

Unconventional Protein Functions through Liquid-Liquid Phase Separation in Stress Responses and Aging

Yuan Gao

Department of Chemistry and Molecular Biology

Faculty of Science and Technology

University of Gothenburg



UNIVERSITY OF GOTHENBURG

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Separation in Stress Responses and Aging

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yuan.gao@gu.se

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***If seeing all forms as not forms,
then seeing the Thus Come One.***

若见诸相非相，则见如来。

- *The Diamond Sutra*

- 《金刚经》

ABSTRACT

Just as human society relies on individuals specializing in distinct roles to ensure its proper functioning, a cell depends on organelles to coordinate essential biological processes. Traditionally, organelles have been defined as membrane-bound structures that establish distinct biochemical microenvironments through physical compartmentalization. However, in recent decades, the discovery and growing recognition of membraneless organelles, such as stress granules (SGs) and the pre-autophagosomal structure (PAS), have reshaped our understanding of intracellular organization and biological processes. These dynamic, non-membranous biomolecular condensates maintain their functional integrity through liquid-liquid phase separation (LLPS), a specialized phase transition in which a homogeneous solution spontaneously demixes into two immiscible liquid phases: a dense phase and a dilute phase. This biophysical process is driven by weak, multivalent interactions among macromolecules, such as proteins and nucleic acids. LLPS enables the reversible formation of spatially and functionally distinct compartments, allowing cells to dynamically regulate essential processes such as stress responses and aging, all without the need for lipid membranes. LLPS has thus opened a new dimension for scientific inquiry, enabling researchers to both observe and influence life's fundamental processes.

This thesis provides new perspectives on traditionally well-studied proteins through the lens of LLPS, focusing on stress responses and aging. Specifically, it uncovers new roles for Lsm7 and thioredoxin reductase 1 (Trr1) in stress responses and aging, respectively, mediated through LLPS. For Lsm7, the mechanism of SG initiation via its phase separation, coupled with a conserved signaling pathway identified in this study, provides new insights into SG formation and their involvement in SG-associated human diseases. For Trr1, our findings reveal an unexpected connection between the autophagy process and the antioxidant system, with Trr1's phase separation playing a key role in initiating ER-phagy during aging. These discoveries offer fresh perspectives on aging, age-related diseases, and the regulation of autophagy. Altogether, these findings offer new insights into fundamental biological processes and lay the

groundwork for future research aimed at leveraging LLPS to better understand and potentially manipulate life itself.

Keywords: Liquid-liquid phase separation, Biomolecular condensates, Stress granules, Lsm7, Aging, Autophagy, Trr1.

SAMMANFATTNING

Precis som människosamhället förlitar sig på att individer specialiserar sig på olika roller för att säkerställa dess rätta funktion, är en cell beroende av organeller för att samordna essentiella biologiska processer. Traditionellt har organeller definierats som membranbundna strukturer som skapar distinkta biokemiska mikromiljöer genom fysisk kompartmentalisering. Under de senaste decennierna har dock upptäckten och den växande erkännandet av membranlösa organeller, såsom stressgranuler (SGs) och den pre-autofagosomala strukturen (PAS), omformat vår förståelse av intracellulär organisation och biologiska processer. Dessa dynamiska, icke-membranösa biomolekylära kondensat bibehåller sin funktionella integritet genom vätskefas-separation (LLPS), en specialiserad fastransition där en homogen lösning spontant delvis separeras i två oförenliga vätskefaser: en tät fas och en utspädd fas. Denna biofysiska process drivs av svaga, multivalenta interaktioner mellan makromolekyler, såsom proteiner och nukleinsyror. LLPS möjliggör den reversibla bildningen av rumsligt och funktionellt distinkta kompartment, vilket gör det möjligt för celler att dynamiskt reglera essentiella processer som stressreaktioner och åldrande, allt utan behov av lipidmembran. LLPS har därmed öppnat en ny dimension för vetenskaplig forskning, vilket gör det möjligt för forskare att både observera och påverka livets grundläggande processer.

Denna avhandling ger nya perspektiv på traditionellt välstuderade proteiner genom LLPS, med fokus på stressreaktioner och åldrande. Specifikt avslöjar den nya roller för Lsm7 och tioredoxinreduktas 1 (Trr1) i stressreaktioner respektive åldrande, medierade genom LLPS. För Lsm7 ger mekanismen för SG-initiering via dess fas-separation, tillsammans med en bevarad signalväg som identifierades i denna studie, nya insikter i SG-bildning och deras involvering i SG-associerade människosjukdomar. För Trr1 avslöjar våra resultat en oväntad koppling mellan autofagiprocessen och antioxidationssystemet, där Trr1:s fas-separation spelar en nyckelroll i att initiera ER-fagi under åldrande. Dessa upptäckter erbjuder nya perspektiv på åldrande, åldersrelaterade sjukdomar och reglering av autofagi. Sammanfattningsvis erbjuder dessa resultat nya insikter i grundläggande biologiska processer och lägger grunden

för framtida forskning som syftar till att använda LLPS för att bättre förstå och potentiellt manipulera själva livet.

Nyckelord: Vätske-vätskefas-separation, Biomolekylära kondensat, Stressgranulat, Lsm7, Åldrande, Autofagi, Trr1.

LIST OF PUBLICATIONS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. M. Lindström, L. Chen, S. Jiang, D. Zhang, **Y. Gao**, J. Zheng, X. Hao, X. Yang, A. Kabbinala, J. Thoma, L. C. Metzger, D. Y. Zhang, X. Zhu, H. Liu, C. M. Gustafsson, B. M. Burmann, J. Winderickx, P. Sunnerhagen and B. Liu.
Lsm7 phase-separated condensates trigger stress granule formation.
Nature Communications, 2022.
- II. Lihua Chen*, **Yuan Gao***, Xinxin Hao, Xiaoxue Yang, Michelle Lindström, Shan Jiang, Xiuling Cao, Huisheng Liu, Thomas Nyström, Per Sunnerhagen, Beidong Liu.
Stress granule formation is regulated by signaling machinery involving Sch9/Ypk1, sphingolipids, and Ubi4.
Theranostics, 2025. * Contributed equally.
- III. **Yuan Gao**, Per Sunnerhagen, Beidong Liu.
Thioredoxin Reductase 1 Modulates ER-Phagy in Aging via Liquid–Liquid Phase Separation.
Manuscript, 2025.

Not included in this thesis:

M. Lindström, L. Chen, **Y. Gao** and B. Liu. FUS-induced abnormal protein assembly formation and cell cycle delay in *Saccharomyces cerevisiae*.
Manuscript, 2025

CONTRIBUTION REPORT

PAPER I:

I performed the bioinformatic analysis and western blotting assays, analyzed the corresponding data, and contributed to writing the manuscript.

PAPER II:

I optimized the draft, carried out the majority of experiments and analysis in the revision, and managed the submission and the peer-review process.

PAPER III:

I conceived, designed, and performed all the experiments, analyzed the results, and wrote the manuscript under the supervisions.

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LIST OF ABBREVIATIONS

2-DG	2-deoxy-D-glucose
3D-SIM	Three-dimensional structured illumination microscopy
AIMs	Atg8-interacting motifs
ALS	Amyotrophic lateral sclerosis
CHX	Cycloheximide
DHS	Dihydrosphingosine
DTT	Dithiothreitol
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
FRAP	Fluorescence recovery after photobleaching
FUS	Fused in Sarcoma
IDRs	Intrinsically disordered regions
LCBs	Long-chain base sphingolipids
LLPS	Liquid-liquid phase separation
PAS	Pre-autophagosomal structure
PBs	Processing bodies
PHS	Phytosphingosine
PLA	Proximity ligation assay
PLDs	Prion-like domains
RBPs	RNA-binding proteins
RLS	Yeast replicative lifespan
RNP	Ribonucleoprotein particle

SGs	Stress granules
SPT	Serine palmitoyltransferase
Tm	Tunicamycin
TOR	Target of rapamycin
Trr1	Thioredoxin reductase 1
Trr2	Thioredoxin reductase 2
Trx1	Thioredoxin 1
Trx2	Thioredoxin 2
Trx3	Thioredoxin 3
TXNRD1	Human thioredoxin reductase 1
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system

AIM OF THE THESIS

The emergence and rapid development of liquid-liquid phase separation (LLPS) in biology have introduced a new dimension to observe and potentially influence life's fundamental processes. This thesis aims to provide new insights into traditionally well-characterized proteins, examining their roles within the context of LLPS in stress responses and aging. We uncover a novel role for Lsm7 in the context of LLPS, along with a conserved signaling pathway in which Lsm7 participates to regulate stress granules (SGs) formation (**Paper I and II**). We further reveal a new function of thioredoxin reductase 1 (Trr1) under LLPS during aging, contributing to the process of ER-phagy (**Paper III**).

