ClipR-59 Interacts with Elmo2 and Modulates Myoblast Fusion*

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Recent studies using ClipR-59 knock-out mice implicated this protein in the regulation of muscle function. In this report, we have examined the role of ClipR-59 in muscle differentiation and found that ClipR-59 knockdown in C2C12 cells suppressed myoblast fusion. To elucidate the molecular mechanism whereby ClipR-59 regulates myoblast fusion, we carried out a yeast two-hybrid screen using ClipR-59 as the bait and identified Elmo2, a member of the Engulfment and cell motility protein family, as a novel ClipR-59-associated protein. We showed that the interaction between ClipR-59 and Elmo2 was mediated by the atypical PH domain of Elmo2 and the Glu-Pro-rich domain of ClipR-59 and regulated by Rho-GTPase. We have examined the impact of ClipR-59 on Elmo2 downstream signaling and found that interaction of ClipR-59 with Elmo2 enhanced Rac1 activation. Collectively, our studies demonstrate that formation of an Elmo2-ClipR-59 complex plays an important role in myoblast fusion.

Elmo (engulfment and cell motility) proteins were identified based on their homology to Caenorhabditis elegans Ced-12, a protein that is required for apoptotic cell engulfment and cell migration (1). Elmo proteins are characterized by the presence of a Ras GTPase-binding domain, a region that is present only in Elmo proteins and ElmoD protein (Elmo domain), an atypical PH domain (aPH)4 and a proline-rich region with a P

Significance: Identification of ClipR-59 as a novel promoter of muscle differentiation through its ability to orchestrate Rac signaling.
ClipR-59 also interacts with TNF receptor to regulate TNFα signaling (28). Inactivation of ClipR-59 in mice resulted in perinatal lethality in part due to muscle malfunction, implying that this gene plays a crucial role in muscle development (29). However, how ClipR-59 affects muscle function remains unexplored.

In this report, we have examined the role of ClipR-59 in muscle differentiation and found that ClipR-59 is required for myoblast fusion. Moreover, we also found that ClipR-59 interacts with Elmo2 to enhance Rac1 activation.

### EXPERIMENTAL PROCEDURES

**Reagents**—Insulin, DAPI, mouse monoclonal anti-FLAG antibody, HRP-conjugated anti-FLAG, and anti-HA antibodies were from Sigma. Mouse monoclonal anti-HA antibody was from Covance. Mouse monoclonal anti-Elmo2, anti-GST and anti-Myc antibodies, anti-myogenin, anti-myosin heavy chain, and anti-β-tubulin antibodies were from Santa Cruz. Rabbit monoclonal anti-Akt and phospho-Akt antibodies were from Cell Signaling. Rabbit anti-ClipR-59 antibody has been described previously (26). TnT kit was from Promega.

**Plasmids and Virus Production**—ClipR-59 and its mutants have been described (27). Myc-tagged Elmo expression vectors and FLAG-Dock 180 have been described (6). FLAG-180 was a gift of Dr. Michiyuki Matsuda of Kyoto University. GST-CRIB was a gift of Dr. Ralph Isberg, Tufts University School of Medicine (30). Rac1 and RhoG expression vectors were a gift of Dr. Ralph Isberg, Tufts University School of Medicine (31). The sequence of ClipR-59 shRNA has been described (26). The lentiviral vectors and subcloning strategy to generate shRNA lentiviral expression vectors and produce the lentiviral particles have been described (32). These lentiviral vectors encode a GFP protein independent of shRNA expression so that the transduced cells can be seen through green fluorescence.

The deletion mutants of Elmo2 were generated by using convenient restriction sites in the Elmo2 cDNA. To generate mammalian GST-Elmo2 expression vectors, mouse Elmo2 cDNA was amplified by PCR with primers 5’-GGAGATCTATGCGCTCCTCGTCTGACA-3’ and 5’-ctcttagcactgata-gac-3’ and cloned into BamHI and SpeI site of pEBG. To generate Elmo2pHA, the sequences after the PH domain were amplified with primers 5’-GGAGATCTATGCGCTCCTCGTCTGACA-3’ and 5’-ctcttagcactgata-gac-3’ and cloned into BamHI and SpeI site of pEBG. To generate Elmo2pMYC, the full-length Elmo2 cDNA was amplified with primers 5’-CCTCTAGACTAGCCATAGTAGTGACTAGAC-3’ and 5’-CCCTCGAGTCAGCCATAGTAGTGACTAGAC-3’ and digested with EcoRI and XhoI. The full-length Elmo2 cDNA was amplified with primers 5’-CCTCTAGACTAGCCATAGTAGTGACTAGAC-3’ and 5’-CCCTCGAGTCAGCCATAGTAGTGACTAGAC-3’ and digested with BglII and MfeI. Then, the EcoRI-Xho fragment and BglII-MfeI fragments were together ligated to either pCMV-Myc vector or pCMV HA vectors. Finally, the EcoRI and XhoI fragment was also cloned into pc-FLAG and pCHA vectors to obtain HA and FLAG-tagged Elmo2, respectively. To generate GST expression vector that express the aPH domain of Elmo2, aPH (aa 534–677) were amplified with primer 5’-GGATCCCTGGAGATCTGGAGCGTGC-3’ and 5’-CCTCTAGACTAGTAGTGACTAGAC-3’ digested with BamHI and XhoI and cloned into pEGX 5-1 expression vector.

