From Monomers to Condensates:

Golgi-Associated plant Pathogenesis Related protein 1 (GAPR-1) Oligomerization in Saccharomyces cerevisiae

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IBSN: 978-94-6483-577-9

Cover: Nafiseh Sirati

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Printed by: Thesis Specialist Ridderprint, ridderprint.nl

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Van Monomeren tot Condensaten:

Oligomerisatie van GAPR-1 in *Saccharomyces cerevisiae*

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof. dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

maandag 11 december 2023 des middags te 2.15 uur

door

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Introduction

1.1-Protein folding: pathological and functional amyloids

Protein folding, the process by which proteins fold into specific three-dimensional structures, has been studied extensively over the past decades [1]. Native states of proteins are typically considered as relatively stable structures under various physiological conditions [2]. However, when a protein successfully achieves its biologically active state following translation, this often does not represent the end-point of its folding/unfolding life. Many proteins undergo cycles of unfolding and refolding due to a variety of factors, such as transport across a membrane, cellular secretion, or exposure to stress conditions (e.g. changes in pH, temperature) [3]. The folding and unfolding of proteins are crucial for regulating their biological activity and targeting proteins to different cellular locations [2]. However, when this tightly regulated protein plasticity becomes imbalanced, proteins can undergo structural rearrangements that result in misfolding. In this process, proteins, which are typically soluble, can transform and assemble into insoluble aggregates called amyloids [4,5]. Various factors contribute to the amyloid formation, including mutations [6], environmental conditions (such as changes in pH, high salt concentration, temperature, or the presence of specific ions or molecules) [7,8], ageing [8,9] and the intrinsic amyloidogenic propensity [6] can drive proteins to adopt an amyloid conformation.

The association between amyloids and human disease was first identified in a pathology named amyloidosis, where proteins and peptides misfold into amyloid fibrils. In the context of amyloidosis, more than 50 different proteins have been identified to misfold and form amyloids, including amyloid-β (Aβ) in Alzheimer's disease, α-synuclein in Parkinson's disease, huntingtin in Huntington's disease, and β2-microglobulin (β2m) in dialysis-related amyloidosis (DRA) (Figure 1). The strong correlation with human diseases has contributed to the perception of amyloid fibril assembly as a pathological process [10,11]. However, it is important to realise that the formation of amyloid fibrils is not always detrimental [12]. Research has identified a variety of functional amyloid fibrils that perform important physiological roles in organisms ranging from prokaryotes to humans. This suggests that amyloid assembly can serve physiological roles in certain contexts, highlighting the complexity of the amyloid phenomenon and its diverse implications in biological systems [12–16]. Examples of physiologically relevant functional amyloids include pigmentation [17], peptide hormone storage [13,18], fertilization of oocytes by sperm [19–21], antimicrobial responses [22], regulated necrosis [23], cellular stress responses [24,25], bacterial stress protection [26], regulation of fungal-host and fungal-fungal interactions [27], modulation of epigenetic heritable phenotypes in yeast [28], and persistence of long-term memory in Drosophila

[29] (Figure 1). Although the precise mechanisms by which amyloid toxicity is prevented in these cases remain unclear, several possibilities have been proposed. These include regulating the levels of amyloidogenic peptides and proteins, reducing the production of prefibrillar oligomers during amyloid assembly, sequestering amyloids within membrane-bound organelles, controlling amyloid assembly through other molecules, and disassembling fibrils under physiological conditions [30]. These examples highlight the diverse and complex biochemical regulation of amyloid structures.

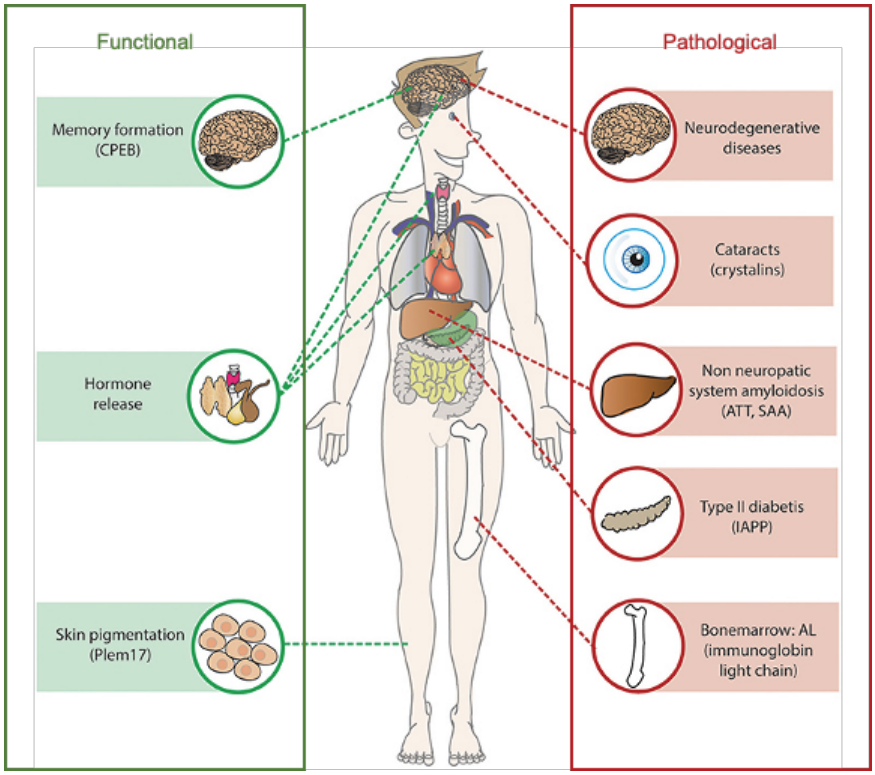


***Figure 1**. Pathological and functional amyloids in human biology, adapted from Frontiers in Neuroscience, 2017 [31].*

1.2- Biomolecular condensates

In recent years, amyloid-related processes such as protein misfolding, protein oligomerization, and fibril formation have been suggested to contribute to the formation of biomolecular condensates. These condensates are specialized compartments within the cell that are rapidly assembled and contain various biomolecules [32]. Within the cellular context, the formation of biomolecular condesates offers a rapid compartmentalization creating specific microenvironments to regulate the efficiency of biological processes. Traditionally, cellular compartmentalization is described as enclosed by membrane organelles that serve distinct functions and tasks. They are called membrane-bound organelles and can be exemplified by the mitochondria, nucleus, endoplasmic reticulum and vacuole. Biomolecular condensates on the other hand are a novel form of cellular compartmentalization without surrounding membranes, also known as membrane-less organelles. Unlike traditional compartments, condensates are characterized by their ability to form dynamic and transient assemblies of biomolecules without the presence of a surrounding membrane. These condensates play important roles in various cellular processes, such as signalling, gene expression, and phase separation, offering a new dimension to our understanding of the cellular organization and function [33–37]. Close observation showed that different condensates differ by location (cytoplasm, nuclei, membranes), number, volume, and functional complexity [38]. The formation of protein condensates is facilitated by a phenomenon called liquid-liquid phase separation (LLPS), which is predominantly driven by protein concentration and weak multivalent interactions among proteins. Intriguingly, these condensates can exhibit liquid-like properties, resembling droplets of liquid within the cellular environment. This liquid-like behavior allows for dynamic and reversible interactions between biomolecules, contributing to cellular processes, organization and regulation [39,40]. Furthermore, the formation of condensates provides an optimal environment for specific biochemical reactions to occur. Different molecules can diffuse within these droplets, facilitating a new interaction platform [41]. Early work in the field has primary focused on identifying various types of condensates and understanding how their physical properties and regulation arise from molecular components. Recent years have focused on understanding condensate functions, revealing their fundamental roles in a wide range of cellular events, from RNA metabolism to signalling to gene regulation (Figure. 2).

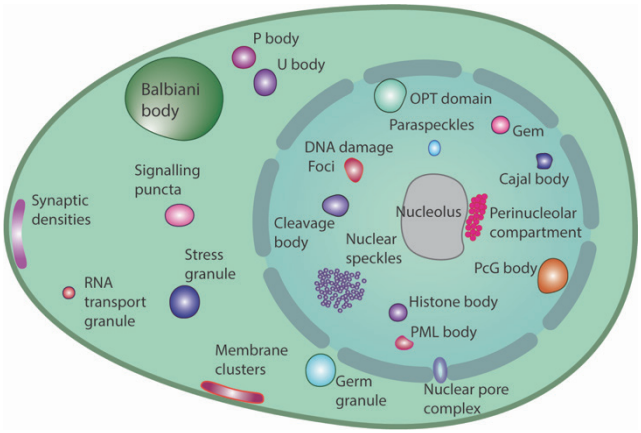


***Figure 2**. Biomolecular condensates in the cell.*

The formation and regulation of biomolecular condensates is influenced by a variety of factors (reviewed in [42,43]), which include:

- Specific sequence motifs: Biomolecular condensates are often formed through multivalent interactions between proteins. These interactions can involve specific domains, such as intrinsically disordered regions (IDRs) [44–46], low-complexity domains (LCDs), [44,47] or prion-like domains (PrLDs) [48]. The presence and affinity of these interaction domains within proteins can determine their ability to participate in condensate formation.
- Protein concentration: When the protein concentration of such proteins exceeds a threshold, the likelihood of their interaction increases, leading to phase separation and the formation of biomolecular condensates. These condensates can differ in size and composition, and their formation can be further regulated by surrounding cellular factors and post-translational protein modifications, [49].
- Post-translational modifications: Various post-translational modifications, including phosphorylation, acetylation, methylation, and ubiquitination, can modulate the formation and dissolution of condensates. For example, phosphorylation can directly modulate the LLPS properties of proteins by altering their multivalent interactions or affecting the degree of their intrinsic disorder. The negative charges introduced by the phosphorylation can influence the protein-protein interactions leading to repulsion and disrupting condensate assembly. Conversely, phosphorylation can also promote condensate formation by enhancing attractive interactions between proteins [50,51].

- RNA molecules: RNA molecules, such as messenger RNA (mRNA) or non-coding RNAs, can contribute to the assembly and regulation of condensates. RNA-binding proteins (RBPs) often interact with RNA molecules to form ribonucleoprotein (RNP) complexes, which can undergo phase separation and contribute to the formation of RNA-protein condensates [52].
- Environmental conditions: Cellular conditions, including temperature, pH, ionic strength, and the presence of molecular crowding agents, can impact condensate formation. Changes in these environmental factors can disrupt or promote the stability and dynamics of condensates [53].
- Chaperones and molecular disaggregases: Molecular chaperones and disaggregases play crucial roles in maintaining protein homeostasis and preventing the accumulation of misfolded or aggregated proteins. These factors can influence the formation and dissolution of condensates by regulating the folding state and solubility of proteins involved in condensate assembly [48].
- Molecular crowding: The cellular environment is highly crowded, with high concentrations of macromolecules. Molecular crowding can influence the formation and properties of condensates by affecting protein-protein interactions, molecular diffusion, and phase separation kinetics [53,54].

Understanding the interplay between these factors is essential for interpreting the regulation and functions of biomolecular condensates in cellular processes.

1.3-Biomolecular condensates and amyloid proteins

H In addition to protein concentration and weak multivalent interactions, misfolding of amyloid-like proteins can also plays a significant role in the formation of protein condensates through LLPS. Studies have revealed that proteins that tend to form aggregates with age exhibit a significantly higher propensity of undergoing liquid phase separation and granule formation [55]. This finding suggests a potential link between LLPS and protein aggregates, possibly shedding light on the mechanisms of underlying disorders, such as in amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) and inclusion body myopathy (IBM) [36,55–61]. Protein segregation in certain condensates is tightly regulated by the protein quality control (PQC) system [62]. The failure in PQC can lead to unusual forms of condensates, which are increasingly linked to age-related diseases, such as neurodegeneration, myopathy, and cancer [56,63,64]. For example, the abnormal aggregation of tau and amyloid beta (Aβ) proteins is a key feature of Alzheimer's disease, and these proteins have been shown to undergo liquid-liquid phase separation *in vitro* and *in vivo* [23,34]. Similarly, α-synuclein, the protein that forms Lewy bodies in Parkinson's disease, has been shown to undergo phase separation and

form cytosolic condensates [35]. In the case of ALS, mutations in several RNA-binding proteins, such as TDP-43 and FUS, have been linked to the formation of abnormal condensates and the accumulation of protein aggregates [36]. Furthermore, it has been hypothesized that the protein aggregation process in neurodegenerative disorders involves a transition from a liquid-like to a solid-like state [34]. This transition is driven by kinetic and thermodynamic mechanisms, leading to the formation of liquid condensates undergoing maturation and solidification, ultimately resembling the irreversible formation of highly stable amyloid fibrils [48,65]. Various factors, such as changes in protein concentration, pH, salt, and other physical-chemical parameters, can trigger this transition by perturbing the delicate balance between attractive and repulsive forces among the protein molecules [66,67]. For instance, high protein concentration emerges as a particularly significant factor in initiating aggregation-prone processes or jamming. As protein concentration reaches a critical threshold, proteins become supersaturated, leading to self-assembly and clustering. These events can ultimately result in the formation of solid gels or even crystals [67] (Figure. 3). Given the close association between the liquid-to-solid phase maturation of protein condensates and the onset of numerous diseases, the phase transition of these droplets has emerged as a new avenue for exploration in cell biology [65,67]. Ultimately, gaining a comprehensive understanding of protein phase separation mechanisms and their implications in disease pathology will offer valuable insights for the development of therapeutic strategies targeting neurodegenerative disorders.

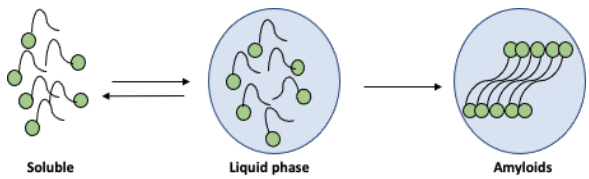


***Figure 3**. Liquid to solid transition in protein droplets.*

1.4-Yeast as a model system

In recent years, *Saccharomyces cerevisiae* has emerged as a preferred model organism for studying reversible protein aggregation, including amyloid-like aggregation. Budding yeast species have historically been used for centuries in producing beer, bread and wine, and also has become a highly tractable eukaryotic model system in biology [68]. Despite being a unicellular organism, S. cerevisiae offers several distinct advantages over higher model organisms. These advantages include high sequence homology with human genes, rapid growth rate, and ease of genetic modification capabilities, enabling efficient scale-up and facilitating high-throughput genetic and small-molecule screening approaches [69]. *S.*

cerevisiae is one of the most intensively studied model organisms, with a fully sequenced genome and various knockout and complementation plasmid libraries available [70]. As a unicellular eukaryote, it has provided invaluable insight into fundamental cellular mechanisms such as DNA replication, protein folding, quality control and degradation, vesicular trafficking, mitochondrial dysfunction and oxidative stress, and even cell death and survival mechanisms. These studies have revealed the conservation of key cellular processes between yeast and humans [70,71].

The remarkable 60% homology between yeast and humans makes yeast an attractive model system for investigating the molecular mechanisms underlying a range of human disorders [72]. Notably, approximately 17% of yeast genes belong to orthologous gene families associated with human diseases, and conversely, around 30% of known genes implicated in human diseases may have a yeast orthologue [73,74]. Initially, classical complementation assays were used to substitute yeast protein with homologous proteins from another organism to elucidate the biological role of human proteins with a yeast homologue. However, a new trend has emerged, namely, the development of yeast cell-based assays, or "humanized yeast systems" that allow to study protein functions without the presence of a yeast homologue [75].

As such, yeast has proven to be a highly versatile model system for studying neurological disorders and identifying genes that regulate processes associated with amyloidosis [69,70] (Figure. 4). This approach has yielded fruitful outcomes in the study of Huntington's disease [71,76,77], Alzheimer's disease [78–81], Parkinson's disease [82–84], Amyotrophic lateral sclerosis (ALS) [85], and proteinopathies such as cystic fibrosis [86]. Protein misfolding, a hallmark of numerous neurodegenerative diseases, can lead to the formation of large protein inclusions or aggregates that are detectible through microscopy and biochemical assays [87–91]. Interestingly, protein deposition also frequently occurs during recombinant expression in simple organisms such as unicellular fungi or bacteria [92,93]. The aggregates formed in these microorganisms resemble those involved in human disorders, suggesting a common process of protein self-assembly into amyloid-like structures [80,94].

Numerous studies have reported the formation of reversible aggregates on a genome-wide scale, revealing that a surprisingly large number of proteins can undergo reversible aggregation in yeast [95]. Examples of such protein aggregates include Balbiani bodies in oocytes [96], nuclear amyloid bodies (A-bodies) [25], and cytoplasmic Cdc19 aggregates in yeast cells [24,95]. These findings have led to the hypothesis that reversible aggregates may constitute a previously unrecognized level of cellular organization that is widely used [95,97,98]. It is tempting to speculate that some proteins in mammalian cells may form reversible, functional, amyloid-like aggregates and that these structures may be regulated similarly. Indeed, several proteins involved in metabolism and stress responses form

cytoplasmic liquid-like droplets in yeast and Escherichia coli, C. elegans, Thermus thermophilus, Xenopus and Human cells [98–101]. Understanding the regulation of such structures could also provide insights into treating and preventing aggregation-based diseases. At the same time, studying the formation of phase-separated protein droplets in yeast by overexpressing amyloid-prone proteins allows to understand the underlying mechanisms of protein phase separation and its potential link to neurodegenerative diseases [79]. For instance, studies have demonstrated that overexpression of α-synuclein in yeast leads to the formation of cytoplasmic condensates that resemble those seen in Parkinson's disease [82,102–104]. Similarly, overexpression of Tau, Aβ, Htt, and TDP-43 in yeast also induces the formation of cytoplasmic condensates [78,105–111]. Dissolving these condensates or preventing their accumulation has been a major therapeutic target in fundamental research initiatives and clinical trials.

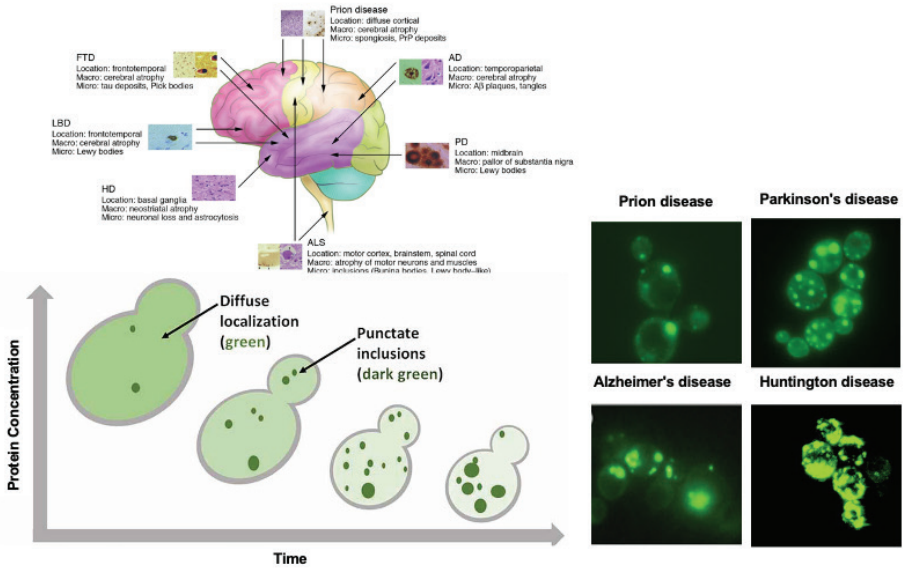


Figure 4*. Humanized yeast model system to study neurodegenerative diseases. The upper panel shows proteins involved in in neurodegenerative diseases. The lower panel shows inclusion formation of some of these proteins upon overexpression in yeast cells, adapted from Frontiers in Molecular Neuroscience, 2018 and The Journal of clinical investigation, 2005 [112,113].*

2- GAPR-1

Golgi-associated plant pathogenesis-related protein 1 (GAPR-1) is a peripheral membrane protein located at the cytosolic leaflet of the Golgi apparatus in mammalian cells [114]. GAPR-1 belongs to the CAP (cysteine-reach secretory proteins, antigen 5, pathogenesis related-1) superfamily of proteins [115]. The CAP superfamily is characterized by the presence of a structurally conserved domain called the CAP domain. The CAP domain is 17 to 21 kDa in size and contains four evolutionarily conserved elements, the CAP motifs (CAP1- 4) [115,116]. The CAP domain is characterized by a tertiary structure, an α-β-α sandwich fold. This fold consists of a central β-sheet, flanked by one α-helix on one side, and three α-helices on the other side [117]. Despite the structural conservation, a common function for the CAP domain in the different family members has yet to be identified (Figure. 5). GAPR-1 is considered to be one of the earliest mammalian CAP proteins in evolution [118]. Whereas many CAP proteins contain additional amino acids at the N-terminus or C-terminus, GAPR-1, with a size of 17 kDa, consists almost exclusively of a CAP domain [119]. Therefore, it is a highly suitable protein to study the CAP domain and its function [116]. Analyzing primary sequences has revealed that the CAP domains of at least eighteen CAP proteins, including GAPR-1, contain predicted amyloidogenic regions, most notably in the CAP1 and CAP2 motifs [120]. Amyloidogenic regions facilitate a protein aggregation pathway from monomers to mature amyloid fibrils with a cross-β sheet structure [121,122]. The amyloidogenic aggregation pathway consists of multiple reversible reaction steps, during which intermediates, namely amyloid oligomers and protofibrils, are formed [123].

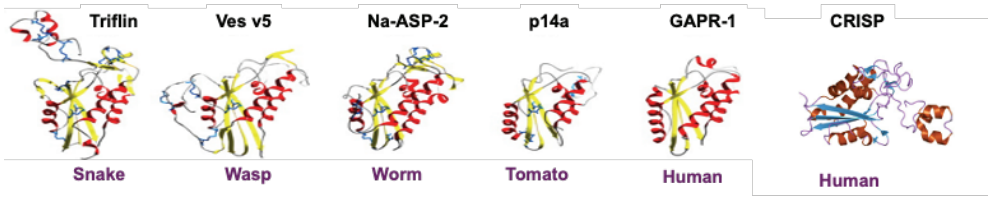


Figure 5*. Crystal structure of various family members of the CAP superfamily of proteins, adapted from Endoc Re. 2008 [115].*

As discussed earlier, even though amyloid formation of many proteins is associated with toxic effects and diseases, amyloid-like behavior of a growing number of proteins is shown to be functional [12–16]. Aggregation of these so-called functional amyloids might, for instance, regulate the protein's stability, the availability of active sites, and

protein-protein interactions (PPIs) [95,122]. As a results of the predicted amyloidogenic regions in the CAP domain, facilitating the amyloid-like aggregation pathway has been suggested to be a general feature of the CAP domain [116,124]. In line with this, the model CAP protein, GAPR-1, has been found to bind the amyloid oligomer-specific A11 antibody [120,125] and forms amyloid-like fibrils *in vitro* [120,126,127]. Although GAPR-1 has an intrinsic tendency for oligomer formation, progression to amyloid-like fibrils requires additional factors. Incubation with negatively charged lipids and inositol hexaphosphate (IP6) have both been found to induce GAPR-1 amyloid-like fibrillation [114]. Other factors identified are the divalent cations Zn^{2+} and Cu^{2+}. Co-incubation with either of these cations and heparin, a negatively charged molecule that is routinely used to catalyze amyloid formation of amyloidogenic proteins, also induces amyloid-like fibrillation of GAPR-1 [124,126,127]. The mechanism underlying the stimulation of aggregation differs per cation. Zn^{2+} binds GAPR-1 at His^{54} and His^{103}, causing a slight conformational change, which is responsible for the fibrillation [127]. The Zn^{2+}-binding residues are located in a central cavity that is highly conserved in CAP proteins, suggesting that metal binding might be important for the general function of the CAP domain [127]. Cu^{2+}-induced fibrillation, on the other hand, is independent of the conserved metal binding site. Additionally, unlike Zn^{2+}-induced fibrillation, it is dependent on the redox state. Cu^{2+} oxidizes residues Cys^{32} and Cys^{63}, thereby inducing the formation of intermolecular disulfide bonds [126]. The oligomers that form due to the establishment of these disulfide bonds likely function as nuclei to elicit further aggregation under oxidative conditions. Notably, the oxidation of Cys^{32} and Cys^{63} causes a conformational change similar the one caused by Zn^{2+} [126].

GAPR-1 has been demonstrated to act as a negative regulator of autophagy by retaining a key autophagy-inducing protein, Beclin 1, at the Golgi membrane [117,128]. Autophagy, a lysosomal degradation pathway, plays a vital role in maintaining cellular homeostasis, facilitating differentiation, and promoting development. Consequently, dysfunctions in autophagy have been associated with various diseases, including cancers, neurodegenerative disorders, cardiovascular diseases, and infectious diseases. Beclin 1 is an integral component of the vacuolar protein sorting 34 (Vps34) complex, which promotes autophagy [129–131]. Numerous proteins, such as various Bcl-2 homologs, are involved in the regulation of autophagy by binding to Beclin 1 and modulating its function [132,133]. A previous study showed the co-immunoprecipitation of GAPR-1 and Beclin 1 [128]. Additionally, a cell-permeable peptide derived from Beclin 1 (residues 267–284) efficiently pulled down GAPR-1 and induced autophagy, possibly by competing with Beclin 1 for binding to GAPR-1 [117,128]. However, the precise mechanism by which GAPR-1 interacts with Beclin 1 to down-regulate autophagy remains unknown. The objective of this research was to employ a humanized yeast model system to characterize

and investigate GAPR-1 oligomerization *in vivo*. These studies allowed to investigate the mechanism of interaction between GAPR-1 and Beclin 1, which may have implications for the role of GAPR-1 in autophagy (Figure. 6).

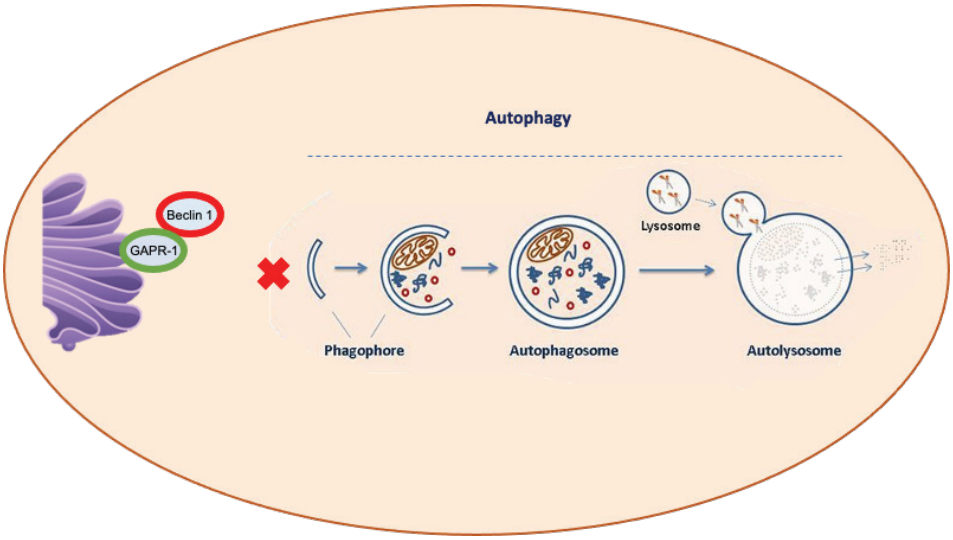


Figure 6. *GAPR-1 is a negative regular of autophagy.*

3- Aims and outline of the thesis

This study employs the yeast *S. cerevisiae* as a model system to investigate the molecular dynamics of GAPR-1 oligomerization *in vivo*. The aim is to explore the factors contributing to and regulating GAPR-1 oligomers and amyloid-like structures *in vivo*.

Chapter 2 focuses on the assembly of GAPR-1 into biomolecular condensates in yeast cytosol. The role of metal binding and membrane association in the formation of GAPR-1 oligomers and biomolecular condensates is investigated. Results show that N-myristoylation of GAPR-1 is a crucial determinant in early stages of protein condensate formation. The conserved metal-binding site (His54 and His103) prevents uncontrolled protein sequestration, and the addition of Zn^{2+} metal ions enhance protein condensate formation.

Chapter 3 introduces the yeast model as a platform for studying protein-protein interactions (PPIs) and explores how GAPR-1 may act as a negative regulator of autophagy by interacting with Beclin 1. Overexpression of human Beclin 1 in yeast also results in the formation of protein condensates. Co-expression of GAPR-1 and Beclin 1 leads to a significant reduction of Beclin 1 condensates. This chapter explores the correlation between amyloid-like interactions and discusses btheir potential impact on the regulation of autophagy.

Chapter 4 studies the effect of phosphorylation on the oligomeric states of GAPR-1 abnd Beclin 1 and on the molecular interaction between both proteins. Phosphorylation of Ser55 is required for GAPR-1 condensate formation, and phosphorylation of Ser15/Ser30 is required for Beclin 1 condensate formation. Co-expression experiments reveal that phosphorylation of Ser55 (GAPR-1) and Ser15/30 (Beclin 1) is also required to interfere with the mutual condensate formation of both proteins upon co-expression. ULK1/Atg1 was shown phosphorylate GAPR-1 and Beclin 1, affecting their amyloidogenic properties and enhancing their interaction.

Chapter 5 extends the yeast model to screen for interactions between different amyloidogenic proteins using protein condensate formation as a readout. The study highlights the versatility of yeast as a model system for investigating various molecular mechanisms and interactions involved in protein oligomerization.

Chapter 6 discusses various factors influencing protein oligomerization *in vivo*, focusing on GAPR-1 oligomerization and biomolecular condensate formation in yeast cytosol.

References

[1] P.C. Ke, R. Zhou, L.C. Serpell, R. Riek, T.P.J. Knowles, H.A. Lashuel, E. Gazit, I.W. Hamley, T.P. Davis, M. Fändrich, D.E. Otzen, M.R. Chapman, C.M. Dobson, D.S. Eisenberg, R. Mezzenga, Half a century of amyloids: past, present and future, Chem. Soc. Rev. 49 (2020) 5473–5509. https://doi.org/10.1039/C9CS00199A.

[2] C.M. Dobson, Protein folding and misfolding, Nature. 426 (2003) 884–890. https://doi.org/10.1038/nature02261.

[3] H. Ecroyd, J.A. Carver, Unraveling the mysteries of protein folding and misfolding, IUBMB Life. 60 (2008) 769–774. https://doi.org/10.1002/iub.117.

[4] S.K. Chaturvedi, M.K. Siddiqi, P. Alam, R.H. Khan, Protein misfolding and aggregation: Mechanism, factors and detection, Process Biochem. 51 (2016) 1183–1192. https://doi.org/10.1016/j.procbio.2016.05.015.

[5] S.Y. Ow, D.E. Dunstan, A brief overview of amyloids and Alzheimer's disease, Protein Sci. 23 (2014) 1315–1331. https://doi.org/10.1002/pro.2524.

[6] N. Guthertz, R. van der Kant, R.M. Martinez, Y. Xu, C.H. Trinh, B.I. Iorga, F. Rousseau, J. Schymkowitz, D.J. Brockwell, S.E. Radford, The effect of mutation on an aggregation-prone protein: An *in vivo*, *in vitro*, and in silico analysis, Proc. Natl. Acad. Sci. 119 (2022). https://doi.org/10.1073/pnas.2200468119.

[7] S.-Y. Ow, D.E. Dunstan, A brief overview of amyloids and Alzheimer's disease, Protein Sci. 23 (2014) 1315–1331. https://doi.org/10.1002/pro.2524.

[8] N. Frankowska, K. Lisowska, J.M. Witkowski, Proteolysis dysfunction in the process of aging and age-related diseases, Front. Aging. 3 (2022). https://doi.org/10.3389/fragi.2022.927630.

[9] J.-H. Wen, X.-H. He, Z.-S. Feng, D.-Y. Li, J.-X. Tang, H.-F. Liu, Cellular Protein Aggregates: Formation, Biological Effects, and Ways of Elimination, Int. J. Mol. Sci. 24 (2023) 8593. https://doi.org/10.3390/ijms24108593.

[10] R.N. Rambaran, L.C. Serpell, Amyloid fibrils: abnormal protein assembly., Prion. 2 (2008) 112–117. https://doi.org/10.4161/pri.2.3.7488.

[11] C. Sachse, M. Fändrich, N. Grigorieff, Paired β-sheet structure of an Aβ(1-40) amyloid fibril revealed by electron microscopy, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 7462–7466. https://doi.org/10.1073/pnas.0712290105.

[12] E. Stroo, M. Koopman, E.A.A. Nollen, A. Mata-Cabana, Cellular regulation of amyloid formation in aging and disease, Front. Neurosci. 11 (2017). https://doi.org/10.3389/fnins.2017.00064.

[13] R.S. Jacob, S. Das, S. Ghosh, A. Anoop, N.N. Jha, T. Khan, P. Singru, A. Kumar, S.K. Maji, Amyloid formation of growth hormone in presence of zinc: Relevance

to its storage in secretory granules, Sci. Rep. 6 (2016) 23370. https://doi.org/10.1038/srep 23370.2016.00069.

[14] E. Grignaschi, G. Cereghetti, F. Grigolato, M.R.G. Kopp, S. Caimi, L. Faltova, S. Saad, M. Peter, P. Arosio, A hydrophobic low-complexity region regulates aggregation of the yeast pyruvate kinase Cdc19 into amyloid-like aggregates in vitro, J. Biol. Chem. 293 (2018) 11424–11432. https://doi.org/10.1074/jbc.RA117.001628.

[15] J. Asencio-Hernández, C. Ruhlmann, A. McEwen, P. Eberling, Y. Nominé, J. Céraline, J.P. Starck, M.A. Delsuc, Reversible amyloid fiber formation in the N terminus of androgen receptor, ChemBioChem. 15 (2014) 2370–2373. https://doi.org/10.1002/cbic.201402420.

[16] A. Anoop, S. Ranganathan, B.D. Dhaked, N.N. Jha, S. Pratihar, S. Ghosh, S. Sahay, S. Kumar, S. Das, M. Kombrabail, K. Agarwal, R.S. Jacob, P. Singru, P. Bhaumik, R. Padinhateeri, A. Kumar, S.K. Maji, Elucidating the role of disulfide bond on amyloid formation and fibril reversibility of somatostatin-14: Relevance to its storage and secretion, J. Biol. Chem. 289 (2014) 16884–16903. https://doi.org/10.1047/jbc.M114548354.

[17] C. Bissig, L. Rochin, G. van Niel, PMEL Amyloid Fibril Formation: The Bright Steps of Pigmentation, Int. J. Mol. Sci. 17 (2016) 1438. https://doi.org/10.3390/ijms17091438.

[18] S.K. Maji, M.H. Perrin, M.R. Sawaya, S. Jessberger, K. Vadodaria, R.A. Rissman, P.S. Singru, K.P.R. Nilsson, R. Simon, D. Schubert, D. Eisenberg, J. Rivier, P. Sawchenko, W. Vale, R. Riek, Functional Amyloids As Natural Storage of Peptide Hormones in Pituitary Secretory Granules, Science (80-.). 325 (2009) 328–332. https://doi.org/10.1126/science.1173155.

[19] N.R. Roan, N. Sandi-Monroy, N. Kohgadai, S.M. Usmani, K.G. Hamil, J. Neidleman, M. Montano, L. Ständker, A. Röcker, M. Cavrois, J. Rosen, K. Marson, J.F. Smith, C.D. Pilcher, F. Gagsteiger, O. Sakk, M. O'Rand, P. V. Lishko, F. Kirchhoff, J. Münch, W.C. Greene, Semen amyloids participate in spermatozoa selection and clearance, ELife. 6 (2017) 1–22.

[20] N. Egge, A. Muthusubramanian, G.A. Cornwall, Amyloid properties of the mouse egg zona pellucida, PLoS ONE. 10 (2015) 1–19.

[21] A. Hewetson, H.Q. Do, C. Myers, A. Muthusubramanian, R.B. Sutton, B.J. Wylie, G.A. Cornwall, Functional Amyloids in Reproduction, Biomolecules. 7 (2017) 46. https://doi.org/10.3390/biom7030046.

[22] H. Jang, F.T. Arce, M. Mustata, S. Ramachandran, R. Capone, R. Nussinov, R. Lal, Antimicrobial Protegrin-1 Forms Amyloid-Like Fibrils with Rapid Kinetics Suggesting a Functional Link, Biophys. J. 100 (2011) 1775–1783. https://doi.org/10.1016/j.bpj.2011.01.072.

[23] S. Liu, H. Liu, A. Johnston, S. Hanna-Addams, E. Reynoso, Y. Xiang, Z. Wang, MLKL forms disulfide bond-dependent amyloid-like polymers to induce necroptosis, Proc. Natl. Acad. Sci. 114 (2017) E7450–E7459. https://doi.org/10.1073/pnas.1707531114.

[24] S. Saad, G. Cereghetti, Y. Feng, P. Picotti, M. Peter, R. Dechant, Reversible protein aggregation is a protective mechanism to ensure cell cycle restart after stress, Nat. Cell Biol. 19 (2017) 1202–1213. https://doi.org/10.1038/ncb3600.

[25] T.E. Audas, D.E. Audas, M.D. Jacob, J.J.D. Ho, M. Khacho, M. Wang, J.K. Perera, C. Gardiner, C.A. Bennett, T. Head, O.N. Kryvenko, M. Jorda, S. Daunert, A. Malhotra, L. Trinkle-Mulcahy, M.L. Gonzalgo, S. Lee, Adaptation to Stressors by Systemic Protein Amyloidogenesis, Dev. Cell. 39 (2016) 155–168. https://doi.org/10.1016/j.devcel.2016.09.002.

[26] N. Van Gerven, R.D. Klein, S.J. Hultgren, H. Remaut, Bacterial amyloid formation: Structural insights into curli biogensis, Trends Microbiol. 23 (2015) 693–706. https://doi.org/10.1016/j.tim.2015.07.010.

[27] P.N. Lipke, M.C. Garcia, D. Alsteens, C.B. Ramsook, S.A. Klotz, Y.F. Dufr??ne, Strengthening relationships: Amyloids create adhesion nanodomains in yeasts, Trends Microbiol. 20 (2012) 59–65. https://doi.org/10.1016/j.tim.2011.10.002.

[28] S. Alberti, R. Halfmann, O. King, A. Kapila, S. Lindquist, A Systematic Survey Identifies Prions and Illuminates Sequence Features of Prionogenic Proteins, Cell. 137 (2009) 146–158. https://doi.org/10.1016/j.cell.2009.02.044.

[29] A. Majumdar, W.C. Cesario, E. White-Grindley, H. Jiang, F. Ren, M.R. Khan, L. Li, E.M.L. Choi, K. Kannan, F. Guo, J. Unruh, B. Slaughter, K. Si, Critical role of amyloid-like oligomers of Drosophila Orb2 in the persistence of memory, Cell. 148 (2012) 515–529. https://doi.org/10.1016/j.cell.2012.01.004.

[30] M.P. Jackson, E.W. Hewitt, Why are functional amyloids non-toxic in humans?, Biomolecules. 7 (2017). https://doi.org/10.3390/biom7040071.

[31] E. Stroo, M. Koopman, E.A.A. Nollen, A. Mata-Cabana, Cellular Regulation of Amyloid Formation in Aging and Disease, Front. Neurosci. 11 (2017). https://doi.org/10.3389/fnins.2017.00064.

[32] D.M. Mitrea, M. Mittasch, B.F. Gomes, I.A. Klein, M.A. Murcko, Modulating biomolecular condensates: a novel approach to drug discovery, Nat. Rev. Drug Discov. 21 (2022) 841–862. https://doi.org/10.1038/s41573-022-00505-4.

[33] I.A. Sawyer, D. Sturgill, M. Dundr, Membraneless nuclear organelles and the search for phases within phases, Wiley Interdiscip. Rev. RNA. 10 (2019) e1514. https://doi.org/10.1002/wrna.1514.

[34] Y. Shin, C.P. Brangwynne, Liquid phase condensation in cell physiology and disease, Science (80-.). 357 (2017) eaaf4382. https://doi.org/10.1126/science.aaf4382.

[35] S.F. Banani, H.O. Lee, A.A. Hyman, M.K. Rosen, Biomolecular condensates: organizers of cellular biochemistry, Nat. Rev. Mol. Cell Biol. 18 (2017) 285–298. https://doi.org/10.1038/nrm.2017.7.

[36] S. Alberti, S. Carra, Quality Control of Membraneless Organelles, J. Mol. Biol. 430 (2018) 4711–4729. https://doi.org/10.1016/j.jmb.2018.05.013.

[37] S. Boeynaems, S. Alberti, N.L. Fawzi, T. Mittag, M. Polymenidou, F. Rousseau, J. Schymkowitz, J. Shorter, B. Wolozin, L. Van Den Bosch, P. Tompa, M. Fuxreiter, Protein Phase Separation: A New Phase in Cell Biology, Trends Cell Biol. 28 (2018) 420–435. https://doi.org/10.1016/j.tcb.2018.02.004.

[38] M. Dundr, T. Misteli, Biogenesis of Nuclear Bodies, Cold Spring Harb. Perspect. Biol. 2 (2010) a000711–a000711. https://doi.org/10.1101/cshperspect.a000711.

[39] Y. Jo, Y. Jung, Interplay between intrinsically disordered proteins inside membraneless protein liquid droplets, Chem. Sci. 11 (2020) 1269–1275. https://doi.org/10.1039/C9SC03191J.

[40] D.S.W. Protter, R. Parker, Principles and Properties of Stress Granules, Trends Cell Biol. 26 (2016) 668–679. https://doi.org/10.1016/j.tcb.2016.05.004.

[41] C.P. Brangwynne, C.R. Eckmann, D.S. Courson, A. Rybarska, C. Hoege, J. Gharakhani, F. Julicher, A.A. Hyman, Germline P Granules Are Liquid Droplets That Localize by Controlled Dissolution/Condensation, Science (80-.). 324 (2009) 1729–1732. https://doi.org/10.1126/science.1172046.

[42] S. Alberti, A.A. Hyman, Biomolecular condensates at the nexus of cellular stress, protein aggregation disease and ageing, Nat. Rev. Mol. Cell Biol. 22 (2021) 196–213. https://doi.org/10.1038/s41580-020-00326-6.

[43] S.F. Banani, H.O. Lee, A.A. Hyman, M.K. Rosen, Biomolecular condensates: organizers of cellular biochemistry, Nat. Rev. Mol. Cell Biol. 18 (2017) 285–298. https://doi.org/10.1038/nrm.2017.7.

[44] M.M. Babu, The contribution of intrinsically disordered regions to protein function, cellular complexity, and human disease, Biochem. Soc. Trans. 44 (2016) 1185–1200. https://doi.org/10.1042/BST20160172.

[45] M. Belli, M. Ramazzotti, F. Chiti, Prediction of amyloid aggregation in vivo, EMBO Rep. 12 (2011) 657–663. https://doi.org/10.1038/embor.2011.116.

[46] A.B. Ahmed, A. V. Kajava, Breaking the amyloidogenicity code: Methods to predict amyloids from amino acid sequence, FEBS Lett. 587 (2013) 1089–1095. https://doi.org/10.1016/j.febslet.2012.12.006.

[47] T.M. Franzmann, S. Alberti, Prion-like low-complexity sequences: Key regulators of protein solubility and phase behavior, J. Biol. Chem. 294 (2019) 7128–7136. https://doi.org/10.1074/jbc.TM118.001190.

[48] M.L. Sprunger, M.E. Jackrel, Prion-Like Proteins in Phase Separation and Their Link to Disease, Biomolecules. 11 (2021) 1014. https://doi.org/10.3390/biom11071014.

[49] D.S. Vazquez, P.L. Toledo, A.R. Gianotti, M.R. Ermácora, Protein conformation and biomolecular condensates, Curr. Res. Struct. Biol. 4 (2022) 285–307. https://doi.org/10.1016/j.crstbi.2022.09.004.

[50] J. Li, M. Zhang, W. Ma, B. Yang, H. Lu, F. Zhou, L. Zhang, Post-translational modifications in liquid-liquid phase separation: a comprehensive review, Mol. Biomed. 3 (2022) 13. https://doi.org/10.1186/s43556-022-00075-2.

[51] H.-X. Zhou, X. Pang, Electrostatic Interactions in Protein Structure, Folding, Binding, and Condensation, Chem. Rev. 118 (2018) 1691–1741. https://doi.org/10.1021/acs.chemrev.7b00305.

[52] A. Cochard, M. Garcia-Jove Navarro, L. Piroska, S. Kashida, M. Kress, D. Weil, Z. Gueroui, RNA at the surface of phase-separated condensates impacts their size and number, Biophys. J. 121 (2022) 1675–1690. https://doi.org/10.1016/j.bpj.2022.03.032.

[53] A.A.M. André, E. Spruijt, Liquid–Liquid Phase Separation in Crowded Environments, Int. J. Mol. Sci. 21 (2020) 5908. https://doi.org/10.3390/ijms21165908.

[54] J. Hochmair, C. Exner, M. Franck, A. Dominguez-Baquero, L. Diez, H. Brognaro, M.L. Kraushar, T. Mielke, H. Radbruch, S. Kaniyappan, S. Falke, E. Mandelkow, C. Betzel, S. Wegmann, Molecular crowding and RNA synergize to promote phase separation, microtubule interaction, and seeding of Tau condensates, EMBO J. 41 (2022). https://doi.org/10.15252/embj.2021108882.

[55] G. Vecchi, P. Sormanni, B. Mannini, A. Vandelli, G.G. Tartaglia, C.M. Dobson, F.U. Hartl, M. Vendruscolo, Proteome-wide observation of the phenomenon of life on the edge of solubility, Proc. Natl. Acad. Sci. (2019) 201910444. https://doi.org/10.1073/pnas.1910444117.

[56] M. Ramaswami, J.P. Taylor, R. Parker, Altered Ribostasis: RNA-Protein Granules in Degenerative Disorders, Cell. 154 (2013) 727–736. https://doi.org/10.1016/j.cell.2013.07.038.

[57] A. Aulas, C. Vande Velde, Alterations in stress granule dynamics driven by TDP-43 and FUS: a link to pathological inclusions in ALS?, Front. Cell. Neurosci. 9 (2015). https://doi.org/10.3389/fncel.2015.00423.

[58] E. Bentmann, C. Haass, D. Dormann, Stress granules in neurodegeneration - lessons learnt from TAR DNA binding protein of 43 kDa and fused in sarcoma, FEBS J. 280 (2013) 4348–4370. https://doi.org/10.1111/febs.12287.

[59] C.M. Dewey, B. Cenik, C.F. Sephton, B.A. Johnson, J. Herz, G. Yu, TDP-43 aggregation in neurodegeneration: Are stress granules the key?, Brain Res. 1462 (2012) 16–25. https://doi.org/10.1016/j.brainres.2012.02.032.

[60] Z. Monahan, F. Shewmaker, U.B. Pandey, Stress granules at the intersection of autophagy and ALS, Brain Res. 1649 (2016) 189–200. https://doi.org/10.1016/j.brainres.2016.05.022.

[61] B. Wolozin, Regulated protein aggregation: stress granules and neurodegeneration, Mol. Neurodegener. 7 (2012) 56. https://doi.org/10.1186/1750-1326-7-56.

[62] S. Alberti, D. Mateju, L. Mediani, S. Carra, Granulostasis: Protein Quality Control of RNP Granules, Front. Mol. Neurosci. 10 (2017). https://doi.org/10.3389/fnmol.2017.00084.

[63] S. Alberti, A.A. Hyman, Are aberrant phase transitions a driver of cellular aging? BioEssays. 38 (2016) 959–968. https://doi.org/10.1002/bies.201600042.

[64] P. Anderson, N. Kedersha, P. Ivanov, Stress granules, P-bodies and cancer, Biochim. Biophys. Acta - Gene Regul. Mech. 1849 (2015) 861–870. https://doi.org/10.1016/j.bbagrm.2014.11.009.

[65] A. Patel, H.O. Lee, L. Jawerth, S. Maharana, M. Jahnel, M.Y. Hein, S. Stoynov, J. Mahamid, S. Saha, T.M. Franzmann, A. Pozniakovski, I. Poser, N. Maghelli, L.A. Royer, M. Weigert, E.W. Myers, S. Grill, D. Drechsel, A.A. Hyman, S. Alberti, A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation, Cell. 162 (2015) 1066–1077. https://doi.org/10.1016/j.cell.2015.07.047.

[66] G.L. Dignon, R.B. Best, J. Mittal, Biomolecular Phase Separation: From Molecular Driving Forces to Macroscopic Properties, Annu. Rev. Phys. Chem. 71 (2020) 53–75. https://doi.org/10.1146/annurev-physchem-071819-113553.

[67] Y. Lin, D.S.W. Protter, M.K. Rosen, R. Parker, Formation and Maturation of Phase-Separated Liquid Droplets by RNA-Binding Proteins, Mol. Cell. 60 (2015) 208–219. https://doi.org/10.1016/j.molcel.2015.08.018.

[68] D. Botstein, G.R. Fink, Yeast: An Experimental Organism for 21st Century Biology, Genetics. 189 (2011) 695–704. https://doi.org/10.1534/genetics.111.130765.

[69] V. Franssens, T. Bynens, J. Van Den Brande, K. Vandermeeren, M. Verduyckt, J. Winderickx, The benefits of humanized yeast models to study Parkinson's disease, Oxid. Med. Cell. Longev. (2013). https://doi.org/10.1155/2013/760629.

[70] J.M. Laurent, J.H. Young, A.H. Kachroo, E.M. Marcotte, Efforts to make and apply humanized yeast, Brief. Funct. Genomics. 15 (2016) 155–163. https://doi.org/10.1093/bfgp/elv041.

[71] J. Winderickx, C. Delay, A. De Vos, H. Klinger, K. Pellens, T. Vanhelmont, F. Van Leuven, P. Zabrocki, Protein folding diseases and neurodegeneration: Lessons learned from yeast, Biochim. Biophys. Acta - Mol. Cell Res. 1783 (2008) 1381–1395. https://doi.org/10.1016/j.bbamcr.2008.01.020.

[72] V. Khurana, S. Lindquist, Modelling neurodegeneration in *Saccharomyces cerevisiae*: why cook with baker's yeast?, Nat. Rev. Neurosci. 11 (2010) 436–449. https://doi.org/10.1038/nrn2809.

[73] F. Foury, Human genetic diseases: a cross-talk between man and yeast, Gene. 195 (1997) 1–10. https://doi.org/10.1016/S0378-1119(97)00140-6.

[74] D.E. Bassett, M.S. Boguski, P. Hieter, Yeast genes and human disease, Nature. 379 (1996) 589–590. https://doi.org/10.1038/379589a0.

[75] W.H. Mager, J. Winderickx, Yeast as a model for medical and medicinal research, Trends Pharmacol. Sci. 26 (2005) 265–273. https://doi.org/10.1016/j.tips.2005.03.004.

[76] S. Krobitsch, S. Lindquist, Aggregation of huntingtin in yeast varies with the length of the polyglutamine expansion and the expression of chaperone proteins, Proc. Natl. Acad. Sci. 97 (2000) 1589–1594. https://doi.org/10.1073/pnas.97.4.1589.

[77] A.B. Meriin, X. Zhang, X. He, G.P. Newnam, Y.O. Chernoff, M.Y. Sherman, Huntingtin toxicity in yeast model depends on polyglutamine aggregation mediated by a prion-like protein Rnq1, J. Cell Biol. 157 (2002) 997–1004. https://doi.org/10.1083/jcb.200112104.

[78] A. Porzoor, I.G. Macreadie, Application of Yeast to Study the Tau and Amyloid-β Abnormalities of Alzheimer's Disease, J. Alzheimer's Dis. 35 (2013) 217–225. https://doi.org/10.3233/JAD-122035.

[79] G. Fruhmann, D. Seynnaeve, J. Zheng, K. Ven, S. Molenberghs, T. Wilms, B. Liu, J. Winderickx, V. Franssens, Yeast buddies helping to unravel the complexity of neurodegenerative disorders, Mech. Ageing Dev. 161 (2017) 288–305. https://doi.org/10.1016/j.mad.2016.05.002.

[80] A. Villar-Piqué, S. Ventura, Protein aggregation propensity is a crucial determinant of intracellular inclusion formation and quality control degradation, Biochim. Biophys. Acta - Mol. Cell Res. 1833 (2013) 2714–2724. https://doi.org/10.1016/j.bbamcr.2013.06.023.

[81] M. Verduyckt, H. Vignaud, T. Bynens, J. Van den Brande, V. Franssens, C. Cullin, J. Winderickx, Yeast as a Model for Alzheimer's Disease: Latest Studies and Advanced Strategies, in: 2016: pp. 197–215. https://doi.org/10.1007/978-1-4939-2627-5_11.

[82] D. Petroi, B. Popova, N. Taheri-Talesh, S. Irniger, H. Shahpasandzadeh, M. Zweckstetter, T.F. Outeiro, G.H. Braus, Aggregate Clearance of α-Synuclein in

Saccharomyces cerevisiae Depends More on Autophagosome and Vacuole Function Than on the Proteasome, J. Biol. Chem. 287 (2012) 27567–27579. https://doi.org/10.1074/jbc.M112.361865.

[83] T.F. Outeiro, Yeast Cells Provide Insight into Alpha-Synuclein Biology and Pathobiology, Science (80-.). 302 (2003) 1772–1775. https://doi.org/10.1126/science.1090439.

[84] I.C. Brás, B. Popova, G.H. Braus, T.F. Outeiro, Yeast-Based Screens to Target Alpha-Synuclein Toxicity, in: 2019: pp. 145–156. https://doi.org/10.1007/978-1-4939-9124-2_12.

[85] E.L. Bastow, C.W. Gourlay, M.F. Tuite, Using yeast models to probe the molecular basis of amyotrophic lateral sclerosis, Biochem. Soc. Trans. 39 (2011) 1482–1487. https://doi.org/10.1042/BST0391482.

[86] P. Faller, C. Hureau, O. Berthoumieu, Role of metal ions in the self-assembly of the Alzheimer's amyloid-β peptide, Inorg. Chem. 52 (2013) 12193–12206. https://doi.org/10.1021/ic4003059.

[87] C.A. Ross, M.A. Poirier, Protein aggregation and neurodegenerative disease, Nat. Med. 10 (2004) S10. https://doi.org/10.1038/nm1066.

[88] R. Halfmann, S. Lindquist, Screening for Amyloid Aggregation by Semi-Denaturing Detergent-Agarose Gel Electrophoresis, J. Vis. Exp. (2008). https://doi.org/10.3791/838.

[89] A.T.N. Tebbenkamp, D.R. Borchelt, Protein Aggregate Characterization in Models of Neurodegenerative Disease, in: 2009: pp. 85–91. https://doi.org/10.1007/978-1-59745-562-6_6.

[90] B. DEMEULE, R. GURNY, T. ARVINTE, Detection and characterization of protein aggregates by fluorescence microscopy, Int. J. Pharm. 329 (2007) 37–45. https://doi.org/10.1016/j.ijpharm.2006.08.024.

[91] M. Lindgren, P. Hammarström, Amyloid oligomers: spectroscopic characterization of amyloidogenic protein states, FEBS J. 277 (2010) 1380–1388. https://doi.org/10.1111/j.1742-4658.2010.07571.x.

[92] M. Morell, N.S. de Groot, J. Vendrell, F.X. Avilés, S. Ventura, Linking amyloid protein aggregation and yeast survival, Mol. Biosyst. 7 (2011) 1121. https://doi.org/10.1039/c0mb00297f.

[93] G. Invernizzi, E. Papaleo, R. Sabate, S. Ventura, Protein aggregation: Mechanisms and functional consequences, Int. J. Biochem. Cell Biol. 44 (2012) 1541–1554. https://doi.org/10.1016/j.biocel.2012.05.023.

[94] C.M. Dobson, The structural basis of protein folding and its links with human disease, Philos. Trans. R. Soc. London. Ser. B Biol. Sci. 356 (2001) 133–145. https://doi.org/10.1098/rstb.2000.0758.

[95] G. Cereghetti, S. Saad, R. Dechant, M. Peter, Reversible, functional amyloids: towards an understanding of their regulation in yeast and humans, Cell Cycle. 17 (2018) 1545–1558. https://doi.org/10.1080/15384101.2018.1480220.

[96] J.B. Woodruff, A.A. Hyman, E. Boke, Organization and Function of Non-dynamic Biomolecular Condensates, Trends Biochem. Sci. 43 (2018) 81–94. https://doi.org/10.1016/j.tibs.2017.11.005.

[97] E.W.J. Wallace, J.L. Kear-Scott, E. V. Pilipenko, M.H. Schwartz, P.R. Laskowski, A.E. Rojek, C.D. Katanski, J.A. Riback, M.F. Dion, A.M. Franks, E.M. Airoldi, T. Pan, B.A. Budnik, D.A. Drummond, Reversible, Specific, Active Aggregates of Endogenous Proteins Assemble upon Heat Stress, Cell. 162 (2015) 1286–1298. https://doi.org/10.1016/j.cell.2015.08.041.

[98] R. Narayanaswamy, M. Levy, M. Tsechansky, G.M. Stovall, J.D. O'Connell, J. Mirrielees, A.D. Ellington, E.M. Marcotte, Widespread reorganization of metabolic enzymes into reversible assemblies upon nutrient starvation, Proc. Natl. Acad. Sci. 106 (2009) 10147–10152. https://doi.org/10.1073/pnas.0812771106.

[99] C.K. Park, N.C. Horton, Structures, functions, and mechanisms of filament forming enzymes: a renaissance of enzyme filamentation, Biophys. Rev. 11 (2019) 927–994. https://doi.org/10.1007/s12551-019-00602-6.

[100] A. Molliex, J. Temirov, J. Lee, M. Coughlin, A.P. Kanagaraj, H.J. Kim, T. Mittag, J.P. Taylor, Phase Separation by Low Complexity Domains Promotes Stress Granule Assembly and Drives Pathological Fibrillization, Cell. 163 (2015) 123–133. https://doi.org/10.1016/j.cell.2015.09.015.

[101] K.E. Handwerger, J.A. Cordero, J.G. Gall, Cajal Bodies, Nucleoli, and Speckles in the Xenopus Oocyte Nucleus Have a Low-Density, Sponge-like Structure, Mol. Biol. Cell. 16 (2005) 202–211. https://doi.org/10.1091/mbc.e04-08-0742.

[102] V. Franssens, T. Bynens, J. Van den Brande, K. Vandermeeren, M. Verduyckt, J. Winderickx, The Benefits of Humanized Yeast Models to Study Parkinson's Disease, Oxid. Med. Cell. Longev. 2013 (2013) 1–9. https://doi.org/10.1155/2013/760629.

[103] S. Tenreiro, M.M. Reimão-Pinto, P. Antas, J. Rino, D. Wawrzycka, D. Macedo, R. Rosado-Ramos, T. Amen, M. Waiss, F. Magalhães, A. Gomes, C.N. Santos, D. Kaganovich, T.F. Outeiro, Phosphorylation Modulates Clearance of Alpha-Synuclein Inclusions in a Yeast Model of Parkinson's Disease, PLoS Genet. 10 (2014) e1004302. https://doi.org/10.1371/journal.pgen.1004302.

[104] B. Popova, A. Kleinknecht, P. Arendarski, J. Mischke, D. Wang, G.H. Braus, Sumoylation Protects Against β-Synuclein Toxicity in Yeast, Front. Mol. Neurosci. 11 (2018). https://doi.org/10.3389/fnmol.2018.00094.

[105] A. De Vos, J. Anandhakumar, J. Van den Brande, M. Verduyckt, V. Franssens, J. Winderickx, E. Swinnen, Yeast as a Model System to Study Tau Biology, Int. J. Alzheimers. Dis. 2011 (2011) 1–16. https://doi.org/10.4061/2011/428970.

[106] G. Ciaccioli, A. Martins, C. Rodrigues, H. Vieira, P. Calado, A Powerful Yeast Model to Investigate the Synergistic Interaction of α-Synuclein and Tau in Neurodegeneration, PLoS One. 8 (2013) e55848. https://doi.org/10.1371/journal.pone.0055848.

[107] X. Chen, D. Petranovic, Amyloid-β peptide-induced cytotoxicity and mitochondrial dysfunction in yeast, FEMS Yeast Res. 15 (2015) fov061. https://doi.org/10.1093/femsyr/fov061.

[108] S. Hofer, K. Kainz, A. Zimmermann, M.A. Bauer, T. Pendl, M. Poglitsch, F. Madeo, D. Carmona-Gutierrez, Studying Huntington's Disease in Yeast: From Mechanisms to Pharmacological Approaches, Front. Mol. Neurosci. 11 (2018). https://doi.org/10.3389/fnmol.2018.00318.

[109] R.P. Mason, F. Giorgini, Modeling Huntington disease in yeast: Perspectives and future directions, Prion. 5 (2011) 269–276. https://doi.org/10.4161/pri.5.4.18005.

[110] M. Armakola, M.P. Hart, A.D. Gitler, TDP-43 toxicity in yeast, Methods. 53 (2011) 238–245. https://doi.org/10.1016/j.ymeth.2010.11.006.

[111] B.S. Johnson, J.M. McCaffery, S. Lindquist, A.D. Gitler, A yeast TDP-43 proteinopathy model: Exploring the molecular determinants of TDP-43 aggregation and cellular toxicity, Proc. Natl. Acad. Sci. 105 (2008) 6439–6444. https://doi.org/10.1073/pnas.0802082105.

[112] Z.T. Monahan, S.N. Rhoads, D.S. Yee, F.P. Shewmaker, Yeast Models of Prion-Like Proteins That Cause Amyotrophic Lateral Sclerosis Reveal Pathogenic Mechanisms, Front. Mol. Neurosci. 11 (2018). https://doi.org/10.3389/fnmol.2018.00453.

