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NUP98 and CBP/p300 in normal development and leukemia

Lawryn Heath Kasper
NUP98 and CBP/p300 in normal development and leukemia

een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

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Cover: Blood smear from a p300\textsuperscript{lox/lox} mutant mouse exhibiting variation in erythrocyte size and increased numbers of platelets, some of abnormal size (see Chapter 6.)
For Mom, Dad and Ed.
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Chapter 1

Outline of this Thesis
The studies in this thesis began with the observation that the nucleoporin NUP98 was at the breakpoint of t(7;11)(p15;p15) chromosomal translocations found in patients with acute myeloid leukemia. We set out to determine the molecular mechanism by which the translocation-generated fusion protein NUP98-HOXA9 might contribute to the generation of leukemia. To this end, we developed an in vitro cell transformation assay and determined which domains of the fusion protein provide critical oncogenic properties. We found that NUP98-HOXA9 had transformation potential that was dependent on the presence of the (phenylalanine-glycine) FG repeat-containing domain of NUP98 and that this potential correlated with the ability of the NUP98 portion to bind the transcriptional coactivators, CBP (CREB-binding protein) and p300. In addition, inhibition of CBP caused repression of transactivation activity associated with the NUP98 portion of the fusion. Thus, NUP98-HOXA9 appears to behave as a transcription factor and this aberrant function may be critical for its ability to contribute to leukemogenesis (Chapter 3).

To obtain additional insight into the mechanisms by which the NUP98 proto-oncogene contributes to the development of various leukemias, we then performed two distinct studies. First, we identified proteins that normally interact with the amino-terminal portion of NUP98 that is present in all fusion proteins, arguing that such proteins could contribute critical oncogenic properties, just like CBP and p300. We found that Rae1, a protein of previously unknown function, binds to the GLEBS (Gle2p binding sequence)-like motif of NUP98. We performed a detailed functional analysis of the Rae1 protein, which revealed that Rae1 is a nuclear export factor for mRNA (Chapter 5). Second, we analyzed the physiological role of NUP98 by using a gene knockout approach in the mouse. Because NUP98 is essential for mouse embryogenesis, we studied cells derived from homozygous mutant embryos to determine how the nuclear pore complex (NPC) and transport through it was affected by the loss of NUP98 (Chapter 4).

We were further intrigued by three observations: first, the potential interaction between the FG repeats of NUP98 and CBP in the leukemic process, second, that CBP and p300 have been found to bind multiple transcription factors involved in hematopoiesis (reviewed in Blobel, 2000) and third, that CBP and p300 themselves can occur as fusion proteins found in leukemias (Borrow et al., 1996; Ida et al., 1997; Kitabayashi et al., 2001; Panagopoulos et al., 2001; Taki et al., 1997). These observations prompted us to investigate the normal roles of CBP and p300 in blood cell development and function. To this end we made mice bearing specific mutations in a transcription factor-binding domain of p300 and CBP, termed KIX, which were predicted to interfere with their ability to bind to CREB and c-Myb, two transcription factors whose knockouts in mice produce hematopoietic defects, but not with the overall structure and function of the proteins (Mucenski et al., 1991; Rudolph et al., 1998; Sumner et al., 2000) (Chapter 6). This approach allowed us to dissect out the function of a specific
domain of p300 and CBP by studying the effect of disrupting its interaction with two factors involved in hematopoiesis.

References


Chapter 2
Introduction
2.1 Hematopoiesis

The constant renewal of cells required in hematopoiesis makes it particularly susceptible to acquired genomic mutations that can cause dysregulation that leads to diseases such as leukemia. Studies of myeloid leukemia-associated chromosomal translocations have shown that many of the genes involved have functions in the regulation of transcription, frequently as transcription factors, but also as coactivators and tyrosine kinases that can modulate transcription factor function. Fusion proteins coded by these translocations often retain transcriptional activity, and in some cases may gain new functions not normally possessed by either of the fusion partners (Scandura et al., 2002). Thus, the fusion proteins can lead to dysregulated transcription and subsequent leukemogenesis.

Regulation and dysregulation in hematopoiesis

The maintenance and regulation of the hematopoietic process is critical for the health of an organism. In this section, I will first summarize three aspects of hematopoiesis where regulation is most critical: cell proliferation, survival, and differentiation. I will then outline the various hematopoietic lineages that are derived from pluripotent hematopoietic stem cells. Finally, I will briefly describe the kinds of diseases that can result from dysregulation of hematopoiesis.

Hematopoiesis requires appropriate cell proliferation, survival and differentiation.

Normal hematopoiesis is regulated by complex signal transduction pathways that transmit extracellular signals from the cell surface to the nucleus. These extracellular signals, such as hormones, cytokines, and growth factors, bind to specific receptors on the cell surface and thereby initiate intracellular signaling pathways. In response to the binding of their ligands, most receptors undergo a conformational change that either allows them to interact with or activate a kinase or causes the receptor itself to gain a kinase activity. This associated or intrinsic kinase activity begins a phosphorylation cascade, ending in the activation of one or more transcription factors. These transcription factors in turn induce the transcription of their target genes. Indeed, cell fate is largely determined by the type of transcription factors expressed in response to extracellular signals. In this manner, the key aspects of hematopoiesis, proliferation, survival and differentiation are regulated.

Cell proliferation is controlled through regulation of the cell cycle.

The appropriate proliferation of hematopoietic cells is necessary for an organism to maintain normal hematopoiesis throughout its lifespan. Hematopoietic stem cells must be able to replicate themselves effectively without losing their pluripotent status. Additionally, an organism must be able to produce sufficient
numbers of mature blood cells to meet its ongoing needs, as well as more immediate needs brought about by illness or injury. Experimental evidence suggests that the ability to proliferate is greatest in the most pluripotent stem cells and is gradually lost as a cell becomes more differentiated (Zhu and Emerson, 2002). Thus, hematopoiesis involves a constant balancing act between the requirements of proliferation and differentiation in the blood cell population.

Cell proliferation is controlled through regulation of the cell cycle. The cell cycle consists of four phases, S phase during which DNA is replicated, M phase during which the cell undergoes mitosis, and G1 and G2 phases in which the cell prepares to enter S and M phases respectively. In addition, non-dividing cells are said to be in phase G0. Non-proliferating progenitor cells remain arrested in G0 until an extracellular signal sends them into active cell cycling. Checkpoints during G1 and G2 phases are present to ensure that cells with DNA damage are not replicated unless the damage is repaired. This helps to prevent mutations from accumulating in the genome. Once a cell has entered S phase it is committed to a round of cell division and the presence or absence of the cytokine or growth factor signal is no longer relevant. However, when the cell again reaches G1, if it is to undergo another round of division, the appropriate signals must be present or the cell will again move into G0 (Sherr, 1996). In living organisms, the amount of time it takes to progress through one round of the cell cycle does not appear to be regulated by external signals (although it can be in vitro), thus the ability to shift cells from G0 to G1 and vice versa provides the primary means by which the rate of cell proliferation is controlled (Pardee, 1989)

A stem cell that has committed to proliferation can produce daughter cells, which have the same pluripotent potential as itself (self-renewal) or which have lost the ability to contribute to some lineages (differentiation). Cells respond to both proliferation and differentiation signals during the G1 phase of the cell cycle. Changes in the dynamics of the cell cycle caused by transcription factors could influence the decision of a stem cell to self-renew or to differentiate. For instance, the transcription factor, Notch-1, when expressed constitutively in human hematopoietic progenitor cells cultured in vitro, produces a shortening of the G1 phase of the cell cycle and also inhibition of cell differentiation, which could be explained by the reduced time frame during which cells can respond to pro-differentiation signals (Carlesso et al., 1999). Moreover, transcription factors, such as c-Myb, that are upregulated in early hematopoietic cells, favor cell proliferation by increasing progression into the cell cycle, while other transcription factors which are expressed in more mature hematopoietic cells have the opposite effect (Friedman, 2002).
Cell survival is mediated by extracellular signals.

Also critical to the maintenance of appropriate numbers of differentiated and progenitor hematopoietic cells is the regulation of cell survival. Appropriate cytokine and growth factor expression is necessary for cells to survive, although expression of certain genes can increase (e.g. the pro-apoptotic protein, Bax) or decrease (e.g. the anti-apoptotic factor, bcl-2) the requirement for these signals. Although it is crucial to the organism that healthy, viable hematopoietic cells survive, it is also necessary that old, damaged or superfluous cells be destroyed. Cells that die accidentally, through necrosis, can cause harm to surrounding cells by releasing their intracellular contents and causing an inflammatory response. Programmed cell death or apoptosis provides a means of destroying unnecessary or unwanted cells without causing harm to neighboring cells, by inducing the cell to shrink, the nucleus to fragment, and a signal to be produced that is recognized by macrophages which then consume the cellular remnants (Wickremasinghe and Hoffbrand, 1999). It is clearly advantageous to an organism to be able to rid itself of unwanted cells in a planned manner that does not cause collateral damage to adjacent cells.

Extracellular signals and the transcription factors they induce are responsible for determining cell differentiation fates.

In addition to being able to control the overall number of hematopoietic cells, organisms must be able to regulate the differentiation and renewal of hematopoietic stem cells. Hematopoietic stem cells are capable of both renewing themselves and differentiating into all of the mature hematopoietic cell lineages. The differentiation process is believed to involve multiple decision points at which a cell will undertake a certain differentiation pathway and consequently lose its ability to progress down other differentiation paths. Once a cell has begun to differentiate, it cannot go back; differentiation is a one-way street. There is, however, evidence that some signs of lineage-specificity, such as expression of lineage-specific genes, precede actual lineage commitment (Rothenberg, 2000). As cells become more differentiated, they lose their ability to self-renew and to proliferate and begin a maturation process that is specific for a particular lineage. The processes by which hematopoietic stem cells become specific lymphoid, myeloid or erythroid lineage cells are only partially understood.

It has been observed that a specific set of cytokines, cell surface receptors, and transcription factors is associated with the differentiation of each of the mature hematopoietic lineages; however, the understanding of the exact role of each of these factors in the commitment and differentiation processes is still being debated. Cytokines and growth factors have been proposed to have either an instructive or a selective role in lineage commitment (Morrison et al., 1997). In the instructive model, cytokines and growth factors directly influence the cell to become specific for a certain lineage by inducing the expression of lineage-
associated transcription factors. In the selective model, cells specify for a certain lineage by a cell autonomous and possibly stochastic process, and the presence of cytokines and growth factors determines only which lineages can survive without contributing to the decision to commit (Cross and Enver, 1997; Morrison et al., 1997).

The prevailing view of the role of transcription factors in lineage commitment has changed somewhat in recent years. Although many transcription factors have been considered to be lineage-specific, studies have indicated that the earliest progenitor cells express RNA transcripts of multiple lineage-associated transcription factors, albeit at low levels (Hu et al., 1997; Krause, 2002). It has been speculated that this low-level transcription of genes coding for transcription factors associated with many different lineages represents a primed state from which the cell can choose any of several different fates. Differentiation to a specific cell lineage would then require the up-regulation of the relevant lineage-specific transcription factors and the repression of others (Hu et al., 1997). The means by which this specification would take place is unknown; however, most theories fall into either a stochastic or deterministic view of differentiation. One stochastic model predicts that the expression of one set of transcription factors may exceed a threshold level by chance and that the increased expression of these transcription factors would have an inhibitory effect on the expression of other transcription factors (Enver and Greaves, 1998). Alternatively, extracellular signals could cause the upregulation of certain transcription factors over others and thus determine cell fate.

**All hematopoietic lineages have a common stem cell precursor.**

The earliest progenitor in the hematopoietic lineage is the hematopoietic stem cell, which is capable of infinite self-renewal and of differentiating into all of the hematopoietic lineages. One of the earliest differentiation choices made by a hematopoietic stem cell is thought to be the decision to commit to either the lymphoid lineages or the myeloid/erythroid lineages. This is supported by the discovery of two types of progenitor cells, the common lymphoid precursor (CLP) and the common myeloid precursor (CMP). In bone marrow transplantation assays in mice, the CLP was found to give rise to only lymphoid lineage cells, while the CMP was found to repopulate only the myeloid and erythroid lineages (Akashi et al., 2000a; Akashi et al., 2000b; Kondo et al., 1997). CLPs and CMPs can be distinguished from each other by the transcription factors and cytokine receptors they express. CLPs have the interleukin-7 (IL-7) receptor on their surface and express the transcription factors PU.1 and c-Myb strongly, while CMPs express the erythropoietin (EPO), and c-mpl or thrombopoietin (TPO), receptors and show upregulation of the transcription factors, SCL, GATA-2, NF-E2, and c-Myb (Akashi et al., 2000a; Akashi et al., 2000b). Other research on hematopoietic stem cells implies that lymphoid T- and B-cells come from separate myeloid/T-cell and myeloid/B-cell progenitors (Katsura, 2002). This model may be supported by findings that
suggest that under certain conditions B-cells can be induced to become macrophages and by the existence of mixed myeloid/B-cell and myeloid/T-cell leukemias (Katsura, 2002). A third model explains these potentially conflicting results by hypothesizing two pathways for macrophage commitment, one through myeloid precursors and an alternate pathway from a B-cell precursor (Rothenberg, 2000). It remains to be seen whether any of these models truly represent the commitment steps taken by hematopoietic stem cells.

Figure 1. Schematic of hematopoietic lineage differentiation from a common hematopoietic stem cell. Abbreviations used: CLP, common lymphoid precursor; CMP, common myeloid precursor; GMP, granulocyte/monocyte precursor; MEP, megakaryocyte/erythroid precursor; NK cell, natural killer cell. Adapted from Akashi et al., Nature (2000).

**Lymphoid Lineage**

Whether or not they come from the same immediate precursor cell, B- and T-lymphocytes resemble each other in both function and certain structural characteristics. B- and T-cells are both central players in the immune response, although they act in different ways. B-cells contribute to the immune response
by producing antibodies to foreign antigens, while T-cell act as either CD4+ helper cells, which assist other cells in making their response to a foreign antigen, or CD8+ cytotoxic cells, which recognize and kill infected cells. The antigen receptors on B- and T-cells bear striking similarities. In order for the mature receptor to be expressed, both the B-cell receptor, immunoglobulin, and the T-cell receptor (TCR) genes must undergo V(D)J recombination, which requires the rearrangement of the V (variable), D (diversity), and J (joining) gene segments (Schatz, 1999). This rearrangement allows an amazing degree of receptor diversity to be encoded in a compact manner in the genome; however, it may provide an increased opportunity for chromosomal translocations to occur, which could lead to leukemia (Scandura et al., 2002). In addition, both B- and T-cells undergo an extensive selection process during maturation to ensure that the cells that are released into the periphery do not react against the organism itself. Cells that do not pass this selection process die by apoptosis and as a result, the number of lymphoid lineage cells produced by an organism is very large compared to the number of cells that actually become mature lymphocytes.

T-cells are derived from progenitors that have relocated from the bone marrow to the thymus and undergo their commitment and maturation there. T-cells go through a CD4 CD8 double-negative stage, followed by a double-positive stage prior to the selection of mature single-positive T lymphocytes. The TCR on a single positive T lymphocyte recognizes either major histocompatibility complex (MHC) I or II. MHC I molecules are expressed on virtually all cells with nuclei, while MHC II molecules are expressed primarily on cells such as macrophages and B-cells that recognize foreign antigens. Both MHC I and II present peptides (derived from proteins that have been degraded inside the cell) on the cell surface, which alerts other cells that recognize MHC I or II to the presence of foreign peptides in these cells. CD4 and CD8 act as co-receptors, which help T-cells to bind specific MHC molecules; CD8 is specific for MHC I, and CD4 is specific for MHC II. Interestingly, while the processes of MHC specification and single-positive T-cell selection occur in parallel, they do not appear to be linked mechanistically (Keefe et al., 1999). The combination of specificities provided by these receptors enables the helper and cytotoxic T lymphocytes to accomplish their different tasks. Specificity for MHC I allows cytotoxic T-cells to recognize virus infected cells and assist directly in their destruction, while MHC II specificity enables helper T-cells to recognize and activate macrophages or B-cells that have been sensitized by the same antigen as the T-cell. The strength of a developing T-cell's reaction to the MHC determines its fate. Either too strong or too weak of a reaction leads to negative selection via apoptosis. Only T-cells that have an appropriate response to the MHC are selected to continue in the differentiation process (Wolfer et al., 2001). Most maturing T-cells fail to meet the rigorous standards necessary to continue to the mature single-positive stage and die by apoptosis as double-positive cells.
In mammals, B-cells differentiate and mature in the bone marrow of adults and in the livers of embryos. B-cells go through several stages before they are fully mature. These stages are marked by the expression of specific antigen receptors and other cell surface proteins. Only about 10% of the B-cells produced in the bone marrow survive to become mature peripheral B-lymphocytes (Cariappa and Pillai, 2002). The remaining cells are either negatively selected and destroyed in the bone marrow for a defect such as self-recognition or else they fail to attain a place in a splenic follicle and apoptose because of survival signal deprivation. B-cells that do survive can become either memory cells or plasma cells upon activation by antigen with the assistance of helper T-cells. Memory cells retain specificity to a specific antigen and can activate other B-cells or differentiate into plasma cells in case of reexposure to the same antigen. Plasma cells are terminally differentiated antibody-secreting B-cells.

Natural killer (NK) cells also belong to the lymphoid lineage, and probably share a common precursor with T-cells. Unlike T-cells they do not have rearranged TCR genes and they do not undergo MHC class specification. NK cells are important in defending against foreign and cancerous cells with reduced expression of MHC I that might not be detected by cytotoxic T-cells.

Myeloid Lineages

The myeloid lineage is comprised of monocytes and granulocytes. Monocytes, or macrophages as they are known once they leave the bloodstream and enter the tissue, are responsible for destroying old, damaged or apoptotic cells as well as foreign organisms via phagocytosis. Granulocytes include neutrophils, whose primary role is the phagocytosis of small organisms such as bacteria; eosinophils, which are involved in tempering allergic responses and destroying some parasites; and basophils, which secrete histamine to control inflammatory reactions. The granulocyte subcategory is actually based on the appearance of these cells under a microscope and may not reflect the degree to which these cells are related. Eosinophils may develop from the CMP along a separate pathway from that taken by neutrophils and monocytes, which are believed to share a common intermediate precursor (Ward et al., 2000; Zhu and Emerson, 2002). There is also some evidence that basophils may actually derive from the megakaryocytic rather than the myeloid lineage (Arock et al., 2002). Further study will be necessary to clarify the origins of these cells.

Erythroid/Megakaryocytic Lineage

Erythrocytes, the most numerous hematopoietic cells, develop in the bone marrow, where during the normal maturation process they lose their nuclei, ribosomes and mitochondria before moving into the blood. The function of red blood cells is to transport oxygen, which binds to the hemoglobin contained in erythrocytes. Erythrocyte production is stimulated by EPO, which is produced by the kidneys. EPO production is increased in response to hypoxia or blood
loss, causing more erythrocytes to be made. The number of erythrocytes produced is a tightly regulated process in normal individuals, which may indicate that a high level of redundancy exists in the regulatory elements responsible for erythropoiesis.

Megakaryocytes, the largest of the hematopoietic cells, are found in the bone marrow, and in the mouse, also in the spleen. During the maturation process, megakaryocytes undergo endomitosis, rounds of chromosomal replication without cell division. This results in a highly polyploid nucleus with a large cytoplasm. The DNA content of megakaryocytes varies between 8N and 128N with the modal ploidy in both humans and most mice being 16N (Jackson et al., 1990; Long, 1998). When megakaryocytes are sufficiently mature, they shed platelets, which are actually fragments of the cytoplasm of the megakaryocyte. Platelets act at sites of blood vessel damage to help seal the break and cause the blood to clot. Platelet production is stimulated by TPO, which is produced in the kidneys, liver, smooth muscle and bone marrow (Kaushansky, 1998). Serum TPO levels are found to vary inversely with platelet count, because platelets bind TPO and remove it from the serum, providing a feedback loop for regulation of platelet production (Kaushansky, 1998).

**Loss of regulation in hematopoiesis can lead to a variety of diseases.**

Dysregulation of hematopoiesis at any stage may have serious consequences for an organism. While some defects can be compensated for, others may lead to disease or even death. Improper regulation can lead to underproduction or overproliferation of one or more blood cell types, a loss of the ability to destroy defective cells, or an inability of cells to mature beyond a certain stage, causing a deficit of one or more mature hematopoietic lineages. An example of a disease marked by underproduction of a specific lineage is anemia. Anemia is characterized by a decrease in the number of red blood cells, or a reduction or defect in the hemoglobin they contain. Anemia has many causes including blood loss, iron deficiency and genetic defects. Anemia is also a common symptom of leukemia. Depending on the source of the anemia, the disease can be either short term or chronic. In response to anemia, red blood cells are frequently rushed into the blood before they are fully mature and in severe cases, erythropoiesis may begin to occur in organs outside the bone marrow, such as the spleen or liver (termed extramedullary hematopoiesis), in an attempt to meet the demand.

On the opposite end of the spectrum are chronic myeloproliferative disorders, in which too many cells of one or more non-lymphoid lineages are produced. In these diseases the maturation process of the affected lineage may or may not be intact. In some cases, one lineage is produced at the expense of another lineage and in many cases the cells being produced possess some defect of physiology or function. Myeloproliferative disorders are frequently clonal in nature, that is, a mutation or mutations acquired in a single stem cell gives its
progeny a growth advantage over normal cells and allows them to overproliferate (Solberg, 2001). Polycythemia vera, in which the erythrocyte count and often the leukocyte count is increased, and essential thrombocythemia, in which the platelet count is elevated, are examples of myeloproliferative disorders. Interestingly, all chronic myeloproliferative disorders display an increase in megakaryocyte numbers, size and ploidy (Jacobsson et al., 1999). Sometimes these disorders can progress into a myelodysplastic syndrome, typified by an overproliferation of abnormal cells or even leukemic disease (Solberg, 2001).

Leukemia combines the overproliferation of one or more hematopoietic cell lineages with blocks in the differentiation and apoptotic pathways. This leads to the uncontrolled expansion of a cell population without the brakes that programmed cell death or the loss of proliferative ability associated with terminal differentiation would provide. Like chronic myeloproliferative disorders, leukemia is usually clonal. Leukemias are generally classified as either acute or chronic and most often affect either the lymphoid or myeloid lineages, although erythroid and megakaryocytic leukemias exist as well. Chromosomal translocations are frequently found in leukemia and the aberrant gene products encoded have been the focus of much study. Two types of leukemia, chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) are of particular interest in this thesis.

CML is most often associated with the chromosomal rearrangement known as the Philadelphia (Ph) chromosome. This translocation results in a fusion between the BCR and ABL genes. The fusion protein encoded by this translocation, p210BCR/ABL is a tyrosine kinase that is constitutively active and has been shown to cause cancer in transgenic animals and transformation in cell culture assays (Cross and Reiter, 2002; Shet et al., 2002). Other fusion proteins that function as tyrosine kinases have been found in the small subset of CML that does not involve the Ph chromosome, indicating that the deregulation of tyrosine kinase function is a critical factor in the genesis of CML (Bain, 2002). CML has two phases, a long chronic phase during which immature and mature myeloid cells are overproduced and found in the periphery, followed by a blast crisis characterized by a block in maturation of one or more lineages (Shet et al., 2002). Which lineage is blocked during blast crisis may be determined by additional mutations that have been acquired; loss of p53 in CML patients has been associated with myeloid blast crisis, while loss of p16INK4a has been found in patients with lymphoid blast crisis (Shet et al., 2002). One interesting difference between CML and non-malignant chronic myeloproliferative disorders has been found regarding megakaryocyte ploidy. While in chronic myeloproliferative disorders the megakaryocytes are found on average to have a greater modal ploidy than in normal individuals, in CML, patients have been found to have megakaryocytes with a modal ploidy of 8N rather than the 16N found normally, indicating a shift towards immaturity (Jacobsson et al., 1999).
The significance of this finding has not been determined, but it is of interest that we have found that disrupting the ability of c-myb to bind p300 also leads to a decreased modal ploidy in megakaryocytes (Chapter 6).

AML is the most common form of acute leukemia in adults. It encompasses eight subgroups, which differ according to the myeloid lineages involved and the degree of maturation those cells attain. AML is also the most frequent type of leukemia to arise from mutations caused by prior cancer therapy (Scandura et al., 2002). AML differs from CML in that it presents with an overproliferation of immature myeloid cells and consequently progresses much faster than CML. Many of the translocations associated with AML involve transcription factor genes, implying that transcriptional dysregulation may be a primary factor in the generation of AML (Alcalay et al., 2001; Scandura et al., 2002). The partner genes found in these translocations have a diversity of functions, but some are thought to have roles in apoptosis and cell survival regulation (Alcalay et al., 2001).

Domains that may contribute to transcriptional activity are often present in fusion proteins associated with AML including transactivation domains, DNA binding domains, domains with protein acetyltransferase activity, and domains that allow interaction with other factors that possess chromatin-remodeling activity (Scandura et al., 2002). In the context of the fusion protein, the aberrant nuclear localization of certain domains may alter their transcriptional regulatory properties or reveal novel transcriptional properties. This potential for gain or alteration of transcriptional function could help to explain the role of proteins involved in leukemia-associated translocations, such as NUP98, that may seem otherwise to have little or no function in hematopoiesis.

2.2 Nucleoporins

All transport of proteins and RNA between the cytoplasm and the nucleus occurs via pores in the nuclear envelope. These pores allow both active transport and passive diffusion of molecules into and out of the nucleus. Passage through these pores is regulated by structures within the pores called nuclear pore complexes (NPC), which are made up of proteins called nucleoporins. In this section I will discuss some features of nucleoporins and their roles in the NPC and then describe the involvement of two specific nucleoporins, NUP98 and NUP214, in chromosomal translocations associated with leukemia.

Structure of the NPC

The NPC has an octagonal symmetry around a central pore. Within this pore is a transporter structure which allows the passive diffusion of small molecules and
the active transport of larger molecules through the central channel (Rout and Aitchison, 2001). Parallel with the nuclear envelope around the transporter is a ring structure from which eight filaments extend into the cytoplasm and eight filaments extend in to the nucleus joined by a ring at their distal ends to form a basket. The NPC is anchored to the nuclear lamina, a filamentous meshwork, which acts as a support structure for the nuclear envelope. Localization of specific nucleoporins within the NPC varies, with some being located throughout the NPC, while others are located either primarily or entirely at the nuclear or cytoplasmic side of the NPC. Structures called annulate lamellae, which also contain nucleoporins and have structural similarity to the NPC are found in the cytoplasm (Lyman and Gerace, 2001). The function of annulate lamellae is not clear, but they may represent stores of nucleoporins not currently required for NPCs, or alternatively, they may consist of accumulations of nucleoporins, which are awaiting breakdown (Daigle et al., 2001; Lyman and Gerace, 2001).

The number of distinct nucleoporins that make up the NPC has been a topic of speculation. A proteomics study of the yeast NPC has defined 29 proteins in the NPC, although the definition of nucleoporin used in this study may exclude some
proteins with important NPC functions (Rout et al., 2000; Vasu and Forbes, 2001). Because the mammalian NPC is known to be larger and more complex than the yeast NPC, it has widely been assumed that there are more nucleoporins in mammalian NPCs and estimates have been in the range of 50 to 100 proteins (Stoffler et al., 1999; Wente, 2000). A recent study, however, has used a proteomics approach to define the proteins in the mammalian NPC and has also identified 29 nucleoporins (Cronshaw et al., 2002). Whether this is the exact number of nucleoporins in the yeast and mammalian NPCs remains to be verified by other approaches, but it seems likely that the number for yeast or mammals will not turn out to be vastly larger.

