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Functional Interactions Between Chromatin Modifying Complexes and the Nuclear Pore in the Yeast *Saccharomyces cerevisiae*

By

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Doctor of Philosophy

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Functional Interactions Between Chromatin Modifying Complexes and the Nuclear Pore in the Yeast *Saccharomyces cerevisiae*

By

Shana C. Kerr
B.S., Georgia Institute of Technology, 2002

Advisor: Anita H. Corbett, Ph.D.

An abstract of
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Abstract

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By Shana C. Kerr

The nucleus is functionally organized by the arrangement of the chromosomes, the nuclear periphery, and spatial regulation of transcription. Though the nuclear periphery has been historically viewed as transcriptionally repressive, recent work in the budding yeast *Saccharomyces cerevisiae* has revealed that some genes physically relocate from the nuclear interior to the nuclear periphery upon transcriptional activation, where they associate with nuclear pore complexes (NPCs). This dissertation focuses on elucidating the mechanism and physiological significance of this phenomenon.

Our work reveals functional interactions between actively transcribed genes, chromatin modifying complexes, and the NPC. Specifically, we identify the SAGA histone acetyltransferase as a necessary link between the NPC and active *GAL* genes. Interestingly, this association requires the physical presence of SAGA rather than transcriptional activation by SAGA, suggesting that gene interaction with the NPC is mediated by protein-protein interactions between NPC subunits and transcriptional activators. Our studies also reveal that functional interactions between SAGA and the NPC regulate global transcript levels, particularly for highly transcribed genes. These findings suggest a role for NPC-gene interactions in regulating the global transcription of highly induced genes. In addition, we find that interactions between NPC and SAGA subunits are required for the retention of the *GAL1* gene at the NPC, and defects in gene retention due to loss of NPC and SAGA subunits correlate with reduced ability of these cells to metabolize galactose. These findings suggest that gene relocation is comprised of two steps, recruitment and retention, and that gene relocation makes a significant contribution to transcriptional regulation. We also identify new factors potentially involved in gene relocation based on functional interactions between INO80 chromatin remodeling complex components and NPC subunits. Interestingly, we find that interactions between the NPC and INO80, as well as interactions between the NPC and SAGA, may play a role in DNA damage repair. These observations are consistent with a physiological role for relocation of damaged DNA to the NPC, analogous to relocation of transcribed genes. Taken together, these results suggest that the NPC is an important regulator of chromatin dynamics that promotes an open chromatin structure permissible to active DNA transactions.

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CHAPTER 1

Introduction and Background

Overview of Nuclear Organization

Eukaryotic cells are named for what is arguably their most striking and distinguishing feature: the presence of the nucleus, the cellular compartment which separates the genetic material and transcriptional machinery from the metabolic processes in the cytoplasm. Though the nucleus was first described approximately 200 years ago (1), the organization of this double membrane-bound organelle has only recently begun to be appreciated. Extensive work in the last 20 years has begun to reveal the nuanced organization within the nuclear compartment (2). Much of this work largely points to the conclusion that there is a relationship between gene location and transcriptional activity (3-5). The factors which impart this location and organization include the 3-dimensional arrangement of the genome into chromosomes territories and the distinct domains of the nuclear membrane, as well as recently proposed smaller, dynamic structures such as transcription factories (2, 4, 5).

The chromosomes are perhaps the most significant organizational coordinators within the nucleus. Anatomist Carl Rabl observed over 100 years ago that chromosomes maintain specific orientations following cell division and proposed that each chromosome occupies a distinct region in the interphase nucleus (6). These distinct regions were later termed chromosome territories (CTs) by Theodor Boveri based on his studies in blastomere nuclei of the nematode *Parascaris equorum* (7). Though it would take 80 years for these early observations to be unequivocally confirmed by chromosome-wide fluorescence *in situ* hybridization (FISH) (8), the arrangement of the chromosomes into CTs is now a widely accepted model for nuclear organization (9-12). The chromosomes

are radially distributed with 'gene-rich' chromosomes located toward the nuclear periphery and 'gene-poor' chromosomes located toward the nuclear interior in human cells (13-15). This organization may have functional relevance, as tumor cells are likely to exhibit a loss of this radial organization (16). Moreover, the positions of particular chromosomes within the nucleus are evolutionarily conserved across primates (17), again suggesting that this organization is functionally significant. Intriguingly, these positions may even be conserved between primates and the chicken, *Gallus gallus domesticus*, indicating a persistence of this arrangement for over 300 million years (18); however, these conclusions are tentative given the high chromosome divergence between primates and chicken.

Regulation of transcription is another major mediator of nuclear organization. While the textbook explanation for transcriptional activation describes genes recruiting the transcriptional machinery (19), studies over the last 15 years suggest that the opposite actually occurs; genes poised to become activated are relocated to sites enriched for the transcriptional machinery (4, 20-22). These sites have been deemed "transcription factories," and are thought to contain multiple active RNA polymerase II molecules and accommodate up to 20 active genes each (23-26). Association with transcription factories is dynamic and correlates with a gene's transcriptional state, and highly expressed genes are more consistently localized to transcription factories (22). Genes in the same transcription factory need not be adjacent, but rather can be widely separated across the chromosome (22). In fact, these genes can reside on entirely different chromosomes; there is considerable contact between adjacent CTs and this contact is transcription-dependent (27). Interestingly, independent transcription factories may be

specialized for distinct functional roles, as the *Myc* and *Igh* genes are preferentially recruited to the same transcription factory during mouse B lymphocyte maturation even though these genes are on different chromosomes (28). Moreover, studies using plasmid expression constructs have revealed that plasmids with similar promoters tend to colocalize to the same transcription factory, but inclusion of an intron into one of these constructs will cause it to associate with a different transcription factory (29), suggesting that specific transcription factories may be optimized for particular transcription factors or intron splicing.

Another critical structure which dictates nuclear organization is the nuclear envelope, the double-membrane boundary which separates the nucleoplasm from the rest of the cell. The nuclear envelope contains two distinct domains: the nuclear pore complexes (NPCs), the large, proteinaceous channels which mediate macromolecular traffic in and out of the nucleus, and the nuclear lamina, a network of intermediate filaments which occupies the regions between NPCs (30-33). Both of these structures are thought to play a role in transcriptional regulation. The lamins associate with many transcriptionally repressed regions of the genome (3, 34, 35). In addition, targeting a reporter gene to the nuclear lamina results in transcriptional repression, even when that gene is driven by a strong viral promoter (36). Moreover, naturally occurring lamin mutations can disrupt the association of heterochromatin with the nuclear periphery (37, 38), suggesting that the lamins play a key role in locating transcriptionally silent chromatin at the nuclear periphery. Interestingly, the budding yeast *Saccharomyces cerevisiae* displays no evidence of containing a nuclear lamina, and yet transcriptionally silent genes still associate with the nuclear periphery in this model organism (39).

Nuclear membrane-associated silenced loci include the telomeres, which can be visualized as several clusters at the nuclear periphery, as well as silent mating-type loci (40). Consistent with a role for the nuclear periphery in silencing, artificial tethering of a reporter gene to the budding yeast nuclear membrane results in transcriptional repression (41), suggesting an evolutionarily conserved role for the nuclear envelope, even in the absence of the nuclear lamina, in transcriptional silencing. While the nuclear membrane and lamins appear to promote transcriptional repression, recent findings suggest that the NPC may regulate transcriptional activation (34, 42-44). Multiple active genes associate with the NPC in a transcription-dependent manner in the budding yeast *S. cerevisiae*, and transcriptional upregulation of the single male X chromosome is dependent upon NPC subunits in the fruit fly *Drosophila melanogaster* (34, 42, 44). These observations suggest that there are differential roles for the NPC and the nuclear membrane/lamina in transcriptional regulation.

The Nuclear Pore Complex

The NPC is an evolutionarily conserved proteinaceous structure of approximately 44 MDa (~60-120 MDa in vertebrates) which perforates the nuclear envelope and mediates macromolecular traffic between the nucleus and the cytoplasm (45-47). Vertebrate cells have between 1,000 and 16,000 NPC per nucleus, depending on species and cell type, while the much smaller nuclei of budding yeast possess ~65-180 (48, 49). The NPCs in both budding yeast and vertebrates are non-randomly distributed across the nuclear envelope, in arrangements which may reflect cell cycle stage or chromosome

organization (49, 50). Although the vertebrate NPC is larger than that of budding yeast, both are comprised of approximately 30 protein components called nucleoporins (Nups), which are present in at least 8 copies per NPC and are arranged in 8-fold radial symmetry (45, 46).

Though many of the individual Nups have only minimal evolutionary conservation or only conserved domains, and the vertebrate NPC is larger than that of budding yeast, the overall structure of the NPC is highly conserved across the eukaryotic lineage (51). Some Nups are asymmetrically localized across the NPC, giving the complex three distinct evolutionarily conserved substructures: a nuclear basket, a central core spanning the nuclear envelope, and cytoplasmic fibrils (33, 46) (Figure 1.1, Table 1.1). Both faces of the NPC as well as the central core are lined with a specific class of Nups, collectively termed FG-Nups, which are characterized by at least one domain of glycine-leucine-phenylalanine-glycine (GLFG), phenylalanine-variable-phenylalanine-glycine (FxFG), and/or phenylalanine-glycine (FG) amino acid repeats (33, 52). The FG Nups play a critical role in mediating traffic through the NPC *via* direct interactions with export and import factors (53, 54), and are thought to form a mesh-like barrier based on weak hydrophobic interactions that prevents unregulated passage through the NPC (55, 56).

There is growing evidence that all NPCs are not identical. In budding yeast, the nuclear basket proteins, Mlp1 and Mlp2, are not present in those NPCs which are adjacent to the nucleolus (57). This differential localization may have functional relevance, as the Mlp proteins have been implicated in both quality control of mRNA export and interaction with actively transcribed genes (57-62), perhaps suggesting that

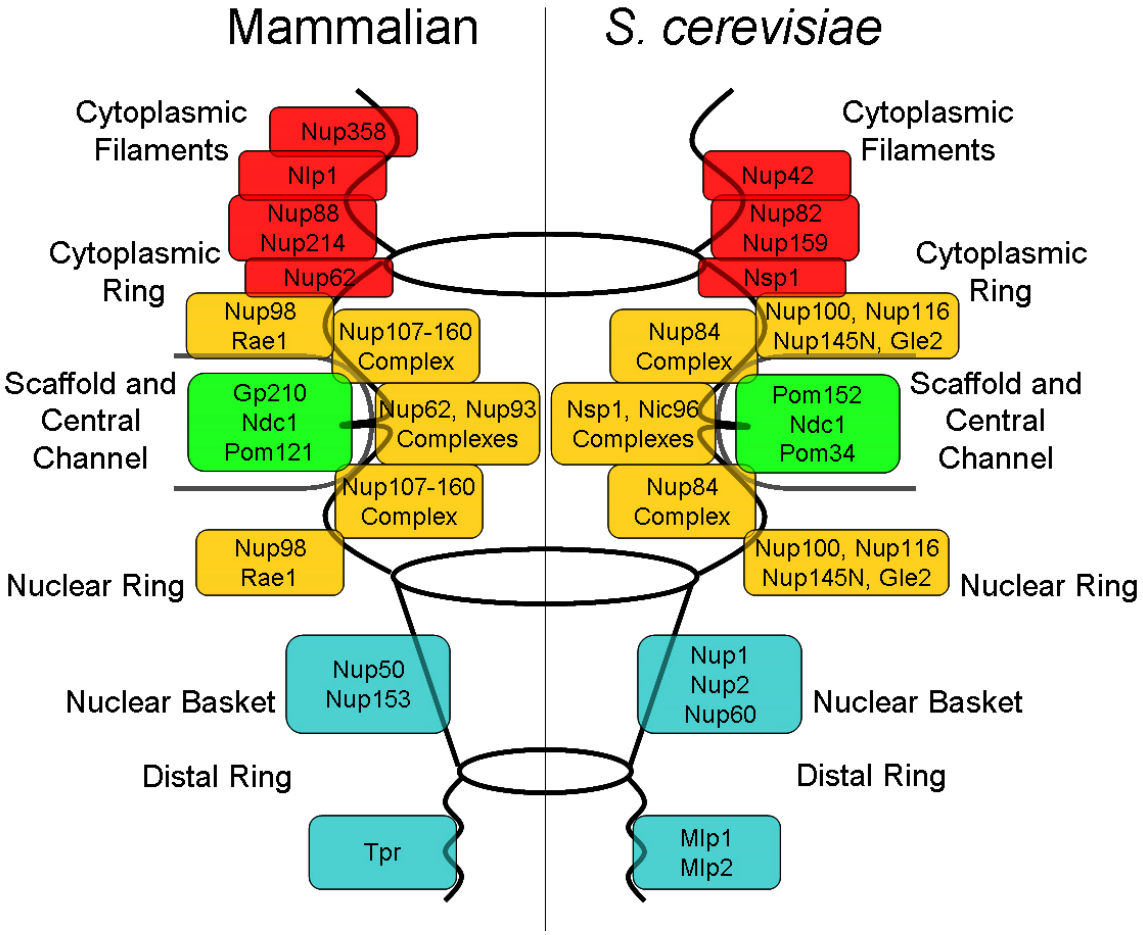

Figure 1.1 **Schematic of nuclear pore structure and composition.** Nuclear (blue), symmetric (orange), transmembrane (green), and cytoplasmic (red) subunits of the mammalian (left) and budding yeast (right) NPC. Figure design based on (30, 46, 63-65).

Table 1.1 **NPC subunit homologs based on similarities in sequence and function**[a,b,c]

Mammalian	***S. cerevisiae***	**Association with NPC**	**Distribution across NPC**	**Motifs/ domains**	**Predicted Function**
Nup50/Npap60	Nup2	Dynamic	Nuclear	FG	Transport
Nup62 complex Nup45, Nup54, Nup58, Nup62[d]	**Nsp1 complex** Nsp1[d], Nup49, Nup57	Intermediate	Symmetric	FG, Coiled-coil	Structure; transport
Nup88	Nup82	Stable	Cytoplasmic	β-propeller, Coiled-coil	Structure
Nup93 complex Nup35/53, Nup93, Nup155, Nup188, Nup205	**Nic96 complex** Nic96, Nup53, Nup59, Nup157, Nup170, Nup188, Nup192	Intermediate	Symmetric	β-propeller, α-solenoid, FG	Structure; transport
Nup98	Nup100, Nup116, Nup145N	Dynamic	Symmetric	FG, Nup98 fold	Transport
Nup107-160 complex ALADIN, Nup37, Nup43, Nup75/85, Nup96, Nup107, Nup133, Nup160, Sec13, Seh1	**Nup84 complex** Nup84, Nup85, Nup120, Nup133, Nup145C, Sec13, Seh1	Stable	Symmetric	β-propeller, α-solenoid	Scaffold
Nup153	Nup1, Nup2, Nup60	Dynamic	Nuclear	FG	Structure; transport
Nup214	Nup159	Stable	Cytoplasmic	β-propeller, Coiled-coil, FG	Structure
Nup358	-	ND	Cytoplasmic	FG	Structure; transport
Gp210	Pom152	Dynamic	Transmembrane	TMH	Transport
Ndc1	Ndc1	ND	Transmembrane	TMH	Structure
NLP1/CG1	Nup42	Intermediate	Cytoplasmic	FG	Structure; transport
Pom121	-	Stable	Transmembrane	TMH, FG	Structure
RAE1/Gle2	Gle2	Dynamic	Symmetric	β-propeller	Transport
Tpr	Mlp1, Mlp2	ND	Nuclear	Coiled-coil	Structure; transport
-	Pom34	ND	Transmembrane	TMH	Structure

Table compiled from (63, 66).

[a]Some Nups share sequence similarity with multiple Nups.

[b]A dash (-) indicates that no homolog has been identified.

[c]Abbreviations: FG, FG repeat domains; ND, not determined, TMH, transmembrane helix.

[d]Nsp1 (Nup62) is symmetrically located as part of the Nsp1 (Nup62) complex, but also localizes to the cytoplasmic face individually

Mlp-containing NPCs are specialized for mRNA export while Mlp-absent NPCs are optimized for transport of ribosome biogenesis factors. There is also evidence of tissue-specific expression of NPC subunits during development in metazoans. The vertebrate gp210 subunit and the *Drosophila melanogaster* Nup88 homolog are differentially expressed during mouse and fly organogenesis, respectively (67, 68). In addition, the vertebrate Nup133 subunit is expressed in a tissue- and stage-specific manner during mouse embryogenesis, and deletion of the *NUP133* gene disrupts neuronal differentiation (69). There are also examples of tissue-specific Nup expression in adult organs. Nup50 (Npap60) is significantly more abundant in rat testis than in somatic tissues, and its sub-cellular localization changes during spermatogenesis from NPC-associated in spermatocytes to nucleoplasmic in spermatids (70). Taken together, these data suggest that differential NPC composition may be related to cell-fate determination during differentiation.

DNA Transactions at the NPC

Twenty-five years ago, Gunter Blobel proposed his seminal gene gating hypothesis that active genes associate with NPCs to facilitate mRNA export and contribute to three-dimensional nuclear organization (71). This proposal coincided with the discovery that DNase I hypersensitive sites, presumably correlating with regions of active chromatin, are located near the nuclear periphery in mouse fibroblast cells (72). In spite of these two provocative works, the nuclear periphery has historically been viewed as repressive to transcription largely due to the effects of the lamins and nuclear

membrane in transcriptional silencing (40) as previously discussed. These effects manifest in two visual observations which helped to establish this view of the nuclear periphery as repressive. First, electron microscopy of interphase nuclei reveals that heterochromatin is enriched at the nuclear periphery (73). Second, chromosomes in mammalian nuclei are radially distributed with 'gene-poor' chromosomes located toward the nuclear periphery and 'gene-rich' chromosomes toward the nuclear interior (13-15). Both of these visually arresting observations implicate the nuclear periphery in mediating transcriptional repression; however, a model for a repressive nuclear periphery based on these observations does not distinguish between the nuclear lamina and the NPCs at the nuclear periphery. In fact, though it would take nearly twenty years to validate Blobel's gene gating hypothesis, there is now overwhelming evidence that the NPC plays a role in transcriptional activation of at least some specific genes. This phenomenon has been well documented in the budding yeast *S. cerevisiae*, and there is strong evidence suggesting that a similar, though not identical, effect may occur in the fruit fly *Drosophila melanogaster* (34, 42-44, 74).

The association of active genes with the NPC was first identified *via* a genome-wide chromatin immunoprecipitation (ChIP) assay, which revealed that actively transcribed genes tend to associate with specific NPC subunits in *S. cerevisiae* (62). Locus-specific fluorescence *in situ* hybridization (FISH) revealed that this association is dynamic, such that specific genes are located in the nuclear interior when repressed but then relocate to the NPC when induced (62), suggesting a causative relationship between location and transcriptional activity. These finding came as a surprise to the authors, who undertook the study anticipating the opposite result given the historical view of the

nuclear periphery as transcriptionally repressive: that repressed genes would be more likely to associate with the NPC. Subsequent studies have found that the relocation of these genes seems to be largely dependent on specific transcriptional activators and co-activators, rather than a result of the locus being "dragged" to the NPC by co-transcriptional export of mRNA through the NPC (59, 60). In fact, gene relocation to the NPC occurs prior to transcriptional activation (75), suggesting physical interactions between transcriptional activators and subunits of the NPC.

More recently, the NPC has also been implicated in repair of DNA damage based on the observation that persistent DNA double strand breaks (DSBs) relocate to the nuclear periphery where they associate with NPCs (76, 77). This finding is perhaps less surprising than recognition of active genes at the NPC, as NPC subunits have been linked to DNA damage repair through a considerable amount of circumstantial evidence. First, cells lacking any of a number of NPC subunits are hypersensitive to DNA damaging agents (78-80). In addition, the DNA damage response desumoylating enzyme, Ulp1, associates with the NPC, and disruption of this association results in increased numbers of endogenous DSBs (81-83). The finding that persistent DSBs relocate to the NPC enhances and extends these circumstantial observations suggesting links between the NPC and DNA damage response. The factors which relocate persistent DSBs to the NPC have only begun to be elucidated, but relocation appears to be linked to mediators of both homologous recombination (HR) and non-homologous end-joining (NHEJ), the two different pathways which can process a DSB (Figure 1.2, Table 1.2). These findings suggest that relocation of DSBs to the NPC is not specific to one repair pathway, but may be a general feature of DSB repair.

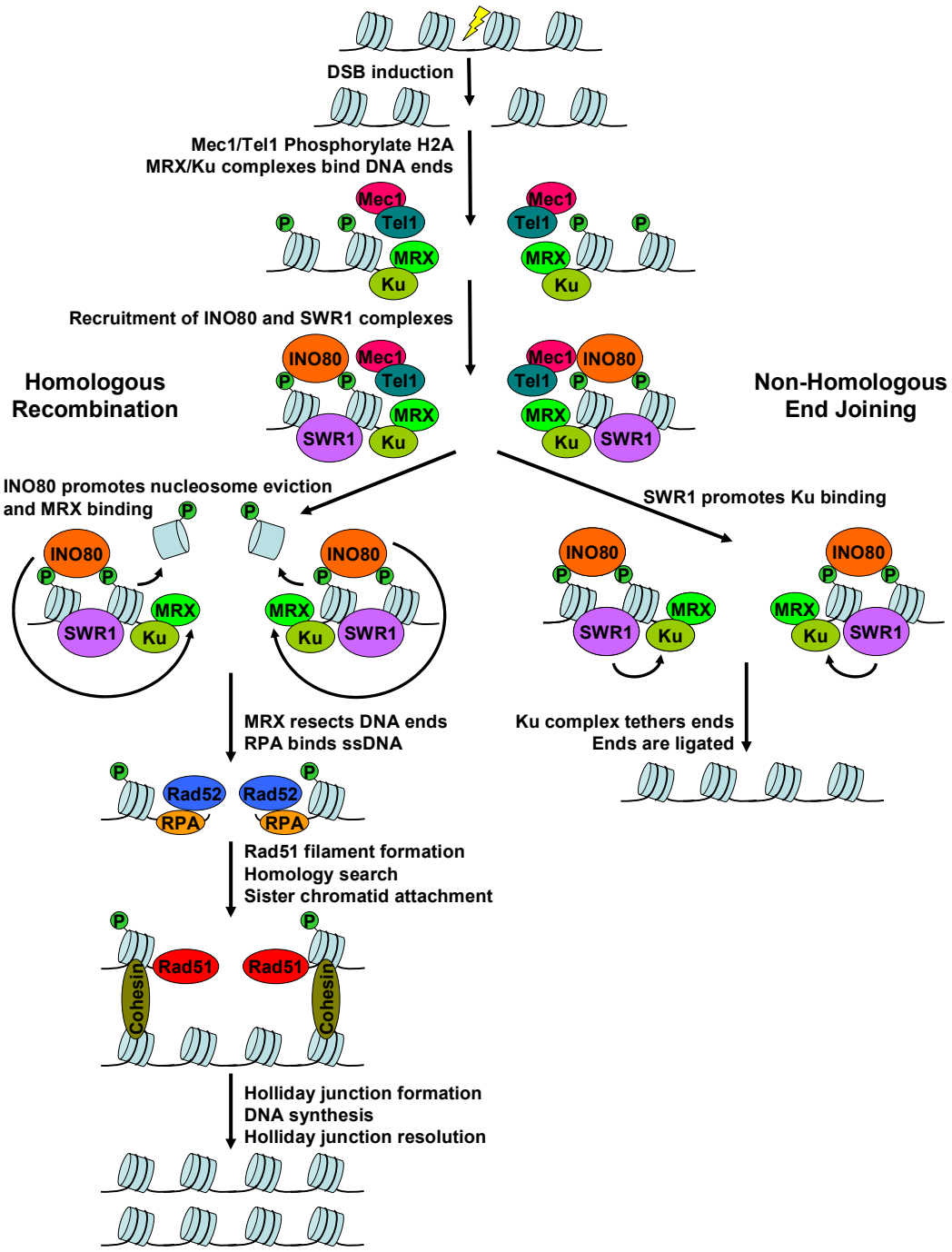
DSB induction
Mec1/Tel1 Phosphorylate H2A
MRX/Ku complexes bind DNA ends
Mec1
Tel1
MRX
Ku
P
Recruitment of INO80 and SWR1 complexes
INO80
SWR1
Homologous Recombination
Non-Homologous End Joining
INO80 promotes nucleosome eviction and MRX binding
SWR1 promotes Ku binding
MRX resects DNA ends
RPA binds ssDNA
Rad52
RPA
Ku complex tethers ends
Ends are ligated
Rad51 filament formation
Homology search
Sister chromatid attachment
Rad51
Cohesin
Holliday junction formation
DNA synthesis
Holliday junction resolution

Figure 1.2 **Double strand break repair model.** Repair of DSBs can occur through homologous recombination (HR) or non-homologous end joining (NHEJ). Following induction of a DSB, DNA ends are recognized by the Mre-11-Rad50-Xrs2 (MRX) complex and the Yku70-Yku80(Ku) complex. The Tel1 and Mec1 kinases phosphorylate H2A, and the phosphorylated histones then recruit the INO80 and SWR1 complexes. For HR (left), INO80 evicts nucleosomes adjacent to the break site and promotes binding of the MRX complex which resects the ends to produce single-stranded DNA (ssDNA) overhangs with the help of the Sae2 endonuclease. Further resection occurs by either Sgs1 and Dna2 or Exo1 nucleases, and the exposed ssDNA is coated by replication protein A (RPA). RPA recruits the Rad52 epistasis group (Rad52, Rad54, Rad55, Rad57, Rad59, and Rdh54), which in turn enables Rad51 filament formation and the search for homologous DNA sequences. Cohesin mediates interactions between homologous DNA strands. A Holliday junction is formed between the two DNA strands, DNA is synthesized, and the junction is resolved. For NHEJ (right), SWR1 promotes the association of the Ku complex with the broken DNA ends, which are then ligated together to resolve the DSB. Figure design based on (84-86).

Table 1.2 **A selection of evolutionarily conserved DSB response proteins**[a]

S. cerevisiae	**Mammalian**	**Function**
Cohesin complex Irr1, Mcd1, Smc1, Smc3	STAG1, STAG2, STAG3, RAD21 SMC1, SMC1B, SMC3	Mediates sister chromatid cohesion during mitosis, meiosis, and DSB repair
Dna2	DNA2	ssDNA endonuclease; ATPase; helicase
Exo1	EXO1	5'-3' exonuclease and flap endonuclease
Mec1	ATR	PIKK required for DNA damage and replication checkpoint
MRX complex Mre11-Rad50-Hrs2	MRE11-RAD50-NBS1	Mre11, ssDNA endonuclease and 3'-5' exonuclease; Rad50, DNA binding and tethering activity; Xrs2, interacts with Tel1
Rad51	RAD51	RecA homolog, binds ssDNA and dsDNA, promotes homologous pairing and strand exchange
Rad52 epistasis group Rad52, Rad54, Rad55, Rad57, Rad59, Rdh54	RAD52, RAD54, RAD51B, RAD51C, RAD52B, RAD54B	Recruit and function with Rad51 to form Rad51 filaments for DNA homology searching
RPA Rfa1-Rfa2-Rfa3	RPA1-RPA2-RPA3	Heterotrimeric ssDNA binding proteins
Sae2	CtIP	Interacts with MRX complex on DNA; *S. cerevisiae* homolog has ssDNA endonuclease activity
Sgs1	BLM	3'-5' helicase of the RecQ family
Tel1	ATM	PIKK required for DNA damage checkpoint and telomere maintenance
Yku70-Yku80	KU70-KU80	Heterodimeric DNA end binding proteins

Table compiled from (87, 88).

[a]Abbreviations: PIKK, phosphatidyl inositol 3' kinase-related kinase

As the majority of the evidence linking actively transcribed genes to the NPC is based on studies in the budding yeast *S. cerevisiae*, one especially intriguing question is whether this phenomenon is evolutionarily conserved. There is evidence to suggest that at least some aspects of this phenomenon may occur in metazoans. First, fusions between Nup98 and Hox family transcriptional regulators result in potent oncogenic transcriptional activators linked to acute leukemia (89). In the fruit fly *Drosophila melanogaster*, nuclear pore components were recently found to be required for proper transcriptional upregulation by the dosage compensation complex on the peripherally located male X chromosome (90). In addition, the mouse nuclear pore component Nup96 is required for proper activation of interferon genes (91). Interestingly, these interactions between NPC components and active genes may not necessarily occur at the nuclear periphery. Very recently, multiple *Drosophila* Nups including Nup98, Nup153, Sec13, and the Mlp/Tpr homolog were found to associate with up to 42% of the genome, including actively transcribed developmental and cell cycle regulation genes (92-94). Moreover, transcription of these genes is dependent on this association (92-94). However, many of these interactions occur in the nucleoplasm rather than at the nuclear periphery (92-94), suggesting that NPC association is not required for these Nups to function in transcriptional activation. These observations suggest that NPC subunits play an evolutionarily conserved role in gene activation which may be independent of their function at the NPC.

Characterized Loci and Mechanisms of Gene Relocation to the NPC

The galactose-responsive *GAL* genes were the first genes recognized to relocate to the NPC upon transcriptional activation (62), and are among the most extensively studied. The *GAL* genes collectively encode the biochemical machinery that imports and converts galactose to glucose-1-phosphate such that it can proceed into glycolysis following interconversion to glucose-6-phosphate by the constitutively expressed phosphoglucomutase, Pgm2 (95). These tightly co-regulated genes, which traditionally include *GAL1*, *GAL2*, *GAL7*, *GAL10*, and *MEL1*, are strongly repressed in the presence of glucose, but rapidly and profoundly induced in the absence of glucose and presence of galactose (96). Most notably, *GAL* gene regulation is not only very precisely controlled within the cell, but also extremely well characterized (95-99), making them excellent model loci for studying the dynamics of gene relocation to the NPC.

Transcription of the *GAL* genes is regulated by the evolutionarily conserved Spt-Ada-Gcn5 acetyltransferase (SAGA) histone acetyltransferase complex (Figure 1.3, Table 1.3) (97-99). SAGA is recruited to chromatin by gene-specific transcription factors *via* the Tra1 subunit; in the case of the *GAL* genes this gene-specific transcription factor is the canonical Gal4 activator (98-101). Once recruited to a gene for activation, SAGA functions as a transcriptional co-activator through at least two distinct mechanisms. First, the Gcn5 subunit acetylates multiple lysine residues on the N-terminal tails histones H2B and H3, and this activity is modulated by the associated Ada2 and Ada3 subunits (102-105). Second, for some genes such as the *GAL* genes, the acetylation activity of SAGA is not required for transcriptional activation; rather, SAGA activates these genes through

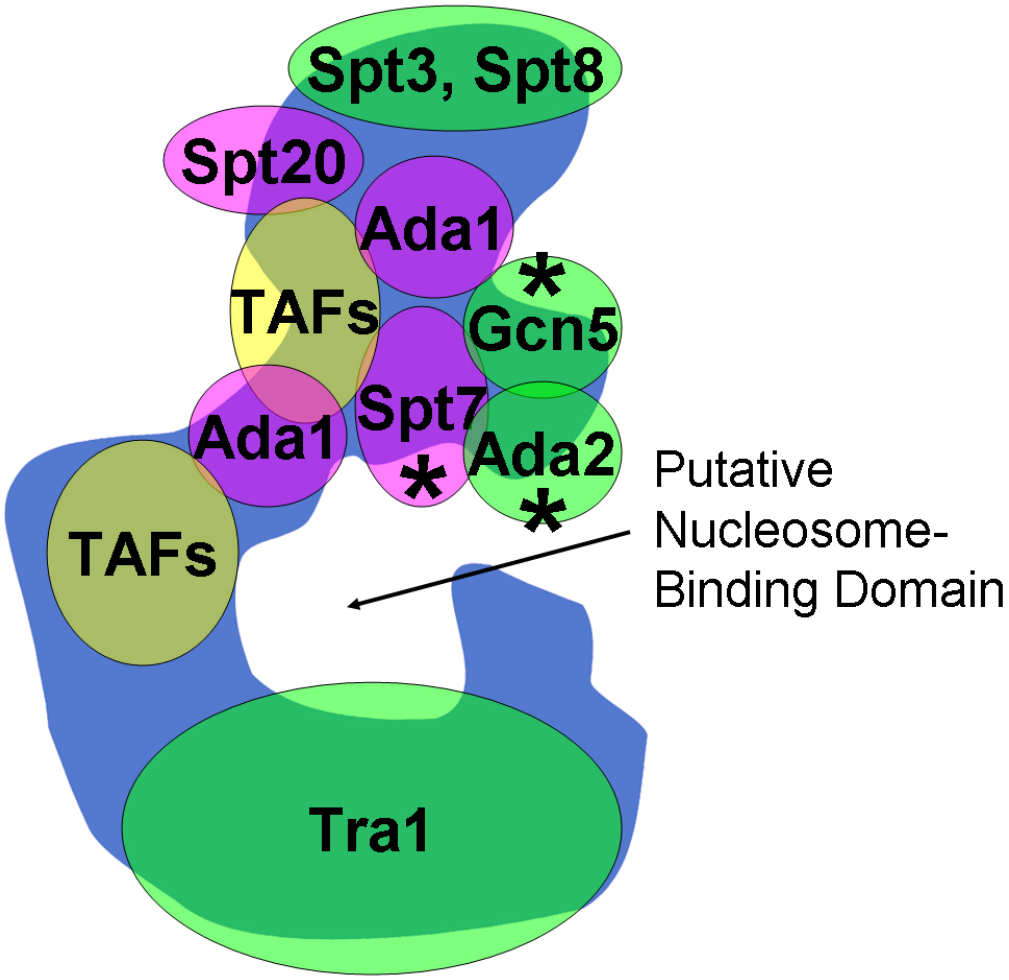

Figure 1.3 **Schematic of the *S. cerevisiae* SAGA complex.** SAGA subunits are represented as cartoons against an approximate outline of the SAGA structure as determined by electron microscopy and immunolabeling (blue). TAF subunits (orange), structural subunits (pink), and subunits involved in transcriptional regulation (green) are identified in their approximate locations within the SAGA structure. Subunits which have been implicated in gene relocation to the NPC are denoted with an asterisk. The Sus1 subunit has also been linked to gene relocation, but its location within the SAGA complex is not known. The putative nucleosome-binding domain is predicted to precisely accommodate a disk-shaped nucleosome. Figure design based on (106).

Table 1.3 **SAGA subunit homologs and functions**[a,b,c].

S. cerevisiae	**Mammalian**	**Motifs/domains**	**Function**
Ada1(Hfi1)	ADA1/*STAF42*	Histone fold	Complex stability
Ada2	ADA2b	SANT domain	Required for Gcn5 activity
Ada3	ADA3	-	
Chd1	-	Chromodomain	Recognition of histone H3 lysine 4 methylation; potentiation of histone acetylation by Gcn5
Gcn5(Ada4)	GCN5/*PCAF*	Bromodomain HAT domain	Acetylation of lysines on histones H3 and H2B; transcriptional activation; NER recognition of acetylated lysines
Rtg2	-	Actin-like ATPase domain	Stability of SAGA-like (SLIK) complex; involved in retrograde response pathway
Sgf11	ATXN7L3	Ataxin box zinc finger	Required for association of Ubp8 and Sus1
Sgf29	SGF29(*STAF36*)	-	Unknown
Sgf73(Sca7)	ATXN7	Ataxin box zinc finger	Complex stability; poly(Q) expansion inhibits nucleosomal acetylation by Gcn5
Spt3	SPT3	Histone-fold	Recruitment of TBP; transcriptional repression at specific loci in budding yeast (including *HIS3* and *ARG1)*
Stp8	-	WD40 repeats	
Spt7	SPT7/*STAF65γ*	Bromodomain Histone-fold	Complex stability
Spt20(Ada5)	SPT20/*FAM48A*/p38IP	-	
Sus1	ENY2	α-helix	mRNA export
TAF5	TAF5L	WD40 repeats	Shared components of TFIID general transcriptional factors; structural integrity, interaction with basal transcription machinery
TAF6	TAF6L	Histone-fold	
TAF9	TAF9/*TAF9b*	Histone-fold	
TAF10	TAF10	Histone-fold	
TAF12	TAF12	Histone-fold	
Tra1	TRRAP	Armadillo repeat	Interaction with transcriptional activators
Ubp8	USP22	UCH domain	De-ubiquitination of histone H2B lysine 123; transcriptional activation

Table compiled from (107, 108).

