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The N-terminal leucine-zipper motif in PTRF/cavin-1 is essential and sufficient for its caveolae-association

Zhuang Wei [a,b,1], Xinle Zou [a,1], Hongzhong Wang [a,b], Jigang Lei [a,b], Yuan Wu [a,b], Kan Liao [a,b,*]

[a] State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
[b] Laboratory of System Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

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ABSTRACT

PTRF/cavin-1 is a protein of two lives. Its reported functions in ribosomal RNA synthesis and in caveolae formation happen in two different cellular locations: nucleus vs. plasma membrane. Here, we identified that the N-terminal leucine-zipper motif in PTRF/cavin-1 was essential for the protein to be associated with caveolae in plasma membrane. It could counteract the effect of nuclear localization sequence in the molecule (AA 235–251). Deletion of this leucine-zipper motif from PTRF/cavin-1 caused the mutant to be exclusively localized in nuclei. The fusion of this leucine-zipper motif with histone 2A, which is a nuclear protein, could induce the fusion protein to be exported from nucleus. Cell migration was greatly inhibited in PTRF/cavin-1$^{-/-}$ mouse embryonic fibroblasts (MEFs). The inhibited cell motility could only be rescued by exogenous cavin-1 but not the leucine-zipper motif deleted cavin-1 mutant. Plasma membrane dynamics is an important factor in cell motility control. Our results suggested that the membrane dynamics in cell migration is affected by caveolae associated PTRF/cavin-1.

1. Introduction

PTRF/cavin-1 is originally identified as a soluble nuclear factor that regulates Pol I transcription process [1,2]. It interacts with both transcription-termination factor I and Pol I and functions as a *trans*-factor for dissociation of Pol I transcripts [1,3]. Therefore, it is called polymerase I and transcript release factor (PTRF). However, this early nomenclature differs from the reports of later studies in which the protein is found to be associated with caveolae and is named cavin-1 [4–6]. To release Pol I transcripts in nuclei and to form caveolae at plasma membrane are two different cellular functions played at different cellular locations. The nomenclature discrepancy indicates that the protein (PTRF/cavin-1) has dual functions and cellular localizations. It implies that multiple structural elements are present in the protein to determine its cellular localizations.

Cavin-1 belongs to a family of caveolar proteins, which has four members, cavin-1, 2, 3 and 4 [7–9]. Interestingly, none of the cavin family proteins is originally identified as caveolar protein [7]. They are all discovered in other studies and have names not related to caveolae: cavin-1, polymerase I and transcript release factor (PTRF); cavin-2, serum deprivation-response protein (SPDR); cavin-3, protein kinase c delta binding protein (PRKCDBP); and cavin-4, muscle-related coiled-coil protein (MURC) [10]. All the cavins share a well-conserved typical N-terminal domain containing heptad repeats of hydrophobic amino acids [11]. The involvement of cavin-1 in caveolae formation has been demonstrated by the results from knockout mice as well as cells with reduced cavin-1 expression [12–14]. In both cases, loss of cavin-1 causes loss of caveolae. Cavin-1 is recruited by caveolins to plasma membrane caveolae. The expression patterns of cavin-1 and caveolin-1 are strictly parallel in mouse tissues [9]. In cavin-1 knockout mice, the expression level of all three caveolins (caveolin-1, 2 and 3) were markedly reduced as result of protein degradation [12]. These results suggest that cavin-1 is essential for caveolin stabilization and caveolae formation *in vivo*.

In caveolae, cavin-1 and caveolin-1 are found to be in close proximity by FRET experiments [14]. However, whether there is direct interaction between cavin-1 and caveolin-1 remains to be a controversial issue. It is generally believed that cavin-1 contributes to the last steps of caveolae biogenesis, because it only associates with caveolae but not with non-caveolar caveolins [14,15]. Cavin complexes in caveolae form a peripheral protein layer around the membrane scaffold formed by caveolins.

* Corresponding author at: Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China. Fax: +86 21 54921167.
E-mail address: kliao@sibs.ac.cn (K. Liao).
[1] These authors contributed equally to this work.

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Although most early studies of PTRF/cavin-1 are about its role in ribosomal RNA synthesis as PTRF [1–3], PTRF/cavin-1 is now considered a caveolar protein and the studies are focused on its caveolae related functions. In PTRF/cavin-1, there are 2 nuclear localization sequences, which should be responsible for its nuclear localization. However, the structural element that determines its caveolae association is not fully understood. In the present study, we reported that the N-terminal leucine-zipper motif was essential for cavin-1 to associate with caveolae. Without this motif the cavin-1 mutant was localized in nuclei. The attachment of this leucine-zipper motif to histone was sufficient to export the fusion protein from nuclei. In cavin-$1^{-/-}$ MEFs, the inhibited cell motility could only be restored by the expression of exogenous wild type cavin-1 but not the cavin-1 mutant in nuclei.

