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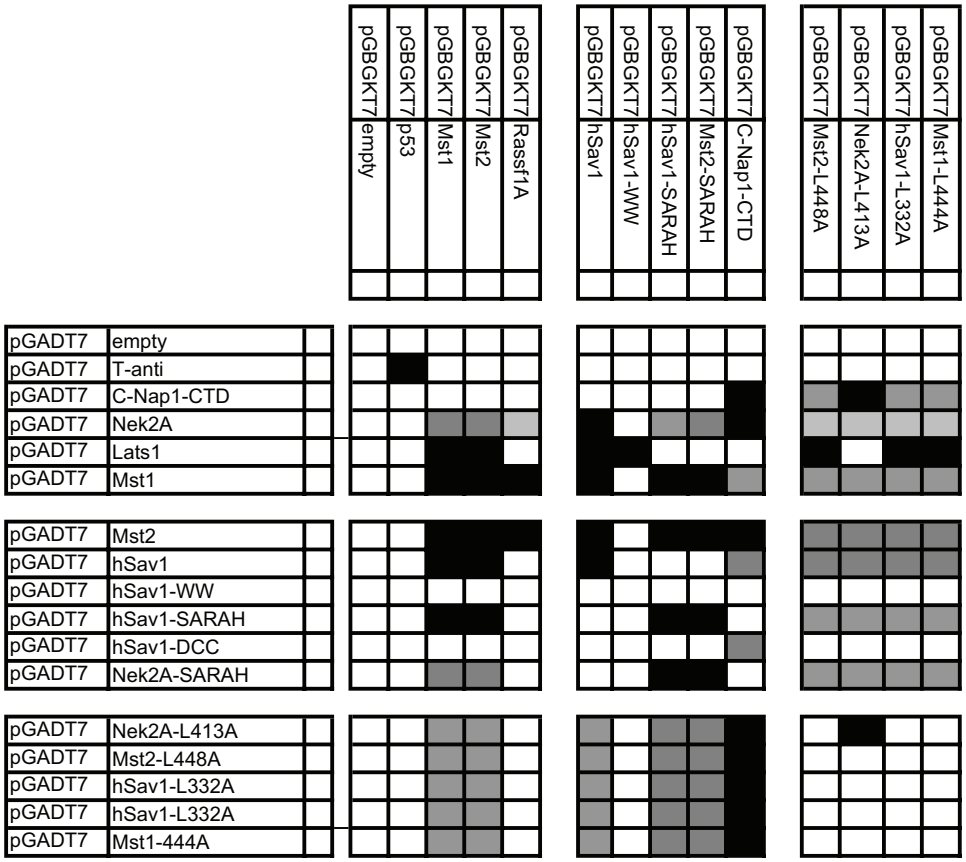

Figure S1 Yeast-Two-Hybrid analysis. Complete table of the yeast-two-hybrid analyses. Intensity of the grey boxes correlate with the strength of interactions. Strength of interactions is defined by β-galactosidase activity. Black boxes show interactions that gave blue color after 30 min, dark grey boxes after 1 h, and light grey boxes after > 2 h.

