

## II. 研 究 成 果 一 覽

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kunita R, Otomo A, Mizumura H, Suzuki-Utsunomiya K, Hadano S, Ikeda JE	The Rab5 activator ALS2/alsin acts as a novel Rac1 effector through Rac1-activated endocytosis	J Biol Chem	282(22)	16599-16611	2007
Hadano S, Kunita R, Otomo A, Suzuki-Utsunomiya K, Ikeda JE	Molecular and cellular function of ALS2/alsin: Implication of membrane dynamics in neuronal development and degeneration	Neurochem. Int	51(2-4)	74-84	2007
Tanaka K, Okada Y, Kanno T, Otomo A, Yanagisawa Y, Shouguchi-Miyata J, Suga E, Kohiki E, Onoe K, Osuga H, Aoki M, Hadano S, Itoyama Y, Ikeda JE	A dopamine receptor antagonist L-745,870 suppresses microglia activation in spinal cord and mitigates the progression in ALS model mice	Exp Neurol	in press		2008
Otomo A, Kunita R, Suzuki-Utsunomiya K, Mizumura H, Onoe K, Osuga H, Hadano S, Ikeda JE	ALS2/alsin deficiency in neurons leads to mild defects in macropinocytosis and axonal growth	Biochem Biophys Res Commn	in press		2008
Suzuki K, Katsuno M, Banno H, Takeuchi Y, Atsuta N, Ito M, Watanabe H, Yamashita F, Hori N, Nakamura T, Hirayama M, Tanaka F, Sobue G	CAG repeat size correlates to electrophysiological motor and sensory phenotypes in SBMA	Brain	131	229-239	2008
Niwa J, Yamada S, Ishigaki S, Sone J, Takahashi M, Katsuno M, Tanaka F, Doyu M, Sobue G	Disulfide bond mediates aggregation, toxicity, and ubiquitylation of familial amyotrophic lateral sclerosis-linked mutant SOD1	J Biol Chem	282	28087-28095	2007
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Ishigaki S, Niwa J, Yamada S, Takahashi M, Ito T, Sone J, Doyu M, Urano F, Sobue G	Dorfin-CHIP chimeric proteins potently ubiquitylate and degrade familial ALS-related mutant SOD1 proteins and reduce their cellular toxicity	Neurobiol Dis	25	331-341	2007

Kayasuga Y, Chiba S, Suzuki M, Kikusui T, Matsuwaki T, Yamanouchi K, Kotaki H, Horai R, Iwakura Y, Nishihara M	Alteration of behavioural phenotype in mice by targeted disruption of the progranulin gene	Behav Brain Res	185	110-118	2007
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Ishigaki A, Aoki M, Nagai M, Warita H, Kato S, Kato M, Nakamura T, Funakoshi H, Itoyama Y	Intrathecal delivery of hepatocyte growth factor from amyotrophic lateral sclerosis onset suppresses disease progression in rat amyotrophic lateral sclerosis model	J Neuropathol Exp Neurol	66(11)	1037-1044	2007
Yamagishi S, Koyama Y, Katayama T, Taniguchi M, Hitomi J, Kato M, Aoki M, Itoyama Y, Kato S, Tohyama M	An <i>in vitro</i> model for Lewy body-like hyaline inclusion/astrocytic hyaline inclusion: induction by ER stress with an ALS-linked SOD1 Mutation	PLoS ONE	2(10)	e1030	2007
Mizuno H, Warita H, Aoki M, Itoyama Y	Accumulation of chondroitin sulfate proteoglycans in the microenvironment of spinal motor neurons in ALS transgenic rats	J Neurosci Res	in press		2008
Suzuki-Utsunomiya K, Hadano S, Otomo A, Kunita R, Mizumura H, Osuga H, Ikeda JE	ALS2CL, a novel ALS2-interactor, modulates ALS2-mediated endosome dynamics	Biochem Biophys Res Commun	354(2)	491-497	2007
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Ishigaki S, Niwa J, Yamada S, Takahashi M, Ito T, Sone J, Doyu M, Urano F, Sobue G	Dorfin-CHIP chimeric proteins potently ubiquitylate and degrade familial ALS-related mutant SOD1 proteins and reduce their cellular toxicity	Neurobiol Dis	25(2)	331-341	2007
Tanaka F, Niwa J, Ishigaki S, Katsuno M, Waza M, Yamamoto M, Doyu M, Sobue G	Gene expression profiling toward understanding of ALS pathogenesis	Ann N Y Acad Sci	1086	1-10	2006

Katsuno M, Adachi H, Minamiyama M, Waza M, Tokui K, Banno H, Suzuki K, Onoda Y, Tanaka F, Doyu M, Sobue G	Reversible disruption of dynactin 1-mediated retrograde axonal transport in polyglutamine-induced motor neuron degeneration	J Neurosci	26(47)	12106-12117	2006
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Matsuki T, Nakae S, Sudo K, Horai R, Iwakura Y	Abnormal T cell activation caused by the imbalance of the IL-1/IL-1R antagonist system is responsible for the development of experimental autoimmune encephalomyelitis	Int Immunol	18(2)	399-407	2006
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Matsumoto A, Okada Y, Nakamichi M, Nakamura M, Toyama Y, Sobue G, Nagai M, Aoki M, Itoyama Y, Okano H	Disease progression of human SOD1 (G93A) transgenic ALS model rats	J Neurosci Res	83(1)	119-133	2006

Hadano S, Benn SC, Kakuta S, Otomo A, Sudo K, Kunita R, Suzuki-Utsunomiya K, Mizumura H, Shefner JM, Cox GA, Iwakura Y, Brown RH Jr, and Ikeda JE	Mice deficient in the Rab5 guanine nucleotide exchange factor ALS2/alsin exhibit age-dependent neurological deficits and altered endosome trafficking	Hum Mol Genet	15(2)	233-250	2006
Hadano S, Ikeda JE	Purification and functional analyses of ALS2 and its homologue	Methods in Enzymology	403	310-321	2005
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Matsuki T, Nakae S, Sudo K, Horai R, Iwakura Y	Abnormal T cell activation caused by the imbalance of the IL-1/IL-1R antagonist system is responsible for the development of experimental autoimmune encephalomyelitis	Int Immunol	18(2)	399-407	2006
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Matsumoto A, Okada Y, Nakamichi M, Nakamura M, Toyama Y, Sobue G, Nagai M, Aoki M, Itoyama Y, Okano H	Disease progression of human SOD1 (G93A) transgenic ALS model rats	J Neurosci Res	83(1)	119-133	2006
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Ikeda K, Aoki M, Kawazoe Y, Sakamoto T, Hayashi Y, Ishigaki A, Nagai M, Kamii R, Kato S, Itoyama Y, Watabe K	Motoneuron degeneration after facial nerve avulsion is exacerbated in presymptomatic transgenic rats expressing human mutant Cu/Zn superoxide dismutase	J Neurosci Res	82(1)	63-70	2005
Kato S, Kato M, Abe Y, Matsumura T, Nishino T, Aoki M, Itoyama Y, Asayama K, Awaya A, Hirano A, Ohama E	Redox system expression in the motor neurons in amyotrophic lateral sclerosis (ALS): immunohistochemical studies on sporadic ALS, superoxide dismutase 1 (SOD1)-mutated familial ALS, and SOD1-mutated ALS animal models	Acta Neuropathol	110(2)	101-112	2005
Chang-Hong R, Wada M, Koyama S, Kimura H, Arawaka S, Kawanami T, Kurita K, Kadoya T, Aoki M, Itoyama Y, Kato T	Neuroprotective effect of oxidized galectin-1 in a transgenic mouse model of amyotrophic lateral sclerosis	Exp Neurol	194(1)	203-211	2005

### III. 研究成果の刊行物・別刷

# The Rab5 Activator ALS2/alsin Acts as a Novel Rac1 Effector through Rac1-activated Endocytosis<sup>\*[5]</sup>

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Ryota Kunita<sup>†§1</sup>, Asako Otomo<sup>‡2</sup>, Hikaru Mizumura<sup>§</sup>, Kyoko Suzuki-Utsunomiya<sup>†1</sup>, Shinji Hadano<sup>†§¶3</sup>, and Joh-E Ikeda<sup>†§¶4</sup>

From the <sup>†</sup>Department of Molecular Life Sciences, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan, the <sup>§</sup>Solution Oriented Research for Science and Technology, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan, the <sup>‡</sup>Department of Molecular Neuroscience, The Institute of Medical Sciences, Tokai University, Isehara, Kanagawa 259-1193, Japan, and the <sup>¶</sup>Department of Paediatrics, Faculty of Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

Mutations in the *ALS2* gene cause a number of recessive motor neuron diseases, indicating that the ALS2 protein (ALS2/alsin) is vital for motor neurons. ALS2 acts as a guanine nucleotide exchange factor (GEF) for Rab5 (Rab5GEF) and is involved in endosome dynamics. However, the spatiotemporal regulation of the ALS2-mediated Rab5 activation is unclear. Here we identified an upstream activator for ALS2 and showed a functional significance of the ALS2 activation in endosome dynamics. ALS2 preferentially interacts with activated Rac1. In the cells activated Rac1 recruits cytoplasmic ALS2 to membrane ruffles and subsequently to nascent macropinosomes via Rac1-activated macropinocytosis. At later endocytic stages macropinosomal ALS2 augments fusion of the ALS2-localized macropinosomes with the transferrin-positive endosomes, depending on the ALS2-associated Rab5GEF activity. These results indicate that Rac1 promotes the ALS2 membranous localization, thereby rendering ALS2 active via Rac1-activated endocytosis. Thus, ALS2 is a novel Rac1 effector and is involved in Rac1-activated macropinocytosis. All together, loss of ALS2 may perturb macropinocytosis and/or the following membrane trafficking, which gives rise to neuronal dysfunction in the ALS2-linked motor neuron diseases.

Mutations in the *ALS2* gene account for a number of recessive motor neuron diseases, including a juvenile recessive form

of amyotrophic lateral sclerosis (ALS2),<sup>5</sup> a juvenile-onset primary lateral sclerosis, and an infantile-onset ascending hereditary spastic paralysis (1–7). The *ALS2* gene encodes a protein (ALS2 or alsin) of 1657 amino acid residues with three predicted GEF domains, *i.e.* regulator of chromosome condensation 1-like domain (RLD), the Dbl-homology and pleckstrin-homology (DH/PH) domain, and the vacuolar protein sorting 9 (VPS9) domain (see Fig. 2A), implicated in membrane trafficking and cytoskeletal dynamics (1, 2). ALS2 also contains eight consecutive membrane occupation and recognition nexus (MORN) motifs, which are known to mediate the plasma membrane binding (8, 9) in the region between PH and VPS9 domains (see Fig. 2A).

The DH/PH and VPS9 are hallmarks of GEFs for the Rho-type GTPases (Rhos) and Rab5, respectively (10, 11). ALS2 indeed activates Rab5 by its Rab5GEF activity and is implicated in endosome dynamics in cultured cells (12–15). Recently, two independent studies of the *Als2*-knock-out (KO) mice have revealed that a loss of ALS2 results in the delayed fusion of epidermal growth factor (EGF)-positive endosomes in mouse embryonic fibroblasts (MEFs) (16) and the reduced Rab5-dependent fusion activity in the brain cytosol (17). Furthermore, small interference RNA-mediated knockdown of rat *Als2* in cultured motor neurons led to smaller-sized early endosome autoantigen 1 (EEA1)-positive endosomes (18). Thus, endogenous ALS2 certainly plays an important role in Rab5-dependent endosome fusion. Additionally, several studies have shown that ALS2 also activates a Rho member Rac1, thereby facilitating the neurite outgrowth (18, 19) and suppressing cell death (16, 18). However, the ALS2-associated Rac1GEF activity is still controversial (12, 14, 19, 20). In any case, it is currently believed that

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–4.

