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RESEARCH PAPER

A nuclear proteome localization screen reveals the exquisite specificity of Gpn2 in RNA polymerase biogenesis

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ABSTRACT

The GPN proteins are a conserved family of GTP-binding proteins that are involved in the assembly and subsequent import of RNA polymerase II and III. In this study, we sought to ascertain the specificity of yeast GPN2 for RNA polymerases by screening the localization of a collection of 1350 GFP-tagged nuclear proteins in WT or *GPN2* mutant cells. We found that the strongest mislocalization occurred for RNA polymerase II and III subunits and only a handful of other RNAPII associated proteins were altered in *GPN2* mutant cells. Our screen identified Ess1, an Rpb1 C-terminal domain (CTD) prolyl isomerase, as mislocalized in *GPN2* mutants. Building on this observation we tested for effects of mutations in other factors which regulate Rpb1-CTD phosphorylation status. This uncovered significant changes in nuclear-cytoplasmic distribution of Rpb1-GFP in strains with disrupted RNA polymerase CTD kinases or phosphatases. Overall, this screen shows the exquisite specificity of GPN2 for RNA polymerase transport, and reveals a previously unappreciated role for CTD modification in RNAPII nuclear localization.

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GPN2; RNA polymerase; functional genomics; yeast; protein complex biogenesis

Introduction

The assembly and nuclear transport of RNA polymerases from the cytoplasm is critical for all eukaryotes. Despite the fundamental role of RNA polymerases in all cells, the process by which they are assembled and imported into the nucleus remains poorly understood. Studies over the past several years have identified factors required for function in RNA polymerase biogenesis. In mammalian cells, the R2TP prefoldin complex [1] and HSP90 have been identified as required co-factors for RNA polymerase assembly and nuclear import [2], in concert with a family of three GTPases known as the GPN proteins [2,3]. In addition, research in the budding yeast Saccharomyces cerevisiae has also revealed a requirement for the prefoldin Bud27 [4] in the assembly of all three RNA polymerases while Iwr1 and the karyopherin-like protein Rtp1 mediate nuclear import [5–7].

The GPN proteins were originally identified through the physical interaction of MBDin/XAB1/GPN1 with both the DNA repair protein XPA chromatin modifier MBD2 [8,9]. X-ray crystallography revealed that the archaeal GPN1 ortholog PAB0955 is a GTPase that forms homodimers [10], yet little was understood about the biological function of GPN1. More recently, mutation of the yeast GPN family members were shown to promote chromosomal instability [11,12] and defects in sister chromatid cohesion [13,14]. All three yeast GPN proteins, Gpn1, Gpn2 and Gpn3 have been shown to play a role in the assembly and subsequent nuclear import of RNA polymerase II (RNAPII), with Gpn2 and Gpn3 also required for correct assembly of RNA polymerase III (RNAPIII) complexes [12,15]. While the GTPase activity of GPN1 is required for correct RNAPII biogenesis, the precise role of each GPN protein remains unclear.

Physical interactions between GPNs and RNA polymerases are likely transient and were first observed under conditions that result in the accumulation of RNA polymerase assembly intermediates [2]. Additionally, global analysis of protein complexes in yeast revealed few physical

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interactions for GPN proteins [16]. Thus, GPNs act somewhat like molecular chaperones, aiding the biogenesis and localization of RNA polymerase complexes somehow, but not participating in the activity of the final assembled complex. Based on this idea, we sought to determine the potential substrate repertoire of the Gpn2 protein by asking whether Gpn2 regulates the assembly and subsequent nuclear import of any other protein complexes. Here we present a comprehensive screen of over 1350 GFP-tagged nuclear associated proteins for mislocalization in a *gpn2-2* mutant background. These studies show strong evidence that Gpn2 function is exquisitely specific to RNAPII and RNAPIII biogenesis. While we show that not all reported players in RNA polymerase assembly have clear roles, the activity of the GPN proteins is an ancient and highly conserved factor specifically required for RNA polymerase maturation.

Methods and materials

GFP strain construction

Yeast crossing was done to generate a starter yeast strain containing the *gpn2-2* mutation, a *LYP1* deletion for haploid selection and a *LEU2* marker under the control of a Ste2 promoter. Additionally, an HTA2::mCherry marker was introduced to provide a fluorescent nuclear marker. Three hundred and eighty-four spot arrays of GFP-tagged strains annotated as nuclear, nuclear periphery or nucleolar were prepared from the GFP tagged collection [17] and crossed with the starter strain (**Table S1**) using a Singer ROTOR robot. Following sporulation, haploid selection was performed by plating spores onto selective media lacking leucine, histidine and uracil, supplemented with thialysine. Sporulation and subsequent tetrad dissection was used to generate all validation strains and query strains to investigate RNA polymerase subunit localization.

Microscopy and image scoring and validation

Ninety-six well plates of query strains and controls were grown to log phase at 30°C in SC-LEU supplemented with hygromycin and thialysine. Imaging was done in 12 sample batches for paired mutant and control strains using Teflon coated multi sample slides (TekDon). Imaging was done on a Leica fluorescence microscope. Images were visually scored for obvious differences between mutant and controls using Metamorph software. Validation of hits was done for haploid strains from tetrad dissection and quantitated as described previously [12]. Temperature sensitive (ts) mutant strains were obtained from the Hieter and Boone lab ts collections [11,18].

Cross suppression analysis

High copy plasmids representing the genes of interest were isolated from the MoBY plasmid collection [19]and transformed into yeast mutants using standard lithium acetate transformation [20]. Transformants were cultured overnight, serially diluted and plated on SC-LEU + G418 plates to assay complementation of the mutant growth defect.

