Supplemental Materials

Molecular Biology of the Cell

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Supplemental figure legends

Figure S1. Arf6 acts downstream of Rab35 and ACAP2. (A, C) Cells were transfected with an siRNA for control, Rab35 (A), or ACAP2 (C), allowed to differentiate for three days, and lysed to be used for an affinity-precipitation of active GTP-bound Arf6. Immunoblots for each total protein are also shown. (B, D) Bands corresponding to each GTP-bound Arf6 protein were scanned and semi-quantified (n=4). Data were evaluated with the Student's *t* test (*, $p<0.01$).

Figure S2. Rab35/ACAP2/Arf6 is required for oligodendrocyte differentiation. (A) Primary OPCs infected with an shRNA for control, Rab35, ACAP2, or Arf6 were allowed to differentiate for three days and stained with an anti-MBP antibody (red) and DAPI (blue). Representative images are shown. The scale bar indicates 100 μm. (B) The percentages of the MBP-positive cells were counted (n=10 fields in three experiments). Data were evaluated with one-way ANOVA (*, $p<0.01$). (C-E) Cells were transfected with an shRNA for control, Rab35, ACAP2, or Arf6 and immunoblotted with an antibody against Rab35, ACAP2, Arf6, MBP, or β-actin.

Figure S3. Analysis of Rab35/ACAP2/Arf6 in spinal cord development. (A) Postnatal mouse spinal cords were lysed to be used in an affinity-precipitation to detect active GTP-bound Rab35 or Arf6. Immunoblotts for total Rab35, ACAP2, Arf6, cytohesin-2, and MBP are also shown.

Figure S4. Cytohesin is required for FBD-102b cell differentiation. (A) Cells were allowed to differentiate with or without 10 μM SecinH3 for three days. Representative images are shown. The scale bar indicates 100 μm. (B) The percentage of cells in stage 3 was counted (n=10 fields in three experiments). (C) Cells were lysed and immunoblotted with an antibody against MBP or β-actin. (D) The bands of each MBP protein were semi-quantified (n=4). Data were evaluated with the Student's *t* test (*, $p<0.01$). (E) Immunoblotting for cytohesin members in FBD-102 cells and 293T (positive control) were performed. Immunoblots for β-actin are also shown.

Figure S5. Cytohesin-2 is activated following differentiation in FBD-102b cells. (A) Cells were allowed to differentiate for 0-3 days and lysed to be used for an affinity-precipitation to detect active cytohesin-2 with recombinant GST-tagged nucleotide-free Arf6. Immunoblots for total cytohesin-2 and β-actin are also shown. (B) Bands corresponding to each active cytohesin-2 protein were scanned and semi-quantified (n=3). Data were evaluated with one-way ANOVA (*, $p<0.01$, **, $p<0.05$).

Figure S6. Cytohesin-2 is required for oligodendrocyte differentiation. (A) Primary OPCs were allowed to differentiate with or without 10 μM SecinH3 for three days and stained with an anti-MBP antibody (red) and DAPI (blue). Representative images are shown. The scale bar indicates 100 μm. (B) The percentages of the MBP-positive cells were counted (n=10 fields in three experiments). (C) Cells were lysed and immunoblotted with an

antibody against MBP or β-actin. Data were evaluated with the Student's *t* test (*, $p<0.01$).

Figure S7. Rab35 forms a complex with ACAP2/Arf6/cytohesin-2. (A) FBD-102b cells were allowed to differentiate for 0 or 3 days, lysed, and immunoprecipitated with an anti-cytohesin-2 antibody. Immunoprecipitates were immunoblotted with an anti-Rab35, ACAP2, or Arf6 antibody. Immunoblotts for total Rab35, ACAP2, Arf6, and cytohesin-2 are also shown.

