ALPHA ACTININ 4: AN INTERGRAL COMPONENT OF TRANSCRIPTIONAL
PROGRAM REGULATED BY NUCLEAR HORMONE RECEPTORS

By
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May 2011

*We also certify that written approval has been obtained for any proprietary material contained therein.
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LIST OF ABBREVIATIONS

ABD    Actin binding domain
ABP    Actin binding protein
ACTN   Actinin
ACTN1  Actinin-1
ACTN4  actinin-4
ACTR   Activator of thyroid and retinoic acid receptor
AR     Androgen receptor
AT-RA  All-trans retinoic acid
CaM    Calmodulin like domain
CARM-1 Coactivator associated arginine methyltransferase
CBP    Cyclic AMP binding protein
CD2AP  CD2-associated protein
CH     Calponin homology
ChIP   Chromatin immunoprecipitation
CHK1   Checkpoint kinase 1
CRM1   Chromosome region maintenance-1
DAPI   4’6-diamidino-2-phenylindole
DBD    DNA binding domain
Dex    Dexamethasone
DHA    Docosahexenoic acid
DMEM   Dulbecco’s Eagle Modified Medium
DRIP   Vitamin D receptor interacting protein
E2     Estradiol
EPA    Eicosapentaenoic acid
ERE    Estrogen response element
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<td>Estrogen receptor</td>
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<td>ESRD</td>
<td>End stage renal dialysis</td>
</tr>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>FLI-1</td>
<td>Flightless-1</td>
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<td>FLN-A</td>
<td>Filamin-A</td>
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<tr>
<td>FOXC1</td>
<td>Forkhead box C1</td>
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<tr>
<td>FP</td>
<td>Foot processes</td>
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<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
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<td>FSGS</td>
<td>Focal segmental Glomerulosclerosis</td>
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<tr>
<td>GBM</td>
<td>Glomerular basement membrane</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>GRE</td>
<td>Glucocorticoid response element</td>
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<tr>
<td>GRIP-1</td>
<td>Glucocorticoid receptor interacting protein 1</td>
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<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
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<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
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<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<tr>
<td>HK-2</td>
<td>Human kidney-2</td>
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<tr>
<td>hnRNPs</td>
<td>Heterogeneous ribonuclear proteins</td>
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<td>HPCs</td>
<td>Human podocytes cells</td>
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<tr>
<td>HRE</td>
<td>Hormone response element</td>
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<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
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<tr>
<td>ITS</td>
<td>Insulin transferrin selenite</td>
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<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
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<td>Ligand binding pocket</td>
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<td>LXR</td>
<td>Liver X receptor</td>
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<td>MCK</td>
<td>Muscle creatine kinase</td>
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<tr>
<td>MMP10</td>
<td>Matrix metalloproteinase 10</td>
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<td>MPCs</td>
<td>Mouse podocytes cells</td>
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<td>MRTFs</td>
<td>Myocardin related transcription factors</td>
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<td>MYPT-1-PP1B</td>
<td>Myosin phosphatase targeting subunit-1 protein phosphatase 1B</td>
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<td>NCoR</td>
<td>Nuclear receptor co-repressor</td>
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<tr>
<td>NES</td>
<td>Nuclear export signal</td>
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<td>NF-Y</td>
<td>Nuclear factor-Y</td>
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<tr>
<td>NGFIB</td>
<td>Nerve growth factor induced factor B</td>
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<td>NLS</td>
<td>Nuclear localization signal</td>
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<td>NR</td>
<td>Nuclear receptor</td>
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<tr>
<td>NS</td>
<td>Nephrotic syndrome</td>
</tr>
<tr>
<td>P/CAF</td>
<td>p300/CBP associated factor</td>
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<tr>
<td>PAN</td>
<td>Puromycin aminonucleoside</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3</td>
<td>phosphatidylinositol 3</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
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<td>PR</td>
<td>Progesterone receptor</td>
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<td>PRMT-1</td>
<td>Protein arginine methyltransferase</td>
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<td>RAR</td>
<td>Retinoic acid receptor</td>
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<td>RARE</td>
<td>Retinoic acid response element</td>
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<td>RIPA</td>
<td>Radioimmune precipitation assay</td>
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<td>RNPs</td>
<td>Ribonuclear proteins</td>
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<tr>
<td>RPMI 1640</td>
<td>Rosewell Park Memorial Institute 1640</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>-----------</td>
</tr>
<tr>
<td>SD</td>
<td>Slit diaphragm</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator of retinoic acid and thyroid receptor</td>
</tr>
<tr>
<td>SR</td>
<td>Spectrin Repeat</td>
</tr>
<tr>
<td>SRC</td>
<td>Steroid receptor coactivator</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thyroid hormone receptor-associated protein</td>
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<td>TRPC6</td>
<td>Transient receptor potential cation channel 6</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
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<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
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<td>VDRE</td>
<td>Vitamin D 3 response element</td>
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<td>ZO-1</td>
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<td>β-gal</td>
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Overexpression and mutations of the actin-binding protein alpha actinin 4 (ACTN4) are linked to tumorigenesis. However, the mechanisms by which ACTN4 links to oncogenesis are not completely understood. The role of ACTN4 protein in maintaining cytoskeletal integrity is well known yet little is known about the nuclear function of ACTN4. Our study dissects a novel role of ACTN4 in nuclear receptor-mediated transcriptional regulation. Our results demonstrate that ACTN4 potentiates transcriptional activity of the estrogen receptor by antagonizing and displacing histone deacetylase 7 (HDAC7) from the selected estrogen receptor (ERα) target genes. Furthermore, we show here that ACTN4 plays an important role in the proliferation of MCF-7 breast cancer cells by transcriptionally regulating selected estrogen receptor target genes.

In humans, another important phenotype associated with ACTN4 mutations (K228E, T232I and S235P) is linked to a kidney disease known as familial focal segmental glomerulosclerosis (FSGS), characterized by proteinuria and effacement of podocytes (highly differentiated kidney cells). Despite the intense investigation the role of ACTN4 in normal podocyte development as well as the role of ACTN4 mutations in disease
pathogenesis is not well understood. Here we have shown a mechanism by which 
ACTN4 mutants might contribute to the disease. We demonstrate that FSGS-linked 
ACTN4 mutants are defective in regulating the transcription mediated by nuclear 
receptors due to their aberrant localization and defect in their ability to interact with 
nuclear hormone receptors including glucocorticoid receptor (GRα) and retinoic acid 
receptor (RARα). In summary, our work identifies a previously unknown function of 
ACTN4 in nuclear receptor mediated transcription. Our results have implications for 
understanding the role of ACTN4 in the pathophysiology of oncogenesis and FSGS.
CHAPTER 1: INTRODUCTION

Family of Nuclear Receptors

Nuclear hormone receptors (NRs) are a family of sequence specific and ligand activated transcription factors that control several aspects of homeostasis, differentiation, proliferation, metabolism and development [1]. In humans, there are 48 members identified through human genome sequencing. Dysfunction of signaling cascades regulated by NRs results in various reproductive, proliferative and metabolic diseases. The ability of some NRs to bind ligands make them excellent pharmaceutical targets, for example, retinoic acid (a ligand for retinoic acid receptor α), the synthetic antagonist tamoxifen (a ligand for estrogen receptor α), dexamethasone (a ligand for glucocorticoid receptor) or thiazolidinediones (ligands for peroxisome proliferator-activated receptor) are used in acute promyelocytic leukemia, breast cancer, inflammatory disease and type II diabetes respectively [2].

NRs have been categorized into four classes based on their ligand binding, DNA binding and dimerization properties. NRs bind to their response elements (motifs of 5-10 base pairs) either as monomers, dimers or heterodimers. Class I receptors bind to the DNA inverted repeats as homodimers. This class includes estrogen receptor (ER), glucocorticoid receptor (GR), progesterone receptor and androgen receptors (PR and ARs). Class II receptors bind to DNA direct repeats and heterodimerize with retinoid X receptor (RXR). Type II receptors include the thyroid hormone receptors (TRs), retinoic acid receptors (RAR) and retinoic X receptors (RXRs), peroxisome proliferator-activated receptors (PPARs), vitamin D3 receptors (VDRs), Liver X receptors (LXRs). Receptors
belonging to class III are also known as orphan receptors, since the natural ligands for these receptors have not been described. These NRs bind to the DNA direct repeats as homodimers. Class IV receptors are also orphan receptors but they bind to DNA as monomers [2]. All the members discovered in human are shown in Table 1.

All the NRs have a common modular structure consisting of four domains (Figure 1). Among these domains, the DNA binding domain and (DBD, region C) and the ligand binding domain (LBD, region E) are the most conserved whereas N-terminal A/B domain and D region are comparatively less well conserved [2].

The N-terminal A/B domain harbors a transcriptional activation domain designated as activation domain and can function in a ligand independent manner. In different NRs, both the length and the sequence of the A/B region vary. The central or C-domain of NRs is a DBD and highly conserved. Crystallography and nuclear magnetic studies have provided insight into the binding properties of NRs with specific DNA sequences known as hormone response elements (HRE) [3-6]. These studies provide evidence that the DBD consists of two cysteine rich zinc finger motifs, two α-helices and a COOH extension. The DBD of NRs also plays an important role in nuclear localization of NRs and in the interaction with other transcription factors [2]. The D region serves as a linker between the DBD and the LBD. This region also contains a nuclear localization signal (NLS). The LBD is comprised of a C-terminal E region and is less well conserved than the DBD. Functionally, it is involved in ligand binding, dimerization and ligand dependent transactivation.
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<th>Name</th>
<th>Abbreviation</th>
<th>Ligand</th>
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<td>TR-α</td>
<td>Thyroid hormones</td>
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<td><strong>tailless gene</strong></td>
<td><strong>Photoreceptor cell-specific nuclear receptor</strong></td>
<td><strong>PNR</strong></td>
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<td><strong>Chicken ovalbumin upstream promoter-transcription factor I</strong></td>
<td><strong>COUP-TFI</strong></td>
<td><strong>Orphan</strong></td>
</tr>
<tr>
<td><strong>Chicken ovalbumin upstream promoter-transcription factor II</strong></td>
<td><strong>COUP-TFII</strong></td>
<td><strong>Orphan</strong></td>
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<tr>
<td><strong>V-erbA-related</strong></td>
<td><strong>EAR</strong></td>
<td><strong>Orphan</strong></td>
</tr>
<tr>
<td><strong>Estrogen receptor-α</strong></td>
<td><strong>ER-α</strong></td>
<td>Estradiol-17, tamoxifen, raloxifene</td>
</tr>
<tr>
<td><strong>Estrogen receptor-β</strong></td>
<td><strong>ER-β</strong></td>
<td>Estradiol-17, various synthetic compounds</td>
</tr>
<tr>
<td><strong>Estrogen-related receptor-α</strong></td>
<td><strong>ERR-α</strong></td>
<td><strong>Orphan</strong></td>
</tr>
<tr>
<td><strong>Estrogen-related receptor-β</strong></td>
<td><strong>ERR-β</strong></td>
<td>DES, 4-OH tamoxifen</td>
</tr>
<tr>
<td><strong>Estrogen-related receptor-γ</strong></td>
<td><strong>ERR-γ</strong></td>
<td>DES, 4-OH tamoxifen</td>
</tr>
<tr>
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<td><strong>GR</strong></td>
<td>Cortisol, dexamethasone, RU486</td>
</tr>
<tr>
<td><strong>Progesterone receptor</strong></td>
<td><strong>PR</strong></td>
<td>Progesterone, medroxyprogesterone acetate, RU486</td>
</tr>
<tr>
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<td>Testosterone, flutamide</td>
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<tr>
<td><strong>Mineralocorticoid receptor</strong></td>
<td><strong>MR</strong></td>
<td>Aldosterone, spiro lactone</td>
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<td><strong>NGF-IB</strong></td>
<td><strong>Orphan</strong></td>
</tr>
<tr>
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<td><strong>NURR1</strong></td>
<td><strong>Orphan</strong></td>
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<tr>
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<td><strong>NOR1</strong></td>
<td><strong>Orphan</strong></td>
</tr>
<tr>
<td><strong>Steroidogenic factor 1</strong></td>
<td><strong>SF1</strong></td>
<td><strong>Orphan</strong></td>
</tr>
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<td><strong>Liver receptor homolog-1</strong></td>
<td><strong>LRH1</strong></td>
<td><strong>Orphan</strong></td>
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<td><strong>Germ cell nuclear factor</strong></td>
<td><strong>GCNF</strong></td>
<td><strong>Orphan</strong></td>
</tr>
<tr>
<td><strong>Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1</strong></td>
<td><strong>DAX-1</strong></td>
<td><strong>Orphan</strong></td>
</tr>
<tr>
<td><strong>Small heterodimer partner</strong></td>
<td><strong>SHP</strong></td>
<td><strong>Orphan</strong></td>
</tr>
</tbody>
</table>

The table shows the list of 48 nuclear receptors in human [2].
**Figure 1:** A schematic representation of nuclear receptors: Nuclear receptors consist of four domains (A-F): The N-terminal ligand-independent transactivation domain (A/B), the DNA binding domain (C), hinge region (D), and C-terminal E/F domain including LBD and ligand dependent transactivation domain. Functions of specific domains are indicated in the text boxes.
The LBD harbors four functionally interconnected regions including a dimerization surface, the ligand binding pocket (LBP), a co-regulator binding surface and an activation helix known as AF-2 which mediates ligand dependent transactivation.

NRs regulate transcription through the recruitment of accessory proteins known as co-regulators (co-activators and co-repressors). Some of the NRs bind to transcriptional co-repressors through their LBD’s, whereas others including GR, MR, PR, AR, and ER require LBD to interact with heat-shock proteins until ligand binding [7]. The crystal structures of several NRs with or without their cognate ligands have been solved and have provided intricate detail of the mechanism involved in transcriptional activation, repression and co-regulator interaction.

**Mechanism of transcriptional regulation by co-repressors and co-activators:**

In the absence of ligand, NRs such as RAR and TR, and steroid receptors after binding to antagonists, recruit co-repressors to repress transcription [8, 9]. Repression is carried out by an interaction between NRs and complexes known as nuclear receptor co-repressor (NCoR) or silencing mediator for retinoid and thyroid hormone receptors (SMRT). Both were originally identified as proteins involved in repression associated with unliganded RAR and TR [10-13]. Unliganded steroid hormone receptors such as GR and ER do not interact with NCoR or SMRT, but interact strongly with them in the presence of antagonists [14, 15]. Both NCoR and SMRT contain a region at their C-termini that specifically binds to a hydrophobic groove in the surface of the LBD of unliganded RAR and TR. NCoR and SMRT interact with unliganded receptors through a conserved helical motif (I/L) XX (I/V) I (L=leucine, I=isoleucine, V=valine and X=any amino acid) [16-
Both NCoR and SMRT do not possess intrinsic enzymatic activity; however, they recruit transcriptional complexes containing histone deacetylases (HDACs). HDACs repress the transcription by deacetylating lysine residues at the N-terminal tails of histone proteins. This condenses the chromatin which in turn restricts access of the basic transcriptional machinery to a target promoter.

After ligand binding to a receptor, the first step is the conformational change in the LBD of the receptor [19]. This conformational change is accompanied by release of the co-repressors and recruitment of coactivator complexes. The latter containing chromatin-modifying enzymes [9] (Figure 2). Recruitment of co-activators allows the subsequent recruitment of RNA polymerase II and general transcription machinery to the targeted promoters [20]. Structurally, helix 12 or the AF-2 region of the LBD plays an important role in the recruitment of co-activators. Upon ligand binding, there is reorientation of helix 12 which results in the formation of a hydrophobic groove that accommodates co-activators (Figure 3). This co-regulator exchange controls transcription by the ability to modify the local chromatin structure through modification of histone tails.
Figure 2: Ligand dependent conformation change and transactivation of a nuclear receptor. In the absence of ligand, nuclear receptors are associated with a co-repressor complex such as mSin3A, SMRT and HDACs and inhibit transcription by keeping the chromatin tightly bound around the promoter. Ligand binding induces a conformational change in the structure of nuclear receptors which exchanges the co-repressors with co-activators. The co-activators including CBP/p300, PCAF, ACTR, and SRC-1 loosen the chromatin by acetylating histone tails. Acetylation of histone tails opens up the chromatin which in turn allows basal transcriptional machinery to the target promoter.
Figure 3: A picture showing the ligand binding domain (LBD) of hERα with bound estradiol. A) Unbound hERα structure is shown in blue. PDB (3OS8) B) hERα structure after binding to estradiol (green). There is reorientation of helix 12 of AF-2 after estradiol binds to ERα, PDB (1QKU). Arrows point out the position of helix 12. C) Superimposed picture of A and B. Note the position of unaligned helix 12 after superimposing pictures A and B.
Importance of LXXLL motif of co-activators in NR mediated transcription

Co-activators interact with NRs through highly the conserved NR interaction domain known as the NR interaction box. The NR interaction domain consists of a short $\alpha$-helical LXXLL motif (where L is leucine and X is any amino-acid). The number of the LXXLL motif varies among different co-activators and also accounts for the preferential binding of some coactivators to the selective NRs [21-23]. NR boxes have been categorized into four classes depending on the amino acid present at the -1 and -2 positions of the LXXLL motif (Figure 4A). The First three classes were identified using a phage display approach and the fourth class consists of naturally occurring motifs within co-activators (Figure 4A).

The region in NRs which binds to co-activators is relatively conserved and consists of charged and hydrophobic residues. The NR interaction motif is positioned in the groove by hydrophobic interactions between leucine residues and hydrophobic residues of the groove. Also, hydrogen bonding between a lysine residue in helix 3 and a glutamic acid in helix 12 with peptide bond of LXXLL motif plays an important role in the formation of “charge clamp” (Figure 4B) [24].

There have been a number of co-activators discovered so far. Among them, the p160 family of proteins, cAMP response element-binding protein (CBP) and p300 are known to be early recruiters by activated NRs [25-29].
Figure 4: A diagram showing the charge clamp. A) The amino acid sequences in the four classes of NRs boxes. The first three classes were identified based on a phage display approach and the fourth was identified as being common to all naturally occurring co-activators. Basic amino acids are shown by (+) and hydrophobic residues are represented by (Φ) B), The charge clamp is composed of two charged residues in the NR LBD. A lysine residue from helix 3 (labeled as K) and glutamic acid residue from helix 12 (labeled as E) positions the LXXLL motif from a co-activator in a groove by forming hydrogen bonds with the peptide backbone of the LXXLL motif. Blue line represents the polypeptide backbone.
The p160 family of co-activator consists of three family members: SRC-1, the first identified nuclear receptor coactivator (also known as p160-1 and N-CoA1), SRC-2 (TIF-2, GRIP1, and N-CoA2) and SRC3 (also known as P/CIP, ACTR, AIB1, RAC3, and TRAM1) [30, 31]. All three p160 family members share a common domain structure. The N-terminus harbors a period/aryl/hydrocarbon receptor/single minded (PAS)-A-basic helix-loop-helix (bHLH) homology domain which is required for dimerization. All the family members have three equally spaced conserved LXXLL motifs to interact with NRs. SRC co-activators harbor two separate transcription activation domains AD-1 and AD-2. AD-1 is involved in recruitment of CBP/p300 co-activator and acetyltransferases and AD-2 is involved in recruitment of other protein known as coactivator associated arginine methyltransferases (CARM1) and protein arginine methyltransferase (PRMT1).

All together, these observations suggest that SRC co-activators function by recruiting chromatin modifying enzymes to the liganded receptors on the HREs. The C-termini of SRC-1 and SRC-3 exhibit weak lysine acetyltransferase activity [2].

Mice lacking individual p160 family members are viable suggesting that there is functional redundancy among them. All SRCs have been linked to cancer including breast, ovarian and prostate cancers [32]. SRC-3 is further associated with lung, colorectal, esophageal, gastric, pancreatic and oral squamous cells cancers [33]. Despite of playing an important role in tumor initiation, SRCs are also known to play a critical role in cancer metastasis [33].

Other co-activators include CBP/p300 and p300/CBP-associated factor (P/CAF). The latter is homologous to the yeast transcriptional factor GCN5. Both CBP/p300 and p/CAF
display strong acetyltransferase (HAT) activities, whereas the p160 family members exhibit weak HAT activity [34]. Coactivator complexes also include other factors in addition to the p160 family members. They often contain ATP-dependent remodeling proteins or histone arginine methyltransferase activities [35-38]. Promoter occupancy by p160 family members is followed by recruitment of RNA polymerase II (holoenzyme). Activated NRs recruit the transcription machinery through their association with members of the mammalian mediator (thyroid hormone receptor-associated protein (TRAP), vitamin D receptor-interacting protein (DRIP) complex) which make direct contact with the components of the basal transcription machinery [39]. In summary, the activated NRs play at least two roles in transcription 1) recruitment of co-activators to mediate chromatin changes and 2) association with mediator to contact general transcription factors.

**Cyclic recruitment of co-regulators on target promoters:**

The association of NRs and co-regulators on target promoters was previously thought to be static but recently the dynamic nature and cyclic recruitment of NRs and coactivators on target gene promoters has been established (through chromatin immunoprecipitation (ChIP) assays and fluorescence recovery after photobleaching (FRAP) experiments). The intricate detail of dynamic recruitment of various cofactors has been shown for ERα responsive genes employing ChIP assays [9, 40-42]. These studies confirm that assembly and the disassembly of the distinct factors takes place in a cyclic and ordered manner on NR regulated promoter [40]. One study showed that ERα and SRC family members are recruited first to the pS2 promoter followed by other transcription factors and
transcriptional machinery [40]. The event of recruitment and release of the NRs from target promoter correlates with proteasome-dependent degradation of NRs and co-regulators [41, 43-45]. NRs and most co-regulators are ubiquitinylated and degraded and this degradation is essential for the gene activation [46-49]. Turnover of GR on synthetic promoters has been studied by FRAP (with a resolution of events in several seconds). FRAP studies revealed that NRs are highly mobile in the nucleus with a rapid exchange of receptor molecules on DNA which can be measured in the second range [44, 45, 50-53].

Interestingly, new co-regulators are still being discovered but some appear unlikely to play a role in transcription. Examples include the RNA transcript for the steroid receptor-RNA activator-1 coactivator, the NAD/NADH sensor C-terminal binding protein of E1A, and several actin binding protein [54-59].

**Actin and actin related proteins (ABPs) in transcription regulation**

Apart from being a cytoskeletal protein, actin is also a major component of nuclear protein complexes. Nuclear actin is involved in diverse aspects of transcription. 1) All three RNA polymerases require actin for transcription 2) Nuclear actin interacts with nuclear ribonucleoproteins (RNPs) and is important for nuclear export of RNA 3) Nuclear actin is known to form complexes with heterogeneous nuclear ribonucleoproteins (hnRNPs) 4) Actin and actin binding proteins (ABPs) are present in complexes containing chromatin remodelers and histone acetyltransferases [60]. Although these observations suggest that actin and ABPs play a critical role in regulating transcription, the mechanisms demonstrating how ABPs regulate gene transcription are not understood.
Actin dynamics regulate the transcription of some genes by controlling the subcellular localization of important transcription factors such as myocardin-related transcription factors (MRTFs), the developmentally regulated PREP2 homeoprotein and the transcription repressor, YY1 [61, 62]. The best example of a transcription factor regulated by actin are the MRTFs. MRTF-A associates with G-actin in the cytoplasm of NIH3T3 cells in the absence of serum and accumulates in the nucleus upon serum stimulation. MRTFs associate with serum response factor (SRF) and stimulate transcription of SRF-dependent gene [63-65]. Actin related Rho activation is also known to cause actin polymerization and release of MRTF-A, which in turn translocates to the nucleus and binds to SRF to regulate SRF dependent transcription [66].

Flightless-1 (FLi-1) is another example of an ABP present in the nucleus as well in the cytoplasm [67]. Fli-1 interacts with AR and enhances the transcription of AR-regulated genes in cooperation with other co-activators [68-70]. Other ABPs involved in transcription regulated by AR include gelsolin, Filamin-A (FLN-A), and supervillin [68-71]. Both gelsolin and Filamin-A are known to facilitate the translocation of AR to the nucleus. FLN-A also interacts with transcription factor forkhead box C1 (FOXC1) and inhibits FOXC1 activity [72]. We have previously shown that ACTN4 (Iso), a spliced form of ACTN4, stimulates transcriptional activity mediated by MEF2 [73]. All these data suggest that actin and actin binding proteins including ACTN4 play an important role in transcription however the mechanisms involved in such regulation have not been fully explored.
**Figure 5:** A schematic diagram of ACTN4 (full-length) and ACTN4 (Iso). Full-length ACTN4 harbors two calponin-homology domains (CH) (CH1-amino acids 50-153, CH2-163-266), four spectrin repeats (SR1-292-401, SRII: 421-518, SRIII: 528-639, SR IV: 550-753), two EF hands (EF 1:769-790, EF2: 819-831) and a C-terminal calmodulin like domain. Bottom panel shows the schematic structure of ACTN4 (Iso). Amino acids 89-478 are absent in ACTN4 (Iso).
α-actinin family

α-actinin belongs to a family of actin cross-linking proteins that includes fimbrin, dystrophin and spectrin [74, 75]. There are four members of the α-actinin subfamily expressed in a tissue specific manner. Among them, α-actinin-2 and -3 are specifically expressed in muscle whereas α-actinin-1 and -4 are ubiquitously expressed [76-78]. All four proteins share extensive sequence homology with a conserved organization of functional domains including an N-terminal actin binding domain (ABD) composed of two calponin homology (CH) domains, a central rod domain consisting of 4 spectrin repeats (SR), followed by a C-terminal calmodulin (CaM)-like domain consisting of two calcium binding motifs (EF) (Figure 5). Calcium inhibits binding of non-muscle isoforms to actin whereas muscle forms are calcium insensitive. In non-muscle cells, α-actinins colocalize with actin filaments and are also found in focal adhesion contacts suggesting a role in anchoring actin filaments to the plasma membrane. The ABD of the α-actinins is the most evolutionary conserved domain in the family. Cross-linking and NMR studies have shown that there are three major actin binding sites (ABS1-3) in ACTN4 (ABS1: residues 48-57, ABS2: 123-147, ABS3: 153-172) [79-81].