Results and discussion

The GPN family members do not appear to exhibit functional redundancy

The GPN proteins sequence similarity and shared functions in RNA polymerase biogenesis raises the question of whether overexpression of any family member or related protein could complement loss of another GPN family protein. Since our focus here is *GPN2*, we transformed *GPN2* mutant strains with 2μ plasmids from the MoBY collection [19], with related genes under control of their own promoter. We chose *GPN1, GPN2, GPN3*, and *IWR1* based on their confirmed roles in RNAPII and RNAPIII biogenesis [5,12]. We observed that only *GPN2* could complement the temperature sensitivity of a *gpn2-1* mutant and in fact, overexpression of *GPN3* resulted in moderately impaired growth (Figure 1a). As shown in Figure 1b, we also observed Rpb1-GFP localization in *gpn2-2* mutants containing these same plasmids and did not observe any improvement in RNAPII nuclear localization, with the exception of wild-type *GPN2* expression. Thus, *GPN2* must have a unique essential function not complemented by

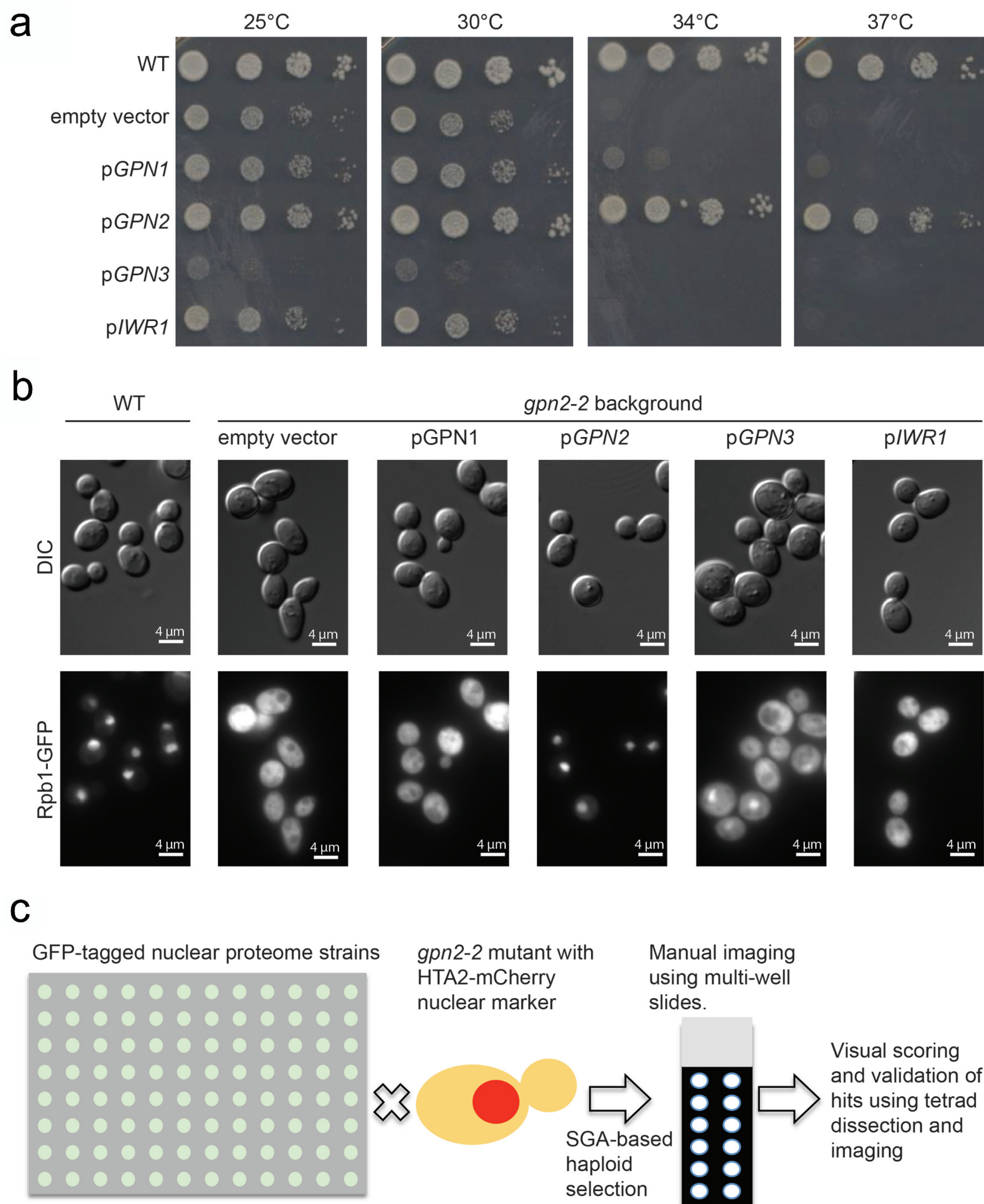

Figure 1. The Gpn2 protein has a unique essential role in growth and RNAPII import in budding yeast. (a) Serial dilution plating assays of WT or *gpn2-1* mutant yeast (lower five rows) carrying the indicated plasmids and grown at the indicated temperatures. Only the *GPN2* insert complements the ts-allele. (b) Localization of Rpb1-GFP in *gpn2-2* mutants complemented with the indicated plasmids. DIC images are shown on top, with GFP on the bottom. (c) Schematic of nuclear proteome GFP array construction. A *gpn2-2* mutant with an HTA2-mCherry nuclear marker was mated to the entire array, before sporulation and haploid selection to produce an output array suitable for manual imaging on 12-well slides.

other GPN family members or regulators of RNA polymerase biogenesis like Iwr1.

