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Title	Characterization of intracellular dynamics of inoculated PrP-res and newly generated PrPSc during early stage prion infection in Neuro2a cells
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Supplemental materials

Construction of expression plasmids encoding EGFP-Rab GTPase fusion proteins.

Expression plasmids encoding EGFP-tagged Rab GTPases were prepared as described elsewhere with some modifications (1). Template cDNA was synthesized with a First Strand cDNA Synthesis Kit (GE Healthcare) using total RNA extracted from N2a-3 cells with TRIzol Reagent (Life Thechnologies). The cDNA fragments, which included the entire open reading frame of each of the following wild-type mouse genes (Rab4a, Rab5a, Rab7, Rab9, Rab11a and Rab22a), were amplified from the template cDNAs using the corresponding pair of oligonucleotide primers (Table S2) in Table S2. The PCR products were cloned into pCRII-topo with TA Cloning Kit (Invitrogen). The cDNA fragments of a GTP-binding deficient dominant-negative mutant of each of six Rab GTPases, Rab4aS22N, Rab5aS34N, Rab7T22N, Rab9S21N, Rab11aS25N and Rab22aS19N (2-7), were produced by site-directed mutagenesis by overlap extension PCR (8) using mutagenic oligonucleotide primers (Table S3). The resulting PCR products were cloned into pCR-BluntII-topo with Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Thereafter, the cloned cDNA fragments encoding the wild-type Rab GTPase or a dominant-negative mutant were excised by digestion with *Xho* I and then cloned into the *Xho* I site of pEGFP-C1.

Purification of PrP-res

Brains of Jcl:ICR mice infected with the Obihiro or 22L prion strain (about 2 g) were used as starting materials. The brains were homogenized in citrate-buffered saline (CBS, 137 mM NaCl and 20 mM citrate, pH 6.0). Brij-96V (Fluka) was added to the brain homogenate to a

final concentration of 0.5%, and the homogenate was incubated for 30 min at 4˚C. Each sample was then adjusted to 26% OptiPrep (Axis-shield) in CBS and stepwise OptiPrep gradients were prepared by overlaying 23% and 8% OptiPrep in CBS, and CBS in an ultra-centrifugation tube (Beckman). The stepwise OptiPrep gradients were centrifuged at 18,000 rpm for 2 h at 4˚C using a SW32Ti rotor (Beckman). The lipid band at the 8%-23% interface was collected and mixed with 1/2 volume of 5 M NaCl, 0.15 M Tris-HCl (pH 8.0). The sample was adjusted to 26% OptiPrep and stepwise OptiPrep gradients were prepared by overlaying 23% and 8% OptiPrep (in PBS), and PBS in an ultra-centrifugation tube. The gradient was centrifuged at 18,000 rpm for 2 h at 20˚C using a SW32Ti rotor. The lipid band at the 8%-23% interface was collected and adjusted to 2% sarkosyl and incubated with 25 units/ml Benzonase (Novagen) for 30 min at 37˚C. Each sample was then digested with 10 μg/ml of PK for 1 h at 37˚C, and the PK digestion was terminated by incubation with 100 μM Pefabloc SC (Roche) for 15 min at 4˚C. Each sample was adjusted to 30 mM EDTA, and thereafter, 1/1.8 volume of 5 M NaCl solution was mixed in to the sample. The sample was overlaid onto a sucrose pad solution [1 M sucrose, 0.1 M NaCl, 0.5% Zwittergent 3-14 (Calbiochem), and 10 mM Tris-HCl (pH 7.5)] in an ultracentrifuge tube and centrifuged at 32,000 rpm for 2 h at 20˚C using a SW32Ti rotor. The pellet was washed twice with 0.5% Zwittergent 3-14 in PBS followed by centrifugation at 64,000 rpm for 30 min at 20˚C using a S80AT3 rotor (Hitachi). The final pellet was resuspended in PBS containing 0.5% Zwittergent 3-14.

Co-localization statistics

Quantitative co-localization analysis was performed as described elsewhere (9). The

co-localization ratio of inoculated PrP-res with each organelle marker that represents a percentage of weighted co-localization coefficient was quantified using ZEN2009 software. Briefly, the weighted co-localization coefficient was calculated using the following formula;

$$\frac{\sum_i PrPres_{i,\,coloc}}{\sum_i PrPres_{i,\,total}}$$

where $PrPres_{i,colocl}$ represents the intensity of a pixel of inoculated PrP-res signal co-localized with an organelle marker, whereas, $PrPres_{i,total}$ represents the intensity of a pixel of inoculated PrP-res signal regardless of whether it co-localized with an organelle marker or not.