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<sup>4</sup> To whom correspondence should be addressed: Dept. of Molecular Life Sciences, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan. Tel.: 81-463-91-5095; Fax: 81-463-91-4993; E-mail: jeikeda3@is.icc.u-tokai.ac.jp.

<sup>5</sup> The abbreviations used are: ALS2, amyotrophic lateral sclerosis 2; GEF, guanine nucleotide exchange factor; RLD, regulator of chromosome condensation 1-like domain; DH/PH, Dbl-homology/Pleckstrin homology; VPS9, vacuolar protein sorting 9; EGF, epidermal growth factor; MEF, mouse embryonic fibroblast; EEA1, early endosome autoantigen 1; CME, clathrin-mediated endocytosis; PBS, phosphate-buffered saline; Ab, antibody; pAb, polyclonal Ab; mAb, monoclonal Ab; CRIB, Cdc42/Rac interactive binding domain; Trio-GEF1, Trio-GEF domain 1; EGFP, enhanced green fluorescent protein; PDGF, platelet-derived growth factor; WT, wild type; Tf, transferrin; MORN, membrane occupation and recognition nexus; GST, glutathione S-transferase; HBSS, Hanks' balanced salt solution; KO, knock out; HA, hemagglutinin; GTP-γS, guanosine 5'-3-O-(thio)triphosphate; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium.



## Rac1-mediated Regulation of the Rab5 Activator

ALS2 is involved in either the Rab5- or the Rac1-mediated cellular process or both.

Two GTPases, Rab5 and Rac1, generally control distinct cellular processes. Rab5 is a key regulator in both endocytosis and following membrane trafficking events, such as clathrin-mediated endocytosis (CME), macropinocytosis, phagocytosis, fusion and motility of early endosomes, and transcriptional regulation (21–23). On the other hand, Rac1 generally controls cell spreading and migration via regulating actin dynamics. Remarkably, it has been recently reported that Rab5, Rac1, and phosphatidylinositol-3-OH kinase are simultaneously required for the induction of dorsal ruffle, the prerequisite for a mode of endocytosis called macropinocytosis (24, 25). Thus, it is conceivable that ALS2 takes part in cellular processes such as macropinocytosis, which is enhanced by both Rab5 and Rac1, although no such experimental evidence has been provided thus far.

Although the current evidence supports that ALS2 is involved in endosome fusion *in vivo*, it is also equally important to note that ALS2 is mostly sequestered in the cytoplasm and certain ALS2 molecules are usually localized onto EEA1-positive endosomes and are thereby involved in endosome dynamics (12, 15). Notably, Panzeri *et al.* (26) have identified a pathogenic missense mutation in the RLD (the N-terminal half of ALS2; see Fig. 2A) that results in the ALS2 mislocalization. These findings suggest that proper regulation of the ALS2 subcellular localization is crucial to exert the physiological ALS2 functions. However, the underlying mechanisms are currently unknown.

In this study, to gain a better understanding of the physiological ALS2 roles, we aimed to determine when and how cytoplasmic dormant ALS2 turns to participate in membrane dynamics. Here we show that Rac1 induces the recruitment of the ALS2 molecules to the plasma membrane, and then ALS2 is relocated to macropinosomes via Rac1-regulated macropinocytosis. Subsequently, macropinosomal ALS2 augments fusion of the ALS2-localized macropinosomes with the transferrin (Tf)-positive endosomes. Thus, ALS2 is the first Rab5GEF that acts as a novel Rac1 effector and plays important roles in Rac1-activated macropinocytosis and fusion of macropinosomes. These findings raise the interesting possibility that an unfamiliar endocytic mechanism, macropinocytosis, and/or the following membrane trafficking might be indispensable for the integrity of motor neurons.

### EXPERIMENTAL PROCEDURES

**Antibodies and Materials**—Anti-ALS2\_RLD (HPF1–680) and anti-ALS2\_MORN/VPS9 (MPF1012–1651) polyclonal antibodies (pAbs) were previously described (12, 15). Monoclonal antibodies (mAbs) used in this study included anti-FLAG M2 (Stratagene), anti-HA (Sigma), anti-GST (Santa Cruz), anti-Rab5 (BD Biosciences), anti-EEA1 (BD Biosciences), and anti-transferrin receptor (Molecular Probes). All other reagents were from commercial sources and of analytical grade.

**Plasmids**—All the expression constructs were obtained by subcloning the PCR- or the reverse transcriptase-PCR-amplified human cDNAs into the appropriate expression vectors.

The DNA sequence of the insert as well as the flanking regions in each plasmid construct was verified by sequencing. Plasmids expressing mutant proteins were generated by a PCR-based method. The cDNA fragments of ALS2 and TRIO were subcloned into the original or modified pCI-neo Mammalian Expression Vectors (Promega), allowing the production of the N-terminal-FLAG-, HA-, or 2XHA-tagged or untagged proteins. RAC1, RHOG, and RAB5A and their mutants were subcloned into the p3XFLAG-CMV<sup>TM</sup>-10 Expression Vector (Sigma), modified p3XFLAG-CMV<sup>TM</sup>-10 (p2XHA-CMV<sup>TM</sup>-10), or pCI-neo, generating plasmids expressing 3XFLAG- or 2XHA-tagged or untagged small GTPases. The cDNA fragment of PAK1 was subcloned into pGEX-6P vector (Amersham Biosciences) to generate pGEX6P-Cdc42/Rac interactive binding domain (CRIB) expressing PAK1 fragment (PAK1 53–165). Constructs generated were as follows: pCIneo-ALS2\_L (untagged full-length ALS2), pCIneoFLAG-ALS2\_L<sup>P1603A</sup>, pCIneoFLAG-ALS2\_L<sup>E697A</sup>, pCIneoFLAG-ALS2\_L<sup>T701A</sup>, pCIneoFLAG-ALS2\_L<sup>Q869A/D870A</sup>, pCIneoFLAG-ALS2\_L<sup>W996A</sup>, pCIneoFLAG-ALS2\_340–680, pCIneoFLAG-ALS2\_1018–1657 ( $\Delta$ 1280–1335), pCIneo2XHA-ALS2\_L, pCIneo2XHA-ALS2\_1018–1657, pCIneoHA-ALS2\_1–680, pCIneoHA-Trio-GEFD1 (1233–1628), p3XFLAG-CMV10-Rac1, p3XFLAG-CMV10-Rac1<sup>Q61L</sup>, p3XFLAG-CMV10-Rac1<sup>T17N</sup>, p3XFLAG-CMV10-RhoG<sup>Q61L</sup>, p2XHA-CMV10-Rac1<sup>Q61L</sup>, p2XHA-CMV10-Rac1<sup>Y40C,Q61L</sup>, pCIneo-Rab5<sup>Q79L</sup> (untagged), and pGEX6P-CRIB. The previously generated plasmids (12, 15) including pCIneoFLAG-ALS2\_L, pCIneoFLAG-ALS2\_1–680, pCIneoFLAG-ALS2\_1018–1657, pCIneoFLAG-ALS2\_S (ALS2 short form), pCIneoFLAG-Trio-GEFD1 (1233–1628), pCIneoFLAG-Rabex-5, pCIneoHA-ALS2\_L, pCIneoHA-ALS2\_1018–1657, pGEX6P-RhoA, pGEX6P-Cdc42, pGEX6P-Rac1, pGEX6P-Rab5A, pEGFP-ALS2\_L, and pEGFP-ALS2\_695–1657 were also utilized.

**Preparation of the GST Fusion Proteins**—GST, GST-fused CRIB, and GST-fused small GTPases were purified as previously described (12), with a minor modification in which Ezview<sup>TM</sup> Red Glutathione Affinity Gel (Sigma) instead of glutathione-Sepharose 4B was used.

**In Vivo Rac1-GTP Loading Assay**—COS-7 or HeLa cells were transfected with p3XFLAG-CMV10-Rac1 along with either pCIneo empty vector, pCIneoHA-ALS2\_L, pCIneoHA-ALS2\_1018–1657 (as a negative control), or pCIneoHA-Trio-GEFD1 (as a positive control) by using Effectene Transfection Reagent (Qiagen) as previously described (12). Twenty-four hours after transfection, the cells were washed twice with PBS(–) and lysed in ice-cold buffer A consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 4% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, and Complete protease inhibitor mixture tablet/EDTA-free (Roche Applied Science). After gentle rotation for 30 min at 4 °C, samples were centrifuged at 12,000 × g for 15 min. The resulting supernatants were mixed with Ezview<sup>TM</sup> Red Glutathione Affinity Gel (Sigma) conjugating GST-CRIB for 30 min at 4 °C. Then the gels were washed three times with ice-cold buffer A. Appropriate amounts of pulldown products were analyzed by Western blot analysis using anti-FLAG M2 mAb as previously described (12).

**In Vitro GST Pulldown Assay**—COS-7 cells transfected either with pCIneoFLAG-ALS2<sub>L</sub>, pCIneoFLAG-ALS2<sub>L</sub><sup>T701A</sup>, or pCIneoFLAG-ALS2<sub>1–680</sub> were lysed in buffer B consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 2% Tween 20 (Sigma), and Complete protease inhibitor mixture tablet/EDTA-free (Roche Applied Science). After centrifugation at 12,000 × g for 15 min, supernatants were recovered and mixed with glutathione gels conjugating GDP- or GTPγS-preloaded GST-fused small G proteins. The reaction was carried out for 2 h at room temperature in buffer C consisting of 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 15 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.2% Tween 20, 5% glycerol, and protease inhibitors/EDTA-free (Roche Applied Science). After washing three times with buffer C, appropriate amounts of the pull-down products were analyzed by Western blot analysis. In the case of the pull-down with nucleotide-free GST fusion proteins, supernatants were prepared in buffer B containing 10 mM EDTA instead of 10 mM MgCl<sub>2</sub>, and the reaction was carried out in buffer C containing 10 mM EDTA instead of 15 mM MgCl<sub>2</sub>. To confirm a direct interaction between ALS2 and Rac1, the FLAG-tagged ALS2 protein was obtained by the affinity purification with Ezview™ Red ANTI-FLAG M2 affinity gel (Sigma) followed by elution with 3XFLAG peptide (Sigma), as previously described (15), and was used for *in vitro* GST pull-down experiments.

**Co-immunoprecipitation**—COS-7 cells co-transfected with constructs expressing FLAG-tagged and HA- or 2XHA-tagged proteins were washed twice with 150 mM NaCl and lysed in buffer D consisting of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 10% glycerol, 1% Tween 20, and protease inhibitors/EDTA-free (Roche Applied Science). For co-immunoprecipitation of the N-terminal with C-terminal ALS2 fragments, buffer E consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630 (Sigma), 100 μM phenylmethylsulfonyl fluoride, and protease inhibitors/EDTA-free (Roche Applied Science) instead of buffer D was used for lysis and washing. After gentle rotation for 4 h at 4 °C, samples were centrifuged at 12,000 × g for 15 min. Supernatants were immunoprecipitated either with anti-FLAG M2 Affinity Gel (Sigma) or with anti-HA Affinity Gel (Sigma). The affinity gels were washed three times with buffer D or E. Appropriate amounts of the immunoprecipitates were analyzed by Western blot analysis using either the anti-FLAG M2 or anti-HA mAb. In the case of co-immunoprecipitation of ALS2 with either Rac1<sup>Q61L</sup> or Rac1<sup>T17N</sup>, COS-7 cells were cultured on a suspension culture dish (Corning) instead of a tissue culture dish for adherent cells.