To generate retroviral expression vectors for ClipR-59 and its mutants, cDNA fragments were cloned into pMigR1 retroviral vector between HpaI and XhoI sites. The viral particles were produced as described (32). To generate GST-E/P/ClipR-59, the E/P domain was amplified with primers 5’-GTGGATCCCATGGACTAAGACAGATCTCGT-3’ and 5’-CCTCTAGACTAGTGACTAGAC-3’, digested with BamHI and XhoI, and cloned into pGEX 5-1 expression vector.

**Cell Culture and Transfection**—COS-7 cells were grown in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen). C2C12 cells were grown in the same DMEM but supplemented with 20% bovine serum instead of FBS. The differentiation of C2C12 cell was as follows. Briefly, C2C12 cells were cultured to 60–80% confluence. Then, the medium was changed to differentiation medium (DMEM supplemented with 2% horse serum). 72–96 h later, myoblast formation was examined.

**Cell Imaging**—C2C12 cells transduced with the indicated viruses were differentiated for 72–96 h. Then the cells were fixed and stained with DAPI. The cells were mounted on a glass coverslide. The fluorescence imaging was captured with confocal microscopy (Olympus).

**Quantification of Myoblast Fusion**—The myoblast fusion is quantified as the ratio of differentiated cells and total cells, which is referred to as the fusion index. Briefly, after differentiation, C2C12 cells were fixed and stained with DAPI. Then, the total number of viral transduced cells (with green fluorescence) and the fluorescence cells that consist of more than three nuclei (based on DAPI staining) were counted, respectively. Then, the ratio of the number of fluorescence cells, which has more than three nuclei, and total fluorescence cells are calculated as myoblast fusion index.

**Immunoprecipitation Assays**—Transfected COS-7 cells or C2C12 cells were extracted with immunoprecipitation buffer (150 mM NaCl, 25 mM Tris, pH 7.6, 0.5 mM EDTA, 10% glycerol, 0.5% Nonidet P-40 plus a protease inhibitor mixture). 500 μg of total proteins from the cell lysates were subjected to immunoprecipitation with the corresponding antibodies.

**GST Pulldown Assay**—GST-ClipR-59 fusion peptide was expressed in COS-7 or HEK293 cells from pEBG expression vector and purified with glutathione-Sepharose 4B according to the manufacturer’s instruction. For examining Elmo2 and ClipR-59 interaction, purified GST-ClipR-59 or Elmo2 was mixed with the total cell lysate of COS-7 cells that had been transiently transfected with proper expression vectors in immunoprecipitation buffer and incubated for 4 h. Then, the beads were washed 3 times, and the proteins associated with GST beads were analyzed in Western blot with the corresponding antibodies. Also, before washing, 2% volume of the mixtures was taken out for Western blot to determine the input level of each component. In some cases, cells were cotransfected with pEBG expression vectors with the indicated Elmo2 or ClipR-59 expression constructs.

**Rac1 Activation Assay**—The assay to measure GTP-loaded Rac1 was carried out as previously described (30). Briefly, GST-CRIB was produced in Escherichia coli BL21 and purified on...
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Figure 1. ClipR-59 modulates myoblast fusion. A, ClipR-59 expression during C2C12 muscle differentiation. Total cell lysates from C2C12 cells that had undergone differentiation at the indicated time points were analyzed in Western blot with anti-ClipR-59 (i), anti-myogenin (ii), and anti-β-tubulin (iv) antibodies, respectively. B, ClipR-59 shRNA expression in C2C12 cells suppressed myoblast fusion. C2C12 cells were transduced with lentiviral vectors that expressed luciferase, and ClipR-59 shRNA, respectively. 48 h post-transduction, cells were switched into differentiation medium (DMEM supplemented with 2% horse serum). 72 h later, the cells were fixed and stained with DAPI. The scale bar represents 50 μm. C, quantification of C2C12 myoblast fusion. The fusion index is the ratio of GFP positive cells that contains more than 3 nuclei and total GFP cells. Bar graphs show mean ± S.D., n = 9 (fields). D, Western blot analysis of total cell lysates collected from the cells in B with anti-myogenin (Myog), ClipR-59, and β-tubulin antibodies, respectively. E, densitometric analysis of ClipR-59 expression in D. The amount of ClipR-59 in luciferase shRNA expressing C2C12 cells before differentiation was set as 1 after normalized to β-tubulin. The bar graphs show ± S.D. (n = 3). In all cases, p < 0.05.

