University of Groningen

Poor old pores

Rempel, Irina Lucia

Chapter 1

A general introduction into aging and nuclear pore complexes in baker's yeast

Baker's yeast as a model for aging studies

One commonly used definition to describe the biology of aging is that aging is the progressive accumulation of damage throughout the lifespan of an organism (Fontana et al., 2010; Hayflick, 2007; Kirkwood, 2008; Rattan, 2006). For the aging organism, the changes result in a decrease in reproductive success and an increased risk for disease and mortality (Kirkwood, 2008; Tosato et al., 2007). Consequently, delaying the aging process, holds promise to increase the health- and lifespan of the organism (Crimmins, 2015; Tosato et al., 2007).

On the cellular level, the accumulation of damage during aging is characterized as nine universal hallmarks. Four hallmarks reflect intracellular changes that hold potential to be causal of aging: (1) genomic instability, (2) telomere attrition, (3) epigenetic alterations and (4) loss of proteostasis. The consequence of those four primary hallmarks are observed as (5) deregulated nutrient sensing, (6) mitochondrial dysfunction and (7) cellular senescence. Ultimately, these cellular changes cause (8) stem cell exhaustion, and (9) altered intercellular communication (López-Otín et al., 2013). With few exceptions (e.g. some sponges, jellyfish, corals and hydras show great longevity and are potentially immortal (Petralia et al., 2014)), these universal hallmarks of aging are present in almost all animals, as well as many plants. Even single-celled organisms show several of these hallmarks, while they age (López-Otín et al., 2013; Petralia et al., 2014).

Differently from humans, model organisms can be genetically modified, held under constant conditions and have significantly shorter lifespans than humans. Therefore, the plethora of our mechanistic insights into the biology of aging stems from model organisms. One of the simplest model organisms used for aging studies is baker's yeast. Indeed, this single cell organism is suitable to study the four primary hallmarks of aging, as well as the three secondary hallmarks of aging (López-Otín et al., 2013). Several molecular pathways that change in aging are evolutionary conserved from yeast to humans (Janssens and Veenhoff, 2016a). Aging in baker's yeast can be evaluated as the time that the cell survives in a non-dividing state (chronological lifespan), reminiscent of aging in non-dividing cells over time. Alternatively, aging in baker's yeast can be evaluated as the number of times the cell divides (replicative lifespan), reminiscent of aging in stem cells and highly proliferative tissues (Longo et al., 2012).

Chronological aging in baker's yeast is induced by the depletion of nutrients either in a culture grown to stationary phase, or by transferring the cells to water

(Hu et al., 2013). Those environmental conditions are fundamentally different from chronological aging of differentiated, and therefore postmitotic, cells in multicellular organisms, where cells are provided with nutrients. Nevertheless, the process of aging bears resemblance and several environmental and genetic interventions, as well as drugs found to extend the chronological lifespan of baker's yeast are conserved in higher eukaryotes (Pitt and Kaeberlein, 2015). For example, lifespan extension through dietary restriction, and the pathways that mediate it (e.g. sirtuins, mechanistic Target of Rapamycin (mTOR), insulin-like signaling pathways) are conserved in higher model organisms (Fontana et al., 2010) and were initially discovered in baker's yeast (Longo et al., 1999).

Replicative aging of baker's yeast is an established model system for aging of other mitotic cells in higher eukaryotes and even organismal aging (Reviewed in: Denoth Lippuner et al., 2014; Janssens and Veenhoff, 2016a; Wasko and Kaeberlein, 2014). The mean lifespan of BY4741, a commonly used yeast strain, is ~ 25 divisions, for haploid cells (Crane et al., 2014; Huberts et al., 2013, 2014). A yeast cell divides asymmetrically and the mother cell selectively retains damaged components. Factors that are asymmetrically inherited include extrachromosomal rDNA circles (ERCs), nuclear pore complexes, carbonylated proteins, protein aggregates, mitochondria and vacuoles (Higuchi-Sanabria et al., 2014). The asymmetrical distribution of these factors causes the mother cell to progressively age with each division, while for the major part of the mother's lifespan, each of its daughter cells is born rejuvenated (Box 1)(Henderson and Gottschling, 2008).

The asymmetrical distribution of factors is established by the cytoskeleton, and a special membrane composition at the site where mother and daughter cells are connected, the bud neck. Here, a sphingolipid diffusion barrier at the endoplasmic reticulum (ER) prevents misfolded proteins in the ER, to be passed on to the daughter cells (Clay et al., 2014). Furthermore, the inheritance of components at the outer nuclear membrane, the ER and the plasma membrane is limited by a septin (Barral et al., 2000; Luedeke et al., 2005; Mostowy and Cossart, 2012; Shcheprova et al., 2008; Takizawa et al., 2000). A major constituent of the cytoskeleton, actin, nucleates into cables at the bud tip and the bud neck and mediates, in combination with the motor protein Myo2, the transport of mitochondria and vacuoles into the new daughter cell, ensuring that only functional organelles are inherited (Henderson et al., 2014; McFaline-Figueroa et al., 2011; Spokoini et al., 2012). Furthermore, aggregates associate

with actin cables, which prevents them to diffuse freely into the daughter cells (Liu et al., 2010, 2011).

Box 1: The replicative aging challenge – Methods to study replicative aging cells

Budding yeast cell cultures grow exponentially, in the presence of sufficient amounts of nutrients. During each division, the mother cell produces a rejuvenated daughter cell. Therefore, old cells are diluted out by their progeny (Figure 1). Hence, techniques that allow us to enrich aged cells are essential to study replicative aging. Several techniques are available to enrich aged cells, advantages and disadvantages of those techniques are briefly discussed below:

Dissection

During dissection a needle is used to move each daughter cells away from the mother cell after each division (Mortimer and Johnston, 1959). This method is laborious and has a very low throughput. Yet the technical requirements for this technique are very low and it is one of the most robust and widely accepted ways to measure the lifespan of cells. A prerequisite is that sufficient numbers of cells (at least 100 per experiment) are measured (Huberts et al., 2014).

Mother enrichment program (MEP)

The mother enrichment program (Lindstrom and Gottschling, 2009) is an inducible system, that can selectively prevent daughter cells (age 0) from dividing. Consequently, the aging cells are diluted out more or less linearly with cell-cycle arrested daughter cells. A disadvantage of this method is that it involves several genetic modifications: The fusion of the cre-recombinase with an estradiol binding domain, which is under the control of a daughter specific promotor and the flanking of two essential cell cycle genes (cdc20 and ubc9) with cre sites. Another challenge when using the MEP is that mutations can cause a daughter cell to become insensitive to estradiol and start growing exponentially. This happens relatively frequently at a rate of 1.4×10^{-6} per cell divison (Lindstrom and Gottschling, 2009).

