UBL/BAG-domain co-chaperones cause cellular stress upon overexpression through constitutive activation of Hsf1

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Abstract As a result of exposure to stress conditions, mutations, or defects during synthesis, cellular proteins are prone to misfold. To cope with such partially denatured proteins, cells mount a regulated transcriptional response involving the Hsf1 transcription factor, which drives the synthesis of molecular chaperones and other stress-relieving proteins. Here, we show that the fission yeast *Schizosaccharomyces pombe* orthologues of human BAG-1, Bag101, and Bag102, are Hsp70 co-chaperones that associate with 26S proteasomes. Only a subgroup of Hsp70-type chaperones, including Ssa1, Ssa2, and Sks2, binds Bag101 and Bag102 and key residues in the Hsp70 ATPase domains, required for interaction with Bag101 and Bag102, were identified. In humans, BAG-1 overexpression is typically observed in cancers. Overexpression of *bag101* and *bag102* in fission yeast leads to a strong growth defect caused by triggering Hsp70 to release and activate the Hsf1 transcription factor. Accordingly, the *bag101*-linked growth defect is alleviated in strains containing a reduced amount of Hsf1 but aggravated in *hsp70* deletion strains. In conclusion, we propose that the fission yeast UBL/BAG proteins release Hsf1 from Hsp70, leading to constitutive Hsf1 activation and growth defects.

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Introduction

Various conditions, such as cellular stress, mutation, or defects during protein synthesis, may cause partial denaturation of cell proteins. Such proteins are either shielded from aggregation and refolded to the native state by molecular chaperones (Hartl and Hayer-Hartl 2009) or targeted for degradation via the ubiquitin-proteasome system (UPS) (Cyr et al. 2002; Esser et al. 2004; Kettern et al. 2010; Le Goff et al. 2016; McClellan et al. 2005; Powers et al. 2009; Wiederkehr et al. 2002). To mount this, stress response involves activation of the heat shock factor (HSF) transcriptional regulator, Hsf1, which drives the synthesis of molecular chaperones and other stress-relieving proteins (Zobeck et al. 2010). The ability of Hsf1 to respond to cellular stress is under negative regulation by molecular chaperones and, in higher eukaryotes, by transition from a monomeric to trimeric form (Anckar and Sistonen 2011). Hsf1 activity is essential in yeast cells, while constitutive Hsf1 activity is known to retard cell growth (Espinet et al. 1995; Vjestica et al. 2013).

A body of evidence supports that chaperones can participate both in protein refolding and protein degradation, but what triggers a chaperone to commit to a folding or a degradation mode is still uncertain. However, studies suggest that certain regulatory co-chaperones contribute to this process (Arndt et al. 2007; Kettern et al. 2010). The human BAG-1 protein is one such cofactor for Hsp70-type chaperones (Hohfeld and Jentsch 1997).

In general, BAG-1 is expressed at low levels in human tissues (Takayama et al. 1998), but is frequently overexpressed in invasive cancers (Papadakis et al. 2016; Takayama et al. 1998; Tang

et al. 1999). Accordingly, in the clinic BAG-1 mRNA is a prognostic biomarker for breast cancer (Paik et al. 2004). However, the underlying mechanisms and cellular consequences of BAG-1 overexpression remain unclear.

As a co-chaperone, BAG-1 functions as a nucleotide exchange factor for the chaperone and triggers the release of bound clients (Brehmer et al. 2001; Sondermann et al. 2001). This substrate release is mediated by the BAG domain in BAG-1, which binds to the ATPase domain of Hsp70 (Luders et al. 2000; Sondermann et al. 2001). Thus, while BAG-1 associates with the 26S proteasome via its N-terminal ubiquitin-like (UBL) domain, chaperone-bound clients are released and degraded (Demand et al. 2001; Luders et al. 2000; Sondermann et al. 2001). Before degradation, chaperone clients are first ubiquitinated. In mammalian cells, this primarily occurs via the E2 ubiquitin-conjugating enzyme Ubc4, and the E3 ubiquitin-protein ligase CHIP (Connell et al. 2001; Demand et al. 2001; Murata et al. 2001; Nielsen et al. 2014).

Curiously, the budding yeast genome does not encode any UBL/BAG-domain proteins. However, the fission yeast, *Schizosaccharomyces pombe*, contains two BAG-1 homologs, Bag101 and Bag102. Recently, Bag101 was shown to regulate turnover of native Rad22, a protein involved in homologous recombination (Saito et al. 2013), while Bag102 was shown to co-ordinate the transfer of mutant, and presumably structurally perturbed, kinetochore proteins to the 26S proteasome (Kriegenburg et al. 2014). In the case of Bag102, the target ubiquitylation was catalyzed by the E3s, Ubr11, and San1 (Kriegenburg et al. 2014).

