through the chamber. Cells were maintained at 37°C using the chamber controller and objective heater controller (Bioptechs). Time-lapse images were captured every 10-30 seconds for 20-30 minutes using the Zeiss Axioplan 2 microscope and a 63× Zeiss Plan-Apochromat objective with a 1.4 N.A. Exposure times for EGFP fusion proteins were 100-200 milliseconds and for the Mitotracker Red dye were 100-500 milliseconds. Adobe Photoshop, MetaMorph, Microsoft PowerPoint and Adobe Illustrator software were used to prepare the digital images.

Fluorescence recovery after photobleaching analysis

SaOS-2 cells were plated in T-25 flasks at a density of 1.2×10^6 cells/flask and cultured until 70% confluency. Expression plasmids (2 μg) were transiently transfected using 5 μl of Lipofectamine and 4 μl Plus reagents (Life Technologies). Transfected cells were incubated at 37°C for 6 hours, trypsinized using 1 ml Trypsin-EDTA (Life Technologies) and plated in coverslip live cell chambers. Cells were incubated overnight at 37°C. The Zeiss Axiovert-10 light microscope was used with a Zeiss 100× Plan-Neofluor N.A. 1.30 lens, adapted with a Roper Scientific (Trenton, NJ) cooled CCD camera with a ST-133 controller and an EEV Type 57 back-illuminated frame transfer chip to capture images. Pre-bleached images were captured using a 200 millisecond exposure time. A small area of the nucleus was photobleached using a 476.5 nm Argon ion laser at 100 mW of power for 100 milliseconds. Images of fluorescence recovery were captured every second for 45 seconds using 200 millisecond exposure times. The half-time of recovery ($t^{\frac{1}{2}}$) was determined by plotting ln (i_∞-i_t) vs. time, where i_∞ is the fluorescent intensity at infinity, i_t is the fluorescent intensity in the bleached area at time (t) and then was calculated as $t^{\frac{1}{2}}$=ln 2 * (–1/slope). The percentage immobile fraction (F) was calculated using the formula:

$$F = \frac{i_{pre}\left(\frac{I_{post}}{I_{pre}}\right) - i_\infty}{i_{pre}\left(\frac{I_{post}}{I_{pre}}\right) - i_\infty + e^b}.$$

The ratio (I_{post}/I_{pre}), where I_{pre} is the pre-bleached intensity over the whole cell and I_{post} is the post-bleached intensity of the whole cell, was used to correct for the extent of photobleaching. i_{pre} is the fluorescent intensity in the pre-bleached area of the nucleus and b is the y-intercept of the graph ln (i_∞-i_t) vs. t. We calculated the recovery rates for both the entire photobleached area and for specific foci in the bleached area. Adobe Photoshop, Microsoft PowerPoint and Adobe Illustrator were used to assemble the digital images. Standard errors were determined as the standard error of the mean (s.e.m.).

Online supplemental information

QuickTime Movies 1A-D (available at jcs.biologists.org/supplemental) show the time-lapse images corresponding to Fig. 5A-D. Time-lapse images were captured for Movie 1A, every 20 seconds for 20 minutes; Movie 1B, every 10 seconds for 30 minutes; Movie 1C, every 20 seconds for 30 minutes; and Movie 1D, every 30 seconds for 30 minutes. QuickTime Movies 2A-D show cells corresponding to those captured in Fig. 6A-D for FRAP analysis. Movie 2D (EGFP alone) shows sequential images captured before bleaching and for every second for the first 10 seconds after photobleaching. Movies 2A-C (EGFP-RUNX fusion proteins) show sequential images captured before bleaching and every second for 45 seconds after photobleaching.