Labeling of the cell wall

The cell wall of *S. cerevisiae* is newly synthesized by the daughter cell during division. Since the cell walls are not shared between mother and daughter cell, the cell wall of a cohort of cells can be labelled with fluorophores or magnetic beads (Kennedy et al. 1994). Subsequently the cells are allowed to divide for several generations. FACS sorting or a magnet can be used to distinguish and/or physically separate the aged cells from their progeny. The labeling of the cell wall can further be combined with the MEP to increase the number of aged cells per experiment (Thayer et al. 2014). Another approach to enrich for aged using cells labelled with magnetic beads was used by Janssens and colleagues (Janssens et al., 2015) to do transcriptome and proteome studies on replicative aged yeast cells. They aged large cohorts of cells in magnetic columns, which retained the bead-labeled mother cell, while flushing out the daughter cells with a constant flow of medium. Taken together, the advantage of those techniques

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is, that they can be adapted to age large cohorts of cells and to perform system wide studies.

Microfluidic chips

Microfluidic chips for yeast aging studies are made from glass and PDMS, for use in combination with a microscope. Those microfluidic devices that allow the user to follow cells throughout their lifespans are based on trapping single yeast cells, when they are young. The flow of medium inside the chip provides the cells with nutrients, while selectively flushing away the daughter cells. Several designs that allow the analysis of full lifespans are published (Crane et al., 2014; Fehrmann et al., 2013; Jo et al., 2015; Lee et al., 2012; Zhang et al., 2012), but none are commercially available at present. The cells are imaged regularly, following each division. Disadvantage of microfluidic devices are the low to medium throughput in cell numbers per experiment and that they require extensive training to use them. However, microfluidic devices allow us to study subcellular localization and abundance of proteins, cell cycle kinetics, cell size and shape as well as organelle function in correlation to the life expectancy of a cell.
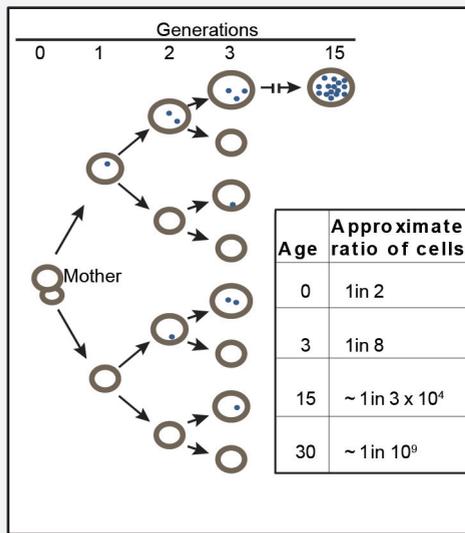

Age	Approximate ratio of cells
0	1 in 2
3	1 in 8
15	~ 1 in 3 x 10^4
30	~ 1 in 10^9

Figure 1 Replicative aged cells are outnumbered by their progeny. A schematic representation that illustrates how the the frequency of replicative aged cells decreases as a function of replicative age. Dots in the yeast cell represent bud scars that appear at the division site in each round of division and that show the replicative age of a mother cell. The ratio of cells in the table is an approximation, based on exponential growth as a function of 2^N, with N being the replicative age of the cell, however this function does not take into account that daughter cells take longer for their first division than mother cells and that mother cells eventually die.

Occasionally, yeast cells fail to perform asymmetric divisions, causing the daughter cell to inherit the age of the mother cell (Kennedy et al., 1994). The frequency of symmetric divisions increases during aging. These divisions are likely caused by a compromised diffusion barrier between mother and daughter, since genetic interventions that compromise the diffusion barrier can also cause the loss of age asymmetry between mother and daughter (Shcheprova et al., 2008). Similar to yeast, human stem cells repeatedly perform asymmetric

divisions, resulting in a stem cell and a progenitor cell. Symmetric divisions of stem cells, can result in either two stem cells, or two differentiated cells. Therefore symmetric divisions can either lead to an increase in stem cell number, as is typical for aging hematopoietic stem cells (de Haan et al., 1997) and intestinal stem cells (Choi et al., 2008), or a decrease in stem cell number, typical for aging in satellite cells (Gibson and Schultz, 1983) and germline stem cells (Killian and Hubbard, 2005; Zhao et al., 2008). Studies in hematopoietic stem cells and germline stem cells have shown that these stem cells perform symmetric divisions more frequently at increased age (Cheng et al., 2008; Kohler et al., 2009). Such unbalances in divisions disturbs the balance between stem cells and somatic cells, as well as the functionality of both cell types and therefore contributes to the decline in regenerative potential of tissues and organs in aging (Schultz and Sinclair, 2016).

The potentially conserved primary causes of aging
That aging and lifespan are partly genetically regulated is evident by the fact that species differ in their average lifespan, that human lifespan expectancy is partly genetically determined (Ruby et al., 2018) and that genetic interventions can alter lifespan. Another important factor that influences aging and lifespan expectancy is the environment (Passarino et al., 2016). Yet, even within a genetically identical population, cultured in the same constant environmental conditions, there is large variation in the lifespan of single yeast cells, as well as worms, flies, or mice (Kirkwood et al., 2005; Mortimer and Johnston, 1959). This indicates that aging is intrinsically variable with a large impact of stochastic processes.

Which factors determine the lifespan and cause aging of a single baker's yeast cell within a genetically identical population? Telomere attrition is not a causal factor for aging in baker's yeast, because the telomeres do not progressively shorten during aging in baker's yeast (D'Mello and Jazwinski, 1991). Differently from most somatic cells in higher eukaryotes, telomerase is constitutively expressed in yeast, allowing the telomeres to be extended when they become critically short (Mozdy and Cech, 2006; Strecker et al., 2017). Increased genomic instability is another hallmark of aging, that can be observed in yeast during aging (Moskalev et al., 2013; Novarina et al., 2017; Szilard, 1959). However, genomic instability cannot be a primary cause of aging in the majority of cells as it is not compatible with the observation that daughter cells are born rejuvenated (Johnston, 1966; Müller, 1971) and that during gametogenesis the lifespan is reset (Unal et al., 2011). Furthermore, the average

mutation rate in yeast is too low to explain aging of individual cells, and one study finds that mutation load is not linked to lifespan in wild type yeast cells (Kaya et al., 2015). Although, we cannot exclude that genomic instability and the accumulation of mutations drive aging in a subset of individual cells, it can be excluded that genomic instability could cause aging for a majority of baker's yeast cells.

Epigenetic alterations and loss of proteostasis are intertwined processes that both have the potential to cause aging in baker's yeast. Aging is characterized by increased acetylation of histones, in combination with loss of histones in the subtelomeric regions and transcriptional desilencing (Dang et al., 2009; Feser et al., 2010; Gehlen et al., 2011). These changes can either be cause or consequence of the loss of proteostasis, that we observe in aging. The loss of proteostasis in aging affects the control over correct folding of proteins and the degradation of non-functional proteins (Ben-Zvi et al., 2009). Several studies highlighted the roles of chaperones in replicative and chronological aging, showing that chaperones contribute to lifespan extension and the maintenance of mother-daughter asymmetry in aging (Hanzén et al., 2016; Harris et al., 2001; Hill et al., 2016; Speldewinde and Grant, 2017). Hsp104, a chaperone that facilitates the refolding of denatured and aggregated proteins (Parsell et al., 1994), has been used as a marker for protein aggregation (Winkler et al., 2012). The appearance of asymmetrically retained Hsp104 foci during aging further indicates problems with correct folding and increased aggregate formation during aging (Zhou et al., 2011). In a broader sense protein homeostasis also includes the correct sorting and targeting of proteins and the formation of macromolecular complexes (Juszkiewicz and Hegde, 2018). Network analysis predicts that protein biogenesis is one of the most causal drivers of aging (Janssens et al., 2015), from this perspective it makes sense that the downregulation of protein synthesis by caloric restriction is one of the most conserved and best understood lifespan extending interventions.

