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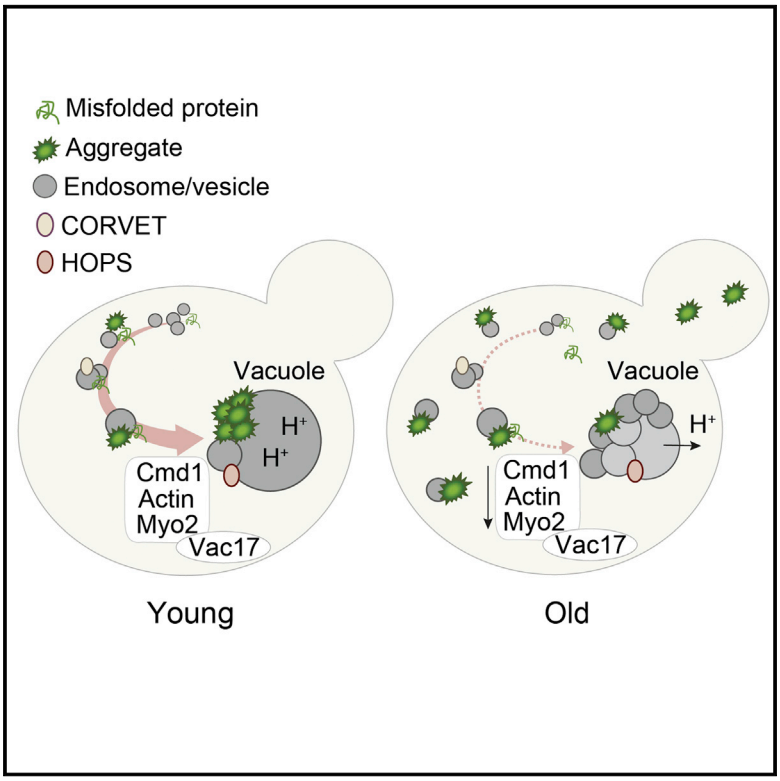
Asymmetric Inheritance of Aggregated Proteins and Age Reset in Yeast Are Regulated by Vac17-Dependent Vacuolar Functions

Graphical Abstract



Highlights

- Vesicle trafficking and fusion control asymmetric inheritance of aggregated proteins
- Vac17 increases vacuole-proximal fusion of aggregated proteins and extends lifespan
- Hsp104 interacts physically with endomembrane trafficking components, including Vps1
- Quality control by Vac17 requires Myo2 binding, membrane tethering, and Vps1

Authors

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In Brief

Cellular rejuvenation is enabled by asymmetrical inheritance of damaged proteins. Using a genome-wide imaging screen to identify asymmetry-generating genes, Hill et al. demonstrate a role for vesicle trafficking, membrane fusion, and the myosin-dependent adaptor protein Vac17 in the asymmetric inheritance of misfolded proteins and consequently in the regulation of lifespan.

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Asymmetric Inheritance of Aggregated Proteins and Age Reset in Yeast Are Regulated by Vac17-Dependent Vacuolar Functions

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SUMMARY

Age can be reset during mitosis in both yeast and stem cells to generate a young daughter cell from an aged and deteriorated one. This phenomenon requires asymmetry-generating genes (AGGs) that govern the asymmetrical inheritance of aggregated proteins. Using a genome-wide imaging screen to identify AGGs in *Saccharomyces cerevisiae*, we discovered a previously unknown role for endocytosis, vacuole fusion, and the myosin-dependent adaptor protein Vac17 in asymmetrical inheritance of misfolded proteins. Overproduction of Vac17 increases deposition of aggregates into cytoprotective vacuole-associated sites, counteracts age-related breakdown of endocytosis and vacuole integrity, and extends replicative lifespan. The link between damage asymmetry and vesicle trafficking can be explained by a direct interaction between aggregates and vesicles. We also show that the protein disaggregase Hsp104 interacts physically with endocytic vesicle-associated proteins, such as the dynamin-like protein, Vps1, which was also shown to be required for Vac17-dependent sequestration of protein aggregates. These data demonstrate that two physiognomies of aging—reduced endocytosis and protein aggregation—are interconnected and regulated by Vac17.

INTRODUCTION

Asymmetric cell division is key to cellular differentiation, and one intriguing aspect of such asymmetry is that a young and immaculate cell can be generated from an aged and deteriorated one (Aguilaniu et al., 2003; Higuchi et al., 2013; Hughes and Gottschling, 2012; Liu et al., 2010; Shcheprova et al., 2008). In bacteria, yeast, and specific stem cells, the generation of such rejuvenated progeny includes an asymmetrical inheritance of oxidized and aggregated proteins (Aguilaniu et al., 2003; Ogrodnik et al., 2014; Rujano et al., 2006). The retention of protein aggregates in the yeast progenitor (mother cell) requires the protein remodeling factor Hsp104 (Erjavec et al., 2007; Liu et al., 2010; Tessarz et al., 2009), the protein deacetylase Sir2 (Aguilaniu et al., 2003; Orlandi et al., 2010), and actin cables together with actin cytoskeletal organizational proteins such as formins, CCT, and calmodulin (Liu et al., 2010; Liu et al., 2011; Song et al., 2014).

The control of damage inheritance is dependent also on spatial deposition of damaged/unfolded proteins into specific protein inclusions (PIs), such as IPOD (Insoluble-Protein-Deposit), JUNQ (JUxta-Nuclear-Quality-control), and INQ (Intra-Nuclear-Quality-control) (Burri and Lithgow, 2004; Kaganovich et al., 2008; Miller et al., 2015; Spokoini et al., 2012). Formation of such PIs are factor-dependent processes, requiring calmodulin and functional actin cables alongside Hsp104 (Liu et al., 2011; Song et al., 2014), suggesting that the actin cytoskeleton is imperative for both mother cell-biased segregation of aggregates (Liu et al., 2011; Song et al., 2014) and the formation of specific inclusions (Specht et al., 2011). In addition, two members of the Hook family of proteins, Btn2 and Cur1, control the spatial deposition of misfolded proteins into discrete PIs together with chaperones (e.g., Sis1) and small heat shock proteins (e.g., Hsp42) (Malinovska et al., 2012; Park et al., 2013; Specht et al., 2011). However, the exact mechanisms of how misfolded proteins and aggregates are distinguished and sorted to the different PIs and quality-control compartments are not identified (Miller et al., 2015). In addition, the identification of asymmetry-generating genes (AGGs) controlling inheritance of aggregates and genes required for PI formation has so far been restricted to candidate approaches, suggesting that many components remain to be discovered.

In this work, we report the results of an unbiased, high-throughput screen for AGGs and present a catalog of an organism's complement of genes involved in generating age-asymmetry. We discovered a previously unknown role for vesicle trafficking, membrane fusion to the vacuole, and vacuole inheritance proteins in the sequestration and fusion of aggregated proteins into cytoprotective IPOD sites that are retained in mother cells. Furthermore, we found that the vacuole inheritance adaptor protein Vac17 is a limiting member of the AGGs and show that this protein regulates asymmetry and replicative

