Supplemental Materials and Methods

Antibodies and plasmids

Other antibodies used in this study include polyclonal rabbit anti-CP110 [1], anti-Cep97 [2], anti-Cep76 [3], anti-centrin2 mouse monoclonal 20H5 (J. Salisbury and Millipore), anti-C-Nap-1 (E. Nigg and Bethyl), anti-Sas6 (Santa Cruz), anti-centrobin (Q. Gao), anti-Cep170 (Invitrogen), anti-Cep290 (Bethyl), anti-cyclin F (Santa Cruz)[4], anti-α-tubulin, anti-Flag, and anti-γ-tubulin (all from Sigma-Aldrich), anti-glutamylated tubulin GT335 (C. Janke and Axxora), mouse and rabbit anti-GFP antibodies (Sigma), and goat anti-GFP (Abcam). myc-Plk4 was obtained from E. Nigg.

RNA interference

The 21-nucleotide siRNA sequence for the non-specific control was 5'-AATTCTCCGAACGTGTCACGT-3'. The 21-nucleotide siRNA sequences used were as follows: Neurl4-1 and Neurl4-2, 5'-CCAUCAUGCAAGACGGUAAUU-3' and 5'-GUGCAGGUGCGGAUAGAUUUU-3'; Centrin-2: 5'-GAGCAAAAGCAGGAGAUCCdTdT-3' and Cyclin F, 5'-UAGCCUACCUCUACAAUGAUU-3' [4]. CP110, Cep97 [2] and Cep76 [3] silencing has been described previously.

Immunostaining

Indirect immunofluorescence was performed as described [1]. Briefly, cells were grown on glass coverslips and fixed with cold methanol for 2 minutes. Cells were then permeabilized with 1% Triton X-100/PBS for 5 minutes. Slides were blocked with 3%

BSA in 0.1% Triton X-100/PBS prior to incubation with primary antibodies. Secondary antibodies used were Cy3- or Alexa488-conjugated donkey anti-mouse or anti-rabbit IgG (Jackson Immunolabs). Cells were also stained with DAPI. Image acquisition was performed on a Zeiss Axiovert 200M microscope (63X objective lens, 1.6x Optovar) equipped with a cooled Retiga 2000R CCD (QImaging) and Metamorph Software (Molecular Devices). Some fluorescence images were collected as 0.5 μm *Z*-stacks and are presented as maximal-intensity projections. Post–acquisition image processing was done using the Metamorph software. Neurl4 antibody recognizing C-terminal residues was used for immunofluorescence.

FACS analysis and Cell cycle synchronization

FACS analyses were performed as described previously [2].

U2OS cells were synchronized by double thymidine block. Cells were released from this block and harvested at several time points during G1/S phase and M phase.

Immunoprecipitation, immunoblotting, and in vitro binding experiments

Cells were lysed with buffer containing 50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA/pH 8, 0.1% NP-40, 1 mM DTT, 0.5 mM AEBSF, 2 μg/ml leupeptin, 2 μg aprotinin, 50 mM ß-glycerophosphate, and 10% glycerol at 4^{o}C for 30 minutes. Immunoprecipitation process was described previously [2].

For expression and mapping studies, 293T cells were transfected with Flag-tagged constructs using calcium phosphate. Cells were harvested 48-72 hours after transfection.

In vitro binding studies were performed by incubating in vitro translated Neurl4 with a

purified GST fusion protein containing CP110 residues 1-629 (Chen et al., 2002) as described previously, except that 100 mM KCl was used in the binding and wash buffer [5]

Electron microscopy (EM)

U2OS cells were washed with PBS followed by fixation with 0.1M sodium cacodylate buffer (pH7.4) supplemented with 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1% ruthenium red. The cells were then detached with a cell scraper and centrifuged. Cell pellets were fixed for 2 additional hours, post-fixed with 1% osmium tetroxide for 1.5 hours at room temperature, and stained with 1% uranyl acetate, then processed in a standard manner and embedded in EMbed 812 (Electron Microscopy Sciences, Hatfield, PA) for transmission electron microscopy (TEM). Serial thin (60 nm) sections were cut, mounted on 200 mesh or slotted copper grids, and stained with uranyl acetate and lead citrate. Stained grids were examined using a Philips CM-12 electron microscope (FEI; Eindhoven, Netherlands) and photographed with a Gatan (4k x2.7k) digital camera (Gatan, Inc., Pleasanton, CA).

Ubiquitylation assays

HEK293 cells were transiently transfected with pcB6-His Ubiquitin (gift from R. Baer) together with HA-CP110 and either Flag-Neurl4, Flag-cyclin F (gift from M. Pagano), or Flag-Fbxo32. After 36 h, cells were treated with 10 μm MG132 (Peptides International) for 4 h, and the cell lysates were subjected to affinity-purification with nickel-NTA resin (Qiagen) to precipitate His-tagged ubiquitylated proteins. Briefly, cells were lysed in

Buffer A (100 mM NaH_2PO4, 10 mM Tris-Cl, 6M guanidine-HCl, 10 mM imidazole pH 8.0), sonicated briefly, and incubated with Ni-NTA resin for three hours at room temperature. Ni-NTA beads were washed with Buffer A twice, followed by two washes with Buffer A/TI (1 volume Buffer A and 1 volume TI Buffer) (TI buffer, 25 mM Tris-Cl pH6.8 and 20 mM imidazole), and then one wash with TI Buffer. Finally, ubiquitylated proteins were eluted in 2X SDS buffer (60 mM Tris pH 6.8, 2% SDS, 100 mM DTT) containing 200 mM imidazole, separated by SDS-PAGE, and detected by immunoblotting with anti-CP110 antibody.

Supplemental Figure Legends

Figure S1. Neurl4 and CP110 interact, most likely directly.

(A) Alignment of Neuralized family members in *Drosophila melanogaster* and *Homo sapiens*. NHR (green), RING (purple), and SOCS (red) domains are shown [6, 7]. In humans, four Neuralized family members have been identified. Neurl1, 2, and 3, which encode either a RING or SOCS domain, function as E3 ubiquitin ligases involved in diverse processes such as Notch signaling (Neurl1)[8-10], myogenesis (Neurl2)[11], and inflammation (Neurl3)[12]. **(B)** 293T cells were transfected with empty vector (EV), Flag-tagged full length Neurl4, NHR1-2, NHR2-3, NHR1-3, NHR3-4, NHR3-5, NHR4-6, or C-terminal domain of Neurl4. Cells were harvested and subjected to anti-Flag immunoprecipitation. Immunoprecipitated samples were analyzed by western blotting with anti-CP110 and anti-Flag antibodies. **(C)** Fragments encoding Neurl4 NHR3-5 (residues 462-988) and Neurl4 C-terminal residues 1201-1562 were translated *in vitro* using reticulocyte lysate. The resulting ^{35}S-methionine-labeled proteins were mixed with

either GST protein or a GST-CP110 fragment spanning residues 1-629 [5]. The ^{35}S-labeled proteins were detected by autoradiography before and after purification with glutathione agarose beads. GST-CP110 protein can pull down Neurl4 NHR3-5, which was shown to interact with CP110 *in vivo*, but the Neurl4 C-terminal domain does not, and therefore this protein serves as a negative control. **(D)** U2OS cells were transfected with indicated siRNAs and were stained with anti-centrin2 (green) or anti-CP110 (red) antibody. Representative images are shown (*left*). The fluorescent intensity of CP110 was quantified by Metamorph Software, and data were plotted after normalization (*right*). **(E)** U2OS cells were triply stained for centrin2 (blue, distal centriolar marker), C-Nap1 (green, proximal centriolar marker), and Neurl4 (red). We conclude that Neurl4 localized to the middle portion of the daughter centriole, more distal to C-Nap1.

Figure S2. Neurl4 domain NHR3 contains a novel centrosome localization signal.

(A) Upper: U2OS cells were transfected with indicated siRNAs and were stained with anti-Centrin2 (green) and anti-Neurl4 (red) antibodies. Scale Bar: 2 μm; Lower: western blotting of Neurl4, CP110, and Cep76 in U2OS cells treated with indicated siRNAs. Western blotting with antibodies against Neurl4, CP110, Cep76, and α-tubulin was performed to verify knock-down efficiency. (**B**) U2OS cells transfected with GFP-NHR3, encoding Neurl4 NHR repeat 3, were visualized through GFP fluorescence and by immunostaining with the indicated antibodies.

Figure S3. Neurl4 depletion does not affect cell cycle progression.

(A) U2OS cells were transfected with non-specific (NS) siRNA or siRNA targeting Neurl4. 48 hours after transfection, cells were fixed, and cell cycle status was examined by FACS analysis. The percentages of cells in G1, S, or G2/M phases were analyzed by ModFit. **(B)** Western blot analysis to monitor the knock-down efficiency in (A).

Figure S4. Depletion of Neurl4 results in supernumerary C-Nap1 and GT335 foci but not Sas6 dots.