2. Materials and methods

2.1. Materials

Anti-caveolin-1 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-cavin-1 antibody was from BD Transduction Laboratories (San Jose, CA, USA). Anti-pCavin-1 was generated against peptide SDAVLDL(pS)D(pS)D by GL Biochem Corporation Ltd. (Shanghai, China). HRP-conjugated secondary antibodies were from Sigma (St Louis, MO, USA).

2.2. Plasmids construction

Mouse cavin-1 cDNA (NM_008986.2) and H2Ax cDNA (NM_010436.2) were cloned from 3T3-L1 cell and constructed into pEGFP-C1 vector. Truncation, deletion or point mutations were carried out by PCR and the mutated cDNA was tagged by eGFP at the C-terminus. All the mutations were confirmed by sequencing.

2.3. Cell culture and transient transfection

Cells (3T3-L1, CHO, COS 7 and MEF) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. The transfection was performed with Lipofectamine 2000 (Invitrogen) following manufacture's protocol. Experiments were conducted 48 h after transfection.

Primary mouse embryonic fibroblasts were isolated from the embryos of cavin-$1^{+/-}$ heterozygous crossed mice. In brief, E12.5 embryos were obtained from the pregnant mice. The embryonic body was cut into small pieces and digested with collagenase A (7 units/ml) in DMEM. The dispersed cells were incubated in petri dish and non-adherent cells were discarded after 1 h incubation. Cells that adhere to the culture dish were collected and propagated [16,17]. The genotype of MEFs was determined by PCR.

2.4. Extraction of lipid microdomains and sucrose density gradient flotation centrifugation

Cultured cells were collected in 2 ml ice-cold MBS buffer containing 0.5% Triton X-100, 25 mM Mes (pH 6.8), 0.15 M NaCl, 5 mM EDTA, 1 mM PMSF and 2 μl/ml protease inhibitors cocktail 1 and 2, and mixed with equal volume of 90% sucrose in MBS buffer. The mixture was placed at the bottom of a 12 ml ultra-centrifuge tube and overlaid with 4 ml 35% sucrose and 4 ml of 5% sucrose in MBS buffer. The gradient was centrifuged at 180,000×*g* for 20 h in a SW41 rotor (Beckman) at 4 °C and fractions were collected after centrifugation [18].

2.5. Western blotting and confocal microscopy

Western blotting was performed as previously described [19]. For immunofluorescence imaging, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeated with 0.1% Triton X-100 and 3% bovine serum albumin in PBS. The cells were then incubated with primary antibody and fluorescein conjugated secondary antibody and photographed by Leica SP8 laser scanning confocal microscope. For cells expressing eGFP tagged protein, the paraformaldehyde fixed cells were visualized directly by confocal microscope [20].

2.6. Cell migration assays

For wound-healing assay, cells reached approximately 90% confluence were scratched by a wound making tool and incubated with fresh DMEM. Images were taken every hour in IncuCyte high throughput living cell culture equipment at 20× magnification. For living cell tracking assay, transfected cells were placed on living cell imaging block with fresh DMEM. Cells were tracked using Operetta high throughput cell analysis system (PerkinElmer). EGFP positive cells were identified by eGFP fluorescence.

2.7. Animal experiments

All animals were kept in the animal facility of Institute of Biochemistry and Cell Biology and experiments were performed under the ethical guidelines of the institute. Cavin-1 knockout mice (B6.129S6-Ptrftm1Pfp/J) were purchased from The Jackson Laboratory in Bar Harbor USA. Heterozygous mice (cavin-$1^{+/-}$) were crossed to breed homozygous mice (cavin-$1^{-/-}$).

3. Results

3.1. The N-terminal leucine-zipper motif was essential and sufficient for the association of cavin-1 with caveolae

The isolation of detergent-insoluble membranes by sucrose-density gradient centrifugation is a widely used method to separate caveolae and caveolar proteins from other membrane and membrane proteins [18]. Proteins associated with caveolae as well as noncaveolar detergent-insoluble membrane microdomains are floated to the low-density region. As shown in Fig. 1A, caveolin-1, the major component protein in caveolae, was all floated to the low-density region in sucrose-density gradient centrifugation. In contrast, cavin-1 was only partially floated to the low-density region. A large fraction of the cellular cavin-1 did not associate with caveolae or detergent-insoluble membrane microdomains (Fig. 1A). Same as the endogenous cavin-1, the exogenous eGFP-tagged cavin-1 expressed in CHO cells was also partitioned between detergent-resistant membranes and soluble fractions (Fig. 1A and D).

