Nuclear localization of the *Saccharomyces cerevisiae* ribonucleotide reductase small subunit requires a karyopherin and a WD40 repeat protein

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Ribonucleotide reductase (RNR) catalyzes the reduction of ribonucleotides to the corresponding deoxyribonucleotides and is an essential enzyme for DNA replication and repair. Cells have evolved intricate mechanisms to regulate RNR activity to ensure high fidelity of DNA replication during normal cell-cycle progression and of DNA repair upon genotoxic stress. The RNR holoenzyme is composed of a large subunit R1 (α, oligomeric state unknown) and a small subunit R2 (β_2). R1 binds substrates and allosteric effectors; R2 contains a diferric-tyrosyl radical [$(Fe)_2$-Y·] cofactor that is required for catalysis. In *Saccharomyces cerevisiae*, R1 is predominantly localized in the cytoplasm, whereas R2, which is a heterodimer ($\beta\beta'$), is predominantly in the nucleus. When cells encounter DNA damage or stress during replication, $\beta\beta'$ is redistributed from the nucleus to the cytoplasm in a checkpoint-dependent manner, resulting in the colocalization of R1 and R2. We have identified two proteins that have an important role in $\beta\beta'$ nuclear localization: the importin β homolog Kap122 and the WD40 repeat protein Wtm1. Deletion of either *WTM1* or *KAP122* leads to loss of $\beta\beta'$ nuclear localization. Wtm1 and its paralog Wtm2 are both nuclear proteins that are in the same protein complex with $\beta\beta'$. Wtm1 also interacts with Kap122 *in vivo* and requires Kap122 for its nuclear localization. Our results suggest that Wtm1 acts either as an adaptor to facilitate nuclear import of $\beta\beta'$ by Kap122 or as an anchor to retain $\beta\beta'$ in the nucleus.

DNA-damage checkpoint | subcellular redistribution

The levels and relative ratios of dNTP pools are important for high-fidelity DNA replication and repair (1). Failure to increase dNTP levels at the G_1-to-S transition of the cell cycle is a lethal event at cellular level (2, 3). Conversely, elevated dNTP pools throughout the cell cycle lead to increased mutation rates (4–6). Imbalance in dNTP pools also contributes to mutagenesis by reducing the fidelity of DNA polymerases (7–9). Eukaryotic cells have evolved complex surveillance mechanisms (i.e., checkpoints) to ensure proper dNTP pool sizes during the normal cell-cycle progression and in response to genotoxic stress (3, 10–14). A major target of such checkpoint regulation is ribonucleotide reductase (RNR), which catalyzes the reduction of ribonucleoside diphosphate to deoxyribonucleoside diphosphate, an essential step in *de novo* biosynthesis of dNTPs (15).

Class I RNRs were identified originally in *Escherichia coli* and are conserved from yeast to mammal (16). The mechanisms of enzymatic catalysis (17) and allosteric regulation (18, 19) have been studied extensively in *E. coli* and, more recently, in mice (20, 21). The archetype RNR holoenzyme consists of a large subunit R1 (α, whose oligomeric state in eukaryotes is not completely understood) (22) and a homodimeric small subunit (β_2) (20). The eukaryotic R1 contains the catalytic site, an effector site that controls substrate specificity, an activity site that controls turnover, and a weak ATP-binding site that controls the R1 oligomeric state (15). R2 houses a diferric-tyrosyl radical [$(Fe)_2$–Y·] cofactor that is essential for catalysis. In *Saccharomyces cerevisiae*, there are two genes encoding R2: *RNR2* (β) and *RNR4* (β') (23–25). Recent studies (26–28) have established the fact that the active form of R2 is the $\beta\beta'$ heterodimer (formerly designated Rnr2–Rnr4). Only β is capable of forming the $(Fe)_2$–Y· cofactor (26, 27), whereas β' facilitates cofactor assembly and stabilizes the resulting holoheterodimer (26–30).

The mechanisms underlying RNR regulation are complex and multilayered. Regulation of RNR has been reported to occur by control of transcription (10, 31, 32); mRNA stability (33); translational efficiency (34, 35); protein degradation (36); protein–protein interactions (3, 37); posttranslational modification, including cofactor assembly (38, 39) and phosphorylation (40, 41); and subcellular localization (12–14, 42, 43). The details of regulation vary between organisms, although some common mechanisms (such as allostery) are conserved. In *S. cerevisiae*, in addition to allosteric regulation, there are three characterized mechanisms that modulate RNR activity, all of which depend on the DNA-damage checkpoint kinases Mec1, Rad53, and Dun1. First, transcription of the *RNR2–4* genes are induced by genotoxic stress by Mec1/Rad53/Dun1-mediated removal of the transcriptional repressor Crt1 from its target promoters (10). Second, DNA damage leads to Mec1/Rad53/Dun1-mediated degradation of Sml1, which is an inhibitor of α (3, 11, 44, 45). The third mechanism is Mec1/Rad53/Dun1-mediated colocalization of α and $\beta\beta'$ upon genotoxic stress (12), which is the subject of this article.

