UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Nuclear Pore Proteins in Cell Differentiation

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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Professor Jing Yang

2013

The Dissertation of Yun Liang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

To my parents and my husband

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ABSTRACT OF THE DISSERTATION

The Role of Nuclear Pore Proteins in Cell Differentiation

by

Yun Liang

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2013

Professor Martin W. Hetzer, Chair

Professor Arshad Desai, Co-Chair

Nuclear pore proteins (Nups) form the only channels on the nuclear envelope that allow macromolecule transport between the cytoplasm and the nucleoplasm. Initial studies in the unicellular organism yeast have confirmed that

deletions and/or mutations of multiple Nups result in deficiency in bulk transport between cellular compartments and subsequently lethality. Recent investigations regarding multicellular organisms, however, have revealed that mutations in Nups are often linked to cell-type specific defects and diseases. Therefore, there might be cell-type specific functions of Nups, for example in the context of differentiation.

One potential cell-type specific function of Nups could be related to the regulation of genes that are activated in a cell-type dependent manner. Such hypothesis stems from the observation that microscopically, Nups appear to selectively interact with active genomic regions. To test this hypothesis, chromatin immunoprecipitation experiments for Nup98 have been performed in multiple cell types, including human cells at different stages of neural differentiation. It has been found that the patterns of Nup98-genome interaction are highly cell-type specific in the neural differentiation system. Further functional studies have demonstrated a role of Nup98 in regulating the expression of a cohort of genes active during neural development.

As an alternative approach to investigate into the potential cell-type dependent behavior of Nups, dynamics of Nups were analyzed at different stages of muscle differentiation by live cell imaging. One Nup, Nup50, was found to exhibit decreased dynamics upon differentiation, serving as the first example of a

Nup that has altered dynamics during development. Kifc1, a kinesin-superfamily protein that is predicted to be a nuclear motor, was found to confer the higher dynamics of Nup50 by physical interaction before differentiation. Upon differentiation, Kifc1 is degraded, which could explain the decrease in Nup50 mobility. Knocking down Nup50 decreased efficiency of muscle differentiation, whereas knocking down Kifc1 accelerated muscle differentiation. This suggests that Nup50 is differentially regulated during muscle development and is functionally important for differentiation.

Together these studies revealed previously unidentified roles of Nup98 and Nup50 in differentiation, which might shed light on the cell-type specific diseases related to Nup mutations.

Chapter 1 Background

In eukaryotic cells, the genetic material is enclosed by the double lipid bilayer known as the nuclear envelope [1]. The outer membrane of the nuclear envelope is continuous with the endoplasmic reticulum and the inner membrane of the nuclear envelope hosts a set of proteins that provides attachment sites for the nuclear lamina and chromatin [2-4]. The two membranes of the nuclear envelope are joined at the sites of the nuclear pore complexes, through which macromolecules are transported between the cytoplasm and the nucleoplasm [5,6]. Each nuclear pore complex is typically composed of multiple copies of around 30 different proteins named the nuclear pore proteins (Nups), or nucleoporins [5,7]. The majority of Nups are evolutionarily conserved and have the well-characterized function of mediating macromolecular transport [5,8]. In human, mutations of several Nups have been linked to diseases, suggesting the importance of proper functioning of those proteins [8]. Somewhat surprisingly, the phenotypes of such Nup-linked diseases are manifested in specific cell types [8-16]. This implies that Nups may have cell-type specific functions in addition to general transport that is thought to occur in all cell types.

1.1 Nuclear Pore Proteins and Their Function in Transport

The nuclear pore complex exhibits an eightfold-symmetrical structure that forms a channel for macromolecule transport across the nuclear envelope [17-20]. The core of the nuclear pore complex is composed of the scaffold Nups, which are assembled into two multiprotein subcomplexes: Nup107-160 and Nup93-205 [8,20] (Figure 1-1). The Nup107-160 complex (Nup160, Nup133, Nup107, Nup96, Nup85, Nup43, Nup37, Seh1, and Sec13 in vertebrates) is essential for nuclear pore complex assembly upon mitotic exit, and depletion of its components resulted in reduced NPC density in the nuclear envelope [21,22]. Several members of the Nup93-205 complex (Nup205, Nup188, Nup155, Nup93, and Nup35) are also required for proper nuclear pore biogenesis as well as maintenance of nuclear size and morphology [22-24].

In addition to the scaffold Nups, pore components include transmembrane Nups that may tether the core of the nuclear pore complex to the nuclear envelope. Transmembrane Nups identified to date include Pom121, NDC1 and gp210 [5]. Pom121 is critical specifically for pore assembly during interphase of the cell cycle [25]. By contrast, NDC1 is required for postmitotic nuclear pore complex assembly and directly interacts with Nup53 that is tightly associated with the nuclear envelope membrane, potentially functioning to anchor the nuclear

pore complex in the membrane [26]. Gp210 is not expressed in all cell types, and is not required for incorporation of core components of the nuclear pore or maintenance of pore stability [27].

A third group of nuclear pore proteins are the peripheral Nups, which associate with the scaffold of the nuclear pore complex and extend into the cytoplasm, the nucleoplasm or the central channel of the pore [8]. Many of the peripheral Nups contain FG-repeats, domains with multiple phenylalanineglycine (FG), GLFG (where L is leucine), or FxFG (where x is any amino acid) repeat motifs separated by charged or polar spacer sequences [28,29]. FG-repeat containing Nups include Nup358, Nup214, Nup153, Nup98, Nup62, Nup58, and Nup54 [5,30-32]. They are crucial for the active translocation through the nuclear pore complex as transport receptors engage in series of stochastic and low-affinity interaction with FG-repeats [29,33]. FG-repeats are also thought to fill the channel of the nuclear pore and thereby preclude entry of molecules larger than 40kDa and contribute to the permeability barrier [34]. In addition to FG-Nups, there are distal Nups such as Tpr, Nup153 and Nup50 on the nuclear side and Nup88 on the cytoplasmsic side of the nuclear pore complexes [22]. These Nups can function at the initial and final steps of export and import [8].

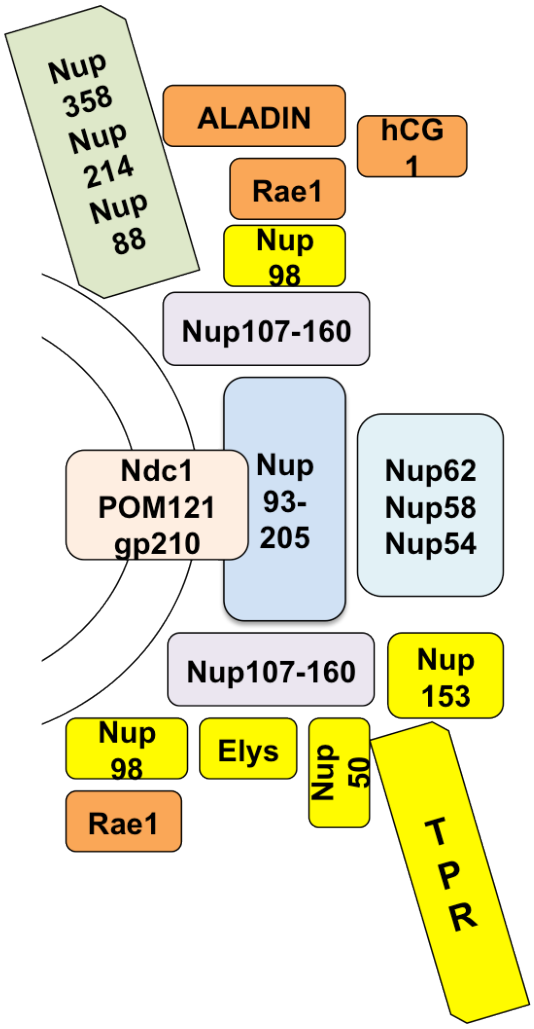


Figure 1-1 Structure of nuclear pore complexes

The nuclear pore complex has been viewed as a 'transport machine'[35]. Yeast genetics have revealed that most nuclear pore proteins are required for nucleocytoplasmic trafficking of RNA and/or protein, and as expected, deletions of a number of Nups are lethal [36]. Somewhat surprisingly, functional studies in higher organisms demonstrated that Nup mutations are often associated with cell

type specific defects or diseases, which cannot be simply explained by deficiency in general transport that should occur in all cell types [8]. Those observations are further detailed below.

1.2 Cell-type Specific Defects Linked to Mutations of Nuclear Pore Proteins

Recently, it has been found that mutations of multiple Nups cause a variety of tissue-specific diseases in human [8]. For example, a missense mutation of Nup155 leads to atrial fibrilliation and early sudden cardiac death, although non-cardiac tissues appear non-affected [9]. Genomic translocations that result in fusion of Nup98 fragment to other genes underlie acute myeloid leukaemia and other haematological malignancies, whereas no abnormal proliferation of non-haemotapoietic stem cells has been documented [10] [37]. Nup62 has been detected as an autoimmune antigen in primary biliary cirrhosis [12], and a missense mutation of Nup358 has been associated with familial cases of infection-triggered acute necrotizing encephalopathy [15].

The cell-type specific deficiencies linked to Nup mutations are not limited to human, but also observed in other multicellular organisms including zebrafish, fruit fly, and mouse [8].

1.3 Hypotheses on the Dynamic Functions of Nuclear Pore Proteins

Based on the above observations, it appears that the functions of Nups are dynamic.

Firstly, a given Nup may have various functions in different cell types, implied by the findings that some Nup mutations seem to affect only certain tissues in multiple diseases [8]. Such functions may be related to the well-characterized transport roles of Nups (i.e. 'transport-dependent' functions). For example, there are transport factors known to be expressed in a tissue-specific manner [38,39], and they may interact with a certain Nup so that deletion of this Nup causes specific defects in tissues where the transport factors are expressed at high levels. Alternatively, the tissue-specific functions of Nups may not be directly linked to transport (i.e. 'transport-independent' functions). One such possibility can be the regulation of gene expression by Nups [8]. Initial electron microscopy studies showed that the nuclear envelope is underlined by the electron dense heterochromatin where genes are largely silenced, with the exceptions of nuclear pore complexes that seem to selectively interact with euchromatin where genes are actively transcribed [40-42]. Based on these observations, it has been proposed that Nups may interact with active genes to

promote the export of their transcripts, known as the ‘gene gating hypothesis’ [42]. Later it was found that in yeast and in *Drosophila* embryonic culture cells, several Nups do preferentially associate with active genes, and are required for optimal expression of genes they interact with [43-51]. Reported evidences for the roles of Nups in gene regulation are further detailed in Chapter 1.4. An implication of the reported gene activation function of Nups is that Nups might regulate the expression of different gene sets in various cell types, and this would be consistent with the observed tissue-specific phenotypes related to Nup mutations [8]. This hypothesis of cell-type specific gene regulation by Nups is tested in the thesis project.

Secondly, different Nups may have functions independent of the entire nuclear pore complex as a whole. This is implied by the observations that deficiencies in different Nups give rise to distinct phenotypes [8]. Consistent with this idea, Nups can shuttle off the nuclear pore complexes, and Nups exhibit a wide range of residence times at the pores from seconds to days and even possibly to the lifetime of an organism [52-54]. Reported evidences for Nup dynamics are further discussed in detail in Chapter 1.4. It is not clear whether such dynamic behavior of Nups can alter in different cell types. In addition, the mechanism of regulation of Nup dynamics remains unexplored and players involved have not been identified. Those questions are addressed in the thesis

project.

In summary, the following hypotheses are tested in the thesis project:

1) Nup has cell-type dependent functions in gene regulation (described in Chapter 2 and Chapter 3);

2) Nup has cell-type specific dynamics that is regulated by a developmentally modulated Nup-interacting protein (described in Chapter 4).

1.4 Evidences for Transport-Independent Functions of Nups

One of the first hints pointing to the possibility of transport-independent functions of Nups was the finding of their shuttling behavior. Some components of the nuclear pore complexes, especially the scaffold Nups such as members of the Nup107-160 subcomplex, are stably anchored in the nuclear envelope [55]. By contrast, several Nups have been shown to shuttle between the nuclear pore complexes and other cellular compartment such as the cytoplasm and nucleopalsm [50,56]. Systematic analysis of the dynamic organization of nuclear pore complexes revealed that Nups exhibited a range of residence times at the nuclear pore complexes from seconds to hundreds of hours [52]. Generally speaking, the scaffold Nups such as the Nup107-160 subcomplex components associate with the nuclear pore complexes very stable. By contrast, most of the peripheral Nups such as Nup153 and Nup50 shuttle frequently on and off the

nuclear pore complexes. The shuttling behavior of Nups could be related to their function in transport, for example capturing and delivery of transport cargos away from the nuclear pore complexes. It is also possible that Nups could have additional functions that are independent of the transport role of the nuclear pore complexes. This speculation is consistent with the observation that the shuttling behavior of some Nups is responsive to transcription inhibition using small molecules [57,58].

The connection between transcription and Nups is further suggested by the microscopic observation that nuclear pore complexes are aligned with euchromatin in the otherwise heterochromatic nuclear periphery, which has led to the ‘gene-gating’ hypothesis which proposes a preferential link between Nups and gene activation [42]. Many studies have been carried out in lower organisms to test this hypothesis. In yeast, several nuclear pore components are found to preferentially associate with active genes from ChIP-chip studies on a genome-wide level, and detailed characterization on single inducible genes has illustrated the relocation of these genes from the nucleoplasm to the nuclear periphery upon activation [43,45,46,49,59,60] (Figure 1-2). The only study on this topic regarding cells of higher organisms, however, suggests that the scaffold nuclear pore component Nup93 mainly interacts with genes carrying silent histone modifications in the human cancer cell line Hela [61] (Figure 1-2). These

seemingly contradictory results suggest that the pattern of Nup-gene interaction may vary depending on cellular and extracellular environments, and need to be analyzed with careful control of cell status.

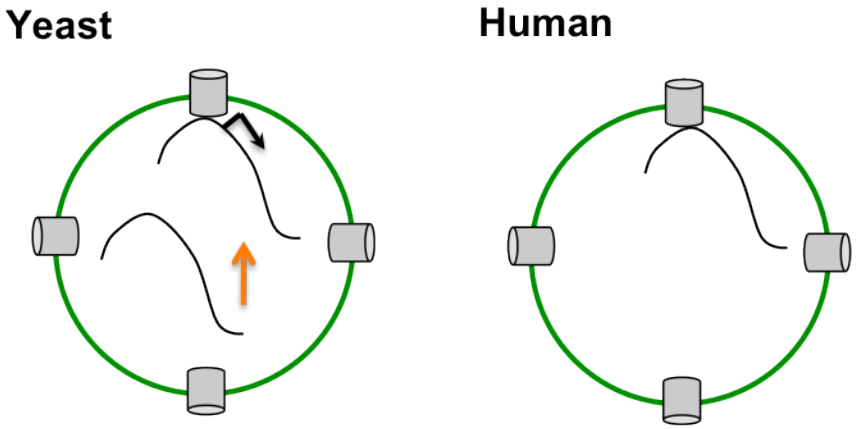


Figure 1-2 Reported evidences for Nup-gene interaction

Not all genetic materials interact with the nuclear periphery, and there are substantial numbers of active genes inside the nucleoplasm (i.e. away from the nuclear periphery) [62]. The shuttling behavior of Nups reveals the possibility that Nups can interact with genes inside the nucleoplasm, away from the nuclear pore complexes (Figure 1-3). If true, this extends the functional reach of Nups in gene regulation and partially explains the presence of active genes inside the nucleoplasm.

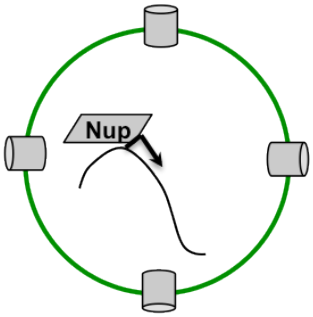


Figure 1-3 Intranuclear Nup-gene interaction

What is the functional relevance of Nup-gene interaction? For example, is Nup-gene association a cause or consequence of transcription activation? There are evidences suggesting that Nup association can enhance transcription, at least in some cases. For instance, fusion of several scaffold Nups of a DNA binding domain of a known transcription factor increased expression levels of a reporter gene [63]. This might be due to recruitment of transcription factors by Nups that were tethered to the reporter gene. In addition, Drosophila Nup153 and Megator are required for gene upregulation on the male X chromosome and the chromosome's association with the nuclear pore complexes at the nuclear periphery [64]. However, there are also examples where failure of gene targeting at the nuclear pore complexes did not correlate with significant loss of transcription, suggesting that in these cases, gene positioning was not essential for optimal expression [43].

A second potential functional relevance of Nup-gene interaction could be promoting the export of transcripts from those loci by nuclear pore components. In yeast, two complexes on chromatin have been implicated in gene tethering to the nuclear pore complexes and coordination of transcription and export, the SAGA and THO complexes [65]. SAGA was originally discovered as a histone acetyltransferace complex, and was later found to be linked through Sus1 to the TREX-2 complex (Sac3/Thp1/Sus1/Cdc31) that is positioned at the nuclear pore complexes [66]. Mutations of SAGA and TREX-2 components caused deficiency in stimuli-induced relocation of the gene GAL1 from the nuclear interior to the periphery, and Sus1 was found to be required for GAL1 induction and mRNA export [66-68].

The THO complex (Tho2/Hpr1/Mft1/Thp2) functions in transcriptional elongation [69]. Two additional proteins, Sub2 which is a helicase and Yra1 which is the adaptor for the mRNA exporter Mex67, associate with THO to form the THO/TREX complex [70]. Genetic studies illustrated that each THO/TREX component was required for efficient mRNA export [70]. In addition, Sub2 and Yra1 are indispensable for transcription in some cases [70].

It is not entirely clear if the SAGA and THO/TREX complexes have evolutionarily conserved function in coordinating transcription and export in higher organisms [65].

Collectively, there are evidences suggesting preferential association between Nups and active genes in yeast. Examples have also been described where interaction with Nups is important for the expression and export of specific genes studied. However, there exist cases in which Nups can be linked to silent genes or not required for optimal gene expression. In addition, the interaction between Nups and genes has not been characterized in higher organisms as extensively as in yeast. It is also not known if Nup-gene interaction is restricted at the nuclear pore complexes, or can happen in intranuclear sites as implicated by the shuttling behavior of Nups. Therefore, more studies need to be performed to analyze the seemingly dynamic interaction between Nups and genes in a variety of scenarios and its functional relevance in terms of transcription and RNA export.

Chapter 2 Function of Drosophila Nuclear Pore Proteins in Gene Regulation

2.1 Introduction

As discussed in Chapter 1, in addition to the well-established roles in mediating transport, Nups have been implicated in chromatin organization and gene regulation [43]. Studies in yeast revealed that Nups can associate with promoters of active genes [60] and that the expression of inducible genes is increased by interactions with nuclear pores [46]. Furthermore, a genome-wide analysis in *S. cerevisiae* demonstrated that a subset of Nups can occupy regions of highly transcribed genes [45]. Additionally, Nups have been shown to function as chromatin boundaries in *S. cerevisiae* [71,72]. Boundary activity involves protection from nearby activating or repressive signals and constitutes another plausible function for nuclear pore complexes in the organization of the genome into discrete chromatin domains. As further evidence for the role of the nuclear pore complex in regulation of active chromatin, Nups have been found to participate in X chromosome transcriptional hyperactivation in dosage compensation of *Drosophila melanogaster* [64].

Interestingly, the only genome-wide study of Nup-chromatin association in animal cells revealed a correlation between the binding sites of Nups and regions

enriched in repressive histone modifications [61], which exhibited characteristics of sequences known to associate with the nuclear periphery in human cells [73]. The observed discrepancy between yeast and human data suggests that the genome-binding pattern of the nuclear pore complex may be quite different or more complex in metazoa. Furthermore, many of the peripheral Nups in mammalian cells have been shown to be mobile and to move dynamically on and off the pore [52]. Therefore, it seems possible that Nup-chromatin interactions could occur at distant sites from the nuclear envelope, a notion that has some experimental support from the observation of intranuclear Nups (i.e., not associated with the nuclear envelope) in mammalian cells [57,58,74]. Thus, the functional role of Nup-chromatin interactions and whether they occur exclusively at the nuclear periphery remain unresolved.

Given the functional implications of yeast Nups in gene regulation, the potential involvement of Nups in gene expression of multicellular organisms was tested in the model organism *Drosophila melanogaster* [44]. Immunofluorescence experiments using a panel of Nup antibodies on polytene chromosome spreads demonstrated that different Nups bind to distinct regions of the *Drosophila* genome in salivary gland cells [44]. For example, the Nup107-160 subcomplex component Sec13 and the nucleoplasmic pore component Nup98 are targeted to sites of active transcription overlapping the staining of phosphorylated RNA

polymerase II. By contrast, the cytoplasmic filament component Nup88 is recruited to non-expressing sites. By immunofluorescence analysis on intact salivary gland nuclei using Nup antibodies in combination with anti-lamin antibody to mark the nuclear periphery, it was demonstrated that many of the chromatin-bound Nup loci did not co-localize with the nuclear envelope, suggesting that these sites are occupied by an intranuclear pool of Nups [44]. Furthermore, RNAi-mediated knockdown of Nups including Sec13 and Nup98 in the glands resulted in decreased expression of gene loci bound by the Nups, suggesting functional roles of Sec13 and Nup98 in gene activation [44].

Based on the observed presence of Sec13 and Nup98 on polytene chromosomes in the salivary gland cells from immunofluorescence experiments, we decided to employ an independent approach, ChIP-chip (chromatin immunoprecipitation coupled with microarray), to examine the interaction between Nup98 and the *Drosophila* genome on a genome-wide level at higher resolution in a diploid cell type. We observed that using this approach, Nup98 is also associated with active genes in *Drosophila* embryonic culture cells. In addition, many of the Nup98-associated sites do not interact with lamin, suggesting binding away from the nuclear envelope.

2.2 Interaction between Nups and Drosophila Genome in Embryonic Culture Cells

ChIP (chromatin immunoprecipitation) was employed as an independent approach to test the interaction between Nup98 and the *Drosophila* genome in the diploid, embryonic culture cell S2 cell line. To examine the consistency between different approaches (polytene immunofluorescece vs. ChIP), target genes that bound Nup98 and Sec13 on polytene chromosomes were selected. Three loci identified in this manner, loci CG6014 at 78D on polytene chromosomes, CG13800 at 62E, and Hph at 82EF, were successfully immunoprecipitated by both Nup98 and Sec13 antisera from S2 cells (Figure 2-1, Figure 2-2). By contrast, control neighboring genes CG32440, CG12163, and CG15877 were not enriched by Nup98 and Sec13 antisera by ChIP (Figure 2-1, Figure 2-2).

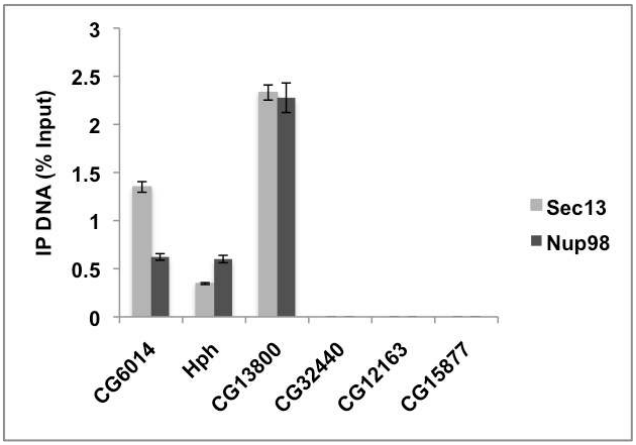


Figure 2-1 ChIP analysis of target and control genes of Sec13 and Nup98 in S2 cells. Immunoprecipitated DNA by Nup antiseraas percentage of input DNA was shown. Error bars show standard error from triplicates.

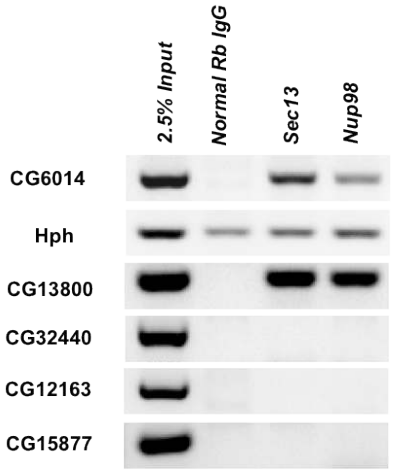


Figure 2-2 Example of ChIP analysis of target and control genes of Sec13 and Nup98 in S2 cells

Furthermore, the same three target genes were selectively immunoprecipitated using a Myc antibody from cells transfected with a Nup98-Myc fusion construct, but not from mock-transfected cells, which validates the Nup98 antisera (Figure 2-3).

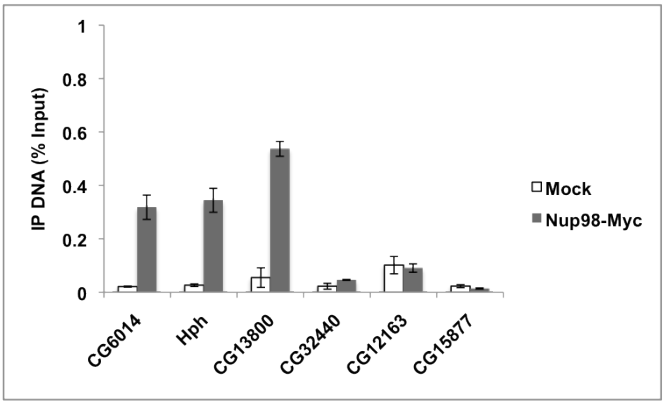


Figure 2-3 ChIP analysis by Myc antibody of target and control genes of Nup98 in transfected S2 cells. Immunoprecipitated DNA by Myc antibody as percentage of input DNA was shown. Error bars show standard error from triplicates.

In order to analyze the interaction between Nup98 and the *Drosophila* genome on a genome-wide level in the diploid S2 cells, ChIP-chip (chromatin immunoprecipitation-microarray) experiment was performed using the validated Nup98 antisera and a normal rabbit IgG as negative control. Briefly, cells were crosslinked with formaldehyde to capture the association between chromatin and interacting proteins. Antibodies against Nup98 and the control normal rabbit IgG were used to immunoprecipitate Nup98-associated chromatin or background signals. DNA was purified from immunoprecipitants and hybridized to *Drosophila* whole-genome array.

