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CHF1/Hey2 suppresses SM-MHC promoter activity through an interaction with GATA-6

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Abstract

The bHLH transcription factor CHF1/Hey2 has been previously shown to regulate neointimal formation after vascular injury, but the mechanisms have not been fully elucidated. The zinc-finger protein GATA-6 has also been shown to regulate vascular smooth-muscle phenotype through regulation of smooth-muscle contractile protein gene expression. To address the potential mechanisms by which CHF1/Hey2 regulates vascular smooth-muscle phenotype switching, we investigated the effect of CHF1/Hey2 on GATA-6-dependent smooth-muscle myosin heavy chain promoter activity. When cotransfected into NIH3T3 cells, CHF1/Hey2 reduced GATA-6-dependent activation of the promoter by 90%. Exogenous p300 was not sufficient to overcome this repression effect, demonstrating that the inhibitor effect did not involve coactivation by p300. Coimmunoprecipitation studies demonstrated that CHF1/Hey2 interacts directly with GATA-6. Mutational analysis demonstrated that the bHLH domain is required for transcriptional repression. Our findings highlight an important transcriptional mechanism by which CHF1/Hey2 may affect smooth-muscle cell phenotype.

Keywords: CHF1/Hey2; GATA-6; Smooth-muscle myosin heavy chain; Transcription factor; Vascular smooth muscle; bHLH protein

Occlusive vascular diseases including atherosclerosis, transplant arteriopathy, and in-stent restenosis are the leading causes of death in Western society. Their pathogenesis involves the complex interaction of multiple cell types in response to physical stimuli and soluble factors that results in the formation of a plaque in the vessel wall. Ultimately, these lesions predispose the injured vessel to gradual or sudden occlusion and downstream ischemia. The vascular smooth-muscle cell (VSMC) plays a critical role in the formation and maintenance of these lesions (reviewed in [1,2]). In normal vessels, VSMCs are differentiated and quiescent. In this state, they function to maintain vascular tone and blood pressure by contracting appropriately in response to the needs of the surrounding tissue or the whole organism. Remarkably, unlike cardiac or skeletal muscle, these cells retain the capacity to de-differentiate and assume a different phenotype in response to injurious stimuli. This second phenotype is characterized by distinct behaviors, including proliferation, migration, and the secretion of paracrine and autocrine factors into the local environment [2,3]. Unfortunately, the movement of these phenotypically transformed cells from the tunica media to the intima of vessels after vascular injury is thought to be maladaptive as these cells contribute both to the overall size of the plaque and to the burden of soluble factors that induce plaque progression. Although the intracellular signals and the genetic programs that mediate this phenotype switch are of great interest as potential therapeutic targets for occlusive vascular disease, they are not well characterized.

CHF1/Hey2 is a basic-helix–loop–helix (bHLH) protein that is restricted to the cardiovascular system [4–6]. During embryonic development, it is expressed primarily in the developing ventricle and vasculature, and in the latter, the importance of CHF1/Hey2 has been demonstrated by studies of the zebrafish ortholog, *gridlock* (*grl*). In this organism, a hypomorphic mutation in *grl* results in

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the development of aortic coarctation [7]. Further study using gene knockdown experiments implicated *gridlock* in the patterning of the arterial and venous systems [8]. Interestingly, mice with deletion of CHF1/Hey2 do not develop coarctation, but rather develop cardiomyopathy, ventricular septal defects, valvular abnormalities, and thin-walled arteries, as we and others have previously reported [9–13].

In addition to playing a role in development, we and others have investigated a potential role for CHF1/Hey2 in the adult vasculature [14–16]. We have previously shown that CHF1/Hey2 knockout mice demonstrate significantly decreased neointimal formation compared to WT controls after femoral artery injury. In culture, VSMCs derived from the knockout mice showed decreased proliferation as well as decreased migration in response to PDGF and HB-EGF in a Boyden chamber assay. Finally, the knockout cells did not demonstrate appropriate lamellopodia formation and membrane ruffling after PDGF or HB-EGF stimulation. These observations suggested that CHF1/Hey2 plays a significant role in VSMC phenotype switching in response to injury.