Recently, we showed that in *S. cerevisiae*, $\beta\beta'$ is predominantly localized in the nucleus, whereas α is localized in the cytoplasm. When cells encounter DNA damage or replicational stress, $\beta\beta'$ undergoes checkpoint-dependent nucleus-to-cytoplasm redistribution to be colocalized with α (12), which facilitates RNR holoenzyme formation. DNA damage-induced RNR redistribution has also been reported in fission yeast (13), plant (42), and mammalian cells (14), suggesting that it is an evolutionarily conserved mechanism.

The movement of most proteins across the nuclear envelope is facilitated by the karyopherin (Kap)-β family of soluble transport receptors (46, 47), also known as importins or exportins, depending on the direction of transport (48). The Kap-β proteins bind specific cargo proteins either directly or by means of interactions with adaptor proteins (47). Also, Kap-β proteins bind RanGTP and interact with the nuclear pore complex (46,

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Abbreviations: RNR, ribonucleotide reductase; Kap, karyopherin; IMF, immunofluorescence; IP, immunoprecipitation; HA, hemagglutinin; NES, nuclear export sequence; NLS, nuclear localization signal.

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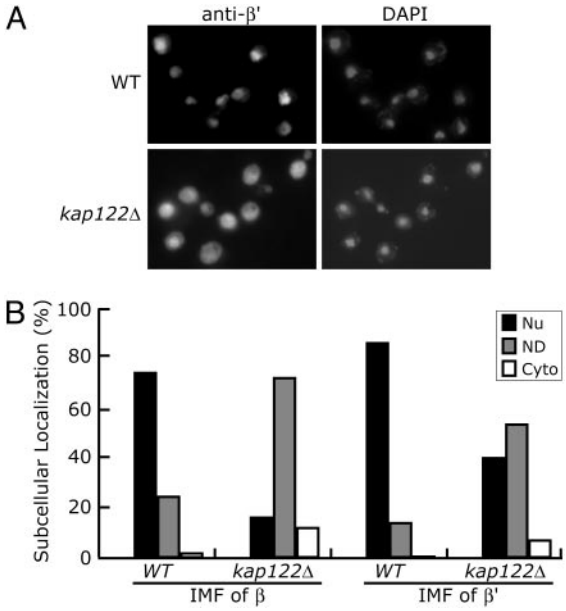

Fig. 1. KAP122 is required for proper nuclear localization of β and β'. (*A*) IMF staining of WT and *kap122*Δ cells from early logarithmic-phase cultures by using anti-β Abs. (*B*) Quantitative analysis of β and β' subcellular localization patterns in WT and *kap122*Δ cells. At least 200 cells were counted for each experiment. Percentages of cells with distinct localization patterns were represented as follows: black bar, cells with a predominantly nuclear signal (Nu); white bar, cells with a predominantly cytoplasmic signal (Cyto); gray bar, cells with no difference (ND) in signal intensity between the nucleus and the cytoplasm.

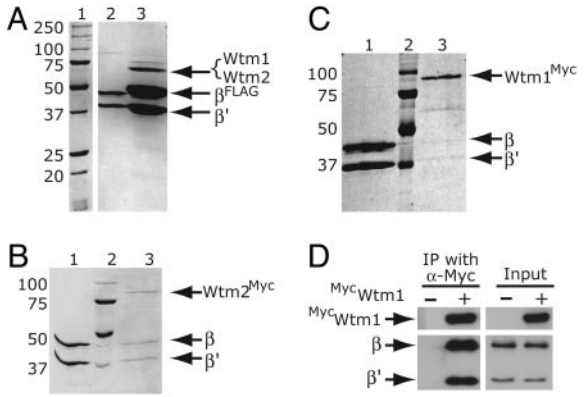

Fig. 2. Wtm1 and Wtm2 are found in the same protein complex as β and β'. (*A*) The $^{Flag}\beta\beta'$ complex was purified from MHY341 by an anti-Flag affinity column. We resolved 40 μg of protein samples on a 5–15% gradient PAGE and stained by Coomassie brilliant blue (lane 3). The positions of β, β', and Wtm1 and Wtm2, as identified by MS/MS are indicated. Lane 1, molecular weight (MW) markers; lane 2, purified $\beta\beta'$ heterodimer. (*B*) Copurification of β and β' with $Wtm2^{Myc}$. Total protein extract from 2.5 g of the *wtm2:WTM2–18xMyc* cells was loaded to an anti-Myc agarose column. After extensive wash, the eluted proteins were resolved by SDS/PAGE and stained by Coomassie brilliant blue (lane 3). Lane 1, purified $\beta\beta'$ heterodimer; lane 2, MW markers. (*C*) Copurification of β and β' with $Wtm1^{Myc}$. Total protein extract from 1.5 g of the *wtm1:WTM1–18xMyc* cells was processed by the same procedure as in *B*. Lanes 1 and 2 are the same as in *B*. (*D*) Co-IP of β and β' with ^{Myc}Wtm1. Protein extract was prepared from cells that contain a centromeric plasmid expressing an N-terminally tagged ^{Myc}WTM1 from its native promoter. The immune complex from anti-Myc IP and total protein extract (Input) were probed with Abs against Myc, β, and β' on Western blots.

47). A major effort in the nucleocytoplasmic transport field has been directed at identifying cargo proteins for individual karyopherins. Considering the large difference in number between karyopherins and cargo proteins, it is expected that there is much redundancy in Kap–cargo association (46).

