Supplementary Information

Dimerization and direct membrane interaction of Nup53 contribute to nuclear pore complex assembly

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Supplementary information contains:

Supplementary Figures S1–S7 & Table S1–S2

Supplementary Methods

Supplementary References

Supplementary Figure S1

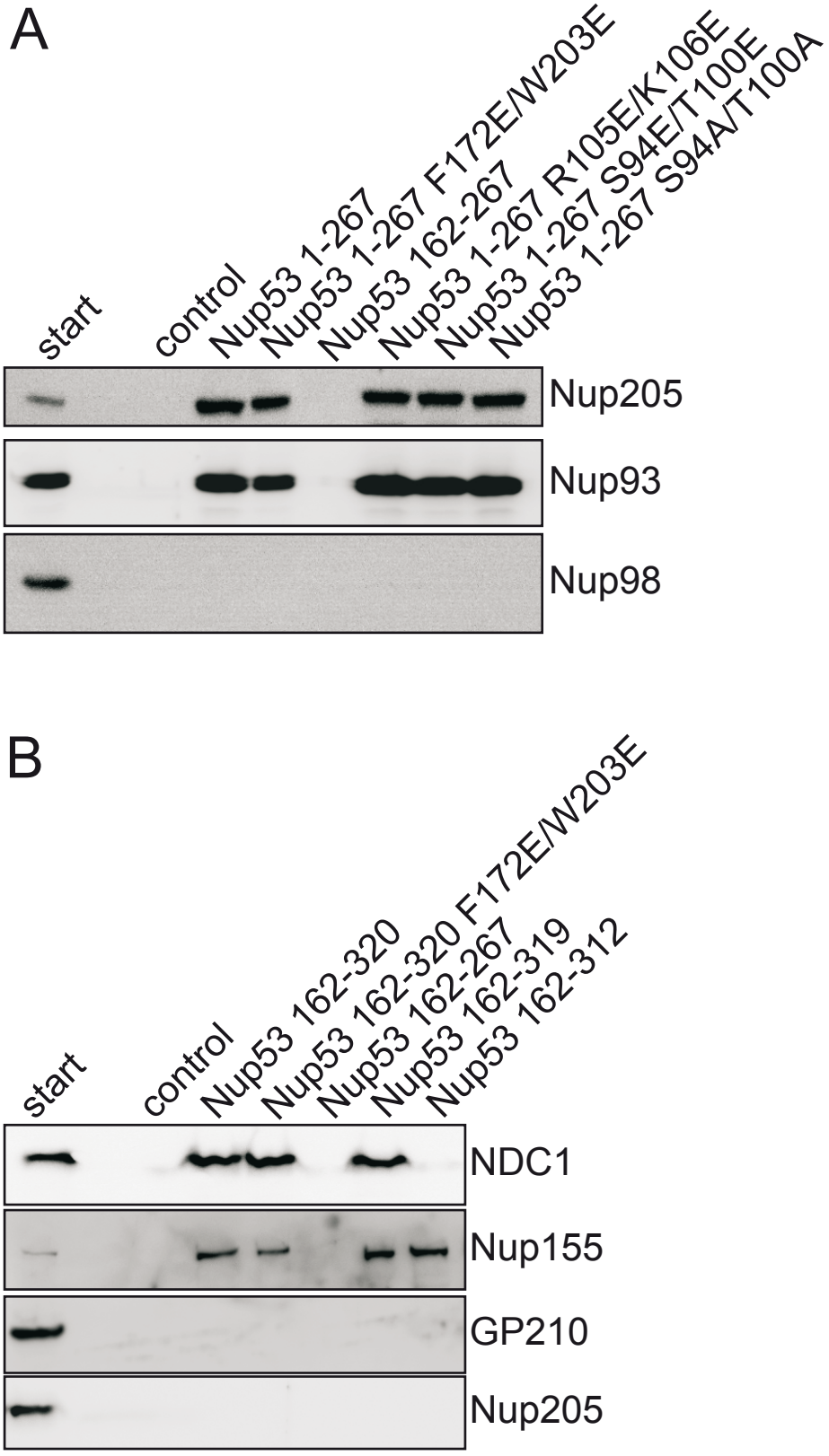


Figure S1

Nup53 has different binding sites for membrane and protein interaction.

(A) GST fusions of an N-terminal fragment of Xenopus Nup53 as well as Xenopus Nup98 (aa 487-634) (control) were incubated with cytosol from Xenopus egg extracts. Eluates were analyzed by western blotting with antibodies against the nucleoporins Nup205 and Nup93, known to bind this region, as well as Nup98 as a negative control. Please note that introducing amino acid changes causing the monomerization of Nup53 (F172E/W203E) also negatively influenced the interaction with Nup205 and Nup93. In contrast, mutations that inactivate the N-terminal membrane binding region (R105E/K106E and S94E/T100E) as well as the S94A/T100A control mutation did not interfere with Nup205 and Nup93 binding.

(B) GST fusions Xenopus Nup98 (aa 487-634) (control), the C-terminal fragment of Xenopus Nup53 (162-320), the RRM mutant (F172E/W203E) and C-terminal truncations were incubated with cytosol (for detection of Nup155 and Nup205) or Triton X-100 solubilized membranes (for NDC1 and GP210 detection) from Xenopus egg extracts. Eluates were analyzed by western blotting with antibodies against the nucleoporins Nup155 and NDC1, known to bind this region as well as Nup205 and GP210 as negative controls. Please note that mutation of the RRM domain (F172E/W203E) did not influence the interaction with NDC1 but results in a decreased binding to Nup155. The C-terminal truncations weakening the C-terminal membrane binding region (162-319 and 162-312) did not interfere with Nup155 binding and the 162-319 fragment was still able to interact with NDC1.

