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Nucleoporins Directly Stimulate Expression of Developmental and Cell-Cycle Genes Inside the Nucleoplasm

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SUMMARY

Nuclear pore complexes (NPCs) mediate transport across the nuclear envelope. In yeast, they also interact with active genes, attracting or retaining them at the nuclear periphery. In higher eukaryotes, some NPC components (nucleoporins) are also found in the nucleoplasm, with a so far unknown function. We have functionally characterized nucleoporin-chromatin interactions specifically at the NPC or within the nucleoplasm in *Drosophila*. We analyzed genomic interactions of full-length nucleoporins Nup98, Nup50, and Nup62 and nucleoplasmic and NPC-tethered forms of Nup98. We found that nucleoporins predominantly interacted with transcriptionally active genes inside the nucleoplasm, in particular those involved in developmental regulation and the cell cycle. A smaller set of nonactive genes interacted with the NPC. Genes strongly interacting with nucleoplasmic Nup98 were downregulated upon Nup98 depletion and activated on nucleoplasmic Nup98 overexpression. Thus, nucleoporins stimulate developmental and cell-cycle gene expression away from the NPC by interacting with these genes inside the nucleoplasm.

INTRODUCTION

In eukaryotes, the nuclear envelope forms a barrier between the cytoplasmic and nucleoplasmic compartments of the cell. It consists of a double lipid bilayer permeated by nuclear pore complexes (NPCs). Inner and outer nuclear membranes contain specific sets of proteins, of which some of those interacting with the inner nuclear envelope function in gene regulation, both by regulation of transcription factors (Heessen and Fornerod, 2007) and by directly regulating chromatin (Stewart et al., 2007). In higher eukaryotes, the inner nuclear membrane is lined with a meshwork of intermediate filaments, the nuclear lamina. Transport across the nuclear envelope occurs through NPCs (Tran and Wente, 2006). Chromatin interaction with the nuclear envelope can be identified both at the nuclear lamina and at NPCs (Marshall, 2002). Recent data clearly indicate that the nuclear lamina interacts with inactive chromatin (Guelen et al., 2008; Pickersgill et al., 2006) and plays a role in gene silencing (Finlan et al., 2008; Reddy et al., 2008), as genes tethered to the nuclear lamina were silenced and depletion of Lamin in *Drosophila* caused a Lamin-associated, repressed gene to become active (Shevelyov et al., 2009). Less is known about a direct role of the NPC in gene expression, particularly in higher eukaryotes.

The ''gene-gating'' hypothesis proposed that active genes associate with NPCs to increase the efficiency of nuclear export of transcribed mRNA (Blobel, 1985). Indeed, in yeast it was found that the constituents of the NPC, nucleoporins, interact with active genes (Casolari et al., 2004) and that certain genes are more frequently observed at the nuclear envelope upon activation (Brickner and Walter, 2004; Casolari et al., 2005; Casolari et al., 2004; Taddei et al., 2006). However, this is certainly not always the case (Taddei et al., 2006). Also, the spatial restriction of chromatin movement in the interphase nucleus (Chubb et al., 2002; Marshall et al., 1997) makes it improbable that genes must relocalize to the NPC in order to be activated, which conceptually limits the usage of gene gating.

Furthermore, a complicating factor is that, at least in higher eukaryotes, many nucleoporins are mobile and found inside the nucleoplasm, with so far unknown function. In particular, this has been shown for Nup50 and Nup153 (Daigle et al., 2001; Lindsay et al., 2002; Rabut et al., 2004; Smitherman et al., 2000), Nup98 (Enninga et al., 2002; Powers et al., 1995), and the *Drosophila* nucleoporin Tpr/Megator (Zimowska et al., 1997). This makes it difficult to ascertain whether nucleoporin-chromatin interactions actually take place at the NPC.

Several observations indicate that the nucleoplasmic pool of nucleoporins has a role in transcription. First, the mobility of nucleoplasmic GFP-Nup98 is decreased by addition of the RNA polymerase II inhibitor actinomycin D (Griffis et al., 2002). Second, oncogenic fusion proteins of nucleoporins and several different nuclear proteins, which can cause human leukemias, are able to activate or repress target genes within the nucleoplasm (Bai et al., 2006; Kasper et al., 1999; Lam and Aplan, 2001; Wang et al., 2007).

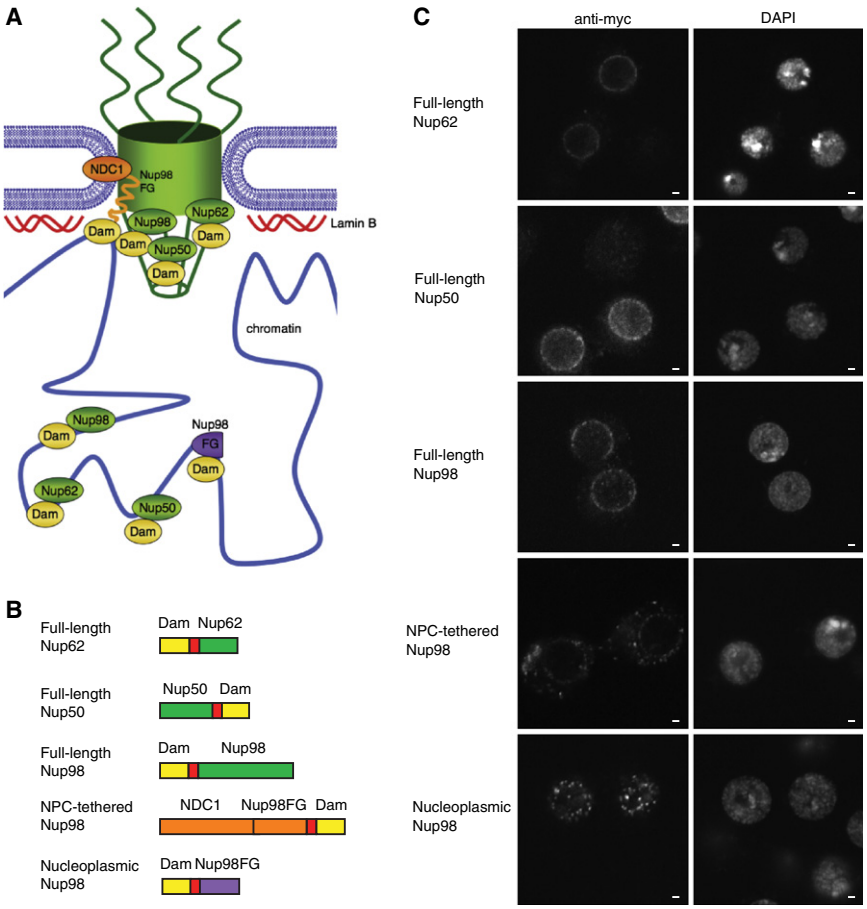


Figure 1. Expression of Fusions of Nucleoporins and *E. coli* Dam Methylase

(A) Experimental setup.

(B) Nucleoporin-Dam fusion constructs used for the generation of nucleoporin-chromatin maps. Fusion proteins were detected by a myc tag (red box).

(C) Localization of the nucleoporin-Dam fusion proteins upon transfection. Nucleoporin-Dam fusion proteins were detected with anti-myc antibody (left panels), and cells were counterstained with DAPI (right panels). Nucleoplasmic Nup98 is in part present in nuclear "GLFG bodies," specific nuclear compartments with unknown function (Griffis et al., 2002). These bodies do not overlap with known nuclear body markers, and may be a specialized recycling compartment. We did not find enrichment of RNA polymerase II in these bodies (data not shown). Scale bars represent 1 μm.

See also Figure S1.

