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# Familial Focal Segmental Glomerulosclerosis (FSGS)-linked α-Actinin 4 (ACTN4) Protein Mutants Lose Ability to Activate Transcription by Nuclear Hormone Receptors*[S]

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**Background:** Mutations in α-ACTN4 are linked with familial FSGS.
**Results:** FSGS linked α-ACTN4 mutants fail to activate transcription mediated by nuclear hormone receptors.
**Conclusion:** The inability of FSGS-linked ACTN4 mutants to potentiate transcriptional activation might be because of their more cytoplasmic localization compared with wild type.
**Significance:** Our results may have implications for understanding the role of α-ACTN4 in the pathophysiology of the kidney disease.

**Mutations in α-actinin 4 (ACTN4) are linked to familial forms of focal segmental glomerulosclerosis (FSGS), a kidney disease characterized by proteinuria due to podocyte injury. The mechanisms underlying ACTN4 mutant-associated FSGS are not completely understood. Although α-actinins are better known to cross-link actin filaments and modulate cytoskeletal organization, we have previously shown that ACTN4 interacts with transcription factors including estrogen receptor and MEF2s and potentiates their transcriptional activity. Nuclear receptors including retinoic acid receptor (RAR) have been proposed to play a protective role in podocytes. We show here that ACTN4 interacts with and enhances transcriptional activation by RARα. In addition, FSGS-linked ACTN4 mutants not only mislocalized to the cytoplasm, but also lost their ability to associate with nuclear receptors. Consequently, FSGS-linked ACTN4 mutants failed to potentiate transcriptional activation by nuclear hormone receptors in podocytes. In addition, overexpression of these mutants suppressed the transcriptional activity mediated by endogenous wild-type ACTN4 possibly by a cytoplasmic sequestration mechanism. Our data provide the first link between FSGS-linked ACTN4 mutants and transcriptional activation by nuclear receptor such as RARα and peroxisome proliferator-activated receptor γ.**

The α-actinins (ACTNs)[3] are a family of cytoskeletal proteins that bind actin filaments to maintain cytoskeletal structure and cell morphology (1, 2). Four actinins encoded by different genes have been identified. Among the four members of the family, ACTN2 and ACTN3 are highly expressed in muscle, whereas ACTN1 and ACTN4 are expressed ubiquitously (2). All four actinins share extensive sequence homology with a number of conserved functional domains including an N-terminal actin-binding domain, two calponin homology (CH1 and CH2) domains, a central domain consisting of four spectrin repeats (SR), two EF hand calcium-binding domains and a C-terminal calmodulin -like domain (3, 4). Although, localized predominantly in the cytoskeleton, ACTN4 is also found in the nucleus of certain cell types. It is also able to translocate from the cytosol into the nucleus in response to extracellular stimuli (5, 6). Indeed, we have demonstrated previously that ACTN4 potentiates transcriptional activation by MEF2 transcription factors, estrogen receptor α,, and vitamin D3 receptor (7, 8).

An elaborate actin cytoskeleton plays an important role in maintaining the unique architecture of the kidney podocyte (9). Podocytes are highly differentiated epithelial cells that extend lamellapodia (known as primary processes) that wrap the capillary walls of the glomerulus. Primary processes further branch into smaller, actin-rich secondary processes known as foot processes (10–13) which interdigitate with foot processes of neighboring podocytes to form a unique cell-cell junction known as the slit diaphragm (13–15). The slit diaphragm is a part of the physical barrier that prevents the passage of larger blood pro-

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[3] The abbreviations used are: ACTN, α-actinin; AT-RA, all-*trans*-retinoic acid; CH, calponin homology; FSGS, focal segmental glomerulosclerosis; Luc, luciferase; PPAR, peroxisome proliferator-activated receptor; RAR, retinoic acid receptor; RARE, retinoic acid response element; TK, thymidine kinase; PPRE, peroxisome proliferator response element.

teins into the urine (11). There are a number of proteins involved in the assembly and function of the slit diaphragm such as nephrin, podocin, CD2-associated protein (CD2AP), P-cadherin, α- and β-catenin, zonula occludens-1 (ZO-1), and transient receptor potential channel (TRPC6). The maintenance of the slit diaphragm is critical for proper glomerular filtration and kidney function. Notably, mutations in nephrin, CD2AP, podocin, and TRPC6 are associated with glomerular dysfunction (11, 15–17). ACTN4 is present in foot processes and is responsible for bundling actin filaments. Mutations in *ACTN4* are linked with late onset autosomal dominant form of familial FSGS (18). The mechanisms by which these mutants contribute to FSGS are emerging but far from complete (19).

Nuclear hormone receptors are a family of sequence-specific, ligand-dependent transcription factors that control cell homeostasis, differentiation, proliferation, metabolism, and animal development (20–24). Transcriptional regulation by nuclear receptors is controlled through the exchange of co-repressors and co-activators. Ligand binding induces a conformational change in the receptor, leading to an exchange of co-repressor complexes with co-activator proteins. The hormone-induced interaction of co-activators is mediated through multiple copies of a conserved motif, L*XX*LL (where L is leucine, *X* can be any amino acid) (25). This motif is present in many nuclear receptor co-activators including p160 family of co-activators (NCoA 1, 2, and 3) and histone acetyltransferases (CBP/p300) (26–31). We have previously identified ACTN4 as a novel transcriptional co-activator that harbors a L*XX*LL motif required for its ability to potentiate transcriptional activation by ERα and vitamin D receptor (8). Increasing evidence from studies in animals and cultured podocytes indicates that natural and synthetic hormones including retinoids, glucocorticoids, pioglitazone, vitamin D3, and WY-14643 may protect and/or rescue podocytes from injury (32–39). However, the mechanisms underlying these effects are not clearly understood. Podocyte injury is generally accompanied by cytoskeletal reorganization, loss of expression of slit diaphragm components such as nephrin, and effacement of foot processes (40). Treatment of podocytes after injury with the above-mentioned ligands restores cytoskeletal architecture and enhances expression of nephrin (33, 36, 38, 39, 41–43). However, the molecular mechanisms by which nuclear receptors and transcriptional co-regulators regulate transcription in podocytes remain largely unknown.