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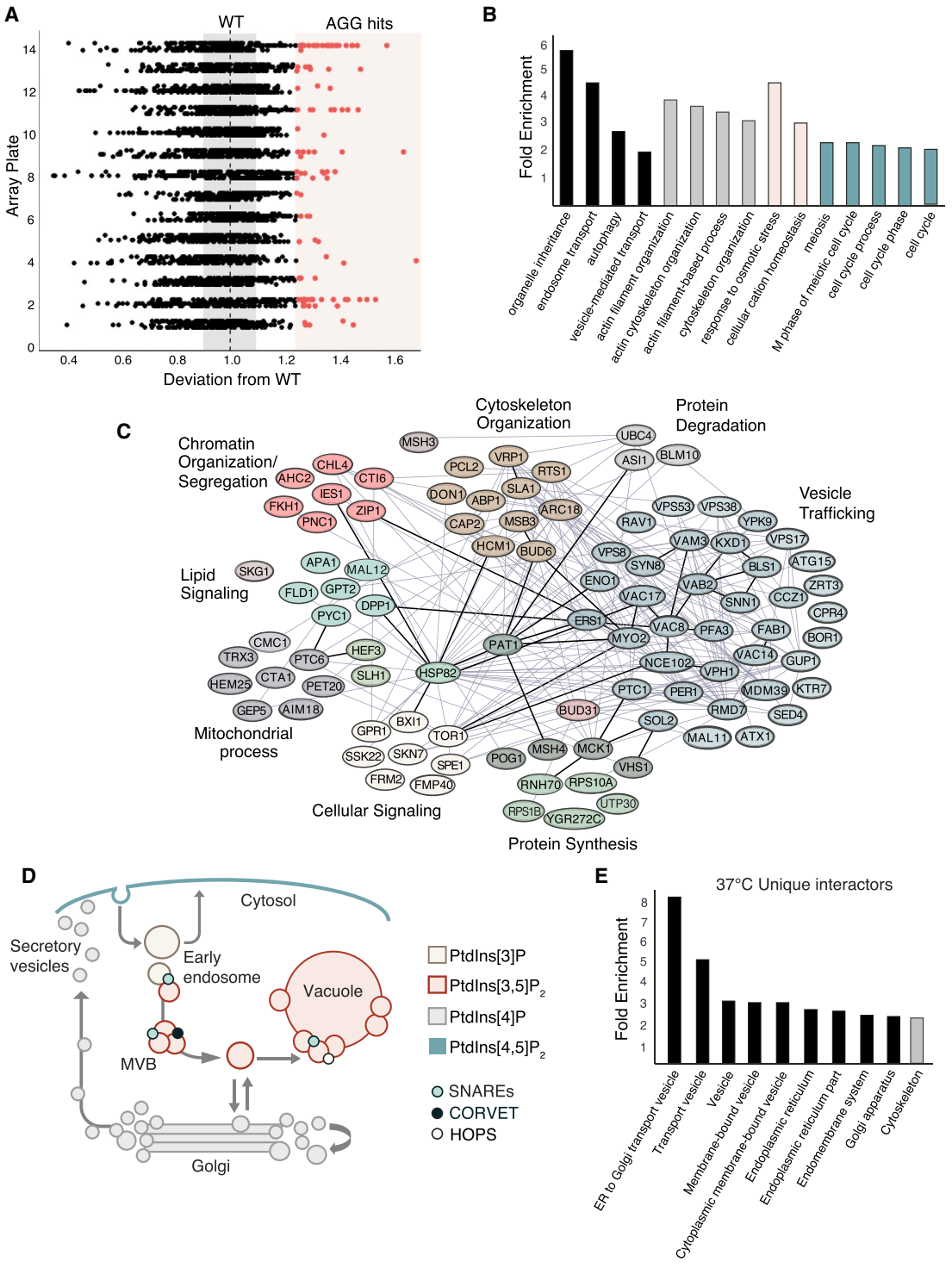

Figure 1. Identification of Age Asymmetry-Generating Genes on a Genome-wide Scale

(A) Deviation from wild-type (WT) in aggregate asymmetry for all mutants tested (array plates 1–14 in A–D). The average value for WT is normalized to 1, with SD (±0.09) depicted in gray. Deviation above 1 corresponds to an increased proportion of daughter cells inheriting protein aggregates. Mutants with a deviation of at least 1.25 (red dots) are well outside the WT variation interval (gray area) and are considered to be AGGs.

(B) The top 111 AGG hits identified were tested for enrichment of GO annotations in Biological Processes using the online tool at DAVID ($p < 0.01$ modified Fisher's exact).

(legend continued on next page)

lifespan through Myo2-dependent effects on endocytosis and spatial protein quality control. In addition, an unbiased proteomics approach identified members of the endosomal vesicle trafficking process, including the dynamin-like protein Vps1, as physical interactors of the protein disaggregase Hsp104. We further established that Vps1 is involved in aggregate retention and formation of protein inclusions and that Vac17 fails to affect these activities in the absence of Vps1, indicating that these two proteins function along the same pathway. The data highlight that endosomal trafficking to the vacuole is an integral part of a cytoprotective spatial protein quality control affecting the rate of cellular aging.

RESULTS

A Genome-wide Imaging Screen for Yeast AGGs

To embark on unbiased, automatized, screens for AGGs, we generated a mutant library, using synthetic genetic array (SGA) technology (see Experimental Procedures), in which the *HSP104* gene was replaced by the functional *HSP104-GFP* fusion. The *HSP104* gene encodes the heat shock protein Hsp104 that binds to protein aggregates (Glover and Lindquist, 1998), and Hsp104-GFP serves as an efficient reporter of such aggregates that can be observed as microscopic intracellular foci and inclusions (Erjavec et al., 2007; Spokoini et al., 2012). After robot-assisted transfer of cells of the ordered *HSP104-GFP*-containing arrays to liquid 96-well plates, aggregate formation was elicited by a transient heat shock (42°C for 30 min; Figure S1A). Upon returning to 30°C, after which no more aggregates were formed, the mother cells were allowed to generate new daughter cells, and the aggregate content in the progenitor and progeny was detected by an online granularity analysis (Molecular Devices) after obtaining images by high-content microscopy (HCM) (Figure S1A). Values of the aggregate content in mothers and their daughters were retrieved and mutants (with deletions in AGGs) deviating from the wild-type value identified. Among the 4,600 mutants analyzed, 111 were scored as AGGs when a deviation of 25% from the wild-type was used as the cutoff, meaning a 25% increase in daughters inheriting one or more aggregates (Figure 1A; Figure S1A; Table S1). The reduction in aggregate asymmetry in the set of mutants identified was not correlated to a general increase (or decrease) in aggregation (Figure S1B). The screen also identified mutants with an enhanced aggregate asymmetry (Table S2), and gene ontology annotation analysis suggests that alterations in the processes related to lipid metabolism and DNA repair can boost the mother cells' ability to retain aggregates during cytokinesis (Figure S1C). This set of mutants was not further characterized in this study, which instead focuses on genes (AGGs) required to generate proper aggregate asymmetry.

Functions in Vesicle Trafficking Are Enriched among AGGs

AGGs were found on all yeast chromosomes but were markedly enriched (25% of the hits) on the right-hand arm of chromosome III (Figure S1D). This is interesting considering that duplications of chromosome III occur reproducibly in yeast cells grown under heat stress and provide the cell with an enhanced tolerance to proteostatic stress (Yona et al., 2012). Cross scoring with gene ontology (GO), genetic/physical interaction, and keyword databases established that AGGs are enriched for actin cytoskeletal functions/binding and intracellular vesicle trafficking processes (Figure 1B; Figures S1E and S1F), specifically, genes required for membrane tethering and fusion to the vacuole. Such genes included *CCZ1* (the guanine nucleotide exchange factor required for membrane tethering and fusion events at the late endosome and vacuole) (Nordmann et al., 2010), the syntaxin-related SNAREs *VAM3* and *SYN8* (mediating docking/fusion of late transport intermediates with the vacuole) (Burri and Lithgow, 2004), and *VPS8* of the CORVET multisubunit tethering complex (involved in endosomal vesicle tethering and fusion of endosomes to the vacuole) (Balderhaar and Ungermann, 2013) (Table S1; Figures 1B–1D). Consistently, *fab1Δ* mutant cells devoid of phosphatidylinositol-3,5-bisphosphate ($PI(3,5)P_2$), a resident signaling lipid on late endosomes, multivesicular bodies (MVB), and vacuoles, required for vesicle fusion to the vacuole (Shaw et al., 2003) (Figures 1C and 1D), were also identified in the screen as an AGG. Moreover, both *VPH1*, required for vacuolar proton pumping as a subunit of the vacuolar-ATPase (V-ATPase), and *RAV1*, a member of the RAVE complex involved in assembly of the V-ATPase, were scored as AGGs. These AGGs may also be linked to vesicle trafficking as vacuole fusion has been shown to require vacuolar acidification by the V-ATPase (Baars et al., 2007; Coonrod et al., 2013) (Figure 1C; Table S1). That AGGs are involved in vesicle and vacuolar fusion is noteworthy as misfolded proteins have been shown to become spatially recruited to IPOD inclusion sites at the proximity of the vacuole surface (Spokoini et al., 2012) (see also below). In addition, we found that the Hsp90 chaperone Hsp82 acts as an AGG and is a central interactor (physical and genetic) of several asymmetry-generating processes, including vesicle trafficking, the Tor1/Skn7 signaling pathway, cytoskeletal organization, chromatin organization/segregation, and lipid signaling (Figure 1C).

The Protein Disaggregase Hsp104 Interacts with Proteins of the Endomembrane Trafficking Route

To test whether the quality-control system of protein aggregates interacts directly with endosomal trafficking systems (or other systems), we performed an unbiased screen for Hsp104 interactions during normal growth conditions and heat shock.

