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Organization of transcriptional regulatory machinery in nuclear microenvironments: implications for biological control and cancer

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Introduction

There is growing appreciation for functional interrelationships between nuclear structure and gene expression. Equally important, it is well documented that modifications in nuclear architecture are a hallmark of tumor cells, and there is growing evidence for a functionally compartmentalized assembly and organization of regulatory machinery for combinatorial control of gene expression in nuclear microenvironments (Figure 1). Further understanding of mechanisms modulating the regulation and regulatory parameters of nuclear architecture can provide necessary insight into: 1) proliferation and tissue-specific gene expression, and 2) aberrant gene expression that is linked to the onset and progression of cancer. Such understanding can serve as a platform for novel approaches to cancer diagnosis and therapy.

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We are characterizing cell and tissue microenvironments that contribute to transformation and tumor progression. Our approach is to functionally define intranuclear microenvironments that require structural and functional fidelity for biological control and exhibit striking aberrations with the onset and progression of tumorigenesis.

Focused on obligatory relationships of nuclear structure with gene expression we are experimentally addressing components of nuclear organization that are causally linked to modified transcriptional control in transformed and tumor cells. In this chapter we will provide an overview of our strategies to explore the working hypothesis that parameters of nuclear structure support cell growth and phenotypic properties of normal and tumor cells by facilitating the organization of chromosomes, chromatin, genes, transcripts and regulatory complexes within the dynamic three-dimensional context of nuclear architecture.

Nuclear architecture and subnuclear organization of regulatory functions

The nucleus is highly compartmentalized and contains a multiplicity of specialized functional domains involved in gene expression, DNA replication and DNA repair (reviewed in (Zaidi *et al*., 2004)) (Figure 1). A specific repertoire of proteins and nucleic acids is associated with each domain (e.g., nucleoli/rDNA, splicing speckles/RNA, cajal bodies/U7snRNP/histone genes, Barr body/X chromosome). The nuclear matrix (Berezney and Coffey, 1975) is a principal component of nuclear architecture that has been functionally associated with DNA replication (Vaughn *et al*., 1990, Jackson and Cook, 1986); gene localization (Robinson et al., 1982); imposition of physical constraints of chromatin structure which support formation of loop domains (Nelkin *et al.*, 1980, Ciejek *et al.*, 1983, Mirkovitch *et al.*, 1984, Cockerill and Garrard, 1986); concentration and targeting of transcription factors (Merriman *et al.*, 1995, van Wijnen *et al*., 1993, Bidwell *et al*., 1993, Dworetzky *et al*., 1992, Dickinson *et al.*, 1992); RNA processing and transport of gene transcripts (van Eekelen and van Venrooij, 1981, Xing *et al*., 1993, Jackson *et al.*, 1981, Herman et al., 1978, Blencowe *et al*., 1994, Mortillaro *et al*., 1996, Grande *et al*., 1996); post-translational modifications of chromosomal proteins (Hendzel *et al*., 1994); as well as imprinting and modifications of chromatin organization (Brown *et al.*, 1992) and chromatin remodeling (Reyes *et al*., 1997). Our studies have been focused on the nuclear matrix as a specialized component of nuclear architecture that facilitates localization of genes and cognate gene regulatory factors at specific subnuclear sites (Zaidi *et al.*, 2004).

The classical view of the nuclear matrix, which is based on seminal ultra-structural studies using electron microscopy (Berezney and Coffey, 1975, Nickerson *et al*., 1989), indicates that the nuclear matrix is a heterogeneous internal fibrogranular network surrounded by the nuclear lamina-pore complex. The dynamic properties of the nuclear matrix are illustrated by the rapid disassembly and assembly of the nucleus during mitosis. Furthermore, data from our group and others that were obtained by in situ immunofluorescence microscopy and biochemical approaches have shown that the nuclear matrix during interphase is a dynamic structure that reversibly associates with gene regulatory factors under different physiological circumstances (reviewed in (Zaidi *et al.*, 2004; Stein *et al*., 2003; Zaidi *et al*., 2005; Lian *et al*., 2004)). While it is recognized that the nuclear matrix is an organized composite of nuclear regulatory machineries with modifications in composition and organization that reflect cell function, the extent to which the nuclear matrix is architecturally or activity driven is unresolved.

