LRCH1 interferes with DOCK8-Cdc42–induced T cell migration and ameliorates experimental autoimmune encephalomyelitis

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Directional autoreactive CD4+ T cell migration into the central nervous system plays a critical role in multiple sclerosis. Recently, DOCK8 was identified as a guanine–nucleotide exchange factor (GEF) for Cdc42 activation and has been associated with human mental retardation. Little is known about whether DOCK8 is related to multiple sclerosis (MS) and how to restrict its GEF activity. Using two screening systems, we found that LRCH1 competes with Cdc42 for interaction with DOCK8 and restrains T cell migration. In response to chemokine stimulation, PKCα phosphorylates DOCK8 at its three serine sites, promoting DOCK8 separation from LRCH1 and translocation to the leading edge to guide T cell migration. Point mutations at the DOCK8 serine sites block chemokine- and PKCα-induced T cell migration. Importantly, Dock8 mutant mice or Lrch1 transgenic mice were protected from MOG (35–55) peptide–induced experimental autoimmune encephalomyelitis (EAE), whereas Lrch1-deficient mice displayed a more severe phenotype. Notably, DOCK8 expression was markedly increased in PBMCs from the acute phase of MS patients. Together, our study demonstrates LRCH1 as a novel effector to restrain PKCα–DOCK8–Cdc42 module–induced T cell migration and ameliorate EAE.

INTRODUCTION

Multiple sclerosis (MS) is considered a T cell–mediated inflammatory and demyelinating disease of the CNS with a complex genetic background (McFarland and Martin, 2007). Autoreactive CD4+ T lymphocyte extravasation and infiltration into the CNS is a finely regulated cascade of steps that is controlled by integrins, chemokines, or inflammatory cytokines (Sigal et al., 2000; Vajkoczy et al., 2001; Kerfoot and Kubes, 2002; Ransohoff et al., 2003). Chemokines such as SDF-1 (also termed CXCL12) and CCL5 are increased in MS lesions, which attract T lymphocytes across the endothelial cell monolayer (Sorensen et al., 1999; dos Santos et al., 2005; Krumbholz et al., 2006). Data from human MS genome–wide association studies and murine experimental autoimmune encephalomyelitis (EAE) models suggest that the dysregulation of T cell extravasation by key signaling proteins, such as S1P1 (sphingosine-1-phosphate receptor 1) and VCAM-1 (vascular cell adhesion molecule 1), might mediate MS development (De Jager et al., 2009; Sawcer et al., 2011; Damotte et al., 2014). Targeting VCAM-1 or S1P1 by antibodies or small molecules represents a novel approach for treating MS (Steinman, 2005; Chun and Hartung, 2010). However, given their side effects, it is critical to explore new proteins that restrain T cell infiltration into the CNS and ameliorate MS.

Cdc42, a member of the Rho small GTPases, orchestrates the cell cytoskeleton for T cell migration. Cdc42 activity is precisely controlled temporally and spatially (Haddad et al., 2001; Etienne-Manneville, 2004). When responding to chemokines, Cdc42 switches from the GDP-bound inactive to the GTP-bound active state in the presence of guanine–nucleotide exchange factors (GEFs; Randall et al., 2009). Several GEFs in T cells, including the VAV (dedicator of cytokinesis) family proteins, ensure the proper activation of Cdc42 (Harada et al., 2012). Studies suggest that Vav1–deficient mice are prevented from EAE induction (Tybulewicz, 2005), and mice having Dock8 mutations or the loss of the Dock8 gene exhibit impaired immune responses to...
clear viral infection (Randall et al., 2009, 2011; Lambe et al., 2011; Jabara et al., 2012). Importantly, human DOCK8 mutations or SNPs are associated with immunodeficiency and mental retardation (Griggs et al., 2008; Zhang et al., 2009). Despite this, it is unknown whether DOCK8 is engaged in MS, and which negative regulators restrict DOCK8 GEF activity to prevent immune cell migration.

In this study, we identified LRCH1 as a novel binding partner to sequester DOCK8 from Cdc42. Upon chemokine stimulation, DOCK8 is phosphorylated by PKCζ to separate from LRCH1 and relocate at the leading edge for T cell migration. By generation of Lrch1 transgenic, Lrch1 knockout α stimulation, DOCK8 is phosphorylated by PKCζ partner to sequester DOCK8 from Cdc42. Upon chemokine despite this, it is unknown whether DOCK8 is engaged in mental retardation (Griggs et al., 2008; Zhang et al., 2009). (Fig. 1 C). This suggests that DOCK8 expression levels are elevated in the CNS at the peak stage than those at the presyndrome or remission stage (Fig. 1 B). Dock8 levels in the blood CD4+ T cells were significantly increased at the peak stage of EAE compared with those at the presymptomatic or remission stage (Fig. 1 C). This suggests that DOCK8 expression levels are correlated with EAE severity.

Next, we elucidated whether DOCK8 was a susceptible gene for the induction and development of EAE. As previously reported (Randall et al., 2009), Dock8’s gene contains a serine-to-proline substitution in the DHR-2 (DOCK homology region 2) domain of Dock8, which abolishes the GEF activity for Cdc42 activation. In response to immunization with a MOG (35–55) peptide, all of the Dock8 mice developed EAE, whereas less than half of the Dock8 mice manifested EAE symptoms, which also reduced the disease severity (Fig. 1 D). Consistently, hematoxylin and eosin (H&E) or luxol fast blue staining revealed a decreased number of immune cells and a lower degree of demyelination in the spinal cord sections of the Dock8 mice compared with those of Dock8 mice (Fig. 1, E and F). Additionally, the percentages of the CNS-infiltrated CD4+ T cells were remarkably decreased in the Dock8 mice when EAE developed at the peak phase (Fig. 1 G). In contrast, the percentages of CD8+ T cells or B220+ B cells were apparently not affected (Fig. 1, G and H).

Because Dock8 regulates naïve T cell apoptosis (Hara et al., 2012), we injected BrdU into the MOG (35–55)–induced Dock8 and Dock8 EAE mice 1 d before the lymphocytes were collected to see whether Dock8 and Dock8 CD4+ T cells showed abnormal proliferation or apoptosis in vivo. Compared with the Dock8 CD4+ T cells, the Dock8 CD4+ T cells showed both increased proliferation and apoptosis in vivo (Fig. 1 I), resulting in the same percentages of CD4+ T cells in the draining LNs and spleen (Fig. 1 J). Moreover, IFN-γ and IL-17A production in the Dock8 CD4+ T cells, as well as the percentages of Foxp3+CD4+ regulatory T (T reg) cells in the Dock8 mice, was not changed (Fig. 1 K). These results led us to speculate whether Dock8 regulates the induction of EAE mainly via affecting CD4+ T cell migration.