In a previous study [12], we observed that mutation of *GPN2* did not appear to have global effects on nuclear import. However, these experiments only investigated a small sampling of nuclear proteins, leaving open the possibility that other Gpn2 substrate proteins exist. To gain a more complete understanding of the potential scope of Gpn2 functions, we surveyed the localization of over 1350 proteins with annotated nuclear, nucleolar or nuclear periphery localization (**Supplementary Table S2**) in a *gpn2-2* mutant background (Figure 1c). We initially completed a visual screen for mis-localization across the entire nuclear proteome collection produced by SGA. Proteins with putative changes in localization were selected for tetrad dissection to generate strains with a clean genetic background and avoid any contamination from diploid strains that escaped the SGA selection steps. Tetrad dissected haploid strains encoding *gpn2*-2 and a GFP fusion of interest were then subject to manual validation by GFP fluorescence imaging to carefully assess any potential changes in localization.

Gpn2 activity is specific for RNA polymerase biogenesis and associated factors

Gpn2 seems to play a role in assembly or transport of RNAPII and RNAPIII, and its disruption leaves unincorporated subunits accumulating in the cytoplasm [3,12]. The *gpn2-2* mutant is unique among the GPN mutants we previously screened as it has only a single-point mutation near the N-terminus (C19S) that confers hypomorphic growth and ts-phenotypes. Remarkably, among 1350 GFP fusion proteins screened manually in *GPN2* and *gpn2-2* cells, we observed strong mis-localization only for multiple RNAPII and RNAPIII subunits (Figure 2 and **Figure S1**); however, the vast majority of nuclear proteins were completely unaffected by mutations in *GPN2* (**Supplementary Table S2**). These data strongly support the tight linkage of GPN proteins to RNA polymerase biogenesis and transport as opposed to any sort of general nuclear transport or chaperone role.

To confirm our observations by analysis of orthogonal functional data, we mined publicly available Cellmap genetic interaction data (https://thecellmap.org) to determine which yeast mutants had a synthetic genetic array (SGA) profile most similar to *GPN2*. We arbitrarily set a Pearson's coefficient cutoff of 0.15, which identified 55 genes with similar SGA patterns to the *GPN2* mutant included in the Cellmap study. As expected, the majority of these genes include RNA polymerase subunits and transcription associated factors, supporting a specific role of Gpn2 in driving RNA polymerase assembly and subsequent transcriptional activity (Figure 2c).

Gpn2 defects partially mislocalize a subset of transcription and splicing associated factors

We were able to validate subtle changes in the localization of nine other proteins emerging from the primary screen, most of which have reported physical or functional connections to RNA polymerase (Table 1 and Figure 3). For the seven genes with clear changes in nuclear:cytoplasmic GFP ratios (Figure 3), six had decreased nuclear intensity and one had increased nuclear intensity. One explanation for the mis-localization of these other nuclear proteins is that the cytoplasmic accumulation of RNA polymerase subunits results in the sequestration of proteins that would not normally interact with RNA polymerase until the assembled complex reaches that nucleus. In fact, the spliceosome is known to associate with the RNAPII CTD, so this may drive the mis-localization of Cwc22 and Slu7 splicing factors in the *gpn2-2* strain [21,22], (Figure 3a).

YAP1, a transcription factor involved in the stress response, interacts with Bud27, a chaperone involved in RNAPII assembly, and associates with the RNAPII subunit RPB2 (Biogrid). Fkh2 is another transcription factor that we observed mislocalized and has previously reported dynamic localization [23]. Thus, transcription factors could be mislocalized either due to stresses placed upon the cell in *gpn2-2* mutants, or due to associations with RNA polymerase itself.

Lif1, and Ess1 were the two other proteins that had a decreased nuclear:cytoplasmic ratio confirmed following our screen. Ess1 has clear links to RNAPII that we describe below. Lif1 is a DNA repair protein that functions as part of the Ligase