INTRODUCTION

Liquid-liquid phase separation and Biomolecular condensates

All matter is composed of atoms, the fundamental building blocks of the universe. These atoms interact and bond to form molecules, giving rise to various chemical compounds with unique properties. For instance, a water molecule (H_2O) consists of two hydrogen atoms and one oxygen atom, creating a substance with distinct physical and chemical characteristics^[1]. Regardless of their molecular composition, all materials typically exist in one of three primary phases—solid, liquid, or gas—each defined by the arrangement and movement of their constituent particles^[2].

Phase

A phase is defined as a homogeneous region within a system where both the composition and physical properties remain uniform. In the solid phase, molecules are tightly packed and held together by strong intermolecular forces, giving solids a fixed shape and volume^[3]. In contrast, the liquid phase is characterized by weaker intermolecular forces; while the molecules remain in close proximity, they can move past one another, allowing liquids to maintain a constant volume while conforming to the shape of their container^[2]. In the gas phase, intermolecular forces are negligible, enabling molecules to move freely and disperse to occupy any available space^[1].

Phase transition

In physics, a phase transition refers to the process by which a system undergoes a transformation from one state of matter to another, such as from a solid to a liquid or from a liquid to a gas^[4]. This transformation is typically accompanied by an abrupt change in physical properties such as density, heat capacity, or magnetization when external conditions like temperature or pressure cross a critical threshold^[5].

Liquid-liquid phase separation

Liquid-liquid phase separation (LLPS) is a specialized type of phase transition in which a homogenous molecular solution spontaneously segregates into two distinct liquid phases: a dense phase enriched with specific molecules and a dilute phase in which these molecules are depleted (Figure 1). The interface of the dense droplets serves as a selective boundary, permitting the passage of certain molecules while restricting others, thereby allowing these liquid droplets to function as dynamic compartments within a system^[6].

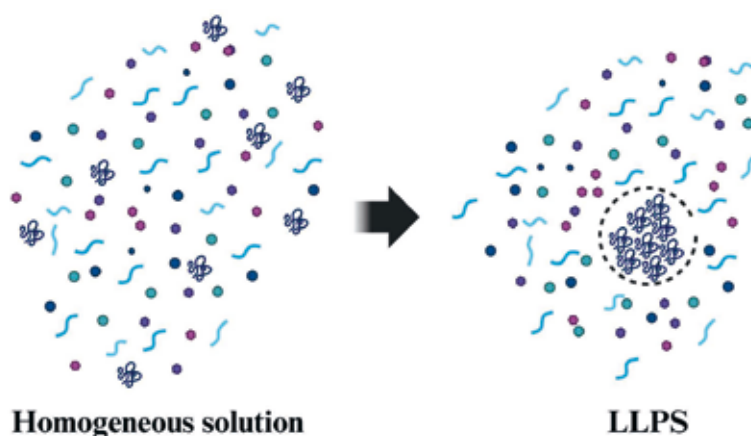


Figure 1. LLPS of a specific molecule from a homogeneous solution. In a homogeneous solution, all components are evenly distributed. Under certain circumstances, a specific molecule may spontaneously undergo LLPS (highlighted by a dashed-line circle), resulting in the formation of a dense phase enriched with this molecule (inside the dashed-line circle) and a dilute phase depleted of it (outside the dashed-line circle). Created with BioRender.com.

LLPS is a widespread phenomenon in soft matter and is commonly observed in systems containing polymers, organic molecules, and proteins. Its behavior is influenced by factors such as temperature, ionic strength, molecular interactions, and the intrinsic properties of its components, for example, proteins enriched in glutamine

(Q) and asparagine (N) repeats within their amino acid sequences^[7]. The growing recognition of LLPS in biological systems has underscored its crucial role in the formation of membrane-less cellular compartments, which facilitate the spatial and temporal coordination of complex biochemical processes^[8].

Biomolecular condensates

In biology, a canonical organelle is traditionally defined as a distinct intracellular compartment, typically enclosed by a lipid bilayer membrane, that is specialized to perform specific cellular functions^[9]. For instance, the nucleus serves as the repository for genetic material and regulates gene expression^[10]; mitochondria generate ATP through oxidative phosphorylation; and the endoplasmic reticulum (ER) is responsible for protein synthesis, folding, and processing^[9]. The presence of lipid membranes in these organelles provides a crucial means of compartmentalization, enabling the precise spatial and temporal regulation of biochemical reactions necessary for maintaining cellular homeostasis^[11]. Meanwhile, an increasing number of cellular compartments lacking a surrounding lipid membrane have been identified^[12]. These compartments, known as membrane-less organelles, exhibit distinct intracellular organization and play essential roles in various biological processes^[13] (Figure 2).

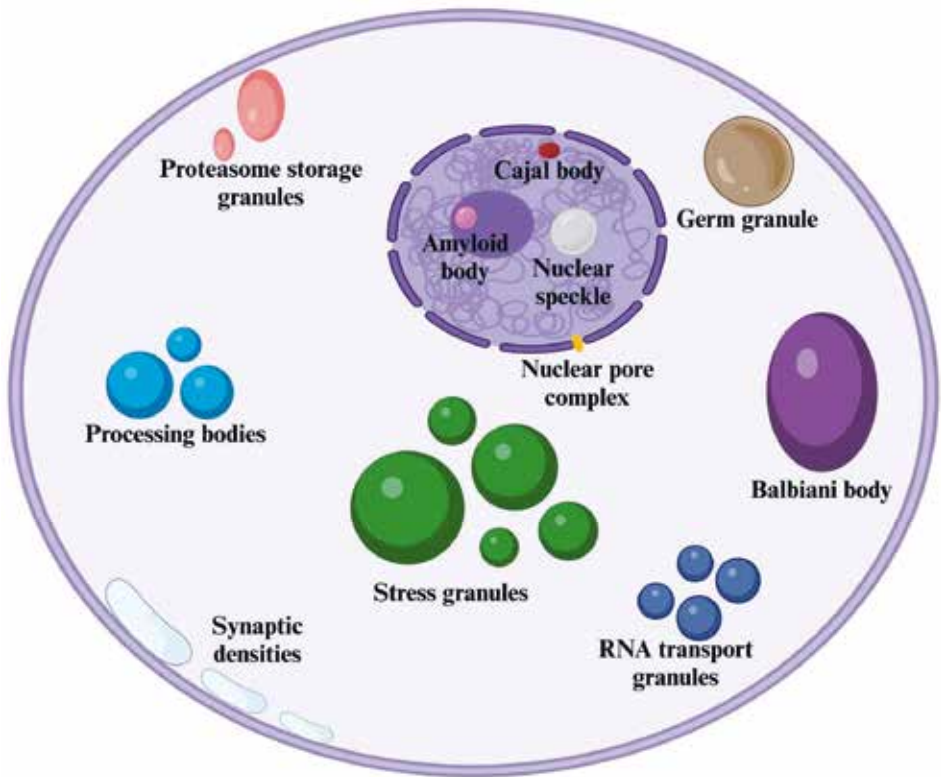


Figure 2. Illustration of diverse membrane-less organelles present in the nucleus, cytoplasm, and membranes of a eukaryotic cell. Certain compartments are exclusive to specific cell types but are included here for completeness. For instance, Balbiani body and germ granule are found in germ cells, while RNA transport granules and postsynaptic densities are unique to neuronal cell types. Created with BioRender.com.

As early as 1889, E. B. Wilson hypothesized that the cytoplasm of a cell might consist of a complex mixture of liquids interspersed with suspended droplets of varying chemical compositions^[14]. In 1999, researchers proposed that high background concentrations of macromolecules can induce macromolecular crowding effects, promoting LLPS in mixtures of two incompatible macromolecules, such as proteins. They further suggested that LLPS is highly likely to occur in the cytoplasm due to its high total macromolecular concentration^[15]. A pivotal breakthrough came in 2009 when

Brangwynne and Hyman demonstrated that P granules in *Caenorhabditis elegans* exhibit liquid-like behaviors, dynamically dissolving and condensing in response to environmental conditions^[16]. This discovery provided the first direct evidence that certain cellular compartments form through LLPS. Since then, numerous membrane-less organelles formed by LLPS have been identified across different organisms and biological processes, expanding our understanding of cellular organization and function^[17-19]. In 2017, these non-membrane-bound compartments were collectively termed biomolecular condensates, highlighting two key characteristics: their ability to concentrate specific molecules and their composition of biological macromolecules, independent of all other structural properties^[20].