Together, these data suggest that nucleoporins may regulate gene expression at the NPC as well as within the nucleoplasm. In this study, we aimed to characterize nucleoporin-chromatin interactions and distinguish between those at the NPC and within the nucleoplasm. We find that Nup98, Nup50, and Nup62 associate directly with a similar set of active genes inside the nucleoplasm. Depletion of Nup50 or Nup98 reduced gene expression preferentially of genes associated with these nucleoporins, while overexpression of a nucleoplasmic version of Nup98 led to preferential upregulation of these genes. Nup98-responsive genes are strongly enriched in genes involved in developmental regulation and the cell cycle, in particular the mitotic spindle checkpoint. We conclude that nucleoporins have a functional role inside the nucleoplasm in regulating expression of these genes.

RESULTS

Three Nucleoporins Interact with a Similar Set of Genes

To investigate nucleoporin-chromatin interactions, we generated genome-wide interaction maps of three nucleoporins, Nup98, Nup62, and Nup50, in embryonic *Drosophila* cells using the in vivo mapping technique DamID. In this technique, proteins of interest are fused to *E. coli* Dam methylase and expressed in living cells at very low (trace) levels. This leads to adenine methylation of genomic loci bound by the protein of interest, which are subsequently identified by microarray analysis (van Steensel et al., 2001). This technique has been extensively used as an alternative to ChIP-on-CHIP methods to map the interactions of, e.g., c-Myc, Polycomb, and Lamin B (Greil et al., 2006).

For the DamID chromatin interaction experiments, only trace levels of the Dam fusion proteins were expressed to avoid overexpression effects (i.e., heat shock promoter without heat shock). For visualization of expression and localization by immunofluorescence microscopy, the constructs were overexpressed by heat shock at much higher levels. All three Dam-nucleoporin fusion proteins (Figures 1A and 1B) localized at the nuclear periphery and throughout the nucleoplasm (Figure 1C). This is not unexpected, as vertebrate Nup98 and Nup50 are known to localize both at the NPC and in the nucleoplasm (Powers et al., 1995; Rabut et al., 2004; Smitherman et al., 2000). Indeed, endogenous *Drosophila* Nup98 and Nup50 (we could not obtain utilizable antibodies to *Drosophila* Nup62) were specifically detected both in the nucleoplasm and at the nuclear periphery by immunofluorescence (Figure S1 available online). In highly overexpressing cells, the Dam-Nup98 fusion was also seen in intranuclear bodies, which have been described in vertebrate cells as GLFG bodies (Griffis et al., 2002). We conclude that the Dam-nucleoporin fusions mimic the normal localization of the respective nucleoporins.

We next examined which genomic loci had been methylated by the trace levels of nucleoporin-Dam fusion proteins by means of complementary DNA (cDNA) microarrays, representing 60% of *Drosophila* genes (Pickersgill et al., 2006) (Table S2). Inspection of linear maps of Nup98, Nup62, and Nup50 interaction along the chromosome revealed a high degree of correspondence in chromatin interactions between the three nucleoporins (Figure S2A). Indeed, pairwise correlations between the datasets

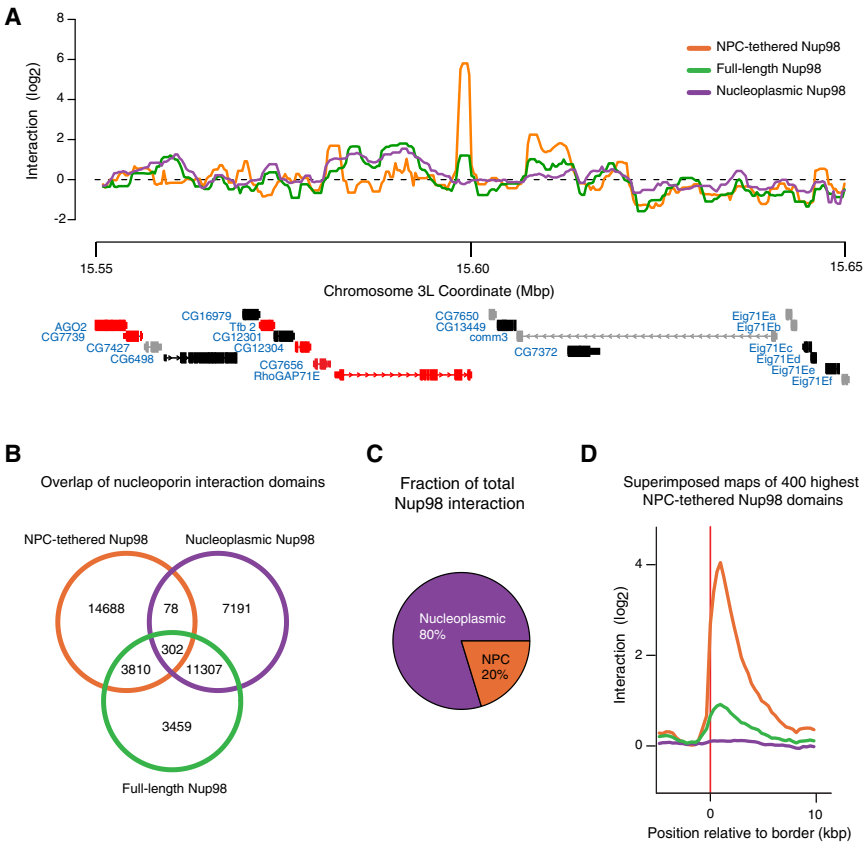


Figure 2. Two Predominantly Distinct Pools of Nucleoporin-Chromatin Interactions at the NPC and in the Nucleoplasm

(A) Interactions of full-length Nup98 (green), nucleoplasmic Nup98 (purple), and NPC-tethered Nup98 (orange) on a 100 kb region on chromosome 3L. Data were obtained using high-density oligonucleotide arrays and smoothed according to the Experimental Procedures. Genes present in this region are depicted with active genes in red (mRNA levels > median) and other genes in black or gray (not on expression array).

(B) Overlap between the 5% highest scoring probes reporting interaction with full-length Nup98, nucleoplasmic Nup98, and NPC-tethered Nup98.

(C) Proportion of Nup98 interaction in NPC and nucleoplasmic chromatin domains. The two types of domain within the Nup98 data were separated and interactions were calculated as described in Figure S2D.

(D) Superimposed chromatin interaction maps of 400 highest scoring NPC-tethered Nup98-interacting domains, aligned at left and flipped right borders.

See also Figures S2 and S3.

are high to very high (r = 0.51 to 0.78, Figure S2B). Thus, the three nucleoporins interacted with similar sets of genes.

Two Pools of Nucleoporin-Chromatin Interactions: At the NPC and Inside the Nucleoplasm

To distinguish between interactions that take place at the NPC and inside the nucleoplasm we generated (1) a fusion of Dam with a nucleoplasmic version of Nup98, lacking the NPC-interacting domain (Griffis et al., 2002), and (2) a fusion of Dam with a NPC-tethered version of Nup98. The latter consisted of the N-terminal part of Nup98 fused to the integral membrane nucleoporin NDC1 (Figure 1A) (Stavru et al., 2006). Nucleoplasmic Nup98 only detectably localized in the nucleoplasm, partly throughout the nucleoplasm and partly in Nup98 bodies (as described above for full-length Nup98) (Figure 1C) (Griffis et al., 2002). NPC-tethered Nup98 only detectably localized at the nuclear envelope and was absent from the nuclear interior (Figure 1C).