In both yeast and mammals, roughly one third of nucleoporins in the NPC have domains containing multiple repeats of phenylalanine (F) and glycine (G), known as FG repeats (Cronshaw et al., 2002; Wente, 2000). Besides repeats of the FG dipeptide alone, the family of FG repeat-containing nucleoporins also can have the variant FG repeats GLFG (where L is leucine) or FXFG (where X is any amino acid). FG repeat-containing nucleoporins interact with transport factors, called karyopherins, and some of these karyopherins have been shown to bind directly to FG repeats (Bayliss et al., 2000; Bayliss et al., 2002; Fribourg et al., 2001; Strasser et al., 2000). The localization of these FG repeat-containing nucleoporins throughout the NPC provides evidence for a model in which transport factors move proteins or RNA through the NPC by means of docking interactions with FG repeat-containing domains.

**Transport through the NPC**

Import and export of molecules through the NPC occur in similar but distinct manners. In both protein import and export, a karyopherin recognizes a targeting signal on the protein, for import a nuclear localization signal (NLS) and for export a nuclear export signal (NES). In some cases this karyopherin may both recognize the targeting signal and interact with nucleoporins; in other instances a pair of karyopherins work together, with one recognizing the targeting signal and one binding to nucleoporins. This interaction of karyopherins with nucleoporins is thought to direct the import or export complex through the NPC; however the specifics of this process are not known. It may involve the binding of the transport complex to nucleoporins with progressively higher affinity until the other side of the NPC is reached, or it may be an essentially stochastic process in which the passage of some molecules through the NPC is restricted more than that of others (Ben-Efraim and Gerace, 2001; Ribbeck and Gorlich, 2001). The manner in which the transport complex is disassembled upon reaching its destination differs between import and export, but involves Ran, a small GTP-binding protein in both cases. When an import complex reaches the nuclear side of the NPC, Ran-GTP binds to the karyopherin causing the complex to disassociate. In the case of an export complex, Ran-GTP travels from the nucleus as part of the export complex and upon reaching the cytoplasm is converted to Ran-GDP by Ran GTPase.
activating protein causing Ran to be inactivated and the complex to destabilize and disassemble. Export of RNA from the nucleus occurs in much the same way as export of protein; however, before the RNA can be exported from the nucleus, it must form a complex with RNA-binding proteins. These proteins provide the NES and interact with RNA transport factors, such as Rae1, which facilitate the export process (Pemberton et al., 1998).

**NUP98 and NUP214 are involved in multiple leukemia-associated fusion proteins.**

Two nucleoporins, NUP98 and NUP214 have been found to be involved in multiple chromosomal translocations associated with leukemia (Table 1). Both NUP98 and NUP214 are FG repeat-containing nucleoporins that have been directly implicated in NPC transport (Radu et al., 1995a; Radu et al., 1995b; van Deursen et al., 1996). In all of the reported leukemias associated with translocations involving NUP98 or NUP214, RNA fusion transcripts are found which include the FG repeat domain, although the reciprocal fusion transcripts are also found in some cases (Lam and Aplan, 2001). The ubiquitous presence of the FG repeat-containing fusion transcripts argues for the importance of these repeats in leukemogenesis. A common theme for the fusion partners of NUP98 and NUP214 is less evident. NUP98 is involved in chromosomal translocations with numerous homeobox genes, but little similarity has been found between its many other fusion partners and those of NUP214. A recent study; however, indicates that the non-homeobox fusion partners of NUP98 and NUP214 all have motifs that are predicted to form coiled-coil domains (Hussey and Dobrovic, 2002). It is speculated that these coiled-coil regions might act as oligomerization domains, which could allow interaction with transcription factors or cofactors. In addition, there is some evidence that several of these fusion partners may have roles in transcription themselves. This would argue that alterations in normal transcriptional regulation might be the critical factor in the leukemogenic potential of these fusion proteins. A closer inspection of these nucleoporins and their fusion partners provides some clues to their roles in leukemogenesis.

NUP98 localizes in the NPC to the midpoint of the nuclear basket and is also found in the nucleoplasm and in distinct structures within the nucleus, termed GLFG bodies (the GLFG domain has been shown to be required for this specific nuclear localization (Griffis et al., 2002)). NUP98 contains a total of 37 FG, FXFG and GLFG repeats and is the only known vertebrate GLFG repeat-containing nucleoporin (Griffis et al., 2002; Lam and Aplan, 2001; Radu et al., 1995b). In addition to the FG repeat domain, NUP98 also contains a Gle2p-binding sequence (GLEBS)-like domain, which is a binding site for the RNA export factor, Rae1 (Bharathi et al., 1997; Brown et al., 1995; Murphy et al., 1996; Whalen et al., 1997). The gene encoding NUP98 also codes for another protein, NUP96, which is not a member of the FG repeat-containing family of nucleoporins. The two proteins can be produced as a single transcript or
NUP98 can be synthesized alone from an alternatively spliced RNA; in both cases the protein produced is cleaved by autoproteolysis (Fontoura et al., 1999; Rosenblum and Blobel, 1999). Studies indicate that NUP98 is primarily involved in RNA export from the nucleus, although a function in the import and export of proteins has not been ruled out (Powers et al., 1997). Some recent studies have helped to elucidate the role of NUP98 in RNA export. NUP98 has been shown to be the target through which vesicular stomatitis virus matrix protein inhibits the expression of host genes by blocking mRNA export (Enninga et al., 2002). Treatment with interferon γ has been shown to increase NUP98 expression through a STAT1-dependent pathway and this increased NUP98 expression relieves the block in mRNA export from the nucleus. Another study has shown that the various localizations of NUP98 in the nucleus (NPC, nucleoplasm, and GLFG bodies) exchange in a transcription-dependent manner and that blocking transcription arrests this mobility (Griffis et al., 2002). This could be evidence that NUP98 may physically escort RNA to the NPC for export.

<table>
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<th>Fusion partner</th>
<th>HD</th>
</tr>
</thead>
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<td>HOXA11</td>
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<td>HOXC13</td>
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<tr>
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<td>T-ALL</td>
<td>RAP1GDS1</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 1. Summary of chromosomal translocations involving NUP98. Shown are the malignancies the translocation is associated with, the fusion partner of NUP98 and whether a homeodomain is contributed by the partner gene. Abbreviations used: HD, homeodomain; AML, acute myeloid leukemia; t-AML, therapy-related acute myeloid leukemia; MDS, myelodysplastic syndrome; t-MDS, therapy-related myelodysplastic syndrome; CML, chronic myeloid leukemia; T-ALL, T-cell acute lymphoid leukemia.
Chromosomal translocations involving NUP98 have been found in \textit{de novo} and therapy-related AML, CML and myelodysplastic syndrome (MDS). To date, fourteen genes have been identified in leukemia-associated chromosomal translocations with NUP98. In all cases, the N-terminus of NUP98 is found by reverse transcription polymerase chain reaction (RT-PCR) to be fused to the C-terminus of the partner gene; in some cases the reciprocal transcript is also detected. The N-terminal portion of NUP98 contained in these fusions includes the GLEBS-like domain and the majority of the FG repeats, and all of the translocations result in in-frame fusions of NUP98 and its partner gene.

\textbf{Homeobox-domain containing proteins are the most common fusion partners of NUP98.}

Eight of the fourteen genes fused to NUP98 in leukemia-associated chromosomal translocations are members of the homeobox gene family. Homeobox genes are transcription factors, which contain a 61 amino acid helix-turn-helix DNA-binding homeodomain, and function primarily in embryonic patterning, but have also been found in some adult tissues, including blood cells (Lawrence \textit{et al.}, 1997; Magli \textit{et al.}, 1997). Homeobox genes are divided into two types; Class I homeobox (HOX) genes, which have a highly conserved homeodomain sequence and Class II homeobox genes, which contain degenerate homeodomain sequences. Class I HOX genes in both mice and humans are arranged in four clusters (A, B, C and D) of 9 to 11 genes with each cluster on a different chromosome (Cillo \textit{et al.}, 2001). The ordering of HOX genes within each cluster corresponds to their temporal and spatial expression in the embryo, with the most 3' HOX genes being expressed earliest and in the most anterior positions in the developing embryo (Cillo \textit{et al.}, 2001; van Oostveen \textit{et al.}, 1999). HOX genes of the A, B and C clusters have been found to be expressed in normal hematopoietic cells, with the A cluster predominantly expressed in myeloid cells and the B and C clusters expressed mostly in erythroid cells, but little evidence of expression of HOX genes of the D cluster in hematopoietic cells has been found (Lam and Aplan, 2001). Class II homeobox genes are found scattered throughout the genome and some of them have also been found to be expressed in hematopoietic cells (Lam and Aplan, 2001; Shivdasani and Orkin, 1996; van Oostveen \textit{et al.}, 1999).

In all of the translocations between NUP98 and homeobox family members, the N-terminus of NUP98 is fused to the homeodomain-containing C-terminus of the homeobox gene, which may indicate that this domain has a role in the leukemogenic function of the fusion proteins. Class I HOX genes belonging to the A, C and D, but not the B clusters have been found in translocations with NUP98. HOXA9 was the first identified fusion partner of NUP98 and the NUP98-HOXA9 fusion is the most common fusion involving NUP98, accounting for approximately 1% of AML cases, as well as having been reported in some CML cases (Ahuja \textit{et al.}, 2001; Look, 1997). Mice nullizygous for HOXA9 have hematopoietic defects including reduced numbers of lymphocytes and
granulocytes and smaller than normal spleens and thymuses (Lawrence et al., 1997). In addition they were found to have fewer myeloid, B-cell and erythroid progenitors, although no attendant anemia was reported (Lawrence et al., 1997). The NUP98-HOXA9 translocation, as well as the other translocations involving NUP98, is often found in therapy-related secondary leukemias.

Other homeobox genes that have been found as fusion partners of NUP98 in leukemia are HOXA11, HOXA13, HOXC11, HOXC13, HOXD11, HOXD13 and PMX1 (Fujino et al., 2002; Nakamura et al., 1999; Panagopoulos et al., 2003; Raza-Egilmez et al., 1998; Suzuki et al., 2002; Taketani et al., 2002a; Taketani et al., 2002b; Taketani et al., 2002c). Knockouts and transgenic overexpression of these genes in mice caused skeletal abnormalities; however, no hematopoietic defects were reported (Davis and Capecchi, 1994; Davis et al., 1995; Fromental-Ramain et al., 1996; Godwin and Capecchi, 1999; Hostikka and Capecchi, 1998; Martin et al., 1995; Papenbrock et al., 2000; Patterson et al., 2001; Small and Potter, 1993; Sur and Toftgard, 2000). Interestingly, PMX1 has been shown to interact with Maf proteins, some of which are known to regulate the expression of certain hematopoietic-specific genes (Kataoka et al., 2001). This could point to an indirect dysregulation of hematopoiesis caused by expression of the NUP98-PMX1 fusion protein. Alternatively, and perhaps more likely, the similarities between this group of NUP98-containing fusion proteins may indicate that the primary contribution of the homeobox-containing fusion partners is their DNA-binding capabilities, while the original function of the homeobox protein may be lost entirely rather than being misregulated.

The other proteins that have been found in fusions with NUP98; DDX10, LEDGF, NSD1, NSD3, RAP1GDS1 and TOP1 have few similarities although several have been suggested as possible transcription cofactors. DDX10 is a ubiquitously expressed member of the DEAD box family of putative RNA helicases. Two variants of the NUP98-DDX10 fusion protein have been found in AML, with slightly different breakpoints in NUP98 and also differing in whether the C terminus of DDX10 fused to NUP98 contains the DEAD box domain or not; however both variants retain the RNA helicase domain and a charged amino acid region (Arai et al., 1997). There is some evidence based on the amino acid sequence of DDX10 that it may have activity as a transcriptional activator, but there is currently no in vivo or in vitro data to back this up (Arai et al., 1997). LEDGF, lens epithelium-derived growth factor, and p52, which is coded by an alternatively spliced RNA from the same gene, are both found in translocations with NUP98 in AML as well. The C termini of p52 and LEDGF both contain highly charged regions of amino acids, which are included in the fusion product (Ahuja et al., 2000b). Both p52 and LEDGF are reported to be transcriptional coactivators and in addition LEDGF functions as a survival factor (Ahuja et al., 2000b; Shinohara et al., 2002). NSD1 and NSD3 are members of a family of proteins whose function has not been determined, but which contain domains that are found in development- and chromatin reorganization-
associated proteins (Arai et al., 1997). In addition, studies have shown NSD1 to possess both activation and repression domains (Huang et al., 1998).

Haploinsufficiency for NSD1 in humans has been linked to Sotos syndrome, a disease that is typified by large body size early in life and mental retardation (Kurotaki et al., 2002). NSD3 has been found to be overexpressed in breast cancer cell lines and the third member of the family, NSD2, which has not been found in a translocation with NUP98, is associated with multiple myeloma (Angrand et al., 2001). RAP1GDS1, which has been found in an acute lymphoblastic leukemia-associated translocation with NUP98, codes for SmgGDS, a guanine nucleotide exchange factor, which regulates the interaction of GDP and GTP with GTPases (Cimino et al., 2001; Hussey et al., 1999; Vikis et al., 2002). Whether SmgGDS might interact with Ran in nuclear transport has not been shown (Hussey et al., 1999). The entire coding region of RAP1GDS1, except for the initial methionine is included in the fusion with NUP98, including a large region of armadillo repeats that may mediate protein-protein interactions (Hussey et al., 1999). The NUP98-TOP1 fusion transcript has been found in a number of therapy-related AML and MDS cases. Topoisomerases act to separate the two strands of DNA and all of the domains needed for this function appear to be retained in the NUP98 fusion (Ahuja et al., 1999, 2000a, 2000b; Panagopoulos et al., 2002; Pommier, 1998). Analysis of the breakpoints of some of the NUP98-TOP1 fusions, combined with the therapy-related nature of the disease, has led to the view that treatment with topoisomerase inhibitors plays an important role in the generation of this chromosomal translocation (Ahuja et al., 2000a).

Besides NUP98, one other nucleoporin, NUP214 (CAN), has been found to be involved in leukemia-associated chromosomal translocations. NUP214 is located at the cytoplasmic face of the NPC, and has been implicated in protein import and RNA export (Kraemer et al., 1994; van Deursen et al., 1996). NUP214 is also a member of the family of FG repeat-containing nucleoporins and both FG and FXFG repeats are found in its C terminus (Kraemer et al., 1994). Chromosomal translocations have been found that fuse NUP214 to DEK in AML and to SET in acute undifferentiated leukemia (von Lindern et al., 1992a; von Lindern et al., 1992b). In both cases, the FG-repeat containing C terminus of NUP214 is fused to the N-terminus of DEK or SET and the resulting fusion proteins localize to the nucleus (Fornerod et al., 1995; von Lindern et al., 1992a; von Lindern et al., 1992b). DEK is a chromatin-associated protein that induces positive supercoiling in DNA and has also been found to bind RNA (Fornerod et al., 1995; Kappes et al., 2001; McGarvey et al., 2000; Waldmann et al., 2002). SET has been reported to have functions in chromatin remodeling, as well as cell cycle regulation and has recently been shown to act as a transcription factor (Compagnone et al., 2000; Zhang et al., 2001). The involvement of FG repeat-containing nucleoporins in so many different leukemia-associated fusion proteins is very suggestive. The importance of the
FG repeats in the binding of transport factors could indicate that the contribution of these nucleoporins to the leukemogenic process primarily involves a dysregulation of transport. Our work, however, indicates that the contribution of NUP98 to these leukemia-associated fusion proteins represents a gain of function caused by aberrant localization within the nucleus that allows the interaction of the FG repeats with the transcriptional co-activators, CBP and p300. This interaction, which we show to confer increased transactivation potential, may be critical in causing dysregulation of transcription that could lead to leukemogenesis.

2.3 CBP and p300

CBP and p300 are transcriptional coactivators with a high degree of similarity that have been shown to interact with numerous transcription factors, including many that have been implicated in hematopoiesis. In this section I will discuss the normal functions of p300 and CBP, the effect of the loss of one or both alleles of CBP or p300 in mice and humans and briefly describe some of the transcription factors that are known to interact with p300 and CBP. I will also review the evidence for the participation of CBP and p300 in leukemic fusion proteins.

p300 and CBP function as transcriptional coactivators.

CBP and p300 carry out their functions as transcriptional coactivators in at least two distinct ways. First, CBP and p300 are believed to act as adaptor molecules that facilitate the interaction between transcription factors and the basal transcription machinery. CBP and p300 have been shown to interact or colocalize with several components of the basal transcription machinery including RNA Polymerase II (Pol II), Transcription Factor II B (TFIIB) and TATA Binding Protein (TBP) (Blobel, 2002). In addition, E1A, a viral protein that acts as an inhibitor of p300 and CBP coactivator functions, has been suggested to produce this inhibitory effect by competing with Pol II and TFIIB for binding to CBP and p300 (Goodman and Smolik, 2000).

A second way in which p300 and CBP are thought to function as coactivators is through their protein acetyltransferase activity. Acetylation of histones opens up the chromatin structure and is usually linked to transcriptional activation. CBP and p300 have been shown to acetylate all four core histones in vitro (Blobel, 2002; Chan and La Thangue, 2001). In addition, transcription factors and other nuclear proteins have also been shown to be acetylated by CBP and p300. Acetylation of some transcription factors has been shown to increase their ability to bind DNA or stabilize their interaction with p300 and CBP (Blobel, 2002; Chan and La Thangue, 2001).
There is also increasing evidence that p300 and CBP may act as scaffolds for the assembly of transcriptional complexes (Chan and La Thangue, 2001; Giles et al., 1998). By recruiting multiple transcription factors and other transcriptional activators such as acetyltransferases to the basal transcription machinery, p300 and CBP not only facilitate transcription, but also may influence which genes are expressed. This influence could be exerted through competition between transcription factors for binding to CBP and p300 or through the cooperative interaction of transcription factors that are bound to p300 and CBP.

p300 and CBP are required for normal embryo development.

Homozygous null mutations in either p300 or CBP result in similar embryonic lethal phenotypes. Mice lacking p300 die between embryonic days 9 and 11.5 and analysis of the embryos showed that they were smaller than their wild type littermates and had defects in neural tube closure and heart development (Yao et al., 1998). Mice nullizygous for CBP or homozygous for a truncated CBP protein both died at a similar embryonic stage to the p300 knockouts and also displayed neurulation defects. In addition, some traits in common with human Rubinstein-Taybi syndrome (RTS), such as skeletal abnormalities were found, and defective blood vessel formation and hematopoiesis were also noted (Oike et al., 1999a; Oike et al., 1999b; Tanaka et al., 2000). The CBP nullizygous embryos appeared pale, and colony-forming assays using cells from yolk sacs or embryos produced reduced numbers of colonies compared to wild-type controls (Tanaka et al., 2000). It is unknown whether the p300 knockout embryos possess a similar hematopoietic defect. Mice compound heterozygous for both p300 and CBP null mutations died during embryogenesis and analysis of the embryos showed that they were smaller than their wild type littermates and exhibited neural tube closure defects similar to the homozygous mutants (Yao et al., 1998).

Heterozygous mutations in p300 or CBP lead to distinct phenotypes.

The comparability of the phenotypes of the homozygous knockouts of p300 and CBP, as well as the compound heterozygous knockout, indicate that p300 and CBP must have many redundant functions as would be predicted by the similarity of the proteins. However, studies of animals heterozygous for CBP or p300 have revealed some phenotypic differences. Although both p300 and CBP heterozygous mice are born at below the expected Mendelian frequencies, implying that they both possess abnormalities, long term study of mice heterozygous for CBP has revealed an increase in hematologic diseases including leukemia, plasmacytoma, and histiocytic sarcoma in CBP, which are not observed in aged p300 heterozygotes (Kung et al., 2000; Tanaka et al., 2000; Yao et al., 1998). In some cases these diseases were accompanied by a loss of heterozygosity. Further studies of CBP and p300 heterozygotes utilizing serial transplantation of bone marrow indicate that cells from CBP heterozygotes have a reduced ability to contribute to hematopoiesis compared to p300
heterozygous or wild-type cells (Rebel et al., 2002). Studies of chimeric mice derived from ES cells null for either p300 or CBP confirmed this result and gave additional evidence for different roles of p300 and CBP in hematopoiesis (Rebel et al., 2002). Analysis of these chimeric mice showed that neither the p300\(^-\) nor the CBP\(^-\) cells made major contributions to the peripheral blood in the chimeric animals, although transplantation assays showed that p300\(^-\) cells, but not CBP\(^-\) cells could reconstitute the bone marrow in recipient mice. In vitro experiments using null ES cells showed that CBP, but not p300 null ES cells could differentiate into hematopoietic cells. Taken together, these results seem to indicate that CBP has a more critical role than p300 in hematopoietic stem cell renewal, while in the differentiation of hematopoietic cells, the reverse seems to be true (Rebel et al., 2002).

In humans, loss of one allele of p300 or CBP also results in distinct phenotypes. Loss of a single allele of CBP results in RTS, a disease characterized by mental retardation and skeletal abnormalities (Petrij et al., 1995). RTS patients have also been found to have a high incidence of certain types of tumors (Miller and Rubinstein, 1995). No hereditary human disease has been found that is linked to loss of a single p300 allele; however, inactivating mutations of p300, often accompanied by loss of heterozygosity, have been reported in human breast, colorectal, gastric and pancreatic tumors and cancer cell lines (Gayther et al., 2000; Muraoka et al., 1996). Inactivating mutations in CBP have been reported in human cancers cell lines, but not in primary tumors (Ozdag et al., 2002).

**Transcription factors bind to CBP and p300 via at least five main protein interaction domains.**

CBP and p300 each have at least five distinct protein-interaction domains, which are highly conserved between the two proteins and through evolution. The most N-terminal of these domains is the RID (receptor interaction domain) domain, which has been found to bind a variety of nuclear receptors. p300 and CBP also possess three cysteine-histidine rich domains; two of which, CH1 and CH3, contain zinc fingers and have been found to bind numerous transcription factors. The KIX domain interacts with cyclic AMP response element (CRE) binding protein (CREB) and c-Myb, among other transcription factors, and its structure has been solved in complex with the phosphorylated kinase inducible domain (KID) of CREB and shown to contain three helices, which provide critical contacts with CREB (Parker et al., 1998; Parker et al., 1999; Radhakrishnan et al., 1997). The most C-terminal protein-binding domain is the Interferon regulatory factor-3 binding domain (IbiD) that is in a glutamine rich region of p300 and CBP (Lin et al., 2001). It is likely that at least some aspects of the phenotypes associated with the loss of p300 or CBP may be attributed to transcription factors that normally bind to p300 and CBP through these domains. Loss of p300 or CBP may affect the ability of these transcription factors to induce expression of their target genes; therefore, the phenotypes associated with knockouts of CBP- and p300-binding transcription factors may have some
similarities to the phenotypes of CBP and p300 mutants. Of special interest in our study were the phenotypes of mice with mutations in transcription factors that have been shown to bind to the KIX domain of CBP and p300, in particular CREB and c-Myb.

**Figure 3. Domains of p300 and CBP.** Abbreviations used: RID, receptor interaction domain; CH1,2 and 3, cysteine/histidine-rich domains; Br, Bromo domain; IBiD, IRF3-binding domain; HAT, histone acetyltransferase.

**p300 and CBP bind to transcription factors implicated in hematopoiesis.**

CREB is a transcription factor that activates its target genes by binding to a CRE near the promoter in response to an increase in intracellular cyclic AMP. CREB binds to p300 and CBP in a phosphorylation-dependent manner, and has been shown to be involved in glucose regulation, cell survival and memory (Mayr and Montminy, 2001). In addition, CREs have been found in several T-cell genes and CREB knockout mice have been shown to have T-cell defects (Barton *et al.*, 1996; Rudolph *et al.*, 1998). CREB nullizygous mice are smaller than their wild-
<table>
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<td>GATA-1</td>
<td>CH3</td>
<td>E10.5-11.5</td>
<td>erythrocytes, megakaryocytes</td>
<td></td>
<td>(Pevny et al., 1991)</td>
</tr>
<tr>
<td>MLL</td>
<td>KIX</td>
<td>E10.5</td>
<td>all primitive lineages</td>
<td>developmental defects</td>
<td>(Hess et al., 1997)</td>
</tr>
<tr>
<td>NF-E2</td>
<td>KIX</td>
<td>No</td>
<td>megakaryocytes</td>
<td></td>
<td>(Andrews, 1998)</td>
</tr>
<tr>
<td>PU.1</td>
<td>CH3</td>
<td>E17-perinatal</td>
<td>myeloid, B-cells, T-cells, erythroid?</td>
<td></td>
<td>(Scott et al., 1994)</td>
</tr>
<tr>
<td>STAT5</td>
<td>KIX?</td>
<td>No</td>
<td>NK cells, T-cells</td>
<td>embryonic anemia?</td>
<td>(Teglund et al., 1998)</td>
</tr>
</tbody>
</table>

Table 2. Summary of hematopoietic transcription factors thought to interact with p300 and CBP. Summarized are the domain(s) of p300 and CBP through which interaction is thought to occur, and the phenotype of mouse knockout models of these transcription factors including lethality, hematopoietic lineages affected and other relevant phenotypes.
type littermates and die immediately after birth from respiratory problems, but analysis of embryos revealed that in addition to brain defects, they possess reduced numbers of fetal thymocytes and a severely diminished αβ T-cell lineage, although the γδ T-cell lineage is unaffected (Rudolph et al., 1998). It is possible that the severity of the CREB phenotype may be attenuated by the upregulation of other CREB gene family members, such as CREM and ATF-1 (Mayr and Montminy, 2001).

c-Myb is a transcription factor that is expressed in hematopoietic precursors including the HSC, CLP and CMP (Akashi et al., 2000b). Mice lacking c-Myb die from anemia at approximately embryonic day 15 and further evaluation of the fetal livers of c-Myb nullizygous embryos has shown that they are severely deficient in all hematopoietic lineages except megakaryocytes, which are less severely affected (Mucenski et al., 1991; Sumner et al., 2000). Studies of chimeric mice derived from c-Myb-/- ES cells showed that c-Myb-/- ES cells do not contribute to any mature definitive (hematopoiesis occurring in the fetal liver or adult bone marrow) hematopoietic lineages and that while cells derived from the c-Myb-/- ES cells populate the fetal liver they do not proliferate (Sumner et al., 2000). The authors of this study theorize that the megakaryocytes seen in the fetal liver are just the remaining cells derived from primitive (occurring in the yolk sac) hematopoiesis, not derived from definitive hematopoietic precursors, which might also explain the mature phenotype of the megakaryocytes.

Evidence exists that the interaction of certain transcription factors with other transcription factors may be influenced by the presence of CBP or p300 as a binding partner. CBP has been shown to increase the cooperative interaction between Myb and C/EBPβ in vitro (Mink et al., 1997). In this case, an inability of Myb to interact with p300 or CBP might cause a decrease in activity of C/EBPβ. By contrast, c-Myb and GATA-1 have been shown to compete for CBP binding, even though they bind to different CBP domains, and this competition has been shown to contribute to their mutual inhibition of transcriptional activity (Takahashi et al., 2000). In this situation, loss of interaction between CBP or p300 and c-Myb could result in more CBP or p300 being available to interact with GATA-1, which could result in an increase in transcription of GATA-1 target genes. The possibility of other such p300/CBP-mediated transcription factor interactions makes a brief summary of the phenotypes associated with other hematopoietic transcription factors that interact with CBP and p300 relevant to this thesis (Table 2).

