Regulation of the Histone Chaperone Molecules Nap1p and Nucleoplasmin by Phosphorylation

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A Dissertation presented to the Graduate Faculty
of the University of Virginia for the Degree of
Doctor of Philosophy

Department of Microbiology

University of Virginia
July, 2007

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Abstract

The dynamic interaction of histones with DNA is regulated by histone chaperones, providing a mechanism for chromatin-dependent transcriptional control. I examined two disparate histone chaperones, mouse NPM2 and yeast Nap1p and determined that both are phosphoproteins and substrates for phosphorylation by CK2. The histone chaperone Nucleoplasmin is a maternally expressed protein in *Xenopus laevis* oocytes and is involved in histone transport and sperm chromatin decondensation in early embryogenesis. In a proteomic analysis of mouse oocyte maturation I identified the mouse homolog of nucleoplasmin (NPM2) and cloned and characterized the gene encoding NPM2. I determined that expression of NPM2 mRNA and protein is oocyte specific, and decreases rapidly following fertilization. NPM2 undergoes a change in localization during oocyte maturation that correlates with a reversible shift in molecular weight, suggestive of phosphorylation.

In *S. cerevisiae* Nap1p has multiple functions, being a cofactor for the nuclear import of H2A and H2B, a chromatin assembly factor and a mitotic factor involved in regulation of bud formation. I identified Nap1-interacting factors including a novel bud-neck associated protein, Nba1p, and showed that Nap1p and Nba1p are phosphoproteins in vivo and are phosphorylated by the mitotic kinase Cdc28p/Clb2p. I determined that the interaction of Nap1p with some of its binding partners is cell-cycle dependent, suggesting that its different functions may be regulated by the protein complexes with which it is associated. Nap1p, like NPM2, is phosphorylated by CK2 and I determined that this phosphorylation promotes its import into the nucleus. Mutation of CK2 phosphoserines

renders Nap1p able to rescue normal bud formation, but unable to restore normal cell cycling in a Clb2-dependent strain, resulting in prolonged S phase. A constitutively charged mutant is unable to assemble chromatin, implying that reversible phosphorylation of Nap1p is required for release of histones onto chromatin. Thus, phosphorylation of Nap1p by CK2 may regulate both its localization and cellular functions. These studies show that localization of both Nap1p and NPM2 may be dependent upon phosphorylation, and these signaling events are likely to be involved in cell cycle progression. Phosphorylation of histone chaperones by CK2 may therefore represent a conserved mechanism for chromatin-dependent regulation of gene expression.

Acknowledgements

I would most of all like to acknowledge Lucy Pemberton for being such a wonderful mentor. Lucy has taught me so much about biochemistry and the working with the yeast model in a relatively short time, but has also become a great friend. I also thank Nima Mosammaparast for helping me get started in the lab and Brian Del Rosario for being a great friend and colleague, helping me experimentally and through great scientific discussions. Thanks to all the Pemberton lab members past and present: Jeffrey Blackwell, Jenny Hodges, Kristin Keck, Jen Leslie, and Amy Shaw, for making the lab a great place to work, and for help and support along the way. I would like to thank everyone on my committee, Todd Stukenberg, Mitch Smith, Ian Macara, and particularly David Brautgian for assistance with kinase assays and experimental design in general. Thanks to my former mentors, John Herr and Scott Coonrod for the training and support I received during my first project, and to Laura Digilio for teaching me everything I needed to know during my work with mouse oocytes. I thank both of my parents, Christina Kennedy and Paul Calvert for all the scientific training they provided during my formative years, for being friends and role models throughout my life and for all the support along the way. I would like to thank the friends I made here in graduate school, particularly Colin DeBakker and Tiffany Keepers for offering a welcome distraction when needed. I would like to thank all the wonderful instructors at Bikram Yoga Charlottesville, and particularly Lizzie and Micheala for allowing me to practice yoga through work study, and providing some much needed balance and harmony in my life over the last few years. Finally, thanks to Alex Ward, my lab mate, collaborator, best friend and husband, for supporting me in every way. I couldn't have done it without him.

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AC	Adenylate cyclase
ADDS	Adenylsuccinate synthase
AF	Antral follicle
AMP	Adenosine monophosphate
APC	Anaphase promoting complex
ATP	Adenosine triphosphate
AU	Arbitrary units
Bl	Blastocyst
BSA	Bovine serum albumin
CBB	Coomassie blue
CSF	Cytostatic factor
CK2	Casein kinase 2
DDCT	Delta delta cycle threshold
DIC	Differential interference contrast
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced Chemiluminescence
FSH	Follicle stimulating hormone
FMOC	9-fluorenylmethyl carbamate
FRGY	Frog Y-box protein
G1	Gap 1

G2	Gap 2
GFP	Green fluorescent protein
GST	Glutathion S-tranferase
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
HSP	Heat shock protein
HU	Hydroxyurea
IF	Immunofluoresence
IgG	Immunoglobulin G
kDa	Kilodalton
KLH	Keyhole limpet hemocyanin
LH	Luteinizing hormone
LMB	Leptomycin B
M	Mitosis
MII	Metaphase II
MAPK	Mitogen-activated protein kinase
MBP	Maltose binding protein
MPF	Mitosis (or maturation) promoting factor
Mo	Morula
MS	Mass spectrometry
MW	Molecular weight
ND	Not detectable
NES	Nuclear export sequence

NGS	Normal goat serum
NLS	Nuclear localization sequence
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDCD6IP	Programmed cell death six-interacting protein
PF	Primary follicle
Ph	Phase
pI	Isoelectric point
PKA	Protein kinase A
PmF	Primodial follicle
PMSF	Phenylmethanesulphonylfluoride
PMSG	Pregnant mare serum gonadotropin
PN	Pronuclear
PrA	Protein A
PVA	Polyvinyl alcohol
PVP	Polyvinyl pyrrolidone
RNA	Ribonucleic acid
SC	Synthetic complete (medium)
SDS/PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	Secondary follicle
STI1	Stress-inducible phosphoprotein
TACC3	Transforming acidic coiled-coil protein 3
TAP	Tandem affinity purification

TBS (TBS-T)	Tris buffered saline (+0.1% Tween-20)
TCTP	Translationally controlled tumor protein
TRITC	Texas red isothiocyanate
YPD	Yeast extract/Peptone/Dextrose

CHAPTER I: General Introduction

A. Regulation of Gene Expression

The ability of an organism to adapt to its environment is the primary determinant of evolutionary fitness, and in Darwin's words, "it is not the strongest species that survive, nor the most intelligent, but the ones most responsive to change". Adaptation or specialization is the response to the pressures of natural selection, the driving force behind evolution, and this demands that the genetic blueprints must be open to interpretation and encode for a variety of outcomes. Gene regulation provides a mechanism by which cells carrying identical sets of chromosomes are able to differentiate, and this process is the fundamental course of action of by which a cell can modify its structure and function.

Gene expression is a multi-step process, and different regulatory mechanisms exist at each step. Chemical modification of the DNA molecule itself through methylation of cytosine (CpG) residues by methyltransferases is a principal method of gene silencing (Scarano et al., 1965). Structural changes to chromatin, accomplished through modifications on the DNA-associated histone molecules such as acetylation, phosphorylation and methylation, can affect the accessibility of the DNA to polymerase and transcription factors (Brownell et al., 1996; Mahadevan et al., 1991; Taunton et al., 1996). The level of expression of a particular gene can be controlled by regulating initiation of transcription. Repressors, activators and other regulatory proteins are trans-acting factors that bind to DNA (frequently non-coding promoter and enhancer sequences) and alter the ability of RNA polymerase to transcribe a specific region of the

chromosome (for review, see (Gaston and Jayaraman, 2003)). Regulation at the level of transcription initiation is the primary method utilized by prokaryotic organisms (Struhl, 1999). In eukaryotes, more complex mechanisms for controlling of gene expression are available. Following transcription, regulation at the level of mRNA can be achieved through capping, alternative splicing mechanisms, and directed degradation. The availability of transcripts to ribosomal machinery can also be affected through regulated export of mRNA and other pathways (for review, see (Kozak, 1983; Rodriguez et al., 2004). Finally, post-translational modifications to the proteins themselves can alter the stability of a protein by promoting degradation or persistence, or by changing the tertiary structure such that a single gene product can perform a diversity of functions. The complexity of these coinciding levels of regulation provides the cell with a multiplicity of responses to a huge variety of signals.

B. Chromatin Structure

Chromatin is structurally composed of the double stranded DNA (dsDNA) molecule complexed with an octamer of histone molecules. This octamer consists of two dimers of histones H2A and H2B, and one tetramer of dimers of histones H3 and H4. One hundred and forty-six base pairs of dsDNA are wrapped twice around each octamer, and this unit is the nucleosome. Nucleosomes are spaced evenly along the DNA, forming a 10nm fiber of chromatin, which is further compacted by the incorporation of the H1 linker histone between individual nucleosome particles (Luger et al., 1997). The nucleosomal structure is highly organized, as revealed by the solved crystal structure, and incredibly well conserved throughout eukaryotes.

Histone molecules have positively charged N-terminal tails which extend out from the compact core of the nucleosome. These histone tails are the site of post-translational modifications that alter the net charge and regulate the access of other proteins to the DNA. In general, acetylation of lysine residues reduces the affinity of the histones for negatively charged DNA and hyperacetylation is believed to locally promote transcriptional activation by reducing chromatin compaction (Brownell et al., 1996). Conversely, deacetylation is frequently associated with increased compaction and repressed and silenced regions of the genome (Taunton et al., 1996). The interplay of histone acetyl transferases (HATs) and histone deacetylases (HDACs) maintains the boundaries between regions of silent heterochromatin and actively transcribed euchromatin (Bulger, 2005). Methylation of histone tails has been associated with multiple effects on transcription and the outcome of methylation, whether transcriptional repression or activation, appears to be system and site specific. Histone tail phosphorylation can inhibit methylation at specific sites and promote acetylation at others, or act synergistically in combination with other adjacent modifications to activate gene expression (for review, see (Jenuwein and Allis, 2001).

In eukaryotes, most histone synthesis is DNA synthesis during the S phase of the cell cycle, and the majority of nucleosomal assembly occurs immediately follows replication. Chromatin assembly can also be replication-independent, such as during DNA damage repair, histone replacement and in the case of sexual reproduction, during fertilization and syngamy (Altheim and Schultz, 1999; Kimmins and Sassone-Corsi, 2005). There are a number of histone variants, such as H2A.Z and H3.3, whose synthesis is uncoupled from S phase (White and Gorovsky, 1988; Wu et al., 1982). The

incorporation of histone variants into chromatin has been shown to alter gene expression. In budding yeast, H2A.Z has been isolated as both a positive and negative regulator of transcription. It has functional redundancies with the chromatin remodeling complexes Swi/Snf and SAGA, suggesting it is involved in transcriptional activation (Santisteban et al., 2000). It is enriched in the promoter region of silenced genes, and loss of the variant occurs following the activation of expression (Adam et al., 2001; Guillemette et al., 2005; Santisteban et al., 2000). It also appears to be important in the maintenance of silencing at the mating-type locus and telomeric regions (Meneghini et al., 2003). Conversely, incorporation of the H3 variant H3.3 is most concentrated in transcriptionally active regions of the genome, and its deposition can occur throughout the cell cycle (Ahmad and Henikoff, 2002). In yeast, in which the majority of the genome is maintained in a transcriptionally active state, this is the predominant H3 subtype.

C. Chromatin assembly factors

At the onset of DNA synthesis parental histones are removed as H3/H4 and H2A/H2B dimers resulting in two pools of histone dimers in the cell: disassembled, parental, nuclear histones and nascently translated, cytoplasmic histones. The process of chromatin assembly therefore requires both cytoplasmic and nuclear factors capable of binding histones, transporting them into the nucleus, and ordering the histones onto DNA. Two of the proteins involved in this process, the histone chaperones Nucleoplasmin and Nap1p, are the focus of this dissertation. Following deposition, histones must slide along DNA and transiently dissociate and reassemble until regular arrays of nucleosomes are formed. The correct assembly and spacing of intact

nucleosomes onto DNA involves ATP-dependent chromatin remodeling complexes. In general, all chromatin remodeling complexes must contain an ATP-ase subunit, since nucleosome remodeling is an energy-demanding process and requires a large amount of ATP hydrolysis (for review, see (Kingston and Narlikar, 1999). Among the other subunits are frequently HATs, helicases and histone chaperones.

Histone chaperones and chromatin remodeling complexes can regulate gene expression by altering histone availability, exchanging differently modified histones, and incorporating histone variants at targeted regions of the genome. The high affinity of positively charged histones with negatively charged DNA could lead to the formation of irregular aggregates of nucleosomal proteins, and histone chaperones shield these charge-charge interactions and, in combination with chromatin remodeling complexes, this allows sequential, organized deposition. In the course of replication, the histones removed during nucleosome disassembly may remain in the immediate vicinity of the DNA. Histone chaperones can incorporate these parental histones into newly replicated DNA, thus transferring any existing modifications. This provides a mechanism for epigenetic inheritance, the exchange of heritable information beyond the nucleotide sequence alone. Histone chaperones play a key role in maintaining epigenetic information, by aiding in replication-coupled displacement of parental histones and promoting the assembly of both nascent and parental histones onto newly synthesized DNA (Jin et al., 2005). Some histone chaperones have specific replication-independent roles. Replication-independent chromatin remodeling is required during homologous recombination and DNA repair, and also permits histone replacement at transcriptionally active regions of the genome, such as during incorporation of the H3.3 histone variant.

There are a variety of histone chaperones and chromatin remodeling proteins. While there are some redundancies, they also appear to have discrete functions, exhibit binding preferences for the different histone subunits, and are sometimes associated with distinct regions of the genome.

i. CAF-1 (chromatin assembly factor 1)

CAF-1 is a complex of three subunits. It associates with nascent H3/H4 on newly replicated DNA, and its assembly activity is specific to DNA synthesis and repair (Krude, 1995; Smith and Stillman, 1989). Through its different subunits (in yeast, Cac1p, Cac2p and Cac3p) CAF-1 interacts with the DNA polymerase complex component, PCNA (Pol30p in yeast), and this interaction physically places it at the site of replication (Shibahara and Stillman, 1999). CAF-1 also binds to the histone chaperone ASF-1 and the two complexes appear to cooperatively assemble nascent H3/H4 dimers onto newly replicated DNA (Tyler et al., 2001). Yeast strains lacking Cac1p, a subunit of the CAF-1 chromatin assembly complex, show similar sensitivity to DNA damage, and when combined with mutations in other checkpoint proteins led to defects in G1/S progression .(Harkness et al., 2005). Yeast CAF1 mutants have decreased silencing at telomeres and HM1 and HMR mating-type loci. Since CAF1 exhibits reduced affinity for hyperacetylated H4 histones, it has been proposed that the silencing defects in CAF1 mutants might be due to incorporation of inappropriately modified histones at these loci (Enomoto and Berman, 1998). *Rtt106* encodes another histone chaperone, and was recently identified in a mutant screen for silencing defects. Rtt106p interacts physically and genetically with CAF-1, but not with ASF-1 (Huang et al., 2005).

ii. ASF1 (anti-silencing function 1)

ASF1 genes are well conserved among higher eukaryotes. Asf1p deposits H3 and H4 histones onto chromatin in a replication-dependent manner, and requires the combined action of a H2A/H2B chaperone (such as Nap1p) for the completion of nucleosome assembly (Tyler et al., 1999a). ASF1 preferentially binds newly synthesized H3 and H4, though in vitro this preference is only observed in the presence of CAF1. ASF1 also binds directly to CAF1, and this interaction may target ASF1 to the replication fork (Mello and Almouzni, 2001; Mello et al., 2002). Asf1p mutations in yeast have been associated with cell cycle delays at G1/S (the DNA damage checkpoint) due to accumulation of DNA damage during DNA replication. It is conceivable that generally decreased histone chaperone activity could delay DNA synthesis, but interestingly these mutants also exhibit delays at G2/M, implying that ASF1 may also have replication-independent remodeling activity (Prado et al., 2004).

iii. HIR1 and HIR2 (histone regulation)

HIR1 and HIR2 genes are non-essential repressors of histone gene expression. When mutations in the *HIR* genes are combined with *CAC* mutations, silencing defects were observed at telomeres, kinetochores and mating type loci (Kaufman et al., 1998). In human cells, ectopic expression of HIRA, a repressor of histone gene expression, caused cells to arrest in S phase, probably due to kinetochore silencing defects and the resulting activation of the spindle checkpoint (Nelson et al., 2002). HIRA binds exclusively to the H3.3 histone variant. Whereas CAF-1 primarily mediates replication-coupled deposition

of H3, HIRA-mediated incorporation of H3.3 to transcriptionally active regions of the genome is replication-independent (Tagami et al., 2004).

iv. ACF (ATP-utilizing chromatin assembly and remodeling factor)

ACF is a multi-subunit complex that, in association with a chromatin assembly factor, is able to assemble periodic arrays of nucleosomes onto chromatin in vitro (Ito et al., 1997). The largest subunit of ACF is ISWI (imitation switch), an ATP-ase and the catalytic component of ACF, CHRAC and NURF remodeling complexes (Ito et al., 1999). In Drosophila, ACF also contains the subunit Acf1, which contributes to the establishment of transcriptional silencing. Mutations in Acf1 result in an accelerated S phase, due to reduced histone availability (Fyodorov et al., 2004; Ito et al., 1997). As a complex, ACF1 cooperatively catalyzes nucleosome assembly and disassembly by the other histone chaperones NAP1 and CAF-1 (Ito et al., 2000).

v. CHD1 (chromo-ATPase/helicase-DNA binding protein 1)

Chd1p is a highly conserved DNA-stimulated ATPase that facilitates nucleosome assembly in vivo. Chd1p also has a role in transcriptional activation and is detected at promoter regions. In yeast *CHD1* interacts with genes involved transcriptional elongation such as the FACT components (Costa and Arndt, 2000; Kelley et al., 1999; Krogan et al., 2002; Simic et al., 2003; Tran et al., 2000). A recent study found that the Chd1p homologs in fission yeast, Hrp1 and Hrp3, have an in vivo role in chromatin remodeling at active regions of the genome where they are primarily involved in nucleosome disassembly at promoters and coding regions (Walfridsson et al., 2007).

vi. FACT (facilitates chromatin transcription)

FACT is a heterodimeric complex consisting of Spt16 and Pob3 (Ssrp1 in humans) subunits (Orphanides et al., 1999). FACT binds to H2A/H2B dimers, assists RNA Polymerase II in elongating transcripts, and is required for transcription in vivo. It has been proposed that FACT has two distinct elongation activities, removal of H2A/H2B dimers from nucleosomes in front of the elongating polymerase, and reassembly of nucleosomes in its wake (Mason and Struhl, 2003; Saunders et al., 2003). Spt6p is a similar protein that binds histone H3 and promotes histone deposition in vitro. Spt6p interacts with the other FACT subunits, and is also thought to mediate chromatin assembly during elongation (Bortvin and Winston, 1996; Kaplan et al., 2003).

vii. Nucleosome assembly protein 1 (Nap1p)

Nap1p, the focus of chapters 3 and 4, is a histone chaperone in yeast that preferentially binds to H2A/H2B dimers, though it has also been found to interact with the histone variant Htz1p and can facilitate H2A.Z/H2B histone dimer exchange in vitro (Mosammaparast et al., 2002; Park et al., 2005). In a genome-wide expression study in yeast about 10% of all genes showed altered transcription in a *Δnap1* strain, with targets exhibiting either upregulation or downregulation of expression (Ohkuni et al., 2003). Additionally, in association with p300 activator and other transcription factors, Nap1p can enhance the transcription of reporter constructs two-fold (Rehtanz et al., 2004; Shikama et al., 2000). In association with the RSC chromatin remodeling complex, Nap1p promotes stepwise chromatin disassembly through removal of H2A/H2B from nucleosomes (Lorch et al., 2006a). It can also promote nucleosome assembly in

association with Chd1p, which transfers nucleosomes from Nap1p onto DNA (Lusser et al., 2005). In *S.pombe* Nap1 and Chd1 paralogues Hrp1 and Hrp2 share in vivo genomic binding targets. These shared targets tend to be active promoter regions of low nucleosome density, suggesting that Nap1p and Chd1p may be cooperatively regulating disassembly (Walfridsson et al., 2007). FACT and Nap1p both bind to H2A/H2B dimers and promote dimer release in the presence of RNA polymerase II, and bind to subunits of the RNA polymerase II elongator complex (Krogan et al., 2006; Orphanides et al., 1998; Orphanides et al., 1999). It has been proposed that Nap1p, Chd1p and the FACT complex all associate with the kinase CK2, and phosphorylation of these substrates may influence transcriptional activity (Krogan et al., 2002; Li et al., 1999b; Walfridsson et al., 2007). Taken together, these studies suggest that Nap1p may have both replication-dependent and independent roles in facilitating nucleosome remodeling, and may have a cooperative role in transcription elongation in association with the FACT complex.

Nap1p is a member of the Nap/SET superfamily of proteins, which have in common the ability to interact with chromatin and a role in the regulation cell proliferation through interactions with the B-type cyclins (Park and Luger, 2006a). The first human Nap1 family member was originally purified from HeLa cell extracts, where it was found to copurify with nucleosome assembly activity in vitro (Ishimi et al., 1984; Ishimi et al., 1985). Since then, other Nap1p homologues have been identified in humans, mice, *Drosophila*, the nematode *S.feltiae*, rice (*Oryza sativa*) and tobacco (*Nicotiana tabacum*) and in some species there are multiple isoforms exhibiting different tissue specificities (Dong et al., 2003; Gal et al., 2005; Ito et al., 1996b; Rodriguez et al., 1997; Watanabe et al., 1996). In addition, members of the Nap1 protein family interact with a

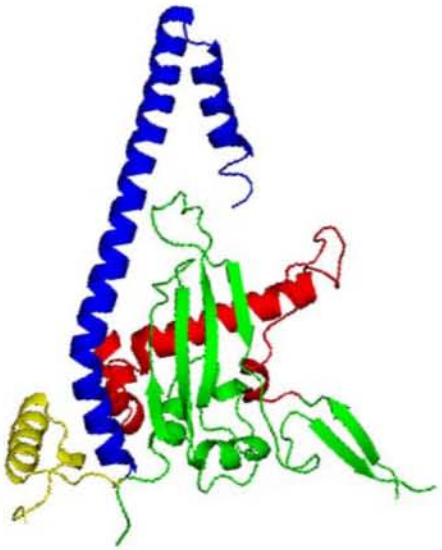
large number of proteins with diverse cellular functions. Taken together, this implies that members of the Nap/SET superfamily are multi-functional proteins and may be important mediators for a wide range of cellular events.

In general, Nap1 family members are highly acidic proteins and this promotes their ability to bind to basic histone molecules. All of the family members share a structurally conserved central Nap domain, and the crystal structure for this domain from yeast Nap1p was solved recently (Fig.1A). In the published structure of Nap1p four distinct regions of the protein were characterized, labeled subdomains A-D. (Park and Luger, 2006b). They found that the protein dimerizes via a long α-helix, in subdomain A, through which the protein monomers interact to form a homodimer with a hydrophobic core. The tip of the dimerization domain in Nap1p contains the nuclear export signal (NES). Subdomain B consists of an accessory domain of the protein that contributes to formation of the dimer, but appears to mask the NES of the dimerization partner. Subdomain C consists of an anti-parallel β-hairpin sheet, and this structural feature is shared among many histone chaperones, including nucleoplasmin, and is the most highly conserved region among members of the Nap/SET protein family (Park and Luger, 2006a; Zlatanova et al., 2007)**.** Subdomain D consists of an α-helix that is thought to stabilize the hydrophobic core formed by the β-sheet. A conserved C-terminal acidic domain of variable length is also shared among Nap/SET protein family members. The acidic C-terminal tail is disordered and not defined in the solved crystal structure, and thus is probably unstructured or highly flexible.

Figure 1

The structure of the central Nap domain, crystallized from yeast Nap1p.

Subdomain A (blue) is the dimerization domain and contains the NES. Subdomain B (yellow) is the accessory domain and may regulate access to the NES. Subdomain C (green) is highly conserved and shared among many histone chaperones. Subdomain D (red) is thought to stabilize the hydrophobic core of the anti-parallel β-sheet and is required for the proper folding of Nap1p.



Nap1p is primarily cytoplasmic throughout the cell cycle and localizes to the bud neck, but mutation of the NES causes a GFP reporter construct of the protein to be sequestered in the nucleus, implying it is a nucleocytoplasmic shuttling protein (Mosammaparast et al., 2002). In the cytoplasm, Nap1p binds to H2A/H2B dimers and facilitates histone import into the nucleus via the karyopherin Kap114p. H2A/H2B can also bind to the karyopherins Kap121p and Kap123p, but Nap1 promotes the specific interaction of H2A/H2B with Kap114p (Mosammaparast, Ewart et al. 2002. In general, high levels of RanGTP in the nucleus interact with karyopherins and this causes a conformational change that promotes cargo release. However, the interaction of Nap1p with Kap114p is Ran-insensitive, and this renders the Kap114p/H2A/H2B complex resistant to Ran-mediated disassociation (Mosammaparast et al., 2002). Additionally, Kap114p directly inhibits chromatin assembly by Nap1p in vitro, implying that once inside the nucleus, Nap1p must first dissociate from Kap114p before assembling nucleosomes (Mosammaparast et al., 2005). This suggests that the chromatin assembly activity of Nap1p is not inherently coupled to import and release of the H2A/H2B dimer, but is undergoing additional regulation once inside the nucleus. The mechanism by which the Kap114p/H2A/H2B/Nap1p complex dissociates inside the nucleus has not been determined, and nuclear factors involved in promoting or inhibiting nucleosome assembly by Nap1p have yet to be characterized. Since Nap1p has both chromatin assembly and disassembly activity, and incorporation of histones into chromatin by Nap1p can be replication-dependent and independent, this suggests that additional regulation of Nap1p nuclear function is required.

Vps75p is the most recently described member of the Nap1/SET protein family, and is a histone chaperone with preference for binding to H3/H4 tetramers. It can assemble chromatin in vitro, and associates with chromatin at transcriptionally active and silent regions of the genome, suggesting its assembly activity may not be replication-dependent (Selth and Svejstrup, 2007). Vps75p also associates with the Rtt-109 HAT complex, though does not appear to have histone acetylation activity (Han et al., 2007). Vps75p protein bears more sequence homology to human SET and TSPY proteins than to yeast Nap1p. Interestingly, Vps75p, SET and TSPY each contain a putative NLS, but lack a defined NES, and also lack the subdomain proposed to regulate NES accessibility in Nap1p. Consistent with this, Vps75p localizes to the nucleus (Huh et al., 2003).

viii. Nucleoplasmin

Nucleoplasmin, the focus of chapter 2, was the first histone chaperone to be discovered. It associates with histones H2A/H2B and is able to import histone dimers into the nucleus and has chromatin assembly activity in vitro. Nucleoplasmin is oocyte specific, highly abundant and is involved in syngamy, the process of pronuclear fusion following fertilization that results in a diploid embryo (Laskey et al., 1978). In order for syngamy to occur, the sperm chromatin must first be remodeled, and this is accomplished by nucleoplasmin. Sperm chromatin is structurally unique, consisting of DNA packaged with histones H2A and H2B, in complex with highly basic sperm-specific protamine molecules. Following membrane fusion, the sperm nuclear envelope breaks down and the disulfide bonds between protamines are reduced by high levels of glutathione in the

oocyte. The protamines are then removed from the DNA entirely and replaced by maternally transcribed H2A and H2B molecules. The sperm chromatin recondenses as nucleosomes are assembled and the male pronucleus is formed. Nucleoplasmin is required for both protamine removal and subsequent nucleosome assembly (Philpott et al., 1991).

The structure of nucleoplasmin is well conserved among higher eukaryotes. The monomer consists of an N-terminal core domain that folds into an eight-stranded β-barrel, and in *Xenopus* this region contains a short acidic tract responsible for oligomerization and histone binding. Each monomer contains a long C-terminal acidic tract that stretches outside the core and carries the exposed NLS; five monomers fit stably into a pentamer, and two pentamers into a decamer (Dutta et al., 2001). Nucleoplasmin also appears to have a role in nucleolar organization, since mice lacking Npm2 show impaired chromatin condensation around the nucleolus (Burns et al., 2003). N1/N2 nucleolar protein is another oocyte-specific and abundant histone chaperone with a preference for H3/H4 dimers. Like nucleoplasmin, N1 is believed to function as a repository or 'histone sink' for excess histones in the oocyte, and these two proteins probably work cooperatively to assemble chromatin in the fertilized egg (Kleinschmidt et al., 1985).

D. Cell Cycle Regulation

Tightly regulated gene expression interprets external cues in order to guide differentiation, but it is also crucial to guiding cell division. Each cell division requires a single and complete replication of the chromosomes, the correct distribution of the duplicated chromomes between the parental and daughter nuclei, and the coordination of the structural events required for the completion of cytokinesis. These processes are driven by an interplay of proteins whose expression levels fluctuate in an organized pattern across the cell cycle, and the molecular events at each stage in the cell cycle are coordinated by the exact milieu of proteins present in the cell at a given point in the cycle. Precise control of the protein levels is accomplished by coordinate regulation of gene expression and protein degradation.

