Maximiliano D'Angelo *Editor*

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Preface

The main hallmark of eukaryotic cells is the segregation of the genome within a membrane-enclosed organelle known as the nucleus. From its discovery more than two centuries ago, the nucleus has continuously marveled scientists for being the largest of all eukaryotic organelles, for its behavior during mitotic cell division, for the discovery that it houses and protects most of the cell's DNA, for its highly organized sub-compartmentalization, and more recently for its role in the regulation of genome functions and genome integrity. One of the main findings of the last two decades is the identification that the genome is not just randomly "bagged" inside the nucleus, but highly organized in a three-dimensional structure. How cells organize their genomes within the nuclear space, how genome architecture differs among different cell types, how it is faithfully maintained in cells, and how it contributes to the control of genome functions are some of the questions that are actively being investigated in the field of the cell nucleus.

The "*wall*" that holds the genome inside the nucleus is known as the nuclear envelope. It is formed by two concentric membranes, an outer and inner nuclear membrane, and a large meshwork of intermediate filaments, known as the nuclear lamina, which provides mechanical stability to the nucleus and helps to organize the genome. The separation of the genome from the rest of the cell naturally requires a system that allows cells to access their genetic information. Nuclear pore complexes (NPCs), the giant protein channels that penetrate the nuclear envelope connecting the nucleus to the cytoplasm, are the principal components of this system and act as gatekeepers of the genetic information. NPCs are the only gateway into the nucleus and, thus, they control the passage of molecules into and out of this compartment. Because of this essential cellular function, for a long time after their discovery most efforts were devoted to understanding the structural properties of NPCs and to dissecting the mechanisms of nucleo-cytoplasmic molecule exchange. But in the last two decades, the exciting discovery that NPCs have several transport-independent functions has completely transformed our view of these structures. We know now that these channels are not just static structures that facilitate nuclear transport; they are actually highly dynamic complexes with multiple cellular functions including

central roles in nuclear organization and genome safeguarding. Considering that NPCs are the largest protein complexes of the nucleus, the identification that these structures have multiple independent functions should perhaps not come as a surprise. But, what is extraordinary and definitely exciting, is the wide range of functions recently unearthed for these complexes, which include roles in chromatin organization, genome architecture, gene expression regulation, DNA damage repair, chromosome segregation, cell cycle control, modulation of signaling pathways, and cytoskeletal organization.

This book provides both, an overview of the organization of the eukaryotic cell nucleus, and a more detailed description of the transport-independent functions of NPCs and their components in genome organization and gene expression. While Chaps. 1 and 2 introduce the different compartments and components of the nucleus and the nuclear envelope, Chap. 3 gives a detailed description of the nuclear pore complex structure and properties. The aim of Chaps. 4–7 is to collect and review all the existing evidence for NPC regulation of genome organization and gene expression in different organisms, while the final chapters describe other novel nuclear functions of NPCs, including their role in HIV infection and genome integration (Chap. 8), DNA repair and telomere maintenance (Chap. 9), and mitosis and chromosome segregation (Chap. 10). We believe this book presents a clear vision of the importance of NPCs beyond their role as mediators of nucleocytoplasmic transport, and we hope the readers will find it a valuable a source of evidence for the emerging roles of nuclear pore complexes as key players in the regulation of genome integrity and function. I would like to thank all the co-authors for their stimulating contributions to this book, Monique Zwang for helping to consolidate our writings into a real book, and Meran Owen for initiating this exciting project. Last, but no least, I would like to thank my family for their endless support, patience, and love, without which this book would not have been possible.

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Contents

Chapter 1
Spatial Organization of the Nucleus Compartmentalizes and Regulates the Genome

Michael I. Robson, Andrea Rizzotto and Eric C. Schirmer

Abstract The nucleus must simultaneously orchestrate DNA replication, transcription, splicing, signalling, and directional transport of proteins into the nucleus and RNA out of the nucleus. Yet the nucleus has no internal membranes to compartmentalize these functions as the cytoplasm does. In fact, such compartmentalization would necessarily be detrimental because particular genes at different locations on the linear chromosomes need to be made at different times while others on the same chromosome need to be tightly shut off. Moreover, expressed genes need to be accessible to a feedback mechanism to determine when to modulate transcription. To accommodate these additional needs the nucleus appears to form microdomains by co-assembly of functional complexes. Thus, microdomains can either form around activated regions on a chromosome or regions on a linear chromosome could be fed into such microdomains for activation. Findings that genome encoded regulatory elements such as enhancers can be hundreds of kb and even Mb apart further highlights the need for such a system as these distal elements must come together in the 3D space of the genome for their efficient functioning. While this much is understood, there is much still to be learned about mechanisms that the nucleus uses to regulate the genome and much more to be learned about how these microdomains come into being. As there is no stable structure within the nucleus except for the nuclear envelope, much recent research has been focusing on potential roles of this subnuclear organelle in establishing 3D nuclear architecture and orchestrating the regulation of these various functions.

Keywords Nucleus · nuclear envelope · NET · gene position · genome organization.

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1.1 Introduction

As the largest organelle within the cell the nucleus was visualized in the earliest days of microscopy over 300 years ago by Dutch microscopist Antonie van Leeuwenhoek, although it was another 100 years before Scottish botanist Robert Brown named it. Both were able to visualize the nucleus with their crude microscopes due to studying organisms with large nuclei such as salmon and plants. As microscopes evolved to reach ~1 μm resolution by the mid-1800s it became apparent that there were substructures inside the nucleus, the most predominant of which are the nucleoli that were first described in the 1830s (Lo et al. 2006). A body of work by several scientists evolving the theory of heredity and connecting this to the distinct worm-like chromosomes observed in mitosis resulted in German zoologist Theodor Boveri's postulating the existence of chromosome territories around the next turn of the century (Boveri 1909). Other indications of nuclear organization came in the same era from Austrian anatomist Carl Rabl's observations that the centromeres of salamander chromosomes were located at the nuclear periphery (Rabl 1885) and Spanish cytologist Santiago Ramon y Cajal's identification of nucleolar accessory bodies subsequently called Cajal bodies (Cajal 1903).

With the advent of electron microscopy (EM) many additional nuclear substructures were observed based on characteristic electron densities. One of the most obvious was the separation of most of the denser staining chromatin, defined at the time as heterochromatin, to the nuclear periphery, particularly in resting lymphoblasts (Mirsky and Allfrey 1960; Hirschhorn et al. 1971). Additionally, Promyelocytic leukaemia (PML) nuclear bodies were identified as electron dense spheres of 0.1–1.0 μm diameter (de et al. 1960). EM also enabled visualization of processes such as the massive ribosomal RNA transcriptional "trees" of amphibian oocyte satellite DNA associated with the nucleolus (Miller and Beatty 1969). The combination of ^{3}H-5-uridine labelling with EM yielded more information about where in the nucleus functions occur, associating more rapidly labelled regions with the perimeter of inter-chromatin granules (Fakan and Bernhard 1971). Finally, the recognition that the nuclear envelope (NE) is a double membrane structure and of the nuclear pore complexes (NPCs) embedded within it first came using EM in large amphibian oocyte nuclei (Callan and Tomlin 1950) and shortly after in mammalian nuclei (Watson 1954). Subsequent work demonstrated the unique eight-fold symmetry of the NPCs (Gall 1967).

At the same time as these early ultrastructural observations there were a number of seminal papers indicating many levels of organization of the genome. For example Huberman and Riggs demonstrated the existence of specific mammalian origins of replication (Huberman and Riggs 1968). Also critical in this period was the development by the team of Joe Gall and Mary Lou Pardue of methods for in situ hybridization that have formed the basis for determining the position of specific genes and transcriptional activity in the nucleus (Gall and Pardue 1969; Pardue and Gall 1969). This was quickly used to determine the chromosomal localization of mouse satellite DNA (Pardue and Gall 1970) and subsequently developed to paint whole chromosomes (Lichter et al. 1988). These tools have been used to

compare the positioning of different nuclear structures with respect to one another and also with respect to their positioning within the 3D nucleus as a whole. The much more recent development of genome-wide sequencing technologies quantifying DNA-DNA proximity, such as chromosome conformation capture (Dekker et al. 2002; Lieberman-Aiden et al. 2009) and Genome Architecture Mapping (GAM) (Beagrie et al. 2017), or DNA-protein proximity, such as DNA adenine methyltransferase identification (DamID) (Guelen et al. 2008; Pickersgill et al. 2006), has significantly expanded these visual observations concerning the spatial positioning and/or the three-dimensional organization of the genome. For example, chromosome conformation capture and GAM methods, have revealed that chromosomes fold along their length into delimited structures termed topologically-associated domains (TADs) which subsequently assemble into higher order compartments (Dixon et al. 2012; Nora et al. 2012). Similarly, DamID has revealed the organization of the fraction of TADs at the NE in lamina-associated domains (LADs) (Gonzalez-Sandoval and Gasser 2016; Vogel et al. 2007). Understanding the functions of the spatially distinct regions of the genome has been greatly aided by the ability to investigate the relationship of these genome domains to different proteins in the nucleus. This in turn was greatly aided by the development of antibodies to proteins in these structures that enabled both their labelling by immunogold EM and their visualization under the light fluorescence microscope.

Most of the first antibodies obtained to these structures were fortuitous, coming from autoimmune sera, and thus providing also the first indications of the importance of these nuclear subdomains to human health and disease. Of particular note, serum from patients with the autoimmune disease systemic lupus erythematosus stained nucleosomes (Rothfield and Stollar 1967). Several other nuclear domains and proteins were subsequently found to also be linked to autoimmune diseases such as autoimmune sera from primary billiary cirrhosis patients identifying the first PML/ND10-associated protein Sp100 (Szostecki et al. 1990). At this time, the number of developmental defects and disease states associated with proteins of nuclear substructures are far to great to detail in a single review, but it is worth noting particularly the links between the NE and disease as this can in many ways be linked specifically to nuclear organization. Mutations in the intermediate filament lamin proteins that line the inner nuclear membrane cause several muscular dystrophies and lipodystrophies along with neuropathy, dermopathy and other disorders including the premature ageing Hutchison-Gilford Progeria syndrome. Moreover, mutations in NE transmembrane proteins (NETs) that interact with lamins often cause variants of the same diseases (Bonne and Quijano-Roy 2013; Worman and Schirmer 2015). The distribution of the dense peripheral heterochromatin was disrupted in cells from patients with both lamin and NET-linked muscular dystrophy (Sewry et al. 2001; Verga et al. 2003; Fidzianska et al. 1998; Maraldi et al. 2002; Ognibene et al. 1999), progeria (Goldman et al. 2004), mandibuloacral dysplasia (Maraldi et al. 2006) and familial partial lipodystrophy, Dunnigan-type (Maraldi et al. 2006). Differences in chromosome territories and their spatial positions within the nucleus were also observed in cells with specific lamin A mutations (Meaburn et al. 2007; Mewborn et al. 2010) and a progeria

mutation further yielded an abnormal distribution of telomeres and clustering of centromeres (Taimen et al. 2009). The specific organization of the genome with respect to regulatory elements and higher order chromosome structure is also important for human disease as disruption of this organization leads to developmental defects and disease (Guo et al. 2015). As Francis Collins has suggested that most disease-causing mutations yet to be identified likely fall in non-coding regions (Manolio et al. 2009), understanding spatial genome organization and its control are fundamentally important.

1.2 Subdomains of the Nucleus

1.2.1 Nuclear Envelope

The NE is a complex system of outer (ONM) and inner (INM) nuclear membranes separated by a ~50 nm lumen in mammalian cells and both connected and perforated at sites of nuclear pore complex (NPC) insertion (Callan and Tomlin 1950). The ONM is continuous with the endoplasmic reticulum (ER) and contains both ER proteins such as ribosomes and also a set of unique NETs, of particular note the KASH domain nesprins that connect the NE to cytoplasmic filaments (Luxton and Starr 2014). The INM harbors its own unique set of NETs (de Las Heras et al. 2013) and the lamins that form a polymer directly under the NE (Gruenbaum and Foisner 2015). Both lamins and most NETs tested bind chromatin proteins (Harr et al. 2016; Kind and van Steensel 2014; Wong et al. 2014). This is particularly noteworthy in context that the lamin polymer confers structural support to the nucleus (Lammerding et al. 2004) and is accordingly the most stable of the nuclear subdomains. With an estimated 9 million copies of lamins per mammalian cell nucleus (Schwanhausser et al. 2013), they are well positioned to serve as the scaffolding of the nucleus. Finally, ONM nesprins interact with INM SUN-domain NETs to connect the lamin polymer to cytoplasmic filaments (Crisp et al. 2006; Padmakumar et al. 2005). This maintains the 50 nm spacing of the lumen and enables force transmission and mechanosignal transduction between the cytoplasm and lamins and associated NETs in the nucleus (Ho et al. 2013; Swift et al. 2013). In addition to its obvious structural function, the NE has been implicated in a wide range of functions that range from the integration of many additional non-mechanical signalling pathways to DNA replication, transcriptional regulation, gene and chromosome positioning, and many others (de Las Heras et al. 2013). It is thus not surprising that the NE is linked to over two dozen diseases (Bonne and Quijano-Roy 2013; Worman and Schirmer 2015) (Fig. 1.1).

1.2.2 Nuclear Pore Complexes

The eight-fold symmetrical NPCs are the largest protein complexes in a typical cell, starting at a minimum of >40 MDa in yeast (Rout and Blobel 1993; Yang et al. 1998) and up to 125 MDa in higher vertebrates (Akey and Radermacher 1993; Hinshaw et al. 1992; Reichelt et al. 1990). The diameter of NPCs is ~120 nm

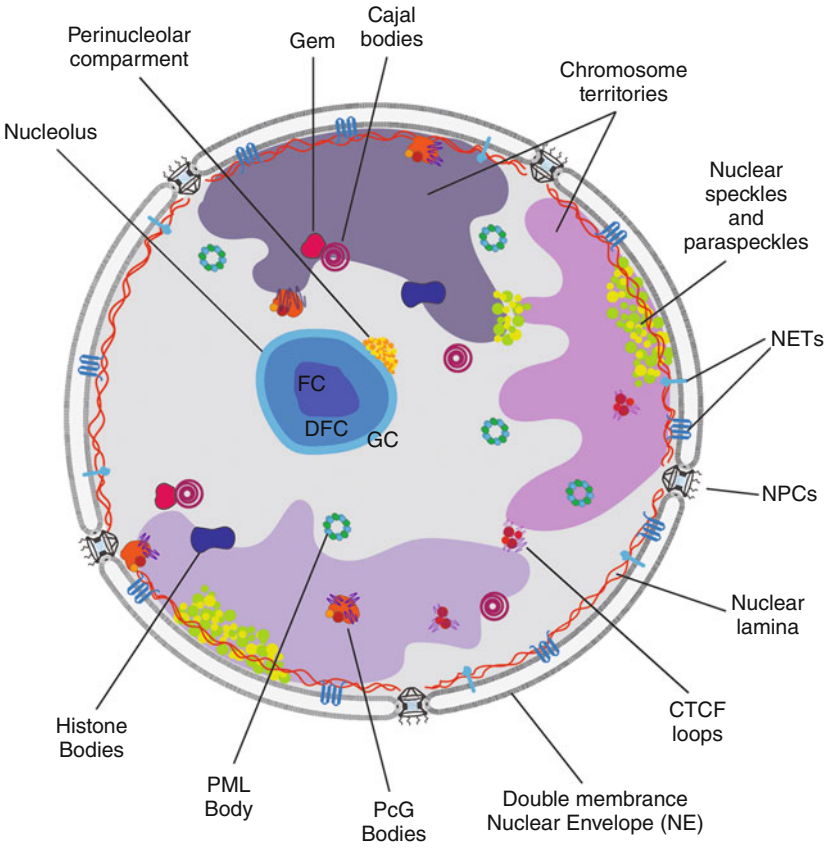

Fig. 1.1 Overview of nuclear subdomains. Most nuclear subdomains shown occur in the interchromatin regions between chromosome territories and are often involved in transcriptional regulation and RNA processing. Some domains such as PcG bodies and CTCF loops are more focused on generating structure within the chromosome. These loop structures can contribute to silencing genes, segregate domains within chromosomes, and promote interactions between domains within or between chromosomes for gene activation. The nucleoli are the largest nuclear subdomain besides the nuclear envelope and is broken up into a fibrillar center (FC), dense fibrillar component (DFC) and granular component (GC)

and not only they span the ~50 nm distance between the ONM and INM, but also comprise filaments that extend into the cytoplasm and a more structured nuclear basket extending into the nucleoplasm that make their total length greater than their diameter. Together with an army of transport receptors and other associated proteins, they direct the regulated transport of proteins and RNA in and out of the nucleus (Dickmanns et al. 2015). Transport of soluble macromolecules appears to occur through an ~39 nm central channel that is filled with phenylalanine-glycine repeat motifs in unstructured regions of many of the core protein components termed nucleoporins (Rexach and Blobel 1995). However, there are also peripheral channels of ~10 nm that have been shown to be involved in transport of

NETs from the ER to the INM (Ohba et al. 2004; Soullam and Worman 1995; Ungricht et al. 2015; Zuleger et al. 2011a). There are roughly 2,000–3,000 NPCs in the nuclei of cycling mammalian cells (Gerace and Burke 1988; Maul and Deaven 1977). Super-resolution microscopy approaches reveal a clear segregation of NPCs from other NE components (Schermelleh et al. 2008; Xie et al. 2016). Nonetheless, NPCs appear to have roles separate from their transport function in genome regulation (Harr et al. 2016; Heessen and Fornerod 2007). Similar to the rest of the NE, an ever-increasing number of NPC proteins are being linked to functions in development and human diseases, particularly cancer (Cronshaw and Matunis 2003; Lupu et al. 2008; Simon and Rout 2014).

1.2.3 Chromosomes

Although it is obvious from their condensation in mitosis that chromosomes are discrete gigadalton entities, the idea that they maintain discrete territories in interphase was only theorized for nearly 100 years after Boveri initially postulated this (Boveri 1909). The first proofs of interphase chromosome territories came from a combination of technological advances and creative experimental approaches driven by the brothers Thomas and Christoph Cremer. They clearly showed that interphase chromosomes organize into discrete chromosome territories (Cremer et al. 1982; Schardin et al. 1985). Certain tendencies were subsequently observed such as that active genes tend to be located at the boundary of a chromosome territory that is facing the inside of the nucleus while genes at the boundary against the NE tend to be repressed (Kurz et al. 1996). It was also observed that some small chromosome regions could loop out so that a small portion of a more internal chromosome could touch the periphery (Zink et al. 2004) and that, once established, the positions of chromosomes in interphase tend to be relatively stable (Strickfaden et al. 2010). This led to a whole new field on the relationship between gene position and expression (see below).

1.2.4 Centromeres

As the largest individual molecules in the cell, chromosomes are themselves effectively segregated into multiple subdomains. Centromeres occur at the primary constriction of mitotic chromosomes and exhibit specialized chromatin and associated proteins at the kinetochore. This includes the assembly into centromeric nucleosomes of the histone H3 homolog CenpA and assembly of dozens of other centromere proteins into a scaffolding and support structure for the binding of mitotic microtubules (Moreno-Moreno et al. 2017). Some of this structure is dynamically assembled when the chromosomes condense at mitosis while most is maintained in both mitosis and throughout interphase. Centromeres range in size from several Mbp out of a total of ~50–250 Mbp on a typical mammalian chromosome to 125 bp in the much smaller chromosomes of the yeast *Saccharomyces cerevisiae*.

1.2.5 Telomeres

The ends of the linear chromosome are capped by telomeres. These range typically from several kbp in humans to 300 bp in *S. cerevisiae*. They are known to be functionally important for maintaining the integrity of chromosomes, but they shorten as cells divide so that loss of gene contents can more easily occur from the chromosome ends in "older" cells (Wu et al. 2017).

1.2.6 Nucleolus

The nucleoli were the first noted nuclear substructure because they can be observed by light microscopy as visible circular structures within the nucleus by their darker appearance and large size (from 0.5 to 8 μm in diameter). This darker appearance correlates with their dense staining in EM. There can be multiple nucleoli in a nucleus and they form around tandem repeats of ribosomal DNA (rDNA). They function primarily in synthesis of rRNA and in ribosome biogenesis, first clarified with the identification of a *Xenopus laevis* mutant lacking nucleoli that also lacked rRNA synthesis (Brown and Gurdon 1964). Recent studies suggest that nucleoli have additional roles in RNA transport, RNA modification, and cell cycle regulation (Stepinski 2016).

1.2.7 Perinucleolar Compartment

The perinucleolar compartment (PNC) is a dynamic compartment that forms in cancer cells adjacent to nucleoli and much smaller at 0.2–1 μm in diameter. It was first identified from observations that the pyrimidine tract-binding protein (PTB) accumulated adjacent to nucleoli (Ghetti et al. 1992). It was subsequently named the PNC after the discovery that it also contains several polymerase III RNAs (Matera et al. 1995). However, its composition appears to be highly dynamic with both RNAs and proteins rapidly exchanging between the compartment and the surrounding nuclear area and its function has not been fully elucidated (Pollock et al. 2011).

1.2.8 Cajal Bodies

Cajal bodies tend to be in the range of 0.3–1.0 μm in diameter, but can be much smaller or get as big as 2 μm (Cioce and Lamond 2005). They were first identified by Santiago Ramón y Cajal in 1903 and called nucleolar accessory bodies. Their characterization much later by EM led to the new name of Coiled bodies because the higher resolution yielded the appearance of a coiled string and subsequently they came to be referred to as Cajal bodies in honor of their discoverer. However, the

core protein constituent was named p80/coilin based on the Coiled body name. A typical nucleus contains 1–10 Cajal bodies with the larger numbers found in metabolically active cells, but they are most prevalent in highly proliferative cells such as embryos and tumors. Cajal bodies are principally involved in assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs), but have many different roles relating to RNA processing including also small nucleolar RNA (snoRNA) maturation, histone mRNA modification and telomere maintenance (Cioce and Lamond 2005).

1.2.9 Gemini of Coiled Bodies

Gemini of Cajal bodies, also called gems for short, are "twins" to Cajal bodies with similar size and shape discovered by Liu and Dreyfuss in 1996 (Liu and Dreyfuss 1996). However, unlike Cajal bodies, gems do not contain snRNPs. They are readily distinguished by immunofluorescence microscopy because Cajal bodies are positive for both coilin and the survival of motor neuron (SMN) protein, while gems are SMN positive and coilin negative (Navascues et al. 2004). Gems are believed to be involved in pre-mRNA splicing and assist Cajal body function.

1.2.10 PML/ND10 Bodies

Promyelocytic leukaemia bodies (PML bodies) are known by many names including nuclear domain 10 (ND10), Kremer bodies, and PML oncogenic domains (Bernardi and Pandolfi 2007). They range in size from 0.1–1.0 μm in diameter and a typical nucleus has between 5 and 30. Their primary name comes from their primary component, the PML protein, though there are also non-PML body stores of PML protein (De Vos et al. 2011). There are many functions ascribed to PML bodies, but these all seem to reflect a core unifying function contributing to cellular responses to stress. In keeping with this the PML protein appears to be dispensable under normal cellular conditions as PML knockout mice develop normally in the absence of major stress conditions. In addition to PML there are a few other core components of PML bodies including Daxx (Ishov et al. 1999), Sumo (Muller et al. 1998) and Sp100 (Szostecki et al. 1990). Interestingly, PML bodies accumulate at centromeres through an interaction with CenpC during viral infection (Everett et al. 1999) and can also associate with telomeres (Chung et al. 2011) and may have functions with other nuclear subdomains.

1.2.11 Speckles

Speckles contain pre-messenger RNA splicing factors and are located in interchromatin regions. Their association and function with splicing has resulted in their also being called splicing speckles, nuclear speckles, splicing factor compartments,

interchromatin granule clusters (IGCs), and B snurposomes. They were first visualized by EM as clusters of interchromatin granules and by fluorescence microscopy appear highly irregular, typically varying in size from 0.8–1.8 μm and so being one of the largest structures in the nucleus after the chromosomes themselves and the nucleoli (Lamond and Spector 2003). They are highly dynamic and their composition and location changes in response to modifications in mRNA transcription (Handwerger and Gall 2006). These structures are also reported to function as storage sites for splicing factors (Matera et al. 2007).

1.2.12 Paraspeckles

The interchromatin space contains irregularly shaped structures that tend to be in close proximity to splicing speckles named paraspeckles (Fox et al. 2002). These are generally observed in human tissues and studies in HeLa cells show that they are highly dynamic. Paraspeckles existence depends on RNA Pol II transcription and these structures change rapidly in response to changes in cellular metabolism (Fox et al. 2005). Typically 10–30 paraspeckles can be observed in a HeLa cell nucleus. Several roles have been suggested for paraspeckles, including regulation of apoptosis and mitosis (Gao et al. 2016) and RNA sequestration in the nucleus (Hu et al. 2015) and DNA damage response (Gao et al. 2014). However, their protein composition would suggest RNA editing and regulation as their core function. Paraspeckles differ from most nuclear subdomains in having a more regular size of ~0.5 μm.

1.2.13 Histone Bodies

Also called histone locus bodies, histone gene synthesis occurs in these bodies formed around the histone gene cluster. Histone transcription is unusual in that histone genes produce the only known cellular mRNAs that are not polyadenylated and instead form a conserved stem loop (Duronio and Marzluff 2017). Histone bodies contain scaffolding protein NPAT, FLASH and U7 SnRNP and are 0.2–1.2 μm in diameter (Sleeman and Trinkle-Mulcahy 2014). NPAT is a substrate for Cyclin E/Cdk2 phosphorylation to activate histone synthesis at the G1/S transition and interacts with the small heat shock protein Cpn10/HSPE for its function. Loss of Cpn10 specifically disrupts these bodies and histone transcription without affecting neighboring nuclear bodies (Ling Zheng et al. 2015).

1.2.14 Polycomb Bodies

Polycomb proteins assemble visible structures involved in mediating gene pairing and silencing called Polycomb group (PcG) bodies. These structures are 0.3–1 μm in diameter and are defined by the presence of Bmi1 and Pc2 proteins. Loci

targeted to PcG bodies become compacted, presumably because of the functions of polycomb repressive complex methylation of histone H3K27 (Margueron and Reinberg 2011). However, PcG bodies also appear to contribute to long-range chromosomal interactions through maintaining multi-looped chromatin structures (Tolhuis et al. 2011; Bantignies et al. 2011). Unlike other nuclear subdomains, however, PcG bodies appear to be visible more due to the local concentration of chromatin fibers rather than protein and RNA assemblies (Smigova et al. 2011).

1.3 Composition of Nuclear Structures

As noted above, the first identifications of protein components of nuclear subdomains came from autoimmune sera that were used to identify the proteins and to reveal their association with a particular structure by immunoelectron miocroscopy. The standard approach was then to use various means ranging from 2-hybrid studies to co-immunoprecipitations to identify other components. Sometimes this was revealing for the function of these bodies. For example, in Cajal bodies, Coilin and its interaction partners were found to associate with pre-mRNA splicing, pre-ribosomal RNA processing, and histone pre-mRNA 3' maturation, increasing our understanding of the function of these bodies in assembly of spliceosomal snRNPs and small nucleolar ribonucleoproteins (snoRNPs) (Gall 2000; Matera 1999).

In other cases identification of subdomain components led to even more confusion. PML/ND10 bodies are an example of this situation. Once the first core components including PML protein, Sp100, and Daxx had been discovered, there were over 100 more to follow such as TDG, PIASy, HIPK2, LEF1, p53, Akt, ChK2, p53, multiple poly-glutamine proteins and many viral proteins. These proteins suggested functions ranging from replication and transcriptional regulation, to cell signaling, cell cycle, anti-viral responses and apoptosis (Lallemand-Breitenbach and de The 2010). The finding that sumoylation regulates the intranuclear partitioning of PML (Muller et al. 1998) and other cofactors such as Daxx (Ishov et al. 1999) shortly led to the hypothesis that PML/ND10 bodies functioned as a nuclear trash depot for proteins that needed to be degraded (Negorev and Maul 2001). This view dominated the field for several years until many studies revealed that there were distinct PML/ND10 bodies with different composition beyond their core components that have individual functions (Lallemand-Breitenbach and de The 2010). These functions include, stress responses to upregulation of interferons, heavy metals, proteasome inhibition, and DNA damage among others.

1.3.1 Mass Spectrometry Opens a Whole New Dimension in Defining Nuclear Subdomains

Though many nucleolar proteins had been identified over the years, it was not until 35 years later that a comprehensive proteomic analysis of the nucleolus was

undertaken. Careful isolation of nucleoli from HeLa cells and analysis by mass spectrometry revealed 271 proteins make this complex structure (Andersen et al. 2002). Roughly 30% of these proteins were previously uncharacterized, raising the possibility that there might be additional functions for nucleoli. A subsequent study increased the number of nucleolar proteins to 489 (Andersen et al. 2005). Treatment of cells with metabolic inhibitors caused changes in the relative amounts of many of the identified nucleolar proteins accompanied by changes in nucleolar morphology (Andersen et al. 2005). When nucleosome-association was tested for identified proteins, the Lamond group found that one uncharacterized protein targeted to a new nuclear subdomain that they called paraspeckles, thus naming this protein paraspeckle protein 1 (PSP1) (Fox et al. 2002). They later identified additional components, such as PSP2 and p54/nrb, that also localized to paraspeckles and found an average of 10 to 20 of these subdomains in a typical HeLa cell nucleus.

The NPC proteome turned out to be a little less exciting, but this is in large part because the structure of the NPC made it easier to study and accordingly more of its constituent proteins called nucleoporins were identified prior to the application of proteomics. The first nucleoporin discovered was the transmembrane gp210 (originally called gp190) that was isolated from a crude NE/NPC fraction and used to generate antibodies that labeled NPCs by immunogold EM (Gerace et al. 1982). Many other nucleoporins were soon identified largely because of their abundance and easy fractionation from Xenopus oocytes. Once the first nucleoporins were identified, the discovery that they tend to form subcomplexes rapidly facilitated further identifications by co-immunoprecipitation e.g. Nup62 was identified due to its abundance and strong antigenicity (Starr et al. 1990) and Nup54 and Nup58 were found by their interaction with Nup62 (Hu et al. 1996; Kita et al. 1993). In the two decades between the first identification of gp210 and the first proteomic study in yeast, 26 nucleoporins had already been identified. The yeast proteomics made 174 protein identifications of which many were transport factors, chaperones and obvious contaminants, but it increased the number of nucleoporins to 30 (Rout et al. 2000). Shortly thereafter, the Matunis laboratory determined the composition of the mammalian NPC that revealed the remarkable conservation of this nuclear structure (Cronshaw et al. 2002). The results of both studies were a surprise to many as the general expectation had been that, there would be 50–100 nucleoporins, based on the mass estimated by cryo-EM (Akey and Radermacher 1993; Hinshaw et al. 1992; Reichelt et al. 1990). However, relative protein abundance estimated by spectral counts suggested that while some nucleoporins may be only represented in eight copies due to the eight-fold symmetry of the NPC (Gall 1967), others have 16 or 32 copies (Cronshaw et al. 2002; Rout et al. 2000). The number of copies estimated for nucleoporins does not account for the total predicted mass: 44 MDa was accounted for yeast out of 55–72 MDa expected (Rout and Blobel 1993; Yang et al. 1998) and ~60 MDa accounted in mammals out of 125 MDa expected (Akey and Radermacher 1993; Reichelt et al. 1990). A subsequent study of the whole NE proteome (see below) additionally found a third transmembrane nucleoporin, NET3/Ndc1, most likely because of special approaches applied for the identification of membrane proteins (Schirmer et al. 2003; Mansfeld et al. 2006). It

is likely that the rest of the mass is provided by a combination of transport factors and proteins in transit *i.e.* the NPC reflects a general characteristic of nuclear subdomains in that they are largely dynamic in structure and composition and highlights the importance of establishing the core for each nuclear body/subdomain.

In contrast to the NPC, the NE proteome was greatly expanded by proteomics. A study using the multi-dimensional protein identification technology approach (MudPIT; (Washburn et al. 2001, 2003; Wolters et al. 2001)), that avoids gel separation steps used in an earlier study (Dreger et al. 2001), increased the number of proteins associated with this nuclear subdomain by roughly 10-fold (Schirmer et al. 2003). Subsequent testing of individual proteins identified for NE targeting confirmed the vast majority, but also revealed that many also have other subcellular localizations (Malik et al. 2010). This is somewhat consistent with the dynamic behavior of PML/ND10 bodies, nucleoli and the NPC.

1.4 Self-Assembly of Nuclear Structures

The entire nucleus disassembles and reassembles in each mitosis of higher organisms (Schellhaus et al. 2016). While this dynamic behavior could be viewed as an obstacle to maintaining spatial genome organization, it is also an opportunity for a cell to change its genome organization by starting from scratch. In post-mitotic cells larger genome organization changes such as the inversion of heterochromatin that occurs in retina cells takes weeks (Solovei et al. 2013), but with the disassembly and rebuilding of the nucleus that occurs in mitosis large-scale global repositioning of whole chromosomes can take place in under an hour. For some whole chromosomes, such changes can occur by an interaction with the NE (Finlan et al. 2008; Reddy et al. 2008). But, many nuclear subdomains are placed in between chromosome territories and far away from the NE and need an independent mechanism for self-assembly.

With its eight-fold symmetry and composition of over 30 different nucleoporins in copies from 8 to 32, the NPC is probably the most complex individual structure in biology. The identification of nucleoporin interaction partners revealed that all nucleoporins associate into specific subcomplexes (Alber et al. 2007). These subcomplexes self-assemble and are stable enough to obtain both EM and crystal structures. The subcomplexes can in turn self-assemble to form the whole NPC in a stepwise LEGO-like fashion (Floch et al. 2014). Interestingly, many nucleoporins have been shown to have separate roles in mitosis. For example, Nup107 associates with the spindle assembly checkpoint protein MAD1 and recruits also one of its subcomplex members, Nup133 to the kinetochore in mitosis (Rodenas et al. 2012). It would be interesting if these separate mitotic roles evolved as effective "place holders" to partly maintain subcomplexes in an inactive state until breaking these interactions would allow reassembly of the NPCs.

Many other individual nuclear subdomains assemble due to specific affinity interactions between components. For example, with PNCs the three core

components mitochondrial RNA-processing (MRP), PTB and CUG-binding protein (CUGBP) have all been shown to interact by pulldown assays (Pollock et al. 2011). In the case of paraspeckles, multiple components share the characteristic of having complexity prion-like domains that promote their association (Hennig et al. 2015). In this respect they could be argued to be more prone to aggregate than to assemble. Nonetheless, this particular method of interaction might have the advantage of segregating paraspeckle assembly from adjacent splicing speckle assembly.

In other cases, assembly may combine specific affinity interactions with a driver process, such as transcription. For example Cajal bodies require active transcription for the U1, U2, U4/U6 and U5 snRNPs to assemble and colocalize in the structures (Carmo-Fonseca et al. 1992). The dynamics of these bodies and association with chromatin also requires ATP (Platani et al. 2002). Likewise, the structural cohesion of the nucleolus depends on its activity and, accordingly, inactivation of rDNA results in a loss in the cohesion of nucleolar structures (Hernandez-Verdun 2006). A similar concept may apply for the different types of PML/ND10 bodies. Some PML/ND10 bodies appear to be associated with transcriptional foci. An elegant live-cell imaging study showed a modified gene locus and a PML/ND10 body moving until they co-localized. The gene locus then changed from a condensed to an open structure and transcription was observed to initiate (Tsukamoto et al. 2000). PML/ND10 bodies have been found to also associate with telomeres, where the PML and Sp100 proteins appear to similarly form a ring wrapping around the telomere. Details on how these structures are nucleated and whether their assembly differs significantly from standard PML/ND10 body nucleation remain unclear. It is thought that free PML and Sp100 might bind particular proteins on the telomeres such as TRF1/2, the DNA repair protein NBS1, or the SUMO ligase MMS21 to initiate assembly of these structures (Chung et al. 2011).

1.5 Genome Organization Patterns

Mirroring the compartmentalization of the nuclear structures described above, the genome is non-randomly organized within the three-dimensional space of interphase nuclei (Bickmore 2013; Bickmore and van Steensel 2013; Lanctot et al. 2007; Zuleger et al. 2011b). This organization exists at multiple scales, ranging from whole chromosomes to individual genes, and is defined by the relative spatial proximity of specific DNA sequences to distinct nuclear structures and/or to each other.

1.5.1 Loops and Topologically-Associated Domains

At a locus level, the transcriptional output of specific genes can be controlled by regulatory elements hundreds of kilobases or even megabases distal along linear chromosomes through physically looping through nuclear space (Dekker and

Mirny 2016). Such looping provides additional regulatory information to gene promoters in a manner necessary to achieve the diversity of transcriptional outputs required in complex multicellular organisms (Rubinstein and de Souza 2013). Much of this looping occurs locally and is the basis of forming so-called TADs. Chromosomes are divided along their length into TADs defined by preferential local interactions that were determined using the chromosome conformation capture (C) and GAM technologies (Beagrie et al. 2017; Dixon et al. 2012; Sexton et al. 2009). Like LADs, the organization of the genome into TADs appears to be conserved from flies to humans, and perhaps also budding yeast, (Dixon et al. 2012, 2015; Eser et al. 2017), where they function to limit the interactions of enhancers to only their target genes and to create independent regulatory domains. Indeed, genes within TADs display coordinated expression changes between different cell types and cell states (Flavahan et al. 2016; Nora et al. 2012; Shen et al. 2012). Consequently, disruption of TAD boundaries induces ectopic interactions between enhancers and inappropriate genes, resulting in gene misexpression and disease (Hnisz et al. 2016; Flavahan et al. 2016; Ibn-Salem et al. 2014). For example, a duplication, inversion or deletion encompassing the boundaries of the *Epha4* TAD results in a limb bud-specific enhancer inappropriately associating with and activating the *Pax3*, *Ihh* or *Wnt6* loci that are normally located in neighboring TADs. This misregulation ultimately results in limb malformations (Lupianez et al. 2015). As a result of these critical functions it is perhaps not surprising that breaks in synteny between species are frequently found proximal to TAD boundaries in a manner that conserves TADs during evolution (Vietri Rudan et al. 2015). This, combined with the observation that the majority of TADs are conserved between different cell types (Dixon et al. 2012; Fraser et al. 2015), suggests that TADs represent a fundamental unit of genome organization. Supporting this, significant overlap is observed between TADs and other organizational units of the genome, including isochores (Jabbari and Bernardi 2017), LADs (Dixon et al. 2012; Fraser et al. 2015), and replication timing domains (Pope et al. 2014). It is also important to note that these are likely not fixed structures because, although their external boundaries appear largely invariant, their internal configurations can undergo significant remodelling during development (Andrey et al. 2017; Javierre et al. 2016).

1.5.2 Compartments

TADs that share similar functional states can also be adjacent in the higher order compartmentalization of the genome, even when separated by megabases along the same linear chromosome or when occurring on different chromosomes (Lieberman-Aiden et al. 2009). Initially, these preferential associations between TADs identified in Hi-C contact maps were delimited into two regimes, referred to as A and B, that correlate with active and repressed chromatin, respectively (Lieberman-Aiden et al. 2009). However, analysis on a higher resolution Hi-C contact map further

segregated these compartments into the A1, A2, B1, B2, B3 and B4 sub-compartments, each of which shares a unique set of properties and spatial localizations (Rao et al. 2014). For example, while all the B sub-compartments represent heterochromatic states displaying diminished gene expression, the B1 compartment is specifically enriched for H3K27me3 polycomb domains, the B2 compartment for LADs and nucleolar-associated domains, and the B3 compartment specifically for only LADs. Similarly, while enriched for active chromatin modifications and transcribing genes, the A sub-compartments can be differentiated with respect to their distance to lamina-associated regions with A2 regions being significantly closer to LADs along linear chromosomes than A1 regions (Robson et al. 2017). Intriguingly, the presumed spatial segregation of such compartments has also been confirmed independently using super-resolution FISH microscopy and single-cell Hi-C combined with 3D-modelling (Stevens et al. 2017; Vietri Rudan et al. 2015). Significantly, the composition of compartments, in contrast to that of TADs, is altered significantly during processes such as differentiation and senescence and between different cell types in a manner correlated with gene activity (Criscione et al. 2016; Dixon et al. 2015; Fraser et al. 2015). While the fundamental associations within TADs and compartments appear to be independent of spatial position within the nucleus, this compartment switching appears to be associated with the spatial repositioning of loci and occurs concomitantly with altered lamina-association during differentiation (Fraser et al. 2015). Hence, the genome is spatially organized into functionally distinct genomic regions that can be reorganized to accommodate changes to gene activity when necessary.

1.6 Structure-Function Relationships

1.6.1 Layers of Functional Separation

Early immunogold-EM studies revealed that the PML protein yields a ring-like staining around the PML/ND10 bodies (Weis et al. 1994) that appears donut shaped by high-resolution immunofluorescence microscopy (Boisvert et al. 2000). The application of super resolution 4Pi microscopy revealed that major components PML and Sp100 protein occur in largely distinct alternating patches in the outer shell of PML/ND10 bodies and that this shell is 50–100 nm thick (Lang et al. 2010). It appears that the integrity of the interactions forming the shell depends on sumoylation of the proteins. Moreover, some of the sumo modifications face inwards toward the center of the shell, presumably to associate with proteins in the core and further facilitate assembly, amongst which heterochromatin protein 1 (HP1) was observed. FRAP and FCS studies further demonstrated that the outer shell did not prevent the diffusion of proteins through the PML structure because GFP conjugated to an NLS was able to freely move through PML/ND10 bodies (Lang et al. 2010). However, the GFP-NLS failed to concentrate on the inside of the PML/Sp100 ring whereas HP1 did. These data argue that specific interactions

build the PML/ND10 bodies in layers with the outer layer relatively constant while the inner material varies. It remains unclear whether the ring forms around the inner material or the inner material is recruited into an already formed ring.

Immunogold EM staining for other nuclear subdomains reveals a similar layered organization. The advent of EM further enabled the subdivision of the nucleolus into fibrillar centers (FCs) that are surrounded by the dense fibrillar component (DFC), which in turn has the granular component as the outermost layer. Finally these three rings are surrounded by perinucleolar heterochromatin (Nemeth and Langst 2011). It is thought that this organization enables the sequential processing of RNAs through the nucleolus. When there is high rDNA transcription, multiple FCs can be observed embedded in the DFC, which helped ascertain that transcription preferentially occurs in the FC subregion. More processed rRNAs accumulate in the outermost subdomains suggesting that their processing occurs sequentially as they move from the FCs outward through the other nucleolar regions (Lamond and Sleeman 2003). The nucleolus initially forms around genome regions containing the genes encoding the large ribosomal RNAs, so-called nucleolar organizing regions, suggesting that the layers also reflect a mechanism for self-association in their assembly. Nuclear speckles also clearly have a layered structure (Nemeth and Langst 2011) (Fig. 1.1).

1.6.2 Loops

TAD formation appears to be driven by the interactions of proteins present on chromatin. In mammals, many TAD boundaries are demarked by a number of classical insulator elements, including interspersed repeats of the SINE family, the promoters of housekeeping genes, cohesin, and the zinc finger protein CCCTC-binding factor (CTCF) (Dixon et al. 2012; Sexton et al. 2009). Of these boundary elements CTCF is the most studied with deletions or modifications abrogating individual CTCF sites causing a collapse of TAD boundaries and/or creation of novel domains and, in some cases, gene miss-expression (Dixon et al. 2012; Dowen et al. 2014; Guo et al. 2015; Lupianez et al. 2015). The mechanism of TAD formation by CTCF is yet to be fully demonstrated, however, an increasing body of evidence supports a model of loop extrusion. In this model loop-extrusion factors such as cohesin or RNA polymerase II, continuously produce increasingly large DNA loops that ultimately stall at boundary elements such as CTCF (Fudenberg et al. 2016). This model is supported by its ability to computationally recapitulate a number of TAD structures as well as numerous data including a recent single-cell Hi-C study demonstrating significantly greater variability of TADs between cells than LADs or compartments (Stevens et al. 2017; Flyamer et al. 2017). Consequently, across short time scales, TADs represent population and temporally averaged ensembles of multiple loops forming and collapsing within the confines of these boundaries. By contrast, LADs and compartments represent less dynamic and more uniform spatially segregated domains within a cell population.

Although the boundaries of TADs correspond significantly to specific CTCF sites, many contacts observed within TADs or between compartments do not, suggesting different additional regimes of chromatin folding may exist. Supporting this, a growing body of evidence now suggests such physical CTCF-independent proximities are driven instead through homotypic interactions between regions possessing similar chromatin states. For example, during limb development and haematopoiesis a number of tissue-specific and CTCF-independent looping interactions were instead enriched for shared active histone modifications such as H3K27ac and H3K4me2 (Andrey et al. 2017; Javierre et al. 2016). As many of these tissue-specific associations were between enhancers and promoters, it is tempting to think that such changing interactions may be integral to the regulation of gene activity. Suggesting a common fundamental property of genome organization, similar repressive associations have also been observed for loci possessing PRC1 and PCR2 polycomb proteins together with several repressive chromatin marks such as H3K27me3 and HP1 (Schoenfelder et al. 2015; Sexton et al. 2012; Tolhuis et al. 2011; Wijchers et al. 2016) and for transcriptionally active genes and enhancers at transcriptional hubs (Beagrie et al. 2017; Schoenfelder et al. 2010). Of particular note, one study targeted transcriptional factor Nanog, repressor SUV39H1 or boundary histone EZH2 to a lacO array inserted into a TAD by fusing them to Lac repressor and found that changing epigenetic marks was sufficient to redirect the locus to a different chromosome compartment (Wijchers et al. 2016). Taken together, these data support a model where genome organization is driven by the self-association of genomic regions that are biochemically similar or share similar components for their function. Such associations likely have the effect of localizing and thus effectively concentrating factors required for specific functions, thus improving efficiency. However, if correct, such a model presents a fundamental question: how is the self--association of the genome limited to prevent its complete non-functional aggregation? In this regards, anchoring of genomic regions such as LADs and nucleolar-associated regions to fixed nuclear structures may be the answer.

1.6.3 Scaffolds

The NE is probably the most important structure from which the genome is spatially organized. Early EM revealed an asymmetric distribution of chromatin within nuclei, with euchromatin dominating the nuclear interior and electron dense heterochromatin dominating regions proximal to the NE and nucleoli (Moses 1956). The advent of fluorescence in situ hybridization (FISH) subsequently revealed that individual loci, differently timed replication origins, and even certain whole chromosomes also have preferred radial positions with respect to the NE (Bickmore 2013; Zink et al. 1999; Zuleger et al. 2011b). Interestingly, the preference towards positioning whole chromosomes at the nuclear periphery corresponded to their gene density (Croft et al. 1999). Together with the concentration of heterochromatin at the NE, this suggested a relationship between locus position and their distinct

transcriptional requirements. Entire chromosomes were also found to reposition during differentiation and to exhibit tissue-specific variation in radial position, possibly reflecting differences to the transcriptional output of their constituent genes (Kim et al. 2004; Parada et al. 2002, 2004; Szczerbal et al. 2009).

It remains unclear if changes to the radial positioning of a locus direct alterations to gene expression at that locus or *vice-versa*. On the one hand, anchoring of specific genomic regions to the periphery through artificial DNA-NE interactions was sufficient to induce repression of tethered genes (Finlan et al. 2008; Reddy et al. 2008). However, different experiments using the same system yielded considerable differences in transcriptional effects (Finlan et al. 2008; Kumaran and Spector 2008; Reddy et al. 2008). On the other hand, targeted transcriptional activation and local chromatin unfolding using viral proteins was sufficient to release individual loci from the periphery suggesting the chromatin state can in some cases determine the position of a gene (Chuang et al. 2006; Therizols et al. 2014; Tumbar and Belmont 2001; Tumbar et al. 1999). Hence, evidence supports both an affinity-tethering mechanism and a gene activation/chromatin unfolding mechanism. Whether different genes use distinct mechanisms or these mechanisms function redundantly is not known.

The advent of high-throughput genome-wide technologies enabled taking these initial EM and FISH-based observations and interrogating their global use. One such technology is DamID, which employs the bacterial Dam methylase. When fusing the Dam methylase to lamin B1 to precisely and globally map DNA regions within tens of nanometres from the NE (Vogel et al. 2007), it was found that ~35–45% of the genome is positioned at the NE in discrete blocks termed LADs. These LADs were found to be largely conserved between cell types and mammalian species (Meuleman et al. 2013; Peric-Hupkes et al. 2010; Robson et al. 2016; Robson et al. 2017). Although depleted in genes relative to non-LADs, both endogenous and ectopically introduced reporter genes present within LADs display 5–10 fold lower expression compared to their non-LAD counterparts (Akhtar et al. 2013; Guelen et al. 2008), supporting EM and FISH studies demonstrating the repressive capacity of the NE. Correspondingly, LADs are significantly depleted of genes, early-replication domains, and active histone modifications such as H3K4me2, H3K4me3, H3K9ac, H3K27ac and H3K36me3, while they are enriched in late-replication domains, pericentric satellite repeats, A- and T-rich sequences, and repressive modifications such as H3K9me2, H3K9me3 and H3K27me3 (Guelen et al. 2008; Pope et al. 2014). Accordingly, loss of repressive histone modifications, such as H3K9me2/3 and H3K27me3 disrupts lamina association (Demmerle et al. 2013; Harr et al. 2015; Kind et al. 2013; Zullo et al. 2012).

The logical mechanism for establishing such genome organization patterns would be the affinity of NE proteins for these silencing marks. Biochemical evidence for the physical interaction of chromatin with the NE goes back over three decades (Bouvier et al. 1985). The loss of certain NE proteins specifically disrupts the NE positioning of specific loci (Robson et al. 2016; Zullo et al. 2012). The NET LBR, for example, binds to HP1 (Ye and Worman 1996) and the depletion of LBR or lamin A from rod cells of nocturnal mammals causes a complete inversion of heterochromatin clusters with their repositioning to the nuclear interior and

euchromatin accumulation at the NE (Solovei et al. 2009; Solovei et al. 2013). As a number of NETs display interactions with enzymes that add heterochromatic modifications to chromatin (Demmerle et al. 2012; Somech et al. 2005) it seems likely that this peripheral association is self-propagating/re-enforcing.

The finding of specific NETs involved in tethering specific genes to the NE enabled testing the effects of gene positioning on gene regulation without artificial systems. Specifically manipulating the position of endogenous genes by manipulating levels of NETs that direct their positioning revealed that peripheral association contributed roughly 50% of the repression normally observed (Robson et al. 2016). However, these gene expression changes were only observed when releasing a locus from the periphery by NET knockdown in differentiating cells. When NET overexpression was performed in a system where the NET was not normally expressed, it inappropriately recruited a locus to the periphery but it did not change the expression, suggesting that gene regulation is a complex interaction of specific transcriptional regulators and the gene repositioning (Robson et al. 2016).

While it is easy to understand how recruitment of a locus and transcriptional regulators to the generally silencing environment of the periphery could contribute to locus repression, there are also more complex effects of the NE on genome organization and regulation. When focusing on their organization into territories, TADs and compartments, it is easy to forget that chromosomes are even more fundamentally linear strands of DNA. As such, a LAD tethering part of a chromosome at the periphery can restrict the nuclear position of genes further down the linear length of the chromosome. As there are many LADs along the linear chromosome, one could view inter-LAD regions as loops reaching into the nucleoplasm from the NE of differing sizes according to the linear length of DNA between the LADs. How particular genes can find one another within the large 3-dimensional space of the nucleus is a major question for the field, especially as modelling studies indicate that it is unlikely for loci to find each other in a single cell cycle by diffusion alone if they are 10 Mb apart and thus potentially >1 μm away from one another (Dekker and Mirny 2016). In contrast, a 0.5–0.8 μm space could be sampled by a locus in 1 h. In a recent study identifying LADs that change during lymphocyte activation, it was noted that loci that were released from changing LADs, but flanked by LADs that were maintained, remained typically much less than 0.8 μm from the periphery (Robson et al. 2017). Critically, multiple loci with similarly flanked LADs that were kept apart when at the periphery were able to find one another upon this "constrained release" from the NE. When comparing the LAD data with compartment data it was found that the released genes associate in the similarly regulated A2 subcompartment (Robson et al. 2017). Thus the limited space to sample increases the likelihood of incorporation of a released locus into a similarly regulated active compartment.

In addition to heterochromatin and gene interactions, telomeres and centromeres also can be oriented to the NE. This was first noted with the Rabl conformation of chromosomes that is often directed at supporting chromosome alignment for synaptonemal complex associated recombination in meiosis (Scherthan et al. 1996). While telomeres associate at least transiently with the NE in organisms

from yeast to man, the mechanisms appear to be somewhat varied. In the budding yeast *S. cerevisiae* the Ku proteins on the telomeres were first implicated in the association (Laroche et al. 1998) followed by the NPC protein TPR (Galy et al. 2000). Subsequently, SUN domain NETs were found to also contribute to the telomere association with the periphery in yeast (Antoniacci et al. 2007; Bupp et al. 2007; Chikashige et al. 2006); however, this association is slightly more complex in mammals. In spermatocyte meiosis, one study implicated SUN1 (Ding et al. 2007) while another implicated SUN2 (Schmitt et al. 2007), possibly indicating that the multiple SUN proteins of higher organisms have partially redundant and partially distinct functions. While SUN proteins appear to be the one unifying player, there are likely to be significant differences in telomere tethering complexes because budding yeast maintain telomeres at the NE while, apart from spermatocytes, most mammalian cells just anchor telomeres transiently in meiosis. Nonetheless, there are other specialized NE-telomere interactions in mammals. For example, telomeres are tethered to the NE during post-mitotic NE reassembly and this association involves both SUN1 and the shelterin subunit RAP1 (Crabbe et al. 2012). A specialized interaction with lamins is also indicated due to the abnormal distribution of telomeres in cells expressing a lamin mutation that causes Hutchison-Gilford progeria syndrome (Taimen et al. 2009).

1.6.4 Boundary Elements

NPCs in yeast contribute to genome regulation by creating boundary elements where NPC connections segregate active and silent regions (Ishii et al. 2002). It appears that associations of certain nucleoporins with the specific silencing-associated histone variant H2AZ form regions of transcriptionally repressed DNA, but directly adjacent to these regions there are also transcriptionally active regions. Yeast nucleoporins also bind transcription factors and thus the large size and complex structural organization of the NPC could segregate the silenced regions from regions with transcription factors that promote gene activation (Schmid et al. 2006; Taddei et al. 2006). Mammalian nucleoporins also have both silencing and activating interactions with chromatin. However, some of the historical findings about interactions are confunded by the fact that some nucleoporins have separate nucleoplasmic pools that are not integrated into the NPC structure and can have distinct effects on genes in the nucleoplasm (Capelson et al. 2010; Kalverda et al. 2010).

1.7 General versus Tissue- or State-Specific Functions

A number of developmentally regulated loci reposition to or from the nuclear periphery concomitantly with changes to expression during differentiation. For example, the *MyoD* locus repositions from the nuclear periphery to the nuclear

interior when it becomes transcriptionally active during myogenesis (Meister et al. 2010; Yao et al. 2011) as does the *IgH* (Kosak et al. 2002) and the Mash1 loci (Williams et al. 2006) during B cell and neuronal differentiation, respectively. Conversely, *Nid1* and *c-maf* reposition to the periphery upon transcriptional repression during myogenesis (Robson et al. 2016) and T-cell differentiation (Hewitt et al. 2004), respectively. Hence, the radial position of a locus is frequently related to its transcriptional state, suggesting a relationship. The application of DamID to systems following differentiation and changes in cell states has also revealed changes in ~5% of LADs. DamID on cells during neurogenesis, myogenesis and T-cell activation all revealed that a small but significant fraction of the genome enriched in developmentally regulated genes display gain or loss of lamina-association in a manner correlating with transcriptional activation and repression, respectively (Peric-Hupkes et al. 2010; Robson et al. 2016, 2017). These findings indicate that differentiation involves the selective reorganization of specific developmentally regulated genes in a manner that correlates with changes to their transcriptional status.

In the case of myogenesis, it was further shown that tissue-specific NETs direct changes in genome organization. Interestingly, it was found that the NE proteome is highly tissue-specific. Strikingly, studies of the NE proteomes of liver (Schirmer et al. 2003), lymphocytes (Korfali et al. 2010) and skeletal muscle (Wilkie et al. 2011) showed that, only ~17% of the proteins identified were shared by all three tissues (liver, lymphoctyes and muscle). Moreover, many of these proteins were uniquely expressed in the particular tissue investigated (Korfali et al. 2012). Thus, tissue-specific NETs likely direct many aspects of tissue-specific genome organization patterns.

Nucleolar composition also varies with the metabolic condition of the cell and throughout different interphase cell cycle stages (Leung and Lamond 2003). It also changes composition during adenovirus infections with 24 nucleolar proteins out of 351 identified by mass spectrometry showing a greater than two-fold change in abundance based on SILAC ratios. Fifteen out of these 24 proteins were directly tested by immunofluorescence microscopy and of these eleven also exhibited altered localization during adenovirus infection (Lam et al. 2010).

1.8 Conclusions and Open Questions

While much has been learned about nuclear subdomains and genome organization there are many open questions for the field. For example, while recent studies demonstrate the existence of tissue-specific differences in both the nuclear subdomains and in genome organization, very few tissues have been analyzed. Establishing if tissue differences reflect common or distinct mechanisms and identifying the relevant players in these processes will require sampling of a much greater set of tissues and should be investigated for all nuclear subdomains.

1.8.1 Mechanism for Establishment and Function of Different Organizational Patterns

There is clear evidence for general heterochromatin interactions directing aspects of spatial genome organization and there is also clear evidence for tissue-specific NETs directing a subset of tissue-specific aspects of spatial genome organization. However, there is no understanding currently of whether the two interact or one directs the other. For example, a high-affinity tissue-specific interaction set up during NE reassembly could position chromosomes so that heterochromatin facing the periphery subsequently engages with LBR and lamin A while that facing the nuclear interior engages with the nucleolus. Alternatively, heterochromatin interactions could facilitate the tissue-specific genome organization patterns or both may work together. Testing such issues will require developing methods not yet in existence for measuring kinetics and relative binding affinities of all the proteins involved. Also, though it is clear that lamins and NETs can contribute to spatial genome organization through tethering chromatin at the periphery, the relative requirements for withstanding forces from gigadalton chromosomes have not been worked out and there may be many additional proteins involved that have yet to be identified.

There are also many standing inconsistencies in the literature such as lamin A plays a major role in the radial organization of heterochromatin (Solovei et al. 2013), but at the same time, though LADs are identified by lamin interactions, LAD organization was mostly unaffected with lamin knockout (Amendola and van Steensel 2015). Thus, there are likely many other factors contributing to these processes that have yet to be identified. We have only just begun investigating the relationship between radial genome organization (LADs) and how it can influence the organization of TADs into compartments.

1.8.2 Zip Codes and Dynamics

One of the biggest outstanding questions is how proteins are targeted within the nucleus and how their dynamics are controlled. While much is now known about how affinity interactions direct self-assembly of complexes, this information is only available for a tiny subset of the many nuclear proteins and subdomains. Though it has been nearly two decades since observations of the *gypsy* insulator DNA sequence being able to re-target an internal locus to the periphery (Gerasimova et al. 2000), questions remain for what DNA sequences contribute to most other aspects of genome organization.

What is clear is that nuclear subdomain and genome dynamics are considerable and likely needed for most of these self-assembly and other aspects of nuclear subdomain function. Individual loci in the nuclear interior can move rapidly

over large distances during interphase, especially when they become activated (Chuang et al. 2006). In one elegant study live cell co-labeling of PML bodies, a gene locus, and its product revealed that upon transcriptional activation the locus decondensed and moved until it associated with PML bodies to maximize transcriptional output (Tsukamoto et al. 2000). It is noteworthy that the different classes of PML bodies include a subclass that moves by a metabolic-energy-dependent mechanism (Muratani et al. 2002).

NE contacts appear to at least partly inhibit such mobility as loci at the nuclear periphery tend to be much less mobile (Chubb et al. 2002). Though whole chromosome territories are generally maintained during interphase, there is greater loss of defined photoactivated regions in the interior compared to the periphery (Strickfaden et al. 2010). Nonetheless, some dynamic exchange of peripheral loci occurs with recently developed single-cell DamID approaches revealing that only 2/5 of LADs are universally observed and some LADs actively change during interphase (Kind et al. 2013). In fact, LADs may move up to 1 μm away from the periphery over 5–20 hours and many LADs were observed to shuffle between the NE and nucleoli. NETs might contribute to this as FRAP and photoactivation experiments on NETs involved in chromosome repositioning revealed that some are very dynamic while others are not (Zuleger et al. 2011a).

1.8.3 Conclusions

The dynamics of nuclear subdomains makes particular sense in conjunction with the absence of intranuclear membranes. The same genes need to be active or shut down both temporally and in different tissues and different genes on the same linear chromosome need to be active or repressed. How would one segregate the same chromosome into distinct compartments? While it might be possible to segregate RNA processing into a membrane bound compartment, there are aspects of quality control that appear to occur co-transcriptional that could not be engaged if this process were segregated by membranes. It makes more biological sense to dynamically move self-assembling factories around the nucleus to where they are needed when they are needed in each cell type.

Other critical questions remaining include what are the fundamental prinicples of nuclear assembly and re-assembly after each mitosis? How do mutations in the vast majority of non-coding genome affect genome structure and subsequently cause disease? How universal are nuclear organization patterns and mechanisms? When and how did it arise in evolution? Is it a fundamental property of complex multicellular organisms? It is interesting in this regard that the tissue-specific NETs tend to be poorly conserved in evolution (de Las Heras et al. 2013) and thus it could be speculated that the appearance of nuclear subdomains and their variation may have enabled an ever increasing complexity in genome regulation that has driven tissue-specification in evolution.