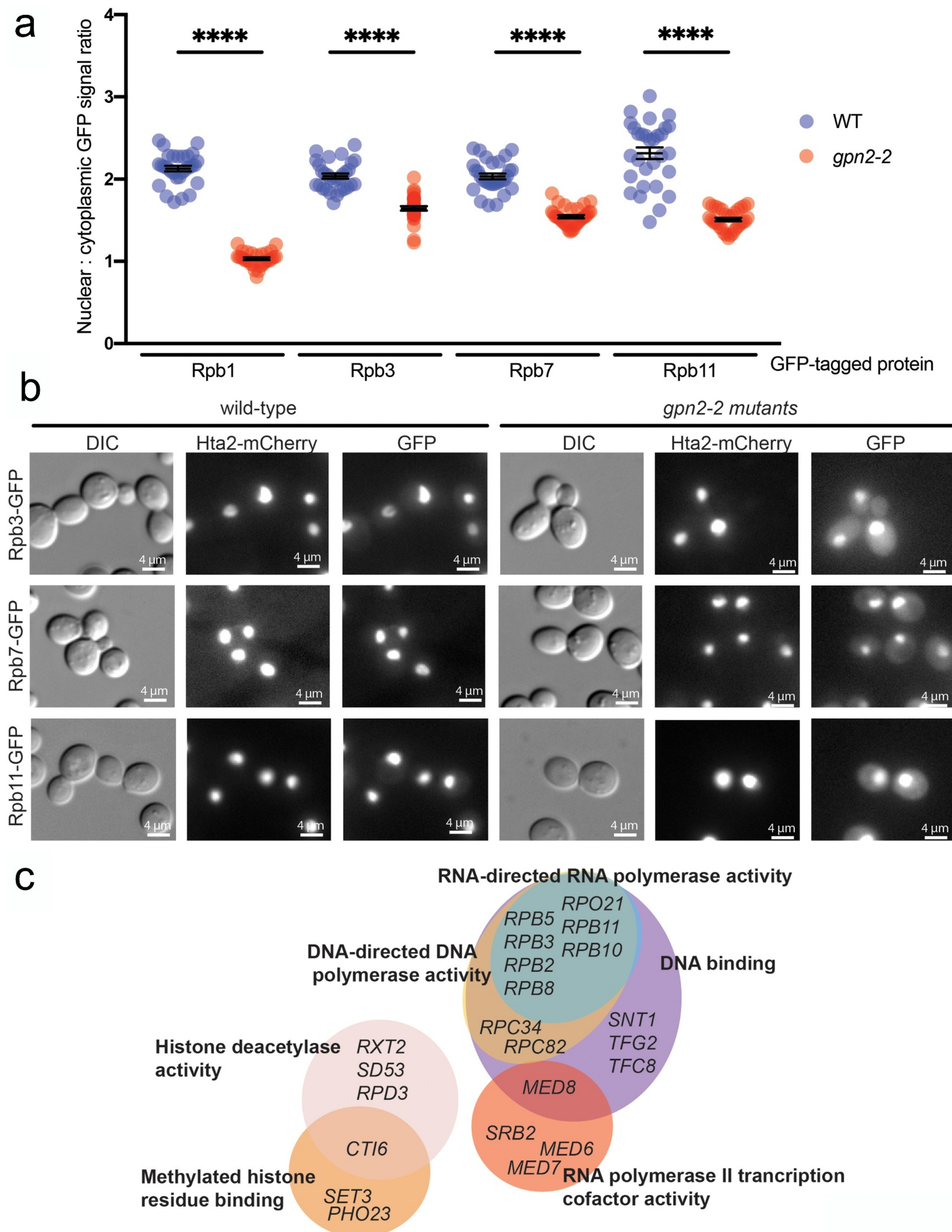

Figure 2. RNAPII localization is strongly affected by *gpn2-2*. (a) Quantification of nuclear-to-cytoplasmic GFP signal ratios in WT or *gpn2-2* mutant yeast strains bearing an RNAPII subunit GFP fusion. The GFP fusion is named on the *X*-axis and scores color-coded by the WT (blue) or *gpn2-2* (red) genotype. Triplicates were conducted, in total $n > 90$, **** $p < 0.0001$ Student's t-test. (b) Representative images of Rpb3-, Rpb7- and Rpb11-GFP in WT or *gpn2-2* cells at the semi-permissive temperature of 30°C. For all figures HTA2-mCherry is included to mark the position of the nucleus. (c) *GPN2* correlated SGA genetic interaction profiles from the Cellmap. Gene ontology terms are noted on the sides of each circle encompassing gene names that have high correlations with the genetic interaction profile of *GPN2* mutants. This illustrates the strong transcriptional bias of *GPN2* genetic interactions.

Table 1. Non-RNA polymerase subunits that have altered localization in a *gpn2-2* mutant.

Protein	Function	Physical Interactions	Change in localization
CWC22	Spliceosomal protein	Other spliceosomal proteins	Decreased nuclear localization
ESS1	Peptidylprolyl-cis/trans-isomerase	Rpb7, Rpo21	Decreased nuclear localization
FKH2	Transcription factor	Swi4,5,6 CDC5, CDC20	Decreased nuclear localization
YAP1	Transcription factor	CRM1, TRX1, TRX2	Decreased nuclear localization
LIF1	DNA ligase IV, DNA repair	YKU70, YKU80, RAD51	Decreased nuclear localization
SLU7	Splicing factor	CWC22, spliceosome	Decreased nuclear localization
MLP2	Nuclear envelope associated protein	Nuclear pore complex and mRNA export factors	Reduced signal intensity and altered localization
SQT1	Chaperone, replication stress factor	RPL10, RRB2	Increased nuclear signal
FOB1	Nucleolar fork blocking	Complex, interacts with numerous DNA binding proteins including splicing	Decreased nuclear signal intensity

IV complex. Sqt1 had an increased nuclear:cytoplasmic intensity ratio, and functions as an assembly chaperone for the large ribosomal subunit Rpl10. Why Lif1 and Sqt1 were specifically affected we do not know, although we do investigate RNA polymerase I (RNAPI) localization below, which may influence ribosome biogenesis and thus Sqt1 localization. Interestingly, we also saw a notable decrease in signal intensity for the Fob1 protein (Table 1, **Figure S2**). Fob1 serves as a replication fork barrier between units of rDNA transcription, further supporting potential effects on ribosome biogenesis in these *gpn2* mutant cells.

In addition to potential shifts from nucleus to cytoplasm, we also validated significant mislocalization of MLP2 from the nuclear envelope (**Figure S2**) which may have consequences for nucleocytoplasmic transport of mRNAs [24]. Notably, MLP2 binds components of the spindle pole body [25] and helps regulate telomere length [26]. Mlp2 has also been implicated, with Mlp1, in preventing R-loop associated genome instability [27]. Therefore, the defective Mlp2 localization we observe may explain part of the reported genome instability phenotype in *gpn2-2* mutants [11].

Overall, we validated a small set of non-RNA polymerase proteins with disrupted localization in *gpn2-2* cells. We did not find a coherent pattern in these changes, except that many of the changes are in proteins linked to transcription. Therefore, we favor a model in which the nine altered proteins are being impacted indirectly either through mislocalization in complex with mislocalized RNA polymerases or through cellular stress caused by the *gpn2* allele.

