Supplemental Information

NDR2-mediated Rabin8 phosphorylation is crucial for ciliogenesis by switching binding specificity from phosphatidylserine to Sec15

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Supplemental Materials and Methods

Reagents and antibodies

Okadaic acid and l-phosphatase were purchased from Sigma and New England Biolabs, respectively. Antibodies were purchased as follows: rabbit polyclonal antibodies against Ki-67 (Abcam) and mouse monoclonal antibodies against Myc (Roche; 9E10), Flag (Sigma; M2), poly-Glu-tubulin (Enzo; GT335), Cep170 (Invitrogen; 72-413-1), and ninein (Millipore; 79-160). Rabbit polyclonal antibodies specific to pRabin8 were raised against the peptides CGHTRNK<u>S</u>(PO_4)TSSAMSG (Hokkaido System Science).

Sequences of siRNA duplex

The Stealth siRNAs targeting human NDR1, NDR2, Rabin8, Sec15A and Sec15B were purchased from Invitrogen. The siRNA sequences used were as follows:
siNDR1-1 (5'-GGCAGACAGUUUGUGGGUUGUGAAA-3'),
siNDR1-2 (5'-GGAAGGAAACAGAGUUUCUUCGUUU-3'),
siNDR1-3 (5'-GGCAGACAGUUUGUGGGUUGUGAAA-3'),
siNDR2-1 (5'-CAGAAUUGGAAAUAGUGGAGUAGAA-3'),
siNDR2-2 (5'-GGCCAGCAGCAAUCCCUAUAGAAAU-3'),
siNDR2-3 (5'-ACGUCGAUCACAACACGCUCGCAAA-3'),
siRabin8 (5'-CCUCAGUGUGAUACAGCCAAUUGUA-3'),
siSec15A (5'-GACCAGAACUUUGAGUAGCUGUUUA-3'),
and siSec15B (5'-GCGUGUAUGUCAGCUUGCAAGCAUU-3').

Immunofluorescence microscopy and time-lapse imaging

For staining cenexin, ninein and centrin, cells were pre-extracted with PHEM buffer (100 mM HEPES, pH 6.8, 20 mM PIPES, pH 6.8, 5 mM EDTA, and 2 mM $MgCl_2$) containing 0.1% Triton X-100 and fixed with cold methanol. For time-lapse imaging, RPE1 cells (1.0×10^5 cells) were transfected with indicated constructs using FuGENE HD (Promega)

and cultured for 24-30 h in DMEM/F12 supplemented with 10% FCS. Cells were further cultured for 1 h in Reibovitz's L-15 medium (Invitrogen) supplemented with 10% FCS, and then serum-starved for 1 h before imaging. Time-lapse fluorescent images were taken using a Leica DMIRBE fluorescent microsope, equipped with a PL Apo 63×oil objective lens (NA 1.3) and a cooled charge-coupled device camera (CoolSNAP HQ, Roper Scientific) driven by Metamorph imaging software (Molecular devices).

Rabin8-Rab11(QL, 2CA) binding assay

GFP-Rab11(QL) and (QL, 2CA) were expressed in 293T cells. To generate the post-nuclear supernatants, cells were homogenized in subcellular fractionation buffer (SFB) (250 mM Sucrose, 20 mM HEPES-NaOH, pH. 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 10 mg/ml leupeptin) by passing through a 27G needle 10 times using a 1 ml syringe. Homogenates were centrifuged for 5 min at 1,000 x g to remove nuclei, and supernatants were centrifuged for 5 min at 5,400 x g. The resulting supernatants were incubated with Flag-Rabin8 in SFB and immunoprecipitated with an anti-Flag antibody. After removal of unbound proteins by centrifugation at 200 x g, beads were washed with SFB. Bound proteins were subjected to SDS-PAGE and analyzed by immunoblotting with anti-Rab11 and anti-Rabin8 antibodies.

GDP-dissociation assay

GDP displacement activity was measured as described (Hattula *et al*, 2002; Sato *et al*, 2007), with minor modifications. Purified Rab8 (10 pmol) was preloaded with 100 pmol [^{3}H]GDP (57 MBq/ml; Perkin Elmer) in preloading buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM EDTA, 1 mg/ml BSA, and 1 mM DTT) for 20 min at 30°C. The concentration of $MgCl_2$ was then adjusted to 10 mM and the proteins were incubated for 45 min at 30°C. Reactions were started by adding 30 μl of [^{3}H]GDP-loaded Rab8 to an equal volume of preloading buffer containing 10 pmol of Rabin8(WT, S272A or S272E), 0.5 mM GDP, and 10 mM $MgCl_2$. Reaction mixtures were incubated at 10°C and aliquots (9 μl) were diluted into 2 ml of ice-cold stop buffer (20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT) and applied to wet PVDF membrane. The membranes were washed twice with 4 ml ice-cold stop buffer. After drying, 4 ml of the scintillation fluid (Ultima Gold, PerkinElmer) was added to the membranes and the amounts of [^{3}H]GDP remaining were measured in a scintillation counter.

Supplemental References

Hattula K, Furuhjelm J, Arffman A, Peränen J (2002) A Rab8-specific GDP/GTP exchange factor is involved in actin remodeling and polarized membrane transport.