Nap1p and the Regulation of Mitosis

The eukaryotic cell cycle consists of four stages: G1, S, G2 and M. Successful cell division will yield a mother and daughter cell, and as each begins a new cycle of division, this event is followed by the G1 (Gap 1) interval during which the cell increases in size. S phase begins at the onset of DNA synthesis and lasts until replication of the chromosomes is complete. A second interval, G2 (Gap 2), follows S phase and this allows the cell to monitor the products of replication and ensure that synthesis has been completed. Once complete, the cell enters M phase (mitosis). During mitosis, chromosomes condense and microtubular spindle fibers connect to chromosomes at the kinetochores. The chromosomes are then pulled into alignment along the axis of cytokinesis at the metaphase plate. Once the mitotic spindle is fully assembled, the sister

chromatids are separated. Mitosis is completed at cytokinesis, the mother and daughter cells are separated and the cell cycle is completed following division.

The cell cycle is tightly regulated by a biochemical signaling network that provides feedback and can drive or delay mitotic progression in response to both internal and external signals. Among eukaryotes, the proteins involved in regulating the cell cycle progression are generally well conserved. In budding yeast, cell cycle control is primarily accomplished by a cyclin-dependent protein kinase, Cdc28p, that induces specific events by phosphorylating downstream targets. There are different cyclins that activate Cdc28p and direct it to specific targets at the appropriate cell cycle transitions. The G1 cyclin Cln1 binds to Cdc28p and this active kinase (often referred to as Start kinase) is required for entry into S phase. Activation of a G1 checkpoint can be triggered by cell size defects, limited nutrients, or the detection of DNA damage, and this will delay activation of Start kinase and delay the onset of DNA synthesis (Tyers et al., 1991). After completion of S phase, the mitotic cyclin Clb2p begins to accumulate during G2, and in complex with Cdc28p this forms the mitotic kinase that is required for entry into mitosis (Grandin and Reed, 1993). Another kinase, Swe1p, phosphorylates and negatively regulates Cdc28p. This phosphorylation is removed by the phosphatase Mih1p, and this occurs concurrently with degradation of Swe1p at the G2/M transition and allows the mitotic cyclin Clb2p to accumulate and activate Cdc28p (Fig. 2A) (Booher et al., 1993).

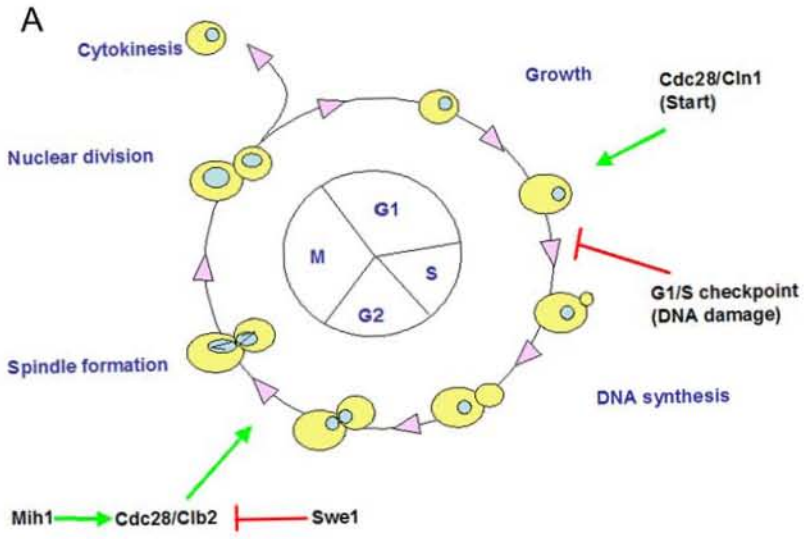
Figure 2

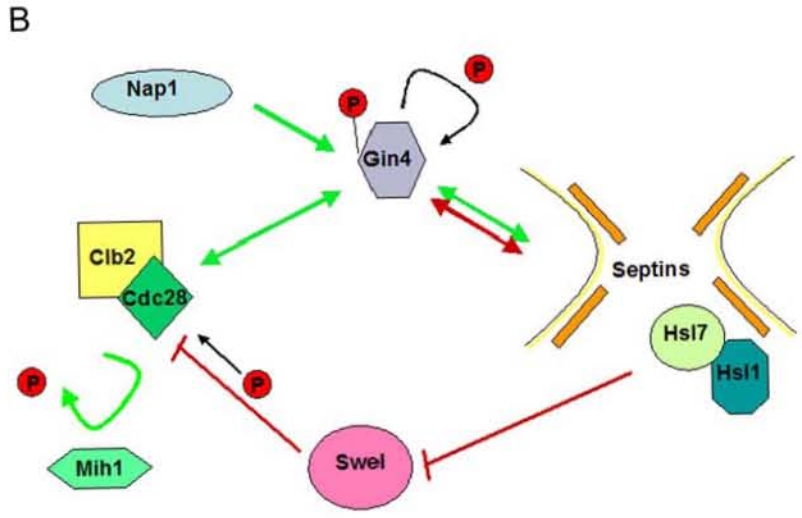
The regulation of mitosis in S.cerevisiae

Green arrows are positive regulatory events, red lines show potential inhibitory signals, P indicates phosphorylated residue

A: Regulation of the budding yeast cell cycle.

B: Mitotic signaling cascade regulates the organization of bud neck septins and bud growth.


A
Cytokinesis
Growth
Cdc28/Cln1
(Start)
Nuclear division
G1
M
S
G2
G1/S checkpoint
(DNA damage)
Spindle formation
DNA synthesis
Mih1
Cdc28/Clb2
Swe1


B
Nap1
P
P
Gin4
Clb2
Cdc28
Septins
Hsl7
Hsl1
P
P
Mih1
Swe1

During S phase in budding yeast the daughter cell grows from the mother cell and bud growth is polar and unidirectional. At the entry into mitosis, growth expands isotropically across the surface of the bud (Lew and Reed, 1993b). If Cdc28p/Clb2p kinase activation is inhibited, or if the Swe1p kinase persists, the cell cycle will be delayed at the G2/M transition, polar bud growth will persist and the resulting cell exhibits an elongated bud phenotype. Clb2p and the histone chaperone Nap1p are involved in regulating the switch from polar to isotropic bud growth. Both Clb2p and Nap1p are required for hyperphosphorylation and activation of another kinase, Gin4p (Altman and Kellogg, 1997a). Once activated, Gin4p binds to septins at the bud neck. A mitotic signaling cascade is initiated, resulting in organization of the bud neck septins, and organized septins are required for activation of Hsl1p kinase. Hsl1p kinase then recruits Swe1p to the bud neck and ultimately triggers its degradation. This is a positive feedback loop; the cascade initiated by the activation of Clb2p ultimately results in degradation of its inhibitor, Swe1p, and this results in the rapid rise in Cdc28p/Clb2p activity required for entry into mitosis (Kellogg, 2003). In the absence of Nap1, the hyperphosphorylation and activation of Gin4p is prevented, the mitotic signaling cascade is terminated and resulting in a G2/M delay and in *Δnap1* mutant strains elongated buds are observed (Kellogg et al., 1995; Kellogg and Murray, 1995a); (Altman and Kellogg, 1997a); (Kellogg and Murray, 1995a; Shimizu et al., 2000) (Fig. 2B). At the end of mitosis, Clb2p is degraded by the anaphase promoting complex (APC), a multi-subunit complex that is critical in cell cycle regulation (Irniger and Nasmyth, 1997). During M, anaphase promoting complex (APC) associates with microtubules of the cytoskeleton and helps to orient the mitotic spindle (Muhua et al., 1998). The cohesion of sister chromatids

is maintained by the cohesin complex and Securin (Pds1p), and APC also mediates the degradation of these factors prior to chromosome segregation (Cohen-Fix et al., 1996). Following chromosome segregation, Cdc28p activity diminishes as the G1 cyclins reappear and resume activity, and the cell enters a subsequent division cycle.

Among eukaryotes, the mechanism of mitotic regulation is well conserved. In higher eukaryotes, the Mih1p phosphatase homolog Cdc25 relieves an autoinhibitory phosphorylation of the mitotic cyclin-dependent kinase Cdc2 that, in complex with cyclin B1, forms the mitosis promoting factor (MPF) heterodimer. MPF is transiently downregulated at the exit of M due to proteosomal degradation of the cyclin B1 subunit by the anaphase promoting complex (APC) (Hoffmann et al., 1993). *Xenopus* homologs of Nap1 also bind to cyclin B1, and in mammals mutations in Nap1 family members are associated with a variety of hyperproliferative disorders, providing further evidence for a role in cell cycle regulation. In mice, deletion of Nap1L-2 is embryonic lethal due to overproduction of neuronal precursor cells (Rogner et al., 2000), and mutations and translocations in Nap/SET family members are associated with a variety of human disorders such as Mixed Lineage Leukemia, gonadoblastoma, and Wilms' tumors associated with Beckwith-Wiedemann syndrome (Adachi et al., 1994a; Adachi et al., 1994b; Fornerod et al., 1995a; Fornerod et al., 1996b; Li et al., 1996; Tsuchiya et al., 1995; von Lindern et al., 1992). In some species Nap family members undergo cell-cycle dependent changes in localization. In Drosophila embryos, Nap1 is predominantly nuclear during S phase and relocalizes to the cytoplasm during G2, and human Nap2 is similarly only observed in the nucleus during S phase (Ito et al., 1996b; Rodriguez, 2000). In contrast, *S.cerevisiae* Nap1p remains predominantly cytoplasmic throughout the

cell cycle. Additionally, it is detected at the actin cap following bud site selection, and at the bud neck throughout bud growth (Fig.1B).

The regulation of Nap1p localization and activity has not been previously described, though there is evidence that phosphorylation may be involved. Both *O.sativa* and *D.melanogaster* Nap1 are substrates for phosphorylation by recombinant human CK2 in vitro, and in a yeast 2-hybrid assay *S. feltiae* Nap1 interacted with endogenous CK2 (Dong, 2005;Li et al., 1999b; Gal, 2005). The testis-specific Human Nap1 homolog TSPY predominantly occurs in a phosphorylated form, and phosphorylation of a C-terminal CK2 consensus serine is required for nuclear localization (Krick et al., 2006). Interestingly, localization of human Nap2 to the nucleus during S phase is associated with a net loss of phosphorylation, though regulation of import by phosphorylation was not demonstrated (Rodriguez et al., 2000b). Taken together, these studies have led to speculation that Nap1p localization may be regulated by phosphorylation.

Meiosis, Oocyte Maturation and Nucleoplasmin

Mitosis is the process by which a diploid cell replicates its genetic material and then divides to produce two genetically identical diploid cells; during meiosis, two successive cell divisions follow a single round of replication, resulting in four genetically distinct haploid cells. It is during meiosis that genetic recombination occurs, yielding a novel assortment of genes and leading to greater genetic diversity, and this is the evolutionary advantage provided by sexual reproduction. In contrast to mitosis, the two meiotic divisions occur without an intervening S phase, though the molecular events controlling meiosis are very similar to those in mitosis. During the first meiotic division,

meiosis I, the homologous chromosomes segregrate and the two sister chromatids remain attached. This is a reductive division, since the tetraploid cell is then divided into two diploid cells, each containing one of the homologous chromosomes. During meiosis II, sister chromatids segregate, and each of the diploid cells divide to produce four haploid cells. As in mitosis, accumulation of Cdc2 occurs at the onset of meiosis I; APC-mediated degradation of cyclin causes the activity to decline between the two divisions, but does not prevent it entirely (Frank-Vaillant et al., 2001). This residual activity may be required to prevent an intervening round of DNA synthesis from occurring.

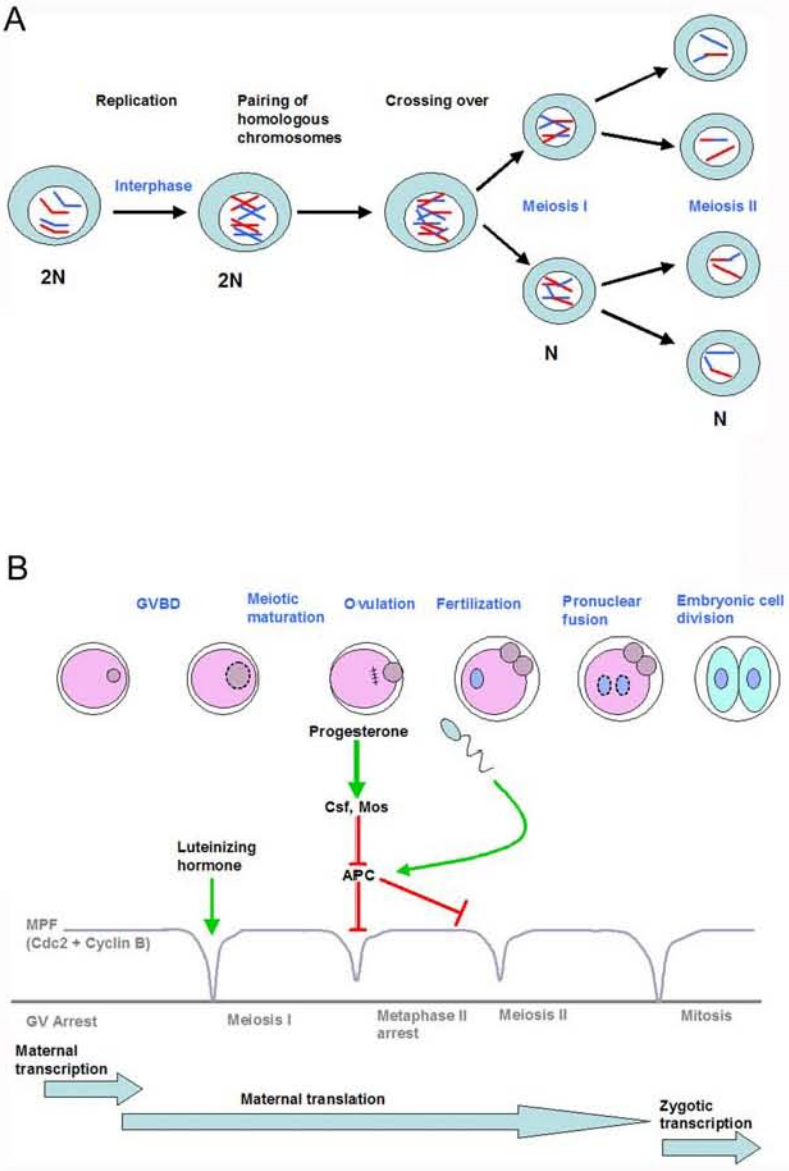
Figure 3

The regulation of meiosis in higher eukaryotes

Green arrows are positive regulatory events, red lines show potential inhibitory signals.

A: Regulation of meiosis. N indicates DNA content; N = haploid, 2N = diploid.

B: Regulation of oocyte maturation.


A
Replication
Pairing of homologous chromosomes
Crossing over
Interphase
Meiosis I
Meiosis II
2N
2N
N
N
B
GVBD
Meiotic maturation
Ovulation
Fertilization
Pronuclear fusion
Embryonic cell division
Progesterone
Csf, Mos
Luteinizing hormone
APC
MPF
(Cdc2 + Cyclin B)
GV Arrest
Meiosis I
Metaphase II arrest
Meiosis II
Mitosis
Maternal transcription
Maternal translation
Zygotic transcription

In the vertebrate ovary, the oocytes are maintained from birth in an immature, cell-cycle arrested state, and the resumption of meiosis is coordinated through a highly specialized cell cycle. Prior to ovulation, oocytes are arrested in the early prophase I of meiosis I with an intact oocytic nucleus, the germinal vesicle. These germinal vesicle oocytes are fertilization incompetent, until a mid-cycle surge of lutenizing hormone triggers one or more immature oocytes within follicles to resume meiosis (Tsafriri et al., 1996). The germinal vesicle breaks down (GVBD), the chromatin condenses and the metaphase spindle forms. In mammals, the first meiotic division is asymmetrical, forming a small polar body containing the inactivated X-chromosome (or Barr body) (Barr and Carr, 1962). Following formation of the second metaphase spindle, the oocyte arrests again at metaphase II (MII) and meiosis II will only be completed following fertilization. In adult vertebrate females each estrus cycle is marked by the release of one or more MII arrested, fertilization competent oocytes from an ovarian follicle into the oviduct or fallopian tube. If viable sperm are present, fertilization will occur in the ampulla of the oviduct, and (following pronuclear fusion) the fertilized egg will begin the first zygotic mitosis.

The process of oocyte maturation is also tightly regulated by an interplay of kinases and phosphatases, and primarily driven by fluctuating MPF levels (Barr and Carr, 1960). Until meiosis II is completed to form the pronuclear zygote, both the fully matured oocyte and the fertilized egg are transcriptionally inactive. Any regulation of gene expression must occur post-transcriptionally, and thus mRNA degradation, translational repression and post-translational modifications are the primary regulatory mechanisms available to the oocyte at this stage (Bettegowda et al., 2006). Immature oocytes are

marked by low levels of MPF, and maturation and ovulation are triggered by increasing levels of MPF. Prior to MII arrest, cyclin B1 is partially degraded and thus MPF levels drop again. If degradation of cyclin B1 is blocked, oocytes will not exit M-phase and no chromosomal segregation or cytokinesis occurs (Ledan et al., 2001). Progesterone signaling at the onset of oocyte maturation also upregulates translation of c-Mos, a germ-cell specific protein kinase. C-Mos, also known as cytostatic factor, activates MAP kinase and stabilizes MPF by inhibiting cyclin B1 degradation and maintaining the MII arrest . C-Mos mutant oocytes therefore fail to arrest at MII, and are often parthenogenetically (asexually) activated . At fertilization, an increase in intracellular calcium activates APC and causes MPF levels to drop, meiosis II is completed, and rapid rounds of embryonic cell divisions commence (Fig. 3B).

During the resumption of Meiosis I at the onset of oocyte maturation, nucleoplasmin becomes hyperphosphorylated and this phosphorylation is required for its decondensation activity. It is believed that phosphorylation increases the affinity of nucleoplasmin for basic sperm proteins (Leno et al., 1996)**.** The binding of phosphorylated nucleoplasmin to sperm protamines leads to a conformational change in the complex, resulting in the dissociation of H2A/H2B dimers. H3/H4 tetramers are released from N1/N2, another oocyte-specific histone chaperone, and the histones are assembled into nucleosomes on the paternal DNA (Dutta et al., 2001). Nucleoplasmin persists in a phosphorylated form until zygotic transcription is initiated at the mid blastula transition, at which point dephosphorylation occurs and the protein is ultimately degraded (Leno et al., 1996). As with many other NLS-containing proteins, phosphorylation of nucleoplasmin regulates its nucleocytoplasmic distribution. In *Xenopus*, nucleoplasmin

hyperphosphorylation at the resumption of meiosis correlates with a relocalization of the protein from the nucleus to the cytoplasm (Sealy et al., 1986). It copurifies with the kinase CK2 and is a substrate for CK2 phosphorylation in vitro, though inhibition of CK2 activity decreases nuclear accumulation of nucleoplasmin in oocytes (Vancurova et al., 1995).

E. Project Rationale

The first evidence that cytoplasmic factors are involved in the regulation of the cell cycle was provided by the *Xenopus* oocyte; Masui and Markert showed that cytoplasm from hormone-stimulated oocytes could be injected into quiescent, unstimulated oocytes and induce the resumption of meiosis (Masui and Markert, 1971). The cytoplasmic factor responsible for this activity was named maturation-promoting factor (MPF), and MPF was soon found to be critical to the regulation of the somatic cell cycle as well. This discovery led to the *Xenopus* oocyte becoming a key model system for exploring the regulation of both meiosis and mitosis on a biochemical level. The genetic counterpart to the MPF experiments of Masui and Markert was provided by Lee Hartwell, who combined mutagenesis and time-lapse microscopy to identify three genes involved in the regulation of the cell division cycle, cdc1, cdc2 and cdc3 (Hartwell et al., 1970). We now know that Cdc2 encodes Cdc28p, the kinase subunit of MPF; the other subunit is the mitotic cyclin, Clb2. These early experiments demonstrated that easy manipulation of the yeast genome makes yeast an ideal model system for exploring cell cycle regulation at the genetic level.

While general mechanisms for the regulation of meiosis are well conserved among vertebrates, many of the specific proteins regulating mammalian oocyte maturation are not well characterized. This is of increasing interest recently, particularly with respect to the epigenetic reprogramming of histone modifications and the potential therapeutic uses for pluripotent embryonic stem cells. I began my graduate work with the proteomic investigation of mouse oocyte maturation, and this led to the identification of

the previously uncharacterized murine ortholog of nucleoplasmin, NPM2. Nucleoplasmin was the first histone chaperone identified, and also contains the first bipartite nuclear localization signal ever characterized (Dingwall et al., 1987). I set out to determine if NPM2 was functionally orthologous to *Xenopus* nucleoplasmin with respect to its mRNA and protein stability, expression pattern and activities during the resumption of meiosis. I also began to investigate how post-translational modification of NPM2 regulates these activities

The histone chaperone Nap1p was originally identified in yeast as a Clb2p-binding protein, and in the absence of Nap1p, Clb2p is unable to induce the switch from polar to isotropic bud growth and exhibits delayed entry into mitosis (Kellogg and Murray, 1995a). Nap1 orthologs in other species were initially characterized with respect to their histone assembly activity, now known to be shared by all Nap1 family members. Yeast Nap1p is an apparently pleiotropic protein. It regulates bud formation through interactions with cytoplasmic and bud neck associated proteins, and promotes nucleosome assembly/disassembly through nuclear interactions with chromatin remodeling and elongation complexes. Within the cell these functions seem to be temporally and spatially separate. It is possible that the cell cycle defects observed in *Δnap1* mutants are a consequence of Nap1-mediated regulation of transcription or elongation. In this case, the observed cell-cycle phenotypes are downstream effects of altered expression of Nap1p genomic targets. An alternative model is that there are two separate pools of Nap1p, one shuttling histones between the nucleus and cytoplasm and regulating transcription in the nucleus, with a second cytoplasmic pool involved in the mitotic signaling cascade at the bud neck. In this case, we expect that Nap1p/H2A/H2B

and Nap1p/Clb2p/Gin4p would only exist as discrete complexes, possibly during different phases of the cell cycle. A third possibility is that there is a more complex pathway in which septin assembly signals directly to the transcriptional machinery via Nap1p, and thus the two functions of Nap1p are interconnected. In support of this, Cdc28p, the bud-neck associated kinase Hsl1p and its binding partner Hsl7p were originally identified in a screen for genes that are synthetic lethal in combination with deletion of the H3 amino terminus (Ma et al., 1996). Additionally, HSL7 interacts genetically with the histone acetyltransferases GCN5 and ESA1, and the histone deacetylases RPD3 and the interactions are dependent upon the morphogenetic checkpoint (Ruault and Pillus, 2006). This suggests that activation of the morphogenetic checkpoint involves chromatin remodeling enzymes, and that the role of Nap1p in this pathway is intimately linked to its histone chaperone activity.

Nap1p is highly abundant, and since Nap1 mRNA and protein levels remain stable throughout the cell cycle, regulation must be occurring independently of transcription and translation. Since Nap1 family members in other species are phosphoproteins, we hypothesized that phosphorylation of Nap1p might be regulating its function. Phosphorylation could be directing Nap1p to different transcriptional targets, altering Nap1p cellular localization and thus physically segregating two pools of Nap1p, or regulating Nap1p association with different protein complexes. I studied the phosphorylation of Nap1p and investigated the functional significance of specific phosphosites with respect to Nap1p localization, interaction with binding partners and cell cycle regulation.

CHAPTER 2: Characterization of Mammalian NPM2 during Murine Oocyte Maturation

This chapter is based on published work.

Proteomic Profiling of Murine Oocyte Maturation. Alejandra M. Vitale*, Meredith E.K. Calvert*, Mallika Mallavarapu, Piraye Yurttas, Julie Perlin, John Herr and Scott Coonrod**.** MOLECULAR REPRODUCTION AND DEVELOPMENT 74:608–616 (2007)

* Alejandra M. Vitale and Meredith E. Kennedy Calvert contributed equally.

Introduction

During growth, the oocyte increases its volume more than 100-fold due, in part, to the accumulation of mRNA, protein, and organelles that are required for embryonic development. Once the oocyte reaches its full size (80 mm in mice) it arrests at prophase I of meiosis, at the germinal vesicle (GV) stage. At this point, mRNA synthesis ceases and does not resume again until the two-cell stage. Regulation of the oocyte to embryo transition period is therefore mediated by proteins that have been synthesized from stored mRNAs and by posttranslational protein modification. Given how little is known about mammalian oocyte maturation compared to oocyte maturation in Xenopus, we decided to take a proteomic approach to identify molecules that are differentially expressed during murine oocyte maturation. Mammalian oocytes contain a large percentage of mRNA that is not polyadenylated and therefore not translated (de Moor and Richter, 2001; Richter, 1991). Furthermore, many of the proteins in the oocyte maturation pathway are activated

or inactivated by post-translational modifications, which would not be detected by mRNA analysis. Proteomics therefore holds several advantages over mRNA expression profiling with respect to identifying proteins that are important for oocyte maturation.

We compared the overall protein expression pattern between GV-stage oocytes and metaphase II (MII) arrested oocytes and found that 12 proteins appeared to be differentially expressed. Mass spectrometry identified the proteins as transforming acidic coiled-coil protein 3 (TACC3), heat shock protein 105 (HSP105), programmed cell death six-interacting protein (PDCD6IP), stress-inducible phosphoprotein (STI1), importin α2, adenylsuccinate synthase (ADDS), nudix, spindlin, lipocalin, lysozyme, translationally controlled tumor protein (TCTP), and a previously uncharacterized protein with high sequence identity with *Xenopus* nucleoplasmin.

Nucleoplasmin is a maternally expressed protein that is highly abundant in oocytes and involved in histone transport and sperm chromatin decondensation in early embryogenesis. We cloned and characterized the mouse ortholog of nucleoplasmin (NPM2) and determined that NPM2, like nucleoplasmin, is oocyte specific. We show that Npm2 mRNA is present throughout follicular development, is oocyte specific and that mRNA levels, while high in GV and MII-arrested oocytes, rapidly decrease upon fertilization. At the protein level, NPM2 persists until the blastocyst stage, undergoing changes in both subcellular localization and electrophoretic mobility at the transition from GV to MII-arrested oocyte. We demonstrate that the change in electrophoretic mobility is phosphatase-sensitive, suggesting that phosphorylation of NPM2 occurs during oocyte maturation, and we show that NPM2 is a substrate for CK2 phosphorylation *in vitro*. Dynamic targeting of the protein from the nucleus to the cortical

region correlates with presence of the high molecular weight form of NPM2, and thus transient phosphorylation of NPM2 may regulate its relocalization. Due to the high level of sequence identity and conserved expression pattern shared by Xenopus nucleoplasmin and NPM2, we propose that NPM2 is the mammalian ortholog of nucleoplasmin.

Materials and Methods

Collection and Preparation of Oocytes and Preimplantation Embryos

All oocytes and embryos were obtained from 25 to 30 g CD1 female mice, GV oocytes were obtained as described previously (Wright et al., 2003) from PMSG stimulated mice. MII were isolated from the oviducts of superovulated (PMSG and hCG) mice. Pronuclear zygotes (PN), two-cell, four- to eight-cell, morula (Mo) and blastocyst (Bl) embryos were isolated from the oviduct and uterus of superovulated and mated female mice.

Two-Dimensional Electrophoretic Analysis of Oocyte Proteins and Identification of Differentially Expressed Proteins by Mass Spectrometry

Proteins from 500 zona-intact GV and MII arrested oocytes were extracted in Celis lysis buffer (containing 2% (v:v) NP-40, 9.8 M urea, 100 mM dithiothreitol (DTT), 2% ampholines (pH 3.5–10), and protease inhibitors) for 30 min at room temperature as previously described (Wright et al., 2003). Isoelectric focusing (IEF) was performed by using the BioRad Protean II Multi-Cell apparatus with an ampholine mixture (Pharmacia Biotech, Uppsala, Sweden) of pH 3.5–5 (30%), 3.5–10 (40%), 5–7 (20%), and 7–9 (10%). The tube gels were placed on 12% slab gels (16 cm plates, 1.5mmdiameter), and the focused proteins were separated in the second dimension. The gels were then stained with silver as previously described (Coonrod et al., 2002). Protein spots that were found to be differentially expressed between the GV and MII-arrested stage were cored from the gels, fragmented, destained in methanol, reduced in 10 mM dithiothreitol, and alkylated in 50mM iodoacetamide in 0.1M ammonium bicarbonate. The gel pieces were then incubated with 12.5 ng/ml trypsin in 50 mM ammonium bicarbonate overnight at

37˚C. Peptides were extracted from the gel pieces in 50% acetonitrile and 5% formic acid and analyzed by liquid chromatography mass spectrometry using a Finnigan LCQ ion trap mass spectrometer. The data were analyzed by database searching using the Seaquest search algorithm. The obtained peptide sequence information was then compared against database sequences using the NCBI Blast Website (http://www.ncbi.nlm.nih.gov/BLAST/).