System wide proteome and transcriptome studies have revealed that proteome and transcriptome uncouple in aging baker's yeast (Janssens et al., 2015) and rat brain and liver tissues (Ori et al., 2015). Protein complexes are especially affected by this uncoupling and changes in protein complex stoichiometry are observed (Janssens et al., 2015; Ori et al., 2015). In baker's yeast tubulin, the vacuolar proton ATPase and the nuclear pore complex (NPC) are among the most substoichiometric complexes in replicative aging cells (Janssens et al., 2015). The NPC is particularly interesting in this context, because

substoichiometric NPCs have the potential to cause, or at least contribute to the loss of proteostasis in aging.

The structure of the nuclear pore complex

The overall structure and function of the nuclear pore complex (NPC) is conserved from yeast to human (Kim et al., 2018; Kosinski et al., 2016; Lin et al., 2016). NPCs are embedded into the double membrane of the nuclear envelope (NE), where they facilitate controlled exchange between the nucleus and the cytoplasm. On one hand, the NPC acts as a size dependent diffusion barrier, which allows small molecules to diffuse freely between nucleus and cytoplasm, while large molecules diffuse slowly (Kapinos et al., 2017; Lowe et al., 2015; Popken et al., 2015; Timney et al., 2016). On the other hand, NPCs in cooperation with nuclear transport receptors (NTRs), also facilitate targeted exchange of macromolecules between nucleus and cytoplasm, in a rapid and energy driven transport reaction.
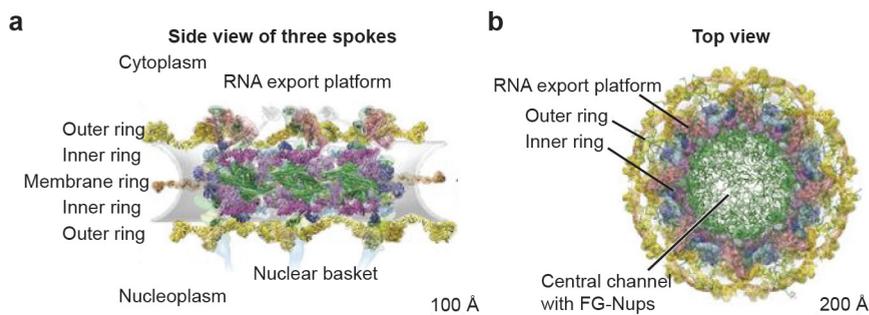

Figure 2 Integrative structure of the native *S. cerevisiae* NPC. The structure was solved by cryo-electron tomography (cryo-ET), at a resolution of 28 Å. Subsequently, individual Nups and sub-complexes of the NPC, were fitted into the cryo-ET structure based on published crystallographic structures, integrative structures and comparative models. The final structure shows the positions of 552 individual Nups, at a resolution of 9 Å
a) Side view of three consecutive spokes.
b) Top view of the cytoplasmic side of the complete NPC. The following structures can be seen: The cytoplasmic ring (yellow), the RNA export platform (pink), the inner ring (purple), the Nic96 complex (blue), the membrane ring (brown), the FG-Nups (green). This figure is adjusted from (Kim et al., 2018) and reprinted with permission.

The NPC is an exceptionally large protein complex. The native yeast NPC has a mass of 52 MDa, excluding the surrounding membrane, the transport factors and cargo, which collectively add another 35 MDa (Kim et al., 2018). Each NPC is composed of ~30 different proteins, called Nucleoporins or Nups. Nups

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are divided into two groups with fundamentally different properties. A group of stably folded proteins forms the scaffold of the NPC, and another group of proteins contain intrinsically disordered (ID) domains. In baker's yeast, each nup is present in 8, 16 or 32 copies per NPC (Kim et al., 2018; Mi et al., 2015). The vast majority of NPCs show an eight fold rotational symmetry formed by eight spokes, that form a cylindrical assembly (Hinshaw and Milligan, 2003; Unwin and Milligan, 1982). This cylindrical assembly is divided into the core scaffold (comprised of inner and outer rings), a membrane ring, the RNA export platform and the nuclear basket. The different substructures (inner and outer rings, membrane ring, RNA export platform, nuclear basket, as well as spokes) of the NPC are held together by flexible elements, which give the structure strength and flexibility at the same time (Fischer et al., 2015; Kim et al., 2018).

The core scaffold of the NPC is a symmetrical structure of two inner rings, which are flanked by outer rings (a total of two in yeast and four in humans) (Kim et al., 2018; Lin et al., 2016). The inner ring is formed by two protein complexes, the inner ring complex and the Nic96 complex. The inner ring complex protein Nup192 interconnects the spokes and functions at the same time together with Nup188 as a spacer between the spokes. The other members of the inner ring complex Nup157, Nup170, Nup53 and Nup59 serve a vital role in anchoring the NPC to the NE. NPCs are anchored to the NE, via membrane binding motifs of those proteins, as well as interactions with three different transmembrane proteins (Ndc1, Pom152 and Pom34) that form one membrane ring around the equator of the core scaffold. Additionally, Nup157 and Nup170 form, together with Nic96, a diagonally oriented column, within each spoke, that connects the inner ring to the Nic96 complex. The Nic96 complex comprises Nsp1, Nup49, Nup57 and Nic96.

Each outer ring is formed by eight highly conserved Y-shaped complexes (also called Nup84 complex). The Y-shaped complexes are arranged head-to-tail, where the interaction between the complexes is established by Nup120 at the head of the complex and Nup133 at the tail of the next complex. Additionally, Nup120 and Nup133 anchor the Y-shaped complex to the NE, via membrane binding motifs.

Positioned over the core scaffold on the cytoplasmic side is a structure called the RNA export platform (formerly known as cytoplasmic filaments) (Fernandez-Martinez et al., 2016). The RNA export platform is connected to the outer ring, via connections to the Nup85-Seh1 arm, at the head of the Y-shaped complex. The RNA export platform is necessary for mRNA export and the final

messenger ribonucleoprotein (mRNP) remodeling steps. These remodeling steps involve the non NPC component DEAD-box protein Dbp5p, which interacts with Nup159, to remove the transport factors from the mRNA and preventing it from diffusing back into the nucleus (Lund and Guthrie, 2005; Montpetit et al., 2011).

Attached to the core scaffold on the nuclear side are eight filaments that form a cage-like structure, called the nuclear basket. Here, the Nup85-Seh1 arm of the outer ring at the nuclear side connect to the nuclear basket proteins Mlp1 and Mlp2, to anchor the basket to the core scaffold. The basket is a dynamic structure (Niño et al., 2016), that serves as an anchoring point for various processes in the nucleus. RNA processing and export, as well as preventing the export of unprocessed RNA are mediated by the nuclear basket. Additionally, the basket structure tethers silencing factors, chromatin and cell-cycle regulators and proteasome (Albert et al., 2017a). Mlp1 and Mlp2 are large, evolutionary conserved coiled-coil proteins that contribute to NPC positioning, nuclear stability and NE morphology (Niepel et al., 2013). The nuclear basket contributes to the passive permeability barrier and nucleocytoplasmic transport (Bogerd et al., 1994; Denning et al., 2001; Jani et al., 2014).