### p300 and CBP are partners in several leukemic fusion proteins.

p300 and CBP have been found to be involved in several rare AML-associated chromosomal translocations. These translocations are frequently found in leukemias that develop after treatment of prior disease, although de novo cases have also been reported.
Both p300 and CBP are found in fusions with MLL, a gene that is involved in a number of chromosomal translocations. MLL is a homolog of the *Drosophila* gene, *trithorax*, which regulates the expression of HOX genes and thus plays a role in embryonic patterning (Yu *et al*., 1998). In addition to a multi-lineage defect in primitive hematopoiesis, embryos nullizygous for MLL display loss of HOX gene expression, while mice heterozygous for MLL display disrupted HOX gene expression in addition to growth abnormalities (Hess *et al*., 1997; Yu *et al*., 1995). Translocations between MLL and CBP or p300 are found in therapy related cases of AML, and in all of them the N-terminus of MLL, including a DNA binding domain, a methylation domain and a transcriptional repression domain is joined to the C-terminus of p300 or CBP. In the MLL-p300 fusion, the p300 portion of the fusion includes a region just N-terminal of the HAT domain through the C-terminus (Iida *et al*., 1997). In the case of the MLL-CBP fusion, two different fusions have been detected, the most common of which fuses MLL to almost the entirety of CBP, and a rare fusion which includes a similar portion of CBP as that seen in the p300-MLL fusion (Lavau *et al*., 2000; Sugita *et al*., 2000). In at least some cases, the RNA transcripts of the reciprocal fusions are also detected, making it unclear which is responsible for the leukemogenic phenotype; however, transplant experiments in mice using bone marrow transduced with an MLL-CBP fusion caused a long myeloproliferative phase, followed by AML in all of the recipient mice (Lavau *et al*., 2000). This result indicates that at least the MLL-CBP and MLL-p300 fusion proteins probably possess leukemogenic properties.

CBP and p300 are also found in translocations with MOZ, and CBP has been found in a translocation with a related gene, MORF. MOZ and MORF are putative histone acetyltransferases and are homologous to SAS2, which is involved in transcriptional silencing in yeast (Borrow *et al*., 1996; Panagopoulos *et al*., 2001; Roth, 1996). The fusions that join the putative acetyltransferase domain containing N-terminus of MOZ or MORF to CBP or p300 have been found to include almost the entire coding sequence of CBP or p300 (Borrow *et al*., 1996; Kitabayashi *et al*., 2001; Panagopoulos *et al*., 2001). The reciprocal fusions, which are sometimes detected as RNA transcripts, would possibly place the C-terminus of MOZ or MORF under the control of the p300 or CBP promoter, but would contain very little coding sequence from p300 or CBP.

The contributions of p300 and CBP to the leukemogenic properties of these fusion proteins have not been fully determined. Colony assays performed using myeloid precursors transduced with MLL-CBP fusions containing different domains of CBP showed that the bromo and HAT domains of CBP fused to the N terminus of MLL are sufficient for transformation, suggesting that chromatin remodeling properties contributed by CBP are important for the leukemogenic potential of the MLL-CBP fusion protein (Lavau *et al*., 2000). In addition to the HAT domain, the CH3 domain of CBP or p300 is present in all of the leukemia-associated fusion proteins found to date. It is possible that in some of the fusion
proteins, the CH3 domain could be important for allowing interaction with the basal transcription machinery. Further study will be necessary to determine the actual causes of the leukemogenesis associated with these fusion proteins.

Conclusions

Transcriptional control of gene expression is one of the primary means by which hematopoiesis is regulated and loss or gain of transcriptional activity can severely disrupt this process. This is seen in the case of gene knockout experiments in mice, in which loss of expression of hematopoietic transcription factors can cause a disruption in hematopoietic cell numbers, the loss of a specific hematopoietic cell lineage, or even a complete ablation of hematopoiesis. In addition, loss or reduction of factors which assist in transcription, such as the transcriptional coactivators p300 and CBP, can lead to hematopoietic specific defects in spite of the wide expression of these proteins. Finally, mutations such as chromosomal translocations, can lead to a gain of transcriptional activity. This may be caused by the loss of a repressive domain or the gain of a domain that leads to an increase in activity for an already transcriptionally active fusion partner such as a transcription factor, as is probably the case for the p300-MLL and CBP-MLL fusions, or it may involve a gain of a completely novel transcriptional function for an existing domain, as seems to be the case for the FG-repeat containing domains of nucleoporins NUP98 and NUP214. This inappropriate increase in transcription is likely to be a major cause of some leukemias.

References


Chapter 3

CREB Binding Protein Interacts with Nucleoporin-Specific FG Repeats That Activate Transcription and Mediate NUP98-HOXA9 Oncogenicity

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CREB Binding Protein Interacts with Nucleoporin-Specific FG Repeats That Activate Transcription and Mediate NUP98-HOXA9 Oncogenicity

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Genes encoding the Phe-Gly (FG) repeat-containing nucleoporins NUP98 and CAN/NUP214 are at the breakpoints of several chromosomal translocations associated with human acute myeloid leukemia (AML), but their role in oncogenesis is unclear. Here we demonstrate that the NUP98-HOXA9 fusion gene encodes two nuclear oncoproteins with either 19 or 37 NUP98 FG repeats fused to the DNA binding and PBX heterodimerization domains of the transcription factor HOXA9. Both NUP98-HOXA9 chimeras transformed NIH 3T3 fibroblasts, and this transformation required the HOXA9 domains for DNA binding and PBX interaction. Surprisingly, the FG repeats acted as very potent transactivators of gene transcription. This NUP98-derived activity is essential for transformation and can be replaced by the bona fide transactivation domain of VP16. Interestingly, FG repeat-containing segments derived from the nucleoporins NUP153 and CAN/NUP214 functioned similarly to those from NUP98. We further demonstrate that transactivation by FG repeat-rich segments of NUP98 correlates with their ability to interact functionally and physically with the transcriptional coactivators CREB binding protein (CBP) and p300. This finding shows, for the first time, that a translocation-generated fusion protein appears to recruit CBP/p300 as an important step of its oncogenic mechanism. Together, our results suggest that NUP98-HOXA9 chimeras are aberrant transcription factors that deregulate HOX-responsive genes through the transcriptional activation properties of nucleoporin-specific FG repeats that recruit CBP/p300. Indeed, FG repeat-mediated transactivation may be a shared pathogenic function of nucleoporin-implied human AML.

An expanding subgroup of chromosomal translocation-generated oncoproteins in human acute myeloid leukemias (AML) involve the FG repeat-containing nuclear pore complex (NPC) proteins NUP98 (39) and CAN/NUP214 (13, 22). The NUP98 gene is found at the breakpoints of two distinct chromosomal rearrangements: t(7;11)(p15;p15) (7, 18, 33), and inv(11)(p15; q22) (2), which link NUP98 to the class I homeotic transcription factor HOXA9 and the putative RNA helicase DDX10, respectively. In each rearrangement, the chromosomal breakpoints are located within two flanking introns of the NUP98 gene that separate the FG repeat-rich N terminus of NUP98 from its C terminus, which contains a ribonucleoprotein (RNP)-binding motif (39). Although each translocation generates two reciprocal chimeric products, only those driven by the NUP98 promoter and containing the FG repeat region are predicted to mediate leukemogenesis (2, 7, 33). Another nucleoporin gene, CAN/NUP214, is found at the breakpoint of two independent chromosomal rearrangements: t(6; 11)(p23;q14), which fuses CAN/NUP214 to DEK (49), and inv(11)(p15;q22), which links it to SET (50). The leukemia-specific transcripts, DEK-CAN/NUP214 and SET-CAN/NUP214, both encode nuclear fusion proteins. The proteins contain identical C-terminal portions of CAN/NUP214, including its FG repeat-rich region, and a coiled-coil domain (13, 22). DEK and SET are both nuclear proteins that have no sequence similarity other than the presence of acidic motifs that may participate in DNA binding (13, 14, 31).

The involvement of two FG repeat-containing nucleoporins in multiple translocations associated with human leukemia raises intriguing questions about their role in leukemogenesis. In particular, the consistent presence of FG repeat regions suggests that such domains could serve a common function in the transformation of hematopoietic cells. Many of the known components of the NPC have regions rich in FXFG, GLFG (amino acids are given in single-letter code, with X indicating any amino acid). Such repeats (called FG for simplicity) are presumed contact sites for soluble nucleocytoplasmic transport factors carrying different kinds of cargo; however, their precise functions in vivo remain to be determined (35, 36).

HOXA9, expressed in both the primitive pluripotent precursors and the myeloid progenitors of human bone marrow (42), is the only nucleoporin fusion partner with an established physiological role in hematopoietic development. HOXA9 knockout mice have multiple hematopoietic defects, including reduced numbers of peripheral blood granulocytes and lymphocytes, as well as myeloid and pre-B-cell progenitors, and their spleens and thymuses are smaller than normal (25). Besides its involvement in (7;11)-mediated myeloid leukemogenesis, HOXA9 has been implicated in the formation of myeloid leukemias in the BXH-2 strain of mice (29). BXH-2 mice carry an endogenous murine leukemia virus that acts as a viralogenous predisposing the animals to myeloid malignancies (4, 5). In this experimental tumor model, about 3% of all leukemias in BXH-2 mice display proviral activation of HOXA9 (34). Constitutive expression of HOXA9 alone is not sufficient for efficient transformation of murine hematopoietic cells; it requires coexpression of MEIS1 (23, 34), a PBX1-related diver-
gent homeodomain-containing protein that cooperatively binds DNA with HOXA9 in vitro (44).

In this study, we show that the t(7;11)-derived fusion gene generates two chimeric proteins via alternative splicing within NUP98. Investigation of the structural and functional regions of the chimeric NUP98-HOXA9 proteins demonstrated that HOXA9-mediated DNA binding and interaction with PAX are essential for transformation of NIH 3T3 fibroblasts. In both chimeras, the NUP98 portions contained very potent transcription activation domains, which replace a strong transcriptional repressor domain within the amino-terminal half HOXA9. Interestingly, the transcriptional coactivators CREB binding protein (CBP) and potentially p300 interacted and functionally cooperated with the NUP98 FG-repeat-rich portions. Abbrogation of NUP98-HOXA9-mediated transformation corresponded to the loss of NUP98-mediated transcriptional activity and CBP binding. Thus, NUP98-HOXA9 seems to recruit CBP/p300 as part of its oncogenic mechanism. Because CBP and p300 are coactivators for a number of gene-specific transcription factors, they could also be critical accessory factors for other fusion proteins that deregulate transcription.

**MATERIALS AND METHODS**

**RT-PCR.** First-strand cDNA was synthesized from 1 μg of total RNA with avian myeloblastosis virus reverse transcriptase (Promega) and primer p1 or p4 in a total volume of 20 μl. The mixtures were incubated 1.5 h at 42°C. Reverse transcription RT mixture was used at 2 μl per PCR (total volume, 50 μl). The PCR primer combinations were as indicated in Fig. 1A (p1, 5' GCCGCTGCT ACGTATGAAATCCAGGGG-3'; p2, 5' GATTATACGGTAAACAGAAG GC-3'; p3, 5' CCAAACACTGTTGTGGTGGT-3'; p4, 5' ATGGAAGGCA GTTGCTTGC-3'). The PCRs were performed with AmpliTaq Gold (Perkin-Elmer Cetus) in buffer supplied by the manufacturer and supplemented with 2.5 mM MgCl2. The PCR cycles were 30 s at 94°C, 1 min at 50°C, and 3 min at 72°C.

**Expression constructs and mutagenesis.** All cDNA fragments were initially cloned in pTZ19 containing the synthetic sequence GAATTCGCCGCCACCA TGTATGAGCTCCAGATTACGCAAGTTTG (NUP98a sequence) or TTATGCCGTCCCAGATTACGCAAGTTTG (NUP98b sequence). The cDNA so that codon 469 is followed by a stop codon, sequences encoding two consecutive influenza virus HA1 epitopes (51) (indicated in italics), and a 3' NheI site. NUP98a-cDNA was isolated from a 3' to the human bone marrow (BM) cDNA library (no. HL1160a; Clontech, Palo Alto, Calif.) full-length NPC98a-cDNA was reconstituted from NPC98a-cDNA and sequences encoding amino acids 254 to 444 obtained from a partial NPC98a-cDNA clone that was isolated from a human placental library (Hu2002B#220). A cDNA encoding the HOXA9 portion of the t(7;11)-generated fusion protein was produced by PCR amplification of DNA extracted from our t(7;11) human BM cDNA library. cDNA clones encoding NPC98a- and NUP98a-HOXA9 were created by insertion of an oligonucleotide in the NPC98a-cDNA so that exon 409 is followed by a stop codon. NPC98b-HOXA9(-Δ323) cDNA was generated from NPC98b-HOXA9 by inserting the oligonucleotide TTTATAAAGATC between codons 47 and 48 of the homeodomain. NPC98a(-Δ323) HOXA9(-Δ4)-cDNA was obtained by standard PCR-based site-directed mutagenesis. NPC98a(Δ25–223)-HOXA9 and NPC98b(Δ25–223)-HOXA9 mutants were constructed by deleting a 819-bp HindIII fragment from NPC98a-HOXA9 and NPC98b-HOXA9 cDNA, respectively. To generate

**FIG. 1.** NUP98 and NUP98-HOXA9 genes generate an alternatively spliced transcript in human BM cells. (A) Overview of the RT-PCR procedure with representations of relevant NUP98-NUP98a cDNA portions and the expected PCR products. The positions of the primers used in the various RT reactions and PCRs are indicated by horizontal bars. To detect NUP98a and NUP98b transcripts in normal BM cells, we used primer 1 (p1) to generate cDNA and primer set p1 and p2 for PCR amplification (40 cycles). Seminested PCR was used to detect NUP98-HOXA9-specific transcripts in BM cells from a patient with t(7;11) positive leukemia; p4 was used for the RT step; p2 and p4 were used in the first round of amplification (40 cycles), and p1 and p2 were used in the seminested round of PCR (30 additional cycles). The oligonucleotide probe for detection of NUP98a- and NUP98b-derived PCR fragments is depicted as p3. (B) Autoradiogram of RT-PCR products detected for PCR amplification (40 cycles). Seminested PCR was used to detect

![Diagram](attachment:image.png)
NUP98(Δ1–499)/HOXA9, NUP98(Δ1–223)/HOXA9, CAN/NUP214/1184–2096)/HOXA9, NUP153(1212–1479)/HOXA9, and VP16(Δ43–499)/HOXA9 cDNAs, we cloned PCR-amplified Nel fragments containing the desired nucleoporin and VP10 portions into the Nhel site of our clone encoding the HOXA9 portion of NUP98-HOXA9. For NUP98(Δ1–499)/HOXA9, we generated a 275-bp Nhel fragment encoding the first 20 amino acids of NUP98, which was PCR amplified from NUP98 cDNA. For NUP98(Δ1–223)/HOXA9, we PCR amplified a 175-bp Nhel fragment from NUP98 cDNA encoding amino acids 224 to 253 and 449 to 469. For CAN/NUP214(1184–2096)/HOXA9, a 1018-bp Nhel fragment encoding the last 226 amino acids of human NUP153 was obtained by PCR amplification from vector pHA-NUP153(3). For VP16(Δ43–499)/HOXA9 a 220-bp Nhel fragment encoding amino acids 413 to 499 of the herpes simplex virus type 1 VP10 protein was amplified from plasmid pCMV-VP10(12). All DNA fragments generated by PCR amplification were sequenced. pT7-3 cDNA fragments were cloned into pBSMvIAcd(CD for transformation studies), pHIP or pHIP7 (for in vitro transcription/translation purposes), and the pHLud-1 derivative (16) termed pCHD13 (13) (for overexpression in HT1080 cells).

Retroviral stocks and transcription analysis. cDNA insertions were cloned into the EcoRI-SmaI sites of retrovirus expression vectors pSRMIAacDH(17, 20)T cells were cotransfected with various pSRMIAacDH vectors and constructs expressing GAL4-SRE-E9-W MUS(30). Culture supernatants containing viral particles were harvested at 6–8 hr intervals from days 2 to posttransfection, filtered (Acrodisc 13 syringe filters [pore size, 0.45 μm], Gelman Sciences, Ann Arbor, Mich.), and supplemented with 8 μg of Polybrene per ml. Low-passage-number NIH 3T3 cells were seeded at low cell density and infected the next day with virus at 6 to 12-mla for 48 h. Infected NIH 3T3 cells were harvested 24 to 72 h later and immunostained with anti-CAN antibodies. CD8-positive cells were then isolated by fluorescence-activated cell sorting (17). To test for anchorage-independent growth, we suspended 2 × 10^5 CD8 NIH 3T3 cells in a 0.3% medium supplemented with 5% fetal bovine serum and 0.5% agar and plated this mixture in a 3-cm-diameter dish. The cells were then cultured at 37°C under 8% CO2, and colonies were counted after 3 weeks. The cells were plated in triplicate for each experiment, with total number of colonies reported as the average of the counts from the three dishes. BM transformation assays were performed as described previously (12).

Indirect immunohistochemistry. NIH 3T3 or HT1080 cells were seeded on 12-well microtiter plates at 10^5 cells/ml. Cells were fixed at 12 h, stained in 3% paraformaldehyde for 15 min on ice, washed three times with phosphate-buffered saline (PBS), permeabilized in PBS–0.2% Triton X-100 for 10 min, washed five times in PBS–2% nonfat milk (Koger Cv., Cincinnati, Ohio), and incubated overnight with the first antibodies. Monoclonal antibody 12CAS (Boehringer Mannheim) was incubated at 4 μg per ml PBS–0.05% Tween 20 for 1 h. Affinity-purified anti-NUP98 polyclonal antibodies (raised in rabbits against the peptide sequence EREI [Superior] per 50 ml of protein A-Sepharose (50% slurry) preincubated with 50 μl of our CBP/p300 antiserum cocktail [see above]). After five washes with PBS–0.2% Triton X-100, the beads were boiled in SDS-PAGE sample buffer and the precipitated proteins were analyzed by SDS-PAGE and fluorography. Transactivation assays. GAL4 fusion proteins were tested for transcriptional activation properties on the GAL4-responsive reporter construct G5BpGL2. This reporter was constructed by inserting a 6.8-kb fragment from BAC017 (27), containing the GAL4-responsive reporter construct that contained five tandem copies of the GAL4 DNA binding site upstream of the simian virus 40 (SV40) early promoter (a kind gift from C. Abate-Shen). Blunt-ended DNA fragments encoding relevant portions of FG repeat-rich nucleoporins or HOXA9 were cloned into the Smal sites of either pm1 or pm2 such that in-frame fusions were created between sequences encoding the GAL4 DNA binding domain and the FG repeat-rich nucleoporin motifs or HOXA9 sequences. NIH 3T3 cells (2 × 10^6) were seeded in 24-well plates. The next day, cells in each well were cotransfected (with Superfect) with 0.6 μg of GAL4 fusion protein expression plasmid, 0.3 μg of G5BpGL2 reporter plasmid, 25 or 2.5 ng of CMV-EUA vectors (where applicable), and 10 ng of pGCR-MV internal control plasmid (Promega Corp.). The cells were harvested after about 16 h, and enzyme assays were performed to assess reporter gene expression. Reporter gene-derived luciferase activity was normalized to Renilla luciferase derived from pRL-CMV. Transfections and analyses with the GAL4-NUP and GAL4-HOXA9 fusion constructs were performed in triplicate. To test for a potential effect of E1A on GAL4-NUP98 protein levels, 1 × 10^6 NIH 3T3 cells were seeded in 10-cm dishes. A day later, the cells were cotransfected with 15.8 μg of GAL4 fusion protein expression plasmid and 60 ng of CMV-EUA vectors [these DNA concentrations correspond to the 0.06 μg of CMV-EUA vector per 2 × 10^6 NIH 3T3 cells used in our transformation assay]. At 24 h posttransfection, the cells were harvested and immunoprecipitations and Western blot analysis with 12CAS antibodies were performed as described above.

For CBP potentiation experiments NIH 3T3 cells were cotransfected with 0.1 μg of GAL4-NUP98(Δ1–499) or 0.5 μg of GAL4-CREB(160–284), 0.25 μg of G5BpGL2 reporter plasmid, 0.5, 0.2, or 0.16 ng of RSV-CBP (or equimolar amounts of empty Ross sarcoma virus [RSV] vector as a negative control), and 10 ng of pGCR-TK internal control plasmid (Promega Corp.). Transfection assays in B95.8 cells were performed as previously described (15). For nuclear run-on experiments, the transfection experiments were done either in duplicate or in triplicate and repeated several times.

In vitro DNA binding assays. Proteins for electrophoretic mobility shift assays (EMSA) were produced in vitro from SP6 expression plasmids with a coupled reovirus rabies system as previously described (26). To ensure that approximately equal amounts of each lysate were added to the DNA binding reaction mixtures, proteins were synthesized in parallel in the presence of [35S]methionine, subjected to SDS-PAGE, quantitated on a PhosphorImager (Molecular Dynamics), and normalized for the number of methionine residues. DNA binding reactions were performed as previously described (11). Single-stranded oligonucleotides were labeled with [γ-32P]ATP, annealed, purified, and used with core sequences matching the consensus TGTTATT.

RESULTS

The NUP98-HOXA9 fusion gene encodes two proteins. To construct a NUP98-HOXA9 fusion cDNA encoding the predicted chimeric oncoprotein (7, 33), we first isolated NUP98 cDNA clones from a human BM library. We identified two independent cDNA clones containing the 5′ and 3′ ends of the human NUP98 coding sequence (7); however, both cDNAs lacked a segment that encoded amino acids 754 to 1218 of the NUP98 protein, including 18 FG repeats. We named this al-
Alternatively spliced product NUP98b. The full-length NUP98 cDNA, referred to as NUP98a, was reconstructed from partial BM and placental cDNAs (see Fig. 2A). To confirm expression of both mRNA transcripts from the NUP98 locus, we analyzed human BM RNA by RT-PCR (Fig. 1A). In this assay, NUP98a and NUP98b RNAs should generate diagnostic PCR fragments of 754 and 181 bp, respectively. Both amplification products were obtained (Fig. 1B, lane 1). Sequencing of the fragments indicated that they indeed differed in the 573-bp segment encoding amino acids 254 to 444 of the full-length NUP98 protein (not shown).

We then adapted the above RT-PCR strategy to screen for alternative NUP98-HOXA9 fusion transcripts. A nested RT-PCR assay based on RNA from a patient with t(7;11)(p15; p15)-positive acute myeloid leukemia (AML) yielded the expected amplification products of 754 and 181 bp (Fig. 1B, lane 3). Subcloning and DNA sequence analysis of the fragments revealed that they had originated from NUP98a-HOXA9 and NUP98b-HOXA9 fusion transcripts (data not shown), indicating that the leukemic cells express both chimeric isoforms.
NUP98-HOXA9 chimeras are nuclear proteins that transform NIH 3T3 fibroblasts. Hemagglutinin (HA1) epitope-tagged versions of cDNAs containing NUP98a, NUP98b, NUP98a-HOXA9, NUP98b-HOXA9, and the separate NUP98b and HOXA9 portions of NUP98b-HOXA9 (Fig. 2A) were cloned into the pSRαMSV/αCD8 retroviral vector (17) and expressed in NIH 3T3 cells by retroviral gene transfer. To determine the subcellular localization of the HA1-tagged proteins, NIH 3T3 fibroblasts were immunostained with 12CA5 monoclonal antibody against the HA1 epitope. HA1-NUP98a and HA1-NUP98b both showed punctate staining of the nuclear rim (Fig. 3A and B), typical of NPC-associated proteins (39). By contrast, the HA1-NUP98a-HOXA9 and HA1-NUP98b-HOXA9 chimeras were found located in the nuclei of NIH 3T3 cells (Fig. 3C and D). Typically, such nuclei displayed a very fine granular staining pattern whereas the nucleoli did not stain at all. To determine whether the HA1-NUP98-HOXA9 chimeras also associate with NPCs, cells were double stained for HA1 and CRM1, a transport factor localized mainly at the nuclear envelope (NE). Our HA1-specific signals did not co-localize with CRM1 signals at the NE, indicating that there is no detectable localization of chimeric protein at the NE (data not shown). Like the fusion proteins, the HOXA9 portion without NUP98 showed nuclear localization but the staining pattern was less granular (Fig. 3F). The NUP98b fragment derived from the chimera was concentrated at the NE, al-
Although a substantial proportion of this truncated protein was found in the nucleus, even at low to moderate levels of expression (Fig. 3E). In all cases, protein expression was confirmed by performing immunoprecipitations with anti-HA monoclonal antibodies on lysates of metabolically labeled cells (data not shown).

To determine the oncogenic potential of the NUP98-HOXA9 proteins, we used an NIH 3T3 fibroblast transfection assay. This assay is commonly used in the field for structure-function studies on leukemia oncoproteins, and the results with fibroblasts are generally in agreement with those of other transformation assays. NIH 3T3 fibroblasts expressing NUP98a-HOXA9 or NUP98b-HOXA9 formed colonies efficiently in soft agar (Fig. 2C and D), indicative of cellular transformation. In contrast, NIH 3T3 fibroblasts over-expressing NUP98a, NUP98b, or the NUP98b or HOXA9 portions of the chimeric constructs, did not form colonies above background levels observed with NIH 3T3 cells infected with retroviral stocks prepared from the empty vector pSRaMSVtkCD8 (Fig. 2C and D). These results indicate that the in vitro cellular oncogenicity of the NUP98-HOXA9 proteins requires both the C-terminal HOXA9 sequences and the N-terminal region of NUP98 containing the nucleoporin-specific FG repeats.

NUP98-HOXA9-mediated transformation depends on its ability to bind DNA and heterodimerize with PBX. The nuclear localization of NUP98-HOXA9 proteins suggested that DNA binding activity might be important for their oncogenic potential. The HOXA9 chimeras contained within the NUP98-HOXA9 oncoprotein has two distinct regions implicated in DNA binding: (i) the homeodomain and (ii) a short tryptophan-containing motif required for binding to the PBX transcriptional cofactors. This motif is located immediately N-terminal of the homeodomain. HOX-PBX heterodimers have increased DNA binding affinity and specificity, allowing selective recognition and activation of genes containing HOX response elements (9–11, 26, 37, 40, 47, 48). We determined the in vitro DNA binding activity of the NUP98-HOXA9 oncoproteins by EMSA (Fig. 2A and C). However, without a consensus DNA binding site for HOXA9 is unknown, we selected the PBX1-HOXA10 consensus DNA binding sequence as a target for interaction with the HOX-PBX heterodimers. This consensus sequence has a TTAT core motif, preferentially recognized by homeobox proteins of the Abd-B-like gene family, including HOXA9 (6, 45). DNA binding of in vitro-synthesized HOX9, NUP98-HOXA9 isoforms, or their truncated derivatives to the PBX1-HOXA10 bipartite probe was determined in the presence or absence of in vitro-translated PBX1a protein. Both full-length HOX9 and the HOX9 portion of NUP98-HOXA9 bound the DNA target alone, while addition of PBX1a to the binding-reaction mixtures increased their activities (Fig. 4A, compare lane 2 with lane 3 and lane 8 with lane 9). Both NUP98-HOXA9 chimeras displayed cooperative DNA binding with PBX1a; however, the DNA binding of PBX1a/NUP98b-HOXA9 chimeras to the consensus site was considerably greater than that of PBX1a/NUP98a-HOXA9 dimeric complexes (compare lanes 4 and 5 with lanes 6 and 7). As expected, the NUP98b portion failed to demonstrate any activity by itself (lanes 10 and 11). To show formation of PBX/NUP98-HOXA9 heterodimers in vivo, we prepared lysates from NIH 3T3 cells expressing HA1-NUP98b-HOXA9 and performed EMSAs with HOX10 and PBX1-HOXA10 probes. Typically, a slower-migrating complex was formed with the PBX1-HOXA10 probe than with the HOXA10 consensus probe (Fig. 4D, compare lanes 1 and 5), indicative of the formation of PBX/NUP98b-HOXA9 heterodimers in vivo (Fig. 4D).