Figure S1

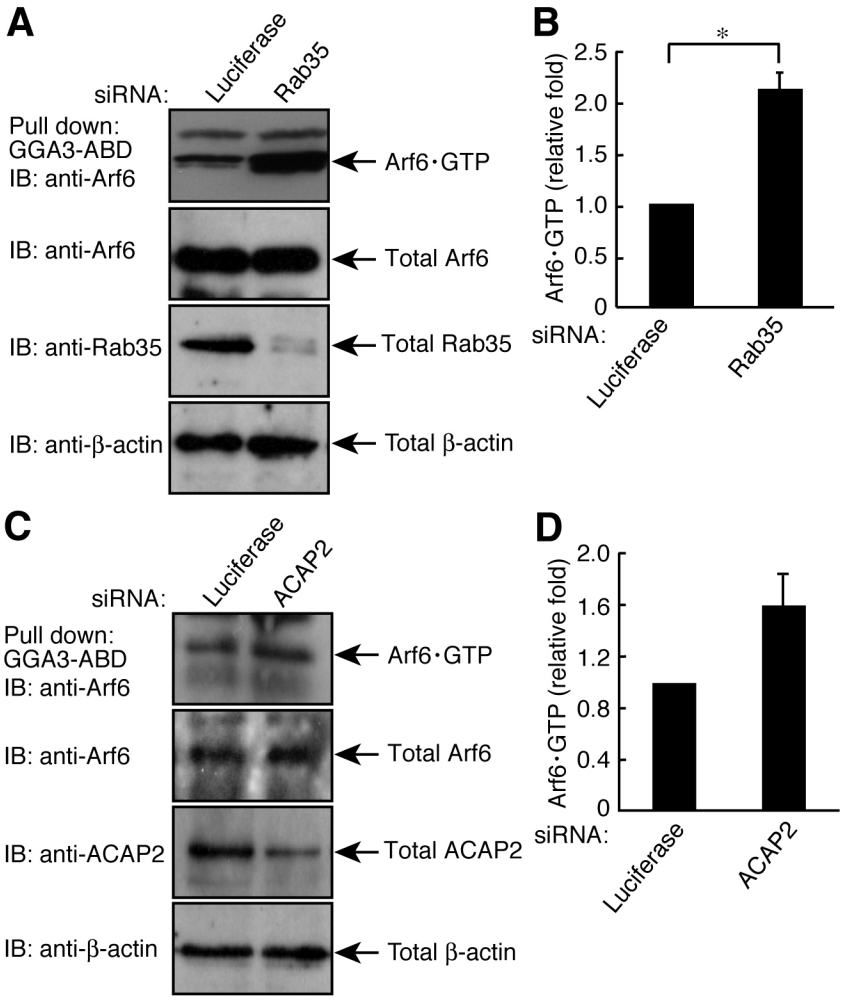

A
siRNA:
Luciferase
Rab35
Pull down:
GGA3-ABD
IB: anti-Arf6
Arf6・GTP
IB: anti-Arf6
Total Arf6
IB: anti-Rab35
Total Rab35
IB: anti-β-actin
Total β-actin
B
*
Arf6・GTP (relative fold)
2.5
2.0
1.5
1.0
0.5
0
siRNA:
Luciferase
Rab35
C
siRNA:
Luciferase
ACAP2
Pull down:
GGA3-ABD
IB: anti-Arf6
Arf6・GTP
IB: anti-Arf6
Total Arf6
IB: anti-ACAP2
Total ACAP2
IB: anti-β-actin
Total β-actin
D
Arf6・GTP (relative fold)
2.0
1.6
1.2
0.8
0.4
0
siRNA:
Luciferase
ACAP2