(C) Physical (thick lines) and genetic (thin lines) interaction network of top AGG hits with known functions, as well as the known AGG, Myo2. *vac8Δ* and *fab1Δ* showed an asymmetry defect but were filtered from the screen data due to the number of cells being below the set cutoff value. These mutants were manually verified and therefore added to the network.

(D) Overview of vesicle trafficking within the cell, the presence of lipid signaling molecules (phosphatidylinositols; PtdIns) on different membranes, and the dependence on SNAREs and tethering complexes (CORVET, HOPS) for fusion of vesicles at different stages.

(E) Enrichment of GO annotations among proteins that were interacting with Hsp104 exclusively under heat shock (37°C).

See also Figures S1 and S2.

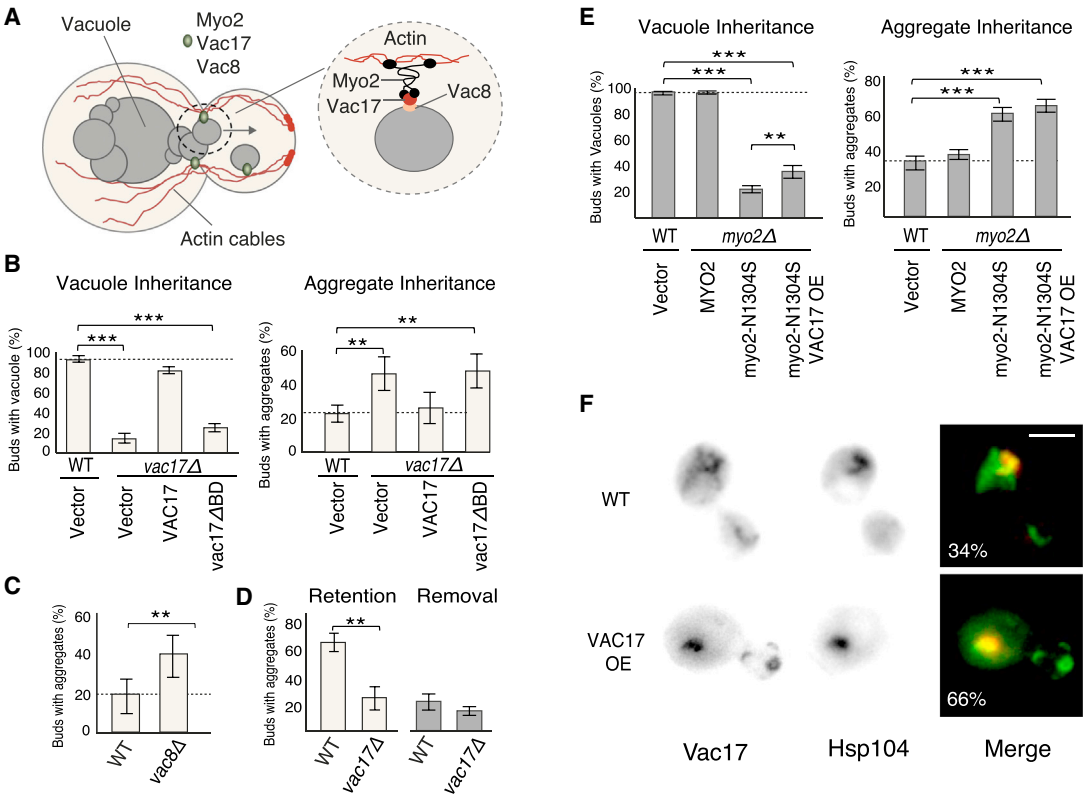

Figure 2. Role of Vac17 in Limiting Aggregate Inheritance Is Dependent on Interactions with the Motor Protein Myo2

(A) Overview of the components involved in tethering vacuoles to actin cables for inheritance.

(B) Vacuole and aggregate inheritance upon continuous heat shock (all mother cells form aggregates) in WT and *vac17Δ* cells containing empty vector, the wild-type *VAC17*, or a *VAC17* allele lacking its Myo2 binding site (*vac17ΔBD*).

(C) Aggregate inheritance in vacuole inheritance mutant *vac8Δ*.

(D) Aggregate retention and aggregate removal efficiency for *WT* and *vac17Δ*, distinguished by the use of concanavalin A staining of the cell wall.

(E) Vacuole and aggregate inheritance in WT and *myo2Δ* cells containing either empty vector, the wild-type *MYO2*, or a *MYO2* allele mutated in its Vac17 binding site (*Myo2-N1304S*).

(F) Colocalization of Vac17-3xGFP and Hsp104-mcherry during heat shock in WT (n = 511 cells) and in *VAC17 OE* (n = 758 cells). Only cells with a visible signal in both channels (green and red) were scored for co-localization.

All data in the bar graphs presented are an average of n ≥ 3 replicates ±SD. *p < 0.05, **p < 0.005, ***p < 0.0005, unpaired two-tailed t test. Scale bar, 5 μm. See also Figure S3.

Hsp104-GFP was immunoprecipitated under mild conditions along with an untagged control both at 30°C and 37°C. Immunoprecipitation was confirmed by SDS-PAGE (Figure S2A), and peptides were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Hsp104 was the most abundant protein present by spectral count as expected (Table S3). Spectral counts in each of the four experiments were compared to the PeptideAtlas database as described (Miao et al., 2013; Michelot et al., 2010). Several components of the vesicle transport system were found to be enriched among Hsp104 interactors, including the guanine exchange factor Sec7, the SNARE disassembly chaperone Sec18, and the vacuole anchor Vac8 (Table S3). Analysis of the Hsp104 interactions that were seen only during heat shock revealed an even more significant enrichment for proteins involved in vesicle trafficking (Figure 1E), suggesting that Hsp104 generally associates with the endomembrane system with a more pronounced association during heat shock. These data indicate that the involvement of membrane trafficking genes in the establishment of damage asymmetry could be linked to an Hsp104-mediated interaction between aggregated proteins and components of the vesicle trafficking machinery.

The Vacuole Adaptor Protein Vac17 Is a Limiting Factor in Creating Damage Asymmetry

Among the vacuolar trafficking-related genes identified in the screen, we found that *VAC17* and *VAC8*, required for actin cable-dependent vacuole inheritance (Weisman, 2006), act as AGGs (Figure 1C; Table S1). *ACT1*, *MYO2*, *VAC8*, and *VAC17* encode the four key components of the vacuole inheritance machinery (Figure 2A): Vac17 serves as an adaptor protein recruiting vacuole vesicles to the actin cable tracks by its dual interaction with Vac8 (on vacuole vesicles) and the Myo2 motor protein (on actin cables) (Weisman, 2006). Manual quantification and complementation of aggregate inheritance defects (Liu et al., 2011; Spokoini et al., 2012) demonstrated that both

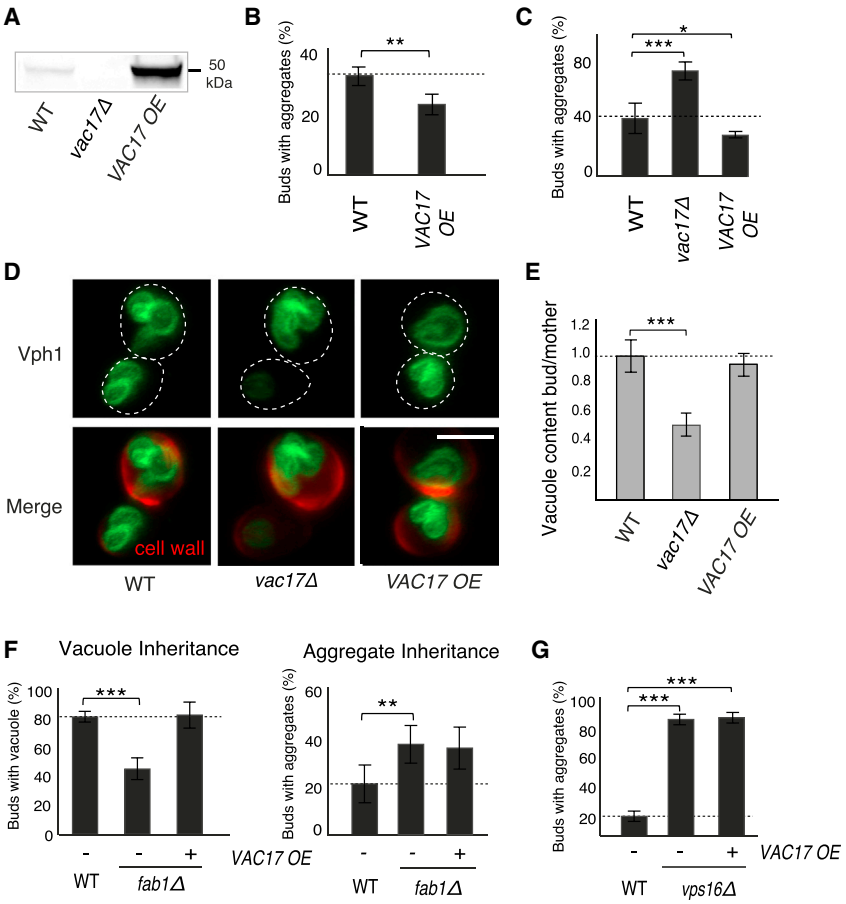

Figure 3. Functions of Vac17 in Damage Asymmetry Is Not Solely Explained by Its Role in Vacuole Inheritance

(A) Levels of Vac17 upon integration of the P_{GPD} promoter.

