Fine-tuning of actin dynamics by the HSPB8-BAG3 chaperone complex facilitates cytokinesis and contributes to its impact on cell division

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Abstract The small heat shock protein HSPB8 and its co-chaperone BAG3 are proposed to regulate cytoskeletal proteostasis in response to mechanical signaling in muscle cells. Here, we show that in dividing cells, the HSPB8-BAG3 complex is instrumental to the accurate disassembly of the actin-based contractile ring during cytokinesis, a process required to allow abscission of daughter cells. Silencing of HSPB8 markedly decreased the mitotic levels of BAG3 in HeLa cells, supporting its crucial role in BAG3 mitotic functions. Cells depleted of HSPB8 were delayed in cytokinesis, remained connected via a disorganized intercellular bridge, and exhibited increased incidence of nuclear abnormalities that result from failed cytokinesis (i.e., bi- and multi-nucleation). Such phenotypes were associated with abnormal accumulation of F-actin at the intercellular bridge of daughter cells at telophase. Remarkably, the actin sequestering drug latrunculin A, like the inhibitor of branched actin polymerization CK666, normalized F-actin during cytokinesis and restored proper cell division in HSPB8-depleted cells, implicating deregulated actin dynamics as a cause of abscission failure. Moreover, this HSPB8-dependent phenotype could be corrected by rapamycin, an autophagy-promoting drug,

whereas it was mimicked by drugs impairing lysosomal function. Together, the results further support a role for the HSPB8-BAG3 chaperone complex in quality control of actin-based structure dynamics that are put under high tension, notably during cell cytokinesis. They expand a so-far underappreciated connection between selective autophagy and cellular morphodynamics that guide cell division.

Keywords Cytokinesis · BAG3 · HSPB8 · Actin · Autophagy

Introduction

Fine-tuned protein quality control (PQC) systems are governed by molecular chaperones of the heat shock protein (HSP) family. Chaperones of the small HSP family (HSPB) form an eclectic family of proteins, which differ in their expression profiles and biochemical properties (Bakthisaran et al. 2015). Nonetheless, they are proposed to act mainly as ATPase-independent holdase or unfoldase by virtue of their dynamic quaternary structure, to segregate damaged proteins and prevent their irreversible aggregation (Finka et al. 2016; Haslbeck and Vierling 2015). In addition to their cytoprotective function under proteotoxic stress conditions, they appear to be instrumental to the normal functioning of complex proteinous structures of the cytoskeleton during biological processes relying on acute cellular remodeling (Guilbert et al. 2015). For instance, a phosphorylation-regulated function of HSPB1 has been reported, which may regulate actin polymerization and cancer cell migration (During et al. 2007; Kostenko and Moens 2009; Lavoie et al. 1993). Furthermore, HSPB8 has been involved in the recognition of damaged filamin, an actin-binding protein, during muscle cell contraction and its segregation for degradation by selective autophagy (Ulbricht et al. 2013). The later mechanism is proposed to maintain muscle fiber

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integrity by promoting clearance of actin-binding proteins undergoing partial misfolding in response to mechanical strain. Expectedly, several HSPB chaperones are up-regulated in cancer cells where they could contribute to the fitness of actin-based structures that support the tumor phenotype. Thus, by enabling targeted protein storage, recycling, or degradation, HSPB proteins would play important roles in cytoskeletal PQC, to maintain the integrity of cytoskeletal structures during stress, as well as to facilitate cellular morphodynamics that guide cell division and differentiation. In line with this concept, we have recently uncovered a novel role for HSPB8 in mitotic cell remodeling (Fuchs et al. 2015).

In spite of their known implications in human diseases, such as cancer, the mechanisms underlying the contribution of HSPB proteins to cytoskeletal dynamics/proteostasis are poorly understood. In that regard, the co-chaperone and scaffold protein BCL2-associated athanogene 3 (BAG3) is of particular interest. BAG3 can associate with several HSPB proteins but show a preferred association with and regulate the stability of HSPB8 (Carra et al. 2008; Fuchs et al. 2010; Taipale et al. 2014). In the context of cancer cells, BAG3 is viewed as a molecular scaffold owing to its interactions with signaling proteins and several regulators of actin dynamics and its implication in key biological processes, including apoptosis, development, cell motility, and cell division (Behl 2016; Rosati et al. 2011). Yet, the significance of distinct chaperone partners for BAG3 functions in intracellular signaling has remained unexplored. BAG3 also acts as a co-chaperone for HSC70/HSP70 chaperone system and modulates its function in proteasomal degradation, autophagy, and signaling (Chen et al. 2013; Colvin et al. 2014; Gamerdinger et al. 2009; Gamerdinger et al. 2011; Minoia et al. 2014). Accumulating evidence suggests that BAG3 serves to bridge important components of the chaperone network (Rauch et al. 2017). Nonetheless, BAG3 may function without recruiting HSPB8 in autophagy (Minoia et al. 2014). In contrast, its interaction with HSPB8 seems to be instrumental in typifying BAG3 functions in cytoskeletal remodeling. This is supported by our recent findings that BAG3 regulates remodeling of the mitotic actin-based structures that guide spindle orientation in a manner that requires binding to HSPB8, but not to HSC70/HSP70 (Fuchs et al. 2015). Because the same mitotic phenotype can be recapitulated by depletion of HSPB8, BAG3, or the autophagic receptor p62/SQSTM1, which shows enhanced mitotic association with BAG3, we have proposed that they act within a so-far unrecognized cytoskeletal PQC mechanism during mitosis. Evidence suggests that targeted cytoskeletal protein degradation can provide a mechanism to limit signaling pathways in time and space during mitotic cell remodeling (Belaid et al. 2013; Werner et al. 2013). Little is known, however, on the mechanism of quality control autophagy that provides “built in” selectivity for components of cytoskeletal remodeling pathways. Whether the HSPB8-BAG3

chaperone complex may contribute for such mechanism during mitosis remains to be demonstrated.

