The nucleoporin Nup50 activates the Ran guanyl-nucleotide exchange factor RCC1 to promote NPC assembly at the end of mitosis – APPENDIX FIGURES

Guillaume Holzer (1), Paola de Magistris (1,2,5), Cathrin Gramminger (2), Ruchika Sachdev (2), Adriana Magalska (2), Allana Schooley (2), Anja, Scheufen (1), Birgitt Lennartz (1), Marianna Tatarek-Nossol (1), Hongqi Lue (1), Monika I. Linder (3, 6), Ulrike Kutay (3), Christian Preisinger (4), Daniel Moreno-Andres (1), Wolfram Antonin (1)

(1) Institute of Biochemistry and Molecular Cell Biology, Medical School, RWTH Aachen University, 52074 Aachen, Germany

(2) Friedrich Miescher Laboratory of the Max Planck Society, Spemannstraße 39, 72076 Tübingen, Germany

(3) Institute of Biochemistry, ETH Zurich, Otto-Stern-Weg 3, 8093 Zurich, Switzerland

(4) Proteomics Facility, Interdisciplinary Centre for Clinical Research (IZKF), Medical School, RWTH Aachen University, Aachen, Germany

(5) Current address: Kavli Institute of Nanoscience, Department of Bionanoscience, van der Maasweg 9, 2629HZ Delft, the Netherlands

(6) Current address Department of Pediatrics, Dr. von Hauner Children's Hospital and Gene Center, University Hospital, LMU, 80337 Munich, Germany

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Appendix Figure S 1: Characterization of Nup50 antisera, recombinant protein and depletion phenotype

(A) Western blot against Xenopus Nup50 on Mock treated (control, left lane) or Nup50 depleted (right lane) Xenopus egg extract.

(B) Confocal microscopy images of nuclei assembled for 120 min in mock depleted, Nup50 depleted (ΔNup50), and Nup50 depleted *Xenopus* egg extracts supplemented with recombinant Nup50. Then, 5 µM recombinant EGFP-M9-M10 as import substrate or EGFP-M9-NES as export substrate [71] were added, the later in the absence or presence of 300 nM leptomycin. After 120 min, nuclei were fixed in 4 % PFA and 0.5 % glutaraldehyde, membranes were pre-labelled with DiIC18 (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate, red) and chromatin was stained with DAPI (4',6-Diamidin-2-phenylindol, blue).

(C) Coomassie gels showing recombinant Nup50 purifications. The respective proteins are marked with an arrow.

(D) Coomassie gels showing the input of the GST-pulldown experiment presented in Fig. 5D.

(E) Coomassie gels showing the input of the GST-pulldown experiment presented in Fig. 7E.

Appendix Figure S2: Nup50 downregulation delays NPC assembly at the end of mitosis in living cells. (A) Live cell imaging of mitotic HeLa cells stably expressing EGFP-Nup107 showing the accumulation of EGFP-Nup107 signal on chromatin during mitotic exit. Chromatin is stained with SiR-DNA, time is normalized to the last metaphase frame. White arrows indicate when the EGFP-Nup107 signal appears on the chromatin surface during late mitosis, distinct from the kinetochore labeling. (B and C) Box and whiskers as well as cumulative frequency plots showing time of appearance of the first EGFP-Nup107 signal on the chromatin surface as in (A) for control (CTRL) (10 cells) and siNup50 (16 cells) treated cells from three independent experiments. (D) Quantitation of EGFP-Nup107 signal at the surface of the daughter chromatin masses 15 min after last metaphase. Truncated violin plots show the median (red line) and interquartile range (black lines). P-values were determined by two-tailed t-test. (E-H) Same analysis as in A-D in HeLa cells stably expressing EGFP-Nup133. Control (12 cells) and siNup50 (18 cells) from four independent experiments were used.

Appendix Figure S3: Nup50 interacts with MEL28/ELYS

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(A) Western blot analysis of Mock treated, MEL28/ELYS depleted or Nup50 depleted Xenopus egg extracts with MEL28/ELYS and Nup50 antibodies. The importin α-export factor CAS serves as loading control.