Cloning full-length Npm2

Primers were designed to the 5' and 3' ends of the EST sequence [gi:16448986] which contained cDNA corresponding to the peptide sequences identified by TMS. Full-length Npm2 was amplified by both 5'-RACE and 3'-RACE PCR from an adaptor-ligated mouse oocyte cDNA library, prepared according to the manufacturers protocol (BD Marathon™ cDNA Amplification Kit, Clontech, Palo Alto, CA). Briefly, two micrograms of mouse oocyte poly(A)+ mRNA, isolated using from 300 oocytes using the FastTrack 2.0 kit from Invitrogen (Carlsbad, CA), was used as the template for the construction of a Marathon adaptor ligated cDNA library. Oligo(dT) primers and avian myeloblastosis virus (AMV) reverse transcriptase were used to construct the first strand of cDNA. The RNA was digested and the second strand of cDNA synthesized, The cDNA ends were then blunted by using T4 DNA polymerase, and Marathon cDNA adaptors were ligated to both ends of the cDNA by the addition of T4 DNA ligase. RACE PCRs were performed using the Amplitaq Gold DNA polymerase from Perkin-Elmer (Norwalk, CT) with oocyte library cDNA templates. Cycling parameters were: 94°C, 10 min; 94°C, 15 s; 60°C, 30 s; 72°C, 2 min; for 40 cycles, and 72°C, 10 min. The full-length PCR products were 624bp, the size of the predicted Npm2 ORF. Full-length

Npm2 was cloned into the TOPO vector (Invitrogen), purified and confirmed by sequencing.

Fluorescent In Situ Hybridization of Npm2 mRNA

Fixed, paraffin-embedded ovaries from female adult mice were sectioned, affixed to slides, deparaffinized and rehydrated in 3 changes each of xylene, absolute alcohol, and 95% and 80% alcohol, and then placed in 1x phosphate-buffered saline (PBS) for 5 minutes. Cell conditioning was achieved through the use of microwaving in citrate buffer for 15 minutes, followed by cooling of the slides in the citrate solution at room temperature for 20 minutes. Sections were then dehydrated in graded alcohols. The full-length Npm2 ORF was subcloned into pGEM vector (Promega) and used as a template for in vitro transcription of anti-sense and sense labeled probe. In vitro transcription to generate 3H-labeled riboprobes was carried out in the presence of 3H-UTP and 3H-CTP. Hybridization was carried out at Tm -25°C = 45°C. Following hybridization, the specimens were washed at high stringency (0.1x SSC at 65°C)(1x SSC = 0.15 mol/L NaCl, 0.0015 mol/L Na-citrate). The slides were then overlaid with Kodak NTB-2 autoradiography emulsion, exposed for 2 weeks at 4°C and developed photographically.

Generation of NPM2 Antibodies

The KLH-coupled peptide PSPQDKSPWKKEKFTPR, corresponding to a likely antigenic region (as predicted by a Kyte-Doolittle hydrophobicity plot), was synthesized using FMOC solid-phase peptide synthesis. This peptide was used to generate anti-NPM2 polyclonal antisera in adult male guinea pigs. For the primary immunization, KLH-conjugated peptide (500 mg total antigen) was emulsified with an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO; 30 ml) and injected subcutaneously

into guinea pigs. The two subsequent boosts were performed with 500 mg antigen emulsified with an equal volume of Freund's incomplete adjuvant. Whole sera were collected by allowing each bleed to coagulate at room temperature and centrifuging at 1,500 rpm in a Sorvall RT tabletop centrifuge. NPM2 antiserum was IgG purified using PrA-agarose beads (Roche, Nutley, NJ) and dialyzed against PBS.

Western Blot Analysis of NPM2 Expression During Oocyte Maturation and Early Embryo Development

For analysis of NPM2 protein expression by Western blotting, 20 oocytes/embryos from each stage were boiled for 5 min in Laemmli buffer and directly loaded onto a 10% SDS-PAGE gel. Proteins were separated at 200 V for 50 min and then transferred to a nitrocellulose membrane by applying current of 90 V for 90 min. All blots were blocked with 5% nonfat dry milk in TBS with 0.1% Tween-20 (TBS-T), washed, and incubated with a 1:5,000 dilution of anti-NPM2 guinea pig IgG. The blots were then washed three times for 10 min in TBS-T and incubated with 1:10,000 dilution of peroxidase-conjugated goat anti-guinea pig IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 hr. Following incubation in secondary antibody, the membranes were washed three times for 10 min TBS-T, and ECL reagent (Amersham Corp. Buckinghamshire, UK) was applied for 2 min and developed as previously described (Wright et al., 2003). The experiments were repeated three times.

Confocal microscopy

To determine the subcellular localization of NPM2, GV and MII oocytes, and embryos were collected as previously described, immediately fixed in 4% paraformaldehyde (in PBS) for 30 min at room temperature. The oocytes/embryos were then washed five times

in IF buffer (PBS +1% BSA +0.5% NGS), permeabilized with 0.5% Triton X-100 in PBS for 30 min, washed again and incubated with a 1:500 dilution of anti-NPM2 guinea pig preimmune and immune antiserum in IF buffer for 1 hr at room temperature. Samples were then washed, incubated in TRITC-labeled donkey anti-guinea pig at 1:200 (Molecular Probes, Eugene, OR) for 1 hour at room temperature, treated with RNAase (Sigma) for 30 minutes and stained with 200μM Sytox nucleic acid (Molecular Probes) for twenty minutes and cover-slipped with Slo-Fade (Molecular Probes). Images were obtained on a Zeiss 410 Axiovert 100 micro systems LSM confocal microscope. For each developmental panel, attenuation, contrast, brightness and pinhole aperture remained constant. For all panels, four-second scans were averaged four times per line using a 40X oil lens equipped with a zoom capacity of two. Z-steps were 5μm and approximately 15-25 sections were analyzed per oocyte or embryo and the relevant optical sections are shown.

Real-Time RT-PCR Quantitation of mRNAs

Total RNA was isolated from pools of 35 GV oocytes, MII oocytes, PN zygotes, 2-cell and 8- to 32-cell embryos using Trizol and chloroform. Then the RNA contained in the aqueous solution was purified using RNeasy Mini Kit (Qiagen, Valencia, CA), reverse transcribed, and subjected to real-time polymerase chain reaction (PCR) using sequence-specific primers (Mm00663 755_m1) from TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). PCR was performed by using the TaqMan PCR Master Mix and the ABI 7700 thermal cycler (Applied Biosystems) using the following parameters: 50˚C, 2 min; 95˚C, 10 min, followed by 40 cycles at 95˚C, 15 sec and 60˚C, 1 min. The expression level of each mRNA was normalized to Gapdh, and each stage of

development was compared against the mRNA of GV oocytes using the 2 DDCT method.

Phosphatase Inhibition

GV and MII oocytes, isolated as described above, were washed in PBS/PVA and frozen at 80˚C. Upon thawing, oocytes were resuspended in 15 ml of PP1/PP2A purified enzymes in Protein Phosphatase Dilution buffer from PP1/PP2A Toolbox (Upstate, Lake Placid, NY) for 30 min of in vitro dephosphorylation at 30˚C. Thirty oocytes were used per treatment and then run on a 10% SDS-PAGE gel as described before for the Western blot analysis of NPM2.

In vitro kinase assay

1 μg of purified α-NPM2 antibody was coupled to IgG-sepharose in PBS + 1% BSA at 4 C for 1 hour. GV oocytes, isolated as described above, were washed in PBS/PVA and frozen at 80˚C. Upon thawing, oocytes were resuspended in PBS + 1% BSA and incubated with IgG-sepharose 0.2mM ATP, 1μCi of [γ-^{32}P]ATP, and 0.2 μl human recombinant CK2 (NEB, Beverley, MA) at 30˚C for 30 min. or as indicated. The reaction was stopped by addition of SDS/PAGE sample buffer. The proteins were separated by SDS/PAGE, stained with Coomassie blue and ^{32}P-labeled proteins were detected by autoradiography on a PhosphorImager.

Results

Mass Spectroscopic Identification of Murine Oocyte Proteins that are Differentially Expressed During Oocyte Maturation. Proteins were extracted from 500 GV and MII arrested oocytes, separated on 2D electrophoretic gels and stained with silver (Fig. 4). The overall protein-staining pattern between the two gels was remarkably similar, with most proteins spots having a molecular weight (MW) and isoelectric point (pI). However, 12 protein spots in the GV-stage oocyte gel did not show a corresponding spot at the same MW and pI in the MII-stage oocyte gel (Fig. 4A). Six of those protein spots appeared to be absent in the MII-stage oocyte gel, whereas five appeared to be located at a different MW and pI in the MII- stage oocyte. In addition, one protein spot in the MII-stage oocyte gel did not display the corresponding spot in the GV-stage oocyte gel. These spots were cored from their respective gels, digested with trypsin, and analyzed by mass spectrometry. The protein that appeared to be upregulated between the GV and MII-stage gels was identified as TCTP (Fig. 4B and Table 1). The apparently downregulated proteins were identified as TACC3, HSP105, STI1, importin α2, ADDS, lipocalin, and lysozyme. Five proteins showed mobility shifts in both MW and pI suggesting they are post translationally modified during oocyte maturation. These were identified as PDCD6IP, spindlin, Nudix, importin α2, and NPM2 (Fig. 4B and Table 1). A summary of the identified protein's identification number (ID), observed and theoretical molecular weight and isoelectric point, NCBI number, and predicted function is shown in Table 1. Of the proteins detected in our proteomic screen, NPM2 is the only oocyte-restricted protein. Accordingly, we decided to clone the gene encoding NPM2 and further

characterize its mRNA and protein dynamics during oocyte maturation and early embryonic development.

Figure 4.

The oocyte maturation proteome.

Comparative silver-stained proteome of whole extracts from mouse from GV and MII oocytes.

A: Differentially expressed proteins (labeled 1-12) were cored from silver-stained 2D gels of proteins isolated from 500 GV and MII oocytes and identified by mass spectrometry.

B: The identified proteins fell into three categories; ‘‘downregulated’’ proteins which were present in the GV stage but absent from the MII stage, post translationally modified proteins that migrated at a different mass and pI between the two stages, and ‘‘upregulated protein’’ that was absent from the GV stage and present at the MII stage.

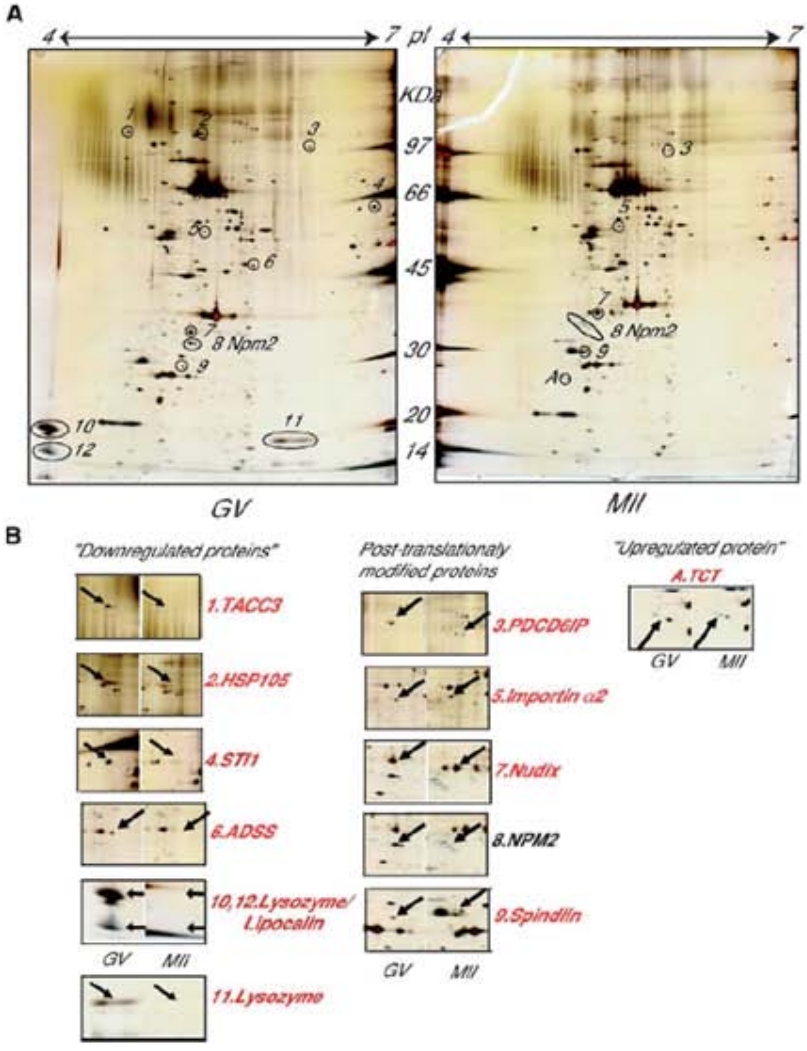

A
4
7
pI
KDa
97
66
45
30
20
14
8 Npm2
GV
MII
B
"Downregulated proteins"
1.TACC3
2.HSP105
4.STI1
6.ADSS
10, 12.Lysozyme/ Lipocalin
11.Lysozyme
Post-translationaly modified proteins
3.PDCD6IP
5.Importin α2
7.Nudix
8.NPM2
9.Spindlin
"Upregulated protein"
A.TCT

Table 1. *Peptide mass identification of proteins from 2D gel of mouse oocytes*.

ID: identification number on the 2D gel (Fig. 4), gi NCBI : protein accession number.

A: Proteins isolated from GV oocytes

		Observed		***Theoretical***			
ID	***Protein name***	***MW (KDa)***	***pI***	***MW (KDa)***	***pI***	***gi NCBI #***	***Localization/Function***
1	TACC3	100	4.7	71.2	4.57	13096904	Centrosomal/mitotic spindle dynamics (Still et al., 2004)
2	HSP105	100	5.4	96.4	5.39	1001009	Suppress the chaperone activity of Hsc70 in vivo and in vitro (Yamagishi et al., 2000)
3	PDCD6IP	97	6.1	96	6.15	6755002	Signal transduction protein, substrate of tyrosine kinases (Che et al., 1999)
4	STI1	60	6.8	62.6	6.4	881485	Mediates the heat shock response of some HSP70 genes (Nicolet and Craig, 1989)
5	Importin α2	50	5.5	58	5.51	2829434	Nuclear import of proteins containing specific nuclear localization signals; associates with membrane and participates in nuclear envelope assembly in vitro (Hachet et al., 2004)
6	ADSS	50.1	5.9	50.2	6.13	1083200	AMP biosynthesis (Borza et al., 2003)
7	Nudix	35	5.2	24	5.34	8393853	hydrolyze X-linked nucleoside diphosphates (Yang et al., 2000)
8	Npm2	32	5.2	23.4	5.07	31077089	Nuclear chaperone
9	Spindlin	27	5	27.1	5.31	6755620	Regulates cell cycle during the gamete to embryo transition
10	Lipocalin	25	4.3	19.3	5.4	4504963	Extracellular proteins that are able to bind lipophiles by enclosure within their structures to minimize solvent contact (Wojnar et al., 2003)
11	Lipocalin	18	4.1	19.3	5.4	4504963	

12	Lysozyme	15	4.1	16.7	9.61	7305247	Secretory enzyme that catalyzes the hydrolysis of specific kinds of polysaccharides comprising the cell walls of bacteria

B: Proteins isolated from MII oocytes

ID	***Protein***	***Observed***		***Theoretical***		***gi NCBI #***	***Function***
		MW (KDa)	***pI***	***MW (KDa)***	***pI***		
A	TCTP	20	5.1	4.76	19.5	6678437	Tubulin-binding protein that dynamically interacts with microtubules during the cell cycle (Gachet et al., 1999)
3	PDCD6IP	100	6.9	6.15	96	6755002	As above
5	Importin α2	63	5.5	5.51	58	12741729 4501885 2829434	As above
7	Nudix	38	5.3	5.34	24	8393853	As above
8	Npm2	34-36	4.4	5.07	23.4	31077089	As above
9	Spindlin	32	5.3	5.31	27.1	6755620	As above

Cloning of Npm2 from mouse GV and MII oocytes. A single protein spot from the GV oocyte proteome and a diagonal train of three protein spots in the MII proteome, all with approximate MW/pI of 30/5.0, yielded peptide sequence corresponding to the same translated cDNA in the mouse EST database. This sequence had been isolated from 2-day pregnant mouse ovary (BB559308, RIKEN full-length enriched, cDNA clone E330038E07). The translated EST sequence was homologous to the *Xenopus* oocyte protein, nucleoplasmin. The full-length gene corresponding to the EST sequence was amplified from an adaptor-ligated mouse oocyte cDNA library using RACE PCR. The gene encoding mouse nucleoplasmin was recently characterized as Npm2 and shares 65% sequence identity and 78% sequence homology with human Npm2 and 46% sequence identity, 67% sequence homology with *Xenopus* nucleoplasmin (Burns et al., 2003). The protein sequence shares the key features with *Xenopus* nucleoplasmin, namely the conventional basic, bipartite NLS, and two C-terminal acidic domains (Fig. 5).

Figure 5

Sequence alignment of Npm2.

The amino acid sequences encoding orthologues Npm2 from mouse, human and *Xenopus* were aligned using CLUSTAL X (Thompson et al., 1997). Conserved residues are highlighted in red. The phosphorylated serine identified by MS is marked with an asterisk; the CK2 consensus motif is underlined. The bipartite nuclear localization signal is boxed in blue and the two conserved acidic domains are boxed in black.


*
Mouse MSRHSTSSVTETTAKNMLWGSELNQEKQTCTFRGQCEKKDSCKLLLSSTICLGEKAKEEV 37
Human MNLSSASSTEEKAVTTVLWGCELSQERRTWTFRP-QLEGKQSCRLLLHTICLGEKAKEEM 59
Xenopus MASTVSNTSKLEKPVSLIWGCELNEQNKTFEFKVEDDEEKCEHQLALRTVCLGDKAKDEF 60
Mouse NRVEVLS---QEGRK-PPITIVTLKASVLPMVTVSGIELSPPVTFRLRTGSGPVFLSGLE 87
Human HRVEILPPANQEDKKMQPVTIASLQASVLPMVSMVGVQLSPPVTFQLRAGSGPVFLSGQE 119
Xenopus HIVEIV---TQEEGAEKSVPIATLKPSILPMATMVGIELTPPVTFRLKAGSGPLYISGQH 117
Mouse CYETSDLTWEDDEEEE----EEEEEEDEDEDADISLEEI-PVKQVKRVAPQKQMSIAKKK 142
Human RYEASDLTWEEEEEEEGEEEEEEEDDEDEDADISLEEQSPVKQVKRLVPQKQASVAKKK 179
Xenopus VAMEEDYSWAEEEDEGEAEGEEEEEEEEDQES--------PPKAVKRPAATKKAGQAKKK 169
Mouse KVEKEEDETVVRPSPQDKSPWKKEKFTPRAKKPVTKK 179
Human KLEKEEEE--IRASVRDKSPVKKAKATARAKKPGFKK 214
Xenopus KLDKEDE------SSEEDSPTKKGKGAGRGRKPAAKK 200

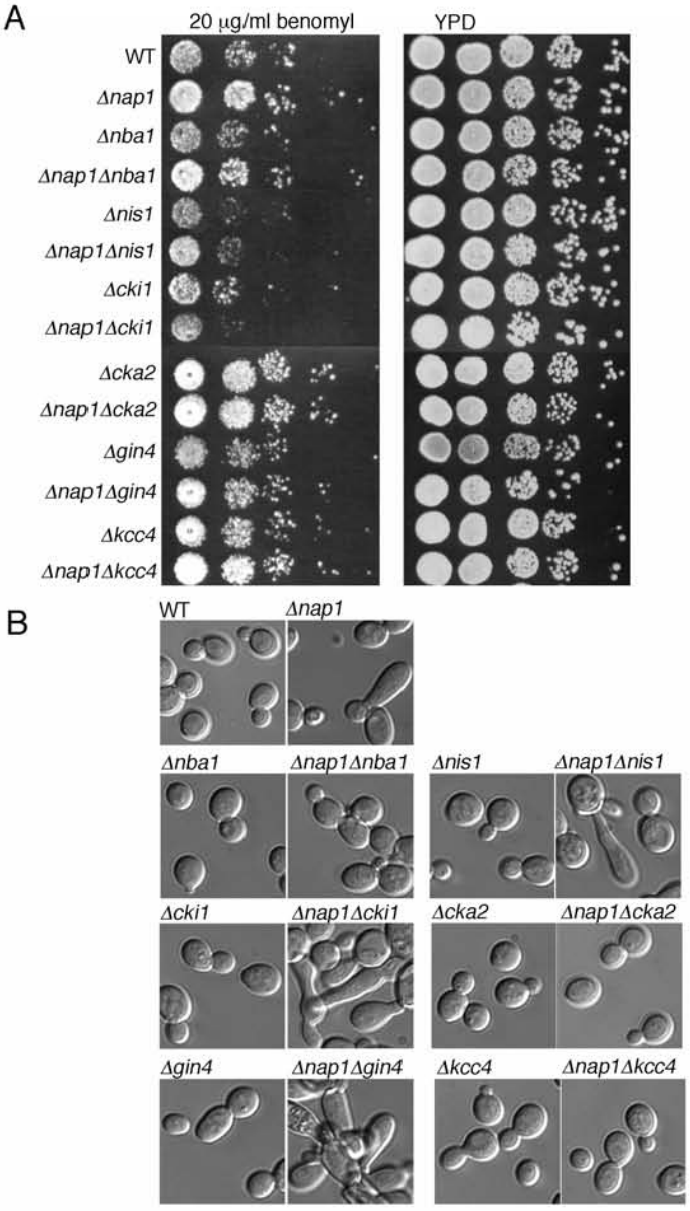
Developmental Expression of Npm2 mRNA. We first decided to investigate Npm2 mRNA expression during oocyte maturation. Npm2 was used as a template for in vitro transcription of anti-sense and sense labeled probe. In situ hybridization on fixed, paraffin-embedded ovarian sections revealed that Npm2 RNA is present throughout follicular oocyte growth. Npm2 mRNA was detected within primordial, primary, secondary and antral follicles, and was oocyte-specific; no signal was observed using the sense labeled probe (Fig. 6A). These data show that Npm2 mRNA expression, similar to that of *Xenopus* nucleoplasmin, is oocyte-specific. We then decided to look at the stability of Npm2 mRNA during oocyte maturation, fertilization and preimplantation development. Therefore, mRNA was isolated from GV and MII-arrested oocytes, pronuclear stage zygotes, 2-cell embryos, and 8- to 32-cell stage embryos, and real-time quantitative RT-PCR was used to evaluate the Npm2 expression during these stages. Results showed that Npm2 mRNA was expressed predominantly in GV and MII oocytes, while its levels decreased dramatically, by approximately 80%, at the PN zygote stage and remained low in the 8- to 32-cell embryo stage (Fig. 6B). Gapdh and Npm2 displayed similar efficiency of amplification, validating the 2 DDCT method. These results support the hypothesis that the differential expression of Npm2 during oocyte maturation is not directly due to changes in Npm2 mRNA synthesis.

Figure 6

Expression of Npm2 mRNA is oocyte specific and decreases following fertilization.

A: Fixed, paraffin-embedded ovarian sections were hybridized with radiolabeled probe anti-sense or sense to Npm2 mRNA as described. PmF; primordial follicle; PF; primary follicle, SF; secondary follicle, AF; antral follicle.

B: Total RNA from germinal vesicle (GV), metaphase II oocytes (MII), pronuclear zygotes (PN), 2-cell and 8- to 32-cell embryos were extracted for quantitative real time PCR. Specific primers based on the Npm2 open reading frame were used in PCR reactions and the relative quantitation (RQ) analysis of Npm2 mRNA levels was performed. Values are expressed as the mean SEM of triplicates of three independent experiments. *$P<0.05$ versus GV oocytes by ANOVA and Dunnett's Test.


A
20 μg/ml benomyl
YPD
WT
Δnap1
Δnba1
Δnap1Δnba1
Δnis1
Δnap1Δnis1
Δcki1
Δnap1Δcki1
Δcka2
Δnap1Δcka2
Δgin4
Δnap1Δgin4
Δkcc4
Δnap1Δkcc4
B
WT
Δnap1
Δnba1
Δnap1Δnba1
Δnis1
Δnap1Δnis1
Δcki1
Δnap1Δcki1
Δcka2
Δnap1Δcka2
Δgin4
Δnap1Δgin4
Δkcc4
Δnap1Δkcc4

Developmental Expression and Subcellular Localization of Npm2 During Oocyte Maturation and Early Embryo Development. We next investigated NPM2 protein expression during oocyte maturation and preimplantation development by immunoblotting, using an antibody generated against an NPM2-specific peptide. Results show that NPM2 is expressed in oocytes and preimplantation embryos until the blastocyst stage (Fig.7A). No expression was detected in Day 10 embryos (data not shown). Interestingly, two predominant molecular weight NPM2 species of approximately 32 kDa and 35 kDa are found in oocytes and early embryos. In GV stage oocytes, the major immunoreactive species is the 32 kDa form, while in MII-arrested oocytes the 35 kDa isoform predominates. In the PN embryo, both bands were observed at approximately equal intensity. At the two-cell and four cell stage the predominant form was the lower molecular weight species. In the morula and blastocyst stage only the lower weight form persists (Fig. 7A). No signal was detected when oocyte/embryo extracts were blotted with pre-immune sera (data not shown). The increase in mass of NPM2 during oocyte maturation correlates well with results obtained from our 2D analysis and suggests that NPM2 undergoes a post-translational modification, most likely phosphorylation, during oocyte maturation. Furthermore, the conversion of the predominant 35 kDa form to the 32 kDa form during subsequent developmental stages suggests that the modification is transient. Additionally, staining for the 35 kDa species appears weaker than the 32 kDa isoform. It is unclear whether this weaker staining is actually due to a decrease in NPM2 protein levels or possibly due to changes in the NPM2 epitope following posttranslational modification.

In order to investigate NPM2 localization during oocyte maturation and early development, we next collected immature GV and MII oocytes, PN zygotes, two-cell embryos (2-cell), four-cell embryos (4-cell), morulae (Mo) and blastocysts (Bl) for immunofluorescence analysis. In the immature GV oocyte, NPM2 was primarily concentrated in the nucleus and was completely excluded from the nucleolus, though some localization to the cortex was observed (Fig. 7B). Little staining was observed in the cytoplasm. Following GVBD, however, NPM2 was localized throughout the cytoplasm, becoming highly concentrated at the cortical region below the plasma membrane Fig. 7B). After fertilization, the protein again showed nuclear localization within the pronuclei. At the two- and four-cell stage, NPM2 localized to the nucleus, however, during these two stages, NPM2 appeared to preferentially associate with the nuclear lamina. By the blastocyst stage, the NPM2 signal decreases significantly and is detected diffusely throughout the embryo (Fig. 7B).

Figure 7

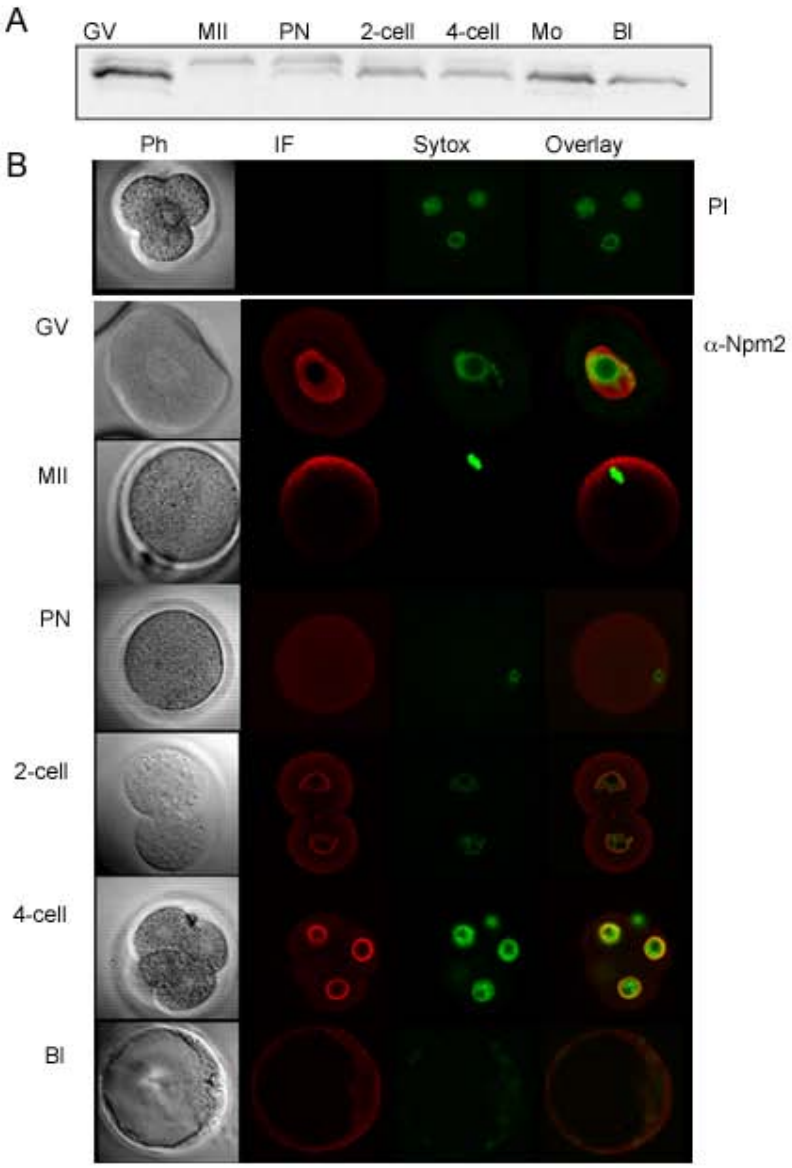
NPM2 protein expression undergoes a maturation-specific change in mobility and localization.