The α-actinins associate with the cytoplasmic tail of various cadherins [82, 83] and intercellular adhesion molecules such as intercellular adhesion molecule-1(ICAM-1) [84]. In addition they interact with signaling molecules including extracellular signal-regulated kinase 1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, PKN, a fatty acid and Rho-activated serine/threonine protein kinase and p85 subunit of phosphatidylinositol 3 kinase [75, 85-87]. The interaction of α-actinins with a
broad range of proteins suggests it may play role as a scaffold protein to promote protein-protein interactions. The $\alpha$-actinins form an antiparallel dimer with an actin binding domain at both the ends. Dimer formation is essential for its function as a cross linker of actin filaments [88]. Altogether, these data suggest that $\alpha$-actinins form anti-parallel dimers to coordinate cytoskeletal organization and signal transduction through association with adhesion molecules and cytoplasmic proteins [89].

The $\alpha$-actinins are known to be regulated by four major mechanisms 1) processing by proteases such as calpain 2) binding to phosphatidylinositol intermediates 3) phosphorylation by tyrosine kinases and 4) by calcium binding. All of these mechanisms are important in cell movement suggesting an involvement of $\alpha$-actinins in cell motility. Calpain regulates the detachment step for cell migration by cleaving several proteins found in focal adhesions [90]. $\alpha$-actinins bind to MEKK1, a MAPK kinase kinase, at focal adhesions and regulates calpain activity [86, 91]. Importantly, MEKK1 helps in recruitment of $\alpha$-actinins to the focal adhesions. Another signaling molecule that binds to $\alpha$-actinin is phosphatidyl 3, 4, 5-triphosphate, a lipid product of phosphatidylinositol 3 kinase (PI3 kinase). It has been shown that interaction between $\alpha$-actinins and phosphatidyl 3, 4, 5-triphosphate decreases the affinity between $\alpha$-actinins and integrin [92, 93]. This leads to disassembly of focal adhesions and promote the detachment step during migration. Regulatory unit (p85) of PI3 kinase also binds directly to $\alpha$-actinins suggesting $\alpha$-actinin involvement in chemotactic cell movement because recently it has been shown that PI3 is the key player in controlling chemotactic cell movement [94]. Calcium is an important divalent cation that binds to EF hands of non-muscle $\alpha$-actinin,
decreasing their affinity to bind to actin and in turn regulating α-actinin’s activity [95].

Another mechanism which regulates α-actinins is tyrosine phosphorylation through integrin activated focal adhesion kinase (FAK), a regulator of adhesion plaques. FAK phosphorylates the ABD domain of the non-muscle α-actinin on tyrosine 12 and reduces its affinity to the actin cytoskeleton suggesting a role in cell adhesion [75]. Altogether, these data suggest that α-actinins play an important role in cell migration and motility.

Alpha actinin 4

Alpha actinin 4 (ACTN4), was cloned from a tumor cell line and showed to have 80% sequence similarity to α-actinin 1(ACTN1) [96]. Although both actinin-4 and actinin-1 are highly similar at the amino acid level (~86%), they exhibit different characteristics in vivo and in vitro [96]. ACTN-1 is highly sensitive to the calcium concentration whereas ACTN-4 shows lower calcium sensitivity [97, 98]. ACTN-1 is located in all F-actin structures including dorsal and peripheral edge ruffles, phagocytic cups and basal podosomes, whereas ACTN-4 is predominantly present in the circular ruffles at the dorsal surface [99]. These observations suggest that the two α-actinins are regulated in a distinct manner.

Localization of ACTN4

Although predominantly localized in the cytoplasm, ACTN4 is also found in the nucleus of certain cell types and is able to translocate into the nucleus in response to extracellular stimuli. Treatment with PI3 kinase inhibitors or actin polymerization results in nuclear accumulation of ACTN4 in several cancer cell lines [96]. ACTN4 accumulates in the
nucleus and associates with p65 subunit of transcription factor NF-kB after treatment with tumor necrosis factor (TNF). Epidermal growth factor (EGF) treatment also results in nuclear accumulation of ACTN4 [100]. Many other structural proteins that play an important role in the cytoplasm are also known to be present in the nucleus such as spectrins, nesprins, and nuclear mitotic apparatus (NuMA) [101] by an unknown nuclear localization mechanism. All these proteins including ACTN4, lack a NLS and the precise mechanism regulating nucleocytoplasmic import of these molecules is not yet known. Recently it has been shown that ACTN4 not only undergoes nucleocytoplasmic shuttling [102] but also associates with the rRNA transcriptional machinery in a cell cycle dependent manner. This nucleocytoplasmic shuttling is nuclear export signal (NES) dependent and mediated by chromosome region maintenance-1 (CRM1) protein. CRM-1 mediates nuclear export and requires a NES. There are two NES sequences at amino acid residues 141-152 and 523-532 respectively in ACTN4 [102].

In a yeast two hybrid screen, a novel spliced isoform of ACTN4, designated as ACTN4 (Iso) was isolated [73]. ACTN4 (Iso) contains an internal deletion from nucleotides 263-1433 from the translation initiation codon. This deletion results in the loss of amino acids 89-478 of ACTN4 that contains part of the calponin homology 1 (CH1) domain, all of the CH2 domains and spectrin repeats 1 and 2 (Figure 5). The presence of this spliced isoform at the protein level was confirmed in HeLa, HEK293 and MCF-7 cells [73].

Despite numerous studies, the cellular functions of the ACTN4 are not well understood. It was originally isolated as a protein that binds filamentous actin (F-actin) capable of modulating cytoskeletal organization and cell motility [96]. There have been reports
suggesting a role in apoptosis and also an association with transcription factors such as nuclear factor Y (NF-Y) [103, 104]. However, there is no functional data suggesting that ACTN4 is involved in transcription regulation.

It is well known that the actin cytoskeleton plays an important role in cell motility and migration and that α-actinins are among the proteins that link actin filaments. Functionally, α-actinins are known to play an important role in metastasis in which both the cell motility and adhesion are affected.

**Alpha actinin 4 and cancer**

ACTN4 has been shown to be predominantly present at the leading edge of the cytoplasm of motile cells in cell surface ruffles [96, 99, 105]. Studies have shown overexpression of ACTN4 in various human carcinomas including invasive breast cancer, lymph node metastasis of colorectal cancer, non-small cell lung cancer and pancreatic cancer [96, 106, 107]. Also, a study showed amplification of the ACTN4 gene in an ovarian cancer [108]. In invasive bladder cancer, there is a direct correlation between ACTN4 expression and cell growth and invasiveness [109]. The mechanisms by which ACTN4 regulates tumorigenicity are not fully understood. One likely mechanism is cross talk between ACTN4 and the phosphatidylinositol 3-kinase (PI3K)/AKT pathway in ovarian cancer since deregulation of AKT pathway has been reported in this cancer. Inhibition of PI3K or AKT results in decreased ovarian cell migration, invasion and proliferation *in vivo* [110-113]. ACTN4 interacts with the p85 regulatory subunit of PI3K and treatment with wortmannin, a PI3K3 inhibitor, affects the translocation of ACTN4 [96, 114]. ACTN4
also interacts with AKT1 and knockdown of \textit{ACTN4} down regulates AKT phosphorylation and inhibits cell proliferation by blocking AKT translocation to the cell membrane [115]. This evidence supports the idea that ACTN4 may be playing an important role in tumorigenesis. It will be important to study the regulatory mechanisms that lead to increased levels of ACTN4 in cancer cells and what role ACTN4 plays in cancer progression. Furthermore, it will be important to investigate whether transcriptional regulation by ACTN4 along with NRs plays a role in breast cancer.

ACTN4 is also known to play an important role in the kidney. It has been shown that ACTN4 deficient mice have severe glomerular disease due to abnormal morphology of the podocytes (highly differentiated kidney cells) and in humans, the phenotype associated with ACTN4 mutations manifests only in kidney [116].

\textbf{Podocytes and proteins involved in the maintenance of their characteristic structure}

One of the important functions of the kidney is to remove toxins and metabolic waste from the body, while preventing proteins larger than albumin from entering the urine. There are many mechanisms regulating this process; among them, a group of highly differentiated cells, known as podocytes [117, 118]. Podocytes are highly specialized epithelial cells covering the outer layer of the glomerular basement membrane (GBM) (Figure 6A). Podocytes consist of a cell body, major processes and foot processes (FPs). Major processes arise from cell body and ramify into small processes known as FPs [117] (Figure 6A). FPs are composed of highly ordered parallel contractile actin filament bundles [119, 120]. FPs from neighboring podocytes are connected by slit diaphragms (SD). The SD is a modified adherens junction that spans on 30-50nm wide filtration slits.
The SD of podocytes is made of extracellular domains from number of transmembrane proteins such as nephrin, P-cadherins and FAT, to form zipper like structure [120] (Figure 6C). The integrity and the function of podocytes to act as a filtration barrier depend on its highly co mplexed structure [118]. There are three membrane domains regulating the structure of FPs, the apical membrane domain, the SD and the GBM. All these domains are physically and functionally associated with actin cytoskeleton of FPs [119]. Any type of oxidative and inflammatory stress leads to effacement of foot processes of the podocytes which further leads to deterioration of the filtration barrier and eventually results in uncontrolled loss of protein into the urine (Figure 6B). Another route resulting in destruction of FPs are mutations in genes encoding SD proteins or in proteins regulating actin dynamics in podocytes.

**Nephrotic syndrome (NS) – Focal segmental glomerulosclerosis**

Nephrotic syndrome (NS) is the condition characterized by heavy proteinuria, edema and hypoalbuminemia and is categorized as either steroid-sensitive or steroid resistant [121]. Focal segmental glomerulosclerosis (FSGS) is found in 5-10% of NS patients. Proteinuria results in podocyte effacement in FSGS [120]. FSGS generally progresses to end stage renal failure (ESRD) and requires renal transplantation. Depending on underlying cause, FSGS can be divided into three categories: idiopathic, genetic and secondary as a result of injury, medication or drug abuse [122, 123]. Genetic linkage studies have identified several genes that contribute to the development of FSGS.
**Figure 6A:** Structure of a podocyte. Left panel shows the scanning electron micrograph (SEM) of a highly differentiated podocyte consisting of cell body (CB), major processes (MPs) and foot processes (FPs). MPs sit on capillaries and FPs interdigitate among themselves to form filtration slits. Right panel (Top) shows a transmission electron micrograph (TEM) of podocytes sitting on the glomerular basement membrane (GBM) and FPs covering the fenestrated epithelium. This picture is adapted from [120]. Right panel (Bottom) shows foot processes from two adjacent podocytes interdigitating with each other.

**Figure 6B:** Structure of podocyte in nephrotic syndrome conditions. In nephrotic syndrome, the FPs lose their normal interdigitating pattern. This picture is adapted from [120].
Figure 7: A diagram showing the various proteins involved in the formation of the slit diaphragm (SD) [118].
Nephrin (NPHS1)

Nephrin was the first gene identified associated with nephrotic syndrome of the Finnish type (CNF) [124]. In Finland, CNF has an incidence of 1:10,000 births but it is less in other countries. There are several missense mutations in the nephrin gene found in CNF patients however, two mutations, the Fin major (deletion nucleotides 121 and 122) and Fin minor (premature stop at amino acid 1109) are responsible for around 90% of the cases in Finland [124]. Nephrin directly links the podocyte junctional complex to actin cytoskeletal dynamics [125]. Nephrin is one of the main proteins involved in the maintenance of the structure of the slit diaphragm in podocytes. Nephrin is a transmembrane protein with eight immunoglobulin like domains. Neighboring nephrin molecules from adjacent foot processes interact with each other to form a zipper like structure. Nephrin deficient mice develop proteinuria and show extreme foot process effacement [126]. Furthermore, injection of anti-nephrin antibody in mice leads into foot process effacement [126]. Nephrin is also involved in signaling. It interacts with the p85 subunit of the PI3K to activate the AKT signaling pathway and thereby regulates cell survival, migration and cell growth [127]. Furthermore, nephrin is associated with lipid rafts in the slit diaphragm [128]. A study has shown that tyrosine phosphorylation of the nephrin protein leads to its subcellular redistribution [128]. Fyn, a Src protein kinase family member, tyrosine phosphorylates the intracellular domain of the nephrin and Fyn deficient mice develop proteinuria and foot process effacement [121, 129] suggesting that Fyn dependent phosphorylation of nephrin is important for the maintenance of the differentiated state.
Evidence from mice and immortalized cultured mouse podocytes (MPCs) indicate that synthetic hormones including retinoids, glucocorticoids, pioglitazone, vitamin D3 and WY14643 may protect/restore podocytes from injury [130-135] by restoring the cytoskeletal architecture and stimulating the expression of nephrin [130, 134, 136]. However, the underlying mechanisms are not well understood.

**CD2AP associated protein (CD2AP)**

Another adaptor protein associated with congenital nephrotic syndrome is CD2-associated protein (CD2AP) [137]. The importance of CD2AP in kidney is evident by the fact that CD2AP knockout mice die from renal failure. CD2AP \(^{-/-}\) mice die at an early age (6-7 weeks) due to renal failure [137]. Electron microscopy shows extensive foot process effacement in these mice. CD2AP \(^{+/-}\) mice show no proteinuria but they are more susceptible to glomerular injury by immune complexes and nephrotoxic antibodies than wild type [138]. Breeding of heterozygous mice for CD2AP (CD2AP\(^{+/-}\)) with heterozygous mice for synaptopodin or Fyn (Syn/Fyn\(^{+/-}\)), which alone does not show any kidney pathophysiology, results in glomerular damage suggesting haploinsufficiency of CD2AP [138]. However, in humans, not all heterozygous CD2AP mutations cause kidney diseases.

CD2AP is an adaptor protein localized in the cytoplasm, membrane ruffles and the leading edge of the cells [139]. It plays an important role in cytoskeletal remodeling, cell survival and endocytosis [140-142]. The C-terminus of CD2AP directly interacts with both F-actin [142] and synaptopodin, an actin-binding protein [143]. It also interacts with nephrin and podocin [144, 145]. It functions as a linker as it connects SD proteins to the
actin cytoskeleton in podocytes. Functionally, CD2AP and nephrin together activate serine threonine kinase AKT signaling after binding to the p85 regulatory subunit of PI3K to promote apoptosis [127].

Podocin

Mutations in another gene NPHS2, account for autosomal recessive steroid-resistant nephrotic syndrome. NPHS2 encodes for podocin, a member of stomatin protein family, and is exclusively expressed in the podocytes [120]. Podocin is an integral membrane protein consisting of a hairpin-like structure in which the N-terminal and C-terminal cytoplasmic domains fold back near one another [121, 146-148]. Podocin associates with lipid rafts and oligomerizes in the slit diaphragm. It is responsible for recruiting nephrin and CD2AP to these lipid rafts [149]. Podocin deficient mice develop proteinuria and die a few days after birth from kidney failure [150].

Other structural proteins associated with glomerular dysfunction include transient receptor potential cation channel family (TRPC) and ACTN4. TRPC-6 is involved in Ca$^{2+}$ influx regulation. TRPC-1, TRPC-2, TRPC-5, TRPC-6 are expressed in podocytes [148]. TRPC6 is localized to the podocyte cell body, primary processes and interacts with nephrin and podocin [148]. Recently, TRPC6 has been shown to be mutated in families with an autosomal dominant form of FSGS [147, 148]. Mutation P112Q in TRPC6 shows an increase influx of Ca$^{2+}$ after activation of the G-protein-coupled receptor AT1 by angiotensin II. The mutations may also alter interaction with other slit diaphragm proteins resulting in disruption of glomerular cell function [121].
**ACTN4 and FSGS**

Transgenic mouse model studies and inborn errors in human have provided evidence for a special role of actin dynamics in podocytes. Although ACTN4 is ubiquitously expressed, the phenotype related to ACTN4 mutations only manifests in the kidney suggesting that ACTN4 plays a specific role in kidney development [121]. Deletion of ACTN4 in mice leads to progressive proteinuria, glomerular disease and death by several months of age, again indicating a critical role of ACTN4 in kidney [116]. Electron microscopy has shown that there is significant effacement of foot processes in these mice. Lymphocytes from homozygous ACTN4 deficient mice show an increase in lymphocyte chemotaxis establishing a role of ACTN4 in cell motility [116]. Also, the podocyte cell lines from these mice display less adherence to GBM components such as collagen IV and laminins-10 and laminins-11 supporting its role in maintaining glomerular structure [151].

In humans, mutations in ACTN4 are associated with the autosomal dominant form of familial FSGS. Three missense mutations at residues K228E, T232I and S235P in ACTN4 have been linked to FSGS so far [152, 153]. The mechanisms by which these mutations result in FSGS are not very well defined however these ACTN4 proteins mutants show a high affinity to F actin and that might be responsible for the altered mechanical properties of affected podocytes [152].

Recently, transgenic mice and cellular studies have provided important information about FSGS-linked ACTN4 mutants. mACTN4 (mouse ACTN4) (K255E), corresponding to human ACTN4 (K255E), is the best characterized. In contrast to humans where FSGS-
linked ACTN4 mutations are autosomal dominant, only one in nine knock-in mice heterozygotes for ACTN4\textsuperscript{WT/K255E} developed a mild proteinuria phenotype. By contrast most of the homozygous ACTN4\textsuperscript{K255E/K255E} animal exhibited severe kidney disease [154]. These data indicate that the phenotype of heterozygous mice does not fully reflect the phenotype of K288E heterozygotes in humans. The distinction between humans and mice could be due to the relatively short life of the mice compared to that of the humans, suggesting that humans likely require a second mutation in order to develop FSGS [154]. Transgenic studies indicate that higher expression of ACTN4 (K255E) correlates with a more severe FSGS phenotype [155]. Furthermore, mice expressing high levels of mACTN4 (K255E) show decreased mRNA and protein accumulation for other slit diaphragm components such as nephrin. ACTN4 (K255E) also showed increased affinity of the ACTN4 for actin [155]. Furthermore, mACTN4 (K255E) is mislocalized in podocytes which affects cell adhesion, spreading and migration [156].

Nephrotic syndrome and Nuclear Receptors

Glucocorticoids, the ligands for glucocorticoid receptors, are very effective drugs used for anti-inflammation. However, their mechanisms of action in nephrotic syndrome have not been well defined. All types of cells in the glomerulus express glucocorticoid receptor protein [157]. Several studies from mice and conditionally immortalized mouse podocyte cell lines have shown that the dexamethasone, a synthetic glucocorticoid, may protect or restore podocyte from injury [136]. However, the underlying mechanisms have not been elucidated. Upon injury, podocytes undergo cytoskeletal changes resulting in foot process effacement and proteinuria accompanied by loss of slit diaphragm components such as
nephrin [158]. After treatment of injured podocytes with dexamethasone, the cytoskeletal architecture is restored and expression of nephrin is enhanced [130, 134, 136]. This observation suggests that glucocorticoid may regulate the expression of nephrin by directly binding to response elements in its promoter. Also, dexamethasone is the well-known therapeutic drug for the treatment of nephrotic syndrome in immune-mediated or inflammation associated glomerular disease [132].

Ligands of PPARs effectively inhibit proinflammatory cytokines including vascular cell adhesion molecule (VCAM-1) and IL-6 expression in various cell types [159, 160]. Both eicosapentaenoic acid (EPA) and docosahexcanoic acid (DHA) (ligands for PPARs) reduce lipopolysaccharide (LPS) induced activation of NF-κB in PPARγ dependent pathway in human kidney-2 (HK-2) cells [161]. Additionally, several reports have suggested that PPARs and GRα inhibit the expression of proinflammatory genes by antagonizing the action of transcription factors such as NF-κB, AP-1 and STATs [161, 162]. An increase in the expression of PPARγ has been observed in both rat and human kidney sclerotic conditions in vivo suggesting compensatory regulatory role of PPARγ in response to podocyte injury. Furthermore, in vitro data has suggested that PPARγ activation protects against puromycin aminonucleoside (PAN) induced apoptosis and necrosis of podocytes [163]. Several other studies suggested a reverse correlation between the degree of proteinuria and nephrin expression levels [124, 164, 165]. Also, it has been shown that PPARα agonists upregulate nephrin expression in human immortalized podocytes and embryonic kidney epithelial cells [133].
There are abnormalities in the fetal kidney and a reduced number of nephrons in retinoic acid receptor deficient mice [166, 167]. Retinoids are known to ameliorate glomerular proliferation, glomerular lesions and albuminuria in established models of renal damage [168]. Ligand induced activation of retinoic acid receptors prevents puromycin aminonucleoside nephrosis (PAN) and oxidative stress induced apoptosis in podocytes and mesangial cells (special cells around the blood vessels in kidney) [169]. Another important nuclear receptor involved in mesangial cell proliferation is the vitamin D receptor [170]. It is known that vitamin D3 inhibits mesangial cell proliferation through the vitamin D receptor [168]. Furthermore, nerve growth factor (NGF) induced factor B (NGFIB), an orphan receptor is also known to regulate apoptosis in mesangial cells [168].

All together these data suggest that RAR, GR, VDR, and PPARγ play an important role in kidney pathophysiology. Physiological ligands of nuclear hormone receptors including all-trans retinoic acid (AT-RA), vitamin D3 and dexamethasone significantly rescue or protect podocytes from injury by restoring cytoskeletal changes and gene expression changes of SD components including nephrin. The detailed mechanism by which NRs regulate the expression of SD components is less explored.

**Histone Deacetylases**

Histone deacetylases (HDACs) are well known for their functions as regulators of the transcription machinery. HDACs play an important role as transcriptional co-repressors in different aspects of physiology. In mammals, HDACs are grouped into four distinct classes (class I, II, III and IV) depending on sequence homology, subcellular localization,
and the chemistry of their enzymatic activity [171]. In mammals, class I members include HDAC1, HDAC2, HDAC3 and HDAC8 and share homology with yeast HDAC known as Rpd3 [172-174]. These HDACs are expressed ubiquitously, localized predominantly in the nucleus and show high enzymatic activity towards histone substrate [171]. They possess a conserved N-terminal conserved deacetylase domain and a C-terminal tail. HDAC1 and HDAC2 are highly similar and mostly found in large repressive complexes such as Sin3, NuRd, CoREST and PRC2 [173]. HDAC3 form complexes with N-CoR and SMRT. To date, HDAC8 has not been found in any complexes [172].

Class II HDACs are homologous to yeast HDAC Hda1 and have been further subdivided into two groups Class Ila and Class Iib. Class Ila HDACs include HDAC4, HDAC5, HDAC7 and HDAC9 whereas HDAC6 and HDAC10 are in class Iib. The precise mechanism by which class Ila HDACs repress transcription is not fully understood because highly purified recombinant class Ila HDACs show very little catalytic activity with histone substrates [175]. The class Ila HDACs has been shown to recruit the class I HDACs through their C-terminal domains which might be responsible for their repressive activity [175].

Class Ila HDACs have large N-terminal domains with conserved binding sites for myocyte enhancer factor 2 (MEF2) and the chaperone protein 14-3-3. They are present in the both the cytoplasm and the nucleus [171]. An important property of class Ila HDACs is their ability to shuttle between the nucleus and the cytoplasm [176-178]. All class Ila HDACs contain a conserved nuclear localization signal (NLS) at the N-terminus and a nuclear export sequence (NES) at the C-terminus. The activity of the NLS and the NES is
modulated by many factors including chaperone protein 14-3-3, CRM1 (exportin1), and calcium dependent calmodulin kinases (CaMK I and/or IV) [179]. It has been shown that cytoplasmic retention of class II HDACs requires phosphorylation of serines residues that are required for 14-3-3 protein binding [179, 180]. One of the best studied is HDAC4 which contains three 14-3-3 binding sites (S246, S467 and S632). All these sites are highly conserved in HDAC5, HDAC6, HDAC7, and HDAC9 [173]. Binding of 14-3-3 proteins to HDACs promotes cytoplasmic retention by various mechanisms 1) one of the 14-3-3 binding sites overlaps the NLS, so 14-3-3 binding masks the NLS and inhibits access of importins α/β [181] 2) 14-3-3 binding protects HDAC7 from proteasomal degradation [182].

There are five groups of kinases known to phosphorylate 14-3-3 binding residues on HDACs including CaMKs,[183-185], protein kinase D [186], microtubule affinity-regulating kinases [187, 188], salt inducible kinases [189] and checkpoint kinase-1 (CHK1) [173, 190]. Most of these kinases have orthologues in invertebrates and among the three HDAC residues undergoing phosphorylation, the S246 site is the most conserved. This site is present in HDACs from C.elegans to mammals suggesting that common regulatory mechanisms control nucleocytoplasmic shuttling in many species [171].

On the other hand, there are two phosphatases known to dephosphorylate class II HDACs, MYPT1-PP1B (myosin phosphatase targeting subunit-1-protein phosphatase-1B) complex and PP2A [191]. MYPT1-PP1B is a part of complex known as myosin phosphatase that dephosphorylates myosin light chains resulting in relaxation of
contracting smooth muscle cells. PP2A is known to interact and dephosphorylate HDAC7 in T cells and in endothelial cells [192, 193].

Class IIa HDACs are differentially expressed in an organs dependent manner. For example HDAC5 and HDAC9 are highly expressed in muscle, heart and the brain [178, 179, 194], while HDAC4 is highly enriched in the brain and skeleton growth plates. HDAC7 is enriched in endothelial cells and thymocytes [186]. Functionally, class IIa HDACs are important regulators in cell differentiation and development partly due to their association with MEF2 transcription factors [178, 194].

