The histone chaperone Nap1 is a major regulator of histone H2A-H2B dynamics at the inducible *GAL* locus

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Running Head: Nap1 and histone dynamics in vivo

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Abstract:

Histone chaperones, like Nucleosome Assembly Protein 1 (Nap1), play a critical role in the maintenance of chromatin architecture. Here, we use the *GAL* locus in *Saccharomyces cerevisiae* to investigate the influence of Nap1 on chromatin structure and histone dynamics during distinct transcriptional states. When the *GAL* locus is not expressed, cells lacking Nap1 show an accumulation of histone H2A-H2B but not histone H3-H4 at this locus. Excess H2A-H2B interacts with the linker DNA between nucleosomes, and the interaction is independent of the inherent DNA-binding affinity of H2A-H2B to these particular sequences as measured in vitro. When the *GAL* locus is transcribed, excess H2A-H2B is reversed and levels of all chromatin-bound histones are depleted in cells lacking Nap1. We developed an *in vivo* system to measure histone exchange at the *GAL* locus, and observe considerable variability in the rate of exchange across the locus in wild type cells. We recapitulate this variability with *in vitro* nucleosome reconstitutions, which suggests a contribution of DNA sequence to histone dynamics. We also find that Nap1 is required for transcription-dependent H2A-H2B exchange. Altogether, these results indicate that Nap1 is essential for maintaining proper chromatin composition and modulating the exchange of H2A-H2B in vivo.

Introduction:

The basic unit of chromatin is the nucleosome, which forms when DNA is wrapped around two copies each of the four core histones arranged as two histone H2A-H2B dimers and a histone H3-H4 tetramer (1). Nucleosomes are highly dynamic, capable of multiple structural transitions between completely assembled, and entirely disassembled (2). Indeed, H2A-H2B and H3-H4 are actively exchanged during both DNA replication-dependent and -independent events (3-9). Chromatin transitions have the potential to

48 profoundly affect gene expression, and a diverse spectrum of factors including histone
49 chaperones, participate in this process (10).
50
51 Histone chaperones are histone-binding proteins that facilitate nucleosome assembly
52 and/or disassembly in an ATP-independent fashion (11-13). The histone chaperone
53 Nucleosome Assembly Protein 1 (Nap1) is a highly conserved chaperone that binds
54 H2A-H2B in vitro with nanomolar affinity (12, 14) in a conformation that shields
55 interfaces required for nucleosome assembly (15). Although functional in assembly of
56 nucleosomes in vitro (16), a number of studies support a role for Nap1 in transcription-
57 dependent processes of disassembly of nucleosomes. Nap1 is critical for the eviction of
58 histones during transcription in a mammalian in vitro system (17), and Nap1 (with the
59 ATP-dependent chromatin remodeler RSC) can facilitate elongation of RNAPII on
60 chromatin templates using yeast in vitro systems (18, 19). Our previous in vivo studies
61 indicated that Nap1 prevents excess H2A-H2B accumulation on chromatin (20), and
62 here we expand our analysis to investigate the role of Nap1 in histone exchange and
63 occupancy. As a model system, we use the *GAL* locus in yeast under transcriptionally
64 repressed and activated conditions.
65
66 The *S. cerevisiae GAL* gene cluster (*GAL7*, *GAL1*, and *GAL10*) is a powerful system for
67 studying chromatin dynamics in vivo under variable levels of gene expression.
68 Transcription of *GAL* genes is repressed in the presence of glucose and coordinately
69 activated in the presence of galactose (21, 22). The region is characterized by high
70 levels of nucleosome occupancy in the repressed state (23-25). Upon activation,
71 histones are acetylated (26-33), and a majority are evicted (24, 34-36) with the help of
72 chromatin remodeling factors (37) and histone chaperones (38).
73

74 Although chromatin structure and histone modifications are well characterized at the
75 *GAL* locus, probing histone dynamics has been confounded by the fact that typical
76 exchange studies utilize a tagged histone expression system regulated by a galactose-
77 inducible promoter (4-9). We therefore constructed a novel exchange system in which
78 expression of a HA-tagged histone (^{HA}H2B or H3^{HA}) is regulated by the antibiotic
79 doxycycline. This allowed us to examine histone exchange at these model genes without
80 altering the carbon source. We found that certain nucleosomes are highly dynamic and
81 require Nap1 for high levels of histone H2A-H2B exchange. Nap1 is also needed for
82 maintaining normal histone density and preventing excess H2A-H2B accumulation.
83

84 **Materials and Methods:**

85 **Yeast strains, plasmids and culture conditions:**

86 The following *S.cerevisiae* yeast strains were purchased from Thermo Scientific Open
87 Biosystems: the wild type strain (BY4741, *MAT*a *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0,* cat.
88 #YSC1048) and *nap1Δ* strain (BY4741, *nap1Δ::Kan^R*, cat. #YSC6273-201936599). The
89 strain containing TAP-tagged Htz1 was purchased from GE Dharmacon (*BY4741, Htz1-*
90 *TAP::HIS3MX6, cat* #YSC1178-202233238*)*. The *TAP-tagged Htz1* in the *nap1Δ* strain
91 was generated by homologous recombination of a PCR product derived from the *TAP-*
92 *tagged Htz1* in wild type strain. For replication-independent histone exchange studies,
93 alpha factor (Genscript, cat. #RP01002) was added to arrest cells in G1 stage. To
94 prevent alpha factor degradation by Bar1 (39), a *BAR1* gene deletion strain (*bar1Δ,*
95 Thermo Scientific, cat. #YSC6273-201920294) was used as the wild type, and *nap1Δ*
96 was generated in this background using established protocol (40).
97 Plasmids for doxycycline-regulated ^{HA}H2B and H3^{HA} expression were generated by sub-
98 cloning from the galactose-regulated plasmids pGAL1 HA-H2B and *pGAL$_1$* H3-HA (both
99 containing 3 contiguous HA sequences, or 3XHA), which were generously provided by