One potential mechanism by which CHF1/Hey2 may promote the development of neointima would involve antagonizing proteins important in maintaining the differentiated phenotype. GATA-binding protein 6 (GATA-6), a member of the GATA family of zinc-finger transcriptional regulators, is expressed in vascular smooth-muscle cells and is rapidly downregulated in proliferating VSMCs in culture [17] and in injured vessels [18]. Furthermore, forced expression of GATA-6 induces expression of the cyclin-dependent kinase inhibitor p21 and causes G1 cell cycle arrest in VSMCs and glomerular mesangial cells [19,20]. In addition, reversing the downregulation of GATA-6 in injured arterial vessels through intravascular injection of an adenoviral vector carrying GATA-6 cDNA resulted in significantly reduced neointimal formation in the mouse [18]. These studies suggest that GATA-6 is a lineage-restricted regulatory protein that maintains the quiescent and differentiated phenotype in VSMCs, although a later study questions this hypothesis [21].

In this study, we tested the hypothesis that CHF1/Hey2 may interact with GATA-6 to suppress expression of smooth-muscle differentiation genes. The plausibility of such an interaction is based on the known interaction between another member of the GATA family, GATA-4, and CHF1/Hey2 [22] (our unpublished observations). In these experiments, CHF1/Hey2 was found to physically associate with GATA-4 and suppress GATA-4-dependent transactivation of cardiac gene promoters. The related protein CHF2/Hey1 has also been shown to interact with GATA-1 to inhibit erythroid differentiation in vitro [23], further suggesting that interactions between these two families of transcription factors may be a more general phenomenon. Therefore, we hypothesized that CHF1/Hey2 may directly interact with GATA-6 and inhibit GATA-6-dependent activation of a smooth-muscle specific gene, and thus provide a novel mechanism for regulation of smooth-muscle differentiation.

Materials and methods

Construction of plasmids. The mouse GATA-6 cDNA was subcloned by reverse transcription of mouse VSMC mRNA followed by amplification of cDNA by the polymerase chain reaction using the PFx DNA polymerase (Invitrogen). The resulting fragment was further amplified with nested primers containing restriction sites for *Bam*HI and *Xho*I. This amplified fragment was digested with *Bam*HI and *Xho*I, and cloned into the multiple cloning site of the commercially available vector pCMV-Tag3B (Stratagene).

The p300 expression vector and the human CHF1/Hey2 expression vectors pCMV2-Flag-hCHF1 and pCDNA3-hCHF1 have been described previously [4,24]. Deletion mutants of hCHF1 were generated by amplifying select regions of the pcDNA3-hCHF1 plasmid with primers tagged with restriction sites for *Hin*dIII and *Xba*I; the 5′ primer also included an NH_2-terminal methionine-Flag epitope. The amplified fragments were cloned into the multiple cloning site (*Hin*dIII and *Xba*I) of the pcDNA3 vector. Expression of these vectors was confirmed by transient transfection of NIH3T3 cells followed by Western blotting.

The mouse smooth-muscle myosin heavy chain promoter vector, pGL3-basic-SM-MHCPr, was constructed by amplification of −1.8 kb of the 5′-untranslated region of the smooth-muscle myosin heavy chain gene from mouse genomic DNA by the polymerase chain reaction. The amplified fragment was digested with *Acc*65I and *Nhe*I and cloned into the *Acc*65I and *Nhe*I sites of the commercially available firefly luciferase reporter vector, pGL3-basic (Promega).

Transient transfection and luciferase assay. NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells were plated onto six-well trays one day prior to transfection. Following confirmation of 50–70% confluency, the cells were transfected with the indicated plasmids and an internal control plasmid encoding *Renilla*-luciferase mixed with FuGENE6 transfection reagent according to the manufacturer's protocol (Roche). Cells were harvested with passive lysis buffer after 48 h and luciferase assays were performed according to the manufacturer's protocol (Promega).