The DNA damage-induced, nucleus-to-cytoplasm redistribution of R2 could be mediated by decreased nuclear import, increased nuclear export, disruption of a nuclear anchoring mechanism, or a combination of these mechanisms. In *Schizosaccharomyces pombe*, R2 redistribution appears to involve both nuclear export and degradation of the nuclear anchor protein Spd1, an S-phase inhibitor (13, 49–52). In this study, we set out to determine the mechanism of DNA damage-induced $\beta\beta'$ redistribution by identifying proteins that have important roles in $\beta\beta'$ nuclear localization. We have identified the Kap-β homolog Kap122 in a candidate screen for $\beta\beta'$ mislocalization by immunofluorescence (IMF). We have also identified by MS two nuclear WD40 repeat proteins, Wtm1 and Wtm2, which copurify with $\beta\beta'$. Removal of either *KAP122* or *WTM1* causes $\beta\beta'$ mislocalization. By using immunoprecipitation (IP) and IMF, we have shown that Wtm1 interacts with Kap122 and requires *KAP122* for its nuclear localization. Our findings provide insights into the mechanism of nuclear import of $\beta\beta'$ and DNA damaged-induced $\beta\beta'$ redistribution.

Results

Nuclear Localization of $\beta\beta'$ Is Defective in the *kap122*Δ Mutant. We have recently shown that β and β' are transported across the nuclear envelope as one protein complex (X.A., Z.Z., K.Y., and M.H., unpublished data). Tagging either β or β' with a nuclear export sequence (NES) leads to cytoplasmic localization of both β and β'. Moreover, mutations at the β–β' heterodimer interface affect $\beta\beta'$ localization without disrupting the $\beta\beta'$ complex. Because nuclear transport of most proteins is mediated by the Kap-β proteins (46, 47), we decided to investigate whether $\beta\beta'$ was mislocalized in Kap-β mutants. There are 14 Kap-β proteins in *S. cerevisiae* (53). We examined $\beta\beta'$ IMF patterns in null mutants of the eight nonessential *KAP* genes (*KAP108*, *KAP114*, *KAP119*, *KAP120*, *KAP122*, *KAP123*, *KAP142*, and *LOS1*) and found that only *kap122*Δ exhibits significant $\beta\beta'$ mislocalization (Fig. 1*A*). Whereas 75–85% of WT cells in a logarithmic-phase culture display a predominantly nuclear $\beta\beta'$ signal, only 20–40% of *kap122*Δ cells do so (Fig. 1*B*). Most of the *kap122*Δ cells ($\approx$75% for β and $\approx$55% for β') display $\beta\beta'$ signals that are equally distributed between the nucleus and the cytoplasm (Fig. 1*B*). For the six essential *KAP* genes (*KAP95*, *KAP104*, *KAP109*, *KAP111*, *KAP121*, and *KAP124*), we compared the $\beta\beta'$ IMF patterns in temperature-sensitive (ts) mutants of each gene at permissive temperatures (PT) and nonpermissive temperatures (NPT). We observed partial loss of $\beta\beta'$ nuclear localization in most of these ts mutants even at PT (data not shown). However, we could not discern significant difference in the $\beta\beta'$ IMF patterns of these mutants between PT and NPT (data not shown). These results, in addition to suggesting the important role of *KAP122* in $\beta\beta'$ nuclear localization, also reflect the likely functional redundancy among the Kap-β proteins in $\beta\beta'$ transport.

Wtm1 and Wtm2 Are Found in the Same Protein Complex with $\beta\beta'$. The Kap proteins are known to interact with their cargo proteins either by direct binding or by indirect association via an adaptor protein (46, 47). To determine whether Kap122 interacts with $\beta\beta'$ and to identify any adaptor protein(s) that may be involved in $\beta\beta'$ transport, we used an N-terminally tagged $^{Flag}\beta$ expressed from the chromosomal *RNR2* locus to facilitate rapid purification of $\beta\beta'$ from yeast cell extract (28). SDS/PAGE analysis of the proteins purified by immunoaffinity chromatography subsequent to extensive washing revealed one major protein band and three or four minor bands (Fig. 2*A*). The amount of the major band accounts for 5–10% of the total protein in the sample. To

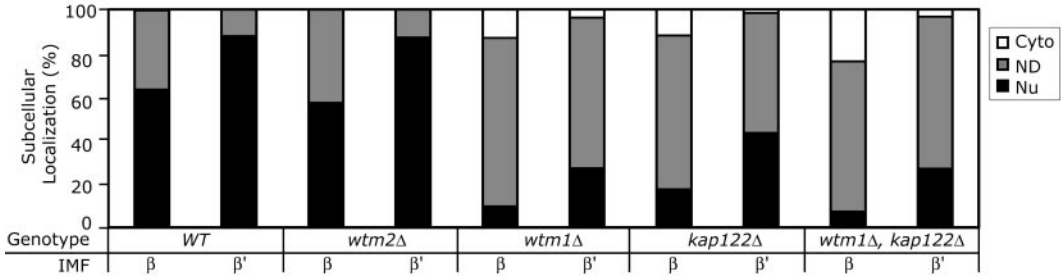

Fig. 3. *WTM1* is required for proper nuclear localization of β and β'. Quantitative analysis of the β and β' subcellular localization patterns in WT, *wtm1*Δ, *wtm2*Δ, *kap122*Δ, and *wtm1*Δ *kap122*Δ mutant cells by using IMF staining. The symbols for bar representation are the same as in Fig. 1*B*.