To confirm that NUP98-HOXA9 requires an intact homeodomain as well as a functional N-terminal PBX-interaction motif for binding to the bipartite DNA probe, we constructed two NUP98b-HOXA9 mutants. In one [NUP98b-HOXA9 (FIKI)] (Fig. 2A)), the oligopeptide FIKI was inserted in the third helix of the HOXA9 homeodomain, while in the other [NUP98b-HOXA9(W→A)] (Fig. 2A)), the conserved tryptophan in the PBX-interaction motif was replaced with an alanine. The NUP98b-HOXA9(FIKI) mutant completely failed to interact with the DNA probe (Fig. 4A, lanes 12 and 13). The DNA-binding capacity of the NUP98b-HOXA9(W→A) mutant was unaffected in the absence of PBX, but its cooperative binding to the probe was severely impaired (compare lane 6 with lane 14 and lane 7 with lane 15).

We then asked whether NUP98-HOXA9 proteins mediate their oncogenic effects by a specific DNA-binding mechanism. When the NUP98b-HOXA9(FIKI) and NUP98b-HOXA9 (W→A) mutants were assayed for their ability to induce NIH 3T3 colonies in soft agar, both mutants showed a complete loss of transforming potential (Fig. 2C), despite their effective expression (data not shown) and localization in the nucleus (Fig. 3G and H). Like NUP98b-HOXA9, both mutants displayed the fine granular nuclear staining pattern. Thus, HOXA9-mediated DNA binding and interaction with PBX are essential for transformation, suggesting that NUP98-HOXA9 chimeras are altered transcription factors rather than altered transport factors.

NUP98 FG repeats are critical for transcriptional and transformation. To evaluate the role of the NUP98 portions in NUP98-HOXA9-mediated transformation, we prepared a series of N-terminal deletion constructs and tested their ability to transform NIH 3T3 cells. Mutant NUP98b[Δ51–223]HOXA9, which lacks 8 of 37 FG repeats in the NUP98a segment (Fig. 2A), retained its ability to transform NIH 3T3 fibroblasts (Fig. 2C). Moreover, mutant NUP98b[Δ51–223]HOXA9, which lacked the same 8 FG repeats and had only 11 repeats left, remained transforming, although the number of colonies formed was only half of that induced by NUP98b-HOXA9. We reasoned that if NUP98-HOXA9 proteins operate as deregulated transcription factors in HOX-PBX-mediated transformation of NIH 3T3 cells with a reporter plasmid containing five GAL4 DNA binding sites (Fig. 5A). The HOXA9 portion of the chimeras fused to the GAL4 DNA binding domain displayed very little ability to activate transcription of this reporter. By contrast, both the NUP98a and the NUP98b portions, when fused to GAL4, were very strong activators of gene transcription. GAL4-NUP98a[Δ51–223] and GAL4-NUP98b[Δ51–223], which both transformed NIH 3T3 cells when linked to the
HOXA9 C terminus, were also efficient transcriptional activators (Fig. 5A). However, NUP98b(Δ51–469) and NUP98b(Δ1–223), the two short segments derived from NUP98b that failed to transform fibroblasts as fusion partners of HOXA9, lacked transcriptional activation potential as GAL4 fusions (Fig. 2C).

Furthermore, a selection of the above GAL4 fusion constructs stimulated the GAL4 reporter in Ba/F3 cells (myeloid progenitors that are dependent on interleukin-3 for growth [Fig. 5B]), demonstrating that FG repeats of NUP98 can act as transactivators in myeloid cells, where NUP98-HOXA9 chimeras are implicated in myeloid leukemia. Taken together, these studies suggest that the FG repeat-rich portions of NUP98a and NUP98b function as novel transactivation domains critical for NUP98-HOXA9-mediated transformation.

FG repeats of CAN/NUP214 and NUP98 are functionally exchangeable. FG repeats from the other nucleoporin implicated in AML, CAN/NUP214 (49, 50), might function similarly to those from NUP98 in our transformation and transactivation assays. To investigate this possibility, we prepared two CAN/NUP214 cDNA fragments, one encoding the last 227 amino acids of CAN/NUP214 [CAN/NUP214(1684–2090)] and including 19 FG repeats (2 FXFG and 17 FG) and another encoding the last 469 amino acids [CAN/NUP214(1605–2090)] and including all 34 FG repeats from DEK-CAN/NUP214 and SET-CAN/NUP214 (3 FXFG and 32 FG). In addition, to test the generality of FG repeats in these assays, we generated a cDNA fragment encoding the last 359 amino acids of NUP153 [NUP153(1121–1479)], which contains 16 FG repeats (8 FXFG and 8 FG). The NUP153 and NUP214 segments prepared showed no significant sequence similarity to NUP98 other than FG repeats and a prevalence for Asn, Gln, Ser, and Thr residues in the spacer sequences between the FG repeats (the nucleoporin portion of NUP98a-HOXA9 consists of 17 GLFG, 2 FXFG, and 18 FG repeats; that of NUP98b-HOXA9 has 10 GLFG, 1 FXFG, and 8 FG repeats). CAN/NUP214(1605–2090)-HOXA9, CAN/NUP214(1684–2090)-HOXA9, and NUP153 (1121–1479)-HOXA9 were all nuclear proteins (Fig. 3M and N), displayed the predicted molecular weight (data not shown), and efficiently bound DNA (Fig. 4C, lanes 2 to 5). When they

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**FIG. 4.** NUP98-HOXA9 fusion proteins bind cooperatively with PBX1a to a PBX1-HOXA10 bipartite DNA sequence. (A to C) EMSAs of in vitro-translated proteins whose identities are indicated above the gel lanes. EMSAs were performed with a radiolabeled probe containing a PBX1-HOXA10 bipartite binding site in the absence (−) or presence (+) of in vitro-translated PBX1a. Cooperative DNA binding was observed for fusion proteins with an intact HOXA9 homeodomain. Typically, the binding affinity for the bipartite probe increased when the NUP98 portion fused to HOXA9 became smaller. The differences in intensities of the protein-DNA complexes within each panel (A or B) represent true variations in DNA binding activity. FP, free probe. (D) NUP98b-HOXA9 and PBX form heterodimers in NIH 3T3 cells. Radiolabeled probe containing the PBX1-HOXA10 or HOXA10 binding site was added to lysates of cells expressing the HA1-tagged NUP98-HOXA9 fusion proteins, and the formation of protein-DNA complexes in the absence (−) or presence (+) of 12CA5 monoclonal antibody was studied. For each lysate, the shifted complexes formed with the PBX1-HOXA10 probe (arrow to the left of lane 5) are larger than those formed with the HOXA10 probe (arrow to the left of lane 1), indicating that NUP98-HOXA9 and PBX form heterodimers in vivo. The shifted complex ablates when incubated with 12CA5 antibody, confirming the presence of HA1-NUP98b-HOXA9 in such complexes.
were assayed for their colony-forming properties, CAN/NUP214 (1605–2090)-HOXA9 and NUP153(1121–1479)-HOXA9, but not CAN/NUP214(1864–2090)-HOXA9, induced colony formation in soft agar [Fig. 2C; the average number of colonies generated by CAN/NUP214(1605–2090)- and NUP153(1121–1479)-HOXA9 expression was 78 and 35%, respectively, of that seen with NUP98b-HOXA9]. We then generated fusion proteins of GAL4 and the CAN/NUP214 and NUP153 portions to determine their transactivation potential. GAL4-CAN/NUP214(1605–2090)-HOXA9 strongly activated reporter gene transcription [Fig. 5A], whereas GAL4-CAN/NUP214(1864–2090)-HOXA9 had very little transactivation potential (comparable to the HOXA9 portion of the NUP98-HOXA9 fusion). GAL4-NUP153(1121–1479) displayed moderate transactivation of the reporter. We noticed that the levels of GAL4-NUP153(1121–1479) protein were about 5- to 10-fold lower than those of the other
GAL4-NUP fusion proteins (data not shown), possibly accounting for its lower effect. Thus, our observations suggest that FG repeat-rich regions from heterologous nucleoporins can replace NUP98 in the NUP98-HOXA9 chimeras if they contain appreciable transcriptional activation potential.

We also swapped the NUP98 portion of NUP98b-HOXA9 for the transcriptional activation domain of VP16 (amino acids 413 to 490) [Fig. 2A] (12). The resulting VP16(413–490)-HOXA9 protein showed nuclear localization (Fig. 3O), bound to the PBX1-HOXA10 DNA probe with PBX1a (Fig. 4C, lanes 6 and 7), and transformed NIH 3T3 cells (about 40% of the efficiency of NUP98b-HOXA9 [Fig. 2C]). Thus, a heterologous transcription domain fused to HOXA9 DNA binding and PBX heterodimerization domains is sufficient for transformation of the NIH 3T3 cells. This result supports the idea that NUP98-HOXA9 proteins are oncogenic transcription factors rather than oncogenic transport factors.

The aberrant function of NUP98-HOXA9 could also be due to loss of a transcriptional repression function present in full-length HOXA9. Indeed, the similar HOX7 is a repressor of gene transcription (43). To test for HOX9-dependent transcriptional repression activity, we cotransfected into NIH 3T3 cells GAL4-HOXA9 and a reporter plasmid containing five GAL4 DNA binding sites upstream of a luciferase gene driven by an SV40 promoter. As shown in Fig. 5C, GAL4-HOXA9 functioned as a very strong transcriptional repressor. For comparison, the HOX9 portion from NUP98-HOXA9 fused to GAL4 did not display appreciable transcriptional repression whereas GAL4-NUP98b-HOXA9 activated the SV40 promoter severalfold. These data suggest that the N terminus of HOXA9 contains a motif that functions as an inhibitor of transcription and is replaced by a nucleoporin-mediated transcriptional activation function in t(7;11) leukemia cells.

CBP/p300 is necessary for NUP98-mediated transactivation.

To further elucidate the mechanism whereby NUP98 FG repeats act as transactivators, we first examined the role of the CBP and p300 in this process. CBP and p300 are direct targets of chromosomal rearrangements in human leukemias, and both function as coactivators of a variety of gene-specific activators (20). To this end, we tested if the adenovirus 12S E1A protein inhibits GAL4-NUP98 function (12S E1A binds to the CREB residues 160 to 284 [GAL4-CREB(160–284)]) (Fig. 6A) (52), confirming that 12S E1A specifically targeted CBP/p300-dependent transactivation in our assay.

These results suggested that CBP would potentiate GAL4-NUP98 activity. We determined this by cotransflecting GAL4-NUP98b(1–469) expression vector with increasing amounts of CBP/p300. CBP was found to stimulate GAL4-HOXA9 activation, which is not transcriptionally active when fused to GAL4, and is replaced by a nucleoporin-mediated transactivation function in t(7;11) leukemia cells.

To confirm that the NUP98-HOXA9 proteins do interact with CBP/p300 in vivo, we prepared lysates of [35S]methionine-labeled NIH 3T3 cells expressing either HA1-NUP98b-HOXA9, HA1-HOXA9, or the HA1-tagged HOXA9 portion and performed two sequential immunoprecipitations, first with 12CA5 monoclonal antibody then with an CBP/p300-specific antibodies (52). An in vivo interaction between each of the NUP98-HOXA9 chimeras and CBP/p300 (seen as a broad band) was detected (Fig. 7B; the results shown are representative of three independent experiments). CBP/p300 was not detectable in an immunocomplex with the HOX9 portion of the fusion protein that lacks FG repeat-rich portions of NUP98 mediate binding of CBP/p300 to NUP98b-HOXA9 and NUP98b-HOXA9. Confirming our findings in NIH 3T3 cells, a specific interaction between NUP98 and CBP/p300 was also detected in lysates of [35S]methionine-labeled HTA cells transiently transfected with an HA1-NUP98b-HOXA9 expression vector (Fig. 7B; the results are representative of two experiments). Western blot analysis with 12CA5 antibody revealed that the various HA1-tagged proteins studied for communoprecipitation of CBP/p300 were expressed at comparable levels. This excludes the possibility that gross variations in HA1-tagged protein levels underlie the observed differences in binding to CBP/p300.

**DISCUSSION**

Although several distinct chromosomal translocations in human leukemias disrupt FG repeat-containing nucleoporins, a role for these components in the transformation of blood cells has not been established. Remarkably, we demonstrate that FG repeat-rich segments, which normally function to transport macromolecules across the NE, perform a critical role in NUP98-HOXA9-mediated transformation, where they function as potent activators of transcription that physically and functionally interact with the transcriptional coactivators CBP and p300. Additional evidence to support a model in which NUP98-HOXA9 proteins act as oncogenic transcription factors includes (i) their nuclear localization, (ii) the specific
DNA binding activity of the chimeras, and (iii) the requirement for intact HOXA9 DNA binding and PBX cofactor interaction. Interestingly, FG repeats from NUP98-HOXA9 could be replaced by CAN/NUP214 FG repeats, indicating that FG repeat-mediated transactivation may be a shared pathogenic function of nucleoporins in AML.

**Deregulation of HOX activity by NUP98-HOXA9.** Vertebrate HOX genes are developmental regulators that also mediate key steps in the proliferation and differentiation of fetal and adult hematopoiesis in a lineage- and stage-specific manner (25). Although the repertoire of HOX gene expression during hematopoiesis is beginning to emerge, the genetic targets regulated by HOX proteins remain elusive, as is the case for the PBX and MEIS family members, which may cooperate with the implicated HOX proteins. NUP98-HOXA9-mediated transformation depends on its ability to bind DNA with members of the PBX family of HOX cofactors, implying that NUP98-HOXA9 affects the control of PBX/HOXA9-regulated target genes.

Like HOXA7, HOXA9 can function as a very efficient inhibitor of gene transcription. The HOXA9 portion of the NUP98-HOXA9 chimeras displayed no such repressor function, indicating that residues in the N terminus of full-length HOXA9 mediate gene repression. It is unlikely that the mere loss of HOXA9 repressor status is sufficient for the development of AML, because overexpression of a carboxy-terminal HOXA9 fragment does not transform NIH 3T3 cells. However, to gain oncogenic properties, the carboxy-terminal HOXA9 portion requires a transcriptional activator, which favors a model of leukemogenesis in which the FG repeats of NUP98-HOXA9 strongly activate HOXA9-responsive target genes whose deregulated expression interferes with proper execution of myelopoiesis. Transformation driven by coexpression of HOXA9 and MEIS1 does not seem to require a gain of transactivation function of HOXA9, suggesting that the mechanism of target gene deregulation involved may be different from that of NUP98-HOXA9-induced leukemias.

**FG repeat-rich nucleoporins as potent oncogenic transactivators.** Most striking is the finding that FG repeat-containing portions from the N terminus of NUP98a and NUP98b can...
function as transactivation domains. Indeed, the oncogenic potential of NUP98-HOXA9 correlates with NUP98-mediated transcriptional competence. A certain threshold level of transactivation function is apparently required for oncogenicity, after which the correlation between transactivation and transformation is qualitative. However, we cannot exclude the possibility that NUP98 contributes another function to the NUP98-HOXA9 chimeras beside transcriptional activation. Nevertheless, our finding that the completely unrelated VP16 transactivation domain can functionally replace NUP98 suggests that other potential NUP98 functions are not necessary for oncogenesis in NIH 3T3 cells. Moreover, specific FG repeat-rich domains of two heterologous nucleoporins, CAN/NUP214 and NUP153, were oncogenic and contained transcriptional activation properties, further strengthening the correlation between transformation and transactivation for these domains. The transactivating FG repeat-rich segment of CAN/NUP214 is retained in the DEK-CAN/NUP214 and SET-CAN/NUP214 leukemic fusion proteins (49, 50). This suggests that the shared pathogenic mechanism of FG repeat-containing nucleoporins is to activate gene transcription. Although the precise function of DEK remains to be established, there is evidence to suggest that DEK is a sequence-specific DNA binding protein (15). Interestingly, the nuclear protein SET interacts directly with MLL (also called HRX) (1), which binds to DNA and positively regulates HOX gene expression (53). The MLL region that binds SET is consistently retained in all MLL leukemic fusion proteins (1). Thus, SET-CAN/NUP214 may contribute to leukemogenesis by interfering with the proper expression of MLL-controlled HOX proteins.

CBP/p300: the link between NUP98 FG repeats and the transcription machinery. How does a protein that mediates macromolecule transport through the nuclear pores act as a regulator of transcription? Thus far, no evidence supporting a direct connection between NPC proteins and the basal transcription machinery has been reported, suggesting that the FG repeat segments of NUP98 may be fortuitous rather than genuine activators of gene transcription. Our results show that transactivation of FG repeat-rich segments of NUP98 correlates with their ability to interact and functionally collaborate with the transcriptional coactivators CBP/p300. Thus far, NUP98-HOXA9 is the first translocation-generated fusion protein known to recruit CBP/p300 as part of its oncogenic mechanism. However, there is additional evidence to suggest a more central role for CBP/p300 in both hematopoiesis and leukemia. First, CBP and p300 are direct targets of at least three independent chromosomal rearrangements in human leukemias. Both CBP and p300 are fused to MLL through, respectively, the t(11;16) (41, 46) and t(11;22) (19) translocations. In addition, CBP is linked to MOZ through the t(10;16) translocation associated with AML (8). Second, CBP/p300 in-

FIG. 7. Transcriptionally active FG repeat-rich portions of NUP98 bind CBP/p300. (A) GAL4-NUP98 transcription activity correlates with binding to CBP in vitro. GST pull-down assays were performed with the indicated [35S]methionine-labeled NUP98 or HOXA9 portions produced in vitro and GST-CBP fusion proteins purified from E. coli. The 25% input (lane) shows 25% of the NUP98 or HOXA9 segments used in each pull-down assay. Typically, in vitro-translated NUP98 portions appear as doublets, representing fragments with and without a HA1 tag (the NUP98 portions were cloned in pSP73 as HA1 fusion genes that retained the endogenous NUP98 translation initiation codon). GST acts as a negative control for binding. Comparable amounts of GST and GST-CBP fusion proteins were used in each pull-down assay. The experiment shown is representative of three independent experiments. (B) In vitro interaction between NUP98-HOXA9 oncoproteins and CBP/p300. Total-cell lysates were prepared from [35S]methionine-labeled HtTA or NIH 3T3 cells expressing the indicated HA1-tagged proteins. Two sequential immunoprecipitations were performed as detailed in Materials and Methods. The first and second immunoprecipitations (IP) were performed with the indicated antisera. A CBP/p300 antiserum cocktail was used for most efficient detection of these transcriptional coactivators. The positions of CBP/p300 and a molecular mass marker are indicated.
teracts and functionally cooperates with the AMLI/CBFβ transcription factor complex in myeloid cell differentiation (21), underscoring a role for CBP/p300 as a hematopoietic transcriptional coactivator. Moreover, the AMLI/CBFβ transcription factor complex is the most frequent target of chromosomal translocations in human leukemias. Thus, aberrant activation of this complex by nuclear relocation of the FG repeats and/or their fusion NUP98 by using NUP98-specific antibodies (8a). This suggests that nuclear pores. However, in preliminary studies, we could not detect colocalization of NUP98 and CBP/p300 at nuclear pores or coimmunoprecipitate CBP/p300 with endogenous NUP98 by using NUP98-specific antibodies (8a). This suggests that nuclear relocation of the FG repeats and/or their fusion with a specific DNA binding domain may be essential for their interaction with CBP/p300.

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Chapter 4

Disruption of the FG Nucleoporin NUP98 Causes Selective Changes in Nuclear Complex Stoichiometry and Function
Disruption of the FG nucleoporin NUP98 causes selective changes in nuclear pore complex stoichiometry and function

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The NUP98 gene encodes precursor proteins that generate two nucleoplasmically oriented nucleoporins, NUP98 and NUP153. By using gene targeting, we have selectively disrupted the murine NUP98 protein, leaving intact the expression and localization of NUP153. We show that NUP98 is essential for mouse gastrulation, a developmental stage that is associated with rapid cell proliferation, but dispensable for basal cell growth. NUP98 disruption selectively eliminates nuclear pore complexes. Typically, NUP98-deficient cells contained on average approximately 5-fold more cytoplasmic annulate lamellae than control cells. We found that a set of nucleoplasmically oriented nucleoporins, including NUP358, NUP214, NUP98, and p62, assembled inefficiently into nuclear pores of NUP98−/− cells. Instead, these nucleoporins were prominently associated with the annulate lamellae. By contrast, a group of nucleoplasmically oriented nucleoporins, including NUP153, NUP50, NUP96, and NUP93, had no affinity for annulate lamellae and assembled normally into nuclear pores. Mutant pores were significantly impaired in transport receptor-mediated docking of proteins with a nuclear localization signal or M9 import signal and showed weak nuclear import of such substrates. In contrast, the ability of mutant pores to import ribosomal protein L23a and spliceosome protein U1A appeared intact. These observations show that NUP98 disruption selectively impairs discrete protein import pathways and support the idea that the transport of distinct import complexes through the nuclear pore complex is mediated by specific subsets of nucleoporins.

Transport between the nucleus and the cytoplasm occurs through nuclear pore complexes (NPCs) embedded in the nuclear envelope (NE). NPCs have an 8-fold rotational symmetry in the plane parallel to the NE (reviewed in ref. 1). Each NPC contains a membrane-embedded central framework that embraces a central pore. The central framework consists of a ring-like spoke complex that is sandwiched between a cytoplasmic and a nuclear ring. Each cytoplasmic ring supports eight fibrils that extend into the cytoplasm, whereas each nuclear ring carries eight filaments that join distally to form a basket-like assembly (1, 2). The vertebrate NPC has an estimated molecular mass of ∼125 MDa and is composed of ∼80–100 different proteins called nucleoporins, of which 16–20 have been cloned (3). In contrast, the Saccharomyces cerevisiae NPC is only ∼66 MDa (4) and is composed of ∼35–50 nucleoporins, of which more than 30 have now been identified (5).

Nucleocytoplasmic transport is mediated by soluble transport factors that belong to the karyophilin family of transport receptors, whose members can be subdivided into importins and exportins. Typically, a given transport receptor binds to an import or export signal-containing cargo in one compartment, guides it to and accompanies it through an NPC, releases it in the opposite compartment, and then shuttles back to the first compartment to repeat the cycle (see refs. 3, 6, and 7). The small GTPase Ran and its regulatory factors play a central role in this process (6, 8). Ran is presumably maintained as RanGTP in the nucleus by the nucleotide exchange factor RCC1, and as RanGDP in the cytoplasm by the GTPase-activating protein RanGAP. RanGTP is bound to cargo-containing export factors as they dock to and translocate through the NPC. At the cytoplasmic face of the NPC, RanGTP becomes exposed to RanGAP activity and converts to RanGDP, which triggers disassembly of the trimeric export complex. In contrast, cargo-containing import factors dock and translocate through the NPC without Ran. At the nuclear face, import factors associate with RanGTP and release their cargo.

Several interactions between individual FG (Phe-Gly) repeat-containing nucleoporins and transport factors have been reported, leading to the idea that such interactions could play a pivotal role in the docking, translocation, and/or termination steps of the transport process (6, 7, 9). In yeast, most FG nucleoporins are symmetrically located on both the nuclear and the cytoplasmic sides of the NPC, but the position of some nucleoporins is asymmetric (1, 5). In mammalian cells, both NUP153 and NUP214 are positioned at the tips of cytoplasmic fibrils, whereas NUP98 and NUP153 are located near the midsection of the nuclear basket. A so-called p62 subcomplex, which consists of four FG nucleoporins (p62, p58, p54, and p45), is found at both the cytoplasmic and nuclear periphery of the central gated channel. Additionally, p62 binds to the distal and nuclear basket.

To obtain insight into the in vivo role of the murine FG-repeat nucleoporin NUP98, we disrupted it by a genetic approach. Our analyses show that NUP98 is required for proper NPC assembly and function.

Materials and Methods

Generation of NUP98 Knockout Mice and Genotyping. The NUP98 targeting vector contained an 8.0-kb NUP98 129Sv/J genomic DNA fragment in which an ∼500-bp SpeI/Stul fragment [containing 3′ sequences of NUP98 exon 3 (encoding amino acids 33–118) and 5′ sequences of NUP98 intron 3] had been replaced by a PGK-neo cassette. We electroporated this targeting vector into embryonic stem (ES) cells and performed drug selections as previously described (10). We identified correctly targeted ES cell clones by Southern blot analysis using a 5′ external probe on PstI-cut genomic DNA. Mutant mice were derived from these targeted ES clones as described in detail (11).

Abbreviations: NPC, nuclear pore complex; AL, annulate lamellae; NE, nuclear envelope; ES, embryonic stem; En, embryonic day; RT-PCR, reverse transcription-PCR; NLS, nuclear localization signal; TEM, transmission electron microscopy; rp, ribosomal protein.

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Generation of Embryo-Derived Cell Lines. Embryos [embryonic day 8.5 (ES.5)] from NUP98−/−;− intercrosses were grown on a
monolayer of mitotically inactivated mouse embryonic fibroblasts (MEFs) in ES cell medium (11). Cell lines derived from
those embryos were grown without MEFs and genotyped by Southern blot analysis. To reestablish NUP98 expression, we
cloned human HA1-tagged NUP98 (1–920) cDNA (for details, see ref. 13) into the ectopic retroviral expression vector
pSRMOSCV. Retroviral stocks were prepared and used for
retroviral gene transfer as described (12).

Western Blot Analyses and Reverse Transcription–PCR (RT-PCR). Western blot analyses were performed as described (12, 13). RT-
PCR were performed with a ThermoScript RT-PCR system
(Life Technologies, Grand Island, NY). cDNA was synthesized
from 1 μg of total RNA with NUP96 primer 5′-GGGGTG-G-
GGGAGCAAGCAGAGGG-3′. PCR and nested-PCR primer sets were p1/p2 and p3/p4, respectively: p1, 5′-GTTTAACAATCTATGGAAACCCCT-3′; p2, 5′-TTAG-
CAAGGGTCACTAGATGGG-3′; p3, 5′-GGGGGGCTTTGGA-
GTACAACGTC-3′; p4, 5′-TACTGGGGGCTTGGG-GGCGCA-3′. The PCRs were performed as recommended by
the manufacturer. Nested-PCR products were cloned into
pGEM-T Easy vector (Promega) and sequenced by standard
methods.

Indirect Immunofluorescence and Electron Microscopy. Immunosta-
ingens were carried out essentially as described (13, 14). Dilutions of primary antibodies were as follows: purified anti-
NUP98 (1:50–1:200), 1:100; mAbA414, 1:250; Kap-α (importin α),
Kap-β (importin β), importin 7, Kap-β3 (importin 5; N. Yasen),
NUP535 (B. Burke), Kap-β2 (transportin; G. Dreyfuss), GLE1 (S.
Wente) and 1CA5 (Roche Molecular Biochemicals), all
1:200; p62, 1:25; NUP358 (J. Wu), NUP214, CRM1 (L. Mattaj),
NUP98 (B. Burke), NUP50 (B. Clurman) and NUP96, all
1:1,000. Images were collected on a Carl Zeiss confocal micro-
scope LSM 510 using a 100× objective. To quantify NPCs,
confocal images of multiple mAbA414-labeled cells were collected
(we used a 100× objective and 4× digital magnification), and
the number of pores within a frame of 138 × 144 pixels was counted
and statistically analyzed.