Analysis of the promoter-associated binding sites identified 841 genes in the fly genome that were specifically immunoprecipitated by the Nup98 antiserum. To further determine how chromatin contact sites of Nups correlate with the nuclear periphery, Nup98 target loci were compared to the previously characterized genome-wide binding sites of Lamin [75]. Consistent with the observations in polytene nuclei, Nup98-binding sites were found preferentially (~98%) outside of reported Lamin sites (Figure 2-4). This suggests that Nup98 can interact with genes inside the nucleoplasm in *Drosophila* diploid cells.

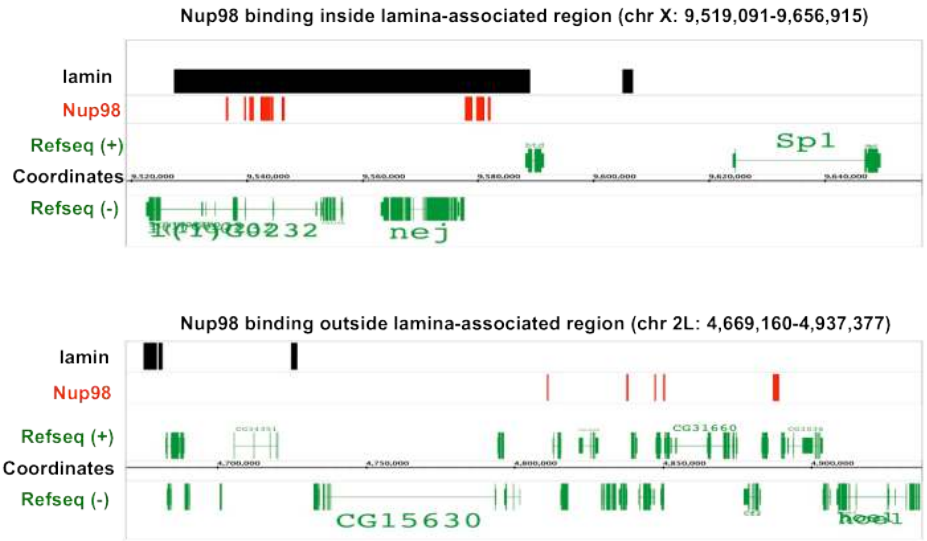


Figure 2-4 Example of Nup98 target sites plotted relative to Lamin-binding sites and annotated genes.

2.3 Correlation between Nup Binding and Gene Expression

To determine whether Nups may be associated with active genes in S2 cells, Nup98-bound genes were compared to genome-wide expression, obtained by hybridizing cellular mRNA to Affymetrix *Drosophila* 2.0 gene arrays. Nup98 target genes exhibited high expression levels when compared to nontarget genes (Figure 2-5), demonstrating that Nup98 is preferentially targeted to active genes.

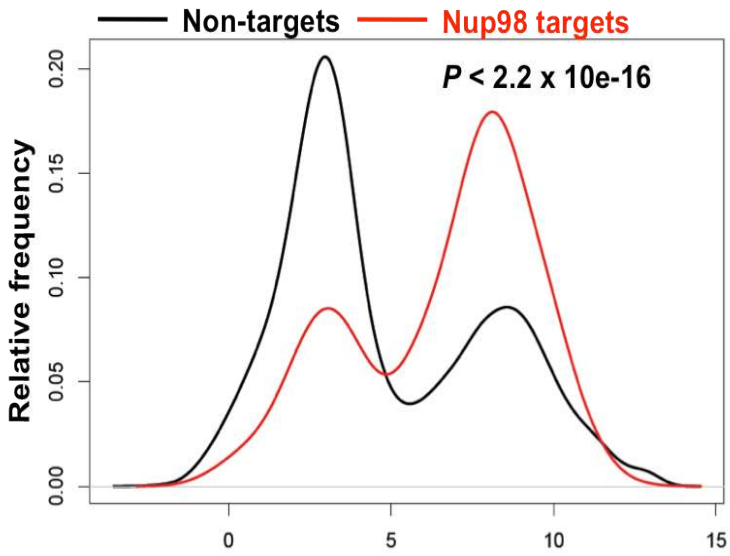


Figure 2-5 Distribution of expression levels of Nup98 target and nontargets. P-value was obtained by Mann-Whiteney U test.

In addition, we knocked down Nup98 by dsRNA in S2 cells and analyzed mRNA levels in knockdown and control cells by expression microarray. Cross analysis of Nup98 ChIP-chip and microarray datasets revealed a small but significant reduction in expression of Nup98 target genes compared to nontarget genes upon Nup98 knockdown (Figure 2-6). This suggests that Nup98 is required for normal expression of its target genes in S2 cells.

Nups have been reported to have a role in dosage compensation in *Drosophila* [64]. However, in our system, Nup98 ChIP targets or genes whose expression levels were downregulated by Nup98 knockdown do not show preferential linkage to the X chromosome, compared to autosomes (Figure 2-7).

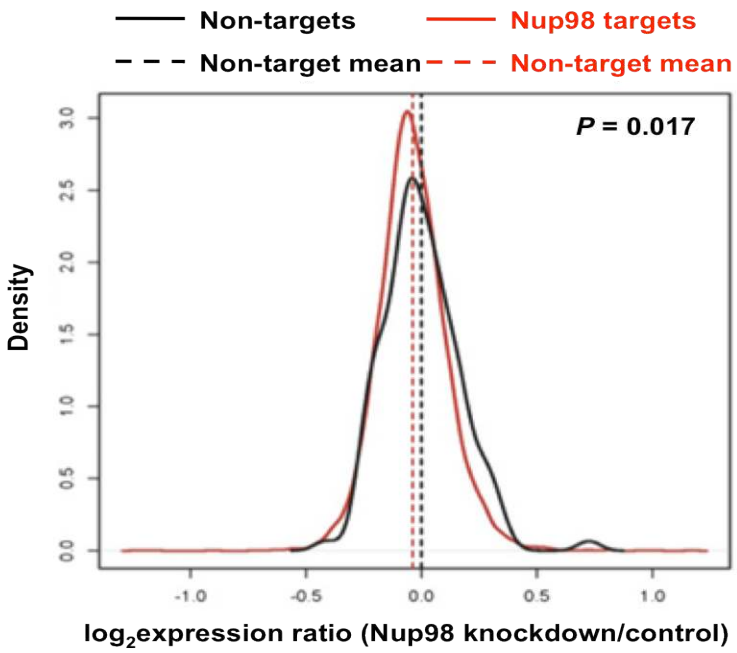


Figure 2-6 Expression changes of Nup98 targets and nontargets upon Nup98 knockdown. For any given gene, expression ratio was calculated as the ratio of transcript level in Nup98 knockdown over that in the control expression microarrays. Dash lines show the mean values of expression ratios of Nup98 targets (red) and nontargets (black), respectively. P-value was obtained by Mann-Whiteney U test.

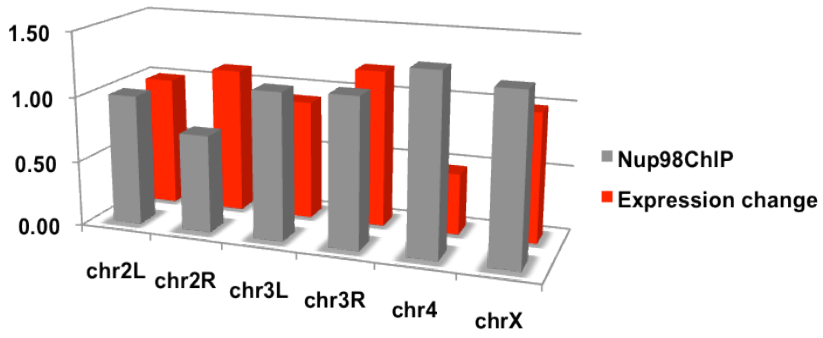


Figure 2-7 Absence of preferential linkage between Nup98 targets and the X chromosome. For each chromosome, the ratio of the number of Nup98 ChIP targets out of the total number of Refseq genes was normalized to chromosome 2L (Nup98 ChIP in grey); the percentage of the number of genes repressed by Nup98 RNAi out of the total number of Refseq genes was normalized to chromosome 2L (Expression change in red).

2.4 Discussion

We provide evidence for a role of chromatin-associated Nup98 in gene expression of a multicellular organism. In *Drosophila* embryonic culture cells, Nup98 associates with 841 genes on promoters. In addition, Nup98 preferentially binds actively expressed genes. RNAi knockdown of Nup98 affected expression levels of Nup98-associated genes to a slight but statistically significant level. The lack of dramatic changes upon Nup98 RNAi could be due to incomplete knockdown of Nup98. Another possibility is that there exist additional factors that maintain the expression of Nup98 target genes.

Combining observations in culture cells and in salivary glands, the role of *Drosophila* Nups in gene regulation is carried out to a great extent by an intranuclear, nuclear envelope-independent pool of Nups. For example, in the S2 culture cells, ~98.5% of Nup98 binding sites do not interact to significant extent with Lamin, a marker of the nuclear periphery. This suggests that in metazoa there is no strict requirement of genes to move to peripheral pores because Nups have the capacity to interact with genomic sites independently of the nuclear envelope. It remains to be determined whether these intranuclear chromatin-binding Nups shuttle between genomic sites and the nuclear periphery, which would be consistent with the dynamic behavior of some pore components

[52]. The former possibility is supported by previously reported observations that the dynamic shuttling of mammalian Nup98 and Nup153 is dependent on active transcription by RNA polymerase I and II [57,58]. It is tempting to speculate that the mobility of Nups may establish a mechanism of communication between sites of production of mRNA and sites of its final exit. This is consistent with the observation that Nup98 is primarily targeted to actively transcribed sites in S2 cells.

Intriguingly, Nup98 has been implicated in tumorigenesis [8]. Genomic fusions of Nup98 to transcription regulators have been shown to be the underlying mutations behind multiple types of leukemia [37]. The uncovered association of Nup98 with chromatin opens new directions for understanding the roles of Nup98 in cancer.

2.5 Materials and Methods

Nup Antibodies

Rabbit polyclonal antibodies were raised against and affinity purified with recombinant GST-fusion of *Drosophila* Nup98 amino acids 770-939. Rabbit polyclonal antibodies were also raised against purified recombinant protein of 6x histidine-tagged full-length human Sec13 and affinity purified using GST full-length human Sec13 protein coupled to sepharose beads.

S2 Cell Culture

S2 cells were maintained in Schneider's media (Invitrogen) with 10% FBS (GIBCO).

Chromatin Immunoprecipitation

S2 cultures were cross-linked with 1% formaldehyde solution for 10min, lysed in RIPA buffer and sonicated to generate DNA fragments <1kb. Chromatin was pre-cleared and immunoprecipitated using Nup98 antibody at 1:50 dilution and Sec13 antibody at 1:200 dilution. DNA was eluted, purified and subjected to PCR.

ChIP-chip

For ChIP-on-chip analysis, the Nup98 antibody- or normal rabbit IgG-immunoprecipitated DNA in duplicates was labeled and co-hybridized with input DNA to 2.1M Drosophila whole genome arrays by NimbleGen Systems, Inc. (http://www.nimblegen.com). ChIP peak data were generated by NimbleGen, and genes with peaks of scaled $\log_2$-ratio ≥ 1 and FDR score ≤ 0.05 were selected as Nup98 targets. Chromosome views were generated using Affymetrix Integrated Genome Browser. Lamin peaks are as previously reported by Pickersgill et al.[75] Analyses of Nup98 binding and expression correlation were performed using the R package for statistical computing (http://www.r-project.org).

Expression Analysis

Genes that show Nup98 ChIP peaks but not normal rabbit IgG ChIP peaks in promoter regions were selected from expression microarray datasets as Nup98 targets. Other genes in the expression microarray datasets were assigned as non-targets. For comparison of expression level between Nup98 targets and non-targets, density plots for the expression levels of these two groups of genes, obtained from the control expression microarrays, were generated using the R package for statistical computing (http://www.r-project.org). For comparison of expression level change upon Nup98 RNAi, expression level ratio between Nup98 RNAi and control microarrays was calculated for each gene and density plots for the expression level ratios of Nup98 targets and non-targets were generated using R. Mean expression level or expression level ratio values as well as P value from Mann-Whitney U test, were also obtained using R functions.

Chromosome Views of Nup98- and Lamin-Chromatin Association

Chromosome views were generated using Affymetrix Integrated Genome Browser. Nup98 ChIP peaks were from the whole-genome Nup98 ChIP-on-chip peak reports. Lamin peaks are as previously reported by Pickersgill et al. [75]

Chapter 2, in part, is a reprint of the material as it appears in Cell 2010. Capelson, Maya; Liang, Yun; Schulte, Roberta; Mair, William; Wagner, Ulrich; Hetzer, Martin W. The dissertation author was the primary investigator/author of this paper.

Chapter 3 Interaction between Nup98 and Human Genome

3.1 Introduction

Following the discussion in Chapter 1 and Chapter 2, several reports have demonstrated that Nups bind active regions of the genome in *S. cerevisiae* and more recently in *Drosophila melanogaster* [43-49,51,59,71,72] (also see Chapter 2). This is consistent with the 'gene gating hypothesis' that proposes nuclear pore complexes may selectively interact with active genes in the genome [42]. In yeast, all Nup-genome interactions identified so far are thought to occur at nuclear pore complexes at the nuclear periphery (i.e. 'on-pore' interaction). Interestingly, evidence of intranuclear Nups that bind specific regions of the genome has been found in *Drosophila* suggesting that Nups can also bind chromatin away from the nuclear pores (i.e. 'off-pore' interaction) [44,48,51] (also see Chapter 2). In *Drosophila* embryonic culture cells, Nups predominantly interacted with active genes inside the nucleoplasm, whereas the nuclear pore complexes at the nuclear periphery was associated with repressed genes [51]. The intranuclear gene regulation function of Nups could be related to their reported dynamic behavior of shuttling on and off the nuclear pore complexes [52].

Limited studies have been carried out to address whether Nups play an

important role in transcription in the mammalian genome. In neonatal rat ventricular cardiomyocytes, NUP155 was found to interact with the histone deacetylase HDAC4 and nuclear pore components associate with a number of HDAC4-target genes [76]. The only study that addressed the potential role of Nups in gene regulation in human cells has shown that nuclear pore complexes preferentially associate with repressive chromatin domains [61]. Combined with studies from fungi and flies, it appears that Nups can interact with both active and silent loci, depending on the cell type or the type of Nups investigated. Therefore, it is tempting to speculate that Nups may dynamically associate with the genome according to developmental stages during differentiation. Accumulating evidence suggests that the organization of the genome is highly dynamic during development [77-79]. For example, on a global level, the hyperdynamic and open chromatin organization has been correlated to the differentiation potential of pluripotent cells, and this property is lost upon differentiation. Moreover, on the single-gene level, repositioning of developmental genes and tissue-specific promoters relative to the nuclear periphery during differentiation has been well documented [80-86]. The potential involvement of Nups in chromatin-related aspects of developmental regulation is further supported by the findings that mutations in multiple Nups caused specific developmental defects rather than a global deficiency that would have been predicted if the sole role of Nups was to

mediate transport in all cell types [8].

Several studies suggest that Nups are involved in developmental gene regulation in lower organisms. In yeast, the mating pheromone alpha factor induces alterations in the association between Nups and specific genomic regions [59]. In *Drosophila* salivary glands, a subset of Nups including the mobile NUP98 can dissociate from nuclear pores and activate a number of ecdysone-induced genes in the nuclear interior (i.e. ‘off-pore’ Nup-gene interaction). These findings raise several key questions regarding the chromatin-related function of Nups during development. For instance, are Nups involved in establishing gene expression programs in diploid cells of mammalian organisms, especially human, during differentiation of pluripotent cells and establishment of cell fate? Do Nups relocate to developmentally induced genes on a genome-wide level in human cells? What are the differences between ‘on-pore’ and ‘off-pore’ Nup-gene interactions in the context of development, and do nuclear pores at the nuclear periphery have a role in developmental gene regulation?

We decided to determine if NUP98, a nuclear pore complex component that is located on both the cytoplasmic and the nucleoplasmic faces of the nuclear pore complex and has the capacity to move on and off the nuclear pore [25,52], interacts with the human genome. Using chromatin immunoprecipiation sequencing (ChIP-seq) we show that NUP98 associates with developmentally

regulated genes in stem cells and progenitor cells. In neural progenitor cells, overexpression of full-length NUP98 increases expression levels of a subset of its binding targets, and overexpression of a dominant negative fragment of NUP98 decreases mRNA levels of NUP98-associated genes. In addition, we found that developmental NUP98-gene interactions occur both on nuclear pore complexes and in the nuclear interior. The ‘on-pore’ interactions seem to be enriched for genes involved in the initial stage of developmental induction, whereas the ‘off-pore’ (i.e. intranuclear) targets are comprised of genes mediating later stages of developmental induction. We concluded that during human stem cell differentiation, NUP98 associated with specific regions of the genome in a manner that was tightly coupled to the developmental stage. In addition, the nuclear pores appeared to function as a transient platform that supported the initial induction of developmental genes.

3.2 Interaction between Nup98 and Human Genome during Cell Differentiation

To test whether NUP98 can bind to the mammalian genome during cell differentiation, we performed ChIP-Seq experiments on cultured human embryonic stem cells (ESCs), neural progenitor cells (NeuPCs) that were differentiated in vitro from ESCs, and neurons that were differentiated in vitro from

NeuPCs. We also determined the presence of chromatin-bound NUP98 in lung fibroblast IMR90 cells as an example of another differentiated cell type. As expected, in all four cell types, NUP98 was found both on nuclear pores at the nuclear periphery and intranuclear sites, consistent with its reported capacity to move on and off the nuclear pores (Figure 3-1) [25,52]. We first validated the ChIP-Seq method using IMR90 cells with two independent antibodies against human NUP98. As expected, both antibodies stained nuclear pores and a few intranuclear sites in IMR90 cells (Figure 3-2). Additionally, both proved efficient and specific in western blot and immunoprecipitation experiments (Figure 3-3, Figure 3-4). Since Nups were not expected to bind directly to DNA, we employed two cross-linking conditions for the ChIP-Seq experiment, formaldehyde single cross-linking and formaldehyde-disuccinimidyl glutarate double-crosslinking in order to more efficiently crosslink indirect Nup-chromatin contacts. After crosslinking, we immunoprecipitated NUP98 using the two antibodies, purified DNA that was immunoprecipitated, and had DNA amplified and subjected to deep sequencing. Sequencing reads were then mapped to the human genome (Figure 3-5). The results from the four ChIP-Seq experiments, using two antibodies and two cross-linking conditions, were highly consistent (Figure 3-6, Figure 3-7, Figure 3-8), with 73% NUP98-binding regions from pull-down using the first antibody overlapping with 78% NUP98-binding regions using the second antibody. We

further validated our results by randomly selecting several NUP98-binding regions called from the ChIP-Seq experiment and confirming the interaction between NUP98 and these regions by ChIP-qPCR (Figure 3-9).

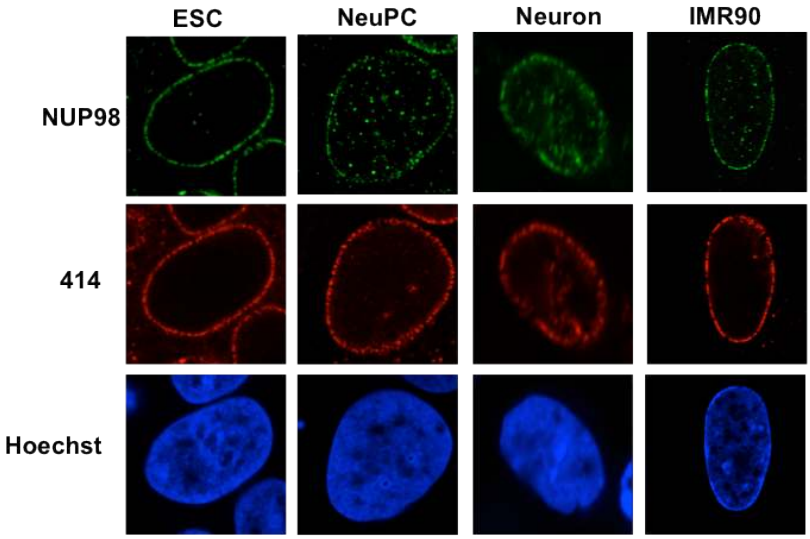


Figure 3-1 NUP98 immunostaining in various human cell lines. Human embryonic stem cells (ESC), neural progenitor cells (NeuPC), neurons, and IMR90 cells were stained with anti-human NUP98 antibodies (green), mAb414 (red), and Hoechst (blue).

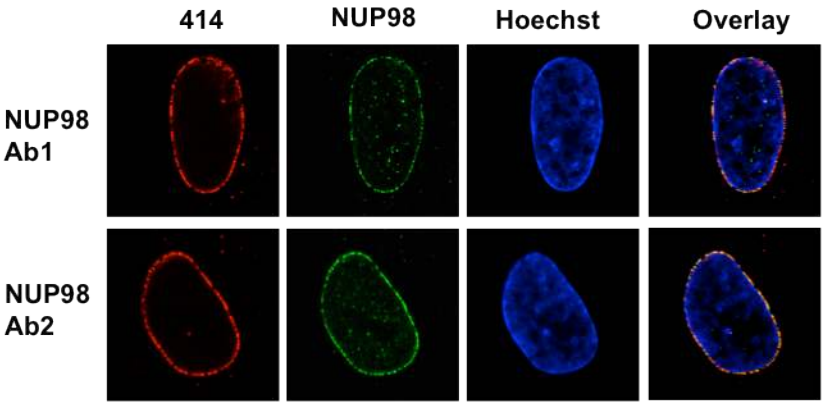


Figure 3-2 NUP98 immunostaining using two antibodies in IMR90 cells. IMR90 cells were stained with two independent anti-human NUP98 antibodies NUP98 Ab1 and NUP98 Ab2 (green), mAb414 (red), and Hoechst (blue).

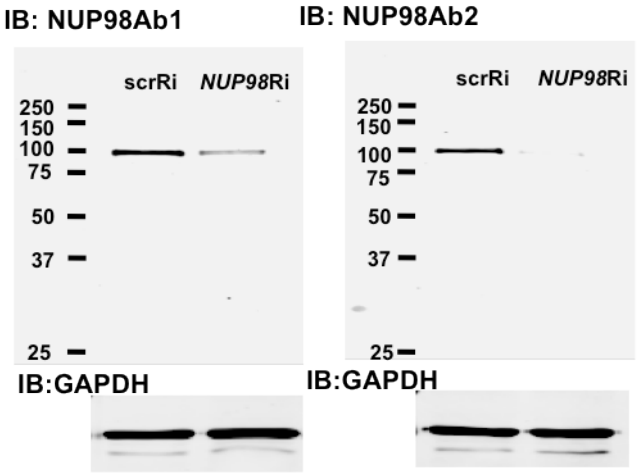


Figure 3-3 Validation of NUP98 antibodies by western blot. IMR90 cells with scrambled RNAi (scrRi) or *NUP98* RNAi (*NUP98*Ri) were lysed according to the ChIP-Seq protocol and proteins in the lysate were transferred to membrane and blotted with two NUP98 antibodies used for ChIP-Seq (NUP98Ab1 and NUP98Ab2). GAPDH was used as loading control.

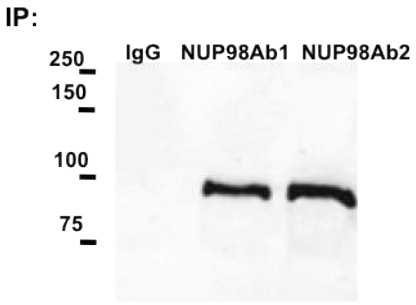


Figure 3-4 Validation of NUP98 antibodies by immunoprecipitation. Immunoprecipitation was performed according to the ChIP-Seq protocol using normal rabbit IgG (IgG) or two NUP98 antibodies (NUP98Ab1, NUP98Ab2) and immunoblotted with NUP98 antibody.

Experiment	Number of Reads	Number of Mappable Reads
ESC-NUP98 ChIP-Seq	26,065,791	24,281,339
ESC-IgG ChIP-Seq	22,964,085	17,087,363
NeuPC-NUP98 ChIP-Seq	27,184,945	24,941,780
NeuPC-IgG ChIP-Seq	25,245,472	18,477,127
Neuron-NUP98 ChIP-Seq	19,357,254	18,418,463
IMR90-NUP98Ab1 ChIP-Seq	28,899,517	27,897,841
IMR90-NUP98Ab2 ChIP-Seq	28,777,670	27,589,069
IMR90-IgG ChIP-Seq	29,217,673	28,150,220

Figure 3-5 Number of reads from ChIP-Seq experiments. Number of total reads and mappable reads from each ChIP-Seq experiment was shown.

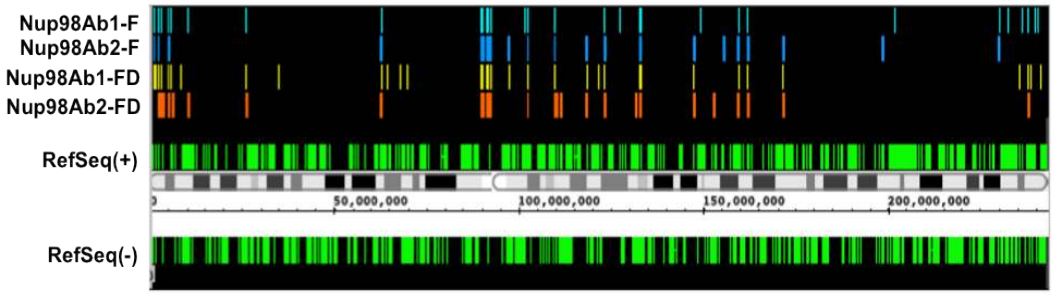


Figure 3-6 Example of chromosomal view of NUP98 binding regions. Chromosomal view of NUP98 binding regions on chromosome 1 from four independent ChIP-Seq experiments using two NUP98 antibodies under formaldehyde crosslinking condition(NUP98Ab1-F, NUP98Ab2-F) and under formaldehyde-disuccinimidyl glutarate double crosslinking condition (NUP98Ab1-FD, NUP98Ab2-FD) was shown.