[113] L. Bertram, The genetic epidemiology of neurodegenerative disease, J. Clin. Invest. 115 (2005) 1449–1457. https://doi.org/10.1172/JCI24761.

[114] J. van Galen, N.K. Olrichs, A. Schouten, R.L. Serrano, E.N.M. Nolte-'t Hoen, R. Eerland, D. Kaloyanova, P. Gros, J.B. Helms, Interaction of GAPR-1 with lipid bilayers is regulated by alternative homodimerization, Biochim. Biophys. Acta - Biomembr. 1818 (2012) 2175–2183. https://doi.org/10.1016/j.bbamem.2012.04.016.

[115] G.M. Gibbs, K. Roelants, M.K. O'Bryan, The CAP Superfamily: Cysteine-Rich Secretory Proteins, Antigen 5, and Pathogenesis-Related 1 Proteins—Roles in Reproduction, Cancer, and Immune Defense, Endocr. Rev. 29 (2008) 865–897. https://doi.org/10.1210/er.2008-0032.

[116] N. K. Olrichs, J. Bernd Helms, Novel insights into the function of the conserved domain of the CAP superfamily of proteins, AIMS Biophys. 3 (2016) 232–246. https://doi.org/10.3934/biophy.2016.2.232.

[117] Y. Li, Y. Zhao, M. Su, K. Glover, S. Chakravarthy, C.L. Colbert, B. Levine, S.C. Sinha, Structural insights into the interaction of the conserved mammalian proteins GAPR-1 and Beclin 1, a key autophagy protein, Acta Crystallogr. Sect. D Struct. Biol. 73 (2017) 775–792. https://doi.org/10.1107/S2059798317011822.

[118] A. Abraham, D.E. Chandler, Tracing the Evolutionary History of the CAP Superfamily of Proteins Using Amino Acid Sequence Homology and Conservation of Splice Sites, J. Mol. Evol. 85 (2017) 137–157. https://doi.org/10.1007/s00239-017-9813-9.

[119] H.B. Eberle, R.L. Serrano, J. Füllekrug, A. Schlosser, W.D. Lehmann, F. Lottspeich, D. Kaloyanova, F.T. Wieland, J.B. Helms, Identification and characterization of a novel human plant pathogenesis-related protein that localizes to lipid-enriched microdomains in the Golgi complex., J. Cell Sci. 115 (2002) 827–38. http://www.ncbi.nlm.nih.gov/pubmed/11865038.

[120] N.K. Olrichs, A.K. Mahalka, D. Kaloyanova, P.K. Kinnunen, J. Bernd Helms, Golgi-Associated plant Pathogenesis Related protein 1 (GAPR-1) forms amyloid-like fibrils by interaction with acidic phospholipids and inhibits Aβ aggregation, Amyloid. 21 (2014) 88–96. https://doi.org/10.3109/13506129.2014.882304.

[121] M. Stefani, S. Rigacci, Protein Folding and Aggregation into Amyloid: The Interference by Natural Phenolic Compounds, Int. J. Mol. Sci. 14 (2013) 12411–12457. https://doi.org/10.3390/ijms140612411.

[122] Z.L. Almeida, R.M.M. Brito, Structure and Aggregation Mechanisms in Amyloids, Molecules. 25 (2020) 1195. https://doi.org/10.3390/molecules25051195.

[123] S. Giorgetti, C. Greco, P. Tortora, F. Aprile, Targeting Amyloid Aggregation: An Overview of Strategies and Mechanisms, Int. J. Mol. Sci. 19 (2018) 2677. https://doi.org/10.3390/ijms19092677.

[124] J. Sheng, N.K. Olrichs, B.M. Gadella, D. V. Kaloyanova, J.B. Helms, Regulation of Functional Protein Aggregation by Multiple Factors: Implications for the Amyloidogenic Behavior of the CAP Superfamily Proteins, Int. J. Mol. Sci. 21 (2020) 6530. https://doi.org/10.3390/ijms21186530.

[125] N. Sirati, B. Popova, M.R. Molenaar, I.C. Verhoek, G.H. Braus, D. V. Kaloyanova, J.B. Helms, Dynamic and Reversible Aggregation of the Human CAP Superfamily Member GAPR-1 in Protein Inclusions in *Saccharomyces cerevisiae*, J. Mol. Biol. 433 (2021) 167162. https://doi.org/10.1016/j.jmb.2021.167162.

[126] J. Sheng, N.K. Olrichs, W.J. Geerts, D. V. Kaloyanova, J.B. Helms, Metal ions and redox balance regulate distinct amyloid-like aggregation pathways of GAPR-1, Sci. Rep. 9 (2019) 15048. https://doi.org/10.1038/s41598-019-51232-7.

[127] J. Sheng, N.K. Olrichs, W.J. Geerts, X. Li, A.U. Rehman, B.M. Gadella, D. V. Kaloyanova, J.B. Helms, Zinc binding regulates amyloid-like aggregation of GAPR-1, Biosci. Rep. 39 (2019) 15048. https://doi.org/10.1042/BSR20182345.

[128] S. Shoji-Kawata, R. Sumpter, M. Leveno, G.R. Campbell, Z. Zou, L. Kinch, A.D. Wilkins, Q. Sun, K. Pallauf, D. MacDuff, C. Huerta, H.W. Virgin, J. Bernd Helms, R. Eerland, S.A. Tooze, R. Xavier, D.J. Lenschow, A. Yamamoto, D. King, O. Lichtarge, N. V. Grishin, S.A. Spector, D. V. Kaloyanova, B. Levine, Identification of a candidate therapeutic autophagy-inducing peptide, Nature. 494 (2013) 201–206. https://doi.org/10.1038/nature11866.

[129] S. Tran, W.D. Fairlie, E.F. Lee, BECLIN1: Protein Structure, Function and Regulation, Cells. 10 (2021) 1522. https://doi.org/10.3390/cells10061522.

[130] R.C. Russell, Y. Tian, H. Yuan, H.W. Park, Y.-Y. Chang, J. Kim, H. Kim, T.P. Neufeld, A. Dillin, K.-L. Guan, ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase, Nat. Cell Biol. 15 (2013) 741–750. https://doi.org/10.1038/ncb2757.

[131] M.B. Menon, S. Dhamija, Beclin 1 Phosphorylation – at the Center of Autophagy Regulation, Front. Cell Dev. Biol. 6 (2018). https://doi.org/10.3389/fcell.2018.00137.

[132] H.-D. Xu, Z.-H. Qin, Beclin 1, Bcl-2 and Autophagy, in: 2019: pp. 109–126. https://doi.org/10.1007/978-981-15-0602-4_5.

[133] Á.F. Fernández, S. Sebti, Y. Wei, Z. Zou, M. Shi, K.L. McMillan, C. He, T. Ting, Y. Liu, W.-C. Chiang, D.K. Marciano, G.G. Schiattarella, G. Bhagat, O. M.C. Hu, B. Levine, Disruption of the beclin 1–BCL2 autophagy regulatory complex promotes longevity in mice, Nature. 558 (2018) 136–140. https://doi.org/10.1038/s41586-018-0162-7

Abstract

Many proteins that can assemble into higher order structures termed amyloids can also concentrate into cytoplasmic inclusions via liquid−liquid phase separation. Here, we study the assembly of human Golgi- associated plant Pathogenesis Related protein 1 (GAPR-1), an amyloidogenic protein of the Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 proteins (CAP) protein superfamily, into cytosolic inclusions in *Saccharomyces cerevisiae*. Overexpression of GAPR-1-GFP results in the formation GAPR-1 oligomers and fluorescent inclusions in yeast cytosol. These cytosolic inclusions are dynamic and reversible organelles that gradually increase during time of overexpression and decrease after promoter shut-off. Inclusion formation is, however, a regulated process that is influenced by factors other than protein expression levels. We identified N-myristoylation of GAPR-1 as an important determinant at early stages of inclusion formation. In addition, mutations in the conserved metal-binding site (His54 and His103) enhanced inclusion formation, suggesting that these residues prevent uncontrolled protein sequestration. In agreement with this, we find that addition of Zn^{2+} metal ions enhance inclusion formation. Furthermore, Zn^{2+} reduces GAPR-1 protein degradation, which indicates stabilization of GAPR-1 in inclusions. We propose that the properties underlying both the amyloidogenic properties and the reversible sequestration of GAPR-1 into inclusions play a role in the biological function of GAPR-1 and other CAP family members.

Keywords: GLIPR-2; Condensates; Amyloids; Zinc; Myristoylation

Introduction

The CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 proteins) superfamily of proteins are widely spread in a variety of species and different kingdoms of life [1]. The highly conserved CAP domain characterises the CAP family members and forms a distinct core tertiary structure with a conserved metal-binding site. Golgi-Associated Plant Pathogenesis Related protein-1 (GAPR-1) is a unique member of the CAP superfamily, which branched off early in evolution and might well be the first mammalian CAP protein [2,3]. GAPR-1 is the only mammalian CAP protein that lacks a signal sequence and localises to the Golgi complex [4]. By retaining Beclin 1 at the Golgi complex, GAPR-1 functions as a negative regulator of autophagy [5–7]. GAPR-1 has a strong tendency to form homodimers and oligomers [7,8]. We showed that GAPR-1 can form amyloid-like fibrils and that this process is induced by the presence of negatively charged lipids or heparin and metal ions [6,9,10]. These results show that different stimuli regulate amyloid-like oligomerisation of GAPR-1 *in vitro,* and we speculated that the amyloidogenic scaffold contributes to the regulation of GAPR-1 function *in vivo* [9–11].

Structured cross β-sheet fibrils, known as amyloids, are highly resistant to degradation and are associated with more than 50 human diseases known as "amyloidoses" [12–14]. Well known examples include neurodegenerative diseases like Alzheimer's and Parkinson's, where misfolding of Aβ, tau and α-synuclein is associated with progression of the disease [15]. The ability to form amyloid structures is, however, not limited to proteins causing pathology but seems to be a universal property of proteins [16]. Proteins and peptides forming amyloid-like oligomers with beneficial effects are often referred to as functional amyloids. The formation of these oligomers is tightly regulated and is characterised by their reversibility and plasticity [11,13,17–19]. Functional amyloids are implicated in a range of different physiological processes, including compartmentalisation, regulation of protein activity, storage and degradation [20–25].

Recently it has become clear that proteins with amyloidogenic properties can cluster into cytoplasmic inclusions [25–29]. Evidence suggests that liquid-liquid phase separation (LLPS) underlies the formation of these cellular inclusions [30–32]. Although much remains to be elucidated about the driving forces and the nature of these biomolecular condensates, it has become clear that they represent distinct membraneless organelles in cells and that components can reversibly and dynamically associate with these structures. Transition from dynamic LLPS-mediated membraneless organelles to more static ones has been reported for several proteins associated with neurodegenerative disorders such as TDP-43, FUS, α-synuclein, tau and also functional amyloid-related proteins, including elastin, yeast prion-like protein Sup35, Rim4, and

yeast pyruvate kinase Cdc19 [28,31,41,33–40]. These observations suggest a link between the assemblies of membraneless organelles and amyloid-like protein behaviour in the cell.

Saccharomyces cerevisiae has been instrumental in the discovery and characterisation of pathological and functional amyloids [42,43]. Initially, so-called "humanised yeast" model systems were developed to study the oligomerisation properties of proteins involved in human amyloidosis like Aβ, tau and α-synuclein [44–51]. Expression of amyloidogenic proteins in yeast has several features in common such as the partial SDS-resistance of protein aggregates and the formation of inclusions or protein aggregates [40,51,52]. These properties have also been used to verify amyloid-like oligomerisation of sequence-predicted amyloid-prompt proteins in yeast model systems [51,53–55]. *Saccharomyces cerevisiae* does not express GAPR-1 and we use this model system to study human GAPR-1 amyloid-like oligomerisation *in vivo.* We show that GAPR-1 clusters into inclusions in yeast cytosol resembling GAPR-1 amyloid-like oligomerisation *in vitro*. We discuss the potential implications for the regulation of GAPR-1 function in autophagy.

Results

GAPR-1 clusters into fluorescent inclusions in yeast cytosol

To study the oligomerisation process of GAPR-1 *in vivo*, C-terminally tagged GAPR-1 with either GFP (GAPR-1-GFP) or mCherry (GAPR-1-mCherry) was expressed from 2µ plasmid in yeast under the control of the galactose-inducible promoter (*GAL1*). Overexpression of both GAPR-1-tagged variants did not affect yeast growth (Supplementary Fig.1A). Live-cell fluorescence microscopy was performed with GAPR-1-GFP and representative images were taken 3h, 6h and 24h post-induction of protein expression. After 3h, the majority of expressed protein was detected in the cytosol, with ~30% of all fluorescent cells displaying formation of cytosolic inclusions (Fig.1A, B). The number of inclusions significantly increased over time, affecting both the number of inclusion-positive cells (Fig.1B) and the number of inclusions per cell (Fig.1C). At 24h post induction, about 94% of all cells contained multiple inclusions (Fig.1B) with an average of >5 inclusions per cell (Fig.1C). The intracellular distribution of fluorescent inclusions also changed over time from being in close proximity to the plasma membrane at early stages and a more central cellular location at later stages. After 24h, almost 60% of the yeast cells contained more than 6 inclusions per cell (Fig.1C). GAPR-1-GFP and GAPR-1-mCherry fusions displayed similar behaviour in live-cell fluorescence microscopy (Supplementary Fig.1B). To address whether the formation of inclusions is related to the level of protein expression,

we assessed GAPR-1-GFP levels in cell lysates by Western blot analysis using an anti-GAPR-1 antibody. Although we observe a modest increase in expression levels between 3h and 24h (Fig.1D), it seems likely that additional factors affect the observed changes in inclusion formation (Fig.1A-C).

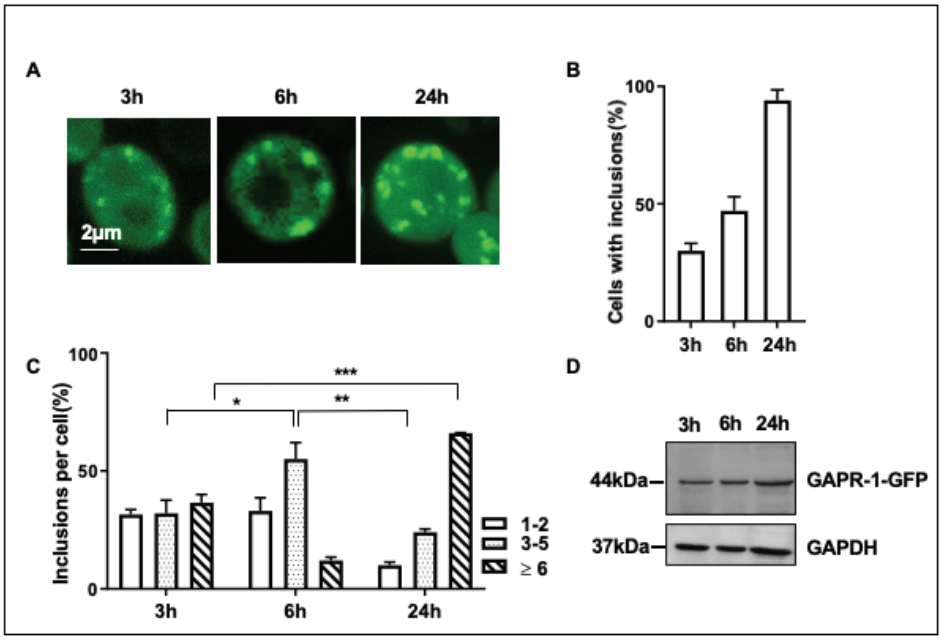


Figure 1. GAPR-1 clusters into fluorescent inclusions. A) Representative live-cell fluorescence microscopy images of yeast cells expressing GAPR-1-GFP from 2µ plasmid (3h, 6h and 24h after induction of protein expression); B) Quantification of cells displaying GAPR-1-GFP inclusions. For each time point, the number of cells displaying cytoplasmic inclusions is quantified as described in Materials and Methods and presented as a percentage of the total number of the cells. Results are expressed as mean ± SD, n=5; C) Quantification of the number of inclusions per cell. The number of inclusions per cell were categorised into three groups (1-2, 3-5 and ≥6 per cell). At least 100 cells were counted per experiment. Results are expressed as mean ± SD, n=3. Significance of differences was calculated with two-tailed unpaired t-test (∗p < 0.05, ∗∗p < 0.01 and ∗∗∗p < 0.001); D) WB analysis of GAPR-1-GFP expressing cells at 3h, 6h and 24h post-induction. Equal amounts of yeast homogenate (20 µg total protein) were

separated by SDS-PAGE and analysed by WB for the presence of GAPR-1-GFP and GAPDH (loading control).

GAPR-1-GFP forms oligomeric structures in yeast

To investigate the oligomeric properties of GAPR-1-GFP after expression in yeast, we performed chemical cross-linking experiments using the amine-to-amine crosslinker BS3. Expression of GAPR-1-GFP was induced for 6h or 24h, and the crosslinker was added for 30 minutes at room temperature after mechanical disruption of the cells. Western blot analysis revealed that at early time points (6h), the majority of overexpressed protein is in a monomeric state (Fig.2A). In contrast, at 24h post-induction, higher-molecular weight species are observed in the BS3-treated sample. A hallmark of amyloid-like oligomerisation *in vivo* is the partial protein resistance to solubilisation by SDS. To determine the amyloid-like characteristics of GAPR-1-GFP oligomerisation in yeast, a protein homogenate was separated into a detergent-soluble and detergent-insoluble fraction using a mild zwitterionic detergent. The detergent-insoluble fraction was then treated with 2% SDS and separated into a SDS-soluble and SDS-insoluble fraction. Western blot analysis showed that GAPR-1-GFP is partially soluble in mild detergent (Fig.2B). After subsequent SDS treatment of the detergent-insoluble fraction, about 50% of GAPR-1-GFP remained insoluble (Fig.2B; Supplementary Fig.2A). Under these conditions, expression of GFP alone did not result in any detergent-insoluble material, even in the presence of only mild detergent (Fig.2B).

To obtain additional evidence for the amyloidogenic behaviour of GAPR-1-GFP, we performed Thioflavin T (ThT) fluorescence imaging of yeast cells. The ThT reagent becomes fluorescent upon intercalation into β-sheet structures of amyloid-like oligomers *in vivo* and in vitro [56,57]. Empty vector p423, GFP and GAPR-1-GFP expression was induced for 24h and after staining of cells with ThT, total fluorescent intensity of cells was measured using 96 well plate reader. As it is shown in Fig.2C, there was a significant increase in total ThT signal in the presence of GAPR-1-GFP comparing with empty vector and GFP. Live-cell microscopy of the ThT stained cells showed ThT labelling of GAPR-1-GFP and GAPR-1-mCherry inclusions (Fig.2D). The ThT signal was no due to spectral overlap with GFP as in the absence of ThT, no ThT-positive inclusions could be observed (Supplementary Fig.2B).

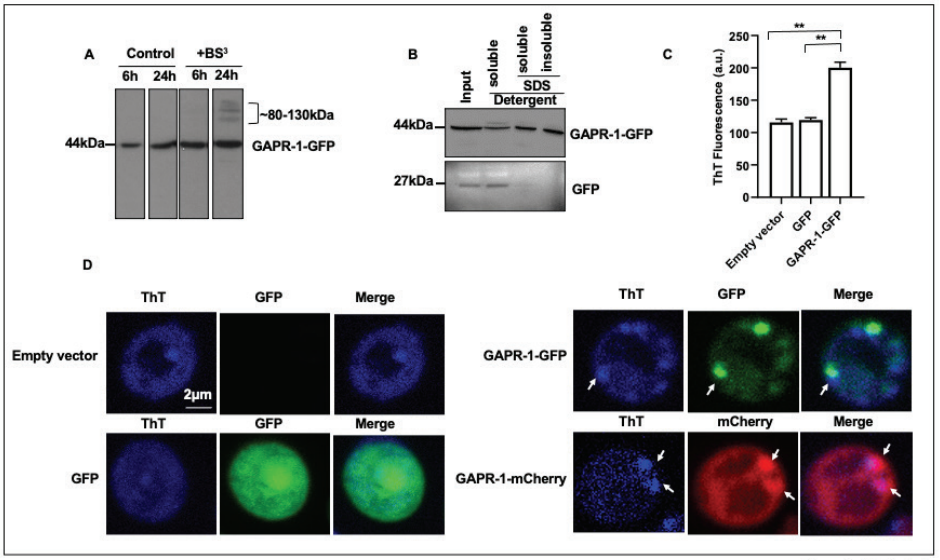


Figure 2. GAPR-1-GFP has oligomeric properties *in vivo*. A) Equal number of GAPR-1-GFP expressing cells were collected 6h and 24h post-induction of protein expression. Yeast homogenates (20 µg protein) were treated without (Control) or with 2.5 mM BS3 (final concentration) for 30 minutes at room temperature. Proteins were separated by SDS-PAGE and analysed by WB using an anti-GAPR-1 antibody. High molecular weight species (12% of total, based on densitometric analysis, see Supplementary Fig.1C) with a mass of 80-130 kDa are indicated; B) GAPR-1-GFP or GFP expression was induced for 24h. Cells (10 OD600) were collected, homogenised and homogenates were prepared using Y-PERTM reagent. After centrifugation, the pellet was treated with 2% SDS and separated into a SDS-soluble and SDS-insoluble fraction as described in Materials and Methods. The same volume (20 µl) of each sample was separated by SDS-PAGE and analysed by WB using an anti-GFP antibody; C) Total fluorescence intensity of Thioflavin T-stained cells after 24h empty vector, GFP and GAPR-1-GFP expression. The results are expressed as the mean ± SEM, n=4. Significance of differences was calculated with Welch t-test, corrected for multiple testing with Benjamini/Hochberg (∗∗$p < 0.01$); D) Representative microscopic images of cells carrying empty vector, GFP, GAPR-1-GFP and GAPR-1-mCherry proteins after 24h expression and stained with 5 µM Thioflavin T.

N-myristoylation of GAPR-1-GFP promotes inclusion formation

Membrane binding of numerous amyloidogenic proteins has been shown to promote amyloid nucleation and subsequent fibril formation *in vivo* and *in vitro* [11,58–61]. The membrane association of GAPR-1-GFP with yeast membranes was investigated by isopycnic density-gradient centrifugation of yeast homogenates obtained by mechanical homogenisation. GAPR-1-GFP was found mainly enriched in the high-density fractions 10-14 (37-41% sucrose density) (Fig.3A). About 20 % of GAPR-1-GFP migrated with the low-density sucrose fractions 1-9 (25-36% sucrose density), suggesting that GAPR-1 is partially associated with membranes. When 1% Triton X-100 was added to the homogenate prior to the flotation, GAPR-1-GFP disappeared from the membrane fractions (1-9) (Fig.3A), supporting the conclusion that GAPR-GFP is partially associated with membranes. To assess the role of N-myristoylation in membrane association of GAPR-1-GFP and in the formation of protein inclusions, a GAPR-1 mutant lacking the N-terminal myristoylation consensus sequence (G2A; Δmyr-GAPR-1-GFP) was constructed by site-directed mutagenesis. Isopycnic density-gradient centrifugation showed that Δmyr-GAPR-1-GFP is found only in the high-density fractions (10-14), both in the presence and absence of 1% TX-100, suggesting that Δmyr-GAPR-1-GFP does not associate with membranes (Fig.3B; Supplementary Fig.2C). The cellular distribution of Δmyr-GAPR-1-GFP was studied in time by live-cell fluorescence microscopy and representative images were taken at 3h, 6h and 24h post-induction (Fig.3C). At early time points (3h and 6h), Δmyr-GAPR-1-GFP remained cytosolic and only after 24h single inclusions were found in about 36% of the cells (Fig.3D). Under these conditions, the protein levels of Δmyr-GAPR-1-GFP are similar to the expression levels of GAPR-1-GFP (Supplementary Fig.2D) and they did not significantly change between the different time points (Fig.3E). These results indicate that the inclusion formation is subject to regulation by myristoylation of GAPR-1. To exclude the possibility that the myr-sequence itself plays a role in the aggregation process, independent of myristoylation, we overexpressed GAPR-1-GFP and Δmyr-GAPR-1-GFP in a yeast strain deficient in N-myristoyl transferase (ΔNMT1). The phenotype of both GAPR-1-GFP and Δmyr-GAPR-1-GFP in ΔNMT1 cells (Supplementary Fig.2E) resembled the phenotype of Δmyr-GAPR-1-GFP in wildtype yeast cells (Fig.3C). These results suggest that i) the myr-sequence itself does not play a role in the aggregation process, and ii) GAPR-1-GFP is myristoylated in wildtype yeast cells.

Interestingly, Δmyr-GAPR-1-GFP had similar amyloid-like characteristics as compared to GAPR-1-GFP based on its ThT staining (Fig.3F, cf. Fig.2D) and SDS-insoluble

characteristics (Fig.3E, cf. Fig.2B and Supplementary Fig.2A). Despite these similar amyloid-like characteristics, Δmyr-GAPR-1-GFP is hampered in the formation of inclusions. Myristoylation likely accelerates the formation of protein inclusions by promoting membranes to function as a seeding platform for the assembly of GAPR-1

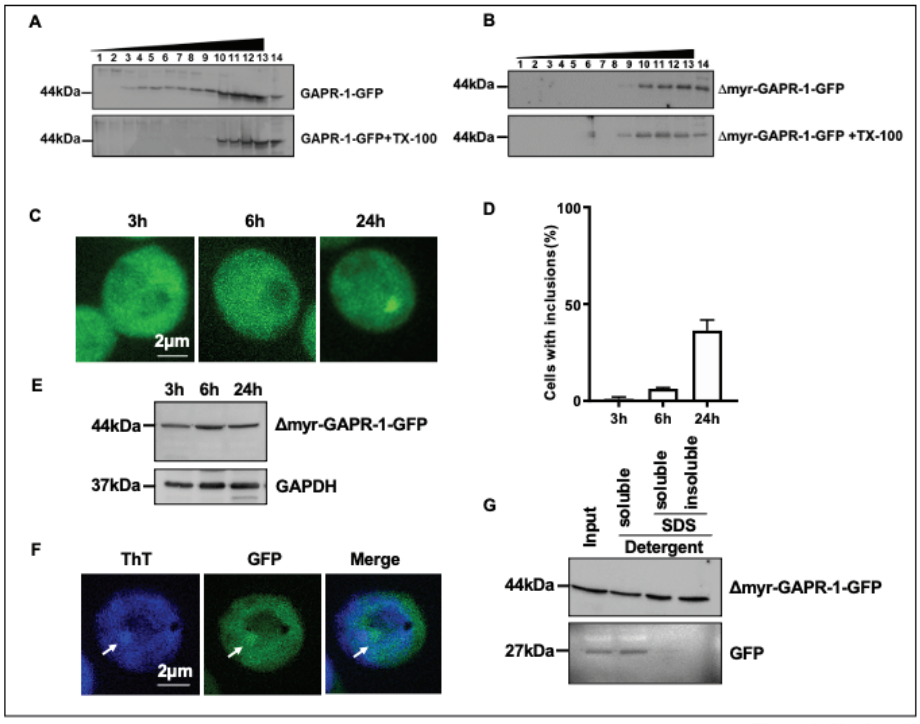


Figure 3, Sirati et al.

into oligomers and protein inclusions.

Figure 3. N-myristoylation of GAPR-1-GFP promotes formation of inclusions.A) GAPR-1-GFP expressing cells (24h) were collected, homogenised and treated without or with 1% TX-100 for 30 minutes on ice, followed by isopycnic density-gradient centrifugation. Fractions were collected from top to bottom and proteins in each fraction were separated by SDS-PAGE and analysed by WB using an anti-GAPR-1 antibody; B) Δmyr-GAPR-1-GFP expressing cells (24h) were collected and analysed by isopycnic density-gradient centrifugation and WB analysis as described for panel A; C) Representative live-cell fluorescence microscopy images of cells after induction of Δmyr-GAPR-1-GFP expression (3h, 6h and 24h); D) Quantification of cells displaying Δmyr-GAPR-1-GFP fluorescent inclusions as described in Materials and Methods. Results are expressed as mean ± SD, n=5; E) Δmyr-GAPR-1-GFP expressing cells were collected at 3h, 6h and 24h post-induction and total homogenates were prepared. Equal amounts of homogenate (20 μg protein) were separated by SDS-PAGE and analysed by WB for the presence of

GAPR-1 and GAPDH (loading control); F) Representative images of cells carrying Δmyr-GAPR-1-GFP protein after 24h expression and stained with 5 μM Thioflavin T; G) Δmyr-GAPR-1-GFP or GFP expression was induced for 24h. Total homogenates were prepared using Y-PERTM reagent. After centrifugation, the pellet was treated with 2% SDS and separated into a SDS-soluble and SDS-insoluble fraction as described in Materials and Methods. The same volume (20 μl) of each sample was separated by SDS-PAGE and analysed by WB using an anti-GFP antibody.

Metal-ions affect the formation of GAPR-1-GFP inclusions

We previously showed that zinc and copper ions induce GAPR-1 amyloid-like aggregation *in vitro* in the presence of heparin [9;10]. Therefore, we assessed the role of zinc in the formation of GAPR-1-GFP inclusions in yeast. Concentrations up to 10 mM $ZnCl_2$ are well tolerated by yeast cells (Supplementary Fig.3A), in agreement with results obtained by others [62]. We conducted live-cell fluorescence microscopy of cells in the absence and presence of 5 mM zinc ions and quantified the number of inclusion forming cells at three time points (3h, 6h and 24h). The percentage of fluorescent inclusions forming cells at 3h post-induction almost doubled in the presence of zinc, reaching about 60% of the cells carrying fluorescent inclusions (Fig.4A). Representative images after 3h post-induction are shown in Figure 4B. The number of inclusions per cell also significantly changed at this early time point and showed a predominance of the "≥ 6 inclusions pool" in comparison to the control cells (Fig.4C). These alterations were not triggered by different protein levels, as shown by Western blot analysis (Fig.4D). Preliminary evidence shows similar effects in the presence of 2 mM Cu^{2+} (Supplementary Fig. 3B,C). The data reveal that GAPR-1-GFP amyloid-like oligomerisation *in vivo* is influenced by zinc, similar to GAPR-1 amyloid aggregation *in vitro* [9].

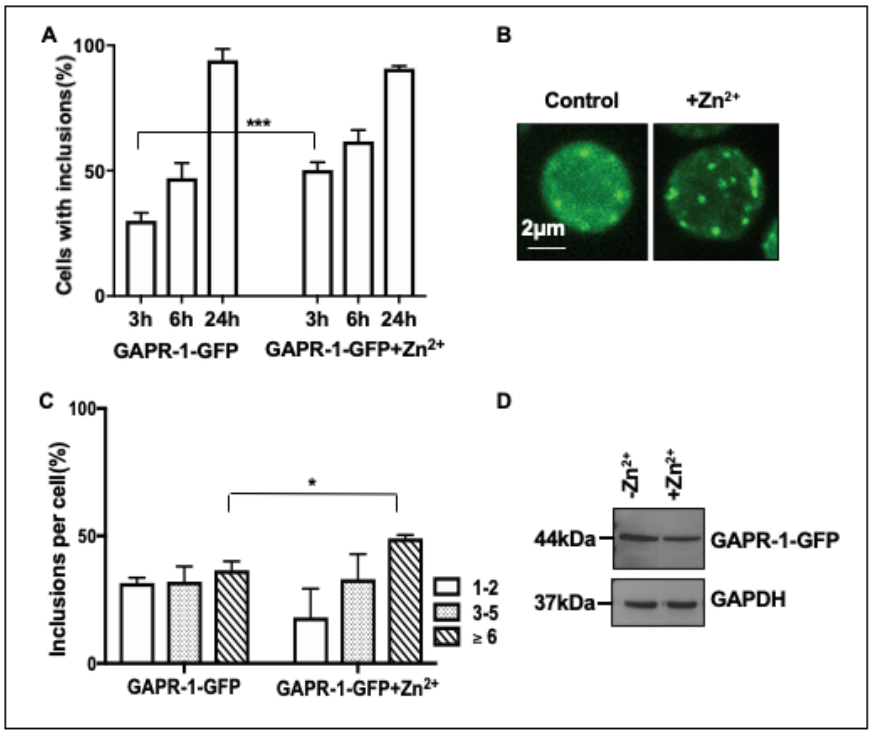


Figure 4. Metal-ions affect the formation of GAPR-1-GFP inclusions. Cells were incubated in the absence or presence of 5mM Zn^{2+} for 3h, 6h, and 24h after induction of GAPR-1-GFP expression A) Quantification of the percentage of cells displaying GAPR-1-GFP inclusions at 3h, 6h and 24h post-induction. For each time point, the number of cells displaying cytoplasmic inclusions is quantified as described in Materials and Methods and presented as percentage of the total number of the cells. The results are expressed as mean ± SD, n=5. Significance of differences was calculated with two-tailed unpaired t-test (***$p < 0.001$); B) Representative live-cell fluorescence microscopy images of cells, 3h after expressing GAPR1-GFP in the absence or presence of 5 mM $ZnCl_2$; C) Number of fluorescence inclusions per cell were quantified as described in Materials and Methods and plotted in three groups (1-2, 3-5 and ≥6 inclusions per cell). The results are expressed as mean ± SD, n=3. Significance of differences was calculated with two-tailed unpaired t-test (*$p < 0.05$); D) WB analysis of homogenates (20 µg total protein) from GAPR-1-GFP expressing cells (3h) in the absence or presence of 5 mM $ZnCl_2$ using an anti-GAPR-1 antibody. Anti-GAPDH antibody served as a loading control.

Zn^{2+} stabilises GAPR-1 protein inclusions

Next, we performed pulse-chase experiments to study the dynamics of GAPR-1 inclusions. To this end, GAPR1-GFP expression was first induced for 24h in galactose-containing medium to generate GAPR-1 inclusions. Subsequently, the cells were shifted to glucose-containing medium to repress the promoter and to decrease the concentration of GAPR-1. Yeast cells were imaged at different time points after promoter shut-off and the percentage of cells with inclusions was determined. After promoter shut-off, both GAPR-1-GFP inclusions (Figs.5A, 5E control) and total GAPR-1 concentrations (Fig.5B) gradually decreased over time, which was accompanied by an increased cytosolic signal (Fig.5A). To investigate the dynamics of GAPR-1 protein inclusions in more detail, Fluorescence Recovery After Photo-bleaching (FRAP) analysis was performed on single fluorescent inclusions. After bleaching, GAPR-1-GFP inclusions were partially recovered in less than 2 minutes, indicating a dynamic association of GAPR-1-GFP with protein inclusions (Fig.5C, top panel). The fluorescence recovery in the presence of zinc ions was slightly faster as compared to the control cells (Fig.5C, bottom panel), suggesting that Zn^{2+} tweaks the equilibrium and stabilises GAPR-1 in inclusions. Indeed, the kinetics of protein degradation was much slower in the presence of Zn^{2+}, resulting in significantly higher GAPR-1-GFP levels at 6h post-shut-off (Fig.5D, left panel). Under these conditions, Zn^{2+} did not affect the degradation of GFP (Fig.5D (right panel), showing that the presence of Zn^{2+} does not affect the protein degradation machinery itself. These results indicate that Zn^{2+} reduces the protein dynamics and stabilises GAPR-1-GFP inclusions, thus preventing it from degradation. In agreement with this, we find that after promotor shut-off, the amount of fluorescent inclusions positive cells (Fig.5E) as well as the number of inclusions per cell (Fig.5F) is significantly increased in the presence of Zn^{2+}.

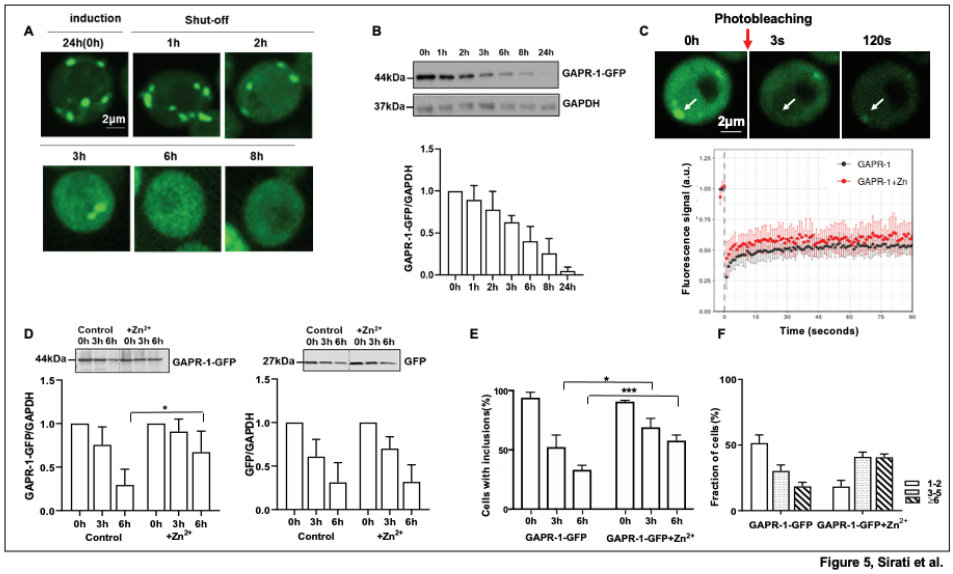


Figure 5, Sirati et al.

Figure 5. Zn^{2+} stabilises dynamic GAPR-1 protein inclusions. Pulse-chase analysis of protein expression. A) Live-cell fluorescence microscopy of yeast cells expressing GAPR-1-GFP after 24h induction (time point 0h) and after promoter shut-off (1h, 2h, 3h, 6h and 8h; B) WB analysis of GAPR-1-GFP levels. Equal amounts of homogenates (20 ug total protein) were separated by SDS-PAGE and analysed by WB (top panel). Densitometric analysis of the immuno-detection of GAPR-1-GFP was determined relative to the intensity of GAPDH (bottom panel). Results are shown as one representative experiment from a total of three independent experiments; C) Fluorescence Recovery After Photo-bleaching (FRAP) time lapse recording of a GAPR-1-GFP inclusion, and FRAP recovery curves of cells expressing GAPR-1-GFP in SC-galactose medium in the absence (control) and presence of 5 mM $ZnCl_2$, at the indicated time points. At least 15 inclusions in different cells were analysed. Each plot represents mean ± SD for each time point for all FRAP experiments; D) Pulse-chase analysis in absence or presence of zinc ions. Protein expression of GAPR-1-GFP (left panel) or GFP (right panel) was induced for 24h (time point 0h), followed by promoter shut-off. Cells from 0h, 3h and at 6h post-shut-off were collected and analysed by WB for the presence of GAPR-1-GFP and GAPDH (loading control). Densitometric analysis of the immuno-detection of GAPR-1-GFP and GFP were determined relative to the intensity of GAPDH ($*p < 0.05$; n=3); E) Cells expressing GAPR-1-GFP from (B) were analysed by live-cell fluorescence microscopy and percentage of cells displaying fluorescence inclusions at 0h, 3h and 6h post-shut-off GAPR-1-GFP expression in the absence or presence of Zn^{2+} were quantified in at least 200 cells. The results are expressed as means ± SD ($**p < 0.01$, $***p < 0.001$; n=3). Differences between groups were statistically determined by using two-tailed

unpaired t-test; F) Quantification of the number of fluorescence inclusions per cell after 6h promoter shut-off. Number of fluorescence inclusions per cell (from condition E) were categorised into three groups (1-2, 3-5 and ≥6 inclusions). At least 100 cells were counted per experiment. The results are expressed as mean ± SD, n=3.

The conserved metal-binding pocket of GAPR-1 affects the formation of inclusions

Zinc-dependent amyloid-like oligomerisation of GAPR-1 *in vitro* requires two conserved histidine residues (H54 and H103) [9]. To investigate the requirement of these histidines for the formation of GAPR-1 inclusions, GAPR-1-GFP mutants were constructed using site-directed mutagenesis (H54V, H103V, and H54V/H103V). Spotting assays revealed unperturbed growth of yeast cells expressing either of these mutants GAPR-1-GFP (Supplementary Fig.1A). The expressed histidine GAPR-1-GFP mutant proteins remained soluble after 100.000 xg centrifugation of yeast homogenates, indicating that the mutations did not disrupt protein folding (Supplementary Fig.4A). Live fluorescence microscopy of yeast cells after expression of H103V and H54V/H103V mutants showed that both mutants reached more than 80% inclusion-positive cells within 3h (Fig.6A,B; Control). These mutants did not show an additional sensitivity to zinc treatment anymore. Only the H54V mutant showed a moderate effect on inclusion formation in comparison to wild type GAPR-1-GFP and still showed a small but significant zinc effect on inclusion formation (Figs.6A, B). The steady-state protein levels of all expressed mutants were comparable in absence and presence of zinc (Fig.6C), showing that the observed phenotypes are not due to different levels of protein expression. To exclude a valine-specific effect on the phenotype of GAPR-1 histidine mutations, we found that the formation of protein inclusions and the sensitivity to zinc were similarly affected after replacement of histidine by alanine (H54A, H103A, and H54A/H103A) (Supplementary Fig.4B).

To explore whether myristoylation is still required for the enhanced inclusion formation in GAPR-1 His54V or His103V mutants, mutants lacking both the consensus sequence for myristoylation (∆myr) and the metal-binding pocket (H54V/H103V) were created. As shown in Figure 6D, all three mutants (ΔmyrH54V, ΔmyrH103V and ΔmyrH54V/H103V) remained predominantly soluble and insensitive to zinc treatment (Figs.6D, 6F) under conditions of similar levels of protein expression (Fig.6E). Thus, myristoylation remains a strong determinant for inclusion formation.

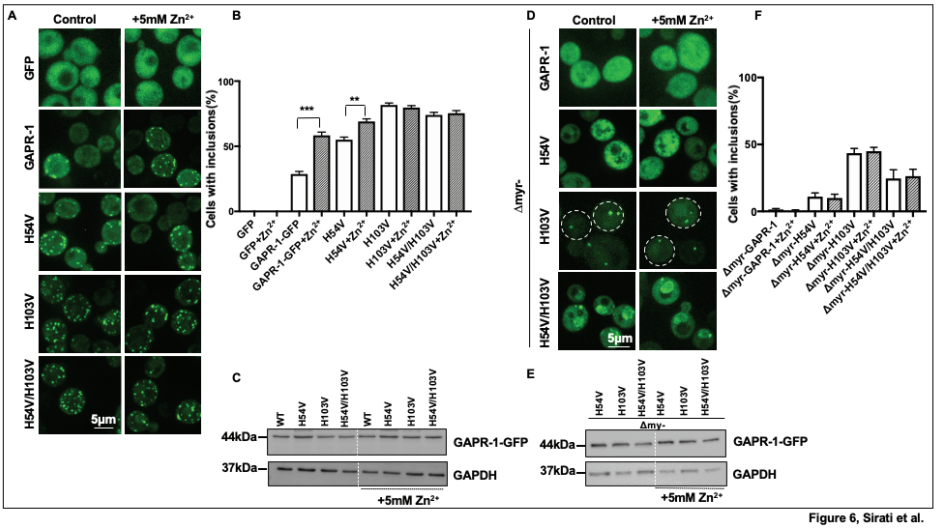


Figure 6. The conserved metal-binding pocket of GAPR-1 affects the formation of inclusions. Cells were transformed with GFP, GAPR-1-GFP, H54V, H103V and H54V/H103V (zinc ion binding sites-mutants) plasmids and protein expression was induced for 3h in the absence and presence of 5 mM $ZnCl_2$. A) Live-cell fluorescence microscopy images of representative cells; B) Quantification of fluorescent inclusions in cells that were incubated as described for panel A as described in Materials and Methods; C) Homogenates (20 µg protein) from each condition (A) were separated by SDS-PAGE and analysed by WB for the presence of GAPR-1-GFP and GAPDH (loading control); D) Representative live-cell fluorescence microscopy images of cells expressing myristoylation mutants of GAPR-1 (Δmyr-GAPR-1, Δmyr-H54V, Δmyr-H103V and Δmyr-H54V/H103V). Protein expression was induced for 3h in the absence or presence of 5 mM $ZnCl_2$; E) Homogenates (20 µg protein) from each condition (D) was analysed by WB using an anti-GAPR-1 and anti-GAPDH (loading control) antibodies; F) Quantification of cells displaying fluorescent inclusion as described in Materials and Methods. The results from B and F are expressed as mean ± SD, n=3. Significance of differences was calculated with two-tailed unpaired t-test (**p < 0.01; ***p < 0.001).

Discussion

GAPR-1 possesses amyloidogenic properties *in vitro* that are regulated by various factors such as negatively charged membranes and metal ions [6,7,9,10]. Here, we used the yeast *S. cerevisiae* to investigate the amyloid-like oligomerisation properties of GAPR-1-GFP *in vivo*. The involvement of the metal-binding site (His54 and His103) in the formation of GAPR-1 inclusions *in vivo* is strikingly similar to their involvement in Zn^{2+}-dependent GAPR-1 amyloid-like aggregation *in vitro* [43].

Hallmarks for the amyloidogenic behaviour of proteins overexpressed in yeast include SDS-resistance, ThT fluorescence, and formation of protein inclusions [50,52–55,63–67]. In the current study, we show that GAPR-1-GFP expression in yeast fulfils these criteria and results in the formation of SDS-resistant aggregates, elevated ThT fluorescence and formation of intracellular protein inclusions. In contrast to pathological amyloids [43,48,51,68–71] and similar to functional amyloids [20,72,73], GAPR-1-GFP expression is not toxic for the cells. These observations support the concept that *in vivo* amyloid-like oligomerisation of GAPR-1 may have functional relevance [11]. GAPR-1 is bound to Golgi membranes and multiple factors may control its oligomeric status, such as the biophysical properties of the membranes that provide a specific surface catalysing structural changes (discussed below), specific negatively charged lipids interacting with GAPR-1 [4], and specific metal ions in the cytosol [9,10]. The oligomeric status of GAPR-1 could control GAPR-1 function and/or affect protein-protein interactions. GAPR-1 was identified as a negative regulator of the autophagic pathway. In agreement with our current findings, we recently suggested that GAPR-1 oligomerisation could be involved in the interaction with other components of the autophagic machinery and thus be essential for the GAPR-1 dependent autophagy regulation [11].

Membranes can serve as catalytic sites that promote the (mis)folding and aggregation of bound amyloidogenic proteins [74,75]. In the past, we showed that GAPR-1 is stably bound to Golgi membranes by a mechanism which involves myristoylation of the N-terminus [2]. The myristoyl-group is, however, not essential for the formation of GAPR-1 amyloid-like fibrils *in vitro*, as recombinant non-myristoylated GAPR-1 aggregates to amyloid-like fibrils in the presence of a seeding platform [6,9,10,76]. In agreement with this, we now show that expression of Δmyr-GAPR-1-GFP in yeast cells also results in the formation of inclusions, albeit at much reduced efficiency as compared to GAPR-1-GFP. Myristoylation, together with relatively strong electrostatic interactions (GAPR-1 has an IP of 9.4), causes efficient interaction with membranes in mammalian cells [77]. In yeast cells, the partial distribution of overexpressed GAPR-1 between membranes and cytosol may be the result of suboptimal N-myristoylation due to high overexpression levels of

GAPR-1-GFP or differences in substrate specificity of orthologous N-myristoyl-transferases [78].

Protein binding to membranes results in increased local concentrations and hence improved conditions for seed formation, which in turn accelerates amyloid-like oligomerisation and the formation of protein inclusions. Interestingly, there is no difference in SDS-solubility between Δmyr-GAPR-1-GFP (Fig.3) and GAPR-1-GFP (Fig.2), suggesting that the amyloidogenic properties are an intrinsic property of the GAPR-1 protein. In agreement with this, we previously reported that GAPR-1 possesses intrinsic amyloidogenic properties based on the finding that purified recombinant GAPR-1 binds to an A11 antibody that recognises the common fold of pre-fibrillar oligomers [6]. The difference in efficiency of inclusion formation between Δmyr-GAPR-1-GFP and GAPR-1-GFP strongly suggests that myristoylation catalyses the slowest step in inclusion formation, namely the clustering of GAPR-1 monomers and/or oligomers into protein inclusions. In addition, myristoylation acts upstream of the formation of protein inclusions as the effects of mutations to the metal-binding site on inclusion formation are largely prevented when myristoylation of GAPR-1 is prohibited (Fig.6). Of course, this does not exclude a potential role for myristoylation in the protein folding process itself, affecting the seeding process independent of membranes.

The process of *in vitro* amyloid-like aggregation of GAPR-1 requires small structural changes at early stages and large structural rearrangements for the formation of amyloid-like fibrils at later stages in the process [9,10]. The small structural changes can be induced by metal-ion-binding to the highly conserved metal-binding site that involves His54 and His103, resulting in enhanced sensitivity of proteases and the specific cleavage of a C-terminal fragment [9,10]. *In vivo*, we now confirm the sensitivity to zinc and show that the addition of zinc ions as well as a mutation of the GAPR-1 metal-binding site leads to an increase of inclusions (Fig.6). Together, these results suggest that small conformational changes in the GAPR-1 structure enhances protein clustering in inclusions and that inclusion formation resembles the early stages of GAPR-1 amyloid-like aggregation *in vitro* (Supplementary Fig.5). The enhanced accumulation of GAPR-1 in inclusions indicates stabilization of GAPR-1 in inclusions, resulting in reduced GAPR-1 dynamics into (FRAP) and from (protein degradation) inclusions (Fig.5). Stabilization of these structures that resemble early stages in amyloid-like aggregation formation are then predicted to inhibit amyloid-like fibril formation that occurs later during the process [12,14,16]. Indeed, a reduced amyloid-like fibril formation of GAPR-1 was observed upon mutations in the GAPR-1 metal-binding site [9,10].

In summary, our results suggest a sequence of events that results in the formation of amyloid-like oligomers and fibrils summarised in Supplementary Fig.3: i) myristoylation acts early in the formation of protein inclusions as the effects of Zn^{2+} and mutations to the metal-binding site are largely prevented by mutations to the myristoylation site; ii) the metal-binding site is involved in the induction of small structural changes resulting in the formation of protein inclusions; iii) large structural changes required for the formation of GAPR-1 amyloids occur at later stages and that have (so far) only been observed *in vitro* with non-myristoylated GAPR-1. GAPR-1 consists almost exclusively of a CAP domain without additional extensions [1,2,8]. We propose that metal-binding, in combination with a specific seeding platform, triggers oligomerisation to regulate the biological functions of various CAP family members. The yeast system may provide a beneficial model to elucidate the molecular mechanisms underlying protein oligomerisation of other CAP family members as well.

Materials and Methods

Yeast strain and plasmids

Wild type yeast strain W303 was transformed by Frozen-EZ yeast kit from Zymo research (Zymo Research, Orange, CA, USA) with a high copy (2µ) plasmid p423 carrying different constructs with C-terminally-tagged GFP (Table 1). Yeast strains BY4741 (wild type and ΔNMT1-181 were obtained from EUROSCARF (Oberursel, Germany).

To construct P*GAL1*-GAPR-1-GFP and P*GAL1*-GAPR-1-mCherry fragments, GAPR-1, GFP and mCherry sequences (Supplementary Table.1 for primers) were amplified by PCR and were inserted into the plasmid p423, previously linearised at SmaI restriction site, by using Geneart® seamless cloning assembly kit (Invitrogen, California, USA). All mutants were constructed by using PhusionTM site-directed mutagenesis kit (Thermo Scientific, Massachusetts, USA). The primers are listed in Supplementary Table.1.

Yeast culture media

Cells were pre-grown overnight in synthetic complete medium lacking the corresponding marker (SC-His) and supplemented with either 2% glucose or 2% galactose at 30^{o}C with orbital agitation (200 rpm) for 18 hours (overnight). The next day, optical density at 600 nm (OD600 nm) was measured, and the cells were placed in selective SC medium (OD600=0.1) supplemented with 2% galactose to induce protein expression. After 3, 6 and 24h, cells were collected to obtain total cell homogenates or were observed under live-cell microscope Nikon Eclipse Ti-E (Nikon).

Table 1. Plasmids used in this study

Plasmid	Description	Source
p423-*GAL1*	*2µm; HIS3; GAL1pr; CYC1term; AmpR*	D. Mumberg, R. Muller, M. Funk, Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression, Nucleic Acids Res. 22 (1994) 5767–5768. https://doi.org/10.1093/nar/22.25.5767. [79]
pME3759	*p426-GAL1-GFP*	D. Petroi, B. Popova, N. Taheri-Talesh, S. Irniger, H. Shahpasandzadeh, M. Zweckstetter, T.F. Outeiro, G.H. Braus, Aggregate Clearance of α-Synuclein in *Saccharomyces cerevisiae* Depends More on Autophagosome and Vacuole Function Than on the Proteasome, J. Biol. Chem. 287 (2012) 27567–27579. https://doi.org/10.1074/jbc.M112.361865. [80]
pNS 1000	*p423-GAL1-GAPR-1-GFP*	This study
pNS 1001	*p423-GAL1-* Δmyr *GAPR-1-GFP*	This study
pNS 1002	*p423-GAL1- H54V GAPR-1-GFP*	This study
pNS 1003	*p423-GAL1-H103V GAPR-1-GFP*	This study
pNS 1004	*p423-GAL1-H54V/H103V GAPR-1-GFP*	This study
pNS 1016	*p423-GAL1-* Δmyr *H54V GAPR-1-GFP*	This study
pNS 1017	*p423-GAL1-* Δmyr *H103V GAPR-1-GFP*	This study
pNS 1018	*p423-GAL1-* Δmyr *H54V/H103V GAPR-1-GFP*	This study

Spotting assay

All spotting assays were performed under the same conditions. Ten-fold serial dilutions starting with an equal number of cells (OD600=0.1) were performed in sterile water. Drops of 10 μl were then spotted on SC plates lacking the corresponding marker (SC-His) and supplemented with either 2% glucose or 2% galactose. Three independent experiments from fresh transformants were performed, followed by a 3 days incubation at 30°C, after which the plates were scanned.

Fluorescence microscopy

Cells were visualised at different time points after induction (3h, 6h, and 24h) using Nikon Eclipse Ti-E microscope (Nikon) equipped with the Perfect Focus System (Nikon) Nikon Apo TIRF 100x N.A. 1.49 oil objective (Nikon), a spinning disk-based confocal scanner unit (CSU-X1-A1, Yokogawa), and the ET-GFP filter set (49002, Chroma). For quantification, at least 300 cells were counted per condition and per experiment. For each condition, the number of cells displaying cytoplasmic inclusions were calculated relative to the total number of cells counted (with inclusions or cytoplasmic distribution). The number of inclusions per cell was determined by counting 100 cells and the inclusion positive cells were categorised in 3 groups: 1) 1-2 inclusions per cell, 2) 3-5 inclusions per cell, and 3) 6 or more than 6 inclusions per cell. At least three independent experiments with freshly transformed constructs were performed.

Protein extraction and Western blotting

10 OD units of cells were collected by centrifugation, washed with sterile water and lysed with 200 μl of Y-PERTM reagent (Thermo Scientific, Massachusetts, USA) containing fresh protease inhibitors (Aprotinin, Leupeptin, Pepstatin, PMSF) (Sigma Aldrich, St. Louis, USA). Lysed cells were incubated for 20 minutes at room temperature, followed by a 15 minute centrifugation at 13000 rpm. The supernatant containing total yeast homogenates were collected for further analysis. Yeast homogenates (20 μg protein) was mixed with Laemmli sample buffer, incubated for 5 minutes at 100°C and separated by SDS-PAGE (12% polyacrylamide). Proteins were transferred onto 0.45 μm nitrocellulose membranes (Amersham Protran GE Healthcare) by Western blot at 90 V for 1h. The presence of GAPR-1 was analysed using a rabbit polyclonal anti-GAPR-1 antibody [2]. A monoclonal anti-GAPDH antibody (Thermo Scientific, Massachusetts, USA) was used as a loading control. Peroxidase-conjugated goat anti-rabbit antibody (Nordic-Mubio, Susteren, The Netherlands) was used as a secondary antibody. Western blot analysis was performed using the SuperSignalTM reagents (Thermo Scientific, Massachusetts, USA) and ChemiDocTM MP Imaging system (BioRad, Hercules,

California, USA). For quantification of Western blots, pixel density values from TIFF files were analysed with Image Lab SoftwareTM. Sample density values were normalised to the corresponding GAPDH loading control. The statistical significance was calculated using two-tailed unpaired t-test.

SDS Solubility test

10 OD unites of cells were collected after 24h induction, homogenised and total protein homogenate was prepared using Y-PERTM reagent. 1/10 of the homogenate was collected as input. 9/10 of the sample was centrifuged at 100,000 x g for 30 minutes and the detergent-soluble supernatant was collected. The detergent-insoluble pellet was treated with 2% SDS (180 µl for 30 minutes at 4^{o} C) and separated by centrifugation (100,000 x g for 30 minutes) into a SDS-soluble and SDS-insoluble fraction. The same volume (20 µl) of each sample was separated by SDS-PAGE and analysed by Western blot using an anti-GFP antibody.For the dot blot analyses, 50 µl (30 µg) of each sample was loaded directly onto 0.45 µm nitrocellulose membranes (Amersham Protran GE Healthcare) and analysed by an anti-GAPR-1 and anti-GFP antibodies.

Crosslinking assay

10 OD units of cells were collected at 6 and 24h post-induction of GAPR-1-GFP protein expression. A homogenate was prepared by mechanical homogenisation with glass beads (0.25-0.5 mm, Carl Roth, Kalrsruhe, Germany). The homogenate (100 µg) was divided equally into two groups. Bis (sulfosuccinimidyl) suberate (BS3) (Pierce Biotechnology, Rockford, IL, USA) was added in a 30-fold molar excess to the protein concentration to one of the groups and both groups were incubated for 30 minutes at room temperature. 0.5 M Tris pH 7.4 was added to quench the reaction. The proteins and cross-linked products were analysed by SDS-PAGE and Western blot.

Promoter shut-off study

Yeast cells containing GAPR-1-GFP constructs were cultured in SC selective medium supplemented with 2% raffinose overnight. The medium was changed to SC selective medium supplemented with 2% galactose to induce the GAPR-1-GFP expression for 24h. The cells were pelleted, washed two times with water and shifted to SC medium supplemented with 2% glucose to shut-off the *GAL1* promoter [80]. Cells were visualised at different time points after promoter shut-off by fluorescence microscopy. The number of cells displaying GAPR-1-GFP inclusions were counted. Western blot analysis was performed as described above with homogenates prepared from cells collected at different time points after promoter shut-off.

Thioflavin T staining

Yeast cells were stained with Thioflavin T according to a protocol adapted from Johnson et al [81] with minor modifications. Briefly, yeast cells were pre-grown overnight in SC-2% raffinose and then induced for 24h in SC-2% galactose. Cells were harvested with a final OD of 0.25-1.0 and washed in 5 ml of PM [0.1 M KPO4 (pH 7.5) and 1 mM MgCl2], then re-suspended in PMST [0.1 M KPO4 (pH 7.5), 1 mM MgCl2, 1 M Sorbitol and 0.1% Tween 20] to a final OD of 10. To make spheroplasts, 100 μl of the cell suspension was incubated with 0.6 μl of 2-mercaptoethanol and 1 mg/ml Zymolyase (Zymo Research, Orange, CA, USA) for 15 minutes. Spheroplasted cells were washed once in PMST and re-suspended in 100 μl of PMST. Cells were then incubated 30 minutes at room temperature with 5 μM Thioflavin T (Sigma Aldrich, St. Louis, USA), followed by washing 3 times with PMST for 30 minutes. Cells were observed using Nikon Eclipse Ti-E microscope (Nikon) equipped with the Perfect Focus System (Nikon) Nikon Apo TIRF 100x N.A. 1.49 oil objective (Nikon) and a spinning disk-based confocal scanner unit (CSU-X1-A1, Yokogawa). GFP, mCherry and Thioflavin T signals were detected in GFP, mCherry and CFP channels, respectively. For measuring total ThT fluorescence intensity by a microplate reader, 100 μl of cells was pipetted into a 96-well plate (black plastic plate with glass bottom, Greiner Bio-One, Frickenhausen, Germany). ThT (excitation at 380-420 nm, emission between 471-569, step size 5 nm) was measured with a microplate reader (CLARIOstar, BMG Labtech, Offenburg, Germany) using preset filter settings. Data were visualised and analysed in RStudio (v1.0.153, R v3.4.4) using R-package ggplot2 (v2.2.1). Total fluorescence per sample was calculated by integrating the areas under the curve between 480-510 nm (ThT).

Isopycnic sucrose density gradients and TX-100 treatment

Expressions of GAPR-1-GFP, Δmyr-GAPR-1-GFP and GFP (as control) were induced for 24h in SC selective medium supplemented with 2% galactose. Cells were mechanically lysed with glass beads (0.25-0.5 mm, Carl Roth, Kalrsruhe, Germany). After centrifugation (13000 rpm for 15 minutes at 4^{o}C), supernatants were collected and treated with or without 1% TX-100 for 30 minutes on ice. A sucrose gradient (37-35-29% sucrose in PBS buffer) was layered on top of 500 μl samples (adjusted to 37% sucrose and 50 μg total protein) in SW60-2 tubes (Beckman Coulter, Brea, California, USA). The samples were centrifuged at 60,000 rpm for 18 hours in an ultracentrifuge. Fractions (350 μl) were collected from top to bottom of the gradients and were precipitated with Chloroform/Methanol (1/2). Samples were analysed by SDS-PAGE and Western blotting for the presence of GAPR-1 using specific antibodies (anti-GAPR-1 polyclonal antibody).