identify the proteins that copurified with $\beta\beta'$, the bands migrating slower than $\beta\beta'$ were cut from the gels and digested with trypsin, and the resulting peptides were subjected to MS/MS analysis. From the major band, 15 different peptides covering 48% of the Wtm1 protein sequence and 10 peptides covering 40% of the Wtm2 protein sequence were identified. Attempts to detect the proteins present in the minor bands resulted in identification of a small number of peptides matching the chaperones Ssa1 and Ssb1. Because these are highly abundant proteins that are found to copurify with numerous tagged proteins (54, 55), it is unclear whether they have any role in RNR regulation *in vivo*. Thus, Wtm1 and Wtm2 under normal growth conditions are tightly associated with $\beta\beta'$. No Kap122 was observed under these conditions, suggesting that the Kap122-$\beta\beta'$ interaction is either nonexistent or relatively weak.

A Wtm2–β interaction has been reported using an overexpressed Flag-tagged Wtm2 (54). To independently confirm the interaction among Wtm1, Wtm2, and $\beta\beta'$, C-terminally $(Myc)_{18}$-tagged Wtm1 and Wtm2 expressed from their own chromosomal loci (56) were purified from WT cell extract by an anti-Myc Ab affinity column. After extensive washing and SDS/PAGE, β and β' were shown to copurify with $Wmt2^{Myc}$ (Fig. 2*B*). Densitometry analysis of the Coomassie brilliant blue-stained bands revealed that $Wmt2^{Myc}$, β, and β' were present in approximately equal amounts. A similar experiment carried out with a $Wtm1^{Myc}$-containing strain revealed substantially less $\beta\beta'$ relative to Wtm1 (Fig. 2*C*). We believe that the apparently weaker Wtm1–$\beta\beta'$ association is due to interference by the $(Myc)_{18}$ tag at the C terminus of Wtm1, as an N-terminally $(Myc)_3$-tagged Wtm1 easily pulls down $\beta\beta'$ in IP (Fig. 2*D*). These results indicate that Wtm1 and Wtm2 are present in the same protein complex with $\beta\beta'$ *in vivo*.

β and β' Are Mislocalized in the *wtm1*Δ but Not the *wtm2*Δ Mutant. The observation of Wtm1 and Wtm2 in a complex with $\beta\beta'$ suggested that they might be involved in the nuclear localization of $\beta\beta'$. To test this possibility, we examined the subcellular localization patterns of β and β' in *wtm1*Δ and *wtm2*Δ by IMF. β and β' are mislocalized in *wtm1*Δ cells; 70–80% of *wtm1*Δ cells have ubiquitous β and β' signals in the nucleus and the cytoplasm, in comparison with the 10–35% observed in WT cells (Fig. 3). Interestingly, *wtm2*Δ cells display WT localization patterns of β and β' (Fig. 3). The degree of $\beta\beta'$ mislocalization is similar between the *wtm1*Δ and the *kap122*Δ mutants. Also, the $\beta\beta'$ localization patterns are not altered in the *wtm1*Δ *kap122*Δ double mutant in comparison with the single mutants (Fig. 3). Together, these results suggest that *WTM1* and *KAP122* function in the same pathway for determining the subcellular localization patterns of β and β'.

Wtm1 and Wtm2 Are Mislocalized in the *kap122*Δ Mutant. Wtm1 and Wtm2 have been shown to be nuclear proteins (57). The findings of Wtm1 and Wtm2 in complex with $\beta\beta'$ and of $\beta\beta'$ mislocalization in *wtm1*Δ suggest that Wtm1 (and probably, to a lesser extent, Wtm2) may act as an adaptor to help Kap122 import $\beta\beta'$ into the nucleus. If this hypothesis is the case, deletion of *KAP122* will lead to mislocalizations of not only $\beta\beta'$ but also Wtm1 and Wtm2. To test this possibility, we compared the subcellular localization patterns of a C-terminally tagged $Wtm1^{Myc}$ between WT and *kap122*Δ by IMF. $Wtm1^{Myc}$ is predominantly localized to the nucleus in WT cells (Fig. 4*A*). In contrast, $Wtm1^{Myc}$ is ubiquitously distributed between the nucleus and the cytoplasm in >90% of the *kap122*Δ cells and is cytoplasmic in the rest of the population (Fig. 4*B*). The loss of Wtm1 nuclear localization