Biomolecular condensates are often enriched with multivalent molecules—those that contain multiple functional elements capable of modulating intra- or intermolecular interactions^[21-24]. This multivalency is widely regarded as a critical driving force behind the phase separation of biomolecular condensates^[20, 25, 26]. Proteins with large intrinsically disordered regions (IDRs), which lack a defined three-dimensional structure but often contain repeated sequence elements, form a prominent class of macromolecules capable of phase separation under physiological conditions^[27, 28]. These sequence elements serve as the basis for multivalent, weakly adhesive intermolecular interactions^[29]. Another major class consists of proteins with specific binding motifs, which have been shown to undergo phase separation through multivalent interactions^[30-33].

Over the past decade, the growing identification of biomolecular condensates—such as stress granules (SGs) and the pre-autophagosomal structure (PAS)—has dramatically expanded and deepened our understanding of numerous biological processes^[34, 35]. Despite these advances, many questions remain unanswered, leaving the field ripe with exciting and challenging opportunities for further exploration.

LLPS in cellular stress responses

Cells continuously encounter a wide range of environmental and physiological stresses throughout their lifespan, and their ability to effectively manage these challenges is critical for survival and maintaining proper function^[36-40]. As LLPS has been shown to play a crucial role in various physiological and pathological processes^[17, 41], its involvement in cellular stress responses remains a key area of investigation. One of the best-characterized examples of LLPS-driven structures in stress adaptation is the formation of SGs, which serve as dynamic and protective biomolecular condensates in response to adverse conditions^[34, 42].

Stress granules

Stress granules (SGs) were first identified in 1983 when they were observed in cultures of Peruvian tomato cells exposed to heat shock^[43]. Today, they have been recognized as highly dynamic, membrane-less biomolecular condensates ranging in size from 100 to 2000 nm that form transiently in the cytoplasm of eukaryotic cells through LLPS in response to various stress conditions, including oxidative stress, heat shock, nutrient deprivation, and viral infection^[44, 45]. These condensates serve as critical hubs for cellular adaptation, functioning as triage centers that sequester untranslated mRNAs and RNA-binding proteins (RBPs) to facilitate the reprogramming of gene expression during stress. By selectively pausing the translation of non-essential transcripts and reallocating cellular resources toward stress-response pathways, SGs play a crucial role in promoting cell survival while minimizing potential damage^[42].

SGs exhibit considerable compositional diversity, being enriched in proteins that play key roles in translation regulation, small ribosomal subunits, and non-canonical RNA-binding factors^[46, 47]. Among these, the poly(A)-binding protein, referred to as Pab1 in budding yeast, is a highly conserved RNA-binding protein present across eukaryotic species. It has consistently been observed to localize to SGs in response to a broad spectrum of environmental stressors^[48-50].

In addition to their role in translation regulation, SGs also function as modulators of cellular signaling pathways. For instance, during heat shock in yeast, SGs transiently recruit Kog1 (the yeast homolog of raptor in mammalian cells), a crucial component of the target of rapamycin complex 1 (TORC1). This sequestration delays the reactivation of TORC1 signaling during recovery, thereby safeguarding cells against DNA damage^[51]. Similarly, in mammalian cells, raptor is sequestered into SGs in response to metabolic challenges and redox stress, preventing excessive mTORC1 activation and mitigating apoptosis^[52].

Dysregulation of SGs has emerged as a key factor in the pathogenesis of several neurodegenerative disorders. Under normal conditions, SGs serve as protective structures, shielding cells from acute stress and allowing for rapid recovery once homeostasis is restored^[53]. However, when stress becomes chronic or mutations occur in critical RNA-binding proteins such as TDP-43 and FUS, SGs can become aberrantly persistent. This persistence leads to the formation of stable, insoluble aggregates that interfere with cellular RNA metabolism and proteostasis, thereby contributing to neurotoxicity and the progression of diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD)^[54]. Moreover, post-translational modifications—such as phosphorylation and ubiquitination—appear to further stabilize these pathological assemblies, suggesting that the dynamic equilibrium between SG assembly and disassembly is finely regulated. When this balance is disrupted, it may drive disease pathogenesis^[17, 34].

SGs assembly and disassembly

Diverse cellular and environmental conditions activate the integrated stress response (ISR), a pathway that rapidly reduces overall protein synthesis while selectively sustaining or enhancing the translation of specific transcripts that support adaptive stress responses. The ISR is regulated by various stress-sensing kinases that converge on a common target: phosphorylation of the serine 51 residues on the alpha subunit of eukaryotic initiation factor 2 (eIF2 α). Phosphorylated eIF2 α (eIF2 α -P) inhibits the eIF2B complex, which is essential for recycling eIF2 from its GDP-bound

state to its active GTP-bound form. In its GTP-bound state, eIF2 associates with methionyl-tRNA (Met-tRNA_i) to form the ternary complex (eIF2-GTP-Met-tRNA_i), a key component required for ribosome recruitment to mRNA. However, when eIF2 α is phosphorylated, ternary complex formation is blocked, stalling translation initiation. Without the ternary complex, ribosomes fail to assemble on mRNAs, leading to the accumulation of 48S preinitiation complexes (consisting of the small ribosomal subunit, eIF3, eIF4F, mRNA, and other factors) that become "stuck" at the initiation phase. These stalled 48S complexes dissociate from polysomes, releasing untranslated mRNAs. Through RNA-RNA and RNA-protein interactions, these mRNAs condense into cytoplasmic foci. RNA-binding proteins (RBPs) such as G3BP1, TIA-1, TTP, and PABP recognize and bind these untranslated mRNAs via their IDRs or RNA-binding domains, facilitating the subsequent assembly of SGs^[42, 55, 56].

Not only does the composition of stress granules (SGs) vary, but the exchange dynamics of individual SG components also differ significantly^[57, 58]. The highly concentrated RNA or protein regions, such as Pab1 in yeast and G3BP in mammalian cells, are often referred to as "cores," while the surrounding, less concentrated material is known as the "shell." SGs exhibit a dynamic shell-like structure encasing more stable cores^[47]. Their assembly follows a multistep process, beginning with the stable association of untranslated mRNAs and proteins into core structures. These cores generate high local concentrations that facilitate localized LLPS that driven by IDRs on RBPs. This process nucleates an initial platform that serves for the growth of a more dynamic shell around the cores. Ultimately, individual core-shell assemblies merge to form large, mature stress granules^[59].

Compared to the mechanisms of SGs assembly, the process of SGs disassembly has received less attention^[60]. In contrast to their formation, SGs disassemble when external stress subsides under normal conditions^[42, 59, 61], while dysregulated disassembly of SGs has been observed in neurodegenerative diseases^[62-64]. Similar to assembly, disassembly occurs in a multistep manner, beginning with the dissolution of the unstable shell, followed by the breakdown of the core structure. Shell dissipation likely involves the exchange of weakly associated shell mRNAs and proteins back into

the recovering translational mRNA and protein pool. More stable core assemblies may then be disassembled by ATP-dependent remodeling complexes or autophagy^[59]. Additionally, studies have shown that SG disassembly is facilitated by molecular chaperones such as heat shock protein 70 (Hsp70), which may promote SG disintegration by preventing the accumulation of misfolded proteins within SGs^[50].

Although the precise mechanisms governing SGs formation remain incompletely understood, substantial progress has been made in identifying key factors involved in their assembly. Notably, the ubiquitin-based machinery has been implicated in the regulation of SGs formation^[65, 66]. Moreover, the yeast deubiquitinase Ubp3—and its human ortholog USP10—has been identified as both a component and regulator of SGs^[67-69]. Additionally, it has also been shown that during glucose deprivation in yeast, SGs form after processing bodies (PBs), another class of biomolecular condensates which serve as sites of both RNA degradation and RNA storage^[70]. PBs are constitutively present in unstressed cells, and further expand under stress conditions that inhibit translation initiation^[58, 71]. SGs primarily assemble on pre-existing PBs and rely on them for efficient formation^[72].