Results

ClipR-59 Is Involved in Myoblast Fusion—Recent studies revealed that ClipR-59 knock-out mice display embryonic lethality in part because of impaired muscle function (29). This implied that ClipR-59 could play a role in muscle development and prompted us to examine the role of ClipR-59 in muscle differentiation. First, we examined ClipR-59 expression during differentiation of C2C12 cells into myoblasts. This cell line is widely used to study muscle differentiation and is amenable to dissect myoblast fusion (34). As shown in Fig. 1A, expression of ClipR-59 was increased following C2C12 differentiation. This change in expression of ClipR-59 is correlated to muscle differentiation, as the expression of myogenin (Fig. 1, panel ii) and myosin heavy chain (MHC) (panel iii), markers of muscle cell differentiation, were also increased. Together, these data demonstrate that ClipR-59 expression is induced during muscle cell differentiation. We next introduced a validated ClipR-59 shRNA into C2C12 cells via lentiviral gene transfer. As a control, cells were transduced with a lentiviral expression vector that expresses an shRNA targeting luciferase. As shown in Fig. 1, B and C, interfering with ClipR-59 expression in C2C12 cells reduced the number of myoblasts (>3 nuclei in each fiber) by more than 80%.

Next, we examined the expression of myogenin in control and ClipR-59 shRNA expressing C2C12 cells. As shown in Fig. 1D, a marginal difference in the expression of myogenin between control and ClipR-59 shRNA-expressing C2C12 cells was observed. In these experiments, we also examined the expression of ClipR-59. As expected, the expression of ClipR-59 is induced during muscle cell differentiation (Fig. 1, panel ii) and suppressed by ClipR-59 shRNA from 90 to 60% depending on the time of differentiation. Taken together, these data demonstrate that ClipR-59 plays a role in muscle differentiation at the myoblast fusion step.

ClipR-59 Interacts with Elmo2—ClipR-59 is a membrane-associated protein without enzymatic activity. To gain mechanistic insights on the role of ClipR-59 in muscle differentiation, we set out to identify its interacting proteins. Toward this end, we carried out a yeast two-hybrid screen using a murine embryonic fibroblast F422A-3T3 cDNA library using ClipR-59 as the bait.

Glutathione beads. Total lysates from COS-7 cells that were transiently transfected with proper expression vectors as indicated in the figure legends were incubated with GST-CRIB beads. After 4 h incubation, GST beads were washed extensively and analyzed in Western blot with the proper antibodies.

Western Blotting—After treatments, cells were washed twice with PBS and extracted with cell lysis buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, protease and phosphatase inhibitors). For cellular fractionation experiments, the cellular fractions were directly dissolved into lysis buffer. Equal amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The quantification of Western blot was determined with Image J software.

Yeast Two-hybrid Screen—The yeast two-hybrid screen was carried out with full-lengthClipR-59 as the bait as described previously (33).

Statistical Analysis—Mean ± S.D. were calculated and statistically significant differences among groups were determined by one-way analysis of variance analysis followed by post hoc comparisons, or by two-tailed unpaired Student’s t test between two groups as appropriate, with significance at p < 0.05.
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Several potential ClipR-59-interacting proteins were identified in this screen. One of them, Elmo2, was particularly interesting because it was recently reported to play an essential role in myoblast fusion (6).