Here, we show that Bag101 and Bag102 both associate with 26S proteasomes and Hsp70. In total, fission yeast encodes eight different Hsp70-type chaperones. Co-precipitation experiments revealed that the BAG co-chaperones exclusively interact with Ssa1, Ssa2, and Sks2, but fail to interact with the remaining Hsp70s Pdr13, Pss1, Bip1, Lhs1, and Ssc1. Accordingly, the Hsp70 ATPase domain sequences cluster in three subfamilies of which two interact with the BAG-domain proteins. While null mutants in *bag101* and *bag102* do not display any obvious phenotypes, we found that overexpression leads to a strong, BAG-domain dependent, growth defect and triggers a stress response resembling that observed upon deleting the Hsp70 chaperone Ssa2. Accordingly, we found that Bag101 releases Hsf1 from Hsp70, leading to constitutive Hsf1 activation and growth defects.

Methods

S. pombe strains and techniques

Fission yeast strains used in this study are derivatives of the wild type heterothallic strains 972h⁻ and 975h⁺. The *pabp-*

mCherry strain was kindly provided by Dr. Shao-Win Wang (Wang et al. 2012). The *hsp104-mCherry*, 5'UTR^{*hsp104*}/*GFP*/3'UTR^{*hsp104*}, *hsf1-GFP*, and *P^{urg1}-hsf1* strains were kindly provided by Dr. Aleksandar Vjestica and Dr. Snezhana Oliferenko (Vjestica et al. 2013). The *bag101Δbag102Δ* strain has been described before (Kriegenburg et al. 2014). Standard genetic methods and media were used, and *S. pombe* transformations were performed using lithium acetate (Moreno et al. 1991).

Plasmids

To generate Bag101 (SPBC16G5.11c) constructs, full length (FL), BAG-domain (amino acids 78–190) and UBL-domain (1–77) complementary DNAs (cDNAs) were amplified from *S. pombe* genomic DNA and inserted into pDONR221 (Invitrogen) and pGEX-6P1 (GE Healthcare). Full-length *bag102*⁺ (SPBC530.03c), ΔTM (31–206), BAG (122–206), and UBL (1–121) cDNAs were also inserted into the pDONR221 and pGEX-6P1 vectors. For expression in *S. pombe*, the inserts from the pDONR221 vectors were transferred to the pDUAL vector system (Matsuyama et al. 2004) using Gateway cloning technology (Invitrogen).

Binding assays

The GST fusion proteins were expressed in *Escherichia coli* BL21 (DE3) or *E. coli* Rosetta (DE3) and bound to glutathione Sepharose 4 beads (GE Healthcare). The protein/bead ratio was 1 mg/mL. Binding experiments were carried out using 20 μL of beads in 1 mL of buffer A (buffer A (25 mM Tris/HCl at pH 7.5, 50 mM NaCl, 10% glycerol, 0.1% Triton X-100, 2 mM DTT, 1 mM PMSF, and complete protease inhibitors (Roche)) containing Hsp70 at a concentration of approximately 0.1 mg/mL. After 4–16 h tumbling at 4 °C, the beads were washed four times in 10 mL buffer A and resuspended into 30 μL SDS sample buffer. Twenty microliters of the samples were analyzed on 12% SDS-PAGE gels and subjected to Western blotting. The purified Hsp70 and Rhp23 proteins were kindly provided by Dr. Colin Gordon (MRC Human Genetics Unit, Edinburgh, UK).

Immunoprecipitations were performed from 50 mL cultures in mid-exponential phase. Cells were lysed using glass beads in buffer B (25 mM Tris/HCl at pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM PMSF, and complete protease inhibitors (Roche)) and cleared by centrifugation (13,000×g, 30 min). The soluble fraction was tumbled end-over-end for 4 h at 4 °C with GFP-trap beads (Chromotek) and 5A5 anti-Hsp70-loaded ProteinG Sepharose (GE Healthcare). The beads were then washed four times in 10 mL of buffer B and resuspended into 30 μL SDS sample buffer. Twenty microliters of the samples were separated on 12% SDS-PAGE gels and subjected to Western blot analysis.