Intrinsically disordered proteins form the passive permeability barrier of the NPC
In the center of the scaffold are ID proteins (also called phenylalanine-glycine repeat nucleoporins or FG-Nups) that form the diffusion barrier of the NPC and interact with the transport factors. All FG-Nups have multiple clustered FG repeats separated by characteristic spacer sequences, that are further categorized as FxFG, GLFG or FG repeats. The FG-Nups are further characterized as asymmetric FG-Nups that localize (mainly) to the RNA export platform (Nup159 and Nup42), or the basket (Nup1, Nup2 and Nup60) and symmetric FG-Nups, that fill the core scaffold of the NPC (Nup100, Nup116, Nup49, Nsp1, Nup145N and Nup57).

How the diffusion barrier is formed and functions in vivo has been studied intensively and fiercely debated (Lemke, 2016; Li et al., 2016). Purified, FG-Nups in solution can phase separate to form a liquid-liquid demixed state (Lemke, 2016), a gel state (Frey and Görlich, 2007; Frey et al., 2006; Labokha et al., 2012), form amyloids (Ader et al., 2010; Halfmann et al., 2012; Milles et al., 2013), or form a polymer brush, when fixed to a surface (Lim et al., 2007). In vitro formed gels (Frey and Görlich, 2007), as well as surface anchored FG-Nups in artificial nanopores (Jovanovic-Talisman et al., 2009; Kowalczyk et al.,

2011), can form selective barriers in the sense that NTRs can pass more rapidly than control proteins.

It remains challenging to determine, in which of those in vitro states of FG-Nups might be found under physiological conditions. However, in vivo the disordered phase is not purely composed of FG-Nups, but additionally hosts a significant proportion of NTRs, and (non-)cargo molecules. Especially the presence of NTRs is important for the functional properties of the NPC, as will be discussed in the next section (Kapinos et al., 2017; Lowe et al., 2015).

The FG-Nup permeability barrier facilitates the rapid passage of large protein complexes, when bound to a NTR (discussed in detail below). This passage is enabled by several low affinity binding events of NTRs to FG-Nups (Rexach and Blobel, 1995). NTRs accept to different extend FG-, GF- and F-binding motifs in the ID domain of the FG-Nups. Each FG-Nup typically therefore has several binding sites that the NTR can bind to and the total concentration of binding sites in the central channel is estimated to be up to 260 nM (Aramburu and Lemke, 2017). Vice versa, each NTR has several binding pockets that recognize binding motifs (Bayliss et al., 2000, 2002; Morrison et al., 2003; Otsuka et al., 2008; Port et al., 2015). In the crowded in vivo environment, the interaction of one individual nup with one individual shuttling NTR is limited to one or few binding sides. At the same time, each FG-Nup will bind several NTRs and each NTR will interact with several FG-Nups. On the other hand, NTRs show high binding affinities to FG-Nups in vitro, as those binding assays measure the affinity of the sum of all binding sites of one individual NTR to one individual FG-Nup, which results in high binding affinities up to nM range (Pyhtila and Rexach, 2003). However, binding affinities in this range would not allow the several binding and unbinding events that facilitate nucleocytoplasmic transport to happen within the timescale of milliseconds.

Consequently, both FG-Nups and NTRs present highly reactive surfaces for binding, where each collision between two different proteins creates a binding event. In vivo, the binding affinity between NTRs and FG-Nups is lowered, by the competition of NTRs for binding sites and weakly binding competitors (Tetenbaum-Novatt et al., 2012), by Ran-GTP and by a non-mobile population of NTRs (Lowe et al., 2015) in the center of the NPC, which jointly reduce the number of interactions between individual FG-Nups and shuttling NTRs. This lowers the affinity of the binding interactions to the mM range, as the binding between individual FG-Nups and a NTRs is limited to one interaction site (Milles et al., 2015; Tetenbaum-Novatt et al., 2012).

Nuclear pore complex function as a macromolecular transport machinery

Although, the NPC's function as gateways between nucleus and cytoplasm is understood best, it is important to acknowledge, that NPCs are multifunctional complexes. NPCs are known to participate in gene regulation, the repair double strand breaks, cell cycle progression, and the anchoring of telomeres and extra chromosomal rDNA circles (ERCs) (Denoth-Lippuner et al., 2014; Ibarra and Hetzer, 2015; Raices and D'Angelo, 2017).

Active transport allows rapid and energy dependent transport of macromolecules between nucleus and cytoplasm and is facilitated by the NPC, in combination with several nuclear transport factors (NTRs, 17 in yeast (Allen et al., 2002)) and a gradient of Ran guanosine di-/tri-phosphate (RanGDP/RanGTP), with high concentrations of RanGTP in the nucleus and high concentrations of RanGDP in the cytoplasm (Reviewed in: Fiserova and Goldberg, 2010). These mechanisms support rapid translocation of NTR-cargo complexes through the NPC, in the order of ~1000 molecules/second (Ribbeck et al., 2001; Yang et al., 2004) with multiple parallel import and export events occurring at the same time.

Proteins that require transport to the nucleus carry a Nuclear Localization Signal (NLS) (Dingwall and Laskey, 1991) that is recognized by transport factors called importins. A screen performed in baker's yeast revealed that 25.8 % of the proteins localize to the nucleus, when tagged with GFP, and grown under standard growth conditions (Huh et al., 2003), it is reasonable to assume, that the a large fraction of those proteins will have a NLS target signal. NLSs vary in length and features, the most commonly studied NLSs is called classical NLS and is recognized by the NTRs Kap60 and Kap95. Classical NLSs are characterized by a short stretch of basic amino acids e.g. KKKRK, which is sufficient for protein targeting to the nucleus (Lange et al., 2007).

Importins recognize and bind their NLS containing cargo in the cytoplasm (Moroianu et al., 1996). The rate limiting factors for the transport is the formation of the importin complex, or in other words affinity of the importin to bind an NLS, and the abundance of importin (Hodel et al., 2006; Riddick and Macara, 2005; Timney et al., 2006). The importin-cargo complex interacts with FG-Nups in the central channel of the NPC, as discussed earlier through low affinity interactions between the importin and the FG-Nups. The RanGDP/RanGTP gradient ensures the directionality of the transport. Inside the nucleus, RanGTP binds to the importin-cargo complex and the cargo is released from its importin (Rexach and Blobel, 1995). The importin-RanGTP complex

shuttles via the NPC back to the cytoplasm, where Ran GTPase-Activating Protein (RanGAP) binds to the complex, hydrolyses GTP to GDP and releases the importin from RanGDP (Becker et al., 1995).

