Supplemental Figure S1. *Characterization of additional Nup98 peptide antibodies and their use in post-embedding immuno-EM*. (A) Immunoblotting of HeLa cell proteins using antibodies against Nup98 aa 206-220 and 596-618. Lanes include total cell proteins (TP), proteins first extracted with 0.4% Triton X-100 (T) and then with 0.36 M NaCl (N) in PBS, and residual cell sediment proteins (S). Lanes T, N, S were loaded with the same percentage of each fraction. Antibodies against aa 596-618 were from different immunizations in two animals (I and II). Antibodies from animal I that did not crossreact with other proteins in immunoblotting were used for immunolocalizations shown in Figures 2 and 4. In contrast, both α-Nup98 aa 206-220 and 596-618 [animal II] showed some crossreactions with other yet uncharacterized proteins (asterisks). (B) Confocal IFM of HeLa cells immunolabeled with α-Nup98 aa 206-220 and 596-618 [animal II]. Double-labelings were with α-Tpr 2063-2084. Cells were either permeabilized with detergent after or before fixation. For α-Nup98 aa 596-618 [animal I], corresponding IF micrographs have been shown earlier (Hase and Cordes 2003) or are shown in Supplemental Figure S3. -100, the epitope for α-Nup98 aa 206-220 was not or hardly accessible. Under these conditions, cross-reactions with cytoplasmic organelles were noted, whereas NE staining was only seen in prophase (arrow) and telophase cells (arrowheads). However, when cells were first extracted with Triton X-100, the epitope was unmasked, allowing clear NE-staining with α-Nup98 aa 206-220. In contrast, using α-Nup98 aa 596-618 [animal II], weak NE staining was already clearly seen in interphase cells mildly permeabilized with digitonin. This also differs from IFM with α-Nup98 aa 596-618 from animal I where NE-staining of interphase NEs was not or barely visible under these conditions (Supplemental Figure S3). (C) Distribution of immunogold particles (IGP) relative to the NE midplane (dashed vertical). Data collection was from randomly chosen gold-labeled NEs from encoded specimens. Values were normalized for NE width variations as described in Figure 3A. Histograms include all gold grains detected in proximity to perpendicularly sectioned NPCs, up to 100 nm from the NE midplane at the

cytoplasmic and 150 nm at the nuclear side (negative values). Sample sizes (n) are 70 (α-Nup98 aa 206-220) and 56 (α-Nup98 aa 596-618 [animal II]). Normalized mean distances of gold grains located up to 50 nm on each side of the NE, considering the algebraic signs of the distance values for nuclear and cytoplasmic gold grains (see Table I), are –5,2 nm (α-Nup98 aa 206-220) and +0,9 nm (α-Nup98 aa 596-618 [animal II]). Means resulting from calculations that ignore the algebraic signs are ±12,8 nm (α-Nup98 aa 206-220) and ±13,3 nm (α-Nup98 aa 596-618 [animal II]). Note that immunogold grain distribution patterns with α-Nup98 aa 596-618 [animal I; Figure 2B and Table 1] and α-Nup98 aa 206-220 are highly similar, with slight preference for the nuclear side of the NE midplane. IF and immunogold labeling with α-Nup98 aa 596-618 [animal II] resulted in some additional labeling of the cytoplasmic side (blue bracket) more distant from the midplane. Whether this represents Nup labeling still needs to be clarified. Clearly, however, none of these Nup98 immunogold grain distribution patterns are indicative of Nup98 being an architectural element of the nuclear basket fibers.

Supplemental Figure S2. *Non-normalized distribution of gold grains relative to the NE midplane.* Histograms correspond to those shown in Figure 2B (Nups; **A**) and Figure 5B (Tpr segments; **B**) but give the measured values prior to their normalization for NE width variations. Following normalization, the steepness of the nuclear and cytoplasmic slopes of the distribution gradients for some Nups has become more similar, emphasizing a bilateral symmetrical arrangement of these proteins (Figure 2B).

Supplemental Figure S3. *Confocal IFM of semi-permeabilized HeLa cells.* Immuno-EM indicated that some Nups are arranged within two planes, i.e. at the cytoplasmic and nuclear side of the NPC, either more distant or close to the NPC midplane. To test by an independent approach whether these Nups are accessible for antibodies on both sides of the NPC, we studied cells by IFM after permeabilization with low concentrations of digitonin prior to immunolabeling.