**Immunocytochemistry and Confocal Microscopy**—Immunofluorescence studies were carried out as previously described (10). Briefly, HeLa or COS-7 cells transfected with appropriate plasmids were washed with PBS(–) twice, fixed with 4% paraformaldehyde in PBS(–) for 20 min, and permeabilized with 0.5% Triton X-100 in PBS(–) for 30 min. HeLa cells transfected with pEGFP-ALS2<sub>L</sub> were serum-starved for 12 h and treated with EGF (DIACLONE) (100 ng/ml, 10 min) before fixation. Anti-ALS2 (HPF1–680 and MPF1012–1651), anti-FLAG M2, anti-HA, anti-Rab5, anti-EEA1, or anti-transferrin receptor Ab, diluted in PBS(–) containing 1.5% normal goat serum and 0.05% Triton X-100, was added to the fixed cells and incubated for 4 h at room temperature. Alexa 488- or 594-

conjugated goat anti-mouse or rabbit IgG (Molecular Probes) was used for the detection of signals of either tag epitopes or proteins of interest. F-actin was visualized by Alexa 594-conjugated phalloidin (Molecular Probes). Finally, images of serial optical section with 0.6–1-μm thickness were captured and analyzed by Leica TCS\_NT confocal microscope system (Leica).

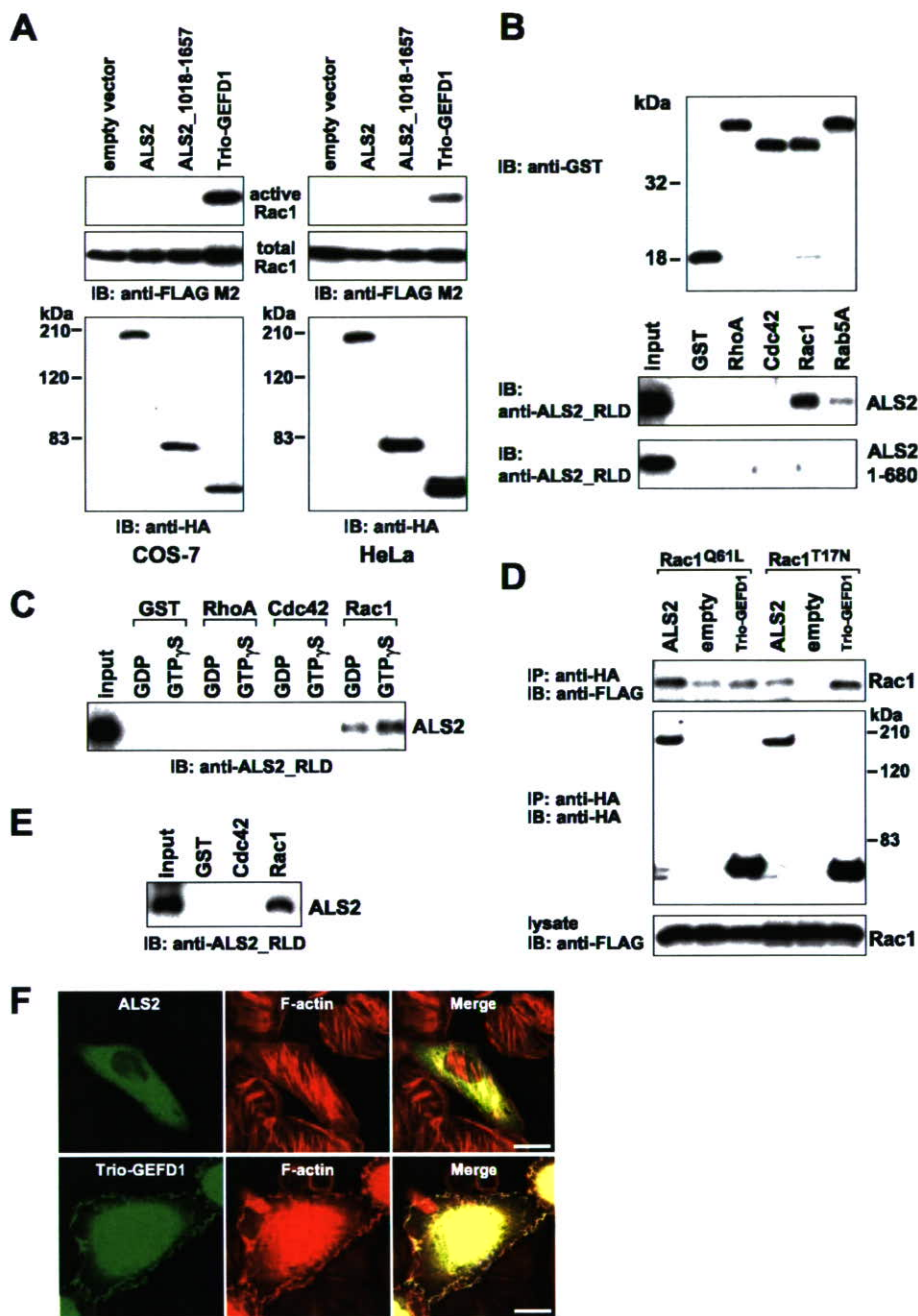
**Primary Culture of Hippocampal Cells**—Primary hippocampal cell cultures were established from WT or *Als2*-KO P1 or P2 infants (16) in B6(N6);129 background. Briefly, hippocampal tissues were dissected out and placed into ice-cold HBSS(–) (pH 7.0) (Sigma). After removing HBSS(–), the tissues were treated with trypsin, washed with HBSS(–). After DNase I treatment, DMEM/F-12/1:1 (pH 7.0) (Invitrogen) containing 20% heat-inactivated FBS was added to the reaction. After centrifugation at 150 × g, the resulting pellets were dissociated in DMEM/F-12/1:1 (pH 7.0) supplemented with 20% FBS by pipetting using a Pasteur pipette. After counting the living cell numbers, the cells were maintained on poly-D-lysine-coated round glasses (BD biosciences) in neuronal cell culture media (DMEM/F-12/1:1 (pH 7.0) containing 20% FBS, 1XB27 supplement (Invitrogen), 25 mM insulin (Sigma), 50 μg/ml streptomycin, and 50 units/ml penicillin G) for 12 h. Medium was changed with the fresh medium containing 5% FBS, and the cells were cultured for another 36 h.

**Primary Culture of Mouse Embryonic Fibroblasts and Circular Ruffle Induction**—Primary-cultured MEFs were established from WT or *Als2*-KO E14.5 embryos (16) in B6(N9);129 background. Briefly, the skin tissues were washed with HBSS(–), treated with trypsin and DNase I, and cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin G, and 100 μg/ml streptomycin. To induce circular ruffles, MEFs were serum-starved for 20 h and treated with platelet-derived growth factor (PDGF-BB; Diaclone) (10 ng/ml, 5 min). Fixation and F-actin staining were carried out immediately afterward. Images were captured and analyzed as described above.

**Dextran Uptake**—For HeLa cells, cells were transfected with either p3XFLAG-CMV10-Rac1<sup>Q61L</sup> or pCIneo-ALS2<sub>L</sub> or co-transfected with p3XFLAG-CMV10-Rac1<sup>Q61L</sup> and pCIneo-ALS2<sub>L</sub> as described above. Twenty-four h after transfection, the cells were washed twice with PBS(–) and cultured in DMEM containing 10% FBS, antibiotics, and 2 mg/ml 70-kDa tetramethylrhodamine-dextran (Molecular Probes) for 30 min. After washing with PBS(–), the cells were fixed and analyzed as described above. For hippocampal neurons, primary hippocampal cell cultures were maintained as described above. After 48 h the cells were incubated in neuronal cell culture media containing 2 mg/ml 70-kDa fluorescein-dextran (Molecular Probes) for 30 min. Then the cells were fixed, permeabilized, and stained with Alexa 594-conjugated phalloidin (Molecular Probes) to visualize neuronal cell shapes and analyzed.

**Transferrin Uptake**—HeLa cells were transfected with pCIneo-ALS2<sub>L</sub> alone or co-transfected either with pCIneo-FLAG-ALS2<sub>L</sub> and p3XFLAG-CMV10-Rac1<sup>Q61L</sup> or with pCIneoFLAG-ALS2<sub>L</sub><sup>P1603A</sup> and p3XFLAG-CMV10-Rac1<sup>Q61L</sup>. Twenty-four h after transfection the cells were washed with

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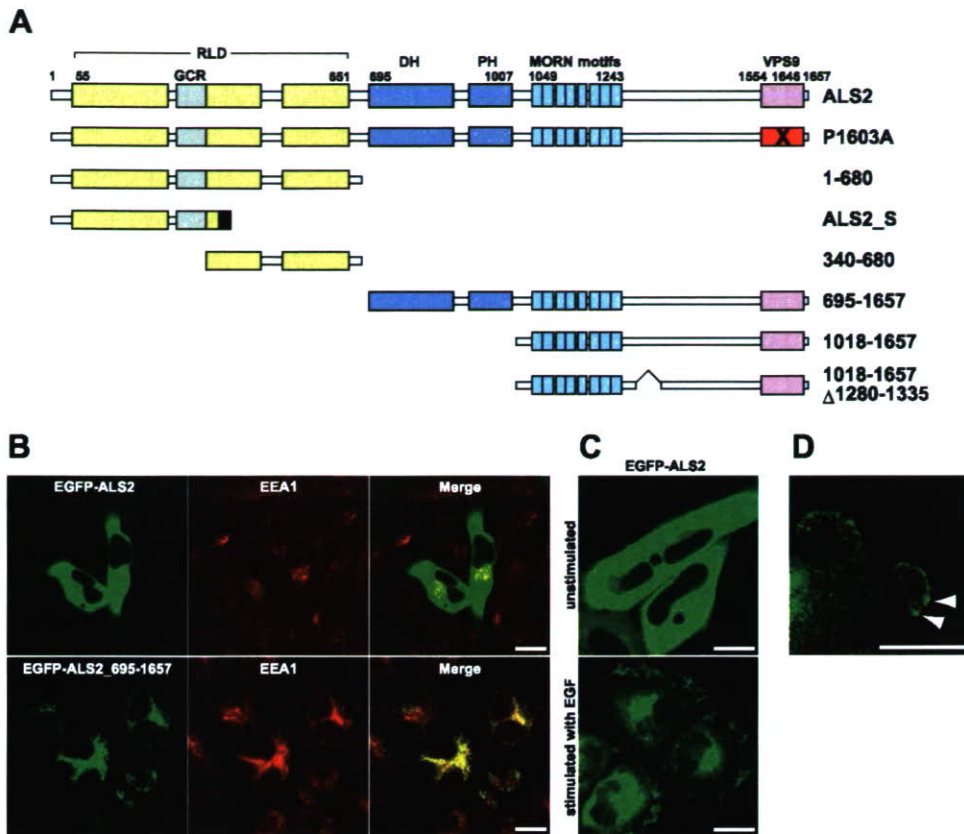