Here, we investigate the impact of HSPB8 in cytoskeletal PQC during cytokinesis, a process that is orchestrated by highly organized actin-based structures that are put under high tensile forces. We show that HSPB8 regulates BAG3 levels during mitosis and the accurate dynamics of the cytokinetic actin ring, via a mechanism that may involve effects on branched actin nucleation and autophagy signaling. Importantly, strong evidence is provided that first link HSPB8 function in cytoskeletal PQC to its modulation of mitotic cell division in cancer cells.

Material and methods

Cell culture, synchronization, drug treatments, and siRNA experiments

Parental HeLa cells, HeLa-GFP-H2B cells (gift from Laurence Pelletier), and HeLa-RFP-H2B cells (gift from Sabine Elowe) (Klebig et al. 2009) were maintained in α -minimal essential medium supplemented with 10% fetal bovine serum. All cell lines were grown in a humidified atmosphere with 5% CO₂ at 37 °C. Cells were synchronized by a double thymidine block and were released for 8 to 10 h in fresh medium, as described previously (Fuchs et al. 2015). For knockdown experiments, HeLa cells were transfected with 25 to 50 nM siRNA duplexes in calcium-phosphate transfection buffer (125 mM MgCl₂, 140 mM NaCl, 25 mM HEPES, 0.75 mM Na₂HPO₄) overnight. Analyses were performed 48 to 88 h later by Western blot, immunofluorescence, or live cell imaging. For cell cycle analyses of the impact of HSPB8-specific siRNAs on BAG3 protein levels, HeLa cells were synchronized in G2/M or M-phase by a 16-h treatment with the selective CDK1 inhibitor (RO3306, 8 μ M) or with nocodazole (400 ng/ml), respectively, and mitotic cells were recovered by a mitotic shake-off, as described before (Fuchs et al. 2015). Cells were lysed by 3 cycles of freezing/thawing in 4 volumes of lysis buffer (20 mM TRIS-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.1% IGEPAL, 1 mM NaVO₄, 10 mM NaF, 40 mM β -glycerophosphate, 1X Complete [Roche], 1 mM DTT), centrifuged at 15,000g for 15 min, and the supernatants were processed for Western blot analyses. The following drugs were added to cells that have been synchronized in mitosis with a double thymidine block, during the last hour of the second release period before cell fixation as followed: latrunculin A, 20 nM; CK666, 40 μ M; rapamycin, 150 nM; E-64D and pepstatin A, 10 μ g/ml; bafilomycin A1, 200 nM. For multi-nucleation assays, unsynchronized HeLa-GFP-H2B cells were grown in the presence of 1 nM latrunculin A for 48 h before cell fixation. The siRNA duplexes were based on human sequences and were purchased

from Qiagen (HPP grade siRNA) or Thermo Fisher Scientific (standard A4 grade). Sequences of the sense strands are as follows: siBAG3 #1: CGAAGAGTATTTGACCAAA-3';

siBAG3 #2: 5'-GCAAAGAGGTGGATTCTAA-3';

siHSPB8 #1: 5'-CAGATAGGCTAGTGGTATT-3';

siHSPB8 #2: 5'-GCAGTGAATGCAAGGGTTATT-3';

siHSPB8 #3: 5'-CAGAGGAGTTGATGGTGAATT-3'.

si AllStars Negative Control, target sequence: 5'-CAGG GTATCGACGATTACAAA-3'.

Transfection, infection, adenovirus, and vectors

HeLa cells were seeded on fibronectin-coated dishes and were synchronized by the double thymidine block method. During the second release period, cells were transfected with GFP-Arp3 (human Arp3 in pEGFP-N1 vector, gift from Dr John A. Cooper, ST Louis, USA) (Welch et al. 1997) using Lipofectamine 2000 at the ratio Lipo/DNA of 2:1 and following the manufacturer's instructions (Invitrogen) for a 10-h period before cell fixation. To follow the dynamics of actin, cells were seeded on fibronectin-coated glass dishes (MatTek Corp.) and synchronized by the double thymidine block method. Adenofection was performed during the first thymidine block using Ad-LifeAct-TagGFP2 (60121, IBIDI) at a multiplicity of infection (MOI) of 1 plaque-forming units per cell (pfu/cell), BacMam 2.0 reagents at 4 pfu/cell in α -MEM-minus, and siRNAs in calcium-phosphate transfection buffer, using the method described in Fuchs et al. (2016). Sixteen hours after adenofection, cells were washed 3 times with HEPES (6.7 mM KCl, 150 mM NaCl, 10 mM HEPES, pH 7.3) and were released in fresh medium for 8 h before adding back 2 mM thymidine.

Antibodies and chemicals

The following antibodies were used for Western blot analyses: rabbit anti-BAG3 LP11 was raised against full length recombinant human BAG3 fused with glutathione S transferase (LP11) (Fuchs et al. 2015), rabbit anti-HSPB8 against a C-terminal peptide (NELPQDSQEVCT) (Carra et al. 2008); anti-Vinculin (V9131) was from Sigma-Aldrich; anti-Arp3 (ab49671) was from Abcam; anti-Calreticulin (#612136) was from BD Biosciences; and anti-GAPDH Clone 6C5 (Fitzgerald, #10R-G109a) was from Millipore. Secondary antibodies (anti-mouse-HRP 115-035-146 and anti-rabbit-HRP 111-055-003) were from Jackson ImmunoResearch. The following antibodies were used for immunofluorescence: anti-Aurora B (ab2254) was from Abcam; Alexa-Fluor® 488 Phalloidin (A12379) and Texas Red®-X Phalloidin (T-7471) were from Molecular Probes/Thermo Fisher. Goat-anti-rabbit-594 (A11037) was from Molecular Probes. Hoechst Bisbenzimidazole H 33342 (B2261), thymidine (T9250), fibronectin (F1141-5 mg), RO-3306 (SML0569), E-64D

(E8640), pepstatin A (P5318), bafilomycin A1 (B1793), and CK666 (SML0006) were from Sigma-Aldrich; latrunculin A (428021-100UG) was from Calbiochem.