In this study, we demonstrate that wild-type, but not FSGS-linked ACTN4 mutants, potently stimulate transcription mediated by RARα. The inability of FSGS-linked ACTN4 mutants to activate transcription correlates with both their failure to translocate to the nucleus and a binding defect with nuclear receptors. Together, we have uncovered a novel function of ACTN4 in nuclear receptor-mediated transcriptional activation in kidney podocytes, and this function is altered by ACTN4 mutations linked to FSGS (9, 44, 45).

## MATERIALS AND METHODS

*Plasmid Construction*—CMX-HA-ACTN4 has been described previously (7). GST-ACTN4 (WT) and ACTN4 (L*XX*AA) expression plasmids were described previously (8). Point mutations of ACTN4 were generated by site-directed PCR mutagenesis according to the manufacturer's protocol (Stratagene). Expression plasmid PPARγ and RARE-TK-Luc and PPRE-TK-Luc were generous gifts from Dr. Ron Evans (The Salk Institute, La Jolla, CA). HA-PPARγ was generated by PCR PPARγ and cloning to CMX-1H vector. HA-RARα was described previously (46).

*Antibodies and Chemicals*—The anti-ACTN4 antibody has been described previously (7). For immunoprecipitation assays, anti-HA-conjugated anti-horseradish peroxidase (Roche Applied Science) and anti-FLAG (Sigma-Aldrich) antibodies were used. For immunostaining, anti-HA (Sc7392; Santa Cruz Biotechnology), and anti-FLAG (F7425; Sigma) antibodies were used. The secondary antibodies were from Molecular Probes (α-mouse or α-rabbit Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 594). Anti-RARα (Sc-20), anti-HDAC1, and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnologies. All-*trans*-retinoic acid (AT-RA) and ciglitazone was purchased from Biomol (BML-GR100-0500 and BML-GR205-005).

*Cell Culture*—HEK293, HeLa, and CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate at 37 °C in 5% $CO_2$. Human immortalized podocyte cells were cultured as described previously (47) and used for experiments under permissive conditions. Primary mouse podocytes were prepared from freshly isolated kidney glomeruli using an established sieving technique (48). Isolated glomeruli were plated under conditions that allow podocyte outgrowth as described previously (49). Cells were used at <5 passages. All animal studies were conducted in accordance with Case Western Reserve University animal care and use committee requirements.

*Subcellular Fractionation*—Subcellular fractionation of podocytes was carried out by a protocol described previously (50). Nuclear and cytoplasmic fractions were resolved on SDS-polyacrylamide gels and followed by Western blotting with the indicated antibodies.

*In Vitro Protein-Protein Interaction Assays*—Glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* DH5α strain, affinity-purified, and immobilized on glutathione-Sepharose 4B beads. *In vitro* pulldown assays were carried out using immobilized GST-ACTN4 (WT) with whole cell extracts expressing nuclear receptors in the presence or absence of 100 nM AT-RA or 1 μM ciglitazone for 1 h at 4 °C. After extensive washes with NETN buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0), 0.1% Nonidet P-40, 10% glycerol, and 1 mM dithiothreitol), SDS-PAGE sample buffer was added to the beads, boiled, and separated by 8% SDS-polyacrylamide gel and immunoblotted with the indicated antibodies.

*Immunoprecipitations and Western Blotting*—HEK293 cells were grown on 10-cm plates and transfected with indicated expression plasmids (10 μg of total DNA) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 48 h, the cells were washed with 1× PBS and resuspended in radioimmune precipitation assay buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) along with protease inhibitors. After a 30-min incubation on ice, lysed cells were centrifuged at 4 °C at 14,000 rpm for 10 min, and the

supernatant was collected and kept at −80 °C. Immunoprecipitations were performed using the indicated antibodies (Sigma) for 4 h at 4 °C with or without 1 μM AT-RA. The immunoprecipitated fractions were analyzed by Western blotting as described previously (8) using the indicated antibodies. Detection was done by chemiluminescence using a kit from Thermo Scientific as directed by the manufacturer.

*Transient Transfection Reporter Assays*—For reporter assays, HEK293, HeLa, or CV-1 were co-transfected with equal amounts of either 100 ng of RARE-TK-Luc or PPRE-TK-Luc with or without pCMX-ACTN1 or pCMX-ACTN4 along with 100 ng of pCMX-β-gal using Lipofectamine 2000. The amount of DNA was kept constant (<1 μg) by the addition of pCMX vector. After 5 h, the medium was replaced with DMEM supplemented with 10% FBS, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate. Forty-eight h after transfection, the cells were treated with either 100 nM AT-RA or 20 μM ciglitazone for 24 h as indicated in the figures. Cells were harvested, and luciferase and β-gal activities were measured according to the manufacturer's protocol using a luciferase assay system (Promega). Luciferase activity was normalized to the level of β-gal activity. Each reaction was performed in triplicate, and triplicates were averaged before statistical analysis. All studies were repeated at least three times.