**FIGURE 1. ALS2 is a novel Rac1 effector.** *A*, *in vivo* Rac1-GTP loading assay. Lysates from COS-7 or HeLa cells transfected with plasmids expressing HA-tagged full-length ALS2, ALS2<sub>1018-1657</sub> (as a negative control), or Trio-GEFD1 (as a positive control) along with plasmid expressing 3XFLAG-tagged Rac1 were incubated with GST-CRIB, and subsequently the pulled-down samples (top) and lysates (middle and bottom) were immunoblotted with the indicated Abs. IB, Ab used for Western blot analysis. ALS2 exhibited no observable Rac1GEF activities both in COS-7 and HeLa cells. *B*, *in vitro* GST pull-down from cellular lysates. Lysates from COS-7 cells expressing FLAG-tagged ALS2 or ALS2<sub>1-680</sub> were incubated with GST or GST-fused/nucleotide-free GTPases indicated, and subsequently the pulled-down samples were immunoblotted with anti-ALS2\_RLD pAb (middle and bottom). GST proteins used were also detected with anti-GST mAb (top). ALS2 preferentially interacts with Rac1 and Rab5A. *C*, *in vitro* GST pull-down assay using the guanine-nucleotide preloaded GTPases. Lysates from COS-7 cells expressing ALS2 were used for GST pull-down assay as in *B* except that all GST proteins were preloaded with either GDP or GTP $\gamma$ S. ALS2 preferentially interacts with GTP $\gamma$ S-preloaded Rac1. *D*, co-immunoprecipitation of ALS2 and Rac1. Lysates from COS-7 cells co-transfected with either p3XFLAG-CMV10-Rac1<sup>Q61L</sup> or p3XFLAG-CMV10-Rac1<sup>T17N</sup> along with either pCneoHA-ALS2\_L (ALS2), pCneo-empty vector (empty), or pCneoHA-Trio-GEFD1 (Trio) were immunoprecipitated with anti-HA affinity gels. Subsequently, immunoprecipitates and lysates were immunoblotted with either anti-FLAG M2 (top) or anti-HA (middle), and anti-FLAG M2 (bottom) Abs, respectively. IP, Ab used for immunoprecipitation. ALS2 preferentially interacts with a constitutively active form of Rac1 (Rac1<sup>Q61L</sup>) in the cells. *E*, *in vitro* binding between ALS2 and GTPases. All steps were performed as in *B* except that the purified ALS2 instead of lysates was used. ALS2 directly interacts with Rac1 *in vitro*. *F*, ALS2 overexpression does not induce any actin-reorganizing phenotypes. HeLa cells transfected with plasmid expressing either 2XHA-tagged ALS2\_L (ALS2) or 2XHA-tagged Trio-GEFD1 were stained with anti-HA mAb and phalloidin. Bars indicate 20  $\mu$ m.

PBS(-) and serum-starved for 30 min followed by the incubation with serum-free DMEM containing 3  $\mu$ g/ml Alexa 594-conjugated Tf (Molecular Probes) and 0.1% bovine serum albumin for the indicated periods of time. After washing, the cells were fixed, permeabilized, stained with Abs, and analyzed as described above.

## RESULTS

**ALS2 Acts as a Novel Rac1 Effector rather than a Rac1GEF**—It has been reported that ALS2 interacts with Rac1 (14) and that ALS2 overexpression enhances the level of active Rac1 in the cells in an ALS2-DH/PH-dependent manner (14, 19, 20), alluding to the ALS2 function as a Rac1GEF. Even so, both we and others have not detected the ALS2-associated Rac1GEF activity *in vitro* (12, 14). Thus, there is a discrepancy in the results of ALS2-associated Rac1GEF activity between *in vitro* and in the cells. To clarify whether ALS2 was indeed a Rac1GEF or not, we performed the pull-down using its Rac1 binding domain, CRIB, is commonly used to monitor the activation status of Rac1 in cells. Surprisingly, as opposed to the previous reports (14, 19, 20), ALS2 overexpression did not result in a significant increment of activated Rac1, GTP-bound Rac1, both in HeLa and COS-7 cells, whereas Trio-GEFD1, a well characterized Rac1GEF module, strongly activated Rac1 under the same experimental conditions (Fig. 1A). ALS2 specifically interacted with either nucleotide-free form of Rac1 or Rab5A but not with RhoA or Cdc42 *in vitro*, whereas the N-terminal RLD bound to none of these GTPases (Fig. 1B), consistent with the earlier results demonstrating that the ALS2-DH/PH directly and specifically interacts with Rac1 (14). Remarkably, ALS2 was preferentially associated with a GTP $\gamma$ S-rather than the GDP-bound form of Rac1 (Fig. 1C). Furthermore, co-immunoprecipitation revealed that



**FIGURE 2. ALS2 is sequestered by the RLD-mediated mechanism and redistributed by EGF.** *A*, schematic representation of the full-length ALS2 protein (ALS2), the alternatively spliced short form of ALS2 (ALS2<sub>S</sub>), and the ALS2 mutants used in this study. The ALS2 mutants, including ALS2<sup>P1603A</sup>, ALS2<sub>1-680</sub>, ALS2<sub>340-680</sub>, ALS2<sub>695-1657</sub>, ALS2<sub>1018-1657</sub>, and ALS2<sub>1018-1657(Δ1280-1335)</sub>, are abbreviated to P1603A, 1-680, 340-680, 695-1657, 1018-1657, and 1018-1657(Δ1280-1335), respectively. ALS2<sup>P1603A</sup>, a Rab5GEF-defective ALS2 mutant (12), and ALS2<sub>1018-1657(Δ1280-1335)</sub>, an oligomerization/Rab5GEF-defective mutant (15), have been previously described. ALS2 contains three predicted GEF domains; *i.e.* the RLD, the DH/PH, and the VPS9 domains. GCR stands for glucocorticoid receptor homologous region. *B*, distribution of the transiently expressed ALS2 or mutant ALS2. ALS2 (EGFP-ALS2) was mainly sequestered in the cytoplasm, whereas an ALS2 mutant lacking the RLD (EGFP-ALS2<sub>695-1657</sub>) was exclusively localized onto endosomes and induced their prominent enlargement. HeLa cells transfected with plasmid expressing either EGFP-ALS2 or EGFP-ALS2<sub>695-1657</sub> were stained with anti-EEA1 mAb. Note that although EGFP-ALS2 was mostly localized to cytoplasm in HeLa cells, a small proportion showed endosomal localization and induced moderately enlarged EEA1-positive endosomes. *C*, effect of EGF on the ALS2 distribution. HeLa cells were transfected with plasmid expressing EGFP-ALS2, cultured for 24 h, and treated with EGF. Representative images for the EGF unstimulated and stimulated HeLa cells are shown. *D*, subcellular distribution of endogenous ALS2. The EGF-stimulated HeLa cells were stained with anti-ALS2<sub>RLD</sub> pAb. Endogenous ALS2 is localized to membrane ruffles and vesicle-like structures. Arrowheads indicate vesicle-like structures. Bars indicate 20 μm.

ALS2 preferentially interacted with a constitutively active Rac1 (Rac1<sup>Q61L</sup>) over a dominant negative Rac1 (Rac1<sup>T17N</sup>), whereas Trio-GEFD1 rather preferentially bound to Rac1<sup>T17N</sup> (Fig. 1D). We also confirmed the direct interaction between ALS2 and Rac1 by using purified recombinant proteins (Fig. 1E). Finally, ALS2 overexpression did not induce any actin-reorganizing phenotypes in HeLa cells, whereas Trio-GEFD1 strongly induced filamentous actin (F-actin)-positive ruffles (Fig. 1F). These results together with the fact that ALS2 did not activate Rac1 *in vitro* (12, 14), indicate that ALS2 is not a Rac1GEF but, rather, acts primarily as a Rac1 effector.

**ALS2 Is Sequestered in Cytoplasm by the N-terminal RLD-mediated Mechanism**—We have previously shown that non-neuronal ALS2 is mostly sequestered in cytoplasm, and a small proportion is usually localized to EEA1-positive endosomes (12, 15), whereas a relatively larger proportion of neuronal

ALS2 is localized to endosomes (12, 14). This suggests that the ALS2 subcellular localization is controlled by its inherent domains and/or other regulatory molecules in a cell context-dependent manner. To clarify this issue, we first examined impacts of the ALS2 domains on the ALS2 subcellular localization and endosome dynamics. Transiently expressed full-length ALS2 (EGFP-ALS2) was mostly localized to cytoplasm in HeLa cells, whereas a small proportion showed endosomal localization and induced moderately enlarged EEA1-positive endosomes (Fig. 2B; compared with the neighboring untransfected cells). By contrast, the ALS2 mutant lacking the RLD (EGFP-ALS2<sub>695-1657</sub>) was exclusively localized onto endosomes and induced their prominent enlargement (Fig. 2B), which was dependent upon the ALS2-associated Rab5GEF activity (12). Because the *in vitro* Rab5GEF activity of full-length ALS2 is comparable with that of the ALS2 mutant lacking the RLD (12), the N-terminal RLD might function as an inhibitory domain for the endosomal localization but not for the Rab5GEF enzymatic activity. Furthermore, because overexpressed ALS2<sub>695-1657</sub> is localized solely onto the enlarged endosomes, it is unlikely that association of full-length ALS2 with early endosomes is saturated, but rather, full-length ALS2 may be actively sequestered in cytoplasm due to a shortage of the upstream activator(s) or the presence of inhibitory factor(s) that

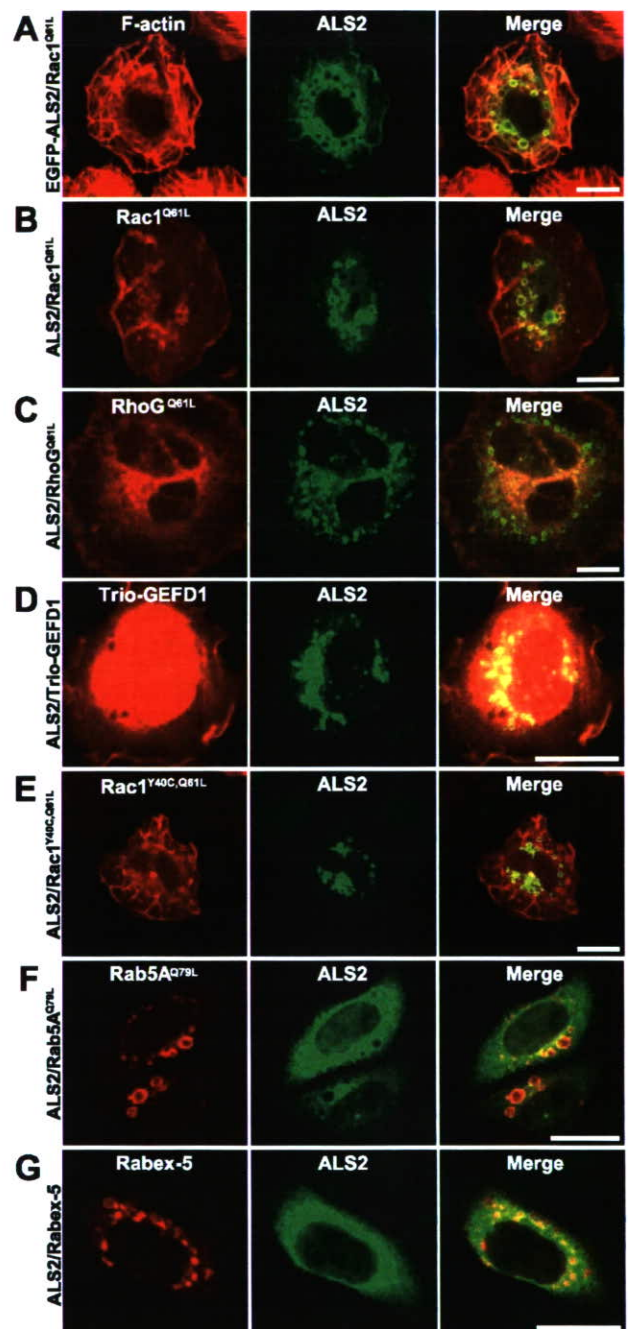
associates with the RLD. In any case the ALS2-associated Rab5GEF activity is likely to be regulated by the alteration of the intracellular localization of ALS2. In an effort to identify regulators for ALS2 that could induce the ALS2 membranous localization,<sup>6</sup> we found that EGF signaling induced the redistribution of overexpressed ALS2 from cytoplasm to EGF-induced membrane ruffles and perinuclear vesicles (Fig. 2C). Similarly, use of pAb (HPF1-680) revealed that endogenous ALS2, despite its low level expression, was also localized to membrane ruffles and large vesicle-like structures in the EGF-stimulated HeLa cells (Fig. 2D). Collectively, the intracellular localization of ALS2 may be regulated by the RLD-mediated mechanism, and upstream activator(s), such as EGF, can cancel the RLD-

<sup>6</sup> R. Kunita, A. Otomo, H. Mizumura, K. Suzuki-Utsunomiya, S. Hadano, and J.-E. Ikeda, unpublished results.