(A) U2OS cells were transfected with non-specific (NS) siRNA or siRNA targeting Neurl4 as indicated. Representative images are shown, and the percentage of cells with more than two C-Nap1 foci is analyzed. Averaged data obtained from three independent experiments are shown. At least 100 cells for each siRNA transfection were scored each time. Error bars represent ± standard deviation (SD). *, $p<0.05$. Scale bar: 10 μm. **(B)** U2OS cells were transfected as in (A) and GT335 foci were analyzed. *, $p<0.05$. Scale bar: 10 μm. **(C)** U2OS cells transfected with non-specific, Neurl4, or cyclin F siRNAs were harvested after 48 hours and subjected to immunofluorescent detection with antibodies against Sas6 (red) or CP110 (green). DNA was stained with DAPI (blue). Scale bar: 10 μm. **(D)** Quantification of cells with more than four CP110 dots or two Sas6 dots after control, Neurl4, or cyclin F depletion. Mean data obtained from two independent experiments are shown. >100 cells for each siRNA transfection were scored in each experiment. Error bars represent +/- SEM. **, $p<0.01$. **(E)** Western blots gauging the knock-down efficiency in this experiment are shown.

Figure S5. Electron microscopic analysis of Neurl4 depleted cells

U2OS cells were transfected with control siRNA or siRNA targeting Neurl4. After 48 hours, cells were fixed and subjected to electron microscopic analysis as described in the Supplemental Materials and Methods section. **(A-B)** Two representative sets of images of U2OS cells treated with Neurl4 siRNA. A series of adjacent sections are shown. White arrows point to the extra electron dense material observed after Neurl4 depletion. Black arrowheads point to normal centrioles. **(C)** Two electron microscopy images from U2OS cells treated with control siRNA or Neurl4 siRNA are shown. In both case, centriole structures were intact, suggesting that Neurl4 depletion does not fragment centrioles.

Figure S6. The phenotype associated with Neurl4 depletion is dependent on CP110 up-regulation.

(A) HeLa cells were transfected with control siRNA or siRNAs targeting Neurl4, CP110, Sas6, Neurl4/CP110, or Neurl4/Sas6. Quantification of cells with > 4 centrin foci is shown. More than 100 cells for each siRNA transfection were scored each time. Error bars represent +/- SEM. *, $p<0.05$; **, $p<0.01$. **(B)** Western blotting to monitor the knock-down efficiency in (A). **(C)** Depletion of Neurl4 in synchronized Hela cells stabilizes CP110. HeLa cells were transfected with control or Neurl4 siRNA and were synchronized by double thymidine block. Cells were harvested at the indicated time points after release from the double-thymidine block into medium containing 100 ng/ml nocodazole and processed for western blotting with indicated antibodies.

Figure S7. Neurl4 depletion leads to an increase in mitotic defects (related to Fig. 4E).

(A) U2OS cells were transfected with non-specific (NS) siRNA or siRNA targeting Neurl4 for 72 hours before immunofluorescence detection with CP110 and α-tubulin antibodies and DAPI. Representative images of mitotic cells with normal bipolar, pseudo-bipolar, mono-polar, and multi-polar spindles and lagging chromosomes are shown (channels: red, CP110; green, α-tubulin; blue, DNA). Scale bar: 10μm. A composite with merged channels is shown in Fig. 4E. **(B)** Hela cells were transfected with non-specific (NS) siRNA or siRNA targeting Neurl4 for 72 hours before immunofluorescence detection with CP110 and α-tubulin antibodies and DAPI. A quantification of mitotic cells with normal bipolar, pseudo-bipolar, mono-polar, and multi-polar spindles and lagging chromosomes are shown in the graph. Error bars represent +/- SEM. *, $p<0.05$; **, $p<0.01$.

Figure S8. Neurl4 and Cyclin F assemble different complexes with CP110

293T cells were lysed and immunoprecipitated with rabbit IgG (control), anti-Neurl4, or anti-CP110 antibody in **(A)** or immunoprecipitated with rabbit IgG (control), anti-cyclin F, or anti-Cep97 antibody in **(B)** and probed with the indicated antibodies. In **(A)**, CP110 can precipitate cyclin F, but antibodies against Neurl4 do not. In **(B)**, anti-cyclin F antibodies can precipitate CP110 but not Neurl4 or Cep97. This result indicates that CP110 may form different complexes with Neurl4 and cyclin F and could suggest that Neurl4 and cyclin F regulate CP110 levels through independent pathways.

Figure S9. A model for how Neurl4 regulates centrosome duplication.

Neurl4 is a daughter centriole-specific protein. In G1 phase, Neurl4 localizes to daughter centrioles. In wild-type cells, when each centriole begins to duplicate, Neurl4 is specifically recruited to newly formed pro-centrioles, permitting normal centrosome duplication and mitosis. However, in Neurl4 depleted cells, similar to cyclin F depleted cells, elevated levels of CP110 accumulate near the existing centrioles, forming CP110-positive structures that can recruit other centriolar proteins and components of the pericentriolar matrix (PCM), including γ-tubulin. A subset of these CP110-positive assemblies can function as MTOCs, leading to various mitotic defects.

Supplemental References

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12. Hu Y, Nguyen TT, Bui KC, Demello DE, Smith JB (2005) A novel inflammation-induced ubiquitin E3 ligase in alveolar type II cells. *Biochemical and biophysical research communications* **333:** 253-263

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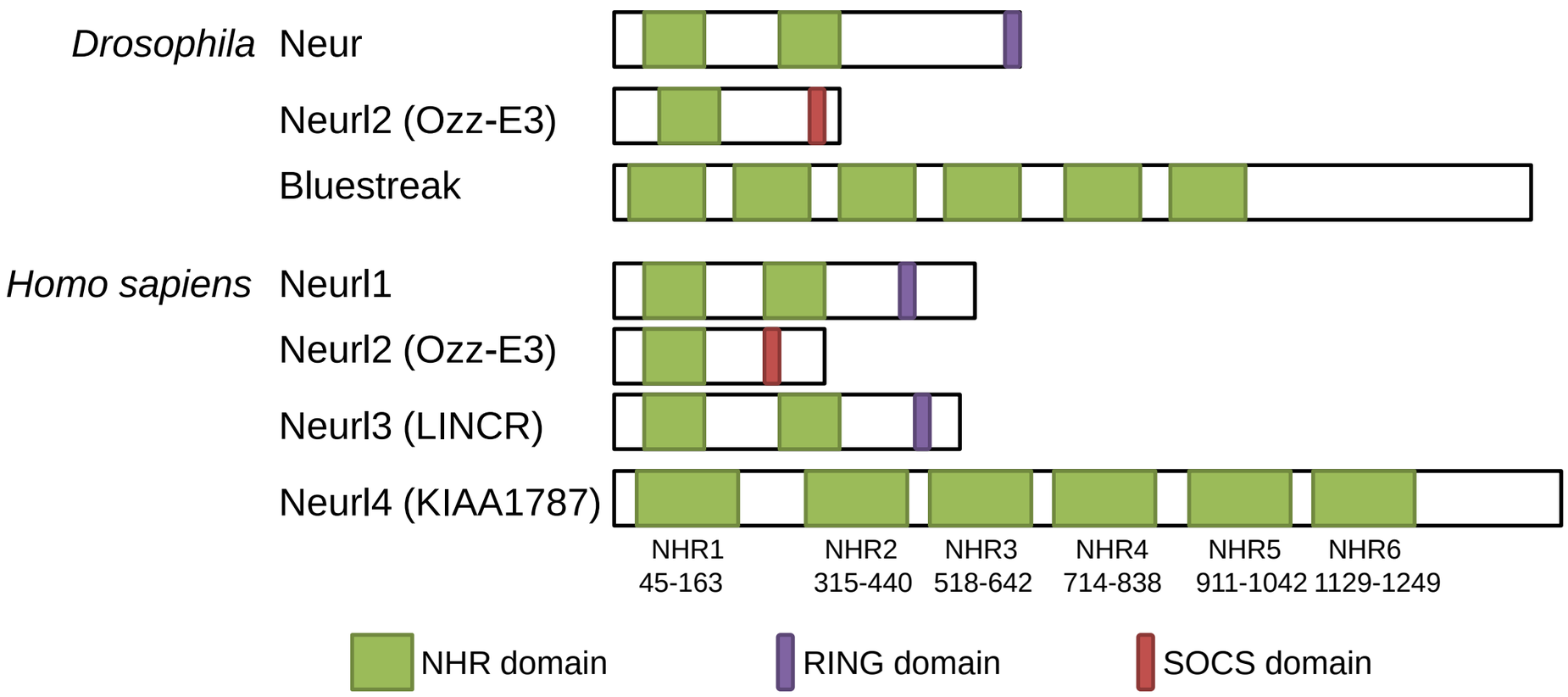
Drosophila Neur
Neurl2 (Ozz-E3)
Bluestreak
Homo sapiens Neurl1
Neurl2 (Ozz-E3)
Neurl3 (LINCR)
Neurl4 (KIAA1787)
NHR1 45-163
NHR2 315-440
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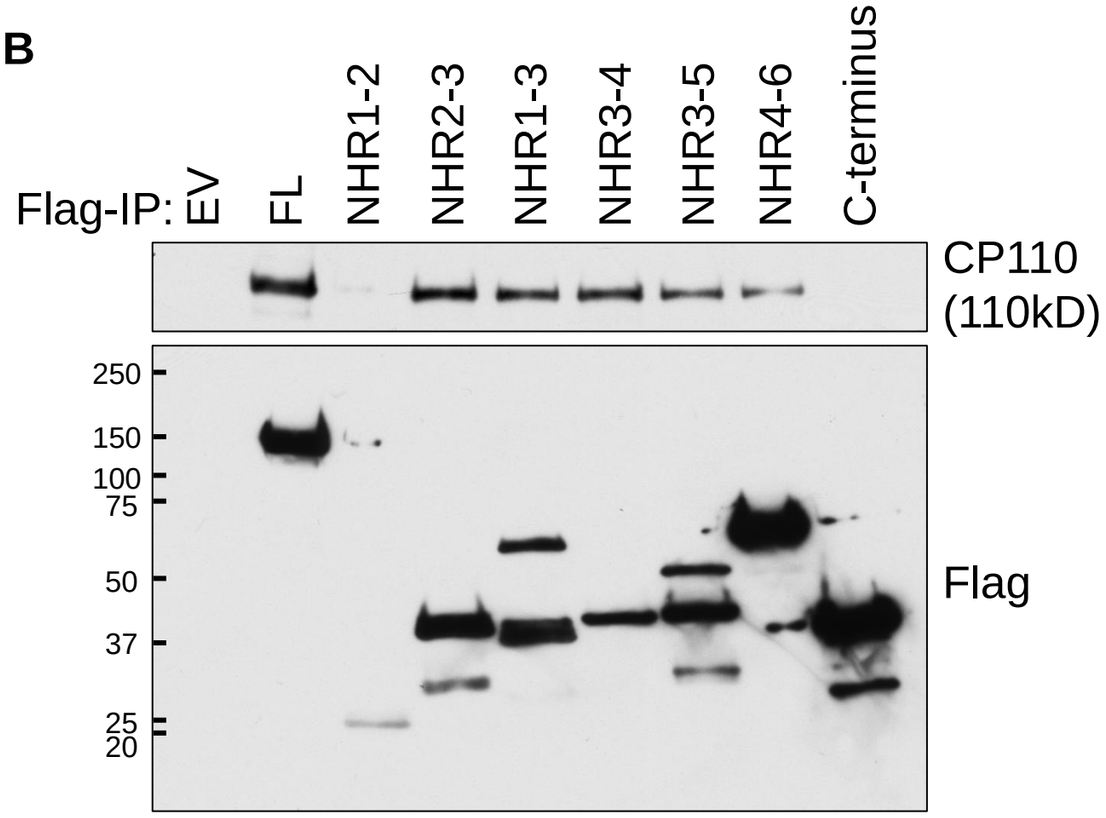
NHR4 714-838
NHR5 911-1042
NHR6 1129-1249
NHR domain
RING domain
SOCS domain