100 Michel Strubin (4). A Not1-BamH1 DNA fragment from pGAL1 HA-H2B encoding ^{HA}H2B
101 was cloned into the doxycycline-regulated pCM188 plasmid (ATCC, cat. #87660) using
102 standard techniques (41). The H3^{HA} gene was amplified from the pGAL1 H3-HA plasmid
103 via PCR using Pfu turbo. The primers used in the PCR were designed to incorporate a
104 BamH1 or Eag1 site near the ends of the PCR product. The resulting product, the H3^{HA}
105 encoding DNA, was cleaved with BamH1 and Eag1, and cloned into the doxycycline-
106 regulated pCM188 plasmid.
107 To analyze histone density and transcription levels of the *GAL* genes after continuous
108 transcriptional activation, wild type and *nap1*Δ strains were grown overnight in either YP
109 Glucose (2%) medium or YP Galactose (2%) medium, diluted to OD_{600} about 0.2, then
110 allowed to undergo 2 cell doublings. When cultures reached an OD_{600} of 0.8 - 1.0, the
111 cells were collected and subjected to ChIP, immunoblot or RNA abundance analysis. For
112 the histone exchange experiments, wild type and *nap1*Δ (both in *bar1*Δ background)
113 were cultured in YP Glucose or YP Galactose media continuously (>24 hours) to early
114 log phase (OD_{600} = 0.2). Alpha factor was then added to the medium to a final
115 concentration of 5 μM and after 90-120 minutes, cells become arrested in G1 phase.
116 Shmooing was confirmed visually using a microscope. Doxycycline was added to a final
117 concentration of 1 μg/ml in YP Glucose or 3 μg/ml in YP Galactose to maintain a
118 consistent histone degradation rate. Following doxycycline treatment, cells were
119 collected every hour for five hours and subjected to chromatin immunoprecipitation.
120
121 **Chromatin immunoprecipitation:**
122 Chromatin immunoprecipitation (42) assays were performed as previously described
123 (20). The cells were cross-linked with 1% formaldehyde and lysed by bead beating. The
124 cross-liked chromatin was then sheared to small fragments of approximately 200 bp with
125 sonication. Antibodies and sepharose beads were added to immunoprecipitate the

126 cross-linked proteins of interest. Cross-linking of the immunoprecipitated material was
127 reversed by overnight incubation at 65˚C, and then samples were treated with
128 proteinase K and RNaseA, followed by DNA extraction using the phenol:chloroform
129 method. The isolated DNA was analyzed by quantitative real-time PCR (qPCR). The
130 commercially available antibodies used in this study were anti-H2B (Active Motif, cat.
131 #39237), Anti-H3 (Abcam, cat. #1791) and anti-HA (Santa Cruz, cat. #sc-7392). ChIP
132 assays were performed in biological triplicate. Unnormalized occupancy was directly
133 calculated from the ΔCq between the immunoprecipitated DNA and the input control
134 DNA. For the exchange assays, the ratio of the unnormalized HA-tagged histone
135 occupancy over the respective unnormalized total (endogenous plus HA-tagged) histone
136 occupancy at each region for each time point of doxycycline treatment was calculated.
137 The average ratio from three biological replicates at the 0 hour time point (T_0) was set as
138 100% histone persistence and the average ratios at the remaining time points were
139 calculated as the percent of histone persistence relative to T_0. The histone persistence
140 percentages are shown as mean ± standard deviation in supplementary Figure S7 for
141 comparison between wt and *nap1*Δ. Heat maps (Figure 5) were generated by assigning
142 colors to the blocks representing each region during the time course according to the
143 histone persistence percentages. For all other ChIP assays, the total occupancy of each
144 protein at the *GAL* region was normalized to its respective occupancy at a telomere
145 proximal position (Chromosome VI 269571-269488), which served as the internal control.
146

147 **RNA abundance**

148 S1 nuclease protection assays to quantify the expression levels of ^{HA}*H2B*, *GAL7*, *GAL10*,
149 *GAL1*, and *tRNA*^{w} were performed as previously described (43). RNA was extracted
150 with the traditional hot phenol method. For each reaction, ^{32}P-labeled S1 probes were
151 incubated with 40μg of RNA at 55°C overnight. The hybridized samples were digested