Nucleocytoplasmic shuttling of class II HDACs is an important mechanism that regulates MEF2 activity [194]. During muscle differentiation and thymocyte development, class IIa HDACs are sequestered in the cytoplasm. As a result, transcriptional repression by MEF2 is relieved, leading to up-regulation of MEF2 target genes such as Nur77, MMP10, and muscle creatine kinase (MCK). Up-regulation of these genes controls muscle differentiation, formation of blood vessels and thymocyte apoptosis [186, 195]. HDAC4 plays an important role in the development of the skeleton [186]. Runt related transcription factor 2 and the MEF2C transcription factor interact with HDAC4 and play a pivotal role in the control of chondrocyte hypertrophy and bone formation. Deletion of HDAC4 leads to excessive bone formation due to uncontrolled transcriptional activation by MEF2C and RUNX2 [196]. Knockout mice of either HDAC5 or HDAC9 are viable however, the double knockout shows lethal ventricular septal defects and thin walled myocardium due to abnormalities in growth and maturation of cardiomyocytes [179]. HDAC7 is specifically expressed in the endothelial cells that form the inner lining of the
cardiovascular system [195]. Genetic deletion of HDAC7 in mice leads to embryonic lethality as a result of loss of integrity of endothelial cell interaction and dilation and rupture of blood vessels [195]. HDAC7 knockout mice shows upregulation of matrix metalloproteinase 10 (MMP10), an endoprotease secreted by endothelial cells that degrades the extracellular matrix perturbing endothelial-cell and smooth muscle cell interactions thereby disrupting blood vessels.

In addition to site specific phosphorylation, classes IIa HDACs are also targets for other post translational modifications including ubiquitylation and sumoylation [182, 197]. Ubiquitylation regulates protein degradation of class IIa in slow-twitch/oxidative skeletal muscle fibres, allowing MEF2 dependent transcription of genes that contribute to the slow-twitch muscle type [198, 199]. Sumoylation of HDAC4 and HDAC9 enhances transcriptional repression and is associated with HDAC4 nuclear import. It is inhibited by CaMK-mediated HDAC4 phosphorylation [197]. Although this modification is highly efficient the functional consequences sumoylation are not well understood.
OUR RESEARCH FOCUS

There is increasing evidence suggesting that actin and actin binding proteins are also nuclear and associated with transcriptional machinery. However, the detailed mechanisms by which these proteins regulate transcription are not well understood. We have isolated ACTN4 (full-length) and a splice variant of ACTN4 known as ACTN4 (Iso), as HDAC7 interacting proteins in yeast two hybrid screening assay. This observation suggested that ACTN4 might be involved in process regulated by HDAC7.

During our analysis we noticed that ACTN4 harbors a LXXLL nuclear receptor interaction motif, a motif generally present in NR co-activators. Therefore, we hypothesized that ACTN4 might act as a co-activator for NRs. We observed that ACTN4 associates with and enhances transcription mediated by NRs including VDR and ERα in a ligand dependent manner. It also regulates the proliferation of MCF-7 breast cancer cells in a ligand independent manner. Mechanistically, we have shown that ACTN4 acts as a co-activator for ERα mediated transcription and that it exerts this role through antagonizing HDAC7 function.

Also, we observed that ACTN4 (Iso) is predominantly present in the nucleus and is more potent transcriptional co-activator than ACTN4 (full-length). This led us to hypothesize that the ACTN4 (Iso) might regulate transcription mediated by ERα by a second mechanism in MCF-7 cells. We have found that the ACTN4 (Iso) regulates NRs mediated transcription by interacting with co-activators including p160 family members and PCAF.
We explored the functional significance of the interaction between ACTN4 and NRs in podocytes. Point mutations (K228E, T232I, and S235P) in ACTN4 are associated with FSGS characterized by proteinuria and podocyte injury. The mechanisms by which these mutations cause FSGS are not very well defined. We hypothesized that disease linked mutations in ACTN4 might interfere with the transcriptional program mediated by nuclear receptors, So far, our data suggested that FSGS-linked ACTN4 mutants are defective in regulating transcription mediated by nuclear receptors because of a loss in their ability to bind to the nuclear receptors and to localize to the nucleus.
CHAPTER 2: THE ACTIN-BINDING PROTEIN, ACTININ ALPHA 4 (ACTN4), IS A NUCLEAR RECEPTOR COACTIVATOR THAT PROMOTES PROLIFERATION OF MCF-7 BREAST CANCER CELLS

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Abstract

Alpha actinins (ACTNs) are known for their ability to modulate cytoskeletal organization and cell motility by crosslinking actin filaments. We show here that ACTN4 harbors a functional LXXLL receptor interaction motif, interacts with nuclear receptors in vitro and in mammalian cells, and potently activates transcription mediated by nuclear receptors. While overexpression of ACTN4 potentiates estrogen receptor alpha (ERα)-mediated transcription in transient transfection reporter assays, knockdown of ACTN4 decreases it. In contrast, histone deacetylase 7 (HDAC7) inhibits estrogen receptor alpha (ERα)-mediated transcription. Moreover, the ACTN4 mutant lacking the CaM (calmodulin)-like domain that is required for its interaction with HDAC7 fails to activate transcription by ERα. Chromatin immunoprecipitation (ChIP) assays demonstrate that maximal associations of ACTN4 and HDAC7 with the pS2 promoter are mutually exclusive. Knockdown of ACTN4 significantly decreases the expression of ERα target genes including pS2 and PR and also affects cell proliferation of MCF-7 breast cancer cells with or without hormone, whereas knockdown of HDAC7 exhibits opposite effects. Interestingly, overexpression of wild-type ACTN4, but not the mutants defective in interacting with ERα or HDAC7, results in an increase in pS2 and PR mRNA accumulation in a hormone-dependent manner. In summary, we have identified ACTN4
as a novel, atypical coactivator that regulates transcription networks to control cell growth.
INTRODUCTION

The alpha actinins (ACTNs) belong to a family of cytoskeletal proteins that bind actin filaments to maintain cytoskeletal structure and cell morphology [200]. Among the four members of the family, ACTN2 and ACTN3 are expressed primarily in muscle, while ACTN1 and ACTN4 are ubiquitously expressed [105]. All four members of the actinin family share high sequence homology with conserved functional domains including an N-terminal actin-binding domain containing two highly conserved 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 (CaM)-like domain [201]. Although predominantly localized in the cytoskeleton, ACTN4 is also found in the nucleus of certain cell types and is capable of translocating to the nucleus in response to extracellular stimuli [96]. In addition to its role in the cytosol, we have recently identified a novel function for ACTN4 in transcriptional regulation by myocyte enhancer factor 2 (MEF2) [73]. Another study has also demonstrated an association between ACTN4 and nuclear factor κB (NF-κB) [100]. These findings suggest that ACTN4 may play an unexpected role in transcriptional regulation. Nuclear hormone receptors, including vitamin D receptor (VDR) and steroid hormone receptors such as estrogen receptors (ER) are ligand-activated transcription factors that control aspects of homeostasis, cell differentiation, proliferation, and development [1, 202, 203]. Transcriptional regulation by nuclear receptors is thought to occur through the exchange of associated corepressors and coactivators. Ligand binding induces an allosteric change in the nuclear receptor,
leading to dissociation of corepressor complexes and recruitment of coactivator proteins followed by transcriptional activation of target genes.

The hormone-induced interaction between nuclear receptors and coactivators is mediated through one or more copies of highly conserved signature sequence designated as a nuclear receptor interaction (NR) box. The NR box is comprised of a short α-helical LXXLL motif (where L is leucine and X can be any amino acid) [21]. NR boxes and surrounding residues have been grouped into four classes depending on the amino acid residue present at the -1 and -2 positions upstream of the LXXLL motif. The first three classes were identified based on the phage display approach and the fourth class was identified following analysis of naturally occurring motifs among coactivators [24]. This motif is present in many nuclear receptor coactivators including p160 family of coactivators (NCoA 1, 2 and 3), histone acetyltransferases (CBP/p300) and p300/CBP-associated factor (PCAF) [23, 100, 203]. The integrity of this motif is essential for the ability of the coactivators to potentiate transcriptional activation by NRs. We have previously shown that the full-length ACTN4 and its isoform potentiate transcriptional activation by MEF2 through antagonizing HDAC [73]. In this study, we focus on the full-length ACTN4 and provide evidence that ACTN4 has a broad role in transcriptional regulation including its role as a transcriptional coactivator for nuclear receptor-mediated transcription. Additionally, we demonstrate that ACTN4 potentiates transcriptional activity of ERα, in part, through its association with HDAC7.
EXPERIMENTAL PROCEDURES

Plasmid construction. CMX-HA-ACTN4, CMX-HA-ACTN4 (LXXAA) and CMX-HA-ACTN4 (Δ831-869) expression plasmids were generated by site directed PCR mutagenesis according to the manufacturer’s protocol (Stratagene). For the glutathione S-transferase (GST) constructs, full-length ACTN4 cDNA was PCR amplified and subcloned into pGEX4T vector using standard techniques. Expression plasmids for nuclear receptors and reporters were generous gifts from Ron Evans (The Salk Institute, La Jolla, CA).

Antibodies and chemicals. α-ACTN4 and α-HDAC7 antibodies have been previously described. Anti-HA conjugated α-horseradish peroxidase was purchased from Roche Applied Science. α-VDR (C-20), α-ERα (D-12), α-Lamin B (sc 6216), α-GAPDH (sc 25778) and α-HDAC1 (sc 7872) antibodies were purchased from Santa Cruz Biotechnology. Anti-α-tubulin (T-5168) antibody was purchased from Sigma- Aldrich. 1-(24R)-24, 25-Dihydroxy-vitamin D3 (705861) and β-estradiol (E2, E8875) were purchased from Sigma-Aldrich.

Cell culture. HEK293, CV-1 and MCF-7 cells were grown in Dulbecco’s Eagle modified medium (DMEM) supplemented with 10% Fetal Bovine Serum, 50 units/ml penicillin G and 50 µg/ml streptomycin sulfate at 37 °C in 5% CO2.

In vitro protein-protein interaction assays. Glutathione S-transferase (GST) fusion proteins were expressed in E. coli DH5α strain, affinity purified and immobilized on glutathione Sepharose 4B beads. In vitro pulldown assays were carried out using
immobilized GST-ACTN4 (WT) or GST-ACTN4 (LXXAA) mutant with whole cell extracts expressing nuclear receptors in the absence or presence of vitamin D3 (100 nM) or E2 (10 nM) for 2 h at 4 °C. For GRIP1 and ACTN4 interaction, GST-ACTN4 (WT) was incubated with whole cell lysates expressing GRIP1. After extensive washes with NETN buffer (100 mM NaCl, 1mM EDTA, 10 mM Tris-Cl (pH 8.0), 0.1% Nonidet P-40, 10% glycerol, and 1 mM dithiothretol), SDS-PAGE sample buffer was added to the beads, boiled and separated by 10% SDS-PAGE gel and immunoblotted with the indicated antibodies.

Coimmunoprecipitation. HEK293 cells were grown on 10-cm plates and transfected with indicated plasmids (10 μg of total DNA) using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol. After 48 h, the cells were washed with 1X PBS and resuspended in NETN buffer along with protease inhibitors. 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 α-VDR antibodies with or without vitamin D3 for 12 h at 4 °C. The immunoprecipitated fractions were analyzed by immunoblotting using α-ACTN4 antibodies. For interaction between endogenous ACTN4 and overexpressed ERα in HEK293 cells, immunoprecipitations were carried out using α-ACTN4 antibodies in the presence or absence of 10 nM E2 for 4 h at 4 °C followed by immunoblotting with α-ERα antibodies.

Transient transfection reporter assays. For reporter assays, CV-1 or MCF-7 cells were co-transfected with equal amounts of either 100 ng of VDRE-TK-Luc or ERE-TK-Luc with or without pCMX-ACTN1 or pCMX-ACTN4 along with 100 ng pCMX-β-gal in 200 μl
Opti-MEM I using Lipofectamine 2000 (Invitrogen). The amount of DNA was kept constant (1µg) by the addition of pCMX vector. After 5 h, the medium was replaced with DMEM supplemented with 10% charcoal stripped fetal bovine serum, 50 units/ml penicillin G and 50 µg/ml streptomycin sulfate. After 24 h, medium was replaced with or without hormones as indicated. For all cell types, cells were harvested 48 h after transfection and luciferase and β-galactosidase (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. The data is representative of at least three separate trials. For siRNA transfection, MCF-7 cells were transfected a control siRNA (siCtrl, 1027415), siACTN4-1 (SI02779973), or siACTN4-2 (SI02779980) at a final concentration of 10 nM using GeneSolution siRNA (Qiagen) according to manufacturer’s protocol. After 48 h, cells were split into 48 well plates and transfected with ERE-TK-Luc along with 100 ng of pCMX-β-gal plasmids as mentioned above for luciferase assays.

Transgenic transfections and RNA analysis: MCF-7 cells were transfected with HA-ACTN4 (WT), HA-ACTN4 (LXXAA) mutant and HA-ACTN4 (Δ831-869) mutant along with GFP expression plasmids using Lipofectamine 2000 (Invitrogen). An aliquot of cells were used for mRNA analyses by qRT-PCR. An aliquot of cells were subjected to protein analyses. For protein analyses, whole cell extracts were prepared using NETN buffer (100 mM NaCl, 1mM EDTA, 10 mM Tris-Cl (pH 8.0), 0.1% Nonidet P-40, 10% glycerol, and 1 mM dithiothreitol). SDS-PAGE sample buffer was added to the lysates,
boiled and separated by 10% SDS-PAGE gel and immunoblotted with the indicated antibodies.

Transient transfections and immunofluorescence. MCF-7 cells were transfected either with appropriate ACTN4 (WT) and/or ACTN4 mutant plasmids using Lipofectamine 2000 (Invitrogen) or with siACTN4 or siCtrl in 12 well culture plates. For siRNA transfection, cells were treated with or without 10 nM of E2 as indicated after 48 h, followed by immunostaining. For plasmid transfection, cells were subjected to immunostaining 24 h post transfection. Transfected cells were fixed in 3.7% paraformaldehyde (PFA) in PBS for 30 minutes 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%) + 0.1% Tween-20 solution (ABB) for 60 min. Incubation with primary antibodies was carried out for 120 min in ABB. The cells were washed three times in PBS, and the secondary antibodies were added for 60 min in the dark, at room temperature in ABB. Cover slips were mounted to slides using Vectashield mounting medium with DAPI (H-1200, Vector Laboratories, Inc.) The primary antibodies used were: purified α-ACTN4 polyclonal, and α-HA and α-ERα mouse monoclonal antibodies (Santa Cruz). The secondary antibodies used are from Molecular Probes (α-mouse or α-rabbit Alexa Fluor 594 or α-mouse Alexa Fluor 488).

Subcellular fractionation. Subcellular fractionation of MCF-7 cells was carried out according to a published protocol [204]. MCF-7 cells were treated with 10 nM of E2 for
indicated periods of time. Nuclear and cytoplasm fractions were resolved on SDS-PAGE gel and immunoblotting was done with the indicated antibodies.

RNA extraction and quantitative real time PCR. Transient transfection of siRNA of ACTN4 was performed as described above. For siRNA transfection of HDAC7, MCF-7 cells were transfected with a non-targeting siRNA siCtrl (D-001810-01-50), siHDAC7-1 (J-009330-07), or siHDAC7-2 (J-009330-08) at a final concentration of 100 nM according to manufacturer’s protocol (Dharmacon). Forty-eight hours post transfection, cells were treated with 10 nM E2 or ethanol as a vehicle control for 24 h prior to harvesting RNA. Total RNA was extracted from MCF-7 cells according to the manufacturer’s protocol (USB). cDNA was synthesized from 1 µg of total RNA according to the manufacturer’s instructions (Invitrogen). Gene expression levels were determined by qRT-PCR using Real Time PCR System (BioRad) with the following primers:

GAPDH: 5’-GAAGGTGAAGGTCGGAGT-3’, 5’-GAAGATGGTGATGGGATTTC-3’;
P R (progesterone receptor): 5’- CCATGTGGCAGATCCCACAGGAGTT-3’, 5’- TGGAAATTTCAACACTCAGTGCC-3’; pS2: 5’-GAGAACAAGGTGATCTGCGCCC-3’, 5’-CCCACGAACGTTGCGAAACA-3’.

Relative changes in gene expression were calculated using the ΔΔCt method. Each value is representative of three replicates and all the experiments were repeated twice.
Chromatin Immunoprecipitation Assays. MCF-7 cells were treated with 100 nM of E2 and ChIP assays were performed according to our published protocol except that α-HDAC7, α-ACTN4, and α-ERα antibodies were used [205].

Cell proliferation assay. MCF-7 cells were transfected with siRNA targeted against non-targeting siRNA (siCtrl), ACTN4 (siACTN4) or HDAC7 (siHDAC7). Forty-eight hours after transfection, cells were counted and 1,500 cells were plated into each well in a 96-cell plate. Cells were treated with either 10 nM E2 or ethanol as a vehicle control. The day 0 time point was taken 5 h after plating. Cell proliferation was measured at days indicated using CyQUANT NF Cell Proliferation Assay kit (Invitrogen).

Statistical analysis. Statistical analysis was performed using two-tailed student’s t-test.

RESULTS

ACTN4 is localized in both nucleus and cytoplasm of MCF-7 cells. ACTN4 is thought to play a role in coordinating cytoskeletal architecture but several reports have suggested additional activities [73, 100]. To explore whether ACTN4 also plays a role in transcriptional regulation, we examined the subcellular distribution of endogenous ACTN4 in MCF-7 breast cancer cells by immunofluorescence microscopy (Figure 8A) and Western blotting following fractionation of cell lysates (Figure 8B). In both assays, we found that ACTN4 was predominantly present in the cytoplasm. However, a significant fraction of the ACTN4 was localized in the nucleus. ACTN4 potentiates nuclear receptor-mediated transcription. Sequence analysis indicated that ACTN4 harbors a putative nuclear receptor interacting motif, LXXLL, found in all four classes of nuclear
receptor coactivators (Figure 9A). This observation raised the possibility that ACTN4 functions as a transcriptional co-activator for nuclear receptors. To test transcriptional response with nuclear receptors, we carried out transient transfection reporter assays using reporter constructs harboring vitamin D3 response element (VDRE) in CV-1 cells. We found that ectopic overexpression of ACTN1, ACTN4 or VDR alone modestly activated VDR-mediated transcription (Figure 9B, lanes 2-4). However, co-expression of ACTN1 or ACTN4 with VDR stimulated hormone (vitamin D3) driven VDR-mediated transcription activity (lanes 5-6). We further investigated whether ACTN4 enhances the transcriptional activity of ERα in transient transcription assays in MCF-7 cells, which express ERα. As shown in Figure 9C, ACTN4 increased the ERE (estrogen response element)-luciferase reporter activity in a hormone (E2) and dosage-dependent manner (lanes 2-4). No significant changes were observed in the reporter activity in the absence of hormone.
Figure 8. Subcellular distribution of ACTN4 in MCF-7 cells. A, MCF-7 cells were grown and immunostained with α-ACTN4 antibodies and the images were taken by Immunofluorescence microscopy. DNA was visualized by 4’, 6-diamidino-2-phenylindole (DAPI) staining. B, Subcellular fractionation of MCF-7 cells was carried out followed by Western blotting with indicated antibodies.
Figure 9. ACTN4 potentiates transcriptional activation by nuclear hormone receptors. A, An alignment of the LXXLL nuclear receptor interacting motif of ACTN4. Consensus nuclear receptor interacting motifs of all four classes of coactivators are shown. B-C, ACTN1 and ACTN4 potentiate reporter activity of VDR in CV-1 cells (B) and ERα in MCF-7 cells (C). Transient transfection assays were conducted as described in “Experimental Procedures”. Each data point represents the mean and standard deviation (S.D.) of the results from triplicate wells. D-E, MCF-7 cells were transiently transfected with control (siCtrl) or with ACTN4-1 siRNA (siACTN4-1). Forty-eight hours after transfection, cells were split for whole cell extract preparation (D), transient transfection reporter assays (E) or proliferation assays. Cells were harvested for whole cell extract preparation 24 h after split. The ACTN4 protein levels were examined by Western blot analyses, quantified and normalized to α-tubulin protein levels. The protein level of siCtrl treated with vehicle is set to 1. E, Knockdown of ACTN4 disrupts E2 driven ERE mediated transcription activity. MCF-7 Cells transfected with siCtrl or siACTN4-1 were subjected to transient transfection reporter assay as described in C. The reporter activity of siCtrl treated with vehicle is set to 1.
Figure 10. Knockdown of ACTN4 disrupts E2 driven ERE-mediated reporter activity. A, The ACTN4 protein levels after ACTN4 knockdown using siACTN4-2 in MCF-7 cells. ACTN4 expression levels were normalized to α-tubulin expression with siCtrl treated with vehicle being set to 1. B, Knockdown of ACTN4 decreased ER-mediated reporter activity. The experiments were performed as described in Figures 9D-E except that siACTN4-2 was used to knockdown ACTN4.
Figure 11. ACTN4 knockdown does not affect subcellular localization of ERα in MCF-7 cells. MCF-7 cells were transfected with siCtrl or siACTN4 followed by treatment with either 10 nM E2 or vehicle for 24 h. Immunostaining was performed using α-ACTN4 and α-ERα antibodies followed by immunofluorescence microscopy. DNA was visualized by 4’, 6-diamidino-2-phenylindole (DAPI) staining.
In order to determine the effects of endogenous ACTN4 in E2-mediated transcriptional activation, we performed knockdown experiments using two separate ACTN4 siRNAs. Knockdown of ACTN4 with siACTN4-1 or siACTN4-2 potently decreased its protein accumulation (Figure 9D & Figure 10A). However, the mRNA level of ACTN1 was only marginally affected (data not shown). For both siRNAs, we found that knockdown of ACTN4 significantly decreased E2-induced ERE-luciferase reporter activity (Figure 9E & Figure 10B). Furthermore, in order to investigate whether ACTN4 has an effect on the subcellular distribution of ERα we carried out immunostaining following ACTN4 knockdown using siACTN4. As shown in Figure 11, we did not observe any significant change in the subcellular localization of ERα when ACTN4 was knocked down, indicating that ACTN4 does not affect the subcellular distribution of ERα. Taken together, our results indicate that ACTN1 and ACTN4 can function as transcriptional co-activators, though the degree of activation varies for different nuclear receptors.