[a]Homologous proteins encoded by paralogous genes are separated by a forward slash. Alternative names for the same protein are shown in parentheses.

[b]A dash (-) indicates that no homolog/domain has been identified.

[c]Abbreviations: HAT, histone acetyltransferase; SANT, Swi3-Ada2-NcCor-TFIIIB; UCH, ubiquitin carboxy-terminal hydrolase

recruitment of the basal transcription machinery *via* the Spt3 and Spt8 subunits (98, 99, 109, 110). SAGA-regulated genes comprise approximately 10% of the yeast genome, including many highly induced and stress-responsive genes (111, 112). Multiple SAGA subunits have been implicated in the relocation process including Ada2, Sus1, and Spt7 (60, 113). Consistent with the fact that Gcn5 activity is dispensable for *GAL* activation, Gcn5 is not required for *GAL* relocation to the NPC, and it appears to be the presence of the SAGA complex rather than its acetyltransferase activity that is required for relocation of active *GAL* genes (60).

Other factors have also been implicated in the relocation of the *GAL* genes to the NPC, suggesting that multiple levels of regulation mediate gene relocation. These factors include the mRNA export factors Mex67, Sac3, and the Sus1 protein, which is also a component of the SAGA complex (59, 113, 114). Interestingly, although these factors are involved in mRNA export, multiple studies have found that the presence of mRNA and active transcription are not required for gene relocation (59, 75, 115), suggesting that relocation to the NPC is an extremely early event in gene activation. In addition, the Nup1, Nup2, and Mlp1 components of the NPC have been implicated in the relocation of the *GAL* genes (59, 75, 113). These interactions are mechanistically consistent with the exclusively nuclear localization of these NPC subunits (33). As Nup2 is a highly mobile component of the NPC (116, 117), it is conceivable that Nup2 may traffic within the nucleoplasm to mediate initial steps in the relocation of genes to the NPC. This potential step-wise process of locus relocation could help to explain the observations that both transcriptional activators, such as SAGA, and mRNA processing factors, such as Mex67 and Sac3, are required for *GAL* gene relocation.

An emerging model locus for the study of gene relocation is the *INO1* gene, whose relocation is dependent upon the Nup2 subunit of the NPC and two newly defined DNA elements upstream of the promoter, for which protein binding partners are yet to be identified (75, 118, 119). *INO1* encodes inositol 1-phosphate synthase, which catalyzes the rate-limiting step in inositol synthesis, and its transcription is strongly repressed in cells grown in the presence of inositol (120). The regulation of *INO1* transcription is fairly well characterized. The Ino2 and Ino4 transcriptional activators recognize the *INO1* promoter (121) and, in the absence of environmental inositol, recruit the INO80 chromatin remodeling complex to activate *INO1* transcription (122). INO80 is an evolutionarily conserved chromatin remodeling complex which remodels nucleosomes at promoters of regulated genes (123-127) (Table 1.4).

Although INO80 has not been directly implicated in relocation of *INO1* to the NPC, there are hints that INO80 may play a role in DNA transactions at the NPC. First, persistent DNA double strand breaks (DSBs) are relocated to the NPC (76, 77), and INO80 plays a critical role in repair of DSBs (128-132). In addition, INO80 subunits show synthetic genetic interactions with components of the NPC (Chapter 4), suggesting that these two complexes share functional roles (133). Although the mechanisms of *INO1* relocation to the NPC remain to be elucidated, *INO1* may be a particularly useful locus for the study of gene relocation as its dynamics at the NPC are intriguingly distinct from those of the *GAL* genes. Specifically, while both the *GAL* genes and *INO1* remain associated with the NPC following transcriptional shutoff, this retention is associated with more rapid re-induction for the *GAL* genes and slower re-induction for *INO1* (75).

Table 1.4 **INO80 subunit homologs and functions[a,b,c].**

S. cerevisiae	**Mammalian**	**Domains/Notes**
Act1	β-actin (ACTB)	Actin
Arp4	Baf53a (Arp4, ACTL6A)	Actin-related protein; Actin-like ATPase domain
Arp5	Arp5 (ACTR5)	
Arp8	Arp8 (ACTR8)	
Ies1	-	-
Ies2	Ies2 (INO80B, PAPA-1)	Zinc finger-HIT domain
Ies3	-	-
Ies4	-	-
Ies5	-	-
Ies6	Ies6 (INO80C, c18orf37)	-
Ino80	INO80	Snf2-like ATPase
Nhp10	-	HMG type-II domain
Rvb1	RuvB-like 1 (Tip48 RUVBL1)	AAA^+ ATPase
Rvb2	RuvB-like 2 (Tip49, RUVBL2b)	
Taf14	-	YEATS domain

Table compiled from (85, 134).

[a]Alternative names for the same protein are shown in parentheses.

[b]A dash (-) indicates that no homolog/domain has been identified.

[c]Abbreviations: AAA, ATPases associated with diverse cellular activities; HIT, His-triad; HMG, high mobility group; YEATS, Yaf9 ENL AF9 Taf14 Sas5

These observations suggest that association with the NPC does not impact all genes universally and warrant the investigation of multiple model loci at the NPC.

Numerous other genes relocate to the NPC when transcriptionally active, and, similar to the *GAL* and *INO1* genes, many of these genes are highly regulated and sensitive to strong, rapid induction and repression. These loci include the α-factor induced *FIG2*, the heat shock-induced *HSP104*, the stress-induced *TSA2*, and the galactose-responsive *HXK1* and *SUC2*, (59, 61, 119, 135, 136). Though the relocation mechanisms and dynamics of these genes have not been as well investigated as the *GAL* genes, a number of different factors have been implicated in their relocation to the NPC including gene-specific transcription factors and elements of the mRNA export machinery. Given the myriad of factors implicated in relocating different genes to the NPC, it is likely that different relocation mechanisms exist for distinct genes in a manner similar to regulation by different transcription factors and co-activators. This possibility is supported by several additional observations. First, altering either the promoter or the 3' end of a gene can dictate whether that gene is relocated to the NPC when active (135, 137). In addition, induction of specific transcriptional programs results in genome-wide changes in NPC association, consistent with a model where these changes occur as a result of interactions between the NPC and the transcriptional regulators specific to these genes (61). Finally, three distinct DNA elements are associated with relocation to the NPC. Genome-wide analysis found that DNA sequences recognized by the transcriptional regulator, Rap1 (138, 139), are enriched in genes which associate with the NPC (62, 140). Recently, two newly defined, evolutionarily conserved DNA elements, termed gene recruitment sequences (GRSs) were found upstream of *INO1* and *TSA2*, both

of which relocate to the NPC when active, and these elements are both necessary and sufficient for gene relocation (119). Though the regulatory factors which recognize these sequences have yet to be identified, the identification of these DNA elements suggests there are as yet unrecognized gene-specific mechanisms of locus relocation.

In addition to interacting with actively transcribed genes, there is emerging evidence that the NPC is also associated with repair of DNA damage. Though the physiological relevance is unknown, several studies have revealed that persistent, DSBs relocate to the NPC (76, 77). This relocation requires multiple components of the DSB repair pathway, including the Mec1 and Tel1 DNA damage checkpoint kinases, the recombination factors Rad51 and Rad52, the chromatin remodeling complex Swr1, and SUMOylated histone variant H2A.Z (76, 77). As Rad51 and Rad52 are essential for repair by homologous recombination (141), and Swr1 has been implicated in repair by non-homologous end joining (132), the requirement for these multiple factors suggests that relocation to the NPC may be a general feature of DSB repair rather than specific to one repair pathway. Interestingly, H2A.Z is also required for retention of the *INO1* locus at the NPC following transcriptional shutoff (75), suggesting that some level of conservation exists between the mechanisms of locus association with the NPC for either expression or repair.

Physiological Relevance of Gene Interactions with the NPC

The physiological relevance of locus relocation to the NPC largely remains an open question. Certainly NPC association is not required for transcriptional activation, as

normally-relocated genes can be activated without interacting with the NPC (59, 60, 113). However, different studies have reached conflicting conclusions regarding the relationship between gene relocation and transcript level regulation of those genes. In two studies, impairment of gene relocation did not impact *GAL* induction or had only minimal impact on transcript levels (60, 113). In contrast, *INO1* and *HXK1* require relocation to the NPC for full induction (118, 119, 135). Studies in *Drosophila* reveal that NPC components are required to upregulate transcription of developmental and cell-cycle regulation genes, although these interactions mostly occur in the nucleoplasm rather than at the NPC (92-94).

While it is unresolved as to whether relocation to the NPC directly affects transcript levels, recent evidence indicates that gene association with the NPC mediates transcriptional memory of recently activated genes (75, 142). This transcriptional memory, which is dependent upon both retained association with the NPC following transcriptional shut-off and the persistence of a gene loop, facilitates a more rapid re-activation of relocated genes (75, 142, 143). Gene loops are formed during active transcription based on physical interactions between the general transcription machinery and mRNA processing factors, resulting in a looped chromatin structure (144). In addition, the Mlp1 subunit of the NPC plays a direct role in gene loop maintenance, as cells lacking *MLP1* can initiate but not maintain gene loops following transcriptional shutoff (142). Interestingly, not all genes form gene loops, and the lack of this chromatin structure correlates with an absence of transcriptional memory even though such genes are retained at the NPC following transcriptional repression (75, 142). Although more studies are needed to fully elucidate these mechanisms, taken together, these observations

suggest that the NPC may play two distinct roles in gene expression: regulating transcript levels and mediating transcriptional memory of relocated genes.

To date, most studies of gene relocation to the NPC have focused on specific highly regulated loci such as the *GAL* and *INO1* genes. However, there are intriguing suggestions that gene relocation to the NPC may be a global phenomenon. First, genome-wide studies have found that highly transcribed genes are more likely to associate with the NPC than genes transcribed at lower levels, (62), and these gene-NPC associations globally change upon induction of a differential transcriptional program (61). In addition, a sophisticated study of the accessibility of endogenous chromatin to cleavage by micrococcal nuclease-fused Nup2 suggests that the majority of promoters in the yeast genome may be capable of interacting with the NPC (115). However, as Nup2 is a mobile subunit of the NPC (116, 117), it is possible that at least some of these interactions occur in the nucleoplasm as was recently demonstrated for many *Drosophila* genes (93, 94). These observations suggest a model whereby the mobile Nups, such as Nup2, identify nucleoplasmic genes which are poised for relocation and subsequently mediate their relocation to the NPC.

The recent finding that persistent DNA DSBs relocate to the NPC suggests a role for the NPC in DNA damage repair; however, the physiological relevance of this relocation is unexplored. One possibility is that relocating persistent DSBs to the NPC is a tolerance mechanism whereby the cell attempts to treat a DSB analogously to telomeres, which associate with the nuclear periphery (145). However, the observation that the Ku complex, which is required for telomere anchoring (146), is dispensable for DSB relocation (76), suggests that this tolerance hypothesis is likely incorrect. Another

possibility is that the NPC may be DNA damage repair center, consistent with the observation that the Ulp1, Slx5, and Slx8 DNA damage response factors associate with the NPC (76, 81-83). Although the physiological relevance of this phenomenon remains to be determined, together the observations of relocation of both active genes and DSBs to the NPC suggest that NPC may be site which facilitates open chromatin structure permissible to DNA transactions necessary for high levels of transcription and DNA damage repair.

Scope of this Dissertation

As described above, the nucleus is functionally organized by the chromosomes, the nuclear periphery, and regulation of transcription. The NPC plays a very recently-recognized and largely unanticipated role in transcriptional activation. At the time we began this investigation, several studies had determined that multiple genes relocate to the nuclear periphery when transcriptionally active, where they associate with the NPC (61, 62, 113, 115, 135). Given the location of the Mlp proteins at the nuclear basket of the NPC (147), we hypothesized that the factors which relocate active genes to the NPC would be likely to directly associate with the Mlp proteins. Using a combination of protein purification and *in vitro* binding assays, we show in Chapter 2 that the SAGA complex, which regulates transcription of the *GAL* genes (97-99), physically interacts with the Mlp1 subunit of the NPC. In addition, ChIP analysis at the *GAL1* and *GAL2* loci reveals that Mlp1 associates with the same region of the *GAL* genes to which SAGA binds during *GAL* gene activation (98, 99). Moreover, using ChIP and quantitative real-

time PCR (QRT-PCR) approaches, we demonstrate that the association of Mlp1 with the active *GAL* genes is dependent on the physical presence of SAGA, and not the transcriptional activity of the *GAL* genes. Taken together, these results suggest that relocation of active genes to the NPC depends upon physical interactions between NPC subunits and the factors which regulate transcription of those genes.

Though the mechanisms of gene relocation have been fairly well characterized for specific genes such as the *GAL* loci, the physiological significance of this phenomenon is still largely undetermined. Specifically, both the global relevance of locus relocation and the relationship between relocation and transcript levels remain to be fully elucidated. Given that highly transcribed genes are more likely to associate with the NPC than those expressed at lower levels, and that all gene promoters may occasionally contact the NPC (62, 115), we hypothesized that relocation to the NPC may be a global phenomenon which regulates transcription of highly transcribed genes. Using a genetic approach in order to gain insight into these unresolved questions, we report in Chapter 3 functional interactions between multiple genes encoding NPC subunits and components of the SAGA complex as indicated by growth defects in cells lacking these NPC and SAGA components. FISH analysis to localize total poly(A) RNA in these cells reveals that these growth defects are not due to grossly impaired mRNA export. Microarray analysis indicates that these growth defects correlate with genome-wide changes in transcript levels. In particular, many genes that are highly transcribed in wildtype cells have significantly reduced transcript levels in these double mutant cells, consistent with a role for locus relocation in regulating highly transcribed genes. Finally, a combination of ChIP and single-locus time-lapse imaging in live cells (148, in preparation) reveal that

while cells lacking NPC or SAGA subunits can recruit the *GAL1* locus to the NPC, these cells appear to be defective in retaining the *GAL1* locus at the NPC. Moreover, cells lacking both NPC and SAGA subunits show an enhanced relocation defect compared to single mutant cells, and this defective relocation correlates with reduced fitness when these cells are grown with galactose as the sole carbon source. Taken together, these results not only suggest that gene relocation to the NPC includes distinct steps, perhaps requiring distinct factors, in relocation and retention, but also that gene relocation is a primary mechanism of global transcriptional regulation.

In addition to interacting with actively transcribed genes, the NPC also associates with persistent DSBs (76, 77). The physiological relevance of this phenomenon is essentially unknown. Given our finding that functional interactions between the NPC and SAGA regulate genome-wide transcript levels described in Chapter 3, we hypothesized that interactions between the NPC and chromatin modifying complexes (CMCs) that regulate DNA damage repair would be important in managing DNA damage response. Using a genetic approach, we identify in Chapter 4 functional interactions between the NPC and the INO80 complex, a complex that regulates transcription as well as repair of DSBs (123-132). These functional interactions are revealed by cell growth defects in cells lacking NPC components and mutant for the INO80 complex. Moreover, these cells, as well as cells lacking NPC subunits in combination with SAGA subunits, show increased sensitivity to various DNA damaging agents compared to single mutant cells, suggesting that interactions between the NPC and CMCs are important for mediating DNA damage repair. Finally, utilizing a Rad52-YFP assay to quantify endogenous DSBs, we find that cells lacking a SAGA component in combination with an

NPC subunit show significantly elevated levels of DSBs. These results suggest that interactions between the NPC and SAGA are important in mediating DNA damage repair and implicate the SAGA complex in repair of DSBs. Taken together, these results suggest a role for CMCs in creating an environment at the NPC which facilitates open DNA transactions such as transcription and repair of DNA damage.

These findings lead to several important conclusions regarding the relocation of active genes to the NPC. First, the association between active genes and the NPC is mediated by physical interactions between NPC subunits and gene-specific transcriptional regulators. Second, gene relocation consists of two stages: initial recruitment and subsequent retention, and interactions between multiple factors mediate these steps. Third, interactions between CMCs and the NPC play a critical role in the regulation of global transcript levels, particularly for highly transcribed genes. Fourth, interactions between CMCs and the NPC are also important in the repair of DNA damage. Taken together, these results not only reveal mechanistic details for gene relocation to the NPC, but also uncover the physiological relevance of this recently recognized and potentially evolutionarily conserved phenomenon. Moreover, these results are consistent with a model where chromatin associates with the NPC *via* interactions between CMCs and specific Nups, and this association promotes an open chromatin structure permissive to DNA transactions at the NPC.

CHAPTER 2

Actively Transcribed *GAL* Genes can be Physically Linked to the Nuclear Pore by the SAGA Chromatin Modifying Complex

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Tandem affinity purification (TAP) tag purification and chromatin immunoprecipitation experiments were performed by Roopa Luthra, Suneela Ramineni, and Shyam Chaurasia. *In vitro* binding assays were performed by Luciano H. Apponi.

Introduction

Chromatin in the interphase nucleus is highly organized into discrete chromosome territories (10, 149), and the position of a particular genetic locus both within a chromosome territory and within the nucleus can influence its transcriptional state (150, 151). In the yeast *Saccharomyces cerevisiae*, specific genetic loci physically relocalize from the nuclear interior to the nuclear periphery upon transcriptional activation (61, 62, 113, 115, 118, 135). These transcribed genes interact with components of the nuclear pore complex (NPC) (61, 62, 113, 115, 135), perhaps facilitating efficient mRNA processing and export. Although this phenomenon of locus association with the nuclear periphery has been reproducibly observed in yeast (61, 62, 113, 115, 118, 135), critical questions remain unanswered regarding the mechanism of locus recruitment to the NPC. Transcription factors, chromatin modifying complexes, and the transcription machinery itself have each been independently implicated in directing active loci to the NPC (90, 113, 115, 135, 152), perhaps suggesting a recruitment mechanism dependent upon transcription initiation. In addition, some interactions between components of the NPC and active loci are RNA-dependent (61), and mRNA processing and export factors have also been implicated in tethering loci to the NPC (59, 113, 114), suggesting that the interaction between active genes and the NPC may instead be dependent upon ongoing transcription and mRNA maturation. Recent, independent studies of *GAL* and *HXK1* recruitment have yielded different results regarding the role of the transcription machinery and transcriptional co-activators in this process (113, 115, 135), raising the possibility that distinct mechanisms of recruitment may operate for individual loci.

Notably, one caveat of these studies is the predominant use of microscopy to assess locus recruitment to the nuclear periphery, without biochemical confirmation of a physical association between a recruited locus and the NPC.

To further characterize the mechanism of gene recruitment to the NPC, we sought to identify proteins which physically interact with NPC components that had been linked to actively transcribed loci. These components of the NPC include the Mlp proteins, Nic96, Nup1, Nup2, Nup60, and Nup116 (61, 62, 113). We focused on the two Mlp proteins as they are localized to the nuclear basket of the NPC (147), have roles in mRNA processing and export (57, 58, 153), and have been implicated in the recruitment of multiple and diverse genes including the *GAL* genes, α factor-responsive genes, and many other genes which are highly expressed under normal growth conditions (61, 62). The two evolutionarily conserved Mlp proteins, Mlp1 and Mlp2, are large (218 kD and 195 kD, respectively), coiled-coil domain proteins localized to the nuclear side of the NPC where they are hypothesized to form the intranuclear filaments of the inner basket of the NPC (147). The Mlp proteins, which are 66% similar, consist of extensive N-terminal coiled-coil domains and globular acidic carboxy-terminal domains (147), the latter of which have been implicated in mRNA export and quality control via interactions with mRNA export factors (57, 58, 153, 154). Several observations have suggested that the Mlp proteins are linked to actively transcribed genes (61, 62, 90, 154, 155). First, Mlp1 and Mlp2 interact with highly transcribed loci in genome-wide chromatin immunoprecipitation (ChIP)-chip experiments (62). In fact, a recent study demonstrated a requirement for Mlp1 in *GAL* locus recruitment to the nuclear periphery (59). Second, Mlp1 has been indirectly linked to the Spt-Ada-Gcn5-Acetyltransferase (SAGA) histone

acetyltransferase complex through interactions between the Sac3-Thp1 mRNA export complex and the Sus1 component of SAGA (154, 155). Finally, the *Drosophila* Mlp homolog, Mtor, was found to be required for proper localization of the MSL histone acetyltransferase complex to the male X chromosome for transcription upregulation of X-linked genes on the single copy of the male X chromosome (90).

In this study, we expressed the C-terminal globular domain of Mlp1 as a tandem affinity purification (TAP)-tagged protein in yeast, purified the associated proteins, and identified these proteins via mass spectroscopy. With this approach, we identified three components of the evolutionarily conserved SAGA complex, a histone acetyltransferase complex which regulates transcription of approximately 10% of the yeast genome (111, 112) including the *GAL* genes (97-99). SAGA interacts with gene-specific transcriptional activators that recruit SAGA and additional transcription machinery to the promoters of target genes (156). In the case of the *GAL* genes, SAGA is recruited to the *GAL* Upstream Activating Sequence (UAS) by the constitutively bound Gal4 protein when cells are grown in the absence of glucose and the presence of galactose (157). The SAGA complex then facilitates recruitment of the general transcription factors and RNA polymerase II (157). As the SAGA complex regulates transcription of the *GAL* genes (97-99), and the *GAL* genes relocalize to the nuclear periphery upon transcriptional activation (62), we hypothesized that a physical interaction between Mlp proteins and the SAGA complex might mediate the recruitment of the *GAL* genes to the NPC. We verified the interaction between the Mlp proteins and SAGA components, and we found that both Mlp1 and Mlp2 interact with the *GAL* upstream activating sequence (UAS), the region of the *GAL* genes which interacts with the SAGA complex. This interaction

between the Mlp proteins and the *GAL* UAS only occurs in the presence of galactose, when the *GAL* genes are active, and is dependent upon the integrity of the SAGA complex. These results suggest that the SAGA histone acetyltransferase complex at the *GAL* UAS may help to direct active *GAL* genes to the NPC, lending support to a model where chromatin modifying complexes facilitate interactions between the NPC and actively transcribed genes.

Results

Previous studies have revealed that the Mlp proteins interact with actively transcribed loci at the NPC (61, 62). To further examine the interactions between the Mlp proteins and genetic loci, we expressed the C-terminal globular domain of Mlp1 in yeast cells as a C-terminally tandem affinity purification (TAP)-tagged protein. We then purified the CT-Mlp1-TAP protein through a standard two step TAP purification (158) and identified specific bound proteins through mass spectrometric analysis of protein bands that were present in the bound fraction for CT-Mlp1-TAP, but not in the bound fraction for lysate from control cells expressing only the TAP tag. This analysis revealed a number of proteins that have previously been identified as Mlp-interacting proteins, including Nab2 (153). Among the other proteins identified in this analysis, we found three members of the SAGA histone acetyltransferase complex: the catalytic subunit, Gcn5 (103), a modulator of acetyltransferase activity, Ada2 (102-104), and a structural component, Spt7 (159). Recent structural analysis reveals that these members of the SAGA complex are all located in close proximity to one another in the complex within a

region designated Domain III (106). The identification of three members of the SAGA complex, which are located within the same subcomplex, led us to hypothesize that Mlp1 could interact with the core components of the SAGA complex.

To confirm the results of our global binding analysis, we carried out several co-purification experiments to examine the interactions of Mlp1 and the homologous protein, Mlp2, with components of the SAGA complex. We first examined the interaction between the C-terminal domain of the Mlp proteins and the SAGA component, Gcn5. Each CT-Mlp was fused to GST and expressed from a galactose-inducible promoter in yeast cells that express a C-terminally TAP-tagged Gcn5 protein from the *GCN5* genomic locus. Following induction, the yeast cells were lysed and the GST fusion proteins were purified on glutathione beads. The bound fractions were washed then analyzed by immunoblotting and probed with PAP antibody to detect TAP-tagged Gcn5. As shown in Figure 2.1*A*, Gcn5 is enriched in the bound fraction (B) of the GST-CT-Mlp1 and GST-CT-Mlp2 samples but not in the GST alone control sample. A control TAP-tagged protein did not co-purify with any of the GST proteins (data not shown). To address the question of whether Gcn5 can also interact with the full length Mlp proteins, we expressed Gcn5-GFP in yeast strains expressing genomic C-terminally TAP-tagged Mlp1, Mlp2, or the control TAP tag alone. Cells were grown to log phase and then lysed, and the TAP-tagged proteins were purified on IgG beads. The lysate (L), unbound (U), and bound (B) fractions were analyzed by immunoblotting and probed for the Gcn5-GFP fusion protein (Figure 2.1*B*). Results show that Gcn5-GFP is enriched in the bound fraction with both Mlp1-TAP and Mlp2-TAP, but not with the TAP tag alone (Figure 2.1*B*). To extend our analysis to another member of the SAGA complex, we next

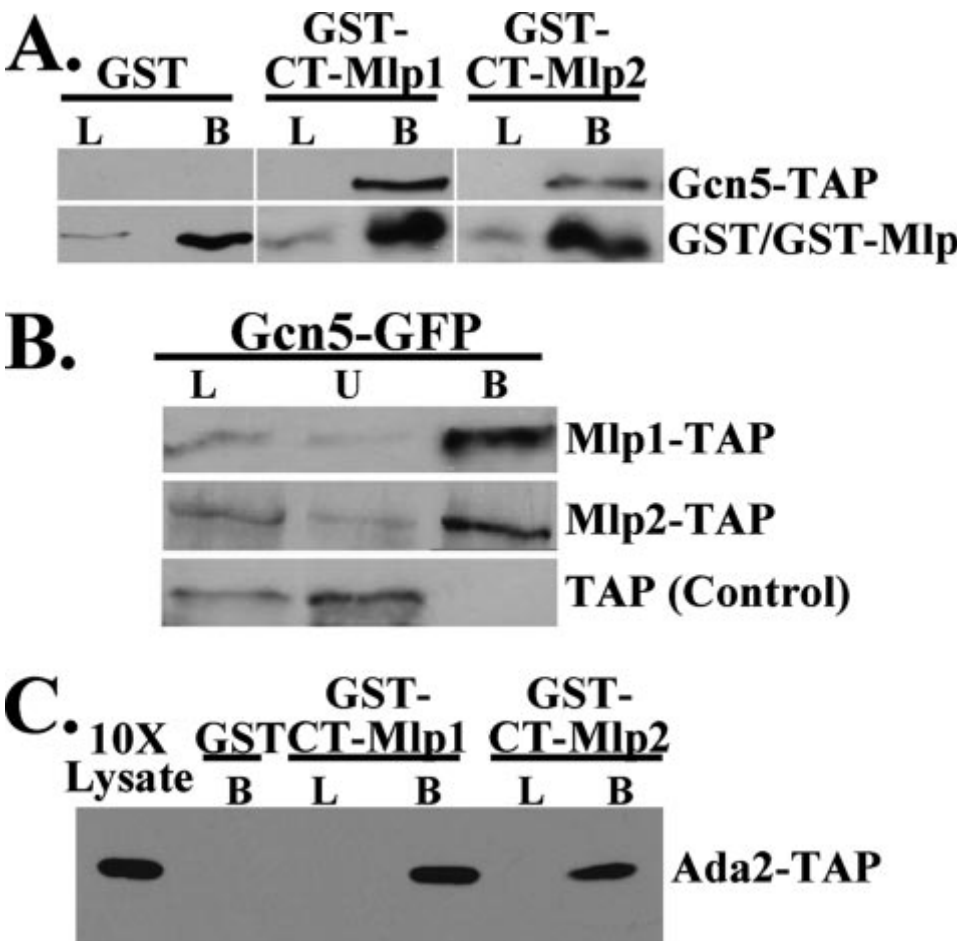

Figure 2.1 **The Mlp proteins interact with components of the SAGA complex.** ***A,*** Gcn5-TAP interacts with the C-terminal domains of both Mlp1 and Mlp2. Yeast cells encoding a C-terminal TAP tag at the *GCN5* open reading frame were transformed with galactose-inducible plasmids encoding GST fusion proteins, consisting of GST fused N-terminal to the C-terminal domain (amino acids 1490-1875) of Mlp1 or the C-terminal domain of Mlp2 (amino acids 1411-1679) or the control GST alone. Cell lysates were prepared from cultures induced with galactose and the GST-fusion proteins were purified as described in Experimental Procedures. A sample (10 μg total protein) of the input lysate (L) and the fraction bound to the GST protein (B) were analyzed by immunoblotting with an anti-PAP antibody, which detects the TAP tag on the Gcn5

protein (Gcn5-TAP). The amount of GST fusion protein in the bound lysate was also analyzed by immunoblotting with an anti-GST antibody (GST). Note that Gcn5-TAP, which is not detectable in cells lysates, is concentrated in the bound samples for the two Mlp proteins, but not for GST alone. ***B,*** Gcn5-GFP co-immunoprecipitates with full-length Mlp proteins. Cells expressing genomically tagged Mlp1-TAP or Mlp2-TAP were transformed with a plasmid expressing Gcn5-GFP. The TAP-tagged proteins were then purified from cell lysates as described in Experimental Procedures, and the bound fraction was analyzed for co-purifying Gcn5-GFP. The input lysate (10 μg total protein) (L), the unbound (U), and the bound (B) fractions were probed with an anti-GFP antibody. Gcn5-GFP is enriched in the bound lane with both Mlp1-TAP and Mlp2-TAP. As a control, the TAP tag alone does not interact with Gcn5-GFP. ***C,*** Ada2 interacts with the C-terminal domain of the Mlp proteins. Yeast cells where the genomic *ADA2* open reading frame was tagged at the C-terminus with TAP were transformed with galactose-inducible plasmids encoding GST-CT-Mlp1, GST-CT-Mlp2, or control GST alone. Cells were induced with galactose, lysed, and the GST fusion proteins were purified using glutathione beads. Ada2-TAP was detected with an anti-PAP antibody. Using this antibody, Ada2-TAP cannot be detected in the lysate (L) when 10 μg of total protein is loaded; however, it is enriched in the bound fraction (B) for both CT-Mlp1 and CT-Mlp2. No Ada2-TAP was detected in the bound fraction for the GST control protein. The left lane (10X lysate) shows that Ada2-TAP can be detected in cell lysate when 10-fold more lysate (100 μg total protein) is analyzed.

examined the interaction between the Mlp proteins and Ada2. Each GST-CT-Mlp protein was expressed in yeast cells that express C-terminally TAP-tagged Ada2. Cells were grown to log phase, lysed, and GST fusion proteins were purified on glutathione beads. Figure 2.1*C* shows that Ada2-TAP is enriched in the bound fraction (B) of both GST-CT-Mlp1 and GST-CT-Mlp2 samples but not the control GST protein. These results confirm that components of the SAGA complex can be co-purified with the Mlp proteins.

Since both the Mlp proteins and SAGA are implicated in gene expression (61, 62, 111, 112) and aspects of mRNA transcription and/or export (57, 58, 153, 155), one possibility is that the interaction between the Mlps and SAGA components is mediated by either DNA or RNA. To address this point, we repeated the co-purification of GST-CT-Mlp proteins with Gcn5-TAP and treated samples with DNase and/or RNase before analyzing the bound samples (Figure 2.2*A*). Our results indicate that treatment with DNase, RNase, or both did not affect the interaction between Gcn5-TAP and GST-CT-Mlp1 (Figure 2.2*A*) or GST-CT-Mlp2 (data not shown). The results of this experiment indicate that the interaction between Gcn5 and Mlp proteins does not depend on associated RNA or DNA, suggesting that this is a protein-protein interaction. In order to investigate whether the Mlp proteins could interact directly with components of the SAGA complex, we performed *in vitro* binding assays with Mlp proteins and the Gcn5 subunit of the SAGA complex (Figure 2.2*B*). Purified, recombinant GST-CT-Mlp1, GST-CT-Mlp2, GST alone (negative control), or Gst-Ada2 (positive control) was bound to glutathione-Sepharose beads and incubated with purified, recombinant His-Gcn5. Results of this experiment indicate that the GST-CT-Mlp1 and GST-CT-Mlp2 can

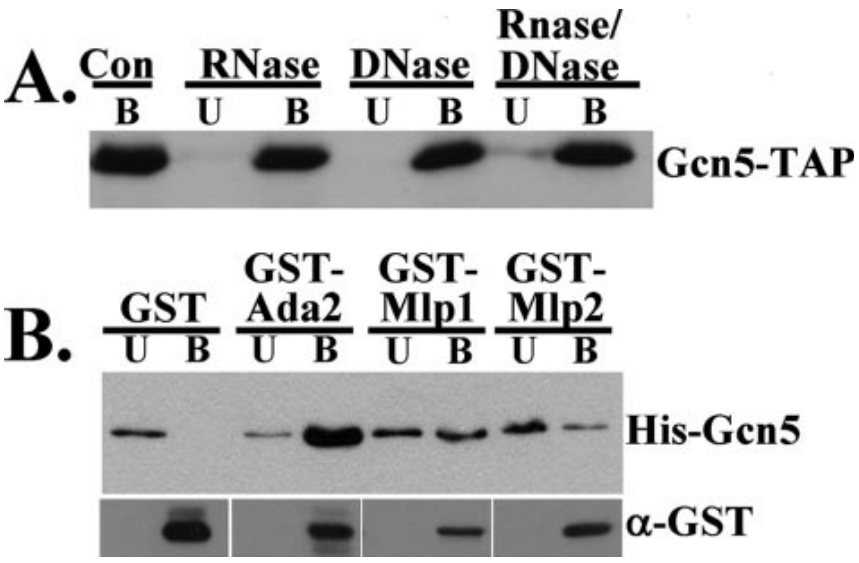

Figure 2.2 **The Mlp proteins can interact directly with Gcn5**. ***A,*** The interaction between Gcn5 and the Mlp proteins does not depend on DNA or RNA. Yeast cells expressing Gcn5-TAP were transformed with the galactose-inducible GST-CT-Mlp1 plasmid. Expression of GST-CT-Mlp1 was induced with galactose and cell lysates were prepared. Prior to purification of GST-CT-Mlp1, lysates were treated with RNase, DNase, both RNase and DNase, or were left untreated as a control (Con), as described in Experimental Procedures. GST-CT-Mlp1 was then purified with glutathione beads and the bound fractions were analyzed with anti-PAP to detect co-purification of Gcn5-TAP. Gcn5-TAP can be detected in the bound fraction for GST-CT-Mlp1 under all conditions. ***B,*** *in vitro* binding assay. Purified recombinant GST-CT-Mlp1, GST-CT-Mlp2, GST-Ada2, or GST alone was bound to glutathione Sepharose beads and incubated with purified recombinant His-Gcn5. Samples were washed extensively, and bound and unbound fractions were analyzed with anti-His to detect co-purification of His-Gcn5 (upper panel). His-Gcn5 can be detected in the bound fractions for GST-CT-Mlp1, GST-CT-Mlp2, and the control protein GST-Ada2, but not GST alone. Purified GST and GST-fusion proteins were detected with anti-GST (lower panel).

directly interact with His-Gcn5, while His-Gcn5 does not interact with the control GST alone. However, the binding between CT-Mlp and Gcn5 is not as robust as the interaction between Gcn5 and the positive control, Ada2, another component of the SAGA complex which directly interacts with Gcn5 (160). The relatively weak interaction between the Mlp proteins and Gcn5 may suggest that Gcn5 participates in the association between the SAGA complex and the Mlp proteins, but that additional members of the SAGA complex or the intact complex itself are required for a robust interaction between SAGA and the Mlp proteins.