B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FL
NHR1-2
NHR2-3
NHR1-3
NHR3-4
NHR3-5
NHR4-6
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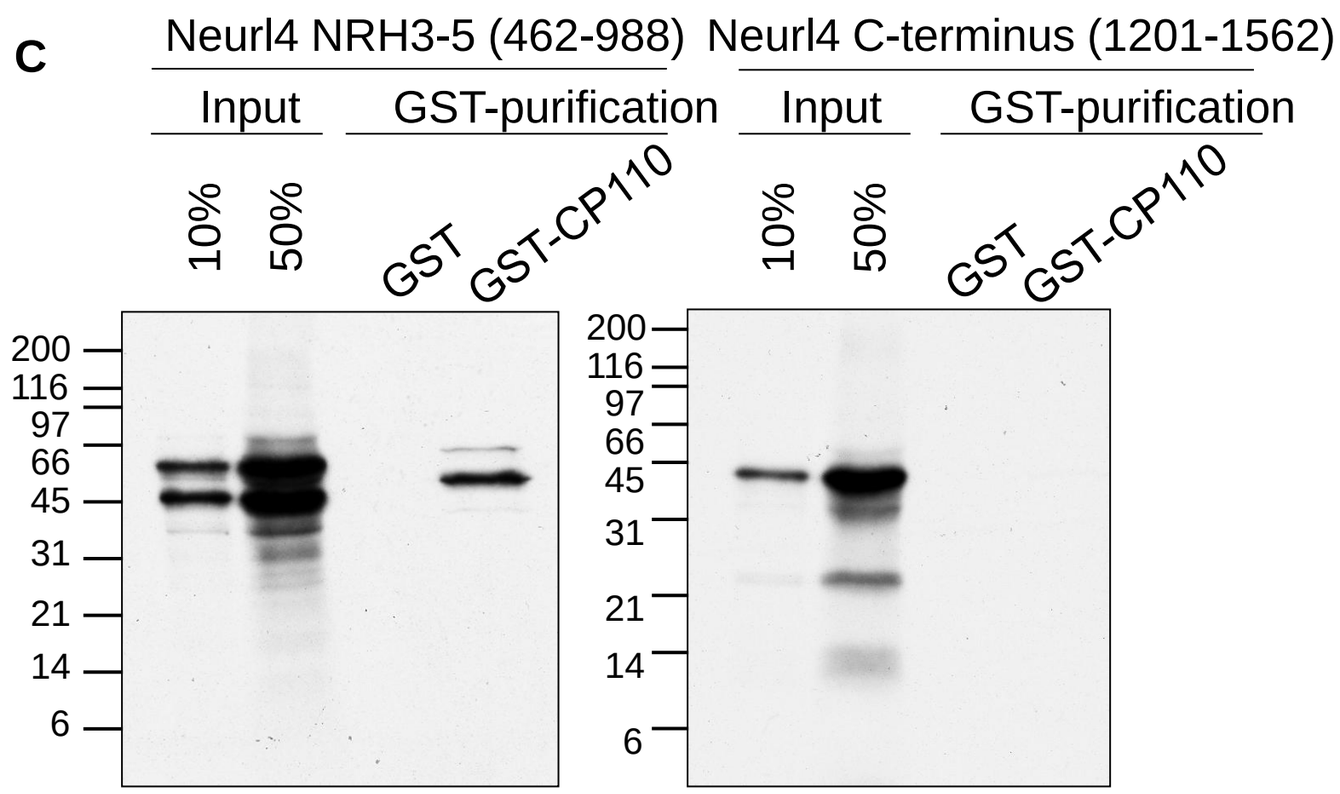
Flag
250
150
100
75
50
37
25
20

C


Neurl4 NRH3-5 (462-988)
Neurl4 C-terminus (1201-1562)
Input
GST-purification
Input
GST-purification
10%
50%
GST
GST-CP110
10%
50%
GST
GST-CP110
200
116
97
66
45
31
21
14
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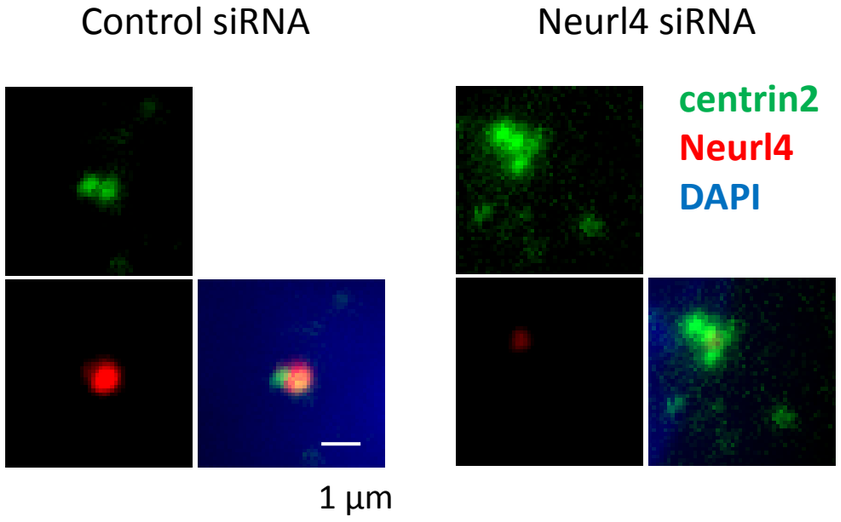
200
116
97
66
45
31
21
14
6