Because the inoculated PrP-res was labeled with Alexa Fluor 555, signals acquired from Alexa Fluor 555 represent the inoculated PrP-res. Signals obtained from indirect immuno-staining with mAb132 followed by Alexa Fluor 488-conjugated secondary antibodies included signals both from inoculated PrP-res and newly generated PrP^{Sc}. Therefore, in the case of PrP^{Sc}-specific staining using mAb 132, signals of newly generated PrP^{Sc} were determined by subtracting the intensity of inoculated PrP-res signal from the intensity of the signal from PrP^{Sc} in the same pixels according to the following formula:

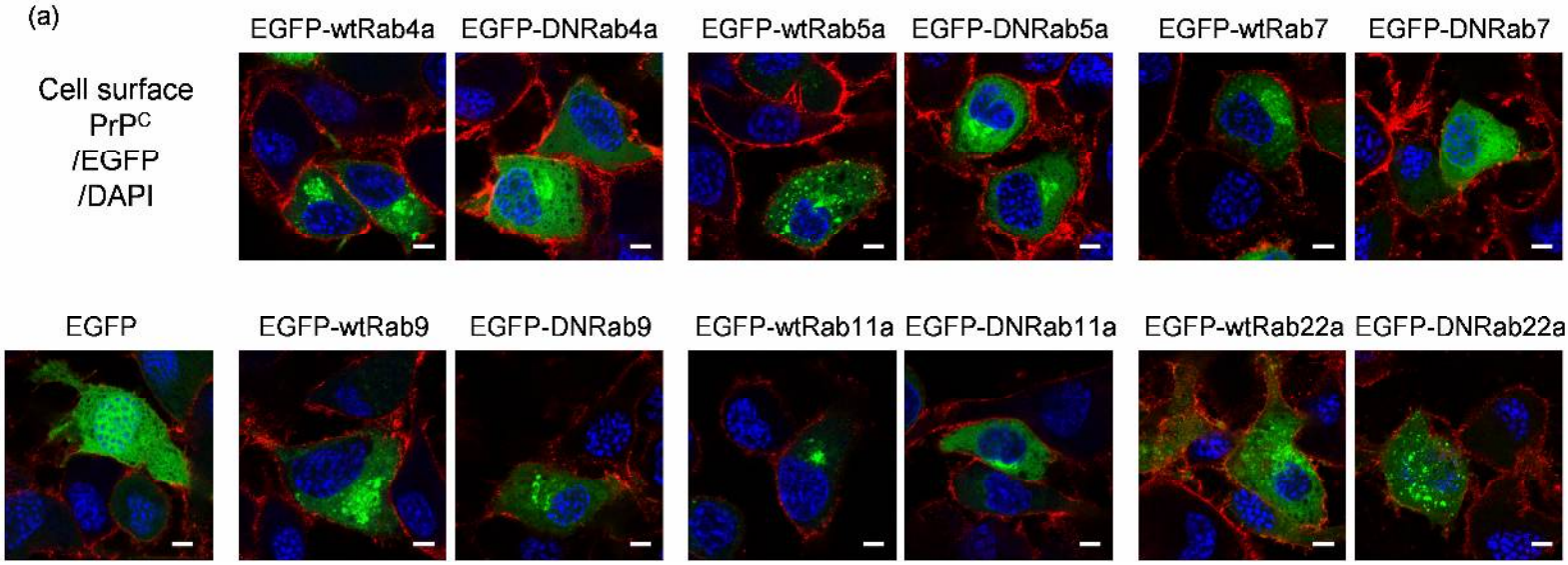
$$newPrP^{Sc}{}_i = PrP^{Sc}{}_i - PrPres_i$$

