Supplementary Information

Supplementary information consist of nine supporting figures.

Figure S1.

(A) Coomassie staining of the Nek9 immunoprecipitates used in the LC/MS/MS analysis of phosphopeptides. *Exp*., exponentially growing cells; *M*, mitotic cells. *NIgG*, normal IgG.

(B) Phosphopeptides and their corresponding unphosphorylated counterparts identified in the LC/MS/MS analysis of Nek9. The most probable phosphorylation site is marked in each case with and asterisk (*). Peak intensities for each peptide and sample are shown and used to infer the percentage of total peptide that is phosphorylated in each case (*phosphopeptide, % of total*), as well as the fold increase of this percentage in mitotic cells as compared to exponential cells (*Fold increase in M*).

Figure S2.

(A) normal IgG (*NIgG*) or a-Nek9 immunoprecipitates from exponentially growing (*Exp*.) or nocodazole-arrested mitotic (*M*) embryonic mouse fibroblasts extracts were analyzed by western blot (*WB*) using the indicated antibodies. Plk1 in the corresponding extracts is shown in the lower panel.

(B) The ability of full length Plk1 or Plk1 PBD (Plk1[345-603]) to interact with the different domains of Nek9 (kinase domain: Nek9[1-346]; RCC1 domain: Nek9[347-726]; C-terminal tail:Nek9[721-979]) was assessed using two hybrid by histidine and adenine prototrophy plus expression of α-galactosidase activity (right plates). *AD*, Gal4 activation domain; *BD*, Gal4 DNA binding domains; *C+*, positive control (BD-p53 and AD-SV40); *C-*, negative control (BD-lamin and either AD-Plk1 or AD-Plk1[345-603].

(C) HeLa cells were transfected with empty FLAG vector (-), FLAG-Nek9 wild type or FLAG-Nek9[S869A]. anti-FLAG immunoprecipitates were obtained from exponentially growing (*Exp*) or

nocodazole-arrested mitotic (M) cells and immunobloted with the indicated antibodies. Plk1 in the corresponding extracts is shown in the lower panel.

Figure S3.

Kinase-defective FLAG-Nek9[D176A] was expressed and purified from 293T cells and incubated for 60 minutes at 30 °C with [γ-^{32}P]ATP/Mg^{2+} in presence or absence of Plk1. After SDS-PAGE, Nek9 was visualized by Coomassie staining, and ^{32}P incorporation was visualized by autoradiograph (upper panel). Identical samples were analyzed by western blot (*WB*) using the indicated antibodies (lower panel).

Figure S4.

(A) Representative HeLa cell with multiple centrosomes. The cell has been transfected with Nek9 siRNA, and after 48 hours, fixed and stained with antibodies against γ-tubulin (red), centrin (green) and DAPI (blue). Insets show magnified centrosomes (γ-tubulin, centrin and overlap).

(B) HeLa cells were transfected with control siRNA or alternative siRNAs directed against Nek6 (Nek6_2, 5'-AGAGGCAUCCCAACACGCUGUCUUU-3', Invitrogen) or Nek7 (Nek7_2, 5'-AUAAACUUCACUAAAUUGUCCGCGA-3', Invitrogen), and after 48 hours fixed, stained and scored as in Figure 4. with antibodies against γ-tubulin (red) and DAPI (blue). The percentage of prophase cells showing 2 unseparated centrosomes (*together*), 2 centrosomes separated less than 2 μm (< *2μm*), or fully separated centrosomes (> *2μm*) is shown in the upper graphic (mean ± SEM of 3 independent experiments; ~50 cells counted in each experiment). The efficiency of the different RNAi treatments used as determined by western blot of total cell extracts is shown.

(C) Levels of endogenous and recombinant Nek9 as determined by western blot for the different conditions used in Figure 4C. An asterisk marks an unspecific band.

Figure S5

DNA content of cells transfected with the indicated FLAG-tagged proteins (Nek9ΔRCC1, Nek9 [Δ346-732]). 24h after transfection, the DNA content of FLAG-positive cells was analyzed by FACS. There is no significant difference in cell-cycle distribution between different conditions. Similar results were obtained when control siRNA was cotransfected.

Figure S6.

(A) HeLa cells were transfected with the indicated siRNAs, and after 48 hours, Eg5 was immunoprecipitated from the corresponding lysates. Eg5[Ser1033-P] and total Eg5 were detected by western blot (*WB*) of the immunoprecipitates. The efficiency of the different RNAi treatments used was determined by western blot of total cell extracts. *Exp*., exponentially growing cells; *I*, interphase cells (2mM thymidine, 16h); *M*, mitotic cells (0.25 mM nocodazole, 16h).

(B) HeLa cells where incubated with nocodazole (*ND,* 0.25 mM) or BI2536 (100 nM) for 16 hours. Cells in mitosis (*M*) were collected after mitotic shake off, and cell extracts were analyzed by western blot (*WB*) using the indicated antibodies. Mitotic arrest was confirmed by FACS (*not shown*) and the phosphorylation state of Cdc27. Untreated cells (*Exp*.) are shown in the first lane as a control. Asterisks mark protein bands with altered mobility due to phosphorylation.