D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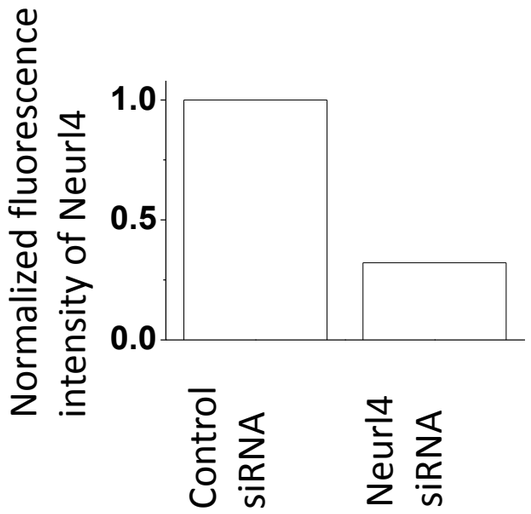
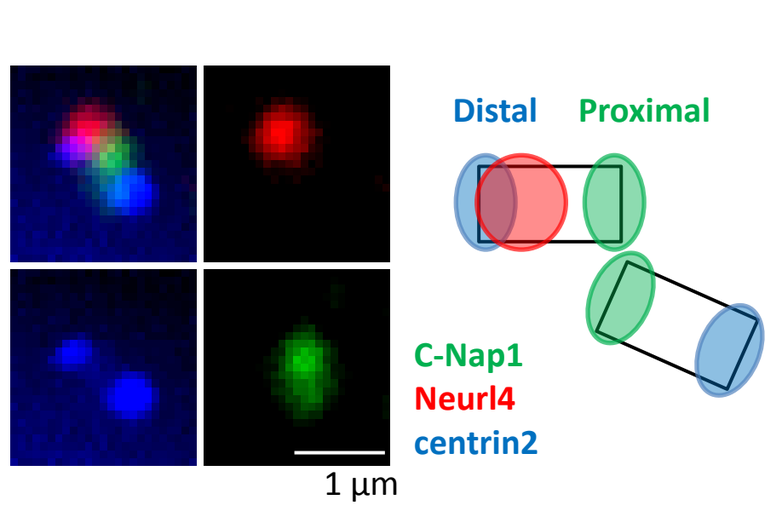
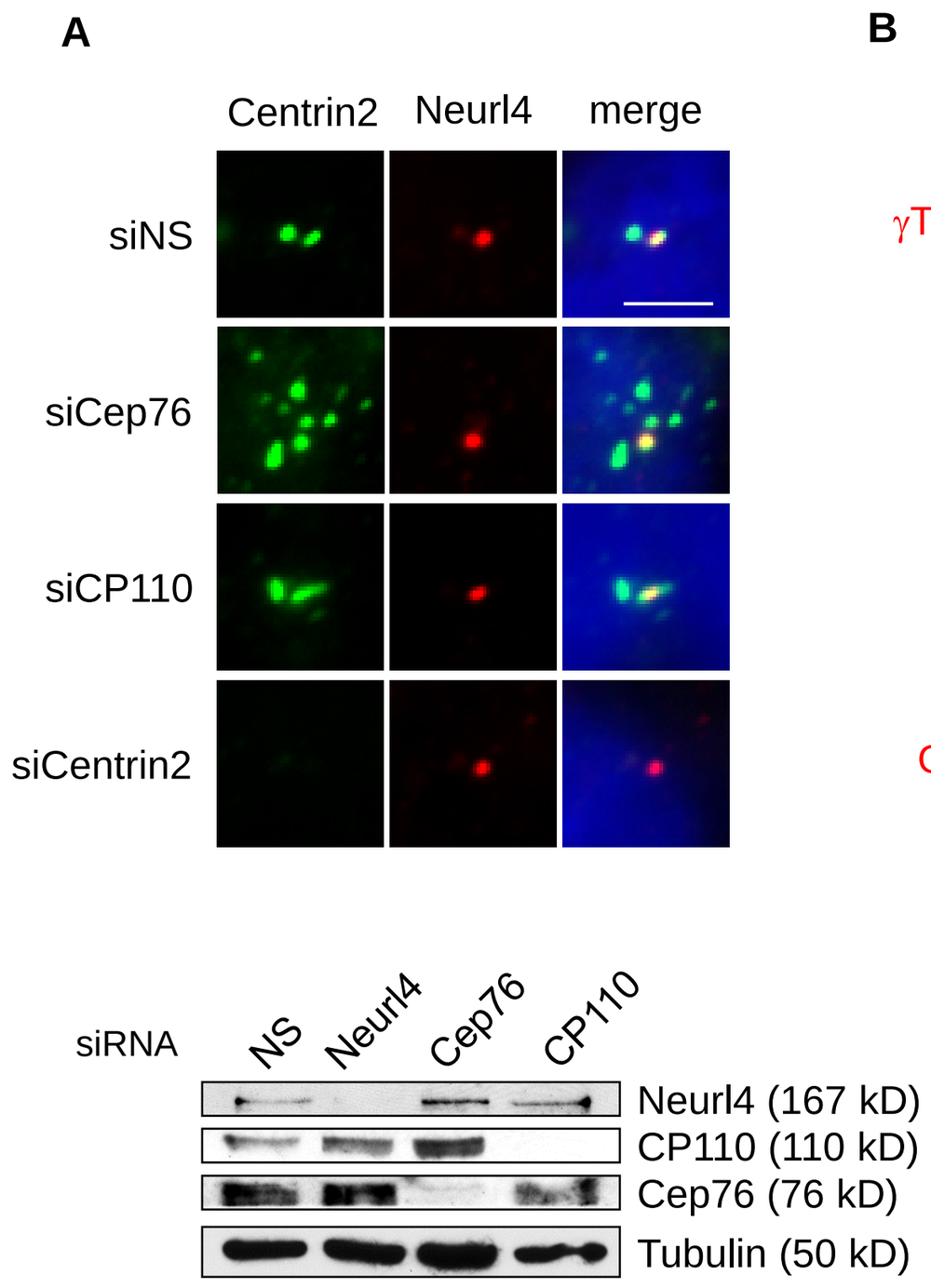
Neurl4 siRNA
centrin2
Neurl4
DAPI
1 µm


Normalized fluorescence intensity of Neurl4
1.0
0.5
0.0
Control siRNA
Neurl4 siRNA

E


Distal
Proximal
C-Nap1
Neurl4
centrin2
1 µm


A
Centrin2
Neurl4
merge
siNS
siCep76
siCP110
siCentrin2
siRNA
NS
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Cep76
CP110
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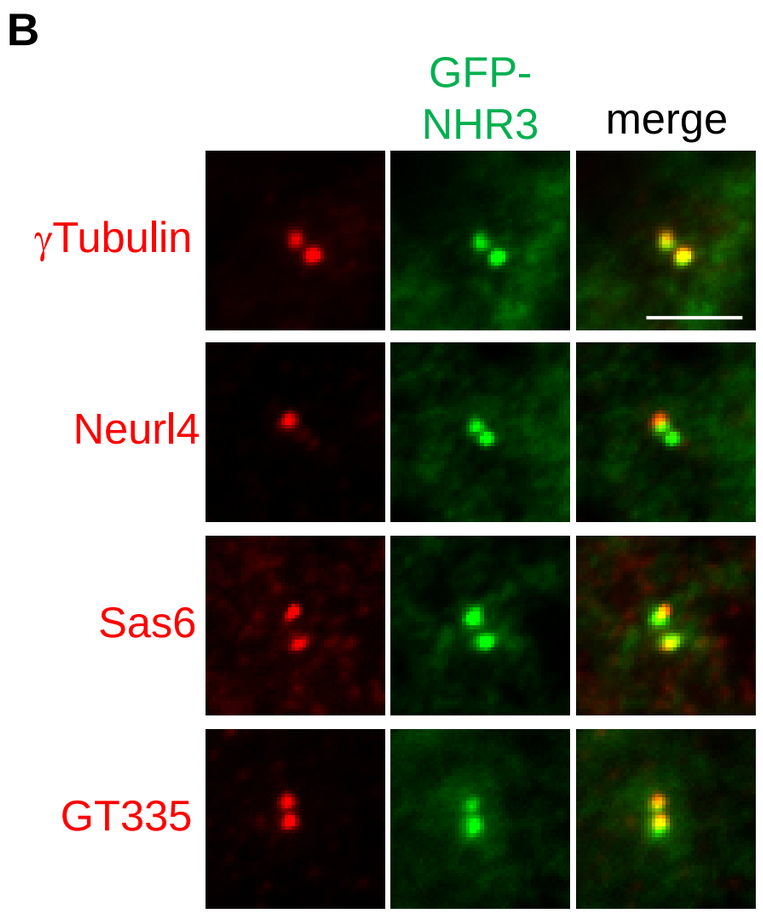
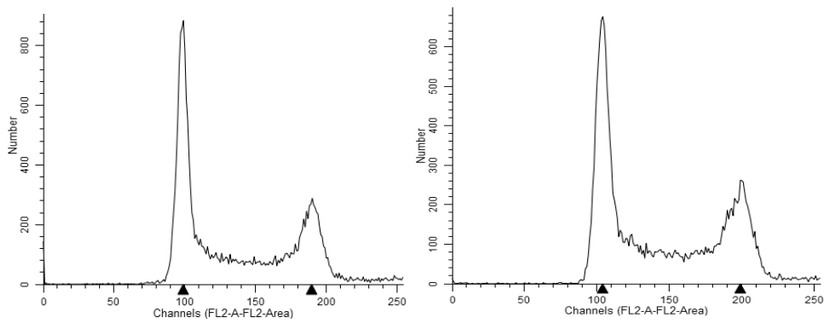
CP110 (110 kD)
Cep76 (76 kD)
Tubulin (50 kD)


B
GFP-NHR3
merge
γTubulin
Neurl4
Sas6
GT335

A



siRNA	Non-specific	Neurl4
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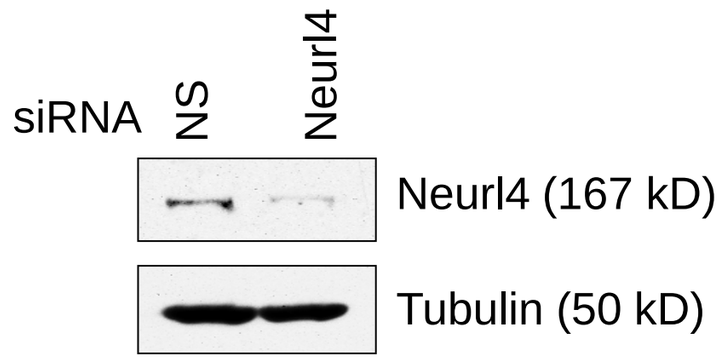
	S: 39.9%	S: 40.9%
	G2/M:16.8%	G2/M: 16.9%