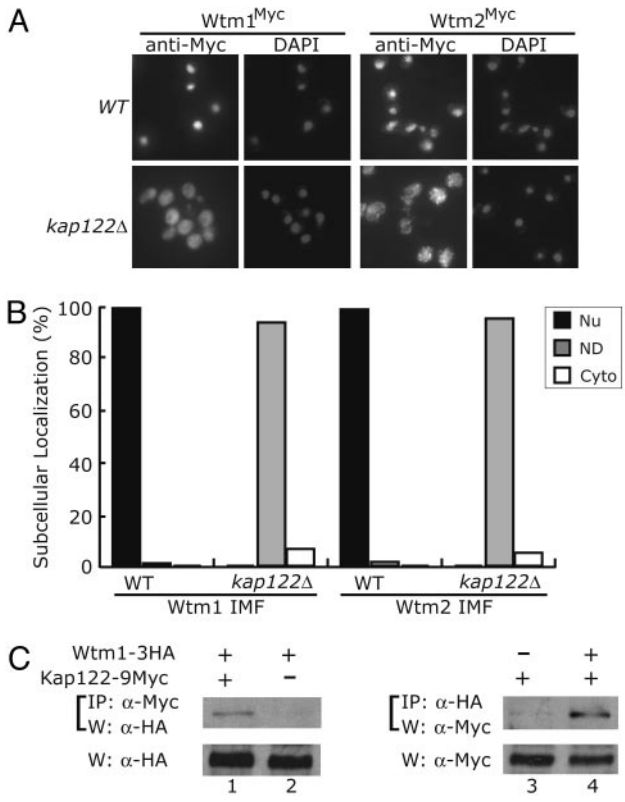

Fig. 4. Wtm1 and Wtm2 are nuclear proteins, and both require *KAP122* for their nuclear localization. (*A*) IMF images of $Wtm1^{Myc}$ and $Wtm2^{Myc}$, both integrated into their respective chromosomal loci, in WT and *kap122*Δ cells by using an anti-Myc Ab. (*B*) Quantitative analysis of $Wtm1^{Myc}$ and $Wtm2^{Myc}$ subcellular localization patterns in WT and *kap122*Δ cells. The symbols for bar representation are the same as in Fig. 1*B*. (*C*) Co-IP of Wtm1 and Kap122. Protein extracts were prepared from cells containing centromeric plasmids that express $Wtm1^{HA}$ and $Kap122^{Myc}$ from their native promoters. The immune complexes from anti-HA and anti-Myc IPs (*Upper*) and total protein extracts (*Lower*) were probed with the indicated Abs on Western blots (W).

in *kap122*Δ was independently confirmed by using an N-terminally tagged ^{Myc}Wtm1 (data not shown). Similarly, we found that a Wtm2^{Myc} also change from being predominantly nuclear in WT cells to being ubiquitously localized in *kap122*Δ cells (Fig. 4 *A* and *B*). Based on these findings, we conclude that *KAP122* is needed for the proper nuclear localization of Wtm1 and Wtm2.

Wtm1 Interacts with Kap122 *in Vivo*. Although *KAP122* is needed for the nuclear localization of $\beta\beta'$, no Kap122-$\beta\beta'$ interaction is detected in a yeast two-hybrid system or by co-IP (data not shown). To examine the possibility that Wtm1 acts as an adaptor between $\beta\beta'$ and Kap122, we focused our attention on determining whether there is any physical interaction between Wtm1 and Kap122 *in vivo* by co-IP. Abs to neither Wtm1 nor Kap122 were available. Therefore, we made centromeric (low copy number) plasmids that express C-terminally epitope-tagged Wtm1-hemagglutinin $(HA)_3$ and Kap122-$(Myc)_9$ under their respective native promoters. These two plasmids were transformed either individually or together into the WT strain. Immune complexes were generated by IP using anti-Myc and anti-HA Abs and probed for Wtm1^{HA} and Kap122^{Myc} by Western blotting. In cells expressing both Wtm1^{HA} and Kap122^{Myc}, Wtm1^{HA} was detected in the immune complex precipitated by anti-Myc (Fig. 4*C*, lane 1) and Kap122^{Myc} was detected in the immune complex precipitated by anti-HA (Fig. 4*C*, lane 4). In contrast, no Wtm1^{HA} was precipitated by anti-Myc when Kap122^{Myc} is absent, nor was Kap122^{Myc} precipitated by anti-HA when Wtm1^{HA} was absent (Fig. 4*C*, lanes 2 and 3). These results indicate that Wtm1 and Kap122 interact *in vivo*, which is consistent with the hypothesis that Wtm1 acts as an adaptor between Kap122 and $\beta\beta'$.

Both β' and Wtm1 Shuttle Between the Nucleus and the Cytoplasm. Although $\beta\beta'$ and Wtm1 are predominantly nuclear proteins, they may shuttle between the nucleus and the cytoplasm. The finding that β and β' are partially localized to the cytoplasm during the S phase (12) suggests that they shuttle. A standard test for shuttling proteins is the heterokaryon assay, in which a WT (*KAR1*) strain bearing a *GAL1* promoter-controlled β'-GFP- or Wtm1-GFP-expressing plasmid is crossed with a strain containing a *kar1–1* mutation that prevents nuclear fusion in zygotes (58). Movement of a GFP fusion protein from the *KAR1* nucleus to the *kar1–1* nucleus, in the absence of new protein synthesis, indicates nucleus-and-cytoplasm shuttling. Synthesis of β'-GFP or Wtm1-GFP was induced for 90 min in the *KAR1* cells and terminated by change of carbon source. The GFP signals were found exclusively in the *KAR1* nucleus 2 h after promoter shutoff, at which time mating was initiated and the GFP signals were monitored in zygotes. In some zygotes, the GFP signal had decreased beyond detection by the time the new buds emerged. In zygotes in which GFP was visible, a fraction of them contain β'-GFP (5/21) and Wtm1-GFP (4/16) in the newly introduced *kar1–1* nuclei (Fig. 5). As a control, we monitored the localization of a GFP fusion to histone 2B (Htb1-GFP), which has been shown to be nonshuttling (59, 60). In the same assay, Htb1-GFP was found to be limited to a single nucleus in all observed zygotes ($n = 23$; Fig. 5). Together, these results suggest that both β' and Wtm1 shuttle between the nucleus and the cytoplasm.