Figure S2

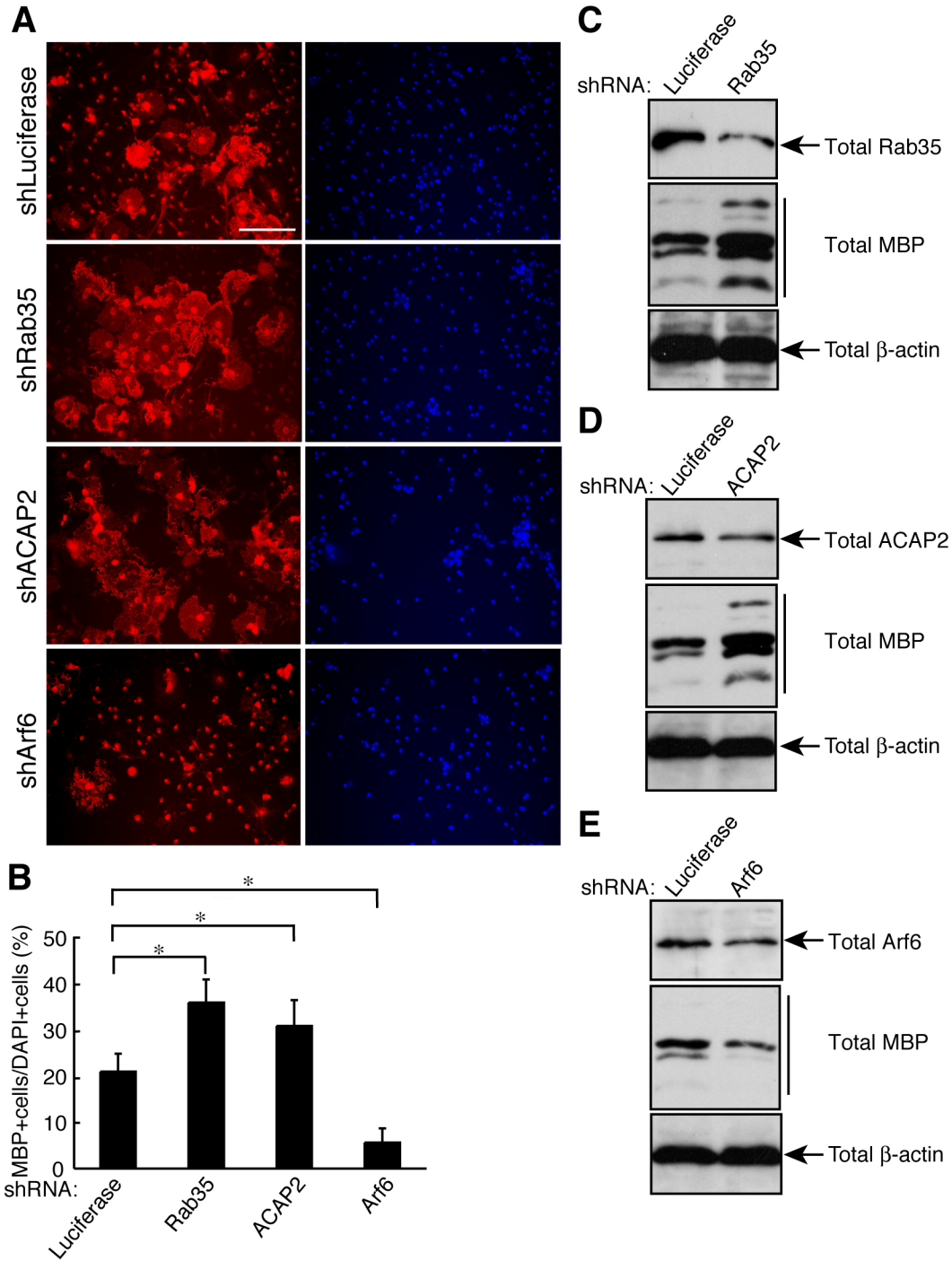

A
shLuciferase
shRab35
shACAP2
shArf6
B
MBP+cells/DAPI+cells (%)
0
10
20
30
40
50
*
*
*
shRNA:
Luciferase
Rab35
ACAP2
Arf6
C
shRNA:
Luciferase
Rab35
Total Rab35
Total MBP
Total β-actin
D
shRNA:
Luciferase
ACAP2
Total ACAP2
Total MBP
Total β-actin
E
shRNA:
Luciferase
Arf6
Total Arf6
Total MBP
Total β-actin