The antisera, used in Western blots, were affinity purified rabbit polyclonal anti-Mts4 (Cabrera et al. 2010), mouse monoclonal MCP231 (Mathiassen et al. 2015) to 20S proteasome α subunits (Enzo), 5A5 mouse monoclonal anti-Hsp70 (Abcam), mouse monoclonal anti-6His (Qiagen), goat anti-GST (Abcam), anti-GFP (Chromotek), and TAT1 mouse monoclonal anti-tubulin (Abcam). Secondary antibodies were purchased from DAKO Cytomation.

Growth assays

Growth assays on solid media were performed essentially as described (Andersen et al. 2011; Penney et al. 2012). Briefly, the *S. pombe* strains to be assayed were grown to an OD_{600 nm} of 0.4–0.8. The cells were then diluted in media to an OD_{600 nm} of exactly 0.40. Serial 5-fold dilutions of this culture were prepared before 5 μ L of each dilution were spotted onto solid media plates (EMM2 for plasmid selection) and incubated at the indicated temperature until colonies formed.

Microscopy

Cells were cultured in minimal media until mid-exponential phase and fixed with 6% formaldehyde for 30 min. The fixed cells were then washed extensively with PBS and mounted on coverslips using VectaShield Hard Set mounting media containing DAPI (Vector Laboratories) and analyzed in a fluorescence microscope (Zeiss AxioImager Z1) and CCD camera (Hamamatsu ORCA-ER).

RNA sequencing

For sequencing and real-time PCR, total RNA was isolated from wild-type *S. pombe* cells transformed to overexpress *bag101*⁺ or, as a control, with an empty vector using the hot phenol method (Lyne et al. 2003). Five independent biological repeats were performed for each condition. The isolated RNA was then converted to cDNA and sequenced by the Beijing Genome Institute.

The Illumina sequencing was performed as paired-end with final read lengths of 49 bp after demultiplexing and produced 15.6–16.1 million reads per sample. Adaptor and low-quality sequences were trimmed from the raw paired-end data using Trimmomatic v.0.32 (default settings except: ILLUMINACLIP:/adapters/TruSeq3-PE-2.fa:2:30:10:8:true LEADING:20 TRAILING:20 SLIDINGWINDOW:4:15 MINLEN:0) (Bolger et al. 2014). Sequence trimming was followed by mapping to the *S. pombe* genome using STAR (default settings except: outFilterMismatchNoverLmax 0.1, outFilterMatchNmin 18, and outFilterMismatchNmax 3) (Dobin et al. 2013) giving 13.2–14.3 million uniquely mapped reads and an estimated insert size in the range 166–190 bp for all ten samples.

For *S. pombe* reference, the ASM294 v2.21 genome assembly and gene annotation were downloaded from Ensembl (<ftp://ftp.ensemblgenomes.org/pub/release-21>). Gene ontology (GO) (ftp://ftp.geneontology.org/pub/go/gene-associations/gene_association.pombase.gz) and GO slim (only available for the “Biological Function” domain; <http://amigo1.geneontology.org/cgi-bin/amigo/slimmer>) annotation terms were also downloaded. Chen et al.’s (2003) supplementary data tables were downloaded from <http://128.40.79.33/projects/stress/>. Data used from Vjestica et al. (2013) are mainly from Electronic supplementary Table S4.

Our annotation also included rRNA, tRNA, ncRNA, snoRNA, and mitochondrial genes, many of which were not present in the older annotations used by Chen et al. (2003) and Vjestica et al. (2013). Thus, the expression levels of many, especially non-coding, genes could not be compared across studies.

The statistical analyses for identification of differentially expressed genes were made with the DESeq2 Bioconductor package in R (Anders et al. 2015). The Venn diagrams, hierarchical clustering, heatmaps and PCA were also performed in R.

Results

The *S. pombe* UBL/BAG-domain proteins associate with Hsp70 and 26S proteasomes

The human BAG-1 protein associates directly with both Hsp70-type chaperones and the 26S proteasome (Luders et al. 2000). The fission yeast, *S. pombe*, encodes two BAG-1 orthologues, Bag101 and Bag102. Similar to human BAG-1 (hBAG-1), both contain an N-terminal UBL domain and a C-terminal BAG domain. To test if these proteins also interact with Hsp70-type chaperones and 26S proteasomes, we expressed full-length and truncated, GFP-tagged versions of Bag101 and Bag102 (Fig. 1a) in wild type *S. pombe* cells and immunoprecipitated the proteins with antibodies to GFP. Both Bag101 and Bag102 interacted with 26S proteasomes and with Hsp70 (Fig. 1b). For both Bag101 and Bag102, association with 26S proteasomes depended on the N-terminal UBL domain (Fig. 1b). Binding to Hsp70, on the other hand, depended on the C-terminal BAG domain (Fig. 1b).