*Transient Transfections and Confocal Microscopy*—Transient transfections for confocal microscopy were performed in 12-well culture plates. Cells were transfected with 1 μg of ACTN4 (WT) and/or ACTN4 mutant plasmids with Lipofectamine 2000 according to the manufacturer's protocol. Twenty-four h after transfection, cells were subjected to immunostaining. Transfected cells were fixed in 3.7% paraformaldehyde in PBS for 30 min at room temperature and permeabilized in PBS with the addition of 0.1% Triton X-100 and 10% goat serum for 10 min. The cells were washed three times with PBS and incubated in a PBS-goat serum (10%) with 0.1% Tween 20 solution for 60 min. Incubation with primary antibodies was carried out for 120 min in PBS-Tween 20. The cells were washed three times in PBS, and the secondary antibodies were added for 60 min in the dark, at room temperature in PBS-Tween 20. Coverslips were mounted to slides using Vectashield mounting medium with DAPI (H-1200; Vector Laboratories). Confocal images were acquired using a Zeiss LSM 510 inverted laser-scanning confocal microscope.

*Statistical Analysis*—All studies are representative of at minimum three separate trials. Data are presented as mean ± S.D.

## RESULTS

*ACTN4 Potentiates Nuclear Receptor-mediated Transcription*—Mutations in ACTN4 are associated with familial FSGS characterized by proteinuria and effacement of podocytes. To examine the role of ACTN4 in transcriptional regulation mediated by RARα, we tested whether ACTN4 interacts with RARα in human embryonic kidney cells (HEK293). We transfected an HA-RARα expression plasmid in HEK293 cells and carried out immunoprecipitation assays. We found that AT-RA, a RARα agonist, potently enhanced the association between HA-RARα and endogenous ACTN4 (Fig. 1*A*). This interaction was confirmed with GST pulldown assays using GST or GST-ACTN4 in lysates expressing HA-RARα in the presence or absence of AT-RA. Consistent with the immunoprecipitation experiments, AT-RA enhanced the association between ACTN4 and RARα (Fig. 1*B*). To evaluate a functional effect of ACTN4 on RARα-mediated transcription, we carried out transient transfection reporter assays using a reporter construct harboring RARE. Fig. 1*C* shows that ectopic expression of ACTN1, ACTN4, or RARα modestly activated RARα-mediated transcription (*lanes 2–4*), and co-expression of ACTN1 or ACTN4 further activated hormone-driven RARα-mediated transcription activity (*lanes 5* and *6*). We also observed hormone-dependent activation of RARE reporter activity by ACTN1, ACTN4, and RARα in HeLa cells (supplemental Fig. 1). We have shown previously that ACTN4 harbors a functional nuclear receptor interaction motif L*XX*LL (8). To investigate whether the L*XX*LL motif of ACTN4 is required for its binding to RARα, GST pulldown assays were employed showing that an ACTN4 mutant with two lysines changed to alanine (L*XX*AA) showed much weaker interactions with RARα even in the presence of hormone (Fig. 1*D*). Furthermore, reporter assays demonstrated that the ACTN4 (L*XX*AA) mutant significantly lost the ability to potentiate hormone-dependent transcriptional activity mediated by RARα (Fig. 1*E*). Similarly, ciglitazone enhanced the interaction between PPARγ with the wild-type ACTN4, but not the ACTN4 (L*XX*AA) mutant (supplemental Fig. 2*A*). Furthermore, both ACTN1 and ACTN4 activate transcription mediated by PPARγ (supplemental Fig. 2*B*). Finally, the ACTN4 (L*XX*AA) mutant lost its ability to stimulate transcription mediated by PPARγ (supplemental Fig. 2*C*). Taken together, these data demonstrate that ACTN1 and ACTN4 can function as transcriptional co-activators.

*Subcellular Distribution of ACTN4 in Podocytes*—ACTN4 is well known to organize cytoskeleton (1, 3); however, the observation that ACTN4 is capable of potentiating RARα-mediated transcription suggested that ACTN4 may localize to, and have a function in, the nucleus as well. To test this, we examined the subcellular distribution of endogenous ACTN4 in primary mouse podocytes (Fig. 2*A*) and a cultured human podocyte cell line (Fig. 2*B*) with immunostaining followed by confocal microscopy. ACTN4 was distributed in both the nucleus and the cytoplasm in primary podocytes and the podocyte cell line. We further confirmed this finding by subcellular fractionation. Cytoplasmic and nuclear fractions were prepared from human podocytes, separated on SDS-PAGE, and analyzed by Western blotting using anti-ACTN4, anti-HDAC1 (nuclear marker), and anti-GAPDH (cytoplasmic marker) antibodies. As shown in Fig. 2*C*, ACTN4 was present in both cytoplasmic and nuclear fractions of human podocytes. Thus, we conclude that ACTN4 is localized in both the nucleus and the cytoplasm of primary and immortalized podocytes.

*FSGS-linked ACTN4 Mutants Failed to Activate Transcription Mediated by Nuclear Receptors*—Three known FSGS-linked ACTN4 mutations, K228E, T232I, and S235P, are located in the actin binding domain, downstream of the nuclear receptor interacting motif L*XX*LL (Fig. 3*A*). To determine whether these mutations affect the nuclear function of ACTN4, we examined the ability of FSGS-linked ACTN4 mutants to potentiate transcriptional activation mediated by RARα.

