Blm10 facilitates nuclear import of proteasome core particles

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Short-lived proteins are degraded by proteasome complexes, which contain a proteolytic core particle (CP) but differ in the number of regulatory particles (RPs) and activators. A recently described member of conserved proteasome activators is Blm10. Blm10 contains 32 HEAT-like modules and is structurally related to the nuclear import receptor importin/karyopherin β . In proliferating yeast, RP-CP assemblies are primarily nuclear and promote cell division. During quiescence, RP-CP assemblies dissociate and CP and RP are sequestered into motile cytosolic proteasome storage granuli (PSG). Here, we show that CP sequestration into PSG depends on Blm10, whereas RP sequestration into PSG is independent of Blm10. PSG rapidly clear upon the resumption of cell proliferation and proteasomes are relocated into the nucleus. Thereby, Blm10 facilitates nuclear import of CP. Blm10-bound CP serves as an import receptor–cargo complex, as Blm10 mediates the interaction with FG-rich nucleoporins and is dissociated from the CP by Ran-GTP. Thus, Blm10 represents the first CP-dedicated nuclear import receptor in yeast.

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Introduction

A typical feature of many neurodegenerative disorders is the accumulation of misfolded proteins in inclusion bodies, which results from failures in quality control mechanisms and is often found in non-dividing cells such as mammalian neurons. As misfolded and aggregation prone polypeptides are potentially toxic, their sequestration into protein inclusions may not be detrimental *per se* but it may rather facilitate

their sorting and clearance. The key protease complex responsible for the degradation of misfolded proteins is the proteasome. Its inhibition causes cell-cycle arrest and the formation of protein aggregates (Kaganovich *et al*, 2008).

Proteasomes exist in different configurations. All configurations contain a proteolytically active core particle (CP), but differ in the number of accessory proteins and regulatory particles (RPs). The association of the RP with the CP is required to open the CP and to activate the degradation of poly-ubiquitylated substrates (Peth *et al*, 2010).

The CP consists of a stack of four coaxial rings, two outer rings composed of α subunits and two inner rings of β subunits (Groll *et al*, 1997). CP assembly starts with two inactive precursor complexes, called half-CP, that contain the CP-dedicated chaperone Ump1. Upon their dimerization, the active sites are liberated by β -propeptide processing and Ump1 is degraded (Ramos and Dohmen, 2008). CP maturation is completed with α ring closure (Groll *et al*, 2000). In addition to the constitutive proteasome in yeast and mammals, mammalian cytokine-inducible isoforms of the proteasome serve immune-specific tasks (Kloetzel, 2004; Huber *et al*, 2012).

In proliferating mammalian and yeast cells, constitutive proteasomes primarily localize in the nucleus, indicating that the nucleus is the major site for proteasomal proteolysis during cell-cycle progression (Tanaka, 2009). The classical transport of proteins through nuclear pore complexes generally requires nuclear localization sequences (NLSs) and NLS receptors, called importins/karyopherins. These karyopherins use the chemical potential of the nucleocytoplasmic Ran-GTP gradient to act as unidirectional cargo pumps (Fried and Kutay, 2003; Wentz and Rout, 2010).

In dividing yeast, the classical NLS pathway is responsible for nuclear import of proteasomal subcomplexes (Lehmann *et al*, 2002; Wendler *et al*, 2004; Isono *et al*, 2007; Chen *et al*, 2011). Classical NLS is present within the α rings of the CP (Wang *et al*, 1997; Lehmann *et al*, 2002). Two conformations of the CP were proposed to exist, one import-competent conformation with exposed NLS and one import-incompetent with masked NLS (Tanaka *et al*, 1990). Since proteasomal precursor complexes exist in the nucleus of yeast and mammalian cells, precursor complexes rather than mature CP are imported into the nucleus (Lehmann *et al*, 2002; Kremer *et al*, 2010). This conclusion was supported by different reconstitution systems of nuclear import showing that mature CP is not imported into the nucleus by the classical NLS pathway (Mayr *et al*, 1999; Lehmann *et al*, 2002; Savulescu *et al*, 2011). Possibly, only precursor complexes exhibit accessible NLS due to disordered gates in the α rings, whereas the NLS of mature CP is buried once the α rings are closed (Lehmann *et al*, 2002).

In contrast to dividing yeast cells, the majority of CP and RP are not associated (Bajorek *et al*, 2003) and do not localize in the nucleus of non-dividing cells. Instead, CP and RP are sequestered into motile cytosolic clusters, named proteasome

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storage granuli (PSG) (Laporte *et al*, 2008). Cell cycle-arrested proteasomal mutants or wild-type cells exposed to proteasome inhibitors also sequester proteasomes into juxtanuclear quality control compartments (JUNQ) (Kaganovich *et al*, 2008). JUNQ and PSG have in common that they rapidly clear when cell growth resumes (Kaganovich *et al*, 2008; Laporte *et al*, 2008). Reversible cytosolic clusters of proteasomes are observed in both yeast and mammalian cells, suggesting that the mechanism underlying their formation and clearance is conserved (Kaganovich *et al*, 2008; Enenkel, 2013).

Blm10, a conserved high molecular mass protein with 32 HEAT repeat-like modules (Kajava *et al*, 2004), has recently been identified as an activator of CP. The function of Blm10 is not yet understood (Savulescu and Glickman, 2011; Stadtmueller and Hill, 2011). Blm10 binds precursor complexes (Fehlker *et al*, 2003; Li *et al*, 2007; Marques *et al*, 2007) suggesting a function in CP maturation. It is also associated with mature CP as Blm10-CP, Blm10-CP-Blm10 and RP-CP-Blm10 hybrids (Schmidt *et al*, 2005) with a preference for CP with disordered α ring gates (Lehmann *et al*, 2008). Furthermore, Blm10 induces opening of the CP gate (Sadre-Bazzaz *et al*, 2010). Hence, peptide hydrolysis is enhanced within Blm10-CP but repressed in Blm10-CP-Blm10 (Lehmann *et al*, 2008). The presence of Blm10-CP-RP hybrids is required for the degradation of specific transcription factors (Dange *et al*, 2011; Lopez *et al*, 2011).