152 by S1 nuclease at 37°C for 30 minutes, seperated by 8% acrylamide sequencing gels.
153 The gels exposed to phosphor screens, visualized by Typhoon PhosphorImager and
154 analyzed using ImageQuant software.
155
156 **Immunoblotting of total protein level**
157 Cells were harvested at log phase, suspended in lysis buffer (120 mM Tris-HCl pH 6.8,
158 12% (v/v) glycerol, 3.4% (w/v) SDS, 200 mM DTT, 0.004% (w/v) bromophenol blue) and
159 incubated at 95°C and on ice for 5 min each. Insoluble cell debris was removed by
160 centrifugation and total protein separated on a 15% SDS-PAGE. The same antibodies
161 against HA, H2B, and H3 that were used for ChIP analysis were used for protein
162 detection. In the total protein level quantitation during histone exchange, the protein level
163 of each strain at T_0 was set as 100; the protein levels of the remaining time points were
164 calculated as the percentage relative to T_0.
165
166 **In vitro H2A-H2B dimer binding competition assay**
167 FRET-based competition assays were conducted in a 396-well format using Alexa 488-
168 labeled *Xenopus* histone H2A-H2B (T118C) as described (44). The final reaction buffer
169 was 20 mM Tris-HCl pH 7.5, 300 mM NaCl, 5% (v/v) glycerol, 0.01% (v/v) NP40, 0.01%
170 (v/v) CHAPS, 1 mM DTT, 1 mM EDTA pH 8.0 and the reaction volume was 40 µL. Each
171 PCR-prepared fragment was titrated against 10 nM histone H2A-H2B in the presence 50
172 nM 59 bp DNA. Each reaction was performed in duplicate and control titrations
173 containing only one labelled component were used to correct the raw FRET signal to
174 produce $FRET_{corrected}$. Each experiment contained an internal control titration of
175 recombinant 147 bp Widom 601 DNA (data not shown). Reactions were pipetted into a
176 microplate and scanned using a Typhoon 8600 variable mode fluorimager (GE). Three
177 scans were performed using excitation/emission wavelengths of 488/520 nm, 633/670

178 nm or 488/670 nm. Fluorescence was quantified using ImageQuant software. *GAL*
179 fragment titration produced a sigmoidal curve from which an IC50 was determined using
180 GraphPad Prism software. Data were fit with a Y_{min} of 0 and normalized. At least two
181 independent titrations were performed for each fragment and all IC50 values used in the
182 mean calculation were derived from data with an $R^2 > 0.98$.
183
184 ***In vitro* micrococcal nuclease analysis of chromatin**
185 *In vitro* micrococcal nuclease (MNase) digestion assays were performed according to
186 the established protocol (45). Canonical mononucleosomes and trinucleosomes were
187 reconstituted by salt dilution using purified recombinant *Xenopus laevis* octamer on a
188 207 bp DNA fragment containing the 147 bp Widom 601 nucleosome positioning
189 sequence with 30 bp flanking linker DNA (46). Nucleosomal DNA or free DNA (5 µg;
190 0.17 µM) was used for each MNase digestion. MNase digestion was performed in the
191 absence and presence of excess recombinant *Xenopus laevis* histone H2A/H2B dimer.
192 A 3-fold molar excess of histone H2A-H2B dimer was added to a final concentration of
193 0.51 µM, incubated for 5 minutes, followed by MNase digestion at room temperature for
194 the indicated time. Reactions were quenched with EDTA to a final concentration of 12.5
195 mM. The DNA was purified by adding SDS (0.5%) and proteinase K (0.2 mg/ml),
196 incubated at 50°C for 30 minutes, phenol-chloroform extracted and ethanol precipitated.
197 The samples were separated on a 6% native polyacrylamide gel and stained with SYBR
198 Gold.
199
200 **Micrococcal nuclease analysis of chromatin structure in vivo.** Detailed protocols
201 can be found in the supplement. Nuclei were isolated from wild type (BY4741) and
202 *nap1Δ* cells essentially as described (47). The indirect end labeling analysis was
203 completed as described (24) and the primer extension analysis as described (48). The

204 membranes and gels were exposed to phosphor storage screens, visualized on a
205 Typhoon PhosphorImager and quantitated using ImageQuant TL software.
206
207 **Reconstitution studies on *GAL* fragments**
208 Nucleosome reconstitution was performed with salt dilution method (49). *Xenopus laevis*
209 histone octamers were titrated against each PCR-prepared DNA fragment. Reactions
210 were separated on 5% native gels, stained with ethidium bromide and visualized by UV.
211
212 **Results:**
213 **Nap1 prevents excess histone H2A-H2B accumulation on linker DNA**
214 We previously showed that a knockout of the histone chaperone *NAP1* in *S. cerevisiae*
215 results in an atypical chromatin structure with excess H2A-H2B at most positions across
216 the *GAL* locus (20). We next asked whether this was a general feature of Nap-family
217 proteins, by assaying a strain deleted for Vps75, a histone chaperone with similar
218 structure to Nap1 (50). We utilized chromatin immunoprecipitation (ChIP) assays to
219 visualize H2A-H2B (via H2B) and H3-H4 (via H3) occupancy at six different regions at
220 the *GAL* locus. The relative positions of the sites were selected based on extensive
221 studies of nucleosome positioning, histone density, and histone variant H2A.Z
222 occupancy (51-55), and includes three promoter (PRO) and three open reading frame
223 (ORF) sites (Supplemental Figure S1). The *GAL10* and *GAL1* genes are divergently
224 transcribed and share one upstream activating sequence (UAS) (23, 56, 57). As shown
225 previously (20), loss of Nap1 (*nap1*Δ) causes a general increase in H2A-H2B compared
226 to the wild type strain at all but one site (Figure 1A). At these sites, the occupancy of H3-
227 H4 remains at or slightly below wild type levels (Figure 1B). In contrast, deletion of
228 *VPS75* (*vps75Δ*) does not significantly change H2A-H2B or H3-H4 occupancy across the
229 *GAL* locus. We also deleted *ASF1* (*asf1Δ*), a histone chaperone structurally distinct from