We then compared chromatin interactions of full-length Nup98 with those of NPC-tethered Nup98 and nucleoplasmic Nup98 using high-density arrays, covering the nonrepetitive *Drosophila* genome at 300 bp resolution (Choksi et al., 2006) (Table S2). Chromatin interaction profiles of full-length Nup98 showed similarities with those of both nucleoplasmic Nup98 (r = 0.75) and NPC-tethered Nup98 (r = 0.57, Figure 2B and Figure S2C), while chromatin interactions of NPC-tethered Nup98 showed a low correlation with those of nucleoplasmic Nup98 (r = 0.26). We observed large chromatin domains (Figure 2A, median 9 kb, Figure S3A) where both nucleoplasmic Nup98 and full-length Nup98 interacted. These domains usually (69%) spanned genes (Figure S3B) that were rather large (Figure S3C) and were not significantly enriched at the 5′ or 3′ end of genes (Figure S3D). In contrast, NPC-tethered Nup98 and full-length Nup98 interaction overlapped on other, much smaller domains (median 2 kb, Figure S3A). These small domains occur with equal probability in intergenic regions (57%) or within genes (43%, Figure S3B), which are very large (Figure S3C). In genes, NPC-interacting domains preferentially occur at their 3′ ends (Figure S3D). To estimate the fractions of chromatin interacting with Nup98 at the NPC versus in the nucleoplasm, we defined Nup98-interacting domains (see the Experimental Procedures), and we observed that they are easily separable into NPC-bound and nucleoplasmic groups (Figure S2D), which account for approximately 20% and 80% of Nup98 interaction, respectively (Figure 2C). As a further illustration, when we align the 400 strongest NPC-tethered Nup98 domains, these are clearly enriched in full-length Nup98 interaction but not in nucleoplasmic Nup98 interaction (Figure 2D). Thus, there are two distinct pools of nucleoporin-chromatin interactions, one at the NPC and one inside the nucleoplasm.

To confirm the existence of the two distinct pools of nucleoporin-chromatin interactions, we performed double-blind fluorescent in situ hybridization (FISH) using four probes containing chromosomal regions interacting with nucleoplasmic Nup98 and four probes containing regions interacting with NPC-tethered Nup98 (Table S1). Nucleoplasmic, NPC-tethered, and full-length Nup98 interaction profiles within the FISH probes are depicted in Figure S4A, and representative single-cell pictures are shown in

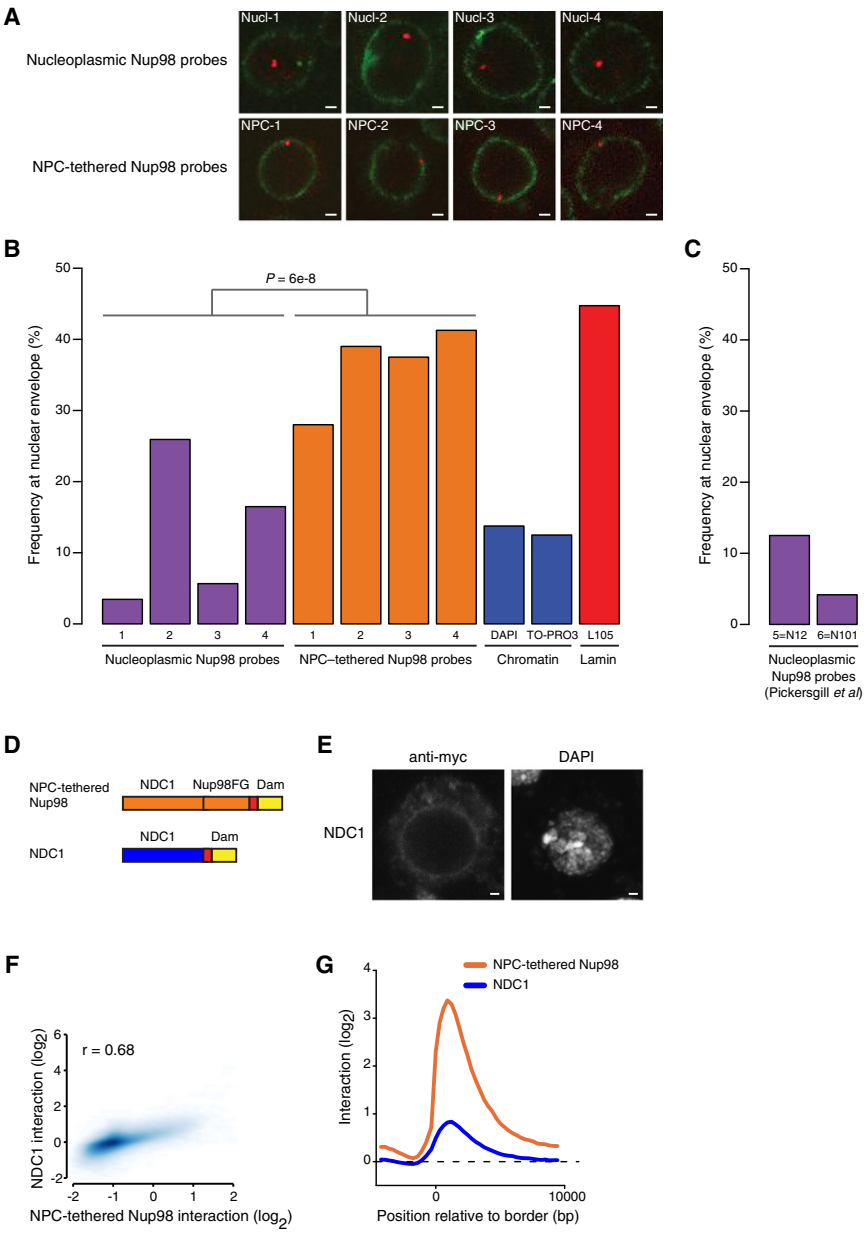


Figure 3. Nuclear Envelope Contact Frequencies of Nucleoplasmic and NPC-Tethered Domains and Chromatin Interactions of NDC1

(A) FISH was performed for four nucleoplasmic Nup98 target loci and four NPC-tethered Nup98 target loci (Table S1, Figure S4A). FISH signal is shown in red, and nuclear envelope (green) is visualized by monoclonal antibody 414 (Davis and Blobel, 1986). Scale bars represent 1 μm.

(B) Frequency of nuclear envelope contacts of the nucleoplasmic and NPC-tethered Nup98 target loci. Signals were considered to localize at the nuclear envelope if localized within 0.2 μm of the nuclear rim. Controls (performed within the same experiments) are random chromatin localization, measured by the intensity of DNA dye signal DAPI and TO-PRO3 (Figures S4B–S4E) and the frequency at the nuclear envelope of a Lamin-interacting region taken along in the same experiment (Probe L5/L105) (Pickersgill et al., 2006).

(C) The frequency at the nuclear envelope of two additional nucleoplasmic Nup98 target loci (data from Pickersgill et al., 2006). The p value was obtained by Fisher's exact test on the combined data from the four nucleoplasmic Nup98 probes and four NPC-tethered Nup98 probes within the same experiment. NE contacts of nucleoplasmic probes were not significantly different from random DNA (Chi square test), while NE frequencies of NPC probes were not significantly different from those of the Lamin probe (Fisher's exact test).

(D) NDC1-Dam fusion construct used for the generation of an NDC1-chromatin map as in Figure 1B. NPC-tethered Nup98 is for reference.

(E) Localization of the NDC1-Dam fusion proteins upon transfection detected using anti-myc antibody (left) and counterstained with DAPI (right).

(F) Comparison of genomic interactions of NDC1 and NPC-tethered Nup98 by a bivariate scatter plot. Pairwise correlation was calculated using Pearson's correlation test.

(G) Superimposed chromatin interaction maps of 400 highest-scoring NPC-tethered Nup98-interacting domains, aligned at left and flipped right borders.

See also Figure S4.

Figure 3A. As a positive control for nuclear envelope localization, we used a probe detecting a Lamin-interacting chromosomal region (Probe L5/L105) (Pickersgill et al., 2006). As a control for random chromatin localization, we measured the distribution of the DNA dyes DAPI or TO-PRO 3 (Figures S4B–S4E). On average, the frequency of the NPC-tethered Nup98 probes localized at the nuclear envelope at a similar ($p > 0.05$) frequency as did the Lamin probe (Figure 3B, Table S1), whereas the nucleoplasmic Nup98 probes localized at the nuclear envelope at the same frequency ($p > 0.05$) as did random chromatin. When we directly compared the NPC-tethered and nucleoplasmic Nup98 probes, we found that chromatin targets of NPC-tethered Nup98 much more frequently localized at the nuclear envelope than did the targets of nucleoplasmic Nup98 ($p = 6e\text{-}08$) (Figure 3B). The two FISH probes from a previous study (Pickersgill et al., 2006) that detected nucleoplasmic Nup98 domains also were predominantly nucleoplasmic (Figure 2C). Taking our FISH and DamID data together, we conclude that Nup98 has a propensity to interact with two predominantly distinct pools of chromatin, one at the NPC and one within the nucleoplasm.

