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Review

Nuclear Periphery Takes Center Stage: The Role of Nuclear Pore Complexes in Cell Identity and Aging

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In recent years, the nuclear pore complex (NPC) has emerged as a key player in genome regulation and cellular homeostasis. New discoveries have revealed that the NPC has multiple cellular functions besides mediating the molecular exchange between the nucleus and the cytoplasm. In this review, we discuss non-transport aspects of the NPC focusing on the NPC-genome interaction, the extreme longevity of the NPC proteins, and NPC dysfunction in age-related diseases. The examples summarized herein demonstrate that the NPC, which first evolved to enable the biochemical communication between the nucleus and the cytoplasm, now doubles as the gatekeeper of cellular identity and aging.

Introduction

The nuclear pore complex (NPC) is central to all forms of eukaryotic life as it mediates biomolecular communication between the nucleoplasm and the cytoplasm. The NPC consists of ~30 subunits called nucleoporins (Nups). Some of them are very static and spend most of their time stably incorporated in the NPC, while others are soluble and shuttle between the NPC and the nucleoplasm. For its foundational role, the NPC is directly or indirectly involved in essentially all cellular activities. Surprisingly, starting in the late 2000s, two major biological processes, genome regulation and aging—long thought to be indirectly affected by NPCs—have been shown to be under the direct control of these nuclear transport channels (Ahmed et al., 2010; Brown et al., 2008; Capelson et al., 2010; Casolari et al., 2005; D'Angelo et al., 2009; Kalverda et al., 2010; Taddei et al., 2006). Recent studies have revealed that the NPC is, in fact, a key cellular component for the transcriptional control of cell identity genes and cellular health.

Evidence that the NPC might directly regulate gene activity dates back to the 1950s. Pioneering electron microscopy studies uncovered that the genome at the nuclear envelope is condensed into heterochromatin with the exception of the regions below NPCs. The discovery of these so-called "heterochromatin exclusion zones" was the first evidence that hinted at the role of the NPC in genome organization and by extension gene regulation (Swift, 1959; Watson, 1959). In 1985, Günter Blobel proposed the "gene gating" hypothesis, which stated that these patches of open euchromatin are generated by the interactions between NPCs and transcriptionally active portion of the genome (Blobel, 1985). Subsequent studies in *Saccharomyces cerevisiae* have demonstrated that NPCs not only determine the three-dimensional locations of the genes within the nucleus but also affect their activities (Randise-Hinchliff and Brickner, 2018). In *Drosophila* and mammalian cells, the NPC has evolved to enable more intricate genome regulation. Soluble Nups that have significant residence time in the nucleoplasm can regulate gene activity even when they are away from the NPC. In other words, Nups function both at and away from the NPC as genome-regulating proteins (Figure 1A) (Raices and D'Angelo, 2017). Latest studies on the genome regulatory roles of Nups illustrate two convergent themes: (1) Nups are mostly involved in the activation or silencing of cell-type-specific genes, and in line with this function, (2) Nup expression levels vary greatly in different cell types. We discuss how these features enable cell differentiation and safeguard the identity of a cell.

In addition to the dual functionality in nucleocytoplasmic transport and transcriptional control, another major focus of the studies on the NPC in the last two decades has been the dynamics of Nups. Soon after the gene-activating capability of Nup98 was reported in the context of acute myeloid leukemia (AML) in 1999, it was revealed that Nup98 is very mobile (Griffis et al., 2002; Kasper et al., 1999). This observation raised a question whether the NPC comprises subunits of widely varying residence times. It is now well established that the dwell time of the ~30 Nups that make up the NPC ranges from tens of seconds to several years, spanning seven orders of magnitude in time (Rabut et al., 2004; Toyama et al., 2013). The discovery of long-lived Nups, together with the finding that NPC biogenesis is minimal in post-mitotic cells (D'Angelo et al., 2009), suggested that NPCs may accrue significant amount of biochemical damage over time and be a major player in aging (Figure 1B). Unlike other long-lived proteins that have been shown to deteriorate with age, such as eye lens crystallin, myelin, and collagen, Nups are strictly intracellular and thus can have greater impact on cellular health. The NPCs in Physiological Aging section details the results that have confirmed the age-dependent decline in the NPC's transport and barrier functions.

Physiological aging is not the only biological context in which disruption of the NPC is observed. During neurodegeneration, progeria, oncogene-induced senescence, viral infection, and apoptosis, the NPC can be compromised via proteolysis, phosphorylation, displacement, and/or mislocalization of Nups.

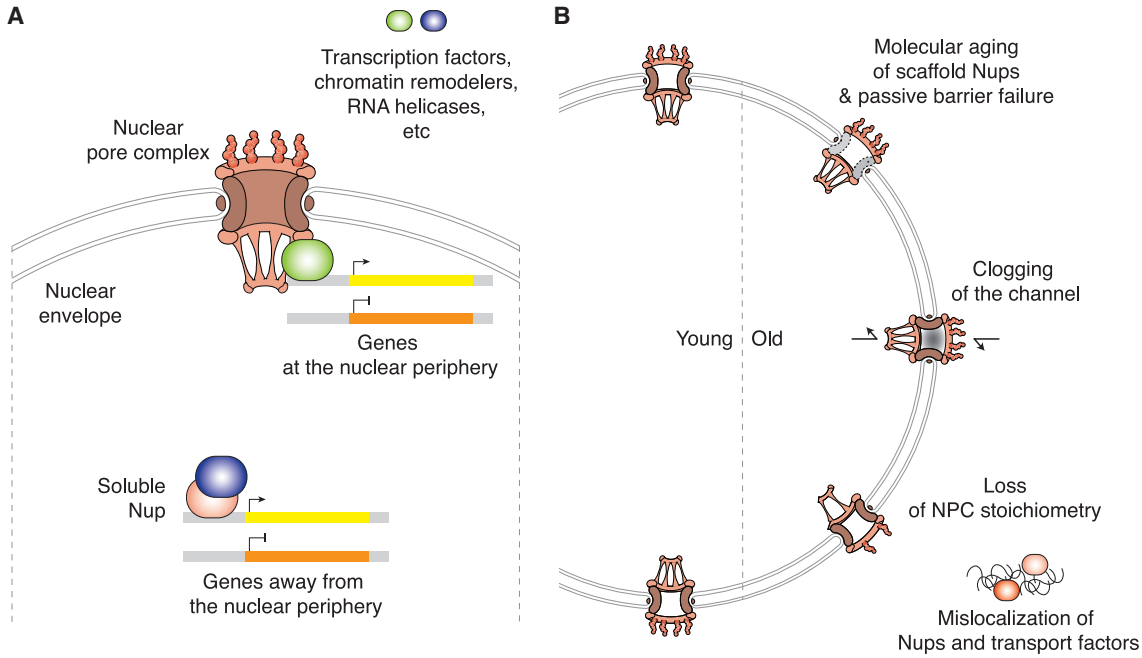

Figure 1. NPC in Gene Regulation and Aging
(A) Nups can regulate genes both at and away from the NPC, typically by recruiting transcription factors, chromatin remodelers, RNA helicases, or other auxiliary proteins. They can either activate or suppress genes.
(B) The NPC can be compromised by various mechanisms during aging.

