Mapping ultrastructural effects of proteotoxic stress on *S. cerevisiae*

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To those who lay the foundation we walk on

"It's getting hot in here." – *S. cerevisiae*

Abstract

Cells are the basic building blocks of any living being, whether the organism consists of only one, or many cells. The different cellular components interact with one another to maintain the viability of the cell. An essential element of cells are proteins – peptide chains folded into a specific conformation unique to every protein. The structure of the protein is important for its function. Proteins execute a range of tasks across the cell, but can misfold during stress, which can lead to inactivity or even toxicity. Stressors, such as heat, are often used to induce proteotoxic stress and simulate certain aspects of ageing-related disease. Upon exposure to stress, genetic programmes, as well as biophysical and biochemical effects contribute to change and adaptation of the cell. This has far-reaching consequences on the cell, which is reflected in numerous ways.

The articles discussed in this thesis examine the effect of different stressors, primarily heat-stress on the budding yeast (*Saccharomyces cerevisiae*) and other organisms. The articles reveal that heat-stress leads to large changes in organellar conformation. In addition, vacuolar pH as well as contact sites with the nucleus increase. Aggregates of misfolded proteins are sequestered in the cytoplasm and within organelles, to then be re-folded correctly or be degraded. Our results indicate that there are differences in aggregation and clearance of proteins, even when they are mixed in the same aggregate. We have also observed new structures, such as electron-translucent clusters that form near the plasma membrane, however their contents and role in the heat-shock response are still undetermined. An unexpected result was that virus-like particles, that contain viral capsid proteins but no viral nucleic acids, become less clustered as heat-shock progresses.

In two of the papers we focus on material accumulating in the nuclear envelope, which then causes the outer nuclear membrane to bud out toward the cytoplasm. We show that it occurs under normal physiological conditions, but also more frequently in several stress conditions which all cause the increase of misfolded proteins in the cell. The results indicate that budding of the nuclear envelope is used for protein transport between the nucleus and the cytoplasm. The last paper is a review that summarises scattered publications of observations of nuclear envelope budding from the last 70 odd years and points out gaps of knowledge in this novel research field.

Overall, this thesis creates a wide, yet detailed overview of how the cell responds to stress, specifically addressing its proteotoxic aspect. This will

contribute to the understanding of cellular mechanisms in ageing-related disease.

Sammanfattning

Celler är alla levande organismers basala byggstenar, oavsett om organismen består av endast en eller flera celler. De olika cellulära komponenterna, till exempel organeller, interagerar med varandra för att upprätthålla cellers livskraft. En av cellens grundläggande beståndsdelar är proteiner, de är peptidkedjor som är vikta på ett visst sätt som är speciellt för varje protein; man brukar kalla detta proteinets struktur och det är viktigt för proteinets funktion. Proteiner utför en rad uppgifter inom cellen, men när cellen är stressad kan en del proteiner vikas fel, vilket kan leda till att de inaktiveras eller till och med att de antar en ny funktion som kan vara giftigt för cellen. Ofta används stressfaktorer, såsom värme, för att framkalla proteotoxisk stress och simulera vissa aspekter av åldersrelaterade sjukdomar. Vid exponering för stress bidrar genetiska program, biofysiska och biokemiska effekter till förändring och anpassning hos cellen. Detta har långtgående konsekvenser för cellen, vilket återspeglas på flera sätt.

Artiklarna som diskuteras i denna avhandling undersöker effekten av olika stressfaktorer, främst värme, på vanlig bakjäst (*Saccharomyces cerevisiae*) och andra organismer. I artiklarna framkommer att värme-stress leder till stora förändringar i organellernas struktur. Dessutom ökar pH-värdet i vakuolen såväl som dess kontaktytor med cellkärnan. I cytoplasman samt inom organellerna avskiljs anhopningar av felvikta proteiner för att antingen vikas rätt igen eller skickas till vidare för nedbrytning. Våra resultat visar att det skiljer sig hur snabbt felvikta proteiner ansamlas i aggregat och rensas bort beroende på vilka proteiner som har vikts fel. Vi observerade också nya strukturer, såsom elektron-transparent kluster som bildas nära plasmamembranet, men deras innehåll och funktion i värmechockreponsen är fortfarande inte fastställd. Ett oväntat resultat var att virusliknande partiklar som innehåller virala kapsidproteiner, men inga virala nukleinsyror, blir glesare efterhand som värme-chocken fortskrider.

I två av artiklarna fokuserar vi på ansamlingar av material mellan kärnmembranen som får dem att böja sig ut in i cytoplasman, i en struktur som påminner om vesikulär avknoppning. Vi visar att detta sker under normala cellulära förhållanden, men att stressförhållanden som orsakar en ökning av felveckade proteiner i cellen även ökar dessa strukturer. Resultaten tyder på att avknoppningen från kärnmembranet används för proteintransport mellan kärnan och cytoplasman. Den sista artikeln är en översiktssammanfattning

över utspridda publikationer från de senaste omkring 70 åren, i vilka observationer av avknoppningar av kärnmembranen gjorts. Denna artikel belyser kunskapsluckorna inom detta nya forskningsområde.

Sammanfattningsvis är denna avhandling en detaljerad översikt över hur jästceller reagerar på stress, vilket kan bidra till en ökad förståelse av cellulära mekanismer vid åldersrelaterade sjukdomar.

Publication List

Paper I **Keuenhof, Katharina S.**, L. Larsson Berglund, S. Malmgren Hill, K. L. Schneider, P. O. Widlund, T. Nyström, and J. L. Höög, "Large organellar changes occur during mild heat shock in yeast", *J. Cell Sci.*, vol. 135, no. 5, 2022. DOI: 10.1242/jcs.258325.

Paper II K. L. Schneider, X. Hao, **Keuenhof, Katharina S.**, L. L. Berglund, A. Fischbach, D. Ahmadpour, P. Gomez, J. L. Höög, P. O. Widlund, and T. Nyström, "Heat-induced protein aggregates co-localize with mitochondria and virus-like particles", *Manuscript*, 2023.

Paper III K. L. Schneider, D. Ahmadpour, **Keuenhof, Katharina S.**, A. M. Eisele-Bürger, L. L. Berglund, F. Eisele, R. Babazadeh, J. L. Höög, T. Nyström, and P. O. Widlund, "Using reporters of different misfolded proteins reveals differential strategies in processing protein aggregates", *J. Biol. Chem.*, vol. 298, no. 11, p. 102 476, 2022. DOI: 10.1016/j.jbc.2022.102476.

Paper IV D. Panagaki, J. T. Croft, **Keuenhof, Katharina**, *et al.*, "Nuclear envelope budding is a response to cellular stress", *Proc. Natl. Acad. Sci.*, vol. 118, no. 30, 2021. DOI: 10.1073/pnas.2020997118.

Paper V **Keuenhof, Katharina S.**, V. Kohler, F. Broeskamp, D. Panagaki, S. D. Speese, S. Büttner, and J. L. Höög, "Nuclear envelope budding and its cellular functions", *Nucleus*, vol. 14, no. 1, 2023. DOI: 10.1080/19491034.2023.2178184.

Other Publications

These publications are not included in the thesis:

Keuenhof, Katharina, P. Heimel, L. Zopf, *et al.*, "Multimodality imaging beyond CLEM: Showcases of combined in-vivo preclinical imaging and ex-vivo microscopy to detect murine mural vascular lesions", in *Methods Cell Biol.* 2020. DOI: 10.1016/bs.mcb.2020.10.002.

Keuenhof, Katharina S., A. Kavirayani, S. Reier, S. H. Geyer, W. J. Weninger, and A. Walter, "High-Resolution Episcopic Microscopy (HREM) in Multimodal Imaging Approaches", *Biomedicines*, vol. 9, no. 12, p. 1918, 2021. DOI: 10.3390/biomedicines9121918.

S. K. Anand, M. Caputo, Y. Xia, *et al.*, "Inhibition of MAP4K4 Signaling Initiaties Metabolic Reprogramming to Protect Hepatocytes from Lipotoxic Damage", *J. Lipid Res.*, vol. 63, no. 7, p. 100 238, 2022. DOI: 10.1016/j.jlr.2022.100238.

Contribution report

Paper I I have prepared all samples for electron microscopy except for the deletion strains (fig. 3E-F), and the first replicate of the heat-shock time-course. I have modelled all electron micrographs and analysed all data except that from panels fig. 2D-F, 3C-I. I have learned and applied R for the statistics and created all figures except for the panels mentioned above and fig. 5I. I wrote a majority of the first draft and contributed to all subsequent revisions. I have managed the submission and peer-review process.

Paper II I have prepared the samples for fig. 2B, 3A-B,E-G, including immuno-EM labelling for fig. 2B and 3A-B. I have performed the data analysis for fig. 3E-G.

Paper III I have performed immuno-labelling experiments, imaging, and analysed the acquired data for fig. 6B. In the process, I have developed a data analysis method for fig. 6B.

Paper IV I have prepared and imaged the samples for fig. 1A-C,F, including immuno-labelling for fig. 1C,F. I have also performed the data analysis for the respective panels, and taken part in writing the manuscript.

Paper V I have written the majority of the initial draft, created a fig. 1, and in the final version have written the introduction, the sections "A neglected child with many names?", "NEB to transport protein aggregates", and the discussion. I have managed the submission and the peer-review process.

Nomenclature

2D two-dimensional

3D three-dimensional

AZC Azetidine-2-carboxylic acid

EDC Electron-Dense Content

ER Endoplasmic Reticulum

ERMES ER-Mitochondria Encounter Structures

ESCRT Endosomal Sorting Complex Required for Transportation

ETC Electron-Translucent Clusters

FLM Fluorescence Light Microscopy

FS Freeze Substitution

H_2O_2 Hydrogen Peroxide

HMC-1 Human Mast Cell line 1

HPF High-Pressure Freezing

HS Heat Stress/Shock

ILV IntraLumenal Vesicle

INM Inner Nuclear Membrane

LD Lipid Droplet

MCS Membrane Contact Site

NE Nuclear Envelope

NEB Nuclear Envelope Budding

NEC Nuclear Egress Complex

NPC NucleoPore Complex

Nups Nucleoporins

NVJ Nucleus-Vacuole Junction

ONM Outer Nuclear Membrane

PQC Protein Quality Control

TEM Transmission Electron Micrscopy

UA Uranyl Acetate

UPS Ubiquitin-Proteasome System

V-ATPase Vacuolar Adenosinetriphosphatase

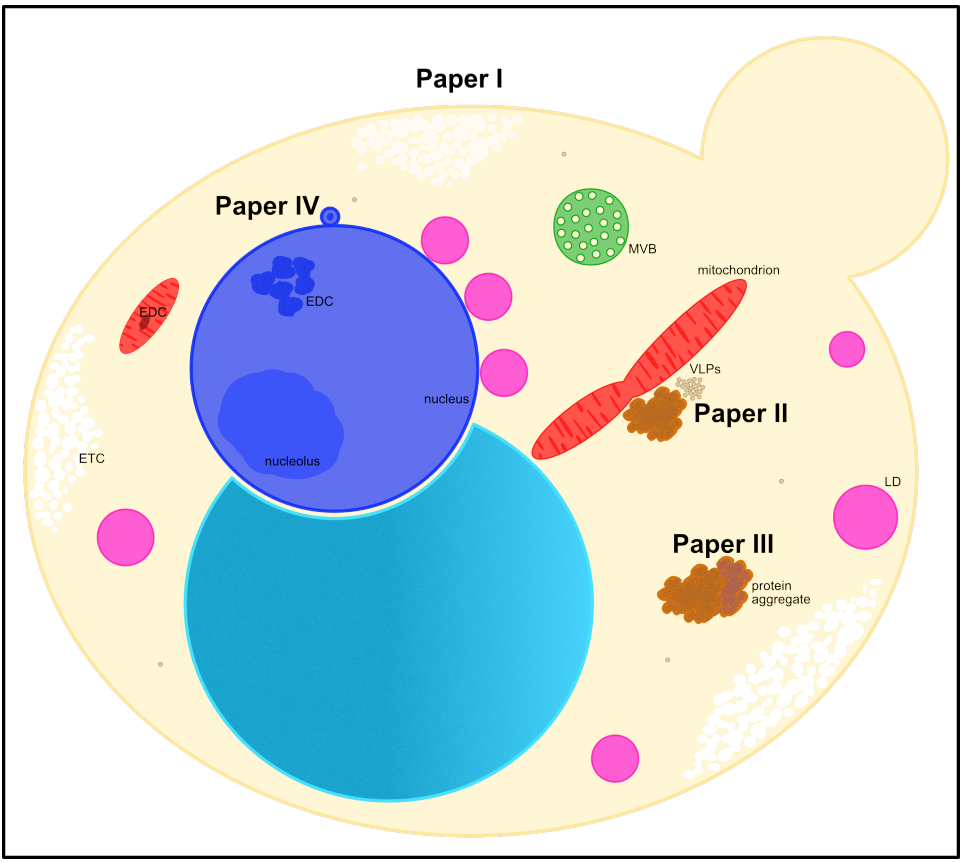
vCLAMP VaCuoLe And Mitochondria Patch

VLP Virus-Like Particle

Contents

Chapter 1

Aim of the Thesis



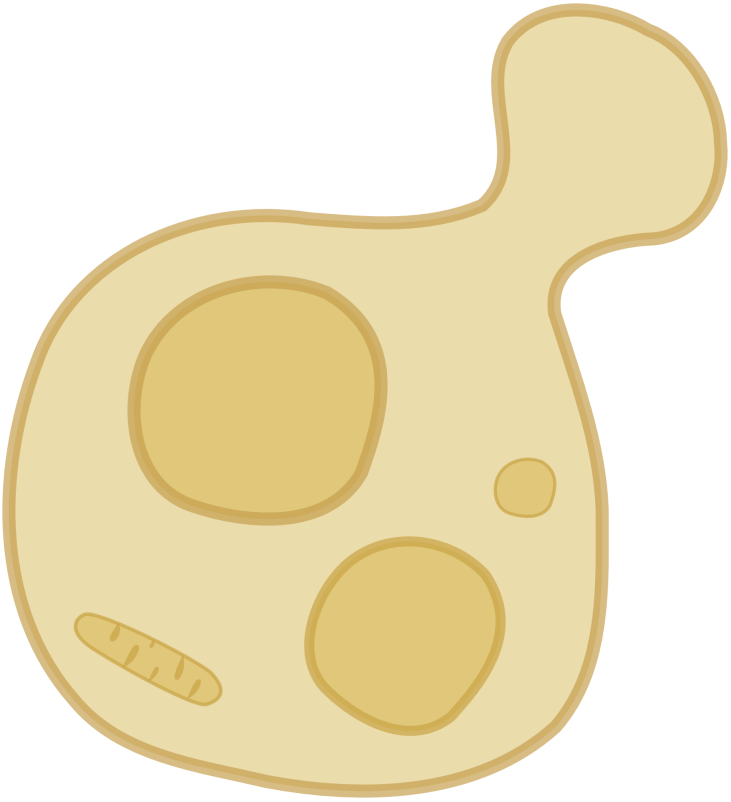
A predominant goal within life sciences is to understand, and in the process alleviate, the burden that various diseases place on society. Even the most complex machines are made of individual parts and mechanisms complementing each other. As such, when considering biological organisms and elucidating disease origin and progression, it is helpful to start simple. Insight can be gained by the observation of unicellular organisms, such as the budding yeast *Saccharomyces cerevisiae*, under stress conditions which simulate disease mechanisms in the cell. The aim of this thesis is to map organellar changes that occur during heat-stress and place them into the context of spatial protein quality control, and to a certain extent, intracellular transport.

