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EXTRA VIEWS

Hitchhiking vesicular transport routes to the vacuole: Amyloid recruitment to the Insoluble Protein Deposit (IPOD)

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ABSTRACT. Sequestration of aggregates into specialized deposition sites occurs in many species across all kingdoms of life ranging from bacteria to mammals and is commonly believed to have a cytoprotective function. Yeast cells possess at least 3 different spatially separated deposition sites, one of which is termed "Insoluble Protein Deposit (IPOD)" and harbors amyloid aggregates. We have recently discovered that recruitment of amyloid aggregates to the IPOD uses an actin cable based recruitment machinery that also involves vesicular transport.[1] Here we discuss how different proteins known to be involved in vesicular transport processes to the vacuole might act to guide amyloid aggregates to the IPOD. These factors include the Myosin V motor protein Myo2 involved in transporting vacuolar vesicles along actin cables, the transmembrane protein Atg9 involved in the recruitment of large precursor hydrolase complexes to the vacuole, the phosphatidylinositol/phosphatidylcholine (PI/PC) transfer protein Sec 14 and the SNARE chaperone Sec 18. Furthermore, we present new data suggesting that the yeast dynamin homolog Vps1 is also crucial for faithful

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delivery of the amyloid model protein PrD-GFP to the IPOD. This is in agreement with a previously identified role for Vps1 in recruitment of heat-denatured aggregates to a perivacuolar deposition site.[2]

KEYWORDS. actin, amyloid aggregates, Atg9 vesicles, Cytoplasm-to-vacuole targeting (CVT) pathway, Insoluble Protein Deposit (IPOD), Myosin motor protein Myo2, prions, Phagophore Assembly Site (PAS), Sec 14, Sec 18, vesicular transport, Vps1, yeast (*Saccharomyces cerevisiae*)

YEAST PROTEIN QUALITY CONTROL COMPARTMENTS (PQC's)