(C) Levels of endogenous and recombinant Eg5 as determined by western blot for the different conditions used in Figure 7B.

Figure S7.

HeLa cells were transfected, fixed and stained as in Figure 8B. Representative examples of the observed phenotypes in prophase cells are shown (Eg5, red; γ-tubulin, green; FLAG, yellow; DAPI, blue). Centrosomes are noted with arrowheads. Bar, 5 μm.

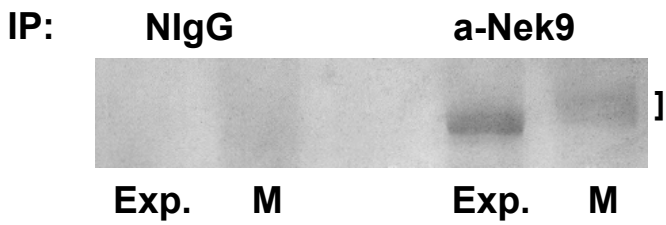
Figure S8.

(A) HeLa cells were transfected with the indicated expression plasmids, and after 24h fixed and stained as in Figure 8B. Representative examples of Eg5 distribution in interphase cells are shown. Images show the same field stained with Eg5 (red), γ-tubulin (green), FLAG (yellow) and DAPI (blue), and a composite of Eg5 (red) plus γ-tubulin (green). Centrosomal accumulation of Eg5 is noted with arrowheads. Bar, 5 μm. (B) Levels of Eg5[S1033-P], Eg5 and FLAG-tagged proteins as determined by western blot of cell extracts for the different conditions used in (A). First two lanes show untransfected cell extracts from untreated (*Exp*.) and nocodazole-arrested mitotic cells (*M*). Note that FLAG-transfected cells are growing exponentially.

Figure S9.

As in Figure 9. Percentage of cells with separated and unseparated centrosomes (distance < 2μm) in RO-3306 arrested cells (*left*) or prometaphase cells (60 min postrelease from RO-3306 arrest, *right*). Mean ± SEM of 3 independent experiments (~30 cells counted in each experiment) is shown.