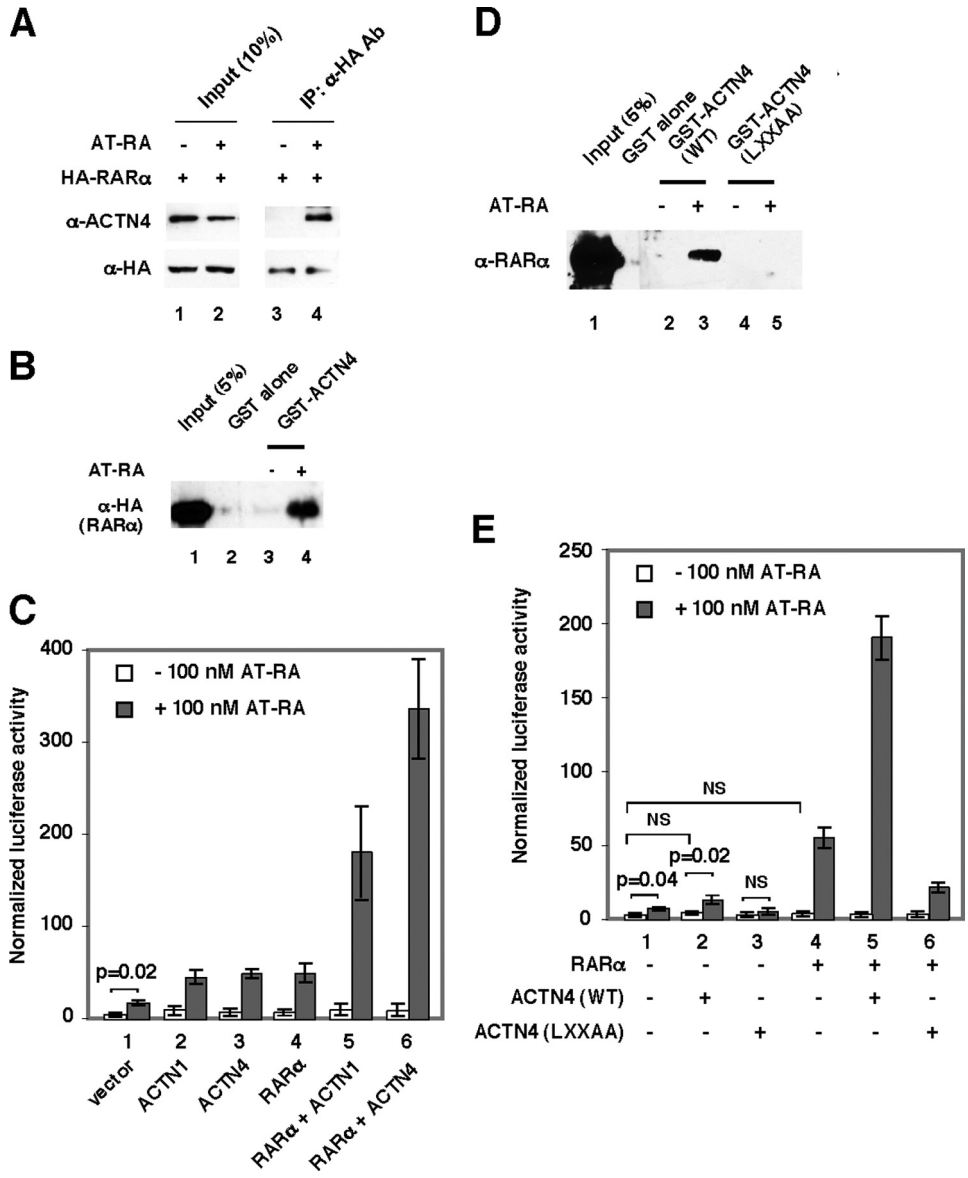


FIGURE 1. **ACTN4 binds and potentiates transcriptional activation by RARα.** *A*, Western blot of co-immunoprecipitated samples demonstrating an interaction between ACTN4 (WT) and RARα. HEK293 cells were transfected with HA-RARα expression plasmids. After 48 h, the extracts were prepared and immunoprecipitated with anti-HA antibodies in the presence or absence of 100 nM AT-RA followed by Western blotting with anti-ACTN4 and anti-HA antibodies. *B*, Western blot of GST pulldowns showing a physical interaction between ACTN4 (WT) and RARα. HEK293 cells were transfected with an HA-RARα expression plasmid. Whole cell extracts were prepared and incubated with bacterially expressed with GST-ACTN4 (WT) in the presence or absence of 100 nM AT-RA. Pulldown fractions were subjected to Western blotting with anti-HA antibodies. *C*, transcriptional reporter assay demonstrating ACTN1 and ACTN4 potentiate transcriptional activity mediated by RARα in CV-1 cells. The expression plasmids for ACTN1 (WT) or ACTN4 (WT) were co-transfected with or without expression plasmids for RARα along with the reporter construct harboring a RARE and β-gal in CV-1 cells. Forty-eight h after transfection, cells were treated with or without 100 nM AT-RA. Luciferase activity was measured and normalized to β-gal activity. Data are mean ± S.D. (*error bars*) ($n = 3$). *D*, Western blot of GST pulldowns showing the ACTN4 (L*XX*AA) mutant loses its ability to interact with RARα. HEK293 cells were transfected with an HA-RARα expression plasmid, and GST pulldown assays were carried out by incubating lysates with immobilized purified, bacterially expressed GST-ACTN4 (WT) or with GST-ACTN4 (L*XX*AA) fusion proteins in the presence or absence of 100 nM AT-RA. Pulldown fractions were subjected to Western blotting using anti-HA antibodies. *E*, transcriptional reporter assay demonstrating ACTN4 (L*XX*AA) mutant functionally loses its ability to potentiate RARα activity. Expression plasmids for ACTN4 (WT) or ACTN4 (L*XX*AA) mutant were co-transfected with or without an expression plasmid for RARα along with a reporter construct harboring an RARE in CV-1 cells. Transient transfection was carried out as described in *C*. Data are mean ± S.D. ($n = 3$).