Germinal vesicle (GV) and metaphase II oocytes (MII), pronuclear zygotes (PN), 2-cell, 4–8-cell, morula (Mo), and blastocyst (Bl) embryos were collected as described in Materials and Methods and fixed immediately after collection for immunofluorescent analysis or proteins were extracted for western blot analysis. NPM2 protein was expressed in all the stages studied.

A: Representative immunoblot of NPM2 protein expression.

B: At the subcellular level, NPM2 predominantly localized to the nucleus and associated with the inner lamina, except at the MII stage were it localized to the cortical region and the cytoplasm. The preimmune serum (PI) was not reactive at any stage studied.

IF; indirect immunofluorescence, Ph; phase contrast, Sytox; DNA stain


A
GV
MII
PN
2-cell
4-cell
Mo
Bl
B
Ph
IF
Sytox
Overlay
PI
GV
MII
PN
2-cell
4-cell
Bl
α-Npm2

MII-oocyte associated NPM2 is phosphatase-sensitive and is phosphorylated by CK2. There is a correlation between the increase in mass of NPM2 and oocyte maturation, suggesting that NPM2 undergoes a post-translational modification (Fig. 7A). In order to assess if this change in mass was due to phosphorylation, proteins extracted from GV and MII-arrested oocytes were treated with phosphatase. Results showed that there was little mobility change of the NPM2 band in extracts from GV stage oocytes following treatment. Interestingly, the NPM2 band from MII oocytes was reduced significantly, suggesting that the protein was phosphorylated (Fig. 8).

NPM2 homologs in other species have also been shown to be phosphorylated (Cotten et al., 1986), and consistent with this, MS results determined that one of the NPM2 peptides sequenced from the MII oocyte proteome was phosphorylated on a serine residue within a CK2 consensus sequence (Fig. 5). In total, there are six serines and two threonines in NPM2 that are contained within CK2 consensus sequences. To determine whether NPM2 is a substrate for CK2, we assessed the ability of CK2 to phosphorylate NPM2 in vitro. NPM2 was immunoprecipitated from MII oocyte extracts, and incubated it with recombinant CK2 and γ^{32}P-labeled ATP. CK2 was able to phosphorylate a band corresponding to the size of NPM2, and also a larger band, most likely representing autophosphorylation of the CK2 α-subunit. No phosphorylation was detected in extracts immunoprecipitated with pre-immune sera, suggesting that the NPM2 is being specifically phosphorylated by CK2. (Fig. 9).

Figure 8

NPM2 undergoes a phosphatase-sensitive mobility shift during maturation.

PP1/PP2A treatment of GV oocytes and MII-arrested oocytes followed by SDS-PAGE; PPtase: phostatase.

Figure 9

CK2 phosphorylates immunoprecipitated NPM2.

α-NPM2 or pre-immune (PI) sera immunoprecipitated MII oocyte extracts were incubated with purified CK2 and [γ-^{32}P]ATP. The proteins were separated by SDS/PAGE, and phosphorylation was measured by ^{32}P incorporation detected using a PhosphorImager.

Figure 8

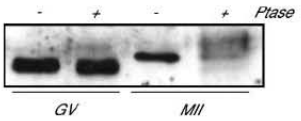
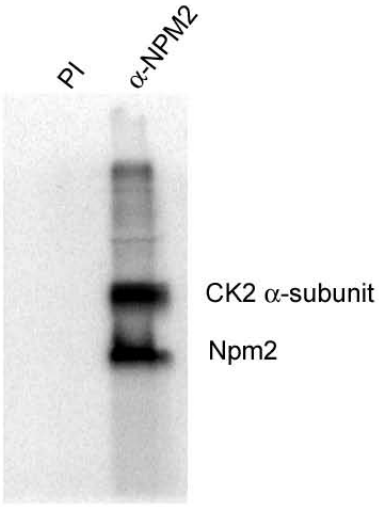


Figure 9



Discussion

In an effort to better understand the molecular processes behind mammalian oocyte maturation, we performed a proteomic analysis and identified for the first time, a selection of oocyte proteins that appear to be differentially regulated during the resumption of meiosis. Most of these proteins have previously been found to play important roles in mitotic events in *Xenopus* and invertebrate oocyte maturation, yet their role in mammalian oocyte maturation has not been previously investigated. In this analysis we identified the previously uncharacterized mammalian ortholog of nucleoplasmin, NPM2. We found that in oocytes, NPM2 exhibits nuclear localization, but it migrates to the cortex during maturation, following breakdown of the nuclear envelope. Similarly to *Xenopus* nucleoplasmin, NPM2 exhibits a shift in molecular weight likely due to phosphorylation during oocyte maturation, and this correlates with its change in cellular localization.

Much of what is known about the molecular forces driving mammalian oocyte maturation comes from studies of meiosis in *Xenopus* and invertebrates. In these species, the extracellular initiation signal is mediated by steroids (either progesterone or androgen) which are secreted from surrounding cumulus cells upon luteinizing hormone (LH) stimulation (Ferrell, 1999). The steroids are then thought to bind to a poorly defined nonconventional steroid receptor, leading to the inhibition of adenylate cyclase (AC) and a reduction in cAMP levels. This, in turn, leads to reduced protein kinase A (PKA) levels and ultimately results in activation of Mos (Ferrell, 1999). Mos is an oocyte-specific serine/threonine kinase that stabilizes MPF, the master cell cycle switch, through a pathway that involves the mitogen-activated protein kinase (MAPK) cascade (Gebauer

and Richter, 1997). Another known target of Mos includes spindlin, an oocyte-abundant mouse protein that associates with the meiotic spindle (Oh et al., 1997). The finding that spindlin is phosphorylated in a cell-cycle dependent manner during oocyte maturation correlates well with our finding that spindlin appears to be posttranslationally modified during oocyte maturation (Oh et al., 1997). Tyrosine phosphorylation has also been implicated in steroid-induced *Xenopus* oocyte maturation; however, neither the tyrosine kinases nor their substrates have been well defined. One putative substrate for tyrosine phosphorylation is Xp95, which was recently found to be phosphorylated during oocyte maturation in *Xenopus* (Che et al., 1999). In our study, we identified programmed cell death 6 interacting protein (PDCD6IP) as a likely candidate for posttranslational modification during oocyte maturation. A comparison of the XP95 and PDCD6IP amino acid sequence finds that these proteins are 80% identical, suggesting that PDCD6IP may be a mammalian ortholog of XP95.

MPF, composed of Cdc2 kinase complexed with cyclin B, is activated at meiosis I by a balance between its activator Cdc25C and its inactivators Wee1 (homologous to Swe1p) and Myt1 (Palmer and Nebreda, 2000). Exit from the first meiotic division is brought about by a decrease in MPF activity due to the degradation of cyclin B by the 26S proteosome (Josefsberg et al., 2000). MPF activity begins to increase again after meiosis I and soon stabilizes resulting in MII arrest. This stabilization is brought about by a cytostatic factor (CSF) of which Mos is thought to be an essential component (Masui, 2000). During mitosis, MPF activity is regulated, in part, by importin-mediated nucleocytoplasmic shuttling (Hagting et al., 1998). Our novel finding that importin α2 undergoes a mobility shift between the GV and MII stage suggests that posttranslational

modification of this molecule may play a role in mammalian oocyte maturation, possibly by regulating MPF activity. Recently, Aurora kinases have been found to play an important role in chromosome segregation during anaphase in oocytes during meiosis (Monje-Casas et al., 2007). TACC3 is targeted to centrosomes by Aurora A-dependent phosphorylation and enhances the number of microtubules emanating from mitotic centrosomes (Kinoshita et al., 2005). TCTP, which we show is up-regulated during oocyte maturation, has also been shown to associate with microtubules. TACC3 is abundantly expressed in oocytes, and here we show that TACC3 expression appears to be downregulated between the GV and MII stage, suggesting that TACC3 expression is tightly regulated during oocyte maturation (Hao et al., 2002)..

Our study also identified several proteins that have not previously been identified as playing a role in some aspect of mitosis or meiosis: STI1, HSP150, Nudix, ADDS, Lipocalin, and lysozyme. Two heat shock-related proteins STI1 and HSP105 appear to be downregulated during oocyte maturation. Stress inducible protein 1 (STI) is a phosphoprotein co-chaperone that regulates the protein folding activity of HSP70 and HSP90 (two of the most abundant HSPs in cells) (Wegele et al., 2003). HSP105 also functions as a co-chaperone and regulates the chaperone activity of Hsp70 (Yamagishi et al., 2004). Two metabolic enzymes, Nudix and ADSS were also identified in our screen. Nudix hydrolyses a wide range of organic pyrophosphates, while ADSS is required for AMP biosynthesis (Bessman et al., 1996; Borza et al., 2003; Yang et al., 2000b). While ADSS appears to be downregulated during oocyte maturation, Nudix was also identified in MII arrested oocytes, suggesting that this protein may be posttranslationally modified during maturation. The proposed functions of the Nudix family of proteins are to

eliminate potentially toxic nucleotide metabolites from the cell and to regulate the concentrations of nucleotide cofactors and signaling molecules for optimal cell growth and survival. NUDT5 is proposed to hydrolyze the ADP-ribose, thus removing a potentially deleterious metabolite and recycling it into AMP and ribose 5-phosphate (Yang et al., 2000b). Interestingly, complete inhibition of oocyte maturation has been reported in the presence of ADP-ribose and FSH in the rat (Miller and Behrman, 1986). Nudix could therefore be functioning as a ‘‘housecleaning’’ protein and preventing the meiotic arrest of the oocyte due to toxic metabolites. Finally, two secreted proteins, lipocalin and lysozymeC, were found to be downregulated during oocyte maturation. Lipocalins function in the transport of lipids and hydrophobic molecules such as retinol, while lysozyme C (P variant) has been characterized in the gut epithelium and catalyzes the hydrolysis of specific polysaccharides (Irwin et al., 1992; Wojnar et al., 2003).

In *Xenopus*, mRNAs encoding Mos, cyclin B, and other signaling factors involved in oocyte maturation and early development are synthesized during oocyte growth and are translationally repressed by the TACC3 homolog, maskin, and other maternal proteins such as FRGY and Xp54. Following hormonal stimulation, these mRNAs are unmasked and translated (Stebbins-Boaz et al., 1999). While the biochemical pathways linking hormonal stimulation to unmasking and the translation of stored maternal transcripts are poorly defined, recent reports suggest that NPM2 may also remodel masked mRNAs to facilitate translational activation. Nucleoplasmin has long been known to play an important role in decondensation in *Xenopus* by removing the arginine rich protein, protamine, from sperm DNA. Meric et al. propose that nucleoplasmin may play a similar role in removing the arginine rich masking protein, FRGY2, from maternal

RNA, thereby facilitating translation of stored maternal messages (Meric et al., 1997). Recently, Npm2 knockout mice were generated and it was found that females displayed reduced fertility due to failed preimplantation development. Interestingly, while sperm decondensation was not affected in oocytes derived from Npm2-null females, NPM2 did appear to play an important role in nuclear and nucleolar organization in oocytes and zygotes (Burns et al., 2003).

In *Xenopus*, nucleoplasmin is stored in a hypophosphorylated form that becomes hyperphosphorylated during oocyte maturation and is released into the cytoplasm following GVBD. Phosphorylation of nucleoplasmin is thought to play an important role in both its function and in its nuclear to cytoplasmic targeting. Our finding that murine NPM2 localizes to the nucleus in GV stage oocytes and is then targeted to the cortex in MII-arrested oocytes correlates well with *Xenopus* nucleoplasmin. Furthermore, the finding that there is a slight mass increase in NPM2 during murine oocyte maturation as detected by both 2D silver-staining and by Western blot analysis strongly suggests that, similar to *Xenopus* nucleoplasmin, murine NPM2 is phosphorylated during meiosis. We also treated murine GV and MII extracts with phosphatase in order to more directly test the hypothesis that NPM2 is phosphorylated during maturation. Results showed that following phosphatase treatment of MII oocytes, there was a significant reduction in detectable NPM2 levels. This suggests that NPM2 is phosphorylated during oocyte maturation. The reason for loss of detection is not clear, though possibly following phosphatase treatment the epitope recognized by our site specific anti-NPM2 antibody is altered. This may have also had an impact on our detection of NPM2 localization by IF, and demands further analysis. With respect to NPM2 expression during preimplantation

development, the transient shift in species from the 32 kDa form to the 35 kDa form during oocyte maturation, followed by the reversion back to the 32 kDa form (which is complete by the two-cell stage) suggests that the MII and pronuclear stages may be particularly important stages for NPM2 function. This prediction is supported by the Npm2 knockout findings showing that Npm2-null embryos arrest at the pronuclear stage. The reversion of the 35–32 kDa form at the two cell stage also correlates with the increased localization of NPM2 to the nuclear lamina, suggesting that phosphorylation may directly regulate the localization of NPM2.

In summary, our proteomic analysis of GV and MII stage oocytes has identified for the first time a selection of oocyte proteins that appear to be differentially regulated during oocyte maturation. Most of these proteins have previously been found to play important roles in mitotic events in *Xenopus* and invertebrate oocyte maturation, yet their role in mammalian oocyte maturation has not been previously investigated. We also further characterized the oocyte restricted protein identified in the screen, NPM2, and found that it is dynamically targeted from the oocyte nucleus to the cortex following oocyte maturation. Furthermore, we show that this dynamic targeting is likely due to transient phosphorylation of NPM2, which persists throughout the MII to pronuclear stage of development. We demonstrate the NPM2 is a phosphoprotein and an in vitro substrate for phosphorylation by CK2. Future studies will determine which residues within NPM2 are targets for CK2, and whether this phosphorylation event is directly regulating NPM2 relocalization during oocyte maturation.

CHAPTER 3: Cell-cycle Dependent Regulation of Nap1p

The majority of this work is based on recent, unpublished data.

The first four figures in this chapter are from **Casein Kinase 2 Phosphorylation Regulates the Localization and Chromatin Assembly Activity of Nucleosome Assembly Protein 1.** Meredith E.K. Calvert, Kristin M. Keck, Celeste Ptak, Jeffrey Shabanowitz, Donald F. Hunt and Lucy F. Pemberton*. Submitted to Molecular and Cellular Biology, 2007.

* The mass spectrometry analyses were performed by Celeste Ptak, Jeffrey Shabanowitz, Donald F. Hunt. Kristin M. Keck assisted with experiments presented in Figures 10-12.

Introduction

Nap1p, or nucleosome assembly protein 1, is a member of the Nap/SET family of proteins and is highly conserved among eukaryotes (Ito et al., 1996a). The first human homolog to be cloned was purified from HeLa cell extracts and characterized by its ability to assemble chromatin in vitro (Ishimi et al., 1984; Ishimi et al., 1985). In mice, deletion of Nap1L2 is embryonic lethal due to hyper proliferation of neuronal precursor cells. Mutations and chromosomal translocations involving Nap/SET family members are associated with a variety of human cancers (Adachi et al., 1994a; Adachi et al., 1994b; Fornerod et al., 1995b; Fornerod et al., 1996a; Li et al., 1996; Tsuchiya et al., 1995; von Lindern et al., 1992). These disorders may be due to Nap/SET-associated defects in cell cycle regulation, or could be the result of transcriptional misregulation, since in a genome-wide expression study in yeast about 10% of all genes showed altered transcription in a *Δnap1* strain (Ohkuni et al., 2003). In *S. cerevisiae,* Nap1p was

originally identified as a Clb2-interacting protein. In complex with the cyclin-dependent kinase complex Clb2p/Cdc28p and a number of other proteins, Nap1p initiates the switch from polar to isotropic bud growth and elongated buds are observed in *Δnap1* mutants (Lew and Reed, 1993a). (Altman and Kellogg, 1997b). This is thought to be due to the role of Nap1p in regulating phosphorylation and activation of the Nim1-related kinase Gin4p, which is a prerequisite for the normal assembly of a septin complex at the bud neck (Altman and Kellogg, 1997b). Although Nap1p and Gin4p remain in complex throughout the cell cycle, at the G2/M transition Gin4p undergoes Nap1p-dependent hyperphosphorylation. Both Nap1p and Gin4p localize to the bud neck at G2/M, though in the case of Gin4p, the localization and association with the septin cortex are dependent upon Nap1p. (Altman and Kellogg, 1997b; Longtine et al., 2000; McMillan et al., 1999; Mortensen et al., 2002; Okuzaki and Nojima, 2001; Okuzaki et al., 1997).

At steady state, Nap1p is primarily cytoplasmic, but mutation of a leucine-rich NES sequesters Nap1p in the nucleus, suggesting it is a nucleocytoplasmic shuttling protein (Mosammaparast et al., 2002). In vivo, Nap1p binds to histones H2A and H2B in the cytoplasm, facilitates histone import into the nucleus via the karyopherin Kap114p, and acts to promote the formation of the Kap114p/H2A/H2B complex (Mosammaparast et al., 2002). Once in the nucleus, Nap1p is presumably released from Kap114p and is able to assemble histones H2A and H2B onto DNA. The assembled chromatin then recruits the Ran nucleotide exchange factor Rcc1 (Prp20p in yeast) leading to the production of RanGTP at the chromatin surface (Arnaoutov and Dasso, 2003; Carazo-Salas et al., 1999; Li and Zheng, 2004; Nemergut et al., 2001). Kap114p directly inhibits chromatin assembly by Nap1p in vitro, suggesting that facilitated import of the H2A/H2B

by Nap1p is not sufficient for chromatin assembly, and that Nap1p undergoes additional regulation inside the nucleus (Mosammaparast, 2005).

Nap1p appears to have two quite distinct roles in key cellular processes, namely nucleosome assembly and the regulation of bud formation, and these functions occur in the nucleus and the cytoplasm, respectively. We wanted to gain insight into how the apparently pleiotropic functions of Nap1p might be regulated. *NAP1* mRNA and protein levels remain stable throughout the cell cycle suggesting the regulatory mechanism is independent of transcription or translation. We hypothesized that distinct functions of Nap1p might be assigned by different Nap1p-interacting proteins. We further considered that discrete complexes of Nap1p and its associated proteins might form at specific stages of the cell cycle, and set out to identify and characterize Nap1p binding partners. These partners included the previously uncharacterized Yol070cp, designated here Nba1p, which localizes to the septin cortex prior to bud emergence and to the bud neck. We found a number of kinases in association with Nap1p, and analysis of Nap1p phosphorylation determined that Nap1p is phosphorylated at eleven sites in vivo. We were also able to identify phosphorylated residues on some of the more abundant Nap1p binding partners. We determined that both Nap1p and Nba1p are phosphorylated by Cdc28p/Clb2p in vitro and we have identified target residues for this kinase within each protein. Cells were then arrested at S phase or at the G2/M transition and we observed cell-cycle dependent changes in Nap1 protein interactions, indicating that Nap1p forms cell-cycle specific complexes that may independently regulate its cellular functions.

Materials and Methods

Yeast strains and plasmids. The yeast strains used in this study were derived from DF5 or BY4741 as noted, and construction of the mutant strain *Δnap1* has been described previously (Mosammaparast, 2005). The Nap1- PrA strain was constructed by integration of a PrA tag at the C-terminal end of *NAP1* in the strain as described (Aitchison et al., 1995). The inducible mutant pds1-mbd strain was made by integration of pr305-P_{GAL1}-PDS1-MDB (from Dr. D. Burke) into the Nap1-PrA strain. *Δnap1* double mutants were made by mating BY4741 deletion strains from Open Biosystems with the *Δnap1::cloNAT* strain (derived from strain Y2454 from Dr. C. Boone, *MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 MFA1pr-HIS3 can1Δ0*) and performing tetrad dissections. Cdc28p/Clb2p was purified from strain DOM0077 from Dr. D. Morgan, w303 *MATa ura3-1 trp1-1 leu2-3,112, his3-11 ade2-1,can1-100 GAL+ Δsic1::LEU2 + pAB1234-2m Gal-Clb2-TAP-URA3*. Expression of GFP-tagged proteins was performed using the $pGFP_2$-C-FUS vector (Mosammaparast et al., 2002). Amino acid substitutions were made using oligonucleotide site-directed mutagenesis of a dsDNA plasmid template and verified by sequencing. For recombinant protein expression, *NAP1, NBA1* and mutant derivatives were cloned into pGEX4T1, and GST fusion proteins were purified as previously described (Mosammaparast et al., 2001).

Purification of Nap1 binding partners and identification of phosphorylation sites. Post-ribosomal whole cell extract was prepared from 1L Nap1- PrA-tagged, or untagged exponentially growing yeast. Cultures were harvested by centrifugation, pretreated with protease/phosphatase inhibitor buffer (1mM peroxovanadate, 10nM Calyculin, 1mM

PMSF, 1μg/ml pepstatin in 100mM Tris pH 8.0) at 30˚C for 30 min, followed by addition of 10mM DTT for 10 min. Cells were lysed in 2% PVP using the French press. Lysates were cleared by centrifugation to remove ribosomes and incubated with IgG-Sepharose. After washing Nap1-PrA and copurifying proteins with 50mM $MgCl_2$, Nap1-PrA interacting proteins were eluted with 1M $MgCl_2$ and identified by MS as described (Fernandez et al., 1994; Gharahdaghi et al., 1996). Proteins from which a minimum of four unique peptides were isolated were included in the analysis. Proteins present in both experimental and control fractions were only included if the total number of peptides present in the experimental fraction was at least 50% higher than the total number in the control fraction. To detect Nap1p phosphopeptides, the Nap1-PrA bound to Sepharose beads was subjected to proteolytic digest by trypsin, Glu C and Asp N. The resulting peptides were separated using a C18 column and esterified in acetyl chloride and MeOH. Phosphopeptides were enriched from the sample using an immobilized metal affinity column and analyzed by RP-HPLC/uESI/MS/MS interfaced with a Finnigan LTQ-FT ion trap mass spectrometer, and identified using SEQUEST. For the coprecipitation of Nap1 binding partners, lysates from 500mL of each tagged strain were prepared as above, and 50mg of total protein was incubated with IgG-Sepharose. Coprecipitating proteins were eluted with an $MgCl_2$ gradient, separated by SDS–PAGE and visualized by Western blotting.

Purification of cell-cycle specific Nap1 binding partners. From the 1L culture, 500mL of cells were then released into 2% raffinose and 2% galactose for 3 hrs. The remaining 500mL was pelleted and stored at -70˚C. After induction, whole cell extracts from both the induced and uninduced cultures were prepared as described above. For this analysis

we compared the peptides to the original control dataset from the untagged strain, and proteins present in both experimental and control fractions were only included if the total number of peptides present in the experimental fraction was at least 50% higher than the total number in the control fraction. Peptides from all proteins present in the original unsynchronized experiment were included in the analysis.

In vitro binding assays. Nba1p and Nap1p binding assays were performed using either GST-Nap1 or mutant immobilized on glutathione–Sepharose (GE Health Care) or MBP-Nba1 immobilized on amylose resin (NEB). In each case, beads were pre-blocked with 10% BSA in buffer containing 1% Tween 20 at the protein concentrations described.

Purification of Cdc28p/Clb2p. Purfication of Cdc28p/Clb2p was performed as described previously (Ubersax et al., 2003). Briefly, 2L of cells carrying a galactose-inducible Clb2p-TAP were grown in 2% raffinose overnight and induced in 2% galactose for 3 hrs. Cell lysates were made using the French press and the active complex was purified by tandem affinity purification (TAP) (Puig et al., 2001). Ten 200µl elution fractions were collected and assayed for activity, and of these fractions 2 and 3 were able to phosphorylate GST-Nba1, as described below. The active fractions were pooled and stored at -70˚C.

In vitro kinase assay. 1µg of GST-Nap1, GST-Nba1, or a mutant derivative were incubated with 0.2mM ATP, 1µCi of [γ-^{32}P]ATP, and 2µl of purified Cdc28p/Clb2p complex at 30˚C for 30 min. The reaction was stopped by addition of SDS/PAGE sample buffer. The proteins were separated by SDS/PAGE, stained with Coomassie blue and ^{32}P-labeled proteins were detected by autoradiography on a PhosphorImager.

Cell culture and microscopy. Cell culture methods and microscopy were performed as described previously (Mosammaparast et al., 2001) using a Nikon Microphot-SA microscope (Melville, NY) and images were captured using OpenLab software (Improvision, Lexington, MA) with a 100X objective. Strains containing reporter GFP constructs were induced in SC-ura-met for 2h. Cells were photographed using identical exposure settings, and all image manipulation was performed identically using Adobe Photoshop. For the benomyl sensitivity assay, 10-fold serial dilutions of each strain were spotted onto YPD, YPD + 20μg/ml Benomyl in 0.4% DMSO, or YPD + 0.4% DMSO and incubated at 30˚ for four days.

Results

Identification of Nap1p interacting proteins. To identify proteins involved in the regulation of Nap1p function, Nap1-PrA was first immunoprecipitated from whole cell lysates prepared from exponentially growing yeast. Nap1p binding proteins were eluted and MS was used to identify the protein constituents. Non-specific interactions were excluded by comparison with a control experiment using an untagged strain. The remaining Nap1p-interacting proteins were evaluated based upon the number of total and unique peptides identified, resulting in the identification of twenty-two Nap1p-interacting proteins (Table 2). Several heatshock proteins, a ribosomal protein, and proteins involved amino acid biosynthesis were present after filtering. These proteins are highly abundant in cellular lysates, and commonly regarded as contaminants in proteomic screens (Gavin et al., 2002). The interacting proteins identified in our screen included known binding partners of Nap1p: H2A, H2B, Kap114p, Htz1p and the kinase Gin4p, as well as some previously unreported interacting proteins and two proteins of unknown function.

Among the Nap1p-interacting proteins found in the screen, four kinases were identified: the Nim1-related kinases Gin4p and Kcc4p, one of the catalytic subunits of CK2, Cka2p, and choline kinase, Cki1p. Phosphorylation of Nap1p by interacting kinases presents a simple and reversible mechanism by which its distinct functions could be regulated, and we were interested to find these kinases in association with Nap1p . Additionally, two largely uncharacterized proteins were identified, Nis1p and Yol070cp. Nis1p (Neck protein Interacting with Septins) has been shown by yeast 2-hybrid screening to interact with septins, the Nim1 kinases Gin4p and Kcc4p, and Nap1p, and it

localizes to the bud neck at G2/M in a septin-dependent manner (Iwase and Toh-e, 2001). Yol070cp was the product of an uncharacterized ORF identified in a screen for potential Cdc28p substrates (Ubersax et al., 2003). Based on these data, we decided to further investigate the roles of Gin4p, Kcc4p, Cka2p, Cki1p, Nis1p and Yol070cp in Nap1p function.

To verify these interactions in vivo, we evaluated the ability of Nap1p to coprecipitate with each protein. Nis1p and Gin4p have previously been shown to interact with Nap1p by co-immunoprecipitation (Altman and Kellogg, 1997b; Iwase and Toh-e, 2001). The TAP or PrA tagged fusion of each protein of interest was purified from whole cell extracts, and co-precipitating proteins were eluted with $MgCl_2$ and western blotted for Nap1p (Fig. 10). We found as expected that Nap1p coprecipitated with Gin4p, and also with Kcc4p, Cka2p, Cki1p and Yol070cp but not with the unrelated karyopherin, Sxm1p. Since Nap1p migrates at a similar size as the Cka2-TAP fusion protein, this membrane was initially probed with IgG to identify Cka2-TAP, followed by a Nap1p antibody. Only Nap1p is visible in the 50 mM $MgCl_2$ fraction, whereas both Nap1p and Cka2-TAP are detected in the 1 M $MgCl_2$ fraction (Fig. 10). These results validated our MS data and showed that these interactions occur either directly or within a shared complex in yeast lysates. The interaction of Nap1p with these proteins suggests they may be involved in the regulation of Nap1p function.

