Autoregulatory Control of Smooth Muscle Myosin Light Chain Kinase Promoter by Notch Signaling

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Smooth muscle myosin light chain kinase (SM-MLCK) is the key enzyme responsible for phosphorylation of regulatory myosin light chain (MLC 20), resulting in actin-myosin cross-bridging and force generation in vascular smooth muscle required for physiological vasoreactivity and blood pressure control. In this study, we investigated the combinatorial role of myocardin/serum response factor (SRF) and Notch signaling in the transcriptional regulation of MLCK gene expression. Promoter reporter analyses in rat A10 smooth muscle cells revealed a bimodal pattern of MLCK promoter activity and gene expression upon stimulation with constitutively active Notch1 in presence of myocardin or by Jagged1 ligand stimulation. An initial Notch1-induced increase in MLCK transcription was followed by loss in promoter sensitivity, which could be restored with further Notch1 dose escalation. Real-time PCR analyses revealed that endogenous levels of Hairy Related Transcription (HRT) factor 2 (HRT2) peaked concurrently with inhibitory concentrations of Notch1. Forced expression of HRT2 demonstrated simultaneous repression of both myocardin- and Notch1-induced MLCK promoter activity. HRT2-mediated repression was further confirmed by HRT2 truncations and siHRT2 treatments that rescued MLCK promoter activity and gene expression. Chromatin immunoprecipitation studies revealed both Jagged1 ligand- and Notch1-enhanced myocardin/SRF complex formation at the promoter CArG element. In contrast, heightened levels of HRT2 concomitantly disrupted myocardin/SRF and Notch transcription complex formation at respective CArG and CSL binding elements. Taken together, SM-MLCK promoter activity appears highly sensitive to the relative levels of Notch1 signaling, HRT2, and myocardin. These findings identify a novel Notch-dependent HRT2 autoregulatory circuit coordinating transcriptional regulation of SM-MLCK.

The contractile activity of vascular smooth muscle cells (VSMCs) 2 serves to regulate physiological vascular resistance and blood pressure and dysfunctional vasoconstriction or relaxation underlies peripheral hypertensive disease, the most common diagnosis and leading risk factor of cardiovascular mortality (1). Mature, differentiated VSMCs express a number of tissue-specific contractile genes such as smooth muscle α-actin (SM α-actin), SM22a, calponin, h-caldesmon, smoothelin-B, smooth muscle-myosin heavy chain (SM-MHC), and regulatory genes including myosin light chain kinase (MLCK) critical for effective constrictor function (2). These genes are known to be transcriptionally regulated in part by serum response factor (SRF) and its co-activator, myocardin (3–5). SRF belongs to the MADS box family of transcription factors, which have the characteristic feature of mediating homodimerization and DNA binding and recruiting a variety of transcriptional cofactors that influence DNA binding affinity, transcriptional activity, and target gene specificity (6, 7). Myocardin is a member of the SAP domain family of nuclear proteins responsible for activation of many smooth muscle genes by forming a ternary complex with SRF on CArG box [CC(A/T) n GG] DNA sequences within their promoter or enhancer regions (8–11).

Smooth muscle (SM-) MLCK is a Ca 2+ /calmodulin-regulated enzyme that catalyzes the phosphorylation of myosin light chain (MLC 20), the penultimate step triggering actin-myosin cross-bridging and force generation (12). Genetic and functional studies reveal that heterozygous loss-of-function mutations in the human MLCK gene, MYLK, leads to reduced SMC contractile function rendering the aorta susceptible to biomechanical stress and dissection (13). Recent studies have shown that a 50% loss in SM-MLCK resulted in 40% inhibition of MLC 20 phosphorylation and aortic contractile responses, whereas a 90% reduction profoundly inhibited both activities (12). We discovered that the SM-MLCK promoter is also a direct transcriptional target of Notch signaling, and inhibition of Notch signaling results in blunted blood pressure responses and reduced force generation in animals and arteries (14).

Notch signaling is a key evolutionarily conserved pathway that controls a number of binary cell functions including proliferation and differentiation, cell fate decisions, and also triggers pro- or anti-angiogenic/tumorigenic processes in a cell context-dependent manner (15, 16). Interaction of cell surface-bound Notch receptors (Notch 1–4) with their ligands (Delta-like or Jagged) on adjacent cells leads to proteolytic cleavage of the transmembrane receptor, resulting in release and nuclear translocation of the Notch intracellular domain (ICN). ICN binds to the transcriptional co-activator, Mastermind (MAM), and CSL (CBF1/Su(H)/Lag-1)/RBP-J, which provides DNA

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2 The abbreviations used are: VSMC, vascular smooth muscle cell; SM-MLCK, smooth muscle myosin light chain kinase; siRNA, small interfering RNA; SM-MHC, smooth muscle-myosin heavy chain; ICN, Notch intracellular domain; HRT, hairy-related transcription.
binding specificity for the ICN-CSL complex targeting gene promoters containing the consensus element, TGGAAT (17, 18). A subset of Notch target genes include the HES (Hairy-and-enhancer of split) and HRT (Hairy-related) bHLH transcription factor families (including the members HRT1, HRT2, and HRT3), which confer inhibitory activities via transcriptional repression or through protein-protein interaction (19). Double knock-out of HRT1 and HRT2 genes in mice leads to defective angiogenesis and embryonic lethality at embryonic day 9.5–10, whereas loss of HRT2 alone results in partial perinatal lethality due to cardiac and outflow tract defects (20–22). In zebrafish, haplo-deficient gridlock (HRT2 homologue) mutants display improper aortic assembly (23). Furthermore, HRT2 is capable of suppressing contractile gene promoters including SM-MHC, SM α-actin, SM22α, and smoothelin (24–27).