(B and C) Inheritance of heat-induced (B) and age-induced aggregates (C) in the Vac17 overproduction strain.

(D) Vacuole content in mother and daughter cell is visualized using intensity of Vph1-mcherry signal.

(E) Vph1-mcherry signal is normalized to cell size, and the ratio of vacuole content in daughter cell versus mother cell is quantified for denoted strains.

(F) Vacuole and aggregate inheritance upon a heat shock in cells lacking *FAB1* with and without overproduction of Vac17 (*VAC17 OE*).

(G) Aggregate inheritance in a *vps16Δ* mutant with and without overproduction of Vac17.

See also Figure S5 and Movies S1, S2, S3, S4, S5, S6, and S7.

Vac17 (Figure 2B) and Vac8 (Figure 2C), similar to Act1 and Myo2 (Erjavec et al., 2007; Liu et al., 2010; Song et al., 2014), are required for mother cell-biased segregation of aggregates. By using a protocol previously described to discriminate between effects on aggregate retention and aggregate removal (Hill et al., 2014; Song et al., 2014), we found that Vac17 is predominantly affecting aggregate retention (Figure 2D). Moreover, while the wild-type allele of *VAC17* complemented both vacuole and aggregate inheritance defects when reintroduced into *vac17Δ* cells, the *vac17ΔBD* allele, encoding a Vac17 protein unable to interact with Myo2 (Tang et al., 2003), did not (Figure 2B). Consistent with a role for Vac17-Myo2 interaction for aggregate inheritance, cells containing the Myo2-N1304S allele of Myo2, which lacks the Vac17-binding domain (Eves et al., 2012), displayed a reduced ability to retain protein aggregates in mother cells (Figure 2E). These data indicate that the role of actin cables in establishing aggregate asymmetry (Aguilaniu et al., 2003; Erjavec et al., 2007; Liu et al., 2010) may be linked to the recruitment of misfolded/aggregated proteins to actin cables by the Vac17/8 proteins and Myo2. Vac17 and Hsp104 aggregates co-localized in about one-third of the cells suggesting that Vac17 is, at least to some degree, associated with misfolded/aggregated proteins further strengthening a role for this protein in spatial control of heat-induced aggregates (Figure 2F). However, the absence of Vac17 did not affect inclusion formation or retention of the amyloid, disease-related, Huntingtin protein Htt103QP (Dehay and Bertolotti, 2006; Wang et al., 2007) (Figure S2B). Previous studies demonstrated that amyloidic proteins and misfolded proteins formed upon heat shock follow different routes for their deposition (Specht et al., 2011), and our results suggest that *VAC17* is only regulating the latter process (see also data below concerning $Ubc9^{ts}$).

The Vac17 vacuole-adaptor protein is only present at around 20 copies/cell and competes with other adaptor proteins for recruitment of cargo to the Myo2 motor protein and actin cables (Eves et al., 2012). To approach whether Vac17 might be limiting for aggregate retention in mother cells, we constructed a Vac17-overproducing strain by exchanging the weak *VAC17* promoter with the strong P_{GPD} promoter. The levels of Vac17 were markedly elevated by this promoter exchange (Figure 3A), and the retention of both heat-induced and aging-induced aggregates in mother cells were even more pronounced than in wild-type cells (Figures 3B and 3C). This effect was not due to an altered localization of Vac17, as the overproduced protein displayed the same localization pattern as endogenously expressed Vac17: during growth at 30°C Vac17 is predominantly found at the bud neck or within the bud (Figures S3A and S3B), consistent with previous observations (Eves et al., 2012; Jin et al., 2009). Upon a shift to 38°C, however, both endogenously and overexpressed Vac17 were relocalized to the mother cell, where 34% and 66% co-localized with Hsp104-associated aggregates, respectively (Figures 2F, S3A, and S3B). The localization of the low-abundant Vac17 was not the result of background fluorescence or signal bleed-through, as evidenced by a non-GFP control (Figure S3A).

Overproduction of Vac17 did not affect aggregate inheritance in cells carrying the Myo2-N1304S protein unable to bind Vac17 suggesting that Vac17 acts through its interaction with Myo2 also in boosting aggregate asymmetry (Figure 2E). Interestingly,

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inheritance of vacuolar content was not boosted above the level of wild-type cells by overproducing Vac17 (Figures 3D and 3E), and while overproduction of Vac17 completely restored vacuole inheritance in a *fab1Δ* mutant it failed to re-establish aggregate retention (Figure 3F). Furthermore, while overproduction of Vac17 could not limit aggregate inheritance in the Myo2-N1304S mutant, vacuole inheritance was modestly improved (Figure 2E). Together, these results suggest that vacuolar inheritance defects alone cannot explain the defects in aggregate segregation, which therefore may be linked to additional functions of the Myo2-Vac17 complex (see below).

Considering the large number of hits in late endosomal trafficking and membrane fusion, we contemplated the possibility that Vac17 might affect aggregate segregation by acting in these pathways. Consistent with this notion, defects in the HOPS/CORVET (*vps16Δ*: Vps16 is a subunit of both HOPS and CORVET; Figure 1D) multi-subunit tethering complexes required for fusion to the vacuole caused a drastic defect in aggregate retention, and Vac17 overproduction failed to affect aggregate inheritance in the absence of these complexes (Figure 3G). These data, along with the identified requirement for SNARE proteins (Figures 1C and 1D; Table S1), show that fusion to the vacuole might be required for proper Vac17-dependent retention of protein aggregates in mother cells. However, we cannot rule out the possibility that the mutants analyzed suffer from additional defects in vacuole functions that might impact on aggregate inheritance.

Vac17 Boosts Fusion of Small Aggregates into IPOD Sites

The aggregate segregation defect in *vac17Δ* cells was accompanied by a reduced ability to deposit misfolded proteins into discrete inclusions (Kaganovich et al., 2008; Spokoini et al., 2012). Upon a shift from 30°C to 38°C, the immediate occurrence of multiple stress foci (SF [Spokoini et al., 2012]; also called Q-bodies [Escusa-Toret et al., 2013], peripheral aggregates [Specht et al., 2011], and CytoQs [Miller et al., 2015]) and the subsequent deposition of misfolded/aggregated proteins in IPOD and the JUxta-Nuclear-Quality control compartment (JUNQ) can be time resolved (Figure S3C). Using Hsp104-GFP together with the SF/IPOD/JUNQ-reporter Ubc9^{ts}-RFP, we found that the advancement to the inclusion stage was affected in Vac17-deficient cells as these cells displayed multiple Hsp104-associated Ubc9^{ts} SF (Figures 4A–4D). In contrast, overproduction of Vac17 increased inclusion formation (Figures 4A, 4C, and 4D). As for aggregate inheritance, the effect of Vac17 on inclusion formation required its Myo2-binding domain (Figure 4B). Single-cell analysis demonstrated that Vac17 affected the fusion of small SF into inclusions such that the merging of SF was retarded in *vac17Δ* cells and accelerated in Vac17-overproducing cells (Figure 4D; Figure S4; Movies S1, S2, and S3). Co-staining with FM4-64 established that the inclusions of wild-type cells and Vac17-overproducing cells often localized to the vicinity of the vacuole (IPODs [Kaganovich et al., 2008]) and that the SF of *vac17Δ* cells were accompanied by a fragmented vacuole morphology of this mutant (Figures 4E and 4F; Figure S4). Analysis of the small heat shock protein Hsp42, which is specifically localized to SF and IPODs (Escusa-Toret et al., 2013; Specht et al., 2011), confirmed that Vac17 is increasing the efficiency of IPOD formation (Figure S5A).