Results

RUNX domains are associated with sites of active transcription

To assess the intranuclear dynamics of RUNX proteins in living cells, we prepared a panel of enhanced green fluorescent protein (EGFP)-RUNX fusion proteins (Fig. 1A). These fusion proteins were expressed at the expected molecular masses (Fig. 1B,C) and functionally activated the osteocalcin-promoter reporter gene (Fig. 1D). To determine the fidelity of subnuclear targeting, we expressed the EGFP-RUNX fusion proteins in SaOS-2 cells and examined their subnuclear distribution by fluorescence microscopy (Fig. 2). Cells were analyzed following either detergent extraction (whole cell, WC) or high salt extraction and nuclease

digestion of chromatin (nuclear-matrix–intermediate-filament preparations, NMIF). The results show that EGFP-RUNX1 and EGFP-RUNX2 proteins produce a punctate nuclear pattern (Fig. 2, WC). Both proteins remain in the nucleus following the removal of soluble proteins and chromatin (NMIF). By contrast, the EGFP-RUNX2Δ361 protein, which lacks the subnuclear targeting signal, produces a diffuse fluorescence pattern in both the cytoplasm and the nucleus (WC). Moreover, EGFP-RUNX2Δ361 is not associated with the nuclear matrix (NMIF). These results indicate that our panel of EGFP-RUNX fusion proteins is functional and that the EGFP moiety does not interfere with the transcriptional activity of RUNX proteins, nor does it interfere with subnuclear targeting.

We determined whether EGFP-RUNX fusion proteins localize to sites of active transcription. Nascent transcripts were labeled with BrUTP in SaOS-2 cells transfected with EGFP-RUNX1 and EGFP-RUNX2. Using confocal microscopy we found that the majority of both the EGFP-RUNX1 and EGFP-RUNX2 foci colocalize with sites of BrUTP incorporation (Fig. 3A,B). Matching red and green peaks in the same positions of the line scans (Fig. 3) demonstrate points of colocalization between EGFP-RUNX1 or EGFP-RUNX2 and BrUTP labeling. These results indicate that active transcription occurs at a significant subset of the punctate RUNX foci.

RUNX1 and RUNX2 are targeted to common subnuclear domains

RUNX1 and RUNX2 have analogous subnuclear targeting signals in their C-termini that are highly conserved. Therefore, in situ immunofluorescence microscopy was used to assess whether RUNX1 and RUNX2 are directed to the same subnuclear domains. We also examined, as a control, whether RUNX2 proteins with different epitope tags are localized in the same subnuclear foci. EGFP- and Xpress (XPR)-tagged RUNX2 proteins (Fig. 4A) or EGFP-RUNX1 and XPR-RUNX2 proteins (Fig. 4B) were co-expressed in SaOS-2 cells and their subnuclear distribution in WC and NMIF preparations was monitored. XPR-tagged RUNX proteins were visualized using a Texas-red-conjugated secondary antibody, and this signal was compared with the intrinsic green fluorescence of EGFP-RUNX proteins. Randomly selected transfected cells (50) were quantified using a dual band pass fluorescence filter (Chroma Technology Corp., Brattleboro, VT, #51006) and evaluated for the extent of signal overlap. The analysis included only those cells that exhibited comparable fluorescence intensities. The