Fluorescence Recovery After Photo-bleaching (FRAP) analysis

Yeast cells harbouring GAPR-1-GFP were pre-grown overnight in SC selective medium containing 2% raffinose followed by 24h induction in SC medium containing 2% galactose supplemented with or without 5 mM $ZnCl_2$ (Sigma Aldrich (St. Louis, USA). Cells were collected by centrifugation and re-suspended in PBS containing 0.5% low melting agarose on a microscope slide. FRAP experiments were performed using a Nikon A1Rsi microscope (Nikon) at room temperature. Images were acquired using a PlanApochromat 63x/1.4 gNA objective with frame size 256×256, pixel width 91 nm and pixel time 4.44 µs at intervals of 1 seconds with pinhole set to 1.5 Airy unit. In each FRAP experiment, a single focus at the central focal plane was bleached using the 488 nm laser line at 100% laser transmission on a circular region of interest (ROI) with a diameter of 8 pixels (0.7 µm radius) for 30 minutes. For imaging, the transmission of the 488 nm laser was set to 1% of the bleach intensity. Regions of interests (ROI) were defined for bleached spots, as well as for non-bleached spots. Mean fluorescence intensities for all ROIs were quantified over time using ImageJ (v1.50b). For every time point, signals from bleached spots were normalised to the signals from non-bleached spots to correct for fluorophore fading caused by image acquisition.

Statistical Analysis

Data were analysed using GraphPad Prism 8 (San Diego, CA, USA) Software. The significance of differences was calculated using two-tailed unpaired t-test or Welch t-test. P-values lower than 0.05 were considered to indicate a significant difference.

Acknowledgements

We thank Ruud Eerland and Ilse Lgerwaard for technical support, Richard Wubbolts and Ilya Grigoriev (Ana Akhmanova) for help with fluorescence microscopy. GB acknowledges the financial support of Deutsche Forschungsgemeinschaft (DFG: BR1502/18-1).

References

[1] G.M. Gibbs, K. Roelants, M.K. O'Bryan, The CAP Superfamily: Cysteine-Rich Secretory Proteins, Antigen 5, and Pathogenesis-Related 1 Proteins—Roles in Reproduction, Cancer, and Immune Defense, Endocr. Rev. 29 (2008) 865–897. https://doi.org/10.1210/er.2008-0032.

[2] H.B. Eberle, R.L. Serrano, J. Füllekrug, A. Schlosser, W.D. Lehmann, F. Lottspeich, D. Kaloyanova, F.T. Wieland, J.B. Helms, Identification and characterization of a novel human plant pathogenesis-related protein that localizes to lipid-enriched microdomains in the Golgi complex., J. Cell Sci. 115 (2002) 827–38. http://www.ncbi.nlm.nih.gov/pubmed/11865038.

[3] A. Abraham, D.E. Chandler, Tracing the evolutionary history of the CAP superfamily of proteins using amino acid sequence homology and conservation of splice sites, J. Mol. Evol. 85 (2017) 137–157.

[4] J. Van Galen, B.W.M. Van Balkom, R.L. Serrano, D. Kaloyanova, R. Eerland, E. Stüven, J.B. Helms, Binding of GAPR-1 to negatively charged phospholipid membranes: Unusual binding characteristics to phosphatidylinositol, Mol. Membr. Biol. 27 (2010) 81–91. https://doi.org/10.3109/09687680903507080.

[5] S. Shoji-Kawata, R. Sumpter, M. Leveno, G.R. Campbell, Z. Zou, L. Kinch, A.D. Wilkins, Q. Sun, K. Pallauf, D. MacDuff, C. Huerta, H.W. Virgin, J. Bernd Helms, R. Eerland, S.A. Tooze, R. Xavier, D.J. Lenschow, A. Yamamoto, D. King, O. Lichtarge, N. V. Grishin, S.A. Spector, D. V. Kaloyanova, B. Levine, Identification of a candidate therapeutic autophagy-inducing peptide, Nature. 494 (2013) 201–206. https://doi.org/10.1038/nature11866.

[6] N.K. Olrichs, A.K. Mahalka, D. Kaloyanova, P.K. Kinnunen, J. Bernd Helms, Golgi-Associated plant Pathogenesis Related protein 1 (GAPR-1) forms amyloid-like fibrils by interaction with acidic phospholipids and inhibits Aβ aggregation, Amyloid. 21 (2014) 88–96. https://doi.org/10.3109/13506129.2014.882304.

[7] J. Van Galen, N.K. Olrichs, A. Schouten, R.L. Serrano, E.N.M. Nolte-'t Hoen, R. Eerland, D. Kaloyanova, P. Gros, J.B. Helms, Interaction of GAPR-1 with lipid bilayers is regulated by alternative homodimerization, Biochim. Biophys. Acta - Biomembr. 1818 (2012) 2175–2183. https://doi.org/10.1016/j.bbamem.2012.04.016.

[8] R.L. Serrano, A. Kuhn, A. Hendricks, J.B. Helms, I. Sinning, M.R. Groves, Structural analysis of the human Golgi-associated plant pathogenesis related protein

GAPR-1 implicates dimerization as a regulatory mechanism, J. Mol. Biol. 339 (2004) 173–183. https://doi.org/10.1016/j.jmb.2004.03.015.

[9] J. Sheng, N.K. Olrichs, W.J. Geerts, X. Li, A.U. Rehman, B.M. Gadella, D. V. Kaloyanova, J.B. Helms, Zinc binding regulates amyloid-like aggregation of GAPR-1, Biosci. Rep. 39 (2019). https://doi.org/10.1042/BSR20182345.

[10] J. Sheng, N.K. Olrichs, W.J. Geerts, D. V. Kaloyanova, J.B. Helms, Metal ions and redox balance regulate distinct amyloid-like aggregation pathways of GAPR-1, Sci. Rep. 9 (2019) 15048. https://doi.org/10.1038/s41598-019-51232-7.

[11] J. Sheng, N.K. Olrichs, B.M. Gadella, D. V. Kaloyanova, J.B. Helms, Regulation of Functional Protein Aggregation by Multiple Factors: Implications for the Amyloidogenic Behavior of the CAP Superfamily Proteins, Int. J. Mol. Sci. 21 (2020) 6530. https://doi.org/10.3390/ijms21186530.

[12] J. Tyedmers, A. Mogk, B. Bukau, Cellular strategies for controlling protein aggregation, Nat. Rev. Mol. Cell Biol. 11 (2010) 777–788. https://doi.org/10.1038/nrm2993.

[13] F. Chiti, C.M. Dobson, Protein Misfolding, Functional Amyloid, and Human Disease, Annu. Rev. Biochem. 75 (2006) 333–366. https://doi.org/10.1146/annurev.biochem.75.101304.123901.

[14] F. Chiti, C.M. Dobson, Protein Misfolding, Amyloid Formation, and Human Disease: A Summary of Progress Over the Last Decade, Annu. Rev. Biochem. 86 (2017) 27–68. https://doi.org/10.1146/annurev-biochem-061516-045115.

[15] M.G. Iadanza, M.P. Jackson, E.W. Hewitt, N.A. Ranson, S.E. Radford, A new era for understanding amyloid structures and disease, Nat. Rev. Mol. Cell Biol. 19 (2018) 755–773. https://doi.org/10.1038/s41580-018-0060-8.

[16] T.P.J. Knowles, M. Vendruscolo, C.M. Dobson, The amyloid state and its association with protein misfolding diseases, Nat. Rev. Mol. Cell Biol. 15 (2014) 384–396. https://doi.org/10.1038/nrm3810.

[17] E. Chuang, A.M. Hori, C.D. Hesketh, J. Shorter, Amyloid assembly and disassembly, J. Cell Sci. 131 (2018) 1–18.

[18] D. Otzen, R. Riek, Functional Amyloids, Cold Spring Harb. Perspect. Biol. 11 (2019) a033860. https://doi.org/10.1101/cshperspect.a033860.

[19] D.M. Fowler, A. V. Koulov, W.E. Balch, J.W. Kelly, Functional amyloid – from bacteria to humans, Trends Biochem. Sci. 32 (2007) 217–224.

https://doi.org/10.1016/j.tibs.2007.03.003.

[20] S.M. Ulamec, S.E. Radford, Spot the Difference: Function versus Toxicity in Amyloid Fibrils, Trends Biochem. Sci. 45 (2020) 635–636. https://doi.org/10.1016/j.tibs.2020.04.007.

[21] S. Saad, G. Cereghetti, Y. Feng, P. Picotti, M. Peter, R. Dechant, Reversible protein aggregation is a protective mechanism to ensure cell cycle restart after stress, Nat. Cell Biol. 19 (2017) 1202–1213. https://doi.org/10.1038/ncb3600.

[22] T.E. Audas, D.E. Audas, M.D. Jacob, J.J.D. Ho, M. Khacho, M. Wang, J.K. Perera, C. Gardiner, C.A. Bennett, T. Head, O.N. Kryvenko, M. Jorda, S. Daunert, A. Malhotra, L. Trinkle-Mulcahy, M.L. Gonzalgo, S. Lee, Adaptation to Stressors by Systemic Protein Amyloidogenesis, Dev. Cell. 39 (2016) 155–168. https://doi.org/10.1016/j.devcel.2016.09.002.

[23] S.K. Maji, M.H. Perrin, M.R. Sawaya, S. Jessberger, K. Vadodaria, R.A. Rissman, P.S. Singru, K.P.R. Nilsson, R. Simon, D. Schubert, D. Eisenberg, J. Rivier, P. Sawchenko, W. Vale, R. Riek, Functional Amyloids As Natural Storage of Peptide Hormones in Pituitary Secretory Granules, Science (80-.). 325 (2009) 328–332. https://doi.org/10.1126/science.1173155.

[24] A. Hewetson, H.Q. Do, C. Myers, A. Muthusubramanian, R.B. Sutton, B.J. Wylie, G.A. Cornwall, Functional Amyloids in Reproduction, Biomolecules. 7 (2017) 46. https://doi.org/10.3390/biom7030046.

[25] M.S. Rubel, S.A. Fedotov, A. V. Grizel, J. V. Sopova, O.A. Malikova, Y.O. Chernoff, A.A. Rubel, Functional Mammalian Amyloids and Amyloid-Like Proteins, Life. 10 (2020) 156. https://doi.org/10.3390/life10090156.

[26] A. Patel, H.O. Lee, L. Jawerth, S. Maharana, M. Jahnel, M.Y. Hein, S. Stoynov, J. Mahamid, S. Saha, T.M. Franzmann, A. Pozniakovski, I. Poser, N. Maghelli, L.A. Royer, M. Weigert, E.W. Myers, S. Grill, D. Drechsel, A.A. Hyman, S. Alberti, A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation, Cell. 162 (2015) 1066–1077. https://doi.org/10.1016/j.cell.2015.07.047.

[27] T. Amen, D. Kaganovich, Dynamic droplets: the role of cytoplasmic inclusions in stress, function, and disease, Cell. Mol. Life Sci. 72 (2015) 401–415. https://doi.org/10.1007/s00018-014-1740-y.

[28] A. Zbinden, M. Pérez-Berlanga, P. De Rossi, M. Polymenidou, Phase Separation and Neurodegenerative Diseases: A Disturbance in the Force, Dev. Cell. 55

(2020) 45–68. https://doi.org/10.1016/j.devcel.2020.09.014.

[29] S. Elbaum-Garfinkle, Matter over mind: Liquid phase separation and neurodegeneration, J. Biol. Chem. 294 (2019) 7160–7168. https://doi.org/10.1074/jbc.REV118.001188.

[30] E. Gomes, J. Shorter, The molecular language of membraneless organelles, J. Biol. Chem. 294 (2019) 7115–7127. https://doi.org/10.1074/jbc.TM118.001192.

[31] S. Alberti, A. Gladfelter, T. Mittag, Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates, Cell. 176 (2019) 419–434. https://doi.org/10.1016/j.cell.2018.12.035.

[32] S. Alberti, A.A. Hyman, Biomolecular condensates at the nexus of cellular stress, protein aggregation disease and ageing, Nat. Rev. Mol. Cell Biol. 22 (2021) 196–213. https://doi.org/10.1038/s41580-020-00326-6.

[33] T.M. Franzmann, M. Jahnel, A. Pozniakovsky, J. Mahamid, A.S. Holehouse, E. Nüske, D. Richter, W. Baumeister, S.W. Grill, R. V. Pappu, A.A. Hyman, S. Alberti, Phase separation of a yeast prion protein promotes cellular fitness, Science (80-.). 359 (2018) eaao5654. https://doi.org/10.1126/science.aao5654.

[34] N. Sanchez de Groot, M. Torrent Burgas, C.N. Ravarani, A. Trusina, S. Ventura, M.M. Babu, The fitness cost and benefit of phase-separated protein deposits, Mol. Syst. Biol. 15 (2019). https://doi.org/10.15252/msb.20178075.

[35] S. Alberti, A.A. Hyman, Are aberrant phase transitions a driver of cellular aging?, BioEssays. 38 (2016) 959–968. https://doi.org/10.1002/bies.201600042.

[36] S. Boeynaems, S. Alberti, N.L. Fawzi, T. Mittag, M. Polymenidou, F. Rousseau, J. Schymkowitz, J. Shorter, B. Wolozin, L. Van Den Bosch, P. Tompa, M. Fuxreiter, Protein Phase Separation: A New Phase in Cell Biology, Trends Cell Biol. 28 (2018) 420–435. https://doi.org/10.1016/j.tcb.2018.02.004.

[37] S. Alberti, S. Carra, Quality Control of Membraneless Organelles, J. Mol. Biol. 430 (2018) 4711–4729. https://doi.org/10.1016/j.jmb.2018.05.013.

[38] P.C. Ke, R. Zhou, L.C. Serpell, R. Riek, T.P.J. Knowles, H.A. Lashuel, E. Gazit, I.W. Hamley, T.P. Davis, M. Fändrich, D.E. Otzen, M.R. Chapman, C.M. Dobson, D.S. Eisenberg, R. Mezzenga, Half a century of amyloids: past, present and future, Chem. Soc. Rev. 49 (2020) 5473–5509. https://doi.org/10.1039/C9CS00199A.

[39] L.D. Muiznieks, S. Sharpe, R. Pomès, F.W. Keeley, Role of Liquid–Liquid Phase Separation in Assembly of Elastin and Other Extracellular Matrix Proteins, J. Mol.

Biol. 430 (2018) 4741–4753. https://doi.org/10.1016/j.jmb.2018.06.010.

[40] L.E. Berchowitz, G. Kabachinski, M.R. Walker, T.M. Carlile, W. V. Gilbert, T.U. Schwartz, A. Amon, Regulated Formation of an Amyloid-like Translational Repressor Governs Gametogenesis, Cell. 163 (2015) 406–418. https://doi.org/10.1016/j.cell.2015.08.060.

[41] A. Balistreri, E. Goetzler, M. Chapman, Functional Amyloids Are the Rule Rather Than the Exception in Cellular Biology, Microorganisms. 8 (2020) 1951. https://doi.org/10.3390/microorganisms8121951.

[42] V. Khurana, S. Lindquist, Modelling neurodegeneration in Saccharomyces cerevisiae: Why cook with baker's yeast?, Nat. Rev. Neurosci. 11 (2010) 436–449. https://doi.org/10.1038/nrn2809.

[43] D.F. Tardiff, V. Khurana, C.Y. Chung, S. Lindquist, From yeast to patient neurons and back again: Powerful new discovery platforms, Mov. Disord. 29 (2014) 1231–1240. https://doi.org/10.1002/mds.25989.

[44] G. Fruhmann, D. Seynnaeve, J. Zheng, K. Ven, S. Molenberghs, T. Wilms, B. Liu, J. Winderickx, V. Franssens, Yeast buddies helping to unravel the complexity of neurodegenerative disorders, Mech. Ageing Dev. 161 (2017) 288–305. https://doi.org/10.1016/j.mad.2016.05.002.

[45] L. McGurk, N.M. Bonini, Yeast Informs Alzheimer's Disease, Science (80-.). 334 (2011) 1212–1213. https://doi.org/10.1126/science.1216073.

[46] A. Porzoor, I.G. Macreadie, Application of Yeast to Study the Tau and Amyloid-β Abnormalities of Alzheimer's Disease, J. Alzheimer's Dis. 35 (2013) 217–225. https://doi.org/10.3233/JAD-122035.

[47] T.F. Outeiro, Yeast Cells Provide Insight into Alpha-Synuclein Biology and Pathobiology, Science (80-.). 302 (2003) 1772–1775. https://doi.org/10.1126/science.1090439.

[48] I.C. Brás, B. Popova, G.H. Braus, T.F. Outeiro, Yeast-Based Screens to Target Alpha-Synuclein Toxicity, in: 2019: pp. 145–156. https://doi.org/10.1007/978-1-4939-9124-2_12.

[49] S. Tenreiro, M.C. Munder, S. Alberti, T.F. Outeiro, Harnessing the power of yeast to unravel the molecular basis of neurodegeneration, J. Neurochem. 127 (2013) 438–452. https://doi.org/10.1111/jnc.12271.

[50] S. Tenreiro, M.M. Reimão-Pinto, P. Antas, J. Rino, D. Wawrzycka, D. Macedo, R.

Rosado-Ramos, T. Amen, M. Waiss, F. Magalhães, A. Gomes, C.N. Santos, D. Kaganovich, T.F. Outeiro, Phosphorylation Modulates Clearance of Alpha-Synuclein Inclusions in a Yeast Model of Parkinson's Disease, PLoS Genet. 10 (2014) e1004302. https://doi.org/10.1371/journal.pgen.1004302.

[51] B. Popova, A. Kleinknecht, P. Arendarski, J. Mischke, D. Wang, G.H. Braus, Sumoylation Protects Against β-Synuclein Toxicity in Yeast, Front. Mol. Neurosci. 11 (2018). https://doi.org/10.3389/fnmol.2018.00094.

[52] S. Alberti, R. Halfmann, S. Lindquist, Biochemical, Cell Biological, and Genetic Assays to Analyze Amyloid and Prion Aggregation in Yeast, in: 2010: pp. 709–734. https://doi.org/10.1016/S0076-6879(10)70030-6.

[53] D. Laor, D. Sade, S. Shaham-Niv, D. Zaguri, M. Gartner, V. Basavalingappa, A. Raveh, E. Pichinuk, H. Engel, K. Iwasaki, T. Yamamoto, H. Noothalapati, E. Gazit, Fibril formation and therapeutic targeting of amyloid-like structures in a yeast model of adenine accumulation, Nat. Commun. 10 (2019) 62. https://doi.org/10.1038/s41467-018-07966-5.

[54] S. Rencus-Lazar, Y. DeRowe, H. Adsi, E. Gazit, D. Laor, Yeast Models for the Study of Amyloid-Associated Disorders and Development of Future Therapy, Front. Mol. Biosci. 6 (2019). https://doi.org/10.3389/fmolb.2019.00015.

[55] G. Cereghetti, S. Saad, R. Dechant, M. Peter, Reversible, functional amyloids: towards an understanding of their regulation in yeast and humans, Cell Cycle. 17 (2018) 1545–1558. https://doi.org/10.1080/15384101.2018.1480220.

[56] M. Biancalana, S. Koide, Molecular mechanism of Thioflavin-T binding to amyloid fibrils, Biochim. Biophys. Acta - Proteins Proteomics. 1804 (2010) 1405–1412. https://doi.org/10.1016/j.bbapap.2010.04.001.

[57] Y. Ohhashi, Y. Yamaguchi, H. Kurahashi, Y.O. Kamatari, S. Sugiyama, B. Uluca, T. Piechatzek, Y. Komi, T. Shida, H. Müller, S. Hanashima, H. Heise, K. Kuwata, M. Tanaka, Molecular basis for diversification of yeast prion strain conformation, Proc. Natl. Acad. Sci. 115 (2018) 2389–2394. https://doi.org/10.1073/pnas.1715483115.

[58] A.M. Fanni, C.M. Vander Zanden, P. V. Majewska, J. Majewski, E.Y. Chi, Membrane-mediated fibrillation and toxicity of the tau hexapeptide PHF6, J. Biol. Chem. 294 (2019) 15304–15317. https://doi.org/10.1074/jbc.RA119.010003.

[59] A. Khondker, R.J. Alsop, M.C. Rheinstädter, Membrane-accelerated Amyloid-β

aggregation and formation of cross-β sheets, Membranes (Basel). 7 (2017). https://doi.org/10.3390/membranes7030049.

[60] J. McLaurin, T. Franklin, X. Zhang, J. Deng, P.E. Fraser, Interactions of Alzheimer amyloid-β peptides with glycosaminoglycans, Eur. J. Biochem. 266 (1999) 1101–1110. https://doi.org/10.1046/j.1432-1327.1999.00957.x.

[61] S. Mehra, D. Ghosh, R. Kumar, M. Mondal, L.G. Gadhe, S. Das, A. Anoop, N.N. Jha, R.S. Jacob, D. Chatterjee, S. Ray, N. Singh, A. Kumar, S.K. Maji, Glycosaminoglycans have variable effects on -synuclein aggregation and differentially affect the activities of the resulting amyloid fibrils, J. Biol. Chem. 293 (2018) 12975–12991. https://doi.org/10.1074/jbc.RA118.004267.

[62] A. Ishii, K. Kurokawa, M. Hotta, S. Yoshizaki, M. Kurita, A. Koyama, A. Nakano, Y. Kimura, Role of Atg8 in the regulation of vacuolar membrane invagination, Sci. Rep. 9 (2019) 14828. https://doi.org/10.1038/s41598-019-51254-1.

[63] T.A. Chernova, Y.O. Chernoff, K.D. Wilkinson, Yeast Models for Amyloids and Prions: Environmental Modulation and Drug Discovery, Molecules. 24 (2019) 3388. https://doi.org/10.3390/molecules24183388.

[64] R.B. Wickner, D. Kryndushkin, F. Shewmaker, R. McGlinchey, H.K. Edskes, Study of Amyloids Using Yeast, in: 2018: pp. 313–339. https://doi.org/10.1007/978-1-4939-7816-8_19.

[65] Z.T. Monahan, S.N. Rhoads, D.S. Yee, F.P. Shewmaker, Yeast Models of Prion-Like Proteins That Cause Amyotrophic Lateral Sclerosis Reveal Pathogenic Mechanisms, Front. Mol. Neurosci. 11 (2018). https://doi.org/10.3389/fnmol.2018.00453.

[66] A.V. Sergeeva, J.V. Sopova, T.A. Belashova, V.A. Siniukova, A.V. Chirinskaite, A.P. Galkin, S.P. Zadorsky, Amyloid properties of the yeast cell wall protein Toh1 and its interaction with prion proteins Rnq1 and Sup35, Prion. 13 (2019) 21–32. https://doi.org/10.1080/19336896.2018.1558763.

[67] D. Kryndushkin, N. Pripuzova, B.G. Burnett, F. Shewmaker, Non-targeted Identification of Prions and Amyloid-forming Proteins from Yeast and Mammalian Cells, J. Biol. Chem. 288 (2013) 27100–27111. https://doi.org/10.1074/jbc.M113.485359.

[68] M. Lindström, B. Liu, Yeast as a Model to Unravel Mechanisms Behind FUS Toxicity in Amyotrophic Lateral Sclerosis, Front. Mol. Neurosci. 11 (2018). https://doi.org/10.3389/fnmol.2018.00218.

[69] Y.O. Chernoff, A. V. Grizel, A.A. Rubel, A.A. Zelinsky, P. Chandramowlishwaran, T.A. Chernova, Application of yeast to studying amyloid and prion diseases, in: 2020: pp. 293–380. https://doi.org/10.1016/bs.adgen.2020.01.002.

[70] A. Villar-Piqué, S. Ventura, Protein aggregation propensity is a crucial determinant of intracellular inclusion formation and quality control degradation, Biochim. Biophys. Acta - Mol. Cell Res. 1833 (2013) 2714–2724. https://doi.org/10.1016/j.bbamcr.2013.06.023.

[71] B. Sampaio-Marques, A. Guedes, I. Vasilevskiy, S. Gonçalves, T.F. Outeiro, J. Winderickx, W.C. Burhans, P. Ludovico, α-Synuclein toxicity in yeast and human cells is caused by cell cycle re-entry and autophagy degradation of ribonucleotide reductase 1, Aging Cell. 18 (2019). https://doi.org/10.1111/acel.12922.

[72] F. Rueda, B. Gasser, A. Sánchez-Chardi, M. Roldán, S. Villegas, V. Puxbaum, N. Ferrer-Miralles, U. Unzueta, E. Vázquez, E. Garcia-Fruitós, D. Mattanovich, A. Villaverde, Functional inclusion bodies produced in the yeast Pichia pastoris, Microb. Cell Fact. 15 (2016) 166. https://doi.org/10.1186/s12934-016-0565-9.

[73] M. Jackson, E. Hewitt, Why are Functional Amyloids Non-Toxic in Humans?, Biomolecules. 7 (2017) 71. https://doi.org/10.3390/biom7040071.

[74] S.M. Butterfield, H.A. Lashuel, Amyloidogenic Protein-Membrane Interactions: Mechanistic Insight from Model Systems, Angew. Chemie Int. Ed. 49 (2010) 5628–5654. https://doi.org/10.1002/anie.200906670.

[75] G. Musteikytė, A.K. Jayaram, C.K. Xu, M. Vendruscolo, G. Krainer, T.P.J. Knowles, Interactions of α-synuclein oligomers with lipid membranes, Biochim. Biophys. Acta - Biomembr. 1863 (2021) 183536. https://doi.org/10.1016/j.bbamem.2020.183536.

[76] N. K. Olrichs, J. Bernd Helms, Novel insights into the function of the conserved domain of the CAP superfamily of proteins, AIMS Biophys. 3 (2016) 232–246. https://doi.org/10.3934/biophy.2016.2.232.

[77] M.D. Resh, Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins, Biochim. Biophys. Acta - Mol. Cell Res. 1451 (1999) 1–16. https://doi.org/10.1016/S0167-4889(99)00075-0.

[78] T.A. Farazi, G. Waksman, J.I. Gordon, The Biology and Enzymology of Protein N - Myristoylation, J. Biol. Chem. 276 (2001) 39501–39504. https://doi.org/10.1074/jbc.R100042200.

[79] D. Mumberg, R. Muller, M. Funk, Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression, Nucleic Acids Res. 22 (1994) 5767–5768. https://doi.org/10.1093/nar/22.25.5767.

[80] D. Petroi, B. Popova, N. Taheri-Talesh, S. Irniger, H. Shahpasandzadeh, M. Zweckstetter, T.F. Outeiro, G.H. Braus, Aggregate Clearance of α-Synuclein in *Saccharomyces cerevisiae* Depends More on Autophagosome and Vacuole Function Than on the Proteasome, J. Biol. Chem. 287 (2012) 27567–27579. https://doi.org/10.1074/jbc.M112.361865.

[81] B.S. Johnson, J.M. McCaffery, S. Lindquist, A.D. Gitler, A yeast TDP-43 proteinopathy model: Exploring the mo**lecul**ar determinants of TDP-43 aggregation and cellular toxicity, Proc. Natl. Acad. Sci. 105 (2008) 6439–6444. https://doi.org/10.1073/pnas.0802082105.

Supplemental information

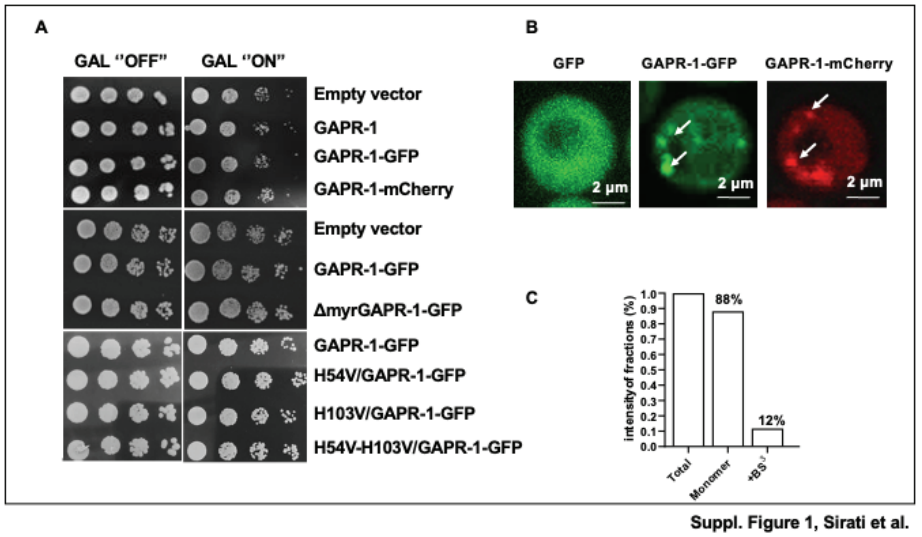
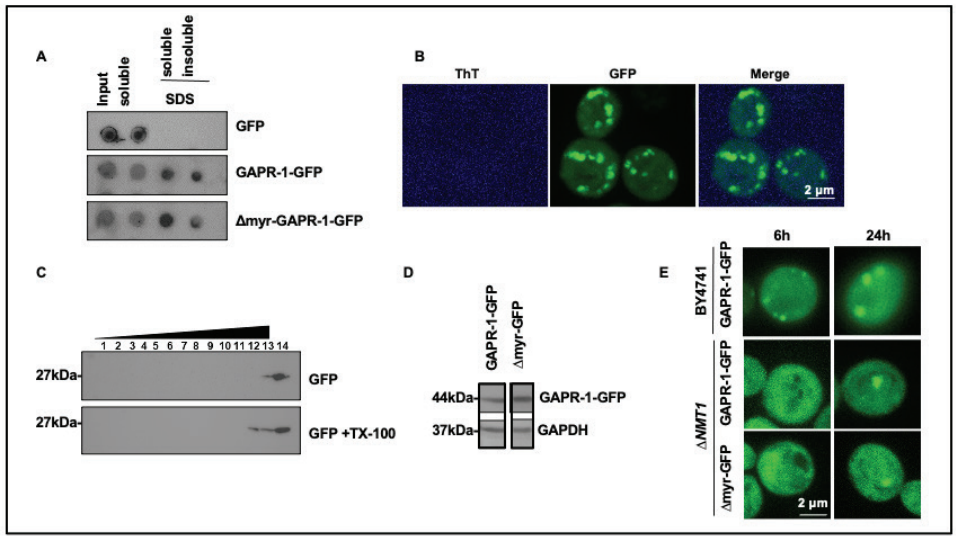
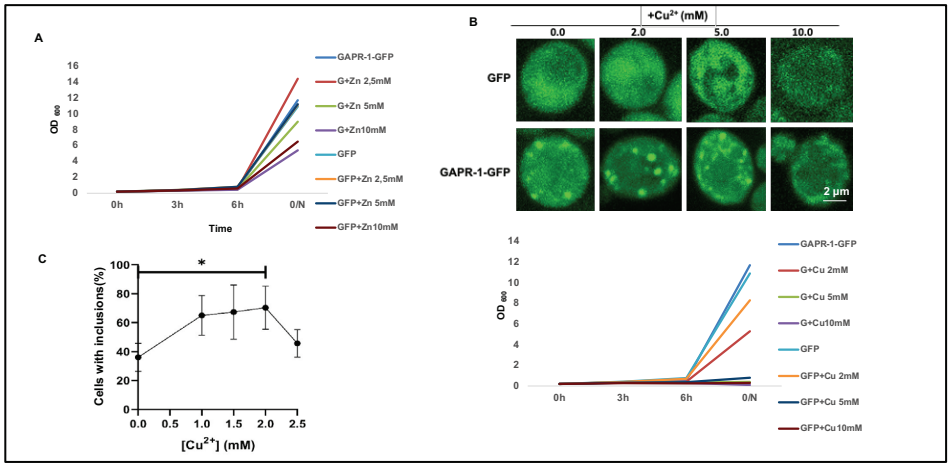


Figure S1. A) Ten-fold dilutions of exponentially growing cultures were spotted using selection agar plates (SC-His) with 2% glucose (*GAL1* promoter "OFF") and 2% galactose (*GAL1* promoter "ON"). The cells were incubated at 30^{o}C for 3 days; B) Wild type yeast cells (W303) were transformed with a plasmid carrying GFP, GAPR-1-GFP or GAPR-1-mCherry under the control of *GAL1* promoter. Live-cell fluorescence microscopy of after 6h induction, arrows indicate GAPR-1 fluorescence inclusions; C) Densitometric analysis of the immuno-detection of GAPR-1-GFP monomer fraction (Monomer) and the 80-130kDa high molecular weight fraction (+BS^3) relative to the intensity of total GAPR-1-GFP at 24h.



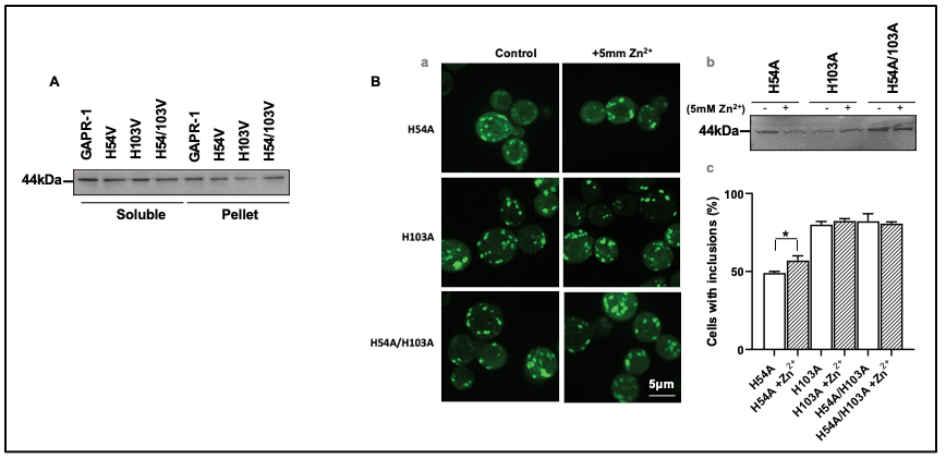
Suppl. Figure 2, Sirati et al.

Figure S2. A) Dot blot analysis of GFP, GAPR-1 and Δmyr-GAPR-1-GFP. Protein expression was induced for 24h. Total homogenates were prepared using Y-PERTM reagent. After centrifugation, the pellet was treated with 2% SDS and separated into a SDS-soluble and SDS-insoluble fraction as described in Materials and Methods. The same volume (50 µl) of each sample was loaded onto nitrocellulose membranes and analysed using an anti-GFP (top panel) and anti-GAPR-1 antibodies (middle and lower panel); B) Representative image of yeast cells with GAPR-1-GFP expression after 24h without ThT staining; C) GFP expressing yeast cells (24h) were collected, homogenised and treated without or with 1% TX-100 for 30 minutes on ice, followed by isopycnic density-gradient centrifugation. Fractions were collected from top to bottom, and proteins in each fraction were separated by SDS-PAGE and analysed by WB using an anti-GFP antibody; D) Homogenates (20 µg protein) from yeast cells expressing GAPR-1-GFP or ∆myr-GAPR-1-GFP for 24h were separated by SDS-PAGE and analysed by WB for the presence of GAPR-1-GFP and GAPDH (loading control); E) Representative images of 6h and 24h expression of GAPR-1-GFP and Δmyr-GAPR-1-GFP in ΔNMT1-181 (N-myristoyltransferase 1 deletion) in yeast strain BY4741. Inclusion formation of GAPR-1-GFP in wildtype yeast strain BY4741 is shown as control.



Suppl. Figure 3, Sirati et al.

Figure S3. A) Growth curve yeast cells harboring GAPR-1-GFP or GFP in presence of (0- 2.5- 5 and 10 mM $ZnCl_2$) in three different time points (OD600=0.2); B) Representative images (3h, top panels) and growth curves (3h, 6h, 24h, bottom panel) of GFP and GAPR-1-GFP expressing cells in the presence of different concentrations of CuCl2; C) Quantification of yeast cells with inclusions in presence of different concentrations of Cu2+. The number of cells displaying cytoplasmic inclusions is quantified as described in Materials and Methods and presented as percentage of the total number of the cells. The results are expressed as mean ± SD, n=3. Significance of differences was calculated with two-tailed unpaired t-test ($*p < 0.5$).



Suppl. Figure 4, Sirati et al.

Figure S4. A) Homogenates (20 μg protein) from yeast cells expressing GAPR-1-GFP, H54V, H103V and H54/H103V for 24h were separated into soluble and pellet fractions by centrifugation (100.000xg, 30 min) and analysed by WB using an anti-GAPR-1 antibody; B) Cells were transformed with H54A, H103A and H54A/H103A (zinc ion binding site-mutants) plasmids and protein expression was induced for 3h in the absence and presence of 5 mM $ZnCl_2$; a) Representative images from live-cell fluorescence microscopy; b) Homogenates (20 μg protein) from each condition described in (a) were separated by SDS-PAGE and analysed by WB using an anti-GAPR-1 antibody; c) Quantification of fluorescent inclusions in cells that were incubated as described for panel (a), Quantification was performed as described in Materials and Methods.

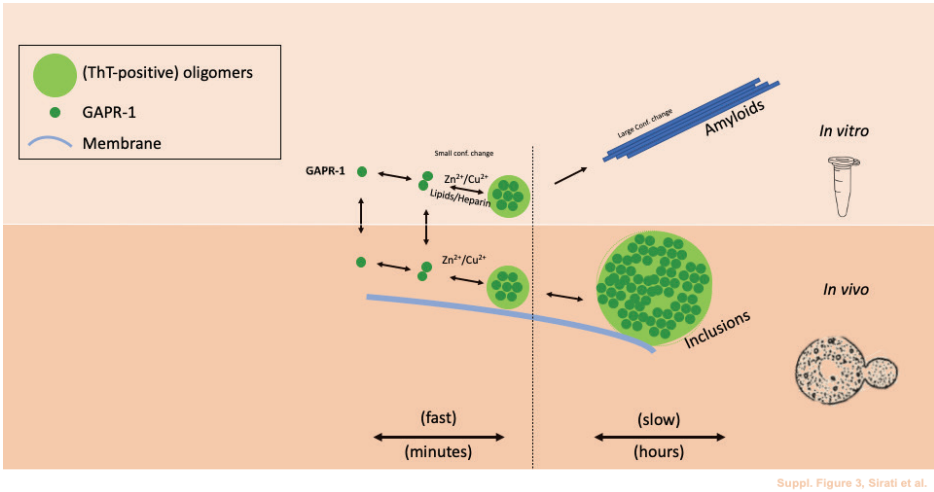


Figure S5. Model- GAPR-1 amyloid-like oligomerisation *in vitro* and *in vivo*.

Abstract

Golgi-Associated plant Pathogenesis Related protein 1 (GAPR-1) acts as a negative regulator of autophagy by interacting with Beclin 1 at Golgi membranes. The molecular mechanism of this interaction is largely unknown. We recently showed that GAPR-1 has amyloidogenic properties resulting in the formation of protein condensates upon overexpression in *Saccharomyces cerevisiae*. Here we show that Beclin 1 has several predicted amyloidogenic regions and that overexpression of Beclin 1-mCherry in yeast also results in the formation of fluorescent protein condensates. Surprisingly, co-expression of GAPR-1-GFP and Beclin 1-mCherry results in a strong reduction of Beclin 1 condensates. Mutations of the known interaction site on the GAPR-1 and Beclin 1 surface abolished the effect on condensate formation during co-expression without affecting the condensate formation properties of the individual proteins. Similarly, a Beclin 1-derived B18 peptide that is known to bind GAPR-1 and to interfere with the interaction between GAPR-1 and Beclin 1, abolished the reduction of Beclin 1 condensates by co-expression of GAPR-1. These results indicate that the same type of protein-protein interactions interfere with condensate formation during co-expression of GAPR-1 and Beclin 1 as previously described for their interaction at Golgi membranes. The amyloidogenic properties of the B18 peptide were, however, important for the interaction with GAPR-1, as mutant peptides with reduced amyloidogenic properties also showed reduced interaction with GAPR-1 and reduced interference with GAPR-1/Beclin 1 condensate formation. We propose that amyloidogenic interactions take place between GAPR-1 and Beclin 1 prior to condensate formation.

Keywords: CAP superfamily, Amyloid-like proteins, Protein-Protein interactions (PPIs), Autophagy, Protein inclusions.

Introduction

Beclin 1 is one of the major autophagy-related proteins that induce and up-regulate autophagy as a core component of the Class III Phosphatidylinositol 3-Kinase (PI3K)/vacuolar protein sorting 34 (Vps34) complex [1,2]. It functions as a molecular platform assembling an interactome with stimulating and suppressive components that bind to different Beclin 1 domains [3–5]. Some of these interactions are regulated by Beclin 1 homo-oligomerization, which favors binding to the antiapoptotic and Beclin 1-interacting protein Bcl-2 (B cell lymphoma 2) and disfavors binding to the Vps34-containing class III PI3K complex [6–9]. For many other Beclin 1-interacting proteins [2–4,10,11], it remains to be established whether or how Beclin 1 oligomerization affects their interaction with Beclin 1.

Golgi-associated plant pathogenesis-related protein 1 (GAPR-1), also known as GLIPR2, has been identified as a negative regulator of Beclin 1 [12]. GAPR-1 belongs to the Cysteine-rich secretory proteins, Antigen 5 and Pathogenesis-related (CAP) protein superfamily [5]. GAPR-1, a small peripheral membrane protein, is localized to lipid-rich microdomains of the Golgi apparatus [13]. By binding to GAPR-1, Beclin 1 is retained at the cytosolic leaflet of the Golgi complex and autophagy is downregulated [12]. The interaction between Beclin 1 and GAPR-1 can be disrupted by a Beclin 1-derived peptide (residues 267-284, Tat-Beclin 1), which corresponds to the potential GAPR-1/Beclin 1 binding interface [12]. Interaction of the Tat-Beclin 1 peptide with GAPR-1 resulted in the redistribution of Beclin 1 from the Golgi to the cytosol and activation of autophagy [12].

Crosslink studies showed that GAPR-1 has a strong propensity to form homodimers *in vivo* and *in vitro* [14]. GAPR-1 oligomerizes in the presence of different stimuli like negatively charged lipids, IP6, heparin and metal ions [15,16] and oligomerization results in the formation of amyloid-like fibrils in time [15–17]. Recently, the amyloidogenic properties of GAPR-1 were supported by *in vivo* studies in *Saccharomyces cerevisiae*, showing that expression of human GAPR-1 results in the formation of protein condensates [18]. We hypothesized that these amyloidogenic properties could be involved in the regulation of autophagy [19,20]. This is in line with recent findings revealing biological functions of amyloidogenic proteins or peptides, therefore often referred to as functional amyloids [21–26].

In this study, we used *Saccharomyces cerevisiae (S. cerevisiae)* as a eukaryotic model system to study mechanisms related to protein folding and quality control [27–29]. In *S. cerevisiae*, the amyloidogenic properties of proteins can result in self-templating and formation of protein condensates in cytosol [30]. This system has been widely used to investigate the oligomeric/amyloid properties of known amyloid-like peptides or proteins, such as Aβ, Tau, α synuclein, prions, and huntingtin (Htt), as well as proteins

and peptides with predicted amyloidogenic behavior [31–35]. We previously used this yeast model system to elucidate the characteristics of amyloid-like oligomerization and protein condensate formation of GAPR-1 [18]. Here, we investigated the amyloidogenic properties of Beclin 1 and characterized protein-protein interactions of Beclin 1 with GAPR-1 in co-expression experiments. Our result imply that this assay system can be expanded to investigate other protein-protein interactions as well.

Results

Beclin 1 clusters into fluorescent condensates in yeast cytosol

To access the amyloidogenic propensity of human Beclin 1, we first analyzed its primary sequence using the amyloid prediction software Amylpred2 [36]. Several regions with potential amyloidogenic properties were found (Fig. 1A). The *in vivo* oligomerization properties of Beclin 1 were analyzed by investigating its propensity to form protein condensates upon overexpression in yeast. To this end, C-terminally tagged Beclin-1 with either GFP (Beclin 1-GFP) or m-Cherry (Beclin 1-mCherry) was expressed under the control of the galactose-inducible promoter (*GAL1*) from a high copy (2µ) plasmid to allow a rapid and synchronized protein expression. Overexpression of the tagged Beclin 1 constructs did not affect yeast growth (Supplementary Fig. 1A). Both Beclin 1-GFP and Beclin 1-mCherry displayed similar behavior in live-cell fluorescence microscopy by forming protein condensates (Supplementary Fig. 1B). Beclin 1-mCherry construct was selected for follow up experiments. Condensate formation over time (3h, 6h and 24h) post-induction was monitored (Fig. 1B) and quantified (Fig. 1C) using live-cell fluorescence microscopy. After 3h, the majority of expressed protein was present in the cytosol and 18% of the cells contained protein condensates, which increased to 52% after 24h (Fig. 1C). During this period, Beclin 1 protein expression levels were comparable (Fig. 1D), indicating that additional factors affect condensate formation.

A hallmark of amyloid-like oligomerization is the partial resistance to solubilization by SDS. After expression of Beclin 1-mCherry, the yeast homogenate was therefore treated with a mild zwitterionic detergent and separated into a detergent-soluble (supernatant) and detergent-insoluble (pellet) fraction. The detergent-insoluble fraction was then treated with 2% SDS and separated into a SDS-soluble and SDS-insoluble fraction. Western blot analysis revealed that Beclin 1-mCherry is partially SDS-insoluble (Fig. 1E). These findings are consistent with observations by others, suggesting a link between assemblies of membraneless organelles and the amyloid-like properties of proteins [34,37,38].

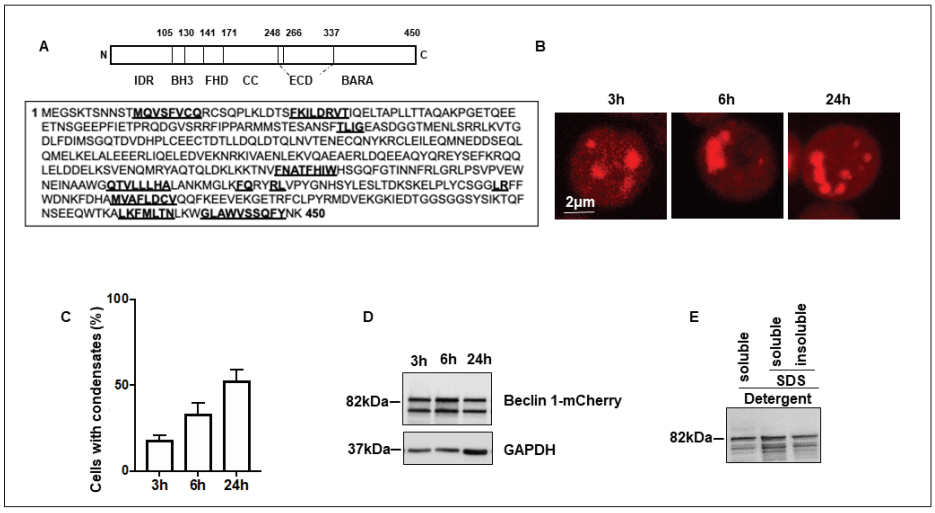


Figure 1, Sirati et al.

Figure 1. Beclin 1 clusters into fluorescent condensates in yeast cytosol. A) Human BECN1 sequence and domains: the intrinsically disordered region (IDR), the BCL-2 homology-3 motif (BH3), the flexible helical domain (FHD), the coiled-coil domain (CC), the evolutionarily conserved domain (ECD), and the β/α-repeated, autophagy-related domain (BARA). In silico analysis of Beclin 1 performed with amyloid prediction software (Amylpred2) identified eleven amyloidogenic regions (underlined sequences); B) Live-cell fluorescence microscopy of yeast cells expressing Beclin 1-mCherry after 3h, 6h and 24h in SC-galactose induction medium; C) Quantification of yeast cells displaying Beclin 1-mCherry condensates. For each time point, the number of cells displaying cytoplasmic condensates is presented as a percent of the total number of cells. For quantification, at least 200 cells were counted per time point and per experiment. Results are expressed as mean ± SD, n=5; D) Beclin1-mCherry expressing cells were collected at 3h, 6h and 24h post-induction and total protein homogenates were prepared. Equal amounts of total protein homogenates were separated by SDS-PAGE gel, transferred onto nitrocellulose membrane and analyzed using an anti-Beclin 1 antibody. Anti-GAPDH antibody served as loading control; E) Beclin 1-mCherry expression was induced for 24h. Total protein homogenate was prepared using Y-PERTM reagent. Pellet was treated with 2% SDS and separated into a SDS-soluble and SDS-insoluble fraction as described in Materials and Methods. The same volume (20µl) of each sample was separated by SDS-PAGE and analyzed by Western blot using an anti-Beclin 1 antibody.

GAPR-1 affects Beclin 1 protein condensate formation

We previously showed that GAPR-1 acts as a negative regulator of autophagy by binding to Beclin 1 on Golgi membranes [12]. Since both GAPR-1 [15,19,20] and Beclin 1 (Fig. 1) have amyloidogenic properties and cluster into protein condensates, we hypothesized that co-expression of both proteins might affect condensate formation of each other in *S. cerevisiae.* Indeed, co-expression of GAPR-1-GFP and Beclin 1-mCherry resulted in a significant reduction of both protein condensates (Fig. 2A). In the presence of GAPR-1, the majority of cells showed cytosolic Beclin 1, suggesting that Beclin 1 is not able to cluster in condensates in the presence of GAPR-1. After 24h of co-expression, the number of cells with Beclin 1 condensates dropped from 52% (in the absence of GAPR-1) to 13% (Fig. 2B). Condensate formation of GAPR-1 was affected differently by co-expression of Beclin 1. The number of cells with GAPR-1 condensates increased in the presence of Beclin 1, especially at 3h and 6h of protein expression (Fig. 2B). After 24h, almost all cells contained GAPR-1 condensates, both in the absence or presence of Beclin 1. However, quantification of the number of condensates per cell after 24h revealed that 70% of the GAPR-1 expressing cells contained ≥6 condensates, which was strongly reduced to 15% by co-expression of Beclin 1 (Fig. 2C). To exclude the possibility that different expression levels of these two proteins in co-expressing cells affect condensate formation, we performed Western blot analysis. Results showed that the intensity of the bands related to GAPR-1-GFP and Beclin 1-mCherry were comparable in single and co-expression conditions, indicating that the expression levels of GAPR-1 and Beclin 1 were not affected by the different experimental conditions (Fig. 2D). In support of this, we found that the effect of GAPR-1 co-expression on Beclin 1 condensate formation was independent of the type of yeast promoter: similar results were obtained with the alcohol dehydrogenase I (ADH1) promoter of *Saccharomyces cerevisiae,* a constitutive promoter resulting in high protein level production, or when using a combination of the *GAL1* and *ADH1* promoter (Supplementary Fig. 2A, B).

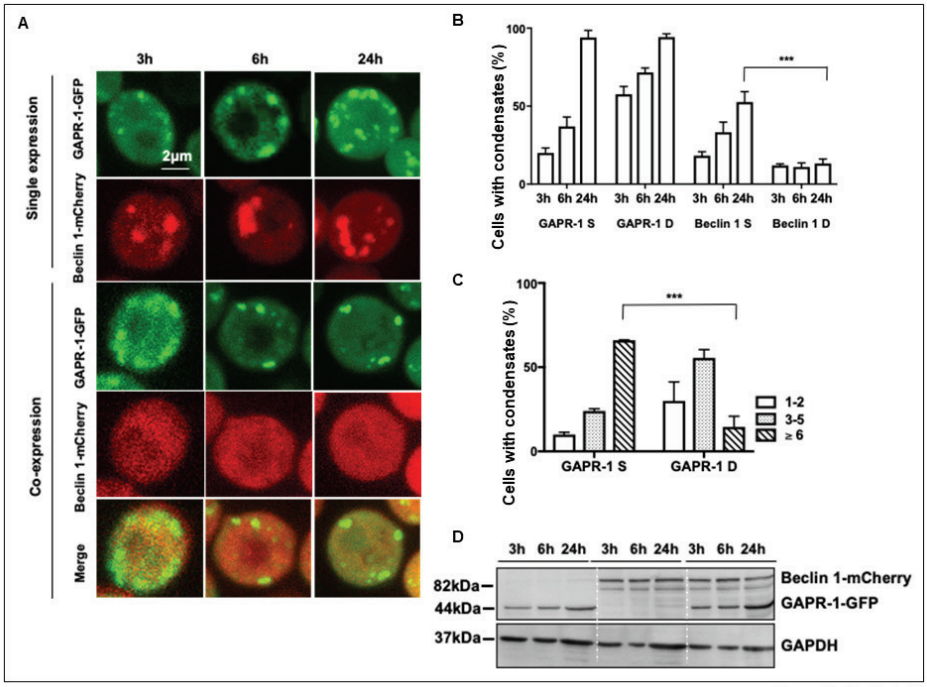


Figure 2. GAPR-1 affects Beclin 1 protein condensate formation. A) Live-cell fluorescence microscopy of yeast cells upon single expression or co-expression of GAPR-1-GFP and Beclin 1-mCherry after 3h, 6h and 24h of induction; B) Quantification of yeast cells displaying GAPR-1 and Beclin 1 condensates upon single expression (S) or co-expression (D). For each time point, the number of cells displaying cytoplasmic condensates is presented as a percent of the total number of cells. Results are expressed as mean ± SD, n=5. Significance of differences was calculated with a two-tailed unpaired t-test (***p<0.001); C) Quantification of the number of GAPR-1-GFP condensates in single and co-expression experiments. The number of condensates per cell were categorized in three groups (1-2, 3-5 and ≥6 per cell). At least 100 cells were counted per experiment. Results are expressed as mean ± SD, n=3. The significance of differences was calculated with two-tailed unpaired t-test (***p<0.001); D) Single and co-expressing cells were collected for total protein homogenates 3h, 6h and 24h post-induction and protein expression levels were assessed by Western blot using anti-GAPR-1 and anti-Beclin 1 antibodies. Anti-GAPDH antibody served as a loading control.

Specific protein-protein interactions are involved in GAPR-1/Beclin 1 interaction

We first confirmed that human GAPR-1 and human Beclin 1 can interact directly in yeast by performing a Bimolecular Fluorescence Complementation (BiFC) assay [39,40]. We tagged non-fluorescent complementary fragments of Venus yellow fluorescent protein (VN and VC) to the N-terminus of Beclin 1 (VN-Beclin 1) and N- or C-terminus of GAPR-1 (VN-GAPR-1 and GAPR-1-VC, respectively). In the absence of either Beclin 1 (VN-GAPR-1 + empty VC) or GAPR-1 (VN-Beclin 1 + empty VC), no fluorescent signal was observed (Supplementary Fig. 3). As a positive control, we co-expressed VN-GAPR-1 and GAPR-1-VC. As expected, the homo-dimerization/oligomerization properties of GAPR-1 [14,15] resulted in a YFP fluorescent signal. Co-expression of VN-Beclin 1 and GAPR-1-VC also resulted in an increased fluorescent signal (Supplementary Fig. 3). These results show that upon overexpression in yeast, the human proteins GAPR-1 and Beclin 1 directly interact.

To investigate the nature of GAPR-1 interference with Beclin 1 condensate formation, we made use of the fact that GAPR-1-GFP condensate formation in yeast cells is strongly delayed when GAPR-1 is not myristoylated (Δmyr-GAPR-1-GFP) [18]. Indeed, at early time points of expression, Δmyr-GAPR-1-GFP is mainly cytosolic, and after 24h expression, only single condensates were found in 36% of cells (Fig. 3A, B, single expression). Interestingly, Δmyr-GAPR-1-GFP is as efficient in reducing Beclin 1-mCherry condensate formation as myristoylated-GAPR-1-GFP (Fig. 3A, B, cf. Fig. 2B). The amount of Δmyr-GAPR-1-GFP condensates was reduced from 36% of positive cells (single expression) to 5% upon co-expression of Beclin 1 (Fig. 3A, B). Western blot analysis of total protein homogenates revealed similar expression levels of both proteins in single or co-expression experiments (Fig. 3C). These results show that myristoylation is not required for the interaction between GAPR-1 and Beclin 1. Our data also suggest that GAPR-1 interference of Beclin 1-mCherry condensate formation is independent of the presence of GAPR-1 condensates as high (wt-GAPR-1) or strongly reduced (Δmyr-GAPR-1) GAPR-1 condensates are equally efficient. As Δmyr GAPR-1 is a cytosolic protein in yeast [18], the GAPR-1 interaction with Beclin 1 is likely to take place in the cytosol, prior to condensate formation.

These findings prompted us to investigate the nature of the protein-protein interactions between GAPR-1 and Beclin 1 in more detail. The GAPR-1 surface area that interacts with Beclin 1 has been mapped to 5 amino acids lining the equatorial groove (H54/E86/G102/H103/N138) [41]. To investigate whether the same amino acids interfere with condensate formation during co-expression experiments, we mutated these 5 amino acids (5m GAPR-1-GFP, H54A/E86A/G102K/H103A/N138G) and expressed 5m GAPR-1-GFP in the absence or presence of Beclin 1 (Fig. 3D,E). Expression of 5m GAPR-1-GFP still resulted in condensate formation. Interestingly, under co-

expression conditions, Beclin 1 condensate formation was not affected anymore (Fig. 3D,E). Western blot analysis showed similar expression levels of the proteins involved, ruling out effects on condensate formation due to different protein expression levels (Fig. 3F).

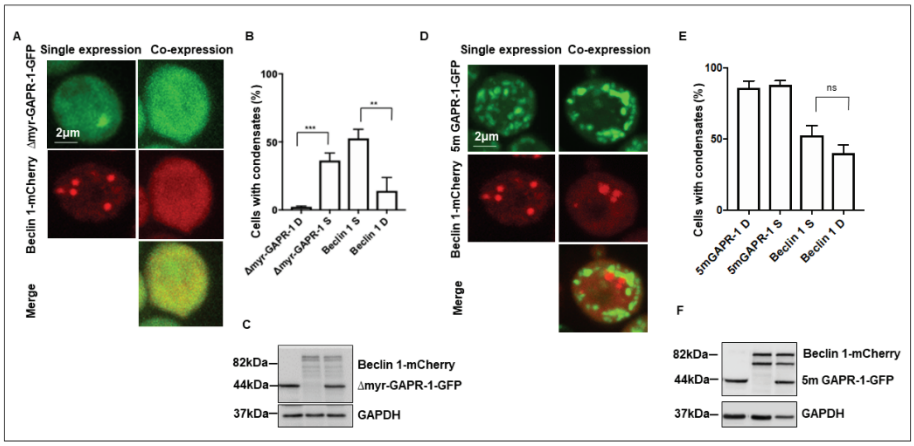


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Figure 3. Specific protein–protein interactions are involved in GAPR-1/Beclin 1 interaction. A) Live-cell fluorescence microscopy of yeast cells upon single expression of Δmyr-GAPR-1-GFP or Beclin 1-mCherry or co-expression of the two proteins 24h post-induction; B) Quantification of yeast cells displaying Δmyr-GAPR-1-GFP condensates (left two bars) or Beclin 1-mCherry (right two bars). Cells were single (S) expressed with either Δmyr-GAPR-1-GFP (S) or Beclin 1-mCherry (S), or double (D) expressed with Δmyr-GAPR-1-GFP and Beclin 1-mCherry (Δmyr-GAPR-1-GFP D and Beclin 1-mCherry (D); C) Western blot analysis of total protein homogenates of Δmyr-GAPR-1-GFP and Beclin 1-mCherry 24h post-induction. Left lane: Δmyr-GAPR-1-GFP expressing cells; Middle lane: Beclin 1-mCherry expressing cells; Right lane: Cells expressing both Δmyr-GAPR-1-GFP and Beclin 1-mCherry. An anti-GAPR-1 and an anti-Beclin 1 antibodies were used for immunoblotting and an anti-GAPDH antibody served as a loading control; D) Live-cell fluorescence microscopy of yeast cells upon single expression of 5mGAPR-1-GFP or Beclin 1-mCherry and co-expressing the two proteins 24h post-induction; E) Quantification of yeast cells displaying GFP condensates (left two bars) upon 5mGAPR-1-GFP single expression (S) or co-expression with Beclin 1-mCherry (5mGAPR-1-GFP D), and m-Cherry condensates (right two bars) upon single expression of Beclin 1-mCherry (S) or co-expression with 5mGAPR-1-GFP (Beclin 1-mCherry (D). All quantifications were done 24h post-induction. Results are expressed as mean ± SD, n=3. The significance of differences was calculated with a two-tailed unpaired t-test (**p<0.01, ***p<0.001); F) Western blot analysis of total protein homogenates of cells expressing 5mGAPR-1-GFP

and/or Beclin 1-mCherry 24h post-induction. Left lane: 5mGAPR-1-GFP expressing cells; Middle lane: Beclin 1-mCherry expressing cells; Right lane: Cells expressing both 5mGAPR-1-GFP and Beclin 1-mCherry. An anti-GAPR-1 and anti-Beclin 1 antibodies were used for immunoblotting and an anti-GAPDH antibody served as a loading control.

Comparable results were obtained when mutating the Beclin 1 surface that interacts with GAPR-1. We previously described a binding interface of Beclin 1 to GAPR-1 comprising residues 267-284 of Beclin 1 (termed B18, see [12]). We deleted residues 267-284 in Beclin 1 (ΔB18). Expression of ΔB18 in yeast cells showed that in the absence of these residues, ΔB18-mCherry was still able to cluster into condensates (Fig. 4A, single expression) with similar efficiency as compared to wild-type (wt) Beclin 1-mCherry (Fig. 4B, cf. Fig. 2B and Fig. 3). However, co-expression of GAPR-1-GFP did not affect the condensate formation of the Beclin 1 mutant protein ΔB18 (Fig. 4A,B), despite equal expression levels under the various conditions (Fig. 4C). Next, we used a membrane-permeable B18 peptide (Tat-B18) that efficiently competes the Beclin 1/GAPR-1 interaction on Golgi membranes in mammalian cells [12,41] to challenge the interference of condensate formation by co-expression of GAPR-1 and Beclin 1 in yeast. Tat-scrambled B18 peptide (Tat-Scr) was used as a control. Cells expressing wt Beclin 1-mCherry with or without wt GAPR-1-GFP were treated with each peptide at a concentration of 5 μM. The Tat-B18 peptide efficiently competed with the Beclin 1/GAPR-1 interaction, resulting in Beclin 1-mCherry condensate formation in the presence of GAPR-1 (Fig. 4D, E). The Tat-Scr peptide did not show this effect.