Our previous identification of SM-MLCK as a novel Notch target gene included the demonstration that its promoter can be independently activated by either Notch or SRF/myocardin signals. However, it remained undetermined whether these two activation pathways functionally interact to coordinate SM-MLCK transcription. Herein, we show for the first time that SM-MLCK promoter activity is cooperatively regulated by both pathways and furthermore is highly sensitive to the relative levels of Notch1 signaling and its target effector, HRT2, uncovering a complex molecular mechanism in the transcriptional regulation of SM-MLCK gene expression. Activated Notch1 or direct Jagged1 ligand stimulation enhances myocardin-induced SRF complex formation on the SM-MLCK promoter, but at relatively high levels can raise HRT2 content, which antagonizes both myocardin- and Notch-induced promoter activity through disruption of respective transcription complexes. Together, these findings suggest the operational presence of a Notch-dependent HRT2 autoregulatory loop coordinating transcriptional control of SM-MLCK gene expression.

**Experimental Procedures**

**Cell Culture and Transfections**—Rat A10 aortic smooth muscle cells and C3H10T1/2 (10T1/2) myofibroblasts (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Den ville). Transient plasmid transfections were performed using Xtremegene transfection reagent (Roche Applied Science) with a 3:1 Xtremegene/DNA ratio, and cells were collected after 48 h. Where indicated, 24 h after plasmid transfection, A10 cells were incubated with 300 nM trichostatin-A (TSA, Sigma) or DMSO in medium and harvested 24 h later as described previously (26).

**Transfection of Small Interfering RNAs (siRNAs)**—A10 aortic smooth muscle cells were transfected with rat HRT2 or control siRNA according to the manufacturer’s protocol (Santa Cruz Biotechnology).

**Plasmid Constructs**—Expression plasmids for intracellular Notch1 (pcDNA3-ICN1), flag-tagged myocardin (pFLAG-myocardin) and MLCK-luciferase reporter constructs (p6476 and p389) were described previously (14, 26, 28). Expression plasmids for wild-type HRT2 (pHRT2-V5) and HRT2 mutants (HRT2:BHO, HRT2:BHO+, and HRT2[B-]) were described previously (26, 29).

**Real-time PCR Analyses**—Total RNA was harvested from A10 cells using TRIzol reagent (Invitrogen). Two micrograms of total RNA was used as template for oligo (dt)-primed reverse transcription using Super-Script III reverse transcriptase (Invitrogen). Real-time PCR analyses were performed on a StepOnePlus quantitative PCR system (Applied Biosystems). 50 ng of cDNA was added to each reaction mixture containing 1× FS Universal SYBR Green master mix (Roche) and 300 nM of forward and reverse gene specific primers. Expression levels of target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. All experiments were performed in triplicate and quantification was performed using the 2−ΔΔCT method (30). Primer sequences for smooth muscle MLCK were 5′-GACGTGTTCACCCTGTTC-T-3′ (forward) and 5′-TTTGTGACCATGATCGACA-3′ (reverse). Primer sequences for HRT1, HRT2, HRT3, and GAPDH were described previously (26, 31).

**Luciferase Assay**—A10 and 10T1/2 cells were seeded in 6-well tissue culture plates and co-transfected with MLCK-luciferase reporter constructs and indicated ratios of pcDNA3-ICN1 and/or pFLAG-myocardin or pHRT2-V5 or HRT2 mutant plasmids. At 48 h post-transfection, cells were lysed and reporter activity measured using luciferase assay system (Promega) according to manufacturer’s protocol in a Veritas plate reader (Turner BioSystems). Readings were normalized to total protein. Experiments were performed in triplicate.

**Chromatin Immunoprecipitation Assay (ChiP)**—Chromatin immunoprecipitation was performed following the manufacturer’s instructions (EMD Millipore). Briefly, 48 h after indicated plasmid transfections, 10T1/2 or A10 cells were fixed by addition of formaldehyde (1%) to Dulbecco’s modified Eagle’s medium for 10 min at 37 °C, washed in PBS, scraped, and resuspended in lysis buffer for 10 min at 4 °C. Following DNA shearing by a Bioruptor apparatus, a fixed volume of lysate was removed as DNA input control. The remaining suspension was diluted 10-fold with dilution buffer and precleared with protein A-agarose/salmon sperm DNA slurry. Supernatants were incubated with 2 μg of Notch1 antibody (sc-6014, Santa Cruz Biotechnology) or 2 μg of SRF antibody (sc-335x) or rabbit IgG overnight at 4 °C. Immunoprecipitated complexes were collected by pulldown with protein A-agarose/salmon sperm DNA. The precipitates were extensively washed and incubated in elution buffer. Cross-linking of protein-DNA complexes was reversed, and DNA recovered by phenol/chloroform extraction and ethanol precipitation. Both input and immunoprecipitated DNAs were subjected to semi-quantitative PCR using primers 5′-TTTGTACCTCAGATGTC-3′ (forward) and 5′- AACCTTCACTGTTGACCC-3′ (reverse) encompassing the CSL binding site (−3687) in the MLCK promoter and primers 5′-GCCCAAGAGAAGATTGGAAG-3′ (forward) and 5′-GCTGTGTATTATAGAAGGAC-3′ (reverse) flanking the promoter CArG box binding element.

