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## Coronary Disease Associated Gene *TCF21* Inhibits Smooth Muscle Cell Differentiation by Blocking the Myocardin-Serum Response Factor Pathway

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### Abstract

**Rationale:** The gene encoding transcription factor *TCF21* has been linked to coronary artery disease (CAD) risk by human genome wide association studies (GWAS) in multiple racial ethnic groups. In murine models, *Tcf21* is required for phenotypic modulation of smooth muscle cells (SMC) in atherosclerotic tissues and promotes a fibroblast phenotype in these cells. In humans, *TCF21* expression inhibits risk for CAD. The molecular mechanism by which *TCF21* regulates SMC phenotype is not known.

**Objective:** To better understand how *TCF21* affects SMC phenotype, we sought to investigate the possible mechanisms by which it regulates the lineage determining myocardin (*MYOCD*)-serum response factor (SRF) pathway.

**Methods and Results:** Modulation of *TCF21* expression in HCASMC revealed that *TCF21* suppresses a broad range of SMC markers, as well as key SMC transcription factors *MYOCD* and SRF, at the RNA and protein level. We conducted chromatin immunoprecipitation (ChIP)-sequencing to map SRF binding sites in HCASMC, showing that binding is colocalized in the genome with *TCF21*, including at a novel enhancer in the *SRF* gene, and at the *MYOCD* gene promoter. In vitro genome editing indicated that the SRF enhancer CARG box regulates transcription of the SRF gene, and mutation of this conserved motif in the orthologous mouse SRF enhancer revealed decreased SRF expression in aorta and heart tissues. Direct *TCF21* binding and transcriptional inhibition at co-localized sites were established by reporter gene transfection assays. Chromatin immunoprecipitation and protein co-immunoprecipitation studies provided evidence that *TCF21* blocks *MYOCD* and SRF association by direct *TCF21*-*MYOCD* interaction.

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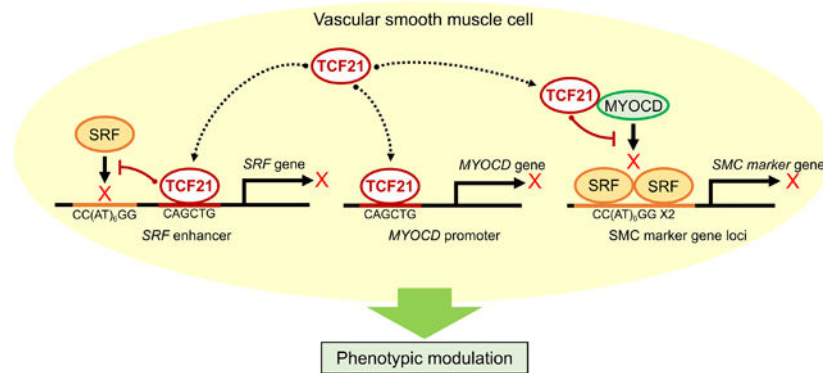
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DISCLOSURES

None.

**Conclusions:** These data indicate that *TCF21* antagonizes the MYOCD-SRF pathway through multiple mechanisms, further establishing a role for this CAD associated gene in fundamental SMC processes and indicating the importance of smooth muscle response to vascular stress and phenotypic modulation of this cell type in CAD risk.

### Graphical Abstract



TCF21 is a highly validated coronary artery disease (CAD) gene that is protective toward coronary artery disease risk. We have shown previously that this gene promotes de-differentiation, proliferation, and migration of SMC into the developing lesion, where they contribute to the protective fibrous cap. A critical question is how does TCF21 mediate these effects on the SMC? Our hypothesis was that TCF21 functions, at least in part, by blocking the MYOCD-SRF lineage determining pathway. By performing chromatin-immunoprecipitation-sequencing we were able to show that TCF21 binds in many of the same loci as MYOCD-SRF. Further, TCF21 binding was associated with decreased binding of MYOCD-SRF, suggesting an epigenetic effect. Interestingly, TCF21 was found to bind at a novel enhancer in the SRF gene and in the promoter region of MYOCD, and binding of TCF21 to these sequences inhibited transcription. Finally, TCF21 was found to disrupt the interaction of MYOCD and SRF in solution, thus blocking the ability of this complex to bind DNA. These studies identify a regulatory pathway in SMC that modulates the risk for CAD, thus providing a possible mechanism of directly inhibiting atherosclerosis through targeting SMC phenotype.

### Keywords

Atherosclerosis; smooth muscle; genetics; genomics; differentiation; myocardin; differentiation; gene expression; regulation; coronary circulation

### Subject Terms:

Atherosclerosis; Coronary Artery Disease; Gene Expression and Regulation; Smooth Muscle Cell Proliferation and Differentiation; Vascular Disease

## INTRODUCTION

There is a growing appreciation for the role of vascular smooth muscle cells (SMCs) in coronary artery disease (CAD) risk<sup>1-6</sup>. In response to atherogenic stimuli in culture, SMCs

de-differentiate, proliferate, display increased migratory behavior, and adopt a “synthetic” phenotype in which they secrete extracellular matrix components and inflammatory cytokines in a process known as “phenotypic modulation”<sup>7–11</sup>. In mouse models of atherosclerosis, contractile SMCs de-differentiate and migrate into the developing lesion, where they comprise as many as 30% of all cells in the lesion<sup>12</sup>. In this context, phenotypically modulated SMCs have been shown to contribute to multiple areas of the lesion including the fibrous cap that is thought to protect against plaque rupture and myocardial infarction<sup>11</sup>. However, the effect of these SMC cell state changes on human atherosclerosis risk has not been elucidated.

We have recently shown that TCF21, a basic helix-loop-helix transcription factor and causal CAD gene, is required for de-differentiation, proliferation and migration of medial SMC into mouse model atherosclerotic lesions, and is also expressed by human coronary artery SMC undergoing phenotypic modulation<sup>5, 6, 13–16</sup>. Human genetic data indicates that *TCF21* expression is protective toward human CAD risk, and by inference suggests that these functions in SMC inhibit primary disease processes<sup>6</sup>. Although TCF21 has a marked effect on SMC phenotype, the mechanisms by which it exerts this control are not known. Myocardin (MYOCD) is a potent transcriptional co-activator found primarily in SMCs. MYOCD is unable to bind DNA on its own, but instead binds to the widely-expressed DNA-binding transcription factor serum response factor (SRF) and this complex regulates the fundamental SMC gene expression program<sup>17</sup>. In particular, MYOCD has been shown to function as a master regulator of SMC differentiation. Its expression is correlated with the quiescent SMC phenotype and ectopic expression of MYOCD activates expression of SMC differentiation markers such as myosin heavy chain 11 (*MYH11*), calponin1 (*CNN1*) and transgelin (*TAGLN*)<sup>18–21</sup>.

Given the marked effect of TCF21 on SMC phenotypic modulation, we hypothesized that TCF21 exerts its effect by inhibiting the MYOCD-SRF pathway. In this study, we demonstrate that TCF21 inhibits expression of multiple SMC differentiation markers, that TCF21 and SRF binding are co-localized in HCASMC genome wide, and that TCF21 inhibits *SRF* and *MYOCD* expression. Further, we show that TCF21 blocks MYOCD-induced transactivation of SMC target genes, and provide evidence that TCF21 inhibits association of the MYOCD-SRF transcriptional complex, possibly through a direct interaction with MYOCD. Through this work, we have identified several molecular mechanisms by which a highly replicated CAD-associated gene modulates SMC phenotype to influence risk of disease.

## METHODS

### Data availability.

SRF ChIP-seq data has been deposited to the Gene Expression Omnibus (GEO) database, accession number GSE124011.

Detailed experimental materials and methods are described in the Online Supplement.

## RESULTS

### TCF21 regulates expression of SMC differentiation markers in vitro.

As a first step to defining the extent to which TCF21 is able to block the SMC contractile phenotype, we have investigated in detail its effect on expression levels for a number of SMC lineage markers. We conducted both *TCF21* siRNA knockdown and *TCF21* overexpression in human coronary artery smooth muscle cells (HCASMC) and assessed these effects at both the RNA and protein levels (Figs. 1A–1C). We found that *TCF21* overexpression led to decreased SM22 $\alpha$  (*TAGLN*), SM  $\alpha$ -actin (*ACTA2*), Calponin (*CNN1*), and SM MHC (*MYH11*) mRNA (Fig. 1D) and protein levels (Fig. 1E). Conversely, we confirmed that *TCF21* knockdown resulted in increased expression of these markers (Figs. 1F, 1G). For these data and all subsequent analyses where representative images and other types of data are presented, examples were chosen that represent values closest to the mean.