Although the mechanisms vary, the consequences are invariably catastrophic, reflecting the paramount importance of this protein complex in eukaryotic cells. NPC dysfunction is also seen during replicative aging of yeast, suggesting that the NPC has long been a weak spot in eukaryotic cellular homeostasis. Recently, a few NPC surveillance mechanisms have been discovered in yeast, raising an exciting possibility that metazoans may have developed similar measures to cope with the same problem. We will mainly focus on the NPC failure in age-related conditions and diseases (Figure 1B) but also briefly describe the NPC modification and disintegration by viral proteins and caspases for comparison. As our understanding of the molecular details of NPC disruption deepens, we will be able to better prevent, slow down, or cure the fatal human pathologies involving the NPC, like amyotrophic lateral sclerosis (ALS) and Huntington's disease. Altogether, we are now beginning to comprehend the fundamental importance of the NPC in both development and aging.

NPCs in Maintaining and Changing Cell Identity

While many aspects of NPC-mediated transcriptional control remain to be determined, we have come a long way in our understanding of gene regulation at the nuclear envelope. Like many other research topics in the field of biology, NPC-mediated genome regulation was first investigated in yeast and flies. For example, the very first genome-wide screening for NPC-interacting genes (Casolari et al., 2004), the identification of genes that are recruited to the NPC upon induction and their DNA sequence requirements (Ahmed et al., 2010; Brickner and Walter, 2004; Cabal et al., 2006; Casolari et al., 2005; Taddei et al., 2006), and the discovery of the NPC-mediated transcriptional memory and subtelomeric gene silencing (Galy et al., 2000; Light et al., 2010) were carried out in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *Drosophila melanogaster* was used to demonstrate that gene gating occurs via SAGA (Spt-Ada-Gcn5-Acetyltransferase) complex (Kurshakova et al., 2007), to study nuclear pore basket Nup-interacting genes (Mendjan et al., 2006; Vaquerizas et al., 2010), and to show that Nups interact with the genome not only at the nuclear periphery but also in the nuclear interior (Capelson et al., 2010; Kalverda et al., 2010). One common theme that emerged from these studies in the 2000s was that Nups, in general, target cell identity genes. Here, we discuss how Nups activate or silence these genes in mammalian systems. Genome regulation by Nups in yeast, flies, and *Caenorhabditis elegans* (*C. elegans*) has been reviewed elsewhere (Kuhn and Capelson, 2019).

Genome Regulation by Nup93 at the NPC

Nup93 was among the first mammalian Nups whose genome regulatory function was examined (Brown et al., 2008). Nup93 is a scaffold Nup that forms the core module (Nup93-205 complex) of the NPC. Unlike mobile Nups such as Nup98, Nup153, and Nup50 that shuttle between the NPC and the nucleoplasm, Nup93 resides almost exclusively at the nuclear envelope and is suitable for studying NPC-genome interactions confined to the nuclear periphery. Soon after the interaction between Nup93 and CREB-binding protein was confirmed (Ryan et al., 2006), the Silver group, who used ChIP-chip (chromatin immunoprecipitation with DNA microarray) to find genes that come in contact with nucleoporins in yeast (Casolari et al., 2004), employed the same technique to map the interactions between Nup93 and

chromosome 5, 7, and 16 in HeLa cells (Brown et al., 2008). Among 86 genes adjacent to Nup93-binding sites were *HOXA1*, *-3*, and *-5*. The role of Nup93 in *HOXA1* gene regulation was investigated in more depth several years later (Labade et al., 2016, 2019). In mice, *Hoxa1* is transiently expressed in the presumptive hindbrain and is necessary for proper development of the brainstem, inner ear, and heart (Makki and Capecchi, 2011). In DLD1 (diploid colorectal cancer) cells, when Nup93 was depleted by small interfering RNA, *HOXA1* transcript level increased ~4-fold and histones became modified with activation marks. At the same time, the gene loci moved away from the nuclear edge. Taken together, these results suggest that Nup93 on the NPC is involved in gene silencing. A recent study in fact have shown that Nup93 preferentially associates with Polycomb-silenced regions of the genome in flies, whereas Nup107 largely targets active genes (Gozalo et al., 2020). Interestingly, repression and de-repression of the *HOXA1* gene through Nup93 binding is observed during neuronal differentiation. When NTERA2/D1 (pluripotent embryonal carcinoma) cells were treated with retinoic acid, the *HOXA1* loci moved away from the nuclear edge early during differentiation but returned back to their initial positions as cells matured into neurons. Accordingly, *HOXA1* transcript level surged ~15-fold upon retinoic acid addition and decreased afterward. Current understanding is that the majority of cell-identity-related genes are controlled by Nup93 and other NPC components. Nup93 DNA adenine methyltransferase identification—a ChIP-chip equivalent that does not require ChIP-grade antibody—revealed that 30.1% of the super-enhancers (SEs) in U2OS cells are associated with Nup93 at the nuclear periphery (Ibarra et al., 2016). Given that SEs regulate genes that determine cell fate (Hnisz et al., 2013), Nup93 can be considered a structural platform for cell identity maintenance and change.