Mol Biol Cell 13: 3268-3280

Sato Y, Shirakawa R, Horiuchi H, Dohmae N, Fukai S, Nureki O (2007) Asymmetric coiled-coil structure with guanine nucleotide exchange activity. Structure 15: 245-252

Supplemental Figure Legends

Figure S1. NDR2 phosphorylates Rabin8 at Ser-272. (**A**, **B**) Specificity of anti-Rabin8 antibody. In (**A**), GST-Rabin8(WT) or the S272A or S272E mutant was expressed in Sf-21 cells and cell lysates were analyzed by immunoblotting with anti-Rabin8 and anti-pRabin8 antibodies. In (**B**), YFP-Rabin8(WT) and the S272A mutant expressed in 293T cells were immunoprecipitated with anti-GFP antibody and incubated with λ-phosphatase (λppt). The precipitates were analyzed by immunoblotting with anti-GFP and anti-pRabin8 antibodies. (**C**) NDR2-mediated phosphorylation of Rabin8 at Ser-272. Myc-NDR2(WT) or the kinase-dead (KD) mutant was immunoprecipitated with anti-Myc antibody, subjected to *in vitro* kinase assays using GST-Rabin8(WT or S272A) as a substrate. Reaction mixtures were immunoblotted with anti-pRabin8, anti-GST, and anti-Myc antibodies. (**D**) Substrate specificity of NDR1. NDR1 was immunoprecipitated with anti-NDR1 antibody and subjected to *in vitro* kinase assays using 2 μg of histone H1 or GST-Rabin8 as a substrate.

Figure S2. Effect of NDR1 and NDR2 depletion on ciliogenesis. (**A**) Effect of NDR1/2 siRNAs on ciliogenesis. RPE1 cells transfected with indicated siRNAs were cultured for 48 h in growth medium and serum-starved for 48 h. Cells were fixed and stained for Ac-tubulin (red) and DNA (blue). Scale bar, 10 μm; insets magnification, x 2.7. (**B**) Dose-dependent effects of NDR1/2 siRNAs on ciliogenesis. RPE1 cells were transfected with 12.5-100 nM of control, NDR1 or NDR2 siRNA, cultured for 36 h in growth medium and serum-starved for 36 h. Data are means ± SEM (n = 3; > 150 cells per experiment). (**C**) Effect of double knockdown of NDR1 and NDR2 on ciliogenesis. RPE1 cells were exposed to the indicated concentrations (nM) of siRNAs and serum-starved as in (**B**). Data are means ± SEM (n = 3; > 100 cells per experiment). *, $p < 0.05$; ***, $p < 0.001$, by one-way ANOVA followed by Turkey test. (**D**) Subcellular localization of NDR kinases. RPE1 cells were transfected with Myc-NDR1 or Myc-NDR2, fixed and stained with a Myc antibody. Scale bar, 20 μm. (**E**) Subcellular localization of centrosomal proteins in NDR2-depleted cells. RPE1 cells were treated with control or NDR2 siRNA and serum-starved. Cells were fixed and costained with the indicated antibodies. Scale bar, 5 μm. (**F**) Ki-67 staining. RPE1 cells transfected with control or NDR2 siRNA were serum-starved as in (**A**) and stained for Ki-67 (red) and Ac-tubulin (green). DNA was stained with DAPI (blue). Scale bar, 10 μm.

Figure S3. Time-dependent changes in the number of cells with Rabin8 localization on the pericentrosome (black bar) and the number of ciliated cells after serum starvation

(open circle). RPE1 cells stably expressing YFP-Rabin8(WT) (**A**) or YFP-Rabin8(S272A) (**B**) were serum-starved. At the indicated times post serum withdrawal (p.s.w.), cells were fixed and imaged as shown in Figure 3A. At least 100 cells were counted at each time point.

Figure S4. Characterization of the interaction between Rabin8 and Rab11. (**A**) Rabin8 binds to the GTP-bound form of Rab11. Lysates of cells expressing GFP-Rab11 (WT, QL or SN) were subjected to GST pull-down assays using GST-Rabin8(WT). (**B**) Rabin8 colocalizes with Rab11(WT) and Rab11(QL), but not Rab11(SN). RPE1 cells were transfected with Flag-Rabin8(WT) and GFP-Rab11(WT, QL or SN), cultured for 48 h, and serum-starved for 24 h. Cells were fixed and imaged by GFP fluorescence (green) and staining for Flag (red). Bar, 10 μm. (**C**) Rab11-GTP binds to Rabin8 and the S272A and S272E mutants to a similar extent. Lysates of cells expressing GFP-Rab11(QL) were subjected to pull-down assays using GST or GST-Rabin8(WT, S272A or S272E). (**D**) Effect of C-terminal geranylgeranyl modifications of Rab11 on the binding to Rabin8. GFP-Rab11(QL, 2CA) was constructed from GFP-Rab11(QL) by replacing Cys-212 and Cys-213 by alanine. The post-nuclear supernatants of cells expressing Rab11(QL) or Rab11 (QL, 2CA) were incubated with Flag-Rabin8 and immunoprecipitated with an anti-Flag antibody. The precipitates were analyzed by immunoblotting with anti-Rab11 and anti-Rabin8 antibodies.

