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GIT1 mediates HDAC5 activation by angiotensin II in vascular smooth muscle cells

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Abstract

Objective—The G-protein-coupled receptor (GPCR)-kinase2 interacting protein1 (GIT1) is a scaffold protein involved in angiotensin II (AngII) signaling. Histone deacetylase-5 (HDAC5) has emerged as an important substrate of calcium/calmodulin-dependent protein kinase II (CamKII) in GPCR signaling. Here we investigated the hypothesis that angiotensin II (AngII)-mediated vascular smooth muscle cell (VSMC) gene transcription involves GIT1-CamKII dependent phosphorylation of HDAC5.

Methods and Results—AngII rapidly stimulated phosphorylation of HDAC5 at Ser498 in VSMC. Knockdown of GIT1 significantly decreased HDAC5 phosphorylation induced by AngII. The involvement of Src, phospholipase γ (PLCγ) and CamKII in GIT1-mediated HDAC5 phosphorylation was demonstrated. The association of GIT1 and CamKII was constitutive, but increased after stimulation with AngII. Moreover, the interaction of GIT1 and CamKII through the ARF GTPase-activating protein (ARF-GAP) and coiled-coil domains of GIT1 was essential for the phosphorylation of HDAC5. Finally, knockdown of GIT1 decreased myocyte enhancer factor 2 transcriptional activity induced by AngII.

Conclusions—This study identifies a novel function for GIT1 as a mediator of AngII-induced VSMC gene transcription via a Src- PLCγ -CamKII-HDAC5 signaling pathway.

Keywords

G-protein-coupled receptor-kinase interacting protein1; GIT1; Angiotensin II; histone heacetylase 5; Ca^{2+}-calmodulin-dependent protein kinase II; vascular smooth muscle cell

Introduction

The importance of the renin angiotensin system (RAS) in cardiovascular disease has been dramatically shown by the beneficial effects of inhibiting the RAS with angiotensin-converting enzyme inhibitors and angiotensin II (AngII) type 1 receptor (AT_1R) blockers. The AT_1R mediates vascular smooth muscle cell (VSMC) migration, hypertrophy, proliferation, and vascular remodeling[1-3]. The G-protein-coupled receptor (GPCR)-kinase 2 interacting protein-1 (GIT1) is a multi-domain scaffold protein involved in multiple GPCR signal pathways including endocytosis, cell adhesion and migration[4]. Our laboratory was the first to show that c-Src phosphorylates GIT1[5] in VSMC following stimulation by AngII. Furthermore,

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we showed that GIT1 binds key signaling mediators including PLCγ and MEK1[5-7]. Conformational changes in GIT1 induced by tyrosine phosphorylation lead to the activation of PLCγ[5]. Activation of PLCγ is responsible for the elevation of intracellular calcium, which results in the autophosphorylation (activation) of calcium/calmodulin-dependent protein kinase II (CamKII) [8, 9].

Histone acetylation/deacetylation has emerged as a fundamental mechanism for the control of gene expression. Histone acetyltransferases stimulate transcription through acetylation of histones, resulting in relaxation of nucleosomes; and histone deacetylases (HDACs) antagonize this activity and repress transcription[10]. Class II HDACs (HDACs 4, 5, 7, and 9) appear to be dedicated to the control of tissue growth and development. Phosphorylation of the amino termini of class II HDACs by CamKII creates docking sites for the 14-3-3 family of chaperone proteins, which promote shuttling of these HDACs from the nucleus to the cytoplasm, thereby derepressing HDAC target genes [11-13]. HDAC5 acts as a negative regulator of cardiac growth. Transgenic mice lacking either HDAC5 or HDAC9 develop extremely enlarged hearts in response to pathological signals [14, 15]. CamKII plays an important role in HDAC5 phosphorylation induced by GPCR ligands[16]. Based on these findings, we hypothesized that GIT1 mediates HDAC5 phosphorylation stimulated by Ang II via a pathway dependent on c-Src, PLCγ and CamKII. Furthermore we propose that derepression of HDAC5 increases MEF2 transcriptional activity in VSMC.

Materials and Methods

Materials

Antibodies to glutathione *S*-transferase (GST) monoclonal, GIT1 polyclonal, CamKII polyclonal, 14-3-3 ploYclonal and ERK1 monoclonal were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). c-Src(for chicken) and PLCγ antibody was from BD Transduction Laboratories (Lexington, KY). FLAG M2 monoclonal antibody was from Sigma. Xpress monoclonal antibody was from Invitrogen. Phosphospecific-ERK1/2 was from Cell Signal. Phospho-Ser498 HDAC5 and Ser498 HDAC5 antibodies were from Signal Antibody Technology. AngII was from ICN Biomedicals. PP2, U73122 and KN93 were from Calbiochem. Other chemicals were purchased from Sigma.

Cell culture and transfection

VSMC were obtained from rat aorta as described [17], and passage 6-10 were used in the experiments. HEK293 cells and VSMC were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37 °C in 5% CO_2. HEK293T cells were transfected by Lipofectamine/plus (Invitrogen). For cotransfection with AT1R, a ratio of 3:1 was used. After allowing protein expression for 24 h, cells were serum-deprived for 16 h and stimulated with 100 nM AngII. GIT1 siRNA (AAGCTGCCAAGAAGAAGCTAC) and control non-silencing siRNA (AATTCTCCGACACGTGTCACT) were designed as described and synthesized by Ambion. VSMC were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol as described previously [18]. GIT1 siRNA were prepared and transfected at 1 μM for 48 h as previously described [6], then serum-deprived for 16 h, and stimulated with 100 nM AngII.

Plasmid Constructs

The mGIT1 expressed sequence tag clone (GenBank™ accession number AI414223) was purchased and completely sequenced. Then, full-length mGIT1 (GIT1(WT)) was cloned into the NotI and Xbal sites of Xpress-pcNDA3.1 vector (Invitrogen)(Xpress-GIT1(WT)). Using PCR, GIT1(1-420aa), GIT1(420-770aa) were cloned into Xpress-pcDNA3.1 vector (Xpress-

GIT1(1-635aa), Xpress-GIT1(1-420aa), Xpress-GIT1(420-770aa)). pEBB-Flag-CamKII was a generous gift from Dr. Richard Mauzer.

Immunoprecipitation and immunoblotting

For immunoprecipitations, cells were lysed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl, pH 8.0). Protein concentrations in the lysates were determined as described [14, 19]. The protein samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with appropriate primary antibodies. After incubating with fluorescence-conjugated secondary antibodies, immunoreactive proteins were visualized by an Odyssey infrared imaging system (LI-COR Biotechnology, Nebraska). Densitometric analysis of the blots was performed with Odyssey software (LI-COR Biotechnology). Results were normalized by arbitrarily setting the densitometry of control samples to 1.0.

Immunofluorescence

VSMC cells were starved with serum-free DMEM overnight and then stimulated with AngII in 0, 5, 10, 30, 60min. Cells were fixed with 4% formaldehyde for 10 min, washed with phosphate-buffered saline three times, permeabilized with 0.05% Triton for 5 min, and blocked with 10% normal goat serum for 1 h. Cells were incubated with GIT1, and CamKII antibodies diluted in phosphate-buffered saline followed by Alexa Fluor 546 anti- rabbit IgG for red fluorescence or by Alexa Fluor 488 goat anti-mouse for green fluorescence (Molecular Probes, Inc.) in phosphate-buffered saline at a final concentration of 1.5-2 μg/ml each.