As a further control for the specificity of NPC-tethered Nup98 domains, we mapped NDC1-interacting genes, omitting the Nup98 domain (Figures 3D and 3E). We hypothesized that this would result in a Dam enzyme much more buried inside the NPC, with much lower or no chromatin accessibility. Indeed, compared to NPC-tethered Nup98, interaction signals were greatly reduced (more than 5-fold); however, in general, the

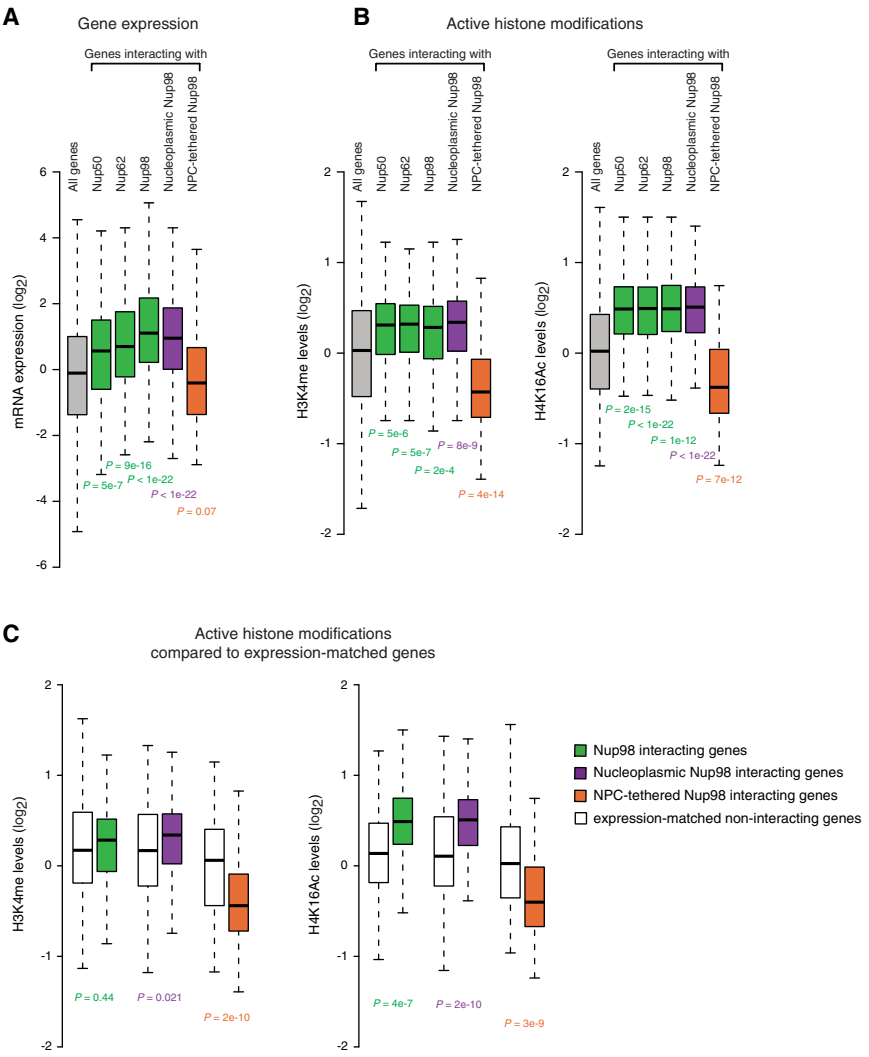


Figure 4. Nucleoplasmic Nup98 Preferentially Interacts with Active Genes, whereas NPC-Tethered Nup98 Does Not

(A and B) Transcriptional activity levels and active histone modification levels of all genes (gray), full-length nucleoporins (green), nucleoplasmic Nup98-interacting genes (purple), and NPC-tethered Nup98-interacting genes (orange), represented in box plots (50% of the data are within the box, the median is represented by a horizontal line, and the whiskers indicate the maximum and minimum value [outliers omitted]). P values were obtained by Mann-Whitney U tests on the distributions of nucleoporin-interacting genes versus all genes. Modification levels are from embryonic *Drosophila* cell chromatin immunoprecipitation (ChIP) data (Schübeler et al., 2004).

(C) Histone modification levels of nucleoporin-interacting genes, depicted as in (B), but now compared to an equally sized set of randomly selected noninteracting genes that match the gene expression level of the interacting genes.

same genomic regions were targeted (Figures 3F and 3G), which provides further support that we are indeed detecting NPC-interacting chromatin domains. Together, we demonstrate two pools of nucleoporin-chromatin interactions, one the NPC and one inside the nucleoplasm.

Genomic Sites Interacting with the NPC Are Devoid of Lamin

To examine the relationship between Lamin-interacting and NPC-interacting chromatin, we extended our previously generated Lamin-chromatin interaction maps (Pickersgill et al., 2006) to 100 bp resolution on chromosome 2L. NPC-interacting chromatin showed low correlation ($r = -0.11$) with Lamin-interacting chromatin, indicating very little direct overlap between the two (Figure S4F). As expected, low correlations were found between Lamin and full-length or nucleoplasmic Nup98 binding chromatin (Figure S4F). Overlay of the NPC-interacting chromatin domains showed that they are devoid of Lamin (Figure S4G). These findings are consistent with electron micrographs of nuclei from many different cell types showing that the ultrastructure of chromatin in the proximity of the nuclear lamina is different from that close to the NPC (Marshall, 2002).

Nucleoplasmic Nucleoporin-Binding Genes Are Actively Transcribed

To investigate whether genes interacting with nucleoporins are more actively transcribed, as has been found in yeast (Casolari et al., 2004), we analyzed activity levels of the full-length nucleoporin-interacting genes using existing gene expression and histone modification profiles of embryonic *Drosophila* cells (Pickersgill et al., 2006; Schübeler et al., 2004). We found that the group of genes interacting with Nup50, Nup62, or Nup98 displayed substantially higher transcriptional activity than average (Figure 4A). This was accompanied by higher levels of active histone marks, such as H3K4me2 and H4K16Ac (Figure 4B).

To determine which pool of nucleoporin-interacting genes (the NPC-interacting or nucleoplasmic pool) showed this active signature, we analyzed NPC-tethered and nucleoplasmic Nup98-interacting genes for expression levels and active histone marks. We found that genes interacting with nucleoplasmic Nup98 resembled full-length nucleoporin-interacting genes in having significantly higher mRNA expression levels and high levels of active histone modifications (Figures 4A and 4B). In contrast, genes interacting with Nup98 at the NPC were not different from noninteracting genes in mRNA expression, indicating that there is no enrichment for gene activity at the NPC (Figure 4A). In addition, NPC-interacting genes had lower levels of active histone modifications (Figure 4B). mRNA levels are known to strongly correlate with H3K4 methylation and H4K16 acetylation of the gene (Schübeler et al., 2004), and we wondered whether

the increased active histone marks were only a reflection of their active state. For H3K4 methylation, equally expressed gene sets indeed showed methylation levels comparable to nucleoplasmic and full-length nucleoporin-interacting genes (Figure 4C and data not shown). However, levels of H4K16 acetylation of nucleoplasmic and full-length nucleoporin-interacting genes were much higher than expected from their gene activity (Figure 4C). NPC-tethered Nup98-interacting genes contained lower levels of both H3K4 methylation and H4K16 acetylation than expected from their mRNA expression level. Thus, on average, only genes interacting with the nucleoplasmic nucleoporins are transcriptionally active and high in active histone marks, in particular histone H4K16 acetylation, whereas genes interacting with the nucleoporins at the NPC are not.