230 Nap1 and Vps75 (58) and detected no significant changes in H2A-H2B or H3-H4
231 occupancy. Thus, excess H2A-H2B accumulation is not a shared feature of strains
232 deleted for Nap-family proteins or other histone chaperones, but is unique to the *nap1*Δ
233 strain. Together, these data indicate that preventing this atypical chromatin is a unique
234 function of Nap1.
235
236 We next characterized chromatin with H2A-H2B enrichment biochemically. To mimic the
237 atypical chromatin observed in vivo in the *nap1Δ* strain, mono-nucleosomes were
238 incubated in the presence (and absence) of excess H2A-H2B and the Micrococcal
239 Nuclease (MNase) digestion patterns were compared (Figure 2A). We used a 207 bp
240 DNA fragment carrying the well-characterized 147 bp Widom 601 nucleosome
241 positioning sequence (59) and 30 bp of flanking linker DNA. As expected, free DNA was
242 rapidly digested by MNase. Incubation of DNA with H2A-H2B resulted in modest
243 protection from MNase. A clearly defined protected region was not observed, consistent
244 with non-specific binding of H2A-H2B to DNA. MNase digestion of mono-nucleosomes
245 revealed a well-defined 140-180 bp region (depending on digestion time) of protection,
246 indicating that the linker DNA is digested more rapidly than nucleosomal DNA (45).
247 Mono-nucleosomes with excess H2A-H2B displayed significantly increased resistance to
248 MNase digestion, and broader DNA protection surrounding the nucleosome site. This
249 suggests that excess H2A-H2B binds to the flanking linker DNA.
250
251 We also performed MNase digestion assays on two other nucleosome templates. First, a
252 621 bp DNA fragment capable of forming tri-nucleosomes with three consecutive 207 bp
253 Widom 601 nucleosome-positioning sequences with linker DNA (Supplemental Figure
254 2A); and second, a 588 bp native promoter DNA fragment without detectable positioning
255 sequences (60) (Supplemental Figure 2B). Similar results are observed for all DNA

256 fragments: the addition of excess H2A-H2B to reconstituted nucleosomes significantly
257 increased resistance to MNase digestion. These results indicate that excess H2A-H2B
258 binds to linker DNA in the context of both mono- and multi-nucleosome templates.
259
260 We next assayed how excess H2A-H2B affects chromatin architecture in vivo by
261 measuring MNase digestion patterns of chromatin from wild type and *nap1Δ* cells.
262 Cleavage patterns within the *GAL* locus were assessed by high resolution (single base
263 pair) reiterative primer extension analysis. The *GAL1* and *GAL10* promoters were
264 analyzed, since by ChIP the *GAL1* promoter had normal and the *GAL10* promoter had
265 excess levels of H2A-H2B in the *nap1Δ* strain (Figure 1). As expected, at the *GAL1*
266 promoter there were no differences observed in the MNase digestion pattern of
267 chromatin from wild type or *nap1Δ* cells (Figure 2B and Supplemental Figure 3A). The
268 *GAL10* promoter region is hypersensitive to MNase cleavage and this is reflected in the
269 amount of product that terminates within the hypersensitive region (Figure 2B and
270 Supplemental Figure 3B). Although subtle, MNase cleavage of the chromatin from the
271 *nap1Δ* cells is reproducibly reduced in the region of *GAL10* that exhibits excess H2A-
272 H2B by ChIP when compared to the chromatin from wild type cells. The minor reduction
273 in MNase cleavage is not unexpected. Nucleosomes in the *GAL* locus evaluated at high
274 resolution in fact occupy multiple positions (36). Thus, the excess dimer is most likely
275 binding at multiple positions, leading to the subtle difference seen in the primer
276 extension products. Taken together, the in vitro and in vivo MNase results suggest that
277 the excess H2A-H2B is binding to the linker DNA.
278
279 **Occupancy of the histone variant H2A.Z is unchanged in the *nap1Δ* strain**
280 Histone H2A.Z (Htz1 in yeast) is a variant of histone H2A that substitutes for the
281 canonical major H2A in a wide, but nonrandom, genomic distribution (61). H2A.Z is

282 involved in transcription regulation, gene activation and silencing, DNA repair and
283 chromosomal stability (62). Since Nap1 also interacts with H2A.Z (63), we investigated
284 whether occupancy of H2A.Z is altered in the absence of Nap1. Chromatin-tandem
285 affinity purification (TAP) was performed using TAP-tagged Htz1 (Htz1-TAP) in wild type
286 or *nap1Δ* strains with analyses of the *GAL* locus genes. In wild type cells, significant
287 occupancy of Htz1 is detected across the region and this occupancy is unaffected by the
288 loss of Nap1 (Supplemental Figure 4). These results indicate that Nap1 is not required
289 for normal levels of Htz1 occupancy at these specific, nor does Htz1 accumulate in the
290 same manner as major H2A in this region in the absence of Nap1.
291