To analyze the molecular domains that determine its caveolar association, cavin-1 was cut into two halves and tagged with eGFP (Fig. 1B). These protein fragments were expressed in CHO cells and could be detected on Western blot with either anti-GFP antibody or anti-cavin-1 antibody (Fig. 1C). By sucrose-density gradient centrifugation, the N-terminal part containing the first 220 amino acids all associated with caveolar membranes, while the C-terminal part was completely in soluble fractions (Fig. 1D). The partition of cavin-1 between caveolae and soluble fractions appeared to be determined by two elements that compete for the protein's cellular localization. When these two elements were separated, each became 100% itself: a caveolae associated fragment and a completely soluble fragment (Fig. 1D). Further analysis by immunoflu-

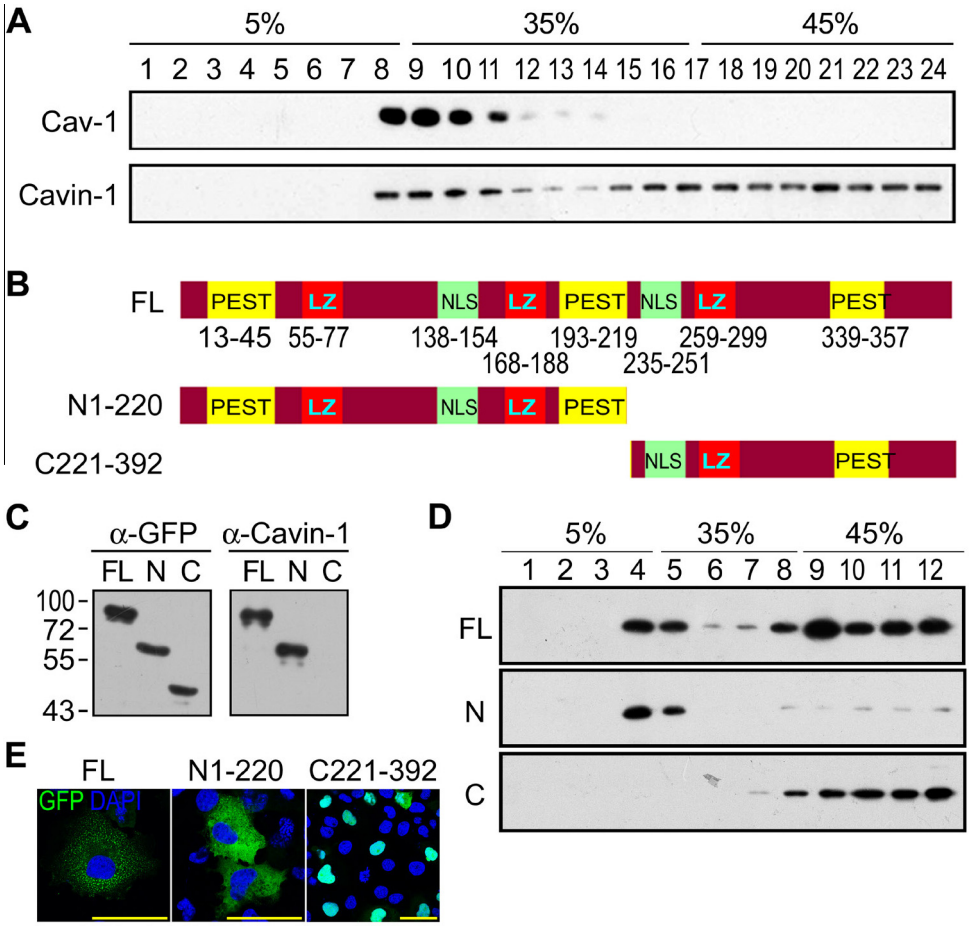

Fig. 1. The partial association of cavin-1 with detergent insoluble caveolae membranes. (A) The separation of detergent insoluble caveolae membrane by sucrose density gradient centrifugation. 5%, 35% and 45% indicate sucrose concentration and the umber 1–24 are the fractions from the density gradient (0.5 ml/fraction). (B) The construction of eGFP tagged cavin-1 fragment. The numbers indicate amino acid positions. *PEST*, PEST sequence; *LZ*, leucine-zipper motif; *NLS*, nuclear localization sequence. (C) The expression of eGFP tagged cavin-1 fragments in CHO cells. *FL*, full length cavin-1; *N*, N-terminal fragment amino acid 1–220; *C*, C-terminal fragment amino acid 221–392. (D) Sucrose density gradient separation of exogenous cavin-1 fragment. EGFP tagged cavin-1 fragments were expressed in COS 7 cells, separated by sucrose density gradient centrifugation and detected by Western blot with anti-eGFP antibody. (E) EGFP fluorescence images of eGFP-tagged cavin-1 fragments in COS 7 cells. The bar is 50 μm.

orescence staining indicated that the C-terminal fragment was actually localized in nuclei but not in cytoplasm (Fig. 1E). The partition of cavin-1 between caveolae and nuclei supported its dual functions as a membrane caveolar component and a nuclear ribosomal RNA synthesis protein.