Supplementary Figure S2

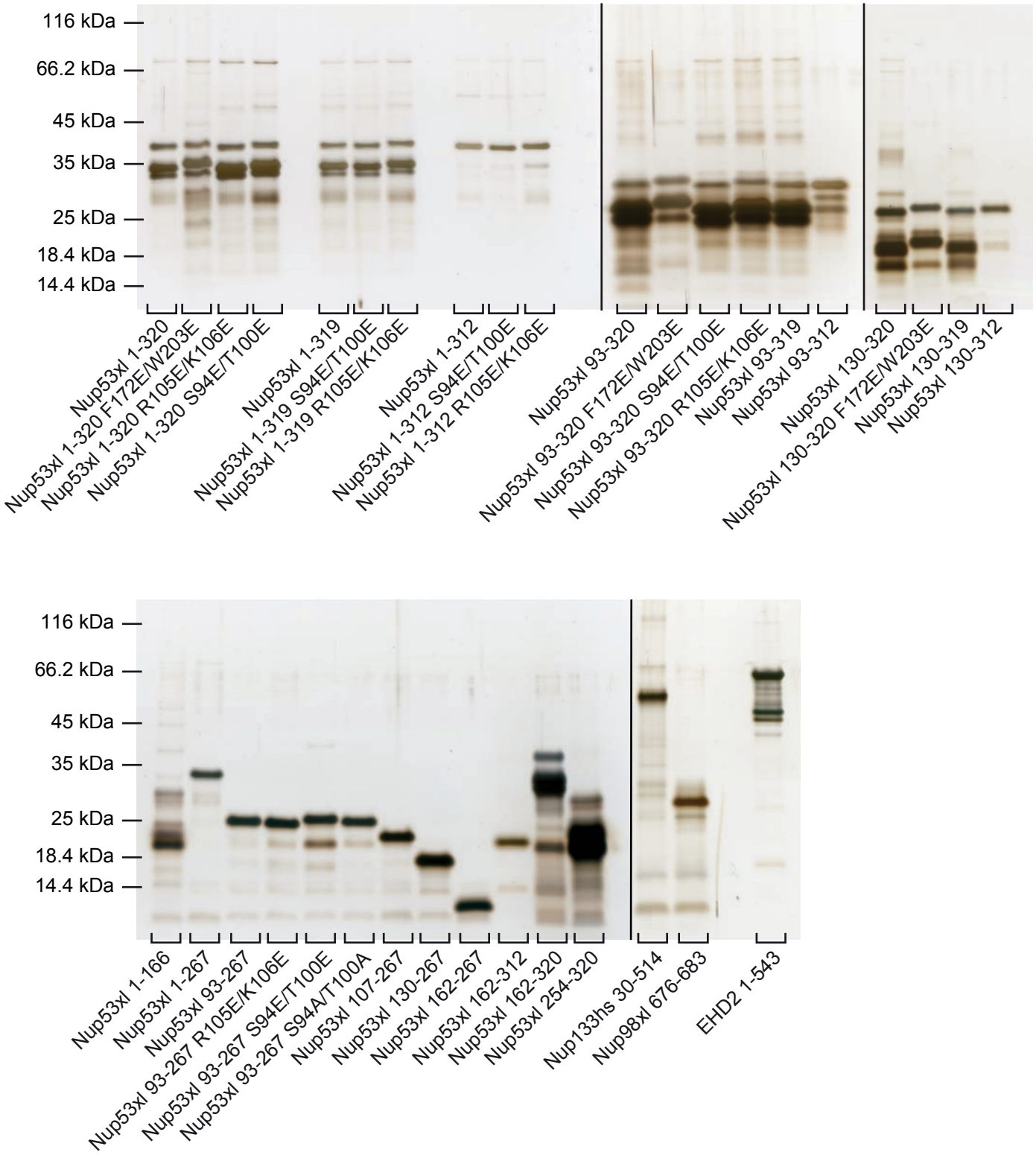
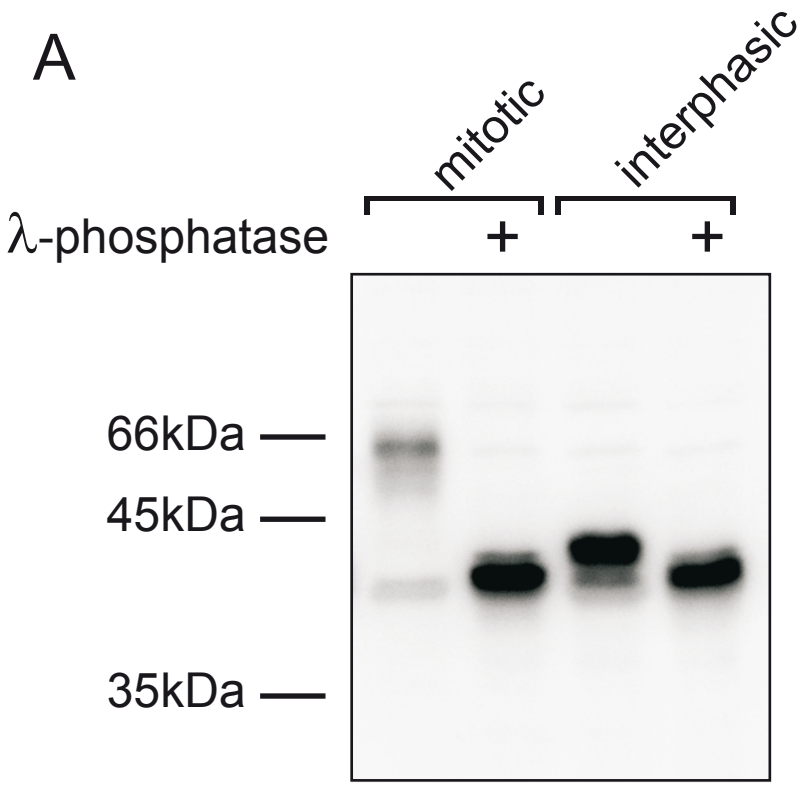


Figure S2

Proteins were separated on Tricine-SDS-PAGE Schägger gels (Schagger & von Jagow, 1987) followed by silver staining.