Luciferase reporter assay

To assess myocyte enhancer factor 2 (MEF2) transcriptional activation, we used 3×MEF2-dependent reporter gene (generous gift from Dr. Joseph Miano) in which three tandem repeats of MEF2 sites were located upstream of the thymidine kinase gene promoter. VSMC cultured in 24-well dishes were cotransfected with 3×MEF2 luciferase reporter gene, thymidine kinase-renilla-luciferase (an internal control from Promega), Myc-HDAC5-WT plasmid, control siRNA or GIT1 siRNA in each experiment using electroporation (Bio-Rad). At 32 h post-transfection, cells were treated with Ang II for another 16 h. The luciferase activities in cell lysates were determined using the Dual-Luciferase Reporter Assay kit (Promega) and Wallac 1420 multilabel counter (PerkinElmer).

Statistical Analysis

All values are expressed as means ± SD from three to six independent experiments. The significance of the results was assessed by t-test. A p value < 0.05 was considered statistically significant.

Results

AngII stimulates phosphorylation of HDAC5 in rat VSMC

Because HDAC5 is phosphorylated by CamKII and we showed that GIT1 phosphorylation by AngII is required for PLCγ mediated calcium mobilization and CamKII activation, we studied HDAC5 phosphorylation in VSMC in response to AngII. Phosphorylation of HDAC5 was determined by phospho-HDAC5-specific antibody, which recognizes phosphorylation at Ser498. In response to 100 nM AngII, HDAC5 phosphorylation rapidly increased by 2.6-fold within 2 min, and reached a maximum at 5 min (3.3-fold; Fig. 1A). HDAC5 phosphorylation returned to baseline after 30 min (Fig. 1A). HDAC5 expression did not change during this time course.

GIT1 is required for phosphorylation of HDAC5 by AngII

To show a role for GIT1 in HDAC5 phosphorylation, we used rat GIT1 siRNA to decrease GIT1 expression in VSMC as previously described[6]. Rat control siRNA and rat GIT1siRNA were designed based on unique sequences and ability to inhibit mRNA expression. Transfection of GIT1 siRNA significantly decreased GIT1 protein expression at 48 h, whereas HDAC5 expression was not altered (Fig. 1B). Treatment with rat GIT1 siRNA significantly decreased HDAC5 phosphorylation induced by AngII (80% inhibition), while control siRNA had no significant effect (Fig. 1B).

AngII stimulates HDAC5 phosphorylation via Src dependent pathway

Previous data from our laboratory suggested an essential role for c-Src in AT_1R signal transduction[20]. To investigate the role of c-Src in HDAC5 phosphorylation, the c-Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) was administrated for 30 min in VSMC, prior to stimulation with 100 nM AngII for 5 min. Phosphorylation of HDAC5 was significantly decreased by PP2 treatment (Supplemental Fig. 1A). To further confirm the role of c-Src, the dominant negative (DN) chicken c-Src adenovirus (Ad.DN-Src) was used to infect VSMC [20]. Infection of VSMC with Ad.DN-Src resulted in robust expression of chicken Src (Supplemental Fig. 1B). Infection at MOI of 100 and 300 almost completely blocked AngII-induced HDAC5 phosphorylation, whereas Ad. Lac-Z infection had no significant effect. In contrast to the dramatic effect of Ad.DN-Src on p-HDAC5, there was significantly less inhibition of p-ERK1/2 (Supplemental Fig. 1B).

PLCγ and CamKII are required for HDAC5 phosphorylation by AngII

c-Src phosphorylates GIT1, which is bound to PLCγ in a complex under basal conditions. Conformational changes in GIT1 induced by tyrosine phosphorylation lead to the activation of PLCγ which is required for elevation of intracellular calcium, and autophosphorylation of CamKII. To determine the involvement of PLCγ and CamKII in HDAC5 phosphorylation in VSMC, the effects of the PLCγ inhibitor, U73122 and the CamKII inhibitor KN93 were investigated. Both U73122 and KN93 dose dependently inhibited the phosphorylation of HDAC5 (Supplemental Fig. 1C-D). These results suggest that PLCγ and CamKII play important roles in the AngII stimulated HDAC5 signaling pathway.

Association of CamKII, HDAC5 and 14-3-3 is increased by AngII

Phosphorylation of HDAC5 creates binding sites for the 14-3-3 proteins, which escort p-HDAC5 from the nucleus to the cytoplasm, with consequent activation of HDAC5 target genes. To investigate the association of CamKII with HDAC5 and 14-3-3 after stimulation of AngII in VSMC, co-immunoprecipitation was performed. The interaction of CamKII with HDAC5 and 14-3-3 increased rapidly (within 1 min, Fig. 2A), and peaked at 5 min, similar to the peak phosphorylation of HDAC5.

The interaction of GIT1 and CamKII is increased by AngII

Because GIT1 is a multidomain scaffold protein [4], we hypothesized that GIT1 functions as a scaffold for CamKII by binding to CamKII. The association of GIT1 and CamKII was assayed by immunoprecipitating CamKII from VSMC lysates. While the binding of GIT1 and CamKII was constitutive (Fig. 2B, time 0, Supplemental Fig.2), in response to AngII binding rapidly increased and peaked at 5 min (2.6 fold increase, Fig. 2B, Supplemental Fig.2), similar to the HDAC5 phosphorylation time course. The time course for GIT1-CamKII binding was similar to the time course for CamKII binding to HDAC5 and 14-3-3 (Fig.2A, Supplemental Fig.2), suggesting a multiprotein complex. To investigate further the potential interaction of HDAC5 and GIT1, we co-expressed them in HEK293 cells (which have low expression of CamKII). There was no basal interaction, nor increase in response to AngII (Supplemental Fig. 3).

GIT1 recruited CamKII and PLCγ to form a "Calciosome" complex, which mediated HDAC5 phosphorylation by AngII

Since GIT1 can associate with both CamKII and PLCγ, we hypothesized that GIT1, CamKII and PLCγ could form a complex required for phosphorylation of HDAC5, which we will term the " Calciosome". The association of GIT1, CamKII, PLCγ and HDAC5 was assayed by immunoprecipitating PLCγ and GIT1 from VSMC lysates. In VSMC, GIT1, CamKII and PLCγ were present in the same complex as shown by the findings that any one of these proteins co-precipitated the other two proteins (Fig.2C-D) if GIT1 was present. This presumed role of GIT1 as a scaffold molecule for CamKII and PLCγ was proved using 293 cells, which express no detectable GIT1. In 293 cells transfected with vector control (pcDNA), there was no interaction between CamKII and PLCγ (supplemental Fig.4). However, when cells were transfected with GIT1 cDNA co-precipitation of PLCγ and CamKII was readily apparent (supplemental Fig.4). The binding of HDAC5 to the calciosome increased in response to AngII stimulation through binding to CamKII (Fig.2C-D, Supplemental Fig.2). The peak time was at 5min, similar to peak time of HDAC5 phosphorylation. These data suggested that the calciosome is essential for HDAC5 phosphorylation.