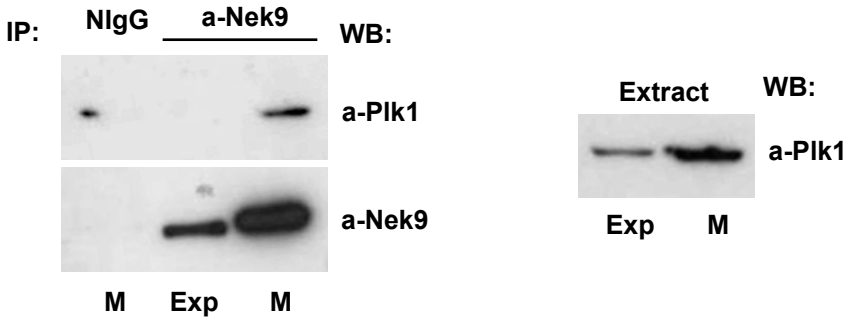
A



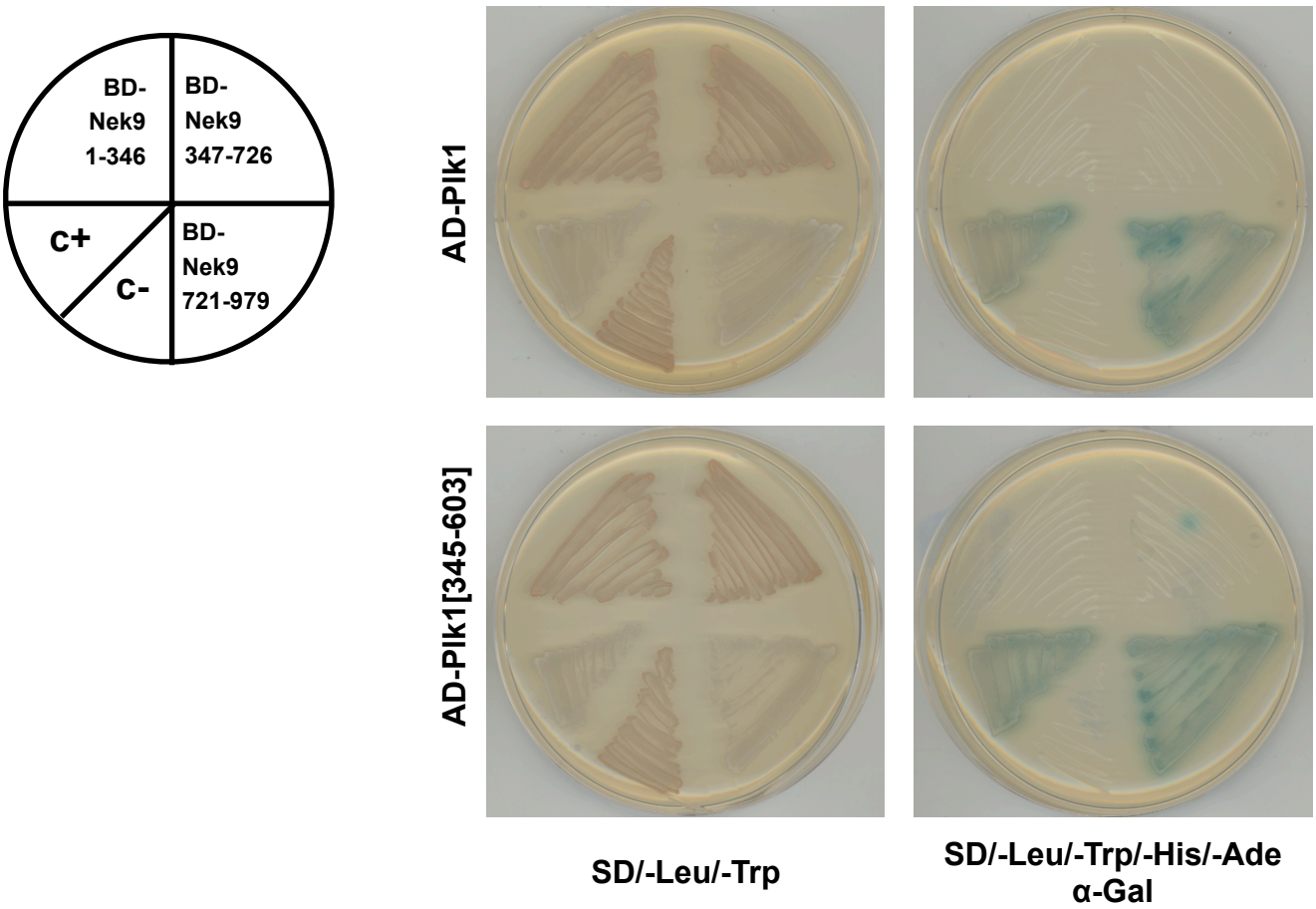
B

Site	Identified peptide	Peak intensity		phosphopeptide (% of total)		Fold increase in M
		Exp.	M	Exp.	M	
Ser29	$_{10}$HCDSINSDFGSESGGCGDSSPGP SASQGPR$_{39}$	7.16 E5	1.80 E5	2.4	67.3	28 x
	$_{10}$HCDSINSDFGSESGGCGDSS*PG PSASQGPR$_{39}$	1.74 E4	3.70 E5			
Thr333	$_{331}$SSTVTEAPIAVVTSR$_{345}$	1.43 E6	6.21 E5	2.1	18.7	9 x
	$_{331}$SST*VTEAPIAVVTSR$_{345}$	3.12 E4	1.43 E5			
Ser750	$_{735}$SNSSGLSIGTVFQSSSPGGGGGG GGGEEEDSQQESETPDPSGGFR$_{779}$	4.14 E5	4.35 E4	3.4	56.3	17 x
	$_{735}$SNSSGLSIGTVFQSSSPGGGGGG GGGEEEDSQQES*ETPDPSGGFR$_{779}$	1.46 E4	5.60 E4			
Ser827	–	-	-	-	-	-
	$_{815}$ELENAEFIPM#PDS*PSPLSAAF SESEKDTLPYEELQGLK$_{852}$					
Ser869	853VASEAPLEHKPQVEASSPR$_{871}$	1.01 E6	1.52 E5	12.4	82.7	7 x
	$_{853}$VASEAPLEHKPQVEAS*SPR$_{871}$	1.43 E5	7.29 E5			
Thr885	–	-	-	-	-	-
	$_{882}$GTPLT*PPACACSSLQVEVER$_{901}$					

A


IP:
NIgG
a-Nek9
WB:
a-Plk1
a-Nek9
M
Exp
M
Extract
WB:
a-Plk1
Exp
M

B


BD-Nek9 1-346
BD-Nek9 347-726
C+
C-
BD-Nek9 721-979
AD-Plk1
AD-Plk1[345-603]
SD/-Leu/-Trp
SD/-Leu/-Trp/-His/-Ade α-Gal