Paper **I** provides an overview of changes that a number of organelles undergo throughout a heat-shock time course. Several thousands of electron micrographs were used to analyse changes in number, size, morphology and connectivity of major organelles. After assessing quantitative and qualitative changes in the cell, we focus on proteins, which occupy a large amount of space in the cell. After synthesis, they are folded to achieve their configuration. This process can be disturbed by external stress, or age. It leads to improper folding (misfolding) of native proteins, which can even be toxic to the cell. It is necessary for the cell to manage the accumulation of misfolded protein in the cytoplasm and inside some organelles. There are cellular pathways to sequester those proteins – either degrade or re-fold them – and the cell's health can depend on their efficiency. A group of diseases, so-called proteopathies, are related to misfolded proteins accumulating in the cell. Thus, understanding cellular protein quality control is an important contribution to understand these disease mechanisms. Paper **II** reveals the location of protein aggregates to certain organelles and other stress-induced structures, such as virus-like particles (VLPs). Paper **III** elaborates on spatial protein quality control in the cell by proposing the use of new misfolding reporter proteins, assessing their clearance, and their organisation within a protein aggregate.

Movement within the cell, is not only inevitable, but also desired. Biomolecules are constantly transported across the cell and in and out of organelles. So far, the only known way out of the nucleus are the nuclear pore complexes (NPCs). Nevertheless, paper **IV** shows that when challenging cells with different stressors, misfolded proteins appear to be transported across the nuclear envelope via budding. As with many crucial processes in the cell, it seems likely that an alternative to transport via the NPC exists and nuclear envelope budding has indeed been observed in various forms, as reviewed in paper **V**.

Chapter 2

Biological background



2.1 Cellular Arrangement

Eukaryotes, a cell type like our own, are defined by having a nucleus and a set-up of organelles. The cell is a crowded space where biomolecules are constantly produced, degraded, and fulfill different tasks during their lifecycle. Moreover, all kinds of different components of the cell interact with each other. These interactions can be promoted or inhibited, depending on the cellular arrangement and composition. Here, I focus on certain organelles and some of the membrane-membrane contact sites between them. Using TEM, we can visualize a section of the entire cell at once and map how for example stress affects cellular morphology.

2.1.1 Membranes

Delineating many organelles from the rest of the cell is a membrane. In the case of the nucleus and mitochondria, this is a double lipid bilayer. For other organelles, it is just an individual one. Membranes keep the insides of an organelle contained, ensuring the right composition, osmotic pressure, phase and pH, but also enable transport between the organelle and either the cytoplasm or another organelle it has formed a contact-site with [1]. This compartmentalisation of organelles allows them to have individual pH levels, and perform various tasks in specialised environments away from the rest of the cell [2]. One example is DNA transcription, that only occurs in the nucleus, another is ATP generation using ATP-synthase inside the mitochondrial membranes.

2.1.1.1 The lipid bilayer

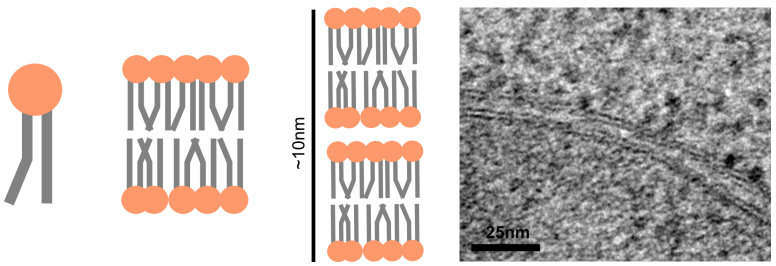


Figure 2.1: Phospholipid, bilayer, double bilayer and electron micrograph of the nuclear envelope. Scale bar 25 nm

Lipid bilayers consist of two layers of phospholipids "facing" each other (fig. 2.1). Their hydrophobic tails (grey) are orientated towards each other,

whereas their hydrophilic heads (orange) face outwards. Lipid bilayers are fluid and their properties change depending on the lipid composition. Additionally, those membranes are also decorated with proteins that are either on the surface, or pass through the membrane itself.

2.1.1.2 Transport across membranes

Lipid bilayers are ideal to separate an organelle from the cytoplasm and control incoming or outgoing passive diffusion of molecules. As the lipid bilayer has a hydrophobic middle, charged molecules, such as ions, have a hard time passing through. They must be transported through specialised channels, such as ion channels that select what can pass depending on size and charge of the transported molecule. Another type of membrane proteins, called transporters or pumps, offer the possibility of active transport across membranes for molecules that otherwise would not pass through the membrane [3]. In the case of the nucleus, the most common route of transport across the nuclear envelope is the nucleopore complex (NPC). Another way for material to be transported across the plasma membrane is via endo- and exocytosis. The membranes bud around the material and release it further along.

2.1.2 Organelles

Organelles, or the "little organs" of the cell, are various entities, each fulfilling specific roles. Below I introduce some organelles that are separated from the cytosol by a membrane that are the main focus of this thesis. However, there are also membrane-less organelles, such as the nucleolus, where ribosome genesis takes place, or stress granules, that consist of proteins and mRNA [4]. All organelles are minutiously orchestrated with each other. This communication and interplay is important during normal physiological processes and especially when responding to stress, for example environmental stressors, as I will discuss later in 2.3.

2.1.2.1 Nucleus

The nucleus (n in fig. 2.2) is the core of the cell, containing most of its genetic information [5]. It is where the DNA is replicated, transcribed and the resulting mRNA is further modified. In baker's yeast (*Saccharomyces cerevisiae*), the species this thesis focuses on, it takes up about $0.9\mu m^3$ of the cell's volume ($15\mu m^3$) and is therefore one of the largest intracellular compartments (Paper **I** and [6], [7]).

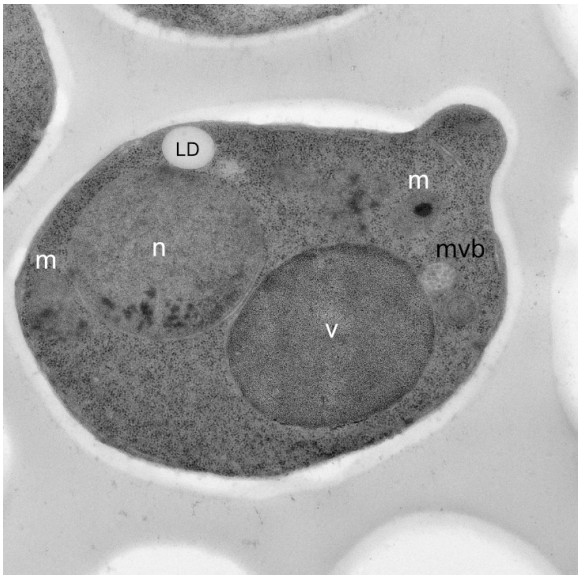


Figure 2.2: Yeast with labelled organelles. n: nucleus, v: vacuole, m: mitochondria, mvb: multi-vesicular body, LD: lipid droplet. Adapted from paper **I**.

The nuclear volume itself is further compartmentalised. The nucleolus is made of proteins, and regional accumulations of DNA and RNA, called the nucleolar organising regions. It is mostly known as site for ribosome biogenesis and also involved in the cell's stress response [8]. For example the nucleolar proteins nucleolin and nucleophosmin can act as chaperones to RNA and proteins and are also able to migrate to the nucleoplasm upon stress to actively participate in DNA repair [9], [10].

Chromosomes are also located in the nucleus, either as euchromatin or in condensed form, as heterochromatin. Chromatin consists of a long strand of DNA and DNA-binding proteins. One class of these proteins are histones, around which the DNA is tightly wrapped to form nucleosomes. The structure of nucleosomes is what allows the condensed packaging of the large amount of DNA in a space as small as the cell nucleus [11]–[13].

The nucleus is surrounded by the nuclear envelope (NE), separating it from the rest of the cell and shielding the genome from many sources of damage. It consists of two phospholipid bilayers: the inner nuclear membrane (INM) and the outer nuclear membrane (ONM), the latter of which is continuous with the endoplasmic reticulum (ER). The two membranes are separated by the perinuclear space. Both then INM and ONM are populated with many different membrane proteins that each fulfil their own function

[14], [15]. Proteins found at the INM assist the localisation of chromatin [16] and NPC [17] and those at the ONM are involved in the nucleus' connection to the cytoskeleton [18]. In yeast, about 200 NPCs mostly responsible for traffic in and out of the nucleus are interspersed in the NE [7].

2.1.2.2 Vacuoles

Vacuoles (v in fig. 2.2) are present in plant cells and also in yeast cells, our main organism of interest. They can be considered the equivalent of lysosomes, found in mammalian cells, as both organelles' task is to rid the cell of waste products and recycle their components. Vacuoles are however much larger than lysosomes, and able to accumulate autophagic bodies, delivered to the vacuole by the autphagosome, which mammalian lysosomes cannot [19]. Nevertheless, both organelles contain specialised enzymes that break down their contents [20], [21]. This enables the recycling of cellular components [22] and can reduce the harm that some structures, for example toxic protein aggregates, may cause to the cell. Furthermore, vacuoles also have a role in storage, including of certain elements such as calcium (Ca) [23] and phosphorus (P) [24]. Besides the storage and circulation of ions, the vacuolar ATP-ases on the single vacuolar membrane control the flux of protons, thus regulating vacuolar and cellular pH. This is crucial as the pH level of the cell can induce expression of certain genes, e.g. the heat-shock response [25]. Additionally, vacuoles play another regulating role when it comes to maintaining osmotic pressure within the cell by controlling their water uptake [26], [27]. They are thus variable in size and in budding yeast, they are between $0.5\,\mu\mathrm{m}^3$ and $4\,\mu\mathrm{m}^3$ (Paper **I** and [28]).

2.1.2.3 Mitochondria

Mitochondria (m in fig. 2.2) are responsible for the cell's respiration and partially its catabolic metabolism. They have an outer and inner membrane, with space in between. The inner membrane is folded inside the mitochondrion, to form what is called cristae, and the mitochondrion's interior is called its matrix. A major role is to phosphorylate adenosine-diphosphate (ADP) to adenosine-triphosphate (ATP), the major chemical energy currency of the cell. Mitochondria also play a role in calcium storage of the cell [29], along with the vacuole and the ER. Their ability to store ions is used to modulate the cell's membrane potential, which is used to import proteins into the mitochondrial matrix space [30]. Due to mitochondia's origin as an engulfed bacteria that remained and provided an ancestral eukaryotic

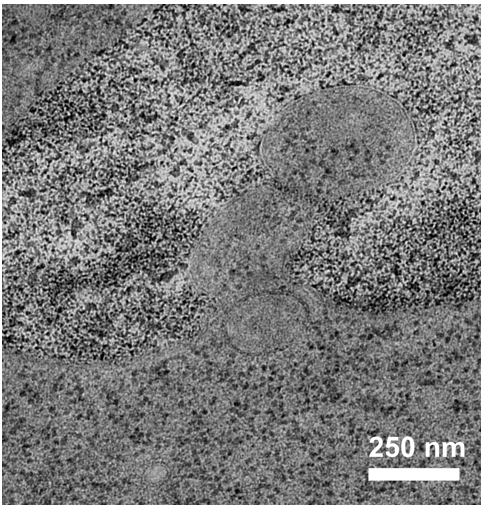


Figure 2.3: An MVB being engulfed by a vacuole.

cell with an evolutionary advantage, they carry their own DNA as a small circular genome.

2.1.2.4 Multivesicular bodies

MVBs (mvb in fig. 2.2) are round organelles with an outer membrane and many, smaller intralumenal vesicles (ILVs) within and have an area of $0.01\,\mu\text{m}^2$ in electron micrographs. MVBs are late endosomes, which take part in the intracellular transport and sorting of biomolecules before their potential delivery to the lysosome, or in the case of yeast, the vacuole [31]. To rid the cell of waste, MVBs fuse with the vacuole, and are then engulfed (fig. 2.3). They carry ubiquitin-tagged cargo that is taken in by an invagination of the endosomal membrane, forming ILVs. In addition, they also transport material to and from the Golgi apparatus. Both of these processes occur with the help of the endosomal sorting complex required for transportation (ESCRT) [32].

2.1.2.5 Lipid Droplets

As spheres of neutral lipids, surrounded by a phospholipid monolayer, lipid droplets (LD in fig. 2.2) are responsible for lipid storage and lipid metabolism in the cell. They are produced in the ER, by enzymes synthesising neutral lipids [33], but their production can also occur at the inner nuclear membrane [34]. Lipid metabolism occurs at the hands of proteins attached to

the surface of the LD's membrane. One of these proteins is Pet10, which is suggested to interact with another protein: Iml2. This protein is essential for the clearance of aggregates in the cell, showing that LDs are involved in protein degradation. Indeed, deletion mutants that lack LDs exhibit deficits in clearance of misfolded protein aggregates [35]. LDs can be degraded in two different ways: by lipolysis or lipophagy. During lipolysis, the contained lipids, mostly triacylglycerols, are broken down into fatty acids and glycerol. The resulting free fatty acids are subsequently catabolised by mitochondria for ATP production. In recent years, the hypothesis of lipophagy, the degradation of LDs by autophagy has also gained traction. The LDs, or parts thereof, are engulfed and then degraded directly, for example by the vacuole [36].

2.1.3 Interconnectivity of organelles

Organelles are not only connected to the cytoplasm of the cells, but also directly to each other. Various types of direct interorganellar connections are used to directly signal to each other. These contact sites, where organelles are in direct physical contact, are a platform for the exchange on ions, lipids, and also of signals between the organelles [37], [38]. There areas of connection are typically referred to as membrane contact sites (MCSs). In yeast, one of the first described MCS in the nucleus-vacuole junction (NVJ). There, the nucleus and vacuoles are tethered to each other by a complex involving a membrane protein from each respective organelle, Nvj1 and Vac8. The junction is a site that enables piecemeal microautophagy of the nucleus (PMN), a process that increasingly occurs in nutrient-deprived cells [39], [40]. Additionally, it is a site for regulation of lipid homeostasis [41], [42].

Another MCS in yeast is the vacuole and mitochondria patch (vCLAMP), it is one of the contact sites necessary for phospholipid transport [43]. Due to the involvement of the vacuolar protein Vps39 in the formation of VCLAMPs, which has been observed to depend on cellular metabolism [44], this MCS has been speculated to play a role in autophagy and in the cell's stress response during starvation [45]. Further MCS in the yeast cell include for example the ER-mitochondria encounter structures (ERMES), a system operating in parallel to vCLAMP. It is also responsible for phospholipid and calcium exchange between the ER and mitochondria [46]. Studies have shown that loss of either ERMES or vCLAMP leads to an enlargement of the respective other contact site, and that at least one of them is necessary for the cell's viability [43].

2.2 Protein Quality Control in the Cell

As previously mentioned, one of the methods the cell uses to maintain functionality and health is protein quality control (PQC). This is vital as misfolded proteins can cause harm to the cell and the rest of the organism [47]. PQC can be divided into temporal and spatial quality control, involving different molecular players and fulfilling different tasks. Below, I will outline major players in PQC, and some will be described more specifically as they are the focus of papers included in this thesis.

2.2.1 PQC Systems

Newly synthesised proteins must be folded, and misfolded proteins must either be re-folded to a correct configuration or, should this not be possible, degraded. Temporal PQC is responsible for these tasks, mainly through the ubiquitin-proteasome system (UPS) or autophagy. In UPS, the 26S proteasome, together with Hsp70 and Hsp40, recognises proteins marked with ubiquitin and unfolds them. In parallel, the spatial PQC system deposits misfolded proteins into inclusions sites within the cell. This happens early on in stress, and even in healthy cells. Misfolded proteins are aggregated and sequestered to different sites in the cytoplasm [48]. Where in the cell these depositions sites are is discussed further below in 2.2.3.

Autophagy, a part of temporal PQC, involves a double-membrane organelle called the autophagosome which engulfes cellular material for transportation to the vacuole. This is also known as macroautophagy. In microautophagy on the other hand, cellular components in the vicinity of vacuoles are recruited to be engulfed by the vacuole directly. The vacuolar membrane invaginates to take in whatever target is destined for degradation. Similarly, in micronucleophagy, also known as piecemeal microautopahgy of the nucleus (PMN), parts of the nucleus are directly taken in by the vacuole. This requires components to establish a contact site between the two organelles [39]. Autophagy in yeast is further elaborated on in [49].