Colocalization of GIT1 and CamKII

To further confirm the interaction of GIT1 and CamKII, immunofluorescence experiments were performed. After serum starvation for 24 hours, VSMC were incubated with AngII for up to 60min. GIT1 and CamKII location was assayed by immunohistochemistry. In the absence of AngII, GIT1 distributed across the entire cell (Fig 3A). The localization of CamKII was similar to GIT1 (Fig 3B). This is consistent with a previous report that CamKIIδ_c locates in cytoplasm while CamKIIδ_b is nuclear [8, 21]. Dual immunofluorescent detection revealed that GIT1 colocalized with CamKII basally (Fig.3C). After administration of AngII for 5-10 min, GIT1 translocated to the perinuclear and nuclear area (Fig.3D, G). CamKII also translocated to the perinuclear and nuclear areas (Fig.3E, H). GIT1 and CamKII were mostly colocalized during this period (Fig.3F, I), consistent with the imunoprecipitation results (Fig.2). From 30-60 min, both GIT1 and CamKII returned to the cytoplasmic compartment (Fig.3J-O). The similar translocation of GIT1 and CamKII suggests a significant functional interaction.

Domains of GIT1 that mediate interaction with CamKII

GIT1 is composed of an ARF GAP domain, an ankyrin repeat region, two carboxyl paxillin-binding subdomains, a Spa2 homology domain (SHD), synaptic localization domain(SLD) and three putative coiled-coil (CC) domains (Fig. 4A). To define the domains responsible for the GIT1-CamKII interaction, we transfected HEK 293 cells with Flag-CamKII and the GIT1 deletion mutants described in methods. Immunoprecipitation of CamKII with anti-Flag antibody co-precipitated GIT1(1-770), GIT1 (1-635), but not GIT1 (1-420), GIT1 (250-770) or GIT1(420-770)(Fig 4B, C). These results suggest that both the ARF-GAP and CC2 domains are required for GIT1-CamKII interaction since the only GIT1 (1-635) has both the ARF-GAP domain and the CC2 domain, while the other mutants lack one of the these domains. GIT1 functions as a scaffold for CamKII by binding to CamKII, which is essential for phosphorylation of HDAC5

To determine the functional significance of GIT1 binding with CamKII, HDAC5 phosphorylation induced by AngII was studied in 293 cells transfected with Flag-CamKII, Xpress-GIT1 or both (note the AT1R was also co-transfected)(Fig.5). Overexpression of GIT1 or CamKII alone had no significant effect on HDAC5 phosphorylation (Fig. 5A-B). However, when both were overexpressed, HDAC5 phosphorylation significantly increased (2.8-fold, Fig. 5A-B). Based on the finding that the ARF GAP domain and CC2 domain are essential for GIT1 and CamKII interaction, we determined the ability of GIT1 mutants to increase phosphorylation of HDAC5. As shown in Fig.5C-D, GIT1 mutants lacking the ARF-GAP

domain (e.g. GIT1 (420-770)) or CC2 domain (e.g. GIT1 (1-420)) had significantly less effect on phosphorylation of HDAC5 compared with WT GIT1 ($p<0.05$, Figure5C-D), but still substantial effect on phosphorylation of HDAC5 compared to pcDNA group ($p<0.05$, Figure5C-D). However in Fig. 4 we showed that these mutants could not bind to CamKII. How can the mutants that do not bind to CamKII still induce HDAC5 phosphorylation? Our hypothesis is that these two mutants may bind to CamKII through one binding site. However, with loss of one required binding domain, this binding is much weaker than the binding of GIT1 (WT) or GIT1 (1-635) (including required two binding domain) to CamKII. This weak interaction is difficult to detect by immunoprecipation. All these data suggest that GIT1 functions as a scaffold for CamKII, thereby increasing phosphorylation of HDAC5.

GIT1 is required for Ang II-induced MEF2 transcriptional activity in VSMC

In the nucleus, HDAC5 associates with the myocyte enhancer factor-2 (MEF2), which represses MEF2 transcriptional activity [22, 23]. To determine the role of GIT1 in HDAC5-mediated regulation of MEF2 transcriptional activation in VSMC, we transfected VSMC with a 3× MEF2-luciferase reporter plasmid and myc-HDAC5 WT. We then determined the effect of decreasing GIT1 expression with GIT1 siRNA on MEF2 activity. Ang II significantly increased MEF2 transcriptional activity in VSMC (Fig. 6), that was decreased by knockdown of GIT1 (Fig.6).

Discussion

The major finding of this study is that GIT1 is a novel mediator of AngII-mediated VSMC gene transcription. Specifically we show that GIT1 participates in an AngII signaling pathway that involves phosphorylation of HDAC5 and activation of MEF2, downstream of a pathway that requires Src, PLCγ and CamKII (Supplemental Fig.5). Our results suggest that GIT1, via its multi-domain scaffolding function, coordinates AngII signaling events that control calcium-dependent signaling (PLCγ and CamKII).

The focus of the present study is on CamKII which is a family of cytosolic serine/threonine protein kinases that exist as multimers consisting of α, β, δ, or γ subunits, each encoded by a different gene[24, 25]. Whereas CamKIIα and β are mainly expressed in neuronal tissues, CaMKIIδ_b, CaMKIIδ_c and CaMKIIγ are abundant in the heart [8, 21, 26] and VSMC[27]. CaMKII can phosphorylate type II HDACs[11]. These HDACs (HDAC 4, 5, 7, and 9) normally repress transcriptional activity (e.g., activation driven by MEF2) and favor condensed DNA. When HDAC is phosphorylated in response to neurohumoral stimuli, it is exported from the nucleus (in association with the chaperone protein 14-3-3), MEF2 is derepressed, and a hypertrophic program of gene expression is activated [28]. Indeed, genetic knockout of HDAC5 results in marked cardiac hypertrophy [14, 15]. AngII is an important cardiovascular hormone that induces cardiomyocyte and VSMC hypertrophy[3, 29, 30].

Here we investigated the role of HDAC5 phosphorylation in MEF2 activation induced by AngII. AngII rapidly induces phosphorylation of HDAC5 in rat VSMC, with a peak at 5 min. In VSMC, AngII binding to the AT1R activates c-Src. c-Src phosphorylates GIT1, which is bound to PLCγ in a complex under basal conditions. Conformational changes in GIT1 induced by Src-dependent tyrosine phosphorylation lead to the activation of PLCγ. Activation of PLCγ is responsible for elevation of intracellular calcium, which results in the autophosphorylation of CamKII. Activation of CamKII causes HDAC5 phosphorylation and changes in target gene expression. Data to support this pathway include the finding that knockdown of GIT1 by siRNA significantly inhibited phosphorylation of HDAC5. Furthermore, DN-Src adenovirus infection, Src inhibitor PP2, PLCγ inhibition and CamKII inhibition decreased phosphorylation of HDAC5 induced by AngII. These data show that GIT1 plays an important role in HDAC5-dependent signaling by regulating the activation of PLCγ.

We previously showed that GIT1 acted as a scaffold protein for the MEK1-ERK1/2 pathway. Specifically GIT1 exhibits four scaffold characteristics[4, 31] including 1) assembly of specific modules; 2) excluding (or insulating) other molecules; 3) promoting sequential activation of enzymes by physical interaction; and 4) providing feedback regulation of cell surface receptors. Here we show that GIT1 acts as a scaffold protein for CamKII. The interaction of GIT1 and CamKII was constitutive and increased after AngII stimulation. The interaction of GIT1 and CamKII was further confirmed by immunofluorescence co-localization. Both GIT1 and CamKII (CamKIIδb or CamKIIδc) translocated from cytoplasm to nucleus in a similar manner after stimulation with AngII. In contrast, there was no direct interaction between GIT1 and HDAC5, nor between PLCγ and CamKII. Rather GIT1 acts as a scaffold protein to assemble a "calcium signaling complex" (eg "Calciosome"), that includes PLCγ as well as CamKII. This complex facilitates phosphorylation of HDAC5 by CamKII. Although it has been reported that PLCγ translocates to the nucleus in VSMC[32], it is unclear how this complex could translocate from cytoplasm to nucleus.