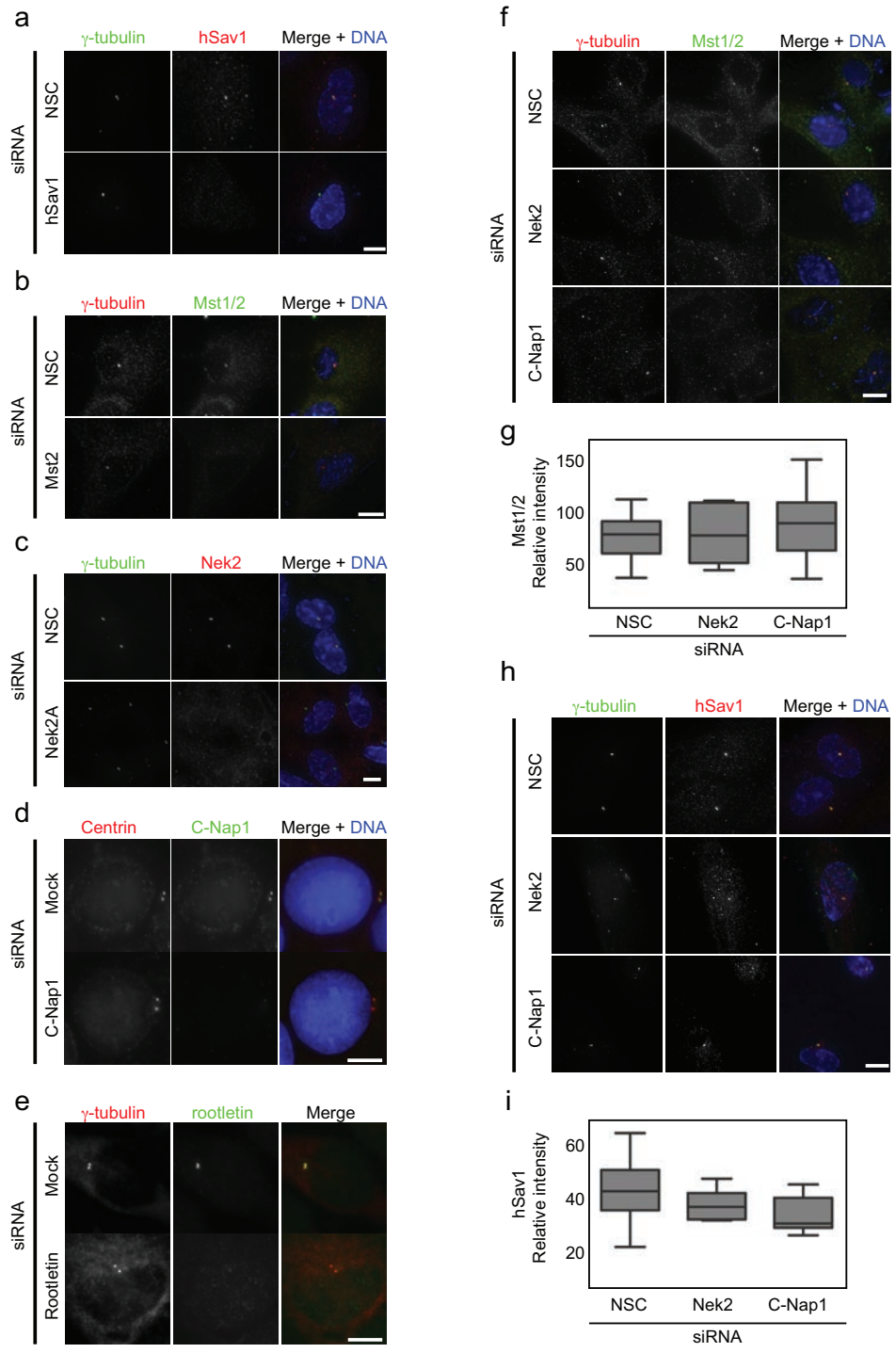

Figure S2 Specificity of antibodies used in this study and control of centrosomal localizations of hSav1 and Mst1/2 upon Nek2 and C-Nap1 depletion. a-e- Non-specific control (NSC) or siRNA-treated RPE-1 cells (as indicated in the figure) were collected after 72 h of transfection, fixed and stained with antibodies directed against hSav1 (a), Mst2 (b), Nek2 (c), C-Nap1 (d) or rootletin (e). Anti-γ-tubulin (a,b,c,e) or anti-centrin (d) antibodies were used to mark centrosomes. Scale bars, 10 μm. f- Non-specific control (NSC), Nek2 or C-Nap1 siRNA-treated RPE-1 cells were collected after 72 h of transfection, fixed and stained with anti-Mst2 and γ-tubulin antibodies. Scale bar, 10 μm. g- Intensities of Mst2 and γ-tubulin at centrosomes from cells in Fig. S2f were measured for each condition and the average background intensity was subtracted. Mst2 signals were then normalized to corresponding γ-tubulin signals. n = 20 cells counted for each condition. Box-and-whiskers plots: boxes show the upper and lower quartiles (25-75 %) with a line at the median, whiskers extend from the 10 to the 90 percentile and dots correspond to the outliers. h- Non-specific control (NSC), Nek2 or C-Nap1 siRNA-treated RPE-1 cells were collected after 72 h of transfection, fixed and stained with anti-hSav1 and -γ-tubulin antibodies. Scale bar, 10 μm. i- Intensities of hSav1 and γ-tubulin at centrosomes from cells in Fig. S2h were measured for each condition and the average background intensity was subtracted. hSav1 signals were then normalized to corresponding γ-tubulin signals. n = 20 cells counted for each condition. Box-and-whiskers plots: boxes show the upper and lower quartiles (25-75 %) with a line at the median, whiskers extend from the 10 to the 90 percentile and dots correspond to the outliers.