A set of cavin-1 deletion mutations were systematically constructed to determine which sequence motif in the N-terminal region mediates caveolar association (Fig. 2A and B). After deletion of N-terminal 80 amino acids, the mutant protein dissociated from caveolae and became a nuclear protein (Fig. 2C). Eventually, it was determined that the N-terminal leucine-zipper motif (amino acids 55–77) was the key element for cavin-1 to associate with caveolae (Fig. 2C and D). Without these 23 amino acids, cavin-1 Δ55–77 mutant dissociated from caveolae and localized in nuclei. Thus, this leucine-zipper motif could counteract the effect of the nuclear localization sequence in cavin-1 and enabled the protein to stay in cytoplasm for caveolae formation.

Of three leucine-zipper motifs in cavin-1, only the N-terminal leucine-zipper motif (amino acids 55–77) had the ability to retain cavin-1 in cytoplasm (Fig. 2C and D). To ascertain if this leucine-zipper motif is sufficient to prevent protein from entering nucleus, the N-terminal 1–80 amino acid sequence was tagged to nuclear protein, histone 2Ax (1-80-H2Ax) (Fig. 2E). It was clear that the tagged leucine-zipper motif exported some histone 2Ax from nucleus into cytoplasm (Fig. 2E). Again, the deletion of leucine-zipper motif from the fusion histone 2Ax abolished its exportation from nucleus. Taken together, the N-terminal leucine-zipper motif in cavin-1 was essential and sufficient to prevent cavin-1 from entering into nucleus.

3.2. Cavin-1 phosphorylation was related to its cellular localization

Protein modifications are often linked to its cellular localizations. To ascertain if caveolar cavin-1 and nuclear cavin-1 had any difference in protein modification, we analyzed cavin-1 phosphorylation. The endogenous cavin-1 protein was present in both caveolar membrane and nucleus, whereas the C-terminal fragment was only localized in nucleus (Figs. 1A and E and 3A). Thus, the analysis of serine phosphorylation of full length cavin-1 and C-terminal fragment would reveal the difference in phosphorylation between cytoplasm and nucleus. At C-terminal end, there were two Ser phosphorylation sites: Ser389 and Ser391 (Fig. 3B). An antibody against peptide with both Ser389 and Ser391 phosphorylation was generated. The detection of endogenous and exogenous cavin-1 by the antibody was abolished by alkaline phosphatase treatment, indicating the antibody's phosphorylation specificity (Fig. 3C). Mutation of both Ser389 and Ser391 into Ala residues also abolished the antibody detection (Fig. 3D). Mutation of either Ser389 or Ser391 in full length cavin-1 did not affect the remaining Ser residue phosphorylation, which could be weakly detected by