Figure S3

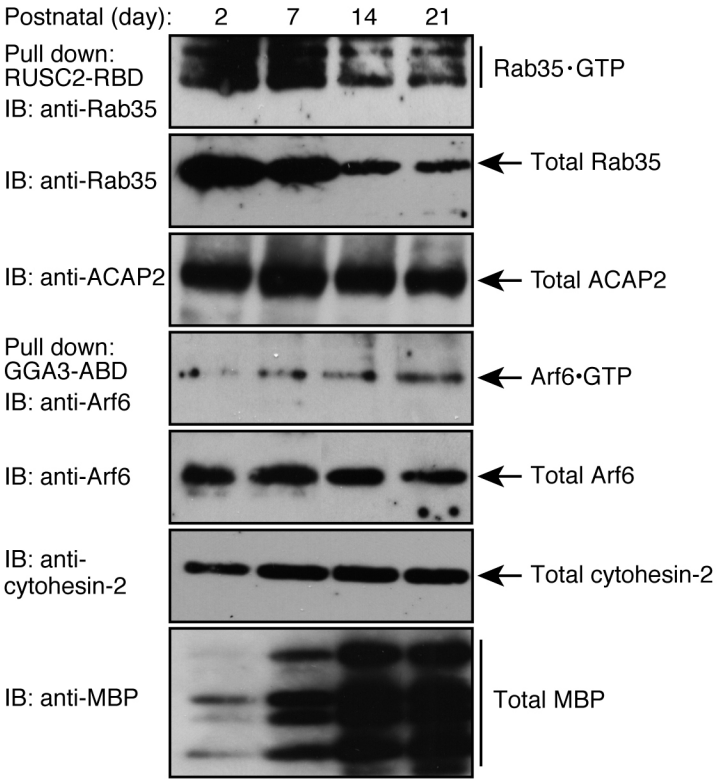

Postnatal (day): 2 7 14 21
Pull down: RUSC2-RBD
IB: anti-Rab35
Rab35·GTP
IB: anti-Rab35
Total Rab35
IB: anti-ACAP2
Total ACAP2
Pull down: GGA3-ABD
IB: anti-Arf6
Arf6•GTP
IB: anti-Arf6
Total Arf6
IB: anti-cytohesin-2
Total cytohesin-2
IB: anti-MBP
Total MBP