ACTN4 binding to nuclear hormone receptors requires the LXXLL receptor interaction motif. The observation that ACTN4 harbors a LXXLL motif suggested that ACTN4 potentiates transcriptional activity mediated by nuclear receptors through a physical interaction with nuclear receptors. In order to test this hypothesis, we first carried out immunoprecipitation experiments. HEK293 cells were transfected with a VDR expression plasmid and immunoprecipitations were carried out on whole cell lysates using α-VDR antibodies in the presence or absence of vitamin D3 followed by immunoblotting with α-ACTN4 antibodies.
Figure 12. Hormone-dependent association of ACTN4 and nuclear receptors. A, Interaction between ACTN4 and overexpressed VDR. HEK293 cells were transfected with a VDR expression plasmid. The cells were harvested 48 h post transfection. Extracts were immunoprecipitated with α-VDR antibodies in the presence or absence of 100 nM of vitamin D3 followed by Western blotting with α-ACTN4 and α-VDR antibodies. Five percent input is shown. B, Association between endogenous ACTN4 and overexpressed ERα in HEK293 cells. HEK293 cells were transfected with an ERα expression plasmid, whole cell lysates prepared and immunoprecipitated with α-ACTN4 antibodies in the presence or absence of 100 nM E2. The immunoprecipitated pellets were analyzed by immunoblotting with α-ERα and α-ACTN4 antibodies. Ten percent input is shown. C, In vitro interaction between ACTN4 and VDR. Lysates from HEK293 cells overexpressing VDR were incubated with immobilized GST or GST-ACTN4 in the presence or absence of 100 nM of vitamin D3. Pulldown fractions were subjected to Western blotting with α-VDR antibodies (top panel). Comassie Blue staining is shown to demonstrate equal loading for GST-beads in each lane (bottom panel). The arrow shows the full-length GST-ACTN4 fusion protein. Lane 1 shows 5% of the input for pulldown assays. D, In vitro interaction of ACTN4 and ERα. Whole cell extracts overexpressing ERα were used in this assay. GST pulldown assays were carried out in the presence or absence of 100 nM of E2. Pull-down fractions were subjected to Western blotting with α-ERα antibodies. Five percent input is shown in lane 1.
As shown in Figure 12A, ACTN4 interacted with VDR in a hormone-dependent manner (lanes 2-3). In order to analyze the interaction between ACTN4 and ER\(\alpha\), whole cell extracts prepared from HEK293 cells overexpressing ER\(\alpha\) were immunoprecipitated with \(\alpha\)-ACTN4 antibodies and immunoblotted with \(\alpha\)-ACTN4 and \(\alpha\)-ER\(\alpha\) antibodies. We found that E2 enhanced the association between ACTN4 and ER\(\alpha\) (Figure 12B, lanes 2-3, bottom panel). Furthermore, we verified the interaction between nuclear receptors and ACTN4 in vitro by GST pulldown assays. Bacterially purified immobilized GST-ACTN4 (WT) was incubated with whole cell lysates expressing VDR or ER\(\alpha\) in the presence or absence of their respective ligands. As a control, little or no binding to GST alone was observed with either of the nuclear receptors while GST-ACTN4 interacted with these receptors in a ligand-dependent manner (Figures 12C-D, lanes 2-4). From these data we concluded that hormone enhances the association of ACTN4 with VDR and ER\(\alpha\) in mammalian cells and in vitro. In order to further investigate whether the LXXLL motif of ACTN4 is required for its hormone dependent interaction with nuclear receptors, we generated an ACTN4 (LXXAA) mutant where Leu-87 and Leu-88 were substituted by Ala and carried out GST pulldown assays. Figures 13A-B (lanes 2-3) show that the ACTN4 (LXXAA) mutant displayed reduced interactions with nuclear receptors, even in the presence of hormone. We next investigated whether disruption of the LXXLL motif affects ACTN4-mediated transcriptional activation in reporter assays using wild-type ACTN4 or the ACTN4 (LXXAA) mutant in CV-1 or MCF-7 cells.
Figure 13: LXXLL motif is essential for the ACTN4 to potentiate nuclear receptor mediated transcriptional activity. A, ACTN4 (LXXAA) mutant loses its ability to interact with VDR. HEK293 cells were transfected with a VDR expression plasmid. Forty-eight hours post-transfection, the lysates were prepared and incubated with bacterially expressed GST-ACTN4 (WT) or with GST-ACTN4 (LXXAA) fusion proteins in the presence 100 nM of vitamin D3. Pulldown fractions were subjected to Western blotting with α-VDR-antibodies. The arrow indicates the full-length GST-ACTN4 fusion protein. Lane 1 shows 10% of the input for pulldown experiments. B, ACTN4 (LXXAA) mutant loses its ability to interact with ERα. GST pulldown assays were carried out as described in Figure 13A, except ERα expression plasmids were used for transfection and pulldowns were carried out in the presence of 100 nM of E2. Lane 1 shows 5% input. C, The LXXLL motif is essential for VDR-mediated transcriptional activation. Expression plasmids for ACTN4 (WT) or the ACTN4 (LXXAA) mutant were co-transfected with or without expression plasmids for VDR along with a reporter construct harboring a VDRE in CV-1 cells. Reporter assays were carried out as described in Figure 9B. D, ACTN4 (LXXAA) loses its ability to potentiate ERα activity. The experiment was performed as described in Figures 9C. Each data point represents the mean and standard deviation (S.D.) of results from triplicates.
Figure 14: Subcellular fractionation of wild type and mutant (LXXAA) ACTN4 in MCF-7 cells. MCF-7 cells were transfected with expression plasmids for HA-ACTN4 (WT) and HA-ACTN4 (LXXAA) mutant. A, Immunostaining was performed using α-HA antibodies followed by immunofluorescence microscopy. DNA was visualized by 4’, 6-diamidino-2-phenylindole (DAPI) staining. B, Cell fractionation was done as described in “Experimental Procedures” followed by Western blotting with the indicated antibodies.
As demonstrated in Figure 13C-D, wild-type ACTN4 potentiated VDR- and ERα-mediated transcriptional activity in a hormone-dependent manner (lanes 5 and 2-4), whereas the ACTN4 (LXXAA) mutant showed a significant decrease in the ability to potentiate hormone-dependent transcriptional activity mediated by VDR or ERα (lanes 6 and 5-7). We also addressed whether the reduced activity of the ACTN4 (LXXAA) mutant was due to its mis-localization by carrying out immunofluorescence microscopy in MCF-7 cells. As shown in Figure 14, the ACTN4 (LXXAA) mutant exhibited a similar subcellular distribution pattern to that of the wild-type protein. Taken together, we have identified that LXXAA motif in ACTN4 is required for its interaction with and transcriptional activity by VDR and ERα. In order to further dissect the role of ACTN4 in ERα-mediated transcriptional activation, we examined whether ACTN4 associates with the ERα target gene promoter by chromatin immunoprecipitation (ChIP) assays in MCF-7 cells. Because we have previously shown that ACTN4 interacts with HDAC7, we also examined the recruitment of HDAC7 to the promoter of the pS2 gene, a well-studied ERα target gene. As shown in the Figure 15A, ERα was recruited to the pS2 promoter at all the time points (lane 2-5), while ACTN4 and HDAC7 were recruited in distinct patterns (lane 2-5). A faint signal for the former was detected at 30’ and a strong signal at 95’. For HDAC7, a weak signal was visible at 75’ and a strong signal at 140’. Moreover, when ACTN4 was most abundant at the pS2 promoter, HDAC7 was absent and vice versa (lane 4 vs. lane 5). This observation raised the possibility that HDAC7 may also regulate ERα-mediated transcriptional activation. To test this possibility, we performed transient transfection reporter assays to determine the effects of overexpressing HDAC7 on ERE-
mediated reporter activity. Figure 15B shows that HDAC7 is capable of repressing E2-induced ERE reporter activity in a dose- and hormone-dependent manner (lane 1-3).

We have previously mapped an HDAC7 interacting domain in ACTN4 to 38 residues between amino acids 831-869 [73]. This region also corresponds to the calmodulin (CaM)-like domain of ACTN4, which is highly conserved in the other family members (Figure 15C). Unexpectedly, deletion of amino acids 831-869 (Δ831-869) resulted in a greater nuclear distribution (Figure 16) and loss of the ability to potentiate ERE reporter activity (Figure 15D). In summary, these data indicate that ACTN4 associates with an endogenous ERα target, the pS2 promoter and is capable of stimulating transcription of an ERE reporter construct in the presence of hormone in MCF-7 cells.

Knockdown of ACTN4 decreased the expression of several endogenous ERα target genes. In order to determine the biological function of ACTN4 in the transcriptional regulation of estrogen responsive genes, we knocked down ACTN4 by two separate siRNAs in MCF-7 cells and determined the effects on the expression of selected estrogen responsive genes. Transfection of siACTN4 into MCF-7 cells reduced ACTN4 by approximately 40% to 60% at both the mRNA (data not shown) and protein levels (Figure 17A), respectively. Knockdown of ACTN4 by siACTN4-1 did not affect pS2 mRNA accumulation in the absence of E2, while significantly inhibited E2-induced increases in pS2 transcript (Figure 17B). Knockdown of ACTN4 by siACTN4-2 led to decreases in pS2 mRNA in the presence or absence of E2 (Figure 18A).
Figure 15. ACTN4 associates with the pS2 promoter. A, Association of ERα, ACTN4 and HDAC7 with the pS2 promoter. Chromatin prepared from MCF-7 cells treated with 10 nM E2 for the indicated time periods was subjected to chromatin immunoprecipitation assays as detailed in “Experimental Procedures”, with antibodies against ERα, HDAC7 and ACTN4. B, The effects of overexpressing HDAC7 on ERE reporter activity. MCF-7 cells were transfected with reporter constructs harboring an ERE and β-gal along with increasing amounts of an HDAC7 expression plasmid. Luciferase reporter assays were performed as described in Figure 9C. C, An alignment between the ACTN4 CaM-like domain and parts of calmodulin (CaM2). The CaM-like domains of all four ACTNs are similar to each other. D, ACTN4 (∆831-869) loses its ability to potentiate ERE reporter activity. The reporter assays were (panels B & D) were performed as described in Figure 9C.
Figure 16. Subcellular localization of wild type and mutant (Δ831-869) ACTN4 in MCF-7 cells. Wild-type or mutant ACTN4 expression plasmids were transiently transfected into MCF-7 cells followed by immunostaining using α-HA antibodies. DNA was visualized by 4', 6-diamidino-2-phenylindole (DAPI) staining.
Figure 17. Knockdown of ACTN4 reduces expression of endogenous ERα regulated genes. 

A, Knockdown of ACTN4 in MCF-7 cells. Western blot analysis of ACTN4 protein levels in MCF-7 cells following transfection of control siRNA (siCtrl) or siRNAs targeting ACTN4 (siACTN4-1) after treatment with or without 10 nM E2 for 24 h. Quantification of ACTN4 level after normalization to α-tubulin is shown below the blot with siCtrl level treated with vehicle being set to 1. B-C, mRNA expression levels of pS2 (B) and PR (C) in MCF-7 cells following ACTN4 knockdown by siACTN4-1. RNA was isolated as described in “Experimental Procedures” and relative expression levels of pS2 and PR were analyzed by qRT-PCR. The mRNA levels of pS2 and PR were normalized to GAPDH mRNA expression levels. NS represents no significant change compared to control siRNA. 

D, MCF-7 cells were transfected with an expression plasmid for HA-ACTN4 (WT), HA-ACTN4 (LXXAA) or HA-ACTN4 (Δ831-869) along with a GFP expression plasmid. An aliquot of the samples was used to prepare total cell lysates for Western blotting (D) or total RNA for real-time PCR (E-F). E, mRNA expression level of pS2 in ACTN4 overexpressing cells. F, mRNA expression of PR in ACTN4 overexpressing cells. The mRNA level of pS2 and PR in MCF-7 cells overexpressing vector alone treated with vehicle is set to 1.
Similarly, transient transfection of siACTN4-1 or siACTN4-2 decreased PR mRNA in the absence and presence of E2 (Figures 17C and 18B). We also determined whether overexpression of ACTN4 had an effect on pS2 and PR mRNA accumulation. We overexpressed ACTN4 wild-type, LXXAA, and Δ831-869 in MCF-7 cells. A GFP expression plasmid was co-transfected for normalization purposes. The expression levels of wild-type and mutant ACTN4s were monitored by Western blotting (Figure 17D). Real-time PCR was carried out to determine the expression levels of pS2 and PR mRNA. We found that wild-type increased pS2 mRNA levels two fold in a hormone-dependent manner, while overexpression of mutants LXXAA and Δ831-869 had little effect (Figure 17E). Overexpression of wild-type or mutant ACTN4s resulted in a similar effect on PR mRNA expression (Figure 17F). Taken together, these data demonstrate that ACTN4 positively regulates expression of ERα target genes, pS2 and PR.

siRNA targeting of HDAC7 enhances the expression of selected endogenous ERα target genes. Our previous data suggested that HDAC7 antagonizes ACTN4 function (Figure 15). In order to investigate the role of HDAC7 in ERα-mediated transcription, we tested whether knockdown of HDAC7 has effects on the expression of ERα target genes. HDAC7 was knocked down by siHDAC7-1 and siHDAC7-2 by 80% and 40%, respectively (Figure 19A). Knockdown of HDAC7 by either siRNA led to a significant increase in pS2 mRNA expression in the absence or presence of E2 (Figures 19B and 20A). Similarly, PR mRNA levels were up-regulated when HDAC7 was knocked down with or without E2 (Figures 19C and 20B). These observations demonstrate that in
contrast to ACTN4, HDAC7 negatively regulates the expression of pS2 and PR and this regulation is independent of the presence of E2. The fact that ACTN4 and HDAC7 regulate transcriptional activation by ERα suggests that ACTN4 and HDAC7 may play a role in E2-mediated regulation of cell proliferation. We found that transient knockdown of ACTN4 significantly decreased MCF-7 cell proliferation in an E2-independent manner (Figure 21A & 22A). By contrast, knockdown of HDAC7 increased MCF-7 cell proliferation regardless of the presence of E2 (Figure 21B & 22B). In conclusion, these data indicate that ACTN4 and HDAC7 play important roles in multiple cellular activities including gene expression and cell proliferation.
Figure 18. Knockdown of ACTN4 reduces expression of endogenous ERα regulated genes. A-B, mRNA expression level of estrogen responsive genes in MCF-7 cells following ACTN4 knockdown by siACTN4-2. RNA was isolated as described in “Experimental Procedures” and relative expression levels of pS2 and PR were determined by qRT-PCR. The mRNA levels of pS2 and PR were normalized to mRNA level of GAPDH with siCtrl treated with vehicle being set to 1.
Figure 19. HDAC7 knockdown enhances the expression of endogenous ERα regulated genes. A, Knockdown of HDAC7 in MCF-7 cells. Western blot analysis of HDAC7 protein levels in MCF-7 cells following transfection of control siRNA (siCtrl) or siRNA targeting HDAC7 (siHDAC7) after treatment with or without 10 nM E2 for 24 h. HDAC7 protein levels are normalized to α-tubulin and are shown below the figure with the siCtrl treated with vehicle being set to 1. B-C, mRNA expression level of estrogen responsive genes pS2 and PR in MCF-7 cells following HDAC7-1 knockdown. RNA was isolated as described in “Experimental Procedures” and relative expression levels of pS2 and PR mRNA levels were determined by qRT-PCR. The mRNA levels of ps2 and PR were normalized to GAPDH mRNA expression levels.
Figure 20. Knockdown of HDAC7 increases expression of endogenous ERα regulated genes. A-B, mRNA expression level of estrogen responsive genes in MCF-7 cells following HDAC7 knockdown by siHDAC7-2. RNA was isolated as described in “Experimental Procedures”. Relative expression levels of pS2 and PR were analyzed by qRT-PCR. The mRNA levels of pS2 and PR were normalized to mRNA level of GAPDH with siCtrl treated with vehicle being set to 1.
Figure 21. The effects of ACTN4 or HDAC7 knockdown on MCF-7 cell proliferation. A, Knockdown of ACTN4 decreases MCF-7 cell proliferation in E2 independent manner. MCF-7 cells transfected with siRNA as described in Figure 9D-E were counted, split evenly into 96 well plates and treated with or without 10 nM E2 for the indicated times. Cell proliferation was measured at the days indicated. Error bars represent ± SD. The knockdown efficiency of ACTN4 is shown in Figure 9D. B, Cell proliferation assays were conducted as described in Figure 22A except that an HDAC7 siRNA-1 was used. The knockdown efficiency of HDAC7 is shown in Figure 19A.
Figure 22. The effects of ACTN4 or HDAC7 knockdown on MCF-7 cell proliferation. A, The experiment was carried out as described in Figure 21A. The effect of a second ACTN4 siRNA (siACTN4-2) on MCF-7 cell proliferation is shown. Fewer number of MCF-7 cells were used as compared to that in Figure 21A. B, Left, Western blotting of HDAC7 protein levels. A control siRNA (siCtrl) or siRNA against HDAC7 (siHDAC7-2) was transiently transfected into MCF-7 cells. An aliquot of cells were used for whole cell extract preparation and an aliquot of cells were used for proliferation assays. Right, The effect of a second HDAC7 siRNA on MCF-7 cell proliferation is shown. Proliferation assays were carried out as described in Figure 21B.
DISCUSSION

In this study, we demonstrate that ACTN4 potentiates transcriptional activity mediated by VDR and ERα, while knockdown of ACTN4 decreased E2-induced reporter activity and the expression of endogenous ERα target genes, pS2 and PR. ChIP assays established a physical association between ACTN4 and the pS2 promoter and that E2 induced this interaction. Finally, we showed that an ACTN4 mutant defective in interacting with HDAC7 loses its ability to potentiate the activity of an ERE-driven reporter. These observations support a model in which ACTN4 plays an important role in transcriptional regulation, in addition to its well-established function in maintaining cytoskeletal architecture.

By immunofluorescence microscopy, we show that ACTN4 is localized in both the nucleus and cytoplasm in MCF-7 cells. This result is consistent with a recent observation that ACTN4 localizes to the nucleus during G1 and G2 phases in HeLa cells [102], implying a nuclear role of ACTN4. The ability of ACTN4 to shuttle between the nucleus and cytoplasm depends on a functional nuclear export sequence (NES), CRM1, and its spectrin repeats (SRs). We also tested the possibility that E2 potentiates nuclear import of ACTN4 in MCF-7 cells by immunofluorescence microscopy and Western blotting analyses and found that E2 had little effect on subcellular distribution of endogenous ACTN4 (Figure 23). Interestingly, the ACTN4 mutant, Δ831-869, exhibited increased nuclear staining compared to the wild-type protein (Figure 16). It should be noted that this region of ACTN4 contains a CaM-like domain.
Figure 23. The effect of E2 on subcellular localization of ACTN4 in MCF-7 cells. A, MCF-7 cells were treated with or without 10 nM of E2 for 24 h followed by immunostaining and immunofluorescence microscopy for ACTN4 and ERα using α-ACTN4 and α-ERα antibodies. DNA was visualized by 4’, 6-diamidino-2-phenylindole (DAPI) staining. B, The subcellular distribution of ACTN4 in cytoplasm and nuclear fractions after E2 treatment. MCF-7 cells were treated with E2 and harvested at the indicated periods of time followed by fractionation using the protocol described in “Experimental Procedures”. Fractionated cytoplasmic and nuclear fractions were resolved on the SDS PAGE and immunoblotted with α-ACTN4, α-HDAC1 and α-GAPDH antibodies.
These data suggest that the CaM-like domain negatively regulates nuclear import or positively regulates nuclear export of ACTN4. Further investigation will be needed to elucidate how extracellular signaling may regulate nucleocytoplasmic shuttling of ACTN4.

Actin-binding proteins other than ACTN4 have been shown to play roles in other cellular processes including transcriptional regulation, in addition to their ability to maintain cytoskeletal architect or cell morphology [206-208]. The simplest explanation for what ACTN4 is doing in the nucleus is binding nuclear actin. This may globally affect nuclear structure, architecture or volume. Our data indicate that E2 does not alter ACTN4 shuttling, thus the E2-dependent effects are not due to global nucleocytoskeletal changes. We also show that ACTN4 physically associates with VDR and ERα in a hormone- and NR box-dependent manner (Figures 12-13). Furthermore, we demonstrate that ACTN4 is recruited to the pS2 promoter (Figure 15A). These observations argue for a more direct role of ACTN4 in E2-mediated transcriptional regulation. Indeed, several studies have indicated that actin and actin-related proteins can associate with chromatin remodeling complexes, suggesting a role for these proteins in transcriptional regulation [60, 63, 208].

ACTN4 harbors a conserved CaM-like domain (Figure 15C) that is essential for its interaction with HDAC7 [73]. Notably, removal of this domain significantly abolishes the ability of ACTN4 to potentiate E2-mediated transcription (Figures 15D, 17E-F). As described earlier, the lack of activation activity is not due to exclusion of this mutant from the nucleus (Figure 16). Intriguingly, our ChIP data suggest that maximal
associations of ACTN4 and HDAC7 with the pS2 promoter are mutually exclusive (Figure 15A), suggesting that the interaction between ACTN4 and HDAC7 on the pS2 promoter is likely to be transient and regulated. Based on these data, we propose a model in which ACTN4 activates E2-induced transcription, in part, through its association with HDAC7. The interaction leads to the displacement of one of the partners from the promoter.

Several lines of evidence suggest that ACTN4 has a broader role as a transcriptional regulator than originally anticipated. We have previously shown that ACTN4 potentiates MEF2-mediated transcriptional activation. In this study, we further demonstrate that knockdown of ACTN4 decreased E2-induced activity of an ERE-driven reporter, while overexpression of ACTN4 increased it, implying a positive role of ACTN4 in E2-dependent transcriptional activation. However, knockdown of ACTN4 also resulted in decreases in mRNA levels of pS2 and PR, regardless of the presence of E2, implying that ACTN4 is also required for ERα-independent transcriptional activation of pS2 and PR. One plausible explanation for these observations is that ACTN4 is required for induction of pS2 and PR mRNA by other transcription factors. Lastly, it has been previously shown that ACTN4 physically interacts with NF-κB, though the functional significance of this association is not clear. In light of our findings, we speculate that ACTN4 may regulate NF-κB transcriptional activity.

Our data showing that knockdown of ACTN4 impaired transcriptional activation by ERα both in transient transfection studies as well as endogenous expression of E2-induced target genes suggest that ACTN4 is required for optimal ERα-mediated transcriptional
activation. ACTN4 does not possess any known histone modifying or chromatin remodeling activity. One possible mechanism by which ACTN4 could potentiate transcription is through an association with histone acetyltransferases, histone methyltransferases or chromatin remodeling complexes. A previous report suggested that GRIP1 interacts with ACTN2, a member of the ACTN protein family [209]. However, we were unable to detect a similar association between ACTN4 and GRIP1 under conditions in which ACTN4 interacts with VDR by GST pulldown assays (Figure 24). Of note, we found that ACTN4 associates with the arginine methyltransferase CARM1 and that an ACTN4 splice variant interacts with several transcriptional coactivators (Khurana et al., manuscript in preparation). Moreover, it has been recently shown that ACTN4 associates with the INO80 chromatin-remodeling complex. Together, these data suggest that ACTN4 might regulate transcription through multiple mechanisms including an association with histone modifying enzymes and chromatin remodeling complexes.

Gene amplification and overexpression of the transcription coactivator SRC family proteins are associated with oncogenesis [33]. In this report, we present evidence that ACTN4 enhances MCF-7 cell proliferation because knockdown of ACTN4 decreases MCF-7 cell proliferation (Figure 21A-22A). This growth suppressive effect is independent of the presence of ERα ligands and suggests that ACTN4 may promote MCF-7 cell cycle progression independently of its role in potentiating expression of ERα target genes. We speculate that ACTN4 mediates transcriptional activation of other transcription factors such as NF-κB to control cell proliferation. Alternatively, but not
exclusively, ACTN4 cytoskeletal functions may underlie its effect on proliferation.

Further investigation will be necessary to distinguish these two possibilities.
Figure 24: GRIP-1 does not interact with ACTN4 in pulldown assays. HEK293 cells transfected with either VDR or HA-GRIP1 expression plasmids. Forty-eight hours post-transfection, whole cell lysates were prepared and incubated with bacterially expressed GST-ACTN4 (WT). GST-pulldown assays were carried out in the presence or absence of vitamin D3 as indicated in the figure. Pulldown fractions were subjected to Western blotting with α-VDR or α-HA antibodies. The arrow indicates the full-length GST-ACTN4 fusion protein.
CHAPTER 3: THE LXXLL MOTIF OF ALPHA ACTININ 4 (ACTN4) IS CRITICAL FOR INTERACTION WITH ESTROGEN RECEPTOR ALPHA AND COACTIVATORS

ABSTRACT

Alpha actinins (ACTNs) are a family of proteins crosslinking actin filaments to maintain cytoskeletal organization and cell motility. We have previously isolated a splice variant of ACTN4, ACTN4 (Iso) and shown that it can potentiate transcriptional activation by myocyte enhancer factor 2 (MEF2). Both ACTN4 and its isoform ACTN4 (Iso) possess a functional, yet distinct, LXXLL nuclear receptor interacting motif with unique C-terminal flanking sequence to the LXXLL. When brought to the promoter, both ACTN4 and ACTN4 (Iso) potentiate basal transcription activity, suggesting that ACTN4 may interact with coactivators. Furthermore, we found that while ACTN4 (Iso) interacts efficiently with transcriptional co-activators such as p160 protein co-activators and PCAF, while the full-length ACTN4 protein does not or interact weakly with these coactivators. Domain mapping studies show that the intrinsic activation activity correlates with its ability to interact with coactivators. More importantly, the flanking sequences of the LXXLL motif are also important not only for interacting with the nuclear receptors but also for its association with the coactivators. We further mapped ACTN4 interaction domain to the histone acetylase domain (HAT) of PCAF. Taken together, our data indicate that ACTN4 regulates the transcriptional activity by interacting with corepressor such as HDAC7 and coactivators. Our data also show for the first time that the LXXLL motif and its flanking sequences are critical for interacting with coactivators.
INTRODUCTION

The nuclear hormone receptors (NRs) are a family of ligand-activated transcriptional factors that include receptors for thyroid hormones (TR), vitamin D (VDR), retinoids (RAR and RXR) and steroid hormones [1, 210]. Nuclear hormone receptors regulate transcription of a network of genes associated with many aspects associated of organ homeostasis, cell differentiation, and development processes.

The mechanisms underlying transcriptional regulation by NRs are thought to occur through their association with co-repressors or co-activators. The unliganded receptors adopt a conformation that favors their association with co-repressors. Upon binding to ligands, receptors undergo an allosteric change, leading to dissociation of co-repressor complexes and concomitant recruitment of co-activator proteins. The transcriptional co-activators are responsible for marking histones and remodeling the chromatin, leading to the recruitment of RNA polymerase II to initiate transcription [211, 212]. The coactivator proteins often function as component of large complex that collaborate to potentiate transcriptional activation. There are three main classes of coactivators associated with NRs, the p160 family, cAMP-responsive element-binding protein (CBP)/p300 and p300/CBP associated factor (PCAF). The well-characterized p160 family proteins include steroid receptor coactivator 1(SRC1), glucocorticoid receptor interacting protein1 (GRIP1) and the activator of thyroid and retinoic acid receptor (ACTR) [213]. The functional importance of p160 family lies in the fact that mice lacking p160 genes are defective in nuclear receptor-mediated developmental processes [214-216]. Several studies indicated that the hormone-induced interaction between NRs and coactivators is
mediated through a single or multiple copies of conserved motif LXXLL (where L is leucine, X can be any amino acid) [21] termed the nuclear receptor interaction box or nuclear receptor signature motif [23, 217-221]. NR boxes and surrounding residues have been grouped into four classes depending on the amino acid residue present at the -1 and -2 positions upstream of the LXXLL motif. The first three classes were identified based on the phage display approach and the fourth class was identified following analysis of naturally occurring motifs among coactivators [203].

The alpha actinins (ACTNs) are actin-binding proteins that are important for the maintenance of cytoskeletal structure and cell morphology [200]. Four ACTNs have been identified and among them, ACTN2 and ACTN3 are expressed exclusively in muscle while ACTN1 and ACTN4 are widely expressed [105]. All ACTNs harbor several conserved functional domains including an N-terminal actin-binding domain that contains two highly conserved calponin homology (CH1 and CH2), a central domain consisting of four spectrin repeats (SR), two EF hand calcium-binding domains and a C-terminal calmodulin (CaM)-like domain [201]. Notably, although originally identified as an actin-binding protein, ACTN4 is localized in both the cytoplasm and the nucleus [73, 96]. Indeed, we have previously shown that ACTN4 interacts with and potentiates MEF2 transcription factors as well as estrogen receptor alpha (ERα) and VDR [222]. The ability of ACTN4 to interact with and potentiate NR activity depends on an intact LXXLL motif present in ACTN4. Functionally, we demonstrated that knockdown of ACTN4 significantly decreased the ability of estrogen to induce the expression of ERα target
genes. Together, these observations indicate that ACTN4 is an integral component in ERα mediated transcriptional activation.