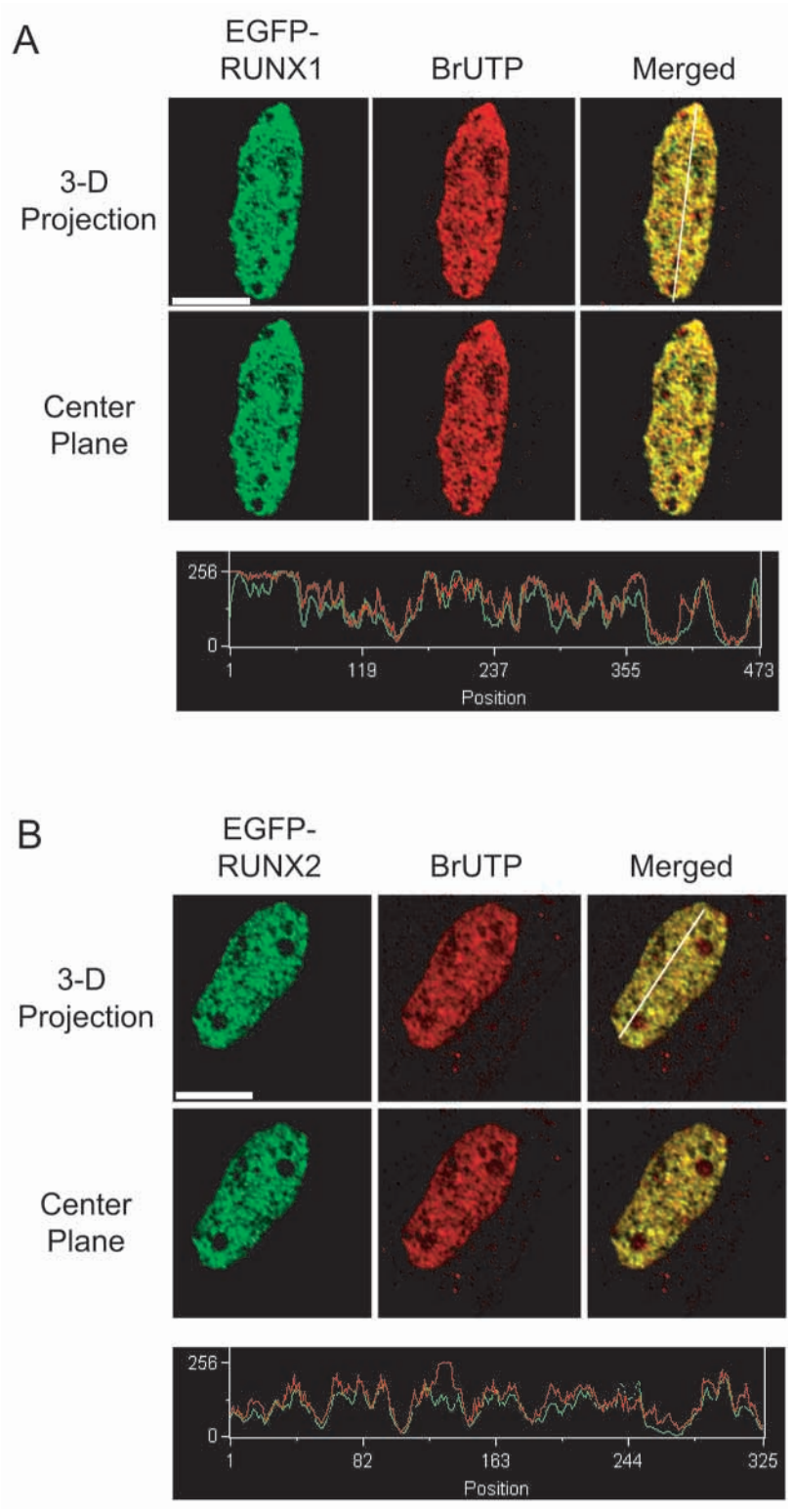

Fig. 3. RUNX proteins localize to sites of active transcription. SaOS-2 cells were transiently transfected with (A) EGFP-RUNX1 and (B) EGFP-RUNX2. Nascent transcripts were labeled with BrUTP for 30 minutes. Confocal microscopy was used to capture images of the intrinsic green fluorescence of EGFP and BrUTP labeling using a rat α-BrdU antibody (red). Merged images show, in NMIF preparations, colocalization of EGFP-RUNX1 or EGFP-RUNX2 with BrUTP incorporation (yellow) in a significant subset of foci. The images shown are 3D projections (top) and a center section (bottom). The scale bars equal 10 μm. Line scans below the images show the extent of colocalization across the center of the nucleus indicated by the white lines of the merged 3D projections in panels A and B.

results show that RUNX2 proteins with two different epitope tags are extensively colocalized in WC preparations (40% of cells: >90% signal overlap; 60% of cells: 60-90% signal overlap) and NMIF preparations (30% of cells: >90% signal overlap; 70% of cells: 60-90% signal overlap). The extent of colocalization of RUNX1 and RUNX2 proteins is very similar to that observed for RUNX2 proteins carrying two distinct tags (compare Fig. 4A with B). All cells in which both proteins were expressed displayed extensive or complete signal overlap. Taken together, these results demonstrate that RUNX1 and RUNX2 are targeted to common subnuclear domains, which is consistent with the amino-acid sequence similarities of their targeting signals (Zeng et al., 1997; Zaidi et al., 2001).