Although the plasma membrane is thereby perforated, the NE usually remains impermeable for antibodies, preventing them from reaching the nuclear interior (e.g., Belgareh et al. 2001; Vasu et al. 2001).

The presented panel of images shows fixed cells that were treated with 0.005% digitonin and double-labeled with the same Nup antibodies used for Figure 2. Tpr staining was with mAb 203-37 and α-Tpr aa 2063-2084. Bar, 20 μm. Independent of whether cells were permeabilized with 0.005% digitonin prior to or after fixation, staining for Nup96 and 107 at the NE was seen in almost all cells. In contrast, Tpr-labeling, as a marker for accessibility of the nuclear interior, was usually seen only in mitotic cells. In a few non-mitotic cells in which the nuclear side of the NE had become accessible, staining for Nup96 and 107 was sometimes more intense, indicative that antibody binding to additional Nup96 and 107 polypeptides at the nuclear NPC side contributed to staining intensity (data not shown).

The pattern of Nup93 and 205 immunogold labeling indicated that these Nups are embedded more deeply within the NPC core and sandwiched between the flanking planes containing Nup96 and 107. In fact, Nup93 was only accessible for several different Nup93 antibodies (see Materials and Methods) when the NPC core had been cross-sectioned, or when unfixed cells were first extracted with strong detergents such as Triton X-100 that partially disrupt NPC structure (Grandi et al. 1997; Hase and Cordes 2003). Permeabilization of non-mitotic cells with mild detergents such as digitonin or saponin did not give access to Nup93. Even when using higher digitonin concentrations that disrupt the NE but still leave NPC structure largely intact, no or only traces of Nup93 staining at the NE were visible; Nup153 and Tpr were brightly labeled under such conditions (data not shown). The epitope recognized by the Nup205 antibody was slightly better accessible. Weak NE staining was visible in cells that had been fixed and then permeabilized with either digitonin, or Triton X-100 (data not shown). Similar to Nup93, however, bright Nup205 staining was only seen in cells extracted with Triton X-100 prior to fixation (see Figure 9 and Supplemental Figures S4-S6).

As for Nup93, NE-staining for Nup98 in non-mitotic cells was not not visible when cells were permeabilized with low concentrations of digitonin and incubated with α-Nup98 aa 206-220 (Supplemental Figure S1) and α-Nup98 aa 596-618 [animal 1; this image]. When using α-Nup98 aa 596-618 from animal 2, weak NE staining was clearly noted under these conditions (Supplemental Figure S1). Different from Nup93, however, higher concentrations of digitonin resulted in clear Nup98 staining at both the NE and intranuclear GLFG bodies with both batches of Nup98 aa 596-618 antibodies (data not shown). Nevertheless, most intense NE staining with all Nup98 antibodies was seen when cells were extracted with Triton X-100 prior to fixation (Supplemental Figure S1 and Hase and Cordes 2003). This indicated that the epitopes recognized by our Nup98 antibodies first had to be unmasked by harsh detergent treatment to become fully accessible.

For Nup153, the results were essentially the same as for Tpr: In cells fixed first and then permeabilized with 0.005% digitonin, Nup153-staining was never seen in non-mitotic cells; staining at the NE was only visible when cells were permeabilized with Triton X-100 or high concentrations of digitonin (Hase and Cordes 2003, and data not shown). The location of a certain number of gold grains at the cytoplasmic side of NPCs immunolabeled with α-Nup153 aa 21-36 (Figures 2 and 4) was therefore regarded as partly resulting from antibody rotation around target sites located at the nuclear NPC side. In addition, a certain level of background staining seen throughout the cytoplasmic compartment on GA-fixed specimen (Figure 2B) might have contributed to this cytoplasmic pool of gold grains as well. Occassionally, however, a gold grain may also label an individual Nup153 polypeptide in the process of nuclear import, and we also do not exclude the possibility that sporadic Nup153 molecules may attach to the cytoplasmic side of the NPC in a transport-independent manner under certain conditions.