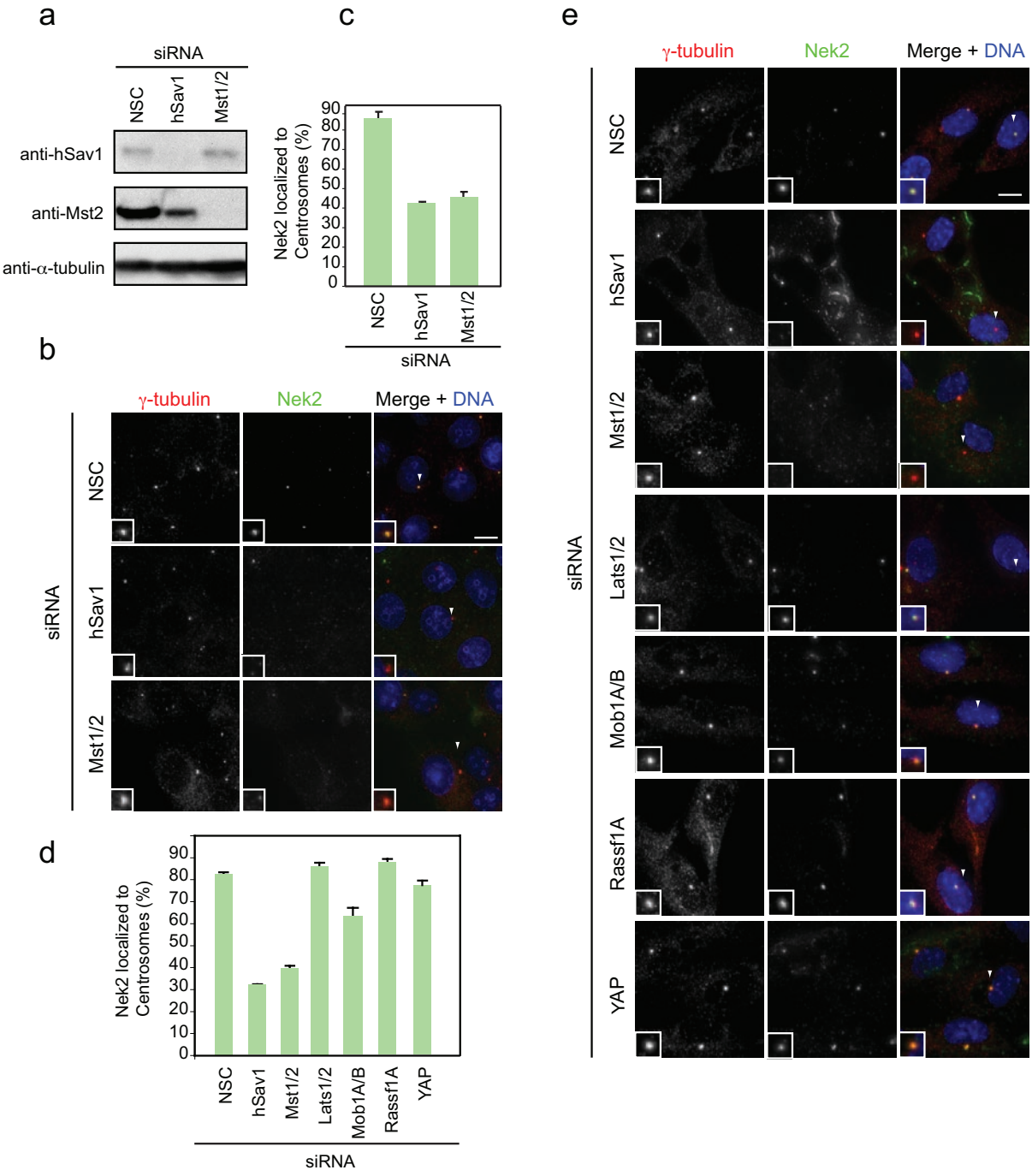

Figure S3 Nek2 is recruited to centrosomes by hSav1 and Mst1/2 in MCF-7 cells and centrosomal localization of Nek2 does not require Lats1/2, Rassf1 or YAP**.** a- MCF7 cells were transfected with non-specific control (NSC), hSav1 or Mst1/2 siRNA oligos and collected after 72 h of transfection. Whole cell extracts were analysed by immunoblotting using anti-hSav1, -Mst1/2 and -α-tubulin antibodies. b- Cells were fixed and stained with anti-Nek2 and -γ-tubulin antibodies. Arrows indicate the centrosomes in insets. Scale bar, 10 μm. c- Cells of Fig. S3b were quantified for Nek2 localizaton. Results are from three independent experiments. n = 50 cells for each condition. Data are represented as mean ± SEM. d- RPE-1 cells transfected with the indicated siRNAs were collected after 72 h and quantified for Nek2 localizaton. Results are from three independent experiments. n = 75 (NSC), 50 (siRNA treatment) cells for each condition. Data are represented as mean ± SEM. e- RPE-1 cells treated as above were fixed and stained with anti-Nek2 and -γ-tubulin antibodies. Scale bar, 10 μm.