Taken together, these results indicate that Mlp proteins, which are located at the nuclear face of the nuclear pore complex, can interact with components of the SAGA complex. This finding, in combination with published work showing that the Ada2 component of the SAGA complex is required for localization of the active *GAL* genes at nuclear pores (113), raises the possibility that this interaction between the Mlp proteins and the SAGA complex could contribute to the recruitment of transcriptionally active SAGA-dependent *GAL* genes to the nuclear periphery. If SAGA does mediate the interaction of Mlp proteins with chromatin, we would hypothesize that the Mlp proteins should interact with the same regions of the chromatin as the SAGA complex. Regulation of the *GAL* genes has been extensively studied, and the SAGA complex interacts with the well-defined *GAL* upstream activating sequence (UAS) but not the TATA box within the core promoter (98, 99). Thus, we hypothesized that the Mlp proteins would interact with *GAL* UAS but not the TATA box. To test this hypothesis, we used ChIP to analyze the interaction of the Mlp proteins with the UAS and the TATA box of *GAL1/10* and *GAL2* promoters, which are schematized in Figure 2.3*A*.

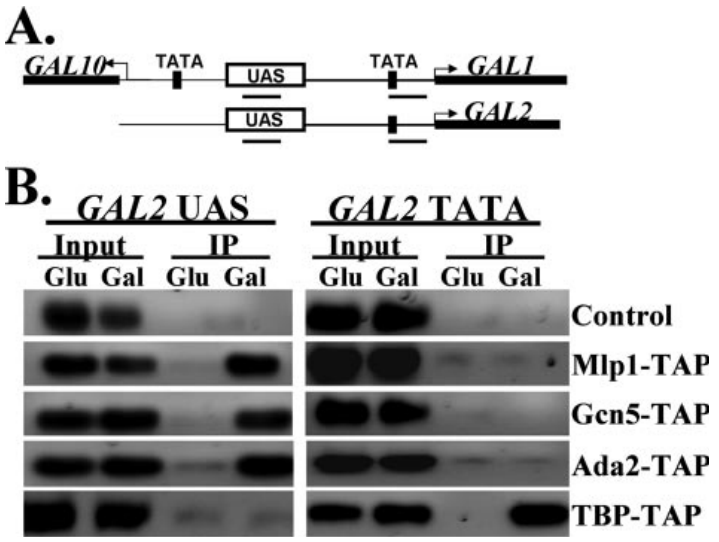

Figure 2.3 **Mlp1 interacts with the upstream activating sequence (UAS) within the GAL genes.** ***A,*** Schematic of the *GAL1/10* and *GAL2* loci. *GAL1/10* is located on chromosome II and *GAL2* is located on chromosome XII. The schematic indicates the position of the UAS and the TATA box. The regions amplified by the UAS-specific and TATA box-specific primers are indicated by the lines below the schematic. ***B,*** ChIP assay to detect Mlp1 interaction with the *GAL2* locus. Yeast cells expressing genomically encoded TAP-tagged Mlp1, Gcn5, Ada2, or TATA binding protein (TBP) or control cells with no TAP tagged protein were analyzed by ChIP as described in Experimental Procedures. Cells were either grown in glucose (Glu) where the galactose genes are not induced or galactose (Gal) where the galactose genes are induced. Primers were designed to detect either the *GAL2* UAS (left panel) or the *GAL2* TATA box (right panel). The total input sample (Input) or the fraction of the TAP-tagged protein immunoprecipitated with IgG beads (IP) was analyzed by PCR. Mlp1 and SAGA components interact with the *GAL2* UAS while TBP does not. In contrast, TBP interacts with the TATA box but neither the SAGA components nor Mlp1 interact with this region.

We first tested whether Mlp1 interacts with the UAS region of both the *GAL1/10* and *GAL2* promoters. Figure 2.3*B* (*left panel*) shows that the *GAL2* UAS can be immunoprecipitated with Mlp1 in this assay. Similar results were obtained for the *GAL1/10* locus (data not shown). As controls, both Gcn5 and Ada2, components of the SAGA complex (103), could also immunoprecipitate the UAS. In contrast, TATA binding protein, TBP, which binds to the TATA box (161, 162) and not the UAS, did not immunoprecipitate the UAS region. As a control for the specificity of this interaction, we next examined whether Mlp1 interacts with the *GAL2* TATA box, which is located ~230 base pairs from the *GAL2* UAS. Results show that neither the SAGA components, Gcn5 and Ada2, nor Mlp1 interact with the TATA region (Figure 2.3*B*, *right panel*). As a control, TBP does bind to the TATA region. These results suggest that Mlp1 and the components of the SAGA complex bind to the same region of the chromatin upstream of *GAL1/10* and *GAL2*.

In order to test whether the interaction of Mlp1 with the *GAL* UAS depends on the SAGA complex, we examined this interaction in cells that lack the SAGA complex. To disrupt the SAGA complex, we deleted the *SPT7* gene, which encodes a protein required for the structural integrity of the SAGA complex (159). However, disruption of the SAGA complex due to the deletion of the *SPT7* gene also results in a severe slow-growth phenotype when galactose is the sole carbon source (159) and decreased recruitment of TBP to the *GAL1* promoter (97), suggesting that transcription of the *GAL* loci might be decreased in *spt7*Δ cells. Thus a decreased association between the *GAL* loci and the Mlp proteins in *spt7*Δ cells could be due to either the physical absence of the SAGA complex at the *GAL* UAS or to a decrease in transcription of the *GAL* genes. In order to

differentiate between these two possibilities, we used semi quantitative RT-PCR to analyze *GAL* transcript levels in *spt7*Δ cells and identify conditions under which *GAL* transcript levels could recover to near wildtype levels. As shown in Figure 2.4*A*, when *spt7*Δ cells were permitted to grow to an optical density (OD) of 0.9, the steady-state level of the *GAL1* transcript in *spt7*Δ cells was approximately equal to the level in wildtype cells grown to the same OD. Quantitation of RT-PCR data revealed only ~20% decrease in *GAL1* or *GAL2* transcript levels in *spt7*Δ cells as compared to wildtype cells grown under these conditions (data not shown), suggesting that the level of transcription of these loci does not differ significantly in the absence or presence of SAGA when the cells are permitted to grow to this density. To further confirm that *GAL* transcript levels are similar under the conditions analyzed, we used quantitative real time PCR (qRT-PCR) to assess *GAL* transcript levels in wildtype, *spt7*Δ, and *spt20*Δ cells at early (0.3) and late (0.9) ODs. We included *spt20*Δ cells in this analysis as Spt20 is another SAGA subunit that contributes to the integrity of the SAGA complex (159). The qRT-PCR experiments revealed that both *spt7*Δ and *spt20*Δ cells display a severe *GAL1* transcript defect compared to wildtype at the early OD (Figure 2.4*B*), consistent with previous reports (98, 163). However, both *spt7*Δ and *spt20*Δ cells show recovery of *GAL1* transcript levels when allowed to grow to OD 0.9, with the *GAL1* transcript in *spt7*Δ cells approaching wildtype levels (Figure 2.4*B*). Similar results were obtained for *GAL2* transcript levels (data not shown). These results are consistent with a previous report that loss of the Ada2 and Sus1 components of the SAGA complex does not drastically affect the transcript levels produced from the *GAL* genes under similar experimental conditions (113).

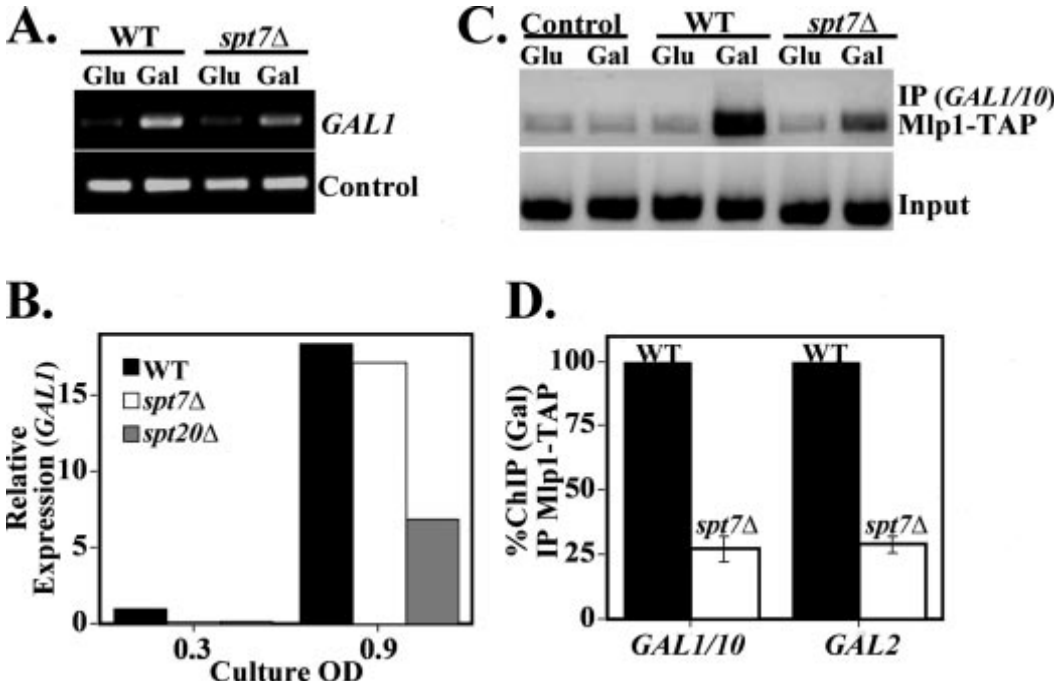

Figure 2.4 **The SAGA complex is required for proper interaction between Mlp1 and the *GAL* loci.** ***A,*** The steady-state level of the *GAL1* transcript is similar in wildtype and *spt7*Δ cells under the conditions used for the ChIP assay. RT-PCR was used to examine the expression of the *GAL1* transcript in wildtype (WT) and *spt7*Δ cells. Cells were grown in glucose (Glu) or galactose (Gal). A transcript that does not change in response to the carbon source (*YRB1*) was used as a control. Quantitation of these data revealed that *GAL1* transcript levels in *spt7*Δ cells were 81.7% ± 6.2 of wildtype. Similar results (data not shown) were obtained for *GAL2* transcript levels (76.4% ± 6.6). ***B,*** The steady-state level of *GAL1* transcript in *spt7*Δ cells recovers to levels similar to wildtype over time. QRT-PCR was used to examine the expression of the *GAL1* transcript in wildtype (WT), *spt7*Δ and *spt20*Δ cells. Cells were grown to saturation in raffinose, then diluted into galactose and grown for either a single doubling (OD 0.3) or until cultures reached a cell density of OD 0.9. RNA was prepared as described in Experimental Procedures. A transcript that does not change in response to the carbon source (*SNR17A*) was used as a

control. Data shown is representative of three independent experiments. Similar trends were observed for *GAL2* (data not shown). ***C,*** ChIP reveals that the interaction between Mlp1 and the *GAL1/10* UAS is drastically decreased in *spt7*Δ cells. Either wildtype or *spt7*Δ cells expressing Mlp1-TAP were examined by ChIP analysis. Cells were either grown in glucose (Glu) (no induction of *GAL1/10*) or galactose (Gal) (induction of *GAL1/10*) to ~OD 0.9. Primers are designed to amplify the region surrounding the *GAL1/10* UAS. The Input is shown in the bottom panel and the Mlp1 IP is in the top panel. ***D,*** Quantitation of association of Mlp1 with the *GAL* loci in *spt7*Δ cells. To quantitate the results of the ChIP analysis, bands in the linear range of PCR reactions as determined by a standard curve with known amounts of DNA were scanned by densitometry to give a semi-quantitative measure of the amount of DNA in both the Input and IP fractions for the galactose-induced sample. Results for the IP fraction were then normalized to the band obtained for the Input fraction for each sample. Results of three independent ChIP experiments were analyzed for both *GAL1/10* and *GAL2*. For each sample, the amount of ChIP obtained in the *spt7*Δ cells was calculated as a percentage of the wildtype level (set to 100%) for both *GAL1/10* (23.9% of wildtype) and *GAL2* (24.6% of wildtype). Standard deviations in the data are indicated by the error bars.

Having identified conditions that should allow us to distinguish between a requirement for SAGA-dependent transcription or the physical presence of the SAGA complex, we next employed ChIP analysis to compare the interaction of Mlp1 with either the *GAL1/10* (Figure 2.4*C,D*) or the *GAL2* UAS (Figure 2.4*D*) in wildtype versus *spt7*Δ cells, under conditions where *GAL* transcript levels were near wildtype in *spt7*Δ cells. Results indicate that the loss of the intact SAGA complex decreases the interaction of Mlp1 with the *GAL1/10* UAS significantly (Figure 2.4*C*). Quantitative analysis of the ChIP data reveals that loss of the SAGA complex leads to a greater than 75% decrease in the association of Mlp1 with either the *GAL1/10* or the *GAL2* UAS (Figure 2.4*D*). This result is consistent with previous reports that loss of components of the SAGA complex results in loss of association of active *GAL* genes with the nuclear periphery (113).

The primary catalytic function associated with the SAGA complex is the histone acetyltransferase activity of the Gcn5 component (103). Deletion of *GCN5* results in a catalytically inactive yet structurally intact SAGA complex (159, 164). In order to determine whether histone acetyltransferase activity was important to link the SAGA-dependent *GAL* loci to the nuclear pore, we tested whether deletion of *GCN5* decreases the interaction of Mlp1 with the *GAL1/10* locus using ChIP. As shown in Figure 2.5, the ChIP data revealed no significant decrease in the association of the Mlp1 with the *GAL1/10* locus upon deletion of *GCN5*. When the data were quantitated, results revealed only an ~10% decrease in the association of Mlp1 with the *GAL1/10* locus in *gcn5*Δ cells as compared to wildtype cells. These results confirm that the acetyltransferase function of SAGA is not required to link actively transcribed genes to the nuclear pore, which is consistent with previous reports that Gcn5 is not required for expression of the *GAL*

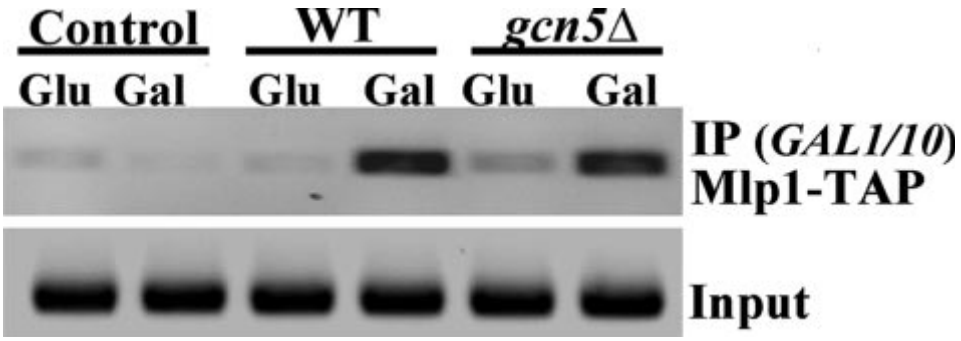

Figure 2.5 **Gcn5 is not required for the interaction between *GAL* genes and the nuclear periphery.** The ChIP interaction between Mlp1-TAP and the *GAL1/10* UAS was analyzed in wildtype (WT) cells and *gcn5*Δ cells, which both express Mlp1-TAP. As a negative control (Control), the ChIP interaction was also analyzed in cells that do not express Mlp1-TAP. Cells were grown in glucose (Glu) or galactose (Gal) and the ChIP interaction between Mlp1-TAP and the *GAL1/10* was analyzed as described in Experimental Procedures. The Input is indicated in the lower panel and the IP (*GAL1/10* UAS) is in the upper panel.

genes (97, 98), integrity of the SAGA complex (164), or visual detection of the *GAL* genes at the nuclear periphery (113).

Discussion

Our results reveal a SAGA-dependent, physical association between the Mlp proteins at the NPC and the actively transcribed *GAL* UAS, as determined by ChIP analysis. While these results support and complement previous reports that components of the SAGA complex are required for visual detection of localization of the *GAL* genes to the nuclear periphery (113), they are significant in identifying the SAGA complex as required for a physical association between NPC components and the actively transcribed *GAL* genes. Furthermore, our results define the requirement for SAGA in the interaction between the NPC and the active *GAL* genes as physical rather than functional through two separate findings. First, we determined that the acetyltransferase activity of the SAGA complex is not required for the physical association between the Mlp proteins and the *GAL* genes, which is consistent with previous reports (113). Second, we found that the physical interaction between the Mlp proteins and the *GAL* genes is significantly reduced in the absence of the SAGA complex, even under conditions where transcription of the *GAL* genes is likely to be largely unaffected. Taken together, these findings indicate that it is the physical presence of the SAGA complex at the *GAL* promoter which is required for association of the *GAL* genes with the NPC. Our findings are complemented by recent results suggesting that Mlp1 is an important connection between

the NPC and actively transcribed genes (59), which suggests that the Mlp/SAGA link analyzed here could be a key element of locus recruitment to the NPC.

Previous reports have implicated diverse aspects of transcription and mRNA processing in the recruitment of active loci to the NPC (59, 90, 113-115, 135, 152), including transcription factors, chromatin modifying complexes, the pre-initiation complex, and mRNA processing and export factors. As the SAGA complex regulates only approximately 10% of the yeast genome (111, 112), our data together with these previous reports strongly suggest that distinct mechanisms of recruitment exist for individual loci. Indeed, a previous study found that altering the induction mechanism of the yeast *HXK1* locus with an artificial promoter abrogated recruitment of the locus to the NPC (135). Furthermore, the findings that both the SAGA complex [(113) and this study] and the mRNA export factor Sac3 (114) are required for *GAL* locus recruitment to the NPC indicates that the interactions that link an activated locus to the NPC may depend upon a host of functionally diverse transcription and mRNA processing factors, some of which are locus-specific. Our data suggest that chromatin modifying complexes, which are recruited to individual loci through specific transcriptional activators, may be important components of this combinatorial, physical interaction between active loci and the NPC.

As the recruitment of actively transcribed genes to the NPC has been thus far documented only in *S. cerevisiae*, a major question is whether this phenomenon also occurs in higher eukaryotes. The evolutionary conservation of both the SAGA complex [human TBP-free TAF-containing complex (TFTC), p300- and CBP-associated factor (PCAF), and SPT3-TAF31-GCN5 acetyltransferase (STAGA) complexes (165-168)] and

the components of the NPC including the Mlp proteins [human translocated promoter region (Tpr) (147)] implies that locus recruitment to the NPC could in principle occur in higher eukaryotes by mechanisms similar to those in yeast. Furthermore, the *Drosophila* Mlp homolog, Mtor, is required for proper localization of the MSL histone acetyltransferase complex to the male X chromosome, where the MSL complex upregulates transcription of X-linked genes on the single copy of the male X chromosome at the nuclear periphery (90). These results tantalizingly hint that the interactions between the NPC and actively transcribed genes may represent an evolutionarily conserved mechanism for gene regulation across the eukaryotic lineage.

Experimental Procedures

Strains, plasmids, and chemicals: All DNA manipulations were performed according to standard methods (169) and all media were prepared by standard procedures (170). All yeast strains and plasmids used are described in Table 2.1. All chemicals were obtained from Ambion (Austin, TX), Sigma Chemical Co. (St. Louis, MO), US Biological (Swampscott, MA) or Fisher Scientific (Pittsburgh, PA) unless otherwise noted.

Tandem affinity purification of proteins: The C-terminal globular domain of Mlp1 (amino acids 1490-1875) was expressed from a galactose-inducible promoter (pAC1657) as a TAP-CT-Mlp1 fusion protein. The tandem affinity purification method was adapted from established protocols (158, 171). Two liter cultures of cells expressing TAP-tagged Mlp1 were grown to late log phase, and the cells were resuspended in 10 mL

Table 2.1 **Strains and plasmids used in Chapter 2**

Strain	**Description**	**Reference/Source**
FY23 (ACY192)	*MATa ura3-52 leu2Δ1 trp1Δ63*	(172)
ACY984	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MLP1-TAP::HIS3*	Open Biosystems
ACY98	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MLP2-TAP::HIS3*	Open Biosystems
ACY1016	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ADA2-TAP::HIS3*	Open Biosystems
ACY1017	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 GCN5-TAP::HIS3*	Open Biosystems
ACY1131	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SPT15-TAP::HIS3*	Open Biosystems
ACY1121	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MLP1-TAP::HIS3 gcn5Δ::KAN*	This Study
ACY1205	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MLP1-TAP::HIS3 spt7Δ::KAN*	This Study
Plasmids	**Description**	**Reference/Source**
pAC1656	*pGAL1-TAP*, 2μ *TRP1*, AMP^R	This Study
pAC1657	*pGAL1-CT-MLP1-TAP*, 2μ, *TRP1*, AMP^R	This Study
pPS892 (pAC403)	*pGAL1-GST*, 2μ, *URA3*, AMP^R	(173)
pAC2069	*pGAL1-GST-CT-MLP1*, 2μ, *URA3*, AMP^R	This Study
pAC1660	*pGAL1-GST-CT-MLP2*, 2μ, *URA3*, AMP^R	This Study
pAC2256	*GCN5-GFP*, 2μ, *URA3*, AMP^R	This Study
pGEX4T-3 (pAC736)	*GST,* AMP^R*,* bacterial expression vector	Amersham Pharmacia
pAC1430	*GST-CT-MLP1*, AMP^R	(153)
pAC1682	*GST-CT-MLP2*, AMP^R	This Study
pET28a (pAC762)	*HIS,* KAN^R bacterial expression vector	Novagen
pAC1781	*HIS-GCN5*, KAN^R	This Study
pAC1851	*GST-ADA2,* AMP^R	This Study

of extract buffer (40 mM HEPES pH 7.4, 350 mM NaCl, 10% glycerol, 0.1% Tween, 2 μg/mL pepstatin A, 1 mM PMSF). Lysate was prepared using a French press at a pressure of 1200 psi. Cell debris was removed by spinning the lysate at 5000 rpm for 15 min, followed by ultracentrifugation for 1 hr. Protein collected in the supernatant fraction was dialyzed (4 hrs, 4°C) against dialysis buffer (20 mM K-HEPES pH 8.0, 50 mM KCl, 0.2 mM EDTA pH 8.0, 0.5 mM DTT, 0.5 mM PMSF, 2 mM benzamidine, 20% glycerol). This clarified lysate was used to bind TAP-tagged proteins to IgG Sepharose beads (Amersham Biosciences). Beads were incubated with clarified lysate for 3 hrs at 4°C, and TEV cleavage was performed with 30 u TEV for 4 hrs at 4°C followed by 1 hr at 18°C. Subsequent elution and purification was performed as described (158). Mass spectrometry identification was performed by the Emory University Microchemical and Proteomics Core Facility.

Co-purification of GST fusion proteins: Yeast cells were transformed with plasmids expressing fusion proteins (GST-CT-Mlp1, GST-CT-Mlp2), or control vector (GST alone). Cultures were grown to log phase on 2% galactose minimal media and then harvested by centrifugation at 3000 rpm. Cells were lysed in PBSMT (PBS, 5 mM $MgCl_2$, 0.5% Triton X-100, pH 7.4) supplemented with protease inhibitor cocktail (1 mM PMSF, 3 ng/mL pepstatin A, leupeptin, aproptinin, and chymostatin). GST-tagged proteins were purified from protein extracts by incubation overnight at 4°C with Glutathione beads (Amersham Biosciences). The bound faction was washed 3 times and eluted from beads with loading buffer (125 mM Tris-HCl, pH 6.8, 250 mM DTT, 5% SDS, 0.25% Bromophenol blue, 25% glycerol). Lysate (25 μg protein) and bound fractions were resolved by SDS/PAGE and analyzed by immunoblotting according to

standard procedures (169). TAP-tagged proteins were detected with PAP antibody (Sigma). For experiments where nuclease treatment was used, lysate prepared from a common culture was divided into four aliquots and aliquots were treated with RNase (10 U/ml), DNase(10 U/ml), both nucleases, or left untreated (control) for 10 min at 37°C followed by incubation at 4°C for 50 min.

Purification of recombinant proteins: Cell lysate preparation and the purification of recombinant proteins were performed as recommended by the resin manufacturer (Amersham Biosciences). Briefly, recombinant proteins were expressed in BL21 *E. coli* by standard IPTG induction, and lysate was prepared from log-phase cultures. GST and GST-fusion proteins were purified from lysate by affinity chromatography on glutathione Sepharose. Six-histidine-tagged Gcn5 (His-Gcn5) was purified with nickel-nitrilotriacetic acid Sepharose.

In vitro binding assay: For *in vitro* binding assays, 5 μg of GST or GST-fused protein was bound to glutathione Sepharose in PBS buffer for 30 min at 25°C. After three washes with 1 mL PBS buffer, 1 μg purified, recombinant His-Gcn5 fusion protein was added to a volume of 1 mL Buffer A (20 mM Tris-HCl pH 8.0; 0.5% Triton X-100). The mixtures were then incubated for 1 hr at 4°C. Unbound fractions were collected and the beads were washed three times with 1 mL Buffer A containing 300 mM NaCl. The bound fraction was eluted by incubation with SDS sample buffer for 5 min at 95°C. Bound and unbound fractions were separated by 12% SDS-PAGE followed by immunoblotting with anti-GST (Sigma) or anti-His (Santa Cruz) antibodies.

ChIP analysis: ChIP was performed essentially as described (174). Briefly, 100 mL cultures were grown to log phase (OD600 ~0.8-0.9). Formaldehyde was added to a

final concentration of 1% for 20 min. Cross-linking was quenched by addition of 270 mM glycine for 10 min. Cells were washed twice with chilled TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl) and once with lysis buffer [50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 1 mM DTT, protease inhibitors cocktail (0.2 mM PMSF, 1 mM benzamidine, 1 μg/mL pepstatin A)]. The pellet was resuspended in lysis buffer and cells were lysed using glass beads. Chromatin was collected in the supernatant fraction and sheared by sonication to ~200 bp fragments. The chromatin solution was bound to prewashed, IgG beads for 2 hrs at room temperature. Immunoprecipitated chromatin was eluted from beads by heating for 10 min at 65°C in elution buffer (25 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS). To reverse crosslinking, samples were incubated with proteinase K for 1 hr at 42°C followed by overnight incubation at 65°C. Samples were purified using Qiagen columns and analyzed by quantitative PCR using gene-specific primer pairs. The fraction of immunoprecipitated material for a specific fragment was calculated by dividing the amount of PCR product obtained from immunoprecipitated DNA by the amount obtained from total DNA (IP/ Input).

RNA isolation: Cells grown in 10 mL cultures of glucose or galactose were pelleted and washed twice with chilled DEPC water. Pellets were resuspended in 200 μL LET buffer (100 mM LiCl, 25 mM Tris pH 8, 20 mM EDTA), and then 200 μL of phenol and 100 μL of glass beads were added to the resuspension. Cells were subjected to a brief heat shock at 65°C and were lysed by bead beating for 4 min with 2 min intervals on ice. Debris was removed by brief centrifugation. The aqueous layer was transferred to a fresh tube containing 200 μL of phenol, vortexed, and centrifuged for 5 min. RNA

was isolated from the aqueous layer by chloroform extraction and was precipitated at -80°C for 30 min using 40 μL 3M NaOAc and 1 mL EtOH. The RNA pellet was collected by spinning the mixture at 12,000 rpm for 10 min. The pellet was washed with 70% EtOH and dried on ice, then resuspended in RNase-free water for further analysis.

RT-PCR: synthesis of cDNA from samples grown in glucose and galactose was performed with an Invitrogen SuperScript III Reverse Transcriptase kit. Amplification reactions to detect relative cDNA levels included 1 μL of cDNA and 300 nM gene-specific forward and reverse primers. Samples were amplified with 35 cycles of denaturation (95°C, 30 sec), annealing (60°C, 30 sec), and extension (72°C, 1 min). Each PCR reaction was performed in triplicate and products were analyzed by agarose gel electrophoresis.

Quantitative real-time PCR: *GAL* transcript levels were determined by two-step quantitative real-time PCR (qRT-PCR) analysis. Synthesis of cDNA from cells grown in glucose and galactose was performed with the Quantitect reverse transcription kit (Qiagen). Amplification reactions to detect relative cDNA levels were performed with the Quantitect SYBR Green PCR kit (Qiagen) using the iCycler iQ Real-Time PCR Detection System (Bio-Rad). Results were analyzed using the iCycler Optical System Version 3.0a software, and data were normalized by the ΔΔCt method to a control transcript (175).

CHAPTER 3

The SAGA Complex and the Nuclear Pore Control Global Transcription and Mediate Retention of the *GAL1* Locus at the Nuclear Periphery

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This chapter is a collaborative paper in progress for co-submission with a related study.

Synthetic genetic array screens were conducted by Milo B. Fasken and Kellie E. Barbara-Haley. Microscopy on wildtype and *ada2*Δ cells was performed by Terry M. Haley. Statistical analyses of microarray results were performed by Kristine A. Willis.

Introduction

Researchers observed over twenty years ago that DNase I hypersensitive sites, presumably representing regions of active chromatin, are located toward the nuclear periphery in mouse fibroblast cells (72). This observation was published the very same month that Blobel communicated his seminal gene gating hypothesis proposing that actively transcribed genes interact with nuclear pore complexes (NPCs) to facilitate transcript export and mediate three-dimensional nuclear organization (71). Despite these two prominent works, the nuclear periphery has been predominantly viewed in the intervening years as repressive to transcription (40); however, recent work has indeed confirmed that at least some active genes are localized at the nuclear periphery in metazoan systems. For instance, the highly expressed, tissue-specific *PLP* gene maintains a perinuclear position throughout differentiation of rat oligodendrocytes (176), as do several active cytokine genes during maturation of mouse T helper cell (177). In addition, a reporter gene can be transcriptionally activated when artificially tethered to the nuclear periphery in human tissue culture cells (178)

Although these studies did not specifically investigate whether these active genes associate with the NPC when at the nuclear periphery, other work suggests that this complex may have a central role in transcriptional activation of perinuclear genes. In the fruit fly *Drosophila melanogaster*, nuclear pore components are required for proper localization and transcriptional upregulation by the dosage compensation complex on the perinuclear male X chromosome (90), and the activated *hsp70* locus also associates with the NPC (43). Recent work in *Drosophila* has also revealed that specific NPC subunits

physically associate with specific actively transcribed genes on a genome-wide level, and these interactions are required for the transcriptional upregulation of these genes (92-94). In addition, the mouse nuclear pore component Nup96 is required for proper transcriptional activation of interferon genes (91), and LexA fusions with nuclear pore proteins can activate transcription in the yeast *Saccharomyces cerevisiae* (152). Finally, treatment of HeLa cells with a histone deacetylase inhibitor results in association of the NPC component Nup93 with gene promoter-regions, active genes, and chromatin bearing active histone modifications (179). Taken together, these results indicate that the nuclear periphery is not necessarily transcriptionally repressive in metazoan cells, and suggest that the NPC is involved in gene activation.

In *S. cerevisiae*, specific genes physically relocate from the nucleoplasm to the nuclear periphery concomitant with transcriptional activation, where they associate with the NPC (34, 73, 180, 181). These inducible loci include *FIG2*, *GAL1-10*, *GAL2*, *HSP104*, *HXK1*, *INO1*, *SUC2*, and *TSA2* (59, 61, 62, 75, 113-115, 118, 119, 135, 136). While relocation has been directly observed only for these identified loci, there are intriguing suggestions that gene relocation to the NPC may be physiologically relevant to the cell on a global level. There is a strong correlation among highly transcribed genes and NPC-association, (62), and these gene-NPC associations globally change upon induction of a differential transcriptional program (61). Moreover, an elegant study of the accessibility of endogenous chromatin to cleavage by micrococcal nuclease suggests that the majority of promoters in the yeast genome may be capable of temporarily interacting with the NPC (115). However, as the nuclease was fused to the mobile

nucleoporin Nup2, (116, 117) it is difficult to eliminate the possibility that at least some of these interactions occur in the nucleoplasm.

Studies of inducible model loci in budding yeast have implicated multiple factors in this phenomenon of gene relocation to the NPC, including both transcriptional activators and co-activators such as Gal4 and the SAGA histone acetyltransferase complex (60, 113, 115), mRNA processing and export factors such as Sac3 and Mex67 (59, 113, 114), and two recently identified DNA promoter elements (119). The fact that independent studies have implicated so many different factors in the process of gene relocation may suggest that individual genes rely on specific rather than universal protein complexes for relocation to the NPC, reminiscent of the distinct transcription factors and co-regulators that regulate transcription for different genes. Another, non-mutually exclusive, possibility is that these different factors are involved in distinct stages of gene relocation: some factors initially recruit active genes, while others retain them at the NPC. These possibilities could help to explain the apparently contradictory findings that gene association with the NPC is RNA-dependent, yet relocation to the NPC occurs prior to transcriptional activation and does not require an mRNA coding region (59, 61, 75).

In addition to elucidating the mechanism of gene relocation, recent studies have begun to identify the physiological relevance of relocation to the NPC, as this phenomenon is required for gene looping and transcriptional memory of recently active genes (75, 142, 143). The Mlp1 subunit of the NPC is required for gene loop persistence following transcriptional shutoff but not gene loop initiation (142), suggesting that the NPC plays a direct role in the maintenance of transcriptional memory. While a role for the NPC in transcriptional memory is unambiguous, the relationship between gene

relocation and transcript levels of relocated genes is less clear. Although some studies have found that loss of factors involved in gene relocation does not impact transcript levels of targeted loci (113), or that impact on transcript levels is measurable but not severe (60), other investigations have reached the conflicting conclusion that gene relocation does significantly impact transcript levels (118, 119, 135). Thus aspects of the physiological significance of this phenomenon are unresolved.

We took a genomics approach in order to gain insight into the universality and physiological relevance of the interaction between active genes and the NPC. We focused on the Mlp proteins, which are localized to the nuclear basket of the NPC (147) and are thus ideally situated to interact with actively transcribed genes, as well as the Nup60 nuclear pore subunit that anchors the Mlp proteins to the NPC (182). Nup60 and the Mlp proteins interact with actively transcribed loci (60-62), and are required for perinuclear localization of several inducible loci (59, 142). Using synthetic genetic array (SGA) analysis (183), we identified interactions between components of the SAGA histone acetyltransferase complex and the Mlp and Nup60 subunits of the NPC. Cells lacking these SAGA and NPC components display growth defects under optimal growth conditions, in which cells are grown on rich medium containing the preferred sugar glucose as a carbon source (184) and incubated at their optimal temperature (185). That growth defects were observed under these non-stress conditions suggests that these interactions are indicative of defects in normal cell physiology. These genetic interactions are not universal to all NPC subunits, suggesting specificity in the interactions between SAGA and components of the NPC. FISH experiments to localize total poly(A) RNA indicate that these slow growth phenotypes are not due to a synthetic

defect in mRNA export. Microarray analysis reveals that the growth defect in these double mutants is correlated with a synthetic reduction in steady-state transcript levels for numerous genes that are strongly expressed in wildtype cells. We thus considered the possibility that a functional interaction between SAGA and the NPC is necessary for efficient gene expression on a global scale. However, it is difficult to determine the relationship between gene position and expression by measuring transcript levels in a population of cells. We therefore utilized a recently developed single-cell assay for measuring both parameters in real time *in vivo* (148, in preparation). By applying this assay to the known SAGA-target *GAL1* (98, 99, 186), we have found that deletion of subunits of either SAGA or the NPC results in a defect in the retention, rather than the recruitment, of *GAL1* to the NPC; combined deletion of NPC and SAGA components results in a synthetic defect in both retention and regulation. These cells have reduced fitness when grown with galactose as the sole carbon source, indicating that defects in gene retention at the NPC result in physiological consequences. Taken together, these results suggest that gene relocation to the NPC includes distinct recruitment and retention steps and is a primary component of transcriptional regulation.