### SRF co-localizes with TCF21 at regions of SMC open chromatin.

To investigate the mechanism by which TCF21 inhibits SMC marker expression, we investigated the interaction of TCF21 with known regulators of SMC differentiation, MYOCD and SRF. We hypothesized that TCF21 preferentially colocalizes in SRF target loci where it independently inhibits expression of SMC contractile state genes, or regulates binding of MYOCD-SRF. To address this hypothesis, we performed chromatin immunoprecipitation-sequencing (ChIP-seq) in HCASMC to define SRF target regions of the genome, where it primarily interacts with MYOCD to promote SMC-specific gene expression<sup>18, 19, 22, 23</sup>. While SRF ChIP-seq has been performed with other cell types<sup>24</sup>, it has not been performed in HCASMC. HCASMC have a unique origin from mesodermal cells in the pro-epicardial organ, exhibit unique physiological features<sup>25</sup>, and are the specific cell type that has been implicated in the causal mechanism of the *TCF21* effect on disease risk<sup>2, 4, 6, 26, 27</sup>. We thus reasoned that SRF ChIP-seq in these cells was required for comparison to TCF21 binding. With SRF ChIP-seq, we obtained 2779 significant peaks with p-value cutoff 1e-5. 22.5% of the peaks were distributed in promoter regions while 36.9% were located in introns and 34.9% in intergenic regions. Most peaks, 72%, were located in open chromatin regions of HCASMC as identified by previous genome-wide Assay for Transposase-Accessible Chromatin using sequencing (ATACseq)<sup>2</sup>.

Intersection of SRF and TCF21 ChIP-seq data revealed significant co-localization in HCASMC regions of open chromatin. This is illustrated with a genome-wide heatmap centered on the SRF ChIP-seq peaks (Fig. 2A, left panel), which are aligned with previously identified TCF21 binding regions (Fig. 2A, middle panel)<sup>28, 29</sup>, and previously identified ATACseq regions of open chromatin (Fig. 2A, right panel)<sup>2</sup>. Intersection of peaks from the TCF21 and SRF datasets showed that 25.7% of the SRF binding sites localized within 200 bp of the nearest TCF21 peak ( $p=1.75e-25$ ) (Fig. 2B), and 22.6% of the SRF sites co-localized with at least one base pair of peak overlap ( $p=1.65e-319$ , for > 1 bp overlap) (Fig. 2C). Scanning of SRF only peaks for known transcription factor binding sites found the SRF cognate binding motif termed a CArG box in 43% of SRF peaks, an approximately 20-fold enrichment over background (Fig. 2D). Performing the same analysis for intersected peaks

showed a 15-fold enrichment for SRF sites and a 3-fold enrichment for TCF21 sites (Fig. 2E). To compare and investigate the pathways enriched for genes in the target loci, we used the Genomic Regions Enrichment of Annotations Tool (GREAT)<sup>30</sup> to assign genes to the common target SRF-TCF21 binding regions and investigated the pathways that were found to be associated with this gene list. The majority of the SRF loci genes were identified in regions that also bound TCF21 (93%, Fig. 2F).

Gene ontology analysis with the DAVID algorithm for genes located at SRF and TCF21 co-regulated loci identified biological process terms “cell adhesion,” “regulation of cell migration,” and “cell-cell adhesion” (Fig. 2F). The identified KEGG pathways were also relevant for vascular smooth muscle cell functions, and included “focal adhesion,” and “MAPK signaling pathway”. Most importantly, the disease terms included “coronary artery disease,” “arteries,” and “myocardial infarction.” While some of these terms were also identified in the analyses with SRF peak genes and TCF21 peak genes alone, the terms identified for the co-localized regions were more appropriate for the cell type function and disease relevance. To further characterize the pathways co-regulated by SRF and TCF21, we investigated the overlap of genes that are differentially regulated by TCF21, as identified by RNAseq of siRNA knockdown in HCASMC<sup>31</sup>, with genes that are direct targets of SRF as identified by ChIP-seq studies reported here. There were 486 genes identified. Gene Ontology analysis provided highly significant terms related to blood vessel development, including “blood vessel development,” “blood vessel morphogenesis,” “angiogenesis.” Genes identified through these analyses provide information regarding how genes regulated by both factors are important for both CAD and developmental processes, and the fundamental link between these has been highlighted with CAD GWAS studies<sup>14</sup>.

### **SRF and TCF21 binding appear counter-regulatory at colocalizing sites.**

To further investigate a possible relationship between binding of SRF and TCF21 in colocalizing target regions, we performed an analysis aimed to compare simultaneous binding of these two TFs. We analyzed the relative binding of each factor by normalizing the number of reads for peaks in SRF and TCF21 ChIP-seq datasets compared to background counts in the region<sup>4</sup>. We focused on those binding sites which showed a greater than two-fold difference in normalized read counts, as a measure of relative binding (Fig. 3). For the directly overlapping ChIP-seq binding sites for SRF and TCF21, more than half of the sites showed greater than a 2-fold discrepancy in binding. Out of 469 biased binding sites, 170 (36.2%) showed higher relative binding of TCF21 and 68 (14.5%) showed higher relative binding for SRF (Fig. 3). This pattern of SRF compared to TCF21 binding at shared loci is much different than expected for factors that bind with equal affinity, as we have shown previously for this type of analysis<sup>4</sup>. These findings suggest that SRF and TCF21 interact at these loci in a fashion that is counter-inhibitory or showing opposing responses to external signaling pathways.

### **Characterization of an auto-regulatory enhancer in the SRF gene that is inhibited by TCF21.**

Analysis of overlapping regions of binding by SRF and TCF21 identified an enhancer region in the second intron of the *SRF* gene (Chr6: 43141913–43142212, hg19) (Fig. 4A, Online

Fig. I). This enhancer was identified by H3K27ac chromatin modification and ATACseq chromatin accessibility datasets developed previously in HCASMC<sup>2, 4</sup>. Analysis of this region identified a single SRF canonical CC(AT)6GG (CArG) binding sequence located 71 base-pairs from the characterized TCF21 E-box binding site as defined by previous ChIP-seq studies (Online Fig. I)<sup>6, 28</sup>. Interestingly, there is only one CC(AT)6GG found in this region, while typically CArG boxes function in pairs to promote heterodimer binding<sup>18, 19, 22, 23</sup>. However, the high degree of sequence conservation in mammalian species ranging from human to opossum (Fig.4B) is consistent with this CArG sequence being functional.

Using ChIP-qPCR with both HEK293 cells transfected with *TCF21* and HCASMC that express native *TCF21*, we verified that TCF21 binds this *SRF* enhancer region (Fig. 4C). Because SRF and TCF21 co-localize at this *SRF* enhancer, we determined the effect of *TCF21* knockdown on SRF binding by ChIP-qPCR. Interestingly, *TCF21* knockdown resulted in a significant increase in SRF binding (Fig. 4D, 4E), suggesting that TCF21 inhibits the ability of SRF to bind to this intronic enhancer. These protein binding data correlated with decreased *SRF* mRNA expression in HCASMC expressing increased *TCF21*, and increased *SRF* mRNA expression in cells with decreased *TCF21* (Fig. 4F). To further validate the functionality of the CArG box in this enhancer region, we performed genome editing in HEK293 cells with the CRISPR/Cas9 approach. A single guide RNA was co-transfected along with a CRISPR/Cas9 plasmid using standard methods, and clones analyzed for genome editing and mRNA expression. The six clones investigated in detail showed various deletions in and around the CArG motif of the SRF enhancer along with significantly decreased expression levels of SRF (Fig. 4G, Online Fig. II). Finally, to investigate the in vivo role of this enhancer region we took advantage of the high degree of phylogenetic conservation, and used CRISPR/Cas9 genome editing to generate a transgenic mouse line with a mutated *Srf* enhancer CArG box, and the mutant allele bred to homozygosity (Online Fig. III). The germline gtgcCCATATAAGG sequence was mutated to gcGGGGATATAAGG, with a resulting decrease in *Srf* expression in aorta and heart, but no significant change in expression in other tissues including bladder and skeletal muscle (Fig. 4H, Online Fig. IV). Taken together these data suggest that the *SRF* enhancer is a functional mechanism of autoregulation for this gene, and is likely modulated by binding of TCF21.