Immunofluorescence and live-cell imaging

Cells were gently washed once with LUFTIG (0.2 M sucrose, 35 mM Pipes, pH 7.4, 5 mM EGTA, 5 mM MgSO₄) and fixed with 3.7% formaldehyde in LUFTIG or 4% PFA in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄) for 20 min at room temperature. DNA was stained with cell permeable Hoechst (150 ng/ml). Specimens were processed for immunofluorescence as described (Lavoie et al. 2000) and were post-fixed with 3.7% formaldehyde in PBS for 20 min at room temperature. Phenotypes were monitored routinely by at least two independent investigators by visual inspection of fixed specimens and were scored in a manner blind to treatment, using an AxioObserver Z1 system using a 60 × 1.25NA objective and a charged-coupled device (CCD) camera AxioCam MRm controlled by the Zen software (Carl Zeiss). Confocal microscopy in live cells and fixed samples was performed with a PerkinElmer Life Science Ultraview Spinning Disk Confocal microscope, equipped with a 40 × 0.75NA objective, an EMCCD cooled charge-coupled camera at −50 °C (Hamamatsu Photonics K.K.), and driven by the Volocity software version 6.01. The system was equipped with a humidified/5% CO₂/thermoregulated chamber. Bi- and multi-nucleation was scored as the number of cells presenting 2 or >2 nuclei per cell; single cells were delineated by staining of the cortex with F-actin. To quantify abnormal F-actin accumulation at the intercellular bridge of daughter cells, we scored the number of daughter cells connected by a bridge labeled by Aurora B staining that had completely de-condensed DNA (late telophase) and abnormal F-actin structures at the bridge.

Long-term live-cell imaging was performed using a Nikon TE-2000 inverted microscope equipped with CO₂/thermoregulated chamber with a 40 × 0.6 NA objective. Images were captured as 16-bit TIFF files with a photometrix Collsnap FX cooled CCD camera (−30 °C) (Rooper Scientific, Tuscon AZ) driven by the Metaview software version 4.5 (Universal Imaging Corp., Downingtown, PA). Images were acquired at 90 s intervals for a 48-h period or at 10 min intervals for a 72-h period, starting at 40 h after siRNAs transfection. Phenotypes associated with cytokinesis were estimated by visual inspection of time sequences. Spreading of daughter cells and disappearance of the intercellular bridge were evaluated by monitoring the time spent between closure of the actin ring and cell spreading and/or disappearance of the dense midbody ring, respectively. Defects in daughter cell spreading were considered as follows: (1) prolonged cortex blebbing, (2) uncoordinated spreading of daughter cells, and (3) no spreading. To quantify the number of cells that re-entered mitosis

synchronously, we determined that cells are derived from the same mother cell by visual inspection of time sequences; cells were considered synchronized if they entered mitosis at the same time point or separated by less than 2 time points. Actin ring (AR) thickness and width were expressed as the mean intensities from F-actin staining and were evaluated by delineating AR manually on confocal slices determined as the center of the cell, using the Volocity software version 6.0 (Quorum Technologies); cells were considered only after AR closure and DNA de-condensation. To quantify GFP-Arp3 recruitment, AR and daughter cells were delineated manually using F-actin staining as the reference for cell shape using the same software. The mean fluorescence intensities of GFP-Arp3 at AR and in the cytoplasm were measured from confocal image stacks acquired at 0.5 μm z-steps and expressed as a ratio of AR over cytoplasm. The Volocity software version 6.0 (Quorum Technologies) and ImageJ 1.48v (National Institute of Health) were used for processing entire images before cropping to emphasize the main point of the image. Processing was limited to background subtraction and brightness/contrast adjustment.

Western blotting

Cells were lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.05% bromophenol blue, 1 mM phenylmethylsulfonyl fluoride); equal amounts of protein from whole cell lysates were separated by SDS-PAGE on 10% acrylamide gels and transferred onto nitrocellulose membranes. Immunoblotting was performed as described (Champagne et al. 2004). Protein concentrations were determined with DC protein assay reagent, and densitometric analyses were performed from FluorS MAX MultiImager-captured images using the QuantityOne software version 4.5.0 (Bio-Rad Laboratories).

Statistical analyses

For experiments examining (a) the time spent for daughter cells spreading or (b) AR thickness/width, the mean values from individual cells from at least three independent experiments were analyzed using the Mann-Whitney test, which is a non-parametric test that compares two unpaired groups of ordinal or numerical variables. For experiments examining the proportions of cells with (a) cell spreading defects, (b) ICB defects, (c) persistent connections, (d) synchronized daughter cell mitosis, (e) nuclear abnormalities, and (f) abnormal F-actin accumulation at the ICB, data from individual experiments were analyzed using the Fisher's exact test, which is used for small sample sizes with nominal/categorical variables. Statistical calculations were performed using Prism 6.0 (GraphPad Software) statistical software. p values <0.05 considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Results

Silencing of HSPB8 or BAG3 perturbs abscission of daughter cells

We sought to characterize the function of HSPB8-BAG3 chaperone complex in the extreme cell morphodynamic changes that guide cytokinesis. To that end, unsynchronized HeLa-GFP-H2B were treated with control small interfering RNA (siRNA), or HSPB8- or BAG3-specific siRNAs that achieved $>75\%$ depletion of the protein in these cells, and were imaged for a period of 72 h starting at 40 h after transfection of siRNAs (Fig. 1a). In agreement with our previous findings, cells depleted of HSPB8 or BAG3 eventually progressed through anaphase, but exhibited higher incidence of defects during cytokinesis (Fuchs et al. 2015). Such defects were associated with two major phenotypes: delayed cell spreading at telophase and delayed abscission of daughter cells. HSPB8-depleted cells showed a ~ 1.3 -fold increase in the mean time required to achieve spreading (35 min; siHSPB8) relative to control cells (26 min; siCtl) (Fig. 1b, c), frequently associated with prolonged cortex blebbing (Fig. 1b, siBAG3 490'; supplemental movies S1–S3). This phenotype was observed in 24 to 30% of HSPB8- or BAG3-depleted cells, respectively, as compared to 12% of the control cell population (Fig. 1c). The data are consistent with previous work involving BAG3 in modulation of cell adhesion, a function that may engage its association with HSPB8 at mitotic exit (Iwasaki et al. 2007; Iwasaki et al. 2010; Kassis et al. 2006).