Intranuclear targeting of RUNX2 in living cells is dependent on the C-terminal domain

To evaluate whether RUNX1 and RUNX2 are localized to punctate foci in living cells, we examined the subnuclear organization of EGFP-RUNX1 and EGFP-RUNX2 fusion proteins in SaOS-2 cells using time-lapse microscopy. For comparison, the subnuclear distribution of EGFP alone was analyzed. To assess movement of the foci, we captured time-lapse images every 10-30 seconds for 20-30 minutes using exposure times of 100 or 200 milliseconds. Only those cells that exhibited a significant signal above background were analyzed. The results show that cells expressing EGFP alone produce a diffuse fluorescence signal with comparable intensity in the cytoplasm and nucleus (Fig. 5A; Movie 1A, available at jcs.biologists.org/supplemental). The movement of EGFP proteins is most clearly observed in the time-lapse video micrographs (see online supplemental information). In cells expressing EGFP-RUNX1 or EGFP-RUNX2, we observe punctate domains in the nucleus (Fig. 5B,C), which are very similar to those in fixed preparations (Fig. 2). Furthermore, these foci remain stationary within the nuclear space throughout the 30 minute time of observation (Fig. 5B,C; Movies 1B,C, available at jcs.biologists.org/supplemental). In the timelapse movies of cells expressing EGFP-RUNX1 and EGFP-RUNX2 the foci appear to move slightly. However, this apparent movement is limited relative to the diameter of the nucleus and may represent either changes in the shape of the foci and/or nuclei or movement of cells relative to the plane of focus. These results show that the punctate foci observed in fixed cells are bona fide subnuclear domains that can be visualized in living cells and that these punctate domains are relatively stable in the nucleus over time.

To determine whether the C-terminus of RUNX2 is necessary for the targeting of RUNX proteins to punctate foci in living cells, we analyzed a RUNX2 deletion mutant lacking the C-terminus (EGFP-RUNX2Δ361). This fusion protein displays a diffuse fluorescence signal throughout the cytoplasm and nucleus (Fig. 5D; Movie 1D), which is similar to EGFP alone (compare Fig. 5A and Fig. 5D; Movies 1A,D). However, the fluorescence intensity of EGFP-RUNX2Δ361 was greater in the nucleus than in the cytoplasm (Fig. 5D). Moreover, the subnuclear distribution of EGFP-RUNX2Δ361 is very different from that of wild-type RUNX1 and RUNX2. Thus, our results indicate that the C-terminus is required for localization of RUNX2 into punctate subnuclear domains.

Deletion of the C-terminus of RUNX2 increases the intranuclear mobility of RUNX2 proteins