Proteins that require transport to the cytoplasm carry a Nuclear Export Signal (NES) (Wen et al., 1995) that is recognized by transport factors called exportins (Reviewed in Cautain *et al.,* 2015). NESs are less well characterized than NLSs, and probably many NESs remain to be identified. The classical NES motif is recognized by the exporting Crm1 and contains three to four hydrophobic amino acids, often leucine, which is intercepted by one or several small, charged or polar amino acids. Exportins recognize and bind their NES containing cargo in the nucleus, as well as RanGTP (Stade et al., 1997). Also this complex interacts with the FG-Nups, to allow rapid transition from the nucleus to the cytoplasm. In the cytoplasm the complex dissociates through binding to RanGAP and GTP hydrolysis (Becker et al., 1995). The RanGTP/GDP gradient is maintained by binding of RanGDP to its importin NTF2 (Bayliss et al., 1999; Oki and Nishimoto, 1998) so that it is transported back into the nucleus, where the RanGDP interacts with Srm1, which replaces GDP for GTP (Oki and Nishimoto, 2000).

NPC assembly and quality control; a role for Nups
NPCs can be assembled by two distinct ways. (1) Postmitotic (re-)assembly of NPCs occurs in higher eukaryotes at the end of mitosis, into the reforming NE and is described elsewhere (Otsuka and Ellenberg, 2018). (2) Interphase assembly (or de novo assembly) of NPCs requires the assembly of NPCs into the intact nuclear envelope. Organisms with closed mitosis, such as baker's yeast, exclusively perform interphase assembly.

How NPCs assemble into the intact nuclear envelope, without perturbing the nucleocytoplasmic barrier, is still largely unknown (Otsuka and Ellenberg, 2018). A major challenge in the study has been to distinguish NPC assembly sites, from the majority of fully assembled NPCs inside the cell. Single NPCs are at a resolution below normal fluorescence microscopy, which adds to the challenge of NPC assembly site identification. Since the order in which the Nups assemble into NPCs is still unknown for most parts, our knowledge on NPC assembly is often limited to our knowledge about the Nups that are essential in supporting the structural integrity of the NPC. Surprisingly few of the NPC components are essential (Table 1.) in baker's yeast. For those essential Nups it is not always clear, whether they perform an essential function,

e.g. facilitating RNA export, or whether they are essential in warranting the structural integrity of the NPC.

A scanning EM study using *Xenopus* egg extract suggested that that assembly begins with the formation and stabilization of a hole (pore) through the nuclear envelope (Goldberg et al., 1997). A more recent study showed transmission EM images of NPC interphase assembly intermediates in mammalian cell lines. These revealed that interphase assembly occurs through the evagination of the INM, which further deforms, until it fuses with the flat ONM. At the site of the deformed membrane is a mushroom shaped, electron dense mass of growing size. The exact protein composition of this mass at different stages remain to be determined, but the authors showed that proteins of the nuclear basket associated early with the assembly site and proteins of the RNA export complex joined later (Otsuka et al., 2016).

It is likely, that also in baker's yeast, the proteins of the nuclear basket play a role during early NPC assembly steps and that the RNA-export machinery is only involved in the later assembly steps. The nuclear basket proteins Nup1 and Nup60 contain amphipathic helix domains that are sufficient to induce membrane bending and their deletion is synthetic lethal, suggesting a crucial role for those amphipathic helices during NPC assembly in curving the NE (Mészáros et al., 2015). The mislocalization of members of the RNA export complex to the cytoplasm is a commonly used indicator for problems with NPC assembly (Hodge et al., 2010; Makio et al., 2009; Onischenko et al., 2017). This indicates, that the RNA export complex may be one of the last steps of NPC assembly and that the most of the essential members of the RNA export complex might be essential due to their function in RNA export, rather than the structural integrity of the NPC. An exception is Dyn2, which is needed to assemble the RNA export platform itself (Gaik et al., 2015). However, Dyn2 is not an exclusive member of the NPC, but also part of the dynein motor complex (Rao et al., 2013), therefore it is hard to tell, if the Dyn2 is an essential factor in NPC assembly, as part of the dynein motor complex, or in both functions.

The essential proteins of the outer ring Nup85 and Seh1 are probably essential, because they anchor the RNA export platform to the NPC scaffold. However, Seh1, as well as Sec13, are not uniquely at the NPC, both proteins are also part of the vacuolar-associated SEA complex (Dokudovskaya et al., 2011) and Sec13 is additionally part of the COPII vesicle coat (Barlowe et al., 1994). Also Ndc1, the only essential protein of the membrane ring, does not uniquely function as a NPC protein, but also contributes to spindle pole body duplication.

Therefore the final proof that those proteins are essential for the assembly of functional NPCs is still missing. At the inner ring, Nic96 alone interacts with every other protein in the inner ring, holding in place much of the scaffold of the NPC, therefore it can be assumed that Nic96 is crucial, already during early steps of NPC assembly.

Nups have remarkable functional redundancy. Therefore, many NPC components are only synthetic lethal, in combination with other components e.g. deletion of nup157 is lethal in combination with nup170, and the deletion of the GLFG domains in nup116 is synthetic lethal with nup188Δ, as well as the deletion of nup116 and nup100. In the case of nup116ΔGLFG nup188Δ and nup157Δ nup170Δ mutations it is known that the combination of those mutations is synthetic lethal, because the cells are not able to assemble new NPCs (Makio et al., 2009; Onischenko et al., 2017).

The deletion of FG-domains is surprisingly well tolerated by yeast cells. Even cells with all FG-domains at the basket and the RNA export platform removed (nup42ΔGLFG nup159ΔGLFG nup1ΔFxFG nup2ΔFxFG nup60ΔFxF) remain viable. Notably, also the deletion of the FG-domains of Nsp1, Nup145N, or Nup49, as well as the deletion of several combinations of FG-domains that belong to essential proteins are viable (e.g. nsp1ΔFxFG and nup49ΔGLFG, nsp1ΔFGΔFxFG and nup145ΔGLFG, nup159ΔGLFG and nsp1ΔFxFG), even when further FG-domains in non-essential proteins are deleted. The deletion of FG-domains alters the passive permeability barrier and nucleocytoplasmic transport in the mutants, which probably is sufficient to explain reduced fitness of some of those mutants (Strawn et al., 2004). Overall, this indicates that many of the ID domains of the NPC do not play a crucial role in NPC assembly.

A notable exception is the Nup116 FG-domain, which has an essential N-terminal region in its FG domain (Iovine et al., 1995; Strawn et al., 2004). This might seem contradictory at first, since the full deletion of nup116 is sick, but viable in most strains. Nup116 is one of five GLFG-repeat Nups (Nup100, Nup116, Nup49, Nup145N and Nup57), and the Nup100 and Nup116 GLFG repeat patterns have been shown to interact with the NPC scaffold during NPC assembly, potentially stabilizing NPC structural intermediates (Onischenko et al., 2017). It has been speculated that the full deletion of nup116 is viable, because Nup100 can take over some of the functions of Nup116, in this background. Additionally, despite the fact, that the FG-domains of Nup145N are not essential, the disordered region of Nup145N enables the interaction with Nup145C at the outer ring, as well as interaction with the Nup157 at the inner

ring and thereby supports the connectivity of the NPC scaffold (Fischer et al., 2015). Nsp1 and Nup49 form a complex with Nup57 and all three proteins contain triple coiled-coil domains, that form a part of the inner rings. The fact that the FG-domains of Nsp1 and Nup49 are not essential, suggests, that the coiled-coil domains are important for the structural integrity of the inner ring and therefore also for NPC assembly.