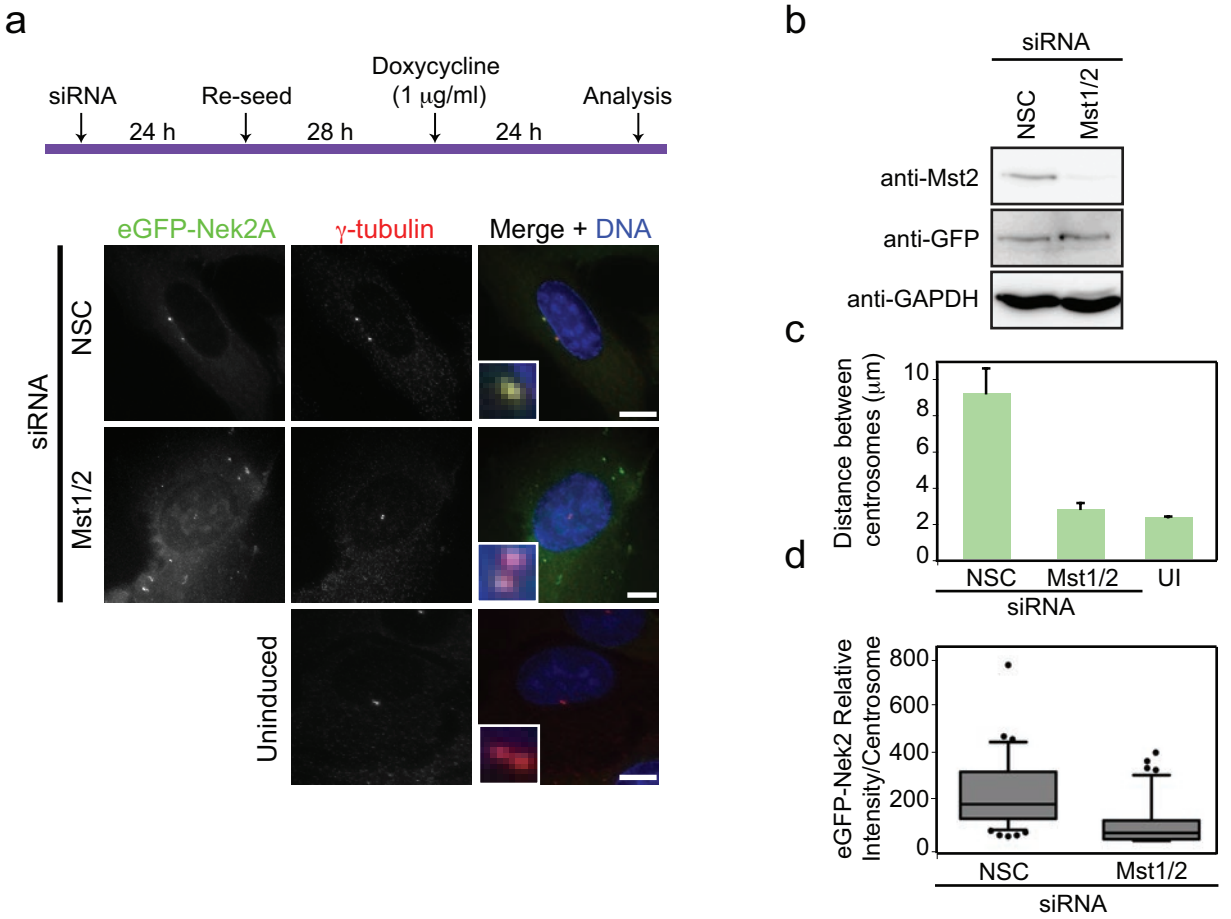

Figure S4 Confirmation of premature centrosome splitting phenotype by U2OS-eGFP-Nek2A cell line. a- U2OS cells expressing tetracycline-inducible eGFP-Nek2A were treated with the indicated siRNA oligos for 52 h and subsequently treated with 1 μg/ml of doxycycline for 24 h to induce centrosome splitting. Cells were fixed and stained with antibodies against γ-tubulin. Insets are magnifications of the centrosomal signals. Scale bar, 10 μm. b- Whole cell extracts were analysed by immunoblotting with the indicated antibodies. c- Distances between the two centrosomes of cells in Fig. S4a. Results are from two independent experiments; n = 30 cells were analysed for each condition. Data are mean ± SEM. d- Intensity of centrosomal association of eGFP-Nek2 was quantified from cells shown in Fig. 4a. Relative intensities were obtained by normalizing the eGFP-Nek2A signal against the γ-tubulin signal. Results are from two independent experiments. n = 30 cells analysed for each condition. Box-and-whiskers plots: boxes show the upper and lower quartiles (25-75 %) with a line at the median, whiskers extend from the 10 to the 90 percentile and dots correspond to the outliers ($p<0.0001$).

a

Protein	Sequence	Peptidemass	m/z
Nek2A-ΔN	NYSLLK	816,4	409,2
	ENIMRSENSESQLTSKSK	2146,98	716,66
	ERKFLSLASNPELLNLPSSVIKK	2663,49	888,83
	NYQLKSRQILGMR	1685,88	562,96