We next aimed to validate Elmo2 as a bona fide ClipR-59-interacting protein in mammalian cells. Toward this goal, a co-immunoprecipitation assay with differentiated C2C12 cells was immunoprecipitated with anti-Elmo2 antibody. The anti-Elmo2 immunoprecipitates were analyzed in Western blot with anti-ClipR-59 antibody (top). The middle and bottom panels show the levels of Elmo2 and ClipR-59 C2C12 total cell lysates. B, co-immunoprecipitation assay to show that transiently expressed Elmo2 and ClipR-59 interact. Total cell lysates from COS-7 cells transiently co-transfected with HA-tagged ClipR-59 expression vectors plus vectors expressing either FLAG-tag alone or FLAG-Elmo2 were subjected to immunoprecipitation with anti-FLAG antibody. The anti-FLAG immunoprecipitates were analyzed in Western blot with HRP-conjugated anti-HA antibody (top). The middle and bottom panels show the levels of FLAG-Elmo2 and HA-ClipR-59 in TEL. C, GST-pulldown assay with GST alone or GST-ClipR-59 purified from HEK293T cells and the total cell lysates from COS-7 cells that transiently expressed Myc-tagged Elmo2. The GST beads were analyzed in Western blot with anti-Myc (top) and anti-GST antibodies (bottom), respectively. The middle shows the total cellular levels of Myc-Elmo2. These experiments were repeated 2–4 times with similar results. IB, immunoblot.

ClipR-59 was produced in 293T cells and were incubated with COS-7 cells lysates that expressed Myc-Elmo2. As shown in Fig. 2C, Myc-Elmo2 was robustly detected in GST-ClipR-59 beads. The middle and bottom panels represent the cellular levels of Myc-Elmo2 and GST fusion proteins, respectively. Collectively, these data demonstrate that ClipR-59 interacts specifically and directly with Elmo2 in mammalian cells.

ClipR-59 Preferentially Interacts with Elmo2 and Elmo3—There are three Elmo proteins in mammals including Elmo1, Elmo2, and Elmo3 (5). We investigated whether ClipR-59 would also interact with other isoforms of Elmo proteins. To test this, a GST pulldown assay was carried out with purified GST-ClipR-59 isolated from HEK293T cell lysate and incubated with total cell lysates of COS-7 cells that were transiently transfected with expression vectors coding for Myc-tagged Elmo1, Elmo2, or Elmo3. As shown in Fig. 3A, all Elmo proteins were retained on GST-ClipR-59 beads, but the amount of Elmo2 and Elmo3 was markedly higher than that of Elmo1. The Elmo proteins retained on the beads were specific to GST-ClipR-59 as no detectable Elmo proteins were found on GST beads alone. The variation among Elmo proteins retained on GST-ClipR-59 beads could not be ascribed to sample variations as comparable levels of Elmo proteins and GST proteins were observed.

To examine this differential association further, we carried out GST pulldown assays with GST-Elmo proteins and FLAG-tagged ClipR-59 expressed in COS-7. As shown in Fig. 3B, ClipR-59 was retained on all GST-Elmo beads. However, the amount of ClipR-59 on GST-Elmo2 and Elmo3 beads was 6-fold higher than that on GST-Elmo1 beads. The ClipR-59 retained on GST-Elmo was specific to Elmo proteins as no ClipR-59 was found on GST beads alone (lane 1, top panel) and comparable levels of ClipR-59 (middle) and GST fusion proteins were observed in each sample.

To rule out the possibility that the lower affinity of Elmo1 for ClipR-59 is due to non-functional Elmo1, we examined the association of Elmo1, Elmo2, and Elmo3 with Dock180, a known Elmo-interacting protein. As expected, all three Elmo proteins exhibited a comparable capability of interacting with Dock180 (Fig. 3C). Taken together, these data demonstrate that ClipR-59 preferentially interacts with Elmo2 and Elmo3.

The Interaction between ClipR-59 and Elmo2 Enhances the Levels of Active Rac1—Elmo2 interacts with the Dock family protein to activate downstream targets including Rac1 (35). To determine whether Elmo2, ClipR-59, and Dock protein forms a tertiary complex, a co-immunoprecipitation assay was carried out with COS-7 cell lysates that express FLAG-Dock180 alone or HA-ClipR-59 plus FLAG-Dock2, or HA-ClipR-59 plus FLAG-Dock180 and Myc-Elmo2, with anti-FLAG antibody. As shown in Fig. 4A, FLAG-Dock180 was only significantly immunoprecipitated with anti-HA antibody in the presence of both HA-ClipR-59 and Myc-Elmo2, indicating that ClipR-59, Elmo2, and Dock180 are present in the same complex.