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mediated sequestration of ALS2, thereby triggering the ALS2 redistribution.

**ALS2 Is Redistributed to Membrane Ruffles and Large Endocytic Vesicles by Activated Rac1**—It has been recently demonstrated that the DH/PH of Cool-2 and Dbs interacts with activated Cdc42 and Rac1, respectively. Consistently, these RhoGEFs act as effectors for Cdc42 and Rac1, respectively (27, 28). Likewise, ALS2 preferentially interacted with activated Rac1 (Fig. 1, C and D). Furthermore, we also showed that EGF triggers the membranous localization of ALS2 (Fig. 2, C and D). These findings together with the fact that EGF signaling activates Rac1 led us to speculate that activated Rac1 is a direct upstream activator for ALS2 and alters the ALS2 subcellular localization. To prove this hypothesis, we examined whether the ALS2 subcellular localization was controlled by Rac1 in HeLa cells. In this study we have mainly analyzed the intracellular behavior of the transiently expressed ALS2 protein due to the low level expression of endogenous ALS2 in HeLa cells. Co-expression of EGFP-fused ALS2 with Rac1<sup>Q61L</sup> revealed drastic recruitment of ALS2 to the Rac1-induced ruffles (Fig. 3A). Because an ALS2 mutant, ALS2<sub>1018–1657</sub> ( $\Delta$ 1280–1335), showed a much weaker ruffle localization upon the overexpression of Rac1<sup>Q61L</sup> compared with the case of WT ALS2 (see Fig. 7B), the ruffle localization of ALS2 seems to be genuine. In addition, EGFP-fused ALS2 was also relocated onto large vesicles, reminiscent of Rac1-induced macropinosomes (Fig. 3A). Unlike the case of EGFP-fused ALS2, the untagged (Fig. 3B) or small epitope-tagged (data not shown) versions of ALS2 were exclusively localized to large vesicles and to membrane ruffles on very rare occasions (see supplemental Figs. 2 and 3B). In addition to this difference, the frequency in the redistribution of the untagged ALS2 upon the Rac1 activation is much higher than that of the EGFP-fused ALS2 (data not shown). Thus, we mostly used the untagged ALS2 in the following experiments. Activation of endogenous Rac1 by expressing either a constitutively active RhoG (RhoG<sup>Q61L</sup>) (29) or Trio-GEFD1 resulted in the ALS2 relocation onto large vesicles (Fig. 3, C and D). It has been shown that Rac1<sup>Y40C, Q61L</sup>, a Rac1 effector mutant that does not activate some of the CRIB-containing Rac1 effectors, could activate the CRIB-free Rac1 effectors (30, 31). Consistent with the fact that ALS2 does not contain any typical CRIB domain, Rac1<sup>Y40C, Q61L</sup> redistributed ALS2 as efficiently as did Rac1<sup>Q61L</sup> (Fig. 3E). On the other hand, ALS2 was rarely redistributed by Cdc42<sup>Q61L</sup> and never by RhoA<sup>Q63L</sup> (data not shown). Next we examined whether ALS2 was redistributed onto the Rab5-induced large endosomes. Rab5 signaling induces enlarged endosomes (21), seemingly quite similar to the Rac1<sup>Q61L</sup>-induced large vesicles, but the mechanism for their induction is thought to be different. Because Rab5 seems to be essential for the induction of dorsal ruffles, a prerequisite for a mode of endocytosis called macropinocytosis (25), we could not preclude the possibility that Rab5 signaling activates Rac1 by unknown mechanism, thereby redistributing ALS2 to intracellular vesicles through macropinocytosis. However, when coexpressed with a constitutively active Rab5A (Rab5A<sup>Q79L</sup>), the distribution of a major-



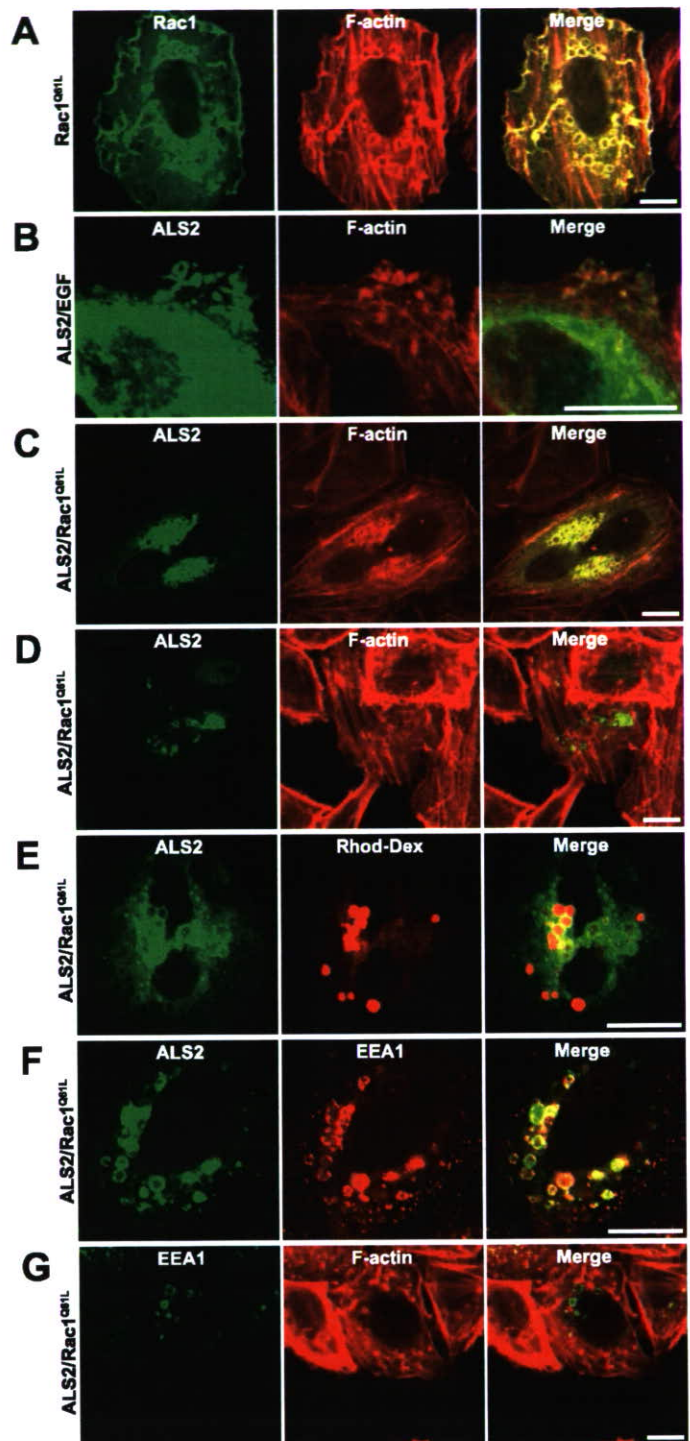
**FIGURE 3. ALS2 is redistributed to membrane ruffles and large endocytic vesicles upon Rac1 signaling.** A–G, changes in the intracellular ALS2 localization elicited by the activation of different GTPases. HeLa cells were transfected with expression construct for EGFP-fused (A) or untagged (B–G) ALS2 along with the plasmid expressing 3XFLAG-tagged Rac1<sup>Q61L</sup> (A and B), RhoG<sup>Q61L</sup> (C), Trio-GEFD1 (D), Rab5A<sup>Q79L</sup> (F), Rabex-5 (G), or 2XHA-tagged Rac1<sup>Y40C, Q61L</sup> (E). After fixation and permeabilization, the cells were stained with either a combination of phalloidin and anti-ALS2\_RLD pAb (A), anti-FLAG mAb and anti-ALS2\_RLD pAb (B–D and G), anti-HA mAb and anti-ALS2\_RLD pAb (E), or anti-Rab5 mAb and anti-ALS2\_RLD pAb (F). Only activated Rac1 but not Rab5 can induce the redistribution of ALS2 to membrane ruffles and endocytic vesicles. Bars indicate 20  $\mu$ m.

ity of cytoplasmic ALS2 was unchanged, although a small proportion was recruited to the enlarged endosomes (Fig. 3F), and its frequency was increased with culture duration (data not shown). Similarly, when coexpressed with Rabex-5, the best-studied Rab5GEF, ALS2 was not efficiently codistributed with Rabex-5 on large vesicles (Fig. 3G). These

results suggest that Rac1 activation by Rab5 is, if any, not effective enough to fully activate ALS2. Instead, it is conceivable that a small proportion of ALS2 is activated by endogenous Rac1 and then relocalized to endosomes that could fuse with the Rab5-induced large endosomes. Taken together, Rac1, a direct ALS2 interactor, immediately alters the ALS2 subcellular localization. Thus, ALS2 is a *bona fide* Rac1 effector redistributing onto Rac1-induced vesicles through Rac1 signaling.

**ALS2 Is Redistributed to Macropinosomes via Macropinocytosis**—We next characterized the nature of ALS2-relocalized endosomes. It has been shown that activated Rac1 induces membrane ruffles and macropinocytosis, resulting in the formation of large F-actin positive pinosomes filled with solute macromolecules, called macropinosomes (32). Subsequently, most F-actin molecules are dissociated from macropinosomes during the vesicle maturation (33). First, we confirmed the colocalization of Rac1<sup>Q61L</sup> with F-actin in dorsal ruffles and large vesicles (Fig. 4A) and found that Rac1<sup>Q61L</sup> indeed induced macropinocytosis. We also confirmed that when treated with EGF, the ALS2-localized ruffles and vesicles were F-actin-positive (Fig. 4B). When coexpressed ALS2 with Rac1<sup>Q61L</sup>, ALS2 was localized onto F-actin-positive ruffles and large vesicles (Figs. 3A and 4C). However, in a majority of the cells, ALS2 was present on F-actin-negative vesicles (Fig. 4D) or showed a mosaic distribution in which ALS2 was localized onto both F-actin (Rac1)-positive and -negative vesicles (Fig. 3, A, B, and E), supporting the notion that F-actin is rapidly dissociated after internalization. Furthermore, most of the ALS2-positive vesicles were strongly labeled with dextran, a fluid-phase endocytic marker (Fig. 4E). Thus, ALS2 may be recruited to ruffles by Rac1 and then relocalized onto the macropinosomes via Rac1-activated macropinocytosis.