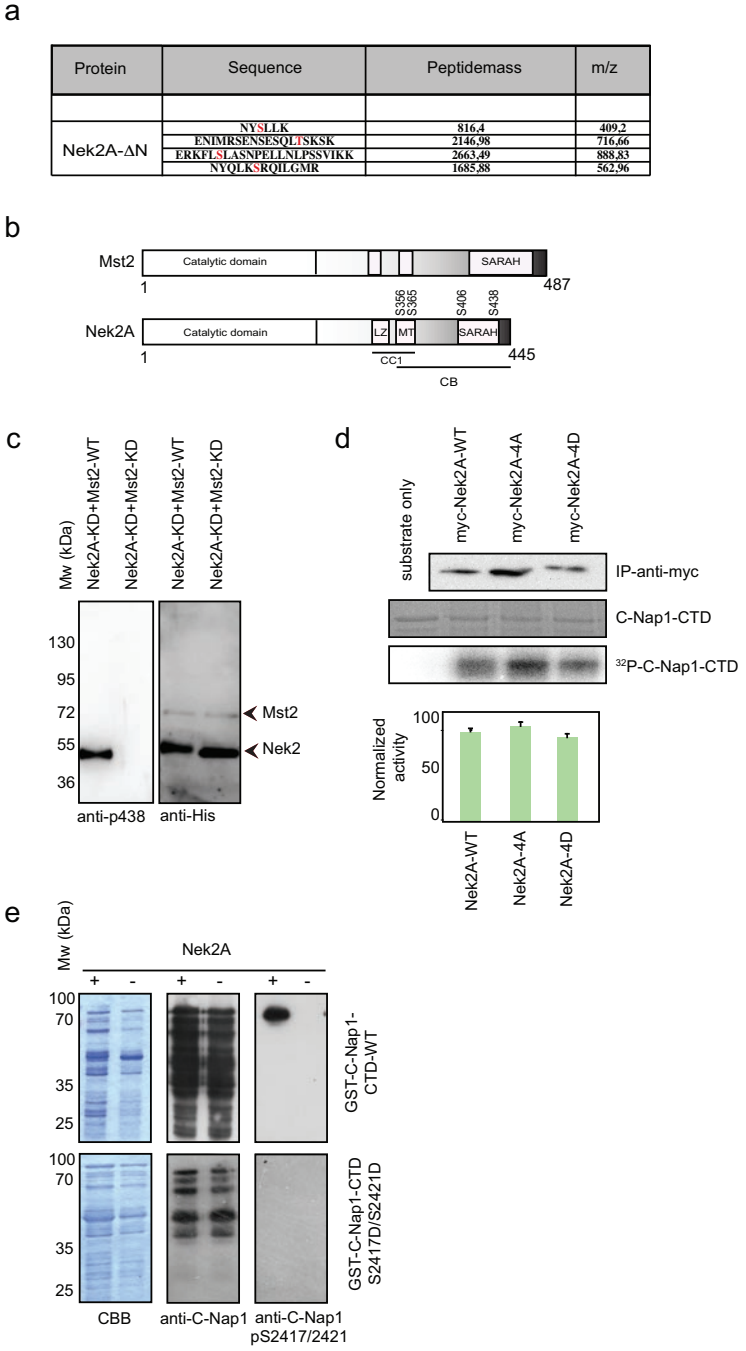

Figure S5 *In vitro* analysis of Mst2 phosphorylation and specificity of phospho-specific antibodies. a- Recombinant Mst2 was incubated with Nek2A-ΔN in the presence of ATP (Fig. 4a). The substrate proteins were subsequently analyzed by LC-MS/MS mass spectrometry for phospho-peptides. The identified phosphorylation sites are indicated as red letters. b- Domain structure of Mst2 and Nek2A. Shown are the C-terminal SARAH domains, centrosomal binding regions of Nek2A (CB), and the catalytic domains of the kinases Mst2 and Nek2A. The serine and threonine residues of Nek2A that are phosphorylated by Mst2 are indicated. c- Specificity of anti-Nek2A-pS438 antibodies. Recombinant kinase dead His-Nek2A-KD was incubated with either kinase-dead or wild-type His-Mst2 in the presence of ATP. Samples were analysed by immunoblotting using anti-His and anti-pS438 antibodies. The Nek2A-K37R (Nek2A-KD) mutation was introduced to prevent autophosphorylation of Nek2A. The pS438 antibodies recognized the *in vitro* phosphorylated Nek2A-KD, whereas it failed when Nek2A-KD was incubated with Mst2-KD. **d**- Myc-tagged Nek2A wild-type, Nek2A-4A and Nek2A-4D mutants were transfected into HEK293 cells. Cells were then lysed and Nek2A proteins were immunoprecipitated with anti-myc antibodies and their activities analysed *in vitro* with recombinant GST-C-Nap1-CTD as substrate. Normalized activity was determined as the incorporated ^{32}P-C-Nap1-CTD in relation to the input myc-Nek2A kinase. Data are shown from 3 independent experiments as average normalized activities with standard deviation. e- Recombinant GST-C-Nap1-CTD proteins purified from *E.coli*, either wild-type (top panel) or S2417D/2421D (bottom panel), were incubated in the presence (+) or absence (-) of Nek2A kinase for 30 min at 30°C before analysis by SDS-PAGE and Coomassie Blue staining (left panels) or immunoblotting with antibodies against total C-Nap1 (middle panels) or phosphorylated (S2417/S2421) C-Nap1 (right panels). Note that the purified GST-C-Nap1-CTD proteins are partially degraded explaining the protein bands below the main band at 90 kDa.