**Jagged1 Ligand Stimulation Assay**—Notch signaling was induced in A10 cells cultured in dishes displaying immobilized Fc-Jagged1 fusion peptide (Fc-J1) as described previously with the following modifications (14, 26): To achieve a Jagged1 dose
response, Fc-J1 (versus control, Fc-only) conditioned medium (2.5 ml standard volume) was diluted in DMEM to generate a spectrum of relative levels (from 1x to 15x) of ligand display, where 1/11003 and 15/11003 represent a 25- and 0.6-fold dilution, respectively, of the standard volume.

**Statistical Analysis**—All data are presented as mean ± S.E. unless indicated in the figure legends. Significance of differences between groups was determined by analysis of variance or by Student's t test. A probability value of 0.05 was considered statistically significant.

**Results**

**Activated Notch1 Modulates the Regulation of SM-MLCK Transcription in a Concentration-dependent Manner**—We previously identified a conserved and functional CSL binding element in the MLCK promoter that mediates Notch-induced transcriptional activity and demonstrated the pathway could function independently of transduction by myocardin/TFII-D (14). However, potentially important functional interactions between the pathways remained unexplored. To assess combined pathway regulation of MLCK gene expression, we performed a series of luciferase reporter assays using a full-length MLCK promoter construct containing sequence -6476 to +115 driving luciferase expression (p6476; gift from Dr. Paul Herring; Fig. 1A). The p6476 construct contains both established CArG and CSL binding elements (Fig. 1A) (14, 32, 33).

A10 aortic smooth muscle cells were co-transfected with p6476, escalating doses of myocardin expression plasmid with constitutively active Notch1 (ICN1) expression. We employed Notch1 in these experiments since we previously identified this receptor in transcription complexes bound to the functional CSL element of the endogenous MLCK promoter in aortic

![Diagram](image-url)
smooth muscle cells (14). The p6476 MLCK promoter reporter was sensitive to 10 ng of ICN1 plasmid and its activity approximately doubled with inclusion of 10 ng of myocardin expression plasmid (Fig. 1B). Thereafter, a dose-dependent increase in MLCK promoter activity was observed with increasing myocardin expression in the presence of a fixed (10 ng) ICN1 expression plasmid level (Fig. 1B). Conversely, to understand the effect of incremental doses of ICN1 in presence of myocardin, we co-transfected A10 cells with 10 ng of myocardin expression plasmid and increasing quantity of ICN1 expression plasmid (10 to 320 ng). Initially, 10 ng of myocardin expression plasmid induced promoter activity ~3-fold in the presence of 10 ng ICN1 compared with reporter-only conditions (Fig. 1C, Myoc 10 ng/ICN1 10 ng). Unexpectedly, a bimodal dose response was observed with increasing ICN1 plasmid levels. Specifically, an initial up-titration to 40 ng of ICN1 plasmid maximally inhibited the p6476 reporter, but subsequently greater ICN1 plasmid content (>80 ng) restored promoter activity (Fig. 1C). An identical bimodal pattern was observed for endogenous MLCK transcript levels in response to ICN1 dose escalation (Fig. 1D). These results suggest that the level of Notch1 signaling might coordinate both induction (10 ng ICN1 or 1:1 myocardin:ICN1) and repression (1:4 myocardin:ICN1) of SM-MLCK promoter activity.

To determine whether the inhibitory effect of ICN1 required the upstream CSL binding element, A10 cells were co-transfected with p6476 or p389 luciferase reporter plasmids along with myocardin expression plasmid. The p389 reporter contains a truncated SM-MLCK promoter spanning −389 to +115 that excludes the functional CSL site (−3681) but maintains the promoter CArG element (gift from Dr. Paul Herring, Fig. 1A, and ref (14, 33)). Both reporters were equally reactive to myocardin alone (Fig. 1E, Myoc). However, co-transfection of ICN1 expression plasmid with the reporters induced p6476 promoter activity (Fig. 1E, ICN1 open bar) but failed to effectively transduce p389 (Fig. 1E, ICN1 black bar). Both reporters were equally sensitive to the stimulatory (1:1) and inhibitory (1:4) dosing of combined myocardin:ICN1 expression plasmids, confirming that Notch inhibition of myocardin-induced promoter activity is functionally independent of CSL element binding. Collectively, these studies suggest a complex bimodal mechanism of MLCK transcriptional regulation by Notch signaling.