Several *S. pombe* UBL domain proteins, including Rhp23 (Rad23 in budding yeast), are known to associate with the 26S proteasome (Madsen et al. 2007; Seeger et al. 2003; Stone et al. 2004; Wilkinson et al. 2001). To check if these UBL domain proteins might compete for proteasome binding, we performed co-precipitation experiments using GST-tagged Bag101 in the presence of increasing amounts of Rhp23. Indeed, we found that Rhp23 and Bag101 compete for proteasome binding (Fig. 1c). This effect was lost when the UBL domain in Rhp23 was deleted

Fig. 1 Bag101 and Bag102 interact with 26S proteasomes and Hsp70. **a** Domain organization (shown to scale) of human BAG-1S and the *S. pombe* homologs Bag101 and Bag102. The truncations, used for the precipitation experiments, are shown. **b** Cells transformed with the indicated GFP-tagged Bag101 and Bag102 constructs were used for immunoprecipitation experiments using antibodies to GFP. The precipitated material was analyzed by SDS-PAGE and blotting for Hsp70 (*top*), the 26S proteasome subunit Mts4/Rpn1, and 20S particle

α subunits (*middle*). Equal loading was checked by blotting with antibodies to GFP (*bottom*). **c** GST-tagged Bag101, bound to glutathione Sepharose beads, was used to precipitate 26S proteasomes in the presence of increasing amounts (0-, 5-, or 50-fold molar excess) of either Rhp23 or, as a control, Rhp23 Δ UBL. The precipitated material was analyzed by SDS-PAGE and blotting for the 26S proteasome subunit Mts4/Rpn1 (*top*). Equal loading was checked by staining with Coomassie Brilliant Blue (CBB) (*bottom*)

and suggests that the proteasome binding of Bag101 and Rhp23/Rad23 is mutually exclusive.

Bag101 and Bag102 are specific for certain Hsp70-type chaperones

The fission yeast genome encodes eight different Hsp70-type chaperones. We expressed each of the Hsp70s, fused to a 6His-tag, in *E. coli* or *S. pombe* cells. The fusion proteins were purified and used for in vitro binding assays with purified, GST-tagged Bag101 and Bag102. Bag101 and Bag102 interacted with Ssa1, Ssa2, and Sks2. However, Bag101 appeared to bind stronger to Ssa1 and Ssa2 than Bag102 (Fig. 2a). None of the other Hsp70 chaperones interacted with the BAG-domain proteins (Fig. 2a), revealing that the BAG-domain proteins are specific for certain Hsp70 family members.

Since previous studies have shown that BAG-1 interacts with the ATPase domain of Hsp70 (Sondermann et al. 2001), we performed bootstrapping of the *S. pombe* Hsp70 chaperone ATPase domains (Fig. 2b). Interestingly, the bootstrapping tree revealed that the different Hsp70 ATPases clustered in three major subfamilies (Fig. 2b). Members of subfamily I do not bind Bag101 and Bag102, whereas

members of subfamily II (Ssa1 and Ssa2) bind preferably to Bag101, and the single member of subfamily III (Sks2) binds both Bag101 and Bag102. To our knowledge, this is the first demonstration of BAG-domain proteins displaying specificity for certain Hsp70-type chaperones.

The amino acid residues of Hsp70 that form contacts to BAG-1 can be identified in the published structure of mammalian HSC70 in complex with BAG-1 (Williamson et al. 2009) (PDB: 3FZF). Accordingly, these residues are conserved primarily in Sks2, Ssa1, and Ssa2 that bind Bag101 and Bag102 but are not conserved in those Hsp70 ATPase domains that fail to interact with BAG-domain proteins (Electronic supplementary material, Fig. S1).