Table 1 Essential NPC components, according to https://www.yeastgenome.org/

NPC sub-structure	Essential genes
FG-Nups	nsp1, nup145 (N-terminus), nup49, nup116 (only essential in BY4741)
Membrane ring	ndc1
RNA export complex	nup82, nup159
Outer ring	nup85, sec13, nup145 (C-terminus)
Inner ring	nup192, nup57, nic96
Nuclear basket	nup1 (controversial)

Cells that lack non-essential Nups are still able to assemble enough NPCs and these NPCs are sufficiently functional to support viability of the cells. Deletion of some non-essential nucleoporins causes accumulation of misassembled NPCs (Wente and Blobel, 1993; Yewdell et al., 2011). Apart from the earlier mentioned Nup82 mislocalization, misassembled NPCs are characterized by NE herniations, clustering of NPCs, or NPCs that are covered by membrane that can be seen in transmission Electron Microscopy (EM) images. For example, transmission EM images of pom152Δ and nup116Δ are known to show misassembled NPCs at the NE at high frequencies, while other NPCs appear to be structurally normal (Madrid et al., 2006; Webster et al., 2014, 2016; Wente and Blobel, 1993).

NPC assembly and quality control; a role for non-NPC components
In *S. cerevisiae* several proteins are known to assist with NPC assembly, which are not part of the final structure of the NPC. Among those proteins are several proteins that localize primarily to the ER and regulate ER morphology. Rtn1 and Rtn2 belong to a conserved family of proteins called reticulons, which induce membrane curvature through amphipathic helix membrane binding motifs. The deletion of Rtn1 and Rtn2, in combination with the reticulon interacting protein Yop1 was shown to block NPC assembly and be synthetic lethal (Dawson et al., 2009). One possible direct function of Rtn1/Rtn2 and Yop1 during NPC assembly is to assist in bending the membrane at the pore

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assembly side. This membrane bending could either promote the fusion of INM and ONM during NPC assembly, or stabilize membrane curvature during NPC assembly (Dawson et al., 2009). However, reticulons are not the only ER proteins that assist in NPC assembly. Two proteins that determine the structure of the tubular ER, Lpn1 and Sey1 have also been shown to be important for NPC distribution at the NE envelope and are therefore likely to have a role in NPC assembly. Sey1 has a known role in promoting the a physical interaction of Ndc1 with Rtn1. For Lpn1 on the other hand, it remains to be determined, whether changes in ER morphology cause the potential NPC assembly defects, or whether Lpn1 has a direct role in shaping the NE during NPC assembly (Casey et al., 2015).

The assembly of NPCs in baker's yeast is directly or indirectly supported by Apq12, Brl1 and Brr6, because misassembled pores accumulate in (conditional) knockout strains (Scarcelli et al., 2007; Zhang et al., 2018). How these proteins influence NPC assembly is still controversial. Previous studies suggested, that Apq12, Brl1 and Brr6 are primarily involved in lipid homeostasis (Hodge et al., 2010; Lone et al., 2015) and suggested that changes in lipid composition could cause NPC assembly defects. Though, another, more recent study suggested a more direct involvement in NPC assembly (Zhang et al., 2018). Structurally, these three proteins are similar, consisting of two transmembrane domains (Figure 3). Functionally, these proteins have been suggested to act in a complex (Lone et al., 2015) and they are partially functionally redundant. Brl1 and Brr6 are essential proteins, but overexpression of Apq12, or either Brl1 or Brr6, depending on the deletion can partially rescue defects in mRNA export and cold sensitivity (Hodge et al., 2010; Lone et al., 2015). Apq12, Brl1 and Brr6 are conserved proteins in eukaryotes with closed mitosis. Although these proteins are not conserved on the sequence level in higher eukaryotes, it has been speculated that functional homologues exist (Laudermilch et al., 2016; Thaller and Lusk, 2018).
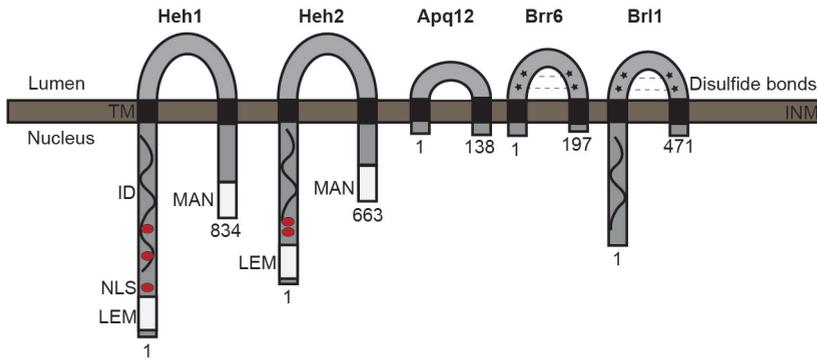
Figure 3 Schematic representation of transmembrane proteins involved in NPC assembly.
Each protein is shown in N-to C-terminal orientation, the length of each protein in amino acids is written at the C-terminus of each protein. Transmembrane domains (TM) are shown as black squares, the location of NLSs are indicated as red dots, ID domains as black scribble within the protein. LEM and MAN domains are indicated as white boxes. Stars with black dashed stripes shows the localization of disulfide bonds in Brr6 and Brl1, these disulfide bonds are important for the functioning of those proteins. The given orientation of Apq12 is only speculative. All proteins are depicted at the Inner Nuclear Membrane (INM) in this cartoon, as all proteins can reach the INM. However, only Heh1, Heh2 and Brl1 are known to accumulate at the INM. Apq12 and Brr6 are enriched at the NE (compared to the ER).

Box 2: The ESCRT machinery

Eukaryotic cells have evolved several systems to facilitate the budding of vesicles into the cytoplasm (COPI, COPII and Clathrin) (Bonifacino and Glick, 2004). However, the ESCRT machinery is the only system inside eukaryotic cells that facilitates membrane bending and budding into the lumen (Figure 4). This kind of membrane remodeling is needed for various processes inside the cell, such as the formation of multivesicular bodies (MVBs), NPC quality control, NE repair (Denais et al., 2016; Raab et al., 2016), NE reformation after open mitosis (Gu et al., 2017), plasma membrane repair, virus budding and the formation of microvesicles. These processes, which require ESCRT function at various intracellular localizations are facilitated by various site specific adaptors, that signal for the ESCRT dependent membrane remodeling (Reviewed in Alonso Y Adell et al., 2016) and the six protein complexes (ESCRT-0, ESCRT-I, ESCRT-III, Vps4-Vta1 and Bro-Alix) which are part of the ESCRT machinery.