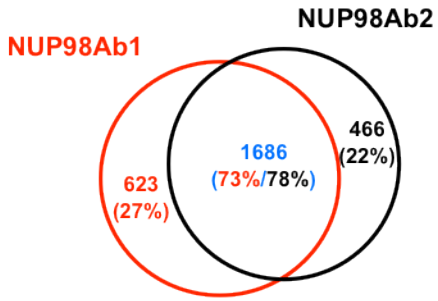


Figure 3-7 Overlap between NUP98 binding regions from ChIP-Seq experiments using two NUP98 antibodies.

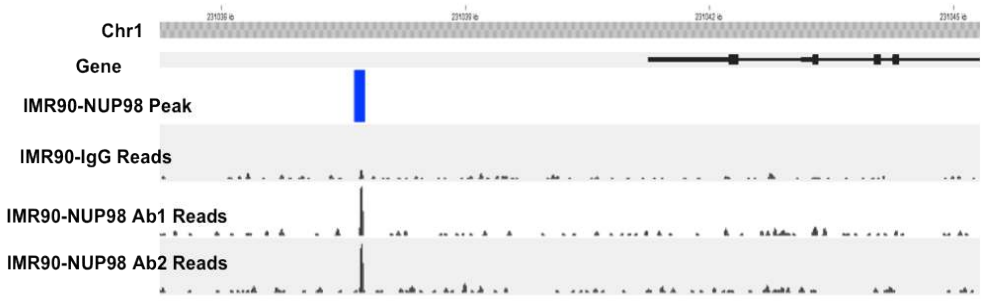


Figure 3-8 Example of peak calling using the Genomatix software. Reads from two NUP98 antibody ChIP-Seq experiments (NUP98Ab1 and NUP98Ab2) and normal rabbit IgG ChIP-Seq experiment (IgG) were shown. Region called as peak by the Genomatix software was indicated by the block in blue (NUP98 Peak).

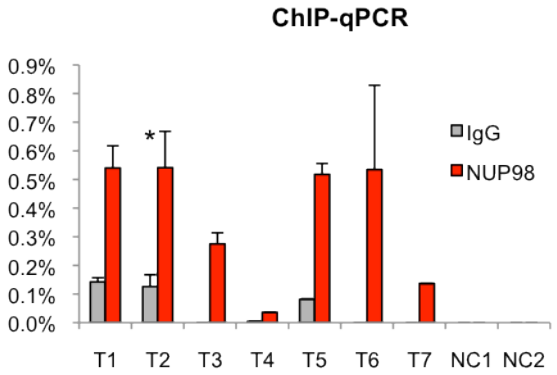


Figure 3-9 ChIP-qPCR validation of ChIP-Seq peaks. Randomly selected seven ChIP-Seq peaks (T1 from T7) called by Genomatix and two non-NUP98 binding regions (NC1 and NC2) were tested for NUP98 binding by target ChIP-qPCR using independent batch of IMR90 cells and independent lot of NUP98 antibody. Error bars were computed as standard deviation from triplicates. P value was obtained from Student's t-test and comparisons with P value <0.05 indicated with asterisks.

After validation of the ChIP-Seq method, we extended the ChIP-Seq analysis to human embryonic stem cells, human embryonic stem cell-derived NeuPCs that were ~90% positive for the neural progenitor cell marker Nestin

(Figure 3-10), and postmitotic neurons. Interestingly, the genome-binding pattern of NUP98 varied greatly depending on the developmental stage of the cells. NUP98 occupied more genomic regions in ESCs and NeuPCs than in differentiated cells. Further analysis revealed that 71% of NUP98-chromatin sites in ESCs and 74% in NeuPCs were specific for the respective cell-type (i.e. not found in the other cell types) (Figure 3-11, Figure 3-12). The most dramatic difference was found in neurons where essentially no significant enrichment for NUP98 binding could be identified (Figure 3-13 and data not shown). Together, these findings suggest that Nup98's ability to interact with the human genome is developmentally regulated.

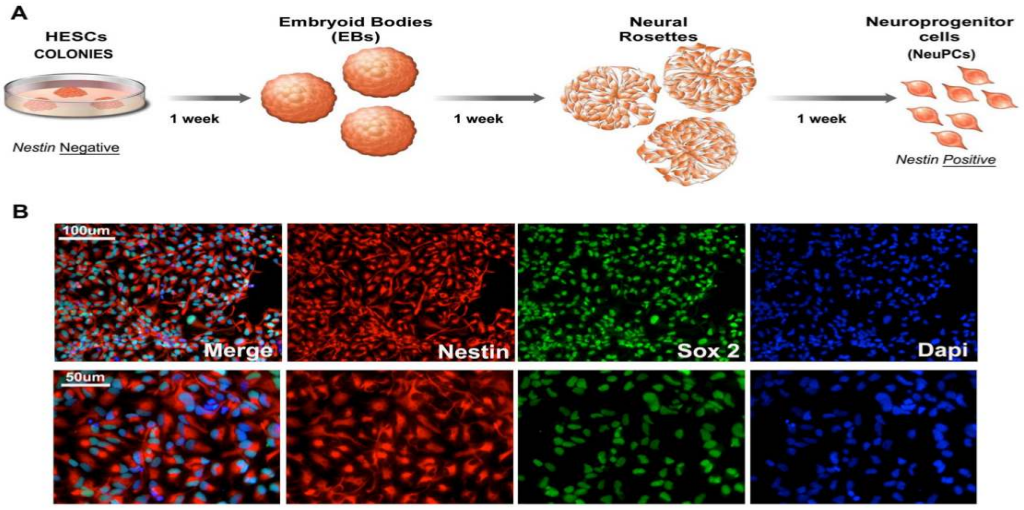


Figure 3-10 Differentiation of human embryonic stem cells into neural progenitor cells. (A) Scheme showing differentiation of human embryonic stem cells (HESCs) into Embryoid Bodies (EBs), neural rosettes and neural progenitor cells (NeuPCs). The neural progenitor cell cultures are grown as monolayers after neural rosette dissociation. (B) Markers for homogeneous NPC population (Nestin and Sox2) at lower (upper panel) and higher (lower panel) magnification.

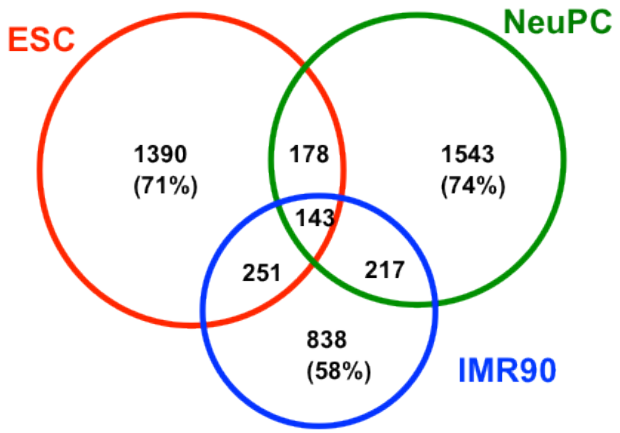


Figure 3-11 Overlap of NUP98 binding regions in different cell types. Venn diagram of the overlap between NUP98-binding regions in human embryonic stem cells (ESC), neural progenitor cells (NeuPC), and lung fibroblast cells (IMR90) was shown.

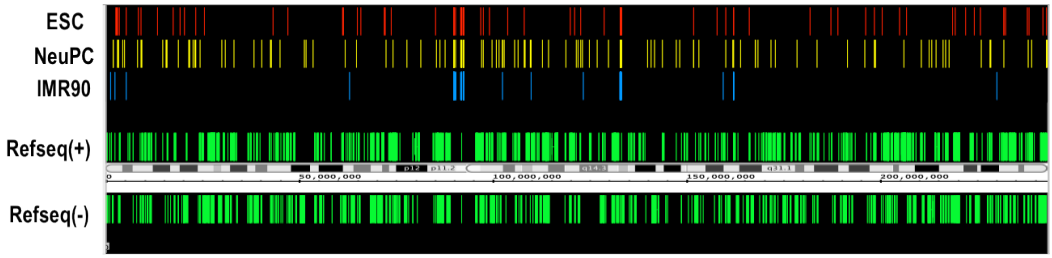


Figure 3-12 Chromosomal view of NUP98 binding regions in different cell types. Chromosomal view of NUP98 binding regions on chromosome 1 in ESCs, NeuPCs, and IMR90 cells was shown. Refseq(+) indicates Refseq genes on the (+) strand, and Refseq(-) indicates Refseq genes on the (-) strand.

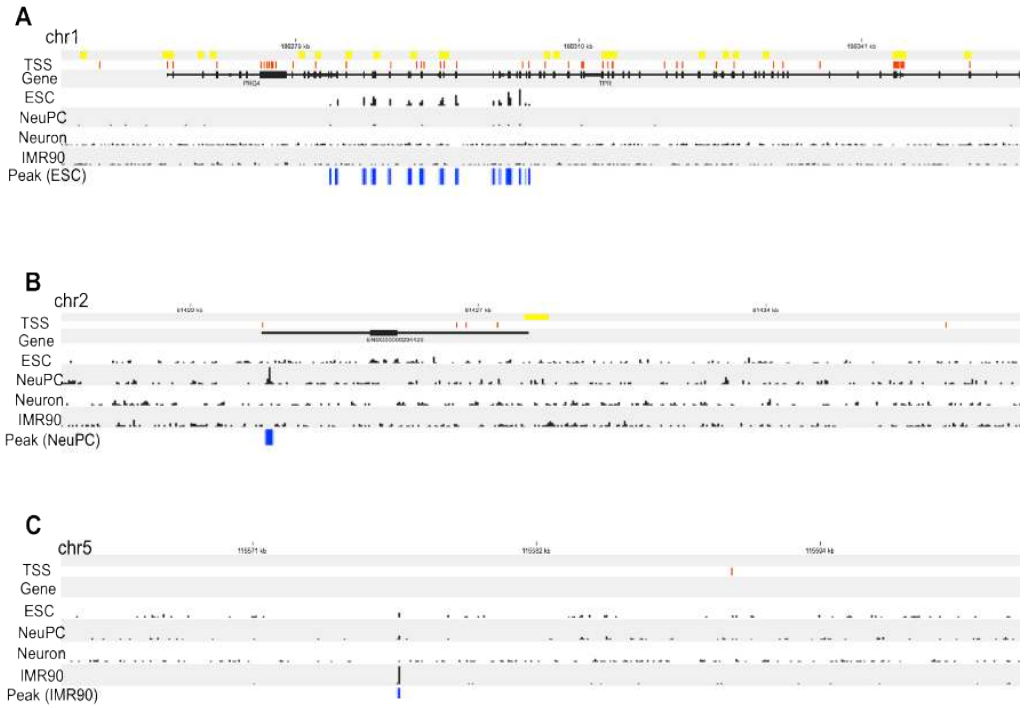


Figure 3-13 Examples of cell type specific NUP98-binding regions. Reads from NUP98 ChIP-Seq experiments were shown for embryonic stem cells (ESC), neural progenitor cells (NeuPC), neurons (Neuron), and IMR90 cells (IMR90). Peak assigned were indicated in blue. Transcriptional start sites as from the Genomatix database were shown in red. Peaks found in ESCs, NeuPCs and IMR90 cells were shown in (A), (B), and (C), respectively.

We further analyzed whether NUP98-DNA interaction occurred on gene regulatory elements and/or coding regions in ESCs and NeuPCs by assigning NUP98 binding regions to promoters, exons, introns, and intergenic regions. In both ESCs and NeuPCs, NUP98 bound to 500-600 genes (Figure 3-14) and exhibited a significant enrichment in promoter regions (Figure 3-15). It is important to note that the few NUP98 binding sites in IMR90 cells were preferentially found

in intergenic regions (Figure 3-16), providing additional evidence for a dynamic and developmentally-controlled association of NUP98 with the human genome. Although we cannot rule out that NUP98 binding in IMR90 has functional significance, we decided to focus our analysis on NUP98-bound genes in ESCs and NeuPCs.

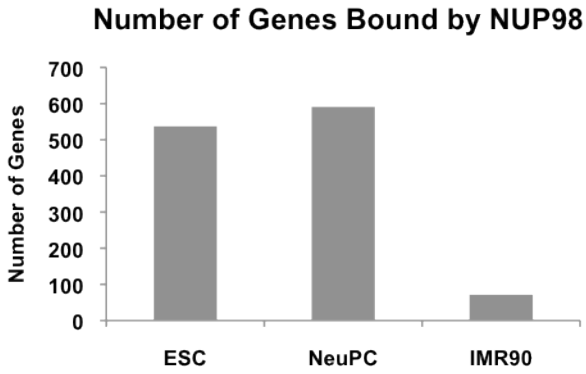


Figure 3-14 Number of genes bound by NUP98 in ESCs, NeuPCs, and IMR90 cells.

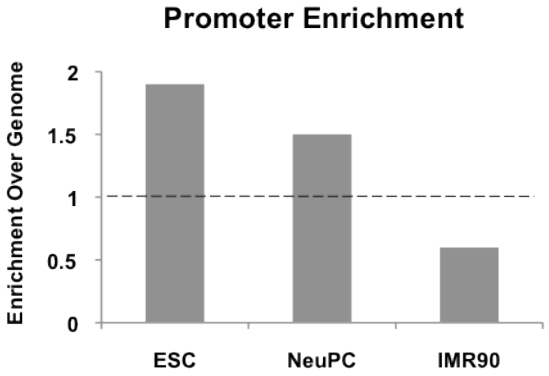


Figure 3-15 Promoter enrichment of NUP98 binding regions in ESCs, NeuPCs, and IMR90 cells. The percentage of NUP98 binding regions that overlap with promoters was normalized against the percentage of promoters in human genome.

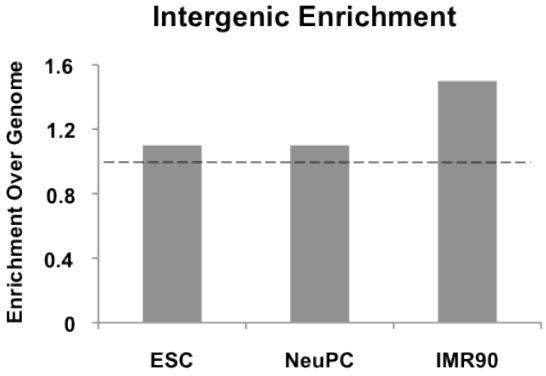


Figure 3-16 Intergenic enrichment of NUP98 binding regions in ESCs, NeuPCs, and IMR90 cells. The percentage of NUP98 binding regions that overlap with intergenic regions was normalized against the percentage of intergenic regions in human genome.

In order to identify potential DNA sequence motifs and/or potential NUP98-interacting transcription factors that direct NUP98-DNA binding, we analyzed the transcription factor motifs overrepresented in NUP98-binding sequences found in ESCs and NeuPCs. We found that GA-boxes were an evolutionarily conserved NUP98-associated motif. This motif was not only overrepresented in human NUP98-binding genomic regions, but also in published *Drosophila* NUP98 binding sequences (Figure 3-17, Figure 3-18)[44,51]. In *Drosophila*, GA-boxes are recognized by GAGA factor, which is a transcriptional activator that is crucial for the proper expression of several homeotic genes[87]. This suggests that the interaction between NUP98 and GA-box motifs, potentially related to the regulation of developmental genes, is evolutionary conserved and further validates our ChIP-Seq results.

Cell Type	TF family	Z-score
Drosophila embryonic culture cells (Dam-ID and ChIP-chip)	GA-boxes	30.7
Human embryonic stem cells	GA-boxes	57.24
Human neural progenitor cells	GA-boxes	79.11

Figure 3-17 Conserved Nup98-interacting motif. GA-boxes is an over-represented transcription factor motif in *Drosophila* from published NUP98 Dam-ID and ChIP-chip datasets [44,51] and in human ESCs and NeuPCs. Z-score represents the distance from the population mean in units of the population standard deviation.

A

Genes:

Cell Type	TF family	Z-score
Human embryonic stem cells	GA-boxes	45.78
Human neural progenitor cells	GA-boxes	69.86

B

Promoter:

Cell Type	TF family	Z-score
Human embryonic stem cells	GA-boxes	40.32
Human neural progenitor cells	GA-boxes	78.42

Figure 3-18 Conserved Nup98-interacting motif on genes and promoters. GA-boxes were over-represented in NUP98-binding genes (A) and NUP98 binding promoters (B) in ESCs and NeuPCs.

We also identified YY1 binding site as NUP98-associated motif in ESCs and NeuPCs (Figure 3-19). Both GAGA factor and YY1 have been linked to boundary activities, in line with the potential role of Nups in the compartmentalization of chromatin into active and silent domains[8,88-90]. The binding motif of nuclear DEAF-1 related (NURD)/homolog to *Drosophila* DEAF-1 is also a NUP98-associated motif enriched in both ESCs and NeuPCs (Figure 3-19). NURD displays homology to the protein SP100, a component of the promyelocytic leukemia-associated nuclear body, implying that NUP98 might be

involved in the regulation of nuclear bodies and is consistent with the reported link of NUP98 to leukemia [91-93].

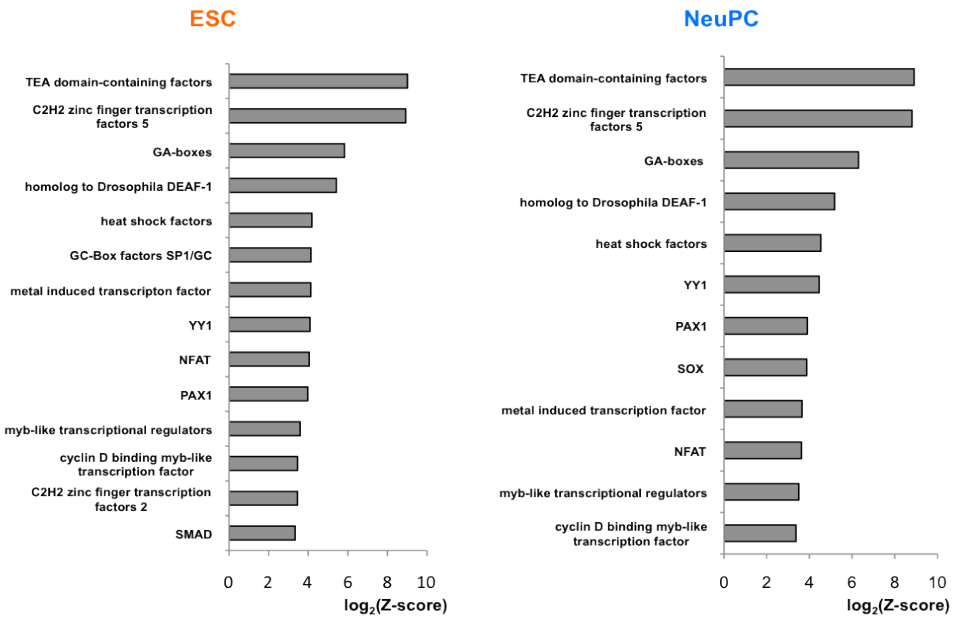


Figure 3-19 Over-represented transcription factor motifs enriched in NUP98-binding regions in ESCs and NeuPCs. Transcription factor motifs were ranked by Z-score and motifs with Z-score more than 10 were listed.

Moreover, we have found that in ESCs specifically, NUP98 binding sequences were enriched for motifs recognized by GC-Box factors SP1, C2H2 zinc finger transcription factors and SMAD (Figure 3-19). These findings raise the exciting possibility that NUP98 is linked to the core transcription circuitry that is crucial for the maintenance of pluripotency in ESCs [94,95].

To further understand the dynamic DNA-binding behavior of NUP98, we investigated the functional categories of genes bound by NUP98 in ESCs and

NeuPCs by gene ontology analysis. In ESCs, the top functional category enriched in NUP98 targets was found to be cytoskeleton organization (Figure 3-20). This is consistent with recent reports showing that in Drosophila embryonic culture cells NUP98 binding targets were also enriched for cytoskeleton genes [51]. As discussed later, NUP98 targets in ESCs could be divided into two groups, one associated with active histone marks and one associated with silent histone marks. The active group of NUP98 targets in human ESCs was enriched for genes in the functional categories of cell cycle regulation, cell communication and metabolism. Such genes were also enriched in Drosophila NUP98 targets in embryonic cells [51] (and data not shown).

Biological Processes Enriched in Nup98 Targets

ESC

GO-Term	P-value	# Genes observed
cytoskeleton organization	5.00e-04	26
calcium ion transport	8.39e-04	13
divalent metal ion transport	1.17e-03	13
metal ion transport	2.09e-03	23
protein amino acid dephosphorylation	2.17e-03	10

NeuPC

GO-Term	P-value	# Genes observed
signaling	1.90e-05	137
cell projection organization	2.11e-05	29
nervous system development	5.99e-05	54
neuron projection development	7.63e-05	22
neuron development	1.05e-04	25

Figure 3-20 Biological processes enriched in NUP98 binding genes in ESCs and NeuPCs by gene ontology analysis.

Interestingly, NUP98 targets were specifically enriched for neurogenesis

genes in NeuPCs, including genes in functional categories of nervous system development, neuron projection development, and neuron development (Figure 3-20). Examples of NUP98-interacting neurogenesis genes include NRG1, ERBB4, SOX5, and ROBO. Furthermore, analysis of disease terms enriched in NUP98 targets in NeuPCs revealed that NUP98 is linked to genes involved in multiple diseases of the nervous system (Figure 3-21). Such diseases include neurodegenerative disorders such as Alzheimer disease and tumors such as glioma and neoplasms of the nerve tissue. The latter finding might be relevant for the previously reported role of NUP98 in tumorigenesis [93]. These results suggest that NUP98 is recruited to neural developmental genes in a developmentally controlled manner.

Disease Terms Enriched in NUP98 Targets in NeuPC

MeSH-Term	P-value	# Genes observed
Chromosome aberrations	1.77e-04	187
Disease susceptibility	2.99e-04	208
Tauopathies	3.47e-04	89
Glioma	5.19e-04	121
Alzheimer disease	5.62e-04	87
Neoplasms, neuroepithelial	8.33e-04	152
Genetic predisposition to disease	1.27e-03	197
Neoplasms, nerve tissue	1.45e-03	186
Chromosome breakage	1.91e-03	40
Translocation, genetic	1.94e-03	98

Figure 3-21 Disease terms enriched in NUP98 binding genes in NeuPCs by MeSH term analysis.

3.3 Correlation between Nup98 Binding and Gene Expression

The specific association between NUP98 and neurogenesis genes in NeuPCs raised the possibility of a positive correlation between NUP98 binding and the activation of these genes during neural differentiation. To test this possibility, we compared the expression levels of genes bound by NUP98 to those of the same number of randomly selected genes in ESCs and NeuPCs using published RNA-Seq datasets [96,97] (Figure 3-22). We found that genes bound by NUP98 had higher expression levels in NeuPCs compared to randomly selected gene sets, suggesting that NUP98-binding was associated with elevated gene expression levels. As an independent test, we correlated the genomic localization of NUP98-binding regions to that of expressed mRNA in NeuPCs (Figure 3-23). We were able to detect a positive correlation between the location of NUP98 binding on the genome and the location of mRNA production, indicating the positive correlation between NUP98 binding and mRNA expression.

Having established a link between NUP98 binding and active gene expression in NeuPCs, we asked if NUP98 binding to its target genes in NeuPCs would coincide with their transcriptional induction during neural differentiation. We found that NUP98-bound loci in NeuPCs had higher expression levels than either ESCs or IMR90 cells (Figure 3-24). By contrast, for randomly selected genes,

there was no statistically significant difference in expression levels in any of the analyzed cell types. Together, these findings support the notion that NUP98 gains association with developmental genes as they are undergoing transcriptional activation during development.

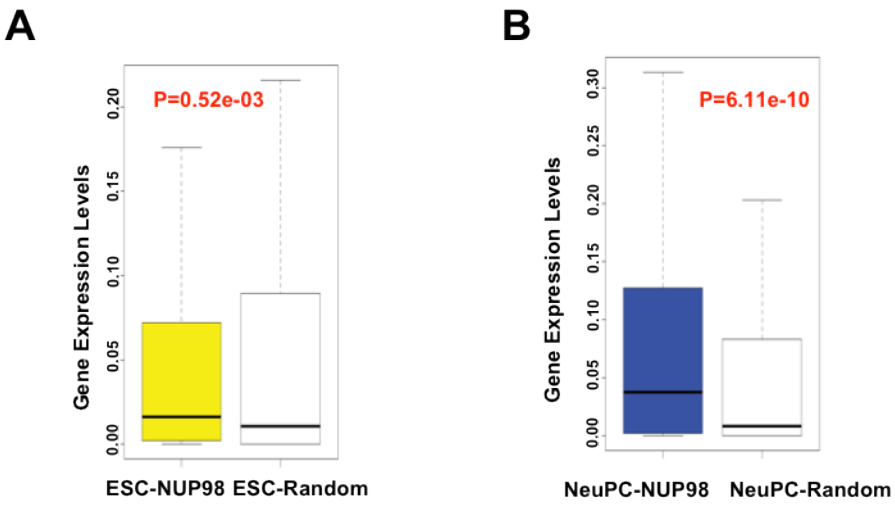


Figure 3-22 Correlation between NUP98 binding and gene expression in ESC and NeuPC. Expression levels of NUP98 binding genes (-NUP98) and same number of randomly selected genes (-Random) in embryonic stem cells (ESC-) (A) and neural progenitor cells (NeuPC-) (B) were plotted. P value was obtained by Mann-Whitney U tests. Randomization was conducted for at least 10 times and similar results were obtained (data not shown). Gene expression values were obtained from [96,97]. Top and bottom of the boxes in the plot are 25th and 75th percentile, centerline is the 50th, and whiskers extend to 1.5 interquartile range from the upper and lower quantile.

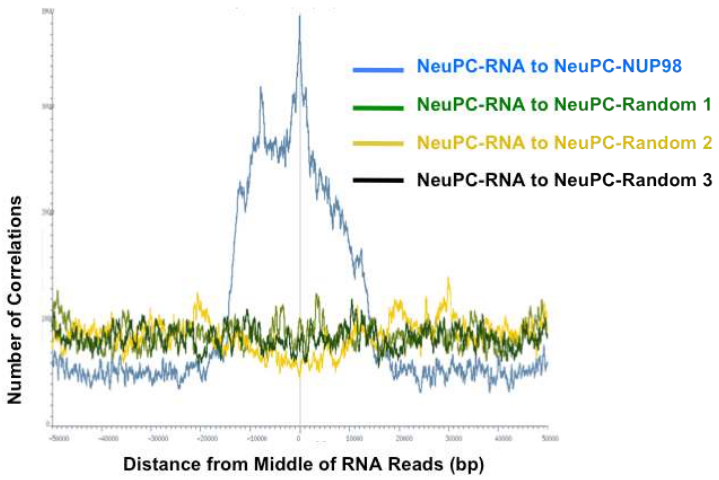


Figure 3-23 Positional correlation between NUP98 binding and gene expression in NeuPC. Positional correlation between expressed mRNA and NUP98-binding (blue) or three sets of same number- and size-matched, randomly selected regions (green, yellow, and black) in NeuPCs were plotted. mRNA expression data were from [97].