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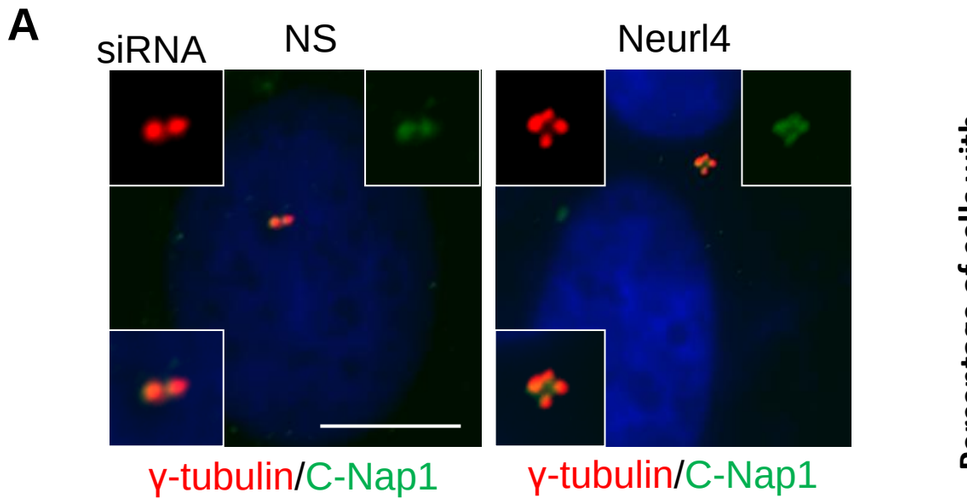
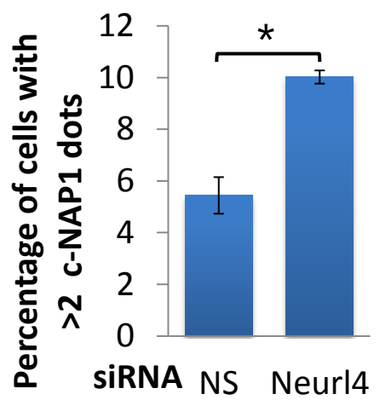
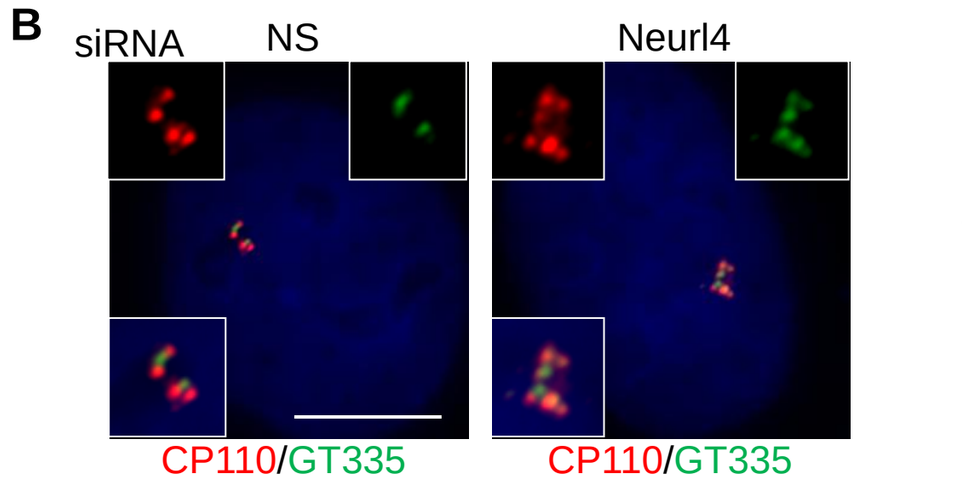

siRNA
NS
Neurl4
γ-tubulin/C-Nap1
γ-tubulin/C-Nap1


Percentage of cells with >2 c-NAP1 dots
12
10
8
6
4
2
0
*
siRNA
NS
Neurl4

B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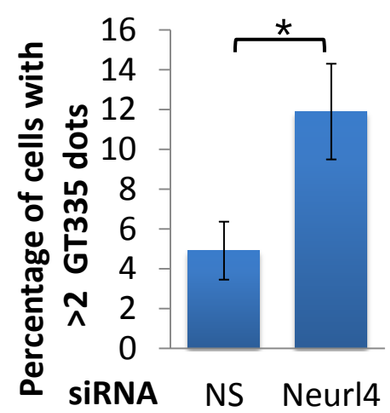
NS
Neurl4
CP110/GT335
CP110/GT335


Percentage of cells with >2 GT335 dots
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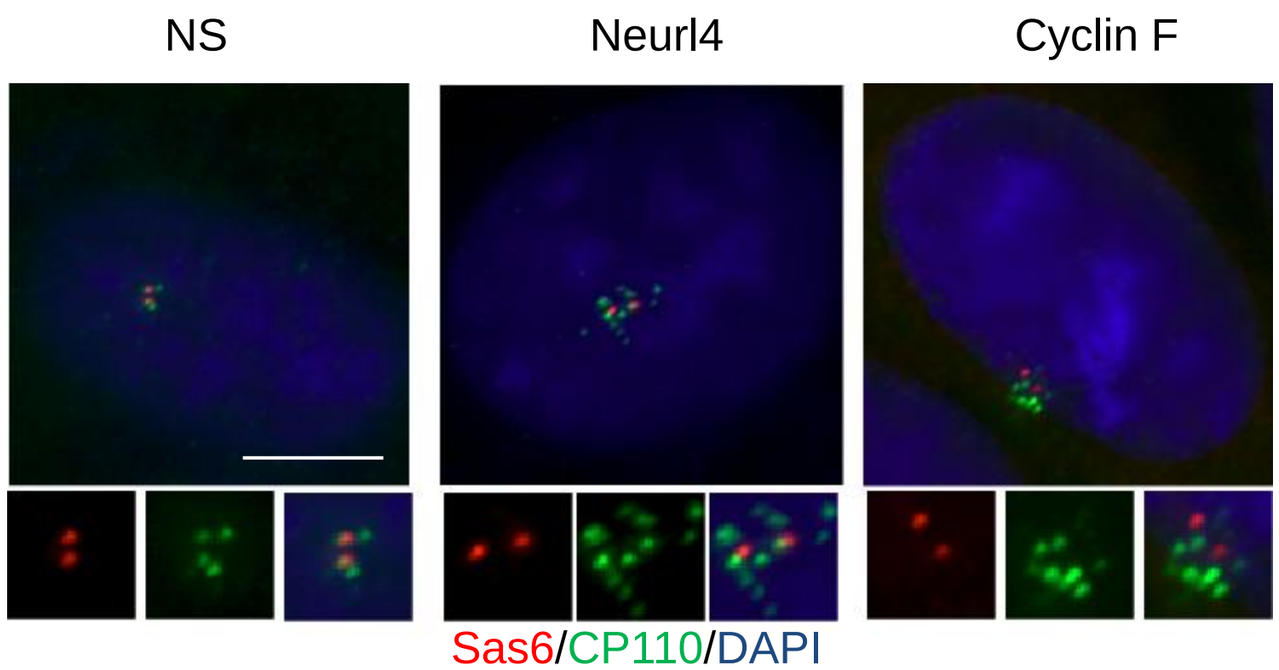
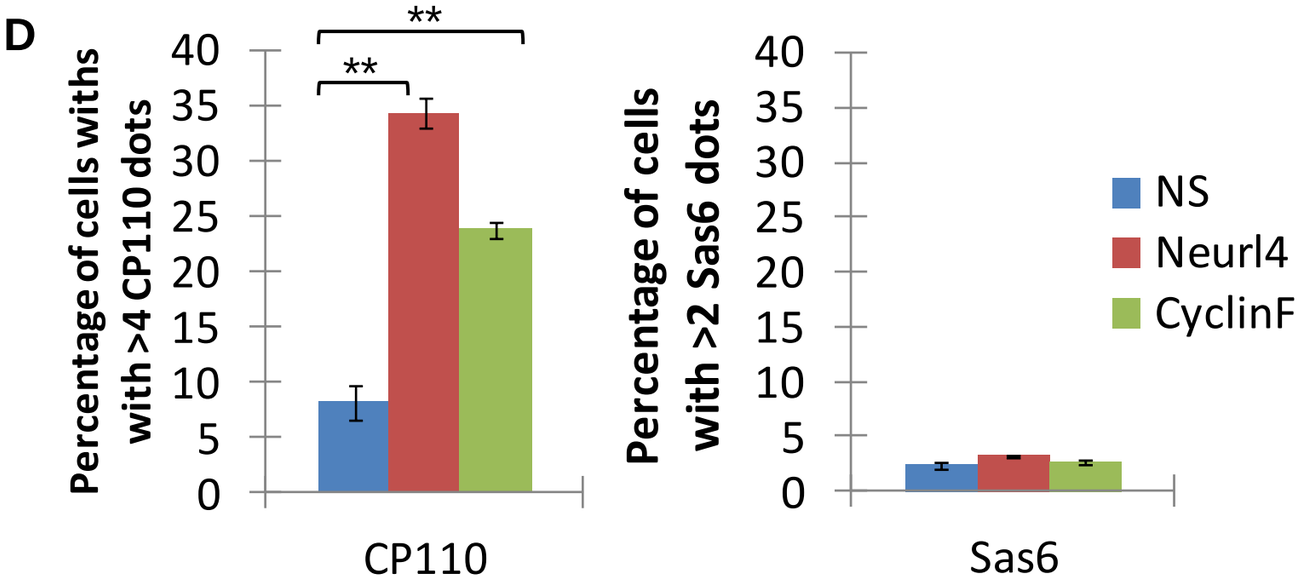
14
12
10
8
6
4
2
0
*
siRNA
NS
Neurl4