Table 2. *Nap1p-Interacting Proteins*

Nap1-PrA interacting proteins were identified by Mass Spectrometry. The number of peptides identified from a control experiment using an untagged strain is shown for comparison.

Function	Protein name	Unique Peptides		Total Peptides		Protein Localization
		Nap1 IP	Ctrl.	Nap1 IP	Ctrl.	
Chromatin associated:						
	Nap1p	19	6	425	11	Cytoplasm, nucleus, bud neck
	Htb2p	7	3	95	6	Nucleus
	Kap114p	6	0	7	0	Cytoplasm, nucleus
	Hta2p	4	4	108	5	Nucleus
	Htz1p	4	0	26	0	Nucleus
Kinases:						
	Gin4p	32	0	81	0	Cytoplasm, bud neck, bud
	Cki1p	23	0	52	0	Cytoplasm
	Kcc4p	21	0	81	0	Bud neck, cell periphery, bud

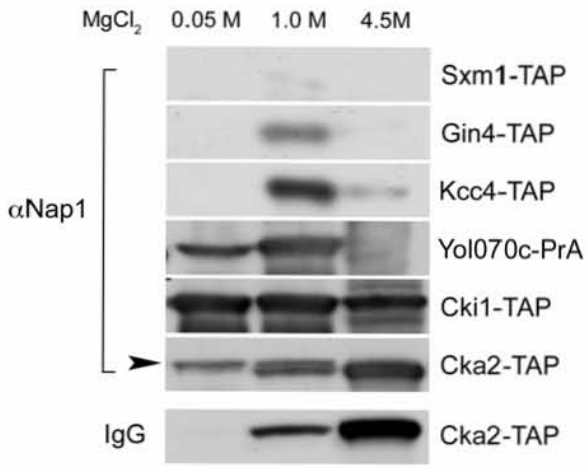
Function	Protein name	Unique Peptides		Total Peptides		Protein Localization
		Nap1 IP	Ctrl.	Nap1 IP	Ctrl.	
Kinases (cont.):						
	Cka2p	5	0	7	0	Cytoplasm, nucleus
Protein synthesis/Metabolic:						
	Tef2p	11	11	40	18	Cytoplasm
	Rpl18bp	7	0	14	0	Cytoplasm
	Shm1p	18	0	38	0	Mitochondria
	Sip5p	7	0	8	0	Cytoplasm
	Tco89p	6	0	6	0	Vacuolar membrane
	Fol1p	5	0	6	0	Cytoplasm, mitochondria
Heat shock:						
	Ssa1p	35	20	115	37	Cytoplasm
	Ssb1p	28	0	92	0	Cytoplasm
	Ssa2p	8	7	15	8	Cytoplasm, nucleus

Function	Protein name	Unique Peptides		Total Peptides		Protein Localization
		Nap1 IP	Ctrl.	Nap1 IP	Ctrl.	
Heat shock (cont.):						
	Ssc1p	6	2	6	2	Cytoplasm, mitochondria
	Hsc82p	4	0	6	0	Cytoplasm
Unknown function:						
	Yol070cp/Nba1p	26	0	111	0	Cytoplasm, bud neck, cell periphery
	Nis1p	2	0	2	0	Bud neck, cell periphery

Figure 10

Nap1p interacting proteins.

PrA and TAP-tagged proteins as indicated were isolated from whole cell lysates using IgG sepharose and eluted with $MgCl_2$. The co-precipitation of Nap1p with these proteins was analyzed by Western blotting with ą Nap1p antibody. For Cka2-TAP the blot was probed with rabbit IgG to detect Cka2-TAP followed by Nap1p antibody. Arrowhead indicates Nap1 band.


MgCl2
0.05 M
1.0 M
4.5M
αNap1
Sxm1-TAP
Gin4-TAP
Kcc4-TAP
Yol070c-PrA
Cki1-TAP
Cka2-TAP
IgG
Cka2-TAP

***YOL070c* encodes a new bud-neck associated protein, Nba1p.** To further validate our candidate Nap1p-interacting proteins we decided to characterize the new Nap1p partner encoded by *YOL070c.* Yol070cp has a predicted molecular weight of 56 KD. The protein shares no significant homology with other known protein families and is conserved within fungi, although close homologs are not found in higher eukaryotes. The most extensive homology within fungi is observed in the C- terminal domain of Yol070cp, and a lysine-rich region (residues 405-421) may represent a classical NLS. Various global proteomic screens have identified potential partners for Yol070cp including Nis1p, Nap1p, Sua7p, Clb2p and Cdc28p (http://db.yeastgenome.org). We observed that recombinant Nap1p and Yol070cp interact directly (Fig. 11A) and we propose that Yol070cp be designated Nap1p and bud neck associated protein (Nba1p). We determined that Nba1p interacts with the central domain of Nap1p (amino acids 143-362; Fig 11A). *NBA1* does not encode an essential gene and *Δnba1* deletion strains have no obvious growth defects (see Fig.12). Interestingly, when we expressed Nba1p as a GFP fusion protein, its localization was seemingly identical to that of Nap1p, primarily cytoplasmic and excluded from the nucleus in many cells (Fig.11B). In addition, bud neck localization was observed in G2/M cells, as well as localization to the actin cap in unbudded cells; this localization was unaltered in *Δnap1* cells (Fig.11B). We also observed that the C-terminal half of Nba1p (amino acids 275-501) contains determinants necessary for the normal cellular distribution and bud neck targeting of Nba1p and an amino terminal fragment (amino acids 1-275) did not contain this activity (Fig. 11C). We hypothesize that Nba1p functions with Nap1p at the septin scaffold and perhaps helps regulate G2/M progression. This characterization of Nba1p, together with our

identification of known Nap1p-interactors, suggests that our screen identified authentic Nap1p binding partners.

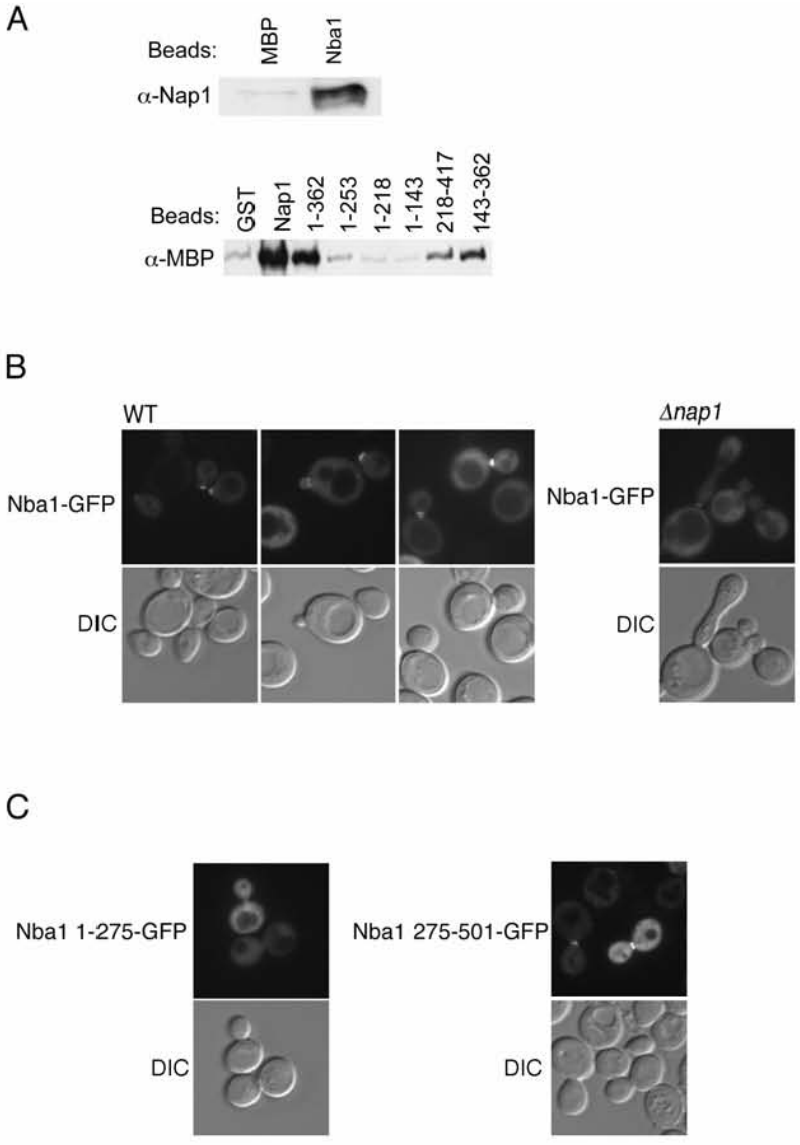
Figure 11

Nap1p interacts directly with the bud neck associated protein, Nba1p.

A: MBP-Nba1 (200nM), MBP (200nM), GST (1μM), GST-Nap1 (1μM) and GST-Nap1 fragments (indicated by amino acid numbers; 1μM) were immobilized, and incubated with 250nM GST-Nap1 (upper) or 500nM MBP-Nba1 (lower panels). Bound proteins were visualized by Western blotting with Nap1p or MBP (for Nba1p) antibodies.

B: Nba1-GFP_2 fusion protein was expressed in wild type or *Δnap1* cells and visualized by fluorescence microscopy. The coincident DIC image is also shown.

C: Nba1p fragments (as indicated by amino acid number) were expressed as GFP_2 fusion proteins in wild type yeast and visualized as above.


A
Beads:
MBP
Nba1
α-Nap1
Beads:
GST
Nap1
1-362
1-253
1-218
1-143
218-417
143-362
α-MBP
B
WT
Δnap1
Nba1-GFP
DIC
Nba1-GFP
DIC
C
Nba1 1-275-GFP
DIC
Nba1 275-501-GFP
DIC

***NAP1* interacts genetically with several kinases.** In order to understand the relationship in vivo between the Nap1p-interacting proteins and Nap1p, we examined strains in which both genes were deleted. None of the double deletion strains showed any obvious growth defect on YPD (Fig. 12A). In *S. cerevisiae, Δnap1* mutants have increased resistance to benomyl, a microtubule-destabilizing drug that causes mitotic arrest at high concentrations, demonstrating a role for Nap1p in regulating microtubule dynamics (Kellogg and Murray, 1995b). As expected, *Δnap1* cells grew better than wild type cells on plates containing 20μg/ml benomyl (Fig. 12A). Strains lacking *NBA1*, *GIN4* and *KCC4* grew similarly to wild type cells suggesting they are not resistant to benomyl. When these mutations were combined with the *NAP1* deletion, *Δnap1Δnba1, Δnap1Δgin4*, and *Δnap1Δkcc4* deletion strains grew similarly to *Δnap1*, suggesting that the resistance to benomyl observed in *Δnap1* strains is not dependent on these three genes. Both *Δnis1* and *Δcki1* strains showed increased benomyl sensitivity compared to wild type. However, unlike the *Δnap1Δnis1* strain, deletion of *NAP1* in combination with *CKI1* caused increased sensitivity to benomyl indicating a genetic interaction. Lastly the *Δcka2* strain showed increased resistance to benomyl in the presence and absence of *NAP1*, suggesting that these mutations may be epistatic, and that both proteins function in microtubule stability (Fig. 12A).

A proportion of *Δnap1* cells exhibit an elongated bud phenotype, indicative of a delayed switch from polar to isotropic bud growth (Kellogg and Murray, 1995b). We therefore examined the morphology of the deletion strains, and elongated buds were observed in *Δnap1* and *Δgin4* cells, but not in other strains bearing a single deletion (Fig. 12B). The long bud phenotype was greatly exacerbated in the *Δnap1Δgin4* and

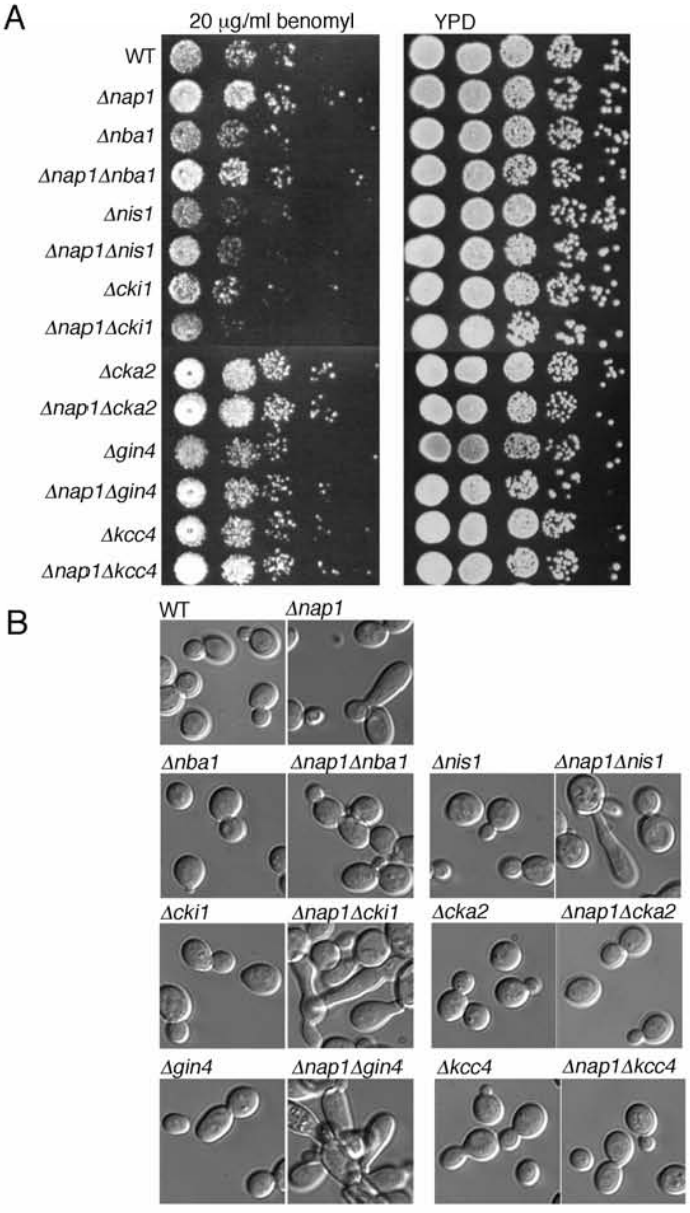
Δnap1Δcki1 strains, further suggestive of a genetic interaction (Fig. 12B). No morphological defect was seen in singly deleted *Δcka2* cells, and no additive interaction was observed in the *Δnap1Δcka2* mutant (Fig. 12B). CK2 contains two catalytic subunits encoded by *CKA1* and *CKA2,* and although disruption of both genes is lethal, depletion of CK2 using conditional alleles is known to cause defects in cell cycle progression, cell polarity, and elongated bud morphology. Whether deletion of *NAP1* exacerbates the phenotypes observed with conditional strains remains to be tested. Collectively these results suggested that *NAP1* interacts genetically with *GIN4* and *CKI1* and may have an overlapping role with *CKA2* in regulating microtubule stability. This also suggests that the elongated bud phenotype seen in *Δnap1* cells, and the resistance of *Δnap1* mutants to benomyl are independent phenotypes, and may indicate that Nap1p has multiple functions in the regulation of entry into mitosis.

Figure 12

Genetic interactions of NAP1.

A: Strains of the indicated genotype were equalized, spotted at 10-fold serial dilutions and grown on YPD plates with and without benomyl.

B: Strains of the indicated genotypes were examined by DIC microscopy.

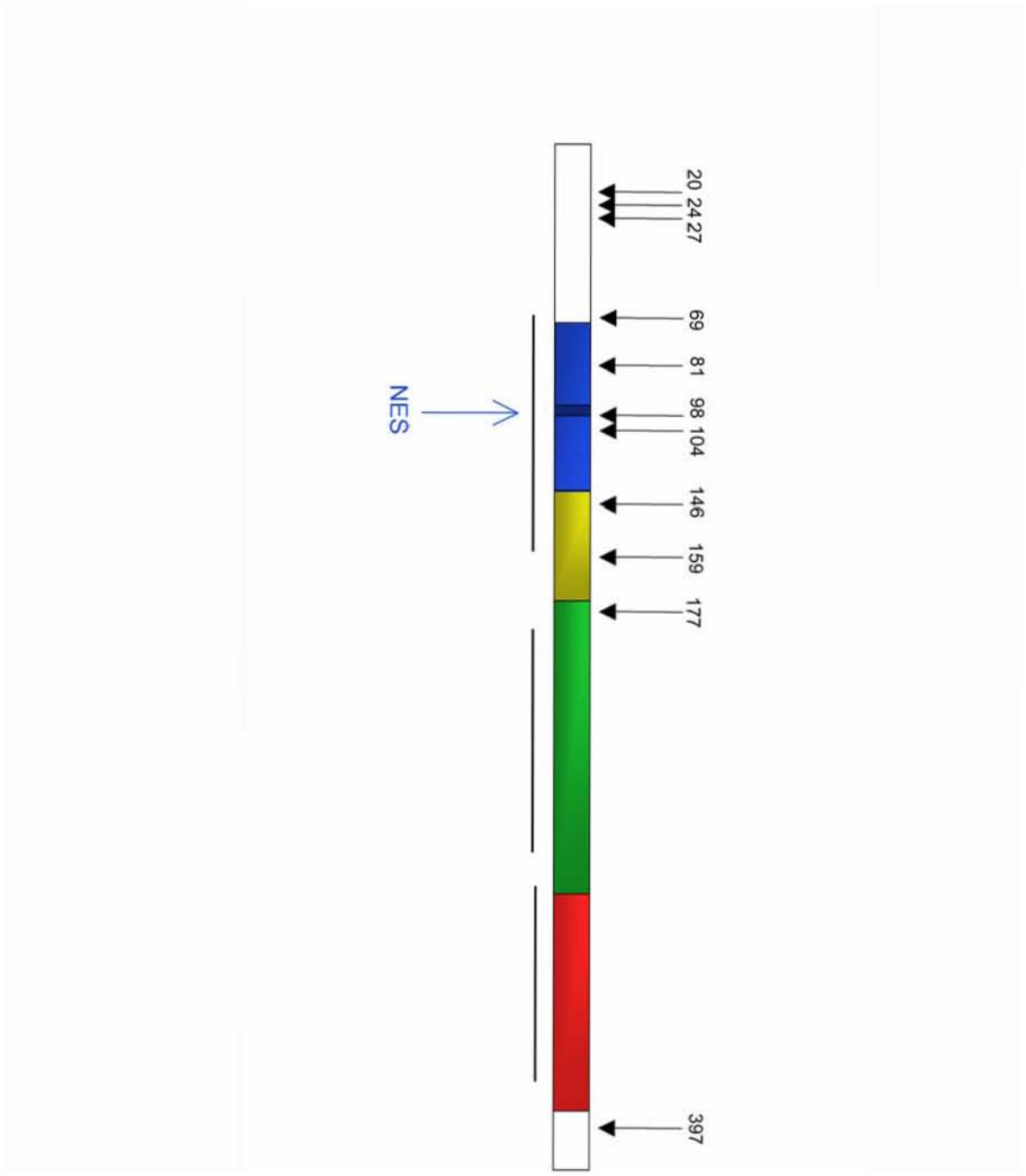

A
20 μg/ml benomyl
YPD
WT
Δnap1
Δnba1
Δnap1Δnba1
Δnis1
Δnap1Δnis1
Δcki1
Δnap1Δcki1
Δcka2
Δnap1Δcka2
Δgin4
Δnap1Δgin4
Δkcc4
Δnap1Δkcc4
B
WT
Δnap1
Δnba1
Δnap1Δnba1
Δnis1
Δnap1Δnis1
Δcki1
Δnap1Δcki1
Δcka2
Δnap1Δcka2
Δgin4
Δnap1Δgin4
Δkcc4
Δnap1Δkcc4

Nap1p is a phosphoprotein in vivo. Phosphorylation of Nap1p has not been demonstrated in *S. cerevisiae*, so we utilized MS to determine whether yeast Nap1p is a phosphoprotein. Nap1-PrA was purified from exponentially growing cells and after protease digestion phosphopeptides were enriched using an immobilized metal affinity column (IMAC) and sequenced by MS/MS. Eleven phosphorylated serines and threonines were identified and all but one were clustered in the amino terminal half of the protein (Fig.13). Six of these sites were visible in the published crystal structure, while the sites at the extreme N and C terminal parts of the protein were not included in the fragment that was crystallized. In addition another site (S177) was contained in a flexible loop which was unstructured in the published structure of Nap1p (Park and Luger, 2006b). The Nap1p structure consists of a long α-helix, through which the protein monomers interact to form a homodimer with a hydrophobic core, and this dimerization domain contains the nuclear export signal (NES). Nap1p also has an accessory domain, which has been proposed to regulate access to the NES. Interestingly several sites were clustered around the dimerization helix and the NES, suggesting they may regulate nuclear transport and the conformation of the dimer. Additional analysis of Nap1p phosphosites revealed that two N-terminal phosphothreonines, T20 and T24, are followed by a proline residue that conforms to the minimal consensus site for phosphorylation by Cdc28p/Clb2p mitotic kinase (Fig. 14, Table 3) (Ubersax et al., 2003).

Figure 13

Nap1p is a phosphoprotein in vivo.

Nap1-PrA phosphosites were identified by mass spectrometry. Schematic of Nap1p, the arrows indicate the eleven identified phosphosites and corresponding amino acids are numbered. Bars beneath the schematic show the regions contained within the solved structure and the subdomains identified in the crystal structure are boxed and color coded: Subdomains A, B, C, and D are boxed in blue, yellow, green, and red, respectively (see Fig. 1 for the corresponding structure)(Park and Luger, 2006b). The NES is identified by the blue arrow (Mosammaparast et al., 2002).


20 24 27
69
81
98 104
146
159
177
397
NES

Performing the IMAC enrichment of protease-treated PrA-Nap1p also permitted us to identify phosphopeptides from a number of the more abundant Nap1p-associated proteins (Table 3). In validation of these findings, we observed phosphorylation of H2A at serine 129, which is a well-established modification that is upregulated in response to DNA damage (Downs et al., 2000). Nba1p was isolated after phosphopeptide enrichment by IMAC, and one of the peptides sequenced was also phosphorylated within a minimal consensus site for Cdc28p. In a recent global screen of budding yeast kinases and their substrates, Nba1p was identified as a potential target for phosphorylation by Cdc28p/Clb2p. Nba1p and Clb2p both interact with Nap1p, and all three proteins localize to the bud neck at G2/M (see Fig. 11, (Hood-DeGrenier et al., 2007)). Given this, we hypothesized that phosphorylation of Nap1p and Nba1p by Cdc28p/Clb2p and their association at the bud neck could be a mitosis-specific signaling event. We decided to determine whether both Nap1p and Nba1p represent bona fide targets for phosphorylation by Cdc28p/Clb2p in vitro.

Nap1p and Nba1p are phosphorylated by Cdc28p/Clb2p in vitro. Nap1p associates with Clb2p, though phosphorylation of Nap1p by Cdc28p/Clb2p has not been shown previously (Kellogg and Murray, 1995a). Based on this evidence, we predicted that threonines 20 and 24 within Nap1p were Cdc28p/Clb2p target residues. In order to determine whether Nap1p is a substrate for Cdc28p/Clb2p, we assessed the ability of this kinase to phosphorylate Nap1p in vitro. Recombinant GST-tagged Nap1p was incubated with the Cdc28p/Clb2p affinity-purified from yeast and γ^{32}P-labeled ATP and became phosphorylated, demonstrating that GST-Nap1 is a substrate for this kinase complex in vitro (Fig. 14A). To confirm the Cdc28p/Clb2p target sites, mutants were constructed in

which one or both of the target threonines were mutated to uncharged, unphosphorylatable valine residues. Mutation of both T20 and T24 to valine reduced phosphorylation of Nap1p to approximately one-third that of wild type Nap1, as measured by ^{32}P incorporation (Fig. 14A). This suggested that both of these sites were recognized by Cdc28p/Clb2p, but that additional target sites for this kinase complex may exist. We then wanted to determine whether Nba1p is a substrate for Cdc28p/Clb2p, and assessed the ability of Cdc28p/Clb2p to phosphorylate Nba1p in vitro. GST-tagged Nba1p was incubated with Cdc28p/Clb2p and $\gamma^{32}P$-labeled ATP, and was shown to be phosphorylated by this kinase in vitro (Fig. 14B). Phosphorylation of Nba1p was reduced by 50% relative to wild type in a mutant in which the Nba1p Cdc28p/Clb2p target threonine, T403, was mutated to valine, as measured by ^{32}P incorporation (Fig. 14B). This suggested that residue T403 in Nba1p was recognized by Cdc28p/Clb2p, but that additional target sites for this kinase complex may be present. We also demonstrated that the phosphorylation of both proteins was specifically dependent upon the presence of substrate and kinase, and that the phosphorylated proteins presumed to be Nap1p or Nba1p were not present in the purified kinase complex (Fig. 14B).

Table 3. *Cell-cycle specific Nap1p-Interacting Proteins*

Cells were arrested during the cell cycle in G2/M or S phase as described in the text and Nap1-PrA interacting proteins were identified by Mass Spectrometry.

Function	Protein name	Unique Peptides		Total Peptides		Protein Localization
		G2/M	S	G2/M	S	
Chromatin associated:						
	Nap1p	22	22	573	608	Cytoplasm, nucleus, bud neck
	Htb2p	0	6	0	12	Nucleus
	Kap114p	0	0	0	0	Cytoplasm, nucleus
	Hta2p	0	3	0	9	Nucleus
	Htz1p	4	0	6	0	Nucleus
Kinases:						
	Gin4p	35	53	56	100	Cytoplasm, bud neck, bud
	Cki1p	31	36	130	200	Cytoplasm
	Kcc4p	20	27	35	48	Bud neck, cell periphery, bud

Function	Protein name	Unique Peptides		Total Peptides		Protein Localization
		G2/M	S	G2/M	S	
Kinases (cont.):						
	Cka2p	0	1	0	1	Cytoplasm, nucleus
	Hsl1p	8	7	8	7	Cytoplasm, bud neck
Unknown function:						
	Yol070cp/Nba1p	2	2	3	3	Cytoplasm, bud neck, cell periphery
	Nis1p	8	0	23	0	Bud neck, cell periphery

Figure 14

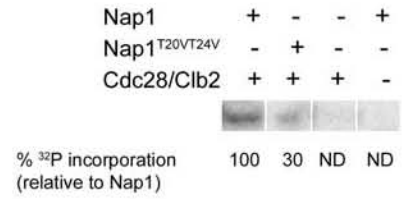
Nap1p and Nba1p are phosphorylated by Clb2p/Cdc28p in vitro.

Recombinant, GST-Nba1 and the mutant derivatives of these proteins were incubated with affinity-purified Clb2p/Cdc28p and [γ-^{32}P]ATP. The proteins were separated by SDS/PAGE and phosphorylation was measured by ^{32}P incorporation detected using a PhosphorImager and compared relative to wild type. ND: not detectable

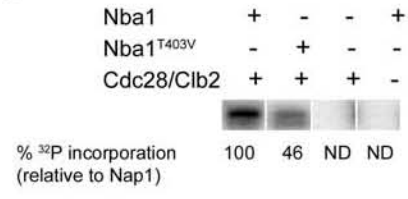
A: In vitro kinase assay of GST-Nap1 and GST-Nap1^{T20VT24V}

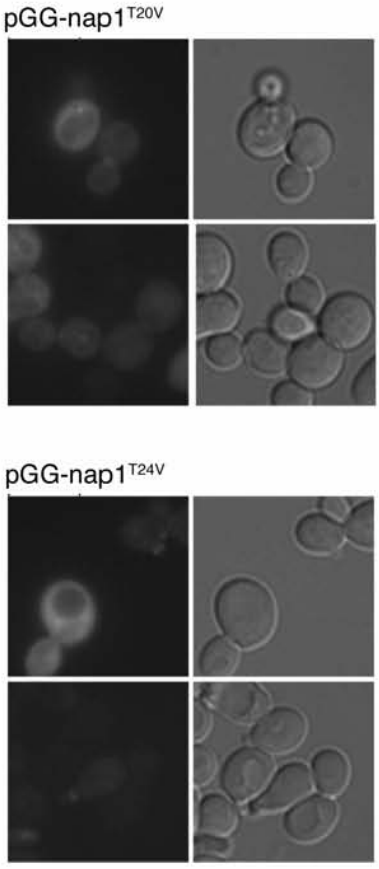
B: In vitro kinase assay of GST-Nba1 and GST-Nba1^{T403V}

A


Nap1 + - - +
Nap1T20VT24V - + - -
Cdc28/Clb2 + + + -
% 32P incorporation (relative to Nap1) 100 30 ND ND

B


Nba1 + - - +
Nba1T403V - + - -
Cdc28/Clb2 + + + -
% 32P incorporation (relative to Nap1) 100 46 ND ND


pGG-nap1T20V
pGG-nap1T24V

Phosphorylation of Nap1p by Cdc28p/Clb2p is not required for localization to the bud neck. Since Nap1p and Clb2p localize to the bud neck during the G2/M transition, and Nap1p is phosphorylated by Cdc28p/Clb2p in vitro, we wanted to determine if phosphorylation of Nap1p may be required for its localization to the bud neck. We made GFP_2 reporter constructs of wild type Nap1p, and mutants lacking each of the Cdc28p/Clb2p target sites. GFP fusions of Nap1p-T20V and T24V mutants and wild type Nap1p were all predominantly cytoplasmic and able to localize to the bud neck in G2/M cells (Fig.15). This suggested that loss of Cdc28p/Clb2p phosphorylation at the individual sites was not required for localization of Nap1p to the bud neck. Since phosphorylation of Nap1p by Cdc28p/Clb2p did not appear to regulate its localization to the bud neck, we hypothesized that it might be required for the interaction of Nap1p with mitosis-specific binding partners. We predicted that Nap1p would be phosphorylated by Cdc28p/Clb2p at the entry into mitosis, and sought to determine if any of the interactions of Nap1p with other proteins were specific to G2/M.