Molecular and structural determinants of subnuclear targeting of Runx(AML) transcription factors

The overall protein composition of the nuclear matrix is tissue-specific, modified during neoplastic transformation, and developmentally regulated (Stein *et al*., 1995). Furthermore, specific proteins are differentially associated with the nuclear matrix in distinct cell types (van

Wijnen *et al.*, 1993) and during differentiation (Lindenmuth *et al*. 1997; Choi *et al.*, 1998). We have demonstrated that the Runx(AML) class of transcription factors, which are key regulators of cell growth and differentiation during myeloid lineage maturation and mesenchymal tissue development, is associated with the nuclear matrix. We have defined the molecular basis for Runx(AML)/nuclear matrix interactions by identifying a specific (C terminal) subnuclear targeting signal (nuclear matrix targeting signal, NMTS). We showed that the NMTS is a unique and autonomous 30–35 amino acid protein motif that is conserved in Runx(AML) proteins and is both necessary and sufficient for localizing Runx(AML) proteins to nuclear matrix associated subnuclear foci. We determined the crystal structure of the NMTS, which is composed of two loops connected by a flexible linker (Tang *et al*., 1999). This structural model was used as the foundation for mutagenesis studies that targeted a putative protein/protein interface composed of basic and aromatic residues in the two loops to define subnuclear trafficking specificity at single amino acid resolution (Zaidi *et al*., 2006). We observed that a highly conserved Y motif in the C-terminal loop is critical for subnuclear targeting of Runx (AML) and transcriptional control (Li *et al.*, 2005; Zaidi *et al*., 2006, 2001).

Runx1(AML1) related subnuclear targeting defects in Acute Myelogenous Leukemia

The NMTS is located in the C-terminus of Runx1(AML1) and this segment is deleted in the t (8;21) related Runx1(AML1)/ETO fusion protein which results from one of the most prevalent chromosomal aberrations in human acute myelogenous leukemia. Indeed, we have demonstrated that Runx1(AML1)/ETO is misrouted to intranuclear locations that are distinct from normal Runx(AML) foci (McNeil *et al*., 1999) through two ETO-specific targeting signals we discovered (Barseguian *et al*., 2002). Expression of Runx1(AML1)/ETO also alters the subnuclear organization of PML domains (McNeil *et al*., 2000), and changes in subnuclear organization appear to be reversible in leukemia patients that are in remission (Gordon *et al*., 2000). The expression of the Runx1(AML1)/ETO fusion protein may cause a pathological phenotype because (i) the C-terminus of Runx1(AML1) is removed (loss-of-function), (ii) the DNA binding domain of Runx1(AML1) is fused to the unrelated ETO protein (gain-of-function), or (iii) the normal function of Runx(AML) foci is perturbed due to the subnuclear targeting defect (dominant negative effect). We have recently shown that expression of mutant Runx(AML) proteins with a subnuclear targeting defect (Runx(AML) *std* point-mutants) causes a profound alteration of cellular phenotypes in both myeloid progenitor cells and metastatic breast cancer cells (Javed *et al.*, 2005, Vradii *et a*l., 2005). Significantly, the co-expression of a Runx1(AML1) *std* mutant during myeloid differentiation in the presence of the endogenous Runx1(AML1) protein results in a maturation arrest (Vradii *et al*., 2005). These data demonstrate that subnuclear targeting defects of Runx1(AML1) may have dominant negative effects that contribute to the pathology of acute myelogenous leukemias.