RESULTS
Dock8 expression is enhanced in the acute phase of murine EAE
Great efforts have been made to identify critical signaling proteins involved in T lymphocyte adhesion and migration (Wang et al., 2010; Zhang and Wang, 2012; Yu et al., 2015). Some of these signaling proteins, including VAV1, ADAP, SKAP55, Rap1, RapL, Mst1, and DOCK8, also regulate T cell activation, apoptosis, or inflammation (Wang et al., 2003, 2004, 2007, 2009; Jo et al., 2005; Katagiri et al., 2006, 2011; Wang and Rudd, 2008; Li et al., 2015a,b,c). Considering the central role of myelin-specific CD4+ T cell activation and infiltration into the CNS in the pathogenesis of MS, we asked whether the expression levels of these molecules were associated with human MS patients. The mRNA levels of Rap1, WASP, VAV1, ADAP, talin, RapL, Mst1, or DOCK8 (but not SKAP55) were significantly enhanced in PBMCs from MS patients compared with age-matched healthy volunteers (Fig. 1 A, left). In agreement with our observation, previous studies suggest that a deficiency of VAV1 or ADAP ameliorates myelin oligodendrocyte glycoprotein peptide (MOG 35–55)–induced EAE, a mouse model that mimics human MS (Korn et al., 2003; Engelmann et al., 2013). Because Mst1 binds to the RapL–Rap1 complex, whereas Dock8 is the key downstream effector of Mst1 (Mou et al., 2012), we asked whether Dock8 influenced the pathogenesis of MS/EAE. First, we confirmed that the mRNA and protein levels of Dock8 were significantly elevated in the PBMCs from MS patients, compared with those from healthy controls and neuromyelitis optica (NMO) patients who displayed similar symptoms to those of MS, but with a distinct etiology (Fig. 1 A, right). Furthermore, during the development of murine EAE model, we noticed that more CD4+ T cells circulated in the blood and infiltrated in the CNS at the peak stage than those at the presyndrome or remission stage (Fig. 1 B). Dock8 levels in the blood CD4+ T cells were significantly increased at the peak stage of EAE compared with those at the presymptomatic or remission stage (Fig. 1 C). This suggests that DOCK8 expression levels are correlated with EAE severity.

CD4+ T cells from Dock8 mice ameliorate EAE with reduced CNS infiltration and migration
To answer this question, equal numbers of encephalitogenic CD4+ T cells were harvested from the Dock8 and Dock8 mice 8 d after MOG (35–55) immunization, and were transferred into the sublethally irradiated WT recipient mice. Before adoptive transfer, we confirmed that Dock8 and Dock8 encephalitogenic CD4+ T cells expressed the same levels of the T cell activation markers CD44 and CD25, as well as secreted similar amount of IL-2 (Fig. 2, A and B). More than 60% of the recipient mice developed EAE after being reconstituted with the Dock8 encephalitogenic CD4+ T cells. In contrast, no recipient mice showed the EAE phenotype after being injected with the Dock8 encephalitogenic CD4+ T cells (Fig. 2 C), which showed less immune cell infiltration (Fig. 2 D, top) and a lower degree of demyelination in the spinal cord (Fig. 2 D, bottom). Furthermore, we revealed a reduced number of the adoptively transferred Dock8 encephalitogenic CD4+ T cells in the CNS or blood (Fig. 2 E), which indeed showed impaired migration...
Figure 1. **DOCK8 expression is positively associated with the peak phase of murine EAE.** (A) The relative mRNA expression levels of the candidate genes in the PBMCs from MS patients and healthy volunteers (top left; n = 4). DOCK8 mRNA levels in the PBMCs (top right) from healthy volunteers (n = 42), NMO patients (n = 24), or MS patients (n = 38). DOCK8 expression in the PBMCs from healthy volunteers and MS patients by immunoblotting (bottom). (B) The total number of CD4+ T cells circulating in the blood (left) or infiltrating in the CNS (right) at different stages of murine EAE. n = 6. (C) Dock8 mRNA levels in CD4+ T cells from murine EAE at the presyndrome, peak, or remission stages. n = 3. (D) Clinical scores (top) and EAE incidence (bottom) of the Dock8pri/+ and Dock8pri/pri mice immunized with MOG (35–55). n = 10. (E and F) H&E and Luxol blue staining of the representative tissue sections of the spinal cords from the Dock8pri/+ and Dock8pri/pri mice on day 18 after EAE induction. Bars, 70 µm. (G and H) Frequency of CD4+ T, CD8+ T, and B220+ cells in the CNS by flow cytometry from the EAE-induced mice. n = 3. (I) The percentages of BrdU+CD4+ T cells and Annexin V+ CD4+ T cells in draining LNs from Dock8pri/+ or Dock8pri/pri mice after EAE induction. n = 3. (J) The percentages of CD4+ T cells in the draining LNs and spleen. n = 3. (K) The percentages of IFN-γ+, IL-17A+,...
toward SDF-1α or CCL5 in a transwell assay ex vivo (Fig. 2 F, left). Nevertheless, the Dock8pri/pri encephalitogetic CD4+ T cells did not affect expression of the chemokine receptors CXCR4 and CCR5 (Fig. 2 F, right). In addition, Dock8pri/pri encephalitogetic CD4+ T cells secreted IFN-γ and IL-17A at levels that were similar to the control cells (Fig. 2 G).

To accomplish directional migration to the sites of inflammation in the CNS, T cells need to polarize and transmigrate through the blood vessels. A polarized migrating T cell displays a typical hand-mirror–like morphology with key molecules being redistributed at the front region or the back tail (i.e., CD44 accumulation in the uropod; Ransohoff et al., 2003; Engelhardt and Ransohoff, 2005). We overexpressed wild-type DOCK8 or the mutant pri into the T cell line T8.1 cells. T8.1 cells expressed endogenous DOCK8, that Dock8pri/pri CD4+ T cells are defective in chemokine-migration (Fig. 2 J, right). Collectively, we have demonstrated only 30% of the mutant pri-expressing T8.1 cells showed cell migration in the transwell assay (Fig. 2 I, left). In contrast, pod (Fig. 2 H); this was in agreement with the enhanced T expressing T8.1 cells polarized with the DOCK8 translocation in T cell migration and EAE/MS, we searched DOCK8's interaction in T cells. T8.1 expressing the full length of LRCH1 decreased cell migration toward SDF-1α, whereas the L305-763 fragment (i.e., 305–763 aa of LRCH1) failed to interact with DOCK8 (Fig. 3 D). We further found that the DHR-2 domain (i.e., 1635–2099 aa) of DOCK8 interacted with LRCH1 (Fig. 3 F), whereas the deletion of the two α-helixes in the DHR-2 domain (i.e., 1687–2099 aa) or the N-terminal fragment (i.e., 1–1634 aa) lost this interaction (Fig. 3 E). The DHR-2 domain is conserved in the DOCK-C family, which contains DOCK6, DOCK7, and DOCK8. We found that LRCH1 also interacted with the DHR-2 domain of DOCK6 and DOCK7 (Fig. 3 G). Whether LRCH1 could affect DOCK6- or DOCK7-induced cell function is to be defined. We have elucidated that the DHR-2 domain of DOCK8 interacts with the nine LRRs of LRCH1.