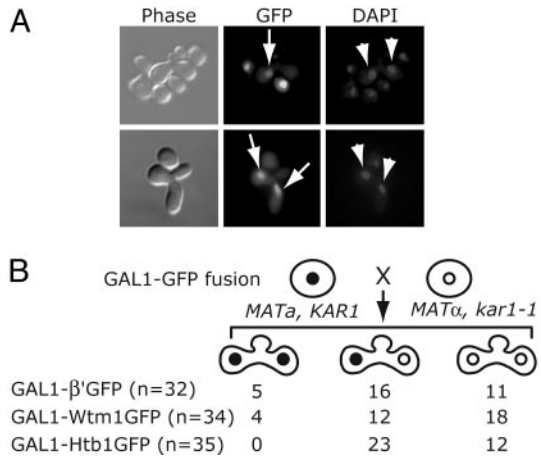

Fig. 5. Wtm1 and β' both can shuttle into an introduced nucleus in heterokaryons. *GAL1* promoter-driven expression of the GFP-tagged proteins were induced for 90 min in *MATa*, *KAR1* cells in a galactose-containing medium and then shut off by glucose addition. Mating with *MATα*, *kar1* cells was performed 2 h after promoter shutoff, when >97% of the *MATa*, *KAR1* cells had bright green nuclei. After zygotes were formed and a new bud had emerged from each zygote (2–3 h), the GFP-tagged proteins were visualized in live cells. (*A*) Images of zygotes with a single GFP-positive nucleus (*Upper*) or two GFP-positive nuclei (*Lower*). (*B*) Locations of β'-GFP, Wtm1-GFP, and Htb1-GFP in heterokaryons.

Discussion

The maintenance of genome stability requires high fidelity in DNA replication and repair processes, which is influenced by the sizes and relative ratios of the intracellular dNTP pools. Eukaryotic cells have evolved complex mechanisms to maintain adequate and balanced dNTP pools by regulating the activity of RNR, a key enzyme in dNTP biosynthesis (15). In addition to allosteric regulation, RNR is regulated by transcriptional repression (10), protein inhibitor association (3, 11), and subcellular compartmentalization (12, 13). In *S. cerevisiae*, the RNR small subunit $\beta\beta'$ undergoes nucleus-to-cytoplasm redistribution to be colocalized with the large subunit upon DNA damage (13). In this work, we demonstrate that the $\beta\beta'$ nuclear localization is defective in cells missing either the Kap-β homolog *KAP122* or the WD40 repeat protein *WTM1*. Wtm1 and its paralog Wtm2 are found in the same protein complex as the $\beta\beta'$ heterodimer. Interestingly, only the *wtm1*Δ mutant is defective in $\beta\beta'$ nuclear localization. Although *KAP122* is needed for proper nuclear localization of $\beta\beta'$, no Kap122-$\beta\beta'$ interaction was detected. Instead, Kap122 interacts with Wtm1 *in vivo* and is need for proper nuclear localization of Wtm1 and Wtm2, suggesting that Wtm1 and Wtm2 may be cargo proteins of Kap122. Also, we show that both β' and Wtm1 can shuttle between the nucleus and the cytoplasm.

Our results suggest two possible mechanisms by which Wtm1 and Kap122 could participate in $\beta\beta'$ nuclear localization. Wtm1 (and probably also Wtm2) may act as an adaptor between Kap122 and $\beta\beta'$ to facilitate the nuclear import of $\beta\beta'$ (Fig. 6*A*). Alternatively, Wtm1 and Wtm2 may be imported directly by Kap122, whereas $\beta\beta'$ may be imported by means of an unidentified mechanism (Fig. 6*B*). When imported into the nucleus, Wtm1 and Wtm2 may act as an anchor to prevent $\beta\beta'$ from being exported back to the cytoplasm (Fig. 6*C*). The two models are not mutually exclusive, because Wtm1 and Wtm2 may participate in both import and nuclear anchorage of $\beta\beta'$. Our result that Wtm1 and β' shuttle between the nucleus and the cytoplasm is consistent with the import adaptor model. The similarity in $\beta\beta'$ mislocalization phenotypes between the *wtm1*Δ*kap122*Δ double mutant and each single mutant is consistent with both models. However, the finding that the *kap122*Δ mutant exhibits a more severe mislocalization of Wtm1 and Wtm2 than mislocalization of $\beta\beta'$ supports the nuclear anchor model.