Supplemental Figure S4. *Post-transcriptional Nup96 gene silencing results in NPC disintegration and mislocalization of Tpr, Nup153, and other nucleoporins*. HeLa cells were

studied by confocal IFM at day 3.5 post-transfection with Nup96 siRNAs. Double-labelings were with antibodies against Nup50 aa 126-147, recombinant full-length murine Nup62, Nup93 aa 350-369, Nup96 aa 880-900, Nup107 aa 33-51, Nup153 aa 21-36 (left panel), Nup205 aa 1784-1803 and Nup358 aa 2285-2314, with Nup153 mAb PF190x7A8 (right), Tpr mAb 203-37, and mAb 414. DNA-staining with TO-PRO-3 is in blue. Some images are identical to those in Figure 9. Cells labeled for Nup50, 93 and 205 were permeabilized with Triton X-100 prior to fixation; for other double-labelings, cell fixation came first. Cells transfected with siRNAs show no or just traces of Nup96 staining at the NE. The usually bright NE staining is visible only in few cells that have remained untransfected. Note that Nup96 deficiency impairs the recruitment of all Nups and Tpr to the NE. In part this can be explained by their reduced *de novo* synthesis. As a consequence of lower NPC numbers, likely to result in diminished net transport of corresponding mRNAs across the NE, translation rates can be expected to be low as well. Sometimes, however, residual amounts of Tpr and Nup153 molecules are still visible in Nup96- and 107-deficient cells. There they are usually found entrapped within the nuclear interior, forming aggregates (arrowheads) similar to the intranuclear Tpr foci seen in Nup153-deficient cells (Hase and Cordes 2003), indicative that NE anchor sites for Tpr and Nup153 are no longer available (see also Supplemental Figure S5). The residual pools of the other Nups rather accumulate within cytoplasmic foci reminiscent of annulate lamellae (AL; Cordes et al. 1996). In fact, thin section EM analysis has revealed that AL are frequent in HeLa cells depleted of Nup133, another component of the Nup160 subcomplex (Walther et al. 2003). Bars, 20 μm (left panel), 20 μm (center), 10 μm (right).

Supplemental Figure S5. *Post-transcriptional Nup107 gene silencing results in NPC disintegration and mislocalization of Tpr, Nup153, and other nucleoporins.* HeLa cells were studied by confocal IFM at day 3.5 post-transfection with Nup107 siRNAs. Fixation and IFM were as for Supplemental Figure S4. Some images are identical to those in Figure 9. Phenotypes caused

by Nup107 deficiency are similar to those in Nup96-depleted cells. As in the latter, intranuclear Tpr aggregates are only occassionally seen in Nup107-deficient cells of the HeLa strain mostly used in this study (strain 1). In another HeLa substrain with kidney-shaped nuclei and a longer cell-cycle (strain 2), the intranuclear Tpr aggregates in Nup107-deficient nuclei are more frequent. Occassional cytoplasmic Tpr aggregates (Hase et al. 2001) are indicative of impaired NLS-mediated nuclear import of Tpr (Cordes et al. 1998) in Nup107-deficient cells. Arrowheads mark individual cells double-labeled for Nup107 and Tpr shown at higher magnification (upper right panel). In cells only partially depleted of Nup107, residual traces of Tpr-staining at the NE (16x magnification, green) are usually found adjacent to residual staining for Nup107 (red). In the absence of Nup107, traces of Tpr staining at the NE are seen only sporadically, indicating that interactions with other non-NPC proteins in general do not suffice to durably secure Tpr at the NPC (see also Hase and Cordes 2003). Bars, 20 μm (upper left panel, all micrographs), 20 μm (bottom and upper right panel, strain 2), 10 μm (upper right, strain 1).

Supplemental Figure S6. *Post-transcriptional Nup205 and Nup93 gene silencing results in temporally distinct loss of different NPC components.* HeLa cells were studied by confocal IFM at day 3-3.5 post-transfection with Nup205 and 93 siRNAs. Fixation, IFM, and most antibodies were as for Figure 9C-C''. Nup93 was stained with α-Nup93 aa 2-218 (left panel) and α-Nup93 aa 350-369 (right); Nup153 with mAb PF190x7A8, and Tpr with α-Tpr 2063-2084. (**A**) Nup205 depletion by RNAi can result in varying phenotypes at 3-3.5 days post-transfection. Reduction of NE-labeling intensity for Tpr and other Nups, such as Nup153 and Nup50, can be very pronounced (left panel of images), but often also only moderate in other areas (right panel). Seldomly, residual amounts of Tpr can be found entrapped in the nuclear interior where they form aggregates (arrowhead) similar to the intranuclear Tpr foci seen in Nup153-, Nup107- and Nup96-deficient cells. (**B**) Tpr aggregates (arrow) are sometimes also found in the cytoplasm, indicative of impaired nuclear import. (**C**) Following early loss of Nup93 and NE-staining with mAb 414 (see

Figure 9C), RNAi of Nup205 ultimately results in loss of all Nups, here exemplified by Nup358 and 96. (**D**) As for Nup205 RNAi, even partial loss of Nup93 by RNAi causes a strong reduction in NE-staining intensity with mAb 414 throughout the entire cell population, with only mild effects on Tpr and Nup153 localization at the NE. Bars, 20 μm.