Some organelles have also been shown to play a role in protein degradation, namely the mitochondrion [50] and LDs [51], [52]. In mitochondria, this mechanism is called mitochondria as guardian in the cytosol (MAGIC). It suggest that cytosolic misfolded proteins are imported into the mitochondria for degradation. Conveniently, protein aggregates induced by heat-shock have been found to accumulate in proximity to mitochondria [53]–[55]. Lipid droplets, whose production is increases under certain types of stress have been shown to act as vehicle to transport misfolded protein away from the

ER. Indeed, cells lacking LDs have impaired management of misfolded proteins [35].

2.2.2 Molecular players

A variety of biomolecules are involved in protein quality controls. Molecular chaperones perform the tasks necessary to either aggregate, disaggregate, or shuttle proteins to their correct destinations.

2.2.2.1 The heat-shock response

In response to the cell sensing heat, it actives a genetic programme that is responsible for the synthesis of the molecular players outlined below. In yeast, this gene is called Heat Shock Factor 1 (HSF1) [56]. In normal physiological conditions, transcription of HSF1 is not active and it is only induced by certain forms of stress, including, most obviously, heat stress. Interestingly, a drop in cytoplasmic pH is necessary for the induction of HSF1.

2.2.2.2 Chaperones

Several molecular chaperones mediate processes during normal proteostasis and also during the heat-stress response. In ascending order of weight, I will briefly describe some classes of heat-shock proteins (HSPs). The smallest are appropriately named small heat-shock proteins (sHSPs), their task is to bind unfolded proteins during stress conditions to prevent them from misfolding irrecoverably and to sequester them to later be refolded [57]. The Hsp40 family takes part in deciding the fate of misfolded proteins, whether they will be refolded or degraded. It does so together with the Hsp70 proteins. These fulfil many different roles, from protein folding to protein degradation. About half of the Hsp70 proteins in yeast are found in the cytosol and form the stress seventy sub-families. The Ssa family for example, is essential to yeast as an organism but only two of the genes are constitutively expressed, and the other two induced under stress [58]. Lastly, the Hsp100 family are disaggregases, participating in the disaggregation of proteins. In yeast, it is the chaperone is Hsp104 which fulfills this function, underregulation of Hsp70 [59].

2.2.2.3 Misfolding Protein Reporters

To track the behaviour and fate of misfolded proteins in the cell, especially if using fluorescence microscopy, it is helpful to observe proteins that are

known to misfold unter certain conditions. Very commonly, the disaggregase Hsp104 is tagged with green fluorescent protein (GFP) and used as a reporter as it is involved in most spatial recruitment of misfolded proteins [60], [61]. Nevertheless, it is sensible to assess the cell's stress response and sensitivity using other reporters. For this, it is useful to know that those proteins do misfold at higher temperatures, that they are non-toxic and also easy to visualise using common fluorescent markers. An example for these would be the proteins guk1-7, gus1-3, and pro3-1, which do not impact cellular fitness when over-expressed but can be visualised independently of the engagement of Hsp104 [62].

2.2.3 Spatial arrangement

An approach to manage misfolded protein aggregates in the cell, is to sequester them to various un-membranated compartments where they can either be stored or processed further. Below, I will focus on the major quality control compartments in the yeast *Saccharomyces cerevisiae*.

2.2.3.1 In the Cytoplasm

We find two major quality control compartments in the cytoplasm. There exist the cytosolic quality control compartments (cytoQs). They are small, peripheral inclusions of misfolded protein in the cell, which coalesce to fewer, larger ones under stress [63]–[65]. In general, it can be said that the sHsp Hsp42 is necessary for their formation, and for their dissolution, Hsp104 along with its co-chaperones from the Hsp70 family.

The other quality compartment in the cytoplasm is the insoluble protein deposit (IPOD) which localises in proximity of the vacuole [66]. It is mostly considered a storage for amyloid proteins, although non-amyloidogenic misfolded protein also accumulate there. Pre-autophagosomal structures localise in vicinty of the IPOD, suggesting clearence by autophagy.

2.2.3.2 In the Nucleus

Misfolded proteins also occur in the nucleus, and need to be sequestered there as well. A compartment formally known as juxta-nuclear quality compartment (JUNQ) that was assumed to be placed adjacent to the nucleus, but in the cytoplasm has since been re-named as the intra-nuclear quality compartment as it has been found to be within the nucleus [67]. It is however, possible that INQ and JUNQ still represent two separate compartments with independent functions.

2.3 Cell stress

Cells, just like larger organisms, experience perturbations which are referred to as stress. Stress evokes different responses and reactions within a cell, that may affect its morphology [68] or also its molecular make-up [56]. A stress can be environmental, e.g. a change in temperature or pressure, chemical, e.g. exposure to toxins, or also temporal, for example process of ageing. One of the ways a cell is affected by stress is a disruption in proteostasis. Proteins synthesised undergo folding before being assembled into functional subunits. Errors in the folding can cause the proteins to dysfunction, and as a result either cause harm to the cell or simply aggregate [69]. The cell is equipped with quality control systems to maintain health during and after exposure to stressors [70]–[72]. One is the protein quality control (PQC) system, discussed in section 2.2.

2.3.1 Heat-shock

A very commonly used stressor when researching stress responses, especially relating to PQC is heat stress (HS). In the 1960s, it had been discovered that there exist regulatory mechanisms that allow adaptation to heat [73]. Besides triggering the PQC, heat is relevant in other contexts. Mammals respond to infection with fevers, increasing the body temperature. Also, global warming is an alarming problem affecting crops, fauna, and humans. The effects of heat-shock on the cell are wide-reaching. DNA damage, an increase in the production of reactive oxygen species, un- and misfolding of proteins and changes in membranes permeability [74] are consequences of heat-shock on the cell. As I show in Paper I, heat-shock also has consequences on the entire cell's architecture.

2.3.2 Chemical Stressors

Besides heat, chemical stressors can greatly impact the cell. These could consist of either chemical drugs, or other toxins such as heavy metals.

Arsenite (As(III)) is a form of arsenic, as well as other heavy metals like cadmium cause misfolding protein aggregates in yeast cells [75], [76]. Prolonged exposure of humans to those metals is linked to neurodegenerative diseases, for instance Alzheimer's and Parkinson's diseases [77]–[80]. Thus, researching their effects in the model organism yeast is a way to better understand the origins of those diseases, and the mechanisms involved dealing

with protein toxicity.

Azetidine-2-carboxylic acid (AZC) is an amino acid homologue to the amino acid proline. Because it can act as an analogue, cells synthesising proteins may substitute proline for AZC. This leads to reduced thermal stability and misfolding of newly produced proteins [81], [82]. As a result, AZC treatment is useful for understanding how cell's deal with large amounts of misfolded proteins, and which pathways could be involved in their management and degradation.

Hydrogen peroxide (H_2O_2) is a reactive oxygen species (ROS), that in acidic solutions acts as oxidiser. It is toxic to cells as it oxidises proteins, lipids, and DNA. This can lead to an aggregation of proteins [83] and activate the PQC mechanism [84]. ROS can cause irreversible damage to the cell, which is not to be confused with the products of physiological reduction-oxidation processes that occur in biochemical pathways, such as signalling.

2.3.3 Ageing

Not often thought of as a stressor, ageing has wide-reaching effects on almost any organism. We, as humans, notice it by the gradual decrease of certain abilities and a higher penchant for disease, however, it starts at cellular or even molecular level [85]. Unicellular organisms also experience ageing. In the case of the budding yeast *Saccharomyces cerevisiae*, this can be categorised as either replicative or chronological ageing [86]. Replicative ageing refers to the number of cell divisions the cell has gone through, and how many daughters one mother cell has produced. This is interesting as daughters are rejuvenated and protein damage is retained in the mother cell after division [87], [88]. Chronological ageing refers to cultures kept alive over time in stationary phase, without the addition of new nutrients [89], [90]. Researching the complex effects of ageing on cells can reveal the mechanisms of ageing-related diseases, for example Alzheimer's disease.

2.4 Implications in disease

Why are all these misfolded protein interesting? Aggregates of misfolded proteins have been connected to various diseases, falling under the umbrella term of proteopathies. Examples for these are neurodegenerative diseases are for example Alzheimers' disease, Parkinson's disease, and Huntington's disease.

2.4.1 Proteopathies

When proteins change conformation, from their correctly folded state into a misfolded state, they can lose normal function and gain toxicity. Following their abnormal folding, the affected proteins aggregate within the cell; a phenotype that has been observed in various neurodegenerative diseases [91], [92]. In Alzheimer's disease, amyloid proteins aggregate between neuronal cells and are then referred to as plaques. This leads to neuron loss, and the deterioration of cognitive decline [93]. Within the cell, aggregates of tau-proteins can accumulate, additionally qualifying Alzheimer's disease as tauopathy [93], [94]. There, tau proteins are highly phosphorylated, and form neurofibrillary tangles. This has been shown to happen downstream of the formation of amyloid-β plaques [95].

Inclusions of the protein alpha-synuclein, a pleiotropic protein, are found in aggregates of the brain stem [96]. These aggregates, also called Lewy bodies, aggregate into amyloid fibrils in the cell, and are a mark of illnesses like Parkinson's disease or Lewy body disease [97]. In amyotrophic lateral sclerosis, a disease that causes involuntary muscle twitching, the protein TDP-43 has been found to accumulate in neuronal cells containing neither alpha-synuclein, nor tau-protein, but ubiquitin [98], [99].

Lastly, prion diseases, such as Creutzfeldt-Jakob disease or bovine spongiform encephalopathy (mad cow disease), stem from the accumulation of prions in the nervous system. These types of proteins are exceptional in their ability to self-propagate, resist cellular clearance mechanisms and ability to propagate from one cell to another [100], [101]. The proteins that are major players in those diseases, and others, can be expressed and studied in the yeast *Saccharomyces cerevisiae* [102].

2.4.2 Yeast as a tool to understand disease

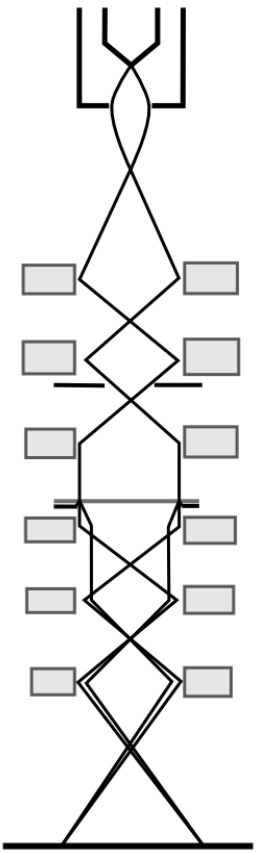
Proteostasis, which includes the sequestration and degradation of misfolded proteins in the cell, declines during ageing. It has been suggested that this is both a cause and a result of age-related diseases [103]. The processes involved in the genesis of those diseases and protein interactions can be studied in budding yeast, and has previously been extensively done [104]–[107]. Misfolding and aggregation of proteins can be triggered by exposure to one of the stressors above (2.3), during which they accumulate at specific sites in the cytoplasm, but also the nucleus [108], [109]. The proteins of interest can be expressed in yeast, even though they are not necessarily native to the organism. Further, model misfolding proteins have facilitated

the creation of pathoneurological models [110].

Additional aspects to be considered are that the genome of budding yeast has been fully sequenced and there exist comprehensive libraries of strains, e.g. deletion collections in which specific genes have been deleted, or a GFP-tagged collection [111]. Budding yeast is non-toxic, has a short generation time, and is widely available and possible to culture. All these factors ensure that the budding yeast *Saccharomyces cerevisiae* is a suitable model organism in the study of ageing-related diseases.

Chapter 3

Methodological background



For this thesis, transmission electron microscopy (TEM) has been the main method of data acquisition [112], [113], despite fluorescence light microscopy (FLM) being one of the most commonly used imaging tools in the field of cell biology. There are several reasons for the preferential use of EM, most importantly the possibility of acquiring nanometre-resolution data without the use of specific labelling on the structure(s) of interest. Here, I give a brief introduction to the method in order to place relevant results into context and facilitate their interpretation.

3.1 Transmission Electron Microscopy

Almost a century ago, the researchers Knoll and Ruska began developing electron microscopes to circumvent the limitations of visible light in microscopy [114]. The resolution of ordinary wide-field visible light microscopes is, among other factors, limited by the wavelength λ of light used, ranging around 380 nm to 750 nm. According to Abbe's diffraction limit d, where $d = \frac{\lambda}{2n \sin\theta}$, this limits the achievable resolution. Although many helpful super-resolution visible-light microscopy techniques have been developed in the recent years [115]–[119], narrowing the resolution gap between EM and LM, the need for specific labelling and limited spatial resolution can be restrictive. A shorter wavelength can be achieved by using higher velocity (v) beams, as described by de Broglie in the early 1920s: $\lambda = \frac{h}{mv}$. This could be achieved by higher-frequency light beams, such as X-rays, or a different source of electro-magnetic radiation altogether: electrons.

Electrons, negatively charged particles of very small mass (9.11×10^{-31} kg), can be accelerated across a potential difference (a voltage). When applying a voltage of 120kV, the electrons will travel at almost 60% of the speed of light and, accounting for relativistic effects, have a wavelength of approximately 4 picometres (4×10^{-12} m). The final resolution of the acquired image will be in the Ångstrom-nanometre range, due to for example limitations by sample preparation [112].

Similar to visible-light microscopes, electron microscopes require sets of lenses to direct, focus and magnify the electron beam. To suitably modify the travel path of the electrons in the microscope column, electrostatic and electromagnetic lenses are employed. These lenses generate either an electric or magnetic field respectively, which steers the electrons as they travel through the sample, and later on to the display screen or camera detector. This way, the beam of electrons generated at the electron source will be condensed and focused by a set of condenser lenses, magnified by further

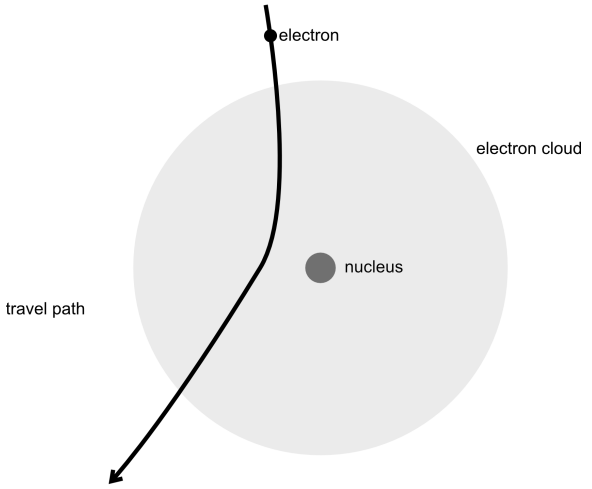


Figure 3.1: Elastic scattering of an electron as it passes through the electron cloud of an atom.

objective, intermediate and projector lenses, resulting in a magnified image of the sample.

To understand how the image itself is created, let's delve into how electrons interact with the sample. First, the sample needs to be sufficiently thin for electrons to traverse it. The main interaction relevant for this type of imaging is called elastic scattering (fig. 3.1). When elastically scattered, the electron passing through the sample will change the direction of its travel path, but not its speed, and will also not lose any, or only a minimal amount of energy in the process. A greater scattering angle will result in higher contrast in the final two-dimensional (2D) image. This is referred to as amplitude-contrast, which we will focus on here. As electrons are not visible to the human eye, the image can be seen on specially coated screens or at the hand of a camera. Cameras may either convert electrons into photons to create an image (charge-coupled devices), or directly detect the incoming electrons (direct detectors).