Binding of Elmo2 to Dock family proteins enhances the levels of active Rac1 (GTP-bound Rac1). Active Rac1, in turn, binds Pak1 and activates it (36). The notion that ClipR-59, Elmo2, and Dock180 are in the same complex raises the possibility that ClipR-59 enhances Rac1 activation. To test this, we carried out
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A GST pulldown assay to examine the impact of ClipR-59 and Elmo2 co-expression on Rac1 GTP loading using recombinant GST-CRIB. GST-CRIB is a GST fusion protein that consists of Cdc42/Rac1 interactive-binding (CRIB) domain of the p21-activated kinase 1 and only interacts with active Rac1 or Cdc42 (30). As shown in Fig. 4B, the levels of Rac1 associated with GST-CRIB under forced expression of either ClipR-59 or Elmo2 were not different from that of control cells. In marked contrast, the amount of Rac1 associated with GST-CRIB increased about 3-fold with ClipR-59 and Elmo2 co-expression (Fig. 4B, top panel). As a control, we also examined the association of Rac1 and GST-CRIB with Dock180 expression. As expected, forcing expression of Dock180 strongly enhanced the association of Rac1 and GST-CRIB.

To further verify the impact of ClipR-59 on Rac1 activation, we next examined the impact of ClipR-59 on Rac1 activation in C2C12 cells. As shown in Fig. 4D, active Rac1 were readily pulled down with GST-CRIB in luciferase shRNA expressing C2C12 cells (Fig. 4, panel i). In contrast, a 40% reduction of Rac1 pulled by GST-CRIB was observed in ClipR-59 shRNA expression cells (Fig. 4D, panel i, for quantification). As expected, ClipR-59 shRNA was effective to suppress ClipR-59 expression (Fig. 4D, panel iii). Together, these data demonstrate that interaction of ClipR-59 with Elmo2 enhances the cellular levels of active Rac1.

The aPH Domain of Elmo2 Is Required for Interaction between Elmo2 and ClipR-59—Among three Elmo proteins, Elmo1 and Elmo2 are the most studied. Because ClipR-59 preferentially interacts with Elmo2, we set out to characterize the interaction between ClipR-59 and Elmo2 in more detail. Toward this, we generated a series of Elmo2 mutants in which different portions of Elmo2 were deleted (Fig. 5A). We tested the ability of each mutant to interact with ClipR-59 in a GST pulldown assay. As shown in Fig. 5B, removal of the first 107 (ΔN1) and 307 aa (ΔN2) from the amino terminus of Elmo2 did not prevent binding but actually increased the amount of Elmo2 retained on GST-ClipR-59 beads. Removal of the last 60 aa (ΔC1) from the carboxyl terminus of Elmo2 also increased the amount of Elmo2 retained on GST-ClipR-59 beads (Fig. 5B2, top panel, compare lanes 2 and 4). However, removal of the last 190 aa from the carboxyl terminus of Elmo2 (ΔC2) abolished Elmo2 precipitation by GST-ClipR-59 beads (Fig. 5B2, top panel, compare lanes 2 and 5). The failure to detect ΔC2 Elmo2 on GST-ClipR-59 beads was not because of sample variation as the levels of Elmo2 and Elmo2 mutants (Fig. 5B2, middle panel) as well as that of the GST protein (Fig. 5B2, bottom panel) were observed in each sample. The last 190 aa of Elmo2 consists of a putative PH domain, EAD, and a PXXP motif. EAD and PXXP motifs are localized in the last 60 amino acids. Because removal of the EAD and PXXP motifs had no effect on the interaction between Elmo2 and ClipR-59, we reasoned that the aPH domain of Elmo2 likely mediates the interaction of Elmo2 with ClipR-59. To test this, the aPH domain of Elmo2 was internally deleted and the resultant mutant (designated as Elmo2ΔPH) was tested for interaction with ClipR-59 in a GST pulldown assay. As shown in Fig. 5C, wild-type Elmo2 was readily detected in GST-ClipR-59 beads but in contrast no ΔaPH Elmo2 was found on GST-ClipR-59 beads (top panel) despite comparable levels of wild-type and Elmo2ΔPH (middle panel) as well as GST-ClipR-59 among these samples.

To confirm that the aPH domain of Elmo2 is sufficient to mediate the interaction between Elmo2 and ClipR-59, we next carried out a GST pulldown assay with GST-aPHElmo2 and ClipR-59 coexpressed in COS-7 cells. As shown in Fig. 5D, ClipR-59 was readily detected in GST-aPHElmo2 beads, but not in GST beads (top panel). As expected, removal of the first 300 aa from Elmo2 had no impact on the interaction between...
ClipR-59 and Elmo2 (top panel). Together, we conclude that the aPH domain of Elmo2 mediates the interaction between Elmo2 and ClipR-59. In addition, these data suggest that binding of Elmo2 to ClipR-59 is in part dependent on the Elmo conformation state.