Results

The *NUP60* and *MLP* genes show synthetic genetic interactions with genes encoding components of the SAGA histone acetyltransferase complex: Given their location at the nuclear basket of the NPC (147), the Mlp proteins and the nucleoporin, Nup60, are ideally positioned to play a fundamental role in the physical association between NPCs

and actively transcribed loci; in fact, both physical and functional links have been identified (59-62, 90, 142). However, these studies primarily focused on selected highly transcribed or specific inducible genes, and the question of whether gene relocation to the NPC is a general feature of transcriptional control remains largely unanswered. Because the set of genetic interactions observed for a particular gene is indicative of shared function (133), we expected that NPC subunits would show genetic interactions with regulators of actively transcribed genes if this phenomenon occurs on a global level. We therefore conducted synthetic genetic array (SGA) analysis (183) with *mlp1*Δ, *mlp2*Δ, *mlp1*Δ *mlp2*Δ, and *nup60*Δ cells in order to identify classes of genes that functionally interact with components of the NPC under optimal growth conditions. Included in the results of the screens were several genes encoding components of the Spt-Ada-Gcn5-Acetyltransferase (SAGA) histone acetyltransferase complex (Figure 3.1), a complex that has been implicated in gene relocation of the galactose-inducible *GAL* genes (60, 113). Because this analysis is conducted under optimal growth conditions, these results are consistent with a model where interactions between the Nup60 and Mlp proteins and the SAGA complex are important for global regulation of transcription. Moreover, because Nup60 is required for Mlp anchoring at the NPC (182), these results suggest that it is the specific location of the Mlp proteins at the NPC that is critical for these functional interactions.

In order to confirm the functional link between the *MLP* genes and components of the SAGA complex, we created mutant strains lacking both *MLP* genes in combination with deletion of either the *ADA2* or *GCN5* gene, both of which encode components of the

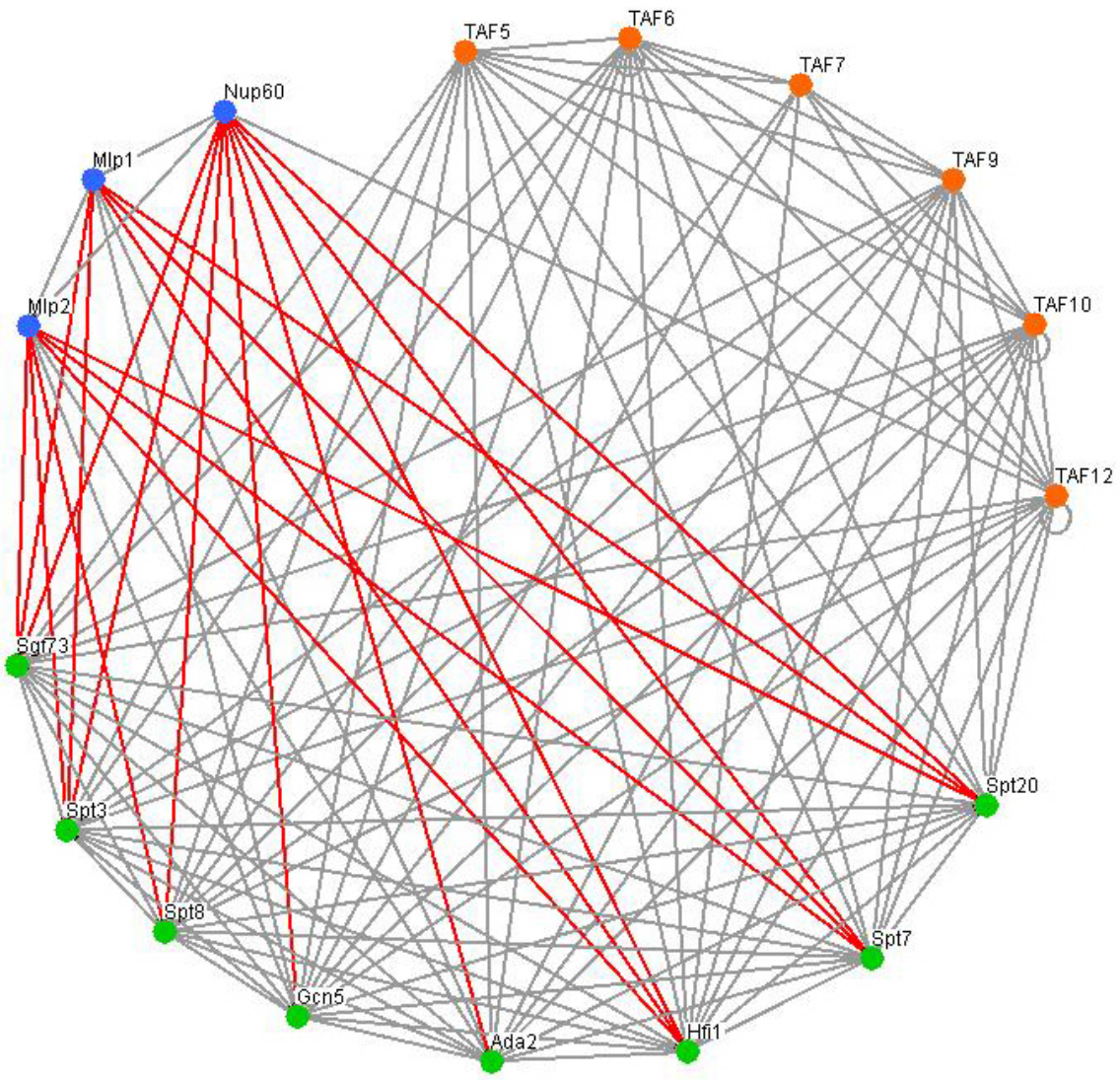


Figure 3.1 **Interactions between the *MLP* and *NUP60* genes and genes encoding SAGA subunits identified *via* SGA screens.** Osprey diagram (187) depicting interactions between Mlp1, Mlp2, Nup60 (blue nodes), SAGA subunits (green nodes), and SAGA/TFIID-shared TAFs (orange nodes). Red lines represent novel interactions identified in this study, and grey lines represent published interactions.

SAGA complex (103). We then performed serial dilution assays under optimal growth conditions to investigate cell fitness. Triple mutant cells (*mlp1*Δ *mlp2*Δ *ada2*Δ and *mlp1*Δ *mlp2*Δ *gcn5*Δ) grow normally in the presence of a wildtype *MLP1* maintenance plasmid, but display growth defects in the absence of this plasmid compared to either wildtype, single mutant, or double mutant cells (Figure 3.2*A*), confirming a functional link between the Mlp proteins and the SAGA complex. Consistent with our SGA results, *nup60*Δ *ada2*Δ and *nup60*Δ *gcn5*Δ cells also show growth defects compared to wildtype and single mutant cells (Figure 3.2*B*). Growth curve analysis *via* liquid growth assays confirmed these results (Figure 3.2*C*,*D*).

Synthetic genetic interactions with components of the SAGA complex are specific to distinct NPC components: Cell fitness analyses indicate that Nup60 and the Mlp proteins have functional interactions with components of SAGA, perhaps indicative of the importance of interactions between the NPC and actively transcribed genes. Multiple other NPC subunits have been linked to actively transcribed genes, including Nic96, Nup1, Nup2, Nup116, and the Nup84 subcomplex of the NPC (59, 61, 62, 75, 113, 115, 152). Whether these nucleoporins all interact with active loci *via* SAGA or through other transcriptional activators is unknown. We therefore tested for genetic interactions between *NUP133*, a component of the Nup84 subcomplex (188), and genes encoding SAGA subunits. Serial dilution assays of *nup133*Δ *ada2*Δ and *nup133*Δ *gcn5*Δ cells reveal a significant growth defect in double mutant cells compared to wildtype and single mutant cells (Figure 3.3*A*). As a control, we combined a deletion of *NUP133* with deletion of the transcriptional activator *GCR2* (189). *nup133*Δ *gcr2*Δ cells do not show a

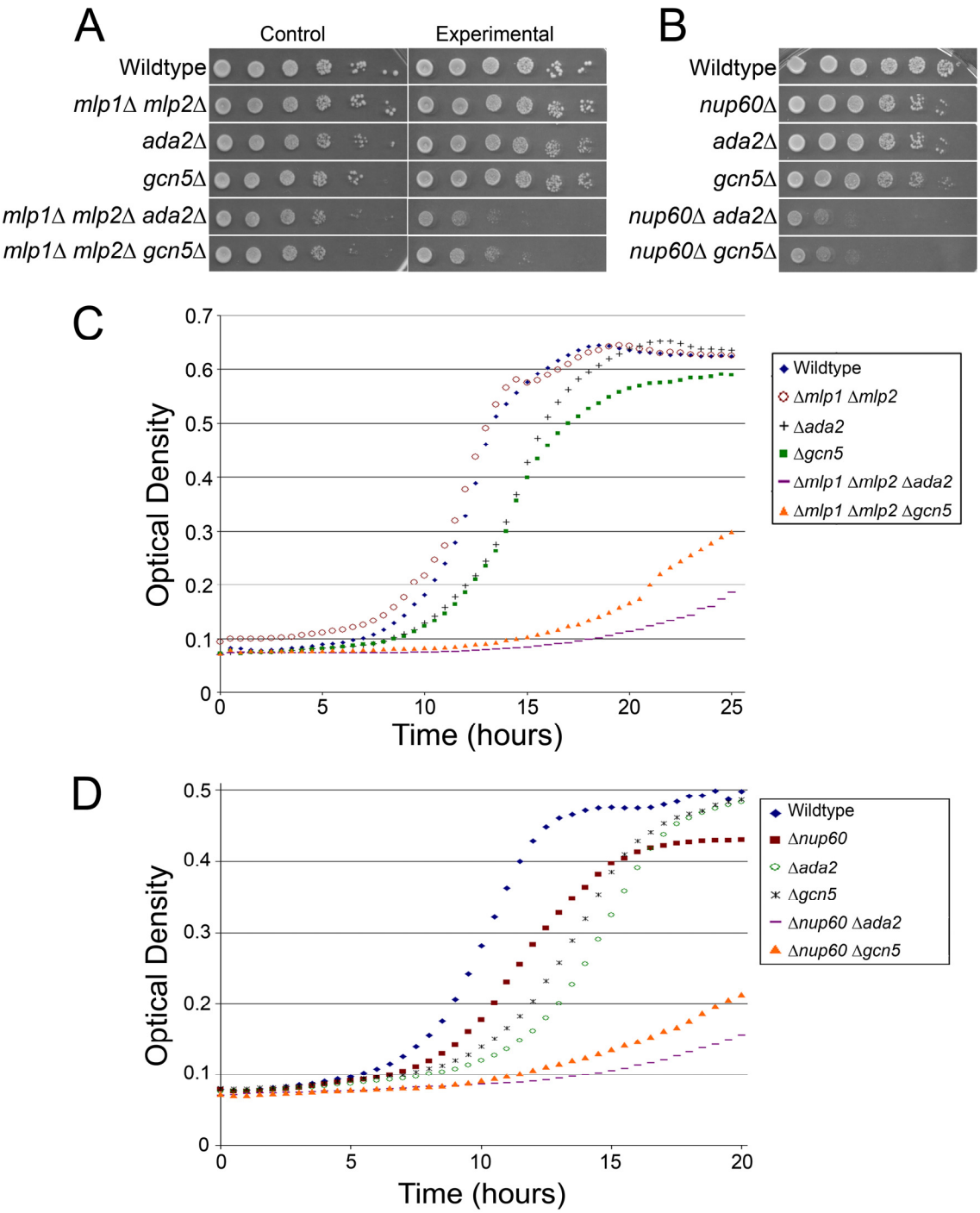

A
Control
Experimental
Wildtype
mlp1Δ mlp2Δ
ada2Δ
gcn5Δ
mlp1Δ mlp2Δ ada2Δ
mlp1Δ mlp2Δ gcn5Δ
B
Wildtype
nup60Δ
ada2Δ
gcn5Δ
nup60Δ ada2Δ
nup60Δ gcn5Δ
C
Optical Density
Time (hours)
Wildtype
Δmlp1 Δmlp2
Δada2
Δgcn5
Δmlp1 Δmlp2 Δada2
Δmlp1 Δmlp2 Δgcn5
D
Optical Density
Time (hours)
Wildtype
Δnup60
Δada2
Δgcn5
Δnup60 Δada2
Δnup60 Δgcn5

Figure 3.2 **The *MLP* and *NUP60* genes functionally interact with genes encoding SAGA subunits.** ***A,*** Wildtype, *mlp1*Δ *mlp2*Δ, *ada2*Δ, *gcn5*Δ, and triple mutant cells containing a wildtype copy of *MLP1* on a *URA3* plasmid were grown to saturation in selectable media then 10-fold serially diluted onto Ura^- or 5-FOA glucose medium and incubated for 2 days at 30ºC. ***B,*** Wildtype, *nup60*Δ, *ada2*Δ, *gcn5*Δ, and double mutant cells were grown to saturation then 10-fold serially diluted onto glucose medium and incubated at 30ºC for 2 days. ***C,D*** Wildtype, *mlp1*Δ *mlp2*Δ, *ada2*Δ, *gcn5*Δ, and triple mutant cells ***C,*** or wildtype, *nup60*Δ, *ada2*Δ, *gcn5*Δ, and double mutant cells ***D,*** were grown to saturation, normalized to equal starting concentrations, and diluted 100-fold into 96-well plates. Plates were incubated at 25ºC in an ultra microplate reader and OD_{600nm} was recorded every 30 min. Data are plotted as OD *versus* time.

growth defect compared to single mutant cells, indicating that the growth defect observed in *nup133*Δ *ada2*Δ and *nup133*Δ *gcn5*Δ cells is not a nonspecific effect due to the NPC clustering which occurs in *nup133*Δ cells (190).

Given that the Nup60, Mlp, and Nup133 proteins functionally interact with components of the SAGA complex, we wondered whether such interactions with SAGA might be a general feature of nuclear pore proteins that associate with actively transcribed genes. To investigate this possibility, we tested for genetic interactions between genes encoding SAGA subunits and *NUP1* or *NUP2*. Serial dilution assays indicate that *nup2*Δ *ada2*Δ and *nup2*Δ *gcn5*Δ cells do not display a growth defect compared to single mutant cells (Figure 3.3*B*). We also do not observe growth defects in *nup1*Δ *ada2*Δ cells (Figure 3.3*C*). Taken together, these results indicate that functional interaction with the SAGA complex is not a general feature of all NPC subunits which interact with actively transcribed genes, but that SAGA selectively interacts with specific components of the NPC.

***nup60*Δ *ada2*Δ cells do not show severe nuclear accumulation of poly(A) RNA:** Both the Mlp proteins and Nup60 have been linked to mRNA export (57, 153, 154, 191). The SAGA complex, which interacts with the Sac3-Thp1 mRNA export complex through its Sus1 subunit, has also been implicated in this process (155). Thus to investigate the possibility that the growth defect exhibited by these double mutants may be due to severely compromised mRNA export, we utilized fluorescence *in situ* hybridization (FISH) to compare poly(A) RNA localization in single mutant, double mutant, and wildtype cells. As expected, the wildtype control shows a diffuse poly(A) signal

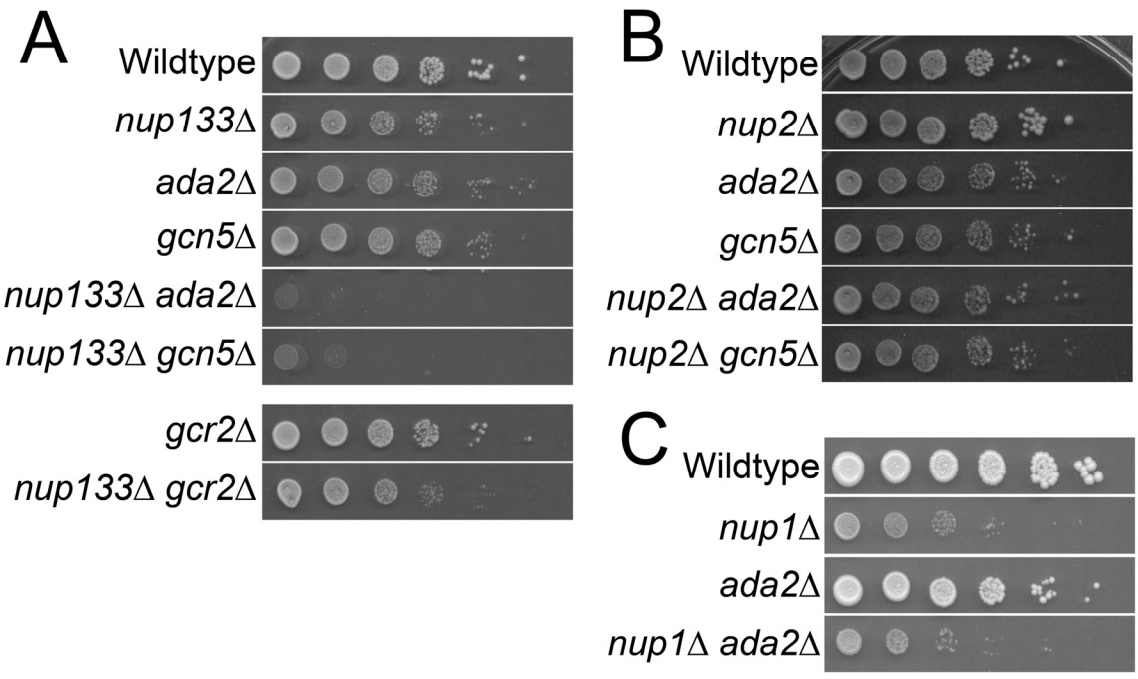


Figure 3.3 **The *NUP133*, *NUP2* and *NUP1* genes display differential functional interactions with genes encoding SAGA subunits.** ***A,*** Wildtype, *nup133*Δ, *ada2*Δ, *gcn5*Δ, *ada2*Δ *gcn5*Δ, and *gcr2*Δ and *nup133*Δ *gcr2*Δ control cells were grown to saturation then 10-fold serially diluted onto rich medium and incubated at 30ºC for 2 days. ***B,*** Wildtype, *nup2*Δ, *ada2*Δ, *gcn5*Δ, and double mutant cells were assayed as in ***A*** except that cells were grown on glucose medium. ***C,*** Wildtype, *nup1*Δ, *ada2*Δ, and double mutant cells were assayed as in ***B***.

throughout the cell (Figure 3.4*A*). As a control, export-defective *nab2-1* mutant cells show dramatic accumulation of poly(A) RNA in the nucleus (192), indicating a severe mRNA export defect. *ada2*Δ and *gcn5*Δ cells show poly(A) signal similar to wildtype cells, and *nup60* cells show some nuclear accumulation of poly(A) signal, consistent with previous results (191). While *nup60*Δ *ada2*Δ and *nup60*Δ *gcn5*Δ cells show accumulation of poly(A) signal in the nucleus, this accumulation is not more severe than *nup60* alone, indicating that the growth defect in these double mutant cells is not due to a gross synthetic mRNA export defect. Similar results were observed for *mlp1*Δ *mlp2*Δ *ada2*Δ and *mlp1*Δ *mlp2*Δ *gcn5*Δ cells (Figure 3.4*B*). Though we cannot rule out an export defect for one specific mRNA, these results indicate that the fitness defect in these double mutant cells is not due to global impairment of poly(A) RNA export.

Regulation of SAGA-dependent genes is defective in *nup60*Δ and *nup60*Δ *ada2*Δ cells: Our results are consistent with a model where physical interactions between the NPC and SAGA are important for transcription of constitutively expressed genes. To test this hypothesis, we first used microarray analysis to assess changes global transcript levels in the absence of Nup60. Because Nup60 anchors the Mlps to the NPC (182), and the Mlps physically interact with SAGA (60), we expect this experiment to report any changes in gene expression that result from displacing SAGA-dependent genes from the nuclear periphery.

Previously reported computational analysis identified genes whose expression is specifically dependent on SAGA versus those dependent on TFIID, which shares the structural TAF subunits Taf5, Taf6, Taf9, Taf10, and Taf12 with SAGA (112). We find

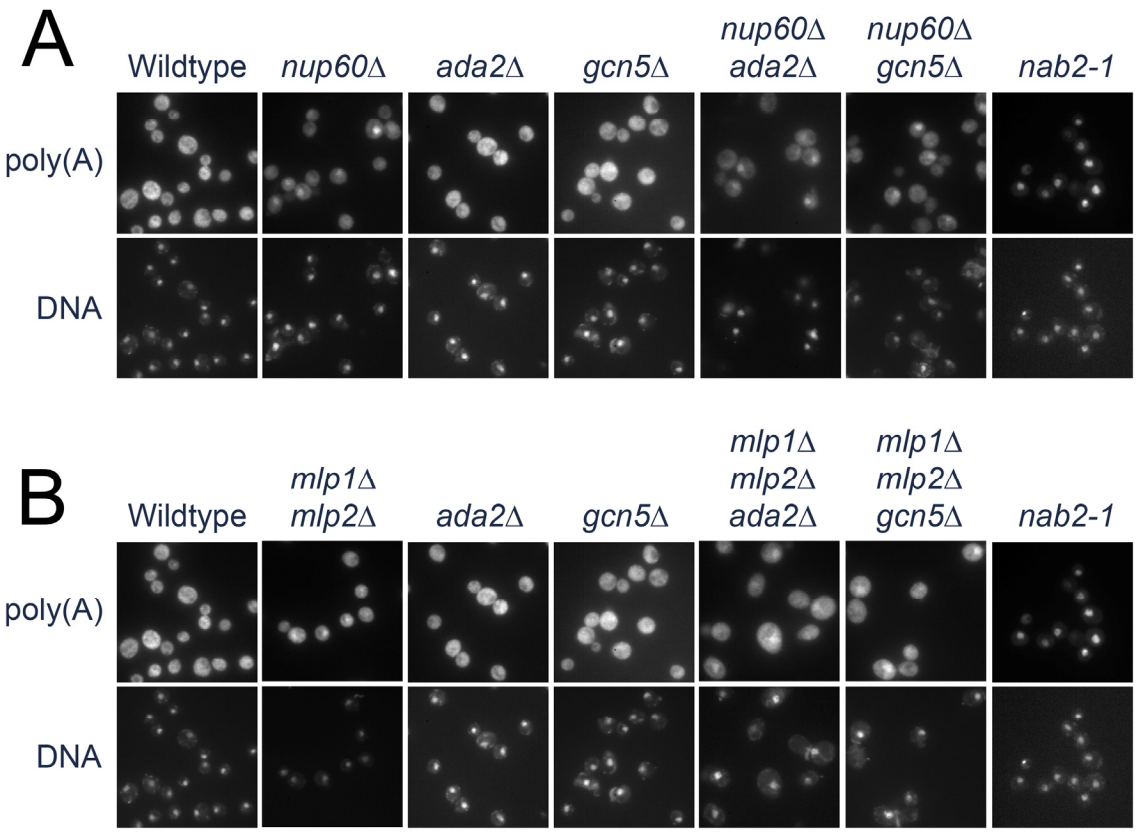


Figure 3.4 **Cells lacking *NUP60* and *ADA2* or *GCN5* do not show a severe nuclear accumulation of poly(A) RNA.** ***A,B,*** Wildtype, *nup60*Δ, *ada2*Δ, *gcn5*Δ, and *nup60*Δ *ada2*Δ cells ***A,*** or wildtype, *mlp1*Δ *mlp2*Δ, *ada2*Δ, *gcn5*Δ, and triple mutant cells ***B,*** were grown to log phase at 30ºC and subjected to FISH as described in Experimental Procedures. Panels are shown for poly(A) RNA and DAPI to visualize chromatin.

that >25% of SAGA-regulated genes (p<1e-14) are affected in *nup60*Δ cells (Figure 3.5*A*, Table 3.1). In contrast, only ~4% of genes regulated by TFIID are affected in *nup60*Δ cells, and this lack of overlap is statistically significant (p<1e-14, Figure 3.5*B*, Table 3.1). These results suggest that changes in gene expression in *nup60*Δ cells are not due to non-specific defects in transcription, but rather due to specific effects on SAGA-regulated genes.

Given the overlap in genes affected by *nup60*Δ and SAGA-regulated genes, we hypothesized that growth defects observed in cells lacking *NUP60* and a SAGA component may be correlated with synthetic defects in global, SAGA-regulated gene expression. In order to investigate this possibility, we performed microarray analysis on cells lacking both Nup60 and Ada2. As a control, we analyzed *ada2*Δ single mutant cells. As expected, genes affected in *ada2*Δ cells show a high degree of overlap with previously identified SAGA-dependent genes, good overlap with genes dependent on both SAGA and TFIID, and less overlap with genes regulated by TFIID alone (Table 3.1). Genes downregulated in *ada2*Δ also show a much higher degree of overlap with those genes downregulated in other SAGA mutants than with genes downregulated in a mutant of the TFIID-specific Taf1 (Table 3.2).

Interestingly, we find that ~25% of the genes represented on the microarray are affected in *nup60*Δ *ada2*Δ cells (Table 3.3). Not surprisingly, we find greater overlap between genes affected in *nup60*Δ *ada2*Δ cells and *nup60*Δ or *ada2*Δ cells than between *nup60*Δ cells and *ada2*Δ cells (Table 3.4). We also find that >47% of SAGA-regulated genes (p<1e-14) are affected in *nup60*Δ *ada2*Δ cells (Figure 3.5*C*, Table 3.1). Only ~18% of TFIID-regulated genes are affected in *nup60*Δ *ada2*Δ cells, and this lack of

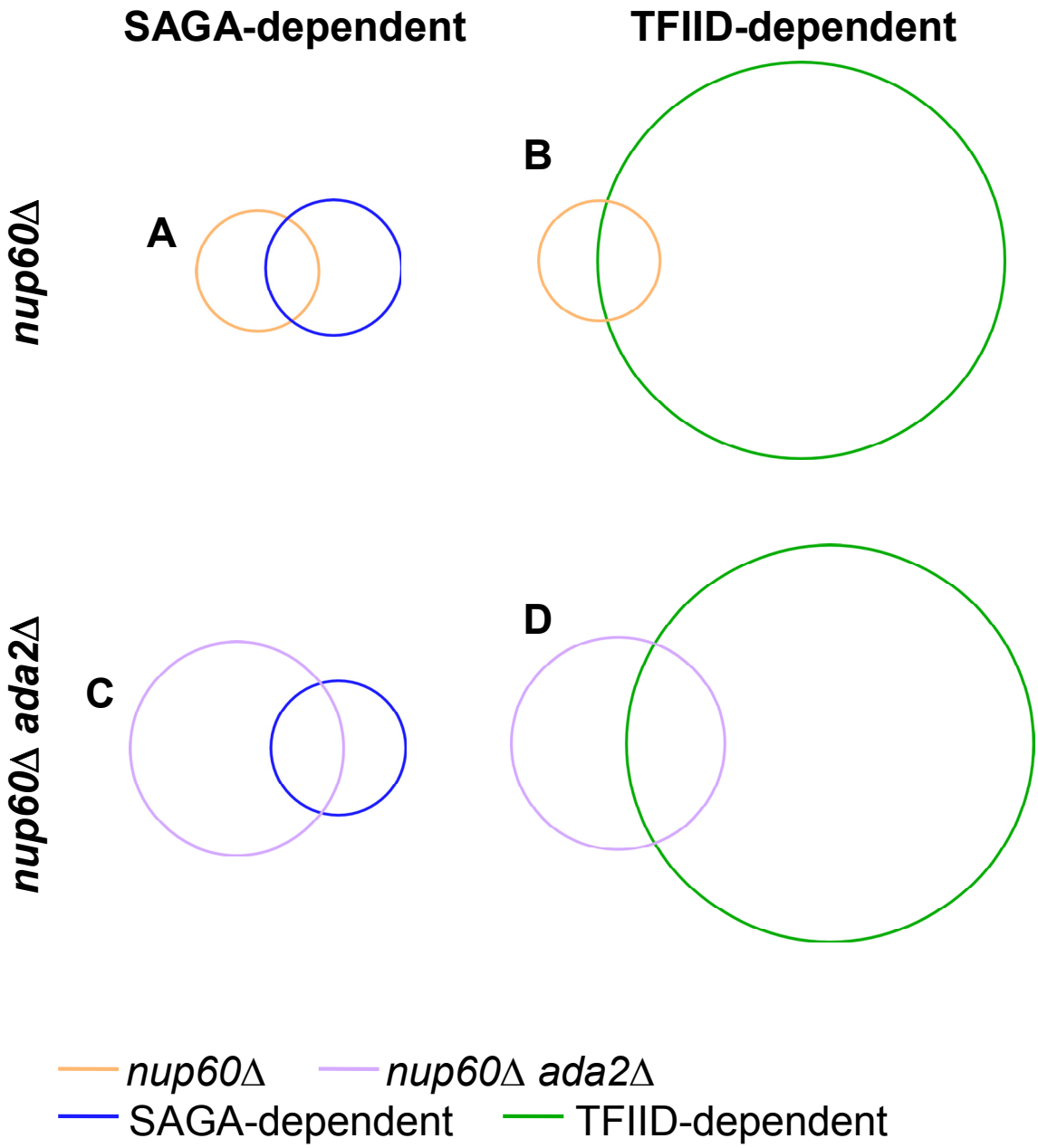


Figure 3.5 **Regulation of SAGA-dependent genes is defective in the absence of *NUP60* and is synthetically defective in the absence of both *NUP60* and *ADA2*. *A-D,*** Venn diagrams illustrating the overlap between genes whose expression is affected in *nup60*Δ (orange circles, ***A,B***) or *nup60*Δ *ada2*Δ (purple circles, ***C,D***) cells versus those identified as regulated by SAGA (blue circles) or TFIID (green circles) (112).

Table 3.1 **Genes affected in *nup60*Δ and *nup60*Δ *ada2*Δ cells are SAGA targets**[a]

		SAGA	TFIID
	n[b]	577	5130
***nup60*Δ**	# overlap	149	219
	% overlap	25.8	4.3
	p[c]	0.00E+00	0.00E+00
***ada2*Δ**	# overlap	126	221
	% overlap	21.8	4.3
	p[c]	0.00E+00	0.00E+00
***nup60*Δ *ada2*Δ**	# overlap	273	952
	% overlap	47.3	18.6
	p[c]	0.00E+00	0.00E+00

[a]genes whose expression is dependent on *nup60 ada2* or *nup60 ada2 versus* those previously determined to be SAGA targets (112).

[b]n, number of genes regulated by SAGA or TFIID

[c]p, statistical significance was calculated using a hypergeometric distribution function (193) and indicates the likelihood that the observed overlap is due to chance

Table 3.2 **TFIID *vs.* SAGA-regulated genes**[a]

	TAF1 (145)	TAF12 (61)	TAF10 (25)	TAF 6 (60)	TAF9 (17)	SPT20	ADA2	SPT3	GCN5
TAF145 (1)	-	< 1.0 E-14	< 1.0 E-14	NA[b]	NA[b]	7.97E-03	5.82E-06	5.28E-03	5.12E-02
TAF61 (12)	< 1.0 E-14	-	< 1.0 E-14	< 1.0 E-14	1.42E-12	1.30E-06	6.09E-02	7.98E-02	9.26E-03
TAF25 (10)	< 1.0 E-14	< 1.0 E-14	-	< 1.0 E-14	< 1.0 E-14	1.32E-10	5.15E-04	3.37E-03	NA[b]
TAF60 (6)	NA[b]	< 1.0 E-14	< 1.0 E-14	-	< 1.0 E-14	7.49E-04	1.95E-02	1.76E-02	6.73E-02
TAF17 (9)	NA[b]	1.42E-12	< 1.0 E-14	< 1.0 E-14	-	7.85E-03	1.61E-08	1.67E-10	3.21E-03
SPT20	7.97E-03	1.30E-06	1.32E-10	7.49E-04	7.85E-03	-	< 1.0 E-14	< 1.0 E-14	6.70E-10
ADA2	5.82E-06	6.09E-02	5.15E-04	1.95E-02	1.16E-08	< 1.0 E-14	-	2.95E-07	1.53E-12
SPT3	5.28E-03	7.98E-02	3.37E-03	1.76E-02	1.67E-10	< 1.0 E-14	2.95E-07	-	1.47E-09
GCN5	5.12E-02	9.26E-03	NA[b]	6.73E-02	3.21E-03	6.70E-10	1.53E-12	1.47E-09	-

[a]pairwise comparison of the genes downregulated in the absence of SAGA or TFIID subunits; p-values are reported to show the similarity between subunits and were calculated using a hypergeometric distribution function (193). Data for *ada2*Δ, this study, All other data from (111); temperature-sensitive alleles were used for essential genes.

[b]NA, no significant overlap.