Supplementary Figure S3



B

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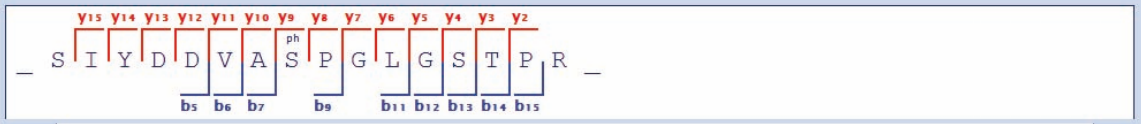
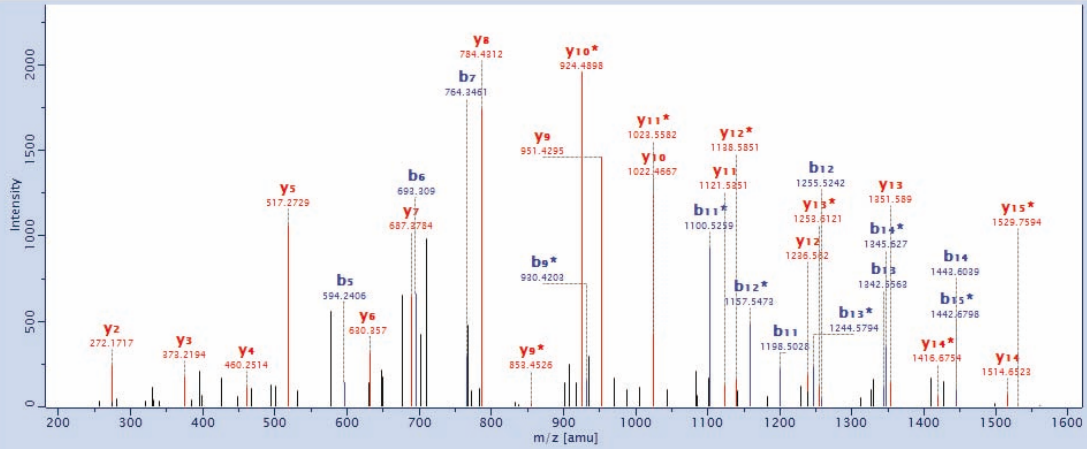
Method: CID; ITMS





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general information

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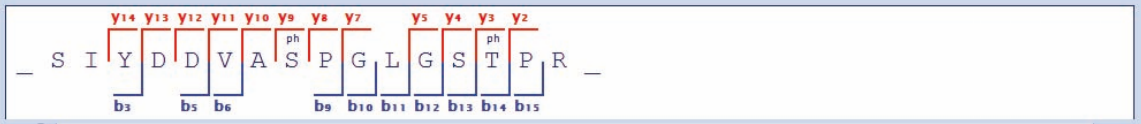

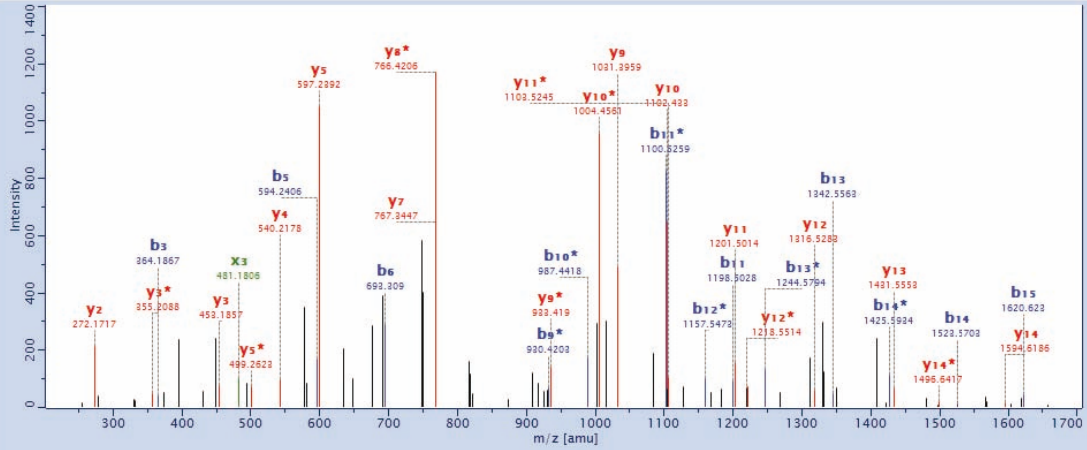

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general information

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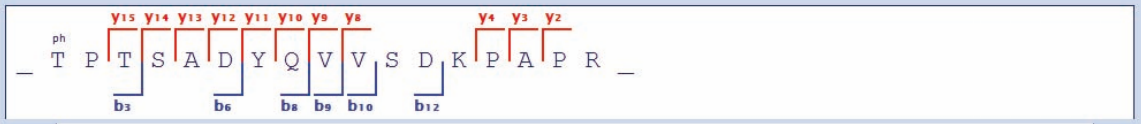

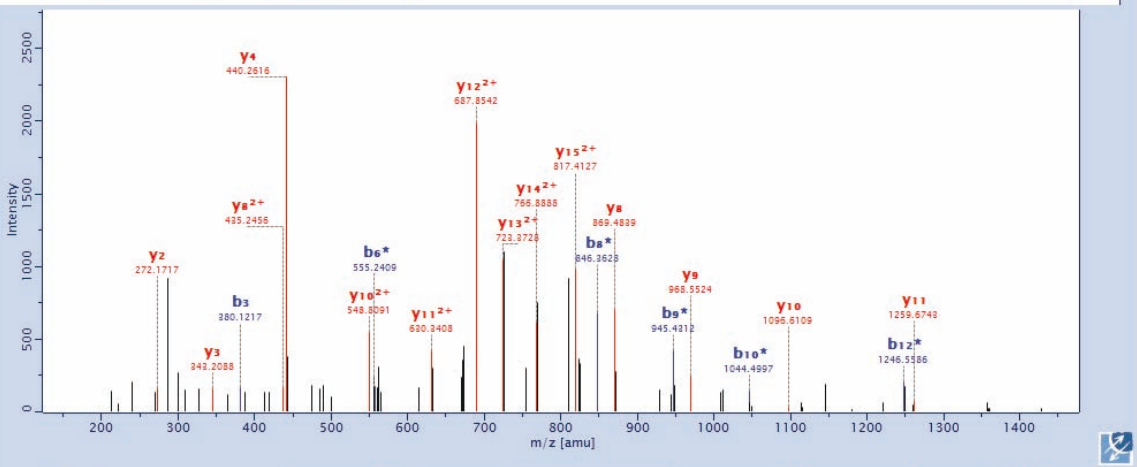

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general information

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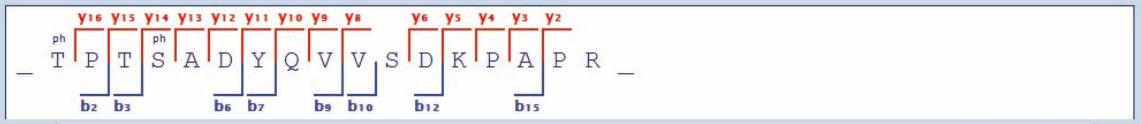