Most remarkably, depletion of HSPB8 or BAG3 was associated with increased occurrence of abnormally thick and long intercellular bridges (ICB) connecting nascent daughter cells, which persisted for longer periods of time (Fig. 1d, arrowheads; Fig. 1e; supplemental movies S4–S6). Indeed, BAG3-depleted cells showed a ~ 1.7 -fold increase in the mean time required for ICB disappearance relative to control cells (Fig. 1e). Moreover, a larger proportion of HSPB8-depleted cells remained connected by an ICB comprising a typical midbody ring (Fig. 2a, red arrowhead and inset) and even re-entered mitosis synchronously (Fig. 2b, c). These phenotypes are typical of cells that fail abscission and were also observed in BAG3-depleted cells (not shown) (Belaïd et al. 2013; Schiel et al. 2012). In further support, a significant proportion of cells transfected with two distinct HSPB8-specific siRNA sequences exhibited bi-nucleation, a defect that arises upon cytokinetic failure and collapsing back into a single bi-nucleated cell, which may be due to impairment of abscission (Fig. 2d, e). Together, these observations suggest that HSPB8, in complex with BAG3, facilitates some late steps during cytokinesis that control the abscission of daughter cells.

Fig. 1 Depletion of HSPB8 or BAG3 perturbs daughter cells spreading and ICB disappearance during cytokinesis. **a** Representative Western blot of HeLa-GFP-H2B cells lysed in SDS-sample buffer, showing the efficiency of HSPB8 and BAG3 depletion after transfection with HSPB8- and BAG3-specific siRNAs. Depletion was estimated at >75% by loading increasing amounts of control extracts (siCtl). Western blot analysis was performed with anti-HSPB8 and anti-BAG3 antibodies; GAPDH: loading control. **b** Unsynchronized HeLa-GFP-H2B cells were depleted of HSPB8 or BAG3 using the indicated siRNAs and were imaged starting 40 h post-transfection for 72 h at 10-min intervals. First row of panels: representative time sequence of a control cell showing normal daughter cells spreading; second and third rows of panels: representative time sequences of HSPB8- or BAG3-depleted cells showing delayed spreading. Scale bar, 10 μ m. **c** On the left: graph showing the average time of daughter cells spreading for control (siCtl) versus HSPB8-depleted cells (siHSPB8). Data are the means $\pm$ SE from three independent experiments; the Mann-Whitney's test was performed

relative to control **** p < 0.0001. On the right: graph depicting the percentage of cells with abnormal daughter cells spreading in HSPB8- (siB8) or BAG3-depleted cells (siBAG3) compared to control cells (siCtl), means $\pm$ SE from two or three independent experiments. * p < 0.05 compares siHSPB8 #3 to siCtl by the Fischer exact test. **d** Representative time-sequences of HeLa-GFP-H2B cells transfected with the indicated siRNAs and imaged for 72 h starting at 40 h after transfection. Enlarged views of the boxed regions emphasize the structure of the ICB, which is abnormally long and/or thick in cells depleted of HSPB8 or BAG3, as designated by the white and red arrows, respectively; time sequences are in min; bars, 10 μ m. **e** Graphs depicting the percentages of cells with abnormal ICB (on the left) or the time spent between early telophase and disappearance of the ICB (on the right); the data are the means $\pm$ SE of two to three independent experiments as indicated. ** p < 0.01 and **** p < 0.0001 compare siHSPB8 to siCtl by the Fisher exact test

Fig. 2 Silencing of HSPB8 interferes with cytokinetic abscission and markedly decreases the mitotic levels of BAG3. **a** Unsynchronized HeLa-GFP-H2B cells transfected with various HSPB8-specific siRNA sequences were imaged for 72 h at 10-min intervals. The red arrowhead designates a persistent ICB connecting sister cells that comprises a dense midbody (emphasized in the enlarged view); bar 10 μm. The graph shows the percentage of cells remaining connected by an ICB; means ± SE. * $p < 0.05$ compares siHSPB8 #3 to siCtl by the Fisher exact test. **b** Time sequences showing sister cells entering mitosis synchronously (red arrowheads); bar 20 μm; P prophase, M metaphase, T telophase. **c** Graph depicting the percentages of cells undergoing synchronous mitosis; means ± SE. *** $p < 0.001$ compares siHSPB8 #3 to siCtl by the Fisher exact test. **d** Representative Western blot of HeLa-GFP-H2B cells that have been lysed in SDS-sample buffer extracts,

showing the efficiency of HSPB8 depletion, 88 h after transfection of the indicated siRNA sequences; calreticulin: loading control. **b** Confocal image of HeLa-GFP-H2B cells transfected with HSPB8-specific siRNAs, showing multi-nucleation; F-actin was stained with Texas-Red-Phalloidin; bar 30 μm. The graph shows the incidence of bi- and multi-nucleation; means ± SE from three independent experiments. **** $p < 0.0001$ compares siHSPB8 #2 to siCtl, and ** $p < 0.01$ compares siHSPB8 #3 to siCtl by the Fisher exact test. **f** Representative Western blot of HeLa cell extracts isolated from unsynchronized cells (As), or cells that have been synchronized at the G2/M border (G2/M), or M-phase (M), after transfection with HSPB8-specific siRNA or control siRNA (siCtl) for 88 h, showing the levels of BAG3, HSPB8, and GAPDH (loading control). Cells have been lysed by 3 cycles of freezing/thawing in 4 volumes of 0.1% IGEPAL lysis buffer