In this study, we dissect the mechanisms underlying the ability of ACTN4 and an alternatively splices product called ACTN4 (Iso) to potentiate transcriptional activation. We found that unlike full-length ACTN4, ACTN4 (Iso) is capable of interacting with selective coactivators such as the p160 family members and PCAF. Unexpectedly the sequence immediately to C-terminal to LXXLL motif of ACTN4 (Iso) is important for both its association with ERα and coactivators. Together, our data define a novel function for the LXXLL motif and its C-terminal flanking sequence in ACTN4.

EXPERIMENTAL PROCEDURES

Plasmid construction: CMX-HA-ACTN4 and its spliced isoform equivalent have been previously described [73]. Point mutations in the ACTN4 (Iso) were generated using ACTN4 (Iso) cDNA as a template by site directed PCR mutagenesis according to the manufacture’s protocol (Stratagene). For glutathione S-transferase (GST) constructs, ACTN4 (Iso) cDNA was PCR amplified and subcloned in the pGEX4T vector using standard cloning techniques. Expression plasmids for nuclear receptors and reporter constructs were generous gifts from Dr. Ron Evans (The Salk Institute, La Jolla, CA). Expression plasmids of FLAG-PCAF, HA-SRC-1, HA-GRIP1, and FLAG-ACTR were generated by PCR reactions and confirmed by sequencing.

Antibodies and chemicals: Anti-ACTN4 antibody has been previously described [73, 222]. Anti-HA conjugated α-horseradish peroxidase was purchased from Roche Applied
α-VDR (C-20) and α-ERα (D-12) antibodies were purchased from Santa Cruz Biotechnology. 1-(24R)-24, 25-Dihydroxy-vitamin D3 (705861) and β-estradiol (E2, E8875) were purchased from Sigma-Aldrich.

Transient transfection reporter assays: For reporter assays, CV-1 or MCF-7 cells in 48 well plates were co-transfected with equal amounts of either 100 ng of VDRE-TK-Luc or ERE-TK-Luc with or without pCMX-ACTN4 (full-length) or pCMX-ACTN4 (Iso) along with 100 ng pCMX-β-gal in 200 μl Opti-MEM I using Lipofectamine 2000 (Life Technologies). The amount of DNA was kept constant by the addition of pCMX vector. After 5 h, the medium was replaced with DMEM supplemented with 10% charcoal stripped fetal bovine serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate. After 24 h, medium was replaced with or without hormones as indicated. In all experiments, cells were harvested 48 h after transfection, and luciferase and β-galactosidase (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.

In vitro protein-protein interaction assays: Glutathione S-transferase fusion proteins GST-ACTN4 (Iso) was expressed in E.coli DH5α strain, affinity purified and immobilized on glutathione Sepharose 4B beads. In vitro pull-down assays were carried out in which purified immobilized GST-ACTN4 (Iso) or GST-ACTN4 (Iso, LXXAA) or GST-ACTN4 (Iso, M1/M2/M3) were incubated with whole cell extracts expressing nuclear receptors in the presence or absence of vitamin D3 (1uM), or E2 (1uM) for 1 h at 4°C. For coactivator binding, immobilized, purified GST-ACTN4 (Iso), GST-ACTN4 (Iso, 1-102) and GST-
ACTN4 (95-521) fusion proteins were incubated with whole cell lysates expressing coactivators. After extensive washing with NETN buffer (100 mm NaCl, 1 mM EDTA, 10 mm Tris-Cl, 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 SDS-PAGE. Western blots were probed either with anti-HA-conjugated anti-horseradish peroxidase antibody (Roche Applied Science) or antibodies against nuclear receptors (Santa Cruz Biotech).

Coimmunoprecipitations: To detect the interaction between ACTN4 (full-length) or ACTN4 (Iso) and coactivators, HEK293 cells were grown on 10 cm plates and transfected with 10 µg of total DNA with Lipofectamine 2000 (Life Technologies). After 48 h, the cells were washed with 1X PBS. Whole cell lysates were prepared in NETN buffer along with protease inhibitors. For interaction with ACTN4 (Iso) and PCAF, immunoprecipitations were carried out with anti-FLAG antibody M2 affinity gel (Sigma) for 4 h at 4°C followed by Western blotting with α-HA-conjugated anti-horseradish peroxidase antibody (Roche Applied Science). To detect the interaction between SRC1 and ACTN4 (Iso), whole cell lysates expressing HA-SRC1 and FLAG-ACTN4 (Iso) were incubated with anti-HA affinity gel (Sigma) for 4 h at 4°C followed by Western blotting with α-FLAG antibodies.

Transient transfections and immunofluorescence: MCF-7 cells were transfected either with wild-type or mutant ACTN4 (Iso) expression plasmids using Lipofectamine 2000 (Life Technologies) followed by immunostaining with the indicated antibodies. Transfected cells were fixed in 3.7% paraformaldehyde (PFA) in PBS for 30 minutes 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%) + 0.1% Tween-20 solution (ABB) for 60 min. Incubation with primary antibodies was carried out for 120 min in ABB. The cells were washed three times in PBS, and the secondary antibodies were added for 60 min in the dark, at room temperature in ABB. Cover slips were mounted to slides using Vectashield mounting medium with DAPI (H-1200, Vector Laboratories, Inc.) The primary antibody used was: purified α-HA mouse monoclonal antibodies (Santa Cruz). The secondary antibodies used were from Molecular Probes (α-mouse Alexa Fluor 594).

Transient transfections and qRT-PCR: MCF-7 cells were transfected with HA vector, HA-ACTN4 (Iso, WT) or HA-ACTN4 (Iso, LXXAA) along with GFP expression plasmids using Lipofectamine 2000 (Life Technologies). An aliquot of cells were used for mRNA analyses by qRT-PCR. A second aliquot of cells was subjected for Western blotting. For protein analyses, whole cell extracts were prepared using radioimmune precipitation assay (RIPA) buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) along with protease inhibitors. SDS-PAGE sample buffer was added to the lysates, boiled, and separated by 10% SDS-PAGE gel and immunoblotted with α-HA and α-GFP antibodies. For total RNA isolation, forty-eight hours post transfection, cells were treated with 10 nM E2 or ethanol as a vehicle control for 24 h, prior to harvesting RNA. Total RNA was extracted from MCF-7 cells according to the manufacturer’s protocol (USB). cDNA was synthesized from 1 µg of total RNA according to the manufacturer’s instructions (Life Technologies). Gene expression levels
were determined by qRT-PCR using Real Time PCR System (Bio Rad) with the following primers:

GAPDH: 5’-GAAGGTGAAGGTCGGAGT-3’, 5’-GAAGATGGTGATGGGATTTC-3’;
c-Myc: 5’-ATGAAAAGGCCCCCAAGGTAGTTAT-3’), 5’-GCA-TTTGATCATGCATTGAAACAA-3’; pS2: 5’-GAGAACAAGGTGATCTGCGCCC-3’, 5’-CCCACGAACGCTGCGAAACA-3’.

Relative changes in gene expression were calculated using the ΔΔCt method. Each value is representative of three replicates and all the experiments were repeated twice. An aliquot of cells were subjected to protein analyses.

Statistical analysis: Statistical analysis was performed using two-tailed student’s t-test.

RESULTS

ACTN4 (Iso) potentiates NR-mediated transcriptional activation. We have previous identified an alternatively spliced ACTN4 product, ACTN4 isoform, ACTN4 (Iso), and shown that both the full-length and the isoform accumulate in cells and are capable of potentiating MEF2 transcription activity [73]. We have recently shown that ACTN4 (full-length) is also capable of potentiating transcriptional activation by VDR and ERα and that knockdown of ACTN4 significantly compromised the ability of estrogen to induce ERα target genes [222]. In addition to missing part of SR2, SR3 and SR4 due to exon exclusion (Figure 5), ACTN4 (Iso) harbors a putative LXXLL nuclear receptor interaction motif with distinct amino acid sequences C-terminal to the LXXLL motif when compared to ACTN4 (full-length) (Figure 25A).
Figure 25. ACTN4 (Iso) potentiates transcriptional activation by nuclear hormone receptors. A, An alignment of the nuclear receptor interactin motif of ACTN4 (full-length) and ACTN4 (Iso). The LXXLL motifs are highlighted. Note that the sequences C-terminal to LXXLL are different between ACTN4 (full-length) and its isoform due to alternative splicing at this site. The LXXLL motif is also aligned with the four known classes of co-activators [24]. B, ACTN4 (Iso) activates VDR reporter activity. Transient transfection assays were carried out according to our published protocol [222]. CV-1 cells were transfected with reporter construct harboring VDRE with or without ACTN4 (Iso) along with β-gal. Forty eight hours after transfection cell were treated with or without 100nM of vitamin D3. Luciferase activities were measured and normalized to β-gal activities. C, GST-ACTN4 interacts with VDR in GST pull-down assays. HEK293 cells were transfected with a plasmid expressing VDR and forty eight hours post transfection cells were lysed and whole cell lysates were prepared and incubated with either GSTA1 alone or with GST-(Iso) in the presence or absence of 1uM vitamin D3. Pull-down fractions were subjected to SDS-PAGE followed by Western blotting with anti-VDR antibodies (top panel). Bottom panel shows coomassie staining of the GST fusion proteins D, ACTN4 (Iso, LXXAA) loses the ability to potentiate transcriptional activity mediated by VDR. Transient transfection assays were carried out as described in Figure 1B except that both ACTN4 (Iso, WT) and ACTN4 (Iso, LXXAA) were used for the transfection. Each data point represents the mean and standard deviation (S.D.) of results from triplicates.
To test whether ACTN4 (Iso) modulates transcriptional activation by NRs, we carried out transient transfection reporter assays in CV1 cells using a reporter construct harboring a vitamin D3 response element (VDRE). Figure 25B shows that in the absence of the VDR expression plasmid, ACTN4 (Iso) weakly activated VDRE reporter activity, regardless of vitamin D3 status. In the presence of VDR, ACTN4 was capable of activating VDRE reporter activity in a VDR dose-dependent manner. Most NR co-activators such as CBP/p300, PCAF, and the p160 family proteins utilize its LXXLL receptor interacting motifs to mediate their association with NRs. The motif is required for transcriptional activation. To test whether ACTN4 (Iso) potentiates NR-mediated transcriptional activation by interacting with NRs through LXXLL motif, we generated mutant ACTN4 (Iso, LXXAA) and examined its ability to interact with receptors by GST pull-down assays. As shown in Figure 25C, we detected hormone-induced association of GST-ACTN4 (Iso, LXXAA) with VDR. In contrast, GST or GST-ACTN4 (Iso, LXXAA) mutant failed to bind VDR even in the presence of the vitamin D3 (lanes 4 and 6). We further determined the functional significance of the LXXLL motif by transient transfection reporter assays and found that ACTN4 (Iso, LXXAA) mutant dramatically lost the ability to potentiate transcriptional activation of a VDRE reporter (Figure 25D). Together, these data show that ACTN4 (Iso) interacts with and potentiates VDR activity in a ligand-dependent manner. To test whether ACTN4 (Iso) can function as a coactivator for estrogen receptor (ERα) mediated transcriptional activation, we carried out transient transfection assays using a reporter construct harboring an estrogen response element (ERE).
Figure 26. ACTN4 (Iso) potentiates ERα transcriptional activity. A, The LXXLL motif is critical for the ability of ACTN4 (Iso) to potentiate transcription from of an ERE reporter. MCF-7 cells were transfected with reporter plasmid harboring an ERE element, expression plasmid for ACTN4 (Iso) or ACTN4 (Iso, LXXAA) and β-gal. Cells were treated with or without 10nM of E2 and luciferase activities were determined and normalized to β-gal activities. Each data point represents the mean and standard deviation (S.D.) of results from triplicates. B, The LXXLL motif is required for ACTN4 (Iso) and ERα association. HEK293 cells were transfected with expression plasmid for ERα and whole cells lysates were incubated with bacterially expressed GST-ACTN4 (Iso) or with GST-ACTN4 (Iso, LXXAA) in the presence or absence of 1uM of E2 for 1 h. Pull-down fractions were subjected to SDS-PAGE followed by Western blotting with α-ERα antibodies (top panel). Coomassie blue staining is shown to demonstrate equal loading for GST beads (bottom panel). Lane 1 shows 10% of the input for pull-down assays C, ACTN4 (Iso) enhances the expression of endogenous ERα target genes. MCF-7 cells were transfected with vector alone, ACTN4 Iso (WT) and ACTN4 (Iso, LXXAA) mutant along with GFP expression plasmids. The GFP plasmids were used for normalization. After 24 h the medium was replaced with charcoal stripped serum containing medium and after 48 h cells were treated with or without E2 for 48 h. An aliquot of the samples were used to prepare total cell lysates for Western blotting (Figure 28) and other aliquot was used to extract RNA. Total RNA was used to prepare cDNA as mentioned in “Experimental Procedures”. Real-Time qRT-PCR was carried out to analyze the changes in mRNA expression level of ERα target genes including pS2 and c-Myc after overexpression of ACTN4 (Iso) and ACTN4 (Iso, LXXAA). The mRNA levels of target genes were normalized to GAPDH. The mRNA expression level of pS2 and c-Myc in MCF-7 cells overexpressing vector alone treated with vehicle is set to 1.
Figure 27. Subcellular distribution of endogenous HA-ACTN4 (Iso, WT) and HA-ACTN4 (Iso, LXXAA). MCF-7 cells were transfected with HA-ACTN4 (Iso) or ACTN4 (Iso, LXXAA) expression plasmids followed by immunostaining using α-HA antibodies and immunofluorescence microscopy. DNA was visualized by 4′, 6-diamidino-2-phenylindole (DAPI) staining.
Figure 28. HA-ACTN4 (Iso, WT) and HA-ACTN4 (Iso, LXXAA) were expressed at similar levels. MCF-7 cells were transfected with vector alone, HA-ACTN4 (Iso) and HA-ACTN4 (Iso, LXXAA) along with a GFP expression plasmid. Forty eight hours post-transfection whole cell lysates were prepared and subjected to Western blotting with α-HA and α-GFP antibodies.
Figure 26A shows that ACTN4 (Iso) was also capable of potentiating transcriptional activation by ERα in a dose-dependent manner. However, the LXXAA mutant failed to potentiate ERα activity (lanes 1-4 vs 5-7). Indeed, overexpression of LXXAA partially inhibited basal and E2-induced reporter activity. The inability of ACTN4 (Iso, LXXAA) to potentiate transcriptional activation by NRs was not due to its aberrant subcellular localization (Figure 27). GST-ACTN4 (Iso, LXXAA) also failed to interact with ERα in a hormone-dependent manner (Figure 26B). We further tested whether ACTN4 (Iso) was capable of inducing the expression of endogenous ERα target genes. MCF-7 cells were transfected with vector alone, ACTN4 (Iso) and ACTN4 (Iso, LXXAA) along with a GFP expression plasmid and qRT-PCR was performed to determine the relative expression of selected ERα target genes. The expression levels of ACTN4 (Iso, WT) and ACTN4 (Iso, LXXAA) were relatively similar (Figure 28). As shown in Figures 26C-D, in the presence of E2, a 4- and 3-fold increase in pS2 and c-Myc mRNA levels was observed in vector transfected cells, respectively. Transient overexpression of ACTN4 (Iso, WT) further induced pS2 and c-Myc mRNA levels by 5- and 3-fold, respectively. In contrast, overexpression of ACTN4 (Iso, LXXAA) failed to potentiate E2-induced mRNA expression level of pS2 and c-Myc, indicating that ACTN4 (Iso) potentiates E2-induced selected endogenous ERα target genes in MCF-7 cells and that the LXXLL motif is critical for this activity.
A

ACTN4 (FL)  77-FQGKMLMLEVISGERLHPK- 98
ACTN4 (Iso) 77-FQGKMLMLELNELDYDS- 98
ACTN4 (Iso, WT)  -FQGKMLMLELNELDYDS-
M1  ----------------------------VIS-------------
M2  ----------------------------GER-------------
M3  ----------------------------RLP-------------

B

![Graph showing normalized luciferase activity with bars for different samples.]

C

![Image showing western blot analysis with input, wild-type, LL, AA, M1, M2, and M3 lanes.]

1 2 3 4 5 6
Figure 29. The effects of the downstream sequence of the LXXLL motif on ERE reporter activity. A, An alignment of the receptor interacting motif LXXLL in ACTN4 (full-length) and ACTN4 (Iso) is shown and the substitutions are described in the text. B, Effect of ACTN4 (Iso) and ACTN4 (Iso) downstream mutants on reporter activity described by ERα. MCF-7 cells were transfected with ACTN4 (Iso) or the mutants indicated in the Figure 29A, in dose dependent manner along with reporter construct bearing an ERE and β-gal. Transient transfection assays were carried out as mentioned in Figure 26A. Luciferase activity was determined and normalized to β-gal activity. C, The ACTN4 (Iso) mutants fail to bind ERα. GST pull-down assays were carried out by incubating GST-ACTN4 (Iso) or GST-ACTN4 (Iso, LXXAA), GST-ACTN4 (Iso, M1) or GST-ACTN4 (Iso, M2), or GST-ACTN4 (Iso, M3) with whole cell lysates prepared from HEK293 cells overexpressing ERα. Pull-down fractions were subjected to SDS-PAGE followed by Western blotting with α-ER antibodies (top panel). Coomassie blue staining is shown at the bottom panel demonstrating equal expression of GST beads. Ten percent of input is shown in lane 1. D, Effect of ACTN4 (full-length) and ACTN4 (full-length) downstream mutants shown in the diagram, on the transcriptional assay mediated by ERα. An alignment showing the mutants made in ACTN4 (full-length) and transient transfection assays were carried out as described in Figure 26A, except that MCF-7 cells were transfected with ACTN4 (full-length) and ACTN4 (full-length) downstream mutants. Each data point represents the mean and standard deviation (S.D.) of results from triplicates.
Figure 30. Subcellular distribution of wild-type and mutant HA-ACTN4. MCF-7 cells were transfected with HA-ACTN4 (Iso, WT), HA-ACTN4 (Iso, M1), HA-ACTN4 (Iso, M2) and HA-ACTN4 (Iso, M3) expression plasmids. Twenty four hours post transfection the cells were fixed and immunostaining was carried out using α-HA antibodies according to protocol described in “Experimental Procedures”. The pictures were taken using immunofluorescence microscopy. DNA was visualized by 4’, 6-diamidino-2-phenylindole (DAPI) staining.
Figure 31. The wild-type and downstream mutants ACTN4 (Iso) interact with HDAC7 in a similar manner. Whole cell lysates prepared from HEK293 cells overexpressing HA-HDAC7 were incubated with GST-ACTN4 (Iso, WT), GST-ACTN4 (Iso, M1), GST-ACTN4 (Iso, M2) and GST-ACTN4 (Iso, M3). Pull-down fractions were Western blotted with α-HA antibodies after running them on 10% SDS-PAGE.
Although the sequences N-terminal to the LXXLL receptor interacting motifs of ACTN4 (full-length) and ACTN4 (Iso) are identical, the sequences C-terminal to the LXXLL motif are distinct (Figure 29A). In order to dissect the molecular basis of the associations between ACTN4 (full-length) or ACTN4 (Iso) and nuclear receptors, we replaced three amino acids of the sequences C-terminal to the LXXLL motif with that of the full-length protein and examined their ability to potentiate transcription. Figure 29B shows that ACTN4 (Iso, WT) potently activated an ERE reporter activity, whereas all three triple mutants (M1, M2 and M3) significantly lost their ability to activate the reporter activity and among them, the M1 mutant exhibited the most severe defect. Immunofluorescence microscopy indicated that these mutants displayed similar subcellular localization to that of ACTN4 (Iso, WT) (Figure 30). Furthermore, these mutants interacted with histone deacetylase (HDAC7) similarly to that of the wild-type protein (Figure 31), suggesting that the global folding of these mutants were not significantly altered. Since these ACTN4 downstream mutants were defective in activating an ERE reporter, we speculated that they might also be defective in their abilities to interact with ERα. In order to address this question, we generated GST-ACTN4 (Iso) mutant fusion proteins and carried out GST pull-down assays. As shown in Figure 29C, all three ACTN4 mutants significantly lost their ability to interact with ERα. These data indicate that the C-terminal sequences to the LXXLL motif are also important for the interaction of ACTN4 (Iso) with ERα. Similar approaches were used to examine the sequence in ACTN4 (full-length). We found that ACTN4 (full-length) mutants M1 and M2 that were replaced by the ACTN4 (Iso) amino acids, significantly lost their ability to activate transcription by ERα whereas M3 retained most of the activity (Figure 29D).
Figure 32. Transcriptional activation by ACTN4 through an intrinsic transcriptional activation domain. A, Gal4-ACTN4 activates basal transcription. CV-1 cells were transfected with MH100 encoding luciferase controlled by Gal4 response elements and expression plasmids encoding Gal4 DBD or Gal4 DBD fused to ACTN4 (full-length) or to ACTN4 (Iso) along with CMX-β-gal as an internal control. Transient transfection reporter assays were performed as described in Figure 26A. Each data point represents the mean and standard deviation (S.D.) of results from triplicates. B, Coactivators potentiate transcriptional activity of Gal4-ACTN4. CV-1 cells were transfected with Gal4-ACTN4 (full-length) and Gal4-ACTN4 (Iso) along with expression plasmids for encoding coactivators GRIP1, PCAF, and SRC-1. Transient transfections were carried out as described in Figure 26A. Note that GRIP1 could not potentiate Gal4-ACTN4 (full-length) activity but did activate reporter activity mediated by Gal4-ACTN4 (Iso). Each data point represents the mean and standard deviation (S.D.) of results from triplicates.
Together these data indicate that sequences C-terminal to the LXXLL motif also regulate the ability of ACTN4 to potentiate ERα binding and ERE reporter activity. To further understand the molecular mechanism underlying ACTN4 (Iso) coactivation activity, we used transient transfection assays to test whether ACTN4 is able to activate transcription when tethered to a promoter region. We generated proteins in which ACTN4 (full-length) and ACTN4 (Iso) were fused C-terminal to the yeast Gal4 DNA binding domain (DBD). Gal4-ACTN4 (Iso) or Gal4-ACTN4 (full-length) expression constructs were co-transfected with a reporter construct harboring four copies of Gal4 binding sites (MH100) [223]. Figure 32A shows that Gal4-ACTN4 (Iso) potently activated basal transcription activity, while Gal4-ACTN4 (full-length) weakly activated the reporter activity. Since neither ACTN4 (full-length) nor ACTN4 (Iso) harbor motifs known to modify histones or remodel chromatin, we hypothesized that ACTN4 may activate transcription through an interaction with other co-activators. To test this, we first examined whether p160 family members or PCAF were able to potentiate reporter activity of MH100 reporter gene by Gal4 ACTN4 (full-length) or ACTN4 (Iso). Since these coactivators harbor intrinsic transcriptional activation domain, an interaction between these coactivators and ACTN4 would further enhance reporter activity. We found that coexpression of both the p160 coactivators and PCAF significantly enhanced the ability of ACTN4 (Iso) to activate reporter activity (Figure 32B). However, only PCAF was capable of weakly activating Gal4-ACTN4 (full-length) activity. In summary, these results suggest that transcription activation function of ACTN4 (Iso) may be attributed to its association with co-activators.
Figure 33. Interaction between ACTN4 (Iso) with coactivators in vitro and in vivo. A-B, ACTN4 interacts with co-activator SRC-1 and PCAF in vitro. HEK293 cells were transfected with either HA-SRC-1 or FLAG-PCAF expression plasmids. Whole cell lysates were prepared and incubated with either GST-ACTN4 (Iso) and or GST-ACTN4 (full-length) for 1 h. Pull down fractions were subjected to SDS-PAGE followed by Western blotting with either α-HA and α-FLAG antibodies. Lane 1 shows 10% input. C, ACTN4 (Iso) associates with SRC-1 in HEK293 cells C, ACTN4 (Iso) interacts with PCAF in HEK293 cells. Expression plasmids for FLAG-ACTN4 (Iso) were cotransfected with HA-PCAF in HEK293 cells. Whole cell lysates were prepared and immunoprecipitations were carried out with α-FLAG antibodies followed by Western blotting with α-HA or α-FLAG antibodies.
Figure 34. Association of GST-ACTN4 (Iso) with GRIP1/SRC-2 and ACTR/SRC-3. HEK293 cells were transfected with expression plasmids for FLAG-ACTR or HA-GRIP-1. Whole cell lysates were prepared and incubated with GST-ACTN4 (Iso). Pull-downs were carried as described in “Experimental Procedure” Pull-down fractions were subjected to SDS-PAGE followed by Western blotting with α-HA or α-FLAG antibodies.
Figure 35. Amino acids of 501-727 of PCAF are essential to interact with ACTN4. Different fragments of FLAG-PCAF were overexpressed in HEK293 cells. Lysates were prepared and pull-down assays were carried out by incubating whole cell lysates with bacterially expressed GST-ACTN4 (Iso) followed by Western blotting with α-FLAG antibodies. Inputs for pull-downs are shown in lanes 1, 4, 7 and 10 respectively. Bottom panel shows coomassie blue staining demonstrating equal expression of beads.
To determine whether ACTN4 physically interacts with co-activators, we first used GST pull-down assays. Immobilized, purified GST-ACTN4 ( Iso) and GST-ACTN4 (full-length) fusion proteins were incubated with whole cell lysates overexpressing p160 coactivator family members or PCAF. We found that GST-ACTN4 (Iso) bound efficiently to all three p160 coactivators (Figure 33A and Figure 34) as well as the PCAF (Figure 33B). In contrast, GST-ACTN4 (full-length) did not interact with either SRC-1 or PCAF (Figure 33A-B, lane 4). In order to validate these interactions in mammalian cells, HA-PCAF and FLAG-ACTN4 (Iso) were coexpressed in HEK293 cells and immunoprecipitations were carried out using α-FLAG antibodies followed by Western blotting. We found that ACTN4 (Iso) associates robustly with PCAF (Figure 33C). PCAF harbors a protein acetylation enzymatic domain (HAT) and a Bromo domain that binds to acetylated protein. We further explored the molecular basis of the interaction between ACTN4 (Iso) and PCAF. We carried out GST pull-down assays using HEK293 whole cell lysates expressing different fragments of PCAF and GST-ACTN4 (Iso, WT). As shown in Figure 35, ACTN4 (Iso) interacted with amino acids 501-831 and only modestly with amino acids 2-607, suggesting that the interaction domain of ACTN4 (Iso) in PCAF lies within HAT domain in order to find out which region in ACTN4 (Iso) interacted with co-activators. CV-1 cells were transfected with Gal4-ACTN4 (Iso), Gal4-ACTN4 (Iso, 1-102) and Gal4-ACTN4 (95-521) along with the Gal-4 reporter construct MH100. We found that both Gal4-ACTN4 (Iso, 1-102) and Gal4-ACTN4 (Iso, 95-521) showed autonomous transcriptional activation, although Iso (1-102) was more potent (Figure 36). Furthermore, we tested whether these two fragments interact with co-
Figure 36. ACTN4 (Iso) harbors two independent activation domains. A, CV-1 cells were transfected with MH100 reporter plasmid harboring Gal4-responsive elements along with expression plasmids encoding Gal4-DBD or Gal4 DBD fused to Gal4 (Iso), or Gal4 (Iso,1-102) or Gal4 (Iso, 95-521). Transient transfection reporter assays were performed as described in Figure 26A. B, Both ACTN4 (Iso) activation domains interact with co-activators. HEK293 cells overexpressing HA-SRC-1 and HA-PCAF were incubated with immobilized GST-ACTN4 (Iso), GST-ACTN4 (Iso, 1-102) and ACTN4 (95-521) for 1 h. Pull-down fractions were subjected to SDS-PAGE followed by Western blotting with α-HA antibodies. C, The LXXLL motif and its flanking sequences are critical for coactivator binding. Immobilized, purified wild-type or mutant GST-ACTN4 (Iso) fusion proteins were incubated with cell lysates expressing HA-SRC-1 or HA-PCAF and pulldown was carried out as described in 26B, followed by immunoblotting with anti-HA antibodies. Coomassie blue staining is shown to demonstrate equal loading of beads. Ten percent and five percent of input are shown for SRC-1 and PCAF respectively.
activators by GST pulldown assays. As shown in Figure 36B, both these two independent activation domains were capable of interacting with co-activators SRC-1 and PCAF although again, Iso (1-102) was stronger. We conclude that there are two independent activation domainin ACTN4 (Iso). The observation that ACTN4 (Iso, 1-102) is capable of interacting with SRC-1 and PCAF raised the question of whether the C-terminal sequences to the LXXLL motif in the ACTN4 (Iso) are critical for this binding activity because amino acids 1-95 of ACTN4 (Iso) and ACTN4 (FL) are identical. To test this possibility, we used ACTN4 (Iso) mutants generated in Figure 29 and carried out GST pull-down assays. Figure 36C shows that surprisingly, the ACTN4 (LXXAA) mutant significantly lost its ability to bind co-activators SRC-1 and PCAF (lanes 3 & 4). Furthermore, M1 and M3 mutants lost their ability to interact with PCAF. Taken together, these data indicate that the LXXLL motif and its flanking sequences are critical for ACTN4 (Iso) co-activators binding.