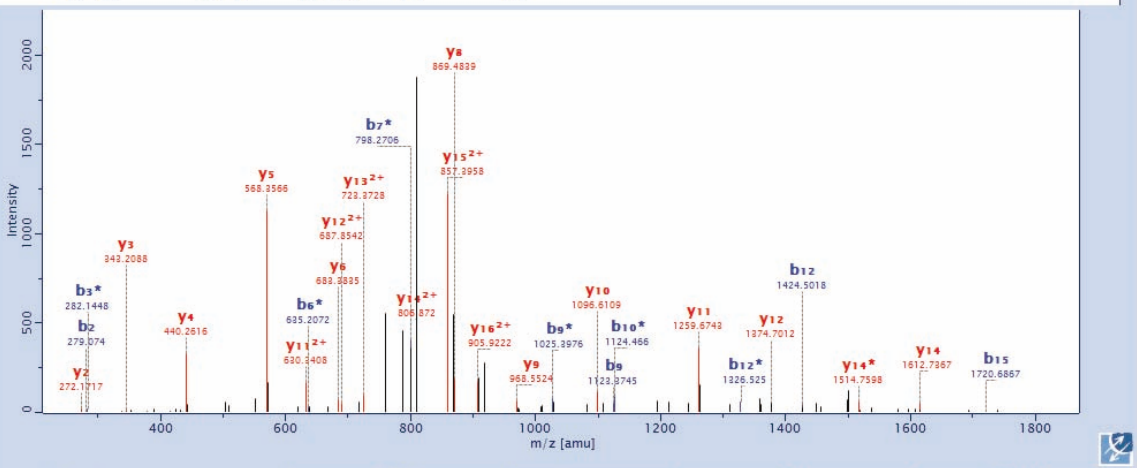

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general information

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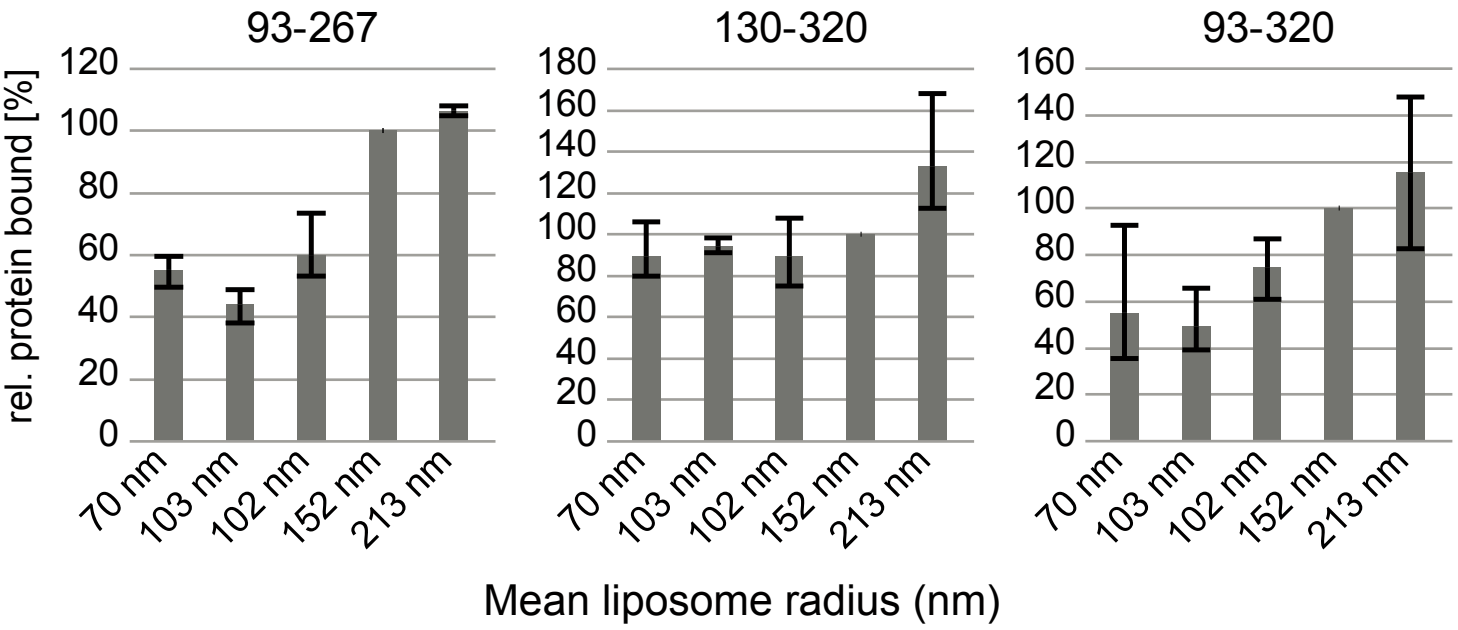
Figure S3

(A) Nup53 is phosphorylated in interphase and mitosis

4 µl of mitotic (CSF arrested) or interphasic Xenopus egg extracts were diluted in 100 µl of phosphatase buffer (NEB) and incubated where indicated with 400 U λ-phosphatase for 30 min at 30°C. Samples were analyzed by 12% SDS-PAGE and Western blotting using the Xenopus Nup53 antibody. Please note the different shifting of mitotic and interphasic Nup53 after phosphatase treatment indicating that Nup53 is a phosphoprotein throughout the cell cycle but hyperphosphorylated during mitosis.