For electron microscopic examinations, cells were trypsinized,
washed three times with PBS, fixed for 4 h with 2.5% (vol/vol)
glutaraldehyde/0.6% formaldehyde (buffered with sodium ca-
corboxymethylglycine, pH 7.2), and then postfixed with 2% (wt/wt) buffered
Oso4. Samples were dehydrated in a graded ethanol, and
then embedded in Spurr resin. Thin sections were examined by
transmission electron microscopy (TEM) by using a JEOL 1200
EXII. Immunogold labeling of cells with mAbA414 antibody was
carried out as described by Hetzer and Mat-
taj (18).

Results

NUP98 is Essential for Mouse Gastrulation but Dispensable for Basal Cell Growth. The NUP98 gene was disrupted by replacing an exon
3 fragment that encodes for NUP98 amino acids 33–118 and part of
intron 3, with a neo cassette (Fig. L4). After transfection, selection of targeted clones, and blastocyst injection, chimeric
mice were obtained that passed the disrupted NUP98 gene
through the germ line (Fig. 1B). F1 heterozygote mice were intercrossed, but no homozygous NUP98 mutant mice were
found among 198 offspring. Dissections of 8.5-day-old embryos
from heterozygous intercrosses revealed that knockout embryos
(n = 5) were grossly retarded in their development (Fig. 1C),
having the size of a normal 6.5- to 7.5-day-old gastrulation-stage
embryo. Histological analysis of these embryos revealed that
they were highly disorganized, with major defects in morpho-
genesis, proliferation, and pattern formation (data not shown).
When E8.5 NUP98−/− embryos were cultured in vitro (n = 3), they developed into epithelium-like cell lines over a period of 80–100 days (Fig. 1B). By contrast, E8.5 NUP98+/− (n = 2) and NUP98+/+ embryos (n = 1) developed into epithelium-like cell lines within 10–20 days after embryo explantation. We conclude that NUP98 is essential for proper gastrulation, but not for basal cell growth.

NUP98 Disruption Does Not Abrogate Expression and NPC Targeting of NUP96. The NUP98 gene encodes a 186-kDa precursor protein that is proteolytically cleaved into two nucleoporins, NUP98 and NUP96 (13). Analysis of NUP98 and NUP96 gene transcripts from NUP98+/+ and NUP98−/− cells by RT-PCR (Fig. 1D) revealed that the mutated allele generated transcripts that were ∼300 bp shorter than those that were derived from the wild-type allele (Fig. 1E). Cloning and sequencing of the wild-type and mutant RT-PCR products showed that the mutant product specifically lacked sequences encoded by exon 3 (encoding for NUP98 amino acids 26–118, Fig. 1F). When these Western blots were probed with antibody raised against NUP98(150–221), NUP98 could easily be detected in NUP98+/+ cells (Fig. 1G). However, both full-length NUP98 and NUP98(Δ26–118) protein were undetectable in NUP98−/− cells (Fig. 1G), even when long exposure times were applied. When embryonic cells were immunostained with antibody against NUP96, NEs of both control (Fig. 2C) and NUP98−/− cells (Fig. 2D) were labeled in a similar fashion, demonstrating proper NPC targeting of NUP96 in NUP98-deficient cells. Taken together, the above studies show that the NUP98 targeting vector effectively disrupted NUP98 expression without abrogating the expression and NPC targeting of NUP96.

NUP98 Disruption Causes Annullate Lamellae (AL) Formation. Embryonic cells were double-stained with antibody against NUP98 (151–221) and monoclonal antibody mAb414, a commonly used NE marker that mainly labels p62 and, to a minor degree, NUP153, NUP214, and NUP358. Control cells showed a typical rim-like NUP98 labeling of the NE (Fig. 2A), however, such staining was not detected in NUP98-deficient cells (Fig. 2B). Like control cells (Fig. 2A), NUP98−/− cells exhibited uninterrupted rim-like labeling of the NE with mAb414 (Fig. 2B), indicating that NUP98-deficient nuclear pores were evenly distributed over the NE. However, mAb414-labeled NUP98-deficient cells typically displayed two remarkable features: (i) a considerably reduced label intensity at the NE compared with control cells, and (ii) numerous dot-like cytoplasmic aggregates that were very similar in appearance to AL.

To examine the nature of the reduced mAb414 labeling at the NE in more detail, we analyzed the punctate nuclear-surface labeling of mAb414-stained control and knockout cells by high-resolution confocal microscopy. We found that individual NUP98-deficient nuclear pores (Fig. 2F) showed much lower intensity staining than did pores of control cells (Fig. 2E). Fig. 2F and I display the same image, with the difference that in I, the intensity of the nuclear pore signals is increased by using computer enhancement to allow for quantification of NPC density in control and NUP98−/− cells. This technique demonstrated that NPC densities were, indeed, not significantly different in control and NUP98-deficient cells (Fig. 2G). Ultrastructural analysis by transmission electron microscopy (TEM) revealed that neither NPC herniations and invaginations nor other alterations in the morphology of the NE or the NPCs could be identified in cells that lack NUP96 (compare Fig. 2H and I). To determine whether the cytoplasmic mAb414-positive aggregates represented AL, we performed the following analyses. First, we prepared ultrathin sections of control and NUP98−/− cells, and used TEM to quantify the number of AL-containing cells within these sections. We found that 2 of 50 control sections contained AL. By contrast, 8 of 50 knockout sections were positive for AL (Fig. 2J). Second, we immunostained control and knockout cells with mAb414 and quantitated the number of aggregates by high-resolution confocal microscopy. We found that knockout cells contained (on average) 12–13 large aggregates (n = 58 cells), whereas control cells had (on average) 2–3
such aggregates (n = 27 cells). This approximately 5-fold increase in the number of aggregates correlated well to the 4-fold increase in AL revealed by TEM analysis. Last, we applied preembedding immunogold electron microscopy with monoclonal antibody mAb414. This analysis revealed that the only cytoplasmic structures enriched in gold particles in knockout cells were AL (Fig. 2K). From these analyses, we conclude that NUP98 knockout cells form significantly more AL than normal cells.

**NUP98 Disruption Changes the Stoichiometry of Cytoplasmically Oriented Nucleoporins.** The decreased mAb414 labeling of the NE in NUP98−/− cells prompted us to test whether the stoichiometry of FG nucleoporins other than NUP98 was affected. To this end, we immunostained control and NUP98−/− cells for individual FG nucleoporins. First we looked at NUP214 and NUP358, two cytoplasmically oriented nucleoporins. We found that in NUP98-deficient cells, a substantial amount of each of these proteins had relocated from the NE to AL (Fig. 3A, B, F, and G, and Table 1). A large proportion of the NUP214 binding partner NUP88 had also relocated from the NE to AL (Table 1). p62, an FG nucleoporin located at both the cytoplasmic and nuclear face of the central gated channel complex and at the tip of the nuclear basket, was also redistributed from the NE to AL. NUP98−/− cells were permeabilized with digitonin to specifically stain p62 at the cytoplasmic face of the NPC, revealing a lower intensity of NE labeling than in control cells (data not shown). These data suggest that the p62 level at the cytoplasmic face of the NPC is reduced. Anti-p62 and anti-NUP214 Western blots (Fig. 1H) revealed that the overall p62 and NUP214 levels were similar in NUP98−/− and control cells, confirming that the reduction of nucleoporin levels at the NE results from a redistribution of protein rather than from a decline in protein production. In contrast to the above-mentioned nucleoporins, NUP153 and NUP50, two nucleoplasmically oriented FG nucleoporins, did not relocate from the NE to AL in NUP98−/− cells. Furthermore, NUP95, a nucleoplasmically oriented nucleoporin without FG repeats, was also normally distributed in NUP98−/− cells (Table 1). Ectopic expression of HA1-NUP98 in NUP98−/− cells restored NE levels of NUP214 (Fig. 3C), NUP358, p62, and NUP88 (data not shown) to normal, and strongly reduced formation of AL.

**Mutant Pores Have Reduced Affinity for a Subset of Transport Receptors.** Several shuttling transport receptors are known to bind to FG nucleoporins. To test their ability to target to mutant pores, we immunostained control and knockout cells for a variety of different transport receptors. We found that NE labeling of Kap-α (importin α; Fig. 3F and G), Kap-β (importin β), CRM1, importin 7, Kap-β2 (transportin), and GLE1 (Table 1), was much lower in knockout cells than in control cells. Moreover, these factors were all prominently associated with AL. Efficient NPC docking of these transport factors was restored when we expressed HA1-NUP98 in the knockout cells (Fig. 3M and data not shown). In contrast to the above receptors, Kap-β3 (importin 5), a transport receptor impli-
Table 1. Effects of NUP98 disruption on NPC binding of nucleoporins and transport factors

<table>
<thead>
<tr>
<th>Transport component*</th>
<th>NE localization</th>
<th>AL binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>FG repeat nucleoporins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NUP153 (NB)</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>NUP50 (NB)</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>p62 (CCC, NCC, NB)</td>
<td>Reduced</td>
<td>Yes</td>
</tr>
<tr>
<td>NUP358 (RanBP2) (CF)</td>
<td>Reduced</td>
<td>Yes</td>
</tr>
<tr>
<td>NUP214 (CAN) (CF)</td>
<td>Reduced</td>
<td>Yes</td>
</tr>
<tr>
<td>Non-FG repeat nucleoporins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NUP96 (NB)</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>NUP93 (NCC, NB)</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>NUP88 (NUP98) (CF)</td>
<td>Reduced</td>
<td>Yes</td>
</tr>
<tr>
<td>Soluble transport factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kap-α (importin α)</td>
<td>Reduced</td>
<td>Yes</td>
</tr>
<tr>
<td>Kap-β (importin β)</td>
<td>Reduced</td>
<td>Yes</td>
</tr>
<tr>
<td>Kap-β2 (transportin)</td>
<td>Reduced</td>
<td>Yes</td>
</tr>
<tr>
<td>Kap-β3 (importin 5)</td>
<td>Normal</td>
<td>No</td>
</tr>
<tr>
<td>Importin 7</td>
<td>Reduced</td>
<td>Yes</td>
</tr>
<tr>
<td>CRM1</td>
<td>Reduced</td>
<td>Yes</td>
</tr>
<tr>
<td>GLE1</td>
<td>Reduced</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Components listed in this column showed pronounced NE staining in control cells. NB, nuclear basket; CF, cytoplasmic fibrils; CCC, cytoplasmic face of the central channel.

NUP98 Disruption Impairs Distinct Protein Import Pathways. Cells lacking NUP98 were viable and proliferated in vitro. This fact implied that none of their major transport pathways was completely blocked. To determine whether the steady-state localization of nuclear proteins would be altered in NUP98−/− cells, we performed immunostainings for CBP, p300, RNA polymerase II, cyclin E, and p21. All these proteins were correctly localized to the nucleus (data not shown), suggesting that NUP98 disruption does not cause overt changes in the steady-state distribution of nuclear proteins. To determine whether the rate of nuclear-protein import through mutant pores was altered, we carried out several well standardized in vitro protein-import assays on control and knockout cells. These assays were typically performed in a transport buffer containing specific soluble transport receptors, an energy-regenerating system, and a well characterized fluorescein-labeled protein acting as a substrate for import into the nuclei of digitonin-permeabilized cells. Nuclear import of substrates was monitored by fluorescence microscopy. We found that nuclear import of proteins with an NLS (Fig. 4A and B) or M9 (Fig. 4C and D) import signal (mediated by Kap-α/β and Kap-β2, respectively) was substantially lower in mutant cells than in control cells. By contrast, Kap-β3-mediated nuclear import of the rpL23a (Fig. 4E and F) was very similar in knockout and control cells. Nuclear import of the spliceosome protein U1A, which seems to be independent of cytosolic-transport factors (18), was also comparable in control and knockout cells (Fig. 4G and H).

We also studied how efficiently the above transport complexes were able to dock to mutant nuclear pores. To this end, we performed the above import reactions at 4°C, because it is known that at this temperature import complexes can dock to nuclear pores, but cannot translocate through them. We found that mutant pores were impaired in both Kap-α/β-mediated docking of NLS-BSA (Fig. 4J and J) and Kap-β2-mediated docking of M9-core (Fig. 4K and L). Docking of rpL23a and spliceosome protein U1A to the cytoplasmic face of normal pores was very weak in comparison to that of NLS-BSA and M9 core. Nevertheless, there was no discernable difference in the NPC docking of these two substrates in control and mutant cells (data not shown).

Discussion

Here we report a selective disruption to the NUP98-NUP96 precursor protein via a genetic approach. Our study shows that NUP98 is required for mouse gastrulation, yet it is not essential for cell viability and basal cell growth. We show that the disruption of the nucleoplasmically oriented NUP98 protein triggers dramatic changes in nucleoporin stoichiometry at the nuclear pores of NUP98 knockout cells.
cytoplasmic face of the NPC, causing distinct protein import pathways to decay. We showed that the observed defects can be corrected by ectopic expression of HA1-NUP98 in NUP98−/− cells, indicating that they are reversible and caused by the disruption of NUP98.

Nucleoporins Selectively Relocate from Nuclear Pores to AL. The formation of vast numbers of AL is a striking feature of virtually all cells that lack NUP98. AL are defined as porous—often stacked—parallel membranes that contain pore complexes (reviewed in ref. 20). The function of AL is not well understood. Thus far, the only other genetic alteration that is known to promote AL formation is the disruption of the Drosophila nuclear lamin Dm0 gene (21). Characteristically, lamin Dm0-deficient cells form large numbers of cytoplasmic AL, but do not exhibit major changes in the NPC density of their NEs. Consistent with this observation, we found that NUP98-deficient cells also form AL while keeping their NPC density at a level that is similar to that of control cells. Thus, it seems unlikely that an NPC embedding defect would trigger AL formation in NUP98-deficient cells.

In this study, we compared the steady-state distributions of eight different nucleoporins in knockout and control cells. From our analysis, two distinct groups of nucleoporins have emerged. One group, consisting of NUP153, NUP50, NUP93, and NUP96, assembled poorly into mutant pores and wrongly localized to AL. Intriguingly, all nucleoporins in the second group, with the exception of p62, were positioned at the cytoplasmic face of the NPC. It seems, therefore, that loss of NUP98 causes nucleoporins to be selectively displaced from the NPC and deposited in the cytoplasmic AL. Whether nucleoporins are displaced seems to depend on their position within the NPC. Our observations further imply that AL in NUP98-deficient cells are not membrane-spanning cisternae that contain pores identical to those embedded in the NE.

Selective Impairment of Protein Import Pathways. We have genetically removed one of an estimated ~80–100 different nucleoporins that compose the mammalian NPC, and shown that this disruption results in pleiotropic changes in NPC stoichiometry. Therefore, functional changes displayed by mutant nuclear pores could be a direct consequence of disrupted NUP98 function(s) or result from impairment of other nucleoporins. Notwithstanding this complexity, which is inherent to the complexity of the NPC itself, our genetic study provides evidence for the idea that transport of distinct import complexes through nuclear pores is mediated by specific subsets of nucleoporins, and shown that this

Chapter 5

RAE1 is a Shuttling mRNA Export Factor that Binds to a GLEBS-like NUP98 Motif at the Nuclear Pore Complex through Multiple Domains

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RAE1 is a Shutting mRNA Export Factor that Binds to a GLEBS-like NUP98 Motif at the Nuclear Pore Complex through Multiple Domains

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Abstract

Gle2p is implicated in nuclear export of poly(A)⁺ RNA and nuclear pore complex (NPC) structure and distribution in Saccharomyces cerevisiae. Gle2p is anchored at the nuclear envelope (NE) via a short Gle2p-binding motif within Nup116p called GLEBS. The molecular mechanism by which Gle2p and the Gle2p-Nup116p interaction function in mRNA export is unknown. Here we show that RAE1, the mammalian homologue of Gle2p, binds to a GLEBS-like NUP98 motif at the NPC through multiple domains which include WD-repeats and a C-terminal non-WD-repeat extension. This interaction is direct, as evidenced by in vitro binding studies and chemical cross-linking. Microinjection experiments performed in Xenopus laevis oocytes demonstrate that RAE1 shuttles between the nucleus and the cytoplasm and is exported from the nucleus in a temperature-dependent and RanGTP-independent manner. Docking of RAE1 to the NE is highly dependent on new mRNA synthesis. Overexpression of the GLEBS-like motif also inhibits NE binding of RAE1 and induces nuclear accumulation of poly(A)⁺ RNA. Both effects are abrogated either by the introduction of point mutations in the GLEBS-like motif or by overexpression of RAE1, indicating a direct role for RAE1 and the NUP98-RAE1 interaction in mRNA export. Together, our data suggest that RAE1 is a shuttling transport factor that directly contributes to nuclear export of mRNAs through its ability to anchor to a specific NUP98 motif at the NPC.
Introduction

Nucleocytoplasmic transport is a signal-mediated process in which soluble carriers bind to a specific cargo in one compartment, guide it to and through the NPC, release it in the other compartment and finally recycle to the original compartment (Cole and Hammell, 1998; Nigg, 1997; Ohno et al., 1998; Weis, 1998). This model is based primarily on our knowledge of the protein import machinery (Gorlich, 1997). However, there are several lines of evidence to suggest that it holds true for protein and RNA export pathways as well. Kinetic competition studies in *Xenopus* oocytes have indicated that export of several classes of RNA, including 5S rRNA, U snRNAs, tRNAs and mRNAs, seems to be mediated by distinct and class-specific transport factors (Izaurralde and Mattaj, 1995; Jarmolowski et al., 1994). The first direct mediator of RNA export identified is the HIV-1 protein REV, which specifically binds to a REV-responsive element (RRE) in non-spliced viral mRNAs (Fischer et al., 1995). REV contains a leucine-rich-type nuclear export signal (NES) that is recognized by the nuclear export receptor CRM1, which mediates export of the viral RNA (Fornerod et al., 1997a; Fukuda et al., 1997; Stade et al., 1997). CRM1 also mediates export of U snRNAs, probably via direct binding to the U snRNP-associating protein CBC, or via an unknown NES-containing bridging factor (Izaurralde et al., 1995a). Recently, a second export receptor, hLos1p or exportin-t, was shown to bind tRNA molecules and promote their nuclear export (Arts et al., 1998; Kutay et al., 1998).

It is generally believed that mRNA molecules are exported from the nucleus as mRNA-protein (mRNP) particles (Nakielny and Dreyfuss, 1997). The protein composition of these complex structures is unclear, although a series of abundant nuclear RNA-binding proteins has been identified (Dreyfuss et al., 1993; Misteli and Spector, 1998). There is some evidence to suggest that important signals for export of mRNA are provided by its interacting proteins (Izaurralde et al., 1997a; Michael et al., 1995; Michael et al., 1997; Nakielny et al., 1997). However, it is unclear whether multiple proteins provide independent signals for export of mRNP, whether these signals would be additive, cooperative or redundant, and whether they occur at different stages of the export pathway (Nigg, 1997). Several laboratories have screened for mutations in yeast that result in nuclear accumulation of poly(A)-containing RNAs (outlined by Doye and Hurt [1997]). Accordingly, a variety of NPC proteins and soluble factors (often referred to as mRNA transport factors) have been identified with possible roles in nuclear export of mRNA. The majority of these yeast mutants display additional defects mostly in NPC structure and distribution but also in NLS-mediated protein import, RNA processing or nucleolar organization (summarized in Doye and Hurt, 1997). Therefore, it is very difficult to be sure whether the observed nuclear accumulation of poly(A)+ RNA is the primary defect (Ohno et al., 1998; Weis, 1998). Some NPC mutants might be expected
to induce an mRNA defect, because mRNPs are relatively large and their passage through the NPC channel may require a nucleoporin-dependent remodeling process (Mehlin et al., 1995). Mutations in three yeast proteins induce nuclear accumulation of poly(A)+ RNA in the absence of impaired nuclear protein import or gross structural NPC defects: Gle1p, an essential RNA export mediator that interacts with Rip1p (Murphy et al., 1996; Segref et al., 1997); Mex67p, a poly(A)+ RNA binding protein (Segref et al., 1997); and Dbp5p, a cytosolic RNA helicase (Snay-Hodge et al., 1998; Tseng et al., 1998). Human Gle1p (hGlep1) seems to be involved in mRNA export as well, although there is evidence to suggest that yeast and human Gle1p function differently (Watkins et al., 1998). The human homologue of Mex67p, TAP, has recently been shown to function in the nuclear export of retroviral RNAs with a constitutive transport element (CTE) (Gruter et al., 1998).

Genetic studies in yeast have implicated the *Schizosaccharomyces pombe* (*S. pombe*) Rae1p (Brown et al., 1995; Whalen et al., 1997) and its *Saccharomyces cerevisiae* (*S. cerevisiae*) homologue Gle2p (Murphy et al., 1996) in mRNA export. Gle2p is known to bind the nuclear envelope (NE) via Nup116p, which contains a short Gle2p-binding motif called GLEBS, that is conserved in the mammalian NUP98 protein (Bailer et al., 1998). However, the precise role of Gle2p and the Gle2p-Nup116p interaction in mRNA export remains a mystery. Here we report the identification and characterization of the interaction between human RAE1 and NUP98, and outline a molecular mechanism by which RAE1 and the RAE1-NUP98 interaction can function in nuclear export of mRNA.

**Materials and Methods**

*Expression constructs of NUP98 and mouse RAE1 fragments*

Human NUP98 cDNA was obtained as described (Kasper et al., 1999). A full-length mouse RAE1 cDNA was generated from two partial cDNA clones, 473342 and 465642, obtained from the I.M.A.G.E. consortium. HA1-NUP98, mouse HA1-RAE1 and mutants thereof were generated by PCR using Pfu DNA polymerase (Stratagene), and cloned in pUHD10S for expression in HTA cells (Kasper et al., 1999). All constructs were verified by DNA sequence analysis. HA1-RAE1 cDNA was cloned into vector pSP73 (Promega) for *in vitro* translation purposes. [35S]-methionine labeled HA1-RAE1 protein was produced using the Promega TNT-coupled rabbit reticulocyte lysate system as indicated by the manufacturer. To isolate pure populations of transiently transfected HTA and BHKgrβ cells HA1-NUP98(150-224) was cloned into the EcoRI-XhoI sites of vector MSCV-IRES-GFP (Persons et al., 1997).
Cell culture, transfections and electron microscopy

HtTA-1 and BHKgrβ cells (Bastos et al., 1996) were grown in DMEM containing 10% fetal bovine serum (FBS). Cells were transfected with Superfect transfection reagent (Qiagen) according to the manufacturer's instructions. Metabolic labeling of HtTA cells was as described (Kasper et al., 1999). For indirect immunofluorescence studies HtTA cells were transiently transfected in 24-well dishes, seeded on microscope slides 6-h post-transfection, and stained 16-18 h later. To study the effect of the RNA-polymerase II inhibition on the RAE1 distribution in HtTA cells, the culture medium was supplemented with 0.04 or 5.0 µg/ml actinomycin D (AMD; Boehringer Mannheim), or 50 µg/ml DRB (Fluka). To obtain pure populations of transiently transfected HtTA or BHKgrβ cells, GFP-positive cells were isolated by fluorescence-activated cell sorting (Persons et al., 1997). Electron microscopy was as previously described (van Deursen et al., 1996).

Antibody production

To generate RAE1-specific antibodies we cloned cDNA sequences encoding mouse RAE1 amino acids 188 to 347 into pQE30 (Qiagen). HIS-tagged recombinant mouse RAE1 protein was produced in E. coli DH12S cells, purified with Ni-NTA agarose beads (Qiagen) according to the manufacturer’s instructions, and injected into rabbits. NUP98- and CRM1-specific antisera were generated as described (Kasper et al., 1999). Antibodies were affinity-purified using recombinant antigen bound to ProBlott (Perkin-Elmer) as previously reported (van Deursen et al., 1996).

Immunofluorescence, coimmunoprecipitations and Western blotting

Indirect immunofluorescence, coimmunoprecipitations and Western blot analyses were carried out as previously described in detail (Kasper et al., 1999).

Generation of recombinant proteins

A cDNA fragment encoding NUP98(150-224) was cloned in pQE31 and pGEX-5X-1, respectively for expression of HIS- and GST-tagged recombinant NUP98(150-224). Recombinant protein expression in E. coli strain DH12S was induced by addition of 1 mM IPTG followed by incubation at 25°C for 4 to 5 h. Harvested bacteria were suspended in 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 2 mM PMSF, 1 mM leupeptin, 2 mM aprotinin and 1 mM pepstatin. Following 30 min of lysozyme treatment at 4°C, bacteria were lysed by sonication. HIS-NUP98(150-224) and GST-NUP98(150-224) were then purified from bacterial lysates using Ni-NTA agarose or glutathione beads (Sepharose 4B), respectively (according
to standard procedures), and used in pull-down assays. A cDNA fragment encoding RAE1(1-368) was cloned in pGEX-5X-1 to express GST-RAE1 in *E. coli*. Bacterial pellets were suspended in PBS with 2 mM PMSF, 1 mM leupeptin, and 2 mM aprotinin and 1 mM pepstatin. Lysis was performed by sonification and GST-RAE1 was purified with glutathione beads using standard procedures. Purified RAE1 was separated from the GST-affinity tag using Factor Xa protease (Pharmacia).

**Pull-down assays and chemical cross-linking**

Glutathione beads with 100 ng purified GST-NUP98(150-224) were washed 3 times with binding buffer (20 mM HEPES [pH 7.9], 100 mM KCl, 5 mM MgCl₂, 0.1% Tween-20, 20% glycerol, 0.01% bovine serum albumin, 1 mM dithiothreitol, 1 mM PMSF, 1 mM leupeptin, 2 mM aprotinin and 1 mM pepstatin), preblocked for 10 min with rabbit serum, washed with binding buffer and resuspended in 60 µl binding buffer. Then, 10 µl *in vitro*-transcribed and –translated [³⁵S]-methionine-labeled HA-RAE1 was added to the beads and the mixture was incubated at 4°C for 1 h (vortexed every 5 min). Beads were washed 6 times with binding buffer and boiled in 15 µl SDS sample buffer. Samples were analyzed by SDS-PAGE (10% polyacrylamide) followed by autoradiography. Pull-down assays with Ni-NTA agarose aliquots containing 100 ng of HIS-NUP98(150-224) were performed in the same way. For chemical cross-linking, pellets were resuspended in 10 µl PBS with 1 mM disuccinimidyl suberate (DSS; Pierce) following the last wash in our pull-down protocol. Cross-linking was at 4°C for 20 min. Finally, 10 µl SDS sample buffer was added and boiled samples were analyzed by SDS-PAGE (9% polyacrylamide) followed by autoradiography.

**Tryptic digestion and RNA degradation**

Trypsin digestion of *in vitro*-translated or recombinant RAE1 was as previously described (Audigier, 1994). The reaction was stopped with 2 µl of 100 mM benzamidine. For enzymatic RNA degradation, 10 µl *in vitro*-transcribed and –translated [³⁵S]-methionine-labeled HA1-RAE1 was incubated with either 0.1 unit *Micrococcal* nuclease (Sigma) or 10 µg RNase A. Both incubations were at 37°C for 30 min and in the presence of 1 mM PMSF, 1 mM leupeptin, 2 mM aprotinin and 1 mM pepstatin.