Overexpression of Bag101 and Bag102 causes growth defects

In mammals, the orthologue BAG-1 is essential and BAG-1 overexpression is observed in some cancers (Gotz et al. 2004; Takayama et al. 1998). Previously, we generated *bag101* and *bag102* deletion mutants (Kriegenburg et al. 2014) and found that neither *bag101* Δ nor *bag102* Δ cells display any obvious growth phenotypes at 25 or 37 °C. A *bag101* Δ *bag102* Δ double

Fig. 2 The UBL/BAG-domain proteins are specific for certain Hsp70-type chaperones. **a** To determine the Hsp70 specificity of the fission yeast UBL/BAG proteins, GST, GST-Bag101, and GST-Bag102, lacking the N-terminal transmembrane domain, were produced in *E. coli* and bound to Sepharose beads. These beads were then used to precipitate purified 6His-tagged Hsp70s. The precipitated material was analyzed by SDS-PAGE and blotting for the 6His-tag on the Hsp70s (*top*). Equal loading was checked by blotting with antibodies to GST (*bottom*). **b** A bootstrap tree of the fission yeast Hsp70 ATPase domains cluster in three subfamilies (I, II, and III). Members of subfamily I do not interact with any BAG-domain proteins, while members of subfamily II interact preferably with Bag101. Finally, the single member of subfamily III, Sks2, interacts with both Bag101 and Bag102

mutant also appeared as wild type (Kriegenburg et al. 2014). Therefore, as an alternative approach to explore the function of Bag101 and Bag102, we decided to over-express the *bag101*⁺ and *bag102*⁺ genes. To accomplish this, *bag101*⁺ and *bag102*⁺ were subcloned into the *S. pombe* expression vectors carrying the thiamine-repressible promoter *nmt1*. Fluorescence microscopy revealed that the proteins were expressed (Fig. 3a) and localized as expected (Kriegenburg et al. 2014), with Bag101 partially co-localizing with Pabp-containing stress granules (supplemental material, Fig. S2) and Bag102 along the nuclear envelope. Overexpression of *bag101*⁺ and to a lesser extent *bag102*⁺ led to a strong growth defect that was especially pronounced at higher temperatures (Fig. 3b). This phenotype was clearly caused by the overexpression of *bag101*⁺ and *bag102*⁺, since the

phenotype was lost when expression was repressed by addition of thiamine. When the *bag101*⁺ and *bag102*⁺ genes were expressed from the attenuated *nmt1* promoter, *nmt41*, a similar, albeit blunted, response was observed (Fig. 3b). With the strongly attenuated *nmt81* promoter, no growth inhibition was observed (Fig. 3b), suggesting that the growth inhibition caused by *bag101*⁺ and *bag102*⁺ overexpression depends on the expression level.

Next, we decided to test if the growth defect was caused by the UBL domains, the BAG domains, or both. Truncated versions of *bag101* and *bag102* were inserted in the *S. pombe* expression vector, carrying the strong *nmt1* promoter, and growth assays were performed as above. Surprisingly, only overexpression of the BAG domains, and not the UBL domains, caused the growth defect (Fig. 3c), although the effect appeared less strong than for the full-length proteins.

Fig. 3 BAG-domain overexpression causes a growth defect. **a** Differential interference contrast (DIC) and fluorescence micrographs of wild type *S. pombe* transformed to express GFP-tagged Bag101 and Bag102. DAPI staining was used to mark the nucleus. **b** The growth of wild-type cells expressing GFP-tagged *bag101*⁺ or *bag102*⁺ or, as a control, an empty vector, from the thiamine-repressed promoters *nmt1*, *nmt41*, and *nmt81*. **c** The growth of wild-type cells expressing *bag101*⁺ or *bag102*⁺ full length (FL), UBL domain, BAG domain or, as a control, an empty vector, from the thiamine repressed promoter *nmt1*

Overexpression of Bag101 induces a cellular stress response

In order to determine why *bag101*⁺ and *bag102*⁺ overexpression inhibits cell growth, we compared the transcriptomes of vector (control) and full-length *bag101*⁺ overexpressing cells by RNA sequencing. To this end, total RNA was purified from vector and full-length *nmt1 bag101*⁺ overexpressing *S. pombe* cells in quintuplicates, reverse transcribed, and subjected to next-generation sequencing. Analysis of global gene expression data showed that the five repeats of vector and *bag101*⁺ overexpression each clustered in two separate groups (Fig. 4a), suggesting that the replicate samples were highly similar, and that the two groups clearly differ in their gene expression patterns. In addition, principal component analysis (PCA) confirmed this difference between the sequences obtained from vector and *bag101*⁺ overexpressing cells (Fig. 4b). In total, our RNA sequencing could assign read counts to 2192 of 7017 annotated genes in the *S. pombe* genome. Applying a significance cutoff of 8-fold change between the groups and adjusted *p* values $\leq e^{-13}$, our dataset contained 139 significantly up-regulated genes and 42

significantly down-regulated genes. All sequencing data have been uploaded to Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>; accession no. GSE86512), and the differentially expressed genes are listed in the supplemental material (Electronic supplementary material, File 1).