Genome Regulation by Nup98 Away from the NPC

The gene regulatory functions of some Nups were discovered through the studies of human diseases. For example, in hematopoietic malignancies, Nup98, Nup214, and Nup358 genes are often found to be fused with genes that encode DNA-binding domains (Fahrenkrog, 2014; Franks and Hetzer, 2013; Gough et al., 2011; Mendes and Fahrenkrog, 2019). The fusion proteins aberrantly activate genes to promote abnormal proliferation of blood cells. More specifically, in AML, a Nup98-Hoxa9 fusion protein is generated by chromosomal translocation, and the mutation and deletion studies of the fusion protein confirmed that the NUP98 FG-repeat domain recruits CREB-binding protein and p300 to function as a transactivator (Kasper et al., 1999). The gene activation by Nup98 gained more interest when it was shown to be a soluble Nup that spends significant amount of time in the nucleoplasm (Griffis et al., 2002). Since then, four other co-activators were identified: Exportin-1 (Oka et al., 2016), Histone-lysine *N*-methyltransferase 2A (Shima et al., 2017), DExH-Box Helicase 9 (Capitanio et al., 2017), and Wdr82 (Franks et al., 2017). However, histone deacetylase (HDAC) 1 was also found to interact with Nup98 (Bai et al., 2006), suggesting that Nup98 can mediate both gene activation and silencing. Nup98 ChIP-seq (chromatin immunoprecipitation sequencing) and DNA FISH (fluorescence *in situ* hybridization) data from human embryonic stem cells (ESCs) and neural progenitor cells (NeuPCs) explain how both gene up- and downregulation can be achieved by the same protein (Liang et al., 2013). During ESC-to-NeuPC differentiation, the expression of Nup98 target genes remains stable in one subset ("group I"), and increases >5-fold in the other subset ("group II"). Interestingly, DNA FISH revealed that group I gene loci were mostly near the nuclear edge in NeuPCs, while those of group II were predominantly in the interior of the nucleus. This indicates that the NPC provides a biochemical environment that promotes gene silencing by Nup98, and the nucleoplasm supports the gene activation capability of Nup98. Accordingly, it was later discovered that Nup98 binds to intranuclear promoters, recruits Wdr82-Set1A/COMPASS complex, and deposits H3K4 trimethylation activating marks (Franks et al., 2017). Of note, out of 1,962 and 2,081 Nup98-binding regions in ESCs and NeuPCs, only 321 overlapped, validating that Nup98 dynamically associates with developmental genes as Nup93. Nup98 targets in NeuPCs were enriched for neurogenesis genes, such as *NRG1*, *SOX5*, *ROBO*, and *ERBB4*. In line with the findings in mammalian cells, in *Drosophila*, Nup98 regulates genes under the control of ecdysone, an insect hormone that is critical for molting and metamorphosis (Pascual-Garcia et al., 2017).

Nups in Differentiation

Generally, Nups modulate gene activity by recruiting transcription factors, chromatin remodelers, RNA helicases, or other types of auxiliary proteins (Figure 1A; Table 1). The latest example is the recruitment of Olig2 and Brd7 by the Y-complex Nup Seh1 during oligodendrocyte differentiation (Liu et al., 2019). Olig2 is a transcription factor and Brd7 is a bromodomain protein that binds to acetylated histones. Upon induction of differentiation, Seh1 in oligodendrocyte progenitor cells interacts with the two proteins at the NPC and enables the expression of myelination-related genes such as *Mbp*, *Cnp*, *Plp1*, and *Mog*. Accordingly, mice lacking Seh1 in the progenitor cells show impaired adult myelinogenesis and remyelination after injury. In C2C12 cells undergoing myoblast-to-myotube transition, a similar but slightly more complex mechanism was observed (D'Angelo et al., 2012; Raices et al., 2017). Nup210, also known as gp210 and one of the three transmembrane Nups, is absent in C2C12 myoblasts but is rapidly upregulated during differentiation into myotubes. Subsequently, Nup210 recruits the transcription factor Mef2C and the transcriptional co-regulator Trip6 to the nuclear periphery and induces myogenic genes such as *Tnnt1*, *Myl2*, *Tmod1*, *miR133a1*, and *miR206*. Nup210-depleted myoblasts fail to become myotubes. While Seh1 in oligodendrocytes and Nup210 in myotubes upregulate new genes, in other systems, Nups repress yet-to-be-expressed developmental genes in progenitor cells. For example, in mouse NeuPCs, Nup153 and Sox2 co-occupy genes necessary for the stemness of the cells at both nuclear periphery and interior. The shRNA-mediated knockdown of Nup153 induced neurite extension and neuronal genes such as *Syn1*, *Tubb3*, and *Hes5*, suggesting that Nup153 is critical for the cell identity homeostasis of NeuPCs (Toda et al., 2017). In mouse ESCs, Nup153 represses developmental genes by recruiting the Polycomb-repressive complex 1 (PRC1) (Jacinto et al., 2015). As in the case of NeuPCs, Nup153 knockdown induced differentiation. In fact, both transcript and protein levels of Nup153 are significantly reduced during ESC-to-NeuPC differentiation. Intriguingly, Polycomb proteins Pc and E(z) interact with Nup93 for

Table 1. Genome-Regulating Nups and Their Binding Partners

Nucleoporin	Binding Partner	Cell Type	Reference
Nup98	CREB-binding protein p300	NIH 3T3 and HeLa	Kasper et al., 1999
	Exportin-1	Embryonic stem cell	Oka et al., 2016
	Histone-lysine *N*-methyltransferase 2A	HEK293T	Shima et al., 2017
	DExH-Box Helicase 9	HEK293T	Capitanio et al., 2017
	WDR82	Hematopoietic progenitor cell	Franks et al., 2017
	CREB-binding proteinHistone deacetylase 1	HEK293T	Bai et al., 2006
	sPom121	HeLa, U2OS, and IMR90	Franks et al., 2016
Seh1	Olig2 and Brd7	Oligodendrocyte	Liu et al., 2019
Nup210	Mef2C and Trip6	Myotube	Raices et al., 2017
Nup153	Sox2	Neural progenitor cell	Toda et al., 2017
Nup153	Polycomb-repressive complex 1	Embryonic stem cell	Jacinto et al., 2015
Nup153	Histone deacetylase 5	Cardiomyocyte	Nanni et al., 2016
Nup155	Histone deacetylase 4	Cardiomyocyte	Kehat et al., 2011

gene silencing in *Drosophila*, hinting that the partnership between Nups and Polycomb proteins arose early during metazoan evolution (Gozalo et al., 2020). Lastly, in resting cardiomyocytes, sarcomeric and Ca^{2+}-handling genes are repressed by Nup155 and HDAC4; however, when hypertrophic growth starts, HDAC4 is exported from the nucleus and Nup155/HDAC4 target genes are de-repressed (Kehat et al., 2011). Unlike the aforementioned Nup98-HDAC1 interaction, Nup155 and HDAC4 work together predominantly at the nuclear periphery. In addition to the Nup155 and HDAC4 pair, in cardiomyocytes, Nup153 and HDAC5 downregulate *Cacna1c* ($Ca_v1.2$ calcium channel) and other genes that can cause cardiac dysfunction when de-repressed (Nanni et al., 2016). To summarize, Nups interact with various transcription factors and coregulators to achieve cell-type-specific control of the genome.

Compositional Variation of the NPC in Various Cell Types

The role of Nups in modulating the activities of developmental genes is to some extent reflected in the compositional variation of the NPC in different cell types. As mentioned above, Nup210, which enables the expression of myogenic proteins, is absent in myoblasts but highly induced during their differentiation into myotubes. In this section, we summarize the recent efforts to quantitatively determine NPC stoichiometry in various cells and tissues.