(B) Relative quantification of MEL28/ELYS and Nup50 proteins in depleted extract. Quantitation in mock treated extracts, MEL28/ELYS depleted extracts and Nup50 depleted extracts are respectively plotted from the left to the right. The white bar represents the amount of Nup50 protein and the grey bar represents the amount of MEL28/ELYS from n=4 independent experiments. Individual data points are indicated, data normalized to the mock condition.

Appendix Figure S4: Time course of Nup50 and MEL28/ELYS chromatin recruitment

(A, B) Demembranated sperm chromatin was preincubated in Xenopus egg extract for 10 min. At time point zero, membranes were added to the reaction. Reactions were stopped at the indicated time points by fixation and analyzed by confocal microscopy after immunofluorescence with α-Nup50 (A), α-

MEL28/ELYS (B), and mAb414 (A, B). Chromatin was stained with DAPI (left column in A and B). Scale bar: 10 µm.

Appendix Figure S5: Alignment of metazoan Nup50 144-189 with Nup2 from different yeast species

Sequence alignment of Nup50 144-189 (*X. laevis* numbering) with Nup2/Nup61, species are indicated on the left. The color scheme indicates the type of amino acids according to the alignment software default setting.

Appendix Figure S6: Localization of Nup50 mutants in in vitro nuclear assembly assays

Confocal microscopy images of nuclei assembled for 120 min in Nup50 depleted *Xenopus* egg extracts supplemented with recombinant wild type Nup50 or different mutants. Nuclei were fixed in 4% PFA and 0.5 % glutaraldehyde, stained for NPCs (mAB414), Nup50 and the chromatin (DAPI). Scale bar: 10 µm.

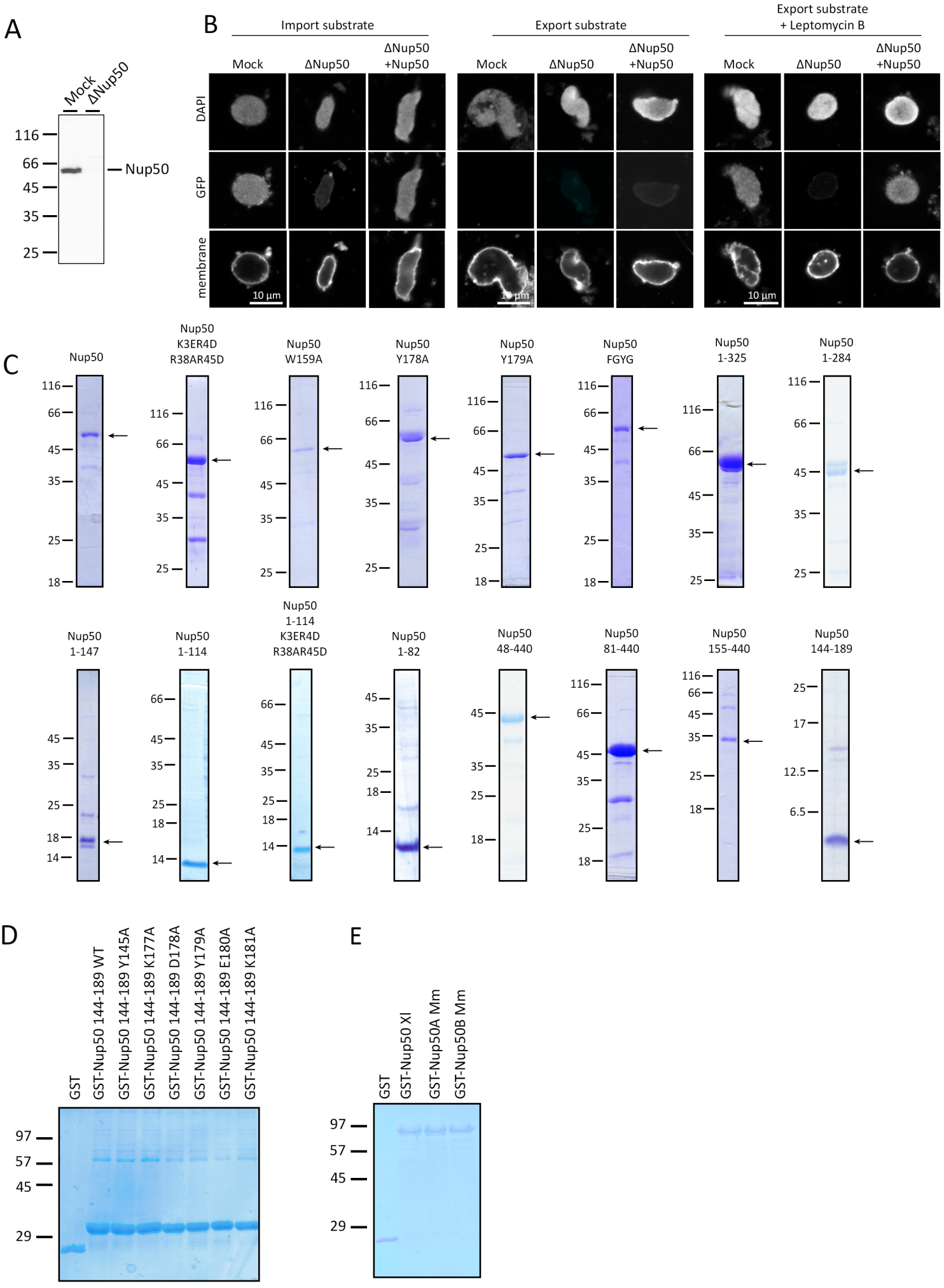
Appendix Figure S7: Nup50 N-terminus interacts with RCC1