C

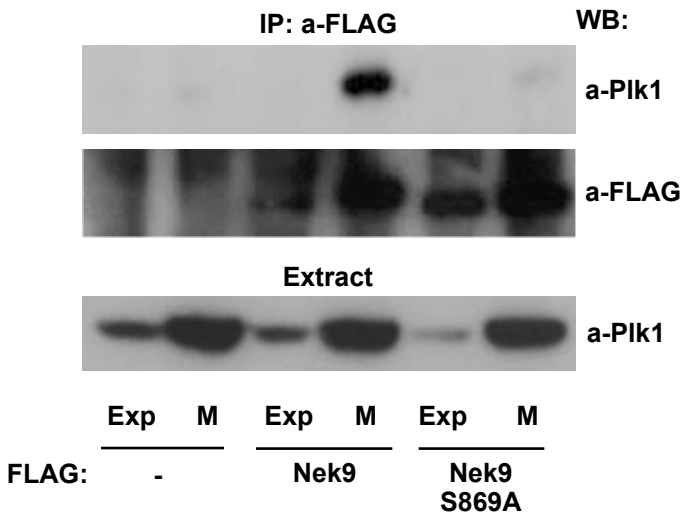
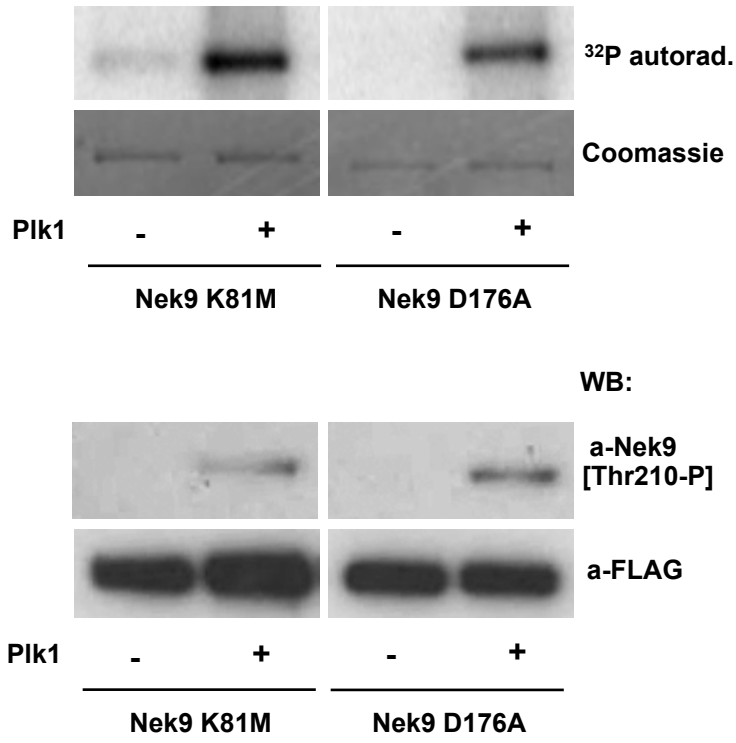
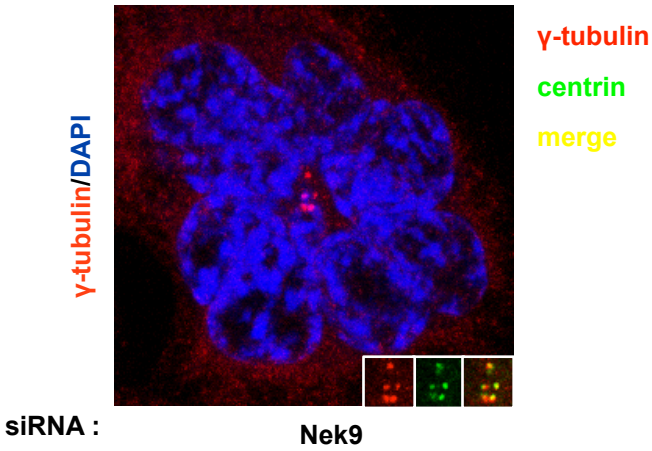

IP: a-FLAG
WB:
a-Plk1
a-FLAG
Extract
a-Plk1
Exp
M
Exp
M
Exp
M
FLAG:
-
Nek9
Nek9 S869A

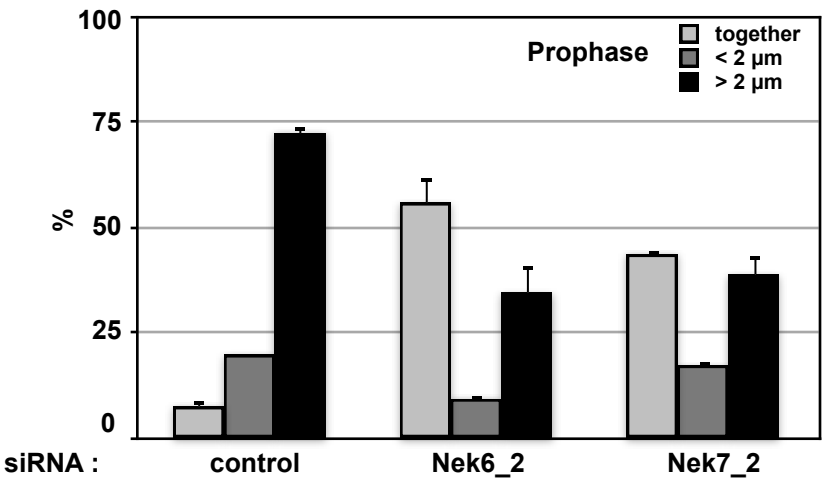
Figure S3

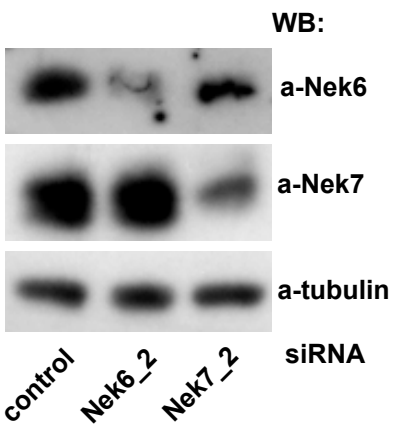

32P autorad.
Coomassie
Plk1
-
+
-
+
Nek9 K81M
Nek9 D176A
WB:
a-Nek9 [Thr210-P]
a-FLAG
Plk1
-
+
-
+
Nek9 K81M
Nek9 D176A