It was known as early as the late 1990s that the expression level of Nups can be cell- and tissue-type dependent. For example, during the characterization of Nup45, which turned out to be an isoform of Nup58 generated by alternative mRNA splicing, Nup45 and Nup58 protein levels were found to be dissimilar in murine melanoma, teratocarcinoma, hepatoma, and liver cells (Hu and Gerace, 1998). Similarly, Nup50 expression was found to be significantly higher in testis than in kidney, liver, and spleen and was undetectable in heart (Guan et al., 2000). *In situ* hybridization for Nup210 mRNA in a whole-mouse embryo section further validated the tissue-specific expression of NPC subunits (Olsson et al., 1999). Before the 2010s, only low-throughput methods were available for examining NPC assembly variation; however, with the advent of advanced mass spectrometry and transcriptomic technologies, it became possible to study the cell-type specificity of the NPC not only with greater throughput but also with better precision (also see Box 1).

In 2013, the relative copy numbers of 29 Nup proteins in human cells were analyzed using mass spectrometry (Ori et al., 2013). Seven Nups (Nup214, Aladin, Nup210, Pom121, Tpr, Nup50, and Nup37) showed varying protein abundance in HEK293 and four cancer cell lines (Figure 2A). The mRNA levels of these Nups were analyzed in 44 tissue types and 30 disease states and also exhibited significant variance. A similar survey was carried out for tens of other protein complexes, including the anaphase-promoting complex, RNA polymerase I and III core complexes, cohesion complex, linker of nucleoskeleton and cytoskeleton complex, and spliceosomes. About two-thirds showed stable stoichiometry across the aforementioned five human cell lines, and among those whose composition was cell-type dependent like the NPC were transcription-export complex, nucleosome remodeling deacetylase complex, and PRC1. (A more comprehensive study on the compositional variation of 182 mammalian protein complexes was published three years later [Ori et al., 2016].) Taken together, these results establish that the NPC is a cellular machinery tailored to meet the particularities of a cell.

While the study described above clearly demonstrates the differential expression of Nups across cell lines, a similar survey for human tissues, which can be more meaningful from a developmental biology point of view, is still lacking. Therefore, we turned to publicly available transcriptomics and proteomics data to compile this information. Using the RNA expression consensus dataset from the Tissue Atlas curated by the Human Protein Atlas consortium (http://www.proteinatlas.org) (Uhlén et al., 2015), we generated a heatmap visualizing the mRNA abundance of 30 Nups in various cells and tissues (Figure 2B). As a control, we included *GAPDH*. Similarly, using the proteomics data that are available through the European Bioinformatics Institute Expression Atlas (https://www.ebi.ac.uk/gxa/) (Kim et al., 2014), the expression patterns of 29 Nups were plotted onto a heatmap (Figure 2C). The data are less comprehensive

Box 1. Visualizing NPC Stoichiometry with Super-Resolution Microscopy

With the maturation of super-resolution microscopy techniques, in the 2010s, it became feasible to directly probe the quaternary structure of the NPC. In other words, the NPC composition can now be examined by counting the copy numbers of Nups in a single pore. Individual Nups within the NPC were successfully resolved for the first time in 2012 by dSTORM (direct stochastic optical reconstruction microscopy) imaging of *Xenopus* oocytes (Löschberger et al., 2012). Super-resolution micrographs of Nup210 showed clearly the 8-fold rotational symmetry of the NPC ring. Its luminal diameter was determined to be 146 nm and the innermost diameter to be 40 nm. In addition, Nup210 dSTORM imaging demonstrated that both luminal and central channel ring diameters decrease by 3–7 nm as *Xenopus* oocytes develop (stage II versus stage VI) (Sellés et al., 2017). The NPC density concomitantly reduces from 54 to 37 NPC per μm^2, suggesting that not only the quaternary structure but also the density of the NPC can change to meet the needs of a cell.

In 2013, the Ellenberg group conducted a more exhaustive study (Szymborska et al., 2013). They, too, adopted dSTORM but imaged Nup133, Nup107, Nup96, Nup160, Seh1, and Nup62; the first five are components of the Y-complex (the Nup107-Nup160 complex) and the last is a central channel Nup. The radii of the rings formed by eight copies of each Nup was calculated, and this information was utilized to determine their locations on the three-dimensional model of the NPC generated by cryogenic electron microscopy. Furthermore, it enabled them to map the stacking orientation of the Y-complexes on the NPC. Of note, central channel Nup62 appeared as a dot, rather than a ring, in the super-resolution micrographs, validating their method. The latest technology to image the NPC at a sub-diffraction limit involves the use of SNAP or Halo-tagged Nups in conjunction with an O^6-benzylguanine- or O^2-benzylcytosine-functionalized fluorophore or GFP-tagged Nups in conjunction with GFP nanobody (Schlichthaerle et al., 2019; Thevathasan et al., 2019). Compared to antibodies, these labeling modules enable a smaller offset between the protein of interest and the imaging probe, enabling a greater localization precision. In short, latest imaging approaches allow us to spatially probe the NPC at a single-protein level.

compared to its RNA counterpart, presumably due to the low abundance and solubility of Nup proteins. Nevertheless, the two heatmaps reveals a few intriguing points. (1) In adults, testis, ovary, pancreas, monocytes, and T cells have high levels of Nup proteins overall, suggesting that these tissues and cells require a greater number of NPCs. (It should be noted that not all cell types in testis, ovary, and pancreas will be abundant in NPCs.) (2) In blood cells, although the transcript levels are low in general, the protein levels are relatively high, hinting that Nup transcripts can be efficiently translated or that Nup proteins have long half-lives in these cells. (3) The expression profile of Nups drastically diverges during the hematopoietic cell differentiation (B cell, T cell, monocyte, versus NK cell). Analogous analyses can be performed for many different biological contexts—such as human brain development, aging, and dementia—with the data generated from the Allen Brain Atlas project (http://portal.brain-map.org). While one needs to use caution in interpreting these large-scale data, they can be utilized as an initial guide for exploring the cell and tissue specificity of the NPC.