LRCH1 is identified as a new binding partner of DOCK8

To understand the underline mechanism of DOCK8 function in T cell migration and EAE/MS, we searched DOCK8's binding partners using mass spectrometry and yeast two-hybrid assays (see Materials and methods). Immunoprecipitation using an anti-FLAG antibody was performed and the pull-down proteins at 95 kD were identified by the mass spectrometry analysis from T8.1 cells, which was stably transfected to overexpress FLAG-tagged DOCK8. A specific band at a molecular weight 95 kD was identified as LRCH1 (Leucine-rich repeat [LRR] and calponin homology [CH] domain-containing protein 1; Fig. 3 A). In the yeast two-hybrid assay, DOCK8 was used as bait for screening a human cDNA library encoding >12,794 human genes (hORFeome V5.1). Positive clones were selected and LRCH1 was again identified in this method (Fig. 3 B). Currently, very limited studies have been reported regarding the function of LRCH1. The function of LRCH was initially reported in Drosophila, which has only one isoform named dLRCH. dLRCH colocalizes with F-actin and functions as a cytoskeleton regulator (Foussard et al., 2010). However, it is still a controversy about whether a C/T transition single-nucleotide polymorphism (SNP; rs912428) in LRCH1 is a risk factor for human osteoarthritis. We were therefore excited to explore whether and how LRCH1 functioned together with DOCK8 in the regulation of EAE.

We first verified the interaction between DOCK8 and LRCH1 by immunoprecipitation using 293T cells that were transfected with a FLAG-tagged DOCK8 and an HA-tagged LRCH1. The FLAG-tagged DOCK8 could pull down the HA-tagged LRCH1 and vice versa (Fig. 3 C). Next, we truncated DOCK8 and LRCH1 to map their binding regions. LRCH1 contains nine leucine-rich repeats (LRRs) and a single CH domain. We found that the N-terminal fragment containing all nine LRRs (i.e., LRR1-9, and 1–304aa) bound to DOCK8. In contrast, after losing two LRRs, the first seven LRRs (i.e., LRR1-7, and 1-238aa) lost this interaction (Fig. 3 D), suggesting the importance of the whole nine LRRs for this interaction. Additionally, the fragment containing 305–763 aa of LRCH1 failed to interact with DOCK8 (Fig. 3 D). We further found that the DHR-2 domain (i.e., 1635–2099 aa) of DOCK8 interacted with LRCH1 (Fig. 3 F), whereas the deletion of the two α-helixes in the DHR-2 domain (i.e., 1687–2099 aa) or the N-terminal fragment (i.e., 1–1634 aa) lost this interaction (Fig. 3 E). The DHR-2 domain is conserved in the DOCK-C family, which contains DOCK6, DOCK7, and DOCK8. We found that LRCH1 also interacted with the DHR-2 domain of DOCK6 and DOCK7 (Fig. 3 G). Whether LRCH1 could affect DOCK6- or DOCK7-induced cell function is to be defined. We have elucidated that the DHR-2 domain of DOCK8 interacts with the nine LRRs of LRCH1.

Lrch1 transgenic mice are resistant to EAE with reduced T cell migration

We next investigated the function of LRCH1 in T cells. T8.1 cells expressed endogenous murine LRCH1 (i.e., 90 kD), which were stably transfected to overexpress human wild-type LRCH1 (Fig. 4 A, middle) or its fragments LRR1-9, and L305–763 (305–763aa of LRCH1 that does not interact with DOCK8). To our surprise, T8.1 expressing the full length of LRCH1 decreased cell migration toward SDF-1α in a transwell assay (Fig. 4 A, left). The overexpression of LRR1-9, the fragment that binds DOCK8, impaired T cell migration toward SDF-1α, whereas the L305–763 fragment showed no interference (Fig. 4 A, left). Despite this,
Figure 2. CD4+ T cells from Dock8pri/pri mice ameliorate EAE with reduced CNS infiltration and migration. (A and B) The encephalitogenic CD4+ T cells were purified from Dock8pri/+ or Dock8pri/pri mice to check surface expression of CD44 and CD25 by FACS (A). IL-2 concentrations were checked by ELISA in the supernatants of Dock8pri/+ or Dock8pri/pri encephalitogenic CD4+ T cells stimulation with MOG for 3 d (B). n = 6. (C–E) Adoptive transfer of encephalitogenic Dock8pri/+ and Dock8pri/pri CD4+ T cells into the sublethally irradiated WT mice (n = 6) to assess clinical scores & EAE incidence (C). (D) H&E staining and Luxol blue staining of the representative tissue sections of the spinal cords. Bars, 70 μm. (E) Total number of CD4+ T cells in the CNS and in the blood. (F) The encephalitogenic CD4+ T cells were isolated from the Dock8pri/+ and Dock8pri/pri mice at day 18 after EAE induction for SDF-1α- or CCL5-induced migration (left), or for a FACS assay to check the surface expression of CXCR4 and CCR5 (right). n = 4–6. (G) The percentages of IFN-γ+ cells and IL-17A+ cells in CD4+ T cells in spleen from the recipient mice presented in C. n = 5. (H–I) T8.1 cells were transfected with FLAG-tagged DOCK8 or...
LRCH1 overexpression did not change the expression levels of the chemokine receptor CXCR4 (Fig. 4 A, right).

To examine the in vivo role of LRCH1, we generated Lrch1 transgenic mice (termed Lrch1 Tg) using a CD2 promoter–based vector that overexpressed FLAG-tagged Lrch1 only in T and B cells. Expression of FLAG-tagged Lrch1 was confirmed in primary T cells by Western blotting (Fig. 4 B). WT mice and Lrch1 transgenic mice were then immunized with the MOG (35–55) peptide to induce EAE. Compared with their WT littermates, Lrch1 transgenic mice were resistant to the development of EAE and showed reduced clinical scores (Fig. 4 C). This was in agreement with a reduced numbers of the Lrch1 CD4+ Tg cells in the CNS or in the blood (Fig. 4 D). Importantly, we confirmed that this was not a result of the abnormal proliferation, apoptosis, or numbers of Lrch1 Tg CD4+ cells in response to the MOG (35–55) peptide stimulation in vivo (Fig. 4 E). Additionally, the Lrch1 Tg mice showed normal numbers of lymphocytes in spleens and draining LNs (Fig. 4 F). The percentages of CD4+ FoxP3+ T reg cells were also normal in Lrch1 transgenic mice after EAE induction (Fig. 4 G).

Next, encephalitogenic CD4+ T cells were purified from the Lrch1 transgenic mice when EAE was developed at the peak stage. Compared with the WT controls, encephalitogenic Lrch1 Tg CD4+ T cells impaired the ex vivo transmigration toward to SDF-1α (Fig. 4 H). Similar to our observation in the T8.1 cell line, encephalitogenic Lrch1 Tg CD4+ T cells did not affect the expression levels of CXCR4 (Fig. 4 I). These data together suggest that as a new binding partner of DOCK8, LRCH1, might play a distinctive role in T cell migration and the development of EAE.