Expression plasmids for ACTN4 (WT) or FSGS-linked ACTN4 mutants were transfected with or without RARα along with a reporter construct harboring a RARE. Fig. 3*B* shows that wild-type ACTN4 alone modestly activated RARα-mediated reporter activity (*lane 2*), whereas the FSGS-linked ACTN4 mutants did not (*lanes 3–5*). Co-expression of the wild-type ACTN4 with RARα significantly enhanced the reporter activity (*lane 7*). In contrast, FSGS-linked ACTN4 mutants inhibited ligand-induced transcriptional activation mediated by RARα (*lanes 8–10*), suggesting that FSGS-linked ACTN4 mutants are defective in enhancing RARα-mediated transcriptional activation. Both wild-type ACTN4 and FSGS-linked ACTN4 mutants were expressed at the similar levels (data not shown). Interestingly, the level of transcriptional activation with any of the overexpressed FSGS mutants was lower than that of the endogenous ACTN4 (*lanes 6* and *8–10*), suggesting a dominant

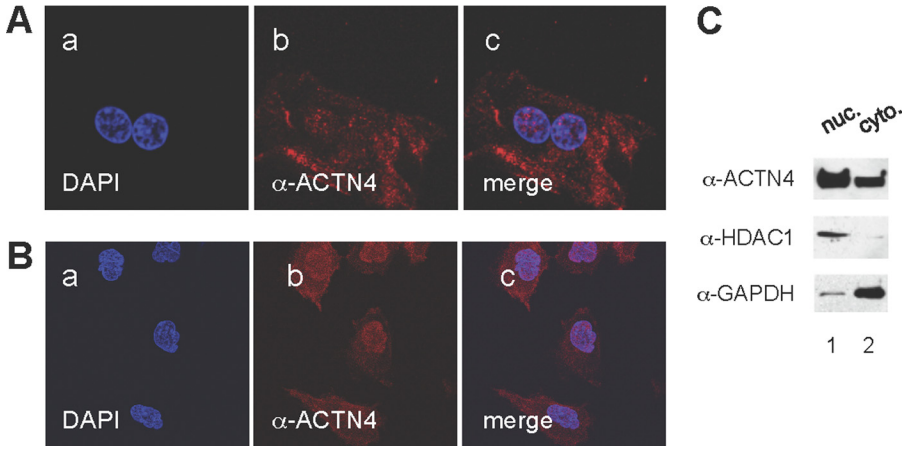


FIGURE 2. **Subcellular distribution of ACTN4 in podocytes.** *A* and *B*, subcellular distribution of ACTN4 in mouse primary podocytes (*A*) and in a conditionally immortalized human podocyte cell line (*B*). Confocal microscopy of cells were performed after immunostaining with anti-ACTN4 antibodies and DNA was visualized by DAPI staining. *C*, subcellular fractionation of endogenous ACTN4 in the podocytes. Subcellular fractionation was carried out as described under "Materials and Methods" followed by immunoblotting with indicated antibodies. GAPDH and HDAC1 were used as a cytoplasmic (*cyto.*) and a nuclear (*nuc.*) marker, respectively.