(B) Fragmentation mass spectra of Xenopus Nup53 peptides carrying mitotic specific phosphorylations

Supplementary Figure S4



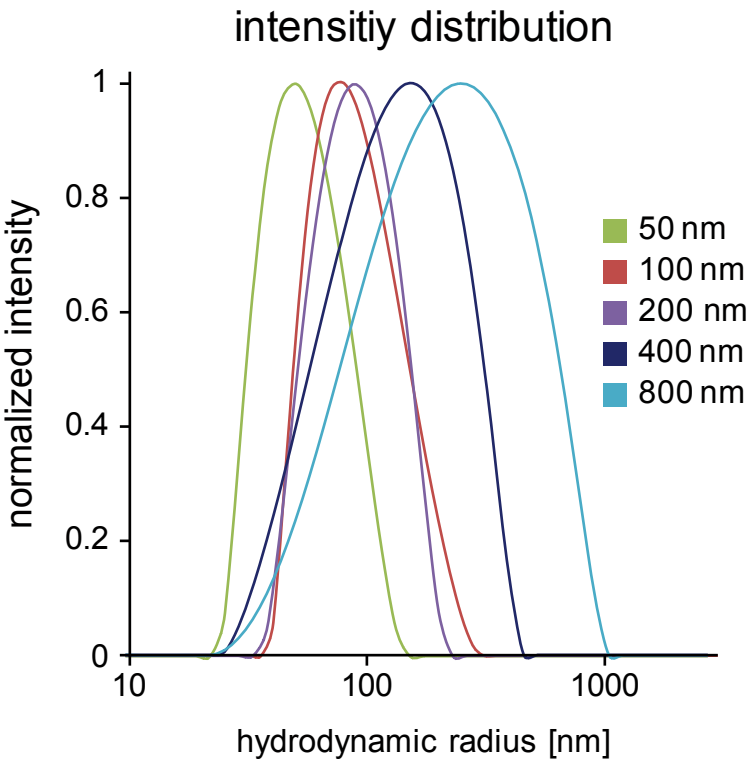


Figure S4

The two Nup53 membrane binding regions show different sensitivity to membrane curvature. Fragments comprising the N-terminal (93-267), C-terminal (130-320) or both (93-320) membrane binding sites, respectively, including the RRM domains were incubated with differently sized liposomes as indicated by the determined mean radii. Liposome binding was quantified as in Figure 2C. Whereas fragments which include the N-terminal membrane binding site (93-267 and 93-320) showed a significantly reduced binding to smaller liposome diameters and thus to higher membrane curvature this effect was not seen for the C-terminal membrane binding site (130-320). The averages of three independent experiments, normalized to the binding of the respective fragments to 150 nm liposomes, are shown. Error bars represent the range. Liposome radii were determined by light scattering after extrusion through membranes of different pore size as indicated for the different measurements. The lower panel shows one exemplary measurement done to determine the average radius of the respective preparation. Please note the rather similar average radii of liposomes prepared using 100 nm and 200 nm membranes.

Supplementary Figure S5

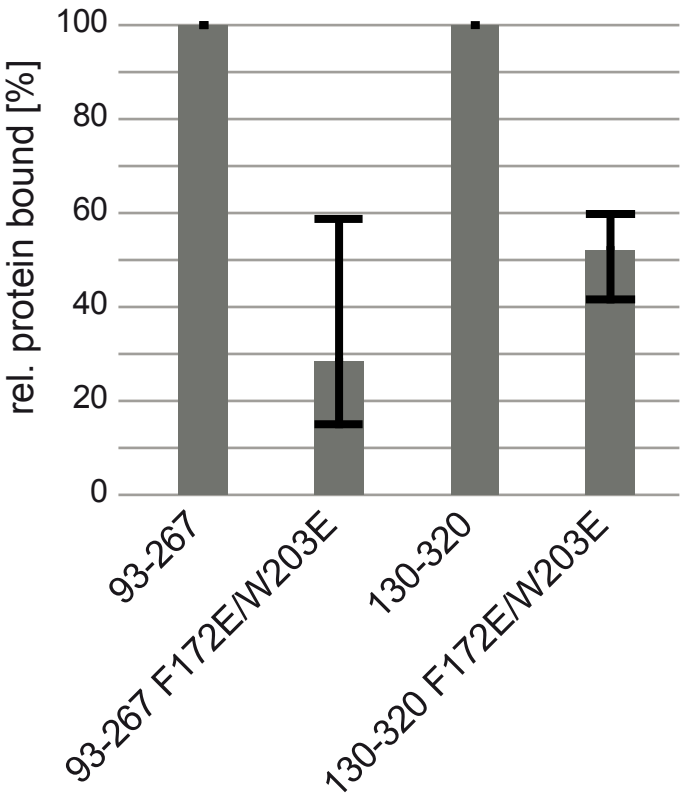


Figure S5

Both Nup53 membrane binding regions require dimerization by the RRM domain.

Nup53 fragments containing the first (93-267) or second (130-320) membrane binding region including the RRM domain were quantitatively assayed for liposome binding as in Figure 2C. Whereas fragments containing the wild type RRM domain bound to liposomes, introduction of two amino acid changes (F172E/W203E), which render the RRM domain incapable of dimerization, reduced liposome binding for both fragments. The averages of three independent experiments, normalized to liposome binding of the wild type protein, are shown. Error bars represent the range.