Phospho-regulators of the RNAPII CTD effect its nuclear localization

Ess1 is a prolyl isomerase involved in regulation of the phosphorylation of the RNAPII c-terminal domain (CTD) located on the largest subunit, Rpb1 [28]. CTD phosphorylation at Tyr1, Ser2, Thr4, Ser5, or Ser7 control the transcription cycle. Our data show that there is a distinct change in the localization of Ess1-GFP in the *gpn2-2* mutants (Figure 3b and c). Since Ess1 binds the CTD, the retention of Ess1 in the cytoplasm may be due to binding to mis-localized Rpb1. Nevertheless, we chose to investigate whether *ESS1* mutation had any effect on Rpb1 localization by testing whether an *ess1-1* ts-mutant had defective Rpb1 localization. We found that Rpb1-GFP nuclear signal does exhibit a small but significant difference between wild-type and *ess1* mutant backgrounds after 3 hours at the non-permissive temperature of 37°C (**Figure S2C**)

Since Ess1 binding with the CTD occurs most strongly when Serine-5 is phosphorylated [29] and *ess1-1* mutants led to changes in Rpb1-GFP, we wondered if other changes in CTD phosphorylation status might impact Rpb1 localization. In cultured mammalian cells depletion of RPAP2 results in cytoplasmic accumulation of RNAPII [3]. The yeast ortholog of RPAP2, Rtr1, is a phosphatase that targets the CTD [30,31], so we first assessed Rpb1-GFP localization in yeast lacking *RTR1* or its paralog *RTR2*. Both *rtr1*Δ and *rtr2*Δ resulted in increased nuclear Rbp1-GFP, suggesting that they may have a negative regulatory role in RNAPII import through phosphorylation of the CTD (**Figure S3**). We were unable to

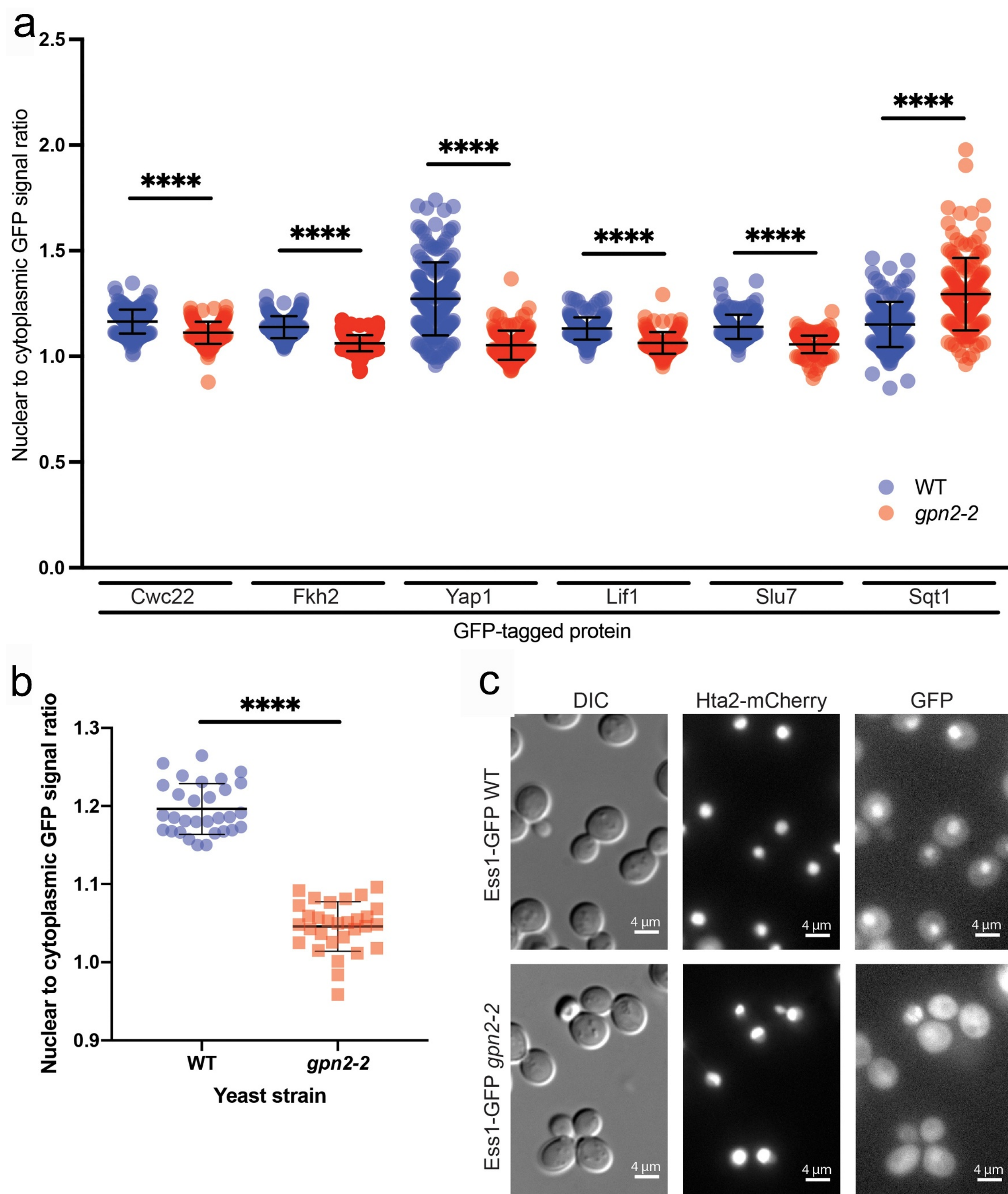

Figure 3. Subtle changes in non-RNA polymerase nuclear protein localizations in *gpn2-2* cells. (a) Quantification and statistical analysis of proteins with decreased or increased nuclear accumulation in *gpn2-2* yeast. The GFP fusion is named on the *X*-axis and scores color-coded by the WT (blue) or *gpn2-2* (red) genotype. (b and c) Quantification (b) and representative images (c) of Ess1-GFP fusions in WT or *gpn2-2* cells. For analysis in A and B, triplicates were conducted, in total $n > 90$, **** $p < 0.0001$ Student's t-test.