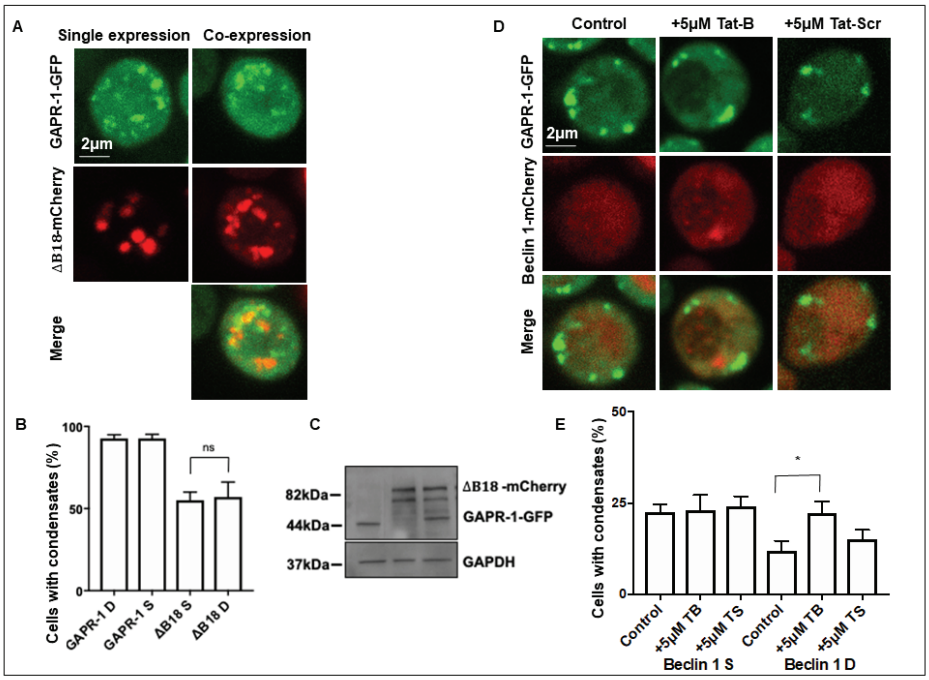


Figure 4. Specific protein–protein interactions are involved in GAPR-1/Beclin 1 interaction. A) Live-cell fluorescence microscopy of yeast cells upon single or co-expression of GAPR-1-GFP and ΔB18-mCherry after 24h induction; B) Quantification of yeast cells displaying i) GFP condensates (left two bars) in GAPR-1-GFP single (GAPR-1 S) and co-expression with ΔB18-mCherry (GAPR-1 D) and ii) m-Cherry condensates (right two bars) in single ΔB18-mCherry single (ΔB18 S) and co-expression with GAPR-1-GFP (ΔB18 D). Quantifications were done after 24h induction. Results are expressed as mean ± SD, n=3. The significance of differences was calculated with two-tailed unpaired t-test (∗p<0.5); C) Western blot analysis of total protein homogenates of cells expressing GAPR-1-GFP and/or ΔB18-mCherry 24h post-induction. Left lane: GAPR-1-GFP expressing cells; Middle lane: (ΔB18-mCherry expressing cells; Right lane: cells expressing GAPR-1-GFP and Beclin 1-mCherry. An anti-GAPR-1 and anti-Beclin 1 antibodies were used for immunoblotting. Anti-GAPDH antibody served as a loading control; D) Live-cell fluorescence microscopy of yeast cells co-expressing GAPR-1-GFP and Beclin 1-mCherry in the absence (control) or presence of 5 μM Tat-Beclin 1 peptide or Tat-Scrambled peptide after 6h co-expression. To induce peptide entry, cells were treated as indicated in Materials and Methods; E) Quantification of yeast cells displaying Beclin 1-mCherry condensates upon single expression (S) or co-expression with GAPR-1-GFP (D) in absence or presence of Tat-Beclin or Tat-Scrambled peptides. After 6h

induction, the number of cells displaying cytoplasmic mCherry condensates was quantified and presented as a percent of the total number of the cells. Results are expressed as mean ± SD, n=3. The significance of differences was calculated with two-tailed unpaired t-test (∗p<0.5).

Amyloid-like interactions between Beclin 1 and GAPR-1 *in vitro* and *in vivo*

Our combined data strongly suggest that the same type of PPIs interfere with condensate formation during co-expression of GAPR-1 and Beclin 1 as previously described for the GAPR-1/Beclin1 interaction at Golgi membranes [12,41]. This raises the question of whether the amyloidogenic properties of GAPR-1 and Beclin 1 are important for the PPI between the two proteins or whether they only play a role during subsequent condensate formation. The B18 peptide overlaps exactly with a region in the ECD domain of Beclin 1 that is predicted to have amyloidogenic characteristics according to Amylpred2 (Figs. 1A, 5A). The amyloid-like behavior of the Tat-B18 peptide was investigated using the anti-amyloid-oligomer antibody A11 [15,33]. This antibody recognizes the common fold of pre-fibrillar oligomers that are early kinetic intermediates, but it does not recognize monomers or mature protein amyloid fibrils [34,35]. Amyloid-like oligomer formation of Tat-B18 after different incubation times was monitored using the A11 antibody in a dot blot assay. Strikingly, Tat-B18 showed a strong affinity to A11, even without prolonged incubation (Fig. 5B). This behavior is in contrast to the behavior of the Aβ peptide (Aβ(1-40)), which showed a transient formation of pre-fibrillar oligomers after 2-4h (Fig. 5B), as also shown by others [15,33,35]. Tat-Scr-B18 peptide was not recognized by the A11 antibody under these conditions. Similarly, a single point mutation in the B18 peptide (F274S) that was shown to abrogate the GAPR-1/Beclin 1 interaction [12], severely hampered the interaction with the A11 antibody (Fig. 5B). To investigate whether the oligomeric properties of Tat-B18 peptide allows interaction with GAPR-1, we incubated Tat-B18, Tat scrambled and Tat F274S for different times and spotted them onto a nitrocellulose membrane. The membrane was subsequently incubated with GAPR-1 recombinant protein and binding of GAPR-1 was analyzed using an anti-GAPR-1 antibody. GAPR-1 showed a high affinity for Tat-B18 under all conditions, a strongly reduced affinity for Tat-F274S, and no affinity for the Tat-Scr-B18 peptide (Fig. 5C, right panel). These binding properties closely correlate with the affinity of these peptides for the A11 antibody (Fig. 5B) and suggest that the amyloid-like behavior of these peptides is important for the interaction with GAPR-1. As a positive control we used the Ab peptide, previously shown to interact with GAPR-1 [15]. The Ab peptide also interacts with the A11 antibody and GAPR-1, but with different kinetics (Fig. 5B, C).

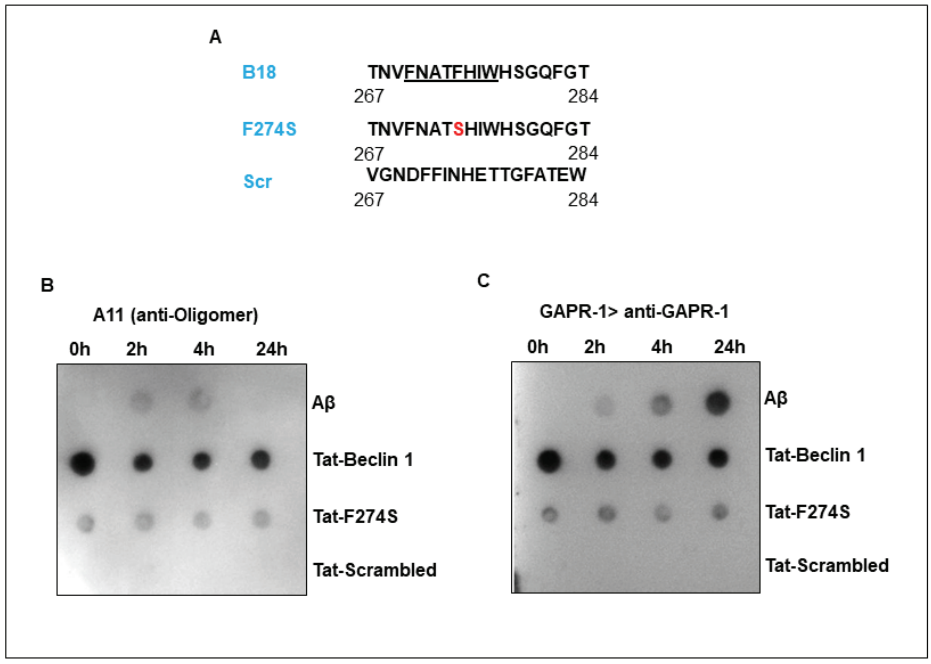


Figure 5. Amyloid-like interactions between Beclin 1 and GAPR-1 *in vitro* and *in vivo*. A) Amyloid prediction using Amylpred2 software of the B18 peptide (AA 267-284), within the ECD domain. A major portion of the peptide region was predicted to support amyloid characteristics. The corresponding amino acid sequence of the B18 peptide with a point mutation (F274S) and the B18 scrambeled peptide (Scr) is also shown; B) Aliquots of Aβ peptide, Tat-B18, Tat-F274S and Tat-Scr were taken after different incubation time points (0-24h), spotted onto a nitrocellulose membrane and analyzed for A11 reactivity; C) Aliquots of Aβ peptide, Tat-B18, Tat-F274S and Tat-Scr were taken after different incubation time points (0-24h) and spotted on a nitrocellulose membrane. Subsequently, the membrane was incubated with recombinant GAPR-1 protein and binding was analyzed using an anti-GAPR-1 antibody.

GAPR-1 interacts with the amyloidogenic Beclin 1 derived peptide (B18) *in vivo*

In line with the *in vitro* amyloidogenic behavior of the B18 peptide, *in vivo* expression of B18 with C-terminally tagged mCherry in yeast cells also resulted in a time-dependent increase of condensate formation (Fig. 6A). After 24h induction of B18-mCherry, 44% of the cells contained protein condensates (Fig. 6B). In contrast, expression of scrambled

B18-mCherry peptide resulted in around 6% of cells with protein condensates that did not increase after 24h of expression. Interestingly, co-expression of the B18 peptide with GAPR-1 showed a reduction of the number of cells containing B18 condensates to 18%, which was comparable to the reduction of Beclin 1 condensates after GAPR-1 co-expression (Fig. 6A, B). Moreover, quantification of GAPR-GFP condensates per cell indicated a comparable decrease due to B18-mCherry and due to Beclin 1-mCherry co-expression (Fig. 6C). No significant differences were observed in co-expression of GAPR-GFP with scrambled B18-mCherry peptide (Fig. 6C). The protein and peptide expression levels remained constant during single and co-expression experiments, ruling out artificial effects due to differences in protein concentrations (Fig. 6D).

To investigate a role of the amyloidogenic properties of the B18 peptide in competing with Beclin 1/GAPR-1 binding [12], we performed triple expression experiments with GAPR-1-GFP, Beclin 1-mCherry and B18-CFP. In the presence of all three components, GAPR-1 and Beclin 1 formed condensates, indicating that B18-CFP efficiently competes with the GAPR-1/Beclin 1 interaction (Fig. 6E, F). When all three components were expressed, B18-CFP was not found in condensates. Beclin 1 condensate formation was dependent on the presence of B18, as in the absence (empty vector) of B18-CFP, the presence of Beclin1-mCherry was strongly reduced (Fig. 6E, F). In the presence of scrambled B18 peptide, condensate of Beclin 1 remained inhibited by GAPR-1. The effect of B18 on Beclin 1 condensate formation correlated strongly with the amyloidogenic properties of the B18 peptide, as the B18-scrambled peptide had no effect on Beclin 1 condensate formation and the B18-F274S peptide had a reduced effect on Beclin 1 condensate formation (Fig. 6E,F).

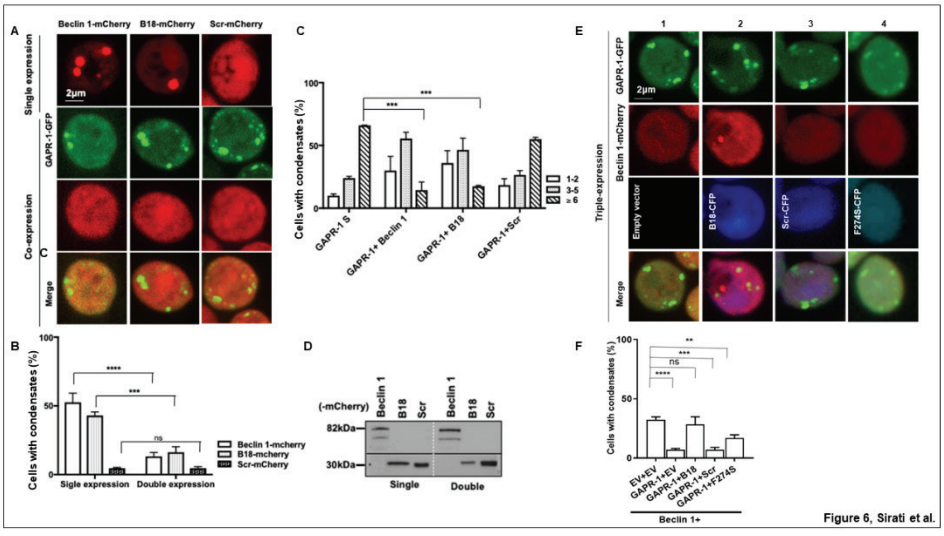


Figure 6, Sirati et al.

Figure 6. GAPR-1 interacts with the amyloidogenic Beclin 1 derived peptide (B18) *in vivo*. A) Live-cell fluorescence microscopy of yeast cells upon single expression of Beclin-mCherry, B18-mCherry or scrambled-mCherry, and upon co-expression with GAPR-1-GFP after 24h; B) Quantification of yeast cells displaying mCherry condensates in single and co-expression experiments with GAPR-1 after 24h. Results are expressed as mean ± SD, n=3. The significance of differences was calculated with two-tailed unpaired t-test (∗p<0.5); C) Quantification of the number of GAPR-1-GFP condensates in single or co-expression experiments with Beclin 1, B18 and Scrambled (-mCherry) cells. The number of condensates per cell was categorized in three groups (1-2, 3-5 and ≥ 6 per cell). At least 100 cells were counted per experiment. Results are expressed as mean ±SD, n=3. The significance of differences was calculated with two-tailed unpaired t-test (∗∗∗p<0.001); D) Western blot analysis of samples from (C), using an anti-mCherry antibody; E) Live-cell fluorescence microscopy of yeast cells triple-expressing i) GAPR-1-GFP, Beclin 1-mCherry and Empty Vector p425 (panels lane 1); ii) GAPR-1-GFP, Beclin 1-mCherry and B18-CFP (panels lane 2); iii) GAPR-1-GFP, Beclin 1-mCherry and Scrambled-CFP (panels lane 3); iv) GAPR-1-GFP, Beclin 1-mCherry and F274S-CFP (panels lane 4) after 24h induction; F) Quantification of yeast cells displaying Beclin 1-mCherry condensates in single and triple-expression with GAPR-1-GFP/Empty Vector p425, GAPR-1-GFP/B18-CFP, GAPR-1-GFP/Scrambled-CFP, and GAPR-1-GFP/F274S-CFP after 24h induction. Results are expressed as mean ±SD, n=3. The significance of differences was calculated with two-tailed unpaired t-test (∗∗p<0.01, ∗∗∗p<0.001).

Discussion

GAPR-1 is a negative regulator of autophagy by interacting with Beclin 1 [12]. We recently showed that GAPR-1 contains amyloidogenic properties *in vitro* [15–17] and *in vivo* by forming reversible amyloid-like oligomers [18]. We now identified Beclin 1 as a second amyloidogenic protein involved in autophagy. Expression of Beclin 1-mCherry in *S. cerevisiae* results in the formation of condensates, and the expressed protein is partially resistant to SDS solubilization. Beclin 1 consists of an N-terminal intrinsically disordered region, a flexible helical domain, a coiled-coil domain and a C-terminal β/α repeated, autophagy-related (BARA) domain [2,11]. Several studies showed that full-length Beclin 1 can form stable homo-oligomers under various conditions and that the N-terminal domain, the coiled-coil domain and the BARA domain facilitate this self-assembly in different ways [2,11,41]. Oligomerized Beclin 1 is proposed to provide a platform for many Beclin 1-associating proteins [2–4,10,11]. Our results indicate that Beclin 1 contains several predicted amyloidogenic regions that may contribute to Beclin 1 condensate formation in yeast upon overexpression. The amyloidogenic region within the ECD domain was confirmed by overexpression of the B18 peptide, resulting in the

formation of condensates (Fig. 6). The amyloidogenic region of the B18 peptide is, however, not required for Beclin 1 condensate formation, as overexpression of Beclin ΔB18 still resulted in protein condensate formation (Fig. 4). These results suggest that the other predicted amyloidogenic regions in Beclin 1 contribute significantly to its amyloidogenic behavior.

Our results indicate that the amyloidogenic region located at the N-terminal region of the BARA domain (the ECD domain) contributes to the interaction with GAPR-1. An interaction between GAPR-1 and Beclin 1 has been shown in direct and indirect ways [12,41,42]. In this study, we confirmed a direct interaction between GAPR-1 and Beclin 1 using the BiFC method, which allows visualization and analysis of protein-protein interactions in live cells [43,44] by complementary reconstitution of a fluorescent protein [43,45]. The interaction area on the Beclin 1 protein has been mapped to amino acids 267–284 (corresponding to the B18 peptide), as the B18 peptide interferes with the GAPR-1/Beclin 1 interaction [12]. The interaction area of the B18 peptide on the GAPR-1 protein has been mapped to 5 amino acids in the equatorial surface groove of GAPR-1 [41]. Mutation of the five conserved residues lining this groove, H54A/E86A/G102K/H103A/N138G abrogated Beclin 1 binding. Remarkably, mutating these amino acids resulted in a shift of monomeric to dimeric GAPR-1 in solution [41]. This may suggest that the oligomeric state of GAPR-1 is involved in the GAPR-1/Beclin 1 interaction.

Various publications have reported monomeric, dimeric, and homo-oligomeric GAPR-1 complexes [13,15,16,41,46], implying that different oligomeric states may have different mechanisms and impact on biological functions. We recently showed that GAPR-1 also has amyloidogenic properties and that several characteristics of GAPR-1 amyloid-like fibril formation *in vitro* closely resemble the characteristics of condensate formation *in vivo* [18]. We now show that in addition to GAPR-1 [18], also Beclin 1 has amyloidogenic properties under *in vivo* conditions, which offers the intriguing possibility that the GAPR-1/Beclin 1 interaction is (partially) based on amyloidogenic oligomeric interactions. Indeed, when GAPR-1 and Beclin 1 are co-expressed in *S. cerevisiae,* they affect condensate behavior of each other. As we did not find any evidence for co-localization of the co-expressed proteins to the same condensate, we consider it likely that the interactions between GAPR-1 and Beclin 1 take place prior to condensate formation. What type of protein-protein interactions takes place during these interactions? When the Beclin1 interaction area with GAPR-1 (amino acids 267–284) is deleted, Beclin 1 still forms condensates when expressed in *S. cerevisiae* (Fig. 4). Likewise, when the GAPR-1 interaction area with Beclin 1 is mutated (H54A/E86A/G102K/H103A/N138G), GAPR-1 still forms condensates when expressed in *S. cerevisiae* (Fig 3). Both types of mutations, however, abrogate the interaction between GAPR-1 and Beclin 1, and as a result these

proteins do not affect the condensate formation of each other anymore. These observations suggest that specific protein-protein interactions are maintained during the process of condensate formation. Nevertheless, the nature of the PPI between Beclin 1 and GAPR-1 correlates with the amyloidogenic properties of the interphase as defined by the B18 peptide. With decreasing amyloidogenic behavior (based on the interaction with the A11 antibody), the B18 peptides show decreasing affinity for GAPR-1 (Fig. 5), decreasing condensate formation (Fig. 6), and decreasing effect on Beclin 1 condensate formation in the presence of GAPR-1 (Fig. 6). These results indicate that amyloidogenic properties play a role at the interphase of GAPR-1 and Beclin 1. We propose that these amyloidogenic properties play a role during oligomeric interactions between these two proteins that lead up to the formation of protein condensates (Supplementary Fig. 4).

Mutual interference with condensate formation in *S. cerevisiae* by co-expression of proteins or peptides (Fig. 6) is an exciting new possibility to identify and/or to study protein-protein interactions of amyloidogenic proteins. The aggregation of proteins into pathological amyloid fibrils and their deposition into plaques and intracellular condensates is the hallmark of amyloid disease [47]. During the last decade, baker's yeast has been extensively used to study several human diseases, including neurodegenerative disorders in which protein misfolding and amyloid formation are shared characteristics [48–52]. One of the most surprising discoveries in recent years was the identification of functional amyloid-like assemblies that are required to carry out physiological functions [26–32]. The yeast system has also been one of the established model systems to study functional amyloids and to investigate how physiological aggregation is regulated [34]. We previously showed that GAPR-1-GFP expression in yeast results in the formation of protein condensates in the cells without any toxic effects [18]. These findings are in contrast with pathological amyloid proteins that do show toxicity in *S. cerevisiae* upon overexpression and condensate formation [21,51,53]. This may limit the use of protein condensate formation in yeast to study protein-protein interactions of amyloidogenic proteins and restrict the use to protein functional amyloid-like characteristics. In this study, we showed that overexpression of Beclin 1-mCherry and subsequent condensate formation do not show any toxicity to the yeast cells (Supplementary Fig. 1). These results are consistent with the possibility that amyloidogenic oligomeric interactions between GAPR-1 and Beclin 1 have a functional role in autophagy.

Materials and Methods

Yeast strain and plasmids

Wild-type yeast strain W303 was transformed using the Frozen-EZ yeast kit from Zymo Research (Zymo Research, Orange, CA, USA) with high copy (2µ) plasmids p423, p425 and p426 carrying different constructs which were tagged C-terminally by GFP, CFP and m-Cherry respectively. To construct P*GAL1*-Beclin 1-GFP and P*GAL1*-Beclin 1-mCherry fragments, Beclin 1, GFP and mCherry sequences were amplified by PCR and were inserted into the plasmid p426, previously linearized at SmaI restriction site, by using Geneart® seamless cloning assembly kit (Invitrogen, California, United States). Beclin 1 mutant was constructed by using PhusionTM site-directed mutagenesis kit (Thermo Scientific, Massachusetts, United States). B18 and scrambled B18 oligonucleotides were synthesized by Integrated DNA Technologies (IDT), and cloned with Geneart® seamless cloning assembly kit (Invitrogen, California, United States) in SmaI restriction site. Peptides were cloned with mCherry tag in p426 and with CFP tag in p425 vectors. All constructs were confirmed by DNA sequencing and the plasmids and yeast strains are listed in Table 1 and the primers in Table 2.

Yeast culture media

Cells were pre-grown overnight in a synthetic complete (SC) medium lacking the corresponding markers (-His, -Ura or -Leu) and supplemented with either 2% glucose or 2% galactose at 30^{o}C with orbital agitation (200 rpm) for 18h (overnight). The day after optical density at 600 nm (OD600 nm) was measured, and the cells were shifted in selective SC medium supplemented with 2% galactose to induce protein expression (OD600=0.1). After 3h, 6h and 24h, cells were collected to obtain total cell homogenates or were observed by live cell microscopy (Nikon Eclipse Ti-E (Nikon)).

Table 1. Plasmids used in this study.

Plasmid	Description	Source
p423-*GAL1*	2µm; HIS3; *GAL1*pr; CYC1term; AmpR	D. Mumberg, R. Muller, M. Funk, Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression, Nucleic Acids Res. 22 (1994) 5767–5768. https://doi.org/10.1093/nar/22.25.5767. [54]
pME3759	p426-*GAL1*-GFP	D. Petroi, B. Popova, N. Taheri-Talesh, S. Irniger, H. Shahpasandzadeh, M. Zweckstetter, T.F. Outeiro, G.H. Braus, Aggregate Clearance of α-Synuclein in *Saccharomyces cerevisiae* Depends More on Autophagosome and Vacuole Function Than on the Proteasome, J. Biol. Chem. 287 (2012) 27567–27579. https://doi.org/10.1074/jbc.M112.361865. [31]
pNS 1000	p423-*GAL1*-GAPR-1-GFP	This study
pNS 1001	p423-*GAL1*- ΔmyrGAPR-1-GFP	This study
pNS 2000	p426-*GAL1*-Beclin 1-mCherry	This study
pNS 2001	p426-*GAL1*-Δ B18-mCherry	This study
pNS 2002	p426-*GAL1*- B18-mCherry	This study
pNS 2003	p426-*GAL1*-Scrambled B18-mCherry	This study
pNS 3001	p425-*GAL1*- B18-CFP	This study
pNS 3002	p425-*GAL1*-Scrambled B18-CFP	This study
pNS 1003	p423-*GAL1*-GAPR-1-GFP-CAAX	This study

Spotting assay

All spotting assays were performed under the same conditions. Ten-fold serial dilutions starting with an equal number of cells (OD600=0.1) were performed in sterile water. Drops of 10 μl were then spotted on SC plates lacking the corresponding marker (-His-Ura) and supplemented with either 2% glucose or 2% galactose. Three independent experiments from fresh transformants were done and followed by 3 days of incubation at 30°C, the plates were scanned.

Fluorescence microscopy

Cells were visualized at different time points after induction (3h, 6h, and 24h) using Nikon Eclipse Ti-E microscope (Nikon) equipped with the Perfect Focus System (Nikon) Nikon Apo TIRF 100x N.A. 1.49 oil objective (Nikon), a spinning disk-based confocal scanner unit (CSU-X1-A1, Yokogawa), and the ET-CFP filter set (49001, Chroma), ET-GFP filter set (49002, Chroma), and ET-mCherry filter set (49008, Chroma). For quantification, at least 300 cells were counted per condition and per experiment. For each condition, the number of cells displaying cytoplasmic protein condensates was calculated relative to the total number of cells counted (with condensate or cytoplasmic distribution). The number of condensates per cell was determined by counting 100 cells and the condensate-positive cells were categorized into 3 groups: 1) 1-2 condensate(s) per cell, 2) 3-5 condensates per cell, and 3) 6 or more condensates per cell. At least three independent experiments with freshly transformed constructs were performed.

Protein extraction and Western blotting

10 OD units of cells were collected by centrifugation, washed with sterile water and lysed with 200 μl of Y-PERTM reagent (Thermo Scientific, Massachusetts, United States) containing fresh protease inhibitors. Lysed cells were incubated for 20 minutes at room temperature, followed by 15 minutes centrifugation at 13000 rpm. Total protein homogenates were collected for further analysis. A total of 20 μg of yeast protein in Laemmli sample buffer was incubated for 5 minutes at 100°C and separated by SDS-PAGE in a 12% polyacrylamide gel. Proteins were electrically transferred onto 0.45 μm nitrocellulose membranes (Amersham Protran GE Healthcare) by Western blot at 90 V for 1 h and were probed with monoclonal anti-Beclin 1 (Santa Cruz, Dallas, Texas, United States), and polyclonal anti-GAPR-1 antibodies [5]. Monoclonal anti-GAPDH antibody (Thermo Scientific, Massachusetts, United States) was used as a loading control. Peroxidase-conjugated goat anti-rabbit antibody (Nordic-Mubio, Susteren, The Netherlands) was used as a secondary antibody. Binding was detected with the SuperSignalTM reagents (Thermo Scientific, Massachusetts, United States) and ChemiDocTM MP Imaging system (BioRad, Hercules, California, United States).

SDS Solubility test

10 OD units of cells were collected after 24h induction, homogenized and total protein homogenate was prepared using Y-PERTM reagent. 1/10 of the homogenate was collected as input. 9/10 of the sample was centrifuged at 100,000 x g for 30 minutes, and the detergent-soluble supernatant was collected. The detergent-insoluble pellet was treated with 2% SDS (180µl for 30 minutes at 4^{o}C) and separated by centrifugation (100,000 x g for 30 minutes) into a SDS-soluble and SDS-insoluble fraction. The same volume (20µl) of each sample was separated by SDS-PAGE and analysed by Western blot using an anti Beclin 1 antibody.

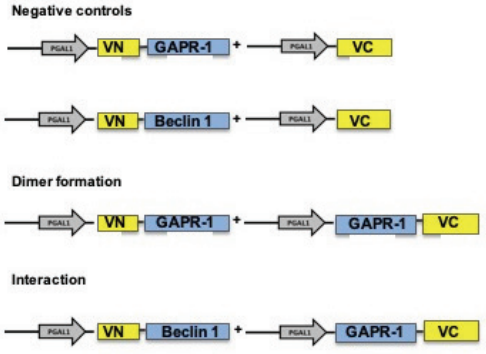
Table 2. Primers used in this study.

Primer	Sequence
FW GAPR-1	5'-GAACTAGTGGATCCCCCATGGGCAAGTCAGC-3'
REV GAPR-1 4 GFP	5'-CTTCTCCTTTACTATCGATAAGCTTCTTCTTCGGCGGC5-3'
FW GFP 4 GAPR-1	5'-CCGCCGAAGAAGAAGCTTATCGATAGTAAAGGAGAAGAAC-3'
REV GFP	5'-GAATTCCTGCAGCCCTTATTTGTATAGTTCATCC-3'
FW Beclin 1	5'-GAACTAGTGGATCCCCCATGGAAGGGTCTAAGAC-3'
REV Beclin 1 4 mCherry	5'-CCTTGCTCACCATATCGATAAGCTTTTTGTTATAAAATTGTGA-3'
FW mCherry 4 Beclin 1	5'-CTCACAATTTTATAACAAAAAGCTTATCGATATGGTGAGCAAG-3'
REV mCherry	5'-GAATTCCTGCAGCCCCTACTTGTACAGCTC-3'
REV GAPR-1 4 VC	5'- CCG TTC TTC TCG AGC TTC TTC GGC GG -3'
FW VC 4 GAPR-1	5'- CCG CCG AAG AAG CTC GAG AAG AAC GG -3'
REV VC	5'- CGA ATT CCT GCA GCC CTT ACT TGT ACA GC -3'
FW VN	5'- CTA GTG GAT CCC CCA TGG TGA GCA AG -3'
REV VN 4 Beclin 1	5'- CGT CTT AGA CCC TTC CTT AAG GGA CCC -3'
FW Beclin 1 4 VN	5'- GGG TCC CTT AAG GAA GGG TCT AAG ACG -3'
REV Beclin 1	5'- CGAATTCCTGCAGCCCTCATTTGTTATAAAATTG -3'

Bimolecular Fluorescence Complementation (BiFC)

In this assay, we used Venus YFP protein as a fluorescence protein and tagged its non-fluorescent halves Venus N (VN) in N-terminal domain of Beclin 1 in p423 plasmid and Venus C (VC) in C-terminal of GAPR-1 in p426 plasmid. All constructs and controls are depicted in Table 3. All VN fragments were cloned in p423 and VC fragments in p426 plasmids and constructs were verified by DNA sequencing. For live cell imaging microscopy, wild-type (W303) yeast cells harbouring different constructs were grown in SC medium -His -Ura containing 2% raffinose at 30^{o}C overnight and shifted to 2% galactose medium for induction of proteins. Cells were visualized after 24h induction by Zeiss Observer. Z1 microscope (Zeiss) was equipped with a CSU-X1 A1 confocal scanner unit (YOKOGAWA), QuantEM:512SC digital camera (Photometrics) and Slide Book 6.0 software package (Intelligent Imaging Innovations). The values of mean fluorescence intensity of 150 cells per condition were measured in three independent experiments.

Table 3. Constructs used in the BiFC assay



Treatment with peptides

Cells carrying different constructs were grown in a culture tube containing 5 ml of minimal medium supplemented with proline as the nitrogen (N) source at 30°C overnight. Proline was added as a poor N source in order to induce the synthesis of the peptide uptake system in *S. cerevisiae* (protocol adapted from [55]). Cells were transferred to a minimal medium with 2% galactose, 5μM Tat-Beclin 1 or Tat-scrambled peptides ((96.6% and 95.8% purity respectively, GenScript (Nanjing) , Jiangsu, China) were added and incubated on a rotor at 30°C (OD600 =0.1). Cells were collected after 6h, washed 3 times with fresh medium to remove the peptides and were directly

visualized by a spinning disc microscope. Quantifications were performed by counting 300 cells per each condition in three independent experiments.

Immuno-Dot Blots

Aβ (1-40) (Innovagen AB, Lund, Sweden), Tat-Beclin 1 and Tat-Scrambled B18 were incubated at a concentration of 50μM in 25mM Tris-HCl, 50mM NaCl, pH 7.4 (NT-50) at 37°C with agitation for 18h. Samples (4μg), were taken at various time points during fibril formation and stored frozen at -20°C then they were spotted onto nitrocellulose membranes (Amersham Protran GE Healthcare) and allowed to air dry. The membranes were then blocked with 5% (w/v) BSA in Tris-buffered saline containing 0.05% Tween-20 (TBS-T). The blocked membranes were used to study Aβ interaction with GAPR-1 or with the A11 antibody. To study the interaction with GAPR-1, blocked membranes were incubated for 2h at room temperature in NT-50 containing 50μg/ml GAPR-1 before being washed with TBS-T. Bound GAPR-1 was detected using specific rabbit polyclonal antibodies as described above. To study interaction of Aβ with A11 antibody, blocked membranes were incubated with rabbit polyclonal anti-oligomer antibody A11 (Invitrogen, California, United States) (0.5μg/ml in TBS-T, 1% (w/v) BSA) for 1h at RT followed by HRP-labeled goat anti-rabbit secondary antibodies with SuperSignalTM reagents (Thermo Scientific, Massachusetts, United States) detection.

Different promoters

WT GAPR-1-GFP and WT Beclin 1-mCherry were cloned in SmaI restriction site in pBP496 (-His) and pBP499 (-Ura) plasmids, respectively. Both plasmids contained the constitutive alcohol dehydrogenase I (ADH1) promoter.
W303 yeast cells carrying GAPR-1-GFP or Beclin 1-mCherry expressing plasmids were grown in a selective media lacking histidine and uracil and containing 2% raffinose at 30°C overnight. Cells shifted to a fresh SC medium with 2% glucose as a carbon source and lacking selective amino acids. After 6h, cells were visualized by microscopy and quantified by counting 300 cells per condition in three independent experiments.
Amyloid prediction software
Amino acid sequences of human Beclin 1, B18, F274S and Scrambled B18 were given as input to the web server-based program AmylPred2 (http://biophysics.biol.uoa.gr/AMYLPRED2) [36] using all methods except Amyloid Mutants.

Statistical Analysis

Data were analyzed using GraphPad Prism 9 (San Diego, CA, USA) Software and are presented as mean ± SD of at least three independent experiments. The significance of differences was calculated using a two-tailed unpaired T-test. P values lower than 0.05 were considered to indicate a significant difference.

Acknowledgements

We thank Ruud Eerland for technical support Ilya Grigoriev (Anna Akhmanova) and Richard Wubbolts for help with fluorescence microscopy. GB acknowledges the financial support of Deutsche Forschungsgemeinschaft (DFG: BR1502/18-2).

References

[1] M.P. Jackson, E.W. Hewitt, Why are functional amyloids non-toxic in humans?, Biomolecules. 7 (2017). https://doi.org/10.3390/biom7040071.

[2] M.J. Ranaghan, M.A. Durney, M.F. Mesleh, P.R. McCarren, C.W. Garvie, D.S. Daniels, K.L. Carey, A.P. Skepner, B. Levine, J.R. Perez, The Autophagy-Related Beclin-1 Protein Requires the Coiled-Coil and BARA Domains To Form a Homodimer with Submicromolar Affinity, Biochemistry. 56 (2017) 6639–6651. https://doi.org/10.1021/acs.biochem.7b00936.

[3] B. Ku, J.-S. Woo, C. Liang, K.-H. Lee, J.U. Jung, B.-H. Oh, An insight into the mechanistic role of Beclin 1 and its inhibition by prosurvival Bcl-2 family proteins, Autophagy. 4 (2008) 519–520. https://doi.org/10.4161/auto.5846.

[4] R. Kang, H.J. Zeh, M.T. Lotze, D. Tang, The Beclin 1 network regulates autophagy and apoptosis, Cell Death Differ. 18 (2011) 571–580. https://doi.org/10.1038/cdd.2010.191.

[5] H.B. Eberle, R.L. Serrano, J. Füllekrug, A. Schlosser, W.D. Lehmann, F. Lottspeich, D. Kaloyanova, F.T. Wieland, J.B. Helms, Identification and characterization of a novel human plant pathogenesis-related protein that localizes to lipid-enriched microdomains in the Golgi complex., J. Cell Sci. 115 (2002) 827–38. http://jcs.biologists.org/content/115/4/827.abstract.

[6] Á.F. Fernández, S. Sebti, Y. Wei, Z. Zou, M. Shi, K.L. McMillan, C. He, T. Ting, Y. Liu, W.-C. Chiang, D.K. Marciano, G.G. Schiattarella, G. Bhagat, O.W. Moe, M.C. Hu, B. Levine, Disruption of the beclin 1–BCL2 autophagy regulatory complex promotes longevity in mice, Nature. 558 (2018) 136–140. https://doi.org/10.1038/s41586-018-0162-7.

[7] M.C. Maiuri, A. Criollo, G. Kroemer, Crosstalk between apoptosis and autophagy within the Beclin 1 interactome, EMBO J. 29 (2010) 515–516. https://doi.org/10.1038/emboj.2009.377.

[8] S.M. Hill, L. Wrobel, D.C. Rubinsztein, Post-translational modifications of Beclin 1 provide multiple strategies for autophagy regulation, Cell Death Differ. 26 (2019) 617–629. https://doi.org/10.1038/s41418-018-0254-9.

[9] S. Tran, W.D. Fairlie, E.F. Lee, BECLIN1: Protein Structure, Function and Regulation, Cells. 10 (2021) 1522. https://doi.org/10.3390/cells10061522.

[10] M. Su, Y. Li, S. Wyborny, D. Neau, S. Chakravarthy, B. Levine, C.L. Colbert, S.C.

Sinha, BECN2 interacts with ATG14 through a metastable coiled-coil to mediate autophagy, Protein Sci. 26 (2017) 972–984. https://doi.org/10.1002/pro.3140.

[11] S. Adi-Harel, S. Erlich, E. Schmukler, S. Cohen-Kedar, O. Segev, L. Mizrachy, J.A. Hirsch, R. Pinkas-Kramarski, Beclin 1 self-association is independent of autophagy induction by amino acid deprivation and rapamycin treatment, J. Cell. Biochem. 110 (2010) 1262–1271. https://doi.org/10.1002/jcb.22642.

[12] S. Shoji-Kawata, R. Sumpter, M. Leveno, G.R. Campbell, Z. Zou, L. Kinch, A.D. Wilkins, Q. Sun, K. Pallauf, D. MacDuff, C. Huerta, H.W. Virgin, J. Bernd Helms, R. Eerland, S.A. Tooze, R. Xavier, D.J. Lenschow, A. Yamamoto, D. King, O. Lichtarge, N. V. Grishin, S.A. Spector, D. V. Kaloyanova, B. Levine, Identification of a candidate therapeutic autophagy-inducing peptide, Nature. 494 (2013) 201–206. https://doi.org/10.1038/nature11866.

[13] J. van Galen, N.K. Olrichs, A. Schouten, R.L. Serrano, E.N.M. Nolte-'t Hoen, R. Eerland, D. Kaloyanova, P. Gros, J.B. Helms, Interaction of GAPR-1 with lipid bilayers is regulated by alternative homodimerization, Biochim. Biophys. Acta - Biomembr. 1818 (2012) 2175–2183. https://doi.org/10.1016/j.bbamem.2012.04.016.

[14] R.L. Serrano, A. Kuhn, A. Hendricks, J.B. Helms, I. Sinning, M.R. Groves, Structural analysis of the human Golgi-associated plant pathogenesis related protein GAPR-1 implicates dimerization as a regulatory mechanism, J. Mol. Biol. 339 (2004) 173–183. https://doi.org/10.1016/j.jmb.2004.03.015.

[15] N.K. Olrichs, A.K. Mahalka, D. Kaloyanova, P.K. Kinnunen, J. Bernd Helms, Golgi-Associated plant Pathogenesis Related protein 1 (GAPR-1) forms amyloid-like fibrils by interaction with acidic phospholipids and inhibits Aβ aggregation, Amyloid. 21 (2014) 88–96. https://doi.org/10.3109/13506129.2014.882304.

[16] J. Sheng, N.K. Olrichs, W.J. Geerts, X. Li, A.U. Rehman, B.M. Gadella, D. V. Kaloyanova, J.B. Helms, Zinc binding regulates amyloid-like aggregation of GAPR-1, Biosci. Rep. 39 (2019) BSR20182345. https://doi.org/10.1042/BSR20182345.

[17] J. Sheng, N.K. Olrichs, W.J. Geerts, D. V. Kaloyanova, J.B. Helms, Metal ions and redox balance regulate distinct amyloid-like aggregation pathways of GAPR-1, Sci. Rep. 9 (2019) 15048. https://doi.org/10.1038/s41598-019-51232-7.

[18] N. Sirati, B. Popova, M.R. Molenaar, I.C. Verhoek, G.H. Braus, D. V. Kaloyanova, J.B. Helms, Dynamic and Reversible Aggregation of the Human CAP Superfamily Member GAPR-1 in Protein Inclusions in *Saccharomyces cerevisiae*, J. Mol. Biol.

433 (2021) 167162. https://doi.org/10.1016/j.jmb.2021.167162.

[19] N. K. Olrichs, J. Bernd Helms, Novel insights into the function of the conserved domain of the CAP superfamily of proteins, AIMS Biophys. 3 (2016) 232–246. https://doi.org/10.3934/biophy.2016.2.232.

[20] J. Sheng, N.K. Olrichs, B.M. Gadella, D. V. Kaloyanova, J.B. Helms, Regulation of Functional Protein Aggregation by Multiple Factors: Implications for the Amyloidogenic Behavior of the CAP Superfamily Proteins, Int. J. Mol. Sci. 21 (2020) 6530. https://doi.org/10.3390/ijms21186530.

[21] D.M. Fowler, A. V. Koulov, W.E. Balch, J.W. Kelly, Functional amyloid - from bacteria to humans, Trends Biochem. Sci. 32 (2007) 217–224. https://doi.org/10.1016/j.tibs.2007.03.003.

[22] A. Hewetson, H.Q. Do, C. Myers, A. Muthusubramanian, R.B. Sutton, B.J. Wylie, G.A. Cornwall, Functional Amyloids in Reproduction, Biomolecules. 7 (2017) 46. https://doi.org/10.3390/biom7030046.

[23] M. Jackson, E. Hewitt, Why are Functional Amyloids Non-Toxic in Humans?, Biomolecules. 7 (2017) 71. https://doi.org/10.3390/biom7040071.

[24] D.S. Eisenberg, M.R. Sawaya, Structural Studies of Amyloid Proteins at the Molecular Level, Annu. Rev. Biochem. 86 (2017) 69–95. https://doi.org/10.1146/annurev-biochem-061516-045104.

[25] F. Chiti, C.M. Dobson, Protein Misfolding, Functional Amyloid, and Human Disease, Annu. Rev. Biochem. 75 (2006) 333–366. https://doi.org/10.1146/annurev.biochem.75.101304.123901.

[26] A. V. Sergeeva, A.P. Galkin, Functional amyloids of eukaryotes: criteria, classification, and biological significance, Curr. Genet. 66 (2020) 849–866. https://doi.org/10.1007/s00294-020-01079-7.

[27] J.M. Laurent, J.H. Young, A.H. Kachroo, E.M. Marcotte, Efforts to make and apply humanized yeast, Brief. Funct. Genomics. 15 (2016) 155–163. https://doi.org/10.1093/bfgp/elv041.

[28] J. Winderickx, C. Delay, A. De Vos, H. Klinger, K. Pellens, T. Vanhelmont, F. Van Leuven, P. Zabrocki, Protein folding diseases and neurodegeneration: Lessons learned from yeast, Biochim. Biophys. Acta - Mol. Cell Res. 1783 (2008) 1381–1395. https://doi.org/10.1016/j.bbamcr.2008.01.020.

[29] W.H. Mager, J. Winderickx, Yeast as a model for medical and medicinal research,

Trends Pharmacol. Sci. 26 (2005) 265–273. https://doi.org/10.1016/j.tips.2005.03.004.

[30] S.E. Di Gregorio, M.L. Duennwald, Yeast as a model to study protein misfolding in aged cells, FEMS Yeast Res. 18 (2018). https://doi.org/10.1093/femsyr/foy054.

[31] D. Petroi, B. Popova, N. Taheri-Talesh, S. Irniger, H. Shahpasandzadeh, M. Zweckstetters, T.F. Outeiro, G.H. Braus, Aggregate clearance of α-synuclein in Saccharomyces cerevisiae depends more on autophagosome and vacuole function than on the proteasome, J. Biol. Chem. 287 (2012) 27567–27579. https://doi.org/10.1074/jbc.M112.361865.

[32] V. Franssens, T. Bynens, J. Van Den Brande, K. Vandermeeren, M. Verduyckt, J. Winderickx, The benefits of humanized yeast models to study Parkinson's disease, Oxid. Med. Cell. Longev. (2013). https://doi.org/10.1155/2013/760629.

[33] R. Krishnan, J.L. Goodman, S. Mukhopadhyay, C.D. Pacheco, E.A. Lemke, A.A. Deniz, S. Lindquist, Conserved features of intermediates in amyloid assembly determine their benign or toxic states, Proc. Natl. Acad. Sci. 109 (2012) 11172–11177. https://doi.org/10.1073/pnas.1209527109.

[34] G. Cereghetti, S. Saad, R. Dechant, M. Peter, Reversible, functional amyloids: towards an understanding of their regulation in yeast and humans, Cell Cycle. 17 (2018) 1545–1558. https://doi.org/10.1080/15384101.2018.1480220.

[35] D. Laor, D. Sade, S. Shaham-Niv, D. Zaguri, M. Gartner, V. Basavalingappa, A. Raveh, E. Pichinuk, H. Engel, K. Iwasaki, T. Yamamoto, H. Noothalapati, E. Gazit, Fibril formation and therapeutic targeting of amyloid-like structures in a yeast model of adenine accumulation, Nat. Commun. 10 (2019) 62. https://doi.org/10.1038/s41467-018-07966-5.

[36] A.C. Tsolis, N.C. Papandreou, V.A. Iconomidou, S.J. Hamodrakas, A Consensus Method for the Prediction of 'Aggregation-Prone' Peptides in Globular Proteins, PLoS One. 8 (2013) e54175. https://doi.org/10.1371/journal.pone.0054175.

[37] S. Alberti, R. Halfmann, S. Lindquist, Biochemical, Cell Biological, and Genetic Assays to Analyze Amyloid and Prion Aggregation in Yeast, in: 2010: pp. 709–734. https://doi.org/10.1016/S0076-6879(10)70030-6.

[38] L.E. Berchowitz, G. Kabachinski, M.R. Walker, T.M. Carlile, W. V. Gilbert, T.U. Schwartz, A. Amon, Regulated Formation of an Amyloid-like Translational Repressor Governs Gametogenesis, Cell. 163 (2015) 406–418.

https://doi.org/10.1016/j.cell.2015.08.060.

[39] V. Citovsky, L.Y. Lee, S. Vyas, E. Glick, M.H. Chen, A. Vainstein, Y. Gafni, S.B. Gelvin, T. Tzfira, Subcellular Localization of Interacting Proteins by Bimolecular Fluorescence Complementation in Planta, J. Mol. Biol. 362 (2006) 1120–1131. https://doi.org/10.1016/j.jmb.2006.08.017.

[40] T.K. Kerppola, Bimolecular Fluorescence Complementation (BiFC) Analysis as a Probe of Protein Interactions in Living Cells, Annu. Rev. Biophys. 37 (2008) 465–487. https://doi.org/10.1146/annurev.biophys.37.032807.125842.

[41] Y. Li, Y. Zhao, M. Su, K. Glover, S. Chakravarthy, C.L. Colbert, B. Levine, S.C. Sinha, Structural insights into the interaction of the conserved mammalian proteins GAPR-1 and Beclin 1, a key autophagy protein, Acta Crystallogr. Sect. D Struct. Biol. 73 (2017) 775–792. https://doi.org/10.1107/S2059798317011822.

[42] P. Zhao, X.-M. Zhou, L.-L. Zhao, A.Y. Cheung, M.-X. Sun, Autophagy-mediated compartmental cytoplasmic deletion is essential for tobacco pollen germination and male fertility, Autophagy. 16 (2020) 2180–2192. https://doi.org/10.1080/15548627.2020.1719722.

[43] T.K. Kerppola, Bimolecular Fluorescence Complementation (BiFC) Analysis as a Probe of Protein Interactions in Living Cells, Annu. Rev. Biophys. 37 (2008) 465–487. https://doi.org/10.1146/annurev.biophys.37.032807.125842.

[44] T.K. Kerppola, Visualization of molecular interactions by fluorescence complementation, Nat. Rev. Mol. Cell Biol. 7 (2006) 449–456. https://doi.org/10.1038/nrm1929.

[45] K. Ohashi, K. Mizuno, A novel pair of split venus fragments to detect protein-protein interactions by in vitro and *in vivo* bimolecular fluorescence complementation assays, Methods Mol. Biol. 1174 (2014) 247–262. https://doi.org/10.1007/978-1-4939-0944-5_17.

[46] J. Van Galen, B.W.M. Van Balkom, R.L. Serrano, D. Kaloyanova, R. Eerland, E. Stven, J.B. Helms, Binding of GAPR-1 to negatively charged phospholipid membranes: Unusual binding characteristics to phosphatidylinositol, Mol. Membr. Biol. 27 (2010) 81–91. https://doi.org/10.3109/09687680903507080.

[47] M.G. Iadanza, M.P. Jackson, E.W. Hewitt, N.A. Ranson, S.E. Radford, A new era for understanding amyloid structures and disease, Nat. Rev. Mol. Cell Biol. 19 (2018) 755–773. https://doi.org/10.1038/s41580-018-0060-8.

[48] R.P. Mason, F. Giorgini, Modeling Huntington disease in yeast: Perspectives and future directions, Prion. 5 (2011) 269–276. https://doi.org/10.4161/pri.5.4.18005.

[49] I.C. Brás, B. Popova, G.H. Braus, T.F. Outeiro, Yeast-Based Screens to Target Alpha-Synuclein Toxicity, in: 2019: pp. 145–156. https://doi.org/10.1007/978-1-4939-9124-2_12.

[50] J.H. Soper, S. Roy, A. Stieber, E. Lee, R.B. Wilson, J.Q. Trojanowski, C.G. Burd, V.M.-Y. Lee, α-Synuclein–induced Aggregation of Cytoplasmic Vesicles in Saccharomyces cerevisiae, Mol. Biol. Cell. 19 (2008) 1093–1103. https://doi.org/10.1091/mbc.e07-08-0827.

[51] F. D'Angelo, H. Vignaud, J. Di Martino, B. Salin, A. Devin, C. Cullin, C. Marchal, A yeast model for amyloid- aggregation exemplifies the role of membrane trafficking and PICALM in cytotoxicity, Dis. Model. Mech. 6 (2013) 206–216. https://doi.org/10.1242/dmm.010108.

[52] V. Franssens, E. Boelen, J. Anandhakumar, T. Vanhelmont, S. Büttner, J. Winderickx, Yeast unfolds the road map toward α-synuclein-induced cell death, Cell Death Differ. 17 (2010) 746–753. https://doi.org/10.1038/cdd.2009.203.

[53] B. Popova, A. Kleinknecht, P. Arendarski, J. Mischke, D. Wang, G.H. Braus, Sumoylation Protects Against β-Synuclein Toxicity in Yeast, Front. Mol. Neurosci. 11 (2018). https://doi.org/10.3389/fnmol.2018.00094.

[54] D. Mumberg, R. Muller, M. Funk, Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression, Nucleic Acids Res. 22 (1994) 5767–5768. https://doi.org/10.1093/nar/22.25.5767.

[55] M. Hauser, H. Cai, F. Naider, J. Becker, Uptake Assay for Radiolabeled Peptides in Yeast, BIO-PROTOCOL. 6 (2016). https://doi.org/10.21769/bioprotoc.2026.

Supplemental information

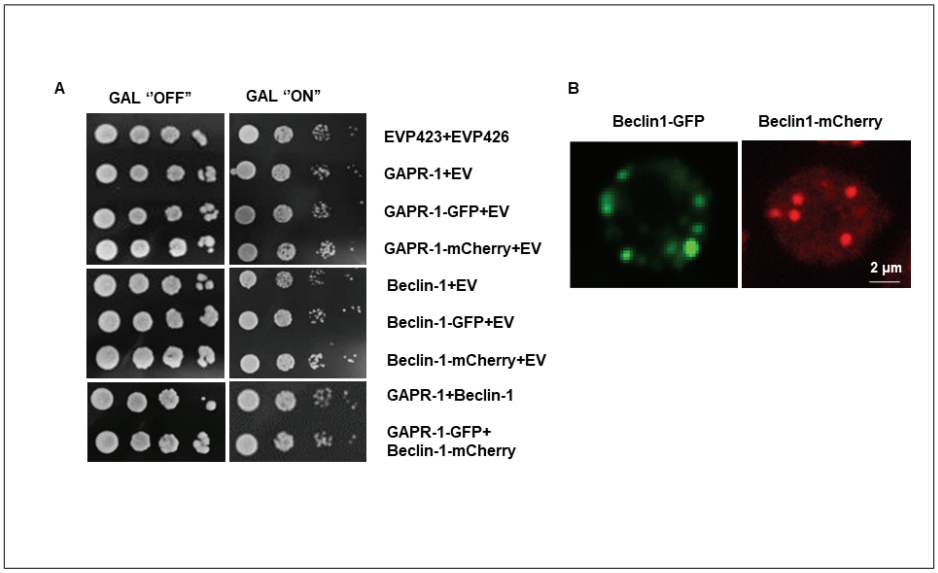


Figure S1. A) Wild-type yeast cells (W303) were transformed with a high copy plasmid carrying different constructs under the control of *GAL1* promotor. Ten-fold dilutions of exponentially growing cultures of W303 cells, transformed with plasmids carrying the different constructions, were spotted using selection plates containing 60 mg/l leucine, and 40 mg/l tryptophan, together with 2% glucose (*GAL1* promoter "OFF") or 2% galactose (*GAL1* promoter "ON"). The cells were incubated at 30^{o}C for 3 days; B) Live-cell fluorescence microscopy after 24h induction. Wild-type yeast cells (W303) were transformed with a plasmid carrying Beclin 1-GFP or Beclin 1-mCherry under the control of the *GAL1* promoter.

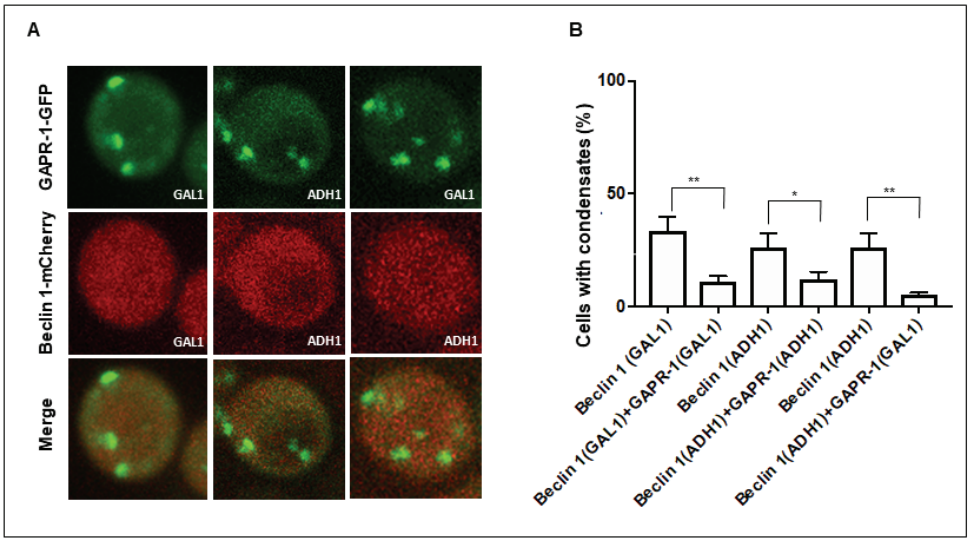


Figure S2. A) Live-cell fluorescence microscopy of yeast cells co-expressing GAPR-1-GFP and Beclin 1-mCherry in *GAL1* (inducible) and *ADH1* (constitutive) promoters after 6h co-expression; B) Quantification of the yeast cells displaying Beclin 1 protein condensates. For each time point, the number of cells displaying protein condensates is presented as a percent of the total number of cells. For quantification of protein condensates at least 200 cells were counted per time point and per experiment. Results are expressed as mean ± SD, from at least three independent experiments.

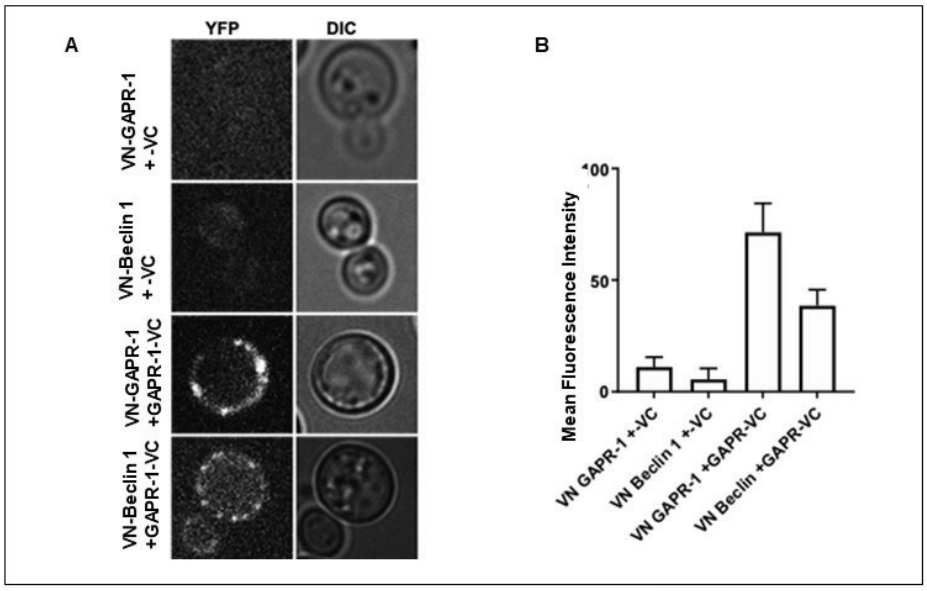


Figure S3. A) Bimolecular Fluorescence Complementation assay (BiFC) using the non-fluorescent N-terminal and C-terminal fragments of Venus yellow fluorescent protein (VN and VC respectively). GAPR-1 and Beclin 1 were tagged with VN or VC (VN-GAPR-1, VN-Beclin 1 and GAPR-1-VC) and fluorescent complementation by co-expression of different VN and VC constructs (as indicated in the figure and in the Results section) was determined by fluorescent microscopy; B) Mean YFP fluorescence intensity of cells after 24h expression. The results are expressed as the mean ±SD of three independent experiments. The significance of differences was calculated with a two-tailed unpaired t-test (***$p < 0.001$; n=150).

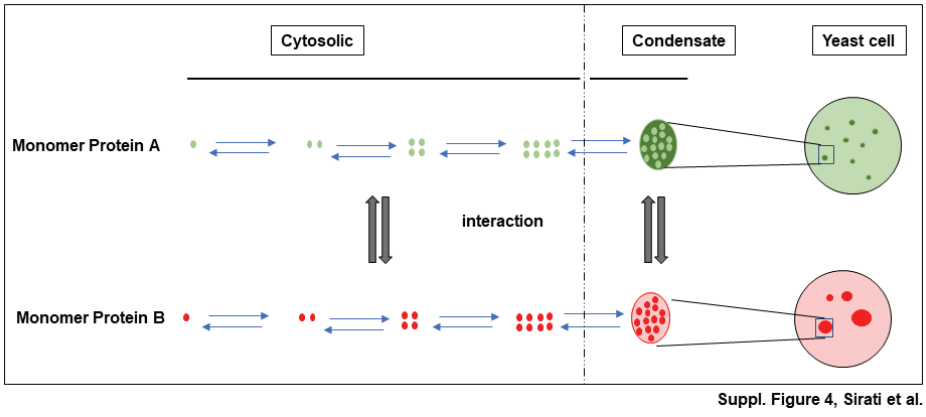


Figure S4. Amyloidogenic PPI in protein condensate formation

Abstract

GAPR-1 is a negative regulator of autophagy and acts by retaining Beclin 1 at the Golgi apparatus in mammalian cells. To study the molecular interactions between GAPR-1 and Beclin 1, we recently developed an *in vivo* protein-protein interaction assay in *Saccharomyces cerevisiae* that is based on mutual interference of condensate formation upon co-expression of the amyloidogenic proteins GAPR-1 and Beclin 1. Here, we used this model system to study the effect of phosphorylation on the oligomeric states of GAPR-1and Beclin 1 and to determine the role of phosphorylation in the GAPR-1/Beclin 1 interaction. We find that phosphorylation of Ser55 is required for GAPR-1 condensate formation. Similarly, phosphorylation of Ser15/Ser30 is required for Beclin 1 condensate formation. Co-expression experiments show that phosphorylation of Ser55 (GAPR-1) as well as phosphorylation of Ser15/Ser30 (Beclin 1), is required for interference with condensate formation of both proteins. Deletion of ULK1/Atg1 in yeast was sufficient to observe the same condensate phenotypes after co-expression of wt GAPR-1 and wt Beclin 1. Thus, ULK1/Atg1 is capable of phosphorylating both GAPR-1 and Beclin 1, thereby affecting their amyloidogenic properties and enhancing their interaction. Our combined results suggest that ULK1/Atg1 can regulate the interaction between GAPR-1 and Beclin 1, providing a potential novel molecular switch acting in autophagy.