Supplementary Figure S6

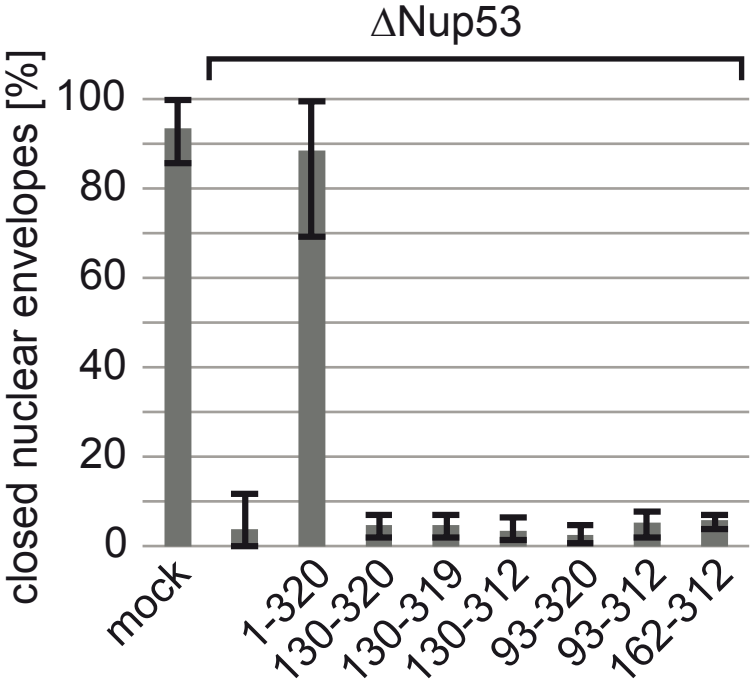


Figure S6

The interaction of Nup53 to Nup93 is necessary for nuclear envelope formation.

Nuclei were assembled in mock, Nup53 depleted extracts or Nup53 depleted extracts supplemented with wild type protein (1-320) or various fragments of Nup53 for 120 min, fixed with 2% PFA and 0.5% glutaraldehyde and analyzed for chromatin and membrane staining. Shown is the quantitation of chromatin substrates with a closed nuclear envelope as done in Figure 2F. Please note that all fragments lacking the N-terminal region of Nup53 necessary for Nup93 interaction (Figure S1A) and especially fragment 162-312 which has the ability to interact with Nup155 (Figure S1B) did not support nuclear envelope formation.

Supplementary Figure S7

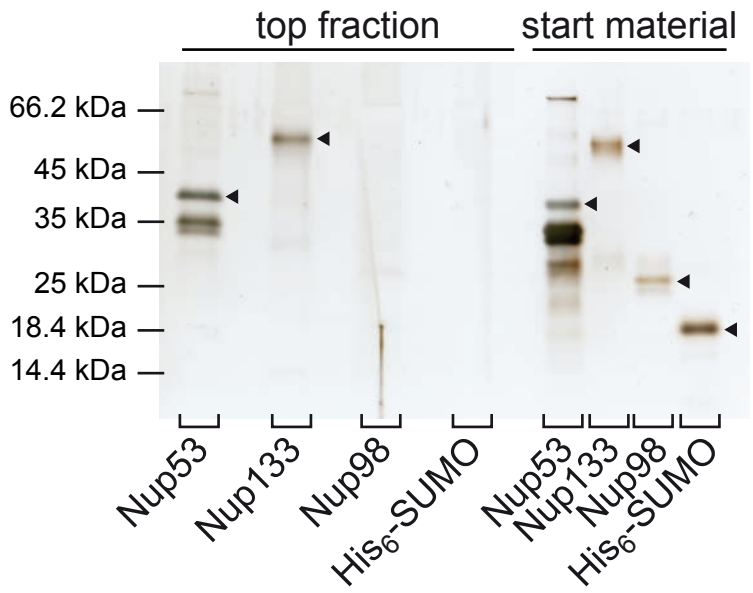


Figure S7

Recombinant Nup53 binds to liposomes mimicking the ER / nuclear envelope lipid composition

3 µM recombinant Xenopus Nup53 (Nup53) a fragment of Nup133 (aa 67-514) as positive control and Nup98 (aa 676-863) and His_6-tagged SUMO as negative controls, were incubated with 6 mg/ml fluorescently labeled liposomes prepared from a lipid mixture mimicking the ER/nuclear envelope lipid composition (see materials and methods). Flotation was done as described in Figure 1A.

Table S1: Peptides and phosphorylation sites identified in Xenopus Nup53 by mass spectrometric analysis

phosphopeptide	amino acid
PSAGAQFLPGFLLGDIPTPV**T**PQPR	T46
SPLH**S**GG**S**PPQPVLPTHK	S60, S64, S67
SPLHSGG**S**PPQPVLPTHK	S67
SIYDDVA**S**PGLGSTPR	S94
SIYDDVA**S**PGLGS**T**PR	S94, T100
MASFSVLHTPLSGAIPS**S**PAVFSPATIGQSR	S124
MASFSVLHTPLSGAIPSSPAVF**S**PATIGQSR	S131
V**S**TPSVSSVFTPPVK	S249
VS**T**PSVSSVFTPPVK	T250
VSTP**S**VSSVFTPPVK	S252
VSTP**S**VSSVF**T**PPVK	S252, T258
VSTPSVSSVF**T**PPVK	T258
SIRTPTQSVGTPR	S263
SIR**T**PTQSVGTPR	T266
SIRTPTQ**S**VGTPR	S270
TPTQSVG**T**PR	T273
TPTSADYQVVSDKPAPR	T288
TPT**S**ADYQVVSDKPAPR	T288, S291

Phosphorylation sites mapped in XenopusNup53 (genebank accession JQ747515) after immunoprecipitation from mitotic or interphase Xenopus egg extracts. Phosphorylation sites are indicated in red. Position T100, T288 and S291 were phosphorylated on Nup53 isolated from mitotic, but not interphasic extracts.

Table S2: DNA constructs used in this study

Constructs
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pET28a SUMO Nup53xl 162-312
pET28a SUMO Nup53xl 162-320
pET28a SUMO Nup53xl 254-320
pET28a SUMO Nup59sc 1-528
pET28a SUMO Nup53sc 1-475
pET28a PP Nup133hs 30-514
pET28a NusA Nup98xl 676-863
pET28a GST Nup53xl 1-267
pET28a GST Nup53xl 1-267 S94A/T100A
pET28a GST Nup53xl 1-267 S94E/T100E
pET28a GST Nup53xl 1-267 R105E/K106E
pET28a GST Nup53xl 1-267 F172E/W203E
pET28a GST Nup53xl 162-267
pET28a GST Nup53xl 162-312
pET28a GST Nup53xl 162-319
pET28a GST Nup53xl 162-320
pET28a GST Nup53xl 162-320 F172E/W203E
pET28a GST Nup98xl 487-634