X-ray structure of Blm10-CP-Blm10 revealed a superhelical dome-like structure of Blm10 on top of the CP α ring that appeared to be consistent with possible functions as an adaptor, an assembly factor, or an inhibitor (Sadre-Bazzaz *et al*, 2010). The arrangement of Blm10's α helices in a toroid fold is similar to the solenoid fold of karyopherin β (Glickman and Raveh, 2005; Huber and Groll, 2012). However, the shared structural features do not necessarily mean that HEAT-repeat proteins like Blm10 function as a β karyopherin.

Here, we show in non-dividing yeast cells that CP sequestration into PSG depends on the presence of Blm10, whereas RP sequestration into PSG occurs independently of Blm10. When quiescent cells resume proliferation, Blm10 facilitates nuclear uptake of mature CP. Reconstituted import assays of yeast CP into *Xenopus* nuclei and localization studies in yeast mutants with defective Ran-GTPase cycle revealed that Blm10 behaves like a CP-specific transport receptor. Blm10 mediates the interaction of the CP with Nup53, a phenylalanine-glycine-rich nuclear pore protein (FG-nucleoporin). As a nuclear import receptor-cargo complex Blm10-bound CP is dissociated by Gsp1-GTP, the yeast homologue of Ran-GTP, whereupon Blm10 remains associated with Gsp1-GTP. The facilitated nuclear import of the CP by Blm10 upon the exit from quiescence allows the rapid reactivation of proteasomal proteolysis in the nucleus.

Results

CP sequestration into PSG is mediated by Blm10

Green fluorescent protein (GFP) labelling of proteasomal subunits is a well-established approach to monitor CP movements between the nucleus and PSG in living *Saccharomyces cerevisiae* cells (Laporte *et al*, 2008). In particular, the amenability of yeast to homologous recombination techniques facilitates the chromosomal replacement of CP

subunits by GFP-tagged versions. Almost all CP subunits can be replaced by functional GFP-tagged versions, but we favour $\alpha 4$ and $\beta 5$ as GFP targets, since we have previously confirmed that their GFP fusion proteins are fully incorporated into the CP (Lehmann *et al*, 2008). Independent of which CP subunit was chosen as a GFP target, wild-type cells displayed the same phenotype, namely the sequestration of $\alpha 4$ and $\beta 5$ into PSG of quiescent yeast cells (Figure 1A; see also Supplementary Figure S1A). As soon as resting cells resume growth, $\alpha 4$ and $\beta 5$ rapidly move into the nucleus, where proteasomes are mainly localized during cell proliferation (Enenkel *et al*, 1998; Laporte *et al*, 2008).

As we were working on Blm10 with regard to proteasome assembly, we were puzzled by the observation that PSG phenotypes were not displayed by *blm10* Δ cells grown to stationary phase.

Instead, the CP showed no selective localization and only a faint decoration of NE/ER-resident CP, suggesting that most CP were exported into the cytoplasm but not sequestered into PSG (Figure 1B). As seen in wild-type cells before, GFP-labelled $\beta 5$ was primarily nuclear in logarithmic phase confirming that *BLM10* deletion has no impact on CP localization during cell proliferation (Fehlker *et al*, 2003). Blm10 was also discovered in a synthetic genetic array in the yeast null mutant collection which we performed in search for proteins regulating PSG formation and clearance (CE and Brenda Andrews, personal communication).

Next, we examined whether the sequestration of the RP into PSG is dependent on Blm10. To test this, wild-type and *blm10* Δ strains were created that express GFP-labelled Rpn11 (RP lid) or Rpn1 (RP base) instead of the endogenous subunits. These GFP-labelled subunits of the RP lid and base are fully incorporated into the RP, thus suited to report on RP lid and base localizations (Wendler *et al*, 2004). Direct fluorescence microscopy confirmed that RP is nuclear in proliferating wild-type and *blm10* Δ cells. In both wild-type and *blm10* Δ cells grown to stationary phase, GFP-labelled Rpn1 and Rpn11 were sequestered into PSG suggesting that the sequestration of RP components into PSG does not depend on Blm10 (Supplementary Figure S1B).

We then investigated whether Blm10 itself is sequestered into PSG during stationary phase. Wild-type cells expressing GFP-labelled Blm10 were monitored by direct fluorescence microscopy. Blm10-GFP localized to the nucleus during logarithmic phase as shown before (Fehlker *et al*, 2003; Schmidt *et al*, 2005) and primarily localized to PSG during stationary phase (Figure 1C). The protein level of Blm10 increased in stationary phase compared with logarithmic phase as anticipated by gene transcription arrays (Lopez *et al*, 2011).

To test whether an excess of Blm10 results in the sequestration of CP into PSG, we analysed the localization of GFP-labelled $\alpha 4$ upon induced overexpression of Blm10 in *blm10* Δ cells. Indeed, GFP-labelled $\alpha 4$ was targeted into PSG upon Blm10 overexpression (Figure 1D). Comparable to quiescence, induced overexpression of Blm10 results in RP-CP dissociation and cell-cycle arrest (Lehmann *et al*, 2008).

RP-CP assemblies dissociate and Ump1-containing precursor complexes are depleted during quiescence

Since RP-CP assemblies dissociate in cells grown to stationary phase (Bajorek *et al*, 2003) and most CP and RP are found within PSG in these cells (Laporte *et al*, 2008), it is reasonable

to assume that PSG do not contain RP-CP assemblies. To test this hypothesis, we subjected lysates of wild-type and *blm10Δ* cells expressing GFP-labelled β5 and haemagglutinin-epitope (HA)-tagged Ump1 to native PAGE. CP configurations were visualized by fluoroimaging of the GFP moieties. Ump1-containing precursor complexes, Blm10-bound CP species, and RP were detected by immunoblotting.

Lysates of wild-type and *blm10Δ* cells grown to logarithmic phase were analysed first to categorize CP configurations under these conditions. Consistent with previous work, RP-CP-RP, Blm10-CP-RP, RP-CP, Blm10-CP-Blm10, Blm10-CP, CP and precursor complexes were present in wild-type cells (Li *et al*, 2007; Lehmann *et al*, 2008). All CP configurations except of those bound to Blm10 were present in *blm10Δ* cells (Figure 2A).