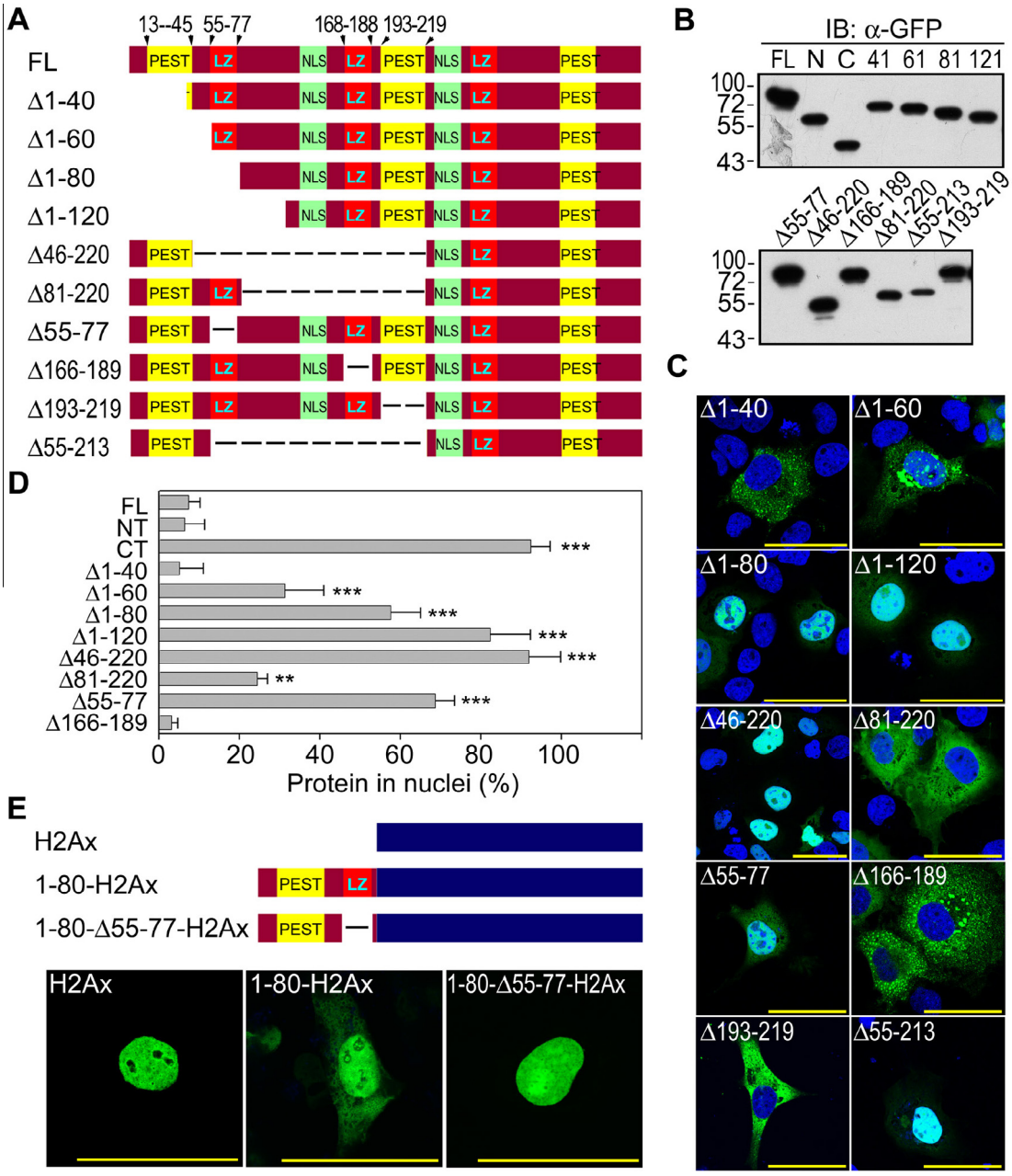

Fig. 2. Identification of N-terminal leucine-zipper motif as caveolae membrane association domain. (A) Construction of eGFP tagged cavin-1 mutants. (B) Expression of cavin-1 mutants in COS 7 cells. (C) EGFP fluorescence images of eGFP-tagged cavin-1 mutants in COS 7 cells. The nucleus was revealed by DAPI staining. The bar is 50 μm. D. The relative amount of EGFP tagged cavin-1 mutant in nuclei. The relative EGFP fluorescence intensity of cavin-1 mutant in nucleus and in whole cell was determined and the percentage of EGFP fluorescence in nucleus was calculated. For each cavin-1 mutant, at least 50 cells were calculated and averaged. $^{***}P < 0.001$, $^{**}P < 0.01$. (E) Construction and expression of leucine-zipper motif and histone fusion protein. The N-terminal 80 amino acids of cavin-1 were fused to the N-terminus of histone 2A and tagged by eGFP at the C-terminus. The fusion protein was expressed in COS 7 cells.

the antibody (Fig. 3D). In nuclear localized C-terminal cavin-1 fragment, however, the remaining Ser389 residue could not be phosphorylated after Ser391 mutation (Fig. 3D). These results suggested that the cellular localization affected cavin-1 phosphorylation.