Cells lacking the *Vps16* subunit of the HOPS/CORVET complexes displayed a drastic defect in inclusion formation, and this defect could not, similar to aggregate retention (Figure 3C), be suppressed by Vac17 overproduction (Figure 5G; Figure S5C; Movies S4 and S5). These data suggest that defects in vacuole tethering/function inhibit fusion of misfolded proteins/SF into vacuole-proximal IPODs in the mother cell, leading to an increased inheritance of damaged proteins by the daughter cell. Cells lacking Fab1, which display defects in vacuole fusion, fission, and tethering and contain one large vacuole rather than fragmented ones (Gary et al., 1998), also display a defect in inclusion formation that was not affected by Vac17 overproduction (Figures S5C and S5D; Movies S6 and S7). This indicates that vacuolar fragmentation is not a prerequisite for a failure to form inclusions. Indeed, salt-stress-induced vacuole fragmentation (Bonangelino et al., 2002; Li and Kane, 2009) demonstrated no significant correlation between the number of vacuoles and the number of heat-induced aggregates (Figures S6A and S6B). Similarly, we found no correlation between vacuole numbers and aggregate numbers in aged mother cells (Figure S6C).

Vac17 Acts in the Same Pathway as the Hsp104 Interactor Vps1 to Ensure Proper Damage Asymmetry

The yeast dynamin homolog, Vps1, a resident protein of endosomes required for proper endocytosis to the vacuole (Smaczynska-de Rooij et al., 2010) was identified as an interactor of Hsp104 in both untreated and heat-shocked cells (Table S3), and the interaction between Vps1 and Hsp104 was confirmed by immunoprecipitation (Figure 5A). Similar to the trafficking genes identified in the screen, deletion of *VPS1* resulted in defects in both damage asymmetry and in the formation of protein inclusions, although the effect on inclusion formation was modest (Figures 5B–5D). Moreover, Vac17 failed to affect these processes in cells lacking Vps1 (Figures 5B–5D), suggesting that Vps1 and Vac17 might act in the same pathway of spatial protein quality control.

Vac17 Controls Management of Age-Induced Aggregates and Extends Replicative Lifespan

Vac17 molecules are progressively lost from mother cells during the generation of daughter cells as the protein is transported into the protruding bud and degraded to allow for proper vacuole inheritance (Weisman, 2006). Thus, the Vac17 pool needs to be replenished after each cell cycle, and, if not properly compensated for by de novo synthesis, Vac17 levels could decline during replicative aging of mother cells. Indeed, we found this to be the case (Figure 6A) and wondered whether Vac17 might become limited in old cells for the management of protein aggregates. In support of this notion, overproduction of Vac17 reduced the fraction of mother cells displaying aggregates as well as the number of aggregates per aged cell (Figures 6B–6D), while the opposite result was observed for cells lacking Vac17 (Figures 6B–6D). Elevation of Vac17 levels also counteracted age-related vacuolar fragmentation (Figure 6E) and the decline in endocytotic capacity (Hughes and Gottschling, 2012; Tang et al., 2008) (Figure 6F), supporting the notion that endocytosis is closely linked to IPOD formation and that endocytosis and vesicle fusion to the vacuole

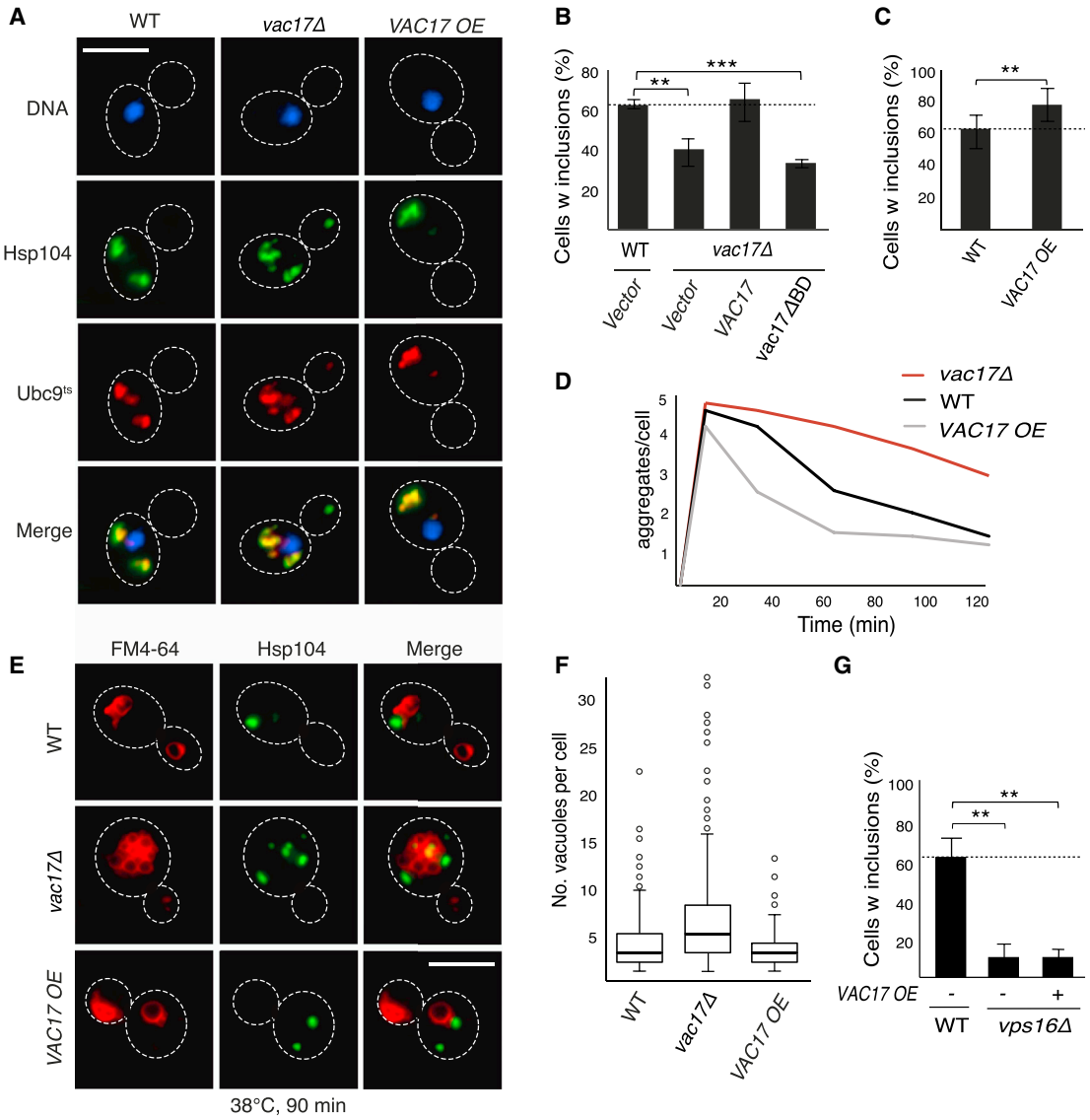

Figure 4. Overproduction of Vac17 Counteracts Vacuolar Fragmentation and Accelerates Aggregate Fusion into Inclusions

(A) Hsp104-GFP colocalizes with Ubc9^{ts}-RFP in quality-control compartments IPOD and JUNQ upon continuous heat shock (38°C, 90 min).

(B) Percentage of heat-treated WT and *vac17Δ* cells able to fuse aggregates into inclusions (maximum two Hsp104 foci per cell).

(C) Percentage of heat-treated WT and Vac17-overproducing cells displaying successful inclusion formation.

(D) Single-cell time-lapse analysis of inclusion formation in WT, *vac17Δ*, and Vac17-overproducing cells (50 cells per strain and time point, n = 3).

(E and F) Vacuolar fragmentation upon heat shock visualized by FM4-64 staining and quantified in (F); n_{WT} = 413 cells; $n_{vac17\Delta}$ = 370 cells, p = 0.0021; $n_{VAC17\ OE}$ = 396 cells, p = 1.0E-08; Mood's median test statistics.

(G) Inclusion formation upon heat shock in a *vps16Δ* mutant with and without overproduction of Vac17.