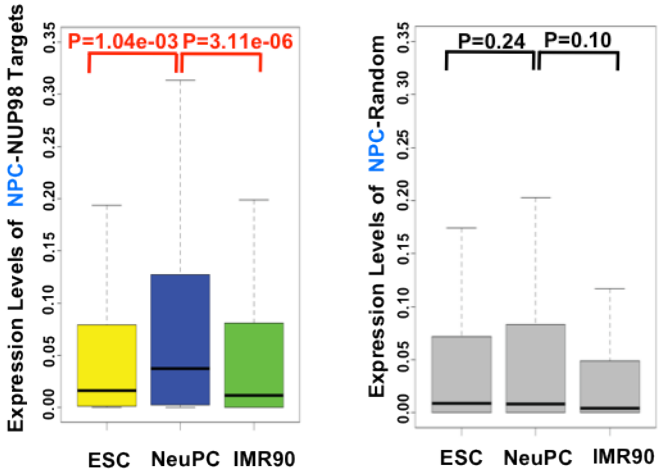


Figure 3-24 Expression level change of neural progenitor cell-NUP98 targets during development, i.e. in ESCs, NeuPCs, and IMR90 cells (left). All NUP98 binding regions detected in neural progenitor cells that overlap with genes (promoters/exons/introns) were used. Expression level change of same number of randomly selected genes during development, i.e. in ESCs, NeuPCs, and IMR90 cells, were shown as negative control (right). Randomization was conducted for at least 10 times and similar results were obtained (data not shown). P values were obtained by Mann-Whitney U tests. Gene expression values were obtained from [96,97]. Top and bottom of the boxes in the plot are 25th and 75th percentile, centerline is the 50th, and whiskers extend to 1.5 interquartile range from the upper and lower quantile.

Considering all genes in the human genome, from published RNA-Seq datasets, there are a total of 8388 genes activated during differentiation of ESCs into NeuPCs. They were defined as genes whose expression levels were not detectable in ESCs but detectable in NeuPCs or were upregulated by more than two-folds in NeuPCs compared to ESCs [96,97]. 2.7% of these genes gained NUP98 binding in NeuPCs compared to ESCs, suggesting that NUP98 is associated with specific subset of developmentally regulated genes.

In addition, we found 138 genes that lost NUP98 binding and also became inactivated in terms of expression levels upon differentiation from ESCs to NeuPCs. The expression levels of these genes were detectable in ESCs but undetectable in NeuPCs or were downregulated by more than two-folds in NeuPCs compared to ESCs from published RNA-Seq datasets [96,97]. This suggests that NUP98 might also be linked to genes active in pluripotent cells.

In contrast to the direct correlation between NUP98 binding and gene activation in NeuPCs, the scenario in ESCs appears more complicated. To gain additional insight into the type of chromatin environment that NUP98 interacts with, we compared NUP98 binding to the levels of different histone modifications by comparing our ChIP-Seq datasets to published ChIP-Seq datasets of histone modifications in ESCs [96]. Specifically, we examined H3K79me2 and H3K36me3 that are linked to active transcription, as well as H3K27me3 and H3K9me3 that

are linked to repressed chromatin domains [98]. We compared histone modification levels for NUP98-binding regions and randomly selected regions as negative controls. We found that, in ESCs, NUP98 binding showed positive correlation with both active and silent histone marks. In contrast, NUP98 binding in IMR90 cells, which does not target promoter regions, was exclusively linked to high H3K9me3 levels (Figure 3-25). This observation is consistent with the idea that NUP98 is preferentially, if not exclusively, involved in developmental gene regulation in pluri-/multi-potent cells whereas in differentiated cells either associates with repressive chromatin (e.g. IMR90 cells) or lacks chromatin association altogether (e.g. neurons).

The finding that NUP98 associates with both active and silent chromatin domains in ESCs could be due to two reasons: 1) NUP98 is directed to bivalent domains that exhibit both active and silent histone marks or 2) there are two subsets of NUP98 targets, one active and one silent. To distinguish between these two possibilities, we determined the extent to which pairs of histone marks were found at NUP98 binding regions by Pearson's Correlation analysis (Figure 3-26). Specifically, we examined the extent of correlation between 4 pairs of histone marks, H3K36me3 (active histone mark) – H3K27me3 (silent histone mark), H3K36me3 (active) –H3K9me3 (silent), H3K79me2 (active) – H3K27me3 (silent), and H3K79me2 (active) – H3K9me3 (silent). The result showed that the

correlation between active and silent histone marks for NUP98 targets was low, suggesting NUP98-binding regions can be divided into at least two distinct subgroups, the group with active histone marks and the group with silent marks. In order to examine the types of genes included in each group, for each histone mark we ranked the genes bound by NUP98 by the levels of the histone mark found at that loci, selected the top 40% of the genes and performed gene ontology analysis on those genes (Figure 3-27). We found that NUP98 targets with high levels of active histone marks (H3K79me2 or H3K36me3) were uniquely enriched for genes involved in macromolecule and nucleic acid metabolism and various aspects of the cell cycle such as nuclear division and mitosis. On the other hand, NUP98 targets, which were characterized by high levels of repressive histone mark H3K27me3, were uniquely enriched for genes involved in transmembrane transport. Furthermore, we confirmed that NUP98 targets with high levels of active histone marks were actively transcribed, whereas the ones with high levels of silent histone marks were repressed (Figure 3-28). These observations are reminiscent of the findings in Drosophila embryonic culture cells in which NUP98 associates with both active and repressed genes as well as cell cycle and nucleic acid metabolism genes ([51]; (data not shown). Combining the observations in Drosophila and human cells, it is possible that NUP98 exhibits an evolutionally conserved role in associating with and potentially regulating cell cycle and nucleic

acid metabolism genes.

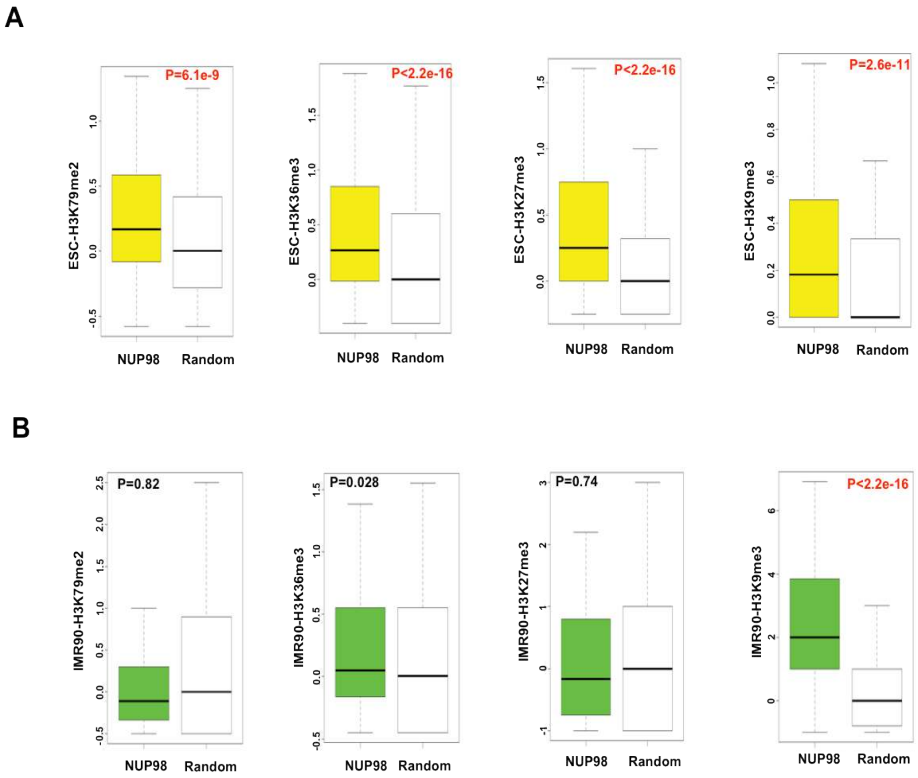


Figure 3-25 NUP98 loses association with active chromatin domains in post-differentiation IMR90 cells. Histone modification levels of NUP98 binding genes (-NUP98) and same number of randomly selected genes (-Random) in embryonic stem cells (ESC-) (A) and lung fibroblasts (IMR90-) (B) were plotted. P values were obtained by Mann-Whitney U tests. Randomization was conducted for at least 10 times and similar results were obtained (data not shown). Histone modification levels were calculated from [96], GSM605321, and GSM605309. Top and bottom of the boxes in the plot are 25th and 75th percentile, centerline is the 50th, and whiskers extend to 1.5 interquartile range from the upper and lower quantile.

Pearson's Correlation Between Pairs of Histone Marks for NUP98 Binding Regions

	H3K27me3	H3K9me3
H3K36me3	R=0.10	R=0.02
H3K79me2	R=0.22	R=0.13

Figure 3-26 Pearson's correlation between pairs of histone modifications for NUP98 binding regions in ESCs. Histone modification levels were calculated from [96], GSM605321, and GSM605309.

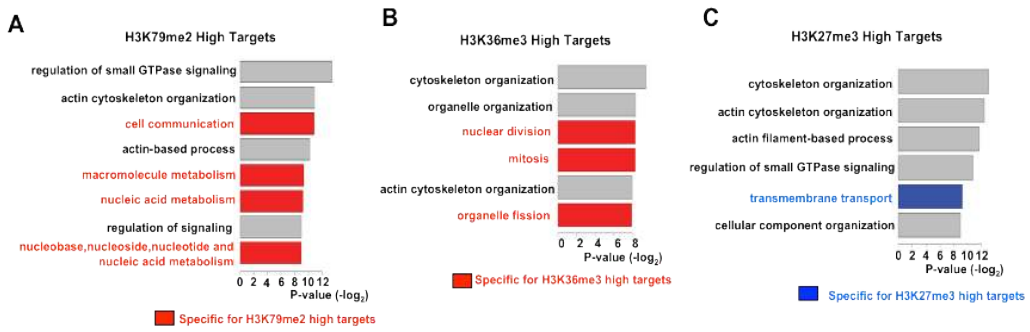


Figure 3-27 Gene ontology analysis categorized by histone modification. For each histone modification type, NUP98 binding genes were ranked by their histone modification levels and top 40% genes were selected for gene ontology analysis. Biological process categories that are uniquely enriched for specific histone modification types were shown in red for active histone marks and in blue for silent histone mark.

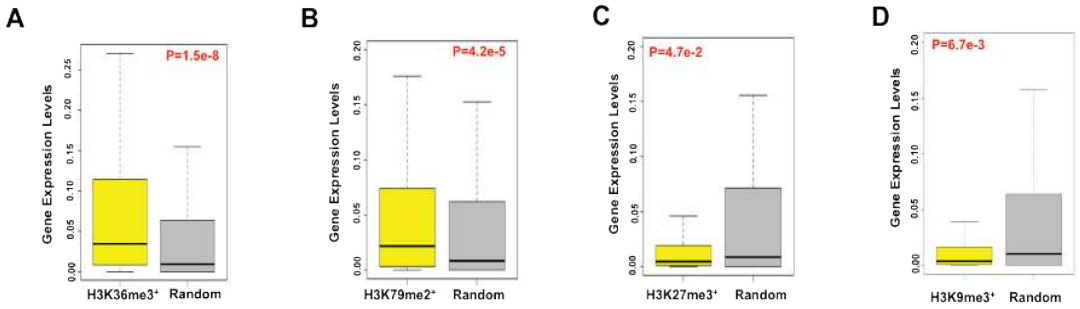


Figure 3-28 Gene expression levels for each histone modification analyzed. Expression levels of NUP98 binding genes that were high in each of the four histone modifications were compared to those of same number of randomly selected genes. P values were obtained by Mann-Whitney U tests. Top and bottom of the boxes in the plot are 25th and 75th percentile, centerline is the 50th, and whiskers extend to 1.5 interquartile range from the upper and lower quantile.

Together these data suggest that in undifferentiated ESCs, NUP98 associates with one subgroup of active genes including cell cycle and nucleic acid metabolism genes as well as with one group of silent chromatin regions.

3.4 Perturbation of Functional Nup98 Levels and Target Gene Expression

Since NUP98 associated with neural development genes during neural differentiation, we asked if this nuclear pore complex component plays a role in their expression. We randomly selected 24 genes from the 54 genes in the 'nervous system development' gene ontology category that showed specific enrichment in NeuPCs (Figure 3-20) together with GAPDH as negative control, and examined how their expression levels were affected by NUP98 overexpression in neural progenitor cells using qRT-PCR (Figure 3-29). To do this, NeuPCs were transfected with NUP98 and the overexpressed NUP98 localized to both nuclear pores and nucleoplasm (Figure 3-30, Figure 3-31). Strikingly, we found that 12 NUP98-associated neural developmental genes showed statistically significant increase in expression levels upon NUP98 overexpression, indicating that NUP98 regulates the transcription of these genes. Since not all genes responded to NUP98 overexpression, we suspect that NUP98 might not be rate-limiting in all its target genes. We then overexpressed a fragment of NUP98

(amino acid 1-504) in NeuPCs, which lacks a C-terminal domain of NUP98 that is no longer capable of binding to the nuclear pore complex (Figure 3-30, Figure 3-31). We were interested in this region of NUP98 because this is the same fragment as appeared in multiple NUP98-involved leukemic fusions and this fragment has been found to interfere with the differentiation of haematopoietic progenitor cells [93]. Given reported evidences for a role of NUP98 in gene regulation [44,51] and our observation of the association between NUP98 and developmental genes at the progenitor cell stage, we hypothesized that this NUP98 fragment might interfere with the expression of NUP98 targets required for neural differentiation. We found that overexpression of this fragment of NUP98 had a dominant negative effect on the expression of NUP98-binding neural developmental genes, and 20 of the 24 genes exhibited statistically significant decrease in expression levels (Figure 3-32). This suggests that the C-terminal domain of NUP98 is required for the expression of NUP98 target genes because the fragment lacking this domain could not stimulate expression of target genes as the full length NUP98 protein did. As an additional negative control, we overexpressed NUP35 using the same vector and found no effects on the expression of NUP98-binding genes (Figure 3-33). We did not examine the effect of NUP98 knockdown on gene expression because NUP98 is encoded on the same mRNA with a core component of the nuclear pore, NUP96, which is

essential to nuclear pore biogenesis [99]. Knockdown of NUP98 causes simultaneous knockdown of NUP96 and a failure in nuclear pore formation and cell death (data not shown). Therefore, it was not possible to specifically analyze the gene regulatory function of NUP98 from such knockdown experiments.

Collectively, these results indicate that NUP98 is functionally relevant for the expression of neural developmental genes it associates with in NeuPCs.

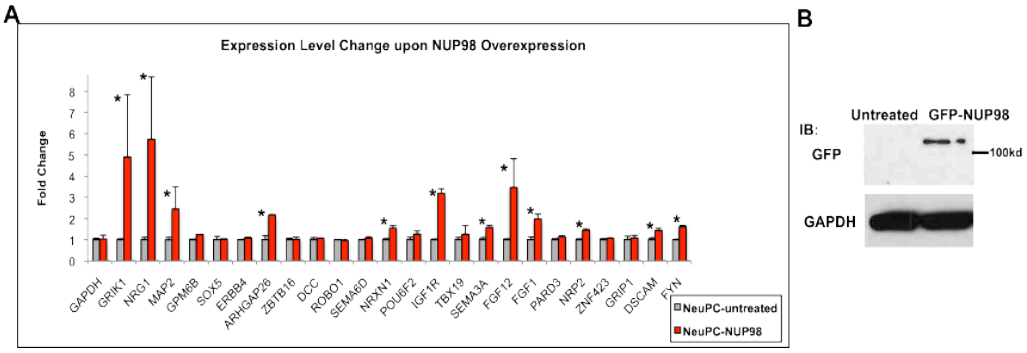


Figure 3-29 Gene expression changes upon overexpression of NUP98 in NeuPCs. (A) Fold change in expression levels upon full length NUP98 (-NUP98) overexpression in NeuPCs. Error bars were computed as standard deviation from triplicates. P value was obtained from Student's t-test and comparisons with P value <0.05 indicated with asterisks. (B) Western blot GAPDH and GFP in NeuPCs with overexpression of GFP-NUP98 or untreated condition as negative control.

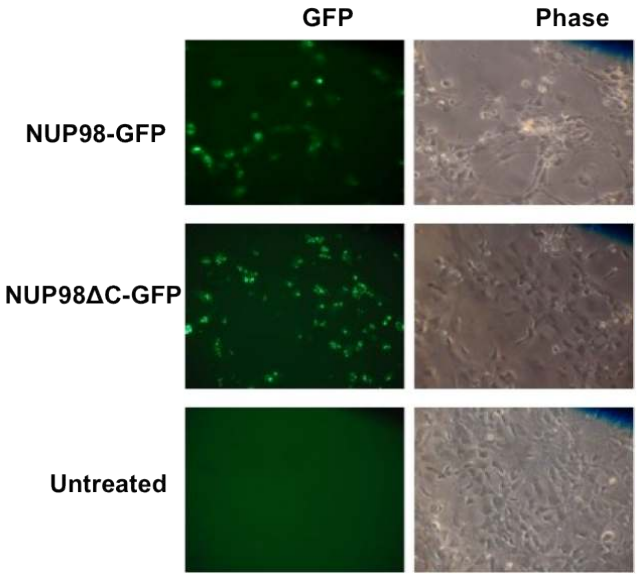


Figure 3-30 Low magnification images of NeuPCs trasnfected with control or NUP98 constructs. Live cell images of neural progenitor cells electroporated with plasmids encoding GFP-tagged full length NUP98 (NUP98-GFP), GFP-tagged NUP98 fragment (NUP98ΔC-GFP), and mock transfected (untreated). GFP channel (left) and phase (right) images were shown.

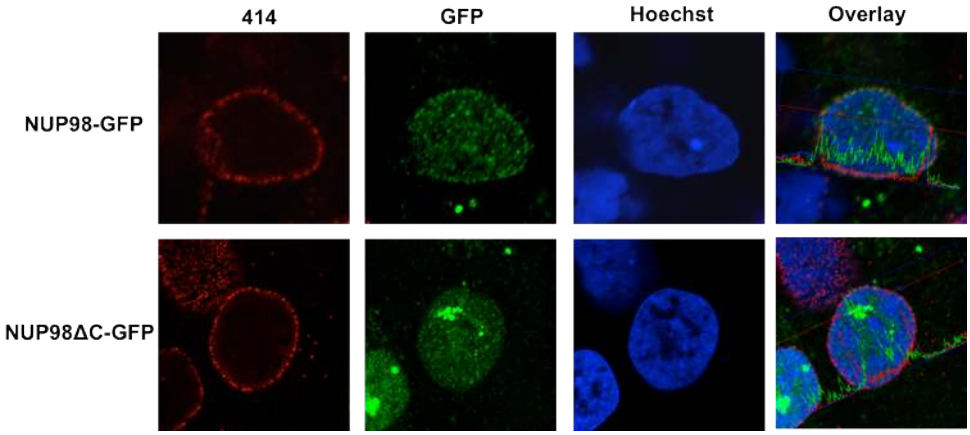


Figure 3-31 High magnification images of NeuPCs trasnfected with control or NUP98 constructs. Confocal images of neural progenitor cells fixed after electroporation with plasmids encoding GFP-tagged full length NUP98 (NUP98-GFP) and GFP-tagged NUP98 fragment (NUP98ΔC-GFP) were shown. Cells were stained with the nuclear pore marker 414 in red, anti-GFP antibody in green, and Hoechst in blue. In the overlay pictures, a line was drawn across the nuclei and 414 and GFP signals were plotted along the line.

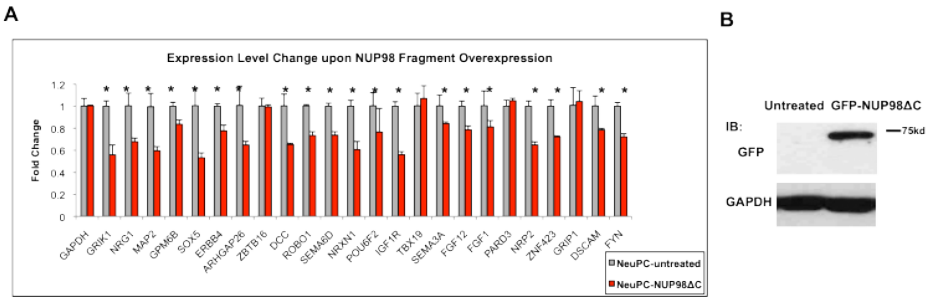


Figure 3-32 Gene expression changes upon overexpression of NUP98 fragment in NeuPCs. (A) Fold change in expression levels upon NUP98 fragment (-NUP98ΔC) overexpression in NeuPCs. Error bars were computed as standard deviation from triplicates. P value was obtained from Student's t-test and comparisons with P value <0.05 indicated with asterisks. (B) Western blot of GAPDH and GFP in NeuPCs with overexpression of GFP-NUP98 fragment (GFP-NUP98ΔC) or untreated condition as negative control.

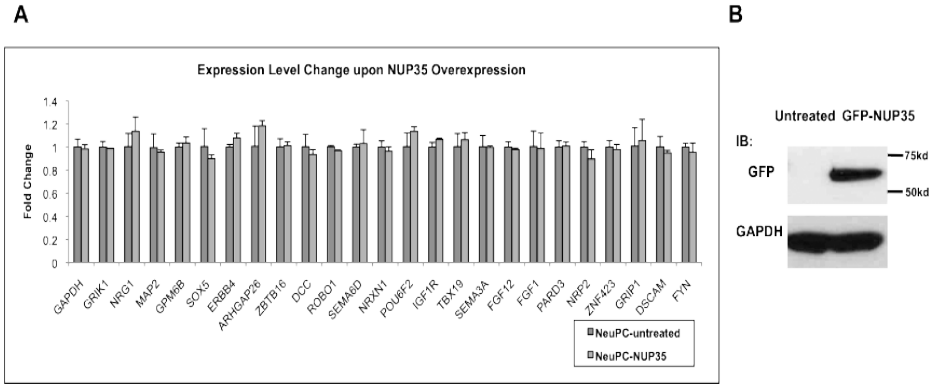


Figure 3-33 Gene expression changes upon overexpression of NUP35 in NeuPCs. (A) Fold change in expression levels upon NUP35 overexpression in NeuPCs. Error bars were computed as standard deviation from triplicates. (B) Western blot GAPDH and GFP in NeuPCs with overexpression of GFP-NUP35 or untreated condition as negative control.

3.5 Intranuclear Positioning of Selected Nup98 Targets during Differentiations

To obtain further insights into the role of NUP98 during differentiation we monitored the mRNA levels of 24 NUP98 target genes that were in the neural development gene ontology category through differentiation from ESCs to NeuPCs, and subsequently to postmitotic neurons in which Nup98 does not seem to bind the genome (Figure 3-34). We found that all 24 genes were upregulated when ESCs were differentiated to NeuPCs, consistent with the genome-wide correlation analysis and supporting a role of NUP98 in the induction of transcription. When NeuPCs were further differentiated to neurons, the majority of genes (20 genes) showed continued transcriptional induction. Among those 20 genes, we focused on 6 genes that exhibited the most dramatic increase in expression in neurons. We observed that these genes could be largely divided into two groups (Figure 6). Group I genes (GRIK1, NRG1, and MAP2; colored in red) showed modest transcriptional induction in NeuPCs compared to ESCs. However, this cohort of genes underwent a robust increase in expression during the transition from NeuPCs to neurons. Group II genes (GPM6B, SOX5, and ERBB4; colored in green) underwent a dramatic activation in the initial commitment of ESCs to NeuPCs and only slight upregulation during subsequent

neuronal differentiation. This suggests that NUP98 associates with both genes starting to be developmentally induced (Group I genes) and genes that are at a later stage of induction (Group II genes) in NeuPCs.

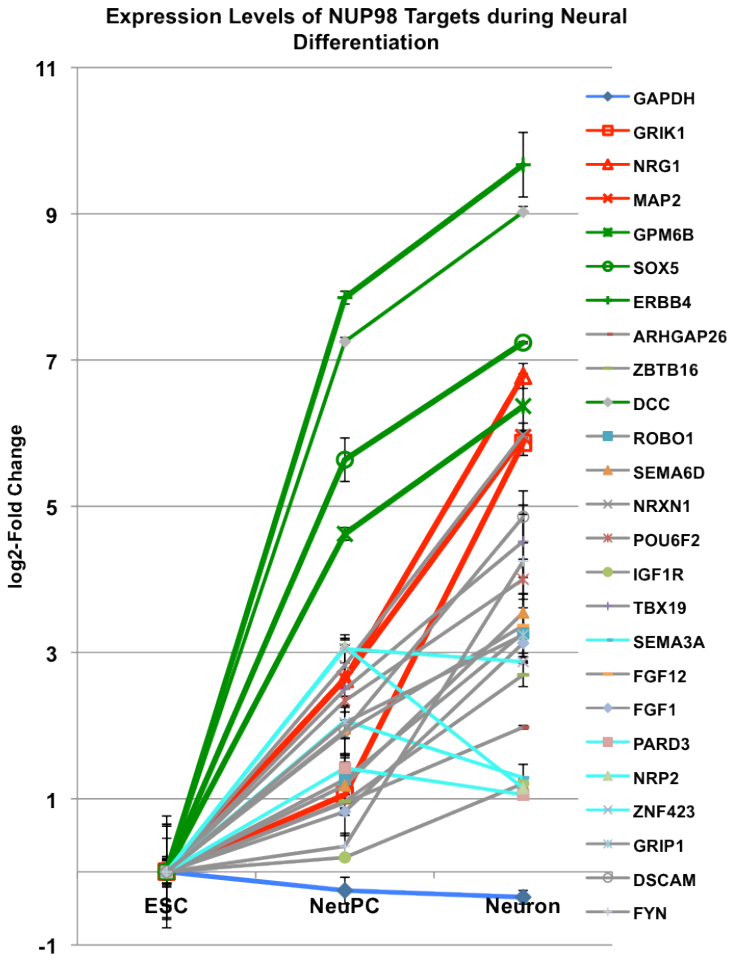


Figure 3-34 Expression level changes of NUP98 target genes during neural differentiation. Fold change in expression levels of neural progenitor cell-NUP98 binding genes from embryonic stem cells (ESC) to neural progenitor cells (NeuPC) and to neurons (Neuron) on a $\log_2$-scale. Different groups of developmentally regulated NUP98 binding genes were labeled in red, green, aqua or grey. Error bars were computed as standard deviation from triplicates.