3.1.1 Electron Tomography

In thicker samples, the information contained can be obtained by using three-dimensional (3D) EM. In electron tomography, the sample slice is tilted at different angles and an image is acquired at each tilt angle. After imaging the sample from various angles, these images make up the tilt series. Computa-

tional processes then allow the calculation of the corresponding tomograms, which is a reconstruction of the sample itself. This is done for instance using back-projection of the acquired tilt series. Tomograms can then be visualised, modelled and where desired, quantified. Using tomography, it possible to gain insight into a sample up to several hundred nanometres in thickness. For the works in this thesis, the IMOD software package was used for tomogram reconstruction and quantitative data analysis [120].

3.2 Sample preparation

Certain technical aspects of the microscope column, for example the vacuum and the intensity of the electron beam are detrimental to any biological organism in its physiological state. Therefore, biological samples must be carefully prepared and made to even fit into a microscope. Here, all samples prepared for EM are high-pressure frozen, freeze-substituted, embedded in plastic and sectioned before imaging in the microscope. High-pressure freezing enables sample preparation with high physiological accuracy and satisfactory resolution for use in TEM [121]–[124]. Below, I will elaborate on the steps necessary to achieve sample preparation.

3.2.1 Cryo-fixation

To avoid deformation of the sample before and during imaging, it must be fixed. The vacuum and intense radiation in the TEM, as well as sectioning it to an adequate thinness, would otherwse largely damage the sample. Sample fixation can either be done chemically, which often introduces distortion and dehydration artefacts, clustering of structures, or poor resolution of bilayer membranes [125], [126]. Alternatively, samples can be fixed using a cryo-fixation method, such as high-pressure freezing (HPF) [28], [125], [127]–[130]. In this process, the sample, sometimes along with some culturing media or cryo-protectant is deposited into a small sample carrier, and covered with another (fig. 3.2). This sample-"sandwich" is then frozen under a pressure of approximately 2100 bar[1], whilst rapidly being enveloped in a liquid nitrogen slurry of ca. -175°C. Within the 25 ms in which the sample is frozen, the ice that forms is vitreous instead of crystalline [131], so it does not expand. Due to the high-pressure applied and the speed of the process, nucleation centres that would normally instigate crystal formation in the ice and destroy cellular

[1]This pressure is comparable to high-powered pressure washers

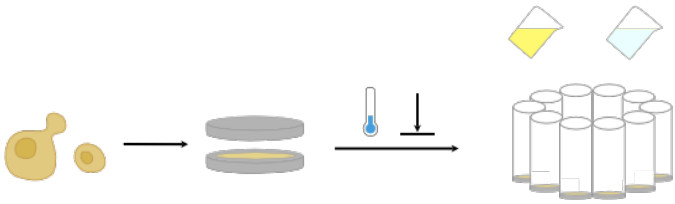


Figure 3.2: Preparation steps in the HPF-FS process. Cells (here *S. cerevisiae*) are filled in aluminium carriers, high-pressure frozen and in the last step undergo FS.

structures are repressed [132]. As opposed to ice that typically expands when frozen, the sample's volume will remain the same.

Following HPF, the sample needs further processing to later be handled at room temperature. Whilst still frozen, a solution termed "freeze substitution cocktail" is added. The goal is to substitute all water in the sample with other, anhydrous, components. Simultaneously, the cocktail acts as fixative, creating crosslinks between molecules that keep them in place. Heavy metals additionally improve the sample's contrast by binding to select structures. In the papers discussed in the thesis, all samples are prepared with a freeze-substitution cocktail in which the only heavy metal contained is uranyl acetate (UA) [133]. It is a depleted uranium salt, that for example preferentially binds to phospholipids and nucleic acids [112], [134]. It acts both as fixative, and contrast agent. As a result, electrons hitting the specimen at theses structures will scatter in larger angles. During this freeze-substitution (FS) step, the samples can be brought to higher temperatures, as they are now fixed.

3.2.2 Room-temperature processing

For visualisation of samples, they need to be sufficiently thin. First, not too many structures of interest should overlap during imaging and the beam needs to be able to penetrate the sample. Additionally, a sample that is too thick promotes inelastic scattering (electrons losing energy when interacting with sample), which causes further damage to the sample. Thus, for further processing the sample is embedded in plastic and then sectioned to an appropriate thickness. There exist different types of resin for embedding, and I will here focus on Lowicryl resin, which was used for all samples analysed because it allows subsequent immuno-gold labelling on any sample (see also 3.2.3) [133], [135], [136]. Following the chemical cocktail, the biological samples are gradually infused with plastic, by increasing the ratio of plastic to solvent (acetone) in every step. When using Lowicryl, this occurs as part

of the FS, at $-50°C$. The pure resin is finally polymerised under UV light until hardened. The sample encased in resin, similar to a prehistoric insect encased in amber, can then be sectioned using microtomes. For thin-section imaging, thicknesses of 50-70 nm are common, and for tomography (consequentially performed with higher-power microscopes), 150-350 nm. The sections to be imaged are then collected on copper grids.

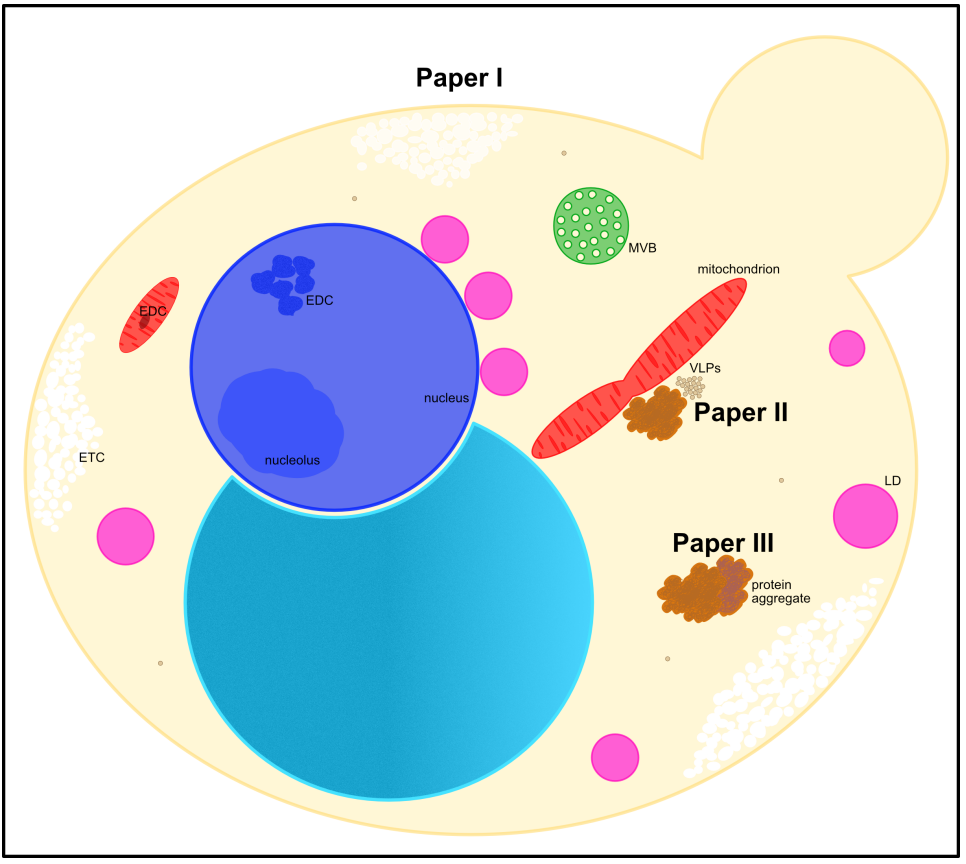
As a last step before imaging, grids can be post-stained to further add contrast to the final image. This usually happens by sequentially applying uranyl acetate and lead citrate solutions onto the section-containing grids directly. The grids are then ready to go into the microscope!

3.2.3 Immuno-EM

EM is a great tool to see many sub-cellular structures without labelling them directly. This way, spatial relationships between organelles and even membranes can be explored. When researching the localisation of for example a protein of interest, immuno-EM is a helpful tool that can be applied before post-staining [135]. Lowicryl resin preserves the antigenicity of the sample, and thus enables the use of immuno-EM labelling. This is performed before post-staining for added contrast. After brief on-grid fixation and blocking of the sample, primary antibodies that bind to the target structure are applied, followed by secondary antibodies binding to the primary ones. Attached to those secondary antibodies are small spheres of gold, around 10 nm across. These gold particles, due to their higher mass, are well-visible in the final image and allow localisation of the structure of interest. This can also be done on different protein types simultaneously, using gold particles of various sizes [137].

Chapter 4

Cellular Ultrastructure in Heat-Stress



4.1 Introduction

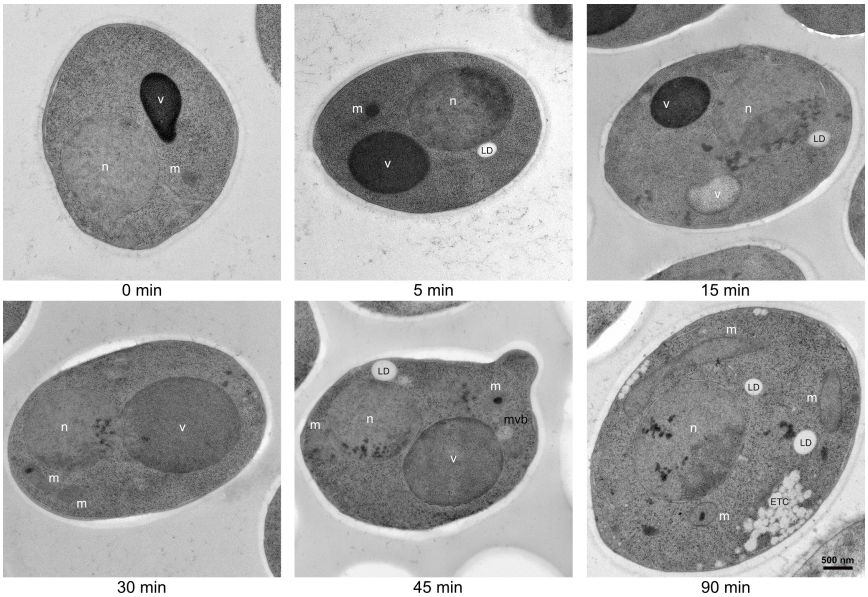


Figure 4.1: Representative TEM images of the yeast *Saccharomyces cerevisiae* from the HS time course at the selected time-points. Scale bar: 500 nm. Adapted from paper **I**.

Stress of different kinds impacts uni- and multi-cellular organisms in various ways. Many of the previously described approaches in yeast, explore changes that occur at molecular level. The more is uncovered about the biology of the cell during stress, the more previously unknown interactions, as well as morphological and molecular changes and components, are revealed. Large changes in the structure require input at a molecular level, and in turn cellular architecture can affect molecular components. Therefore, it is vital to place all those details within the larger picture. In papers **I** to **III**, various ultrastructural and mechanistic changes are explored and within the frame of this thesis, I shall place them into context. Paper **I** observes and quantifies changes at the organellar scale using TEM as primary imaging method. Yeast cells subjected to different lengths of HS at 38°C were cryo-immobilised, and processed for TEM (fig. 4.1). Thus, an insight into the cellular architecture unhindered by any labelling was possible. Paper **II** assesses the use of different reporters of misfolded protein to examine how the PQC handles misfolded proteins, their impact on cellular fitness and whether they are cleared in a similar manner. Lastly, in paper **III**, a work in progress, but near completion, virus-like particles (VLPs) and their role

in the cellular stress response are examined.

4.2 Organelles change in number and size

As basis for a quantification of changes in the cellular architecture, we have considered the number and size of organelles. This is based on a total of 2134 electron micrographs, and the analysis of up to approximately 2900 specimens per considered organelle (fig. 4.2 and 4.4). Below, I iterate the results of the quantified membrane-enclosed organelles.

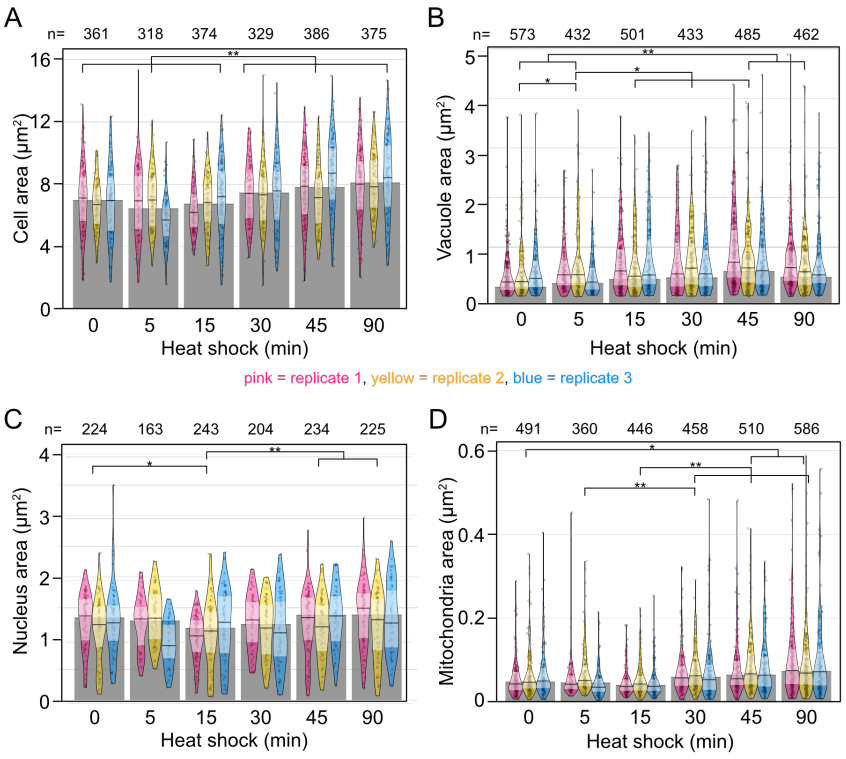


Figure 4.2: Development of cell and organelle cross-sectional area as measured in TEM micrographs. Cell (A), vacuole (B), nucleus (C), and mitochondria (D) area are shown in μm^2. Each colour shows a different biological replicate, individual dots represent individual cells, thick black line is the median, lighter areas the i.q.r., and grey bars are the time-point average. Adapted from paper **I**.

Throughout HS, the average area of the cell's cross-section increases by almost 20% over the course of 90 minutes (fig. 4.2). This change in size

mainly occurs between the 15 and 30-min marks, which are the time-points between which a significant difference begins to show.

When it comes to the nucleus, no significant changes in size have been observed between the beginning and end of the HS time course. A notable difference lies only in the 15 min time-point, at which the nuclear area in the section is reduced by 11% in comparison to untreated cells (time-point 0). As the HS progress, the nucleus returns to about its original size (fig. 4.2).

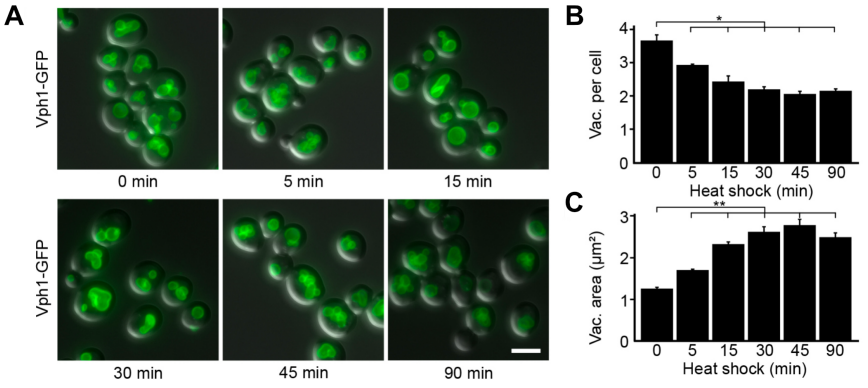


Figure 4.3: (A) Time-course of yeast cells expressing Vph1-GFP. Scale bar is 5 μm. (B) Number and (C) area of vacuoles throughout a 38°C HS at indicated time-points. Adapted from paper **I**.