A

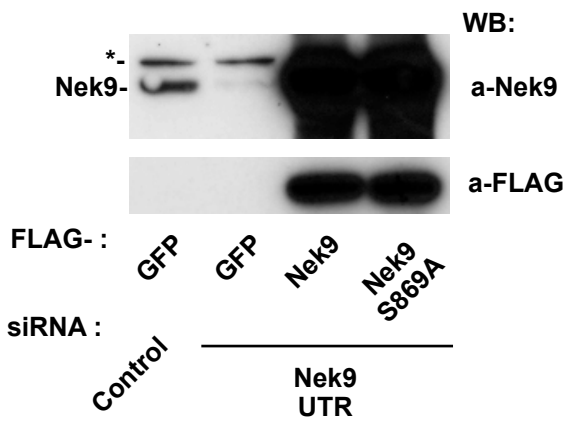

γ-tubulin/DAPI
γ-tubulin
centrin
merge
siRNA :
Nek9

B


100
75
50
25
0
%
Prophase
together
< 2 µm
> 2 µm
siRNA :
control
Nek6_2
Nek7_2

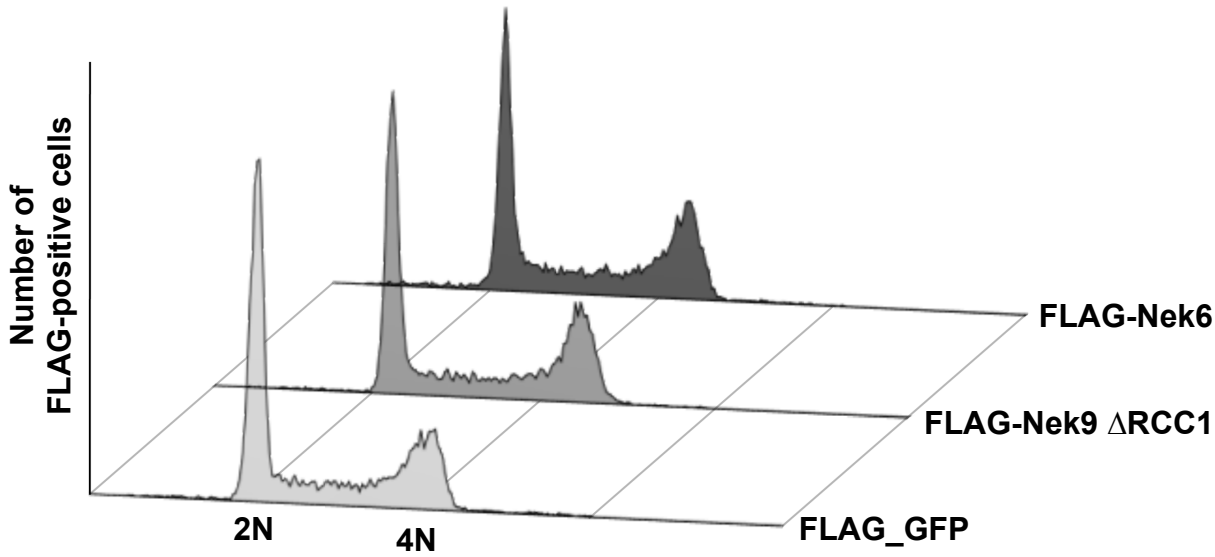

WB:
a-Nek6
a-Nek7
a-tubulin
siRNA
control
Nek6_2
Nek7_2

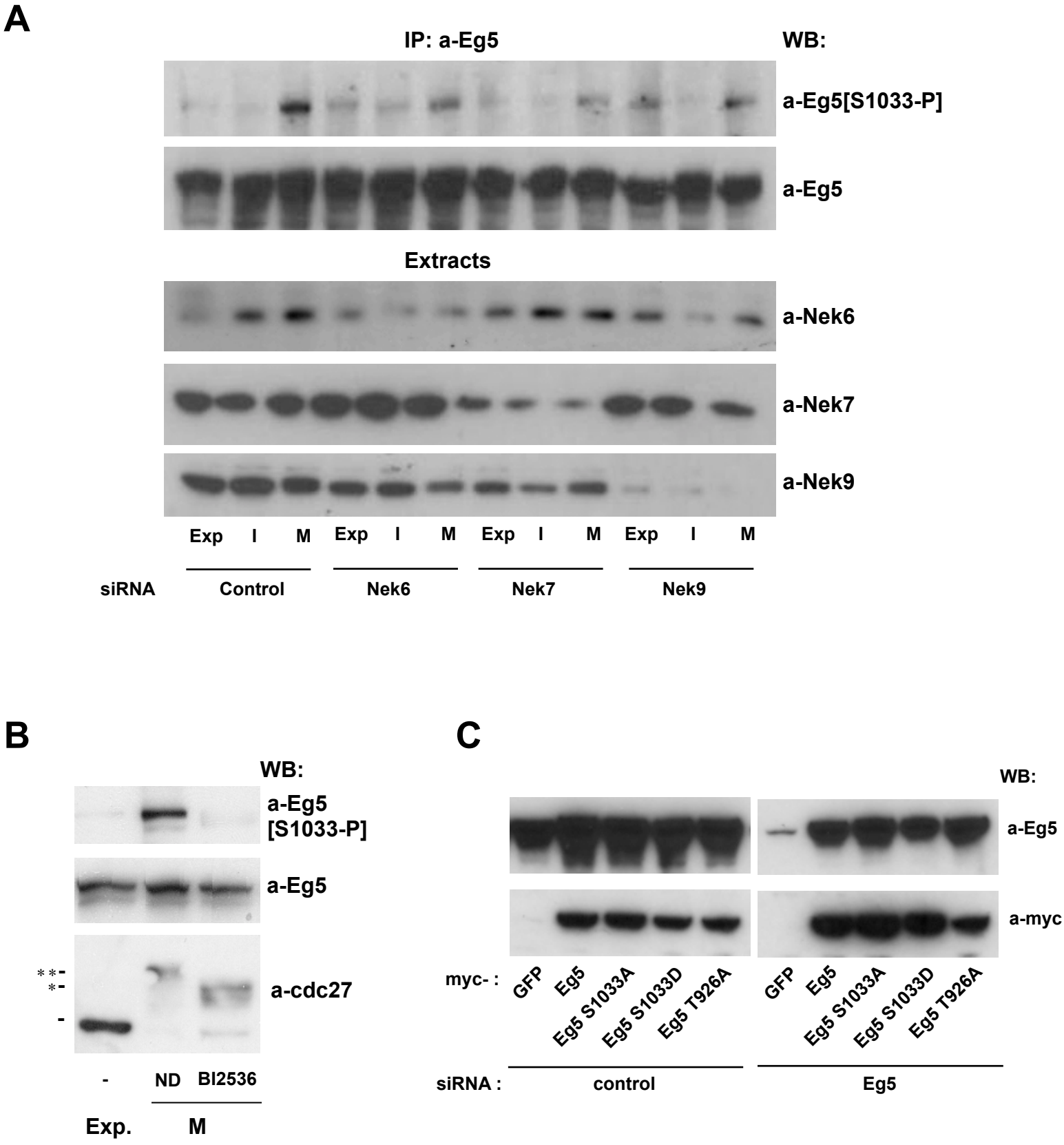
C


WB:
*-
Nek9-
a-Nek9
a-FLAG
FLAG- :
GFP
GFP
Nek9
Nek9 S869A
siRNA :
Control
Nek9 UTR