Coimmunoprecipitation assay. COS7 cells were cotransfected with pCMV-Tag3-myc-GATA-6 and pCMV2-Flag-CHF1/Hey2 or pCMV-Tag3-myc-GATA-6 and empty vector with FuGENE 6 according to the manufacturer's protocol (Roche). After 48 h, cells were washed with 1× PBS and lysed with ice-cold immunoprecipitation assay buffer (20 mM sodium phosphate, pH 7.5, 140 mM NaCl, 1% Nonidet P-40, and 0.25% sodium deoxycholate) with protease inhibitors (Roche Complete). Lysates were cleared by centrifugation for 15,000*g* for 10 min. 2.5 μg of 9E10 anti-Myc monoclonal antibody (Santa Cruz) was added to 500 μl clarified lysate containing 500 μg of total cellular protein and incubated overnight at 4 °C with rotation. Forty microliters of protein G–agarose was added and incubated for 6 h at 4 °C with rotation. The immunoprecipitates were collected by centrifugation (4000*g* for 1 min) and washed with radioimmunoprecipitation assay buffer a total of five times. The pellets were then resuspended in an equal volume of 1× SDS–PAGE sample buffer, incubated for 5 min at 95 °C, and were collected by centrifugation (4000*g* for 1 min). The supernatants were analyzed by SDS–PAGE. The separated proteins were then electrophoretically transferred to nitrocellulose and subjected to Western blotting with horseradish peroxidase-conjugated M2 anti-Flag antibody (Sigma) or horseradish peroxidase-conjugated 9E10 anti-Myc antibody (Santa Cruz). Bands were visualized by enhanced chemiluminescence according to the manufacturer's protocol (PerkinElmer Life Sciences).

For the reverse coimmunoprecipitation, lysing of cells was performed with immunoprecipitation buffer without sodium deoxycholate. The cleared lysates were incubated with 40 μl agarose-conjugated M2 anti-Flag antibody (Sigma). The immunoprecipitates were collected, washed, and analyzed as described.

Results

CHF1/Hey2 inhibits GATA-6 transactivation of the smooth-muscle myosin heavy chain promoter

To test the hypothesis that CHF1/Hey2 negatively regulates GATA-6-driven smooth-muscle specific gene expression, we assayed smooth-muscle myosin heavy chain (SM-MHC) promoter activity through transient transfection of NIH3T3 cells with a SM-MHC luciferase reporter and expression plasmids for CHF1/Hey2, GATA4 or pcDNA3 in various combinations. As shown in Fig. 1A, cotransfection of GATA-6 with the SM-MHC promoter led to a 35-fold induction in reporter gene activity. When CHF1/Hey2 was cotransfected with GATA-6, the GATA-6-induced increase in promoter activity was decreased sixfold. To verify that the effect on the SM-MHC chain gene was dose-dependent, increasing doses of CHF1 were transfected, keeping total DNA constant by the addition of empty vector DNA (pcDNA3). As shown in Fig. 1B, the repression of GATA-6-driven SM-MHC promoter activity was dose-dependent.

CHF1/Hey2 forms a heterodimer with GATA-6

To elucidate whether CHF1/Hey2 could physically associate with GATA-6, we performed coimmunoprecipitation experiments using the Myc- and Flag-tagged vectors encoding mouse GATA-6 and human CHF1/Hey2. As shown in Fig. 2, immunoprecipitation of Myc-tagged GATA-6 led to coimmunoprecipitation of Flag-tagged CHF1 when cells were cotransfected with both plasmids. As a control, the anti-Myc antibody did not immunoprecipitate Flag-tagged CHF1 in the absence of Myc-GATA-6. The reverse coimmunoprecipitation experiment gave similar results; immunoprecipitation of Flag-tagged CHF1/Hey2 led to coimmunoprecipitation of Myc-GATA-6. These findings demonstrate that CHF1/Hey2 can physically associate with GATA-6 to form a heterodimer.

The bHLH region of CHF1/Hey2 is necessary for inhibition of GATA-6

To determine the regions of CHF1/Hey2 responsible for GATA-6 inhibition, we generated a series of mutations in CHF1/Hey2. These mutations targeted the bHLH, orange, and C-terminal YRPW motifs. In transient transfection, full-length CHF1/Hey2 repressed SM-MHC promoter activity (Fig. 3). Deletion of 12 carboxy-terminal residues, including the YRPW motif, had no effect on this repression. Further deletion of the carboxy terminus to amino acid 169, leaving the bHLH and orange domain, also had no effect on repression. Deletion of the first 140 amino terminal amino acids, which left the orange domain and the carboxy terminus, did abolish repression. These results