### **TCF21 binds the MYOCD promoter and inhibits its expression.**

We next investigated TCF21 binding and transcriptional regulation at the *MYOCD* gene locus. We identified a region of TCF21 ChIP-seq binding within the *MYOCD* promoter, and found that this peak localized to a region of open chromatin as determined by colocalization with our previously generated ATAC-seq, H3K27Ac histone modification, and TCF21 ChIP-seq data<sup>2, 4, 6, 28</sup> (Fig. 5A, Online Fig. V). ChIP-qPCR analysis confirmed that recombinant TCF21 bound this *MYOCD* promoter region in transfected HEK293 cells and similar results were obtained with native TCF21 in HCASMC (Fig. 5B). si *TCF21* knockdown attenuated TCF21 binding to the *MYOCD* promoter region by ChIP-qPCR (Fig. 5C), reinforcing the specificity of our initial ChIP-seq findings at this site. To determine the effect of TCF21 on *MYOCD* expression, we perturbed *TCF21* expression in HCASMC and measured *MYOCD* expression at the mRNA level. Overexpression of *TCF21* in HCASMC with lentiviral



transduction resulted in a decrease in *MYOCD* mRNA (Fig. 5D). On the other hand, si*TCF21* knockdown in HCASMC resulted in an increase in *MYOCD* expression (Fig. 5E). Interestingly, there was no evidence for SRF binding at the *MYOCD* locus (Fig. 5A). Together, these experiments provide evidence that TCF21 binds to the *MYOCD* gene promoter region and inhibits *MYOCD* expression.

### **TCF21 inhibits MYOCD transactivation of SMC genes.**

In addition to its effect on *MYOCD* expression, we sought to determine whether TCF21 affects the ability of MYOCD to transactivate its target genes. Thus, we conducted reporter gene transfection studies. We transfected different amounts of *TCF21* expression construct DNA, along with a *MYOCD* expression construct and an *SRF* enhancer reporter plasmid into A7R5 cells. Transfection of *MYOCD* produced a robust activation of reporter expression as measured by dual luciferase assay (Fig. 6A, Online Table II), and this effect was abrogated when the CArG box was deleted from the reporter (Online Fig. VI, Online Table III). Expression of *TCF21* resulted in a dose-dependent decrease in *SRF* enhancer reporter expression. The inhibitory effect was also observed in cultured HCASMC treated with the highest concentration of *TCF21* DNA (800ng) (Fig. 6B, Online Table II). This effect was not due to changes in MYOCD levels, as shown by evaluating the transfected cells by western blot with an antibody validated to detect recombinant protein in these cells (Online Fig. VII). The inhibitory effect of TCF21 was also seen at endogenous levels, as MYOCD transactivation of *SRF*-luc was perturbed by knocking down native *TCF21* in HCASMC (Online Fig. VIII). Similar experiments were conducted with reporter gene constructs containing the SMC-restricted promoters of the *TAGLN* and *CNN1* genes (Methods)<sup>32-34</sup>. The reporters for these genes showed similar ability to respond to MYOCD mediated stimulation of transcription with a similar inhibition produced by *TCF21* concomitant expression in both A7R5 and HCASMC (Fig. 6C-F, Online Table II).

The fact that TCF21 was able to block the transcriptional activity of the *TAGLN* promoter was surprising, given that the *TAGLN* promoter sequence in the reporter plasmid did not contain a classical TCF21 binding site<sup>28</sup>. To investigate further the possibility that TCF21 was affecting reporter gene expression in the absence of its canonical binding sequence, we deleted the TCF21 binding motif (CAGCTG) from the *SRF* enhancer-luciferase construct. Co-transfection of *MYOCD* and *TCF21* with this modified luciferase reporter vector showed a significant reduction in enhancer activity, with the 20-fold effect of MYOCD decreasing to ~3-fold in HCASMC (Online Fig. IX, Online Table IV). These results are not conclusive, but suggest that the inhibitory effect of TCF21 is independent of DNA binding and may exert its affect by interacting directly with the MYOCD-SRF complex to block assembly in solution or its binding to DNA.

### **TCF21 directly interferes with formation and function of the MYOCD-SRF complex.**

To further investigate mechanisms by which TCF21 interacts with MYOCD and SRF to regulate gene expression, we investigated whether expression of *TCF21* interfered with their known protein-protein interaction. In the first experiments, HEK293 cells were transfected with plasmid constructs encoding *MYOCD*, *SRF*, and increasing amounts of *Myc*-tagged *TCF21* plasmid DNA (0, 1, 2  $\mu$ g). In the presence of increasing concentrations of TCF21,

we demonstrated a reduced concentration of SRF in the protein fraction immunoprecipitated by the anti-MYOCD antibody (Fig. 7A). As shown in these studies, expression of *TCF21* did not affect expression levels of MYOCD or SRF, so we speculated that the decrease in co-immunoprecipitated SRF was due to a disruption of the MYOCD-SRF complex. We thus pursued further experiments to investigate the possibility that TCF21 mediates this effect by directly interacting with MYOCD or SRF. For these studies, we expressed recombinant MYOCD and Myc-tagged TCF21 in HEK293 cells and found that immunoprecipitated MYOCD was accompanied by Myc-tagged TCF21 as detected by western blot (Fig. 7B). Further, the immunoprecipitation of Myc-tagged TCF21 was accompanied by MYOCD immunoreactivity by western blot (Fig. 7C). To ensure this interaction is found at endogenous levels of MYOCD, the experiments were repeated in A7r5 cells, which showed Myc-tagged TCF21 co-immunoprecipitated with native Myocd (Fig. 7D). Similar experiments failed to provide evidence for MycTCF21 interacting with Srf (Fig. 7E). Further, we have performed ChIP-PCR assays using MYOCD antibody and showed there was approximately a 3-fold increase in MYOCD binding at the *SRF* enhancer in HCASMC with siTCF21 knockdown (Fig. 7F). Taken together, these data are suggestive that TCF21 interacts with MYOCD to block interaction of MYOCD and SRF in solution and binding to the CArG motif in SMC (Fig. 7G).

Unfortunately, we were not able to detect reproducible native TCF21 binding to native MYOCD, due in part to the anti-correlated expression patterns of the two proteins in HCASMC (Online Fig. X) and the inability of available MYOCD antibodies to reliably detect endogenous MYOCD protein at the expected molecular weight in HCASMC<sup>35</sup>. Nevertheless, our data do show convincing evidence for native Myocd interacting with recombinant TCF21 in the A7R5 cell line, and knockdown of native TCF21 was shown to increase the binding of native MYOCD to the SRF enhancer in the HCASMC nucleus by ChIP-PCR. The data showing co-immunoprecipitation of recombinant MYOCD and TCF21 are suggestive that protein-protein interaction of these two factors impairs their association in solution, and thus the ability of the complex to bind DNA.

## DISCUSSION

To date, GWAS have identified over 160 genomic loci associated with CAD<sup>13–15</sup>. Through replication of these findings in an independent cohort and evidence for a validated mechanism in vascular cells, these findings provide an unparalleled opportunity to identify causal mechanisms for this disease process in the context of vascular wall pathology. However, because the majority of these associations reflect allelic differences in gene expression levels, due to complex enhancer gene-promoter interactions that are affected by functional differences between the causal and protective variants, progress in understanding mechanisms of genetic CAD risk remains quite limited<sup>2, 36</sup>. The genomic locus at 6q23.2 is one of the most highly replicated CAD loci, in multiple racial ethnic groups<sup>13–15, 37</sup>, and extensive genomic and genetic studies have identified TCF21 as the causal gene in this region of the human genome<sup>5, 16, 27</sup>. Using lineage tracing and single cell RNA sequencing, we have recently characterized the transcriptomic phenotype of modulated SMC *in vivo* in mice with SMC-specific knockout of *Tcf21*, and showed that loss of *Tcf21* expression leads to fewer modulated SMC within the lesion and the protective fibrous cap<sup>6</sup>. *TCF21*



expression was also strongly associated with SMC phenotypic modulation in diseased human coronaries. In human CAD-relevant tissues, *TCF21* expression was associated with decreased CAD risk, establishing a protective role for both *TCF21* and SMC phenotypic modulation in this disease. Studies described here were aimed at investigating the mechanism by which this transcription factor regulates the de-differentiated SMC phenotype, focusing on the SMC lineage determining MYOCD-SRF pathway.