292 **Histone H2A-H2B accumulation in the *nap1Δ* strain is independent of DNA**
293 **sequence**

294 The varying degrees of H2A-H2B accumulation across the *GAL* locus in *nap1*Δ cells
295 (Figure 1), prompted us to test the role of DNA sequence. Some studies suggest that
296 DNA sequence is a major factor in nucleosome positioning (64, 65), while others argue it
297 may only be a partial factor (66-69). To test the role of sequence in vitro, we used a
298 Förster resonance energy transfer (Fret) competition assay (44) to compare H2A-H2B
299 affinity for the six different *GAL* promoter (PRO) and open reading frame (ORF)
300 sequences, all of approximately 150 bp. We competed FRET of a complex between
301 H2A-H2B and a 59 bp DNA fragment containing the H2A-H2B-binding region of the well-
302 characterized Widom 601 nucleosome positioning sequence (59). We titrated each of
303 the *GAL* locus DNAs and measured the half-maximal inhibitory concentration (IC50)
304 values as the FRET signal decreases to zero (Figure 3A). A lower IC50 reflects a lower
305 $K_{d,}$ or a tighter interaction between the *GAL* locus DNA and H2A-H2B. All *GAL* locus
306 DNAs were able to fully compete with the 59 bp DNA for H2A-H2B, indicating efficient
307 H2A-H2B-*GAL* DNA interactions. Slight differences in IC50s were observed among the
308 *GAL* DNAs (10-30 nM), which do not correlate with H2A-H2B accumulation in *nap1*Δ
309 cells. For example, the lowest IC50 was measured with the $GAL10^{PRO}$ and $GAL1^{PRO}$

310 DNAs, which in fact possess the highest and lowest levels respectively of H2A-H2B
311 accumulation in vivo. We did notice, however, that the in vitro binding of H2A-H2B
312 correlates with the DNA GC content (Figure 3B). DNA with a lower CG content is more
313 flexible (70), consistent with lower K_d for H2A-H2B binding. Overall, these data indicate
314 that the DNA sequences themselves do not account for the varied H2A-H2B
315 accumulation across the *GAL locus* in the absence of Nap1 in vivo. This implies the
316 involvement of other extrinsic factors.
317

318 **Transcription removes accumulated histone H2A-H2B in the *nap1Δ* strain**

319 The aforementioned accumulation of H2A-H2B in the *nap1Δ* strain is observed under
320 repressed conditions for *GAL* gene expression (glucose medium). We next asked if this
321 atypical H2A-H2B distribution remained when the *GAL* genes are actively transcribed.
322 Histone occupancy was assayed using ChIP after growth under inducing conditions
323 (galactose medium) for 24 hours or approximately 10 generations. The ratio of H2A-H2B
324 occupancy (H2B) to H3-H4 occupancy (H3) at each location revealed no significant
325 difference between the wild type and *nap1*Δ strains, nor between different *GAL* loci
326 (Figure 4A and B, Supplemental Figure 5A). This indicates that excess H2A-H2B does
327 not stably accumulate during active transcription. It is important to note, however, that in
328 the *nap1Δ* strain, occupancy of both H2B and H3 is lower than wild type at every site
329 tested except $GAL10^{PRO}$, where occupancy is similar to wild type. Analysis of extracts
330 confirmed that this decrease in histone occupancy is not due to lower overall levels of
331 histones in *nap1Δ* strain (Supplemental Figure 5B). Lower nucleosome occupancy in
332 galactose in cells lacking Nap1 thus reflects a change in histone deposition, supporting a
333 nucleosome assembly role for Nap1. Since the transcribing *GAL* locus in the *nap1*Δ
334 strain has lower histone occupancy, we tested whether this impacted gene expression

335 using S1 nuclease protection assays (Figure 4C). Higher expression levels are observed
336 from the *GAL* genes in the *nap1*Δ cells relative to the wild type strain. Thus, the reduced
337 histone density at the *GAL* locus observed in the absence of Nap1 is linked to increased
338 transcription levels, implicating Nap1 in repression of gene expression via maintenance
339 of histone occupancy, potentially through nucleosome assembly in vivo.
340

341 **Establishment of an in vivo doxycycline-regulated histone exchange system**

342 To further investigate the role of Nap1, we developed a method to measure histone
343 exchange at the *GAL* locus. We created an in vivo system in which expression of
344 epitope-tagged histones is controlled using doxycycline (Figure 5A). Previously
345 published histone exchange studies utilized a system in which tagged histone
346 expression is *GAL* promoter–regulated and therefore induced by the presence of
347 galactose (4-9). Since we wanted to evaluate histone exchange specifically at the *GAL*
348 locus, we needed a different means to control histone expression. We sub-cloned HA-
349 tagged versions of histone H2B (^{HA}H2B) and H3 (H3^{HA}) (4) into a doxycycline-controlled
350 expression vector. In this system, expression of the HA-tagged histones is repressed
351 when doxycycline is present. These plasmids were introduced into both wild type and
352 *nap1*Δ strains, in a *bar1Δ* background. The *bar1Δ* background increases alpha-factor
353 sensitivity, which arrests cells in G1 (39), such that subsequent studies are replication-
354 independent. As expected, addition of doxycycline results in transcriptional repression of
355 the HA-tagged histone and does not affect *GAL* gene expression in either wild type or
356 *nap1*Δ cells (Supplemental Figure 6). After addition of doxycycline, the level of total H2B
357 protein (endogenous plus ^{HA}H2B, detected by anti-H2B antibody) is not affected,
358 whereas the level of ^{HA}H2B protein (detected by anti-HA antibody) gradually diminishes
359 (Supplemental Figure 7A and B). After 5 h of repression by doxycycline, the ^{HA}H2B level