Table 3.3 **Percent of genome affected in *ada2*Δ, *nup60*Δ, and *nup60*Δ *ada2*Δ cells**

Comparison	Number of genes affected	approximate % of genome[a]
Wildtype *vs.* *ada2*Δ	493	8.44
Wildtype *vs.* *nup60*Δ	464	7.94
Wildtype *vs.* *nup60*Δ *ada2*Δ	1446	24.76

[a]5841 genes are represented on the Affymetrix Yeast Genome 2.0 Array

Table 3.4 **Overlap of genes downregulated in *ada2*Δ, *nup60*Δ, and *nup60*Δ *ada2*Δ cells**

		***nup60*Δ**	***ada2*Δ**	***nup60*Δ *ada2*Δ**
	n[a]	95	244	540
***nup60*Δ**	# overlap	-	15	38
	% overlap	-	6.1	7.0
	p[b]	-	1.36E-06	0.00E+00
***ada2*Δ**	# overlap	15	-	89
	% overlap	15.8	-	16.5
	p[b]	1.36E-06	-	0.00E+00
***nup60*Δ *ada2*Δ**	# overlap	38	89	-
	% overlap	40.0	36.5	-
	p[b]	0.00E+00	0.00E+00	-

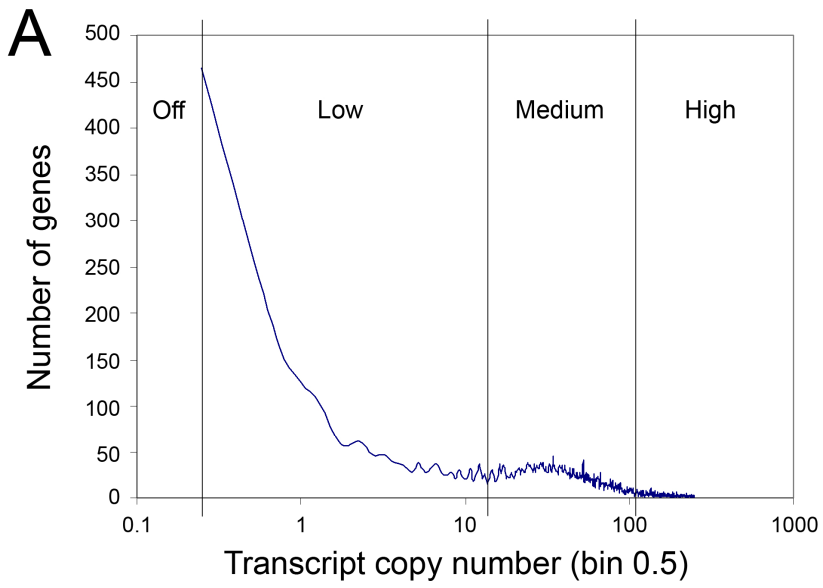
[a]n, number of genes downregulated in *ada2*Δ, *nup60*Δ, or *nup60*Δ *ada2*Δ cells

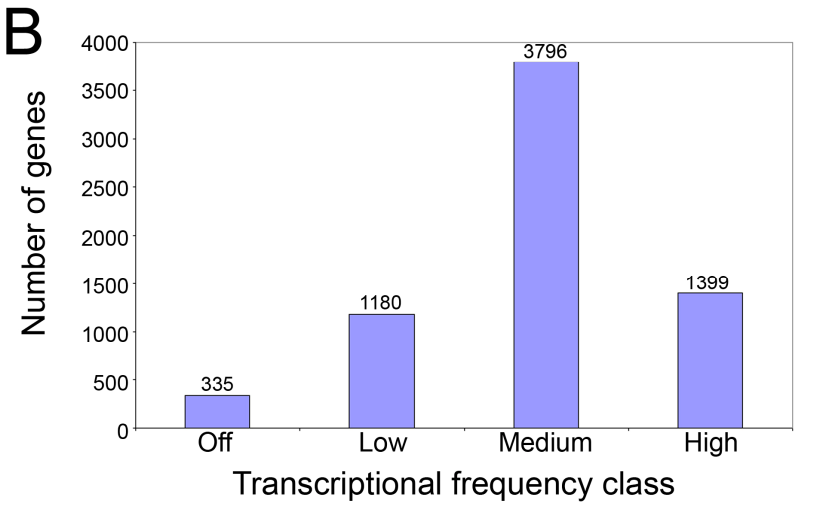
[b]p, statistical significance, calculated using a hypergeometric distribution function (193)

overlap is statistically significant (p<1e-14) (Figure 3.5*D*, Table 3.1). These results further suggest that SAGA and Nup60 cooperate in transcriptional regulation, and are consistent with a model for global regulation of SAGA-dependent transcription at the NPC.

Genes which associate with the NPC tend to be highly transcribed (62), and so we wondered whether genes downregulated in *nup60*Δ *ada2*Δ cells share this characteristic. To functionally classify the budding yeast transcriptome according to transcriptional frequency, we utilized a previously reported genome-wide transcript level analysis (194) (Figure 3.6*A*). We then compared the genes in each transcriptional category (Figure 3.6*B*) to the genes downregulated in *nup60*Δ, *ada2*Δ, and *nup60*Δ *ada2*Δ cells. Interestingly, genes downregulated in *nup60*Δ *ada2*Δ cells tend to be highly expressed genes in wildtype cells while genes downregulated in *nup60*Δ and *ada2*Δ cells do not show particular enrichment for highly transcribed genes (Figure 3.6*C*). Taken together, these results further suggest that interactions between SAGA and the nuclear basket of the NPC are important for upregulation of genes recruited to the NPC on a global level.

***nup60*Δ *ada2*Δ cells are defective in retention of the *GAL1* locus at the nuclear periphery:** The profound fitness defect in *nup60*Δ *ada2*Δ cells correlates with genome-wide changes in steady state transcript levels. These results are consistent with a model where physical interactions between the Nup60 and the SAGA complex are important for relocation of actively transcribed genes to the NPC. The SAGA-regulated *GAL* genes (98, 99, 186) were the first genes found to relocate from the nuclear interior to the nuclear periphery upon induction by galactose (62), where they interact with NPCs *via* the


A
Number of genes
500
450
400
350
300
250
200
150
100
50
0
Off
Low
Medium
High
0.1
1
10
100
1000
Transcript copy number (bin 0.5)


B
Number of genes
4000
3500
3000
2500
2000
1500
1000
500
0
335
1180
3796
1399
Off
Low
Medium
High
Transcriptional frequency class

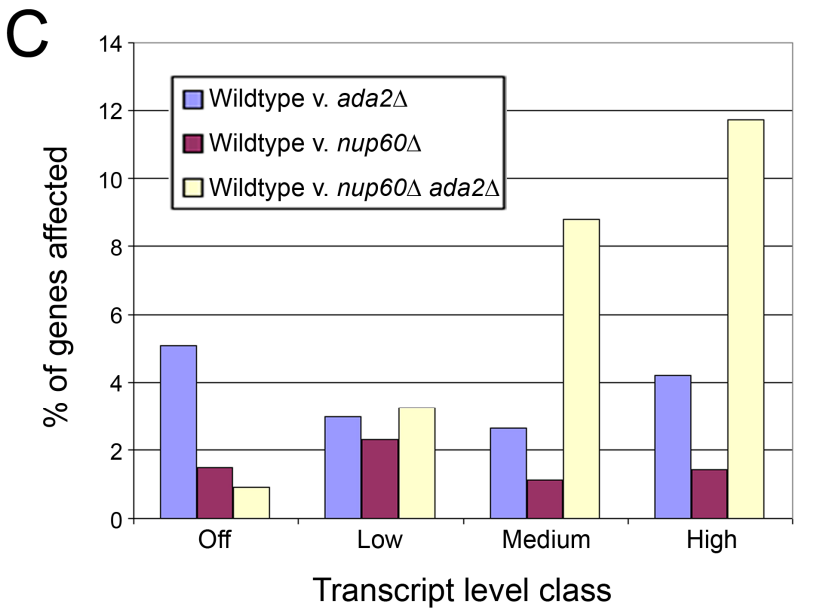

C
% of genes affected
14
12
10
8
6
4
2
0
Wildtype v. ada2Δ
Wildtype v. nup60Δ
Wildtype v. nup60Δ ada2Δ
Off
Low
Medium
High
Transcript level class

Figure 3.6 **Highly transcribed genes are preferentially downregulated in *nup60*Δ *ada2*Δ cells.** ***A,*** Histogram illustrating average transcript level distribution of the yeast transcriptome as previously determined (194) with a bin width of 0.5 units. Transcript level categories (Off, Low, Medium, and High) were determined by visual analysis of the data distribution on a log scale. ***B,*** The number of genes in each transcript level category as determined by histogram analysis. ***C,*** Genes identified as downregulated in *ada2*Δ, *nup60*Δ, and *nup60*Δ *ada2*Δ cells were categorized by their average transcript level in WT cells determined in ***A***. Data are plotted as transcript level class (Off, Low, Medium, or High) *versus* the % of genes in that class identified as downregulated in *ada2*Δ, *nup60*Δ, and *nup60*Δ *ada2*Δ cells.

upstream activating sequence (UAS) and promoter regions (60, 113, 115). In order to investigate whether *nup60*Δ *ada2*Δ cells are defective in the process of gene relocation to the NPC, we utilized a recently developed live-cell microscopy analysis technique to investigate the association the *GAL1* locus with the NPC under activating conditions (148, in preparation). We integrated a tandem array of 256 repeats of the Lac operator (LacO) sequence upstream of the *GAL1* ORF and simultaneously expressed LacI-GFP to visualize the location of the *GAL1* gene (Figure 3.7*A*, arrows). We also expressed a nucleoporin tagged with RFP or YFP to visualize the nuclear periphery. This system allows us to assess whether the *GAL1* locus is adjacent to the nuclear periphery or more centrally located within the nucleus (Figure 3.7*A*). In order to confirm that the *GAL1* promoter is active in these cells, we replaced the *GAL1* ORF with a Ras2-GFP reporter. Ras2-GFP tightly associates with the plasma membrane, allowing us to confirm that *GAL1* is active without masking the nuclear LacI-GFP signal (Figure 3.7*A*).

In order to analyze the association of the active *GAL1* locus with the NPC in live wildtype, *nup60*Δ, *ada2*Δ, and *nup60*Δ *ada2*Δ cells, we visualized these cells using time-lapse microscopy over a four-minute period and classified *GAL1* as attached to or detached from the nuclear periphery in each frame of the time-lapse image. We limited our analysis to those cells where the *GAL1* gene was attached to the nuclear periphery at time zero. Using this approach, we find that *GAL1* remains attached to the nuclear periphery throughout the four minute period in ~90% of wildtype cells (Figure 3.7*B*). In the ~10% of wildtype cells where *GAL1* is not attached, the gene is detached in only one frame of the five-frame time series. In contrast, *GAL1* is detached in at least one frame in ~50% of *ada2*Δ and ~65% of *nup60*Δ cells (Figure 3.7*B*). These results are consistent

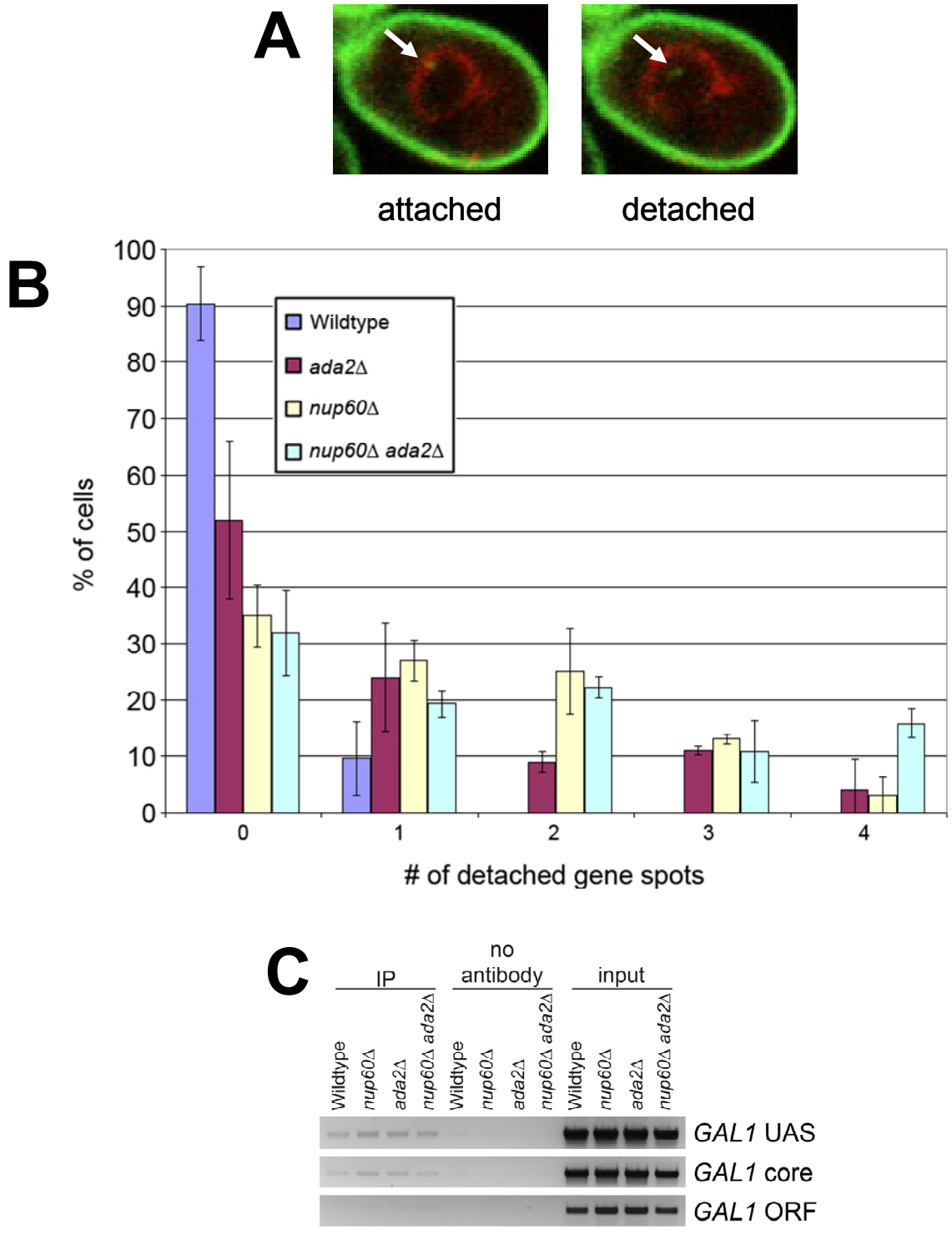

A
attached
detached
B
100
90
80
70
60
50
40
30
20
10
0
% of cells
Wildtype
ada2Δ
nup60Δ
nup60Δ ada2Δ
0
1
2
3
4
# of detached gene spots
C
IP
no antibody
input
Wildtype
nup60Δ
ada2Δ
nup60Δ ada2Δ
GAL1 UAS
GAL1 core
GAL1 ORF

Figure 3.7 ***nup60*Δ *ada2*Δ cells are defective in retention of *GAL1* at the nuclear periphery.** ***A,*** Association of the *GAL1* locus with the nuclear periphery. The LacO array was integrated adjacent to the *GAL1* locus, and LacI-GFP was expressed to visualize gene location (green spot, arrow). Fluorescently tagged NPC subunits were used to visualize the nuclear periphery (yellow ring) to permit categorization of the *GAL1* gene as attached or detached relative to the nuclear periphery. Cells were grown in galactose to induce *GAL1* expression. To verify that cells expressing *GAL1* were analyzed, the *GAL1* ORF was replaced by the Ras2-GFP reporter, which tightly associates with the plasma membrane (green ring). ***B,*** Association of the *GAL1* gene with the nuclear periphery in live wildtype, *ada2*Δ, *nup60*Δ, and *nup60*Δ *ada2*Δ cells. Time-lapse images were analyzed by two independent operators, which a minimum of 100 images analyzed for each cell type. The *GAL1* gene was scored as attached or detached relative to the nuclear periphery in cells expressing the Ras2-GFP reporter and where the *GAL1* gene was attached in the first frame. Cells were categorized by the number of detached gene spots observed over 4 minutes. Error bars represent standard deviation. ***C,*** ChIP to analyze the ability of the active *GAL1* gene to associate with the NPC. Wildtype, *ada2*Δ, *nup60*Δ, and *nup60*Δ *ada2*Δ cells expressing Nup133-TAP were grown in 2% galactose to induce *GAL1* expression and analyzed by ChIP as described in Experimental Procedures. Primers were designed to detect the *GAL1* UAS, core promoter, or ORF.

with the observation that *ada2*Δ cells are defective for retention of the *GAL1* gene at the NPC (148, in preparation). In addition, *nup60*Δ *ada2*Δ cells show even greater defects in gene relocation than the single mutant cells, with *GAL1* detached from the nuclear periphery in four of five frames in ~15% of cells (Figure 3.7*B*). These results suggest that both Nup60 and SAGA play a critical role in the anchoring of active *GAL1* to the NPC. Interestingly, the *GAL1* UAS and core promoter can associate with TAP-tagged Nup133 in *nup60*Δ *ada2*Δ similarly to wildtype, *nup60*Δ, and *ada2*Δ cells as determined by chromatin immunoprecipitation (ChIP) (Figure 3.7*C*), indicating that the *GAL1* locus can physically interact with the NPC in *nup60*Δ *ada2*Δ cells. Consistent with previous reports, the *GAL1* ORF does not associate with TAP-tagged Nup133 (Figure 3.7*C*) (60). Taken together, these results indicate that *GAL1* can physically interact with the NPC in *nup60*Δ *ada2*Δ cells but does not remain associated with the NPC as in wildtype cells, and suggest that *nup60*Δ *ada2*Δ cells are defective for retention, but not recruitment, of *GAL1* to the NPC.

***nup60*Δ *ada2*Δ cells display severe growth defects on galactose media:** The apparent gene retention defect in *nup60*Δ *ada2*Δ cells correlates with genome-wide changes in SAGA-regulated gene expression. This correlation suggests physiological consequences for defects in gene relocation to the NPC. In order to investigate whether defects in *GAL1* relocation to the NPC correlate with physiological consequences, we analyzed the ability of *nup60*Δ *ada2*Δ to metabolize galactose by serial dilution assays with media containing galactose as the sole carbon source (Figure 3.8*A*). Compared to results on glucose-containing plates, *nup60*Δ *ada2*Δ cells show an even more severe growth defect

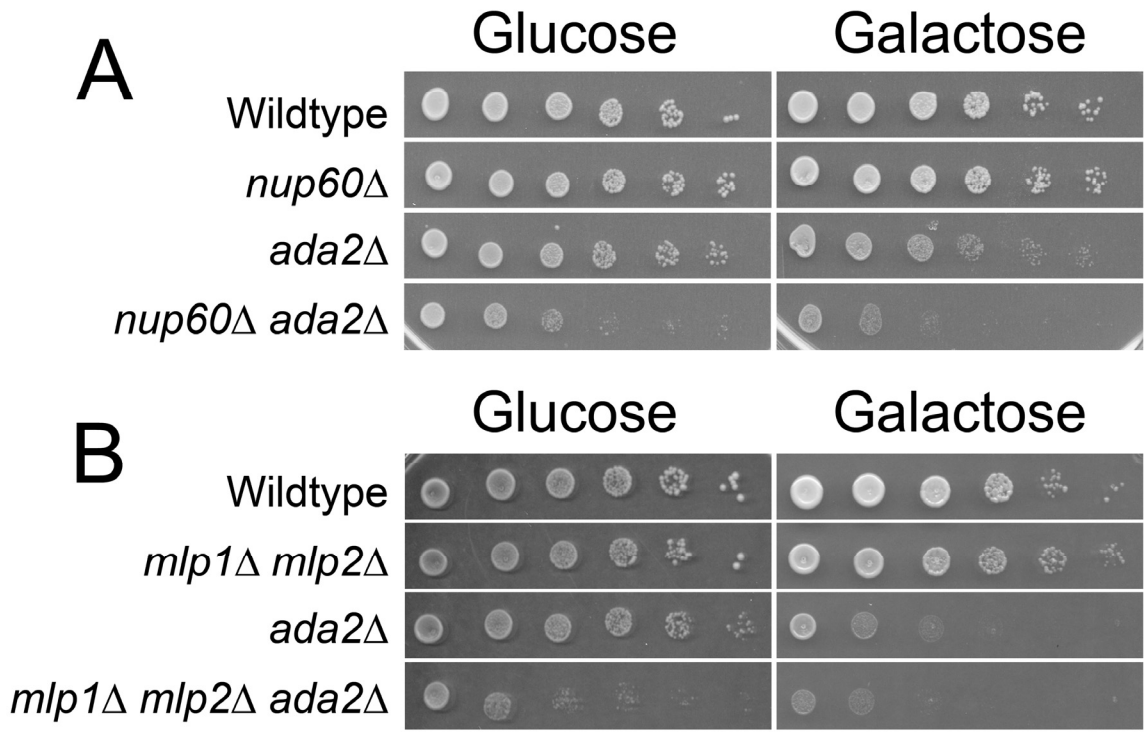


Figure 3.8 ***nup60Δ ada2Δ* cells display severe growth defects on galactose medium.** ***A,B*** Wildtype, *nup60*Δ, *ada2*Δ, and double mutant cells ***A,*** or wildtype, *mlp1*Δ *mlp2*Δ, *ada2*Δ, and triple mutant cells ***B,*** were grown to saturation in raffinose media then 10-fold serially diluted onto glucose or galactose medium and incubated at 30ºC for 2 days.

compared to wildtype and either single mutant cell, indicating that cells lacking *NUP60* and a component of the SAGA complex have a reduced ability to metabolize galactose. Similar results were obtained for cells lacking the *MLP* genes and *ADA2* (Figure 3.8*B*). Taken together, these results are consistent with a model where gene relocation to the NPC is required for proper gene expression.

Discussion

Though the phenomenon of gene relocation to the NPC is well established in the budding yeast *S. cerevisiae*, the physiological significance and global relevance of gene relocation is poorly understood. We provide evidence that interactions between the SAGA histone acetyltransferase complex and components of the NPC are important for regulation of global gene expression. Moreover, these interactions are specifically required for retention of a reporter locus at the NPC, suggesting that global gene expression is dependent on gene association with the NPC. These results are particularly interesting in light of the recent findings that multiple *Drosophila* nucleoporins associate with actively transcribed developmental and cell cycle regulatory genes, and that the upregulation of these genes requires association with particular nucleoporins (93, 94). In addition, this transcriptional regulation by NPC subunits may occur on a global level, as specific *Drosophila* nucleoporins associate with up to 42% of the genome (92). These findings are consistent with our observation of global changes in transcriptional profiles in cells lacking SAGA and NPC components. Taken together, these observations suggest

an evolutionarily conserved role for genome-wide, nucleoporin-mediated transcriptional activation.

Our findings that *GAL1* can associate with the NPC in *nup60*Δ *ada2*Δ as assayed by ChIP, yet *GAL1* does not remain stably associated with the NPC in these cells as observed by live cell time-lapse microscopy, suggest that *GAL1* can contact but cannot remain associated with the NPC in these double mutant cells. This possibility suggests that gene relocation to the NPC consists of multiple stages. Moreover, these findings also potentially explain the apparent discrepancy that gene relocation occurs prior to transcriptional activation, yet requires mRNA processing and export factors such as the THO/TREX elongation and export complex and the Mex67 export factor (59, 181, 195). According to a recruitment-retention model for gene relocation, initial recruitment to the NPC occurs before the activation of transcription and may be mediated by interactions between NPC subunits and transcriptional activators such as Gal4 and SAGA. Subsequent retention of the gene at the NPC may be mediated by these NPC association with transcriptional activators as well as additional NPC interactions with mRNA processing and export factors. According to this model, a requirement for SAGA in gene retention may reflect both protein-protein interactions between SAGA and the NPC, as well as NPC interactions with the mRNA processing and export machinery as a results of SAGA-driven gene activation.

Interestingly, a recruitment-retention model is consistent with recent work in *Drosophila* which identified differential association between NPC subunits and active loci during different stages of transcriptional activation (93). Specifically, *Drosophila* Nup98 and Sec13 associate with genes prior to and during transcriptional activation,

while nucleoporins containing FG-repeats associate once transcription has been established (93). These observations imply different functional roles in transcriptional regulation for different nucleoporins. First, Nup98 and Sec13 may help to recruit the transcriptional machinery and initiate transcriptional activation. Second, the FG nucleoporins may help to establish and maintain efficient transcriptional elongation based on interactions with mRNA processing and export factors (191, 196). Taken together with our findings, these observations suggest an evolutionarily conserved mechanism for transcriptional regulation based on associations of distinct nucleoporins during different stages of gene activation.

In addition, there are intriguing hints that movement of specific active genes to or from sites at the nuclear periphery may also be evolutionarily conserved. The transcriptionally active mouse β-globin gene is located at the nuclear periphery in early erythroid maturation before relocalizing to the nuclear interior (197), and the *var* genes of *Plasmodium falciparum* are shuffled from one distinct perinuclear location to another upon induction (198). These data are consistent with the larger body of work which indicates that the position of a gene within its chromosome territory and its overall location within the nucleus can be correlated with its transcriptional state (20, 150, 151, 199). Our findings extend this work by suggesting that the NPC is a nuclear location involved in global regulation of gene expression.

Experimental Procedures

Strains, Plasmids, and Chemicals: All media were prepared by standard procedures (170). *S. cerevisiae* strains and plasmids used are described in Table 3.5. Deletion mutants were purchased from the *Saccharomyces* Genome Deletion Project (200) unless otherwise noted. Yeast strains containing double and triple deletions were constructed by standard mating and tetrad dissection (170). All chemicals were obtained from Ambion (Austin, TX), Sigma Chemical Co. (St. Louis, MO), US Biological (Swampscott, MA) or Fisher Scientific (Pittsburgh, PA) unless otherwise noted.

Synthetic Genetic Array analysis: Screening of consortium strains with *mlp1*Δ, *mlp2*Δ, *mlp1*Δ *mlp2*Δ, and *nup60*Δ query strains was performed as described (183). After final selection, double- and triple-mutant colonies that displayed synthetic growth defects were scored. Genes that were identified in two independent screens were scored as hits.

Cell fitness analysis: For serial dilution spotting assays, single colonies of wildtype and mutant strains were grown to saturation in liquid culture, normalized to equal starting concentrations, and serially diluted (1:10) in dH_2O and spotted onto selective ura^-, synthetic complete (SC) glucose, SC galactose, or yeast extract peptone dextrose (YPD) medium. Plates were incubated at 30ºC for 2 days. For growth curve analysis, single colonies were grown to saturation overnight, normalized to equal starting concentrations, diluted 1:100 in a 96-well plate, and monitored for growth over time using an ultra microplate reader (Bio-Tek Instruments, Inc). Cells were grown at room temperature with shaking, and the optical density (OD) was measured at 600 nm every 30 min. Data were plotted as OD *versus* time.

Table 3.5 **Strains and plasmids used in Chapter 3**

Strain	Description	Reference/Source
FY23 (ACY192)	*MATa ura leu trp1*	(172)
BY4741 (ACY402)	*MATa his3 leu2 met15 ura3*	(200)
ACY1285	*mlp1Δ::NAT^R can1Δ::STE2pr-Sp-HIS5 his3 leu2 ura3 met15 lyp1Δ::STE3pr-LEU2 cyh2 leu2 MATα*	This Study
ACY1287	*mlp2Δ::NAT^R can1Δ::STE2pr-Sp-HIS5 his3 leu2 ura3 met15 lyp1Δ::STE3pr-LEU2 cyh2 leu2 MATα*	This Study
ACY1289	*mlp2Δ::URA3 mlp2Δ::NAT^R can1Δ::STE2pr-Sp-HIS5 his3 leu2 ura3 met15 lyp1Δ::STE3pr-LEU2 cyh2 leu2 MATα*	This Study
KB0001	*nup60Δ::NAT^R mfa1Δ::MFA1pr-HIS3 can1Δ ura3 leu2 his3 lys2*	This Study
ACY1535	*MATa his3 leu2 met15 ura3 ada2Δ::KAN^R*	(200)
ACY1536	*MATa his3 leu2 met15 ura3 gcn5Δ::KAN^R*	(200)
ACY730	*MATα his3 leu2 ura3 Δmlp1::LEU2 mlp2Δ::HIS3*	Gift from O. Cohen-Fix
ACY1591	*his3 leu2 ura3 mlp1Δ::LEU2 mlp2Δ::HIS3 ada2Δ::KAN^R*	This Study
ACY1593	*his3 leu2 ura3 mlp1Δ::LEU2 mlp2Δ::HIS3 gcn5Δ::KAN^R*	This Study
ACY601	*MATa his3 leu2 met15 ura3 nup60Δ::KAN^R*	(200)
ACY1639	*MATα his3 leu2 met15 ura3 nup60Δ::KAN^R*	This Study
ACY1950	*MATα his3 leu2 met15 ura3 nup60Δ::KAN^R ada2Δ::KAN^R*	This Study
ACY1668	*his3 leu2 met15 ura3 nup60Δ::KAN^R gcn5Δ::KAN^R*	This Study
ACY1029	*MATa his3 leu2 met15 ura3 nup133Δ::KAN^R*	(200)
ACY1917	*his3 leu2 met15 ura3 nup133Δ::KAN^R ada2Δ::KAN^R*	This Study
ACY1918	*his3 leu2 met15 ura3 nup133Δ::KAN^R gcn5Δ::KAN^R*	This Study
ACY721	*MATα his3 leu2 met15 ura3 trp1 nup2Δ::KAN^R*	Gift from K. Belanger
ACY1642	*his3 leu2 ura3 nup2Δ::KAN^R ada2Δ::KAN^R*	This Study
ACY1643	*his3 leu2 ura3 nup2Δ::KAN^R gcn5Δ::KAN^R*	This Study
KEB3052	*MATa his3 leu2 ura3 LacO@GAL1::LEU2 gal1Δ::RAS2-GFP-URA3 NUP49-tDimer2::URA3(5FOA selected) NUP60-tDimer2::KAN^R*	(148, in preparation)
KB1024	*MATa his3 leu2 ura3 LacO@GAL1::LEU2 gal1Δ::RAS2-GFP-URA3 NUP49-tDimer2::URA3(5FOA-selected) NUP60-tDimer2::KAN^R ada2Δ::NAT^R*	(148, in preparation)
ACY2002	*his3 leu2 ura3 LacO@GAL1::LEU2 gal1Δ::RAS2-GFP-URA3(5FOA-selected) nup60Δ::KAN^R*	This Study
ACY2000	*his3 leu2 ura3 LacO@GAL1::LEU2 gal1Δ::RAS2-GFP-URA3(5FOA-selected) nup60Δ::KAN^R ada2Δ::KAN^R*	This Study
ACY2109	*MATa his3 leu2 met15 ura3 gcr2Δ::KAN^R*	(200)
ACY2112	*MATa his3 leu2 met15 ura3 nup133Δ::KAN^R gcr2Δ::NAT^R*	This Study
Plasmids	**Description**	**Reference/Source**
pAC2721	pHKB *LACI-GFP + pGAL-GAL1*, *CEN*, *HIS3*, *AMP^R*	This Study
pAC2722	YCp50 *NSP1-YFP*, *CEN*, *URA3*, *AMP^R*	This Study

Fluorescence *in situ* hybridization (FISH): The intracellular localization of poly(A) RNA was assayed as described (201, 202). Briefly, cells were grown to saturation overnight at 25ºC and subsequently diluted and incubated for 2 h to allow cells to re-enter growth phase. Cells were then shifted to 30ºC for 2-4 h. Cells were fixed with 4.2% formaldehyde. The cell wall was digested with 0.5 mg/mL zymolase, and cells were applied to multi-well slides (Thermo Electron Corporation) pre-treated with 0.1% polylysine. Cells were then permeabilized with 0.5% NP-40, equilibrated with 0.1 M triethanolamine, pH 8.0, and incubated with 0.25% acetic anhydride to block polar groups. Cells were then incubated in prehybridization buffer [50% deionized formamide, 10% dextran sulfate, 4X Sodium Chloride-Sodium Citrate buffer (SSC), 1X Denhardt's solution, 125 µg/mL tRNA] and hybridized overnight to digoxigenin-labeled 50-mer oligo(dT) probe (IDT DNA). Wells were washed several times and blocked in 0.1 M Tris pH 9.0, 0.15 M NaCl, 5% heat-inactivated fetal calf serum, and 0.3% Triton X-100. Cells were incubated 2 hours with fluorescein isothiocyanate (FITC)-conjugated α-digoxigenin antibody (1:200, Roche). Wells were then washed several times and stained with 1 µg/µL 4',6-diamidino-2-phenylindole-dihydrochlorine (DAPI) to detect chromatin. Cells were mounted in antifade medium (0.1% *p*-phenylenediamine, 90% glycerol in phosphate-buffered saline). Slides were stored at -20ºC until visualization. Samples were visualized using filters from Chroma Technology (Brattleboro, VT) and an Olympus BX60 epifluorescence microscope equipped with a photometric Quantix digital camera.

RNA isolation: Cell pellets were collected from mid-log phase cultures, washed with chilled water and stored at -80ºC. Total RNA was isolated using TRIzol reagent

(Invitrogen) according to the manufacturer's protocol, with modification for yeast cells. Briefly, TRIzol reagent and glass beads were added to the cell pellet, and cells were subjected to bead beating in a Mini-Beadbeater-16 (BioSpec Products, Inc) for 2 min to disrupt the cell wall. To induce phase separation, 1-Bromo-3-chloropropane was added to the lysate, and the tubes were vortexed and subjected to centrifugation. RNA was precipitated from the aqueous layer with isopropanol, collected by centrifugation, and washed with 70% ethanol. The RNA pellet was allowed to air-dry before resuspension in RNase-free water.

Gene expression analysis: Total RNA isolated from three biological replicates was treated with DNase (Invitrogen) according to the manufacturer's protocol. RNA was labeled for microarray analysis and hybridization was performed using the GeneChip Yeast Genome 2.0 Array (Affymetrix) by the Emory BioMarker Service Center. Misregulated genes in single and double mutant cells were identified by an average expression change of at least 1.5 fold relative to the isogenic wildtype value. Statistical significance of overlap between gene sets was determined using the hypergeometric distribution as previously described (193). For transcriptional frequency analysis, a histogram was generated based on the average transcript level for each gene as previously determined (194) with a bin width of 0.5 units. Transcriptional frequency categories (off, low, medium, and high) were determined based on histogram distribution.

Single cell time-lapse microscopy: *In vivo* time-lapse microscopy was performed essentially as described (136, 148, in preparation). A Zeiss LSM510 META confocal microscope with a 100× αPlan-Fluar 1.45 NA oil objective lens was used to capture a series of 6 frames with an image taken ever 60 sec of cells grown in selective media

containing 2% galactose. The imaged cells contained 256 repeats of the Lac operator integrated adjacent to the *GAL1* locus, the *GAL1* locus was replaced with a construct encoding Ras2-GFP as a marker for *GAL1* activation, and the cells contained plasmid-borne LacI-GFP and Gal1 as well as plasmid-borne Nsp1-YFP or integrated Nup49-RFP and Nup60-RFP as markers for the NPC. GFP, YFP, and RFP were excited with the 488, 514, and 543 nm lasers and detected with 505-530 BP, 530 LP, and 585 LP filters, respectively. Imaging was performed using a αPlan-Fluar 100×/1.45 NA objective with a depth of focus of 1 μm; resolution was 0.04 μm/pixel. Time lapse was performed over 6 min with an image taken every 60 sec starting at time zero. Cells in which the LacI-GFP spot was attached to the nuclear periphery in the first frame and visible in at least 5 consecutive frames were scored for attachment to or detachment from the NPC using the LSM 5 Image Examiner v3.1.099 software (Carl Zeiss GmbH) as described (148, in preparation). A minimum of 100 images for each cell type were scored by two independent operators.

Chromatin immunoprecipitation (ChIP): Investigation of association of the *GAL1* locus with the NPC by Chip was performed essentially as described (136, 203). Briefly, 100 mL cultures were grown to log phase in galactose media. Formaldehyde was added to a final concentration of 1% and cells were fixed at room temperature for 20 min under gentle shaking. Cross-linking was quenched by addition of glycine (125 mM final concentration) for 10 min. Cells were washed twice with ice-cold water and stored at -80°C until further processing. The pellet was then resuspended in ice-cold lysis buffer (300 mM NaCl, 1 mM EDTA, 1% Triton X 100, 0.1% sodium deoxycholate, 50 mm Hepes-KOH, pH 7.5) containing a protease inhibitor cocktail (0.2 mM PMSF, 1 mM

benzamidine, 1 μg/mL pepstatin A). Glass beads were added to the cell pellet, ad cells were lysed by shaking in a Mini-Beadbeater-16 (BioSpec Products, Inc). Lysed cells were sonicated at maximum power with a 50% duty cycle using a Bioruptor (Diagenode) for 40 minutes in an ice-cold water bath. The supernatant was recovered following centrifugation, and the chromatin solution was cleared by mixing with protein-A Sepharose beads for 1 h at 4°C. Prior to use, protein A-Sepharose was blocked with 0.1 μg/μL salmon sperm DNA, 0.1 μg/μL tRNA, and 1 μg/μL bovine serum albumin for 15 min at 4°C. For the input fraction, 20 μL of cleared chromatin solution was saved and stored at 4°C in elution buffer(1% SDS, 100 mm NaH_2CO_3) before processing with other samples. Cleared chromatin solution was incubated with 5 μg α-TAP antibody (Open Biosystems), or without antibody for the no antibody control, and rotated overnight at 4°C. Protein-A Sepharose was added and suspensions were incubated 2 hours at 4°C. Beads were recovered by centrifugation at 2,000 rpm for 2 min and the supernatant was discarded. Beads were washed sequentially in lysis buffer, lysis buffer containing 500 mM NaCl, and LiCl solution (250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, 10 mM Tris-Cl, pH 8.0). Immunoprecipitated chromatin was eluted from beads by rocking 20 min at in elution buffer. Elution was repeated once and fractions were pooled. To reverse crosslinking, samples were incubated overnight at 65°C then incubated with proteinase K for 1 hr at 42°C. Samples were purified using Qiagen columns, and PCR detection was performed with primers specific to the *GAL1* upstream activating sequence (UAS) (-550 to -250), core promoter (-278 to +79), and the ORF (+350 to +650).