Figure S5. Rabin8 binds to PS in phospholipid-cosedimentation assays. (**A**) Dose-dependent increase in Rabin8 binding to PS multilamellar vesicles. Purified Rabin8 was incubated with multilamellar vesicles composed of different molar ratios (0:100 to 50:50 molar ratio) of PS/PC. After centrifugation, pellets were analyzed by Coomassie Brilliant Blue (CBB) staining. Quantitative analysis is shown in the right (n = 3, error bars are mean ± SD). (**B**) Rabin8, but not Sec2p, binds to PS. Purified GST-Sec2 and GST-Rabin8 were incubated with different amounts of PS/PC multilamellar vesicles (15:85 molar ratio) and analyzed as in (**A**). (**C**, **D**) Mapping of the PS-binding region of Rabin8. Purified Rabin8 deletion mutants were incubated with multilamellar vesicles composed of PC (100%) or PS/PC (15:85 molar ratio) and analyzed as in (**A**).

Figure S6. Localization of Rabin8 mutants, Rab11(QL) and PS. RPE1 cells were cotransfected with YFP-Rabin8(WT) or the indicated mutants, CFP-Rab11(QL) and mCherry-evt2(PH), cultured for 36 h, and analyzed by YFP (green), CFP (blue) and mCherry (red) fluorescence. Arrowheads indicate the accumulation of Δ3 and ΔN2 on the centrosome. Scale bar, 20 μm. Magnified images are shown in the four right panels. Scale

bar in the magnified images, 5 μm.

Figure S7. Effect of Rabin8 mutations on the Rab8-GEF activity. (**A**) Purification of Rabin8(WT, S272A and S272E) and Rab8. Proteins were expressed using a baculovirus expression system and purified from Sf-21 insect cells. Proteins were visualized by CBB staining. (**B**) Ser-272 mutations do not affect the Rab8-GEF activity of Rabin8. Purified Rab8 was loaded with [^{3}H]GDP and incubated with Rabin8(WT) or the S272A or S272E mutant. Aliquots of each reaction were taken at different time points and filtered on PVDF membranes. The amounts of [^{3}H]GDP on the membrane were measured by a scintillation counter (n = 3, error bars are mean ± SD).

Figure S8. A proposed model for the role of NDR2-mediated Rabin8 phosphorylation in ciliary membrane formation. Molecular interactions and the sequence of events occuring in the early phase of ciliogenesis are schematically depicted. See details in Discussion.

Supplemental Movie Legend

Movie S1. Time-lapse fluorescence imaging of YFP-NDR1 and mCherry-centrin2 in serum-starved RPE1 cells. Scale bar: 2 μm.

Movie S2. Time-lapse fluorescence imaging of YFP-NDR2 and mCherry-centrin2 in serum-starved RPE1 cells. Scale bar: 2 μm.

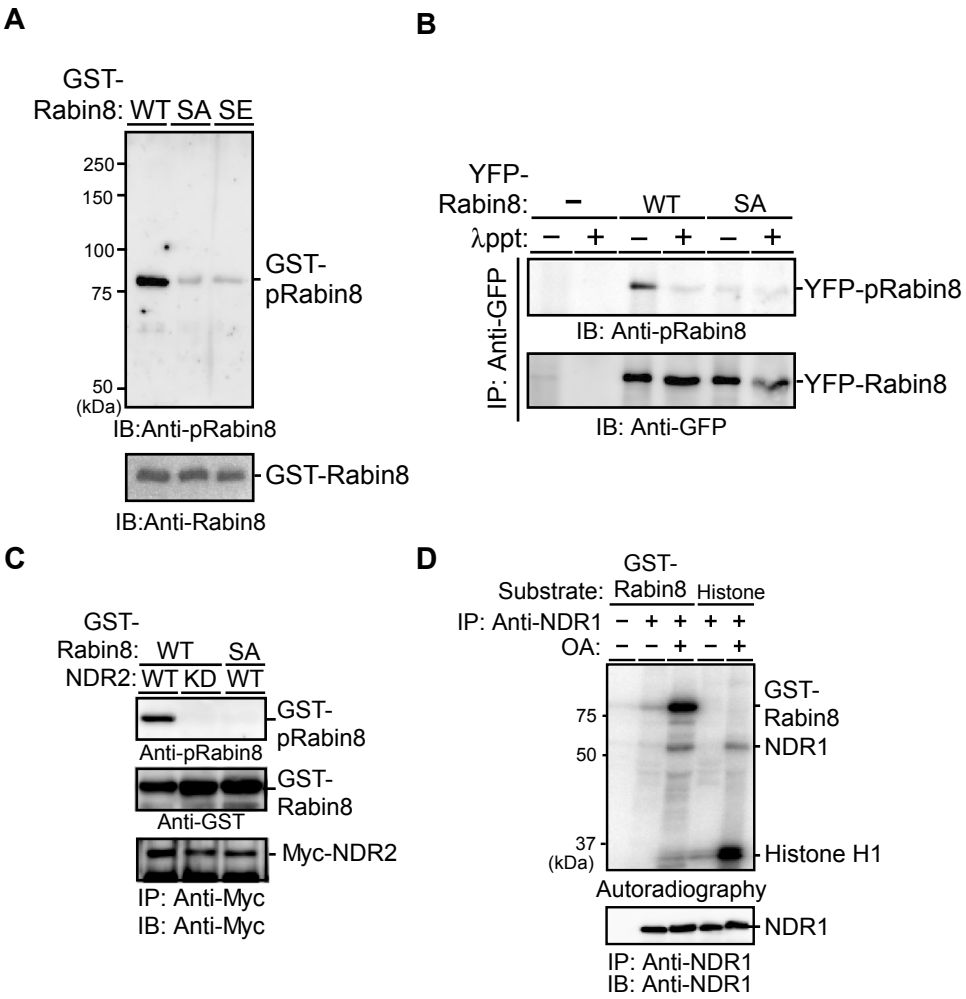
A
GST-
Rabin8: WT SA SE
250
150
100
75
50
(kDa)
GST-
pRabin8
IB:Anti-pRabin8
GST-Rabin8
IB:Anti-Rabin8
B
YFP-
Rabin8: – WT SA
λppt: – + – + – +
IP: Anti-GFP
YFP-pRabin8
IB: Anti-pRabin8
YFP-Rabin8
IB: Anti-GFP
C
GST-
Rabin8: WT SA
NDR2: WT KD WT
GST-
pRabin8
Anti-pRabin8
GST-
Rabin8
Anti-GST
Myc-NDR2
IP: Anti-Myc
IB: Anti-Myc
D
GST-
Substrate: Rabin8 Histone
IP: Anti-NDR1 – + + + +
OA: – – + – +
75
50
37
(kDa)
GST-
Rabin8
NDR1
Histone H1
Autoradiography
NDR1
IP: Anti-NDR1
IB: Anti-NDR1