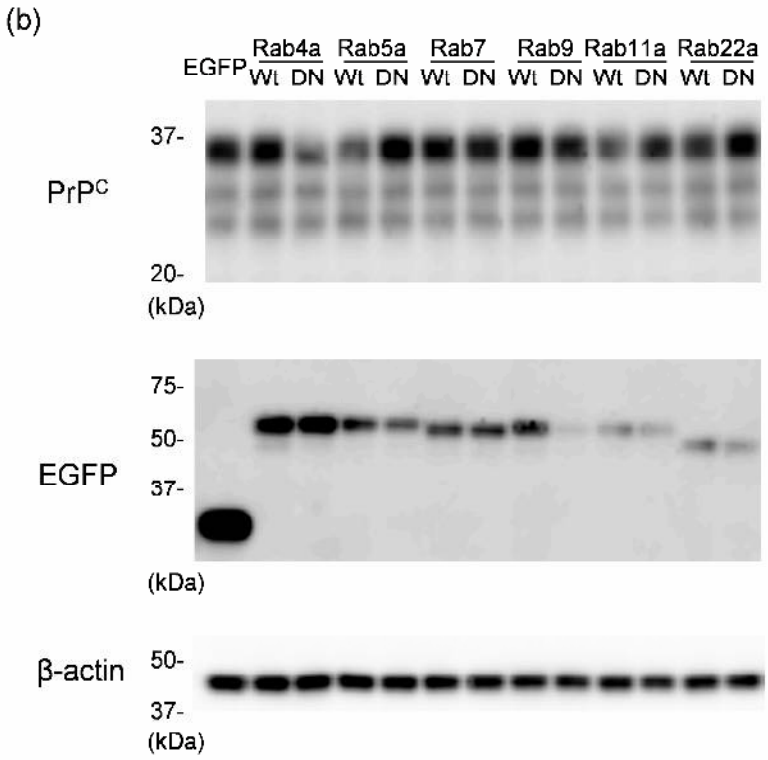
where $newPrP^{Sc}{}_i$, $PrP^{Sc}{}_i$ and $PrPres_i$ represent intensities of pixels of newly generated PrP^{Sc} signals, PrP^{Sc} signals by Alexa Fluor 488 and inoculated PrP-res signals by Alexa Fluor 555, respectively.

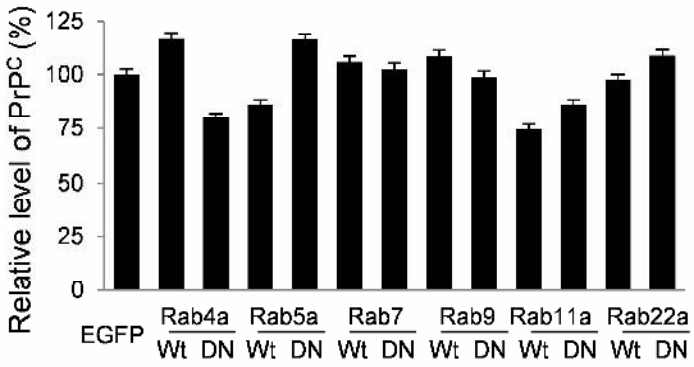
The co-localization ratio of newly generated PrP^{Sc} with an organelle marker represented a percentage of the weighted co-localization coefficient that was calculated according to the following formula;

$$\frac{\sum_i newPrP^{Sc}{}_{i,\,coloc}}{\sum_i newPrP^{Sc}{}_{i,\,total}}$$

where $newPrP^{Sc}_{i,coloc}$ represents the intensity of a pixel of newly generated PrP^{Sc} signal co-localized with an organelle marker, whereas, $newPrP^{Sc}_{i,total}$ represents the intensity of a pixel of newly generated PrP^{Sc} signal regardless of whether it co-localized with an organelle marker or not.







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Supplementary Figure 1. Influence of overexpression of Rab GTPases on expression of PrP^C.

(a) Expression of PrP^C on cell surface. To label cell surface PrP^C, living N2a-3 cells were incubated with mAb 44B1 for 15 min beginning 24 h after transfection of the expression plasmid encoding EGFP-tagged wild-type Rab GTPase or dominant-negative Rab GTPase mutant. Cells transfected with an expression plasmid encoding EGFP were used as the control. The cells were fixed and then counterstained with DAPI. Merged images of cell surface PrP^C (red), EGFP (green), and DAPI (blue) are shown. Scale bars: 5 μm. (b) Immunoblotting. N2a-3 cells were processed for immunoblotting 24 h after transfection to monitor PrP^C, EGFP, and β-actin. The graph on the right shows the levels of PrP^C relative to control cells expressing EGFP. Means of 2 independent experiments with variation are shown.

There was no difference in the staining of cell surface PrP^C among cells that overexpressed EGFP, EGFP-tagged wild-type Rab GTPase, or EGFP-tagged dominant-negative Rab GTPase mutant (a). Expression of any EGFP-tagged wild-type GTPase or dominant-negative GTPase, except for the dominant-negative mutant of Rab9 and Rab22a mutants, were reproducible based on immunoblotting (b). The PrP^C levels were not markedly affected by overexpression of any of the EGFP-Rab GTPase fusion proteins. Although a slight decrease in the PrP^C level was observed with the overexpression of wild-type Rab5a, Rab11a, or the dominant-negative Rab4a mutant, these tagged proteins did not influence the *de novo* generation of PrP^{Sc} (Fig. 7).