C


NS
Neurl4
Cyclin F
Sas6/CP110/DAPI

D


Percentage of cells withs with >4 CP110 dots
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35
30
25
20
15
10
5
0
**
**
CP110
Percentage of cells with >2 Sas6 dots
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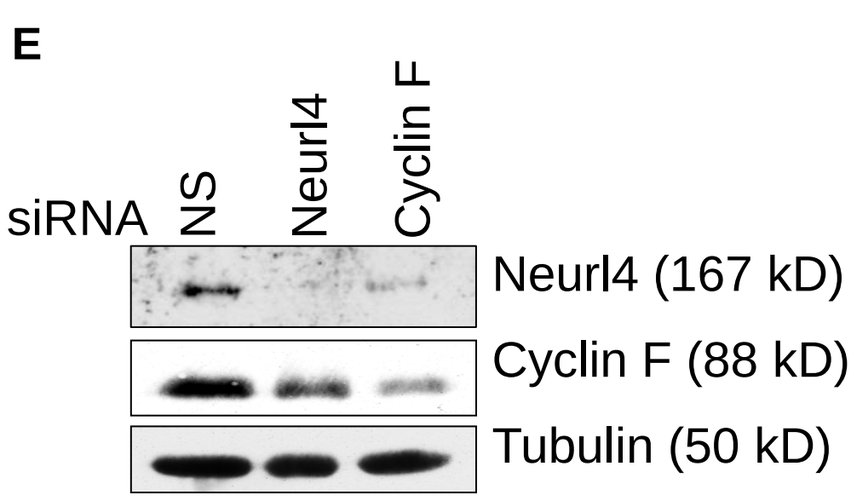
35
30
25
20
15
10
5
0
Sas6
NS
Neurl4
CyclinF

E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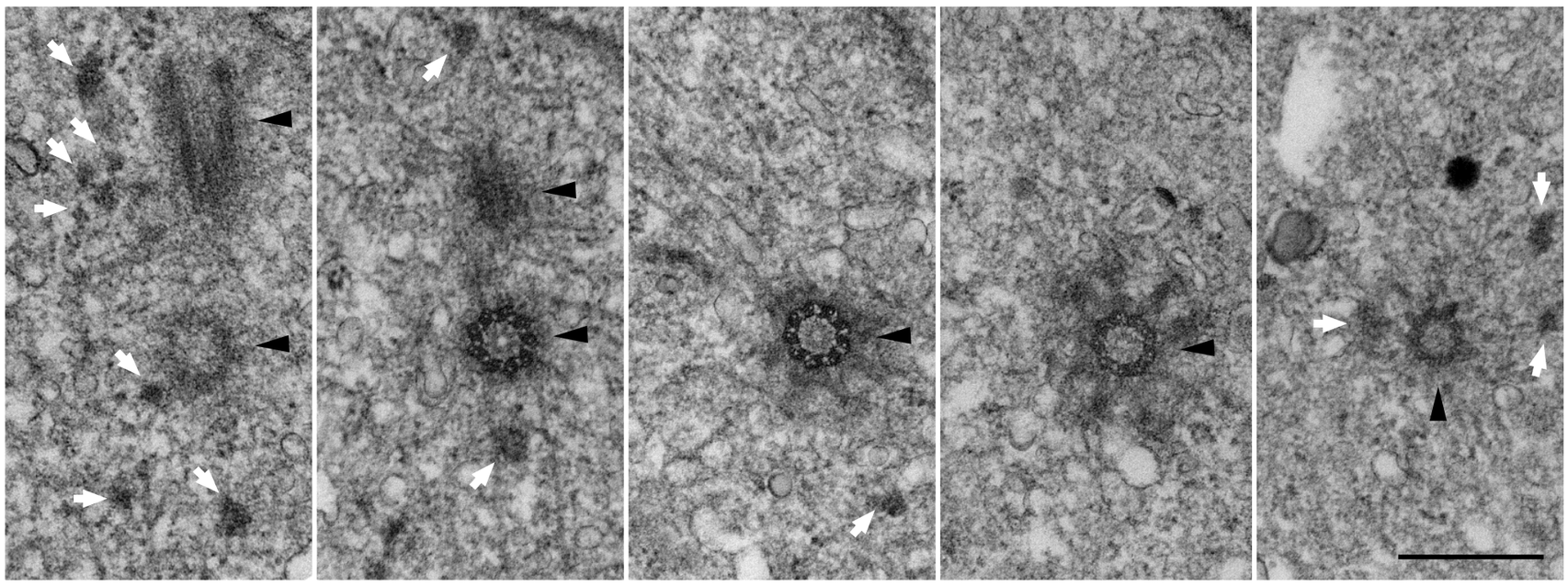
Cyclin F
Neurl4 (167 kD)
Cyclin F (88 kD)
Tubulin (50 kD)

A



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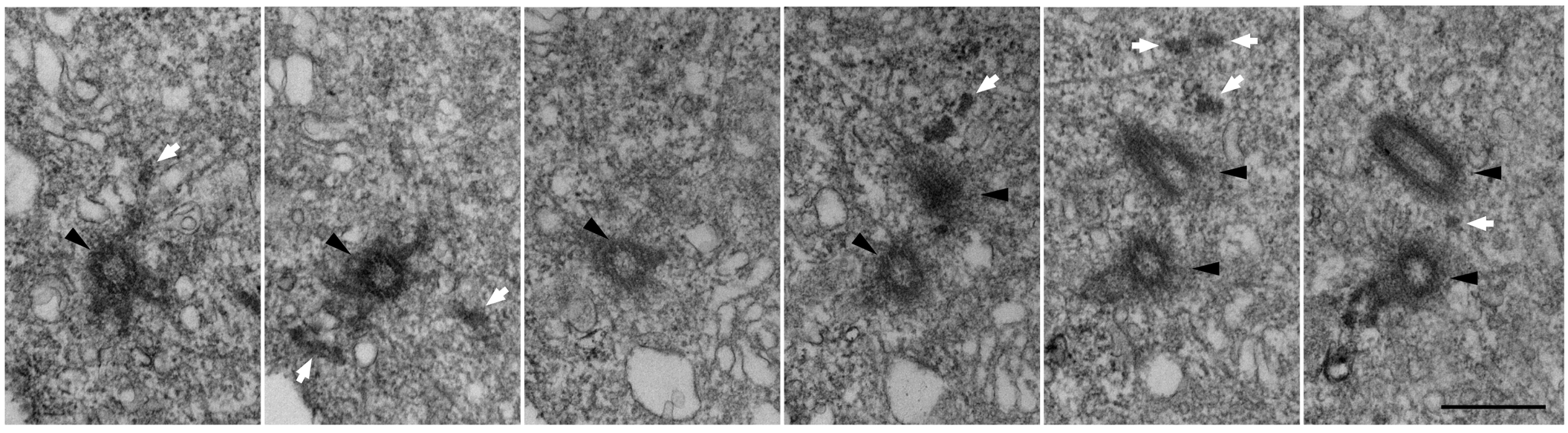

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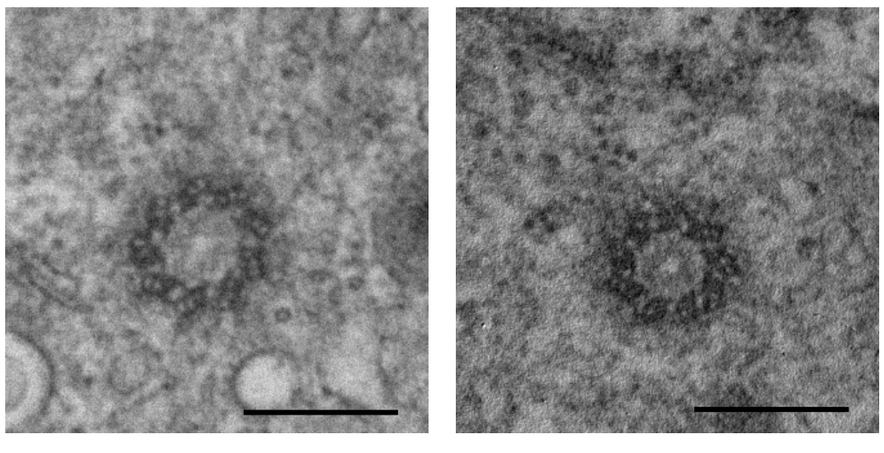