Consistent with previous studies of cells grown to stationary phase (Bajorek *et al*, 2003), we found that the equilibrium between RP-CP assemblies and RP-free CP shifted towards RP-free CP such that only a few RP-CP assemblies remained in stationary phase. These RP-CP remnants may account for

the faint nuclear staining that is still visible in quiescent *blm10Δ* and wild-type cells (Figure 1B). RP-CP assemblies are fully dissociated in prolonged quiescence (Bajorek *et al*, 2003) and sequestered into PSG (Laporte *et al*, 2008). As shown in Figure 2B, most CP occur as free particles or bound to Blm10 as in the case of wild-type cells. During quiescence, CP precursor complexes were not detectable due to repressed protein synthesis (see Supplementary Figure S1C). Most RP seem to migrate as RPnn and RPn oligomers as reported by previous studies (Kleijnen *et al*, 2007) (for the assignment of CP and RP species, see Supplementary Figure S2).

Blm10 facilitates nuclear import of CP upon the resumption of cell growth

Previous localization studies revealed that the addition of fresh medium to quiescent wild-type cells results in the mobilization of GFP-labelled CP from PSG into the nucleus within a few minutes (Laporte *et al*, 2008) (Figure 3A; upper panel). In the absence of Blm10, CP import into the nucleus is delayed for 2 h (Figure 3A, lower panel). At this time point, GFP-labelled CP reappeared in the nucleus of *blm10Δ* cells, because new CP precursor complexes were synthesized that can be imported into the nucleus independently of Blm10 (Lehmann *et al*, 2002).

Then, we tested whether the mobilization of RP from PSG into the nucleus is affected by the absence of Blm10. We found that nuclear re-import of GFP-labelled Rpn1 and Rpn11, representing RP base and lid, respectively, does not depend on Blm10 (shown for RP lid, Supplementary Figure S3A). Due to the delay of CP import, the formation of RP-CP assemblies is significantly slower in *blm10Δ* cells than in wild-type cells (Supplementary Figure S3B).

When quiescent cells resume growth, pre-assembled and mature CP must be imported into the nucleus and reassembled into active RP-CP-RP holoenzymes as precursor complexes are not available due to the repression of protein

Figure 1 CP sequestration into PSG is mediated by Blm10. (A) Wild-type cells expressing cherry Red (RFP)-labelled histone H2A and GFP-tagged β5 were grown to stationary phase and monitored by direct fluorescence microscopy using Nomarski optics, RFP and GFP filter sets (upper panels). Upon the addition of fresh YPD medium, cells exit quiescence and resume proliferation. The CP rapidly moves into the nucleus where it is primarily localized during cell proliferation (lower panels). (B) Wild-type and *blm10Δ* cells expressing GFP-tagged β5 were grown to stationary phase and monitored by direct fluorescence microscopy using Nomarski optics and GFP filter sets (left panels). Quiescent *blm10Δ* cells expressing GFP-tagged β5 and RFP-labelled histone H2A were monitored by direct fluorescence microscopy (right panels). (C) Wild-type cells expressing GFP-tagged Blm10 were grown to logarithmic and stationary phases, respectively, and monitored by direct fluorescence microscopy. Lysates were analysed by SDS-PAGE followed by immunoblotting against Blm10-GFP, CP subunit α4, RP base subunit Rpt1 and RP lid subunit Rpn5; lane 1, logarithmic phase; lane 2, stationary phase. (D) Direct fluorescence microscopy of *blm10Δ* cells expressing GFP-labelled α4 upon overexpression of Blm10 (left panel). Note that galactose-induced overexpression of Blm10 is induced during logarithmic phase. Thus, CP is nuclear before induction. During induction of Blm10 overexpression cells cease to grow and behave like quiescent cells (Lehmann *et al*, 2008). Overexpression of Blm10 is confirmed by western blotting (right panel). Bars, 2 μm; protein molecular weight (kDa). See also Supplementary Figures S1B and C for *in vivo* localization studies of RP and Ump1-associated precursor complexes in proliferating and quiescent wild-type and *blm10Δ* cells. Source data for this figure is available on the online supplementary information page.

Figure 2 Dissociation of RP-CP assemblies and depletion of Ump1-containing precursor complexes during stationary phase. CP configurations were resolved by native PAGE in lysates of wild-type and *blm10 Δ* cells expressing CP with GFP-tagged β 5 and HA-tagged Ump1. (A) Cells were grown to logarithmic phase and disintegrated. Equal amounts of proteins were loaded. The GFP moieties were visualized in gel by fluoroimaging (upper left panel) and CP configurations were assigned (see Supplementary Figure S2). Blm10-, Ump1-, and RP (Rpt1)-containing protein complexes were detected by immunoblotting (middle and right panels). As a loading control, cell lysates were subjected to SDS-PAGE followed by immunoblotting against β 5-GFP, Ump1-HA, and Blm10 (lower left panel); matured (m-) and unprocessed (pro-) β 5-GFP. (B) Wild-type and *blm10 Δ* cells described above were grown to stationary phase and analysed by native PAGE followed by fluoroimaging and immunoblotting against Ump1-HA, Blm10, and Rpt1. RPnn and RPn oligomers were assigned according to Kleijnen *et al* (2007). As a loading control, cell lysates were subjected to SDS-PAGE followed by immunoblotting against β 5-GFP; protein molecular weights (kDa). Source data for this figure is available on the online supplementary information page.

synthesis. Thus, pre-assembled and mature CPs are most likely imported by an alternative pathway than what has been described for newly synthesized precursor complexes. Since reconstituted nuclei from *Xenopus* eggs were successfully used to study nuclear import of pre-assembled *Xenopus* CP (Savulescu *et al*, 2011), we examined whether Blm10 is able to promote nuclear import of yeast CP into reconstituted *Xenopus* nuclei. Eggs do not divide, thus share with quiescent yeast cells that they need rapid import of mature CP into the nucleus upon the resumption of cell proliferation.