NPCs in Physiological Aging

During the last decade, the NPC was revealed to be a major weak spot of aging eukaryotic cells. It has been shown that NPC integrity and functions deteriorate during physiological, pathological, and premature aging. This is reminiscent of the NPC disintegration by caspases and viral proteins and presumably signifies that undermining NPC integrity is among the most unfailing ways to make cells dysfunctional. In cells undergoing apoptosis, the core functions of the NPC, nucleocytoplasmic transport and passive permeability barrier, are abolished by caspases that can cleave at least seven Nups (Tpr, Nup358, Nup214, Nup153, Nup96, Nup93, and Nup50) (Faleiro and Lazebnik, 2000; Ferrando-May et al., 2001; Patre et al., 2006). The NPC is similarly compromised by viruses (Gomez et al., 2019; Le Sage and Mouland, 2013; Yarbrough et al., 2014). They quickly disrupt NPCs to inhibit host mRNA export and protein import using proteases, kinases, and other mechanisms. For example, poliovirus 2A protease cleaves Nup98, Nup153, and Nup62, and encephalomyocarditis virus L protein hyperphosphorylates Nup214, Nup153, and Nup62. Severe acute respiratory syndrome coronavirus nonstructural protein 1 disrupts Nup93 localization. In short, NPC integrity needs to be well maintained for a cell to function normally. In this section, we describe how NPCs become defective during normal aging and briefly summarize the NPC quality control mechanisms identified in yeast, which can guide the discovery of similar protective and repair measures in metazoans.

Extreme Longevity of Scaffold Nups

Scaffold Nups (Nup93, Nup155, and Nup205) have extremely long residence times and low degradation rates. In fact, they are the most long-lived proteins alongside histone H3.1 (Toyama et al., 2013). These Nups have to persist for the entire lifespan of an animal in neurons, cardiomyocytes, myotubes, etc., since the biogenesis of new NPCs is negligible in post-mitotic cells (D'Angelo et al., 2009). Although not as long lived as these, a few Y-complex Nups (Nup96, Nup160, and Nup107) also have long half-lives. The minimal turnover of long-lived Nups in the rat brain and heart was shown by labeling newborns with ^{15}N until 6 weeks after birth and switching to ^{14}N diet afterward. In neurons of 1-year-old rats, one-fourth of scaffold Nups still contained ^{15}N, proving their extreme longevity. Considering these samples also contained non-post-mitotic cells from brain and heart that continue to divide in adulthood, the actual replacement rate in post-mitotic cells could be even lower. In cultured cells, the lifetimes of scaffold Nups were determined to be about 100 h, much shorter than in a whole animal (Mathieson et al., 2018; Rabut et al., 2004). However, they are still longer than the average lifespan of the whole proteome, which is 1–2 days depending on the cell lines (Cambridge et al., 2011). Consistently, using the recombination-induced tag exchange technology (Verzijlbergen et al.,

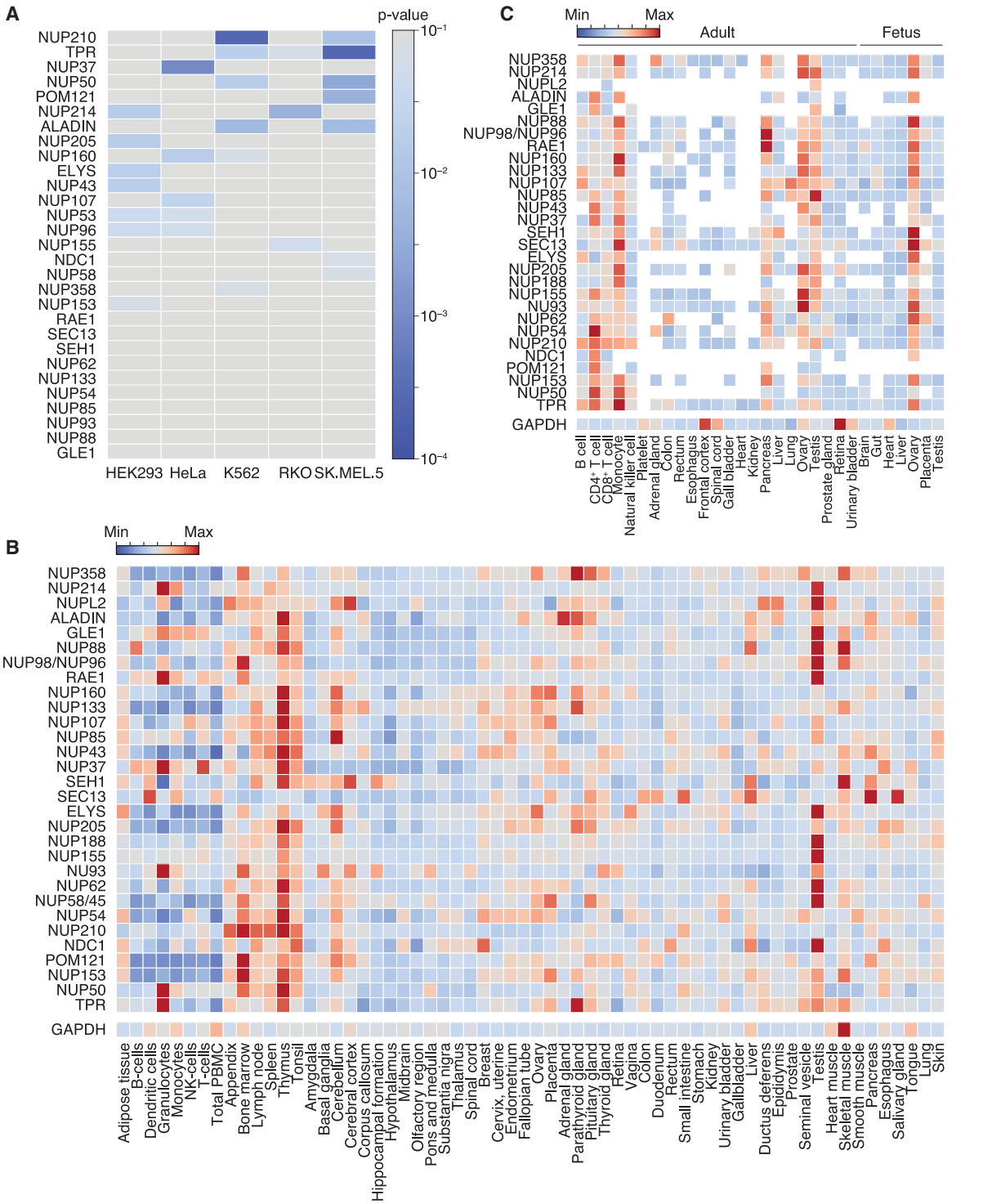

Figure 2. NPC Stoichiometry and Nup Expression Vary in Different Cell Types and Tissues

(A) A p value heatmap that visualizes the differential expression of Nups in HEK293 and four different human cancer cell lines. A low p value means that the Nup protein level in a given cell line significantly differs from that in others. Image reproduced from Ori et al. (2013) under a Creative Commons license (https://creativecommons.org/licenses/by/4.0/).