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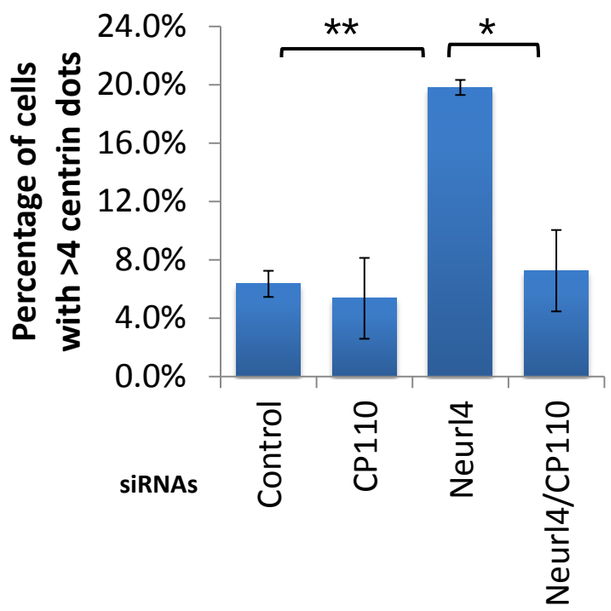
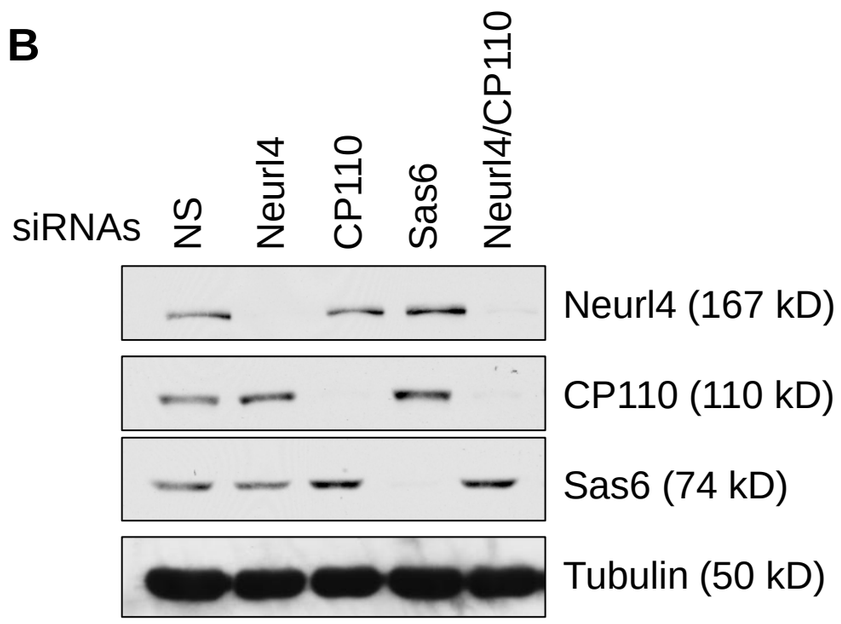
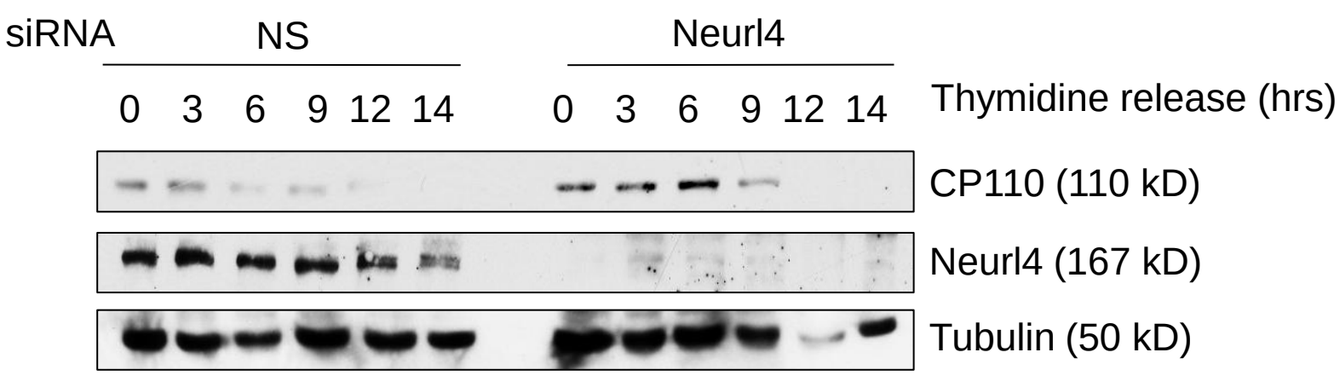

Percentage of cells with >4 centrin dots
24.0%
20.0%
16.0%
12.0%
8.0%
4.0%
0.0%
**
*
siRNAs
Control
CP110
Neurl4
Neurl4/CP110

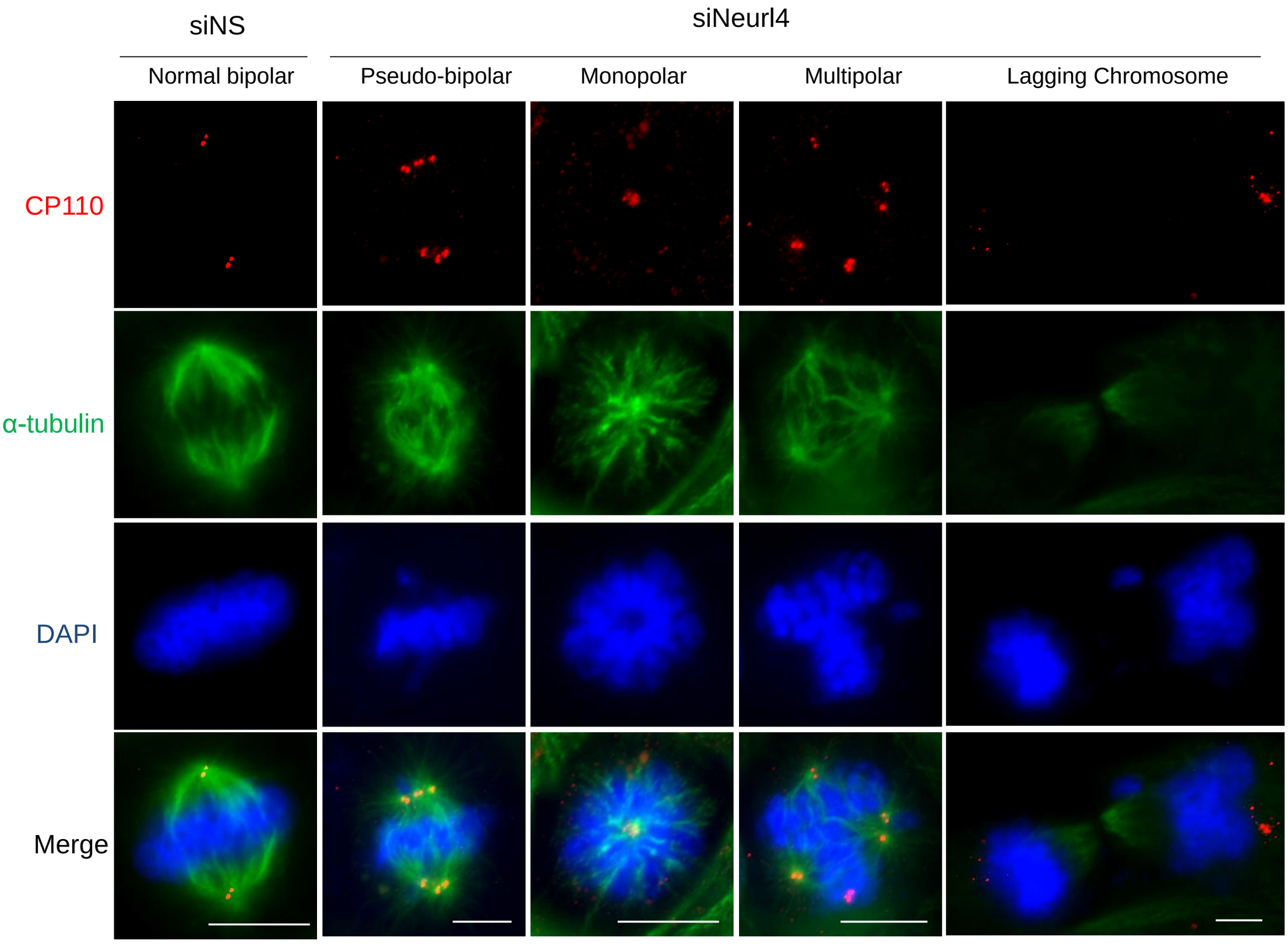
B


siRNAs
NS
Neurl4
CP110
Sas6
Neurl4/CP110
Neurl4 (167 kD)
CP110 (110 kD)
Sas6 (74 kD)
Tubulin (50 kD)