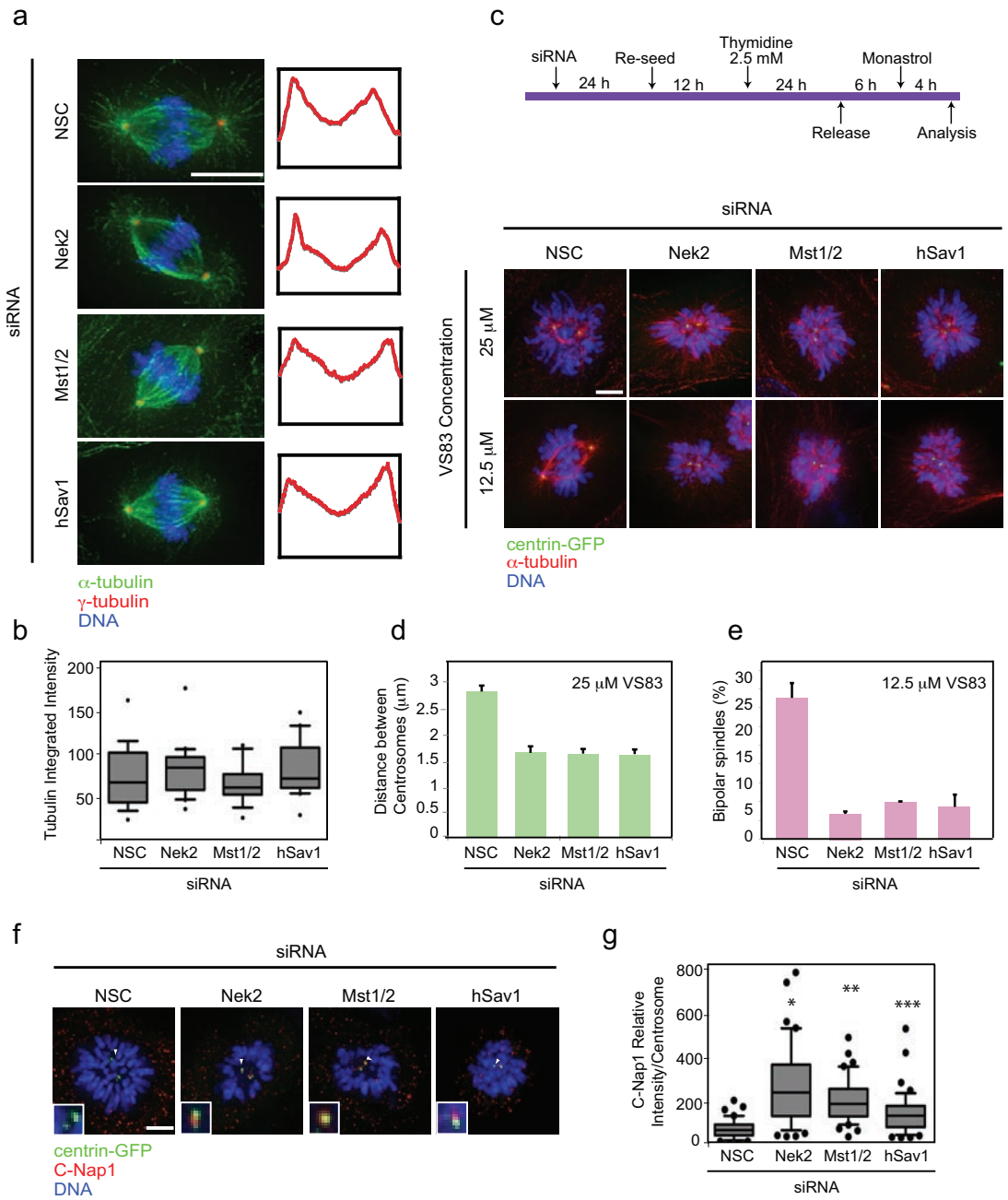

Figure S6 Formation of normal bipolar spindles in the presence of active Eg5 in Nek2-Mst1/2-hSav1 depleted cells and confirmation of monastrol phenotypes by an additional Eg5 inhibitor, VS83. a- RPE-1 centrin-GFP cells were treated with the indicated siRNA oligos, enriched in G2 by single thymidine block/release and collected at different time points. Metaphase cells were analysed. Left panels: Cells were fixed and stained with α-tubulin and γ-tubulin antibodies. Right panels: line scans from the corresponding metaphase cells. Tubulin intensities were measured from pole-to-pole. Note that there is no significant difference between control and siRNA treated cells. Scale bar, 5 μm. b- Overall tubulin intensities from cells in Fig. S6a were measured for each condition and the average background intensity was subtracted. N = 30 cells counted for each condition. Box-and-whiskers plots: boxes show the upper and lower quartiles (25-75 %) with a line at the median, whiskers extend from the 10 to the 90 percentile and dots correspond to the outliers. c- RPE-1-centrin-GFP cells were treated with the indicated siRNA oligos, enriched in G2 by a single thymidine block/release and subsequently treated with 12.5 μM and 25 μM VS83 for 4 h to inhibit Eg5-dependent centrosome splitting. Cells were fixed and stained with α- and γ-tubulin antibodies. Scale bar, 5 μm. d- Distances between the two centrosomes were analysed from the data in Fig. S6c of cells treated with 25 μM VS83. Results are from two independent experiments. n = 30 cells counted for each condition. Data are mean ± SEM. e- Cells treated with 12.5 μM VS83 were analysed for their ability to form bipolar spindles. Results are from two independent experiments. n = 122 (NSC), 106 (siNek2), 120 (siMst1/2), 102 (sihSav1) cells counted for each condition. Data are mean ± SEM. f- RPE-1 centrin-GFP cells treated as in Fig. S6a, arrested with 25 μM of VS83, fixed and stained with C-Nap1 antibodies. Scale bar, 5 μm. g- Intensity of centrosomal C-Nap1 of cells from Fig. S6f was measured. The average background intensity was subtracted and the intensities were normalized to corresponding centrin signal. Results are from two independent experiments. n = 30 (NSC and siMst1/2), 20 (siNek2, sihSav1) cells counted for each condition. Box-and-whiskers plots: boxes show the upper and lower quartiles (25-75 %) with a line at the median, whiskers extend from the 10 to the 90 percentile and dots correspond to the outliers (*NSC/Nek2 $p<0.0001$, **NSC/Mst2 $p<0.0001$, ***NSC/hSav1 $p<0.0001$).

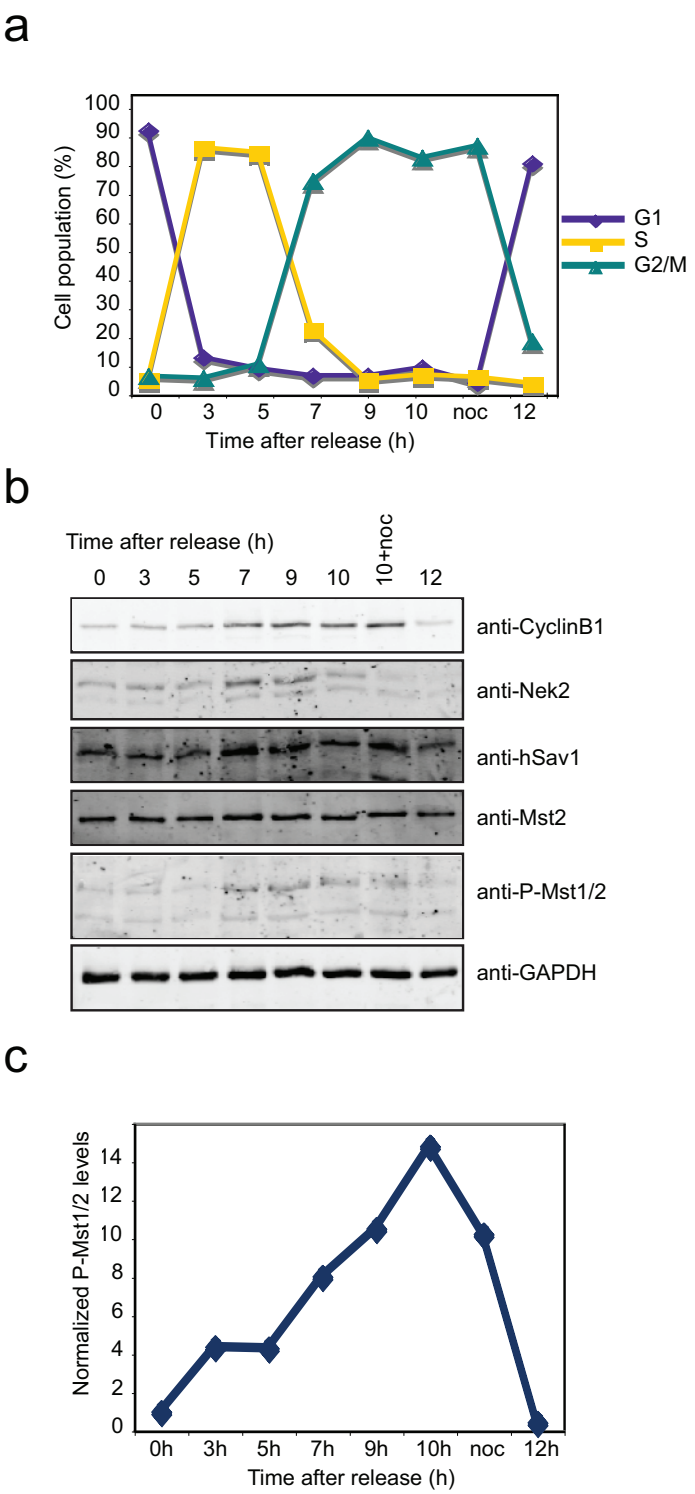

Figure S7 Cell cycle regulation of Mst1/2. a-c- HeLa Kyoto cells synchronized at the G1/S boundary by a double thymidine block were released and harvested at the indicated times. One pool of cells was released from double thymidine block into nocodazole in order to trap cells in prometaphase state (indicated as noc). a- Cell populations were determined by Flow cytometry analysis of the cells taken at the indicated time points. b- Whole cell extracts were analysed by SDS-PAGE and Western blot with the indicated antibodies. Anti-P-Mst1/2 antibodies were used as a measure of the autophosphorylation of Mst2, which is proportional to the kinase activity of Mst1/2. c- P-Mst1/2 levels were quantified from Fig. S7b and normalized to Mst2 and GAPDH levels.