Figure S1

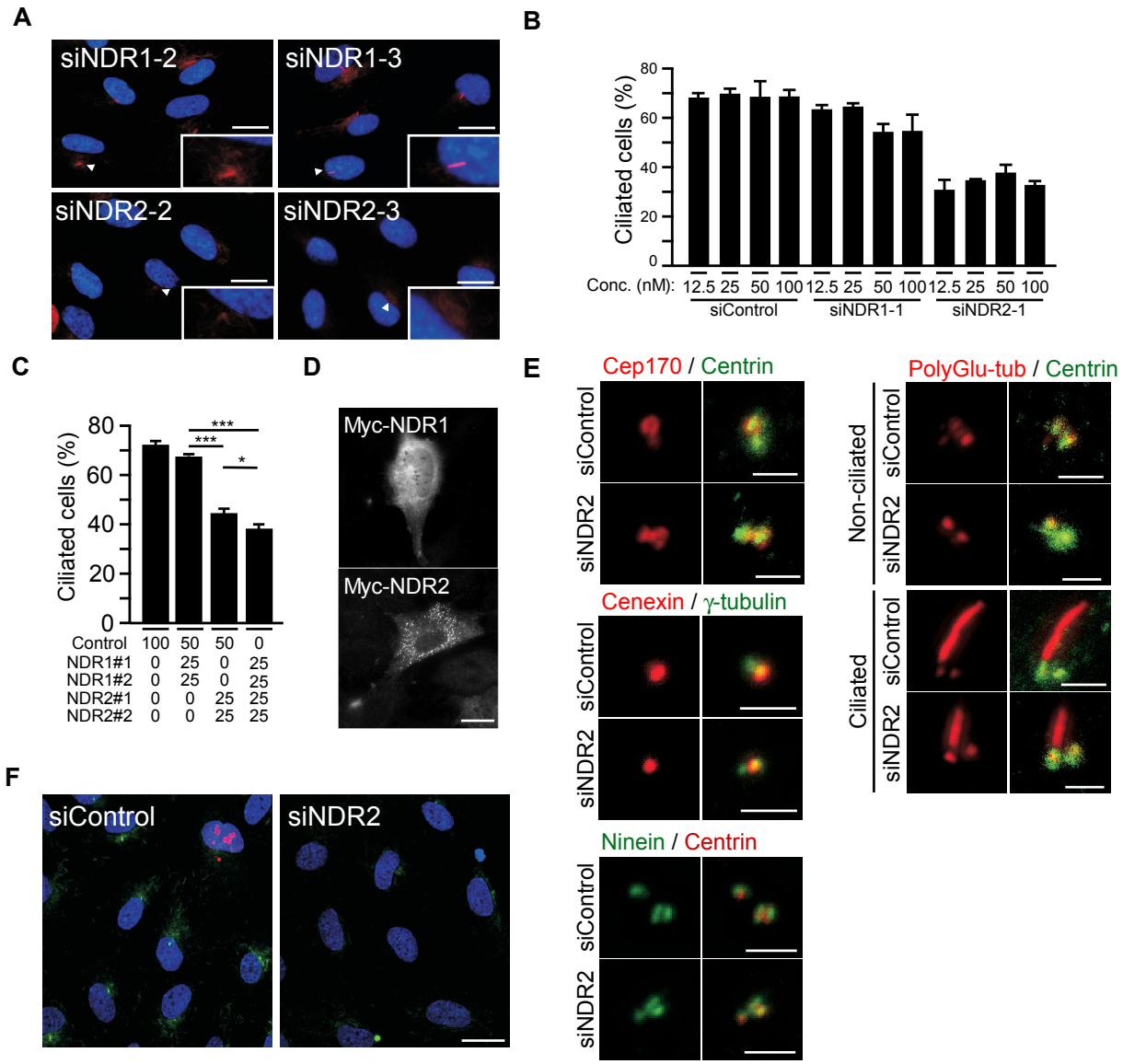
A
siNDR1-2
siNDR1-3
siNDR2-2
siNDR2-3
B
Ciliated cells (%)
80
60
40
20
0
Conc. (nM): 12.5 25 50 100 12.5 25 50 100 12.5 25 50 100
siControl
siNDR1-1
siNDR2-1
C
Ciliated cells (%)
80
60
40
20
0
***
***
*
Control 100 50 50 0
NDR1#1 0 25 0 25
NDR1#2 0 25 0 25
NDR2#1 0 0 25 25
NDR2#2 0 0 25 25
D
Myc-NDR1
Myc-NDR2
E
Cep170 / Centrin
siControl
siNDR2
PolyGlu-tub / Centrin
Non-ciliated
Ciliated
Cenexin / γ-tubulin
Ninein / Centrin
F
siControl
siNDR2