**DISCUSSION**

Previous studies have suggested that actin and actin binding proteins play an important role in transcription in addition to their function as cytoskeleton proteins [206, 224]. Actin and actin related proteins have been shown to be present in complexes associated with transcriptional machinery [208, 225]. We have also previously shown that both ACTN4 (full-length) and ACTN4 (Iso) are capable of potentiating MEF2 transcription factors [73]. Furthermore, we have reported that ACTN4 (full-length) regulates nuclear receptor mediated transcription by antagonizing HDAC7 function [222]. Here we report a distinct role played by ACTN4 (Iso) in nuclear receptor mediated transcription that is due
to its ability to interact with NRs and co-activators. In this manuscript we have dissected the mechanisms by which ACTN4 (Iso), a spliced isoform of ACTN4, potentiates transcriptional activation mediated by ERα. Major findings from our study include 1) ACTN4 (Iso) is a more potent transcriptional coactivator of nuclear hormone receptors than ACTN4 (full-length), 2) Both ACTN4 (Iso) and ACTN4 (full-length) activate basal transcription when tethered to promoter region, 3) ACTN4 (Iso) harbors two independent activation domains, 4) While ACTN4 (Iso) interacts strongly with co-activators, ACTN4 (full-length) associates weakly with co-activators, 5) The nuclear interacting motif LXXLL is critical for ACTN4 to interact with and potentiate transcriptional activation by nuclear receptors, 6) The HAT domain of PCAF is essential for PCAF to interact with ACTN4 (Iso). Taken together, we have defined ACTN4 (Iso) as a potent transcriptional co-activator for nuclear hormone receptors. We have shown that the LXXLL motif present in both ACTN4 (Iso) and ACTN4 (full-length) plays an important role in transcriptional regulation mediated by nuclear receptors [222] and Figure 25A). Mutations of LXXLL to LXXAA lead to disruption of physical interaction of ACTN4 with NRs and also abolish its ability to regulate the transcriptional activation mediated by NRs (Figure 25B-C and Figure 26A-B). Furthermore, we show here that ACTN4 (Iso, WT) is required for efficient hormonal induction of endogenous ER target genes (Figure 26C-D). There is a significant reduction in the mRNA expression level of these genes when ACTN4 (Iso, LXXAA) is overexpressed suggesting that ACTN4 (Iso) plays an important role in hormonal activation of these genes.
Molecular determinants regulating NRs mediated transcriptional activity: One of the interesting findings in this study is that the amino acids C-terminal to LXXLL motif in ACTN4 (Iso) are also critical for its ability to bind to ERα and to activate transcription (Figure 29B-C). We have shown that exchanging C-terminal residues of ACTN4 (Iso) with the corresponding residues in ACTN4 (full-length) reduces the ability of ACTN4 (Iso) to activate transcription of genes regulated by ERα. Furthermore, we have also shown that LXXLL, M1, M2, and M3 mutants of ACTN4 (Iso) are defective in binding with PCAF (Figure 36C) although only LXXAA mutant could not bind to SRC-1. These results suggest that different co-activators interact with ACTN4 (Iso) through distinct surfaces. Taken together, our data suggests that ACTN4 (Iso) interacts with several co-activators but whether these interaction are responsible for recruitment of ACTN4 (Iso) to selected ERα target promoters remains to be determined.

Distinct coactivation activity: Both ACTN4 (full-length) and its isoform possess LXXLL receptor interaction motifs. However, the mechanisms by which these two isoform activate ERα-mediated transcription are distinct. First, based on the expression levels of ACTN4 and the potency of coactivation, it is clear that ACTN4 (Iso) is a more potent ERα coactivator than ACTN4 (full-length) when both are expressed at the same level. Several reasons may account for ACTN4 (Iso) being a more potent coactivator as compared to ACTN4 (full-length) for transcription mediated by ERα. For example, ACTN4 (full-length) is predominantly cytoplasmic, whereas ACTN4 (Iso) is primarily nuclear ([73]Chakraborty 2006 and Figure 27). Second, ACTN4 (Iso) interacts with higher affinity with co-activators than ACTN4 (full-length). The interaction between
ACTN4 (Iso) and coactivators is specific since they show relatively no interaction or weak interaction with ACTN4 (full-length). In fact, deletion of the actin-binding domain of ACTN4 (full-length) led to a predominantly nuclear distribution and a higher co-activation activity (data not shown). Third, ACTN4 (Iso) interacts more strongly with ERα than ACTN4 (full-length). These data argue that the ability of ACTN4 (Iso) and ACTN4 (full-length) to activate ERα activity requires a high affinity association with ERα. Our data suggest that when brought to the promoter region, Gal4-ACTN4 (Iso) possesses potent transcriptional activity (Figure 32) and this activity correlates with its ability to associate with other coactivators including PCAF and p160 family members (Figure 33-35). Indeed, ACTN4 (full-length) is a weaker coactivator and only binds weakly to most coactivators. Thus our data suggest that ACTN4 (Iso) interacts with GRIP-1 and PCAF in order to regulate the transcription mediated by ERα. It is possible that another difference between ACTN4 (full-length) and ACTN4 (Iso) is that the latter may be a substrate for a HAT enzyme. GCN5 and PCAF have been shown to acetylate several other transcription factors directly in addition to their global and gene specific acetylation of histone proteins [226-229]. The acetylation of non-histone proteins such as chromatin remodelers, sequence specific transcription factors, transcriptional activators, and nuclear receptor cofactors can affect nuclear localization, inhibition of nuclear export of transcription factor, enhanced DNA binding, stimulation of transcription mediated by some factor or enhanced co-activators association [230]. Our binding studies suggest that the region required for binding of PCAF to ACTN4 (Iso) harbors the HAT domain (Figure 35). However, whether HAT activity of PCAF is essential for ACTN4 (Iso) to
enhance the transcriptional activity mediated by nuclear hormone receptor is under investigation.
CHAPTER 4: FAMILIAL FOCAL SEGMENTAL GLOMERULOSCLEROSIS (FSGS)-LINKED α-ACTININ4 (ACTN4) MUTANTS LOSE THE ABILITY TO ACTIVATE TRANSCRIPTION BY NUCLEAR HORMONE RECEPTORS.

ABSTRACT

Alpha actinins (ACTNs) are known for their ability to modulate cytoskeletal organization and cell motility by crosslinking actin filaments. Mutations in ACTN4 are linked to familial focal segmental glomerulosclerosis (FSGS), a kidney disease characterized by proteinuria. We have previously shown that ACTN4 interacts with HDAC7 and MEF2s to regulate transcription mediated by MEF2s. Also, we have shown that ACTN4 harbors a functional LXXLL receptor interaction motif, interacts with nuclear receptors \textit{in vitro} and in mammalian cells, and potently activates transcription by nuclear receptors. Mutations in the LXXLL motif abolish the ability of ACTN4 to interact with or enhance the transcriptional activation by nuclear receptors. We show here that FSGS-linked ACTN4 mutants not only mis-localize to the cytoplasm, but also lose the ability to associate with nuclear receptors in a hormone-dependent manner. Consequently, FSGS-linked ACTN4 mutants fail to potentiate hormone driven transcriptional activation by nuclear hormone receptors. We hypothesize that the inability of FSGS-linked ACTN4 mutants to activate transcription by nuclear receptors contributes to the development of FSGS.
INTRODUCTION

The actin cytoskeleton plays a critical role in podocyte function. Mutations in genes encoding the components of the cytoskeleton are linked to several kidney diseases. Podocytes are highly differentiated kidney cells that develop long branches from the cell body known as primary processes, which further send out several small secondary foot processes. Secondary processes of neighboring cells interdigitate to form unique cell-cell junctions known as the slit diaphragm [119] which act as a physical sieve to prevent the passage of proteins (larger than 65KD) into the urine [125]. There are a number of proteins involved in the assembly of the slit diaphragm such as nephrin, CD2 associated protein (CD2AP), podocin, P-cadherin, α and β catenin, zonula ooculdens -1 (ZO-1) and transient receptor potential channel (TRPC6). Notably, mutations in nephrin, CD2AP, podocin, and TRPC6 are associated with familial focal segmental glomerulosclerosis (FSGS) [231], a kidney disease characterized by proteinuria, a common consequence of podocyte injury [152]. Mutations in ACTN4, including K228E, T232I, and S235P, are also linked to FSGS [152]; however, the mechanisms by which these mutants contribute to FSGS are currently not well understood [154].

Nuclear hormone receptors, including retinoid acid receptor (RAR), the vitamin D receptor (VDR), peroxisome proliferator-activated PPARγ receptor (PPARγ) and glucocorticoid receptor (GRα), are ligand-activated transcription factors that control aspects of homeostasis, cell differentiation, proliferation, and development [1, 202, 203]. The mechanism of transcriptional regulation by nuclear receptors is thought to occur through the exchange of associated co-regulators including co-repressors and co-
activators. Ligand binding induces an allostERIC change in the nuclear receptor, leading to
dissociation of corepressor complexes and recruitment of coactivator proteins. The
hormone-induced interaction of coactivators is mediated through multiple copies of a
conserved motif, LXXLL (where L is leucine, X can be any amino acid) [21]. This motif
is present in many nuclear receptor coactivators including p160 family of coactivators
(NCoA 1, 2 and 3) and histone acetyltransferases (CBP/P300) [23, 218-220].

We have recently identified a novel function for ACTN4 in transcriptional regulation
mediated by nuclear receptors including ERα and VDR in MCF-7 breast cancer cell line
[222]. In this study, we demonstrate that ACTN4 also interacts with and regulate
transcription mediated by RARα and GRα through its LXXLL motif. Also, we show that
wild-type ACTN4, but not FSGS-linked ACTN4 mutants, potently stimulate transcription
mediated by nuclear receptors. The inability of FSGS-linked ACTN4 mutants to activate
transcription correlates with their inability to localize to the nucleus and defects in
binding to nuclear receptors. Together, we have uncovered a novel function of ACTN4 in
nuclear receptor-mediated transcriptional activation that may play a role in the
development of FSGS in addition to known effects on podocyte cytoskeleton
structure/function [151, 153, 155, 156].
EXPERIMENTAL PROCEDURES

Plasmid construction: CMX-HA-ACTN4 has been previously described [73]. CMX-HA-ACTN4 (K228E), CMX-HA-ACTN4 (T232I) and CMX-HA-ACTN4 (S235P) expression plasmids were generated by site directed PCR mutagenesis according to the manufacturer’s protocol (Stratagene). For the glutathione S-transferase (GST) constructs, ACTN4 was PCR amplified and subcloned into pGEX4T vector. Expression plasmids for nuclear receptors and reporters were generous gifts from Ron Evans (The Salk Institute, La Jolla, CA).

Antibodies and chemicals: ACTN4 antibody has been previously described [73]. Anti-HA conjugated anti-horseradish peroxidase and anti-FLAG antibodies were purchased from Roche Applied Science and Sigma. Anti-GRα (Sc-8992) anti RARα (Sc-551) antibodies were purchased from Santa Cruz Biotech. Anti-HDAC1 and anti-GAPDH antibodies were purchased from Santa Cruz and Trivegen. All trans retinoic acid (ATRA) and dexamethasone were purchased from Sigma-Aldrich.

Cell culture: HEK293 and CV-1 cells were grown in Eagle Dulbecco’s modified medium (DMEM) medium supplemented with 10% Fetal Bovine Serum, 50units/ml penicillin G and 50µg/ml streptomycin sulfate at 37 °C in 5% CO2. Conditionally immortalized human podocyte (HPC) cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 50 units/ml penicillin G and 50 µg/ml streptomycin sulfate and insulin, transferring, selenite (ITS) (Sigma Chemicals). Cells were grown at permissive temperature of 33°C to promote cell propagation as undifferentiated cells.
Subcellular Fractionation: Subcellular fractionation of undifferentiated HPCs was carried out by previously published protocol [204]. Nuclear and cytoplasm fractions were resolved on SDS-PAGE gel and subjected to immunoblotting with the indicated antibodies.

*In vitro* protein-protein interaction assays: Glutathione S-transferase (GST) fusion proteins GST-ACTN4 (WT) was expressed in E.coli DH5α strain, affinity purified and immobilized on glutathione Sepharose 4B beads. *In vitro* pull-down assays were carried out using immobilized GST-ACTN4 (WT) with whole cell extracts expressing nuclear receptors including HA-RARα and HA-GRα in the presence or absence of AT-RA (100 nM) and dexamethasone (100nM) for 1 h at 4 °C. After extensive washes with NETN buffer (100 mM NaCl, 1mM EDTA, 10 mM Tris-Hcl (pH 8.0), 0.1% Nonidet P-40, 10% glycerol, and 1 mM dithiothretol), SDS-PAGE sample buffer was added to the beads, boiled and separated by 10% SDS-PAGE gel and probed with the indicated antibodies.

Coimmunoprecipitation assays: HEK293 cells were grown on 10-cm plates and transfected with 10 μg of plasmid DNA using Lipofectamine 2000 (Life Tehnologies) according to manufacturer protocol. After 48 h, the cells were washed with 1X PBS and resuspended in radioimmune precipitation assay (RIPA) buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) along with protease inhibitors. After 1 h 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 anti-FLAG antibody M2 affinity gel (Sigma) for 4 h at 4 °C. The
immunoprecipitated fractions were analyzed by Western blotting using HA-conjugated anti-horseradish peroxidase antibody.

Transient transfection reporter assays: For reporter assays, HEK293 and CV-1 cells were co-transfected with equal amounts of the following constructs: 100 ng RARE-TK-Luc and GRE-TK-luc with or without pCMX-ACTN4 or pCMX-ACTN4 (mutants) and 100 ng pCMX-β-gal in 200 μl Opti-MEM I using Lipofectamine 2000 (Life Technologies). The amount of DNA was kept constant (<1μg) by addition of pCMX vector. After 5 h, the medium was replaced with DMEM medium supplemented with 10% charcoal stripped fetal bovine serum, 50 units/ml penicillin G and 50 μg/ml streptomycin sulfate for HEK293 cells and CV-1 cells. After 24 h, the cells were treated with or without 100 nM of all-trans retinoic acid and 100 nM of dexamethasone. Cells were harvested 48 h after transfection and luciferase assays and β-gal activity was measured by using luciferase assay system (Promega). Luciferase activity was normalized to the level of β-gal activity. Each reaction was performed in triplicate. Transient transfections were repeated three times.

Confocal Microscopy: Transient transfections for confocal microscopy were performed in 12-well culture plates. Cells were transfected with appropriate ACTN4 (WT) and /or ACTN4 mutant plasmids using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol. Twenty-four hours post transfection, cells were subjected to immunostaining. Transfected cells were fixed in 3.7% paraformaldehyde (PFA) in PBS for 30 minutes 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%) + 0.1% Tween-20 solution (ABB) for 60 min. Incubation with primary antibodies was carried out for 120 min in ABB. The cells were washed three times in PBS, and the secondary antibodies were added for 60 min in the dark, at room temperature in ABB. Cover slips were mounted to slides using Vectashield mounting medium with DAPI (H-1200, Vector Laboratories, Inc.) Confocal images were acquired using a Zeiss LSM 510 inverted laser-scanning confocal microscope. A 63X numerical aperture of 1.4 oil immersion planapochromat objective was used for all experiments. For endogenous and transiently transfected ACTN4, images of Alexa Fluor 594 were collected using a 633-nm excitation light from a He/Ne2 laser, a 633-nm dichroic mirror and 650-nm long pass filter. For endogenous ACTN4, images of Alexa Fluor 488 were collected using a 488-nm excitation light from an argon laser, a 488-nm dichroic mirror and 500-550-nm band pass barrier filter. All DAPI stained nuclear images were collected using a Coherent Mira-F-V5-XW-220 (Verdi 5W) Ti:sapphire laser tuned at 750-nm, a 700-nm dichroic mirror and a 390-465 nm band pass barrier filter. The primary antibodies used were: purified α-ACTN4 polyclonal, α-FLAG mouse monoclonal (Sigma), α-HA rabbit (Sigma), α-GR polyclonal (Santa Cruz), and α-synaptodendrin polyclonal (Santa Cruz). The secondary antibodies used are from Molecular Probes (α-mouse or α-rabbit Alexa Fluor 488, α-rabbit Alexa Fluor 594 or mouse Alexa Fluor 594).

In vitro differentiation assay: Conditionally immortalized human podocytes (HPCs) were grown at 33°C to proliferate. For differentiation assays, the HPCs were moved to 37°C in order to inactivate the SV40 large T antigen. At 37°C, these cells usually take 1-2 days
before they start differentiation. After twenty four hours, the cells were subjected to siRNA transfection with either a non-targeting siRNA (siCtrl) or with siRNA against GR (GR-1(J003424-07) or siGR-2 (J003424-09)) according to the manufacturer’s protocol (Dharmacon). After 24 h of transfection the medium was replaced with RPMI1640 supplemented with 10% charcoal stripped fetal bovine serum, 50 units/ml penicillin G, and 50ug/ml streptomycin sulfate and ITS (Sigma). The next day, the cells were treated with or without 100nM of dexamethasone. After 24 h, pictures were taken using a phase contrast microscope and medium was replaced with fresh medium containing hormone for another 24 h. After 96 h of transfection the cells were subjected to either Western blotting or immunostaining with indicated antibodies.

Real time PCR assays: The undifferentiated HPCs (33°C) were moved to differentiation (37°C) conditions. After 24 h, the medium was replaced with RPMI1640 supplemented with 10% charcoal stripped fetal bovine serum, 50 units/ml penicillin G, and 50ug/ml streptomycin sulfate and ITS (Sigma). After 24 h, the cells were treated with dexamethasone or with vehicle for 48 h. Total RNA was extracted according to the manufacturer’s protocol (USB). cDNA was synthesized from 1 µg of total RNA according to the manufacturer’s instructions (Invitrogen). Gene expression levels were determined by qRT-PCR using Real Time PCR System (Bio Rad) with the following primers:

GAPDH: 5’-GAAGGTGAAGGTCGGAGT-3’, 5’-GAAGATGGTGATGGGATTTC-3’;
Nephrin: 5’-GACCCCACCAATCGCTACTACAAC-3”, 5’-GGGTGTCATACGCCTCATATGG-3’.
Relative changes in gene expression were calculated using the \( \Delta \Delta \text{Ct} \) method. Each value is representative of three replicates and all the experiments were repeated twice.

**RESULTS AND DISCUSSION**

Subcellular localization of ACTN4 in immortalized human podocytes (HPCs): FSGS-linked ACTN4 mutations acquire increased F-actin binding activity[156]. We hypothesized that the increased F-actin binding activity affects the subcellular localization of ACTN4. In order to address this issue, we first examined the subcellular distribution of endogenous ACTN4 in conditionally immortalized human podocytes (HPCs) [232]. Cytoplasmic and nuclear fractions were separated on SDS-PAGE and analyzed by Western blotting with anti-ACTN4, anti-HDAC1 (nuclear marker) and anti-GAPDH (cytoplasmic marker) antibodies. As shown in Figure 37A, ACTN4 was localized in the cytoplasm and nucleus of undifferentiated HPCs. Immunostaining using anti-ACTN4 antibodies followed by microscopy also showed both nuclear and cytoplasm distributions of endogenous ACTN4 in undifferentiated podocytes (Figure 37B, panels a-c). We next examined the subcellular distribution of FSGS-linked ACTN4 mutants K228E, T232I, and S235P by immunostaining and confocal microscopy. Figure 37C shows that all three FSGS-linked ACTN4 mutants were excluded from the nucleus (panels g-l, m-r, and s-x).
Figure 37: The subcellular distribution of FSGS-linked ACTN4 mutants in undifferentiated HPCs. A, Expression of ACTN4 protein in cytoplasm and nucleus fractions of undifferentiated HPCs. Fractionation of HPCs was carried out as described in “Experimental Procedures”. The subcellular fractions were separated by SDS-PAGE and Western blots were probed with the indicated antibodies. B, Subcellular distribution of endogenous ACTN4 in HPCs. Immunostaining and immunofluorescence of undifferentiated HPCs was performed with anti-ACTN4 antibodies. The DNA was visualized by 4’, 6-diamidino-2-phenylindole (DAPI) staining (panels a & c). The arrows indicate ACTN4 staining in the nucleus and the cytoplasm. C, Subcellular distribution of FSGS-linked ACTN4 mutants. Expression plasmids for HA-tagged ACTN4 (WT) and FSGS-linked ACTN4 mutants were transiently transfected into undifferentiated HPCs. Immunostaining was carried out using α-HA antibodies followed by confocal microscopy. The DNA was visualized by DAPI. All the images are shown with two z-plane sections; the one is a central section to show nuclear staining, and the other one is a section proximal to the basal membrane showing the cytoskeleton staining. Panels a-f, g-l, m-r and s-x show the subcellular distribution of ACTN4 (WT), K228E, T232I and S235P respectively. The arrows indicate the differential localization of ACTN4 (WT) and FSGS-linked ACTN4 mutants.
FSGS associated with ACTN4 mutations appears to be an autosomal dominant disease [154] indicating these mutants may interfere with the function of wild-type ACTN4. The fact that ACTN4 forms homodimers suggested that FSGS-linked ACTN4 mutants may sequester wild type ACTN4 by 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-tagged ACTN4 (WT) or FSGS-linked ACTN4 mutants. As a control, whole cell extracts expressing HA-ACTN4 (WT) alone were included. Our results demonstrated that FSGS-linked ACTN4 mutants associated with wild-type ACTN4 to a similar extent as wild-type ACTN4 does with itself (Figure 38A, lane 6 versus lane 7-9). These data suggest that FSGS-linked ACTN4 mutants might alter the subcellular localization of the wild-type protein. To test this, we co-transfected expression plasmids for FLAG-ACTN4 (WT) with HA-ACTN4 (WT) or FSGS-linked HA-ACTN4 mutants in undifferentiated HPCs and examined their subcellular localization by immunostaining and confocal microscopy. As shown in Figure 38, when an expression plasmid encoding HA-ACTN4 (K228E) was co-transfected with a FLAG-ACTN4 (WT) expressing plasmid, the wild-type ACTN4 redistributed to the cytoplasm (panels i-p). A similar pattern of localization was observed when an expression plasmid encoding FLAG-ACTN4 (WT) was co-transfected with HA-ACTN4 (T232I) (panels q-x). When expression plasmid for FLAG-ACTN4 (WT) was co-transfected with HA-ACTN4 (S235P), wild-type ACTN4 exhibited more cytoplasmic and actin stress fiber like staining (panels y-ff).
Figure 38: FSGS mutants sequester wild-type ACTN4 in the cytoplasm. A, FSGS-linked ACTN4 mutants associate with wild-type ACTN4. An expression plasmid for FLAG-ACTN4 (WT) was co-transfected either with expression plasmids for HA-ACTN4 (WT) or with HA-tagged FSGS-linked ACTN4 mutants in HEK293 cells. Forty-eight hours post transfection, whole cell lysates were prepared and incubated with FLAG M2 agarose beads and bound fractions were resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies. B, FSGS-linked ACTN4 mutants alter the subcellular distribution of wild-type ACTN4. An expression plasmid for FLAG-ACTN4 (WT) was were co-transfected with expression plasmids for HA-ACTN4 (WT) or HA-tagged FSGS-linked ACTN4 mutants in undifferentiated HPCs followed by immunostaining with anti-FLAG and anti-HA antibodies and confocal microscopy. Panels a-h show subcellular distribution of FLAG-ACTN4 (WT) and HA-ACTN4 (WT) and panels i-p, q-x and y-ff show subcellular distribution of FLAG-ACTN4 (WT) with HA-ACTN4 (K228E), HA-ACTN4 (T232I) and HA-ACTN4 (S235P), respectively. All the images are shown with two z-plane sections; the one is a central section to show nuclear staining, and the other one is a section proximal to the basal membrane showing the cytoskeleton staining.
There was no change in the localization of wild-type ACTN4 when expression plasmids for FLAG-ACTN4 (WT) and HA-ACTN4 (WT) were cotransfected (panels a-h). These data indicate that co-transfected FSGS-linked ACTN4 mutants alter the subcellular distribution of the wild-type ACTN4 protein. In contrast, these mutants did not alter subcellular distribution of GPS2, a component of transcriptional co-repressor complexes (data not shown). Taken together, these data demonstrate that FSGS-linked ACTN4 mutants exhibit staining patterns distinct from that of the wild-type protein and that these mutants likely sequester wild type ACTN4 from the nucleus.