Supplementary Video 1. Time-lapse image of Af555-22L-PrP-res in N2a-3 cells.

N2a-3 cells were incubated with Af555-22L-PrP-res for 6 h at 37˚C and then subjected to

live-cell time-lapse imaging. The video image shows merged signals of Af555-22L-PrP-res (red) with DIC. The video film was sped up 20×.

Supplementary references

1. **Fukuda M.** 2003. Distinct Rab binding specificity of Rim1, Rim2, rabphilin, and Noc2. Identification of a critical determinant of Rab3A/Rab27A recognition by Rim2. J. Biol. Chem. **278**:15373-15380.
2. **Roberts M, Barry S, Woods A, van der Sluijs P, Norman J.** 2001. PDGF-regulated rab4-dependent recycling of αvβ3 integrin from early endosomes is necessary for cell adhesion and spreading. Curr. Biol. **11**:1392-1402.
3. **Stenmark H, Parton RG, Steele-Mortimer O, Lütcke A, Gruenberg J, Zerial M.** 1994. Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. EMBO J. **13**:1287-1296.
4. **Feng Y, Press B, Wandinger-Ness A.** 1995. Rab 7: an important regulator of late endocytic membrane traffic. J. Cell Biol. **131**:1435-1452.
5. **Riederer MA, Soldati T, Shapiro AD, Lin J, Pfeffer SR.** 1994. Lysosome biogenesis requires Rab9 function and receptor recycling from endosomes to the trans-Golgi network. J. Cell Biol. **125**:573-582.
6. **Ren M, Xu G, Zeng J, De Lemos-Chiarandini C, Adesnik M, Sabatini DD.** 1998. Hydrolysis of GTP on rab11 is required for the direct delivery of transferrin from the pericentriolar recycling compartment to the cell surface but not from sorting endosomes. Proc. Natl. Acad. Sci. U. S. A. **95**:6187-6192.
7. **Weigert R, Yeung AC, Li J, Donaldson JG.** 2004. Rab22a regulates the recycling of

membrane proteins internalized independently of clathrin. Mol. Biol. Cell. **15**:3758-3770

8. **Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene. **77**:51-59.
9. **Dunn KW, Kamocka MM, McDonald JH.** 2011. A practical guide to evaluating colocalization in biological microscopy. Am. J. Physiol., Cell Physiol. **300**:C723-C742.

Table S1. Source of commercially available antibodies.

	antibody	company	code number
Primary antibodies	anti-Rab5 rabbit polyclonal antibody	Cell Signaling Technology	#2143
	anti-Rab11a rabbit polyclonal antibody	Cell Signaling Technology	#3539
	anti-Rab7 rabbit monoclonal antibody clone D95F2	Cell Signaling Technology	#9367
	anti-EEA1 rabbit monoclonal antibody clone C45B10	Cell Signaling Technology	#3288
	anti-Rab4a rabbit polyclonal antibody	Proteintech Group	10347-1-AP
	anti-Rab9 rabbit polyclonal antibody	Proteintech Group	11420-1-AP
	anti-Rab22a rabbit polyclonal antibody	Proteintech Group	12125-1-AP
	anti-cathepsin D rabbit monoclonal antibody clone EPR3057Y	abcam	ab75852
	anti-Alexa Fluor 488 rabbit polyclonal antibody	Life technologies	A-11094
	anti-GFP rabbit polyclonal antibody	Life technologies	A-11122
Secondary antibodies	anti-mouse IgG horseradish peroxdase (HRP)-linked sheep F(ab')$_2$ fragment	GE Healthcare	NA9310
	anti-rabbit HRP-linked donkey F(ab')$_2$ fragment	GE Healthcare	NA9340
	Alexa Fluor 488-conjugated goat F(ab')$_2$ fragment anti-mouse IgG	Life technologies	A-11017
	Alexa Fluor 546-conjugated goat F(ab')$_2$ fragment anti-mouse IgG	Life technologies	A-11071
	Alexa Fluor 647-conjugated goat F(ab')$_2$ fragment anti-rabbit IgG	Life technologies	A-21246

Table S2. Oligonucleotide primers used for the amplification of cDNA of Rab GTPases by PCR.