Cell-cycle dependent Nap1p protein-protein interactions. To investigate whether Nap1p forms specific complexes during different phases of the cell cycle, we arrested cells using hydroxyurea (HU), an inhibitor of ribonucleotide reductase that blocks cells in S phase, or by inducing a stabilized mutant of Pds1p with a mutation in the destruction box motif (*pds1-mdb*) that causes cells to arrest at the G2/M transition. Nap1-PrA was immunoprecipitated from treated whole cell lysates. Nap1p binding proteins were eluted and sequenced using MS, and interactions were evaluated as described previously. For comparison purposes, we focused primarily on proteins that had been identified in our initial screen from an unsynchronized population (Table 4). Although Hsl1p was not

detected above threshold levels in our original screen, Hsl1p was detected bound to Nap1p in cells arrested at both S phase and G2/M. Hsl1p is a Nim1-related kinase in the same family as Kcc4p and Gin4p, and is included here due to its known role in the mitotic signaling cascade. Of the histones identified in our initial screen, the core histones H2A and H2B were only detected bound to Nap1p in cells arrested in S phase, whereas binding to the histone variant H2A.Z was only detected during G2/M. Kap114p was not detected in either sample, though in our initial analysis from unsynchronized cells, very few peptides from this protein were detected.

All of the Nap1p-binding kinases were more predominant in cells arrested during S phase relative to those in G2/M. Only a single peptide from Cka2p was detected in S phase arrested cells though, like Kap114p, this was of low abundance in our initial analysis. Gin4p and Kcc4p, like Hsl1p, are Nim1-related kinases that coordinate cell cycle progression with the organization of the bud neck septins, and both of these kinases were detected in association with Nap1p more frequently in S phase than at G2/M (Barral et al., 1999). This difference was most pronounced with respect to Gin4p kinase, for which almost twice as many total peptides were detected bound to Nap1p during S phase. Equivalent numbers of Nba1p peptides were bound to Nap1p during both S phase and G2/M arrest, whereas Nis1p peptides were only detected binding to Nap1p during G2/M. Both Nis1p and Nba1p are Nap1p-associated proteins that localize to the bud neck during G2/M, yet these results suggest that the interactions of these proteins with Nap1p are functionally distinct. Peptides from heat shock, metabolic or protein synthesis proteins were not evaluated in this experiment. Taken together, these results suggest that Nap1p forms cell-cycle specific interactions with different proteins, and that it exists in distinct

Figure 15

Phosphorylation of Nap1p at T20 or T24 by Cdc28p/Clb2p is not required for localization to the bud neck.

$Nap1^{T20V}$ and $Nap1^{T24V}$ mutants were expressed as GFP_2 fusion in *Δnap1* cells and visualized by fluorescence microscopy. The coincident DIC and Hoechst images are shown.

complexes that regulate the different functions of Nap1p during the cell cycle.

Table 4. *Phosphorylated Residues of Nap1p and Nap1p-Interacting Proteins*

Phosphopeptides from Nap1-PrA interacting proteins were enriched by IMAC and identified by Mass Spectrometry. The predicted kinase based on kinase consensus sequence. Phosphosites that have been previously identified or proposed based on experimental evidence are marked with an asterisk and the kinase listed is that identified by the original authors.

Protein name	**Phosphosites**	**Predicted Kinase**	**Reference**
Nap1p	T20	Cdc28p	
	T24	Cdc28p	
	S27	None	
	S81	none	
	S98	PKA/PKC	
	S104	None	
	S146	None	
	S159	CK2	
	S177	CK2	
	S397	CK2	
Hta2p	S129**	Mec1	(Downs, 2000)
Gin4p	S382	PKA	
	S389*	Elm1p	(Asano, 2006)
	T397	Cdc28p	

	T411	PKC	
Protein name	**Phosphosites**	**Predicted Kinase**	
Gin4p (cont.)	S460*	Elm1p	(Asano, 2006)
	T462*	Elm1p	(Asano, 2006)
	S639**	Elm1p	(Asano, 2006)
	S689	Cdc28p	
	S930	none	
Cki1p	S48	CK2	
	S51	None	
	T54	CK2	
Kcc4p	S777	None	
	S822	None	
Nba1p	S328	None	
	T403	Cdc28p	
Nis1p	S300	Cdc28p	
Hsl1p	S157	None	
	S627	Cdc28p	
Cki1p	S48	CK2	
	S51	None	

Discussion

In higher eukaryotes there are several members of the Nap1 superfamily to which different roles in chromatin metabolism, transcription, and cell cycle regulation have been ascribed. (Park and Luger, 2006a). Whether these functions are interconnected, or represent an example gene sharing, whereby a single protein performing multiple, unrelated functions, is not yet understood. *S. cerevisiae* has only two members of this protein family, Nap1p and Vps75p, and we set out to use this simple model organism to elucidate the regulatory mechanisms governing Nap1p's pleiotropic functions. We identified various Nap1p interacting proteins, including most of the expected and previously characterized binding partners such as histones, Kap114p and Gin4p. In addition, some new partners were identified including the kinases CK2, Kcc4p, Gin4p and Cki1p. We showed that Nap1p was phosphorylated in vivo at eleven sites. We also arrested cells at S phase and G2/M in order to see if the any of the interactions between Nap1p and its binding partners were specific to a given stage of the cell cycle. Though the number of peptides identified in a proteomic screen is not directly quantitative, it can be used as a measure of the relative abundance of a protein between two samples by subtractive proteomics, and this allowed us to determine if any Nap1p protein-protein interactions were cell-cycle dependent (Veenstra, 2007).

Many members of the Nap/SET superfamily in higher eukaryotes are known phosphoproteins. In this study, we demonstrate for the first time that in *S.cerevisiae* Nap1p is phosphorylated in vivo, and we have identified specific phosphorylated residues on both Nap1p and on a number of Nap1p-interacting proteins. Although Clb2p is a

known Nap1p-interacting protein, we did not detect it among our Nap1p binding partners. Nap1p was initially observed in association with Clb2p by affinity purification, and the complex was disrupted at relatively low salt concentration, suggesting this is a low-affinity interaction. Additionally, the association was only detected in the presence of Cdc28p (Kellogg et al., 1995). It is possible that we would have detected this interaction under less stringent conditions. Nap1p and Clb2p interact functionally at the entry into mitosis, when the Cdc28p kinase is activated. Two of the phosphorylated threonines identified on Nap1p fell within consensus sites for Cdc28p. Given this, we performed an in vitro kinase assay and demonstrated that Nap1p is a specific substrate for phosphorylation by Cdc28p/Clb2p. Mutation of the two target residues reduced the incorporation of ^{32}P by Nap1, but did not abolish phosphorylation entirely. This implies these sites are recognized by the kinase but that there are other Nap1p substrate sites for Cdc28p/Clb2p that we have yet to identify. None of the other phosphosites on Nap1p identified by MS fit within the minimal consensus motif for this kinase. Of the other serines and threonines, only Nap1p T307 is followed by a proline and thus conforms to the Cdc28p/Clb2p minimal consensus motif. Although not identified as a phosphosite in this analysis, this residue is the principal candidate for the remaining Cdc28p/Clb2p target site and further mutational analyses will determine whether this is the case.

Mutation of the individual Cdc28p/Clb2p target serines of Nap1p did not prevent Nap1p from localizing to the bud neck, and this suggests that phosphorylation may occur subsequent to localization. In this case, the bud neck and associated septins might be acting as a scaffold for this signaling event. Alternatively, since mutation of both T20 and T24 target residues did not abolish phosphorylation of Nap1 by Cdc28p/Clb2p, it is

possible that the remaining level of phosphorylation of Nap1p is sufficient for its localization. In future experiments we hope to identify the remaining target residue(s) and mutate each of the Cdc28p/Clb2p phosphosites within a single reporter construct to make a nap1$^{\Delta Cdc28p}$ phosphorylation-deficient mutant. By examining the localization of such a mutant we can determine whether bud neck localization of Nap1p is directly dependent upon phosphorylation by Cdc28p/Clb2p. We predict that preventing phosphorylation of Nap1p by Cdc28p/Clb2p may inhibit the ability of Nap1p and Clb2p to induce isotropic bud growth, resulting in elongated buds and a delayed entry into mitosis. To address this, we intend to express the nap1$^{\Delta Cdc28p}$ mutant in the Clb2p-dependent strain and assess the ability to rescue normal bud shape.

Three of the phosphoserines identified on Nap1p, S159, S177 and S397, were within a CK2 consensus substrate recognition motif. It is likely that Nap1p is a substrate for this kinase, since the catalytic α' subunit was detected in our screen for Nap1p binding partners. The following chapter will analyze the role of CK2 in Nap1p function. There are six phosphosites in Nap1p that are not within a known consensus sequence for any kinase. Future studies will focus on identifying the other kinases phosphorylating Nap1p and the functional significance of these modifications. For this analysis of Nap1p phosphosites we isolated Nap1p from asynchronous cells, and it is possible that there are additional rare or short-lived phosphorylation events that were not identified. By using cell cycle arrest techniques such as those we employed for our cell cycle dependent binding partner assay, we hope to identify cell-cycle specific phosphorylation events. This approach may also enrich for transient modifications and thus facilitate their detection.

In this study, the identification of the previously uncharacterized bud neck associated protein Nba1p validated our approach for looking at Nap1p-interacting proteins. In addition, we demonstrate Nba1p is a substrate for phosphorylation by Cdc28p/Clb2p in vitro (Ubersax et al., 2003). We identified a substrate threonine for this kinase, but mutating this residue did not completely abolish phosphorylation of Nba1p by Cdc28p/Clb2, implying that other substrate residues remain to be identified. Global analysis of *NBA1* mRNA levels during the cell cycle demonstrated that its expression, like that of Clb2p, is periodic and peaks during mitosis (Spellman et al., 1998). Although no genetic interaction was detected between the two proteins, Nba1p, like Nap1p, is primarily cytoplasmic and localizes to the bud neck at G2/M. We hypothesize that Nap1p and Nba1p interact at the bud neck, possibly in complex with Clb2p, and function in the regulation of G2/M progression.

Another Nap1p-interacting protein identified in this assay was Nis1p. Nap1p has previously been shown to bind to Nis1p, and although the specific function of Nis1p is not well understood it is proposed to be involved in the mitotic signaling network (Iwase and Toh-e, 2001). Compared to wild type, the *Δnis1* strain was highly sensitive to benomyl, implying it plays a role in regulating microtubule stability. Nis1p is a phosphoprotein; only the phosphorylated form of Nis1p is present during G2/M and this correlates with its localization to the bud neck (Iwase and Toh-e, 2001). In this study we identified a phosphopeptide from Nis1p, and the phosphorylated serine is within a consensus site for Cdc28p kinase. Nis1p was only detected binding to Nap1p in cells arrested at G2/M, at the time Nis1p is phosphorylated. We predict that phosphorylation of

Nis1p by Cdc28p/Clb2p may be involved in regulating its association with Nap1p or localizing this protein to the bud neck, or these processes may be coordinately regulated.

Gin4p and three other kinases were identified in association with Nap1p. Gin4p is thought to regulate bud formation through a pathway parallel to Nap1p, since in this study and others the deletion of both genes enhances the elongated bud phenotype seen in both single deletion strains (Altman and Kellogg, 1997b). Another Nim1-like kinase, Kcc4p, was found to associate with Nap1p, although the two proteins did not appear to interact genetically. All three proteins, Nap1p, Kcc4p and Gin4p, cause an elongated bud phenotype when overexpressed (data not shown and (Akada et al., 1997; Okuzaki and Nojima, 2001). Taken together, these results suggest that these proteins function in overlapping, but non-identical pathways in the regulation of bud formation. We sequenced phosphopeptides from both Gin4p and Kcc4p in association with Nap1p. Gin4p is a known target for phosphorylation by Elm1p at S369, and this phosphoserines was detected in our screen. Additionally, three other Gin4p phosphosites described here were identified as potential targets for this kinase; Elm1p also phosphorylates Kcc4p in vitro, though no specific sites have been determined and no consensus target sequence has been identified for Elm1p (Asano et al., 2006).

Gin4p, Kcc4p, Hsl1p and Elm1p are all bud neck associated kinases and work in parallel within the mitotic signaling network. A mutant in which Elm1p is deleted mislocalizes the septin Cdc11p to the bud tip, whereas in *Δgin4 Δkcc4 Δhsl1* triple mutant, Cdc11p forms disorganized septin rings at the bud neck (Bouquin et al., 2000). This suggests that in the mitotic signaling cascade Elm1p kinase activity is required for localization of septins to the bud neck, whereas Gin4p, Kcc4p and Hsl1p function

downstream of Elm1p and are required for the organization of septins following localization to the bud neck. The association of Nap1p with Gin4p and the other kinases may be required for targeting Nap1p to the bud neck, and thus these interactions appear to occur in a specific order. It is not known whether Nap1p is a substrate for phosphorylation by these mitotic regulatory kinases, but all are promising candidate kinases for phosphorylation of Nap1p at the remaining, uncharacterized phosphosites. Any of these phosphorylation events may be part of a signaling cascade that is upstream of the regulation of bud growth by Nap1p.

The fourth kinase we identified in our screen for Nap1p-interacting proteins was Cki1p. Cki1p is involved in the synthesis of the membrane lipid phosphatidylcholine in the Kennedy pathway (Hosaka et al., 1989). Cki1p and Nap1p interact genetically, as indicated by the fact that deletion of *NAP1* greatly increases the benomyl sensitivity observed in *Δcki1* and exacerbates the elongated bud phenotype compared to either single deletion strain. Phosphorylation of Nap1p itself or a Nap1p-interacting substrate by Cki1p may be required for normal bud formation.

With the exception of Hsl1p, all of the kinases that associate with Nap1p in our assays show a decrease in binding to Nap1p during G2/M, though more careful analysis of each interaction will be needed to confirm this observation. . The G2/M-associated decrease in binding was most pronounced with respect to the association of Nap1p with Gin4p. It was previously reported that Nap1p and Gin4p co-immunoprecipitate throughout the cell cycle, whereas the association of Gin4p with the septin Cdc11p was restricted to G2/M. Nap1p is required for hyperphosphorylation of Gin4p and its localization to the bud neck, and this suggests that the association of Nap1p with Gin4p

occurs prior to the association of Gin4p with the septins (Mortensen et al., 2002). It is possible that during G2/M Nap1p and Gin4p are indirectly associated within a complex at the bud neck. In this case, only the direct binding of Nap1p to Gin4p is decreased during G2/M, and this decrease may not have been detectable by co-immunoprecipiation.

Though subtractive proteomic analysis is not a directly quantitative, it was nevertheless of interest to observe the how Nap1p associations appeared to change at different stages in the cell cycle. The most striking observation was that the core histones H2A and H2B were only detected in association with during S phase, whereas the histone variant H2A.Z was only detected in association with Nap1p in cells arrested at G2/M. Since synthesis of histones H2A and H2B occurs only during S phase, there is a significant decrease in free H2A and H2B in the cytoplasm outside of synthesis. H2A.Z synthesis occurs throughout the cell cycle, and it is possible that cell cycle dependent difference in histones bound to Nap1p reflects the stochiometries of available cytoplasmic histones (White and Gorovsky, 1988; Wu et al., 1982). However, this data may also suggest that import of H2A and H2B by Nap1p is replication-dependent. The finding that Nap1p association with H2A.Z is not detected during S phase suggests that nuclear import of H2A.Z by Nap1p may be similarly restricted. It is therefore possible that Nap1p may exhibit different cell-cycle dependent chaperone activity for H2A/H2B dimers relative to H2A.Z/H2B.

The differences between Nap1p binding partners at S phase and at G2/M would suggest that the distinct functions of Nap1p are distinguished by the proteins it associates with. We had hypothesized that Nap1p/H2A/H2B and Nap1p/Clb2p/Gin4p complexes might be exclusively present at S phase and G2/M respectively, but this was not the case.

It is still possible that they exist as mutually exclusive complexes but that complex formation is not cell cycle dependent. In this study we show that Nap1p is phosphorylated by the mitotic kinase Cdc28p/Clb2p, and it will be interesting to determine the functional significance of this event. Phosphorylation of Nap1p by Cdc28p/Clb2p at G2/M may promote its interaction with H2A.Z. The identified Cdc28p/Clb2p phosphosites are outside of the region represented in the crystal structure, thus it is not possible to predict how phosphorylation might physically alter Nap1p histone binding activity. In conclusion, our data show for the first time that Nap1p associates with histones and some of its interacting kinases in a cell-cycle dependent manner. We also show that Nap1p is a substrate for phosphorylation by Cdc28p/Clb2p, and we predict that this phosphorylation may alter the interaction of Nap1p with its binding partners, and thus regulate cell-cycle specific functions.

CHAPTER 4: Casein Kinase 2 Phosphorylation Regulates the Localization and Chromatin Assembly Activity of Nap1

This chapter is based on **Casein Kinase 2 Phosphorylation Regulates the Localization and Chromatin Assembly Activity of Nucleosome Assembly Protein 1.** Meredith E.K. Calvert, Kristin M. Keck, Celeste Ptak, Jeffrey Shabanowitz, Donald F. Hunt and Lucy F. Pemberton. Submitted to Molecular and Cellular Biology, 2007.

Introduction

The *Saccharomyces cerevisiae* nucleocytoplasmic shuttling protein Nap1p is a histone chaperone with chromatin assembly and disassembly activity and a cofactor for the import of histones H2A and H2B. It also binds to the karyopherin Kap114p, and promotes the specificity of the interaction of H2A/H2B dimers with Kap114p. Nap1p, H2A/H2B and Kap114p form a complex that is imported through the nuclear pore complex into the nucleus. Most import complexes are disassembled in the nucleus due to high levels of RanGTP that promotes cargo release. However, the association of Kap114p with Nap1p not sensitive to RanGTP, and this causes the Kap114p/Nap1p/H2A/H2B complex to be resistant to Ran-mediated disassembly (Mosammaparast et al., 2002). The mechanism by which the complex dissociates is not known, though interestingly Kap114p has been shown to inhibit chromatin assembly by Nap1p in vitro (Mosammaparast et al., 2005). This suggests that Nap1p and the H2A/H2B dimers must be released from Kap114p prior to chromatin assembly, and release may be regulated by other as yet unidentified nuclear factors.

In vitro, Nap1p can assemble intact nucleosomes onto chromatin and is associated with large regions of the genome. Nap1p stimulates the binding of coactivator p300, GAL4 and other transcription factors to nucleosomal DNA and can upregulate transcription at these domains (Walter et al., 1995; Rehtanz, 2004; Shikama, 2000). At active promoter regions, Nap1p is found in association with the RSC chromatin remodeling complex, where it promotes chromatin disassembly through removal of H2A/H2B dimers (Lorch et al., 2006a). It can also exchange H2A/H2B dimers for dimers containing the histone variant H2A.Z, and the assembly mechanism for H2A.Z/H2B dimers is indistinguishable (Mazurkiewicz et al., 2006; Park et al., 2005). Nap1p has been shown to promote nucleosome assembly in association with the ATPase Chd1p, which transfers nucleosomes from Nap1p onto DNA (Lusser et al., 2005). In *S.pombe,* Nap1 and the Chd1 paralogues Hrp1 and Hrp2 share genomic binding targets, which tend to be active promoter regions. This suggests that Nap1p and Chd1p may also cooperatively regulate disassembly. (Walfridsson et al., 2007). Nap1p also has a proposed role in transcriptional elongation, since in association with the FACT complex, Nap1p binds to subunits of the RNA polymerase II elongator complex (Krogan et al., 2006; Orphanides et al., 1998; Orphanides et al., 1999). It has been proposed that Nap1p, Chd1p and the FACT complex all associate with the CK2, and phosphorylation of these substrates may influence transcriptional activity (Krogan et al., 2002; Li et al., 1999b; Walfridsson et al., 2007). Due to these various acitivites, deletion of Nap1p results in changes in the level of transcription of about 10% the yeast genome, causing both upregulation and downregulation of expression with target-specific effects (Ohkuni et al., 2003).

Nap1p is highly conserved throughout eukaryotes and is a member of the Nap/SET superfamily of proteins. It was originally identified in *S.cerevisiae* through its interaction with the mitotic cyclin, Clb2p, and was found to have a role in the regulation of microtubule dynamics during mitosis. In association with Clb2p, Nap1p is required for facilitating the switch from polar to isotropic bud growth, and deletion of *NAP1* causes elongated buds and a delayed entry into mitosis (Kellogg and Murray, 1995a). Nap1p and Clb2p cooperatively initiate a mitotic signaling network that promotes the assembly and organization of septins at the bud neck, inhibits polar bud growth and leads to the degradation of the Swe1p kinase (Mortensen et al., 2002). Swe1p kinase, in turn, negatively regulates the activation of the mitotic kinase, Cdc28p/Clb2p. Degradation of Swe1p is required for entry into mitosis, and its persistence causes elongated buds and a G2/M cell cycle delay (McMillan et al., 1999; Sia et al., 1998). Thus, activation of Clb2p in cooperation with Nap1p is the first step in a positive feedback loop regulating the entry into mitosis.

Nap1 orthologs in Drosophila and humans exhibit cell-cycle dependent localization, accumulating in the nucleus during S phase (Ito et al., 1996b; Rodriguez et al., 2000b. In contrast, in S.cerevisiae,Nap1p remains cytoplasmic throughout the cell cycle and localizes to the actin cap and bud neck throughout bud growth. Though it is not observed in the nucleus, the nucleocytoplasmic shuttling of Nap1p is evidenced by nuclear accumulation of Nap1p following a mutation in the NES (Mosammaparast, 2002). The apparently distinct functions of Nap1p as a histone chaperone and a component of the mitotic signaling cascade suggests that there may be separable

cytoplasmic and nuclear functions for Nap1p. In this case, the regulation of Nap1p localization would directly influence its activity.

In a recent study identifying Nap1p binding partners, we identified the catalytic α' subunit of Casein Kinase 2 (CK2), and confirmed this interaction by co-immunoprecipitation of Nap1p and CK2 in vivo (see chapter 3). In a screen for genetic interactions we determined that mutations in *Δcka2* strain showed increased resistance to benomyl in the presence and absence of *NAP1*, suggesting that both proteins function in regulating microtubule stability (see chapter 3). There is evidence that CK2 may phosphorylate Nap1 family members in other species. In the nematode *S.feltiae*, Nap1p and CK2 interact by yeast 2-hybrid (Gal et al., 2005). In both *O.sativa* and *D.melanogaster* Nap1 proteins are substrates for phosphorylation by recombinant human CK2 in vitro (Li, 1999; Rodriguez, 2000; Dong, 2005). Because *Δcka2* and *Δnap1* mutants exhibit overlapping phenotypes and CK2 like Nap1p is present in both the cytoplasm and nucleus, and Nap1p has been implicated as a CK2 substrate in other species, we decided to investigate CK2-mediated regulation of Nap1p function.

We identified three phosphorylated residues on Nap1p that were within the consensus site motif for phosphorylation by CK2. We show that Nap1p is a substrate for phosphorylation by CK2 at these three serines in vitro. We demonstrate that phosphorylation of Nap1p by CK2 appears to promote its import into the nucleus, but does not affect the binding of Nap1p to Kap114p or histones. CK2 phosphorylation also inhibits chromatin assembly by Nap1p in vitro and is required for normal S phase progression, thus representing the first example of regulation of Nap1p localization and function.

Materials and Methods

Yeast strains and plasmids

Yeast strains used in this study were derived from DF5 or BY4741 as noted, and construction of mutant strains *Δnap1* and *Δkap114* have been described previously (Mosammaparast, 2005; Pemberton et al., 1995). Strains DK212 and DK213 were a gift from Dr. D. Kellogg (Altman and Kellogg, 1997b). Strain KWY486 was a gift from Dr. K. Weis. The *Crm1^{LMB} Δmsn5 Δlos1* was derived from KWY486; w303 *XPO1::LEU2 Xpo1^{LMB}::HIS3 LOS1::ClonNAT MSN5::KanMX trp1-1 ura3-1 ade2-1*. Expression of GFP-tagged proteins was performed using the pGFP$_2$-C-FUS vector (Mosammaparast et al., 2002). Amino acid substitutions were made using oligonucleotide site-directed mutagenesis of a dsDNA plasmid template and verified by sequencing. For plasmid rescue experiments, the *NAP1* promoter (400bp upstream) and terminator (296bp downstream) fragments were cloned into pRS316 with an intervening BamHI site, and *NAP1* and mutant derivatives were inserted into this site. For recombinant protein expression, *NAP1* and mutant derivatives were cloned into pGEX4T1, and GST fusion proteins were purified as previously described (Mosammaparast et al., 2001).

In vitro binding assays

For the histone binding assay, 4μg of Nap1p or mutant protein was immobilized on glutathione-Sepharose and incubated with 2μg of chicken erythrocyte core histones. For quantification of Nap1p binding to MBP-Kap114, MBP-Kap114p was immobilized on amylose resin (NEB). In both cases, beads were pre-blocked with 10% BSA in buffer containing 1% Tween 20 at the protein concentrations described. Beads were washed

extensively and bound material was eluted by boiling in loading buffer and separated by SDS–PAGE and visualized as indicated. In both cases, the membrane was incubated with an anti-Nap1p antibody, followed by either anti-GST or anti-MBP antibody, and binding was detected using the Odyssey infrared imaging system (LI-COR, Biosciences). Binding was quantified by comparing the amount of Nap1p or mutant signal relative to the amount of input MBP-Kap114 signal detected by the imaging system.

In vitro phosphorylation of Nap1p

For the in vitro kinase assays,1μg of GST-Nap1 or mutant was incubated with 0.2mM ATP, 1μCi of [γ-^{32}P]ATP, and 0.2μl human recombinant CK2 (NEB, Beverley, MA) at 30˚C for 30 min. or as indicated. The reaction was stopped by addition of SDS/PAGE sample buffer. The proteins were separated by SDS/PAGE, stained with Coomassie blue and ^{32}P-labeled proteins were detected by autoradiography on a PhosphorImager.

For in vitro phosphorylation of Nap1p for use in assaying binding to Kap114p, 2μg of GST-Nap1p was assembled into a kinase reaction as above, but in the absence of [γ^{32}P]ATP. From this, one fifth of the reaction (containing 0.4μg of GST-Nap1) was removed and incubated with 0.5μCi of [γ-^{32}P]ATP in order to determine the stochiometry of the phosphorylation reaction by liquid scintillation counting. In order to maximize phosphorylation of the substrate by CK2, the two reactions were incubated at room temperature for 48 hrs.

Cell culture and microscopy

Cell culture methods and microscopy were performed as described previously (Mosammaparast et al., 2001) using a Nikon Microphot-SA microscope (Melville, NY) and images were captured using OpenLab software (Improvision, Lexington, MA) with a

100X objective. Strains containing reporter GFP constructs were induced in SC-ura-met either overnight (nap1 3S-A-L99S-GFP2), or for 2h (all other constructs). Cells were photographed using identical exposure settings, and all image manipulation was performed identically using Adobe Photoshop. For the benomyl sensitivity assay, 10-fold serial dilutions of each strain were spotted onto YPD, YPD + 20μg/ml Benomyl in 0.4% DMSO, or YPD + 0.4% DMSO and incubated at 30˚ for four days.

Cell cycle analysis

Exponentially growing DK213 cells carrying the pRS-316 *NAP1* constructs were cultured in SC-ura media; DK212 control cells were cultured in CSM. Cells were counted, fixed in 70% EtOH 30% sorbitol, and stained with 1μM Sytox Green (Molecular Probes, Eugene, OR). 10,000 cells per sample were acquired on an ImageStream imaging flow cytometer (Amnis Corporation, Seattle, WA) using INSPIRE software. The Sytox Green signal and corresponding brightfield images were also collected. Spectral overlap of Sytox Green into the brightfield channel was calculated and subtracted using the manufacturer's image analysis software. Random cell images were collected from within a cross-section of the 4N peak on the histogram. Cell cycle analysis was performed on exponentially growing cultures; 500 Hoechst-labeled cells from each sample were assigned to one of three morphological categories based on microscopic analysis: no bud (G1), budded cell with single nuclei (S phase) or budded cell with two nuclei (G2/M). Cells of abnormal morphology were excluded from the analysis. A two-way chi-squared test was performed comparing values calculated for each mutant to the expected values obtained from wild type cells.