**HRT2 Can Repress Myocardin and Notch1-induced SM-MLCK Promoter Transactivation—**Notch effectors HRT1, 2, and 3, have been shown to suppress myocardin-dependent transactivation of myofilament gene promoters including SM-MHC, Smo actin and SM22α (24, 26, 27). Therefore we sought whether HRTs might be contributing to the inhibitory activity of ICN1 on the MLCK promoter. Under conditions used for detecting p6476 reporter activity in A10 cells, relative levels of endogenous HRT1, 2, and 3 mRNA transcripts were measured by real-time PCR following transfection with variable ratios of myocardin:ICN1 expression plasmids. An early dose-dependent increase in HRT1 and HRT2 transcript levels was observed with escalating levels of ICN1 in presence of fixed myocardin expression plasmid (Fig. 2A, HRT1, HRT2). For HRT2, the initial increase in mRNA levels reached a maximum 3.2-fold in the presence of 4:1 ratio of ICN1 to myocardin expression plasmid, and incremental ICN1:myocardin ratios beyond 4:1 revealed near baseline (i.e. un-stimulated) levels (Fig. 2A, HRT2). In contrast, HRT1 levels did not return to baseline while HRT3 transcripts were poorly induced in this system (Fig. 2A, HRT1, HRT3). These intriguing observations revealed that the endogenous level of HRT2 (but not of HRT1 or HRT3) peaked coincident with the 4:1 ICN1:myocardin ratio that results in repression of the MLCK promoter and endogenous transcript levels (Fig. 2, B and C, arrows). These findings raised the possibility that the Notch effector, HRT2, may be particularly relevant for repression of MLCK transcription under these conditions.

To investigate a unique role for HRT2 in this context, loss-of-function studies were performed using inhibitory siRNA against HRT2, which achieved an approximate 75% reduction of baseline HRT2 transcript levels without altering HRT1 or HRT3 expression levels (Fig. 2D, right and data not shown). Whereas HRT2 reduction had no impact on p6476 reporter activity or MLCK mRNA levels under stimulatory conditions (1:1 ICN1:myocardin), loss of HRT2 restored promoter activity and gene expression under otherwise inhibitory conditions (4:1 ICN1:myocardin) (Fig. 2D, left and middle). Furthermore, to determine a dose-dependent relationship between levels of HRT2 and repression of the MLCK promoter, 10T1/2 cells were co-transfected with a fixed concentration of myocardin expression plasmid and increasing quantity of wild-type HRT2 expression vector. As illustrated in Fig. 2E, myocardin-induced activation of the MLCK promoter was progressively repressed by HRT2. Similarly, repression of MLCK promoter activity was observed when 10T1/2 cells were co-transfected with ICN1 expression plasmid along with dose escalation of HRT2 (Fig. 2F). Taken together, these findings confirm a functional role for HRT2 in suppression of SM-MLCK promoter activity.

HRT2-mediated Transcriptional Repression of SM-MLCK Promoter Is Dependent on Its C-terminal Domain—Prior structure-function analyses of HRT2 revealed protein regions important for repressive activity (24, 26, 29). To identify domains in HRT2 that confer repression of myocardin-induced MLCK promoter activity, HRT2 truncation and deletion mutants were assayed for their capacity to repress the MLCK promoter. A10 cells were co-transfected with p6476 luciferase reporter and myocardin and HRT2 (full length or mutant) expression plasmids. A linear representation of domain structure of full length HRT2 is illustrated in Fig. 3A, and includes basic, helix-loop-helix, Orange, and YRPW domains. Relative to myocardin alone, addition of wild-type HRT2 (Myoc + Hrt2) reduced promoter activity to 38% (Fig. 3A, right). A construct expressing only the basic, helix-loop-helix and Orange domains (HRT2:BHO) failed to repress myocardin-induced activation, suggesting the importance of C-terminal sequence for inhibitory function (Fig. 3A, right). HRT2 lacking only the YRPW domain (HRT2:BHO+) retained repressive activity comparable to full-length protein. Finally, a HRT2 construct with a mutation in the basic domain known to abolish DNA binding capacity (HRT2(B-)) also exhibited full repressive capacity (Fig. 3A, right). Together, these results confirm the inhibitory role of HRT2 and demonstrate that repressive activity appears inde-
Transcriptional Autoregulation of SM-MLCK Promoter

FIGURE 2. HRT2-mediated transcriptional repression of the SM-MLCK promoter. A, endogenous HRT1, HRT2, and HRT3 transcript levels in A10 cells containing variable myocardin:ICN1 expression plasmid ratios (*, p < 0.01). B-C, coincident levels of endogenous HRT2 mRNA with either p6476 reporter activity (B) or endogenous MLCK mRNA (C) in A10 cells transfected with variable ratios of myocardin and ICN1 expression plasmids as indicated. Arrows denote nadir of reporter activity and MLCK mRNA content at peak HRT2 levels (myocardin:ICN1 plasmid ratio 1:4). D, left, p6476 reporter activity in A10 cells treated with scrambled (sc) or HRT2-specific inhibitory (siHRT2) antisense RNA under conditions of inductive (myocardin:ICN1, 1:1) or repressive (myocardin:ICN1, 1:4) levels of forced myocardin:ICN1 expression (*, p < 0.005); middle, relative levels of endogenous MLCK mRNA in A10 cells treated with siHRT2 under inductive or repressive ratios of myocardin:ICN1 expression plasmid as indicated (*, p < 0.0001); right, relative levels of endogenous HRT2 mRNA in A10 cells treated with siHRT2 under inductive or repressive ratios of myocardin:ICN1 expression plasmid as indicated. Data normalized to pcDNA3-only treatment. E, myocardin-induced p6476 reporter activity in response to dose-escalating HRT2 levels in 10T1/2 cells. F, ICN1-induced p6476 reporter activity in response to dose-escalating HRT2 levels in 10T1/2 cells. Data represent mean ± S.E., n = 3.
Pendent of DNA binding and requires the C-terminal sequence between Orange and YRPW domains.