		Primer sets
Rab4aWt	sense	5'-TGCTAGCGATGGCGCAGACCGCCATGTC-3'
	antisense	5'-T*CTCGAG*CTAGCAGCCACACTCCTGTG-3'
Rab5aWt	sense	5'-TGCTAGCGATGGCTAATCGAGGAGCAAC-3'
	antisense	5'-T*CTCGAG*TCAGTTACTACAACACTGG-3'
Rab7Wt	sense	5'-TGCTAGCGATGACCTCTAGGAAGAAAGT-3'
	antisense	5'-T*CTCGAG*TCAACAACTGCAGCTTTCTG-3'
Rab9Wt	sense	5'-TGCTAGCGATGGCAGGAAAATCGTCTCT-3'
	antisense	5'-T*CTCGAG*TCAACAGCAAGATGAGTTTG-3'
Rab11aWt	sense	5'-TGCTAGCGATGGGCACCCGCGACGACGA-3'
	antisense	5'-T*CTCGAG*TTAGATGTTCTGACAGCACT-3'
Rab22aWt	sense	5'-TT*CTCGAG*CTATGGCGCTGAGGGAACT-3'
	antisense	5'-AAAAGCTTTCAGCAGCAGCTTCGC-3'

Italics indicate the introduced restriction sites.

Table S3. Oligonucleotide primers used for site-directed mutagenesis by overlap extension PCR

		Primers
Rab4aS22N	sense	5'-GGCAAAAATTGCTTGCTTCATCAGTTCATTGAAA-3'
	antisense	5'-ATTTTTGCCAGTTCCCGCATTTCCGATGACCAA-3'
Rab5aS34N	sense	5'-GGCAAAAATAGCCTGGTTCTTCGCTTTGTGAAA-3'
	antisense	5'-ATTTTTGCCAACAGCAGACTCTCCTAGAAGGAC-3'
Rab7T22N	sense	5'-GGAAAGAACTCTCTCATGAACCAGTATGTGAAC-3'
	antisense	5'-GTTCTTTCCAACACCAGAGTCCCCCAGGATGAT-3'
Rab9S21N	sense	5'-GGCAAGAATTCTCTTATGAACAGATATGTAACC-3'
	antisense	5'-ATTCTTGCCAACTCCACCATCTCCAAGAAGAAT-3'
Rab11aS25N	sense	5'-AATAACCTCCTGTCTCGATTTACTCGAAATGAGTT-3'
	antisense	5'-GAGGTTATTCTTTCCAACACCAGAATCTCCAAT-3'
Rab22aS19N	sense	5'-AATAGCATCGTGTGGCGGTTTGTGGAAGACAG-3'
	antisense	5'-GATGCTATTTTTACCCACACCCGTATCCCCGA-3'

Underlines indicate the mutated nucleotides for substituting amino acids.

Table S4. Number of foci used for co-localization statistics in Figure 3c.

Marker	Incubation time	Number of granules		Number of cells
		Inoculated PrP-res	Inoculated PrP-res co-localized with marker	
Tfn	0 h	1152	328	230
	30 h	902	121	276
LDL	0 h	2060	1581	243
	30 h	770	599	217

Table S5. Number of foci used for co-localization statistics in Figure 6.

Marker	Incubation time	Number of granules				Number of cells
		Inoculated PrP-res	Inoculated PrP-res co-localized with marker	Newly generated PrP^{Sc}	Newly generated PrP^{Sc} co-localized with marker	
PrP^{C}	0 hpi	2843	685	-	-	44
	24 hpi	1110	30	201	77	35
	48 hpi	1685	25	361	33	47
	72 hpi	2407	13	1540	47	77
EEA1	0 hpi	615	78	-	-	62
	24 hpi	264	32	336	56	52
	48 hpi	213	25	1454	323	50
	72 hpi	286	29	1791	553	46
Rab11a	0 hpi	1519	323	-	-	62
	24 hpi	878	79	335	13	56
	48 hpi	560	25	810	95	53
	72 hpi	1701	698	4249	1212	57
Rab7	0 hpi	1772	836	-	-	60
	24 hpi	1502	287	474	97	63
	48 hpi	629	142	1154	450	52
	72 hpi	1550	436	3337	1106	67
Cathepsin D	24 hpi	794	228	236	14	64
	48 hpi	1017	207	1870	428	49
	72 hpi	1322	320	1997	271	53