Keywords: GAPR-1, phosphorylation, Beclin 1, Amyloid-like proteins, Protein-Protein interactions (PPIs), *Saccharomyces cerevisiae*, ULK 1, Atg 1

Introduction

Protein phosphorylation is one of the most common post-translational modifications that play an important role in protein function by regulating their activity, localization, and interaction with other molecules. One possible mechanism to regulate protein function by phosphorylation is by affecting its oligomerization properties [1]. In this way, protein phosphorylation can regulate a broad range of cellular processes, such as protein degradation via the proteasome [2], cell migration and adhesion [3,4], signal transduction [5,6], and cellular stress-related events [7]. Phosphorylation-dependent protein oligomerization and aggregation are also associated with several pathologies [8–12]. Aberrant phosphorylation is observed in neurodegenerative diseases such as Alzheimer's disease with hyperphosphorylation of Tau and phosphorylation of the Aß peptide [13–16] and in Parkinson's disease with phosphorylation of a-synuclein [17–19] causing amyloid formation. In human acquired heart failure, mono-phosphorylation of desmin triggers pre-amyloid oligomer formation and myocyte dysfunction [20]. More recently, several studies have indicated a role of phosphorylation in liquid-liquid phase separation of proteins and the biogenesis of biomolecular condensates [21–24].

Golgi-Associated plant Pathogenesis-Related protein 1 (GAPR-1) is a mammalian protein belonging to the CAP superfamily [25,26] and is known to exist in various oligomeric forms. It localizes to Golgi membranes as a dimer and possesses amyloidogenic properties *in vitro* [27–29]. In line with these observations, we demonstrated that, by using a well-established *Saccharomyces cerevisiae* model system, over-expression of human GAPR-1 (GAPR-1) is associated with the formation of biomolecular condensates [30]. GAPR-1 condensates were shown to be dynamic and reversible organelles, with myristoylation and metal ions as important factors involved in condensate formation. GAPR-1 is also a Beclin 1 interacting protein that negatively regulates autophagy [31–33], and recently we showed that the amyloidogenic properties of both proteins contribute to the condensate formation and their interaction *in vivo* [34].

Interestingly, mutagenesis studies revealed that the same type of protein-protein interactions previously described for the GAPR-1/Beclin 1 interaction at Golgi membranes, also interfere with the condensate formation in yeast during the co-expression of GAPR-1 and Beclin 1 [34]. These observations make the *Saccharomyces cerevisiae* model system an interesting platform to study the involvement of phosphorylation in the GAPR-1/Beclin 1 interaction [31]. GAPR-1 contains multiple putative phosphorylation sites (more than 20 tyrosine/serine/threonine residues). To date, seven of these amino acids have been identified as phosphorylated residues by proteomic mass spectrometry (PhosphoSitePlus [35]). Beclin 1 is a central player in autophagy and constitutes a molecular platform for the regulation of autophagosome

formation and maturation [36–38]. Phosphorylation of Beclin 1 affects its stability, interactions with other proteins, and ability to regulate PI3K activity and initiation of autophagy [39,40]. In response to cells' metabolic needs, autophagy can be initiated by the concerted action of the ULK1/Atg1 kinase complex and the PI3K-III kinase complex. This leads to the nucleation of phagophores, and the generation of double-membranous structures called autophagosomes, which eventually fuse with lysosomes to form autolysosomes and release amino acids and other metabolites.

In this study, we focused on the role of phosphorylation in GAPR-1 and Beclin 1 amyloid-like oligomerization and condensate formation in a yeast model system. We show that both GAPR-1 and Beclin 1 are targets of the autophagy-inducing ULK1/Atg1 kinase complex.

Results

Phosphorylation regulates GAPR-1 condensate formation

Proteomic studies on various tissues and cancer cells have revealed the presence of multiple GAPR-1 phosphorylation sites (Supplementary Fig.1A) [35,41]. To investigate whether phosphorylation of GAPR-1 contributes to its condensate behavior *in vivo*, we used a previously developed yeast S. cerevisiae model system that shows GAPR-1 condensate formation upon overexpression of wild-type (wt) GAPR-1 [30]. Three phosphorylation sites, Tyr42, Ser55, and Ser123, were selected for further investigation based on the frequency of their identification (Tyr42 and Ser55, Supplementary Fig.1A) and accessibility at the surface of GAPR-1 in the crystal structure of GAPR-1 (Ser123). Using mutagenesis, single, double, and triple phospho mutants of C-terminally GFP-tagged GAPR-1 (GAPR-1-GFP) were created by changing Tyr42 to Phe (Y42F) and by changing Ser55 or Ser123 to Gly (S55G and S123G) (Fig.1A). Cytotoxicity and protein expression levels of all mutants were shown to be similar to wt GAPR-1-GFP, as determined by spotting assay and Western blot analyses, respectively (Supplementary Fig.1B, 1C).

The effect of GAPR-1 phosphorylation on condensate formation in yeast was studied by live-cell fluorescent imaging at 3h post-induction of GAPR-1-GFP constructs. The morphology of wt GAPR-1 condensates (Fig.1B, PPP) was severely affected in the GAPR-1 triple mutant in which all three sites were unavailable for phosphorylation, resulting in aberrant fluorescent structures in the yeast cell (Fig.1B, XXX). To identify which of the mutated amino acids contributed to these effects, we expressed GAPR-1 mutants in which only one amino acid was mutated. Preventing the phosphorylation of Tyr42 (XPP) or Ser55 (PXP) altered the protein condensate morphology, whereas mutation of Ser123 (PPX) did not affect condensate morphology (Fig.1B). Double mutants of GAPR-1

(indicated as PXX, XPX, XXP) showed the importance of Ser55 in condensate formation of GAPR-1 *in vivo* as only GAPR-1 XPX maintained a normal condensate morphology (Fig.1B). In addition, GAPR-1 PXP was the only mutant that showed a reduced efficiency in condensate formation as determined by the number of cells containing condensates after 3h of expression (Fig.1C). To confirm that the oligomerization of GAPR-1 is influenced by the phosphorylation of Ser55, a GAPR-1 triple mutant was created that contains aspartic acid (D) at position 55 as a phosphomimetic of phospho-serine (XDX). The morphology and efficiency of cytosolic condensates in this phosphomimetic mutant were shown to be similar to the double mutant in which Ser55 remains available for phosphorylation (XDX versus XPX, Figs.1B,C). These results suggest a role for Ser55 phosphorylation in the oligomerization properties of GAPR-1 *in vivo*.

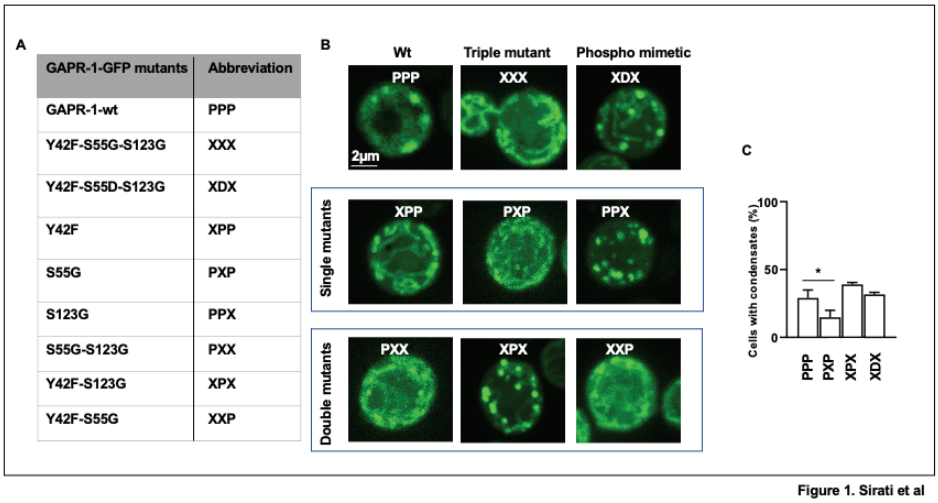


Figure 1. Phosphorylation regulates GAPR-1 amyloid-like oligomerization in vivo. A) Names and abbreviations of the GAPR-1 constructs used in this study; B) Live-cell fluorescence microscopy of yeast cells expressing wt GAPR1-GFP and phospho mutants as indicated in the panels after 3h in SC-galactose induction media; C) Quantification of yeast cells displaying cytoplasmic condensates, presented as percent of the total number of cells. For the quantification of condensates, at least 100 cells were counted per experiment. Results are expressed as mean ± SD, n=3. The significance of differences was calculated with a two-tailed unpaired t-test(***p<0.0001).

Phosphorylation contributes to zinc-enhanced GAPR-1 condensate formation

Ser55 is localized in close proximity to the conserved metal-binding site of GAPR-1 (Supplementary Fig.2A). Zinc binding enhances GAPR-1 protein oligomerization where the two conserved histidine residues (His54 and His103) are essential for zinc-enhanced amyloid-like oligomerization of GAPR-1 *in vitro* and *in vivo* [28,30]. This raised the possibility that phosphorylation of Ser55 interferes with the metal-binding properties of GAPR-1. Molecular dynamics simulations revealed that introducing a negative charge at position 55 might cause a repulsion with the neighboring glutamate (Glu65) and thus affect the orientation of the loop (Arg50-Gly61) that contains His54 (Fig.2A). This rearrangement of His54 could enhance the metal binding properties and stimulate GAPR-1 oligomerization. To test this experimentally, we determined protein condensate formation in the absence and presence of zinc in the Ser55 phosphorylation-deficient (PXP) and phospho-mimetic GAPR-1 (PDP) mutants. In the phosphorylation-deficient mutant (PXP), GAPR-1 protein condensate formation became insensitive to the presence of zinc ions, whereas zinc-enhanced protein condensate formation was maintained in the phosphomimetic (PDP) mutant (Fig.2B,C). To confirm that phosphorylation of Ser55 impacts His54 in zinc-mediated oligomerization, we constructed two double mutants [(His54V/PXP) and (His54V/PDP)] and studied the condensate-forming properties of these double mutants using live-cell imaging (Figs.2B,C). In the absence of His54, protein condensate formation is no longer zinc sensitive, suggesting that phosphorylation of Ser55 enhances the His54-mediated zinc-binding affinity and subsequently promotes oligomerization (Figs.2B,C).

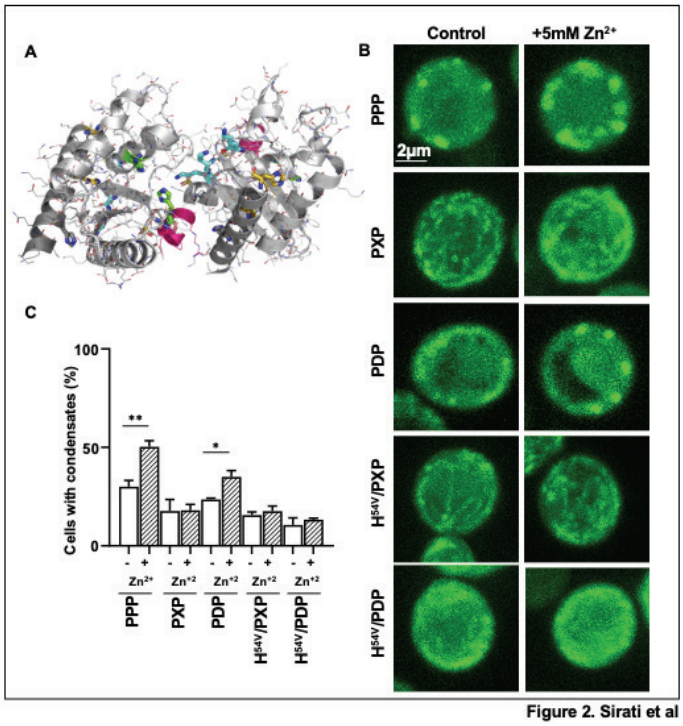


Figure 2. Phosphorylation contributes to zinc-enhanced GAPR-1 condensate formation. A) Molecular dynamics simulations of GAPR-1 in the dimer structure. His54 and His103 are shown in green, the small helix (S55-R60) is shown in pink. B) Live-cell fluorescence microscopy of yeast cells expressing the indicated GAPR-1-GFP constructs (PPP, PXP, PDP, H54V/PXP and H54V/PDP) was performed after 3h in SC-galactose induction media in the absence or presence of 5mM Zn^{2+}; C) Quantification of yeast cells displaying cytoplasmic condensates under conditions presented in (B). The number of cells displaying cytoplasmic condensates is presented as a percent of the total number of cells. At least 100 cells were counted per experiment. The results are expressed as mean ± SD, n=3. The significance of differences was calculated with a two-tailed unpaired t-test (*p<0.05, **P<0.01).

Phosphorylation of GAPR-1 contributes to its interaction with Beclin 1

We recently revealed that GAPR-1 interacts with Beclin 1 and that co-expression of GAPR-1 and Beclin 1 affected the condensate behavior of each other [34]. Therefore, we investigated the contribution of Ser55 phosphorylation of GAPR-1 in the GAPR-1/Beclin 1 interaction. To this end, the number of condensate-forming cells were quantified upon

co-expression of GAPR-1 Ser55 phospho mutant (PXP) with Beclin 1. In contrast to the wt GAPR-1/Beclin 1 co-expression [34] (Supplementary Fig.3), co-expression of the phosphorylation-deficient GAPR-1 mutant PXP with Beclin 1 does not interfere anymore with the protein condensate formation of Beclin 1 (Figs.3A,B). This suggests that phosphorylation-deficient GAPR-1 does not interact with Beclin 1. To exclude that other phosphorylation sites, contribute to the observed effects on condensate formation, we co-expressed the GAPR-1 phosphorylation-deficient mutant XPX with Beclin 1. Expression of this GAPR-1 mutant did result in re-distribution of Beclin 1 to the cytosol, closely resembling the wild-type phenotype. Under all conditions, similar protein expression levels were detected in the single and co-expression experiments using Western blot analyses (Figs.3A,B bottom panels), ruling out effects on condensate formation due to different protein expression levels. Together, our data suggest that phosphorylation of GAPR-1 can regulate the interaction with Beclin 1.

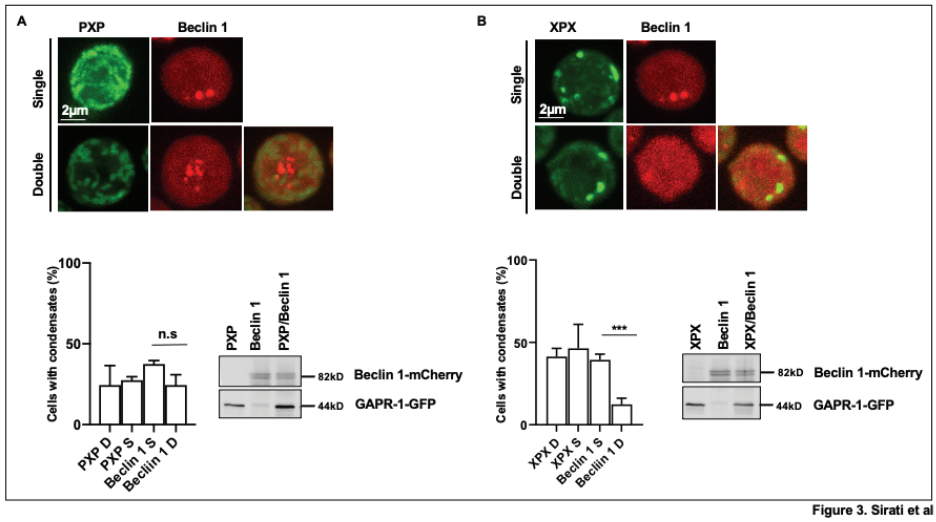


Figure 3. Sirati et al

Figure 3. Phosphorylation of GAPR-1 contributes to its interaction with Beclin 1. A) Live-cell fluorescence microscopy of W303 yeast cells in single and co-expression of GAPR-1-GFP mutant PXP and Beclin 1-mCherry after 24h in SC-galactose induction media. Quantification of cells displaying PXP-GFP and Beclin 1-mCherry condensates in single (S) and co-expression (D) conditions (bottom left). Expression levels of PXP-GFP and Beclin 1-mCherry in single and co-expression conditions were assessed by Western blot, using anti-GAPR-1 and anti-Beclin 1 antibody (bottom right); B) Representative images of single and co-expression of GAPR-1-GFP mutant and Beclin 1-mCherry as indicated in the panels. Quantification of cells displaying XPX-GFP and Beclin 1-mCherry condensates

in single (S) and co-expression (D) conditions. The number of cells displaying cytoplasmic condensates is presented as a percentage of the total number of cells (bottom left). To quantify condensates, at least 100 cells were counted per time point and experiment. Results are expressed as mean ± SD, n=3. The significance of differences was calculated with a two-tailed unpaired t-test ***p<0.001. Expression levels of GAPR-1-GFP mutant XPX and Beclin 1-mCherry in single and co-expression conditions were assessed by Western blot, using anti-GAPR-1 and anti-Beclin 1 antibody (bottom right).

Phosphorylation regulates Beclin 1 condensate formation

Currently, Beclin 1 is known to be phosphorylated at 25 positions (PhosphoSitePlus [35], Supplementary Fig.2B) by various kinases [42]. ULK1/Atg1 is a central regulator of autophagy by phosphorylating Ser15 and Ser30 of Beclin 1 [42–44]. We studied the role of ULK1/Atg1-mediated phosphorylation on Beclin 1 condensates formation by constructing the Beclin 1 mutant Ser15/30A [42,43,45]. Expression of Beclin 1 Ser15/30A in yeast showed a severe effect on condensate formation (Fig.4A). In contrast to wt Beclin 1, Beclin 1 Ser15/30A did not form protein condensates after 3h of expression. Upon prolonged-expression, Beclin 1 Ser15/30A typically clustered into a single condensate, whereas the majority of cells contained more than 3 condensates after expressing wt Beclin 1 (Fig.4A). Protein levels remained comparable in both conditions (Fig.4A). To confirm that the reduction of protein condensate formation in Ser15/30A mutant is related to phosphorylation of Beclin 1 by the ULK1/Atg1 complex, wt Beclin 1 was expressed in the kinase-deficient yeast strain Δatg1. The formation of protein condensates of wt Beclin 1 in this background was identical to the one observed in the Ser15/30A mutant in the wild-type W303 yeast strain. Under all conditions, similar protein expression levels for both constructs (Fig.4B). These data suggest that phosphorylation of Beclin 1 by ULK1/Atg1 influences the amyloidogenic properties of Beclin 1 that cause condensate formation.

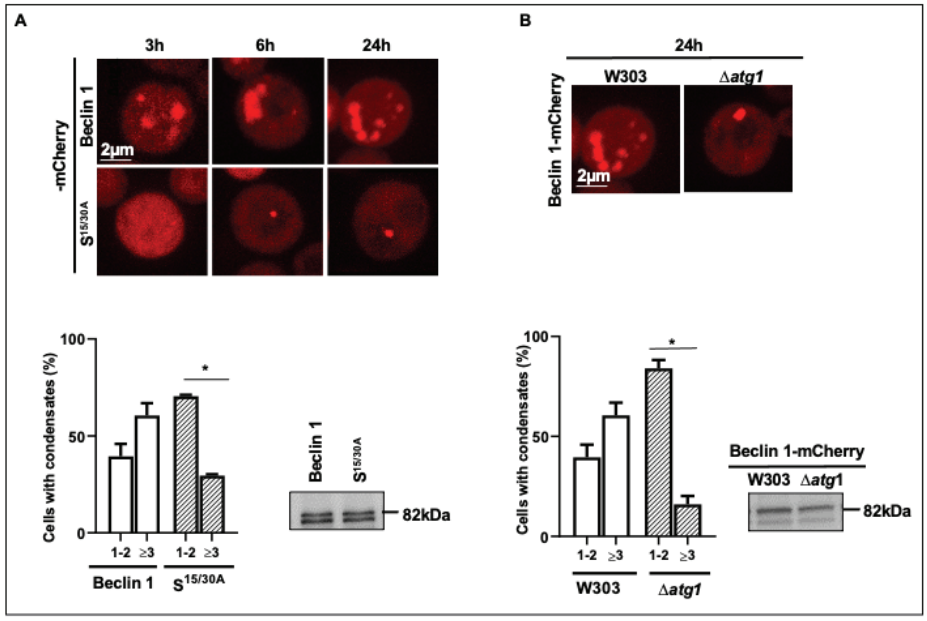


Figure 4. Sirati et al

Figure 4. Phosphorylation regulates Beclin 1 condensate formation. A) Live-cell fluorescent microscopy of W303 cells expressing Beclin 1-mCherry and phospho mutant (S15/30A) after 3h, 6h and 24h of induction. Representative images are shown in the top half panels. Quantification of condensate-positive yeast cells with 1-2 or more than 3 condensates per cell after 24h (bottom left). For the quantification of condensates, at least 100 cells were counted per experiment. Results are expressed as mean ± SD, n=3; Expression levels of Beclin 1-mCherry and phospho mutant were performed by Western blot analysis using an anti-Beclin 1 antibody (bottom left); B) Representative images of Beclin1-mCherry expression in W303 and Δatg1 yeast strains after 24h of induction (top). Quantification of Beclin 1-mCherry condensate-positive yeast cells with 1-2 or more than 3 condensates per cell in W303 and Δatg1 background after 24h (bottom right). For the quantification of condensates, at least 100 cells were counted per experiment. Results are expressed as mean ± SD, n=3; Expression levels of Beclin 1-mCherry in W303 and Δatg1 background after 24h was performed by Western blot analysis using an anti-Beclin 1 antibody (bottom right).

GAPR-1 condensate formation is changed in a Δatg1 yeast strain

To examine whether GAPR-1 is also a substrate of ULK1/Atg1-mediated phosphorylation, we compared the phenotype of GAPR-1-GFP protein condensate formation in the wild-type yeast (W303) with the yeast strain lacking Atg1 (Δatg1). As shown in Fig.5A, expression of GAPR-1-GFP in wild-type yeast results in a time-dependent increase of condensates formation. In the absence of Atg1, GAPR-1-GFP can still form protein condensates, but after 3h, the increase in the number of condensate-forming cells seems halted. GAPR-1 protein levels remained similar in all conditions, suggesting that the difference in the phenotype is not influenced by the Δatg1 background (Fig. 5A, bottom panels). To investigate the role of the Atg1 kinase activity in GAPR-1 oligomerization in more detail, we reconstituted Δatg1 cells harboring GAPR-1-GFP with wild-type Atg1 and an Atg1 kinase-dead mutant (Atg1KD) (KD-K54A/D211A) plasmids, both under the control of the *GAL1* promoter. Wild-type Atg1 but not the mutant Atg1KD fully complemented the effect of Δatg1 background, as shown in Fig. 5B (containing representative images and quantification after 3, 6, and 24h induction). Remarkably, the introduction of a kinase-dead Atg1 mutant in Δatg1 cells showed that condensate formation could be restored to normal levels, but only at late time points (24h). These results suggest an important role of the Atg1 kinase activity at early stages of condensate formation and Atg1 kinase activity-independent effects at later stages of Atg1 overexpression. To identify the GAPR-1 amino acids that are phosphorylated by Atg1, we overexpressed the GAPR-1 Ser55-related mutants PXP and XPX in the Δatg1 yeast strain. As expected, live-cell imaging now showed a similar phenotype of the GAPR-1 (PXP) mutant, in which Ser55 is blocked, as compared to both wt GAPR-1 and the GAPR-1 XPX mutant (Fig.5C). Protein expression levels were comparable under these conditions (Fig.5C, bottom panels). Our combined results predicted that GAPR-1 condensate formation becomes insensitive to the presence of zinc ions in these phosphorylation-deficient cells. Indeed, in contrast to wild-type yeast cells, no zinc sensitivity could be detected anymore in Δatg1 cells (Fig.5D). The zinc sensitivity of Δatg1 cells could be restored by co-expressing Atg1. The kinase-dead mutant Atg1KD could not restore the zinc sensitivity in Δatg1 cells (Fig.5D). Overall, our data revealed that Atg1 kinase activity and phosphorylation in position Ser55 plays an important role in the regulation of zinc-enhanced GAPR-1 oligomerization and condensate formation *in vivo*, especially at early stages.

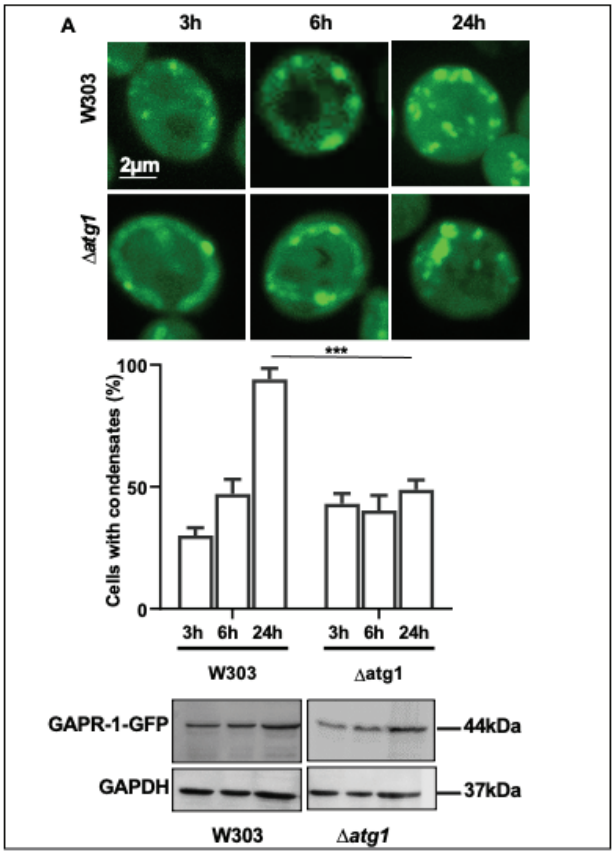


Figure 5A. Sirati et al

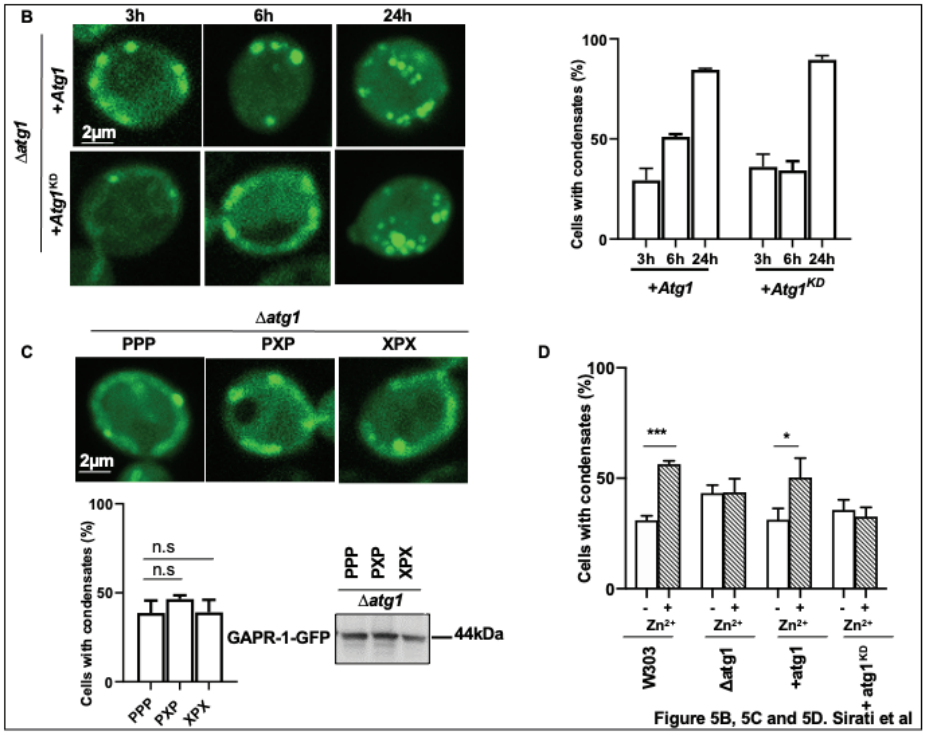


Figure 5. GAPR-1 condensate formation is affected in a Δatg1 yeast strain. A) Representative images of live-cell fluorescent microscopy of W303 and Δatg1 cells expressing GAPR-1-GFP (PPP) are shown after 3h, 6h and 24h of induction (top panels). For quantification, the number of cells displaying cytoplasmic condensates is presented as a percent of the total number of cells (middle panel). Expression levels of GAPR-1-GFP in W303 and Δatg1 backgrounds are determined by Western blot analysis using an anti-GAPR-1 antibody. Expression levels of GAPDH served as loading control (bottom panel); B) Live-cell fluorescence microscopy of cells expressing GAPR1-GFP (PPP) in Δatg1 cells complemented with an Atg1 or Atg1KD after 3h, 6h and 24h. For quantification, the number of cells displaying cytoplasmic condensates is presented as a percent of the total number of cells (right panel); C) GAPR-1-GFP (PPP) and two GAPR-1-GFP phospho mutants (PXP and XPX) were analyzed by live-cell fluorescence microscopy after 3h induction. For quantification, the number of cells displaying cytoplasmic condensates is presented as a percent of the total number of cells. Expression levels of the three GAPR-1-GFP constructs (PPP, PXP and XPX) were assessed by Western blot analysis using an anti-GAPR-1 antibody; D) Quantification of GAPR-1-GFP (PPP) condensate-positive yeast cells in W303, Δatg1, Δatg1+Atg1 and Δatg1+Atg1KD in the presence and in the absence of 5mM zinc. For the quantification, the number of cells displaying cytoplasmic

condensates is presented as a percent of the total number of cells. For all quantifications of condensates, at least 100 cells were counted per time point and per experiment. Results are expressed as mean ± SD, n=3. The significance of differences was calculated with a two-tailed unpaired t-test ***p<0.001.

ULK1/Atg1 regulates the GAPR-1/Beclin 1 interaction

To determine whether phosphorylation of GAPR-1 or Beclin 1 affects the interaction between these two proteins, we investigated the interference with condensate formation during co-expression. As recently described, co-expression of wt GAPR-1 and wt Beclin 1 affected the condensate formation properties of both proteins ([34], and Supplementary Fig.3). When wt GAPR-1-GFP (PPP) and the Ser15/30A Beclin 1-mCherry phosphodeficient mutant were co-expressed in W303 cells, both proteins remain in protein condensates, indicating that they do not interact with each other anymore (Fig.6A). Co-expression of the GAPR-1-GFP phosphodeficient mutant PXP with the Ser15/30A Beclin 1 phosphodeficient mutant also showed no interference with condensate formation (Fig.6B). The protein levels during overexpression remained comparable in both conditions. Our results predict that if the ULK1/Atg1 complex is responsible for both GAPR-1 and Beclin 1 phosphorylation, co-expression of wt GAPR-1 and wt Beclin 1 in a yeast strain that lacks Atg1 would be sufficient to block their interaction. Indeed, in the absence of Atg1, wt GAPR-1 and wt Beclin 1 do not interfere with the condensate formation of each other anymore, indicating a lack of interaction between these two proteins (Fig.6C). Protein expression levels showed similar expression levels of proteins under the various conditions (Fig.6C).

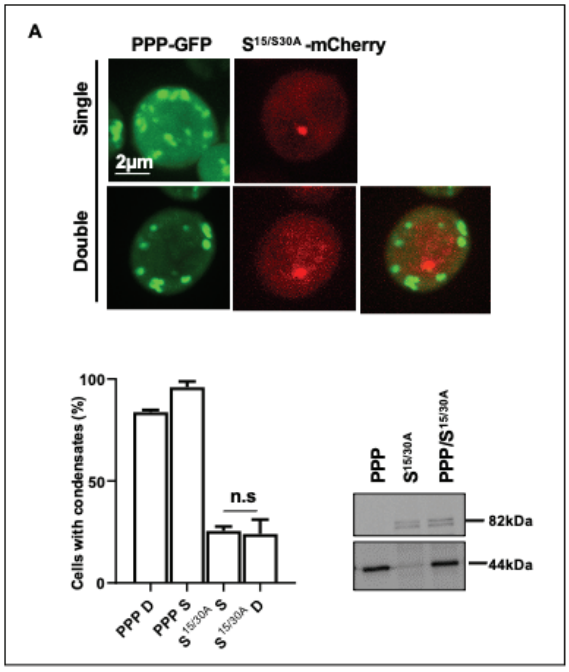


Figure 6A. Sirati et al

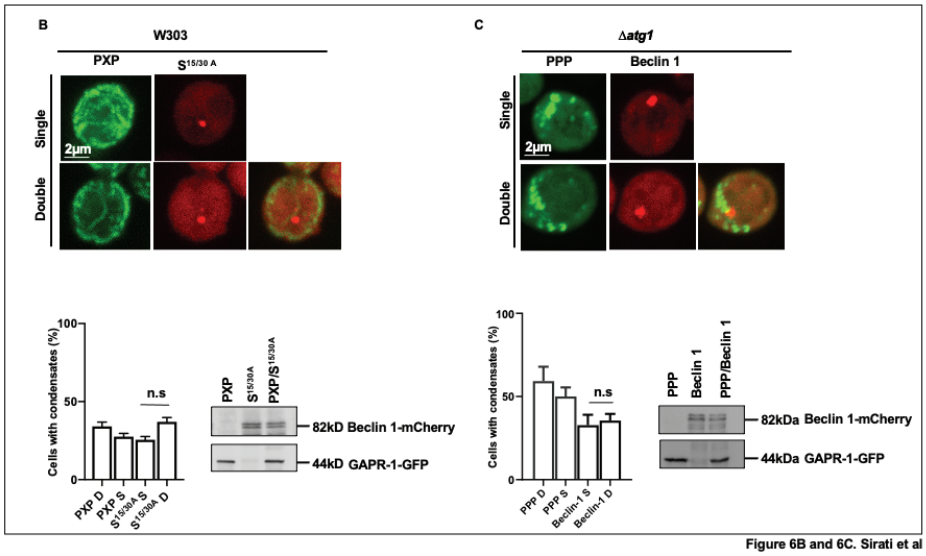


Figure 6B and 6C. Sirati et al

Figure 6. ULK1/Atg1 regulates the GAPR-1/Beclin 1 interaction. A) Single and co-expression of wt GAPR-1-GFP (PPP) and S15/30A Beclin 1-mCherry for 24h in W303 yeast cells. B) Single and co-expression of GAPR-1-GFP (PXP) and S15/30A-mCherry for 24h of expression in W303 yeast cells; C) Single and co-expression of GAPR-1-GFP (PPP) and

Beclin 1-mCherry for 24h in Δatg1 yeast cells. For all conditions: i) top panels: representative live-cell fluorescence microscopy images; ii) bottom left: quantification of cells displaying GFP and/or mCherry condensates of the different constructs in single (S) and co-expression (D) experiments, presented as percentage of the total number of cells displaying cytoplasmic condensates. At least 100 cells were counted per time point and per experiment for all conditions. Results are expressed as mean ± SD, n=3. The significance of differences was calculated with a two-tailed unpaired t-test *p<0.05; iii) bottom right: Western blot analysis of the expression levels of the different GAPR-1-GFP and Beclin 1-mCherry constructs, as indicated in the different panels, after 24h expression in W303 and Δatg1 cells using an anti-GAPR-1 and anti-Beclin 1 antibody.

Discussion

We previously showed that co-expression of human GAPR-1 and human Beclin 1 in yeast interfered with their mutual biomolecular condensate formation and proposed that this interference may be used as a tool to unravel the molecular mechanism of PPIs of amyloidogenic proteins [34]. Here we show the first application of this assay system by identifying protein phosphorylation sites that can act as a regulatory switch in the interaction between GAPR-1 and Beclin 1. Phosphorylation of GAPR-1 at Ser55 and/or phosphorylation of Beclin 1 at Ser15/30 affects the interference with their mutual condensate formation. The precise mechanism of action of phosphorylation in the interaction between GAPR-1 and Beclin 1 remains to be established. We show that phosphorylation regulates the amyloidogenic properties of the individual proteins GAPR-1 and Beclin 1 as phospho-mutants of GAPR-1 and Beclin 1 are hampered in their protein condensate formation. Thus, one possible scenario is that by affecting the amyloidogenic properties, the interaction between GAPR-1 and Beclin 1 is affected. This effect of phosphorylation on condensate formation is reminiscent of the Tau protein, which also forms biomolecular condensates in a phosphorylation-specific manner. Phosphorylation by MARK2 kinase changes the kinetic and dimensions of Tau protein condensates and enhances phase separation at lower protein concentrations [46–49]. Alternatively, in hetero-oligomeric and weak transient homo-oligomeric complexes, phosphorylation sites tend to be located at the binding interfaces [50], similar to what we now observe for GAPR-1. Thirdly, phosphorylation can also affect the protein conformation [50,51], thus affecting condensate formation in a more indirect manner. Finally, phosphorylation could affect the direct PPI between these two proteins as we previously showed that the same type of protein-protein interactions interferes with condensate formation during the co-expression of GAPR-1 and Beclin 1 as described before for their interaction at Golgi membranes [31]. Remarkably, deletion of a single

kinase (ULK1/Atg1), obliterates the interaction between GAPR-1 and Beclin 1, indicating that the molecular mechanism of ULK1/Atg1-mediated autophagy includes regulating amyloidogenic interactions between GAPR-1 and Beclin 1 by protein phosphorylation.

Multiple factors regulate the oligomeric status of GAPR-1, such as the biophysical properties of the membranes, specific negatively charged lipids [52], specific metal ions in the cytosol, and myristoylation [28,29]. Here we show that phosphorylation also contributes to the regulation of the oligomeric properties of GAPR-1, resulting in enhanced protein condensate formation. We previously showed that small but significant conformational changes accompany the formation of GAPR-1 oligomers and amyloid-like assemblies in the presence of zinc ions [28]. Similarly, using molecular dynamics simulations, we now observe that the introduction of a negative charge at position 55 might cause a repulsion with the neighboring glutamate and thus reorients a small loop in the GAPR-1 3D structure. This would allow His54 to rearrange and enhance amyloid-like oligomerization by enhanced zinc binding. Zinc is one of the most abundant micronutrients in the human body, playing a role in cell division, growth, differentiation, signal transduction but also pathology [53]. The combination of disturbed zinc metabolism and (hyper)phosphorylation of e.g. Tau, APP, Aβ and α-synuclein have been shown to play a role in other amyloid-like protein oligomerization and aggregation processes as well, resulting in severe neurodegenerative disorders such as Parkinson's, Alzheimer's and ALS diseases [53]. In pathology, the formation of toxic intermediates is often accompanied by protein hyperphosphorylation and is associated with disturbed zinc homeostasis [54]. Here, we propose that under physiological conditions, the formation of functional amyloidogenic oligomers may be regulated via reversible phosphorylation that can directly impact the metal ion affinity of metal-binding amyloidogenic proteins such as GAPR-1.

Beclin 1 has been reported to form stable homo-oligomers under various conditions and the N-terminal domain, the coiled-coil domain, and the BARA domain facilitate this self-assembly in different ways [8,17,34,55]. It is also known that Beclin 1 can be phosphorylated by various kinases [36,39,42,56]. We now show that these effects may be linked as phosphorylation at amino acids at positions 15 and 30 are essential for protein condensate formation upon overexpression of Beclin 1 in yeast. This offers the intriguing possibility that phosphorylation may indirectly regulate protein-protein interactions by affecting the oligomeric status of Beclin 1. Phosphorylation not only affected the amyloid-like behavior of the individual proteins by affecting their condensate formation, but it also affected their interaction by interfering with the condensate formation during the co-expression of GAPR-1 and Beclin 1. Indeed, the observed interference with condensate formation during co-expression of GAPR-1 and

Beclin 1 may provide a first example of the effect of phosphorylation on the oligomeric status of individual proteins, thus affecting their interaction.

GAPR-1 has been proposed to act as a negative regulator of autophagy by recruiting Beclin 1 to the Golgi complex, thus excluding it from the autophagy signalling cascade [31]. What could be the physiological role of GAPR-1 and/or Beclin 1 phosphorylation in their interaction and in the autophagy process? ULK1/Atg1 induces autophagy by phosphorylating Beclin 1 and activating Vps34 lipid kinase [43,57,58]. We now show that ULK1/Atg1 can also phosphorylate GAPR-1 and that phosphorylation of GAPR-1 and Beclin 1 enhances their interaction. Thus, activating Beclin 1 in the autophagy pathway also enhances the inhibitory effect of GAPR-1 on Beclin 1. We hypothesize that phosphorylation of GAPR-1 provides a negative feedback loop for the activation of Beclin 1 and induction of autophagy. For example, autophagy could be primed by Beclin 1 phosphorylation, and subsequent GAPR-1 phosphorylation could provide a feedback loop preventing the overactivation of autophagy. However, it remains to be established whether GAPR-1 is phosphorylated by ULK1/Atg1 in mammalian cells upon induction of autophagy. Also, we cannot exclude the possibility that other kinases and phosphatases finetune the phosphorylation of GAPR-1 and hence indirectly affect autophagy in a ULK1-independent manner. Irrespective of the possible mechanisms, our results do indicate that phosphorylation may act as a molecular switch to regulate the process of autophagy by amyloidogenic interactions. We recently showed that the interaction between GAPR-1 and Beclin 1 is defined by structural and amyloidogenic protein-protein interactions, and we now show that phosphorylation controls this interaction. In this respect, it is also interesting to note that the kinase-dead mutant of Atg1 could also restore GAPR-1 condensate formation, but only at later stages after overexpression in Δatg1 cells. Analysis of the Atg1 primary sequence using the amyloid prediction software AmylPred2 [59] revealed multiple regions with potential amyloidogenic properties (Supplementary Fig.4). Thus, the autophagy process may be partly regulated by amyloidogenic interactions of other key players as well.

Stimulatory compounds of the autophagy pathway have a strong pharmaceutical potential, e.g. for the clearance of viruses in infectious diseases and for the clearance of protein aggregates in neurodegenerative disorders. The identification of GAPR-1 phosphorylation as an efficient means to regulate the GAPR-1/Beclin 1 interaction may provide novel tools to interfere with autophagy based on the available high throughput screens of kinase and phosphatase inhibitors.

Materials and methods

Yeast strains and plasmids

Wild-type yeast W303 and Δ*atg1* strains were transformed by Frozen-EZ yeast kit from Zymo research (Zymo Research, Orange, CA, USA) with a high copy (2μ) plasmid p423 carrying different constructs, which were tagged C-terminally by GFP, or with a p426 plasmid C-terminally tagged with mCherry (Table 1).

To construct P*GAL1*-GAPR-1-GFP and P*GAL1*-Beclin 1-mCherry fragments, GAPR-1, Beclin 1, GFP and mCherry sequences were amplified by PCR and were inserted into the plasmids at the SmaI restriction site by using Geneart® seamless cloning assembly kit (Invitrogen, California, United States). All mutants were constructed by using a Phusion™ site-directed mutagenesis kit (Thermo Scientific, Massachusetts, United States). The primers are listed in Table 2.

Table 1. Plasmids used in this study

Plasmid	Description	Source
p423-*GAL1*	*2μm; HIS3; GAL1pr; CYC1term; AmpR*	D. Mumberg, R. Muller, M. Funk, Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression, Nucleic Acids Res. 22 (1994) 5767–5768. https://doi.org/10.1093/nar/22.25.5767. [60]
p426-*GAL1*	*2μm; URA3; GAL1pr; CYC1term; AmpR*	D. Mumberg, R. Muller, M. Funk, Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression, Nucleic Acids Res. 22 (1994) 5767–5768. https://doi.org/10.1093/nar/22.25.5767. [60]
pNS 1000	*p423-GAL1-GAPR-1-GFP*	This study
pNS 2000	*p426-GAL1*-Beclin 1-mCherry	This study
pNS 1005	*p423-GAL1-Y42F GAPR-1-GFP*	This study

pNS 1006	*p423-GAL1-S55G GAPR-1-GFP*	This study
pNS 1007	*p423-GAL1-S123G GAPR-1-GFP*	This study
pNS 1008	*p423-GAL1-* S55G/S123G*GAPR-1-GFP*	This study
pNS 1009	*p423-GAL1-* Y42F/S123G *GAPR-1-GFP*	This study
pNS 1010	*p423-GAL1-* Y42F/S55G *GAPR-1-GFP*	This study
pNS 1011	*p423-GAL1-* Y42F/S55G/S123G *GAPR-1-GFP*	This study
pNS 1012	*p423-GAL1-* Y42D/S55D/S123D *GAPR-1-GFP*	This study
pNS 1013	*p423-GAL1-* Y42F/S55D/S123G *GAPR-1-GFP*	This study
pNS 1014	*p423-GAL1*-S55D *GAPR-1-GFP*	This study
pNS 2005	*p426-GAL1-S15A/S30A*-Beclin 1-mCherry	This study

Table 2. Primers used in this study

Primer	Sequence
FW GAPR-1	5’-GAACTAGTGGATCCCCCATGGGCAAGTCAGC-3'
REV GAPR-1 for GFP	5’-CTTCTCCTTTACTATCGATAAGCTTCTTCTTCGGCGGC5-3'
FW GFP for GAPR-1	5’-CCGCCGAAGAAGAAGCTTATCGATAGTAAAGGAGAAGAAC-3’
REV GFP	5’-GAATTCCTGCAGCCCTTATTTGTATAGTTCATCC-3'
FW Beclin 1	5'- GAACTAGTGGATCCCCCATGGAAGGGTCTAAGACG-3'
REV Beclin 1 for mCherry	5’- CCTTGCTCACCATATCGATAAGCTTTTTGTTATAAAATTGTGAG-3’
FW mCherry for Beclin	5’- CTCACAATTTTATAACAAAAAGCTTATCGATATGGTGAGCAAGG-3’
REV mCherry	5'- GAATTCCTGCAGCCCCTACTTGTACAGCTC-3'
FW Y42F-GAPR-1	5’-CAACAGTTTTCTGAGGCCCTG-3'
REV Y42F-GAPR-1	5’-CTCAGAAAACTGTTGAGCCTCCCG-3'
FW S55G-GAPR-1	5’-CAAGCACGGCCCGGAGTCCAG-3'
REV S55G-GAPR-1	5’-CTCCGGGCCGTGCTTGAGGATC-3'
FW S55D-GAPR-1	5’-CAAGCACGACGACCCGGAGTCCAGC-3'
REV S55D-GAPR-1	5’-CTCCGGGTCGTGCTTGAGGATC-3'
FW S123G-GAPR-1	5’-CCGCAGGTGACGGGTCCTCCTTTG-3'
REV S123G-GAPR-1	5’-GTCACCTGCGGACGCCTTCC-3'

Yeast culture media

Cells were pre-grown overnight in synthetic complete (SC) medium lacking the corresponding marker (-His, -Ura) and supplemented with either 2% glucose or 2% galactose at 30^{o}C with orbital agitation (200 rpm) for 18 hours (overnight). The day after, the cells were placed in a selective SC medium supplemented with 2% galactose to induce protein expression (OD_{600}=0.2). After 3h, 6h and, 24h, cells were collected to obtain total cell homogenates or were observed under a live-cell Nikon Eclipse Ti-E microscope (Nikon).

Co-expression study

Yeast strains containing GAPR-1-GFP or/and Beclin 1-mCherry were grown overnight in 10 ml SC medium supplemented with 2% raffinose and lacking histidine and uracil. Two different strains, W303 and Δatg1, were used in this study. Single expression conditions were achieved by supplementing the transformation with empty vectors (P423-His or P426-Ura). To induce the *GAL1* promoter, cells were inoculated into a fresh 50 ml culture of SC-Ura-His containing 2% galactose, with an initial OD600 of 0.1. The cultures were then incubated for 24h under appropriate conditions.

Spotting assay

All spotting assays were performed under the same conditions. Ten-fold serial dilutions starting with an equal number of cells (OD_{600}=0.1) were performed in sterile water. Drops of 10 µl were then spotted on SC plates lacking the corresponding markers (SC-His-Ura) and supplemented with either 2% glucose or 2% galactose. Three independent experiments from fresh transformants were performed and followed by 3 days of incubation at 30°C before scanning the plates.

Fluorescence microscopy

Cells were visualized at different time points after induction (3h, 6h and 24h) using a Nikon Eclipse Ti-E microscope (Nikon) equipped with the Perfect Focus System (Nikon) Nikon Apo TIRF 100x N.A. 1.49 oil objective (Nikon), a spinning disk-based confocal scanner unit (CSU-X1-A1, Yokogawa), and the ET-GFP filter set (49002, Chroma) and ET-mCherry filter set (49008, Chroma). At least 100 cells were counted per condition and experiment for quantifications. The number of cells displaying protein condensates for each condition was calculated relative to the total number of cells counted (with condensates or cytoplasmic distribution) and reported as a percentage on a column chart. The

number of condensates per cell was determined by counting 100 cells, and the condensate-positive cells were categorized into two groups: 1) one or two condensates per cell or 2) three or more condensates per cell. At least three independent experiments with freshly transformed constructs were performed.

Protein extraction and Western blotting

10 OD units of cells after overexpression under *GAL1* promoter were collected by centrifugation, washed with sterile water and lysed with 200 µl of Y-PER™ reagent (Thermo Scientific, Massachusetts, United States) containing fresh protease inhibitors (Aprotinin, Leupeptin, Pepstatin, PMSF) (Sigma Aldrich, St. Louis, USA). Lysed cells were incubated for 20 minutes at room temperature. Total protein homogenates were collected for further analysis. 20 µg of proteins were incubated with Laemmli sample buffer for 5 minutes at 100°C and separated by SDS-PAGE (12% polyacrylamide). Proteins were transferred onto 0.45 µm nitrocellulose membranes (Amersham Protran GE Healthcare) at 100 V for 1h for Western blot analysis and were probed with a rabbit polyclonal anti-GAPR-1 antibody [27] and/or a rabbit monoclonal anti-Beclin 1 antibody (Santa Cruz, Dallas, Texas, United States). A monoclonal anti-GAPDH antibody (Thermo Scientific, Massachusetts, United States) was used as a loading control. Peroxidase-conjugated goat anti-rabbit antibody (Nordic-Mubio, Susteren, The Netherlands) was used as a secondary antibody. Binding was detected with the SuperSignal™ reagents (Thermo Scientific, Massachusetts, United States) and ChemiDoc™ MP Imaging system (BioRad, Hercules, California, United States).

Statistical Analysis

Data were analyzed using GraphPad Prism 9 (San Diego, CA, USA) Software and were presented as mean ± SD of at least three independent experiments. The significance of differences was calculated using a two-tailed unpaired t-test. P-values lower than 0.05 were considered to indicate a significant difference.For quantification of Western blots, pixel density values were obtained from TIFF files and analyzed with Image Lab Software™. Sample density values were normalized to the corresponding GAPDH loading control. The adjusted density values were standardized to the control lane, and the significance of differences was calculated using a two-tailed unpaired t-test.

References

[1] K.W. Barber, J. Rinehart, The ABCs of PTMs, Nat. Chem. Biol. 14 (2018) 188–192. https://doi.org/10.1038/nchembio.2572.

[2] J. Roignot, T. Bonacci, E. Ghigo, J.L. Iovanna, P. Soubeyran, Oligomerization and Phosphorylation Dependent Regulation of ArgBP2 Adaptive Capabilities and Associated Functions, PLoS One. 9 (2014) e87130. https://doi.org/10.1371/journal.pone.0087130.

[3] A. Zuidema, P. Atherton, M. Kreft, L. Hoekman, O.B. Bleijerveld, N. Nagaraj, N. Chen, R. Fässler, A. Sonnenberg, PEAK1 Y635 phosphorylation regulates cell migration through association with Tensin3 and integrins, J. Cell Biol. 221 (2022). https://doi.org/10.1083/jcb.202108027.

[4] Q. Xue, S.R.S. Varady, T.Q.A. Waddell, M.R. Roman, J. Carrington, M. Roh-Johnson, Lack of Paxillin phosphorylation promotes single-cell migration *in vivo*, J. Cell Biol. 222 (2023). https://doi.org/10.1083/jcb.202206078.

[5] N. Kumari, S. Yadav, Modulation of protein oligomerization: An overview, Prog. Biophys. Mol. Biol. 149 (2019) 99–113. https://doi.org/10.1016/j.pbiomolbio.2019.03.003.

[6] R. Maeda, H. Tamagaki-Asahina, T. Sato, M. Yanagawa, Y. Sako, Threonine phosphorylation regulates the molecular assembly and signaling of EGFR in cooperation with membrane lipids, J. Cell Sci. 135 (2022). https://doi.org/10.1242/jcs.260355.

[7] S.S.-Y. Kim, D.C.N. Sim, G. Carissimo, H.-H. Lim, K.-P. Lam, Bruton's tyrosine kinase phosphorylates scaffolding and RNA-binding protein G3BP1 to induce stress granule aggregation during host sensing of foreign ribonucleic acids, J. Biol. Chem. 298 (2022) 102231. https://doi.org/10.1016/j.jbc.2022.102231.

[8] E.P. Barykin, V.A. Mitkevich, S.A. Kozin, A.A. Makarov, Amyloid β Modification: A Key to the Sporadic Alzheimer's Disease?, Front. Genet. 8 (2017). https://doi.org/10.3389/fgene.2017.00058.

[9] N. Georgopoulou, M. McLaughlin, I. McFarlane, K.C. Breen, The role of post-translational modification in ϐ-amyloid precursor protein processing, Biochem. Soc. Symp. 67 (2001) 23–36. https://doi.org/10.1042/bss0670023.

[10] B. György, E. Tóth, E. Tarcsa, A. Falus, E.I. Buzás, Citrullination: A posttranslational modification in health and disease, Int. J. Biochem. Cell Biol. 38

(2006) 1662–1677. https://doi.org/10.1016/j.biocel.2006.03.008.

[11] S.C. Junqueira, E.G.Z. Centeno, K.A. Wilkinson, H. Cimarosti, Post-translational modifications of Parkinson's disease-related proteins: Phosphorylation, SUMOylation and Ubiquitination, Biochim. Biophys. Acta - Mol. Basis Dis. 1865 (2019) 2001–2007. https://doi.org/10.1016/j.bbadis.2018.10.025.

[12] B. Popova, A. Kleinknecht, P. Arendarski, J. Mischke, D. Wang, G.H. Braus, Sumoylation Protects Against β-Synuclein Toxicity in Yeast, Front. Mol. Neurosci. 11 (2018). https://doi.org/10.3389/fnmol.2018.00094.

[13] M.M. Hefti, S. Kim, A.J. Bell, R.K. Betters, K.L. Fiock, M.A. Iida, M.E. Smalley, K. Farrell, M.E. Fowkes, J.F. Crary, Tau Phosphorylation and Aggregation in the Developing Human Brain, J. Neuropathol. Exp. Neurol. 78 (2019) 930–938. https://doi.org/10.1093/jnen/nlz073.

[14] S. Mondragón-Rodríguez, G. Perry, J. Luna-Muñoz, M.C. Acevedo-Aquino, S. Williams, Phosphorylation of tau protein at sites Ser 396-404 is one of the earliest events in Alzheimer's disease and Down syndrome, Neuropathol. Appl. Neurobiol. 40 (2014) 121–135. https://doi.org/10.1111/nan.12084.

[15] G. Šimić, M. Babić Leko, S. Wray, C. Harrington, I. Delalle, N. Jovanov-Milošević, D. Bažadona, L. Buée, R. de Silva, G. Di Giovanni, C. Wischik, P. Hof, Tau Protein Hyperphosphorylation and Aggregation in Alzheimer's Disease and Other Tauopathies, and Possible Neuroprotective Strategies, Biomolecules. 6 (2016) 6. https://doi.org/10.3390/biom6010006.

[16] E.P. Barykin, D. V. Yanvarev, M.A. Strelkova, V.T. Valuev-Elliston, K.B. Varshavskaya, V.A. Mitkevich, A.A. Makarov, Phosphorylation and Dephosphorylation of Beta-Amyloid Peptide in Model Cell Cultures: The Role of Cellular Protein Kinases and Phosphatases, Life. 13 (2023) 147. https://doi.org/10.3390/life13010147.

[17] N. González, T. Arcos-López, A. König, L. Quintanar, M. Menacho Márquez, T.F. Outeiro, C.O. Fernández, Effects of alpha-synuclein post-translational modifications on metal binding, J. Neurochem. 150 (2019) 507–521. https://doi.org/10.1111/jnc.14721.

[18] P.J. Barrett, J. Timothy Greenamyre, Post-translational modification of α-synuclein in Parkinson's disease, Brain Res. 1628 (2015) 247–253. https://doi.org/10.1016/j.brainres.2015.06.002.

[19] S. Zhang, R. Zhu, B. Pan, H. Xu, M.F. Olufemi, R.J. Gathagan, Y. Li, L. Zhang, J.

Zhang, W. Xiang, E.M. Kagan, X. Cao, C. Yuan, S.-J. Kim, C.K. Williams, S. Magaki, H. V. Vinters, H.A. Lashuel, B.A. Garcia, E. James Petersson, J.Q. Trojanowski, V.M.-Y. Lee, C. Peng, Post-translational modifications of soluble α-synuclein regulate the amplification of pathological α-synuclein, Nat. Neurosci. 26 (2023) 213–225. https://doi.org/10.1038/s41593-022-01239-7.

[20] P.P. Rainer, P. Dong, M. Sorge, J. Fert-Bober, R.J. Holewinski, Y. Wang, C.A. Foss, S.S. An, A. Baracca, G. Solaini, C.G. Glabe, M.G. Pomper, J.E. Van Eyk, G.F. Tomaselli, N. Paolocci, G. Agnetti, Desmin Phosphorylation Triggers Preamyloid Oligomers Formation and Myocyte Dysfunction in Acquired Heart Failure, Circ. Res. 122 (2018). https://doi.org/10.1161/CIRCRESAHA.117.312082.

[21] I. Owen, F. Shewmaker, The Role of Post-Translational Modifications in the Phase Transitions of Intrinsically Disordered Proteins, Int. J. Mol. Sci. 20 (2019) 5501. https://doi.org/10.3390/ijms20215501.

[22] M. Hofweber, D. Dormann, Friend or foe—Post-translational modifications as regulators of phase separation and RNP granule dynamics, J. Biol. Chem. 294 (2019) 7137–7150. https://doi.org/10.1074/jbc.TM118.001189.

[23] Z. Monahan, V.H. Ryan, A.M. Janke, K.A. Burke, S.N. Rhoads, G.H. Zerze, R. O'Meally, G.L. Dignon, A.E. Conicella, W. Zheng, R.B. Best, R.N. Cole, J. Mittal, F. Shewmaker, N.L. Fawzi, Phosphorylation of the <scp>FUS</scp> low-complexity domain disrupts phase separation, aggregation, and toxicity, EMBO J. 36 (2017) 2951–2967. https://doi.org/10.15252/embj.201696394.

[24] S.R. Kundinger, E.B. Dammer, L. Yin, C. Hurst, S. Shapley, L. Ping, S. Khoshnevis, H. Ghalei, D.M. Duong, N.T. Seyfried, Phosphorylation regulates arginine-rich RNA-binding protein solubility and oligomerization, J. Biol. Chem. 297 (2021) 101306. https://doi.org/10.1016/j.jbc.2021.101306.

[25] N. K. Olrichs, J. Bernd Helms, Novel insights into the function of the conserved domain of the CAP superfamily of proteins, AIMS Biophys. 3 (2016) 232–246. https://doi.org/10.3934/biophy.2016.2.232.

[26] J. Sheng, N.K. Olrichs, B.M. Gadella, D. V. Kaloyanova, J.B. Helms, Regulation of Functional Protein Aggregation by Multiple Factors: Implications for the Amyloidogenic Behavior of the CAP Superfamily Proteins, Int. J. Mol. Sci. 21 (2020) 6530. https://doi.org/10.3390/ijms21186530.

[27] H.B. Eberle, R.L. Serrano, J. Füllekrug, A. Schlosser, W.D. Lehmann, F. Lottspeich, D. Kaloyanova, F.T. Wieland, J.B. Helms, Identification and characterization of a

novel human plant pathogenesis-related protein that localizes to lipid-enriched microdomains in the Golgi complex., J. Cell Sci. 115 (2002) 827–38. http://jcs.biologists.org/content/115/4/827.abstract.

[28] J. Sheng, N.K. Olrichs, W.J. Geerts, X. Li, A.U. Rehman, B.M. Gadella, D. V. Kaloyanova, J.B. Helms, Zinc binding regulates amyloid-like aggregation of GAPR-1, Biosci. Rep. 39 (2019) BSR20182345. https://doi.org/10.1042/BSR20182345.

[29] J. Sheng, N.K. Olrichs, W.J. Geerts, D. V. Kaloyanova, J.B. Helms, Metal ions and redox balance regulate distinct amyloid-like aggregation pathways of GAPR-1, Sci. Rep. 9 (2019) 15048. https://doi.org/10.1038/s41598-019-51232-7.