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Chapter 2
The Molecular Composition and Function of the Nuclear Periphery and Its Impact on the Genome

C. Patrick Lusk and Megan C. King

Abstract The nuclear periphery is an essential element of nuclear architecture that contributes to the organization and function of the genome. Over the last few decades, remarkable molecular insight from many model systems has contributed to a dynamic and nuanced view of the nuclear periphery, which had previously been considered a static, transcriptionally-silent nuclear subcompartment. While modern genomic analyses have confirmed that the nuclear periphery is home to repetitive, gene-poor chromatin rich in repressive histone marks, specific genic regions either leave or associate with the nuclear periphery in response to external environmental or developmental inputs in a way that correlates with transcriptional output. Recently, work suggests surprisingly that transcription per se is not a determinant of gene position in relation to the nuclear periphery; an emerging view instead supports that peripheral tethering may reflect mechanisms to promote genome stability while being dispensable for gene silencing. Here, we review our current understanding of the molecular components that form the nuclear periphery, including integral inner nuclear membrane proteins and the nuclear lamins, while overviewing the key studies that are contributing to our evolving view of this important nuclear subcompartment.

Keywords Inner nuclear membrane · lamina · LADs · heterochromatin · histone · genome stability

2.1 The Conservation of the Spatial Positioning of the Genome Across Eukaryotes

The genome is encased in the nuclear envelope (NE) – a double membrane that is contiguous with the endoplasmic reticulum (ER). The biochemical (and thus,

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functional) specialization of the NE is conferred by a discrete proteome that includes nuclear pore complexes (NPCs) and specific membrane and membrane-associated proteins that directly interface with the genome and the inner nuclear membrane (INM; Fig. 2.1). Indeed, it is widely accepted that the nuclear periphery is a major component of nuclear architecture that contributes to the non-random organization of chromosomes within "territories" in the nucleus, a term first coined by Boveri in 1909 (Boveri 1909) but which was not directly visualized until many decades later (see (Cremer and Cremer 2010) for a more extensive historical overview). Moreover, most eukaryotic cells display a distinct segregation of (largely) transcriptionally silent heterochromatin at the nuclear periphery with more active euchromatin within the interior. This observation, made first by Rabl over 130 years ago (Rabl 1885), has been revisited time and again with ever increasing technological advances in many model organisms.

The obvious tethering of heterochromatin to the nuclear periphery from yeast to man has captivated our imaginations and given rise to long-standing hypotheses that posit a central role for peripheral tethering in regulating gene expression, whether to "gate genes" (Blobel 1985) or (conversely) to silence gene expression. Perhaps not surprisingly, decoupling gene recruitment to the nuclear periphery and processes linked to transcriptional up or down-regulation has proven extremely challenging. Nonetheless, work over the last several decades has delineated mechanisms by which the nuclear periphery acts as a critical platform for modulating transcriptional output, maintaining genome stability and regulating coordinated differentiation programs during development in multicellular eukaryotes. Here, we will overview the proteome and interactome of the NE and describe our molecular understanding of how the nuclear periphery, particularly the INM, impacts these critical genomic processes.

2.2 Integral INM Proteins

The nuclear periphery can be conceptualized as being "built" upon the resident components of the INM. It is well understood that the INM has a distinct proteome made up of integral membrane and membrane-associated proteins (Fig. 2.1), although a complete cataloguing of the INM has remained elusive. This is due to several experimental limitations: (1) the continuity of the NE and ER make biochemically isolating the INM a so-far insurmountable hurdle; (2) the relatively low abundance of many integral INM proteins, which is exacerbated by their insolubility; and (3) the likely ability of many ER proteins to sample the INM, without accumulating or functioning there (Deng and Hochstrasser 2006; Smoyer et al. 2016). As a consequence, a major fraction of the INM proteome at steady state is likely identical to that of the outer nuclear membrane (ONM)/ER, making even successful subtractive proteomics approaches (Schirmer et al. 2003) ineffective at conclusively differentiating between ONM and INM without additional experimental evidence. Indeed, while hundreds of NE transmembrane

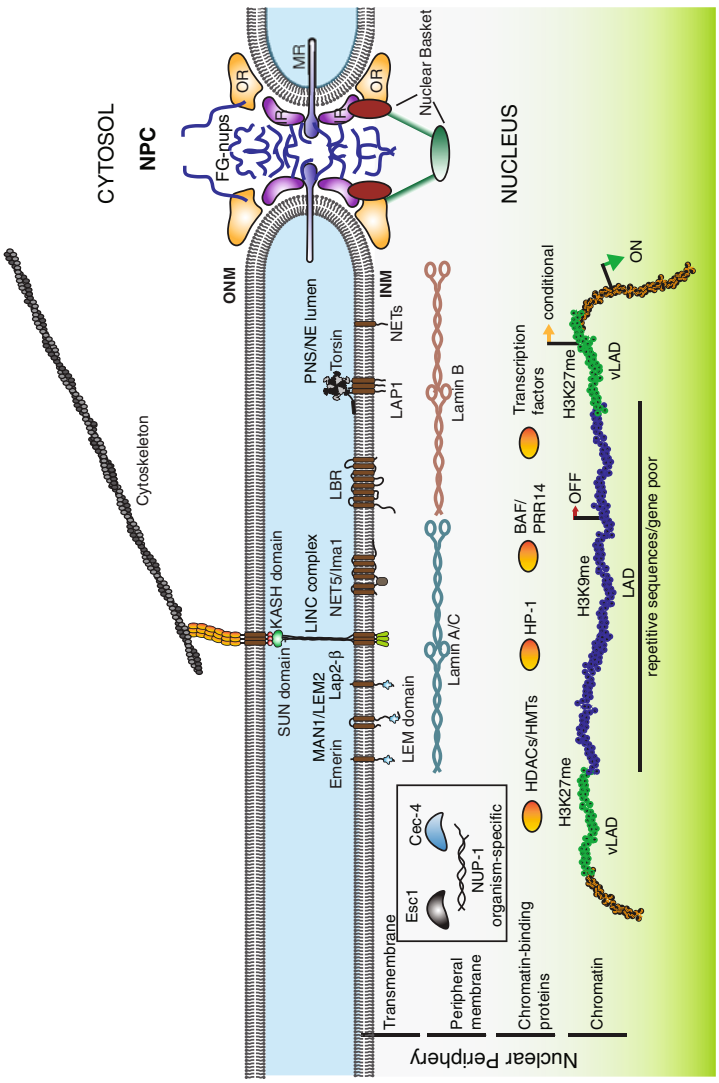

Fig. 2.1 Schematic of major components of the nuclear periphery required for genome tethering. The nuclear periphery is built on the inner and outer nuclear membranes (INM and ONM) with embedded nuclear pore complexes (NPC). NPCs are lined with FG-rich proteins scaffolded by subcomplexes like the outer ring (OR), inner ring (IR) and membrane ring (MR); the nuclear basket extends into the nucleoplasm. Well established integral INM proteins are shown including the SUN and KASH domain proteins that make up the linker of nucleoskeleton and cytoskeleton (LINC) complexes that span the perinuclear space (PNS)/nuclear envelope (NE) lumen and the Lap2-Emerin-MAN1 (LEM) domain proteins. Several peripheral membrane proteins have been identified, the most notable of which are the A- and B-type lamin networks. There are also other peripheral tethers in disparate model systems (Esc1, *Saccharomyces cerevisiae*, Cec-4, *Caenorhabditis elegans*, NUP-1, *Trypanosoma brucei*). The tethering and/or recruitment of lamina-associated domains (LADs) requires several factors including histone methyltransferases (HMTs) and/or histone deacetylases (HDACs), heterochromatin-binding proteins (HP-1; which might also require binding to PRR14), transcription factors like YY1 or cKROX and barrier to autointegration factor (BAF). In general LADs are transcriptionally silent and rich in H3K9me marks (blue) with H3K27me (green) at LAD borders. The LAD borders are often conditionally associated with the nuclear periphery and have been termed variable (v) LADs

proteins (NETs) have now been identified, some of which show tissue-specific expression (Schirmer et al. 2003; Korfali et al. 2010, 2012; Wilkie et al. 2011), there remains a laborious task of testing how many of these ultimately localize (and function) at the INM. The latter is particularly challenging as reagents such as specific antibodies are largely unavailable, necessitating a reliance on heterologous tagging/overexpression strategies that often lead to aberrant accumulation of the excess protein in the ER, therefore altering its steady-state distribution. Moreover, many heterologous tags, particularly large proteins such as GFP, can often interfere with membrane integration, and/or their targeting to the INM (Khmelinskii et al. 2014). Most critically, standard immunofluorescence microscopy cannot discern the INM from the ONM due to the inherent diffraction limited resolution of light microscopes, making immunoEM the gold standard for confirming INM localization (which is itself difficult and far from perfect given the decrease in effective localization accuracy when using secondary gold conjugated antibodies). Fortunately, technological innovations such as single molecular FRAP (Mudumbi et al. 2016) and super-resolution microscopy promise to supplant immunoEM to precisely localize integral membrane proteins (Korfali et al. 2016), suggesting that our understanding of the INM proteome will likely continue to expand with time.

2.3 A Brief History of INM Targeting

Like most subcellular compartments, the biochemical identity of the INM is assured by mechanisms that control protein targeting and quality control to turnover damaged or mistargeted proteins (Boban et al. 2014; Webster et al. 2014; Foresti et al. 2014; Khmelinskii et al. 2014; Turner and Schlieker 2016; Webster and Lusk 2016). While various hypothetical models for accumulating integral membrane proteins at the INM have been proposed (see (Katta et al. 2014) for discussion), there is a general consensus that membrane proteins travel along the continuous bilayer from the ONM/ER across the nuclear pore membrane to the INM (Lusk et al. 2007; Antonin et al. 2011; Laba et al. 2014) (Fig. 2.2). For example, several studies support that disruption of specific NPC components (nucleoporins/nups) influences the kinetics and/or steady-state distribution of integral INM proteins (Ohba et al. 2004; King et al. 2006; Deng and Hochstrasser 2006; Theerthagiri et al. 2010; Mitchell et al. 2010; Zuleger et al. 2011; Meinema et al. 2011; Boni et al. 2015; Ungricht et al. 2015; Lokareddy et al. 2015).

But, what is the mechanism of INM targeting? Moreover, do all INM proteins utilize the same mechanism or do subsets of INM proteins access distinct mechanisms? Early studies examining the localization of the Lamina Associate Polypeptide-1 (LAP1) and others revealed that binding to elements of the nuclear architecture (particularly nuclear lamins) plays a critical role in determining the steady-state distribution and immobilization of most integral membrane proteins at the INM (Powell and Burke 1990; Smith and Blobel 1993; Soullam and Worman

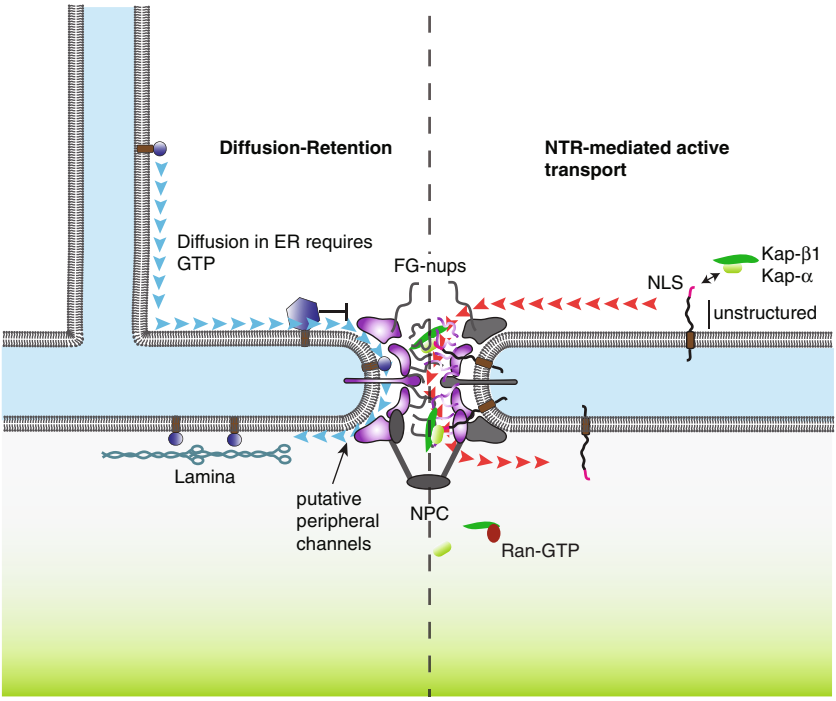

Fig. 2.2 Models of integral membrane protein targeting to the INM. In the diffusion-retention model (left; blue arrows), GTP is required to remodel the ER in a way that promotes membrane protein diffusion and thus the probability of reaching the nuclear pore membrane is increased; passage by the NPC is likely through putative peripheral channels that impose a molecular weight cut-off (small blue circles pass whereas large hexagons do not). Retention is mediated by binding to nuclear factors like the lamins or chromatin. In the NTR-based model, GTP is required for the Ran cycle with Ran-GTP dissociating INM protein cargo from the NTRs Kap-β1/Kap-α in a mechanism directly analogous to soluble nuclear transport. Such a model requires that extralumenal domains reach into the central transport channel of the NPC to allow NTR binding to FG-nups. This is thought to be achieved by a long ~120 amino acid unstructured region that is capped by a high affinity NLS. NPC subcomplexes shaded purple have been shown to be required for either diffusion-retention or NTR-mediated transport. Key in Fig. 2.1

1993, 1995; Ellenberg et al. 1997; Ostlund et al. 1999; Vaughan et al. 2001; Gruenbaum et al. 2002; Ohba et al. 2004). These data, in combination with those remarking on the free diffusion of viral proteins that access the INM (Torrisi et al. 1987; Torrisi et al. 1989), were suggestive of a model of INM accumulation in which "diffusion-retention" is sufficient for INM targeting (Fig. 2.2). In such a model, a molecular weight cut-off of ~60 kD is established for extralumenal domains of membrane proteins (likely imposed by steric hindrance by the scaffold of the NPC); exposure of nucleoplasmic domains to the nuclear interior then allow for subsequent binding to (and retention by) a nuclear factor (typically thought to be nuclear lamins or chromatin). However, the simplicity of such a mechanism was challenged by work supporting the existence of an energy-dependent targeting

step; the requirement for energy was postulated to be essential to remodel the NPC scaffold to allow passage of membrane proteins along constrictive peripheral channels that line the nuclear pore membrane (Ohba et al. 2004).

The energy-requirement to accumulate a reporter at the INM stimulated consideration of potential active INM targeting pathways. As membrane proteins must pass the NPC, a logical hypothesis was that Ran-GTP and nuclear transport receptors (NTRs; a.k.a. karyopherins/importins/exportins) might, in addition to supporting soluble nuclear transport, also promote membrane protein targeting. Indeed, it was recognized that the integral INM protein lamin B receptor (LBR) has a nuclear localization signal (NLS; (Soullam and Worman 1993, 1995), as do many other INM proteins (Lusk et al. 2007)) that could, in principle, be recognized by NTRs. However, the classical SV40 large T-antigen NLS (recognized by the karyopherin/importin α/β1 heterodimer) or the nucleoplasmin NLS (recognized by transportin/karyopherin β2) fused to a heterologous type II ER membrane protein was insufficient to confer INM localization (Soullam and Worman 1995), putting this idea aside until the discovery of conserved integral INM proteins of the LAP2, emerin, MAN1 (LEM) family in budding yeast, Src1/Heh1 and Heh2 (King et al. 2006). Importantly, molecular insights into the ability of NTRs to promote targeting of yeast LEM domain proteins across the NPC explain the failure of these engineered constructs to localize to the INM (Meinema et al. 2011).

A stand out feature of Heh1 and Heh2 is the presence of a bipartite NLS just downstream of the conserved LEM domain. The Heh2 NLS directly binds the NTR Kap α in the absence of Kap-β1 (King et al. 2006), an atypical result for NLSs transported by the Kap α/β1 heterodimer, which usually require Kap-β1 to bind and remove an inhibitory domain of Kap-α that prevents NLS binding (Rexach and Blobel 1995; Fanara et al. 2000). Thus, this observation suggested that the Heh2-NLS binds to Kap-α with an unusually high-affinity that can effectively compete with its inhibitory domain, a hypothesis that was directly confirmed by subsequent biochemical and structural analyses (Lokareddy et al. 2015). In addition, this NLS is required for Heh2 to gain access to the INM; the molecular necessity of this high-affinity, Kap-α-specific NLS for INM targeting remains enigmatic to this day.

Consistent with the ability of an NLS and NTRs to promote the efficient targeting of LEM domain proteins to the INM in yeast, genetic ablation of Ran, Kap α/β1 and several nups also inhibited INM targeting of Heh2 (King et al. 2006; Meinema et al. 2011). Together these results support a model in which Heh2 uses the soluble transport machinery to gain access to the INM, a confounding result when one considers that the nuclear domains of Heh1 and Heh2 are 40–50 kD; the addition of Kap-α (and its binding partner Kap-β1) would contribute an additional ~200 kD of mass, making passage through the size-restricted channel along the nuclear pore membrane likely impossible.

Interestingly, consistent with earlier studies in mammalian cells (Soullam and Worman 1995), an NLS fused to a transmembrane domain was also insufficient to drive INM accumulation in yeast, suggesting that other sequence determinants are required to get membrane proteins, including Heh1 and Heh2, across the nuclear

pore membrane (Meinema et al. 2011). Surprisingly, virtually the entire N-terminal domain of Heh2 is necessary for efficient INM targeting; a breakthrough was the recognition that this domain is largely unstructured (Meinema et al. 2011) (Fig. 2.2). Indeed, sufficiency of targeting a multipass transmembrane component of the ER translocon to the INM could be achieved by addition of a high affinity NLS coupled to an extended, completely artificial unstructured linker of at least 120 amino acids (Meinema et al. 2011). Thus, the passage of this ~200 kD complex through the NPC is likely facilitated by the ability of the unstructured linker to cut through the pore membrane-proximal scaffold, with the NLS-associated NTR moving through the central transport channel (Fig. 2.2).

But, how universal is an active INM-targeting mechanism? Apart from a mammalian nuclear pore membrane protein (POM121) that shares a similar NLS-requirement for INM accumulation (Doucet et al. 2010; Funakoshi et al 2011; Kralt et al. 2015), this question remains to be fully answered. For example, a recent study where interpretation of an extensive analysis of the steady state and kinetics of the localization of multiple reporters (both modeled on native integral INM proteins and completely artificial reporters) in a permeabilized mammalian cell system firmly supports a diffusion-retention model (Ungricht et al. 2015). Similarly, the results from an RNAi-based screen examining the genetic requirements that contribute to the kinetics of INM targeting (with mathematical modeling) were again most consistent with diffusion-retention being the major determinant of INM protein distribution, although NTRs were among those factors identified that influenced INM targeting kinetics, albeit potentially indirectly (Boni et al. 2015). Moreover, the energy requirement for INM targeting could be attributed to the necessity of energy-dependent ER dynamics required for the lateral mobility of membrane proteins to increase the likelihood that they reach the nuclear pore membrane (Ungricht et al. 2015).

How can we reconcile these two, potentially antagonistic, views of the INM targeting pathway? One possibility is that with the evolution of an open mitosis (which might allow larger extralumenal domains access to the nucleus without having to travel past NPCs) coupled to a more elaborate nuclear architecture that includes the lamina, the advantages of an active targeting pathway were supplanted by other functional priorities. In addition, while the NPC itself is compositionally near-identical from yeast to man (Rout et al. 2000; Cronshaw et al. 2002), it is much larger in vertebrates (Yang et al. 1998) owing to a doubling of the stoichiometry of the scaffold nups (Bui et al. 2013). Indeed, while the major "Y-complex" is likely organized in a single head-to-tail ring in the yeast NPC (Alber et al. 2007), the second "Y" in humans overlaps the other resembling a brick wall (Bui et al. 2013; von Appen et al. 2015). Thus, it is possible that any plasticity that might allow an unfolded peptide to weave through the scaffold in yeast was lost as the NPC became more elaborate through evolution. Lastly, there may be specific proteins, such as the LEM domain proteins of yeast, for which a rapid, active mechanism is beneficial to prevent these factors from residing in the cytoplasmic compartment; in this context, such a pathway may not be a requirement for passage through the NPC, but instead a mechanism to promote import efficiency

immediately upon biosynthesis. Importantly, no one has yet directly visualized the passage of INM proteins across the nuclear pore membrane in yeast or in mammalian cell lines, leaving the door open for additional new discoveries.

2.4 The Nuclear Lamina

Virtually all the well-determined integral INM proteins in metazoans interact with the lamin network that lines the INM, made up of A- and B-type lamin proteins, which are members of the type V intermediate filament family (Fig. 2.1). The A-type lamins, lamin A and lamin C, are derived by alternative splicing of the single *LmnA* gene (Burke and Stewart 2013; Gruenbaum and Foisner 2015), while the broadly expressed B-type lamins, lamin B1 and lamin B2, are encoded by separate genes. While most lamins associate with the INM, there is also a soluble pool of A-type lamins within the nucleoplasm. INM association of lamins can be reinforced by post-translational farnesylation at their C-terminal CAAX box, although in lamin A additional processing by the protease Zmpste24 removes the C-terminus, leading to production of "mature" lamin A (Davies et al. 2009; Burke and Stewart 2014). The function of the processing of lamin A remains ill-defined, as a mouse model expressing only the (unmodified) lamin C splice variant is viable and without phenotype (Sullivan et al. 1999; Fong et al. 2006), while mutations that disrupt maturation leading to constitutive farnesylation of lamin A (a form of the protein called progerin) lead to severe human disease (Burke and Stewart 2013). In contrast, the farnesylation of lamin B1 is required for its function and contributes to its association with the INM (Moir et al. 2000; Burke and Stewart 2014).

Until recently, our understanding of the organization of the lamin network was derived from iconic electron microscopy images of an interlocking 10 nm-diameter thick orthogonally-organized filament network in frog oocytes observed over 30 years ago (Aebi et al. 1986). More recently, with the advent of super resolution light microscopy and the revolution in detector technology that has improved the resolution of cryo-EM, we are approaching a clearer in situ picture of the lamina in model systems and in human cells. For example, super-resolution microscopy studies provide a compelling description of distinct (yet interdependent) A- and B-type lamin filament networks (Shimi et al. 2015; Xie et al. 2016), which had been inferred from lower resolution approaches (Shimi et al. 2008; Taimen et al. 2009; Kolb et al. 2011). These networks, while distinct, rely on each other to form a cohesive nuclear lamina, although how they interface with one another remains unclear (Shimi et al. 2015). In addition, it is not well understood how other NE landmarks like NPCs might contribute to the formation and/or organization of distinct lamin networks. Intriguingly, NPCs are specifically recruited to filaments formed through the overexpression of lamin C (in a lamin A null background), but not lamin A, suggesting a specific molecular link between lamin C and the NPC, perhaps through the nuclear basket component, Tpr (Xie et al. 2016).

Direct physical links between the lamina and NPCs have been suggested by several studies (Smythe et al. 2000; Hawryluk-Gara et al. 2005; Al-Haboubi et al. 2011), and are likely visualized by cryo-EM views in which lamin filaments appear to directly contact NPCs (Grossman et al. 2012). Very recently, cryoelectron tomography has revealed that the nuclear lamina is predominantly composed of lamin tetramers that give rise to a meshwork of 3.5 nm filaments in somatic cells; this suggests that the organization of the lamins is morphologically distinct from all cytoplasmic cytoskeketal elements (Turgay et al. 2017).

The interdependence of the lamin filament networks supports a model in which the nuclear lamina provides mechanical support to the nucleus. This function is well established, with A-type lamins contributing substantially to nuclear rigidity when subjected to large deformations, which is likely most critical in "stiff" tissues where mechanical strain on the nucleus would be predicted to be high (Davidson and Lammerding 2014). Consistent with this idea, lamin A levels scale with tissue stiffness (Swift et al. 2013) and many of the diseases (the laminopathies; discussed in detail elsewhere (Burke and Stewart 2014)) associated with lamin dysfunction manifest as defects in nuclear shape/integrity. In response to small deformations another lamina component, the chromatin, and particularly the heterochromatin associated with the nuclear periphery, also contributes to the mechanical response of nuclei (King et al. 2008; Schreiner et al. 2015; Furusawa et al. 2015; Stephens et al. 2017). This network not only provides a bulwark that ensures nuclear integrity in cell culture (De Vos et al. 2011; Vargas et al. 2012; Hatch et al. 2013; Maciejowski et al. 2015), but most dramatically serves critical functions as migrating cells move through confined spaces either in vivo or in vitro (Denais et al. 2016; Raab et al. 2016).

These studies highlight that the nucleus is far from an island, but is instead mechanically integrated into the cell (and tissue) in which it resides. A growing body of work demonstrates that the nucleus and its interactions with the cytoskeleton through the Linker of Nucleoskeleton and Cytoskeleton (LINC) complexes, which bridge both INM and ONM to mechanically couple the nuclear lamina to the cytoplasmic cytoskeleton (Fig. 2.1), play important roles in responding to and coordinating forces generated by cells and tissues (Lombardi and Lammerding 2011). Tension exerted onto LINC complexes in isolated nuclei can drive changes in the post-translational modifications of key lamina components such as emerin (Guilluy et al. 2014), suggesting a potential role for the LINC complex in mechanotransduction (although the transcriptional outputs of such a cascade remain poorly defined in vivo). Further, several recent studies highlight that nuclear lamina components, including the conserved integral INM (Sad1p, UNC-84; SUN) proteins that comprise the inner aspect of LINC complexes, play important (and unexpected) roles in regulating cell-matrix and cell-cell adhesions (Stewart et al. 2015; Thakar et al. 2017). Taken together, these studies suggest that signaling between cell junctions at the cell surface, and LINC complexes, which can be envisaged as NE "junctions," given that they couple two lipid bilayers that span a lumenal or extracellular space (Blobel 2010), may provide mechanisms for mechanical communication to (and from) the nucleus that awaits further investigation.

2.5 Lamina Associated Domains

In addition to providing a mechanical scaffold that supports nuclear integrity, the lamins (with INM proteins) organize a network of interactions that both promote the formation of heterochromatic domains and physically link these domains to the INM in most differentiated cell types where it has been examined (Fig. 2.1). A key technological breakthrough that revolutionized our understanding of the chromatin interface with the nuclear periphery was the development of the Dam-ID approach, which relies on the expression (at low levels) of Lamin A or B fused to the bacterially-derived Dam-methylase (Pickersgill et al. 2006; Vogel et al. 2007); Dam specifically methylates adenines within the sequence GATC. Until very recently, adenine methylation within eukaryotic DNA was thought to be completely absent; while it is now recognized that this modification does take place at very low levels (Wu et al. 2016), the Dam-ID approach continues to provide advantages for the characterization of some protein-DNA interactions, such as for the lamina. Sites of adenine methylation can be identified genome wide using microarray chip technologies, or, more recently, next generation sequencing.

As might be expected from the physical enrichment of heterochromatin at the nuclear periphery, lamina-associated domains (LADs) are 0.1–10 Mb chromosomal regions rich in repetitive gene-poor "deserts" covering ~30% of the genome (Guelen et al. 2008). Clues to what might define LADs can be found by analyzing their boundaries, which contain binding sites for insulator elements like CTCF, CpG islands and active promoters transcribing away from the border (Guelen et al. 2008). The enrichment for CTCF is particularly interesting as it is postulated to bring sequence specificity to organize architectural proteins of the SMC family (cohesin and condensin) to define so-called Topologically Associated Domains (TADs; (Dixon et al. 2012; Jin et al. 2013; Fudenberg et al. 2016)) revealed by chromosome conformation capture methods that identify genomic regions that are proximal in space (reviewed in (Dixon et al. 2016)). Indeed, at least in some contexts (like the X-chromosome) it seems clear that LADs likely represent a subset of TADs, and therefore are defined by some of the same topological determinants (Nora et al. 2012).

Interestingly, actively transcribed genes can also be found in LADs, suggesting that peripheral tethering, per se, is not sufficient to inhibit transcription ((Wu and Yao 2013) and more on this below). However, as NPCs have been established to be linked to active transcription in a variety of model systems (see Chap. 3), an alternative possibility is that Dam-ID may not have sufficient resolution to distinguish the differential association of a gene promoter with NPCs rather than the lamina. Lastly, it is important to consider that most Dam-ID (and ChIP) experiments result in population-based metrics, which average out single-cell variability. To this point, the development of single-cell Dam-ID reveals that the majority of LADs are stochastically associated with the nuclear periphery (Kind et al. 2013), with only ~15% of LADs establishing more stable "backbone" interactions (Kind et al. 2015).

2.6 LADs as Developmentally Regulated Regions

The observation that many LADs display high variability within cell populations could be interpreted in two very different contexts. In the first, this observation could reflect a highly stochastic aspect of nuclear compartmentalization, suggesting that gene regulation may, in many cases, be independent of gene position. Indeed, recent studies in *Caenorhabditis elegans* showing that peripheral tethering of heterochromatin is not essential to maintain gene silencing, supports this point of view ((Gonzalez-Sandoval et al. 2015); more below). However, a compelling case can also be made for the second context, in which subnuclear compartmentalization leads to direct functional consequences. Indeed, a wealth of studies have documented that altered subnuclear localization of a given locus (or group of loci responsive to a given input) occurs concurrent with the execution of differentiation programs in cells and organisms; individual examples include the immunoglobulin heavy-chain (IgH)(Kosak et al. 2002; Reddy et al. 2008), β-globin (Ragoczy et al. 2006) and CFTR (Zink et al. 2004) loci. However, altered subnuclear compartmentalization is likely to be much more extensive in some contexts; indeed, genome-wide studies using Dam-ID reveal that two thirds of LADs dissociate from the nuclear periphery upon murine stem cell differentiation (Peric-Hupkes et al. 2010). Such changes can also be recapitulated using repetitive transgenes in multicellular models like *C. elegans*, which are released from the nuclear periphery in fully differentiated tissues (Meister et al. 2010). How the observed changes in subnuclear distribution mechanistically impact gene output in many of these cases still remains largely enigmatic. However, insights can be gleaned from the investigation of the *MyoD* locus, which is released from the nuclear periphery during myogenesis. In this case, regulatory transcription factors exhibited distinct steady-state distributions with respect to the nuclear periphery, which correlated with their occupancy on the *MyoD* promoter during differentiation (Yao et al. 2011). These data suggest that release from the periphery might promote encounters with distinct transcriptional environments.

It may well be that satisfying universal rules for how subnuclear position and transcription are related across the genome will never arise. For example, several landmark studies exploited conditional genomic tethering systems to directly test whether peripheral tethering was sufficient to infer transcriptional silencing (Kumaran and Spector 2008; Reddy et al. 2008; Finlan et al. 2008). While these studies generally support a model in which peripheral tethering leads to a down-regulation of transcription at genic regions surrounding the tether (Reddy et al. 2008; Finlan et al. 2008), it is clear that transgenes driven by high level promoters can be insensitive to repression at the nuclear periphery, as assessed by the recruitment of RNA Pol II and the kinetics of transcriptional activation (Kumaran and Spector 2008). Taken together, these results suggest that gene tethering at the nuclear periphery might be an initial step that provides a platform for the subsequent recruitment of other factors that ultimately confer silencing on specific genes with (potentially) specific promoters.

Consistent with the concept that peripheral tethering is simply a first step in a more elaborate gene inactivation program, conditional tethering of transgene loci resulted in the local recruitment and accumulation of integral INM proteins like LAP2, lamin B and Emerin (Kumaran and Spector 2008; Reddy et al. 2008). As these factors also bind to chromatin modifying enzymes like histone deacetylases (HDACs; (Somech et al. 2005)), it is easy to imagine a scenario in which transcriptionally active acetylated chromatin is locally deacetylated as a transition to a more silenced state. Consistent with such a model, inhibition of deacetylases led to the reversal of the tethering-induced transcriptional down-regulation (Finlan et al. 2008). And indeed, HDAC3 can target LADS (Zullo et al. 2012). Thus, silencing is likely determined by local chromatin structure; consistent with this, the introduction of local chromatin decondensation is sufficient to induce dissociation of genetic loci from the NE (Therizols et al. 2014).

2.7 Histone Modifications at the Nuclear Periphery

In addition to the nuclear periphery being relatively free of "active" histone marks, it is also rich in silent epigenetic signatures like histone 3 lysine 9 (H3K9) and H3K27 methylation (me; Fig. 2.1); H3K27 tri-methylation (me3) tends to be more enriched at LAD borders (Pickersgill et al. 2006; Guelen et al. 2008; Ikegami et al. 2010; Towbin et al. 2012; Kind et al. 2013; Bian et al. 2013), which are more variably associated with the nuclear periphery (so called variable "v" or facultative LADs; Fig. 2.1). As most heterochromatin is rich in H3K9/K27me, this result is not overly surprising. However, a more functional connection is suggested by the observation that the silent epigenetic signature is likely itself essential for the physical association of heterochromatin with the nuclear periphery. For example, while random insertion of the β-globin locus resulted in its targeting to the nuclear periphery, this peripheral association could be prevented by co-inhibition of the methylases required for both H3K9me2 and H3K9me3 (G9a and Suv39H1/2, respectively; (Bian et al. 2013)). Similar results were observed for specific vLAD-sequences (Harr et al. 2015). Indeed, the requirement for H3K9me is likely a conserved feature of genome-INM contacts in all Metazoa; for example, hundreds of tandem arrays of transgenes associate with the INM and accumulate H3K9me and H3K27me marks in *C. elegans*, which also provided a genetic platform to probe the requirements for INM association in a multicellular genetic model (Towbin et al. 2010).

Interestingly, while genetic screens in *C. elegans* identified dozens of factors that could de-repress transgene arrays, only the knockdown of two near-identical S-adenosyl methionine synthetases (SAMs) resulted in both array de-repression and de-localization from the nuclear periphery (Towbin et al. 2012), supporting a critical role for histone methylation as the key nexus of these two aspects: gene output and subnuclear compartmentalization. Consistent with the theme that transcription does not influence nuclear position relative to the periphery,

de-repression of the arrays was not sufficient, nor was it required for peripheral release. Interestingly, the effects of SAM inhibition could be recapitulated by the specific knockdown of two histone methylases, SET-25 (the homologue of mammalian G9a and SUV39h1/2) and MET-2 (homologue of SETDB1), which both target H3K9. Consistent with the idea that H3K9 was the essential histone modification that conferred INM tethering, only H3K9me2 and me3 were globally reduced in set-25/set-2 animals while H3K23, K27 or K36 were largely unaffected. Furthermore, a careful analysis of methyl marks after individual deletion of SET-2 and SET-25 supported a step-wise model, with SET-2 providing the H3K9mono and di-methyl substrate for SET-25. Interestingly, SET-25 itself is enriched at the nuclear periphery through (likely) indirect interactions with the H3K9me3 marks that it produces (Towbin et al. 2012), supporting a model of action in which local tri-methylation is amplified by a self-reinforcing cycle of SET-25 recruitment and catalysis.

2.8 Peripheral Tethers

The growing functional links between H3K9me and peripheral tethering supports the existence of INM proteins capable of mediating direct physical interactions with specific chromatin domains either through histone modifications, transcription factors and/or direct binding to DNA sequence elements (Fig. 2.1). While there is some evidence that lamin A/C might directly bind to DNA ((Kubben et al. 2012) and references therein), it is likely that much of the defective heterochromatin tethering to the nuclear periphery observed in lamin A/C-null cells is due to mislocalization of other integral INM proteins like the LEM proteins, which themselves might directly or indirectly (through effectors like BAF) interact with DNA (Brachner and Foisner 2011). Interestingly, the contribution of lamin A to heterochromatin tethering activity appears to be at least partially redundant with that of LBR (Solovei et al. 2013). This redundancy was elegantly illustrated in specialized murine retinal cells that possess an "inverted" nuclear architecture with heterochromatin concentrated at the center of the nucleus; this adaptation is thought to help focus light to improve night-vision (Solovei et al. 2009). Indeed, many nocturnal animals exhibit this change in global chromosome organization and these morphological changes are correlated with a repression of both lamin A and LBR expression in these cells. Moreover, the experimentally controlled up-regulation of LBR could mitigate these effects (Solovei et al. 2013) supporting a direct role for LBR as a peripheral tether. Indeed, in addition to binding to lamin B, LBR also directly interacts with the H3K9me3-binding protein HP-1 and has a Tudor domain that recognizes the silencing H4K20me2 modification (Hirano et al. 2012).

The redundancy between LBR and lamin A with respect to maintaining heterochromatin at the nuclear periphery could reflect the critical importance of this aspect of nuclear organization; redundancy is also reflected in the evolutionary expansion in the number of the LEM domain protein paralogues (and likely

others) (Brachner and Foisner 2011; Barton et al. 2015). Additional tethers have also been recently identified like proline-rich protein 14 (PRR14), a dynamic soluble protein that serves as a bridge between the lamins and HP-1(Robson et al. 2016), and there are also NETs that modulate peripheral anchoring in specific tissues (Zuleger et al. 2013). Dealing with this level of complexity (and integration) provides a challenge to cleanly defining a function for chromatin tethering to the nuclear periphery, making simpler genetic model systems essential to define the mechanistic paradigms. For example, in *C. elegans* a genetic screen to identify a specific H3K9me2/3-tether of an integrated repetitive transgene array identified the novel factor Cec-4. Cec-4 is not an integral INM protein but nonetheless specifically associates with the INM through a lamin-independent mechanism that remains to completely defined (Gonzalez-Sandoval et al. 2015) (Fig. 2.1). Consistent with its tethering activity, Cec-4 contains a chromodomain that specifically recognizes H3K9me3; mutation of this domain leads to a loss of the peripheral array position and, most importantly, globally affects chromosome tethering to the INM. Interestingly, however, in differentiated larva the effects of Cec-4 depletion on chromosome position were more muted, suggesting that there are likely multiple redundant tethers in differentiated cells of *C. elegans*, perhaps a reflection of a nuclear architecture that becomes more cemented to ensure the maintenance of cell fate. Consistent with this idea, depletion of Cec-4 (and thus peripheral tethering) did not maintain an artificially induced muscle-cell fate in embyros (Gonzalez-Sandoval et al. 2015).

2.9 Lamina Associated Sequences

In a scenario in which there are multiple INM tethers, some of which are tissue specific, one strategy might be to approach mechanisms of peripheral tethering intrinsic to the DNA sequence itself. While "DNA zip codes" that are sufficient to confer gene localization to the nuclear periphery have been long identified in unicellular models like yeasts (Ahmed et al. 2010), these sequences have been more challenging to identify in multicellular eukaryotes. Nonetheless, by focusing on the developmentally regulated regions of LADs that are variably associated with the lamina, some sequence elements sufficient to confer lamina association have been identified. These sequences are rich in GA dinucleotides (unlike most of LADs that are A/T rich) and have been termed lamina associated sequences (LASs) (Zullo et al. 2012; Harr et al. 2015). Interestingly, like in budding yeast, in which nuclear peripheral targeting of the DNA zip codes is conferred by direct binding to transcription factors (Brickner et al. 2012), the GAGA transcription factor cKROX (Zullo et al. 2012) and also "Ying Yang 1" (YY1) (Harr et al. 2015) were identified as key factors that could direct LASs to the nuclear periphery, perhaps (and very intriguingly) specifically through binding to lamin C (Harr et al. 2015). In addition, cKROX association with LASs persists throughout mitosis, suggesting that LAS binding by soluble elements of the transcription machinery

are likely early events that help re-establish nuclear organization as the NE reforms at mitotic exit (Zullo et al. 2012), thus providing compelling evidence for a concept proposed by Blobel 30 years ago (Blobel 1985).

2.10 Beyond Silencing: The Periphery and Genome Integrity

Given the putative myriad of (often redundant) chromatin tethers and the likely multifactorial nature of sequence elements and gene-specific binding proteins in tying gene position with gene output, defining explicit function(s) for anchoring of chromatin to the nuclear periphery will remain a persistent challenge. Moreover, even in systems like *C. elegans*, in which it is possible (at least in early development) to release heterochromatin from the periphery through deletion of Cec-4, this chromatin nonetheless remains silenced in the nuclear interior (because it retains H3K9me2/3 marks; (Gonzalez-Sandoval et al. 2015)). Perhaps most shockingly, however, is the finding that the abrogation of H3K9 methylation, which can be achieved in *C. elegans*, gives rise to completely viable animals, with only rare, mild phenotypic abnormalities (Towbin et al. 2012; Zeller et al. 2016). Thus, H3K9 methylated heterochromatin, much of which is associated with the nuclear periphery, is dispensable for the development of a multicellular organism. Interestingly, however, after a few generations the worms became completely sterile; this arises due to massive, p53-dependent apoptosis in the germline, suggesting persistent DNA damage (Zeller et al. 2016). Consistent with this, use of a reporter construct in somatic cells from these animals revealed an increase in insertion-deletion (indel) rates specifically within a heterochromatic chromatin environment. Taken together, these data suggested that H3K9me (and perhaps peripheral tethering) is perhaps most important for maintaining genome stability within silenced regions of the genome.

2.11 Repetitive DNA and the Nuclear Periphery

Looking back, the connection between repetitive DNA, the nuclear periphery, and genome stability first arose in pioneering work in the relatively "simple" unicellular yeast models. While budding yeast do not utilize the H3K9me modifications, they nonetheless compartmentalize repetitive regions of their genomes at the nuclear periphery, most notably the ribosomal DNA (rDNA) repeats housed in the peripheral nucleolar compartment (Taddei and Gasser 2012). Indeed, the concept that the INM could promote genome stability was first established in work examining the stability of rDNA repeats (Mekhail et al. 2008). rDNA repeats are tethered to the nuclear periphery by a complex of proteins called Chromosome Linkage Inner nuclear membrane Proteins (CLIP). The INM tether for this complex is the conserved integral INM protein Heh1/Src1 (a member of the LEM

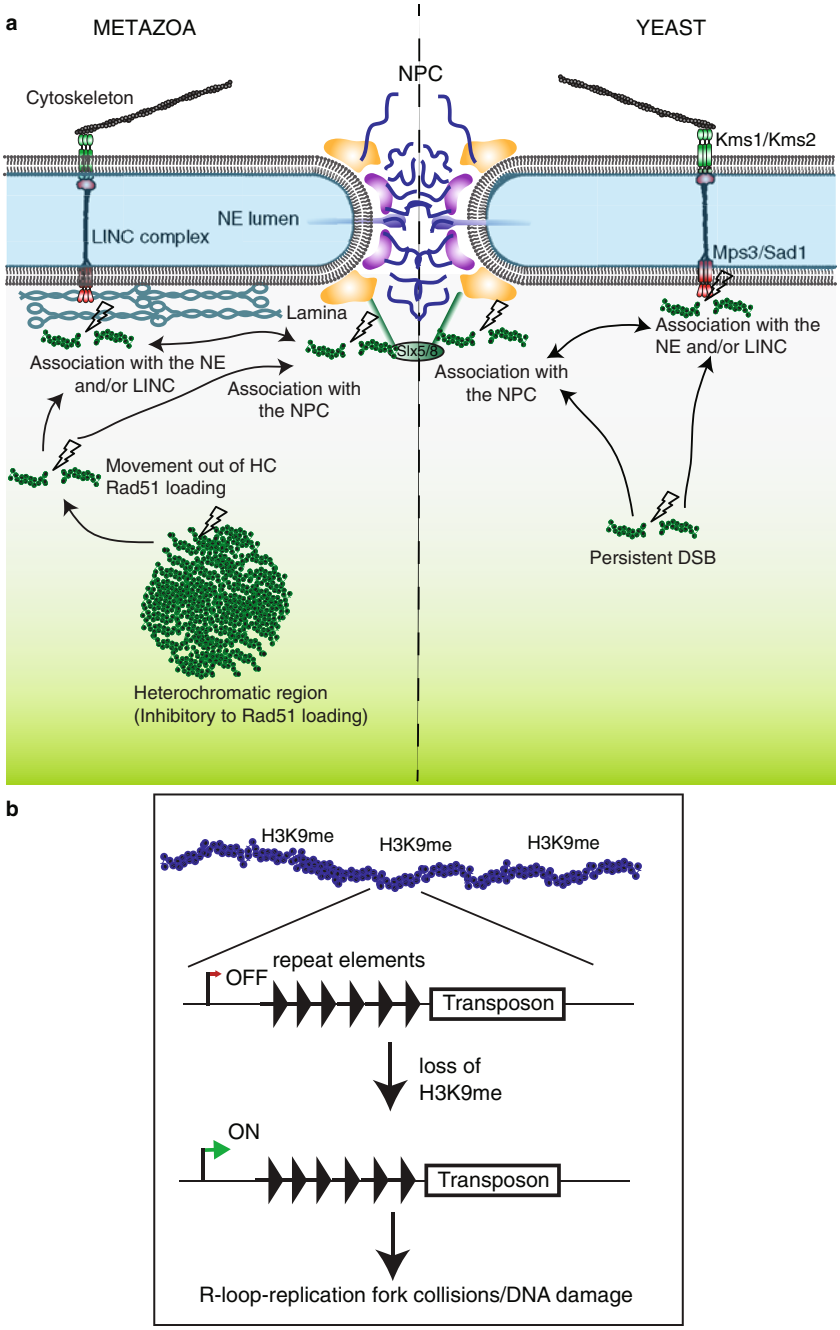

Fig. 2.3 Subnuclear compartments and epigenetic modifications influence genome integrity. (a) Studies in yeast first revealed that persistent DNA double-strand breaks (DSBs) that initially reside in the nuclear interior move to the nuclear periphery to associate with the nuclear pore complex (Continued)

domain family; (King et al. 2006)) and its yeast-specific binding partner Nur1 (Mekhail et al. 2008). Remarkably, deletion of Heh1 leads to a loss of nucleolar structure and an increase in copy number changes within the rDNA repeats, suggesting heightened homologous recombination, which could drive repeat expansion or contraction (Mekhail et al. 2008). Indeed, it was previously established that loading of recombination factors such as Rad52 onto a lesion within the rDNA occurs only once it moves out of the peripheral nucleolar compartment (Torres-Rosell et al. 2007), first suggesting the concept that nuclear compartments can influence DNA repair mechanisms. In addition, recent studies suggest that such regulation is conserved in higher eukaryotes, as the H3K9me-binding protein HP-1 inhibits Rad51 loading onto heterochromatic DNA double strand breaks (DSB) in *Drosophila* (Chiolo et al. 2011) (Fig. 2.3a) while tethering of a site-specific DSB to the nuclear periphery also abrogates loading of homologous recombination factors in human cells (Lemaître et al. 2014).

In addition to influencing repair mechanisms specifically in repetitive regions of the genome, there is ample evidence that the nuclear periphery influences both mechanisms that drive DNA DSBs and the pathways that repair such lesions (Seeber and Gasser 2016) (Fig. 2.3a). Returning to *C. elegans*, the DNA damage observed in the absence of H3K9me was traced to an increase in RNA-DNA hybrids or "R-loops," which can lead to collisions with replication forks to drive fork collapse (Zeller et al. 2016) (Fig. 2.3b). In this way, aberrant transcription could still be the root cause of the genome integrity defects in worms lacking H3K9me. Interestingly, an earlier study linked topological stress at highly transcribed genes associated with the NPC to DNA damage, which was suggested to be normally attenuated through phosphorylation events orchestrated by the Mec1/ATR pathway (Bermejo et al. 2011). In this context, controlled release of these genomic regions from the periphery preserves genome integrity, and could be avoided in the absence of this pathway through deletion of proteins necessary for NPC basket formation (Bermejo et al. 2011). The possibility for DNA damage driven by topological constraints imposed by chromatin-NE tethers remains to be fully characterized, but presents an important area of future research, particularly as several recent reports suggest site-specific DNA damage programs that play critical roles in development (Madabhushi et al. 2015) – a tantalizing clue that such mechanisms might be functionally important and not just unintended by-products of genome organization.

◀ (NPC) or the SUN proteins (Mps3/Sad1). In fission yeast, persistent DSBs that associate with the SUN protein Sad1 form LINC complexes that interact with cytoplasmic microtubules. More recently, studies in Metazoa reveal that irradiation-induced DSBs within internal heterochromatin (HC) do not load Rad51, while movement of these DSBs into the euchromatic environment facilitates Rad51 loading. These DSBs can also go on to associate with either LINC complexes or the NPC at the nuclear periphery. In both yeast and Metazoa, peripheral association is promoted by post-translational modification (SUMOylation) of repair factors. (b) Loss of H3K9me in *C. elegans* leads to derepression of transposable elements. The resulting transcript, in the form of an R-loop, leads to collisions with the replication machinery, driving formation of DSBs.

Considering that DNA damage might occur in specific regions of the genome, are there hotspots that become fragile in the absence of H3K9me? Here, again, the advantages of the *C. elegans* system come into play. While over half of the human genome consists of repetitive elements (REs: SINES, LINES, retrotransposons, TY elements, etc.), most of these cannot be uniquely mapped from next-generation sequencing data due to the lack of unique sequence features. By contrast, while the *C. elegans* genome houses many REs, 80% of these are uniquely mappable, allowing the authors to demonstrate that R-loops accumulate specifically within REs, concomitant with loss of H3K9me marks (Zeller et al. 2016) (Fig. 2.3b). Moreover, large indels in these regions, often adjacent to transposon sequences, suggests that aberrant transcription of transposons might drive the loss of genome stability. In this context, it is the act of transcription rather than the transposon up-regulation itself that drives genome instability, suggesting that transposons need not "jump" to drive losses in genome integrity. Importantly, loss of the H3K9me-binding protein HP-1 leads to loading of the recombination factor Rad51 inside heterochromatic domains in *Drosophila* (Chiolo et al. 2011), suggesting a possible mechanism by which abrogation of H3K9me compromises genome integrity due to illegitimate recombination.

2.12 Inputs of Nuclear Compartmentalization on DNA Repair Mechanisms

Beyond the ability of H3K9me/HP-1 to inhibit Rad51 loading, what else is known about how distinct nuclear compartments influence DNA repair mechanisms when a DNA lesion does occur? Pioneering studies in yeast over the past ten years have unearthed a great deal of insights into this question. While much of this work has been extensively reviewed elsewhere (Seeber and Gasser 2016), here it is important to highlight that both the NPC and LINC complexes (or their constituent parts, such as the factors associated with the NPC basket or the SUN protein Mps3 in budding yeast) have been shown to be repositories for persistent DNA DSBs, each with unique contexts and consequences ((Nagai et al. 2008; Kalocsay et al. 2009; Oza et al. 2009; Swartz et al. 2014; Horigome et al. 2014); Fig. 2.3a).

Combining systems to tag genomic loci with heterologous operator arrays recognized by fluorescent protein fusions of their cognate binding proteins (for example, lacO/lacI or tetO/tetR) with inducible, site-specific DSB induction systems has allowed investigators to monitor the compartmentalization of single DSBs within the nuclear volume; in haploid yeast, such DSBs are irreparable but are recruited to the nuclear periphery, where they colocalize with NPCs (Nagai et al. 2008). The nuclear aspect of the NPC is linked to a SUMO-targeted ubiquitin ligases or STUbL – the Slx5/Slx8 heterodimer; driving association of a DSB with the nup, Nup84, or Slx8 is sufficient to increase rates of homologous recombination through gene conversion, but also promotes errant repair mechanisms such as break-induced replication and alternative non-homologous end joining ((Nagai et al. 2008);

Fig. 2.3a). Extensive further work has revealed that SUMOylation through the ligases Siz2 and Mms21 occurs upstream of peripheral DSB recruitment and Slx5/8, although the key substrates remain enigmatic (Horigome et al. 2016).

Through a molecularly distinct pathway, persistent DSBs are recruited to the SUN protein Mps3 (in budding yeast (Kalocsay et al. 2009; Oza et al. 2009)) or Sad1 (in fission yeast (Swartz et al. 2014); Fig. 2.3a). This pathway is active specifically during S and/or G2, lies downstream of initial processing events that commit the DSB to repair by homologous recombination, and has specific requirements for the Ino80 chromatin remodeling complex and the histone variant H2AZ (Kalocsay et al. 2009; Horigome et al. 2014). Association with Mps3/Sad1 is thought to both inhibit (perhaps non-allelic) DSB repair or errant repair of deprotected telomeres by homologous recombination, and/or to promote repair from alternative homologous templates (Oza et al. 2009; Swartz et al. 2014; Horigome et al. 2014); this pathway may be related to the role that LINC complexes play in promoting proper homologous chromosome pairing in meiosis (Hiraoka and Dernburg 2009).

For some time, the broad conservation of the mechanistic roles for the nuclear periphery in DSB repair had been questioned, derived primarily from the irreparable nature of the DSB models used in many yeast studies and the relatively smaller nuclear volume compared to mammalian cells. However, several recent studies highlight that the same pathways first identified in yeast are active in multicellular eukaryotes. Indeed, the observation that Rad51 loading occurs only after irradiation-induced DNA lesions move out of heterochromatic compartments in *Drosophila* (Chiolo et al. 2011) mirrors the earlier work of Torres-Rossel, who made the same observation for Rad52 loading onto the rDNA (Torres-Rosell et al. 2007). Moreover, it was recently demonstrated that SUMO ligases are necessary for the movement of DSBs out of heterochromatic compartments (Ryu et al. 2015); a subset of these DSBs then move to the nuclear periphery to associate with the NPC and/or LINC complex components in a pathway dependent on STUbLs (Ryu et al. 2015), again similar to results observed in yeast (Horigome et al. 2016). One critical question that remains to be fully investigated is how the chromatin mobility necessary for a DSB to move from an internal heterochromatic compartment to the nuclear periphery is achieved, particularly given the observation that chromatin loci are highly constrained in mammalian cells, as suggested by the inability of genic loci to be effectively tethered to the NE without passage of cells through mitosis (Kumaran and Spector 2008; Reddy et al. 2008; Zullo et al. 2012; Kind et al. 2013).

Again, seminal work in yeast suggests that formation of a DSB significantly increases its mobility within the nucleus, concomitant with a global increase in chromatin mobility (Miné-Hattab and Rothstein 2012; Seeber et al. 2013); these mechanisms have both common and specific genetic requirements, but likely involve chromatin remodeling complexes (such as Ino80 in budding yeast); their conservation remains to be tested. Roles for the LINC complex and the cytoskeleton in mediating an increase in the mobility of DNA lesions also appear to be conserved, as cytoplasmic microtubules act to promote DSB (or critically short

telomere) mobility, supporting interhomologue or ectopic homologous recombination in fission yeast (Swartz et al. 2014) or deleterious end joining reactions in mammalian cells (Lottersberger et al. 2015) (Fig. 2.3a). The LINC complex may play additional roles in regulating repair mechanism choice by suppressing NHEJ at lesions caused by cisplatin treatment in *C. elegans* (Lawrence et al. 2016). Thus, increased mobility of DSBs may facilitate encounters that allow repair to occur, but whether this promotes faithful repair or reactions that drive genome stability may depend on the context (i.e. how many lesions there are in a single nucleus). One open question is whether the LINC complex is associated with DNA lesions (as in fission yeast (Swartz et al. 2014)) or acts "at a distance" (Lottersberger et al. 2015). A recent study suggests that the LINC complex may do both, including roles for regulating repair factor localization and/or function through the nucleoplasmic domain of SUN proteins (Lawrence et al. 2016). It is worth noting that roles for direct physical association of DNA lesions with the NPC or LINC complex could be particularly important in repetitive, heterochromatic regions, as genetic ablation of DSB-nuclear periphery interactions leads to fusions and aneuploidies specifically in regions of the genome rich in H3K9me (Ryu et al. 2015). Taken together, these observations provide an additional rationale for why repetitive, H3K9me regions of the genome are found associated with the nuclear periphery: to poise them for regulation of DNA repair through NPCs or the LINC complex without the need for dramatic chromatin mobility.

2.13 Outlook

The advent of new molecular approaches to investigate the interface between the nuclear periphery and the genome has reinforced the decades-old concept that the INM maintains a tight association with transcriptionally silent chromatin. Surprisingly, however, recent studies suggest that transcription per se has limited impact on this association. Indeed, our understanding of the functional impacts that chromatin tethering to the INM has on its emergent biology is currently in flux. This revolution is being driven by pioneering work in multiple model systems; an emerging theme is that the nuclear periphery plays a critical role in maintaining genome stability. As the number of repetitive elements, many of which are derived from transposons and retroviruses, have infiltrated our genome, it is perhaps not surprising that we have developed effective means to silence these factors (Gasser 2016). How the periphery contributes to silencing repetitive elements is just beginning to come to light, but what is most exciting is that their de-repression in the germline might be deliberate to promote adaptation to environmental stress (Gangaraju et al. 2011). In this context, the nuclear periphery may be most critical during differentiation and when cell fate decisions must be established and maintained. This ongoing reconceptualization of the functions that the nuclear periphery supports provides an essential foundation to further understand the ever growing list of genetic connections between defects in the nuclear lamina and disease.

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Chapter 3
Nuclear Pore Complexes: Fascinating Nucleocytoplasmic Checkpoints

Victor Shahin

Abstract Nuclear pore complexes (NPCs) are elaborate proteinaceous assemblies which span the nuclear envelope of eukaryotic cells at regular distances. They control all bidirectional transport between the cytosol and the nucleus in a highly selective manner thereby turning the nuclear envelope into a selective barrier. NPCs limit passive diffusion to an upper cut-off of ~ 40 kDa but enable selective transport of macromolecules as large as ribonucleoproteins and viral particles in a receptor-mediated manner. NPC selectivity is of profound physiological importance as it protects the DNA and guarantees that biochemical intracellular reactions are kept in separate organelles. The protein composition of NPCs may vary among the same cells and is subject to dynamic changes depending on the stage and metabolic demands throughout the cells lifecycle. The evidence is mounting that NPCs possess remarkable plasticity. The present review reflects on the versatility of NPCs and highlights its highly dynamic nature from multiple aspects. These include changes in NPC structure, composition and density during physiological and pathophysiological processes.