ClipR-59 and Elmo2 Interaction Is Regulated by Rho GTPase—Elmo proteins exist as autoinhibited scaffolds under basal conditions through the intramolecular interaction between the Elmo inhibitory domain and EAD (4) (Fig. 5A). At present, how this inhibitory status is relieved remains elusive. It is postulated that binding of active RhoG GTPase to Elmo proteins targets the Elmo-Dock complex to the membrane, via the Ras GTPase-binding domain, to mediate Rac1 activation (4, 14). As described above, we noticed that the removal of the first 100 aa from the amino terminus as well as 60 aa from the carboxyl terminus enhances the interaction between ClipR-59 and Elmo2 (Fig. 5B), raising the possibility that the interaction between ClipR-59 and Elmo2 is subject to specific regulation. With this in mind, we tested the impact of active RhoG expression on ClipR-59 and Elmo2 in GST pulldown assays. As shown in Fig. 6A, exogenous expression of constitutively active RhoG\textsuperscript{G12V} increased the amount of Elmo2 associated with GST-ClipR-59 by more than 3-fold (top panel). In these experiments, we also examined the impact of active Rac1\textsuperscript{G12V} on the interaction between ClipR-59 and Elmo2 as Rac1 has been reportedly involved in the interaction between Dock180 and Elmo1 (37). Indeed, we observed that expression of constitutively active Rac1\textsuperscript{G12V} also increased the association of ClipR-59 with Elmo2 (Fig. 6A, top panel). In these experiments, we also examined the presence of RhoG and Rac1 in GST-ClipR-59 beads. In agreement with the notion that RhoG is associated with Elmo2 (14), RhoG was detected in GST-ClipR-59 beads (panel iii). Panels ii, iv, and v show the cellular levels of Myc-Elmo2, GST-ClipR-59, and FLAG-RhoG and FLAG-Rac1, respectively.

Rho-GTPases are activated by a range of extracellular stimuli including insulin and growth factors. With this in mind, we tested the impact of insulin and IGF on the interaction between ClipR-59 and Elmo2. As shown in Fig. 6B, treatment of cells with either IGF or insulin enhanced the interaction between ClipR-59 and Elmo2 (panel i, compare lanes 1, 2, and 3). The IGF and insulin used here are active as
they induced Akt phosphorylation (panels iv and v). Panels ii and iii show the levels of Myc-Elmo2 and GST-ClipR-58 in total cell lysates, respectively.

To further evaluate the regulation of ClipR-59 and Elmo2 interaction by insulin, we next examined the impact of insulin on the interaction between ClipR-59 and ΔN2 Elmo2, which showed a stronger interaction with ClipR-59 (Fig. 5B). As shown in Fig. 6C, as expected, ΔN2 Elmo2 still showed a strong interaction with ClipR-59 (compare lanes 1 and 3, panel i). However, insulin treatment appeared to diminish the interaction between ClipR-59 and Elmo2, suggesting that insulin treatment relieved Elmo2 from an autoinhibitory status. Insulin treatment was effective as it induced Akt phosphorylation without an effect on total cellular levels of Akt (panels iv and v). Panels ii and iii show the total cellular levels of Myc-Elmo2 and GST-ClipR-58.

The Domain in ClipR-59 That Mediates the Interaction between Elmo2 and ClipR-59—To determine the domain in ClipR-59 that mediates the interaction between Elmo2 and ClipR-59, ClipR-59 was divided into portions 1–245 (designated as ΔC1) and 245–547 (designated as ΔN1) and the resulting ClipR-59 peptides were tested for their interaction with Elmo2 in a GST pulldown assay. As shown in Fig. 7B, ΔC1-ClipR-59, but not ΔN1-ClipR-59 was retained on GST-Elmo2 beads, suggesting that sequences within the first 245 aa residues of ClipR-59 mediate the interaction between ClipR-59 and Elmo2 interaction.

The first 254 aa of ClipR-59 consists of a Glu-Pro-rich domain and three ankyrin repeats. Both are protein-protein interaction modules. To determine whether ankyrin repeats play a role in ClipR-59 and Elmo2 interaction, we internally deleted ankyrin repeats from ClipR-59 (designated as ΔANK ClipR-59) and examined the interaction of ΔANK ClipR-59 with Elmo2 in a GST pulldown assay. As shown in Fig. 7C, ΔANK ClipR-59 showed a similar capacity to wild-type ClipR-59 in the interaction with Elmo2 (compare lanes 2 and 3, top panel), ruling out the role of ankyrin repeats in the interaction between ClipR-59 and Elmo2. In these experiments, we also examined interaction of the ClipR-59 mutant Δ2CAP ClipR-59 in which both CAP-Gly domains were deleted. No alteration of interaction between Elmo2 and ClipR-59 was observed when both CAP-Gly domains were deleted.