Nucleoporins Colocalize with Transcribed Regions in Polytene Chromosomes Inside the Nucleoplasm

To examine nucleoporin-chromatin interactions in a different cell type and using an unrelated methodology, we stained polytene chromosomes from salivary glands from *Drosophila* larvae with antibodies to Nup50 (Brandt et al., 2006) and Nup98 (Capelson et al., 2010) (for specificity, see Figure S1). Both for Nup50 and Nup98, we observed a specific banding pattern throughout the chromosomes (Figure 5A, Figure S5A). The nucleoporin-positive bands were low in DAPI staining, i.e., interbands containing decondensed chromatin. Consistent with the DamID data, we observed a significant overlap between the Nup98 and Nup50 banding patterns (Figure S5B, Table S3). With the Nup50 antibody, we obtained the clearest banding pattern and found that it was present at sites that were actively transcribed, as shown by colocalization of Nup50 and serine 2-phosphorylated RNA polymerase II (Ahn et al., 2004) (Figure 5B). Nucleoporins Tpr/Megator and Nup153 have been shown to be required for dosage compensation of the male X chromosome (Mendjan et al., 2006). However, we did not observe enrichment of nucleoporins on the male X chromosome in Nup50 polytene stainings or in DamID experiments using a male cell line (data not shown). To assess whether the Nup50-positive chromosomal bands were present at the nuclear periphery or within the nucleoplasm, we compared them to nuclear envelope contact frequencies of these bands recorded in the same tissue (Hochstrasser et al., 1986). This comparison shows that Nup50-positive bands localized mainly within the nucleoplasm and only occasionally were found at high frequency at the nuclear envelope (Figure 5C). This is consistent with our findings in embryonic cells that most of the interactions between the nucleoporins Nup62, Nup50, and Nup98 and chromatin occur within the nucleoplasm.

To investigate whether nucleoporin-chromatin interactions are dynamic, we induced heat shock to the larvae, leading to visible changes in polytene chromosomes at specific sites of high gene expression, so called ''puffs'' (Beermann, 1952). Major heat shock puffs include those at 87A and 87B, which have been shown to localize randomly inside the nucleoplasm (Yao et al., 2007). These puffs, as well as a heat shock puff at 67B, clearly recruited Nup50 and Nup98, as well as serine 2-phosphorylated RNA polymerase II (Figure 5D). This indicates that there is a dynamic interaction of nucleoporins with active genes. Nup50 staining remains prominent on polytene chromosomes under heat shock conditions, when downregulation of non-heat shock genes takes place. This indicates that Nup50 remains present when gene expression is halted. A remarkable observation from the polytene chromosome stainings was that from the nine brightest Nup50-positive bands, eight were developmental puffs, including 68C, 71CE, and 73F (Figure 5E), while one was a constant puff (90C) (Table S3).

To further investigate the dynamics of nucleoporin-chromatin interaction inside the nucleoplasm at high resolution in a developmental setting, we treated embryonic *Drosophila* cells with the steroid hormone ecdysone for 24 hr, causing the cells to partially differentiate (Echalier, 1997). We then examined changes in gene expression and changes in nucleoplasmic Nup98-chromatin interaction in differentiated cells compared to untreated cells. Genes that were upregulated after ecdysone treatment also significantly gained nucleoplasmic Nup98 interaction, whereas genes that were downregulated significantly lost nucleoplasmic Nup98 interaction (Figure 5F). Chromatin accessibility, as measured by unfused Dam interaction (Kladde and Simpson, 1994), was slightly increased in upregulated genes, indicating that part of the gain in nucleoplasmic Nup98 interaction may be driven by changes in chromatin accessibility. These data indicate that, consistent with the polytene chromosome results, dynamic changes in the expression levels of genes go together with their ability to interact with a nucleoplasmic nucleoporin.

Nucleoporin-Chromatin Interactions Preferentially Occur at Developmental Genes

The observation that Nup50 strongly stained developmental puffs (Figure 5E) prompted us to investigate which types of genes interacted with the nucleoporins in the *Drosophila* early embryonic cell line. Interestingly, the genes interacting with nucleoplasmic Nup98 and Nup50 were very highly enriched (almost 50% of the top 400 interacting genes) for development (Figure 6A and Tables S4 and S6). Also enriched were genes involved in signal transduction, regulation, actin cytoskeletal function, and the cell cycle (Figure 6A and Tables S4 and S6). Strikingly, genes that most highly interacted with NPC-tethered Nup98 were also very highly enriched in developmental genes (Figure 6A and Tables S4 and S6); however, this was a very different gene set (Figure 6B). Accordingly, developmental NPC-interacting genes were connected with different biological processes than nucleoplasmic Nup98 and Nup50-interacting genes, in particular cell adhesion, cell communication, and behavior (Figure 6A). Robustness of the gene ontology method was tested by analyzing the 400 highest expressing genes in embryonic cells. As expected, the most highly enriched biological process was cellular metabolism, along with translation and nucleotide metabolism (Tables S4 and S6). We conclude that nucleoporin-chromatin interactions preferentially occur at genes involved in development.

Nucleoplasmic Nucleoporin-Chromatin Interactions Stimulate Expression of Developmental and Cell-Cycle Genes

The positive and dynamic relationship found between gene expression and interaction with nucleoplasmic nucleoporins raised