GIT1 is composed of an ARF GAP domain, an ankyrin repeat region, two carboxyl paxillin-binding subdomains, a Spa2 homology domain (SHD), and three putative coiled-coil (CC) domains. The ARF-GAP domain has been shown to be functionally important for endocytosis[33]. The CC2 region is important for interactions with PAK[34], the CC3 region (including residues 646 to 770) is essential for interactions with paxillin[35], and the SHD region overlaps with binding sites defined for FAK and PIX[35]. The domains of GIT1 required for CamKII interaction are located in the N-terminal ARF GAP domain and CC2 domains. Although binding of CamKII to GIT1 is constitutive, it appears that specific conformational changes occur in GIT1 and/or CamKII in response to tyrosine phosphorylation of GIT1 that enable GIT1 (and CamKII) translocation to the nucleus. It is likely that dephosphorylation contributes to export from the nucleus.

Recently it was reported that AngII-dependent phosphorylation of HDAC5 in VSMC is through a PKC and PKD pathway that is resistant to the calcium chelator BAPTA/AM as well as CaMK inhibitors KN93 and KN62[36]. Our data suggest that GIT1 and CamKII play an essential role in phosphorylation of HDAC5, possibly independent of the PKC-PKD signal pathway. The discrepancy regarding the role of calcium and CamKII could be due to different cell conditions and experimental procedures.

In summary, we have demonstrated that HDAC5 is phosphorylated in rat VSMC by AngII via a pathway that involves PLCγ and CamKII. We also found that GIT1 and HDAC5 are involved in AngII-stimulated MEF2 transcriptional activity (Supplemental Fig.5). These findings suggest novel roles for GIT1 and HDAC5 in Ang II signaling in VSMC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Berk BC. Vascular smooth muscle growth: autocrine growth mechanisms. Physiol Rev 2001;81:999–1030. [PubMed: 11427690]
2. Berk BC. Angiotensin II signal transduction in vascular smooth muscle: pathways activated by specific tyrosine kinases. J Am Soc Nephrol 1999;10:S62–68. [PubMed: 9892142]

3. Berk BC, Haendeler J, Sottile J. Angiotensin II, atherosclerosis, and aortic aneurysms. J Clin Invest 2000;105:1525–1526. [PubMed: 10841510]
4. Hoefen RJ, Berk BC. The multifunctional GIT family of proteins. J Cell Sci 2006;119:1469–1475. [PubMed: 16598076]
5. Haendeler J, Yin G, Hojo Y, Saito Y, Melaragno M, Yan C, Sharma VK, Heller M, Aebersold R, Berk BC. GIT1 mediates Src-dependent activation of phospholipase Cgamma by angiotensin II and epidermal growth factor. J Biol Chem 2003;278:49936–49944. [PubMed: 14523024]
6. Yin G, Haendeler J, Yan C, Berk BC. GIT1 functions as a scaffold for MEK1-extracellular signal-regulated kinase 1 and 2 activation by angiotensin II and epidermal growth factor. Mol Cell Biol 2004;24:875–885. [PubMed: 14701758]
7. Yin G, Zheng Q, Yan C, Berk BC. GIT1 Is a Scaffold for ERK1/2 Activation in Focal Adhesions. J Biol Chem 2005;280:27705–27712. [PubMed: 15923189]
8. Ginnan R, Singer HA. CaM kinase II-dependent activation of tyrosine kinases and ERK1/2 in vascular smooth muscle. Am J Physiol Cell Physiol 2002;282:C754–761. [PubMed: 11880263]
9. Van Riper DA, Schworer CM, Singer HA. Ca2+-induced redistribution of Ca2+/calmodulin-dependent protein kinase II associated with an endoplasmic reticulum stress response in vascular smooth muscle. Mol Cell Biochem 2000;213:83–92. [PubMed: 11129962]
10. Kuo MH, Allis CD. Roles of histone acetyltransferases and deacetylases in gene regulation. Bioessays 1998;20:615–626. [PubMed: 9780836]
11. McKinsey TA, Zhang CL, Lu J, Olson EN. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. Nature 2000;408:106–111. [PubMed: 11081517]
12. McKinsey TA, Zhang CL, Olson EN. Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5. Proc Natl Acad Sci U S A 2000;97:14400–14405. [PubMed: 11114197]
13. Vega RB, Harrison BC, Meadows E, Roberts CR, Papst PJ, Olson EN, McKinsey TA. Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. Mol Cell Biol 2004;24:8374–8385. [PubMed: 15367659]
14. Zhang CL, McKinsey TA, Chang S, Antos CL, Hill JA, Olson EN. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell 2002;110:479–488. [PubMed: 12202037]
15. Chang S, McKinsey TA, Zhang CL, Richardson JA, Hill JA, Olson EN. Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. Mol Cell Biol 2004;24:8467–8476. [PubMed: 15367668]
16. Wu X, Zhang T, Bossuyt J, Li X, McKinsey TA, Dedman JR, Olson EN, Chen J, Brown JH, Bers DM. Local InsP3-dependent perinuclear Ca2+ signaling in cardiac myocyte excitation-transcription coupling. J Clin Invest 2006;116:675–682. [PubMed: 16511602]
17. Melaragno MG, Wuthrich DA, Poppa V, Gill D, Lindner V, Berk BC, Corson MA. Increased expression of Axl tyrosine kinase after vascular injury and regulation by G protein-coupled receptor agonists in rats. Circ Res 1998;83:697–704. [PubMed: 9758639]
18. Caplice NM, Bunch TJ, Stalboerger PG, Wang S, Simper D, Miller DV, Russell SJ, Litzow MR, Edwards WD. Smooth muscle cells in human coronary atherosclerosis can originate from cells administered at marrow transplantation. Proc Natl Acad Sci U S A 2003;100:4754–4759. [PubMed: 12665618]
19. Backs J, Song K, Bezprozvannaya S, Chang S, Olson EN. CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. J Clin Invest 2006;116:1853–1864. [PubMed: 16767219]
20. Ishida M, Ishida T, Thomas S, Berk BC. Activation of extracellular signal-regulated kinases (ERK1/2) by angiotensin II is dependent on c-Src in vascular smooth muscle cells. Circ Res 1998;82:7–12. [PubMed: 9440699]
21. Hoch B, Meyer R, Hetzer R, Krause EG, Karczewski P. Identification and expression of delta-isoforms of the multifunctional Ca2+/calmodulin-dependent protein kinase in failing and nonfailing human myocardium. Circ Res 1999;84:713–721. [PubMed: 10189359]