Dynamics and mechanisms of subnuclear targeting of Runx(AML) proteins in live cells

Based on the major functional defects that arise from compromised subnuclear targeting of Runx(AML) proteins we have observed in two distinct biological models (Javed *et al.*, 2005, Vradii *et al*., 2005), it is now necessary to define the mechanism by which abrogation of subnuclear targeting causes gene regulatory defects and pathological phenotypes. Our working model is that Runx(AML) factors are architectural proteins that provide DNA-bound scaffolds for the nucleation of multiple co-regulatory proteins into macromolecular complexes. Runx (AML) proteins are known to be associated with many co-regulators, some of which have been shown to be nuclear matrix proteins themselves (Durst and Hiebert, 2004; Westendorf, 2006). Interestingly, we have shown that Runx(AML) proteins are required for chromatin remodeling of tissue-restricted genes (Javed *et al*., 1999). Data from our group and others indicate that many Runx(AML) co-factors are components of large macromolecular complexes capable of modifying chromatin structure (e.g., histone/lysine deacetylases [HDACs] and acetyl transferases [HATs], as well as SWI/SNF components) (reviewed in (Durst and Hiebert,

2004; Zaidi *et al.*, 2005), and unpublished data). We have observed that Runx(AML) recognition motifs are frequently clustered in target promoters, and that Runx(AML) responsive genes are clustered in specific genomic regions. We postulate that the super-clustering of multiple Runx(AML) proteins with macro-molecular co-factor complexes at many loci results in the formation of the subnuclear target sites we refer to as Runx(AML) foci.

Our live cell studies with Runx(AML)/GFP fusion proteins have shown that Runx(AML) foci remain in a relatively steady location (> 30 minutes based on time-lapse fluorescence microscopy). Thus, Runx(AML) foci are positionally stable (Harrington *et al.*, 2002). However, photo-bleaching results clearly establish that Runx(AML) foci are dynamic entities and that Runx(AML) proteins are rapidly discharged and recruited (half-time of recovery ~5 to 10 sec). Runx(AML) proteins that are defective for subnuclear targeting, yet competent for DNA binding, exhibit a much faster exchange rate that is comparable to that of GFP (half-time of recovery <500 milliseconds) (Harrington *et al*., 2002).

The alteration in exchange rates of Runx(AML) mutant proteins in live cells is reflected by differences in the detection of wild type and *std* mutant Runx(AML) in subcellular fractions by Western blot analysis. The biochemical phenotype of all *std* mutants is decreased detection (or absence) in the nuclear matrix fraction and increased detection in non-matrix fractions. Most Runx(AML) *std* mutants exhibit a punctate subnuclear distribution in whole cells but are typically not detected in the nuclear matrix as examined by immunofluorescence microscopy. Therefore, we postulate that Runx(AML) *std* mutants form meta-stable complexes and that the recruitment of these mutant complexes at subnuclear foci containing wild type Runx1(AML1) alters the micro-environment and abrogates fidelity of gene regulation.

Mitotic control by Runx(AML) proteins and association with microtubules

We recently demonstrated that Runx(AML) proteins are associated with metaphase chromosomes at multiple distinct foci (Zaidi *et al*., 2003). This key finding is a paradigm shifting observation that indicates a regulatory function for sequence-specific transcription factors at genomic loci in otherwise massively condensed metaphase chromosomes. Our data provide compelling support for the hypothesis, which has been discussed by Workman and colleagues (John and Workman, 1998), that specific genomic regions may maintain a locally active chromatin conformation during mitosis through proteins that support the bookmarking of genes (Young *et al.*, 2005a, 2005b) and mediate their post-mitotic transcriptional regulation. We have observed that Runx(AML) proteins interact with microtubules.

Runx(AML)/microtubule interactions may regulate the cytoplasmic/nuclear equilibrium of Runx(AML) proteins, and nuclear compartmentalization is a prerequisite for subnuclear targeting during interphase. The association with microtubules may also facilitate the interaction of Runx(AML) proteins with the mitotic apparatus during metaphase. Our quantitative microscopic analysis has revealed that Runx(AML) proteins partition equivalently into progeny cells during cell division (Zaidi *et al*., 2003). Therefore, we postulate that the association of Runx(AML) proteins with metaphase chromosomes and the mitotic apparatus may secure the appropriate mitotic distribution of this regulatory protein. We are further assessing the biological significance of the association of Runx(AML) proteins with microtubule-related cytoarchitecture.