As a mobile nuclear pore complex component, NUP98 can act both at the nuclear pore complexes and inside the nucleus at sites that are not attached to the nuclear envelope (NE) [44,51]. Therefore, we wondered if either of the two classes of genes is specifically associated with nuclear pore complexes at the NE. We examined the localization of the group I and group II NUP98 targets by immunofluorescence-fluorescence in situ hybridization (IF-FISH) experiments. We used lamin (LMNB) staining as a marker for the NE, and only counted FISH signals whose center overlaid with the NE (corresponding to <0.5 μm distance from the NE) as 'periphery' localization (Figure 3-35). We found that the two groups of genes also showed distinct intranuclear localization at the progenitor cell stage. In NeuPCs, group I genes that will become transcriptionally active were localized to the periphery, whereas group II genes that were already expressed at high levels were in the interior of the nucleus (Figure 3-36, Figure 3-37, Figure 3-38). Upon differentiation into neurons, group I genes moved into the nuclear interior whereas group II genes maintained their interior localization (Figure 3-36, Figure 3-37, Figure 3-38).

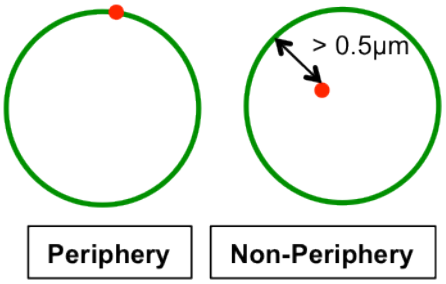


Figure 3-35 Criteria for counting gene localization as 'Periphery' or 'Non-Periphery'. LMNB staining was shown in green and FISH signal in red. Genes counted as 'Periphery' were localized within 0.5μm of the nuclear lamina.

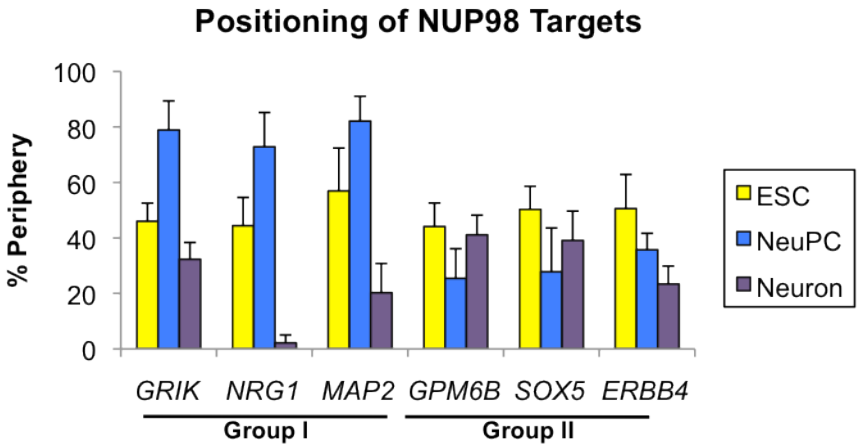


Figure 3-36 Percentage of periphery localization of NUP98 binding genes through development. Percentages were shown for ESC (yellow), NeuPC (blue) and Neuron (purple), determined from IF-FISH experiments. Error bars were calculated as standard deviation from triplicates for a total of at least 100 cells using 3D reconstruction of images.

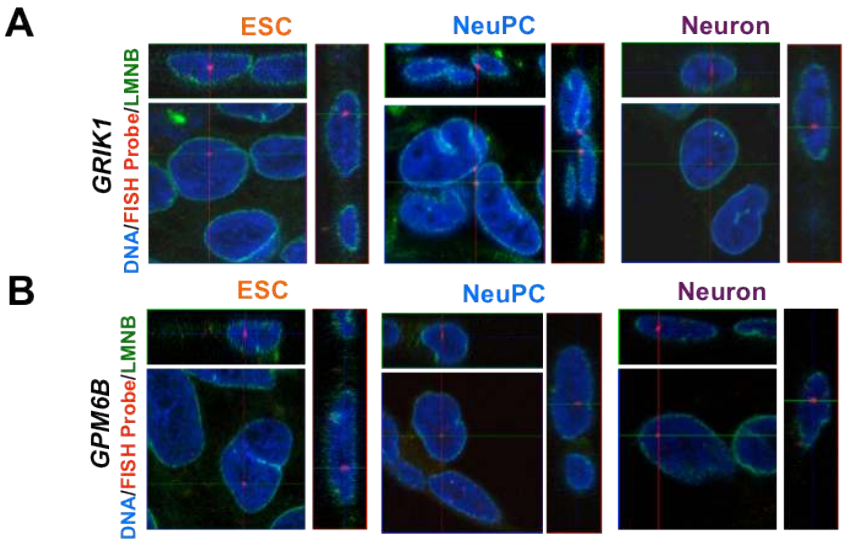


Figure 3-37 Distinct localization of two groups of NUP98-regulated developmental genes. Representative 3D IF-FISH images showing the localization of (A) group I genes (*GRIK1*) and (B) group II genes (*GPM6B*) through development, i.e. in ESC, NeuPC, and Neuron. FISH probes were shown in red, LMNB staining in green, and Hoechst in blue. Each set of images includes the x-y, y-z and x-z planes that cross at the FISH probe signal.

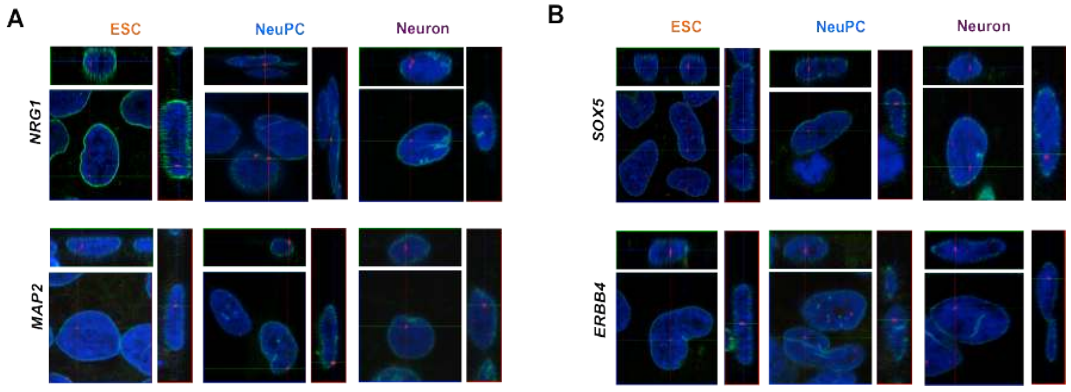


Figure 3-38 More examples of gene localization of two groups of NUP98 targets. Representative 3D IF-FISH images showing the localization of group I genes (*NRG1* and *MAP2*, in A) and group II genes (*SOX5* and *ERBB4*, in B) through development, i.e. in ESC, NeuPC, and Neuron. FISH probes were shown in red, LMNB staining in green, and Hoechst in blue. Each set of images includes the x-y, y-z and x-z planes that cross at the FISH probe signal.

3.6 Perturbation of Functional Nup98 Levels and Efficiency of Differentiation

Given the association between NUP98 and neural developmental genes, we decided to test if overexpression of full length NUP98 and its dominant negative fragment in neural progenitor cells affected efficiency of neuronal differentiation. We examined the efficiency of neuronal differentiation by measuring the expression levels of markers for differentiated neurons (RBFox3, TUBB3, and Syn1) at the end of 1 month's neuronal differentiation from NeuPCs. We observed that overexpression of full length NUP98 increased expression of those neuronal markers, whereas overexpression of the dominant negative fragment decreased their expression levels (Figure 3-39). This is consistent with the findings that overexpression of full length NUP98 increased expression of neural developmental genes, whereas overexpression of the fragment reduced expression of such genes. Collectively these results suggest that NUP98 regulates the efficiency of neuronal differentiation from neural progenitor cells. Based on these observations, we conclude that at the neural progenitor stage, there are at least two modes of gene regulation by NUP98, 1) the 'gene to pore' model where genes relocate to the nuclear pore at the initial stage of transcriptional induction associated with neurogenesis; and 2) the 'Nup to gene'

model where NUP98 acts away from the nuclear pore to interact with genes that are highly activated (Figure 3-40).

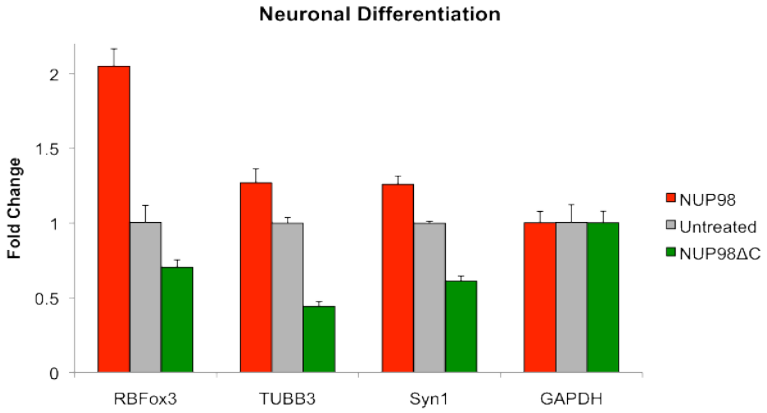


Figure 3-39 NUP98 regulates neuronal differentiation.
Fold change in expression levels of markers for differentiated neurons in cells overexpressing full length NUP98 (NUP98) or its dominant negative fragment (NUP98ΔC), compared to Untreated control.

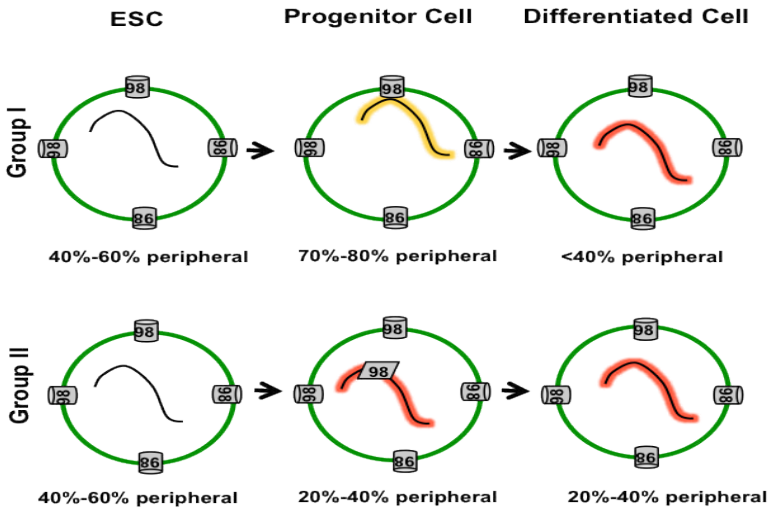


Figure 3-40 Model of two groups of NUP98-gene interaction. Group I genes are at the beginning stage of developmental induction in neural progenitor cells and interact with NUP98 at the nuclear pores in the NE, and subsequently translocate to intranuclear sites upon full induction in neurons. In contrast, group II genes are already greatly activated in NeuPCs and interact with NUP98 at intranuclear sites away from the NE. The percentage of genes observed at the nuclear periphery at each stage was indicated.

3.7 Discussion

In addition to their well established role in mediating transport across the NE, nuclear pore proteins have been implicated in directly regulating gene expression in organisms as diverse as yeast and *Drosophila* [43-48,51,59,72]. However, the functions of Nups during development, especially their roles in gene regulation and in higher organisms such as humans, remain largely unexplored. Here we provide evidence that in human cells, the nuclear pore protein NUP98 binds the nuclear genome in a manner that is tightly linked to differentiation status and developmental gene expression. In embryonic stem cells, NUP98 bound genes include an active subgroup such as genes involved in cell cycle and nucleic acid metabolism regulation and a silent subgroup. In neural progenitor cells, NUP98 shows distinct association with genes activated during neural development, and NUP98 is functionally important for the expression of these genes. In the lung fibroblast IMR90 cells NUP98 mainly interacts with silent chromatin domains. This suggests that besides controlling nucleo-cytoplasmic exchange, NUPs can dynamically interact with the human genome during differentiation, providing an additional layer of genome regulation during development.

From a cell biological point of view, there are at least two modes of

developmental gene regulation by NUP98, the ‘on-pore’ regulation and the ‘off-pore’ regulation. Our findings suggest that at least one of the distinctions of the two modes of regulation might be related to the temporal gene expression dynamics of NUP98 targets. Specifically, during the differentiation of human embryonic stem cells along the neural lineage, nuclear pore-tethered NUP98 acts as a short-term anchoring point for certain developmental genes at the beginning stages of transcription induction.

In progenitor cells, anchorage at the nuclear pores could be especially important for genes at the initial stages of developmental induction because for these genes the activation status may not be stable yet and therefore require the microenvironment of the nuclear pores to maintain chromatin decondensation and gene transcriptional status, especially through repeated cell cycles such as in neural progenitor cells (discussed below). On the other hand, for genes that are at later stages of developmental induction, the chromatin is entirely open and thus does not require the nuclear pore-tethering mechanism to maintain transcription. Under such circumstances, the nuclear interior might be a more optimal microenvironment for those genes that supports robust transcription compared to the nuclear pores which are in proximity to the nuclear lamina which can mediate transcriptional repression [100,101].

The rationale for the involvement of the nuclear pores in developmental

gene regulation, especially at the progenitor stage, probably relates to the necessity of re-establishing chromatin organization after nuclear envelope breakdown and reformation in mitosis. During M phase of the cell cycle, chromatin is condensed, transcription activities are largely diminished and most transcription factors are absent from mitotic chromosomes, which composes a window that allows for cell fate reprogramming [102-104]. Therefore, in progenitor cells, upon mitosis exit, chromatin has to be decondensed in a manner that faithfully restores the ‘open’ or ‘closed’ states for different chromatin domains to ensure that corresponding developmental genes can be activated or repressed correctly. Nups are prime candidates to regulate transcription re-initiation of developmental genes based on ‘transcriptional memory’ from previous cell cycles because during mitosis exit, Nups are among the first proteins to establish contacts with chromatin and it has been found that proper chromatin decondensation requires the functioning of Nups [21,105-107]. Furthermore, association with Nups in yeast has been shown to convey a ‘gene memory’ function so that genes can be rapidly re-induced for repeated transcription stimulation cycles [47,108]. Along these lines of evidence, NUP98 in *Drosophila* is involved in the re-initiation of transcription after heat shock [44] and our study has shown that in the cycling human neural progenitor cells NUP98 associates with and regulates expression of neural development genes. Together these observations point to the role of

Nups in the rapid and faithful re-initiation of expression of developmental genes after each mitosis cycle.

In the search for DNA sequences that might direct NUP98-chromatin interaction, we identified a conserved DNA binding motif, the GA boxes. This motif is overrepresented in NUP98-binding sequences not only in human cells from our study, but also in *Drosophila* cells from published ChIP-chip and Dam-ID datasets. In *Drosophila*, GA-boxes are recognized by the GAGA factor, which is encoded by the *Trithorax-like* gene and is required for the proper development of the organism [87]. Interestingly, GAGA factor has been related to the yeast factor Rap1 because of their similarities in binding to both repetitive sequences and transcriptionally active genes as well as exhibiting boundary activity [87], and the Rap1 binding site has been identified as the nuclear-pore recognizing DNA motif in yeast [45]. Together these lines of evidence suggest that the DNA recognition activity of Nups or Nup-interacting partners is evolutionarily conserved.

Finally, the involvement of NUP98 in developmental regulation sheds light on its involvement in multiple types of leukemia where it is fused to various transcription regulators [93]. Such oncogenic NUP98-fusion proteins have been shown to promote the self-renewal of hematopoietic progenitor cells and inhibit their differentiation [109]. We found that NUP98 is connected to the regulation of genes implicated in neoplasm formation especially at the progenitor stage. In

addition, overexpression of the NUP98 fragment as appeared in the fusion proteins disrupted the expression of endogenous NUP98 targets which, during normal differentiation processes, were activated. Therefore, the misregulation of developmental genes in hematopoietic cells due to genomic fusion of *NUP98* with transcription regulators may be a potential mechanism driving the transformation events in NUP98-fusion protein associated leukemias.

3.8 Materials and Methods

Cell Culture and Differentiation

Human embryonic stem cell line HUES6 were grown under feeder-free conditions in mTeSR1 medium. HUES6-derived neural progenitor cells were grown in DMEM/F12 supplemented with N2/B27. Early passage IMR90 cells were grown in DMEM, 15% FBS and MEM nonessential amino acids.

Human embryonic stem cell line HUES6 was grown in mTeSR1 medium (STEMCELL Technologies). To obtain neuroprogenitor cells, embryoid bodies were formed by mechanically lifting HUES6 cells and transferring them to low-adherence dishes in HUES6 medium without FGF2 for approximately 7 days. After that, embryoid boides were plated onto poly-ornithine/laminin (Sigma)-coated dishes in DMEM/F12 supplemented with N2 and B27 (Invitrogen). Rosettes were visible to collect after 5-7 days. Rosettes were then dissociated

with TripLE (Invitrogen) diluted 1:5 in PBS and plated again onto poly-ornithine/laminin-coated dishes with the same medium but now with the addition of 10ng/ml FGF2. Homogeneous populations of neuroprogenitor cells were achieved after 1-2 passages with TripLE in the same conditions. HUES6-derived neural progenitor cells were grown in DMEM/F12/Glutamax (Invitrogen) supplemented with N-2 (Invitrogen), B-27 (Invitrogen), 20ng/ml FGF2 (Stemgent), and 1μg/ml laminin (Invitrogen). For differentiation into neurons, neural progenitor cells were plated at low density and cultured in DMEM/F12/Glutamax supplemented with N-2, B-27, 20ng/ml BDNF (Brain-derived neurotrophic factor, Peprotech), 20ng/ml GDNF (Glial-derived neurotrophic factor, Peprotech) and 1μg/ml laminin for 1 month. Early passage IMR90 cells were grown in DMEM (Mediatech), 15% FBS (Invitrogen) and MEM nonessential amino acids (Mediatech).

Antibodies

Primary antibodies used include rabbit anti-human NUP98 polyclonal antibody (Cell Signaling 2292; ‘NUP98Ab1’ specified in the experiment), rabbit anti-human NUP98 monoclonal antibody (Cell Signaling 2598), mAb414 (Covance MMS-120R), normal rabbit IgG (Cell Signaling 2729), anti-human Nestin antibody (Chemicon), anti-Sox2 antibody (Chemicon), and rabbit-anti-LMNB antibody (Aviva ARP46357-P050).

Chromatin Immunoprecipitation (ChIP)

For formaldehyde crosslinking, cells were fixed in 1% formaldehyde (Polysciences) for 10min. For formaldehyde-disuccinimidyl glutarate crosslinking, cells were fixed in 2mM disuccinimidel glutarate (Pierce) for 30min and then fixed in 1% formaldehyde for 10min. Fixation was stopped by adding glycine to a final concentration of 125mM. Fixed cells were lysed by Lysis/Wash Buffer (150mM NaCl, 5mM EDTA, 50mM Tris pH7.5, 0.5% NP-40) and spun down. Pellet was resuspended in Shearing Buffer (1% SDS, 10mM EDTA, 50mM Tris pH8.0) and sonicated to DNA fragments of sizes between 150-300 base pairs using Bioruptor (Diagenode). Sheared chromatin was diluted 10 fold using Dilution Buffer (0.01% SDS, 1.1% TX-100, 1mM EDTA, 16.7mM Tris pH8.0, 167mM NaCl) and incubated with primary antibody overnight at 4°C. Antibody-chromatin mixtures were then incubated with pre-blocked Dynabeads M280 sheep-anti-rabbit IgG for 6 hours at 4°C. Beads were collected, washed with Lysis/Wash Buffer for 6 times and DNA was eluted, de-crosslinked, treated with Rnase and protease, and column purified. Purified DNA was subjected to qPCR analysis or ChIP-Seq library construction. NUP98Ab1 was rabbit anti-human NUP98 polyclonal antibody (Cell Signaling 2292), and NUP98Ab2 was rabbit anti-human NUP98 monoclonal antibody (Cell Signaling 2598). NUP98Ab1 and formaldehyde crosslinking was used for ChIP-Seq experiments in ESCs and NeuPCs.

ChIP-Seq and Analysis of Sequencing Data

Cells were fixed in 1% formaldehyde (Polysciences) for 10min. Fixation was stopped by adding glycine to a final concentration of 125mM. Fixed cells were lysed and sonicated. DNA was immunoprecipitated, eluted, de-crosslinked, treated with Rnase and protease, and purified. Procedures were detailed in Supplemental Information. Library was constructed using Illumina ChIP-Seq DNA sample prep kit and sequencing was done on Illumina GAII. Mapping and peak calling of ChIP-Seq data, annotation of NUP98-binding regions, mapping and expression level analysis of RNA-Seq data, transcription factor motif analysis, gene ontology analysis, positional correlation of ChIP-Seq and RNA-Seq were conducted using the Genomatix software. Peak calling was based on Audic-Claverie algorithm for NGSAnalyzer. Chromosomal views of ChIP-Seq data were generated using Affymetrix Integrated Genome Browser and correlation of NUP98 binding with gene expression levels and histone modification levels was performed using the R package for statistical computing.

For peak assignment to genes and promoters, genomic positions of exons/introns were determined by the mapping of cDNAs from different sources (e.g.RefSeq, GenBank, Ensembl) in Genomatix's transcript collection to the reference genome, and promoter was defined as 500bp/100bp up/downstream of the transcriptional start site. Peak was assigned to promoters if it overlapped with

any genomic regions defined as promoters, and to genes if it overlapped with promoters/exons/introns.

For correlation with gene expression levels, gene expression values were normalized expression/enrichment value (NE-value) obtained using the expression analysis for RNASeq data in the Genomatix software. The NE-value was calculated based on the formula: $NE = c * \#reads_{region} / (\#reads_{mapped} * length_{region})$, where $\#reads_{region}$ was the number of reads (sum of base pairs) of falling into the transcript, $\#reads_{mapped}$ was the number of all mapped reads (in base pairs), $length_{region}$ was the transcript length in base pairs, and c was a normalization constant set to 10^7.

For correlation with histone modification levels, for given selected genomic region (NUP98-binding or randomly selected region), histone modification level was calculated as the number of reads falling into the region from histone modification ChIP-Seq experiment normalized to that from input of ChIP-Seq experiment by subtraction.

Immunofluorescence

Cells were fixed in PBS/4%PFA for 5min, blocked in IF Buffer (PBS, 1%BSA, 0.1%TX-100) for 20min, incubated with primary antibody for 1 hour, washed 3 times in IF Buffer, incubated with secondary antibody for 1 hour, washed in IF Buffer, stained with Hoechst and mounted in Vectashield.

IF-FISH

Cells were fixed, stained according to the immunofluorescence procedures, fixed again with 4% PFA for 10min, washed with 2xSSC, permeablized in 0.1M HCl, 0.7% TX-100 on ice for 15min, washed with 2xSSC, denatured in 50% formamide, 2xSSC pH7.2 for 30min at 80°C, and washed with ice-cold 2xSSC. While cells were prepared for hybridization, 200ng of DIG-labeled FISH probes were combined with 5ul human Cot-I DNA (Invitrogen) and 10ug salmon sperm DNA, precipitated, resuspended and incubated with 5ul 100% formamide for 30min at 37°C, denatured for 8min at 85°C, incubated for 1hr at 37°C and combined with 5ul 2xhybridization buffer (4xSSC, 20% dextran sulfate, 2mg/ml BSA). Cells were then incubated with probes overnight at 42°C, re-blocked, stained with anti-DIG antibody (Roche) and Hoechst and mounted in Vectashield. Three-dimensional image stacks were recorded with Zeiss LSM710 scanning scope using a 63x objective, 512x512 resolution, 2x averaging and optimal interval (0.31µm) between stacks in Z-direction and three-dimensional images were reconstructed from the Z-stack images.

Overexpression and RNAi of *NUPs*

For NUP overexpression, plasmids were electroporated into NeuPCs using rat neural stem cell Nucleofector solution (Lonza Amaxa, VPG-1005) or (in differentiation assays) packaged into lentiviruses that were used subsequently to

infect NeuPCs. NUP98 was knocked down by siRNA (oligo sequence: GAG AGA GAT TTA GTT TCC TAA GCA A) in IMR90 cells using Dharmafect 1 siRNA transfection reagent according to the manufacturer's instructions.

RNA Extraction and RT-PCR

RNA was extracted from cells using Trizol (Ambion) and column purified using Rneasy kit (Qiagen). RNA was subsequently reverse transcribed using the QuantiTect reverse transcription kit (Qiagen) and cDNA was subjected to qPCR using SYBR Green PCR Master Mix (AppliedBiosystems). Primer sequences are available upon request.

Chapter 3, in part, has been submitted for publication of the material. Liang, Yun; Franks, Tobias M.; Marchetto, Maria C.; Gage, Fred H.; Hetzer, Martin W. The dissertation author was the primary investigator/author of this paper.

Chapter 4 Regulation and Function of Nup50 during Muscle Differentiation

4.1 Introduction

As the sole gateway between the cytoplasm and nucleoplasm, nuclear pore complexes are responsible for macromolecule transport between cellular compartments [5]. Recent evidence suggests that Nups, components of the nuclear pore complexes, are associated with tissue-specific diseases, pointing to cell type-specific functions of nuclear pore proteins [8]. In addition, mutations in different Nups result in distinct disease phenotypes [8]. For example, a Nup155 mutation causes atrial fibrilliation [9]. Genomic translocations that result in fusion of Nup98 fragment to other genes leads to acute myeloid leukaemia [10] [37]. Nup62 is linked to primary biliary cirrhosis [12].