22. McKinsey TA, Kuwahara K, Bezprozvannaya S, Olson EN. Class II histone deacetylases confer signal responsiveness to the ankyrin-repeat proteins ANKRA2 and RFXANK. Mol Biol Cell 2006;17:438–447. [PubMed: 16236793]
23. Berger I, Bieniossek C, Schaffitzel C, Hassler M, Santelli E, Richmond TJ. Direct interaction of Ca2+/calmodulin inhibits histone deacetylase 5 repressor core binding to myocyte enhancer factor 2. J Biol Chem 2003;278:17625–17635. [PubMed: 12626519]
24. Hudmon A, Schulman H. Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. Biochem J 2002;364:593–611. [PubMed: 11931644]
25. Tombes RM, Faison MO, Turbeville JM. Organization and evolution of multifunctional Ca(2+)/CaM-dependent protein kinase genes. Gene 2003;322:17–31. [PubMed: 14644494]
26. Colomer JM, Mao L, Rockman HA, Means AR. Pressure overload selectively up-regulates Ca2+/calmodulin-dependent protein kinase II in vivo. Mol Endocrinol 2003;17:183–192. [PubMed: 12554746]
27. Schworer CM, Rothblum LI, Thekkumkara TJ, Singer HA. Identification of novel isoforms of the delta subunit of Ca2+/calmodulin-dependent protein kinase II. Differential expression in rat brain and aorta. J Biol Chem 1993;268:14443–14449. [PubMed: 8390994]
28. Olson EN, Schneider MD. Sizing up the heart: development redux in disease. Genes Dev 2003;17:1937–1956. [PubMed: 12893779]
29. Watanabe T, Barker TA, Berk BC. Angiotensin II and the Endothelium: Diverse Signals and Effects. Hypertension 2005;45:163–169. [PubMed: 15630047]
30. Dikalova A, Clempus R, Lassegue B, Cheng G, McCoy J, Dikalov S, San Martin A, Lyle A, Weber DS, Weiss D, Taylor WR, Schmidt HH, Owens GK, Lambeth JD, Griendling KK. Nox1 overexpression potentiates angiotensin II-induced hypertension and vascular smooth muscle hypertrophy in transgenic mice. Circulation 2005;112:2668–2676. [PubMed: 16230485]
31. Claing A, Perry SJ, Achiriloaie M, Walker JK, Albanesi JP, Lefkowitz RJ, Premont RT. Multiple endocytic pathways of G protein-coupled receptors delineated by GIT1 sensitivity. Proc Natl Acad Sci U S A 2000;97:1119–1124. [PubMed: 10655494]
32. Egan CG, Wainwright CL, Wadsworth RM, Nixon GF. PDGF-induced signaling in proliferating and differentiated vascular smooth muscle: effects of altered intracellular Ca2+ regulation. Cardiovasc Res 2005;67:308–316. [PubMed: 15885673]
33. Claing A, Chen W, Miller WE, Vitale N, Moss J, Premont RT, Lefkowitz RJ. beta-Arrestin-mediated ADP-ribosylation factor 6 activation and beta 2- adrenergic receptor endocytosis. J Biol Chem 2001;276:42509–42513. [PubMed: 11533043]
34. Turner CE, Brown MC, Perrotta JA, Riedy MC, Nikolopoulos SN, McDonald AR, Bagrodia S, Thomas S, Leventhal PS. Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: A role in cytoskeletal remodeling. J Cell Biol 1999;145:851–863. [PubMed: 10330411]
35. Zhao ZS, Manser E, Loo TH, Lim L. Coupling of PAK-interacting exchange factor PIX to GIT1 promotes focal complex disassembly. Mol Cell Biol 2000;20:6354–6363. [PubMed: 10938112]
36. Xu X, Ha CH, Wong C, Wang W, Hausser A, Pfizenmaier K, Olson EN, McKinsey TA, Jin ZG. Angiotensin II stimulates protein kinase D-dependent histone deacetylase 5 phosphorylation and nuclear export leading to vascular smooth muscle cell hypertrophy. Arterioscler Thromb Vasc Biol 2007;27:2355–2362. [PubMed: 17823368]

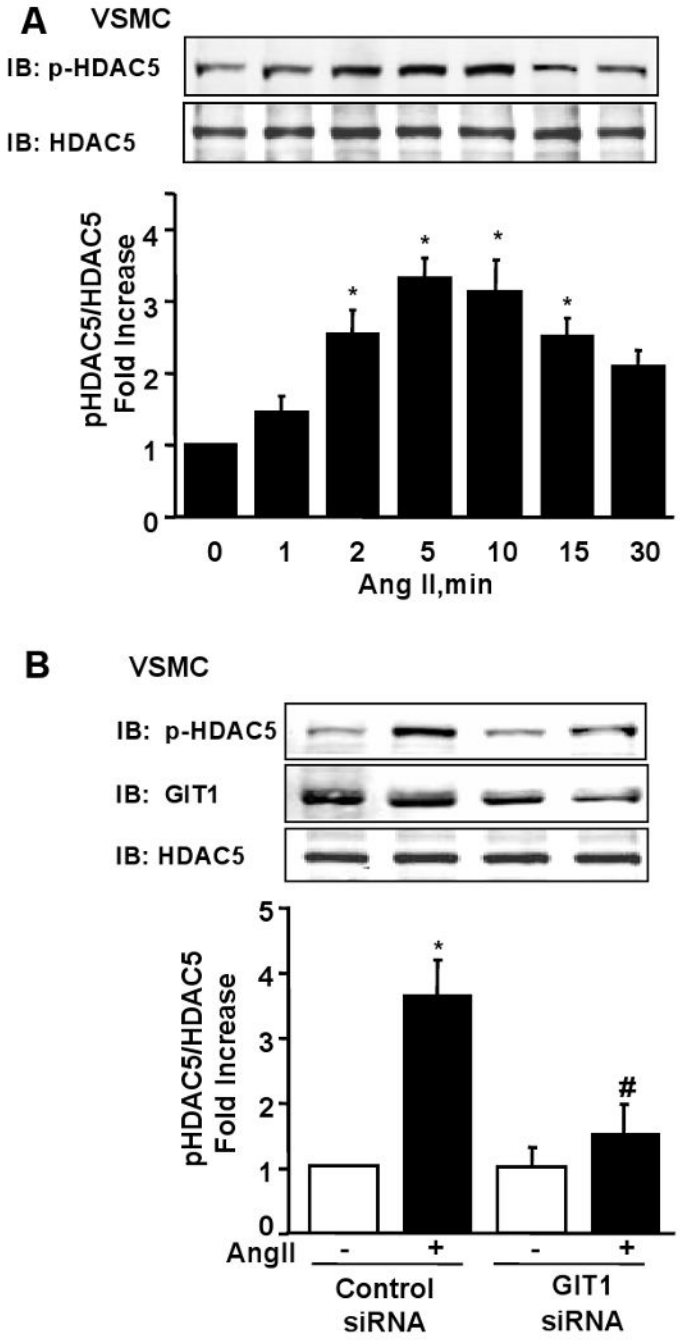


Figure 1. GIT1 mediated HDAC5 phosphorylation is induced by AngII

A. AngII stimulates HDAC5 phosphorylation in VSMC. **B.** Knockdown of GIT1 decreased AngII-stimulated HDAC5 phosphorylation. The lower panel shows relative increase of HDAC5 phosphorylation compared to control **P*< 0.05 (mean ±SD; *n* =3)