The relative mobility of the EGFP-RUNX fusion proteins was

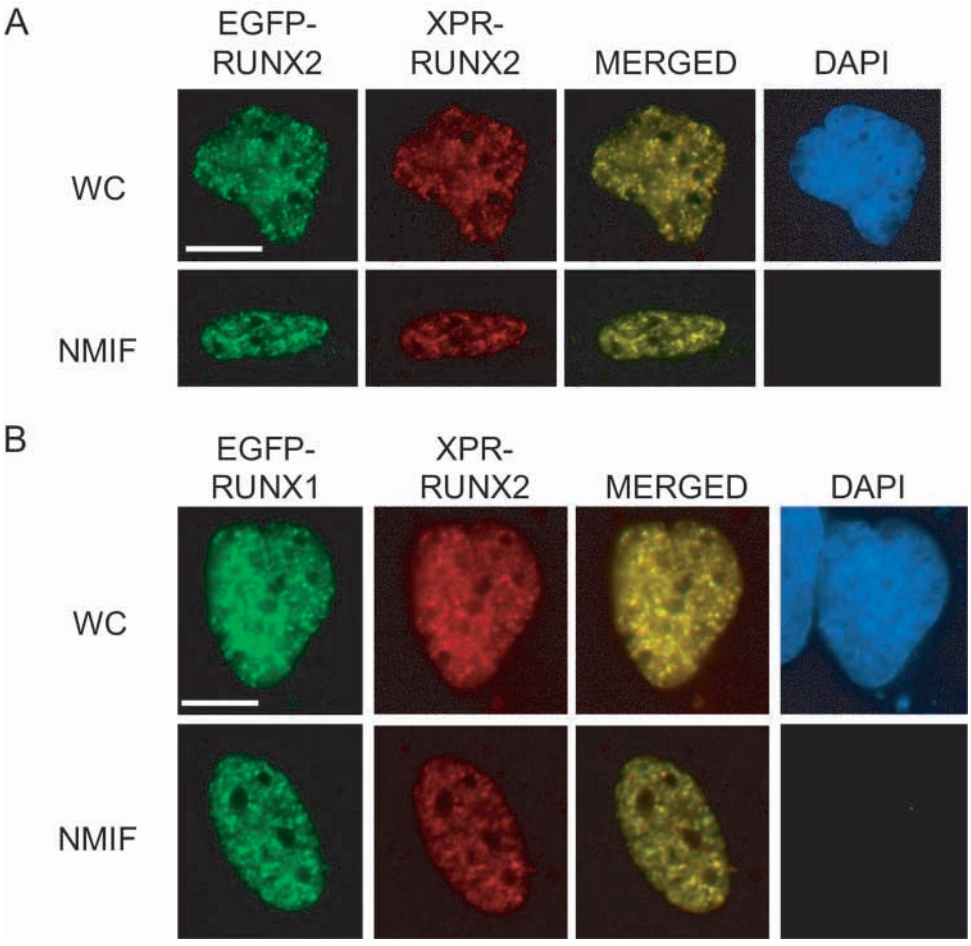

Fig. 4. RUNX1 and RUNX2 colocalize in common subnuclear domains. SaOS-2 cells were co-transfected with (A) EGFP-RUNX2 and Xpress (XPR)-RUNX2 (control) and (B) EGFP-RUNX1 and XPR-RUNX2. Whole cell (WC) and nuclear matrix-intermediate filament (NMIF) preparations were performed. The yellow fluorescence in the merged images indicates colocalization between the EGFP- and XPR-tagged RUNX proteins. Cells were stained with 0.05 μg/ml DAPI. Chromatin-extracted NMIF preparations do not present any DAPI staining as expected. Scale bars equal 10 μm.

determined by using FRAP analysis. SaOS-2 cells were transiently transfected with EGFP-RUNX1, EGFP-RUNX2, EGFP-RUNX2Δ361 or EGFP alone. A defined area in the nucleus of cells expressing each of these proteins was photobleached with a laser beam for 100 milliseconds. Recovery of the fluorescence signal in the entire bleached area was determined by capturing sequential images following photobleaching (Fig. 6). The estimated half-time of recovery of EGFP-RUNX1 and EGFP-RUNX2 proteins, respectively, is calculated to be 10.2±0.6 (n=5) and 10.7±1.1 (n=10) seconds and the mean percent immobile fraction is calculated to be 20.0±2.8% and 26.1±7.1%. These findings were reproduced in independent experiments (n=15). We observed that the punctate domains containing EGFP-RUNX1 and EGFP-RUNX2 proteins recovered after photobleaching with a similar morphology as before photobleaching and in the same location (Fig. 6A,B; see Movies 2A,B, available at jcs.biologist.org/supplemental).

Additionally, we determined the dynamic exchange of RUNX factors at specific foci in the photobleached area (boxed areas in Fig. 6A and 6B show examples). For EGFP-RUNX1 and EGFP-RUNX2 the mean half-time of recovery of the foci was similar to that of the entire photobleached area. The mean percent immobile fraction for the foci is 29.7±4.5% for EGFP-RUNX1 and 32.1±7.9% for EGFP-RUNX2. Thus, these results indicate that RUNX proteins undergo dynamic exchange at the stationary subnuclear punctate domains. RUNX2 proteins that have the C-terminus deleted (EGFP-RUNX2Δ361) exhibit a mobility comparable to that of EGFP alone. EGFP and EGFP-RUNX2Δ361 proteins (Fig. 6C and D; see Movies 2C,D) are completely mobile in comparison with EGFP-RUNX1 and EGFP-RUNX2 proteins (see Fig. 6A,B). The relative recovery curves of EGFP-RUNX1, EGFP-RUNX2, EGFP-RUNX2Δ361 and EGFP proteins are shown in Fig. 6E. The estimated half-time of recovery is <600 milliseconds for EGFP-RUNX2Δ361 and <400 milliseconds for EGFP alone. Both EGFP-RUNX2Δ361 and EGFP are almost completely recovered within 1 second of photobleaching. The increased mobility of RUNX2Δ361 compared with full-length proteins suggests that deletion of the C-terminal domain perturbs the association of RUNX proteins with subnuclear foci in living cells. One plausible interpretation of our finding is that the C-terminus together with its interacting proteins contributes to the stabilization of RUNX subnuclear foci.