Mature CP was purified from yeast, labelled with the fluorescent dye Oregon Green and incubated with and without Blm10. To confirm that Oregon Green-labelled CP associates with Blm10, native PAGE followed by fluoroimaging and peptide cleavage assays were performed (Figure 3C; see Supplementary data). Blm10-bound Oregon Green-labelled CP was added to reconstituted *Xenopus* nuclei. Confocal fluorescence microscopy revealed a significant increase in nuclear rim and intranuclear staining in the presence of Blm10 compared with the control without Blm10 (Figure 3B). The mean fluorescence pixel intensities at the nuclear rim and inside the nucleus were scored from three

independent experiments and compiled in a histogram. In the presence of Blm10, a two-fold increase in Oregon Green-labelled CP inside the nucleus was notified (Figure 3D). The observation that Oregon Green-labelled CP from yeast binds to the NE of the *Xenopus* nucleus in the absence of Blm10 might be due to cross interactions between yeast CP and putative amphibian orthologues of Blm10, which had remained at the nuclear membrane (see Supplementary data).

Blm10 binds to Ran/Gsp1-GTP and nuclear pore protein Nup53

The most common mechanism of protein transport through nuclear pore complexes is driven by β karyopherins and depends on the Ran-GTP cycle between the nucleo- and cytoplasm (Fried and Kutay, 2003; Wentz and Rout, 2010).

Genetic interactions between Blm10 and Prp20, the Ran nucleotide exchange factor, and Blm10 and Rna1, the RanGTPase activating protein, were first hints that Blm10 senses Ran-GTP (Costanzo *et al*, 2010). Before we considered this possibility, we first examined whether Blm10 and pre-assembled CP are possible cargoes of the classical NLS

Figure 3 Nuclear import of mature CP by Blm10. **(A)** Recovery of GFP-labelled CP in the nucleus, when cell growth resumes after stationary phase. Wild-type and *blm10Δ* cells expressing GFP-labelled $\beta 5$ were monitored by direct fluorescence microscopy at different time points. Time point 0 indicates the addition of fresh YPD medium. The ratio of the nuclear to cytoplasmic pixel intensity is indicated as percentage $\pm$ s.d. (Wendler *et al*, 2004). Accordingly, the reassembly of RP and CP was monitored by native PAGE and fluorimaging (see Supplementary Figure S3B). Bar, 2 μ m. **(B)** Nuclear import of yeast CP into reconstituted *Xenopus* nuclei is facilitated by Blm10. Yeast CP was labelled with Oregon Green and incubated without or with Blm10 prior to reconstitution assays. Samples were fixed and analysed by confocal fluorescence microscopy. Three representative nuclei were selected and designated with Roman numerals. Three consecutive 0.37 μ m confocal sections of each representative nucleus are shown. Scale bar 10 μ m. **(C)** Native PAGE and fluorimaging of mock- and Blm10-treated Oregon Green-CP. **(D)** The histogram compiles the result of three reconstitution experiments, which were performed as described above but analysed by epifluorescence microscopy. Mean values of fluorescence pixel intensities at the nuclear rim (white column) and inside the nucleus (black column) were scored from 30 individual nuclei that were either mock or Blm10 treated. Error bars indicate s.e.m. Source data for this figure is available on the online supplementary information page.

receptor karyopherin $\alpha\beta$ upon the exit of quiescence. For this purpose, we monitored GFP-labelled CP in *srp1-49* cells (Yano *et al*, 1992), a mutant in karyopherin α (Enenkel *et al*, 1995). In line with our observations described above, quiescent *srp1-49* cells expressing GFP-labelled $\beta 5$ also displayed PSG formation. Upon the resumption of growth, GFP-labelled $\beta 5$ was rapidly mobilized from PSG into the nucleus at non-permissive temperature (Supplementary Figure S4A). This

finding supports our previous conclusion that karyopherin $\alpha\beta$ has no major impact on nuclear import of mature CP (Lehmann *et al*, 2002). Likewise, GFP-labelled Blm10 was rapidly mobilized from PSG into the nucleus of *srp1-49* mutants following regrowth from stationary phase (Supplementary Figure S4B).

To test whether Blm10 behaves like a CP-specific nuclear transport receptor, we examined the localization of GFP-labelled Blm10 in Ran-GTPase cycle mutants. We chose *gsp1-1* as an appropriate Ran-GTPase cycle mutant as it carries a temperature-sensitive mutant allele of Gsp1, the essential homologue of Ran in yeast (Wong *et al*, 1997). In addition, we analysed the temperature-sensitive Ran-nucleotide exchange factor mutant *prp20-1* (Aebi *et al*, 1990). During cell division at permissive temperature, Blm10-GFP is primarily nuclear in *gsp1-1* and *prp20-1* cells. In stationary phase, PSG formation was observed at permissive and restrictive temperatures (Figure 4A, compare upper panels). At restrictive temperature, quiescent *gsp1-1* and *prp20-1* cells which were incubated with fresh medium failed to relocate Blm10 into the nucleus showing that nuclear import of Blm10 depends on the Ran-GTPase cycle. As a control, quiescent *gsp1-1* and *prp20-1* cells were incubated with fresh medium at permissive temperature. Blm10-GFP was rapidly relocated into the nucleus and cells resumed proliferation. The PSG also rapidly cleared in wild-type cells, if they were allowed to exit quiescence at elevated temperatures (not shown).

Since overexpression of the dominant-negative yeast Ran mutant Gsp1-G21V inhibits nucleocytoplasmic transport (Schlenstedt *et al*, 1995), we tested how Gsp1-G21V affects nuclear import of Blm10. Gsp1-G21V is a Gsp1 mutant that is stabilized in its GTP-bound form. Cells expressing GFP-labelled Blm10 were transformed with a plasmid that expresses Gsp1-G21V under the control of a galactose-inducible promoter. Upon the induction of Gsp1-G21V, the cell cycle was arrested and GFP-labelled Blm10 showed no selective localization (Figure 4A, lower panel). As soon as the expression of Gsp1-G21V was repressed, the cells resumed growth and Blm10 was localized to the nucleus again. Nuclear import of GFP-labelled CP as well as of GFP-labelled RP is also disturbed upon overexpression of Gsp1-G21V (as shown for Blm10-GFP).

In support of this observation, reconstitution experiments with *Xenopus* nuclei using RanQ69L harbouring a mutation that prevents GTP hydrolysis, revealed that nuclear import of Oregon Green-labelled CP from yeast is Ran dependent (Supplementary Figure S5).