Figure S4

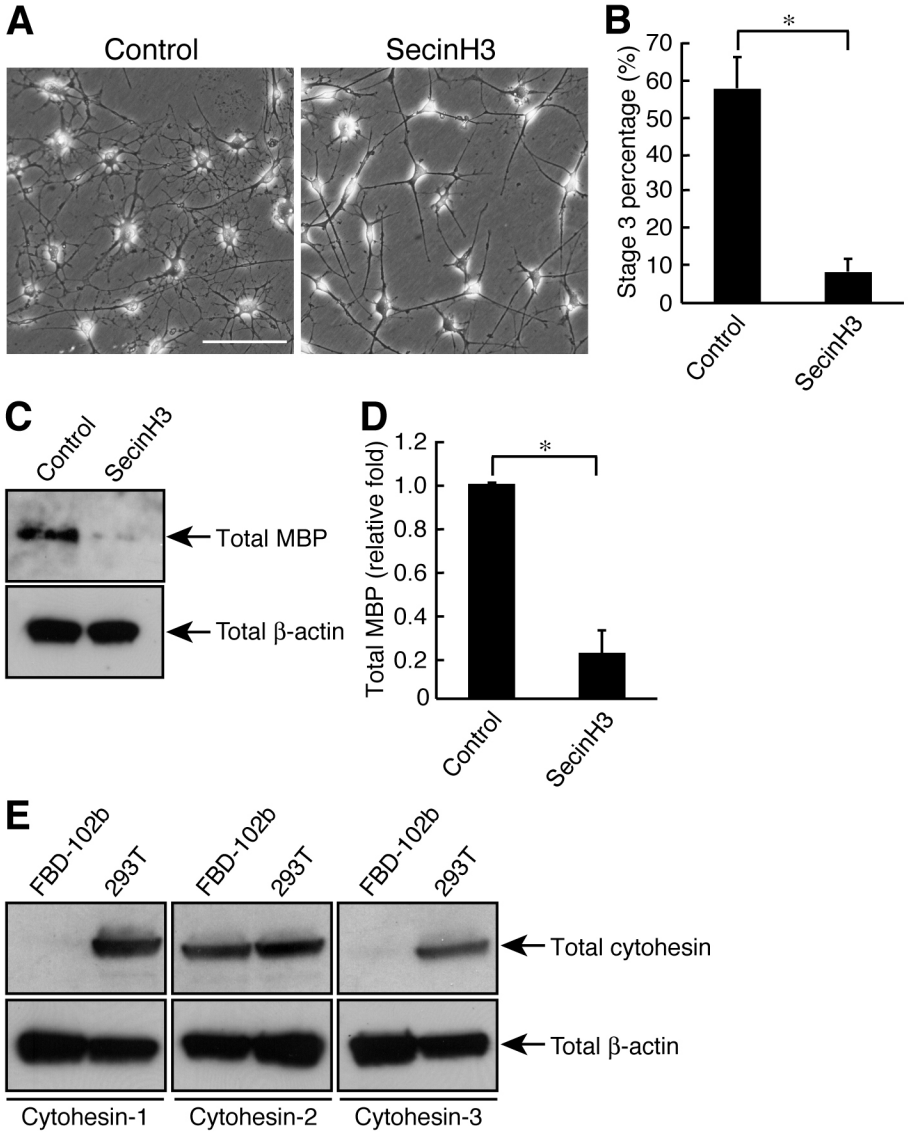

A
Control
SecinH3
B
Stage 3 percentage (%)
70
60
50
40
30
20
10
0
*
Control
SecinH3
C
Control
SecinH3
Total MBP
Total β-actin
D
Total MBP (relative fold)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Control
SecinH3
E
FBD-102b
293T
FBD-102b
293T
FBD-102b
293T
Total cytohesin
Total β-actin
Cytohesin-1
Cytohesin-2
Cytohesin-3

Figure S5

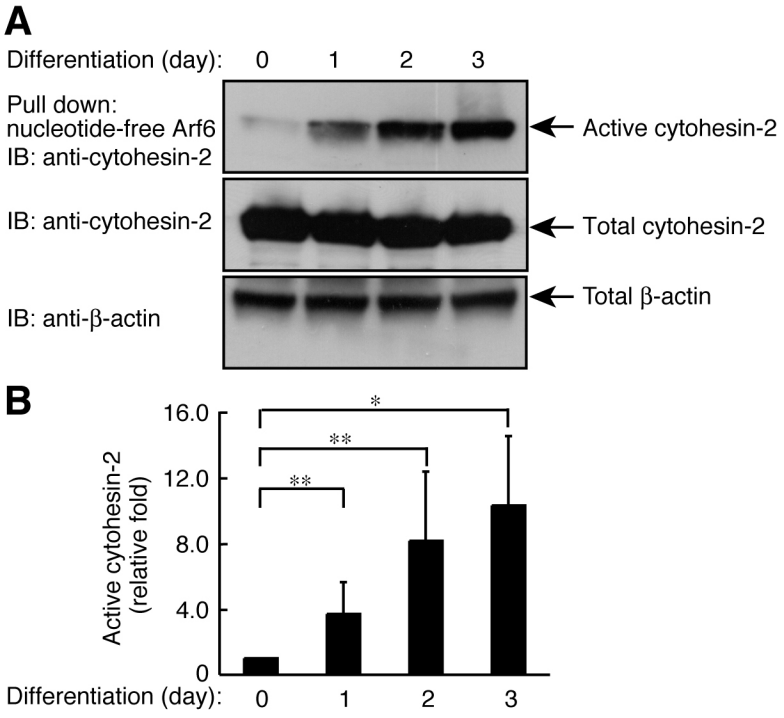

A
Differentiation (day): 0 1 2 3
Pull down:
nucleotide-free Arf6
IB: anti-cytohesin-2
Active cytohesin-2
IB: anti-cytohesin-2
Total cytohesin-2
IB: anti-β-actin
Total β-actin
B
Active cytohesin-2
(relative fold)
16.0
12.0
8.0
4.0
0
*
**
**
Differentiation (day): 0 1 2 3