Keywords Nuclear pore complexes · nucleocytoplasmic transport · nuclear envelope · cell nucleus · atomic force microscopy

3.1 Introduction

The eukaryotic cell nucleus is surrounded by an outer and an inner lipid bilayer membrane, which face the cytoplasm and the nucleus, respectively (Fahrenkrog and Aebi 2003). Both membranes collectively comprise the nuclear envelope (Fig. 3.1). The outer membrane is continuous with the membrane of the rough endoplasmic reticulum and separated from the inner membrane by a fluid compartment, the perinuclear cisternal space (Mazzanti et al. 2001). However, both membranes are

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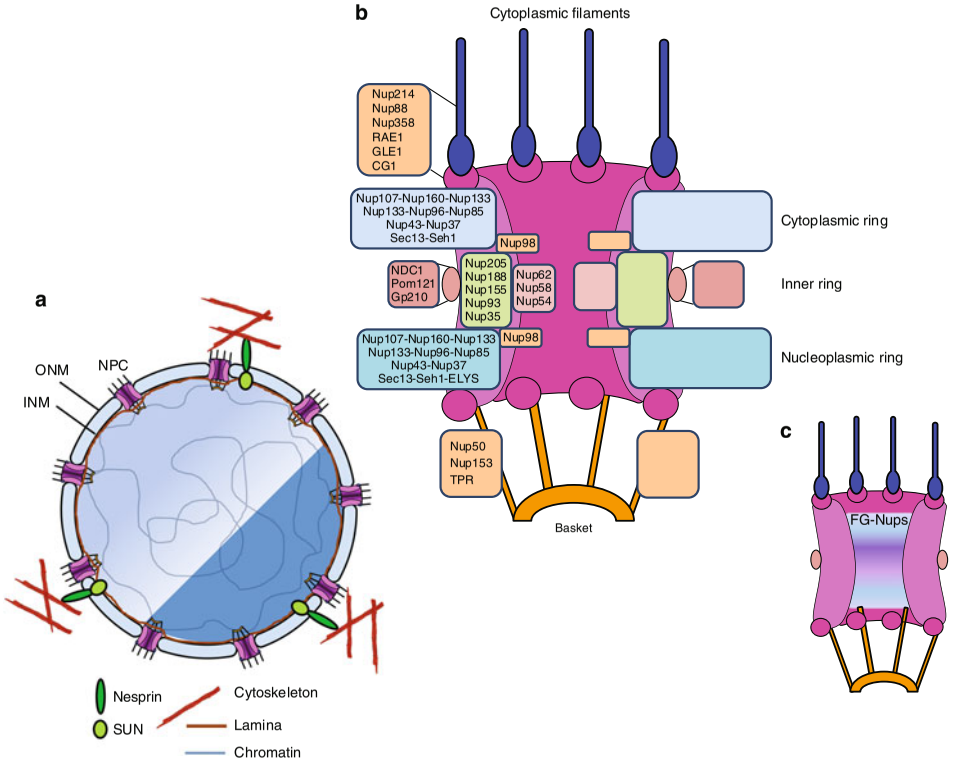

Fig. 3.1 Schematics of the cell nucleus and the nuclear pore complex (NPC). (a) Outer nuclear membrane (ONM) and inner nuclear membrane (INM) are regularly joined by NPCs, which span the nuclear envelope at regular distances. NPCs are connected to the nuclear lamina and chromatin from the nucleoplasmic side. Proteins including SUN and Nesprin amongst others connect the nuclear lamina to the cytoskeleton. (b) Cross-sectional schematic of the NPC displaying the rotational symmetry: central framework (cytoplasmic, inner and nucleoplasmic rings), eight cytoplasmic filaments and 8 nucleoplasmic filaments joined distally to form the NPC basket. Nucleoporins (Nups) are mostly arranged in defined subcomplexes. (c) Nups rich in phenyl-alanine (FG) repeats (FG-Nups) crowd the cavity of the NPC central channel

regularly joined to form gaps occupied by prominent structures termed nuclear pore complexes (NPCs) (Fahrenkrog and Aebi 2003). NPCs are virtually the sole transport pathways between the cytoplasm and the nucleus. Their transport selectivity safeguards the enclosed nuclear DNA and prevents undesired mixing between the two compartments (Conti and Izaurralde 2001; Izaurralde and Adam 1998; Shahin 2006b). The number of NPCs in eukaryotic cells may range from a few hundreds to as many as 40–50 millions depending on the species (Maul and Deaven 1977; Maul et al. 1980; Mazzanti et al. 2001). These elaborate assemblies are built from multiple copies of ~ 30 different proteins termed nucleoporins (Nups) (Knockenhauer and Schwartz 2016; Terry and Wente 2009). They are mostly joined in defined subcomplexes (Knockenhauer and Schwartz 2016) to form the eight-fold rotational cylindrical structure of the NPC (Hoelz et al. 2011). The aqueous central cavity of the NPC, designated NPC central channel (Fahrenkrog and Aebi 2003), is crowded with Nups containing domains rich in the amino acids phenylalanine-glycine (FG). These so-called FG-Nups possess peculiar biochemical and biophysical properties which impart transport selectivity to the NPC. FG-Nups act collectively to form the barrier but it remains a subject of a heated debate how the barrier is generated (Frey et al. 2006; Lim et al. 2006; Macara 2001; Peters 2005; Rout et al. 2003; Yamada et al. 2010). The versatility of NPC is reflected by their remarkable plasticity, heterogeneity, dynamics and diverse fundamental roles in addition to acting as selective gatekeepers between the cytoplasm and the nucleus (Adams and Wente 2013; D'Angelo and Hetzer 2008; Knockenhauer and Schwartz 2016; Maul 1977b; Shahin 2006b; Strambio-de-Castillia et al. 2010; Terry and Wente 2009). They can alter their structural configuration and composition in response to physiological stimuli or in order to help cells adapt to triggered changes in their surroundings (D'Angelo and Hetzer 2008; Maul and Deaven 1977; Maul et al. 1980; Perez-Terzic et al. 1996; Rabut et al. 2004b; Shahin et al. 2001; Shahin 2006a; Stoffler et al. 1999). Their density may change dynamically depending on the metabolic activities of cells, and they disassemble during mitosis and fully reassemble post-mitotically (D'Angelo and Hetzer 2008). Moreover, NPCs are critically engaged in gene expression (Raices and D'Angelo 2017; Strambio-de-Castillia et al. 2010). Several Nups have been shown to regulate tissue-specific and developmental functions through transcriptional modulation (Raices and D'Angelo 2012). NPCs constantly interact with diverse elements of the cytoplasm and the nucleus to regulate the cross-talk between the two compartments (Raices and D'Angelo 2012; Strambio-de-Castillia et al. 1999, 2010). For instance, they are connected to the cytoskeleton and the chromatin on the cytoplasmic and nucleoplasmic sides, respectively (Strambio-de-Castillia et al. 2010). They are also connected to the nuclear lamina (Aebi et al. 1986; Gruenbaum et al. 2000, 2003, 2005; Kramer et al. 2008), which underlies the surface of the inner nuclear membrane and plays key roles in mediating nucleocytoplasmic cross-talk (Strambio-de-Castillia et al. 2010). The bonds between integrins, focal adhesion proteins, the cytoskeleton, inner membrane proteins and the nuclear lamina enable signal transmission from the outside of the cell through the cytoskeleton to the cell nucleus where gene regulation is finally regulated (Wang et al. 2009), and NPCs are actively engaged in this

final process and the preceding steps (Raices and D'Angelo 2012, 2017; Strambio-de-Castillia et al. 2010). Therefore, it comes as no surprise that NPC malfunction or alteration of its composition is associated with diverse diseases including cancer and ageing amongst others (Capelson and Hetzer 2009; D'Angelo and Hetzer 2008; D'Angelo et al. 2009; Simon and Rout 2014). Furthermore, with respect to their central roles, NPC have become an attractive target for drug design (Gasiorowski and Dean 2003; Mor et al. 2014).

3.2 Versatility of the NPCs Structural Configuration

Despite significant evolutionary distances, the basic NPC structure is well conserved in eukaryotes from yeast to humans (Akey 1989; Allen et al. 2000; Bui et al. 2013; Degrasse et al. 2009; Hoelz et al. 2011; Kiseleva et al. 2004; Kramer et al. 2008; Pante and Aebi 1993; Sakiyama et al. 2016; Stoffler et al. 2003). It resembles an hourglass garnished with diverse elements which may vary among species (Field et al. 2014; Neumann et al. 2010). It starts off wide at the top, then gradually narrows to reach a minimum in the centre, before it widens again towards the bottom (Fahrenkrog and Aebi 2003). The complexity and elegance of the NPC assembly comes to light upon high resolution investigations (Alber et al. 2007; Beck et al. 2004; Brohawn et al. 2008; Hoelz et al. 2011; Kiseleva et al. 2004; Sakiyama et al. 2016; Schafer et al. 2002; Stoffler et al. 2003; von et al. 2015). NPCs were first discovered in 1949 in an electron-microscopy and they appeared as holes in the membranes (Callan et al. 1949). This discovery was followed by an impressive progress of our NPC understanding owing to diverse publications, comprehensively described in a timeline fashion in a recent review (Beck and Hurt 2017). The first 3D model of the NPC was proposed in 1992 (Hinshaw et al. 1992) and the first cryo-electron microscopy map followed on within a year (Akey and Radermacher 1993). The configuration of the NPC core scaffold was revealed in 3D reconstructions of both negatively stained (Hinshaw et al. 1992) and frozen-hydrated NPCs (Akey and Radermacher 1993) from *Xenopus laevis* oocytes after treatment of the nuclear envelopes with detergents. Tomographic 3D reconstruction of fully native NPCs embedded in thick amorphous ice unraveled further structural details of the NPC (Stoffler et al. 2003). The NPC core has an eight-fold rotational symmetry made up of three distinct rings stacked up on top of one another (Adams and Wente 2013; Fahrenkrog and Aebi 2003; Fernandez-Martinez et al. 2012; Hoelz et al. 2011; Knockenhauer and Schwartz 2016; Strambio-de-Castillia et al. 2010). Cytoplasmic and nucleoplasmic rings, which face the cytosoplasm and the nucleus, respectively, and a third ring, termed inner or central ring, sandwiched in between at the NPC midplane (Fahrenkrog and Aebi 2003). To lock in place between the convex curvature of the inner and outer nuclear membranes all NPC rings adopt a bent shape with a convex curvature along the outer and inner rings and a concave curvature in the centre. The outer NPC diameter is 80–120 nm as determined by atomic force microscopy (AFM) imaging (Fig. 3.2) and Cryo-electron tomographic

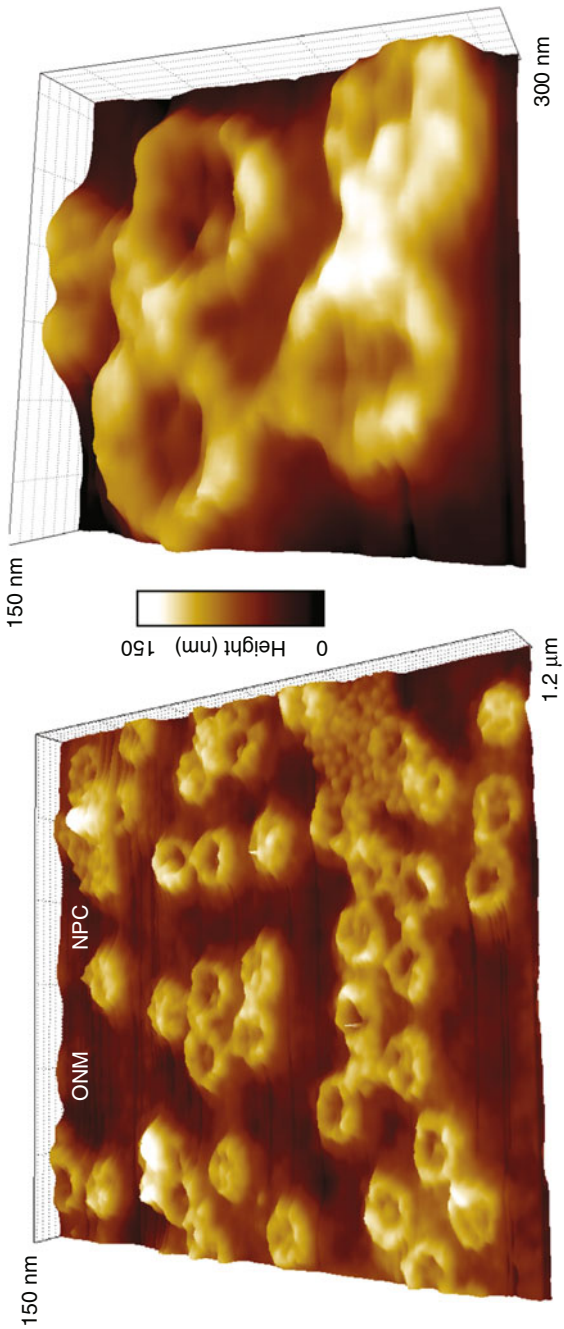

Fig. 3.2 Three-dimensional atomic force microscopy images of the cytoplasmic face of native nuclear envelope and NPCs from *Xenopus laevis* oocytes. The height is colour-coded. NPCs appear as ring-lie structures (yell) spanning the outer nuclear membrane (brown)

reconstruction (Bui et al. 2013; Eibauer et al. 2015; Liashkovich et al. 2012). The structural organisation of the NPC rings creates a tapered lumen, which is narrowest at the NPC midplane (Fahrenkrog and Aebi 2003). The lumen is referred to as the NPC central channel, through which transport of macromolecules proceeds (Fahrenkrog and Aebi 2003). The functional diameter of the lumen can only be estimated and is believed to approximate 40 nm at the NPC equator (Fahrenkrog and Aebi 2003). It is generally inferred from the determination of the upper cut-off for NPC transport in permeability experiments with diverse exogenous and endogenous cargoes (Feldherr and Akin 1997; Johnson et al. 2002; Kohler and Hurt 2007; Pante and Kann 2002). The central channel, when viewed in projection, was repeatedly observed to be plugged by some unidentified material which prompted the term central plug or transporter (Kiseleva et al. 1998). Size and shape of the central plug were highly variable when analyzed in 2D projection images of the NPC from negatively stained and frozen-hydrated nuclear envelopes preparations, and therefore, the identity and function of the plug remained long heatedly discussed (Fahrenkrog and Aebi 2003). The views varied from the plug being simply a cargo caught in transit to it being a functional transporter (Fahrenkrog and Aebi 2003). In 2D projection images it was interpreted to be composed of two co-axial tubes and two globular assemblies located symmetrically in the midplane of the NPC (Kiseleva et al. 1998). However, the lack of the z-dimension in 2D images kept the interpretation questionable. As a matter of fact, 3D reconstruction of the nucleoplasmic face of native NPCs revealed a prominent and large distal structure with a massive size of ~ 8 MDa (Stoffler et al. 2003). It protruded from the NPC scaffold toward the nucleoplasmic side and accounted for two-thirds of the mass which was referred to in 2D projections as the central plug (Stoffler et al. 2003). These 3D observations on native NPCs were in agreement with other observations, which showed that quick-freeze/freeze-dried NPCs visualised from the cytoplasmic side lacked the central plug, whereas, when visualised from the nucleoplasmic side, a prominent distal ring was observed (Jarnik and Aebi 1991). Hence, the central plug described in 2D projection images is rather a large cargo in transit than a transporter. This view receives support from an AFM study showing that the plug is most likely to be a massive nuclear cargo exiting the NPC channel (Schafer et al. 2002). An additional set of eight channels, termed peripheral channels, surround the central channel in the same rotational symmetry as the rest of the NPC and are believed to mediate the transport of small molecules (Fahrenkrog and Aebi 2003; Pante and Aebi 1994; Shahin et al. 2001). Eight filaments emanate from the cytoplasmic NPC ring and extend 50–100 nm into the cytosol (Adams and Wente 2013). They act as docking sites for cytosolic transport cargoes prior to translocation through the NPC (Fahrenkrog and Aebi 2003). Another eight filaments emerge from the nucleoplasmic NPC ring and are joined distally to form a structure termed NPC basket (Allen et al. 2000; Kramer et al. 2008). It is very likely that NPC basket accounted for the structure seen in the NPC channel in 2D projection images which described a central a plug in the NPC central channel (Fahrenkrog and Aebi 2003; Kiseleva et al. 1998). The basket acts as a docking site for nucleoplasmic cargoes intending to exit the nucleus through the NPC (Fahrenkrog and Aebi 2003). Estimates of the

dimensions of the NPC are ~ 40–90 nm core height, ~ 160–290 nm total height, 80–120 nm width, ~ 40 nm channel diameter at the NPC midplane (Adams and Wente 2013; Bui et al. 2013; Eibauer et al. 2015; Fahrenkrog and Aebi 2003; Maimon et al. 2012; Stoffler et al. 2003). They may vary significantly depending on both the species and the utilised NPC investigation approaches (Alber et al. 2007; Allen et al. 2000; Beck et al. 2004; Brohawn et al. 2008; Bui et al. 2013; Eibauer et al. 2015; Kiseleva et al. 1996, 2004; Kramer et al. 2008; Rout and Blobel 1993; Rout et al. 2000; Stoffler et al. 2003). Besides, the NPC dimensions are not static but dynamic (Fahrenkrog and Aebi 2003; Raices and D'Angelo 2012; Shahin 2006b). NPCs have been shown to dilate, contract and constrict under diverse physiological and pathophysiological conditions (Shahin 2006b). For instance, the NPC peripheral channels open up or close in presence or absence of ATP, respectively (Shahin et al. 2001). Depletion of Ca^{2+} stores in the nuclear envelope results in contraction of the NPC central channel while repletion fully restores the original size of the channel (Perez-Terzic et al. 1997). Similarly, the presence and absence of Ca^{2+} induces fully reversible opening and closing of the iris diaphragm-like distal ring topping the NPC basket, respectively (Huang et al. 2010). Exposure of NPCs to specific agonists of the calcium channels (inositol 1,4,5-trisphosphate and ryanodine receptors) which are located in the nuclear envelope, causes a displacement of the NPC central mass towards the nuclear side of the membrane (Erickson et al. 2004; Mooren et al. 2004). In a concentration-dependent manner resembling the alteration of the physiological glucocorticoid levels, NPCs dilate transiently to a remarkable extent (Kastrup et al. 2006; Shahin et al. 2005a, b). Investigation of genomic steroid hormone signaling across the nuclear envelope, using aldosterone as an example, reveals that NPCs dilate remarkably to enable the export of newly synthesized ribonucleoprotein (RNP) particles to the cytoplasm (Schafer et al. 2002). Moreover, export of an RNP particle through the NPC proceeds in sequential steps each paralleled by significant rearrangement of the NPC configuration (Kiseleva et al. 1998). Following RNP release the NPCs restore their original shape (Kiseleva et al. 1998; Schafer et al. 2002). RanGTP, a key player in selective nucleocytoplasmic transport across the NPC causes alterations of NPC structure, including compaction of the NPC and extension of the cytoplasmic filaments (Goldberg et al. 2000). Binding of transport receptors to the NPC channel increases NPC diameter (Jaggi et al. 2003). Not only physiological conditions mentioned so far but also pathophysiological conditions rearrange the NPC configuration transiently. For instances, during an infection with Herpes simplex virus type I (HSV-1), the incoming HSV-1 capsids must overcome a major physical barrier inside the NPC channel in order to deliver the viral genome into the nucleus, a key step in the virus lifecycle. The genome is 45 μm long and has to be translocated through a tight space of ~ 85 nm long NPC channel and the question is how (Liashkovich et al. 2011a). Viruses are well known to evolve ingenious strategies to overcome cellular barriers (Greber and Fassati 2003; Whittaker et al. 2000). In the case of HSV-1, the genome is so tightly packed and restrained inside a merely 125 nm large capsid such that it generates an enormous interior pressure, far greater than the intracellular pressure (Liashkovich et al. 2011a). The evidence is growing that HSV-1 capsids utilise the pressure-gradient to

literally shoot the capsid out through the NPC channel (Liashkovich et al. 2011a). AFM investigations show that NPCs dilate remarkably to translocate the viral genome (Shahin et al. 2006). Elevated levels of CO_2, for instance in hypercapnia, lead to a strong decrease in NPC height and diameter which is reversible upon CO_2 removal (Oberleithner et al. 2000). Casual alcohol consumption within or beyond tolerable levels (0.05–0.2%) causes NPC clustering which is reversed upon alcohol clearance (Schafer et al. 2007). Finally, artificial ways aimed at inflicting structural damage upon NPCs unravel just how flexible and resilient the NPCs are. In an AFM study a loading force was increased in incremental steps to compress the NPC basket with a sharp and hard AFM tip. The force was stepped up to an extent such that the basket was squeezed deep into the NPC central channel. Intriguingly, force removal immediately led to full recovery of the basket shape without any visible damage (Liashkovich et al. 2011b).

3.3 Composition, Heterogeneity and Dynamics of the NPCs

The NPC is a supramolecular proteinaceous assembly with a vast molecular weight which may vary strongly among certain species (Hoelz et al. 2011). Analytical ultracentrifugation on isolated intact NPCs of *Saccharomyces cerevisiae* estimates the molecular weight at ~ 66 MDa (Rout and Blobel 1993), whereas the molecular weight of the NPC in the vertebrate *X. laevis* is approximately ~ 112 MDa based scanning transmission electron microscopy analysis (Reichelt et al. 1990). The NPC is comprised of approximately 30 different proteins termed nucleoporins (Nups), which come in multiple copies of eight reflecting the NPC's eight-fold rotational symmetry. The total number of Nups in a fully assembled NPC is approximately 500–1000 depending on the species (Hoelz et al. 2011). Nups belong to the most divergent intracellular proteins (Knockenhauer and Schwartz 2016). Yet, consistently with the NPC structure, they are remarkably conserved among eukaryotes (Onischenko and Weis 2011) as demonstrated in different studies using several approaches including electron tomography, mass spectrometry, biochemical analysis and structural remodeling amongst others (Brohawn et al. 2008; Bui et al. 2013; Cronshaw et al. 2002; Degrasse and Devos 2010; Rout et al. 2000; Tamura et al. 2010; von et al. 2015). Nups are generally grouped in subcomplexes with specific functions, compositions, biochemical properties and positions in the NPC (Capelson and Hetzer 2009; Cronshaw et al. 2002; D'Angelo and Hetzer 2008; Denning et al. 2003; Denning and Rexach 2007; Frey et al. 2006; Hoelz et al. 2011; Knockenhauer and Schwartz 2016; Lim et al. 2006, 2007; Lord et al. 2015; Milles and Lemke 2014; Patel et al. 2007; Raices and D'Angelo 2012; Strambio-de-Castillia et al. 2010). Nups may be classified in four categories. (1) Structural Nups which determine the basic NPC shape and build a stable core scaffold embedded in the gaps between the nuclear membranes. About 15 out of the 30 different Nups are structured and they form complexes termed Y-complexes owing to their overall shape (Brohawn et al. 2008; Bui et al. 2013).

The Y-complexes, which are arranged in a head-to-tail manner, are generally assembled from ten Nups (Vertebrates: Nup160, Nup37, ELYS, Nup85, Nup43, Seh1, Nup96, Sec13, Nup107 and Nup133) and they are known to form the cytoplasmic and nucleoplasmic rings of the NPC (von et al. 2015). The inner ring of the NPC (Vertebrates: Nups93, Nup188, Nup205, Nup155 and Nup53) is the link to NPC barrier-forming Nups occupying the NPC central channel. For instance, positioning of the large barrier-forming Nup62 complex (Nup62, Nup584, Nup54) inside the NPC central channel is mediated by the inner ring Nup93 complex (Sachdev et al. 2012; Chug et al. 2015). Transmembrane Nups (Vertebrates: Pom 121, Gp210, NDC1) which anchor the NPC scaffold firmly to the nuclear membranes. They function as membrane fusion complexes and may be among the first Nups targeting the nuclear membranes post-mitotically to start off the membrane insertion and reassembly process of NPCs (Imamoto and Funakoshi 2012; Rothballer and Kutay 2013). (2) Flexible, brush-like Nups (Vertebrates: Nup98, Nup62, Nup54, Nup58, Nup153, Nup35, Nup214, Nup358, Nup 50, Pom121) with domains rich in clusters FG-repeats, FG-Nups (Terry and Wente 2009). These proteins impart transport selectivity to the NPC. (3) Linker proteins (Vertebrates: Nup88, Nup93) which connect the flexible FG-Nups to the core scaffold. The evidence is growing that NPCs dynamically change their molecular composition in order to adapt and respond to extra- and intracellular changes (D'Angelo and Hetzer 2008). Experiments in high resolution fluorescence microscopy studies performed at the single molecule level with fluorescently labelled Nups and transport factors underline the dynamics of Nups (Kubitscheck et al. 2005; Morchoisne-Bolhy et al. 2015; Rabut et al. 2004a). The dwell times of Nups in general varies from seconds to hours or days (Rabut et al. 2004a). The times seem to depend on the roles of Nups within and outside the NPC. Nups in the NPC core reveal low dynamical behaviour, consistent with their function as providers of stable structural scaffold. They reveal remarkable longevity approaching time scales from months to years which may outlast the life cycle of the cell (Raices and D'Angelo 2012; D'Angelo et al. 2009). Their exchange may not happen before mitosis and they may not even turn over in post-mitotic cells (Raices and D'Angelo 2012; D'Angelo et al. 2009). Peripheral Nups in contrast, exhibit much higher dynamical behaviour, which may be essential to enable their diverse regulatory functions (Rabut et al. 2004b). At least one third of Nups, predominantly FG-Nups from the NPC channel, exhibit dwell times ranging from merely few seconds to a few hours (Rabut et al. 2004b). Hence, NPCs are obviously capable of rapidly changing their molecular composition, probably in response to the physiological conditions they are exposed to. The NPC scaffold is generally assumed to be rigid in order to provide structural stability to the highly curved NPCs. On the other hand, the NPC scaffold undergoes dynamic changes throughout the cells lifecycle. It disassembles during mitosis and reassembles post-mitotically (Maul 1977a; Tran and Wente 2006; D'Angelo and Hetzer 2008; Fernandez-Martinez and Rout 2009; Glavy et al. 2007; Schooley et al. 2012; Imamoto and Funakoshi 2012; Rothballer and Kutay 2013; Rabut et al. 2004b). At early stages of mitosis the nuclear envelope is broken down and taken

up by the mitotic endoplasmic reticulum membrane network and the NPCs undergo disassembly. At later stages the nuclear envelope is rebuilt and the NPCs are inserted back into the nuclear membranes in sequential steps and the exact order of Nups insertion for NPC reassembly remains a subject of intense research (Ungricht and Kutay 2017; Fernandez-Martinez and Rout 2009; Rothballer and Kutay 2013; Dultz et al. 2008). Post-mitotic reassembly of all NPCs is intriguingly fast taking merely 10 minutes to complete (Dultz et al. 2008). At the same time, reassembly of the NPCs requires that the diverse Nups be targeted to their defined positions defying tight space constraints, which underlines the scaffold plasticity. Hence, once formed the scaffold may remain rather stable while it should retain considerable flexibility necessary for the post-mitotic NPC reassembly. Another aspect underlining the plasticity of NPCs is related to the dynamic changes in their density within the same nuclear envelope. The total number of NPCs in the nuclear envelope correlates with the rate of nucleocytoplasmic transport required to meet the metabolic demands of the cell at any given point in time throughout its life cycle. It may range from 200–400 to a 20000 (Mazzanti et al. 2001) in the nuclear envelope of a typical mammalian cell but can go all the way up to tens of millions in the oocytes of the amphibian *X. laevis* (Mazzanti et al. 2001). This number reflects the very high protein synthesis rates the oocytes need to set the stage for a rapid cell division after fertilisation (Schlune et al. 2006). Like the NPC density, the assembly of NPCs is also regulated by phosphorylation and dephosphorylation cycles (Weberruss and Antonin 2016). Besides, dynamic change in composition and structure, in yeast NPCs exhibit high mobility within the plane of the nuclear envelope. In certain mutant strains of yeast NPCs can move through the nuclear envelope membranes and form clusters. Whether the NPC mobility is due to the lack of a dense nuclear lamina, a loss of tethers on the nuclear side, or by a dissociation of certain Nups which may be responsible for keeping the NPCs in place, remains elusive (Bucci and Wente 1997). The presence of a dense nuclear lamina certainly contributes to keeping the NPCs in place. On the other hand, NPCs are presumably capable of moving laterally within the nuclear envelope plane despite the presence of a very dense nuclear lamina in stage-VI *X. laevis* oocytes (Aebi et al. 1986; Kramer et al. 2008). Injection of glucocorticoids into oocytes triggers gene expression which is paralleled by remodeling of the nuclear envelope structure and NPC clustering but it remains unclear how (Shahin et al. 2005b). NPCs are very actively engaged in gene expression regulation (D'Angelo and Hetzer 2006, 2008; Raices and D'Angelo 2017) and their non-random distribution in the nuclear envelope may reflect the periodic organization of the subjacent genome as suggested by Günther Blobel over three decades ago (Blobel 1985): NPCs may serve as gene-gating organelles capable of interacting specifically with expanded (transcribable) portions of the genome. Their nonrandom distribution in the nuclear envelope may reflect the underlying periodic organization of the genome into expanded and compacted domains, which alternate with each other. It is tempting to speculate that non-randomly distributed, transcribable chromatin domains would be subjacent to NPC clusters whereas repressive chromatin domains would be subjacent to nuclear envelope regions devoid of NPCs clusters.

3.4 NPCs as Fascinating Gatekeepers Between the Cytoplasm and the Nucleus

NPCs are virtually the sole transport pathways between the cytoplasm and the nucleus. An individual NPC can mediate both import and export of nucleocytoplasmic cargoes and it is speculated that one and the same NPC may be engaged simultaneously in both import and export (Feldherr 1998; Ludwig et al. 2006). Moreover, the NPC is capable of transporting cargoes with diverse biochemical and biophysical properties and remarkable sizes in a receptor-mediated manner upon cytoplasmic recognition of a nuclear localisation signal (NLS) displayed by the cargo. The size cut-off for NLS-mediated transport was initially assumed to be ~ 26 nm. It was based on microinjection experiments performed with *X. laevis* oocytes in a study published in 1988 (Dworetzky et al. 1988) (Fig. 3.3). Colloidal-gold particles ranging in diameter from 20 to 28 nm were coated with bovine serum albumin, which was cross-linked to a synthetic NLS rest, were injected into the cytoplasm. The number of NLSs conjugated to BSA was varied; 5, 8, and 11 signals per molecule of carrier protein. Analysis of the distribution and size of the tracers that reached the nucleus revealed that the number of signals per molecule affects both the relative uptake of particles and the functional size of the channels available for translocation. Particles up to the size of ~ 26 nm readily passed the NPCs and therefore the conclusion was drawn that the functional NPC channel diameter was ~ 26 nm (Dworetzky et al. 1988). A study which was published ~ 2.5 decades later raised the functional NPC diameter up to ~ 40 nm. It considered a fact which was left out earlier, namely that the NLS-bearing cargo is transported through the NPC in conjunction with specific import receptors, for instance the importin-α and importin-β heterodimer (Conti et al. 2006a; Izaurralde and Adam 1998). Hence, colloidal-gold particles were coated with NLS-bearing proteins coupled to importin-α-importin-β or hepatitis B virus core capsid of 32-36 nm diameter, and then microinjected in the cytoplasm of oocytes. Based on the observations made the functional NPC diameter was found out to be ~ 40 nm and was therefore concluded to match the physical NPC channel diameter (Fahrenkrog and Aebi 2003). This striking size encompasses cargoes as large and diverse as proteins, mRNAs, tRNAs, ribosomal subunits viruses and viral particles amongst others. The transport capacities of NPCs are equally striking considering the fact that an individual NPC can translocate an estimated total number of 1000 molecules per second (Kubitscheck et al. 2005; Ribbeck and Gorlich 2001; Terry and Wente 2009; Yang et al. 2004). The mass flow of proteins through NPC is also remarkable approximating 100 MDa per second (Ribbeck and Gorlich 2001). It is believed that small molecules are transported preferably through the peripheral channels of the NPC while macromolecules are transported through the central channel (Fahrenkrog and Aebi 2003; Mazzanti et al. 2001; Shahin et al. 2001). However, the NPC central channel is unlikely to provide a physical barrier to small molecules. Hence, it is conceivable that that the presence of peripheral channels may relieve the transport burden on the central channel. Alternatively,

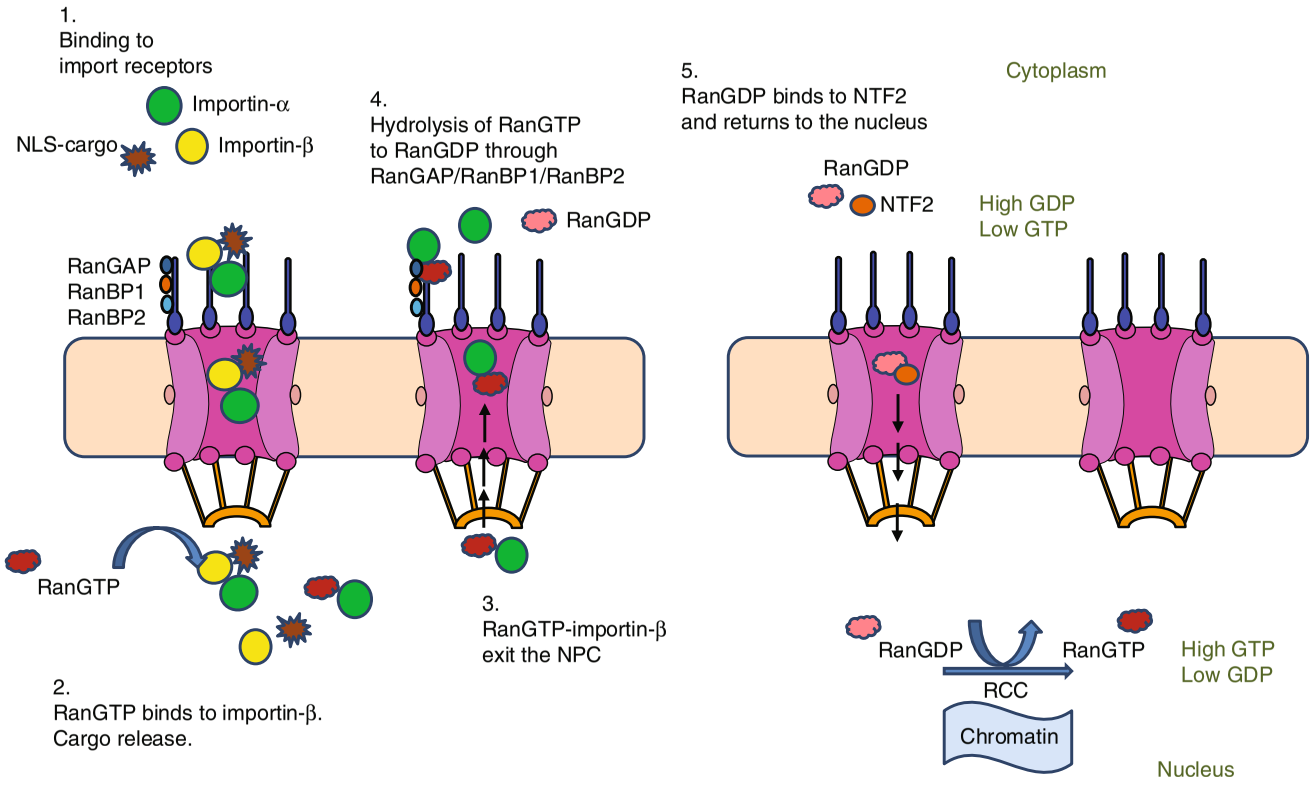

Fig. 3.3 Model of receptor-mediated transport through the NPC. The presence of RanGTP gradient powers the transport cycle. A protein bearing nuclear localisation signal (NLS) is recognised in the cytosol by the import receptor importin-α. Subsequent binding to importin-β targets the resulting cargo-receptor-complex to the NPC and mediates the translocation. Binding of RanGTP to importin-β dissociates importin-β and takes it back through the NPC to the cytoplasm. Hydrolysis of RanGTP to RanGDP at the NPC cytoplasmic filaments through the cooperation of RanGTPase-activating factor (RanGAP), RanBP1 and RanBP2 (parts of the cytoplasmic filaments), releases importin-β. RanGDP return to the nucleus in conjunction with nuclear transport factor 2 (NTF2). Ran guanine nucleotide exchange factor (RCC), which is bound to chromatin, induces the exchange of GDP with GTP on Ran

peripheral channels may act as gateways for specific small molecules. Cargoes up to the size of 40 kDa, equivalent to 5 nm can diffuse freely through NPCs while larger cargoes require selective transport in a receptor-mediated manner. Receptor-mediated nucleocytoplasmic works against a concentration gradient, and it facilitates the translocation of macromolecules with striking individual masses exceeding 1 MDa (Pante and Kann 2002). Selective cargoes display localisation signals which are recognised by specific transport receptors that shuttle between the cyto- and nucleoplasmic compartments (Gorlich and Kutay 1999). Several selective transport pathways are available depending on the types of the cargoes (Conti et al. 2006a). The best understood is the classical import pathway, which utilises the heterodimeric complex of the import receptors importins α and β to mediate the transport of NLS-bearing cytoplasmic protein to the nucleus (Conti et al. 2006a). The presence of this kind of pathway was first proposed in 1984 when a nuclear targeting signal was characterised in the simian virus 40 (SV40) (Kalderon et al. 1984b) which was composed of the amino acids sequence PKKKRKV (Kalderon et al. 1984a). Several NLSs have been identified since (Cook et al. 2007). In the importin-αβ pathway, the NLS-cargo is recognised specifically by importin-α which next binds to importin-β (Cook et al. 2007). The latter mediates both targeting of the resulting complex to the NPC and subsequent translocation through the NPC channel (Cook et al. 2007). Upon nuclear entry the complex must dissociate to release the cargo and return the transport receptors to the nucleus for the next import cycle (Cook et al. 2007). This multi-step process is based on the asymmetric spatial distribution of regulatory proteins of the small RanGTPases between the nucleus and the cytoplasm (Cook et al. 2007). The Ran guanine-nucleotide exchange factor RCC1 is generally bound to chromatin and is therefore located in the nucleus (Cook et al. 2007). In the nucleus, RCC1 exchanges GDP for GTP on Ran consequently rendering Ran predominantly bound to GTP (Cook et al. 2007). RanGTPase-activating protein (RanGAP), which triggers GTP hydrolysis to GDP on Ran, is located in the cytoplasm (Cook et al. 2007). Cytoplasmic Ran is therefore predominantly in its GDP-bound state (Conti and Izaurralde 2001). This asymmetric distribution of the Ran auxiliary proteins builds up a GTP gradient with much higher levels in the nucleus compared to the cytoplasm (Conti and Izaurralde 2001). It is the presence of this gradient that powers the selective nucleocytoplasmic transport and provides the essential directionality (Conti and Izaurralde 2001; Gorlich and Mattaj 1996; Gorlich and Kutay 1999). RanGTP has a high binding affinity to importin-β (Lee et al. 2005). Binding to importin-β in the cargo-receptor-complex upon nuclear entry dissociates importin-β from the complex (Conti and Izaurralde 2001). RanGTP bound to importin-β diffuses through the NPC channel to the cytoplasm (Conti and Izaurralde 2001). Next, RanGAP triggers GTP hydrolysis to GDP in cooperation with two RanGTP-binding proteins, RanBP1 and RanBP2, which form parts of the cytoplasmic NPC filaments (Fahrenkrog and Aebi 2003). Switching Ran from the GTP to the GDP bound state reduces its affinity to importin-β which dissociates and becomes accessible to the next import cycle (Fahrenkrog and Aebi 2003). Return of RanGDP to the nucleus through the NPC channel is mediated by

the nuclear transcription factor 2 (NTF2) (Conti and Izaurralde 2001; Conti et al. 2006b; Ribbeck et al. 1998). The translocation step through the NPC is not energy-dependent but the transport cycle as a whole requires the hydrolysis of 2 GTPs and is therefore energy-dependent and must be considered active. A similar cycle is utilised for selective export of cargoes out of the nucleus to the cytoplasm. Proteins intended to leave the nucleus in a selective fashion display a NLS which is recognised specifically by one of the exportins present the nucleus, such as CRM1 (Conti and Izaurralde 2001; Conti et al. 2006b). Together with RanGTP the formed cargo-complex is targeted to the NPC and exported to the cytoplasm where GTP is hydrolysed and dissociated from the complex in conjunction with CRM1 (Conti and Izaurralde 2001; Conti et al. 2006b). CRM1-RanGDP then diffuses back to the nucleus and RanGEF eventually replaces GDP with GTP rendering CRM1 free the next cycle (Conti and Izaurralde 2001; Conti et al. 2006b). This export mechanism applies to proteins while separate export mechanisms are utilised for RNAs depending on their classes.

3.5 Configuration and Functional Mechanisms of the Selective NPC Barrier: FG-Nups

NPCs prohibit passive diffusion of cargoes larger than 5 nm but permit massive cargoes as large as 40 nm at astonishing flow rate in a receptor-mediated manner. This special feature is bestowed upon NPCs by FG-Nups, which generate a versatile selective barrier. The NPC central channel is crowded with ~ 10 different FG-Nups (Adams and Wente 2013; Cronshaw et al. 2002; Denning and Rexach 2007; Strambio-de-Castillia et al. 2010) which create a microenvironment with peculiar biophysical and biochemical properties imparting a selective barrier function to the NPC for bidirectional nucleocytoplasmic transport. The versatility of the barrier requires that challenging demands are met. It must concurrently be tight enough for passive transport but remarkably flexible for selective transport. To promote the striking flow rate through the NPC the barrier must minimise both the steric hindrance and adhesion of receptor-cargo-complexes during translocation. To accomplish this challenging task FG-Nups must be able to interact with import and export cargo-receptor-complexes in certain biochemical and biophysical ways. While the fact that the NPC barrier is generated by FG-Nups is unequivocal, the exact structural configuration of the FG-Nups which enables their barrier function remains a subject of a heated debate. In the following several aspects will be mentioned which demonstrate just how challenging it is to elucidate the exact structural configuration of FG-Nups in native NPCs. The FG-repeats in FG-Nups are connected to each other through specific spacer sequences and the core repeat units may be classified in the following predominant groups: FG, FxFG (x = any amino acid) and GLFG (L = Leucine) (Terry and Wente 2009). Further classification has been suggested (Denning and Rexach 2007; Patel et al. 2007). The distribution of FG-Nups within the NPC is asymmetric. Besides, FG-repeat regions are not

identical but differ strongly from multiple biochemical and biophysical aspects such as the spacers they are connected to, charges, cohesiveness, flexibility and length (Alber et al. 2007; Denning and Rexach 2007; Field et al. 2014; Knockenhauer and Schwartz 2016; Labokha et al. 2013; Lim et al. 2006; Patel et al. 2007; Raices and D'Angelo 2012; Sakiyama et al. 2016). They also differ in their *N*-acetyl-glycosylation, which reduces the cohesiveness of the FG-repeat domains and thereby increases the transport rate of transport receptor–cargo complexes (Antonin 2013). High resolution imaging techniques fail to unravel the structural configuration of FG-Nups in native NPCs beyond doubt due to their short dwell times and highly disordered structure and dynamics within the NPC. Data from an AFM study carried out at the single molecule level on isolated FG domains demonstrated that they are natively unfolded (Lim et al. 2006) consistently with biochemical studies (Denning et al. 2003; Patel et al. 2007). The FG-motifs with up to 50 repeats in individual FG-Nups render them intrinsically disordered such that FG-Nups crowd the NPC channel and extend into the NPC periphery (Terry and Wente 2009). They occupy a dynamic range of topological positions (Chatel et al. 2012; Fahrenkrog et al. 2002; Sakiyama et al. 2016) and alter their positions in all directions at a striking velocity of milliseconds during transport (Sakiyama et al. 2016). Functional redundancy and divergence of the FG-domains add further to the complexity of elucidating the structural configuration of FG-Nups which enables them to generate the selective barrier. In genetically traceable budding yeast, transport defects were only observed when multiple specific FG-domains were deleted (Strawn et al. 2004; Terry and Wente 2007). This observation received further support from a study testing the effect of FG-Nups deletion on the NPC barrier in *S. cerevisiae* and showing that many FG-Nups genes were not essential to establish the barrier function (Doye and Hurt 1997). Redundancy was observed even within the same FG-domain (Sistla et al. 2007). The evidence is growing that different FG-Nups are required to mediate the transport of different cargoes, for instance mRNA export and protein import (Fiserova et al. 2010; Terry and Wente 2007). This prompts the suggestion that different transport receptors may interact with different FG-Nups and that different multiple transport routes exist within the NPC (Fiserova et al. 2010; Terry and Wente 2007). Several models have been postulated, to explain the transport selectivity through the NPC channel (Frey et al. 2006; Lim et al. 2006; Macara 2001; Peters 2005; Rout et al. 2003; Yamada et al. 2010) (Fig. 3.4). The "hydrogel model" is based on the assumption that cohesive FG-domains engage in weak and dynamic bonds to each other in multivalent ways (Frey and Gorlich 2007; Labokha et al. 2013; Ribbeck and Gorlich 2002). Following the formation of flexible bonds and owing to their assumed high concentration in the NPC channel they build a homogenous hydrogel (Frey et al. 2006). The hydrogel contains hydrophobic FG-domains embedded in otherwise hydrophilic environment. It fills the NPC channel cavity and acts as a sieve. The regular spacing between the FG-bonds is assumed to form a mesh (Frey et al. 2006). Cargoes may slip through the spacing without disrupting the FG-contacts if their size is below the mesh size. Hence, the spacing/mesh size of the hydrogel determines the upper cut-off for unhindered passive diffusion, and hydrophobic cargoes are favoured (Frey and Gorlich 2007).

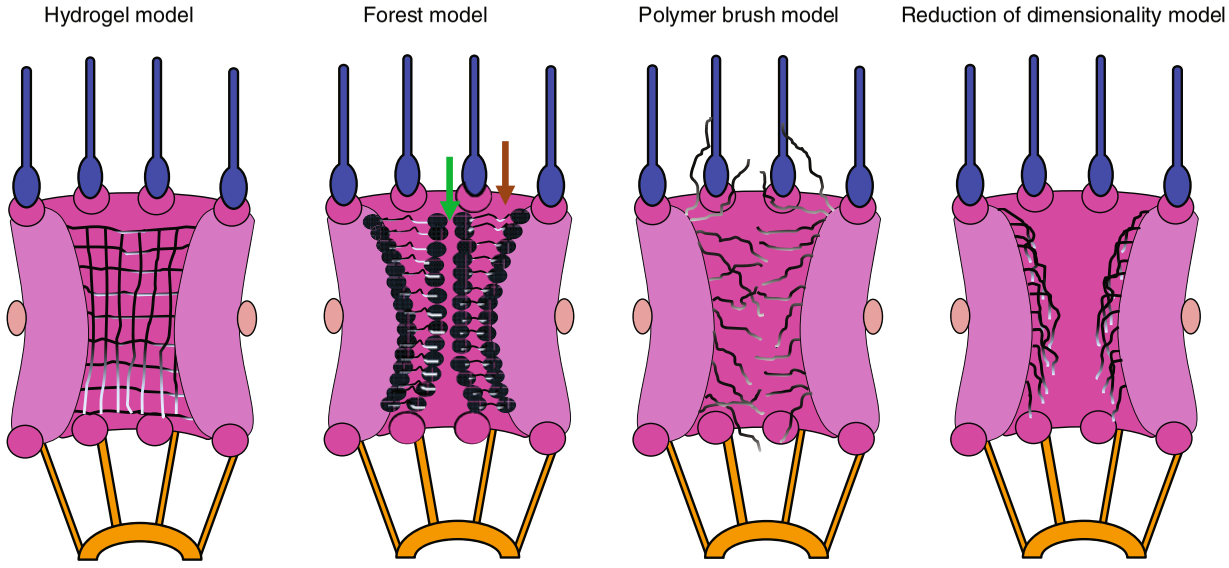

Fig. 3.4 Proposed models for the structural arrangement of FG-Nups that provides NPCs with transport selectivity. The "hydrogel model" presumes that FG-Nups bind to each other to form a homogenous, mesh-like hydrogel; hydrophobic FG-domains are embedded in hydrophilic environment. Cargo-receptor-complexes dissociate the FG-bonds transiently and glide through the hydrogel. The "forest model" proposes two distinct transport zones: hydrophobic zone in the centre (green arrow) for large cargoes and hydrophilic zone in the periphery (brown arrow) for smaller cargoes. The "polymer brush model" assumes that the highly disordered and dynamic FG-Nups create an entropic barrier preventing unselective cargoes from passage based on their size. Cargo-receptor-complexes bind to FG-Nups and reduce the barrier which eventually enables nuclear translocation. The "reduction of dimensionality model" suggests that FG-domains form a continuous surface, which provides binding sites for cargo-receptor-complexes and with FG-domains close to the wall. The resulting opening in the NPC centre acts as a physical barrier for passive transport of unselective cargoes, which will be excluded from passage unless their sizes are below the opening's diameter

This model refers to the ability of isolated FG-Nups domains to form a hydrogel in vitro, which has been shown to reconstruct the selectivity barrier of the native NPC. Import and export receptors possess hydrophobic binding pockets and are known to have intrinsic affinity to FG-Nups (Cook et al. 2007; Lee et al. 2005; Stewart 2003, 2007). The hydrogel model predicts that transport receptors compete transiently for bonds with FG-domains thereby transiently disrupting them. Thus, binding of cargo-receptor-complexes to FG-motifs would compete the FG-FG-bonds due to higher binding affinity. Consequently, the complex would "melt" the hydrogel locally and passes through (Hülsmann et al. 2012). Two studies using different approaches conclude that interactions between transport receptors and cargo are weak and transient, which prevents receptor-cargo-complexes from getting stuck in the NPC channel and promotes rapid translocation (Hough et al. 2015; Milles et al. 2015). The self-healing hydrogel is capable of rapid recovery (Frey and Gorlich 2009). This model is plausible and provides reasonable explanation for the barrier function but still has some drawbacks. For instance, in vitro investigations were performed on FG-repeat domains from single Nups, unlike the in vivo condition in which ~ 10 different types of FG-Nups interact in conjunction. Besides, it remains unclear whether such a hydrogel can be formed in vivo in native NPCs considering the harsh chemical conditions used in vitro to form the hydrogels. In contrast to the "hydrogel model," the "forest" model (Patel et al. 2007; Yamada et al. 2010) presumes that the barrier configuration is heterogeneous. It is based on the fact that some FG-Nups are cohesive while others are not and that some FG-Nups form tighter coiled domains than the others. The differential biochemical and biophysical behaviour of FG-Nups places them in different positions inside the NPC channel thereby creating two distinct zones: a hydrophobic one in the centre which mediates the transport of large cargoes such as ribonucleoproteins and a hydrophilic one in the periphery surrounding the central zone, for transport of smaller cargoes such as certain proteins (Patel et al. 2007; Yamada et al. 2010). This model receives some support from an AFM study on NPCs of *X. laevis* oocytes showing that the NPC centre is not equal but exhibits a central and a peripheral route: Comparison of the binding pattern of an importin β fragment, that binds specifically to FG-domains, with the binding pattern of wheat germ agglutinin that binds elsewhere in the NPC, reveals that FG-domains tend to cluster in the very centre of the NPC (Liashkovich et al. 2012). However, the barrier configuration in the AFM study was tested on fixed NPC which hampered their dynamics.

A recent AFM study tested the barrier configuration on native NPCs of *X. laevis* oocytes (Sakiyama et al. 2016) and the observations made there corroborated the third proposed model, termed entropic "barrier/polymer brush model" (Lim et al. 2006; Rout et al. 2003). This model is based on the fact that FG-Nups are intrinsically disordered and highly dynamic in agreement with previous studies on FG-Nups dwell times, biochemical and biophysical properties (Terry and Wente 2009). The lack of interaction between the FG-Nups and their free Brownian motion are proposed to account for the unfolded highly dynamic conformation. Consequently, the model states that FG-Nups tend to repel but not attract each other in opposite to the "hydrogel model." This resulting entropic barrier hampers

the passage of unselective cargoes. Selective cargoes, however, easily overcome this barrier; binding of receptor-cargo-complexes to FG-Nups collapses the barrier. Several AFM studies lend strong support to this model (Lim et al. 2006, 2007; Sakiyama et al. 2016). High-speed AFM imaging on native NPCs of *X. laevis* oocytes reveal that FG-Nups alter their lateral and vertical positions within the NPC in a highly dynamic manner and within milliseconds (Sakiyama et al. 2016). The high-resolution images obtained disagree with the morphological view of the "hydrogel model." Considering the fact that the AFM images reveal transient convergence of FG-Nups for a very short time in the very centre of the NPC channel, it is well conceivable that the apparent discrepancy between the AFM studies on *X. laevis* NPCs results from static (Liashkovich et al. 2012) vs dynamic (Sakiyama et al. 2016) conditions tested (Shahin 2016).The fourth model termed "reduction of dimensionality" (Peters 2005) presumes that FG-Nups generate a continuous surface along the wall of the NPC channel with FG-domains preferentially extending away from the wall and into the channel. It further assumes that the spacer sequences connected to the FG-domains and other Nups create a physical barrier excluding unselective cargoes based on the size (Peters 2009). Deletion of FG-Nups from the NPC channel should disrupt the barrier if this model is to hold. However, as discussed earlier deletion of several FG-Nups did not affect the barrier function (Strawn et al. 2004). Finally, while all models provide plausible explanations from distinct aspects none received general acceptance yet.

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Chapter 4
Nuclear Pore Complex in Genome Organization and Gene Expression in Yeast

Carlo Randise-Hinchliff and Jason H. Brickner

Abstract The nuclear pore complexes (NPCs) are large, evolutionarily conserved multiprotein channels embedded in the nuclear envelope of all eukaryotes cells. NPCs mediate macromolecular import and export from the nucleoplasm and cytoplasm by an active signal-dependent process. Recent research indicates that the NPCs play many additional roles in gene function and spatial organization of the genome. This chapter highlights our current understanding of NPC in genome-related functions in budding yeast. In yeast, Nups physically interact with a large number of highly expressed genes and active inducible genes. The repositioning of inducible genes to the NPCs leads to stronger expression and is regulated through multiple regulatory strategies including cell cycle regulated phosphorylation of Nup1. Many inactive or poised genes also interact with Nups. The interaction of recently repressed *GAL1* and *INO1* with the NPC is necessary for transcriptional memory. Retention at the NPC for these genes lead to an altered chromatin structure that primes them for rapid transcriptional reactivation. Thus, interactions with the NPC influences the spatial organization of the genome and impacts transcription.

Keywords Chromosomal spatial organization · nuclear pore complex · yeast nucleoporins · transcription control · interchromosomal clustering · transcriptional memory · cell cycle regulation · repositioning of inducible genes · regulation of gene recruitment and clustering · aging

4.1 Introduction

A membrane-bounded nucleus is a defining feature of all eukaryotic cells. The nucleus contains the majority of the genetic material in the cell and isolates nuclear from cytoplasmic functions. The nucleus is delimited by a double lipid

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bilayer membrane called the nuclear envelope (NE) and communication between the cytoplasm and nucleus is mediated by the nuclear pore complex (NPC). The NPC regulates the bidirectional exchange of macromolecules, export of specific RNA molecules, and selective transport of regulatory factors. Thus, the NPC is a critical mediator of cellular processes between the nucleus and the rest of the cell.

Within the nucleus, eukaryotic genomes are organized spatially and some nuclear functions are compartmentalized. Each chromosome occupies a distinct "territory" and can position it's chromatin into subnuclear compartments where loci can cluster with co-regulated regions or interact with stable nuclear structures (Sexton and Cavalli 2015). The spatial position of individual genes often reflects their transcriptional states (Pombo and Dillon 2015). In metazoans, chromosomes fold back onto themselves forming distinct non-overlapping globular territories (Cremer et al. 2006). Transcriptionally active regions tend to position at the edges of the territories in the inter-territorial space. Soluble factors such as transcriptional regulators and RNA polymerase II are non-uniformly distributed within the nucleus (Bartlett et al. 2006). The nucleolus, for example, is a subnuclear compartment that serves as the site for ribosome biogenesis (Boisvert et al. 2007). The nucleolus concentrates factors involved in rRNA production and ribosomal biogenesis (Andersen et al. 2005). Thus, both chromatin and soluble factors are spatially organized within the nucleus.

The organization of chromatin is also dynamic; developmental and physiological signals that alter gene expression also alter chromatin organization (Peric-Hupkes et al. 2010; Randise-Hinchliff et al. 2016). This suggests that the spatial organization of the genome within the nucleus contributes to gene regulation. However, the mechanisms and functional significance of the nuclear organization are not fully understood. What is clear is that stable nuclear structures bind to certain chromosomal regions, imparting organization and influencing transcriptional regulation (Meldi and Brickner 2011; Taddei and Gasser 2012). For example, in metazoans, the nuclear lamina, a filamentous network of lamins and lamin-associated proteins at the nuclear periphery, associates with large, transcriptional repressed regions of the genome (Luperchio et al. 2014). Because the nuclear lamina associates with chromatin modifying proteins and transcriptional repressors, it has been proposed that it is a transcriptional repressive environment (Gruenbaum and Foisner 2015).

However, the nuclear periphery is not exclusively associated with transcriptionally silent heterochromatin. Electron microscopy shows decondensed euchromatin positioned adjacent to NPCs (Belmont et al. 1993). In yeast, repressive regions and NPCs form distinct, non-overlapping foci (Taddei et al. 2004). This suggest that beyond its vital role in nucleo-cytoplasmic transport, the NPC may interact with active regions of the genome. Indeed, in yeast, flies, worms and mammalians, NPC components interact with hundreds to thousands of active genes (Brickner and Walter 2004; Casolari et al. 2004; Brown et al. 2008; Ahmed et al. 2010; Kalverda et al. 2010; Rohner et al. 2013). In yeast, these interactions occur at the nuclear periphery (Ahmed et al. 2010). However, in flies and mammals, such interactions can occur at both the NPC and with soluble nuclear pore proteins, in the nucleoplasm (Capelson et al. 2010; Kalverda et al. 2010). Interaction with

nuclear pore proteins promotes stronger transcription, alters chromatin structure and limits the spread of silencing (Ishii et al. 2002; Dilworth et al. 2005; Ahmed et al. 2010; Kalverda et al. 2010; Light et al. 2010, 2013; D'Urso et al. 2016). In yeast, interaction with the NPC can also lead to interchromosomal clustering of co-regulated genes (Brickner and Brickner 2012; Brickner et al. 2016; Randise-Hinchliff et al. 2016). Additionally, recently repressed genes bound at the NPC are poised for faster reactivation (Brickner et al. 2007; Brickner 2009; Tan-Wong et al. 2009; Light et al. 2010; Botstein and Fink 2011). Thus the NPC plays an important role in both the spatial organization of the nucleus and transcriptional regulation.

Here we review our current understanding of the mechanism and functional significance of the interaction of the NPC with the budding yeast genome. Research in yeast has provided significant conceptual and mechanistic insight into chromosomal organization and its effects on gene regulation. These discoveries have stimulated work in metazoan systems, which has revealed that these mechanisms are largely conserved.

4.2 Spatial Organization of the Yeast Genome

Budding yeast, *Saccharomyces cerevisiae*, has served as an outstanding model for understanding fundamental cell and molecular biology of eukaryotic cells (Taddei et al. 2010; Botstein and Fink 2011). However, budding yeast has several nuclear features that contribute to chromatin organization that are distinct from higher eukaryotes (Taddei et al. 2010; Zimmer and Fabre 2011). The primary difference is that budding yeast undergoes a closed mitosis; the NE does not break down during mitosis. During interphase, the centromeres of the 16 relatively small chromosomes (230–1,500 kb) remain tethered to the spindle pole body (SPB). The SPB, functionally analogous to the microtubule organizing center, is embedded in the NE and is positioned opposite the nucleolus (McBratney and Winey 2002). Chromosome arms emanate away from the SPB towards the opposite pole of the nucleus, where telomeres cluster as well. The 32 telomeres form a small number of foci at the NE by FISH, reflecting their inter-chromosomal clustering (Hediger et al. 2002). Since centromeres remain tethered through interphase, there is a strong determinant for the spatial position of chromosomal regions (Duan et al. 2010; Zimmer and Fabre 2011). In other words, short chromosome arms are unable to explore the same nuclear volume as longer arms. Consistent with this notion, telomeres of chromosomes having short arms (< 300 kb) cluster together near the SPB and telomeres of chromosomes having longer arms cluster together near the nucleolus (Duan et al. 2010). This organization is known as the Rabl configuration and is not specific to yeast. It was first observed by Carl Rabl in 1885 in epithelial salamander larvae and later in *Drosophila melanogaster* embryos and in many cereal species (Marshall et al. 1996; Parada and Misteli 2002). Despite yeast possessing unique features, the morphology and mechanisms that influence the spatial arrangement of yeast chromosomes have been important to understanding genomic organization in all eukaryotes.

4.3 Composition of NPC

The yeast NPC is one of largest and most complex proteinaceous assemblies in the cell, consisting of approximately 400 proteins with a mass of 66 million Daltons (Aitchison and Rout 2012). The NPC is composed of approximately 30 nucleoporins (Nups), each of which are present in multiple copies (usually 8 or 16), reflecting the eight-fold symmetry of the structure. Specific groups of Nups contribute to repetitive subcomplexes that form the NPC (Aitchison and Rout 2012). Based on structure, motifs, and locations, Nups can be classified into distinct groups (Fig. 4.1). Furthermore, many Nups bind dynamically to the NPC, cycling on or off or associating only during certain phases of the cell cycle (Dilworth et al. 2001; Makhnevych et al. 2003; Tran and Wente 2006). Thus, the exact number and definition of Nups is uncertain.

The NPC is a highly conserved structure and the majority of Nups have structural conservation that has been extrapolated to the last common eukaryotic ancestor (Brohawn et al. 2008; Neumann et al. 2010). However, due to a recent whole-genome duplication during *Saccharomyces* evolution, followed by gene divergence and loss, several Nups that are encoded by single genes in vertebrates exist as paralogous pairs in *S. cerevisiae* such as Nup116/Nup100 (Nup98 in vertebrates), Nup157/Nup170 (Nup155 in vertebrates), and Nup53/Nup59 (Nup3 in vertebrates; (Aitchison and Rout 2012). Also, the metazoan cytoplasmic filament Nups, Nup358 and Aladdin, are absent in yeast and the nucleoplasmic yeast Nup60 is absent in vertebrates (Wu et al. 1995; Cronshaw et al. 2002; Hoelz et al. 2011).

The yeast NPC, compared to the vertebrate NPC, is also both significantly smaller (66MDa compared to 125MDa) and less abundant in the NE (200 compared to 2,500–5,000) (Reichelt et al. 1990; Rout and Blobel 1993; Grossman et al. 2012). In metazoan organisms, NPCs are disassembled and reassembled

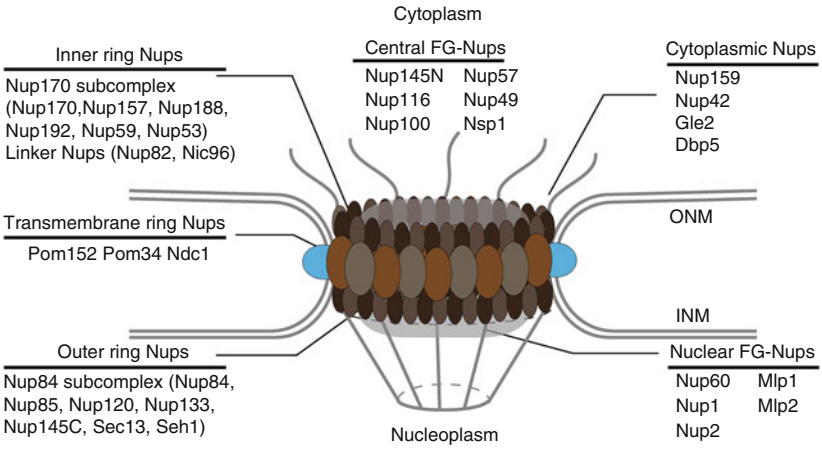

Fig. 4.1 Overall structure of the yeast nuclear pore complex (NPC). Nups are classified into distinct groups by structure and location

during mitosis while in yeast, due to a closed mitosis, the NPC remains assembled through the life cycle of the cell. Besides these differences, the core structure and function of the NPC is conserved between yeast and metazoans.

The cylindrical structure of the NPC is organized with eight-fold symmetry around a central transport channel and pseudo two fold symmetry between the cytoplasm and the nucleoplasm (Fig. 4.1) (Hoelz et al. 2011). The NPC is composed of two main functional regions; a central core and peripheral structures. The NPC core consists of coaxial inner, outer, and transmembrane rings surrounding a central channel, approximately 40 nm in diameter (Hoelz et al. 2011). The core is built from scaffold Nups (outer ring Nups, linker Nups and inner ring Nup), membrane-embedded ring Nups, and central FG-Nups. The core scaffold defines the shape and dimensions of the NPC (Kampmann and Blobel 2009). These Nups are structurally related to vesicular coat proteins and have been proposed to catalyze the formation of the sharply curved pore membrane (Devos et al. 2004). The pore membrane domain harbors three transmembrane proteins, Ndc1, Pom152 and Pom34, that interact with the core proteins and anchor the NPC within a pore in the NE. Finally, 11 Nups rich in phenylalanine-glycine (FG) repeats, are natively unstructured domains that form the permeability barrier of the NPC channel and serve as docking sites for transport receptors (Alber et al. 2007). The peripheral structures are made up of asymmetrical filaments that extend into either the cytoplasm and nucleoplasm. The cytoplasmic filaments are composed of Nup159, Nup42, Gle2 and Dbp5 and function in mRNP remodeling (Okamura et al. 2015). The nuclear basket forms the peripheral structure within the nucleus. It consists of filaments of FG Nups: Nup60, Nup1, Nup2, Mlp1, and Mlp2 (Hoelz et al. 2011). The nuclear basket functions in transport but an accumulating body of evidence also connects the nucleoplasmic basket to transcriptional regulation, modulating chromatin structure and organization of the genome.