[30] N. Sirati, B. Popova, M.R. Molenaar, I.C. Verhoek, G.H. Braus, D. V. Kaloyanova, J.B. Helms, Dynamic and Reversible Aggregation of the Human CAP Superfamily Member GAPR-1 in Protein Inclusions in *Saccharomyces cerevisiae*, J. Mol. Biol. 433 (2021) 167162. https://doi.org/10.1016/j.jmb.2021.167162.

[31] S. Shoji-Kawata, R. Sumpter, M. Leveno, G.R. Campbell, Z. Zou, L. Kinch, A.D. Wilkins, Q. Sun, K. Pallauf, D. MacDuff, C. Huerta, H.W. Virgin, J. Bernd Helms, R. Eerland, S.A. Tooze, R. Xavier, D.J. Lenschow, A. Yamamoto, D. King, O. Lichtarge, N. V. Grishin, S.A. Spector, D. V. Kaloyanova, B. Levine, Identification of a candidate therapeutic autophagy-inducing peptide, Nature. 494 (2013) 201–206. https://doi.org/10.1038/nature11866.

[32] Y. Li, Y. Zhao, M. Su, K. Glover, S. Chakravarthy, C.L. Colbert, B. Levine, S.C. Sinha, Structural insights into the interaction of the conserved mammalian proteins GAPR-1 and Beclin 1, a key autophagy protein, Acta Crystallogr. Sect. D Struct. Biol. 73 (2017) 775–792. https://doi.org/10.1107/S2059798317011822.

[33] Y. Zhao, Z. Zou, D. Sun, Y. Li, S.C. Sinha, L. Yu, L. Bennett, B. Levine, GLIPR2 is a negative regulator of autophagy and the BECN1-ATG14-containing phosphatidylinositol 3-kinase complex, Autophagy. 17 (2021) 2891–2904. https://doi.org/10.1080/15548627.2020.1847798.

[34] N. Sirati, Z. Shen, N.K. Olrichs, B. Popova, I.C. Verhoek, I.M. Lagerwaard, G.H. Braus, D. V. Kaloyanova, J.B. Helms, GAPR-1 Interferes with Condensate Formation of Beclin 1 in *Saccharomyces cerevisiae*, J. Mol. Biol. 435 (2023) 167935. https://doi.org/10.1016/j.jmb.2022.167935.

[35] P. V. Hornbeck, B. Zhang, B. Murray, J.M. Kornhauser, V. Latham, E. Skrzypek, PhosphoSitePlus, 2014: mutations, PTMs and recalibrations, Nucleic Acids Res. 43 (2015) D512–D520. https://doi.org/10.1093/nar/gku1267.

[36] R. Kang, H.J. Zeh, M.T. Lotze, D. Tang, The Beclin 1 network regulates autophagy and apoptosis, Cell Death Differ. 18 (2011) 571–580. https://doi.org/10.1038/cdd.2010.191.

[37] C. He, B. Levine, The Beclin 1 interactome, Curr. Opin. Cell Biol. 22 (2010) 140–149. https://doi.org/10.1016/j.ceb.2010.01.001.

[38] Y. Sun, X. Yao, Q.-J. Zhang, M. Zhu, Z.-P. Liu, B. Ci, Y. Xie, D. Carlson, B.A. Rothermel, Y. Sun, B. Levine, J.A. Hill, S.E. Wolf, J.P. Minei, Q.S. Zang, Beclin-1-Dependent Autophagy Protects the Heart During Sepsis, Circulation. 138 (2018) 2247–2262. https://doi.org/10.1161/CIRCULATIONAHA.117.032821.

[39] X. Qian, X. Li, Q. Cai, C. Zhang, Q. Yu, Y. Jiang, J.-H. Lee, D. Hawke, Y. Wang, Y. Xia, Y. Zheng, B.-H. Jiang, D.X. Liu, T. Jiang, Z. Lu, Phosphoglycerate Kinase 1 Phosphorylates Beclin1 to Induce Autophagy, Mol. Cell. 65 (2017) 917-931.e6. https://doi.org/10.1016/j.molcel.2017.01.027.

[40] Y. Ohashi, Activation Mechanisms of the VPS34 Complexes, Cells. 10 (2021) 3124. https://doi.org/10.3390/cells10113124.

[41] M. Zahn-Zabal, P.-A. Michel, A. Gateau, F. Nikitin, M. Schaeffer, E. Audot, P. Gaudet, P.D. Duek, D. Teixeira, V. Rech de Laval, K. Samarasinghe, A. Bairoch, L. Lane, The neXtProt knowledgebase in 2020: data, tools and usability improvements, Nucleic Acids Res. (2019). https://doi.org/10.1093/nar/gkz995.

[42] M.B. Menon, S. Dhamija, Beclin 1 Phosphorylation – at the Center of Autophagy Regulation, Front. Cell Dev. Biol. 6 (2018). https://doi.org/10.3389/fcell.2018.00137.

[43] R.C. Russell, Y. Tian, H. Yuan, H.W. Park, Y.-Y. Chang, J. Kim, H. Kim, T.P. Neufeld, A. Dillin, K.-L. Guan, ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase, Nat. Cell Biol. 15 (2013) 741–750. https://doi.org/10.1038/ncb2757.

[44] J.-M. Park, M. Seo, C.H. Jung, D. Grunwald, M. Stone, N.M. Otto, E. Toso, Y. Ahn, M. Kyba, T.J. Griffin, L. Higgins, D.-H. Kim, ULK1 phosphorylates Ser30 of BECN1 in association with ATG14 to stimulate autophagy induction, Autophagy. 14 (2018) 584–597. https://doi.org/10.1080/15548627.2017.1422851.

[45] S.M. Hill, L. Wrobel, D.C. Rubinsztein, Post-translational modifications of Beclin 1 provide multiple strategies for autophagy regulation, Cell Death Differ. 26 (2019) 617–629. https://doi.org/10.1038/s41418-018-0254-9.

[46] S. Wegmann, B. Eftekharzadeh, K. Tepper, K.M. Zoltowska, R.E. Bennett, S. Dujardin, P.R. Laskowski, D. MacKenzie, T. Kamath, C. Commins, C. Vanderburg, A.D. Roe, Z. Fan, A.M. Molliex, A. Hernandez-Vega, D. Muller, A.A. Hyman, E. Mandelkow, J.P. Taylor, B.T. Hyman, Tau protein liquid–liquid phase separation can initiate tau aggregation, EMBO J. 37 (2018). https://doi.org/10.15252/embj.201798049.

[47] S. Ambadipudi, J. Biernat, D. Riedel, E. Mandelkow, M. Zweckstetter, Liquid–liquid phase separation of the microtubule-binding repeats of the Alzheimer-related protein Tau, Nat. Commun. 8 (2017) 275. https://doi.org/10.1038/s41467-017-00480-0.

[48] N.M. Kanaan, C. Hamel, T. Grabinski, B. Combs, Liquid-liquid phase separation induces pathogenic tau conformations in vitro, Nat. Commun. 11 (2020) 2809. https://doi.org/10.1038/s41467-020-16580-3.

[49] A. Savastano, D. Flores, H. Kadavath, J. Biernat, E. Mandelkow, M. Zweckstetter, Disease-Associated Tau Phosphorylation Hinders Tubulin Assembly within Tau Condensates., Angew. Chem. Int. Ed. Engl. 60 (2021) 726–730. https://doi.org/10.1002/anie.202011157.

[50] H. Nishi, K. Hashimoto, A.R. Panchenko, Phosphorylation in Protein-Protein Binding: Effect on Stability and Function, Structure. 19 (2011) 1807–1815. https://doi.org/10.1016/j.str.2011.09.021.

[51] F. Ardito, M. Giuliani, D. Perrone, G. Troiano, L. Lo Muzio, The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review), Int. J. Mol. Med. 40 (2017) 271–280. https://doi.org/10.3892/ijmm.2017.3036.

[52] J. Van Galen, B.W.M. Van Balkom, R.L. Serrano, D. Kaloyanova, R. Eerland, E. Stüven, J.B. Helms, Binding of GAPR-1 to negatively charged phospholipid membranes: Unusual binding characteristics to phosphatidylinositol, Mol. Membr. Biol. 27 (2010) 81–91. https://doi.org/10.3109/09687680903507080.

[53] M. Maywald, L. Rink, Zinc in Human Health and Infectious Diseases, Biomolecules. 12 (2022) 1748. https://doi.org/10.3390/biom12121748.

[54] J.-Y. Hu, D.-L. Zhang, X.-L. Liu, X.-S. Li, X.-Q. Cheng, J. Chen, H.-N. Du, Y. Liang, Pathological concentration of zinc dramatically accelerates abnormal aggregation of full-length human Tau and thereby significantly increases Tau toxicity in neuronal cells, Biochim. Biophys. Acta - Mol. Basis Dis. 1863 (2017)

414–427. https://doi.org/10.1016/j.bbadis.2016.11.022.

[55] B.M. Sefton, Overview of Protein Phosphorylation, Curr. Protoc. Cell Biol. 00 (1998) 14.1.1-14.1.3. https://doi.org/10.1002/0471143030.cb1401s00.

[56] S. Tran, W.D. Fairlie, E.F. Lee, BECLIN1: Protein Structure, Function and Regulation, Cells. 10 (2021) 1522. https://doi.org/10.3390/cells10061522.

[57] M. Zachari, I.G. Ganley, The mammalian ULK1 complex and autophagy initiation, Essays Biochem. 61 (2017) 585–596. https://doi.org/10.1042/EBC20170021.

[58] M. Licheva, B. Raman, C. Kraft, F. Reggiori, Phosphoregulation of the autophagy machinery by kinases and phosphatases, Autophagy. 18 (2022) 104–123. https://doi.org/10.1080/15548627.2021.1909407.

[59] A.C. Tsolis, N.C. Papandreou, V.A. Iconomidou, S.J. Hamodrakas, A Consensus Method for the Prediction of 'Aggregation-Prone' Peptides in Globular Proteins, PLoS One. 8 (2013) e54175. https://doi.org/10.1371/journal.pone.0054175.

[60] D. Mumberg, R. Muller, M. Funk, Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression, Nucleic Acids Res. 22 (1994) 5767–5768. https://doi.org/10.1093/nar/22.25.5767.

Supplemental information

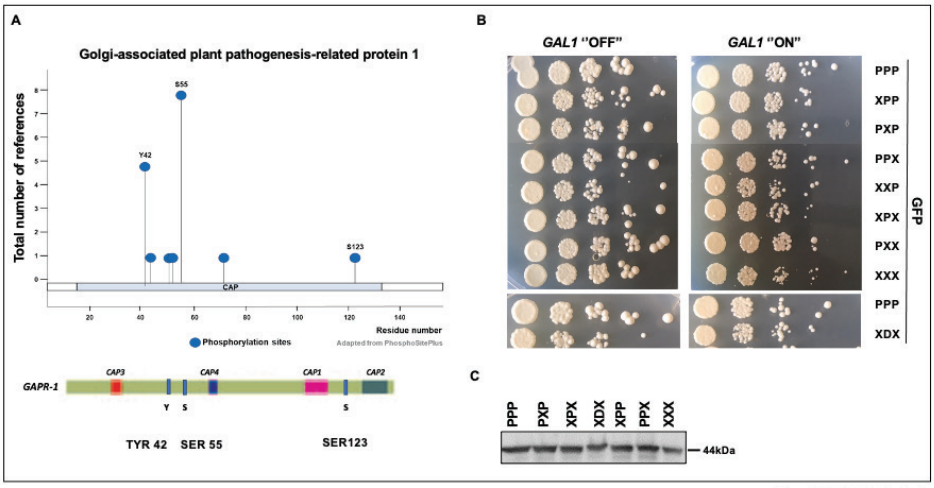


Figure S1. A) Identified phosphorylation sites of GAPR-1 according to PhosphoSitePlus [35]. The phosphorylation sites used in this study (Tyr^{42}, Ser^{55} and Ser^{123}) are labeled; B) W303 cells were transformed with a plasmid carrying GAPR-1-GFP (PPP) and phospho mutants. Ten-fold dilutions of exponentially growing cultures, transformed with plasmids carrying the different constructions, were spotted using selection plates containing 2% glucose (*GAL1* promoter "OFF") and 2% galactose (*GAL1* promoter "ON"). The cells were incubated at 30°C for 3 days; C) Western blot analysis of wt GAPR-1(PPP) and phospho mutants after 24h expression and using an anti-GAPR-1 antibody.

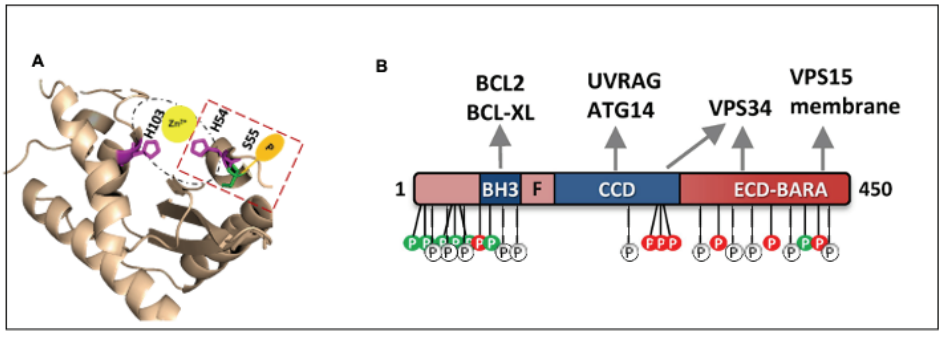


Figure S2. A) 3D structure of GAPR-1, highlighting the amino acids involved in zinc binding (H54, H103), the S55 phosphorylation site, and the suggested Zn^{2+} binding (yellow, based on [28]; B) Primary structure of Beclin 1 showing the different motifs and

the approximate locations of identified phosphorylation sites according to PhosphoSitePlus (pro-autophagy and inhibitory phosphorylation are shown in green and red, respectively, according to Menon et al. [42]).

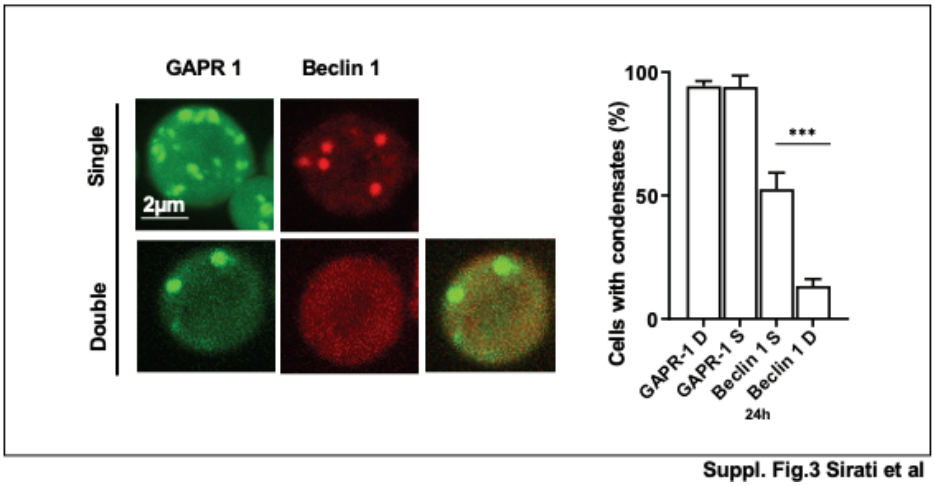


Figure S3. Representative images of GAPR-1-GFP and Beclin1-mCherry single and co-expression in W303 strain after 24h expression. Quantification of GAPR-1-GFP and Beclin 1-mCherry cells displaying condensate in single (S) and double (D) expression in W303 after 24h. For the quantification of condensates, at least 100 cells were counted per experiment. Results are expressed as mean ± SD, n=3.

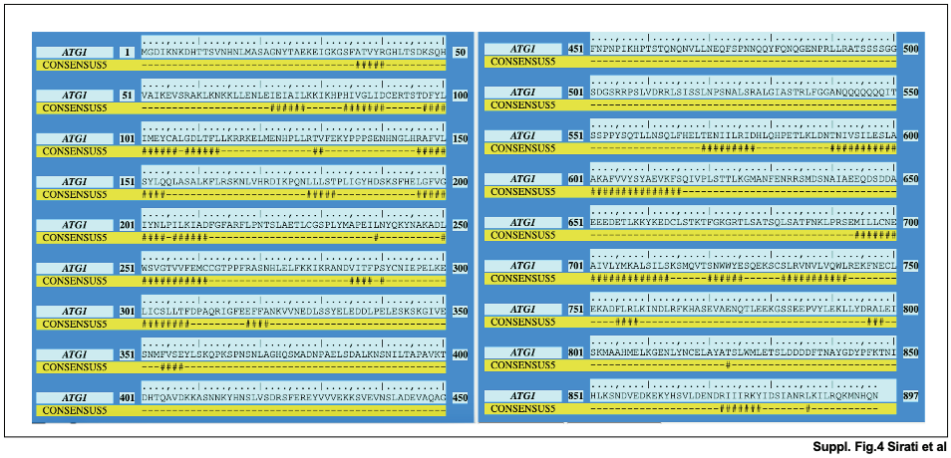


Figure S4. Analysis of the Atg1 primary sequence using the amyloid prediction software AmylPred2 (amyloidogenic regions are shown by #).

Abstract

An important organizing principle of biomolecular condensates is liquid–liquid phase separation, driven by multivalent macromolecular interactions. Overexpression of proteins containing intrinsically disordered regions or amyloidogenic properties are known to induce the formation of condensates. We previously showed that upon co-overexpression of two amyloidogenic proteins in *Saccharomyces cerevisiae*, human GAPR-1 and human Beclin 1, they interfere with their mutual condensate formation. Intriguingly, this interference was based on the same type of protein-protein interactions as described for their *in vivo* interaction at Golgi membranes. Here, we explored this principle of interference with condensate formation to develop a new method for investigating protein-protein interactions. By co-expression of various family members of the CAP superfamily that GAPR-1 belongs to, we identified four different phenotypes of interference with condensate formation. We then used this assay system to confirm our previous *in vitro* observation that GAPR-1 also interacts with the Ab-peptide. In addition, we show that the amyloidogenic Ab-peptide as well as Huntington protein constructs with different polyQ-expansions efficiently compete with the GAPR-1/Beclin 1 interaction, suggesting that these peptides have a higher affinity for GAPR-1 than Beclin 1. We propose that this assay system allows for a new approach to study protein-protein interactions of phase separation-prone and amyloidogenic proteins.

Keywords: Oligomers, Amyloid (-like) proteins, Biomolecular condensates, Protein-Protein interactions (PPIs), *Saccharomyces cerevisiae*.

Introduction

Virtually all processes in living cells are dependent on protein–protein interactions. The protein interactome has been estimated to consist of 650,000 protein interactions [1,2] and characterizing these interactions is critical for understanding protein function in a variety of cellular processes. A plethora of methods have been developed to study protein-protein interactions, both *in vitro* and *in vivo*, based on affinity chromatography, co-immunoprecipitation (co-IP), phage display, crosslinking, label transfer, mammalian and yeast two-hybrid assays, GST-fusion protein pull-down, immune-histochemistry, BiFC , and FRET [1,3–8]. These assays have greatly contributed to our understanding of protein-protein interactions, but in general they do not distinguish between monomeric and multimeric protein interactions. However, recent advances have highlighted the significance of interactions occurring between proteins in their dimeric, trimeric, tetrameric, and/or oligomeric states [9,10].

One intriguing aspect of (patho)biological processes involving protein oligomerization is the formation of amyloid fibrils, which are formed by a nucleation-dependent mechanism based on transient oligomeric states and conformational changes. A detailed understanding of the complex kinetic and thermodynamic changes that occur during the transition from monomer to amyloid fibril is an important challenge for the identification of the agents responsible for disease and hence their treatment [11,12]. Recently, it has become clear that amyloidogenic proteins can assemble into biomolecular condensates [13–25] through a physical process known as liquid–liquid phase separation (LLPS) [13,26–28]. Biomolecular condensates are implicated in a wide range of cellular functions and are found in the nucleus and cytoplasm of eucaryotic cells [29].

In *Saccharomyces cerevisiae*, overexpression of amyloidogenic proteins can result in the formation of protein condensates and this phenomenon has been widely used to investigate the oligomeric/amyloidogenic properties of known amyloid-like peptides or proteins, such as Aβ, Tau, α-synuclein, prions, Huntingtin (Htt), as well as proteins and peptides with predicted amyloidogenic behavior [30–35]. Based on this system, we recently developed an *in vivo* protein-protein interaction assay that is based on the capacity of amyloidogenic proteins to interfere with protein condensates when co-expressed. Using this method we studied the interaction between GAPR-1 and Beclin 1 [36]. When these proteins are individually expressed in yeast, they form protein condensates. Upon co-expression, they interfere with their mutual condensate formation and Beclin 1 relocalizes to the cytosol (Supplementary Fig. 1). These results showed for the first time that the GAPR-1/Beclin 1 interaction has oligomeric properties. Furthermore, introduction of a third component in this assay system (a Beclin 1-derived peptide) resulted in efficient inhibition of the GAPR-1/Beclin 1 interaction [36].

Based on these results we anticipated that the nature of interference and its impact on protein condensate formation may vary, depending on the specific type of protein-protein interaction under investigation. Therefore, in this study we investigated various types of interference with protein condensate formation upon co-expression of different amyloidogenic proteins and identified three additional phenotypes. This assay system was then used to expand and characterize our knowledge of possible protein-protein interactions of GAPR-1, illustrating its potential.

Results

GAPR-1 forms homo-oligomers

We previously showed that GAPR-1-GFP can form protein condensates in yeast cells [13]. This capacity was not influenced by the tag, as GAPR-1-mCherry was equally efficient in condensate formation [13]. It is expected that upon co-expression of GAPR-1-GFP and GAPR-1-mCherry, the two proteins colocalize to the same condensates. The propensity of GAPR-1-GFP and GAPR-1-mCherry to form condensates was confirmed by expression of each protein individually under the control of the galactose-inducible promoter (*GAL1*) from a high copy (2µ) plasmid (Fig. 1A). Overexpression of the tagged GAPR-1 constructs did not affect yeast growth (Supplementary Fig. 2). After 24h of co-expression of GAPR-1-GFP and GAPR-1-mCherry, the expressed proteins indeed localized to the same condensates resulting in the presence of yellow condensates (Fig. 1A). During this period, protein expression levels of single and co-expression experiments remained constant, in agreement with the similar number of cells that have condensates between the different types of conditions (Fig. 1B,C). To obtain independent confirmation for a direct interaction between GAPR-1-GFP and GAPR-1-mCherry, we performed a Bimolecular Fluorescence Complementation (BiFC) assay [37,38]. We tagged non-fluorescent complementary fragments of Venus yellow fluorescent protein (VN and VC) to the N-terminus or C-terminus of GAPR-1 and co-expressed both proteins in yeast cells. As expected, the homo-dimerization/oligomerization properties of GAPR-1 [36] resulted in a YFP fluorescent signal (Fig.1D,E). No fluorescent signal was observed upon co-expression of VN-GAPR-1 and the VC-domain without GAPR-1. Together, these results reveal a second phenotype of the assay system, namely co-localization of amyloidogenic proteins to the same condensates upon co-expression in yeast cells.

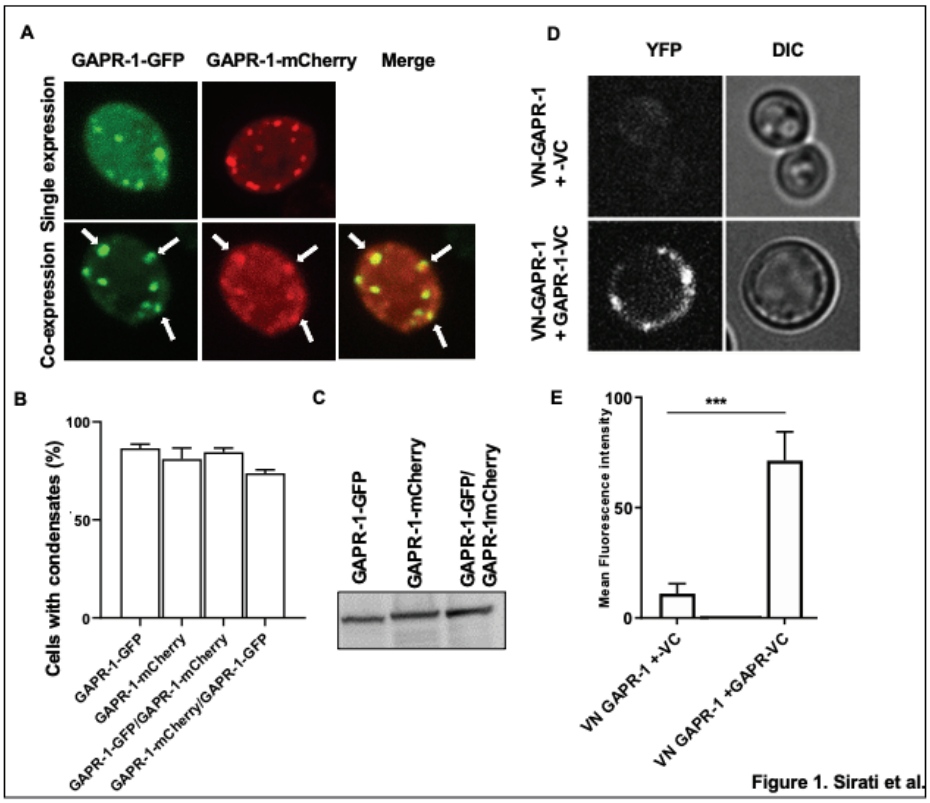


Figure 1. GAPR-1 forms homo-oligomers. A) Live-cell fluorescence microscopy of cells after 24h induction of expression of wt GAPR1 tagged C-terminally with either GFP (GAPR-1-GFP) or mCherry (GAPR-1-mCherry) (single expression) or both constructs together (co-expression); B) Quantification of yeast cells with condensates expressing GAPR-1-GFP, GAPR-1-mCherry (single expression) or both constructs together (co-expression) after 24h induction. The number of cells displaying cytoplasmic condensates is presented as a percent of the total number of the cells. For quantification of condensates at least 100 cells were counted per experiment. Results are expressed as mean ± SD, n=3; C) Western blot analysis of the cells after 24h of single expression and co-expression of GAPR-1-GFP and GAPR-1-mCherry, using an anti-GAPR-1 antibody; D) *In vivo* interaction assay using Bimolecular Fluorescence complementation (BIFC). Live-cell fluorescence microscopy is shown after 24 h induction. GAPR-1 was tagged by VN (N-terminal Venus fragment, VN-GAPR-1) and VC (C-terminal Venus fragment, GAPR-1-VC). Co-expression of VN-GAPR-1 and GAPR-1-VC shows a positive YFP signal as compared to a negative control (VN-GAPR-1 + VC); E) Mean YFP fluorescence intensity of cells described under D) after 24h expression. The results are expressed as mean ±SD

of three independent experiments. Significance of differences was calculated with two-tailed unpaired t-test (***p < 0.001; n=50).

GAPR-1 and CRISP proteins do not interact

GAPR-1 belongs to the CAP superfamily of proteins that is characterized by the presence of the CAP domain, typically found at the N-terminus of the protein [39]. We next addressed the question whether other human family members also localize to the same condensates upon co-expression with GAPR-1. To this end, human CRISP1, CRISP2 and CRISP3 were tagged with mCherry and their intrinsic capacity to form condensates was determined by overexpression of CRISP proteins without their signal sequence in yeast. As shown in Figure 2, CRISP 1 and CRISP 3 cluster to protein condensates already at early time points (3h post-induction) (Fig. 2 single expression). In contrast, CRISP 2 remained localized to the cytosol. To determine whether these CRISP proteins interact with GAPR-1, we performed co-expression studies of mCherry-tagged CRISP proteins with GFP-tagged GAPR-1. As shown in Figure 2, no interaction was detected of GAPR-1 with these proteins. CRISP 1-mCherry and CRISP 3-mCherry clustered in different condensates as GAPR-1-GFP, and CRISP 2-mCherry remained present in yeast cytosol. Altogether, our data suggest that no interactions are taking place between GAPR-1 and CRISP proteins *in vivo*. These results first of all demonstrate the specificity of the assay system as different family members of the same CAP superfamily do not localize to the same condensates or affect their mutual condensate-forming capacity. Secondly, these experiments reveal a third phenotype of the assay system, namely that no interference is detected despite significant homology between co-expressed proteins. In this third phenotype, co-expressed proteins can form separate condensates in the same yeast cell.

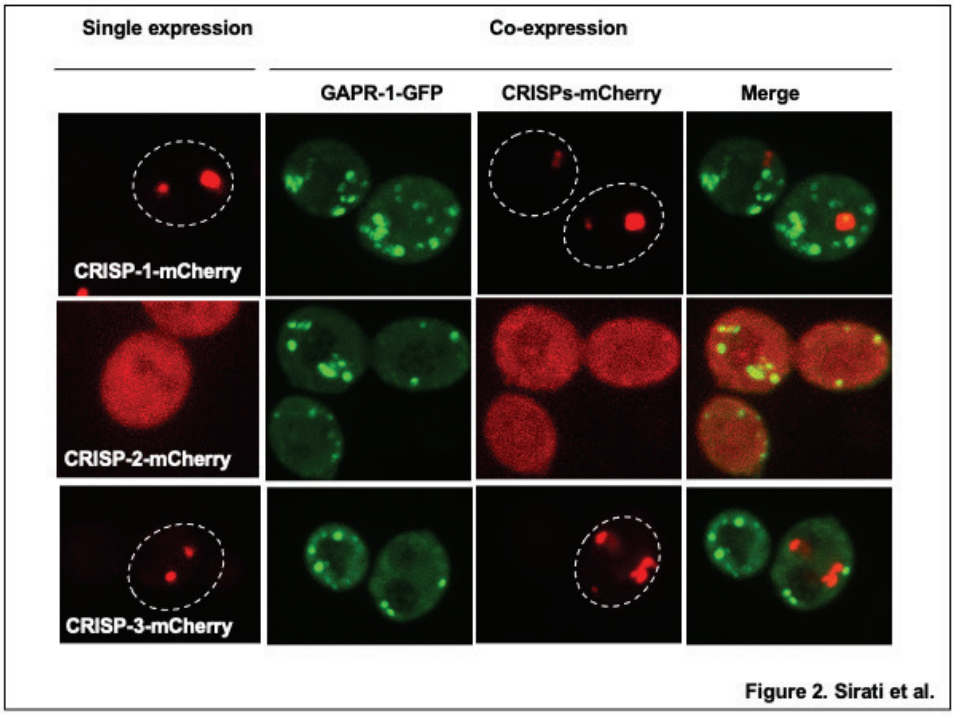


Figure 2. GAPR-1 does not interact with CRISP proteins. Representative images of live-cell fluorescence microscopy of yeast cells after 24h induction of expression of CRISP 1-mCherry, CRISP 2-mCherry and CRISP 3-mCherry without (single expression) or together with expression of GAPR-1-GFP (co-expression). The experiment was replicated three times, and consistent results were obtained in each repetition.

Specificity of interactions between CRISP proteins

CRISP proteins participate in various fertilization steps [28] and several studies have reported that the formation of homo- and hetero-oligomers of CRISPs are essential in these steps [30,40,41]. Therefore, we characterized the interactions between the different CRISP proteins in our protein-protein condensate interaction assay. Co-expression studies showed that CRISP 1 and 3 clusters in the same condensates (Fig.3A) under conditions of similar protein expression levels and number of condensate-forming cells (Fig. 3B,C). Interestingly, CRISP 2 re-localized to CRISP 1 condensates when co-

expressed (Fig. 3D,E). This re-localization is not due to different protein expression levels (Fig.3F). These experiments reveal a fourth phenotype of the assay system, namely re-localization from the cytosol to protein condensates that are formed by another (co-expressed) protein.

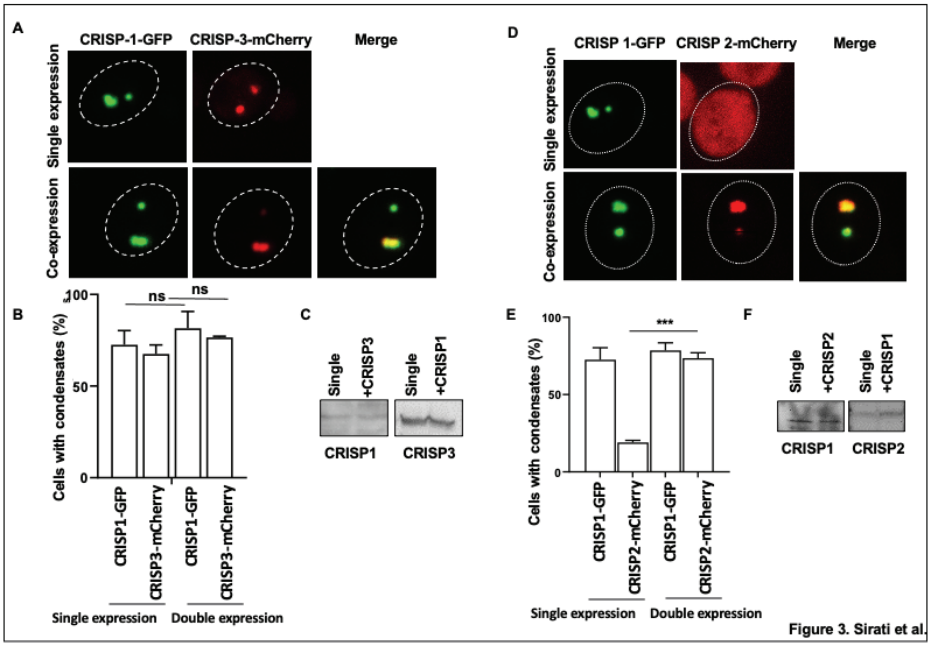


Figure 3. Interaction between CRISP proteins. A) Live cell fluorescent microscopy of cells after 6h induction of expression of CRISP 1-GFP or CRISP 3-mCherry (single expression) or both constructs together (co-expression); B) Quantification of yeast cells with condensates harboring CRISP 1-GFP, CRISP 3-mCherry (single expression) or both constructs together (co-expression) after 6h induction; C) Western blot analysis of the cells after 6h of single expression and co-expression of CRISP 1-GFP and CRISP 3-mCherry, using an anti-CRISP 1 and an anti-CRISP 3 antibody; D) Live cell fluorescent microscopy of cells after 6h induction of expression of CRISP 1-GFP or CRISP 2-mCherry (single expression) or both constructs together (co-expression); E) Quantification of yeast cells with condensates harboring CRISP 1-GFP, CRISP 2-mCherry (single expression) or both constructs together (co-expression) after 6h induction; F) Western blot analysis of the cells after 6h of single expression and co-expression of CRISP 1-GFP and CRISP 2-mCherry, using an anti-CRISP 1 or anti-CRISP 2 antibody. For quantification (C, F), the number of cells displaying cytoplasmic condensates is presented as a percent of the total number of the cells. For quantification of condensates at least 100 cells were counted

per time point and per experiment. Results are expressed as mean ± SD, n=3. Significance of differences was calculated with two-tailed unpaired t-test (***p<0.001).

Aβ peptide interacts with GAPR-1 and inhibits GAPR-1/Beclin 1 interaction

The different phenotypes of the assay system allowed us to investigate GAPR-1 protein-protein interactions in more detail. We previously showed that purified recombinant GAPR-1 inhibits amyloid formation of the Aβ-peptide (1-40) *in vitro* by binding to oligomeric Aβ [31]. We investigated interactions with the Aβ-peptide in a triple co-expression system employing GAPR-1-GFP and Beclin 1-mCherry, and a third component of choice (here Aβ-CFP), cloned under control of a galactose-inducible promoter (*GAL1*) for synchronous expression. In this triple co-expression system, GAPR-1 interferes with Beclin 1 condensate formation, unless a third component efficiently competes for GAPR-1 binding, resulting in restoring Beclin 1 condensates again. We previously showed that expression of a Beclin 1-derived peptide (B18) can successfully compete for GAPR-1 binding to restore Beclin 1 condensates [36]. Individual (single) expression of Aβ-CFP, GAPR-1-GFP and Beclin 1-mCherry after 24h induction showed the formation of condensates of all three constructs (Fig. 4A single expression), in agreement with previous publications [13,36,42,43]. In triple-expression experiments, the number of cells containing GAPR-1-GFP condensates decreased (Fig. 4B), whereas the number of cells containing Beclin-1-mCherry condensate did not change (Fig. 4C). These results suggest that 1) GAPR-1 and Aβ-peptide interact, in agreement with our *in vitro* studies; 2) GAPR-1 has a higher affinity for Aβ than Beclin 1, allowing efficient competition of Aβ with the GAPR-1/Beclin 1 interaction.

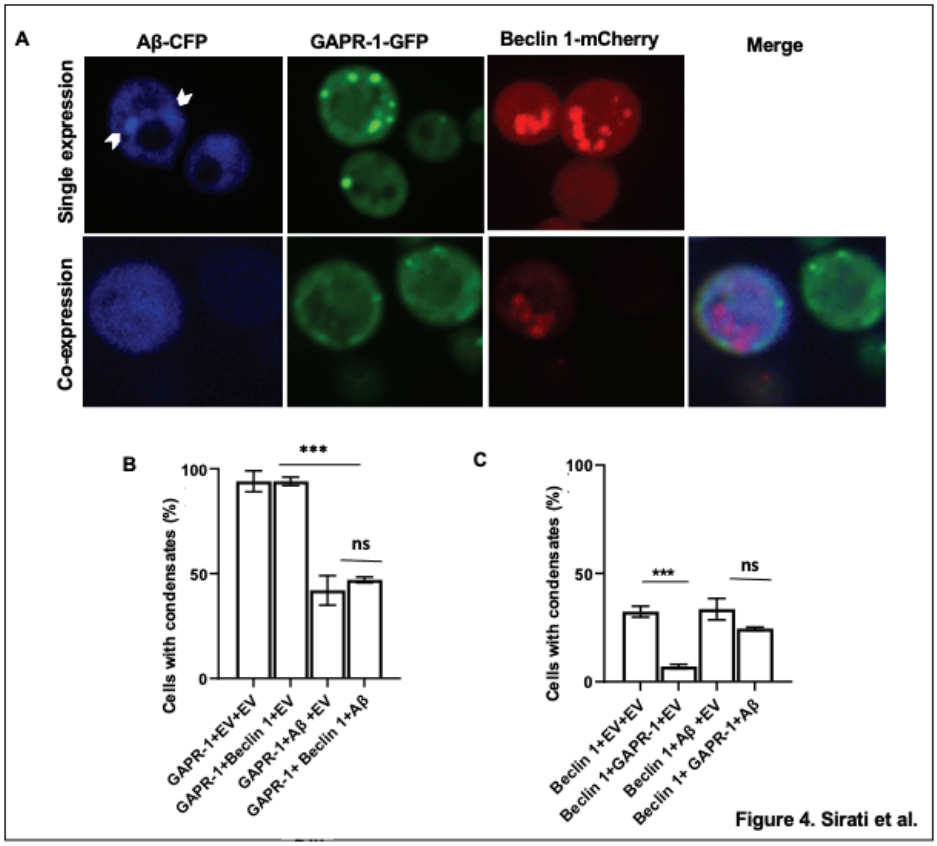


Figure 4. Aβ- peptide interacts with GAPR-1 and inhibits GAPR-1/Beclin 1 interaction. A) Live cell fluorescent microscopy of yeast W303 cells after 24h induction of expression of GAPR-1-GFP, Beclin 1-mCherry and Aβ-CFP (single expression) or all three constructs together (co-expression); B) Quantification of the number of cells with GAPR-1-GFP condensates in single, double and triple-expression after 24h; C) Quantification of the number of cells with Beclin 1-mCherry (Bottom panel) condensates in single, double and triple-expression after 24h. The number of cells displaying cytoplasmic condensates is presented as a percent of the total number of the cells. For quantification of condensates at least 100 cells were counted per time point and per experiment. Results are expressed as mean ± SD, n=3. Significance of differences was calculated with two-tailed unpaired t-test (***$p<0.001$).

Requirements for the interaction between the Aβ peptide and GAPR-1-GFP

To characterize the requirements of GAPR-1 for the interaction with the Aβ-peptide in more detail, we studied the effect of several GAPR-1 mutants in the triple expression system. To this end, we investigated the effect of GAPR-1 myristoylation by expressing a myristoylation-deficient GAPR-1mutant (Δmyr-GAPR-1). Previously, we demonstrated that this mutant exhibited a significant impairment in condensate formation, but it still efficiently reduced the Beclin 1 condensate forming capacity in our assay system [36]. In the triple expression system, we observed the same effects. Co-expression of Δmyr-GAPR-1 with Beclin 1 (along with an empty vector as the third component) markedly reduced the number of cells with Beclin 1 condensates (Fig. 5A). In the absence of Beclin 1 (Δmyr-GAPR-1+Aβ), the formation of GAPR-1 condensates was reduced (Fig. 5A, top half). Conversely, in the absence of GAPR-1 (Beclin 1+Aβ), the presence of Aβ peptide did not impact Beclin 1 condensate formation. Notably, when all three components were co-expressed, the Aβ-peptide efficiently competed with Beclin 1 for interacting with GAPR-1 and it restored Beclin 1 condensate formation, indicating that myristoylation is not required for the interaction between GAPR-1 and the Aβ-peptide (Fig. 5A, bottom half). In a recent study we demonstrated that co-expression of the GAPR mutants [H54V] GAPR-1-GFP and [S55G] GAPR-1-GFP did not affect Beclin 1 condensate formation, indicating an essential role of these amino acids for the GAPR-1/Beclin 1 interaction (this thesis, chapter 4). The Aβ-peptide also did not affect the mutant GAPR-1 nor the Beclin 1 condensate formation in triple-expression systems (Fig. 5B, C), suggesting that the Aβ-peptide is no longer able to interact with mutant GAPR-1. Taken together, these findings suggest that the Aβ-peptide and Beclin 1 compete for the same GAPR-1 binding site. These results are surprising as there is no homology at the level of the primary amino acids sequence or structural level between the Aβ peptide and Beclin 1.

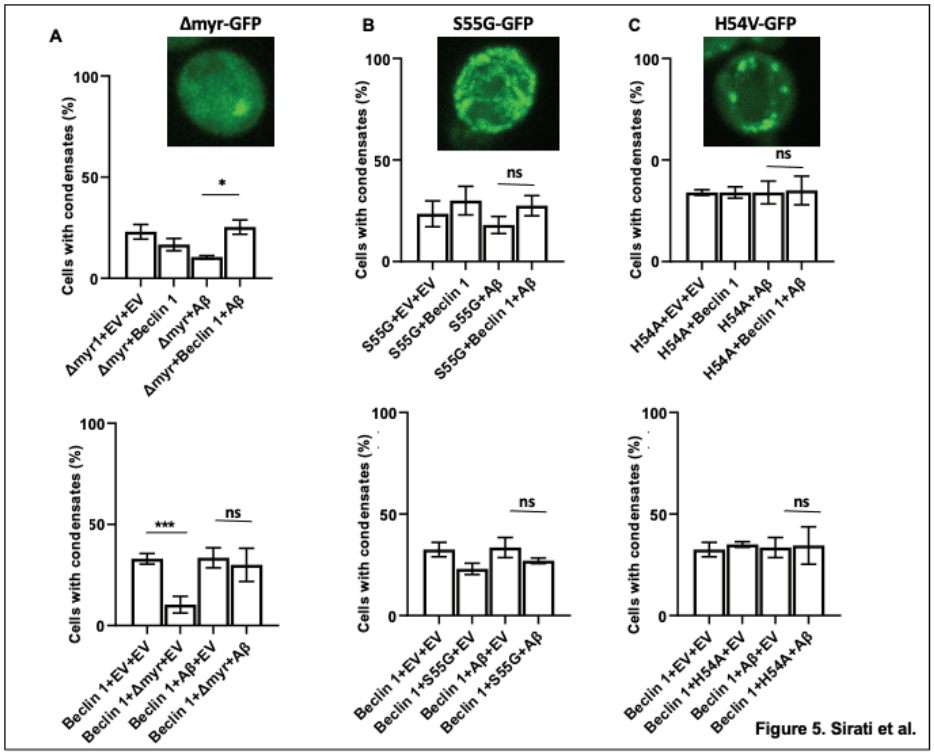


Figure 5. Requirements for the interaction between the Aβ peptide and GAPR-1-GFP. A) Quantification of the number of cells with non-myristoylated GAPR-1-GFP (Δmyr-GFP) condensates (top panel) and Beclin 1-mCherry condensates (bottom panel) after 24h induction of triple-expression of Δmyr-GFP, Beclin 1-mCherry and Aβ-CFP; B) Quantification of the number of cells with phosphomutant [S55G]GAPR-1-GFP (S55G-GFP) condensates (top panel) and Beclin 1-mCherry condensates (bottom panel) after 24h induction of triple-expression of [S55G]GAPR-1-GFP, Beclin 1-mCherry and Aβ-CFP; C) Quantification of the number of cells with GAPR-1 mutant [H54V]GAPR-1-GFP (H54V-GFP) condensates (top panel) and Beclin 1-mCherry condensates (bottom panel) after 24h induction of triple-expression of [H54V]GAPR-1-GFP, Beclin 1-mCherry and Aβ-CFP. The number of cells displaying cytoplasmic condensates is presented as a percent of the total number of the cells. For quantification of condensates at least 100 cells were counted per time point and per experiment. Results are expressed as mean ± SD, n=3. Significance of differences was calculated with two-tailed unpaired t-test (*p<0.5, ***p<0.001).

Htt protein interferes with GAPR-1 and Beclin 1 interaction

We considered the possibility that the shared characteristic of the Aβ peptide and Beclin 1 in their interaction with GAPR-1 is their amyloidogenic behavior, reflected by their capacity to form protein condensates. Rather than recognizing a protein surface defined by the primary amino acid sequence of a protein (e.g. as performed by AlphaFold [44]), GAPR-1 may recognize specific surfaces of oligomeric amyloidogenic proteins. To explore this, we turned our attention to other amyloidogenic proteins, such as the Huntingtin (Htt) protein.

Normal human Htt contains between 9 and 37 glutamines in the N-terminal repeat, whereas pathogenic forms contain ≥37 glutamines in the repeat region [45]. Expression of the N-terminal fragment of Htt with different polyQ repeats resulted in cytosolic aggregation, and the extent of aggregation varied with the length of the polyQ repeat: whereas Ht25Q exhibited no sign of aggregation, Ht103Q did aggregate upon overexpression in yeast cells [46]. We used BY4741 wild-type cells in which exon 1 of full length human huntingtin with 25 or 103 glutamine residues (Ht25Q-CFP or Ht103Q-CFP) are stably integrated into the yeast genome [47].

Using live cell microscopy, we confirmed that H25Q-CFP displayed a cytosolic phenotype 24h post-induction and that Ht103Q-CFP localized to condensates (Fig. 6A), in line with previously published results by Krobitch and Lindquist [46] , Popova et al [47], and Aktar et al [48]. Co-expression of GAPR-1-GFP and Beclin 1-mCherry in BY4741 cells expressing either H25Q-CFP (Fig. 6B,C) and H103Q-CFP (Fig. 6D,E) showed no interference with Beclin 1 condensate formation anymore (comparing with control conditions in BY4741 cells without expressing Ht25Q and Ht103Q), suggesting that both Htt25Q-CFP and Ht103Q-CFP efficiently competed with GAPR-1 to restore Beclin 1 condensate formation. These results confirm that GAPR-1 can interact with a variety of proteins. The correlation with the amyloidogenic behavior of these interacting proteins and their interaction with GAPR-1 will be discussed below.

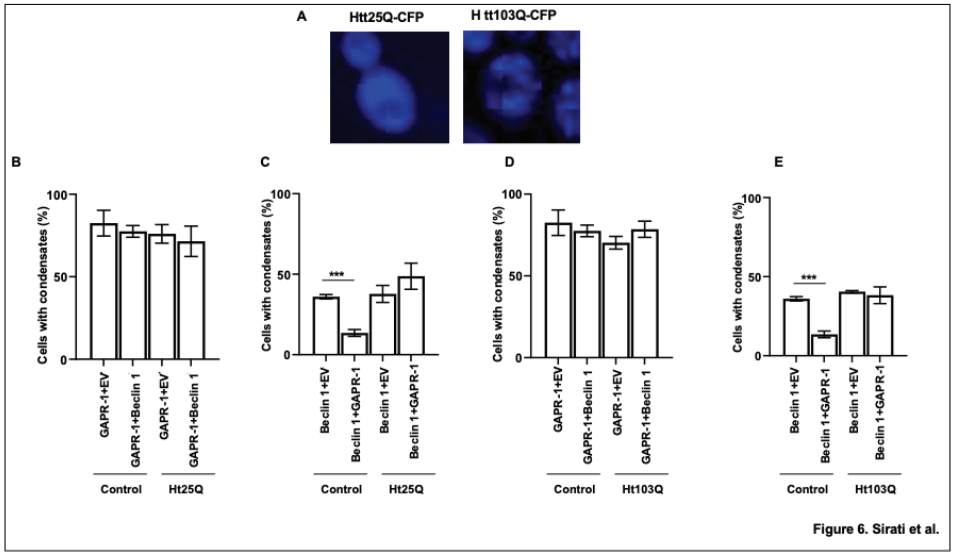


Figure 6. Huntington protein interferes with GAPR-1/Beclin 1 interaction *in vivo*. Representative live cell microscopy images of yeast cells expressing H25Q-CFP or H103Q-CFP; B,C) Quantification of the number of cells with GAPR-1-GFP (B) and Beclin 1-mCherry condensates (C) after 24h induction of expression of GAPR-1-GFP and Beclin 1-mCherry in BY471 cells without (control) and with stably expressing Ht25Q-CFP; D,E) Quantification of the number of cells with GAPR-1-GFP (D) and Beclin 1-mCherry condensates (E) after 24h induction of expression of GAPR-1-GFP and Beclin 1-mCherry in BY471 cells without (control) and with stably expressing Ht103Q-CFP. At least 100 cells were counted per experiment. Results are expressed as mean ±SD, n=3. The significance of differences was calculated with two-tailed unpaired t-test (***p<0.001).

Discussion

Here we describe a novel assay system to study protein-protein interactions that is based on the propensity of proteins to form biomolecular condensates in *Saccharomyces cerevisiae*. This assay system builds upon previous observations of mutual interference of condensate formation when co-expressing the amyloidogenic proteins GAPR-1 and Beclin 1[36]. We now show that this assay system can be expanded to other protein-protein interactions as well, resulting in four different phenotypes, as illustrated in Fig. 7: Type I) the expressed proteins both show the propensity to form condensates and they co-localize to the same condensates upon co-expression; Type II) the expressed proteins both show the propensity to form condensates and they form

different condensates upon co-expression; Type III) the expressed proteins both show the propensity to form condensates and one of them re-localizes to the cytosol upon co-expression; Type IV) only one of the expressed proteins shows the propensity to form condensates and the other one re-localizes to condensates upon co-expression. We expanded these co-expression experiments with triple-expression experiments by expressing a competitor for the protein-protein interaction, allowing the assessment of relative binding affinities as compared to the co-expressed protein. The specificity of these interference phenomena in condensate formation during co-expression is illustrated in various ways. First, we show that despite the large size of the fluorescent tag (GFP or mCherry, 27kD and 28 kD, respectively) compared to GAPR-1 itself (17kD), identical proteins can still co-localize within the same condensates (Type I). Second, even though GAPR-1 and CRISP1-3 belong to the same superfamily of proteins with significant identity and homology, GAPR-1 does not interfere with any of the CRISP condensates (Type II), and also the CRISP proteins show a very different behavior among each other upon co-expression (Type I and Type IV). Third, we previously showed that Type III interference can be influenced by specific mutations of GAPR-1 and/or Beclin 1 during co-expression [36]. Fourth, we show that specific mutations in GAPR-1 ([H54V] and ([S55G]) affect the interaction with Beclin 1 and the Aβ-peptide (Type IV interaction), whereas another mutation, affecting GAPR-1 myristoylation (Δmyr-GAPR-1) can still interact with the Aβ-peptide.

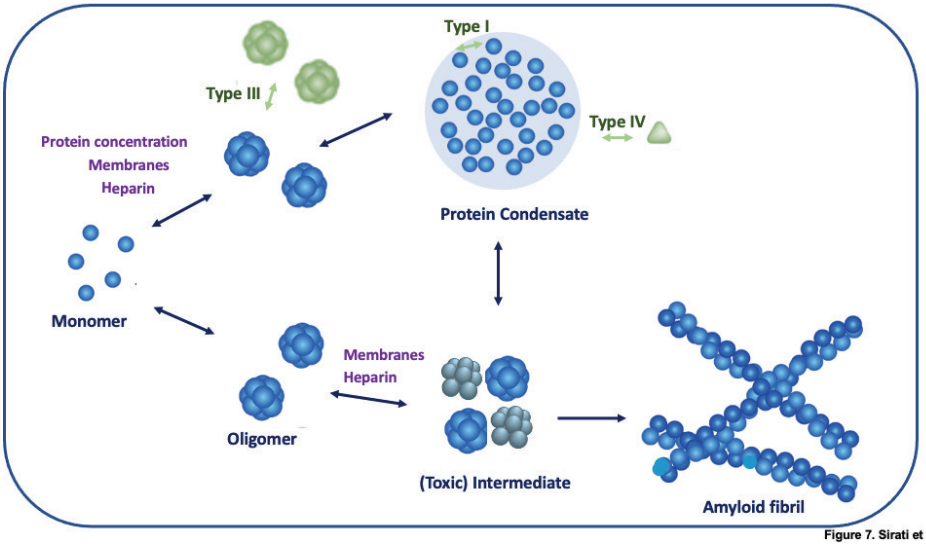


Figure 7. Sirati et al.

Figure 7. Experimental concept: *Saccharomyces cerevisiae* as a model system to study amyloidogenic protein-protein interactions.

The molecular mechanisms underlying these different types of interference in condensate formation upon protein co-expression remain to be established. Condensates are conditionally formed in specific cell types or in specific stress conditions and exhibit dynamic liquid-like properties, driven by weak multivalent interactions between proteins and/or nucleic acids [49–51]. *In vitro*, LLPS is an energetically favorable process that depends on the concentration of macromolecules in aqueous solution and is also affected by the nature of the macromolecules, such as their length, hydrophobicity and charge distribution, and by the surrounding biophysical conditions such as temperature, pH and ionic strength [50,52]. Thus, protein localization to these condensates can be the result of a variety of interactions. Most proteins undergoing LLPS possess domains, such as intrinsically disordered regions (IDRs), that can form multivalent interactions with themselves, other proteins, or nucleic acids [50,52]. LLPS-prone proteins are considered to contribute to the formation of the condensate ‘scaffold’. Additional proteins, known as ‘clients’, which cannot themselves undergo LLPS, are recruited into the scaffold to form the final condensate structure [52]. In the case of amyloidogenic proteins, there is a clear correlation between the propensity of proteins to form amyloids and condensates, but they can be distinct processes [50]. In fact, it has been proposed that amyloid formation is likely to be a secondary consequence of increased protein concentration rather than LLPS being a consequence of limited amyloid-like interactions [49]. The presence of GAPR-1 in condensates could provide a concentration platform, enhancing its amyloidogenic properties, much like we previously observed at lipid membranes [31] and using heparin [53]. Under physiological conditions, however, the cellular concentrations of endogenous GAPR-1 are most likely too low to form cytosolic condensates and/or amyloids [54] and so far, GAPR-1 condensates amyloids have not been observed in cells. Nevertheless, yeast cells with overexpressed proteins provide an excellent model system to study the oligomeric properties of GAPR-1, reflecting the oligomeric behavior of GAPR-1 in eukaryotic cells [13,36].

It remains to be established how GAPR-1 forms condensates in yeast cells upon overexpression. GAPR-1 has a very defined tertiary structure [55,56], whereas intrinsically disordered proteins (IDPs) or regions (IDRs) have no single well-defined tertiary structure under native conditions. Thus, it seems more likely that GAPR-1 forms condensates as a result of weak multivalent transient interactions between GAPR-1 molecules. This interpretation is in agreement with our previous observation that

mutations of the known interaction site on the GAPR-1 and Beclin 1 surface abolished the effect on condensate formation during co-expression of both proteins without affecting the condensate formation properties of the individual proteins [36]. Thus, the overexpression of proteins in our assay system enhances the transient and multivalent interactions between homotypic and/or heterotypic protein-protein interactions by increasing their protein and in this way it becomes a sensitive assay system using condensates as a readout system.

At this stage, it is not possible to predict which phenotype can be expected upon co-expression of different proteins. In case of high identity or homology, proteins are expected to localize to the same condensates (Type I phenotype). For the other phenotypes, much will depend at which stage the interaction takes place and in which form, as a monomer or as an oligomer. In the type II phenotype, homo-oligomeric interactions may be stronger than hetero-oligomeric interactions. In the type III phenotype, the hetero-oligomeric interactions prior to the condensate formation may be stronger. In addition, it will matter whether a protein has the intrinsic capacity to form condensates (i.e. can act as a scaffold) or whether it can be recruited to condensates as a client. This may be defined by the type IV phenotype. An interesting case is offered by the competition of the Huntington peptide with the GAPR-1/Beclin 1 interaction. Ht25Q-CFP as well as Ht103Q-CFP, efficiently competed with GAPR-1 to restore Beclin 1 condensate formation. Others have shown that the propensity for condensate formation in yeast increases with the length of the polyglutamine repeat sequence and that Htt103Q-CFP has the intrinsic capacity to form condensates, whereas H25Q-CFP does not have this capacity [46,48]. Nevertheless, non-toxic polymers of various proteins with glutamine-rich domains could seed polymerization of Htt25Q, which in turn caused toxicity of the interacting proteins [57]. These results indicate that Htt25Q has a tendency to form oligomers, but less pronounced as compared to e.g. H103Q-CFP and not sufficient to form condensates. It seems likely that these oligomers, present in both H25Q and H103Q, are capable of interfering with the GAPR-1/Beclin 1 interaction in the type III phenotype, which is defined by hetero-oligomeric interactions.

We used this novel assay system to screen for novel GAPR-1 interacting partners. GAPR-1 is unique as it consists almost exclusively of a CAP domain without C-terminal extensions and we previously proposed that the amyloidogenic properties of GAPR-1 might represent a general characteristic of the CAP domain. Oligomerization of the CAP domain would allow to regulate the biological functions of various CAP family members [58]. In this respect, it is remarkable that GAPR-1 interacts very differently with different CRISP proteins and even CRISP proteins among themselves interact very differently. CRISP2 and 3 are close homologues, yet CRISP2 does not form condensates itself. The reason for this is not clear. Given the high homology, it is likely that CRISP2 has the

intrinsic capacity to form condensates, but that something may prevent this from happening. This could be the C-terminal extension, the Cys-rich domain (CRD). It could also be that the CRD prevents interaction with GAPR-1. This can be tested by co-expressing the CAP domain of CRISP proteins with GAPR-1. Of note: GAPR-1 is the only intracellular protein of the CAP superfamily and is not expected to interact with CRISP proteins. The C-terminal domain of CAP family members may regulate the specificity of their interactions. This assay system allows for a new approach to study protein-protein interactions and can be expanded to other proteins as well. A deep understanding of protein–protein interactions and their molecular interactions is important for a number of applications, including drug design.

Materials & Methods

Yeast strain and plasmids

Wild-type yeast strain W303 or BY4741 were transformed using Frozen-EZ yeast kit from Zymo research (Zymo Research, Orange, CA, USA) with high copy (2µ) plasmids p423, p425 and p426 carrying different constructs, which were tagged C-terminally by GFP, CFP and m-Cherry respectively. To construct P*GAL1*-X-CFP, P*GAL1*-X-GFP and P*GAL1*-X-1-mCherry fragments, protein sequences were amplified by PCR and were inserted into the plasmids, previously linearized at SmaI restriction site by using Geneart® seamless cloning assembly kit (Invitrogen, California, United States). All mutant was constructed by using Phusion™ site-directed mutagenesis kit (Thermo Scientific, Massachusetts, United States) and all constructs were confirmed by DNA sequencing. Plasmids are listed in Table 1 and primers in Table 2.

