Mitotic partitioning and selective reorganization of tissue-specific transcription factors in progeny cells

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Postmitotic gene expression requires restoration of nuclear organization and assembly of regulatory complexes. The hematopoietic and osteogenic Runx (Cbfa/AML) transcription factors are punctately organized in the interphase nucleus and provide a model for understanding the subnuclear organization of tissue-specific regulatory proteins after mitosis. Here we have used quantitative *in situ* immunofluorescence microscopy and quantitative image analysis to show that Runx factors undergo progressive changes in cellular localization during mitosis while retaining a punctate distribution. In comparison, the acetyl transferase p300 and acetylated histone H4 remain localized with DNA throughout mitosis while the RNA processing factor SC35 is excluded from mitotic chromatin. Subnuclear organization of Runx foci is completely restored in telophase, and Runx proteins are equally partitioned into progeny nuclei. In contrast, subnuclear organization of SC35 is restored subsequent to telophase. Our results show a sequential reorganization of Runx and its coregulatory proteins that precedes restoration of RNA processing speckles. Thus, mitotic partitioning and spatiotemporal reorganization of regulatory proteins together render progeny cells equivalently competent to support phenotypic gene expression.

In the interphase nucleus, many tissue-restricted transcription factors are architecturally organized at punctate subnuclear sites that are associated with the nuclear matrix scaffold (1–18). These nuclear matrix-associated intranuclear foci are linked to transcriptional activation and suppression and contain coregulatory proteins and signaling molecules (19–22, ‡). Compromised nuclear matrix targeting and/or altered gene dosage of regulatory proteins is associated with pathological conditions (23–25). Gross alteration of subnuclear organization (26–30) and relocalization of regulatory complexes occur concomitant with transcriptional silencing during mitosis (31–33); therefore, a fundamental question is how cells restore subnuclear distribution of tissue-specific transcription factors in progeny cells to regulate postmitotic phenotypic gene transcription.

Runx (Cbfa/AML) proteins are tissue-specific transcription factors that control hematopoietic and osteogenic lineage commitment (reviewed in ref. 34). Runx factors bind to DNA in a sequence-specific manner, are targeted to transcriptionally active subnuclear foci, and are required for the maintenance of chromatin architecture of target genes in the interphase nucleus (11–13, 35–37). Perturbed subnuclear organization and/or altered physiological levels of Runx proteins are associated with genetic disorders and tumorigenesis (23–25, 38, 39). Runx protein levels persist through the proliferation of lineage-committed cells (40).

Although the rules that govern mitotic chromosome segregation are longstanding (41), only a limited number of studies have addressed redistribution of regulatory proteins during mitosis (42–46). By the combined use of *in situ* immunofluorescence microscopy and image quantitation, we have documented progressive mitotic changes in the distribution of Runx foci and sequential reorganization of nuclear proteins involved in gene expression. The interphase subnuclear organization of Runx foci is selectively restored in telophase with equal partitioning of the

protein into progeny nuclei. Thus, we show a dynamic spatial distribution of Runx transcription factors in parallel with chromosomal partitioning to sustain balanced expression of phenotypic genes postmitotically.

Materials and Methods

Cell Culture and Cell Synchronization. Hematopoietic (Jurkat lymphoma) and osteogenic (rat osteosarcoma ROS 17/2.8) cells were maintained in F12 medium containing 5% FBS (GIBCO/Life Technologies, Grand Island, NY) and RPMI medium 1640 supplemented with 10% FBS, respectively. ROS 17/2.8 cells were synchronized in early S phase by double thymidine block as described elsewhere (47) and subjected to *in situ* immunofluorescence analyses.