4.4 Nuclear Pore Complex Interacts with the Genome

In addition to its role in regulating nucleo-cytoplasmic transport, the NPC also contributes to transcription and the spatial organization of the genome within the nucleus. Nuclear pore components directly interact with transcriptional regulators, mRNA export factors and chromatin (Table 4.1; (Steglich et al. 2013). The interactions with chromatin provide anchor points along the nuclear periphery to spatially organize and compartmentalize the genome. Using chromatin immunoprecipitation (ChIP) coupled to DNA microarray analysis (ChIP-chip), the interactions of Nups and NPC-associated factors were mapped genome-wide in yeast (Casolari et al. 2004, 2005). For a majority of the NPC components, genomic occupancy strongly correlated with transcriptional activity (Casolari et al. 2004). This included the nuclear basket components Nup2, Nup60, Mlp1 and Mlp2, the scaffold components Nic96 and Nup116, and the karyopherins Xpo1 and Cse1. These Nups also preferentially bound to genes involved in glycolysis and protein biosynthesis (Casolari

Table 4.1 Summary of nucleoporins in genome-related functions in yeast

Name	Location	Functions*
Nup 1	Nuclear basket	• Cell cycle phophorylation of Nup1 is required for periperhallocalization and interchromosomal clustering of *GAL*1 and *IN01* genes • Physically interacts with TREX-2 complex
Nup2	Nuclear basket	• Association with active genes • Required for peripheral localization of *GAL*1, *IN01* and tDNA genes • Required for peripheral localization of recently repressed *IN01* • Role in chromatin boundary activity • Physically interacts with H2A.Z as well as *IN01* and *GAL*1 gene promoters
Nup60	Nuclear basket	• Association with active genes • Required for peripheral localization and clustering of *GAL*1 and *IN01* genes • Required for peripheral localization of recently repressed *IN01*
Mlp1	Nuclear basket	• Association with active genes • Physically interacts with the SAGA complex and Ulp1 • Required for transcriptional memory of *GAL1* • Required for interchromosomal clustering of *GAL*1 gene
Mlp2	Nuclear basket	• Association with active genes • Physically interacts with Ulp1 • Required for peripheral localization of *GAL*1 and *IN01* genes
Nup100	Central FG-Nups	• Physically interacts with *GAL1* • Required for transcription memory and peripheral localization of recently repressed *IN01*
Nup116	Central FG-Nups	• Association with active genes • Physically interacts with *GAL1*
Nic96	Inner ring Nups	• Association with active genes
Nup170 subcomplex	Inner ring Nups	• Required in tethering and silencing of ribosomal and subtelomeric genes
Nup84 subcomplex	Outer ring Nups	• Required for peripheral localization of recently repressed *IN01*

*Refer to text for citations.

et al. 2004). Thus, certain active chromatin regions position and physically interact with the NPC (Table 4.1).

Interaction with Nups does not always correlate with transcription. The genomic occupancy of Nsp1, Nup84, Nup145 and Nup100 had no correlation with expression (Casolari et al. 2004). Thus, the NPC interacts with both active and inactive regions of the genome. The differences in the observed binding profiles for nuclear pore components may either reflect functional distinct molecular interactions with NPC or distinct NPC molecular composition. In support of the idea that different NPCs might be compositionally distinct, Mlp1, Mlp2, Ulp1 and Pml39 are associated with only a subset of NPCs (Zhao et al. 2004; Palancade et al. 2005).

Many inducible genes reposition from the nucleoplasm to the nuclear periphery and physically interact with the NPC in response to different environmental stimuli. For example, the *GAL* genes (*GAL1*, *GAL2*, *GAL7* and *GAL10*) in glucose are transcriptionally repressed and are localized in the nucleoplasm with sub-diffusive constrained movement (Casolari et al. 2004; Cabal et al. 2006). In contrast, in galactose, the *GAL* genes become transcriptionally induced and reposition to the nuclear periphery with more constrained diffusion (Cabal et al. 2006; Brickner 2007). At the nuclear periphery, *GAL* genes physically interact with Nup116, Mlp1, Nup60, Nup2, Cse1, XpoI and Nup100 (Casolari et al. 2004). This interaction depends on gene activity and the transcriptional activator Gal4 and occurs in the gene promoter (Schmid et al. 2006). In strains lacking Nup2, Nup1, Nup60 or Mlp2, *GAL1* remains nucleoplasmic in galactose media (Brickner et al. 2007; Brickner et al. 2016). Furthermore, the Gal genes are not the only region of the genome that is recruited to the NPC in galactose. When media is shifted to galactose, large scale rearrangements occur, repositioning many chromosomal regions to the nuclear periphery through multiple anchor points (Dultz et al. 2016).

Gene recruitment to the NPC has been observed in many environmental stimuli such as nutrient shifts (*INO1, HIS4, HXK1, SUC2*), osmotic stress (*CTT1*, *STL1*), heat shock (*TSA2*, *HSP104*) and mating pheromone treatment (*PRM1*, *FIG2, FUS1*; (Brickner and Walter 2004; Casolari et al. 2005; Dieppois et al. 2006; Taddei et al. 2006; Sarma et al. 2007; Ahmed et al. 2010; Regot et al. 2013; Guet et al. 2015; Randise-Hinchliff et al. 2016). The *INO1* gene (encoding inositol 1-phoshate synthase) repositions to the nuclear periphery upon activation during inositol starvation. The repositioning of *INO1* requires many Nups including Nup1, Nup2, Nup60, Nup157, Nup42, Gle2, and Mlp2 (Ahmed et al. 2010). Interaction of *INO1* and *GAL1* promotes stronger transcription by increasing the fraction of cells that respond to the inducing signal (Brickner et al. 2007; Texari et al. 2013; Brickner et al. 2016).

The interaction of the genome with the NPC is regulated through the cell cycle. Active genes such as *GAL1*, *INO1* and *HSP104* relocalize from the nuclear periphery to the nucleoplasm during S-phase (Brickner and Brickner 2010, 2012). This regulation of peripheral localization is due to oscillating Cdk-mediated phosphorylation of Nup1. Targeting of these genes to the NPC requires Cdk activity and either of two Cdk phosphorylation sites on Nup1. However, substitution of phosphomimetic aspartates in place of the phosphoacceptor residues at either position leads to localization at the periphery throughout the cell cycle and bypasses the requirement for Cdk activity (Brickner and Brickner 2010). Likewise, although tDNA genes encoding tRNAs are generally clustered in the nucleolus, during M phase, they reposition to the NPC (Chen and Gartenberg 2014). This coincides with the peak of tDNA expression. Loss of either Nup60 and Nup2 blocks recruitment to the NPC and leads to reduced transcription of tDNA genes during M-phase (Chen and Gartenberg 2014). Thus, in response to different environmental stimuli or cell cycle signals regions of the genome reposition to the NPC, enhancing transcription.

NPC-DNA interactions also play an important role in chronological aging in yeast, the process by which cells cease to divide after producing a fixed number of

daughter cells (Sinclair and Guarente 1997; Denoth-Lippuner et al. 2014). Aging is asymmetrically inherited; each generation the mother ages, but the daughter cell is born with full longevity (Sinclair and Guarente 1997). Extrachromosomal rDNA circles (ERCs) form spontaneously by homologous recombination within the rDNA locus and accumulate in older cells (Sinclair and Guarente 1997) and these ERCs have been proposed to serve as aging factors for several reasons (Denoth Lippuner et al. 2014). ERCs are asymmetrically inherited, accumulating and being retained in the mother cells. Artificially introducing ERC in daughter cells, or enhancing ERC formation in mother cells, shortens longevity (Sinclair and Guarente 1997). Conversely, reducing the rate of ERC formation increases lifespan (Defossez et al. 1999). Attachment of ERCs to NPC confine the DNA circles to the mother cell and preventing their inheritance (Denoth-Lippuner et al. 2014). Likewise, ERC association affects NPC inheritance to the daughter: ERC-bound NPCs are concentrated as an "NPC cap" in the mother cell and are retained, whereas unbound NPCs freely move into the daughter cell. The mechanism for this retention is not completely understood, however the SAGA complex is involved. Loss of SAGA complex components, such as Gcn5 and Spt3, cause DNA circles to dissociate from the NPC, spread into the daughter cells and lead to shorter lifespan (Denoth-Lippuner et al. 2014).

The NPC interacts with both active and repressed regions of the yeast genome, influencing its spatial organization, transcription and chronological aging. The role for Nups in regulating transcription may be evolutionarily conserved. In flies, mice and humans, expression of certain genes is enhanced by interaction with Nups (Brickner and Walter 2004; Casolari et al. 2004; Brown et al. 2008; Ahmed et al. 2010; Kalverda et al. 2010; Rohner et al. 2013). However, many inactive or poised genes also interact with Nups, so interaction with Nups or NPCs does not always correlate with transcription (Casolari et al. 2004; Brickner et al. 2007; Light et al. 2013). Below we discuss our current understanding of the impact of the NPC on transcriptional regulation, the molecular mechanisms that target genes to the NPC, how the interaction with the NPC leads to interchromosomal interactions and the role of the NPC in promoting epigenetic transcriptional memory in budding yeast.

4.5 Nups Influence Transcription

In 1985, Günter Blobel put forth an attractive "gene gating hypothesis," postulating that the interactions of active genes with NPCs might coordinate transcription with mRNA biogenesis and export out of the nucleus to limit mRNA diffusion rates (Blobel 1985). Indeed, interaction with NPC promotes stronger expression for inducible genes such as *INO1* and *GAL1* (Brickner et al. 2007; Ahmed et al. 2010; Brickner et al. 2016). Single molecule mRNA FISH suggests that this is due to an increase in the fraction of cells that induce these genes, rather than an increase in the amount of mRNA produced per transcription event (Brickner et al. 2016). It remains unclear if mRNA export is affected by this interaction. Promoter mutations that

block interaction of genes with the yeast NPC do not lead to nuclear accumulation of those mRNAs (Ahmed et al. 2010; Brickner et al. 2016). The yeast nucleus is small and mRNA export is rapid (Smith et al. 2015). Live cell imaging of mRNAs does not support the model in which mRNAs are directed to particular NPCs (Smith et al. 2015). Thus, although the transcription of genes is impacted by the interaction with the NPC, it is still unclear if post-transcriptional events are affected.

NPCs may anchor and concentrate transcriptional regulators to promote expression, functioning as a transcriptionally active subnuclear compartment. Consistent with this notion, the kinetics of *GAL1* expression is enhanced by Ulp1 anchored at the NPC (Texari et al. 2013). Ulp1 is a SUMO protease that is maintained at the NPC by association with Mlp1 and Mlp2 (Zhao et al. 2004). Ulp1 enhances the rate of *GAL1* mRNA production by catalyzing the desumoylation and attenuation of two repressors, Tup1 and Ssn6 (Texari et al. 2013). Furthermore, many transcriptional activators and mRNA export factors bind directly to the NPC. For example, the multiprotein complex TREX-2, which is necessary for mRNA export, interacts with Nup1 and localizes to inner nuclear basket of the NPC (Fischer et al. 2002; Kohler and Hurt 2007). The SAGA complex, a transcriptional co-activator, is linked to TREX-2 through a common component, Sus1, and binds to the NPC directly through Mlp1 (Rodriguez-Navarro et al. 2004; Luthra et al. 2007). Finally, the Mediator complex, another transcriptional coactivator, also binds to TREX-2 (Schneider et al. 2015). Therefore, interaction of transcriptional regulators with the NPC might enhance expression of active genes at the NPC.

NPC components may also promote transcriptional repression. Loss of members of the Nup84 subcomplex (Nup84, Nup120, Nup133, and Nup145) detaches telomeres from the nuclear periphery and leads to loss of silencing of subtelomeric reporter gene (Therizols et al. 2006). Likewise, the Nup84 subcomplex participates in glucose-responsive repression of *SUC2* by physically interacting with Mig1 (Sarma et al. 2011). Finally, Nup170 is required for peripheral tethering and silencing of many ribosomal and subtelomeric genes through cooperation with chromatin remodeler RSC and Sir4 (Van de Vosse et al. 2013). These findings suggest NPC components can influence silencing.

One complication in understanding the effects of gene-NPC interactions on transcription is that null mutations can disrupt the spatial organization that is normally being exploited in a wild type cell. For example, the Ulp1 SUMO protease is maintained at the NPC by Mlp1 and Mlp2 and is normally important for promoting *GAL1* derepression (Texari et al. 2013). However, mutants lacking NPC basket components both block targeting of *GAL1* to the nuclear periphery and release Ulp1 into the nucleoplasm. This results in more rapid *GAL1* depression, which has been interpreted as a role for the NPC in negatively regulating *GAL1* (Green et al. 2012). However, in a strain lacking Mlp1 and Mlp2, normal regulation of *GAL1* is restored when Ulp1 is artificially anchored to the NPC (Texari et al. 2013). Thus, interpreting the effects of null mutations of NPC components can be complicated by the change in the spatial organization of NPC-associated factors. For that reason, mutations in *cis*-acting DNA elements that perturb the positioning of a gene in an otherwise normal nucleus can provide important

information about the function of NPC interactions (Ahmed et al. 2010; Light et al. 2010; Brickner et al. 2012, 2016). One caveat to this statement is that, in cases where the *cis*-acting DNA elements that control gene positioning are the same as the elements that control transcription, the effects of interaction with the NPC on gene expression have not been distinguishable from the effects on targeting (Randise-Hinchliff and Brickner 2016).

Finally, another function of the interaction of NPCs with chromatin may be to alter chromatin structure to insulate active and silent regions. Studies using a "boundary trap" identified several NPC components capable of inducing boundary activity (Ishii et al. 2002; Dilworth et al. 2005). A boundary factor blocks the spread of heterochromatin without inducing transcription. Tethering of the nuclear pore protein Nup2, Exportins Cse1, Mex67 and Los1 and the RAN GEF Prp20 beside a reporter gene prevented the spread of silencing from the *HML* locus without activating an adjacent gene (Ishii et al. 2002; Dilworth et al. 2005). Endogenous Nups may also possess boundary active. Loss of endogenous Nup2 alleviates telomeric repression (Dilworth et al. 2005). Also Nup2 physically interacts with chromatin-modifying proteins and histone variant H2A.Z and binds to intergenic regions near telomeres (Dilworth et al. 2005).

4.6 Mechanisms of Gene Recruitment

The molecular mechanisms underlying gene recruitment to the nuclear periphery and interactions with NPC are not completely understood. Consistent with the gene gating hypothesis, factors involved in early transcription and mRNA export are required for recruitment of genes to the NPC. For example, peripheral localization of *INO1* requires components of both SAGA (Gcn5, Spt7 or Spt20) and TREX-2 (Sac3, Thp1, Sus1; (Ahmed et al. 2010). Likewise, recruitment of *GAL* genes to the NPC is blocked in strains lacking components of SAGA, Mediator (Med31, Cdk8), TREX-2 and the mRNA export receptor Mex67 (Luthra et al. 2007; Schneider et al. 2015; Brickner et al. 2016). SAGA and Mediator complexes mediate two complementary pathways for transcriptional activation (Bhaumik 2011). Mediator stabilizes the transcription factor TFIID, which is involved in general housekeeping genes, whereas SAGA-dependent genes are involved in environmental stress responses. It is conceivable that TREX-2 and Mex67 are recruited to active genes, acting as a bridge that anchors genes to NPC by interacting with components of the SAGA or mediator complexes bound to the genes. However, several observations are not consistent with this model. For example, recruitment of both *INO1* and *GAL1* to the nuclear periphery occur independent of either the transcriptional activator or RNA polymerase II, suggesting that transcription is not required for repositioning to the NPC (Schmid et al. 2006; Brickner et al. 2007, 2016). Thus, although the requirement for these factors is clear, the interpretation of their role is not.

Gene recruitment to the NPC is controlled by *cis*-acting elements in promoters of these genes (Randise-Hinchliff and Brickner 2016). For example, recruitment

of *INO1* to the nuclear periphery is controlled by two DNA Gene Recruitment Sequences called GRSI and GRSII. GRSI and GRSII redundantly control targeting of *INO1* to the NPC and a mutation that disrupts both elements blocks *INO1* recruitment to the nuclear periphery (Ahmed et al. 2010). When inserted at an ectopic locus that is normally nucleoplasmic (*URA3*), each GRS is sufficient to promote recruitment to the nuclear periphery. Thus, these GRS elements function as *DNA zip* codes; being both necessary and sufficient to control interactions with the NPC and contribute to the spatial organization of the genome.

GRS elements are binding sites for transcription factors (TFs; (Brickner et al. 2012; Brickner and Brickner 2012; Randise-Hinchliff et al. 2016). The TFs Put3 and Cbf1 bind to GRSI and GRSII, respectively, and are both necessary for *INO1* gene recruitment (Fig. 4.2a; (Randise-Hinchliff et al. 2016). Interestingly, neither Put3 or Cbf1 control *INO1* transcription. *INO1* expression is regulated by the Ino2

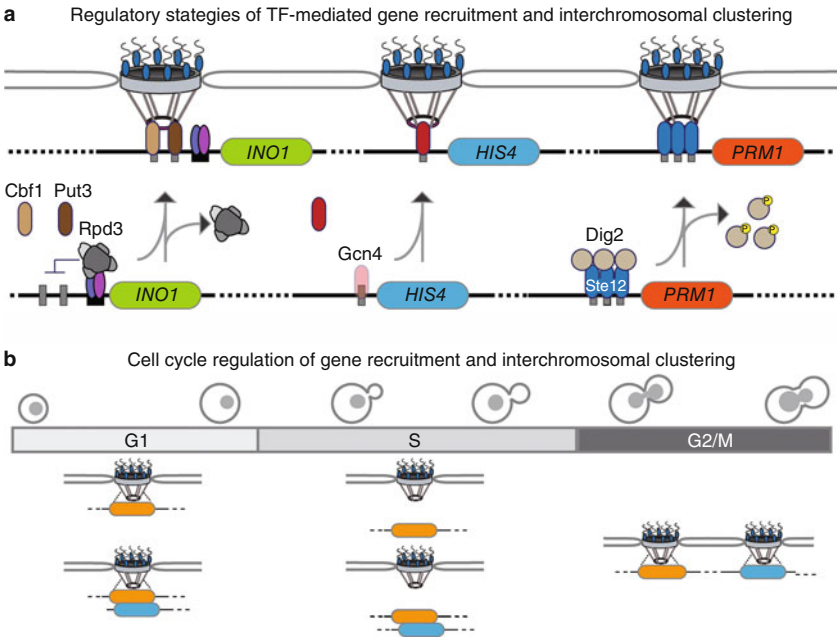

Fig. 4.2 Regulation of gene recruitment and interchromosomal clustering at the NPC. (a) Transcription factor (TF)-mediated recruitment and clustering are regulated by multiple strategies. (Left) Put3 and Cbf1 bind to GRSI and GRSII respectively and mediate *INO1* recruitment to the NPC upon inositol starvation. Recruitment of *INO1* is regulated by the local recruitment of Rpd3(L) histone deacetylase. (Middle) Gcn4-mediated recruitment of *HIS4* to the NPC is controlled by Gcn4 protein abundance. Gcn4 is translationally regulated. (Right) Upon mating pheromone stimulation, Ste12 mediates recruitment of *PRM1* to the NPC. Ste12 is regulated by MAPK phosphorylation of the inhibitor Dig2. (b) Gene recruitment and interchromosomal clustering are regulated through the cell cycle. During G1, gene recruitment and interchromosomal clustering of two loci (orange and blue) are localized at the NPC. During S-phase, phosphorylation of Nup1 blocks recruitment to the periphery but clustering is maintained in the nucleoplasm. During G2/M-phase the two loci are repositioned to the nuclear periphery, unclustered

and Ino4 TFs, neither of which are necessary to promote peripheral localization (Graves and Henry 2000; Randise-Hinchliff and Brickner 2016). Thus, genes such as *INO1* have separate elements and TFs controlling their transcription and positioning. In contrast, recruitment of *HIS4* and *PRM1* to the nuclear periphery is controlled by the same TFs that regulate their expression (Arndt and Fink 1986; Hagen et al. 1991; Randise-Hinchliff et al. 2016). *HIS4* repositions to the nuclear periphery upon activation by amino acid starvation and *PRM1* repositions upon mating pheromone stimulation and this requires Gcn4 and Ste12, respectively (Fig. 4.2a; (Randise-Hinchliff et al. 2016). The binding sites of Gcn4 (Gcn4BS) and Ste12 (pheromone-response element, 3xPRE) also function as DNA zip codes to target *URA3* to the nuclear periphery. This suggests that some, but not all, TFs function in mediating gene recruitment to the NPC. What distinguishes TFs that mediate gene recruitment from ones that do not? Put3, Cbf1, Gcn4 and Ste12 are not obviously similar in structure; representing four different families of TFs (Randise-Hinchliff and Brickner 2016). Furthermore, it is unclear how these factors mediate recruitment to the NPC. Is it through direct interaction with NPC components or through intermediate anchors such as TREX-2 or Mediator?

4.7 Interchromosomal Clustering at the NPC

Zip code-mediated targeting to NPC leads to interchromosomal clustering of genes. This can be observed by comparing the position of two loci that are targeted to the nuclear periphery in either haploid or diploid yeast cells (Brickner and Brickner 2012). Active *INO1* clusters at the NPC with another GRSI-containing gene, *TSA2* and with ectopic GRSI inserted at the *URA3* locus, but does not cluster with these loci in the nucleoplasm when repressed (Brickner et al. 2012). In diploid cells, two active alleles of *INO1* also cluster together. GRSI-mediated clustering requires the Put3 TF, which binds to GRSI. In contrast, *INO1* does not cluster with genes recruited to the nuclear periphery by different zip codes such as the *HSP104* gene (targeted by a different zip code called GRS3). Importantly, GRS3 inserted at *URA3* is sufficient to induce clustering with *HSP104*. Thus, clustering is zip code-specific. Interchromosomal clustering at the NPC has been observed for many genes such as *INO1*, *GAL1*, *HIS4*, *PRM1* and *HSP104* (Brickner et al. 2012, 2016; Randise-Hinchliff et al. 2016). Therefore, zip code-mediated targeting to the NPC leads to interchromosomal interactions and likely impacts the spatial organization of the yeast genome.

Targeting to the NPC is a prerequisite for zip-code mediated clustering. However, the molecular mechanisms controlling targeting to the NPC and interchromosomal clustering are distinct. For example, the recruitment of *GAL1* to the NPC, like *INO1*, is controlled by two redundant zip codes GRS4 and GRS5 (Brickner et al. 2016). Although both GRS4 and GRS5 are sufficient to target *URA3* locus to the nuclear periphery, GRS4 alone controls *GAL1* clustering (Brickner et al. 2016). Likewise, GRSI is both necessary and sufficient for *INO1* clustering whereas GRSII is not

(Brickner et al. 2012). Therefore, not all zip codes that are sufficient to target *URA3* to the nuclear periphery are sufficient to induce interchromosomal clustering. Clustering, unlike gene targeting, requires both transcription and transcriptional activators such as Gal4 (Brickner et al. 2016). Finally, the set of NPC components required for clustering are overlapping, but distinct, from the set required for targeting. Loss of Nup1, Nup60 and Mlp2 block both targeting to the nuclear periphery and clustering of *GAL1*, whereas loss of Mlp1 specifically blocks *GAL1* clustering without affecting peripheral targeting (Brickner et al. 2016).

4.8 Regulation of Gene Recruitment and Clustering

Gene recruitment to the NPC and interchromosomal clustering of many genes are conditional and occur under specific environmental stimuli (Randise-Hinchliff et al. 2016). This reflects how each zip code and the TF that binds to them are regulated. Put3-, Cbf1-, Ste12-, and Gcn4- mediated recruitment are regulated through different strategies (Randise-Hinchliff and Brickner 2016). Put3 and Cbf1 are regulated by a context-dependent mechanism. While Put3 and Cbf1 conditionally recruit *INO1* upon inositol starvation, when the GRSI and GRSII are inserted at an ectopic site, recruitment to the nuclear periphery is constitutive. This suggests that Put3 and Cbf1 have the capacity to recruit chromatin under repressing conditions, but are negatively regulated in the context of the *INO1* promoter. Indeed, Put3 and Cbf1 are regulated at *INO1* by local recruitment of Rpd3(L) histone deacetylase complex by transcriptional repressors, Ume6 and Opi1 (Fig. 4.2a; (Randise-Hinchliff et al. 2016). Loss of Rpd(L) leads to constitutive binding of Put3 and constitutive recruitment and interchromosomal clustering of *INO1* at the NPC. Many transcriptional repressors are sufficient to block GRSI- and GRSII-mediated recruitment to the NPC as well as GRSI-mediated clustering (Randise-Hinchliff et al. 2016). Sixteen of twenty one transcriptional repressors tested were able to block GRSI zip code activity (Randise-Hinchliff et al. 2016). This suggests that this is a general function of transcriptional repressors, which may provide multiple, alternative strategies to regulate the recruitment to the NPC mediated by a particular TF. For example, the *TSA2* gene is recruited to the NPC by Put3. *TSA2* is induced by protein folding stress, is not regulated by Ume6 or Opi1, and is recruited by different environmental stimuli (Ahmed et al. 2010; Brickner and Brickner 2012). Therefore, the context contributes to zip code regulation.

Gcn4- and Ste12-mediated gene recruitment are regulated through context-independent mechanisms (Fig. 4.2a; (Randise-Hinchliff and Brickner 2016). The zip code activity of the Gcn4 and Ste12 binding sites inserted at an ectopic site in the genome are still regulated (Randise-Hinchliff et al. 2016). Ste12-mediated recruitment is regulated downstream of DNA binding by MAPK phosphorylation of the inhibitor Dig2. Loss of Dig2 or a phosphomimetic mutation in Dig2 led to constitutive Ste12-mediated recruitment of both *PRM1* and the 3xPRE at *URA3*. Gcn4-mediated targeting is regulated by its abundance. Upregulating Gcn4 protein

levels led to an increased level of peripheral localization of *HIS4* and ectopic GCN4BS at *URA3*. Thus, in addition to regulation by local recruitment of transcriptional repressors, targeting to the NPC can be regulated by other mechanisms.

TF-mediated gene positioning and interchromosomal clustering is regulated by at least three different mechanisms that operate on different time scales (Randise-Hinchliff et al. 2016). MAPK signaling is rapid and leads to repositioning and clustering within 15–30 minutes. Changes in Gcn4 protein levels lead to slower repositioning and clustering of Gcn4 targets over 30–60 minutes. *INO1* recruitment and clustering occurs even more slowly over 60–120 minutes, consistent with the slow depression of *INO1* transcription. Thus, cells employ different strategies to regulate TF-mediated gene positioning over different time scales.

4.9 Gene Recruitment and Clustering Through the Cell Cycle

The recruitment of inducible genes to the NPC is regulated through the cell cycle. For active *INO1*, *GAL1* and *HSP104* genes, recruitment to the nuclear periphery occurs during G1 and G2/M, but not in S-phase when the genes localize in the nucleoplasm (Fig. 4.2b; (Brickner and Brickner 2010). Importantly, the loss of peripheral localization is not a nonspecific effect of DNA replication, but rather due to phosphorylation of Nup1 by the cyclin-dependent kinase Cdk1 (Brickner and Brickner 2010). Phosphorylation of Nup1 is required for normal targeting to the nuclear periphery; inactivation of Cdk or mutations that block phosphorylation of Nup1 also block targeting of *INO1* and *GAL1* to the periphery. Conversely, mutations in Nup1 that mimic phosphorylation at either of two sites or loss of the Cdk1 inhibitor, Sic1, led to *INO1* and *GAL1* remaining at the nuclear periphery during S-phase. The phosphomimetic mutations bypass the requirement of Cdk1, suggesting that Nup1 is the only protein whose phosphorylation affects peripheral targeting of these genes.

Interchromosomal clustering is also regulated through the cell cycle, but is out of phase with gene recruitment. *GAL1* clustering is maintained in the nucleoplasm through S-phase, but is lost upon repositioning to the periphery during G2/M (Fig. 4.2b; (Brickner et al. 2016). Interestingly, the regulation of peripheral targeting and clustering are interdependent. Loss of phosphorylation of Nup1 leads to loss of interchromosomal clustering and phosphomimetic Nup1 both maintains *GAL1* at the NPC during S-phase and leads to clustering during G2/M. Therefore, Cdk phosphorylation of the NPC coordinates the positioning of individual genes and the organization of chromosomes with respect to each other through the cell cycle.

4.10 Transcription Memory

Several inducible genes such as *INO1* and *GAL1* that are recruited to the NPC upon activation remain anchored to the pore for several generations after

repression (D'Urso and Brickner 2014). Such epigenetic retention leads to an altered chromatin structure and primes genes for rapid transcriptional reactivation. This phenomenon is called transcriptional memory and represents a mitotically heritable state. Furthermore, transcriptional memory leads to a faster or stronger response when cells are confronted with an environmental challenge previously experienced, presumably impacting cellular fitness and survival (D'Urso and Brickner 2014). Nuclear pore components play important roles in transcriptional memory, but not all genes that interact with the NPC when active exhibit memory. Understanding the mechanisms and specific NPC components involved in transcriptional memory can further elucidate the functions of the NPC.

A well-established model for transcriptional memory is *GAL1* (Brickner et al. 2007; Kundu et al. 2007; Brickner 2009). After being repressed, *GAL1* is retained at the nuclear periphery, primed for faster reactivation for up to seven generations (Brickner et al. 2007). During the first few hours, *GAL1* is anchored to the NPC as an intragenic loop between its promoter and 3' end; called a memory gene loop (MGL; (Tan-Wong et al. 2009). MGLs are stabilized at the NPC by Mlp1 and are thought to prime genes for reactivation by retaining transcription initiation factors, such as TBP. Indeed, destabilizing *GAL1* MGL, through loss of Mlp1, significantly reduces both TBP binding and the rate of reactivation (Tan-Wong et al. 2009). However, this is not the sole mechanism of *GAL1* transcriptional memory, since the *GAL1* MGL does not persist as long as memory (Brickner et al. 2007; Tan-Wong et al. 2009). It is possible that MGLs initiate memory and downstream mechanisms maintain transcriptional memory. Consistent with this notion, the chromatin remodeling complex, SWI/SNF1, is required for *GAL1* memory, but not for loop formation (Kundu et al. 2007). Interestingly, the inheritance of *GAL1* memory is not perpetuated by chromatin alone, but through *trans*-acting Gal1 protein itself, which is necessary for epigenetic memory (Zacharioudakis et al. 2007). Ectopic expression of *GAL1* is sufficient to induce faster induction of the other *GAL* genes (Zacharioudakis et al. 2007). Thus, the rapid reactivation of *GAL* genes involves multiple mechanisms including the formation of gene loops, chromatin-based mechanisms and *GAL1* protein itself.

Loss of the histone variant H2A.Z both blocks periphery localization of *INO1* and *GAL1* and causes a dramatic decrease in the reactivation after repression (Brickner et al. 2007) (our unpublished results). This suggests that peripheral localization is coupled to reactivation. Indeed, H2A.Z incorporation after repression depends on the nuclear pore protein Nup100. H2A.Z also physically associates with Nup2 (Dilworth et al. 2005). However, it is unclear how H2A.Z perpetuates memory. Loss of H2A.Z and Nup100 leads to a strong and specific defect in the rate of reactivation of *INO1* (Light et al. 2010), but loss of H2A.Z affects both the rate of activation and reactivation of *GAL1* (Halley et al. 2010). Similar to Nup2, H2A.Z functions to insulate euchromatin from the spread of heterochromatin (Meneghini et al. 2003). It is found in most inducible promoters and facilitates faster induction (Zhang et al. 2005; Albert et al. 2007; Wan et al. 2009). H2A.Z-containing nucleosomes are also less stable and flank nucleosome-free regions in promoters (Albert et al. 2007). Therefore, perhaps chromatin changes like H2A.Z

incorporation generally enhance the rate of transcriptional induction and such changes can be influenced by interactions with the NPC during memory.

INO1 gene remains associated with the nuclear periphery for up to four generations after repression, dependent on H2A.Z incorporation and Nup100 (Brickner et al. 2007; Light et al. 2010). After repression, the *INO1* promoter is marked with another chromatin mark, dimethylated histone H3 lysine 4 (H3K4me2). Memory leads to binding of poised RNA polymerase II (RNAPII) preinitiation complex (PIC), which enhances the rate of future reactivation (Fig. 4.3a; (Light et al. 2010; Light et al. 2013; D'Urso et al. 2016). Many of the NPC components required for active recruitment were also required in memory such as Nup1, Nup2 and Nup60. However, five Nups are specifically required for retention at the nuclear periphery

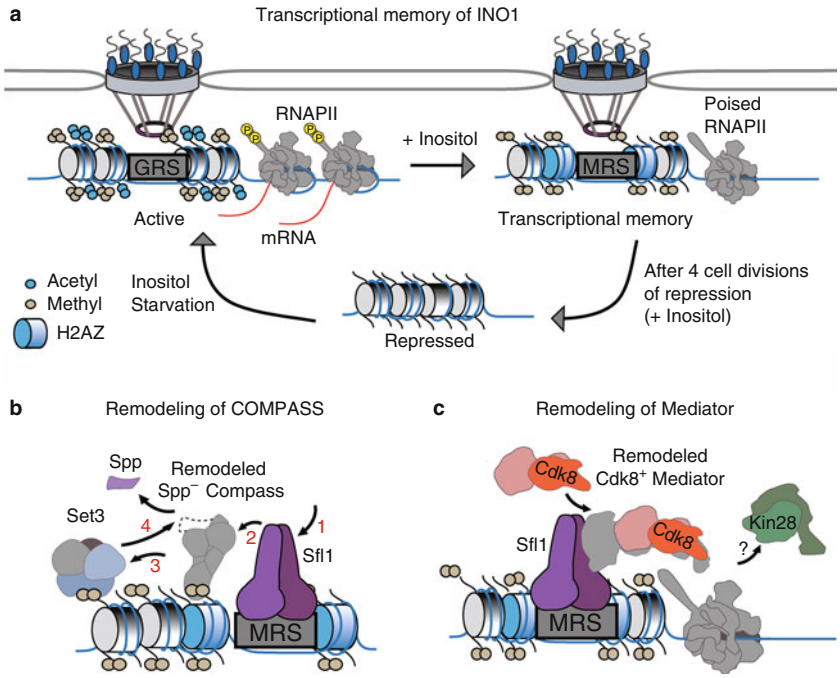

Fig. 4.3 Model of INO1 transcriptional memory at the NPC. (a) *INO1* is recruited to the NPC under inositol starvation, which leads to transcription by RNA polymerase II (RNAPII) as well as hyperacetylation and both di- and trimethylation of histone H3, lysine 4 (H3K4). Upon repression, *INO1* remains associated to the NPC and the preinitiation RNAPII is poised to the promoter for up to four generations. *INO1* transcriptional memory leads to an altered chromatin state involving the incorporation of H2A.Z and dimethylation of H3K4. (b) Transcription factor Sfl1 binds to the MRS upon repression and is required for recruitment and remodeling of Set1/COMPASS (1). The remodeled form of COMPASS lacking the Spp1 subunit is necessary to establish H3K3 dimethylation (2). H3K4me2 recruits Set3C (3). Set3C promotes the persistence of H3K4me2 and potentially the recruitment or remodeling of COMPASS (4). (c) $Cdk8^{+}$ Mediator promotes transcriptional poising. During activation, Mediator lacks Cdk8 and TFIIK (Kin28/Cdk7) phosphorylates Serine 5 of RNAPII to initiate transcription. Upon repression, Kin28 is lost and $Cdk8^{+}$ Mediator is recruited

during transcriptional memory: Nup100 and Nup84 subcomplex components Nup84, Nup120, Nup133, and Nup145C (Light et al. 2010). In contrast to *GAL1*, *INO1* does not require Mlp1 and MGLs do neither form nor are required for *INO1* memory (Tan-Wong et al. 2009; Light et al. 2010). By ChIP, Nup2 binds to the *INO1* promoter both in active and recently repressed conditions, whereas, Nup100 binds specifically during memory (Light et al. 2010). In strains lacking Nup100, the *INO1* promoter loses H2A.Z incorporation, H3K4me2 and poised RNA polymerase II PIC, leading to slower reactivation (Light et al. 2010, 2013).

Targeting of active and recently repressed *INO1* to the NPC is mediated by distinct mechanisms and different zip codes (Light et al. 2010). Recruitment of recently repressed *INO1* to the nuclear periphery does not require GRSI and GRSII. Instead, after repression a zip code called the Memory Recruitment Sequence (MRS) is both necessary and sufficient to recruit *INO1* to the NPC. A mutation in the MRS sequence specifically blocks *INO1* peripheral positioning after repression, but not in active conditions (Light et al. 2010). Finally unlike the GRS, MRS-mediated recruitment is not regulated throughout the cell cycle (Brickner and Brickner 2010).

Transcriptional memory also leads to interchromosomal clustering of *INO1* (Brickner et al. 2015). During memory, two alleles *INO1* remain clustered in diploid cells, which requires the MRS and Nup100. Unlike recruitment during memory, *INO1* clustering during memory also requires GRSI and GRSII zip codes (Brickner et al. 2015). Furthermore, neither GRSI or MRS inserted at *URA3* is sufficient to cause clustering with *INO1* during memory. In contrast, the ectopic GRSI clusters with *INO1* in active conditions (Brickner and Brickner 2012). This suggest clustering during memory requires previous clustering of *INO1* during activation. Therefore, the MRS zip code is necessary, but not sufficient, to induce clustering. Clustering of *INO1* during transcriptional memory is regulated through the cell cycle. In G2/M phase, *INO1* clustering is lost (Brickner et al. 2015). Therefore, MRS- and GRS- mediated recruitment and clustering of *INO1* share some similarities, but function by distinct mechanisms.

4.11 Molecular Mechanism of INO1 Transcriptional Memory

INO1 transcriptional memory is initiated by binding of a TF to the MRS zip code. The TF Sfl1 binds to the MRS upon shifting cells from activating to repressing conditions (Fig. 4.3; (D'Urso et al. 2016). Sfl1 has a genetic interaction with the Nup84 subcomplex component, Nup120, and is both necessary and sufficient to recruit chromatin to the nuclear periphery (Robertson and Fink 1998, D'Urso et al. 2016). Sfl1 and the MRS, like Nup100, are essential for all aspects of transcriptional memory (D'Urso et al. 2016). This suggests that binding of Sfl1 to the MRS initiates *INO1* transcriptional memory and may determine the duration of memory.

INO1 transcriptional memory is associated with histone modifications. When *INO1* is repressed, H3K4 is hypoacetylated and unmethylated whereas during

activation, H3K4 is hyperacetylated and both di- and trimethylated (Fig. 4.3a; (D'Urso et al. 2016). However, upon repression, *INO1* loses histone acetylation and trimethylation, but remains dimethylated (H3K4me2; (D'Urso et al. 2016). H3K4me2 is necessary for memory and is established by remodeling of the Set1/COMPASS methyltransferase complex, ejecting the Spp1 subunit (Fig. 4.3b; (D'Urso et al. 2016). The Spp1$^-$ complex is capable of dimethylation, but not trimethylation of H3K4 (Schneider et al. 2005; Takahashi et al. 2009). H3K4me2 recruits the SET3C histone deacetylase, which is also required for memory (D'Urso et al. 2016). Set3 is the eponymous member of SET3C and binds to H3K4me2 through its PHD domain (Kim and Buratowski 2009). SET3C binding to H3K4me2 is required both to recruit RNAPII and to maintain H3K4me2 during memory (D'Urso et al. 2016). Conditional inactivation of SET3C leads to rapid loss of both H3K4me2 and poised RNAPII from the *INO1* promoter (D'Urso et al. 2016). Thus, SET3C has a direct and continuous role in memory. The maintenance of H3K4me2 may provide a chromatin state that allows recruitment of RNAPII and rapid reactivation.

Changes in chromatin composition (H2A.Z) and histone modifications (H3K4me2) are necessary for transcriptional memory. These changes presumably allow RNAPII PIC to remain bound; poising genes for transcriptional reactivation (D'Urso and Brickner 2014). PIC assembly during memory also requires Cdk8$^+$ form of Mediator (Fig. 4.3c). Mediator binds to the *INO1* promoter both under activating and memory condition (D'Urso and Brickner 2014). However, the Cdk8 module only binds during memory. Inactivation of Cdk8 specifically disrupts RNAPII binding during memory and slows reactivation without affecting *INO1* activation (D'Urso and Brickner 2014). Interestingly, Cdk8$^+$ Mediator physically interacts with both Sfl1 and the NPC-associates TREX-2 complex, both of which are required for memory (Song and Carlson 1998; Schneider et al. 2015). The poised PIC complex during memory is partially assembled, missing both Ctk1 and Kin28, which phosphorylate serine 2 and 5 on the caboxy terminal domain, respectively (Light et al. 2010). Unlike Cdk8, Kin28 is also not required for memory and the poised RNAPII is unphosphorylated on Ser2 and 5. It's conceivable that Cdk8 and Kin28 are mutually exclusive and that Cdk8$^+$ Mediator promotes transcriptional poising by blocking Kin28 association with the PIC (Fig. 4.3c). Further experiments will discern this mechanism.

The mechanism of *INO1* memory is related to the mechanism of stress-induced memory in yeast and IFNγ-induced memory in human cells (D'Urso et al. 2016). In both systems, genes that display memory are marked with H3K4me2, bound by RNAPII and Cdk8. In yeast, 77 of the genes induced by oxidative stress are primed for activation in response to previously experienced salt stress (Guan et al. 2012). This effect persists for four generations. However, unlike *INO1*, salt stress-induced memory does not require Sfl1 or Nup100 and requires a different NPC component, Nup42, for faster reactivation (Guan et al. 2012). In human cells, genes that exhibit IFNγ-induced memory physically interact with Nup98, a homologue of Nup100, and require Nup98 for memory (Light et al. 2013). Unlike in yeast, IFNγ-induced genes interact with Nup98 in the nucleoplasm. Despite these

differences, the core mechanism revealed by studies of *INO1* transcriptional memory is both general and conserved (D'Urso et al. 2016).

4.12 Concluding Remarks

From yeast to humans, the NPC plays essential roles in promoting transcription, regulating chromatin structure, spatially organizing eukaryotic genomes. Research in yeast has guided our understanding of these mechanisms, many of which are evolutionarily conserved. These observations have broadened our understanding of the NPC's role as a regulatory hub for genome organization and function.

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Chapter 5
Nuclear Pore and Genome Organization and Gene Expression in *Drosophila*

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Abstract Regulation of gene expression is central to the cell's ability to respond to external cues and to establish and maintain its developmental identity. The Nuclear Pore Complex (NPC) forms the nuclear envelope-spanning channel that mediates selective nucleo-cytoplasmic transport of macromolecules. In addition to contributing to gene expression via its transport functions, the NPC comes in close contact with the underlying chromatin and plays a role in regulation of gene expression of the associated gene targets. In recent years, studies in *Drosophila* and other organisms have identified numerous physiological roles for NPC components, including functions in immune response, tissue-specific development, epigenetic processes and neurodegeneration. This chapter focuses on the current knowledge of the physiological roles of NPC components and on the relationship between the NPC and chromatin regulation, obtained in the fly model. Findings, described here, demonstrate the far-reaching potential of NPC components to regulate gene expression via both transport and chromatin-binding mechanisms. Furthermore, they reveal *Drosophila* to be a useful experimental system for future dissections of the in vivo phenotypes and gene regulatory functions of the NPC.

Keywords nuclear pore · nucleoporin · genome organization · gene expression · drosophila · gene regulation

5.1 Introduction

The Nuclear Pore Complex (NPC) is a massive nuclear envelope (NE)-embedded protein complex that consists of components termed Nucleoporins (Nups) (D'Angelo and Hetzer 2008; Knockenhauer and Schwartz 2016). The classically

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characterized function of the NPC is to mediate nucleo-cytoplasmic transport. Via its transport function, the NPC is intimately tied to regulation of gene expression, since the entry of cell cycle regulators and transcription factors, and the exit of mature mRNAs are all critical steps of the gene expression regulatory cascade. Yet in addition to transport, the NPC has been linked to gene regulation directly, via interactions with the underlying genome. The link between chromatin and NPC was initially suggested by electron microscopy images of nuclei, which showed a juxtaposition of decondensed chromatin against nuclear pores (Blobel 1985). Such images formed the basis for the "gene gating hypothesis," which proposed that NPCs preferentially associate with decondensed and presumably active genomic regions to couple transcription to export of the generated mRNA. Multiple studies in several organisms have since supported the general idea that NPC components engage in interactions with active genes and contribute to regulation of transcription and chromatin (Ptak and Wozniak 2016; Sood and Brickner 2014). The core structures of the NPC, such as the NE-embedded channel-forming rings, are comprised of Nups that stably associate with the NPC, while many of the peripheral structures, such as the inner channel or the nuclear basket, consist of Nups that can move on and off the NPC relatively rapidly and are thus termed dynamic (Rabut et al. 2004). *Drosophila* contains clear homologues to the known verterbrate Nups, and the general NPC structure and assembly pathways are likely to be conserved. Although NPC biogenesis and structure have not been as thoroughly investigated in *Drosophila* as in yeast and verterbrates, the fly model system has been instrumental in expanding other areas of the NPC research field. One such contribution is revealing in vivo phenotypes and physiological functions of Nups, due to the availability of powerful genetic tools and the existence of well-characterized experimental models of organogenesis and physiology in *Drosophila*. Though mechanisms behind many of these phenotypes remain to be determined, the importance of various NPC components in development, disease models and evolutionary processes has been recently brought forth by studies in the fly system.

Another contribution of the *Drosophila* model system is the characterization of NPC-genome interactions in the context of a developing organism. This characterization has been facilitated by the presence of polytenized tissues such as salivary glands, which contain giant polytene chromosomes that allow for ready cytological analysis of chromatin-binding behaviors (Kuzin et al. 1994). One aspect of the NPC-genome relationship discovered in *Drosophila* is the ability of dynamic Nups to interact with chromatin in the nuclear interior, away from the NE-embedded NPCs (Hou and Corces 2010). This property appears to be conserved in mammalian cells and expands our understanding of the functional roles of Nups in regulation of gene expression, as discussed further below. In this chapter, we provide a brief summary of the known unique features of the fly NPC, outline reported *Drosophila* phenotypes of individual Nups, and discuss the current knowledge of the NPC-chromatin relationship gained from the fly system. Together, these studies highlight the notion that NPC components represent a far-reaching aspect of the gene regulatory cascade, with physiological roles in tissue-specific development and tissue homeostasis.

5.2 Unique Features of *Drosophila* NPC Structure and Assembly

The *Drosophila* genome possesses readily identifiable homologues to components of the mammalian outer ring Nup107-160 sub-complex and associated Nups Elys and Nup98, as well as to components of the cytoplasmic filament Nup88-Nup214 sub-complex, the transport channel Nup62 sub-complex and the nuclear basket Nups Tpr (termed Megator (Mtor) in flies), Nup153, and Nup50. Like in verterbrates, there appears to be three trans-membrane Nups in *Drosophila*, including homologues of Nup210 and Ndc1, as well as what appears to be a distantly related homologue of Pom121, termed *dumpy*. Surprisingly, the fly genome carries two versions of Nup93, the defining component of the inner ring Nup93-Nup205 sub-complex: Nup93-1 (CG11092) and Nup93-2 (CG7262), which share a 65% homology and are located on different chromosomes. Interestingly, although homozygous mutations, generated by P-element insertions into the 5′ Untranslated Transcribed Region (UTR) are adult lethal for the majority of fly *Nup* genes, such mutations in either *Nup93* gene still produce viable adults. This viability suggests that *Nup93* genes may compensate for each other's functions. On the other hand, the reported embryonic and adult expression patterns of *Nup93-1* and *Nup93-2* exhibit several drastic differences from each other (Chintapalli et al. 2007). For instance, while *Nup93-1* is expressed very highly in the adult heart, *Nup93-2* is not and is instead highly enriched in the adult testes, suggesting that the two Nup93 subtypes execute unique tissue-specific functions.

Although NPCs appear to disassemble during mitosis in *Drosophila* similarly to verterbrates (Katsani et al. 2008), mitosis occurs in a "semi-closed" fashion in fly cells, with some remnants of the NE persisting through early anaphase (Katsani et al. 2008; Kiseleva et al. 2001). Furthermore, the mitotic spindle in *Drosophila* embryos appears to be confined by a membranous structure, termed spindle envelope, which is required for faithful chromosome segregation (Harel et al. 1989; Kiseleva et al. 2001; Schweizer et al. 2015). This structure may be related to or associated with the spindle matrix, a conserved filamentous network supporting the mitotic spindle (Jiang et al. 2015; Qi et al. 2004), a defining component of which is the nuclear basket Nup Mtor (Qi et al. 2004). Interestingly, in developmental stages with rapid cell cycles, such as the early pre-gastrulation *Drosophila* embryo, NPC re-assembly has been recently shown to occur by a newly discovered method of pre-assembled nuclear pore insertion (Hampoelz et al. 2016). In such rapidly cycling cells, which rely on maternally supplied factors, ER-embedded storages of NPC scaffolds, termed annulate lamellae, are fed into the expanding NEs as cells progress through their shortened interphases. These findings demonstrate that unique pathways of NPC assembly can occur during specific developmental stages.

5.3 Phenotypes of *Drosophila* Nups

In this section, we highlight some of the best-characterized and recurrent phenotypes of *Drosophila* Nups in physiological and developmental processes. Studies in mammalian models have demonstrated unique functions of specific Nups in tissue-specific development, such as differentiation of neuronal and muscle lineages, as well as in maintenance of stem cell pluripotency (D'Angelo et al. 2012; Jacinto et al. 2015; Lupu et al. 2008; Raices and D'Angelo 2012). As discussed below, due to the widespread use of genome-wide screens in *Drosophila*, fly NPC components have been similarly identified as hits in a number of assays of organismal function and dysfunction. Some of the phenotypes defined for Nups in this manner have been unexpected, given the necessary function of the NPC as a transport channel and its identity as a highly structured protein complex. But identification of such roles in *Drosophila* offers a glimpse of the broad physiological significance of the NPC that is yet to be fully uncovered.

5.3.1 *Components of the Nup107-160 Sub-complex in* Drosophila *Speciation*

Speciation involves the evolution of incompatible gene interactions that cause sterility or lethality in hybrids between related populations, a phenomenon termed hybrid lethality or incompatibility (Presgraves 2007). For example, if two closely related *Drosophila* species are mated together, such as *Drosophila melanogaster* females crossed to *Drosophila simulans* males, the resulting hybrid progeny is inviable (Sawamura et al. 1993). To understand the molecular mechanisms driving speciation, there has been great interest in identifying genes that can rescue or alter hybrid incompatibility. Strikingly, a genome-wide screen, aimed at identifying genes that can reverse the hybrid viability phenotype of the *Lethal hybrid rescue (Lhr)* mutation, identified the *Nup96* gene as being able to restore hybrid inviability between *D. melangaster* and *D. simulans* (Presgraves et al. 2003). Although *Nup96* is encoded in the same gene as *Nup98*, the hybrid lethality phenotype has been narrowed down specifically to *Nup96*, and particularly to the most N-terminal ~100 amino acids of Nup96 (Presgraves et al. 2003).

Furthermore, comparison of amino acid substitutions between species demonstrated a high level of non-silent changes in *Nup96*, indicative of positive selective pressure and adaptive evolution in this NPC component. Given that Nup96 is a core component of the stable Nup107-160 sub-complex, its high degree of divergence between closely related species was somewhat unexpected. However, signs of rapid adaptive evolution were similarly observed in other components of this NPC sub-complex and in Nups known to interact with Nup96, such as Nup107, Nup160, Nup133, Nup75 and Nup98 (Presgraves and Stephan 2007; Tang and Presgraves 2009). Furthermore, another key Nup107-160 sub-complex component, *Nup160* has also been identified as a key speciation gene in subsequent

hybrid incompatibility screens (Tang and Presgraves 2009; Sawamura et al. 2010). Interestingly, the hybrid incompatibility effects of both *Nup96* and *Nup160* were found to depend on an unknown component of the *D. melanogaster* X chromosome, as for instance, hybrid lethality of *Nup96* mutations can be reversed if the *D. melongaster* X chromosome is replaced with the *D. simulans* version (Presgraves et al. 2003; Barbash 2007). It was hypothesized this factor could be the *Nup153* gene encoded on the X-chromosome, although this has not been proven.

Consistently with being components of the same NPC sub-complex, the roles of Nup96 and Nup160 in hybrid incompatibility appear to be linked, yet each Nup may have distinct contributions. For instance, homozygous *D.simulans* version of *Nup96* ($Nup96^{sim}$) in a *D. melanogaster* background restores hybrid inviability independently of the *D. melanogaster* X chromosome if $Nup160^{sim}$ is also hemizygous, indicating that their hybrid incompatibility phenotypes are functionally related (Maehara et al. 2012). However, $Nup160^{sim}$, but not $Nup96^{sim}$, in a *D. melanogaster* background, induces female sterility, and this phenotype is independent of species origin of the X chromosome (Sawamura et al. 2010; Maehara et al. 2012), suggesting a separate and distinct role of Nup160 in oogenesis or gamete compatibility.

Together, these studies revealed that components of the core NPC sub-complex are some of the key factors driving speciation in *Drosophila* evolution. However, how these Nups contribute to speciation or why these genes may be fast-evolving is not entirely understood. One proposed explanation is related to the role of the NPC in regulating viral and retrotransposon nuclear import (Sistla et al. 2007; Yarbrough et al. 2014), enabling diverging species to keep up with evolving pathogens in order to increase defense against them. Another proposed mechanism involves the chromatin-interacting roles of the NPC. Repetitive satellite DNAs within heterochromatin, especially at centromeres, are known to evolve rapidly, which is thought to correlate with rapid evolution of proteins that bind such DNA (Ferree and Barbash 2009; Sawamura 2012). In fact, *Lhr* and several other identified speciation genes code for proteins that bind repetitive heterochromatin (Sawamura 2012), suggesting an interesting possibility that Nup96 and Nup160 may similarly play a role in heterochromatin maintenance.

5.3.2 NPC Components and ALS/FTD Pathogenesis

The *Drosophila* model has been widely utilized for characterization of molecular pathways driving the pathogenesis of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (Mcgurk et al. 2015). Recently, several genome-wide screens looking for genetic modifiers of ALS and FTD pathogenesis, conducted in *Drosophila*, have identified multiple nucleocytoplasmic transport factors, including components of the NPC (Boeynaems et al. 2016a; Zhang et al. 2015; Jovicic et al. 2015; Freibaum et al. 2015). These screens primarily utilized the fly disease model based on the

hexanucleotide repeat expansion in the *C9orf72* gene, which is the most common genetic factor contributing to both ALS and FTD (Dejesus-Hernandez et al. 2011). Although these diseases have very different clinical presentations, one common factor is degeneration of neurons, to which repeat expansion in *C9orf72* gene is known to contribute. The molecular mechanism behind pathogenesis of expanded repeats in *C9orf72* proved to be complicated, with several different models of action in existence. One emerging theme however is the impairment of nucleocytoplasmic transport and of NPC components in ALS and FTD, both in human cells and *Drosophila* disease models (Boeynaems et al. 2016b; Jovicic et al. 2016).

In one model of pathogenicity, toxicity of expanded *C9orf72* repeats is based on non-canonical repeat-associated non-ATG (RAN) translation of the C9orf72 RNA, which produces toxic dipeptide repeat proteins (DPRs) (Ling et al. 2013). Expression of *C9orf72* repeats or specifically of DPRs in the *Drosophila* ALS/FTD model results in pronounced tissue degeneration when expressed in the eye. A screen for modifiers of this degenerative phenotype discovered multiple *Drosophila* Nups, RNAi-meditated depletion of which can suppress or enhance the phenotype (Boeynaems et al. 2016a). Specifically, knock-down of Nup62, Nup93-1 or Nup44A/Seh1 exacerbated DPR toxicity, while in contrast, knock-down of Nup107, Nup50 or Nup154 ameliorated the degenerative phenotype and dramatically rescued morphology in DPR-expressing eyes. Strikingly, other independent screens, using similar expanded repeat-based *Drosophila* models of ALS, also identified multiple Nups as strong modifiers of the degenerative phenotype (Freibaum et al. 2015; Jovicic et al. 2015; Zhang et al. 2015). Once again, RNAi against Nups such as Nup107, Nup160, and Nup96-Nup98 demonstrated powerful suppression of expanded repeat-induced toxicity (Freibaum et al. 2015).

Identification of NPC components as strong suppressors of the ALS-like phenotype offers exciting therapeutic potential, yet the mechanism by which certain Nups can alleviate ALS-associated toxicity remains unclear. This is due in part to the fact the mechanism behind degeneration, induced by expanded repeats, remains controversial. While repeat-produced DPRs are often considered the main source of toxicity, aberrant repeat-produced RNA has also been suggested to be a causal agent (Burguete et al. 2015; Freibaum et al. 2015; Ling et al. 2013). One hypothesis of *C9orf72* toxicity is based on the sequestration of RNA-binding proteins by the *C9orf72* RNA with expanded repeats, which could have a negative impact on mRNA export. In support of this hypothesis, depletion of mRNA export receptors, such as Gle1 and NXF1, enhanced the degenerative phenotype of *C9orf72* repeat-based *Drosophila* models, and RNA export defects have been reported in iPS cells-derived neurons, obtained from ALS patients (Freibaum et al. 2015). Interestingly, Nup50 was similarly identified as a modifier in a screen based on a distinct *Drosophila* ALS model, which overexpresses the nuclear RNA-binding protein TDP-43, cytoplasmic mis-localization of which is a canonical marker of ALS toxicity (Zhan et al. 2013). A loss-of-function mutation in Nup50 was found to result in suppression of toxicity, increasing lifespan of TDP-43-overexpressing flies. Furthermore, administering an inhibitor of mRNA export, Leptomycin B, to flies, overexpressing *C9orf72* repeats, enhanced the toxic effects

of the repeat expression (Freibaum et al. 2015), suggesting that RNA-based toxicity and mRNA export defects at least partly contribute to ALS pathogenesis.

A recent study offered a new insight into the etiology of ALS pathogenesis, showing that DPRs interact with endogenous proteins with low complexity sequence domains (LCDs), which are intrinsically disordered and can form phase-separated structures such as hydrogels (Kwon et al. 2014; Lee et al. 2016). LCDs are often components of membrane-less organelles, such as nucleoli and NPCs, and are exemplified by the Phenylalanine Glycine (FG) repeat domain of Nups. Interactions of DPRs with LCD proteins were shown to disrupt their phase separation and material properties, which may provide a mechanism for the observed transport defects associated with ALS/FTD pathogenesis (Lee et al. 2016). However, opposing effects of specific NPC components on ALS-based toxicity, observed in the fly model, are still to be explained, since for example, depletion of either the FG domain-containing Nup62 or the core Nup107-160 sub-complex component Nup44A/Seh1 enhance the ALS-like degenerative phenotype (Freibaum et al. 2015). Together, these findings expose the critical roles of NPC components in human neurodegenerative diseases and highlight the utility of the *Drosophila* model in identifying disease-relevant roles of NPC components.

5.3.3 Cytoplasmic Nups in Immune Response and Import of NF-κB

One of the initial demonstrations of the functional specificity of individual Nups was the discovery of the specific involvement of Nup88, the fly gene for which is termed *members only* (*mbo*), in *Drosophila* immune function. Nup88 expression was found to be tissue-specific in fly larvae: enriched in imaginal discs, trachea, CNS, and fat body, but low in epidermis, muscles and gut (Uv et al. 2000). While *mbo* loss-of-function mutants did not present obvious defects in nuclear morphology, NPC structure, mRNA export, or nuclear localization of multiple endogenous proteins, a specific defect in nuclear import of the *Drosophila* transcription factors Dorsal and Dif, homologues of the NF-κB/Rel transcription factors, was identified in *mbo* mutant cells (Uv et al. 2000).

NF-κB/Rel transcription factors are critical for response to immune insults in metazoan organisms. Upon immune activation, cytoplasmic inhibitors of NF-κB factors are degraded, physically releasing them and allowing for their translocation into the nucleus (Mitchell et al. 2016). Here NF-κB factors interact with chromatin and promote expression of target immune response genes, and successful nuclear translocation of these transcription factors during immune response is crucial for the expression of downstream immune response. When Nup88 mutant larvae were infected with bacteria, Dorsal and Dif NF-κB/Rel transcription factors were not effectively transported into the nuclei of cells within the fat body, which is the primary organ for anti-bacterial response, yet nucleocytoplasmic transport of several other tested proteins and mRNA products was completely unaffected in these mutants. Accordingly, the normally observed dramatic up-regulation of

target antimicrobial peptide genes *drosomycin* and *diptericin* is completely abolished in the Nup88 mutant background (Uv et al. 2000).