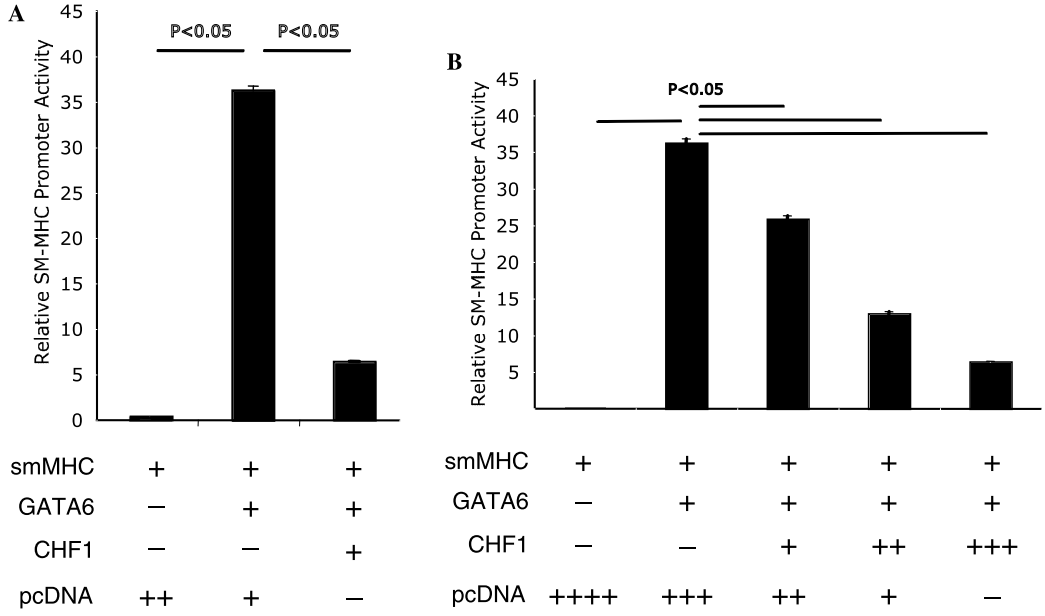


Fig. 1. CHF1 inhibits GATA-6 transactivation of the SM-MHC promoter. (A) NIH3T3 cells were cotransfected with the 1.8 kb SM-MHC heavy chain promoter/firefly luciferase reporter plasmid (2 μg), with CHF1 (0.3 μg), and with GATA-6 (0.6 μg) as indicated. Total DNA was kept constant by addition of empty vector. SM-MHC promoter activity is expressed in arbitrary units representing the ratio of firefly luciferase to *Renilla* luciferase activity. Data are presented as means of three replicates − standard error. *P* values indicating statistically significant differences on the chart were calculated by Student's *t* test. This experiment is representative of three trials. "SM-MHC" refers to the smooth-muscle heavy chain promoter plasmid. (B) Repression of the SM-MHC promoter by CHF1 is dose-dependent. NIH3T3 cells were transfected with the SM-MHC promoter and GATA-6 expression plasmids along with varying amounts of pcDNA-hCHF1 (0, 0.1, 0.3, and 0.6 μg). Total DNA was kept constant by addition of appropriate amounts of empty vector. The data are presented as means of three replicates ± standard error. There is a statistically significant difference among the groups ($P < 0.05$) as measured by one-way ANOVA. "SM-MHC" refers to the smooth-muscle heavy chain promoter plasmid.

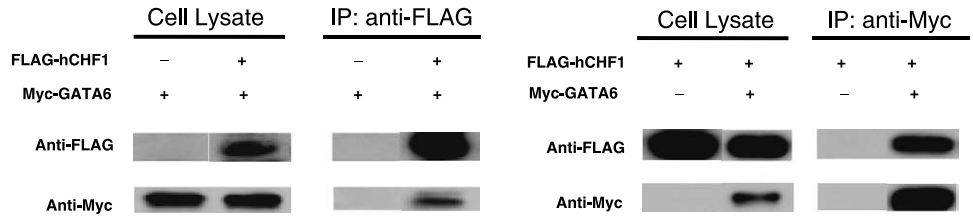


Fig. 2. CHF1 forms a heterodimer with GATA-6. Cos-7 cells were cotransfected with Flag-CHF1 and Myc-GATA-6 vectors, Flag-CHF1 and empty vectors, or Myc-GATA-6 and empty vectors, as described under Materials and methods. Lysates were prepared and immunoprecipitations were performed with indicated antibodies as described. Total cell lysates were analyzed to verify protein expression (rightmost lanes). In the figure, the first two rows beneath the line refer to the transfected plasmids while the second two rows refer to Western blot detection of the indicated epitopes (Flag or Myc). After coimmunoprecipitation with anti-Myc antibody, Flag-CHF1 was detected only in the presence of Myc-GATA-6. Similarly, after coimmunoprecipitation with anti-Flag antibody, Myc-GATA-6 was only detected in the presence of Flag-CHF1.