Discussion

Here we show that RUNX transcription factors dynamically associate with stationary subnuclear foci in living cells. We find that both RUNX1 and RUNX2 proteins, which support development of different tissues and specification of distinct cell types, localize to the same foci. Furthermore, the C-terminal segment of RUNX factors, which contains the nuclear matrix targeting signal (NMTS), regulates intranuclear mobility by increasing the association of RUNX factors at their subnuclear foci in living cells.

The homologous targeting signals present in the C-termini

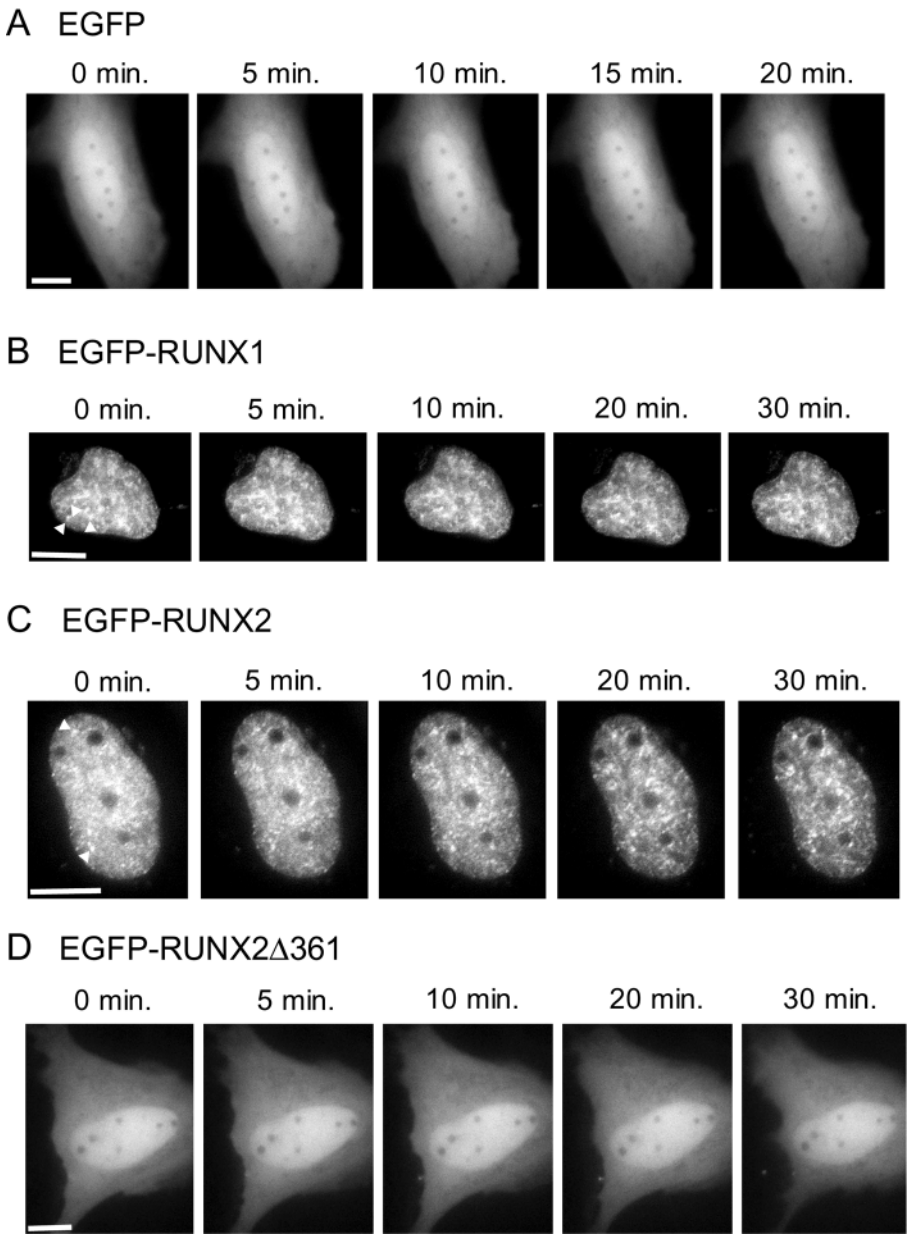

Fig. 5. RUNX1 and RUNX2 subnuclear foci are stationary in living cells within the nuclear space. SaOS-2 cells were transiently transfected with (A) EGFP; (B) EGFP-RUNX1; (C) EGFP-RUNX2 and (D) EGFP-RUNX2Δ361 expression vectors. Time-lapse images were pictures captured at 0, 5, 10, 15 and 20 minutes (A) or 0, 5, 10, 20, and 30 minutes (B-D) using 100 milliseconds (A, B) or 200 milliseconds (C,D) exposure times. Arrowheads in B and C illustrate examples of stationary RUNX subnuclear domains. Scale bars equal 10 μm.