Adaptive transfer of Lrch1 KO CD4+ T cells accelerates EAE with enhanced T cell migration

To further validate the opposite effect of LRCH1 from DOCK8, we generated Lrch1 KO mice by TALEN technology via targeting the first exon of Lrch1. One founder carried a 2-nt deletion and a 1-nt insertion in the ORF of the Lrch1 gene and created a stop code to KO Lrch1 (Fig. 5 A). The homozygous Lrch1-deficient mice (Lrch1−/−) were viable, fertile and showed normal populations of CD4+ cells in the spleen and LNs (Fig. 5 B). We induced active EAE using Lrch1−/− mice, which manifested a greater disease severity compared with WT mice (Fig. 5 C). During the induction of EAE, Lrch1 deficiency did not change the numbers of CD4+ T cells in spleen and draining LNs (Fig. 5 D). We confirmed that TCR usage in CD4+ T cells in response to MOG peptide stimulation was not changed in Lrch1−/− mice relative to that in Lrch1+/+ mice (Fig. 5 E). Moreover, Lrch1-deficient CD4+ T cells also proliferated and produced IL-2 at normal levels, and expressed normal levels of the activation markers CD44 and CD25 (Fig. 5 F).

To verify the role of Lrch1−/− CD4+ T cells in the development of EAE, we isolated equal numbers of encephalitogenic CD4+ T cells from the MOG (35–55) peptide-treated WT mice or Lrch1−/− mice, and transferred them into the sublethally irradiated WT recipient mice. The Lrch1−/− encephalitogenic CD4+ T cell–reconstituted mice showed an earlier disease onset with higher clinical scores (Fig. 5 G). Consistently, more Lrch1−/− encephalitogenic CD4+ T cells migrated into the CNS (Fig. 5 H, left), which also displayed an enhanced migration toward CCL5 in a transwell assay (Fig. 5 H, right). Reconstitution of LRCH1 expression in Lrch1−/− CD4+ T cells restored cell migration to normal levels as that in WT CD4+ T cells (Fig. 5 I, left). Despite of this, Lrch1−/− CD4+ T cells expressed normal levels of CCR5 (Fig. 5 I, right). After collecting our data from the Lrch1 Tg and Lrch1−/− mice, we suggest that LRCH1 protects mice against EAE as a result of the reduced CD4+ T cell migration.

LRCH1 attenuates DOCK8-mediated Cdc42 activation for T cell migration

Considering the opposite role of DOCK8 and LRCH1 in T cell migration without affecting the expression of chemokine receptors, we speculated how LRCH1 and DOCK8 cooperated together in response to chemokine stimulation. As a GEF protein, DOCK8 binds and activates Cdc42 (Harada et al., 2012). Using previously reported methods, we incubated T8.1 cells with the tetanus toxoid peptide (Ttox) peptide-pulsed APCs (i.e., APCs and L625.7) to form cell conjugates, and noticed that DOCK8 co-localized with Cdc42 at the immunological synapses (Fig. 6 A). Moreover, we examined Cdc42 activity in WT and Lrch1−/− CD4+ T cells by transfection of a fluorescent resonance energy transfer (FRET)–based biosensor Raichu-Cdc42, which consists of Cdc42, Cdc42-binding domain of PAK1, and a pair of green fluorescent protein mutants to monitor Cdc42 activation in vivo (Itoh et al., 2002; Shen et al., 2008). In response to SDF-1α treatment, FRET efficiency was increased in Lrch1−/− CD4+ T cells compared with that in WT cells, which indicates higher Cdc42 activation in Lrch1−/− CD4+ T cells (Fig. 6 B, left). Activated Cdc42-GTP interacts with the downstream effector PAK1, and we next determined how LRCH1 could affect the amount of activated Cdc42-GTP by a GST pull down assay.
using the GST-fused CRIB domain of PAK1 (Benard et al., 1999). Overexpression of LRCH1 in T8.1 cells reduced the amount of activated Cdc42-GTP by a GST pull-down assay compared with the control T8.1 cells (Fig. 6 B, right). Collectively, we suggest that LRCH1 functions as a negative regulator of Cdc42 activation. This led us to speculate whether LRCH1 interfered with Cdc42 activation by modulating the GEF activity of DOCK8.

Previous studies found that activated GEFs preferentially bind to guanine nucleotide–free forms of GTPases, suggesting that the Cdc42G15A mutant could be used to measure GEF activity (García-Mata et al., 2006). We therefore overexpressed DOCK8 with or without LRCH1 in the 293T cells and measured the amount of DOCK8 binding to the GST-fused-Cdc42G15A. Interestingly, fewer DOCK8 interacted with Cdc42G15A when LRCH1 was also present (Fig. 6 C). In addition, we transfected the FLAG-tagged catalytic DHR-2 domain of DOCK8 with or without LRCH1, followed by a GST pull-down assay to measure how LRCH1 affected Cdc42 activation. Although DOCK8 DHR-2 enhanced the amount of GTP-bound-Cdc42, the coexpression of LRCH1 substantially decreased this effect (Fig. 6 D). This led us to speculate whether LRCH1 and Cdc42 competed for binding to the DHR-2 domain of DOCK8. Indeed, in the presence of LRCH1, Cdc42 precipitated less DOCK8 DHR-2 (Fig. 6 E). To further confirm this phenotype, we purified the recombinant proteins His-DHR-2, GST-Cdc42G15A from Escherichia coli, and the recombinant proteins FLAG-
Figure 4. *Lrch1* transgenic mice are resistant to EAE with reduced T cell migration. (A) T8.1 cells were transfected with the vector control, LRCH1, or its fragments (LRR1-9, L305-763) for a transwell assay in response to SDF-1α (n = 3). FACS assay was performed to check the cell surface expression of CXCR4. The transfected exogenous human LRCH1 was detected at 130 kD and the endogenous murine LRCH1 in T8.1 cells was detected at 95 kD by Western blot. (B) The expression of FLAG-Lrch1 in thymus from Lrch1 transgenic mice was assessed by immunoblotting. (C) The clinical scores (left) and EAE incidence (right) of WT and Lrch1 transgenic mice induced by the MOG (35–55) peptide. n = 5. (D and E) The total numbers of CD4+ T cells in the CNS, blood (D), spleen, and draining LNs (E; middle right and right) of the WT or Lrch1 transgenic mice; percentages of BrdU+ or Annexin V+ CD4+ T cells in spleen were checked at the peak stage of EAE (E; left and middle left). n = 4–5. (F) The number of lymphocytes in spleen and draining LNs were counted from WT and Lrch1 Tg mice after EAE induction. n = 5. (G) The percentage of Foxp3+ CD4+ T reg cells in draining LNs from the WT or Lrch1 transgenic mice at the peak stage of EAE. n = 4. (H and I) The encephalitogenic CD4+ T cells were purified from the WT or Lrch1 transgenic mice for a transwell assay in response to SDF-1α (H), or for a FACS assay to check the surface expression of CXCR4 (I). n = 4–5. NS, not significant (P > 0.05); *, P < 0.05; **, P < 0.01. Data are representative of four experiments (A, mean ± SD), three experiments (C, mean ± SEM), or two experiments (D–I, mean ± SD). Statistical significance was determined using unpaired Student’s t test.
LRR1-9 or FLAG-L305-763 from 293T cells. The purity and specificity of the purified His-DHR-2 and GST-Cdc42G15A protein from E. coli was shown in a Coomassie blue staining (Fig. 6F). When GST-Cdc42G15A formed a complex with His-DHR-2 in vitro, addition of the increasing amount of FLAG-LRR1-9 could significantly decrease the amount of protein in the complex.