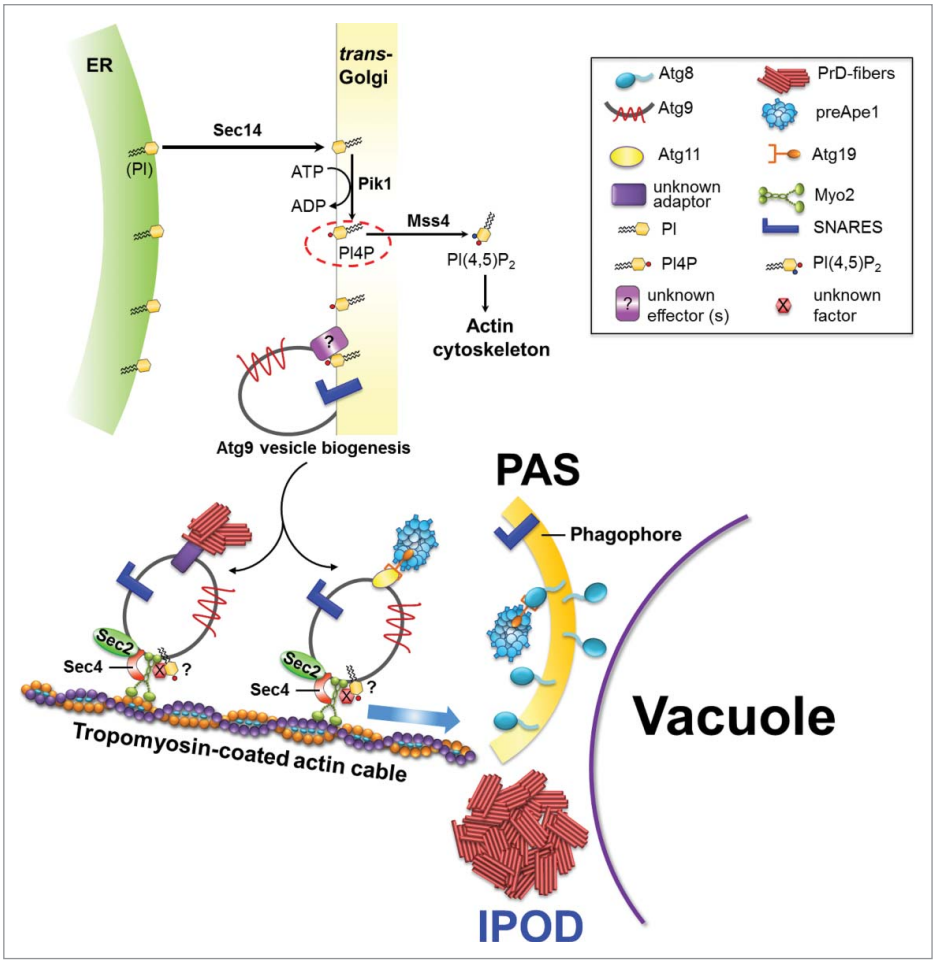
Yeast cells possess at least 3 different spatially separated deposition sites for misfolded and aggregated proteins termed protein quality control compartments (PQC's). Those comprise the peri- or intranuclear quality control compartment JUNQ or INQ,[3-5] respectively, the cytosolic quality control-bodies (Q-bodies),[6] also known as peripheral aggregates[7] or stress foci,[8] and the perivacuolar Insoluble Protein Deposit (IPOD).[3,9] The term IPOD was coined in 2008 by Judith Frydman and coworkers.[3] It was initially identified as a perivacuolar deposition site for terminally aggregated proteins. Identified substrates comprised constantly misfolding proteins such as a temperature-sensitive SUMO-conjugating enzyme ubc9ts and the heterologously expressed "Von Hippel-Lindau tumor suppressor VHL" that cannot fold to it's native state without it's cofactors elongin BC.[3] Those factors were not exclusively found at the IPOD, but also at a perinuclear deposition site for soluble misfolded proteins termed JUNQ (juxtanuclear quality control compartment). They accumulated mainly after inhibition of the proteasome.[3] Another class of substrates for the IPOD are amyloidogenic proteins.[3,9] In contrast to the constantly misfolding substrates, amyloidogenic proteins accumulate constantly at the IPOD even in non-stress conditions and are usually not found in other deposition sites such as JUNQ/INQ or Q-bodies. During isolation of the IPOD formed by the yeast prion domain of the [*PSI*$^+$] prion determinant Sup35, several proteins that are known to be highly sensitive toward the oxidative modification of carbonylation were co-purified. Corresponding GFP fusions were found to aggregate and partially co-localize with the IPOD upon oxidative stress applied to the cells.[9] Besides these substrates, the IPOD was more recently also described to harbor inactive proteasomes.[10-12]

What mechanisms are known to guide the diverse types of aggregates to the different PQC's? Previous searches for signals and factors that target substrates to the JUNQ/INQ or Q-bodies suggested polyubiquitination as a targeting signal.[3] However, it was found not to be essential for deposition of substrates at the JUNQ/INQ[4]. Another mechanism for proper targeting to the INQ/JUNQ and Q-bodies is the association of misfolded and aggregated proteins with specific targeting factors: the small heat shock protein Hsp42 is required for substrate targeting to Q-bodies.[6,7] while the heat shock inducible protein Btn2 and the Hsp40 chaperone Sis1 are involved in targeting to the JUNQ/INQ.[4,13,14] Furthermore, Hsp42 was also suggested to target inactive proteasome subunits to the IPOD.[10-12] Specific targeting factors for amyloid substrates to the IPOD were previously not known.