Lsm7

The LSm (Like-Sm) proteins are a highly conserved family of RNA-binding proteins found in eukaryotes. They are defined by a conserved Sm-like fold—a compact structure composed of a short N-terminal α -helix followed by a five-stranded antiparallel β -sheet—that is critical for the assembly of ring-shaped complexes^[73]. In eukaryotes, LSm proteins participate in multiple RNA metabolic processes, including pre-mRNA splicing, mRNA decapping, and the stabilization of noncoding RNAs such as U6 small nuclear RNA (snRNA) and various small nucleolar RNAs (snoRNAs)^[74, 75]. These proteins typically assemble into toroidal structures—hexameric or heptameric rings—that bind RNA oligonucleotides within their central channel, thereby influencing the structure, processing, and function of the bound RNA^[76].

Two principal classes of LSm complexes have been described in eukaryotes. The nuclear Lsm2-8 complex is essential for stabilizing U6 snRNA and thereby for spliceosome function. By binding the 3' end of U6 snRNA, this complex protects the RNA from exonucleolytic degradation and facilitates its role in splicing^[77]. In contrast, the cytoplasmic Lsm1-7 complex, in association with the decapping activator Pat1, plays a critical role in mRNA decay by promoting removal of the 5' cap and subsequent 5'-3' degradation^[78].

Lsm7 is a small protein composed of 115 amino acids and serves as a core subunit in both the Lsm1-7 and Lsm2-8 complexes^[78] (Figure 3). Dysregulation in the expression or function of LSm proteins, including Lsm7, has been implicated in a range of human diseases^[79, 80]. Recent research has identified specific variants of Lsm7 that disrupt LSm complex assembly, leading to neurodevelopmental abnormalities and leukodystrophy-like phenotypes in animal models^[81].

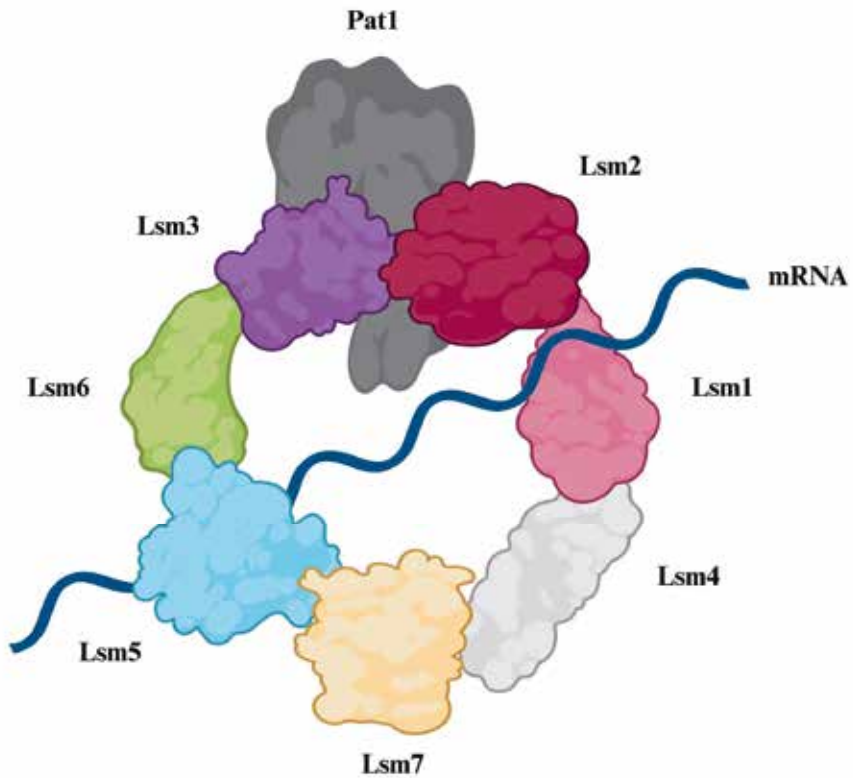


Figure 3. Schematic heptameric ring structure of Lsm1-7/Pat1 complex. The interacting partner Pat1 interacts with Lsm2 and Lsm3. Created with BioRender.com.

Several components of Lsm1-7/Pat1 complex have been shown to co-localize with PBs in yeast (Pat1 and Lsm1)^[82], as well as in human cells (Lsm1 and Lsm4)^[83, 84]. However, it remains unclear whether Lsm7 exhibits similar properties to other LSm proteins that are recruited to PBs or play a role in PBs formation. Furthermore, its potential involvement in the assembly of SGs remains unknown, especially considering the dynamic exchange of mRNAs and proteins between SGs and PBs^[58, 85].

LLPS in aging

Aging is characterized by a gradual decline in physiological function across all somatic cells and is often linked to several key hallmarks, including the loss of proteostasis^[86]. It is widely accepted that aging results from the time-dependent accumulation of cellular damage^[87, 88], which leads to impaired physiological functions and an increased susceptibility to various diseases^[89-91]. Over the past decade, growing evidence has underscored the involvement of LLPS in age-related disorders^[92-94]. For example, aberrant phase transitions in neurons have been implicated in the pathogenesis of ALS^[17]. Recent studies have further elucidated a direct correlation between aging and LLPS^[95-97], leaving this an active and rapidly evolving field of research.

Aging

Humanity's obsession with unraveling the secrets of aging and seeking ways to slow it down—or even achieve immortality—has persisted since the time of ancient alchemists^[98]. In the end, entropy always wins. Every organism harnesses energy from the sun to grow and sustain itself, but only for a limited time. Eventually, breakdown overtakes renewal, leading to aging. Aging is the progressive decline of physiological functions required for survival and reproduction. Unlike age-related diseases such as cancer or neurodegenerative diseases, the characteristics of aging impact all individuals within a species^[99]. However, upon entering modern society, with significant improvements in nutrition level, hygiene condition, and medical treatment, human life expectancy began to rise and continues to increase^[100]. As a consequence, the aging process in the dimension of time has been widely extended, becoming increasingly prevalent and beneficial. Nevertheless, an increased lifespan does not automatically ensure optimal health outcomes. Aging itself has become one of the highest risk factors for human health, with age-related diseases—such as cardiovascular diseases, diabetes, neurodegenerative disorders, and cancer—constituting major challenges to human society^[101]. Estimates suggest that the number of adults over 65 will be over 88.5 million by the year of 2050, inevitably increasing the demand for healthcare providers and hospital systems^[102]. Therefore, getting a better understanding of the process of aging is becoming more and more vital to minimize its negative impact while simultaneously benefiting from the extended lifespan in today's world.

Many theories have been proposed to explain the process of aging, but none of them alone can fully account for all aspects of the phenomenon^[103]. In 2013, nine hallmarks of aging across different organisms were identified. These hallmarks are: loss of proteostasis, deregulated nutrient-sensing, genomic instability, mitochondrial dysfunction, telomere attrition, cellular senescence, epigenetic alterations, stem cell exhaustion, and altered intercellular communication^[86]. With the advancement of related research and science in general, the nine hallmarks of aging were updated in 2023 by adding three additional hallmarks: chronic inflammation, dysbiosis, and disabled macroautophagy. Notably, disabled macroautophagy was initially considered a special case of loss of proteostasis. However, macroautophagy not only affects proteins but also targets entire organelles and non-proteinaceous macromolecules, justifying its classification as a separate hallmark. These hallmarks are categorized into three groups: primary, antagonistic, and integrative^[104].

The primary hallmarks of aging progressively accumulate over time and directly contribute to the aging process. These include genomic instability, epigenetic alterations, telomere attrition, loss of proteostasis, and disabled macroautophagy. The antagonistic hallmarks represent responses to damage and play a more nuanced role in aging, including mitochondrial dysfunction, deregulated nutrient-sensing, and cellular senescence. The integrative hallmarks of aging emerge when the cumulative damage from both primary and antagonistic hallmarks exceeds the body's ability to compensate, leading to stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis^[104] (Figure 4). With the advancement of LLPS-related studies, LLPS has been found to play a cascading role in aging by influencing the formation and progression of the aforementioned aging hallmarks^[105]. One notable example is its involvement in autophagy, a crucial cellular process responsible for maintaining proteostasis and eliminating damaged organelles and macromolecules. LLPS plays a key regulatory role in different stages of autophagy^[106-109].