Defects in RNR regulation often result in activated checkpoint and increased genome instability (3, 23, 61). Overexpression of the yeast β or the human R2 C-terminal domain has been shown to cause increased loss of a supernumerary chromosome in yeast (6). Deletion of the *WTM* genes has been correlated with increased silencing at the *HMR* locus and the telomeres (57), although it is

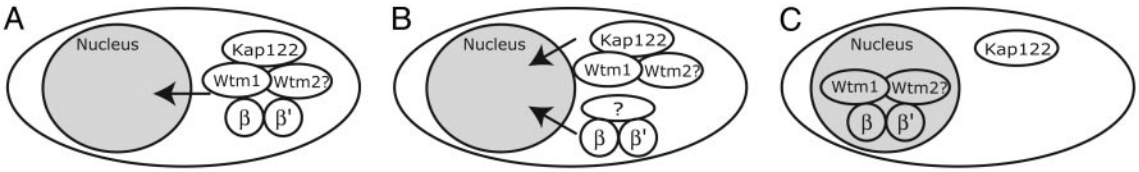

Fig. 6. Models for the roles that Kap122, Wtm1, and Wtm2 have in the nuclear localization of the RNR small subunit. (*A*) Kap122, Wtm1, and Wtm2 directly participate in the nuclear import of $\beta\beta'$, with Wtm1 acting as an adaptor between Kap122 and $\beta\beta'$. (*B*) Wtm1 and Wtm2 are imported by Kap122, whereas $\beta\beta'$ is imported by an unidentified protein. (*C*) In both scenarios as shown in *A* and *B*, Wtm1 and Wtm2 may act as an anchor to retain $\beta\beta'$ in the nucleus.

unclear whether this defect is due to defective RNR regulation. Interestingly, both *kap122*Δ and *rnr1*Δ have been identified in a genetic screen for mutants with elevated Ty1 retrotransposon mobility, a screen that also yielded many genes that are known to be required for maintenance of genome integrity (62).

The Kap-β family of transport receptors facilitate transport of numerous proteins across the nuclear envelope (46, 47). How each Kap recognizes specific cargo proteins has been the subject of intense study. The only characterized cargo proteins of Kap122 are two TFIIA subunits, Toa1 and Toa2 (63). In this study, we have identified four potential cargo proteins of Kap122: Wtm1, Wtm2, β, and β'. The two WD40 repeat proteins, Wtm1 and Wtm2, were originally identified as nuclear proteins involved in transcriptional modulation (57). Wtm1 is more abundant than Wtm2, and both proteins are able to associate with themselves and each other (57). We show that Wtm1 and Wtm2 require the same Kap for nuclear localization. However, we do not know whether all Wtm2 is in complex with Wtm1. It is possible that Wtm2 may have a minor role in comparison with Wtm1 in the nuclear import or anchorage of $\beta\beta'$. This difference may explain why the *wtm2*Δ deletion does not affect $\beta\beta'$ localization. The stoichiometries of the Wtm–$\beta\beta'$ and Wtm–Kap122 complexes remain to be determined.

How is the $\beta\beta'$ subcellular localization regulated by the DNA-damage checkpoint? The levels of Wtm1 and Wtm2 do not change significantly, and both proteins remain mostly in the nucleus upon DNA damage (data not shown). Thus, we surmise that decreased import and/or disrupted anchorage of $\beta\beta'$, which possibly resulted from a decrease in Wtm–$\beta\beta'$ association, could contribute to $\beta\beta'$ redistribution. We show that Wtm1 and β' both can shuttle between the nucleus and the cytoplasm. Proteins undergoing nucleus/cytoplasm shuttling often contain signals for both nuclear import [nuclear localization signal (NLS)] and export, and their steady state localization patterns are determined by the relative activities of the NLS and the NES. Hence, mechanisms that affect the NLS and NES activities of a protein can regulate its nucleocytoplasmic distribution (64). Phosphorylation events adjacent to the NLS or NES of a protein have been shown to affect its nuclear transport (65, 66). It is possible that phosphorylation of Wtm1 or $\beta\beta'$ by a checkpoint kinase changes the relative NES/NLS strength ratio, which leads to $\beta\beta'$ redistribution. Further investigation is needed to address these questions.

Materials and Methods

Strains and Plasmids. The following strains were derived from Y300 (*MATa*; *can1–100*; *ade2-1*; *his3-11,15*; *leu2-3,112*; *trp1-1*; and *ura3-1*) (67): MHY343 (*rnr2*::*Flag-RNR2*), MHY685 [*wtm1*::*WTM1*-$(Myc)_{18}$], MHY686 [*wtm2*::*WTM2*-$(Myc)_{18}$], and MHY688 [*wtm1*::*WTM1*-$(Myc)_{18}$, *kap122*::*Kan*]. The *wtm1*, *wtm2*, and all *kap* deletion strains were obtained from the European *Saccharomyces Cerevisiae* Archive for Functional Analysis (EUROSCARF; Johann Wolfgang Goethe University, Frankfurt); all EUROSCARF strains were derived from BY4741 (*MATa*, *his3*Δ*1*, *leu2*Δ*0*, *met15*Δ*0*, and *ura3*Δ*0*) (68), along with MHY673 (*wtm1*::*Kan* and *kap122*::*kan*). Strain 211 (MATα, kar1–1, ade1, ade2, his7, trp1, leu2, and arg4) was obtained from R. Sclafani (University of Colorado Health Sciences Center). Yeast growth and genetic manipulation were as described in ref. 69.