ROLE OF ACTIN IN HANDLING OF CELLULAR AGGREGATES

The existence of spatially separated deposition sites for different types of aggregates implies that the cell must be able to differentiate between different aggregates and target them to the designated deposition site. In our recent study,[1] we searched for factors that are involved in recruitment of amyloid aggregates to the IPOD. To identify those, we used an amyloid fiber-binding assay. We tagged the prion domain (PrD) of the [*PSI*$^+$] prion determinant Sup35 with a biotin moiety, produced the protein recombinantly in *E.coli and* generated amyloid fibers *in vitro*. Those PrD amyloid

FIGURE 1. Model for an involvement of vesicular transport proteins in the recruitment of PrD-GFP aggregates and preApe1 to the IPOD and PAS respectively. For details, please see text.



fibers were immobilized to an avidin resin, incubated with [*PSI*$^{+}$] yeast cell lysates and proteins bound to the fibers were identified by mass spectrometry.[1] Those studies revealed that proper recruitment of amyloid aggregates to the IPOD requires, among others, an actin cable-based transport machinery comprising tropomyosin and the actin motor protein Myo2 (compare also Fig. 1). Transient depletion of these 2 proteins led to a targeting defect of amyloid aggregates to the single perivacuolar IPOD deposition site, which resulted in an accumulation of aggregates in multiple foci dispersed throughout the cytoplasm.[1]

Actin had been implicated a role in the cellular handling of aggregates before. During the *de novo* induction of yeast prions, interactions of prion aggregates with actin were documented *in vivo*.[15] Later, it was observed that molecules of Sup35 can be linked with actin patches in the cell periphery via the stress-inducible protein Lsb2 that can associate with Sup35 aggregates on the one hand and the actin binding factor Las17 on the other hand.[16] Thus Lsb2 might collect and concentrate misfolded Sup35 molecules, resulting in locally high concentrations that facilitate conformational conversion to the prion state. It was suggested that aggregates captured near actin patches through this mechanism might be further directed to perivacuolar protein quality control compartments such as the IPOD. Since *de novo* formation of another yeast prion termed

[RNQ^+] was also facilitated by Lsb2, this proposed mechanism may be a general one for amyloidogenic proteins.[16] Additionally, actin was also implicated in targeting of other types of aggregates to respective deposition sites when it was observed that an intact actin cytoskeleton was crucial to target constantly misfolding VHL to the JUNQ/INQ and Q-bodies.[7] Another hint for a role of the actin cytoskeleton in aggregate handling came from studies investigating the molecular mechanism of asymmetric retention of protein aggregates in mother cells during cell division.[17,18] It was found that deletion of several components of the actin polarity machinery including the formin Bni1, actin mutants that cause reduced binding of actin cables to myosin and mutants of Myo2, resulted in a severe reduction of damage asymmetry.[19,20] Furthermore, aggregates that had formed in buds of budding mother cells were transported back into the mother cell. This process depended again on the formin Bni1 and the actin cable decorating protein tropomyosin. Both, retention of aggregates in mother cells as well as retrograde clearance of aggregates from the bud involved binding of aggregates associated with the protein disaggregase Hsp104, to actin. These data led to a model where actin cables provide a scaffold for tethering of Hsp104-containing aggregates. Retrograde actin flow away from the polarisome in the bud, initiated through Bni1 dependent actin nucleation, would then allow for net movement of aggregates toward the mother cell.[19,20] The role of Myo2 in this model was proposed to be an indirect one, because Myo2 moves along actin cables in the opposite direction as compared with aggregates. It was suggested to be involved in delivery of components to the polarisome, favoring an indirect effect of impairment of Myo2 function on aggregate asymmetry. In similar experiments, it was shown that aggregates of the amyloidogenic polyQ extension Huntingtin variant Htt103Q are also subject to asymmetric inheritance. In this case, the process is also dependent on the formin Bni1, suggesting that it might be retained by the same or a very similar mechanism.[20] More recently, higher resolution microscopy (3D-SIM) has confirmed an association of heat-denatured aggregates bound by Hsp104 and dispersed Htt103Q aggregates, with actin cables. The nature of these Htt103Q aggregates, however, remained unclear because these aggregates did not stain with the amyloid dye ThioflavinT,[21] opening the possibility that they were of amorphous nature rather than being amyloid like. Furthermore, both types of aggregates co-localized with Myo2 and the actin-organizing protein calmodulin, further substantiating a physical interaction of aggregates with the actin cytoskeleton network.[21]