Constructs
pSI HA Nup53xl
pSI HA Nup53xl F172E/W203E
pSI myc Nup53xl
pSI myc Nup53xl F172E/W203E

Supplementary Methods

Pulldown experiments

Fragments used for the GST pulldown experiments were cloned into a modified pET28a vector with GST tag followed by a recognition site for TEV protease and purified via the N-terminal His_6 tag. 60 µl GSH–Sepharose (GE Healthcare) were incubated with 300 µg of the respective bait proteins, washed and blocked with 5% BSA in PBS. Beads were incubated with cytosol from Xenopus egg extracts (diluted 1:1 with PBS, and cleared by centrifugation for 30 min at 100,000 rpm in a TLA110 rotor (Beckman Coulter) for 2 h and washed six times with PBS. Bound proteins were eluted by cleavage with TEV protease (0.5 mg/ml) for 1 h at RT and analyzed by SDS-PAGE and Western blotting. For detection of NDC1 and GP210, 5 mg of membranes from Xenopus egg extracts (Antonin et al, 2005) were solubilized in 5 ml 50 mM Phosphate buffer pH 7.4, 500 mM NaCl, 1% Triton X-100 and protease inhibitors (Roche), instead of cytosol and the first four washes with PBS were in the presence of 0.1% Triton X-100.

Mass Spectrometry

2 ml interphasic (Hartl et al, 1994) or (CSF arrested) mitotic (Murray, 1991) Xenopus egg extracts were diluted with 1.2 ml wash buffer (10 mM HEPES, 50 mM KCl, 2.5 mM $MgCl_2$ pH 7.4), cleared by centrifugation for 10 min at 100,000 rpm in a TLA110 rotor and incubated with 50 µl Protein A Sepharose (GE Healthcare), to which affinity purified Nup53 antibodies were bound and crosslinked with 10 mM dimethylpimelimidate (Pierce). After 1h incubation the sepharose was washed 10 times with wash buffer. Proteins were eluted with SDS sample buffer (without DTT) and separated by SDS-PAGE. Gel sections from 30-45 kDa were excised and proteins were in-gel digested by trypsin. The resulting peptide mixtures were measured on an LTQ-Orbitrap XL and processed by MaxQuant software as described (Borchert et al, 2010). Multistage activation was enabled in all MS measurements.

Generation of liposomes

A mixture of lipids resembling the ER/nuclear envelope composition (Franke et al, 1970) (60 mol % phosphatidylcholine, 19.8 mol % phosphatidylethanolamine, 10 mol % phosphatidylinositol, 5 mol % cholesterol, 2.5 mol % sphingomyelin, 2.5 mol % phosphatidylserine 0.2 mol % 18:1-12:0 NBD-PE all Avanti polar lipids) dissolved in chloroform were dried on a rotary evaporator and overnight under vacuum. PBS buffer was gently added to result in a final lipid concentration of 6 mg/ml. After 2 h of incubation at

37°C to allow spontaneous liposome formation the flask was agitated to dissolve residual lipids. After ten cycles of freeze/thawing liposomes were extruded as described before.

DNA sequence of Xenopus laevis Nup53 optimized for expression in E. coli

ATGATGGCAGCAGCATTTAGCATGGAACCGATGGGTGCAGAACCGATGGCACTG
GGTAGCCCGACCAGCCCGAAACCGAGTGCCGGTGCACAGTTTCTGCCTGGTTTTC
TGCTGGGTGATATTCCGACACCGGTTACACCGCAGCCTCGTCCGAGCCTGGGTAT
TATGGAAGTTCGTAGTCCGCTGCATAGCGGTGGTAGTCCTCCGCAGCCGGTTCTG
CCGACCCATAAAGATAAAAGCGGTGCACCTCCGGTTCGTAGCATTTATGATGATG
TTGCAAGTCCGGGTCTGGGTAGCACACCGCGTAATACCCGTAAAATGGCAAGCTT
TAGCGTTCTGCATACACCTCTGAGCGGTGCAATTCCGAGCAGTCCGGCAAGCAAT
GTTTTTAGTCCGGCAACCATTGGTCAGAGCCGTAAAACCACCCTGAGTCCGGCAC
AGATGGACCCGTTTTATACCCAGGGTGATGCACTGACCAGTGATGATCAGCTGGA
TGATACCTGGGTTACCGTTTTTGGTTTTCCGCAGGCAAGCGCAAGCTATATTCTGC
TGCAGTTTGCACAGTATGGCAATATTATTAAACATGTGATGAGCAATAATGGCAA
TTGGATGCATATTCAGTATCAGAGCAAACTGCAGGCACGTAAAGCACTGAGCAA
AGATGGTCGTATTTTTGGTGAAAGCATTATGATTGGTGTGAAACCGTGCATTGAT
AAAAGCGTTATGGAAGCAACCGAAAAAGTTAGCACCCCGAGCGTTAGCAGCGTT
TTTACACCTCCGGTTAAAAGCATTCGTACCCCGACCCAGAGCGTTGGTACACCGC
GTGCAGCAAGCATGCGTCCGCTGGCAGCAACCTATCGCACCCCGACCAGCGCAG
ATTATCAGGTTGTTAGCGATAAACCGGCACCGCGTAAAGATGAAAGCATTGTTAG
CAAAGCCATGGAATATATGTTTGGTTGGTGATAG

DNA sequence of Saccharomyces cerevisiae NUP53 optimized for expression in E. coli