**HRT2-mediated Transcriptional Repression of SM-MLCK Promoter Is Independent of HDAC Activity**—Previous reports have shown that the basic domain of HRT family members can interact with and recruit corepressors Sin3 and SIRT1, silencing gene expression through histone deacetylase (HDAC) activity (34, 35). To determine whether HRT2-dependent transcriptional repression of MLCK is mediated via a capacity to recruit HDAC activity, A10 cells were co-transfected with indicated ratios of p6476 luciferase reporter, myocardin, ICN1, and HRT2 expression plasmids in presence or absence of the HDAC inhibitor, Trichostatin A (TSA). Treatment of cells with either TSA or DMSO vehicle (the TSA solvent) failed to rescue HRT2-mediated repression of myocardin- or ICN1-induced MLCK promoter activity (Fig. 3B, Myoc:HRT2 (1:8), ICN1:HRT2 (1:8)). These data suggest that the mechanism of HRT2-mediated repression does not involve recruitment of HDAC activity.

**Activated Notch1 Augments SRF Complex Formation at the SM-MLCK Promoter CArG Element**—Myocardin binds to SRF forming an activated transcription complex at promoter or enhancer CArG elements of multiple smooth muscle contractile genes including MLCK (6, 33). As the addition of constitutively active Notch1 to myocardin in A10 cells induced p6476 and p389 MLCK reporter activities despite absence of the CSL binding element (p389, Fig. 1E), we employed a series of chromatin immunoprecipitation (ChIP) assays to investigate whether Notch1 influences the binding of SRF to the MLCK promoter. Initially, forced expression of myocardin in 10T1/2 cells enhanced immunoprecipitation of the MLCK promoter CArG element using an antibody against SRF (Fig. 4A, lane 4 versus 2). Notably, inclusion of both myocardin and ICN1 (1:1 expression plasmid ratio) in 10T1/2 cells significantly increased CArG DNA precipitation (Fig. 4A, lane 6 versus 4). Control reactions performed with nonspecific polyclonal rabbit antiserum (IgG) failed to precipitate DNA (Fig. 4A, lanes 1, 3, and 5). These findings suggest that activated Notch signaling can augment SRF occupancy at the MLCK promoter CArG element, consistent with observed functional synergy of ICN1 and myocardin (1:1 ratio) transduction of MLCK reporters (Fig. 1E).

**HRT2 Disrupts the DNA Binding Complexes of Both SRF-myocardin and Intracellular Notch1-CSL on the MLCK Promoter**—We next investigated, in A10 smooth muscle cells, the concomitant impact of myocardin, ICN1, and HRT2 on transcription complex formation at CArG and CSL binding sites in the SM-MLCK promoter. In ChIP experiments, A10
cells were transiently co-transfected with empty reporter vector (pGL2b) or p6476 alone or with myocardin expression plasmid (Fig. 4B). Of note, the p6476 promoter reporter was included in cell transfections to augment signal detection from the endogenous MLCK promoter. Immunoprecipitation with anti-SRF antibody revealed an enrichment of MLCK-CArG sequence with forced myocardin expression in contrast to cells expressing only p6476 or empty vector (Fig. 4B, lane 6 versus lanes 2 and 4). As previously observed in 10T1/2 cells, functionally synergistic levels (1:1) of transfected ICN1 and myocardin expression plasmids resulted in enhanced CArG element pull-down (Fig. 4B lane 10 versus 6). Significantly, forced HRT2 expression abrogated both myocardin and myocardin/ICN1-induced SRF content at the CArG element (Fig. 4B, lanes 8 and 12). Under all conditions, control reactions performed with polyclonal rabbit antiserum (IgG) failed to precipitate DNA (Fig. 4B, odd lanes). We also determined that exogenous HRT2 did not alter endogenous myocardin, SRF, CSL, or Notch1 gene expression levels (data not shown).

In parallel ChIP experiments, we determined the effect of myocardin and/or HRT2 on ICN1-containing complex formation at the MLCK promoter CSL element. Forced expression of