(B and C) Nup mRNA (B; Uhlén et al., 2015) and protein expression (C; Kim et al., 2014) in various cells and tissues.

2010), we were able to confirm that the majority of long-lived Nups are not replaced over the course of 2 weeks in C2C12 myotubes (Toyama et al., 2019). During the same time period, short-lived Nups, such as Nup133 and Pom121, were mostly replenished with newly synthesized copies, suggesting that the NPC in old cells are composed of Nups with varying lifespans.

Like other long-lived proteins such as eye lens crystallin, myelin, and collagen, long-lived Nups can accrue damage over time and can be difficult to replace once lost from the complex (Figure 1B) (Toyama and Hetzer, 2013). We indeed discovered an aging phenotype in *C. elegans* and rat brains that can be attributed to Nup degeneration (D'Angelo et al., 2009). Nup93 and Nup205 are essential for NPC passive permeability barrier (Galy et al., 2003), and their breakdown is expected to make nuclei permeable to macromolecules that are normally excluded. Using a fluorescently labeled 70-kDa dextran, we confirmed that the nuclei isolated from post-mitotic adult worms and old rat brains are significantly "leakier" than those obtained from younger counterparts. Impaired permeability barrier can lead to unregulated mixing of nuclear and cytoplasmic contents, jeopardizing cellular homeostasis. This aging phenotype was more pronounced in *C. elegans* grown in the presence of paraquat, a chemical that generates reactive oxygen species, and suppressed in worms with increased lifespan (*daf-2* RNAi), indicating that NPC integrity is strongly linked to organismal longevity. In nuclei isolated from rat brains, Nup93 immunofluorescence signal was 2-fold higher in old-but-intact than in old-and-leaky nuclei, further corroborating that the nuclear barrier decline is due to irreversible Nup damage or loss. In a separate study, the same dextran exclusion assay revealed that cardiomyocyte nuclei from old rats are more permeable than those from young rats (Kajstura et al., 2010). More recently, the age-dependent reduction of Nup93 immunofluorescence intensity and deterioration of permeability barrier were also confirmed in human pancreases (Arrojo e Drigo et al., 2019a). Interestingly, the basal body of the primary cilium, which contains Nup93 (Del Viso et al., 2016; Kee et al., 2012; McClure-Begley and Klymkowsky, 2017), was found to be long lived by ^{15}N pulse-chase labeling in mice (Arrojo E Drigo et al., 2019b). This raises an interesting possibility that primary cilia, analogous to NPCs, may experience an age-related decline in function.

While it is still unknown whether metazoan cells have a dedicated protein quality control mechanism for the NPC, several such processes have been identified in yeast, and it is tempting to assume that similar pathways exist in multicellular organisms. For example, during budding, dysfunctional NPCs are preferentially retained in the mother cells to maximize daughter lifespan (Webster et al., 2014). Defective NPCs in mothers become buried in the nuclear envelope, which presumably is a measure to prevent faulty nucleocytoplasmic transport activities (Rempel et al., 2019). During meiosis of budding yeast, gametes inherit short-lived nuclear basket Nups, but not long-lived core Nups (King et al., 2019). This ensures that progeny start their lives only with fully functional, recently synthesized proteins. It will be interesting to investigate whether the second polar body generated in the course of meiosis II in mammals also sequester away the damaged Nups or NPCs from the mature ovum. In fact, during *C. elegans* gametogenesis, aged proteins are cleared by lysosomes (Bohnert and Kenyon, 2017), and mammals are likely to have a similar renewal checkpoint.

NPC Stoichiometry and Density Change during Aging

Another form of molecular aging for large protein assemblies like the NPC is the loss of optimal stoichiometry. For example, the transcript and protein levels of *NUP88*, *NUP107*, *NUP155*, and *NUP50* decrease with passage number in human fibroblasts (Kim et al., 2010). The decrease in several Nups explains the reduction in nuclear translocation of phosphorylated ERK1/2 and NF-κB p50 and related aging phenotypes such as the insensitivity to growth factors and apoptotic stress. More recently, in a study in which age-dependent changes in the transcriptomes of human fibroblasts, fibroblast-derived induced neurons, and prefrontal cortex cells were analyzed, RANBP17, a transport receptor that belongs to the importin β family (Koch et al., 2000; Kutay et al., 2000), was discovered to decline chronologically at both RNA and protein levels (Mertens et al., 2015). Accordingly, when RFP-NLS and GFP-NES (NLS, nuclear localization signal; NES, nuclear export signal) were co-expressed in fibroblasts, GFP-to-RFP ratio in the nucleus well correlated with the donor age, demonstrating that nucleocytoplasmic transport becomes defective in old cells. Yeast NPCs also suffer from stoichiometry loss during replicative aging (Janssens et al., 2015; Rempel et al., 2019), further testifying to the strong link between the NPC and cellular health. Yet another cellular aging phenotype is an increase in NPC density at the nuclear envelope (Maeshima et al., 2006). It is unclear what induces NPC density to surge and what the long-term consequences are, but in oncogene-induced senescence, NPC density increase is necessary for the formation of senescence-associated heterochromatin foci (SAHFs), which are required for the silencing of several proliferation-related genes (Boumendil et al., 2019). In short, aging brings about changes to NPCs at multiple levels, from individual Nups to their assembly to density.

NPCs in Premature and Pathological Aging

NPC in Premature Aging

In the past decade, the NPC has been studied widely not only in the context of physiological aging but also in premature and pathological forms of aging. It is now well accepted that the NPC is directly involved in the latter as well. Hutchinson-Gilford progeria syndrome (HGPS) is a premature aging genetic disorder caused by a mutant form of *LMNA* that generates a protein called Progerin (De Sandre-Giovannoli et al., 2003). This protein weakens the nuclear lamina and promotes nuclear blebbing. Early studies on HGPS thus had focused on nuclear lamina defects and the concomitant reduction of perinuclear heterochromatin. However, in the 2010s, several studies have shown that nucleocytoplasmic transport is also affected. For example, the ratio of Ran protein in the nucleus versus cytoplasm is normally 3:1, but it is reduced to 1:1 in fibroblasts from HGPS patients (Kelley et al., 2011). As a consequence, the translocation of large cargos like Tpr and ATM is markedly diminished although importin β-dependent nuclear import is still functional (Dworak et al., 2019; Snow et al., 2013). Nup153 import is also decreased as its binding partner Transportin-1 is sequestered by an abnormally stable microtubule network in HGPS cells. Exportin-1-dependent nuclear export, on the other hand, is enhanced

Table 2. Nups and Other Nucleocytoplasmic Transport Proteins in Premature Aging and Neurodegeneration