**Detection of poly(A)+ RNA**

HTA or BHKgrβ cells were stained for specific proteins and poly(A)+ RNA by use of the following combined immunostaining/*in situ* hybridization procedure. At 6 h post-transfection, HTA cells were seeded on microscope slides and
approximately 16 h later they were fixed in PBS/3% formaldehyde for 15 min at 4°C. After 5 washes in PBS, cells were permeabilized in PBS/0.5% Triton X-100 for 5 min at 4°C. They were then washed in PBS (5x) and incubated with either 12CA5 antibodies (9 µg/ml) alone or 12CA5 and anti-RAE1 antibodies in PBS/2% BSA/0.2% Triton X-100/200 units/ml RNasin for 30 min at RT. Cells were then washed in PBS (5x) and incubated with fluorochrome-coupled secondary antibodies for 30 min at RT: RAE1 antibodies were detected with Texas-Red conjugated goat-anti-rabbit antibodies (5 µg/ml) and 12CA5 mouse monoclonal antibodies were detected with R-phycoerythrin conjugated goat-anti-mouse antibodies (CalTag Laboratories). Cells were washed in PBS (5x) and then fixed in PBS/3% formaldehyde for 5 min at RT. After 5 rinses in PBS, the cells were equilibrated in 2X SSC for the in situ hybridization. Hybridization was performed by using an FITC-coupled oligo-(dT) 50 mer probe as detailed by Amberg et al. (Amberg et al., 1992). At the end of the procedure the cells were mounted on the slides in Vectashield (Vector Laboratories, Inc.) and analyzed by using laser scanning confocal microscopy. The above procedure is very well suited for detection of alterations in nuclear poly(A)+ RNA levels. However, due to the relatively short fixation time of 15 min, a fraction of the cytoplasmic poly(A)+ RNA pool is lost (Haruhiko Siomi, personal communication). Therefore, if we wanted to study the cytoplasmic poly(A)+ RNA levels in detail, we extended the 3% formaldehyde fixation step by 15 min and fixed at RT.

**Oocyte injections**

[^35S]-methionine-labeled protein for microinjection into *Xenopus* oocytes was synthesized in a rabbit reticulocyte lysate as indicated by the manufacturer (Promega). Templates were pT7-CBP80 (Izaurralde et al., 1995a), pSP73-HA1-RAE1 (mouse) and pT7-GST-NES, encoding a GST fusion with the HIV-1 Rev nuclear export signal (Fischer et al., 1995). *E.coli*-expressed Rna1p was coinjected at a concentration of 80 mM in the injection mixture as described (Izaurralde et al., 1997b). Microinjections, incubations, and protein extraction and analysis were performed as described (Jarmolowski et al., 1994). Nuclear export of RAE1 was quantified by measuring the nuclear and cytoplasmic fractions of RAE1 with a phosphoimager (we corrected for leakage of RAE1 into the cytoplasm by quantifying the percentage of cytoplasmic CBP80).
Results

Human RAE1 interacts with a GLEBS-like NUP98 motif

We overexpressed an HA1-tagged NUP98 cDNA (Fig. 1A) in HtTA cells and immunoprecipitated the cell lysates with 12CA5 monoclonal antibody against the tag. A protein of approximately 40 kDa specifically coprecipitated with HA1-NUP98 (Fig. 1B). We wanted to test whether this interacting protein was the human homologue of the *S. cerevisiae* Gle2p because of (1) molecular weight similarity and (2) the presence a motif within NUP98 that is similar to the Gle2p-binding sequence of Nup116p (Bailer et al., 1998). We obtained a murine cDNA clone with similarity to yeast *gle2/rae1* cDNA (designated *RAE1*) and generated polyclonal antisera in rabbits against the carboxy-terminal half of RAE1. We overexpressed HA1-NUP98 in HtTA cells, immunoprecipitated the cell lysates with 12CA5 monoclonal antibody against the HA1 tag, and performed a Western blot analysis using affinity-purified antibodies raised against mouse RAE1. As shown in Figure 1C, the RAE1 antibodies recognized the HA1-NUP98 coprecipitating protein, demonstrating that NUP98 indeed interacts with the human homologue of the yeast Gle2p. To ensure that the interaction of NUP98 with RAE1 was not an overexpression artifact, we precipitated NUP98 from non-transfected HtTA cells with affinity-purified NUP98 antibodies and determined whether RAE1 was coisolated by Western blot analysis. RAE1 indeed coprecipitated with NUP98 from HtTA cells (Fig. 1D, lanes 3 and 4). When affinity-purified RAE1 antibodies were used in the immunoprecipitation step, NUP98 was coisolated with RAE1 (Fig. 1D, lanes 1 and 2), thereby confirming that NUP98 and RAE1 are in a complex in human cells. It should be emphasized that our data do not rule out the possibility that the NUP98-RAE1 complex is part of a larger protein assembly.

To test the role of the GLEBS-like motif of NUP98 in RAE1 binding, an HA1-NUP98 mutant lacking amino acids 192 to 221 was generated (Fig. 1E). This mutant, designated as HA1-NUP98Δ(192-221), failed to coimmunoprecipitate RAE1 (Fig. 1F, lanes 2 and 5), although, like full-length NUP98, it localized at the NE (data not shown). Hence, the GLEBS-like motif of NUP98 was necessary for binding RAE1. We then asked whether this region of NUP98 was sufficient for RAE1 binding. We expressed amino acids 150 to 224 of NUP98 as an HA1-tagged fusion protein in HtTA cells and performed a co-IP Western analysis. As shown in Fig. 1G (lane 2), NUP98(150-224) indeed coimmunoprecipitated RAE1. Additional mutagenesis studies revealed that the actual NUP98 interaction motif is located within residues 181-224 (Fig. 1G, lane 4). Computer analysis (using the GCG program PEPTIDESTRUCTURE) identified a potential alpha-helical region from amino acids 187 to 212. Three helix-breaking proline mutations introduced in this region abrogated the NUP98(181-224) interaction.
Figure 1. A GLEBS-like motif within NUP98 is necessary and sufficient for interaction with human RAE1. (A) Schematic of the NUP98 structure. Vertical bars indicate FG repeats; HA1, hemagglutinin tag; NRM, nucleoporin RNA-binding motif (shaded box); gray box, Nup116p homology region. (B) [35S]-methionine-labeled proteins immunoprecipitated with monoclonal antibody 12CA5 from HtTA cells transiently expressing an HA1-tagged version of NUP98, separated by SDS-PAGE (8% polyacrylamide), and visualized by autoradiography. A molecular weight standard is indicated to the right. (C) Western blot analysis (8% polyacrylamide) of the 40 kDa protein coimmunoprecipitated with HA1-NUP98 transiently expressed in HtTA cells (lanes 2 and 4). Non-transfected cells served as a negative control (lanes 1 and 3). The blots were first incubated with 12CA5 antibody (lanes 1 and 2) and then with affinity-purified anti-m(ouse)RAE1 antibodies (lanes 3 and 4). The position of human RAE1 is indicated with an arrow. Molecular weight standards are indicated to the left. (D) Western blot analysis (8% polyacrylamide) of proteins coimmunoprecipitated from HtTA lysates with anti-RAE1 (left panel) or anti-NUP98 antibodies (right panel). The antibodies used to visualize NUP98 or RAE1 proteins are indicated above the lanes. Molecular weight standards are indicated to the left. (E) Structure of NUP98 mutants used to define
with RAE1 (Fig. 1G, lane 5), further confirming that the NUP98(181-224) segment contains the GLEBS-like motif.

The RAE1-NUP98 interaction is direct and mRNA independent

To further characterize the RAE1-NUP98 interaction we produced RAE1 and the GLEBS-like motif \textit{in vitro}, and analyzed whether they bind directly, or indirectly via an adaptor protein or a molecule of mRNA. Presumably, members of the WD-repeat superfamily all form compact globular propeller structures that are resistant to proteolysis (Garcia-Higuera et al., 1996a; Garcia-Higuera et al., 1996b). It has been shown previously that most WD-repeat proteins fold into their native globular structure when synthesized \textit{in vitro} in a rabbit reticulocyte lysate system, but not when synthesized in \textit{E. coli} (Garcia-Higuera et al., 1996a; Neer et al., 1994a). We synthesized RAE1 protein both in a rabbit reticulocyte lysate system (see Fig. 2C, lane 1) and in \textit{E. coli} (not shown) and performed pull-down assays with HIS-NUP98(150-224) or GST-NUP98(150-224) purified from \textit{E. coli} (see Fig. 2A and B). Both HIS- and GST-NUP98(150-224) bind to RAE1 (HA-tagged) generated in a rabbit reticulocyte lysate system (Fig. 2C, lanes 4 and 6), but failed to bind recombinant RAE1 synthesized in \textit{E. coli} (not shown). To test the folding of RAE1 purified from \textit{E. coli} and in a rabbit reticulocyte lysate, we analyzed their sensitivity to tryptic cleavage (Garcia-Higuera et al., 1996a; Garcia-Higuera et al., 1998; Neer et al., 1994a). We found that tryptic cleavage of \textit{in vitro}-translated $[^{35}\text{S}]$-methionine-labeled HA1-RAE1 removes about 3 kDa and leaves a large stable fragment of 42 kDa, despite the presence of many potential cleavage sites (data not shown). By contrast, RAE1 from \textit{E. coli} was extensively degraded because of cleavage at multiple tryptic sites (data not shown). This result implies that in \textit{E. coli} RAE1 cannot fold into a compact, globular structure capable of interacting with NUP98.

To investigate whether RAE1 and NUP98 establish direct contact, we used chemical cross-linkers (Garcia-Higuera et al., 1996b; Neer et al., 1994a; Yi et al., 1991). Cross-linking of residues from \textit{in vitro}-translated $[^{35}\text{S}]$-methionine-labeled HA1-RAE1 and recombinant HIS-NUP98(150-224) or GST-NUP98(150-224) with the GLEBS-like motif. The various NUP98 motifs are as indicated in Figure 1A. (F) Western blot analysis (7% polyacrylamide) of proteins precipitated with 12CA5 antibody from HITa cells transiently transfected with HA1-NUP98 or HA1-NUP98(Δ192-221). Molecular weight standards are indicated to the left. (G) Same as (F) for a set of HA1-tagged GLEBS-like motif mutants. Immunoprecipitated proteins were split in two equal portions, one half was run through a 15% polyacrylamide gel to verify proper expression of HA1-tagged mutant peptides (upper panel), the other half on an 8% polyacrylamide gel to determine coimmunoprecipitation of RAE1. Because HA1-NUP98(150-186) protein (lane 3 upper panel) was expressed at a lower level than the other HA1-tagged mutants, we also collected longer exposures of the RAE1 immunoblot (the lower panel); however, we were still unable to detect a RAE1-specific signal in lane 3. With the transfection protocol applied, levels of HA1-NUP98(150-224) were consistently higher than those of HA1-NUP98(150-224) which causes the difference in intensity of RAE1 signals in lanes 2 and 4. Molecular weight standards for each gel are indicated to the left.
Figure 2. Chemical cross-linking of \textit{in vitro}-translated HA1-RAE1 to \textit{E. coli} purified GLEBS-like motifs. (A) Purified recombinant HIS-NUP98(150-224) (approximately 11 kDa) protein separated by SDS-PAGE (15% polyacrylamide gel) and detected by CBB staining. (B) Purified recombinant GST (29 kDa) and GST-NUP98(150-224) (approximately 38 kDa) protein run on a 10% polyacrylamide gel and stained with CBB. (C) Pull-down assays performed with [\textsuperscript{35}S]-methionine-labeled HA1-RAE1 synthesized \textit{in vitro} (45 kDa), and GST- or HIS-NUP98(150-224) fusion proteins purified from \textit{E. coli}. 5% input shows 5% of the labeled HA1-RAE1 protein used in each pull-down assay. Typically, the \textit{in vitro}-translated RAE1 appears as a doublet, representing fragments with and without a HA1 tag (the RAE1 cDNA was cloned in pSP73 as a HA1 fusion gene that retained the endogenous RAE1 translation initiation codon). GST beads and Ni-NTA agarose acted as negative control for binding in GST-NUP98(150-224) and HIS-NUP98(150-224) pull-down assays, respectively. Comparable amounts of GST, GST-NUP98(150-224) and HIS-NUP98(150-224) proteins were used in each pull-down assay. The experiment shown is representative for two independent experiments. A cross-linked GST-NUP98(150-224)/RAE1 product of approximately 84 kDa and a cross-linked HIS-NUP98(150-224)/RAE1 product of approximately 56 kDa were obtained specifically in DSS-treated samples. Note that cross-linking of RAE1 to the GLEBS-like motif of NUP98 revealed that the interaction is direct and not mediated through another protein.

224) should yield specific cross-linked products of ~56 kDa and ~83 kDa, respectively. The predicted cross-linked products were indeed obtained with DSS (Fig. 2C, lanes 3 and 5), a reagent that cross-links mainly lysine residues, but not with BMH, a sulfhydryl-reactive cross-linker (data not shown). DSS-mediated coupling of RAE1 to the GLEBS-like motif of NUP98 revealed that the interaction is direct and not mediated through another protein.
It has been reported that RAE1 can be UV cross-linked to poly(A)^+ RNA (Kraemer and Blobel, 1997), and theoretically, binding between RAE1 and GLEBS-like motif may be established via mRNA. To investigate this possibility, we synthesized[^35S]-methionine-labeled HA1-RAE1 protein, removed the mRNA from the reticulocyte lysate with either Micrococcal nuclease or RNase A, and performed pull-down assays with GST-NUP98(150-224) beads. Neither the nuclease (Fig. 3A) nor the RNase A treatment (Fig. 3B) had any effects on the binding ability of RAE1 to NUP98 in vitro. Pull-down assays were also performed after addition of various amounts of poly(A)^+ mRNA isolated from HtTA cells. However, the binding efficiency of HA1-RAE1 to the GLEBS-like motif was similar irrespective of the mRNA amount present during the binding reaction (Fig. 3C). In summary, our in vitro-binding studies indicate that the interaction between RAE1 and the GLEBS-like motif of NUP98 is direct and mRNA-independent.
The COOH-terminal non-WD-repeat extension of RAE1 is essential for NUP98 binding

To start investigating how RAE1 binds to the GLEBS-like motif of NUP98 at the NPC, we generated a series of mutants with deletions in the N- or C-terminal non-WD-repeat extensions and tested them for their ability to interact with NUP98 by using a coimmunoprecipitation approach (see Fig. 4A). Mutant HA1-RAE1(33-368), which lacks the entire non-WD-repeat N-terminal extension, was able to coprecipitate NUP98, although with reduced efficiency compared to wildtype RAE1 (Fig. 4B, lanes 1 and 3; n = 3 independent experiments). Extension of this deletion into the first WD repeat [HA1-RAE1(66-368)],
abolished interaction with NUP98 (Fig. 4B, lane 6). HA1-RAE1(1-329), which lacks the C-terminal 39 amino acids, again failed to coprecipitate NUP98 (Fig. 4B, lane 5). By contrast, mutant HA1-RAE1(1-359), which lacks the C-terminal 9 residues containing a highly conserved basic motif that has been shown to be essential for Rae1p function in *S. pombe* (Whalen et al., 1997), could coprecipitate NUP98 (Fig. 4B, lane 4). Hence, the basic motif of RAE1 may attribute a critical cellular function of RAE1 other than binding to NUP98.

In non-transfected HitTA cells, RAE1 was localized prominently at the NE, but substantial amounts of RAE1 were also found in the nucleus and the cytoplasm (see Fig. 6B). A very similar distribution pattern was observed in HitTA cells that moderately overexpress HA1-RAE1 (see Fig. 4C), however, more robust overexpression resulted in a disproportionate increase of RAE1 levels in the nucleus (not shown). As expected, all deletion mutants but the ones that failed to coprecipitate NUP98, displayed overt NE localization when transiently expressed in HitTA cells (see Fig. 4D-F). The above experiments suggest that both the WD repeat propeller and the C-terminal non-WD-repeat extension of RAE1 contribute to NUP98 binding.

**Point mutation’s individual WD-repeats differentially affect NUP98 binding**

The HA1-RAE1(66-368) mutant suggested that the WD repeat propeller of RAE1 is implicated in NUP98 binding. To further define the role of the WD repeats in the RAE1-NUP98 interaction, we mutated single WD-repeats at their highly conserved aspartic acid residue positioned in the turn connecting the β strands b and c of a propeller blade (Fig. 5A and B). We targeted these particular residues because their conservation in 85% of all known WD-repeats indicates that they perform an important role within the propeller structure (Garcia-Higuera et al., 1998; Neer et al., 1994b; Neer and Smith, 1996). Moreover, it has been reported that point mutations at the conserved aspartic acid residues of the WD-repeat proteins Gβ and sec13 can cause local distortions in the structure of individual propeller blades without affecting the overall structure of the propeller (Garcia-Higuera et al., 1998). We expressed the point mutants in HitTA cells and determined their ability to interact with NUP98. We found that the point mutation in propeller blade 4 (Fig. 5C, lane 3), but not the point mutation in blade 2 or 3 (Fig. 5C, lanes 1 and 2, respectively), abolished RAE1’s ability to bind to NUP98. Accordingly, HA1-RAE1-D2 and -D3 (Fig. 5E and F, respectively) displayed a prominent NE localization when transiently expressed in HitTA cells. However, HA1-RAE1-D4 (Fig. 5G) was undetectable at the NE. Together, the above studies underscore that the WD-repeat propeller is implicated in the binding of RAE1 to NUP98. Moreover, they suggest that individual WD repeats differentially support the RAE1-NUP98 interaction.
Figure 5. A single point mutation in a highly conserved WD-repeat residue abrogates RAE1-NUP98 interaction at the NPC. (A) A scale drawing of HA1-RAE1 depicting the positions of the highly conserved D residues within the last three WD repeats that we individually mutated to A. (B) Schematic representation of a blade from a WD repeat propeller structure (Garcia-Higuera et al., 1998). The four β strands within the blade are indicated with a to d. Note that the position of the conserved D residue in the hairpin turn between strand b and c is highlighted (star). (C) Western blot analysis of proteins precipitated with 12CA5 antibody from lysates of HtTA cells transiently transfected with HA1-RAE1 or the point mutants. Precipitated proteins were visualized with 12CA5 (upper panel) and anti-NUP98 antibodies (lower panel). The HA1-RAE1 mutants used are indicated above the lanes. Molecular weight standards are indicated to the left. Results shown are representative for three independent experiments. (D-G) Representative confocal images detailing the subcellular distribution of HA1-RAE1 mutants in HtTA cells (12CA5 antibody staining).

RAE1 shuttles between the nucleus and the cytoplasm

RAE1 could be permanently or transiently bound to NUP98 at the NPC, or both. As a first step to investigate whether RAE1 has dynamic properties, we
Figure 6. RAE1 shuttles between the nucleus and the cytoplasm and its association with the NE requires RNA polymerase II activity. (A) A mixture of in vitro-translated [35S]-methionine labeled CBP80, HA1-RAE1 (seen as a doublet that represents HA1-RAE1 and RAE1), and GTS-NES was injected into Xenopus laevis oocytes nuclei, either in the absence (lanes 1-6) or presence (lanes 7-12) of 80 mM Rna1p. Following injection, oocytes were incubated at 20°C and protein samples from nuclear (N) and cytoplasmic fractions (C) were collected at 10, 30 or 90 minutes. Proteins were separated by SDS-PAGE and detected by fluorography. (B) Kinetics of RAE1 distribution after nuclear injection. Values were obtained from 3 experiments; error bars represent standard deviations. (C) Comparison of nuclear export of RAE1 at 20°C (lanes 1-2) and 0°C (lanes 3-4). (D) Nuclear uptake of RAE1 after cytoplasmic injection after 0.2 (lanes 1-2), 6 (lanes 3-4) or 9 hours (lanes 5-6).

microinjected in vitro-translated [35S]-methionine-labeled mouse RAE1 into Xenopus oocytes and analyzed its ability to shuttle between the nucleus and the cytoplasm. The NLS-containing protein CBP80 was coinjected with RAE1 to serve as a control for nuclear protein import, and proper injection and dissection of the oocytes (Izaurralde et al., 1995b). We injected RAE1 into the oocyte nucleus and quantified the fraction of RAE1 appearing in the cytoplasm at various time points post-injection by phosphoimager analysis. Figure 6A (lanes 1-6) and B demonstrate that approximately 24% (SD ± 4%, n = 3 independent experiments) of the RAE1 molecules injected into the nucleus is present in the cytoplasm within 30 min post-injection and maximal cytoplasmic levels of 31% (SD ± 4%, n = 3) are achieved within 90 min post-injection. These data suggest
(a) that an equilibrium between export and import has been established around 90 min post-injection and (b) that the majority of the microinjected RAE1 molecules seems to be export incompetent. In addition, Export of microinjected RAE1 was completely inhibited at 0°C (Fig. 6C, lanes 3 and 4), indicating that RAE1 export is temperature dependent and not driven by “simple” diffusion. We also investigated whether RanGTP mediates nuclear export of RAE1 by reducing the level of RanGTP via nuclear injection of Rna1p, a normally cytoplasmic GTPase-activating protein for Ran (Izaurralde et al., 1997b). As shown in figure 6A (compare lanes 1-6 with 7-12), coinjection of 80 mM Rna1p (1-10 mM usually induces a significant inhibition in RanGTP-dependent export) did not significantly inhibit nuclear export of RAE1, while, in accordance with previous data (Izaurralde et al., 1997b; Richards et al., 1997) nuclear export of NES-tagged GST substrates was dramatically reduced. As expected from its nuclear localization, RAE1 protein injected into the oocyte cytoplasm was able to migrate rapidly into the nucleus (Fig. 6D, lanes 1-6).

**Association of RAE1 with the NE requires RNA polymerase II activity**

The combination of shuttling and poly(A)^+ RNA-binding properties (Kraemer and Blobel, 1997) led us to hypothesize that RAE1 travels between cellular compartments as an mRNA export factor. To investigate this possibility we stopped mRNA synthesis in HtTA cells by adding RNA polymerase II inhibitors and asked whether the subcellular distribution of RAE1 changed. After 1 h of treatment with 5 µg/ml AMD, a dose which inhibits both RNA polymerase I and II activity, the prominent RAE1 staining at the NE normally seen in HtTA cells was no longer detectable (compare Fig. 7A and C). In contrast, normal levels of RAE1 at the NE were observed in cells exposed to a dose of AMD that only inhibited RNA polymerase I activity (0.04 µg/ml AMD; see Fig. 7B). When we shortened the treatment with 5 µg/ml AMD from 1 h to 15 min, the drop in RAE1 levels at the NE was still detectable, suggesting that the observed effect can be an immediate early response to RNA polymerase II inhibition. HtTA cells exposed to 50 µg/ml of the RNA polymerase II inhibitor 5,6-dichloro-b-D-ribofuranosylbenzimidazole (DRB) showed a RAE1-staining pattern very similar to that observed in cells exposed to a high dose of AMD (Fig. 7D). The inhibitory effect of DRB is reversible and we analyzed whether normal RAE1 levels at the NE would be restored upon reactivation of RNA polymerase II-mediated transcription. As shown in Figure 7E, a prominent NE staining was observed in cells cultured for 6 h in the absence of DRB following a 1h exposure to this component. As expected, the RAE1 levels at the NE were substantially reduced following a second DRB treatment (Fig. 7F). In both AMD- and DRB-treated
Figure 7. RAE1’s association with the NE requires RNA polymerase II activity. (A-H) Images of HtTA cells stained for RAE1 or NUP98 after treatment with RNA polymerase inhibitors. RAE1-stained cells treated with: (A) 0 µg/ml AMD for 1 h, (B) 0.04 µg/ml AMD for 1 h, (C) 5.0 µg/ml AMD for 1 h, (D) 50 µg/ml DRB for 1 h, (E) 50 µg/ml DRB for 1 h and then cultured 6 h in the absence of DRB, (F) 50 µg/ml DRB for 1 h and then cultured 6 h without DRB and finally treated again with 50 µg/ml DRB for 1 h Images shown are representative for results obtained from three independent experiments. (G and H) Respectively non-treated and 5.0 µg/ml AMD treated HtTA cells stained with NUP98-specific antibodies. Note that NUP98 levels at the NE are not dependent on RNA polymerase II activity. (I, J) Effect of AMD treatment on HtTA cells that moderately express HA1-RAE1.

cells, the decrease for NE-associated RAE1 did not coincide with significant alterations in the nuclear and cytoplasmic RAE1 levels. Inhibition of RNA polymerase II activity did not affect NUP98 association with the NE (compare Fig. 7G and H), demonstrating that the absence of substantial amounts of RAE1 at the NE was not the result of NUP98 relocation. As expected, the nuclear export receptor hCRM1, which mediates export of certain viral RNAs and U snRNP, but not cellular mRNA, retained its NE localization in the presence of AMD (5 µg/ml) or DRB (data not shown). Together, the above experiments confirm that the NE association of RAE1 is transient rather than stable. Furthermore, they support the hypothesis that RAE1 associates with the NE as part of a mRNP complex.
It has been demonstrated that an AU1-tagged version of human RAE1 localizes at high levels to the nucleus and at considerably lower levels to the cytoplasm of HeLa cells (Bharathi et al., 1997). When AU1-RAE1 expressing cells were treated with RNA polymerase II inhibitors, no major changes in the distribution of AU1-RAE1 were detected. This result may seem to contradict the data presented in this report; however, it should be stressed that the effects of RNA polymerase II activity on AU1-RAE1 levels at the NE could not be evaluated because the robust nuclear staining masked the NE staining (Bharathi et al., 1997). To re-evaluate the effect of RNA polymerase II inhibitors on the distribution of overexpressed RAE1, we transiently expressed HA1-RAE1 in HtTA cells. Typically, when overexpressed at low to moderate levels, HA1-RAE1 prominently localized to the NE, but significant amounts of RAE1 were also found in the nucleus and the cytoplasm (see Fig. 7I). More robust overexpression of HA1-RAE1 resulted mostly in a disproportionate increase of RAE1 levels in the nucleus, which concealed the NE staining (data not shown). We determined the effect of RNA polymerase II inhibition on the distribution of HA1-RAE1 by focussing on cells with a low to moderate level of expression. As shown in Figure 7J, HA-RAE1 levels at the NE decreased significantly when cells were treated with 5 µg/ml AMD for 1 h, which is consistent with the results that we obtained by using non-transfected HtTA cells.

**Overexpression of the GLEBS-like motif inhibits nuclear export of poly(A)^+**

When HtTA cells transiently expressing HA1-NUP98(150-224) or HA1-NUP98(181-224) were immunostained with 12CA5 and RAE1 antibodies 24-h post transfection, we noticed a considerable reduction of RAE1 levels at the NE. As shown in Figure 8A/A’ and B/B’, NE staining of RAE1 is easily detectable in non-transfected HtTA cells (marked with “nt”) but not in HA1-NUP98(150-224) or HA1-NUP98(181-224) expressing cells (marked with “t”). By contrast, NE staining of RAE1 remained intact when HA1-NUP98(181-224)M (Fig. 8C/C’), a mutated GLEBS-like motif which does not interact with RAE1 (see also Fig. 1G, lane 5), or full-length HA1-NUP98 were overexpressed (Fig. 8D/D’). In all cases NUP98 localization at the pores appeared unchanged (data not shown). The above results suggest that overexpressed GLEBS-like motif of NUP98 acts as a dominant negative inhibitor of RAE1-NPC association by titrating RAE1 from the NPC and/or interfering with RAE1 docking to NUP98 at the NPC.