Figure S2

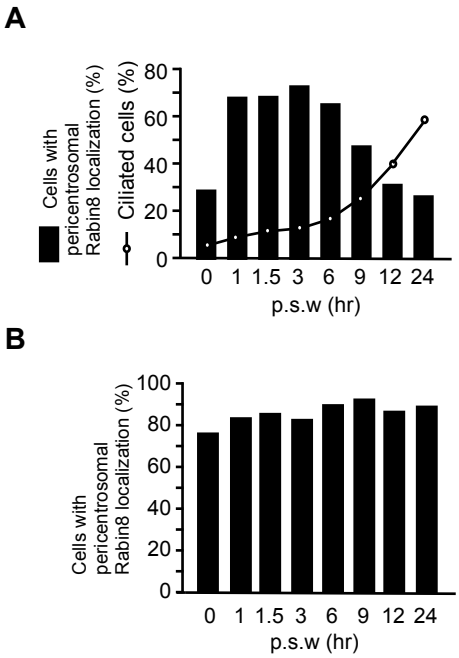
A
Cells with pericentrosomal Rabin8 localization (%)
Ciliated cells (%)
80
60
40
20
0
0 1 1.5 3 6 9 12 24
p.s.w (hr)
B
Cells with pericentrosomal Rabin8 localization (%)
100
80
60
40
20
0
0 1 1.5 3 6 9 12 24
p.s.w (hr)

Figure S3

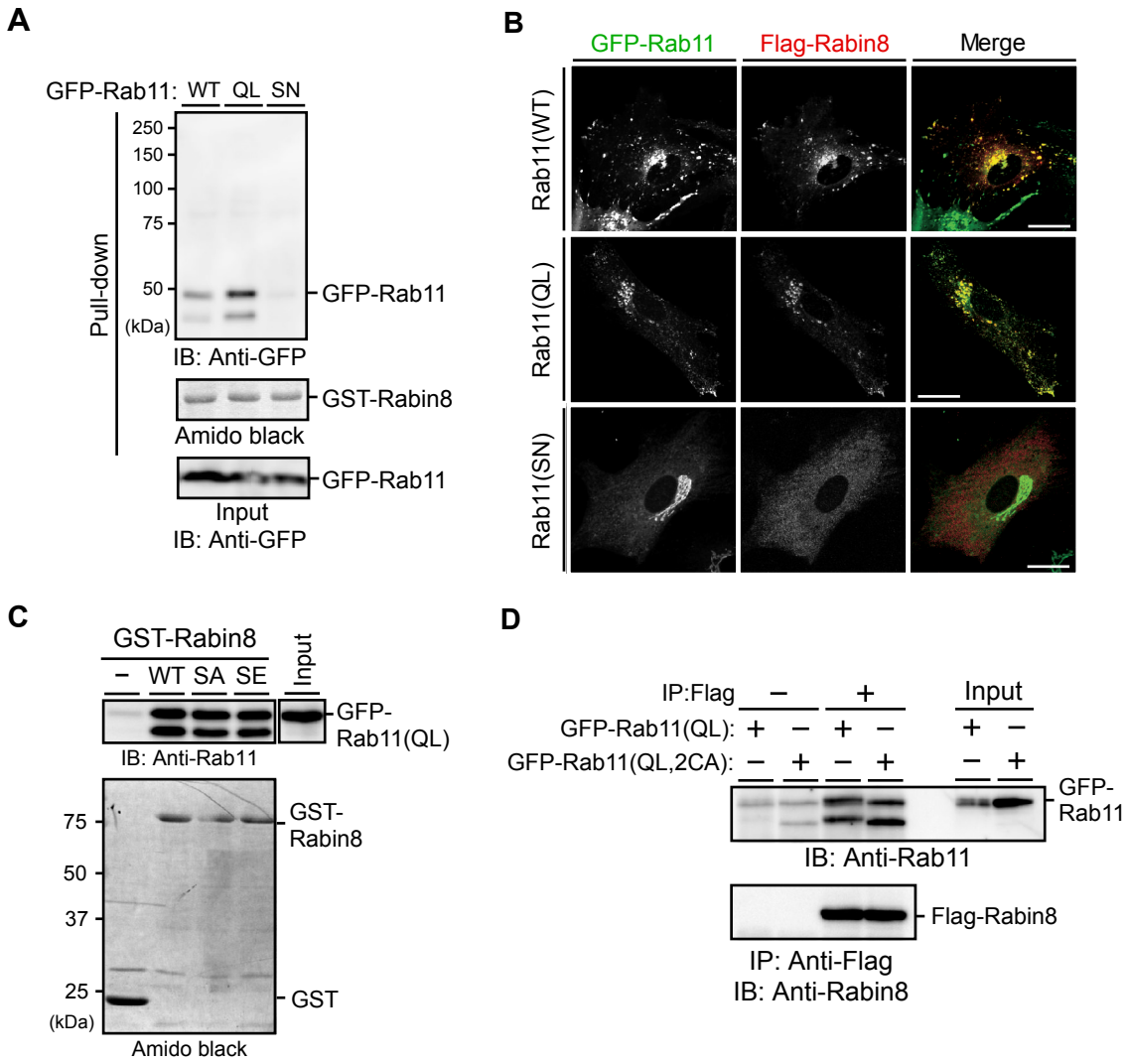
A
GFP-Rab11: WT QL SN
250
150
100
75
50
(kDa)
Pull-down
GFP-Rab11
IB: Anti-GFP
GST-Rabin8
Amido black
GFP-Rab11
Input
IB: Anti-GFP
B
GFP-Rab11
Flag-Rabin8
Merge
Rab11(WT)
Rab11(QL)
Rab11(SN)
C
GST-Rabin8
– WT SA SE
Input
GFP-
Rab11(QL)
IB: Anti-Rab11
75
50
37
25
(kDa)
GST-
Rabin8
GST
Amido black
D
IP:Flag – + Input
GFP-Rab11(QL): + – + – + –
GFP-Rab11(QL,2CA): – + – + – +
GFP-
Rab11
IB: Anti-Rab11
Flag-Rabin8
IP: Anti-Flag
IB: Anti-Rabin8