Supplemental Figure S7. *Latitudinal distortion effects of projected gold grain coordinates that result from indirect immunogold labeling of cross-sectioned NPCs can be minimized by direct labeling procedures using gold-coupled primary antibodies and Fab fragments.* When gold grain coordinates are normalized for non-diametric perpendicular section planes using the equation: $D_y = \sqrt{R^2 - r^2 + d^2}$ (Figure 3B), different types of latitudinal compression of an ideal circular immunogold distribution pattern can arise. (i) For Nups located far from the NPC midaxis, such compressions can occur when gold-labeled grazing sections of the pore are ignored for data collection. Omission of such sections with small ***r*** values can affect the outer margin of the distribution cloud, i.e. the one most distant from the NPC midaxis. (ii) For Nups located closer to the central symmetry axis of the NPC, compressions can arise at the other, i.e. inner side of the distribution cloud as a direct consequence of large IgG rotation radii as in indirect immunogold-labeling. Such distortions, which can manifest themselves as ellipsoid gold grain distribution patterns instead of ideal circular ones, can arise when the actual distance between the NPC y-axis (dashed vertical) and the target site (T) in a cross-sectioned NPC is shorter than the length of the IgG rotation radius (scheme A; the circular area in grey representing the corresponding surface of revolution). In this scenario, an antibody can have labeled its target T on one side of the y-axis whereas its gold grain has collapsed onto the axis' other side (**A**). Such gold grains are nevertheless treated as if being located together with T on the same side of the mid-axis ($\mathbf{A^I}$). This results in underestimation of the true grain distance to T and in plotted distribution clouds in which those parts closest to the mid-axis can appear compressed. At the same time, the calculated mean D_y value for all

gold grains will be larger than the true x value for T, or than mean $\boldsymbol{D_y}$ values derived from labelings with gold-coupled primary antibodies or Fab fragments with smaller rotation radii (exemplified by $[\sum d_{30\text{-}1}^2, d_{30\text{-}2}^2, d_{30\text{-}3}^2, d_{30\text{-}4}^2]x4^{-1} > [\sum d_{15\text{-}1}^2, d_{15\text{-}2}^2, d_{15\text{-}3}^2, d_{15\text{-}4}^2]x4^{-1}$; compare schemes $\mathbf{A^I}$ and **B**). Using the original mean *d* and *r* values from each of the present data sets of gold-labeled NPCs for mathematical simulations testing rotation radii of 30 and 15 nm, the theoretical maximal differences between mean $\boldsymbol{D_y}$ values versus putative real x values ($\boldsymbol{D_{prx}}$) were determined. For an antibody rotation radius of 30 nm, ***Dy*** and smaller ***Dprx*** values could differ by up to 3 nm. In contrast, when considering a rotation radius of 15 nm, values did not differ at all or merely by less than 0.5 nm. Simulations were performed by a geometrical approximation approach (data not shown), and independently confirmed by using the equation: $\boldsymbol{D_{prx} = \sqrt{R^2 - r^2 + d_{nw}^2}}$ in which $\boldsymbol{d_{nw}}$ stands for the corrected mean distance (non-normalized) between midaxis and measured gold grains**.** The value for $\boldsymbol{d_{nw}}$ is obtained by the equation: $\boldsymbol{d_{nw} = d_m(\pi\, r_{ab}^2 - 2CS)(\pi\, r_{ab}^2)^{-1}}$**,** in which $\mathbf{d_m}$ stands for the non-normalized mean distance between midaxis and measured gold grains, $\mathbf{r_{ab}}$ for the presumed antibody rotation radius, and **CS** for the surface of the circle segment representing all values of opposite algebraic sign (scheme **C**). The value for **CS** is given by the equation:

$\mathbf{CS = \pi\, r_{ab}^2 \frac{2 \arccos(\frac{d_m}{r_{ab}})}{360} - d_m\sqrt{r_{ab}^2 - d_m^2}}$. Use of gold-coupled primary antibodies or Fab fragments may be especially advantageous for the topological mapping of Nups located especially close to the NPC midaxis, as may be the case for the components of the Nup62 subcomplex, and others.