generate a viable double *rtr1Δrtr2Δ* mutant with Rpb1-GFP, possibly due to altered function with the GFP tag, so it is unclear whether the effect of deletion of both phosphatases would be additive. If CTD phosphatase loss increased nuclear Rpb1-GFP fluorescence, we reasoned that CTD kinase mutations would decrease Rpb1-GFP fluorescence. Sgv1 is an essential yeast kinase that phosphorylates the RNAPII CTD [32]. Employing a series of three SGV1 ts-alleles we observed a significant decrease in Rpb1 nuclear localization in all *SGV1* mutants (**Figure S3**).

Considering the complexity and redundancy in Rpb1 CTD phosphorylation we elected to test

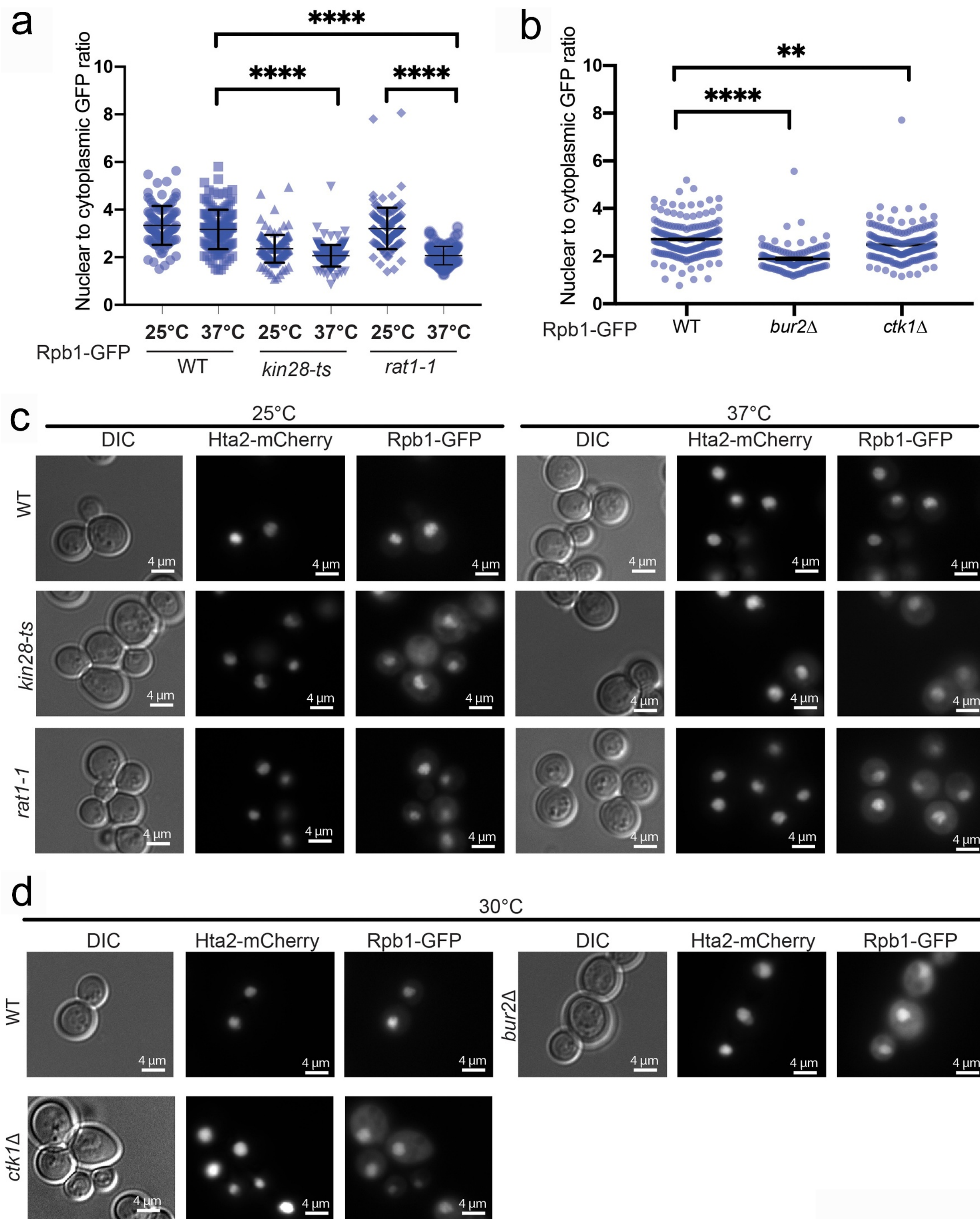

Figure 4. Significant defects in Rbp1-GFP localization result from mutation of CTD phospho-regulatory proteins. (a and b) Quantification of Rpb1-GFP nuclear-to-cytoplasmic ratios in the indicated CTD phosphoregulatory mutants. In A, results from both a permissive (25°C) and non-permissive (37°C) temperature are indicated, while in B standard growth at 30°C was used. For analysis in A and B, triplicates were conducted, in total $n > 90$, **** $p < 0.0001$, ** $p < 0.01$ by a Student's t-test. (c and d) Representative images of the Rpb1-GFP in WT cells and the indicated mutant strains at the indicated temperatures.