CHAPTER 4

Functional Interactions between Chromatin Modifying Complexes and the Nuclear Pore Play a Role in DNA Damage Repair

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Synthetic genetic array screens were conducted by Milo B. Fasken and Kellie E. Barbara-Haley.

Introduction

Recent work in multiple species has implicated the nuclear periphery in influencing chromatin transactions, including transcriptional activation and DNA damage repair (34, 42-44, 76, 77). The majority of this work has been done in the budding yeast *Saccharomyces cerevisiae*, where specific loci physically relocate from the nuclear interior to the nuclear periphery upon induction and associate with the nuclear pore complex (NPC) (34, 42, 44). Though the physiological relevance of this relocation is not entirely understood, these interactions between active genes and the NPC have been proposed to regulate transcription, facilitate mRNA processing and export, and mediate transcriptional memory of previously active gene states [(34, 42, 44) and Chapter 3]. The NPC has also been linked to repair of DNA damage through interactions with key factors: Ulp1, a desumoylating enzyme involved in DNA damage response, and the DNA damage repair checkpoint proteins, Slx5 and Slx8 (76, 81-83). Moreover, persistent DNA double strand breaks (DSBs) relocate to the NPC, and this movement requires a functional DNA damage checkpoint (76, 77). Taken together, these observations suggest that the NPC may be a site which facilitates transcription and repair of DNA damage.

Much work has been done to elucidate the mechanism of relocation for several specific, highly expressed genes (34, 42, 44). Among the better characterized of these model loci are the galactose-responsive *GAL* genes and the inositol-sensitive *INO1* gene (59, 60, 75, 113, 114, 118, 119). The SAGA histone acetyltransferase complex is required for the relocation of the *GAL* genes to the NPC (60, 113). SAGA is an evolutionarily conserved transcriptional co-activator which regulates transcription of

approximately 10% of the budding yeast genome (111, 112). Several subunits of the SAGA complex have been specifically implicated in the relocation of the *GAL* genes to the NPC, including Ada2, Spt7, and Sus1 (60, 113). Interestingly, the relocation of the *GAL* genes to the NPC is dependent upon the physical presence of the SAGA complex at the *GAL1* gene, rather than its acetyltransferase activity (60), suggesting that SAGA components may be involved in direct interaction with NPC subunits. In addition to the established role for SAGA in transcription, there are also potential links between SAGA and DNA damage repair (167, 204, 205). Two of the human SAGA counterparts, STAGA and TFTC, contain a subunit involved in response to UV-induced DNA damage, and TFTC shows greater acetylation activity toward damaged DNA templates *in vitro* (167, 205). More recently, a genome-wide screen of a *Schizosaccharomyces pombe* gene deletion library identified SAGA mutants as sensitive to DNA damaging agents (204). While a role in DNA damage repair is still being elucidated, it is interesting to speculate that SAGA's function at the NPC may regulate both transcriptional activation and DNA damage response.

Although the mechanism of relocation of the *INO1* gene to the NPC is not as well elucidated as for the *GAL* genes, transcriptional regulation of *INO1* is fairly well characterized. *INO1* transcription is controlled by the evolutionarily conserved INO80 complex, an ATP-dependent chromatin remodeling complex which remodels nucleosomes to create an open chromatin structure that is accessible to the transcription machinery (123-125). The nominative Ino80 subunit is responsible for the majority of the ATPase activity of the INO80 complex (123), and the *INO80* gene is essential in some budding yeast genetic backgrounds (200), attesting to the importance of this protein

to normal cell physiology. In addition to regulating transcription of specific genes, the INO80 complex also has a well characterized role in DNA damage repair, particularly repair of double strand breaks (DSBs) (128-132). Analogous to its role in transcriptional activation, Ino80 is thought to evict nucleosomes to permit repair enzymes access to the damaged DNA (129). Thus the INO80 complex functions in multiple contexts to mediate active DNA transactions.

Much research has established links between the INO80 complex and both transcription and DSB repair. As both DSBs and the INO80-regulated *INO1* gene relocate to the NPC, we wondered whether the INO80 complex functionally interacts with the NPC. Moreover, given the recent work implicating both the NPC and SAGA in repair of DNA damage in addition to their roles in transcriptional activation, we wondered whether the SAGA complex also has links to DNA damage repair as part of its role in mediating DNA transactions at the NPC. Here we present data identifying genetic interactions between INO80 subunits and multiple components of the NPC which have been implicated in relocation of actively transcribed genes. These functional links between INO80 and the NPC suggest a global role for INO80 in mediating DNA transactions at the NPC. We also find that cells expressing a mutant *INO80* allele in combination with loss of an NPC subunit show increased sensitivity to DNA damaging agents, as do cells deleted for both a SAGA subunit and an NPC subunit. These data suggest that interactions between chromatin modifying complexes (CMCs) and the NPC are important for facilitating repair of DNA damage. Moreover, we find that double mutant cells lacking a SAGA subunit and an NPC subunit show elevated levels of endogenous DSBs, suggesting that these cells have impaired DNA damage response and

for the first time implicating the *S. cerevisiae* SAGA complex in repair of DSBs. Taken together, these finding suggest a generalized role for CMCs in creating an open DNA state amenable to DNA transactions at the NPC.

Results

Components of the NPC display synthetic interactions with components of the INO80 chromatin remodeling complex: Previous analyses revealed functional interactions between the multiple subunits of the NPC and components of the SAGA histone acetyltransferase complex (Chapter 3). These functional interactions were initially identified by synthetic genetic array (SGA) analysis (183) carried out for cells lacking the *MLP* or *NUP60* genes which encode subunits of the NPC nuclear basket (46, 147). SGA analysis identifies genetic interactions between particular genes as indicated by growth defects in cells lacking those genes (183). Such genetic interactions are often indicative of shared function (133), making this genome-wide analysis a valuable tool for identifying novel functional interactions between genes. In addition to components of the SAGA complex (Chapter 3), among the synthetic interactions identified by both *MLP* and *NUP60* SGA preliminary analyses were subunits from multiple chromatin modifying complexes (CMCs) and other transcriptional regulators (Table 4.1), consistent with a model where CMCs and other transcription mediators recruit actively transcribed genes to the NPC. In particular, these SGA results indicate that multiple components of the INO80 chromatin remodeling complex interact with the NPC (Figure 4.1). It is noteworthy that the nominative subunit of the INO80 complex, Ino80, is essential in

Table 4.1 **Transcriptional regulators identified in *MLP* and *NUP60* SGA screens.**

Complex/function	Gene	Interaction identified with
INO80, chromatin remodeling complex	*ARP5*	*MLP1*, *MLP2*
	ARP8	*MLP1*, *MLP2*, *NUP60*
	NHP10	*MLP1*, *MLP2*
	TAF14	*MLP1*
Mediator, transcriptional co-activator	*CSE2*	*MLP1*, *MLP2*
	ROX3	*MLP1*, *MLP2*
	SOH1	*MLP1*, *MLP2*
	SRB2	*MLP1*, *MLP2*, *NUP60*
	SRB5	*MLP1*, *MLP2*, *NUP60*
	SRB8	*MLP1*, *MLP2*
NuA4, histone acetyltransferase	*YAF9*	*MLP1*, *MLP2*, *NUP60*
Paf1, RNA polymerase II associated factor	*CDC73*	*MLP1*, *MLP2*, *NUP60*
	LEO1	*MLP1*, *MLP2*, *NUP60*
	PAF1	*MLP1*, *MLP2*, *NUP60*
RSC, chromatin remodeling complex	*HTL1*	*MLP1*, *MLP2*
	NPL6	*MLP1*, *MLP2*, *NUP60*
	RSC1	*MLP1*, *MLP2*
	RSC2	*MLP1*, *MLP2*, *NUP60*
	TAF14	*MLP1*
Set1/COMPASS, chromatin remodeling complex	*BRE2*	*MLP1*, *MLP2*
	SPP1	*NUP60*
	SWD1	*MLP1*, *MLP2*, *NUP60*
	SWD3	*MLP1*, *MLP2*, *NUP60*
SWI/SNF, chromatin remodeling complex	*SNF2*	*MLP1*, *MLP2*, *NUP60*
	SNF5	*MLP1*, *MLP2*
	SNF6	*MLP1*, *MLP2*, *NUP60*
	SNF11	*NUP60*
	SWI3	*MLP1*, *MLP2*, *NUP60*
	TAF14	*MLP1*
SWR1, chromatin remodeling complex	*ARP6*	*NUP60*
	SWC3	*NUP60*
	SWC5	*MLP1*, *MLP2*, *NUP60*
	YAF9	*MLP1*, *MLP2*, *NUP60*
RNA polymerase II holoenzyme	*BUR2*	*MLP1*, *MLP2*
	RPB4	*MLP1*, *MLP2*
	RPB9	*MLP2*, *NUP60*
	RTR1	*NUP60*
	SSN3	*MLP1*, *MLP2*
	SSN8	*MLP1*, *NUP60*
Transcription factors	*AFT1*	*MLP1*, *MLP2*, *NUP60*
	FZF1	*MLP1*, *MLP2*, *NUP60*
	GCN4	*MLP1*, *MLP2*, *NUP60*
	INO2	*MLP1*, *MLP2*, *NUP60*
	INO4	*MLP1*, *MLP2*, *NUP60*
	RPN4	*NUP60*
	SFP1	*MLP2*, *NUP60*
	SMP1	*NUP60*
	STB5	*MLP1*, *NUP60*
	STP1	*NUP60*
Other transcriptional regulators	*BRE1*	*MLP1*, *MLP2*, *NUP60*
	MET18	*NUP60*
	RAD6	*MLP1*, *MLP2*, *NUP60*
	SPT4	*MLP1*, *MLP2*, *NUP60*

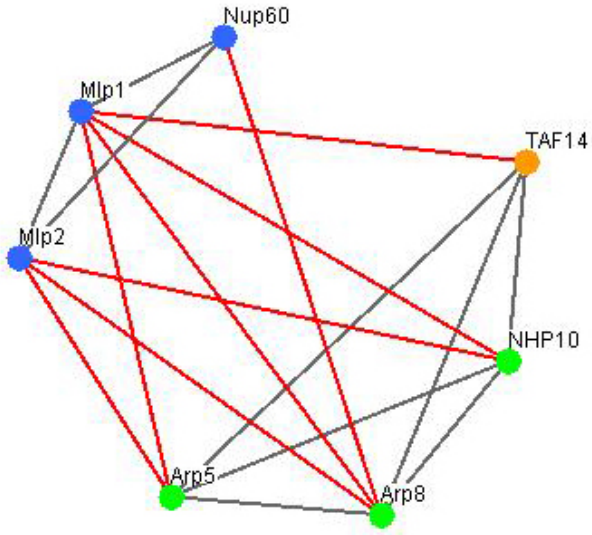

Figure 4.1 **The *MLP* and *NUP60* genes functionally interact with genes encoding INO80 subunits.** Osprey diagram (187) depicting interactions between Mlp1, Mlp2, Nup60 (blue nodes), INO80 subunits (green nodes), and the INO80/TFIID-shared TAF, TAF14 (orange node). Red lines represent novel interactions identified in this study and grey lines represent known published interactions.

some backgrounds and therefore is not included in the SGA screening collection (183, 200). Similar to previous results identifying interactions between SAGA and Nup60 in regulating genome-wide transcript levels (Chapter 3), these results suggest that the Mlp proteins and Nup60 may interact with the INO80 complex to regulate transcription and other DNA interactions on a global scale.

Nup60 anchors the Mlp proteins to the NPC, and disruption of *NUP60* results in mislocalization of the Mlp proteins in the nucleoplasm (182). We took advantage of this phenomenon in *nup60*Δ cells to determine whether the location of the Mlp proteins at the NPC is important for their functional interaction with the INO80 complex by creating double mutant cells combining the deletion of the *NUP60* gene with the *ino80-1* mutant allele of the *INO80* gene (125). We investigated the fitness of these double mutant cells using a serial dilution cell growth assay. Because *ino80-1* cells fail to grow at 37°C on media lacking inositol (ino^-) (125), we assayed fitness of *nup60*Δ *ino80-1* cells on rich media to uncover more subtle genetic interactions that would otherwise be masked by this severe growth defect on ino^- media. Serial dilution assays reveal that *nup60*Δ *ino80-1* cells grow normally compared to wildtype or single mutant cells at room temperature, but display severe growth defects at 37°C even on rich media (Figure 4.2*A*), suggesting that the location of the Mlp proteins at the NPC is important for proper INO80 function.

To extend our investigation into the links between INO80 and the NPC, we tested whether *INO80* displays synthetic interactions with two other NPC subunits that have been linked to transcriptionally active genes, Nup2 and Nup84 (75, 115, 152). Serial dilution assays of double mutant cells deleted for *NUP2* in combination with the temperature sensitive *ino80-1* mutant allele (*nup2*Δ *ino80-1*) reveal a slight growth defect

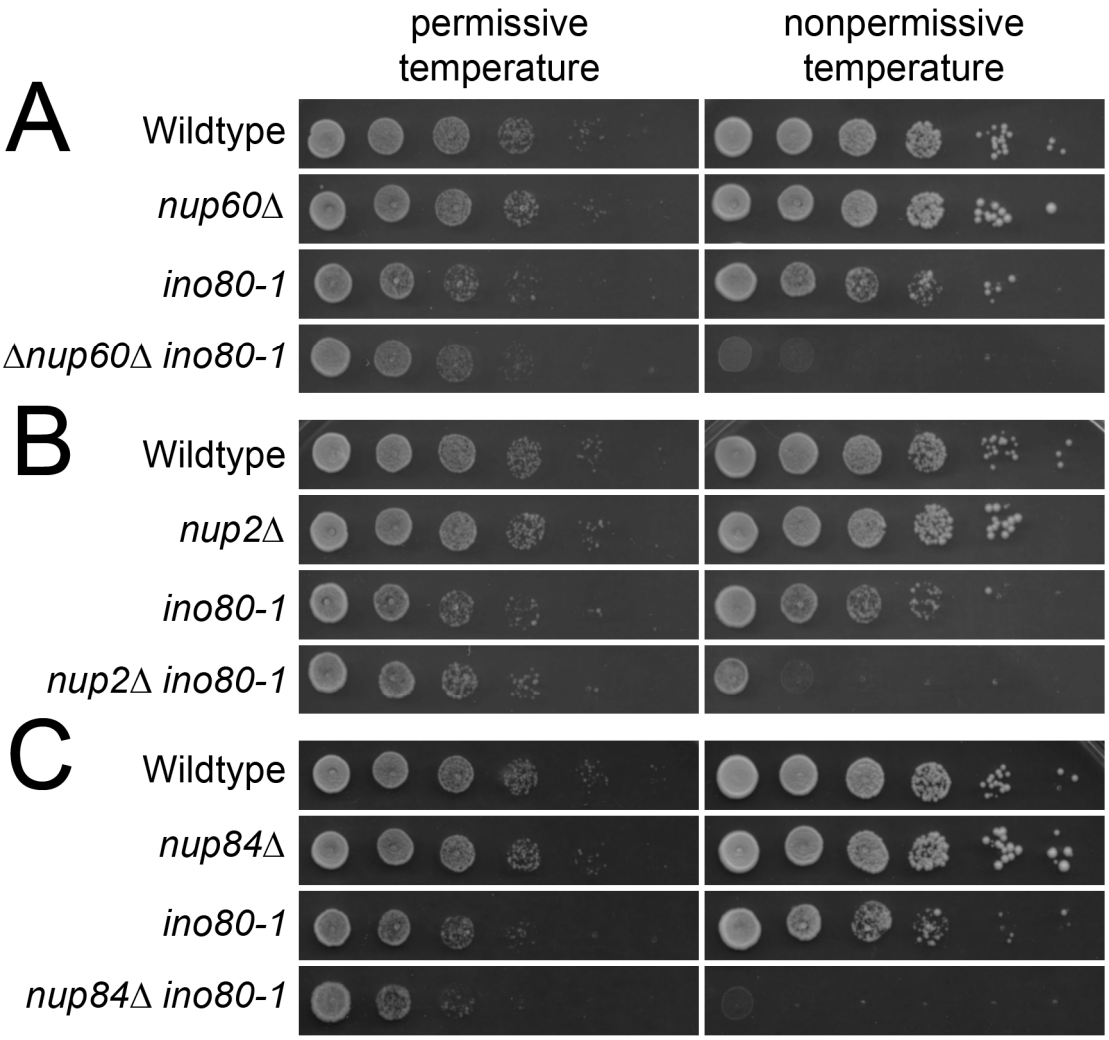

Figure 4.2 **The *NUP60*, *NUP2*, and *NUP84* genes functionally interact with *INO80*.** Wildtype, single, and double mutant cells were grown to saturation in rich media then 10-fold serially diluted onto rich medium and incubated for 2 days at 25ºC (permissive temperature) or 37ºC (nonpermissive temperature). This serial dilution assay was performed to assess fitness of ***A***, Wildtype, *nup60*Δ, *ino80-1*, and *nup60*Δ *ino80-1* cells ***B***, Wildtype, *nup2*Δ, *ino80-1*, and *nup2*Δ *ino80-1* cells, and ***C***, Wildtype, *nup84*Δ, *ino80-1*, and *nup84*Δ *ino80-1* cells.

in these double mutant cells at the permissive temperature and a severe growth defect at the nonpermissive temperature compared to wildtype and single mutant cells (Figure 4.2*B*). In addition, serial dilution assays of *nup84*Δ *ino80-1* cells reveal a significant growth defect in double mutant cells compared to wildtype and single mutant cells at the nonpermissive temperature but not the permissive temperature (Figure 4.2*C*), consistent with results for Nup2 and Nup60.

Double mutant cells are sensitive to DNA-damaging agents: Our results indicate that there are functional interactions between the INO80 complex and multiple components of the NPC which have been implicated in recruitment of actively transcribed genes (62, 75, 115, 152). In addition to playing a role in transcription, the INO80 complex is also a well established modulator of DNA damage response, particularly double strand break (DSB) repair (85, 134). Recently, the NPC has also been implicated in repair of DNA damage (81, 82), and persistent DSBs are relocated to the NPC (76, 77) in a manner similar to some actively transcribed genes. Moreover, the human and more recently the *S. pombe* SAGA homologues have been linked to DNA damage response (167, 204, 205), though their role in repair is far less understood than that of INO80. These observations raised the possibility that disruption of either the INO80 complex or the SAGA complex in combination with deletion of genes encoding NPC subunits might results in cells that are compromised for DNA damage repair. In order to investigate this possibility, we combined either the *ino80-1* mutation or deletion of the gene encoding the Ada2 subunit of SAGA with deletion of genes encoding NPC subunits. We then tested these cells for sensitivity to a variety DNA damaging agents

including bleomycin which induces single strand breaks (SSBs) at low concentrations and DSBs at high concentrations (206), methyl methanesulfonate (MMS) which forms DNA adducts that result in SSBs and DSBs (207), hydroxyurea (HU) which inhibits ribonucleotide reductase, depleting the nucleotide pool and resulting in stalled replication forks (208), and ultraviolet irradiation (UV) which results in bulky DNA lesions that can lead to strand breaks (209).

As expected, wildtype cells grow similarly on control plates and experimental plates exposed to bleomycin, UV, MMS, and HU (Figure 4.3, *Controls*). In contrast, control DNA damage mutants *rad6*Δ and *rad52*Δ show varying sensitivities to bleomycin, UV and MMS, and HU (210-212). Consistent with previous results, *ino80-1* cells show sensitivity to HU and UV (123), as well as slight sensitivity to bleomycin and MMS (Figure 4.3, *Single mutants*). *ADA2* deletion cells show slight sensitivity to bleomycin and sensitivity to UV, consistent with findings that two of the human SAGA homologs, STAGA and TFTC, play a role in UV damage response (167, 205). *NUP60* deletion cells show sensitivity to UV, as has been previously reported (213) and MMS. *NUP2* deletion cells also show sensitivity to UV and severe sensitivity to MMS. Interestingly, *ino80-1* or *ada2*Δ in combination with either *nup60*Δ or *nup2*Δ results in double mutant cells sensitive to nearly all DNA damaging agents tested (Figure 4.3, *Double mutants*). *nup60*Δ *ino80-1* mutants show severe sensitivity to MMS compared to single mutant cells, and *nup2*Δ *ino80-1* are more sensitive to bleomycin than single mutant cells. In addition, *nup60*Δ *ada2*Δ cells are sensitive to bleomycin, MMS, and HU and slightly sensitive to UV compared to single mutant cells. *nup2*Δ *ada2*Δ cells are more sensitive to bleomycin and HU and again show slight sensitivity to UV compared to

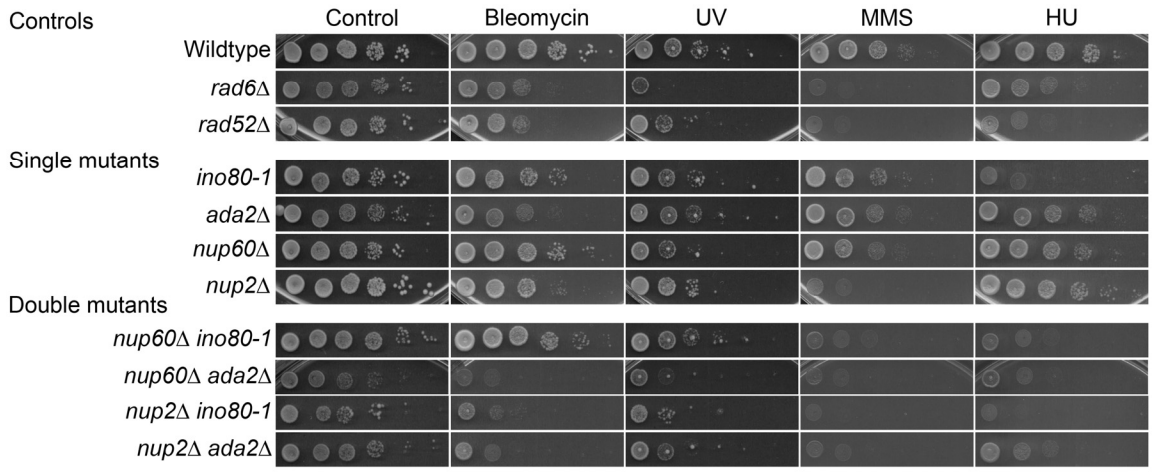

Figure 4.3 **Double mutant cells are sensitive to DNA damaging agents.** Wildtype, *ada2*Δ, *ino80-1*, *nup60*Δ, *nup2*Δ, and double mutant cells were grown to saturation in rich media then 10-fold serially diluted onto rich medium or rich medium containing bleomycin (3 µg/mL), MMS (0.5%), or HU (100 mM) and incubated 2 days at 30ºC. For UV irradiation, plates were exposed to UV (20 mJ/cm^2) following serial dilution.

single mutant cells. Taken together, these results suggest that interactions between NPC subunits and CMCs are important for handling of DNA damage, and that different NPC subunits show different functional interactions with specific CMCs.

***nup60*Δ *ada2*Δ cells show elevated levels of non-induced DSBs:** While the role of the INO80 complex in DNA damage repair has been extensively studied (85, 134), the role of SAGA in DNA damage response is less understood (204, 214). The finding that *nup60*Δ *ada2*Δ cells are sensitive to bleomycin and MMS (Figure 4.3), both of which result in DSB formation, led us to speculate that the SAGA complex may functionally interact with Nup60 to facilitate repair of DSBs. We therefore used a Rad52-YFP reporter assay to investigate whether *nup60*Δ *ada2*Δ cells show elevated levels of non-induced DSBs. Rad52 accumulates at sites of DSBs repaired by homologous recombination, and these sites can be visualized as Rad52-YFP foci (215). Analysis of Rad52-YFP foci in *nup60*Δ *ada2*Δ cells reveals a significant increase in the number of Rad52 foci as compared to wildtype (p=0.004), *nup60*Δ (p=0.01), or *ada2*Δ (borderline significant, p=0.05) cells (Figure 4.4*A*,*B*). Interestingly, *ada2*Δ cells also show significantly greater numbers of Rad52 foci than wildtype (p=0.01) and *nup60*Δ (p=0.04) cells. Moreover, *nup60*Δ *ada2*Δ cells are more likely to exhibit two or more Rad52 foci than wildtype or single deletion cells (Figure 4.4*C*), indicating multiple sites of Rad52-mediated homologous recombination. These results suggest that Nup60 and SAGA together contribute to repair of DNA damage and for the first time implicate the *S. cerevisiae* SAGA complex in DSB repair.

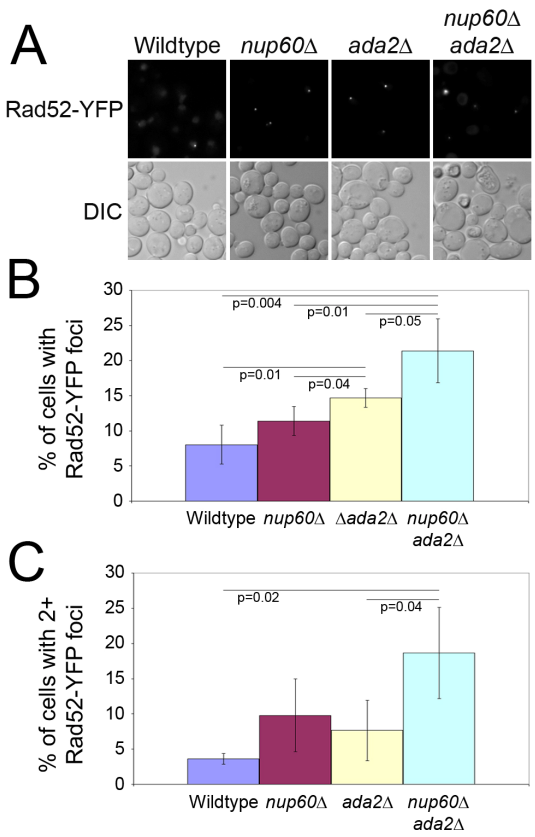

Figure 4.4 ***nup60*Δ *ada2*Δ cells display increased numbers of Rad52-YFP foci.** ***A***, Live wildtype, *nup60*Δ, *ada2*Δ, and *nup60*Δ *ada2*Δ cells expressing Rad52-YFP were examined by direct fluorescence microscopy to visualize Rad52-YFP foci. Corresponding DIC images are shown. ***B***, Quantification of Rad52-YFP foci. A minimum of 3,000 cells were analyzed for each cell type in triplicate. The percentage of cells showing Rad52-YFP foci is plotted for wildtype, *nup60*Δ, *ada2*Δ, and *nup60*Δ *ada2*Δ cells. Error bars indicate standard deviation, and p-values were determined using an unpaired Student's *t*-test assuming unequal variance. ***C***, Quantification of cells containing more than one Rad52-YFP focus. The percentage of cells showing >1 Rad52-YFP focus is plotted for wildtype, *nup60*Δ, *ada2*Δ, and *nup60*Δ *ada2*Δ cells. Error bars indicate standard deviation, and p-values were determined using an unpaired Student's *t*-test assuming unequal variance.

Discussion

Our results reveal functional interactions between two chromatin modifying complexes and the NPC, and, moreover, these interactions are important in repair of DNA damage. These findings complement and extend the growing body of work which links the NPC to actively transcribed genes and repair of DSBs (34, 42, 44, 76, 77) in several important ways. First, functional interactions between NPC components and the SAGA complex have been previously found to be important in regulating genome-wide transcript levels and retention of the *GAL1* locus at the NPC (Chapter 3). Here we report newly identified functional interactions between multiple NPC subunits and the INO80 complex. These findings are consistent with a role for INO80 similar to that of SAGA in genome-wide regulation of transcript levels and relocation of genes to the NPC. Though we did not test for direct interactions between NPC and INO80 subunits, such interactions would be consistent with previous findings of direct interactions between SAGA and the NPC which mediate relocation of active *GAL* genes to the NPC (60).

In addition, we find that cells mutated for *INO80* in combination with loss of an NPC subunit are sensitive to DNA damaging agents. These findings are particularly intriguing given the recent observation that persistent DSBs relocate to the NPC (76, 77), though the physiological relevance of this relocation is unexplored. Considering that INO80 has a well-established role in repair of DSBs (128-132), the finding that these double mutant cells show increased sensitivity to DNA damaging agents which induce DNA DSBs is consistent with a role for INO80 in mediating DSB repair at the NPC. We also find that cells lacking the Ada2 subunit of the SAGA complex in combination with

loss of an NPC component show increased sensitivity to multiple DNA damaging agents, suggesting that interactions between SAGA and the NPC are important for mediating DNA repair. Taken together, these observations point toward a role for both INO80 and SAGA in mediating DNA damage repair at the NPC. Moreover, these results are significant in that they hint that DSB relocation to the NPC may be a physiologically important mechanism for DNA damage repair, rather than an artifact induced by persistent DSBs.

The novel finding that *ada2*Δ cells have elevated levels of endogenous DSBs compared to wildtype cells further implicates the *S. cerevisiae* SAGA complex in repair of this form of DNA damage. Given that many CMCs including the ATP-dependent chromatin remodelers INO80, ISW2, RSC, SWI/SNF, and SWR1, the histone acetyltransferase NuA4, and now more definitively, SAGA, have all been implicated in the DNA damage response (216, 217), these observations suggest that CMCs play dual roles in both regulating the transcription of particular target genes and modulating the accessibility of chromatin for the purpose of DNA damage repair. In addition, the incidence of DSB occurrence is increased in cells lacking both Ada2 and the Nup60 subunit of the NPC, further suggesting that the NPC plays a role with SAGA in facilitating the repair of these DSBs. Taken together, our results are consistent with a model where CMCs mediate interactions between chromatin and the NPC, and provide evidence for the possibility that the NPC may represent a nuclear location that promotes an open chromatin structure to facilitate active DNA transactions such as transcription and DNA repair.

Experimental Procedures

Strains, plasmids, and chemicals: All DNA manipulations were performed according to standard methods (169) and all media were prepared by standard procedures (170). All *S. cerevisiae* strains used are described in Table 4.2. The Rad52-YFP plasmid (pWJ1213) was a gift from R. Rothstein (218). Single deletion strains were purchased from the *Saccharomyces* Genome Deletion Project (200) unless otherwise noted. Yeast strains containing double and triple deletions were constructed by standard mating and tetrad dissection procedures. All chemicals were obtained from Ambion (Austin, TX), Sigma Chemical Co. (St. Louis, MO), US Biological (Swampscott, MA) or Fisher Scientific (Pittsburgh, PA) unless otherwise noted.

Fitness analysis: For serial dilution spotting assays, single colonies of wildtype and mutant strains were grown to saturation in liquid culture overnight, normalized to equal starting concentrations, and serially diluted (1:10) in dH_2O and spotted onto rich media plates. Plates were incubated at 25 or 37ºC for 2-4 days. For drug sensitivity assays, cell cultures normalized to equal starting concentrations were serially diluted onto rich media plates, or rich media plates containing bleomycin (3 μg/mL), methyl methanesulfonate (MMS) (0.5%), or hydroxyurea (HU) (100 mM). For ultraviolet (UV) irradiation sensitivity assays, cells were serially diluted onto rich media plates and irradiated with 20 mJ/cm^2. Plates were incubated at 30ºC for 2-4 days.

Endogenous double strand break analysis: Live cells expressing plasmid-borne Rad52-YFP under the endogenous *RAD52* promoter (218) were grown to log phase and visualized using an Olympus BX60 epifluorescence microscope equipped with Chroma Technology filters (Brattleboro, VT) and a photometric Quantix digital camera. Images

Table 4.2 **Strains and plasmids used in Chapter 4.**

Strain	Description	Reference/Source
BY4741 (ACY402)	*his3 leu2 met15 ura3 MATa*	(200)
ACY1285	*mlpΔ 1::NAT^R can1Δ::STE2pr-Sp-HIS5 his3 leu2 ura3 met15 lyp1Δ::STE3pr-LEU2 cyh2 leu2 MATα*	(Chapter 3)
ACY1287	*mlp2Δ::NAT^R can1Δ::STE2pr-Sp-HIS5 his3 leu2 ura3 met15 lyp1Δ::STE3pr-LEU2 cyh2 leu2 MATα*	(Chapter 3)
ACY1289	*mlp2Δ::URA3 mlp2Δ::NAT^R can1Δ::STE2pr-Sp-HIS5 his3 leu2 ura3 met15 lyp1Δ::STE3pr-LEU2 cyh2 leu2 MATα*	(Chapter 3)
KB0001	*nup60Δ::NAT^R mfa1Δ::MFA1pr-HIS3 can1 ura3 leu2 his3 lys2*	(Chapter 3)
JS95.2-4 (ACY1483)	*ino80-1 ura3 leu2 MATa*	(125)
ACY1639	*nup60Δ::KAN^R his3 leu2 met15 ura3 MATα*	(Chapter 3)
ACY721	*nup2Δ::KAN^R his3 leu2 met15 ura3 trp1 MATα*	Gift from Ken Belanger
ACY1034	*nup84Δ::KAN^R his3 leu2 met15 ura3 MATα*	Gift from Scott Devine
ACY1535	*ada2Δ::KAN^R his3 leu2 met15 ura3 MATa*	(200)
ACY1536	*gcn5Δ::KAN^R his3 leu2 met15 ura3 MATa*	(200)
ACY1823	*nup60Δ::KAN^R ino80-1 ura3 leu2 his3*	This Study
ACY1822	*nup2Δ::KAN^R ino80-1 ura3 leu2 his3 trp1*	This Study
ACY1824	*nup84Δ::KAN^R ino80-1 ura3 leu2 his3*	This Study
ACY1998	*rad6Δ::KAN^R ura3 leu2 his3 MATa*	(200)
ACY1999	*rad52Δ::KAN^R ura3 leu2 his3 MATa*	(200)

were captured using IP Lab Spectrum software. For analyses of Rad52-YFP foci, a minimum of 3,000 cells were analyzed for each strain in triplicate using ImageJ software, and percentages of cells exhibiting at least one Rad52-YFP focus were calculated. Unpaired Student's *t*-test assuming unequal variance was used to determine statistical significance.

CHAPTER 5

Conclusions and Discussion

The discovery approximately six years ago that active genes associate with the NPC in the budding yeast *S. cerevisiae* unraveled years of scientific dogma that the nuclear periphery was exclusively repressive to transcription (40, 62). Though this phenomenon has now been generally accepted into the model for gene activation in budding yeast, there remain critical questions regarding the mechanisms, physiological ramifications, and evolutionary conservation of this gene relocation. The work presented here enhances and extends our current understanding of this phenomenon regarding both mechanism and functional relevance; however, ultimately these studies merely scratch the surface of the remaining unknowns regarding this phenomenon. Here we offer insights and speculations on the mechanism, physiological relevance, and evolutionary conservation of gene association with the NPC.