All data in the bar graphs presented are an average of n ≥ 3 replicates ±SD. *p < 0.05, **p < 0.005, ***p < 0.0005, unpaired two-tailed t test. Scale bar, 5 μM. See also Figures S4 and S5 and Movies S1, S2, S3, S4, S5, S6, and S7.

is diminished in aged cells. The data indicate that Vac17 possesses a previously unknown role in endocytosis, in addition to its role in vacuole inheritance, and we therefore tested whether cells carrying the Myo2 protein (Myo2-N1304S), specifically unable to bind Vac17, display reduced endocytosis and found this to be the case both in young and old cells (Figure 6G). To test whether Vac17 also affected vacuole-to-vacuole fusion, we analyzed vacuolar fusion during salt-stress recovery and found no evidence for the fusion of vacuoles being retarded in cells lacking Vac17 (Figure S5D).

Interestingly, improvement of aggregate management and endocytosis by Vac17 overproduction was accompanied by a robust extension of lifespan, whereas Vac17 deficiency modestly accelerated aging (Figure 7A). To evaluate whether the effects of Vac17 acted through its influence on protein quality control (PQC), we tested whether full activity of cytosolic Hsp70

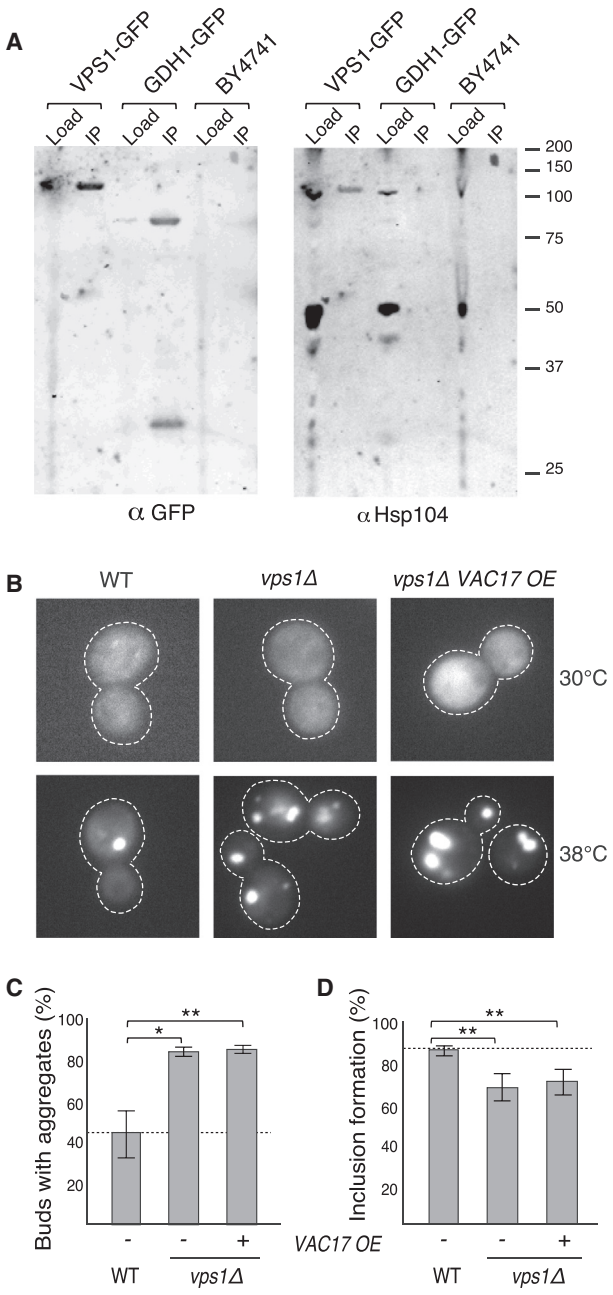

Figure 5. Vps1 Interacts with Hsp104 and Acts Together with Vac17 in a Pathway Regulating Vesicle Trafficking, Inclusion Formation, and Damage Asymmetry

(A) Confirmation of Vps1 and Hsp104 interaction upon immunoprecipitation of Vps1-GFP. Immunoprecipitation of Gdh1-GFP is used as a non-interacting control.

(B) Hsp104-GFP-associated aggregates are formed upon heat treatment (38°C 90 min) in WT, *vps1Δ*, and *vps1Δ VAC17 OE*.

(C and D) Quantification of aggregate inheritance (C) and inclusion formation (D) in denoted strains.

All data in the bar graphs presented are an average of n ≥ 3 replicates ±SD. *p < 0.05, **p < 0.005, ***p < 0.0005, unpaired two-tailed t test. Scale bar, 5 μM.

chaperone activity and/or the protein disaggregase Hsp104 was required for lifespan extension by Vac17. Cytosolic Hsp70s were needed for lifespan extension upon Vac17 overproduction (Figures 7B and 7C), while the Hsp104 disaggregase was not (Figure 7C), suggesting that PQC functions other than protein disaggregation are required for lifespan control by Vac17. Consistent with the requirement for the HOPS/CORVET complexes for Vac17-dependent aggregate fusion (Figure 3G), the *vps16* deletion completely blocked lifespan extension by Vac17 overproduction (Figure 7D). In addition, the myosin-binding domain of Vac17 was required for lifespan extension suggesting that interaction with Myo2-actin is a prerequisite for Vac17-dependent lifespan control, similar to aggregate retention, endocytosis, and IPOD formation (Figure 6E).

The results demonstrating that Vac17 overproduction extend lifespan in a Hsp70-dependent manner indicates that Vac17-dependent sequestration of misfolded proteins and SF into discrete inclusions (Figures 4A–4D) may be an important process in detoxifying aberrant protein species (Cohen et al., 2006; Treusch et al., 2009), and we therefore tested whether $Ubc9^{ts}$ became toxic in *vac17Δ* cells, as in cells deficient in cytosolic Hsp70 chaperone activity (Öling et al., 2014). We found this to be the case; lack of Vac17 reduced the fitness of cells expressing unfolded $Ubc9^{ts}$ (Figure 6F) demonstrating that Vac17 is a physiologically relevant aide-de-camp in spatial protein quality control required for mitigating proteotoxicity.

DISCUSSION

In this paper, using an un-biased imaging approach to discover asymmetry-generating genes (AGGs), we found that the adaptor protein Vac17, which is a limiting factor for vacuole inheritance (Weisman, 2006), is a key factor also in (1) limiting inheritance of protein aggregates, (2) generating protein inclusions in both heat-stressed and aged cells, and (3) maintaining endocytosis and vacuole integrity during aging. Specifically, defects in late endocytosis and vesicle fusion to the vacuole lead to defects in protein inclusion formation and subsequent inheritance of protein aggregates, indicating that the aberrant management of damaged proteins observed in aged cells might be linked to a prior decline in endocytosis (Figures 6F and 7G). Mitigating such a decline in vacuole-directed endocytosis by Vac17 overproduction counteracted the accumulation of multiple aggregates in aged cells and extended lifespan. Such data are in line with work showing that increased vacuole fusion by Osh6 overproduction can retard mother cell aging (Gebre et al., 2012) and that lifespan extension by caloric restriction requires vesicle-vacuole fusion (Tang et al., 2008).

We observed a partial colocalization between Vac17 and Hsp104-associated aggregates and found that Vac17 became increasingly localized to the mother cell during heat stress (Figures 2F and S3). While such data might indicate that Vac17 is affecting inclusion formation and retention by direct interaction with protein aggregates in the mother cell, it is also possible that a fraction of the Vac17 pool is aggregating during heat stress. In fact, a role for Vac17 in inclusion formation does not require a direct interaction with protein aggregates as Vac17 may be acting upstream the final deposition of misfolded proteins at the surface of the vacuole. Vac17 may be doing so by