Knockdown of HSPB8 causes abnormal F-actin accumulation at the intercellular bridge of daughter cells

Having shown that silencing of either BAG3 or HSPB8 causes similar cytokinetic defects, and considering that HSPB8 levels depend on BAG3 (Fig. 1a) (Carra et al. 2008), subsequent analyses were performed in HSPB8-depleted cells. Besides, we found that depletion of HSPB8 reduced BAG3 levels by ~50 to 75% in soluble fractions recovered from cells arrested at the G2/M border and at the M-phase, respectively, without affecting BAG3 levels seen in the insoluble fractions (Fig. 2f, G2/M and M). In mitotic cells, BAG3 was mainly detected as a

supershifted, hyperphosphorylated protein band on SDS-PAGE as previously shown (Fig. 2f, red arrow) (Fuchs et al. 2015). This was contrasting with the modest reduction of BAG3 levels seen upon silencing of HSPB8 in asynchronous cells (~30%), as shown before (Fig. 2f, AS) (Carra et al. 2008). Together with our previous findings that HSPB8 facilitates BAG3 phosphorylation and localization at mitotic entry (Fuchs et al. 2015), these results suggest that cell mitosis offers a unique model to uncover the HSPB8-dependent functions of BAG3.

Based on our previous work linking BAG3-HSPB8 to the remodeling of actin-based structures at mitotic entry (Fuchs et al. 2015), we then sought to determine the impact of

HSPB8 on actin dynamics during cytokinesis. In animal cells, cytokinesis begins with the assembly and contraction of an actomyosin ring that leads to the formation of an intercellular bridge (von Dassow and Bement 2005). Final cleavage and fusion of this intercellular bridge require thinning of the ICB (secondary ingression) that critically relies on actin depolymerization within the ICB (Schiel et al. 2013; Schiel et al. 2012). We hypothesized that the chaperone complex might assist in accurate assembly and/or disassembly of actin-based cytokinetic structures.

To test this idea, we took advantage of LifeAct-GFP—a probe that binds to F-actin (Riedl et al. 2008), to visualize the dynamics of actin at the actomyosin ring by spinning confocal microscopy. HeLa-RFP-H2B cells were adenofected with recombinant adenovirus driving the expression of LifeAct-GFP together with control or HSPB8-specific siRNAs, and then synchronized by a double thymidine block using a protocol developed for knockdown-rescue experiments (Fig. 3a) (Fuchs et al. 2016). Live-cell imaging revealed that assembly and constriction of the actomyosin ring were not significantly perturbed by depletion of HSPB8 (Fig. 3c). Nonetheless, in HSPB8-depleted cells, the AR appeared abnormally enriched in F-actin. This was revealed by an increase of AR thickness at telophase (Fig. 3c, supplemental movies S7–S8). Remarkably, higher proportions of HSPB8-depleted cells ($\sim 43 \pm 2.5\%$; $43 \pm 4.2\%$; $58 \pm 2.5\%$) displayed abnormal F-actin accumulation at the ICB marked by Aurora B (Murata-Hori et al. 2002)—a mitotic kinase that relocates to the ICB at telophase, as compared to cells transfected with control siRNAs ($\sim 24 \pm 1.3\%$). Such disorganized actin structures persisted after cell spreading and DNA de-condensation and were observed using three different HSPB8-specific siRNA sequences in depletion experiments (Fig. 3b, c). The data suggest that HSPB8-BAG3 chaperone complex facilitates accurate AR dynamics to enable timely abscission of daughter cells.

Down-modulation of actin dynamics can normalize F-actin accumulation at the intercellular bridge and correct cytokinetic defects caused by depletion of HSPB8

Abnormal accumulation of F-actin at the ICB suggested that HSPB8 depletion could perturb actin dynamics, i.e., polymerization-depolymerization. To obtain evidence for a causal relationship, we used the actin drug latrunculin A (LatA), which inhibits actin dynamics by sequestering G-actin, hence limiting the actin pool available for F-actin assembly (Coue et al. 1987; Morton et al. 2000). Cells were treated with a low concentration (20 nM) during the last hour of the release period before cell fixation (Fig. 4a). Under these conditions, the organization of F-actin in

control cells was not noticeably affected. Significantly, such treatment inhibited F-actin accumulation at the ICB in HSPB8-depleted cells, bringing it in line with cells transfected with control siRNAs (Fig. 4b, c). The results suggest that HSPB8-depleted cells have deregulated actin polymerization at the ICB that can be normalized by limiting the pool of G-actin.

We then took advantage of the hypersensitivity of HSPB8-depleted cells to LatA to examine the cause-effect relationship between deregulated actin dynamics and cytokinetic failure, by measuring the occurrence of bi- and multi-nucleation. We determined that incubation of HeLa-GFP-H2B cells in the presence of 1 nM LatA for a 48-h period of time modestly affected cell division and increased the incidence of nuclear abnormalities by ~ 1.8 -fold (Fig. 4d, siCtl LatA). However, this treatment reduced the incidence of bi- and multi-nucleation in HSPB8-depleted cells to a level comparable to the one observed in cells transfected with control siRNAs (Fig. 4d, siB8 LatA). Thus, the data suggest that perturbation of actin dynamics upon depletion of HSPB8 contributes to its impact on cell division.