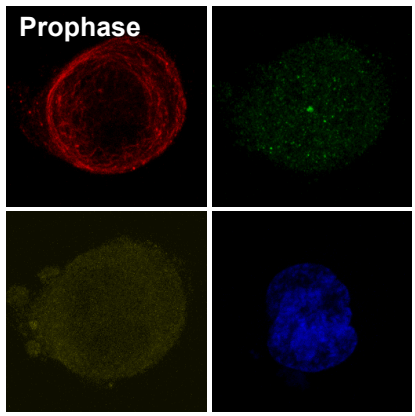
A

Figure S5

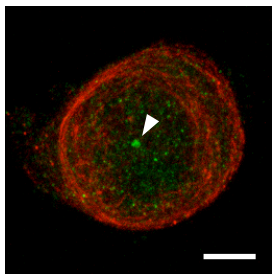

Number of
FLAG-positive cells
FLAG-Nek6
FLAG-Nek9 ΔRCC1
FLAG_GFP
2N
4N


A
IP: a-Eg5
WB:
a-Eg5[S1033-P]
a-Eg5
Extracts
a-Nek6
a-Nek7
a-Nek9
Exp I M Exp I M Exp I M Exp I M
siRNA
Control
Nek6
Nek7
Nek9
B
WB:
a-Eg5 [S1033-P]
a-Eg5
a-cdc27
**-
*-
-
-
ND
BI2536
Exp.
M
C
WB:
a-Eg5
a-myc
myc- :
GFP
Eg5
Eg5 S1033A
Eg5 S1033D
Eg5 T926A
siRNA :
control
Eg5