To independently validate the quality of the dataset, we selected three up-regulated genes, *lsd90*⁺, *hsp16*⁺, and *ssa1*⁺ and quantified their expression by real-time PCR. Indeed, the expressions of these genes were significantly up-regulated (Fig. 4c), suggesting that the RNA sequencing dataset is robust.

Fig. 4 RNA sequencing of *bag101*⁺ overexpressing cells. **a** Total RNA was sequenced from control cells and *bag101*⁺ expressing cells in quintuplicates. Heatmap showing hierarchical clustering on the Euclidian distances between full set of gene counts for all samples (after DESeq2's own log transformation and variance stabilization). **b** Plot showing the first two principal components from a principal component analysis (PCA) on the full set of gene counts. **c** The amounts of *lsd90*⁺, *hsp16*⁺, and *ssa1*⁺ gene products, relative to the constitute actin (*act1*⁺), were compared by real-time PCR between control cells and cells overexpressing *bag101*⁺. **d** Bar diagram showing the relative frequencies of associated GO slim terms for all genes and *bag101*⁺ overexpression differentially regulated genes

Fig. 5 *bag101*⁺ triggers a stress response. **a** Venn diagram of the overlap between *bag101*⁺ overexpression-regulated genes and the five lists of stress response genes as reported by Chen et al. (2003). **b** Venn diagrams of the pairwise overlap between *bag101*⁺ overexpression-regulated genes and each of the five lists of stress response genes as reported by Chen et al. (2003). **c** Heatmap of CESR genes as defined by Chen et al. for *bag101*⁺ overexpression-regulated genes, *mas5Δ*, *ssa2Δ*, heat shock (Vjestica et al. 2013), and *sty1Δ* (Chen et al. 2003). The combined datasets were quantile normalized to make them comparable. Color key including value frequency is shown above heatmap. **d** Plot showing the first two principal components from a principal component analysis (PCA) on the same data shown in (c). **e** Venn diagrams of the overlap between *bag101*⁺ overexpression-regulated and *ssa2Δ* (Vjestica et al. 2013) for up- and down-regulated genes, respectively. To mimic the annotation of Vjestica et al., all non-coding and mitochondrial genes were removed from the list *bag101*⁺ overexpression-regulated genes. Furthermore, all *bag101*⁺ overexpression-regulated genes not found in the annotation of Vjestica et al. were removed

To further analyze and compare the datasets, we first assigned GO to each of the datasets to determine if *bag101*⁺ overexpression specifically causes differential expression (DE) of genes involved in certain biological processes. Indeed, when comparing with all genes in the *S. pombe* genome, we found that *bag101*⁺ overexpression caused differential expression of genes involved in metabolism and transmembrane transport (Fig. 4d). However, since this did not provide any clues as to why *bag101*⁺ overexpression would cause the observed growth defects, we decided to further compare our dataset with previous transcriptomic analyses in fission yeast. Since our biochemical analyses of Bag101 and Bag102 suggest that these proteins interact with Hsp70-type

chaperones via the BAG domains, and the observed growth defect depends on the BAG domain, we compared our data with the environmental stress response described by Chen et al. (2003). In this report, stress was applied using Cd, H₂O₂, heat shock, MMS, or sorbitol. Indeed, we found that the differentially expressed genes in our dataset overlapped with the defined environmental stress response genes (Fig. 5a). In total, out of the 181 differentially expressed genes in *bag101*⁺ overexpressing cells, 87 genes (48%) overlapped with those of Chen et al. (Fig. 5a). Comparing our dataset with the individual Cd, H₂O₂, heat shock, MMS, or sorbitol-regulated genes, however, did not suggest that the *bag101*⁺ responsive genes were particularly biased towards any single

Fig. 6 Bag101 activates Hsf1. **a** Microscopy of cells carrying the 5' UTR^{hsp104}/GFP/3'UTR^{hsp104} reporter for Hsf1 activity expressing either *bag101*⁺ or as a control the empty vector. **b** The growth of cells overexpressing *pdr13* or *skt2* (as indicated) without (vector) or with the *nmt1-bag101*⁺ expression vector was compared by serial dilutions on solid media. **c** The growth of wild-type, *ssa1* Δ , *skt2* Δ , and *hsp104* Δ cells expressing *bag101*⁺ from the thiamine-repressed promoter *nmt1* was compared by serial dilutions on solid media. **d** The growth of wild-