Given that the ~30 different types of Nups are thought to function cooperatively in the context of the nuclear pore complex to mediate transport [110], it is not clear how individual Nups exhibit distinct functions. One possibility is that Nups can be expressed at different levels in various cell types, and as a result, various cell types are differentially sensitive to the impairement in Nup function. In line with this possibility, the expression of several Nups varies dependent on the cell type. For example, the Nup ALADIN is highly expressed in

testis and pancreas, the absence of which causes triple A syndrome [111]. The expression of the transmembrane Nup gp210 is strong in developing epithelia and some other cell types, but not in other embryonic tissue compartments [112]. In addition, the expression of gp210 is elevated during the differentiation of myoblasts into myotubes, and gp210 is required functionally for in vitro muscle differentiation [113].

In addition to variation in expression levels, there might be other mechanisms underlying the tissue-specific functions of Nups, given that not all Nups have been detected to exhibit tissue-specific expression. It remains to be tested whether and how a Nup can exhibit cell type-specific functions if it is expressed in all cell types. Such a possibility could be linked to the observation that the organization of the nuclear pore complex is dynamic and multiple Nups shuttle on and off the nuclear pore complexes at different rates [52]. A Nup may exhibit different dynamic behaviors in different cell types, related to its distinct functions on and/or off the nuclear pore complexes. Furthermore, the evidence that different Nups show distinct rates of shuttling can potentially be associated with the functional differences among Nups in disease scenarios [8].

Based on the reasoning above, it is worth examining closely the dynamics of Nups and how the shuttling processes are regulated. Up to present, the only reported observation regarding the regulation of Nup shuttling is that the

dynamics of Nup98 and Nup153 can be altered upon treatment of RNA polymerase inhibitors [57,58]. It is not yet identified which players can physically interact with Nups and regulate their dynamics.

We used in vitro differentiation of myoblasts into myocytes as a system to investigate whether Nups can exhibit different dynamics during differentiation. We identified Nup50 as a Nup that shows decreased dynamics upon muscle differentiation. In addition, we demonstrated that Kifc1 interacts with Nup50 and is responsible for the fast shuttling behavior of Nup50 in myoblasts. Upon differentiation, Kifc1 is degraded, which could explain the observed decrease of Nup50 dynamics. Importantly, knockdown of Nup50 and Kifc1 affected efficiency of muscle differentiation. We concluded that Kifc1 is a cell-type specific regulator of Nup50 dynamics during muscle differentiation.

4.2 Changes in Nup50 Dynamics during Muscle Differentiation

To test whether Nups change dynamics during muscle differentiation, stable C2C12 cell lines expressing GFP-tagged Nups were established. C2C12 is a mouse myoblast cell line originally obtained from post-injury mouse muscle, and is often used to study the differentiation of myoblast into myocytes. We performed fluorescence-recovery-after-photobleaching (FRAP) experiments in C2C12 cells stably expressing GFP-Nup50, which measured the rate of replacement of

photobleached GFP-Nup50 molecules at the nuclear pore complexes by unbleached GFP-Nup50 (Figure 4-1). We observed slower fluorescence recovery after photobleaching in myocytes compared to myoblasts, suggesting decreased Nup50 mobility after muscle differentiation. By contrast, in C2C12 cells stably expressing GFP-Nup98, FRAP experiments showed accelerated dynamics of Nup98 in myocytes compared to myoblasts, suggesting higher Nup98 mobility after muscle differentiation (Figure 4-2). Together these lines of evidence are in consistence with the hypothesis that Nup dynamics can be altered in different cell types. In addition, not all Nups exhibit the same pattern of dynamics alteration.

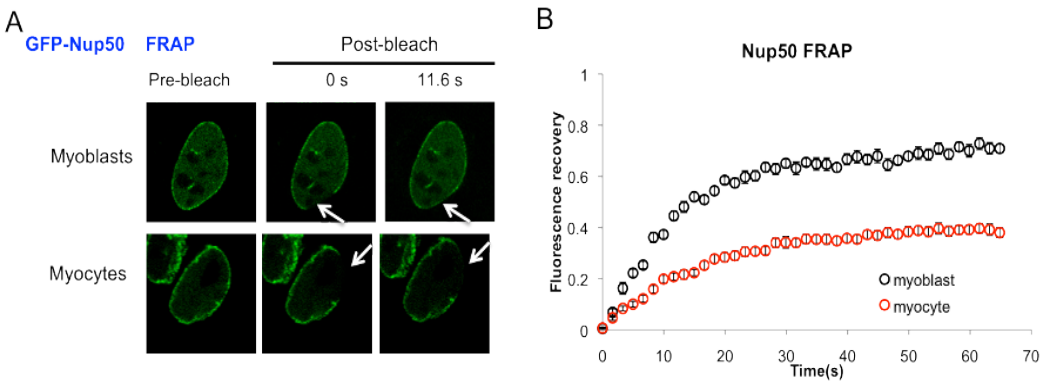


Figure 4-1 Decrease in Nup50 dynamics measured by FRAP after muscle differentiation. (A) Representative images of C2C12 cells stably expressing GFP-Nup50 pre-bleach and at 0s and 11.6s post-bleach. Cells before differentiation (myoblasts) and after differentiation (myocytes) were shown. Arrows point to region of photobleaching. (B) Quantification of fluorescence signals in FRAP experiments in myoblast and myocyte. Error bars show standard deviation.

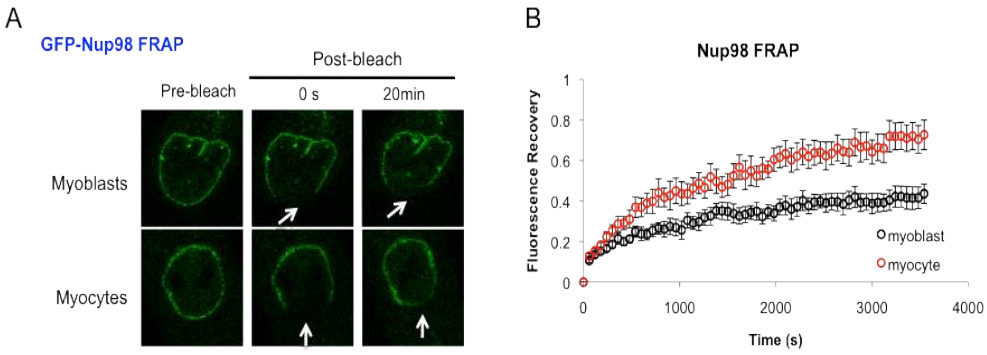


Figure 4-2 Increase in Nup98 dynamics measured by FRAP after muscle differentiation. (A) Representative images of C2C12 cells stably expressing GFP-Nup98 pre-bleach and at 0s and 20min post-bleach. Cells before differentiation (myoblasts) and after differentiation (myocytes) were shown. Arrows point to region of photobleaching. (B) Quantification of fluorescence signals in FRAP experiments in myoblast and myocyte. Error bars show standard deviation.

4.3 Localization and Dynamics of Nup50 fragments

To gain further insight into the domains of Nup50 that mediate its differential dynamics during C2C12 differentiation, we made 3 GFP-tagged fragments of Nup50 that encompass amino acids 1-214, 1-320, and 214-468, respectively. It has been reported that Nup50 interacts with importin-alpha and Nup153 via its N-terminal domain (amino acid 1-214) [114,115]. The middle region of Nup50 (amino acid 214-320) contains FG-repeat and mediates its interaction with importin-beta [114]. The C-terminus of Nup50 (amino acid

320-468) is the Ran-binding domain [114]. Consistent with its interaction with Nup153, the two N-terminal fragments of Nup50 (amino acids 1-214 and amino acids 1-320) were localized to the nuclear pore complexes in addition to the nucleoplasm (Figure 4-3). By contrast, the C-terminal fragment of Nup50 (amino acid 214-468) was localized to the nucleoplasm and cytoplasm, but devoid from the nuclear pores (Figure 4-3).

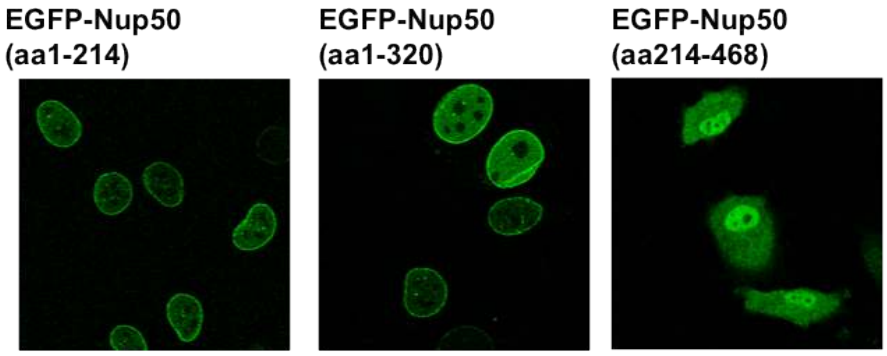


Figure 4-3 Localization of Nup50 fragments in myoblasts. Confocal images of C2C12 cells stably expressing GFP-tagged Nup50 fragments were shown. (aa1-214), Nup50 fragment encompassing amino acids 1-214. (aa1-320), Nup50 fragment encompassing amino acids 1-320. (aa214-468), Nup50 fragment encompassing amino acids 214-468.

We then examined the dynamic behavior of the various Nup50 fragments by FRAP in C2C12 cells before and after differentiation. In undifferentiated myoblasts, the two N-terminal fragments of Nup50 (amino acids 1-214 and amino acids 1-320) shuttled at slower rate compared to the full length Nup50 (Figure 4-4).

In differentiated myocytes, however, the dynamics of the Nup50 fragments were comparable to the full length Nup50 (Figure 4-5). This suggests that the C-terminal domain of Nup50 (amino acids 320-468), which does not bind the nuclear pores, is responsible for the faster dynamics of Nup50 before differentiation, i.e. in myoblasts.

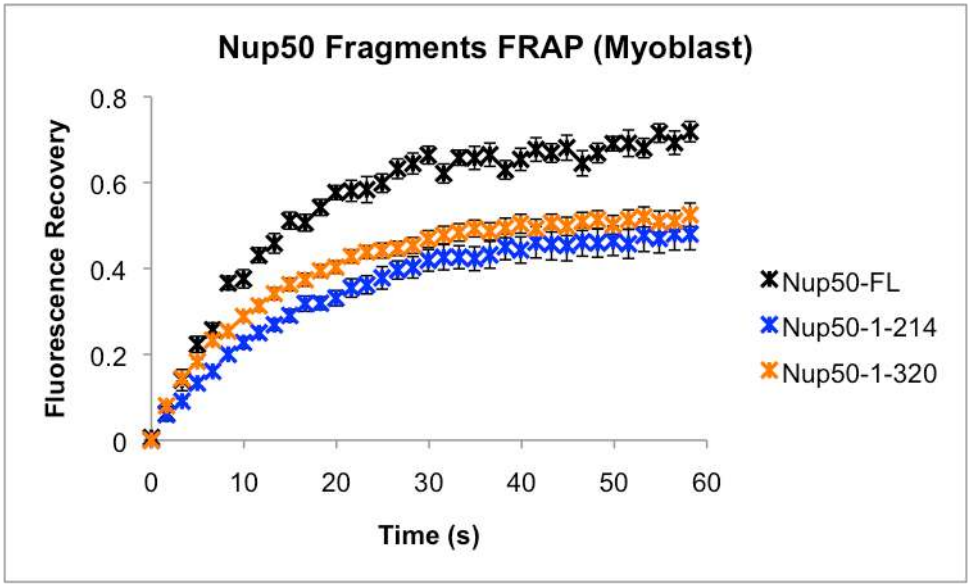


Figure 4-4 Dynamics of Nup50 and fragments in myoblasts. Fluorescence recovery after photobleaching was quantified and shown for full length Nup50 (Nup50-FL), Nup50 amino acids 1-214 (Nup50-1-214), and Nup50 amino acids 1-320 (Nup50-1-320). Error bars show standard deviation.

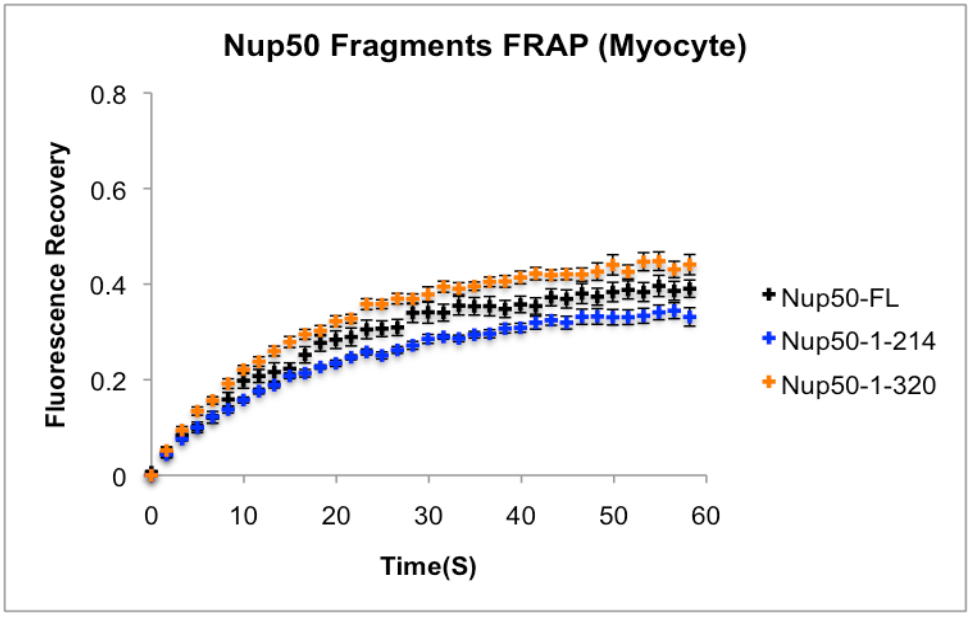


Figure 4-5 Dynamics of Nup50 and fragments in myocytes. Fluorescence recovery after photobleaching was quantified and shown for full length Nup50 (Nup50-FL), Nup50 amino acids 1-214 (Nup50-1-214), and Nup50 amino acids 1-320 (Nup50-1-320). Error bars show standard deviation.

We proposed that the C-terminal domain of Nup50 might interact with a factor that mediated the increased dynamics of Nup50 in myoblasts. For example, this factor might promote the dissociation of Nup50 from the nuclear pore complexes. If this idea is correct, overexpressing the C-terminal domain of Nup50 should saturate this factor and as a result, the shuttling of endogenous full-length Nup50 should be slowed down for lack of interaction with this factor. To test this, in myoblasts stably expressing GFP-tagged full length Nup50, we transiently overexpressed mcherry-tagged C-terminal domain of Nup50 (amino acids

214-468) or mcherry-tagged H2B as a negative control (Figure 4-6). We then performed FRAP experiments to study the dynamics of the GFP-tagged full length Nup50 in those cells. We observed that full length Nup50 dynamics were slower in cells overexpressing the C-terminal domain of Nup50, suggesting a dominant negative effect of the C-terminal Nup50 fragment (Figure 4-7). This is consistent with the idea that the C-terminal domain of Nup50 interacts with a factor that mediates the increased mobility of Nup50 in myoblasts.

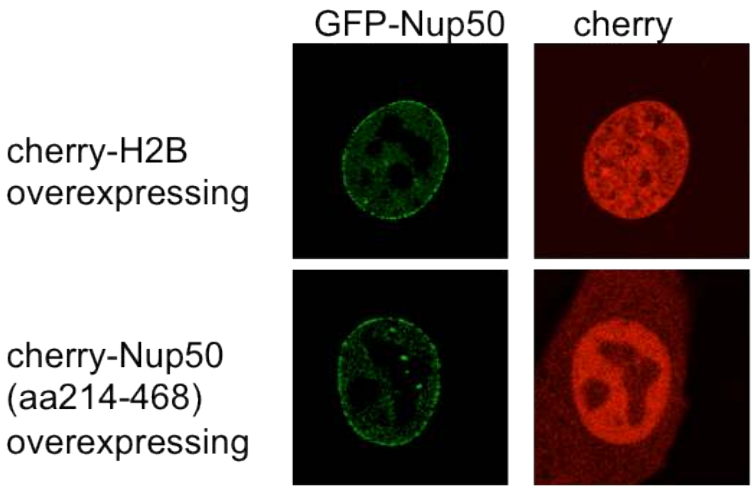


Figure 4-6 Overexpression of C-terminal fragment of Nup50 or control H2B in GFP-Nup50 cells. C2C12 cells stably expressing GFP-tagged full length Nup50 were transiently transfected with plasmids expressing mcherry-tagged H2B (cherry-H2B overexpressing) as a control or mcherry-tagged Nup50 amino acids 214-468 (cherry-Nup50(aa214-468) overexpressing). Confocal images of cells in the green channel (GFP-Nup50) and red channel (cherry) were shown.

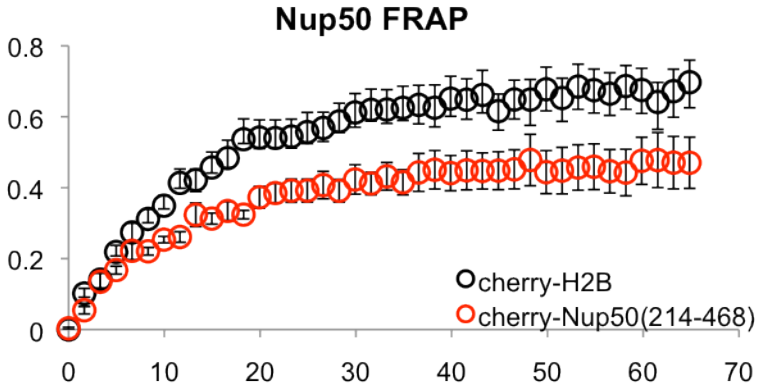


Figure 4-7 Dynamics of full-length Nup50 upon overexpression of Nup50 C-terminal fragment. Fluorescence recovery after photobleaching was quantified and shown for full length Nup50 in cells overexpressing mcherry-tagged Nup50 C-terminal fragment (cherry-Nup50(214-468) or cells overexpressing mcherry-tagged H2B (cherry-H2B) as a negative control. Error bars show standard deviation.

4.4 Perturbation of Functional Nup50 Levels and Efficiency of Differentiation

Given the observation that the C-terminal fragment of Nup50 had a dominant negative effect on Nup50 dynamics (Figure 4-6, Figure 4-7), we tested how overexpression of this and other fragments of Nup50 affected efficiency of muscle differentiation. We established C2C12 cell lines stably overexpressing GFP-tagged Nup50 fragments encompassing amino acids 1-214, 1-320, and

214-468, respectively (Figure 4-8).

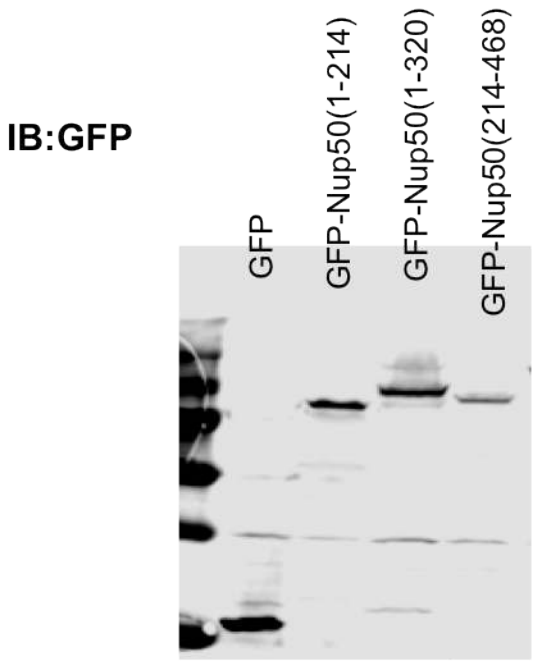


Figure 4-8 Stable overexpression of Nup50 fragments in C2C12 cells. Cells stably overexpressing GFP-tagged Nup50 amino acids 1-214 (GFP-Nup50(1-214)), amino acids 1-320 (GFP-Nup50(1-320)), amino acids 214-468 (GFP-Nup50(214-468)) or GFP as negative control were lysed and lysates were transferred to membrane and immunoblotted with anti-GFP antibody.

We then differentiated these C2C12 cells and performed MyHC staining to visualize differentiated cells [113]. We observed that overexpression of the C-terminal fragment of Nup50 (amino acids 214-468) accelerated muscle differentiation suggested by MyHC staining (Figure 4-9). In addition, quantification

of percentages of cells with multiple nuclei, a feature of myotube formation, showed that overexpression of Nup50 amino acids 214-468 promoted formation of multinucleated myotubes (Figure 4-10). Collectively these observations suggest that overexpression of the C-terminal domain of Nup50 (amino acids 214-468), which decreased dynamics of full length Nup50 endogenously (Figure 4-6 and Figure 4-7) mimicking the alteration of Nup50 dynamics post differentiation, accelerated C2C12 differentiation.

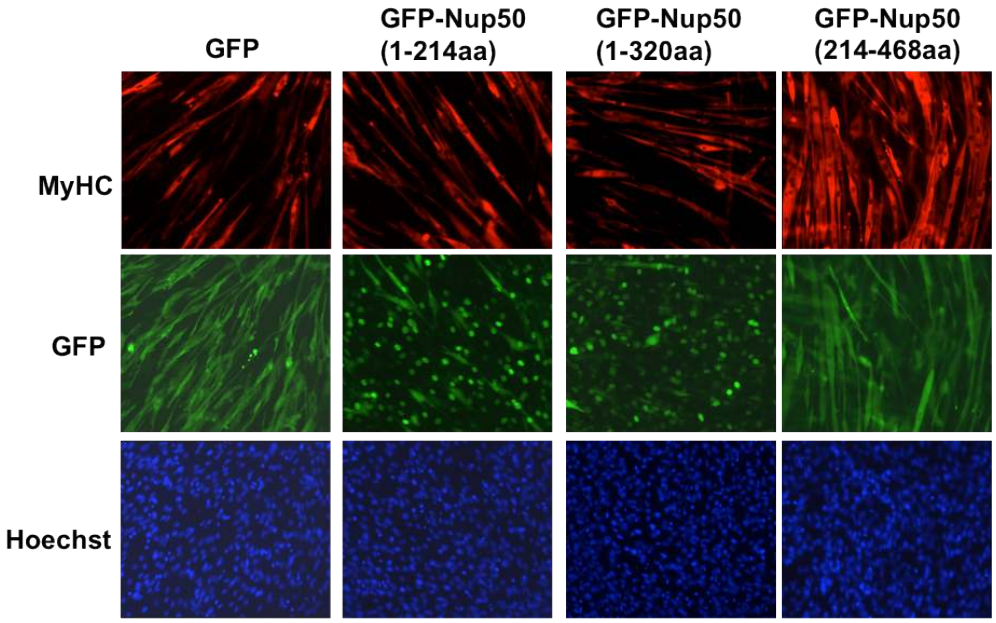


Figure 4-9 Overexpression of Nup50 fragments and efficiency of C2C12 differentiation. Myoblasts stably overexpressing GFP-tagged Nup50 amino acids 1-214 (GFP-Nup50(aa1-214)), 1-320 (GFP-Nup50(aa1-320)), 214-468(GFP-Nup50 (aa214-468)) and GFP as a negative control were differentiated for 72 hours and cells were immunostained for MyHC in red, GFP in green and Hoechst in blue.

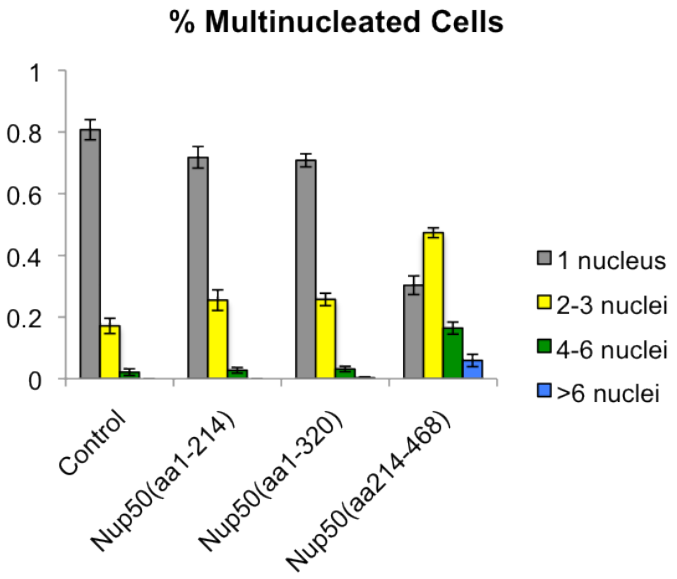


Figure 4-10 Quantification of multinucleated cells upon overexpression of Nup50 fragments. Myoblasts stably overexpressing GFP-tagged Nup50 amino acids 1-214 (GFP-Nup50(aa1-214)), 1-320 (GFP-Nup50(aa1-320)), 214-468(GFP-Nup50 (aa214-468)) and GFP as a negative control (control) were differentiated for 72 hours and percentages of cells with 1 nucleus, 2-3 nuclei, 4-6 nuclei, and more than 6 nuclei were shown.

The differentiation phenotype upon overexpression of the Nup50 fragment revealed the possibility that Nup50 regulates C2C12 cell differentiation. To further test this, we knocked down Nup50 using two shRNA sequences that were able to efficiently knock down Nup50 in C2C12 cells (Figure 4-11 and data not shown). Knock down of Nup50 with these two constructs caused decrease in differentiation efficiency, judged from MyHC staining and quantification of percentages of cells with multiple nuclei (Figure 4-12 and Figure 4-13). On the

other hand, Nup50 knockdown did not lead to significant defects in proliferation of undifferentiated myoblasts (Figure 4-14). Together these lines of evidence suggest that Nup50 regulates C2C12 cell differentiation.

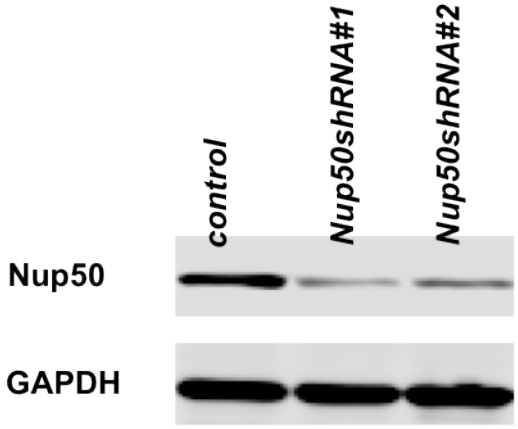


Figure 4-11 Knock down of Nup50 by shRNA in C2C12 cells. Western blot were performed for cells stably transfected with pLKO control vector (control), vector with Nup50 shRNA sequence #1 (Nup50shRNA#1), and vector with Nup50 shRNA sequence #2 (Nup50shRNA#2). Immunoblot was performed using Nup50 antibody and GAPDH antibody as a loading control.