All ESCRT protein complexes are needed for the formation of multivesicular bodies (MVBs), a specialized subset of endosomes that contain membrane-bound intraluminal vesicles. (Katzmann et al., 2002). MVBs sort ubiquitylated

cell-surface receptors and other proteins to the vacuole, where they can be degraded. During this process, the ESCRT-0, ESCRT-I and ESCRT-II complexes, which contain several ubiquitin Ile44 recognition motifs, interact with ubiquitylated proteins and cluster them. ESCRT-II specifically initiates contact with the ESCRT-III protein Vps20 (Yorikawa et al., 2005). Once this interaction is established, Vps20 and interacts with ESCRT-I and a nucleation complex is formed, that drives assembly of the ESCRT-III filament, at the budding side (Henne et al., 2012). The Bro-Alix complex is involved in recruiting, activating or stabilizing the ESCRT-III complex (Christ et al., 2016; Tang et al., 2015; Wemmer et al., 2011).

The ESCRT-III protein Snf7 assembles into spiral filaments on the membrane. The membrane interaction is facilitated by an N-terminal amphipathic helix domain and/or through clusters of basic residues in their core domain (Buchkovich et al., 2013; Yorikawa et al., 2005). The assembly of the filament is driven by the release of autoinhibition, either through the exposure to membranes, or through the exposure to upstream ESCRT components. During this process, the ESCRT-III subunits adopt energetically unfavorable conformations at the membrane, while the spiral expands. In the absence of Vps4, the spiral eventually relaxes, an can indeed cause membrane deformation, comparable to the relaxation of a spring (Chiaruttini et al., 2015).

The exact function of ESCRT-III proteins downstream of Snf7 remains elusive. Did2, Vps2 and Vps24 have been proposed to cap the Snf7 filament, but in vitro work shows that these proteins can form filaments (Ghazi-Tabatabai et al., 2008; Lata et al., 2008; McCullough et al., 2015), which have been proposed to form filaments parallel to Snf7 (Alonso Y Adell et al., 2016). The presence of Vps60 is known to promote the assembly of the Vps4-Vta1 complex and increase its activity (Azmi et al., 2006).

Vps4 belongs to the group of AAA-ATPases (ATPase associated with a variety of cellular activities). Vta1 directly binds to Vps4 to promote Vps4 oligomerization and stimulates its ATPase activity (Azmi et al., 2006). The interaction between the ESCRT-III complex and the Vps4-Vta1 complex is established by MIT (microtubule interacting and transport) motifs of Vps4 and Vta1, that interact with the MIM (MIT interacting motif) elements of Snf7 (Kieffer et al., 2008; Skalicky et al., 2012). The rapid depolimerization of the filament is achieved through possessive unfolding of the filament (Yang et al., 2015). The presence of the Vps4-Vta1 complex, probably potentiates membrane remodeling, while the Snf7 filament is disassembled by the Vps4-Vta1 complex, additionally this process facilitates faster membrane remodeling (Adell et al., 2014) and allows the ESCRT-III components to be recycled.

Other membrane remodeling events are independent of several of the ESCRT protein complexes (Reviewed in Alonso Y Adell et al., 2016). The minimal

components required for efficient ESCRT dependent membrane remodeling are the ESCRT-III complex and the Vps4-Vta1 complex (Hurley, 2010). Membrane remodeling at the NE further depends on the site specific adaptor Chm7, cytokinesis is mediated by ESCRT-I, ESCRT-II and Bro-Alix, and plasma membrane repair is dependent on Bro-Alix and potential other factors, yet to be identified (Alonso Y Adell et al., 2016).
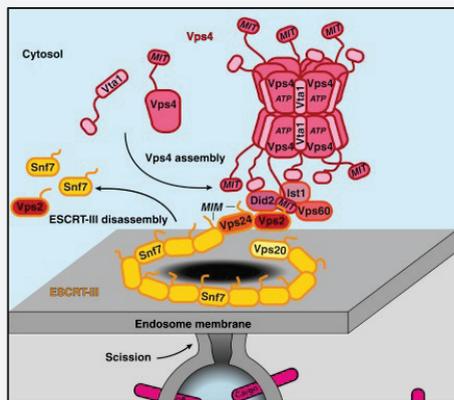

Figure 4 Cartoon of the key components of ESCRT dependent membrane remodeling: ESCRT-III and the Vps4-Vta1 complex. Shown here in the formation of a MVB at the endosome membrane. This process is initiated by ESCRT-0 and further driven by recruitment of ESCRT-I and ESCRT-II complexes to the budding site (not shown). The ESCRT-III complex assembles at the budding site. Here, Vps20 binds to ESCRT-II and subsequently to ESCRT-I to initiate a nucleation complex. This facilitates the assembly of the Snf7 filament. The here shown capping of the assembled filament by Vps24, Vps2 and Vps60 remains controversial. The Vps4-Vta1 complex has the structure of an asymmetric hexameric ring and drives ESCRT-III disassembly. ESCRT-III proteins have MIT-domain Interaction Motifs (MIMs) that engage with the Microtubule Interaction and Trafficking (MIT)-domains of the Vps4-Vta1 complex. Adjusted and reprinted with permission from Schmidt and Teis, 2012.

A dedicated quality control mechanism checks NPCs during their assembly and clears misassembled NPCs from the NE. Heh1 and Heh2, are the proteins that recognize the misassembled intermediate via currently unknown mechanisms. In yeast, Heh1 and Heh2 are the only transmembrane proteins known to require active transport mechanisms to reach their target localization, the Inner Nuclear Membrane (INM). Heh1 and Heh2 have a long and ID linker sequence in between the NLS and the transmembrane domain. Any long ID linker supports transport to the INM (Meinema et al., 2011). Experimental data shows that the transmembrane domain of those proteins stays in the membrane during transport, and suggests that the NLS sequence, bound to its importins Kap60 and Kap95 reaches into the central channel. The linker domain is likely a spacer, that has to dodge into the scaffold of the NPC during transport (Laba et al., 2015; Meinema et al., 2011) (Reviewed in Dixon and Schirmer, 2018; Laba et al., 2014). Whether the ID linker domains of Heh1 and Heh2 are solely needed to facilitate the transport of these proteins to the INM, or whether they

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are also involved in their function in assisting NPC assembly and/or identifying misassembled NPCs is currently unknown.

The data that is currently available supports the following model for the clearance of misassembled NPCs: The NE specific Endosomal Sorting Complexes Required for Transport (ESCRT) adaptor, Chm7, binds to Heh1/Heh2, which allows the ESCRT machinery (See Box 2) to assemble at the site of the misassembled NPC (Webster et al., 2014, 2016). The subsequent clearance of the misassembled NPC from the NE depends on several proteins of the ESCRT-III complex. Vps2, Vps24 and Snf7 are certainly involved, Vps20 does not seem to be part of the nuclear ESCRT-III complex (Webster et al., 2014), although it was previously reported to be part of the core ESCRT-III complex (Hurley, 2010). Snf7 binds directly to Heh2 and Chm7, where it assembles into a polymer. The polymer is capped by Vps24. Vps24 then recruits Vps2 to the complex. Vps2 promotes the assembly of a Vps4 hexamer at the site of the misassembled NPC. Vps4 disassembles the ESCRT-III complex, while hydrolyzing ATP. What kind of membrane remodeling is needed to remove the misassembled NPC from the NE is currently still unknown (Thaller and Patrick Lusk, 2018), but membrane remodeling is achieved through the disassembly of the Snf7 filament (Hurley, 2010). Surprisingly, this process seems to be independent of the Vps4 cofactor, Vta1, based on the absence of synthetic genetic interactions between Vta1 and various tested Nups (Webster et al., 2014). This suggests, that the disassembly of the filament is relatively ineffective (Azmi et al., 2006). Ultimately, the misassembled NPC is degraded by the proteasome (Webster et al., 2014).