Table 1.Plasmids used in this study

Plasmid	Description	Source
p423-GAL1	2µm; *HIS3*; *GAL1*pr; CYC1term; AmpR	D. Mumberg, R. Muller, M. Funk, Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression, Nucleic Acids Res. 22 (1994) 5767–5768. https://doi.org/10.1093/nar/22.25.5767. [59]
pME3759	p426-*GAL1*-GFP	D. Petroi, B. Popova, N. Taheri-Talesh, S. Irniger, H. Shahpasandzadeh, M. Zweckstetter, T.F. Outeiro, G.H. Braus, Aggregate Clearance of α-Synuclein in *Saccharomyces cerevisiae* Depends More on Autophagosome and Vacuole Function Than on the Proteasome, J. Biol. Chem. 287 (2012) 27567–27579. https://doi.org/10.1074/jbc.M112.36186 5 [31]
pNS 1000	p423-*GAL1*-GAPR-1-GFP	This study
pNS 1001	p423-*GAL1*- ΔmyrGAPR-1-GFP	This study
pNS 2000	p426-*GAL1*-Beclin 1-mCherry	This study
pIV 4001	p425-*GAL1*- Aß-CFP	This study
pNS 1000	p423-*GAL1*-GAPR-1-GFP	This study
pNS 1001	p423-*GAL1*- ΔmyrGAPR-1-GFP	This study
pNS 1002	p423-*GAL1*- H54VGAPR-1-GFP	This study
pNS 1006	p423-*GAL1*-S55GAPR-1-GFP	This study
pRE 0001	p423-*GAL1*-CRISP1-GFP	This study
pRE 0002	p426-*GAL1*-CRISP2-mCherry	This study

pIV 4001	p425-*GAL1*- Aß-CFP	This study
pRE 0003	p426-*GAL1*-CRISP3-mCherry	This study
pRE 0004	p426-*GAL1*-CRISP2-mCherry	This study
RH3788	BY474-*GAL1*-FLAG-*ht103QΔ*Pro-CFP in *his3* locus	B. Popova, D. Wang, A. Rajavel, K. Dhamotharan, D.F. Lázaro, J. Gerke, J.F. Uhrig, M. Hoppert, T.F. Outeiro, G.H. Braus, Identification of Two Novel Peptides That Inhibit α-Synuclein Toxicity and Aggregation, Front. Mol. Neurosci. 14 (2021). https://doi.org/10.3389/fnmol.2021.659926.[47]

Table 2-Primers used in this study

Primer	Sequence
FW GAPR-1	5'-GAACTAGTGGATCCCCCATGGGCAAGTCAGC-3'
REV GAPR-1 for GFP	5'-CTTCTCCTTTACTATCGATAAGCTTCTTCTTCGGCGGC5-3'
FW GFP for GAPR-1	5'-CCGCCGAAGAAGAAGCTTATCGATAGTAAAGGAGAAGAAC-3'
REV GFP	5'-GAATTCCTGCAGCCCTTATTTGTATAGTTCATCC-3'
REV GAPR-1 for mCherry	5'-CCTTGCTCACCATATCGATAAGCTTCTTCTTCGGCGGC-3'
FW mCherry for GAPR-1	5'-CCGCCGAAGAAGAAGCTTATCGATATGGTGAGCAAGGG-3'
REV mCherry	5'-GAATTCCTGCAGCCCCTACTTGTACAGCTCGTC-3'
FW G2A-GAPR-1	5'-GATCCCCCATGGCCAAGTCAGCTTCCAAACAG-3'
REV G2A-GAPR-1	5'-GCTGACTTGGCCATGGGGGATCCACTAGTTC-3'
FW H54V-GAPR-1	5'-CAGCACGAGGATCCTCAAGGTCAGCCCGGAGTCCACCCGTG-3'
REV H54V-GAPR-1	5'-CACGGCTGGACTCCGGGCTGACCTTGATCCTCGTGCTG-3'
FW Beclin 1	5'-GAACTAGTGGATCCCCCATGGAAGGGTCTAAGAC-3'
REV Beclin 1 4 mCherry	5'-CCTTGCTCACCATATCGATAAGCTTTTTGTTATAAAATTGTGA-3'

FW mCherry 4 Beclin 1	5’-CTCACAATTTTATAACAAAAAGCTTATCGATATGGTGAGCAAG-3’
REV mCherry	5’-GAATTCCTGCAGCCCCTACTTGTACAGCTC-3’
REV GAPR-1 4 VC	5'- CCG TTC TTC TCG AGC TTC TTC GGC GG -3’
FW VC 4 GAPR-1	5'- CCG CCG AAG AAG CTC GAG AAG AAC GG -3’
REV VC	5'- CGA ATT CCT GCA GCC CTT ACT TGT ACA GC -3’
FW VN	5'- CTA GTG GAT CCC CCA TGG TGA GCA AG -3’
REV VN 4 Beclin 1	5'- CGT CTT AGA CCC TTC CTT AAG GGA CCC -3’
FW Beclin 1 4 VN	5'- GGG TCC CTT AAG GAA GGG TCT AAG ACG -3'
REV Beclin 1	5’- CGAATTCCTGCAGCCCTCATTTGTTATAAAATTG -3'
FW CRISP1	5’GAACTAGTGGATCCCCCATGAAAAAGAAATCTGC 3’
REV CRISP 1 for GFP	5’ CTCCTTTACTATCGATAAGCTTTTTAATTTCAGTATC 3’
FW GFP for CRISP1	5’ GATACTGAAATTAAAAAGCTTATCGATAGTAAAGGAG 3’
FW CRISP3	5’GAACTAGTGGATCCCCCATGAATGAAGATAAAG 3’
REV CRISP1 for mCherry	5’ CCTTGCTCACCATATCGATAAGCTTTTTAATTTCAGTATC 3’
FW mCherry for CRISP1	5’ GATACTGAAATTAAAAAGCTTATCGATATGGTGAGCAAGGG 3’
FW CRISP2	5’ GAACTAGTGGATCCCCCATGGGTAAAGATCCAGC 3’
REV CRISP2 for mCherry	5’ CCTTGCTCACCATATCGATAAGCTTGATCTTATTTTCAC 3’
FW mCherry for CRISP2	5’ GTGAAAATAAGATCAAGCTTATCGATATGGTGAGCAAGG 3’
REV CRISP3 for mCherry	5’ CCTTGCTCACCATATCGATAAGCTTGATAGAATTTGAAC 3’
FW mCherry for CRISP3	5’ GTTCAAATTCTATCAAGCTTATCGATATGGTGAGCAAG 3’

FW Aß	5'- GAA CTA GTG GAT CCC CCA TGG ACG CTG AAT TCC GTC -3'
REV Aß for CFP	5'- CCT TTA CTC ATC CCA TCG ATA AGC TTA GCG ATC ACA ACG CCA CC -3'

Yeast culture media

Cells were pre-grown overnight in a synthetic complete (SC) medium lacking the corresponding markers (-His, -Ura or -Leu) and supplemented with either 2% glucose or 2% galactose at 30^{o}C with orbital agitation (200 rpm) for 18h (overnight). The day after optical density at 600 nm (OD_{600} nm) was measured, and the cells were shifted in selective SC medium supplemented with 2% galactose to induce protein expression (OD_{600}=0.1). After 3h, 6h and 24h, cells were collected to obtain total cell homogenates for Western blot analysis or were observed by live cell microscopy (Nikon Eclipse Ti-E (Nikon)).

Spotting assay

All spotting assays were performed under the same conditions. Ten-fold serial dilutions starting with an equal number of cells (OD_{600}=0.1) were made in sterile water. Drops of 10µl were then spotted on SC plates lacking the corresponding marker (-His-Ura /-Leu) and supplemented with either 2% glucose or 2% galactose. Three independent experiments from fresh transformants were done and followed by 3 days of incubation at 30°C, after which the plates were scanned.

Fluorescence microscopy

Cells were visualized at different time points after induction (3h, 6h, and 24h) using Nikon Eclipse Ti-E microscope (Nikon) equipped with the Perfect Focus System (Nikon) Nikon Apo TIRF 100x N.A. 1.49 oil objective (Nikon), a spinning disk-based confocal scanner unit (CSU-X1-A1, Yokogawa), and the ET-CFP filter set (49001, Chroma), ET-GFP filter set (49002, Chroma) and ET-mCherry filter set (49008, Chroma). For quantification, at least 300 cells were counted per condition and per experiment. For each condition, the number of cells displaying cytoplasmic protein condensates were calculated relative to the total number of cells counted (with condensate or cytoplasmic distribution). At least three independent experiments with freshly transformed constructs were performed.

Protein extraction and Western blotting

10 OD units of cells were collected by centrifugation, washed with sterile water and lysed with 200 µl of Y-PER™ reagent (Thermo Scientific, Massachusetts, United States) containing fresh protease inhibitors. Lysed cells were incubated for 20 minutes at room temperature, followed by 15 minutes of centrifugation at 13000 rpm. Total protein homogenates were collected for further analysis. A total of 20 µg of yeast protein in Laemmli sample buffer was incubated for 5 minutes at 100°C and separated by SDS-PAGE in a 12% polyacrylamide gel. Proteins were electrically transferred onto 0.45 µm nitrocellulose membranes (Amersham Protran GE Healthcare) by Western blot at 90 V for 1 h and were probed with monoclonal anti-Beclin 1 antibody (Santa Cruz, Dallas, Texas, United States), an anti-CRISP1, 2 , 3 antibodies (Abcam, Cambridge, UK) and an anti-GAPR-1 antibody [54]. Peroxidase-conjugated goat, an anti-rabbit antibody (Nordic-Mubio, Susteren, The Netherlands) was used as a secondary antibody. Binding was detected with the SuperSignal™ reagents (Thermo Scientific, Massachusetts, United States) and ChemiDoc™ MP Imaging system (BioRad, Hercules, California, United States).

Bimolecular Fluorescence Complementation (BiFC)

In this assay, we used Venus YFP protein as a fluorescence protein and tagged its non-fluorescent halves Venus N (VN) to the N-terminal domain of GAPR-1 in a p423 plasmid and Venus C (VC) to the C-terminal of GAPR-1 in a p426 plasmid and constructs were verified by DNA sequencing. For live cell imaging microscopy, wild-type (W303) yeast cells harbouring different constructs were grown in SC medium -His -Ura -Leu containing 2% raffinose at 30 ^{o}C overnight and shifted to 2% galactose medium for induction of proteins. Cells were visualized after 24h induction by Zeiss Observer. Z1 microscope (Zeiss) was equipped with a CSU-X1 A1 confocal scanner unit (YOKOGAWA), QuantEM:512SC digital camera (Photometrics) and Slide Book 6.0 software package (Intelligent Imaging Innovations). The mean fluorescence intensity values of 50 cells per condition were measured in three independent experiments

Statistical Analysis

Data were analyzed using GraphPad Prism 9 (San Diego, CA, USA) Software and are presented as mean ± SD of at least three independent experiments. The significance of differences was calculated using a two-tailed unpaired T-test. P values lower than 0.05 were considered to indicate a significant difference.

Acknowledgements

We thank Ilya Grigoriev (Anna Akhmanova) for help with fluorescence microscopy.

References

[1] J. Mehla, J.H. Caufield, P. Uetz, The Yeast Two-Hybrid System: A Tool for Mapping Protein–Protein Interactions, Cold Spring Harb. Protoc. 2015 (2015) pdb.top083345. https://doi.org/10.1101/pdb.top083345.

[2] M.P.H. Stumpf, T. Thorne, E. de Silva, R. Stewart, H.J. An, M. Lappe, C. Wiuf, Estimating the size of the human interactome, Proc. Natl. Acad. Sci. 105 (2008) 6959–6964. https://doi.org/10.1073/pnas.0708078105.

[3] S. Fields, O. Song, A novel genetic system to detect protein–protein interactions, Nature. 340 (1989) 245–246. https://doi.org/10.1038/340245a0.

[4] S. Xing, N. Wallmeroth, K.W. Berendzen, C. Grefen, Techniques for the analysis of protein-protein interactions *in vivo*, Plant Physiol. (2016) pp.00470.2016. https://doi.org/10.1104/pp.16.00470.

[5] T. Berggård, S. Linse, P. James, Methods for the detection and analysis of protein–protein interactions, Proteomics. 7 (2007) 2833–2842. https://doi.org/10.1002/pmic.200700131.

[6] K.H. Rainey, G.H. Patterson, Photoswitching FRET to monitor protein–protein interactions, Proc. Natl. Acad. Sci. 116 (2019) 864–873. https://doi.org/10.1073/pnas.1805333116.

[7] H.-T. Lai, C.-M. Chiang, Bimolecular Fluorescence Complementation (BiFC) Assay for Direct Visualization of Protein-Protein Interaction *in vivo*, BIO-PROTOCOL. 3 (2013). https://doi.org/10.21769/BioProtoc.935.

[8] T. Maculins, J. Garcia-Pardo, A. Skenderovic, J. Gebel, M. Putyrski, A. Vorobyov, P. Busse, G. Varga, M. Kuzikov, A. Zaliani, S. Rahighi, V. Schaeffer, M.J. Parnham, S.S. Sidhu, A. Ernst, V. Dötsch, M. Akutsu, I. Dikic, Discovery of Protein-Protein Interaction Inhibitors by Integrating Protein Engineering and Chemical Screening Platforms, Cell Chem. Biol. 27 (2020) 1441-1451.e7. https://doi.org/10.1016/j.chembiol.2020.07.010.

[9] M. Budayova, F. Bonneté, A. Tardieu, P. Vachette, Interactions in solution of a large oligomeric protein, J. Cryst. Growth. 196 (1999) 210–219. https://doi.org/10.1016/S0022-0248(98)00844-6.

[10] R.C. Garratt, N.F. Valadares, J.F.R. Bachega, Oligomeric Proteins, in: Encycl. Biophys., Springer Berlin Heidelberg, Berlin, Heidelberg, 2013: pp. 1781–1789. https://doi.org/10.1007/978-3-642-16712-6_416.

[11] T. Eichner, S.E. Radford, A Diversity of Assembly Mechanisms of a Generic

Amyloid Fold, Mol. Cell. 43 (2011) 8–18. https://doi.org/10.1016/j.molcel.2011.05.012.

[12] T.P.J. Knowles, M. Vendruscolo, C.M. Dobson, The amyloid state and its association with protein misfolding diseases, Nat. Rev. Mol. Cell Biol. 15 (2014) 384–396. https://doi.org/10.1038/nrm3810.

[13] N. Sirati, B. Popova, M.R. Molenaar, I.C. Verhoek, G.H. Braus, D. V. Kaloyanova, J.B. Helms, Dynamic and Reversible Aggregation of the Human CAP Superfamily Member GAPR-1 in Protein Inclusions in *Saccharomyces cerevisiae*, J. Mol. Biol. 433 (2021) 167162. https://doi.org/10.1016/j.jmb.2021.167162.

[14] A. Aulas, C. Vande Velde, Alterations in stress granule dynamics driven by TDP-43 and FUS: a link to pathological inclusions in ALS?, Front. Cell. Neurosci. 9 (2015). https://doi.org/10.3389/fncel.2015.00423.

[15] A. Zbinden, M. Pérez-Berlanga, P. De Rossi, M. Polymenidou, Phase Separation and Neurodegenerative Diseases: A Disturbance in the Force, Dev. Cell. 55 (2020) 45–68. https://doi.org/10.1016/j.devcel.2020.09.014.

[16] S. Elbaum-Garfinkle, Matter over mind: Liquid phase separation and neurodegeneration, J. Biol. Chem. 294 (2019) 7160–7168. https://doi.org/10.1074/jbc.REV118.001188.

[17] M.S. Rubel, S.A. Fedotov, A. V. Grizel, J. V. Sopova, O.A. Malikova, Y.O. Chernoff, A.A. Rubel, Functional Mammalian Amyloids and Amyloid-Like Proteins, Life. 10 (2020) 156. https://doi.org/10.3390/life10090156.

[18] S. Rhoads, Z. Monahan, D. Yee, F. Shewmaker, The Role of Post-Translational Modifications on Prion-Like Aggregation and Liquid-Phase Separation of FUS, Int. J. Mol. Sci. 19 (2018) 886. https://doi.org/10.3390/ijms19030886.

[19] E.G.P. Stender, S. Ray, R.K. Norrild, J.A. Larsen, D. Petersen, A. Farzadfard, C. Galvagnion, H. Jensen, A.K. Buell, Capillary flow experiments for thermodynamic and kinetic characterization of protein liquid-liquid phase separation, Nat. Commun. 12 (2021) 7289. https://doi.org/10.1038/s41467-021-27433-y.

[20] A. Savastano, D. Flores, H. Kadavath, J. Biernat, E. Mandelkow, M. Zweckstetter, Disease-Associated Tau Phosphorylation Hinders Tubulin Assembly within Tau Condensates., Angew. Chem. Int. Ed. Engl. 60 (2021) 726–730. https://doi.org/10.1002/anie.202011157.

[21] L.D. Muiznieks, S. Sharpe, R. Pomès, F.W. Keeley, Role of Liquid–Liquid Phase

Separation in Assembly of Elastin and Other Extracellular Matrix Proteins, J. Mol. Biol. 430 (2018) 4741–4753. https://doi.org/10.1016/j.jmb.2018.06.010.

[22] T.M. Franzmann, M. Jahnel, A. Pozniakovsky, J. Mahamid, A.S. Holehouse, E. Nüske, D. Richter, W. Baumeister, S.W. Grill, R. V. Pappu, A.A. Hyman, S. Alberti, Phase separation of a yeast prion protein promotes cellular fitness, Science (80-.). 359 (2018) eaao5654. https://doi.org/10.1126/science.aao5654.

[23] E. Grignaschi, G. Cereghetti, F. Grigolato, M.R.G. Kopp, S. Caimi, L. Faltova, S. Saad, M. Peter, P. Arosio, A hydrophobic low-complexity region regulates aggregation of the yeast pyruvate kinase Cdc19 into amyloid-like aggregates in vitro, J. Biol. Chem. 293 (2018) 11424–11432. https://doi.org/10.1074/jbc.RA117.001628.

[24] A. Patel, H.O. Lee, L. Jawerth, S. Maharana, M. Jahnel, M.Y. Hein, S. Stoynov, J. Mahamid, S. Saha, T.M. Franzmann, A. Pozniakovski, I. Poser, N. Maghelli, L.A. Royer, M. Weigert, E.W. Myers, S. Grill, D. Drechsel, A.A. Hyman, S. Alberti, A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation, Cell. 162 (2015) 1066–1077. https://doi.org/10.1016/j.cell.2015.07.047.

[25] T. Amen, D. Kaganovich, Dynamic droplets: the role of cytoplasmic inclusions in stress, function, and disease, Cell. Mol. Life Sci. 72 (2015) 401–415. https://doi.org/10.1007/s00018-014-1740-y.

[26] S. Alberti, A.A. Hyman, Biomolecular condensates at the nexus of cellular stress, protein aggregation disease and ageing, Nat. Rev. Mol. Cell Biol. 22 (2021) 196–213. https://doi.org/10.1038/s41580-020-00326-6.

[27] S. Alberti, A. Gladfelter, T. Mittag, Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates, Cell. 176 (2019) 419–434. https://doi.org/10.1016/j.cell.2018.12.035.

[28] E. Gomes, J. Shorter, The molecular language of membraneless organelles, J. Biol. Chem. 294 (2019) 7115–7127. https://doi.org/10.1074/jbc.TM118.001192.

[29] A.S. Lyon, W.B. Peeples, M.K. Rosen, A framework for understanding the functions of biomolecular condensates across scales, Nat. Rev. Mol. Cell Biol. 22 (2021) 215–235. https://doi.org/10.1038/s41580-020-00303-z.

[30] A.J. Koppers, T. Reddy, M.K. O'Bryan, The role of cysteine-rich secretory proteins in male fertility, Asian J. Androl. 13 (2011) 111–117. https://doi.org/10.1038/aja.2010.77.

[31] N.K. Olrichs, A.K. Mahalka, D. Kaloyanova, P.K. Kinnunen, J. Bernd Helms, Golgi-Associated plant Pathogenesis Related protein 1 (GAPR-1) forms amyloid-like fibrils by interaction with acidic phospholipids and inhibits Aβ aggregation, Amyloid. 21 (2014) 88–96. https://doi.org/10.3109/13506129.2014.882304.

[32] T.F. Outeiro, Yeast Cells Provide Insight into Alpha-Synuclein Biology and Pathobiology, Science (80-.). 302 (2003) 1772–1775. https://doi.org/10.1126/science.1090439.

[33] B. Popova, A. Kleinknecht, P. Arendarski, J. Mischke, D. Wang, G.H. Braus, Sumoylation Protects Against β-Synuclein Toxicity in Yeast, Front. Mol. Neurosci. 11 (2018). https://doi.org/10.3389/fnmol.2018.00094.

[34] D. Petroi, B. Popova, N. Taheri-Talesh, S. Irniger, H. Shahpasandzadeh, M. Zweckstetter, T.F. Outeiro, G.H. Braus, Aggregate Clearance of α-Synuclein in *Saccharomyces cerevisiae* Depends More on Autophagosome and Vacuole Function Than on the Proteasome, J. Biol. Chem. 287 (2012) 27567–27579. https://doi.org/10.1074/jbc.M112.361865.

[35] B. Popova, D. Wang, C. Pätz, D. Akkermann, D.F. Lázaro, D. Galka, M. Kolog Gulko, M.T. Bohnsack, W. Möbius, K.E. Bohnsack, T.F. Outeiro, G.H. Braus, DEAD-box RNA helicase Dbp4/DDX10 is an enhancer of α-synuclein toxicity and oligomerization, PLOS Genet. 17 (2021) e1009407. https://doi.org/10.1371/journal.pgen.1009407.

[36] N. Sirati, Z. Shen, N.K. Olrichs, B. Popova, I.C. Verhoek, I.M. Lagerwaard, G.H. Braus, D. V. Kaloyanova, J.B. Helms, GAPR-1 Interferes with Condensate Formation of Beclin 1 in *Saccharomyces cerevisiae*, J. Mol. Biol. 435 (2023) 167935. https://doi.org/10.1016/j.jmb.2022.167935.

[37] V. Citovsky, L.Y. Lee, S. Vyas, E. Glick, M.H. Chen, A. Vainstein, Y. Gafni, S.B. Gelvin, T. Tzfira, Subcellular Localization of Interacting Proteins by Bimolecular Fluorescence Complementation in Planta, J. Mol. Biol. 362 (2006) 1120–1131. https://doi.org/10.1016/j.jmb.2006.08.017.

[38] T.K. Kerppola, Bimolecular Fluorescence Complementation (BiFC) Analysis as a Probe of Protein Interactions in Living Cells, Annu. Rev. Biophys. 37 (2008) 465–487. https://doi.org/10.1146/annurev.biophys.37.032807.125842.

[39] G.M. Gibbs, K. Roelants, M.K. O'Bryan, The CAP Superfamily: Cysteine-Rich Secretory Proteins, Antigen 5, and Pathogenesis-Related 1 Proteins—Roles in Reproduction, Cancer, and Immune Defense, Endocr. Rev. 29 (2008) 865–897.

https://doi.org/10.1210/er.2008-0032.

[40] M. Battistone, N. Brukman, G. Carvajal, D. Cohen, P. Cuasnicú, L. Curci, V. Da Ros, M. Gómez Elías, Mw. Muñoz, From the Epididymis to the Egg: participation of CRISP Proteins in Mammalian Fertilization, Asian J. Androl. 17 (2015) 711–715. https://doi.org/10.4103/1008-682X.155769.

[41] J. Cohen, J.A. Maldera, A. Dematteis, P.S. Cuasnicu, A Novel Function for CRISP1 in Rodent Fertilization : Involvement in Sperm-Zona Pellucida Interaction, Biol. Reprod. 77 (2007) 848–854. https://doi.org/10.1095/biolreprod.107.061788.

[42] J.P. Connor, S.D. Quinn, C. Schaefer, Sticker-and-spacer model for amyloid beta condensation and fibrillation, Front. Mol. Neurosci. 15 (2022). https://doi.org/10.3389/fnmol.2022.962526.

[43] X. Gui, S. Feng, Z. Li, Y. Li, B. Reif, B. Shi, Z. Niu, Liquid–liquid phase separation of amyloid-β oligomers modulates amyloid fibrils formation, J. Biol. Chem. 299 (2023) 102926. https://doi.org/10.1016/j.jbc.2023.102926.

[44] J. Jumper, R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Žídek, A. Potapenko, A. Bridgland, C. Meyer, S.A.A. Kohl, A.J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A.W. Senior, K. Kavukcuoglu, P. Kohli, D. Hassabis, Highly accurate protein structure prediction with AlphaFold, Nature. 596 (2021) 583–589. https://doi.org/10.1038/s41586-021-03819-2.

[45] K. Kieburtz, M. MacDonald, C. Shih, A. Feigin, K. Steinberg, K. Bordwell, C. Zimmerman, J. Srinidhi, J. Sotack, J. Gusella, Trinucleotide repeat length and progression of illness in Huntington's disease., J. Med. Genet. 31 (1994) 872–874. https://doi.org/10.1136/jmg.31.11.872.

[46] S. Krobitsch, S. Lindquist, Aggregation of huntingtin in yeast varies with the length of the polyglutamine expansion and the expression of chaperone proteins, Proc. Natl. Acad. Sci. 97 (2000) 1589–1594. https://doi.org/10.1073/pnas.97.4.1589.

[47] B. Popova, D. Wang, A. Rajavel, K. Dhamotharan, D.F. Lázaro, J. Gerke, J.F. Uhrig, M. Hoppert, T.F. Outeiro, G.H. Braus, Identification of Two Novel Peptides That Inhibit α-Synuclein Toxicity and Aggregation, Front. Mol. Neurosci. 14 (2021). https://doi.org/10.3389/fnmol.2021.659926.

[48] F. Aktar, C. Burudpakdee, M. Polanco, S. Pei, T.C. Swayne, P.N. Lipke, L. Emtage, The huntingtin inclusion is a dynamic phase-separated compartment, Life Sci. Alliance. 2 (2019) e201900489. https://doi.org/10.26508/lsa.201900489.

[49] E.M. Courchaine, A. Lu, K.M. Neugebauer, Droplet organelles?, EMBO J. 35 (2016) 1603–1612. https://doi.org/10.15252/embj.201593517.

[50] Y. Shin, C.P. Brangwynne, Liquid phase condensation in cell physiology and disease, Science (80-.). 357 (2017). https://doi.org/10.1126/science.aaf4382.

[51] T. Hirose, K. Ninomiya, S. Nakagawa, T. Yamazaki, A guide to membraneless organelles and their various roles in gene regulation, Nat. Rev. Mol. Cell Biol. 24 (2023) 288–304. https://doi.org/10.1038/s41580-022-00558-8.

[52] S.F. Banani, H.O. Lee, A.A. Hyman, M.K. Rosen, Biomolecular condensates: organizers of cellular biochemistry, Nat. Rev. Mol. Cell Biol. 18 (2017) 285–298. https://doi.org/10.1038/nrm.2017.7.

[53] J. Sheng, N.K. Olrichs, W.J. Geerts, X. Li, A.U. Rehman, B.M. Gadella, D. V. Kaloyanova, J.B. Helms, Zinc binding regulates amyloid-like aggregation of GAPR-1, Biosci. Rep. 39 (2019). https://doi.org/10.1042/BSR20182345.

[54] H.B. Eberle, R.L. Serrano, J. Füllekrug, A. Schlosser, W.D. Lehmann, F. Lottspeich, D. Kaloyanova, F.T. Wieland, J.B. Helms, Identification and characterization of a novel human plant pathogenesis-related protein that localizes to lipid-enriched microdomains in the Golgi complex, J. Cell Sci. 115 (2002) 827–838. https://doi.org/10.1242/jcs.115.4.827.

[55] R.L. Serrano, A. Kuhn, A. Hendricks, J.B. Helms, I. Sinning, M.R. Groves, Structural Analysis of the Human Golgi-associated Plant Pathogenesis Related Protein GAPR-1 Implicates Dimerization as a Regulatory Mechanism, J. Mol. Biol. 339 (2004) 173–183. https://doi.org/10.1016/j.jmb.2004.03.015.

[56] J. Van Galen, B.W.M. Van Balkom, R.L. Serrano, D. Kaloyanova, R. Eerland, E. Stüven, J.B. Helms, Binding of GAPR-1 to negatively charged phospholipid membranes: Unusual binding characteristics to phosphatidylinositol, Mol. Membr. Biol. 27 (2010) 81–91. https://doi.org/10.3109/09687680903507080.

[57] G. V. Serpionov, A.I. Alexandrov, Y.N. Antonenko, M.D. Ter-Avanesyan, A protein polymerization cascade mediates toxicity of non-pathological human huntingtin in yeast, Sci. Rep. 5 (2015) 18407. https://doi.org/10.1038/srep18407.

[58] J. Sheng, N.K. Olrichs, B.M. Gadella, D. V. Kaloyanova, J.B. Helms, Regulation of

Functional Protein Aggregation by Multiple Factors: Implications for the Amyloidogenic Behavior of the CAP Superfamily Proteins, Int. J. Mol. Sci. 21 (2020) 6530. https://doi.org/10.3390/ijms21186530.

[59] D. Mumberg, R. Muller, M. Funk, Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression, Nucleic Acids Res. 22 (1994) 5767–5768. https://doi.org/10.1093/nar/22.25.5767.

[60] D. Petroi, B. Popova, N. Taheri-Talesh, S. Irniger, H. Shahpasandzadeh, M. Zweckstetters, T.F. Outeiro, G.H. Braus, Aggregate clearance of α-synuclein in *Saccharomyces cerevisiae* depends more on autophagosome and vacuole function than on the proteasome, J. Biol. Chem. 287 (2012) 27567–27579. https://doi.org/10.1074/jbc.M112.361865.

Supplemental information

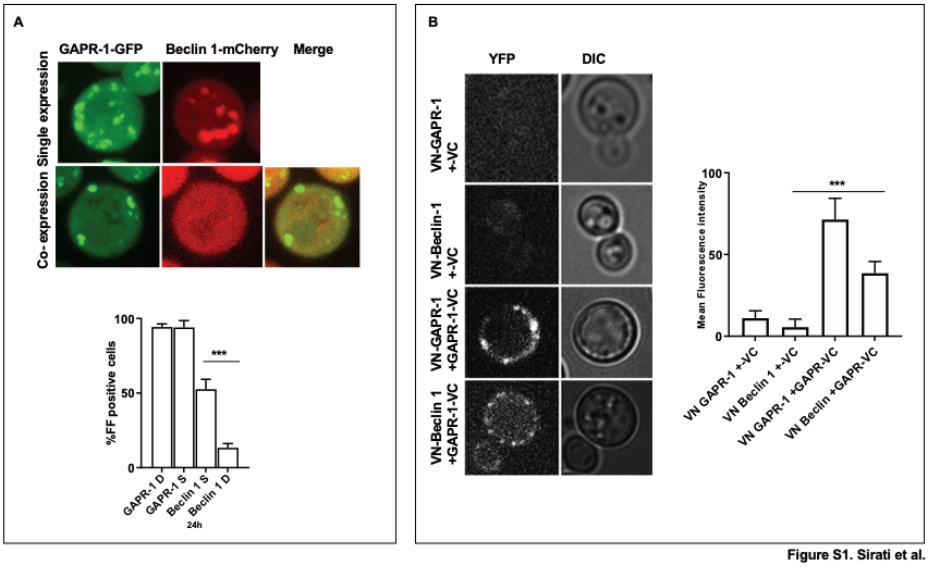


Figure S1. Sirati et al.

Figure S1. A) Live cell fluorescent microscopy of yeast W303 cells after 24h induction of expression of GAPR-1-GFP (single expression), Beclin 1-mCherry (single expression) or both constructs together (co-expression). Quantification of yeast cells with condensates expressing GAPR-1-GFP, GAPR-1-mCherry (single expression, S) or both constructs together (co-expression, D) after 24h induction. For the quantification of condensates, at least 100 cells were counted per experiment. Results are expressed as mean ± SD, n=3; B) Representative images of live-cell fluorescence microscopy in the BIFC studies. GAPR-1 and Beclin 1 were tagged with the N-terminal Venus fragment and co-expressed with i) the C-terminal fragment of Venus protein as negative controls (top 4 panels); ii) with the C-terminal fragment of Venus bound to GAPR-1 (GAPR-1-VC) (bottom 4 panels). Co-expression of VN-Beclin 1with GAPR-1-VC shows a YFP signal as compared to the negative controls. Co-expression of VN-GAPR-1 with GAPR-1-VC served as a positive control. The mean YFP fluorescence intensity of cells described under B) was determined after 24h expression (right panel). The results are expressed as mean ±SD of three independent experiments. Significance of differences was calculated with two-tailed unpaired t-test (***$p < 0.001$; n=50).

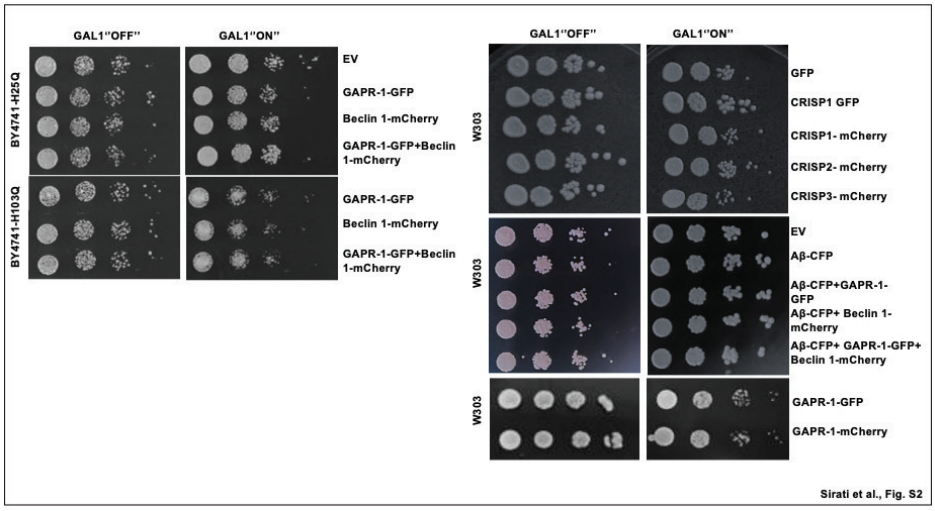


Figure S2. Ten-fold dilutions of exponentially growing yeast cultures expressing different constructs as indicated in the figure were spotted using selection amino acid plates containing 2% glucose (*GAL1* promoter "OFF") and 2% galactose (*GAL1* promoter "ON"). The cells were incubated at 30^{o}C for 3 days and imaged by scanning.

Our previous studies showed that Golgi-associated plant pathogenesis-related protein 1 (GAPR-1) exhibits amyloid-like characteristics *in vitro* [1–4]. In this thesis project, we employed yeast *Saccharomyces cerevisiae* as a model system to investigate the *in vivo* amyloid-like oligomerization properties of GAPR-1. Consistent with this, we found that GAPR-1 localizes to biomolecular condensates, similar to the other pathogenic and functional amyloid proteins. Our combined results confirm for the first time the amyloidogenic properties of GAPR-1 *in vivo* and suggest that the amyloidogenic properties of GAPR-1 may be involved in its biological function, similar to functional amyloids [5,6].

Functional amyloids

Functional amyloids are a class of proteins that have the ability to self-assemble into structured cross-β sheet scaffolds known as amyloid fibrils [7–10]. Traditionally, amyloids have been associated with neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, in which aberrant protein aggregation leads to pathological consequences [11–15]. However, it has become evident in recent years that amyloids can also serve functional roles in various biological systems [16–20]. Functional amyloids are found in various organisms, from bacteria and fungi to humans, indicating their evolutionary significance [21,22]. These amyloid-forming proteins have been identified in diverse contexts, including biofilm formation [23], extracellular matrix assembly [24], and regulation of protein activity. Functional amyloids are characterized by their reversibility, enabling them to assemble and disassemble under controlled conditions. This ability allows functional amyloids to adapt to changing environmental conditions or cellular requirements [9,25]. Unlike pathological amyloids, which are associated with toxicity and cellular damage [26], functional amyloids are generally non-toxic and do not cause harm to the cell or organism. Their controlled formation and non-toxic nature make functional amyloids suitable for physiological processes and for the overall function and survival of organisms. One well-known example of functional amyloids is found in yeast *S. cerevisiae*. In yeast, several prion proteins, such as Sup35 and Ure2, adopt an amyloid conformation, forming functional amyloids. Prions are self-propagating protein conformations that can induce heritable phenotypic changes. These prion amyloids can influence gene expression, stress response, and other cellular processes [27]. Another example of functional amyloids is curli fibrils produced by certain strains of *Escherichia coli* [23]. Curli fibrils are involved in biofilm formation [28], which allows bacteria to adhere to surfaces and establish structured communities. These amyloid fibrils provide structural stability to the biofilm and enable bacterial cells to resist environmental stresses.

Functional amyloids have also been identified in higher organisms. For instance, the protein Pmel17 is involved in the formation of melanin in mammals, the pigment responsible for hair, skin, and eye color [29]. Pmel17 self-assembles into amyloid-like structures in so-called melanosomes, which serve as compartments for melanin synthesis and storage [30].

The functional amyloid concept challenges the notion that amyloids are exclusively pathological. It highlights the dynamic nature of protein aggregation and recognizes that under certain circumstances, amyloid formation can be beneficial and serve important biological functions. Understanding the mechanisms by which functional amyloids are formed, regulated, and function, can have implications for diverse fields, including bioengineering, biotechnology, and medicine. In the past decade, the utilization of baker's yeast as a research tool has significantly contributed to the understanding of various human diseases, particularly neurodegenerative disorders that are characterized by protein misfolding and amyloid formation [31–35]. The recognition of functional amyloid-like assemblies has led to the development of similar yeast model systems for investigating functional amyloids and exploring the regulatory mechanisms underlying physiological aggregation [25].

GAPR-1 may function similarly to a functional amyloid

Our collective results demonstrate that expression of GAPR-1-GFP in yeast fulfils the criteria of a protein with amyloidogenic properties [25,36–44], leading to the generation of SDS-resistant aggregates, increased ThT fluorescence, and the formation of reversible intracellular protein condensates. Unlike pathological amyloids [32,45–50], but like functional amyloids [51–53], the expression of GAPR-1-GFP does not induce toxicity in the cells. These observations support our hypothesis that *in vivo* amyloid-like oligomerization of GAPR-1 may serve a functional purpose [54]. Given that GAPR-1 associates with Golgi membranes, its oligomeric state is likely regulated by multiple factors, such as the biophysical properties of the membranes to facilitate specific surface interactions by specific negatively charged lipids that interact with GAPR-1 and to catalyze structural changes by hydrophobic interactions [55]. Cytosolic factors that are involved in GAPR-1 oligomerization include particular metal ions [3,4].

Factors involved in GAPR-1 oligomerization in yeast

In a previous publication [54], we summarized the regulation of amyloid-like behavior of GAPR-1 *in vitro* by multiple factors. We now validate these findings *in vivo* by confirming the involvement of certain factors in condensate formation in yeast.

Lipid bilayers

Membranes have been identified as catalytic sites that facilitate the (mis)folding and aggregation of amyloidogenic proteins [56,57]. The interaction between oligomeric proteins and cellular membranes is known to play a significant role in the pathogenesis of various diseases. Proteins such as Aβ [58], PrP [59], α-synuclein [60,61], and islet amyloid peptide (IAPP) [62] have been observed to adopt oligomeric states upon interacting with membranes [54,63]. For instance, studies have revealed that wild-type and A53T α-synuclein exhibit an affinity for yeast plasma membranes, which aligns with previous *in vitro* evidence demonstrating the binding of α-synuclein to fatty acids and phospholipids [61]. Its aggregation is also influenced by fatty acids, with polyunsaturated fatty acids promoting aggregation and saturated fatty acids inhibiting it [64]. Yeast models investigating α-synuclein have consistently reported its localization to the cellular membrane [32,65,66].

Earlier studies from our group have demonstrated that GAPR-1 is firmly bound to Golgi membranes through a mechanism involving myristoylation of its N-terminus [67]. Remarkably, the presence of a myristoyl group is not essential for the formation of amyloid-like fibrils by GAPR-1 *in vitro*, as recombinant non-myristoylated GAPR-1 readily aggregates into amyloid-like fibrils when seeded with an appropriate platform [2–4,68]. Consistent with this, our current investigation reveals that the expression of Δmyr-GAPR-1-GFP in yeast cells also leads to the formation of protein condensates, albeit with reduced efficiency compared to GAPR-1-GFP. In mammalian cells, myristoylation, coupled with strong electrostatic interactions (GAPR-1 has an isoelectric point of 9.4), facilitates efficient membrane interaction [69]. In yeast cells, the observed partial distribution of overexpressed GAPR-1 between membranes and cytosol may be attributed to suboptimal N-myristoylation due to the high levels of GAPR-1-GFP overexpression or differences in substrate specificity of orthologous N-myristoyltransferases [70]. Protein binding to membranes leads to increased local concentrations, creating favourable conditions for seed formation [71,72]. Consequently, this accelerates amyloid-like oligomerization and promotes the formation of protein condensates (Figure 1).

Metal ions

Extensive research has focused on investigating the coordination of metal ions with various amyloidogenic proteins. Zinc is one of the most abundant micronutrients in the human body, playing a role in cell division, growth, differentiation, signal transduction and pathology [73]. The combination of disturbed zinc metabolism and (hyper)phosphorylation of e.g. Tau, APP, Aβ and α-synuclein have been shown to play a role in these and other amyloid-like protein oligomerization and aggregation processes, resulting in severe neurodegenerative disorders such as Parkinson's, Alzheimer's and ALS diseases [73]. Interestingly, the involvement of the metal-binding site (His54 and His103) in the formation of GAPR-1 protein condensates *in vivo* mirrors its participation in Zn^{2+}-dependent amyloid-like aggregation of GAPR-1 *in vitro*. Mutations in the conserved metal-binding site enhanced condensate formation, suggesting that these residues prevent uncontrolled protein sequestration. In agreement with this, we find that adding Zn^{2+} metal ions enhance protein condensate formation. Furthermore, Zn^{2+} reduces GAPR-1 protein degradation, which indicates the stabilization of GAPR-1 in condensates (Figure 1). We propose that the properties underlying both the amyloidogenic properties and the reversible sequestration of GAPR-1 leading to condensates play a role in its biological function.

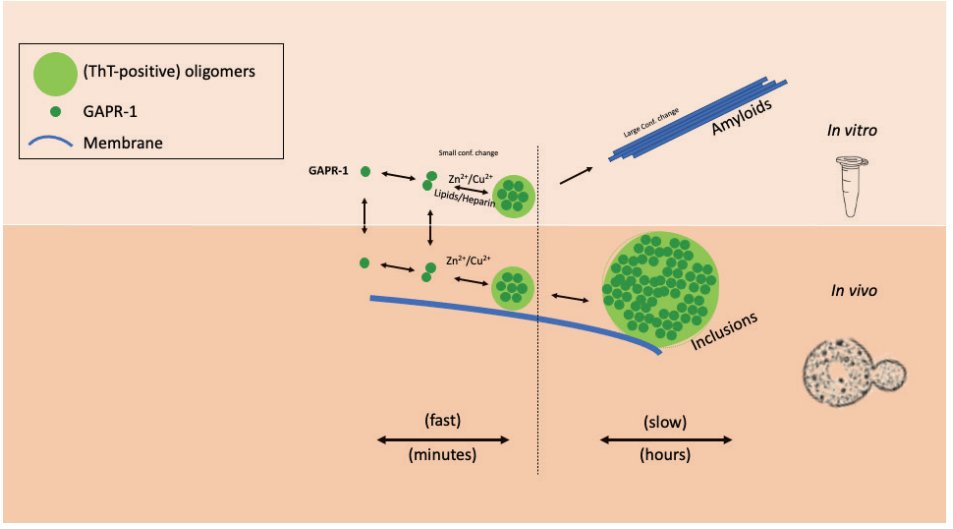


Figure 1. Baker's yeast as an *in vivo* model to study GAPR-1 oligomerization.

GAPR-1 regulates autophagy

GAPR-1 has been identified as a negative regulator of the autophagy [74]. Consistent with our present findings, we have recently proposed that the oligomerization of GAPR-1 could play a role in its interaction with other components of the autophagic machinery, thereby being crucial for the regulation of autophagy [54]. In this study, we revealed that Beclin 1 can also form amyloids, shedding new light on its role in autophagy. Expression of Beclin 1-mCherry in *S. cerevisiae* leads to the formation of protein condensates and exhibits elevated ThT fluorescence levels in yeast cells. Previous studies by others have demonstrated that full-length Beclin 1 can form stable homo-oligomers under various conditions [75–77]. Oligomerized Beclin 1 is suggested to provide a platform for multiple Beclin 1-interacting proteins [75,76,78–80]. Our results indicate that Beclin 1 contains several predicted amyloidogenic regions that likely contribute to its condensate formation in yeast upon overexpression. We confirmed the presence of an amyloidogenic region within the ECD domain through the overexpression of these 18 amino acids region (B18 peptide), which resulted in the formation of protein condensates. However, the amyloidogenic region of the B18 peptide is not essential for Beclin 1 condensate formation, as overexpression of Beclin ΔB18 still leads to protein condensate formation. These findings suggest that the other predicted amyloidogenic regions in Beclin 1 significantly contribute to its amyloidogenic behavior. Previous studies have demonstrated the interaction between GAPR-1 and Beclin 1 through direct and indirect means [74,77,81]. In this study, we have confirmed a direct interaction between GAPR-1 and Beclin 1 using the BiFC (bimolecular fluorescence complementation) method, which allows visualization and analysis of protein-protein interactions in live cells [82,83] by complementarily reconstituting a fluorescent protein [82,84]. The interaction site on the Beclin 1 protein has been mapped to amino acids 267–284 (corresponding to the B18 peptide), as the B18 peptide interferes with the GAPR-1/Beclin 1 interaction [74]. Indeed, co-expression of GAPR-1 and Beclin 1 in *S. cerevisiae* influenced the condensate behavior of both proteins. As we did not observe co-localization of the co-expressed proteins within the same condensates, the interactions between GAPR-1 and Beclin 1 likely occur prior to condensate formation. The specific protein-protein interactions that take place during these interactions remain to be determined (Figure 2). As mentioned earlier, when the interaction region of Beclin 1 with GAPR-1 (amino acids 267–284) is deleted, Beclin 1 still forms condensates upon expression in *S. cerevisiae*. However, the deletion of these 18 amino acids abolishes the interaction between GAPR-1 and Beclin 1, resulting in no effect on the condensate formation of each other anymore. These combined results indicate the involvement of amyloidogenic properties during the oligomeric interactions between GAPR-1 and Beclin 1. We propose that the interaction between GAPR-1 and Beclin 1 in

human (mammalian) cells is based on their amyloidogenic properties during the oligomerization process before the formation of amyloid-like fibrils.

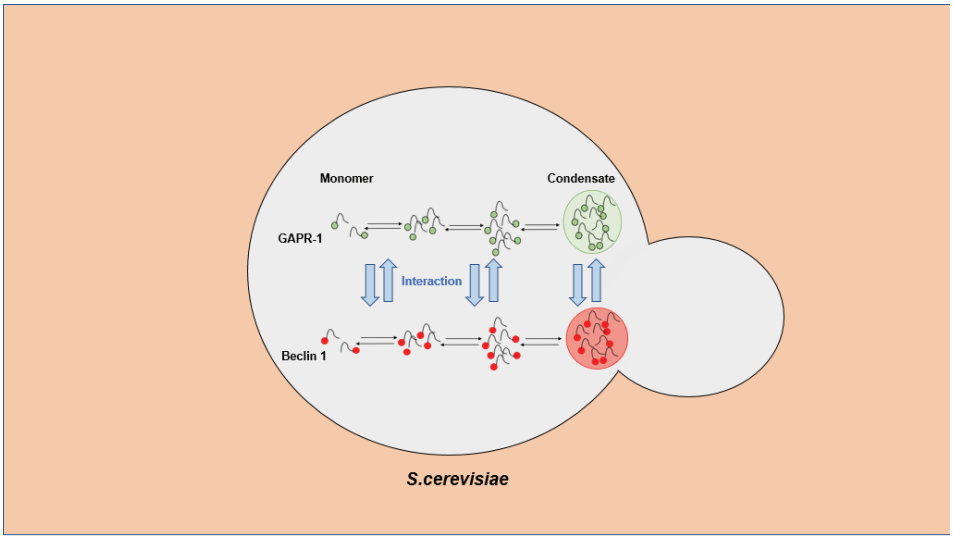


Figure 2. Oligomeric interaction of GAPR-1 and Beclin 1 in the yeast model system.

In the context of autophagy, understanding the regulation of the GAPR-1/Beclin 1 interaction could shed light on the induction of autophagy. In this study, we propose that the oligomeric interactions between GAPR-1 and Beclin 1 occur at Golgi membranes and involve a subset of oligomeric Beclin 1 that exists alongside other cellular Beclin 1. This interaction site might serve as a sensor for small, soluble amyloid-like oligomers. When these intermediates accumulate in the cell, they can compete with Beclin 1 for Golgi binding, releasing Beclin 1 oligomers into the cytosol. Once in the cytosol, these Beclin 1 oligomers revert to monomers and induce autophagy.

Experimental testing of this hypothesis is presented in Chapter 5, where we observe that Aβ peptides and the Huntington protein effectively compete with the GAPR-1/Beclin 1 interaction. Consequently, disrupting the GAPR-1/Beclin 1 interaction may trigger the autophagic flux (see Figure 3). This complex interplay highlights the significance of the GAPR-1/Beclin 1 interaction in the regulation of autophagy and the potential implications for biological processes involving amyloidogenic proteins and e.g. age-related diseases.

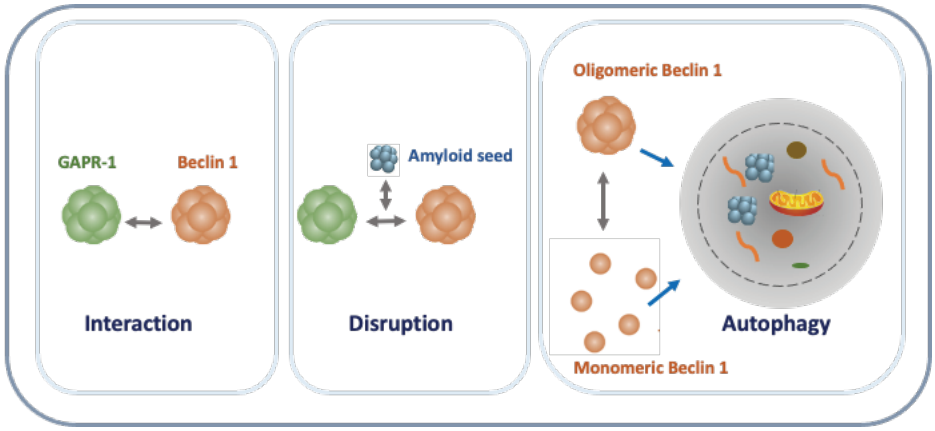


Figure 3. Model of monomeric and oligomeric Beclin 1 in autophagy.

Phosphorylation regulates GAPR-1 and Beclin 1 oligomerization and their interaction

Although significant progress in understanding the formation and dissolution of biomolecular condensates within cells has been made, our knowledge of the intracellular mechanisms that regulate these processes remains limited. One well-known regulatory mechanism that has gained considerable attention is the post-translational modification (PTM) of proteins. Among different PTMs, phosphorylation is of particular interest due to its ability to be rapidly and reversibly added in response to cellular signals. This dynamic modification can profoundly influence protein function, interactions, and localization, thus playing a crucial role in cellular processes [85]. Protein phosphorylation introduces a negatively charged phosphate group, which can significantly impact the chemical, steric, and electrostatic properties of amino acid side chains. This modification has the potential to induce a diverse range of structural changes within the protein. By adding negative charges, phosphorylation can affect the local environment, leading to conformational changes, alterations in protein-protein interactions, and modulation of enzymatic activity [86,87]. The impact of protein phosphorylation on condensate formation can be dual in nature, with the ability to both promote and repress these structures.

A noteworthy example is fused in sarcoma (FUS), an RNA-binding protein associated with neurodegenerative disorders. The multi-phosphorylation of its N-terminal disordered segment has been shown to prevent condensate formation [88]. Conversely, in fragile X mental retardation protein (FMRP), which forms ribonuclear protein granules

in neurons, multi-phosphorylation of its C-terminal disordered region has been found to increase condensation *in vitro* [89]. These observations highlight the complex regulatory role of protein phosphorylation in modulating the formation or dissolution of biomolecular condensates, depending on the specific protein.

In this study, we employed a yeast model system to investigate the impact of phosphorylation on the oligomeric states of GAPR-1 and Beclin 1. Our objective was to understand the role of phosphorylation in the interaction between GAPR-1 and Beclin 1. Phosphorylation of Ser55 is essential for the formation of condensates in GAPR-1. Similarly, phosphorylation of Ser15 and Ser30 is crucial for the formation of condensates in Beclin 1. Remarkably, ULK1/Atg1 kinase mediates phosphorylation of these sites in both proteins. Moreover, phosphorylation of GAPR-1 at Ser55 and/or phosphorylation of Beclin 1 at Ser15/30 affects the interference with their mutual condensate formation upon co-expression. The precise mechanism by which phosphorylation affects the interaction between these proteins has yet to be determined. Here, we demonstrate that phosphorylation plays a role in regulating the amyloidogenic properties of GAPR-1 and Beclin 1, as phospho-mutants of these proteins exhibit impaired condensate formation. Furthermore, deletion of the ULK1/Atg1 kinase abolishes the interaction between GAPR-1 and Beclin 1, suggesting that this kinase plays a critical role in regulating the interactions between these two proteins (Figure. 4). ULK1/Atg1 is a key regulator of autophagy initiation and is involved in the formation of the autophagosome, the double-membrane structure responsible for sequestering cellular components for degradation. We propose that phosphorylation of GAPR-1 may serve as a negative feedback loop regulating the activation of Beclin 1 and the induction of autophagy. According to this hypothesis, Beclin 1 phosphorylation may initiate the priming of autophagy and subsequent phosphorylation of GAPR-1 could act as a feedback loop to prevent excessive autophagy activation. Indeed, stimulatory compounds that modulate the autophagy pathway hold significant pharmaceutical potential for various applications, including targeting clearance of viral infections and protein aggregates associated with neurodegenerative disorders.

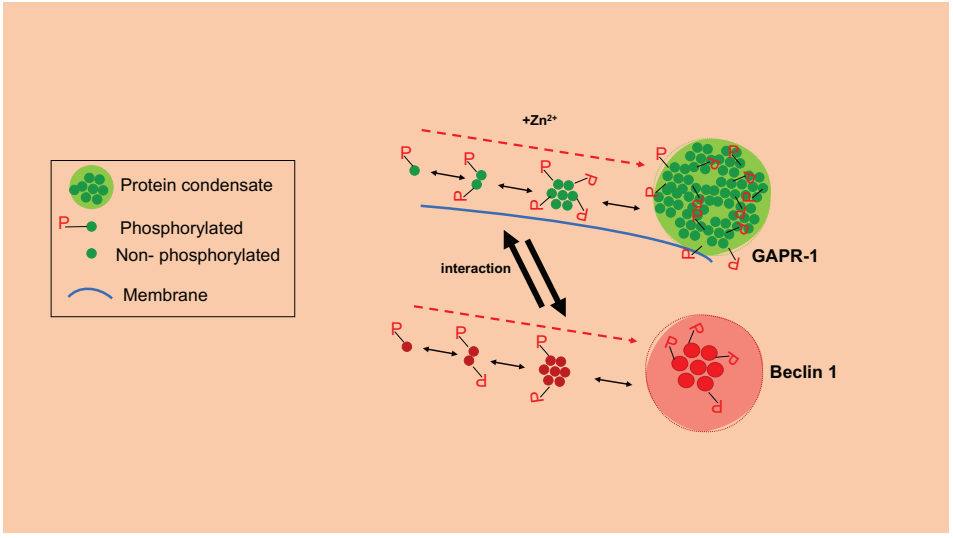


Figure 4. Phosphorylation involved in GAPR-1/Beclin 1 oligomerization and interaction *in vivo.*

Homo-oligomeric and hetero-oligomeric protein-protein interactions

In previous *in vivo* studies, a significant interaction between GAPR-1 and Beclin 1 was observed, indicating a potential involvement of GAPR-1 in the regulation of autophagy [74,77,81]. Moreover, it was demonstrated that purified recombinant GAPR-1 can inhibit amyloid formation of the Aβ peptide (1-40) by binding to oligomeric Aβ species [90]. Building upon these findings, our focus in Chapter 5 was on investigating the affinity of oligomeric GAPR-1 for other proteins. We discovered that GAPR-1 forms hetero-typic interactions not only with Beclin 1 [6] but also with the Aβ peptide (1-40) and Huntington protein. These findings suggest a potential role of GAPR-1 in modulating interactions with other amyloidogenic proteins, which may also have relevance for cellular processes involving autophagy and protein aggregation.

In recent years, cross-seeding among amyloid proteins have gained considerable interest [91–94]. In contrast to homotypic seeding, when proteins with identical (homologous) amino acid sequences or structural motifs come together, cross-seeding occurs when misfolded proteins with different (heterologous) sequences interact and induce hybrid or mixed amyloid structures [95–97]. This phenomenon is also termed heterotypic cross-seeding aggregation. The coexistence of heterologous protein aggregates, such as Aβ and α-synuclein, Aβ and Tau, Aβ and transthyretin, as well as hIAPP and insulin, has been observed in patients affected by several protein misfolding diseases [91,97–99]. The principle of cross-seeding is not limited to pathological

amyloids. It also occurs between functional and pathological amyloid peptides, as has been observed e.g. between bacterial curli and amyloid peptides like SEVI, Aβ, and hIAPP [100]. Notably, the cross-seeding involving bacterial-produced curli and HIV-associated amyloid aggregates indicates that curli cross-seeding may not only influence the nucleation of amyloidogenic aggregation of IAPP and Aβ 1-40, but may also trigger biological exogenous infections [91,100]. These findings offer potential insights into the molecular basis of exogenously triggered amyloid diseases and propose that amyloid cross-seeding aggregation could be a mechanism driving the spread of amyloidosis across different cells and tissues and between different diseases.

Interactions between different amyloid peptides can, however, also lead to cross-amyloid inhibition. It is important to note that cross-amyloid aggregation and inhibition arise from the same origin of cross-amyloid interactions [91]. Understanding cross-seeding and cross-amyloid interactions may shed light on the mechanisms behind a range of protein misfolding diseases as well as functional amyloids [92,94] and may open up opportunities for exploring therapeutic strategies that target these interactions to modulate the formation and progression of amyloid aggregates.

S. cerevisiae as a novel screening model system for oligomeric protein-protein interactions.

In recent years, amyloid fibril formation and amyloidogenic protein oligomerization have been identified as a factor driving the formation of biomolecular condensates [101]. The assay system described in this thesis provides a novel approach to investigate cross-seeding interactions based on the formation of biomolecular condensates in *S. cerevisiae*. By studying the condensate behavior of co-expressed proteins, we observed different phenotypes that can shed light on the nature of cross-seeding interactions. The four phenotypes illustrated in Chapter 5 represent distinct outcomes of the co-expression experiments. In Type I, both expressed proteins have the propensity to form condensates and co-localize to the same condensates upon co-expression. Type II involves the formation of different condensates by the expressed proteins. Type III demonstrates the re-localization of one of the proteins to the cytosol upon co-expression with the other protein that still forms condensates. Lastly, in Type IV, only one of the expressed proteins has the propensity to form condensates, while the other protein re-localizes to condensates upon co-expression (Figure 5). In Chapter 5 these co-expression experiments were also expanded to include triple-expression experiments. By introducing a competitor for the protein-protein interaction of interest, we were able to assess the relative binding affinities of the competitor as compared to the co-expressed protein. This approach provides insights into the strength of the protein-protein interactions and the ability of the competitor protein or peptide to interfere with

these interactions. The described assay system provides researchers with a valuable tool to investigate protein-protein interactions and their dynamics within the context of biomolecular condensate formation. By studying the formation and behavior of these condensates, researchers can gain insights into the mechanisms underlying the binding affinities of protein interactions. This knowledge can be crucial in understanding various cellular processes and pathways.

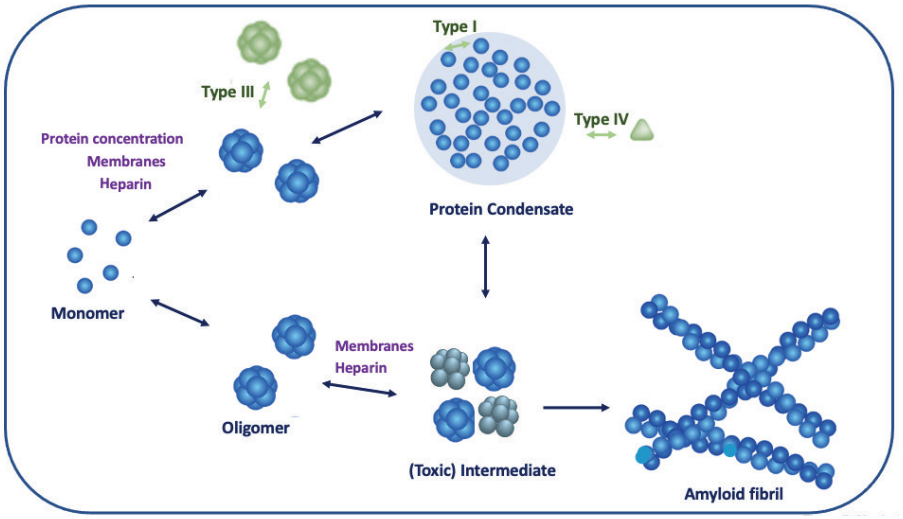


Figure 5. A novel screening assay to study amyloidogenic cross-seeding interaction using *S. cerevisiae*.

Furthermore, this assay system has the potential to contribute to the discovery of novel therapeutic targets or interventions. By studying cross-seeding interactions and identifying molecules or compounds that can modulate these interactions, researchers may uncover new strategies to manipulate cellular processes. Overall, the exploration of cross-seeding interactions and their role in cellular processes holds great potential for advancing our understanding of molecular mechanisms and opening up avenues for therapeutic interventions in various diseases and biological systems.

References

[1] J. van Galen, N.K. Olrichs, A. Schouten, R.L. Serrano, E.N.M. Nolte-'t Hoen, R. Eerland, D. Kaloyanova, P. Gros, J.B. Helms, Interaction of GAPR-1 with lipid bilayers is regulated by alternative homodimerization, Biochim. Biophys. Acta - Biomembr. 1818 (2012) 2175–2183. https://doi.org/10.1016/j.bbamem.2012.04.016.

[2] N.K. Olrichs, A.K. Mahalka, D. Kaloyanova, P.K. Kinnunen, J. Bernd Helms, Golgi-Associated plant Pathogenesis Related protein 1 (GAPR-1) forms amyloid-like fibrils by interaction with acidic phospholipids and inhibits Aβ aggregation, Amyloid. 21 (2014) 88–96. https://doi.org/10.3109/13506129.2014.882304.