**ALS2 Is Not Essential to Macropinocytosis**—Because Rab5 signaling is crucial for the induction of dorsal ruffles (25), a prerequisite for macropinocytosis, we tested whether ALS2 contributed to macropinocytosis by activating Rab5. We utilized three different types of cells, HeLa cells, MEFs, and hippocampal neurons. First, we examined the dextran uptake in HeLa cells expressing ALS2 and/or Rac1. As reported previously, Rac1<sup>Q61L</sup> strongly enhanced the dextran uptake (Fig. 5A, Rac1<sup>Q61L</sup>). However, overexpression of ALS2 alone did not enhance the uptake, supporting a notion that ALS2 is not a Rac1GEF (Fig. 5A, ALS2). When coexpressed ALS2 with Rac1<sup>Q61L</sup>, there was no observable increment in the level of dextran uptake by ALS2 (Fig. 5A, ALS2/Rac1<sup>Q61L</sup>), indicating that enhancement of macropinocytosis by ALS2 is undetectable in HeLa cells overexpressing Rac1<sup>Q61L</sup>. In addition, the dorsal ruffles, which are shown to be tightly connected to macropinocytosis (25), were indistinguishably induced both in WT and *Als2*-KO MEFs upon PDGF stimulation (Fig. 5B). Furthermore, both the WT and *Als2*-KO primary-cultured hippocampal neurons showed at least certain activities for the dextran uptake (Fig. 5C), consistent with the observation that there are no qualitative differences in the dextran uptake between WT and *Als2*-KO cells (17). Thus, macropinocytosis is not completely disturbed by loss of ALS2. In other words, ALS2 is not essential for macropinocytosis. Because Rab5 is crucial for the



**FIGURE 4. ALS2 is redistributed to macropinosomes via macropinocytosis.** HeLa cells transfected with p3XFLAG-CMV10-Rac1<sup>Q61L</sup> (Rac1<sup>Q61L</sup>) (A) or pEGFP-ALS2\_L (ALS2) (B) or co-transfected with p3XFLAG-CMV10-Rac1<sup>Q61L</sup> and pCneo-ALS2\_L (C–G) were stained with either anti-FLAG mAb and phalloidin (A), phalloidin (B), anti-ALS2\_RLD pAb and phalloidin (C and D), anti-ALS2\_RLD pAb (E), anti-ALS2\_RLD pAb and anti-EEA1 mAb (F), or anti-EEA1 mAb and phalloidin (G). HeLa cells were treated with EGF (B) or with rhodamine-dextran (70 kDa) (E) before fixation. Bars indicate 20 μm.

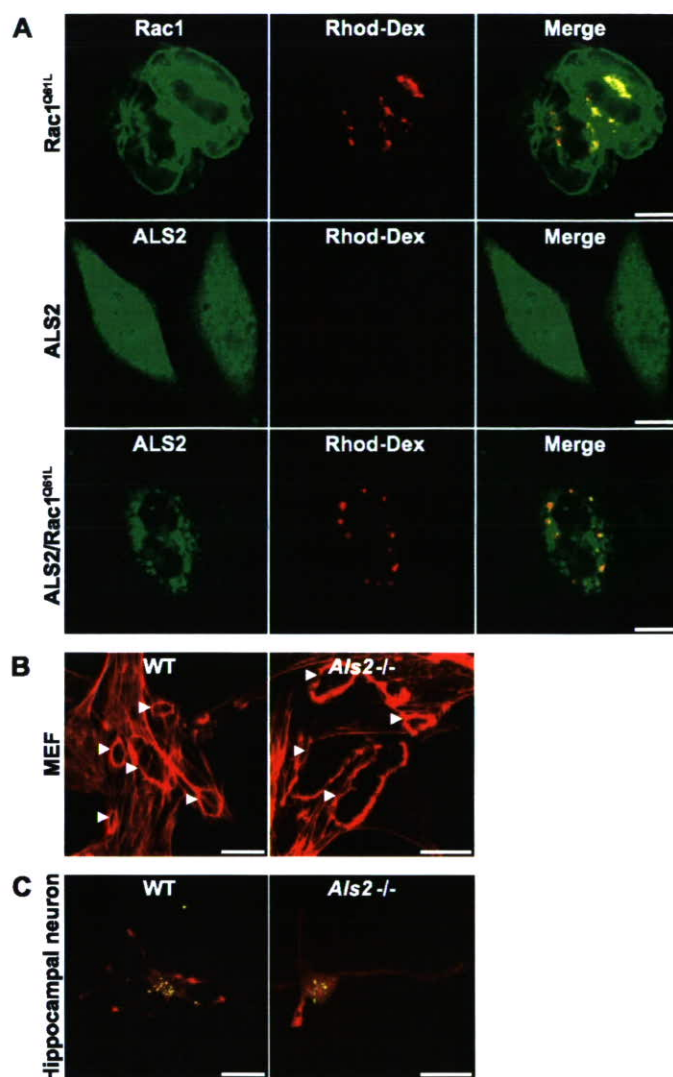
induction of dorsal ruffles (25), there may be redundant Rab5GEF(s) responsible for the dorsal ruffle induction and after macropinocytosis. Nevertheless, our quantitative analyses for the horseradish peroxidase uptake using ALS2-deficient cells suggested that ALS2 slightly but significantly contributed to

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macropinocytosis in certain types of the cells including neurons.<sup>7</sup> Because functional redundancy of the Rab5GEFs responsible for the dorsal ruffle formation and/or macropinocytosis may vary among different cell types, it is reasonable that ALS2 substantially contributes to macropinocytosis *in vivo* in a cell context-dependent manner. Future careful assessment will clarify this issue.

**Macropinosomal ALS2 Enhances Fusion of Macropinosomes with CME-derived Endosomes through Its Rab5GEF Activity**—Because Rab5 regulates not only endocytosis but also fusion and trafficking of endosomes, it is likely that ALS2 affected the maturation status of the ALS2-localized macropinosomes. It has been demonstrated that a symmetrical Rab5 activation on both endosomes is required for endosome-endosome fusion (34). Rab5 activation sequentially recruits EEA1 onto endosomes, and EEA1 is a core component of endosome docking (35, 36). Thus, the EEA1 recruitment to endosomes through Rab5 activation is prerequisite for endosome fusion. Consistently, it has been recently shown that the recruitment of EEA1 to macropinosomes is essential for the fusion of macropinosomes (37). Therefore, EEA1 plays a pivotal role in fusion of not only classical early endosomes but also macropinosomes. Because ALS2 certainly contributes to endosome fusion (16–18), we first examined whether EEA1 was recruited to the ALS2-localized macropinosomes. Indeed, EEA1 was extensively recruited onto the ALS2-localized macropinosomes (Fig. 4F). F-actin and EEA1 were mutually exclusive on macropinosomes (Fig. 4G), consistent with a notion that EEA1 is recruited to macropinosomes after F-actin dissociation (38). Thus, a majority of ALS2-localized vesicles may represent matured EEA1-positive macropinosomes. Furthermore, full-length ALS2 alone (without Rac1 overexpression) also moderately recruits EEA1 onto endosomes and induces their modest enlargement (Fig. 1B; compared with the neighboring untransfected cells). Taken together, it is hypothesized that the ALS2-mediated Rab5 activation on macropinosomes enhances the EEA1 recruitment, thereby augmenting fusion of macropinosomes with other macropinosomes and/or other types of endosome.

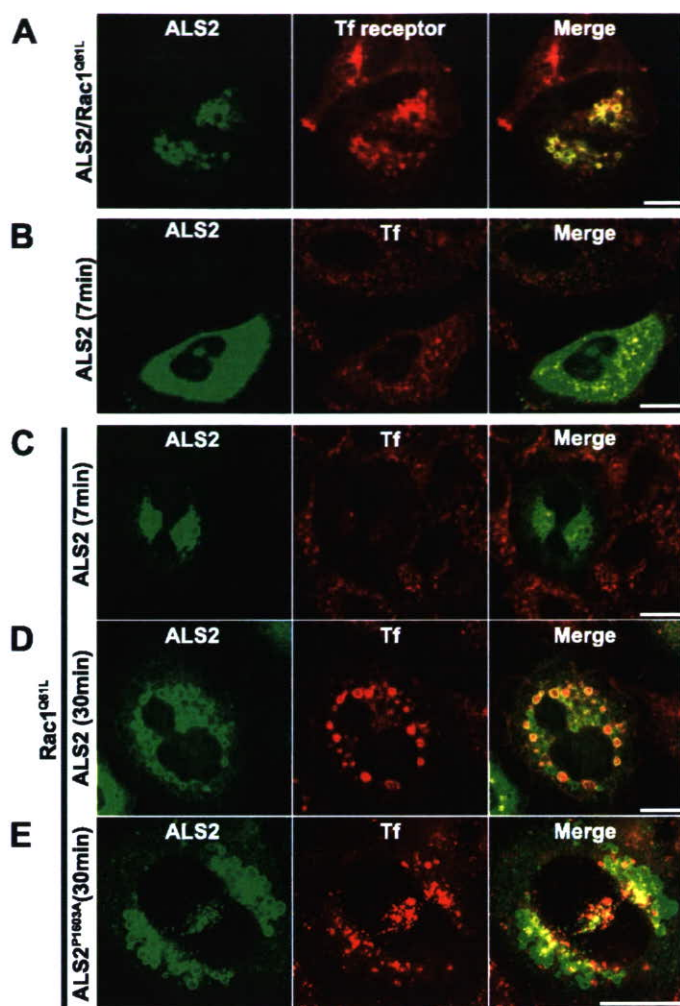
To verify this hypothesis, we first analyzed the localization of Tf receptor, an early and recycling endosomes marker. Consistent with our hypothesis, Tf receptor and ALS2 were extensively colocalized onto the Rac1-induced large vesicles (Fig. 6A), indicating that ALS2-localized macropinosomes extensively fused with CME-derived/Tf receptor-localized endosomes. We next investigated whether macropinosomal ALS2 enhanced fusion between the ALS2-localized and the incoming Tf-positive endosomes. After 24 h of transfection, HeLa cells expressing ALS2 or coexpressing both ALS2 and Rac1<sup>Q61L</sup> were fed with Alexa594-labeled Tf for the indicated duration. Without Rac1 stimulation, expression of ALS2 alone did not alter the Tf uptake (Fig. 6B). Furthermore, consistent with the published finding that a constitutively active Rac1 inhibits CME (39) when coexpressed ALS2 with Rac1<sup>Q61L</sup>, Rac1<sup>Q61L</sup> significantly inhibited the Tf uptake after a 7-min incubation with Tf (Fig. 6, B and C). However, when fed with Tf for 30 min, strong Tf signals on



**FIGURE 5. ALS2 is not vital for macropinocytosis *per se*.** A, dextran uptake analysis in HeLa cells. HeLa cells transfected with plasmid expressing either FLAG-tagged Rac1<sup>Q61L</sup> or untagged ALS2 and those co-transfected with both plasmids were incubated with rhodamine-dextran (*Rhod-Dex*; red) for 30 min followed by immunostaining with either anti-FLAG mAb for Rac1 (*green*) (top row) or anti-ALS2\_RLD pAb (*green*) (middle and bottom rows). B, PDGF indistinguishably induces the circular ruffles in both WT and *AIs2*-KO MEFs. MEFs were serum-starved for 20 h and then treated with PDGF. F-actin staining was carried out immediately afterward. Arrowheads indicate circular ruffles induced by PDGF. C, dextran uptake analysis in neuronal cell cultures. Primary hippocampal cell cultures were incubated in the media containing 2 mg/ml 70-kDa fluorescein-dextran (*green*) for 30 min. After fixation and permeabilization, the cells were stained with Alexa 594-conjugated phalloidin (*red*) to visualize neuronal cell shapes. Merged images are shown. Bars indicate 20 μm.

the ALS2-localized macropinosomes were detected (Fig. 6D), showing intensive fusion between the Tf-positive endosomes and ALS2-localized macropinosomes. In stark contrast, when cotransfected Rac1<sup>Q61L</sup> with a Rab5GEF-defective ALS2<sup>P1603A</sup> (10), a weak labeling with Tf on the ALS2<sup>P1603A</sup>-localized macropinosomes was observed after 30 min of incubation (Fig. 6E), yet Tf signals on the ALS2<sup>P1603A</sup>-localized macropinosomes were gradually increased when incubated with Tf for a longer period of time (>2 h) (data not shown). Remarkably, the EEA1 recruitment to the ALS2<sup>P1603A</sup>-localized macropinosomes was less efficient (supplemental Fig. 1) compared with that to the WT ALS2-localized macropinosomes (Fig. 4F). This is consist-

<sup>7</sup> A. Otomo, R. Kunita, K. Suzuki-Utsunomiya, H. Mizumura, K. Onoe, H. Osuga, S. Hadano, and J.-E. Ikeda, submitted for publication.