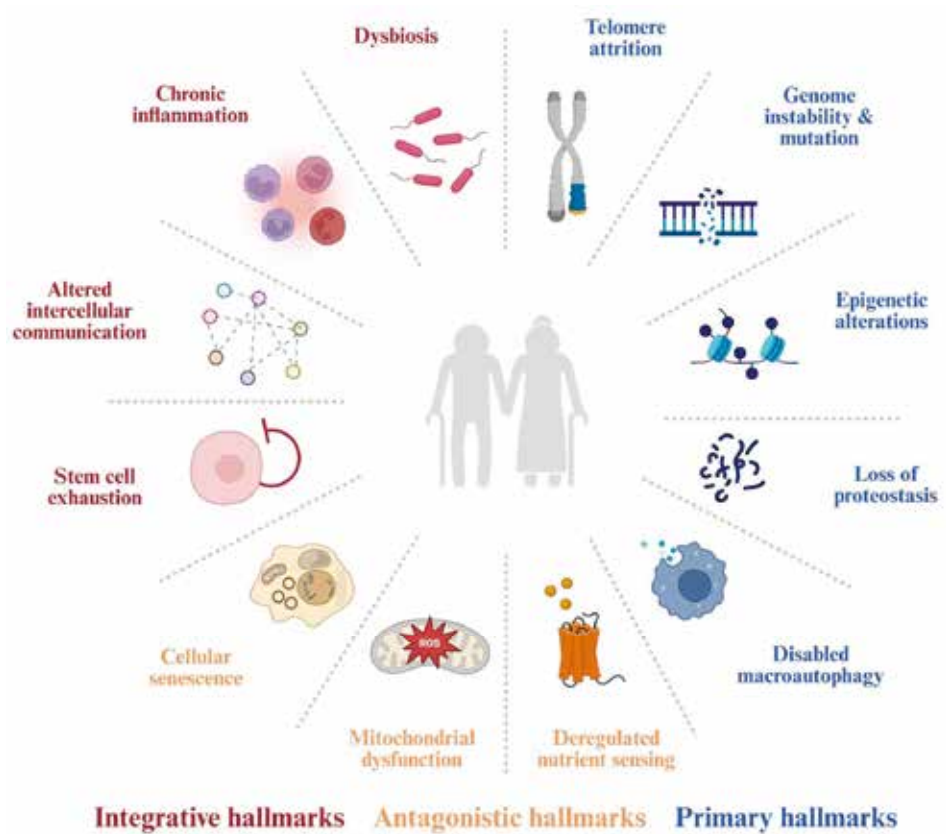


Figure 4. The 12 hallmarks of aging categorized into their respective groups. Created with BioRender.com.

Budding yeast as a model organism to study aging

As a single-celled eukaryote, yeast has proven to be an invaluable model organism for addressing a wide range of challenges in cell biology. This is largely due to the many advantages they offer compared to more complex higher organisms, including a fully sequenced genome, easier genetic editing, simplified experimental manipulation, and lower maintenance, while still sharing significant proteome similarities with human cells^[110, 111]. In addition to many other areas, it has significantly contributed to our

understanding of cell cycle regulation, nutrient signaling, fundamental mechanisms of vesicle trafficking, and autophagy^[112, 113]. It has been nearly four decades since the budding yeast *Saccharomyces cerevisiae* became a key model organism for aging research, and over this time, it has proven to be both a simple and powerful tool in the field^[114]. There are two primary yeast aging assays used: replicative lifespan (RLS) assay and chronological lifespan (CLS) assay^[115] (Figure 5).

The RLS assay takes advantage of the asymmetrical cellular division of the budding yeast, where the larger mother cell produces a smaller daughter cell by budding, to measure the number of times a mother cell can divide. This assay is believed to provide insights into the aging process of individual stem cells in metazoans. The total number of divisions a mother cell can undergo is determined by counting the number of the daughter cells or bud scars produced by a mother cell^[116]. Individual cells do not divide endlessly. Rather, they will stop after a limited number of divisions, typically around 20 to 25 times, and enter a short post-replicative state before undergoing lysis^[117]. The CLS assay was developed to complement the RLS assay, which measures the length of time populations of postmitotic cells remain functional, and assessed through mitotic viability. This assay models the lifespan of nondividing cells in higher organisms^[118-121].

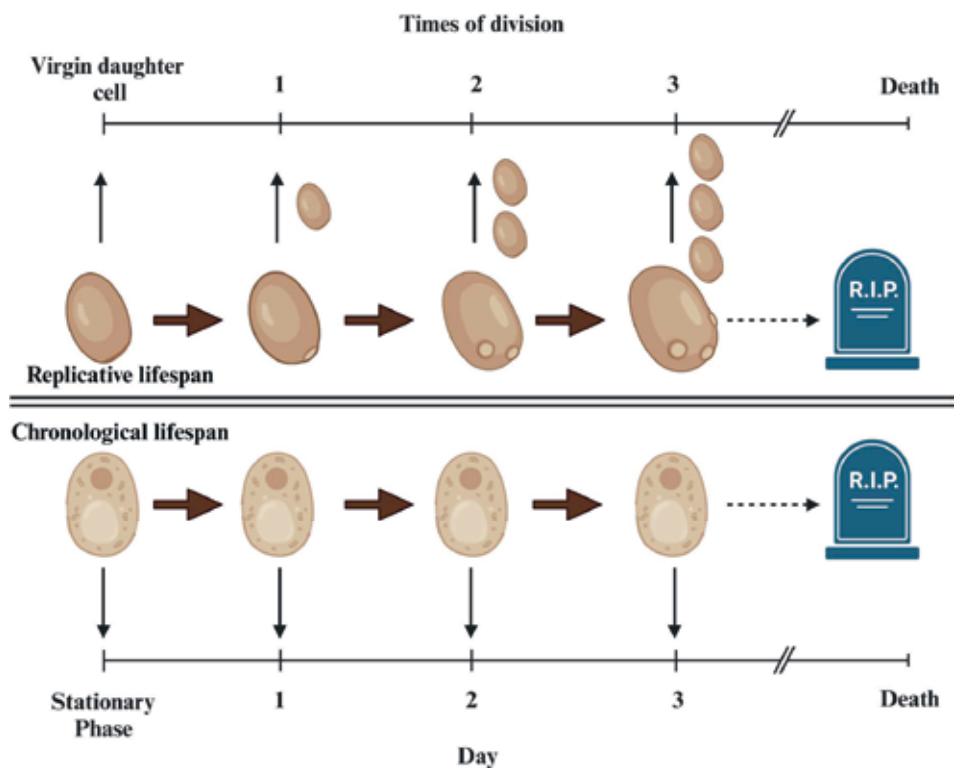


Figure 5. Schematic representation of RLS and CLS in yeast. Created with BioRender.com.

Aging and autophagy

Aging and numerous age-related diseases are closely associated with impaired proteostasis, a condition that results in the accumulation of misfolded, oxidized, glycosylated, or ubiquitinated proteins^[122]. For example, an increase in misfolded proteins within the endoplasmic reticulum (ER) has been observed in aging lung tissue, ultimately triggering prolonged ER stress^[123]. To counteract proteotoxic stress and maintain proteostasis, autophagy—a highly conserved intracellular degradation and recycling process—has been shown to be indispensable^[124, 125]. Meanwhile, disabled

macroautophagy has been shown as a hallmark of aging, contributing to the progressive decline in cellular function observed in aged organisms^[104]. Importantly, autophagy is not only essential for maintaining cellular homeostasis but has also been implicated in lifespan regulation. Studies across a broad range of model organisms, from yeast to great apes, have demonstrated that starvation-induced autophagy plays a crucial role in promoting longevity^[126, 127]. Furthermore, several chemical compounds with lifespan-extending properties, including spermidine, resveratrol, and rapamycin, have been shown to exert their pro-longevity effects through the induction of autophagy^[128-131]. All these findings indicate the central role of autophagy in aging process.

Autophagy (“self-eating” in Greek), was initially termed by Christian de Duve to describe the process that heterogenic intracellular materials delivered to lysosome for digestion In 1963^[132]. This field has been highly explored since the contribution made by Yoshinori Ohsumi, who led a genetic screen to dissect the process of autophagy in yeast, and identified 15 autophagy-related proteins (ATGs) that are essential for autophagic cargo delivery to yeast vacuole (counterpart of lysosome)^[133]. Originally viewed as a fundamental cellular recycling system triggered to counteract starvation by dismantling unnecessary cellular components, autophagy is now better understood to be indispensable for maintaining homeostasis in cells even under nutrient-sufficient conditions^[134]. So far, there are four types of autophagy identified: macroautophagy, microautophagy, chaperone-mediated autophagy (CMA), and crinophagy^[135-137].