Figure S4

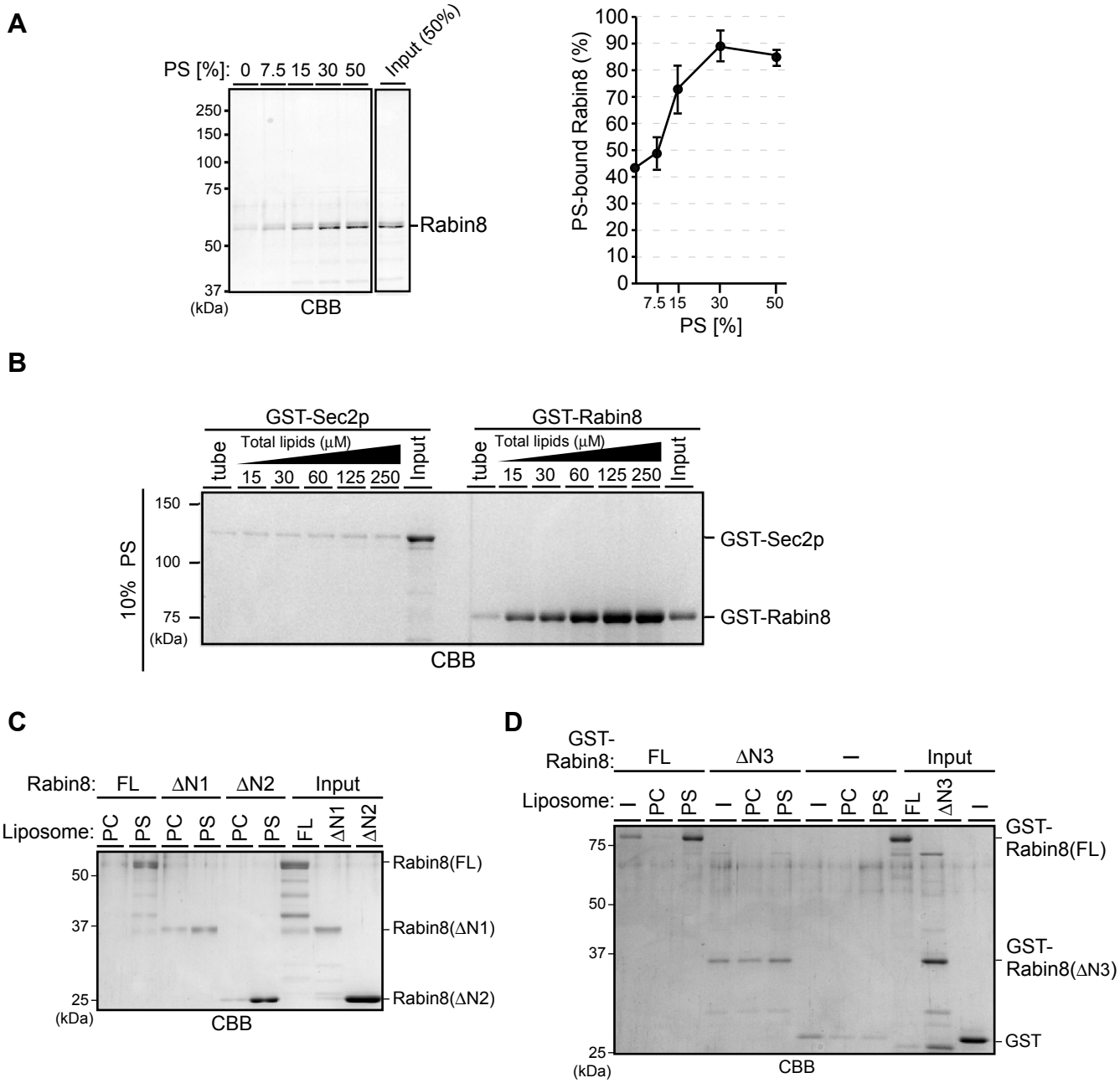
A
PS [%]: 0 7.5 15 30 50
Input (50%)
250
150
100
75
50
37
(kDa)
Rabin8
CBB
PS-bound Rabin8 (%)
100
90
80
70
60
50
40
30
20
10
0
7.5 15 30 50
PS [%]
B
GST-Sec2p
GST-Rabin8
Total lipids (μM)
tube
15 30 60 125 250
Input
10% PS
150
100
75
(kDa)
GST-Sec2p
GST-Rabin8
CBB
C
Rabin8: FL ΔN1 ΔN2 Input
Liposome: PC PS PC PS PC PS FL ΔN1 ΔN2
50
37
25
(kDa)
Rabin8(FL)
Rabin8(ΔN1)
Rabin8(ΔN2)
CBB
D
GST-Rabin8: FL ΔN3 — Input
Liposome: — PC PS — PC PS — PC PS FL ΔN3 —
75
50
37
25
(kDa)
GST-Rabin8(FL)
GST-Rabin8(ΔN3)
GST
CBB