type and *P^{arg1}-hsf1* cells expressing *bag101*⁺ from the thiamine-repressed promoter *nmt1* was compared by serial dilutions on solid media. **e** Wild-type, *bag101* Δ *bag102* Δ , and *bag101*-GFP overexpressing cells all carrying GFP-tagged Hsf1 were used for immunoprecipitation (IP) experiments using antibodies to Hsp70. Input samples and the precipitated material were analyzed by SDS-PAGE and blotting for Hsp70 (top), the GFP-tag on Hsf1 and Bag101 (middle). Blotting with antibodies to α -tubulin served as a loading control (bottom)

stress condition (Fig. 5b). Thus, *bag101*⁺ overexpression causes a general stress response.

To gain further information on the *bag101*⁺-triggered transcriptional response, we compared our dataset with transcriptomic data of cells, deleted for the stress-activated MAP kinase Sty1, the stress-responsive transcription factor, Atf1, the Hsf1 transcriptional repressor, Mas5, and the Hsp70 chaperone Ssa2 (Chen et al. 2003; Vjestica et al. 2013). When comparing these datasets, the *bag101*⁺ overexpression response resembled those observed upon deletion of *mas5* or *ssa2* (Fig. 5c), which was also clear in PCA (Fig. 5d). Specifically, when comparing the *bag101*⁺ response with the *ssa2* Δ response, the overlap was only apparent for the up-regulated genes (Fig. 5e).

Collectively, these data suggest that the growth defect caused by *bag101*⁺ overexpression is akin to the response observed when reducing Hsp70 levels, such as in the *ssa2* Δ strain or upon Hsf1 activation as in the *mas5* Δ strain.

Bag101 activates Hsf1 by prompting its release from Hsp70

Since the *bag101*-triggered gene expression profile resembles that observed when the Hsf1 repressor *mas5* is deleted, we tested if Hsf1 was activated by Bag101. A strain, carrying a GFP reporter expressed under the *hsp104* regulatory elements (Vjestica et al. 2013), was transformed with either vector or a *bag101*⁺ expression construct and fluorescence was monitored by microscopy. Indeed, *bag101*⁺ caused a robust induction of the GFP reporter (Fig. 6a) which, along with the RNA sequencing data, strongly suggests that the *bag101*-mediated transcriptional response is largely or wholly dependent on Hsf1. To test this further, we reasoned that concomitant overexpression of the BAG-domain-binding Sks2 could function as a sink for Bag101 and should therefore blunt the growth defect caused by *bag101* expression. Indeed, the growth defect was slightly alleviated when Sks2 was co-expressed,

Fig. 7 Model of UBL/BAG-regulated Hsf1 activation in fission yeast. The presented data support a model where under normal (unstressed) conditions Hsf1 is repressed by Hsp70s such as Ssa1 and Ssa2 along with a Hsp40 co-chaperone such as Mas5. Under stress conditions, where misfolded proteins are in abundance or when UBL/BAG proteins are overproduced, Hsf1 is released from Hsp70 and activates transcription while the UBL/BAG proteins assist in targeting misfolded proteins to the 26S proteasome for degradation

whereas there was no effect of overproducing Pdr13 which does not bind Bag101 (Fig. 6b). Conversely, we observed that *bag101* overexpression was more adverse in strains lacking the BAG-domain-binding chaperones, Ssa1 and Sks2 (Fig. 6c). This was specific for the BAG-domain-binding chaperones, since there was no effect of deleting the Hsp104 chaperone (Fig. 6c), which does not bind the UBL/BAG-domain proteins or Hsf1.

Since Hsf1 is essential, it is not possible to use deletion mutants to directly assess if the growth defect, caused by the UBL/BAG-domain proteins, is caused by hyperactivation of Hsf1. Instead, we therefore sought to reduce Hsf1 expression using the *P^{urg1}-hsf1* strain where the native *hsf1* promoter is replaced with the weaker *urg1* promoter (Vjestica et al. 2013). Importantly, this down-regulation of *hsf1* partially rescued the *bag101*-mediated growth defect (Fig. 6d), suggesting the growth defect is, at least in part, caused by constitutive activity of Hsf1.