Pathogenic Macromolecules of Interest	Experimental Systems (Excluding Non-mammalian Models)	Mislocalized or Coaggregated Proteins	Reference
HGPS			
Progerin	Patient fibroblasts	Tpr and Ran	Kelley et al., 2011
Progerin	Patient fibroblasts	Nup153 and Transportin-1	Larrieu et al., 2018
ALS			
N/A	iPSC-derived neurons and patient brains	Nup107, Nup205, Ran, and RanGAP1	Zhang et al., 2015
N/A	iPSC-derived neurons	RCC1	Jovičić et al., 2015
$(GR)_n$ and $(PR)_n$	HEK293T	Nup155, Nup205, Ran, RanGAP1, and several karyopherins	Lee et al., 2016
$(GA)_n$	Mouse models	Pom121 and RanGAP1	Zhang et al., 2016
$(PR)_n$	Recombinant proteins	Nup98 and Nup54	Shi et al., 2017
Arginine-containing DPRs	HEK293T lysate	>20 Nups and transport factors	Hayes et al., 2020
$(G_4C_2)_n$ and $(G_2G_4)_n$ RNAs	iPSC-derived neurons and patient brains	Pom121, Ndc1, Nup210, Tpr, Nup50, Nup98, Nup107, and Nup133	Coyne et al., 2020
TDP-43	Neuro-2a cell line and patient brains	>20 Nups and transport factors	Chou et al., 2018
Huntington's			
Polyglutamine-expanded HTT	Mouse models, iPSC-derived neurons, and patient brains	Gle1 and RanGAP1	Gasset-Rosa et al., 2017
Polyglutamine-expanded HTT	Mouse models, iPSC-derived neurons, and patient brains	Nup62, Nup88, Ran, and RanGAP1	Grima et al., 2017
Alzheimer's			
Tau	Mouse models and patient brains	Nup98 and Ran	Eftekharzadeh et al., 2018

(García-Aguirre et al., 2019). Interestingly, small-molecule studies indicate that several cellular defects in HGPS can be rescued simultaneously by correcting one phenotype, whether it be NPC transport defect, permanent farnesylation of Progerin, or anomalously sturdy microtubule network. For example, Leptomycin B, a covalent inhibitor of Exportin-1, not only attenuates nuclear blebbing but also prevents fusiform morphology and nucleolar expansion, both of which are characteristic HGPS fibroblast phenotypes (Buchwalter and Hetzer, 2017; García-Aguirre et al., 2019). Farnesyl transferase inhibitors restore nuclear architecture, the Ran gradient, Tpr import, and heterochromatin level (Capell et al., 2005; Kelley et al., 2011). Remodelin, a small molecule that inhibits N-acetyltransferase 10 and subsequently destabilizes microtubules, reverts Transportin-1 and Nup153 sequestration in the cytoplasm and normalizes nuclear shape, Ran localization, and gene regulation (Larrieu et al., 2014, 2018). Of note, normally aged cells share several phenotypes with HGPS cells, and these small molecules may mitigate physiological aging as well. Taken together, the studies on HGPS demonstrate that maintaining functional NPCs is essential for the longevity and robustness of eukaryotic cells.

NPC in Neurodegenerative Diseases

The vulnerability of the NPC is also seen in neurodegenerative diseases, such as ALS, frontotemporal dementia, Huntington's disease, and Alzheimer's disease (Table 2) (Kim and Taylor, 2017; Li and Lagier-Tourenne, 2018). The convergent theme is that mutated or repeat-expanded proteins or RNAs (hnRNP A1, FUS, TDP-43, huntingtin, C9orf72, etc.) either induce the mislocalization of Nups and transport factors or localize to the NPC and clog the channel (Figure 1B). This has been ascribed to low-complexity domains (LCDs) in NPC-related proteins, which enable passive permeability barrier and active transport through phase separation (Schmidt and Görlich, 2016). In fact, other membrane-less organelles that contain several LCD proteins and make use of phase separation like nucleolus, nuclear speckle, Cajal body, and stress granule also become defective in the presence of these pathogenic macromolecules (Lee et al., 2016). In other words, LCDs are a double-edged sword in disease context.

NPC transport failure has been most extensively studied in *C9orf72* ALS, which is caused by the GGGGCC-repeat expansion in the *C9orf72* gene. The hexanucleotide repeats give rise to dipeptide-repeat proteins (DPRs; $(GA)_n$, $(GP)_n$, $(GR)_n$, $(PA)_n$, or $(PR)_n$) through repeat-associated, non-AUG translation and $(G_4C_2)_n$ and $(G_2G_4)_n$ RNAs from bidirectional transcription. The link between nucleocytoplasmic transport and these gene products was first discovered through unbiased genetic screens in yeast and flies where several Nups and transport-related proteins were shown to enhance or suppress disease phenotypes (Freibaum et al., 2015; Jovičić et al., 2015; Zhang et al., 2015). In addition, reduced mRNA export, protein import, and disrupted Ran gradient were observed in *C9orf72* patient induced pluripotent stem cell (iPSC)-derived neurons, further supporting NPC dysfunction. Since this pioneering work, there have been significant efforts to determine the relative contribution of DPRs versus $(G_4C_2)_n$ and $(G_2G_4)_n$ RNAs to the pathogenesis of ALS. For example, arginine-containing DPRs have been shown to interact with several LCD proteins, including Nups, and to be

cytotoxic in non-neuronal cells and *in vitro* systems (Hayes et al., 2020; Lee et al., 2016; Lin et al., 2016; Shi et al., 2017). Similar results were obtained in primary neurons and mouse brains overexpressing $(GA)_n$ (Khosravi et al., 2017; Zhang et al., 2016). Furthermore, antibodies against DPRs decrease neurodegeneration and increase survival in ALS mouse models (Nguyen et al., 2020; Zhou et al., 2017). The most recent report, however, provides an additional layer suggesting that repeat RNAs might be the root cause and DPRs are likely to be a secondary factor (Coyne et al., 2020). In *C9orf72* patient iPSC-derived spinal neurons and postmortem motor cortex and thoracic spinal cord, the levels of Pom121, NDC1, Tpr, Nup50, Nup98, Nup107, and Nup133 at NPCs were found to be significantly and specifically reduced. This phenotype can be recapitulated in control iPSC-derived neurons by knocking down Pom121 alone or, more importantly, by transfecting a stop codon-optimized plasmid that produces $(G_4C_2)_n$ RNA, but not DPRs. In line with these results, treating patient-derived neurons with antisense oligonucleotides against $(G_4C_2)_n$ RNA for 5 days, which is long enough to induce the degradation of the RNA, but not DPRs, rescues the disease phenotype. Overexpression of Pom121 alone also has the same effect. While this study does not rule out the contribution of DPRs in the later stages of ALS, using a model system that closely mimics neurons in patients and actual postmortem tissues, it demonstrates that the hexanucleotide-repeat RNAs initiate the disease process.