AMYLOID RECRUITMENT TO THE IPOD OVERLAPS WITH SUBSTRATE DELIVERY TO THE ADJACENT PHAGOPHORE ASSEMBLY SITE (PAS)

When we studied an involvement of actin cable-based recruitment of amyloids to the IPOD, we observed that the corresponding recruitment machinery strongly overlapped with that for precursor hydrolases on transit to the Phagophore Assembly Site (PAS).[1] The PAS is the site where the cell initiates the formation of autophagosomes during macroautophagy and CVT vesicles in the so-called cytoplasm-to-vacuole targeting pathway. A precursor form of the aminopeptidase1 (preApe1) is a major substrate of the CVT pathway. It forms homododecamers that assemble into higher order complexes in the cytoplasm. These complexes are recruited to the PAS where they are encapsulated into autophagosome-related double membrane vesicles termed CVT vesicles that subsequently fuse with the vacuolar membrane to release their content into the lumen for activation through cleavage of the propeptide.[22-25] Interestingly, the faithful recruitment of preApe1 complexes to the PAS involves actin cables.[26,27] According to a current model,[26] preApe1 complexes are recruited to the outside of small vesicles termed Atg9 vesicles via the adaptor Atg11 and the

specific receptor Atg19 that can associate with Atg9 vesicles.[28,29] These Atg9 vesicles are generated through Golgi-related secretory and endosomal pathways.[30-32] They are stored in cytoplasmic reservoirs of 20–30 nm vesicles that can be activated for rapid delivery to the PAS if needed. At the PAS, Atg9 resides in a cluster of vesicles and tubules that can fuse to initiate autophagosome/CVT vesicle formation.[31] SNARE proteins are involved in formation and fusion of Atg9 containing vesicles and tubules.[33-35] When it was observed that the transmembrane protein Atg9 also failed to reach the PAS upon impairment of the actin cytoskeleton,[27] it was proposed that Atg9 vesicles loaded with preApe1 are associated with actin cables through an thitherto unknown factor and move either actively along actin cables or are pushed toward the PAS through nucleation and elongation of the actin cables they associate with.[26,36] We observed that the faithful recruitment of preApe1 as well as the PAS marker protein Atg8 to the PAS is also impaired after depletion of the actin cable motor Myo2[1]. This would strongly support the hypothesis that Atg9 vesicles loaded with preApe1 are actively moving along actin cables to the PAS[26] and identifies Myo2 as the missing factor between Atg9 vesicles and actin cables.[1] Such a Myo2 based movement of vesicles along actin cables is in full agreement with the confirmed role for Myo2 in moving vacuolar lobes on actin cables during vacuole inheritance.[37]