Fig. 3. The bHLH region of CHF1 is necessary for inhibition of GATA-6. Deletion mutants were generated as described under Materials and methods. NIH3T3 cells were cotransfected with the 1.8 kb SM-MHC heavy chain promoter/firefly luciferase reporter plasmid (2 μg), GATA-6 (1 μg), and either CHF1 wild type or mutant vector (0.5 μg) as indicated. Relative SM-MHC promoter activity is presented in arbitrary units representing the ratio of firefly luciferase to *Renilla* luciferase (mean of three replicates ± SE). As mentioned under Materials and methods, the second amino acid was used as the starting point for mutants that included the 5′ end of CHF1 to prevent competition with the start codon of the 5′ Flag sequence. Asterisks denote a statistically significant difference ($P < 0.05$) from the first lane (GATA-6 and SM-MHC promoter only) using the student's *t* test.

indicated that an intact bHLH was necessary for repression of GATA-6 activity and that the orange and YRPW domain were dispensable. Distorting the helix–loop–helix region by mutation of the glycine at position 54 to a proline resulted in partial loss of repression, again indicating the necessary role of an intact bHLH region for GATA-6 inhibition. Protein expression was verified by Western blotting (data not shown).

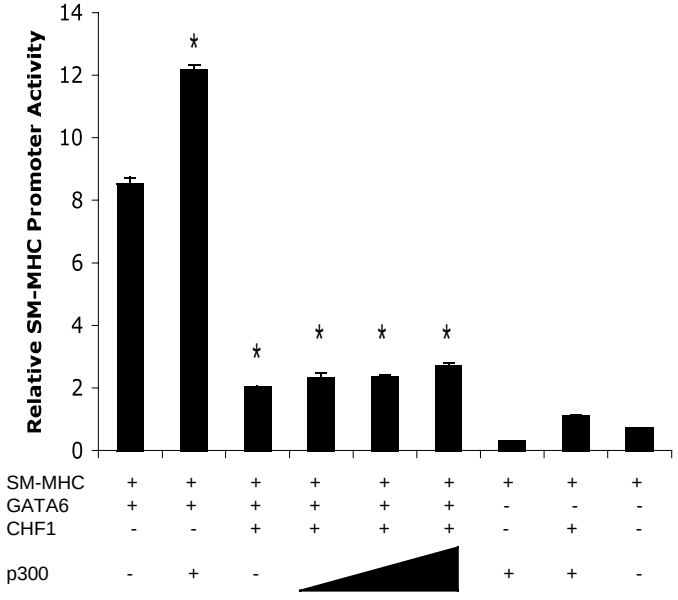
CHF1/Hey2 inhibition of GATA-6 cannot be overcome by p300

An important component of GATA-6 function is its capacity to associate with other transcriptional regulators such as p300 [25], Nkx family members [26], and SRF [26]. The transcriptional activator p300 has been characterized as a GATA-6 coactivator specifically for the SM-MHC promoter [25]. To determine whether the mechanism by which CHF1/Hey2 inhibits transcriptional activation by GATA-6 involves competition for the coactivator p300, SM-MHC promoter activity was assayed in the presence of CHF1/Hey2, GATA-6, and p300. By itself, p300 did not activate the promoter and the combination of p300 and CHF1/Hey2 also did not have a significant effect. p300 was able to increase GATA-6 transactivation of the SM-MHC promoter by approximately 50% when transfected in a 1:1 ratio by mass with GATA-6. These results suggest that p300's effects on the SM-MHC promoter were due to coactivation with GATA-6. CHF1/Hey2 was able to abolish GATA-6 repression as previously shown, and increasing amounts of p300 did not overcome this repression. These findings suggest that the mechanism of repression does not involve competition for p300 (Fig. 4).