360 drops to less than 20% of the pre-doxycycline levels, with no impact on endogenous
361 histone levels.
362
363 We next utilized ChIP assays to measure occupancy of ^{HA}H2B and total H2B at a
364 telomere-proximal location as a standard in the assay. H2B exchange is observed at this
365 location (Supplemental Figure 7C and D). During the 5-hours following doxycycline
366 addition, the decrease in ^{HA}H2B protein levels correlates directly with the decrease in
367 ^{HA}H2B occupancy, whereas the occupancy of total H2B is not affected by the
368 doxycycline treatment. Histone-exchange data at the telomere-proximal control region
369 with doxycycline-regulated H3^{HA} is similar to that obtained with ^{HA}H2B (data not shown),
370 and it is important to note that neither H2B (tagged or untagged) nor H3 (tagged or
371 untagged) levels are impacted by the deletion of Nap1 (Supplemental Figure 8).
372
373 **Histone exchange rates vary across the *GAL* locus**
374 We utilized the doxycycline-regulated histone-exchange system to determine exchange
375 rates at the *GAL* locus. We tested both wild type and *nap1*Δ strains, and compared them
376 under repressed and transcriptionally active conditions (Figure 5B). To visualize and
377 compare the complex histone exchange patterns at the different *GAL* loci, the
378 percentage of HA-tagged histone that persists at each site is shown as a heat map.
379 Under transcriptionally repressed conditions, exchange of both H2B and H3 is observed
380 in the wild type strain at all promoter and open reading frame sites tested (Figure 6A and
381 B). However, the exchange kinetics differ, depending on the site. Histone H2B exchange
382 is the least dynamic at the *GAL1*^{PRO} and *GAL1*^{ORF}, where it is similar to the exchange
383 rate at the telomere-proximal region. The most dynamic exchange is observed at the
384 *GAL10*^{PRO}, where persistence of the tagged derivative is down to 50% within an hour. In
385 general, H2B exchange is significantly more dynamic than H3 across the *GAL* locus, a

386 trend that has also been shown in a previous study (4). The one exception to this trend
387 is the *GAL10*^{PRO}, where exchange of H3 is as dynamic as that of H2B. The rapid
388 exchange of both H2A-H2B and H3-H4 at the *GAL10*^{PRO} indicates a highly dynamic
389 chromatin structure at this location, even when transcription is off. Under transcriptionally
390 repressed conditions, there was very little impact of the loss of Nap1 on histone H2B or
391 H3 exchange at the promoter and open reading frame of all *GAL* sites tested, except for
392 a decrease in exchange of H2B at the otherwise highly dynamic *GAL10*^{PRO}. The
393 absence of Nap1 reduces H2B exchange, but does not affect H3 exchange. This
394 indicates that the "old" or tagged version of H2B is being reassembled into chromatin in
395 the absence of Nap1. This suggests that when present, Nap1 facilitates incorporation of
396 "new" histones and not reassembly of the "old" histones, at least at this highly dynamic
397 position. It is important to note, that these studies were performed in cell-cycle arrested
398 cells, so this Nap1 function is independent of histone deposition during replication.
399
400 When *GAL* gene transcription is activated, the exchange kinetics in the wild type strain
401 of both H2B and H3 are highly dynamic across the locus (Figure 5, and 6C and D), to a
402 level comparable to the *GAL10*^{PRO} in the absence of transcription. This rapid
403 transcription–mediated histone exchange is consistent with previous observations that
404 nucleosomes are highly dynamic at the *GAL* locus upon activation of transcription (20,
405 25, 30, 31, 71). In the *nap1*Δ strain, H2B exchange kinetics are less dynamic across the
406 region, whereas histone H3 exchange is similar to wild type (Figure 6C and D).
407 Therefore, when transcription is active and chromatin is highly dynamic in the wild type
408 strain, H2A-H2B exchange is consistently slower in the absence of Nap1.
409
410 **In vitro nucleosome reconstitution recapitulates some of the in vivo dynamics of**
411 **the *GAL* locus**