Whether and how NPCs are maintained functional, once they have been assembled successfully is a question of ongoing investigation. To this date, no mechanisms that can repair NPCs, or clear nonfunctional NPCs from the NE have been found. Three different theories can be proposed on the maintenance of nuclear pore complexes. First, the same mechanisms that guard NPC assembly and target NPCs for proteasome degradation, could also be used to identify damaged NPCs. Alternatively, or additionally, defective NPCs could be cleared through autophagy, specifically through microautophagy of nuclear vacuolar junctions. A third option, namely that defective NPC scaffolds cannot be cleared from the NE, once assembly is completed, cannot be ruled out, either. Indeed, NPC scaffolds remain intact for at least for 12 month, in postmitotic brain cells (D'Angelo et al., 2009; Savas et al., 2012; Toyama et al., 2013).

Previous studies have suggested, that the loss of stoichiometry of NPCs is likely caused by the accumulation of oxidative damage during aging in postmitotic cells (D'Angelo et al., 2009). Additionally it was shown, that isolated nuclei from old animals are more permeable to dextran molecules than nuclei from young animals. Recent studies propose that protein aggregates found in neurodegenerative diseases are cytotoxic because they impair nucleo-cytoplasmic transport (Freibaum *et al.,* 2015; Jovičić *et al.,* 2015; Woerner *et al.,* 2015; Zhang *et al.,* 2015). Neurodegenerative diseases are age-related, and hence a good understanding of the age-related changes to NPCs may help to understand the disease mechanisms, as well as provide new possibilities to delay the disease's onset. Yet, apart from the above mentioned work by the Hetzer laboratory on NPCs in aging postmitotic cells, at the start of this PhD little was known about NPCs and nucleocytoplasmic transport in aging dividing cells.

Outline of this thesis

This thesis investigates age-related changes in structure and function of NPCs, as well as NPC assembly, in aging *S. cerevisiae* cells. In chapter 2, we ask how NPC structure and function change during replicative aging. We use microfluidic devices to study single cells during the aging process. This allows us to detect changes in protein localization and abundance during aging and to correlate the protein abundance at certain subcellular compartments to the lifespan expectancy of individual cells. Additionally, we mine published data by (Janssens et al., 2015), to analyze the whole cell abundance of NPC, and NPC assembly components. Our results show, that aged cells lose several of the proteins involved in NPC assembly (Apq12, Heh2, Vps4 and Brl1), as well as several of the FG-Nups (Nsp1, Nup2, Nup49 and Nup116). Additionally, we find that aged cells show more frequently Chm7 foci at the nuclear envelope, which supports the hypothesis, that aged cells encounter problems to assemble NPCs. Several proteins studied in the microfluidic device showed, that the loss of NPC and NPC assembly proteins is negatively correlated to the lifespan expectancy of a cell. Consequently, cells that manage to maintain protein levels at the NE are long lived.

We also study the steady state localization of nuclear transport reporter proteins, as a readout of NPC function. We find that nuclear compartmentalization increases during aging and that this phenotype can be mimicked by a strain that is defective in NPC assembly. Furthermore, we show that native Srm1 shows increased nuclear localization in aged cells, reminiscent of what is measured

with the reporter protein. Additionally, we study nuclear transport dynamics by monitoring the shuttling transcription factor, Msn2. We find that nuclear transport dynamics decrease during replicative aging, and that the decrease in nuclear transport dynamics is correlated to lifespan. Overall, our functional data (increased nuclear compartmentalization and decreased nuclear transport dynamics) are consistent with a reduced number of transport competent NPCs in aged cells, which is most likely caused by a problems in NPC assembly.

Since the mechanisms for NPC assembly and quality control appear as an important factor in aging, we set out to further study the mechanisms of NPC quality control, in chapter 3. Here, we focus on one particular protein involved in NPC assembly and quality control, Heh2. Heh2 resides exclusively at the INM and arrives there through a unique transport mechanism that relies on a long ID linker and a bipartite NLS. We entertained the hypothesis that the unusual translocation mechanism of Heh2 might be part of the function of Heh2 in the recognition of misassembled NPCs, where the ID linker probes the integrity of the NPC scaffold during its translocation. In chapter 3, we study the translocation mechanism and ask how changes in the long ID linker domain impacts the ability of transmembrane proteins to reach the INM. We find, that the ID linker is optimized to facilitate highly efficient transport to the INM, but other extended linker domains are also able to facilitate INM localization of Heh2. Recent structural data of the yeast NPC reveals that the approximate distance between the pore membrane and the inner surface of the NPC central channel is about 25 nm. Consistent with a transport mechanism, where the ID linker facilitates the crossing of the NPC scaffold, we find that the frequency at which the linkers are at an extended end-to-end distance larger than 25 nm is indeed a good predictor for the INM targeting efficiency of transmembrane proteins with an NLS. Future studies should aim to investigate, whether the highly efficient transport of Heh2 to the INM, could potentially serve as a sensing mechanism for misassembled NPCs, because only a functional NPC scaffold should allow the efficient translocation of Heh2 to the INM.

NPC assembly has been suggested, to be very limited in postmitotic cells. Therefore, we asked, whether replicative and chronological aging have distinct influence on the structure and function of NPCs. Chapter 4 describes preliminary results on NPC structure and function in chronological aging. The comparison of proteome data from replicative (Janssens et al., 2015) and chronological aging yeast cells (Binai et al., 2014) show, that the loss of FG-Nup abundance is specific for replicative aging cells. We age the cells in water

(extreme caloric restriction) and study the localization of GFP reporter proteins and several Nups tagged with fluorescent proteins over the time course of one week. We find, a significant decrease in the steady state localization of GFP-NLS reporter protein, however the majority of cells are able to maintain nuclear compartmentalization. We confirm that the nuclear accumulation is the result of active import of GFP-NLS, using a Fluorescence Recovery After Photobleaching (FRAP) based assay. Additionally, I describe problems and challenges, of yeast as a model system to study aging of postmitotic cells. The results in chapter 4 indicate that chronological and replicative aging pose distinct challenges, namely that dividing cells face the challenge to assemble NPCs, while non-dividing cells face the challenge to maintain NPCs.

Chapter 5 is a review on our current knowledge about NPC assembly, maintenance and function in aging of mitotic and postmitotic cells. We mine several proteome datasets that analyze age-related changes in protein abundance in different aging model systems. We find that changes in nup abundances are highly variable, but changes at the NPC in yeast replicative aging bear resemblance with the changes found in rat liver and changes at the NPC in chronologically aged yeast bears resemblance with the changes found in mouse brain samples. Additionally, this chapter discusses the potential relevance of our findings