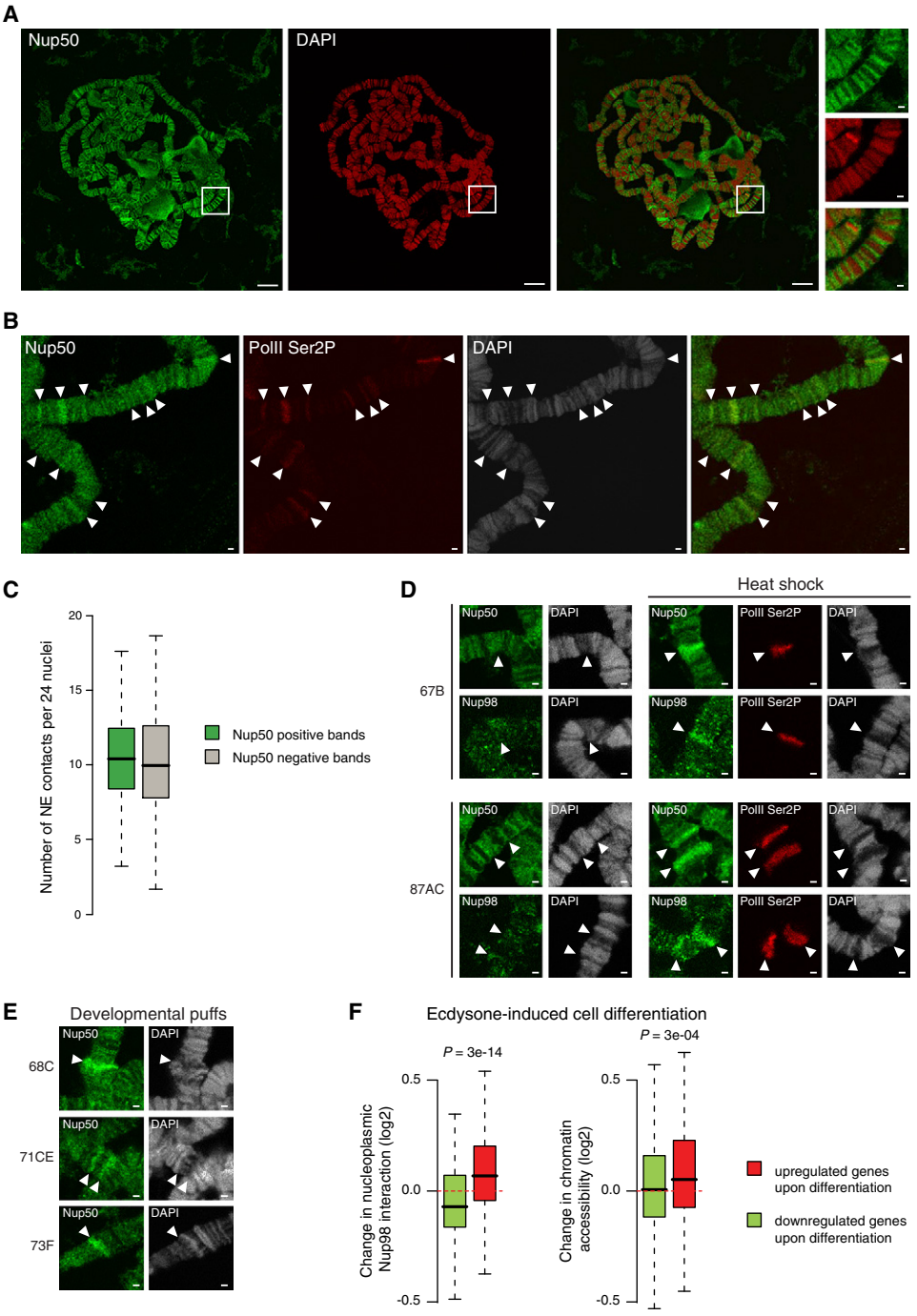


Figure 5. Nucleoporins Colocalize with Transcribed Regions and Developmental Puffs on Polytene Chromosomes

Salivary gland polytene chromosomes from *Drosophila melanogaster* third-instar larvae stained for Nup50, Nup98, active RNA polymerase II, and DAPI. Scale bars represent either 10 μm (large panels in A) or 1 μm (other panels in A, B, D, and E). Regions indicated by white squares are shown in a higher magnification in the panels on the right.

(B) Arrowheads indicate staining of active RNA polymerase II.

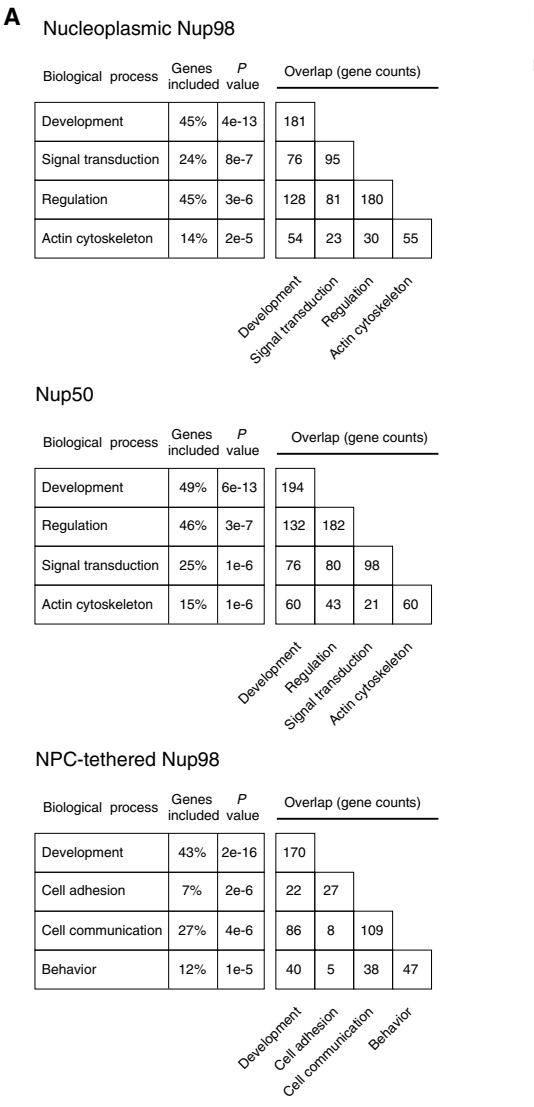
(C) Proximity to the nuclear envelope (Hochstrasser et al., 1986) of Nup50-positive bands and Nup50-negative bands in polytene chromosome stainings.

(D) Polytene chromosomes from larvae that obtained a heat shock of 37°C for 20 min before dissection. Arrowheads indicate major heat shock-induced puffs.

(E) Examples of Nup50 staining on developmental puffs (arrowheads).

(F) Association of nucleoplasmic Nup98 with ecdysone-induced genes. Changes in nucleoplasmic Nup98 interaction (left) and of chromatin accessibility (unfused Dam interaction) (right) after treatment of embryonic *Drosophila* cells with the steroid hormone ecdysone for 24 hr. The 5% most upregulated (red) or downregulated (green) genes upon ecdysone treatment are shown.

See also Figure S5.



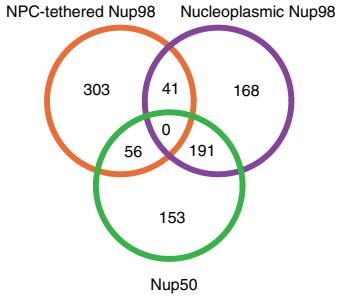


Figure 6. Many Nucleoporin-Chromatin Interactions Preferentially Occur at Developmental Genes

(A) Gene ontology analysis showing the four categories of biological processes with highest enrichment for the top 400 genes interacting with Nup98, Nup50, and NPC-tethered Nup98. Percentages shown are the percentages of the interacting genes in the category. The p values represent the mean p values of enrichment across clustered gene ontology terms (see the Experimental Procedures). Overlap is the number of interacting genes included in both processes.

(B) Overlap between the NPC-tethered Nup98, nucleoplasmic Nup98, and Nup50 genes used in the gene ontology analysis.

the question of whether nucleoplasmic nucleoporins have a functional role in regulation of gene expression. To test this, we depleted Nup98 or Nup50 using RNAi in embryonic *Drosophila* cells for 96 hr (Figure S1). We measured changes in global mRNA expression by microarray analysis as compared to control cells, which were cells with a knockdown of the *White* gene. Interestingly, genes that were the most significantly downregulated by Nup50 knockdown (n = 322) were the ones which interacted more strongly with Nup50 than other genes (Figure 7B). Similarly, genes that were significantly downregulated by Nup98 knockdown (n = 1307) were the ones that interacted more strongly with Nup98, in particular with nucleoplasmic Nup98 (Figure 7A). In contrast, NPC-tethered Nup98 binding genes were hardly affected by Nup98 knockdown, indicating that these genes are not dependent on Nup98 for their expression. Genes dependent on Nup98 for expression were most significantly enriched in developmental and cell-cycle genes (Figure 7E, Figure S6B, and Tables S5 and S6) and showed high levels of Nup98 interaction (Figure 7E). Genes significantly dependent on Nup50 largely overlapped (74%) with those dependent on Nup98 (Figure S6A) and were also enriched in developmental genes and high in Nup50 interaction (Figure S6B and Tables S5 and S6). These results indicate that nucleoplasmic Nup98 and Nup50 are required for normal expression of a set of genes with which they interact and that these are enriched in developmental genes.