(A) Xenopus egg extract were supplemented with 5 µM RanQ69L, RCC1, Nup50 wild type and fragments as well indicated combinations of 5 µM Nup50 and 5 µM RCC1. After 90 min, annulate lamellae were isolated by centrifugation and quantified by western blotting with mAB414 antibody. Quantitation shows the relative Nup62 signal as a mean from at least three experiments, normalized to the buffer control. Individual data points are indicated.

(B) HEK293T cells were transfected with empty control vector, different FLAG-Nup50 N-terminal fragments aa 1-120 and aa 48-120 (of the human sequence, respectively corresponding to the 1-114 or 48-114 residues of the Xenopus sequence, amino acid exchanges are identical between Xenopus and human proteins) or different double mutants designed to compromise RCC1 binding. Please note that with the E32AE33A a third Nup50 mutant, which is not impaired in RCC1 binding, is included. 24h post transfection cells were lysed, FLAG-tagged proteins immunoisolated and analyzed by western blotting, with 10% of the inputs loaded.

(C) GST-fusion constructs of the Xenopus Nup50 N-terminal fragment (aa 1-114) comprising no or the two RCC1-bidning mutations (G25AF27A and K36AR38A) were incubated with Xenopus egg extracts. Starting material (input) as well as bound proteins were analyzed by Western blotting with indicated antibodies. The quantitation shows the average bead bound signal from three independent experiments, normalized to the input. Data points from individual experiments are indicated. GST-Nup53 (aa 162-267) served as a control for unspecific binding (control).