Plk1 siRNA + FLAG-GFP

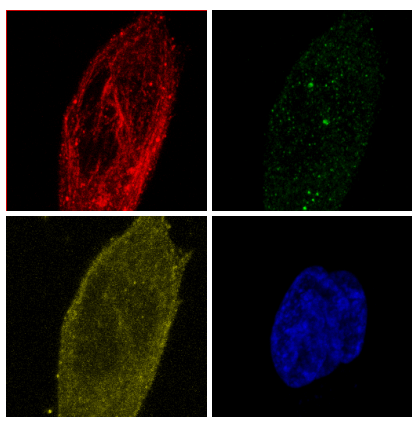


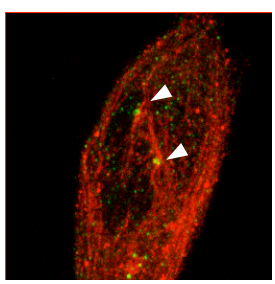
Eg5 FLAG γ-tubulin DAPI



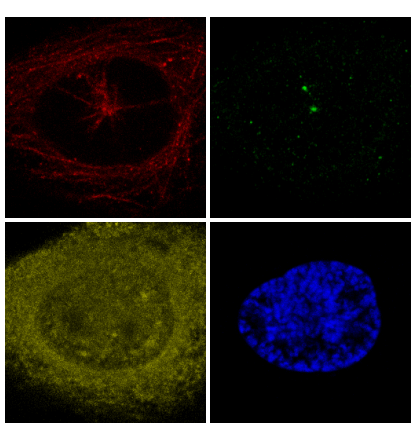
Eg5 + γ-tubulin

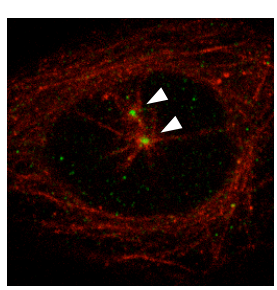
Plk1 siRNA + FLAG-Nek6



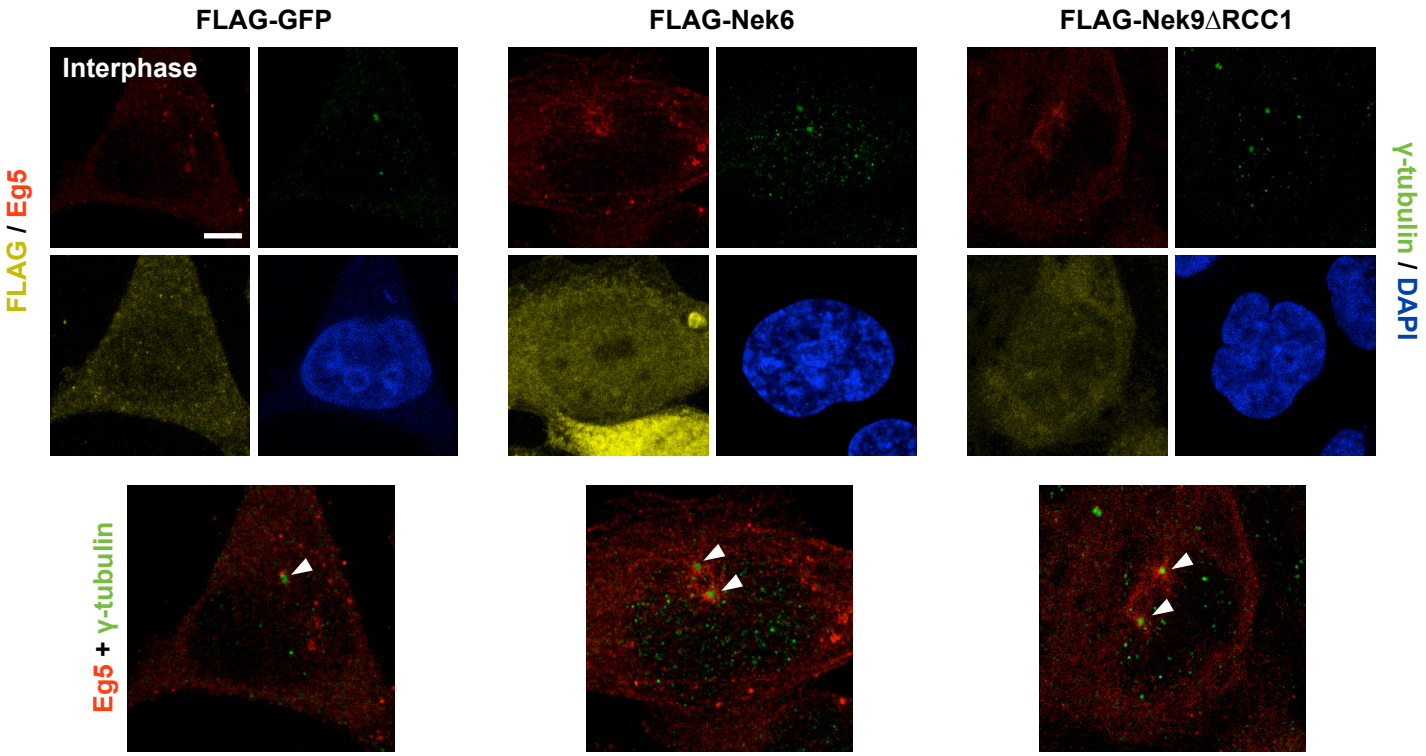


Plk1 siRNA + FLAG-Nek9 ΔRCC1





A


FLAG-GFP
FLAG-Nek6
FLAG-Nek9ΔRCC1
Interphase
FLAG / Eg5
γ-tubulin / DAPI
Eg5 + γ-tubulin