Plasmid pMH1030 contains *KAP122*-$(Myc)_9$ (Ap^r *CEN TRP1*); pMH1332 contains *WTM1*-$(HA)_3$ (Ap^r *CEN HIS3*); pMH1326 and pMH1344 contain *GAL1-RNR4-GFP* and *GAL1-HTB1-GFP*, respectively (Ap^r *CEN URA3*); pMH1351 contains *GAL1-WTM1-GFP* (Ap^r 2μ *URA3*); and pMH1445 contains $(Myc)_3$-*WTM1* under the *WTM1* promoter (Ap^r *CEN TRP1*).

Abs. Polyclonal anti-β and anti-β' Abs are described in ref. 12. Anti-Myc (9E10) and anti-HA (12CA5) mAbs were obtained from Roche Applied Sciences (Indianapolis). Horseradish peroxidase- and FITC-conjugated goat anti-mouse and goat anti-rabbit Abs were obtained from Jackson ImmunoResearch. Anti-Zwf1 was obtained from Sigma.

Indirect IMF. IMF staining of yeast spheroplasts and image acquisition were performed by using an E-800 microscope (Nikon) as described in ref. 12. DNA was visualized by DAPI.

Co-IP and Western Blot Analysis. Yeast cells were harvested from early logarithmic-phase cultures ($\approx 2 \times 10^7$ cells per ml). For Western blotting, protein extract was prepared from trichloroacetic acid-treated yeast cells by using the glass-beads disruption method as described in ref. 12. All IP steps were performed at 4°C. Protein extract was prepared in buffer B (50 mM Hepes·KOH, pH 7.5/140 mM NaCl/1 mM EDTA/1% Triton X-100/0.1% $C_{24}H_{39}O_4Na$/1 mM PMSF/1 μg/ml each of leupeptin and pepstatin A) by using the glass-beads disruption method and centrifuged at 13,000 × *g* for 15 min to remove debris. We diluted 250–500 μg of protein extract to 500 μl in buffer B and incubated with 5–10 μg of primary Ab overnight. The Ab–protein complexes were precipitated by absorption to protein A–Sepharose beads for 2 h. After repeated washes of the beads with buffer B, the immune complexes were eluted into SDS sample buffer and resolved by SDS/PAGE. Western blots were performed as described in ref. 12.

Identification of Proteins Copurifying with ^{Flag}Rnr2 by MS. $^{Flag}\beta$ was purified by using an anti-Flag column as described in ref. 28. Samples of $^{Flag}\beta\beta'$ (40 μg) eluted from the column after extensive washing were analyzed on a 5–15% gradient gel and stained by Coomassie brilliant blue. Several bands that migrated more slowly than $^{Flag}\beta$ were observed. They were cut out of the gel and submitted to the Protein Sciences Facility at the University of Illinois at Urbana–Champaign. Each sample was digested with 25 μl of sequencing-grade modified trypsin (Promega) that was reconstituted in 25 mM NH_4HCO_3 at 12.5 ng/μl for 8 h at 37°C. The resulted peptides were extracted three times by using a solution of 50% CH_3CN/5% formic acid. The pooled extracts were lyophilized, and the sample was redissolved in 12 μl of 5% CH_3CN/0.1% formic acid. The resulting peptides were separated by liquid chromatography and identified by MS/MS using a CapLC system (Waters) with a C-18 NanoEase Symmetry column (150 mm; 3.5-μm particles, 300-Å pore) coupled to a Q-TOF Global Ultima (Micromass, Manchester, U.K.) by

using the software package PROTEINLYNX GLOBAL SERVER (Version 2.1; Waters).

Copurification of Wtm1^{Myc} and Wtm2^{Myc} with $\beta\beta'$. MHY685 and MHY686 were grown in 6 liters of yeast extract/peptone/dextrose to midlogarithmic phase. Protein extract was prepared as described in ref. 28. The extract was incubated with 750 μl of anti-Myc agarose for 1 h at 4°C, and the mixture was poured into a column. After washing the column with 100 ml of buffer A (25 mM Hepes, pH 7.5/5 mM $MgCl_2$/50 mM KCl/10% glycerol/0.1 mM DTT) supplemented with protease inhibitors (28), the bound proteins were eluted with 5 ml of 100 mM NH_4OH. The eluent was neutralized by addition of 1 M HOAc, concentrated with a Centriprep YM10 (Millipore) to <500 μl, and analyzed by SDS/PAGE.

Heterokaryon Analysis. BY4741 cells that were transformed with pMH1326, pMH1344, and pMH1351 were grown to $\approx 2 \times 10^7$ cells per ml in a glucose-containing medium, diluted 1:50, and grown to $\approx 5 \times 10^6$ cells per ml in a raffinose-containing medium. Synthesis of β'-GFP, Wtm1-GFP, and Htb1-GFP was induced by addition of 2% galactose for 90 min and terminated by switching the carbon source to glucose. At 2 h after promoter shutoff, $\approx 5 \times 10^6$ cells of each strain were mixed with an equal number of strain 211 cells in 100 μl of yeast extract/peptone/dextrose liquid before being spotted on a yeast extract/peptone/dextrose plate at 24°C. After 2–3 h, ≈20% of the cells were zygotes. The GFP signal was visualized by using an E-800 microscope (Nikon).

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