Inhibition of Arp2/3 complex can normalize F-actin at the ICB in HSPB8-depleted cells

Accurate assembly and disassembly of the cortical AR during cytokinesis are proposed to rely on the combined action of two F-actin nucleators, the formin diaphanous (Dia) and the Arp2/3 complex, and require dynamic actin turnover at the equatorial AR (Bovellan et al. 2014; Murthy and Wadsworth 2005). We noted that the BAG3 interactome from HeLa cells contains proteins that could link BAG3 to Arp2/3 complex, notably ArpC2, a component of Arp2/3 complex, and ELMO1, which provides a connection with upstream regulators (Chen et al. 2013). To explore a potential relationship with the Arp2/3 complex, we first analyzed its subcellular distribution during cytokinesis. Small amounts of a fluorescent probe, GFP-Arp3, were expressed in HeLa cells for a short period of time after silencing HSPB8 to avoid perturbing actin dynamics. The subcellular distribution of GFP-Arp3 was analyzed by confocal microscopy after cell fixation (Fig. 5a, experimental scheme; Fig. 5b). Cells transfected with control siRNAs undergoing cytokinesis exhibited a rather diffuse localization of Arp3-GFP with no significant enrichment at the ICB (Fig. 5c). This was revealed by quantification of the GFP fluorescence intensity ratio of ICB over cytoplasm (Fig. 5, ~ 0.89). In marked contrast, in cells transfected with HSPB8-specific siRNAs, GFP-Arp3 accumulated at the ICB and co-distributed with aberrant F-actin structures, as revealed by a ~ 1.6 -fold increase in the ICB/cytoplasmic GFP fluorescence intensity ratio (Fig. 5c, arrowhead).

Fig. 3 Silencing of HSPB8 causes abnormal F-actin accumulation at the ICB. **a** Schematic of the protocol used for adenofection of HeLa-RFP-H2B cells with Ad-LifeAct-GFP together with HSPB8-specific siRNAs. **b** Western blot of HeLa-RFP-H2B cell extracts prepared in SDS-sample buffer showing the efficiency of HSPB8 depletion 48 h after transfection of the indicated HSPB8-specific siRNAs; depletion was estimated at >75% by loading increasing amounts of control extracts (1/4, 1/2, 1, siCtl); vinculin: loading control. **c** Live-cell imaging was performed for a 2-h period at 5-min intervals by spinning disk confocal microscopy. Representative time sequences show F-actin organization at the actin ring (pointed by white arrowheads) in cells transfected with control siRNAs (siCtl) compared to cells transfected with HSPB8-specific siRNAs; bar, 10 μ m. Quantification of the actin ring thickness over

width; means $\pm$ SE from three independent experiments. The Mann-Whitney's test was performed relative to siCtl; **** p < 0.0001. **d** Confocal images of HeLa-RFP-H2B cells that have been synchronized in mitosis after transfection with HSPB8-specific siRNAs, showing abnormal F-actin accumulation stained by Alexa-488-phalloidin at the ICB (green) marked by immunostaining of Aurora B (red). White arrowheads designate enlarged views that emphasize actin organization at the bridge of daughter cells; bar, 10 μ m. The graph shows the percentages of cells with abnormal F-actin accumulation at the ICB in late cytokinesis, means $\pm$ SE from three independent experiments. **** p < 0.0001 compares HSPB8-specific siRNAs to siCtl by the Fischer exact test

We then sought to acutely perturb Arp2/3 activity as cells entered cytokinesis and monitor the impact on ICB-associated aberrant F-actin. To that end, HSPB8-depleted cells that have been synchronized in late mitosis were incubated in the presence of the selective Arp2/3 inhibitor CK666 (Nolen et al. 2009) or with the vehicle only, during the last hour of the release period from the thymidine-block. Using this protocol, inhibition of the Arp2/3

complex did not impact cytokinesis in cells treated with control siRNAs, consistent with recent findings (Bovellan et al. 2014; Rosa et al. 2015). Nonetheless, such treatment inhibited the occurrence of abnormal F-actin at the ICB in HSPB8-depleted cells (Fig. 5d). These data suggest that by limiting Arp2/3-mediated actin polymerization, an accurate balance between actin assembly and turnover at the ICB can be restored in HSPB8-depleted cells.

Fig. 4 Down-modulation of actin dynamics can rescue phenotypes caused by depletion of HSPB8. **a** Schematic of the protocol used to modulate actin dynamic in HeLa-RFP-H2B cells synchronized in late mitosis that have been transfected with HSPB8-specific or control siRNAs. **b** Representative confocal images of fixed HSPB8-depleted HeLa-RFP-H2B cells, showing normalized F-actin at the ICB (emphasized in enlarged views pointed by white arrowheads), after treatment with latrunculin A (LatA, 20 nM); immunostaining of Aurora B (red) was performed to detect ICB and F-actin was stained with Alexa-488-phalloidin (green). Bar, 10 μ m. **c** The graph depicts the percentages of cells with abnormal F-actin accumulation

at the ICB; means $\pm$ SE from four independent experiments. **** p < 0.0001 compares siHSPB8 #3 treated with the vehicle (DMSO) to siCtl $\pm$ DMSO by the Fisher exact test; n.s.: non-significant compares siHSPB8 #3 + LatA to siCtl $\pm$ LatA. **d** Unsynchronized HeLa-GFP-H2B that have been transfected with HSPB8-specific or control siRNAs (siB8 vs siCtl) were grown in the presence of 1 nM LatA for 48 h and the percentages of nuclear abnormalities were scored; means $\pm$ SE from three independent experiments. **** p < 0.0001 compares siHSPB8 #2 treated with the vehicle (DMSO) to siCtl $\pm$ DMSO by the Fisher exact test; n.s.: non-significant difference between siHSPB8 #3 + LatA and siCtl $\pm$ LatA.

Drugs that affect lysosomal function recapitulate the HSPB8-dependent phenotype, whereas induction of autophagy signaling can restore the F-actin defects caused by depletion of HSPB8

A specialized role for a multichaperone complex organized by BAG3 has been proposed in the clearance of damaged

proteins undergoing partial misfolding in response to mechanical strain during skeletal muscle contraction (Arndt et al. 2010; Ulbricht et al. 2013). Given that the actin-based AR is also put under high tensile forces during cytokinesis, we hypothesized that HSPB8-BAG3 could be mobilized to facilitate the autophagic clearance of components of actin-based cytokinetic structures.