The oftentimes largest organelle in the yeast cell, the vacuole, is greatly affected by HS. Its size increases throughout the first 45 min of HS (fig. 4.2). At its peak it is on average 69% larger than at time-point 0. Within the next 45 minutes, the cross-sectional area decreases again to almost 150% of its original size. The vacuole is a comparatively large organelle which can fuse or divide into fewer or more vacuoles, and so the results obtained from the TEM data were verified using FLM. There, cells expressing the vacuolar protein Vph1 tagged with GFP [138] were counted and measured. Vph1-GFP is a part of the vacuolar ATPase (V-ATPase) V_0 and found on the vacuolar membrane [139]. Strikingly, FLM showed that after 45 min of HS, vacuoles are 2.2 times as large as at the beginning of the HS time-course (fig. 4.3). Their size recovers towards the 90-min mark, similarly to TEM observations, but eventually they are still twice as large as in the control set. Analysis using FLM also allowed a more accurate evaluation of vacuole number during HS. Although vacuoles become much larger in response to stress, they also become fewer, suggesting a fusion of vacuoles. Overall, when multiplying the average vacuole number by the average vacuolar area as gathered by FLM

data, the total space occupied by the vacuole in the cell increases by 17% over the course of 90 min.

Mitochondria are also known to play a role in the cell's stress response ([50], [53]–[55], papers **II** and **III**). In a similar manner to the nucleus, the average size of the mitochondrion dips at the 15-min time-point at which it is reduced by 25% in size (fig. 4.2). At later time-points, the average size is significantly larger and by the end of the 90-min time-course, mitochondria are 50% larger than in untreated cells. The number of mitochondria in the cell does not vary significantly in TEM observations (Paper **I**, fig. S3A), which suggests that overall mitochondrial volume increases during HS.

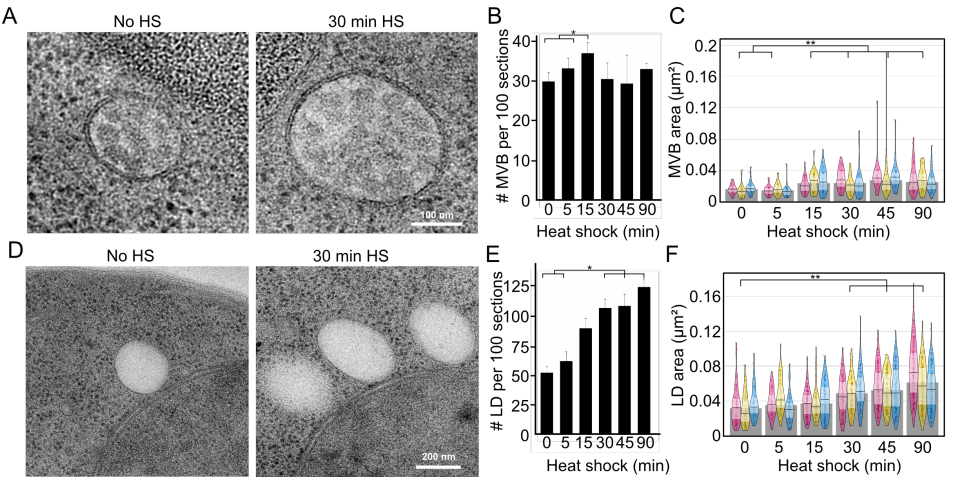


Figure 4.4: (A) MVBs before and after 30 min of HS. Scale bar: 100 nm. (B) Number of MVBs per 100 cell sections. Error bars are s.e.m. (C) Area of MVB cross-sections over a 90-min time-course. (D) LDs before and after 30 min of HS. Scale bar: 100 nm. (E) Number of LDs per 100 cell sections. Error bars are s.e.m. (F) Area of LDs over a 90-min time-course. Adapted from paper **I**.

Another organelle which is affected by HS is the MVB. At the 15-min time-point, MVBs were 24% more numerous than in untreated cells. Then, they are also already significantly larger than at the beginning of the time-course. After 90 min, MVBs are 73% larger (fig. 4.4).

Lastly, LDs undergo a stark change during HS. They are 2.4 times as numerous after 90 min of HS and are first significantly larger after 30 min of stress. They eventually come to be 85% larger than in their initial state after 90 min (fig. 4.4). Taken together, LDs take up more than 4-fold the amount of space after 90 min of HS than when cells are kept at 30°C.

Most organelles become larger throughout HS by an amount that is not

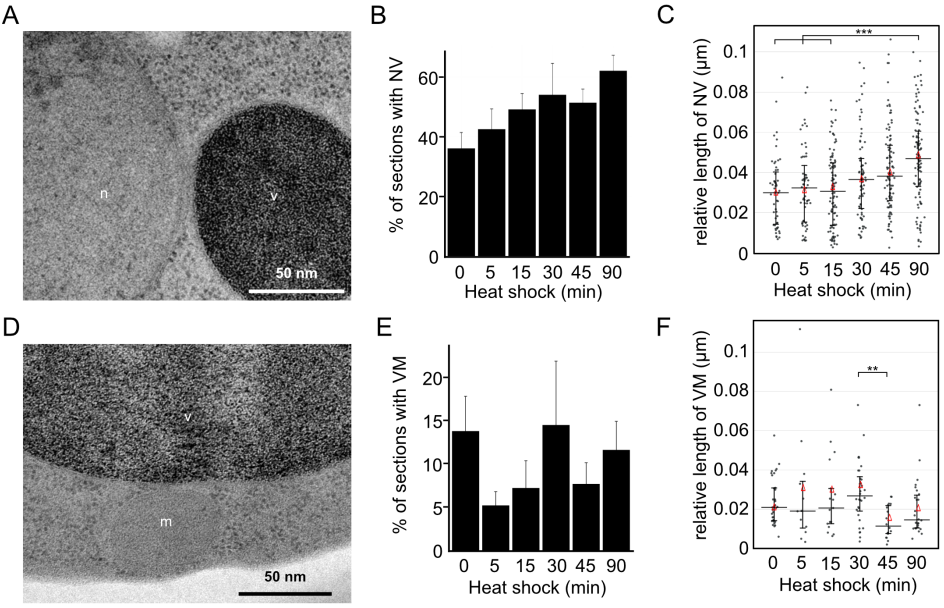


Figure 4.5: (A) MCS between nucleus (n) and vacuole (v). Scale bar: 50 nm (B) Proportion of sections containing both a nucleus and at least one vacuole with a respective MCS. (C) Length of contact site taking into account circumference of nucleus and vacuole. (D) MCS between vacuole (v) and mitochondrion (m). Scale bar: 50 nm (E) Proportion of sections containing both at least one vacuole and one mitochondrion with a respective MCS. (F) Length of contact site taking into account circumference of vacuole and mitochondrion. Adapted from paper **I**.

explicable solely by the increase in overall cell size, and so it can be expected that MCSs would also increase. During the first 15 min of the HS time-course the length of the MCSs between nucleus and vacuole correspond to their expected values (Paper **I**, fig. S5A) when considering the change in size of the respective organelles. However, by 90 min, the length of the MCS has increased beyond their expected value, resulting in a 57% increase taking into account organelle circumferences. Quite similarly, when observing MCSs between vacuoles and mitochondria, during the first 15 min, the change in length of the vacuole-mitochondria contact site corresponds to expected values, considering enlarged organelles. At 45 min, the MCS length is the lowest, even though it is the time-point at which vacuoles are the largest, both in TEM and FLM observations. Due to the shape of both organelles, contact sites between vacuoles and mitochondria are harder to quantify in TEM. Mitochondria are oblong, branched structures and, as well as vacuoles, may undergo fusion or fission. Seeing through only a thin section of the cell can hide or exaggerate interactions between the two organelles, highlighting the importance of a quantitative approach at an ultrastructural scale. Nevertheless, the results presented in paper **I** suggest that there is a specificity in the change of MCS length during the cell's stress response.

Besides the aforementioned changes, certain organelles' internal morphology was also affected, and new structures in the cytoplasm were uncovered.

4.3 Both organellar and cellular morphology is altered

Of the above mentioned organelles, three large ones: the nucleus, mitochondria and vacuoles not only undergo changes in size, and partially number, but also show different morphologies throughout the HS time-course. As the nucleus experiences a size decrease, many dark-looking structures, here termed electron-dense content (EDC), appear in the nucleus. It can be found in around three quarters of nuclei, and its presence peaks at 30 min and decreases slightly by the 90-min mark (fig. 4.6). EDC is different from the nucleolus and heterochromatin present in the nucleus [140]–[142], and is mostly found outside the nucleolus (Paper **I**, fig. S2). When observing the total EDC area over the HS time course, it does not have an impact on the nucleolar area (unpublished observations), which suggests that the EDC's origin does not stem from the nucleolus. Indeed, the reporters of misfolded protein Hsp104-GFP and guk1-7-GFP localise to the EDC in stressed cells (Paper **IV**).

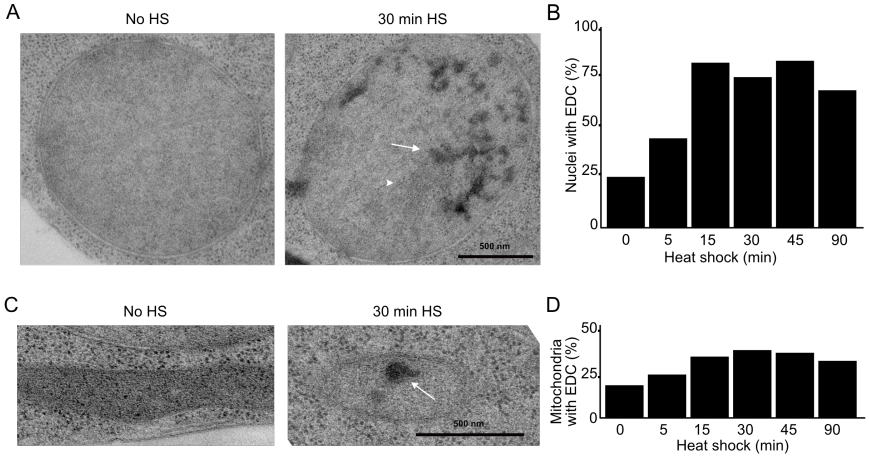


Figure 4.6: (A) A nucleus before and after 30 min HS, displaying EDC (arrow) besides the nucleolus (arrowhead). (B) Proportion of nuclei that contain EDC. (C) A mitochondrion before and after 30 min HS, displaying EDC (arrow). (D) Proportion of mitochondria containing EDC. Adapted from paper **I**.

In the same manner, EDC also accumulates within the mitochondria, although less frequently. The proportion of mitochondria containing EDC increases until it peaks at 30 min and then decreases again slightly. The overall proportion of EDC-containing mitochondria lies at about 40% at its maximum (fig. 4.6).

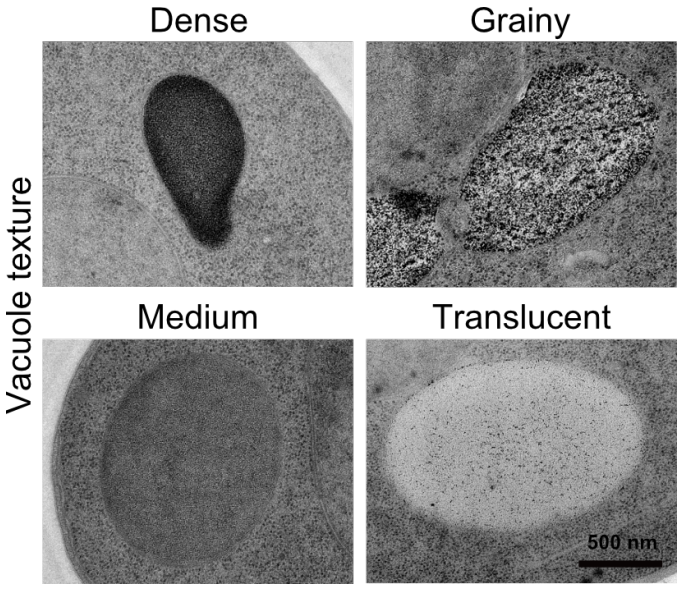


Figure 4.7: Different vacuolar electron densities characterised during the 38°C HS time course. Adapted from paper **I**.

Variations were also found in the electron density of vacuoles. They were classified into 4 categories, comprising dense, medium(dense), translucent and so-called "grainy" vacuoles (fig. 4.7). Those considered grainy did not have a uniform colouration, but a rough-looking texture. On one hand, variations can be quite large between replicates, suggesting that the preparation method influences final vacuolar electron-density. On the other hand, where all samples within one biological replicate belong to the same technical replicate, it was possible to draw conclusions on the change in vacuolar electron-density during HS. It is then noticeable that it fluctuates throughout the time-course and tends to become lighter in appearance. In the same vein, when comparing mother-daughter pairs of cells, vacuoles in the mother-cell always are either equally or, more frequently, more electron translucent than in the daughter cell (paper **I**, fig 3). It has previously been demonstrated that the pH of vacuoles in daughter cells is lower than that of mother cells [143], [144], and so a possible correlation between vacuolar electron-density

and acidity was verified by analysing mutants that possess either abnormally acidic or basic vacuoles. The usual pH of a vacuole lies between 5 to 6.5 [26], [145], [146], with a median value of 5.3. The deletion of *PRB1*, a vacuolar protease, causes more acidic vacuoles than in WT cells [147], which is seen in electron micrographs as highly electron-dense vacuoles. On the other hand, deletion of *VMA2*, part of the V-ATPase subunit V_1, leads to an increase in vacuolar pH, which also reduces vacuolar protease activity [147]. In both mutants proteolytic activity is inhibited, leading to vacuoles full of undigested cellular components [147], [148]. This excludes low vacuolar staining from being caused by a lack of content. Additionally, using pH-sensitive fluorescent dyes, the pH of the vacuoles in FLM have shown a change in the mutants selected for TEM analysis and also in heat-shocked cells. Besides changes in electron-densities, including varying degrees of electron density within the vacuole and the presence of grainy precipitates, vacuoles also show an array of deformations (Paper **I**, fig. S1A). These include indentations, invaginations, presence of cytoplasmic material in the vacuole, engulfing of LDs and MVBs, and leaking. It is unclear whether this points to increased or decreased vacuolar activity in the cell during HS. For UA to be used as pH indicator in TEM, which results around vacuolar electron-density and pH point to, a standardisation of the protocol would be an interesting future development.

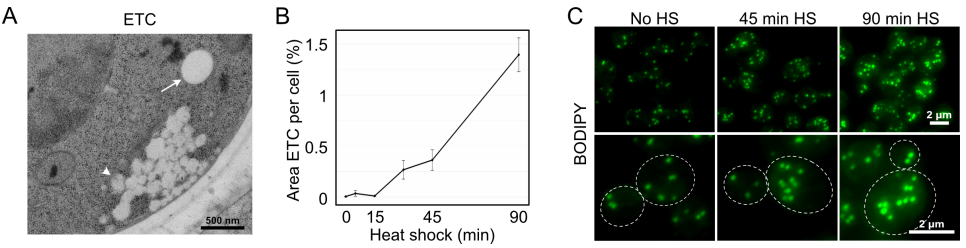


Figure 4.8: (A) ETC (arrowhead) near the plasma membrane, with different morphology from the LD (arrow). Scale bar: 500 nm. (B) Area of the cell occupied by ETC throughout HS progression (C) FLM maximum projection images of yeast cells stained using BODIPY. Scale bar: 2 µm. Adapted from [68].