(D) Coomassie gels showing recombinant proteins employed in the GEF assay (Fig 8E). The respective proteins are marked with an arrow.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ΔNup50
116
66
45
35
25
Nup50
B
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Export substrate
Export substrate
+ Leptomycin B
Mock
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ΔNup50
+Nup50
DAPI
GFP
membrane
10 µm
C
Nup50
Nup50
K3ER4D
R38AR45D
Nup50
W159A
Nup50
Y178A
Nup50
Y179A
Nup50
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Nup50
1-114
Nup50
1-114
K3ER4D
R38AR45D
Nup50
1-82
Nup50
48-440
Nup50
81-440
Nup50
155-440
Nup50
144-189
D
GST
GST-Nup50 144-189 WT
GST-Nup50 144-189 Y145A
GST-Nup50 144-189 K177A
GST-Nup50 144-189 D178A
GST-Nup50 144-189 Y179A
GST-Nup50 144-189 E180A
GST-Nup50 144-189 K181A
97
57
45
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E
GST
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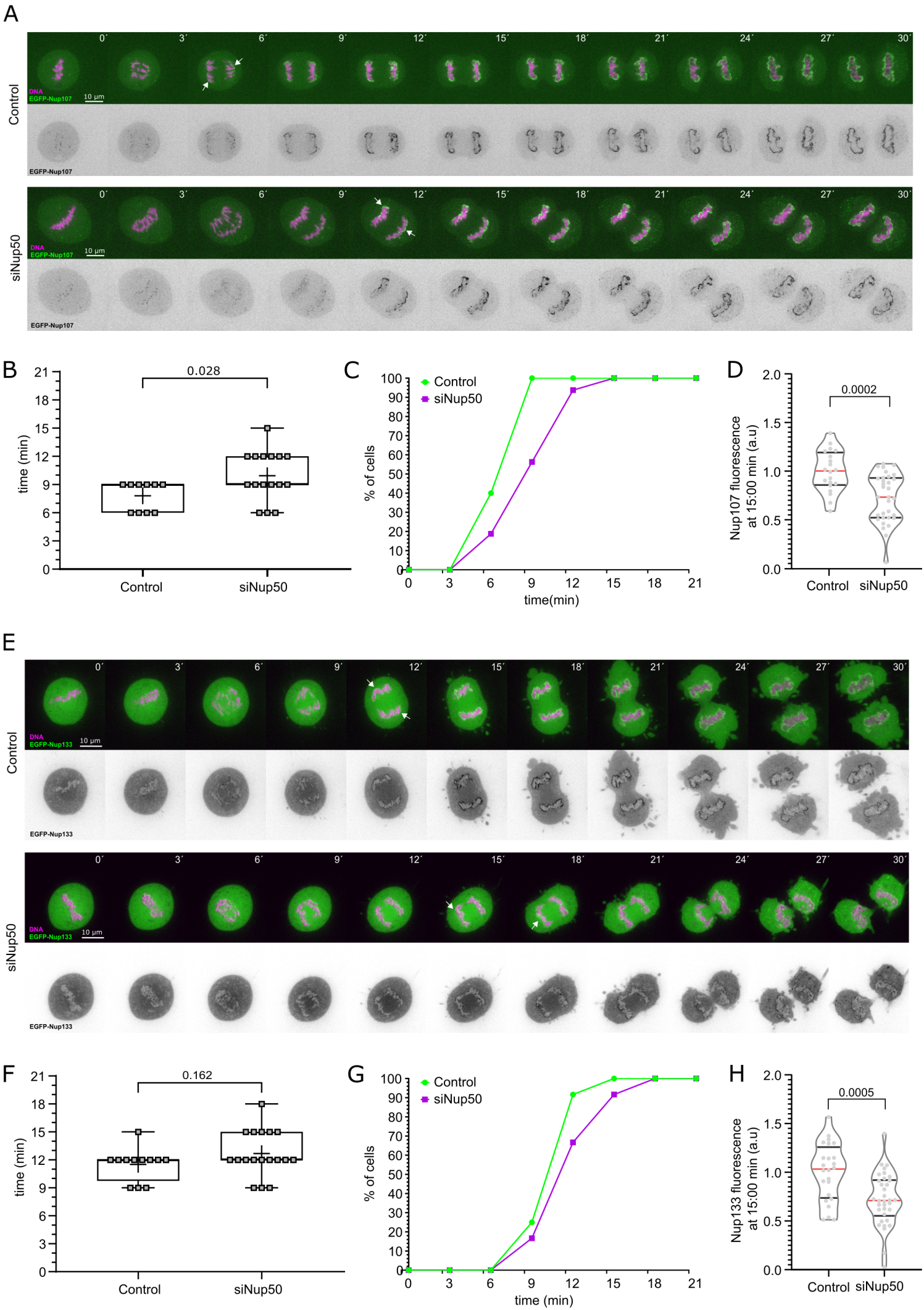
GST-Nup50B Mm
97
57
45
29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siNup50
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EGFP-Nup107
10 µm
EGFP-Nup107
B
time (min)
0.028
Control
siNup50
C
% of cells
Control
siNup50
time(min)
D
Nup107 fluorescence at 15:00 min (a.u)
0.0002
Control
siNup50
E
Control
siNup50
DNA
EGFP-Nup133
10 µm
EGFP-Nup133
F
time (min)
0.162
Control