412 The various regions of the *GAL* locus exhibit distinctive exchange profiles in the wild
413 type strain (Figure 5B). Since DNA sequence can directly affect nucleosome stability and
414 contribute to in vivo nucleosome dynamics (64, 65), we tested the fragments for their
415 intrinsic ability to form nucleosomes. Using the 601 positioning sequence (59) as a
416 control, the *GAL* fragments were analyzed for the formation of stable, positioned
417 nucleosomes by salt reconstitution with native histones (49)(Supplemental Figure 9).
418 This allowed us to group the *GAL* fragments into three classes, represented by 601 (601
419 and $GAL7^{ORF}$), *GAL10* promoter ($GAL10^{PRO}$, $GAL10^{ORF}$, and $GAL7^{PRO}$), and the *GAL1*
420 promoter ($GAL1^{PRO}$ and $GAL1^{ORF}$). In some cases, multiple forms of DNA-histone
421 complexes are evident. To identify the histone composition of the complexes,
422 nucleosome reconstitution was performed with fluorescently labeled histones
423 (Supplemental Figure 10). In addition, heat-shifting and lower salt experiments were
424 used to determine if complexes were well-positioned (Supplemental Figure 11). The
425 results indicate that the 601 class forms hexasome and positioned nucleosome at low
426 concentrations of octamer, with an increase in nucleosome as octamer is titrated. The
427 *GAL1* promoter class formed stable, positioned nucleosomes more efficiently than 601,
428 in that only a single band was observed even at low octamer levels. Both of the
429 fragments in this class (*GAL1* promoter and *GAL1* ORF) had the least dynamic
430 nucleosomes in vivo in the exchange assay for both H2B and H3, suggesting that
431 sequence plays an important role in these properties. The *GAL10* promoter class formed
432 intermediate complexes and different forms of nucleosomes at every ratio in vitro,
433 suggesting that nucleosomes are not stable and/or that there are multiple nucleosome
434 positions. The *GAL10* promoter region was the highly dynamic site in the in vivo
435 exchange assay (Figure 5), but the other two fragments in this unstable in vitro class
436 (*GAL10* ORF and *GAL7* promoter), did not share this behavior in vivo.
437

438 **Discussion:**

439 We previously found that Nap1 prevents the formation of atypical chromatin
440 characterized by excess H2A-H2B, across the *GAL* locus under transcriptionally
441 repressed conditions (20). Here we show that H2A-H2B accumulation does not occur in
442 a strain deleted for the Nap1 family member Vps75, nor the H3-H4 histone chaperone
443 Asf1. In addition, this effect is specific for the major histone variants, as H2A.Z does not
444 accumulate in these same regions. Although H2A-H2B accumulation varies at different
445 locations in the absence of Nap1, we found this is not likely due to variable H2A-H2B-
446 DNA binding, as in vitro affinities did not correlate with in vivo accumulation. The excess
447 H2A-H2B appears to bind between nucleosomes in the linker regions as evidenced by in
448 vitro and in vivo MNase protection assays. Notably, the process of transcription results in
449 loss of the excess H2A-H2B, and in fact, the absence of Nap1 leads to lower overall
450 histone density on chromatin, indicative of critical nucleosome assembly functions of
451 Nap1.

452

453 Nucleosomal histones are readily exchanged into and out of chromatin (4, 5). This
454 histone exchange is a combination of partial nucleosome disassembly and reassembly,
455 and histone chaperones are directly involved in this process (3, 7, 8, 72-74). However,
456 the requirement for histone chaperones in histone exchange within nucleosomes has
457 primarily focused on H3-H4 histone chaperones (7, 9, 75). Also, most histone exchange
458 studies utilize a galactose-inducible promoter for regulation of tagged histone expression
459 (4-9), therefore, *GAL* locus gene transcription is affected when expression of the tagged
460 histones is turned on or off. In this study, we designed a doxycycline-regulated histone-
461 exchange system that does not affect *GAL* gene transcription. Histone exchange is
462 generally observed across the *GAL* locus under repressed conditions, with exchange of
463 both H2B and H3 being highly dynamic at the *GAL10* promoter ($GAL10^{PRO}$). Furthermore,

464 the highly dynamic H2B exchange is Nap1-dependent whereas H3 exchange is not. It is
465 intriguing that even without active transcription, the nucleosome at the *GAL10* promoter
466 is highly dynamic. One unique feature of the chromatin at this site is a lower H2B
467 occupancy in general; the *GAL10* promoter has only half the H2B occupancy of other
468 *GAL* sites (20). Moreover, in the absence of Nap1, this decrease in H2B occupancy is
469 reversed (20) and the H2A-H2B exchange rate is severely reduced. Importantly, the
470 *GAL10* promoter region has the binding site for the Gal4-Gal80 activator complex and
471 the RSC chromatin-remodeling complex in the transcriptionally repressed condition (55).
472 In vitro studies have shown that RSC promotes histone transfer (76). In the presence of
473 Nap1, RSC releases one H2A-H2B from the nucleosome to form a hexasome and the
474 dimer is transferred to Nap1 (19). This combination of highly dynamic Nap1-dependent
475 H2A-H2B exchange, a 2-fold reduction in H2B occupancy, and the presence of a RSC
476 binding site supports a model whereby hexasome formation is likely at the *GAL10*
477 promoter *in vivo*.
478
479 Taken together, the findings presented here advance our understanding of the diverse
480 roles of the histone chaperone Nap1 in chromatin regulation in vivo (Supplemental
481 Figure 12): i) removal of atypical histone H2A-H2B under transcriptionally repressed
482 conditions; ii) maintenance of proper histone density on chromatin during activated
483 transcription (i.e. transcription-coupled assembly); and iii) facilitation of histone H2A-H2B
484 exchange within highly dynamic chromatin regions whether the locus is repressed or
485 activated. Active exchange of H2A-H2B could lead to destabilization of the H3-H4
486 tetramer, thereby Nap1 could also contribute to nucleosome disassembly. This is
487 consistent with the finding that deletion of *NAP1* reverses the cryptic transcript
488 phenotype (interpreted as the by-product of under-assembled chromatin) observed in
489 mutant strains defective for other chromatin regulators (77), and that Nap1 plays an

important function in the eviction of histones during transcription in a mammalian vitro system (17). Importantly, these varied functions of Nap1 are not redundant with other histone chaperones in vivo. Since Nap1 has high affinity for H3-H4 tetramers, as well as H2A-H2B dimers (14), Nap1 has the potential to function independently in these various roles. However, a large body of work indicates collaborative functional activities between Nap1 and other chaperones as well as ATP-dependent chromatin remodelers and histone acetyltransferases (13, 17-19, 60, 77-79). How the combinatorial action of these factors contributes to histone occupancy and dynamics in vivo remains to be elucidated.