[3] J. Sheng, N.K. Olrichs, W.J. Geerts, X. Li, A.U. Rehman, B.M. Gadella, D. V. Kaloyanova, J.B. Helms, Zinc binding regulates amyloid-like aggregation of GAPR-1, Biosci. Rep. 39 (2019). https://doi.org/10.1042/BSR20182345.

[4] J. Sheng, N.K. Olrichs, W.J. Geerts, D. V. Kaloyanova, J.B. Helms, Metal ions and redox balance regulate distinct amyloid-like aggregation pathways of GAPR-1, Sci. Rep. 9 (2019) 15048. https://doi.org/10.1038/s41598-019-51232-7.

[5] N. Sirati, B. Popova, M.R. Molenaar, I.C. Verhoek, G.H. Braus, D. V. Kaloyanova, J.B. Helms, Dynamic and Reversible Aggregation of the Human CAP Superfamily Member GAPR-1 in Protein Inclusions in *Saccharomyces cerevisiae*, J. Mol. Biol. 433 (2021) 167162. https://doi.org/10.1016/j.jmb.2021.167162.

[6] N. Sirati, Z. Shen, N.K. Olrichs, B. Popova, I.C. Verhoek, I.M. Lagerwaard, G.H. Braus, D. V. Kaloyanova, J.B. Helms, GAPR-1 Interferes with Condensate Formation of Beclin 1 in *Saccharomyces cerevisiae,* J. Mol. Biol. 435 (2023) 167935. https://doi.org/10.1016/j.jmb.2022.167935.

[7] S.K. Chaturvedi, M.K. Siddiqi, P. Alam, R.H. Khan, Protein misfolding and aggregation: Mechanism, factors and detection, Process Biochem. 51 (2016) 1183–1192. https://doi.org/10.1016/j.procbio.2016.05.015.

[8] S.Y. Ow, D.E. Dunstan, A brief overview of amyloids and Alzheimer's disease, Protein Sci. 23 (2014) 1315–1331. https://doi.org/10.1002/pro.2524.

[9] J.A. Buchanan, N.R. Varghese, C.L. Johnston, M. Sunde, Functional Amyloids: Where Supramolecular Amyloid Assembly Controls Biological Activity or Generates New Functionality, J. Mol. Biol. 435 (2023) 167919. https://doi.org/10.1016/j.jmb.2022.167919.

[10] M.R. Sawaya, M.P. Hughes, J.A. Rodriguez, R. Riek, D.S. Eisenberg, The expanding amyloid family: Structure, stability, function, and pathogenesis, Cell. 184 (2021) 4857–4873. https://doi.org/10.1016/j.cell.2021.08.013.

[11] K.W. Tipping, P. van Oosten-Hawle, E.W. Hewitt, S.E. Radford, Amyloid Fibres: Inert End-Stage Aggregates or Key Players in Disease?, Trends Biochem. Sci. 40 (2015) 719–727. https://doi.org/10.1016/j.tibs.2015.10.002.

[12] C. Wells, S. Brennan, M. Keon, L. Ooi, The role of amyloid oligomers in neurodegenerative pathologies, Int. J. Biol. Macromol. 181 (2021) 582–604. https://doi.org/10.1016/j.ijbiomac.2021.03.113.

[13] R.N. Rambaran, L.C. Serpell, Amyloid fibrils: abnormal protein assembly., Prion. 2 (2008) 112–117. https://doi.org/10.4161/pri.2.3.7488.

[14] C. Sachse, M. Fändrich, N. Grigorieff, Paired β-sheet structure of an Aβ(1-40) amyloid fibril revealed by electron microscopy, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 7462–7466. https://doi.org/10.1073/pnas.0712290105.

[15] S. Linse, T. Knowles, Amyloids and protein aggregation, Chem. Sci. 14 (2023) 6491–6492. https://doi.org/10.1039/D2SC90225G.

[16] E. Stroo, M. Koopman, E.A.A. Nollen, A. Mata-Cabana, Cellular regulation of amyloid formation in aging and disease, Front. Neurosci. 11 (2017). https://doi.org/10.3389/fnins.2017.00064.

[17] R.S. Jacob, S. Das, S. Ghosh, A. Anoop, N.N. Jha, T. Khan, P. Singru, A. Kumar, S.K. Maji, Amyloid formation of growth hormone in presence of zinc: Relevance to its storage in secretory granules, Sci. Rep. 6 (2016) 23370.

[18] E. Grignaschi, G. Cereghetti, F. Grigolato, M.R.G. Kopp, S. Caimi, L. Faltova, S. Saad, M. Peter, P. Arosio, A hydrophobic low-complexity region regulates aggregation of the yeast pyruvate kinase Cdc19 into amyloid-like aggregates in vitro, J. Biol. Chem. 293 (2018) 11424–11432. https://doi.org/10.1074/jbc.RA117.001628.

[19] J. Asencio-Hernández, C. Ruhlmann, A. McEwen, P. Eberling, Y. Nominé, J. Céraline, J.P. Starck, M.A. Delsuc, Reversible amyloid fiber formation in the N terminus of androgen receptor, ChemBioChem. 15 (2014) 2370–2373. https://doi.org/10.1002/cbic.201402420.

[20] A. Anoop, S. Ranganathan, B.D. Dhaked, N.N. Jha, S. Pratihar, S. Ghosh, S. Sahay, S. Kumar, S. Das, M. Kombrabail, K. Agarwal, R.S. Jacob, P. Singru, P. Bhaumik, R.

Padinhateeri, A. Kumar, S.K. Maji, Elucidating the role of disulfide bond on amyloid formation and fibril reversibility of somatostatin-14: Relevance to its storage and secretion, J. Biol. Chem. 289 (2014) 16884–16903.

[21] A. Balistreri, E. Goetzler, M. Chapman, Functional Amyloids Are the Rule Rather Than the Exception in Cellular Biology, Microorganisms. 8 (2020) 1951. https://doi.org/10.3390/microorganisms8121951.

[22] D. Otzen, R. Riek, Functional Amyloids, Cold Spring Harb. Perspect. Biol. 11 (2019) a033860. https://doi.org/10.1101/cshperspect.a033860.

[23] Ü. Akbey, M. Andreasen, Functional amyloids from bacterial biofilms – structural properties and interaction partners, Chem. Sci. 13 (2022) 6457–6477. https://doi.org/10.1039/D2SC00645F.

[24] M. Ghrayeb, S. Hayet, N. Lester-Zer, Y. Levi-Kalisman, L. Chai, Fibrilar Polymorphism of the Bacterial Extracellular Matrix Protein TasA, Microorganisms. 9 (2021) 529. https://doi.org/10.3390/microorganisms9030529.

[25] G. Cereghetti, S. Saad, R. Dechant, M. Peter, Reversible, functional amyloids: towards an understanding of their regulation in yeast and humans, Cell Cycle. 17 (2018) 1545–1558. https://doi.org/10.1080/15384101.2018.1480220.

[26] E.I. Yakupova, L.G. Bobyleva, S.A. Shumeyko, I.M. Vikhlyantsev, A.G. Bobylev, Amyloids: The History of Toxicity and Functionality, Biology (Basel). 10 (2021) 394. https://doi.org/10.3390/biology10050394.

[27] S. Alberti, R. Halfmann, O. King, A. Kapila, S. Lindquist, A Systematic Survey Identifies Prions and Illuminates Sequence Features of Prionogenic Proteins, Cell. 137 (2009) 146–158. https://doi.org/10.1016/j.cell.2009.02.044.

[28] T. V. Sønderby, N.N. Louros, L. Khodaparast, L. Khodaparast, D.J. Madsen, W.P. Olsen, N. Moonen, M. Nagaraj, V. Sereikaite, K. Strømgaard, F. Rousseau, J. Schymkowitz, D.E. Otzen, Sequence-targeted Peptides Divert Functional Bacterial Amyloid Towards Destabilized Aggregates and Reduce Biofilm Formation, J. Mol. Biol. 435 (2023) 168039. https://doi.org/10.1016/j.jmb.2023.168039.

[29] D.M. Fowler, A. V Koulov, C. Alory-Jost, M.S. Marks, W.E. Balch, J.W. Kelly, Functional Amyloid Formation within Mammalian Tissue, PLoS Biol. 4 (2005) e6. https://doi.org/10.1371/journal.pbio.0040006.

[30] J.S. Hee, S.M. Mitchell, X. Liu, R.M. Leonhardt, Melanosomal formation of PMEL core amyloid is driven by aromatic residues, Sci. Rep. 7 (2017) 44064. https://doi.org/10.1038/srep44064.

[31] R.P. Mason, F. Giorgini, Modeling Huntington disease in yeast: Perspectives and future directions, Prion. 5 (2011) 269–276. https://doi.org/10.4161/pri.5.4.18005.

[32] I.C. Brás, B. Popova, G.H. Braus, T.F. Outeiro, Yeast-Based Screens to Target Alpha-Synuclein Toxicity, in: 2019: pp. 145–156. https://doi.org/10.1007/978-1-4939-9124-2_12.

[33] J.H. Soper, S. Roy, A. Stieber, E. Lee, R.B. Wilson, J.Q. Trojanowski, C.G. Burd, V.M.-Y. Lee, α-Synuclein–induced Aggregation of Cytoplasmic Vesicles in *Saccharomyces cerevisiae*, Mol. Biol. Cell. 19 (2008) 1093–1103. https://doi.org/10.1091/mbc.e07-08-0827.

[34] F. D'Angelo, H. Vignaud, J. Di Martino, B. Salin, A. Devin, C. Cullin, C. Marchal, A yeast model for amyloid- aggregation exemplifies the role of membrane trafficking and PICALM in cytotoxicity, Dis. Model. Mech. 6 (2013) 206–216. https://doi.org/10.1242/dmm.010108.

[35] V. Franssens, E. Boelen, J. Anandhakumar, T. Vanhelmont, S. Büttner, J. Winderickx, Yeast unfolds the road map toward α-synuclein-induced cell death, Cell Death Differ. 17 (2010) 746–753. https://doi.org/10.1038/cdd.2009.203.

[36] S. Alberti, R. Halfmann, S. Lindquist, Biochemical, Cell Biological, and Genetic Assays to Analyze Amyloid and Prion Aggregation in Yeast, in: 2010: pp. 709–734. https://doi.org/10.1016/S0076-6879(10)70030-6.

[37] D. Laor, D. Sade, S. Shaham-Niv, D. Zaguri, M. Gartner, V. Basavalingappa, A. Raveh, E. Pichinuk, H. Engel, K. Iwasaki, T. Yamamoto, H. Noothalapati, E. Gazit, Fibril formation and therapeutic targeting of amyloid-like structures in a yeast model of adenine accumulation, Nat. Commun. 10 (2019) 62. https://doi.org/10.1038/s41467-018-07966-5.

[38] S. Rencus-Lazar, Y. DeRowe, H. Adsi, E. Gazit, D. Laor, Yeast Models for the Study of Amyloid-Associated Disorders and Development of Future Therapy, Front. Mol. Biosci. 6 (2019). https://doi.org/10.3389/fmolb.2019.00015.

[39] T.A. Chernova, Y.O. Chernoff, K.D. Wilkinson, Yeast Models for Amyloids and Prions: Environmental Modulation and Drug Discovery, Molecules. 24 (2019) 3388. https://doi.org/10.3390/molecules24183388.

[40] R.B. Wickner, D. Kryndushkin, F. Shewmaker, R. McGlinchey, H.K. Edskes, Study of Amyloids Using Yeast, in: 2018: pp. 313–339. https://doi.org/10.1007/978-1-4939-7816-8_19.

[41] Z.T. Monahan, S.N. Rhoads, D.S. Yee, F.P. Shewmaker, Yeast Models of Prion-Like Proteins That Cause Amyotrophic Lateral Sclerosis Reveal Pathogenic Mechanisms, Front. Mol. Neurosci. 11 (2018). https://doi.org/10.3389/fnmol.2018.00453.

[42] A.V. Sergeeva, J.V. Sopova, T.A. Belashova, V.A. Siniukova, A.V. Chirinskaite, A.P. Galkin, S.P. Zadorsky, Amyloid properties of the yeast cell wall protein Toh1 and its interaction with prion proteins Rnq1 and Sup35, Prion. 13 (2019) 21–32. https://doi.org/10.1080/19336896.2018.1558763.

[43] D. Kryndushkin, N. Pripuzova, B.G. Burnett, F. Shewmaker, Non-targeted Identification of Prions and Amyloid-forming Proteins from Yeast and Mammalian Cells, J. Biol. Chem. 288 (2013) 27100–27111. https://doi.org/10.1074/jbc.M113.485359.

[44] S. Tenreiro, M.M. Reimão-Pinto, P. Antas, J. Rino, D. Wawrzycka, D. Macedo, R. Rosado-Ramos, T. Amen, M. Waiss, F. Magalhães, A. Gomes, C.N. Santos, D. Kaganovich, T.F. Outeiro, Phosphorylation Modulates Clearance of Alpha-Synuclein Inclusions in a Yeast Model of Parkinson's Disease, PLoS Genet. 10 (2014) e1004302. https://doi.org/10.1371/journal.pgen.1004302.

[45] M. Lindström, B. Liu, Yeast as a Model to Unravel Mechanisms Behind FUS Toxicity in Amyotrophic Lateral Sclerosis, Front. Mol. Neurosci. 11 (2018). https://doi.org/10.3389/fnmol.2018.00218.

[46] Y.O. Chernoff, A. V. Grizel, A.A. Rubel, A.A. Zelinsky, P. Chandramowlishwaran, T.A. Chernova, Application of yeast to studying amyloid and prion diseases, in: 2020: pp. 293–380. https://doi.org/10.1016/bs.adgen.2020.01.002.

[47] A. Villar-Piqué, S. Ventura, Protein aggregation propensity is a crucial determinant of intracellular inclusion formation and quality control degradation, Biochim. Biophys. Acta - Mol. Cell Res. 1833 (2013) 2714–2724. https://doi.org/10.1016/j.bbamcr.2013.06.023.

[48] B. Sampaio-Marques, A. Guedes, I. Vasilevskiy, S. Gonçalves, T.F. Outeiro, J. Winderickx, W.C. Burhans, P. Ludovico, α-Synuclein toxicity in yeast and human cells is caused by cell cycle re-entry and autophagy degradation of ribonucleotide reductase 1, Aging Cell. 18 (2019).

https://doi.org/10.1111/acel.12922.

[49] D.F. Tardiff, V. Khurana, C.Y. Chung, S. Lindquist, From yeast to patient neurons and back again: Powerful new discovery platforms, Mov. Disord. 29 (2014) 1231–1240. https://doi.org/10.1002/mds.25989.

[50] B. Popova, A. Kleinknecht, P. Arendarski, J. Mischke, D. Wang, G.H. Braus, Sumoylation Protects Against β-Synuclein Toxicity in Yeast, Front. Mol. Neurosci. 11 (2018). https://doi.org/10.3389/fnmol.2018.00094.

[51] F. Rueda, B. Gasser, A. Sánchez-Chardi, M. Roldán, S. Villegas, V. Puxbaum, N. Ferrer-Miralles, U. Unzueta, E. Vázquez, E. Garcia-Fruitós, D. Mattanovich, A. Villaverde, Functional inclusion bodies produced in the yeast Pichia pastoris, Microb. Cell Fact. 15 (2016) 166. https://doi.org/10.1186/s12934-016-0565-9.

[52] M. Jackson, E. Hewitt, Why are Functional Amyloids Non-Toxic in Humans?, Biomolecules. 7 (2017) 71. https://doi.org/10.3390/biom7040071.

[53] S.M. Ulamec, S.E. Radford, Spot the Difference: Function versus Toxicity in Amyloid Fibrils, Trends Biochem. Sci. 45 (2020) 635–636. https://doi.org/10.1016/j.tibs.2020.04.007.

[54] J. Sheng, N.K. Olrichs, B.M. Gadella, D. V. Kaloyanova, J.B. Helms, Regulation of Functional Protein Aggregation by Multiple Factors: Implications for the Amyloidogenic Behavior of the CAP Superfamily Proteins, Int. J. Mol. Sci. 21 (2020) 6530. https://doi.org/10.3390/ijms21186530.

[55] J. Van Galen, B.W.M. Van Balkom, R.L. Serrano, D. Kaloyanova, R. Eerland, E. Stüven, J.B. Helms, Binding of GAPR-1 to negatively charged phospholipid membranes: Unusual binding characteristics to phosphatidylinositol, Mol. Membr. Biol. 27 (2010) 81–91. https://doi.org/10.3109/09687680903507080.

[56] S.M. Butterfield, H.A. Lashuel, Amyloidogenic Protein-Membrane Interactions: Mechanistic Insight from Model Systems, Angew. Chemie Int. Ed. 49 (2010) 5628–5654. https://doi.org/10.1002/anie.200906670.

[57] G. Musteikytė, A.K. Jayaram, C.K. Xu, M. Vendruscolo, G. Krainer, T.P.J. Knowles, Interactions of α-synuclein oligomers with lipid membranes, Biochim. Biophys. Acta - Biomembr. 1863 (2021) 183536. https://doi.org/10.1016/j.bbamem.2020.183536.

[58] S. Banerjee, M. Hashemi, K. Zagorski, Y.L. Lyubchenko, Interaction of Aβ42 with Membranes Triggers the Self-Assembly into Oligomers, Int. J. Mol. Sci. 21 (2020)

1129. https://doi.org/10.3390/ijms21031129.

[59] S. Ambadi Thody, M.K. Mathew, J.B. Udgaonkar, Mechanism of aggregation and membrane interactions of mammalian prion protein, Biochim. Biophys. Acta - Biomembr. 1860 (2018) 1927–1935. https://doi.org/10.1016/j.bbamem.2018.02.031.

[60] A.S. Kurochka, D.A. Yushchenko, P. Bouř, V. V. Shvadchak, Influence of Lipid Membranes on α-Synuclein Aggregation, ACS Chem. Neurosci. 12 (2021) 825–830. https://doi.org/10.1021/acschemneuro.0c00819.

[61] N.P. Alza, P.A. Iglesias González, M.A. Conde, R.M. Uranga, G.A. Salvador, Lipids at the Crossroad of α-Synuclein Function and Dysfunction: Biological and Pathological Implications, Front. Cell. Neurosci. 13 (2019). https://doi.org/10.3389/fncel.2019.00175.

[62] B.O.W. Elenbaas, L. Khemtemourian, J.A. Killian, T. Sinnige, Membrane-Catalyzed Aggregation of Islet Amyloid Polypeptide Is Dominated by Secondary Nucleation, Biochemistry. 61 (2022) 1465–1472. https://doi.org/10.1021/acs.biochem.2c00184.

[63] E. Sparr, S. Linse, Lipid-protein interactions in amyloid formation, Biochim. Biophys. Acta - Proteins Proteomics. 1867 (2019) 455–457. https://doi.org/10.1016/j.bbapap.2019.03.006.

[64] R. Sharon, M.S. Goldberg, I. Bar-Josef, R.A. Betensky, J. Shen, D.J. Selkoe, -Synuclein occurs in lipid-rich high molecular weight complexes, binds fatty acids, and shows homology to the fatty acid-binding proteins, Proc. Natl. Acad. Sci. 98 (2001) 9110–9115. https://doi.org/10.1073/pnas.171300598.

[65] T.F. Outeiro, Yeast Cells Provide Insight into Alpha-Synuclein Biology and Pathobiology, Science (80-.). 302 (2003) 1772–1775. https://doi.org/10.1126/science.1090439.

[66] C. Dixon, N. Mathias, R.M. Zweig, D.A. Davis, D.S. Gross, α-Synuclein Targets the Plasma Membrane via the Secretory Pathway and Induces Toxicity in Yeast, Genetics. 170 (2005) 47–59. https://doi.org/10.1534/genetics.104.035493.

[67] H.B. Eberle, R.L. Serrano, J. Füllekrug, A. Schlosser, W.D. Lehmann, F. Lottspeich, D. Kaloyanova, F.T. Wieland, J.B. Helms, Identification and characterization of a novel human plant pathogenesis-related protein that localizes to lipid-enriched microdomains in the Golgi complex., J. Cell Sci. 115 (2002) 827–38. http://www.ncbi.nlm.nih.gov/pubmed/11865038.

[68] N. K. Olrichs, J. Bernd Helms, Novel insights into the function of the conserved domain of the CAP superfamily of proteins, AIMS Biophys. 3 (2016) 232–246. https://doi.org/10.3934/biophy.2016.2.232.

[69] M.D. Resh, Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins, Biochim. Biophys. Acta - Mol. Cell Res. 1451 (1999) 1–16. https://doi.org/10.1016/S0167-4889(99)00075-0.

[70] T.A. Farazi, G. Waksman, J.I. Gordon, The Biology and Enzymology of Protein N - Myristoylation, J. Biol. Chem. 276 (2001) 39501–39504. https://doi.org/10.1074/jbc.R100042200.

[71] K. Jaqaman, J.A. Ditlev, Biomolecular condensates in membrane receptor signaling, Curr. Opin. Cell Biol. 69 (2021) 48–54. https://doi.org/10.1016/j.ceb.2020.12.006.

[72] S. Alberti, A. Gladfelter, T. Mittag, Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates, Cell. 176 (2019) 419–434. https://doi.org/10.1016/j.cell.2018.12.035.

[73] M. Maywald, L. Rink, Zinc in Human Health and Infectious Diseases, Biomolecules. 12 (2022) 1748. https://doi.org/10.3390/biom12121748.

[74] S. Shoji-Kawata, R. Sumpter, M. Leveno, G.R. Campbell, Z. Zou, L. Kinch, A.D. Wilkins, Q. Sun, K. Pallauf, D. MacDuff, C. Huerta, H.W. Virgin, J. Bernd Helms, R. Eerland, S.A. Tooze, R. Xavier, D.J. Lenschow, A. Yamamoto, D. King, O. Lichtarge, N. V. Grishin, S.A. Spector, D. V. Kaloyanova, B. Levine, Identification of a candidate therapeutic autophagy-inducing peptide, Nature. 494 (2013) 201–206. https://doi.org/10.1038/nature11866.

[75] S. Adi-Harel, S. Erlich, E. Schmukler, S. Cohen-Kedar, O. Segev, L. Mizrachy, J.A. Hirsch, R. Pinkas-Kramarski, Beclin 1 self-association is independent of autophagy induction by amino acid deprivation and rapamycin treatment, J. Cell. Biochem. 110 (2010) 1262–1271. https://doi.org/10.1002/jcb.22642.

[76] M.J. Ranaghan, M.A. Durney, M.F. Mesleh, P.R. McCarren, C.W. Garvie, D.S. Daniels, K.L. Carey, A.P. Skepner, B. Levine, J.R. Perez, The Autophagy-Related Beclin-1 Protein Requires the Coiled-Coil and BARA Domains To Form a Homodimer with Submicromolar Affinity, Biochemistry. 56 (2017) 6639–6651. https://doi.org/10.1021/acs.biochem.7b00936.

[77] Y. Li, Y. Zhao, M. Su, K. Glover, S. Chakravarthy, C.L. Colbert, B. Levine, S.C. Sinha, Structural insights into the interaction of the conserved mammalian proteins

GAPR-1 and Beclin 1, a key autophagy protein, Acta Crystallogr. Sect. D Struct. Biol. 73 (2017) 775–792. https://doi.org/10.1107/S2059798317011822.

[78] B. Ku, J.-S. Woo, C. Liang, K.-H. Lee, J.U. Jung, B.-H. Oh, An insight into the mechanistic role of Beclin 1 and its inhibition by prosurvival Bcl-2 family proteins, Autophagy. 4 (2008) 519–520. https://doi.org/10.4161/auto.5846.

[79] R. Kang, H.J. Zeh, M.T. Lotze, D. Tang, The Beclin 1 network regulates autophagy and apoptosis, Cell Death Differ. 18 (2011) 571–580. https://doi.org/10.1038/cdd.2010.191.

[80] M. Su, Y. Li, S. Wyborny, D. Neau, S. Chakravarthy, B. Levine, C.L. Colbert, S.C. Sinha, BECN2 interacts with ATG14 through a metastable coiled-coil to mediate autophagy, Protein Sci. 26 (2017) 972–984. https://doi.org/10.1002/pro.3140.

[81] P. Zhao, X.-M. Zhou, L.-L. Zhao, A.Y. Cheung, M.-X. Sun, Autophagy-mediated compartmental cytoplasmic deletion is essential for tobacco pollen germination and male fertility, Autophagy. 16 (2020) 2180–2192. https://doi.org/10.1080/15548627.2020.1719722.

[82] T.K. Kerppola, Bimolecular Fluorescence Complementation (BiFC) Analysis as a Probe of Protein Interactions in Living Cells, Annu. Rev. Biophys. 37 (2008) 465–487. https://doi.org/10.1146/annurev.biophys.37.032807.125842.

[83] T.K. Kerppola, Visualization of molecular interactions by fluorescence complementation, Nat. Rev. Mol. Cell Biol. 7 (2006) 449–456. https://doi.org/10.1038/nrm1929.

[84] K. Ohashi, K. Mizuno, A novel pair of split venus fragments to detect protein-protein interactions by in vitro and in vivo bimolecular fluorescence complementation assays, Methods Mol. Biol. 1174 (2014) 247–262. https://doi.org/10.1007/978-1-4939-0944-5_17.

[85] L.-N. Schaffert, W.G. Carter, Do Post-Translational Modifications Influence Protein Aggregation in Neurodegenerative Diseases: A Systematic Review, Brain Sci. 10 (2020) 232. https://doi.org/10.3390/brainsci10040232.

[86] S. Sridharan, A. Hernandez-Armendariz, N. Kurzawa, C.M. Potel, D. Memon, P. Beltrao, M. Bantscheff, W. Huber, S. Cuylen-Haering, M.M. Savitski, Systematic discovery of biomolecular condensate-specific protein phosphorylation, Nat. Chem. Biol. 18 (2022) 1104–1114. https://doi.org/10.1038/s41589-022-01062-y.

[87] A. Bah, R.M. Vernon, Z. Siddiqui, M. Krzeminski, R. Muhandiram, C. Zhao, N. Sonenberg, L.E. Kay, J.D. Forman-Kay, Folding of an intrinsically disordered protein by phosphorylation as a regulatory switch, Nature. 519 (2015) 106–109. https://doi.org/10.1038/nature13999.

[88] Z. Monahan, V.H. Ryan, A.M. Janke, K.A. Burke, S.N. Rhoads, G.H. Zerze, R. O'Meally, G.L. Dignon, A.E. Conicella, W. Zheng, R.B. Best, R.N. Cole, J. Mittal, F. Shewmaker, N.L. Fawzi, Phosphorylation of the <scp>FUS</scp> low-complexity domain disrupts phase separation, aggregation, and toxicity, EMBO J. 36 (2017) 2951–2967. https://doi.org/10.15252/embj.201696394.

[89] B. Tsang, J. Arsenault, R.M. Vernon, H. Lin, N. Sonenberg, L.-Y. Wang, A. Bah, J.D. Forman-Kay, Phosphoregulated FMRP phase separation models activity-dependent translation through bidirectional control of mRNA granule formation, Proc. Natl. Acad. Sci. 116 (2019) 4218–4227. https://doi.org/10.1073/pnas.1814385116.

[90] N.K. Olrichs, A.K. Mahalka, D. Kaloyanova, P.K. Kinnunen, J. Bernd Helms, Golgi-Associated plant Pathogenesis Related protein 1 (GAPR-1) forms amyloid-like fibrils by interaction with acidic phospholipids and inhibits Aβ aggregation, Amyloid. 21 (2014) 88–96. https://doi.org/10.3109/13506129.2014.882304.

[91] B. Ren, Y. Zhang, M. Zhang, Y. Liu, D. Zhang, X. Gong, Z. Feng, J. Tang, Y. Chang, J. Zheng, Fundamentals of cross-seeding of amyloid proteins: an introduction, J. Mater. Chem. B. 7 (2019) 7267–7282. https://doi.org/10.1039/C9TB01871A.

[92] J. Wagner, K. Degenhardt, M. Veit, N. Louros, K. Konstantoulea, A. Skodras, K. Wild, P. Liu, U. Obermüller, V. Bansal, A. Dalmia, L.M. Häsler, M. Lambert, M. De Vleeschouwer, H.A. Davies, J. Madine, D. Kronenberg-Versteeg, R. Feederle, D. Del Turco, K.P.R. Nilsson, T. Lashley, T. Deller, M. Gearing, L.C. Walker, P. Heutink, F. Rousseau, J. Schymkowitz, M. Jucker, J.J. Neher, Medin co-aggregates with vascular amyloid-β in Alzheimer's disease, Nature. 612 (2022) 123–131. https://doi.org/10.1038/s41586-022-05440-3.

[93] U. Nowicka, P. Chroscicki, K. Stroobants, M. Sladowska, M. Turek, B. Uszczynska-Ratajczak, R. Kundra, T. Goral, M. Perni, C.M. Dobson, M. Vendruscolo, A. Chacinska, Cytosolic aggregation of mitochondrial proteins disrupts cellular homeostasis by stimulating the aggregation of other proteins, Elife. 10 (2021). https://doi.org/10.7554/eLife.65484.

[94] N. Louros, M. Ramakers, E. Michiels, K. Konstantoulea, C. Morelli, T. Garcia, N. Moonen, S. D'Haeyer, V. Goossens, D.R. Thal, D. Audenaert, F. Rousseau, J.

Schymkowitz, Mapping the sequence specificity of heterotypic amyloid interactions enables the identification of aggregation modifiers, Nat. Commun. 13 (2022) 1351. https://doi.org/10.1038/s41467-022-28955-9.

[95] M.I. Ivanova, Y. Lin, Y.-H. Lee, J. Zheng, A. Ramamoorthy, Biophysical processes underlying cross-seeding in amyloid aggregation and implications in amyloid pathology, Biophys. Chem. 269 (2021) 106507. https://doi.org/10.1016/j.bpc.2020.106507.

[96] S. Subedi, S. Sasidharan, N. Nag, P. Saudagar, T. Tripathi, Amyloid Cross-Seeding: Mechanism, Implication, and Inhibition, Molecules. 27 (2022) 1776. https://doi.org/10.3390/molecules27061776.

[97] K. Konstantoulea, P. Guerreiro, M. Ramakers, N. Louros, L.D. Aubrey, B. Houben, E. Michiels, M. De Vleeschouwer, Y. Lampi, L.F. Ribeiro, J. de Wit, W. Xue, J. Schymkowitz, F. Rousseau, Heterotypic Amyloid β interactions facilitate amyloid assembly and modify amyloid structure, EMBO J. 41 (2022). https://doi.org/10.15252/embj.2021108591.

[98] K.H. Shim, M.J. Kang, Y.C. Youn, S.S.A. An, S. Kim, Alpha-synuclein: a pathological factor with Aβ and tau and biomarker in Alzheimer's disease, Alzheimers. Res. Ther. 14 (2022) 201. https://doi.org/10.1186/s13195-022-01150-0.

[99] K. Konstantoulea, N. Louros, F. Rousseau, J. Schymkowitz, Heterotypic interactions in amyloid function and disease, FEBS J. 289 (2022) 2025–2046. https://doi.org/10.1111/febs.15719.

[100] K. Hartman, J.R. Brender, K. Monde, A. Ono, M.L. Evans, N. Popovych, M.R. Chapman, A. Ramamoorthy, Bacterial curli protein promotes the conversion of PAP 248-286 into the amyloid SEVI: cross-seeding of dissimilar amyloid sequences, PeerJ. 1 (2013) e5. https://doi.org/10.7717/peerj.5.

[101] X. Niu, L. Zhang, Y. Wu, Z. Zong, B. Wang, J. Liu, L. Zhang, F. Zhou, Biomolecular condensates: Formation mechanisms, biological functions, and therapeutic targets, MedComm. 4 (2023). https://doi.org/10.1002/mco2.223.

English summary

Golgi-associated plant pathogenesis-related protein 1 (GAPR-1) is a peripheral membrane protein located on the cytosolic leaflet of the Golgi apparatus in mammalian cells. GAPR-1 belongs to the CAP (cysteine-rich secretory proteins, antigen 5, pathogenesis-related-1) superfamily of proteins, harboring predicted amyloidogenic regions, especially in the CAP1 and CAP2 motifs. The presence of these predicted amyloidogenic regions within the CAP domain implies that the amyloid-like aggregation pathway might be a shared characteristic of the CAP superfamily. Consistent with this, GAPR-1 has been shown to interact with an amyloid oligomer-specific antibody and form amyloid-like fibrils *in vitro*.

In recent years, *Saccharomyces cerevisiae* has become a favored model organism for exploring reversible protein aggregation, including amyloid-like aggregation. A recent trend involves the development of yeast cell-based assays, or "humanized yeast systems," enabling the study of human protein functions in yeast. The induction of phase-separated protein droplets (condensates) in yeast by overexpressing amyloid-prone proteins provides insight into the mechanisms of protein phase separation and its potential link to the formation of functional/pathological amyloids in the human body.

Chapter 2 of the thesis delves into the amyloidogenic properties of GAPR-1 in *S. cerevisiae.* The study illustrates that GAPR-1-GFP expression meets the criteria of a protein with amyloidogenic properties, leading to the generation of SDS-resistant aggregates, heightened ThT fluorescence, and the formation of reversible intracellular protein condensates. Importantly, this behavior resembles functional amyloids rather than pathological amyloids, as GAPR-1 expression does not induce toxicity in yeast cells. The chapter investigates factors influencing GAPR-1 oligomerization in yeast, particularly the role of lipid bilayers and metal ions. Membrane interactions, identified as catalytic sites for amyloidogenic proteins, are explored in the context of GAPR-1's binding to Golgi membranes. The presence of a myristoyl group, while not essential for *in vitro* fibril formation, influences membrane interaction efficiency. The study reveals that Δmyr-GAPR-1-GFP, lacking the myristoyl group, also forms protein condensates in yeast, albeit with reduced efficiency. Metal ions, specifically zinc, are investigated for their role in GAPR-1 oligomerization. The study shows that the conserved metal-binding site in GAPR-1, involving His54 and His103, participates in Zn^{2+}-dependent amyloid-like aggregation *in vitro*. Mutations in this site enhance condensate formation in yeast, indicating a role in preventing uncontrolled protein sequestration. The addition of Zn^{2+} enhances condensate formation and reduces GAPR-1 protein degradation, suggesting the stabilization of GAPR-1 in condensates.

Chapter 3 explores the role of GAPR-1 as a negative regulator of autophagy and its potential interaction with Beclin 1, a key initiator of autophagy. Building on previous findings, the study suggests that the oligomerization of GAPR-1 may be instrumental in its interaction with components of the autophagic machinery, thereby influencing the regulation of autophagy. The investigation extends to Beclin 1 and its ability to form amyloids. Expression of Beclin 1-mCherry in *S. cerevisiae* leads to the formation of protein condensates and elevated ThT fluorescence levels. The study identifies predicted amyloidogenic regions within Beclin 1 that contribute to its condensate formation upon overexpression. An 18-amino acid region (B18 peptide) within the ECD domain is confirmed as an amyloidogenic region, although its deletion does not prevent condensate formation, indicating the involvement of other predicted amyloidogenic regions in Beclin 1. The chapter establishes a direct interaction between GAPR-1 and Beclin 1 using different methods, mapping the interaction site on Beclin 1 to amino acids 267–284 (corresponding to the B18 peptide). Co-expression of GAPR-1 and Beclin 1 influences the condensate behavior of both proteins. Deletion of the interaction region in Beclin 1 does not prevent condensate formation, but it abolishes the interaction with GAPR-1. This suggests that the amyloidogenic properties are involved in the oligomeric interactions between GAPR-1 and Beclin 1. The proposed mechanism indicates that the interaction between GAPR-1 and Beclin 1 in mammalian cells is based on their amyloidogenic properties during the oligomerization process before the formation of amyloid-like fibrils.

Chapter 4 investigates the role of phosphorylation in regulating the oligomeric states of GAPR-1 and Beclin 1 in a yeast model system, with a focus on understanding how phosphorylation influences their interaction. Post-translational modifications, particularly phosphorylation, are explored for their dynamic impact on protein function, interactions, and localization. Phosphorylation introduces negatively charged phosphate groups, influencing amino acid side chain properties and inducing diverse structural changes within proteins. The study identifies specific phosphorylation sites critical for condensate formation in both GAPR-1 and Beclin 1. Phosphorylation of Ser55 in GAPR-1 and Ser15/Ser30 in Beclin 1 is supposed essential for their respective condensate formation. ULK1/Atg1 kinase is identified as the mediator of phosphorylation at these sites in both proteins. Additionally, phosphorylation at these sites affects the interference with mutual condensate formation upon co-expression. Phospho-mutants of GAPR-1 and Beclin 1 show impaired condensate formation, indicating the regulatory role of phosphorylation in their amyloidogenic properties. The deletion of ULK1/Atg1 kinase abolishes the interaction between GAPR-1 and Beclin 1, emphasizing the critical role of this kinase in regulating their interactions. The proposed model suggests that

ULK1/Atg1-mediated phosphorylation of GAPR-1 and Beclin 1 may serve as a negative feedback loop regulating autophagy activation. Phosphorylation of Beclin 1 initiates autophagy priming, and subsequent phosphorylation of GAPR-1 acts as a feedback mechanism to prevent excessive autophagy activation.

Chapter 5 introduces a novel assay system designed to explore protein-protein interactions, based on the formation of biomolecular condensates in *S. cerevisiae*. Expanding on earlier observations of mutual interference in condensate formation during the co-expression of GAPR-1 and Beclin 1, our assay system is extended to investigate various protein-protein interactions. Four distinct phenotypes are determined: Type I) both expressed proteins exhibit condensate formation and co-localize within the same condensates; Type II) both proteins form condensates but in different locations; Type III) both proteins show condensate propensity, but one relocates to the cytosol upon co-expression; Type IV) only one protein forms condensates, causing the other to relocate. Triple-expression experiments introduce a competitor for the protein-protein interaction, enabling to compare how strongly one protein binds to others when expressed together. This innovative assay system offers a versatile platform for investigating diverse protein-protein interactions and their dynamics within cellular condensates. The chapter explores protein-protein interactions involving GAPR-1, extending beyond its known interaction with Beclin 1 and its inhibitory effect on Aβ peptide (1-40) amyloid formation. The investigation reveals that GAPR-1 forms heterotypic interactions not only with Beclin 1 but also with the Aβ peptide (1-40) and Huntington protein. These findings suggest a potential role of GAPR-1 in modulating interactions with other amyloidogenic proteins, with potential implications for cellular processes related to autophagy and protein aggregation.

In conclusion, the **discussion chapter** explores the concept of cross-seeding among amyloid proteins, highlighting the significance of interactions between misfolded proteins with different sequences or structural motifs, leading to hybrid or mixed amyloid structures. This phenomenon, termed heterotypic cross-seeding aggregation, is not exclusive to pathological amyloids but is also observed between functional amyloid peptides. The chapter emphasizes that cross-seeding may not only influence the nucleation of amyloidogenic aggregation but could also be a mechanism driving the spread of amyloidosis across different cells, tissues, and diseases. Understanding cross-seeding and cross-amyloid interactions is positioned as a crucial aspect that may shed light on the mechanisms behind a range of protein misfolding diseases, including functional amyloids. The potential therapeutic opportunities arising from targeting these interactions to modulate the formation and progression of amyloid aggregates are also discussed.

Nederlandse samenvatting

GAPR-1 is een perifeer membraaneiwit dat zich bevindt aan de cytosolische zijde van het Golgi-apparaat in zoogdiercellen. GAPR-1 behoort tot de zgn. CAP eiwit superfamilie, die gekenmerkt wordt door de aanwezigheid van vier CAP (CAP1-CAP4) motieven. Bioinformatica analyses voorspellen dat vooral de CAP1 en CAP2 motieven amyloïde eigenschappen hebben, d.w.z. deze eiwitten de neiging geven tot het vormen van amyloïde eiwit structuren. In overeenstemming hiermee is experimenteel aangetoond dat GAPR-1 bindt aan een antilichaam dat specifiek amyloïde oligomeren herkent en dat het GAPR-1 eiwit onder *in vitro* condities amyloïde-achtige fibrillen vormt. De aanwezigheid van deze amyloïdogene regio's binnen de CAP-domeinen suggereert tevens dat de amyloïde-achtige aggregatie een eigenschap zou kunnen zijn van alle eiwitten in de CAP-superfamilie.

Saccharomyces cerevisiae is een favoriet modelorganisme voor het bestuderen van eiwit aggregatie, waaronder ook amyloïde-achtige aggregatie. Recent zijn "gehumaniseerde gist systemen" ontwikkeld, waarmee de functies van menselijke eiwitten in gist kunnen worden bestudeerd. Overexpressie van amyloïde-gevoelige eiwitten in gist cellen kan fase-scheiding van deze eiwitten induceren waardoor eiwit druppels (condensaten) ontstaan. Dit systeem geeft inzicht in de mechanismen van eiwit fasescheiding en levert daarmee een mogelijke link naar de vorming van functionele/pathologische amyloïden in het menselijk lichaam.

Hoofdstuk 2 van het proefschrift beschrijft de amyloïdogene eigenschappen van het humane eiwit GAPR-1 in *S. cerevisiae*. De studie toont aan dat expressie van GAPR-1-GFP in gistcellen leidt tot de vorming van SDS-bestendige aggregaten, verhoogde ThT-fluorescentie, en de vorming van reversibele intracellulaire eiwit condensaten. Het GAPR-1 eiwit voldoet daarmee aan de criteria voor het hebben van amyloïdogene eigenschappen. De expressie van GAPR-1 induceert geen toxiciteit in gistcellen en lijkt daarmee meer op functionele amyloïden dan op pathologische amyloïden. Het hoofdstuk onderzoekt daarna factoren die de oligomerisatie van GAPR-1 in gist kunnen beïnvloeden, met name de rol van lipide bilagen en metaalionen. Membraan interacties, reeds eerder geïdentificeerd als katalytische oppervlakken voor de vorming van amyloïden door amyloïdogene eiwitten, werden onderzocht in de context van de binding van GAPR-1 aan Golgi-membranen. De aanwezigheid van een myristoyl-groep in GAPR-1, hoewel niet essentieel voor fibrilvorming *in vitro*, beïnvloedde de efficiëntie van de interactie van GAPR-1 met membranen. De studie liet zien dat Δmyr-GAPR-1-GFP, een GAPR-1 eiwit zonder de myristoyl groep, ook eiwit condensaten vormt in gist, zij het met verminderde efficiëntie. Daarnaast werden zink-ionen onderzocht op hun rol in de oligomerisatie van GAPR-1. De studie toont aan dat His^{54} en His^{103}, die deel uitmaken van

de geconserveerde metaal-bindende positie in GAPR-1, deelnemen aan de Zn^{2+} -afhankelijke amyloïde-achtige aggregatie *in vitro*. Mutaties van deze histidines versterkten de vorming van condensaten in gist, wat wijst op een rol van deze histidines bij het voorkomen van ongecontroleerde eiwit condensatie. De toevoeging van Zn^{2+} ionen bevorderde de vorming van condensaten en vermindert de afbraak van GAPR-1-eiwit, wat wederom wijst op de stabilisatie van GAPR-1 in eiwit condensaten.

Hoofdstuk 3 verkent de rol van GAPR-1 en zijn mogelijke interactie met Beclin 1, een belangrijk initiator van autofagie. Expressie van Beclin 1-mCherry in *S. cerevisiae* leidde eveneens tot de vorming van eiwit condensaten en verhoogde ThT-fluorescentie niveaus. Met behulp van bioinformatica analyses werden meerdere amyloïdogene regio's binnen het humane Beclin 1 eiwit geïdentificeerd die mogelijk bijdragen aan de vorming van condensaten bij over-expressie in gist. Een geïdentificeerde regio van 18 aminozuren binnen het ECD-domein van Beclin 1 (het B18 peptide) blijkt inderdaad amyloïdogene eigenschappen te hebben, maar de verwijdering ervan voorkwam niet de vorming van Beclin 1 condensaten, wat wijst op de betrokkenheid van meerdere amyloïdogene regio's in Beclin 1.

Het hoofdstuk bevestigt een directe interactie tussen GAPR-1 en Beclin 1 door middel van co-expressie van GAPR-1 en Beclin 1, dat het condensatiegedrag van beide eiwitten beïnvloedde. De interactie tussen beide eiwitten werd met behulp van verschillende andere methoden bevestigd, waarbij de positie van de interactie op het Beclin 1 eiwit werd geïdentificeerd en bestaat uit de aminozuren 267-284 (overeenkomend met het B18 peptide). Het verwijderen van het interactiegebied in Beclin 1 voorkwam niet de vorming van condensaten, maar verhinderde wel de interactie met GAPR-1. Dit suggereert dat amyloïdogene eigenschappen betrokken zijn bij de oligomere interacties tussen GAPR-1 en Beclin 1. Op basis van deze observaties wordt een moleculair mechanisme beschreven waarbij de oligomere interacties tussen GAPR-1 en Beclin 1 in zoogdiercellen is gebaseerd op hun amyloïdogene eigenschappen. Deze interactie kan essentieel zijn voor de regulatie van autofagie.

Hoofdstuk 4 beschrijft het onderzoek naar de rol van fosforylering bij het reguleren van de oligomere toestanden van GAPR-1 en Beclin 1 in het humane gist model, met een focus op het beïnvloeden van hun interactie. Post-translationele eiwitmodificaties worden veelvuldig onderzocht vanwege hun dynamische impact op eiwitfunctie, interacties en lokalisatie. Fosforylering introduceert negatief geladen fosfaatgroepen, die de eigenschappen van aminozuren beïnvloeden en daarbij diverse structurele veranderingen in eiwitten kunnen induceren. De studie identificeerde specifieke fosforylering plaatsen die essentieel zijn voor condensaat vorming van zowel GAPR-1 als Beclin 1. Fosforylering van Ser^{55} in GAPR-1 en Ser^{15}/Ser^{30} in Beclin 1 bleek essentieel te

zijn voor hun condensaat vorming. Fosfo-mutanten van deze aminozuren in GAPR-1 en Beclin 1 vertoonden verminderde condensaat vorming, wat wijst op de regulerende rol van fosforylering voor hun amyloïdogene eigenschappen. Het eiwit LK1/Atg1 werd geïdentificeerd als de kinase van deze aminozuren in beide eiwitten. Bovendien beïnvloedde de fosforylering op deze plaatsen de interferentie van de wederzijdse condensaatvorming tijdens co-expressie. Het verwijderen van ULK1/Atg1-kinase uit gist cellen verhinderde de interactie tussen GAPR-1 en Beclin 1, wat mogelijk duidt op een cruciale rol van deze kinase bij het reguleren van hun interacties. Op basis van deze observaties wordt een model beschreven dat suggereert dat de fosforylering van GAPR-1 en Beclin 1 door ULK1/Atg1 kan dienen als negatieve feedback die de activering van autofagie reguleert: fosforylering van Beclin 1 initieert autofagie, en de daaropvolgende fosforylering van GAPR-1 fungeert als een terugkoppelingsmechanisme om overmatige activering van autofagie te voorkomen.

Hoofdstuk 5 introduceert een nieuw assay-systeem voor de bestudering van eiwit-eiwit interacties dat gebaseerd zijn op de vorming van biomoleculaire condensaten in *S. cerevisiae*. Voortbordurend op eerdere observaties van wederzijdse interferentie bij de vorming van condensaten tijdens de co-expressie van GAPR-1 en Beclin 1 (hoofdstuk 3), werd het assay-systeem uitgebreid om ook andere eiwit-eiwit interacties te onderzoeken. Vier verschillende fenotypen werden geïdentificeerd: Type I) beide tot expressie gebrachte eiwitten vertonen de neiging tot condensaat vorming en co-lokaliseren in dezelfde condensaten; Type II) beide eiwitten vertonen de neiging tot condensaat vorming maar lokaliseren in verschillende condensaten tijdens co-expressie; Type III) beide eiwitten vertonen de neiging tot condensaat vorming, maar een eiwit verplaatst zich naar het cytosol tijdens co-expressie; Type IV) slechts een eiwit vertoont de neiging tot condensaat vorming, waarheen de ander zich verplaatst tijdens co-expressie.

Tijdens triple-expressie experimenten kan tevens een concurrent voor een eiwit-eiwit interactie geïntroduceerd worden. Hierdoor wordt het mogelijk om de sterkte van eiwit-eiwit interacties te bestuderen in competitie met andere eiwitten. Dit innovatieve assay systeem biedt een veelzijdig platform voor het onderzoeken van diverse eiwit-eiwit interacties en hun dynamiek in cellulaire condensaten. Het hoofdstuk verkende eiwit-eiwit interacties met betrekking tot GAPR-1. De reeds bekende interactie met Beclin 1 werd bevestigd. Het onderzoek onthult tevens dat GAPR-1 hetero-typische interacties vormt met het Aβ-peptide (1-40) en het Huntington-eiwit. Deze bevindingen suggereren een mogelijke rol van GAPR-1 bij het moduleren van interacties met amyloïde-eiwitten, met mogelijke implicaties voor cellulair processen gerelateerd aan autofagie en eiwit aggregatie.

Tot slot verkent het **discussie hoofdstuk** het concept van ‘cross-seeding’ tussen amyloïde eiwitten, waarbij de nadruk wordt gelegd op de betekenis van interacties tussen (gedeeltelijk) verkeerd gevouwen eiwitten, wat kan leiden tot hybride of gemengde amyloïde structuren. Dit fenomeen, ook wel aangeduid als hetero-typische ‘cross-seeding’ aggregatie, beperkt zich niet tot pathologische amyloïden, maar wordt ook waargenomen tussen functionele amyloïden. Het hoofdstuk beschrijft dat ‘cross-seeding’ de nucleatie van eiwit aggregatie van eiwitten met amyloïdogene eigenschappen kan beïnvloeden, maar ook een mechanisme zou kunnen zijn waardoor de verspreiding van amyloïdose over verschillende cellen, weefsels en ziekten gestimuleerd wordt. Het begrijpen van ‘cross-seeding’ en cross-amyloïde interacties wordt beschouwd als een cruciaal aspect dat licht kan werpen op de mechanismen achter een reeks ziekten die veroorzaakt worden door verkeerd vouwen van eiwitten, waaronder functionele amyloïden. De potentiële therapeutische kansen die voortkomen uit het onderzoek naar deze interacties en om de vorming en progressie van amyloïde aggregaten te moduleren, worden ook besproken.

Curriculum Vitae

Nafiseh Sirati was born in Rasht, Iran, on July 2, 1985. After completing high school and participating in the National University Entrance Exam, she was accepted to study Veterinary Medicine at Urmia University in Iran. She successfully obtained her Doctor of Veterinary Medicine degree in May 2011. Following her graduation, she began working as a scientist in a veterinary pharmaceutical company in her hometown of Rasht. After relocating to the Netherlands in 2014, her interest in basic sciences, particularly Biochemistry, led her to join the Cell Biology and Biochemistry department in the Veterinary Medicine Faculty at Utrecht University as a researcher. Subsequently, she pursued a Ph.D. in the Cell Biology, Cancer, and Metabolism department. Her Ph.D. project, supervised by Prof. Dr. J. Bernd Helm and Dr. Dora V. Kaloyanova, also included a period as a guest researcher at Göttingen University in Germany due to collaboration on her project. Her research aimed to expand our understanding of the functional role of the GAPR-1 protein, using Baker's yeast as a model system, which ultimately drew her into the fascinating field of protein condensates. The results from this research are presented in her thesis. As of September 2022, Nafiseh continued her journey in biomolecular condensate research, working as a Postdoc at Schuijers Lab, University Medical Center Utrecht.

List of publications

Tianshu Gui, Cassio Fleming, Caterina Manzato, Benjamin Bourgeois, **Nafiseh Sirati**, Jasper Heuer, lonna Papadionysio, Daniel van Montfort, Merel van Gijzen, Lydia M.M. Smits, Boudewijn M.T. Burgering, Tobias Madl, Jurian Schuijers. Targeted perturbation of signaling-driven condensates. Molecular cell, 2023

Nafiseh Sirati, Ziying Shen, Nick K. Olrichs, Blagovesta Popova, Gerhard H. Braus, Iris C. Verhoek, Ilse M Lagerwaard, Dora V. Kaloyanova, J. Bernd Helms. GAPR-1 interferes with condensate formation of Beclin 1 in *Saccharomyces cerevisiae.* Journal of Molecular Biology, 2022.

Nafiseh Sirati, Blagovesta Popova, Martijn R. Molenaar, Iris C. Verhoek, Gerhard H. Braus, Dora V. Kaloyanova, J. Bernd Helms. Dynamic and reversible aggregation of the human CAP superfamily member GAPR-1 in protein inclusions in *Saccharomyces cerevisiae.* Journal of Molecular Biology, 2021

Nafiseh Sirati, Iris C. Verhoek, Dora V. Kaloyanova, J. Bernd Helms. ULK1/Atg1-mediated phosphorylation regulates the interaction between GAPR-1 and Beclin 1. Submitted for publication.

Nafiseh Sirati, Iris C. Verhoek, Blagovesta Popova, Gerhard H. Braus, Dora V. Kaloyanova, J. Bernd Helms. A novel screening assay using biomolecular condensates in *Saccharomyces cerevisiae* to uncover and characterize amyloidogenic protein-protein interaction. *Identification of novel GAPR-1 interacting proteins.* Ready for submission.

Acknowledgements

Today is the day of writing the note of thanks as the finishing touch of my thesis. What an adventure it has been doing this PhD project! It has been a period of intense learning for me, not only in the scientific arena, but also on a personal level. This would not happen without the positive impression of living in this wonderful country. Thanks to the Netherlands for being such a generous and kind host for me. Living in the Netherlands and doing my PhD at the Utrecht University gave me a fascinating opportunity to make too many new friends who played a significant role in developing my scientific and personal character.

Dear **Bernd**, dear Promoter, I would like to initially express my deep appreciations for all your fruitful inputs, for keeping the PhD project in the correct direction and your never-end support along the PhD adventure. You are the best example of a humble and supportive promoter. Thanks for everything.

Dear **Dora**, dear co-promoter, I was very lucky to be guided by an extraordinary co-supervisor like you. With no doubt I owe the smooth flow of my PhD carrier to you, because of being so precise, supportive and helpful in running the projects forward. You always showed that any single issue and challenge in my PhD carrier or in my personal life is important for you. Thank you for being such a loyal friend outside the serious professional academic life.

Dear **Blaga**, I am so glad that I had the opportunity to know you and work with you in Gottingen university. With your help, I had a chance to expand my network and make friends outside my department. Thanks for all your kind supports.

Dear **Nick**, I will never forget your endless kindness and positive energy. I enjoyed our friendly conversations a lot. Thanks for being such a nice person!

Dear **Jie**, thanks a lot for your endless energy and friendly character and I am so happy of having such a friendly rooom/lab-mate for almost 4 years.

Dear **Maya** and **Iris**, I am very proud and happy of having two of my best friends next to me in my promotion session as my “Paranymphs”. Dear Maya, I know how busy scientific days you had when you accepted to be my Paranymph. It showed me more than ever, your kind heart. Thanks for your unconditional and sincere friendship. Dear **Iris**, thanks for being so helpful, so kind and thanks for all the friendly chats we had together.

Dear **Min** and **Ziying**, you guys are so loyal, generous and supportive. Thanks for all the fascinating moments we had together, thank you for being there when I needed you. **Min**, wishing you lots of success in your new career in UK and **Ziying**, good luck with your PhD life in Utrecht!

Dear **Petra**, I am sure that you are the most warm-hearted person I have met in the Netherlands, thanks for all your supports, helps and friendly attitude.

Dear **Bart** and **Celia**, please accept my deep appreciation for all your helps, supports and friendly chats. Thanks a lot! Dear **Ruud**, **Martijn M** and **Aike**, I do appreciate for all the nice moments we had together, I enjoyed talking with you, learning from you and thanks for your supports. My dear **Alberta** and **Laura**, I know I am such a lucky person for spending the majority of my work time with you guys at B&C. You were the best roommates ever and I enjoyed all the talks about our research, social issues and different cultures. Wishing you all the best.

Heartily, thanks to all my Biochemistry colleagues and friends. Dear **Bas**, **Martin**, **Chris**, **Coos**, **Jeroen**, **Esther.Z**, **Louis, Liz**, **Naomi**, **Ilse**, **Rachid** thanks for the amazing times we had together. My CMC colleagues for all the nice moments, friendly chats and good memories we had together, Dear **Estefenia**, **Xiogang**, **Willem**, **Marca**, **Esther.N**, **Richard**, **Theo**, **Carla, Louise**, **Jillis**, **Esther**, **Ellen**, **Tom**, **Janneke**, **Suzzane**, **Marije**, **Kyra**, **Xinyi**, **Marije.K**, **Martijn**, **Hannah**, **Thi**, **Abbey**, **Demi**, **Maria**, **Anastasia, Jung-Chin** thanks for being so kind and helpful colleagues.

I would like to extend my heartfelt gratitude to all my new friends and colleagues at the UMCU. Dear **Jurian**, **Cassio**, **Caterina**, and **Tianshu**, your support has been invaluable throughout the past year and a half. Here's to many more shared successes and memorable moments together in the future. Dear **Lucie**, **Theo**, and **Mehmet**, I am incredibly grateful to have you as my roommates. Your friendship have added a special dimension to my journey.

Dear **Prof. Fink**, **Saskia** and **Pim**, I will never forget your supports specially in the beginning of my career in the Netherlands. With your help, I had a chance to expand my professional network and make great friends.

Dear **Nasim**, **Naser**, **Shadi, Mahdi**, **Sameh**, **Navid**, **Sara**, **Antoine**, **Farzaneh** and **Hadi** you have showed that true friendship knows no bounds; it is measured by the heart, not miles. I am incredibly grateful for your care and support while I was far from home. I am overjoyed to have established lifelong friendships with all of you.

I was so lucky to have known great Iranian friends who supported me specially in the early days I arrived to The Netherlands. **Sara**, **Ardalan**, **Maryam**, **Mojtaba**, **Kumars**, **Arash**, **Noushin**, **Niloufar**, **Mahsa**, **Hossein**, **Samaneh**, **Naveed**, **Morvarid**, **Ramin**, **Fatemeh**, **Yasaman**, **Hojjat**, **Haniyeh**, **Kamin**, **Elnaz**, **Alireza**, **Kiana**, **Pejman**, **Elnaz** , **Mahdi**, and **Aida**, I truly enjoyed every moment being with you guys. I wish you best of luck I will never forget those wonderful days we spent together. Thanks a lot for all the great times.

Dear **Farshad**, **Samaneh** and **Mazda**; you gusys are awesome. Finding true friends is not always easy, but I and Soheil are so happy that living in the Utrecht, gave us the opportunity to know and build life-long friendship with you.

Dear **Sepideh** and **Saeid**, I am so glad that living in The Netherlands gave me the opportunity to get to know you. Your kind and crystal hearts and trustfulness makes me sure our friendship will continue innthe future.
Dear **Elmira** and **Adel**, having you as friends is an absolute joy. I cherish all the adventures we've shared and the beautiful moments we've created together. Thank you for your unwavering care and the love you constantly spread. I can't wait to meet you again and finally meet my beautiful **Delin** in person.
Dear **Fahimeh** and **Mahdi**, how can I appreciate our years of great friendship. Your calm, friendly and caring personality has always been inspiring for me. I enjoyed every moment of our trips that we had together and all those Persian or international dinner gatherings.
Dear **Hamed** and **Sara**, I always admire your kind and supportive personality. You guys have a solutions for any obstacle that I or Soheil face. Specially during the hard period of time as unexperienced parents, you were there all the time to support us!
Dear **Peyman** and **Arash**; I can write a book if I just want to describe all the nice memories we had in the last 10 years of friendship! How beautiful is that. Thanks for accepting me as a home mate when I joined Soheil in the Netherlands and thanks for the great friendship in all these years! You guys are beyond friends.

Dear dayee **Mohammad**, **Fariba** joon, **Tohid** and **Negar** I wish the world had as many kind people as you. Thanks for all the love and of joy you add to the whole family. Thanks a lot for your hospitality whenever we visited you in Sweden
Special thanks to my wonderful family-in-law: dear **Maman**, **Baba**, **Pooneh** joon, **Amin** agha, **Maman bozorg** and **Parla**ye nazam. I am deeply grateful to have you as my family. Thank you for your endless encouragement, kindness, help, and support. Your presence has enriched my life beyond measure.
To my two amazing sisters, **Ala** and **Maggie**, you are the symbol of kindness and sweetness. Your unwavering care throughout my life has been my anchor, even during the most challenging moments. I can't thank you enough for always being there when I needed you. I love you both dearly. **Milad** joon, your rationality, patience, and kind nature are greatly appreciated. We are incredibly fortunate to have you as a part of our family.
And of course, no achievement would be possible without the presence of such wonderful parents. To my dear **Mahmood**, the best father I could have ever asked for, although I lost you years ago, I've always been thankful for having you in my life. Your strength of character, kindness, and generosity continue to inspire me. To my dear **Mom**, words cannot adequately express how much I love you. Your warm smile, boundless caring, and incredible love have made my life truly amazing. Thank you for your unconditional love **Soudi** joon.

I want to give a big shout-out to my soulmate and best friend, **Soheil**. Without your support, patience, and love, I wouldn't have made it through this adventure. We've tackled plenty of obstacles together, and there are more to come, but I'm grateful for your clear and loving heart. You're the most special person in my life, and I can't wait to see what the future holds for us. Thanks for being such an awesome friend and husband! Finally, to the sunshine of my life, my daily dose of happiness, and the embodiment of unconditional love. **Elara**, your sweet smiles and beautiful soul have been a constant source of inspiration during this challenging journey of completing my thesis. I'm deeply grateful for the love and happiness you bring into our life.

Thank you all for being a part of this incredible journey.

Nafiseh

1.Nov.2023