![FIGURE 4. Activated Notch1 (ICN1) augments SRF occupancy at the SM-MLCK promoter CArG element. A, detection of CArG promoter element by chromatin immunoprecipitation (ChIP) analyses using anti-SRF antibody. 10T1/2 cells co-transfected with p6476 and either pcDNA3 (control lanes 1 and 2), myocardin (lanes 3 and 4), or myocardin and ICN1 (1:1 ratio; lanes 5 and 6) expression plasmids. B, detection of CArG box promoter element by ChIP analysis using anti-SRF antibody. A10 cells were co-transfected with either pGL2b (reporter control, lanes 1 and 2) or p6476 MLCK reporter with pcDNA3 (lanes 3 and 4), myocardin (lanes 5 and 6), myocardin and Hrt2 (lanes 7 and 8), myocardin and ICN1 (lanes 9 and 10), or myocardin, ICN1, and Hrt2 expression plasmids (lanes 11 and 12). C, detection of CSL promoter element by ChIP analysis using anti-Notch1 antibody. A10 cells were co-transfected with either pGL2b (reporter control, lanes 1 and 2) or p6476 MLCK reporter with pcDNA3 (lanes 3 and 4), ICN1 (lanes 5 and 6), ICN1 and myocardin (lanes 7 and 8), ICN1 and myocardin (lanes 9 and 10) or ICN1, myocardin, and Hrt2 expression plasmids (lanes 11 and 12). Precipitating antibodies (α) include αIgG, αSRF (serum response factor), and αN1 (Notch1). Quantitative densitometry data displayed as mean ± S.D. and normalized to lane 2 of each experimental set, n = 3; *, p < 0.002; **, p < 0.02; ***, p < 0.03; ns, not significant; Ip, immunoprecipitate; Inp, input chromatin.](image-url)
ICN1 resulted in enhanced complex formation at the CSL site as determined by Notch1 antibody immunoprecipitation of CSL promoter sequence (Fig. 4C, lanes 6 versus 4 and 2). However, the addition of myocardin overexpression did not alter the level of ICN1 complex (Fig. 4C, lane 10 versus 6). Importantly, forced expression of HRT2 inhibited ICN1-induced complex formation at the CSL site (Fig. 4C, lanes 8 and 12). Immunoprecipitation with nonspecific polyclonal IgG did not yield DNA sequence (Fig. 4C, odd lanes).

Taken together, these data demonstrate (i) activated Notch1 augments SRF occupancy at the MLCK promoter CArG element, (ii) myocardin does not alter ICN1-CSL complex formation at the MLCK promoter CSL site, and (iii) HRT2 is capable of simultaneous disruption of both myocardin-SRF-CArG and Notch1-CSL transcription complexes.

**SRF-CArG Complex Abundance Is Enhanced by Activated Notch1 and Repressed by Hrt2 Independent of SM-MLCK CSL Promoter Element**—In the prior ChIP experiments, p6476 promoter reporter was included in cell transfections in part to augment signal detection from the endogenous promoter. This strategy also lends to comparisons with transcription complex capacity on the p389 promoter, which lacks the CSL element but retains the CArG sequence. In the presence of empty reporter (pGL2b), all immunoprecipitated signal is derived from the endogenous MLCK promoter. Fig. 5A ChIP study displays the relative abundance of SRF antibody-precipitated CArG sequence from the endogenous MLCK locus with forced expression of myocardin, ICN1, or myocardin with ICN1 (1:1 plasmid ratio). The addition of ICN1 to myocardin increased SRF content at the endogenous CArG site (Fig. 5A, lanes 8 and 4 versus 2). This pattern was similarly observed when p389 was substituted for pGL2b though with proportionally greater signal output reflecting exogenous promoter substrate (Fig. 5A, lanes 16 and 12 versus 10). Significantly, these experimental conditions using p389 demonstrated that ICN1-induced SRF-CArG complex does not require the CSL promoter element, which is consistent with functional data (Fig. 1F). Of note, ICN1 in the absence of exogenous myocardin had little inductive capacity of SRF-CArG complex (Fig. 5A, lanes 6 and 14 versus 2 and 10, respectively).

Next, we performed ChIP assays to determine the capacity of HRT2 to repress myocardin- or myocardin/ICN1-induced SRF-CArG complex formation in A10 cells containing p389. The addition of exogenous HRT2 significantly abrogated SRF-CArG immunoprecipitation at the CArG element in the presence of myocardin alone or in combination with ICN1 (1:1) (Fig. 5B, lanes 8 and 12 versus 6 and 10, respectively). These data fortify the notion that HRT2 repression does not intrinsically require the presence of a functional Notch-CSL transcription complex in the SM-MLCK promoter.

**Jagged1 Ligand Modulates Endogenous SM-MLCK Transcriptional Activity in a Dose-dependent Manner**—To extend our regulatory findings using a more physiologic mode of Notch signaling stimulation, we recapitulated the inductive and repressive effects on SM-MLCK transcription by dose-dependent Jagged1 ligand stimulation. Through use of our established ligand induction system, A10 cells were cultured on plastic dishes displaying increasing quantity of Fc-Jagged1 fusion peptide (Fc-J1), resulting in initial rise and peak in endogenous SM-MLCK transcripts followed by decline (Fig. 6A, Fc-J1 10×...
versus 15×, respectively). Concomitantly, HRT2 mRNA was induced and remained elevated with Fc-J1 15× stimulation (Fig. 6A). Parallel experiments using Fc-only (control) did not alter gene expression (data not shown). We then performed ChIP analyses to determine content of both Notch1- and SRF-containing transcription complexes on the SM-MLCK promoter at peak and repressed SM-MLCK transcript levels. Consistent with the pattern of dose-dependent induction and repression of SM-MLCK mRNA levels, Fc-J1 10× promoted SRF complexes at the CArG element and Notch1-containing complexes at the CSL binding site (Fig. 6, B and C; lanes 8 versus 4, respectively); in contrast, the stronger Fc-J1 15× stimulation suppressed complex formation at both elements to near basal levels (Fig. 6, B and C; lanes 12 versus 8 and 4, respectively). Notably, stimulation with control Fc did not affect transcription complex abundance. Hence, modulation of Notch signaling levels by titration of either forced Notch1 expression or Jagged1 ligand stimulation results in a dual pattern of induction and repression of the SM-MLCK promoter.