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Figure legends:

Figure 1. Excess H2A-H2B accumulation is observed with loss of Nap1, but not Vps75 or Asf1. A) Occupancy of Histone H2B or B) Histone H3 at the *GAL* locus by ChIP assay under transcriptionally repressed conditions (glucose) in the wild type (wt), *nap1*Δ, *vps75*Δ, or *asf1*Δ strains. Data is represented as the mean from 3 biological

516 replicates ± standard deviation, normalized to the levels of occupancy at a telomere
517 proximal location.
518
519 **Figure 2. H2A-H2B accumulation protects DNA from MNase digestion.** A) In vitro
520 analysis of chromatin with and without excess H2A-H2B. PAGE analysis of MNase
521 digestion of naked DNA alone (first panel), DNA with H2A-H2B (second panel), mono-
522 nucleosomes (third panel), and mono-nucleosomes with H2A-H2B (fourth panel),
523 assessed on a 207 bp DNA fragment. B) Chromatin from wild type and *nap1Δ* cells was
524 digested with MNase in vivo and the cleavage products analyzed by primer extension.
525 Quantitation of the primer extension products from the *GAL1* promoter (upper panel) and
526 the *GAL10* promoter (lower panel) is shown in black for *nap1Δ* cells and grey for wild
527 type cells. The *GAL10* promoter, which has excess H2A-H2B by ChIP assay, is more
528 protected from MNase cleavage in *nap1Δ* cells.
529
530 **Figure 3. All six *GAL* DNA fragments bind histone H2A-H2B with comparable**
531 **affinities.** H2A-H2B-DNA binding affinities were measured with a FRET-based
532 competition assay. DNA fragments (~150 bp) from the six promoter (PRO) and open
533 reading frame (ORF) sites of *GAL7*, *GAL10*, and *GAL1* were titrated in the presence of
534 50 nM 59 bp dimer binding region of the 601 sequence and analyzed for binding to
535 Alexa 488-labeled histone H2A-H2B. A) Representative competition curves. Each data
536 point reflects the mean of duplicate measurements ± one SEM. The errors bars are
537 small and in many cases not visible. All curves used for statistics had an $R^2 \geq 0.98$. B)
538 The mean IC50 values for at least 2 replicates and the % GC content for the DNA
539 sequences. Error bars reflect one SEM.
540

541 **Figure 4. H2A-H2B accumulation is not maintained in *nap1Δ* at the *GAL* locus in**
542 **the induced condition.** The occupancy of H2B (A) or H3 (B) in the wild type (wt) strain
543 (white bars) and the *nap1*Δ strain (black bars) under active transcription were
544 determined by ChIP assays. C) Transcript levels of *GAL* genes in the wt and *nap1*Δ
545 strains were detected by S1 nuclease protection assay with ^{32}P-labeled probes specific
546 to *GAL7*, *GAL10*, *GAL1*, and $tRNA^{W}$. The transcript levels of $tRNA^{W}$ were used as an
547 internal control for normalization. Bars reflect the means from 3 biological replicates ±
548 standard deviation.
549
550 **Figure 5. Histone exchange rates at the *GAL* locus are distinct and Nap1 is**
551 **involved in H2A-H2B exchange.** A) Schematic of histone exchange assay with
552 representative western blot showing decreasing HA-tagged H2B level and unchanged
553 endogenous H2B level. B) Heat maps showing tagged histone persistence over the 5 h
554 time course. Persistence is defined as the percentage of the tagged histone occupancy
555 (detected with anti-HA) over the endogenous histone occupancy (detected with anti-H3)
556 under transcriptionally repressed ("Off") or activated ('On") conditions in wt and *nap1*Δ
557 strains. The tagged histone persistence at T0 was set as 100% (dark blue). Bright yellow
558 indicates that tagged histone was completely exchanged (0% tagged histone
559 persistence). Promoter (P) and ORF (O) regions are indicated.
560
561 **Figure 6. Histone persistence varies depending upon time of shut-off and specific**
562 ***GAL* region.** Histone persistence is defined as the relative percentage of the tagged
563 histone occupancy (detected with anti-HA) over the endogenous histone occupancy
564 (detected with anti-H2B or anti-H3) under *GAL* gene transcriptionally repressed ("Off") or
565 activated ('On") conditions in wt and *nap1*Δ strains. The histone persistence at T0 was
566 set as 100%. (A) H2B and (B) H3 persistence at *GAL* locus under transcriptionally

repressed condition. (C) H2B and (D) H3 persistence in the continuous transcriptionally active condition. P: promoter. O: ORF. *: P value<0.05. **: P value <0.01

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