a panel of mutants in other factors that control CTD phosphorylation to confirm our observations. Analysis of *BUR2* and *CTK1* deletion strains, *kin28-ts* and *rat1-1* mutants, all of which perturb CTD phosphorylation, all showed defects in Rpb1-GFP nuclear accumulation (Figure 4a–d). Therefore, our data support a model in which Rpb1-CTD phosphorylation status impacts the nucleo-cytoplasmic distribution of the polymerase. Since most CTD phosphorylation presumably takes place co-transcriptionally in the nucleus this raises the possibility that RNA polymerase recycling is regulated by its CTD.

RNAPI nuclear localization increases in gpn2-2 mutants

RNAPI assembly is reported to occur in the nucleolus following nuclear import of the required subunits [33]. Consistent with this we were unable to detect mislocalization of the RNAPI subunit RPA135 in GPN mutant or *iwr1Δ* mutant backgrounds [12]. Several GFP-tagged subunits of RNAPI were included in the nuclear proteome screen and as shown in Figure 5a, there is no decrease in nuclear signal for Rpa12, Rpa34 or Rpa190 in a *gpn2-2* mutant background. Instead, when we quantified the nuclear:cytoplasmic ratio of GFP signal, or the mean nuclear GFP intensity, *gpn2-2* cells appeared to have higher levels of RNAPI subunits in the nucleus (Figure 5b and c). Since *gpn2-2* mutants have altered RNAPII transcription due to mis-localization, we hypothesized that defects in RNAPII activity may affect nucleolar transcription by RNAPI. We obtained a temperature sensitive *RPB1* mutant called *rpo21-1* and subsequently observed increased Rpa190-GFP signal at 37°C (**Figure S4**), similar to the *gpn2-2* mutant background. These data show that defects in RNAPII function can increase RNAPI localization in the nucleolus.

Perspective

Molecular chaperone proteins can be generalists or specialists; interacting with a wide range of non-native substrate proteins or being dedicated to a single protein substrate. The assembly and transport roles of GPN-family proteins seem to fall into the highly-specialized category. Our data confirm that the GPN-family members cannot complement the loss of *GPN2* function, supporting a unique essential function for each family member. In addition, direct tests of >1300 nuclear protein localizations in *gpn2-2* mutants found strong effects only on RNA polymerase subunits, with a handful of weaker effects on a small number of proteins. Thus, Gpn2 protein has a unique essential function that is almost completely restricted to its effects on

RNAPII and RNAPIII assembly and nuclear import. Gpn2 does not have a general nuclear transport function. Our data are consistent with previous reports that Gpn2 has an essential function in the assembly of an Rpb3 sub-complex of RNAPII [34]. Since Gpn2 mutants also have RNAPIII defects it remains to be determined whether an analogous Rpc40 sub-complex exists during assembly of polymerase III.

One of the clearest non-RNAPII/III related hits from our screen was Ess1-GFP, which was displaced to the cytoplasm in our *gpn2-2* mutant. Since Ess1 binds the CTD of Rpb1 we tested a range of CTD modifiers and found that many either increased or decreased nuclear:cytoplasmic ratios of Rpb1-GFP. This suggests that the CTD phosphorylation status is an important contributor to the distribution of RNAPII in cells. Whether the CTD and associated modifications play a role in assembly and import, or turnover and recycling of RNA polymerase subunits is not clear. Human RPAP2 regulates the nuclear import of RNAPII in human cells [3]; however, loss of the yeast orthologs Rtr1 and Rtr2 actually increased nuclear intensity of Rpb1-GFP. Rtr1/2 are phosphatases for the CTD which should increase CTD phosphorylation. Similarly, *rat1-1* alleles have been reported to lead to hyper-phosphorylated CTD, but this allele had the opposite effects in our assay, decreasing nuclear Rpb1-GFP, along with mutations in any of the CTD kinases tested. Thus, we speculate that timing and target residues of these kinases and phosphatases on the CTD is likely important to determine any effects of the CTD on RNAPII import or export.

In our previous study, we demonstrated that *gpn2-2* mutants have an increase in the nuclear to cytoplasmic ratio for Rpa135-GFP [12]. In