There also are changes happening in the cell cytoplasm. For example, electron-translucent clusters (ETC) appear throughout the cell as HS progresses, whereas these structures are barely seen in undisturbed cells. Most ETC localise near the plasma membrane (paper **I**, fig. S1A). By 90 min of HS, 89% of cells analysed contain ETC, and the area they take up increases ten-fold over the time-course (fig. 4.8). Eventually, coming in at about 1.4% of the total cell area, it is comparable to the area occupied by MVBs in

the cell and I suggest that it is non-negligible. UA, which is used in the FS cocktail, preferentially binds to proteins and nucleic acids, and the electron translucency of the clusters indicates that neither of these structures is present. A follow-up question is thus whether these structures are, or come from LDs, which are also electron translucent in TEM images. However, ETC and LDs have different morphologies. LDs are covered by a layer of phospholipids, that clearly delineate them from the cytosol in electron micrographs. This is not the case in ETC, as they appear in cloud-like clusters. Additionally, LDs primarily localise to the cell nucleus (the ER being their site of production [149]) whereas ETC do not (paper **I**, fig. S4). In our observations of FLM images in which neutral lipids, which are contained in LDs, were stained, no structures alike the ETC were visible at the periphery of the cell. ETC appear in the cell as a consequence of HS, but it remains unclear what they are composed of, although some components can be ruled out.

Lastly, data from Paper **II** reveals that the accumulation of virus-like particles (VLPs) in the cell is reduced throughout HS. VLPs appear as small, circular structures with an electron dense periphery of approximately 18 nm in diameter. For the quantification, two VLPs in close proximity with no other cytoplasmic material between them, were considered clustered. Overall, the total number of VLPs is reduced throughout HS (Paper **II**, fig. S1). However, the number of individual VLPs found in the cytoplasm does not vary, but the number of clustered VLPs is reduced. Results from co-localisation studies in FLM shows that both VLPs and protein aggregates localise near mitochondria (paper **II**, fig. 2A, 3C-D, S3F,G). When using immuno-EM, the protein guk1-7-GFP, known to misfold at high temperatures, is found at these aggregates.

4.4 Localisation of protein aggregates

As described in section 2.2.3, there exist several deposition sites for protein aggregates throughout the cell. When monitoring protein aggregate behaviour in the cell based on the localisation of the disaggregase Hsp104-GFP, it becomes apparent that under stress, misfolded or damaged proteins first aggregate to several smaller inclusions. Under continuous stress, these inclusions coalesce to become fewer and larger. In electron micrographs, areas in the cytoplasm immuno-labelled to detect Hsp104-GFP are devoid of ribosomes, and oftentimes also more electron-dense than the surrounding cytoplasm (paper **II**, fig. 1). Furthermore, these aggregates change local-

isation throughout HS. After 15 min, around 30% of aggregates are in the vicinity of mitochondria and ca. 55% are considered cytosolic, with no proximity to any specific cellular structure in the micrographs analysed here. By 90 min, almost every cytoplasmic inclusion is found next a mitochondrion. Albeit less preferentially, the aggregates also localise close to the nucleus (in accordance with [55]), vacuoles, and LDs throughout HS.

Whilst Hsp104-GFP is a useful accomplice to track the genesis and fate of protein aggregates, it is worthwhile to use misfolded proteins directly. Many of the commonly used reporters are proteins not native to yeast, e.g. TDP-43 which is connected to amytrophic lateral sclerosis [150], whose expression requires induction using additional treatments. This can lead to a change in cellular physiology, or toxicity due to the nature of the proteins [110]. Alternatives should ideally be non-toxic to the cell, easily labelled using a common fluorescent tag, misfold under controlled conditions and, where spatial PQC is of interest, aggregate within the cell. A shift in temperature can rapidly induce protein aggregation of misfolding reporters in temperature-sensitive (ts) alleles. The proteins guk1-7-GFP, pro3-1-GFP, and gus1-3-GFP can be constitutively overexpressed without any evidence of toxicity, making them ideal candidates for this task (paper **III**). These contructs permit the investigation of differences in aggregation and disaggregation between proteins with no apparent effects on cellular function.

4.5 Ultrastructural differences between inclusions

The three selected temperature-sensitive proteins, guk1-7, pro3-1, and gus 1-3, are often found in the same aggregate within the cell (Paper **III**, fig. 1D). Interestingly, the protein gus1-3-GFP co-localises with the other two reporter proteins, but also forms individual inclusions (Paper **III**, fig. 1E). The presence of different types of misfolded proteins in the same aggregate brings up the question whether they are intermixed or in distinct compartments in the inclusions. Analysis with structured illumination microscopy (SIM), and verification with double-immuno TEM, showed that the different proteins appear evenly distributed within the inclusion, with no spatial separation between them. It is surprising then, that different misfolding proteins evenly distributed in the same inclusions can be degraded at different rates. The protein pro3-1-GFP is cleared faster than gus1-3-GFP and guk1-7-GFP (paper **III**, fig. 2A). Consistent with this, there are also differences in proteasome-dependent processing of the different reporters for misfolded proteins. All three reporter proteins require Hsp104 for clearance

of their respective inclusions although the efficiency of Hsp104 recruitment differs between reporters. An Hsp70 double deletion mutant (ssa1Δssa2Δ) had a smaller effect on the coalescence of protein aggregates than the lack of Hsp104, but also resulted in much larger inclusions. In the case of pro3-1-GFP, the ssa1Δssa2Δ mutant also showed an increase of aggregates at 30°C, despite being stable in an rpn4Δ mutant that has lower proteasome activity.

4.6 Summary and Discussion

The chosen approaches, specifically the use of TEM for quantitative ultrastructural analysis, allowed us to gain insight into the detailed cellular architecture and mechanics. Thin section TEM of high-pressure frozen, freeze-substituted samples enables the acquisition of nanometre-resolution images of whole cells without the use of any labelling to recognise cellular structures. Where TEM imaging was limiting, for example when studying large organelles such as vacuoles, other complimentary imaging methods, such as wide-field FLM and SIM were used. These techniques allowed the localisation of proteins aggregates in the entire cell (papers **II** and **III**) and the quantification of changes of large organelles (Paper **I**). This highlights the importance of multi-faceted approaches.

Accordingly, using this combination of techniques we could reveal structural changes both on a cellular and organellar level during HS. For example, the cell becomes larger overall, so do vacuoles, mitochondria, MVBs, and LDs. It also reveals that LDs more than double in number, ETCs appear, there exist fewer VLPs in clusters and gives information on the behaviour of protein aggregates throughout HS. Certain structures, such as EDC in the nucleus and mitochondria, recover toward the end of the 90-min HS time-course whereas others experience a dip around the 15-min mark. The nucleus, mitochondria and LDs dip in their size development, even though the latter two later continue to increase in size. Lastly, we have gained information of the localisation and behaviour of protein aggregates, which are a hallmark of proteopathies and an important tool in deciphering the mechanisms behind this group of diseases.

Almost all analysed organelles increase in size (Paper **I**), which could require the cell itself to become larger, but it did so to a lesser extent than what actually was measured. From a biophysical standpoint, it could be expected that upon heat, cells, which mostly contain water, would swell up, especially when taking into account an increase in membrane fluidity. However, from the appearance of electron micrographs, the cell did not seem less crowded as

ribosome-density seemed unchanged, although a future quantification would be interesting. Upon energy-depletion, ribosome density does in fact increase [151], representing an increase of crowding in the cytoplasm. An increase in cell size may also indicate an arrest of the cell cycle as the cell's resources are diverted to the more urgent stress response. Cell stress can cause cell cycle arrest in the G_1 phase [152], which would lead to a higher percentage of G_1-phase cells in the culture, reduced replication and to a reduced population of daughter cells. If cellular replication is slowed down, the average age of a cell in the culture will increase, and with this, the average size.

The nucleus, whose size changes in relation to overall cell size, but in a manner that is cell-cycle dependent under normal growth conditions [6], [153], remains fairly constant throughout the HS time-course. Within the nucleus, an accumulation of misfolded proteins is visible and peaks at 45 min. This reinforces the nucleus' role in PQC. An import of misfolded proteins into the nucleus has been proposed previously [154]. The nucleus' transport pathways and possible roles in PQC will be further elucidated in chapter 5. Interestingly, an increase in protein content of the nucleus does not automatically entail an increase in size [6]. This may suggest that if the total protein mass in the nucleus increases, it is compacted in a space-efficient way. It would be interesting to study the biophysical effect of a change in nuclear protein mass, and whether it relates to their sequestration. The nucleus is in physical contact with the vacuole, and during the first 15 min of HS, nucleo-vacuolar MCSs are merely sustained but further into HS they increase. This may point to an upregulation in transfer between the two organelles, either through the membrane directly or via an increase in PMN [39], as a direct support mechanism by the vacuole in protein management during stress. The NVJ is also a site for LD localisation and involved in lipid metabolism [41], [42]. This reflects the changes that the cell's lipid metabolism undergoes during stress.

Vacuoles undergo many changes during HS [155]. They become fewer, larger, and change in morphology. Additionally, their pH increases during the stress response. Cells in which a V-ATPase subunit is either missing or has been inhibited by the V-ATPase-specific drug concanamycin show few, large vacuoles [156]–[158]. It is the same case in vph1Δ cells, missing the V-ATPase subunit V_0, that are treated with concanamycin to abolish any remaining vacuolar acidification. Baars *et al.* suggest that vacuolar morphology can be dictated by the V-ATPase, which is responsible for proton flux across the vacuolar membrane [159]. Larger vacuoles can also indicate activity of an additional protein degradation machinery: autophagy. Indeed, it is upregulated in various forms of stress [160], [161]. Although this points

to vacuoles supporting the UPS in protein degradation, their subsequent deacidification also impairs their function [143]. This may however be a mechanism to regulate vacuolar function in a biochemical fashion throughout the HS time-course. Total vacuolar area decreases between the 45 and the 90 min time-point as measured in FLM images, which correlates with the time-point where vacuolar electron-density (and inferred pH) decrease again.

It is also around this time that EDC in nuclei and mitochondria are reduced and most Hsp104-GFP labelled protein aggregates have coalesced (Paper **II**, fig. 1A). A previous assumption that we made in paper **I** – that vacuolar deacidification could support a drop in cytoplasmic pH necessary for the induction of HSF1 [25], does not necessarily hold up when comparing to the induction time of HSF1. The gene is induced within the first 20 min of HS [25] whereas vacuolar electron-density, and implicitly its pH is mainly altered after 45 min of HS, when transcription of HSF1 has already plateaued [162]. Nevertheless, it has been suggested that vacuolar ATP-ases do assist the plasma membrane in regulating intracellular pH [163]. A combination of ultrastructural resolution, novel pH probes, and a time-course experiment could shed some more light on the role of the vacuolar membrane and acidity during the HS response.

Organelles whose final destination includes the vacuole are MVBs. Their exact involvement in the HS response is unclear, although they are clearly affected as organelles as their increase in size is steep. In the fission yeast *Schizosaccharomyces pombe*, the size of MVBs has been shown to increase with the number of ILVs [164] and similar observations have been made in the plant *Arabidopsis thaliana* [165]. In *S. pombe*, it possibly correlates with the cell size and/or cycle, as MVBs are also affected by the cell entering the mitotic phase [164]. I suggest that in *S. cerevisiae*, the number of ILVs also correlates with MVB size, although this remains to be shown. MVBs support proteasome-independent protein degradation, the demand for which increases during stress, so why do MVBs become bigger but not more numerous? It is unclear whether endocytosis is partially inhibited in yeast during HS [166], but this could provide a possible explanation for an increase in size (and thus activity) of MVBs, but without the appearance of new early endosomes, their precursor. MVBs become much larger between 30 min to 45 min, which could be due to the impairment of vacuolar function and thus fewer MVBs being engulfed. Further, an increase in autophagic activity could mean that many different cellular components (cytoplasm, LDs, MVBs, ...) are taken in by the vacuole, generating a backlog of material for import into the vacuole and subsequent degradation.

Vacuole-mitochondria MCSs do not increase in correspondence with their

general increase in organelle size and increased crowding within the cell. That not all MCSs are increased during HS is an additional indication for MCS specificity, but could also be to avoid mitochondrial deterioration, which has been linked to vacuolar deacification [143]. Interestingly, it has been observed that specific MCS between vacuole and mitochondria (vCLAMP) and between the ER and mitochondria (ERMES) are on opposite ends of a balance [43]. Previous studies have observed a decrease in vCLAMP by deletion of *VPS39* being followed by an increase in ERMES. This suggests that MCS between the nucleus, and the connected ER, to other organelles may be promoted during HS.

Mitochondria also dramatically increase in size throughout HS. This could be due to an increase in ATP-production, its role in the potential cell cycle arrest, its increased participation in PQC, or a combination of some or all of these factors. A possible explanation for the presence of EDC in the mitochondria could be misfolded mitochondrial proteins [167]–[169], or even an import of cytosolic misfolded proteins, destined for degradation [50]. The involvement of mitochondria in sequestration and degradation of misfolded proteins is supported by the localisation of protein aggregates near the organelle (Paper **III**). Additionally, the tethering of protein aggregates to mitochondria have been proposed as a way for the cell to retain misfolded proteins in the mother cell upon division [170].

Mitochondria are also involved in the cell's metabolism and the synthesis of various phospholipids [171], [172]. The large effect of HS on the lipid metabolism of the cell is also reflected in the considerable change in LDs, which end up doubling in both size and number. Furthermore, the surface of LD are covered in proteins, some of which have been found to facilitate aggregate clearance [35], [36].

ETC have previously been observed [151], [173], [174], and on occasion also likened to LDs, despite their morphology being quite different. The FLM performed did not reveal any indication that the ETC contain neutral lipids. A possible explanation would be the presence of carbohydrates, which are not stained using our TEM preparation protocol. It has also previously been shown that the sugar trehalose accumulates in the cell upon stress [175], [176]. Alternatively, the ETC could be extra-cellular material that has been imported by the cell. For example, it has been shown that the cell relies on the import of extracellular protons for acidification of the cytoplasm that is required to induce the HS response [25]. During stress, the plasma membrane increases in fluidity, meaning fewer unsaturated lipids are part of the lipid bilayer [177]–[179]. Should the ETC consist of lipids, it could be a deposit of unsaturated lipids required to uphold membrane rigidity in

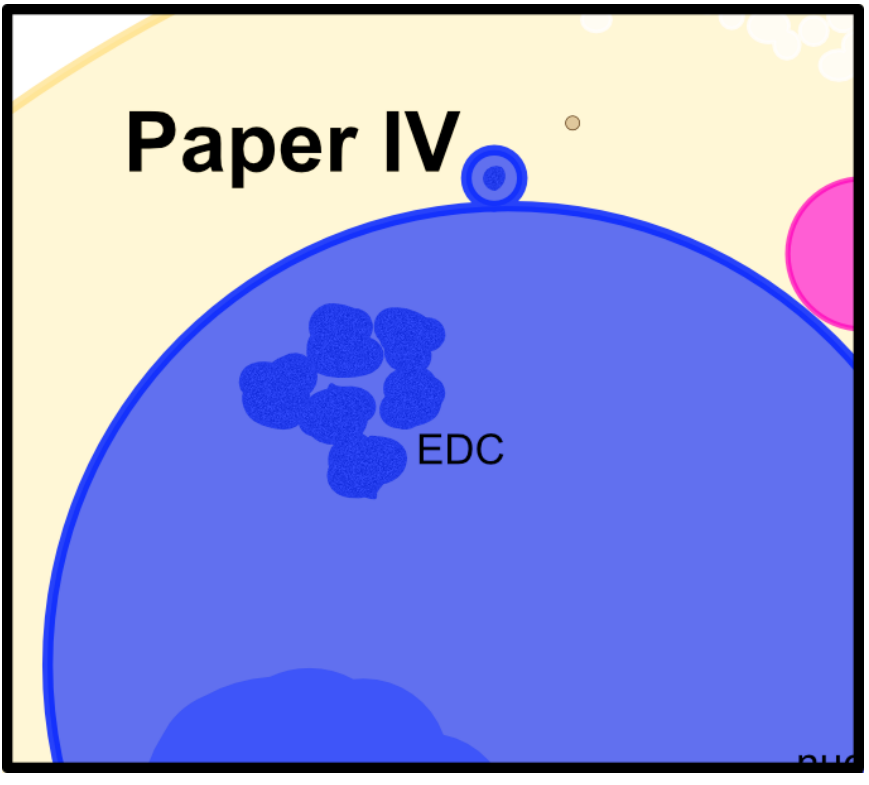
non-stress conditions.