3.3. Membrane associated cavin-1 regulated cell motility

Plasma membrane dynamics is an important factor that affects cell motility. It has been reported that cavin-1 affects cell migration. However, its effect on cell migration is mixed. In NIH3T3 cell and PC3 prostate cancer cell, expression of cavin-1 decreases cell migration [21,22]. In endothelial cells, however, knockdown of cavin-1 by small interference RNA decreases cell migration [23]. From cavin-1 knockout mice we obtained cavin-$1^{-/-}$ MEFs and compared the cell migration with wild type MEFs from the littermate. In wound-healing assay recorded by time-lapse video microscope, cavin-$1^{-/-}$ MEFs exhibited decreased motility in comparison with the wild type littermate MEFs (Fig. 4A and B). Another cavin-$1^{-/-}$ MEF cell line isolated from different mother showed the same decreased cell motility (Fig. 4A and B). To verify if the decreased cell motility was caused by the absence of cavin-1, eGFP-tagged cavin-1 was transfected into cavin-$1^{-/-}$ MEFs and the migration tracks of eGFP positive cells were recorded and analyzed by the instrument software (Fig. 4D). As shown in Fig. 4E, the expression of eGFP-tagged cavin-1 in cavin-$1^{-/-}$ MEFs greatly increased cell

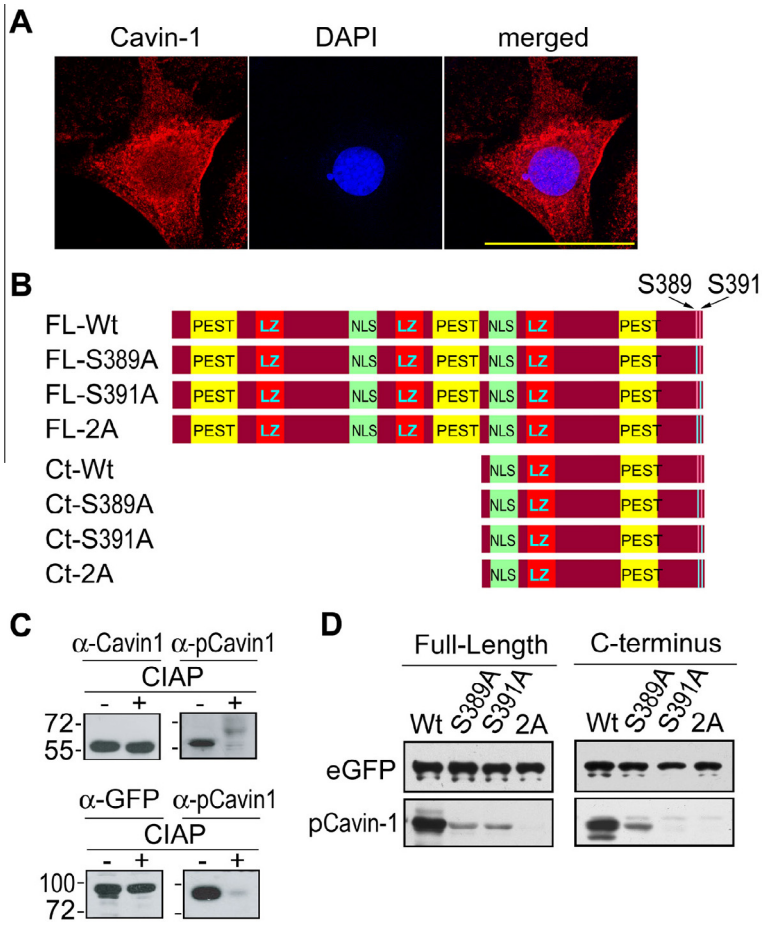

Fig. 3. The different phosphorylation of cavin-1 in cytoplasm and nucleus. (A) Immunofluorescence staining of cavin-1 in CHO cell. (B) Construction of cavin-1 with mutations in Ser389 and 391. *FL*, full length cavin-1; *Ct*, C-terminus (aa221–392); *S389A*, Ala mutation of Ser389; *S391A*, Ala mutation of Ser391; *2A*, Ala mutations of both Ser389 & 391. (C) Verification of anti-phospho-cavin-1 antibody for Ser389 and Ser391 phosphorylation. The upper panel is endogenous cavin-1 and the bottom panel is exogenous eGFP tagged cavin-1. *CIAP*, calf intestinal alkaline phosphatase. (D) Ser389 and 391 phosphorylation in cavin-1 and nuclear localized C-terminus.

motility. Interestingly, the expression of eGFP-tagged cavin-1 Δ55–77 mutant could not restore the decreased motility in cavin-1$^{-/-}$ MEFs (Fig. 4E). As cell migration is a membrane related function and a nuclear bound cavin-1 Δ55–77 mutant could hardly interact with plasma membrane structure, it was not surprise to find that the nuclear bound cavin-1 Δ55–77 mutant was ineffective in rescuing caveolae related functions.