In summary, in addition to our previous report demonstrating direct transcriptional activation of SM-MLCK by Notch through CSL complex formation (14), we have now identified a second mechanism of Notch activation through enhancing SRF occupancy at the promoter CArG element that is independent of CSL complex formation. Moreover, at relatively high signaling levels, Notch-induced HRT2 leads to functional inhibition.
of the MLCK promoter concomitant with reduced transcriptional activation complexes at both the CSL and CArG promoter elements. Together, these findings suggest a Notch-directed autoregulatory control of the SM-MLCK promoter.

Discussion

Notch signaling has been shown to influence contractile gene expression in both myofibroblasts and VSMCs (14, 24–27, 36, 37). We, and others, initially reported that Notch activation represses myocardin-induced contractile gene expression through induction of Notch target effectors, especially HRT2 (24–26). Subsequent studies revealed transcriptional activation of SM-MHC, SM α-actin, and SM-MLCK by Notch through functional CSL binding elements identified in the gene promoters (14, 36, 37). Understanding the mechanistic basis of these paradoxical findings is an important and active area of investigation with implications to pharmacological manipulation of Notch signaling for regulating arterial contractile function.

Myocardin partners with SRF, forming a transcriptional activation complex on promoter/enhancers containing the CSL recognition element. A subset of myocardin-SRF targets in VSMCs includes both myofilament (SM-MHC, SM α-actin, calponin-h1, SM22α) and regulatory contractile genes including SM-MLCK required for optimal force generation and contractile function characteristic of differentiated smooth muscle behavior (38–40). Activated Notch complexes can also transduce SM α-actin, SM-MHC, and SM-MLCK promoters through CSL binding elements independent of myocardin-SRF activity (14, 36, 37). In one study, functional synergism between Notch and myocardin-induced SM-MHC promoter activity was identified (36). This cooperative effect appeared independent of Notch-CSL complex formation, yet the molecular mechanism remained obscure. We previously demonstrated a dependence of a CSL binding element in the SM-MLCK promoter for transduction by Notch1 independent of myocardin (14). We have now uncovered an additional, CSL-independent mode of SM-MLCK promoter activation. Notably, the ability of activated Notch1 to augment myocardin-induced SRF-CArG complex formation and functionality of a SM-MLCK promoter lacking CSL binding capacity (p389) suggests that Notch1 likely stabilizes SRF complex formation as a basis for functional synergism with myocardin.

Hairy-related transcription (HRT) factors are established Notch effectors that generally exert repressive functions in part through HDAC recruitment and gene silencing (35). Increased HRT1, -2, or -3 levels by forced expression or Notch activation reduced contractile gene expression in vascular smooth muscle cells and myocardin-induced 10T1/2 myofibroblasts (24–27). One study suggested that the bHLH domains of HRT2 may interact with SRF and abrogate SRF-CArG complex formation in the SM-MHC promoter (24). Our studies of the SM-MLCK promoter reveal that heightened levels of HRT2 also reduce SRF content at the CArG element. However, the bHLH domains are not sufficient to confer inhibition, which otherwise requires C-terminal sequence between Orange and YRPW domains. A similar dependence on this C-terminal region was determined for HRT2 repression of the SM22α promoter CArG element (26). Functional structural motifs within the C-terminal sequence remain subject to investigation. We also show that HRT2 can inhibit Notch-CSL complex formation in the SM-MLCK promoter independent of HDAC recruitment. These findings extend previous studies of the SM α-actin promoter in which HRT2 disrupted NICD-CSL complex formation without directly binding to either NICD or CSL (37). Finally, it is important to note that HRT2 can regulate its expression through negative feedback inhibition of its own promoter (29). This inhibition appears independent of Notch transcription complex formation, binding to E-box elements, or HDAC recruitment but does require the basic domain. Hence, HRT2 has the intrinsic capacity to suppress both Notch-CSL and myocardin-SRF transcription complexes as well as its endogenous expression likely through distinct molecular mechanisms.

Studies, herein, of the transcriptional regulation of the SM-MLCK promoter have yielded important insights toward reconciling the combined stimulatory and inhibitory effects of Notch activation at a single locus. Critical observations include the relative associations between levels of ICN1 (or Jagged1 stimulation), myocardin, endogenous HRT2, and MLCK promoter/transcript activity. The combination of activated Notch1 and myocardin–expression plasmids in unimolar ratio (1:1) favors synergistic activation while the levels of Notch-induced HRT2 remain relatively low. At a 4:1 (ICN1:myoc) ratio, endogenous HRT2 levels peak in association with functional inhibition of the promoter. With further increase in ICN1: myoc ratio, HRT2 declines to pre-induction levels while MLCK promoter activity is restored. We speculate that the decline of HRT2 content under the latter conditions may be due to HRT2 feedback inhibition of its endogenous promoter as previously described (29). Furthermore, the demonstration that HRT2 knockdown in cells containing the inhibitory (4:1) ICN1:myoc expression ratio rescued SM-MLCK promoter activity and transcript levels suggests a non-redundant functional role for HRT2 among the HRT factor family. Finally, similar to forced Notch1 expression, we demonstrate that a more physiologic strategy of Notch signaling stimulation by Jagged1 ligand was sufficient to induce and repress SM-MLCK promoter activity in a dose-dependent manner.