The Mechanism of Gene Relocation

The characterization of Gal4 and other transcriptional activators nearly twenty years ago seemed to largely answer the fundamental question of how transcription of eukaryotic genes is regulated (219). In what is now considered the textbook model of gene-activation, transcription was proposed to proceed through an ordered series of steps beginning with the binding of a transcriptional activator to regulatory elements and culminating in the ordered recruitment of the general transcription factors and RNA polymerase II (220). We now understand that transcriptional activation is not only much more complex than this step-wise model, requiring a host of different factors to affect

chromatin accessibility and mediate polymerase recruitment and progression, but also highly tailored to each particular gene (221-225).

Though the study of gene relocation to the NPC is a relatively new undertaking, there are already such a large number of factors implicated in the process that it is difficult to imagine any model for gene relocation that is not gene-specific (34, 44, 180). Lending support to this hypothesis, several studies have found that altering the promoter or 3'-end of a gene can affect the relocation of that gene (135, 137); suggesting that the presence of particular transcriptional regulators dictates gene relocation rather than the activity or presence of RNA polymerase II itself. In addition, association of active genes with the NPC is globally altered upon induction of a specific transcriptional program (61), suggesting that changes may occur as a result of interactions between the NPC and the transcriptional regulators specific to these genes. Finally, the active genes which have thus far been found to relocate to the NPC, which include *FIG2*, *GAL1-10*, *GAL2*, *HSP104*, *HXK1*, *INO1*, *SUC2*, and *TSA2*, are not part of one transcriptional program (59-62, 75, 113, 114, 118, 119, 135, 136); rather, their transcription is regulated by distinct factors, consistent with a model where relocation to the NPC is dictated by distinct regulatory factors for different genes.

Although the process of gene relocation is likely specific for different genes, there are some inferences and conclusions which can be made regarding a general mechanism for this phenomenon. First, relocation to the NPC occurs prior to transcriptional activation and does not require the production of mRNA (59, 75). Second, based on work presented here, gene association with the NPC is mediated by protein-protein interactions between Nups and the factors which regulate transcription of relocated genes

(Chapter 2) (Figure 5.1*A*). This generalization is consistent with the observation that relocation to the NPC does not require transcription (75); instead, it is likely to occur prior to transcription initiation as the regulatory factors assemble at the promoter. Here we primarily focus on interactions between the SAGA complex and the NPC, but we anticipate that relocation of other genes is dependent upon interactions with other CMCs and transcriptional regulators. Indeed, this prediction is consistent with our findings that NPC subunits functionally interact with multiple components of other CMCs and transcriptional regulators, including the histone acetyltransferase complex NuA4, the chromatin remodeling complexes INO80, RSC, SWI/SNF, and SWR1, and a host of gene-specific transcription factors (Chapter 4).

The initial recruitment of a gene to the NPC is only one of several apparent stages of gene relocation to the NPC. We find that gene recruitment as measured by chromatin immunoprecipitation (ChIP) can be separated from gene retention at the NPC as assessed by time-lapse microscopy (Chapter 3). These processes are likely mediated by different protein-protein interactions between transcriptional regulators and different NPC components. This hypothesis helps to explain several apparently contradictory observations. First, one study found that the Mlp1 subunit of the NPC is not required for gene relocation, while another found the opposite (59, 113). In addition, another study reported a requirement for Nup2 in *GAL* gene relocation to the NPC, while our analysis found no functional interactions between Nup2 and the SAGA complex which regulates *GAL* gene expression [(75) and Chapter 3]. These discrepancies could potentially be explained by a requirement for these NPC subunits in either retention but not initial recruitment, or recruitment but not retention, of active genes to the NPC.

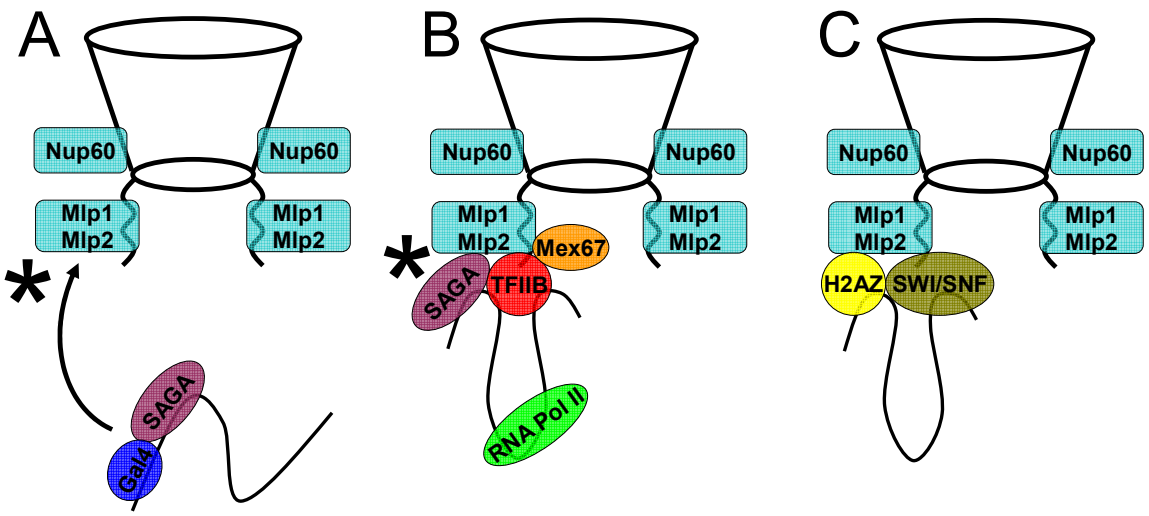

Figure 5.1 **A model for multiple stages of gene relocation to the NPC.** Asterisks denote interactions proposed based on work presented in this dissertation. ***A*,** The *GAL* genes are recruited to the nuclear basket of the NPC based on the physical presence of Gal4, SAGA, and other transcriptional activators (Chapter 2) prior to transcriptional initiation (75). ***B*,** Following transcriptional activation, the recruited gene forms a gene loop facilitated by TFIIB and other components of the transcriptional machinery (144, 226). Retention of relocated genes may be mediated by interactions between NPC subunits and both transcriptional activators such as SAGA (Chapter 3) and between mRNA quality surveillance, processing, and export factors which are co-transcriptionally recruited to the active gene. Such factors include the nuclear exosome which degrades aberrant mRNAs, the THO/TREX transcriptional elongation and mRNA export complex, and the Mex67 mRNA export factor (59, 195, 227). ***C*,** After transcriptional shut-off, genes which exhibit transcriptional memory remain associated with the NPC in the form of a gene loop (142). Persistence of the gene loop and the resulting transcriptional memory require the histone variant H2AZ, the SWI/SNF chromatin remodeling complex, and the NPC subunit Mlp1 (75, 142, 143).

Moreover, one study found that the transcriptional activator Gal4 is required but the SAGA complex is dispensable for association between the *GAL* genes and Nup2, while others have found that SAGA is required for relocation to the NPC (60, 113, 115). The binding of Gal4 to *GAL* gene regulatory elements is the initiating step in *GAL* activation, and the presence of Gal4 leads to subsequent recruitment of SAGA (98-101). These contradictory findings regarding the necessity of SAGA for gene relocation could reflect differential requirements for Gal4 and SAGA at distinct stages of locus relocation. As Nup2 is a mobile NPC subunit (116, 117), nucleoplasmic Nup2 may bind Gal4 to initiate the recruitment process, and SAGA and other transcriptional activators could then mediate interactions with Nups at the nuclear basket. This model is consistent with our finding that cells lacking both the Ada2 subunit of SAGA and Nup60 of the nuclear basket appear to be compromised in retention of the *GAL1* gene (Chapter 3). Moreover, as Nup2 can associate with the majority of promoters, (115), this model provides a general mechanism for the cellular recognition of genes poised for relocation to the NPC *via* interactions between Nup2 and gene-specific transcriptional activators.

The requirement for multiple mRNA export factors such as Mex67, Sac3, and Sus1, may reflect the secondary stage of gene relocation as transcription is established (59, 113, 114) (Figure 5.1*B*). This hypothesis may explain the apparently contradictory findings that gene association with the NPC is RNA-dependent, yet gene relocation precedes transcription initiation and does not require an mRNA coding region (59, 61, 75). In fact, multiple mRNA processing and surveillance factors have been implicated in gene relocation. These factors include the nuclear exosome which degrades aberrant mRNAs, the THO/TREX transcriptional elongation and mRNA export complex, and the

Mex67 mRNA export factor (59, 195, 227). Taken together, these differential requirements for Mlp1, SAGA, transcription, and mRNA export factors are consistent with a stepwise model where initial gene recruitment is mediated by interactions between transcriptional activators and Nup2, then subsequent retention is mediated by interactions between transcriptional co-activators and Mlp1 (Figure 5.1*A,B*). In addition, this model also accommodates the involvement of other as yet unidentified protein-protein interactions between transcriptional regulators and other NPC subunits as part of either recruitment or retention stages of gene relocation.

Finally, some relocated genes exhibit transcriptional memory, a process which facilitates the rapid re-activation of recently active genes (75, 142). Such genes remain associated with the NPC in the form of a gene loop, a process which requires the histone variant H2AZ, the SWI/SNF complex, and the Mlp1 subunit of the NPC (75, 142, 143) (Figure 5.1*C*). Taken together, these observations suggest a three-stage model for recruitment of active genes, including initial recruitment prior to transcriptional activation, stable retention during transcription, and long-term retention following transcriptional shut-off to mediate transcriptional memory.

The Physiological Relevance of Gene Relocation to the NPC

A major question regarding gene relocation to the NPC is why this phenomenon occurs: why does the cell invest in or permit large-scale chromosome movements during transcriptional activation? The immediate answer, based on work presented here as well as that of others, is that this relocation appears to influence transcript levels and mediate

transcriptional memory of recently active genes (Chapter 3) and (44, 118, 119, 142, 181). However, these observations do not address why relocation to the NPC *per se* should have these effects. Though there is no data to support the following hypothesis, it is straightforward to imagine that locating a transcribing gene at the NPC would prioritize the export of its mRNA simply due to its location. If, in addition, the transcriptional machinery, transcriptional activators and co-regulators, and mRNA processing and export factors were enriched at the NPC in transcription factories, then relocating a gene to the NPC would not only prioritize the export of its encoded mRNA based on association with the NPC, but also increase the efficiency of the production of that mRNA. Given the recent finding that persistent double strand breaks (DSBs) also relocate to the NPC (76, 77), and that many of the factors which repair DSBs are also involved in transcriptional activation [(216, 217) and Chapter 4], then this relocation may represent an attempt by the cell to take advantage of this proposed concentration of factors which modulate chromatin accessibility. Moreover, the fact that several NPC-associated factors exhibit boundary activity (228, 229), further supports a model where the NPC is a site conducive to the strong induction of associated genes. Thus the NPC may represent a physical location which is permissive to an open chromatin structure which facilitates both strong transcriptional activation and chromatin accessibility for repair of DNA damage.

The observation that Nup2 can associate with the majority of yeast promoters (115), coupled with our finding that functional interactions between the SAGA complex and Nup60 regulate global transcript levels (Chapter 3), suggests that a large majority of yeast genes may interact with the NPC when activated. If indeed most active yeast genes associate with the NPC, it is an interesting question as to whether there are enough NPCs

on the nuclear envelope to accommodate all active genes. Assuming that the *S. cerevisiae* nucleus contains between 65 and 180 NPCs (48, 49), and that the 8-fold radial symmetry of the nuclear basket accommodates 8 genes, then the budding yeast nucleus can maintain between 520 and 1440 genes at the NPC at any one time. Efforts to quantify the yeast 'transcriptome' have suggested that between ~4,600 and 6,500 loci (including unconfirmed ORFs) are actively transcribed under normal growth conditions (194, 230). However, considering that only ~20% of these loci (~930 to ~1420) are considered highly transcribed (230), and that association with the NPC is more likely observed for highly transcribed genes (62), then the number of yeast NPCs is in fact sufficient to accommodate the total number of highly transcribed genes. Moreover, at least one subunit of the NPC has boundary activity (228, 229), consistent with a role for the NPC in mediating global expression of highly-transcribed genes while preventing the spread of strong transcriptional activation to adjacent genes.

Evolutionary Conservation of Nup Associations with Active Genes

Though not directly addressed by the work presented here, one of the major questions regarding gene relocation to the NPC is the potential evolutionary conservation of this phenomenon. Locus relocation has been directly observed only in the budding yeast *S. cerevisiae*, where it appears to be a general mechanism of transcriptional regulation at least for highly transcribed genes [(34, 44, 100) and Chapter 3]. In addition, many *D. melanogaster* genes associate with the NPC, including the heat shock response gene *hsp70* and the active genes on the perinuclear male X chromosome; however, these

genes appear to be permanently associated with the nuclear periphery rather than undergoing relocation upon activation (43, 90). While the phenomenon has not been observed in vertebrates, it is noteworthy that all of the factors implicated in gene relocation are highly conserved across eukaryotes, including the SAGA histone acetyltransferase complex, the mRNA export factors Mex67, Sac3, and Sus1, and multiple NPC components including Nic96, Nup1, Nup2, Nup60, Nup116, the Nup84 subcomplex, and the Mlp proteins (59-62, 113-115, 152). In fact, association of *hsp70* with the NPC in *D. melanogaster* is SAGA-dependent (43), similar to the mechanism of *GAL* gene relocation in *S. cerevisiae* (60, 113). These observations provide for the possibilities that locus relocation to the NPC is evolutionarily conserved, and that interaction between CMCs and the NPC is a conserved mechanism of this locus relocation.

Interestingly, three recent studies in *Drosophila* found that the transcription of developmentally regulated genes and cell cycle genes depends upon interaction with several NPC subunits, but that these interactions largely occur in the nucleoplasm rather than at the NPC (92-94). These intriguing findings suggest two important points. First, NPC subunits may play a direct role in transcriptional activation, consistent with observations that both naturally occurring vertebrate Nup98-Hox fusion proteins and artificially constructed budding yeast Nup-LexA fusion proteins are strong transcriptional activators (89, 152). Second, transcriptional activation by NPC subunits does not necessarily occur at the NPC, consistent the observation that many NPC subunits are highly mobile and dynamically associate with the NPC (116, 117, 231-234). Moreover, the finding that *Drosophila* Nup98 and Sec13 associate with genes immediately prior to

and during initial transcriptional activation, while other Nups associate once transcription is established (93), suggest that different Nups have evolutionarily conserved roles in the distinct stages of gene activation even though these genes are not relocated to the NPC, analogous to the proposed roles for Nup2 and Mlp1 in budding yeast. Taken together, these observations raise the possibility that association between NPC subunits and active genes is an evolutionarily conserved mechanism for gene activation, but that relocation of these genes to the NPC is not conserved.

If in fact there is evolutionary conservation in the interaction between NPC subunits and active genes, but not in relocation of these genes to the NPC, then one might wonder when in evolutionary history this distinction arose. Did the budding yeast co-opt these Nup-gene interactions to physically relocate such genes to the NPC, or did other eukaryotic lineages abandon gene relocation but maintain the association between Nups and active genes? Though there are no data as of yet to support this hypothesis, it is conceivable that this phenomenon appeared early in eukaryotic evolution as a mechanism to prioritize the transcription, processing, and nuclear export of highly transcribed genes by physically linking them to the NPC. This model would make sense for a small eukaryotic genome such as that of budding yeast, where the nucleus is small enough that each gene may physically sample up to one-third of the nuclear volume (235). According to this model, increasing genome size during eukaryotic evolution would have precluded the maintenance of these potentially dramatic chromatin movements throughout the increasing nuclear space. However, this phenomenon was evidently maintained for select genes in *Drosophila* (43, 90, 92), while in general the NPC-active gene association was evolutionarily adapted such that Nups physically leave the NPC to interact with active

genes in the nucleoplasm (93, 94). Additionally, such an adaptation could be linked to the breakdown of the nuclear envelope during metazoan mitosis, which necessitates the dynamic association of many Nups with the NPC (236).

However, based on the recent evolutionary origins of *S. cerevisiae*, it is also conceivable that association between Nups and active genes is the more ancient phenomenon while physical relocation to the NPC is a more recent adaptation. The budding yeast *S. cerevisiae* is a member of the Saccharomycotina, a relatively young subphyla of organisms which arose as recently as ~200 to ~100 million years ago (Mya) (237). For perspective, the earliest fungi appear in the fossil record ~ 1400 Mya, vertebrate evolution began as early as ~525 Mya, and fossil evidence suggests mammals arose between ~167 and ~195 Mya (238-241). Thus the relatively recent appearance of *S. cerevisiae* in the eukaryotic domain of life suggests that any biological phenomena exclusive to budding yeast are likely to be evolutionary adaptations particular to the Saccharomycotina lineage. However, though gene relocation to the NPC has only been robustly observed in *S. cerevisiae*, there are compelling hints that gene relocation may occur in *S. pombe* as well (119). *S. pombe* is a member of the Taphrinomycotina subphyla, which diverged from the fungal lineage between ~400 and ~600 Mya (237). If in fact gene relocation to the NPC also occurs in *S. pombe*, then gene relocation may be an ancient, though perhaps fungal-specific, mechanism of transcriptional regulation. More research into this phenomenon in *Giardia*, which are considered to be the prototypical early-diverging eukaryotes, (242, 243), as well as vertebrate model organisms such as the zebrafish *Danio rerio* and the mouse *Mus musculus* is critical in

order to fully elucidate the evolutionary history and universality of this fascinating biological phenomenon.

Final Conclusions

The work presented here significantly extends our current understanding of both the mechanism and the physiological ramifications of gene relocation to the NPC. We have not only identified physical links between active genes and the NPC, but also revealed functional consequences for both transcriptional regulation and repair of DNA damage when that association is disrupted. Taken together, these results suggest that the NPC is an important regulator chromatin structure and promotes chromatin accessibility to transcriptional and DNA damage repair factors. Given the tantalizing hints that some aspects of this phenomenon also occur in *Drosophila*, it will particularly interesting to learn if these interactions between active genes and NPC subunits are evolutionarily conserved across the eukaryotic domain of life.

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APPENDIX

The CCR4-NOT Complex Physically and Functionally Interacts with the mRNA Export Pathway

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Tandem affinity purification (TAP) tag purifications performed by Nowel Azzouz. Co-purification experiments were performed R. Nicholas Laribee.

Introduction

Gene expression is regulated at multiple levels, including at the stages of transcriptional and post-transcriptional control, to achieve correct levels and patterns of expression (1). These processes are highly integrated and are controlled by evolutionarily conserved factors and mechanisms which package an mRNA molecule into an export-competent ribonucleoprotein (mRNP) complex (1-3). There is mounting evidence that the steps from transcription to mRNA export are not only sequential, but in fact are highly coupled and interdependent, whereby proteins involved in one step of mRNA biogenesis are subsequently used as adaptor proteins to recruit other processing or export factors (1-6). Among these RNA binding proteins are the historically defined heterogenous nuclear ribonuclear proteins (hnRNPs) which mediate multiple steps in the mRNA lifecycle such as processing, nuclear export, and delivery to the cytoplasm (7, 8). The budding yeast *Saccharomyces cerevisiae* has a number of hnRNPs including Hrp1, which is required for proper mRNA cleavage and polyadenylation (9), the poly(A) binding protein Nab2 required for mRNA export and proper poly(A) tail length (10-12), and Npl3, which is involved in splicing and transcription elongation (13, 14).

Following mRNA maturation and processing, the export-competent mRNP must travel through the nuclear pore complex (NPC) to reach the cytoplasm. The NPC is an evolutionarily conserved structure comprised of approximately 30 protein components called nucleoporins (Nups), which are present in at least 8 copies per NPC and are arranged in 8-fold radial symmetry to form channels that perforate the nuclear envelope and regulate traffic between the nucleus and cytoplasm (15, 16). Some Nups are

asymmetrically localized across the NPC, giving the complex three distinct substructures: a nuclear basket, a central core spanning the nuclear envelope, and cytoplasmic fibrils (16). In order for an mRNA to translocate through the NPC, mRNA export factors in complex with the mRNA interface with a distinct class of Nups called FG-Nups, which contain at least one domain of distinct repeating patterns of phenylalanine (F) and glycine (G) residues (5, 16). Testifying to the importance of the NPC in regulating mRNA traffic, mutations in many distinct Nups result in mRNA export defects and mRNA accumulation in the nucleus (17-22). Interestingly, recent studies have uncovered a physical link between transcriptionally active genes and the NPC (23), reminiscent of Blobel's gene gating hypothesis (24) and further suggesting that every aspect of mRNA maturation may be tightly coupled from biogenesis to nuclear export.

A significant contributor to the lifecycle of an mRNA molecule, from mRNA biogenesis to eventual degradation, is the evolutionarily conserved multi-subunit Ccr4-Not complex. The Ccr4-Not complex is a large protein complex (~0.9-1.0 MDa), containing nine core subunits (Ccr4, Caf1, Caf40, Caf130, and Not1-5) that localizes to both the nucleus and cytoplasm (25, 26). The Caf1 and Ccr4 subunits are mRNA deadenylases, responsible for the major cytoplasmic deadenylase activity in budding yeast (27-29), The Not4 subunit is a RING-domain containing ubiquitin ligase whose only known substrates are the Egd1 and Egd2 proteins involved in translation and the Jhd2 histone demethylase (30-32). The Ccr4-Not complex negatively and positively regulates both transcription initiation and elongation, and it has been suggested that the combined actions of Ccr4-Not members contribute to transcriptional control of ~85% of the *S. cerevisiae* genome (33, 34). This regulation is achieved in part through physical

interactions between Ccr4-Not subunits and components of the basal transcription apparatus and other accessory transcriptional co-regulators, including the SAGA histone acetylransferase complex, the PAF transcription elongation complex, and the proteasome (35-39). Recent studies also demonstrate that Ccr4-Not regulates global histone methylation, acetylation, and ubiquitination, suggesting this complex contributes significantly to the establishment of chromatin states critical for transcriptional regulation (36, 40, 41).

Until recently, the known nuclear functions of Ccr4-Not were confined to transcriptional regulation; however, new studies suggest that Ccr4-Not contributes significantly to other nuclear processes. Cells mutant for Ccr4-Not components were shown to overexpress snRNAs and snoRNAs and accumulate a significant fraction of these RNAs as polyadenylated species (42). Subsequently, Ccr4-Not was found to interact physically and functionally with both the nuclear exosome and the TRAMP complex, components of a nuclear surveillance pathway that degrades aberrantly processed RNAs (42). These results suggest that Ccr4-Not has a role in nuclear RNA turnover through interactions with both the exosome and TRAMP. Ccr4-Not also has been linked to other nuclear, RNA-based processes. For example, one of the two human Caf1 orthologs, hCaf1, was recently determined to associate with the arginine methyltransferase, PRMT1 (43). Both factors localize to nuclear speckles, which are sub-nuclear domains enriched for small nuclear ribonucleoproteins and splicing factors. hCAF1 interaction with PRMT1 regulates PRMT1-mediated methylation of both histone H4 and the RNA binding protein Sam68 *in vitro* and *in viv*o, suggesting that Ccr4-Not may play a significant role in PRMT1-regulated biological processes.

To further define the interactions between Ccr4-Not and processes regulated by arginine methylation, we used budding yeast to determine whether Ccr4-Not members interact with the yeast ortholog of PRMT1, the hnRNP methyltransferase Hmt1 (44). In this study, we demonstrate that Ccr4-Not subunits associate both with Hmt1 and the hnRNPs Hrp1 and Nab2, which are known Hmt1 substrates (45, 46). We also demonstrate that Ccr4-Not association with hnRNPs depends on Hmt1 methyltransferase activity. Tandem-affinity-purification (TAP) of individual Ccr4-Not members identified the S-adenosylmethionine (SAM) synthetases Sam1 and Sam2 (Sam1/2), which regulate cellular levels of the universal methyl donor SAM (47), as stably associating with Ccr4-Not. Furthermore, these TAP-purification results demonstrate that Ccr4-Not co-purifies with the Mlp1 and Mlp2 subunits of the NPC nuclear basket, a region of the NPC critically involved in mRNA quality checkpoint control and nuclear export. We also show genetic interactions between Ccr4-Not, hnRNPs, and NPC components, and demonstrate that overexpression of the Not4 ubiquitin ligase exacerbates mRNA export defects in cells expressing a mutant NPC subunit. These studies suggest a novel functional role for Ccr4-Not in the mRNA export pathway that likely depends on physical interactions with Hmt1, Sam1/2, hnRNPs, and the NPC.

Results

Hmt1 physically interacts with components of the CCR4-NOT complex: A previous study identified one of the human homologs of yeast Caf1, hCAF1, as a regulator of the arginine methyltransferase PRMT1 (43). To determine whether this

functional relationship was evolutionarily conserved in the yeast *Saccharomyces cerevisiae*, we examined whether Hmt1, the budding yeast ortholog of PRMT1 (44), physically associates with components of the Ccr4-Not complex by co-immunoprecipitation. For this analysis, an HA-tag was integrated into the endogenous *HMT1* locus in cells also expressing Myc-tagged Ccr4-Not subunits from their endogenous loci. We then performed co-immunoprecipitation experiments from whole-cell lysates with α-Myc antibody to precipitate individual Ccr4-Not subunits and blotted with α-HA antibody to detect Hmt1 association. Hmt1 association is readily detectable with the Caf1 and Ccr4 subunits, and is more weakly detected with the Not2 subunit (Figure A.1*A*). Interestingly, Hmt1-HA did not co-immunoprecipitate with Not5-Myc, suggesting the possibility that differential interactions occur between Hmt1 and the individual Ccr4-Not members or that Not5 association is less stable than the other subunits. We confirmed these results by performing the reciprocal co-immunoprecipitaions and obtained similar results (Figure A.1*B*).

The CCR4-NOT complex physically interacts with hnRNPs: Among the major physiological targets of the Hmt1 methyltransferase are heterogeneous nuclear ribonucleoproteins (hnRNPs) (44, 45), which bind mRNAs during processing and export from the nucleus (7, 8). In *S. cerevisiae*, hnRNPs include Hrp1, Nab2, and Npl3 (44-46). Given the physical association we identified between Hmt1 and Ccr4-Not subunits, we hypothesized that Ccr4-Not might also interact with hnRNPs. To investigate this possibility, we prepared lysates from cells expressing Myc-tagged Ccr4-Not subunits, performed α-Myc immunoprecipitations and then immunoblotted with antibodies specific for either Nab2 or Hrp1 to determine if they co-precipitated with Ccr4-Not

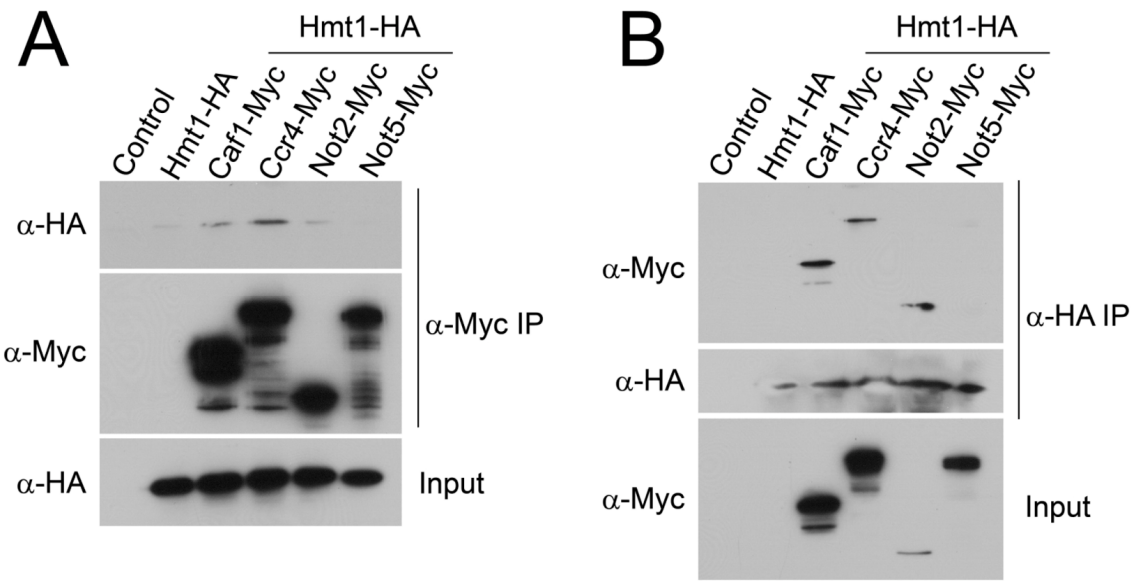

Figure A.1 **Ccr4-Not subunits associate with the arginine methyltransferase Hmt1.** *A*, The indicated strains were grown asynchronously to log phase before harvesting and extracting whole-cell proteins in IP buffer. IPs were performed overnight at 4°C using α-Myc antibody. Immune complexes were captured by incubation with Protein A-conjugated agarose beads. Samples were washed three times with 0.5 mL IP buffer per wash and then resolved by 10% SDS-PAGE, transferred to PVDF membrane and immunoblotted with α-HA antibody. To control for immunoprecipitation efficiency, the α-HA immunoblot was subsequently stripped and reprobed with α-Myc antibody. Input samples correspond to 30 µg of whole cell extract. *B*, As in *A* except Hmt1 was immunoprecipitated using α-HA antibody and the association of Ccr4-Not members was detected by α-Myc immunoblot. The immunoblot was then strippted and reprobed with α-HA to control for immunoprecipitation efficiency. Input samples correspond to 30 µg of whole cell extract.

members. Nab2 was readily detectable in the Caf1, Ccr4, and Not5 immunoprecipitates but not in the Not1 or Not2 immunoprecipitates (Figure A.2*A*). Similar to results for Nab2, we identified Hrp1 in co-immunoprecipitates of Caf1, Ccr4, and Not5. Interestingly, in contrast to the results for Nab2 association, we were also able to detect Hrp1 in the Not2 and Not1 immunoprecipitates (Figure A.2*B*). As a control, Nab2 and Hrp1 did not co-immunoprecipitate from cell lysates that did not express Myc-tagged Ccr4-Not subunits. These experiments reveal that multiple subunits of the Ccr4-Not complex differentially co-associate with the hnRNPs Nab2 and Hrp1, and they further suggest that Ccr4-Not may play a functional role in the mRNA export pathway.

Physical interactions between the CCR4-NOT complex and hnRNPs depend upon Hmt1 methyltransferase activity: To test whether the association between Ccr4-Not members and hnRNPs is Hmt1-dependent, we deleted *HMT1* in cells expressing Caf1-Myc and tested for association with Hrp1 and Nab2. While we detected both Hrp1 and Nab2 in the Caf1-Myc immunoprecipitates, the amount of Hrp1 or Nab2 co-immunoprecipitated with Caf1-Myc was reduced significantly in *hmt1Δ* cells relative to cells expressing *HMT1*, suggesting that the interaction between Caf1 and Hrp1 or Nab2 is Hmt1-dependent (Figure A.2*C*). Furthermore, these interactions depend upon the methyltransferase activity of Hmt1, as Caf1 does not interact significantly with Hrp1 or Nab2 when the catalytically-inactive *hmt1-G68R* mutant is expressed in *hmt1Δ* cells, whereas expression of wildtype *HMT1* does rescue these interactions. These results support the hypothesis that Ccr4-Not members associate both with Hmt1 and hnRNPs, and these associations are dependent on the Hmt1 methyltransferase activity. Using an antibody specific to arginine-methylated Npl3 (48, 49), we find no significant differences

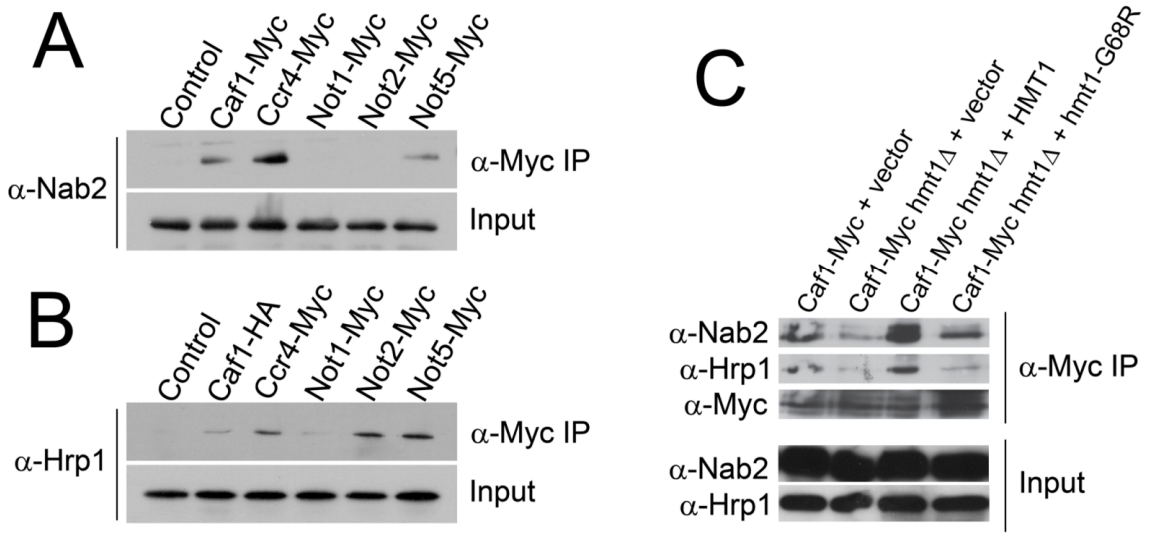

Figure A.2 **Association of the hnRNP proteins Hrp1 and Nab2 with Ccr4-Not subunits depends on Hmt1 arginine methyltransferase activity.** *A*, Nab2 associates with Caf1, Ccr4, and Not5. Whole cell extracts and immunoprecipitations were performed as described in Figure A.1, except that 500 µg of extract was used per immunoprecipitation. Samples were resolved by 8% SDS-PAGE and immunoblotted with α-Nab2 antibody. Input samples represent 30 µg of starting material. *B*, Hrp1 associates with Caf1, Ccr4, Not1, Not2, and Not5. Experiment was performed as in *A*, but samples were immunoblotted with α-Hrp1 antibody. *C*, Caf1 association with Nab2 and Hrp1 depends on Hmt1-mediated arginine methylation. *CAF1-MYC* or *CAF1-MYC hmt1Δ* cells were transformed with empty vector, *HMT1* or *hmt1G68R* expression vectors, and cells were grown in SC-Ura media to select for plasmid maintenance. Log phase cells were harvested and immunoprecipitations performed as described in *A*. The α-Nab2 immunoblot was stripped and reprobed for α-Hrp1 and then again for α-Myc to control for immunoprecipitation efficiency. Input samples correspond to 30 µg of whole cell extract.

in Hmt1-dependent methylation of Npl3 in Ccr4-Not deletion mutants compared to wild-type cells (data not shown), suggesting that perturbation of the Ccr4-Not complex does not impact Hmt1 methyltransferase activity *in vivo*. Taken together, these results suggest that methylation of Hrp1 and Nab2 by Hmt1 is required for interaction with the Ccr4-Not complex.

Previously, we TAP-purified individual Ccr4-Not subunits and identified associated proteins by mass spectrometry (42, 50). Among the proteins which co-purified with multiple Ccr4-Not subunits and which were not previously reported where the S-adenosylmethionine synthetases, Sam1 and Sam2 (Table A.1). These enzymes regulate the cellular pool of S-adenosylmethionine (SAM) which is the universal methyl donor required for numerous biochemical reactions, including protein methylation (47). Ccr4-Not association with these enzymes suggests the possibility that this complex may play an important role in regulating protein methylation, including those methylation events mediated by Hmt1. However, our analysis of individual Ccr4-Not deletion mutants with an antibody specific to arginine-methylated Npl3 (48, 49) found no sigificant change in Hmt1-dependent methylation of Npl3. These results suggest that while Ccr4-Not interacts with Hmt1 and Sam1/2, it does not play a significant role in Hmt1-mediated Npl3-methylation. However, these data do not preclude the possibility that Ccr4-Not may regulate methylation of other Hmt1-specific substrates.