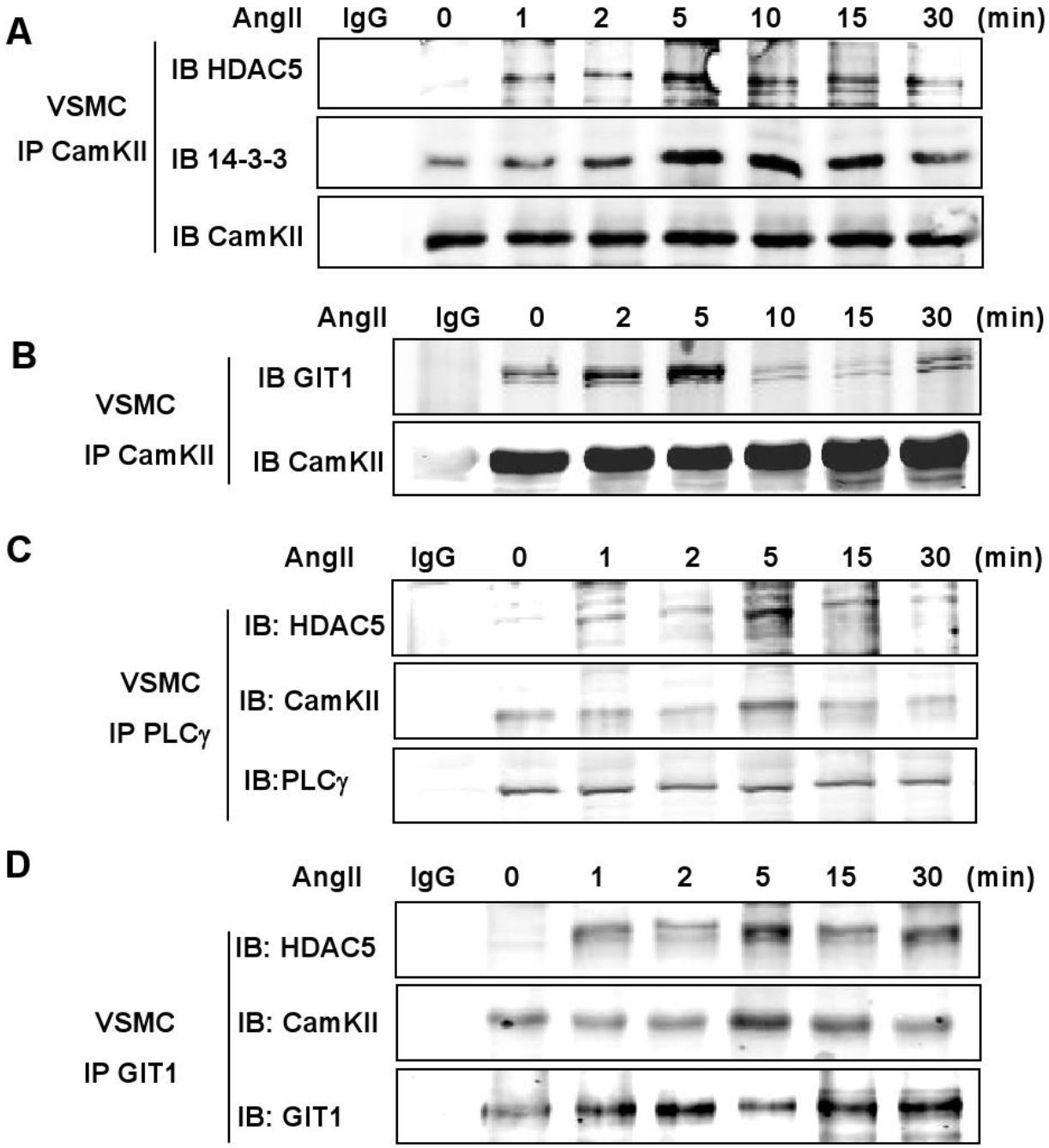


Figure 2. Association of GIT1, CamKII and PLCγ was increased by AngII

The association of endogenous CamKII, HDAC5, 14-3-3, PLCγ and GIT1 in VSMC was assayed by immunoprecipitation (IP) with CamKII antibody and probing for HDAC5 and 14-3-3**(A)**, GIT1 **(B)**. The binding of CamKII, PLCγ and HDAC5 or the interaction of GIT1, CamKII and HDAC5 were assayed by immunoprecipitation respectively with PLCγ antibody **(C)** or GIT1 antibody **(D)**.

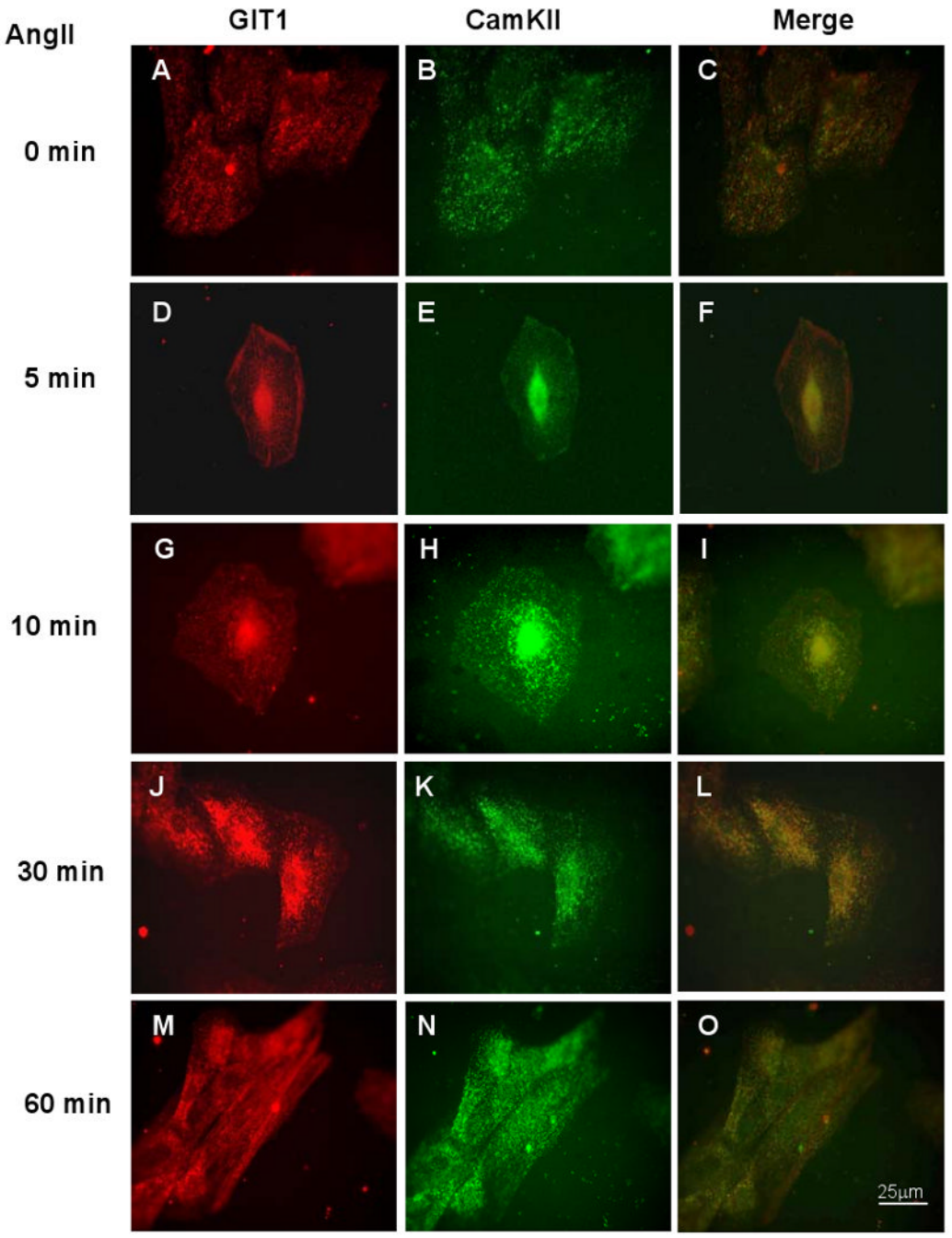


Figure 3. Colocalization of GIT1 and CamKII by immunofluorescence

VSMC were incubated with AngII for the indicated times. The cells were washed, fixed and stained using GIT1 and CamKII antibodies. GIT1 was visualized as red fluorescence, CamKII visualized as green fluorescence, and colocalization of GIT1 and CamKII as yellow fluorescence. Bar represents 25 μm.