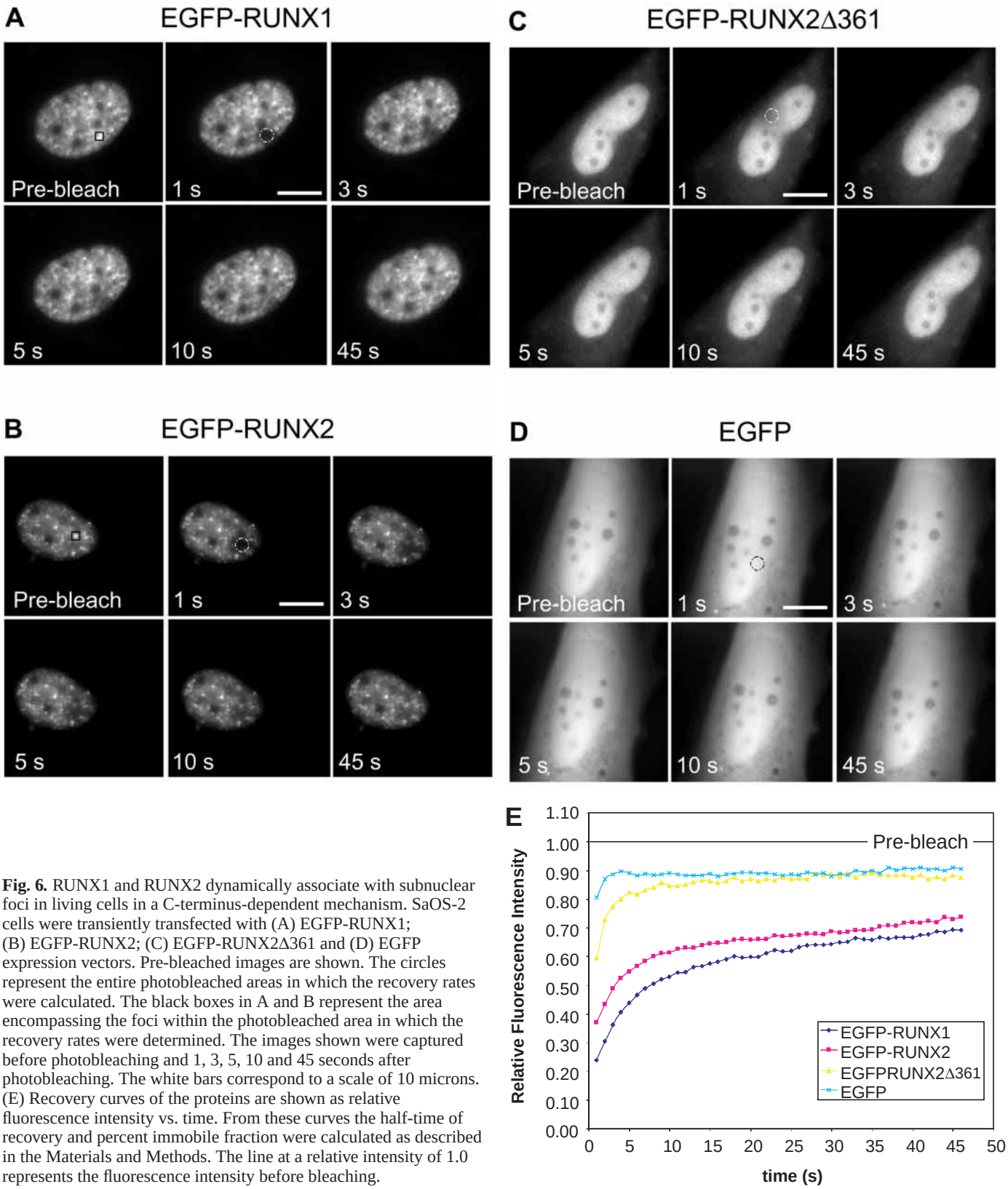

Fig. 6. RUNX1 and RUNX2 dynamically associate with subnuclear foci in living cells in a C-terminus-dependent mechanism. SaOS-2 cells were transiently transfected with (A) EGFP-RUNX1; (B) EGFP-RUNX2; (C) EGFP-RUNX2Δ361 and (D) EGFP expression vectors. Pre-bleached images are shown. The circles represent the entire photobleached areas in which the recovery rates were calculated. The black boxes in A and B represent the area encompassing the foci within the photobleached area in which the recovery rates were determined. The images shown were captured before photobleaching and 1, 3, 5, 10 and 45 seconds after photobleaching. The white bars correspond to a scale of 10 microns. (E) Recovery curves of the proteins are shown as relative fluorescence intensity vs. time. From these curves the half-time of recovery and percent immobile fraction were calculated as described in the Materials and Methods. The line at a relative intensity of 1.0 represents the fluorescence intensity before bleaching.

of RUNX proteins (Zeng et al., 1997; Zaidi et al., 2001) may direct these factors to common subnuclear domains. The C-terminal truncation of RUNX2, which removes the intranuclear targeting signal, results in a lethal phenotype in vivo, suggesting that the C-termini of RUNX proteins are essential for functional activity (Choi et al., 2001). Interactions of the C-termini of RUNX factors with co-repressors and co-activators are important for regulation of transcription (Hanai

et al., 1999; Javed et al., 2000; Lutterbach and Hiebert, 2000). Our results presented here together with previous data suggest that RUNX proteins assemble into macromolecular complexes with co-regulatory proteins at nuclear-matrix-associated sites to regulate gene transcription (Berezney and Wei, 1998; Lutterbach and Hiebert, 2000; Stein et al., 2000a; Javed et al., 2000; Zeng et al., 1997; Zeng et al., 1998; Zaidi et al., 2001). The C-terminal segment of RUNX proteins appears to reduce the mobility of these proteins by mediating association with nuclear architecture, perhaps by supporting in situ formation of complexes. We propose that the functional activity of RUNX proteins at subnuclear foci may critically depend on the spatial-temporal availability of co-factors.