Chromatin Assembly Assay

The plasmid supercoiling assay was performed as described previously, (Mosammaparast, 2005). Briefly, 250ng of supercoiled pBluescript DNA is relaxed by incubating with 1U topoisomerase I (Promega), then added to a binding reaction containing chicken erythrocyte core histones and Nap1p or mutant derivatives at the indicated protein concentrations. After incubation reactions were deproteinized and DNAs were separated by electrophoresis in an agarose gel and visualized with ethidium bromide.

Results

Nap1p is phosphorylated by CK2 in vitro

CK2 has a consensus substrate recognition motif (S/TxxE/D, where x is any amino acid) and analysis of Nap1p phosphosites revealed that three phosphoserines, S159, S177 and S397, were within a CK2 consensus sequence (see chapter 3). Based on this evidence, we predicted that serines 159, 177 and 397 within Nap1p were CK2 target residues. To determine whether Nap1p is a substrate for CK2, we incubated recombinant GST-tagged Nap1p with recombinant CK2 and γ^{32}P-labeled ATP. GST-Nap1 was phosphorylated by CK2 within one minute of incubation with the kinase (Fig. 16A). In order to confirm the CK2 target sites, mutants were constructed in which two or three of the CK2 target serines were mutated to uncharged, unphosphorylatable alanine residues. As predicted, mutation of two of the three serines to alanines reduced phosphorylation of Nap1p as measured by ^{32}P incorporation quantified on a phosphoimager, whereas mutation of all three serines (creating nap1 3S-A) completely eliminated phosphorylation by CK2 (Fig. 16A). This suggested that all three sites were recognized by CK2. We also demonstrated that phosphorylation of Nap1p in this reaction was specifically dependent upon presence of both the substrate and CK2 (Fig. 16B). A time course experiment determined that the in vitro phosphorylation progresses to saturation by 60 min, whereas nap1 3S-A remained unphosphorylated after 120 minutes (Fig. 16C). MS of recombinant GST-Nap1p phosphorylated by CK2 in vitro confirmed the presence of phosphorylated S177 and S397 (data not shown). Taken together, these results show that Nap1p contains three substrate serines for phosphorylation by CK2.

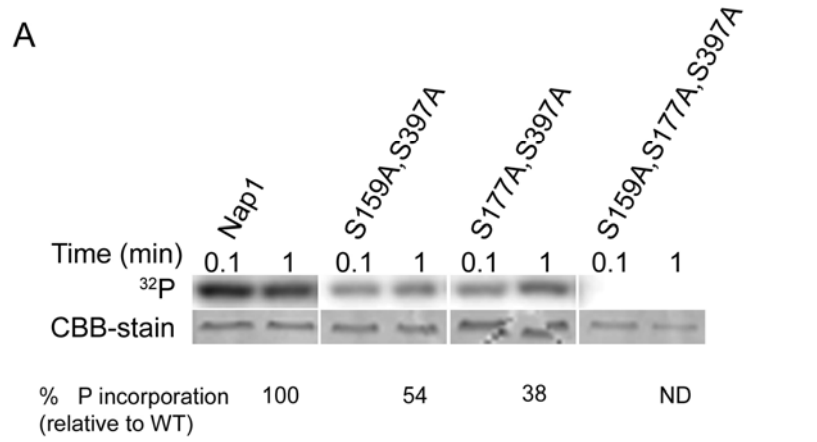
Figure 16

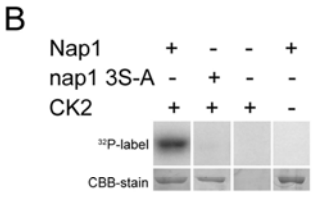
Nap1p is phosphorylated by CK2.

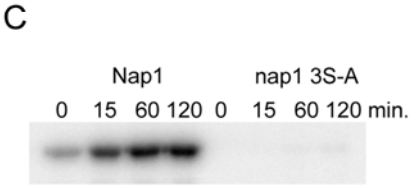
A: Recombinant GST Nap1 and the indicated Nap1 mutants were incubated with purified CK2 and [γ-^{32}P]ATP for the time indicated. The proteins were separated by SDS/PAGE, Nap1p was visualized by coomassie blue staining (CBB) and phosphorylation was measured by ^{32}P incorporation detected using a PhosphorImager. ND: not detectable.

B: In vitro kinase assay of GST-Nap1 or GST- nap1 3S-A was carried out for 30 min. as above, and the proteins included in the reaction are as indicated.

C: In vitro kinase assay of GST-Nap1 or GST- nap1 3S-A was carried out as above for the times indicated.


A
Nap1
S159A,S397A
S177A,S397A
S159A,S177A,S397A
Time (min)
0.1
1
0.1
1
0.1
1
0.1
1
32P
CBB-stain
% P incorporation
(relative to WT)
100
54
38
ND


B
Nap1 + - - +
nap1 3S-A - + - -
CK2 + + + -
32P-label
CBB-stain


C
Nap1
nap1 3S-A
0 15 60 120 0 15 60 120 min.

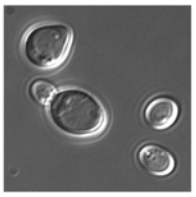
Phosphorylation of Nap1p by CK2 is not required for Clb2-dependent bud formation. To investigate how phosphorylation of Nap1p by CK2 might regulate its various cellular functions, we constructed plasmids expressing wild type *NAP1* or mutants in which the three CK2 target phosphoserines were mutated to alanine (nap1 3S-A) or to negatively charged aspartic acid (nap1 3S-D) to mimic constitutively unphosphorylated or phosphorylated Nap1p, respectively. Each was expressed under the control of the endogenous *NAP1* promoter and terminator, and Western blotting confirmed that the wild type and mutant proteins were expressed at similar levels (data not shown). In order to determine whether phosphorylation of Nap1p by CK2 was necessary for the regulation of correct bud formation, we expressed nap1 3S-A and nap1 3S-D in a *CLB2*-dependent strain, in which the genes encoding *CLB1*, *CLB3*, *CLB4* and *NAP1* were deleted (DK213). In this background the *Δnap1* phenotype is exacerbated, resulting in cells with highly elongated buds and the formation of large, interconnected clumps of cells (Kellogg and Murray, 1995b). In this strain, expression of Nap1p or either CK2 phosphomutants of Nap1p (nap1 3S-A and nap1 3S-D) were able to rescue normal bud shape (Fig. 17). This suggests that CK2 phosphorylation of Nap1p is not required for correct bud formation.

Figure 17

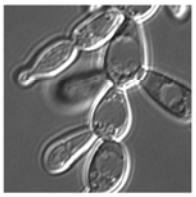
Phosphorylation of Nap1p by CK2 is not required for normal bud formation.

Nap1p, nap1 3S-A or nap1 3S-D were expressed in the Clb2-dependent strain and examined by DIC microscopy.

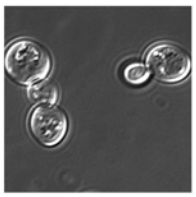
Δclb1Δclb3Δclb4



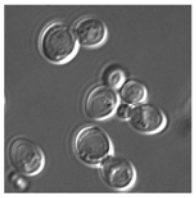
Δnap1Δclb1Δclb3Δclb4
+ vector



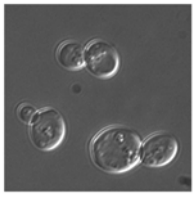
Δnap1Δclb1Δclb3Δclb4
+ Nap1



Δnap1Δclb1Δclb3Δclb4
+ nap1 3S-A



Δnap1Δclb1Δclb3Δclb4
+ nap1 3S-D



Reversible phosphorylation of Nap1p by CK2 is required for normal cell cycle progression. In order to determine whether Nap1p phosphorylation by CK2 regulates cell cycle progression, we observed the cell cycle profile of the Nap1p phosphomutants in the Clb2-dependent strain. Cells were analyzed using Amnis ImageStream allowing the correlation of DNA content with cell morphology of individual cells within the population. In unsynchronized cells, the *Δnap1*mutant has a higher ratio of cells with 2N DNA to those with 1N DNA content (Fig. 18A), indicative of the mitotic delay described previously (Kellogg and Murray, 1995b). In this strain *Δnap1* cells exhibit a large proportion of multinucleate cells, as evidenced by the presence of an additional peak representing 4N DNA content. Morphologically, this peak consisted of interconnected buds representing failed cytokinetic events and Sytox green staining revealed that these interconnected buds had ≥2 nuclei (Fig. 18B). Expression of Nap1p, nap1 3S-A or nap1 3S-D rescued the cytokinetic defect, resulting in a significant reduction in the number of 2N+ cells and a loss of the peak corresponding to 4N DNA content, further suggesting that phosphorylation of Nap1p by CK2 is not required for its role in mediating bud formation (Fig. 18B). However, analysis of the cell-cycle profile for these strains suggested that there was a small increase in cells in G1/S in the nap1 3S-A and nap1 3S-D strains relative to Nap1p expressing cells. To analyze this more thoroughly we used microscopy to characterize the cell cycle profile of each strain morphologically. Random fields were scored for the number of cells in G1 (single nuclei, no bud), S (single nuclei, small bud) and G2/M (two nuclei, large bud). Although the doubling times for strains expressing the different forms of Nap1 were equivalent, expression of nap1 3S-A or nap1 3S-D led to an increase in the total number of cells in S phase and a reduced number of

cells in G1 relative to wild type, a result which was more pronounced with nap1 3S-D (Fig. 18C,D). This suggests that the cells expressing each of the Nap1 mutants (nap 3S-A and nap 3S-D) exhibit a prolonged passage through S phase and a shortened passage through G1, relative to cells expressing wild type Nap1p. As we observed a similar effect with both the nap1 3S-A and nap1 3S-D, these data suggest that reversible phosphorylation of Nap1p by CK2 is required for normal progression through S phase.

Figure 18

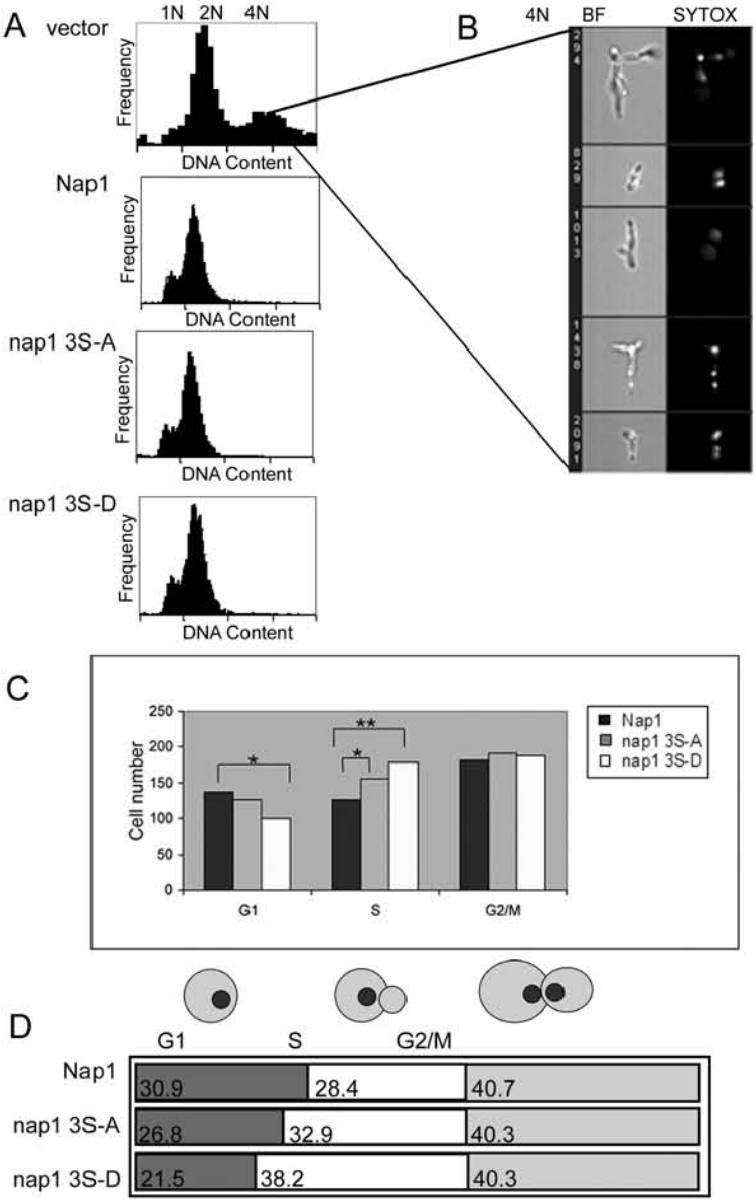
Reversible phosphorylation of Nap1p by CK2 may regulate normal progression through S phase.

A: Nap1p, nap1 3S-A or nap1 3S-D were expressed in the Clb2-dependent strain fixed, and the DNA stained with Sytox Green. Cells were analyzed for DNA content using the Amnis Imagestream instrument.

B: Random images of cells within the peak corresponding to 4N DNA content (vector control sample only) are displayed on right. BF: brightfield image, Sytox: stained DNA.

C: Strains as above were stained with Hoechst and observed by fluorescence and DIC microscopy, and scored morphologically for their cell cycle distribution. Numbers of cells with no bud (G1), small bud, one nuclei (S phase), large bud, two nuclei (G2/M) are indicated. Comparison of strains showed statistically significant differences (* $p < 0.05$, ** $p < 0.01$).

D: Numbers in boxes indicate percentage of cells in each phase of the cell cycle derived from (C).


A
vector
1N 2N 4N
Frequency
DNA Content
Nap1
nap1 3S-A
nap1 3S-D
B
4N
BF
SYTOX
C
Cell number
250
200
150
100
50
0
G1
S
G2/M
Nap1
nap1 3S-A
nap1 3S-D
D
G1
S
G2/M
Nap1
30.9
28.4
40.7
nap1 3S-A
26.8
32.9
40.3
nap1 3S-D
21.5
38.2
40.3

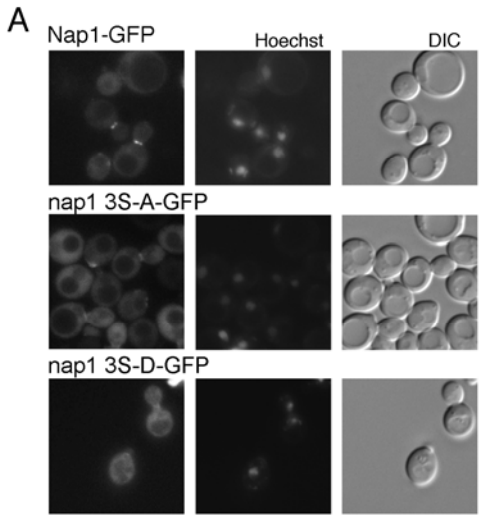
Phosphorylation of Nap1p by CK2 is not required for normal localization. In yeast, Nap1p is predominantly cytoplasmic and no cell-cycle dependent change in nuclear localization has been reported. However Nap1p has been demonstrated to shuttle between the nucleus and cytoplasm in a NES-dependent manner (Mosammaparast et al., 2002). Two of the CK2 phosphorylation sites are located near the NES within the crystal structure, and we hypothesized that CK2 may regulate Nap1p export. GFP_2 fusions of Nap1p, nap1 3S-A and nap1 3S-D were expressed from plasmids in a *Δnap1* strain to prevent dimerization or competition with endogenous Nap1p. In exponentially growing cells, wild type and mutant GFP fusions were predominantly cytoplasmic and all localized to the bud neck in G2/M cells (Fig. 19A). Since in this assay Nap1p is almost undetectable in the nucleus, we decided to assess the ability of the mutants to be imported in the context of an export-deficient reporter. In this way the steady state localization of Nap1p would be shifted to the nucleus making it possible to reveal an import defect. It has previously been demonstrated that mutation of two of the leucine residues in the NES, L99 and L102, is sufficient to make Nap1p predominantly nuclear (Miyaji-Yamaguchi et al., 2003). We constructed a minimal export-deficient mutant, in which a single leucine (L99) within the NES was mutated to a serine, redistributing Nap1-GFP_2 to the nucleus. The L99S mutation was incorporated into the GFP reporter constructs described above. Nap1-L99S-GFP_2 and the phosphomimic nap1 3S-D-L99S-GFP_2 fusion proteins localized to the nucleus (Fig. 19B). However, the unphosphorylatable nap1 3S-A -L99S-GFP_2 mutant was predominantly cytoplasmic, although it was still detectable in the nucleus at low levels. This indicated that nuclear import of the unphosphorylatable mutant was greatly reduced relative to the wild type and phosphomimic forms.

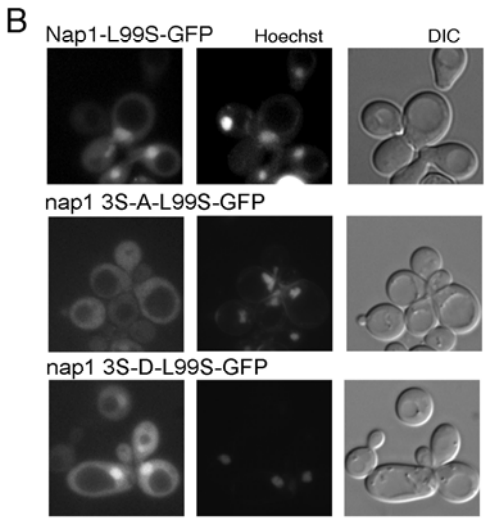
Figure 19

Phosphorylation of Nap1p by CK2 regulates nuclear localization.

A: Nap1 nap1 3S-A or nap1 3S-D mutants were expressed as GFP_2 fusion in *Δnap1* cells and visualized by fluorescence microscopy. The coincident DIC and Hoechst images are shown.

B: Export deficient Nap1 nap1 3S-A and nap1 3S-D GFP_2 fusion proteins were expressed and visualized as above.


A
Nap1-GFP
Hoechst
DIC
nap1 3S-A-GFP
nap1 3S-D-GFP


B
Nap1-L99S-GFP
Hoechst
DIC
nap1 3S-A-L99S-GFP
nap1 3S-D-L99S-GFP

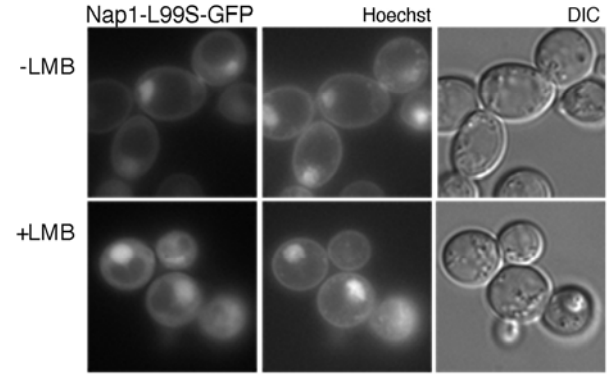
An alternative explanation for the mislocalization of nap1 3S-A -L99S-GFP_2 is that the export of this mutant is upregulated, relative to the phosphomimic and wild type forms of Nap1p. In the recently published crystal structure, Park and Luger identified the predicted CK2 consensus sites S159 and S177 and proposed that phosphorylation of Nap1p by CK2 could affect export of Nap1p by altering the availability of the NES (Park and Luger, 2006b). In order to test this possibility we examined the localization of Nap1-L99S-GFP_2 and nap1 3S-A -L99S-GFP_2 in an export-deficient strain. In yeast, the primary exportin for NES-containing proteins is Crm1p/Xpo1p. Previous work in our lab demonstrated that Crm1 is not directly required for the export of Nap1p, and that multiple export pathways exist for Nap1p (Mosammaparast et al., 2002). There are two other export factors in yeast, Los1p, which exports tRNA from the nucleus, and Msn5p, a transport factor that can import and export, and whose acitivity is frequently dependent upon the phosphorylation status of its cargo (DeVit and Johnston, 1999; Kaffman et al., 1998; Komeili and O'Shea, 1999; Sarkar and Hopper, 1998; Shen et al., 1996). Since there are cargo redundancies observed between these three export factors, we made a strain carrying a leptomycin B (LMB) sensitive allele of Crm1p and in which the export factors Los1p and Msn5p were deleted. In this strain, nap1 3S-A-L99S-GFP_2 localized to the nucleus and the cytoplasm in both the presence and absence of LMB, whereas Nap1-L99S-GFP_2 remained nuclear and the localization was unchanged by the addition of LMB (Fig. 20). This suggests that enhanced export of nap1 3S-A-L99S-GFP_2 by Crm1, Los1p or Msn5p is not the reason for its decreased nuclear accumulation.

Figure 20

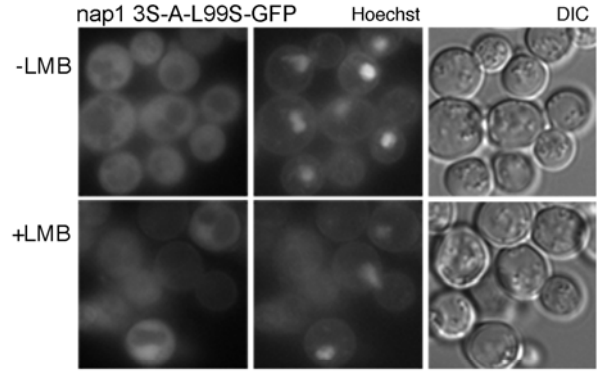
Mislocalization of nap1 3S-A-L99S GFP_2 is not due to upregulated export.

Export deficient Nap1-L99S and fusion proteins were expressed in *$xpo1^{LMB}\Delta msn5\Delta los1$* cells, in the presence and absence of LMB, and visualized by fluorescence microscopy. The coincident DIC and Hoechst images are shown.

A


Nap1-L99S-GFP
Hoechst
DIC
-LMB
+LMB

B


nap1 3S-A-L99S-GFP
Hoechst
DIC
-LMB
+LMB

Nap1p can be imported by the karyopherin Kap114p in a Kap114p-Nap1p-histone complex and the presence of Nap1p in the Kap114p-histone complex increases the association of Kap114p with histones (Mosammaparast et al., 2002). We tested whether phosphorylation of Nap1p by CK2 may promote binding of Nap1p to Kap114p and therefore explain the observed mislocalization of nap1 3S-A-L99S-GFP_2. We initially performed an in vitro binding assay with unphosphorylated recombinant Nap1p or the nap1 3S-D phosphomimic, and quantitated the amount of Nap1p bound to immobilized Kap114p. No difference in binding to Kap114p was detected between the two recombinant forms of Nap1p (Fig. 21A). We then phosphorylated Nap1p with recombinant CK2 and compared its ability to bind to Kap114p with that of unphosphorylated Nap1p. CK2 activity has been shown to be stimulated in vitro by the addition of histones, and we also assessed the ability of lysine-rich H2B histone tail to stimulate phosphorylation of Nap1p by CK2. In the absence of histone, 38% of the substrate was phosphorylated, and preincubation of the substrate with the H2B histone tail stimulated the activity of CK2 and increased the efficiency of the reaction to 50%.We found that the association of recombinant Nap1p with Kap114p was unaffected by in vitro phosphorylation of Nap1p by CK2, and also by preincubation with the H2B tail, showing that CK2 phosphorylation does not affect the interaction between Nap1p and Kap114p. This also suggests that the binding of histones by Nap1p does not alter its association with Kap114p (Fig. 21B). Taken together, these results imply that the decreased import of nap1 3S-A-L99S-GFP_2 was not due to a decreased affinity for Kap114p, though it is possible that CK2 could regulate the affinity of Nap1p for a

Kap114p-independent import pathway. In summary our results suggest that CK2 phosphorylation of Nap1p regulates its import into the nucleus.

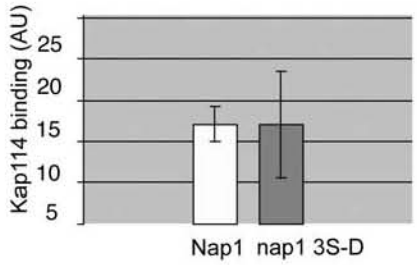
Figure 21

Nap1p binding to Kap114p is not dependent upon CK2 phosphorylation.

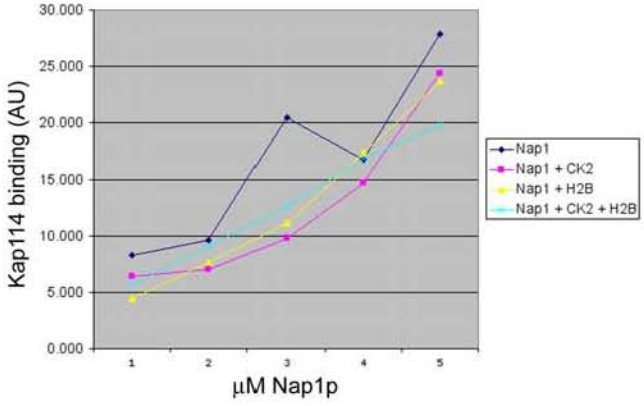
A: MBP-Kap114p (500nM) was immobilized and incubated with recombinant Nap1 or nap1 3S-D (both 250nM). Bound proteins were visualized by Western blotting and quantitated using the Odyssey infrared imaging system as described. The error bars indicate the standard deviation in triplicate experiments. The relative binding of Nap1p to Kap114p is expressed in arbitrary units (AU).

B: GST-Nap1 was preincubated with $H2B_{1-56}$ where indicated, then incubated with CK2 or buffer alone for 48 hours. By stoichiometry calculations, 38% of the substrate was phosphorylated by CK2 in vitro; preincubation with histones increased the efficiency of phosphorylation to 50%. Phosphorylated or unphosphorylated substrate was then incubated with immobilized MBP-Kap114 at the indicated concentrations. Binding was quantitated as above. Data shown are representative of three separate experiments.

A


Kap114 binding (AU)
25
20
15
10
5
Nap1
nap1 3S-D

B


Kap114 binding (AU)
30.000
25.000
20.000
15.000
10.000
5.000
0.000
1
2
3
4
5
μM Nap1p
Nap1
Nap1 + CK2
Nap1 + H2B
Nap1 + CK2 + H2B

Phosphorylation of Nap1p by CK2 inhibits chromatin assembly. The fact that the presence of the H2B tail upregulated the phosphorylation of Nap1p by CK2 suggested that phosphorylation might also influence the interaction of Nap1p with histones. This would represent a mechanism for regulating the assembly or disassembly of the Kap114p-Nap1p-histone import complex and therefore we examined the interaction of Nap1p with histones. Among NAP family members, the central NAP domain (containing S159 and S177 in Nap1p) is the minimal region required for histone binding, and the acidic C-terminal region of Nap1p (containing S397) has been proposed to promote its histone binding activity (Fujii-Nakata et al., 1992). We wanted to determine whether phosphorylation of Nap1p by CK2 might regulate this function. Core chicken erythrocyte histones were incubated with immobilized GST-Nap1p, nap1 3S-A or nap1 3S-D (Fig. 22A). We did not observe a difference in the ability of the recombinant proteins to bind histones in vitro, but there may have been differences in affinity that were not detectable in this assay. To further examine the Nap1p-histone interaction, we used an in vitro plasmid supercoiling assay to compare the ability of Nap1p and nap1 3S-A (both unphosphorylated) with the phosphomimic nap1 3S-D to assemble histones onto DNA. Only recombinant Nap1p and nap1 3S-A were able to introduce negative supercoiling onto relaxed plasmid DNA. The phosphomimic, nap1 3S-D, was unable to assemble chromatin when assayed at two concentrations (Fig. 22B). This suggests that the CK2-phosphorylation of Nap1p is inhibitory for its chromatin assembly activity. It is possible that phosphorylation of Nap1p by CK2 increases its affinity for histones and thus inhibits the release of histones from Nap1p and that a nuclear dephosphorylation event is required prior to histone deposition onto chromatin. It is possible that rounds of CK2

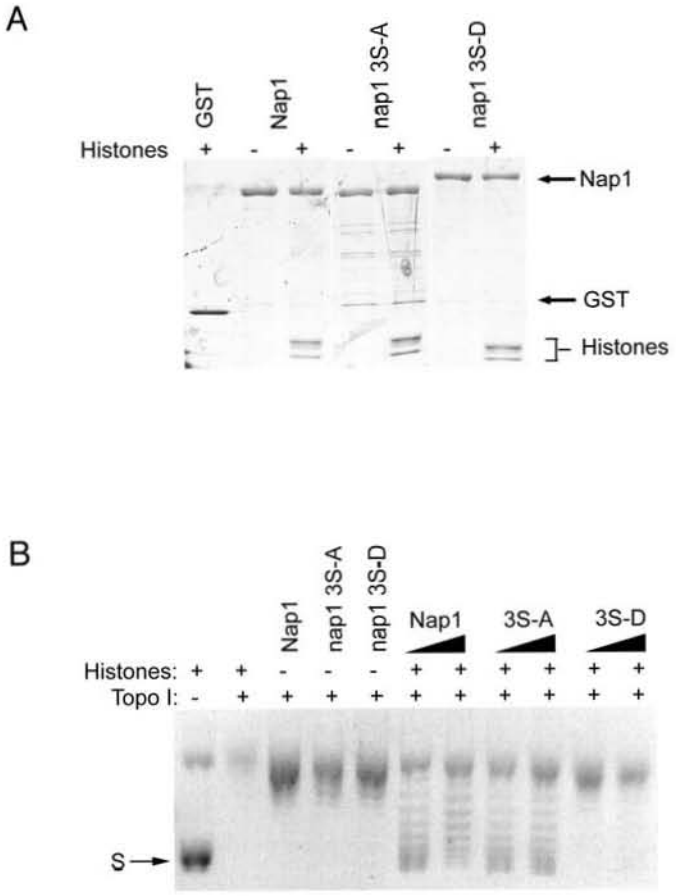
phosphorylation and dephosphorylation at chromatin regulate the ability of Nap1p to act as a histone donor and acceptor during replication and transcription.