To assess whether this effect is associated with changes in mRNA export, we examined the poly(A)^+ RNA distribution in HtTA cells expressing the NUP98 GLEBS-like motif by *in situ* hybridization with an FITC-labeled oligo-(dT) 50 mer probe (Amberg et al., 1992; van Deursen et al., 1996). With this *in situ* hybridization protocol, alterations in nuclear poly(A)^+ levels can easily be
Figure 8. Overexpression of the GLEBS-like motif of NUP98 results in decreased levels of RAE1 at the NE. (A/A'-D/D'). Paired confocal images from HtTA cells that transiently express various forms of the GLEBS-like motif of NUP98. Cells were double-stained for HA1-tagged protein using the 12CA5 monoclonal antibody (left image) and for RAE1 using affinity-purified RAE1 polyclonal rabbit antibodies. Cells shown in the confocal images are representative for results obtained in 3 to 4 independent experiments. High magnification images are given to illustrate detail. (A/A' and B/B') Overexpression of the GLEBS-like motif leads to a subtle but significant decrease in NE localization of RAE1. Compare NE staining of cells that do not overexpress the RAE1-binding (which are marked with "nt") and cells that do (marked by "t"). (C/C' and D/D') Cells expressing a GLEBS-like motif mutant that is unable to interact with RAE1 or full-length NUP98 (D/D'), display an RAE1-distribution pattern comparable to that of wildtype HtTA cells.

detected. Hybridized cells were examined by confocal microscopy. As shown in detail in Figure 9F', poly(A)^+ RNA was detected in both the nucleus and the cytoplasm of non-transfected HtTA cells. In situ hybridization of transiently transfected HtTA cells expressing HA1-NUP98(150-224) (Fig. 9B/B') or NUP98(181-224) (Fig. 9C/C') revealed a dramatic increase in nuclear labeling (for detailed images see Fig. 9G/G' and H/H'). To confirm that the strong nuclear
labeling was indeed due to RNA accumulation, HtTA cells expressing HA1-NUP98(150-224) or HA1-NUP98(181-224) were incubated with RNase for 30 min prior to \textit{in situ} hybridization. As expected, no labeling was detectable after such treatment (Fig. 9J/J'). As an additional control that the signal detected in the nucleus is indeed mRNA, we incubated HA1-NUP98(150-224) expressing cells for 1 h with DRB prior to \textit{in situ} hybridization with the oligo-(dT) 50-mer probe. We quantified the nuclear signal of 20-25 cells by using confocal microscopy and the software program QUANTIFY. We compared the levels
Figure 9. Overexpression of the GLEBS-like motif of NUP98 results in accumulation of poly(A)^+ RNA in the nucleus. (A-J) Paired confocal images from HTA cells that are transiently expressing HA1-NUP98 mutants. These cells were double-stained for HA1-tagged protein by immunohistochemistry using the 12CA5 monoclonal antibody (left image) and for poly(A)^+ RNA by in situ hybridization using a FITC-labeled oligo-(dT) 50 mer (right image). The identity of the HA1-tagged NUP98 mutants is indicated in the left image of each pair. The poly(A)^+ RNA accumulation in B' appears more robust than that in C', which represents a photographic rather than a real difference. The arrow in F' points to one of the sites of preferred poly(A)^+ RNA localization (also reported by Huang and co-workers 1994). (K and L) Shows poly(A)^+ distribution in HTA cells overexpressing mouse RAE1 protein and, at the same time, HA1-NUP98(150-224) or HA1-NUP98(181-224). Each row of three images shows the same field of cells stained for either HA1-tagged protein (left), ectopically expressed mouse RAE1 and native human RAE1 (middle), and poly(A)^+ RNA (right). Note that mouse RAE1 overexpression restores proper mRNA export in cells expressing various forms of the GLEBS-like motif. The combined anti-HA1/poly(A)^+ staining procedure does not allow extensive blocking of non-specific 12CA5 antibody binding. Therefore, non-transfected cells display higher levels of background staining than those shown in Figure 7.

obtained with those measured in the same amount of non-treated HA1-NUP98(150-224) positive cells. In three independent experiments, the nuclear poly(A)^+ signal detected in DRB-treated cells was 29, 30, and 36% lower than in non-treated cells. Thus, a proportion of the nuclear poly(A)^+ RNA is either exported to the cytoplasm or rapidly degraded (or both), suggesting that at least a proportion of the signal obtained with the oligo-(dT) probe represents nuclear mRNA and not just stable nuclear poly(A)^+ RNA (Huang et al., 1994).

Nuclear accumulation of poly(A)^+ RNA induced by HA1-NUP98(150-224) or NUP98(181-224) expression typically coincided with a decrease in cytoplasmic
poly(A)^+ RNA levels; however, considerable amounts of polyadenylated RNA were still found in the cytoplasm (see Fig. 9B/B', C/C', G/G' and H/H'). This was further corroborated by using an in situ hybridization procedure optimized for detection of cytoplasmic polyadenylated RNA (for details see materials and methods), as is illustrated in Figure 9I/I'. No nuclear build-up of poly(A)^+ RNA was found in cells expressing the GLEBS-like motif mutant HA1-NUP98(181-224)M (Fig. 9D/D'), which confirms that binding of endogenous RAE1 to overexpressed GLEBS-like motif is essential poly(A)^+ RNA accumulation. In HtTA cells overexpressing mouse RAE1 in addition to HA1-NUP98(150-224), or HA1-NUP98(181-224) nuclear accumulation of poly(A)^+ RNA was either not seen or hardly detectable (see representative images in Fig. 9K and L). Thus, proper poly(A)^+ RNA export can take place in the presence of transiently expressed GLEBS-like motif if RAE1 levels are increased above normal. RAE1 overexpression probably restores the cellular pool of "free" RAE1 to a level required for proper nuclear mRNA export. We verified that overexpression of HA1-tagged mouse RAE1 (Fig. 9E/E') or non-tagged RAE1 (not shown) alone did not induce any measurable alterations in poly(A)^+ RNA distribution compared to non-transfected HtTA cells.

To verify that the poly(A)^+ defect was not the result of a nuclear import defect we used BHKgrß cells. These cells express a glucocorticoid receptor-β-
galactosidase fusion protein that is strictly localized to the cytoplasm. When exposed to dexamethasone, the fusion protein translocates within 30 min to the nucleus in a quantitative fashion (Bastos et al., 1996). When we transfected BHKgrβ cells with an HA1-NUP98(150-224) construct, they accumulated poly(A)+ RNA in the nucleus (compare Fig. 10A/A’ and B/B’) with no detectable effect on the import of the glucocorticoid receptor-b-galactosidase fusion protein (Fig. 10C/C’ and D/D’). Although only one protein was tested, these results show that the NLS-mediated nuclear import pathway remained intact, and that the mRNA export phenotype is not likely to be the result of a general trafficking defect.

Finally, we wanted to exclude that overexpression of the GLEBS-like motif induced NPC herniations similar to those seen in yeast nup116- and gle2- knockouts (Murphy et al., 1996; Wente and Blobel, 1993) and nup116pD(GLEBS) mutant cells (Bailer et al., 1998). To this end, we purified transiently transfected HtTA cells expressing both HA1-NUP98(150-224) and green fluorescent protein (GFP) by FACS sorting and studied their NPC integrity using an electron microscope. None of the HA1-NUP98(150-224) expressing cells examined displayed any herniated or clustered NPCs (data not shown). Thus, the mRNA-export defect is not likely to be secondary to abnormalities in NPC structure and distribution. Taken together, our results suggest a model in which a dominant negative GLEBS-like motif directly interferes with export of poly(A)+ RNA from the nucleus by targeting RAE1 the NUP98-RAE1 interaction, or both.

Discussion

Details about the mechanism by which mRNA is exported from the nucleus remain a mystery. Here we identified and characterized an interaction between human RAE1 and NUP98, and studied its significance in mRNA export. Our studies support a model in which RAE1 is a shuttling transport factor that permits efficient export of mRNA through its ability to anchor to a GLEBS-like NUP98 motif at the NPC. Specifically, we show that: (1) RAE1 binds to the GLEBS-like motif of NUP98 through multiple domains including the WD propeller and part of the carboxy-terminal non-WD-repeat extension; (2) the RAE1-NUP98 interaction is direct and not via another protein or RNA; (3) RAE1 has the ability to shuttle from the nucleus to the cytoplasm and that its interaction with the NE seems to be transient rather than stable; (4) the GLEBS-like motif, when overexpressed, binds to RAE1 and inhibits poly(A)+ RNA export from the nucleus, but not NLS-mediated import and NPC structure or distribution.
Shuttling and dynamic properties of RAE1

Analysis of the dynamic properties of RAE1 by microinjection of *in vitro*-translated protein into *Xenopus laevis* oocytes revealed that RAE1 has the ability to shuttle between the nucleus and the cytoplasm in a rapid manner. Furthermore, nuclear export of RAE1 appears to be established by a temperature-sensitive, RanGTP-independent, mechanism. Several studies have demonstrated that GTP-bound Ran has an essential role in nuclear RNA export. However, different RNA classes seem to depend differently on RanGTP for their export from the nucleus. Both U snRNA and tRNA export are highly sensitive towards RanGTP depletion. On the other hand, some mRNAs, such as H4 and DHFR mRNA (Izaurralde et al., 1997b), apparently use RanGTP-dependent as well as RanGTP-independent mechanisms for their nuclear export, whereas export of adenovirus major late transcripts (Izaurralde et al., 1997b) and heat shock mRNAs (Saavedra et al., 1997; Stutz et al., 1997) is unaffected by the absence of nuclear RanGTP. Therefore, the RanGTP-insensitivity of RAE1 export does not argue against the idea that RAE1 may be a shuttling nuclear export factor for mRNAs. Indeed, no RanGTP-binding exportin has yet been identified that is directly involved in mRNA export (see also Stutz and Rosbash, 1998), and it remains possible that the effects of RanGTP depletion on mRNA export are indirect.

Two additional findings reported here support RAE1's dynamic properties. First, overexpression of the GLEBS-like motif of NUP98 causes a reduction in the level of RAE1 associated with the NE. If RAE1 is permanently associated with NUP98 at the NE, overexpression of the GLEBS-like motif is expected to have no major effects on RAE1 levels at the NE. On the other hand, if RAE1's association with the NE would be transient rather than permanent, RAE1 molecules released from the NE may form a complex with the overexpressed GLEBS-like motif of NUP98. Once established, such complexes may be defective in docking to NUP98 at the NE and induce a general decline in RAE1 levels at the NE. The second finding that emphasizes the dynamic properties of RAE1 is that the level of RAE1 at the NE appears to be dependent on RNA polymerase II activity. Specifically, we observed that the amount of RAE1 at the NE dropped considerably if cells were exposed to RNA polymerase II inhibitors. When RNA polymerase II activity was restored by removal of the inhibitor, RAE1 levels at the NE returned to normal. Thus, RAE1's translocation from the nucleus to the NE may be dependent on the availability of gene transcripts generated by RNA polymerase II. It can be argued that the effect of RNA polymerase II inhibitors on the NE association of RAE1 is secondary to the depletion or relocation of one or more other cellular factors of unknown identity. Although we cannot exclude this possibility, three of our findings argue against an indirect effect: (1) the RAE1 levels at the NE drop shortly after initiation of AMD treatment (≤ 15 min); (2) the reversibility of the DRB-induced effect on RAE1; and (3) the observation that the inhibitors do not alter the distribution of
the transport factors NUP98 and hCRM1 (Fornerod et al., 1997b). While more detailed analysis need to be done, the RNA polymerase II inhibition experiments are consistent with the notion that RAE1 is a dynamic mRNA export mediator.

On the surface, the observation that the association of RAE1 with the NE depends on RNA polymerase II activity seems to contradict the data from our in vitro binding studies, which indicated that mRNA is not a cofactor in the interaction between RAE1 and the GLEBS-like motif of NUP98. This apparent paradox can be explained if one assumes that docking of a transport substrate from the nucleus to the NPC follows a two-step process. In the first step, the substrate translocates from its site of assembly in the nucleus to the nuclear periphery. In the second step, the substrate anchors to the NPC. In this light, the negative effect of RNA polymerase II inhibitors on the association of RAE1 with the NE may reflect a defect not in the ability to anchor to NUP98 at the nuclear face of the NPC but rather in RAE1’s translocation of from the nucleus to the NPC. Guidance of RAE1 to the NPC may be a signal-mediated process relying on external export signals perhaps provided by proteins within the mRNP transport substrate. Because so many proteins associated with the mRNA export substrate, multiple associated proteins may provide independent signals for export of mRNP.

RAE1 and mRNA export

Our most powerful evidence for a direct role of RAE1 and the RAE1-NUP98 interaction in the mRNA export pathway comes from GLEBS-like motif overexpression studies. Typically, overexpression of the GLEBS-like motif of NUP98 resulted in reduced levels of RAE1 at the NE and nuclear accumulation of poly(A)+ RNA. Presuming that RAE1 is a component of mRNP (Kraemer and Blobel, 1997) two possibilities could explain these data. It seems fair to argue that RAE1 associates with mRNP since RAE1 and poly(A)+ RNA can be cross-linked by UV irradiation in vivo (Kraemer and Blobel, 1997). Thus, at least part of the cellular RAE1 pool is in close proximity to mRNA, although it remains to be determined whether RAE1 and mRNA interact directly (RAE1 sequences lack similarity to any known mRNA-binding motifs), or indirectly via one of the mRNA-associated proteins. In the first possibility, one might propose that the NUP98 GLEBS-like motif binds to a RAE1 molecule that is in a complex with mRNP. The presence of the GLEBS-like motif in the resulting complex would then prevent its anchoring to full-length NUP98 at the NPC. In the second possibility, one might argue that the GLEBS-like motif associates with “free” RAE1 protein to affect its binding to mRNP. Lack of RAE1 within the mRNP particle would then affect its proper anchoring to the NPC.

We found that cells which co-overexpress the GLEBS-like motif together with full-length RAE1 displayed neither reduced levels of RAE1 at the NE nor accumulation of poly(A)+ RNA in the nucleus, indicating that the mRNP export
defect induced by the GLEBS-like motif is not an overexpression artifact but rather a result of impaired RAE1 function. How could RAE1 overexpression neutralize the mRNA export defect induced by the GLEBS-like motif? If the GLEBS-like motif indeed interacts only with RAE1 that is bound to mRNP, as proposed above in possibility one, then RAE1 overexpression is expected to have little or no effect on the formation of anchoring-defective complexes. However, if the GLEBS-like motif only associates with a “free” RAE1 molecule, as outlined in possibility two, then RAE1 overexpression could simply titrate GLEBS-like motifs and restore the pool of “free” RAE1 to a level sufficient for proper mRNP export. Thus, the data from our co-overexpression studies are apparently more consistent with possibility two than with possibility one.

Additional data presented in this report are consistent with the idea that RAE1 and the RAE1-NUP98 interaction directly serve in the nuclear export pathway for mRNA. First, NUP98 fragments with point mutations in the GLEBS-like motif that abolish its association with RAE1 failed to inhibit poly(A)* RNA export from the nucleus. Thus, there is a correlation between nuclear accumulation of poly(A)* RNA and binding of the GLEBS-like motif to RAE1. Second, there is no evidence to suggest that the observed poly(A)* RNA accumulation phenotype results from impaired NLS-mediated protein import, loss, clustering or herniation of nuclear pores, or release of NUP98 from the NE. In particular the absence of any gross defects in NPC structure and distribution is of importance with respect to earlier work in the yeast system. In gle2 mutant yeast, as well as those with gle2 or nup116 null alleles (Murphy et al., 1996; Wente and Blobel, 1993), a block in poly(A)* RNA export is always accompanied by severe clustering and herniation of the nuclear pores. Because of these NPCs perturbations, it has been difficult to ascertain whether Gle2p and Nup116p are mediators of RNA transport. Nup116▵GLEBS mutants (Bailer et al., 1998), which are defective in the docking of Gle2p to Nup116p at the NPC, display very similar spatio-structural NPC defects as gle2 or nup116p mutants. Because of these defects, it is difficult to study the export function of the Gle2p-Nup116p interaction in the nup116▵GLEBS) mutants. An interesting difference between S. cerevisiae Gle2p and S. pombe Rae1p is that a lack of Rae1p function results in poly(A)* RNA accumulation in the absence of any detectable NPC defects (Brown et al., 1995; Whalen et al., 1997). It is conceivable that Gle2p and Rae1p functions have diverged, and that Gle2p may perform separate functions in both mRNA transport and NPC structure. Insight in how Gle2p and Rae1p function in the pathway for nuclear export of mRNA is required in order to address such issues. In S. cerevisiae, it will be important to establish whether the Nup116p-Gle2p interaction at the NPC serves directly in the RNA export pathway. Perhaps GLEBS-overexpression studies similar to those presented in this paper can yield such information. To gain insight in how Rae1p functions in fission yeast, a crucial step will be to identify whether S. pombe contains an S. cerevisiae Nup116p homologue that has a GLEBS-like binding site, and if so, whether this site directly functions in mRNA export.
Could RAE1 serve as the exportin for mRNA or as a factor that provides the NES signal to mRNA (Ohno et al., 1998; Ullman et al., 1997)? Unlike Rae1p in *S. pombe* (Brown et al., 1995), Gle2p in *S. cerevisae* (Murphy et al., 1996) is not a strictly essential protein indicating that mRNA export is impaired rather than completely blocked in the absence of functional Gle2p. Thus, Gle2p does not seem to operate as the sole exportin or NES-containing factor for mRNA export. Instead, Gle2p and its homologues in fission yeast (Rae1p) and mammals (RAE1) more likely participate in mRNA export as mRNP-interacting proteins necessary for efficient anchoring of the transport complex to the NPC.

**Evidence for specialization of WD-repeats in RAE1**

RAE1 is a member of superfamily of WD-repeat proteins. Given the high conservation the WD repeats, it is likely that they all fold into a propeller structure (Garcia-Higuera et al., 1996a). Members of this family do not have an immediately obvious common function. Rather, the common thread between WD repeat proteins seems to be that they make up part of large macromolecule assemblies. The capacity to assemble multiple proteins may be an essential part of their function, including that of RAE1 (Neer et al., 1994a). The signal-transducing WD-repeat protein Gβ binds to Gγ to form a heterodimer that in turn interacts with Gα or a variety of different effector proteins (Clapham and Neer, 1997). The various interactions with Gβ are established through unique as well as overlapping contact sites involving specific blades of the Gβ propeller (Li et al., 1998). Similarly, the blades of the RAE1 propeller might serve as contact sites for multiple distinct RAE1 partners. This idea has some support from the observation that the interaction of RAE1 and the GLEBS-like motif of NUP98 is highly sensitive to a point mutation in blade 4, but not those in blades 2 and 3. Additionally, studies in *S. pombe* have demonstrated that a conserved 10-residue basic motif at the carboxy-terminus of Rae1p is necessary for Rae1p function. Here we found that this motif is not essential for the RAE1-NUP98 interaction, which is in keeping with the idea that RAE1 may be involved in an additional protein-protein interaction. A future goal to provide further insight into the complex mechanism of mRNA export from the nucleus now is to determine whether RAE1 preferentially interacts with specific kinds of mRNA molecules and to define how RAE1 interacts with mRNP particles. Preliminary *in vivo* cross-linking studies suggest that RAE1 indeed interacts with at least one other protein besides NUP98.

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References


Chapter 6

A Transcription-Factor-Binding Surface of Coactivator p300 is Required for Haematopoiesis

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A transcription-factor-binding surface of coactivator p300 is required for haematopoiesis

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The coactivators CBP (Cre-element binding protein (CREB)-binding protein) and its paralogue p300 are thought to supply adaptor molecule and protein acetyltransferase functions to many transcription factors that regulate gene expression. Normal development requires CBP and p300, and mutations in these genes are found in haematopoietic and epithelial tumours. It is unclear, however, which functions of CBP and p300 are essential for haematopoiesis. Here we show that the protein-binding KIX domains of CBP and p300 have nonredundant functions in mice. In mice homozygous for point mutations in the KIX domain of p300 designed to disrupt the binding surface for the transcription factors c-Myb and CREB, multilineage defects occur in haematopoiesis, including anaemia, B-cell deficiency, thymic hypoplasia, megakaryocytosis and thrombocytosis. By contrast, age-matched mice homozygous for identical mutations in the KIX domain of CBP are essentially normal. There is a synergistic genetic interaction between mutations in c-Myb and mutations in the KIX domain of p300, which suggests that the binding of c-Myb to this domain of p300 is crucial for the development and function of megakaryocytes. Thus, conserved domains in two highly related coactivators have contrasting roles in haematopoiesis.

The KIX domain is one of several domains in CBP (also known as Crebbp) and p300 that bind transcriptional regulators (Fig. 1a); it is highly preserved in evolution, with 90% identity in human CBP and p300 (Fig. 1b). The three-dimensional structure of the CBP KIX domain in a complex containing a portion of the CREB activation domain has been determined (ref. 9 and Fig. 1c). Specific hydrophobic residues on the surface of the KIX domain are important for binding the α-helical activation domains of CREB and c-Myb.

We tested the role of the KIX domain transcription factor-binding surface in vivo by mutating three highly conserved residues that lie on one face of the α3 helix in the KIX domain of CBP (Fig. 1b, c); Tyr 650 forms part of the hydrophobic surface that makes crucial contacts with CREB; the small side chain of Ala 654 allows the close packing of CREB; and Tyr 658 forms hydrophobic interactions with CREB and forms hydrogen bonds with phosphorylated Ser 133 of CREB. Thus, we thought that replacing Tyr 650 and Tyr 658 with alanines should disrupt binding with CREB, and replacing the Ala 654 methyl group with a bulkier glutamine side chain should sterically hinder CREB binding. Accordingly, the individual mutations of Tyr650Ala, Ala654Gln, and Tyr658Ala each diminish CREB and c-Myb binding to KIX (refs 7, 8, and data not shown). Structure prediction analyses using AGADIR and PSIPRED predicted that KIX secondary structure would not be affected by α-talin mutations of Tyr650Ala, Ala654Gln, and Tyr658Ala each dimensional mutations of Tyr650Ala, Ala654Gln, and Tyr658Ala each diminished CREB and c-Myb binding to KIX (refs 7, 8, and data not shown).

We introduced this triple mutation into the CBP and p300 loci of embryonic stem (ES) cells by homologous recombination (Fig. 1d, e). For p300, the corresponding residues, Tyr 630, Ala 634 and Tyr 638, were mutated. Correctly targeted ES cells were used to produce mice carrying mutant alleles of the KIX domain of CBP (designated CBPKIX/KIX) or p300 (designated p300KIX/KIX; Fig. 1d, e). CBPKIX/KIX and p300KIX/KIX homozygous mice were viable, although some died of unknown causes before they were 3 weeks old (mostly p300KIX/KIX mice; see Supplementary Information). Surviving 4-week-old p300KIX/KIX mice averaged about 50–70% of the size of wild-type or heterozygous littermates, and CBPKIX/KIX mice also tended to be smaller than their littermate controls. Analysis of the surviving CBPKIX/KIX mice showed increased variation in thymocyte numbers (Supplementary Information). By contrast, p300KIX/KIX mice had a marked reduction in thymocyte numbers (~5% of wild-type) and severe anaemia (haematocrit 20.2 ± 5.3 versus 42.3 ± 1.4% for wild type) and thrombocytosis (15.0 ± 1.1 × 10^11 versus 1.2 ± 0.2 × 10^11 platelets per ml for wild type). Numbers of neutrophils were generally in the normal range (data not shown). The blood from p300KIX/KIX mice showed increased variation in erythrocyte size (compare Fig. 2b with Fig. 2a, c), and the presence of megathrombocytes (Fig. 2b, broken arrow). CBPKIX/KIX and p300KIX/KIX mice were essentially normal (except for a small increase in platelets in p300KIX/KIX mice), which showed that the mutations were not overly dominant (Supplementary Information).

The bone marrow of p300KIX/KIX mice showed megakaryocytic hyperplasia with a corresponding decrease in other bone marrow elements (compare Fig. 2d with Fig. 2e). Megakaryocytosis was evident in the spleens of these mice (compare Fig. 2g with Fig. 2f, h) and was indicated further by widespread staining for acetylcholinesterase enzyme (compare Fig. 2j with Fig. 2i) and factor VIII (data not shown). Megakaryocytosis in p300KIX/KIX mice was also indicated by flow cytometric analysis of bone marrow and spleen cells, which contained an overabundance of CD41-positive cells as compared with CBPKIX/KIX and wild-type mice (Fig. 2k).
concentrations of thrombopoietin (Tpo), which stimulates megakaryopoiesis, were reduced about fivefold in p300/KIX mice (probably owing to Tpo internalization and degradation by excess platelets), which indicated that overproduction of Tpo was not the cause of the megakaryocytosis (Supplementary Information).

We assessed the development of p300/KIX megakaryocytes by quantifying the DNA content in CD42d-positive bone marrow cells (Fig. 2). p300/KIX mice had more immature megakaryocytes with a modal ploidy of 8n, as compared with 16n in control mice.

Thymuses from p300/KIX mice had an apparently normal distribution of single- and double-positive CD4 and CD8 thymocytes (Fig. 2n). Despite the decreased absolute number of p300/KIX thymocytes, peripheral single-positive CD4 and CD8 T-cell populations seemed to be in the normal range (data not shown). By contrast, there was a deficit of B220-positive, immunoglobulin-μ (IgM)-positive, immunoglobulin-μ (IgD)-positive, immature B cells in p300/KIX mice (Fig. 2m).

To determine whether the p300/KIX phenotype was intrinsic to the bone marrow, we transplanted p300/KIX bone marrow cells into wild-type mice that had been lethally irradiated. Mice that received p300/KIX bone marrow cells developed a phenotype comparable to that of p300/KIX mice, with severe anaemia, thrombocytosis and B-cell deficiency, indicating that p300/KIX bone marrow cells were sufficient to confer the disease (Supplementary Information, and data not shown). A mix of p300/KIX bone marrow cells with equal numbers of wild-type cells conferred a milder anaemia with thrombocytosis (Supplementary Information, and data not shown). A mix of wild-type CBP or p300, but not mutant CBP, could restore Gal4–CRE activity in CBP/KIX/KIX MEFs (Fig. 3i, j). Cyclic-AMP-dependent induction of the endogenous CREB target genes encoding c-Fos, junh and the ICER form of CREM were not measurably different in mutant and wild-type MEFs, which suggests that the KIX domain is not limiting for these genes in MEFs (data not shown). Together, these data indicate that a 50% reduction in the expression of wild-type KIX domains results in the specific attenuation of CREB and c-Myb activities in certain promoter contexts.