Our data suggest that a critical component of TCF21 effects on HCASMC phenotype, and likely the disease process in the vessel wall, are related to inhibition of SMC lineage gene expression, mediated in part by inhibition of the MYOCD-SRF pathway. We had previously shown that TCF21 binds the *ACTA2* locus and inhibits expression of this gene, thus indicating that it directly blocks expression of some SMC genes<sup>31</sup>. However, there was no evidence for TCF21 binding in many of the SMC genes that were differentially regulated by *TCF21* expression<sup>28</sup>. Thus, we initiated studies to investigate whether TCF21 affects SMC gene expression indirectly by blocking MYOCD and SRF signaling, which is responsible in large part for determining the SMC differentiated phenotype<sup>18, 19, 21–23</sup>.

These studies suggest multiple mechanisms by which TCF21 blocks MYOCD-SRF function in SMC, underscoring the importance of this interaction in the regulation of SMC phenotype (Fig. 7G). First, we mapped SRF binding genome-wide in HCASMC and intersected the binding sites with those identified by similar studies for TCF21<sup>28</sup>, with the expectation of finding SMC target regions where TCF21 might bind and independently regulate expression of genes that are activated by MYOCD-SRF, or TCF21 might regulate MYOCD-SRF binding and function. This overlap was highly statistically significant. Interestingly, SRF was found to colocalize with TCF21 in the second intron of the *SRF* gene, in a region of HCASMC open chromatin that is also marked by an enhancer mark, H3K27ac histone modification. This finding suggests a positive feedback autoregulatory capability of SRF that is inhibited by TCF21. Importantly, knockdown of *TCF21* led to increased binding of SRF at the intronic enhancer and *SRF* expression. CRISPR/Cas9 genome editing studies in cultured cells, and in a mouse model in the highly conserved murine *Srf* enhancer, showed that mutation of the CArG motif led to decreased *Srf* expression. Also, TCF21 was shown to bind at the *MYOCD* gene promoter and inhibit its gene expression. Previous study of the regulatory mechanisms for Myocd expression identified an enhancer ~30 kilobases upstream of the regulatory region identified in these studies, which also failed to bind SRF but rather was shown to be regulated by Mef2, Tead and Foxo factors<sup>38</sup>. Interestingly, this enhancer conferred an autoregulatory function on the Myocd gene, similar to the SRF enhancer described in these studies.

The inhibitory effects of TCF21 on transcription at the *SRF* and *MYOCD* loci are consistent with TCF21 altering the local epigenetic landscape by recruiting inhibitory histone deacetylases<sup>39, 40</sup> as we have shown for the effect of TCF21 on SMAD3 binding in loci where they colocalize<sup>4, 29</sup>. Alternatively, at the SRF enhancer TCF21 and MYOCD-SRF bind 71 bp apart and are potentially associated with the same nucleosome. Thus the opposing effect of TCF21 on SRF binding might reflect the physical competition for the space necessary to establish DNA interaction. Also, the fact that TCF21 blocks MYOCD transactivation of the *TAGLN* promoter and the mutated *SRF* enhancer, which do not

contain TCF21 binding sequences, is most consistent with direct protein-protein interactions that block MYOCD-SRF binding and/or interaction with the basal transcription apparatus. Our data suggest that direct TCF21 interaction with MYOCD, i.e. protein-protein interaction, that disrupts the MYOCD-SRF complex may be responsible for some of the SMC gene expression inhibitory effects of TCF21. In this regard, TCF21 is similar to the Hippo signaling molecule TEAD1, which also directly interacts with MYOCD and blocks MYOCD-SRF complex formation<sup>41</sup>. We have shown direct protein-protein interaction for recombinant MYOCD and TCF21 in heterologous HEK 293 cells by over-expression, and interaction of native MYOCD with recombinant TCF21 in A7R5 SMC, which do not normally express TCF21. We were also able to show by ChIP-PCR that TCF21 suppresses binding of native MYOCD in HCASMC at the SRF enhancer.

It has been difficult to understand whether phenotypic modulation of HCASMC in the coronary artery promotes or diminishes the risk for CAD. On one hand, de-differentiated cells migrate into the plaque where they secrete stabilizing matrix proteins and contribute to the stabilizing fibrous cap<sup>11, 42</sup>. On the other hand, these cells are also believed to contribute to the macrophage like cells in the plaque, promoting the inflammatory milieu that destabilizes the vessel wall<sup>11, 43</sup>. Through molecular interaction of the MYOCD-SRF pathway with the CAD associated gene *TCF21*, studies reported here provide evidence for involvement of basic HCASMC processes such as lineage restricted gene expression and phenotypic modulation in the risk for CAD. Human genetic and genomic data indicate that expression of *TCF21* promotes protection from CAD<sup>6, 27</sup>, and thus by inference suggest that phenotypic modulation is protective, while factors that promote differentiation, such as the MYOCD-SRF pathway promotes CAD risk<sup>5</sup>. This directionality is strengthened by the fact that CAD associated factors with pro-differentiation effects on HCASMC, such as SMAD3 and TGF $\beta$ 1, appear to promote risk<sup>2, 4</sup>. However, this paradigm is not consistent with *ApoE* knockout model data indicating that *Myocd* negatively regulates SMC inflammatory activation and atherogenesis. The basis of this disparity in directionality of effect is not understood and will require additional study in both animal disease models and humans<sup>44</sup>.

In summary, TCF21 is associated with CAD risk, with protective alleles of this gene being linked to increased expression in HCASMC<sup>2, 5, 27</sup>. In keeping with its embryonic role where expression in the pro-epicardial organ and epicardium inhibits differentiation of coronary artery SMC precursor cells<sup>45, 46</sup>, re-activation of expression of this factor in adult SMC is associated with increased proliferation, migration, and suppressed expression of classical SMC lineage markers, the process of phenotypic modulation<sup>6, 31</sup>. One molecular mechanism by which it mediates this effect is through inhibition of the lineage determining MYOCD-SRF pathway. TCF21 binds in some of the same loci as SRF, including in an intronic enhancer in the SRF gene where it inhibits positive autoregulation. It binds the MYOCD promoter and inhibits its expression, and also blocks transcriptional regulation by MYOCD. Together, these studies provide evidence for epigenetic and transcriptional mechanisms by which TCF21 regulates MYOCD-SRF expression and function. Finally, TCF21 may possibly interact directly with MYOCD to disrupt its functional association with SRF and transcriptional regulation of SMC genes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## SOURCES OF FUNDING

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## Nonstandard Abbreviations and Acronyms:

<b>TCF21</b>	transcription factor 21
<b>SRF</b>	serum response factor
<b>MYOCD</b>	myocardin
<b>SMC</b>	smooth muscle cell
<b>HCASMC</b>	human coronary artery smooth muscle cells
<b>CAD</b>	coronary artery disease
<b>ATAC-seq</b>	(Assay for Transposase-Accessible Chromatin using sequencing)
<b>ChIP-seq</b>	chromatin immunoprecipitation sequencing