Crinophagy is a process where excess secretory granules bypass exocytosis and fuse directly with lysosomes for degradation^[138]. CMA is a proteolytic system that facilitates the degradation of intracellular proteins in lysosomes. During this process, substrate proteins are selectively recognized and transported into the lysosomal lumen through the coordinated action of chaperones on both sides of the membrane, along with a specialized protein translocation complex^[139]. In budding yeast, there are mainly two types of autophagy: macroautophagy and microautophagy^[140]. Microautophagy, involves the direct engulfment of cytoplasmic material by the lysosome or vacuole. This process occurs through random invagination of the lysosomal or vacuolar membrane,

enclosing the cargo for degradation^[141]. Macroautophagy, in contrast, is the most thoroughly researched form of autophagy (will hereafter be referred to as “autophagy” in the text), aimed primarily to eradicate damaged organelles or unneeded proteins^[142]. It involves the formation of a double-membrane sequestering compartment, termed the phagophore, which matures into an autophagosome to encapsulate the organelle designated for degradation. Upon fusing with the vacuole or lysosome, the cargo enclosed within the autophagosome is degraded, and the resulting macromolecules are recycled back into the cytosol for reuse^[143] (Figure 6).

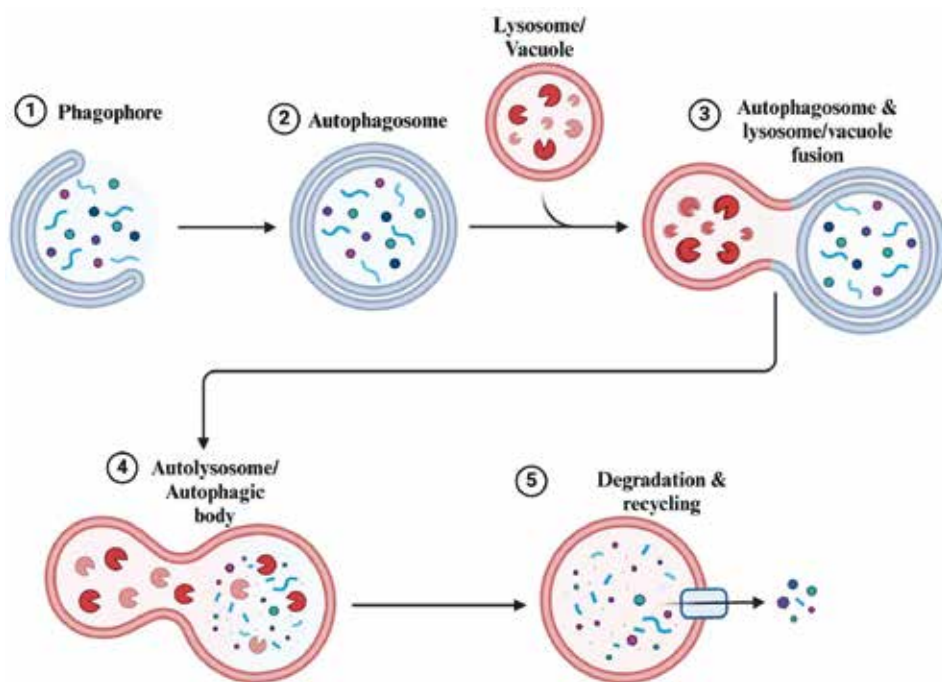


Figure 6. Schematic of autophagy process. Created with BioRender.com.

Autophagy can be further categorized into two forms: bulk autophagy (non-selective) and selective autophagy^[144]. Selective autophagy pathways depend on the involvement of specific autophagy receptors. These pathways are crucial for

maintaining organismal physiology through the regulated and selective degradation of various organelle components, including mitochondria (mitophagy), lipid droplets (lipophagy), lysosomes (lysophagy), peroxisomes (pexophagy), the nucleus (nucleophagy), and the ER (ER-phagy). In selective autophagy, both the binding of soluble autophagy receptors to cargo and membrane-bound receptors activation can trigger the recruitment and activation of the Atg1/ULK kinase complex. This complex then promotes the formation of the phagophore, which forms adjacent to the structure being selectively targeted for degradation, whether it is a macromolecule or part of an organelle^[145, 146]. For instance, during the initiation of ER-phagy, whose receptors are Atg39 (localizes to the perinuclear ER membrane) and Atg40 (localizes to the cortical and cytosolic ER membrane) in budding yeast, these receptors bind to Atg8 through Atg8-interacting motifs (AIMs) and interact with the scaffold protein Atg11. This interaction triggers the activation of the Atg1/ULK kinase complex, which in turn nucleates and expands a double-membrane phagophore to sequester the targeted ER fragment, directing it to the vacuole for degradation^[147, 148].

To date, there are more than 40 ATGs identified in budding yeast, which involve in a variety of bulk and selective autophagic pathways. Among them, around 20 ATGs form a highly conserved core machinery responsible for regulating autophagosome biogenesis across all eukaryotes^[149]. These core ATGs can be divided into six modules based on their functions: 1. the Atg1 complex; 2. the phosphatidylinositol 3-kinase (PI3K) complex; 3. the Atg9-positive vesicles; 4. the Atg2-Atg18 complex; 5. the Atg12 ubiquitin-like conjugation system; 6. the Atg8 ubiquitin-like conjugation system^[149, 150]. The Atg1 complex (comprising Atg1 and Atg13) is a key regulator of autophagosome formation. This complex includes the ternary complex of Atg17, Atg29, and Atg31. The PI3K complex (composed of Atg6, Atg14, Vps15, and Vps34) is a PI3K complex specific to autophagy, responsible for generating Phosphatidylinositol 3-phosphate (PI3P) during the initiation of isolation membrane nucleation. The Atg9 vesicle and the Atg2-Atg18 complex function as lipid transporters, with Atg9 transporting lipids between leaflets and Atg2-Atg18 transporting lipids between membranes, supplying phospholipids from the ER to the isolation membrane. The Atg12 conjugation system (involving Atg5, Atg7, Atg10, Atg12, and Atg16) mediates the covalent attachment of Atg12 to Atg5. Atg12, a ubiquitin-like protein, is activated by Atg7 (an E1-like enzyme),

forms intermediates with Atg7 and Atg10 (E2-like enzymes), and is then conjugated to Atg5. Similarly, the Atg8 conjugation system (involving Atg3, Atg4, Atg7, and Atg8) facilitates the covalent attachment of Atg8 to phosphatidylethanolamine. During this process, the Atg12–Atg5–Atg16 complex acts as an E3-like enzyme, working alongside Atg4 (an Atg8-processing enzyme), Atg7 (an E1-like enzyme), and Atg3 (an E2-like enzyme)^[151, 152].

In yeast, the site where autophagosome formation occurs, is referred to as the pre-autophagosomal structure or the phagophore assembly site (PAS)^[153, 154]. The PAS was initially thought to consist of the Atg1 complex, which associates with the vacuole membrane. This complex is made up of the serine/threonine kinase Atg1, the intrinsically disordered protein Atg13, the scaffold protein Atg17, and two accessory proteins, Atg29 and Atg31^[155]. The Atg1 complex recruits Atg9 vesicles and other downstream autophagy proteins to facilitate the nucleation of the isolation membrane, followed by its expansion and eventual closure to form an autophagosome^[156]. Recent studies revealed that the essence of the PAS is actually a biomolecular condensate formed via LLPS^[35]. Additionally, during selective autophagy, several critical autophagy biogenesis factors form phase-separated initiation hubs on the cargo surface, promoting the assembly of the phagophore^[157]. These findings suggest that precisely regulated LLPS is crucial for the initiation of both bulk and selective autophagy in evolutionarily diverse organisms. Given the strong evidence that the core process of autophagy is relevant to aging^[104], a more comprehensive examination of the interplay among aging, autophagy, and LLPS is needed.