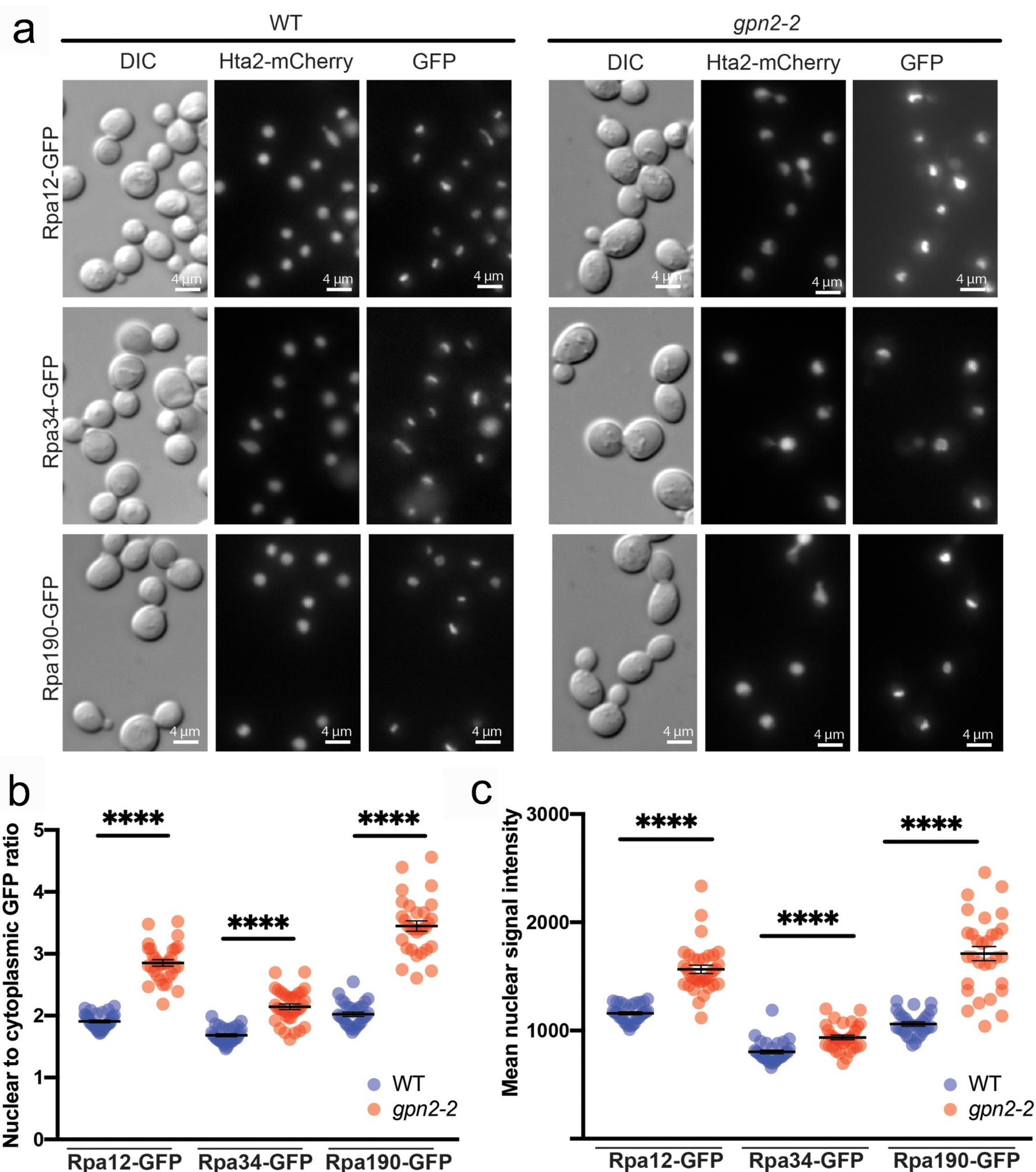

Figure 5. Impacts of *gpn2-2* mutation on RNAPI subunit localization. (a) Representative images of the indicated RNAPI subunit GFP fusion in WT or *gpn2-2* cells. (b) Quantification of the nuclear:cytoplasmic ratio of the indicated GFP fusion in WT or *gpn2-2* cells. (c) Quantification of the total nuclear signal intensity for the indicated GFP fusion in WT or *gpn2-2* cells. For B and C, triplicates were conducted, in total $n > 90$, **** $p < 0.0001$ by a Student's t-test.

extending this work to other RNAPI subunits we show that defects in *gpn2-2* activity result in accumulation of RNAPI at the nucleolus, although we cannot state whether they are part of an assembled complex. This phenotype may be due to altered transcription by RNAPII as an *rpo21-1* mutant allele of *RPB1* exhibited the same phenotype. Previous work has shown that RNAPI accumulates in soybean nuclei upon inhibition of RNAPII activity [35] and a recent paper revealed a role for RNAPII in transcription of rRNA genes [36]. Thus there may be some interplay between RNAPI and RNAPII where increased RNAPI in the nucleus is a compensatory mechanism for reduced RNAPII activity. Notably, stress conditions have been shown to cause increased homodimerization of inactive RNAPI subunits, which could possibly affect GFP signal intensity in *gpn2-2* mutants [37].

Finally, another contributing factor could be altered stoichiometry of RNAPI subunits since four subunits, Rpb5, Rpb8, Rpb10 and Rpb12 are shared by all three RNA polymerases. The sequestration of RNAPII subunits in the cytoplasm could alter the availability of these proteins for integration into RNAPI, leading to accumulation of unassembled RNAPI in the nucleolus. Additional experiments will be required to dissect the indirect effects of disrupting one polymerase on the others.

Several recent publications have investigated the role of GPN family proteins in cancers, including breast cancer [38,39] and small cell lung carcinoma [40]. However, data from the CBio Cancer Genomics Portal [41] indicates that mutations and copy number changes in the GPN proteins are rare in cancers. The essential nature of the GPN family members may limit their potential as a therapeutic target in human cancers; however, we cannot rule out that they may be identified as contributing factors in rare disease. Mutations associated with the genetic disorder Treacher-Collins syndrome have been shown to affect the stability of RNA polymerases I and III [42]. Perhaps alterations of GPN proteins could also contribute to rare disease phenotypes through their key role in RNA polymerase assembly and transcription. Regardless of their involvement in human disease, their ubiquitous presence in eukaryotes and essential function makes the GPN proteins a fascinating and still poorly understood protein family. Based on the data presented here, we suggest that the future of GPN research should focus on the biochemical mechanisms behind GPN activity in promoting RNA polymerase assembly.

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Disclosure Statement

No potential conflict of interest was reported by the author(s).

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