Search for primary sequence similarities between β karyopherins and Blm10 did not reveal structural determinants for Ran-GTP binding within the N-terminal arch of Blm10, that would allow to assign Blm10 as a new classical member of the β karyopherin family (Kutay *et al*, 1997; Vetter *et al*, 1999). However, the C-terminal part of Blm10, the most conserved region of the protein, shows consecutive HEAT modules stacking together in a parallel fashion with a slight clock-wise twist, comparable to the arrangement in the three-dimensional structure of the Ran-binding arch of β karyopherins (Cook *et al*, 2007). Thus, we tested whether the C-terminal part of Blm10 confers Ran dependence on nuclear import of Blm10-bound CP. To test whether the C-terminal region of Blm10 is involved in the contact

between Ran/Gsp1-GTP and Blm10, we performed solution binding assays (for example, see Enenkel *et al*, 1996). Glutathione-S-transferase (GST) fusion proteins of (wild type) Gsp1 and Gsp1Q71L, a Ran mutant defective in GTP hydrolysis, were loaded with GDP and GTP, respectively, and immobilized to glutathione-sepharose beads. Blm10 was purified as His-tagged protein from yeast and allowed to associate in solution with immobilized GST-Gsp1-GDP, GST-Gsp1Q71L-GTP and GST as a control. The bound proteins were resolved by SDS-PAGE followed by immunoblotting against His-tagged Blm10. Blm10 was bound to GST-Gsp1Q71L-GTP, but not to GST-Gsp1-GDP and GST

(Figure 4B, lanes 1–3). Then, we analysed whether the truncation of the C-terminal amino acid residues 1804–2143 abrogates binding of Blm10 to Gsp1Q71L-GTP. His-tagged Blm10ΔC1804–2143 was purified and incubated with immobilized GST-Gsp1Q71L-GTP. SDS-PAGE followed by immunoblotting against His-tagged Blm10ΔC1804–2143 revealed no interaction (Figure 4C, lane 1). His-tagged Blm10-C1804–2143 (Blm10C) was tightly bound to GST-Gsp1Q71L-GTP (lane 2). We also tested conserved amino-acid residues within the C-terminal region of Blm10 for their effect on Ran/Gsp1 binding. Wild-type Blm10C and mutant Blm10C-W2021A were bacterially expressed and purified as His-tagged

proteins (Figure 4C, lanes 3 and 4). Compared with the control (Figure 4C, lane 5) wild-type Blm10C showed higher affinity towards Gsp1Q71L-GTP (lane 6) than mutant Blm10C-W2021A (lane 7).

Then, we examined whether Blm10 directly binds Nup53. Nup53 was bacterially expressed as GST fusion protein, immobilized to glutathione-sepharose beads and incubated with His-tagged Blm10. Solution binding assays revealed that Blm10 interacts with Nup53 (Figure 4D).

Next, we examined whether Blm10 facilitates the interaction of CP with Nup53. Again, GST-Nup53 was immobilized to glutathione-sepharose beads and incubated with CP either in the absence or in the presence of Blm10. During incubation, proteasome inhibitor MG132 was added to avoid proteasomal degradation of intrinsically disordered FG-nucleoporin Nup53. Elution of tightly bound proteins revealed a significant higher amount of CP to be bound to Nup53 in the presence of Blm10 compared with the absence of Blm10 (Figure 4E, lanes 1 and 2).

According to the current model of nuclear import, the encounter of importin-bound cargo with Ran-GTP results in the release of cargo into the nucleoplasm and enables the re-export of Ran-GTP-bound importin to the cytoplasm (Wente and Rout, 2010). If Blm10 serves as an importin-like protein, then the presence of the dominant-negative Ran mutant Gsp1G21V should weaken the interaction between Blm10 and the CP. To test this, Gsp1G21V was expressed behind a galactose-inducible promoter in wild-type cells in which the CP subunit $\alpha 4$ was chromosomally replaced by a proteinA-tagged version. This approach allows a rapid one-step purification of proteinA-tagged CP on IgG-sepharose beads. The purified CP was subjected to SDS-PAGE followed by Coomassie blue staining (Figure 4F, upper panel) and anti-Blm10 western blotting (lower panel). Compared with the control, Blm10 was not associated with the CP in the presence of Gsp1G21V (compare lanes 1 and 2).

To test whether recombinant Ran-GTP is sufficient to dissociate Blm10-bound CP *in vitro*, GFP-labelled CP was purified from yeast under native conditions and allowed to react with His-tagged Blm10 that was purified from yeast as

described before (Lehmann *et al*, 2008). The reconstitution of Blm10-bound CP was verified by a gel shift in native PAGE and the detection of a Blm10-specific band in the western blot. Blm10-bound CP migrates slower than free CP as revealed by fluorimaging of the GFP moieties of the CP. In parallel, Ran/Gsp1-GTP was incubated with Blm10-bound CP. Native PAGE and fluorimaging revealed that Blm10 was ejected from the CP by Gsp1-GTP as indicated by the gel shift towards the fast-migrating CP species. Gsp1 (molecular mass of 25 kDa) and Blm10 (240 kDa) co-migrated in native PAGE as shown by western blotting against Blm10 and Gsp1 allowing the conclusion that Blm10 and Gsp1 associate in solution (Figure 4G).

Discussion

In this work, we report on a new function of Blm10, a conserved 240 kDa protein with 32 HEAT-like modules. Previous studies suggested diverse functions for Blm10. On the one hand, Blm10 was proposed to be involved in CP maturation and quality control, since it is associated with CP precursor complexes (Fehlker *et al*, 2003; Li *et al*, 2007; Marques *et al*, 2007) and mature CP with disordered gates of the α rings (Lehmann *et al*, 2008). Those findings supported the idea that Blm10 registers α ring conformations during CP assembly and serves as a scaffold for CP assembly. On the other hand, Blm10 was found to serve as a specific activator of proteasomal degradation based on the observation that the transcription factor Sfp1 is stabilized in quiescent *blm10 Δ* cells (Lopez *et al*, 2011). How can these findings be related to the present study showing that Blm10 is responsible for CP sequestration into PSG during quiescence and that Blm10 facilitates nuclear uptake of mature CP upon the resumption of growth?