In Situ Immunofluorescence Microscopy. Synchronized cells, grown on gelatin-coated coverslips, were processed for *in situ* immunofluorescence as described (48). In brief, cells were rinsed twice with ice-cold PBS and fixed in 3.7% formaldehyde in PBS for 10 min. on ice. After rinsing once with PBS, the cells were permeabilized in 0.1% Triton X-100 in PBS and rinsed twice with PBSA (0.5% BSA in PBS) followed by antibody staining. Antibodies and their dilutions used were as follows: rabbit polyclonal antibodies against Runx2 [1:200; EMD Biosciences (Oncogene), San Diego], rabbit polyclonal antibody raised against Runx1 (1:25; Geneka Biotechnology, Montreal), tetra-acetylated-histone H4 (1:400; 06-866, Upstate Biotechnology, Lake Placid, NY), p300 (1:400; Santa Cruz Biotechnology), and mouse monoclonal antibody against SC35 (1:200; Sigma-Aldrich). The secondary antibodies used were either anti-mouse Alexa 568 or anti-rabbit Alexa 488 (1:800; Molecular Probes). DNA was visualized by 4',6-diamidino-2-phenylindole staining. Immunostaining of cell preparations was recorded by an epifluorescence Axioplan 2 (Zeiss) microscope attached to a charge-coupled device camera.

Exponentially growing Jurkat cells (6×10^4) were cytopun directly onto slides coated with Cell-Tak (BD Biosciences) and were then subjected to *in situ* immunofluorescence analysis as described above.

Quantitative Image Analysis. We quantitated the relative DNA and protein distribution (mitotic partitioning) in each progeny nucleus. The amount of protein and DNA in each nucleus was measured by using image pixel intensities. The relative protein or DNA distribution between progeny nuclei [the partition coefficient (PC)] was then expressed as the ratio of nuclear signal

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Abbreviation: PC, partition coefficient.

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Fig. 1. Runx proteins partition equivalently in progeny cells following cell division. Jurkat lymphoma cells (A) or ROS 17/2.8 osteosarcoma cells (B) were subjected to *in situ* immunofluorescence microscopy. Runx1 and Runx2 were detected by rabbit polyclonal antibodies followed by the incubation of cells with secondary antibodies conjugated with Alexa 488 fluorochrome. Both Runx1 and Runx2 were distributed at punctate subnuclear foci throughout the interphase nucleus (Upper). A quantitative image analysis was applied to determine the relative levels of Runx in nuclei of the telophase cells ($n = 10$; Lower). We defined a PC that reflects the ratio of integrated signal intensities between progeny nuclei. Both Runx proteins exhibited a PC equivalent to that of DNA, demonstrating that these factors are equally segregated in progeny cells after cell division. Student's *t* test was performed to assess the significance of observed differences.

intensity ($PC = I_1/I_2$, where I_1 and I_2 are integrated pixel intensities of each of the progeny nuclei, i.e., total protein or DNA amount per nucleus); the designation of nucleus 1 versus nucleus 2 was randomly assigned.

We characterized Runx foci in G_2 and telophase nuclei in terms of size, number, and spatial organization. This analysis was carried out in three steps: image deconvolution, determination of the pixel intensity threshold, and image binarization. First, image deconvolution was accomplished by an unsharp mask algorithm. Pixel intensity for image thresholding was defined as the intranuclear pixel intensity level that maximizes the number of detectable foci. In our quantitative analysis, the image binarization is performed by assigning the value of 1 to pixels with grayscale values higher than threshold; all other pixels are assigned the value of 0. Quantitation of nuclear foci from the binary image included determining the number (connected components), the size in pixels (1 pixel = $0.028 \mu m^2$), and the location of each of the foci (centroid image coordinates). The spatial organization of intranuclear foci is expressed as the coefficient of variation of nearest-neighbor distances. For this purpose, the Euclidean nearest-neighbor distance was determined for each focal point by using the centroid coordinates. The mean (M) and SD of foci nearest-neighbor distances within a single nucleus were determined and used to compute the coefficient of variation (CV), where $CV = SD/M$. The image analysis was performed by using the MATLAB image processing toolbox (Mathworks, Natick, MA) and METAMORPH imaging software (Universal Imaging, Downingtown, PA).