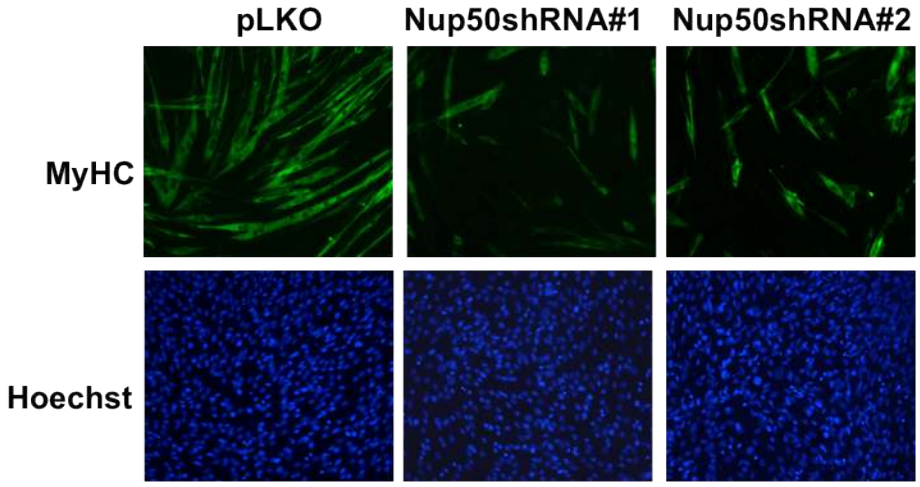


Figure 4-12 Nup50 knockdown and efficiency of C2C12 differentiation. Myoblasts with two independent Nup50 knockdown (Nup50shRNA#1, Nup50shRNA#2) and pLKO knockdown (pLKO) as a negative control were differentiated for 96 hours and cells were immunostained for MyHC in green and Hoechst in blue.

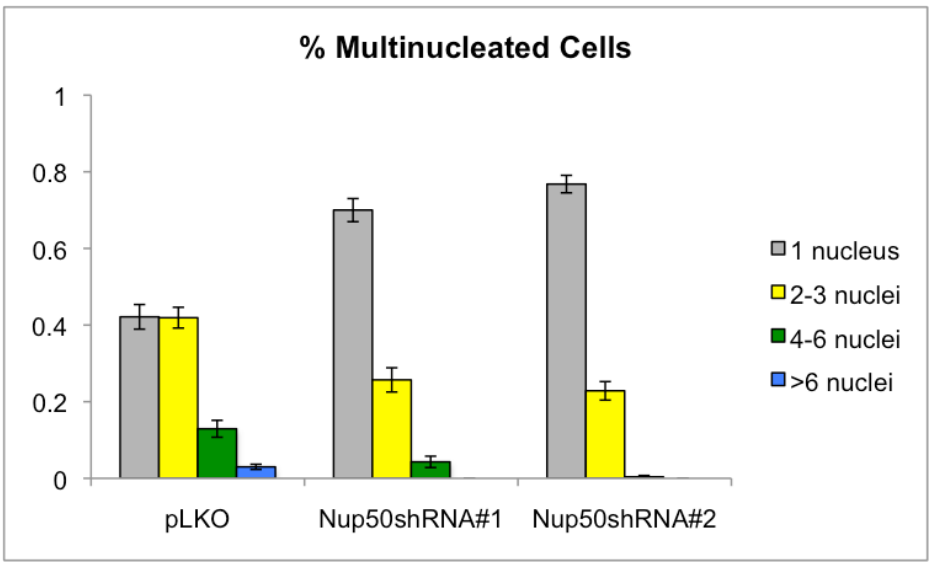


Figure 4-13 Quantification of multinucleated cells upon Nup50 knockdown. Myoblasts with two independent Nup50 knockdown (Nup50shRNA#1, Nup50shRNA#2) and pLKO knockdown (pLKO) as a negative control were differentiated for 96 hours and percentages of cells with 1 nucleus, 2-3 nuclei, 4-6 nuclei, and more than 6 nuclei were shown. Error bars show standard deviation.

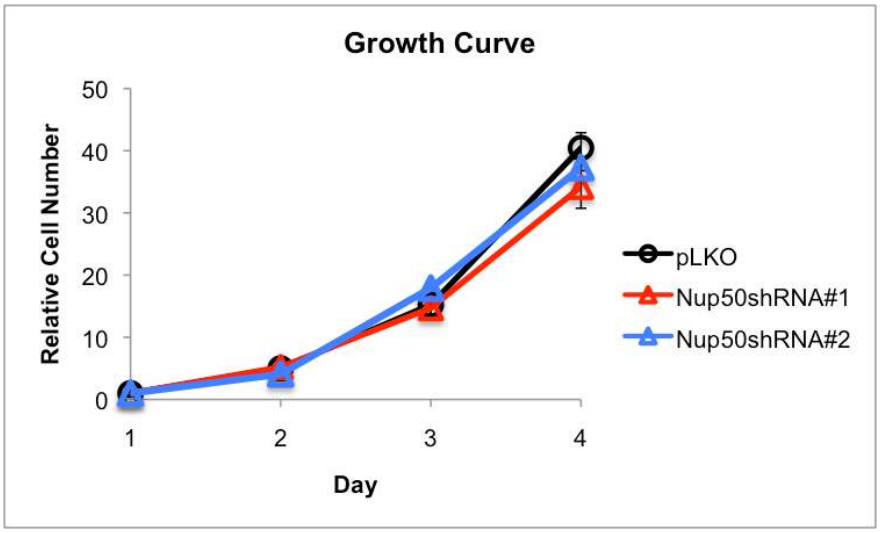


Figure 4-14 Growth curve of myoblasts upon Nup50 knockdown. 1000 myoblasts with two independent Nup50 knockdown (Nup50shRNA#1, Nup50shRNA#2) and pLKO knockdown (pLKO) as a negative control were seeded on Day 1 and cell number relative to Day 1 was shown up to Day 4. Error bars show standard deviation.

4.5 Identification of Kifc1 as Nup50-interacting Protein in Myoblasts

To identify potential interacting factors that mediate the higher dynamics of Nup50 in myoblasts, we performed tandem affinity purification of Streptavidin- and Flag-tagged Nup50 that was stably expressed in myoblasts followed by mass spectrometry to identify proteins co-purified with Nup50 (Figure 4-15). Of the Nup50 interactors found in this manner, we detected reported Nup50-interacting proteins including importin-alpha, importin-beta, and Nup153 (Figure 4-16), which validated the approach [114,115]. In addition, we identified novel Nup50 interactors such as Kifc1, Trpm 4, and Adnp. We focused on Kifc1 in later studies because of the high confidence with the interaction, suggested by high mass spectrometry match scores (Figure 4-16).

Kifc1, kinesis like motor protein 1, is a member of the C-terminal kinesin family [116] [117]. It has a kinesin motor domain at the C-terminus by Pubmed domain search, and a putative NLS (nuclear localization signal) at the N-terminus and a putative NES by software prediction (data not shown). During formation of acrosome in developing spermatids, the intracellular localization of Kifc1 varies from the nucleoplasm to acrosome and to distal cytoplasm, depending on the

developmental stage [118]. The developmental function of Kifc1 is unclear as no Kifc1 knockdown or knockout studies have been reported in developing systems.

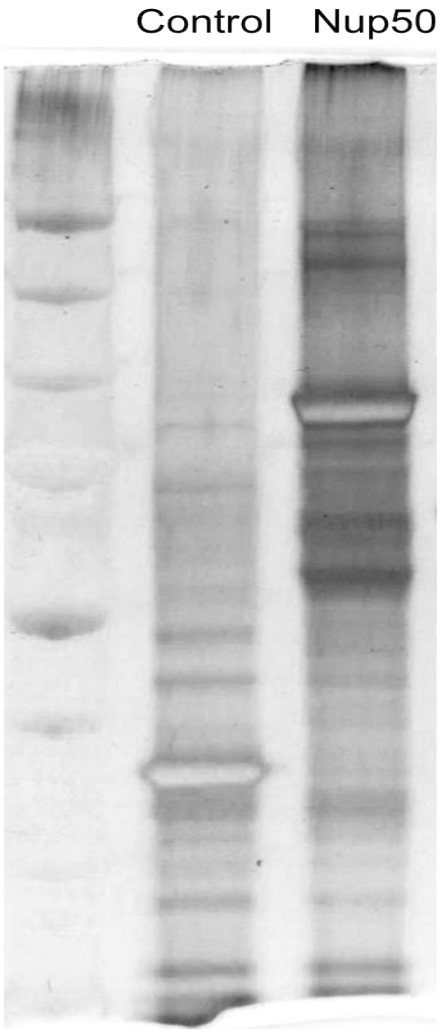


Figure 4-15 Identification of Nup50-interacting proteins by immunoprecipitation-mass spectrometry. Strepavidin- and Flag-tagged GFP as control (Control) or GFP-Nup50 (Nup50) was tandem affinity purified, and immunoprecipitant was loaded on gel and subjected to silver staining as shown.

Protein name	score	Peptide matches
Acetyl-coA carboxylase a	1897	49
Nup50	1563	200
Importin a6/7	1245	87
Importin a1/5	1047	78
Importin a2	911	70
Kifc1	506	12
Importin b1	494	15
Nup153	356	8
Importin a3	235	9
Importin a1/2	93	2
3-methylcrotonyl-CoA carboxylase a	65	1
Trpm4	46	1
Adnp(Activity-dependent neuroprotector homeobox protein)	43	1

Figure 4-16 Nup50-interacting proteins identified from mass spectrometry. Names of the proteins identified as well as score and number of peptide matches were shown.

To verify the interaction of Nup50 and Kifc1 in myoblasts, we expressed Myc- and RFP-tagged Kifc1 in undifferentiated C2C12 cells. We pulled down the overexpressed Kifc1 and found that Nup50 co-purifed with Kifc1 (Figure 4-17). By contrast, none of the other Nups tested, including Nup62, the highly mobile Nups Nup153 and Nup98, as well as the scaffold Nups Nup107 and Nup93 (Figure 4-17). This suggests the interaction of Nup50 and Kifc1 is specific.

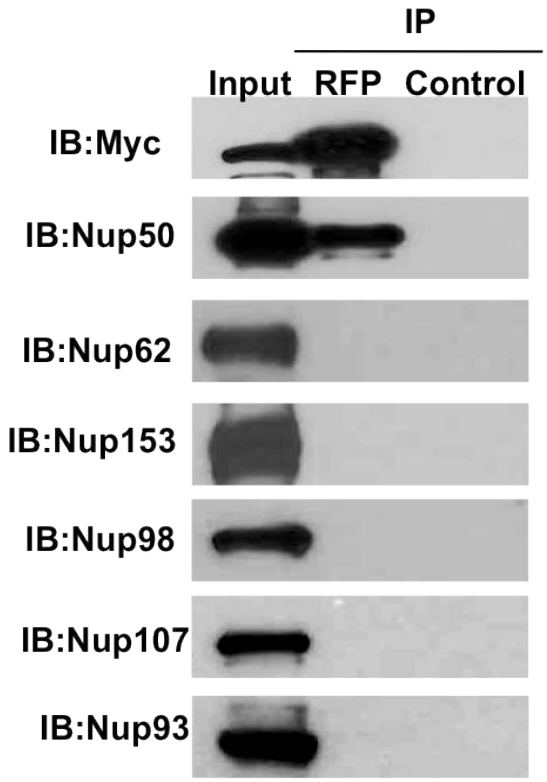


Figure 4-17 Co-IP of Nup50 and Kifc1. Myoblasts expressing Myc- and RFP-tagged Kifc1 were subjected to immunoprecipitation (IP) using the anti-RFP beads. Immunoprecipitant were immunoblotted (IB) with antibodies against Myc to detect Kifc1, and antibodies against various Nups.

Given the predicted NLS of Kifc1 and its interaction with Nup50 which is localized both to the nuclear pore complexes and nucleoplasm [119], we tested the localization of Kifc1 in myoblasts by immunofluorescence. We observed that Kifc1 was present in the nucleoplasm, but not at the nuclear pores (Figure 4-18), suggesting that Kifc1 interacts with Nup50 in the nucleoplasm. In addition, Nup50 knockdown by shRNA did not affect the localization of Kifc1 inside the nucleus, suggesting that Kifc1 localization is not dependent on Nup50 (Figure 4-18).

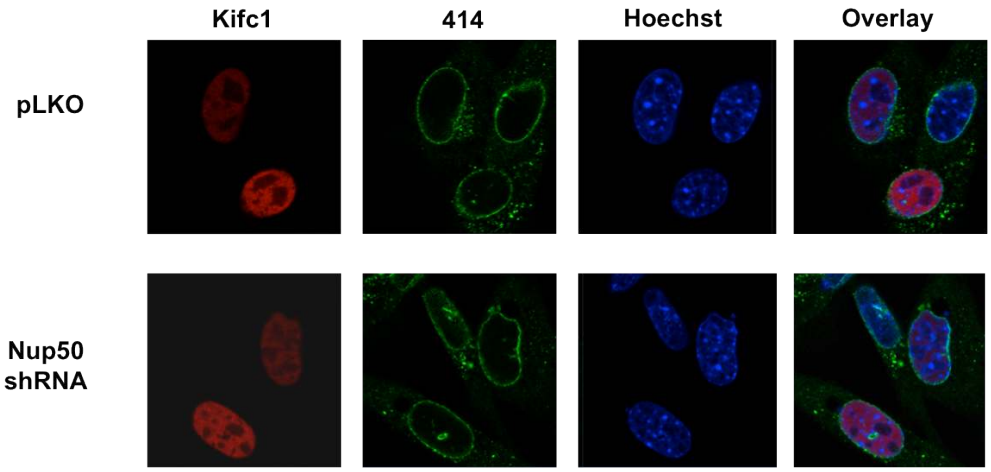
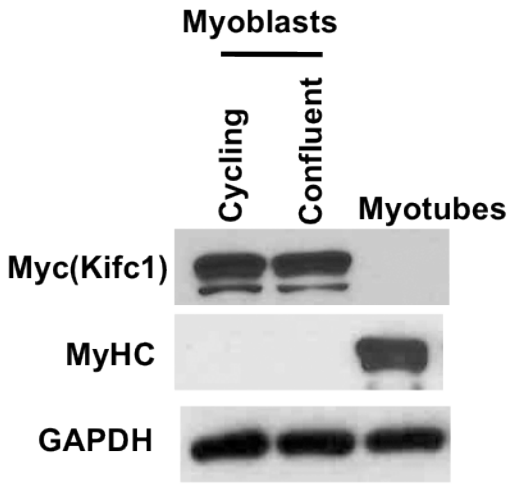


Figure 4-18 Kifc1 localization in C2C12 cells. Control (pLKO) or Nup50 knockdown (shRNA) C2C12 cells expressing RFP-tagged Kifc1 were stained with the nuclear pore marker 414 and Hoechst and visualized by confocal microscopy.

4.6 Kifc1 Level Changes during Muscle Differentiation

We followed Kifc1 during differentiation in C2C12 cells stably expressing

Myc-tagged Kifc1. We found that the protein level of Myc-Kifc1 was decreased after C2C12 differentiation (Figure 4-19). Given that the protein in question was exogenously expressed, it is possible that Kifc1 is degraded at the protein level as opposed to transcriptional regulation. To exclude the possibility that the observed decrease in protein level was due to the nature of the vector used to express the exogenous protein, we made stable C2C12 cell lines expressing GFP using the same vector (Figure 4-20). In such lines we observed no decrease in GFP levels after C2C12 differentiation, suggesting that not all exogenously expressed proteins were degraded post-differentiation. In addition, we tested the RNA level of Kifc1 expressed from the exogenous vector as well as total Kifc1 RNA levels (endogenous and exogenous), and observed no difference before and after C2C12 differentiation (Figure 4-21). This further suggests that Kifc1 is regulated at the protein level instead of the RNA level during differentiation.



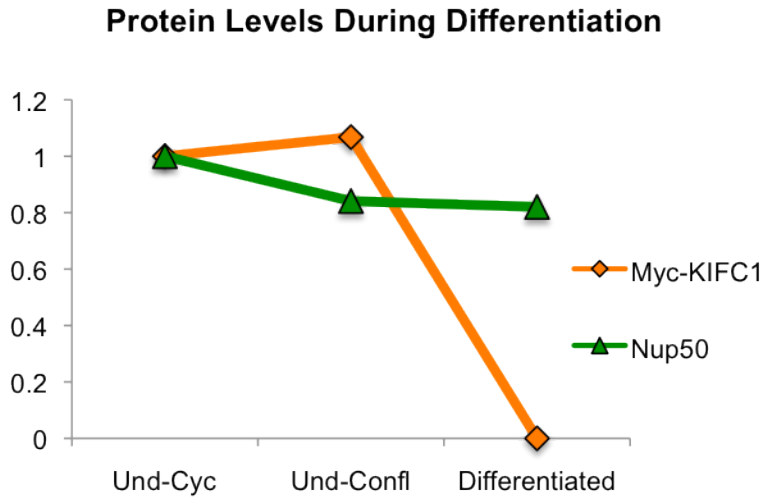


Figure 4-19 Kifc1 protein levels during differentiation. (Top) Cycling or confluent myoblasts and differentiated myotubes expressing Myc-Kifc1 were immunoblotted with anti-Myc, anti-MyHC as a marker of differentiation, and anti-GAPDH for loading control. (Bottom) Protein levels of Kifc1 and Nup50 were quantified for cycling undifferentiated myoblasts (Und-Cyc), confluent undifferentiated myoblasts (Und-Confl), and differentiated myotubes (Differentiated) from western blot experiments.

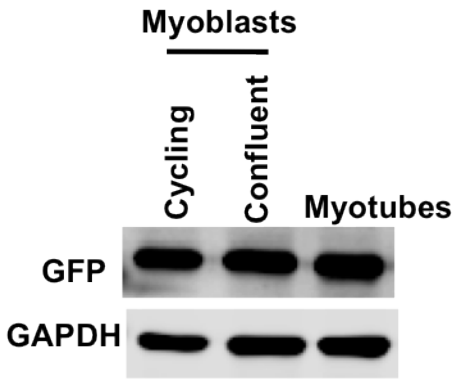


Figure 4-20 GFP protein levels during differentiation. Cycling or confluent myoblasts and differentiated myotubes expressing GFP were immunoblotted with anti-GFP and anti-GAPDH for loading control.

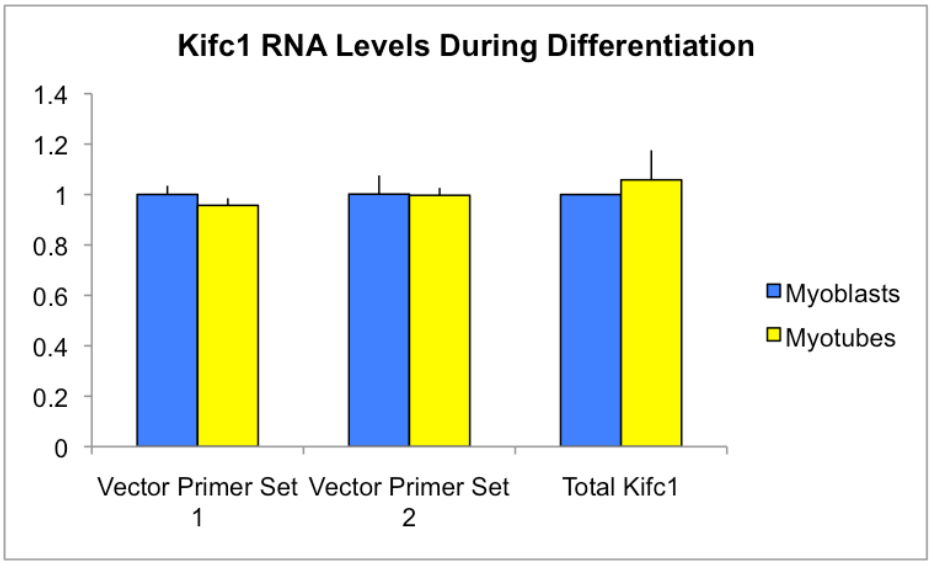


Figure 4-21 RNA levels of Kifc1 during differentiation. RNAs from undifferentiated myoblasts or differentiated myotubes were subjected qRT-PCR using two sets of primers that specifically amplify RNAs transcribed from the exogenous Kifc1 vector (Vector Primer Set1 and Vector Primer Set 2) as well as primers that recognize all Kifc1 RNA. Error bars show standard deviation.

4.7 Perturbation of Kifc1 Levels and Nup50 Dynamics

We have previously observed that 1) Nup50 dynamics is higher in myoblasts (i.e., before differentiation) than in myocytes (i.e., after differentiation); 2) Nup50 interacts with Kifc1 in myoblasts; and 3) Kifc1 protein level decreases upon differentiation. Based on these observation, we hypothesized that Kifc1 mediates the higher dynamics of Nup50 in myoblasts. To test this hypothesis, we knocked down Kifc1 by shRNA (Figure 4-22) and tested its effect on Nup50 dynamics by FRAP in myoblasts. We found that Kifc1 knockdown led to decreased Nup50 dynamics (Figure 4-23), suggesting that Kifc1 is required for high Nup50 mobility in myoblasts.

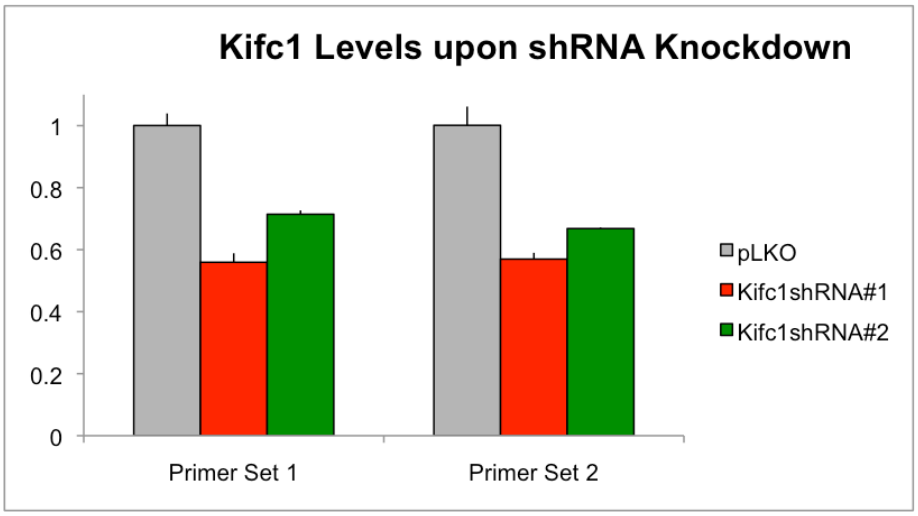


Figure 4-22 Kifc1 knockdown in C2C12 cells. C2C12 cells were knock down by two sets of shRNA (Kifc1shRNA#1, Kifc1shRNA#2) or pLKO vector as negative control (pLKO) and RNA levels of Kifc1 were studied by qRT-PCR using two primer sets (Primer Set 1, Primer Set 2). Error bars show standard deviation.

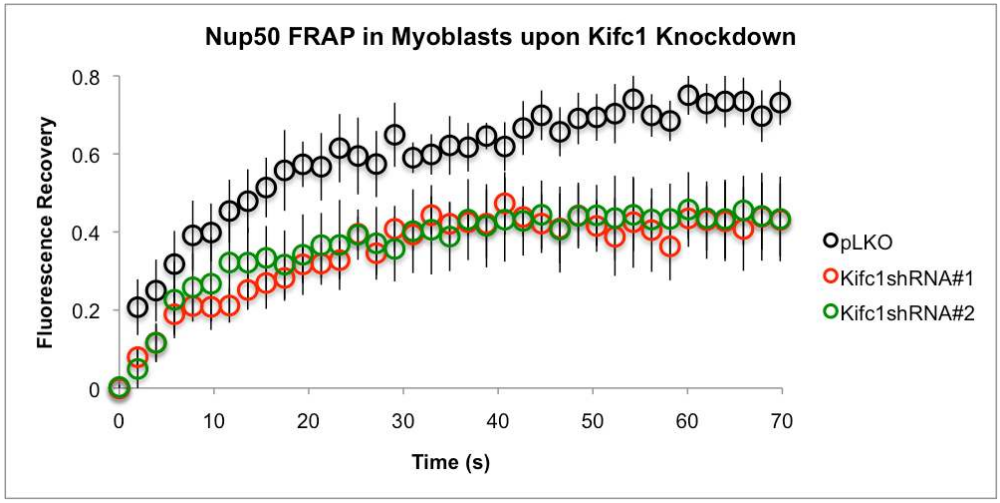


Figure 4-23 Nup50 dynamics upon Kifc1 knockdown. Nup50 dynamics were measured in myoblasts with pLKO for control, and two sets of Kifc1 shRNA (Kifc1shRNA#1, Kifc1shRNA#2) by FRAP. Error bars show standard deviation.

4.8 Perturbation of Kifc1 Levels and Efficiency of Muscle Differentiation

To test if Kifc1 is functionally relevant for C2C12 cell differentiation, we knocked down Kifc1 and examined its effects on myoblast proliferation and the differentiation of myoblast into myotubes. Kifc1 level reduction imparied proliferation of myoblasts (Figure 4-24), indicating that Kifc1 is required for the doubling of myoblasts. In addition, Kifc1 knockdown resulted in accelerated muscle differentiation (Figure 4-25, Figure 4-26), consistent with the reduction in Kifc1 protein levels during differentiation of wild-type C2C12 cells. This suggests

that Kifc1 is required in myoblasts for normal proliferation, and maintains cells in an undifferentiated state.