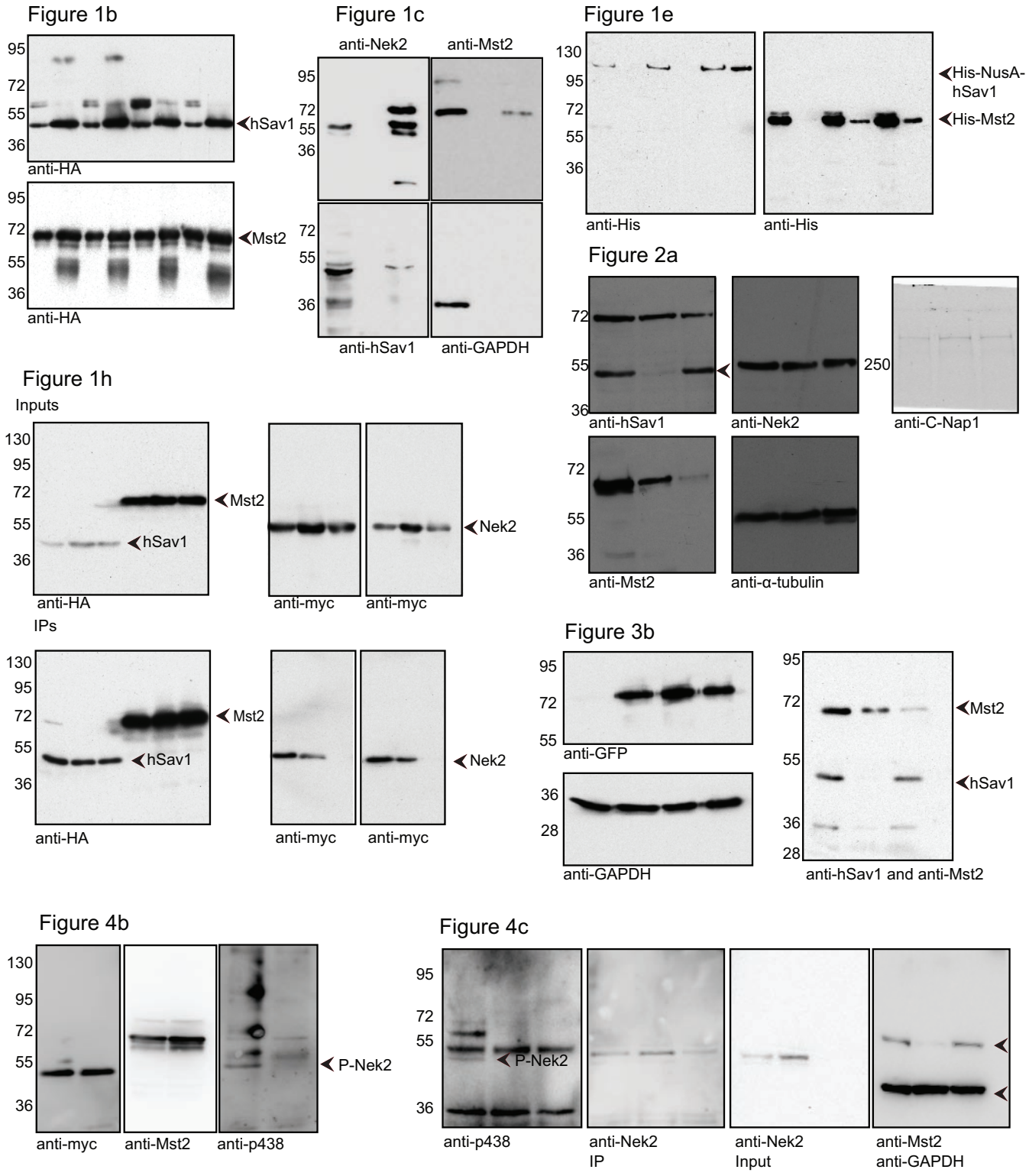

Figure S8 Full scans of blots. Molecular weight markers are indicated in kDa. The Fig. number is indicated. Arrowheads indicate the position of the proteins.