NPC defects in ALS have also been linked to cytoplasmic mislocalization of an RNA-binding protein TDP-43, which is predominantly nuclear in healthy cells. Although mutations in the gene of TDP-43 protein, *TARDBP*, accounts for less than 5% of ALS, cytoplasmic aggregation of wild-type/mutated TDP-43 is observed in 97% of ALS (Ling et al., 2013). Proximity-dependent biotin identification in Neuro-2a cells using a C-terminal fragment of wild-type TDP-43 has revealed that cytoplasmic TDP-43 aggregates are enriched for Nups and nucleocytoplasmic transport factors (Chou et al., 2018). An ALS-associated missense TDP-43 mutant also brings about a similar problem in nucleocytoplasmic transport in the same cell line. These findings require further confirmation since TDP-43 variants were expressed at abnormally high levels in a neuroblastoma cell line rather than at endogenous levels in patient-derived neurons. Nevertheless, in line with the results, the motor cortex from *TARDBP* mutation-associated and sporadic ALS patients shows reduced Nup205 nuclear immunoreactivity and large Nup205-positive cytoplasmic inclusions. The same tissue from *C9orf72* mutation-associated ALS exhibits abnormal perinuclear punctate Nup205 staining. On the other hand, in the *TARDBP* ALS cerebellum and *SOD1* mutation-associated ALS motor cortex, where TDP-43 does not aggregate, Nup205 localization is not perturbed. It remains to be determined whether TDP-43 aggregation represents the earliest event that disrupts NPC or a secondary symptom (for example, in *C9orf72* ALS) that further aggravates NPC failure.

The results from ALS studies prompted researchers to examine NPC integrity in other neurodegenerative diseases as well. Huntington's disease, for instance, has a very similar pathogenic mechanism to *C9orf72* ALS. It is caused by a CAG-repeat expansion in the *HTT* gene and concomitant elongation of the polyglutamine tract. Polyglutamine-expanded HTT, which typically forms a huge insoluble aggregate inside the nucleus, colocalize with Nup62, Nup88, Gle1, and RanGAP1, impairing both active transport and passive permeability barrier (Gasset-Rosa et al., 2017; Grima et al., 2017). Consistently, pharmacological agents like Thiamet-G and KPT-350 that mend NPC transport dysfunction, as well as the overexpression of RanGAP1 and Ran and immunotherapy, were shown to reduce neurotoxicity in Huntington's disease (Denis et al., 2019; Grima et al., 2017). While more studies in animal models and human patients are required, this finding suggests that the NPC can be an effective therapeutic target not only for HGPS but also for neurodegenerative diseases. Tau protein in Alzheimer's disease was confirmed to compromise nucleocytoplasmic transport by interacting with Nup98 (Eftekharzadeh et al., 2018), again illustrating that NPC impairment is central to and shared by most pathological neurodegeneration. One remaining question in the field is whether non-transport functions of the NPC, e.g., gene activation/silencing and genome folding, are comprised as well. This could be another major factor undermining cellular homeostasis in diseased neurons.

Conclusions and Perspectives

In this review, we have summarized non-transport functions of the NPC, which we are just beginning to understand. The NPC is an essential structure for the non-random three-dimensional organization of the nuclear genome, and it is particularly important for the regulation of cell-type-specific genes. In line with this cellular function, the NPC stoichiometry and Nup expression levels vary greatly depending on the cell and tissue types. Nups can control gene activity both at the nuclear periphery and inside the nucleus and have been shown to double as transcription factors in yeast, worms, flies, and vertebrates, suggesting that they have long been multifunctional proteins during eukaryotic evolution. One exciting hypothesis arose soon after the dual role of Nups was revealed: can there be a subpopulation of NPCs in a cell that is specialized for genome regulation? In other words, is there a subset of NPCs that have forsaken their primary function of the NPC as transport channel to better interact with the genome? In fact, it has been shown that NPCs in the same nucleus exhibit varying levels of transport activity (Grünwald and Singer, 2010). Another circumstantial evidence is that gene-regulating Nups such as Nup93 and Nup210 appear virtually inaccessible in the NPC structures obtained by cryo-electron microscopy (Beck and Hurt, 2017; Bui et al., 2013; Kosinski et al., 2016; von Appen et al., 2015). These data are acquired from purified, DNase-treated nuclear envelope and presumably are representations of NPCs that are not interacting with chromatins. We surmise that NPCs participating in gene regulation will adopt a different conformation or stoichiometry to expose Nup93 and Nup210 to their binding partners and may have reduced transport capability. Single-molecule fluorescence resonance energy transfer imaging in live cells can be employed to examine whether NPCs alternate between quaternary structures attuned for nucleocytoplasmic transport versus genome regulation.

Another intriguing hypothesis that has not been addressed is whether the NPC- and/or soluble Nup-genome interaction is mediated by phase separation. SEs, the major target sites for Nups, are often found in phase-separated liquid droplets with

high concentrations of transcription machineries (Sabari et al., 2018). Given that many Nups have LCDs, it is tempting to assume that phase-separating capabilities of Nups enable genome binding. The NPC, with a high local concentration of FG-Nups, indeed seems to be an ideal venue for phase separation. Soluble Nups like Nup98 can also form liquid-like droplets on their own (Celetti et al., 2020), suggesting that phase separation may be utilized inside the nucleus as well.

We have also discussed how the molecular integrity and function of the NPC decline during normal, premature, and pathological aging. The NPC is a huge protein complex with dozens of dynamic and static components, and many of them can molecularly senesce or mislocalize during aging. In addition, Nup expression levels can deviate from the normal range due to genome dysregulation. Since the NPC is a fundamental apparatus for eukaryotic forms of life, it is not too surprising that cells with NPC dysfunction quickly become debilitated. Yet, there remains one poorly understood aspect regarding the NPC-aging link. Multiple studies have shown that NPC passive barrier and transport is compromised by aging conditions, but it has not been examined whether NPC- and/or soluble Nup-genome interactions are disrupted at the same time. Their loss may result in unwanted (de-)differentiation—one of the major aging phenotypes—as they are essential for the regulation of cell identity genes. If this turns out to be the case, it would mean that the NPC can contribute to aging in two different ways. Similarly, in many other biological contexts where the NPC is disturbed, such as viral infection and cellular stress, most research has focused only on transport failure. Given what we have learned from a decade of studies on the genome regulatory roles of the NPC and Nups, alterations in transcriptional programs may be another major mechanism by which cells lose identity and homeostasis upon NPC disruption.

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