FSGS-Linked ACTN4 mutants failed to activate transcription mediated by nuclear receptors. As we have shown previously, ACTN4 interacts with and regulates transcription mediated by VDR and GR in a hormone dependent manner [222] in MCF-7 cells. Similarly, we tested whether ACTN4 regulates the transcription mediated by GR and RARα in HEK293 and CV-1 cells. There is increasing evidence that synthetic hormones including retinoids, glucocorticoids, pioglitazone, and vitamin D3 may protect or restore podocytes from injury [130-135], implying a role for nuclear receptors in maintaining normal podocyte function. We hypothesized that disease-linked mutations in co-activators such as ACTN4 might interfere with the transcriptional program of nuclear receptors. To test this, we examined whether FSGS-linked ACTN4 mutants were defective in regulating transcription mediated by nuclear receptors.
**Figure 39**: FSGS Mutants fail to activate nuclear receptor mediated transcription in HEK293. A, A schematic diagram of ACTN4 showing the position of FSGS mutants. ACTN4 harbors two calponin homology domains (CH), four spectrin repeats domains, two EF hands and a C-terminal calmodulin homology domain. FSGS-linked ACTN4 mutants are indicated. B, FSGS-linked ACTN4 mutants fail to activate transcription mediated by RARα. Expression plasmids for RARE-containing reporter constructs were co-transfected with or without plasmids expressing RARα and ACTN4 (WT) or FSGS-linked ACTN4 mutants along with β-gal as indicated. Expression plasmids for ACTN4 (WT) and FSGS-linked ACTN4 mutants were transfected in increasing amount. After 48 h of transfection, the cells were treated with 100 nM AT-RA. Luciferase activity was measured and normalized to β-gal activity. The expression levels ACTN4 (WT) and FSGS-linked ACTN4 mutants were similar (data not shown). C, FSGS-linked ACTN4 mutants fail to activate transcription mediated by GRα. Transient transfection reporter assays were performed as described in Figure 39B, except that plasmids expressing GRα were cotransfected along with the expression constructs for ACTN4 (WT) and FSGS-linked ACTN4 mutants and cells were treated with or without 100 nM dexamethasone.
Expression plasmids encoding ACTN4 (WT) or FSGS-linked ACTN4 mutants were transfected with or without RARα along with a reporter construct harboring a RARE into HEK293 cells. Figure 39B shows that wild-type ACTN4 alone modestly activated RARα-mediated reporter activity (lanes 2-3) while the FSGS-linked ACTN4 mutants did not (lanes 4-9). Co-expression of wild-type ACTN4 with RARα enhanced the reporter activity significantly (lanes 11 & 12). In contrast, FSGS-linked ACTN4 mutants inhibited ligand-induced transcriptional activation by RARα (lanes 4-9 and 13-18) suggesting that FSGS-linked ACTN4 mutants are dominant-negative to endogenous wild-type ACTN4. Furthermore, we carried out transient transfection assays with a GRE-containing reporter construct. As shown in Figure 39C, ACTN4 (WT) potentiated GRα mediated transcriptional activity (lane 11-12) while the FSGS-linked ACTN4 mutants did not (lanes 13-18). Taken together, these data indicate that expression of FSGS-linked ACTN4 mutants results in inhibition of hormone-mediated RARα and GRα transcriptional activation. FSGS mutations are located C-terminal to the LXXLL receptor interacting motif. Nonetheless, structural studies suggest that the SR repeats 1-4 of ACTN4 form a dimmerization interface. As such, FSGS-linked mutations may alter the quaternary structure of ACTN4 such that FSGS-linked ACTN4 mutants may acquire aberrant interactions. In this case, we expect that the FSGS-linked mutants would interact with nuclear receptors differently from the wild-type ACTN4. To test this hypothesis, we examined the interaction between wild-type ACTN4 and FSGS-linked ACTN4 mutants with nuclear receptors by GST pull-down assays. As shown in Figure 40A-B, FSGS-
linked ACTN4 mutants lost the ability to interact with RARα and GRα. These data indicate that although FSGS-causing mutations are located away from the LXXLL motif these mutations may change more globally the conformation of the protein and disrupt association with nuclear receptors.

The next question we asked was what is the functional and physiological significance of GR and ACTN4 interaction in the context of podocyte cells? Studies from mice and immortalized cultured mouse podocytes (MPCs) indicated that glucocorticoids may protect or restore podocytes from injury and help in maintaining the differentiated state of podocytes [132]. Based on these observations we hypothesized that GR and ACTN4 together are required for the differentiation of podocytes. In order to test this hypothesis, GR was knocked down by two individual siRNA (siGR-1 and siGR-2) after switching HPCs from 33°C to 37°C (from permissive to non-permissive temperature) followed by dexamethasone treatment according to the procedure mentioned in “Experimental Procedures”. The knockdown efficiency is shown by Western blotting and immunostaining in Figure 41A-B. The differentiation status of HPCs was monitored using synaptopodin antibodies, as synaptopodin is a differentiation marker for podocytes. As shown in the Figure 42A, we observed the expected difference in expression level and staining pattern of synaptopodin between undifferentiated and differentiated HPCs. Furthermore, there was a significant difference in the staining pattern of synaptopodin between siCtrl and siGR transfected cells. The HPCs with siCtrl transfected and dexamethasone treated were more spread out with more prominent actin stress fibers compared to siCtrl treated with vehicle alone (Figure 42-43). However, the siGR
knockdown cells are smaller and show a distinct staining pattern for synaptopodin. Altogether, these findings suggest that GR may be required for the differentiation of HPCs. The next question was whether knockdown ACTN4 also showed the similar phenotype during differentiation. This is still under investigation. We further hypothesized that ACTN4 and GR are involved in transcriptional regulation of a subset of genes involved in regulating several aspects of podocyte biology. Nephrin is one of the important and integral components of SD and mutations in nephrin are associated with congenital nephrotic syndrome of the Finnish type [124]. Furthermore, deletion of nephrin in mice results in proteinuria and neonatal lethality [164, 233]. As an integral component of the SD, the expression level of nephrin is critical for the filtration activity of the SD and disease development. Despite intense studies, the mechanism by which nephrin expression in podocytes is regulated is not well understood. Several recent reports suggested that NRs play an important role in regulating nephrin expression in mice and MPCs. Specifically, treatment of MPCs with nuclear receptor ligands protects/rescues cells from injury suggesting a role of nuclear receptors in the kidney diseases. Furthermore, retinoic acid, and dexamethasone up-regulate the expression of nephrin in cultured podocytes [134, 136, 234]. Our observations that ACTN4 acts as co-activator for NRs and the fact that nephrin expression is significantly decreased in ACTN4 (K255E) transgenic mice suggests that ACTN4 and nuclear receptors together form a transcriptional network to regulate the expression of nephrin. In order to analyze whether nephrin expression is regulated by GR and dexamethasone we used undifferentiated human podocytes, allowed them to differentiate followed by treatment
Figure 40: FSGS-linked ACTN4 mutants lose their interactions with nuclear hormone receptors. A, FSGS-linked ACTN4 mutants were unable to interact with RARα compared to wild-type ACTN4 in vitro. GST pull-downs were carried out by incubating GST-ACTN4 (WT) or GST-ACTN4 (K228E), GST-ACTN4 (T232I) and GST-ACTN4 (S235P) with whole cell lysates prepared from HEK293 cells overexpressing HA-RARα. Pull-down fractions were subjected to SDS-PAGE followed by Western blotting with α-HA antibodies (top panel). Five percent of input is shown in lane 1. B, FSGS-linked ACTN4 mutants lose their interaction with GRα in mammalian cells. GST pull-downs were carried out as described in Figure 40A except that HEK293 cells expressing HA-GRα were used. Bottom panles show coomassie blue staining showing the equal expression of the GST beads.
with or without dexamethasone. As shown in Figure 44A, there is an increase in the expression of nephrin after treatment of dexamethasone. In order to find out whether nephrin is regulated by ACTN4, we carried out an ACTN4 knockdown using siACTN4 in HPCs. As shown in Figure 44B, there was a significant reduction in the mRNA expression level of nephrin after ACTN4 knockdown. Our preliminary data together with the previous data in the field suggest that ACTN4 and GR may be involved in the transcriptional regulation of nephrin in HPCs; however, still we are working on number of unanswered questions given below:

1. What is the effect of GR or ACTN4 knockdown on mRNA expression level of nephrin in the presence or absence of dexamethasone? Our observation that the mRNA expression level of nephrin increases after treating HPCs with dexamethasone suggests that GR is involved in the regulation of nephrin mRNA expression level. In order to confirm this observation, it will be important to analyze mRNA expression level of nephrin after knockdown of GR with two individual siRNAs, followed by treatment with or without dexamethasone in differentiated HPCs using a protocol described in “Experimental Procedure”. Furthermore, nephrin expression will also be analyzed after ACTN4 knockdown with or without dexamethasone treatment.

2. What are the sequences in the nephrin promoter that are responsive to GR, and ACTN4? Treatment of HPCs with various ligands including retinoic acid and dexamethasone increases the nephrin mRNA level, but the mechanism underlying this regulation is not known [134, 136, 234]. We hypothesize that nuclear
receptors such as GR and RAR associate with the nephrin promoter and regulate its activity directly. To test our hypothesis we will carry out transient transfection assays using the reporter constructs harboring the 5’regulatory region of human nephrin promoter (2.5kb) fused with the luciferase reporter in HEK293 cells. A series of deletion constructs will also be used to further map the GR binding sites on the nephrin promoter. Furthermore, the same constructs will also be used to check ACTN4 responsiveness in transient luciferase assays. Once putative binding sites are identified, the data will be confirmed using ChIP assays. For ChIP assays, HPCs will be allowed to differentiate for 8-10 days in the presence or absence of dexamethasone. Chromatin immunoprecipitation will be carried out using anti-ACTN4 and anti-GR antibodies. Following immunoprecipitation, the DNA will be extracted and PCR will be carried out using primers flanking the GR binding sites. These experiments will provide a mechanism by which GR and ACTN4 act together to regulate nephrin at transcriptional level.

3. Do FSGS mutants affect nephrin expression at the mRNA level in the presence or absence of dexamethasone in HPCs? In order to answer this question, we will use a HPCs cell line with a stably integrated copy of either HA-ACTN4 (WT) or HA-ACTN4 (K228E). Stable clones will be generated using HA-ACTN4 (WT) or HA-ACTN4 (K228E) cloned in lentiviral vector (pLM-CMV-H4-puro-PL3-seq-verif) and selected using puromycin under undifferentiated conditions. In order to analyze the effect of FSGS mutant on nephrin expression, the HPCs with stably integrated HA-ACTN4 (WT) or HA-ACTN4 (K228E) will be allowed to
differentiate for 8-10 days in the presence or absence of dexamethasone. The RNA will be extracted and analyzed for nephrin mRNA expression level using real time qRT-PCR. Since we have shown that FSGS-linked ACTN4 mutants are defective in regulating transcriptional activity mediated by GREs, we anticipate that stably integrated HA-ACTN4 (K228E) HPCs will show less mRNA expression level of nephrin compared to clones expressing HA-ACTN4 (WT).

4. Are FSGS-linked ACTN4 mutants poorly recruited to the nephrin promoter compared to wild-type ACTN4? This question can be answered using the above mentioned stable cell lines expressing either HA-ACTN4 (WT) or HA-ACTN4 (K228E). These cells will be allowed to differentiate for 8-10 days in the presence or absence of dexamethasone. ChIP assays will be carried out using α-HA antibodies and α-GR antibodies and DNA will be amplified using primers flanking the GR binding sites.

In conclusion, interest in the function of ACTN4 has intensified due to its association with familial forms of the common kidney disease FSGS. To date, many disease causing mutations in ACTN4 have been identified, and research into possible mechanisms of disease have focused on their role in podocyte cytoskeleton dysfunction [151,156]. Our findings provide an alternative or additional pathogenic mechanism, linking aberrant transcriptional regulation by nuclear receptors with FSGS-link ACTN4 mutations. In general, little is known about the important co-regulators involved in nuclear receptor regulation in podocytes despite a long standing association of hormones and nuclear receptors with normal kidney physiology. Evidence from in vitro and in vivo studies have
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 [130, 131, 134, 135, 232]. 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 [130, 134, and 136]. 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 the effect of the FSGS-linked ACTN4 mutants on both cytoskeletal 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 since the FSGS mutants are positioned more than 100 amino acids downstream of the LXXLL receptor interaction motif [222]. These results may support a model in which FSGS-linked ACTN4 mutations alter the global
structure of ACTN4 such that the LXXLL 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 between ACTN4, nuclear receptors and the actin cytoskeleton will be critical in determining ACTN4's role in mediating these integral events in normal podocyte homeostasis and disease responses.

In summary, our observation that ACTN4 potentiates transcriptional activation by nuclear receptors, and that FSGS-linked ACTN4 mutants are defective in these transcriptional responses, suggest the ability of ACTN4 to regulate transcription is critical for normal development and maintenance of podocytes. Our data suggest that FSGS caused by ACTN4 mutations might be partly due to the altered nuclear receptor-mediated transcription, and further investigation in the role ACTN4 in mediating nuclear receptor function in podocytes is needed. This connection to podocyte function may be relevant to a broader range of kidney diseases than those associated with ACTN4 mutations, as therapeutic use of glucocorticoids is effective in the treatment of other forms of kidney disease mediated by injury.
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Figure 41: GR knockdown in HPCs. A, HPCs cells were transfected with either siGRα-1 or siCtrl before differentiation. Forty eight hours post-transfection cells were treated with or without 100 nM of dexamethasone for two days. The cells were allowed to differentiate for a total of five days. After five days of *in vitro* differentiation, the cells were immunostained with α-GR antibodies followed by microscopy. B, Western blot showing the knockdown efficiency of GR in differentiated HPCs. Knockdown was done using two individual siRNA (siGR-1 and siGR-2) against GR.
**Figure 42**: Knockdown of GR affects differentiation of HPCs *in vitro*. HPCs cells were transfected with either siGRα-1 or siCtrl before differentiation. Forty eight hours post-transfection cells were treated with or without 100 nM of dexamethasone for two days. The cells were allowed to differentiate for total of five days. After five days of *in vitro* differentiation, the cells were immunostained with α-synaptotodin antibody followed by microscopy. In order to check the specificity of the antibody undifferentiated HPCs were also stained with α-synaptotodin antibody. Nuclear staining may be due to the cross reactivity of antibody with some nuclear protein.
Figure 43: Knockdown of GR affects differentiation of HPCs *in vitro*. The experiment was carried out as described in Figure 42 except that a second siRNA (siGRα-2) was used to knockdown GRα.
A

![Graph showing fold change for different treatments.]

B

![Bar chart showing fold change for ACTN4 and Nephrin with siCtrl and siACTN4 conditions.]
Figure 44: Dexamethasone treatment enhances the nephrin expression in differentiated HPCs. A, Undifferentiated HPCs were allowed to differentiate followed by treatment with or without dexamethasone for 48 h as mentioned in Experimental Procedures. The RNA was extracted and relative expression level of nephrin was analyzed by qRT-PCR. B, Knockdown ACTN4 decreases the mRNA expression level of nephrin in HPCs. ACTN4 was knocked down in undifferentiated HPCs as mentioned in Experimental Procedures. RNA was isolated and the relative expression level of ACTN4 and nephrin were detected by qRT-PCR.
CHAPTER 5: THE HISTONE DEACYTLASE 7 (HDAC7) REGULATES DIFFERENTIATION BY REGULATING EXPRESSION OF CD2AP, A FSGS-ASSOCIATED GENE

ABSTRACT

Podocytes are highly differentiated cells in the renal glomerulus that possess primary and secondary foot processes responsible for their function as the filtration barrier of the glomerulus. Familial focal segmental glomerulosclerosis (FSGS) is characterized by proteinuria (proteins in the urine), a common phenotype of podocyte injury. Mapping studies have indicated that mutations in several genes, including alpha actinin 4 (ACTN4), are tightly linked to FSGS and likely contribute directly to the disease. Our understanding on the mechanisms underlying ACTN4-linked FSGS have progressed, but are far from complete. Identification of ACTN4-interacting partners in podocytes and elucidating the functional significance of these interactions is a key to a better understanding of the pathogenesis underlying this disease and may have therapeutic implications. We have previously identified novel interactions between ACTN4 and several transcription factors including histone deacetylase 7 (HDAC7). This proposal is aimed at elucidating the functional significance of this association and the role of HDAC7 in podocytes.
INTRODUCTION

HDAC7 is a member of the class IIa histone deacetylases (HDACs) [175] that include HDAC4, -5, -7, and -9 and contain carboxy-terminal catalytic domains with 80% amino acid identity. An important feature of class II HDACs is their ability to shuttle between the nucleus and the cytoplasm [180, 183, 235]. All class II HDACs contain a conserved amino-terminal nuclear localization sequence (NLS) and a putative carboxy-terminal nuclear export sequence (NES). The activity of the NLS and the putative NES is modulated by a mechanism that largely depends on the activity of cellular factors, including 14-3-3, CRM1 (or exportin 1), CaMKs, and PKD [37, 181, 183, 184, 223, 236].

Class IIa HDACs are important regulators in cell differentiation and animal development, partly due to their association with MEF2 transcription factors [175]. Class IIa HDAC shuttling between the nucleus and the cytoplasm is an important regulatory mechanism that controls the activity of transcription factors such as MEF2s [175]. Knockout of HDAC7 causes embryonic lethality, partly due to a failure of cell-cell adhesion of endothelial cells and dilation and rupture of blood vessels [195]. We have identified class IIa HDACs as ACTN4-interacting proteins and demonstrated that ACTN4 can potentiate MEF2 activity, partly by antagonizing HDAC7 activity. We have recently shown that in response to TNFα, class IIa HDACs are capable of potentiating sumoylation of the protein promyelocytic leukemia protein (PML) and are essential for PML nuclear body (NB) formation [237, 238]. We further demonstrated that knockdown of HDAC7 inhibits endothelial cell migration and causes reorganization of the actin cytoskeleton (unpublished data). Based on these data, we hypothesize that HDAC7 may regulate
cytoskeleton architecture by 1) interacting with actin-binding proteins including ACTN4, and/or by 2) regulating genes whose products are critical for maintaining the actin cytoskeleton. Indeed, our gene expression microarray studies in HUVECs indicate that CD2AP, a protein encoded by a gene associated with FSGS, is a HDAC7 target gene.

Mutations in ACTN4 have been linked to FSGS, establishing its role in podocyte development. We have shown that HDAC7 and ACTN4 interact in vitro and in mammalian cells [73] and we hypothesize that this interaction affects ACTN4 function and thus podocyte development.

In this study, we show that: 1) endogenous HDAC7 and ACTN4 interact in podocytes, 2) pS178-HDAC7 (S178-phosphorylated HDAC7) partially colocalizes with synaptopodin in differentiated podocytes, 3) pS479-HDAC7 is localized in the nucleus and plasma membranes of differentiated podocytes, 4) Knockdown of HDAC7 in HPCs results in altered cell morphology. Based on these observations, we conclude that HDAC7 plays a role in maintaining actin cytoskeleton of podocytes. In this chapter, we will characterize the interaction between HDAC7 and ACTN4 and elucidate the physiological significance of this interaction.
EXPERIMENTAL PROCEDURES

Plasmid construction: CMX-HA-HDAC7 and its variants have been previously described [180]. The retroviral expression plasmids pBabe-FLAG-HDAC7 and its variants were generated by PCR using HDAC7 cDNA as a template and subcloned into pBabe-FLAG vector.

Antibodies and chemicals: ACTN4 and HDAC7 antibodies have been previously described [73, 239]. Alexa Fluor® 594 Phalldoin probe was purchased from Life Technologies (AI2381).

Coimmunoprecipitations: To detect the interaction between ACTN4 and HDAC7, undifferentiated SV-40 T antigen immoratlized HPCs were grown at permissive temperature (33°C). The cells were lysed with NETN buffer with protease inhibitors. 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 α-HDAC7 (115-129) antibodies and immunoprecipitated fractions were analyzed with α-ACTN4 antibodies.

Confocal microscopy: Transfected cells were fixed in 3.7% paraformaldehyde (PFA) in PBS for 30 minutes 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%) + 0.1% Tween-20 solution (ABB) for 60 min. Incubation with primary antibodies was carried out for 120 min in ABB. The cells were washed three times in PBS, and the secondary antibodies were added for
60 min in the dark, at room temperature in ABB. Cover slips were mounted to slides using Vectashield mounting medium with DAPI (H-1200, Vector Laboratories, Inc.). Confocal images were acquired using a Zeiss LSM 510 inverted laser-scanning confocal microscope. A 63X numerical aperture of 1.4 oil immersion planapochromat objective was used for all experiments. For endogenous and transiently transfected ACTN4, images of Alexa Fluor 594 were collected using a 633-nm excitation light from a He/Ne2 laser, a 633-nm dichroic mirror and 650-nm long pass filter. For endogenous ACTN4, images of Alexa Fluor 488 were collected using a 488-nm excitation light from an argon laser, a 488-nm dichroic mirror and 500-550-nm band pass barrier filter. All DAPI stained nuclear images were collected using a Coherent Mira-F-V5-XW-220 (Verdi 5W) Ti:sapphire laser tuned at 750-nm, a 700-nm dichroic mirror and a 390-465 nm band pass barrier filter. The primary antibodies used are: purified α-ACTN4 polyclonal, α-HDAC7 (115-129) polyclonal, α-pS178, α-pS344, and α-pS479 polyclonal antibodies. The secondary antibodies used are from Molecular Probes (α-mouse or α-rabbit Alexa Fluor 488, α-rabbit Alexa Fluor 594, and mouse Alexa Fluor 594). Actin fibers were stained using Alexa Fluor® 594 Phalloidin.

Isolation of total RNA and real-time PCR: For siRNA transfection of HDAC7, undifferentiated HPCs were transfected with a non-targeting siRNA siCtrl (D-001810-01-50) or siHDAC7 (smart pool) at a final concentration of 100 nM according to manufacturer’s protocol (Dharmacon). For siRNA transfection of ACTN4, undifferentiated HPCs were transfected with a control siRNA (siCtrl, 1027415), or siACTN4-(mix) at a final concentration of 10 nM using GeneSolution siRNA (Qiagen)
according to manufacturer’s protocol. Seventy-two hours post transfection, RNA was harvested using a kit form USB according to the manufacturer’s protocol. cDNA was synthesized from 1 µg of total RNA according to the manufacturer’s instructions (LifeTechnologies). Gene expression levels were determined by qRT-PCR using Real Time PCR System (BioRad) with the following primers:

GAPDH: 5’-GAAGGTGAAGGTCGGAGT-3’, 5’-GAAGATGGTGATGGGATTTC-3’;
HDAC7: 5’-AGCCCCTACCTCATCCACAG-3’, 5’-TGGGCTTCTGCTTCTTCAAC-3’;
CD2AP: 5’-TCAACACCTCCAGTGCTT-3’, 5’-CCGATCATCCTTTTCAGTGC-3’.

Relative changes in gene expression were calculated using the ΔΔCt method. Each value is representative of three replicates and all the experiments were repeated twice.

Isolation of primary podocytes from mice: Mice were euthanized according to the standard procedures and kidneys were removed from the mice with sterile razor blade on sterile surface and mashed to pulp at room temperature. Mashed pulp was transferred to a first sieve (0.30-0.50mm opening) followed by forcing the pulp to the next sieve (90µm opening) with the help of a syringe plunger. Using the plunger and 1XPBS, the pulp was again pushed through the second sieve to the third sieve (45µm). On the 45µm sieve, the kidney pulp was washed thoroughly with 1XPBS, holding sieve an angle, glomeruli were collected and transferred to 15ml conical tube. The glomeruli were pelleted at 500RPM followed by digestion with 1mg/ml collagenase at 37°C for 10 min. Glomeruli were washed again extensively with 1XPBS to remove debris and collagenase. After washing,
the isolated glomeruli were plated in medium RPMI1640 supplemented with 10% fetal bovine serum and 2% 50 units/ml penicillin G and 50 μg/ml streptomycin sulfate.