ATGGCAGATCTGCAGAAACAAGAAAATTCAAGCCGTTTTACCAATGTTAGCGTTA
TTGCACCGGAAAGCCAGGGTCAGCATGAACAGCAGAAACAGCAAGAACAACAA
GAACAGCAGAAACAGCCGACAGGTCTGCTGAAAGGTCTGAATGGTTTTCCGAGC
GCACCGCAGCCGCTGTTTATGGAAGATCCTCCGAGCACCGTTAGCGGTGAACTGA
ATGATAATCCGGCATGGTTTAATAATCCGCGTAAACGTGCAATTCCGAATAGCAT
TATTAAACGTAGCAATGGTCAGAGCCTGAGTCCGGTTCGTAGCGATAGCGCAGAT
GTTCCGGCATTTAGCAATAGCAATGGCTTTAATAATGTGACCTTTGGCAGCAAAA
AAGATCCGCGTATTCTGAAAAATGTGAGCCCGAATGATAATAATAGCGCCAATA
ATAATGCCCATAGCAGCGATCTGGGCACCGTTGTTTTTGATAGCAATGAAGCACC
TCCGAAAACCAGCCTGGCAGATTGGCAGAAAGAAGATGGTATTTTTAGCAGCAA
AACCGATAATATTGAAGATCCGAATCTGAGCAGCAATATTACCTTTGATGGTAAA
CCGACCGCAACCCCGAGCCCGTTTCGTCCGCTGGAAAAAACCAGCCGTATTCTGA
ATTTTTTTGATAAAAATACCAAAACCACCCCGAATACCGCAAGCAGCGAAGCAA
GCGCAGGTAGCAAAGAAGGTGCAAGCACCAATTGGGATGATCATGCCATTATTA
TTTTTGGCTATCCGGAAACCATTGCCAATAGTATTATTTTTCATTTTGCCAATTTTG
GCGAAATTCTGGAAGATTTTCGCGTGATTAAAGATTTTAAAAAGCTGAACAGCAA
AAATAAAAGCAAAAGCCCGAGCCTGACCGCACAGAAATATCCGATTTATACCGG
TGATGGTTGGGTTAAACTGACCTATAAAAGCGAACTGAGCAAAAGCCGTGCACT
GCAAGAAAATGGCATTATTATGAATGGCACCCTGATTGGTTGCGTTAGCTATAGT
CCGGCAGCACTGAAACAGCTGGCAAGCCTGAAAAAAAGCGAAGAAATTATTAAT
AATAAAACCAGCAGCCAGACCAGCCTGAGCAGCAAAGATCTGAGCAATTATCGT
AAAACCGAAGGCATTTTTGAAAAAGCCAAAGCAAAAGCGGTGACCAGCAAAGTT
CGTAATGCCGAATTTAAAGTGAGCAAAAATAGCACCAGCTTTAAAAATCCGCGTC
GCCTGGAAATTAAAGATGGTCGTAGCCTGTTTCTGCGTAATCGTGGTAAAATTCA
TAGCGGTGTTCTGAGCAGCATTGAAAGCGATCTGAAAAAACGTGAACAGGCAAG
CAAAAGCAAAAAAAGCTGGCTGAATCGCCTGAATAATTGGCTGTTTGGTTGGAAT
GATCTGTAGTGA

DNA sequence of Saccharomyces cerevisiae NUP59 optimized for expression in E. coli

ATGTTTGGTATTCGCAGCGGCAATAATAATGGTGGTTTTACCAATCTGACCAGCC
AGGCACCGCAGACCACCCAGATGTTTCAGAGCCAGAGCCAGCTGCAGCCGCAGC
CGCAGCCTCAACCGCAGCAGCAGCAACAGCATCTGCAGTTTAATGGTAGCAGTG
ATGCAAGCAGCCTGCGTTTTGGTAATAGCCTGAGCAATACCGTGAATGCCAATAA
TTATAGCAGCAATATTGGCAATAACAGCATCAACAATAATAACATCAAAAATGG
CACCAATAACATTAGCCAGCATGGTCAGGGCAATAATCCGAGCTGGGTTAATAAT
CCGAAAAAACGTTTTACACCGCATACCGTTATTCGTCGTAAAACCACCAAACAGA
ATAGCAGCAGCGATATTAATCAGAATGATGATAGCAGCAGCATGAATGCAACCA
TGCGTAATTTTAGCAAACAGAATCAGGATAGCAAACATAATGAACGCAATAAAA
GCGCAGCCAATAATGATATTAATAGCCTGCTGAGCAACTTTAATGATATTCCTCC
GAGCGTTACCCTGCAGGATTGGCAGCGTGAAGATGAATTTGGTAGCATTCCGAGC
CTGACCACCCAGTTTGTTACCGATAAATATACCGCCAAAAAAACCAATCGCAGCG
CCTATGATAGCAAAAATACCCCGAATGTGTTTGATAAAGATAGCTATGTGCGCAT
TGCCAATATTGAACAGAATCATCTGGATAATAATTATAATACCGCAGAAACCAAT
AATAAAGTGCATGAAACCAGCAGCAAAAGCAGCAGCCTGAGCGCAATTATTGTT
TTTGGTTATCCGGAAAGCATTAGCAATGAACTGATTGAACATTTTAGCCATTTTGG
CCATATTATGGAAGATTTTCAGGTTCTGCGTCTGGGTCGTGGTATTAATCCGAATA
CCTTTCGCATTTTTCATAATCATGATACCGGCTGTGATGAAAATGATAGCACCGTG
AATAAAAGCATTACCCTGAAAGGTCGCAATAATGAAAGTAATAACAAAAAATAT
CCGATTTTTACAGGCGAAAGCTGGGTTAAACTGACCTATAATAGCCCGAGCAGCG
CACTGCGTGCACTGCAAGAAAATGGTACAATTTTTCGTGGTAGCCTGATTGGTTG
TATTCCGTATAGCAAAAATGCCGTTGAACAGCTGGCAGGTTGCAAAATTGATAAT
GTGGATGATATTGGCGAATTTAATGTGAGCATGTATCAGAATAGCAGTACCAGCA
GCACCAGCAATACCCCGAGTCCTCCGAATGTTATTATTACCGATGGCACCCTGCT
GCGCGAAGATGATAATACACCGGCAGGTCATGCAGGCAATCCGACCAATATTAG
CAGCCCGATTGTTGCAAATAGCCCGAATAAACGTCTGGATGTGATTGATGGTAAA
CTGCCGTTTATGCAGAATGCAGGTCCGAATAGCAATATTCCGAATCTGCTGCGTA
ATCTGGAAAGCAAAATGCGTCAGCAAGAAGCAAAATATCGTAATAATGAACCGG
CAGGCTTTACCCATAAACTGAGCAATTGGCTGTTTGGTTGGAATGATCTGTAGTG
A

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