Further exploration has led to a likely mechanism, and implicated another NPC component Nup214, in this selective transport of NF-κB factors. Localization of Dorsal within the cell has been shown to rely directly on the nuclear export factor CRM1. In salivary gland cells of CRM1 mutant flies, or S2 cells with CRM1 activity chemically inhibited, the nuclear/cytoplasmic ratio of Dorsal protein is dramatically increased compared to wild type, demonstrating a clear defect in its nuclear export (Roth et al. 2003). CRM1, Nup214, and Nup88 appear to interact directly, and loss of either Nup88 or Nup214 induces re-localization of CRM1 from the nuclear periphery to the nuclear interior (Xylourgidis et al. 2006), suggesting that the Nup88/Nup214 complex normally sequesters CRM1 at the periphery and prevents it from performing its export functions. Without being anchored to the periphery, CRM1 is free to export Dorsal, Dif, and other protein cargo out of nuclei unabated, inhibiting their ability to act as transcription factors and induce expression of antimicrobial peptides. The specificity in transport targets in this system is thought to be accomplished by the previously demonstrated affinity of CRM1 for leucine-rich nuclear export sequences, including those possessed by Dorsal (Xylourgidis et al. 2006). These findings have introduced a mechanistic paradigm for how a specific gene expression program and the immune response can be directly influenced by transport activities of individual Nups. Interestingly, Nup98 has also been identified as a key player in *Drosophila* immunity, but is thought to function by a distinct and likely transport-independent mechanism, discussed further below (Panda et al. 2014).

5.3.4 Roles of Drosophila *Nups in Germ Cell Development*

Early genetic screens for female sterility have identified hundreds of genes that control germ cell development and maintenance in *Drosophila*, and have allowed for sophisticated mechanistic dissection of this critical developmental process. One hit from such screens was termed *tulipano* (*tlp*) and was subsequently found to encode the *Drosophila* homologue of the verterbrate Nup155, Nup154 (Gigliotti et al. 1998). Hypomorphic alleles of *Nup154* were found to affect both female and male sterility (Gigliotti et al. 1998), and stronger loss-of-function mutants demonstrated a severe phenotype, including failure to maintain spermatogenic stem cells in the male gonad and failure to progress into vitellogenic stages of oogenesis in the female gonad, with a complete loss of germ cells in the male testes by adulthood (Kiger et al. 1999).

One mechanistic insight into how Nup154 affects *Drosophila* germ cell development was obtained from the discovery of an interaction between Nup154 and another critical germ cell factor, the Cup protein (Grimaldi et al. 2007). Cup is a germline-specific protein, previously implicated in regulating several aspects of gametogenesis, translational repression and chromatin structure in the egg chamber (Piccioni et al. 2005). Nup154 and Cup were found to enhance each other's

sterility phenotypes and to co-immunoprecipitate together upon Nup154 overexpression (Grimaldi et al. 2007). Furthermore, Cup was found to associate with the nuclear periphery specifically in early stage (stage 4) egg-chambers. Strong mutants of either Nup154 or Cup both resulted in a very similar phenotype of developmentally stalled chromosome structure in stage 5 eggs, where chromosomes did not properly decondense from their polytenized state, suggesting that Nup154 and Cup co-function in regulating chromosome structure at this stage in egg development (Grimaldi et al. 2007). Interestingly, neither general mRNA export nor protein import of tested factors appeared impaired in *Nup154* mutant germ cells or somatic cells of the gonad (Gigliotti et al. 1998). Based on these findings, it was proposed that Cup and Nup154 cooperate at the nuclear periphery to possibly regulate transport of specific developmental factors important for chromatin structure and oogenesis (Grimaldi et al. 2007), though the precise role of Nup154 in these processes remains unclear. Coincidently, another critical regulator of germ cell development, *Germ cell less* (*Gcl*), was similarly found to localize to the nuclear periphery and co-localize with NPCs in very early germ cells (Jongens et al. 1994), suggesting that localization to the NPC is a recurrent regulatory mechanism utilized in germline maturation.

Another defect uncovered in *Nup154* mutant germ cells is a disruption of the TGFβ signaling pathway, manifested as a dramatically reduced nuclear import of active phosphorylated(p)-Mad in germ cells and primary spermatocytes of male testes (Colozza et al. 2011). Nup154 however was not found to interact physically with p-Mad, and the mechanism behind this import defect is not fully understood. Furthermore, this role does not appear to be unique to Nup154, as additional *Drosophila* Nups, such as Nup75, Nup93, Sec13, Nup205, and Nup50 were discovered in an RNAi screen for proteins required for nuclear Mad accumulation in *Drosophila* S2 culture cells (Chen and Xu, 2010). Levels of both p-Mad and Msk, the importin responsible for p-MAD import, were found to remain normal in cells depleted for these Nups, indicating that the observed loss of p-Mad from the nucleus was specifically due to reduced translocation and not protein stability. Similarly to the *Nup154* mutant phenotype in the germline (Gigliotti et al. 1998), general protein import was not disrupted upon knock-down of these Nups in culture cells. Interestingly, addition of a classical NLS to Mad rescued nuclear localization of p-Mad in Nup-depleted cells, suggesting that this select group of Nups play a role in a non-canonical mechanism of import utilized for activation-induced translocation of p-Mad.

In addition to Nup154, several other NPC components have been implicated in *Drosophila* oogenesis and germ cell development. A separate genetic screen for sterility associated with small gonads yielded a mutation in the *Nup96-nup98* gene, which disrupts both Nup96 and Nup98 proteins and results in a progressive loss of germ cells in both male and female gonads during adult lifespan (Parrott et al. 2011). This progressive loss was linked to increased rates of differentiation, at the expense of germ cell self-renewal, suggesting that either Nup96 or Nup98 regulate stem cell maintenance in the adult gonad. Additionally, a mutation in the *Drosophila* Nup107-160 sub-complex component Seh1 was found to specifically

affect oogenesis, similarly resulting in a differentiation defect, which led to an aberrant differentiation of oocytes into a related lineage of nurse cells (Senger et al. 2011). Whether the mechanisms behind these phenotypes are related to specific transport events, such as those suggested for Nup154, or to other Nup-associated functions, such as chromatin regulation, remains to be determined.

5.4 Chromatin-Binding Roles of *Drosophila* Nups in Gene Regulation

Multiple studies in yeast have identified the ability of Nups to interact with chromatin and to recruit inducible genes undergoing activation to the NPC (Ptak et al. 2014; Akhtar and Gasser 2007). Mechanistically, these investigations focused on the connection between transcription and mRNA export, proposed by the "gene gating hypothesis" and on the role of Nups in epigenetic memory of previous transcriptional activation, known as transcriptional memory (Tan-Wong et al. 2009; Rodriguez-Navarro et al. 2004; Light et al. 2013; Kohler et al. 2008; Cabal et al. 2006). These functional implications of yeast Nups in gene regulation have suggested that NPC-genome interactions may also play critical roles in the establishment of gene expression programs during multicellular development. Investigations in *Drosophila*, discussed below, have identified roles for NPC components in transcriptional and epigenetic phenomena, such as dosage compensation of the X chromosome, maintenance of tissue-specific expression by Trithorax Group (TrxG) proteins and transcriptional memory of hormone-inducible genes. Chromatin-binding roles of Nups are likely to underlie some of the reported phenotypes of Nups, described in the previous section, and below we discuss examples, where such roles have been suggested as the main mechanism by which Nups execute a particular physiological function.

5.4.1 Nups Interact with Chromatin On and Off the NPC

Analysis of chromatin binding behavior of *Drosophila* Nups, achieved by various genome-wide methods such as immunostaining of polytene chromosomes, ChIP and Dam-ID, revealed the presence of NPC components at a number of genomic locations, which showed enrichment for active genes (Capelson et al. 2010; Kalverda et al. 2010; Vaquerizas et al. 2010). These studies demonstrated that similarly to its yeast counterparts, multiple *Drosophila* Nups exhibit chromatin-binding behavior, but with an unexpected twist. Surprisingly, Nup-chromatin contacts were commonly found to occur in the nucleoplasm, away from the NE-embedded NPCs. This is possible because as discussed above, a subset of Nups has been classified as dynamic and able to exist in a nucleoplasmic pool (Rabut et al. 2004). Consistently, the Nups identified to participate in intra-nuclear chromatin binding were predominantly dynamic Nups, such as Nup98, Nup62,

Nup50, Nup153 and Tpr, as well as Sec13, which although not considered dynamic, has been reported to have an intranuclear population (Enninga et al. 2003). These findings were revealed by diverse methods in multiple cell types (Capelson et al. 2010; Kalverda et al. 2010; Vaquerizas et al. 2010), arguing for the generality of this phenomenon. For instance, an elegantly designed DamID approach, which mapped genomic binding of full-length Nup98 as well as Nup98 missing its C-terminal NPC-targeting domain in fly embryonic culture cells, was able to distinguish between genomic binding of NPC-associated versus nucleoplasmic Nup98 and to demonstrate the common occurrence of intranuclear Nup98-genome contacts (Kalverda et al. 2010). The ability of Nups to interact with genes in the nucleoplasm has now been also identified in mammalian cells (Franks et al. 2016; Jacinto et al. 2015; Liang et al. 2013; Light et al. 2013), thus it appears to be an evolutionarily conserved phenomenon and may be unique to metazoan cells.

Importantly, these reports also demonstrated a functional role of chromatin-bound Nups in transcriptional activity of its target genes. In polytenized tissues of *Drosophila* larvae, Nup98 and Sec13 were identified at loci of the *Ecdysone-induced protein 74* and *75* (*Eip74* and *Eip75*) genes, which are induced by a developmental steroid hormone ecdysone (Capelson et al. 2010). Tissue-specific RNAi knock-downs of Nup98 and Sec13 during the developmental stage when *Eip74* an *Eip75* are highly induced, resulted in reduced levels of phosphorylated RNA Polymerase II (RNAP II), decreased chromatin de-condensation and reduced mRNA output specifically at the *Eip74* and *Eip75* genes. Functional involvement of Nup98 in gene activation was similarly revealed by modulating levels of Nup98 in culture cells, via over-expression or RNAi depletion, which increased and decreased expression of a subset of its target intranuclear genes, respectively (Kalverda et al. 2010). Thus, the widespread genomic binding of *Drosophila* Nups, a large fraction of which occurs in the nuclear interior, is likely to contribute to functional regulation of genome activity.

Together, these studies suggested that Nups regulate gene activity independently of NPC localization, effectively extending the reach of the previously proposed "gene gating hypothesis." Since the majority of active genes are transcribed in the nuclear interior, these findings can overcome a major limitation of the "gene gating hypothesis," which argues that active genes have to be repositioned to the NPCs at the NE in order to be regulated by NPC components. Instead, dynamic Nups are able to come off the NPC to affect a larger pool of transcribing genes throughout nuclear space. On the other hand, the existence of Nup-chromatin contacts in the nucleoplasm calls into question one of the major premises of the "gene gating hypothesis": the coupling between transcription and mRNA export as the main cellular "reason" for recruiting genes to the NPC. It is less obvious how such a reason would apply to genes bound by Nups in the nuclear interior, suggesting that there is an additional, transport-independent function that Nups perform at active genes. Such functions have been proposed to involve regulation of chromatin, genome architecture and epigenetic memory, as discussed in the sections below.

5.4.2 Drosophila *Nups and Dosage Compensation Machinery*

Dosage compensation is a process by which organisms equalize expression differences derived from the unequal number of X chromosomes between the sexes. Dosage compensation in *Drosophila* occurs via a two-fold transcriptional upregulation of the male X chromosome (Mendjan and Akhtar 2007). The male X chromosome is maintained in this transcriptionally hyper-activated state by a chromatin regulatory complex Males Specific Lethal (MSL), which includes five core protein members and is associated with noncoding RNAs *rox*1 and *rox2*. The enzymatic component of the MSL complex is a HAT, termed Males absent On the First (MOF), which acetylates the histone H4 lysine 16 (H4K16) residue (Gelbart et al. 2009). The MSL core components MSL1, MSL2, MSL3, MOF and MLE were originally identified genetically, based on their phenotypes of being essential for dosage compensation in fly males (Gorman and Baker 1994). Interestingly, subsequent biochemical purifications of the tagged core components of the MSL complex, such as MOF and MSL3, from fly culture cells and embryos consistently yielded substantial amounts of several Nups, including Nup153, Mtor, Nup98, Nup160 and Nup154 (Mendjan et al. 2006). The interaction between MOF and Mtor was found to be evolutionarily conserved, since Tpr, the human homologue of Mtor, was similarly co-purified with MOF from HeLa cells extracts (Mendjan et al. 2006). Furthermore, depletion of either Mtor or Nup153 resulted in compromised localization of MSL proteins to the X chromosome and in lower expression of the X-linked genes in male culture cells, suggesting that Mtor and Nup153 are functionally involved in transcription-promoting activity of the MOF-containing complex (Mendjan et al. 2006). Together, these findings brought forth the possibility that metazoan cells utilize Nups to execute their complex epigenetic phenomena, and that similarly to yeast, these functions appear to involve physical interactions between nuclear basket Nups and HAT complexes.

A subsequent study of the MSL-driven dosage compensation in vivo failed to detect any obvious roles of Nup153 and Mtor in the recruitment of the MSL complex components to the X chromosome (Grimaud and Becker 2009). Specifically, RNAi against Mtor and Nup153 did not disrupt X chromosome targeting of at least one MSL component, MSL2 in several tested tissues of male fly larvae. Thus it remains unclear whether nuclear basket Nups functionally contribute to dosage compensation in the developing organism, or if perhaps reported conserved interactions between MOF and Nups are involved in non-dosage compensating activities of MOF. Interestingly, detailed biochemical characterization revealed that in addition to the MSL complex, MOF is present in a related but distinct chromatin-binding complex, termed Non Specific Lethal (NSL), in both *Drosophila* and human cells (Lam et al. 2012; Raja et al. 2010). The NSL complex contains several unique components, including NSL1 and Methyl Binding Domain Related 2 (MBD-R2), and importantly, targets autosomal genes, where it is involved in transcriptional regulation (Feller et al. 2011; Raja et al. 2010). The relationship between Nups and the MOF-containing NSL complex is supported by recent

findings that *Drosophila* Nup98 physically and genetically interacts with NSL components such as MBD-R2 (Pascual-Garcia et al. 2014). MBD-R2 and Nup98 appear to co-bind many of the same target genes in S2 cells and on polytene chromosomes, and both proteins bind autosomes to a similar degree as the X chromosome. Thus it appears that Nup98 and potentially other Nups may co-function with the NSL complex in regulation of transcription, independently of dosage compensation.

On the other hand, high-resolution chromatin binding analysis demonstrated that Mtor and Nup153 are particularly enriched on the male X chromosome relative to autosomes (Vaquerizas et al. 2010). This binding pattern at least indirectly suggests that Nups do indeed play a role in promoting the specialized transcriptional state of the male X. This notion is further supported by a recent study that remarkably, has also linked dosage compensation in *Caenorhabditis elegans* to the nuclear pore (Sharma et al. 2014). Dosage compensation in *C. elegans* occurs by an entirely different mechanism, where expression from both X chromosomes is down-regulated two-fold in the XX-bearing hermaphrodite, and requires a protein complex that includes condensins (Csankovszki 2009). Interestingly, the un-compensated and more active male X chromosome was found to associate with NPCs in nuclear space and to display enriched binding of the Elys homologue Mel-28, as assessed by genome-wide DamID (Sharma et al. 2014). The interaction between the X chromosome and the NPCs was antagonized by the presence of the silencing dosage compensating machinery in the hermaphrodite, suggesting that the NPC functions as an activity-promoting nuclear environment. A similar role for Nups in promoting global X chromosome activity via providing a permissive nuclear compartment or scaffold may be conserved in *Drosophila* (Fig. 5.1). In support of this idea, Nup153 and Mtor bind chromatin not as discrete peaks, but as large megabase-long domains, termed Nucleoporin Associated Regions (NARs) (Vaquerizas et al. 2010), representative of a potential scaffolding function. Beyond dosage compensation, these studies have highlighted the relationship between Nups and epigenetic machinery, and this connection is likely to be a component of tissue-specific gene regulation.

5.4.3 *Physiological Gene Targets of* Drosophila *Nups*

Genome-wide binding analysis of Nup98 by DamID and ChIP in culture cells identified on the order of 1,000 genes as binding targets of Nup98, which are frequently co-occupied by other Nups such as Nup50 or Nup62 (Capelson et al. 2010; Kalverda et al. 2010; Pascual-Garcia et al. 2014). Aside from the X chromosome dosage compensation mechanisms described above, a key question in the field is what defines the subset of genes that are subject to regulation by Nups. Gene ontology analysis has identified genes that regulate cell cycle and tissue-specific development as enriched categories among genome-wide targets of Nup98 (Kalverda et al. 2010). Consistently, Nup98 has been implicated in direct

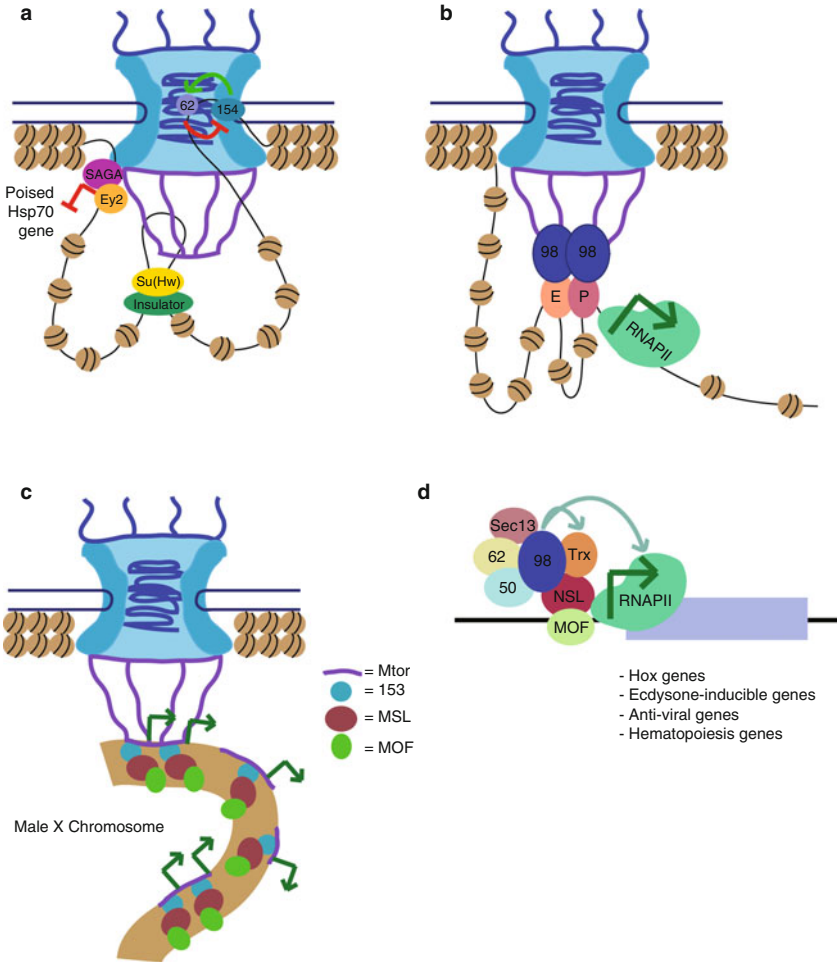

Fig. 5.1 Roles of NPC components in regulation of chromatin and transcription. (a) Schematic summary of NPC-genome interactions in non-transcribing processes, including tethering of poised *hsp70* gene to the NPC, targeting binding sites of insulator proteins, such as Su(Hw), and proposed chromatin binding of Nup154 being regulated by Nup62. (b) Model for the proposed role of Nup98 and the NPC in scaffolding activation-driven enhancer-promoter contacts of inducible genes. (c) Possible model for the relationship between nuclear basket Nups and dosage compensated male X chromosome, showing the binding of Mtor in extended domains along the X chromosome and the identified interactions between the MOF/MSL complex and Mtor and Nup153; these interactions may provide a permissive environment or structural scaffolding for the hyper-activated X chromosome. (d) Schematic summary for the roles of dynamic Nups in transcription, showing the identified interactions between Nup98 and the Trx and MOF/NSL complexes, and their proposed co-functioning in gene activation; below is the list of gene classes that are regulated by Nups in *Drosophila*. Beige circles represent the nucleosomes, green arrows represent active genes, and red lines represent silent genes. Please see text for specific references

regulation of at least two sets of developmentally critical genes. The first set is the ecdysone-inducible genes, discussed above, which exhibit robust binding of Nup98, Sec13 and mAb414-recognized FG Nups, and depend on Nup98 and Sec13 for normal transcriptional activation in larval development (Pascual-Garcia et al. 2017; Capelson et al. 2010). The second set of developmental genes regulated by Nup98 is the *Hox* or *Homeotic* genes, which are conserved regulators of tissue identity. During *Drosophila* development, *Hox* genes are expressed in precise tissue-specific patterns to define future identity of precursor structures known as imaginal discs, which will give rise to adult body parts. In particular, *Hox* gene *Ultrabithorax* (*Ubx*) is highly expressed in the haltere imaginal disc, and this expression is epigenetically maintained by a histone methyl transferase (HMT) Trithorax (Trx) (Schuettengruber et al. 2007; Ringrose and Paro 2007). It has been shown that depleting Trx during development, after the initial specification of *Hox* gene expression patterns, leads to cells "forgetting" the Hox expression profile of their lineage and to the resulting mis-specification of tissue identity, known as homeotic transformations (Grimaud et al. 2006). Interestingly, depletion of Nup98, but not of Nup107, in the haltere imaginal disc was found to result in down-regulation of *Ubx*, in a stochastic pattern reminiscent of the loss of Trx (Pascual-Garcia et al. 2014). Furthermore, over-expression of Nup98 was shown to genetically interact with *trx*, and to exhibit a homeotic transformation of the haltere into wing, a known consequence of insufficient levels of *Ubx* (Pascual-Garcia et al. 2014).

The identified role of *Drosophila* Nup98 in transcriptional regulation of *Hox* genes is particularly relevant to human cases of Acute Myeologenous Leukemia (AML), caused by translocation-derived oncogenic fusions of Nup98, which fuse the N-terminal half of Nup98 to a number of C-terminal partners (Wang et al. 2007; Gough et al. 2011). Aberrant *Hox* gene expression pattern, particularly of the *HoxA* cluster, is a hallmark of AML and is thought to underlie the loss of differentiating ability and the over-proliferation of hematopoietic precursor cells, observed in AML (Alharbi et al. 2013). In agreement with findings in the fly system, binding of fusion-derived Nup98 to *HoxA* gene clusters, as well as a functional role of Nup98 in regulation of *Hox* gene activity have been recently reported in mouse cells (Xu et al. 2016; Oka et al. 2016). These reports support the notion that Nup98 contributes to leukemigenesis and likely to hematopoietic development via direct regulation of *Hox* gene expression. Interestingly, Nup98 was identified as a key player in hematopoietic development in an unbiased screen for factors that regulate stem cell maintenance in the *Drosophila* hematopoietic organ, the lymph gland (Mondal et al. 2014). In this study, depletion of Nup98 was found to result in increased differentiation of hematopoietic stem cells and in decreased expression of key differentiation markers such as *Pvr*. The *Pvr* gene has been detected as a direct binding target of Nup98 in genome-wide studies (Capelson et al. 2010), leading the authors to suggest that Nup98 regulates transcription of key hematopoiesis genes to influence stem cell maintenance.

In addition to genes involved in regulation of tissue-specific development, Nup98 was recently shown to be required for the host-driven induction of

anti-viral genes in *Drosophila* cells (Panda et al. 2014). In response to viral infections, insect cells initiate a rapid transcriptional program, sharply up-regulating around 500 genes, as part of their host defense response (Xu and Cherry 2014). Nup98 was identified as a strong antiviral host factor, such that fly culture cells or adult organisms depleted of Nup98 were found to be more susceptible to infection against a panel of disparate RNA viruses, including human viruses Sindbis virus (SINV), vesicular stomatitis virus (VSV) and West Nile virus (Panda et al. 2014). It is of note that the life cycle of these RNA viruses takes place in the cytoplasm and does not involve nuclear import of the RNA genome or viral particles, making a transport-associated function of Nup98 less likely to play a major role in identified anti-viral activity. In support of this idea, single-molecule RNA FISH against mRNAs of antiviral genes in virus-infected Nup98-depleted cells did not identify a defect in their export, but instead revealed a reduction in their levels (Panda et al. 2014). Furthermore, Nup98 was detected at promoters of a subset of the rapidly induced anti-viral genes, and depletion of Nup98 was found to result in their compromised transcriptional up-regulation upon infection with SINV. Thus it appears that Nup98 functions as an anti-viral host defense factor via its chromatin-binding activity by promoting rapid up-regulation of an anti-viral transcriptional program.

5.4.4 *Roles of* Drosophila *Nups in Non-transcribing Chromatin*

In addition to regulation of actively transcribing genes, NPC components in yeast and mammalian cells have been implicated in binding and influencing non-transcribing regions of the genome (Jacinto et al. 2015; Van de Vosse et al. 2013; Casolari et al. 2004). *Drosophila* similarly exhibits examples of NPC-chromatin interactions that do not involve active genes (Fig. 5.1). Genome-wide DamID analysis revealed that while binding of nucleoplasmic Nups is enriched for highly transcribing genes, chromatin binding of the actual NPCs is not, and instead includes many silenced regions (Kalverda et al. 2010; Kalverda and Fornerod 2010). This observation seems counter to the idea of the NPC being a regulatory hub for actively transcribing genes, yet it is supported by similar findings in other organisms. Studies in yeast and human cells demonstrated binding of stable Nups such as Nup93, Nup155 and Nup107 at chromatin sites that show enrichment for repressive histone marks, silencing proteins or non-transcribing genes (Kehat et al. 2011; Brown et al. 2008; Casolari et al. 2004).

One possible explanation to reconcile these functionally opposing links to active vs. silent chromatin may be the binding of NPCs to poised genes that are held ready for future activation. This idea is supported by identification of certain poised genes, such as the heat shock response *hsp70* gene, being present at the NPC prior to activation in both *Drosophila* (Kurshakova et al. 2007) and yeast cells (Woolcock et al. 2012). Additionally, during developmental progression of the ecdysone-induced transcriptional program in salivary glands, Nup98 and Sec13 were located at the ecdysone-inducible genes before the appearance of

phosphorylated RNAP II, suggesting that they may have a role in pre-marking or poising these genes for upcoming activation (Capelson et al. 2010). Detailed genome-wide analysis in *Drosophila* embryonic S2 cells similarly identified robust binding of Nup98 at silenced ecdysone-inducible genes (Pascual-Garcia et al. 2017). Interestingly, these genes were found to be positioned at the NE-embedded NPCs before activation with ecdysone, and to remain associated with NPCs through activation, supporting the notion that stable NPCs function in tethering silenced genes that may be readily induced (Pascual-Garcia et al. 2017).

NPC binding at *Hox* gene clusters (Pascual-Garcia et al. 2017; Kalverda et al. 2010) also occurs in the context of completely silenced *Hox* genes, which are bound by the repressive Polycomb Group (PcG) proteins in embryonic Kc and S2 cells. Given the repeatedly reported binding of Nups to *Hox* genes, discussed above, it is tempting to speculate that the NPC-*Hox* gene interaction similarly represents the ability of the NPC to function as a scaffold for silent genes that are accessible for future activation, and that this function is a critical part of developmental gene regulation.

5.4.5 *Nups as Mediators of Epigenetic Memory and Genome Architecture*

Insights into the mechanisms by which Nups may regulate transcription have come form studies of a process known as transcriptional memory, which manifests as an enhanced transcriptional response of inducible genes after they have been previously activated by the inducing agent (Light et al. 2013). This enhanced transcriptional output in subsequent rounds of activation is thought to represent the primed or "remembered" state of recently transcribed genes, which may underlie the ability of cells to adapt to previous environmental stimuli. Importantly, the primed state of recently transcribed genes is maintained epigenetically, i.e. through cell divisions (Brickner et al. 2007). Mechanistically, it has been shown to involve the H3 K4 dimethyl (H3K4Me2) histone modification and the persistent binding of an un-phosphorylated poised form of RNAP II at gene promoters, and these events were found to depend on Nup98 in yeast and human culture cells (Light et al. 2013).

Identification of Trx as an interacting partner of *Drosophila* Nup98 (Pascual-Garcia et al. 2014) supports the reported role of Nup98 in H3K4Me2 deposition, since Trx and its human homologue Mixed lineage leukemia 1 (MLL1 or MLL) are specifically involved in catalyzing this modification (Rickels et al. 2016). In further support of this interaction, a sequence analysis of protein domain architecture of Nups across a large range of eukaryotes reported a repeated evolutionary occurrence of Nup98 homologues containing a SET domain, a conserved HMT domain that is present in Trx and MLL (Katsani et al. 2014). Interestingly, aberrant fusions of human MLL are another set of common genetic translocations that lead to leukemia phenotypes that are highly similar to those of Nup98 fusions

(Canaani et al. 2004; Hayashi 2000). Recently, the identified interactions of *Drosophila* Nup98 with the NSL and Trx/MLL complexes were found to be conserved in mammalian cells (Xu et al. 2016). Consistently, therapeutic agents developed against MLL-based leukemias have been shown to also effectively target Nup98 fusion-transformed cells (Deshpande et al. 2014). Together, these findings support the notion that Nup98 is involved in epigenetic regulation across species and that histone methylation is an integral part of the gene regulatory mechanisms of Nups.

Multiple studies have also linked NPC components to regulation of topological genome architecture, which is thought to be a key aspect of gene expression control. Genome architecture involves long-range genomic contacts, which lead to formation of gene loops and of topologically associated domains (Pombo and Dillon 2015). Interestingly, while *Drosophila* nuclear basket Nup Mtor binds chromatin in long NAR domains in fly cells (Vaquerizas et al. 2010), its yeast homologue Mlp1/2 was shown to be required for the formation of a transcriptional 5′-3′ gene loop at a galactose-inducible gene (Tan-Wong et al. 2009). In further support of an architectural role for NPC components, *Drosophila* Nups, such as Elys, Nup93 and Nup98, were identified at promoters and enhancers of multiple genes, and in line with this pattern of binding, Nup98 was found to be required for the formation of enhancer-promoter loops at ecdysone-inducible genes (Pascual-Garcia et al. 2017). This architectural role of Nup98 appears to be again linked to transcriptional memory, in this case of ecdysone-inducible genes in S2 cells, where Nup98 is specifically required for the higher levels of the later as opposed to initial inductions. Intriguingly, the high-frequency enhancer-promoter contact of the *Eip74* gene, formed as a result of ecdysone-driven activation, persisted after the transcriptional shut-off in an Nup98-dependent manner. This observation implies that stabilization of enhancer-promoter loops by NPC components may be a mechanism of epigenetic transcriptional memory (Pascual-Garcia et al. 2017). The role of Nup98 in enhancer-promoter looping is further supported by a recent study of *Drosophila* architectural proteins, which reported the prevalence of Nup98 at the bases of loops, detected by genome-wide chromosome conformation capture experiments (Cubenas-Potts et al. 2017). Furthermore, widespread targeting of strong tissue-specific enhancers, known as super-enhancers, to nuclear pores was recently observed in human cells (Ibarra et al. 2016), suggesting that the enhancer-looping function of Nups may be also be utilized in mammals.

The role of the NPC in genome architecture is also supported by previous links of Nups to insulators or boundary elements. Insulators are regulatory genomic elements that partition chromatin domains of different expression states and help establish topological genome architecture by scaffolding genomic loops (Yang and Corces 2012). Analysis of NPC binding sites in the *Drosophila* genome identified an enrichment of DNA binding motifs of a well-characterized insulator or architectural protein, Su(Hw), among NPC-genome contacts sites (Kalverda and Fornerod 2010). This link is in line with the previously discovered ability of ectopically tethered yeast Nup2 to function as a heterochromatin-impeding boundary (Ishii et al. 2002). Furthermore, ecdysone-dependent physical interactions between Nup98 and a number of architectural proteins, such as Su(Hw) and CTCF, have

been reported (Pascual-Garcia et al. 2017). The biophysical properties of FG domains within transcription-linked Nups, such as Nup98 may be particularly interesting to explore in this context, since recent studies have highlighted the importance of intrinsically disordered proteins in genome architecture and transcriptional control (Dunker et al. 2015; Hnisz et al. 2017). It remains to be determined how the architectural function of Nups is integrated with their connection to histone methylation, but together, these studies implicate Nups as key players in transcriptional memory via maintenance of gene looping conformations and of epigenetic marks.

5.5 Conclusions and Perspectives

Numerous studies in *Drosophila* and other organisms over the last 15 years have demonstrated the roles of Nups in physiological processes, such as tissue-specific development and immune response, and in regulation of transcription and chromatin. In the future, the field is likely to focus on the question of mechanism, i.e. which specific function of Nups, be it gene regulatory, transport-related or mitotic (Bukata et al. 2013), contributes to a given developmental or physiological role. This will be particularly relevant to cases of human disease that are linked to Nup mutations. For instance, while ALS pathogenesis appears to involve transport functions of Nups, AML is likely associated with chromatin-binding roles of Nup98. Specific *Drosophila* Nups have been identified as hits in genome-wide and targeted screens for regulators of key developmental signaling pathways, including TGF-β and Notch (Chen and Xu 2010; Saj et al. 2010). As described above, select Nups appear to contribute to these signaling pathways via their transport functions, but it remains possible that given the abundance of developmental genes among direct binding targets of the NPC, Nups also tune developmental signaling by binding to target genes of nuclear effectors. A particularly intriguing possibility is that metazoan NPCs couple the nuclear entry of signaling effectors to their respective target genes, thus integrating transport and chromatin-binding functions of the NPC, but this notion awaits future investigation.

One functional parallel that may be drawn between known gene targets of fly Nups is the need for rapid induction, suggesting that like in yeast, *Drosophila* NPC components preferentially target inducible genes that have to respond rapidly and robustly to external signals in their environment. In yeast cells, this signal can be an alternative carbohydrate source such as galactose, whereas steroid hormones or viral pathogens can constitute such signals in metazoan cells (Fig. 5.1). The proposed role of Nup98 and other Nups in epigenetic processes, discussed above, suggests that the primary function of Nups at inducible genes may be to enable an optimized transcriptional response and to facilitate the cellular memory of an external or developmental signal. This function of Nups appears to be linked to deposition of specific histone methyl marks and to stabilization of topological gene looping conformations. Whether these represent two distinct roles of NPC components or one role is a consequence of the other will be determined by future

studies, but the function of Nups in epigenetic memory is an intriguing emerging aspect of multicellular development and of organism-environment interactions.

Another question that remains is the dynamic relationship between NPC-bound and intranuclear gene targets of Nups. It is currently unclear whether Nups play a role in re-positioning of genes between the nuclear periphery and the nucleoplasm, or whether dynamic Nups actively shuttle between intra-nuclear chromatin binding sites and the NPC. Furthermore, which NPC components are responsible for gene recruitment to the NE-embedded NPCs is unknown. Interestingly, *Drosophila* Nup154 has been recently implicated as the primary tether of chromatin to the NE-embedded NPCs (Breuer and Ohkura 2015). It appears that recruitment of Nup62 by the Nup154-Nup93 stable sub-complex suppresses excessive chromatin attachment to the NPCs, since depletion of Nup62 in gonadal cells results in aberrant localization of chromatin to the nuclear periphery, while co-depletion of Nup154 rescues this phenotype (Breuer and Ohkura 2015). A related mechanistic question, crucial for further understanding of gene regulatory roles of Nups, is how Nups are actually recruited to chromatin. Since the majority of Nups, including Nup154, lack any identifiable chromatin-binding or DNA-binding domains, they likely employ adaptor proteins to mediate their targeting. Investigation of these questions will allow for better understanding of the functions of NPC components in tissue-specific development, evolutionary processes, immune responses and human disease, all of which appear to utilize Nups as regulatory modules.

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Chapter 6
Caenorhabditis elegans Nuclear Pore Complexes in Genome Organization and Gene Expression

Celia María Muñoz-Jiménez and Peter Askjaer

Abstract The nuclear pore complex (NPC) serves as gateway for transport between the cytoplasm and the nucleus and its structure as well as individual components (nucleoporins or nups) are conserved among all eukaryotes, suggesting they evolved in an ancient common ancestor. In addition to their role in nucleocytoplasmic transport, nups located either at NPCs or in the nucleoplasm participate in regulation of gene expression, DNA repair and chromosome segregation during cell division. Far from being a static structure, recent studies have demonstrated that alterations in NPC composition or function occur as consequences of normal cell differentiation, physiological aging and disease. In this review, we discuss how the popular model organism *Caenorhabditis elegans* has contributed to our understanding of NPC biogenesis and function from single cell resolution in young embryos to organismal homeostasis in adults.

Keywords Caenorhabditis elegans · development · gene expression · NPC · npp · nuclear organization · nuclear pore complex · nucleocytoplasmic transport · nucleoporin

6.1 Introduction

As in other eukaryotes, the nuclear envelope (NE) of the nematode *Caenorhabditis elegans* is a double lipid bilayer composed of the outer nuclear membrane (ONM), which is continuous with the endoplasmic reticulum (ER), and the inner nuclear membrane (INM) (Cohen-Fix and Askjaer 2017) (Fig. 6.1a). The ONM and the ER have similar composition, whereas the INM contains proteins involved in chromatin

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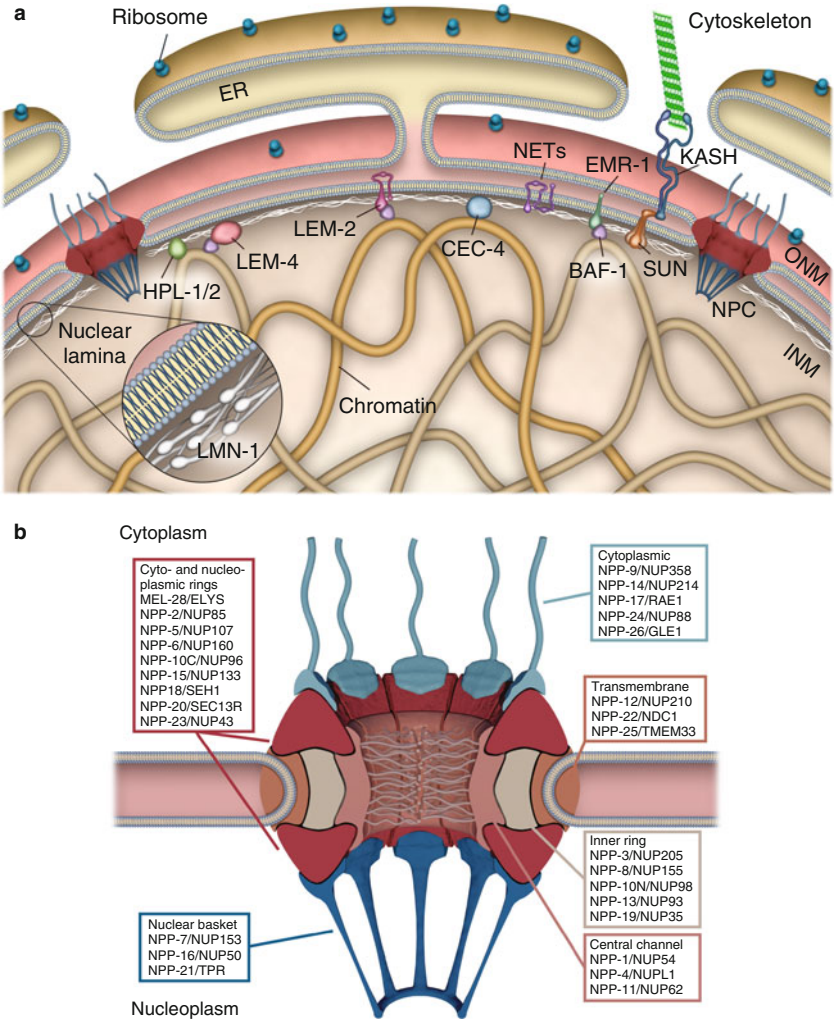

Fig. 6.1 Schematic overview of nuclear envelope and NPC organization in *C. elegans*. (a) The nuclear envelope (NE) consists of outer and inner nuclear membranes (ONM and INM, respectively) that fuse where nuclear pore complexes (NPCs) form transport channels between the cytoplasm and the nucleus. The ONM is continuous with the endoplasmic reticulum (ER) and connects to the cytoskeleton. The nuclear lamina is a network of multimerized LMN-1 protein underneath the INM. Many transmembrane proteins (NETs) accumulate specifically in the INM, including the LEM domain proteins EMR-1 and LEM-2 that associate with chromatin via LMN-1 and the BAF-1 protein. Other proteins involved in anchoring of chromatin to the NE include CEC-4 and HPL-1/2 whereas LEM-4 acts in post-mitotic NE assembly. (b) The NPC is built from multiple copies of ~30 proteins called nucleoporins (nups) that localize to distinct parts of the structure. Overall, transmembrane nups are involved in anchoring of NPCs whereas the symmetric cytoplasmic and nucleoplasmic rings serve as important structural scaffolds. The inner ring anchors the nups of the central channel, which together with the peripheral cytoplasmic and nucleoplasmic structures are involved in translocation of substrates through the NPC. Note that many nups have additional functions outside these categories, including in the nucleoplasm. The relative positions of *C. elegans* nups within the NPC are inferred from their yeast and vertebrate orthologs. (Panels modified from Cohen-Fix and Askjaer (2017))

interactions and in anchoring of transcription factors and signaling molecules. Underlying the INM is the nuclear lamina, a protein meshwork, which is required for the recruitment of INM proteins, the maintenance of nuclear morphology (Dobrzynska et al. 2016a) and the peripheral localization of heterochromatin (Cabianca and Gasser 2016). The NE is perforated at multiple sites by nuclear pore complexes (NPCs) that are gateways for transport in and out of the nucleus (Cohen-Fix and Askjaer 2017). NPCs are among the largest protein structures in the cell and understanding their assembly and mode of action are fascinating challenges. Traditionally, the NE has been considered as a passive barrier that separates the nucleus from the cytoplasm. However, nowadays, the NE and NPCs are known to be involved in a variety of processes, such as transcription, DNA repair and chromatin dynamics as well as cell signaling, mechanosensation and apoptosis.

Several components of the NPC, as well as proteins that localize at the inner NE surface, such us lamins and lamina-associated proteins, interact with chromatin in a dynamic way and regulate chromatin distribution inside the nucleus. Specifically, distal arms of *C. elegans* chromosomes have higher density of repeats and are placed near the nuclear periphery whereas the chromosome centers are positioned in the nuclear interior (Ikegami et al. 2010). Chromatin is organized in domains that share common features, such as lamina-associated domains (LADs) (Gonzalez-Aguilera et al. 2014a). Theses domains are enriched in repressive histone modifications and transcriptional activity of genes within the domains are generally low (Gonzalez-Aguilera et al. 2014a; Ikegami et al. 2010). However, not all chromatin at the NE is repressed. In yeast exist several examples of highly transcribed genes positioned at NPCs (Burns and Wente 2014) and as described below, certain gene classes might also be recruited to NPCs in *C. elegans* (Ikegami and Lieb 2013; Rohner et al. 2013).

C. elegans has many features that make this popular model organism attractive to scientists with an interest in NPC biogenesis and function. These include, but are not limited to, ease of genetic manipulations, scalability for genetic and compound screening, as well as amenability to high resolution, non-invasive live imaging throughout development (Askjaer et al. 2014b)(Fig. 6.2). In this chapter, we will focus on studies in *C. elegans* that have addressed the functions of NPC components and other NE proteins and their implication in controlling gene expression.

6.2 *C. elegans* NPC Composition

Thanks to the combination of genetic interactions, biochemical fractionation and advanced microscopy, the NPC structure has been dissected in several organisms (Hoelz et al. 2011; Knockenhauer and Schwartz 2016). The NPC is composed of several copies of ~30 different proteins, termed nucleoporins or nups. Analyses of the stoichiometry of NPCs in yeast and human cells suggest that nups are present in 8, 16, 32 or 48 copies, forming ~50–100 MDa assemblies of ~450–900 proteins (Alber et al. 2007; Ori et al. 2013). The periodicity in nup copy number reflects an

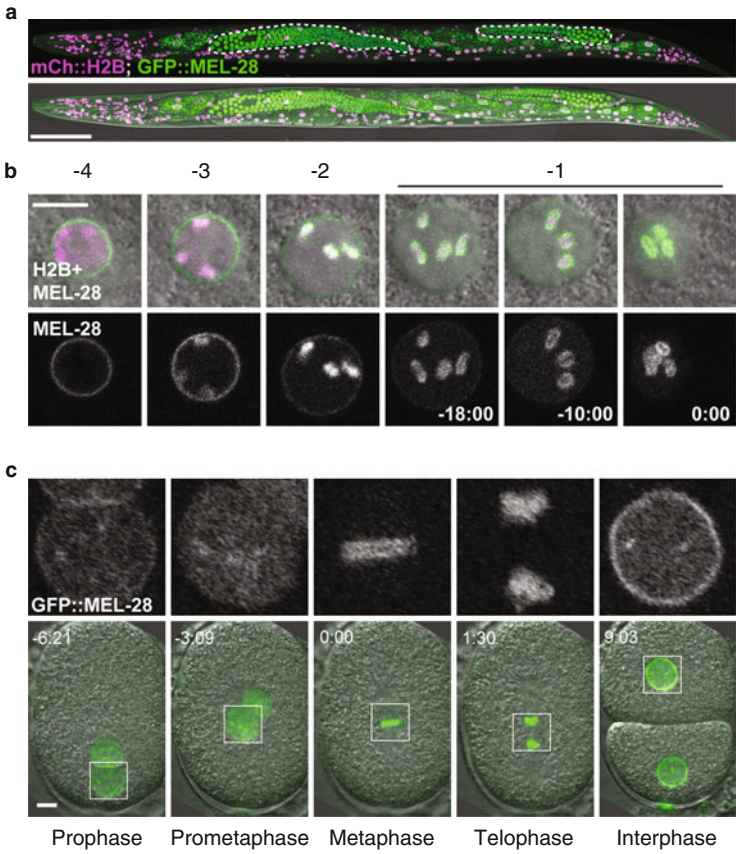

Fig. 6.2 Observation of nucleoporin dynamics from single cell to whole organism level. *C. elegans* is highly amenable to genetic manipulation and high resolution live microscopy as illustrated with these confocal images from a strain that was modified by CRISPR/Cas9 technology to tag endogenous MEL-28 with GFP and transformed to express ectopically mCherry fused to histone H2B (HIS-58). (a) Maximum projection of several confocal images of an adult animal. MEL-28 is ubiquitously expressed and most abundant in the syncytial gonads (indicated with discontinuous lines). Scale bar, 100 μm. (b) Maturing oocytes in the proximal part of the gonad. Numbers above the images refer to relative position within the gonad where −1 is immediately next to the spermatheca. Three images are shown for the −1 oocyte with time indicated relative to NE breakdown (min:sec). MEL-28 relocates gradually from the NE to the kinetochores of condensing meiotic chromosomes. Scale bar, 5 μm. (c) The fast divisions of *C. elegans* embryos make them attractive for studying mitotic processes, including NE breakdown, chromosome segregation and nuclear reassembly. Although only ~15 minutes of early development are represented here (min:sec relative to the onset of the first mitotic anaphase) the entire process of embryogenesis can be accurately observed by time-lapse microscopy. Scale bar, 5 μm. (Images in (a) and (b–c) are from Cohen-Fix and Askjaer (2017) and Gomez-Saldivar et al. (2016), respectively)

eight-fold symmetry of NPCs when observed from the cytoplasmic or nuclear side. In the plane of the nuclear membranes the NPC consists of three concentric rings: an outer ring of transmembrane nups, a middle ring of linker nups and an inner ring of nups enriched in phenylanaline-glycine (FG) di-repeats (Fig. 6.1b). These structures are associated with a cytoplasmic and a nucleoplasmic ring as well as cytoplasmic fibrils and a nucleoplasmic basket structure. Although the primary sequences of nups have diverged during evolution, their secondary structures are generally well conserved across all eukaryotes (Devos et al. 2006; Knockenhauer and Schwartz 2016). Specifically, the majority (28/33) of human nups have conserved homologs in *C. elegans* where most are known as *npp* (*n*uclear *p*ore *p*rotein) genes (Askjaer et al. 2014b; Galy et al. 2003; Gonzalez-Aguilera and Askjaer 2012) (Table 6.1). An interesting exception is NUP188, which is present in yeast, flies and vertebrates but not in nematodes. Vertebrate NUP188 is structurally similar to NUP205 and the two nups interact in a mutually exclusive way with the vertebrate linker nups NUP35, NUP93 and NUP155 to form stable complexes (Vollmer and Antonin 2014). It is therefore likely that NUP188 and NUP205 evolved by gene duplication in a eukaryotic ancestor and only NUP205 was maintained in *C. elegans*. Similarly, metazoan NUP35 and NUP155 each have two homologs in budding yeast (Nup53/Nup59 and Nup157/Nup170, respectively (Vollmer and Antonin 2014).

C. elegans nups are expressed throughout development. Highest expression is observed in embryos and adults (Fig. 6.3b), which are the life stages with most active cell proliferation (mitotic nuclei are very abundant in the gonads of *C. elegans* adults). The relative expression levels among nups are largely maintained during development although fluctuations are observed for a few (e.g. *npp-1/NUP54* and *npp-12/NUP210*; Fig. 6.3c). Whether these changes in mRNA abundance correlate with protein levels remains to be investigated, but they could potentially reflect differences in NPC composition among tissues or developmental stages as reported in other organisms (Raices and D'Angelo 2012). In fact, a recent dissection of transcriptional profiles of individual blastomers of 16-cell stage embryos (Tintori et al. 2016) revealed that *npp-9/NUP358*, *npp-16/NUP50* and *npp-22/NDC1* are enriched in the germline blastomere P4 whereas expression of several other nups is decreased in specific cells, in particular the D cell that give rise to 20 body wall muscle cells (Fig. 6.3a). These observations should be corroborated analyzing protein expression but serve as an interesting starting point for future investigations.

Several studies have focused on the structure and function of *C. elegans* NPCs as well as on the role of different nups in nuclear assembly. Using a combination of RNAi and mutant alleles, experiments in *C. elegans* have indeed been the first to analyze the roles of multiple nups (NUP35, NUP107, NUP155, NUP210, and others) during animal development. This has demonstrated that at least 20 *C. elegans* nups are required for embryogenesis, either alone or in a complex (Table 6.1). For instance, knockdown of NPP-3/NUP205 or NPP-13/NUP93 by RNAi shows defects in NPC distribution in the NE, abnormal chromatin condensation and early embryonic arrest (Galy et al. 2003). Moreover, depletion of

Table 6.1 *C. elegans* nucleoporins

Position[a]	Worm	Human	Frequent phenotypes[b]	Specific phenotypes	References
Cytoplasmic region	NPP-9	NUP358	Emb; Nmo; Pgl; Pvl	RNAi efficiency; spindle assembly; nuclear envelope formation	Kim et al. (2005); Sheth et al. (2010); Voronina and Seydoux (2010); Askjaer et al. (2002)
	NPP-14	NUP214	wt	Regulation of CED-3 caspase; synthetic lethal with NPP-2	Galy et al. (2003); Chen et al. (2016)
	NPP-17/ RAE-1	RAE1	Emb; Hya; Pvl; Ste; Stp	Axon termination and synapse formation	Grill et al. (2012)
	NPP-26	GLE1	wt	Abnormal distribution of recycling endosomes	Winter et al. (2012)
	NPP-24	NUP88	wt		
Cytoplasmic and nucleoplasmic rings	NPP-2	NUP85	Clr; Emb; Lva; Nmo; Pgl; Pvl; Stp	NPC assembly; synthetic lethal with NPP-5, -14, -15, -17	Rodenas et al. (2012); Galy et al. (2003)
	NPP-5	NUP107	Emb; Pgl	Interaction with spindle assembly checkpoint; kinetochore assembly	Franz et al. (2005); Rodenas et al. (2012)
	NPP-6	NUP160	Emb; Lva; Lvl; Nmo; Pgl	NPC assembly	D'Angelo et al. (2009); Rodenas et al. (2012)
	NPP-10C[c]	NUP96	Emb; Lva; Lvl; Nmo; Ste	NPC assembly; nuclear protein import	Galy et al. (2003); Rodenas et al. (2012); Ferreira et al. (2017)
	NPP-15	NUP133	Lvl	Sensitivity to ionizing radiation	D'Angelo et al. (2009); Rodenas et al. (2012); van Haaften et al. (2006)

(continued)

Table 6.1 (continued)

Position[a]	Worm	Human	Frequent phenotypes[b]	Specific phenotypes	References
	NPP-18	SEH1	wt		
	NPP-20	SEC13R	Emb; Lva; Lvl; Nmo; Pgl; Stp	Nonsense Mediated Decay of aberrant mRNA; nuclear protein import	Ferreira et al. (2017); Casadio et al. (2015)
	NPP-23	NUP43	wt		Rodenas et al. (2012)
	MEL-28	ELYS/ AHCTF1	Emb; Lva	NPC assembly; spindle assembly	Fernandez and Piano (2006); Galy et al. (2006)
Inner ring	NPP-3	NUP205	Clr; Emb; Lva; Nmo; Pgl; Ste	NPC exclusion limit; spindle orientation; timing of mitosis	Schetter et al. (2006); Hachet et al. (2012); Galy et al. (2003)
	NPP-8	NUP155	Emb; Lva; Lvl; Nmo; Pgl; Pvl	NPC assembly	Franz et al. (2005)
	NPP-10N[c]	NUP98	Emb; Lva; Lvl; Nmo; Pgl; Ste	NPC assembly; P granule integrity	Galy et al. (2003); Voronina and Seydoux (2010); Rodenas et al. (2012)
	NPP-13	NUP93	Emb; Nmo; Pgl	NPC exclusion limit; spindle orientation; timing of mitosis; nuclear protein import	Schetter et al. (2006); Hachet et al. (2012); Galy et al. (2003); Ferreira et al. (2017)
	NPP-19	NUP35	Emb; Nmo; Pgl; Stp	NPC assembly; nuclear protein import	Rodenas et al. (2012); Rodenas et al. (2009)
Central channel	NPP-1	NUP54	Emb; Lva; Lvl; Nmo; Pgl; Stp	Spindle orientation; RNAi efficiency; nuclear protein import	Kim et al. (2005); Schetter et al. (2006)

(continued)

Table 6.1 (continued)

Position[a]	Worm	Human	Frequent phenotypes[b]	Specific phenotypes	References
	NPP-4	NUPL1	Emb; Stp	Spindle orientation; transposon silencing	Schetter et al. (2006); Franz et al. (2005); Vastenhouw et al. (2003); Updike et al. (2011)
	NPP-11	NUP62	Emb; Lva; Lvl; Nmo	Spindle orientation	Schetter et al. (2006)
Transmembrane nups	NPP-12	NUP210	Emb; Lva	Nuclear envelope breakdown	Cohen et al. (2003); Galy et al. (2008); Audhya et al. (2007)
	NPP-22/ NDC-1	NDC1/ TMEM48	Clr; Emb; Lva; Lvl; Nmo; Ste	NPC assembly; modification of dynein activity	Stavru et al. (2006); O'Rourke et al. (2007)
	NPP-25	TMEM33	wt		Chadrin et al. (2010)
Nuclear basket	NPP-7	NUP153	Emb; Nmo; Lva; Pgl; Ste		Galy et al. (2003); Voronina and Seydoux (2010); D'Angelo et al. (2009)
	NPP-16	NUP50	wt	RNAi efficiency; anoxia-induced prophase arrest	Kim et al. (2005); Hajeri et al. (2010)
	NPP-21	TPR	Clr; Emb; Lva; Ste	Regulation of tumor growth and apoptosis	Pinkston-Gosse and Kenyon (2007)

No clear *C. elegans* homologues were found for the mammalian nups AAAS/ALADIN, NUP37, NUP188, NUPL2/hCG1, POM121

[a] Some nups are reported to localize to several positions within the NPC, e.g. NPP-10N/NUP98 and NPP-17/RAE1 but for simplicity each nup is only listed once

[b] Gross phenotypes, which for most genes were reported in large-scale RNAi studies. See (Galy et al. 2003) and WormBase (http://www.wormbase.org) for details and references. *Clr* clear/ transparent body, *Emb* embryonic lethal, *Hya* hyper active, *Lva* larval arrest, *Lvl* larval lethal, *Nmo* (pro-)nuclear morphology alteration in early embryo, *Pgl* P-granule abnormality, *Pvl* protruding vulva, *Ste* sterile, *Stp* sterile progeny, *wt* wild type. Abnormal P granule distribution (Pgl) was observed for many *npp* genes (Updike and Strome 2009; Voronina and Seydoux 2010)

[c] Because NPP-10N and NPP-10C are produced from a single protein precursor, a given RNAi phenotype will generally reflect the combined effect of depleting both proteins. P granule phenotypes are, however, specific to NPP-10N depletion

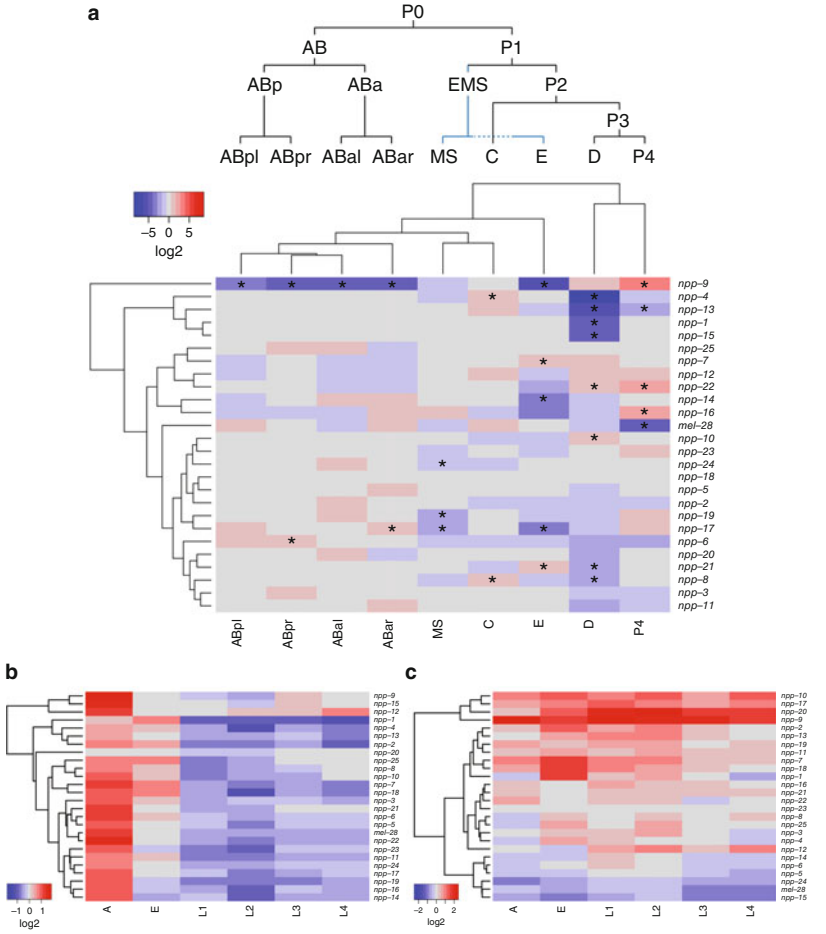

Fig. 6.3 Expression of *C. elegans* nups during development. (a) Heatmap showing average expression in different blastomers of 16-cell stage embryos; D and P4 are present as individual cells whereas other blastomers are present as pairs of sister cells. Shown are log2 values of fold change relative to average expression in whole 16-cell stage embryos. Asterisks indicate *P*-values < 0.05. Note that the unbiased dendrogram generated by the package "gplots" in RStudio correlates very well with the cell lineages indicated above, suggesting that cell-specific transcription profiles are partly inherited from the preceding developmental stages. (Data from (Tintori et al. 2016)). (b–c) Heatmaps showing median expression in adults (A), embryos (E) and larval stages L1–L4. Shown are log2 values of fold change relative to expression across all developmental stages (b) or relative to expression of *npp-23/NUP43* (c). (Data from modENCODE (Gerstein et al. 2010)). The expression levels reported for *npp-26/GLE1* are very low and were omitted from the analysis represented in this figure

NPP-3/NUP205 or NPP-13/NUP93 causes a breach in the NPC permeability barrier. Based on homology to their yeast and vertebrates counterparts, NPP-3/NUP205 and NPP-13/NUP93 presumably form a complex (the NUP93 complex in vertebrates) with NPP-8/NUP155 and NPP-19/NUP35 and together they

constitute the NPC middle ring of linker nups (Fig. 6.1b). Embryos lacking NPP-19/NUP35 or NPP-8/NUP155 are characterized by even stronger defects, including inhibition of NPC and nuclear lamina assembly as well as chromosome missegregation during mitosis, eventually leading to early embryonic death (Franz et al. 2005; Rodenas et al. 2009). Analyzing a temperature-sensitive loss-of-function allele of *npp-19*, it was proposed that NPP-19/NUP35 is particularly important for NPC assembly during the rapid cell divisions of early embryogenesis, suggesting that redundant mechanisms may alleviate the absence of NPP-19/NUP35 when mitosis slows down (Rodenas et al. 2009). In vertebrates, the NUP93 complex is anchored to the NPC via the transmembrane nup NDC1 (Mansfeld et al. 2006) and depletion of NPP-22/NDC1 in *C. elegans* is embryonic lethal and impedes NPC assembly as judged from reduced staining with the general NPC antibody mAb414 (Stavru et al. 2006). The sequence conservation of transmembrane nups between species is generally low, including vertebrate NDC1 and *C. elegans* NPP-22/NDC1 (BLAST e-value at WormBase $\sim 1e^{-21}$). An exception is NPP-12, which is highly similar to vertebrate NUP210 ($\sim 1e^{-135}$). NUP210 is a single-pass transmembrane glycoprotein and is expressed only in certain cell types (Olsson et al. 2004) where it is involved in cell differentiation (D'Angelo et al. 2012). As noted above, expression of *npp-12* differs between life stages of *C. elegans* (Fig. 6.3b), suggesting that it might have a specific role(-s) during development. In addition, depletion of NPP-12/NUP210 affects NE breakdown and depolymerization of the nuclear lamina in 1-cell stage *C. elegans* embryos, resulting in the formation of daughter cells with two haploid nuclei ("twinned nuclei") (Audhya et al. 2007; Galy et al. 2008).