Fig. 5 Co-distribution of abnormal F-actin and Arp2/3, and restoration of F-actin organization at the ICB in HSPB8-depleted cells upon inhibition of Arp2/3 complex. **a** Schematic of the protocol used to visualize Arp2/3 localization in HeLa-RFP-H2B cells during cytokinesis. **b** Western blot of HeLa-RFP-H2B cell extracts prepared in SDS-sample buffer showing the levels of transfected GFP-Arp3 and endogenous Arp3; calreticulin levels are shown as loading control. **c** Representative confocal images of HSPB8-depleted HeLa-RFP-H2B cells, showing accumulation of GFP-Arp3 together with F-actin; staining of Aurora B (purple) was performed to localize ICBs that are emphasized in enlarged views designated by white arrowheads; bar, 10 μ m. Quantification of the fluorescence intensity ratios of GFP-Arp3 is shown on the right graph, means $\pm$ SE of at least 98 cells from four

independent experiments. The Mann-Whitney's test was performed relative to control; **** $p < 0.0001$. **d** Confocal images showing synchronized HeLa-RFP-H2B cells depleted of HSPB8 treated with the vehicle only (DMSO) or with a selective Arp 2/3 inhibitor (CK666, 40 μ M) 1 h before cell fixation. F-actin organization at the ICB (phalloidin staining: green, Aurora B staining: red) is emphasized in the enlarged views designated by white arrowheads; bar, 10 μ m. A schematic of the protocol used is shown below. The graph depicts the percentages of cells with abnormal F-actin accumulation at the ICB; means $\pm$ SE from two or four independent experiments; n.s.: non-significant difference between siHSPB8 #3 + CK666 versus siCtl $\pm$ CK666 by the Fisher exact test

To explore this idea, we used two distinct experimental approaches. We first asked whether impairing lysosomal function could interfere with AR dynamics. HeLa cells that have been synchronized in mitosis by a double thymidine block were incubated in the presence of drugs that are commonly used to disrupt the autophagic flux; these include bafilomycin

A (BafA), which inhibits V-ATPases and blocks autophagosome-lysosome fusion, or pepstatin A and E64D, two inhibitors of lysosomal proteases (Klionsky et al. 2016). The drugs were added during the last hour of the release period before cell fixation, and cells in late cytokinesis were detected by immunostaining of the ICB using anti-Aurora B

antibody. Analysis of F-actin distribution revealed that a larger proportion of cells treated with the drugs accumulated aberrant actin structures at the ICB that persist after cell spreading and DNA de-condensation relative to cells treated with the vehicle only (~1.8-fold increase; Fig. 6a). Thus, the data suggest that inhibition of vacuolar acidification or lysosomal enzymes, like depletion of HSPB8, can recapitulate similar defects in actin dynamics at the ICB.

As a second approach, we submitted HSPB8-depleted cells that have been synchronized in late mitosis to a 1-h incubation period with rapamycin—a drug that inhibits mTORC1 and stimulates autophagy signaling (Crespo and Hall 2002). Remarkably, such treatment corrected F-actin accumulation at the ICB and brought the proportions of HSPB8-depleted cells bearing F-actin defects in line with cells transfected with control siRNAs (Fig. 6b). Collectively, our results suggest that defects in the dynamics of the actin-based AR caused by depletion of HSPB8 may result, at least in part, from its function in the segregation of cytoskeletal protein clients to selective autophagy.

Discussion

BAG3, in association with its chaperone partners, has been linked to the selection of damaged proteins for autophagic degradation during proteotoxic stress and muscle cell contraction (Minoia et al. 2014; Ulbricht et al. 2013). While BAG3 controls the stability of HSPB8, it may also function without HSPB8 during stress, questioning the exact role of HSPB8 in the reported functions of BAG3 (our unpublished data) (Ganassi et al. 2016; Minoia et al. 2014). Recently, we uncovered an HSPB8-dependent function of BAG3 in a basic and fundamental activity of cancer cells: mitosis, in the remodeling of actin-based structures that guide spindle dynamics (Fuchs et al. 2015). In the current study, we present further evidence that in response to mitotic signaling, HSPB8 and BAG3 act together to modulate actin dynamics during cytokinesis and facilitate abscission of daughter cells. Depletion of HSPB8 or BAG3 caused levels of cytokinetic defects, notably multi-nucleation, that compare favorably with those reported in the literature upon silencing of p62/SQSTM1 or key effectors of cytokinesis, using similar approaches based on analyses of hypomorphic phenotypes (5 to 25% of multi-nucleated cells on average) (Belaïd et al. 2013; Ma and Chircop 2012; Schiel et al. 2012). This suggests that the HSPB8-BAG3 complex plays a significant role in the context of HeLa cells. Because drugs that selectively perturb actin polymerization can correct the multi-nucleation phenotype caused by depletion of HSPB8, which also depletes the mitotic forms of BAG3, we suggest that an HSPB8-BAG3 complex promotes accurate cell division mainly by limiting actin polymerization and/or promoting actin turnover. Whether such process may involve HSPB8-BAG3

function in autophagic sequestration is suggested by some results presented here and is further discussed below.

Our finding that HSPB8 maintains BAG3 levels mainly during M-phase suggests that it may serve to stabilize particular conformations of BAG3 induced by mitotic phosphorylation. This could promote interactions between BAG3 and client proteins that would connect the chaperone complex to actin-based structures, perhaps, via BAG3 Pro-rich motifs, which we have identified as crucial determinants of BAG3 mitotic activity in previous work (Fuchs et al. 2015). BAG3 was reported to interact with several proteins that could modulate actin assembly or promote actin turnover, for instance CapZ—an actin-capping protein that prevents filament growth (Cooper and Schafer 2000; Hishiyama et al. 2010; Pollard and Cooper 2009), or non muscle myosin II—the molecular motor that generates forces for constriction of the actin-based AR (Hong et al. 2016; Maupin and Pollard 1986; Schiel et al. 2013). It has been shown that actin at the AR is highly dynamic and myosin II was involved in the dynamic turnover of actin during cytokinesis (Murthy and Wadsworth 2005). Given that BAG3 can interact with a plethora of potential client proteins in HeLa cells (Chen et al. 2013), its mitotic activity likely involves complex interactions with client proteins that could be regulated in time and space by HSPB8 and multisite phosphorylation of BAG3.