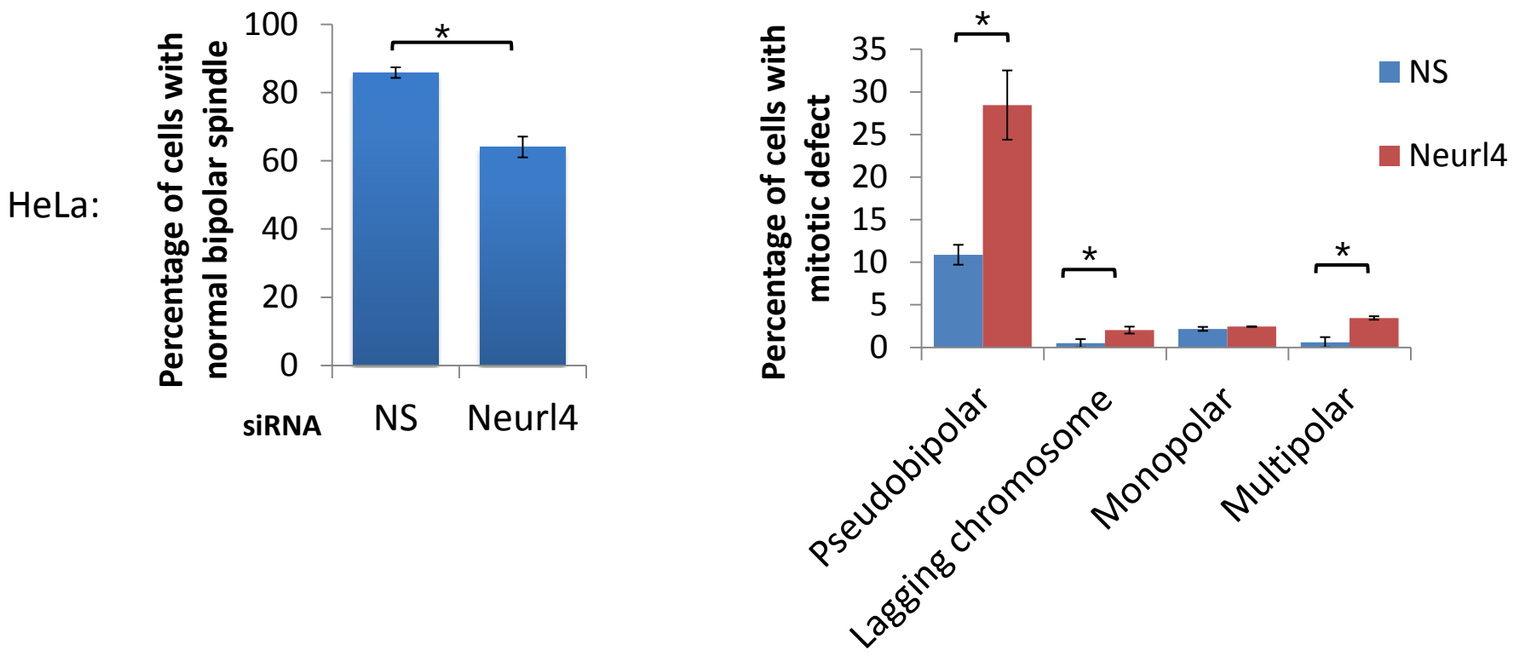
C

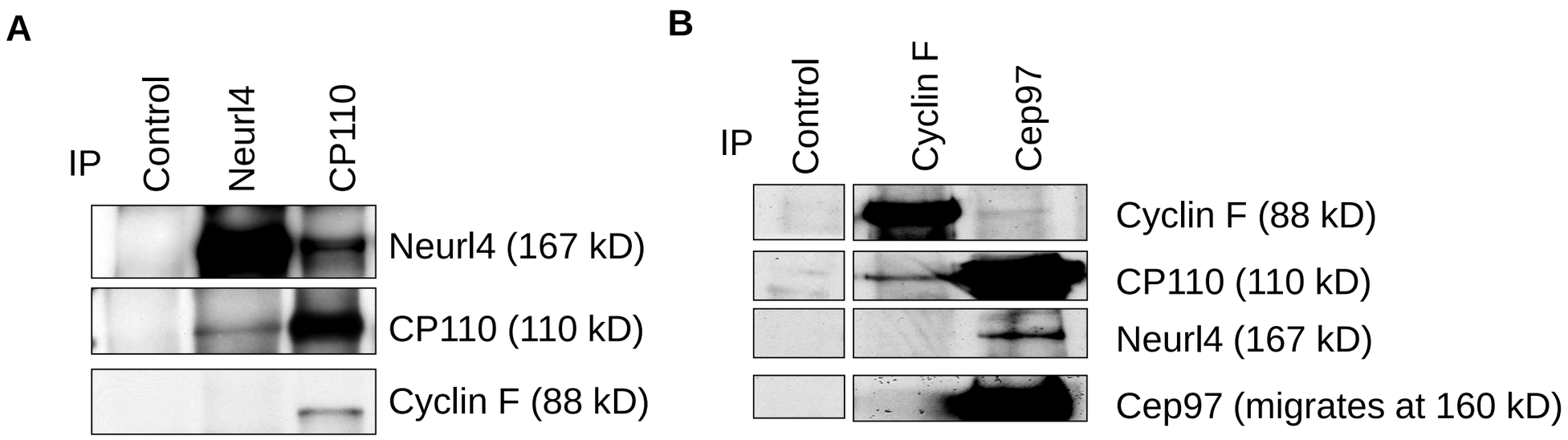

siRNA
NS
Neurl4
0 3 6 9 12 14
0 3 6 9 12 14
Thymidine release (hrs)
CP110 (110 kD)
Neurl4 (167 kD)
Tubulin (50 kD)

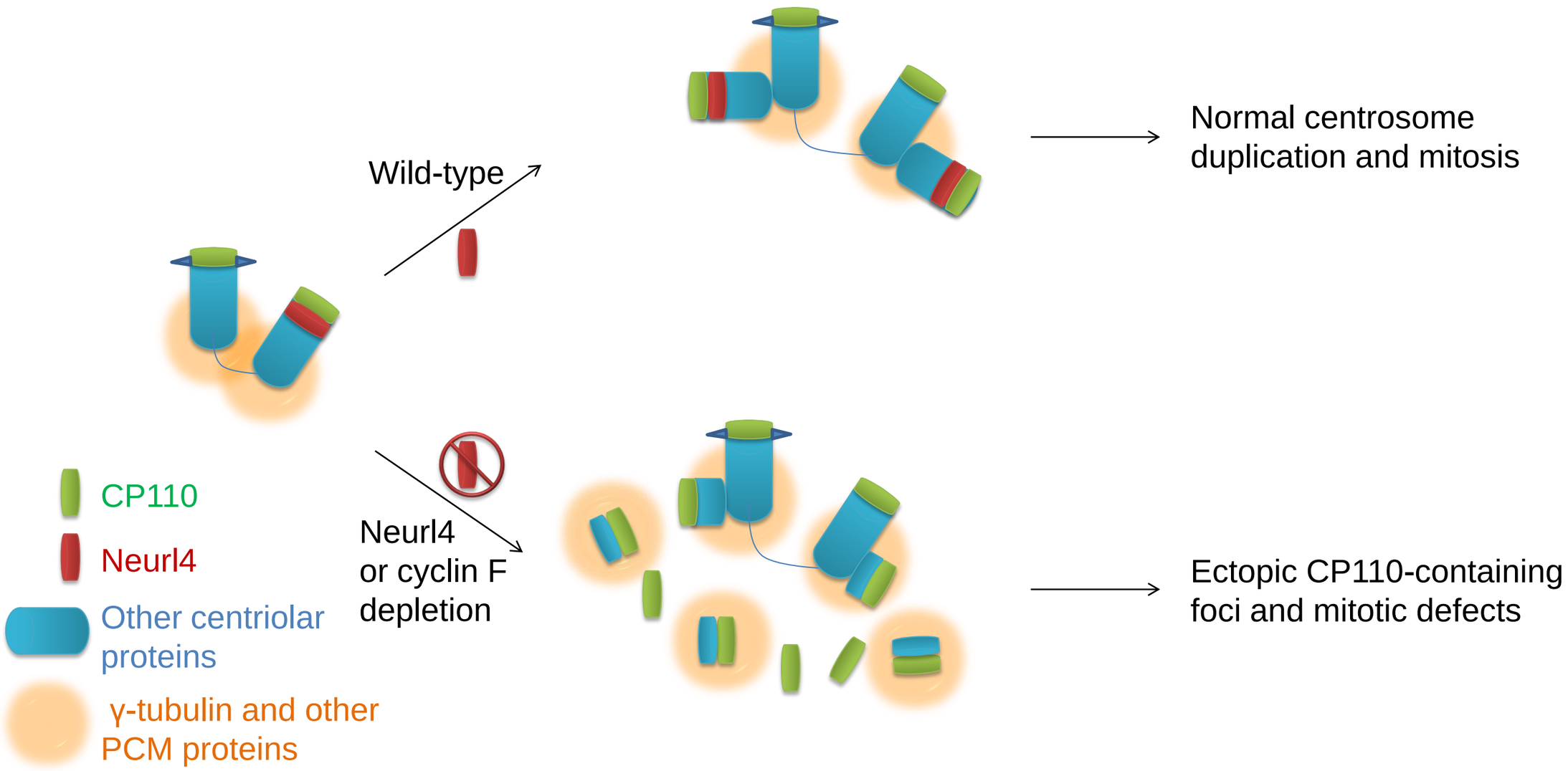
A


siNS
siNeurl4
Normal bipolar
Pseudo-bipolar
Monopolar
Multipolar
Lagging Chromosome
CP110
α-tubulin
DAPI
Merge

B


HeLa:
siRNA
NS
Neurl4
Percentage of cells with normal bipolar spindle
100
80
60
40
20
0
*
Percentage of cells with mitotic defect
35
30
25
20
15
10
5
0
*
*
*
NS
Neurl4
Pseudobipolar
Lagging chromosome
Monopolar
Multipolar


A
IP
Control
Neurl4
CP110
Neurl4 (167 kD)
CP110 (110 kD)
Cyclin F (88 kD)
B
IP
Control
Cyclin F
Cep97
Cyclin F (88 kD)
CP110 (110 kD)
Neurl4 (167 kD)
Cep97 (migrates at 160 kD)


Wild-type
Normal centrosome duplication and mitosis
Neurl4 or cyclin F depletion
Ectopic CP110-containing foci and mitotic defects
CP110
Neurl4
Other centriolar proteins
γ-tubulin and other PCM proteins