Figure 5. Adoptive transfer of Lrch1 KO CD4+ T cells accelerates EAE with enhanced T cell migration. (A) Generation of Lrch1 KO mice. The exon 1 of the Lrch1 gene was specifically targeted by TALEN, and DNA sequencing confirmed the nucleotide mutation in the Lrch1 locus adjacent to the FOKI cleavage site (arrow). (B) Numbers of CD4+ T cells in spleen and LNs from unimmunized mice. n = 3. (C) EAE incidence of WT and Lrch1 KO mice in response to MOG (35–55) treatment. n = 5. (D–F) Numbers of CD4+ T cells in spleen and draining LNs (D); TCR usage analyzed by anti-TCR Vα and anti-TCR Vβ antibodies (E); and percentages of BrdU+ CD4+ T cells, IL-2 secretion, and the surface expression of CD25 and CD44 in CD4+ T cells from the draining LNs (F) of WT and Lrch1 KO mice after EAE induction. n = 5. (G) The sublethally irradiated WT recipient mice were reconstituted with WT or Lrch1−/− encephalitogenic CD4+ T cells to assess their clinical scores (left) and EAE incidence (right). n = 5. (H) The total numbers of WT or Lrch1−/− encephalitogenic CD4+ T cells in the CNS from the sublethally irradiated WT recipient mice presented in G. The encephalitogenic CD4+ T cells were purified from the WT or Lrch1−/− mice for a transwell assay in response to CCL5 (right). n = 5. (I) CD4+ T cells were reconstituted with LRCH1 or the vector control, for a transwell assay in response to CCL5 (left). The surface expression of CCR5 in the spleen CD4+ T cells from WT and Lrch1 KO mice after EAE induction (right). n = 3–4. NS, not significant (P > 0.05); *, P < 0.05; **, P < 0.01. Data are representative of three experiments (G, mean ± SEM), or two experiments (B–F and H–I, mean ± SD). Statistical significance was determined using unpaired Student’s t test.
Figure 6. LRCH1 attenuates DOCK8-mediated Cdc42 activation for T cell migration. (A) T8.1 cells were transfected with FLAG-DOCK8, incubated with Ttox peptide-pulsed L625.7 cells to form cell conjugates, followed by immunostaining to visualize DOCK8 and Cdc42. Bar, 5 µm. (B) The FRET efficiency of biosensor Raichu-Cdc42 between WT and Lrch1−/−CD4+ T cells in response to SDF-1α treatment. FRET efficiency was measured with donor dequenching approach, and was calculated as E = (Post − [Pre/Post]) × 100%, where Post and Pre represents the donor fluorescence before and after photo bleaching (left). n = 20. NS, not significant (P > 0.05); **, P < 0.01. Activated Cdc42 was precipitated by a GST-CRIB-PAK1 pull-down assay in T8.1 cells that over-expressed LRCH1 or the vector control (right). (C) 293T cells were transfected with FLAG-DOCK8 with or without HA-LRCH1, and the amount of DOCK8 binding to the GST-Cdc42G15A–coated beads was used to evaluate the GEF activity of DOCK8. (D) 293T cells were cotransfected with FLAG-DHR-2, HA-Cdc42 with or without HA-LRCH1. The cell lysates were subjected to a GST-CRIB-PAK1 pull-down assay to precipitate the active Cdc42. (E) 293T cells were transfected with HA-Cdc2, Myc-LRCH1, and FLAG-DHR-2, followed by immunoprecipitation with anti-HA and immunoblotting with anti-HA or anti-FLAG. (F) The purity of the purified His-DHR-2 (1632–2068 aa) and GST-Cdc42 G15A protein from E. coli was determined by Coomassie blue staining. (G and H) Increasing amounts of FLAG-LRR1-9 (G) or FLAG-L305-763 (H) were added into the solution containing the purified recombinant proteins His-DHR-2 and GST-Cdc42G15A, incubated and then subjected for precipitation using anti-His antibody. (I) T8.1 cells, which were transfected with FLAG-DOCK8 and HA-LRCH1, were treated with or without SDF-1α to assess localization of DOCK8 (red, top) and LRCH1 (green, middle). Bar, 5 µm. (J and K) T8.1 cells expressing FLAG-DOCK8 and HA-LRCH1 were treated or untreated with SDF-1α and PMA (J). 293T cells were transfected with HA-LRCH1, FLAG-DOCK8, or the mem-
GST-Cdc42G15A binding to His-DHR–2 in a dose-dependent manner (Fig. 6 G). As an important negative control, addition of the purified recombinant protein FLAG–L305–763 did not affect the formation of the complex containing GST-Cdc42G15A and His-DHR–2 (Fig. 6 H). We propose the novel finding that LRCH1 attenuates DOCK8-mediated Cdc42 activation for T cell migration.

Upon SDF-1α stimulation, DOCK8 redistributed from the cytoplasm to the cell membrane. In contrast, LRCH1 was still ubiquitous in the cytoplasm (Fig. 6 I). We questioned whether chemokine signaling induced DOCK8 separation from LRCH1. Indeed, SDF-1α treatment could increase DOCK8 phosphorylation, and substantially reduced its interaction with LRCH1 (Fig. 6 J). To further confirm this, we generated a membrane-bound DOCK8 by fusing the CVIM motif to the C terminus of DOCK8. Critically, the membrane-localized CVIM-FLAG–DOCK8 recruited less LRCH1 and exhibited an enhanced phosphorylation (Fig. 6 K). This suggests that chemokine-induced DOCK8 phosphorylation might release DOCK8 from LRCH1, which is then able to interact with Cdc42 for further activation.