Figure S6

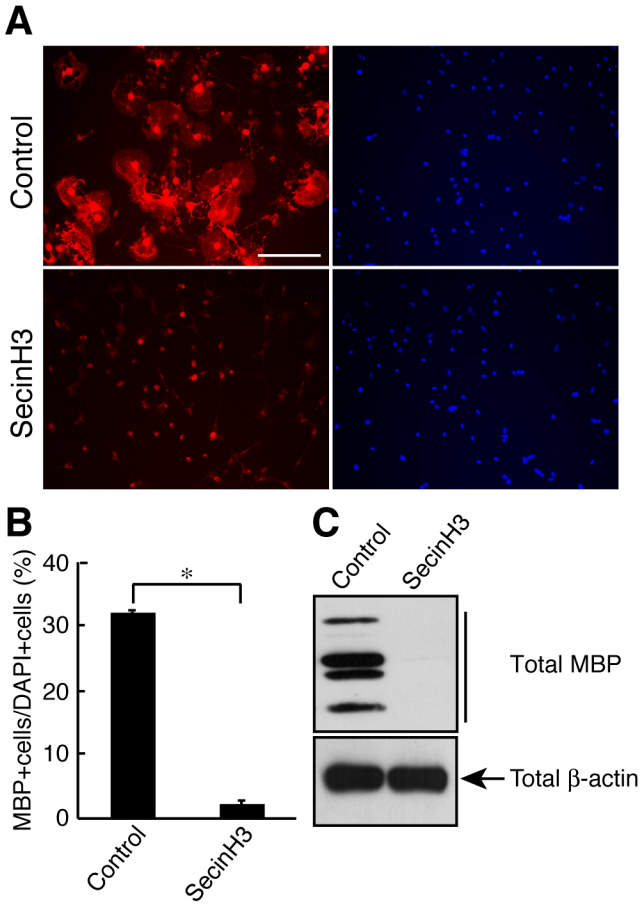
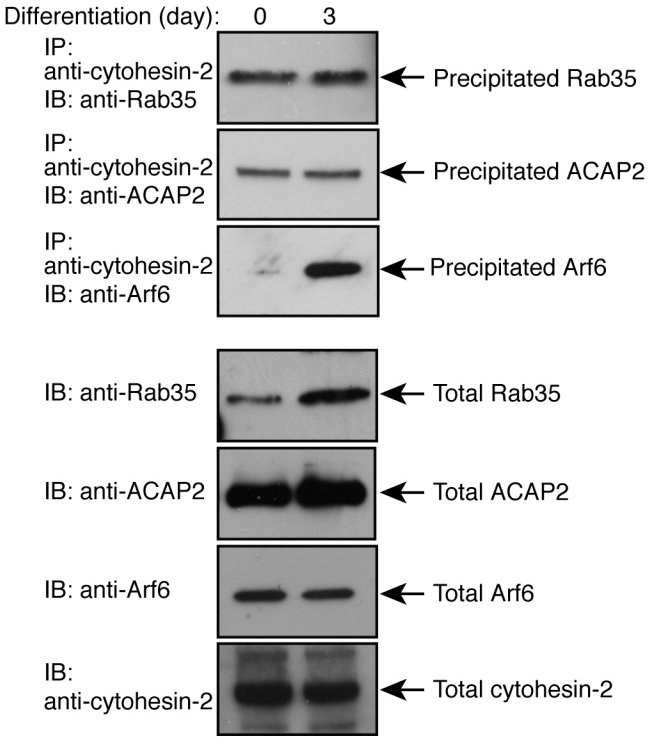

A
Control
SecinH3
B
MBP+cells/DAPI+cells (%)
40
30
20
10
0
*
Control
SecinH3
C
Control
SecinH3
Total MBP
Total β-actin

Figure S7


Differentiation (day): 0 3
IP: anti-cytohesin-2 IB: anti-Rab35
Precipitated Rab35
IP: anti-cytohesin-2 IB: anti-ACAP2
Precipitated ACAP2
IP: anti-cytohesin-2 IB: anti-Arf6
Precipitated Arf6
IB: anti-Rab35
Total Rab35
IB: anti-ACAP2
Total ACAP2
IB: anti-Arf6
Total Arf6
IB: anti-cytohesin-2
Total cytohesin-2