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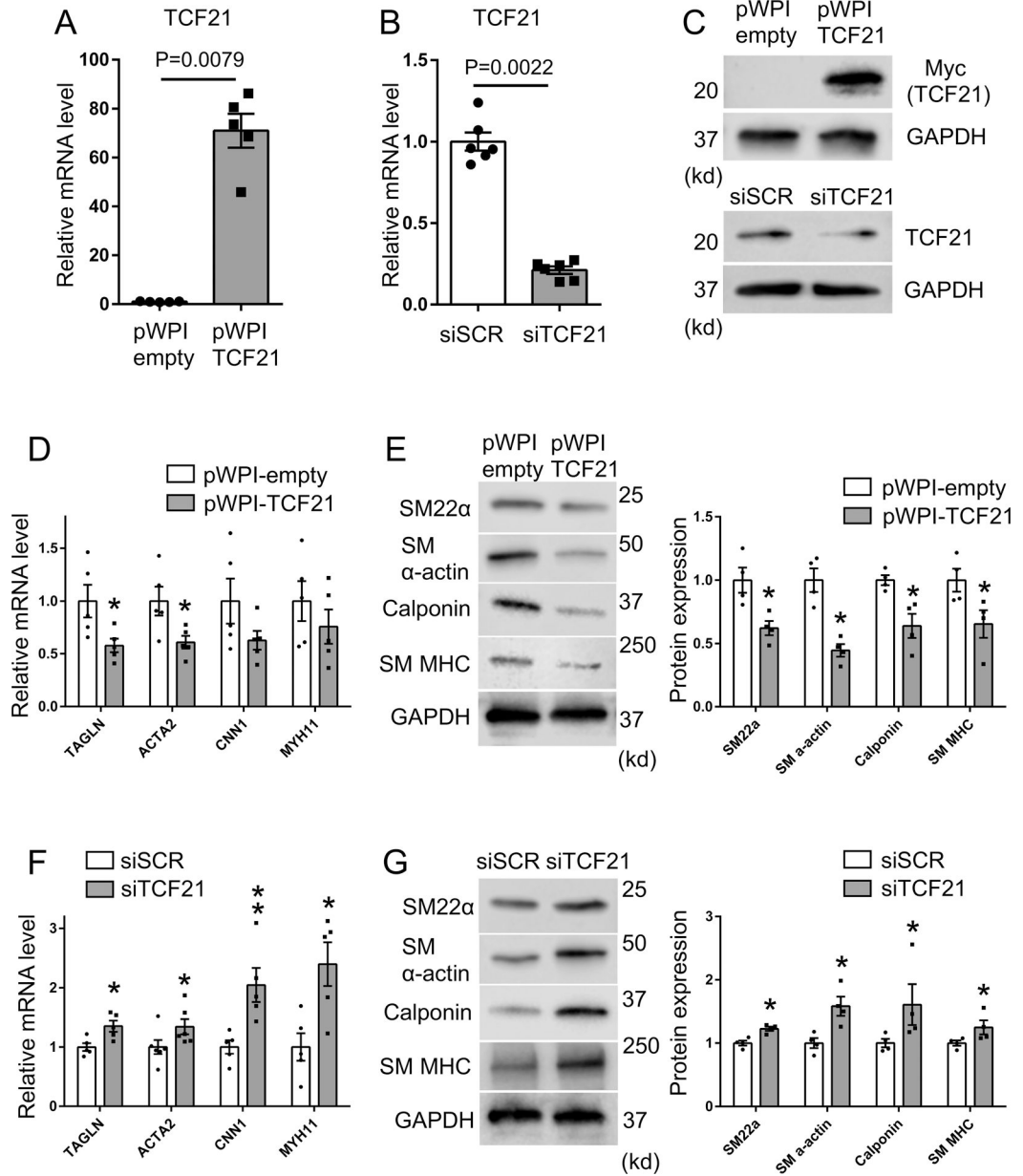
## NOVELTY AND SIGNIFICANCE

### What Is Known?

- Smooth muscle cells constitute an important component of the blood vessel wall and have been implicated in vascular disease because of their loss of mature markers and expansion in the lesion.
- The transcription factor TCF21 has been shown to promote these disease-associated features in SMC, and inhibit disease through this process, but the mechanism of this effect in these cells has not been identified.
- To potentially target this pathway for therapeutic purposes, the molecular mechanism must be examined.

### What New Information Does This Article Contribute?

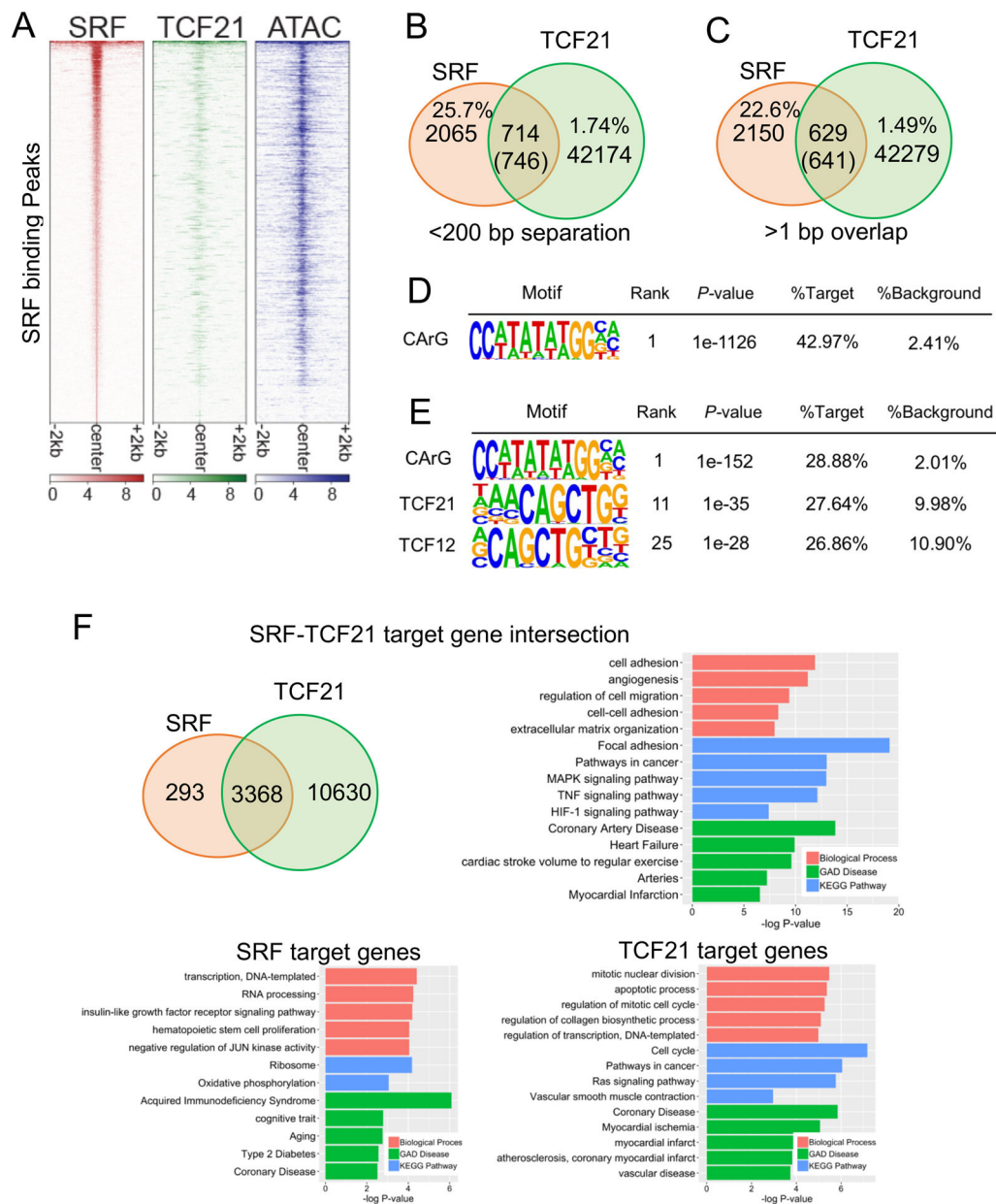
- This work shows that TCF21 binds in the same regions of the genome as the myocardin (MYOCD)-serum response factor (SRF) complex that promotes a mature phenotype in SMC.
- This work has also shown that TCF21 binds in an autoregulatory enhancer in the SRF gene as well as the promoter of the MYOCD gene to block expression of these factors.
- Further, these studies show that TCF21 directly blocks the ability of MYOCD and SRF proteins to associate in solution and bind to DNA, thus further inhibiting the ability of these factors to direct a mature phenotype in the SMC.