Figure 4d

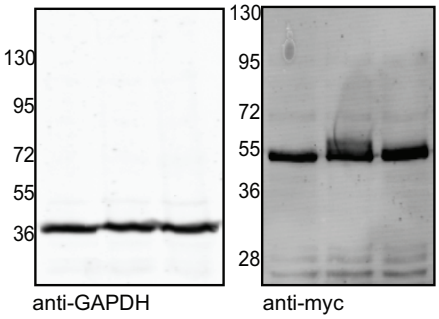

Figure 4h

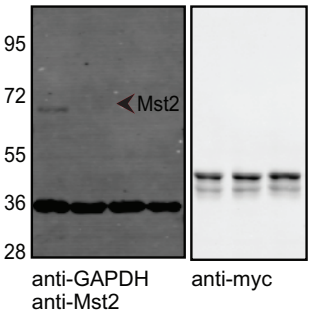

Figure 4k

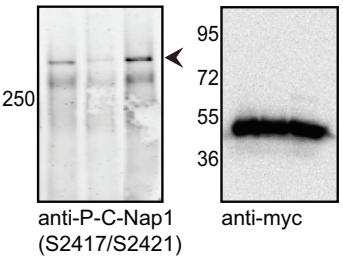

Figure 6c

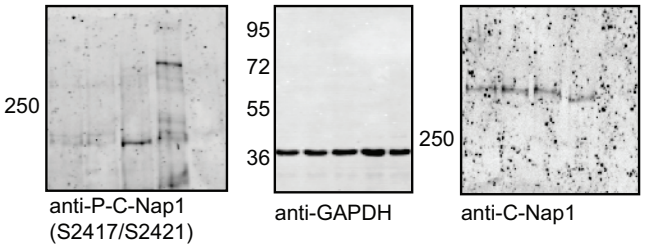

Figure 6d

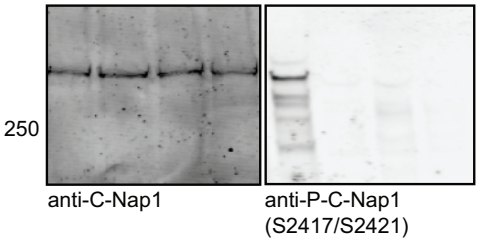

Figure S3a

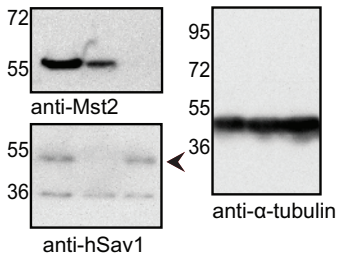

Figure S4b

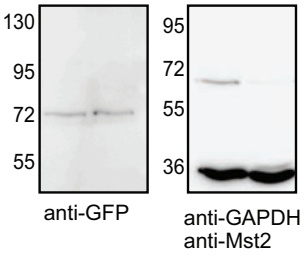

Figure S5d

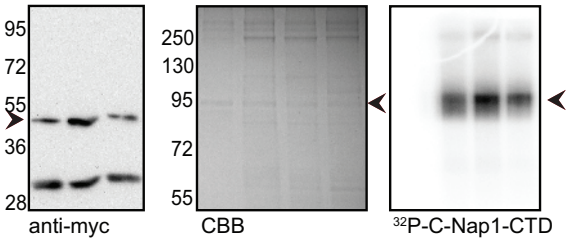

Figure S7b

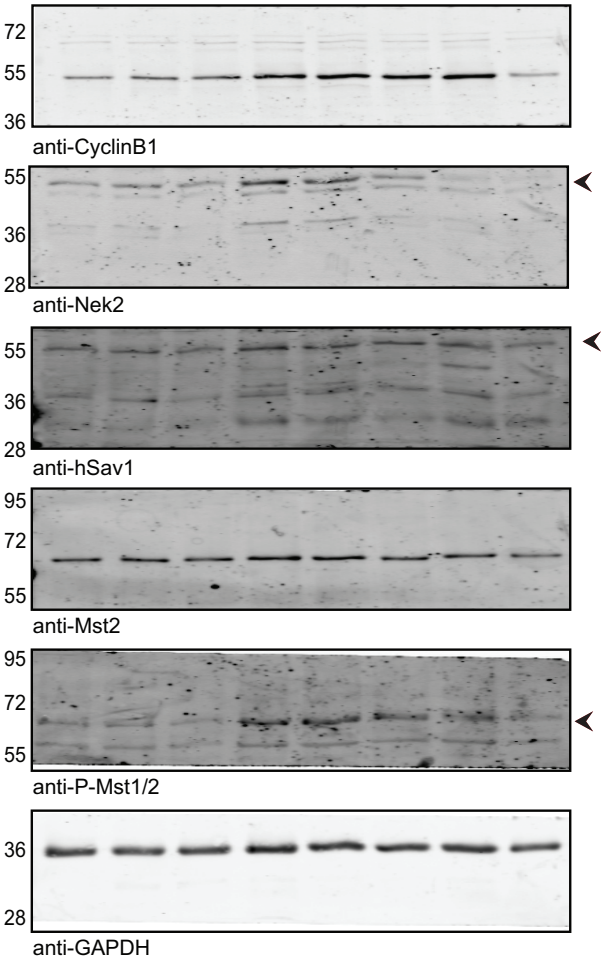

Figure S8 continued

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Supplementary Information

Supplemental Table 1: siRNA duplexes used in this study

Name	Sequence	Over	Company
NSC-47%GC	AGGUAGUGUAAUCGCCUUG	tt	MWG
GFP	GGCUACGUCCAGGAGCGCACC		MWG
Sav1-48	AAAUUCGGAUGACUCAACUCGUUCC		Invitrogen
Sav1-49	UUCAUGUGCACGAUCUCCAAGUGGC		Invitrogen
Sav1-50	UAAAUCUUCAGAACCAUGGUUAGUC		Invitrogen
YAP1	GACAUCUUCUGGUCAGAGA	tt	MWG
Mst1/STK4-si1	ACAGCUUCUUGCUAAUACA	tt	MWG
Mst1/STK4-si2	UCGGACCUGCAGGAGAUAA	tt	MWG
Mst2/STK3-si2	ACCUCCUUAUGCUGAUAUA	tt	MWG
Mst2/STK3-si1	GCCCAUAUGUUGUAAAGUA	tt	Dharmacon
Lats1-si2	UAGCAUGGAUUUCAGUAAU	tt	MWG
Lats1-si1	CUAACAACAGAAGUAUAGA	tt	MWG
Lats2-si1	GGUUCUCUAUAGGAACUAC	tt	MWG
Lats2-si2	GGUUCUCUAUAGGAACUAC	tt	MWG
Mob1A	GCACCAAAGTATATTGATT	tt	MWG
Mob1B	GCAGATGGTACTAATATTA	tt	MWG
Nek2-siGendub8	AAACAUCGUUCGUUACUAU	tt	MWG/
Nek2-siGendub6	GAAAGGCAATACTTAGATG	tt	MWG/
Rootletin-Si-dub1	CAGCCAGGAGAAGAUCAGCAAUU		Dharmacon
Rootletin. Si-dub2	CAGGGAGAUUGUCACCCGCAAUU		Dharmacon
Eg5	CCAUCAACACUGGUAAGAA	tt	Ambion