Thioredoxin reductase 1

Free radicals are highly reactive molecules characterized by an unpaired electron in their outer orbital^[158]. Reactive oxygen species (ROS) include both free radical and non-radical oxygen-containing molecules, such as hydrogen peroxide, superoxide, singlet oxygen, and the hydroxyl radical^[159]. ROS are naturally generated as by-products of cellular metabolism, for example, mitochondria are a major source of endogenous ROS because of their central role in oxidative ATP production^[160], and

play essential roles in intracellular signaling and homeostasis^[161]. Elevated ROS levels can disrupt cellular homeostasis, damage cellular structures, impair cellular functions, and lead to oxidative stress. And the disruption of cellular redox balance is a high risk factor for various human pathologies, including cancer, cardiovascular diseases, neurodegenerative disorders, inflammation, and aging.^[162]

The antioxidant machinery consists of enzymes that detoxify ROS, such as catalases, which break down hydrogen peroxide, and superoxide dismutases, which neutralize superoxide radicals, along with systems that regulate cellular redox balance^[163, 164]. There are two major antioxidant systems: one based on the tripeptide glutathione (GSH), and the other on small proteins called thioredoxins. In the cytosol of yeast, two thioredoxins, thioredoxin 1 (Trx1) and thioredoxin 2 (Trx2), work as reducing equivalent carriers to help restore the redox status of different protein disulfide bonds, which are crucial for the function of several enzymes, including ribonucleotide reductase. This system also includes various peroxiredoxins, such as thioredoxin peroxidase, which play a key role in detoxifying peroxides^[165]. The reducing power of the entire system is derived from the electrons originated from NADPH, and the electrons transfer mediated through a pivotal oxidoreductase flavoenzyme thioredoxin reductase (Trr1), which restores the reduced state of both Trx1 and Trx2^[166, 167] (Figure 7).

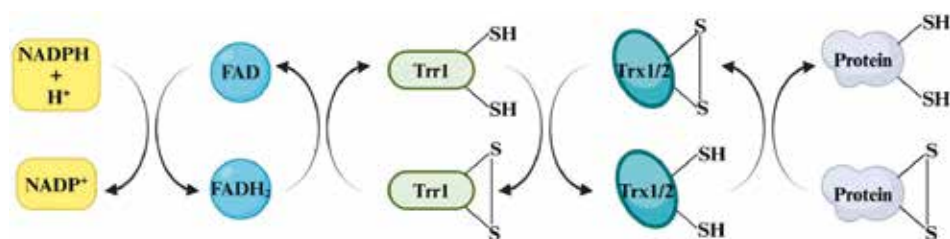


Figure 7. Schematic representation of yeast cytoplasmic thioredoxin system. Created with BioRender.com.

Apart from being a key antioxidant enzyme that regulates cellular redox balance, studies have demonstrated that Trp1 plays an essential role during ER stress^[167, 168]. When exposed to chemical stressors such as dithiothreitol (DTT), oxidative protein folding in the ER is inhibited^[168], leading to the accumulation of misfolded and unfolded proteins in the ER lumen that causes ER dysfunction, which is commonly termed ER stress. In response, the stressed cell will activate various pathways to mitigate ER stress and restore ER function, including the unfolded protein response (UPR) and ER-associated degradation (ERAD)^[169]. Recently, ER-phagy has also been identified as a mechanism that helps maintain ER homeostasis by eliminating damaged ER components, thereby protecting cells from the harmful effects of excessive ER stress^[170-172] (Figure 8). In yeasts lacking Trp1, in addition to being more vulnerable to DTT treatment^[168], the UPR is constitutively activated, indicating an elevated level of protein misfolding and a persistent ER stress condition^[167].

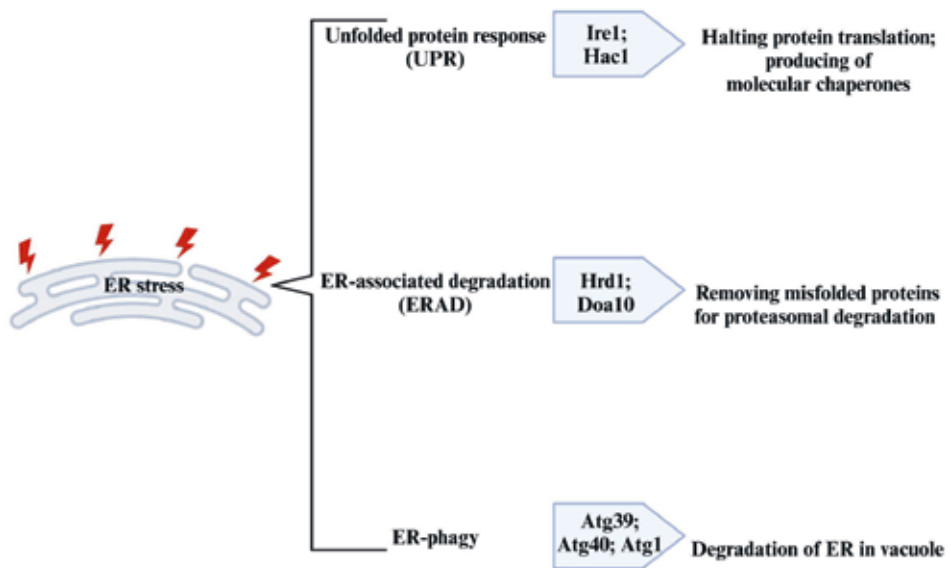


Figure 8. Schematic representation of cellular responses to ER stress in yeast. Created with BioRender.com.

The deletion of the *TRR1* gene has also been shown to lead to a 54% reduction in RLS, whereas the deletion of *TRX1* or *TRX2* does not appear to have any significant effect on RLS ^[173]. As a comparison, yeasts lacking Sir2, a key regulator of yeast longevity, exhibit an approximately 50% decrease in RLS^[174]. Interestingly, while the loss of Trr1 impairs oxidative stress tolerance as anticipated, the cells initiate a compensatory mechanism, leading to an increase in catalase and glutathione levels. Moreover, the overall redox status in cells during the exponential growth phase remains unchanged compared to that of the wild type. At the same time, the induction of autophagy response triggered by rapamycin or nitrogen depletion is dramatically dropped^[166].

Splice variants of Trr1's human homolog, thioredoxin reductase 1 (TXNRD1), have been reported to exhibit dynamics mobility^[175, 176]. Meanwhile, recent studies suggest that TXNRD1 regulates tissue aging independently of its enzymatic activity^[177]. Together, these findings suggest a potential yet-to-be-revealed role for Trr1.

MAIN FINDINGS

PAPER I

Stress granules (SGs) are biomolecular condensates that generated in response to cellular stress and play a critical role in managing stress at the cellular level. The process of liquid-liquid phase separation (LLPS) has been implicated in the biogenesis of these membrane-less organelles. Meanwhile, the precise molecular mechanisms LLPS regulating SGs initiation remain incompletely understood. In this study, we applied a genome-wide, imaging-based screen to identify proteins that colocalize with SGs (Pab1 as a marker) under conditions of 2-deoxy-D-glucose (2-DG, a glycolysis inhibitor) stress. Follow-up investigations on one of the identified SGs-associated proteins, Lsm7, revealed a novel mechanism essential for SGs assembly.

1. The genome-wide imaging-based screen identified RNA-binding protein Lsm7, which had not previously been shown to be required for SG formation, to be critical for the promotion of SGs formation, which is partially independent of its role in processing bodies (PBs).
2. The capacity of Lsm7 to undergo LLPS and form dynamic condensates, driven by the intrinsically disordered regions (IDRs) and hydrophobic clusters within the Lsm7 amino acid sequence, is essential for its function in SGs assembly.
3. Lsm7 condensates appear to act as nucleating scaffolds, facilitating the demixing of Pab1 ("cores" of SGs) and subsequent SGs formation, with this process likely mediated through RNA interactions.

Conclusion: Our findings build a novel model for SGs assembly with the LLPS of Lsm7 serving as a scaffolding structure that seeds SGs initiation likely through modulating RNA interactions.

PAPER II

In **Paper I**, we have demonstrated that Lsm7 plays an important role in the initiation of stress granules (SGs) through Liquid-liquid phase separation (LLPS), which is at least partially independent of its conventional role. At the same time, studies have suggested that several signaling components, RNA-binding proteins, and regulatory factors contribute to SGs initiation. Yet, the potential interactions and crosstalk among the above remain poorly understood. To address this gap, we aimed to identify the possible upstream signaling pathway that activates Lsm7's phase separation, thereby promoting subsequent SGs formation upon 2-deoxy-D-glucose (2-DG) stress. Two genome-wide, imaging-based screens were conducted to uncover genes involved in the regulation of SGs formation. These identified candidates facilitated the discovery of upstream signaling components that form a potential signaling pathway involved in regulating SGs formation.