Further, other non-membraneous structures that occur in the cell are protein aggregates. They are often found in proximity to mitochondria, especially as HS progresses (Paper **II**). Even though the aggregates can contain different intermixed protein species, those may be cleared at different speeds. The three reporter proteins analysed here, guk1-7-GFP, gus1-3-GFP, pro3-1-GFP are cleared by common PQC pathways, notable involving the chaperone system consisting of Hsp104 and Hsp70 (Paper **III**). Nevertheless, the protein pro3-1-GFP is cleared more rapidly from the rest. This could be related to its higher stability, as it is stable at 30°C (Paper **III**), and could be more easily re-folded. Interestingly, the proteins being intermixed in the aggregates did not affect their individual clearance efficiency (Paper **III**), further pointing to processing by different systems.

Overall, this thesis contributes to what could be an atlas of all cellular, organellar and some molecular changes in the cell during HS reveals the interconnectivity of cellular components.

Chapter 5

Nuclear Envelope Budding as a Response to Stress



5.1 Introduction

The previous chapter outlines some of the many ultrastructural changes that occur in response to HS. Organelles fuse or swell, and proteins are misfolded, sequestered, and degraded or re-folded (Papers **I** to **III**). In this chapter I will zoom in to one unexpected cellular reaction to stress. In yeast, the nuclear envelope undergoes a specific remodelling that appears as budding of the ONM towards the cytoplasm. Oftentimes, material is found between the nuclear membranes, which we interpret as cargo (Paper **IV**). These nuclear envelope budding (NEB) events were present during all conditions, but with increased frequency during cell stress. Lastly, in paper **V**, a review article, different forms of NEB are iterated and presented as possible routes for nucleo-cytoplasmic transport (section 5.3).

5.1.1 History

Such deformations of the nuclear membrane have previously been observed in electron micrographs, and some have speculated for them to consist of RNA [180]. As early as the 1950s, they have been observed by Helen Gay in salivary glands of the fruit fly *Drosophila melanogaster* [181]. Throughout the years, more observations were made in unicellular organisms [182], plants [183]–[186], sea urchin [187], rodents [188], [189], and mammalian cells [190], [191]. In the process, NEB events were given many different names (Paper **IV**, Supplemental material, Table 1). Approximately a decade ago, NEB was demonstrated to be a physiological process in fruit fly muscle cells that serves as transport of RNA granules [192]. Most recently, it was observed to be involved in the cellular stress response in *Saccharomyces cerevisiae* (Paper **IV** and [193]). Herein, we will explore the role of NEB during cellular stress, and whether it could be an alternative route of transport to NPCs.

5.2 Nuclear Envelope Budding and Stress

In yeast cells stressed with a mild HS (38°C), 10.3% of sections containing nuclei show NEB events. This is a little more than a four-fold increase compared to the about 2.4% of nuclei in cells grown at 30°C showing similar structures (fig. 5.1A). During a stronger HS (42°C for 30 min), the NEB events are even more frequent (16.6% of observed nuclear sections). Yeast cells behave similarly when applying other types of stressors. Subjecting them to treatment with sodium arsenite, a salt containing the heavy metal arsenic (see 2.3.2), yields an increase in NEB compared to untreated cells by

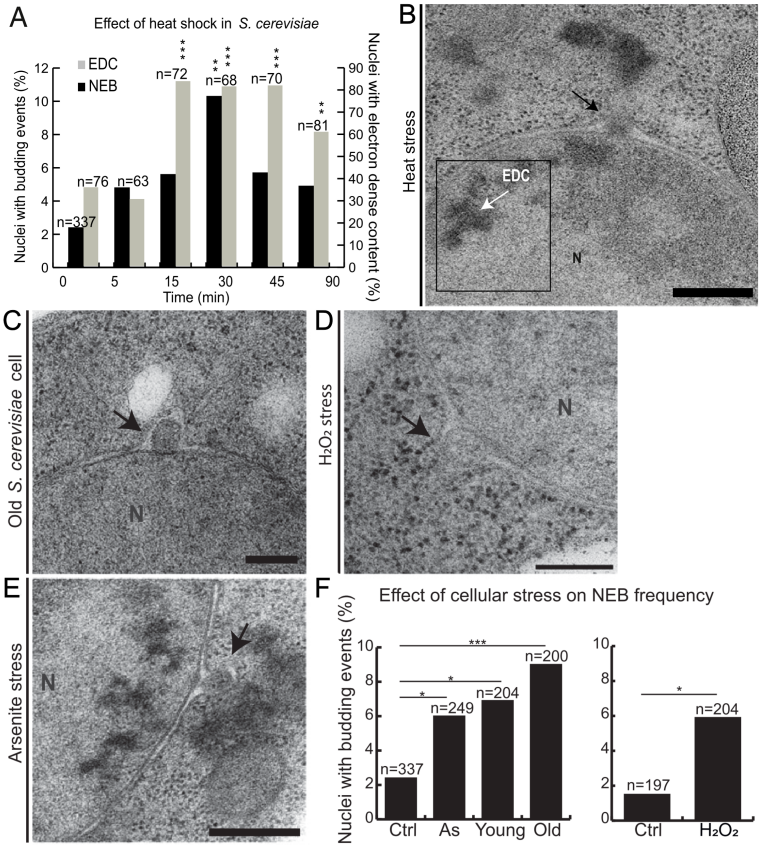


Figure 5.1: NEB is increased in yeast cells exposed to various stressors: heat (A-B), age (C), H_2O_2 (D) and Arsenite (E). In stress, all NEB event frequencies lie above 4% (A and F). Nuclear EDC increases throughout HS (A-B). Adapted from paper **IV**.

almost 3-fold. When stressing cells with H_2O_2, they exhibit similar effects and the frequency of NEB is nearly as high as in sodium arsenite stress. Lastly, aged cells also differ from young cells, and significantly so from entirely untreated cells, coming in at a frequency of about 9% of NEB. For the collection of aged cells, the cellular membrane is biotinylated, cells are left to grow over several days and older cells are magnetically separated from younger cells after the addition of streptavidin-coated magnetic beads of the course of several isolations [194], [195]. During the last isolation process, the budded off, uncoated cells are kept and referred to as the young cell population. Despite the significant difference between aged cells and entirely untreated cells, there is no significant difference to the young population of cells that have undergone the same separation process. The statistically significant difference between young and entirely untreated cells, taken together with previous results and ultrastructural observations, however suggest that young cells have also experienced stress during the isolation process. A common feature of all stressors mentioned above: heat, heavy metal, and oxidative stress as well as ageing is their causation of misfolded proteins. Additionally, a higher level of NEB in young cells compared to entirely untreated cells could be an indicator that mechanical and/or cold stress induces a similar stress response.

5.2.1 Misfolded Protein in the Nucleus

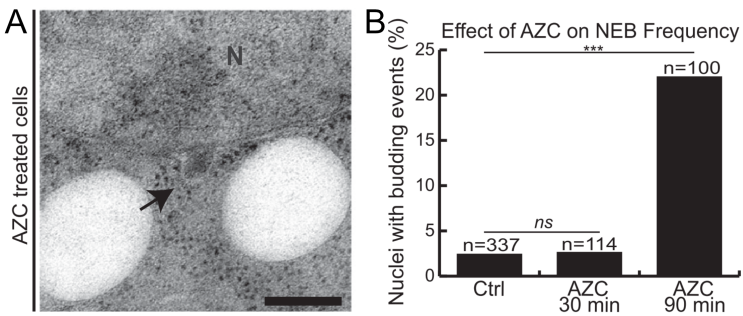


Figure 5.2: Treatment with AZC leads to increased NEB (A-B). Adapted from paper **IV**.

When inducing misfolding of newly synthesised proteins in the cell using AZC (see 2.3.2), the rate of NEB was highest, at 22%.

During HS, the peak increase in frequency of NEB at 30 min coincides with the presence of EDC in the nucleus. It was then investigated whether

the EDC – previously described as structures in the nucleus that are distinct from other nucleolar and nuclear components (see 4.3) – consists of misfolded proteins. Using immuno-labelling (see 3.2.3) on electron micrographs, it was found that the misfolding protein reporter guk1-7-GFP (Papers **II, III**), which is also known to localise to the nucleus [196], and the protein disaggregase Hsp104-GFP were found primarily in the nuclear EDC, and not in the remaining nucleoplasm. This confirms that the EDC consists of protein aggregates.

Ubiquitination marks misfolded proteins for degradation by the proteasome [197]. We have used an antibody against ubiquitin that identifies both free ubiquitin and ubiquinated proteins, and has a stronger affinity to poly-ubiquitin chains than monomeric ubiquitin. This immuno-labelling revealed that both EDC and NEB events had increased labelling in comparison to the negative control (LDs). Additionally, the nucleoplasm had similar labelling density to the negative control (LDs), indicating that ubiquitin is not generally spread out throughout the nucleus but concentrates in the EDC. The HS protein Hsp104-GFP is required during stress and disaggregates misfolded protein for re-folding [198], [199]. It also localises to both EDC and NEB events, with a gold-labelling density at least 70-fold higher than that of the negative control (LDs). This strongly suggests that EDC and NEBs contain misfolded proteins labelled for degradation.

If NEB serves as transport for misfolded proteins, inhibition of the proteasome should cause an increase in NEB events. Indeed, this occurs both during chemical and genetic inhibition of the proteasome. The drug MG132 is frequently used to induce proteasome inhibition, however in yeast cells the gene PDR5 confers multiple-drug resistance, including resistance to MG132 [200], [201]. Upon the addition of MG132 to pdr5Δ cells, the proteasome is inhibited and NEB frequency increases to 7%, whereas untreated pdr5Δ cells have a NEB frequency similar to WT. Comparatively, inhibiting the proteasome by deleting the transcription factor RPN4 that stimulates expression of proteasome genes also significantly increased NEB frequency to 15%. As such, stronger inhibition of the proteasome relates directly to a stronger increase in NEB frequency observed in the cell.

TEM is a static imaging method for which samples are necessarily fixed and cannot be observed under live conditions. This complicates the investigation into NEB as possible transport system using live cell FLM imaging, as it occurs at such a small spatial scale. It is however not unlikely that the cell, and the nucleus specifically, rely on a protein clearance system distinct from the UPS for times in which the primary system would be disabled or overloaded. Furthermore, it could accompany the management of the

intranuclear spatial PQC deposit site INQ. Nevertheless, NEB events have been observed to participate in nucleo-cytoplasmic transport in more ways than one.

5.3 NEB and Transport

Destination degradation – all aboard the NEB train? Transport of misfolded proteins between nucleus and cytoplasm via NEB has yet to be observed in live cell imaging, even though there are strong indications for its existence. It has however been shown that other cargo can be transported across the nuclear envelope, including INM components, RNA granules, and viral material [192], [193], [202]–[206]. For the sake of completeness, I will briefly introduce other cargo that has been found in NEB events by other research groups, and which are summarised in the review, paper **IV**.

5.3.1 INM cargo

Autophagy is another PQC mechanism of the cell, and can be induced in yeast by treating the cells with the drug rapamycin or by nitrogen-starvation. Under both conditions, NEB and other protrusions of the nuclear envelope were observed [202], [204] and the arising structures are later enveloped by the vacuole. Both cases require the autophagy receptor Atg39, a transmembrane protein between the ONM and INM [207] that binds the cargo for transport. At ultrastructural level, either both nuclear membranes bud outwards and are then sectioned off from the nucleus in one single event [204], or the cargo is first sequestered into an INM vesicle that buds off into perinuclear space and later into the cytosol [202] (fig. 5.3). In both cases, a double bilayer vesicle is released into the cytoplasm that is later destined for autophagic removal. However, we have seldomly observed double bilayer vesicles in proteotoxic stresses (paper **IV**). Interestingly, NPCs are not found in regions in which NEB occurs [208], supporting the idea that NEB corresponds to a different PQC pathway.

5.3.2 RNA granules

From the first observation of NEB events onward, it has been suggested that they would serve the transport of nucleic acids [181], [209]. This was later confirmed in neuromuscular junction cells of the fruit fly where foci of the protein DFz2C enveloped by nuclear lamina, a dense filament network underlying the INM, were found at the nuclear periphery [192]. Ultrastructurally,

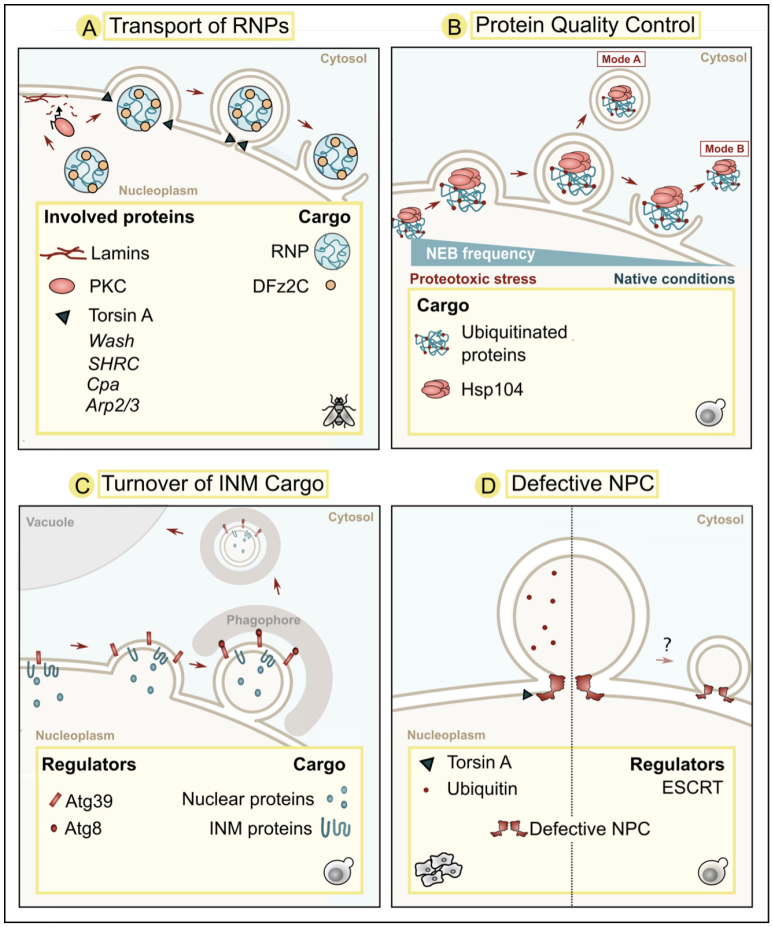


Figure 5.3: Different types of NEB observed to date. (A) Transport of ribonuclear proteins/RNA granules, (B) NEB in PQC, (C) INM cargo transportation for degradation by the vacuole, (D) formation of nuclear envelope herniations around defective NPCs.

these occurrences show as inward deformations of the INM containing round, electron-dense granules surrounded by a membrane and about 200 nm in diameter. The granules contain proteins and RNA transcribing scaffolding proteins required in the neuromuscular junction synapse [192], [210]. It is hypothesised that they serve the delivery of mRNA for on-site protein translation, but it is also possible that they are destined for degradation. In salivary glands of *Drosophila melanogaster*, some molecular players in NEB formation have been identified, including Torsin A [193], [205]. It is worth noting that yeast cells do not posses a nuclear lamina, nor Torsin A, implying that the two NEB mechanisms might be different or have evolved using different factors.