It has been proposed that PSG either serve as storage granules (Laporte *et al*, 2008) or represent major sites for the degradation of misfolded proteins (Kaganovich *et al*, 2008). Examination of proteasome configurations in quiescent yeast cells provided a hint concerning the above possibilities. During quiescence and prolonged stationary

Figure 4 Blm10 binds Ran-GTP and Nup53, an FG-rich nuclear pore protein. (A) Nuclear import of GFP-labelled Blm10 is affected in the temperature-sensitive RanGTPase cycle mutant *gsp1-1* and *prp20-1* (upper panels; bar, 2 μ m) and upon overexpression of the dominant-negative Ran mutant Gsp1-G21V (lower panels; bar, 5 μ m). Blm10 is chromosomally replaced by a GFP-tagged version and monitored by direct fluorescence microscopy. (B) Blm10 binds Ran/Gsp1-GTP in solution binding assays. Wild-type GST-Gsp1-GDP (lane 1), GST-Gsp1Q71L-GTP (lane 2), and GST alone (lane 3) were allowed to react with His-tagged Blm10 (lane 4). Bound proteins were resolved by SDS-PAGE and anti-His immunoblotting (upper panel, lanes 1–3). Protein loads are shown by Coomassie blue stain (lower panel, lanes 1–3; lane 4). His-tagged Blm10 was purified from yeast (Lehmann *et al*, 2008). (C) The C-terminal region of Blm10 binds Ran/Gsp1-GTP. C-terminally truncated Blm10 Δ C1804-2143 (lane 1) and Blm10C-1804-2143 (lane 2) were tested for binding to GST-Gsp1Q71L-GTP. Load and bound fractions of Blm10 Δ C1804-2143 and Blm10C-1804-2143 (abbreviated as Blm10C) are shown by anti-His immunoblotting. Purified wild-type Blm10C (lane 3) and mutant Blm10C-W2021A (lane 4) were resolved by SDS-PAGE and Coomassie blue staining. The affinities of Blm10C towards GST (lane 5), GST-Gsp1Q71L-GTP (lane 6) and of mutant Blm10C-W2021A towards GST-Gsp1Q71L-GTP (lane 7) were tested by solution binding assays using anti-His immunoblotting. (D) Blm10 binds Nup53, an FG-rich nuclear pore protein. GST (lane 1) and GST-Nup53 (lane 2) were immobilized to glutathione-sepharose beads and incubated with His-tagged Blm10. SDS-PAGE and Coomassie blue stain revealed Blm10 load (upper panel), bound Blm10, GST and GST-Nup53 (lower panels). Purified GST-Nup53 is resolved by SDS-PAGE and Coomassie blue staining (lane 3). (E) CP binding to Nup53 is facilitated by the presence of Blm10. Immobilized GST-Nup53 was incubated either with CP in the presence of 200 μ M MG132 (lane 1) or with Blm10-bound CP (lane 2). Purified CP and Blm10 are resolved by SDS-PAGE and Coomassie blue staining (upper panels). Load and bound fractions of Blm10 and CP were detected by immunoblotting against Blm10 and CP subunit $\alpha 4$ (lower panels). (F) Blm10-bound CP is dissociated by the dominant-negative Ran mutant Gsp1G21V *in vivo*. Gsp1G21V was expressed behind the inducible GAL promoter in wild-type cells harbouring the CP as a proteinA-tagged protein complex. CP was affinity purified from non-induced (lane 1) and induced cells (lane 2) and subjected to SDS-PAGE followed by Coomassie blue staining (upper panel) and anti-Blm10 western blotting (lower panel). (G) Recombinant Ran/Gsp1-GTP dissociates Blm10-bound CP *in vitro*. GFP-labelled CP was purified under native conditions and incubated with purified His-tagged Blm10 resulting in the formation of Blm10-bound CP (Lehmann *et al*, 2008). Blm10-bound CP was incubated either with Gsp1-GTP or mock-treated. The reaction was subjected to native PAGE followed by fluorimaging and immunoblotting using antibodies specific for His-tagged Blm10 bound to the CP (upper middle panel), free Blm10 (lower middle panel), and Ran/Gsp1 (right panel); protein molecular weights (kDa). Source data for this figure is available on the online supplementary information page.

phase, RP-CP assemblies are diminished or even ablated (Bajorek *et al*, 2003). This phenomenon is reflected by changes in the metabolome of quiescent yeast. The four-fold decrease in ATP from logarithmic to stationary phase (Laporte *et al*, 2011) parallels with RP-CP disassembly and PSG formation. Thus, PSG may not serve as major sites of proteasomal proteolysis of poly-ubiquitylated proteins, since their degradation requires RP-CP assemblies (Enenkel, 2013). More likely, as suggested before by Sagot and her colleagues, proteasomes are stored within PSG to ensure a quicker outgrowth from stationary to logarithmic phase (Laporte *et al*, 2008), which is according to our present studies achieved by Blm10.

Our finding that Blm10 is required for nuclear import of mature CP nicely supports Tanaka's hypothesis that only CP with specific conformations is imported into the nucleus (Tanaka *et al*, 1990). Accordingly, NLS that is present within α subunits are recognized by the classical NLS receptor karyopherin $\alpha\beta$. The necessity of a regulation in CP translocation proposes the existence of two α ring conformations, one in which the NLS is masked and a second with exposed NLS (Tanaka *et al*, 1990). During cell proliferation, when CP precursor complexes are assembled from newly synthesized subunits, CP conformations with open α rings and accessible NLS are provided, allowing the binding of karyopherin $\alpha\beta$ and the subsequent translocation into the nucleus (Lehmann *et al*, 2002) (Figure 5A). Closed α rings of mature CP may be the reason why mature CP is neither efficiently imported into the nucleus of reconstitution assays nor recognized by karyopherin $\alpha\beta$ in solution (Wang *et al*, 1997; Lehmann *et al*, 2002; Savulescu *et al*, 2011).

In quiescence, Blm10 and CP are sequestered into PSG and no precursor complexes are available (Figure 5B). With the resumption of cell growth the nuclear import of mature CP requires therefore an alternative pathway, in which Blm10 takes over the function of a nuclear import receptor. Apparently, the CP passes the barrier of natively disordered nuclear pore proteins either as an inactive precursor complex or as a Blm10-capped particle.