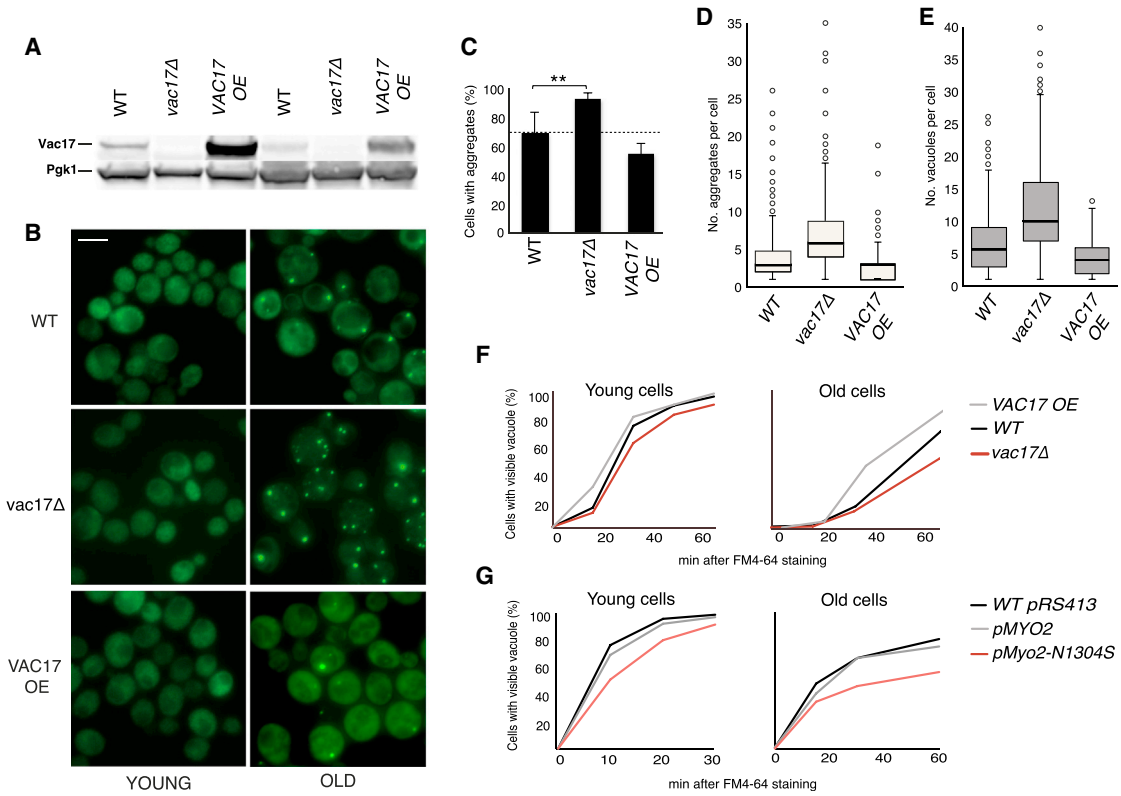

Figure 6. Overexpression of *VAC17* Counteracts Age-Related Aggregation, Loss of Vacuolar Integrity, and Endocytotic Capacity

(A) Levels of Vac17 in young and replicatively old cells (median ten generations) of denoted strains.

(B) Hsp104-GFP-associated aggregates are formed in old cells of *WT*, *vac17Δ*, and *VAC17 OE* cells.

(C) Percentage of aged cells displaying Hsp104-GFP aggregates.

(D) Number of aggregates per aged mother cell of the WT, *vac17Δ*, and Vac17-overexpressing strains *(VAC17 OE)*; $n_{WT} = 760$; $n_{vac17\Delta} = 647$ cells p = 4.67E-43; $n_{VAC17\ OE} = 780$ cells p = 2.14E-06.

(E) Vacuolar fragmentation in aged mother cell of the WT, *vac17Δ*, and Vac17-overproducing strains; $n_{WT} = 318$ cells; $n_{vac17\Delta} = 238$ cells p = 5.11E-18; $n_{VAC17\ OE} = 378$ cells p = 2.41E-7. $n \geq 3$ replicates; median values are shown as thick lines, and p values are determined by Mood's median test.

(F) Rate of endocytosis in young and old cells of WT, *vac17Δ*, and *VAC17 OE* strains. The *VAC17* deletion significantly reduced endocytosis in old cells (p = 0.031) compared to WT, whereas *VAC17* overexpression accelerated endocytosis (p = 0.0013).

(G) Rate of endocytosis in young and old cells of WT and *myo2Δ* cells containing either empty vector, the wild-type *MYO2*, or the *Myo2-N1304S* allele. Cells with the Myo2-N1304S allele display a significant reduction of endocytosis rate in both young (p = 0.0035) and old (p = 0.012) cells.

affecting the rate of vesicle trafficking as discussed, and our data suggest that Vac17 is not required for vacuole-vacuole fusions. In addition, we found that Vac17 overproduction can suppress severe defects in vacuole inheritance without affecting aggregate inheritance in some mutants analyzed (Figures 2E and 3F). However, although *VAC17* overexpression can restore vacuole inheritance in some mutants, we cannot exclude the possibility that the inherited vacuoles are not fully equivalent, functionally, to wild-type (Anand et al., 2009). Therefore, defects in vacuole inheritance may affect aggregate retention not through vacuole inheritance per se, but instead through the generation of daughters and, in turn, mothers with defective vacuoles.

The improvement of endocytosis and extension of lifespan by Vac17 overproduction suggest that this protein becomes limiting upon aging, and that the decline in both endocytosis and formation of protein inclusions during aging may be due to a failure to maintain a proper titer of Vac17 (Weisman, 2006). Indeed, we found that Vac17 levels, normalized to total amount of protein, declined during mother cells aging (Figure 6A). It is possible also that the activity of the endocytic apparatus decline during aging due to an age-related degeneration of actin cytoskeletal dynamics (Gourlay et al., 2004) and a collapse in vacuolar acidification (Hughes and Gottschling, 2012), which is required for vesicle-vacuole fusion (Coonrod et al., 2013). Regardless of the nature of system failure, it is clear that elevated levels of Vac17 alone results in a robust extension of lifespan, pinpointing *VAC17* as a yeast gerontogene.

We found that Vac17 together with the actin-associated protein Myo2 is required for the merging of aggregates into IPODs, which is interesting since actin, and actin-remodeling proteins such as calmodulin (Cmd), is required for heterotypic and homotypic vacuole fusion (Eitzen et al., 2002) (Figure 7G), and actin and Cmd have previously been shown to be required for proper formation and asymmetrical inheritance of protein inclusions (Liu et al., 2010; Song et al., 2014). The concerted effect of Vac17-Myo2 on endocytosis, vacuolar integrity, and aggregate

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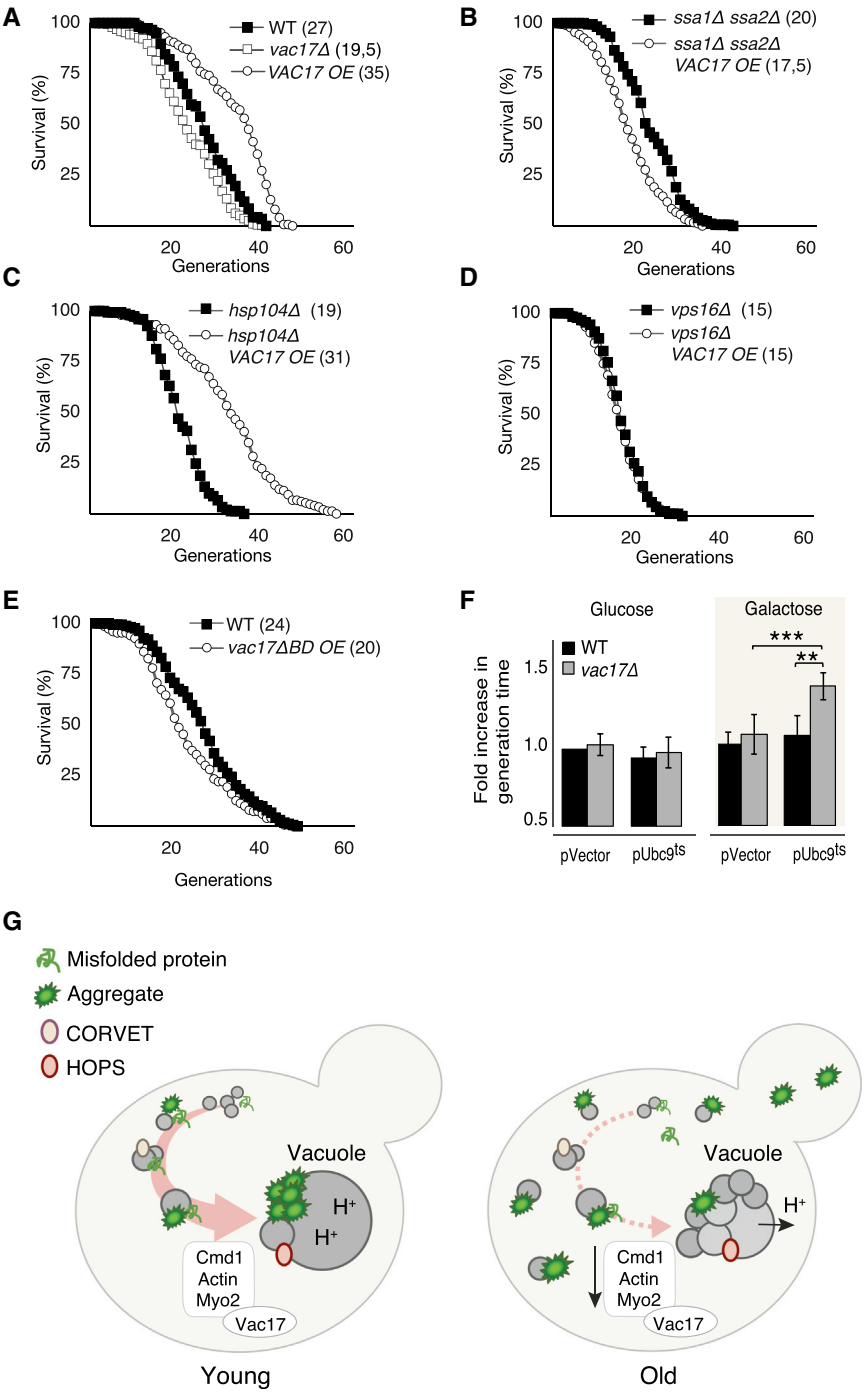