One of the most studied NPC subcomplexes is the NUP107 complex, also known as the Y complex because of its three dimensional structure (Stuwe et al. 2015; Gonzalez-Aguilera and Askjaer 2012). The complex consists of 7-8 nups in yeast, 9 in nematodes and 10 in vertebrates and forms the cytoplasmic and nucleoplasmic rings, each containing 16 copies of the complex (Fig. 6.1b). Biochemical depletion of the NUP107 complex from Xenopus egg extracts prevents NPC assembly, but co-regulation of its individual subunits in mammalian cells has complicated assignment of functions to specific nups (Walther et al. 2003; Harel et al. 2003; Lupu et al. 2008). However, *C. elegans npp-5/NUP107* null mutants express normal levels of other NUP107 complex subunits and their recruitment to NPCs is normal (Rodenas et al. 2012). This was an unexpected finding because NUP107 is located in the "stalk" of the Y, forming a bridge between NUP96 and NUP133 and suggests that extensive head-to-tail interactions between individual NUP107 complexes are sufficient to tether the remaining nups to NPCs in the absence of NPP-5/NUP107 (see also (Stuwe et al. 2015)). During mitosis the NUP107 complex relocates to kinetochores and regulates their composition in vertebrates and nematodes (reviewed in (Gonzalez-Aguilera and Askjaer 2012)). Moreover, the spindle assembly checkpoint is compromised in the absence of NPP-5/NUP107 and *npp-5/NUP107* mutants die during embryonic or larval development (Rodenas et al. 2012). Depletion of another member of the NUP107 complex, the large protein MEL-28/ELYS, leads to a strong block in NPC assembly,

defective chromosome segregation and early embryonic arrest (Fernandez and Piano 2006; Galy et al. 2006). To dissect MEL-28/ELYS's functions, recent studies have identified short conserved elements in MEL-28/ELYS that are required for efficient separation of meiotic chromosomes and nuclear assembly (Hattersley et al. 2016; Gomez-Saldivar et al. 2016). This led also to the identification of the protein phosphatase 1 catalytic subunit GSP-2 as a direct interaction partner of MEL-28/ELYS (Hattersley et al. 2016). Many nups and other NE proteins are phosphorylated in mitosis to facilitate NE breakdown and binding of GSP-2 to MEL-28/ELYS is required in ana- and telophase for kinetochore disassembly and nuclear assembly, possibly coordinating the two processes (Hattersley et al. 2016). Moreover, MEL-28/ELYS has also been implicated in the coordination between chromatin decondensation and DNA replication (Sonneville et al. 2015).

Several studies have identified protein kinases and other enzymes that are required for NPC and NE disassembly. Similarly to the twinned nuclei phenotype in *npp-12/NUP210* embryos described above, depletion of the LPIN-1/LIPIN phosphatidic acid phosphohydrolase delays disassembly of the nuclear lamina and NPCs (Golden et al. 2009; Gorjanacz and Mattaj 2009). Mechanistic insight as to how LPIN-1 controls NE breakdown is still lacking, but might involve the absorption of NE components into the ER. The exact relationships between protein kinases and their targets during NE breakdown are also poorly understood, but inhibition of AIR-1/Aurora A, NCC-1/CDK-1, PLK-1/Polo-like kinase, and VRK-1/Vaccinia-Related kinase 1 all inhibit NEBD (Portier et al. 2007; Boxem et al. 1999; Chase et al. 2000; Hachet et al. 2007; Rahman et al. 2015; Gorjanacz et al. 2007; Noatynska et al. 2010; Tavernier et al. 2015; Martino et al. 2017). Interestingly, AIR-1 acts partially by releasing NPP-3/NUP205, NPP-13/NUP93 and NPP-19/NUP35 from the NE in the vicinity of centrosomes (Hachet et al. 2012), whereas the small DNA-binding protein BAF-1/BANF1 is phosphorylated by VRK-1 at mitotic entry to release NE components (Gorjanacz et al. 2007). Finally, an unexpected connection between Sm proteins, which are known for their role in pre-mRNA splicing, and NPC disassembly was discovered in an RNAi screen of 1870 genes annotated as being essential for embryogenesis (Joseph-Strauss et al. 2012). RNAi against six out of the seven genes encoding Sm proteins caused clustering on NPCs in interphase and time-lapse microscopy demonstrated that nups, but not the nuclear lamina, dispersed from the NE later than in control embryos, suggesting a specific defect in NPC disassembly (Joseph-Strauss et al. 2012).

6.3 Association of Nups with Chromatin

Many studies in different organisms have reported correlations between gene positioning within the nucleus and transcription, hinting at conserved mechanisms (Solovei et al. 2016). Specifically, the position of genes relative to the NE has been extensively compared to their expression levels and histone modifications that determine the level of chromatin compaction, transcription factor binding and

transcription elongation (Harr et al. 2016; Gonzalez-Aguilera et al. 2014b). However, it is not clear if the spatial organization of particular genes is a cause or a consequence of the gene expression status. Moreover, many examples also exist where expression of a locus does not change upon relocation within the nucleus (or vice versa).

Analysis of nuclear organization by electron microscopy has demonstrated that heterochromatin accumulates at the nuclear periphery in regions between NPCs, suggesting that proteins of the lamina participate in gene repression. A group of lamina-associated INM proteins is characterized by the presence of the LAP2-emerin-MAN1 (LEM) domain (Fig. 6.1a). The LEM domain binds to the highly conserved barrier-to-autointegration factor (BAF-1/BANF1), which interacts with histones and DNA in a sequence independent manner and tethers repressive chromatin at the nuclear periphery (Barton et al. 2015). It has been proposed that LEM domain proteins function as hubs that integrate external signals to regulate the gene expression (Brachner and Foisner 2011; Dobrzynska et al. 2016a). For example, the INM proteins lamin B receptor, LAP-2 and emerin bind chromatin modifiers and transcriptional repressors inducing changes in histone modifications, such as deacetylation. Although most NE proteins are widely expressed, their absolute levels vary during development and across tissues (Gomez-Cavazos and Hetzer 2012; Morales-Martinez et al. 2015)(Fig. 6.3). Combined with the ability of NE proteins to interact directly or indirectly with chromatin, this suggests that NE proteins might regulate expression in a highly tissue-specific manner and has gained acceptance as a likely explanation why mutations in human genes encoding nups and other NE components often give rise to clinical symptoms restricted to a single or few tissues (Dobrzynska et al. 2016b; Raices and D'Angelo 2012).

Understanding how NE proteins influence gene expression and cell differentiation requires global analysis of the genomic regions they contact. This can be achieved using DamID (Dam methyltransferase identification) or ChIP (chromatin immunoprecipitation) techniques, which both have been applied to *C. elegans* to identify the genomic regions associated with nups, the nuclear lamina or INM proteins (for review, see (Askjaer et al. 2014a)). For instance, ChIP of NPP-3/NUP205 and NPP-13/NUP93 found an association with a subset of small nucleolar RNAs (snoRNAs) and tRNA genes transcribed by RNA polymerase (pol) III (Ikegami and Lieb 2013). Interestingly, the association of snoRNA genes with NPP-13/NUP93 is required for correct RNA processing, but not for recruitment of RNA pol III. NPP-3/NUP205 and NPP-13/NUP93 are considered stable NPC components but it remains to be demonstrated if the interaction with RNA pol III genes takes place at NPCs. In mammalian cell cultures, interaction of several mobile nups (e.g. NUP50 and NUP98) with RNA pol II genes has been shown to regulate gene expression but mainly through binding inside the nucleoplasm away from NPCs (for review, see (Ibarra and Hetzer 2015)). Taking advantage of the fact that DamID can be performed with little biological material, the association of MEL-28/ELYS with chromatin was recently compared between hermaphrodites and males (Sharma et al. 2014). In *C. elegans*, genes on the single X chromosome in males are expressed to the same level as the sum of expression

from the two X chromosomes in hermaphrodites, which both are repressed by the dosage compensation complex. DamID of MEL-28/ELYS revealed increased contacts with the X chromosome specifically in males, pointing to the possibility that MEL-28/ELYS might be involved in dosage compensation (Sharma et al. 2014). MEL-28/ELYS is enriched at transcriptional active regions throughout the genome (Gómez-Saldivar and Askjaer, unpublished results), which is in contrast to LMN-1/lamin, EMR-1/emerin and LEM-2/LEMD2 that all associate with repressed chromatin and mainly at the arms of chromosomes (Gonzalez-Aguilera et al. 2014a; Towbin et al. 2012; Ikegami et al. 2010).

Interaction of nups with chromatin is also likely to be important for DNA damage repair and genome stability. In yeast, several factors involved in recognition and repair of DNA damage accumulate at NPCs in a NUP107 complex-dependent manner (reviewed in (Ibarra and Hetzer 2015). Moreover, in human cells, chromatin in the vicinity of NPCs is accessible to the DNA damage response machinery whereas chromatin in LADs is not. In concordance with these observations, mutation of *C. elegans* NPP-15/NUP133 leads to increased sensitivity to ionizing irradiation (van Haaften et al. 2006), although the precise implication of NPP-15/NUP133 in this process remains to be described. Moreover, chromatin bridges are frequently observed during mitosis in embryos depleted for MEL-28/ELYS, NPP-8/NUP155, NPP-10/NUP98-96 or NPP-19/NUP35 (Franz et al. 2005; Galy et al. 2006; Rodenas et al. 2012; Rodenas et al. 2009), indicating that several NPC components might be involved in resolving replication-induced DNA structures.

6.4 Gene Repositioning Upon Transcriptional Activation

Organization of the genome is dynamic and non-random during development and relates with cell fate (Burns and Wente 2014). The locus tracking system based on integrated LacO arrays and fluorescent GFP-LacI has been used in many organisms, including *C. elegans* to study the gene positioning in living cells (Askjaer et al. 2014a). This has revealed that many tissue-specific promoters are sequestered at the nuclear periphery when repressed and move to the nuclear interior when activated. For instance, *pha-4* (gut) and *myo-3* (muscle) promoters are inactive and located at the NE during early development but are later found more frequently in the interior of differentiated gut and muscle nuclei, respectively (Meister et al. 2010). Transcriptional activation also leads to chromatin decompaction, as demonstrated for the *myo-2* and *pax-1* promoters in pharyngeal cells upon binding by PHA-4 (Fakhouri et al. 2010). Importantly, ectopic expression in *C. elegans* of a LMN-1 variant mimicking a disease-causing mutation in human lamin A prevents release of *myo-3* arrays in differentiated muscle cells and interferes with muscle activity (Mattout et al. 2011), indicating that the nuclear lamina plays an active role in regulation of gene expression and cell fate execution.

Repressed chromatin is characterized by methylation of lysine 9 of histone H3 (H3K9me) and the nematode-specific CEC-4 protein was recently identified as an anchor for H3K9me at the NE in embryos (Gonzalez-Sandoval et al. 2015). Mutation of *cec-4* releases heterochromatin from the NE but does not lead to derepression, demonstrating that tethering to the NE is not required for repression. In contrast, prevention of H3K9 methylation by deletion of the methyl transferase genes *set-25* and *met-2* causes both, detachment and derepression of heterochromatin (Towbin et al. 2012). Interestingly, these phenotypes are mainly restricted to embryos, suggesting that unknown tethering mechanisms act later in *C. elegans* development.

Recruitment of promoters to NPCs as part of the mechanism of gene activation is well described in yeast, but it remains unclear to which extend this also occur in animal cells, where the increased genome size might impede long range movements (Burns and Wente 2014). However, heat-shock responsive promoters represent an interesting example of chromatin mobility within the *C. elegans* nucleus. Super-resolution microscopy and ChIP experiments with antibodies against NPP-13/NUP93 have revealed that the position of the stress-induced *hsp-16.2/41* promoter differs upon transcriptional activation. In non-stress conditions, the promoter resides at the nuclear periphery in regions lacking NPCs, and upon heat shock, it repositions and interacts with NPCs (Rohner et al. 2013). The recruitment to NPCs depends on transcriptional activation of the *hsp-16.2/41* promoter because mutation of RNA pol II subunit AMA-1 or THO/TREX subunit ENY-2 prevents efficient repositioning. In conclusion, in *C. elegans*, two classes of genes present distinct patterns of gene positioning upon transcriptional activation. Developmentally induced genes relocate from the periphery to the interior when they are transcriptionally active, whereas stress-induced genes reposition within the nuclear periphery to the NPC upon stress conditions, suggesting distinct modes of gene regulation.

6.5 The NPC in Nucleocytoplasmic Transport and Beyond

The conventional role of the NPC is to regulate nucleocytoplasmic transport and thereby facilitate gene expression. For instance, gene transcription depends on nuclear import of transcription factors whereas protein synthesis requires active nuclear export of mRNA in the form of messenger ribonucleoprotein particles (mRNPs). Several nups have unstructured domains rich in phenylalanine-glycine (FG) dipeptide repeats, which form a highly selective permeability barrier in the central channel of the NPC (Knockenhauer and Schwartz 2016). Specific and rapid passage of transport substrates through this barrier is mediated by transport receptors (aka karyopherins or importins and exportins) that interact simultaneously with substrates and FG nups. The recognition of substrates has been

described in details whereas the biophysical properties of the interactions of transport receptor with nups in the central channel are still an area of active research. Examples of substrate-receptor interactions characterized in *C. elegans* include the FOXO-like transcription factor DAF-16 and its nuclear import receptor IMB-2/transportin (Putker et al. 2013) as well as the cell cycle-related phosphatase CDC-14 and its nuclear export receptor XPO-1/CRM1 (Roy et al. 2011).

So far only few studies have analyzed the role of individual *C. elegans* nups in nucleocytoplasmic transport. Early RNAi experiments indicated that several nups are required for nuclear growth after mitosis (Galy et al. 2003). It is reasonable to speculate that the reduced nuclear growth phenotype is caused by impaired nuclear protein import, although this should be investigated in more detail. Nuclei lacking NPP-5/NUP107 are also reduced in size (Rodenas et al. 2012) whereas depletion of NPP-1/NUP54 or NPP-19/NUP35 inhibits nuclear import of PIE-1 (Rodenas et al. 2009; Schetter et al. 2006). Recently, RNAi against *npp-10/NUP98-96*, *npp-13/NUP93* or *npp-20/SEC13R* was reported to interfere with centromere assembly by impeding nuclear import of HCP-4/CENP-C (Ferreira et al. 2017). Based on evidence from yeast and vertebrate systems, we expect that several other *C. elegans* FG nups, such as NPP-4/NUPL1, NPP-7/NUP153, NPP-11/NUP62, NPP-10N/NUP98 and NPP-16/NUP50 might be directly implicated in regulation of transport through the NPC, but future experiments are required to address this. The recruitment of the *hsp-16.2/41* promoter to NPCs upon heat shock induction described above might stimulate both, transcription and mRNA export, by local concentration of transcription factors and facilitated access to the NPC ("gene gating," see (Burns and Wente 2014)).

There is growing evidence that nups have additional roles away from the NPC and this includes also *C. elegans* NPP-9/NUP358 and NPP-10N/NUP98 (note that the *npp-10/NUP98-96* gene produces 3 transcripts, of which the long b isoform encodes a precursor protein, which, similarly to the situation in yeast and vertebrates, is proteolytically cleaved to produce NPP-10N/NUP98 and NPP-10C/NUP96). NPP-9/NUP358 and NPP-10N/NUP98 are present in P granules, which are conserved germline cytoplasmic ribonucleoprotein complexes required for fertility (Sheth et al. 2010; Voronina and Seydoux 2010). RNAi screens for regulators of P granule integrity have retrieved ~10 *npp* genes, which suggest that NPCs have a central role in assembly of P granules (Updike and Strome 2009; Voronina and Seydoux 2010). P granules are located both, in the cytoplasm and at the nuclear periphery juxtaposed to NPCs. Moreover, several P granule components, similarly to nups, are enriched in FG repeats and P granules share certain biophysical properties with NPCs, which led to the notion that they might serve as a physical extension of NPCs in mRNA export and storage (Updike et al. 2011). Moreover, NPP-10N forms a complex with *nos-2* mRNA and is required for translational repression of P granule-associated *nos-2* mRNA (Voronina and Seydoux 2010). An interesting question that remains to be addressed is whether the other nups that were identified in the RNAi screens also relocate (transiently) to P granules or if their role is restricted to export of P granule RNA components through the NPC.

6.6 The NPC During Aging

The discovery that mutations in genes encoding NE components are the cause of Hutchinson-Gilford Progeria Syndrome (HGPS) sparked much interest in understanding the relation between the NE and aging. Cells from HGPS patients are characterized by irregular nuclear morphology, clustering of NPCs and increased levels of DNA damage (Gonzalo et al. 2017). During the short life of *C. elegans* (2–3 weeks under normal laboratory conditions), most post-mitotic cells also undergo dramatic changes in nuclear morphology and distribution of LMN-1/lamin (Haithcock et al. 2005). Throughout larval development nuclei are surrounded by smooth NEs with uniform distribution of LMN-1 and NPP-1/NUP54 but as animals enter adulthood the NE becomes gradually more convoluted, the levels of intranuclear LMN-1 increase at the expense of NE-localized LMN-1, and clusters of NPP-1 appear. Initial observations found a correlation between NE deterioration and lifespan: in long-lived animals the NE remained more uniform for an extended time, whereas a mutation reducing lifespan was accompanied with earlier changes in nuclear morphology (Haithcock et al. 2005). However, this correlation is not universal as later analyses identified mutants that uncoupled lifespan from NE alterations: in two alleles of the insulin receptor *daf-2* the NE changed nuclear morphology with the same kinetics as in wild type animals, yet the mutants lived ~50-150% longer (Perez-Jimenez et al. 2014). Whereas these two studies described the distribution of LMN-1, an analysis in the Hetzer laboratory focused on the changes in expression of *C. elegans* nups during aging. Interestingly, for several nups, in particular those belonging to the NUP107 complex (NPP-5/NUP107, NPP-6/NUP160, NPP-15/Nup133 and NPP-23/NUP43), as well as NPP-3/NUP205 and NPP-8/NUP155, their mRNA was only present in embryos and larval stages but the proteins were detected throughout life (D'Angelo et al. 2009). In contrast, genes encoding nups of the peripheral cytoplasmic and nucleoplasmic NPC structures (NPP-7/NUP153, NPP-9/NUP358 and NPP-16/NUP50), as well as NPP-19/NUP35 and transmembrane NPP-12/NUP210 were transcribed and translated in all life stages. Together with experiments in mammalian cells, this led to the conclusion that once so-called scaffold nups are inserted into post-mitotic NPC, they do not exchange for the rest of the life of the cell (D'Angelo et al. 2009). As a consequence of the lack of protein turnover, the NPCs are prone to accumulation of damage from for instance reactive oxygen species, and indeed, many nuclei isolated from old nematodes had compromised permeability barriers (D'Angelo et al. 2009). The precise implication of these observations for normal and disease-related aging in humans is still unclear, but they have added a novel aspect to the complexity of biological aging. Combined with the age-related alterations in the nuclear lamina described above, changes in NPC composition and/or function in old individuals could have both global and specific impacts on signaling across the NE and gene expression. Moreover, an interesting question is how these observations relate to different tissues. *C. elegans* neurons generally maintain a smooth, regular nuclear morphology throughout the

life of the animal (Haithcock et al. 2005), but whether this correlates with efficient NPC function in nucleocytoplasmic transport and gene expression has not been analyzed yet. Finally, a recent study found that NPP-21/TPR is required for Metformin-induced lifespan increase, possibly through regulation of NPC permeability (Wu et al. 2016).

6.7 Concluding Remarks

Increasing evidences support a critical role of NPCs in different genetic processes in *C. elegans*. However, the specific roles of most nups still remain unclear. Furthermore, although NPC structure is conserved through evolution, nups are likely to have both shared and different roles between species. For example, in yeast, nups recruit genes to the nuclear periphery upon activation to achieve optimal gene expression but this might be the exception rather than the norm in nematodes: *C. elegans* heat shock promoters relocate to NPCs at elevated temperatures whereas developmentally regulated promoters are located in the nuclear interior when they are transcriptionally active. Also, although most studies have reported positive roles of nups in transcriptional activation, there exist several examples of nups involved in gene silencing. This suggests that the function of NPCs and nups in gene expression regulation is quite complex and more experiments in different model organisms are required to clarify this process.

Another interesting topic for future research is that many pathologies, cellular phenotypes and expression patterns associated with metazoan nups are tissue-specific. For instance, it has been shown that specific nups affect neural and muscle differentiation during mouse embryonic development. Although transcriptomic data suggest that several *C. elegans* nups are expressed in a cell type-specific manner, the potential implication during development remains to be explored. Because of the characteristics of *C. elegans*, we envision that many interesting discoveries will be made in this model organism, paving the way to better understand the basis of pathologies caused by nups dysfunctions.

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Chapter 7
Nuclear Pore Complexes in the Organization and Regulation of the Mammalian Genome

Marcela Raices and Maximiliano A. D'Angelo

Abstract In the last decade, the nuclear envelope (NE) has emerged as an important regulator of genome architecture and a central player in gene expression regulation. Nuclear pore complexes (NPCs), the channels that penetrate the NE connecting the nucleus to the cytoplasm, are the largest protein complexes of the NE. Built by multiple copies of roughly 30 different proteins, NPCs were traditionally studied for their role in controlling nucleocytoplasmic transport. But accumulating evidence shows that these massive molecular structures play multiple transport-independent roles that are key for the maintenance of cellular physiology and tissue homeostasis. In this chapter, we will focus on the current knowledge of the role of mammalian NPCs in the regulation of genome organization and gene expression. The recent findings showing that NPCs regulate the activity of specific genes either at the nuclear periphery or inside the nucleus point towards these structures as critical controllers of genome function. Deciphering the molecular mechanism employed by NPCs to modulate specific gene expression programs and to maintain genome integrity are our main challenges for the next decade.

Keywords Nuclear pore complex · nuclear envelope · nucleoporin · transcription · gene expression · nuclear transport

7.1 Introduction

In eukaryotic cells the nucleus is the organelle where the genome is housed. Discovered in 1833 by botanist Robert Brown (Oliver 1913), the nucleus serves as the control center of the cell where all the genetic information is stored and translated. The nucleus is characterized by a double-membrane structure, known as

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the nuclear envelope (NE), that separates the chromosomes from the cytoplasm. In metazoans, the nuclear lamina, which is a filamentous protein meshwork situated underneath the NE, provides mechanical stability to the nucleus and aids in the regulation of many nuclear processes, including genome organization and gene expression regulation (Stancheva and Schirmer 2014). The NE is perforated by large multiprotein channels known as nuclear pore complexes or NPCs. Discovered in 1950 (Callan and Tomlin 1950), NPCs represent the sole gateway into the nucleus and, thus, they are responsible for the entry and exit of most molecules from and into this compartment. Although NPCs have been historically studied for their essential role in controlling nucleocytoplasmic molecule exchange, they have recently emerged as important regulators of diverse cellular processes in a transport-independent manner (Raices and D'Angelo 2012). One of the most studied functions of NPCs is their role in the regulation of genome integrity. Increasing evidence supports a model in which NPCs not only act as organizers of the cellular genome but also as scaffolds for the regulation of specific gene groups confined to the nuclear periphery. Moreover, several nucleoporins have been now found to localized to the nuclear interior where they assist the transcriptional machinery in regulating gene activity. In this chapter, we describe our current knowledge of the role of mammalian NPCs in the regulation of genome organization and gene expression. While our understanding of these functions of NPCs in mammalian cells is not yet as extensive as in yeast, increasing evidence indicates a strong conservation in the genomic processes and mechanisms regulated by NPCs between these organisms.

7.2 Stability, Mobility and Lifespan of Mammalian NPCs

NPCs are one of the largest protein complexes of eukaryotic cells (Raices and D'Angelo 2012). These channels have an eight-fold-symmetrical structure that consists of a nuclear envelope-embedded scaffold that is built to surround a central transport channel through which all nucleocytoplasmic transport takes place (Frenkiel-Krispin et al. 2010; Beck et al. 2004, 2007; Maimon et al. 2012). Attached to this scaffold are two rings, the cytoplasmic and nuclear rings, from where eight filaments emanate (Fig. 7.1a). On the nuclear side, the filaments are joined in a distal ring to form the nuclear basket of the NPC. Even though the overall structure of NPC is conserved among species, its size varies, being the mammalian NPCs the largest complexes of all (estimated molecular mass = 60–125 MDa) (Suntharalingam and Wente 2003; Yang et al. 1998). Interestingly, even though NPCs are massive protein complexes, these channels are composed of roughly 30 different proteins known as nucleoporins or Nups (Rout et al. 2000; Cronshaw et al. 2002; Yang et al. 1998; Reichelt et al. 1990; Hoelz et al. 2016) (Fig. 7.1b). Despite being a membrane embedded structure, most NPCs components are soluble proteins and in mammalian cells only three nucleoporins are transmembrane (D'Angelo and Hetzer 2008). Most nucleoporins have been found to associate in biochemically stable subcomplexes that are believed to act as the building blocks of nuclear pores (Fig. 7.1b) (Hoelz et al. 2016). Due to the eight-fold rotational symmetry of these structures,

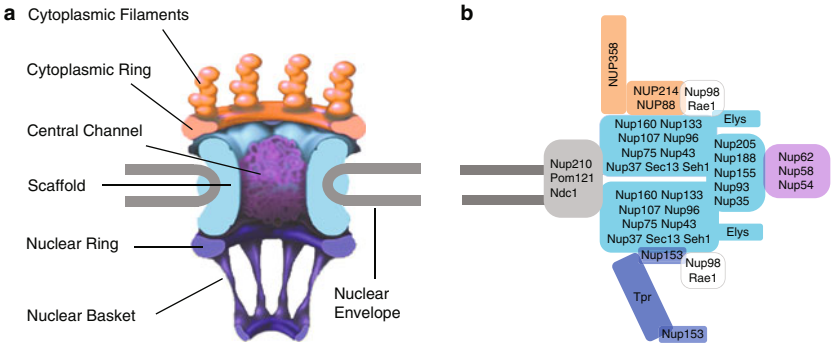

Fig. 7.1 Nuclear pore complex structure and composition (a) Scheme of the NPC structure. (b) Composition of NPCs. Boxes indicate subcomplexes

nucleoporins and subcomplexes are present in eight or multiples of eight copies, and each pore has an estimated 500–1000 total proteins. In addition to their localization within the NPC structure, nucleoporins have also been classified depending on their residence time (Rabut et al. 2004). Stable nucleoporins are those that show very low exchange rates from NPCs and are mostly components of the pore scaffold. Dynamic nucleoporins, on the other hand, are constantly exchanged from NPCs, having residence times at the structure that range from a few seconds to a few hours (Rabut et al. 2004). These dynamic nucleoporins are mostly members of the pore peripheral structures that include the nuclear basket, central channel and filaments.

Mammalian cells divide through open mitosis, a process in which the nucleus is disassembled during the M-phase of the cell cycle to allow the separation of sister chromatids (Kutay and Hetzer 2008). In each cell division, when the nucleus breaks down , NPCs are disassembled in their stable subcomplexes, which are recycled at the end of M-phase to assemble new channels at the daughters' nuclear envelopes (D'Angelo and Hetzer 2008). This disassembly–reassembly cycle ensures that in dividing mammalian cells NPCs are renewed in each cell-division. But NPCs behave differently in non-dividing and postmitotic cells (D'Angelo et al. 2009). As mentioned above, the scaffold components of NPCs have very long residence times at this structure. In fact, studies in dividing cells have identified that these nucleoporins have residence times at NPCs that are longer than the cell cycle (Rabut et al. 2004; Daigle et al. 2001). This provided the first evidence that the core NPC proteins would only exchange when pores disassemble during mitosis. Support for this model came from recent studies showing that scaffold nucleoporins indeed have extremely long lives at NPCs of postmitotic cells (D'Angelo et al. 2009; Savas et al. 2012). Analysis of nucleoporin turnover in postmitotic cells and tissues uncovered extremely low rates of exchange for these nucleoporins, suggesting minimal turnover of NPC structural components. These findings indicate that when mammalian cells exit the cell cycle they maintain their NPC scaffold structures for almost their entire life (D'Angelo et al. 2009; Savas et al. 2012; Toyama et al. 2013). This long life of NPCs does not come without cost, and nuclear pores have been shown to deteriorate as postmitotic cells age, leading to the loss of nuclear compartmentalization in old cells (D'Angelo et al. 2009).

Mammalian NPCs are not only incredibly stable structures at the NE, they are also immobile. Studies using Fluorescent Recovery After Photobleaching (FRAP) have identified that NPCs do not move independently at the NE (Daigle et al. 2001). In fact, in the same way as the nuclear lamina, nuclear pores move as large arrays in response to changes in nuclear shape (Daigle et al. 2001). The low turnover of NPCs, their large size and potential anchor sites for DNA and proteins, and their lack of mobility at the nuclear periphery suggest that these structures play a role in nuclear organization by acting as stationary, long-lived, positional markers at the NE (D'Angelo and Hetzer 2008).

7.3 Chromatin Interactions with Mammalian NPCs

It is has now become clear that chromosomes are not randomly dispersed inside the nucleus (Misteli 2007). A significant amount of data shows that genes and chromosomal domains have unique relative positons within the nucleus of different cell types, and several studies have demonstrated that intranuclear gene position can affect gene activity/regulation (Nguyen and Bosco 2015; Talamas and Capelson 2015; Stancheva and Schirmer 2014). These findings have uncovered that the three-dimensional (3D) organization of the genome plays a key role in gene expression control. However, how genome architecture is faithfully maintained in mammalian cells remains poorly comprehended.

The first studies of the association of mammalian NPCs with the genome identified that the Nup93 nucleoporin associates with chromatin regions enriched in heterochromatin markers (Brown et al. 2008). Nup93 is a scaffold component of NPCs that shows a low exchange rate from nuclear pores during interphase and plays a key role in the maintenance of the nuclear permeability barrier (Rabut et al. 2004; Galy et al. 2003; D'Angelo et al. 2009). The finding that Nup93 associates with heterochromatin fueled the original idea that the nuclear periphery was a repressive environment mostly associated with chromatin condensation and gene silencing (Towbin et al. 2009). Interestingly, in this original study, the genome regions associated with NPCs were found to change when global histone acetylation was modified, indicating that NPC-genome interactions are dynamic (Brown et al. 2008). More recent studies have identified that NPCs also bind active genes (Kehat et al. 2011; Raices et al. 2017), open chromatin domains (Ibarra et al. 2016), and enhancer regions (Ibarra et al. 2016), which is more consistent with the long-time observation by electron microscopy that differently from the nuclear lamina, NPCs are surrounded by euchromatin (Lemaitre and Bickmore 2015; Capelson and Hetzer 2009) (Fig. 7.2). These studies point to NPC surroundings as regions of decondensed, transcription-permissive, chromatin, and suggest that these channels play a role in the positive regulation of gene expression. Notably, the maintenance of these NPC-associated decondensed domains, also known as heterochromatin exclusion zones, has been shown to depend on the nuclear basket nucleoporin Tpr (Krull et al. 2010). This indicates that nuclear pores have an active role in regulating the state of

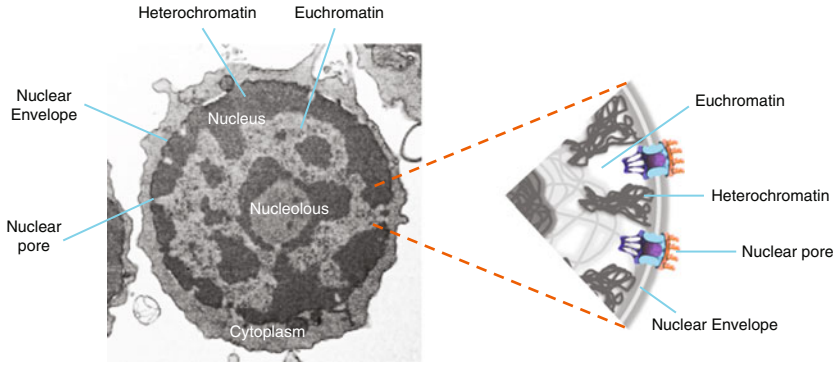

Fig. 7.2 Chromatin organization around nuclear pore complexes Electron micrograph image of a resting T lymphocyte showing the distribution of euchromatin (light gray) and heterochromatin (dark gray) within the nucleus. Right panel: Schematic representation of a zoomed-in region of the nuclear envelope depicting NPCs and their surrounding decondensed chromatin

their surrounding chromatin. The direct binding of different genomic regions, their active role in the regulation of the neighboring chromatin environment, and the fact that mammalian NPCs are immobile due to their interactions with the nuclear lamina, support the current model in which these large structures play a critical role in genome architecture.

7.4 Gene Expression Regulation at NPCs

7.4.1 Genes that Associate with Mammalian NPCs

In budding yeast, many genes have been found to relocate to NPCs when activated, and it is well established that NPC-gene association is important for their efficient expression as well as for their transcriptional memory (Schneider et al. 2015; Sood and Brickner 2014; D'Urso and Brickner 2017). In mammals, the regulation of gene expression by NPCs is just beginning to be exposed, and emerging evidence suggest that similar to its yeast counterparts, nuclear pores play a critical role in the regulation of gene expression at the nuclear periphery. The first description of active genes requiring NPC-association for their efficient transcription came from a study of cardiomyocyte hypertrophic growth (Kehat et al. 2011). This work showed that when cardiomyocyte hypertrophic growth is induced, the proper transcription of several genes, including sarcomeric and calcium-handling genes, requires their relocation to NPCs (Kehat et al. 2011) (Fig. 7.3a). Similarly, the regulation of multiple structural and contraction genes in skeletal muscle has been recently shown to take place at NPCs and to require the presence of a tissue-specific nucleoporin known as Nup210 (Fig. 7.3b). Nup210 was the first nucleoporin identified (Gerace et al. 1982). This transmembrane protein shows cell type- and tissue-specific expression (Olsson et al. 1999,

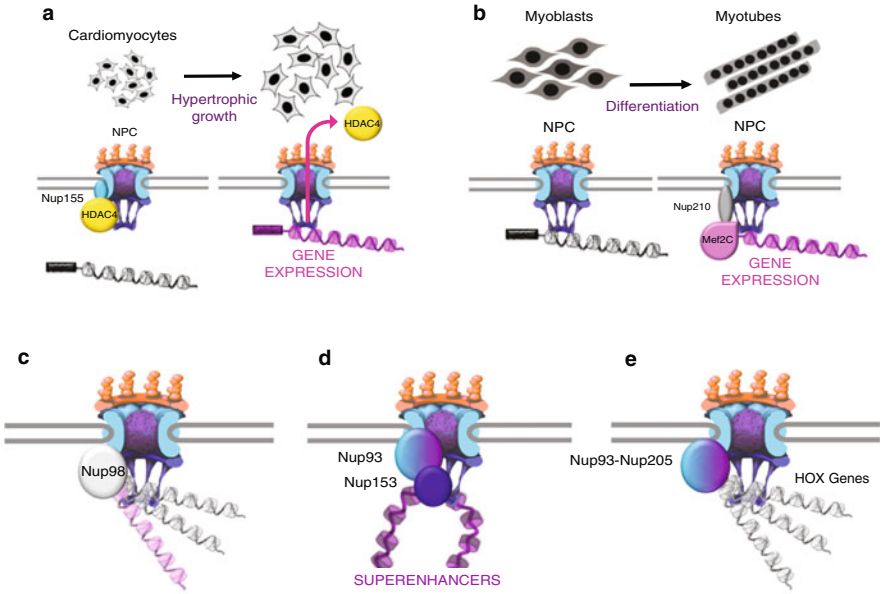

Fig. 7.3 Gene regulation at NPCs (a) In resting cardiomyocytes, the histone deacetylase 4 (HDAC4) binds to Nup155 at NPCs and prevents the association of sarcomeric and calcium-handling genes with nuclear pores. When hypertrophic growth is induced in these cells, HDAC4 is exported from the nucleus, which allows the association of muscle genes with NPCs, promoting their proper activation. (b) During myoblast differentiation, the expression of the transmembrane nucleoporin Nup210 is induced. Nup210 is added to NPCs of differentiating muscle cells, where it recruits the transcription factor Mef2C to regulate the activity of muscle structural genes that were already associated with nuclear pores. (c) In mammalian cells, Nup98 has been shown to bind inactive genes (gray) or genes that have basal/low (light pink) activity at NPCs. (d) Analysis of the genome domains that associate with nucleoporins Nup93 and Nup153 uncovered an enrichment of superenhancer sequences. The binding to superenhancerrs to Nup93 and Nup153 was found to take place at NPCs. (e) Members of the Nup93-Nup205 NPC subcomplex, including Nup93, Nup188 and Nup205, associate with HoxA genes and repress their activity at NPCs. In this figure, silent genes are shown in gray, active genes in pink

2004; D'Angelo et al. 2012). By regulating gene expression, Nup210 has been shown to be important for the differentiation of muscle progenitors and for the maturation and survival of differentiated muscle cells (D'Angelo et al. 2012; Raices et al. 2017). In the absence of this nucleoporin, the activity of many structural genes becomes misregulated, although their association with NPCs is not affected (Raices et al. 2017). The findings of these studies indicate that in cardiac and skeletal muscle the positioning of specific genes at NPCs is critical for their proper regulation and reveal that NPCs act as scaffolds for the regulation of specific gene groups. Notably, Nup210 is not expressed in muscle progenitor cells (Raices et al. 2017; D'Angelo et al. 2012). Its expression is induced and Nup210 is added to NPCs during differentiation (Raices et al. 2017; D'Angelo et al. 2012). This indicates that gene regulation at NPCs can be modulated by changing the composition of this structure (Fig. 7.3b).

The association of genes with NPCs is not restricted to muscle cells and has also been described during neuronal differentiation. Chromatin immunoprecipitation (ChIP) studies of the Nup98 nuclear pore complex member showed that this nucleoporin binds many developmental and cell differentiation genes (Liang et al. 2013). Nup98 is a phenylalanine-glycine (FG) repeat-containing dynamic nucleoporin that also localizes to the nuclear interior (Griffis et al. 2002, 2003). It is expressed from the NUP98 gene as a 98 kDa protein or as a larger 195-kDa precursor encoding Nup98-Nup96 that is autoproteolytically cleaved to produce both nuclear pore complex components (Fontoura et al. 1999; Ratner et al. 2007). At NPCs, Nup98 localizes at the cytoplasmic and nuclear sides (Griffis et al. 2003). Inside the nucleus, the localization of Nup98 varies among different cell types, but cells that have high levels of this nucleoporin, as well as cells overexpressing it, show its accumulation in intranuclear foci known as GLFG bodies, due to the glycine-lysine-phenylalanine-glycine repeats that Nup98 contains (Griffis et al. 2002, 2003). The function of Nup98-containing intranuclear foci is yet to be elucidated. A large amount of evidence accumulated in the past decade indicates that Nup98 plays an important role in the regulation of gene expression at NPCs and inside the nucleus (Franks and Hetzer 2013). During the early stages of neuronal differentiation Nup98 has been shown to bind a subset of non-active or low activity genes at NPCs (Liang et al. 2013) (Fig. 7.3c). On the other hand, the binding of Nup98 to genes that are highly activated during this process occurs in the nucleoplasm and away from NPCs as described below (see Sect. 7.5)(Liang et al. 2013).

Consistent with NPCs acting as hubs for transcriptional regulation, a recent study identified an enrichment of superenhancer sequences within the genomic regions associated with nuclear pores (Ibarra et al. 2016) (Fig. 7.3d). Superenhancers are domains of the genome that contain clusters of enhancers in close proximity (Niederriter et al. 2015) and generally play a role in the regulation genes involved in cell identity/cell type specification. This study found that depletion of Nup153 or Nup93 dramatically affects the transcription of genes regulated by NPC-associated superenhancers (Ibarra et al. 2016) (Fig. 7.3d).

Even though these studies have identified a positive role of NPCs in transcriptional regulation, the Nup93 nucleoporin was found to bind genome regions enriched in silent chromatin markers (Brown et al. 2008). This suggests a role for NPCs in gene repression. Consistent with this idea, a recent study uncovered that several members of the Nup93-Nup205 NPC subcomplex (including Nup93, Nup188 and Nup205) bind to the promoter of HOXA genes and silence their expression (Labade et al. 2016) (Fig. 7.3e). Fluorescent in situ hybridization analyses of the genes regulated by these nucleoporins confirmed their association with the nuclear periphery and uncovered that Nup93 is required for the tethering of HOXA genes to NPCs (Labade et al. 2016).

All these studies demonstrate that similar to the yeast NPCs, mammalian NPCs can bind active and silent chromatin, and act as positive or negative regulators of gene expression. But more importantly, that mammalian NPCs are key players in the regulation of developmental and cell type-specific gene expression by acting as protein scaffolds that allow the local regulation of specific genes confined to the nuclear periphery.

7.4.2 Mechanisms of Gene-Expression Regulation by NPCs

A large amount of evidence has shown that in yeast the dynamic association of genes with NPCs is regulated by transcription factors (Randise-Hinchliff et al. 2016; Brickner et al. 2012). Transcription factors not only regulate NPC-tethering in these organisms but they are also required for the clustering of genes that present their binding sites (Randise-Hinchliff et al. 2016; Brickner et al. 2012). The role of transcription factors in regulating gene expression at NPCs has also been described in flies, where the Ecdysone receptor, a nuclear hormone receptor, is recruited to NPCs upon stimulation to regulate the activity of NPC-associated genes (Pascual-Garcia et al. 2017). How NPCs regulate gene expression at the nuclear periphery in mammals is just starting to be unraveled. Recent findings showed that the nucleoporin Nup210 recruits the transcription factor Mef2C to NPCs to modulate the activity of several muscle structural genes (Raices et al. 2017). This indicates that the role of transcription factors in regulating gene expression at the nuclear periphery might be conserved in mammals. But even though Nup210 is important for the efficient expression of NPC-associated genes, it is not required for gene localization to the nuclear periphery (Raices et al. 2017). Interestingly, during cardiomyocyte hypertrophic growth, the association of genes with NPCs is negatively modulated by the histone deacetylase HDAC4 (Kehat et al. 2011). In these cells, when HDAC4 is anchored to NPCs, it prevents the association and transcription of several sarcomeric genes (Kehat et al. 2011). When hypertrophic growth is stimulated, the release of HDAC4 from NPCs leads to the recruitment and activation of these genes. Because HDAC4 is a key negative regulator of Mef2C activity that is exported from the nucleus during myogenesis (Clocchiatti et al. 2013; McKinsey et al. 2000), these findings indicate that the interplay between transcription factors and chromatin modulators might regulate NPC-gene association and gene expression regulation. Altogether, the existing data allows to propose that: (1) by interacting and recruiting transcriptional modulators, including transcription factors and chromatin regulators, (2) by concentrating super-enhancers in their vicinity, and (3) by tethering genes that share common regulatory domains, NPCs act as hubs for the transcriptional regulation of specific gene groups at the nuclear periphery.

7.5 Gene Expression Regulation by Nucleoporins in the Nuclear Interior

7.5.1 Genes that Associate with Intranuclear Nucleoporins

Despite the emerging evidence that the NPC structure is itself important for gene expression regulation, most nucleoporin-genome interactions described so far in metazoans take place in the nuclear interior and away from NPCs (Kalverda et al. 2010; Capelson et al. 2010; Liang et al. 2013) (Fig. 7.4). This phenomenon was initially identified in flies, where several nucleoporins were found to bind

chromatin inside the nucleus and to play an important role in the regulation of developmental gene expression (Kalverda et al. 2010; Capelson et al. 2010). In mammalian cells, a few nucleoporins have also been shown to regulate gene expression inside the nucleus. The clearest example is Nup98 (Fig. 7.4). As mentioned, Nup98 is a dynamic nucleoporin that shuttles between NPCs and the nuclear interior (Griffis et al. 2002). In mammalian cells, the mobility of Nup98 inside the nucleus has been shown to be transcription-dependent (Griffis et al. 2002), and this nucleoporin has been found to bind developmentally regulated genes during embryonic stem cell (ESC) to neuronal differentiation (Liang et al. 2013). The genes bound by Nup98 in ESCs, which include active cell cycle and nucleic acid metabolism genes as well as some silent genes, differ from the ones bound in neuroprogenitors, which are mostly genes that are activated during neural differentiation (Liang et al. 2013). These findings indicate that Nup98 genome association is cell type-specific and developmentally regulated. Consistent with this idea, in lung fibroblasts Nup98 was found to associate with silent chromatin domains (Liang et al. 2013). Functionally, inhibition of Nup98 activity by expression of a dominant negative mutant has been found to affect the expression of the Nup98-bound developmental genes (Liang et al. 2013). Interestingly, two modes of gene regulation during neuronal differentiation have been described for Nup98. This nucleoporin has been shown to bind genes that are in the initial stage of induction at NPCs (on-pore), while the association of Nup98 with genes that are strongly induced during neuronal differentiation has been found to take place in the nuclear interior (off-pore) (Liang et al. 2013) (Figs. 7.3 and 7.4). These findings indicate that nuclear pore complex components might modulate different genes/gene expression programs depending on their spatial location within the nuclear space.

A detailed analysis of the DNA bound by Nup98 in mammals showed enrichment for GA-box DNA motifs. GA repeat motifs are bound by the GAGA factor (Liang et al. 2013), which in Drosophila and mammals regulates boundary activity at HOX clusters (Srivastava et al. 2015; Adkins et al. 2006; Granok et al. 1995) and modulates the expression of homeotic genes (Adkins et al. 2006; Granok et al. 1995). These findings might help to explain why several of the abnormal Nup98 fusion proteins that result from chromosomal translocations result in the alteration of HOX gene expression (see Sect. 7.6).

In addition to its role in regulating the activity of developmental genes, the binding of Nup98 to interferon gamma (INFγ) target genes is required for transcriptional memory (Light et al. 2013). In human cells, many genes that are induced by INFγ retain a "memory" of the activation and are turned on at a faster rate if cells are re-exposed to the cytokine. This transcriptional memory is maintained for several generations and depends on epigenetic modifications (D'Urso and Brickner 2017). Nup98 has been found to be recruited to the promoter of several INFγ target genes, such as HLA-DRA, only after removal of the cytokine (Light et al. 2013). The association of Nup98 with the promoter of these recently expressed genes is required for their proper re-activation upon INFγ re-exposure (transcriptional memory) (Light et al. 2013). The function of Nup98 in

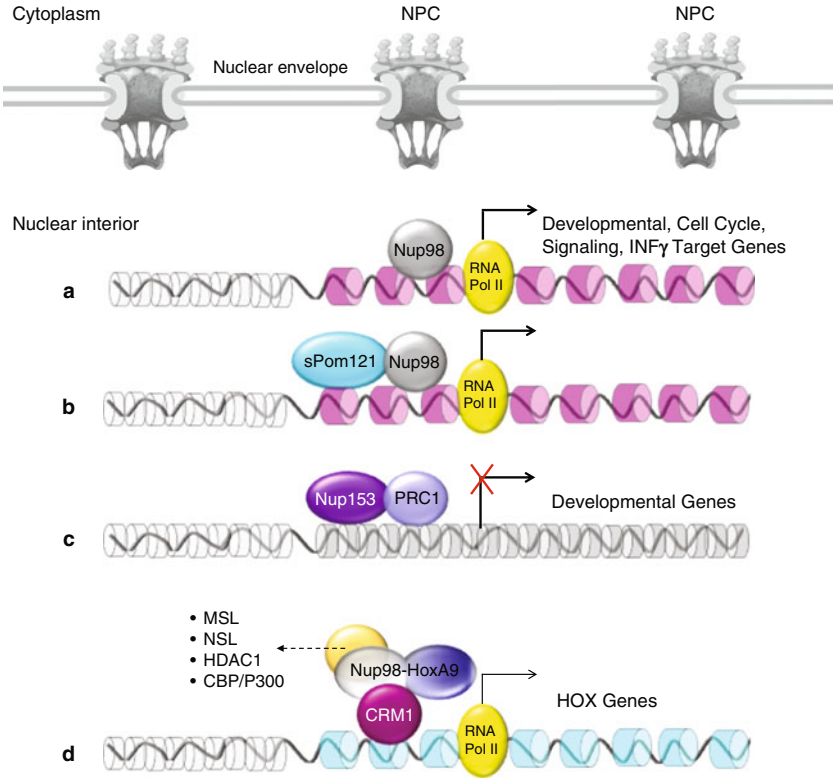

Fig. 7.4 Gene regulation by nucleoporins inside the nucleus. In mammalian cells, several nucleoporins have been found to regulate gene expression away from NPCs. (a) Nup98 has been found to bind multiple genes, including cell cycle, metabolic, cell differentiation, developmental, and INFγ-regulated genes. Some of these genes are co-bound by a soluble version of the transmembrane nucleoporin Pom121 (sPom121) (b). (c) Nup153 has also been shown to bind several developmental/differentiation genes in the nuclear interior. But contrary to Nup98, Nup153 recruits the polycomb complex PRC1 to their promoters to repress their activity in pluripotent stem cells. (d) The oncogenic Nup98-HoxA9 fusion has been found to induce the abnormal expression of HOX genes by recruiting different chromatin modulators to HOX clusters, including NSL, MLL, HDAC1 and CBP/p300. The association of this fusion protein with HOX genes is mediated by the export receptor CRM1, which has been found to be prebound to these genes

transcriptional memory is conserved in yeast, as its homologue Nup100 is required for the transcriptional memory of the inositol-responsive gene *INO1* (Light et al. 2013; D'Urso and Brickner 2014). But differently from yeast, the regulation of transcriptional memory by nucleoporins in human cells takes place inside the nucleus and not at NPCs (Fig. 7.4). Interestingly, ChIP studies using the antibody mAb414, which recognizes the Nup62, Nup153, Nup214 and Nup358 nucleoporins, showed that one or more of these nucleoporins also associate with the HLA-DRA locus. But differently from Nup98, this association is also observed when the gene is activated by INFγ (Light et al. 2013). What role does the association

of the mAb414-recognized nucleoporins with this gene play in its transcriptional regulation remains to be determined.

The NPC Nup153 member is a main component of the nuclear basket (Sukegawa and Blobel 1993; Pante et al. 1994), and another dynamic nucleoporin that shows transcription-dependent mobility (Griffis et al. 2004). Nup153 has been found to interact with the nuclear lamina (Al-Haboubi et al. 2011; Smythe et al. 2000) and has been associated with intranuclear filaments of the Tpr nucleoporin that emanate from NPCs into the nuclear interior (Hase and Cordes 2003; Simon and Wilson 2011). Nup153 has several zinc fingers motifs in its N-terminal region (Sukegawa and Blobel 1993) and has been shown to bind DNA (Sukegawa and Blobel 1993) and RNA (Ullman et al. 1999; Dimaano et al. 2001; Ball et al. 2007). It also has multiple FG repeats in its C-terminal domain through which it interacts with transport receptors (Shah et al. 1998; Moroianu et al. 1995; Nakielny et al. 1999). Several functions have been attributed to Nup153. These include the regulation of mRNA export (Bastos et al. 1996; Ullman et al. 1999), importin α/β-mediated nuclear import (Walther et al. 2001; Shah and Forbes 1998; Ogawa et al. 2012; Makise et al. 2012), NPC assembly (Walther et al. 2001; Vollmer et al. 2015), mitotic checkpoint regulation (Mackay et al. 2009; Lussi et al. 2010), HIV infection and replication, DNA damage repair (Mackay et al. 2017; Duheron et al. 2017; Chow et al. 2012; Lemaitre et al. 2012) and gene expression regulation (Vaquerizas et al. 2010; Mendjan et al. 2006; Jacinto et al. 2015; Nanni et al. 2016). A role for Nup153 in gene expression regulation was originally identified in Drosophila, where Nup153 together with Megator, the homolog of human Tpr and another nuclear basket nucleoporin, were found to be required for the transcriptional regulation of dosage compensation (Mendjan et al. 2006). These proteins were later shown to bind a great portion of the genome (~25%) in continuous domains of 10–500 kilobases that present chromatin markers of active transcription (Vaquerizas et al. 2010). Consistent with a role in transcriptional regulation, downregulation of Nup153 was found to affect the expression a large number of genes (~5,700) in flies (Vaquerizas et al. 2010). In mouse ESCs, Nup153 was recently identified to bind to the transcription start site of several developmental genes (Jacinto et al. 2015). Interestingly, in these cells Nup153 was found to act as a repressor for differentiation genes (Fig. 7.4). The repression by Nup153 is required for the maintenance of the pluripotent state of ESCs, and depletion of this nuclear pore complex component results in early cell differentiation into different linages (Jacinto et al. 2015). The role of Nup153 in gene expression regulation is not restricted to ESCs. In mouse cardiomyocytes, Nup153 has also been found to associate with, and to regulate the activity of, genes involved in cardiac remodeling (Nanni et al. 2016). In this case, the binding of Nup153 correlates with markers of active chromatin but whether they occur inside the nucleus or at the nuclear periphery has not been investigated (Nanni et al. 2016). These findings further support the concept that nucleoporins can regulate different subsets of genes in distinct cell types.

The idea that nuclear pore complex components might play multiple functions depending on their intracellular localization is further reinforced by the findings

that a soluble isoform of the transmembrane nucleoporin Pom121 (sPom121) regulates gene expression inside the nucleus (Franks et al. 2016) (Fig. 7.4). sPom121 is a consequence of a genomic rearrangement during mammalian evolution that generated an alternative transcription initiation in the POM121 loci. The product of this alternative start site is spliced so that it loses exon 4 encoding the transmembrane domain of Pom121. This results in a soluble isoform of Pom121 that does not associate with NPCs (Franks et al. 2016). The soluble sPom121 uses its nuclear localization signal to access the nuclear interior where it interacts with Nup98 at many gene promoters and cooperates to regulate multiple target genes (Fig. 7.4). Like Nup98 and Nup153, sPom121 mobility is affected by the transcriptional state of the cell, and the transcriptional inhibitor Actinomycin D strongly slows down the exchange of Pom121 inside the nucleus (Franks et al. 2016).

7.5.2 *Mechanisms of Gene-Expression Regulation by Intranuclear Nucleoporins*

To date, we have a very limited knowledge of the mechanisms through which intranuclear nucleoporins regulate gene expression in mammalian cells. In the case of Nup98, studies using the Nup98 fusion proteins that result from chromosomal translocations uncovered that through its FG-rich repeats this nucleoporin interacts with several transcriptional and chromatin modulators including CREB binding protein (CBP)/p300 (Kasper et al. 1999), histone deacetylase 1 (HDAC1) (Bai et al. 2006), and mixed lineage leukemia (MLL) (Shima et al. 2017) (Fig. 7.4). Many of these interactions have been shown to play a key role in the deregulation of HOX gene expression that is associated with the malignant transformation of hematopoietic progenitors expressing Nup98 fusion proteins (see Sect. 7.6). Interestingly, wild type Nup98 has also been found to interact with Trx/MLL and NSL in Drosophila, and to regulate HOX gene expression in this organism (Pascual-Garcia et al. 2014). Nup98 interactors suggest that this nucleoporin helps to recruit chromatin modifiers to specific loci, particularly developmental genes, influencing their expression. This may also hold true for Nup98-regulated INFγ target genes. Although the mechanisms through which this nucleoporin regulates transcriptional memory have not been identified, this process requires specific changes in chromatin modifications (D'Urso and Brickner 2017, 2014) that suggest that Nup98 might also work by modulating the activity of chromatin modifying complexes. But how Nup98, which does not contain DNA binding domains per se, recruits these transcriptional regulators to specific DNA sites is still unknown. A key player in this process might be the nuclear export factor Crm1. Crm1 is the major transport receptor for the export of proteins from the nucleus (Fung and Chook 2014). Recently, it was found that in leukemic cells Crm1 is prebound to HOX gene clusters and helps to recruit the Nup98-HoxA9 and CALM-AF10 aberrant fusion proteins to regulate HOX gene expression (Conway

et al. 2015; Oka et al. 2016) (Fig. 7.4). These findings are very exciting because they identify that the coordinated activity of nuclear transport receptors and nuclear pore complex proteins is not just restricted to the regulation of nucleocytoplasmic transport, but is also critical for the assembly of transcriptional complexes that modulate the activity of the mammalian genome.

The regulation of gene expression by Nup98 is not restricted to its function on specific loci. Nup98 has also been shown to bind to the 3' end of a distinct set of p53 target genes and to regulate mRNA stability (Singer et al. 2012). For example, Nup98 binding to the 3'UTR of p21 mRNA prevents its degradation by the exosome and increases its levels in cells. Because certain cancers, such as hepatocellular carcinoma (HCC) show reduced levels of Nup98, this nucleoporin has been suggested to act as a tumor suppressor required for the proper function of p53 in cells (Singer et al. 2012).

Recently, a proteomic screen identified the DExH/D-box helicase DHX9 as a binding partner for Nup98 (Capitanio et al. 2017). Helicases are enzymes that catalyze nucleic acid remodeling. The DHX9 helicase is able to unwind RNA as well as DNA, and has been shown to play critical roles in gene transcription and RNA processing (Lee and Pelletier 2016). Nup98 has been found to recruit DHX9 to specific foci within the nucleus and modifying Nup98 levels affects the intranuclear localization of the enzyme (Capitanio et al. 2017). Interestingly, Nup98 and DHX9 co-bind a subset of messenger RNAs and genomic loci; and the interaction of Nup98 with DHX9 has been shown to stimulate the transcriptional function of the enzyme (Capitanio et al. 2017). Because Nup98 binding to DHX9 increases its ATPase activity, it has been proposed that this nucleoporin acts as a cofactor for DHX9 during transcription. The identification of several additional helicases as interaction partners for Nup98 suggest that a Nup98-helicase complex may be responsible for regulating the activity of a subset of Nup98 target genes, either by modulating the activity of gene loci themselves or by regulating the processing of their transcripts (Capitanio et al. 2017).

In contrast to the transcriptional activator function of Nup98, Nup153 has been shown to negatively regulate the activity of differentiation/developmental genes, promoting in this manner the pluripotency of ESCs (Jacinto et al. 2015). The way Nup153 represses such genes is by directly interacting and recruiting the polycomb repressive complex 1 (PRC1) to their promoters (Jacinto et al. 2015) (Fig. 7.4). The Polycomb-group (PcG) protein complexes, which include PRC1 and PRC2 among others, are responsible for creating and maintaining a repressive chromatin environment that ensures the silencing of many developmental genes (Aloia et al. 2013; Margueron and Reinberg 2011). Polycomb proteins mediate gene silencing mainly by modulating chromatin structure through histone post-translational modifications. The PRC1 complex, for example, induces chromatin condensation by monoubiquitylation of histone H2A (Wang et al. 2004), which leads to the repression of PRC1 target genes. By bringing the PRC1 complex to differentiation-inducing genes, the chromatin-associated Nup153 induces epigenetic gene silencing that maintains stem cell pluripotency.

7.6 Gene Regulation by Abnormal Nucleoporin Fusion Proteins

Many cancers are characterized by chromosomal translocations that lead to gene fusion encoding chimeric proteins with aberrant functions (Zheng 2013). Because chromosomal rearrangements require the interaction of the two translocating chromosomes, it is considered that the nonrandom distribution of chromosomes inside the cell nucleus is a key determinant of this process (Nikiforova et al. 2000; Roix et al. 2003; Soutoglou and Misteli 2008; Zheng 2013). Chromosomal rearrangements are particularly represented in blood cancers, and several chimeric fusion proteins have been found to play critical roles in the transformation of hematopoietic progenitors and to significantly contribute to the development of blood malignancies. Notably, several nucleoporins were found to be part of oncogenic fusions in blood malignancies, particularly in acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), T-cell acute lymphoblastic leukemia (T-ALL) and myelodysplastic syndrome (MDS). These include Nup98, Nup160, Nup214, Nup358 and Tpr (Simon and Rout 2014; Shimozono et al. 2015; Fahrenkrog 2014).

Most studies involving nucleoporin fusions have been centered on Nup98 fusions, and to a minor extent, on Nup214 fusions. Nup98 is the most frequently translocated nucleoporin in leukemia, and at least 30 different fusions partners for this nucleoporin have been identified so far (Fahrenkrog 2014; Saw et al. 2013). The oncogenic strength and the mechanisms of function of different Nup98 fusions depend on the fusion partner (Saw et al. 2013). Leukemias that have Nup98 chimeric fusions are generally highly aggressive and very resistant to therapies (Moore et al. 2007; Gough et al. 2011). In most cases, NUP98 participates in balanced chromosomal translocation that result in fusion proteins of the N-terminal domain of Nup98, which contains its GLFG repeats, and the C-terminal domain of a fusion partner (Gough et al. 2011; Moore et al. 2007). The GLFG repeats of Nup98 have been shown to be essential for the transformation of hematopoietic progenitors by Nup98 fusions (Kasper et al. 1999); and although wild type Nup98 localizes to NPCs and the nuclear interior, Nup98 fusions have been described as intranuclear proteins that do not associate with NPCs (Kasper et al. 1999; Xu and Powers 2010; Fahrenkrog et al. 2016). Of all Nup98 fusions, approximately one third are proteins that contain homeodomains (HD), which are helix-turn-helix DNA-binding domains (Fahrenkrog 2014; Moore et al. 2007; Gough et al. 2011). This class mostly include transcription factors that play key roles in blood development. The rest include non-HD proteins, the majority of which are involved in epigenetic regulation and chromatin remodeling. The ectopic expression of several Nup98 fusion proteins has been shown to promote the proliferation and prevent the differentiation of hematopoietic progenitors (Chung et al. 2006; Calvo et al. 2002; Choi et al. 2009; Yassin et al. 2009; Takeda et al. 2006).