PKC phosphorylates DOCK8 for separation from LRCH1

The next critical question was which kinases could phosphorylate DOCK8. We searched the DOCK8 phosphorylation sites in the PhosphoSitePlus database, which provides the possible in vivo phosphorylation sites of proteins from published studies or from high-throughput phosphorylation site discovery programs. The database showed that the C-terminal motif of DOCK8 (i.e., 2077–2087 aa: SQKRDSFHR SS) is phosphorylated (Fig. 7 A), and kinases including PKCα and AKT are predicted to phosphorylate this motif by the program group base prediction 3.0. We therefore treated cells with either PKCα or AKT inhibitors, and then pulled down the membrane-bound CVIM–DOCK8 to assess DOCK8 phosphorylation. The PKCα inhibitor substantially reduced DOCK8 phosphorylation levels, whereas the AKT inhibitor showed little effect (Fig. 7 B). Moreover, the PKCα inhibitor abolished DOCK8–induced cell migration in response to SDF-1α stimulation (Fig. 7 C). This observation is in agreement with previous studies that PKCα is recruited to membrane for cytoskeleton rearrangement and chemokine-induced cell migration (Sun et al., 2014). Interestingly, PKCα was previously reported as a susceptible gene in MS patients (Barton et al., 2004).

Because PKCα phosphorylates serines (Parekh et al., 2000; Parker and Murray-Rust, 2004), we mutated the three key serines in the C-terminal motif of DOCK8 (Ser2077/2082/2087) to either glutamic acid or alanine, which, respectively, mimicked the phosphorylated DOCK8 (i.e., termed 3S/E) or abolished DOCK8 phosphorylation (i.e., termed 3S/A). Compared with the DOCK8-transfected T8.1 cells, the overexpression of 3S/E further enhanced cell migration in response to SDF-1α stimulation, whereas 3S/A markedly inhibited this effect (Fig. 7 D). Consistently, the 3S/A-transfected cells showed reduced GEF activity in the Cdc42G15A pull-down assay (Fig. 7 E). Moreover, when coexpression of PKCα with DOCK8 further promoted cell migration to SDF-1α, the 3S/A mutant failed to synergize with PKCα to increase migration (Fig. 7 F). Taken our data together, we suggest that PKCα phosphorylates DOCK8 at the Ser2077/2082/2087 sites to promote T cell migration.

Compared with the amount of LRCH1 binding to WT DOCK8, we found that the DOCK8 3S/E mutant (mimicking the phosphorylated DOCK8) recruited less LRCH1 (Fig. 7 G). Interestingly, coexpression of LRCH1 could block DOCK8–induced T cell migration, whereas LRCH1 could not inhibit the DOCK8 3S/E mutant–induced T cell migration (Fig. 7 H). In contrast, the membrane-bound CVIM–3S/A (abolishing DOCK8 phosphorylation) could still recruit more LRCH1 compared with the membrane-bound DOCK8 (Fig. 7 I). Furthermore, given that PMA stimulation triggers PKCα activation, DOCK8 was phosphorylated, which in turn reduced its interaction with LRCH1 (Fig. 7 J, lane 2 vs. lane 1). In contrast, the 3S/A mutation failed to be phosphorylated by PMA and still bound to LRCH1 at similar levels as that in resting T8.1 cells (Fig. 7 J, lane 4 vs. lane 3). Together, we have uncovered a novel mechanism that PKCα phosphorylates DOCK8 for separation from LRCH1, leading to Cdc42 activation and T cell migration.

DISCUSSION

DOCK8 was recently identified as a key modulator for immune cell function, including B cell adhesion and integrin activation (Jabara et al., 2012). In agreement with this phenotype, Cdc42 was suggested to be DOCK8’s binding partner for cytoskeleton rearrangement and T cell migration. In this study, we identified LRCH1 as a novel DOCK8–interacting protein to restrain the GEF activity of DOCK8, resulting in the inhibition of Cdc42 activation and T cell migration. During the in vivo MOG (35–55) peptide–induced EAE, we observed the protective role of LRCH1 against EAE as a result of a blockage of CD4+ T cell migration into the CNS as demonstrated by Lrch1 transgenic or Lrch1 KO mice. Importantly, we also elucidated that LRCH1 deficiency did not affect CD4+ T cell proliferation, apoptosis, activation and production of IL-2. Next, we identified that LRCH1 competes with Cdc42 for binding to the catalytic DHR–2 domain of DOCK8 and restricts the GEF activity of DOCK8. Other members in the DOCK family contain an SH3 domain,
Figure 7. PKCα phosphorylates DOCK8 for separation from LRCH1. (A) The amino acid sequence (2075–2089) of DOCK8 and its mutants depict the three key serine residues. (B) 293T cells expressing CVIM-FLAG-DOCK8 were untreated or treated with the AKT and PKCα inhibitors, followed by immunoprecipitation with anti-FLAG to detect DOCK8 phosphorylation levels. (C and D) Migration of the T8.1 cells expressing the vector control, DOCK8, or the mutant 3S/E or 3S/A were examined by a transwell assay in response to SDF-1α in the presence or absence of the PKC inhibitor. n = 3. (E) 293T cells were transfected with FLAG-DOCK8 or the 3S/A mutant, followed by a GST-Cdc42G15A pull-down assay to measure their GEF activity. (F) The migration of T8.1 cells coexpressing PKCα with GFP, DOCK8, or the 3S/A mutant was assessed by a transwell assay. n = 3. (G) 293T cells were transfected with HA-LRCH1, FLAG-DOCK8, or 3S/E, followed by immunoprecipitation with anti-FLAG to analyze their binding to LRCH1. (H) The vector control, DOCK8, or the mutant 3S/E were coexpressed with or without LRCH1 into T8.1 cells and migration was examined by a transwell assay in response to SDF-1α. n = 3. (I) The local-
which provides a self-inhibition mechanism to restrain their GEF activity (Lu et al., 2005). For example, the SH3 domain in DOCK1 interacts with its own DHR-2 domain and inhibits Rac binding (Meller et al., 2008). In contrast, DOCK8 has no SH3 domain. Our study suggests a possible mechanism that DOCK8 interacts with LRCH1 to lock DOCK8 at low GEF activity in resting T cells.

When T cells are stimulated with external signals such as chemokines, PI3K is activated and generates the product PIP(3,4,5)3 on the membrane, which recruits DOCK8 through its DHR-1 domain (Côté et al., 2005). We observed that DOCK8 was separated from LRCH1 in the cytoplasm, and relocated to the leading edge with Cdc42 after chemokine stimulation. Our findings are in agreement with previous reports that DOCK8 controls Cdc42 activity, specifically on the leading edge of the cell membrane rather than the globally distributed Cdc42 (Harada et al., 2012). Combining these observations, we propose a model that DOCK8 is located in the cytoplasm and interacts with LRCH1 to block Cdc42 binding in a resting T cell. In response to chemokine stimulation, DOCK8 is separated from LRCH1 and exposes its DHR-2 domain for Cdc42 activation, whereas its DHR-1 domain is recruited by PIP(3,4,5)3 to relocate DOCK8 at the leading edge of a migrating T cell.