1. The TORC1/2 complex, through the activation of kinases Sch9 and Ypk1, results in a reduction in the levels of long-chain base (LCB) sphingolipids.
2. The decrease in LCBs triggers the de-repression of the deubiquitinase Ubp3, leading to the subsequent downregulation of the ubiquitin gene UBI4.
3. Further investigation revealed that Ubp3 positively regulates Lsm7's phase separation by suppressing Ubi4, which in turn promotes SGs formation.
4. The signaling pathway addressed is conserved in mammalian cells as well.

Conclusion: This study identifies a conserved regulatory signaling pathway involved in SGs formation under 2-DG stress, which is characterized by the interplay among Sch9/Ypk1, LCBs, Ubp3, Ubi4, and Lsm7.

PAPER III

Aging is associated with various deterioration of biological processes, including disrupted proteostasis and impaired autophagy, which leads to the accumulation of damaged proteins and cellular dysfunction. Autophagy, a highly conserved intracellular degradation and recycling pathway, plays a crucial role in maintaining proteostasis. Multiple anti-aging pathways have been shown to extend lifespan by promoting autophagy. Recent studies have highlighted the involvement of LLPS in both aging and autophagy, suggesting that phase separation may serve as a mechanistic link between these processes. In this study, we identified Trr1 as a key player that phase separates to exert unconventional function in the above interaction, demonstrating its essential role in bridging autophagy and aging through LLPS.

1. Trr1 forms dynamic condensates through LLPS during yeast replicative aging, with an increasing propensity for phase separation as cells age.
2. The formation of Trr1 phase-separated condensates is driven by the accumulation of endoplasmic reticulum (ER) stress during aging, rather than by oxidative stress, which is traditionally associated with its antioxidant function.
3. Trr1-containing condensates participate ER-phagy to relieve ER stress during aging by joining the pre-autophagosomal structure (PAS) to promote the development of autophagosome through affecting the lipidation of Atg8.
4. Optogenetic analysis reveals that the physical states of Trr1 condensates can affect cellular fitness.

Conclusion: This study uncovers a novel function of the classic antioxidant enzyme Trr1 through its LLPS during aging, which plays a crucial role in regulating ER-phagy to ensure a normally aged lifespan.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

With the definition of biomolecular condensates and the discovery of liquid-liquid phase separation (LLPS) in various physiological and pathological processes in this century, a new chapter in biology is gradually revealed. LLPS offers a novel perspective on understanding life and even intervening in biological processes. More than just a conceptual breakthrough, LLPS-based research and therapeutic strategies targeting human diseases are rapidly advancing. For example, β -catenin condensate-perturbing peptides have been identified as potential treatments for Wnt signaling-related cancers^[178, 179]. In such an encouraging and highly developing field, opportunities and challenges are co-existing.

The work included in this thesis presents novel perspectives on traditionally well-studied proteins through the lens of LLPS with a focus on stress responses and aging.

In **Paper I**, through an imaging-based phenomic screen searching for proteins co-localizing with Pab1 (Marker of SGs) under 2-deoxy-D-glucose (2-DG, a glycolysis inhibitor) induced stress conditions, we identified previously unreported proteins that form co-localizing foci with SGs (Pab1). Among these candidates, the highly conserved protein Lsm7 plays an important role in the formation of SGs, which is exerted through the formation of phase-separated condensates that further promote Pab1 nucleation and subsequent SGs assembly. We also identified that the intrinsically disordered regions (IDRs) and the hydrophobic clusters within the Lsm7 amino acid sequence are the driving forces to mediate its LLPS. We further found that Lsm7 can rescue the demixing of Pab1 condensates from the presence of RNA. Last but not the least, the role of Lsm7 in SGs formation we elucidated here, is partially independent of its role in processing bodies (PBs). Notably, PBs do not require Lsm7 for its condensate assembly. These findings collectively propose a novel model that Lsm7 phase-separated condensates work as seeding scaffolds to promote Pab1 demixing and subsequent SGs initiation, a process likely regulated through RNA interactions. Apart from presenting an alternative mechanism for SG initiation compared to the “Cores First” models^[34, 42, 180], our study also reveals an unconventional role of Lsm7 in the

situation of LLPS. Meanwhile, there are several questions remained for further exploration. First, after 120 minutes of 2-DG treatment, we observed a higher number of Pab1 foci compared to Lsm7 condensates. If Lsm7 condensates are essential for the maturation of SGs' solid cores, the mechanism underlying this discrepancy needs further clarification. Additionally, our findings indicate that Lsm1, Lsm8, and Pat1 influence Lsm7 foci formation. Future studies are needed to elucidate the mechanisms behind this regulation and determine whether it involves the Lsm1-7/Pat1 complex. Lastly, further investigations are required to clarify the role of Lsm7 in SGs formation in mammalian cells.

In **Paper II**, based on **Paper I**, we seek to uncover the upstream signaling pathway to regulate the LLPS of Lsm7 in SGs formation. Using an imaging-based phenomic screen, we identified Sch9 and Ypk1 as downstream effectors of the TORC1/2 complexes that regulate SGs formation. The TORC1/2-Sch9/Ypk1 signaling cascade reduces cellular levels of major long-chain base sphingolipids (LCBs), which in turn downregulates the expression of the ubiquitin-proteasome system (UPS) component Ubi4, partially through the upregulation of the deubiquitinase Ubp3. Furthermore, we discovered that the SGs initiation factor Lsm7 functions downstream of the Ubi4-Ubp3 signaling axis, with Ubp3 positively regulating Lsm7 phase separation, thereby promoting SGs formation. Meanwhile, this signaling pathway is conserved in mammalian cells. Together with **Paper I**, we identified an evolutionarily conserved signaling pathway together with its major regulatory components mediating the formation of SGs through the LLPS of the key protein Lsm7. Nevertheless, it remains unknown how exactly the reduced ubiquitin levels can lead to the LLPS of Lsm7. Another question needs further exploration is whether there are additional components involved in the interaction between Ubp3, Ubi4, and Lsm7. Finally, although we validated our findings in mammalian cells, it remains unclear whether stresses other than 2-DG-induced stress produce the same results.

In **Paper III**, we investigated the role of Trr1 in yeast replicative aging. Our findings demonstrate that Trr1 undergoes LLPS under aging conditions, with an increasing propensity for phase separation as cells age. The formation of these phase-separated

biomolecular condensates is driven by the accumulation of endoplasmic reticulum (ER) stress during aging, rather than oxidative stress traditionally associated with its antioxidant function. Furthermore, Trr1 phase-separates to facilitate ER-phagy at the pre-autophagosomal structure (PAS) formation stage through LLPS to ensure proper autophagosome formation and the successful completion of ER-phagy. Utilizing an optogenetic approach, we demonstrate that dysregulation of Trr1's phase separation perturbs cellular fitness. Our work provides new insight not only expands our understanding of Trr1's multifaceted role in cellular homeostasis but also opens up fresh avenues for exploring the interplay between autophagy and aging. At the same time, our findings raise additional questions for future research. First, given the absence of obvious IDRs in Trr1's amino acid sequence, what drives its LLPS? While we propose that the repeated NADPH and FAD binding regions, along with Atg8-interacting motifs (AIMs), could be key areas for further investigation, other yet-to-be-discovered mechanisms may also contribute. Meanwhile, it's unknown if ER stress and ER-phagy are the only stress and response that phase-separated Trr1 involved. Meanwhile, the precise mechanism of how phase-separated Trr1 promote the autophagosome development needs further exploration. In addition, with the optogenetic tools we have developed to investigate Trr1 LLPS, along with the advancing of biomolecular condensates isolation methods, future studies could leverage these to see a bigger picture. Last but not the least, given the important role of human thioredoxin reductase 1 (TXNRD1) as an anticancer therapeutic target^[181], future research needs to explore whether TXNRD1 undergoes LLPS and, if so, which specific splice variants are involved, as well as the cellular conditions under which LLPS is induced.

As Occam's razor states, "Entities are not to be multiplied without necessity", proteins may achieve this through LLPS to cope with different conditions while saving the overall cost. Altogether, the work in this thesis takes a small step forward in uncovering the unconventional roles of proteins we are familiar with through the lens of LLPS. It builds a brick that paves the foundation ultimately leads to a potential revolution in our conceptual perception of life.

Nature and Nature's laws lay hid in night, do not go gentle into that good night without LLPS.

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