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time (min)
H
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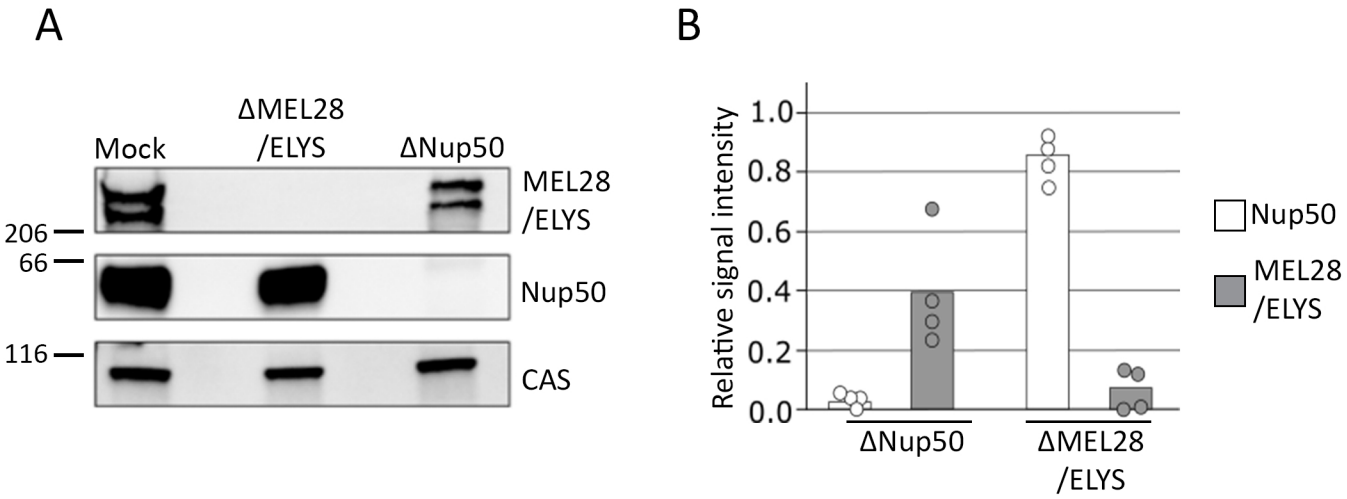
Control
siNup50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/ELYS
ΔNup50
206
66
116
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/ELYS
Nup50
CAS
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0.4
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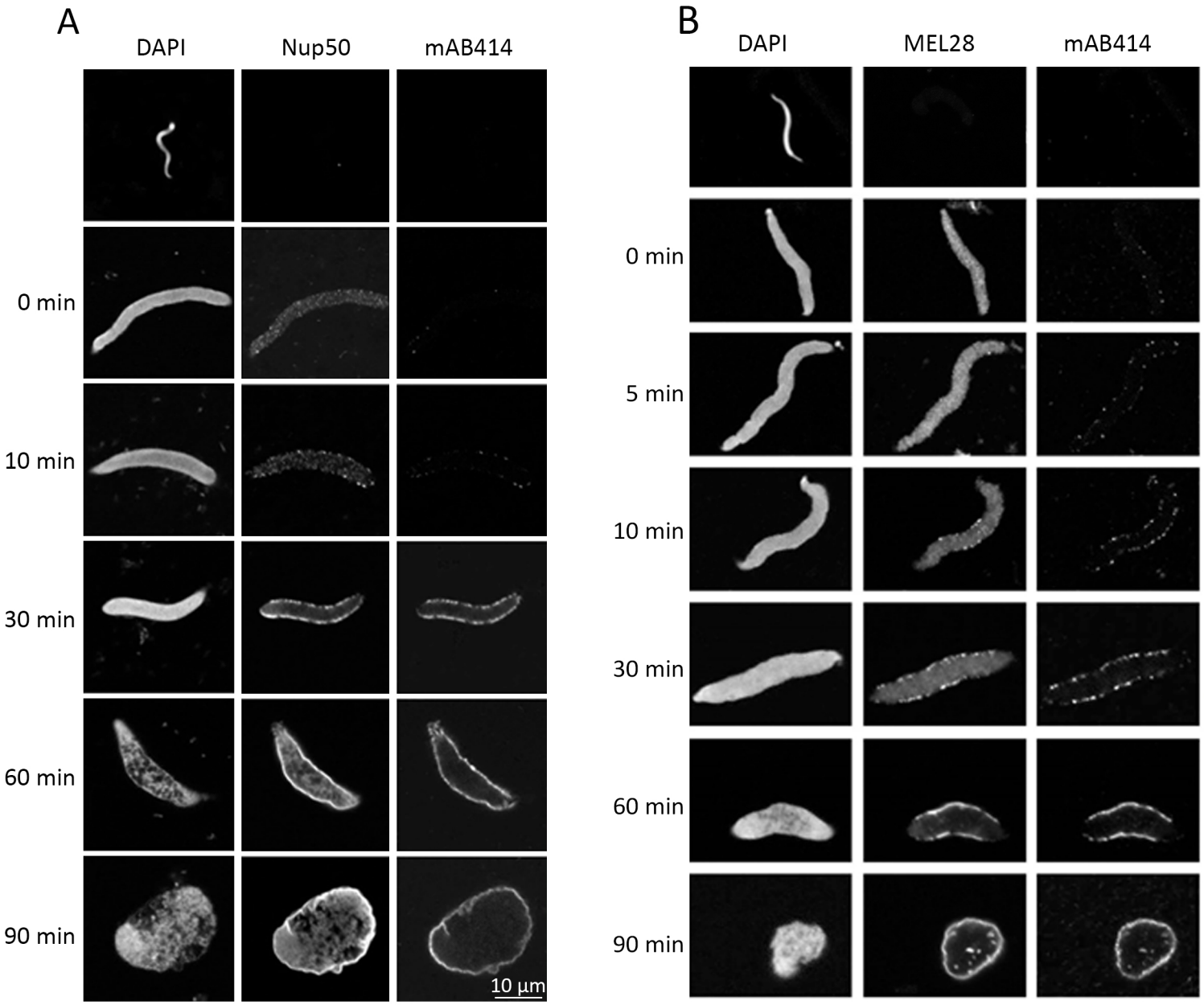
ΔMEL28
/ELYS
Nup50
MEL28
/ELYS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mAB414
0 min
10 min
30 min
60 min
90 min
10 µm
B
DAPI
MEL28
mAB414
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5 min
10 min
30 min
60 min
90 min