Figure 22

The phosphomimic mutant of Nap1p is unable to assemble chromatin in vitro.

A: GST, GST-Nap1p, nap1 3S-A or nap1 3S-D was immobilized and incubated with purified core histones. Bound proteins were visualized by Coomassie Blue staining (nap1 3S-D consistently has slightly reduced mobility).

B: Plasmid supercoiling assays were performed with 500 and 750nM Nap1, nap1 3S-A and nap1 3S-D as indicated. S = supercoiled plasmid


A
GST
Nap1
nap1 3S-A
nap1 3S-D
Histones + - + - + - +
Nap1
GST
Histones
B
Nap1
nap1 3S-A
nap1 3S-D
Nap1
3S-A
3S-D
Histones: + + - - - + + + + + +
Topo I: - + + + + + + + + + +
S

Discussion

We have shown that some of the distinct functions of Nap1p are regulated by phosphorylation and we present evidence that Nap1p is phosphorylated by CK2. CK2 phosphorylation appears to be necessary for efficient nuclear import of Nap1p, but inhibits Nap1p-mediated chromatin assembly. This is the first report on the mechanism of Nap1p regulation. CK2 is ubiquitously expressed in the nucleus and cytoplasm, and thus the phosphorylation of Nap1p is likely reversible, regulated by the combined action of this kinase and a phosphatase. The importance of regulated phosphorylation and dephosphorylation events is further emphasized by the fact that yeast strains expressing either the phosphorylation defective (nap1 3S-A) or constitutively charged (nap1 3S-D) mutants of Nap1p had alterations in the cell cycle consistent with a defect in S phase progression and replication.

CK2 is a ubiquitous kinase that is highly conserved throughout eukaryotes, has a wide range of cellular targets, and is required for viability (for review, see (Litchfield, 2003)). It is made up of four subunits, two catalytic α subunits and two regulatory β subunits. Interestingly, the catalytic subunits are not entirely functionally redundant. In strains carrying a temperature sensitive allele of a single subunit, it was revealed that disruption of *CKA1*, encoding the α subunit, prevents polarization of the actin cytoskeleton and causes a loss of cell polarity (Rethinaswamy et al., 1998). In contrast, loss of *CKA2* (encoding the α' subunit) causes elongated buds in 10-30% of cells and cell cycle arrests in both G1 and G2/M (Hanna et al., 1995; Padmanabha et al., 1990).

It has been previously shown that *Drosophila* Nap1 binds to and is phosphorylated by human CK2 holoenzyme in vitro (Li et al., 1999a; Rodriguez et al., 2000a). The Nap1 homolog in the nematode *S. feltiae* interacts with endogenous CK2 by yeast 2-hybrid (Gal et al., 2005), and the homolog in the rice *O. sativa* is phosphorylated by recombinant CK2 in vitro (Dong et al., 2005). Human TSPY predominantly occurs in a phosphorylated form, and a putative CK2 phosphorylation site is required for nuclear import of this protein (Krick et al., 2006). These studies have led to speculation that Nap1 is a CK2 substrate and that phosphorylation may regulate Nap1p localization, and our studies have demonstrated for the first time that this is indeed the case.

We analyzed the potential impact of phosphorylation on Nap1p conformation using the published crystal structure (Park and Luger, 2006b). Several of the identified phosphorylated residues are located near the dimerization domain, and even though Nap1p has been predicted to be an obligate dimer, it is possible that phosphorylation may affect the interaction of the two monomers in some way (Park and Luger, 2006b). In their recently solved structure of Nap1p, Park and Luger noted three potential CK2 targets based on the kinase consensus sequence (S140, S159, and S177), the first two of which are located on the accessory domain (subdomain B), adjacent to the NES of the opposing molecule (Park and Luger, 2006b). They predicted that phosphorylation of these residues by CK2 could affect localization by altering the interaction of this subdomain with the NES. We demonstrated that S159 and S177 are phosphorylated by CK2, as well as a third residue, S397, and so far we have no evidence that S140 is phosphorylated by CK2. Serine 397 is of particular interest, since in both *Orysa* and *Drosophila* Nap1 homologs a C-terminal serine is proposed as a CK2 target, and in human TSPY phosphorylation of a

C-terminal serine is required for nuclear import (Krick et al., 2006). From the crystal structure of Nap1p, the location of the CK2 consensus sites led the authors to predict that phosphorylation would impact export of Nap1p by altering the availability of the NES to transport factors (Park and Luger, 2006b). We tested whether the mislocalization of nap1 3S-A-L99S was due to upregulated export by examining the localization of the mutant reporter constructs in a variety of export-deficient strains. We found that export was seemingly unaffected when phosphorylation was prevented and that nuclear import was inhibited. CK2 phosphorylation has also been found to regulate import of other substrates; in the case of the SV40 large tumor antigen, CK2 phosphorylation of serine residues flanking the NLS greatly enhances nuclear accumulation of the protein (Hubner et al., 1997).

The mechanism by which CK2 phosphorylation promotes nuclear import of Nap1p is not yet understood. We examined whether phosphorylation could enhance the affinity of Nap1p for its karyopherin Kap114p. In this study, Kap114p binding was unaffected by either mutation of the phosphorylated residues or by in vitro phosphorylation of Nap1p by CK2. This suggests that phosphorylation does not regulate the Kap114p-Nap1p interaction. Previous work has shown that a Nap1p export-deficient mutant is not significantly redistributed to the cytoplasm in strains lacking *KAP114,* suggesting that Nap1p has additional routes to the nucleus (Mosammaparast et al., 2002). These routes may be mediated by other karyopherins or indirectly by piggybacking on other nuclear proteins, and it is possible that phosphorylation could promote these interactions. Another model is that CK2 phosphorylation may modulate the Nap1p-histone interaction and hence regulate the formation of a stable

Kap114p/Nap1p/H2A/H2B complex. Surprisingly there was not an observable difference between the binding of the nap1 3S-A or nap1 3S-D to histones in an in vitro binding assay. We also tested the activity of nap1 3S-A and nap1 3S-D in a chromatin assembly assay, which we predicted would allow a more sensitive analysis of the effects of CK2 phosphorylation on the Nap1p-histone interaction. Although nap1 3S-D was able to bind both Kap114p and histones, it was unable to assemble chromatin when tested at a range of concentrations. This suggests that for Nap1p to assemble chromatin in the nucleus, a phosphatase must dephosphorylate the CK2 sites. CK2 is also present in the nucleus, therefore Nap1p may be rapidly rephosphorylated or, if the CK2 sites are masked in some way, rephosphorylation may occur only in the cytoplasm prior to import.

The nuclear localization of Nap1p is not cell-cycle dependent, as has been shown in higher eukaryotic homologs**.** However, both nap1 3S-A and nap1 3S-D exhibited a shortened G1 and prolonged S phase relative to wild type, with nap1 3S-D (which mimics constitutive phosphorylation by CK2) showing a more pronounced defect. The fact that we saw the defect with both mutants suggests that regulated cycles of phosphorylation and dephosphorylation are important for correct cell cycle progression. Loss of Nap1p is known to lead to an elongated bud phenotype and delayed G2/M progression. Intriguingly, the defect we observed with our CK2 mutants was a prolonged S phase, a defect associated with replication stressors such as impaired dNTP synthesis and defects in the DNA damage response pathway (Longhese et al., 2003). Interestingly this cell cycle defect is also seen in yeast lacking another chromatin assembly factor, Asf1p. *Δasf1* strains exhibit delays in both S phase and G2/M and are hypersensitive to DNA double-strand break-inducing agents, suggesting the chromatin assembly activity of

Asf1p is required during both replication and repair (Tyler et al., 1999b). Although no role in DNA repair has been defined for Nap1p, it was recently shown that Nap1p greatly enhances the disassembly of nucleosomes from chromatin by RSC in vitro (Lorch et al., 2006b). The RSC complex is specifically recruited to regions of double-strand breaks, where the coordinated remodeling activity of Nap1p and RSC makes chromatin more accessible to the repair machinery (Shim et al., 2007).

A recent study investigating the role of histone variant H2A.Z in cell cycle progression determined that *Δhtaz1* yeast exhibit a delayed progression through S phase. The authors propose that there is an introduction of DNA damage in the absence of H2A.Z that is activating the S phase checkpoint and thus prolonging passage through S (Dhillon et al., 2006). In our original screen of Nap1p binding partners, the histone variant H2A.Z was only detected in association with Nap1p during G2/M, implying that nuclear import by Nap1p may be downregulated during S phase. If reversible phosphorylation of Nap1p by CK2 is involved in the import of H2A.Z, then the cell cycle defect exhibited by the Nap1p phosphomutants may be a repair defect due to decreased nuclear levels of of H2A.Z.

There is a wealth of evidence implicating CK2 in the regulation of DNA synthesis and repair mechanisms. Analysis of gene expression in CK2 mutant strains demonstrated that many of the genes showing altered expression encoded chromatin remodeling and chromatin assembly factors, including Asf1p and Cac2p (Barz et al., 2003). CK2 phosphorylation of the FACT subunit, SSRP1, inhibited non-specific DNA binding activity by the FACT complex in vitro, and was upregulated following UV irradiation (Li et al., 2005). UV irradiation also induces the formation of a complex consisiting of FACT

and CK2 that increases specific phosphorylation and activation of p53 by this kinase (Keller et al., 2001). It has been proposed that Nap1p, Chd1p and FACT interact with CK2 within a complex, and phosphorylation of each protein component by CK2 is thought to regulate chromatin remodeling by these proteins (Krogan et al., 2002; Walfridsson et al., 2007).

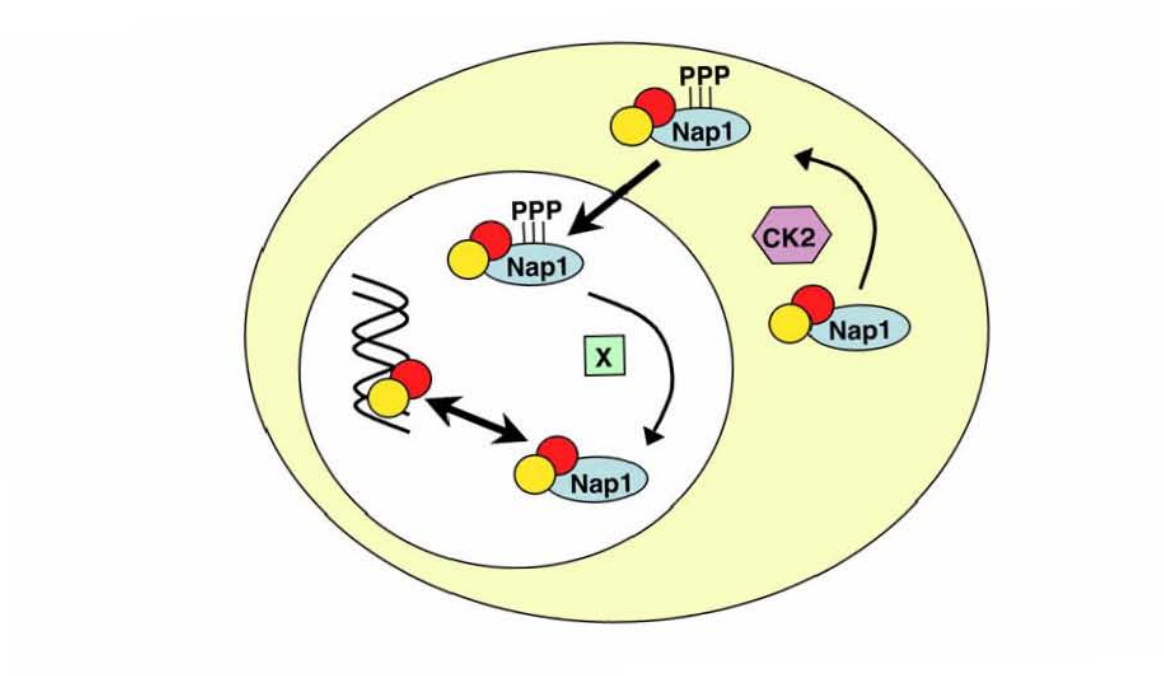
In this study we report that reversible phosphorylation of Nap1p by CK2 may be involved in cell cycle regulation, regulation of Nap1p nuclear import, and Nap1p-mediated chromatin assembly activity. The ability of nap1 3S-A to rescue normal bud morphology in a *Δnap1* strain implies Nap1p phosphorylation is not required for its role in bud formation. Mechanistically, we propose that phosphorylation of Nap1p occurs in the cytoplasm prior to import, though nuclear phosphorylation is also possible since kinase and substrate are abundant in both compartments. Once inside the nucleus, we propose that phosphorylation is removed to allow Nap1p to assemble chromatin, and future studies will identify the nuclear phosphatase involved in dephosphorylating Nap1p (Fig. 23). The Nap1p-Kap114p association has been shown to be insensitive to RanGTP, and a nuclear dephosphorylation event might also provide a mechanism for disassembling the import complex, and releasing histones prior to chromatin assembly. Phosphorylated Nap1p may be functioning in association with the RSC complex, promoting nucleosome disassembly, acting as a histone repository during repair or synthesis, and upon desphosphorylation, facilitating reassembly of nucleosomes (Lorch et al., 2006b). This suggests that cycles of phosphorylation and dephosphorylation may occur in the nucleus. In conclusion, our data show for the first time that Nap1p

phosphorylation by CK2 appears to regulate Nap1p localization and its chromatin assembly activity, and may be required for normal progression through S phase.

Figure 23

Model of Nap1p regulation by CK2.

P = phosphorylated serines, X = phosphatase, red and yellow ovals = histone H2A/H2B dimer.


PPP
Nap1
PPP
Nap1
CK2
Nap1
X
Nap1

Chapter 5: Discussion and Perspectives

The dynamic influence of chromatin structure on the regulation of gene expression was only fully acknowledged within the last ten years, and since then the study of the histone code has become a major focal point in molecular biology. The histone code hypothesis states that the post-translational modifications on histone molecules, in association with both discrete and widespread regions of the genome, actively direct the levels of gene expression and thus affect all cellular processes. To extend the metaphor, the process of packaging and unpackaging histones into chromatin is the process of encrypting and decoding the histone code, thus making sure the message is appropriately communicated, and this is the role of histone chaperones.

A. Comparison of Nucleoplasmin and NPM2

At the time that nucleoplasmin was discovered, it was generally believed that nucleosomes self-assembled, since this could be accomplished in vitro under conditions of high salt and step-wise dialysis. Using cell-free Xenopus oocyte extracts provided the same outcome under physiological conditions, and fractionation of these extracts identified nucleoplasmin as the factor required for this activity (Earnshaw et al., 1980). This was the first protein to be characterized as a histone chaperone. It was later determined that only nucleoplasmin isolated from mature oocytes had nucleosome assembly activity, whereas the same protein purified from immature, arrested oocytes did not, and phosphorylation was the distinguishing difference in activity (Sealy et al., 1986). It is now thought that in the quiescent oocyte, nucleoplasmin binds to the excess of maternal histones and acts as a ‘histone sink’. Upon resumption of meiosis,

phosphorylation reduces its affinity for histones relative to sperm protamines and this facilitates the exchange. The specific phosphorylation event that turns nucleoplasmin from a histone sink into an active assembly factor remains to be established.

In characterizing the mammalian ortholog of nucleoplasmin, I demonstrated that NPM2 is a substrate for CK2 phosphorylation. There are six CK2 consensus sites in NPM2; one is the N-terminal serine I identified as a phosphoserine by MS, and the other five are within amino acids 112-146, surrounded by the polyglutamic tract that is implicated in histone binding. Phosphorylation increases the pI of the protein, as evidenced by the molecular weight shift we observed during oocyte maturation. This would increase its affinity for protamines, which are more basic than histone molecules, and promote sperm chromatin decondensation through electrostatic interactions. CK2 activity is generally stimulated by the presence of basic polypeptides, and both protamines and histones have this effect in vitro (Ohtsuki et al., 1996). Nucleoplasmin is an exception to this, since phosphorylation of nucleoplasmin by CK2 is actually inhibited in the presence of histones (Vancurova et al., 1995). Another study found that CK2 activity was stimulated by poly-Arg peptides and arginine-rich protamines, but not by poly-Lys peptides and lysine-rich histones (Ohtsuki et al., 1996). This suggests that the modulation of CK2 activity by basic polypeptides is dependent on both the substrate and the composition of the polypeptide. It is not known whether histones inhibit the phosphorylation of NPM2 by CK2, but this would provide insight into the mechanism of histone assembly in the mammalian oocyte.

Nucleoplasmin is a member of the nucleophosmin/nucleoplasmin protein family, and family members have been identified throughout higher eukaryotes. NPM2 is the

mammalian ortholog of Xenopus nucleoplasmin, and at the time that I initiated my graduate work, had not been characterized. NPM1 is a highly expressed, nucleolar protein that has a wide tissue distribution and is often upregulated in cancers. NPM3 is also expressed in multiple tissues and concentrated in the nucleolus, and binds to NPM1. Both NPM1 and NPM3 function in ribosome biogenesis, whereas all three family members have histone chaperone activity. The three proteins share structural similarity; they have a highly conserved N-terminus that contains an acidic tract involved in histone binding. The C-terminal domain contains one or two acidic tracts and is more variable between the family members. All three have a classical bipartite NLS and nucleocytoplasmic shuttling activity.

NPM2 appears to be the closest relative of Xenopus nucleoplasmin, yet in mammalian oocytes it is not essential for sperm chromatin decondensation following fertilization. This implies that another histone chaperone, possibly NPM3, may be involved in this process in mammalian eggs. It has been noted that NPM2 does not have the N-terminal acidic tract, and this domain is required for sperm decondensation by Xenopus nucleoplasmin. NPM3 does contain this acidic tract, and antisense mRNA inhibition experiments show that NPM3 is required for sperm chromatin decondensation. This implies that the N-terminal acidic tract is directly involved in sperm decondensation and the exchange of protamines for histones, and mutational analyses of NPM2 and NPM3 would determine this.

Though sperm decondensation occurred normally in the oocytes of *Npm2*-null female mice, it was found that there was a loss of heterochromatin around the nucleolus and an increase in acetylated H3 in this region. There was a significant reduction in

fertility in *Npm2*-null female mice due to high rates of early embryonic lethality, and this may indicate a crucial role for NPM2 in histone deacetylation and silencing during maternal gene translation. In contrast to this, treatment of terminally differentiated somatic cells with *Xenopus* nucleoplasmin has been recently shown to decondense heterochromatin and increase histone acetylation and phosphorylation, and the promotion of chromatin decondensation requires the C-terminal polyglutamic tract (a feature NPM2 shares with nucleoplasmin and Nap1). This suggests that NPM2 remodeling activity, whether promoting deacetylation and silencing in the oocyte, or decondensation and activation at the onset of zygotic transcription, is site specific. We would therefore predict that phosphorylation of NPM2 at oocyte maturation would promote deacetylation and compaction. Zygotic transcription is initiated at the 2-cell stage, and this correlates with a loss of phosphorylation. Dephosphorylated NPM2 would therefore promote chromatin disassembly and transcriptional activation.

B. Coordination of Transcription and Mitosis by Nap1p

The study of Nap1p function has been divided between two camps, those researching mitotic signaling cascades and those investigating the mechanisms of chromatin assembly, and there is little overlap between the two. There are three possibilities for reconciliation, as discussed in the project rationale: 1) cell cycle defects in the absence of Nap1p are exclusively due to misregulation of gene expression, 2) Nap1p is a "shared gene", and there are two separate pools of Nap1p with two separate functions or, 3) there is cross-talk between the mitotic signaling cascade and transcriptional machinery, facilitated by Nap1p. The finding that Nap1p forms cell-cycle

specific associations with molecules from both functional categories would argue for one of the latter two explanations. Our minimal NES mutant, Nap-L99S, is unable to rescue normal bud formation in the Clb2p-dependent strain (data not shown). If this is merely due to decreased cytoplasmic levels of Nap1p, then this would argue for the 'two pools' model. However, it may be that Nap-L99S is shuttling deficient, and the inability to shuttle is causing the mitotic defect. This would support a requirement for cross-talk between the bud neck and the chromatin that is mediated by Nap1p. It would be of interest to construct an NLS-deficient Nap mutant incapable of nuclear import. A specific NLS has not been defined in Nap1p, and the deletion of its importin, Kap114p, does not prevent import of Nap1p. However, recent studies have used Caax-box prenylation motif fusions to tether proteins to the plasma membrane and prevent nuclear import, and this technique could be employed to make a mutant form of Nap1 that is constituitively cytoplasmic (Lu et al., 2003).

It was interesting to observe that some of the bud neck associated proteins interact with Nap1p throughout the cell cycle (e.g. Nba1p), whereas other interactions appear to be restricted to specific stages (e.g. Nis1p). This implies that these interactions occur in a specific order. Presumably the phosphorylation of Nap1p by Cdc28p/Clb2p is restricted to G2/M, following activation of this kinase, and I predict this will influence the order of assembly of these proteins at the bud neck. Mutation of the remaining Cdc28p/Clb2p target residues on Nap1p will allow us to determine which interactions are affected, and how this impacts localization of the different proteins to the bud neck. The Nim1-like kinases Gin4p, Kcc4p and Hsl1p have overlapping roles in regulating bud growth, and Kristin Keck in our lab is using mutational analysis of the phosphosites I identified on

these proteins to further characterize the mitotic signaling cascade. The assembly of the septins and associated proteins may provide a physical scaffold for downstream signaling events. It will be interesting to explore the structural requirements for this, and experimental disruption of this scaffold (e.g. using forchlorfenuron, a drug that specifically perturbs septin structures) would allow us to further examine this.

We determined that CK2 phosphorylation of Nap1p is involved in regulating progression through S phase, and this phenotype is generally associated with the S phase checkpoint and DNA repair pathways. The phosphomutants may be inhibiting normal chromatin assembly, leading to an accumulation of DNA damage and triggering a checkpoint response. In preliminary studies, I did not observe a difference in the sensitivity of the Nap1 CK2-phosphomutants to DNA-damaging agents (methylmethanesulfonate, camptothecin and UV), relative to wild type yeast, though this warrants further investigation (data not shown). A number of checkpoint-defective mutants exhibit the elongated bud phenotype associated with the mitotic signaling cascade. Rad53p is required for the S phase checkpoint in response to DNA damage, and mutations in *RAD53* interact genetically with Cla4p, another bud neck associated kinase (Enserink et al., 2006). There may be genetic interactions between the Nap1 CK2-phosphomutants and other proteins involved in DNA damage repair and the S phase checkpoint (such as Rad53p), and this might elucidate a new role for Nap1p in S phase progression. This would also provide further evidence that there is cross-talk between the regulation of bud formation and the DNA replication machinery.

C. Regulation of Nap1p Import by CK2 in vivo

A study of Nap1 family members in plants found that Nap1 orthologs bound to histones and the mitotic cyclin and had nucleocytoplasmic shuttling activity. Surprisingly, only one of the Nap1 orthologs in rice, Orysa;NAP1;3, was a substrate for phosphorylation by CK2. Orysa;NAP1;3 is the only family member in plants that, like Nap1p, lacks a defined NLS and exhibits both cytoplasmic and nuclear localization; all other plant Nap1 family members are nuclear. Phosphorylation of Nap1 proteins by CK2 may therefore be a conserved mechanism for regulating import in the absence of an NLS. This may also indicate that the cytoplasmic roles of Nap1p and Orysa:NAP1;3 are more crucial to normal cellular function in these organisms, and that phosphoregulation of nuclear import provides a more sophisticated level of control than that provided by NLS-dependent import.

It is conceivable that CK2-mediated import of Nap1p involves other intermediary factors that require the CK2 target phosphoserines for binding. An example of this type of regulation is the 14-3-3 proteins that bind specifically to phosphoserines and have a number of roles in cell-cycle progression (Hermeking and Benzinger, 2006). The phosphatase Cdc25 is phosphorylated by Chk1, both at S phase and in response to DNA damage, and 14-3-3 binds to phosphorylated Cdc25 and inhibits nuclear accumulation by upregulating export of Cdc25 (Peng et al., 1997; Sanchez et al., 1997). At the entry into mitosis this complex dissociates, Cdc25 is activated and accumulates in the nucleus, where it is required for the dephosphorylation and activation of Cdc2/Cyclin B2 (Lopez-Girona et al., 1999; Peng et al., 1997). The regulation of Cdc25 localization by 14-3-3 is similarly involved in oocyte maturation (Yang et al., 1999). Potentially, a phosphosite

specific Nap1p binding protein could be promoting the association of Nap1p with Kap114p and upregulating nuclear import of Nap1p by a similar mechanism. If there were a requirement for an intermediary protein, this would explain why we did not observe a CK2-dependent change in Nap1p binding to Kap114p in our in vitro binding assays with recombinant proteins.

CK2 is present in both the nucleus and the cytoplasm, but promotion of nuclear import of Nap1p by CK2 suggests that this signaling event occurs cytoplasmically. We hope to determine if there is compartmentalization of differently phosphorylated forms of Nap1p in vivo, and phosphospecific antibodies would allow us to detect this either by cellular fractionation or immunofluoresence. Our data indicate that phosphorylation does not prevent histone binding by Nap1p, but is inhibitory to chromatin assembly. This may be due to an altered affinity of phosphorylated Nap1p for histones that was not detectable in our binding assays. Taken together, these data suggest that nuclear dephosphorylation of Nap1p is a prerequisite for assembly. I also hope to identify the nuclear phosphatase involved in dephosphorylating Nap1p, but at this time no phosphatases have been identified in our screens for Nap1 binding partners. Two candidate phosphatases are PP1 and PP2A; both of these proteins are serine phosphatases that localize to the nucleus, and mutational analyses have implicated both proteins in the regulation of the G2/M transition (Hisamoto et al., 1994; Yang et al., 2000a). It is possible that a screen for genetic interactions with the Nap1p phosphomutants would identify prospective phosphatases, and this would significantly expand our understanding of how Nap1p function is regulated.

D. Structural significance of phosphorylation of Nap1p and NPM2

Nucleoplasmin forms a pentamer in vivo, and phosphorylation of nucleoplasmin during oocyte maturation releases the unstructured, acidic C-terminus and polyglutamic tract from the pentamer and results in an open configuration that favors chromatin remodeling (Frehlick et al., 2007). Interestingly, this region is not involved in histone binding, suggesting that chromatin remodeling activity is not coupled to histone binding. The structure of NPM2 has not been determined, though we predict that NPM2 will also be pentameric in vivo and that phosphorylation in the polyglutamic tract, where the five CK2 consensus sites are located, will promote chromatin remodeling.

Nap1p forms a homodimer in solution, and the dimer is found in association with histones, though how dimerization impacts chromatin remodeling has not been determined. One of our identified CK2 sites was on an unstructured region of the accessory domain; this region was proposed to act as a flexible clamp involved in dimerization of Nap1p. Another CK2 site we identified is within the dimerization region, though it faces into the center of the structure and is not at the interface. In a preliminary assay, both nap1 3S-A and nap1 3S-D were able to dimerize in vitro, suggesting that CK2 phosphorylation does not affect dimerization (data not shown).

Could phosphorylation of histone chaperones by CK2 be a conserved signaling mechanism? One of the CK2 phosphoresidues we identified in Nap1p is contained within the polyglutamic tract, the same region that we believe is phosphorylated by CK2 in NPM2. In our assays, we find that phosphorylation of Nap1p by CK2 inhibits chromatin assembly, whereas phosphorylation of nucleoplasmin promotes assembly. It also appears that phosphorylation of both Nap1p and NPM2 by CK2 promotes nuclear import, though

in both cases histone binding was unaffected. Phosphorylation of Nap1p and NPM2 has not been observed in the β-sheet that is highly conserved among most histone chaperones, but occurs within the more variable regions of these proteins. This implies that if phosphorylation of histone chaperones is a conserved mechanism, is not a simple 'on/off' switch, but something more complex, capable of responding to multiple inputs and modifying the signal it conveys accordingly. At the risk of stretching a metaphor beyond its limits, let us think of phosphorylation of histone chaperones as providing a decoder key to our histone chaperone cryptologists.

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