**Figure 1. TCF21 suppresses SMC lineage marker expression.**

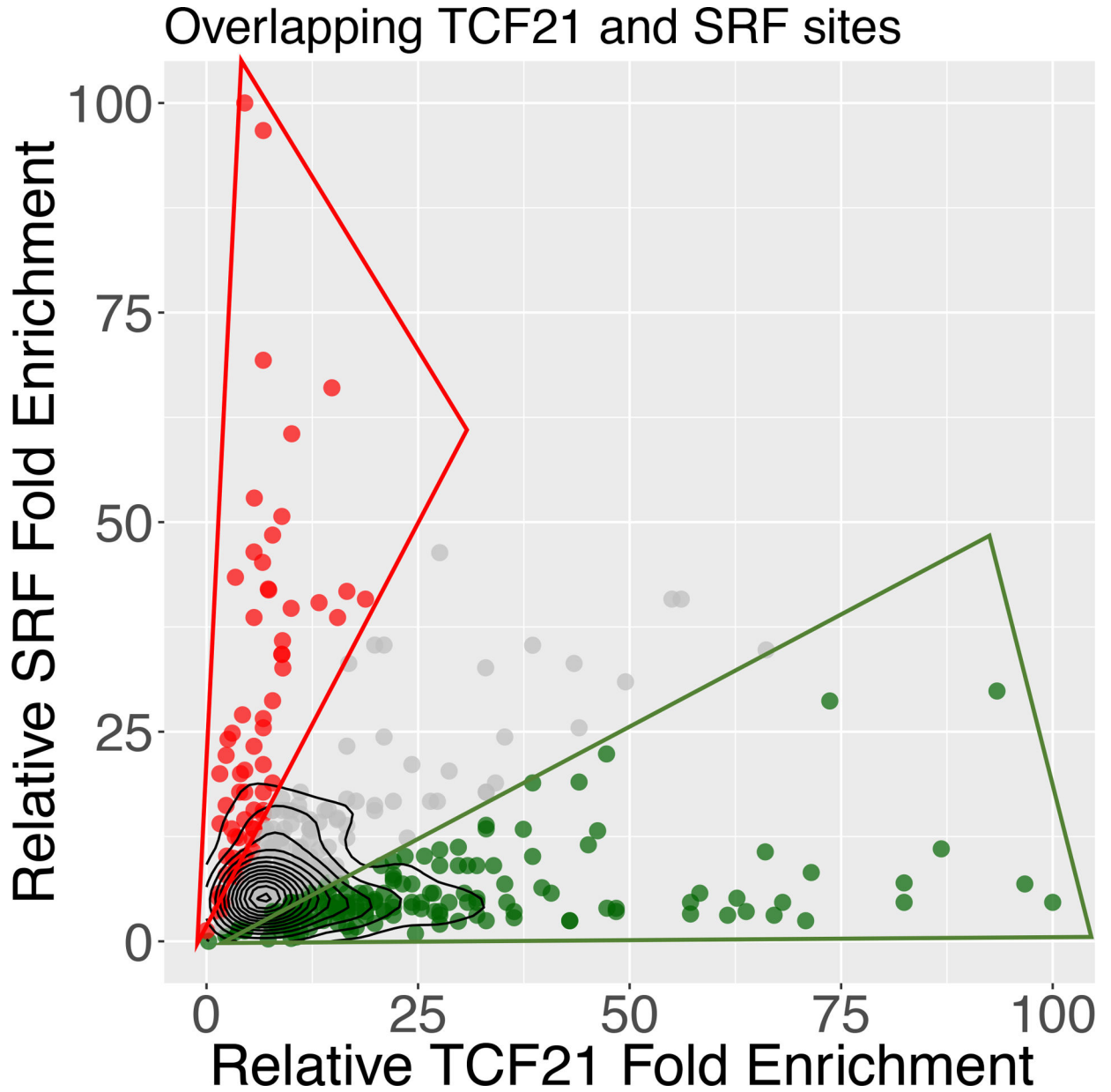
**A-C)** For overexpression or knockdown of TCF21, HCASMC were transfected with *TCF21* overexpressing lentivirus (pWPI-*TCF21*) or si *TCF21*, respectively. mRNA and protein expression of TCF21 was evaluated by qPCR or western blot. **D)** HCASMC were treated with *TCF21* overexpressing lentivirus (pWPI-*TCF21*) or empty control lentivirus (pWPI-empty) for 24 hours, and mRNA was evaluated by qPCR for expression of SMC markers *TAGLN*, *ACTA2*, *CNN1*, *MYH11* (n=5), (p\* values, *TAGLN*; 0.0317, *ACTA2*; 0.0317). **E)** HCASMC were treated with *TCF21* overexpressing lentivirus (pWPI-*TCF21*) or empty lentivirus (pWPI-empty) and protein expression of SMC markers evaluated by western blot. Band intensity of SMC markers was quantified by image J software and normalized to GAPDH (n=4) (all p\* values; 0.0286). **F)** HCASMC underwent TCF21 knockdown with

transfection of si *TCF21* or control siSCR regulatory RNAs and SMC marker expression evaluated by qPCR (n=5–6) (p\* values, TAGLN; 0.0317, ACTA2; 0.026, MYH11; 0.0317; p\*\* value, CNN1; 0.0079). **G**) HCASMC underwent TCF21 knockdown with transfection of si *TCF21* or control siSCR regulatory RNAs and protein expression of SMC markers evaluated by western blot. Band intensity was quantified by image J software and normalized to GAPDH (n=4) (all p\* values; 0.0286). Data analyzed by Mann-Whitney U test.



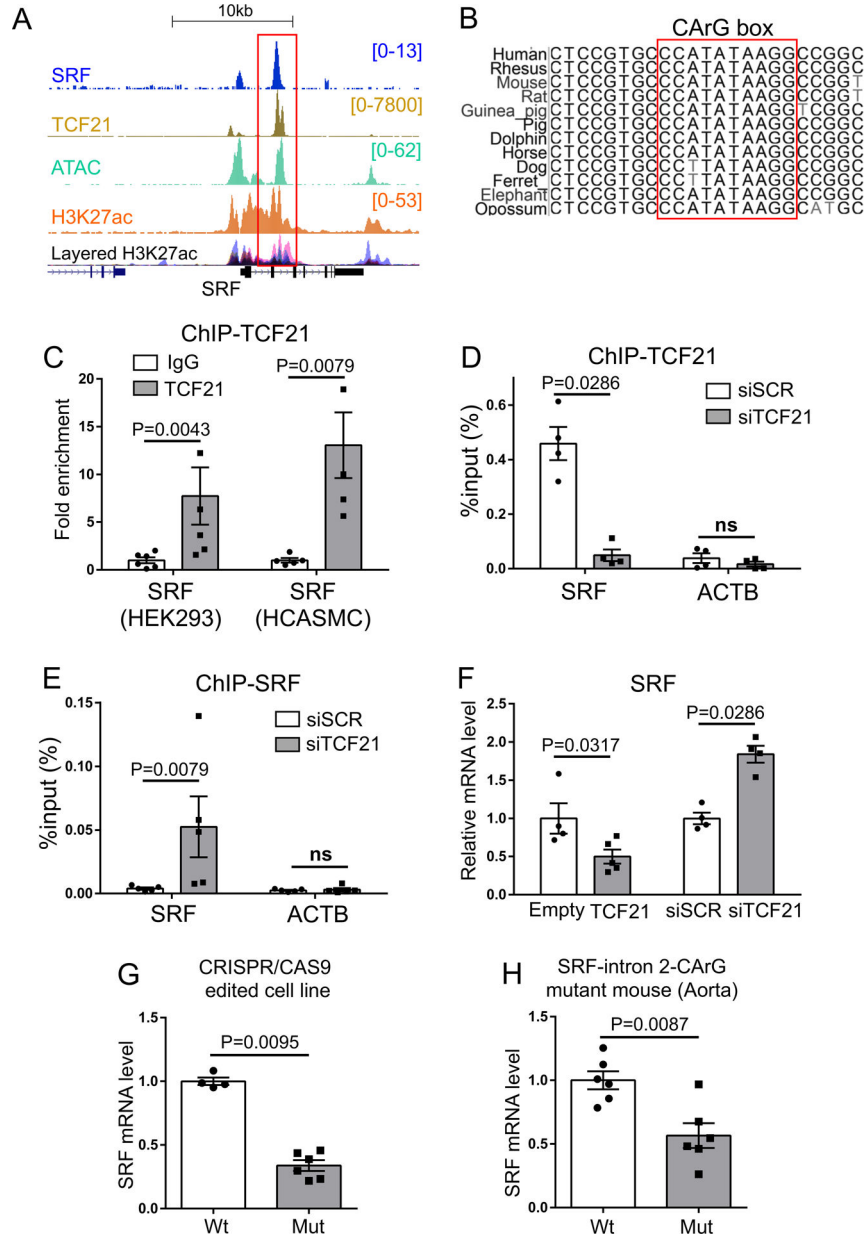
**Figure 2. SRF and TCF21 co-localize genome-wide in HCASMC and regulate HCASMC pathways.**

**A)** Heatmap distribution of SRF binding compared to previously generated TCF21 ChIP-seq and open chromatin regions (ATAC-seq), centered on SRF peaks in HCASMC within a 4-kb window. **B)** Venn diagram showing the number of overlaps of TCF21 and SRF peaks in the single nucleosome (<200bp separation). **C)** Venn diagram showing the number of direct overlaps (>1bp) of TCF21 and SRF peaks. **D)** Homer known motif analysis of SRF peaks identified CARg as the top motif. **E)** Homer known motif analysis of SRF-TCF21 joint loci showing the co-enrichment of TCF21 E-boxes and SRF CARg binding motifs. **F)** Venn diagram showing the number of overlapped target genes of TCF21 and SRF. Barplots show the DAVID Gene Ontology analysis of SRF target genes (bottom left), TCF21 target genes (bottom right), or intersected target genes (top right). Genes were assigned by GREAT.