In line with this scenario, we further demonstrated that in response to chemokine stimulation, PKCa phosphorylates DOCK8 at the Ser2077/2082/2087 sites to promote DOCK8 separation from LRCH1 (green), CVIM-DOCK8, or CVIM-DOCK8 3S/A (red) was examined in 293T cells by immunostaining. Bar, 5 µm. (J) 293T cells were transfected with FLAG-DOCK8 or 3S/A together with HA-LRCH1 and Myc-PKCα, stimulated with or without PMA, followed by immunoprecipitation with anti-FLAG to analyze DOCK8 phosphorylation levels and binding to LRCH-1. NS, not significant (P > 0.05); *, P < 0.05; **, P < 0.01. Data are representative of three experiments. (K) The Dock8pri/pri mice were kindly provided by C.C. Goodnow (John Curtin School of Medical Research, The Australian Nation University, Canberra, Australia). To specifically overexpress Lrchi in T or B lymphocytes, cDNA encoding murine Lrchi was tagged with HA and inserted to the human CD2 plasmid (provided by Paul Love, Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, MD). The Lrchi transgenic mice in the C57BL/6 mouse background were generated by X. Liu’s group at the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (CAS); the mice specifically overexpressed FLAG-tagged Lrchi only in T and B cells using a CD2-promoter based vector. The Lrchi transgenic positive mice were identified by PCR with the following primers.

**MATERIAL AND METHODS**

**Mice**

The Dock8−/− mice were kindly provided by C.C. Goodnow (John Curtin School of Medical Research, The Australian Nation University, Canberra, Australia). To specifically overexpress Lrchi in T or B lymphocytes, cDNA encoding murine Lrchi was tagged with HA and inserted to the human CD2 plasmid (provided by Paul Love, Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, MD). The Lrchi transgenic mice in the C57BL/6 mouse background were generated by X. Liu’s group at the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (CAS); the mice specifically overexpressed FLAG-tagged Lrchi only in T and B cells using a CD2-promoter based vector. The Lrchi transgenic positive mice were identified by PCR with the following primers.
primers: forward, 5′-CACTCGGGACCTATGAACT-3′; reverse, 5′-GATCGTAACTGTGGCTCT-3′. The Lrch1 KO mice were generated by transcription activator-like effector nuclease (TALEN) technology specifically targeting the exon 1 of Lrch1 (SIDANSAI Biotechnology Co.). PCR and DNA sequencing were used to identify the Lrch1 KO founder mice, which were selected and used for further breeding and characterization. All mice were bred under specific pathogenic–free conditions at the Animal Care Facility of Shanghai Institute of Biochemistry and Cell Biology (SIBCB, CAS, Shanghai, China). The animal experiments were performed in compliance with the guidance for the care and use of laboratory animals and were approved by the institutional biomedical research ethics committee of SIBCB.

Enrollment of patients and healthy subjects
Patients clinically diagnosed with MS or NMO and healthy volunteers from the outpatient clinic were enrolled from Huashan Hospital, Shanghai, China. After informed consent, blood samples from the subjects were collected and prepared in accordance with the guidelines of the Review Boards of Huashan Hospital (Shanghai, China).

Cell lines
T8.1 cells, a murine cell line, were stably transfected with an antigen receptor specific for Tox, and the murine fibroblast cell line L625.7 cells were used as APCs that express HLA-DR*1102, CD80, ICAM-2 and ICAM-1 (Michel and Acuto, 1996). T8.1 cells expressed endogenous Dock8 and Lrch1. 293T is a Human Embryonic Kidney Cell line. T8.1 and 293T cells were cultured in DMEM supplemented with 10% (vol/vol) fetal calf serum and 50 U/ml penicillin/streptomycin.

Reagents
The following antibodies were purchased from eBioscience and used for FACS assay: anti–mouse CD3e (145-2C11), anti–mouse CD4 (GK1.5), anti–mouse CD8a (53–6.7), anti–mouse CD45R (B220; RA3–6B2), anti–mouse IL-17A (eBio17B7), anti–mouse IFN-γ (XMG1.2), anti–mouse CD11b (M1/70), anti–mouse CD29 (eBioHMb1-1), and anti–mouse CXCR4 (2B11). The following antibodies were purchased from BD: anti–mouse–Vα2 (B20.1), anti–mouse–Vα3.2 (553219), anti–mouse–Vβ6 (553194), anti–mouse–Vβ8.1/8.2 (118405), anti–mouse–Vβ8.3 (118603), anti–mouse–Vβ11 (125907), and anti–mouse–Vβ14 (553258). The following antibodies were purchased from to be added: anti–mouse CCR5 (HM–CCR5; BD), anti–mouse CD44 (IM7; eBioscience), and anti–mouse Foxp3 (FJK–16S; eBioscience). Anti–human CD4 (SS.3; Invitrogen), anti–human DOCK8 (sc–104911; Santa Cruz Biotechnology, Inc.), and anti–human LRCH1 (sc–84195; Santa Cruz Biotechnology, Inc.) were used for FACS or immunoblotting assay. Anti–Phosphoserine/threonine/tyrosine antibody (ab15556; Abcam), anti–FLAG (F3165; Sigma–Aldrich), anti–HA (H3663; Sigma–Aldrich), anti–c–Myc (C3956; Sigma–Aldrich), and anti–Cdc42 (sc–87; Santa Cruz Biotechnology, Inc.) were used for immunoprecipitation or immunoblot assay.

The induction of EAE
The encephalitogenic peptide MOG (35–55; GL Biochem) used to induce EAE had a purity of 95%. For EAE induction, 8–10-wk-old C57BL/6 mice were immunized s.c. with 200 µg MOG (35–55) in complete Freund’s adjuvant containing heat-killed Mycobacterium tuberculosis (H37Rv strain; 5 mg/ml; Sigma–Aldrich). Pertussis toxin (200 ng per mouse; EMD Millipore) in PBS was administered i.p. on days 0 and 2. Mice were examined daily for disease signs by researchers blinded to experimental conditions and were assigned scores on a scale of 0–5 as follows: 0, no clinical signs; 1, paralyzed tail; 2, paresis (weakness, incomplete paralysis of one or two hindlimbs); 3, paraplegia (complete paralysis of both hindlimbs); 4, paraplegia with forelimb weakness or paralysis; and 5, moribund state or death (Jin et al., 2009). For analysis of CNS infiltrates, brain and spinal cord tissues were collected from perfused mice and mononuclear cells were prepared by Percoll gradient centrifugation. For histological analysis, the same tissue samples were immediately fixed in 4% (wt/vol) paraformaldehyde. Paraffin–embedded sections of spinal cord were stained with H&E or with Luxol fast blue for analysis of inflammation or demyelination, respectively. For adoptive transfer of encephalitogenic CD4+ T cells experiments, splenocytes were isolated from mice 8 d after active MOG immunization and cultured for 3 d in the growth media containing 20 µg/ml MOG; 2 × 10^6 encephalitogenic CD4+ T cells were i.v. injected into the sublethally irradiated recipient mice.