The notion that ankyrin repeats in ClipR-59 play no role in the interaction between ClipR-59 and Elmo2 implies that the
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To better understand the interaction between ClipR-59 and Elmo2, we have characterized the sequences in ClipR-59 and Elmo2 that mediate the interaction between ClipR-59 and Elmo2. We found that the PH domain of Elmo2 and the Glu-Pro-rich domain of ClipR-59 interacted in vitro and regulate Rac1 activation (Fig. 4), indicating the possible mechanism by which ClipR-59 modulates muscle differentiation. There are three Elmo proteins in mammals. Given the sequence similarity, Elmo proteins are believed functionally exchangeable. Interestingly, we found that ClipR-59 preferentially interacted with Elmo2 and Elmo3 among three mammalian Elmo proteins, indicating the possibility that each Elmo protein may have a specific function.

To further understand the interaction between ClipR-59 and Elmo2, we have characterized the sequences in ClipR-59 and Elmo2 that mediate the interaction between ClipR-59 and Elmo2. We found that the PH domain of Elmo2 and the Glu-Pro-rich domain of ClipR-59 mediated the interaction between Elmo2 and ClipR-59 (Figs. 4 and 7). The PH domain of ClipR-59 is highly acidic. The PH domain is often associated with an acidic motif (39). This is also in agreement with the belief that ClipR-59 plays a role in the regulation of membrane dynamics (24).

To illustrate the mechanism by which ClipR-59 regulates muscle differentiation, we set to identify new ClipR-59-interacting proteins and identified Elmo2 using a yeast two-hybrid screening approach. Elmo2 is essential for muscle differentiation. Our data indicate that ClipR-59 and Elmo2 interact in vivo (Fig. 2) and regulate Rac1 activation (Fig. 4), indicating the possible mechanism by which ClipR-59 modulates muscle differentiation. There are three Elmo proteins in mammals. Given the sequence similarity, Elmo proteins are believed functionally exchangeable. Interestingly, we found that ClipR-59 preferentially interacted with Elmo2 and Elmo3 among three mammalian Elmo proteins, indicating the possibility that each Elmo protein may have a specific function.

To better understand the interaction between ClipR-59 and Elmo2, we have characterized the sequences in ClipR-59 and Elmo2 that mediate the interaction between ClipR-59 and Elmo2. We found that the PH domain of Elmo2 and the Glu-Pro-rich domain of ClipR-59 mediated the interaction between Elmo2 and ClipR-59 (Figs. 4 and 7). The PH domain of Elmo2 has no binding activity toward phospholipid (38), therefore this domain is considered an atypical PH domain. The PH domain is often associated with an acidic motif (39). The Glu-Pro-rich domain of ClipR-59 is highly acidic. The finding that the Glu-Pro-rich domain ClipR-59 interacts with the PH domain of Elmo2 is in line with the notion.
**FIGURE 7. The amino terminus of ClipR-59 mediates the interaction between ClipR-59 and Elmo2.**

A. Schematic presentation of ClipR-59 proteins. The functional domains are indicated. E/P, Glu-Pro-rich domain; ankyrin, ankyrin repeats; CC, palmitoylated Cys at 534 and 535.