5.3.3 Herpesviral Egress

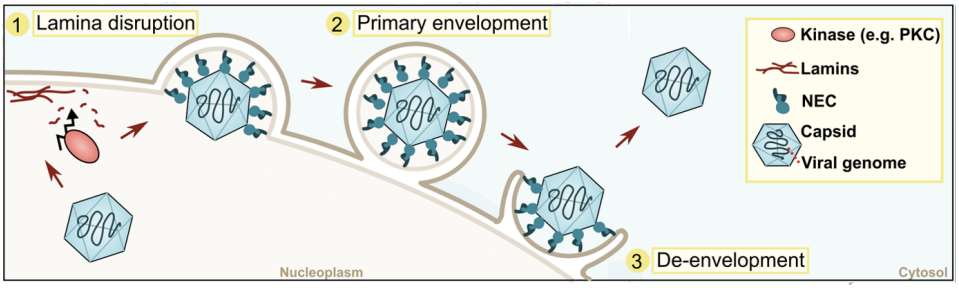


Figure 5.4: Viral egress across the nuclear envelope, from disruption of the lamina (1) to envelopment of the capsid (2) to de-envelopment of teh capsid (3).

Some viruses, specifically Herpesviruses, commonly use budding of the nuclear membrane and subsequent release of a vesicle into the cytoplasm in their replication process, for which they highjack the cellular machinery (fig. 5.4). As a method of reproduction, proteins and the genome making up the viral capsid are assembled inside the nucleus before being exported into the cytoplasm. This is enabled by a nuclear egress complex (NEC), which consists of host and viral proteins [206], [211]–[213] and can then interact with the INM, facilitating membrane curvature for bud formation. At last, the capsid-containing vesicle is released into the perinuclear space, and into the cytosol after fusion with the ONM. Viral egress of herpesviruses has long been considered a virus-specific mechanism, it would however not be unanticipated that also this process makes use of a previously existing physiological cellular function.

5.3.4 NPCs

Last but not least, the currently only accepted method of transport of intracellular material between the nucleus and cytosol are NPCs, first described about 60 years ago [214]–[217]. They are basket-like structures in which the INM and ONM fuse and form a pore that allows bi-directional transport of material [218]–[220]. They consist of several rings and their eight-fold symmetry is made up of proteins called nucleoporins (Nups). Their final structure has an inner diameter of about 40 nm. Transport across the NPC can be passive in the case of sufficiently small molecules, up to about 40 kDa, allowing up to 1000 translocations per second [221]. If sufficiently small, the molecules can be transported in their native configuration without impacting their functionality [220]. Many molecules do however need to be transported actively, these can be up to about 100 kDa [222]–[224]. NPC cargo can be slightly larger than the inner diameter, as structures can be unfolded and refolded after transit [225]. Nevertheless, it does not seem possible for NPCs to transport very large assemblies of molecules, such as protein aggregates, assembled virions, or RNA granules, which would create the need for the existence of an alternative transport pathway.

Should NPCs become defective, they must be recognised and degraded appropriately. In the same vein, in *S. cerevisiae* the mother cell retains dysfunctional NPCs upon cell division via asymmetric aggregation [208], [226]. Ultrastructural observations show that compromised NPCs also form herniations of the nuclear membrane budding outward into the cytoplasm. These herniations especially occur in cells whose genes encode the necessary proteins or factors for correct NPC formation are mutated or deleted [208], [227]–[234]. The herniations are omega-shaped, with an electron density at the INM, caused by the partial NPC [203]. It is not yet clear how defective NPCs are related to other forms of NEB, although there are some parallels. Certain human cell lines lacking Torsin A also show herniations of the nuclear envelope which contain both NPC assembly intermediate and also ubiquitin, similar to the stress NEB events present in yeast. Despite this, immunogold labelling against several Nups in unstressed and aged yeast cells shows that 10 % to 16 % of NEB events are labelled, similar to the negative control (LDs), as opposed to 67 and 81% of NPCs respectively, showing that not all NEB events are failed NPCs. It is possible that the herniation sites at defective NPCs are required for storage of the compromised units. Upon deletion of the protein Nup116, autophagy induced by starvation degraded fewer defective NPCs than in WT. Furthermore, as mentioned above, NPCs are not found on parts of the nuclear membrane where INM cargo is delivered

for autophagy in a double bilayer vesicle. It has not yet been determined how these different mechanisms are interconnected and/or related, nor quantified if and how many different types of NEB events can occur simultaneously.

5.4 Relevance to human health

Just as the management of misfolded protein is vital to the cell's health, so is adequate communication between the nucleus and cytoplasm. Various health issues have already been linked to NPC malfunction [235]–[240], for example in amyotrophic lateral sclerosis [241], [242], frontotemporal dementia, and Huntington's disease [243]. Nups have also been found to play a role in Alzheimer's disease [244], cardiovascular problems [245]–[247], and even cancer [248]–[254].

There are many ways in which NPC can malfunction, from their assembly to their individual components, and this is also seen in stress and disease [236], [255]–[259] and as nucleo-cytoplasmic transport is essential, it is likely that other, possibly parallel, pathways exist.

5.4.1 Disease and Ageing

NEB structures have previously been connected to various forms of disease through different mechanisms. Old yeast cells show an increase in NEB events ([226] and Paper **IV**) and NEB found to serve as transport for RNA granules requires lamins, proteins that make up the nuclear lamina and that are connected to laminopathies [260]. Torsin A, which is required for scission of the bud from the nucleus, is associated with the neurological disease dystonia, characterised by uncontrollable muscular twitching [261]. Although management of the response to proteotoxic stress is crucial under normal physiological conditions, it becomes even more so in cancer cells, which have high protein turnover and proliferate quickly. This in turn means that the HS response is activated in cancer cells [47]. Inhibiting the proteasome by using MG132 which leads to higher frequency of NEB (paper **IV**), also induces apoptosis in cancer cells [262]. All in all, investigating NEB can contribute to better understanding of not just one, but several diseases (Paper **V**).

5.4.2 Evolutionary conservation

When exploring NEB's relevance in stress and disease mechanisms, it is vital to keep an eye on its evolutionary conservation from the parasitic protozoon Trypanosoma brucei all the way to human cell lines (fig. 5.5). NEB was

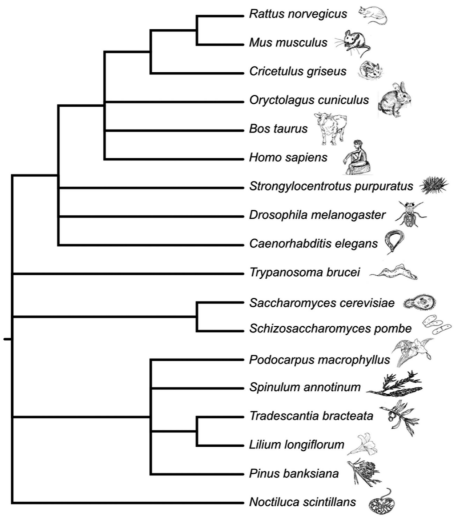


Figure 5.5: Phylogenetic tree of species in which NEB has been observed (refs will be added). Adapted from paper **V**.

found in all the species we investigated: fission yeast (*S. pombe*), *T. brucei*, in the nematode *Caernohabditis elegans* and the human mast cell line HMC-1 (paper **IV**). In all the named organisms, NEB frequency was higher than in budding yeast. In general, most NEB events showed as outward protrusions of the nucleus, as seen in *S. cerevisiae*. In some events in *S. pombe*, as well as *T. brucei*, electron tomography revealed a membrane surrounding the perinuclear cargo and its complete detachment from either nuclear membrane. One NEB event in *S. pombe* and two in HMC-1 formed an "eye", where the cargo was contained between the INM and ONM without exaggerated protrusion of either membrane. It was only in HMC-1 cells that the NEB event also protruded towards the inside of nucleus, as described with RNA granules previously observed [192]. The human cell line NEB events were also the largest, possibly proportional to the overall size of the nucleus.

To determine the importance of NEB in disease and ageing models, it is necessary to assess whether cellular stress in human cells leads to a higher NEB frequency. Many ageing-related diseases, e.g. Alzheimer's disease, are connected to the accumulation of misfolded proteins in the cell. Should NEB serve as a transport route mechanism for aggregates of misfolded proteins in the cell and thus potentially enhanced degradation, it has the potential to offer many unexplored avenues in pathoneurological research.

5.5 Discussion

Is a paradigm shift around nucleo-cytoplasmic transport in order? Since their discovery, NPCs have been assumed to be the only method of transport between the nucleus and the cytoplasm. However, nucleo-cytoplasmic transport is critical to the cell and it is not uncommon that there exist several parallel systems for the same purpose in the cell, for example UPS and autophagy.

In papers **IV** and **V**, NEB has been linked to increased stress, PQC and placed within context of other ultrastructurally similar mechanisms. Treatment with the drug AZC resulted in the highest NEB frequency, and its action on cellular proteins is also known to have the widest effect among the tested stressors [263]. Additionally, severe HS (42°C) caused more NEB events than mild HS (38°C). This elicits the question: is NEB frequency generally proportional to the severity of the stress?

The nucleus also possesses its own proteasome [264], and has also been shown to import protein destined for degradation from the cytosol [265], [266]. Considering its involvement in protein degradation, it is likely that it relies on more ways than one for material transport. Further, inhibition of the proteasome leads to an increase in NEB events, which reveals the necessity for the clearance of protein aggregates within or from the nucleus highlights NEB's role in PQC. This correlates well with the fact that NEB in rpn4Δ cells have distinctly electron-dense content, indicative of a higher concentration of cargo (Paper **IV**).

As outlined in the sections above, different types of NEB events have been described (Paper **IV**). Overall, several observations now confirm that NEB is not restricted to the envelopment and disposal of defective NPC units. This is supported by the fact that NPCs are not found in areas of NE around NEB events that contain atg39-associated cargo [202], [208]. If deformation and fusion/scission of the nuclear membranes enable nucleo-cytoplasmic communication, it would be interesting to explore the presence of different types of NEB within the same cell. However, this may need to be triggered by complex stressors, or a combination thereof, complicating analysing and prying out the individual factors involved. On the other hand, looking for similarities between stressors, e.g. an increase in transcription, would also deliver clues on NEB triggers. Nonetheless, this work has taught me that even unicellular organisms are complex within themselves and use a variety of systems in parallel to ensure the survival and proliferation, as seen by the example of PQC. Besides differences between the cargo of NEB events, there are also morphological differences within these groups. Different proteotoxic stres-

sors may cause NEB with content of different electron-densities, membrane dynamics and sizes. Certain NEB events have also been observed to contain ribosomes, which could hint that this transport is bidirectional, both in and out of the nucleus. Further investigation requires live-cell imaging that can provide the necessary spatial and temporal resolution, to confirm transport of material and NEB dynamics.

The evolutionarily conserved nature of NEB highlights its importance to mammalian cells and organisms. Further, the involvement of some of its molecular factors in disease put this young research field into societal context. A better grasp of NEB could then lead to a more profound understanding of a wide range of diseases, from neurological conditions caused by proteotoxicity to cancer. We are currently exploring if there is a direct correlation between increased NEB frequency and cancer cells.

Chapter 6

Thesis Summary and Outlook

6.1 Main findings

The findings in papers **I** to **III** refer to observations in the budding yeast *Saccharomyces cerevisiae*. Papers **I** to **IV** are research articles whereas **V** is a review article.

Paper I During a mild continuous heat-shock:

- Vacuoles, mitochondria, lipid droplets, multi-vesicular bodies, and the cell overall increase in size
- The number of mitochondria, lipid droplets, and multi-vesicular bodies increases
- Membrane contact sites have a specific response and those between vacuoles and the nucleus increase
- Electron-dense content accumulates in the nucleus and mitochondria
- Vacuolar electron-density and pH varies in response to heat-stress
- Electron-translucent clusters appear and locate close to the plasma membrane

Paper II Our contributions have shown that:

- Virus-like particles become fewer and less clustered upon exposure to heat-shock
- Inclusions of misfolded protein aggregate in proximity to mitochondria
- Virus-like particles localise near mitochondria, together with misfolded protein aggregates

Paper III

- The thermo-sensitive proteins guk1-7-GFP, gus1-3-GFP, and pro3-1-GFP can be used as reporters for misfolded proteins
- All three proteins intermingle in common aggregates, yet are cleared at different rates and show differences in processing by the proteasome
- The heat-shock proteins Hsp104 and Hsp70 are required for aggregate clearance

Paper IV

- Nuclear envelope budding is more frequent during proteotoxic stress and when the proteasome is inhibited
- Electron-dense content in the nucleus and in NEB events contains Hsp104 and ubiquitin
- Most observed buds are different from malformed nucleopore complexes
- Nuclear envelope budding is was present in all species examined (*T. brucei*, *S. cerevisiae*, *S. pombe*, *C. elegans*, Human Mast Cells)

Paper V reviews:

- Nuclear envelope budding events enveloping various types of material:
  - Originating at the inner nuclear membrane
  - Granules of ribonuclear-protein
  - Viral capsids
  - Misfolded protein
  - Misassembled nucleopore complexes
- the connection between issues with nucleo-cytoplasmic communication and disease

6.2 Outlook

As it happens, one question leads to another, or to multiple others. We have uncovered changes in the cellular architecture during heat-stress, established a more precise idea of how proteins aggregate, and that the nuclear envelope buds during proteotoxic stress. We have done this by using room-temperature TEM of plastic-embedded samples for quantitative purposes. Naturally, this has opened up many avenues specked with further questions on our findings. Below I will outline just some directions I deem worth investigating.

The TEM performed here is generally considered a low-throughput method, that delivers nanometre-resolution of many cellular structures simultaneously, without any labelling. Although quite unusual, TEM has been applied in a quantitative manner (e.g. [164], [203], [267]), revealing its potential. We hope our work will demonstrate the strength of TEM as an experimental method when used on its own, and inspire more scientists to use it in a quantitative manner. Of course, anything making electron microscopy more high-throughput would increase the scientific output.

There are several software suites and approaches for automatic segmentation of data in 3D EM, which is the task of separating the structure of interest from its neighbouring structures. In this type of data, several similar images from the same cell are segmented sequentially. Analysing thousands of images of sections from different cells, however, is more challenging. It becomes more difficult to train an algorithm when every cell looks different, as it cannot compare to training data that is similar enough. However, with an automatic process, much more information could be extracted from TEM micrographs and would strengthen the technique. A project led by collaborators, that I am involved in, is currently in progress (not included in this thesis).

It is still unclear what is contained in the ETC observed around the cell periphery. Possible ways to determine their nature could be testing different staining agents using FLM, or further investigating the chemistry of plastic sections. It would also be interesting to compare the presence and prevalence of ETC in TEM images of cells subjected to other stressors.

An interesting observation is the change of vacuolar pH, as reflected by their electron density. When observing mother-daughter pairs of cells, this correlation was quite clear but less so during the HS time-course. A standardisation of UA as pH indicator in TEM could have many advantages, e.g. when observing individual organelle's response to a stressor. To achieve this, it would be necessary to stain cellular material of a specific pH in a controlled

and reproducible manner. This could for example be done by investigating additional outside conditions that affect organellar pH, or examining an organelle with a very small pH range. The pH of the freeze substitution solution would also need to be controlled, as it affects the binding efficiency of UA [112].

Understanding the way proteins that are intermingled in the same aggregates but are processed at different speeds could provide insight into the cell's clearance, and perhaps degradation mechanisms. In the future, this knowledge could be used to target a specific type of protein (examples and refs) within the cell to accelerate its clearance and contribute to cell health. Alternatively, it may also be useful to intentionally slow down the clearance of specific proteins that may be less harmful to the cell. This could free up the disaggregation machinery for clearance and the proteasome for the degradation of other, more toxic proteins.

To elucidate the relevance of NEB in the stress response, it would be interesting to analyse the frequency of NEB events in mammalian cell that have undergone HS. Do NEB events carry or store misfolded protein, and if so, which? Additionally, some of the molecular players that have already been connected to NEB, are also found to play a role in certain diseases. These include for example Torsin A in dystonia, and nuclear lamins in laminopathies. NEB have now been observed to contain different types of cargo, and uncovering whether this is dependent on the stressor would aid anchor its position as a physiological mechanism in the stress response.

Using existing methods in new ways will continue to enable us to find new questions, and new answers.

Chapter 7

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