Our assumption that Blm10 acts as a CP-specific nuclear import receptor is supported by structural similarities between Blm10 and β karyopherins, although the β karyopherin-type Ran-binding domain does not reside in the N-terminal arch of Blm10. In fact, the tertiary structure of the C-terminal part of Blm10, the most conserved region of the protein (Sadre-Bazzaz *et al*, 2010, PDB 1YSY), is similar to the superhelical Ran-binding arch of β karyopherins (Lee *et al*, 2005, PDB 2bku). Ran-GTP binding may induce conformational changes within Blm10 that weaken the interaction of Blm10's penultimate tyrosine required for CP binding.

For being recycled to the cytoplasm importing karyopherins bind Ran-GTP (Hahn and Schlenstedt, 2011). In quiescence, Blm10 lacking the conserved C-terminal region remains nuclear (Doherty *et al*, 2012). Our findings suggest that Blm10 lacking the C-terminal region cannot bind Ran-GTP and fails to be exported to the cytoplasm.

Few HEAT repeat proteins are reported to translocate cargoes without the aid of additional karyopherins raising the question whether HEAT repeat proteins are generally able to interact with nucleoporins (Macara, 2001). At least HEAT repeat proteins such as Blm10 would reduce the reliance on single karyopherins with regard to the mass of CP and other

Figure 5 Model of CP import through nuclear pores. Transport of macromolecules through nuclear pores requires carriers of the HEAT repeat protein family of β karyopherins/importins. (A) In the cytoplasm of dividing yeast cells, CP precursor complexes are assembled from newly synthesized subunits and Ump1, a small CP-dedicated chaperone. The NLS within the α subunits of the CP precursor complexes is accessible for the classical NLS receptor karyopherin/importin $\alpha\beta$, which mediates nuclear import of CP precursor complexes (Lehmann *et al*, 2002). Karyopherin/importin $\alpha\beta$ is dissociated from the import cargo by nuclear Gsp1/Ran-GTP. In the nucleus, the CP is matured, Ump1 is degraded, and the NLS is masked by α ring closure. The α rings of the CP are depicted in purple, and the β rings in blue. Ump1 is depicted in cyan. (B) In quiescence, Blm10 and CP are sequestered into PSG. CP precursor complexes are not available. Upon the exit from quiescence, Blm10 mediates the transport of mature CP through natively disordered nuclear pore proteins that build the entropic barrier between the nucleo- and cytoplasm. Blm10 is dissociated from the CP by nuclear Gsp1/Ran-GTP. Blm10 is depicted in yellow.

proteins that must be imported into the nucleus upon the exit of quiescence.

Parallels can be drawn to previous studies reporting on HEAT repeat proteins that provide the framework of ribosome assembly and nuclear export. Prp12, one of these HEAT repeat containing proteins, was shown to bind Ran-GTP and FG-nucleoporins Nup53 (Oeffinger *et al*, 2004). Binding to Ran-GTP and FG-nucleoporins is mandatory for a HEAT repeat protein to serve as nuclear import receptors and to assure directed transport of macromolecules through nuclear pore complexes.

Finally, we want to discuss our results in the context of the mammalian homologue of Blm10, namely PA200. PA200 was proposed to activate nuclear CP for specific functions during DNA repair, based on the finding that (i) PA200 expression is induced by DNA damaging agents (Ustrell *et al*, 2002), (ii) PA200 and CP co-localize in nuclear speckles upon induced cell-cycle arrest (Blickwedehl *et al*, 2007), and (iii) PA200 knockdown cells showed reduced survival after exposure to

ionizing radiation and PA200 knockout led to impaired fertility (Khor *et al*, 2006). These observations in mammalian cells complement our studies in yeast showing that (i) Blm10 expression is induced by proteotoxic stress (Lehmann *et al*, 2008), (ii) Blm10 and CP are sequestered into PSG during quiescence and (iii) quiescent *blm10Δ* cells exhibit hypersensitivity to bleomycin-induced DNA damage and delayed outgrowth upon the resumption of cell division (Doherty *et al*, 2012; Febres *et al*, 2001) (see Supplementary Figure S6).

Taken together, our results in yeast suggest that Blm10 is the first CP-dedicated nuclear import receptor in yeast. Blm10 is essential for rapid nuclear import of mature CP, when quiescent cells resume proliferation. At this state of the cell cycle, Blm10 accelerates the mobilization of CP from PSG into the nucleus, where proteasomal degradation is reactivated to promote cell-cycle progression.

Materials and methods

Strains and growth

Isogenic yeast strains expressing tagged versions of Ump1, $\alpha 4$ (Pre6), $\beta 5$ (Pre2), Rpn1, and Rpn11 were created as previously described (Lehmann *et al*, 2008). Strains were derived from WCGa (*MATa his3-11,15 leu2-3,112 ura3-52 can GAL*) and *blm10Δ* (*MATa blm10Δ::HIS3 his3-11,15 leu2-3,112 ura3-52 can GAL*), which was originally named *blm3Δ* (Fehlker *et al*, 2003). Cells were grown in yeast extract peptone dextrose (YPD) medium for several hours to logarithmic phase ($OD_{600} \sim 0.7$) or for 3–4 days to stationary phase ($OD_{600} \sim 13$). To counter-stain the nucleus, histone H2A was expressed as a cherry red fluorescent protein (RFP) by fusion of an *RFP-natMX* cassette to the endogenous *HTA2* gene.

Plasmids and strains used in this work are listed in Supplementary Tables S1 and S2.

Direct fluorescence microscopy of living yeast cells

Living yeast cells were washed with water and visualized at RT with a DMR microscope (Leica, Bensheim, Germany) equipped with a $\times 100/1.4$ oil-immersion objective lens (PL Fluotar). To monitor GFP- and CFP-labelled proteins, filters for fluorescein isothiocyanate/GFP (495-nm excitation and 525-nm emission) or CFP (436-nm excitation and 480-nm emission) were used. The cells were visualized by Nomarski optics. Images were taken with a Hamamatsu C5985-10 charge-coupled device camera using the MetaVue Software (Visitron Systems, Puchheim, Germany). Settings were identical for all images in a given experiment.

Living yeast cells expressing GFP- and RFP-labelled proteins were monitored with a ZEISS AxioVision fluorescence microscope equipped with a 100/1.4 oil-immersion objective lens (Plan APOCHROMAT) and filters for DIC, GFP (Alexa Fluor 488-nm excitation and 520-nm emission) and RFP (Texas Red 595-nm excitation and 615-nm emission). Images were taken with a Hamamatsu ORCA-ER digital camera using the Volocity Software (Improvision).