The results presented here provide important mechanistic clue by showing that silencing of HSPB8 causes accumulation of branched actin at the ICB. Assembly of branched actin networks is mediated by the actin nucleator complex Arp2/3 that appeared to accumulate at the ICB of HSPB8-depleted cells (Goley and Welch 2006). Furthermore, inhibition of Arp2/3 using CK666 can prevent abnormal F-actin accumulation at the ICB in these cells, suggesting that HSPB8-BAG3 act by limiting Arp2/3 activity. It has been proposed that a switch from Arp2/3 to diaphanous-mediated actin nucleation is required to drive assembly of a rigid actin cortex in metaphase cells (Rosa et al. 2015). Too much Arp2/3-mediated actin polymerization may also lead to cytokinesis failure (Chircop 2014). Indeed, deregulation of the antagonism between the Rho GTPases Rho and Rac, which is believed to control AR dynamics during cytokinesis, leads to defective cytokinesis and aberrant distribution of F-actin (D'Avino et al. 2004). We have shown that depletion of HSPB8 or BAG3 interferes with cell rounding and assembly of a mechanically rigid actin cortex at mitotic entry, in addition to its perturbing effect during cytokinesis (Fuchs et al. 2015). Based on the overall data, it can be argued that failure to accurately limit the activity of Arp2/3 upon depletion of HSPB8 or BAG3 contributes to defects in mitotic cortical rigidity, spindle positioning, and cytokinesis, leading to impaired cell division and multi-nucleation.

Our findings also suggest that a phosphorylation-activated function of HSPB8-BAG3 would promote actin turnover

Fig. 6 Inhibition of lysosomal function mimics, whereas autophagic signaling can correct the cytokinetic defect in F-actin caused by depletion of HSPB8. **a, b** Representative confocal images of HeLa-RFP-H2B cells untransfected (**a**) or transfected with HSPB8-specific siRNA (siB8, **b**) that have been synchronized in late mitosis and treated with the indicated compounds for a 1-h period before cell fixation; bafilomycin A1 (BafA, 200 nM), pepstatin A and E-64D (Pep + E64D, 10 μ g/mL), rapamycin (150 nM). F-actin was stained with phalloidin

(green), and immunostaining of Aurora B was performed to detect the ICB (red). F-actin organization at the ICB is emphasized in the enlarged views designated by white arrowheads; bars 10 μ m. The percentages of cells exhibiting abnormal F-actin accumulation at the ICB are shown on the right graphs; means $\pm$ SE from three independent experiments. **** p < 0.0001, compare BafA or Pepstatin + E64D to DMSO, or siB8 + DMSO to siCtrl by the Fisher exact test; n.s.: non-significant difference by comparing siB8 + rapamycin to siCtrl $\pm$ rapamycin

rather than enhancing actin polymerization, as it is the case for HSPB1 (During et al. 2007; Lavoie et al. 1995). Such function would be in line with the proposed role of HSPB8-BAG3 within a pathway induced by mechanical cues for the clearance of cytoskeletal protein in the muscle sarcomere—the actin contractile unit of myofibrils (Ulbricht and Hohfeld 2013). This pathway could be mobilized in dividing cells by mitotic signaling, via BAG3 phosphorylation, to facilitate accurate turnover of non-muscle contractile structures that are submitted to high tensile forces. It has been hypothesized that the organization of actin and myosin II in the cytokinetic AR is similar to that seen in muscle sarcomere; recent evidence

supports a sliding filament-based purse string contraction mechanism for AR constriction (Fenix et al. 2016; Pollard and Wu 2010). Although it has been established that the AR needs to be disassembled to allow for the final abscission of daughter cells, the mechanisms remain poorly understood. A role for selective autophagy has been proposed, in the localized inactivation of RhoA involving the autophagic receptor p62/SQSTM1 (Belaid et al. 2014; Mizuno 2013; Piekny et al. 2005; Theriot 1997). In further support of a role for autophagy, we observed here that treatment of cells with commonly used inhibitory drugs recapitulated abnormal accumulation of F-actin at the ICB, similar to the phenotype caused by depletion of

HSPB8. Recently, we have shown that HSPB8, BAG3, and p62/SQSTM1 form a mitotic complex and that depletion of any of the proteins resulted in the same phenotypes in early mitosis (Fuchs et al. 2015). Here, we provide further evidence for a connection between the activity of HSPB8-BAG3 during cytokinesis and autophagy signaling by showing that treatment of HSPB8-depleted cells with rapamycin could prevent F-actin accumulation. This is in line with recent work suggesting that BAG3 modulates the spatial activity of mTORC1—the target of rapamycin and a key regulator of autophagy signaling, in addition to its direct interaction with the selective autophagic machinery via p62/SQSTM1 (Gamerding et al. 2011; Kathage et al. 2017). Overall, though the exact mechanism whereby HSPB8 and BAG3 promote disassembly of cytokinetic actin structures remains to be established, we think that we can reasonably speculate that the chaperone complex could act in the autophagic sequestration of proteins that regulate assembly of actin networks, a function that would be modulated by BAG3 mitotic phosphorylation.

In conclusion, the present study further supports a specialized role for HSPB chaperones as members of a quality control network that would assist in the assembly and disassembly of macromolecular complexes controlling the dynamic architecture of actin during various mechanical processes, including cell-shape changes that guide mitosis in cancer cells (Guilbert et al. 2015). Deciphering the mitotic interactome of BAG3 and the molecular impact of BAG3 multisite phosphorylation is crucial to our understanding of HSPB8-BAG3 function in actin dynamics and is the object of our current work. Given that HSPB8 and BAG3 are expressed in a wide variety of cancer cell types, their activity in cell division may sustain tumor expansion (Rosati et al. 2011). Thus, uncovering their mode of action is of great interest for the development of targeted therapeutic approaches to inhibit the HSPB8-dependent function of BAG3 and further enhance mitotic stress in cancer cells.

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