Intracellular staining and flow cytometry
For intracellular cytokine staining, cells obtained from DLNs of mice with EAE were incubated in a tissue culture incubator for 5 h at 37°C with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma–Aldrich), ionomycin (1 µg/ml; Sigma–Aldrich), and Breifeldin A (10 µg/ml; Sigma–Aldrich). Surface staining was performed with the corresponding fluorescence-labeled surface antibodies in PBS buffer for 30 min. After surface staining, cells were resuspended in Fixation/Permeabilization solution (Cytofix/Cytoperm kit; eBioscience), and intracellular cytokine staining was done according to the manufacturer’s protocol. For Foxp3 staining, cells were isolated from the EAE mice and prepared for intracellular staining using the Foxp3 Staining Buffer set as suggested (eBioscience). FACSCalibur (BD), FlowJo software, or Accuri C6 was used for flow cytometry.

Plasmids and qRT–PCR
Flag–tagged human DOCK8 (WT or pri mutation) were subcloned into the retroviral vector pMX-IRES-GFP; HA–tagged human LRCH1 was subcloned into the MIGR-IRES-GFP vector. These plasmids were transfected to 293T cells using the reagent pCL–10A, and the retroviral superna-
transduced CD4⁺ T cells and the percentages of anti-FLAG or anti-HA. Chemotaxis assay was performed and LRCH1 were examined by intracellular staining with infected with pMX-FLAG-DOCK8 or MIGR-HA-LRCH1, including puromycin and blasticidin. Plat-E cells were transduced with 10% (vol/vol) fetal calf serum and antibiotics, 293T cells overexpressed indicated proteins for 36 h were centrifuged at 100 g for 1 min. 100 ng/ml SDF-1α was added to the bottom well. After 4 h of incubation, cells in the bottom well were collected and the cell number was counted to the top well. After 4 h of incubation, cells in the top and bottom wells were counted. The migration efficiency was calculated by a formula Efficiency = (% GFP⁺ cells x total cells in the bottom well)/(% GFP⁺ cells x total cells before transwell).

**Chemotaxis assay**
The chemotaxis assay was performed using a transwell chamber (5 µm; Corning). 200,000 cells suspended in 100 µl medium were placed into the top chamber, and 600 µl medium containing 50 ng/ml human SDF-1α (PeproTech) was added to the bottom well. After 4 h of incubation, cells in the bottom well were collected and the cell number was counted using an Accuri C6 (BD).

**Polarization and immunofluorescence**
T8.1 cells were placed on ICAM-1–coated plates that were centrifuged at 100 g for 1 min. 100 ng/ml SDF-1α was added into the plates. For T-APC conjugate formation, T8.1 cells were transfected with FLAG-DOCK8 and HA-Cdc42, and incubated with Toxopulsed murine fibroblast L625.7 cells to form cell conjugates. After 10-min incubation at 37°C, cells were fixed with 4% PFA in PBS and stained with anti-CDC44. For intracellular staining, fixed cells were permeabilized with 0.1% Triton X-100 in PBS and stained with anti-FLAG and anti-HA. Images were captured with Olympus BX51 microscope and polarized cells were counted.

**Immunoprecipitation and immunoblotting assay**
293T cells overexpressed indicated proteins for 36 h were immediately washed twice with ice-cold PBS before harvested in ice-cold lysis buffer (PBS containing 1% Triton X-100, 2 mM EDTA, and protease and phosphatase inhibitors). Whole-cell lysate was incubated with anti-FLAG or anti-HA beads and at 4°C for 2 h. The beads were washed three times with lysis buffer and then resuspended in an appropriate amount of SDS-PAGE loading buffer. Proteins were loaded onto SDS-PAGE gel and analyzed via immunoblotting. To measure DOCK8 phosphorylation, the membrane-bound DOCK8 (FLAG-CVIM-DOCK8) was transfected to 293T cells, and treated with the indicated inhibitors for 2 h. Cell lysates were prepared for immunoprecipitation with anti-FLAG antibody to pull-down FLAG-DOCK8, and the phosphorylation levels of DOCK8 were then assessed by immunoblotting with anti-phosphoserine/threonine antibody.

**GST pull-down and Cdc42 activity assay**
GST-PAK1-PBD (amino acids 69–150 of human PAK1) was kindly provided by Z. Chen (SIBCB, CAS). 293T cells transfected with indicated proteins were washed twice with the ice-cold PBS and lysed in Mg²⁺/lysis buffer (MLB) as described by the manual (EMD Millipore). The supernatants were incubated with GST-PBD-agarose beads at 4°C for 2 h. The beads were washed three times by MLB and resuspended in the loading buffer for the immunoblotting assay.

**Fluorescence resonance energy transfer measurement**
FRET efficiency was measured with donor dequenching approach and the filter sets (458 nm for CFP, 514 nm for YFP) were used as previously described (Xu et al., 2008). Images were captured by Leica TCS SP2 AOBS microscope. The FRET efficiency was calculated as E = (Post −[Pre/Post]) × 100%, where Post and Pre represents the donor fluorescence before and after photo bleaching.

**Cloning and purification of recombinant protein**
The DHR-2 domain of DOCK8 (residues 1632–2086) was cloned into the pET-28a plasmid with a N-terminal His·tag (Novagen), and Cdc42G15A was inserted into the pGEX4T1 plasmid (GE Healthcare). Recombinant proteins were expressed in E. coli BL21 (DE3) Codon-Plus strain (Novagen). The transformed cells were grown at 37°C in LB medium containing 0.05 mg/ml ampicillin until OD600 reached 0.8, and then induced with 0.25 mM IPTG at 16°C for 24 h His-DHR-2 was purified by Ni-NTA affinity chromatography (QIAGEN) and GST-Cdc42G15A was purified by glutathione Sepharose beads as previously reported (Yang et al., 2013; Zhang et al., 2014). FLAG-LRR1-9 and FLAG-L305-763 were transfected into 293T cells and purified with anti-FLAG antibody. The increasing amount of the purified FLAG-LRR1-9 or FLAG-L305-763 was added to a solution containing HisDHR-2 and GST-Cdc42G15A. The mixture were incubated at 4°C for 60 min and then subjected to anti-His antibody precipitation.
MS and yeast two-hybrid assays

Immunoprecipitation using an anti-FLAG antibody was performed from T8.1 cells overexpressing FLAG-tagged DOCK8. The pull-down proteins at 90 kD were identified by the mass spectrometry analysis (below panel). DOCK8 was used as bait for screening a human cDNA library encoding over 12,794 human genes (hORFeome V5.1) by a yeast two-hybrid assay, and LRCH1 was found as a positive clone.

Statistics

All statistical analyses were performed with Prism6 software (GraphPad Software). Student's t test was used for comparisons between two groups. P < 0.05 was considered statistically significant.

Study approval

All procedures of animal experiments were conducted in accordance with the institutional guidelines and were approved by the Institutional Animal Care and Use Committee of Shanghai Institute of Biochemistry and Cell Biology (Protocol No. IBC0057). For collecting human MS patients' blood samples, the study was approved by the Ethical Committee for Clinical Research of Huashan Hospital, Fudan University.

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