Scatter plots were generated to illustrate the coincidence of pixel intensities between Runx and DNA images; each data point represents a corresponding pixel in the Runx and DNA images. The y axis value reflects the pixel intensity from the Runx image, and the x axis reflects the pixel intensity from the DNA image. Data points above the red line are pixels that correspond to Runx foci. Intensity profiles for Runx and DNA images were generated by using METAMORPH imaging software in which a pseudocolor map is applied to pixel intensities (e.g., red = 255 and blue = 0).

Statistical Analysis. Statistical computations were performed in SAS (SAS Institute, Cary, NC). Two-tail paired Student's *t* test was used to compare differences between mean DNA and Runx mitotic PCs. To measure intracellular colocalization between

Fig. 2. Runx foci dynamically redistribute during mitosis. Endogenous Runx2 and tubulin were visualized by *in situ* immunofluorescence in synchronized ROS 17/2.8 cells at indicated stages of mitosis. Runx2 foci shown in deconvoluted images (marked by dotted boxes) in Left are distributed throughout the cell in prophase. This distribution of Runx2 foci changes to predominantly extra-chromosomal in metaphase and anaphase. In telophase, Runx2 foci appear to colocalize with DNA. Tubulin shows characteristic staining throughout mitosis and serves as a marker to identify mitotic stages. The DNA boundaries from 4',6-diamidino-2-phenylindole images are drawn as white dotted lines. Specificity is demonstrated by *in situ* preparations in which the primary antibody incubation was omitted (bottom panel).

Fig. 3. Some of Runx2 foci associate with chromosomes throughout mitosis. Images showing mitotic redistribution of Runx2 foci were subjected to quantitative image analysis. Intensity profiles (center panel in each mitotic stage) of images shown on the left were generated by using METAMORPH imaging software. A scatter plot between the signal intensities of Runx2 (y axis) and DNA (x axis) indicates that Runx2 is associated with DNA in interphase and telophase although this association decreases during prophase-metaphase and anaphase. A subset of Runx2 foci (indicated by arrows) is associated with chromosomes during all stages of mitosis. The red line demarcates the level above which all pixels correspond to Runx foci. The bar at the bottom right represents the pseudocolor map for image intensity.

proteins and DNA, the image cross-correlation analysis was performed by using Pearson's coefficient (3). ANOVA and Tukey's multiple comparison test ($\alpha = 0.05$) were performed to assess the significance of observed differences for protein (Runx2, H4, SC35, and p300)–DNA (4',6-diamidino-2-phenylindole) image correlations between mitotic phases, as well as for number of foci, average size of foci, and spatial distribution between telophase and interphase nuclei. Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

Osteogenic and Hematopoietic Runx Proteins Partition Equally into Progeny Cells. Runx transcription factors are required for lineage commitment and retention of phenotype (49, 50). Stringent transcriptional and translational regulation of Runx proteins indicates that the maintenance of Runx cellular levels is critical for their biological activity. Temporal expression and regulation of Runx factors are documented during development and lineage commitment. We find that Runx protein levels remain constant during and after cell division (data not shown). To assess the cellular organization of the Runx regulatory proteins during mitosis, we examined hematopoietic Jurkat lymphoma and

osteoblastic ROS 17/2.8 cells. These cells express Runx1 and Runx2, respectively, as well as Runx-responsive phenotypic genes (49–51). In addition, Runx1 and Runx2 in these cells exhibit characteristic punctate subnuclear distribution during interphase (Fig. 1 *Upper*). We analyzed Runx proteins in telophase by *in situ* immunofluorescence microscopy. As shown in Fig. 1 *Lower*, Runx1 and Runx2 are present in both telophase nuclei. We next assessed the relative distribution of Runx proteins in progeny nuclei by measuring the ratio of the integrated pixel intensity between postmitotic progeny nuclei. We find that both Runx proteins are distributed equivalently between progeny nuclei. Equal segregation of DNA, as assessed by 4',6-diamidino-2-phenylindole staining, serves as a biological frame of reference and supports our conclusion that both Runx proteins are equivalently partitioned to progeny nuclei during mitosis.