Depletion of nucleoporins may lead to changes in nucleocytoplasmic transport, which may indirectly affect gene expression levels, although the affected genes also interact with the nucleoporins, arguing against this indirect effect. However, to more rigorously test this, we analyzed changes in gene expression upon overexpression of nucleoplasmic Nup98 protein. Overexpression of this protein would be expected to positively act on genes that are negatively affected by Nup98 depletion. If so, it would confirm that the influence of nucleoporins takes place inside the nucleoplasm and is not NPC dependent. For this, we transfected GFP-labeled nucleoplasmic Nup98 (GFP-Nup98-1:479) into embryonic *Drosophila* cells and FACS sorted GFP-positive cells. As a reference for changes in mRNA expression, we used the GFP-negative fraction and the GFP-positive fraction of GFP-transfected cells (either reference gave essentially the same result). First, we found that the genes that were most upregulated by GFP-Nup98-1:479 overexpression (n = 327) strongly interacted with Nup98, in particular with nucleoplasmic Nup98. Very little effect was seen with NPC-tethered Nup98 (Figure 7C), again indicating that expression of NPC-tethered Nup98-interacting genes are not dependent on Nup98 for expression. Second, the genes significantly upregulated in cells overexpressing nucleoplasmic Nup98 were the ones that were substantially downregulated upon Nup98 knockdown (Figure 7D). Third, similarly to genes downregulated upon Nup98 knockdown, genes upregulated upon nucleoplasmic Nup98 overexpression

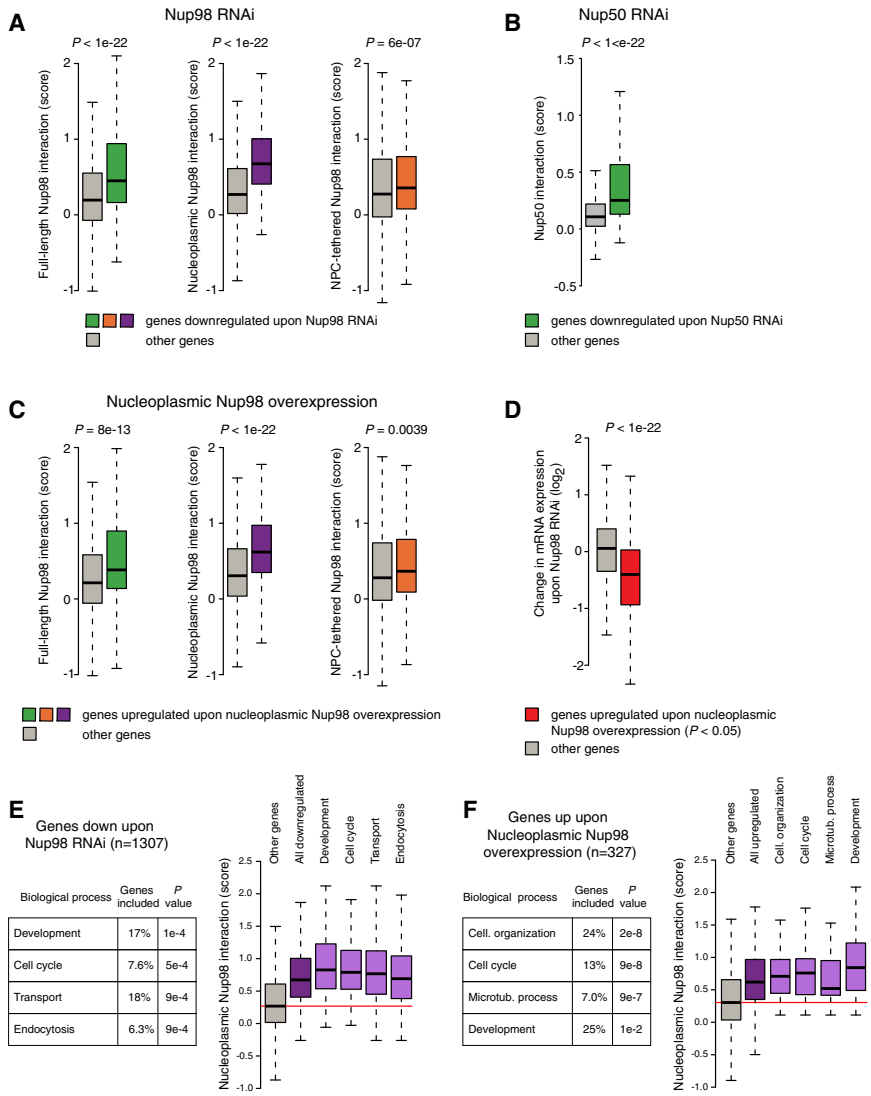


Figure 7. Nucleoplasmic Nucleoporin-Chromatin Interactions Stimulate Gene Expression of Developmental and Cell-Cycle Genes

(A) Nucleoplasmic Nup98-interacting genes are sensitive to Nup98 depletion. Levels of full-length, nucleoplasmic, and NPC-tethered Nup98 interaction of genes downregulated by Nup98 RNAi (*P*fdr < 0.05) compared to other genes (gray).

(B) Nup50-interacting genes are sensitive to Nup50 depletion. Levels of Nup50 interaction of genes downregulated by Nup50 RNAi (green, *P*fdr < 0.1) compared to other genes (gray).

(C) Nucleoplasmic Nup98-interacting genes are upregulated upon nucleoplasmic Nup98 overexpression. Levels of full-length, nucleoplasmic, and NPC-tethered Nup98 interaction of genes upregulated by nucleoplasmic Nup98 overexpression ($p < 0.05$) compared to other genes (gray).

(D) Genes downregulated by Nup98 depletion are upregulated by nucleoplasmic Nup98 overexpression. Changes in mRNA levels caused by Nup98 RNAi of genes upregulated upon nucleoplasmic Nup98 expression (red, $p < 0.05$) and other genes (gray).

(E and F) Gene ontology analysis of genes downregulated upon Nup98 RNAi (E) and genes upregulated upon nucleoplasmic Nup98 overexpression (F). The analysis was performed as in Figure 6. The bar plots show the nucleoplasmic Nup98 interaction for the subgroups of responding genes identified by the gene ontology analysis in light purple, in dark purple and gray are all responding genes and all other genes respectively (same data as in the middle panels of A and C).

Box plots are as in Figure 4, and p values were obtained by Mann-Whitney U tests. See also Figures S6 and S7.

were enriched in developmental and cell-cycle genes (Figure 7F, Figure S6B, and Tables S5 and S6) and showed high levels of Nup98 interaction (Figure 7F). Together, these data demonstrate that Nup98 and Nup50 stimulate mRNA expression in the nucleoplasm at the genomic sites where they interact and that their target genes are enriched in developmental genes and cell-cycle regulators.

Finally, we wondered whether Nup50 and Nup98 depended on each other for gene interaction. We generated a Nup50 interaction map after Nup98 knockdown and a Nup98 interaction map after Nup50 knockdown. Interestingly, we found that both the number of Nup50-interacting genes and the level of interaction per gene decreased upon Nup98 knockdown (Figure S7). On the contrary, upon Nup50 knockdown, there was an increase in the number of Nup98-interacting genes and a slight increase in their level of interaction. This suggests that Nup50 interaction is (partially) dependent on Nup98, but not the other way around.

DISCUSSION

The primary function of the NPC is to mediate transport between the nucleus and cytoplasm. Here, we report that some NPC components also function away from the NPC in the nucleoplasm, where they directly regulate gene expression, particularly of genes involved in development and the cell cycle.

The gene gating hypothesis (Blobel, 1985) predicted that active genes would preferentially associate with NPCs, enabling transcripts to efficiently enter the cytoplasm. Indeed, in yeast, a large body of evidence indicates that genes interacting with the NPC are active and that this interaction is important for gene expression (Casolari et al., 2004; Brickner and Walter, 2004). In *Drosophila*, the situation seems to be different, as we found that genes that associated with the NPC were not particularly active but were also not strongly repressed. One possible explanation is that chromatin in the direct vicinity of the NPC should be shielded from adjacent, Lamin-interacting dense

heterochromatin (Pickersgill et al., 2006) and should not be too active to avoid obstructing access to the NPC with transcription complexes or factories. Consistent with this idea, we found that NPC-interacting domains are present in very large genes, preferentially far away from the promoter. Besides, a recent study using vertebrate Nup93 to monitor structural changes in chromatin organization upon histone deacetylase inhibition also found that Nup93-interacting genes were not particularly active (Brown et al., 2008). Nevertheless, the strong enrichment for genes involved in development among NPC-interacting genes suggests additional regulatory functions that need to be explored. Our data so far indicate that expression of NPC-tethered Nup98-interacting genes is not dependent on Nup98 itself. In contrast to the situation with NPC-binding genes, genes that interact with nucleoplasmic pools of Nup98 or Nup50 did show high gene expression, which, importantly, was dependent on the presence of the nucleoporin. Thus, nucleoporins stimulate gene expression away from the NPC, inside the nucleoplasm.