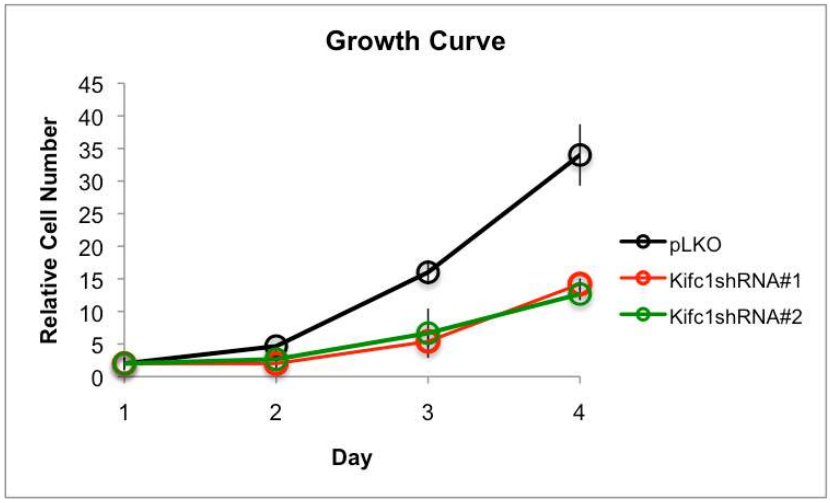


Figure 4-24 Growth curve of myoblasts upon Kifc1 knockdown. 1000 myoblasts with two independent Kifc1 knockdown (Kifc1shRNA#1, Kifc1shRNA#2 and pLKO knockdown (pLKO) as a negative control were seeded on Day 1 and cell number relative to Day 1 was shown up to Day 4. Error bars show standard deviation.

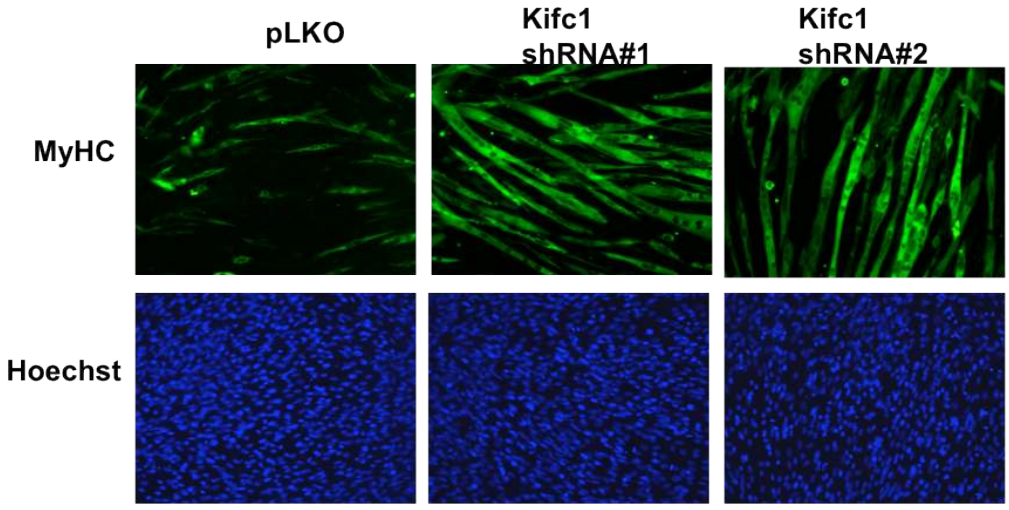


Figure 4-25 Kifc1 knockdown and efficiency of C2C12 differentiation. Myoblasts with two independent Kifc1 knockdown (Kifc1shRNA#1, Kifc1shRNA#5) and pLKO knockdown (pLKO) as a negative control were differentiated for 60 hours and cells were immunostained for MyHC in green and Hoechst in blue.

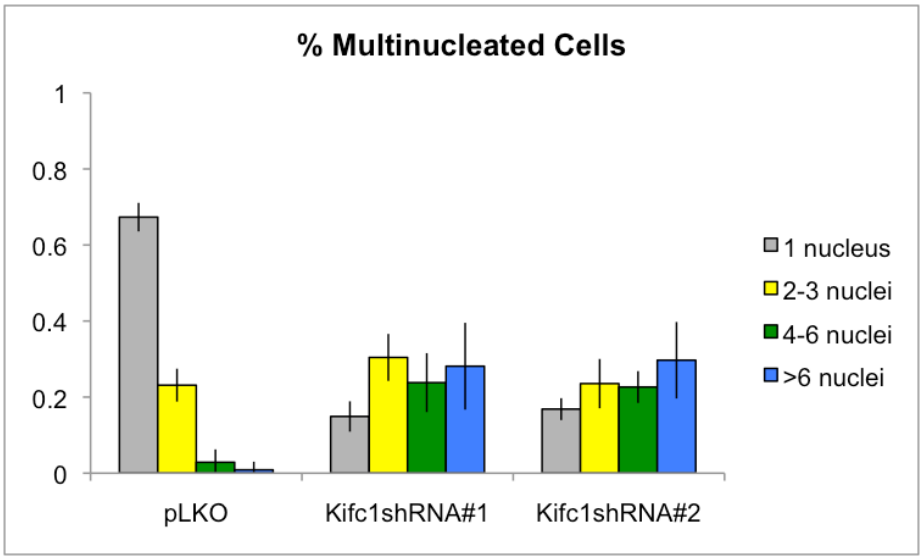


Figure 4-26 Quantification of multinucleated cells upon Kifc1 knockdown. Myoblasts with two independent Kifc1 knockdown (Kifc1shRNA#1, Kifc1shRNA#2) and pLKO knockdown (pLKO) as a negative control were differentiated for 60 hours and percentages of cells with 1 nucleus, 2-3 nuclei, 4-6 nuclei, and more than 6 nuclei were shown. Error bars show standard deviation.

4.9 Discussion

We used the differentiation of C2C12 cells as a system to investigate into the dynamic regulation of Nup50, and found a reduction in Nup50 mobility upon differentiation. Kifc1, a kinesin-superfamily protein that is predicted to be a nuclear motor, interacts with Nup50 in myoblasts and is required for the fast shuttling behavior of Nup50. Kifc1 protein levels are reduced during muscle differentiation, concomitant with a decrease in Nup50 dynamics. Based on these observations, we propose a model in which Kifc1 mediates the high dynamics of Nup50 in

myoblasts specifically (Figure 4-27).

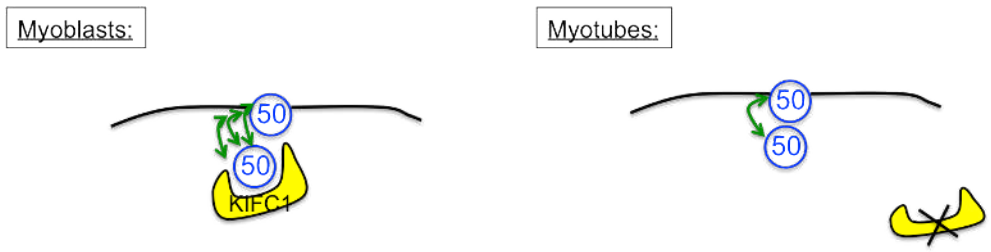


Figure 4-27 Regulation of Nup50 dynamics during muscle differentiation. Before differentiation (i.e., in myoblasts), Nup50 exhibits a fast shuttling profile, mediated by Kifc1 that directly interacts with Nup50. Upon differentiation (i.e., in myotubes), Kifc1 is reduced in protein level, resulting in slower shuttling of Nup50.

This to our knowledge represents the first example of developmentally regulated Nup dynamics as well as identification of a direct protein regulator of Nup dynamics. Intriguingly, Kifc1 specifically interacts with Nup50, but not other Nups of the nuclear pore complexes. This might help explain why Nups within the nuclear pore complex exhibit widely different dynamics [52].

It is worth noting that both Nup50 and Kifc1 are functionally relevant for muscle differentiation at least in the C2C12 system. It is currently unclear how exactly the change in Nup50 dynamics confers its functional relationship to regulation of differentiation. One possibility is the slower shuttling form of Nup50 represents a form that is more stably associated with the nuclear pore complexes and is linked to the pore-associated function of Nup50. Several

differentiation-related proteins including Par6 become associated with the nuclear periphery during the formation of myotubes from myoblasts [120]. It can be tested in the future whether particular Par6 isoforms relocate to the nuclear pore complexes at the nuclear periphery during differentiation and if that is related to the pore-associated Nup50. Alternatively, the slower shuttling form of Nup50 could represent a form that is more stably associated with intranuclear structures and molecules. One example would be the chromatin, as reported previously that Nup50 interacts with and regulates development genes in the nucleoplasm in Drosophila [51]. A third possibility is that the alterations in dynamics indirectly represent a change in the conformation of Nup50, for example change in post-translational modification, which is required for differentiation regulation by Nup50. Consistent with this possibility, several Nups are known to be phosphorylated and/or linked to ubiquitination that are independent of mitotic modifications in the disassembly of the nuclear pore complex during mitotic entry [115,121,122]. Posttranslational modification of Nup50 could potentially affect its interaction with proteins that are key to the regulation of the myogenic program.

Kifc1, a member of the kinesin superfamily, was found to have a putative nuclear localization signal and localize to the nucleoplasm. Increasing evidence supports the presence of myosin- and kinesin-family motor proteins within the cell nucleus, which might be responsible for genome organization and integrity [123].

For example, KIF4A can prevent hypercondensation of metaphase chromosome, and can also localize to DNA repair sites in interphase [124,125]. During spermatid development, nuclear KIF17B promotes export of the transcriptional co-activator ACT, thus regulating gene expression [126]. Our findings that Kifc1 regulates Nup50 dynamics and myoblast differentiation add to the functional spectrum of nuclear kinesin family proteins.

4.10 Materials and Methods

Cell Culture and Differentiation

C2C12 cells were grown in DMEM and 20% FBS. For differentiation, cells were plated at around 90% confluency and differentiated when confluency was reached by switching media to DMEM and 2% horse serum.

293T cells were grown in DMEM and 10% FBS.

Overexpression and Knockdown

For transient overexpression, C2C12 cells were transfected with the appropriate plasmids on 6-well plates at 30% confluency using Optifect according to the manufecturer's instructions.

For establishment of GFP-positive stable cell lines, 2 days after transfection on 6-well plates, C2C12 cells were split into 10cm plates and exposed to 1mg/ml of G418. Untransfected cells were G418-treated in the same

manner as negative control for selection drug. When untransfected cells were all killed by G418 treatment, resistant transfected cells were trypsinized and resuspended in PBS and 2% FBS for FACS-sorting by GFP. GFP positive cells obtained by FACS-sorting were plated in 6-well plate if cell number was less than 10,000. Cells were plated in 10cm plate if cell number was more than 10,000. Selected cells were maintained in DMEM, 20%FBS, and 0.5mg/ml G418.

For shRNA-mediated knockdown, lentiviral vectors with appropriate shRNA sequences were obtained from Open Biosystems. To produce lentiviruses, 293T cells were transfected in 15cm plates with 2ug shRNA vector, 7.5ug pMDLg/pRRE, 7.5g pRSV/REV, and 5ug pMD2.G using 500ul of Optimem II and 88ul PEI at 1ug/ul in PBS, pH4.5. 48 hours post transfection, 2.5ml of media was filtered through a 0.22um low protein binding filter, mixed with 2.5ul of 6mg/ml polybrene, and added to C2C12 cells at 30% confluency on a 6-well plate. This was repeated at 72 hours post transfection of 293T cells. 96 hours post transfection of 293T cells, C2C12 cells were split to 10cm plate and grown with DMEM, 20%FBS, and 2ug/ul puromycin. Uninfected cells were drug selected in the same manner as negative control for puromycin. When uninfected cells were all killed by puromycin treatment, resistant infected cells were tested for knockdown by qRT-PCR or western blot.

Western Blot

Cells were collected by scraping and directly lysed by boiling in 2x Laemmli Sample buffer (126mM Tris-HCl pH6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue) with 200mM DTT at 95°C for 10 minutes. Lysates were run on a SDS-PAGE gel and transferred to membranes. Membranes were blocked with PBS-0.05Tween 20 (PBST) and 5% nonfat milk for 1 hour, incubated in PBST, 5% milk and primary antibody overnight at 4°C. Membranes were washed 3 times with PBST, incubated with PBST, 5% milk, and secondary antibody for 1 hour at room temperature, washed 3 times with PBST, and analyzed with an Odyssey infrared imaging system or developed on a film.

Immunofluorescence

Cells were cultured in 8-well Ibidi plates (#80826), fixed with 4% PFA in PBS for 5 minutes, washed in IF buffer (PBS, 1% BSA, 0.1% Triton-X100) and blocked in IF buffer for 20 minutes. Primary antibody in IF buffer was applied to cells for 1 hour. Cells were washed 3 times with IF Buffer, incubated with secondary antibody in IF buffer for 1 hour, washed 3 times with IF Buffer, and stained with Hoechst in PBS for 5min. After Hoechst staining, cells were kept in PBS and imaged with a Leica SP2 confocal microscope and a Zeiss Axio Observer Z1 motorized microscope.

Fluorescence Recovery After Photobleaching (FRAP)

Cells were cultured in 8-well Ibidi plates (#80826) and FRAP was performed with cells in normal culture condition (DMEM and 20%FBS, 5% CO_2, 37°C) on a Leica SP2 confocal microscope. Region to be bleached was selected on the Leica microscope software. FRAP settings were 4 frames prebleach, 6 frames bleach, and 30 frames postbleach, with time per frame dependent on the specific Nup studied. Fluorescence intensity files were exported and analyzed in Excel.

Tandem Affinity Purification

15 15cm plates of C2C12 cells at 80% confluency were used for tandem affinity purification. Cells were collected in PBS by scraping and lysed in 15ml of ice-cold lysis buffer (50mM Tris-HCl pH8, 150mM NaCl, 10% glycerol, 0.5% Triton X-100) with protease and phosphatase inhibitor tablets on ice for 30 minutes. Lysate was cleared for precipitation by centrifugation and incubated with 250ul of Streptavidin resin pre-balanced in TBS pH7.4 on a turning wheel for 3 hours at 4°C, packed onto a column, washed on column with 10ml of wash buffer (50mM Tris-HCl pH8, 150mM NaCl, 1mM EDTA and 0.1% NP40) at 4°C, and eluted with 1ml of Biotin elution buffer (40mM Tris-HCl pH7.5, 150mM NaCl, 0.1% NP-40, 6mM d-Biotin, and 0.5mM EDTA). Eluate were incubated with 500ul of Flag-M2 agarose pre-balanced in TBS pH7.4 on a turning wheel for 1 hours at 4°C, packed onto a column, washed on column with 10ml of wash buffer at 4°C, and eluted

with 1ml of Flag elution buffer (20mM Tris-HCl pH7.4, 150mM NaCl, 10% glycerol, 0.5mM EDTA, 200ng/ul Flag peptide). To TCA precipitate the proteins in the eluate, 150ul of 100% TCA was added to eluate. Then samples were vortexed vigorously for 1 minute and incubated at 4°C overnight. Samples were spun at maximum speed in a benchtop centrifuge at 4°C for 20 minutes. Supernatant was removed and precipitate was washed with 1ml cold acetone, dried and subjected to subsequent analysis such as SDS-PAGE and silver staining and mass spectrometry.

Co-immunoprecipitation

1 10cm-plate of cells were collected in PBS and lysed in lysis buffer (50mM Tris-HCl pH8, 150mM NaCl, 10% glycerol, 0.5% Triton X-100) with protease and phosphatase inhibitor tablets on ice for 30 minutes. Lysate was cleared for precipitatant by centrifugation and incubated with primary antibody followed by protein-A agarose or GFP/RFP-Trap agarose for 2 hours at 4°C. Agarose was collected by centrifugation and washed with wash buffer (50mM Tris-HCl pH8, 150mM NaCl, 1mM EDTA and 0.1% NP40) at 4°C 3 times. Agarose was boiled in 2x Laemmli Sample buffer (126mM Tris-HCl pH6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue) with 200mM DTT at 95°C for 15 minutes and analyzed by SDS-PAGE and western blot.

RNA Extraction and RT-PCR

RNA was extracted from cells using Trizol (Ambion) and column purified using Rneasy kit (Qiagen). RNA was subsequently reverse transcribed using the QuantiTect reverse transcription kit (Qiagen) and cDNA was subjected to qPCR using SYBR Green PCR Master Mix (AppliedBiosystems).

Chapter 5 Conclusions and Potential Future Directions

5.1 Conclusions

As discussed in Chapter 2 and 3, we have explored the function of nuclear pore proteins (Nups) in differentiation and found that in addition to their well-established roles in mediating transport, Nups can be involved in the regulation of developmental genes in at least *Drosophila* and human cells. Gene regulation by Nups occurs not only at the nuclear pore complexes at the nuclear periphery, but also at intranuclear sites. The nuclear pore complexes appear to act as a platform that supports the initial induction of some developmental genes before the genes move to intranuclear sites when they are fully activated later in development.

Considering one specific nuclear pore component Nup98, which is implicated in gene fusion in multiple types of leukemia [93], its association with the genome is dynamic both in *Drosophila* and human cells. In *Drosophila* salivary gland cells whose chromatin is in the polytene form, Nup98 becomes associated with developmentally activated loci at specific developmental stages [44]. This pattern was also observed in human diploid cells during neural differentiation of embryonic stem cells. Nup98 target genes were found to be enriched for neural

developmental genes at the neural progenitor stage specifically, not in embryonic stem cells, neurons, or lung fibroblasts. This suggests that the interaction between Nup98 and chromatin is dynamic.

It is worth noting that not all Nup98 target genes in all cell types are active. In human embryonic stem cells for example, Nup98 interacts with a group of active genes and a group of silent genes. This is similar to the reported observation in *Drosophila* embryonic cells, Nup98 target genes include not only highly expressed genes that are functionally similar to the active Nup98 targets in human embryonic stem cells, but also repressed genes [51]. In *Drosophila*, the distinction was made based on the localization of Nup98 target genes. The pore-Nup98 associated genes are silent, whereas the nonpore-Nup98 target genes are active. It remains to be tested whether in human cells similar correlation can be made.

In human neural progenitor cells, from the examination of 6 Nup98 binding, developmentally-induced genes, it also seems that the pore-associated Nup98 targets have lower expression induction levels compared to the non-pore targets. The pore targets are at the initial stage of developmental induction (i.e. slightly activated), whereas the non-pore targets are highly activated. This seems reminiscent of the finding in *Drosophila* that pore-targets have lower expression levels compared to nonpore targets [51], and reveals a previously unidentified role

of nuclear pore complexes at the nuclear periphery in supporting the initial induction of developmental genes.

Why are the nuclear pore complexes at the nuclear periphery associated with initial gene induction in progenitor cells? One hypothesis is that the nuclear pore complexes are involved in the re-establishment of chromosome architecture during nuclei reformation in mitosis. During cell cycle, in M phase, most transcription factors are lost from the condensed, mitotic chromosomes and this composes a window allowing for cell fate reprogramming [102-104]. Upon mitosis exit, chromatin has to be decondensed in a manner that faithfully restores the correct states (active or silent) for individual chromatin subdomains to maintain the progenitor status of the cells. Intriguingly, during mitosis exit, many components of the nuclear pore complexes are among the first proteins to contact chromatin and indeed are indispensable for proper decondensation of chromatin [21,105-107]. Therefore, the nuclear pore complex may capture the developmentally induced genes during mitosis exit, and establish decondensed chromatin landscape on those sites, a ‘memory’ of gene activating status in the previous cell cycle. Several evidences are in line with this notion. Association with the nuclear pore complex in yeast was found to convey ‘gene memory’ function, which is defined as rapid re-induction in repeated transcription stimulation cycles [47,108]. In *Drosophila,* Nup98 is also required for faithful re-initiation of

transcription after heat shock [44]. Therefore, nuclear pore complexes might have a role in maintaining transcription memory of developmental genes in cell cycle.

Compared to the nuclear periphery, intranuclear sites could represent environment that is more optimal for highly activated genes because the nuclear lamina is largely associated with silent chromatin regions [127]. That might be one reason why highly activated genes were found to associate with Nup98 inside the nucleoplasm.

Perturbation in functional levels of Nup98 resulted in changes in mRNA levels of Nup98-binding genes. This suggests that Nup98 is functionally relevant for the transcript level of its target. It is not known yet whether the regulation occurs on the rate of mRNA production and/or its stability. In addition, it is not known whether Nup98 regulates the splicing and export of mRNA. Investigation into those various aspects of RNA biology will help understand the role of Nups in gene regulation and its potential function in coordinating transcription with splicing and export in higher organisms, as implicated by yeast studies.

As discussed in Chapter 4, investigation into the dynamics of Nups during muscle differentiation revealed that Nups can exhibit changes in dynamic behavior at different developmental stages. The mobility of Nup50 decreases, whereas that of Nup98 increases during muscle differentiation. This adds a layer to the cell-specific regulation of Nups, in addition to alterations in their expression

levels.

The study also revealed an unexpected link between a nuclear pore protein Nup50 and Kifc1, a nuclear protein of the kinesin superfamily. In myoblasts, Kifc1 interacts with Nup50, but not other Nups tested including the peripheral Nups Nup62, Nup153, Nup98 and the scaffold Nups Nup107 and Nup93. In addition, Kifc1 is localized in the nucleoplasm instead of the nuclear pore complexes, suggesting that Kifc1 specifically interacts with Nup50 inside the nucleoplasm. This is consistent with the hypothesis that Kifc1 interacts with Nup50 to promote its disassociation from the nuclear pore complexes, causing a fast shuttling pattern. The observations that Kifc1 does not interact with other Nups and that Nup98 and Nup50 exhibit distinct changes in dynamics during differentiation are in line with the idea that mobility of different Nups are regulated by distinct mechanisms.

In myoblasts, in addition to the regulation of Nup50 dynamics, Kifc1 has additional functions in maintaining the highly proliferative profile of cells. By contrast, Nup50 knockdown in myoblasts did not cause significant deficiency in proliferation. This suggests that Kifc1 has other partners to mediate its function in proliferation, which could on mitotic spindles as reported [128-130] or nucleoplasmic in interphase that is to be tested.

During differentiation, Kifc1 levels are reduced, allowing differentiation to occur efficiently. The reduction in Kifc1 levels could partially explain the decrease in Nup50 mobility. The slower shuttling form of Nup50 has a differentiation-promoting role, suggested by the observation that Nup50 knockdown impaired differentiation. The mechanism of regulation of muscle differentiation by Kifc1 and Nup50 is unclear. One hypothesis is the slower shuttling form of Nup50 might represent a conformation that allows Nup50 to stably associate with chromatin and regulate expression of genes important for differentiation. This would be consistent with the finding in Drosophila Nup50 is associated with developmental genes [51]. Kifc1 might have functions in gene regulation as well, based on the observations that several nuclear motors have been shown to affect multiple facets of gene expression, from histone modification to movement of gene loci as ‘cargos’ [123]. There also exists the possibility that Nup50 and/or Kifc1 is important for the nuclear import/export of proteins important for differentiation. Although many details of the regulation process remain to be examined, these observations shed light on the dynamic interplay between nuclear pore proteins and nuclear motor proteins during cell differentiation.

5.2 Potential Future Directions

Mechanism of Gene Regulation by Nups

Nups are found to interact with and regulate expression of genes in a development-independent manner. However, it is not known how the gene regulation by Nups is achieved mechanistically. It will be interesting to use biochemical approaches to identify the interacting partners of Nups that are known to be transcription regulators. Similar approaches can also be used for Nups that lack the pore-binding domain to compare the partners of the on-pore and off-pore Nups. It is worth noting that the original gene gating hypothesis proposed that genes interact with Nups to promote export of their transcripts [42]. However, protein complexes containing Nups, transcription factors, and export factors at the same time have not been described. It remains to be investigated what factors bridge or coordinate transcription with export and whether Nups regulate transcription, mRNA stability and/or export at the same time.

Significance and Mechanism of Gene Movement during Development

Several neural developmental genes showed repositioning from the nuclear periphery to the interior during the differentiation of neural progenitor cells to neurons. The observed extent of repositioning was significant, opening the possibility that the repositioning event might be functional relevant for gene expression. Using artificially tethered genes, studies regarding the functional significance of gene movement relative to the nuclear periphery have resulted in ambiguous and somewhat contradictory results [62]. A potentially complicating

factor of these studies is that the locus investigated was mostly random in the sense that any locus inserted with reporter arrays was visualized [101,131,132]. The genetic nature of the locus and its neighborhood may affect the effects of repositioning on gene expression. Therefore, it is worth studying the functional significance of repositioning of an endogenous gene, or at least sequences from the endogenous gene.

Endogenous gene locus could be theoretically visualized by insertion of reporter sequences by zinc finger-mediated homologous recombination [133]. Alternatively, sequences from the locus of interest could be cloned to a plasmid attached to additional reporter sequences. Once an endogenous gene locus could be visualized in real time, one can ask what factors affect gene repositioning during a normally occurring situation such as neural differentiation by shRNA library screening and subsequently, what is the functional consequence of inability of repositioning.

A descriptive question related to gene repositioning that might also be interesting is, for a developmental gene that relocates, are there other genes that co-migrate with it? The 3-dimensional organization of genome allows contact of genes that are not close to each other linearly on the same chromosome [134,135]. It is tempting to speculate that genes clustered spatially are co-regulated. Therefore, to test this idea, it might be interesting to identify the

cluster of genes that are in contact of the relocating developmental genes at different developmental stages and examine their behavior and mode of regulation. Such genes could be identified using high throughput chromosome conformation capture techniques using the target developmental genes that are known to reposition as bait.

Function of Nuclear Motor Proteins

Kifc1 was found to interact with Nup50 and regulate its dynamics. Kifc1 is a member of the kinesin superfamily that is present in the nucleus. There have been some speculations as to the ‘cargo’ of such nuclear motor proteins, for example, chromatin [123]. In our system where Kifc1 regulates the mobility of a nuclear pore protein, it remains to be examined if this role bears any similarity to the cargo-moving function of classical kinesins. For instance, there is no identified nuclear microtubule [123], so it needs to be tested if Kifc1 diffuses freely or ‘walk’ on any nuclear filament structures. In addition, it is unclear to what extent Kifc1 function depends on the ATPase and other domains that are conserved with classical kinesins, and whether they can be swapped with corresponding domains of cytoplasmic kinesins.

One can also search for additional interacting partners of Kifc1 by approaches such as co-IP-mass spectrometry because as mentioned above, chromatin is on top of the list of potential cargoes for the nuclear motors [123].

Nup50 has previously been found to associate with developmental genes and regulate their expression [51]. Therefore, there exists the possibility that Kifc1 could be one of the links between Nups and genes. From the standpoint of differentiation, it is not known how a nuclear motor protein can regulate development. Therefore, it will also be interesting to see how Kifc1 maintains proliferation and prevents differentiation of progenitor cells by identifying its interacting partners.

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