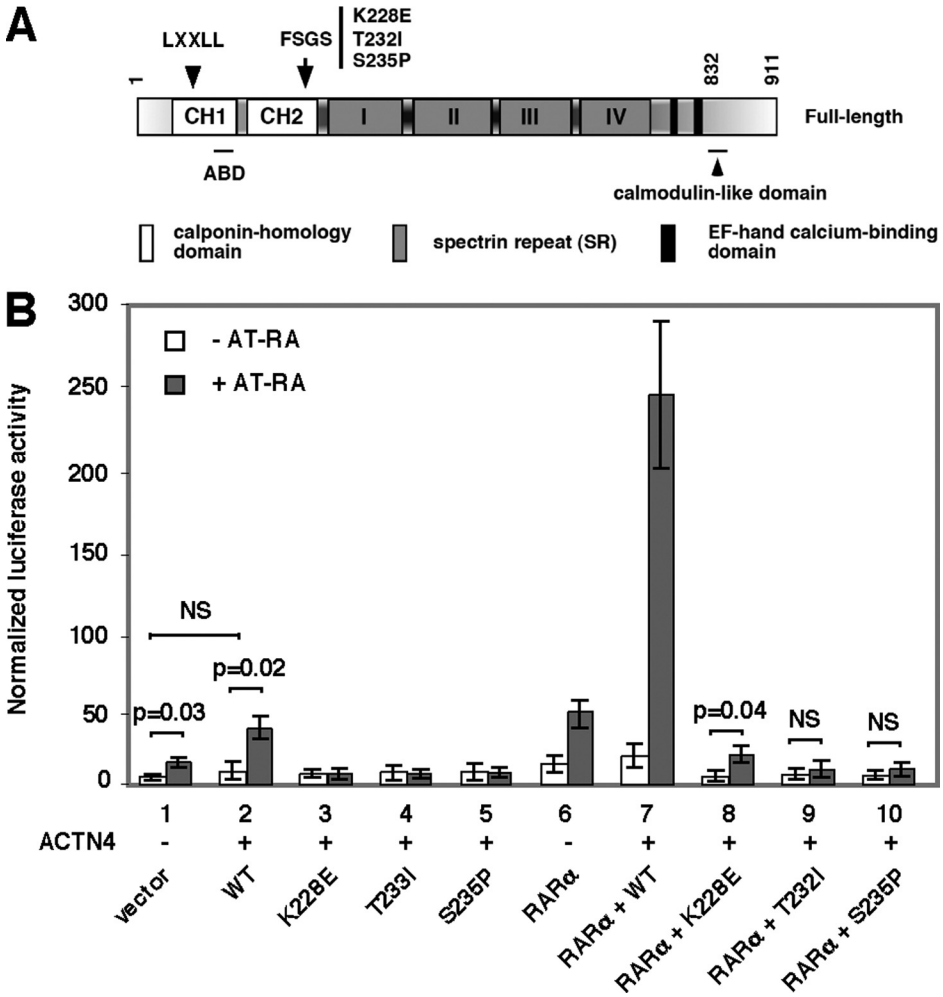


FIGURE 3. **FSGS-linked ACTN4 mutants lose their ability to potentiate RARα-mediated transcription activation.** *A*, schematic diagram of ACTN4 indicating the position of FSGS-linked mutations. ACTN4 harbors two CH domains, CH1 and CH2, four spectrin repeats (I–IV), two EF hand calcium binding domains, and a C-terminal calmodulin-like domain. The positions of the L*XX*LL motif and three FSGS-linked ACTN4 mutants are indicated by *arrows*. *B*, transcriptional reporter assays demonstrating FSGS-linked ACTN4 mutants fail to activate transcription mediated by RARα. An RARE-containing reporter construct was co-transfected with or without plasmids expressing RARα and ACTN4 (WT) or FSGS-linked ACTN4 mutants along with β-gal as indicated. Forty-eight h after transfection, the cells were treated with or without 100 nM AT-RA. Luciferase activity was measured and normalized to β-gal activity. The expression levels of ACTN4 (WT) and FSGS-linked ACTN4 mutants were analyzed by Western blotting and were similar (data not shown). Data are mean ± S.D. (*error bars*) ($n = 3$).

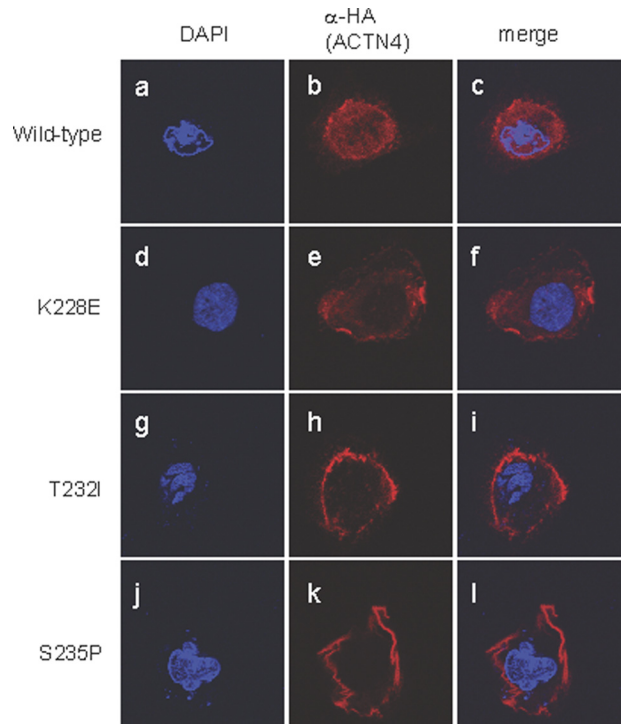


FIGURE 4. **Subcellular distribution of FSGS-linked ACTN4 mutants in podocytes.** Expression plasmids for HA-ACTN4 (WT) and FSGS-linked HA-ACTN4 mutants were transiently transfected in the human podocyte cell line. Immunostaining was carried out using α-HA antibodies followed by confocal microscopy. DNA was visualized by DAPI staining (*a–j*). *a–c*, *d–f*, *g–i*, and *j–l*, subcellular distribution of ACTN4 (WT), K228E, T232I, and S235P, respectively.

negative effect of the mutants on RARα-mediated transcriptional activation. Similarly, FSGS-linked ACTN4 mutants were unable to potentiate transcription mediated by PPARγ (supplemental Fig. 3).

*Subcellular Localization of ACTN4 in Immortalized Human Podocytes*—To dissect the mechanisms by which FSGS-linked ACTN4 mutants are defective in potentiating RARα-mediated transcriptional activation, we first examined the subcellular localization of FSGS-linked ACTN4 mutants by immunostaining and confocal microscopy. Fig. 4 shows that all three FSGS-linked ACTN4 mutants were largely excluded from the nucleus (*a–c versus d–f*, *g–i*, and *j–l*). The transcriptional assays in Fig. 3 suggested that ACTN4 mutants may interfere with the function of wild-type ACTN4. Because ACTN4 is known to form homodimers, this would suggest a mechanism whereby FSGS-linked ACTN4 mutants could potentially affect wild-type protein activity by direct binding and subsequently altering its subcellular localization. To test this hypothesis, we first examined whether FSGS-linked ACTN4 mutants associate with wild-type ACTN4. We performed co-immunoprecipitations on whole cell lysates prepared from HEK293 co-transfected with expression plasmids for FLAG-ACTN4 (WT) and HA-ACTN4 (WT) or FSGS-linked ACTN4 mutants. FSGS-linked ACTN4 mutants interact with wild-type ACTN4 to an extent similar to wild-type ACTN4 interaction with itself (Fig. 5*A*, *lane 6 versus lanes 7-9*). Next, we examined their subcellular localization by immunostaining and confocal microscopy. As shown in Fig. 5*B*, *a–d*, there was no change in the localization of wild-type FLAG-ACTN4 with co-expression of wild-type HA-ACTN4 using both tags (*i.e.* co-transfection of FLAG-ACTN4 and HA-ACTN4), and the observed pattern was similar to the distribution of endogenous ACTN4 shown in Fig. 2. By contrast, co-expression of FLAG-ACTN4 (WT) with HA-ACTN4 K228E mutant resulted in an altered distribution of the wild-type ACTN4 protein, showing enriched staining in the cytoplasm (Fig. 5*B*, *e–h*). A similar pattern of localization was observed with the co-expression of FLAG-ACTN4 (WT) and the HA-ACTN4 T232I mutant (Fig. 5*B*, *i–l*). However, the cytoplasmic staining was concentrated at what appeared to be regions of focal contacts. Interestingly, co-expression of FLAG-ACTN4 (WT) with HA-ACTN4 (S235P) also resulted in wild-type ACTN4 exhibiting a more cytoplasmic localization, but the pattern resembled actin stress fibers (Fig. 5*B*, *m–p*). These data indicate that co-transfected FSGS-linked ACTN4 mutants altered the subcellular distribution of the wild-type ACTN4 protein. In contrast, these mutants did not alter the subcellular distribution of G protein pathway suppressor 2, a component of nuclear receptor transcriptional co-repressor complex (supplemental Fig. 4). This result suggests that the co-expression method did not cause a general disruption of either actin binding or nuclear-cytoplasmic shuttling events. Taken together, these data demonstrate that FSGS-linked ACTN4 mutants exhibit a staining patterns distinct from that of the wild-type protein and that these mutants likely sequester wild-type ACTN4 from the nucleus.