Studies of Nup98 fusion proteins in leukemia have uncovered alterations in the NE structure (Fahrenkrog et al. 2016), nucleocytoplasmic transport (Funasaka

et al. 2011; Saito et al. 2017; Takeda et al. 2010), cell signaling (Qiu et al. 2015), and mitosis (Salsi et al. 2014, 2016). But probably the most common abnormalities observed with the ectopic expression of Nup98 chimeric proteins are the deregulation of gene expression, particularly of HOX genes, and epigenetic alterations (Kasper et al. 1999; Ghannam et al. 2004; Bai et al. 2006; Calvo et al. 2002; Saw et al. 2013; Oka et al. 2016; Rio-Machin et al. 2017). This suggest, that key mechanisms though which these aberrant proteins affect normal cell physiology are by modulating the expression of key differentiation/developmental genes and through changes in chromatin organization. How Nup98 fusions perform these functions is an area of active research. Recently, several Nup98 fusion proteins were shown to interact with the histone modifying complexes mixed lineage leukemia 1 (MLL1) and the non-specific lethal (NSL) (Xu et al. 2016), being these interactions required for leukemogenesis (Xu et al. 2016; Shima et al. 2017). These include NUP98-HOXA9, NUP98-HOXD13, NUP98-NSD1, NUP98-PHF23, and NUP98-TOP1 (Xu et al. 2016). As mentioned, wild type Nup98 in drosophila also interacts with Trx/MLL and NSL to regulate HOX gene expression (Pascual-Garcia et al. 2014). This indicates that the regulation of histone modifications is a normal function of Nup98 that gets hijacked by its fusion partners. Nup98 fusion proteins have also been shown to associate with other chromatin modifiers including the histone deacetylase HDAC1 and CBP/p300 (Kasper et al. 1999; Bai et al. 2006; Rio-Machin et al. 2017). For many interactions between Nup98 and chromatin modulators, the FG repeats of Nup98 have been shown to be essential for protein association and for chromatin modifications (Bai et al. 2006; Kasper et al. 1999). This also supports the idea that wild type Nup98 has a role in epigenetic modulation. Some of these endogenous Nup98 functions might be further potentiated by its fusion with other chromatin modifiers such as the histone methyl transferases NSD1, NSD3 or MLL. Conversely, the endogenous functions of transcriptional/chromatin modulators might be enhanced by fusion with Nup98. For example, in some cases of AML, Nup98 is fused to the plant homeodomain (PHD) domains of JARID1A and NSD1. PHD fingers, which are present in many chromatin-remodeling proteins, bind to specific histone/epigenetic marks and help to recruit transcription factors and chromatin modulators (Musselman and Kutateladze 2009). At the HOXA locus, PHD domains have been found to prevent the spreading of polycomb repressive complexes which promote gene silencing. Consistent with a chromatin boundary activity, NUP98–PHD fusions were found to prevent polycomb-mediated gene silencing and to help maintain chromatin in an active state, stimulating in this manner HOX gene expression (Wang et al. 2009).

As mentioned before, several other nucleoporins participate in cancer associated chromosomal translocations. For most of them, the mechanisms through which this nucleoporin chimeric proteins deregulate cellular physiology is still unknown. So far, the only other nucleoporin fusion that has been shown to also affect gene expression directly is the SET-NUP214 chimera. Nup214 (also known as CAN) is an FG repeat-containing nucleoporin component of the cytoplasmic filaments (Kraemer et al. 1994; Napetschnig et al. 2009). This large nucleoporin

plays important roles in nuclear import and export. Similar to Nup98, the fusions of Nup214 maintain its FG domains and localize to intranuclear foci instead of NPCs (Fornerod et al. 1995; Saito et al. 2004; Simon and Rout 2014). Also like Nup98, Set-Nup214 chimeras bind to the promoter of HOXA genes and deregulate their expression, promoting cell proliferation and inhibiting cell differentiation. At HOXA gene promoters Set-Nup214 interacts with the transport receptor CRM1 and the histone methyltransferase DOTL1 (Van Vlierberghe et al. 2008). This suggests that its mechanisms of recruitment and gene expression regulation might be conserved with the Nup98-HoxA9 fusion (see Sect. 7.5.2) (Conway et al. 2015; Oka et al. 2016).

Another fusion of Nup214, Dek-Nup214, has been shown to affect gene expression but at the translation, instead of transcriptional, level (Ageberg et al. 2008). By stimulating hyperphosphorylation of the oncogene Elf4E, Dek-Nup214 increases overall protein synthesis (Ageberg et al. 2008). This is interesting, because increased activity of Elf4E has been found to modify the configuration of NPCs to stimulate the export of oncogenic RNAs and promote cell proliferation (Culjkovic-Kraljacic et al. 2012). It is worth mentioning, that even though Set- and Dek-Nup214 fusions do not localize to NPCs, they still affect nucleocytoplasmic transport (Saito et al. 2016; Port et al. 2016). The way these fusion proteins affect nuclear transport is through the sequestration of transport receptors and nucleoporins into their highly dynamic intranuclear foci (Saito et al. 2016; Port et al. 2016). Naturally, by affecting nuclear transport, and also by tethering transcription factors to these foci, Nup214 fusions have an indirect impact on gene expression.

7.7 Conclusions

Since their discovery, NPCs have continuously amazed scientist for their unique features. First was their exceptional structure, then was their essential role in controlling the exchange of all molecules between the nucleus and the cytoplasm, and now their emerging contributions to genome integrity and gene expression regulation. Though almost 70 years have passed from their first observation, there are still many mysteries that need to be elucidated. What is the organization of the central channel? Which is/are the definite mechanism/s of nucleo cytoplasmic transport? How many partners work together with NPCs to regulate genome function? are some of many questions that still remain to be answered.

But what has become clear with a large amount of work from many different labs is that NPCs are not just mere channels that sit at the NE passively allowing the exchange of molecules between the nucleus and the cytoplasm. NPCs are highly dynamic and plastic structures that can be modified to change their properties, that can have distinct composition in different cell types, that play multiple transport-independent functions, and that they are central regulators of cellular physiology. The recent findings that NPC components also have off-pore functions not only extends the potential processes modulated by nucleoporins but also

provides a novel perspective on how these structures might contribute to regulate essential cellular functions.

The role of NPCs as critical regulators of genome organization and gene expression was originally described in yeast and flies. Although understanding the function of mammalian NPCs in these processes lagged behind for some time, it has now started to catch up with the emerging roles of mammalian NPCs in all aspects of genome integrity, including genome organization, transcriptional regulation, DNA repair, DNA replication, chromosome segregation, and others (see (Bukata et al. 2013; Raices and D'Angelo 2012) for additional information). The central role that NPCs and nucleoporins play in many of these processes explains the increasing number of alterations in these structures that are being linked to diseases such as neurodegeneration and cancer. Understanding the modes of action of NPCs and nucleoporins in regulating and coordinating genome functions will help us elucidate how these structures contribute to the faithful translation and transmission of the genetic information, and will result in a better understanding of how alterations in their function contribute to disease development.

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Chapter 8
Nuclear Pore Complexes, Genome Organization and HIV-1 Infection

Francesca Di Nunzio

Abstract Nuclear pore complexes (NPCs) are dynamic structures embedded in double lipid layer of the nuclear envelope (NE), which act as guardians of nucleo-cytoplasmic transport, and contribute to genome organization, genome stability and gene expression regulation. Some of these cellular functions orchestrated by NPCs are usurped by viruses that replicate in the nucleus. Non-mitotic cells are one of the major targets of HIV-1, thus the passage of the virus through the NPC is a key step for viral replication. In recent years, research regarding multiple aspects of the early steps of HIV-1 life cycle highlights dynamic and concerted interactions between viral components, NPC and chromatin state. HIV-1 is a member of this host-pathogen activity which ensures favourable conditions for the production of its own progeny. This chapter aims to review the existing and emerging concepts showing how individual nucleoporins (Nups) may be a "cellular code" that dictates HIV-1 fate in infected cells.

Keywords HIV-1 · nucleoporins · nuclear translocation · integration · viral transcription

8.1 Introduction

Eukaryotic chromosomes are protected and enclosed by the NE, a double-lipid bilayer, which allows the communication between the inner and the outer sides of the nucleus. This link is mediated by dynamic windows, called NPCs, which are uniformly or unequally distributed depending on the cell cycle (Maeshima et al. 2006). Nuclear pore components play critical roles in some vital cellular functions and can be also hijacked by viruses. Some Nups are also directly involved in the nuclear entry of viruses that replicate in the nucleus. Recent studies highlighted

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the importance of Nups for viral nuclear import and viral replication of different viruses that replicate in the nucleus: Human Immunodeficiency Virus (HIV), Hepatitis B Virus (HBV), Herpes Simplex Virus (HSV), Influenza Virus, and Adenovirus (Brass et al. 2008; Copeland et al. 2009; Di Nunzio 2013; Di Nunzio et al. 2012, 2013; Konig et al. 2008; Lelek et al. 2015; Matreyek et al. 2013; Schmitz et al. 2010; Trotman et al. 2001). In particular, RanBP2/Nup358, main component of the cytoplasmic fibrils, and Nup153, the most dynamic Nup, are involved in HIV-1 docking at the pore and nuclear translocation respectively (Di Nunzio et al. 2012, 2013; Matreyek et al. 2013; Schaller et al. 2011). The main component of the nuclear basket of the NPC, Tpr, regulates the chromatin landscape around nuclear pores and is critical for HIV-1 replication (Lelek et al., 2015).

This manuscript provides a detailed view of the recent advances in the early steps of HIV-1 infection, taking into account results obtained by crystal structures for interactions between viral components and host factors, next generation sequencing methods to identify integration sites, as well as new cutting-edge microscopy to visualize the viral journey into the host cell.

8.2 Early Steps of HIV-1 Infection

HIV-1 has the peculiarity to infect non-dividing cells and, hence, it enters the nucleus through the NPC channel. The life cycle of HIV-1 begins with binding of the viral envelope glycoproteins to receptor CD4 and co-receptors CCR5 or CXCR4 on the target cell, followed by viral fusion to the cellular membrane and release of the viral core into the cytoplasm. The viral genome packaged into the capsid (CA) shell, the viral core, is formed by two positive strands of RNA that are retrotranscribed in DNA which forms the pre-integration complex (PIC) when coupled with other known, such as the integrase (IN), and unknown factors. The PIC has been estimated to have a size ~ 56 nm (Miller et al. 1997) which far exceeds the size of molecules that can pass through the NPC channel, thus, it is generally accepted that the nuclear import of the HIV-1 PIC is an energy-dependent active process that is governed by different viral and cellular factors. Once inside the nucleus, the PIC can integrate into the host chromatin. This step is considered to be essential for productive replication and ensure the stable insertion of the viral genome sequence in the host chromatin. HIV-1 inserts its genome in the active chromatin located near NPCs (Lelek et al. 2015; Marini et al. 2015). Viral and host factors directly or indirectly influence the selection of the target site. These include nucleoporins, chromatin structure, the viral integrase and chromatin tethering factors like lens-epithelium-derived growth factor/p75 (LEDGF/p75). One of the latest findings shows that chromatin organization around NPCs is determinant for the fate of viral progeny. In fact, the nuclear basket of the pore maintains an open chromatin that is favourable for viral replication (Lelek et al. 2015). Therefore, even if the majority of integrated proviruses are transcribed to ensure their progeny, a small fraction remains silent and forms viral reservoir,

which is the most critical barrier for the eradication of HIV-1. The passage through the pore can determine directly or indirectly HIV-1 integration sites as it has been shown in infected cells depleted for some Nups, such as RanBP2/Nup358, Nup153, Nup98 and Tpr (Di Nunzio et al. 2013; Lelek et al. 2015; Ocwieja et al. 2011; Schaller et al. 2011). A particular euchromatin landmark, H3K36me3, associated to viral integration sites has been found in the chromatin near the nuclear basket. Its location near the pore channel is regulated by the presence of Tpr, in fact cells depleted for Tpr have less H3K36me3 near the NPC (Lelek et al. 2015). Interestingly, infected lymphocytes depleted for Tpr show a silent virus while the global profile of gene expression is not altered. Results obtained by super resolution imaging identified a peak density of H3K36me3 at approximately 500 nm from the NPC position (Lelek et al. 2015), this is in agreement with data obtained by DNA FISH showing that HIV-1 preferentially integrates at the nuclear periphery, within 1 μm from the nuclear envelope (NE) (Marini et al. 2015). In contrast, proviral integrations are disfavoured in the chromatin associated with the nuclear lamina (Marini et al. 2015). This chromatin located between pores is poor in marks of active genes, such as H3K36me3, and weakly associated with integration sites (Lelek et al. 2015). It is clear that the fate of HIV-1 integration is dictated by PIC-host nuclear factor interactions and the chromatin landscape near the NPC, in particular, the chromatin located in the vicinity of the nuclear pore complex creates a favourable environment for HIV-1 integration sites selection.

8.3 Role of NPC Components in HIV-1 Replication

8.3.1 Nups and HIV-1 Uncoating

After fusion to the cellular membrane, HIV-1 releases the viral core into the cytoplasm. The HIV-1 core then travels through the microtubule network of the host cell to traffic towards the nucleus (McDonald et al. 2002). This pathway is used only by HIV-1 carrying an envelope WT, while HIV-1 delta Env pseudoptyped with VSV-G, commonly used for the investigation of the early steps of HIV-1 life cycle, enters in the cytoplasm by endocytosis (Campbell and Hope, 2015). However, only the VSV-G pseudotyped virus is able to escape from endosomes and is highly infectious, giving the virus 20- to 130-fold higher infectivity (Aiken, 1997; Luo et al. 1998). Even if the two viruses enter in contact with different cytoplasmic components, both need the NPC to enter the nucleus (Di Nunzio et al. 2012). Once at the NPC, the viruses dock at the pore through RanBP2/Nup358 (Di Nunzio et al. 2012), spending several hours before translocating inside the nucleus. HIV-1 needs to uncoat (loss of the integrity of core) before integrating. This is one of the most intriguing steps of the HIV-1 life cycle. In the past years, the most accredited model defined the uncoating as the process by which the capsid core dissociates from the rest of the RTC immediately after viral infection (Aiken 2006). Furthermore, some studies suggest that CA remains associated with the RTC before to translocate into

the nucleus (Arhel et al. 2007; Di Nunzio et al. 2012, 2013; Jacques et al. 2016; Lelek et al. 2012; Matreyek et al. 2013; Price et al. 2014). Uncoating may occur following three principal models that have been proposed (Campbell and Hope 2015): (1) immediately after viral fusion with the cellular membrane (Francis et al. 2016; Mamede et al., 2017); (2) during cytoplasmic trafficking (Francis et al. 2016; Hulme et al. 2011); (3) with the aid of nuclear pore factors (Di Nunzio et al. 2012, 2013; Lelek et al. 2015; Price et al. 2014). A recent study using an artificial system for viral imaging observed that the majority of HIV-1 cores uncoat soon after release into the cytoplasm. Less than 5% of viral particles uncoat gradually during their cytoplasmic journey and only a small fraction shows a late uncoating at the pore (Francis et al. 2016). Recent studies attributed the CA shell an important protective role versus the viral genome avoiding its exposure to cytosolic DNA sensors. This observation is not compatible with a model of premature uncoating (Rasaiyaah et al. 2013). Interestingly, results from different teams and techniques have shown that inhibition of reverse transcription delays uncoating (Hulme et al. 2011; Yang et al. 2013). The peak of reverse transcription is around 7–8 hrs from infection (Butler et al. 2001), suggesting a late uncoating. Other studies show that the CA is the viral determinant for HIV-1 nuclear import (Yamashita et al. 2007) through the interaction with cellular host factors (Brass et al. 2008; Di Nunzio et al. 2012, 2013; Konig et al. 2008; Lee et al. 2010; Matreyek et al. 2013; Schaller et al. 2011). Because of the large size (120 nm × 60 nm × 40 nm), the viral core cannot translocate through the NPC channel, which has an estimated diameter of ~ 39nm (Pante and Kann 2002). Thus, current models propose that uncoating begins before to enter in the nucleus (Ambrose and Aiken 2014; Arhel et al. 2007; Chen et al. 2016; Fassati 2012; Hilditch and Towers 2014; Burdick et al., 2017). Nups have been found to have an active role in PIC maturation and in the uncoating processes (Bichel et al., 2013). The peculiarity of lentiviruses with respect to retroviruses in usurping the NPC could be due to the specific interaction of the lentiviruses CA to particular Nups. The first Nup that the viral CA can meet is RanBP2/Nup358. The interaction between them has been well documented (Di Nunzio et al. 2012; Schaller et al. 2011). The C-terminus of RanBP2/Nup358 comprising a cyclophilin (cyp)-homology domain has a major contribution to the *in vitro* cores binding, probably helped by FG repeats dispersed along the entire protein (Di Nunzio et al. 2012). A recent study highlights the possibility that the CA isomerization mediated by the cyclophilin-homology domain of RanBP2/Nup358 may be preserved by HIV-1 to target the nuclear pore and synchronize nuclear entry with CA uncoating (Bichel et al. 2013). Interestingly, the cyclophilin A binding loop is an exclusive feature of lentiviruses capsid, while retroviruses do not have this loop in their CA preventing their docking at nuclear pores. Another critical Nup for HIV-1 infection is Nup153. This Nup plays multiple cellular functions and also participates in the replication of several viruses. HBV, for example, binds Nup153 to help the uncoating and the release of its DNA genome into the nucleus (Schmitz et al. 2010). HIV-1 CA also binds the human Nup153 (Di Nunzio et al. 2013). It is possible that Nup153 participates in HIV-1 uncoating, but the mechanism is still under

investigation. Interestingly, the multifaceted role of viral CA can determine the differences between the two retroviral subfamilies, gamma retroviruses and lentiviruses. In the case of gamma retroviruses, like MLV, the uncoating step is better understood than for lentiviruses, such as HIV-1. MLV uncoating is triggered by an accessory p12 protein that interacts with MLV cores, preventing uncoating and ensuring completion of reverse transcription. The C-terminus tail of p12 binds condensed chromatin, which is typical during mitosis, and tethers the CA-associated PIC to the chromatin helping with the integration step (Elis et al. 2012; Wight et al. 2014). Consistent with this mechanism, when the mitosis ends and the NE reassembles, p12 is released and orchestrates CA uncoating (Schneider et al. 2013). In contrast to retroviruses, lentiviruses have a more unstable core, and *in vitro* studies have failed to show association of CA with PIC (Farnet and Haseltine 1991; Fassati and Goff 2001; Forshey et al. 2002; Miller et al. 1997). The high fragility of HIV-1 CA increases the difficulty of studying the fate of viral CA traveling into the host cell environment. Microscopy studies could overcome the problems correlated with the CA stability, but the limited number of cores that can mature in functional PICs poses a problem for the interpretation of the imaging results. Recent new advances in imaging approaches have led to a better comprehension of the molecular mechanism underlying the uncoating step (Chin et al. 2015; Lelek et al. 2012, 2015; Peng et al. 2014; Burdick et al., 2017) and might aid in the design of new compounds to specifically target this step.

8.3.2 Nups and HIV-1 Translocation

HIV-1 is a component of *Retroviridae* family. Contrary to other subclasses, the lentivirus category, which includes HIV-1, has the ability to infect non-dividing cells as well as dividing cells, likely sharing the same nuclear import mechanism (Katz et al. 2003). Larger complexes are more efficiently translocated in nuclei of actively dividing cells with respect to quiescent cells, probably due to the dependency of the nuclear import on the phosphorylation of some critical factors and on cell metabolism (Feldherr and Akin 1994). NPCs are the exclusive controllers of nucleocytoplasmic trafficking and regulate the passage of macromolecules in and out the nucleus. Recent experiments based on atomic force microscopy suggest that Nups containing FG repeats form condensates in the centre of NPC channel that may transiently dissolve to transport larger cargoes (Stanley et al. 2017). Viruses are also subjected to the nucleocytoplasmic transport rules. Herpesviruses and adenoviruses, possess a large and relatively stable icosahedral capsid (CA), which envelops the viral genome. Since their capsids are larger than the nuclear pore channel, these viruses need to uncoat before entering the nucleus. Viral capsids dock at the NPC cytoplasmic side, and the interaction with Nups is used as a cue to trigger genome nuclear release. Herpesviruses bind to the NPC via an importin β-dependent interaction with RanBP2/Nup358 and the interaction with

Nup214 triggers DNA release in the nucleus of the target cell (Cohen et al. 2011). The CA of adenovirus recruits histone H1, importin β, importin 7 and hsp70 (Mercer et al. 2010) which trigger the disassembly/conformational changes required for the import of viral DNA into the nucleus through the NPC (Cohen et al. 2011; Hsieh et al. 2010). Different retroviruses have different behaviours during nuclear entry. MLV enters the nucleus during mitosis while HIV-1 reaches the chromatin during interphase. Both pathways are regulated by different cellular factors that play critical and indispensable roles in retroviruses nuclear import. The differences between these two retroviruses, MLV and HIV-1, have been one of the most intriguing observations in the field of viral nuclear import. Biochemical data suggest that the divergence between the two retroviruses could be attributed to a different stability of viral CA. In the case of HIV-1, the presence of CA as component of the PIC is still uncertain, however, increasing evidences suggest that this viral protein is a determinant of HIV-1 nuclear import. Low levels of HIV-1 CA has been observed associated with the reverse transcription (RTC) and the PIC (Farnet and Haseltine 1991; Fassati and Goff 2001; Forshey et al. 2002; Miller et al. 1997). For many years it was believed that the traffic through the pore was independent on the viral CA. Recently several teams showed the critical role of viral CA in HIV-1 translocation and integration, highlighting a new role for HIV-1 CA in the early steps of viral infection (Brass et al. 2008; Chen et al. 2016; Dharan et al. 2016; Di Nunzio et al. 2012, 2013; Jacques et al. 2016; Konig et al. 2008; Lelek et al. 2015; Matreyek et al. 2013; Ocwieja et al. 2011; Saito et al. 2016; Sowd et al. 2016). Direct interactions between viral cores and Nups have been shown to be essential for an efficient viral translocation (Di Nunzio et al. 2012, 2013; Lelek et al. 2015; Matreyek et al. 2013; Schaller et al. 2011). Some groups detected the presence of CA inside of the nucleus (Chin et al. 2015; Hulme et al. 2015; Peng et al. 2014) and its interaction with nuclear factors, such as the polyadenylation factor CPSF6 which is a mRNA processing protein that shuttles between the nucleus and the cytoplasm (Chin et al. 2015; Lee et al. 2010). This factor predominantly localizes inside the nucleus due to the presence of a serine/arginine (SR)-rich nuclear localization signal located at the C-terminus of the protein (Lee et al. 2010; Price et al. 2012). The first study showing the critical role of CPSF6 for HIV-1 infection was based on a mouse cDNA-expression screen that identified the truncated form of CPSF6 lacking the C-terminal SR rich domain, which accumulated in the cytoplasm and prevented HIV-1 nuclear import (Lee et al. 2010). The direct binding between CPSF6 and *in vitro* cores was later shown (Fricke et al. 2013). Overall these findings uncovered that HIV-1 usurps the nuclear pore machinery to dock, translocate and integrate into the host chromatin. These steps are concerted to aid the PIC to mature and allow the provirus to integrate into the host genome to replicate (Fig. 8.1). Nups also have a key role in HIV-1 translocation in fact, the depletion of particular Nups induces a block in HIV-1 nuclear entry. Several studies have determined the individual role of Nups in major viral steps, such as viral docking at the pore and nuclear translocation. The depletion of RanBP2/Nup358, which is exclusively located in the outer side of the pore, reduces HIV-1 nuclear entry by up to ten fold (Di Nunzio et al. 2012;

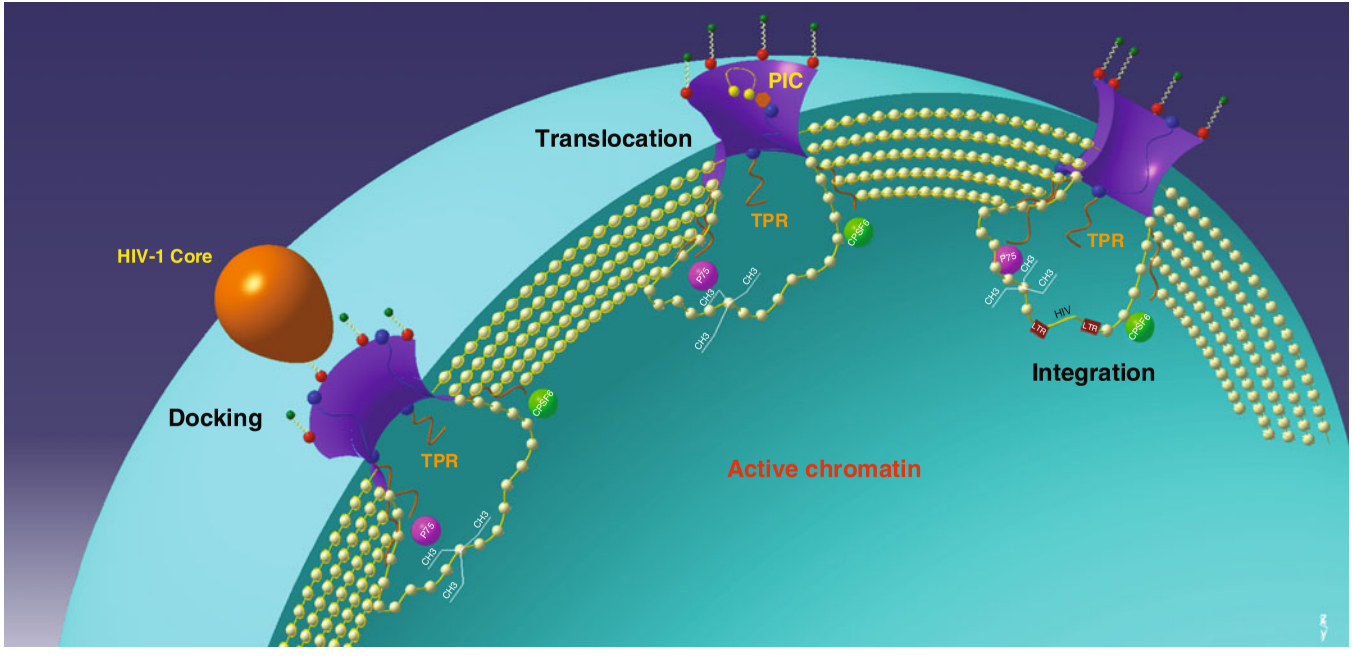

Fig. 8.1 HIV-1 nuclear import and integration in target cells. An intact core is shown before/during interaction with the NPC (left) (docking), in the centre it is represented the PIC translocation, potentially piloted by Nup153 (blue), which is in a collapsed conformation compatible with nuclear import of cargo. The CA hexamer (orange) could be part of this latter complex. On the right, the proviral integration into the host euchromatin located underneath the pore (Lelek et al. 2015; Marini et al. 2015) enriched in open chromatin feature H3K36me3 and nuclear factors LEDGF/p75 (pink), CPSF6 (green)

Schaller et al. 2011). This is similar to the depletion of Nup153, a Nup predominantly located in the nuclear side of the NPC (Di Nunzio et al. 2013; Matreyek et al. 2013). Outwardly, these two Nups showing opposite locations at NPCs seem to participate at the same step of HIV-1 infection. But several differences in their roles have been identified. RanBP2/Nup358 is involved in the docking step of the virus at the pore (Di Nunzio et al. 2012), while Nup153 is required for nuclear translocation, which is mediated by its flexible C-terminus domain (Di Nunzio et al. 2013; Lelek et al. 2015; Matreyek et al. 2013). According to *in vitro* studies, the Cyp loop of CA determines the interaction with the Cyp-like domain of RanBP2/Nup358 (Lin et al. 2013), while a specific region of the C-terminus domain of Nup153 that interacts with the interphase CA (NTD)-CA (CTD) in the CA hexamer is responsible for the binding to the viral core (Matreyek et al. 2013). This CA pocket is present only in assembled or partially assembled core but not in monomers, suggesting that Nup153 interacts and favours HIV-1 nuclear entry through the binding of CA hexamers (Figs. 8.1 and 8.2). Similarly to HIV-1, the yeast ortholog of human Nup153, Nup124p binds *gag* of the retrotransposon Tf1 to help nuclear import (Varadarajan et al. 2005). This Nup contains 29 FG repeats at its terminal tail, which are responsible for this binding as it has been shown *in vitro* (Matreyek et al. 2013) and *in vivo* (Lelek et al. 2015). Even if Nup153 is predominantly located at the nuclear basket, it has also been observed at the cytoplasmic side of the pore aiding nuclear import of cargo (Lim et al. 2007). Nup153 can assume two conformations a "collapse" conformation for cargo transport and a "release" for cargo interaction in the cytoplasmic side of the NPC (Cardarelli et al. 2012). This motion of Nup153 could correlate with the motion of the molecules actively transported through the NPC. This cellular process could be usurped by HIV-1 to translocate inside the nucleus (Fig. 8.1). However, the mechanistic requirements underlying the *in vivo* viral nuclear translocation mediated by Nup153 or other factors and the state of the viral CA during translocation have yet to be formally demonstrated. In summary, the viral core is believed to dock onto the cytoplasmic side of the NPC through interactions with RanBP2/Nup358 (Di Nunzio et al. 2012; Schaller et al. 2011). HIV-1 subsequently interacts with some Nups, such as Nup153, Nup98-Nup96, Tpr, and factors like TNPO3 and CPSF6, directly or indirectly to release the PIC into the nucleoplasm (Di Nunzio et al. 2012, 2013; Krishnan et al. 2010; Lee et al. 2010; Lelek et al. 2015; Matreyek et al. 2013; Price et al. 2014; Valle-Casuso et al. 2012).

8.3.3 Nups and HIV-1 Integration

The regulation of nucleocytoplasmic transport is the major known role of NPC. However, recent evidences show Nups as regulators of different processes in the cell. In particular, Nups can affect fundamental genome functions independently of their nuclear transport function (Ibarra and Hetzer 2015). The multifaceted role of Nups could be attributed to particular domains present in them. In particular,

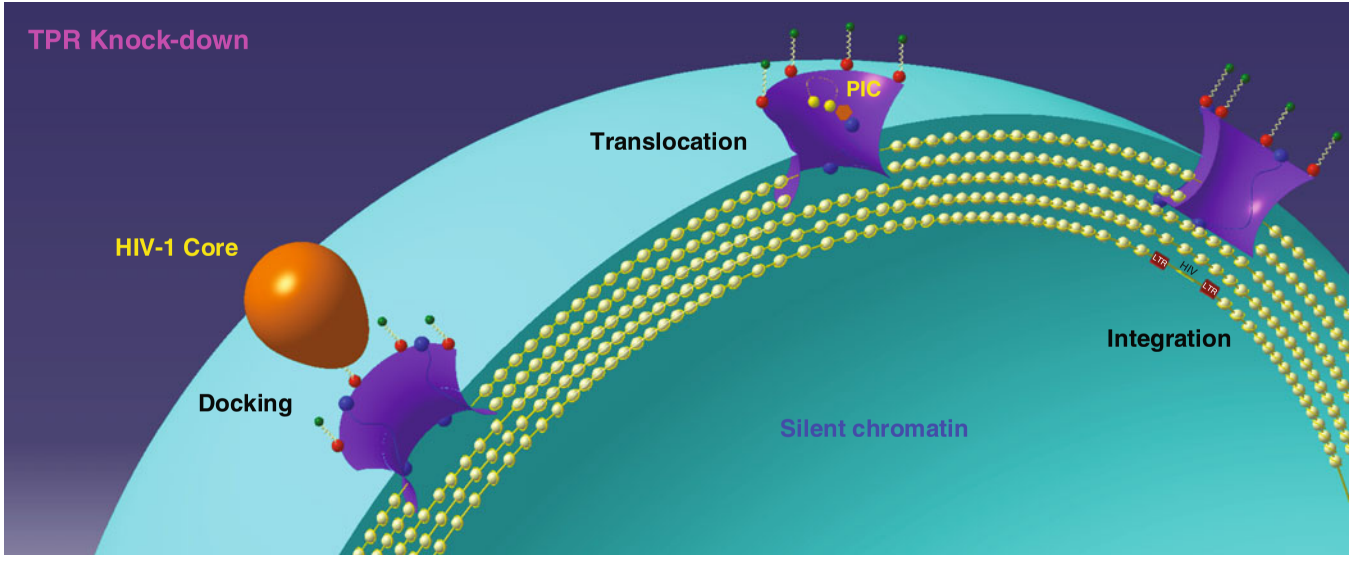

Fig. 8.2 HIV-1 nuclear import and integration in Tpr depleted cells. NPCs represented lack of Tpr, thus the chromatin underneath the nuclear basket is less de-condensed. An intact core is shown before/during interaction with the NPC (left) (docking), in the centre it is represented the PIC translocation, potentially piloted by Nup153 (blue), which is in a collapsed conformation compatible with nuclear import of cargo. The CA hexamer (orange) could be part of this latter complex. On the right, the proviral integration into the host chromatin less de-condensed due to the loss of Tpr fibrils located underneath the pore (Lelek et al. 2015; Marini et al. 2015)

Nups are able to influence the chromatin landscape. The association of active decondensed chromatin to NPCs has been interpreted as the link between the transcription of active genes with mRNA export (Blobel 1985). The functional reason for the presence of active chromatin at NPCs could be the presence of the transcriptional activator SAGA and the mRNA export machinery at the nuclear basket (Cabal et al. 2006; Dieppois et al. 2006; Garcia-Oliver et al. 2012; Iglesias et al. 2010; Rodriguez-Navarro et al. 2004; Taddei and Gasser 2004). In yeast, gene regulation regulated by Nups-chromatin interactions is spatially confined at the nuclear periphery. In metazoans, Nups can exist in two populations, one associated with the pore and the other free in the nucleoplasm. Thus, gene regulation mediated by Nups-chromatin interactions is more complicated. In *Drosophila*, the silencing of nuclear basket Nups, Nup153 and Mtor/Tpr, reduces the expression of thousands of genes. Interestingly, these Nups are associated with active chromatin marks (Vaquerizas et al. 2010). It is possible that the same Nup can participate in different transcriptional regulation events depending on their location at the NPC or off-NPC. For example, in *Drosophila* Nup98 shows preferential binding for active genes only if located out of the pore (Kalverda et al. 2010). However, in human cells, Nup98 also participates in gene activation at the pore (Liang et al. 2013). Nups seem to organize chromatin topology, controlling the accessibility of transcription factors. The complex formed by NPCs and the underlying chromatin could act as a functional hub that recruits the enzymatic machinery that leads to epigenetic reformatting and the transcriptional regulation of particular genes. This model might explain the role of Nups in transcriptional memory, in which Nup98 favours specific chromatin structure changes, such as H2A.Z deposition and H3K4me2 (Ahmed et al. 2010; Brickner et al. 2007; Light et al. 2010, 2013). NPCs also play a critical role in chromatin organization, defining active and silent regions near the pore. During interphase the chromatin is well organized within the nucleus. In particular, condensed chromatin (heterochromatin) is associated with nuclear periphery, interrupted by stretches of less condensed chromatin (euchromatin) at NPCs (Krull et al. 2010; Ma et al. 2015; Raices and D'Angelo 2012) (Fig. 8.1). The chromatin near the pore represents actively transcribed regions and it is also the target of HIV-1 PIC (Lelek et al. 2015; Marini et al. 2015). This might ensure/promote efficient viral gene expression after integration. HIV-1 integrase (IN) binds to LEDGF/p75, which was the first cellular factor identified to help HIV-1 integration into active genes (Cherepanov et al. 2003; Ciuffi et al. 2005). LEDGF/p75 promotes efficient infection (Llano et al. 2006; Shun et al. 2007) and tethers IN to favour target sites (Fig. 8.1). The HIV-1 integration machinery must also interact with many additional host factors during infection, including nuclear trafficking and pore proteins during nuclear entry, histones during initial target capture, and DNA repair proteins during completion of the DNA joining steps. The NPC has been described as the link between HIV-1 translocation and integration into the host chromatin (Di Nunzio 2013; Lelek et al. 2015). It is possible that HIV-1 cores recruit cellular factors that promote or participate in these concerted steps. The viral-host complexes can favour the accessibility to viral components, usually hided in the inner core, or modify

the conformation of the CA influencing the engagement of other host factors that mediate the nuclear translocation and integration of the PIC into the host chromosomal DNA. The viral CA shows some plasticity in the use alternative pathways to aid the PIC nuclear entry and HIV-1 CA mutants insensitive to certain Nups show different integration sites pattern than wild type viruses (Schaller et al. 2011). For example, HIV-1 CA carrying point mutations, like N74D, which is unable to recruit the cellular factors CPSF6 and RanBP2/Nup358, shows a different pattern of integration sites than wild type HIV-1 (Koh et al. 2013; Schaller et al. 2011; Sowd et al. 2016). Other HIV-1 CA mutants, such as N57A, which are more severely defective in arrested cells than dividing cells (Yamashita et al. 2007), also show a different integration pattern than the wild type virus (Schaller et al. 2011). It is possible that HIV-1 CA mutant engages different host factors determining a viral nuclear entry at a different cell cycle step than the wild type virus. The results underscore the plasticity of HIV-1 CA and its ability to recruit different host factors to reach and integrate in cellular chromosomes to guarantee new viral progeny. Beside the role in HIV-1 nuclear import, CPSF6 is also involved in defining the distribution of HIV-1 integration sites. Interestingly, the depletion of CPSF6 provokes changes in the kinetics of viral infectivity and reduces integration into transcriptionally active and spliced genes. CPSF6 binds CA and it has been proposed to drive the PIC to actively transcribed chromatin (Chin et al. 2015; Sowd et al. 2016). The integrase-LEDGF/p75 interaction, on the other hand, promotes integration into gene bodies where nucleosomes are remodelled for an efficient HIV-1 integration (Lesbats et al. 2011; Sowd et al. 2016). A double CPSF6 and LEDGF/p75 knockout diminishes integration into genes below the levels observed for each of them. These observations support a model in which NPC components act in concert with other cellular factor to favour HIV-1 replication.

Studies of different integrating genomic parasites show that their host chromatin targeting preferences have evolved to optimize their coexistence with the host and to favour the release of new viral progeny. One example comes from the yeast Ty retrotransposons, which must coexist with their hosts indefinitely so they integrate into host genomic locations that do not damage the survival of yeast cell (Bushman 2003; Craig and Marszalek 2002). Instead, HIV-1 infected T cells typically survive only a day or two before to be killed by the cellular immune system or by the toxicity of infection (Perelson et al. 1996). Thus, HIV-1 needs to push the release of the newborn viruses within few days from infection. Viral integration within transcription units is usually favourable for efficient transcription (Jordan et al. 2001; Lewinski et al. 2005) potentially explaining the targeting preference. But, HIV-1 can also coexist long time as chimera into the genome of infected patients as latent virus (Razooky et al. 2015). The mechanisms underlying the establishment of latency are under investigation and it is highly possible that chromatin factors associated with nuclear entry proteins can play a critical role on the persistence of HIV-1 in infected cells. Other retroviruses, such as gamma retroviruses favour integration near transcription start sites of cancer genes (Wu et al. 2003) to ensure a favourable environment for proviral transcription with the additional advantage to promote the proliferation and/or survival of the infected cells.

Two recent studies (Lelek et al. 2015; Marini et al. 2015) have shown that the classical nuclear entry pathway adopted by HIV-1, which involves the interaction of the virus to canonical Nups, creates a favourable chromatin environment for viral replication underneath the NPC. These Nups, in particular Tpr, organizes the chromatin near the pore, which is the target of HIV-1 integration. In the NPC surrounding chromatin there is a high density of the epigenetic landmark H3K36me3 (Lelek et al. 2015), which is highly present in HIV-1 integration sites (Wang et al. 2007), and LEDGF/p75 (Fig. 8.1). Depletion of Tpr results in the loss of the nuclear basket and a concomitant loss of euchromatin containing the chromatin feature H3K36me3, into the vicinity of NPCs (Fig. 8.2). Interestingly, the nuclear import and integration of the viral DNA are not affected by Tpr depletion but the viral genes are under expressed. Thus, in the absence of Tpr, HIV-1 integration can occur but in silent chromatin. As a consequence the replication of the HIV-1 genome is attenuated (Lelek et al. 2015) (Fig. 8.2). Because NPC components have a critical role in chromatin organization around pores, depletion of one that changes the chromatin topology promotes the integration of the virus in other available regions dictated by the new NPC composition. The PIC is probably guided by CPSF6 and/or Nup153 to target active genes, spatially available near the pores and maintained by the nuclear basket protein Tpr. Additionally, LEDGF/p75 helps the virus to integrate into the body of active genes (Fig. 8.1). The integration sites are critical for the outcome of viral transcription. HIV-1 integrates within transcriptional units in both acutely and latently infected cells (Wang et al. 2007). The viral persistence is characterized by viral reservoirs where a replication competent virus persists for long time in a quiescent state (Van Lint et al. 2013). This observation increases the complexity of how viral reservoir are established. Interestingly, the loss of the nuclear basket due to the depletion of Tpr reproduces a phenotype similar to that of persistent viral infected cells, in which the virus integrates but does not replicate. According to this scenario, proviral transcription is influenced by chromatin structure and epigenetic features at the viral integration site. It is possible that the virus evolved the ability to establish a viral reservoir to co-exist with the host for a long term, especially in unfavourable environmental conditions (Lucic and Lusic 2016).

More extensive studies will help to elucidate the cellular mechanisms usurped by the virus to promote its own survival and persistence. In fact, persistent infected cells remain one of the most important limit to eradicate HIV-1.

8.4 Concluding Remarks

Studies based on the role of NPC components highlight the emerging role of Nups at the pore and off-pore as organizer of chromatin structure and nuclear topology. Furthermore, it is clear that the link between Nups and the transcriptional machinery is critical for gene expression. A clear challenge consists in a better comprehension of how, at a molecular level, Nups contribute to divergent

genome-associated functions, such as transcriptional activation or repression. An important step will be the understanding of the physical interactions between Nups and chromatin-associated proteins. All these processes regulated by Nups seem to play a role in HIV-1 life cycle. In particular, the NPC is the exclusive pathway adopted by HIV-1 to reach the host chromatin in non-dividing cells and probably also in dividing cells. Nuclear basket Nups bind particular chromatin regions and regulate gene activity, however it is still under investigation how Nups, chromatin factors and genes are concerted to orchestrate HIV-1 replication. Nuclear basket Nups may be another "cellular code" for specifying HIV-1 fate through their contacts with the underlying chromatin. With a deeper understanding of the relationships between HIV-1 components, NPC and chromatin, it will be possible to shed light on the cellular pathways underlying AIDS pathology. Overall, new studies on this topic will undoubtedly gain insights into HIV-1 replication mechanisms and could serve in the development of new antiviral strategies.

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Chapter 9
Nuclear Pore Complexes in DNA Repair and Telomere Maintenance

Marie-Noelle Simon, Alkmini Kalousi, Evi Soutoglou, Vincent Géli and Catherine Dargemont

Abstract Nuclear Pore complexes (NPCs) constitute unique aqueous channels embedded in the nuclear envelope that insure the selective and massive exchange of macromolecules between these cellular compartments. The NPC is one of the largest proteinaceous assemblies in the cell whose overall structural organization and composition is highly conserved from yeast to humans. Studies conducted during the last decade highlighted the function of the NPC not only as a critical actor of nucleocytoplasmic trafficking but also as a "hub" that coordinates chromatin organization, gene regulation and genome integrity. DNA repair and maintenance of genome integrity are essential to cellular and organismal function, and defects in these processes have a profound impact in cancer, stem cell exhaustion and ageing. Besides the involvement of precise molecular actors and pathways responsible for DNA repair, high-order genome organization and nuclear architecture also participate to the DNA damage response and in particular to double strand breaks (DSB) repair. In this context, persistent double-strand breaks, arrested replication forks and eroded telomeres have been shown to relocate to the yeast NPC and some NPC proteins even influence DNA repair both in yeast and mammalian cells.

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9.1 The Nuclear Pore Complex: Overall Organization and Architectural Plasticity

Nuclear Pore complexes (NPCs) are gigantic structures varying in size (from 50 to 120 MDa in size depending on the species), which allow particles of up to 30 nm to traffic between the nucleus and the cytoplasm of eukaryotic cells, in a selective and regulated manner. Precise analysis of protein composition of the NPC from diverse species including mammals, plants, fungi and trypanosomes revealed that NPC is composed by about 30 distinct nucleoporins, with a group of 20 Nups conserved across phyla and 10 more divergent Nups (DeGrasse et al. 2009; Neumann et al. 2010; Obado et al. 2016). Numerous copies of each Nup, with a stoichiometry being a multiple of eight units likely give rise to the eight-fold rotational symmetry of the NPC. NPC is formed by biochemically defined submodules namely the coat nucleoporin complex (or Y complex, see Table 9.1), the inner ring complex (or Nic 96 complex), the central channel nucleoporin complex, the nuclear basket (Table 9.1,

Table 9.1 The Y complex and the nuclear basket of the NPC: composition, post-translational modifications and involvement in DDR

	Yeast	Metazoa	Phosphorylation (DDR kinases)	Ubiquitin	SUMO	Involvement in DDR
	Nup133	Nup133		+	+	+
	Nup120	Nup160		+	+	+
Y complex	Nup85	Nup85		−		
	Nup84	Nup107		+	+	+ +
	Nup145C	Nup96		+		
	Seh1	Seh1		+		
	Sec13	Sec13		+		
		Elys				+
		Nup43				
		Nup37				
	Mlp1	Tpr	Rad53/ATM/ATR	+	−	+
	Mlp2	Tpr		+	+	+
Nuclear basket	Nup2	Nup50	Rad53	+	+	
	Nup60		Rad53	+	+	+
	Nup1		Rad53	+	+	
		Nup153	ATM/ATR		+	+

the cytoplasmic domain (filaments and associated proteins) and the transmembrane proteins that anchors the NPC within the nuclear envelope (Knockenhauer and Schwartz 2016; Hoelz et al. 2016). Combination of high resolution structure of building blocks, cryo -electron microscopy and electron-tomography analysis and in silico computational modeling recently generated major insights into the NPC molecular architecture (Alber et al. 2007; Eibauer et al. 2015; Bui et al. 2013; Kosinski et al. 2016; Lin et al. 2016; Chug et al. 2015; Szymborska et al. 2013; Fischer et al. 2015; von Appen et al. 2015).

Besides these recent structural studies suggesting some conformational flexibility of the submodules, NPC composition can vary as a function of cell type, cell differentiation or aging (D'Angelo et al. 2012; Gomez-Cavazos and Hetzer 2015; Lupu et al. 2008). In addition, some Nups, such as the Mlps from the yeast nuclear basket are asymmetrically localized with an exclusion from NPCs juxtaposed to the nucleolus (Galy et al. 2004; Zhao et al. 2004). However mechanisms responsible for either the diversity within a single cell or for the plasticity of the NPC, such as dynamic nucleoporin associations, posttranslational or conformational changes, or temporal changes in expression have been so far poorly explored. In this respect, peripheral Nups, and in particular nuclear basket components, present a dynamic behavior whereas Nups from the core NPC are very stably associated with the NPC (Rabut et al. 2004; Nino et al. 2016). In agreement with this dynamic association of peripheral Nups with the NPC, few Nups including Nup98, Nup50, Nup153 are able, both in *Drosophila melanogaster* and mammalian cells, to interact with active genes when not associated to NPCs (Capelson et al. 2010; Kalverda et al. 2010; Vaquerizas et al. 2010). Although the molecular basis of such an architectural plasticity remains rarely elucidated, post-translational modifications appear as a conserved strategy to trigger Nups dynamics.

Phosphorylation of Nups by mitotic kinases participates to the NPC disassembly at the onset of prophase (Macaulay et al. 1995; Glavy et al. 1997, 2007; Onischenko et al. 2005; Lusk et al. 2007; Laurell et al. 2011) but Cdks are also required for the proper expression and localization of Nups involved in postmitotic NPC assembly, such as Elys (Maeshima et al. 2010). However, no phosphorylation event has so far been described to alter Nups dynamics in G1 or S phase.

Nucleoporins are also targets of ubiquitin-like proteins, in particular SUMO. Interestingly, Nup358, a cytoplasmic mammalian Nup, presents an E3 SUMO ligase activity, with RanGAP being its major substrate (Pichler et al. 2002). Some mammalian (SENP1, SENP2) and yeast (Ulp1) SUMO proteases are specifically associated with the NPC, at the level of the nuclear basket, the Y complex and cytoplasmic filaments (Hang and Dasso 2002; Zhang et al. 2006; Goeres et al. 2011; Chow et al. 2012; Zhao et al. 2004; Takahashi et al. 2000). Depletion of SENP1 and SENP2 even leads to mislocalization and down regulation of some Nups (Chow et al. 2014). Whether the correct expression level or the localization of SUMO proteases is responsible for such a phenotype remains unclear. This intriguing connection between the SUMO machinery and the NPC correlates with the identification of many mammalian Nups and few yeast Nups (in particular Nups of the nuclear basket, Table 9.1, personal communication) as targets for mono and poly-SUMOylation (Li et al. 2004; Nino et al. 2016; Vertegaal et al.

2006; Blomster et al. 2009; Golebiowski et al. 2009; Matafora et al. 2009; Bruderer et al. 2011). Interestingly, Slx8-Slx5, a yeast SUMO-dependent ubiquitin ligase associates with the NPC (Nagai et al. 2008) suggesting a potential crosstalk of SUMOylation and ubiquitylation at the NPC. However, SUMOylation of Nup60, the unique Nup described to date to be both SUMOylated and ubiquitylated by the Slx5/Slx8 or Uls1 SUMO-dependent ligases, is not sufficient for its ubiquitylation (Nino et al. 2016).

Intriguingly, more than half of yeast Nups are modified by ubiquitin, essentially monoubiquitin, suggesting broad functions of this post-translational modification rather than a degradative role (Hayakawa et al. 2012; Nino et al. 2012). Among these, yeast Nup60 is monoubiquitylated by Rad6 as an E2 ubiquitin conjugation enzyme and Slx5–Slx8 or Uls1 as E3 ubiquitin ligases (Nino et al. 2016). This nuclear basket protein is anchored to the nuclear membrane via its N-terminal amphipathic helix (Meszaros et al. 2015) whereas its monoubiquitylation controls the dynamic association of both Nup60 and its partner Nup2 with the core NPC, via a ubiquitin-mediated interaction with Nup84, a component of the Y complex (Table 9.1). As a consequence, combining deletion of the N-terminal helix and mutation of the ubiquitylation site results in the release of Nup60 in the nucleoplasm (Fig. 9.1). In contrast, interaction of other proteins of the nuclear basket such as Mlps is not altered when Nup60 ubiquitylation is prevented, thus revealing that the nuclear basket does not behave as a homogenous module of the NPC (Nino et al. 2016). These results validate the hypothesis that post-translational modifications can contribute to the plasticity of the NPC at an architectural level and eventually adapt its diverse functions in nucleocytoplasmic transport, gene expression and genome integrity to cell requirements.

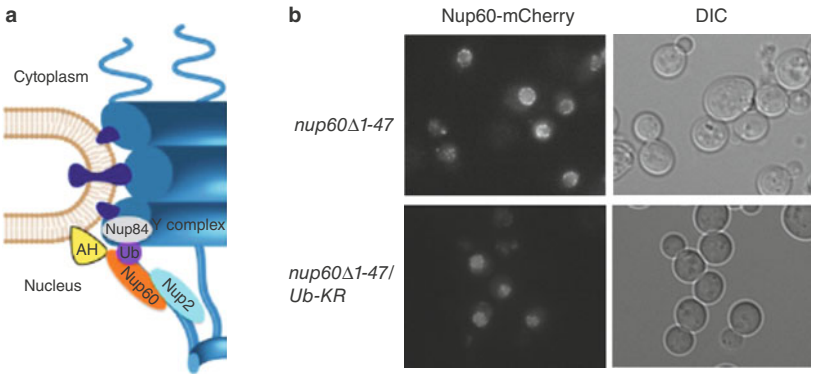

Fig. 9.1 Anchoring of Nup60 to the NPC. (a) Nup60 is anchored to the nuclear membrane via its N-terminal amphipathic helix and Nup60/Nup2 subcomplex to the Y complex via the interaction between monoubiquitylated Nup60 and Nup84. (Adapted from (Meszaros et al. 2015; Nino et al. 2016)). (b) Steady state localization of plasmid-expressed and mCherry-tagged Nup60 deleted for its N-terminus amphipathic helix (*nup60Δ1-47*, generous gift from A. Köhler) without or with combined mutation of Nup60 ubiquitylation site (*nup60Δ1-47UbKR)*

9.2 NPC and DNA Repair in Yeast

DNA damage can occur due to environmental agents, such as UV light or irradiation, and endogenous sources, such as oxidative by-products of cellular metabolism or stalled replication forks (Jackson and Bartek 2009). Impaired DNA repair is a major driver for carcinogenesis and alterations in DNA repair pathways that arise during tumor development can make some cancer cells specifically dependent on a limited set of DNA repair pathways (Helleday et al. 2008). To prevent irreversible mutations that can occur throughout our life span, multiple repair systems have emerged during evolution. DNA double strand breaks (DSBs) are considered to be the most deleterious type of damage that can potentially lead to gross chromosomal rearrangements (Jackson and Bartek 2009; Gospodinov and Herceg 2013). There are two major pathways to repair DSBs: homologous recombination (HR) and non-homologous end joining (NHEJ) (Wyman and Kanaar 2006). HR is functional predominantly when the pairing of sister chromatids occurs (during S/G2) and takes advantage of the information coded by the homologous template to eliminate the DSB in an error-free manner (Wyman and Kanaar 2006; Heyer et al. 2010). In contrast, the NHEJ pathway re-ligates the free DNA ends without the presence of a non-damaged template strand and is active throughout the cell cycle (Lieber 2010). This pathway is required for the repair of most radiation-induced DSBs in mammalian cells but corresponds to a minor mechanism in yeast cells (Lewis and Resnick 2000). Cells respond to DSBs by initiating a signaling cascade, called the DNA damage response (DDR), which leads to the activation of the cell cycle checkpoints. Checkpoint activation arrests the cell cycle and gives the cell time to repair the damage before the cell continues to divide (Lukas et al. 2006). The DDR is initiated by the recruitment and extensive spreading of DDR proteins around the lesions, resulting in the formation of discrete foci (Lukas et al. 2005).

9.2.1 Role of Nucleoporins

A genome-wide screen of yeast mutants affecting the sensitivity to ionizing radiations led to the identification of 130 genes, with 50% presenting homology with human genes, thus illustrating that DDR pathways are universal and the majority of the involved proteins are highly conserved from yeast to humans (Bennett et al. 2001; Lukas et al. 2004). Intriguingly, this screen identified five Nups from the Y complex (Nup84, Nup120, Nup133) and the inner ring (Nup170, Nup188) involved in this process. This result raised the question of the role of the NPC in DNA repair and more generally whether repair can occur anywhere in the nucleus and whether repair efficiency is affected by the location of the lesion. Besides the increased sensitivity of Nups mutants to many DNA damaging agents (Loeillet et al. 2005; Chang et al. 2002; Hediger et al. 2002), depletion of distinct members of the Nup84 complex leads to synthetic lethality when combined with genes that

are required for DSB repair through HR like RAD52 (Loeillet et al. 2005). The same study revealed that depletion of Nup84 leads to accumulation of unrepaired DSBs. In addition to the Nup84 complex, the yeast nuclear basket proteins Nup60, Mlp1 and Mlp2 are essential to efficient DNA repair (Palancade et al. 2007) (Table 9.1). The role of both the Y complex and the nuclear basket in DNA repair is, at least in part, due to the ability of these complexes to ensure the proper localization and expression levels of the Ulp1 SUMO protease at the nuclear envelope (Palancade et al. 2007, Zhao et al. 2004). In particular, overexpression of Ulp1 decreases the number of Rad52 foci (used as an indicator of DNA lesions) observed upon disruption of *NUP60* or *NUP133*. Affecting the levels of Ulp1 leads to decrease in the sumoylation of the NHEJ factor yKU70 and probably of other repair factors that remain to be determined (Palancade et al. 2007). In addition to this Ulp1-mediated DDR, ubiquitylation of Nup60 is also stimulated upon genotoxic stress suggesting that the interaction of Nup60/Nup2 with the Y complex could be stabilized upon DNA damage (Nino et al. 2016). This environmental sensitive modification contributes to the DDR. Preventing ubiquitylation of Nup60 indeed leads to an increased sensitivity to genotoxic agents that correlates with an increased formation of Rad52 foci. However, forcing Ulp1 to localize to the NPC independently of Nup60 or delocalizing Ulp1 by deletion of *MLP1/MLP2* does not alter the effect of Nup60 ubiquitylation thus indicating that the involvement of Nup60 ubiquitylation in the DDR is independent of the SUMO protease Ulp1 (Nino et al. 2016). Ubiquitylated Nup60 rather reinforces DDR downstream Mec1/Rad53. Ubiquitylated Nup60 is indeed a target of Rad53 (human CHK2), one of the key effector kinase of the DDR in *Saccharomyces cerevisiae* activated by Mec1 (ATR) via Mrc1 (CLASPIN) in response to replication stress, and by Rad9 (53BP1) in response to DNA damage (Smolka et al. 2007; Branzei and Foiani 2009; Nino et al. 2016). Although the precise molecular role of Nup60 ubiquitylation in the DDR remains to be clarified, we recently proposed that genotoxic stress could modulate the plasticity of the nuclear basket -by regulating nucleoporin post-translational modifications- thereby regulating NPC microenvironments and favoring an efficient DDR.

Nup1, Nup2, and Nup60 are also phosphorylated by the stress-activated protein kinase Hog1 that, upon osmotic stress, associates with the NPC and coordinates replication and transcription (Regot et al. 2013; Duch et al. 2013). Hog1 indeed phosphorylates the S-phase checkpoint protein Mrc1 independently of Mec1 and Rad53 thereby defining a new checkpoint pathway thought to prevent conflict between DNA replication and transcription (Duch et al. 2013). In addition, Rad53 phosphorylates nucleoporins of the nuclear basket in response to both DNA-damaging agents and replication stress generated by the replication of transcribed genes associated with the NPC (Smolka et al. 2007; Bermejo et al. 2011) (Table 9.1). More precisely, phosphorylation of Mlp1 was proposed to release transcribed genes from their association with the NPC thereby decreasing topological constraints at the vicinity of the replication fork (Jossen and Bermejo 2013). Such an intriguing interplay between transcribed genes associated with the nuclear periphery, replication stress, checkpoint signalling, and posttranslational modifications of the

NPC has certainly to be further explored to understand the role of NPC modifications and plasticity in the coordination between transcription, replication and repair.

9.2.2 NPC Regulates HR at Specific Types of DNA Lesions

In yeast, DSBs formation increases chromatin mobility. This increased mobility of chromatin in response to DSBs is global and dependent on Mec1 activation but whether this mechanism facilitates homology search during HR is a matter of intense debate (Mine-Hattab and Rothstein 2012; Dion et al. 2012; Saad et al. 2014; Strecker et al. 2016). In this context, NPCs have been shown to play a role in anchoring hard-to-repair DNA damage, such as DSBs without a donor template for HR, collapsed replication forks (Nagai et al. 2008), and eroded telomeres (Khadaroo et al. 2009). This observation was recently extended to tandem CAG repeats able to form secondary structures, which relocate to NPCs during their replication in repeat array size-dependent manner (Su et al. 2015). Specialization of specific compartments emerged as a major actor of HR regulation for specific repair and/or protection (Geli and Lisby 2015). Persistently unrepaired DSBs were shown to migrate from their internal nuclear positions to the nuclear periphery, where they associate with nuclear pores (Nagai et al. 2008; Oza et al. 2009). This sequestration to the nuclear periphery requires the Nup84 complex and Nup60, as well as the SUMO-dependent ubiquitin ligase Slx5-Slx8 and the Mec1 (ATR)/Tel1 (ATM) kinases but the precise substrate of Slx5/Slx8 involved in this function is still unknown (Nagai et al. 2008). More recently, factors required to localize unrepairable *homothallic (HO) endonuclease.*-induced DSB to either the membrane anchor site Mps3 or the NPC were determined (Horigome et al. 2014, 2016). Similarly, relocation of expanded CAG repeat tract to the NPC also requires Slx5-Slx8 and the Nup84 subunit of the Y complex (Su et al. 2015). Failure to relocate the CAG repeat tract to the NPC in *slx8*Δ and *nup84*Δ mutants correlates with *RAD52*-dependent instability leading to expansions and contractions. Thus relocation of the expanded CAG repeat to the NPC is proposed to prevent aberrant HR and CAG array fragility and instability (Su et al. 2015). These studies in yeast indicate that the NPC represents either a dedicated DNA-repair center or alternatively an appropriate microenvironment for protecting damaged DNA against degradation, with the nuclear basket and the Y complex playing a central role. Whether this function of NPC could be regulated by nucleoporins post-translational modifications (SUMO, ubiquitin, phosphorylation, etc) remains to be determined.

9.3 NPC and DNA Repair in Mammals

Although the role of yeast nucleoporins in DNA repair is well described in many studies, the impact of mammalian nucleoporins remains largely unknown. In general the mammalian nuclear pore is associated with euchromatin and is permissive

for both main DNA repair pathways NHEJ and HR (Lemaitre et al. 2012). Indeed, relocation of a single DSB at the nuclear pore through tethering of the transmembrane nucleoporin Pom121 to a LacO/LacI/ISceI system showed that both NHEJ and HR are active and important players of each pathways, such as 53BP1, BRCA1 and Rad51 can accumulate to the break (Lemaitre et al. 2012).