B

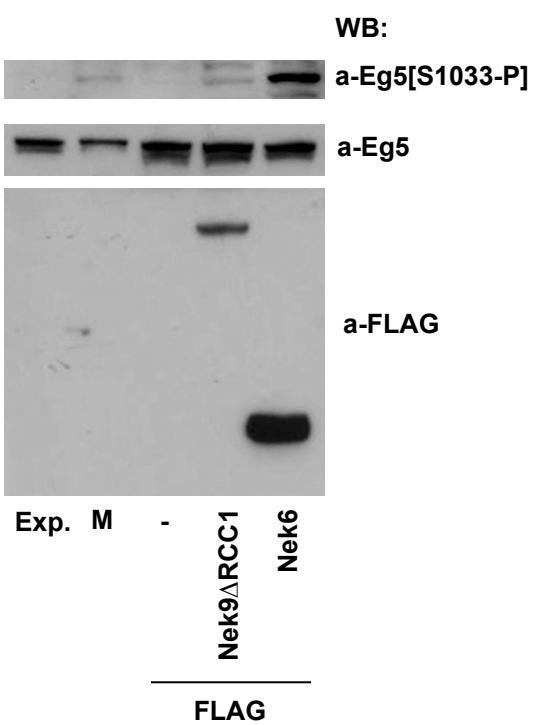
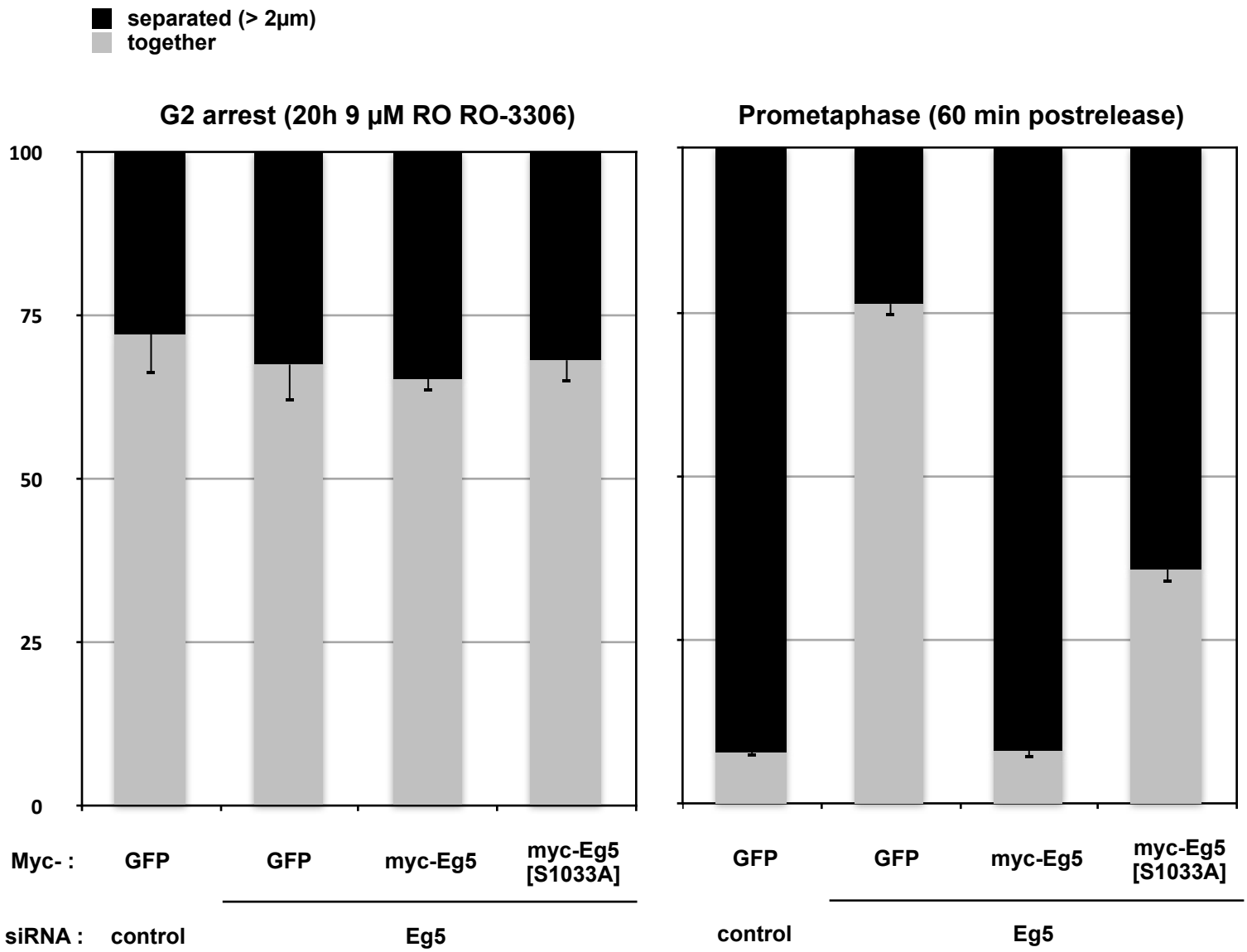

WB:
a-Eg5[S1033-P]
a-Eg5
a-FLAG
Exp.
M
-
Nek9ΔRCC1
Nek6
FLAG

Figure S9


separated (> 2µm)
together
G2 arrest (20h 9 µM RO RO-3306)
Prometaphase (60 min postrelease)
100
75
50
25
0
Myc- :
GFP
GFP
myc-Eg5
myc-Eg5 [S1033A]
GFP
GFP
myc-Eg5
myc-Eg5 [S1033A]
siRNA :
control
Eg5
control
Eg5