Structural studies suggest that the spectrin repeats of ACTN4 form a dimerization interface. Because the FSGS-linked mutants lie within spectrin repeats of ACTN4, these mutations may alter the quaternary structure of ACTN4 resulting in aberrant protein-protein interactions. Thus, it is possible that the FSGS-linked mutants interact with nuclear receptors differently from the wild-type protein. To test this, we first examined the interaction between wild-type ACTN4 and FSGS-linked ACTN4 mutants with nuclear receptors by co-immunoprecipitation assay. We found that wild-type, but not FSGS-linked ACTN4 mutants, bind RARα (Fig. 6*A* and data not shown). Using GST pulldown assays, we further demonstrated that FSGS-linked ACTN4 mutants had a clear reduction in binding to RARα (Fig. 6*B*). Moreover, we also observed a similar reduced binding of FSGS-linked ACTN4 mutants with PPARγ (supplemental Fig. 5).

## DISCUSSION

ACTN4 is best known as an actin-binding protein that functions as a part of the cytoskeleton mediating events associated with cell adhesion, spreading, and motility. We and others, however, have found that ACTN4 can also translocate to the nucleus where it has a functional role involving the transcriptional activity of nuclear receptors (5–8). Studies here revealed that ACTN4 harbors a functional L*XX*LL motif, the conserved protein-protein interaction domain required for nuclear receptor interaction with co-activators. Mutation of this motif in ACTN4 significantly limited both its ability to bind to RARα and PPARγ and to potentiate transcription mediated by RARα and PPARγ agonists. These results indicate that ACTN4 is a *bona fide* nuclear receptor co-activator. In conjunction with our past studies demonstrating ACTN4 potentiates transcrip-