AMYLOID AGGREGATES ARE LIKELY TRANSPORTED TO THE IPOD VIA Atg9 RELATED VESICLES

Depletion of components of the recruitment machinery for substrates to the PAS and IPOD, respectively, led to the co-accumulation of amyloid aggregates with preApe1, Atg8 and Atg9 in the same foci dispersed throughout the cytoplasm.[1] It was proposed that the precursor hydrolase complexes are attached to Atg9 vesicles that move along actin cables toward the PAS.[26] In such a scenario, cargoes that move along actin cables may simply get stuck at particular cellular sites when the integrity or functionality of the actin tracks is impaired or crucial factors are missing, similar to a traffic jam in front of a construction site. If amyloid aggregates were tethered directly to those actin cables, for example through direct interaction of Hsp104 with aggregates on the one hand and actin cables on the other hand, and moved indirectly through actin cable polymerization,[19] it would be curious why they accumulated at the same cellular sites as compared with actively moving Atg9 vesicles. Furthermore, in that case, it would be harder to explain why the depletion of the Myo2 motor protein led to a transport block of amyloid aggregates. Therefore, we hypothesized that amyloid aggregates might be associated with vesicles just like the large preApe1 complexes are. Due to the partial co-localization of PrD-GFP with Atg9, those vesicles might even represent Atg9 containing vesicles. We found several lines of evidence that this might indeed be the case. The first one came from our observation that Myo2 co-localized with PrD-GFP aggregates upon disruption of the recruitment machinery. As a second line of evidence, we observed that the depletion of different proteins involved in vesicular transport processes led to an identical phenotype as compared with Myo2 depletion, namely a block in recruitment of amyloid aggregates to the IPOD and accumulation of the aggregates in multiple dispersed foci. This clearly ties vesicular transport processes with the faithful recruitment of amyloid aggregates to the IPOD (Fig. 1). One of those vesicular transport proteins we identified was Sec 14[1]. It is a phosphatidylinositol/phosphatidylcholine (PI/PC) transfer protein[38] that associates preferentially to Golgi membranes.[39] It transfers phosphatidylinositol (PI) lipid from the ER to the Golgi complex, where PI is phosphorylated to form phosphatidylinositol-4-phosphate (PI4P) by the PI4P kinase, Pik1.[38,40,41] It has been shown that the pool of PI4P lipids generated by Pik1 regulates the formation of Golgi-derived secretory vesicles.[41-43] Therefore, when Sec 14 or Pik1 function is abolished, the amount of PI4P is reduced and *de novo* formation of Golgi-derived vesicles is reduced.[40-42] Remarkably, Atg9 vesicles are also derived from the Golgi apparatus.[30] In line with this, it was shown that inactivation of Pik1

mutants at the nonpermissive temperature blocks the formation of Atg9 vesicles from the Golgi.[44] Since Sec 14 acts upstream of Pik1 in the formation of PI4P lipids,[40,41] its function is likely also crucial for releasing of Atg9 vesicles from the Golgi. Since we hypothesized that PrD-GFP may be recruited to Atg9 containing vesicles, this would plausibly explain why depletion of Sec 14 disturbs recruitment of PrD-GFP aggregates to the IPOD (Fig. 1). However, it should be noted that Sec 14 may also influence the integrity of actin cables: besides the role of PI4P lipids on the formation of Golgi-derived Atg9 transport vesicles,[44] PI4P also serves as a substrate for the PIP5-kinase Mss4 to generate $PI(4,5)P_2$ lipids, which are required for the proper organization of the actin cytoskeleton.[40,45] In agreement with this, it was shown that impaired production of PI4P in pik1–101 mutant cells results in less $PI(4,5)P_2$ generation, which causes disruption (depolarization) of actin cables.[43] Upon Sec 14 depletion, PI is not transported properly to Pik1. This may eventually also reduce the levels of $PI(4,5)P_2$ lipids, causing an impairment of the actin cytoskeleton, which in turn could also contribute to impaired recruitment of PrD-GFP to the IPOD (Fig. 1). Furthermore, the PI4P lipids whose levels are reduced upon Sec 14 depletion, have an additional role in actin cable-based transport of vesicles. It was shown that the association of secretory vesicles with Myo2 for their transport along actin cables to the plasma membrane requires vesicle bound PI4P.[46] Since Atg9 vesicles also originate from the trans Golgi compartment,[30-32] it appears possible that PI4P is also important for binding of Myo2 to Atg9 containing vesicles that mediate recruitment of preApe1 and presumably also amyloid aggregates to the PAS and IPOD, respectively (Fig. 1).

A second protein whose depletion gave the same phenotype of impaired recruitment of amyloid aggregates to the IPOD as compared with Myo2 was the SNARE chaperone Sec 18[47] that acts in disassembly of SNARE proteins and is crucial for different vesicular fusion and transport processes. Stunningly, Sec 18 and SNARE protein function is known to be required for autophagy and the CVT pathway.[33,35,48] Moreover, it was also found to be involved in establishing aggregate inheritance asymmetry during cell division.[21] As generation and trafficking of Atg9 vesicles to the PAS requires the action of several SNARE proteins,[33] it seems plausible that the effect of Sec 18 depletion is due to reduced trafficking of Atg9 vesicles to the PAS/IPOD.