Runx Proteins Undergo Dynamic Alterations in Distribution During Mitosis, and a Subset of Runx Foci Remains Associated with Chromosomes. Runx proteins persist throughout mitosis and are equally partitioned in telophase (Fig. 1 and data not shown). Therefore, we examined the subcellular localization of Runx during suc-

Fig. 4. Runx2 foci are equally segregated to progeny nuclei with restoration of subnuclear organization during telophase. We performed a quantitative image analysis on telophase and G₂ nuclei to assess the number and size of Runx2 domains in parent and progeny cells. The analysis was carried out by image deconvolution (A, left-most panels; for details, see *Materials and Methods*) followed by image thresholding and binarization to define Runx2 domains (A, right panels). The domain number, size, and spatial organization were then calculated in each of the telophase (designated as T1 and T2) and G₂ nuclei analyzed, and mean values are displayed as bar graphs ($n = 10$). Asterisks indicate statistically significant differences between G₂ and telophase nuclei. The error bars represent SEM. We found double the number of Runx2 domains in G₂ nuclei compared with telophase nuclei, while the domain size and spatial distribution remain the same. Conversely, we find equal numbers of SC35 foci, but these foci are smaller and exhibit a different spatial distribution than G₂ nuclei.

cessive mitotic stages by *in situ* immunofluorescence microscopy. Our results show that Runx proteins are distributed as punctate foci during all stages of mitosis (Fig. 2 *Insets*). Concomitant with alterations in nuclear structure during mitosis, a sequential change in the distribution of Runx proteins is observed (see Fig. 2). In contrast to interphase, these foci are no longer completely colocalized with chromosomes during prophase. As mitosis progresses through metaphase and anaphase, Runx foci predominantly exhibit an extra-chromosomal localization. During the anaphase to telophase transition, Runx foci are redistributed, colocalizing with DNA at telophase (Fig. 2). Interestingly, both microscopic observations (Figs. 2 and 3) and image quantitation (Fig. 3) show a subset of Runx foci associated with chromosomes throughout mitosis. We observe similar spatiotemporal redistribution of Runx foci during mitosis of normal diploid cells (data not shown). Specificity of the mitotic localization and chromosomal association of tissue-specific Runx proteins is further indicated by displacement of sequence-specific transcription factors (including Oct1, cFos, SP1, AP2, HSF, etc.) from the chromosomes (52). Thus, Runx proteins are organized as punctate foci throughout mitosis, and these foci are dynamically redistributed during mitotic progression, with consequent equal partitioning of the protein in progeny cells.

Postmitotic Restoration of Runx Subnuclear Distribution. The organization of Runx proteins at subnuclear foci has been linked to

transcriptional control (15, 20). Hence, we determined the extent to which the punctate organization of Runx foci is restored in telophase nuclei. Runx2 was detected by *in situ* immunofluorescence microscopy. Using a quantitative approach, we assessed the number, size, and spatial organization of subnuclear foci (Fig. 4). We detected equal numbers of Runx2 foci in each of the telophase progeny nuclei, and this value was half the number of foci present in G₂ (Fig. 4A). These results are consistent with an equal mitotic partitioning of Runx proteins (Fig. 1). Size and spatial organization of Runx2 foci in telophase nuclei remain equivalent to those in G₂ nuclei (Fig. 4A). It is well established that SC35 subnuclear speckles are associated with RNA processing (reviewed in ref. 53). Therefore, we assessed the parameters of subnuclear organization (i.e., number, size, and spatial organization) for SC35. As shown in Fig. 4B, we do not detect a difference in foci number for SC35, yet these foci are significantly smaller in telophase than G₂ nuclei and exhibit a different spatial organization. Thus, although it has been reported that splicing activity is detectable at this time, the interphase SC35 is not completely restored in telophase (54). Taken together, these findings demonstrate an equivalent partitioning of Runx2 foci into progeny nuclei with selective restoration of Runx2 subnuclear organization.