Active retention of phenotype during cell division: Runx(AML) transcription factors remain associated with gene promoters within the condensed mitotic chromosomes

During cell division there is a cessation of transcription that is coupled with chromosome condensation. Resumption of gene expression post-mitotically requires restoration of nuclear

organization and assembly of regulatory complexes. We have found that transcription factor stability during mitosis and biochemical association with chromatin in mitotic cells suggest that association of regulatory factors with metaphase chromosomes may be a biologically relevant component of gene regulatory functions following mitosis (Young *et al*., 2005a). We propose that transcription factors that include the Runx(AML) proteins have an active role in retaining phenotype during cell division to support lineage-specific gene expression in progeny cells. Such factor-dependent mitotic control provides an epigenetic mechanism for the retention of gene expression patterns and lineage commitment during cell division.

From a fundamental regulatory perspective, the contributions of nuclear organization in control of replication and transcription are evident despite the gaps in our understanding of the rules that govern gene expression. Equally important, insight into regulatory parameters of organization and assembly of machinery for transcription, replication and repair in nuclear microenvironments provide a new dimension to cancer diagnosis and targeted therapy.

Functional implications of nuclear organization for biological control and cancer

Biochemical, in situ microscopic, and in vivo genetic evidence demonstrate the requirement for intranuclear placement of regulatory complexes, which is directly linked with cellular response to physiological cues and is essential for combinatorial control of gene expression. Subnuclear targeting of transcription factors and regulatory proteins provides a mechanistic link between the temporal-spatial regulation of gene expression and architectural organization of regulatory complexes within the nucleus. It also establishes the requirement for delivery of regulatory proteins to the right place at the right time.

Summary

The nucleus is highly compartmentalized and contains a multiplicity of specialized functional microenvironments involved in gene expression, DNA replication and DNA repair. We have demonstrated that the Runx(AML) class of transcription factors, which are key regulators of cell growth and differentiation during myeloid lineage maturation and mesenchymal tissue development, is associated with the nuclear matrix by a specific C terminal subnuclear targeting signal (Nuclear Matrix Targeting Signal, NMTS). We have shown that expression of mutant Runx(AML) proteins with a subnuclear targeting defect causes a profound alteration of cellular phenotypes in both myeloid progenitor cells and metastatic breast cancer cells. We have established that Runx(AML) proteins are associated with metaphase chromosomes at multiple distinct foci indicating a regulatory function for sequence-specific transcription factors at genomic loci and a mechanism for mitotic distribution of regulatory factors. We propose that transcription factors that include the Runx(AML) proteins have an active role in epigenetically retaining phenotype during cell division to support lineage-specific gene expression and cell fate determination in progeny cells. The contributions of nuclear organization in control of replication and transcription are evident despite gaps in our understanding of the rules that govern gene expression. Insight into regulatory parameters of organization and assembly of machinery for transcription, replication and repair in nuclear microenvironments provide a new dimension to cancer diagnosis and targeted therapy.