4. Discussion

PTRF/cavin-1 was first identified as a nuclear polymerase and transcript release factor and the early studies were about its function in ribosomal RNA synthesis [1–3]. Since the identification of PTRF and cavin-1 as the same protein [4,6], its function in plasma membrane caveolae has become the focal point for studying PTRF/cavin-1. The recent works of cavin-1 raise substantial questions about its caveolae-independent functions in nuclei, especially no phenotypic defect related to ribosomal RNA synthesis has been identified in cavin-1$^{-/-}$ mice [12–14,24,25]. Even in human clinical research, Cavin-1 deficient patients are only with muscle related diseases and metabolic diseases [26–29]. In contrast, more and more evidence support the involvement of cavin-1 in caveolae: the localization of cavin-1 at caveolae by electron microscopic observation [4]; the close proximity of cavin-1 and caveolin-1 in caveolae by FRET experiment detection [14]; and the loss of morphologically identifiable caveolae in absence of cavin-1 mice [9,12].

As a caveolar protein, however, cavin-1 is not entirely similar to caveolin-1. A substantial fraction of cavin-1 is not co-isolated with caveolae (Fig. 1A). In fact, after the deletion of caveolae association motif in cavin-1, the mutated cavin-1 becomes a nuclear protein (Fig. 2). On the other hand, cavin-1 fragment without its C-terminal nuclear localization sequence becomes an entirely caveolae associated protein (Fig. 1D). It appears that two sequence elements in cavin-1 compete for its cellular localizations and the caveolae dissociated cavin-1 can enter nucleus for its function. Another line of evidence implicating cavin-1's caveolae-independent functions comes from the studies of caveolin-1$^{-/-}$ mice and cavin-1$^{-/-}$ mice. These two knockout mice share many similar phenotypes such as loss of caveolae, cardiovascular disease and metabolic disorders (dyslipidemia and glucose intolerance) [12,30,31]. However, they breed very differently. Caveolin-1$^{-/-}$ mice breed normally whereas cavin-1$^{-/-}$ mice exhibit a high perinatal lethality [25]. Their phenotypic similarities can be attributed to the loss of caveolae or membrane functional disruption. The high perinatal lethality of cavin-1 knockout mice implies the caveolae-independent functions for cavin-1.

The caveolae-dependent and caveolae-independent functions of cavin-1 can be further clarified in cell motility control. As cell migration is a coordinated action of cell membrane and cytoskeleton, the loss of caveolae can be an important factor to affect membrane dynamics and then cell motility [20,32]. The decreased cell motility in cavin-1 deficient MEFs (cavin-1$^{-/-}$ MEFs) can only be restored by the expression of wild type cavin-1 but not the nucleus localized cavin-1 Δ55–77 mutant (Fig. 4). It is the caveolae associ-