**Figure 3. SRF and TCF21 show opposing binding behavior in many colocalized regions of the genome.**

SRF and TCF21 joint binding regions were evaluated for degree of occupancy by each factor. DNA binding was assessed by comparing peak reads normalized to background reads at each locus and this variable graphed. Most of the colocalized binding regions were biased for either SRF or TCF21 binding, with a greater than 2-fold number of normalized reads for one or the other factor.



**Figure 4. Identification of an intragenic autoregulatory *SRF* enhancer region inhibited by TCF21.**

**A)** Pattern of ChIP-seq mapping of SRF binding as well previously determined TCF21, H3K27ac histone modification and ATAC-seq identification of open chromatin in HCASMC at the human *SRF* locus. ENCODE H3K27ac data are shown as well. **B)** Phylogenetic conservation of DNA sequence in the second intron of the *SRF* enhancer at the CArG box. **C)** Chromatin immunoprecipitation PCR (ChIP-qPCR) for TCF21 binding at the *SRF* enhancer in HEK293 overexpressing TCF21 or HCASMC (n=5–6). **D)** ChIP-qPCR for TCF21 binding (n=4) or **E)** SRF binding (n=5) at the *SRF* enhancer in HCASMC treated with siTCF21 or scrambled siRNA (siSCR). ACTB,  $\beta$ -actin transcription start site, was used as a negative control. **F)** Relative *SRF* expression level evaluated by qPCR with TCF21 over-expression (TCF21) compared to control (Empty) (n=4–5), and *SRF* expression level



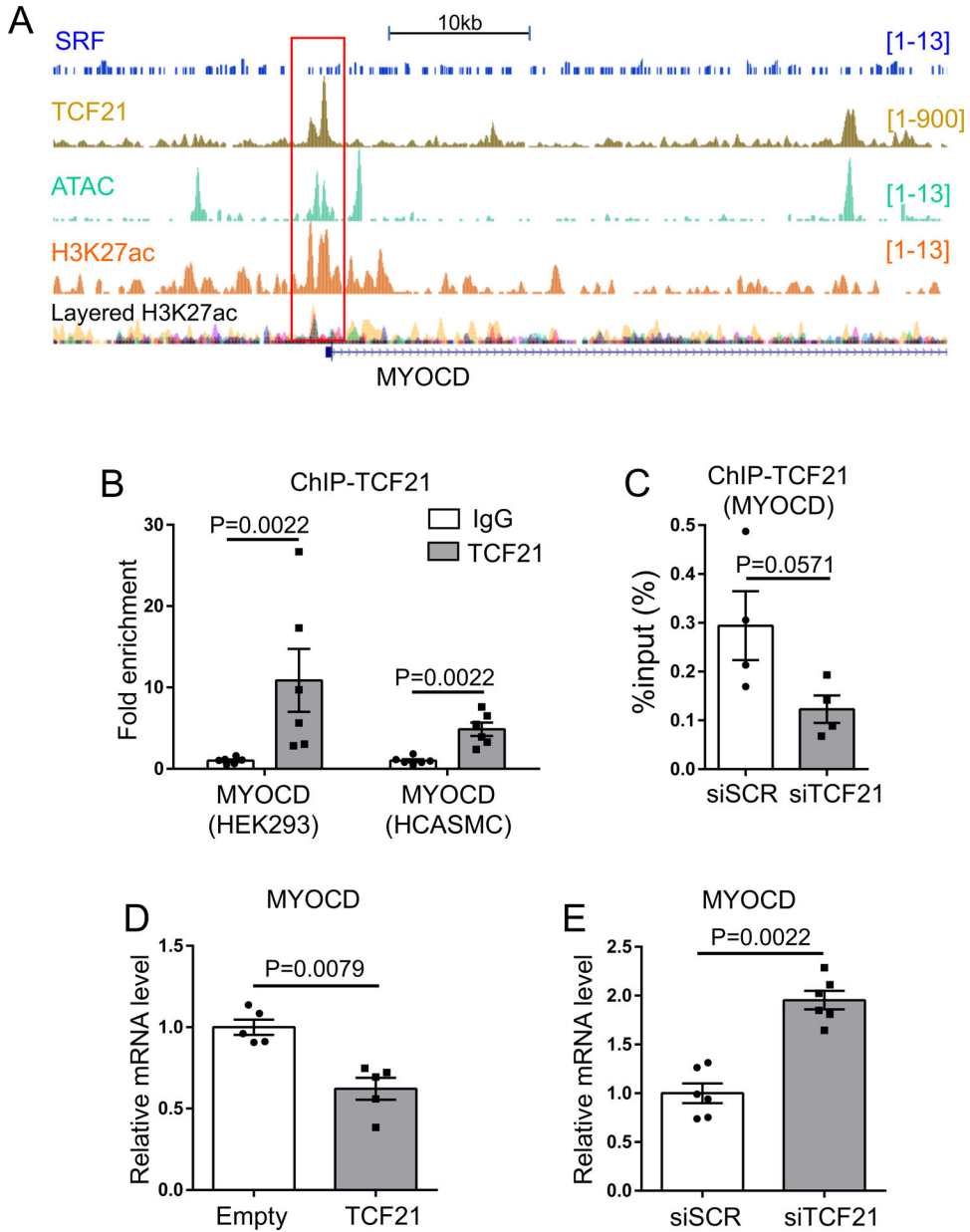
with *TCF21* knockdown (si *TCF21*) or control (siSCR) transfection in HCASMC (n=4). **G**) *SRF* expression levels evaluated in wild type (WT) or CRISPR/Cas9 edited mutant CArG box HEK293 cells (Mut) at the *SRF* enhancer (Wt vs Mut =4 vs 6). **H**) qPCR analysis of *SRF* in wild-type (Wt) vs homozygous mutant CArG box (Mut) at the *SRF* enhancer in mouse aorta (n=6). C-H) Data analyzed by Mann-Whitney U test.

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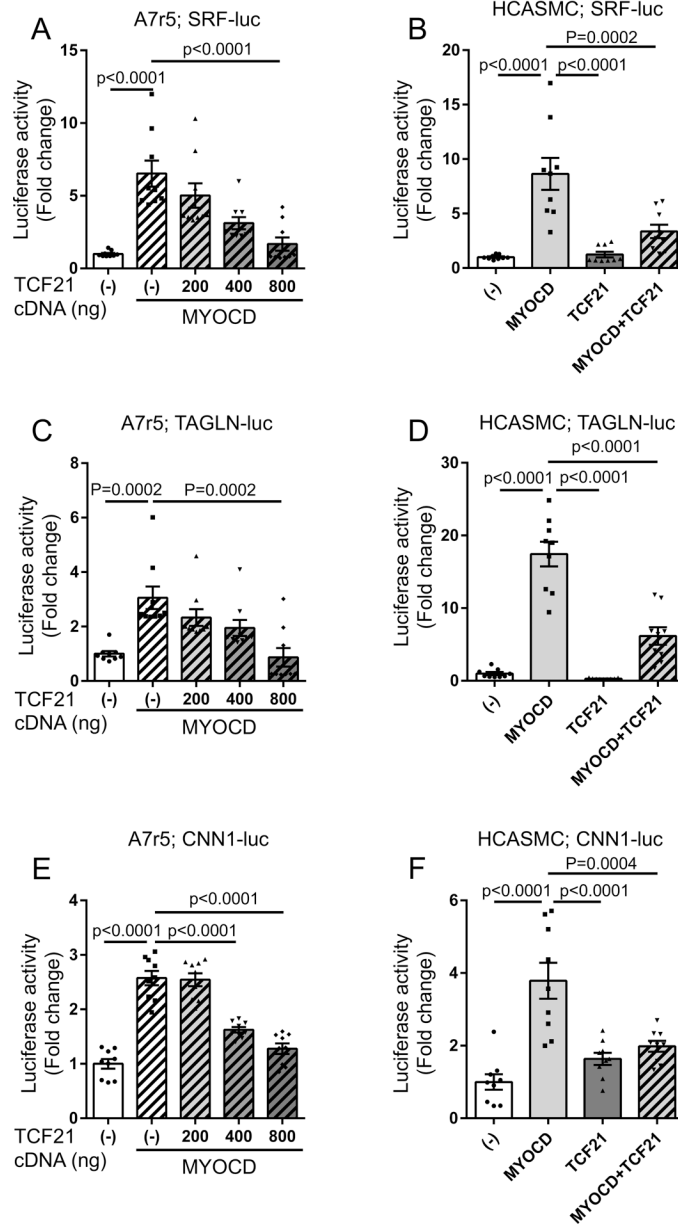
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**Figure 5. TCF21 binds the *MYOCD* promoter and suppresses its expression.**