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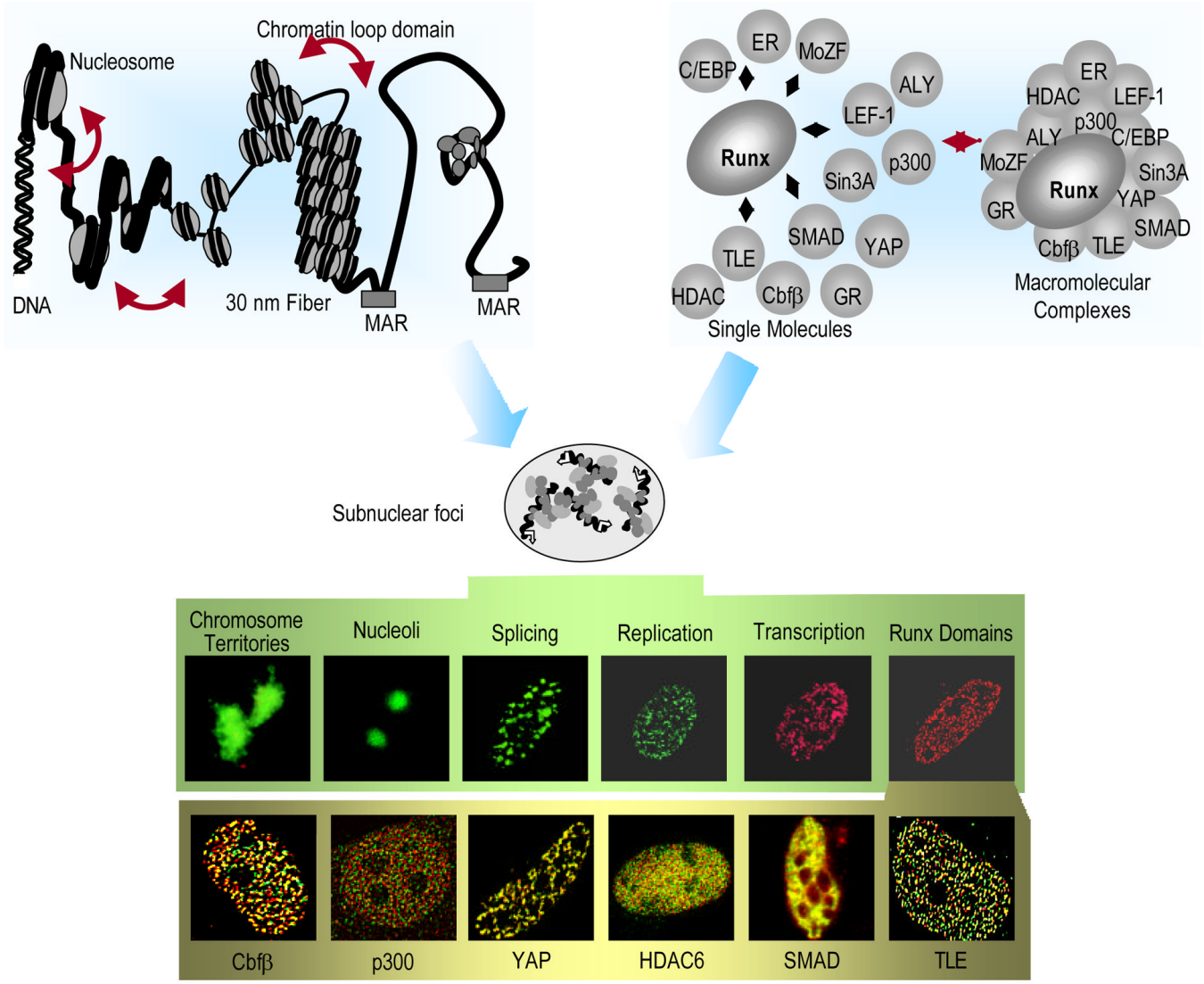

Fig. 1. Levels of nuclear organization

The linear placement of DNA-regulatory elements in gene promoters constitutes the primary level of nuclear organization. The distance between these regulatory sites is intricately regulated by the packaging of DNA into nucleosomes and higher order chromatin structures (left, upper panel). Scaffolding nuclear proteins, such as RUNX, provide structural platforms for the assembly of multiprotein supercomplexes to facilitate the combinatorial control of gene expression (left, bottom panel). Genes and macromolecular regulatory complexes together give rise to dynamic nuclear microenvironments in the nucleus. RUNX bodies are nuclear microenvironments that contain various co-regulatory proteins that are involved in gene activation, as well as repression, chromatin remodeling and cellular signaling (immunofluorescence images on the right, shaded yellow). RUNX was visualized using the Alexa 488 secondary antibody in all images and the proteins were detected using Alexa 568 fluorochrome-conjugated secondary antibodies, as indicated.