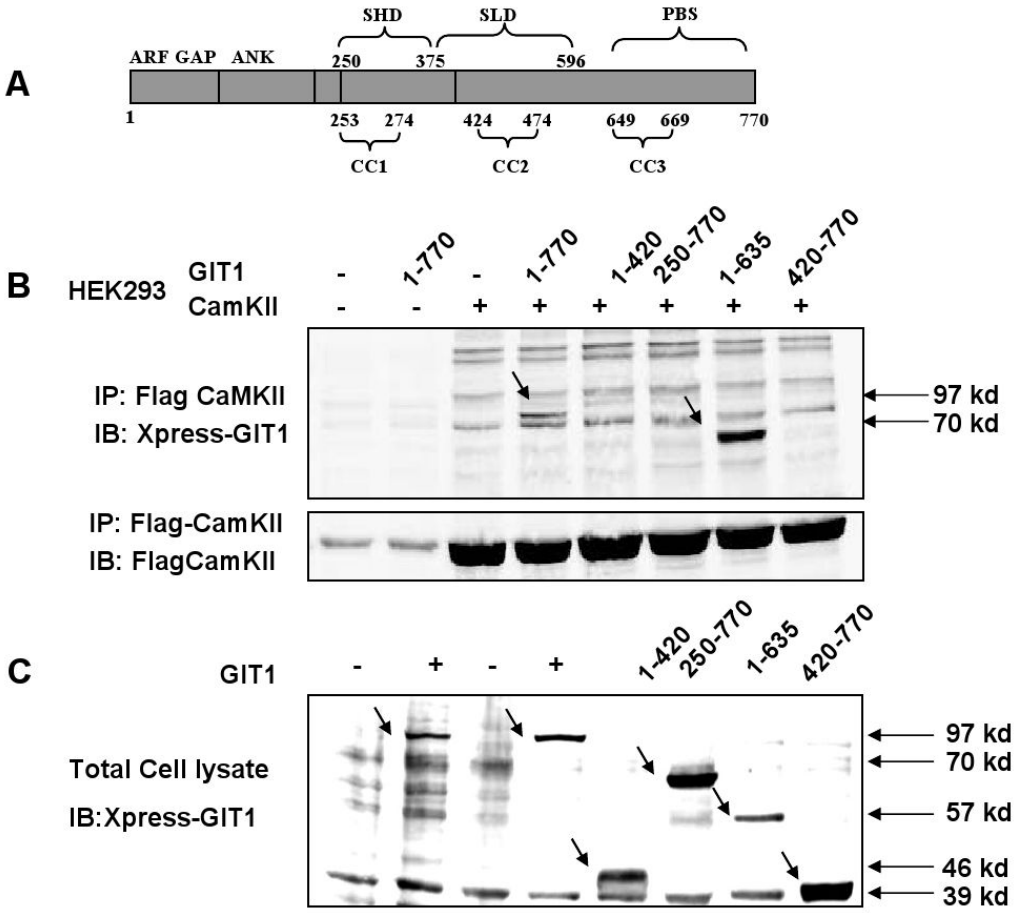
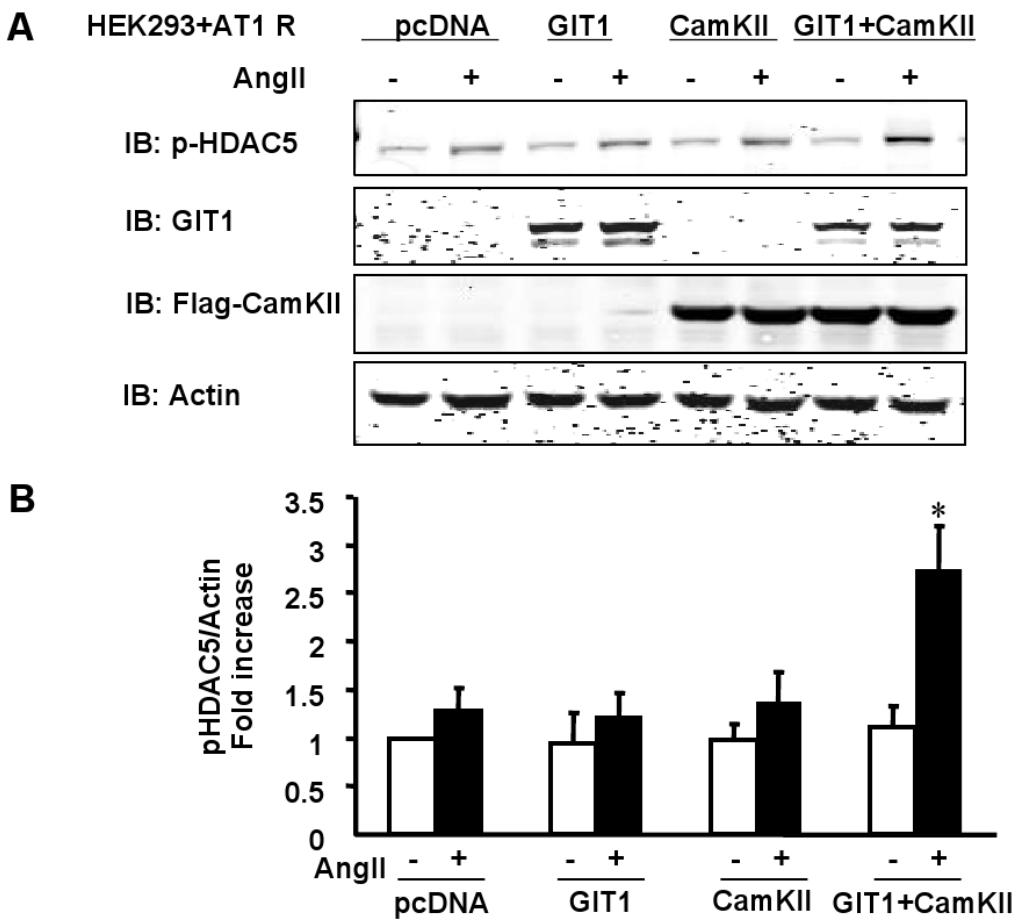


Figure 4. Domains of GIT1 required for interaction with CamKII

A. GIT1 domain structure **B.** Xpress-GIT1 constructs were cotransfected into HEK 293 cells with Flag-CamKII. CamKII was immunoprecipitated (IP). The expression of GIT1 was detected by anti-Xpress antibodies. The arrows show interactions of GIT1 constructs. **C.** Total cell lysate probed with anti-Xpress showed levels of expression of GIT1.