**A)** Pattern of TCF21 binding at the *MYOCD* locus, as identified by previously determined TCF21 ChIP-seq, H3K27ac histone modification and ATAC-seq identification of open chromatin in HCASMC. **B)** Chromatin immunoprecipitation for TCF21 binding to the *MYOCD* promoter in HEK293 overexpressing TCF21 and HCASMC (n=5–6). **C)** Chromatin immunoprecipitation for TCF21 binding to the *MYOCD* promoter in HCASMC treated with siTCF21 or siSCR (n=4). **D, E)** *MYOCD* gene expression analyzed by qPCR. For overexpression or knockdown of *TCF21*, HCASMC were treated with *TCF21* overexpressing lentivirus (pWPI-*TCF21*) or siTCF21, respectively. B-E) Data analyzed by Mann-Whitney U test.



**Figure 6. TCF21 blocks transcriptional regulation by MYOCD at the *SRF* enhancer, the *TAGLN* promoter and the *CNN1* promoter.**

**A, B** Either one or both *MYOCD* (pcDNA3.1-*MYOCD*) and *TCF21* (pCMV6-AC-*TCF21*) expression plasmids were co-transfected with a reporter construct containing the *SRF* enhancer (*SRF-luc*) into A7r5 (**A**) and HCASMC (**B**). Dual luciferase assays were performed, using Renilla luciferase reporter plasmid as an internal control of transfection efficiency. **C**) Dual luciferase assays were conducted using a reporter construct containing the *TAGLN* promoter (*TAGLN-luc*) transfected into A7r5 cells, and **D**) HCASMC with the same methodology. **E**) Dual luciferase assays were conducted using a reporter construct containing the *CNN1* promoter (*CNN1-luc*) transfected into A7r5 cells, and **F**) HCASMC with the same methodology. All experiments, (n>8). Each group was compared to

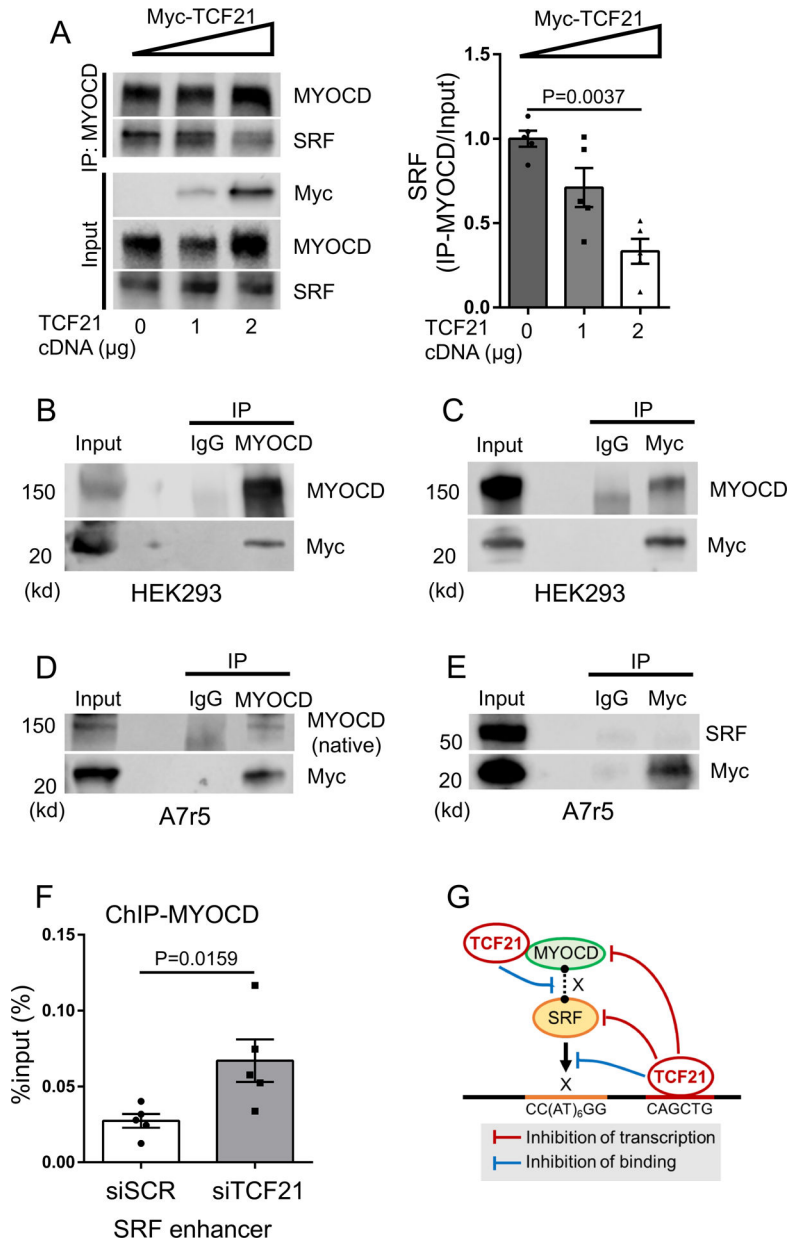
“TCF21(-) with MYOCD” (A,C,E) or “MYOCD” (B,D,F) using one-way ANOVA followed by Dunnett’s post-test (B,D-F), or Kruskal-Wallis test, followed by Dunn’s post-test (A,C).

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**Figure 7. TCF21 binds MYOCD and disrupts the functional MYOCD-SRF complex.**  
**A)** HEK293 cells were transfected with plasmid constructs encoding *MYOCD*, *SRF*, and increasing amounts of *Myc*-tagged *TCF21* plasmid DNA (0, 1, 2 μg). Immunoprecipitation was then performed with anti-MYOCD antibody, followed by western blot with antibodies as indicated. Band intensity of SRF was quantified using image J software and normalized to the input band of SRF (n=5). Data analyzed by Kruskal-Wallis test, followed by Dunn’s post-test. **B, C)** HEK293 cells were transfected with *MYOCD* and *Myc*-tagged *TCF21* expression plasmids and immunoprecipitation was performed with anti-MYOCD antibody or anti-Myc antibody, followed by western blot with antibodies as indicated. **D, E)** To test the physical interaction of TCF21 with native rat Myocd (D) or Srf (E), A7r5 cells were transfected with *Myc*-tagged *TCF21* expression plasmid and immunoprecipitation was

performed with anti-MYOCD or anti-Myc antibody, followed by western blot with antibodies as indicated. **F)** ChIP-qPCR with anti-MYOCD antibody at the SRF enhancer in HCASMC treated with si *TCF21* or scrambled siRNA (siSCR) (n=5). Data analyzed by Mann-Whitney U test. **G)** TCF21 binding is enriched in SRF targeted loci genome-wide, and binds and inhibits transcription in both the SRF and MYOCD loci. TCF21 also blocks SRF binding to DNA at an autoregulatory enhancer region in the SRF gene. Further, direct interaction of TCF21 with MYOCD disrupts the MYOCD-SRF complex leading to decreased MYOCD-SRF association.

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