Nup98 plays a causative yet incompletely understood role in human leukemia. A large number of different chromosome translocations in mainly acute myeloid leukemia (AML) result in chimaeric proteins containing the FG repeat part of Nup98 and a wide set of proteins, including homeobox transcription factors such as HoxA9 (reviewed in Lam and Aplan, 2001). Common to all oncogenic Nup98 fusion proteins is that their localization is inside the nucleoplasm, not at the NPC. Studies on Nup98-homeobox fusions show that the transforming ability of these proteins in vitro and in mouse models of AML is dependent on the Nup98 and homeobox portions (Kasper et al., 1999; Kroon et al., 2001; Pineault et al., 2003). The fusion genes are able to activate Hox target genes (reviewed in Argiropoulos and Humphries, 2007). The Nup98 part has been found to recruit histone acetylases or deacetylases through part of its Nup98 phenylalanine-glycine(FG)-repeat domain (Bai et al., 2006; Kasper et al., 1999; Wang et al., 2007). We have found that genes associating with nucleoplasmic nucleoporins are particularly high in acetylated histones, suggesting a common mechanism between physiological gene activation by nucleoplasmic nucleoporins and oncogenic gene activation by nucleoporin fusion proteins inside the nucleoplasm.

Genes that interact with and respond to nucleoplasmic pools of Nup98 or Nup50 are highly enriched in developmental genes, suggesting an important function of the nucleoplasmic pool of nucleoporins on fly development. Also, genes that interact with and respond to nucleoplasmic Nup98 are enriched in genes that are directly linked to the cell cycle. These include for example *Cyclin B*, *Bub1*, and *Mad2* (Table S6) (reviewed in Musacchio and Salmon, 2007). Interestingly, several of the nucleoporin-regulated cell-cycle genes have also been implicated in human cancer. For example, overexpression of Mad2 leads to tumors in transgenic mice (Sotillo et al., 2007), and the human homologs of several of the group (*Cyclin B*, *Bub1*, *Plk4*, and *Mad2*) are included in ''death-from-cancer'' gene signatures (Glinsky et al., 2005; Glinsky, 2006): high expression of this signature set of genes correlates with an unfavorable outcome in several types of cancer. Therefore, it is tempting to speculate that nucleoplasmic nucleoporins expressed as a consequence of leukemia-associated chromosome translocations may contribute to oncogenesis by promoting expression of these cell-cycle genes.

Together, our study sheds new light on the question of how nucleoporins are involved in regulating gene expression. We show that nucleoporins stimulate gene expression away from the NPC in the nucleoplasm, in particular of a set of developmental and cell-cycle genes. Our data also shed new light on the frequent occurrence of nucleoporins in leukemogenic fusion proteins, as they may perform their regular role in gene activation in a mistargeted fashion and enhance overall levels of genes required for cell-cycle progression.

EXPERIMENTAL PROCEDURES

Plasmid Constructs

Full-length Nup62, full-length Nup50, and full-length Nup98 were cloned into the Dam vector pNDamMyc (van Steensel and Henikoff, 2000) (see the Extended Experimental Procedures for placement of Dam). The Nucleoplasmic Nup98 DamID construct was created by cloning amino acids 1–481 of Nup98 into the pNDamMyc vector. The NPC-tethered Nup DamID construct was created by fusion of full-length NDC1 (Stavru et al., 2006) to amino acids 1–576 of Nup98 and cloning of this into the Dam vector pCASPERGW-MycDam, which was a kind gift of U. Braunschweig and B. van Steensel, with the pENTR Directional TOPO Cloning Kit (Invitrogen). The NDC1-Dam construct was the same, but then without the Nup98 part in between. Overexpression of nucleoplasmic Nup98 was performed with a fusion protein of amino acids 1–479 of Nup98 with GFP in a vector with an actin5C promoter, with the pENTR Directional TOPO Cloning Kit (Invitrogen). The GFP control vector was the same without the Nup98 part.

Cell Culture

Drosophila melanogaster embryonic Kc167 cells were grown in BPYE medium (Shields and Sang M3 Insect medium, Sigma; supplemented with 25% w/v bacto-peptone, 20% w/v yeast extract, 5% heat-inactivated fetal calf serum, all GIBCO) at 23°C.

Immunofluorescence

Plasmids were transfected into *Drosophila* Kc cells by electroporation as described (van Steensel and Henikoff, 2000). Expression was induced by heat shock at 37°C for 2 hr and subsequent recovery at 23°C for 24 hr. Cells were immobilized on poly-L-lysine coated coverslips and fixed and stained as described (van Steensel and Henikoff, 2000). Fusion proteins were detected with cMyc antibody 9E10 (Santa Cruz Biotechnology). Images were recorded with a Leica AOBS confocal microscope using a 63× oil objective, 8× zoom, 512 × 512 resolution, and 4× averaging.

DamID and RNA Expression Profiling

DamID and RNA expression profiling were performed as previously described (Pickersgill et al., 2006). When required, after electroporation the cells were incubated with 1 μM ecdysone (added from a 1 mM stock dissolved in ethanol) or with the equivalent volume of ethanol. All hybridizations were performed in balanced dye orientations to rule out dye bias effects. NPC-tethered Nup98-methylated fragments were normalized to randomly in vitro Dam-methylated fragments of total genomic DNA, using recombinant Dam methylase (Biolabs) instead of in vivo-methylated DNA of cells transfected with unfused Dam. This standard reference corrects for freely diffusing nuclear Dam fusions (van Steensel and Henikoff, 2000). In case of NPC-tethered Nup98, this standard reference leads to overnormalization, as the NPC-tethered Nup98 Dam fusion protein cannot diffuse into the nucleus. Methylated fragments were amplified by PCR and subsequently hybridized to Fly12K cDNA microarrays (Pickersgill et al., 2006) or NimbleGen high-density oligonucleotide arrays containing a 60 bp probe every 300 bp (Choksi et al., 2006). Lamin B-methylated DNA fragments were hybridized to NimbleGen high-density oligonucleotide arrays containing a 60 bp probe every 100 bp, covering the entire chromosome 2L, 10 Mb of chromosome 2R, 2 Mb of the X chromosome, and chromosome 4

(Mito et al., 2005). When necessary, 100 bp spacing data was interpolated to 300 bp by univariate interpolation. For RNA expression profiling and the DamID experiments with nucleoplasmic Nup98 overexpression, Nup98 and Nup50 RNAi and ecdysone inductions, hybridizations were performed using Illumina Fly INDAC 35K Oligo arrays. For the high-density arrays, labeling of methylated DNA fragments, hybridization, and scanning of arrays were performed by NimbleGen (http://www.nimblegen.com/), except the Nup50 and NDC1 high-density DamID, which was handled at the Netherlands Cancer Institute with the exact same procedures.

Gene Ontology Analysis

Target genes sets were analyzed for enriched biological processes (GOTERM_BP_ALL) using DAVID (Huang et al., 2009; Dennis et al., 2003) functional annotation clustering with medium classification stringency (default settings). Annotation clusters were further analyzed when geometric mean modified Fischer exact p value of enrichment across gene ontology (GO) terms was <0.05 (EASE score >1.30), and at least one of the individual GO terms was enriched with a p value of <0.05 when corrected for multiple testing according to Benjamini and Hochberg (Tables S4 and S5). Annotation clusters were categorized in overall biological processes that best described the cluster (Table S6) and unique genes within these groups overlapping with the target sets were counted and compared. For the summary tables in Figures 6 and 7 and Figure S8, only processes with a coverage of >5% of the target gene sets are shown.

ACCESSION NUMBERS

All microarray data used in this study are available in MIAME-compliant format under accession number GSE19307 of the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and six tables and can be found with this article online at doi:10.1016/j.cell.2010.01.011.

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