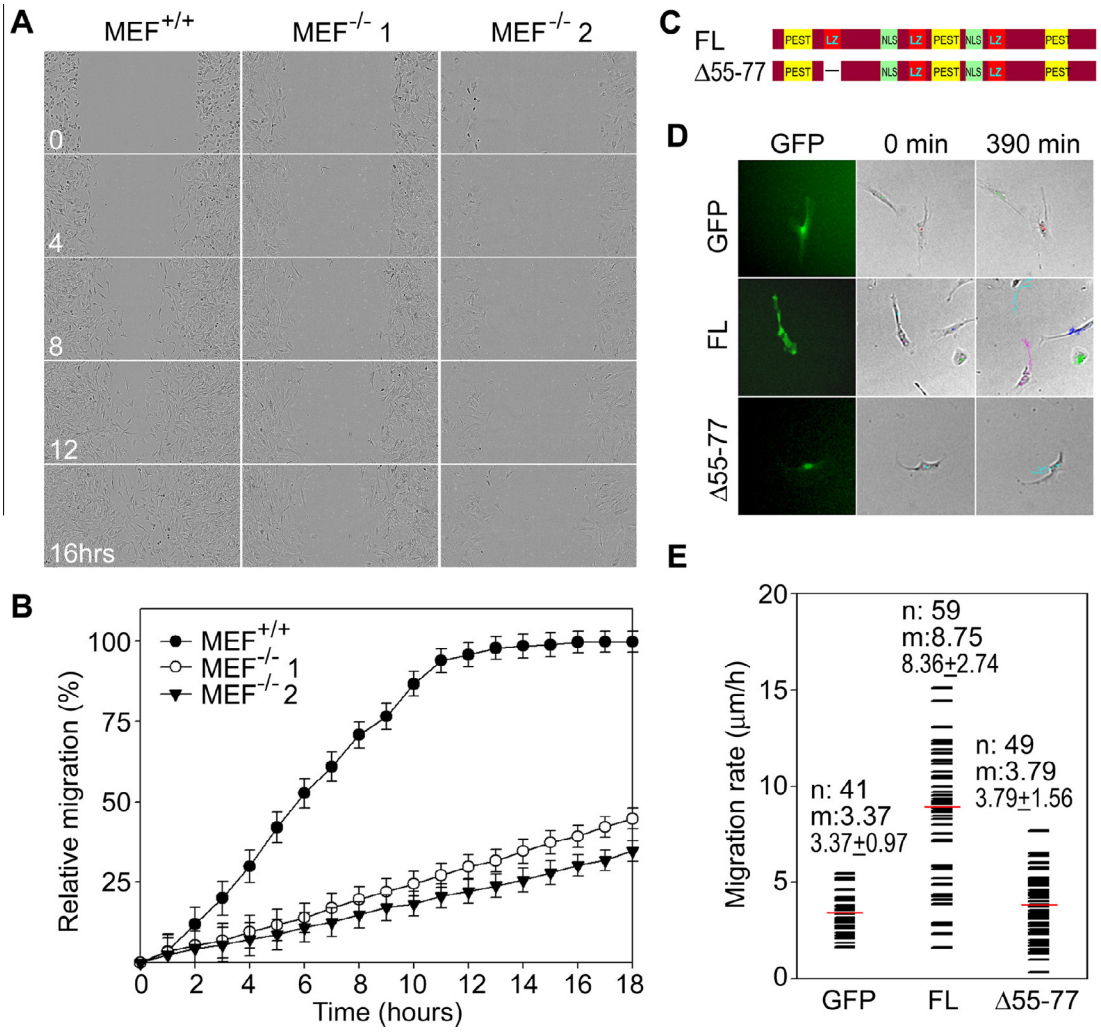

Fig. 4. Cell motility regulated by plasma membrane associated cavin-1. (A) The wound-healing assay of cavin-1$^{-/-}$ MEFs. *MEF*$^{+/+}$, MEFs from the wild type embryo; *MEF*$^{-/-}$*-1*, cavin-1$^{-/-}$ MEFs from the littermate embryo; *MEF*$^{-/-}$*-2*, cavin-1$^{-/-}$ MEFs from different pregnant mouse. (B) The plot of the wound-healing assay. The plot is the average of three experiments ± SD. (C) Construction of eGFP tagged cavin-1 and cavin-1 Δ55–77 mutant. (D) Cell tracking analysis of cavin-1$^{-/-}$ MEFs expressing exogenous wild type cavin-1 or cavin-1 Δ55–77 mutant. *GFP*, cavin-1$^{-/-}$ MEFs expressing control eGFP protein; *FL*, cavin-1$^{-/-}$ MEFs expressing wild type cavin-1; Δ55–77, cavin-1$^{-/-}$ MEFs expressing cavin-1 Δ55–77 mutant. (E) Statistical analysis of cell tracking in panel D. *n*, number of eGFP positive cells tracked; *m*, median. The number is average ± SD.

ated cavin-1 that is important for cell motility regulation. Although cavin-1 Δ55–77 mutant has most functional domains, its nuclear localization separates it from membrane caveolae. Essentially, the cellular localization of cavin-1 determines its function in caveolae or in nuclei.

The sequence motif that determines the association of cavin-1 with caveolae is the N-terminal leucine-zipper (amino acid 55–77). When tagged to histone, this motif is sufficient to export the fusion protein from the nuclei (Fig. 2E). As cavin-1 contains both caveolae motif and nuclear localization sequence, the combined action of these two motifs will balance the protein between membrane and nuclei. In fact, neither of the motifs seems to be strong enough to overcome the other, as cavin-1 is partitioned between membrane caveolae and nuclear fraction (Fig. 1). Nevertheless, the presence of cavin-1 in nuclei represents a possibility for cavin-1 to play function there. The cavin-1 Δ55–77 mutant can be used to analyze its functions related to its PTRF part.

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