To further test the UBL/BAG mediated activation of Hsf1, we analyzed the interaction between Hsf1 and Hsp70 by immunoprecipitation and Western blotting. When Hsp70 was precipitated from wild type *S. pombe* cells, Hsf1 co-precipitated (Fig. 6e), indicating that Hsf1 forms a complex with Hsp70 under normal growth conditions. However, the Hsf1-Hsp70 interaction was reduced when *bag101*⁺ was overexpressed, while more Hsf1 was associated with Hsp70 in a *bag101Δbag102Δ* double mutant (Fig. 6e). This

suggests that the UBL/BAG-domain proteins regulate the Hsf1-Hsp70 interaction, and that the growth defect caused by *bag101* and *bag102* expression triggers Hsp70 to release Hsf1 leading to a constitutive Hsf1 activity.

Discussion

The mammalian BAG-1 protein is an essential (Gotz et al. 2004) anti-apoptotic co-chaperone which is frequently overexpressed in invasive cancers (Takayama et al. 1998; Tang et al. 1999) and functions as a prognostic biomarker for breast cancer (Paik et al. 2004). In this paper, we show that the *S. pombe* UBL/BAG-domain proteins Bag101 and Bag102 are co-factors of both 26S proteasomes and Hsp70-type chaperones, a conclusion that is in line with previous studies of UBL/BAG proteins (Alberti et al. 2002; Kriegenburg et al. 2014).

Surprisingly, we found that the fission yeast BAG proteins exclusively associate with the Sks2, Ssa1, and Ssa2 members of the Hsp70 family, three of five Hsp70s localized in the cytosol and/or nucleus in fission yeast. This suggests that these are the primary chaperones involved in targeting misfolded proteins for degradation via the ubiquitin-proteasome system, while the remaining two, Pdr13 and Pss1, perhaps play more specialized roles. Accordingly, the Pdr13 orthologue in budding yeast,

Ssz1, together with its Hsp40 co-chaperone, Zoutin, associate with the ribosome to assist in folding of nascent polypeptides (Huang et al. 2005). The *S. cerevisiae* orthologues of fission yeast Pss1, called Sse1 and Sse2, belong to the Hsp110 subclass of Hsp70 proteins that appear to mainly function as nucleotide exchange factors for other Hsp70s (Dragovic et al. 2006). We speculate that these more specialized Hsp70s do not require BAG-domain co-chaperones.

Previously, it has been shown that overexpression of the UBL domain protein, Dsk2, is toxic in budding yeast (Funakoshi et al. 2002), presumably by blocking binding of other UBL domain proteins to the proteasome, and thus hindering an efficient targeting of ubiquitylated proteins to the 26S proteasome. In case of the UBL/BAG-domain proteins, we found that the growth defect was triggered by the BAG domains, suggesting a different mechanism than that observed for Dsk2. Presumably, the BAG-domain proteins, when overexpressed, block Hsp70 function, thus in essence phenocopying an *ssk2Δssa1Δssa2Δ* triple deletion mutant. So far, our attempts at constructing this strain have been unsuccessful, which may suggest that such cells are not viable. In agreement with this, the RNA sequencing dataset, presented here, suggests that the observed Bag101-triggered growth defect is linked to a stress response. In eukaryotes, Hsf1 is one of the main stress responsive transcription factors. In fission yeast, Hsf1 activity is kept balanced through binding to Ssa1 and Ssa2 (Vjestica et al. 2013), and similar to the UBL/BAG-domain proteins, Hsf1 causes a growth defect when overproduced (Vjestica et al. 2013). Accordingly, we found that the UBL/BAG-induced growth defect is partly suppressed when the Hsf1 level is reduced. As for the reason why constitutive Hsf1 activity causes growth arrest, this phenotype is probably pleiotropic. However, the Hsf1-triggered transcriptional program has been shown to prevent normal growth patterning connected to insufficient function of the Cdc42 GTPase (Vjestica et al. 2013), revealing an unexpected link between thermal stress adaption, cell cycle control and cytoskeletal rearrangements.

In conclusion, the presented data support a model (Fig. 7) where, under stress conditions, when misfolded proteins are in abundance and the BAG-domain proteins shuttle these to the proteasome, Hsf1 is released from Hsp70 and activates transcription. As mentioned, in *S. pombe*, Bag101 and Bag102 represent the only BAG-domain co-chaperones and both display sequence homology with human BAG-1. However, the human genome encodes multiple BAG-domain proteins (Behl 2016; Bracher and Verghese 2015; Kabbage and Dickman 2008) that are involved in several processes, including apoptosis and tumorigenesis. However, they all function as co-chaperones for Hsp70, and although Hsf1 is more tightly regulated in mammals, the human BAG protein are likely to influence Hsf1 activity in a manner similar to that described here, which may also be relevant in cancers overexpressing BAG-1.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no competing interests.

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