**FIGURE 6. Macropinosomal ALS2 augments fusion between the ALS2-localized macropinosomes and CME-derived endosomes.** *A*, HeLa cells expressing both ALS2 and Rac1<sup>Q61L</sup> were stained with anti-ALS2<sub>RLD</sub> pAb and anti-Tf receptor mAb. *B–E*, HeLa cells were either transfected with plasmid expressing ALS2 (*B*) or co-transfected with those expressing ALS2 and Rac1<sup>Q61L</sup> (*C* and *D*) or ALS2<sup>P1603A</sup> and Rac1<sup>Q61L</sup> (*E*). After 24 h the cells were incubated with Alexa 594-conjugated Tf for either 7 min (*B* and *C*) or 30 min (*D* and *E*). ALS2 (green) and Tf (red) were fluorescently detected. Bars indicate 20  $\mu\text{m}$ .

ent with the fact that the EEA1 recruitment is dependent on the activated Rab5-mediated signaling including the Rab5 binding to EEA1 and Rab5-mediated activation of phosphatidylinositol-3-OH kinase (21). To investigate the involvement of endogenous ALS2 in the fusion of macropinosomes, we conducted a similar experiment using the Rac1<sup>Q61L</sup>-transfected or non-transfected WT or *Als2*-KO MEFs. As a result, even in *Als2*-KO MEFs, a significant colocalization of dextran with Tf was observed (data not shown), indicating that endogenous ALS2 is not essential for the fusion of macropinosomes in MEFs and that redundant Rab5GEF(s) could compensate a loss of the ALS2 functions.

Our results here indicate that macropinosomal ALS2 is active in regard to the Rab5GEF enzymatic activity, thereby augmenting fusion between the ALS2-localized macropinosomes and Tf-positive endosomes by potentiating the fusion competence of macropinosomes through the Rab5 activation and the EEA1 recruitment. At the same time it is also true that

other endogenous redundant Rab5GEF(s) is involved in this cellular process. Because the Rab5 signalings are known to be important for maturation of endosomes in many aspects (21), future studies will clarify the relevance of redundant Rab5GEFs including ALS2 in trafficking of macropinosomes and the homotypic fusion (between macropinosomes) as well as the heterotypic endosome fusion (between macropinosomes and other endosomal compartments).

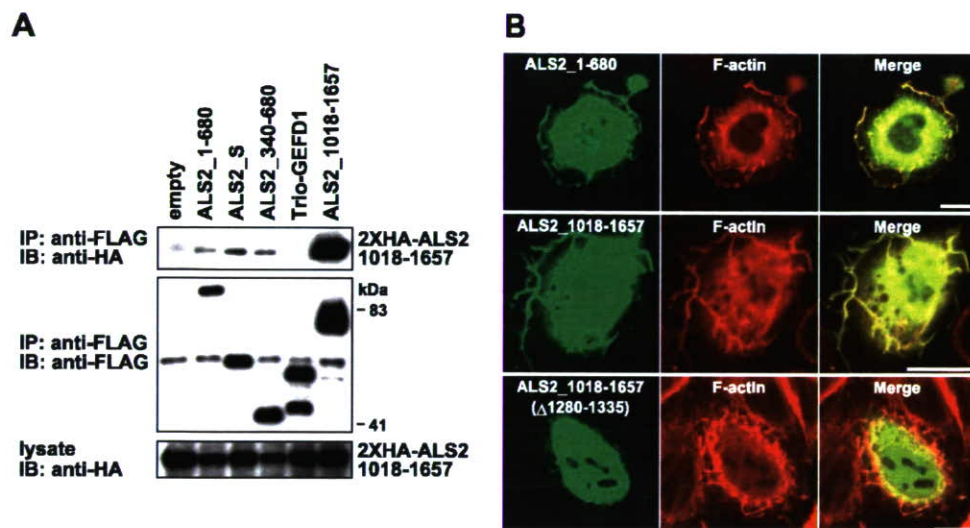
*Each of the ALS2 Domains Plays Important Roles in the Regulation of the ALS2 Subcellular Localization*—Finally, we investigated whether and how the ALS2 domains were involved in the ALS2 sequestration and the association with membrane ruffles and/or macropinosomes. Because ALS2 acts as a Rac1 effector, a regulatory mechanism underlying the activation and/or inactivation of ALS2 might be similar to that of other Rac1 effectors. Thus, we hypothesized as follows. The N and C termini interact with each other, thereby masking the domains responsible for the ruffle localization. Rac1 binding to ALS2 disrupts the interaction between the N and C termini, thereby exposing the domains for the ruffle localization. First, we tested whether the N and C termini interacted with each other. Co-immunoprecipitation analysis showed that the N-terminal RLD (ALS2<sub>1–680</sub>) indeed preferentially interacted with the C-terminal MORN/VPS9 (ALS2<sub>1018–1657</sub>) (Fig. 7A). Interestingly, although the full-length ALS2 is exceptionally non-ionic detergent-soluble, both the isolated RLD and MORN/VPS9 domains are much more insoluble (data not shown). These results suggest that hydrophobic domains within the N and C termini may be hidden by the interaction. Because ALS2 is not present as monomer, but rather forms a homophilic oligomer through C-terminal MORN/VPS9 domains (15), the mode of the interaction between the N and C termini, *i.e.* inter- or intramolecular interaction, is currently undefined.

Next, we searched for the domains responsible for the ruffle localization and found that the MORN/VPS9 (ALS2<sub>1018–1657</sub>) is one of the determinants for the ruffle localization (Fig. 7B), consistent with the published finding that the MORN/VPS9 is important for the ALS2 membranous localization (12). We have previously shown that ALS2 lacking the exon 25-coded region, ALS2<sub>1018–1657</sub> ( $\Delta$ 1280–1335), is oligomerization-defective (15). Despite that this mutant still retained intact MORN and VPS9 domains (see Fig. 2A), it was not efficiently localized to membrane ruffles (Fig. 7B), suggesting that the properly oligomerized C-terminal domain is, rather, a determinant for the ruffle localization of ALS2. We also found that although the RLD (ALS2<sub>1–680</sub>) was important for the cytoplasmic sequestration of ALS2, it was also responsible for the ruffle localization (Fig. 7B). This result implies that after the Rac1 binding, the interaction of the RLD with ruffles prevents ALS2 from re-sequestering by the self-interaction between the RLD and the MORN/VPS9.

Intriguingly, neither the N nor C termini alone was sufficient for the efficient redistribution of ALS2 from membrane ruffles to macropinosomes (Fig. 7B; data not shown). When the full-length ALS2 carrying a non-synonymous mutation (W996A) in the DH/PH domain was coexpressed with Rac1<sup>Q61L</sup>, it was significantly recruited to membrane ruffles but not to macropinosomes (supplemental Fig. 2). Furthermore, none of the sole



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**FIGURE 7. Identification of the domains required for cytoplasmic sequestration (A) and ruffle localization (B) of ALS2.** A, the N-terminal RLD (ALS2\_1–680) interacts with the C-terminal MORN/VPS9 domain (ALS2\_1018–1657). Lysates from COS-7 cells transfected with either pCneo-empty vector, pCneoFLAG-ALS2\_1–680, pCneoFLAG-ALS2\_S (the short form of ALS2; see Fig. 2A), pCneoFLAG-ALS2\_340–680, pCneoFLAG-Trio-GEFD1 (as a negative control), or pCneoFLAG-ALS2\_1018–1657 along with pCneo2XHA-ALS2\_1018–1657 were immunoprecipitated with anti-HA affinity gels. Lysates (bottom) and immunoprecipitates (top and middle) were analyzed by immunoblotting with the Abs indicated. Note that FLAG-tagged ALS2\_1018–1657 strongly interacted with 2XHA-tagged ALS2\_1018–1657, as we have previously shown (15). IP, Ab used for immunoprecipitation; IB, Ab used for Western blot analysis. B, both the RLD and MORN/VPS9 domains were recruited to the Rac1-induced ruffles. HeLa cells transfected with plasmid expressing either FLAG-tagged ALS2\_1–680 (RLD), ALS2\_1018–1657 (MORN/VPS9), or ALS2\_1018–1657 (Δ1280–1335) along with plasmid expressing Rac1<sup>Q61L</sup> were fixed, permeabilized, and stained for ALS2 (green) (top row, anti-ALS2\_RLD pAb; middle and bottom rows, anti-ALS2\_MORN/VPS9 pAb) and F-actin (red; phalloidin). Bars indicate 20 μm.

domains including the DH/PH has been assigned as a determinant for the macropinosomal localization (data not shown). Thus, intact DH/PH domain of ALS2 is indispensable for the ALS2 redistribution from membrane ruffles to macropinosomes but not from cytoplasm.

Last, we tested whether the ALS2-associated Rab5GEF activity could facilitate the ALS2 redistribution from ruffles to macropinosomes. When coexpressed the Rab5GEF-defective ALS2<sup>P1603A</sup> (12) with Rac1<sup>Q61L</sup>, ALS2<sup>P1603A</sup> was efficiently redistributed to Rac1-induced macropinosomes (supplemental Fig. 3A), indicating that the ALS2-associated Rab5GEF activity is not essential for the macropinosomal distribution of ALS2. Nonetheless, there is a slight difference between the WT and the Rab5GEF-defective ALS2 proteins in the intracellular behavior. ALS2<sup>P1603A</sup> tended to be simultaneously localized onto both intracellular macropinosomes and membrane ruffles, whereas the WT ALS2 was exclusively localized to the intracellular macropinosomes (supplemental Fig. 3B), suggesting that the ALS2-associated Rab5GEF activity may provide a positive impact on the ALS2 redistribution from ruffles to macropinosomes.

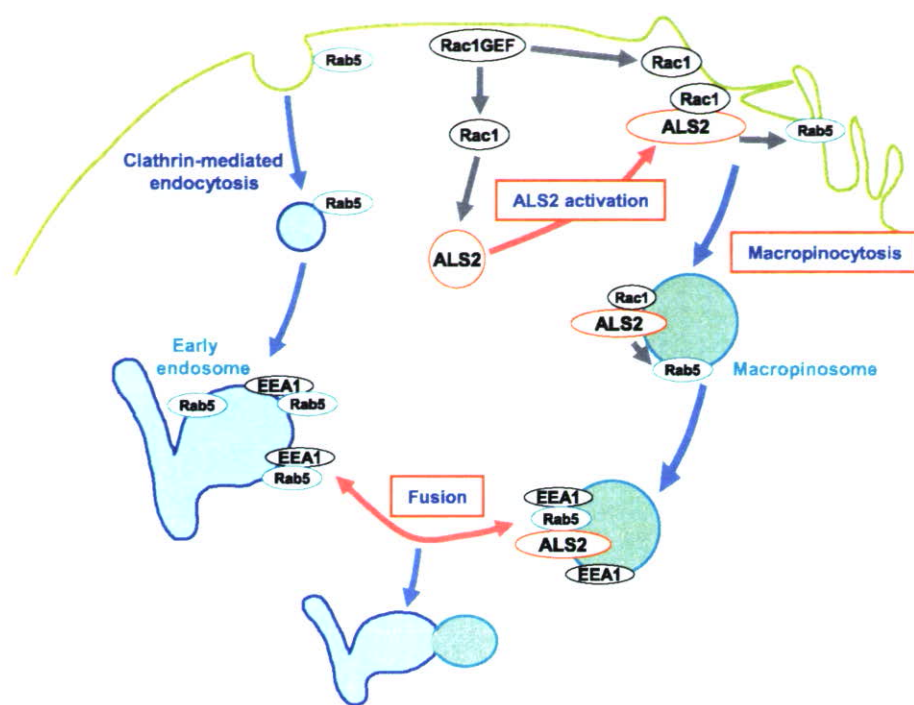
All together, all of the RLD, DH/PH, and MORN/VPS9 domains of ALS2 seem to contribute to the intracellular behavior of ALS2, which is associated with macropinosome-related functions. Here, we propose the spatiotemporal regulation of the ALS2-mediated Rab5 activation as follows. ALS2 is sequestered in cytoplasm in its dormant state through the RLD-mediated inhibitory mechanism. Yet-to-be-identified upstream signals may consequently activate Rac1, and activated Rac1 turns

ALS2 into its active state via the direct interaction, thereby rendering the RLD and MORN/VPS9 to associate with Rac1-induced ruffles. The ruffle-localized ALS2 is then redistributed to macropinosomes via macropinosocytosis. ALS2 may contribute to macropinosocytosis in a cell context-dependent manner. Ultimately, macropinosomal ALS2 mediates endosome fusion (Fig. 8). Future studies are required to fortify this hypothesis.