Hence, for the first time, experimental findings support a model of Notch-induced transcriptional autoregulation of SM-MLCK promoter activity involving simultaneous functional interactions between Notch1, HRT2, and myocardin-SRF (see model, Fig. 7). Relative levels of activated Notch and its effector, HRT2, appear to dictate the balance of promoter stimulation and repression. We suggest that this autoregulatory behavior by Notch signals may be a general feature of transcriptional regulation of myocardin-SRF-dependent genes in vascular smooth muscle. A prior examination of functional linkage between Notch and myocardin regulation of the SM-MHC promoter failed to detect Notch-induced HRT2 repression, suggesting that the levels of HRT2 were insufficient to counter stimulation (36). Nevertheless it is plausible that additional inducers of HRT2 are required in a promoter-specific context and/or that broadened experimental conditions may optimally uncover intrinsic autoregulatory inhibition. Given the capacity for
transcriptional autoregulation of SM-MLCK promoter by Notch signaling. SM-MLCK promoter activity is governed, in part, by the combinatorial activities of available Notch1, myocardin, and Hrt2. Jagged1 (J1) ligand stimulation of Notch1 (N1) receptor leads to cleavage and nuclear translocation of the intracellular receptor domain (ICN1). ICN1 binds to Mastermind (not shown) and its DNA binding partner (CSL) forming a pro-active transcription complex anchored to the CSL promoter element. In parallel, myocardin (Myoc) induces transcription through the binding and stabilization of SRF at the CArG element. ICN1 can further augment SRF occupancy at the CArG site enhancing myocardin-induced transcription. Sustained Notch activity leads to accumulation of Hrt2, which both negatively regulates SM-MLCK promoter activity by abrogating ICN1 and SRF occupancy at CSL and CArG elements, respectively, and represses its own promoter (dashed lines). Hence, Notch1 signaling may function as a rheostat in coordinating and fine-tuning the balance of stimulatory and repressive signals that influence the transcriptional regulation of SM-MLCK.

Notch-induced HRT2 to inhibit Notch complex formation at CSL elements (including its own), this function may be an important autoregulatory feature of other biological systems and tissues modulated by intensity of Notch signals and is consistent with the notion that loss or excess of Notch1 can result in overlapping phenotypes (41).

We previously identified Jagged1 ligand-induced Notch1 transcription complexes at the endogenous SM-MLCK promoter CSL element (14). As such, for the current study, we employed activated Notch1 (ICN1) in cell transfections to stimulate promoter activity. Whether other Notch receptors exhibit similar regulatory control or demonstrate quantitative or qualitative differences remains to be determined. Linkage of individual Notch receptors with functional specificities or capacities to regulate contractile gene and HRT effector expression will require further examination in smooth muscle cells. In myofibroblasts, for example, Jagged1, but not Dll4, induced SM-MHC gene expression despite a similar rise in HRT1 levels (36). Hence, Notch ligand-specific dependence on effector profile warrants careful interpretation of phenotypes in systems modulated by Notch signaling.

Overall transcriptional autoregulation of SM contractile genes by a Notch-HRT-myocardin axis may especially serve a homeostatic constrictor function in the vasculature, however additional regulatory inputs may feed into this circuit. For example, a recent study identified miR-143/145 as a target of Notch activation (42). Notch-induced miR-143/145 can silence mRNAs and protein expression of myocardin repressors KLF4/5, thereby favoring contractile gene expression. A regulatory role for concomitant Notch1-induced HRT2 in miR-143/145 regulation remains to be described.

In mice, transgenic expression of a dominant-negative Mastermind (DNMAML1) in smooth muscle to repress signaling through all Notch receptors impairs SM-MLCK expression (and its phosphorylation of MLC20), arterial force production and blood pressure responses to constrictor challenge (14). DNMAML1 also abrogates Jagged1-induced HRT expression in primary aortic smooth muscle cells (43). As such, a molecular strategy to reduce the sum of Notch signaling output in smooth muscle cells may be particularly effective in disrupting the autoregulatory axis of Notch-HRT2-myocardin transcription regulation for therapeutic gain in vasculopathies including arterial hypertension.

In summary, the transcriptional regulation of smooth muscle contractile gene expression is in part governed by an exquisite interplay between levels of Notch and myocardin-SRF activity. We demonstrate both a direct (CSL site-dependent) and indirect (CSL site-independent) stimulatory function for Notch1 in the transcriptional activation of the SM-MLCK promoter. We show that Notch1 can enhance myocardin-induced promoter activity through augmentation of SRF content at the CArG element. Furthermore, Notch1-induced HRT2 can lead to repression of both Notch and myocardin function through destabilization of respective transcription complexes. Of note, a recent study identified a functional CArG element within intron 15 of SM-MLCK that selectively regulates 130 kDa MLCK isoform expression required for contractile activity in visceral smooth muscle (28). Whether the intronic CArG is additionally sensitive to Notch signals remains to be determined. Nevertheless, we have uncovered a transcriptional autoregulatory feature of Notch activity, which may have broad biological implications in systems in which Notch plays an important modulatory role.

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Transcriptional Autoregulation of SM-MLCK Promoter

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