Finally, some interesting additional findings support the hypothesis that Atg9 related vesicles are involved in recruitment of amyloid aggregates to the IPOD: Sec 4, a Rab family GTPase that is essential for autophagy and that is involved in anterograde transport of Atg9 vesicles to the PAS,[49] was found to bind to immobilized PrD fibers.[1] Since Sec 4 can associate with Myo2 during the transport of secretory vesicles along actin cables to the plasma membrane,[46,50] it may have a similar role in Atg9 containing vesicles (Fig. 1), which would explain why it is important for anterograde transport of Atg9 vesicles to the PAS. Furthermore, Ykt6, a v-SNARE protein that has been implicated in different vesicle trafficking pathways including the CVT pathway and Atg9 vesicle trafficking[33] also bound to immobilized PrD fibers. Thus we may have found these proteins to bind to immobilized PrD fibers[1] because they are components of the vesicles that recruit PrD-GFP and guide it to the IPOD (Fig. 1). In conclusion, many of these findings point to a direct recruitment of amyloid aggregates to Atg9 related vesicles. A more direct proof for this could come from direct visualization of amyloid aggregates with vesicles by EM in the future.

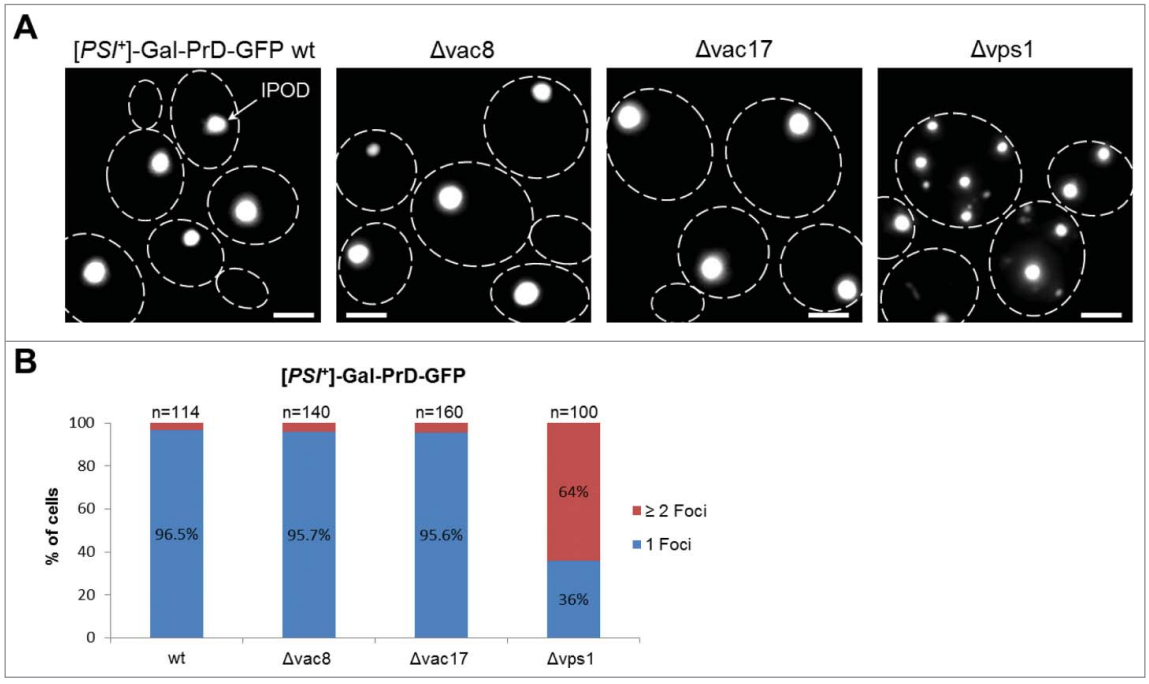
ASSOCIATION OF AGGREGATES WITH VESICULAR STRUCTURES