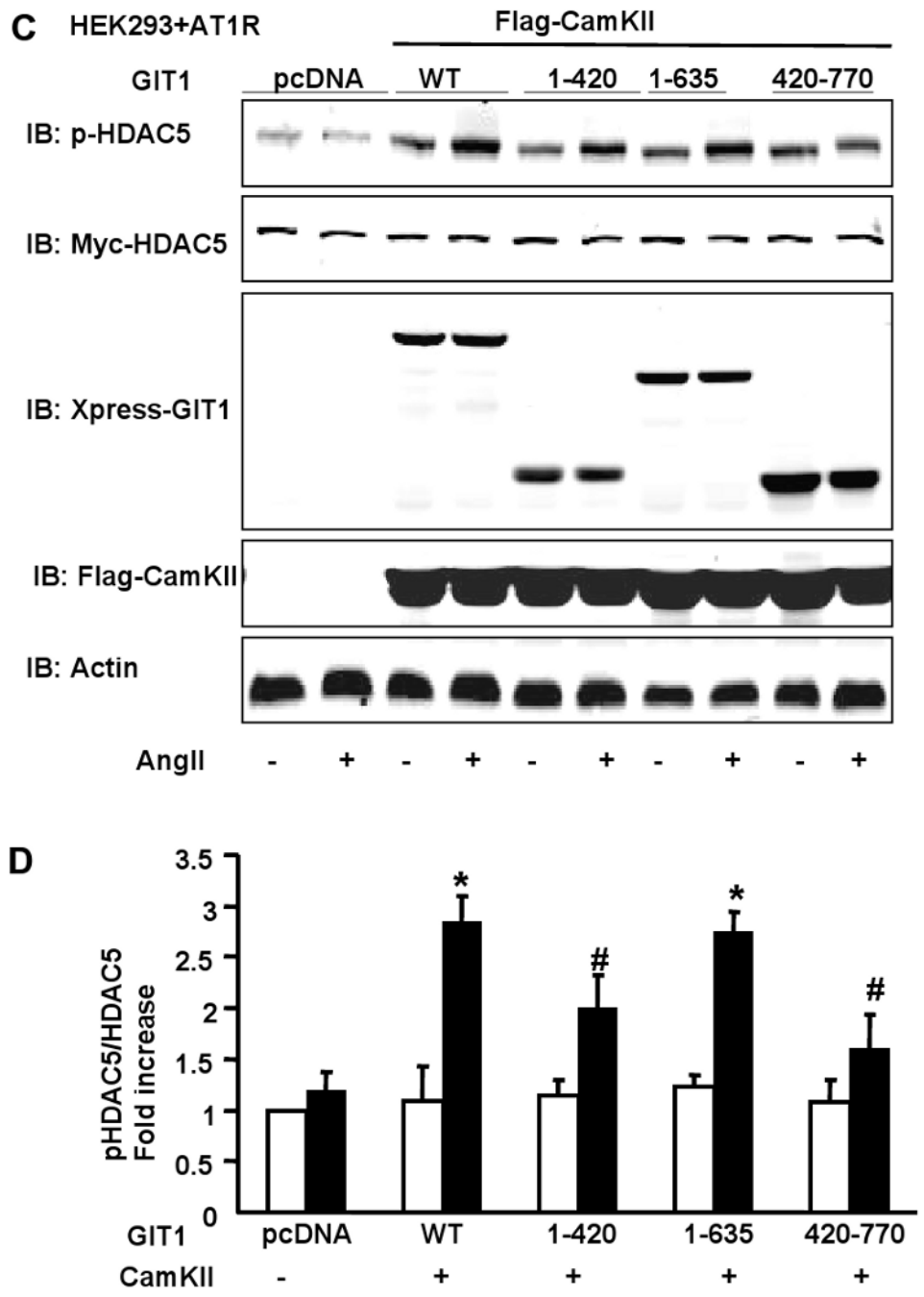


Figure 5. Binding of GIT1 and CamKII is essential for phosphorylation of HDAC5

A-B. HEK293 cells were transfected with pcDNA, Xpress-GIT1(wt), Flag-CamII or Xpress-GIT1(wt)+Flag-CamII and stimulated with AngII for 5min. **C-D.** HEK293 cells were co-transfected with Xpress-GIT1WT or GIT1 mutants with Flag-CamKII and stimulated with AngII for 5min. Lysates was immunoblotted for p-HDAC5, Actin, anti-Xpress and anti-Flag antibody **B, D.** Relative increase of HDAC5 phosphorylation compared to pcDNA group or WT group. **P*< 0.05 compared with pcDNA group; # *P*< 0.05compared with WT group. (means ±SD; *n* =3)

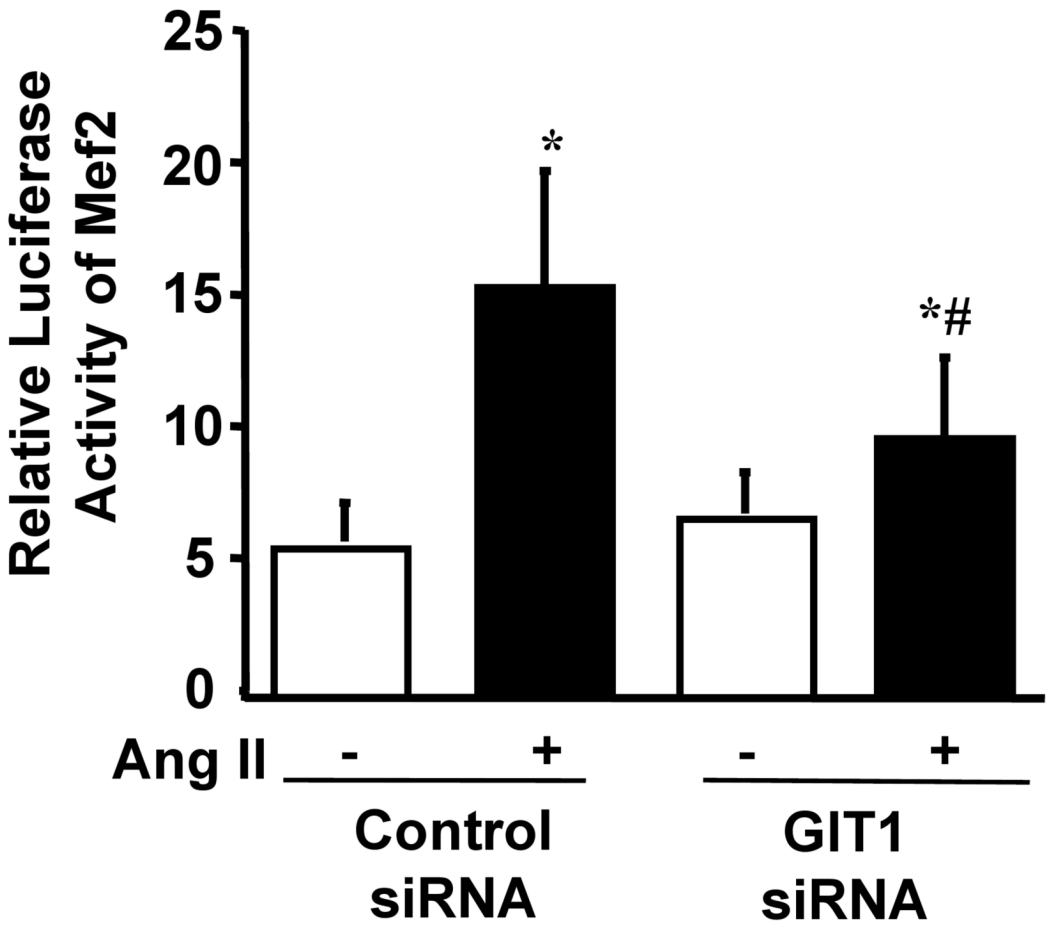


Figure 6. GIT1 altered AngII stimulated MEF2 transcriptional activity

Luciferase activity was determined in VSMC transfected with MEF2 reporter gene and control or GIT1 siRNA. The graphs represent averaged data (means ± SD, n=6). (* $p < 0.05$ vs. control siRNA without AngII; $^{\#}$ $p < 0.05$ vs. control siRNA and AngII group.)