Discussion

We have previously demonstrated that the absence of CHF1/Hey2 attenuates neointimal lesion formation in injured arteries, in part through inhibition of the activation of the small GTPase Rac1 [16]. In the present study, we have demonstrated that CHF1/Hey2 physically associates with the zinc-finger protein GATA-6 and that this interaction result attenuates GATA-6 transactivation of a smooth-muscle specific promoter. These findings suggest an additional mechanism by which CHF1/Hey2 loss-of-function may affect smooth-muscle phenotype in vivo and in vitro.

CHF1 was originally identified as a member of a novel subclass of hairy-related proteins [4–7,27,28]. These factors



*Asterisk denotes statistically significant (P<0.05) difference from SM-MHC+GATA6 (lane 1)

Fig. 4. CHF1 inhibition of GATA-6 cannot be overcome by p300. Transient transfections were performed as described under Materials and methods. NIH3T3 cells were transfected with 1 μg GATA-6, 0.5 μg CHF1, and 0.33 (lane 4), 0.66 (lane 5), and 1.0 μg (lanes 2, 6, and 7) of p300 expression vector. Relative SM-MHC promoter activity is presented in arbitrary units representing the ratio of firefly luciferase to *Renilla* luciferase (mean of three replicates ± SE). "SM-MHC" refers to the smooth-muscle heavy chain promoter plasmid.

are characterized by the presence of several functional domains, including a basic-helix–loop–helix, an orange domain, an alanine-rich central region, and a YRPW motif, which allow direct interaction with transcriptional regulators [4,22,29,30] and DNA [30,31]. Currently, no direct target genes of CHF proteins have been identified in vivo, but several interactions with other transcriptional regulators have been studied including ARNT [4], MyoD [29], mSin3 [30], NCoR [30], GATA4 [22], and myocardin [15]. In the study of ARNT-induced transcription, it was concluded that both the bHLH and the orange domain were required for repression although other domains were thought to also be involved [4]. In contrast, the study of MyoD determined that the alanine-rich region played the most significant role [29]. And in the study of GATA-4, repression could be totally abolished by deletion of the basic region, while neither the deletion of the orange domain nor the alanine-rich domain had an effect [22]. The myocardin study is particularly interesting because it demonstrated a repressive effect of CHF1/Hey2 on myocardin-induced expression of the smooth-muscle-specific genes SM α-actin, SM-MHC, and SM22α, but no direct interaction with myocardin or SRF could be demonstrated, and no effect on SRF binding to CArG boxes was seen. Interestingly, the bHLH domain and the C-terminal domain beyond the orange domain but not including the YRPW motif contributed to this effect [15]. Combined, these studies suggest that the different domains allow a rich spectrum of protein–protein interactions for CHF family members through which they could exert an effect on specific, tissue-restricted genetic programs. The limited expression of CHF1/Hey2 in the ventricles and vasculature presumably takes advantage of some of these structural motifs to interact with transcriptional regulators specific to those tissues.

In this study, we focused on the interaction between CHF1 and GATA-6 because of in vivo phenotypic similarities in the response to vascular injury in a loss-of-function model of CHF1/Hey2 [16] and a gain-of-function model of GATA-6 [18]. In both cases, the response of the arterial wall to injury was marked by decreased proliferation and migration of smooth-muscle cells, which suggests that there may have been a common genetic program underlying the phenotypic similarity. Indeed, our findings show that CHF1 is able to physically associate with GATA-6 and that this association prevents GATA-6 activation of the smooth-muscle heavy chain transcription.

Together, our data suggest that the phenotypic similarity between CHF1/Hey2 −/− and adenoviral-GATA-6 treated smooth-muscle cells is likely to be due to increased activity of GATA-6 through reduction of CHF1/Hey2 inhibition in the former or through increased amounts of GATA-6 protein in the latter. This is significant as GATA-6 is associated with upregulation of nearly all the smooth-muscle marker genes, including smooth-muscle myosin heavy chain [25], smooth-muscle α-actin [26], and SM22α [26]. GATA-6, along with myocardin, is thought to be a key regulator of the differentiated vascular phenotype. CHF1/Hey2, by opposing the actions of GATA-6 and myocardin, is also a key regulator of the smooth-muscle cell phenotype. Further characterization of CHF1/Hey2 structure and function, along with identification of additional protein interactors that form a "transcriptosome" and identification of direct target genes, will provide further insight into the molecular mechanisms underlying smooth-muscle cell behavior in health and in disease.

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