Cell culture: HEK293 were grown in Eagle Dulbecco’s modified medium (DMEM) medium supplemented with 10% Fetal Bovine Serum, 100 U/ml penicillin/streptomycin (GIBCO) and 50μg/ml streptomycin sulfate at 37°C. Human immortalized podocyte (HPC) cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco), 1% 100 U/ml penicillin/streptomycin (Gibco) and insulin, transferring, selenite (ITS) (Sigma Chemicals). Cells were grown at the permissive temperature of 33°C to promote cell propagation as undifferentiated cells. Cells were then moved to 37°C to induce differentiation. Differentiation takes around 10-14 days. A temperature sensitive SV-40 T antigen-mediated conditionally immortalized mouse podocyte cell line was maintained at 33°C in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin (Gibco) with or without recombinant mouse γ-interferon (Sigma) in humidified incubators with air-5% CO₂. MPCs were propagated at 33°C in the presence of recombinant mouse γ-interferon (10 U/ml) to maintain the undifferentiated state. Removal of γ-interferon and temperature switch to 37°C inactivated the SV40 T antigen and induced the podocytes to differentiate.
RESULTS AND DISCUSSION

To determine whether HDAC7 plays a role in podocytes, we first verified its expression in mouse primary podocytes by immunostaining followed by confocal microscopy (Figure 45A). In addition we followed the subcellular localization of HDAC7 during podocyte differentiation. Synaptopodin was used as a marker for differentiated podocytes. Figure 45A (right panel) shows that HDAC7 is present in MPCs and localized in both the nucleus and the cytoplasm of mouse primary podocytes. Phosphorylation has been shown to regulate the subcellular localization of class IIa HDACs in myocyte differentiation [191, 193]. To test whether subcellular distribution of HDAC7 is regulated by phosphorylation in podocytes, we examined the subcellular distribution of phosphorylated HDAC7 by immunofluorescence followed by confocal microscopy. We found that while anti-pS178 and anti-S479 antibodies exhibited primarily cytoplasmic staining while anti-pS344 antibody displayed more nuclear staining (Figure 45B-D). Importantly, these phosphorylated antibodies also cross react with HDAC4 and HDAC5 due to high degrees of conservation of the amino acid sequences flanking the phosphorylated residues. Intriguingly, HDAC7 and synaptopodin partially colocalize. Furthermore, we also used conditionally immortalized mouse and human podocyte cell lines to study podocyte biology (MPCs and HPCs).
**Figure 45.** Localization of HDAC7 in primary podocytes isolated from mice. A, Expression of HDAC7 in primary podocytes. Left Panel shows the glomeruli isolated from mice kidney using the protocol mentioned in “Experimental Procedures”. The picture shows the isolated glomeruli from two different fields after culturing for 4 and 10 days in growth medium. The arrow indicates the FPs from the podocytes. Podocytes can be seen after 10 days of culturing. Right Panel: Primary podocytes were fixed and immunostained using α-HDAC7 (115-129) antibodies and α-synaptopodin antibodies followed by confocal microscopy. Note that HDAC7 is present in both the nucleus and the cytoplasm. B-D, Primary podocytes are immunostained with α-pS178, α-pS344, α-pS478, and α-synaptopodin antibodies. Pictures were taken under two different exposure times. Pictures a-f were taken using longer exposure times whereas pictures g-l were taken using shorter exposure times. Note that all three antibodies against phosphorylated HDAC7 show distinct staining pattern. The arrow indicate the partial co-localization of HDAC7 and synaptopodin.
**Figure 46**: Interaction of HDAC7 and ACTN4 in MPCs. A, Expression of HDAC7 in undifferentiated HPCs. Western blot using α-HDAC7 antibodies show that HDAC7 is present in undifferentiated and differentiated MPCs. B, Subcellular distribution of HDAC7 and ACTN4. Undifferentiated MPCs were harvested and fractionated according to the manufacturer’s protocol (Pierce). C, HDAC7 associates with ACTN4 in undifferentiated MPCs. Whole cell extracts were prepared from undifferentiated MPCs followed by immunoprecipitation with HDAC7 (115-129) antibodies or IgG and Western blotting with α-ACTN4 antibodies.
We further examined the subcellular distribution of HDAC7 in undifferentiated MPC by subcellular fractionation. We found that HDAC7 is primarily localized in the cytosol with a fraction localized in the cytoplasm (C), plasma membranes/organelles (M/O), nucleus (N), and cytoskeletal matrix (CM) (Figure 46A). Similar patterns were observed for ACTN4. As controls, we included HDAC1 and flotillin. Flotillin is a protein often found in cytoskeletal matrix and membranes [240]. HDAC1 is a nuclear marker. To test whether endogenous HDAC7 and ACTN4 interact in MPCs, we carried out coimmunoprecipitation experiments. Figure 46B shows that a fraction of ACTN4 interacts with HDAC7 in MPC. Taken together, these data suggest that HDAC7 is widely distributed in podocytes and that it is found in complexes with ACTN4.

We next examined the subcellular distribution of HDAC7 in undifferentiated and differentiated podocytes by confocal microscopy. Synaptopodin was used as a marker of differentiation. Figure 47A demonstrates that HDAC7 is localized in both the nucleus and the cytosol of undifferentiated and differentiated podocytes. We also observed a fraction of HDAC7 colocalized with synaptopodin. This result is consistent with our data demonstrating that ACTN4 intercats with HDAC7 and is known to interact with with synaptopodin [241, 242]. The subcellular localization of HDAC7 in MPCs is also consistent with primary podocytes. Moreover, anti-pS178 shows nuclear and cytoplasmic staining in both undifferentiated and differentiated MPCs (Figure 47B) in addition to actin stress fiber-like staining in differentiated podocytes.
Figure 47. Subcellular localization of HDAC7 during MPCs differentiation in vitro:
Mouse immortalized podocytes were induced to differentiate for 14 days as described in Experimental Procedures. α-synaptopodin antibodies were used to show the differentiated state. Cells were fixed and stained with anti-HDAC7 (115-129) (panel A), anti-pS178 (panel B), anti-pS344 (panel C), and anti-pS479 (panel D) followed by confocal microscopy. Note that α-pS178 antibodies show actin stress fiber staining, whereas anti-pS344 antibodies exhibit predominantly nuclear staining. In addition to nuclear staining, anti-pS479 antibodies show more cell-cell junction staining (arrow). Both anti-pS178 and anti-pS479 antibodies show partial colocalization with synaptopodin.
Figure 48. Subcellular localization of HDAC7 in undifferentiated HPCs. HPCs were induced to differentiate for 14 days as described in Experimental Procedures. A, Cells were fixed and stained with α-HDAC7 (115-129) antibodies. B), HPCs were immunostained with α-pS178, α-pS344, and α-pS479 antibodies followed by confocal microscopy. All the images are shown with two z-plane sections; the one is a central section to show nuclear staining (a-c, g-I, and m-o), and the other one is a section proximal to the basal membrane (d-f, j-l and p-r) showing the cytoskeleton staining.
In contrast, staining with α-pS344-HDAC7 antibodies was predominantly localized in the nucleus of undifferentiated and differentiated MPCs (Figure 47C). Anti-pS479 antibodies displayed a pattern distinct from that of pS178 and pS344 antibodies, showing pS479-HDAC7 enriched in the nucleus and plasma membrane (Figure 47D). This distribution is similar to that of nephrin and P-cadherin. Together, we conclude that distinct forms of HDAC7 are localized in the nucleus, cytosol, stress fibres and plasma membrane of differentiated MPCs. We have also examined the subcellular distribution of HDAC7 and phosphorylated HDAC7 in immortalized HPCs. Figure 48 shows that the staining patterns of HDAC7 were similar to those found in MPCs (Figure 47) except that more nuclear HDAC7 was observed in the former.

Knockdown of HDAC7 alters cell morphology: Podocytes derived from ACTN4−/− knockout mice are defective in spreading and adhesion [151]. To investigate the role of ACTN4 in human podocytes, we examined whether knockdown of ACTN4 had effects on cell morphology or actin cytoskeleton of undifferentiated cells. Figure 49A shows that anti-ACTN4 antibodies largely co-stain with phalloidin (actin filament marker) at cytoplasmic membranes, in the cytoplasm, and in the nucleus. Knockdown of ACTN4 (marked by a white arrow) resulted in a loss of actin staining in the cytoplasmic membranes (compare panel c to panel i). Knockdown of HDAC7 (Figure 49B, white arrow), on the other hand, resulted in an elongated cell morphology with actin staining enriched in the cytoplasmic membranes. These observations suggest that HDAC7 and ACTN4 have distinct roles in regulating actin cytoskeletons. Indeed, we have demonstrated that while HDAC7 represses transcription,
Figure 49. ACTN4 and HDAC7 knockdown affect the cytoskeleton of HPCs. A, Effect of ACTN4 knockdown on HPCs. Undifferentiated HPCs were transfected with siControl or siACTN4. Seventy two post transfection, the cells were fixed and immunostained with α-ACTN4 antibodies (green) and phallodin stain followed by microscopy. B, Effect of HDAC7 knockdown on HPCs. HPCs cells were transfected with siControl or siHDAC7. Immunostaining was performed using α-HDAC7 (115-129) antibodies and α-phalloidin stain followed by immunoflourescence microscopy. DNA was visualized by 4’, 6-diamidino-2-phenylindole (DAPI) staining. The arrows point out the cell with the knockdown of either ACTN4 or HDAC7.
ACTN4 activates transcription. Furthermore, we found that in endothelial cells, knockdown of HDAC7 inhibits tube formation and migration, whereas knockdown of ACTN4 promotes tube formation and migration (unpublished data). Our model is that HDAC7 and ACTN4 mutually antagonizes each other’s function.

HDAC7 directly inhibits CD2AP (an important SD component) gene transcription. We have previously shown that ACTN4 positively regulates CD2AP expression and its promoter activity (unpublished data) and that ACTN4 antagonizes HDAC7 in regulating MEF2 and ERα transcriptional activity [73, 222]. These observations raised the possibility that HDAC7 might negatively regulate CD2AP expression. To test this, we first knocked down HDAC7 by siRNA and found that knockdown of HDAC7 increased CD2AP mRNA accumulation (Figure 50A). In contrast, overexpression of wild-type HDAC7 decreased CD2AP mRNA levels in HEK293 cells (Figure 50B). Furthermore, overexpression of an HDAC7 mutant defective in cytoplasmic localization resulted in a significant loss of CD2AP mRNA expression.

Stable expression of a constitutive nuclear HDAC7 mutant blocks differentiation of HPCs in vitro: Haploinsufficiency of CD2AP is associated with glomerular disease susceptibility [138] and may affect podocyte development. We reasoned that aberrant expression of CD2AP due to repression by HDAC7 may have an effect on HPCs. To test this, we determined whether stable expression of a constitutively nuclear HDAC7 has an effect on HPCs. We have shown that HDAC7 (mNES, mutation at the C-terminal nuclear export sequence) is confined in the nucleus. To determine whether this mutant has an effect on podocyte differentiation, we established stable podocyte cell lines expressing
FLAG-HDAC7 (WT) or FLAG-HDAC7 (mNES). As controls, a parental cells and stable line expressing vector alone was also included. These stable cell lines were grown and induced to differentiate. Figure 51 shows that while stable expression of HDAC7 (WT) did not effect podocyte differentiation, expression of HDAC7 (S/A) or HDAC7 (mNES) significantly blocked differentiation.

Based on these observations, we conclude that: 1) HDAC7 is expressed in both undifferentiated and differentiated MPC and HPCs, 2) HDAC7 becomes more cytoplasmic as MPCs differentiate, 3) HDAC7 (or HDAC4/5) is phosphorylated at S178, S344, and S479, 4) pS178-HDAC7 (or HDAC4) can be found in the nucleus, cytoplasm, and the plasma membrane, 5) pS344-HDAC7 is predominantly localized in the nucleus, 6) pS479-HDAC7 is localized in the nucleus and cell-cell junctions, similar to that of the Zona Occludin protein (ZO-1), 7) Stable expression of HDAC7 nuclear mutants blocks HPC differentiation (Figure 51), 8) Knockdown of ACTN4 or HDAC7 in HPCs resulted in altered cell morphology (Figure 49). We hypothesize that HDAC7 plays a role in coordinating the cytoskeleton architecture of podocytes and that some cytoskeleton proteins such as ACTN4 may sequester HDAC7 to the cytoplasm. These data suggest that HDAC7 plays a role in differentiation process of podocytes. In order to further test this hypothesis we will address following questions:
Figure 50: HDAC7 knockdown enhances the accumulation of CD2AP mRNA. A, HDAC7 was knocked down in undifferentiated HPCs using siRNA against HDAC7. RNA was isolated as described in “Experimental Procedures” and relative expression level of HDAC7 and CD2AP were determined by qRT-PCR. The mRNA levels of HDAC7 and CD2AP were normalized to 18S mRNA levels B, HEK293 cells were transfected with plasmids expressing F-vector or F-WT-HDAC7 or F-NES-HDAC7 mutant for 48 h. Levels of CD2AP mRNA relative to 18S mRNA were determined by real-time quantitative PCR.
**Figure 51**: NES-HDAC7 mutant is defective in differentiation. HPCs stably expressing FLAG-vector, FLAG-WT-HDAC7 and FLAG-NES-HDAC7 mutant were allowed to differentiate for 5 days followed by immunostaining with α-pS178 HDAC7 antibodies.
1) Does HDAC7 play a role in differentiation in \textit{in vitro}? To test whether HDAC7 is essential for podocyte differentiation, we will determine whether knockdown of HDAC7 has effects on podocyte differentiation. As a control, we will include a non-target shRNA. shRNA against HDAC7 and control shRNA will be used to test the effects of HDAC7 knockdown on podocyte differentiation. These stable cells will be grown and induced to differentiation for 14 days. The ability of the stable clones to differentiate will be examined by immunostaining with synaptopodin antibodies. Actin fibers will be stained using Alexa Fluor® 594 Phalloidin.

2) Since our data suggest that HDAC7 regulates CD2AP expression, it would be important to find out the mRNA and protein expression level of CD2AP in HPCs stably expressing FLAG-pBABE vector, FLAG-pBABE WT-HDAC7 and FLAG-pBABE NES-HDAC7. Also, it will be important to know whether there is any effect of NES-HDAC7 on expression of other important SD proteins including nephrin, podocin, and TRPC6.

3) What region of CD2AP promoter is associated with HDAC7? This question would be addressed using ChIP assays employing \( \alpha \)-HDAC7 (115-129) antibodies, in undifferentiated and differentiated HPCs. Also, it will be important to know how HDAC7 is recruited to the CD2AP promoter. This will be addressed by predicting putative transcription factor binding sites on corresponding fragment associated with HDAC7 by using genomatix software. Furthermore, recruitment of the specific transcription factor to the CD2AP
promoter will be further confirmed by ChIP assays. In another set of experiments, fragments flanking 2kb upstream and 300 base pairs downstream of the transcription start site of human CD2AP gene will be fused with TK-Luc reporter and this construct will be used in luciferase assays. Reporter assays will be done to confirm the regulation of the CD2AP promoter by wild-type HDAC7 and the above described mutants of HDAC7 in HEK293 cells.

4) Whether HDAC7 interacts with proteins involved in formation of the slit diaphragm (SD)? Components of the slit diaphragm play an important role in podocyte development and in the regulation of its morphology. Several components and their interacting proteins including nephrin, P-cadherin, podocin, and CD2AP have been shown to be critical in regulating podocyte function. Mutations in these genes are associated with severe glomerular diseases. Our observations that HDAC7 interacts with ACTN4 and pS479-HDAC7 exhibits a similar staining pattern to that of suggest that HDAC7 is a potential interacting partner with components of the SD. Currently, only few proteins have been identified that interact with components of the SD in podocytes. Identification of HDAC7 as an interacting protein for components of SD will provide an additional avenue of investigation in podocyte biology. To test this, we will carry out immunoprecipitations on primary podocytes from mice and on differentiated HPCs.
5) CD2AP is known to be involved in cell proliferation. In order to define the functional relevance of regulation of CD2AP by HDAC7, proliferation assays will be carried out after knocking down CD2AP and HDAC7 in HPCs.
CHAPTER 6: DISCUSSION AND FUTURE DIRECTIONS

It has been shown by previous studies that actin and actin binding proteins other than ACTN4 play an important role in transcription in addition to functions as cytoskeleton proteins [206, 224]. Also, actin and actin related proteins have been shown to be present in complexes associated with transcriptional machinery in the nucleus [225, 243]. Our data, together with the previous data in the field, show that ACTN4 is present in the nucleus in MCF-7 cells and in podocytes. The first important question is what ACTN4 does in the nucleus and the simplest explanation is that it may bind nuclear actin to affect nuclear structure, architecture or volume. Our data suggest that ACTN4 plays a more direct role in transcriptional regulation rather than just binding to the nuclear actin. Previously, we have shown that both ACTN4 (full-length) and ACTN4 (Iso) are capable of potentiating transcription mediated by MEF2 transcription factors [73]. Furthermore, our data show that ACTN4 physically interacts with nuclear receptors including ERα, VDR, RAR and GRα in a hormone and NR box (LXXAA) dependent manner, suggesting a more direct role for ACTN4 in hormone mediated transcriptional regulation. Furthermore, we report that ACTN4 (Iso) can interact with selective co-activators including SRC-1 and PCAF as a mechanism whereby it potentiates nuclear receptor mediated transcription.
Overexpression and mutations of ACTN4 are associated with human cancers and kidney diseases. There are some reports suggesting a role for ACTN4 in cell motility and cancer invasion [96, 98, 244-246], however, the precise roles and mechanisms by which ACTN4 is involved in tumorigenesis are not completely understood. Our findings that ACTN4 plays a role in transcription regulation mediated by ERα in MCF-7 cells provides a possible additional mechanism by which ACTN4 might contribute to the pathogenesis of this disease. Our data suggest that ACTN4 (full-length) and ACTN4 (Iso) interacts with ERα and promotes transcriptional regulation mediated by ERα in MCF-7 cells. Also, we have shown that ACTN4 knockdown decreases the proliferation of MCF-7 cells. This decrease in the proliferation might be due to change in the mRNA expression level of genes involved in proliferation including cyclin D-1, PR and c-Myc as shown by our data. Estrogens are known to regulate aspects of growth, development and differentiation. Estrogen receptors and estrogen together play an important role in development and progression of breast cancer. Also, it is known that SRC-3 (AIB1) is highly amplified in approximately 10% of the breast cancers and is overexpressed at the mRNA level in 64% of the primary breast tumors [208, 247]. The size of ER positive and PR positive breast tumors are directly correlated with SRC-3 amplification and overexpression [248]. SRC-3 deficient mice show a phenotype of delayed puberty, mammary gland growth retardation and abnormal reproductive function [216]. Our observations that ACTN4 (full-length) and ACTN4 (Iso) enhance the transcriptional activation by ERα in hormone dependent manner. We have also dissected the mechanisms by which ACTN4 (Iso) potentiates transcriptional activation mediated by ERα. We have concluded from our studies that 1) ACTN4 (Iso) is a more potent transcriptional coactivator for nuclear
hormone receptors than ACTN4 (full-length), 2) Both ACTN4 (Iso) and ACTN4 (full-length) activate basal transcription when brought to a promoter region, 3) ACTN4 (Iso) harbors two independent activation domains, 4) While ACTN4 (Iso) interacts strongly with co-activators, ACTN4 (full-length) associates weakly with co-activators, 5) The nuclear receptors interacting motif LXXLL is critical for ACTN4 to bind to and potentiate transcriptional activation by nuclear receptors, 5) The HAT domain of PCAF is essential for its interaction with ACTN4 (Iso). Taken together, we have defined ACTN4 (Iso) as a more potent transcriptional co-activator for nuclear hormone receptors as compared to ACTN4 (full-length). We think that this distinct regulation by ACTN4 (Iso) is due to its ability to interact with p160 family members including SRC-1 and SRC-3 and PCAF. Interaction of ACTN4 (full-length) and ACTN4 (Iso) with ERα and interaction between ACTN4 (Iso) with SRC-1 and SRC-3 suggest that ACTN4 may play a role in tumorigenesis and mammary gland growth development. So, it will be interesting to test our hypothesis on the mouse models using ACTN4 knockout mice.

Our findings that both the ACTN4 (Iso) and the ACTN4 (full-length) interact with HDAC7 and that ACTN4 (Iso) interacts with PCAF suggest that ACTN4 (Iso)/ACTN4 (full-length) might undergo post-translational modification such as acetylation. So, an important issue to analyze is the acetylation of ACTN4. A recent report has provided evidence that ACTN4 is acetylated at five lysine residues (K114, K214, K217, K592 and K625) in a human acute myeloid leukemia cell line [249], however, the mechanistic details and the functional significance of acetylation of ACTN4 are not known. Interestingly, among these five lysine residues, only two (K592 and K625) are present in
ACTN4 (Iso), suggesting that there might be a differential mechanism regulating the acetylation of ACTN4 (Iso) and ACTN4 (full-length). We have preliminary results from immunoprecipitation experiments showing that ACTN4 (full-length) mutant with all of above mentioned lysines mutated to arginines still undergoes acetylation suggesting that there is/are more lysine residues responsible for acetylation of ACTN4. It will be interesting to determine which of the lysine residues is important for acetylation of ACTN4 (full-length). In order to answer these questions, we will do transfections with different HA-tagged smaller fragments of ACTN4 (full-length) followed by immunoprecipitations with α-HA antibodies and Western blotting with anti-acetyl lysine antibodies. Also, it is important to identify the enzyme (acetyltransferase) responsible for ACTN4 (full-length) acetylation. The potential effects of various acetyltransferases including CBP/p300, and PCAF will be evaluated by overexpression and siRNA knockdown experiments. Furthermore, it will be interesting to investigate whether acetylation is important for cytoskeletal functions of ACTN4 (full-length) or for transcriptional regulation function.

Our data suggest that ACTN4 (Iso) interacts with PCAF more strongly than ACTN4 (full-length) and mapping studies suggest that the minimal binding domain of PCAF with ACTN4 (Iso) encompass the HAT domain. Work by others indicates that PCAF5 acetylates several other transcription factors in addition to histones [226-229]. The acetylation of non-histone proteins such as chromatin remodelers, transcription factors, transcriptional activators, and nuclear receptor cofactors can affect nuclear localization, inhibition of nuclear export of transcription factor, enhanced DNA binding, stimulation
of transcription mediated by some factor or enhanced co-activators association [230]. One example of a transcription factor undergoing acetylation in response to hormone is AR. AR is directly acetylated at lysine residues in the hinge region and acetylation has been shown to increase its transcriptional activity, response element binding, and cellular proliferation [250-253]. The data is supported by the fact that acetylation AR interacts with p300 more strongly than unacetylated AR. More importantly, these mutants prompted tumor cell growth in vitro and in vivo suggesting that AR acetylation may play an important role in development and progression of prostate cancer [250, 254]. There are several other NRs known to be acetylated including hepatocyte nuclear factor-4 (HNF-4), Steroidogenic factor 1(SF-1), GR, TR, and ER. In this respect, it will be important to know whether PCAF acetylates ACTN4 (Iso) and which lysine residues are involved. Likewise it will be important to study the functional consequences of acetylation of ACTN4 (Iso).

Role of ACTN4 in podocytes: Mice homozygous for ACTN4 knockout survive in smaller numbers than predicted by Mendelian inheritance. Those that survive to birth die at 3-4 weeks age and display severe kidney pathology from day 0 [152]. These data indicate a key role of ACTN4 in kidney function.. Although it has been known that ACTN4 mutations cause cytoskeletal changes, the exact function of ACTN4 in podocytes has not been elucidated. It has been shown that FSGS-linked ACTN4 (K228E) causes lower expression of nephrin mRNA in transgenic mice [156], suggesting that this mutant might be interfering with the transcriptional regulation of a subset of genes including those encoding for components of the SD. We hypothesized that ACTN4 may be essential for the HPCs.
differentiation. It is important to investigate the role of ACTN4 in conditionally immortalized podocyte cell lines because the podocytes cells from ACTN4 knockout mice are not available. ACTN4 could be involved in cytoskeletal functions through associating with actin filaments or synaptopodin [241, 242]. Alternatively, ACTN4 might play a role in transcriptional regulation mediated by nuclear hormone receptors as suggested by our data. Since in vitro differentiation for HPCs takes around 10-14 days, knockdown by siRNA is not suitable for these kinds of experiments. In order to study the in-depth mechanism by which ACTN4 regulates podocyte biology, a stable cell line using shRNA against ACTN4 will be generated under undifferentiated conditions. A control non-targeting shRNA will be generated as well for comparison. For in vitro differentiation assay, the HPCs stably expressing shRNA against ACTN4 and a non-targeting shRNA will be allowed to differentiate for 8-10 days. The effects of the ACTN4 shRNA on HPCs will be evaluated by the ability of HPCs to express podocyte marker genes including synaptopodin, nephrin and podocin [232].

Likewise, in order to increase our understanding of role of ACTN4 in HPCs, it is important to know target genes regulated are by ACTN4. The information on potential target genes could be achieved using ChIP-on-chip assays on the stable cell lines expressing HA-ACTN4 (Iso), HA-ACTN4 (full-length) and HA-ACTN4 (K-E) in undifferentiated and differentiated HPCs. We anticipate that ACTN4 (full-length) and ACTN4 (Iso) may yield overlapping but not the identical results. Also, we think that ACTN4 may associate with promoter sequences that are controlled by transcriptional regulators other than nuclear receptors. Furthermore, this strategy will provide us with the details of the differential regulation of genes undifferentiated and differentiated.
conditions. Also, it will be interesting to know how HPCs that express FSGS-linked ACTN4 (K228E) respond to hormones. We will treat HPCs stably expressing ACTN4 (K228E) with different concentrations of dexamethasone, AT-RA, ciglitazone and vitamin D3 and examine the expression of podocyte marker genes. We anticipate that a fraction of ACTN4 target genes will also be responsive ligands for NRs. These results will be important in identifying genes that are regulated by ACTN4 and NRs together.
REFERENCES:


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