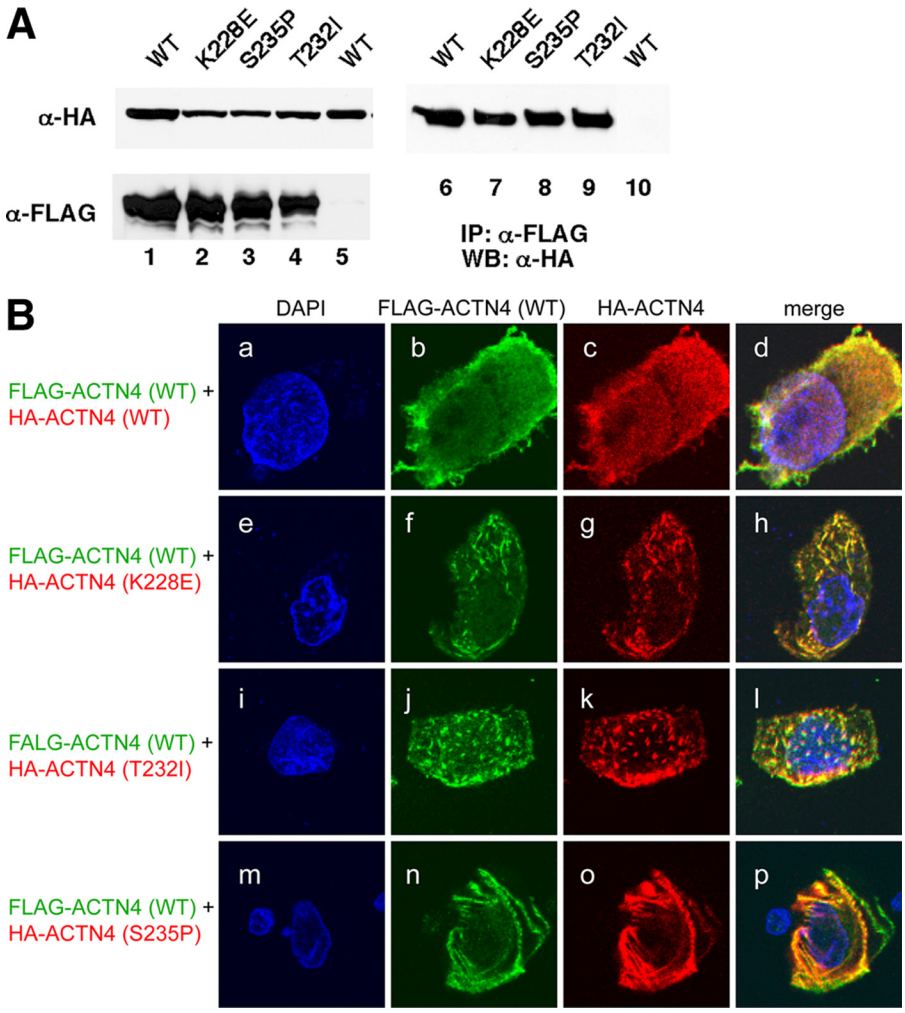


FIGURE 5. **FSGS-associated ACTN4 mutants alter subcellular distribution of wild-type protein in podocytes.** *A*, FSGS-linked ACTN4 mutants associate with wild-type ACTN4. Expression plasmids for FLAG-ACTN4 (WT) were co-transfected either with expression plasmids for HA-ACTN4 (WT) or with FSGS-linked HA-ACTN4 mutants in HEK293 cells. Forty-eight h after transfection, whole cell lysates were prepared and incubated with FLAG M2 agarose beads, and bound fractions were resolved by SDS-PAGE and visualized by immunoblotting with the indicated antibodies. *B*, FSGS-linked ACTN4 mutants alter the subcellular distribution of wild-type ACTN4. Expression plasmids for FLAG-ACTN4 (WT) were co-transfected with expression plasmid for HA-ACTN4 (WT) or FSGS-linked HA-ACTN4 mutants in podocytes followed by immunostaining with anti-FLAG and anti-HA antibodies and confocal microscopy. *a–d* show subcellular distribution of FLAG-ACTN4 (WT) and HA-ACTN4 (WT), and *e–h*, *i–l*, and *m–p* show subcellular distribution of FLAG-ACTN4 (WT) with HA-ACTN4 (K228E), HA-ACTN4 (T232I), and HA-ACTN4 (S235P), respectively.

tional responses from the estrogen and vitamin D3 receptors, these studies suggests ACTN4 may have a general role in regulating nuclear hormone receptor responses.

Recently, interest in the function of ACTN4 has intensified due to its association with familial forms of the common kidney disease FSGS. To date, several disease-causing mutations in ACTN4 have been identified, and research into possible mechanisms have focused on their role in podocyte cytoskeleton dysfunction (9, 45). Our prior studies and the new findings reported here suggest that ACTN4 may have additional functions in podocytes involving transcriptional regulation by nuclear receptors. In general, little is known about the importance of regulation in nuclear receptor function in podocytes despite a longstanding association of hormones and nuclear receptors with normal kidney physiology. Evidence from *in vitro* and *in vivo* studies has consistently shown that natural and synthetic hormones, including retinoids, glucocorticoids, pioglitazone, vitamin D3, and WY-14643, enhance podocyte differentiation and protect or rescue podocytes from disease-induced injury (32–37, 39, 51–53). The typical disease injury response in podocytes involves reorganization of the cytoskeleton resulting in effacement of foot processes, as well as losses in expression of components of the slit diaphragm such as nephrin and synaptopodin. Treatment of injured podocytes with the above hormones restores the cytoskeletal architecture and enhances expression of nephrin (33, 36, 39, 41). Thus, our observations may delineate how ACTN4 can act as a novel bridge between these two effects, being both an actin-binding protein and a nuclear hormone co-activator. However, whether these two functions are directly linked or are independent, concurrent processes will require further investigation.

FSGS caused by ACTN4 mutations is an autosomal dominant disease. We found that FSGS-linked ACTN4 mutants can redistribute the subcellular localization of the wild-type protein, including its exclusion from the nucleus. In addition, expression of mutant protein suppressed the transcriptional responses of the endogenous wild-type protein. This indicates that the effect of FSGS-linked ACTN4 mutants on both cyto-

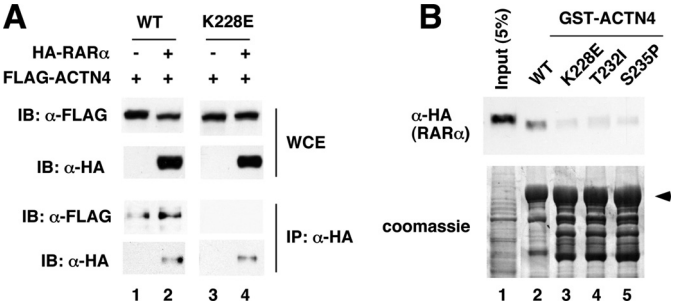


FIGURE 6. **FSGS-linked ACTN4 mutants lose their interactions with nuclear hormone receptors.** *A*, FSGS-linked ACTN4 mutant K228E fails to interact with RARα in HEK293 cells. FLAG-ACTN4 was co-transfected with or without HA-RARα into HEK293 cells. Whole cell extracts were prepared, and co-immunoprecipitation was carried out using anti-FLAG antibodies followed by immunoblotting with anti-HA and anti-FLAG antibodies. *B*, FSGS-linked ACTN4 mutants fail to interact with RARα *in vitro*. HEK293 cells were transfected with an HA-RARα expression plasmid. Lysates were incubated with immobilized bacterially expressed GST-ACTN4 (WT), GST-ACTN4 (K228E), GST-ACTN4 (T232I), or GST-ACTN4 (S235P) fusion proteins in the presence or absence of 100 nM AT-RA. Pulldown fractions were subjected to immunoblotting with anti-HA antibodies. *Bottom panel* shows the Coomassie Blue staining. The *arrow* indicates the full-length, wild-type or mutant GST-ACTN4 proteins.

skeletal changes and transcriptional responses are dominant negative to the wild-type protein. However, our observation that FSGS-linked ACTN4 mutants lost their interactions with nuclear receptors was somewhat unexpected because the FSGS mutants are positioned >100 amino acids downstream of the L*XX*LL receptor interaction motif (8). These results may support a model in which FSGS-linked ACTN4 mutations alter the global structure of ACTN4 such that the L*XX*LL motif alone is no longer sufficient to support the interaction of ACTN4 with nuclear receptors. To our knowledge, this is the first example of a mutation outside the receptor interaction motif that affects receptor binding. Further study on the mechanism of interaction among ACTN4, nuclear receptors, and the actin cytoskeleton will be critical in determining the role of ACTN4 in mediating these integral events in normal podocyte homeostasis and disease responses.

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