Foci that contain RUNX transcription factors remain stationary within the nuclear space, but are dynamic structures with which RUNX proteins continuously associate and disassociate. Our results suggest that immobilization of these subnuclear domains within the nuclear space may reflect association with the nuclear matrix. Previous commentaries have argued that some subnuclear structures could be artifacts resulting from the fixation and/or extraction procedures (Pederson, 2000), as opposed to functional compartments that support gene expression (Penman, 1995; Stein et al., 2000b; Wei et al., 1998; Cook, 1999; Stenoien et al., 2000; Nickerson, 2001). Here, we show that these RUNX transcription factor domains are observed in both fixed and living cells and that a subset of these foci represent active sites of transcription (as revealed by BrUTP labeling). Thus, our findings demonstrate that the RUNX domains are functional subnuclear structures.

Our observation that RUNX proteins continuously and rapidly shuttle into and out of the dynamic, yet spatially stable foci may reflect a mechanism for the organization and reversible formation of transcriptional complexes in situ. The movement of RUNX transcription factors into these stationary domains occurs within the same time scale as the movement of the splicing factor ASF into splicing factor domains (Phair and Misteli, 2000; Kruhlak et al., 2000). It has been well established that processing of gene transcripts occurs within specific domains (SC-35 ‘speckles’) in the nucleus, which reflects the spatial compartmentalization of the splicing machinery (Xing et al., 1993; Spector, 1993; Phair and Misteli, 2000; Kruhlak et al., 2000). Our data indicate that tissue-specific transcription factors are similarly compartmentalized within the nucleus in living cells. Thus, we conclude that the dynamic association of RUNX factors to stationary subnuclear foci through a common C-terminal signal provides a biological mechanism for the formation of essential tissue-related and gene-specific regulatory complexes.

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