Figure S5

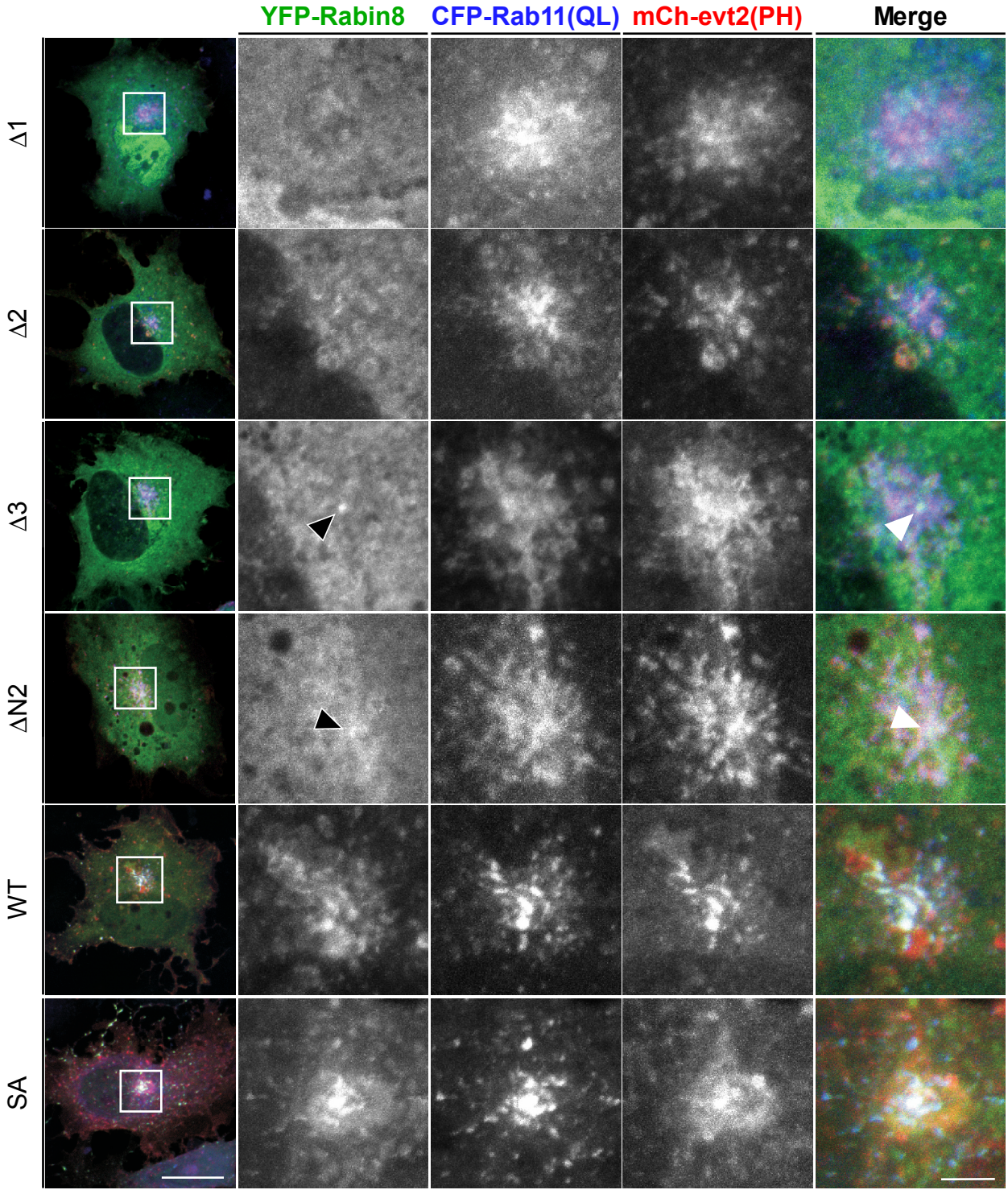

Figure S6

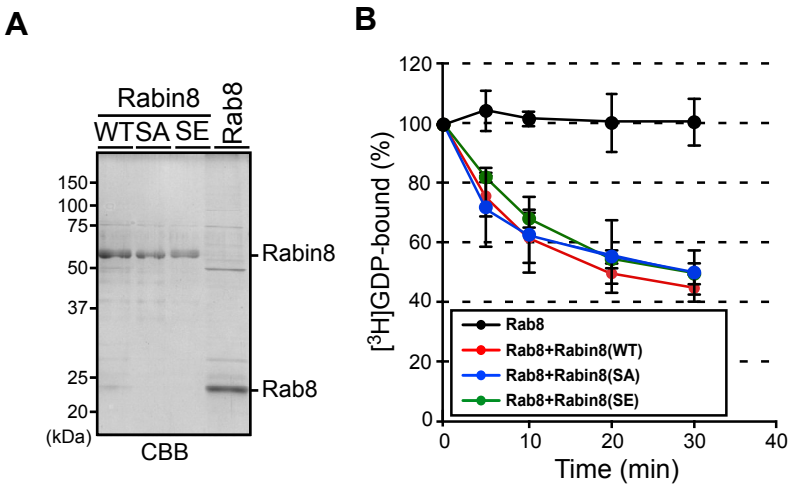
A
Rabin8
WT
SA
SE
Rab8
150
100
75
50
37
25
20
(kDa)
Rabin8
Rab8
CBB
B
120
100
80
60
40
20
0
[3H]GDP-bound (%)
0
10
20
30
40
Time (min)
Rab8
Rab8+Rabin8(WT)
Rab8+Rabin8(SA)
Rab8+Rabin8(SE)

Figure S7

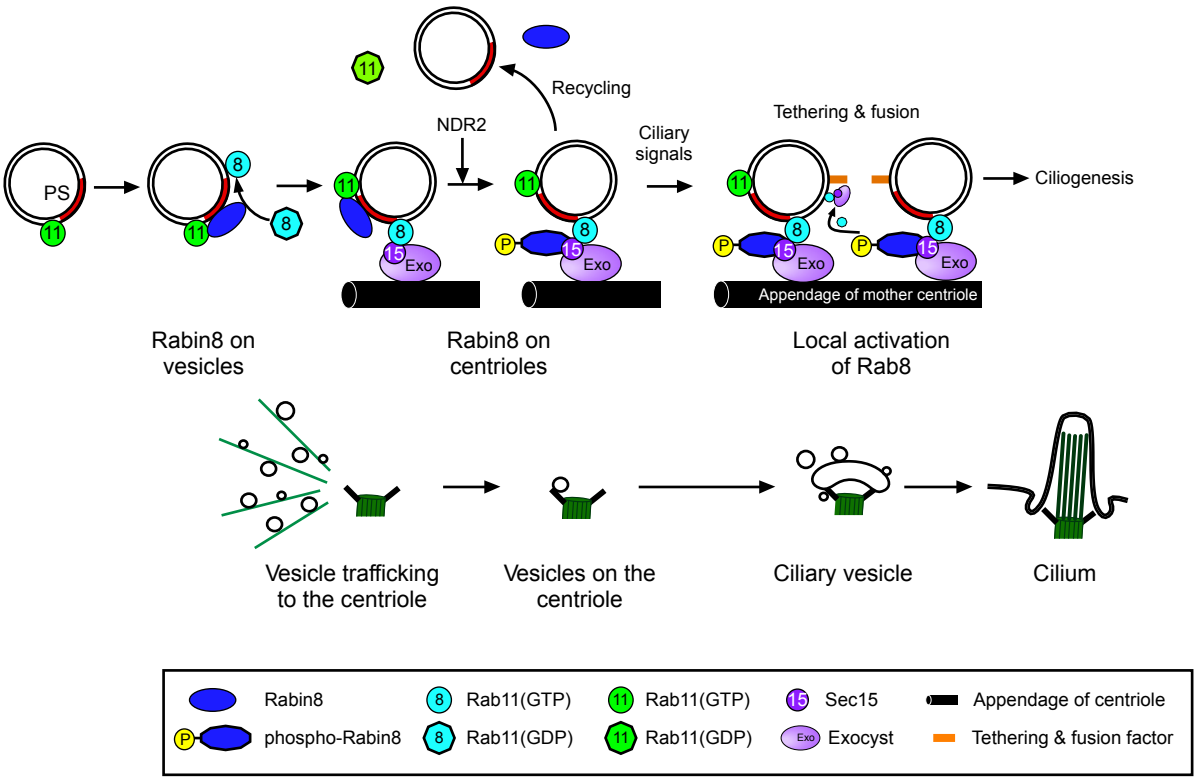
PS
Recycling
NDR2
Ciliary signals
Tethering & fusion
Ciliogenesis
Exo
Appendage of mother centriole
Rabin8 on vesicles
Rabin8 on centrioles
Local activation of Rab8
Vesicle trafficking to the centriole
Vesicles on the centriole
Ciliary vesicle
Cilium
Rabin8
phospho-Rabin8
Rab11(GTP)
Rab11(GDP)
Rab11(GTP)
Rab11(GDP)
Sec15
Exocyst
Appendage of centriole
Tethering & fusion factor

Figure S8