B. GST pulldown assay to show that the amino-terminal sequence of ClipR-59 mediates the interaction between ClipR-59 and Elmo2. Total cell lysates from COS-7 cells were transiently co-transfected with FLAG-ClipR-59 (wild-type and mutants) expression vectors plus either pEBG (GST vector) or pEBG-Elmo2 were subject to GST pulldown assay. The proteins associated with GST beads were analyzed in a Western blot with anti-FLAG antibody (top). The middle panel shows the levels of FLAG-ClipR-59 and its mutant in TCL. The bottom panels show the levels of GST fusion proteins associated with GST beads. The GST pulldown assay to show that ankyrin repeats of Elmo2 are not required for the interaction between ClipR-59 and Elmo2. The experiments were carried out similar to B, except FLAG-ΔANK-ClipR-59 (ΔA) and FLAG-Δ2CAP-ClipR59 (ΔC2) were used. D. GST pulldown assay to show that the E/P domain of ClipR-59 mediates the interaction between ClipR-59 and Elmo2. Total cell lysates from COS-7 cells that were transiently co-transfected with Myc-Elmo2 (wild-type) expression vectors plus either pEBG (GST vector) or pEBG-ClipR-59 or pEBG-ClipR-59ΔE/P were subjected to GST pulldown assay. The proteins associated with GST beads were analyzed in Western blot with anti-FLAG antibody (top). The middle panel shows the levels of Myc-Elmo2 in TCL. The bottom panel shows the levels of GST fusion proteins associated with GST beads. These experiments were repeated 2–4 times with similar results. A representative result is shown. E. GST pulldown assay to show that the E/P domain of ClipR-59 mediates the interaction between Elmo2 and ClipR-59 with recombinant GST-E/P ClipR-59 and in vitro translated FLAG-Elmo2. Top, GST beads associated with FLAG-Elmo2; middle, FLAG-Elmo2 input; bottom, GST fusion protein input. F. Forcing expression of the Elmo2 interaction defective ClipR-59ΔE/P impaired C2C12 cell differentiation. C2C12 cells were transduced with pMigR1 (left, served as a control), pMigR-ClipR-59 (middle), and pMigR ClipR-59ΔE/P, respectively. 48 h post-transduction, cells were switched into differentiation medium (DMEM supplemented with 2% horse serum). 72 h later, the cells were fixed and stained with DAPI. G. Quantification of C2C12 myoblast fusion. The fusion index is the ratio of GFP positive cells that contains more than 3 nuclei and total GFP cells. Bar graphs show mean ± S.D., n = 9 (fields). In all cases, p < 0.05. H. Forcing expression of ClipR-59, but not ClipR-59ΔE/P in ClipR-59 shRNA expressing C2C12 cells rescues C2C12 differentiation. The C2C12 cells were co-transduced with lentiviral vectors that express ClipR-59 shRNA and pMigR1 vectors as indicated. Then, the cells were differentiated and examined as described in F. The numbers under each panel are the fusion index as mean ± S.D., n = 9 (fields). In all cases, p < 0.05. IB, immunoblot.
Interaction between ClipR-59 and Elmo2

The atypical PH domain of the Elmo protein was required for Elmo protein to interact with Dock protein. In our studies, we found that the PH domain was also required for Elmo2 to interact with ClipR-59. The question raised is how the same domain mediates the interaction of Elmo2 with both proteins? Studies of the interaction between Elmo and Dock protein revealed that the aPH domain does not directly contact with Dock, but provides a structural determinant in that the helice (αN and αC) before and after the PH domain along the PXXP motif were arranged to direct contact with Dock protein (35, 38). In our studies, we found that the aPH domain without these helices was sufficient to interact with ClipR-59 (Fig. 5D). These studies argue that the interaction of Elmo2 with ClipR-59 and Dock180 is through different sequences and unlikely mutually excluded. Further analysis of the structural requirement in the Elmo2 aPH domain is required to clarify this issue.

In the current study, we used overexpressed ClipR-59, Elmo2, as well as Dock180 to examine formation of the ClipR-59-Elmo2-Dock180 complex. Although these studies might not provide direct evidence that there is a complex consisting of these three proteins endogenously, the notion that knockdown of ClipR-59 reduced the levels of active Rac1 will argue that an endogenous complex consisting of these proteins is likely presented in cells (Fig. 4D). Further studies are required to clarify this issue.

Elmo2 exists in an autoinhibited status in which an intramolecular interaction between the Elmo inhibitory domain and EAD occurs (4). It is believed that the autoinhibitory status of Elmo2 is relieved following extracellular cues. In our studies, we found that the removal of either the Elmo inhibitory domain or the autoregulatory domain enhanced the interaction between ClipR-59. This suggests that the interaction between ClipR-59 and Elmo2 is subject to regulation. Supporting this notion, we found that expression of active Rho-GTPases such as RhoG to enhance the interaction between ClipR-59 and Elmo2 is subject to regulation. Supporting this notion, we found that the aPH domain without these helices could result from interaction between RhoG and Elmo2 as Elmo2 specifically interacts with active RhoG and was found in the ClipR-59-Elmo2 complex (Fig. 6A). Further studies are required to clarify these issues. Regardless, our studies here present the first experimental evidence that Elmo2 activity is regulated by extracellular stimuli.

To determine whether regulation of muscle differentiation by ClipR-59 required the interaction between ClipR-59 and Elmo2, we examined impact of the Elmo2 interacting defective ClipR-59 on C2C12 muscle differentiation and found that Elmo2 interacting defective ClipR-59 seemed to suppress C2C12 muscle cell differentiation (Fig. 7E), providing evidence that the interaction of ClipR-59 with Elmo2 is required for ClipR-59 to moderate muscle differentiation.

In summary, we have identified Elmo2 as a ClipR-59 interacting protein and showed that ClipR-59 regulates myoblast fusion. Overall, our studies suggest that by interacting with Elmo2, ClipR-59 facilitates Rac1 activation, and thereby muscle differentiation. Further examining the muscle development in ClipR-59 knock-out mice will be required to better define the role of ClipR-59.

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