## DISCUSSION

Here we have provided compelling evidence that ALS2 acted as a Rac1 effector rather than a Rac1GEF. First, consistent with the fact that ALS2 does not exhibit the Rac1GEF activity *in vitro* (12, 14), our pull-down assays revealed that ALS2 overexpression did not activate Rac1 both in HeLa and COS-7 cells. We utilized Trio-GEFD1, a well known Rac1GEF module, as a positive control and conducted assays in parallel, since no positive control was included in all previous

studies. Our results indicate that the ALS2-associated Rac1GEF activity is, if any, certainly negligible in these cells (Fig. 1). Second, ALS2 overexpression did not induce any actin-reorganizing phenotypes, whereas Trio-GEFD1 did strongly. Third, given that ALS2 is a Rac1GEF, it could activate Rac1, thereby significantly enhancing a fluid-phase endocytosis. However, we have not obtained such results by the ALS2 overexpression (Fig. 5). Fourth, we have shown that ALS2 is sequestered in cytoplasm unless Rac1 signaling is activated. If ALS2 acted as a Rac1GEF, ALS2 would be autonomously activated/redistributed through its intrinsic Rac1GEF activity by the mechanism that we have proposed here (Fig. 8). In fact, the ALS2 mutants carrying a mutation(s) within the DH including E697A, T701A, and Q869A/D870A, whose analogous mutations within the prototypic RhoGEF, Dbl, are subversive (40), were redistributed by activated Rac1 as similarly as was WT ALS2 (supplemental Fig. 4A). Furthermore, the ALS2-DH mutant (ALS2<sup>T701A</sup>) could also interact with Rac1 *in vitro* (supplemental Fig. 4B) as does WT ALS2 (Fig. 1B), showing that the evolutionary-conserved residues within the DH domain are dispensable for the ALS2 redistribution. All together, we concluded that ALS2 is not a Rac1GEF but, rather, primarily acts as a Rac1 effector (Figs. 3 and 4), and its intracellular behavior is dependent on the external Rac1 signaling. Previous studies have documented that ALS2 acts as a Rac1GEF (14, 19, 20). Thus far, the reason for the discrepancy remains unclear. Although we could not exclude the possibility that ALS2 acts as a bifunctional protein (Rac1GEF and Rac1 effector) in certain cell types, it may be because the complex formation between ALS2- and GTP-



**FIGURE 8. Models for the ALS2 activation and the potential roles of ALS2 in membrane/endosome dynamics.** ALS2 is sequestered in the cytoplasm when inactivated. Yet-to-be-identified Rac1 activator (*Rac1GEF*) may activate Rac1 in response to upstream signals. Then activated Rac1 binds directly to ALS2, resulting in the ALS2 recruitment to the ruffles followed by relocalization via macropinocytosis, the ALS2 activation. ALS2 may contribute to a mode of endocytosis called macropinocytosis in a cell context-dependent manner. Subsequently, ALS2 is localized to nascent macropinosomes. Macropinosomal ALS2 activates Rab5 and enhances the EEA1 recruitment, thereby promoting maturation (fusion and trafficking) of the ALS2-localized macropinosomes. We propose that macropinocytosis and/or the following maturation of macropinosomes modulated by ALS2 might be essential for the integrity of motor neurons.

bound (activated) Rac1 increases in the stabilization of activated Rac1 presumably by inhibiting its intrinsic GTPase activity, thereby enhancing the level of activated Rac1.

Several RhoGEFs are known to act as effectors for other Rhos. For example, the Rac1GEF complex DOCK180/Elmo, the RhoAGEF Dbs, and the Rac1GEF Cool-2 are RhoG, Rac1, and Cdc42 effectors, respectively (27–29). Additionally, the Rab5GEF RIN1 is regulated by Ras binding (41). Upon Rac1 signaling, ALS2 is initially recruited to ruffles and then relocalized to macropinosomes (Fig. 8). Interestingly, even after both Rac1 and F-actin are dissociated from these membranes, ALS2 still resides onto the matured macropinosomes and commits endosome fusion through its Rab5GEF activity. These results indicate that the ALS2 function is regulated by altering its subcellular localization, although there is the yet-to-be-identified mechanism by which ALS2 is kept activated without Rac1 binding. Rac1 binding to ALS2 may be important for the initiation of the ALS2 redistribution but not for functional modulation, such as potentiation of the Rab5GEF activity. Interestingly, the Rab5GEF RIN1 is recruited to tyrosine-phosphorylated residues in cytoplasmic tails of many receptor-tyrosine kinases upon activation of the receptors (42, 43). Thus, alteration of the subcellular localization of Rab5GEFs, such as ALS2 and RIN1, may be a common means of the spatiotemporal activation of Rab5 signaling in response to upstream signals. Recently, the first ALS2 missense mutation in the RLD domain has been reported (26). Because this mutant ALS2 is hardly localized onto EEA1-positive endosomes, it is interesting to test whether

the mutant is unresponsive to the Rac1 signaling. It has also been shown that ALS2 is involved in endosomal trafficking of insulin-like growth factor 1 receptor (17). Because many trophic factors including insulin-like growth factor 1 are known to activate Rac1, it is possible that the ALS2 function is regulated by trophic factor receptor-mediated Rac1 activation. Indeed, here we showed that EGF induced the ALS2 redistribution in HeLa cells. Future studies will identify *bona fide* upstream trophic factors, which redistribute/activate ALS2 by activating Rac1 *in vivo*.

ALS2 is the Rab5GEF clearly localized onto F-actin-positive macropinosomes and involved in Rac1-activated macropinocytosis. Endocytosis involves multiple internalization mechanisms such as CME, macropinocytosis, caveolin-dependent endocytosis, and other clathrin- and/or caveolin-independent pathways (44, 45). Because most studies have focused on CME or caveolin-dependent endocytosis, little is known about macropinocytosis.

Here we shed light on an unfamiliar endocytosis, macropinocytosis in neurons. Different endocytic systems initially give rise to distinct endocytic vesicles, but depending on the cellular context, they mature into specialized membrane compartments by fusing together, mixing, and/or exchanging internalized materials (46, 47). In fact, macropinosomes may fuse homotypically with other macropinosomes and, in some cases, heterotypically with distinct endosomal compartments, suggesting that there is a Rab5GEF(s) localized on macropinosomes (37). Thus, a newly discovered macropinosomal localization of ALS2 provides the missing link. Interestingly, two key regulators for macropinocytosis, Rac1 and Rab5, function upstream and downstream of ALS2, respectively. In other words, ALS2 converts Rac1 signaling into Rab5 signaling at the sites where ALS2 is recruited. Because ALS2 intrinsically possesses a Rab5GEF activity and Rac1 binding site, the ALS2 role on regulation of Rac1-Rab5 signaling may be universal among the different cell types.

Here we showed that ALS2 was involved in but not vital for both macropinocytosis and fusion of the ALS2-localized macropinosomes with the Tf-positive endosomes in some cell types. However, our quantitative analyses of the uptake of horseradish peroxidase, a fluid-phase endocytosis marker, in different cell types from either WT or *Als2*-KO mice revealed that ALS2 substantially contributes to macropinocytosis in a certain type of neurons but does not in MEFs.<sup>7</sup> In other words, ALS2 is not the sole factor responsible for macropinocytosis and fusion of macropinosomes, and there is

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obviously a cell type-dependent functional redundancy for Rab5GEF(s), which is predictable and reasonable. Because, other than certain neurological defects, ALS2-deficient patients show the normal function of other organs and tissues, it is unlikely that patients completely lack these fundamental cellular processes. Furthermore, we and others have generated *Als2*-null mice and reported that these mice showed no obvious developmental and reproductive abnormalities (16, 17, 48, 49), supporting that the redundant Rab5GEFs may compensate the loss of ALS2 in most tissues *in vivo*. In *Caenorhabditis elegans*, two unrelated Rab5GEFs, RME-6 and Rabex-5, are partially redundant for Rab5 activation at the plasma membrane (50). Thus, multiple Rab5GEFs might be cooperatively/redundantly responsible for endocytic processes in organisms. Importantly, it has been suggested that two Rab5GEFs, RAP6/GAPex-5 and RIN1, both enhance macropinocytosis (43, 51, 52). These Rab5GEFs can be candidates for redundant Rab5GEFs in the ALS2-mediated cellular processes. In fact, ALS2 and RIN1 may be redistributed/activated simultaneously upon the activation of trophic factor receptors by Rac1 activation and tyrosine-phosphorylated cytoplasmic tails of the receptors, respectively. Therefore, it is particularly intriguing to investigate whether their functions are partially redundant in trophic factor-inducing signaling.

As aforementioned, we would emphasize that although ALS2 is not the sole factor responsible for macropinocytosis, it may contribute to this mechanism in certain cell types. Because there is no doubt that only the proteins associated with the plasma membrane but not in cytoplasm or on endosomes could contribute to endocytosis (an internalization step), our two independent findings, the transient membrane ruffle localization of ALS2 upon Rac1 signaling and the contribution to macropinocytosis of ALS2, are fully consistent with this notion.

Mutations in *ALS2* cause a number of recessive motor neuron diseases. This group of diseases is described as a spastic pseudobulbar syndrome with spastic paraplegia involving a dysfunction of upper motor neurons and occasionally associated with several signs of lower motor neuron defects. However, the pathogenesis of these motor neuron diseases remains elusive. In this study, to delineate the normal physiological ALS2 role, we investigated concrete processes within membrane dynamics modulated by ALS2. We show here that ALS2 has potential for contribution to both macropinocytosis and the cross-talk between macropinocytosis and other endocytic mechanisms. Because ALS2 and Rac1 are colocalized to the F-actin-positive vesicles in primary-cultured neurons (19),<sup>7</sup> it can be true that ALS2 is involved in macropinocytosis and the endosome fusion in neurons, thereby maintaining the integrity of neuronal cells, particularly upper motor neurons. In any case ALS2 may be activated via macropinocytic pathway, suggesting that the macropinocytosis-mediated signal(s) preserves motor neurons. Future studies will provide further insights not only into the fundamental role of ALS2 in macropinocytosis and the endosomal cross-talk in neurons but also into pathogenesis underlying the ALS2-linked motor neuron diseases.

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