The most studied mammalian nucleoporin for its role in DNA damage and repair is the nuclear basket protein Nup153. It has been shown that Nup153 and its binding partner at the nuclear basket Tpr, are phosphorylated upon DNA damage by ATM/ATR and this modification was proved to be important for the proper activation of G2/M and intra S checkpoint (Matsuoka et al. 2007) (Table 9.1). Such as its yeast orthologue Nup60, Nup153 is sumoylated and this modification is required for the interaction with the SUMO proteases SENP1 and SENP2 (Chow et al. 2012, 2014). Two independent studies observed that Nup153 plays an important role in DNA repair regulating the localization of the DNA repair factor 53BP1. It was shown that depletion of Nup153 decreased cell survival levels upon treatment with Ionized Irradiation (IR) or radiomimetic drugs (Lemaitre et al. 2012; Moudry et al. 2012). Moreover absence of Nup153 results in substantial decrease in DNA repair by NHEJ when at the same time HR levels were increased (Lemaitre et al. 2012). Under the same conditions, an abnormal localization of 53BP1 is observed and foci formation is impaired. Thus, it was suggested that Nup153 is important for the nuclear localization of 53BP1 and therefore essential for genome integrity. On the other hand, the observed increase in HR levels in the absence of Nup153 suggests that it might have an alternative role in DNA repair independent of 53BP1, which remains to be investigated (Lemaitre et al. 2012).

A recent study implicates Nup153 in the progression of ageing (Cobb et al. 2016). As previously shown, prelamin A, a precursor molecule of Lamin-A whose truncated form is implicated in the premature aging disease Hutchinson–Gilford progeria syndrome (HGPS), is causing an abnormal topological arrangement of Nup153 at the nuclear envelope (Goulbourne et al. 2011). This abnormality in localization of Nup153, combined with the increased expression of prelamin A in aged vascular smooth muscle cells results in increased basal levels of DNA damage through the defective nuclear import of 53BP1 (Cobb et al. 2016). Going further into the mechanism, the authors show that Nup153 is important for the nuclear localization of Ran and that deregulation of Ran gradient may compromise the nuclear import of 53BP1 (Cobb et al. 2016).

As far as the rest of the mammalian nucleoporins are concerned, there have been only a few studies reporting their potential role in DNA repair. The FG-nucleoporin Nup98 was reported to be involved in the progression of myelodysplastic syndrome. The leukemic fusion gene NUP98-HOXD13 (NHD13) leads to acute leukemia development in mice with the occurrence of collaborating mutations. More specifically these mice showed defects in class switch recombination and DNA repair by NHEJ (Puthiyaveetil et al. 2013; Slape et al. 2008). This might suggest the existence of an unknown role of Nup98 in the NHEJ pathway that remains to be elucidated.

The nucleoporin Elys, which constitutes a member of the Y complex of the nuclear pore, was reported to help in the maintenance of genome stability in intestinal epithelial progenitor cells in mice (Gao et al. 2011). Elys was found to be required for the survival of intestinal crypt progenitor cells but not for the NPC assembly in these cells. Moreover, Elys deficient crypt cells exhibited persistent apoptosis accompanied by increased activation of the DDR pathway (Gao et al. 2011). The authors speculate that this increased DNA damage upon Elys depletion arises from replication stress as this nucleoporin was previously found to interact with the Mcm2/7 DNA replication helicase (Gillespie et al. 2007).

Finally, recent data revealed a role of the nucleoporin Nup107 (component of the Y complex of the nuclear pore) in the DDR. It was shown that Nup107 facilitates the rapid nuclear translocation of the apoptotic factor Apaf-1 through their ATR dependent interaction which is triggered upon DNA damage (Jagot-Lacoussiere et al. 2015). The Nup107 dependent nuclear import of Apaf-1 is crucial for genome integrity as it regulates DNA damage-induced cell cycle arrest, thus making this nucleoporin a new player in the DDR.

Together these studies suggest that the mammalian Y complex and the nuclear basket could represent central actors of the DDR but likely through more diverse molecular pathways.

9.4 NPC Regulates Telomere Recombination During Pre-senescence and Survivor Formation

In the absence of telomerase activity, yeast telomeres shorten on average 3–5 bp per population doubling (PD) (Marcand et al. 1999). After about 20–30 PDs, proliferation rate begins to decline until it reaches a minimum that is referred to as crisis caused by the critical shortening of telomeres. Cells then undergo a Mec1-dependent irreversible G2/M arrest (Enomoto et al. 2002; Chen et al. 2001). Few cells can overcome this permanent arrest by regenerating functional telomeres through recombination-based mechanisms. These survivors are classified into two types based on genetic requirements and telomere organization (Le et al. 1999; Chen et al. 2001) (Fig. 9.2). The formation of both types of survivors relies on break-induced replication (BIR) and requires the main HR protein Rad52 (Teng and Zakian 1999; Lydeard et al. 2007). Type I survivor formation is preceded by a Rad59-dependent translocation of Y' elements onto X-only telomeres (Churikov et al. 2014) followed by the spreading and amplification of Y' elements at all telomeres. This second step requires Rad51, Rad54 and Rad55 in addition of Rad52 and Pol32 (Lydeard et al. 2007; Le et al. 1999). Type II survivors carry telomere repeats that are long and heterogeneous in length. The mechanism of telomere elongation observed in type II survivors shares similarity with the ALT (Alternative Lengthening of Telomere) phenotype that accounts for about 15% of human cancers (Pickett and Reddel 2015). The sudden amplification of $TG_{1\text{-}3}$ repeats characterizing Type II recombination relies on the strand annealing

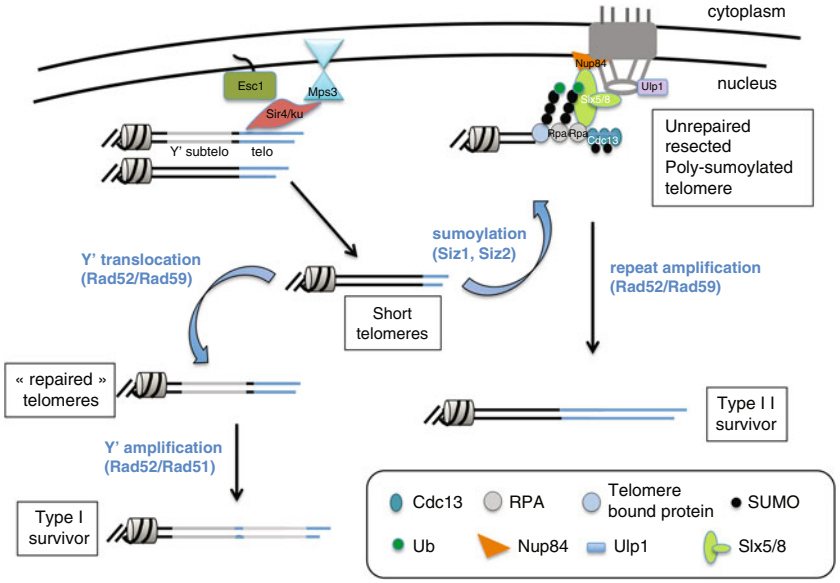

Fig. 9.2 SUMO and NPC localization regulate telomere recombination. Undamaged telomeres cluster adjacent to the nuclear membrane through redundant pathways that involve the Ku (Ku70 and Ku80) and the Sir (Sir2, 3,4) complexes, the inner nuclear membrane protein Esc1 and the protein Mps3 (Taddei and Gasser 2012). Upon inactivation of the telomerase, the shortest telomeres are repaired by copying the information from other telomeres leading to the spreading of Y' subtelomeric sequence at all telomeres (Churikov et al. 2014). This step prepares the background for Rad52/Rad51-dependent Y'amplification observed in type I survivors. When not repaired, short telomeres undergo extended resection up to subtelomeric sequences. Telomere-bound proteins, including RPA, become highly SUMOylated creating a substrate for the SUMO-dependent ubiquitin ligase Slx5–Slx8. Binding of Slx5–Slx8 would promote or participate to tethering eroded telomeres to the NPC through the interaction of Slx5-8 with Nup84 as described by (Nagai et al. 2008). We proposed that relocalization to the nuclear pore would facilitate de-SUMOylation by the SUMO-protease Ulp1 and/or degradation of key proteins allowing repair to resume through type II recombination (Churikov et al. 2016)

function of Rad52 reinforced by Rad59 as well as components of the BIR pathway (McEachern and Haber 2006) (Lydeard et al. 2010). This recombination dependent telomere elongation takes place during crisis (Teng et al. 2000; Chang et al. 2011) when eroded telomeres are recognized and processed as DSBs, consistent with its dependence on the DNA damage checkpoint (Grandin and Charbonneau 2007). Recently, the mechanism that regulates the balance between the two types of telomere recombination was shown to involve the NPC (see below).

In telomerase positive cells, telomeres are clustered in 6 to 8 foci at the nuclear periphery in a zone enriched in Sir proteins that restricts HR (Gotta et al. 1996). Localization of telomeres at the periphery depends on two redundant pathways involving Sir4 and the yKu70/80 heterodimer at the telomere, and the nuclear membrane anchored proteins Esc1 and Mps3, at the nuclear envelope (Hediger et al. 2002; Taddei et al. 2004; Taddei et al. 2010). Although the general telomere

organisation remains, eroded recombinogenic telomeres were found to be relocated from their membrane anchor to the NPC during senescence (Khadaroo et al. 2009). Relocation of telomeres to NPC, as well as formation of type II survivors, depends on Slx5-Slx8 (Churikov et al. 2016). SUMOylation of telomere bound proteins were shown to increase as telomeres shorten and correlated with the telomere recruitment of Slx5-8 (Churikov et al. 2016). Therefore critically short telomeres are tethered to the NPC by a mechanism sharing similarities with other « hard-to-repair DNA damage » (Fig. 9.2). Based on this result, it was proposed that telomere repair in telomerase-negative cells occurs in two steps. In early senescence, telomeres with limited levels of single-stranded overhangs would be repaired by subtelomeric sequence translocation and amplification (Churikov et al. 2014). The role of the NPC during this first step of telomere repair is still elusive, but our recent results suggest that Nup60 ubiquitylation inhibit Type I recombination (Nino et al. 2016). By the time of crisis, highly eroded non-repaired telomeres would undergo extensive resection and excessive SUMOylation thereby becoming « hard to repair telomeres » (Churikov et al. 2016). These non-repairable telomeres would be targeted to the NPC through a pathway involving the Slx5-Slx8-dependent targeting of poly-SUMOylated proteins to clean up of poly-SUMOylated proteins. Our findings suggests that relocation to the NPC may help disassembling dead-end intermediates at resected telomeres to facilitate Type II telomere recombination. Type II recombination can therefore be considered as a rescue pathway. The question rises which SUMOylated targets at critically short telomeres regulate NPC localization and whether NPC components other than Nup84 act as acceptors of the eroded telomeres. Although multiple candidates are likely involved, RPA, which binds resected telomeres, is SUMOylated in response to DNA damage and constitutively interacts with Slx5 (Churikov et al. 2016), appears as a candidate of choice (Fig. 9.2).

Eroded mammalian telomeres do not relocalize to the nuclear pore but to promyelocytic leukemia (PML) bodies, called APB for ALT-associated PML bodies (Yeager et al. 1999). APBs contain a number of DNA repair proteins, including RPA, RAD52 and RAD51 but also the RNF4 STUBL and the SMC5/6 complex. MMS21, a SUMO ligase, which is part of the SMC5/6 complex, SUMOylates several proteins bound to telomeres including the shelterin proteins TRF1 and TRF2 and is required for both targeting telomeres to APB and maintenance of telomeres through HR (Potts and Yu 2007).

Another intriguing aspect of type II recombination is the substrate used for telomere elongation when the reserve of telomeric sequence is exhausted. The fact that NPC promotes type II recombination suggests that NPCs may serve to anchor such templates. Interestingly, extrachromosomal telomeric circles (t-circles) in type II survivors (Larrivee and Wellinger 2006), *Kluyveromyces lactis* mutants (Basenko et al. 2010) and ALT cancer cells (Henson et al. 2009) were previously proposed to act as templates for roll-and-spread mechanism for fast amplification of telomeric repeats (McEachern and Haber 2006; Natarajan and McEachern 2002). Whether the NPC favours Type II recombination by concentrating telomeric circles remains an intriguing open question.

9.5 Concluding Remarks

Collectively, these studies reveal that in yeast as well as in mammalian cells there is a compartmentalization of DNA repair for which the Y complex and the nuclear basket play a central role. Relocalization to the NPC appears to regulate DSB repair and fork restart and involves not only SUMO but also others post-translational modifications of NPC components. The NPC contributes to rescue mechanisms of "difficult to repair" lesions, although it is still elusive which HR pathway is regulated by the NPC for the different types of DNA damage. In addition, NPCs likely provide a protective nuclear microenvironment to avoid more deleterious effect due to these lesions. Overall, the emerging concept is that the NPC regulates backup repair pathways involved in the processing of unrepairable DNA structures that otherwise will be deleterious for the cell.

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Chapter 10
NPCs in Mitosis and Chromosome Segregation

Masaharu Hazawa, Akiko Kobayashi and Richard W. Wong

Abstract In eukaryotic cells, the nuclear envelope (NE) separates thousands of genes and RNAs inside the nucleus from the rest of the cell. The NE consists of two separate membranes, the inner nucleoplasm-facing nuclear membrane and the outer cytoplasm-facing nuclear membrane, which is continuous with the endoplasmic reticulum (ER). These membranes are separated by the perinuclear luminal space. Transport between the cytoplasm and the nucleus occurs through dedicated, cylindrical holes that are present at sites where the inner and outer nuclear membranes connect together. These holes are filled with macromolecular gates termed nuclear pore complexes (NPCs), which are the only gateway between the nucleus and the cytoplasm. In recent years, several nucleoporins have been shown to play important roles in mitosis. In this chapter, we summarize the recent progress in our understanding of the roles of different nuclear pore components at different stages of mitosis, with a focus on their functions within the mitotic machinery and in the inhibition of tumorigenesis.

Keywords Mitosis · NPC · nucleoporin · chromosomes · cancer

10.1 NPC Structure and Composition

The structure of the human NPC was initially determined using transmission electron microscopy, subsequently scanning electron microscopy and most recently cryo-electron tomography (for review, see (Sakiyama et al. 2017)) and atomic

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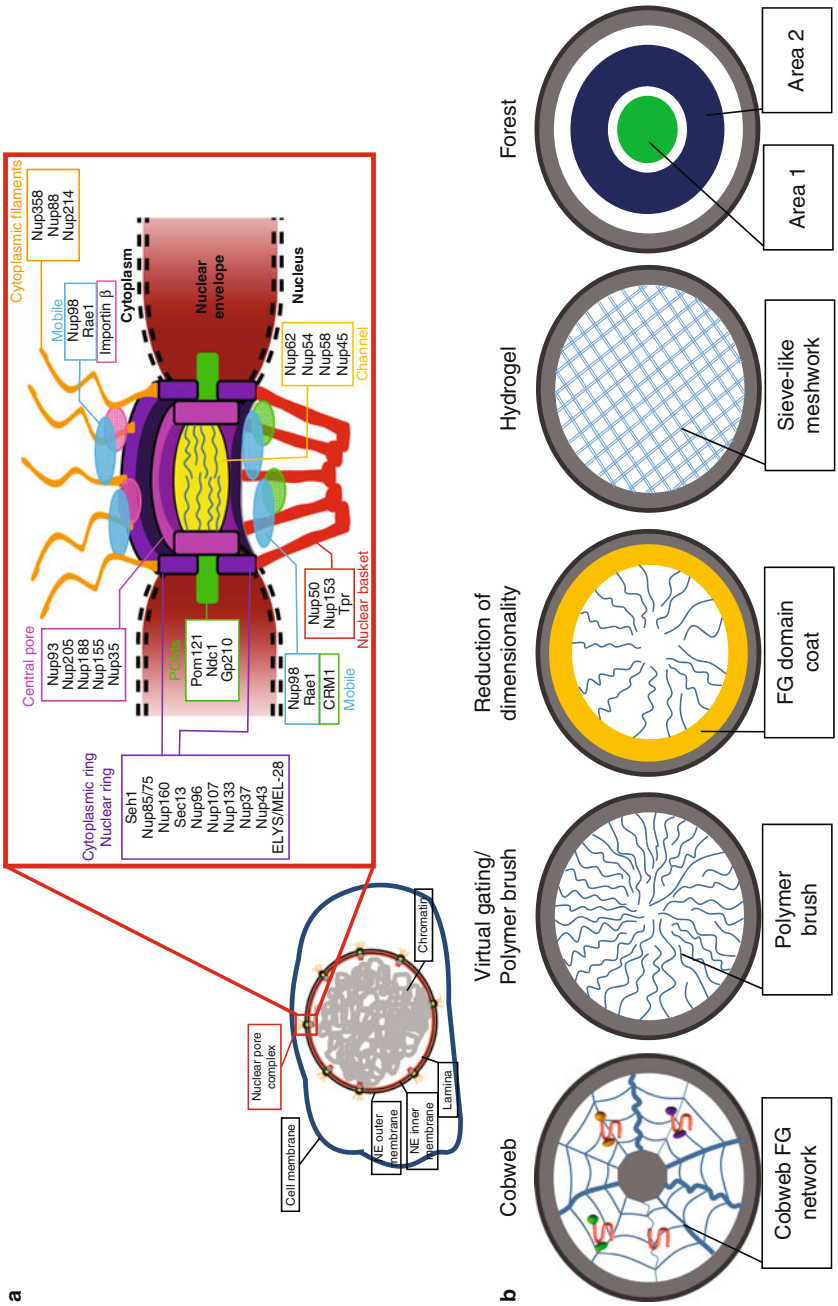

Fig. 10.1 (a) Structure and composition of the vertebrate NPC. (b) A schematic illustration of various Nucleocytoplasmic transport models

force microscopy (AFM) (Dufrene et al. 2017; Liashkovich and Shahin 2017; Stanley et al. 2017; Mohamed et al. 2017). NPCs are large multiprotein channels of around 120 MDa and about 80-120 nm in diameter in vertebrates (Blobel 2010; Doucet and Hetzer 2010). They have eightfold rotational symmetry and comprise multiple copies of ~30 different proteins called nucleoporins or Nups (Fig. 10.1a) (Sakuma and D'Angelo 2017; Hayama et al. 2017; Wong 2015; Nakano et al. 2011). The structures of NPCs consist of a spoke-ring complex, a central transporter, cytoplasmic and nucleoplasmic rings, kinetically movable transporters (e.g., Rae1 and Nup98), attached cytoplasmic filaments and a nuclear basket (Sakuma and D'Angelo 2017). Nups (labeled "Nup" followed by their expected molecular weight) are segmental proteins with a limited number of structural motifs (coiled-coils, α-solenoids, β-propellers) that are used repetitively to build the symmetrical NPC structure. Nearly one-third of Nups consist phenylalanine–glycine (FG) domain motifs interspersed with spacer sequences (Wong 2015; Nakano et al. 2011) (Fig. 10.1b). These FG domains are intrinsically disordered polypeptide chains and serve as interaction sites for transport receptors (karyopherins) that escort cargo through the pore (Sakuma and D'Angelo 2017). These FG-Nups form a selective barrier allowing passive diffusion and active transport with the assistance of transport receptors. Several FG-Nup trafficking models have been proposed (Fig. 10.1b). Recently, by directly observing native nuclei of colon cancer cells via HS-AFM, we found that FG-Nups are short, stiff, hair-like, twisted ropes that together form a broken spider's web pattern (Mohamed et al. 2017) (Fig. 10.1b).

Lately, growing appreciation of the role of NPCs in cell division has emerged. When mitosis starts, the NE and NPCs are disassembled and nucleoporins are detected either alone or in subcomplexes/small building blocks after nuclear envelope breakdown (NEBD) (Webster et al. 2009). Nups are associated with the kinetochore, spindle and centrosome functions. At the end of telophase, Nup building blocks reassemble sequentially and are finally brought to the NPC within NE. Here, we describe the recent progress in research on various NPC components in mitosis.

10.2 Function of NPC Components in Chromosome Segregation and Mitotic Apparatus Orchestration During Mitosis

The precise capture of mitotic chromosomes by spindle microtubules is critical for accurate cell division. During mitosis, microtubules are assembled such that the minus ends are focused into two poles at centrosomes, while the plus ends interact with chromosomes via kinetochores and align them on the metaphase plate. Spindle reorganization is assisted not only by centrosomes and chromatin, but also by microtubule-binding proteins, such as molecular motors (Guttinger et al. 2009; Nakano et al. 2011). Higher eukaryotes form a cytoplasmic spindle, which incorporates NEBD to allow the contact of spindle microtubules to kinetochores in the

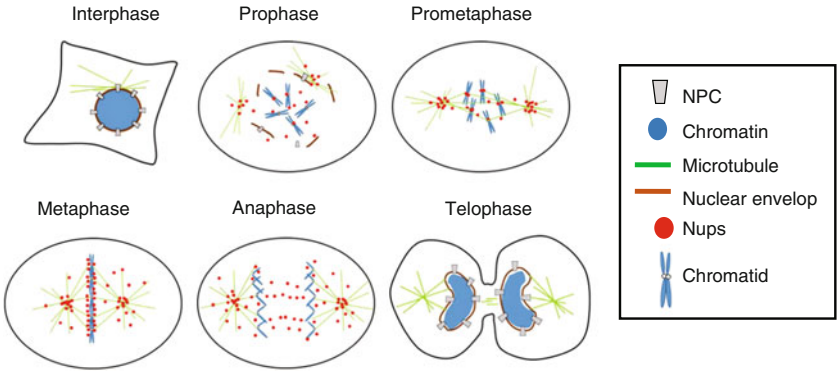

Fig. 10.2 Schematic model of NPC proteins/Nups and chromosome-spindle interactions during the cell cycle

early stage of mitosis. NEBD results in the removal of NE membranes and the disassembly of large macromolecular complexes, such as the lamina and NPCs. After late anaphase, NE is restructured around each mass of chromatin in the daughter cells to reform the nuclear boundary (Guttinger et al. 2009). The most impressive change of the mammalian NPC takes place at the beginning of cell division, when individual Nups become heavily phosphorylated and NPCs are taken into pieces (Tran and Wente 2006; D'Angelo and Hetzer 2008; Guttinger et al. 2009; Strambio-De-Castillia et al. 2010; Wozniak et al. 2010; Hetzer 2010; Ibarra and Hetzer 2015; Wong and D'Angelo 2016). Later, NPC subunits relocate to mitotic substructures, mainly the kinetochores, and many of them show mitotic-specific functions in spindle assembly or anaphase onset (Wozniak et al. 2010). During exit from mitosis, NPCs reassemble into the reforming NE, and their number is approximately doubled during interphase (Guttinger et al. 2009; Nakano et al. 2011). NPC components are also involved in the dynamics of S phase and contribute to the maintenance of genome integrity, preventing the accumulation of DNA lesions (Hetzer 2010; Ibarra and Hetzer 2015) (Fig. 10.2). In the text below, we summarize how these nucleoporins coordinate cell cycle progression in mammalian cells.

10.2.1 Nup358/RanBP2 Subcomplex in Mitosis

Nup358/RanBP2, a Ran binding protein with four Ran binding domains, is an utmost cytosolic component of filaments derived from the cytoplasmic ring of the NPC (Matunis and Pickart 2005; Wu et al. 1995) (Fig. 10.1a). This large protein (3,224 residues in humans) can be divided into several domains: an N-terminal TPR domain, an α-helical region, four Ran-binding domains, eight tandem zinc fingers, a SUMO E3 ligase domain, FG and FxFG repeats that act as binding surfaces for transport receptors, and a C-terminal domain that displays sequence homology to cyclophilins (Fig. 10.1) (Wong and D'Angelo 2016; Hashizume et al. 2013). Surprisingly, Nup358/RanBP2 has recently been shown also to play an

unexpected role in Y-complex oligomerization, a finding that blurs the conventional boundary between scaffold and transport-channel Nup building blocks (von Appen et al. 2015). Nup358/RanBP2 function was initially linked to nucleocytoplasmic transport; however, consistent with its multidomain configuration, Nup358/RanBP2 has been shown to have more pleiotropic functions. This large Nup is now recognized as a regulator of numerous cellular processes. In particular, RanBP2–RanGAP1*SUMO1/Ubc9 was identified as a multisubunit SUMO E3 ligase (Werner et al. 2012). Remarkably, RanBP2 has also been implicated in the delivery and integration of the genomic material of HIV-1 (Wong et al. 2015).

At the beginning of mitosis when the NE breaks down and NPCs disassemble, RanBP2–RanGAP1–SUMO1–Ubc9 subcomplexes diffuse into the mitotic cytosol and gather at the plus ends of free spindle microtubules and at kinetochores of chromosomes that have been captured by spindle microtubules (Hashizume et al. 2013; Joseph et al. 2002, 2004). The nuclear export receptor Crm1 is responsible for kinetochore targeting of RanBP2–RanGAP1–SUMO1–Ubc9 (Arnaoutov et al. 2005). In HeLa and RGG cells, knockdown of RanBP2 causes several mitotic abnormalities, including misalignment of the chromosome in metaphase, mislocalization of several kinetochore-associated proteins and formation of multipolar spindles (Hashizume et al. 2013; Joseph et al. 2004; Salina et al. 2003). When anaphase starts, sister chromatids are disconnected and separated. This comprises decatenation of sister chromatids at centromeres by Topoisomerase II (TOPOII) (Guttinger et al. 2009). Nup358/RanBP2 was found to enrol TOPOII to centromeres by its sumoylation in mammalian cells (Dawlaty et al. 2008), and similar to TOPOII depletion, knockdown of Nup358/RanBP2 enhanced the formation of anaphase bridges (Dawlaty et al. 2008). As a consequence, mice with reduced levels of Nup358/RanBP2 exploit serious aneuploidy phenotypes and are predisposed to cancer (Dawlaty et al. 2008). These findings suggest that Nup358/RanBP2 functions as a tumor suppressor (Guttinger et al. 2009). Moreover, using RanBP2 conditional knockout mouse embryonic fibroblasts and a series of mutant constructs, Hamada et al. showed that transport, rather than mitotic, functions of RanBP2 are required for cell viability (Hamada et al. 2011).

Interestingly, Nup358/RanBP2 has been associated with cancer in different, and possibly contradictory, manners. On the one hand, its downregulation induces G_2/M phase arrest, impairs chromosomal alignment and results in mitotic catastrophe and cell death. Consistent with this, Nup358/RanBP2 participation in chromosomal translocations that result in hematological malignancies suggests its potential oncogenic role in patients. Vecchione et al. (2016) discovered that increased expression of Nup358/RanBP2 protects a subgroup of colorectal cancer (CRC) cells from undergoing mitotic cell death, which is consistent with an oncogenic function. They studied an important subset of CCs that carry the BRAF mutation V600E; this mutation occurs in about 8%–10% of CRC patients and is associated with a poor prognosis, particularly in the metastatic setting (Wong and D'Angelo 2016). Vecchione et al. (2016) discovered that the suppression of Nup358/RanBP2 is selectively lethal to colon cancers having a BRAF-like signature (Fig. 10.3). Consistent with previous findings (Hashizume et al. 2013), they also found that the depletion of Nup358/RanBP2 causes defective kinetochore structure

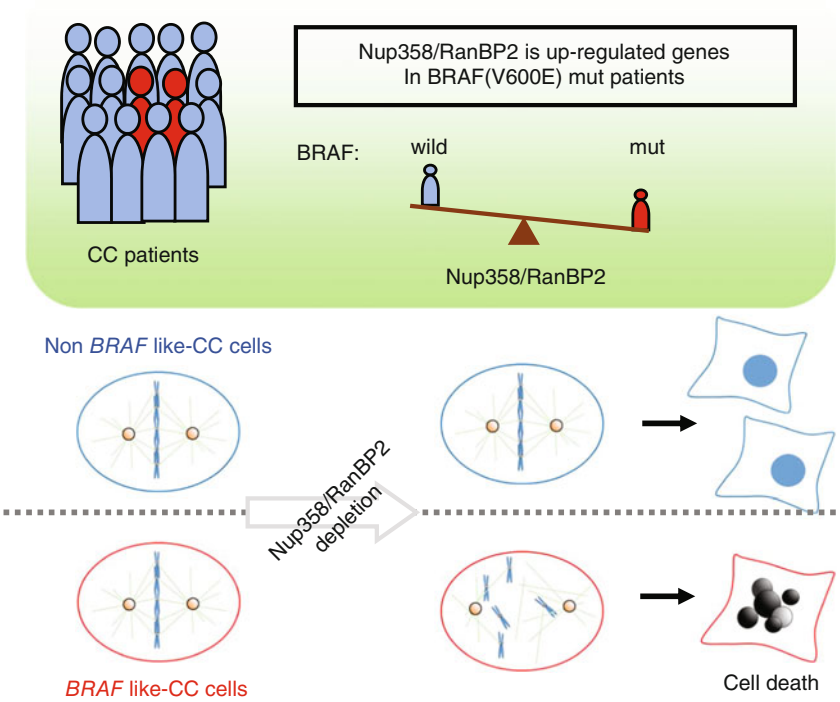

Fig. 10.3 A Model for Nup358/RanBP2 in mitotic progression and faithful chromosomal segregation

and composition, abnormal mitotic progression and abnormal chromosome segregation. BRAF-like CC cells depleted of Nup358/RanBP2 showed prolonged mitosis or mitotic arrest, which eventually triggered mitotic cell death (Hashizume et al. 2013). They also revealed that BRAF-like CC cell lines are defective in kinetochore microtubule outgrowth and that Nup358/RanBP2 depletion further potentiates this abnormality, leading to mitotic cell death (Fig. 10.3). These recent findings prompted the proposal that BRAF-like CC cells depend on Nup358/RanBP2 expression to tolerate the defects in mitosis (Wong and D'Angelo 2016).

10.2.1.1 Nup88

Nup88 localizes between Nup358 and Nup214 and physically interacts with them (Hashizume et al. 2010). Alterations in the expression of Nup88 in mice (Naylor et al. 2016) or cells (Hashizume et al. 2010) enhanced the multinucleation of cells and multipolar spindle formation, leading to aneuploidy and enhanced genomic instability (Hashizume et al. 2010). A potential explanation for the appearance of these cell populations is that disruption of the normal Nup88 expression levels

(by overexpression or depletion strategies) leads to a failure in the kinetochore–spindle microtubule interactions to capture chromosomes, ultimately leading to mitotic exit and nuclear envelope (NE) reformation around dispersed chromosomes or groups of chromosomes. It is enticing to speculate that Nup88 might be involved in cell cycle checkpoints to prevent aneuploidy. Naylor et al. also reported that the Nup88–Nup98–Rae1–APC/Cdh1 axis contributes to aneuploidy. They found that Nup88 overexpression did not alter global nuclear transport, but was a potent inducer of aneuploidy and chromosomal instability in mice (Naylor et al. 2016). The sequential molecular mechanisms underlying Nup and APC (Cdc20–Cdh1) signaling pathways are still not fully understood.

10.2.1.2 Nup214

Another cytoplasmic filament nucleoporin, Nup214/CAN, is a proto-oncogene implicated in leukemia (Saito et al. 2016). RNAi-mediated knockdown of Nup88 disrupted Nup214 expression and localization and caused multipolar spindle phenotypes (Hashizume et al. 2010). The abolition of Nup214 from mitotic spindles results in chromosome separation defects and aneuploidy with multinucleated cells. Similarly, the downregulation of Nup214 by miR-133b, which has been described as a tumor suppressor in esophageal squamous cell carcinoma (SCC) (Bhattacharjya et al. 2015), results in chromosome defects, with some cells appearing like "flowers" with greatly lobulated nuclei (multipolarization and multilobulation of cells) (Bhattacharjya et al. 2015).

10.2.2 Nup62 Subcomplex in Mitosis

The mammalian Nup62 subcomplex assembles from 4 O-glycosylated nucleoporins Nup62, 58, 54 and 45. The 62-kDa component of this complex, Nup62, contains three domains: N-terminal FG-repeat, central threonine/alanine-rich linker and C-terminal α-helical coiled-coil (Guan et al. 1995; Wong 2015). During interpahse, NUP62 as a gatekeeper regulates p63 nuclear transport and cell fate of squamous cell carcinoma (Hazawa et al 2018). During mitosis, Nup62 has been shown to play a novel role in centrosome integrity (Hashizume et al. 2013). Knockdown of Nup62 induces mitotic arrest in the G_2/M phase and mitotic cell death. Depletion of Nup62 also results in abnormal centriole synthesis and maturation, defective centrosome segregation, formation of multipolar centrosomes, dramatic spindle orientation defects, centrosome component rearrangements that impair cell bipolarity and multinucleated cells (Fig. 10.4) (Hashizume et al. 2013). Consistent with these functions, a GFP–Nup62 fusion protein was reported to accumulate at centrosomes (Hashizume et al. 2013) and spindle-like structures, identified by costaining with α-tubulin antibody (Wu et al. 2016). Consistent with this intimate link between centrosomes and cilia, nucleoporins have also been detected in the base of the cilia. Takao and Verhey (2016) developed a system to

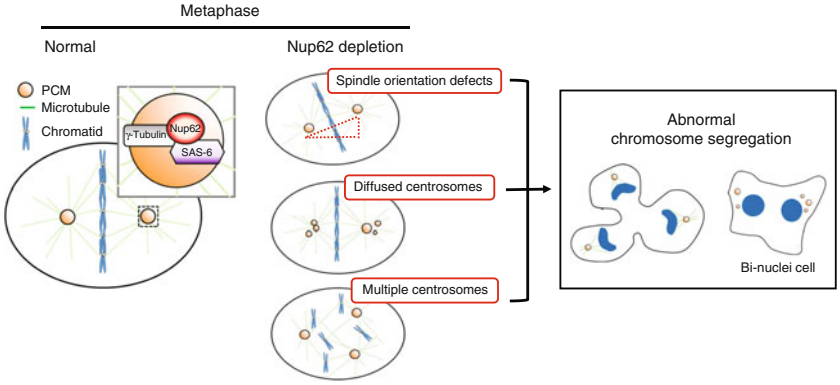

Fig. 10.4 Orchestration of the Nup62 inner ring subcomplex dynamics at the centrosomes

clog the pore by inhibiting Nup62 function via forced dimerization, and showed that forced dimerization of Nup62-Fv attenuated the kinesin-2 motor KIF17 (Wong et al. 2002) into the ciliary compartment, proposing the existence of a "ciliary pore complex" (Takao and Verhey 2016). Whether this model will stand the test of time is yet to be determined (Del Viso et al. 2016), but it offers an interesting mechanistic possibility. The mitotic functions of other members (Nup54, Nup58 or Nup45) of the Nup62 subcomplex remain to be established.

10.2.3 Nup107–Nup160 Subcomplex in Mitosis

Several lines of evidence support the localization of vertebrate Nup107–Nup160 complex (yeast Nup84 complex) at the kinetochores and spindles during mitosis (Belgareh et al. 2001; Loiodice et al. 2004). This subcomplex contains at least 10 members (Nup160, Nup133, Nup107, Nup96, Nup85, Nup43, Nup37, Sec13, Seh1 and ELYS/MEL-28) (Resendes et al. 2008). It is also worth noting that phosphorylation controls the breakdown of the NE at mitosis and the disassembly of the NPC into different subcomplexes. The Nup107–160 subcomplex was shown to interact with CENP-F. Partial depletion of the components ELYS or Nup133 in human cells did not alter spindle assembly or chromosome segregation, but induced cytokinesis defects (Rasala et al. 2008). By combining in vivo and in vitro studies, Mishra et al. (2010) further showed that the Nup107–160 complex promotes spindle assembly through Ran-GTP-regulated nucleation of microtubules by c-TuRC at kinetochores (Wozniak et al. 2010) (Fig. 10.5). Bolhy et al. (2011) also showed that Nup133 exerts this function through an interaction chain via CENP-F and NudE/EL. This molecular network is critical for maintaining centrosome association with the NE at mitotic entry. Moreover, depletion of one of the subunits, Seh1, induced a mitotic delay (Zuccolo et al. 2007). Seh1 also interacts with Mio, a key member of the SEACAT complex in both interphase and

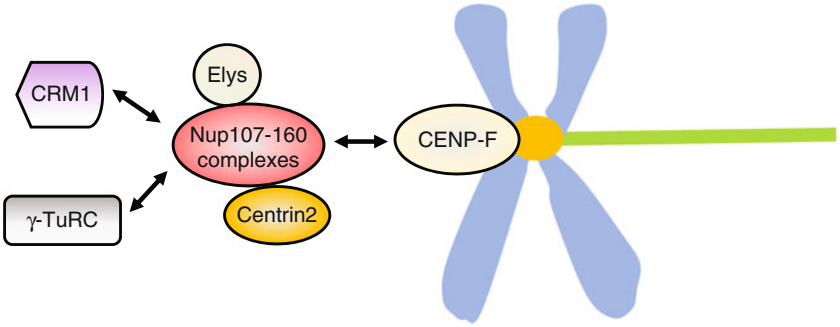

Fig. 10.5 Orchestration of the Nup107-160 core ring subcomplex dynamics in mitosis

mitosis. Mio plays a critical role in activation of the essential mitotic kinases, Aurora A and Plk1, at spindle poles/centrosomes (Platani et al. 2015). In addition, Clever et al. (2011) showed that ELYS/Mel28 plays a role in NE subdomain formation in late mitosis. The depletion of ELYS/Mel28 also accelerates the entry into cytokinesis after the recruitment of emerin to chromosomes. Moreover, Yokoyama et al. (2014)showed that, upon mitotic NPC disassembly, MEL-28 dissociates from chromatin and relocalizes to spindle microtubules and kinetochores. It then directly binds microtubules in a Ran-GTP-regulated manner via its C-terminal chromatin-binding domain. Supporting this notion, Gómez-Saldivar et al. (2016) identified functional domains responsible for NE and kinetochore localization, chromatin binding, mitotic spindle association and chromosome segregation. Phylogenetic profile analysis also suggested that Nup107–160 subcomplex proteins may function in the SAC and that they potentially interact with Mad2 and MadBub/Bub3 (van Hooff et al. 2017).

10.2.4 Tpr–Nup153 Subcomplex in Mitosis

Mitotic arrest deficient 1 (Mad1) and Mad2 proteins, which are critical regulators of the spindle assembly checkpoint (SAC), interact with another Nup, Tpr (Mlp1 and 2 in yeast). Once the cells enter prophase, Mad1 and Mad2 gather on unattached kinetochores and monitor microtubule occupancy to avoid the premature onset of anaphase (Guttinger et al. 2009). Mad1 is accountable for targeting of the Mad1–Mad2 complex to both NPCs during interphase and kinetochores during mitosis (Rao et al. 2009). Tpr, Mad1 and Mad2 coprecipitate in mitotic-enriched HeLa cell extracts, which lack microtubules and intact nuclear pores (Lee et al. 2008; Lince-Faria et al. 2009; Nakano et al. 2010). Tpr-depleted cells show chromosome segregation defects similar to those seen in cells depleted of Mad1 and Mad2 (Nakano et al. 2010). The functional significance of the connection between the basket and the SAC was further emphasized by experiments demonstrating that Tpr is liable for faithful chromosome segregation during mitosis through its

association with the dynein light chain (DLC) (Nakano et al. 2010). Tpr acts as a spatial and temporal regulator of the SAC, maintaining the efficient recruitment of Mad1 and Mad2 to the molecular motor dynein to promote correct anaphase progression (Nakano et al. 2010). When Tpr levels are reduced, many cells show abnormal spindle polarity, bending chromosomes and chromosome lagging (Nakano et al. 2010). These phenotypes suggest a direct role for Tpr in forming spindle structures. Nakano et al. performed a series of assays to rescue the chromosome-lagging defects in cells in which Tpr had been knockdown by siRNA, to confirm the functional role of the Tpr–dynein interaction with the mitotic spindle (Rodriguez-Bravo et al. 2014) (Nakano et al. 2010) (Fig. 10.6). Tpr also associates with A-Kinase Anchoring Protein 95 (AKAP95) during mitosis. AKAP95-depleted cells display more rapid prometaphase-to-anaphase transition, escape from nocodazole-induced mitotic arrest and show partial delocalization from kinetochores of the SAC component Mad1 {Lopez-Soop, 2017 #1950}. Kobayashi et al. (2015) also reported that Tpr depletion enhances the rate of tetraploidy and polyploidy. Mechanistically, Tpr interacts, via its central domain, with Aurora A but not Aurora B kinase. In Tpr-depleted cells, the expression levels, spindle pole/centrosomal localization and phosphorylation of Aurora A were all found to be reduced (Kobayashi et al. 2015). Remarkably, an Aurora A inhibitor, Alisertib (MLN8237), also disrupted the centrosomal localization of Tpr and induced cell death in a time- and dose-dependent manner (Fig. 10.7).

Schweizer et al. (2013) showed that Tpr was normally undetectable at kinetochores and dispensable for the kinetochore localization of Mad1, but not of Mad2, which suggests that SAC robustness depends on Mad2 levels at kinetochores. In addition, Rodriguez-Bravo et al. showed that Mad1–Mad2 complexes tethered to the nuclear basket, which activated soluble Mad2 as a binding partner and inhibitor of Cdc20 in the cytoplasm. Displacing Mad1–Mad2 from nuclear pores hastened the onset of anaphase, prevented the effective correction of merotelic errors and increased the threshold of kinetochore-dependent signaling needed to halt mitosis in response to spindle poisons (Rodriguez-Bravo et al. 2014). They suggested that both nuclear pores and kinetochores emit “wait anaphase” signals that preserve genome integrity (Rodriguez-Bravo et al. 2014). Moreover, Rajanala et al. found that Tpr is phosphorylated at the S2059 residue by CDK1 and the phosphorylated form clearly localizes with chromatin during telophase. Abrogation of S2059 phosphorylation abolishes the interaction of Tpr with Mad1, thus compromising the localization of both Mad1 and Mad2 proteins, resulting in cell cycle defects (Rajanala et al. 2014). It is tempting to surmise that Tpr function impacts on the progression out of metaphase–anaphase transition and/or chromosome segregation itself, and the lack of coordination that follows results in aberrant chromatin morphology (Nakano et al. 2010) (Fig. 10.8). In this context, it is worth mentioning that the Tpr–Met oncogene, a carcinogen-induced chromosomal rearrangement resulting in fusion of a protein dimerization of Tpr to the receptor tyrosine kinase domain of Met, has been described (Peschard and Park 2007). In particular, the N terminus of Tpr undergoes frequent rearrangement with Met, Trk and Raf in gastric and thyroid cancers, resulting in hyperactive tyrosine kinase

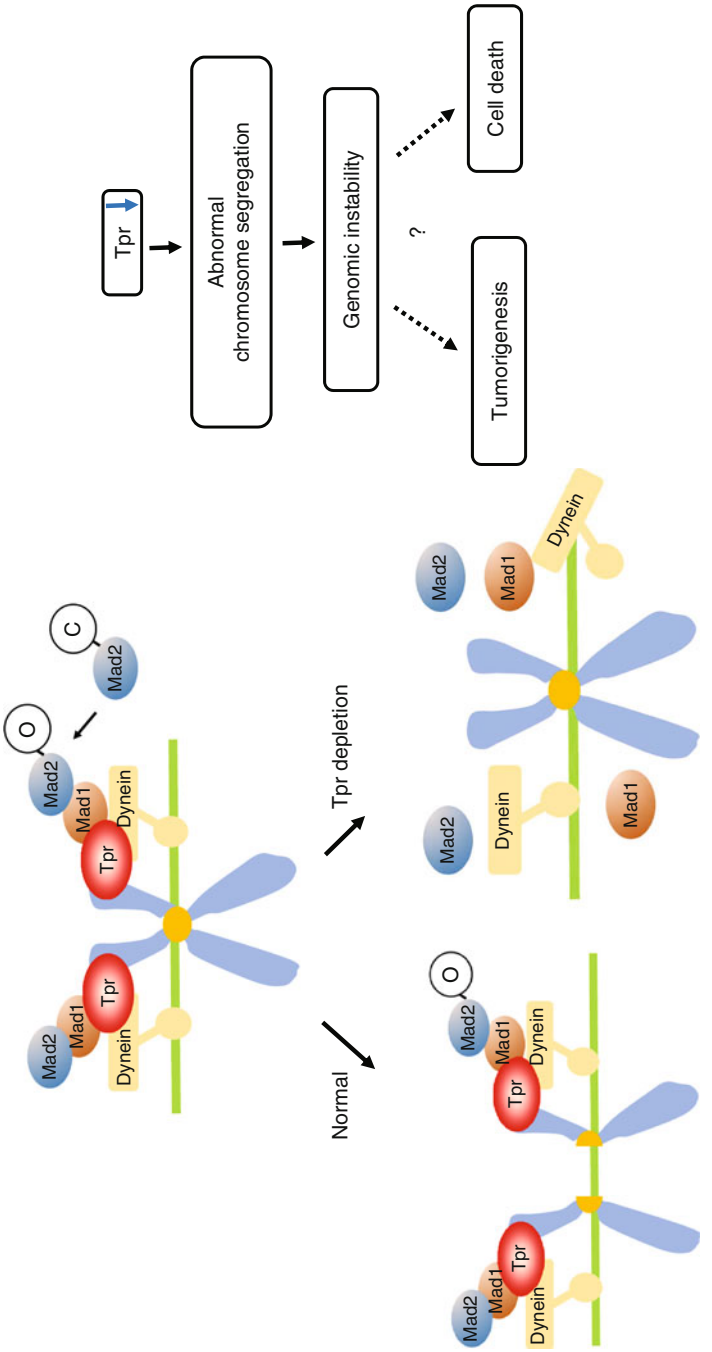

Fig. 10.6 Orchestration of the Tpr-Nup153 nuclear basket subcomplex dynamics in mitosis

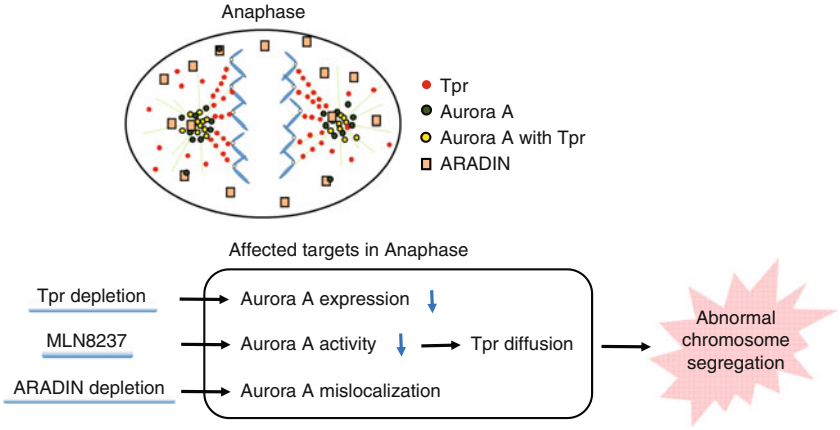

Fig. 10.7 Orchestration of the Tpr-Aurora A subcomplex dynamics in mitosis

fusions that are mislocalized to the cytoplasm (Kohler and Hurt 2010). This segment of Tpr also induces lagging chromosomes when expressed on its own (Nakano et al. 2010), suggesting that these translocations fuel carcinogenesis through increased tyrosine kinase signaling and by subversion of NPC-based defenses against chromosome instability. Given that the data shows chromosome lagging and congregation defects at the metaphase–anaphase transition (Lee et al. 2008; Nakano et al. 2010), one might assume that chromosomal rearrangement of Tpr could lead to chromosomal instability in certain tumors.

10.2.4.1 Nup153

Tpr interacts with another nuclear basket protein, Nup153. Makay et al. (2009) demonstrated that two different phenotypes result from the knockdown of Nup153 to different levels and that rescue of these phenotypes involves distinct domains within Nup153. They suggested that the FG-rich region of Nup153 plays a critical role in mitosis. Besides, when Nup153 levels are reduced further, many cells show abnormal, multilobed nuclei. This phenotype might indicate at a direct role for Nup153 in formation of the nuclear structure (Mackay et al. 2009). By image analysis of live cells in which Nup153 had been knocked down, they also showed that significant delays occur early in mitosis (Mackay et al. 2009; Lussi et al. 2010). Ullman's group also indicated that Nup153 function influences the active state of the Aurora B-mediated abscission checkpoint during cell division (Mackay et al. 2015). Finally, both nuclear basket proteins Tpr and Nup153 were reported to play a role in genome integrity. For example, Tpr siRNA treatment impaired cell growth and proliferation compared with those in control siRNA-treated cells. In Tpr-depleted cells, the levels of p53 and p21 proteins were also increased (Funasaka et al. 2012). Moreover, Tpr depletion increased the nuclear accumulation of p53 and facilitated autophagy (Funasaka et al. 2012).

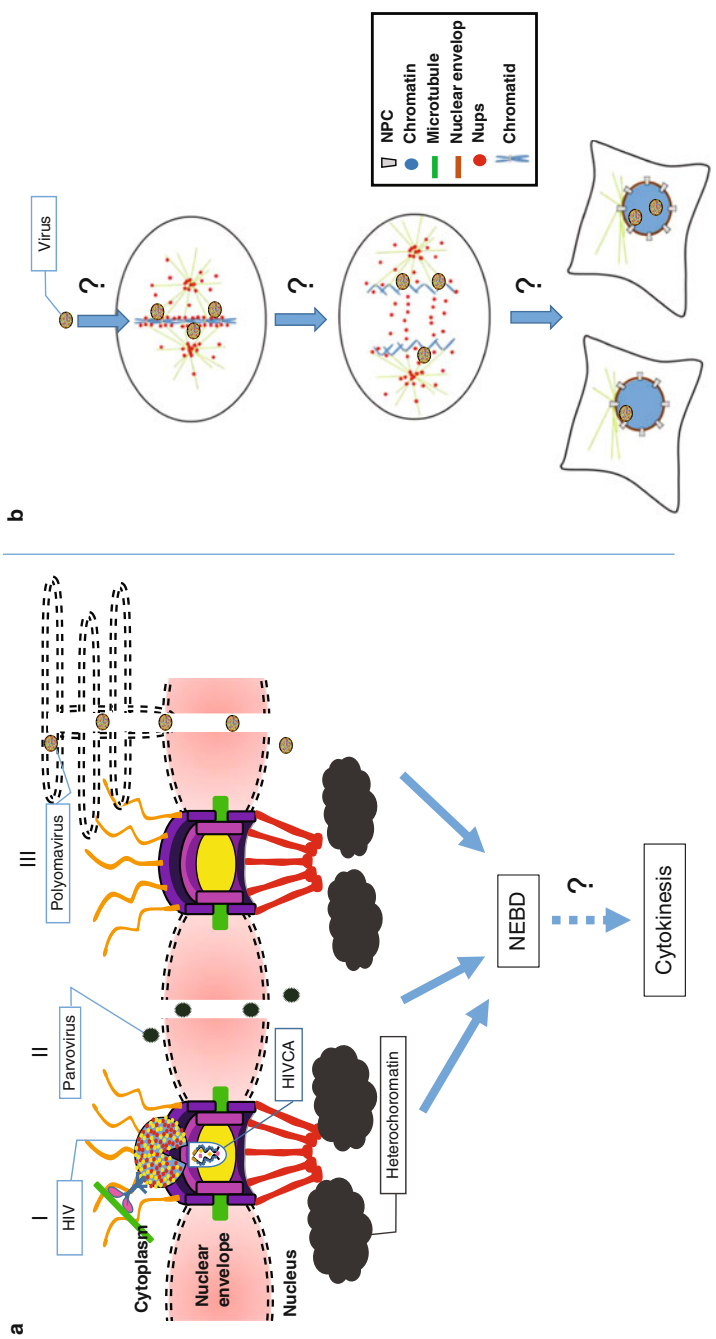

Fig. 10.8 A model for viral (a) nuclear import, integration and (b) mitosis with nucleoporins

10.2.5 Rae1–Nup98 Subcomplex in Mitosis

Another Nup that has been related with spindle assembly is RNA export 1 (Rae1/GLE2/mRNP41). Rae1 has also been linked to the pathophysiology of breast cancer (Chin et al. 2006). Rae1 is a tryptophan–aspartic acid (WD) repeat β propeller protein that is kinetically distributed in NPCs during interphase (Wong et al. 2006). Rae1 forms a complex with Nup98, and both are implicated in RNA export during interphase (Pritchard et al. 1999; Ren et al. 2010; Tran and Wente 2006). Rae1 has been showed to bind to Nup98 and the mitotic checkpoint kinase Bub1 through their Gle2-binding site (GLEBS) domains and to function with Nup98 in securin degradation. Rae1–Nup98 complex has also been reported to inhibit the formation of the anaphase-promoting complex (APC) in a mouse model system (Jeganathan et al. 2005). Several studies have also reported that Rae1 binds to microtubules (MT) (Kraemer et al. 2001; Wong et al. 2006). Using *Xenopus* egg (Blower et al. 2005) and HeLa cell (Wong and Blobel 2008) extract systems for mitotic spindle formation, Rae1 was identified as a essential component for the promotion of microtubule assembly. It was also found to interact and colocalize with nuclear mitotic apparatus protein (NUMA), a microtubule-associated protein that supports microtubule bundling at spindle poles (Wong and Blobel 2008; Wong et al. 2006). Moreover, Rae1 interacts with a subunit of cohesin, SMC1947-967, and it was shown that binding to Rae1 only occurred after the phosphorylation of Ser957 and Ser966 by the spindle pole-localized kinase ATM (Ataxia Telangiectasia Mutated) (Wong and Blobel 2008). Imbalances in SMC1 or Rae1 reactions were also found to cause the formation of multipolar spindles (Wong 2010a,b; Wong and Blobel 2008). In this context, the following question arises: Which signaling pathways regulate the mobile nucleoporin Rae1 in the cell cycle? Jahanshahi et al. (2016) identified that the Hippo pathway targets Rae1 to regulate mitosis and organ size, and provides feedback to regulate the upstream components merlin, hippo and warts. Rae1 loss restricts cyclin B levels and organ size, while Rae1 overexpression has the opposite effect, similar to Hippo pathway overactivation or loss of function, respectively (Jahanshahi et al. 2016). Future work should also define how Rae1 acts in a feedback circuit to regulate pathway homeostasis in cancer cells.

10.2.5.1 Nup98

Chromosomal translocations involving chimeric fusions of the nucleoporin Nup98 protein have often been described in acute myelogenous leukemia (AML). All of the fusion proteins have an identical Nup98 N terminus, which includes the GLEBS motif for interaction with Rae1 and FG repeats that associate with the transcription factors HDAC1 and p300 (Funasaka and Wong 2011). Nup98 RNAi caused severe chromosome segregation defects and disrupted Rae1 but not HDAC1 expression and localization (Funasaka et al. 2011). Interestingly, the wild

type Nup98 and the leukemogenic fusion protein Nup98–HOXA9 behave differently during the cell cycle. In mitosis, only Nup98–HD fusions were found to be concentrated on chromosomes (Funasaka et al. 2011). In Nup98–HOXA9-transfected cells, Rae1 protein is also decreased and mislocalized. These findings were confirmed in Nup98–HOXA9 transgenic mice and a Nup98–HOXA9 AML patient (Funasaka et al. 2011). Moreover, Nup98 stability was shown to be controlled by a PEST sequence, absent in NUP98 oncoproteins, whose deletion reproduced the aberrant chromosome segregation activity of Nup98 oncoproteins (Salsi et al. 2014, 2016).

10.2.5.2 Nup188

Nup188 is a component of the Nup93 subcomplex. Itoh et al. showed that Nup188 localizes to spindle poles during mitosis, through the C-terminal region of Nup188. In Nup188-depleted mitotic cells, chromosomes fail to align to the metaphase plate, which induces mitotic arrest due to the SAC. Nup188 also associates with NuMA, which plays an instrumental role in focusing microtubules at centrosomes, and NuMA localization to spindle poles is disturbed in Nup188-depleted cells (Itoh et al. 2013). Following this line of evidence, del Viso found that Nup188 localized at the bases of cilia that extend from centrioles (Del Viso et al. 2016).

10.3 Viral Nups in Mitosis

Viruses use several strategies to deliver their genomes into the host nucleus (Fig. 10.8). One involves nuclear entry during mitosis, when the NE is disassembled; an example of this is found in gammaretroviruses, the replication of which is dependent on the passage of target cells through mitosis, at which point they are believed to obtain access to chromosomes when the NE dissolves for mitosis (Matreyek and Engelman 2013). Another mechanism is viral genome release in the cytoplasm, followed by entry of the genome through the nuclear pore complex (NPC); an example of this occurs in lentiviruses such as HIV-1, which infect nondividing cells and are believed to enter the nucleus by passing through the NPC. Recent evidence has highlighted the importance of the HIV-1 capsid in this process. Furthermore, the capsid was found to be responsible for the viral requirement of various nucleoporins Tpr, Nup153 and Nup358, during infection (Matreyek and Engelman 2013; Wong et al. 2015). Which mechanism a particular virus uses may depend on its size and structure, the cellular cues that it uses to trigger capsid disassembly and genome release, as well as the phase of the cell cycle (Matreyek and Engelman 2013; Wong et al. 2015) (Fig. 10.8).

10.4 ESCRT-III in NE/NPC Sealing at the End of Mitosis

Recent work has also shed light on the NPC and ESCRT (endosomal sorting complex required for transport)-III membrane remodeling machinery in this process (Vietri et al. 2015) (Fig. 10.9). It has been proposed that ESCRT-III, VPS4 and spastin cooperate to coordinate NE sealing and spindle disassembly at NE–microtubule intersection sites during mitotic exit to ensure nuclear integrity and genome safeguarding, with a striking mechanistic parallel to cytokinetic abscission (Vietri et al. 2015).

10.5 Concluding Remarks

While the functions of NPCs in transport are well established, coupling of the nuclear transport machinery to processes that regulate chromosome segregation during mitosis is still an emerging area of investigation. Our current understanding of this issue can be summarized as follows: (1) during the early stage of mitosis, the cytoplasmic NPC component Nup358 facilitates centrosome anchoring by its association with the molecular motors dynein and kinesin, which localizes at the NPC during G2. (2) Upon NEBD (complete disassembly of the NPC), Nups start to be relocated to the kinetochore, spindles and centrosomes. The NE and NPCs disassemble in prophase, which is most likely triggered by the phosphorylation of NE and NPC proteins. Nucleoporins usually remain in subcomplexes and are found dispersed in the mitotic cytoplasm or associated with mitotic structures, such as the spindle or kinetochores.

How Nups adapt their functions during mitosis and whether their phosphorylation by mitotic kinases is linked to that adaptation remain to be investigated. Moreover, Nups are directly implicated in cancer in several ways: changes in nucleoporin protein expression levels during the cell cycle, single point variants and chromosomal translocations generating fusion proteins. The accurate regulation of Nup levels and functions seems crucial to avoid the accumulation of DNA lesions and aneuploidy and probably to prevent malignant transformation in proliferating cells (Ibarra and Hetzer 2015; Nakano et al. 2011).

A challenging issue to be addressed in the near future involves elucidation of the individual steps, both spatially and temporally, taken by nucleoproteins after the NE breaks down in mitosis. Resolving this issue will involve a combination of developing extremely high-resolution real-time single-molecule imaging microscopy or HS-AFM techniques along with techniques in the fields of biochemistry, genetics, bioinformatics and structural biology. These approaches have provided and should continue to provide intriguing advances in our understanding of the roles of nucleoporins during mitosis. From a clinical perspective, it will also be important to further address the exact contributions of individual nucleoporins to various stages of carcinogenesis.

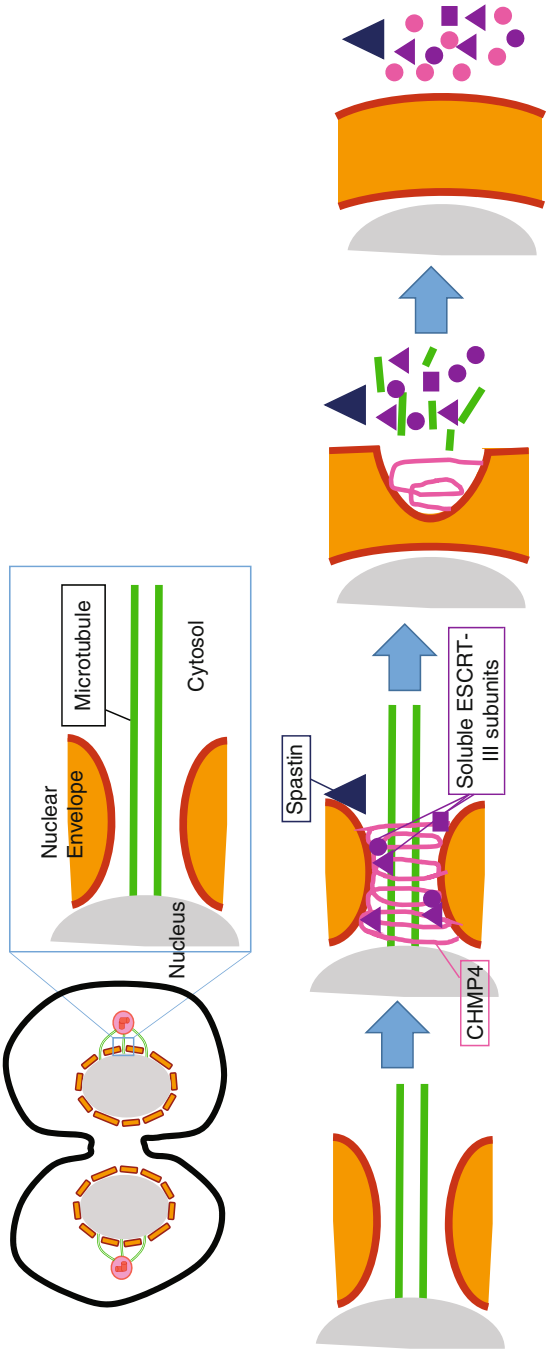

Fig. 10.9 A model for ESCRT-III in NE/NPC sealing at the end of mitosis

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