The concept proposing that vesicular transport is involved in the recruitment of aggregates to different aggregate deposition sites is rather new, but various previous studies exist that report an association of aggregates with membranes. Htt103 aggregates for example were shown to associate with endocytic membranes, thereby impairing endocytic processes.[51] Moreover, interaction of Htt103Q with endocytic membranes facilitated its

aggregation.[52] Furthermore, cytosolic aggregates were found to be associated with the ER and mitochondria, probably constraining aggregate mobility and facilitating retention of aggregates in mother cells during cell division.[6,53]

In a recent study, Hill et al investigated the mechanism of mother cell-biased segregation of aggregates.[2] The authors observed that Hsp104-bound cytosolic aggregates co-precipitated with several proteins involved in vesicular trafficking. More specifically, the 4 components of the vacuole inheritance machinery, Act1, Myo2, Vac17 and Vac8 as well as the yeast dynamin homolog Vps1 were identified to be involved in this process. Moreover, the authors observed that the deletion of Vac17 and Vps1 did not only impair retention of heat-denatured aggregates in the mother cells, but also disturbed the recruitment of these aggregates to a perivacuolar inclusion reminiscent of the IPOD. In line with this, the study also reported that Vac17 overexpression boosted fusion of small aggregates into larger perivacuolar assemblies. It was therefore proposed that aggregates bound by Hsp104 might interact with membrane vesicles, thereby hitchhiking on trafficking routes leading to the vacuole.[2] Interestingly, the deletion of Vac17 did not effect the recruitment of the amyloidogenic IPOD substrate Htt103Q.[2] This different dependency of heat-denatured and amyloidogenic aggregates on the Vac17 function for recruitment to IPOD like inclusions prompted

FIGURE 2. Vps1, but not Vac8 and Vac17, is involved in the sorting of PrD-GFP aggregates to the IPOD. (A) PrD-GFP was integrated into the genome under the galactose-inducible promoter in a [*PSI*$^+$] wild-type (wt) strain or a strain with a deletion of Vac8, Vac17 or Vps1. Cells were grown overnight in YPD and induced with galactose for 6 hours at an OD_{600} of 0.15. Cells were then fixed with 4 % paraformaldehyde (PFA) and analyzed by fluorescence microscopy (xcellence IX81Olympus). Images were acquired with a 100X /NA 1.45 oil immersion objective as Z-stacks with an optical section of 0.2 μm. An overlay of the acquired z-stacks (maximum intensity projection) was then processed with the ImageJ software. Scale bar, 2 μm. (B) Quantification of PrD-GFP foci in the wild-type strain and strains with the deletion of Vac8, Vac17 or Vps1 is shown. Frequencies of cells with one single focus or more than one foci are given in %; n = number of cells analyzed for quantification.



us to ask whether the recruitment machineries for different types of aggregates differ. To approach this question, we tested whether deletion of Vac8, Vac17 or Vps1 also affected the transport of the model amyloid substrate PrD-GFP to the IPOD. Thus we deleted each of these proteins in a strain expressing PrD-GFP under control of the Gal1 promoter. After 6 hours of induction of PrD-GFP expression with galactose, the protein was exclusively found at a perivacuolar IPOD site in the absence of Vac8 or Vac17, just like in a wild type control strain (Fig. 2A, B). To our big surprise, however, the deletion of Vps1 did cause a severe recruitment defect for PrD-GFP to the IPOD (Fig. 2A, B), just like it did for heat-denatured proteins in the study by Hill et al.[2] Thus our data on Vac8 and Vac17 deletion fully confirm the previous results[2] that these proteins do not influence the recruitment of amyloidogenic proteins to the IPOD to a measureable extend. A possible explanation for these results could be that both types of aggregates, heat-denatured proteins and amyloids, use different types of vesicles with different adaptors for their recruitment to IPOD-like inclusions, but both types of vesicles require the dynamin homolog Vps1. Additional future studies, however, have to unravel this issue in more molecular detail to confirm or disprove this hypothesis. Nonetheless, the key finding that recruitment of heat-denatured protein aggregates to a perivacuolar deposition site involves proteins mediating vesicular transport[2] fully supports our concept that vesicular transport is crucial for the sorting of amyloid aggregates to the perivacuolar deposition site IPOD.[1]

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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