Components of CCR4-NOT physically and functionally interact with the nuclear pore complex: We have identified physical associations between the Ccr4-Not complex and two hnRNPs essential for mRNA export. One critical aspect of the mRNA export process is the interaction between mRNA binding proteins and components of the

Table A.1 **Ccr4-Not TAP purifications identify Mlp1/2 and Sam1/2**

Ccr4-Not TAP Purification	**Co-purified NPC Subunits**
Caf40	Mlp1, Mlp2, Sam1, Sam2
Caf130	Mlp1
Not4	Mlp2, Sam1, Sam2
Not2	Mlp1, Sam1

nuclear pore complex (NPC) as they chaperone their mRNA cargoes out of the nucleus (4, 5). Given the association of Ccr4-Not members with Hmt1 and hnRNPs, we hypothesized that Ccr4-Not might also physically interact with components of the NPC as part of this process. In support of this hypothesis, we identified in our TAP-purifications of individual Ccr4-Not subunits the Mlp1 and Mlp2 components of the inner nuclear basket of the NPC (51) [Table A.1 and (42)]. Interestingly, no strictly cytoplasmic NPC components were found in these purifications, suggesting that Ccr4-Not stably interacts with the nuclear face of the NPC, a region that has critical roles in mRNA export (4, 5).

To determine the functional relevance of Ccr4-Not association with the NPC, we tested for genetic interactions between Ccr4-Not subunits and various NPC components. Gene deletions of several Ccr4-Not complex members result in profound growth defects (25, 26); therefore, we assayed for genetic interactions by overexpressing individual subunits in the *nup116Δ* mutant to determine their effects on growth. Interestingly, these experiments revealed that overexpression of *NOT4*, but not *CAF1* or *CCR4*, causes growth *nup116Δ* cells (Figure A.3). As a control, overexpression of Ccr4-Not subunits was found to have no effect on the growth of wild type cells.

Not4 encodes a ubiquitin E3 ligase whose *in vivo* substrates remain largely unknown (30-32, 52). To determine if the Not4 ligase function is important for the overexpression phenotype in *nup116Δ* cells and identify other potential NPC genetic interactions, we overexpressed *NOT4* and the *not4L35A* mutant in a variety of mutants carrying temperature sensitive NPC gene deletions or mutant alleles. The *not4L35A* mutation disrupts interactions between Not4 and its two known E2 ubiquitin-conjugating enzymes,

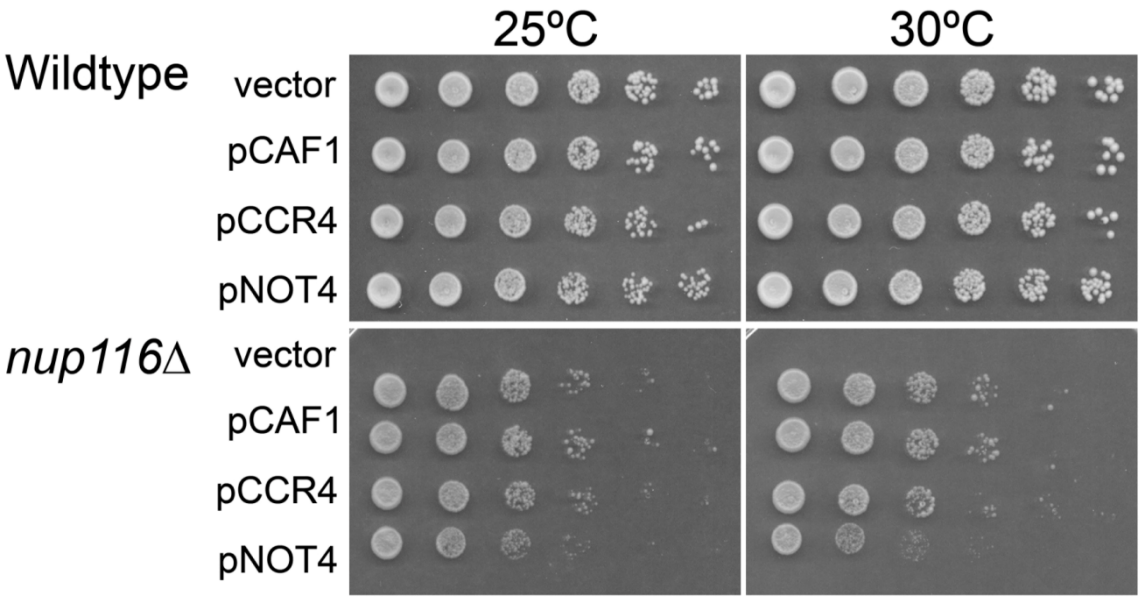

Figure A.3 **Not4 overexpression causes growth defects in NPC mutant cells.** Wild-type and *nup116Δ* cells were transformed with empty vector, *CAF1*, *CCR4* or *NOT4* overexpression constructs. Cells were grown to saturation in SC-Ura media, ten-fold serially diluted, and spotted onto SC-Ura plates. Plates were incubated at 25°C or 30°C.

Ubc4 and Ubc5, thus compromising its ubiquitin ligase function (52). *NOT4* overexpression causes significant growth defects in cells mutated or deleted for NPC components, including *nup1Δ*, *nup116Δ*, *nup120-1*, and *nup133-1* mutant cells (Figure A.4). In contrast, *NOT4* overexpression modestly affected *nup49-313* mutant cells and no effect on wild-type cells. Interestingly, overexpression of *not4L35A* mostly mirrored the effect of *NOT4* overexpression except that it had a slightly less negative effect on growth in *nup1Δ* and *nup120-1* cells and no detectable effect in *nup49-313* cells (Figure A.4). The differences in the effects of *NOT4* and *not4L35A* overexpression in the *nup1Δ*, *nup120-1*, and *nup49-313* cells are not completely surprising as *not4Δ* and *not4L35A* mutants have both overlapping and distinct phenotypic effects (52). Taken together, these results suggest that altered stoichiometry of the Not4 ligase is detrimental for cells with compromised NPCs, and that these negative growth effects are only partially dependent on Not4 interactions with Ubc4 or Ubc5.

Not4 functionally interacts with hnRNPs: Given the importance of Hmt1-mediated methylation of hnRNPs for efficient hnRNP nuclear export (45, 53), and the critical role of the NPC in mRNA export, we next asked whether Ccr4-Not associations with hnRNPs and NPC components might have functional implications for mRNA nuclear export. To investigate this question, we tested for genetic interactions between Not4 and various hnRNPs by overexpressing *NOT4* and *not4L35A* in a variety of hnRNP mutants, some of which are known to give mRNA export phenotypes (19-22). Intriguingly, this analysis revealed that overexpression of *NOT4* is synthetically deleterious to cells expressing mutant versions of Nab2 and Hrp1 but not to wild-type

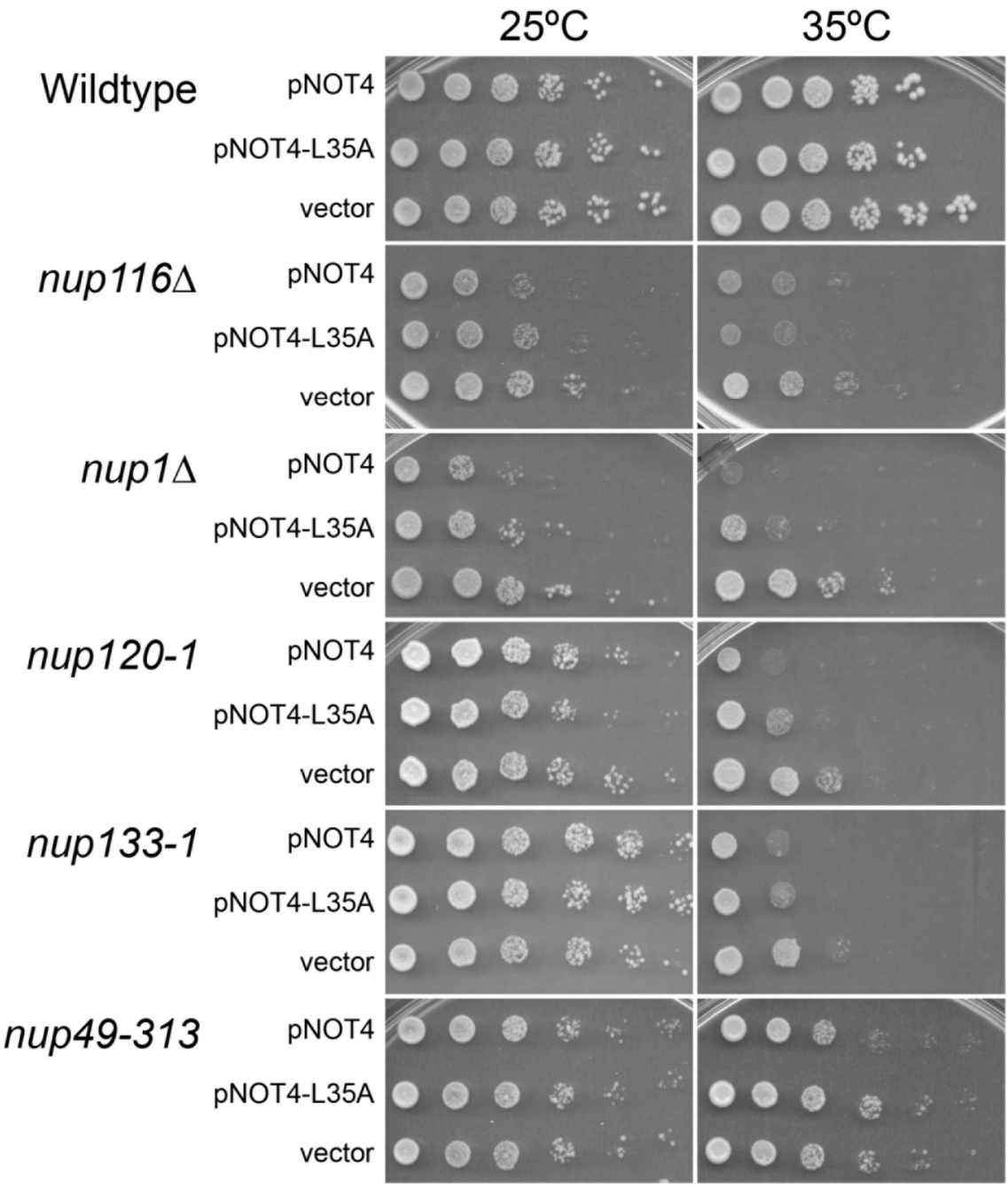

Figure A.4 **Not4 and not4L35A overexpression cause differential growth effects in NPC mutant cells.** Wildtype, *nup1Δ*, *nup116Δ*, *nup120-1*, *nup133-1*, and *nup49-313* cells were transformed with empty vector, *NOT4*, or *not4L35A* overexpression constructs. Cells were grown to saturation in SC-Ura media, ten-fold serially diluted, and spotted onto SC-Ura plates. Plates were incubated 25°C or 35°C.

cells (Figure A.5). In sharp contrast, overexpression of *NOT4* weakly suppresses the severe temperature-sensitive growth phenotype of the *npl3-1* mutant (Figure A.5). Similar to results in NPC mutants, overexpression of *not4L35A* had different effects on growth in different hnRNP mutants, with deleterious effects on Nab2 and Hrp1 mutants, and no effect on the Npl3 mutant (Figure A.5). These results suggest that the Not4 ubiquitin ligase has both ligase-dependent and independent interactions with hnRNPs and NPC mutants essential for mRNA export.

NOT4 overexpression exacerbates the poly(A) export defect in a nuclear pore mutant: The results presented above suggest that Ccr4-Not may have an unrealized role in the nuclear mRNA processing and export pathway. This hypothesis is supported through the physical associations between Ccr4-Not members and either hnRNPs or the NPC and also by the functional interactions between Not4 and many of the temperature sensitive NPC mutants (see Figure A.3) that have poly(A) RNA export defects at the nonpermissive temperature (19-22). We initially assayed for defects in global mRNA export in wild-type and various Ccr4-Not deletion mutants by fluorescence *in situ* hybridization (FISH), but did not detect significant mRNA nuclear accumulation in any of these mutants (data not shown). Because the mRNA export pathway is highly robust and redundant, we speculated that inhibition of Ccr4-Not alone may not result in a detectable defect in global mRNA export, especially if only the export of specific mRNAs are affected. Deletion of *NUP116* results in nuclear global poly(A) RNA accumulation at 37°C (22). The growth defects we observed in *nup116Δ* cells overexpressing *NOT4* (see Figure A.3) suggested the possibility that NOT4 overexpression might exacerbate the mRNA export defect in these cells. To investigate

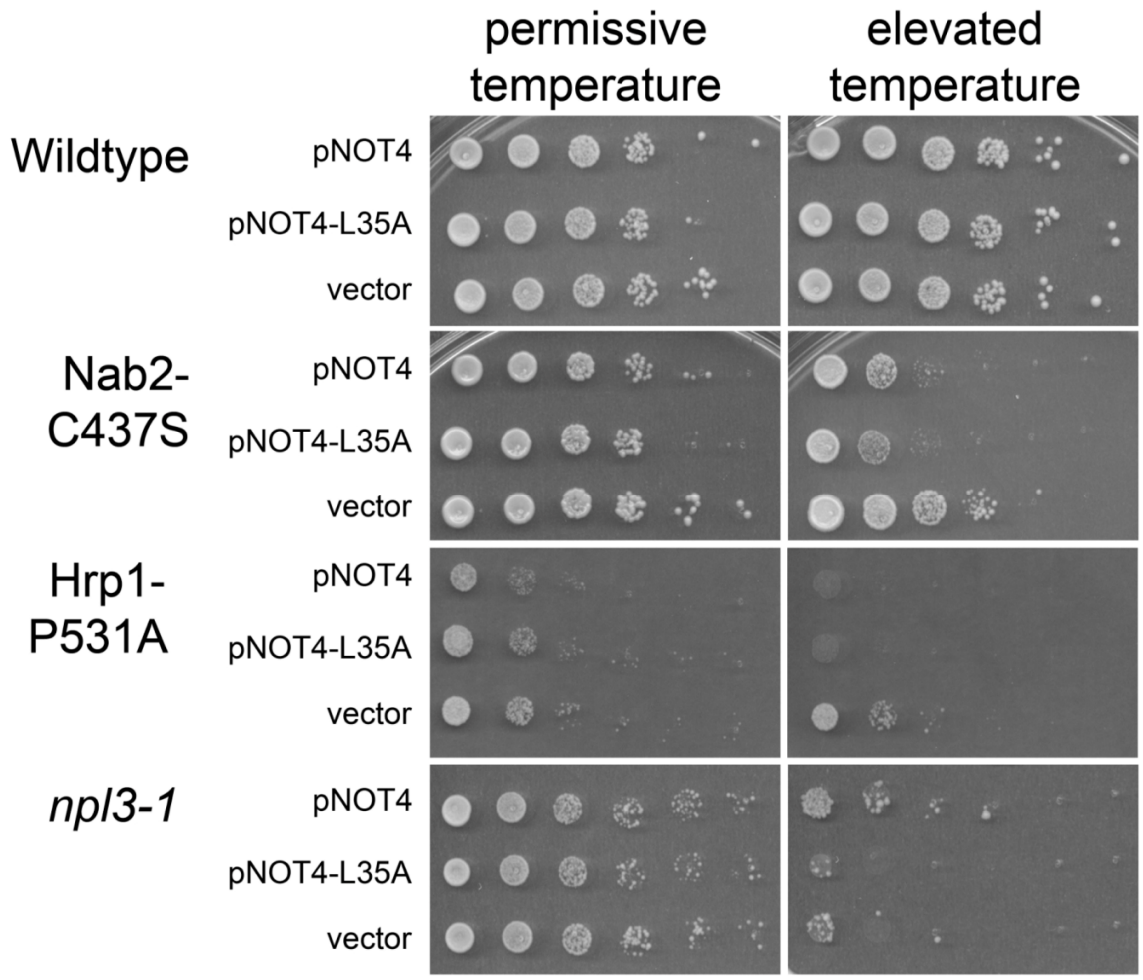

Figure A.5 **Not4 and not4L35A overexpression cause differential growth effects in hnRNP mutant cells.** Growth assays were performed as described for Figure A.4. Plates were incubated at 25°C for the permissive temperature or 39°C (for Nab2-C437S) 37°C (for Hrp1-P531A), or 30°C (for *npl3-1*) for the elevated temperature.

this possibility, we conducted FISH analysis for bulk poly(A) mRNA in cells deleted for *NUP116* and carrying either empty plasmid or plasmid expressing the *NOT4* gene. This analysis revealed a statistically significant increase (p=0.05) in nuclear poly(A) RNA accumulation in *nup116Δ* cells overexpressing *NOT4* (29.84 ± 2.70 %) compared to *nup116Δ* carrying control plasmid (19.00 ± 6.16 %) (Figure A.6*A*,*B*). As controls, wild-type cells overexpressing *NOT4* showed no increase in nuclear poly(A) RNA signal whereas the *nab2-1* mutant results in significant accumulation of poly(A) RNA (73.17 ± 2.79 %), consistent with previous reports (46). These results suggest a functional relationship between Ccr4-Not, components of the mRNA export pathway, and the NPC. Moreover, these results demonstrate that steady-state mRNA export is susceptible to alterations in Ccr4-Not when combined with NPC mutants.

Discussion

Our results identify new connections between the Ccr4-Not complex and the mRNA processing and export pathway through physical and functional interactions both with hnRNPs and the NPC. The identification of Sam1 and Sam2 in our Ccr4-Not TAP-purifications further supports the hypothesis that Ccr4-Not may play important roles in protein methylation, although this effect most likely does not involve global regulation of Hmt1-dependent methylation since no effect on Npl2 methylation was detected in Ccr4-Not mutant cells. We also present the novel finding that altered stoichiometry of the Ccr4-Not complex, specifically by increased expression of the Not4 ubiquitin ligase, causes significant growth defects in cells mutant for Nab2, Hrp1, and NPC subunits. In

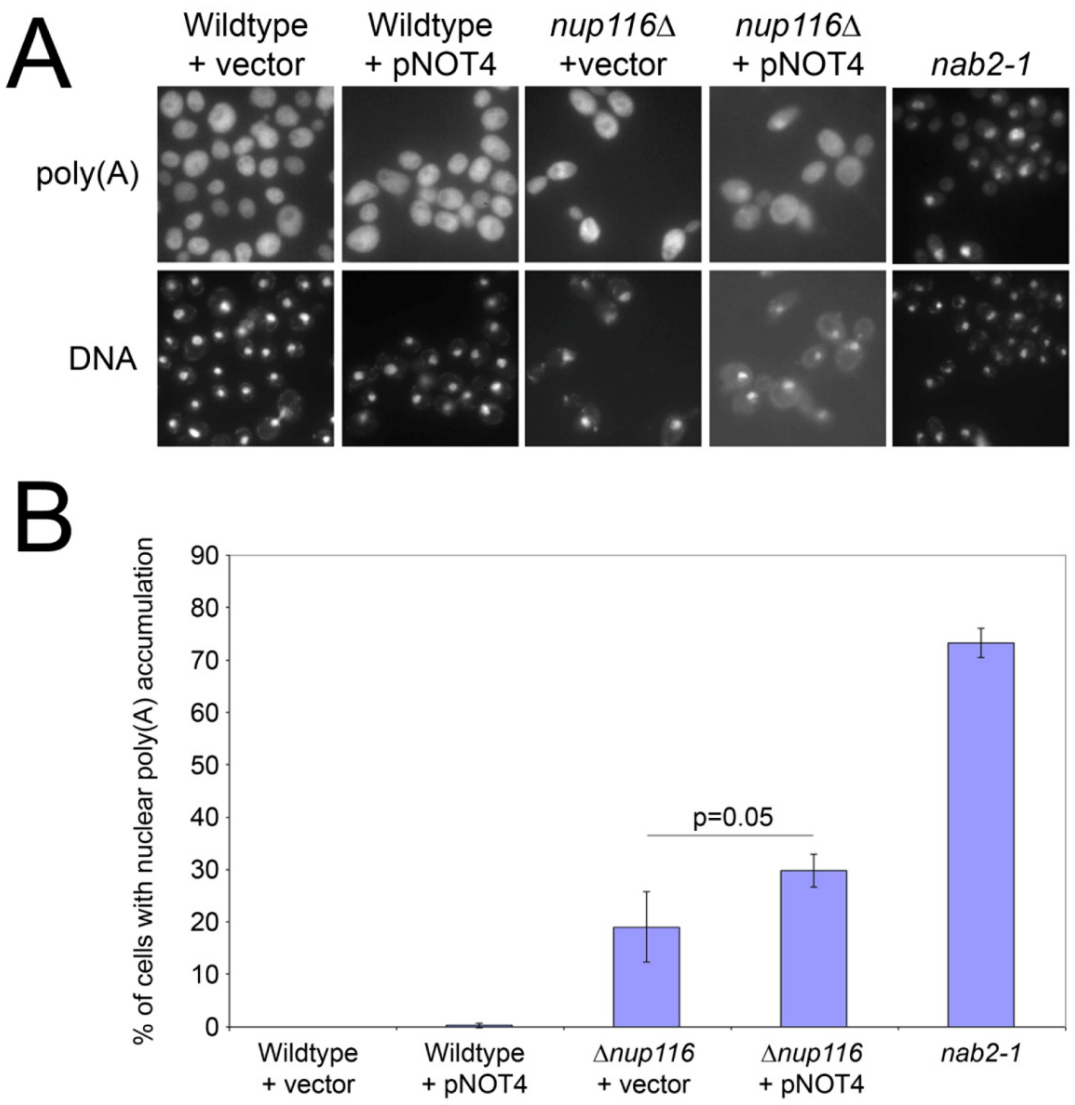

Figure A.6 **Not4 overexpression exacerbates the poly(A) RNA export defect in *nup116Δ* cells.** Wildtype and *nup16Δ* cells were transformed with empty vector or a *NOT4* overexpression construct. Cells were grown to log phase at 30°C and subjected to FISH. *A*, FISH was performed on cells as described in Materials and Methods. Panels are shown for poly(A) RNA and DAPI to visualize chromatin. *B*, Quantification of cells showing nuclear accumulation of poly(A) RNA. Images were analyzed blind, and a minimum of 50 cells were analyzed in triplicate for each condition. Student's *t*-test was used to determine statistical significance.

stark contrast, we find that Not4 overexpression rescues growth of the *npl3-1* mutant at the non-permissive temperature. Furthermore, we demonstrate that increased Not4 expression exacerbates the mRNA export defect seen in *nup116Δ* cells, further suggesting that the Ccr4-Not complex plays a functional role in mRNA processing and export. This point is further supported by the co-purification of Mlp1 and Mlp2 proteins with Ccr4-Not members, including Not4. Taken together, these results extend the functions of the Ccr4-Not complex in the lifecycle of an mRNA from its known roles in transcriptional regulation and mRNA degradation to newly identified connections to mRNA export.

The initial report demonstrating that hCaf1 interacts with PRMT1 and regulates its methyltransferase activity suggested that Ccr4-Not plays a significant role in PRMT1-dependent processes (43). However, the *in vivo* relevance of these interactions was not explored in detail. We significantly extend these preliminary findings by demonstrating that multiple components of the budding yeast Ccr4-Not complex associate with the PRMT1 homolog, Hmt1, and that Ccr4-Not also associates with the Hmt1 substrates, Hrp1 and Nab2 in an Hmt1 methyltransferase-dependent fashion (45, 46). The findings that Ccr4-Not interactions with these hnRNPs depends on Hmt1 methyltransferase activity strongly suggests that Ccr4-Not associates predominantly with methylated, export-competent hnRNPs.

The Ccr4-Not complex has recently been implicated in nuclear RNA quality control through interactions with the TRAMP complex and nuclear exosome (42). In addition, we demonstrate that Ccr4-Not co-purifies with the Mlp proteins of the NPC, which have a well established role in mRNA export quality control (54, 55). The

observation that Ccr4-Not associates, most likely in a transient manner, with methylated, export-competent hnRNPs but appears to stably associate with NPC nuclear basket components is consistent with a model in which Ccr4-Not selectively interacts with methylated hnRNPs as they chaperone their mRNA cargoes through the NPC. The demonstration that Hmt1 methyltransferase activity is required for Ccr4-Not to associat with Nab2 and Hrp1, coupled with the identification of Sam1 and Sam2 as Ccr4-Not co-purifying factors, suggests that Ccr4-Not may act to physically position these factors at the NPC to facilitated methylation and subsequent nuclear mRNA export. Our genetic analysis demonstrating that increased expression of the Not4 ubiquin ligase results in synthetic growth defects in cells mutant for Nab2, Hrp1, and NPC subunits suggests that Ccr4-Not also may have other, as yet undefined roles in the mRNA export pathway that become dysregulated when Not4 exists in excess. This hypothesis is supported by our results demonstrating that Not4 overexpression exacerbates the mRNA export defect in *nup116Δ* cells. Interestingly, Not4 overexpression is not universally detrimental to hnRNP mutants, as it rescues the extreme temperature sensitivity of *npl3-1* cells which also display mRNA processing and export phenotypes (14, 56, 57). These differential effects of Not4 overexpression suggest a complex and nuanced interaction between different components of the mRNA export pathway. As ubiquitination has been implicated in control of mRNA processing and export (58-60), our experiments raise the possibility that some of these factors may be targets of the Not4 ubiquitin ligase. This potential activity of Not4 against mRNA processing and export factors may regulate modification of their function through mono-ubiquitination or target them for degradation through poly-ubiquitination.

We demonstrate that the Not4L35A mutant, which blocks interaction with the ubiquitin conjugating enzymes Ubc4 and Ubc5 (Ubc4/5), has differential effects with different hnRNP and NPC mutants relative to wild-type Not4. These effects suggest the possibility that Ccr4-Not may be part of a complex regulatory cascade that partially depends on interactions between Not4 and Ubc4/5. Interestingly, Ubc4/5 also interact with the Tom1 ubiquitin ligase, a HECT-domain ligase (61) whose E3 ligase function is implicated in mRNA export (58). One possible mechanism by which Ccr4-Not might regulate mRNA export is through interactions with Ubc4/5, which might reduce or prevent Ubc4/5 interactions with Tom1 and thus prevent Tom1-mediated ubiquitination of downstream targets. In addition, it is possible that as yet unidentified targets of Not4 may play a role in mRNA export control. This possibility is consistent with the fact that deletion of *NOT4* results in significant growth defects that are not phenocopied by deletion of known substrates (31, 32), suggesting that a number of important Not4 targets remain to be identified. In addition to its activity as a ubiquitin ligase, Not4 also contains a putative RNA recognition motif (RRM), that has significant sequence similarity to characterized RNA binding domains (25, 62). Although the *in vivo* significance of this domain is unknown, it is possible that Ccr4-Not may bind RNA *via* the Not4 subunit as part of its role in mRNA export. One speculative possibility is that the Not4 overexpression phenotype in cells mutant for hnRNPs and NPC subunits may result from dysregulated interactions of Not4 with specific mRNA classes. However, whether Not4 binds RNA *in vivo*, and the detailed mechanism by which it affects the mRNA processing and export machinery to impact mRNA export, remain to be addressed in future studies.

Experimental Procedures

Strains, Plasmids, and Chemicals: All DNA manipulations were performed according to standard methods (63) and all media were prepared by standard procedures (64). All *S. cerevisiae* strains and plasmids used are described in Table A.2. Plasmid pAC2668 was generated by using the QuikChange Site-Directed Mutagenesis (Stratagene, La Jolla, CA) approach and plasmid pAC2492(*NOT4*) as template. The resulting mutation was confirmed by sequence analysis. All chemicals were obtained from Ambion (Austin, TX), Sigma Chemical Co. (St. Louis, MO), US Biological (Swampscott, MA) or Fisher Scientific (Pittsburgh, PA) unless otherwise noted.

Tandem-affinity purification (TAP): TAP-purifications were performed and analyzed as previously described (42, 50). Briefly, yeast strains expressing TAP-tagged subunits were grown to log phase and whole cell extracts were prepared. Purified proteins were resolved on 4-12% SDS-PAGE gradient gels, stained with Coomassie, detectable bands were excised from the gel, and MALDI-TOF mass spectrometry analysis of the excised bands was performed. The spectra obtained were analyzed using the DATA EXPLORER program and proteins identified using the MASCOT SEARCH website.

Co-immunoprecipitation experiments: Asynchronous cell cultures were grown to log phase before pelleting and lysing cells in immunoprecipitation buffer [10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 10% glycerol containing protease and phosphatase inhibitors, 1mM DTT] using the method of bead beating as previously described (36). Immunoprecipitations were performed using 1 mg of whole-cell extract

Table A.2 **Strains and plasmids used in this study**.

Strain	**Description**	**Reference/Source**
FY23 (ACY192)	*MATa ura3 leu2 trp1*	(65)
BY4741 (ACY402)	*MAT a his3 leu2 met15 ura3*	Open Biosystems
LDY561 (ACY786)	*MAT α nup1Δ::LEU2 ura3 leu2 trp1 his3 ade3*	(66)
SWY27 (ACY542)	*MAT α nup116Δ::HIS3 ura3 leu2 trp1 his3 ade2 can1*	(22)
Dat4-2 (ACY1136)	*MAT a nup120-1 ura3 leu2 trp1*	(67)
Dat3-2 (ACY1135)	*MAT a nup133-1 ura3 leu2 trp1*	(67)
ACY1903	*nup49Δ::KANMX ura3 his3 leu2* (pUN90-LEU2-nup49-313)	This study
ACY427	*MAT a nab2Δ::HIS3 ura3 leu2 his3* (pAC636)	(68)
SVL182/PSY1224 (ACY1571)	*hrp1ΔHIS3 ura3 his3* [*HRP1 CEN URA3*]	S.R. Valentini
ACY71	*MAT α npl3-1 trp1 ura3 leu2*	M. Henry
H3247	*Mat a his3 leu2 met15 ura3 CAF1-MYC13::HIS3 MX6*	(69)
H3239	*Mat a his3 leu2 met15 ura3 CCR4-MYC13::HIS3 MX6*	(69)
H2341	*Mat a his3 leu2 met15 ura3 NOT2-MYC13::HIS3 MX6*	(69)
H3245	*Mat a his3 leu2 met15 ura3 NOT5-MYC13::HIS3 MX6*	(69)
YNL052	*Mat a his3 leu2 met15 ura3 NOT1-MYC13::HIS3 MX6*	This study
YNL068	*Mat a his3 leu2 met15 ura3 HMT1-6XHA::KANMX*	This study
YNL069	*Mat a his3 leu2 met15 ura3 CCR4-MYC13::HIS MX6 HMT1-6XHA::KANMX*	This study
YNL070	*Mat a his3 leu2 met15 ura3 NOT2-MYC13::HIS MX6 HMT1-6XHA::KANMX*	This study
YNL071	*Mat a his3 leu2 met15 ura3 NOT5-MYC13::HIS MX6 HMT1-6XHA::KANMX*	This study
YNL179	*Mat a his3 leu2 met15 ura3 CAF1-MYC13::HIS3 MX6 hmt1Δ::KANMX*	This study
Plasmids	**Description**	**Reference/Source**
pN827	*pADH1 CEN URA3*	(70)
pAC2492	*pADH1-NOT4-FLAG CEN URA3*	(36)
pAC2494	*pADH1-CCR4-FLAG CEN URA3*	This Study
pAC2668	*pADH1-not4-L35A-FLAG CEN URA*	This Study
pAC2816	*pADH1-CAF1-FLAG CEN URA3*	This Study
pUN90-LEU2-nup49-313	*nup49-313 CEN LEU2*	(19)
pAC636	*NAB2 CEN URA3*	(46)
pSW3298 (pAC2307)	*nab2-C437S CEN LEU2*	(71)
pAC2539	*hrp1-P531A CEN LEU2*	(68)
pPS1750 (pAC2813)	*hmt1-G68R CEN URA3*	(72)
pPS1307 (pAC2811)	*HMT1 CEN URA3*	(44)
pRS306 (pAC4)	*CEN URA3*	(73)

(for experiments detecting Hmt1 or NPC associations) or 500 μg (for Hrp1 and Nab2 associations) and 2-3 μL of α-Myc or α-HA antibody (Santa Cruz Biotechnology). Immunoprecipitations were rotated 2 hours to overnight at 4°C before immune complexes were captured using Protein A-conjugated agarose (Santa Cruz Biotechnology). Samples were washed 3 times with 0.5-1 mL immunoprecipitation buffer and then resolved by SDS-PAGE and detected by immunoblot analysis. Nab2 and Hrp1 were detected by α-Nab2 and α-Hrp1 specific antibodies [(46) and gift from M. Swanson].

Fitness analysis: For serial dilution spotting assays, single colonies of wildtype or mutant cells expressing plasmid-borne *CAF1*, *CCR4*, *NOT4*, *not4L35A*, or empty vector were grown to saturation in selective liquid culture lacking uracil (ura^-), normalized to equal starting concentrations, and then serially diluted (1:10) in dH_2O and spotted onto selective ura^- plates. Plates were incubated at 25, 30, 33, 35 or 37ºC for 2-4 days as indicated.

Fluorescence in situ hybridization (FISH): The intracellular localization of poly(A) RNA was assayed essentially as described (46, 74). Briefly, cells were grown to saturation overnight at 25ºC and subsequently diluted and incubated for 2 h to allow cells to re-enter growth phase. Cells were then shifted to 30ºC for 2-4 h. Cells were fixed with 4.2% formaldehyde. The cell wall was digested with 0.5 mg/mL zymolase, and cells were applied to multi-well slides (Thermo Electron Corporation) pre-treated with 0.1% polylysine. Cells were then permeabilized with 0.5% NP-40, equilibrated with 0.1 M triethanolamine, pH 8.0, and incubated with 0.25% acetic anhydride to block polar groups. Cells were then incubated in prehybridization buffer [50% deionized formamide,

10% dextran sulfate, 4X Sodium Chloride-Sodium Citrate buffer (SSC), 1X Denhardt's solution, 125 μg/mL tRNA] and hybridized overnight to digoxigenin-labeled 50-mer oligo(dT) probe (IDT DNA). Wells were washed several times and blocked in 0.1 M Tris pH 9.0, 0.15 M NaCl, 5% heat-inactivated fetal calf serum, and 0.3% Triton X-100. Cells were incubated 2 hours with fluorescein isothiocyanate (FITC)-conjugated α-digoxigenin antibody (1:200, Roche). Wells were then washed several times and stained with 1 μg/μL 4',6-diamidino-2-phenylindole-dihydrochlorine (DAPI) to detect chromatin. Cells were mounted in antifade medium (0.1% *p*-phenylenediamine, 90% glycerol in phosphate-buffered saline). Slides were stored at -20ºC until visualization. Samples were visualized using filters from Chroma Technology (Brattleboro, VT) and an Olympus BX60 epifluorescence microscope equipped with a photometric Quantix digital camera. For quantification of results, blinded images were analyzed for poly(A) nuclear signal using the ImageJ Cell Counter plugin. A minimum of 50 cells were analyzed in triplicate for each condition. Unpaired Student's *t*-test assuming unequal variance was used to determine statistical significance.

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