Figure 7. VAC17 Overexpression Extends Replicative Lifespan in a Chaperone-Dependent Manner

(A) Replicative lifespan of WT (n = 117 cells), *vac17Δ* (n = 194 cells; p = 1.25E-06), and *VAC17 OE* (n = 147 cells p = 2.25E-19).

(B) Lifespan of an *ssa1Δ ssa2Δ* mutant with (n = 178 cells, p = 1.66E-10) and without *VAC17* overexpression (n = 177 cells).

(C) Lifespan of *hsp104Δ* cells with (n = 148 cells p = 3.54E-21) and without *VAC17* overexpression (n = 149 cells).

(D) Lifespan of *vps16Δ* mutant with (n = 177 cells, p = 0.21) and without *VAC17* overexpression (n = 177 cells).

(E) Lifespan of WT (n = 164) and overexpression of truncated vac17ΔBD, lacking the Myo2 binding domain (n = 150, p = 0,00042). n ≥ 3 for lifespans; p values were determined by Mann-Whitney U test.

(F) Fold increase in generation time at 35°C upon galactose induction (right) of misfolding pUbc9^{ts} in WT and *vac17Δ* strain. All data in the bar graphs are presented as an average of n ≥ 3 replicates ±SD. **p < 0.005, ***p < 0.0005, unpaired two-tailed t test.

(G) Illustration of how misfolded proteins/stress foci might be sequestered at the surface of the vacuole by "hitchhiking" on the route of late endocytosis. Such a pathway for the compartmentalization of damaged and misfolded proteins at IPOD (Kaganovich et al., 2008) sites is consistent with the involvement of actin, calmodulin (Cmd1), Myo2, SNAREs, and HOPS/CORVET as these components are required for fusion to the vacuole (Baars et al., 2007; Coonrod et al., 2013; Eitzen et al., 2002). The decline in endocytotic capacity and vesicle fusion seen upon aging (right) could explain both vacuolar fragmentation and the accompanied decline in IPOD formation and aggregate retention. Since acidification of the vacuole is required for fusion to the vacuole (Baars et al., 2007; Coonrod et al., 2013), it is likely that the collapse in vacuolar pH control observed in aging cells (Hughes and Gottschling, 2012) gives rise to failures also in the spatial control of misfolded proteins—i.e., that two identified routes driving yeast aging, vacuolar defects (Burri and Lithgow, 2004; Hughes and Gottschling, 2012) and protein aggregation (Hill et al., 2014; Liu et al., 2010), are interconnected. See text for more details.

fusion/IPOD formation could be a result of misfolded proteins/stress foci interacting with membrane vesicles (Meriin et al., 2003) that could concentrate misfolded proteins at vacuolar-proximal IPOD sites with the aid of SNARE and the HOPS/CORVET tethering complex (Figures 1E and 7G). Indeed, we found that the protein disaggregase Hsp104 interacted with several protein components involved in endomembrane trafficking, suggesting that misfolded/aggregated proteins interacting with this disaggregase might hitchhike on trafficking routes leading to the vacuole. Moreover, Hsp104-endomembrane protein interactions became more pronounced upon heat shock, indicating that that disaggregation machinery and vesicle trafficking systems are increasingly engaged with each other upon protein aggregation (Figure 1E). The proteins interacting with Hsp104 included the dynamin-like protein, Vps1, which appears to act on the same pathway of IPOD formation as Vac17. Vps1 is localized on endosomes, and its absence results in a retarded endocytosis to the vacuole and the accumulation of clusters

of endosomes surrounding the vacuole (Smaczynska-de Rooij et al., 2010).

Although the exact mechanisms remain unclear, the Vac17-dependent route for aggregate deposition appears to provide the cell with two important features of protein quality control. First, it ensures that misfolded and potentially cytotoxic proteins are not inherited by the progeny. Second, the spatial deposition of aggregated proteins appears to be directly cytoprotective. Some misfolded proteins are imported into the nucleus where their deposition serves as a spatial cytoprotective measure and allows for consecutive degradation by the 26S proteasome (Miller et al., 2015; Park et al., 2013). It is possible that the same is true for misfolded/aggregated proteins deposited at the vacuolar surface, i.e., that a proportion of the proteins are translocated into the vacuolar lumen for destruction by vacuolar proteases, a possibility that remains to be elucidated.

EXPERIMENTAL PROCEDURES

Large-Scale Microscopy Screen for Asymmetry Generating Genes

The yeast strains used (S228C background) were grown in YPD or synthetic drop-out media with corresponding antibiotics. SGA mating to introduce HSP104-GFP into the deletion collection was performed according to published protocols (Costanzo et al., 2010; Tong et al., 2001, 2004). Cells were subjected to heat treatment to induce protein aggregation, followed by a recovery period and formaldehyde fixation. Imaging of fixed cells with Hsp104-GFP-bound aggregates was performed using a high-content microscope (ImageXpress^{MICRO}). Acquired images were quantified using MetaXpress, and top hits were analyzed for functional enrichment using Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2007, 2009).

HSP104 Co-immunoprecipitation and Mass Spectrometry Analysis

Immunoprecipitations using anti-GFP beads (Chromotek) were performed on exponentially growing cells expressing Hsp104-GFP as well as an untagged control at both 30°C and 37°C. Eluted proteins were digested with trypsin, peptides were identified by LC-MS/MS, and unweighted spectra were compared to the PeptideAtlas *S. cerevisiae* database to determine enrichment.

Aggregate Inheritance and Fusion

Protein aggregation was induced at 38°C or 42°C, followed by live-cell imaging. Single-cell analysis of aggregate fusion was accomplished through 4D confocal imaging of cells attached to concanavalin A (Sigma)-coated microscope plates. For the aggregation of Ubc9^{ts}-RFP, expression was induced by growing cells in galactose at 30°C and repressed by the addition of glucose before transferring cells to 38°C (Kaganovich et al., 2008). All figure data are based on the average of at least three individual experiments, error bars representing SD. Data were tested using an unpaired two-tailed t test, with p values less than 0.05 considered to be significant.

Isolation of Replicatively Old Cells and Lifespan Analysis

Old cells were obtained using the magnetabind biotin-streptavidin approach (Sinclair and Guarente, 1997; Smeal et al., 1996), consecutively isolating biotin-labeled cells after culturing for 2 days. Replicative lifespan was determined through manual dissection using Singer MSM micromanipulator (Egilmez et al., 1990), and lifespan data were analyzed using a two-tailed Mann-Whitney U test, with p values less than 0.05 considered as significant.

Full methods and associated references are available in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, four tables, and seven movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.016.

AUTHOR CONTRIBUTIONS

B.L. designed the AGG screen, wrote the journal for image analysis, and, together with X.H., optimized and performed the screen. S.M.H. compiled and analyzed the screen data and conducted the follow-up experiments, with the aid of J.G., S.S.-N., A.J., and R.J. P.O.W. performed the biochemical analysis of Hsp104 interactions and the follow-up experiments on Vps1. T.A. and D.K. performed the 4D imaging experiments. 4D imaging of *vps16Δ* was done by S.M.H. T.N initiated and coordinated the study, analyzed the data, and, together with B.L. and S.M.H., designed the experiments. T.N wrote the manuscript with input from B.L. and S.M.H.

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