Sequential Redistribution of Nuclear Proteins Involved in Gene Expression During Cell Division. We assessed the sequential reorganization of Runx2 during mitosis relative to other nuclear

Fig. 5. Chromatin-modifying factor p300 and nucleosomal protein histone H4, but not RNA processing factor SC35, show colocalization with DNA during mitotic progression. Chromatin-modifying factor p300 (A Left), RNA processing protein SC35 (A Right), and tetra-acetylated histone H4 (B) were detected by *in situ* immunofluorescence in synchronized RO5 17/2.8 cells at indicated stages of mitosis. Histone H4 and p300 show a constitutive DNA localization throughout mitosis, while SC35 is excluded from DNA.

proteins that are involved in transcription and RNA processing. We first examined the distribution of Runx coregulatory protein p300 during cell division. In the interphase nucleus, p300 exhibits a punctate distribution and partially colocalizes with Runx2 (data not shown). During metaphase and anaphase, p300 foci, unlike Runx2, remain predominantly localized with the chromosomes (Fig. 5A). The interphase subnuclear localization of p300 is restored in telophase as chromosomes decondense. The extent to which p300 is chromosomally associated may in part be cell type- or reagent-dependent (55). As expected, the nucleosomal protein histone H4 remains tightly associated with chromosomes throughout mitosis (Fig. 5B). In contrast, the SC35 RNA processing factor is not localized with chromosomes during mitosis (Fig. 5A). These results show that a sequential reorganization of Runx2 and its coregulatory protein p300 in progeny nuclei precedes the reappearance of SC35 RNA processing speckles.

The extent to which each of these regulatory factors exhibits a spatiotemporal relationship with DNA during mitosis was quantitated by using image cross-correlation analysis (ref. 3 and Fig. 6). Consistent with our microscopic observations (Figs. 2 and 5), p300 and histone H4 show high correlation with DNA throughout mitosis. By comparison, Runx2 and DNA are highly colocalized only during interphase and telophase, as reflected by maximal correlation coefficient. The Runx2–DNA correlation gradually decreases in prophase and metaphase and increases in anaphase (Fig. 6). In contrast, SC35 and DNA are weakly correlated during mitosis (Fig. 6). Taken together, these findings demonstrate a sequential and selective reorganization of transcriptional regulators and RNA processing factors during progression of cell division.

Fig. 6. Sequential redistribution of nuclear proteins involved in RNA synthesis and processing after cell division. The relationship between DNA and different nuclear proteins during mitosis was confirmed by Pearson correlation analysis. The correlation between image intensities reflects the colocalization of different nuclear proteins with DNA during mitotic progression. Whereas histone H4 and p300 are colocalized to DNA throughout mitosis, SC35 is excluded. Runx2, in comparison, shows a gradual decrease in chromosomal localization until cells enter anaphase. In telophase, Runx2 exhibits a restoration of interphase subnuclear distribution. Asterisks indicate correlations that are significantly different from interphase.

Conclusion

Runx transcription factors provide a model for characterizing the distribution of regulatory proteins to progeny cells during mitosis. Runx proteins are distributed as transcriptionally active subnuclear foci throughout the interphase nucleus that support Runx-dependent integration of regulatory signals, e.g., BMP and Src signals (19, 37, ‡). In this study, we have demonstrated that Runx foci persist throughout mitosis and undergo a spatiotemporal redistribution that results in equal partitioning of the protein into each of the progeny nuclei. Loss of both amount and subnuclear organization of Runx proteins is associated with genetic disorders (23–25). Equal partitioning and a complete restoration of subnuclear organization of Runx foci in telophase provides a mechanism for maintenance of cellular levels and activity of Runx proteins after mitosis. These findings are consistent with a requirement of Runx factors for postmitotic transcriptional control and assembly of multicomponent complexes to regulate Runx-responsive genes. Furthermore, subnuclear organization of Runx foci precedes that of SC35 RNA processing speckles after cell division. Taken together, these findings demonstrate a spatiotemporal partitioning and reorganization of regulatory factors that render progeny cells equivalently competent for the resumption of tissue-specific gene expression.

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