144
189
H. sapiens AYHKQLAALNCSVRDWIVKHVNTNPLCDLTPIFKDYEKYLANIEQQ
X. laevis EYNKQLTSLNCSVRDWIVKHVNANPLCDLTPIFKDYEKHLSAIEQK
M. musculus A AYHKQLAGLNCSVRDWIVKHVNTNPLCDLTPIFKDYERYLATIEKQ
M. musculus B VYHRQLTGLNCSVRDWIVKHVNANPFCDLTPVFKQYEKYLAAIEKQ
X. tropicalis EYNKHLTSLNCSVRDWIVKHVNTNPLCDLSPIFRDYEKHLSAIEQK
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S. cerevisiae ESNSRLKALNLQFKAKVDDLVLSKPLADLRPLFTRYELYIKNILEA
S. pombe DIHLKKRGLNKSFIDAVIKSVDNNPFGNLSPLFDEYRQHFSSIEKK
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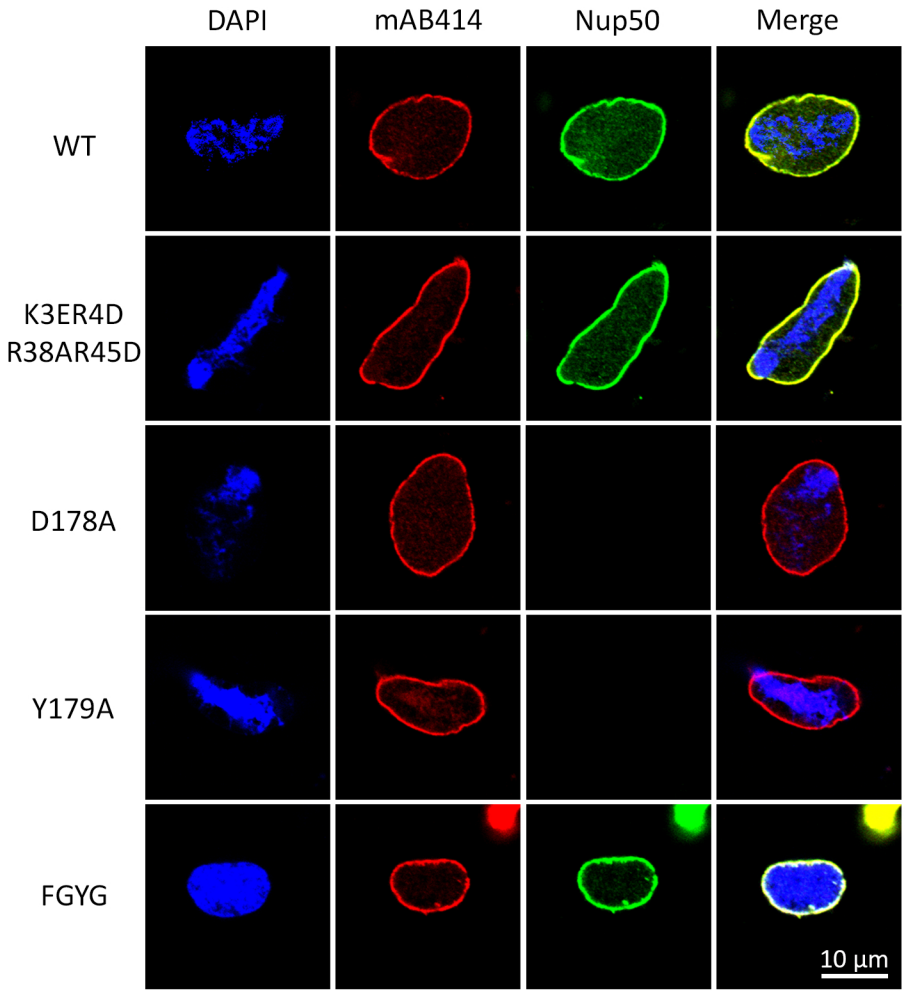
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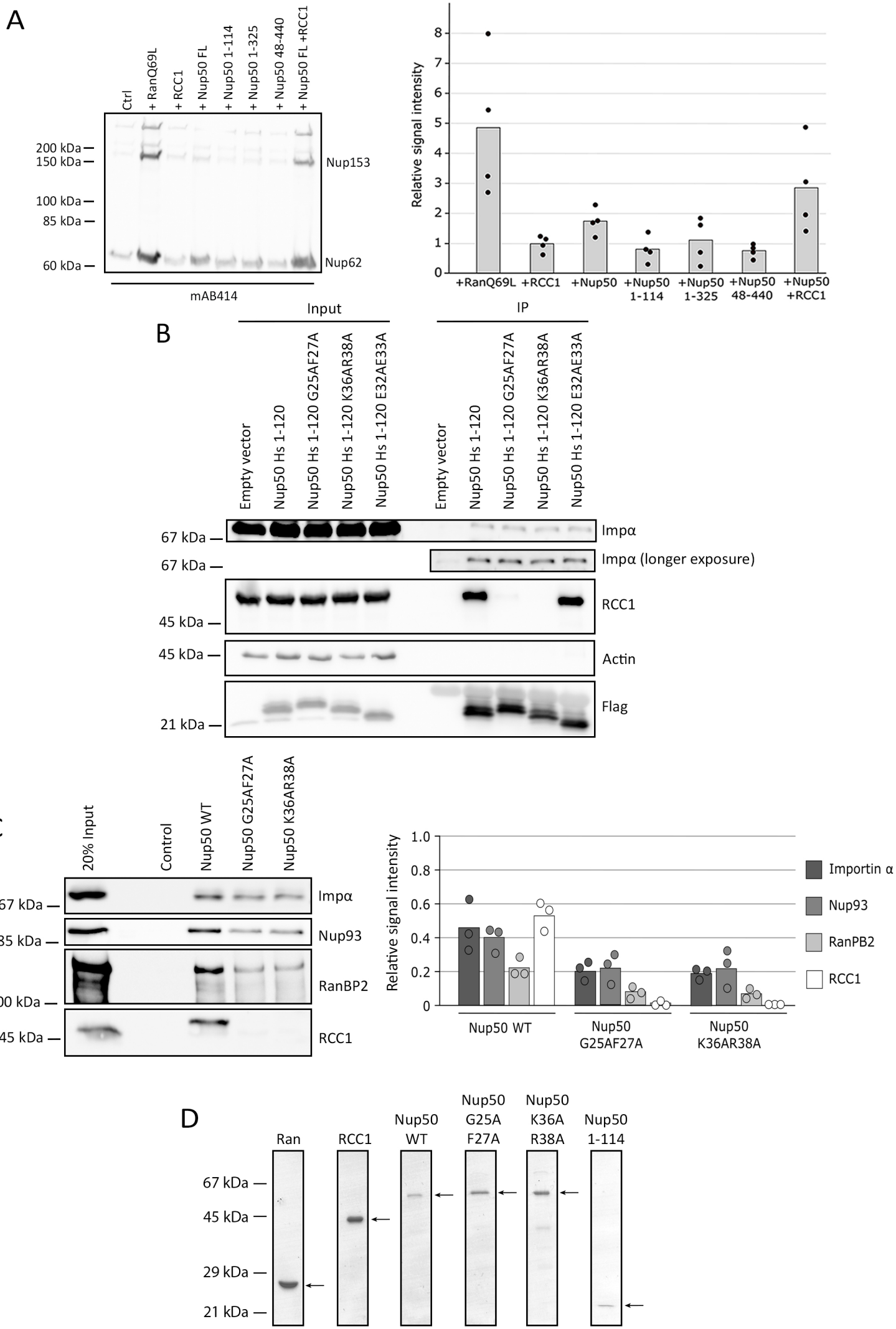
FGYG
10 µm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+ Nup50 FL
+ Nup50 1-114
+ Nup50 1-325
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200 kDa
150 kDa
100 kDa
85 kDa
60 kDa
Nup153
Nup62
mAB414
Relative signal intensity
+RanQ69L
+RCC1
+Nup50
+Nup50 1-114
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+Nup50 48-440
+Nup50 +RCC1
B
Input
IP
Empty vector
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Nup50 Hs 1-120 K36AR38A
Nup50 Hs 1-120 E32AE33A
67 kDa
Impα
Impα (longer exposure)
45 kDa
RCC1
Actin
21 kDa
Flag
C
20% Input
Control
Nup50 WT
Nup50 G25AF27A
Nup50 K36AR38A
67 kDa
Impα
85 kDa
Nup93
200 kDa
RanBP2
45 kDa
RCC1
Relative signal intensity
Importin α
Nup93
RanPB2
RCC1
Nup50 WT
Nup50 G25AF27A
Nup50 K36AR38A
D
Ran
RCC1
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21 kDa