To monitor Blm10-GFP in *gsp1-1* and *prp20-1* mutants, cells were grown to logarithmic phase at permissive temperature (23°C). To monitor Blm10-GFP in quiescent *gsp1-1* and *prp20-1* mutants, cells were grown to stationary phase at permissive and restrictive temperatures (23°C and after shift for 3 h to 30°C). Fresh YPD medium was added at restrictive and permissive temperatures. The localization of Blm10-GFP was monitored after 30 min of incubation.

To monitor Blm10-GFP upon overexpression of Gsp1G21V, GFP-labelled Blm10 was monitored in cells grown in galactose with empty plasmid (GAL), in cells in which Gsp1G21V expression was induced by galactose for 6 h and after repression of Gsp1G21V expression by glucose.

Native PAGE

Cells were disintegrated by glass beads in buffer TB (20 mM HEPES/KOH pH 7.4, 110 mM KOAc, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM dithiothreitol, and 2 mM ATP). The lysate was cleared by

centrifugation and immediately subjected to native PAGE. Native PAGE using 3.5–6.0% polyacrylamide gels, GFP fluorimaging of native gels, and immunoblotting were previously described in detail (Lehmann *et al*, 2008).

Reconstitution assay

Nuclei were assembled from fractionated *Xenopus* egg extract as previously described (Savulescu *et al*, 2011). The egg extract presumably contained putative amphibian orthologues of Blm10, which could not be removed due to the lack of characterized antibodies. Oregon Green-labelled CP was introduced into the reconstitution reactions after nuclear assembly was completed and nuclear import was assessed by epifluorescence or confocal microscopy. (see Supplementary data). Yeast CP was affinity purified from *blm10Δ* cells and labelled with Oregon Green 488 succinimidyl ester (Molecular Probes).

Solution binding assay

The affinity of full-length Blm10 and C-terminal domains of Blm10 for wild-type Gsp1, Gsp1Q71L, and Nup53 was tested by solution binding assays in PBSKMT (25 mM sodium phosphate, 150 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 5 mM β -mercaptoethanol, 0.1% Tween-20, pH 7.3) according to Hahn and Schlenstedt (2011) and Marelli *et al* (1998). GST, GST-Gsp1, GST-Gsp1Q71L, and GST-Nup53 were expressed from pGEX-derived plasmids in *E. coli* BL21 DE3. The GST fusion proteins were isolated by affinity purification on glutathione-sepharose beads. Homogeneity of purified proteins was verified by SDS-PAGE and Coomassie blue staining. Ran variants were loaded with either 2 mM GDP or 2 mM GTP prior immobilization to glutathione-sepharose beads. Ten micrograms of GST fusion protein was immobilized to 30 μ l glutathione-sepharose beads. 6His-tagged Ran/Gsp1 was expressed from a pQE-derived plasmid in *E. coli* BL21 DE3, purified on nickel affinity beads and used in the dissociation experiment of Blm10-CP.

Possible binding partners were allowed to react with glutathione sepharose-immobilized GST fusion proteins for 30 min at 4°C in PBSKMT. The beads (30 μ l) were washed three times with 1 ml PBSKMT in the cold. The washed beads were boiled in SDS-PAGE buffer and the samples were subjected to SDS-PAGE, either stained with Coomassie Blue or blotted and probed with anti-penta-His antibodies (Qiagen).

12His-tagged versions of full-length Blm10 and Blm10 lacking amino acids 1804–2143 were purified upon galactose-induced overexpression in yeast by using pTF155-derived plasmids that either harbour full-length or C-terminally truncated 12His-BLM10 behind the *GAL1* promoter (Iwanczyk *et al*, 2006). The cells were lysed by French pressure cell disintegration in 50 mM imidazole, 50 mM NaCl, 20 mM Tris/HCl pH 7.5, 0.01% (v/v) NP-40 and in the presence of protease inhibitor cocktail. The lysate clarified by centrifugation was passed on Ni-NTA beads. The Ni-NTA slurry was washed three times in batch with 100 bed volumes of lysis buffer containing 2 M NaCl and then 50 mM NaCl. His-tagged Blm10 was eluted by 500 mM imidazole, 50 mM NaCl, 20 mM Tris/HCl pH 7.5. Despite some decay products, the major fraction contains full-length His-tagged Blm10 which allows the reconstitution of Blm10-CP-Blm10 in PBSKMT (Iwanczyk *et al*, 2006). To avoid Blm10 decay, pTF155-driven expression is performed in yeast strain YMW11 harbouring a proteinA-tagged version of CP subunit Pre6 ($\alpha 4$). In this background, full-length Blm10 is co-purified with the CP which is immobilized to IgG-sepharose beads. Full-length His-tagged Blm10 is obtained by washing the beads with 1 M NaCl as shown before (Fehlker *et al*, 2003). The released His-tagged Blm10 is transferred into PBSKMT containing 5 mM β -mercaptoethanol. Blm10 must be used fresh for solution binding studies and reconstitution assays, because storage at 4°C results in rapid decay.

The C-terminal deletion of amino-acid residues 1804–2143 of Blm10 was created by modifying the *PstI/XhoI* fragment within pTF155 by PCR yielding pTF155 Δ C.

Blm10C fragments were created by PCR amplification of *BamHI/XhoI* fragments containing Blm10 codons 1749–2143 and 1804–2143, respectively, fused to six histidine (6His) codons. The expression of 6His-Blm10-C1804–2143 from pET21b in *E. coli* yielded a less soluble protein. The expression of 6His-Blm10-C1749–2143 from pGEX-4T3 in *E. coli* yielded soluble 6His-Blm10C after thrombin cleavage.

Quick-change PCR (Agilent Technologies) was used to generate Blm10C-W2021A from template pGEX-4T3-Blm10C-1749-2143, which yielded pGEX-4T3-Blm10C-W2021.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: CE conceived and designed the project. MW and CE performed the experiments and analysed the data with the help of JJ and TB. AS, AH, and MG provided the reconstitution assays in the *Xenopus* system. CE wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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