We compared CBP and p300 proteins in MEFs, megakaryocytes, thymus and relatively unaffected tissues from wild-type mice. Western blot analysis of nuclear extracts made from highly enriched megakaryocytes showed increased concentrations of p300 relative to CBP when compared with brain, heart and MEFs (Fig. 4a). The thymus also had a higher p300/CBP protein ratio (data not shown). We verified the increased ratio of p300 to CBP in megakaryocytes by analysing mRNA levels in megakaryocyte and brain by quantitative polymerase chain reaction with reverse transcription (RT–PCR), p300 mRNA was about six times more abundant than CBP mRNA in

**Figure 1** Mouse KIX domain mutations. a, Domains in CBP and p300: nuclear receptor interaction domain (RID), cysteine-histidine-rich domains (CH1, CH2, CH3), KIX bromodomain (Br), IRF3-binding domain (IBiD) and HAT. b, KIX domains from mouse, human, Drosophila melanogaster and Caenorhabditis elegans. Conserved residues are indicated. Produced with Boxshade. c, Structure of the KIX domain from CBP (green) and the KIX domain from CREB (magenta) with the side chains of Tyr 650, Ala 654, Tyr 658 and the phosphoserine 133 (P-Ser133) of CREB highlighted. Produced using Protein Explorer and data from the Protein Data Bank. d, e, Targeting strategies for CBP (d) and p300 (e), and Southern blots of mouse tail DNA. Star indicates a mutated exon, filled boxes indicate probes and exons and arrowhead indicates loxP site. X, BamHI; N, NheI; S, SpeI; I, XbaI; H, HinI; HIII; B, BglII; N, NheI; H, HindIII; S, SpeI.
megakaryocytes (t-test, \( P < 0.01 \); Fig. 4b). Protein binding assays using glutathione S-transferase (GST)–Myb and GST–CREB did not identify tissue-specific differences in the binding of CBP and p300 from nuclear extracts, which suggests that the KIX domains of CBP and p300 are functionally equivalent when isolated from different sources (data not shown). Thus, cells with an increased p300/CBP ratio conceivably might be more susceptible to mutations in the KIX domain of p300.

Because c-Myb homozygous null mice have a deficiency in haematopoiesis\(^{15,16}\), we investigated whether defects in the p300\(^{KIX/KIX}\) mice were caused by an attenuation of c-Myb activity. We examined mice with a single c-Myb null allele, either alone or in combination with a single p300\(^{KIX}\) gene. For c-Myb\(^{−/−}\) mice we reasoned that if mutations in KIX lead to a reduction in c-Myb function, then decreased concentrations of c-Myb might result in a p300\(^{KIX/KIX}\)-like phenotype. Similarly, mice heterozygous for

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**Figure 2** Haematopoetic defects in p300\(^{KIX/KIX}\) mice. a–j, Blood (a–c), bone marrow (d, e), spleen (f–j) from wild-type (a, f, i) and p300\(^{KIX/KIX}\) (b, d, g, j) and CBP\(^{KIX/KIX}\) (c, e, h) mice; examples are shown of giant (broken arrow) and normal small arrow platelets and megakaryocytes (large arrows). Acetylcholinesterase-positive megakaryocytes in spleen are shown at low magnification (i, j). CD41-positive megakaryocytes in p300\(^{KIX/KIX}\) bone marrow and spleen. k, p300\(^{KIX/KIX}\) megakaryocytes have low modal ploidy (k). The peaks in the histogram (top) correspond to 2n to 32n cells (left to right). l, B-cell deficiency in p300\(^{KIX/KIX}\) mice. m, Normal population distribution of double-positive CD4 CD8 thymocytes in p300\(^{KIX/KIX}\) mice.
mutations in both c-Myb and the KIX domain of p300 might show a synergistic phenotype if the interaction between c-Myb and the KIX domain of p300 is limiting for c-Myb-dependent transcription and hematopoietic development17,18. Two- to three-week-old c-Myb+/−p300+/+KIX+/− double heterozygous mice had a large increase in platelets ((3.05 ± 0.47) × 10^11 versus (1.22 ± 0.22) × 10^11 platelets per ml for wild type; t-test, P < 0.01) that was more than additive relative to single heterozygotes and approached concentrations seen in p300−/−/KIX−/− mice (Fig. 4c). Notably, platelet counts in c-Myb+/−p300−/−/KIX−/− mice aged 5–6 weeks were similar to those in the single heterozygotes, suggesting that the interaction between c-Myb and the KIX domain of p300 is less crucial in adult thrombopoiesis (data not shown). Thymocyte numbers were not vastly different between wild type and single heterozygotes, but were reduced by about 40% in c-Myb+/−p300−/−/KIX−/− mice as compared with wild type, suggesting that there is an interaction between c-Myb and the p300 KIX domain in these cells (analysis of variance, P < 0.01, n = 13–21 per strain; data not shown). Other aspects of c-Myb+/−p300−/−/KIX−/− and c-Myb+/−p300−/− mice appeared normal (data not shown), except that the single heterozygotes had slightly more platelets than did their wild-type littermates ((1.52 ± 0.29) × 10^11 for c-Myb+/+p300+/+, and (1.55 ± 0.27) × 10^11 platelets per ml for p300−/−/KIX−/−, t-test, P < 0.01; Fig. 4c).

When we examined megakaryocyte ploidy in mice aged 5–6 weeks, c-Myb+/−p300−/−/KIX−/− cells showed a synergistic reduction in DNA content as compared with the single heterozygotes, with fivefold more cells with >8n, similar numbers of cells with 16n, and 12-fold fewer cells with 32n relative to wild type (Fig. 4d, g). c-Myb+/−p300−/−/KIX−/− megakaryocytes had a noticeable but less marked ‘left shift’ (that is, about twofold more 8n and twofold less 32n cells), whereas p300+/−/KIX−/− megakaryocytes seemed to have an intermediate phenotype (Fig. 4d–f). This left shift towards a modal ploidy of 8n, which is reminiscent of p300+/−/KIX−/− megakaryocytes (Fig. 4h), is abnormal19,20. There was also a significant increase in the percentage of megakaryocytes (CD42d+ cells) in the bone marrow of c-Myb+/−/− and c-Myb+/−p300−/−/KIX−/− mice as compared with wild-type mice (t-test, P < 0.01; Fig. 4i), which is consistent with a trend towards megakaryocytosis. Together, these findings suggest that decreased c-Myb-dependent transcription, either by reduction of c-Myb protein (c-Myb+/−/−) or by a transactivation function (p300+/−/−/KIX−/−), results in abnormal megakaryocytosis and predisposes mice to develop thrombocytosis.

Although the p300−/−/KIX−/− phenotype does not simply recapitulate the phenotypes of knockout mouse models of CREB, c-Myb or other known KIX-binding proteins, connections between p300 KIX and c-Myb were identified nonetheless. c-Myb+/−/− mice die in embryogenesis owing to a multilineage failure of hematopoiesis, but megakaryocytes are still present, which suggests that any loss of c-Myb function preferentially favours megakaryocytes over other hematopoietic lineages17,18. Whether binding of the p300 KIX domain to c-Myb or CREB is necessary for the development of other hematopoietic lineages affected by mutations in c-Myb (erythroid, myeloid and B cells) or CREB (thymocytes) remains to be tested17,18. Notably, the hematopoietic phenotype is specific for p300−/−/KIX−/− mice even though previous work has shown that...
CBP−/−, but not p300−/−, mice tend to develop multilineage defects in haematopoietic differentiation. Taking into account our findings that p300 may be relatively more abundant in an affected lineage, a comparison of the CBP−/− and p300−/− phenotypes implies that other domains besides KIX (or other KIX residues) are limiting for haematopoiesis. Finally, our findings imply that mutations in the KIX domain of p300 and in c-Myb may contribute to human haematopoietic diseases, particularly those with abnormal megakaryopoiesis and thrombopoiesis.

**Methods**

**Mice**

Targeting vectors for CBP and p300 (Fig. 14, e) were made from DNA that was isotopic with the E14 ES cells used in this study. We generated point mutations in the KIX exons using PCR with the following primers: CBP, 5′-CATTTCATTGGAGGAAATGCT AAAAGAGAGAGCGGCT-3′ and 5′-GATTTCCTGGGAGTATACGTA TCTCTCTAAAAGAAACAAAT-3′; p300, 5′-GAATTCTTTGTAGGAGAAGATCGCTAAGAT-3′ and 5′-GAATTCTTTGTAGGAGAAGATCGCTAAGAT-3′. The Hartwell Center at St Jude supplied oligonucleotides for transient expression plasmid pRL–SV40 from Promega. Antibodies were from Santa Cruz (CBP, A22; p300, N15; p300, C20; GST, Z5).

**Cell culture and transient assays**

We obtained megakaryocytes from the fetal livers of C57BL/6 X 129Ola mice hybrid wild-type mice (E13.5). After 7 d of culture, highly enriched primary megakaryocytes were isolated using a bovine serum albumin albumin step gradient (R. Shivdasani, personal communication). We isolated MEFs from E12.5–E14.5 embryos; growth rates were comparable between wild-type and mutant cells. For transient transfection assays, 3 × 10^5 cells per well were cultured overnight in 24-well plates. Cells were transfected with 1 μg of plasmid DNA (0.25 μg of CMV–p300) according to the protocol of Gibco-BRL. After 2 h, the DNA was removed and 1 ml of DMEM plus 10% FBS was added. After 24 h, the cells were harvested and treated as described above.

**Flow cytometry**

Cells were stained with antibodies to CD41, CD45R (B220), CD4, CD8 (PharMingen), Igl and Igk (Southern Biotechnology) conjugated to fluorescein isothiocyanate (CD44, IgJ), phycoerythrin (CD8, IgM), allophycocyanin (B220) or biotin (CD4). Biotinylated antibody was used with a streptavidin-PE-D663 conjugate (Becton Dickinson). We carried out megakaryocyte flow analysis as described.

**Plasmids and antibodies**

The reporter and expression plasmids have been described. Gal–Myb–mutant plasmid was cotransfected with Renilla reporter plasmid pRL–SV40 into human embryonal carcinoma cells (NS5) by transfection (In Vitro Transfection System, Promega). Primers were obtained from Santa Cruz (CBP, A22; p300, N15; p300, C20; GST, Z5).

**Figure 4:** Myb and p300 KIX functionally interact to regulate megakaryopoiesis. a, Western blot of CBP and p300 using wild-type nuclear extracts. Meg, megakaryocyte. Results are normalized to CBP. b, Quantitative real-time RT-PCR analysis of CBP and p300 mRNA in wild-type tissues. Shown are the means ± s.d. relative to CBP mRNA in bone marrow (p = 0.12–0.18). e, Patient counts of CCBP/KIX and p300−/− mice. d–h, Ploidy analysis of megakaryocytes from littermates aged 5–6 weeks (the p300−/− mice in h were not littermate). Shown are the means ± s.d. percentages of megakaryocytes (p = 2–5). i, Percentage of CD42d− cells (megakaryocytes) in bone marrow (p = 4–5).
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Supplementary information accompanies the paper on Nature’s website.

(1) 3.5 ml MgCl2, 4 mM (final) KCl, 3% glycerol and 0.2 ml FBS/10 ml buffers contained 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 0.5 mM EDTA and 1 μM pepstatin. Each brain was homogenised in 5 ml of buffer A. Nuclear proteins were fractionated in 2 ml of buffer C. Extracts were not dialysed. We carried out western blot transfers of CBP and p300 in cold transfer buffer containing 0.1% SDS for 2 h at 400 mA. HAT assays were done as described, with the non-specific control antibody GST Z-5 (ref. 11). Western blot analysis of immunoprecipitations following the HAT assays showed that equivalent amounts of CBP or p300 precipitated from mutant and wild-type extracts (data not shown). We used equal amounts of wild-type and mutant total nuclear protein for western blot analysis and HAT assays.

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Supplementary Information

Table 1
Features of CBP<sup>KIX</sup> and p300<sup>KKX</sup> mutant mice and bone marrow transplant recipients

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<td>Platelet Count of BL6 recipient mice 40 days post transplant (x10&lt;sup&gt;9&lt;/sup&gt;/ml)</td>
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ND = not done, Tpo = thrombopoietin; BL6 = C57BL/6 wild type mice.

Mice were produced by breeding heterozygous animals and genotyped at weaning. Weights and hematocrits are shown for 4-week old mice. Data shown as mean ± SD.
Chapter 7

Summary and Discussion
Summary

NUP98 is an FG repeat-containing nucleoporin that has been found to be involved in multiple leukemia-associated chromosomal translocations, of which the most common is the t(7;11)(p15;p15) rearrangement, which links NUP98 to the class I homeotic transcription factor HOXA9. In our initial studies we sought to answer two main questions: First, what is the contribution of NUP98 to the oncogenic potential of the NUP98-HOXA9 fusion protein? Second, what is the normal role of NUP98 in the cell? In the process of investigating these first two questions, our findings led us to pose two more questions: What are the normal functions of the NUP98-interacting protein Rae1? What are the roles in hematopoiesis for the transcriptional coactivators, CBP and p300, which physically and functionally interact with NUP98-HOXA9?

Our first approach was to test the various domains of NUP98-HOXA9 for cellular transformation and transactivation function using transfection assays and DNA constructs of NUP98-HOXA9 bearing specific deletions and mutations (Chapter 3). We found that the FG repeats contributed by NUP98 were required for \textit{in vitro} cellular transformation and gene transcription transactivation potential. We also showed that this transformation and transactivation potential correlates with the ability to interact with CBP and p300. These results suggested that the oncogenicity of the NUP98-HOXA9 fusion protein results from its aberrant transcriptional properties.

To clarify the means by which NUP98 might contribute to the oncogenic potential of the NUP98-HOXA9 fusion, we examined the normal functions of NUP98 via a mouse knockout approach (Chapter 4). NUP98-deficient mice could not be generated indicating that NUP98 is required for normal development. Studies of cells derived from NUP98 knockout embryos revealed that loss of NUP98 did not affect the number of nuclear pore complexes (NPC) per cell, but that the architecture and transport ability of the NPCs was altered. In the absence of NUP98, NUP358, NUP214, NUP88 and p62 were underrepresented in the nuclear pores of mutant cells compared to wild type. In addition, mutant cells showed an increase in annulate lamellae in the cytoplasm and NUP358, NUP214, NUP88 and p62 were shown to be associated with these structures. Some, but not all protein import pathways are disrupted in the NUP98 null cells indicating that specific nucleoporins have roles in specific nuclear import and export pathways.

We also looked for proteins that interact with the N-terminal portion of NUP98, which is found in leukemia-associated fusion proteins, and showed that Rae1 interacts with NUP98 via its GLEBS-like motif (Chapter 5). Further analysis of Rae1 demonstrated that it acts as an mRNA export factor. Our studies showed that the localization of Rae1 to the nuclear envelope is dependent on ongoing
synthesis of mRNA, and overexpression of the GLEBS-like motif, which acts as a competitor for binding of Rae1 to NUP98, causes accumulation of poly(A)^+ RNA in the nucleus and loss of Rae1 localization to the nuclear envelope. These results support the importance of the Rae1-NUP98 interaction in mRNA nuclear export.

Given the important role that CBP and p300 appear to play in the oncogenicity of the NUP98-HOXA9 fusion protein, we wanted to elucidate the role of p300 and CBP in normal hematopoiesis. To this end, we made targeted mutations in the KIX domain of these proteins, which were designed to interrupt the interaction of p300 or CBP with CREB and c-Myb, both transcription factors with known roles in hematopoiesis (Chapter 6). These studies revealed that while mice bearing homozygous mutations in the KIX domain of CBP were relatively normal, p300\textsuperscript{KIX/KIX} mice had defects in multiple hematopoietic lineages including reduced numbers of erythrocytes and B-lymphocytes, small thymuses, and increased numbers of megakaryocytes and platelets. In addition, mice heterozygous for both the p300 KIX mutation and a knockout allele of c-Myb, displayed a more than additive increase in platelets and decreased megakaryocyte ploidy compared to the single heterozygotes. The phenotype of the compound heterozygotes was similar to, but less dramatic than that seen in p300\textsuperscript{KIX/KIX} mice. These results suggest that the interaction between c-Myb and the KIX domain of p300 is important for certain aspects of megakaryocyte development and function.
Discussion

The results of our research bring to mind several interesting questions that future studies may address. First, do proteins that interact with the NUP98-HOXA9 fusion protein contribute to its oncogenicity? In the case of CBP and p300 our data present a clear argument for the importance of this interaction in the leukemogenic potential of the fusion protein, but the question remains open for other interacting proteins. A recent paper by Babu et al. shows that Rae1 functions as a mitotic checkpoint regulator and is important for maintaining correct chromosome segregation (Babu et al., 2003). These findings suggest that chromosomal instability that could potentiate oncogenesis may be altered by the interaction of Rae1 and the NUP98-HOXA9 fusion protein. In our studies, NUP98-HOXA9 constructs lacking the GLEBS-like motif (necessary for binding Rae1) still had transforming and transactivating potential in NIH-3T3 cells; however, the interaction of Rae1 with the NUP98-HOXA9 fusion protein may contribute to oncogenesis at the level of the organism.

Another interesting question is whether other leukemic fusion proteins involving NUP98 (and perhaps the FG repeat-containing nucleoporin, NUP214) also interact with p300 and CBP, and whether this interaction contributes to their leukemogenesis. The presence of the FG repeat-containing region in all the leukemia-associated fusion proteins involving NUP98 (and NUP214) that have been found to date, argue for the importance of this domain and provide the possibility of interaction with CBP and p300. Whether the FG repeats of NUP214 can also interact with CBP and p300 has not been shown; however, our data indicate that these repeats conferred similar transforming and transactivating potential. The fact that of the fourteen known chromosomal translocations involving NUP98, eight also involve a homeobox protein that contributes its DNA binding domain to the fusion is strong evidence that aberrant transcriptional activity is critical to the oncogenic potential of these fusion proteins. In addition, five of the six (the sixth was not tested) remaining fusion partners of NUP98, as well as both fusion partners of NUP214, contribute domains that are predicted to form coiled-coil structures that could allow oligomerization with transcription factors or cofactors (Hussey and Dobrovic, 2002). This evidence of a transcriptional basis for the leukemogenic properties of these fusion proteins fits well with a possible interaction with p300 and CBP.

It has not been determined whether NUP98 might interact with p300 and CBP in the context of a normal cell. Recent studies linking NUP98 to the transcriptional process raise the possibility that NUP98 and p300/CBP could interact during normal transcription. If this is so, then the FG repeat region of NUP98 might form the basis of that interaction. NUP98 has been shown to localize within the nucleus in a transcription and FG motif-dependent manner; however, NUP98 does not seem to colocalize with the active form of RNA Polymerase II (Griffis et
It is possible that NUP98 may have a role in the movement of RNA to the NPC for transport out of the nucleus in addition to its role at the NPC. Our studies showing the interaction of NUP98 and Rae1 support a role in RNA transport, and the studies reported in Griffis et al. indicate that blocking RNA synthesis causes NUP98 to lose its normal mobility within the nucleus. It seems likely that NUP98 may accompany the RNA from the nuclear interior to the NPC and it is conceivable that NUP98 and CBP/p300 may interact via the FG repeats at some point during this process, although it is not immediately clear what the significance of this interaction might be.

Since p300 and CBP act as coactivators for such a wide range of transcription factors, the loss or disruption of p300 or CBP can be expected to have widespread implications for transcription. We sought to dissect out the effect of the interaction of CBP and p300 with specific transcription factors by making a targeted mutation in the KIX domain that was predicted to inhibit binding to CREB and c-Myb without affecting the overall structure of p300 or CBP; however, the effect of our mutation on other transcription factors that bind the KIX domain was not known. Recent studies have made it clear that not all transcription factors that bind to the KIX domain bind to the same surface. MLL has been shown not only to bind to a different portion of KIX than that bound by CREB and c-Myb, but the binding of MLL to KIX has been shown to increase the affinity of CREB and c-Myb for the KIX domain (Goto et al., 2002). Similarly, the immediate early transcription factor, c-Jun, can bind to KIX simultaneously with CREB, by using a distinct surface (Campbell and Lumb, 2002). These studies are important to our results in several regards. First, they indicate that the mutations we made in the KIX domains of p300 and CBP most likely disrupt the interaction of p300 and CBP with only a subset of the transcription factors known to bind to the KIX domain. Second, the studies by Goto et al. bring up an important point with regard to domain-targeted mutations as they showed that MLL and c-Myb bind to distinct areas of the KIX domain in a cooperative manner. This reminds us that a mutation that interrupts binding of p300 or CBP to a single transcription factor could have a wider impact by disrupting cooperative interactions between transcription factors that are mediated by p300 and CBP acting as scaffolds.

A question that has yet to be answered definitively is to what degree do p300 and CBP function redundantly? Our results, which present such different phenotypes for identical mutations in p300 and CBP, can be interpreted in at least two ways. One interpretation is that the strikingly different phenotypes of p300<sup>KIX<sup>−<sup>−</sup></sup></sup> and CBP<sup>KIX<sup>−<sup>−</sup></sup></sup> mice represent separate and distinct functions of p300 and CBP. This view would seem to be supported by the results of studies on mice heterozygous for either p300 or CBP, which indicate that loss of a single allele of p300 results in different hematologic defects and oncogenic potential than loss of a single CBP allele (Kung et al., 2000; Rebel et al., 2002). A multitude of other experiments; however, have indicated redundant roles for
p300 and CBP, including those that showed that a compound heterozygous knockout mutation in both p300 and CBP results in an embryonic lethal phenotype (Yao et al., 1998). Thus, a second possible interpretation of our results is that p300 and CBP are highly redundant and that the relative abundance of p300 and CBP in specific tissues can result in different phenotypes. Our data suggest that the ratio of p300 to CBP protein levels varies in tissues, with p300 appearing to be relatively more abundant compared to CBP in tissues that are affected in p300KIX/KIX mice. It is likely that reality lies somewhere between these two interpretations and that p300 and CBP possess both specific and redundant functions.

There is evidence that p300 and CBP may function as both tumor suppressors and oncoproteins. The loss of a single allele of CBP has been shown to lead to a greater than normal incidence of oncogenesis in both mice and humans, and transplant studies indicate that loss of a p300 allele can also lead to an increase in hematologic neoplasms (Kung et al., 2000; Miller and Rubinstein, 1995; Rebel et al., 2002). The means by which p300 and CBP function as tumor suppressors is not clear, but our research indicates that at least some aspects of this function could be narrowed down to the KIX domain. While the phenotype of the p300KIX/KIX mice does not include oncogenesis, the extreme overproduction of megakaryocytes and the relative immaturity of these cells are clearly abnormal, and such dysplasias could lead to cancer.

In addition to evidence of roles as tumor suppressors, the involvement of p300 and CBP in multiple leukemia-associated fusion proteins indicates that they may also function as oncoproteins. It seems likely that dysregulation of their normal functions as adaptor molecules and acetyltransferases could account, at least in part, for the oncogenic potential of these fusion proteins. In the case of the MLL fusions, the coupling of MLL’s transcriptional activity to p300 or CBP’s ability to interact with the basal transcription machinery and acetylate chromatin to make genes accessible for transcription can be envisioned to lead to dysregulated transcription. When CBP or p300 is fused to MOZ or MORF, the additional acetyltransferase activity could cause increased gene activation when a transcription factor recruits CBP or p300 in the context of the fusion, which again could lead to dysregulated gene expression that results in leukemia. The implications of this dual role for p300 and CBP as both tumor suppressors and oncoproteins are only beginning to be understood.
References


In addition to the involvement of the NUP98-HOXA9 chromosomal translocation in a subset of acute myeloid leukemia (AML), HOXA9 itself has been found to be deregulated in most AML cases. (Golub et al., 1999) The correlation of HOXA9 overexpression with poor prognosis makes it attractive as a target of therapeutic drugs for the treatment of AML. Since the NUP98-HOXA9 fusion protein utilizes the DNA binding domain of HOXA9 in its aberrant transcriptional capabilities, it is likely that the normal targets of HOXA9 are the same genes subject to misregulation by the fusion protein. Both of these findings point to the importance of identifying HOXA9 target genes.

Although accounting for a small subset of leukemias, NUP98, as of this writing, is a partner in at least fifteen leukemia-associated chromosomal translocations. Interestingly, recent studies show that aberrant expression of NUP98 and RAE1 causes mitotic checkpoint failure and chromosomal instability. It will be interesting to explore further whether mitotic checkpoint deregulation is a shared pathogenic effect of NUP98-containing fusion proteins.

I believe that further mouse models mutating other protein binding domains of p300 and CBP or even bearing different mutations in the KIX domain will be of great value in allowing the study of specific interactions involving these rather promiscuous coactivators. This type of approach, particularly when combined with genetic crosses involving predicted binding partners to look for synthetic phenotypes should prove helpful in answering mechanistic questions that are often not easily addressed in vivo.

To address in greater detail biochemical and transcriptional questions regarding the effects of these mutations, it will be necessary to reduce the effect of remaining wild type alleles of p300 or CBP. One possibility is the generation of mouse embryonic fibroblasts (MEFs) with three or four KIX domain mutations in p300 and CBP. Alternatively, a “knock-down” approach using RNA interference technology could be used in MEFs bearing a homozygous mutation in the KIX domains of p300 to reduce the effect of the unmutated CBP domains or vice versa. These cells would be very valuable in assessing the specific interactions of transcription factors with p300 and CBP.

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References
**Abbreviations used in this thesis**

AML  acute myeloid leukemia  
C  carboxy  
CBP  CREB-binding protein  
CLP  common lymphoid precursor  
CML  chronic myeloid leukemia  
CMP  common myeloid precursor  
CRE  cyclic AMP response element  
CREB  CRE-binding protein  
EPO  erythropoietin  
ES  embryonic stem  
FG  phenylalanine-glycine  
FXFG  phenylalanine-any amino acid-phenylalanine-glycine  
GLEBS  Gfe2p-binding sequence  
GLFG  glycine-leucine-phenylalanine-glycine  
HAT  histone acetyltransferase  
HOX  homeobox  
HSC  hematopoietic stem cell  
MDS  myelodysplastic syndrome  
MHC  major histocompatibility complex  
N  amino  
NES  nuclear export signal  
NK  natural killer  
NLS  nuclear localization signal  
NPC  nuclear pore complex  
NUP  nucleoporin  
Ph  Philadelphia  
RT-PCR  reverse transcription polymerase chain reaction  
RTS  Rubinstein-Taybi Syndrome  
T-ALL  T-cell acute lymphoid leukemia  
t-AML  therapy-related acute myeloid leukemia  
t-CML  therapy-related chronic myeloid leukemia  
TCR  T-cell receptor  
t-MDS  therapy-related myelodysplastic syndrome  
TPO  thrombopoietin
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Lawryn Kasper
Publications (in reverse chronological order)


Curriculum Vitae

Lawryn Heath Kasper was born in Covina, California, USA in 1968. She attended American public high schools in Heidelberg, West Germany and Springfield, Virginia, USA and graduated in May 1986 as a valedictorian of her class. From August 1986 through May 1991 she studied at Rice University in Houston, Texas, USA and graduated with a Bachelor of Arts in Biology, Anthropology and German. From June 1991 until June 1994, she worked as a research technician in the laboratory of Dr. J. Arly Nelson in the Department of Experimental Pediatrics, Pharmacology Section at the University of Texas, M.D. Anderson Cancer Center in Houston, Texas, USA. In January 1995 she began working at St. Jude Children’s Research Hospital in Memphis, Tennessee, USA, first in the Genetics Department as a Senior Research Assistant in the laboratory of Dr. Gerard Grosveld, then beginning in 1996 in the laboratory of Dr. Jan van Deursen where her research focused on the nucleoporin, NUP98 and its involvement in the chromosomal translocation t(7;11)(p15;p15). In 1999 she moved to the laboratory of Dr. Paul Brindle in the Department of Biochemistry and was promoted to Research Laboratory Specialist in 2000. Her research through 2002 focused on the role of the coactivators, p300 and CBP, in hematopoiesis. She continues to work in Dr. Brindle’s laboratory on a variety of projects involving p300, CBP and a component of the Mediator coactivator complex, Sur2.