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Modulation of Myocardin Function by the Ubiquitin E3 Ligase UBR5*[S]

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Fully differentiated mature smooth muscle cells (SMCs) are characterized by the presence of a unique repertoire of smooth muscle-specific proteins. Although previous studies have shown myocardin to be a critical transcription factor for stimulating expression of smooth muscle-specific genes, the mechanisms regulating myocardin activity are still poorly understood. We used a yeast two-hybrid screen with myocardin as bait to search for factors that may regulate the transcriptional activity of the myocardin. From this screen we identified a HECT domain-containing protein UBR5 (ubiquitin protein ligase E3 component n-recognin 5) as a myocardin-binding protein. Previous studies have shown that HECT domain-containing proteins are ubiquitin E3 ligases that play an important role in protein degradation. UBR5 has, however, also been shown to regulate transcription independent of its E3 ligase activity. In the current study we demonstrated that UBR5 localized in the nuclei of SMCs and forms a complex with myocardin *in vivo* and *in vitro*. We also show that UBR5 specifically enhanced trans-activation of smooth muscle-specific promoters by the myocardin family of proteins. In addition, UBR5 significantly augmented the ability of myocardin to induce expression of endogenous SMC marker genes independent on its E3 ligase function. Conversely, depletion of endogenous UBR5 by small interfering RNA in fibroblast cells attenuated myocardin-induced smooth muscle-specific gene expression, and UBR5 knockdown in SMCs resulted in down-regulation of smooth muscle-specific genes. Furthermore, we found that UBR5 can attenuate myocardin protein degradation resulting in increased myocardin protein expression without affecting myocardin mRNA expression. The effects of UBR5 on myocardin requires only the HECT and UBR1 domains of UBR5. This study reveals an unexpected role for the ubiquitin E3 ligase UBR5 as an activator of smooth muscle differentiation through its ability to stabilize myocardin protein.

Smooth muscle cells (SMCs)[3] are the major contractile component of the vascular, respiratory, genitourinary, and digestive systems. Differentiated SMCs are characterized by a unique repertoire of contractile proteins such as smooth muscle α- and γ-actin, smooth muscle myosin heavy chain, h-caldesmon, calponin, SM22α, and telokin. In many pathological conditions such as hypertension and atherosclerosis, the expression of these proteins is markedly attenuated (1–4). The mechanisms that result in down-regulation of contractile proteins during phenotypic modulation of smooth muscle are poorly defined. Understanding the mechanisms controlling smooth muscle phenotypic modulation under the pathological conditions is critical for identifying new therapeutic targets to help prevent or treat many cardiovascular diseases.

SRF is a transcription factor that plays a central role in the expression of many different smooth muscle-specific genes (5). It is a multifunctional protein that not only binds a highly conserved *cis*-regulatory element CC(A/T)$_6$GG, termed a CArG box, but also provides a docking surface within its conserved MADS (MDM1, agamous, deficiens, SRF) domain for interaction with a wide variety of accessory cofactors. The physical association of SRF with various cell-restricted and/or signal-dependent accessory factors confers co-activator or co-repressor activity via ternary complex formation. Of the SRF-associated proteins identified, myocardin is perhaps the most potent for stimulating expression of smooth muscle-specific genes (6). Myocardin is restricted to cardiac and smooth muscle lineages in developing mouse embryos and is an extraordinarily potent activator of CArG box-containing cardiac and smooth muscle-specific promoters in a manner that is strictly dependent on its association with SRF (7).

Although several proteins have been identified that can interact with myocardin and modulate its function (8–12), how myocardin activity is regulated is still poorly understood. By using a yeast two-hybrid screen we previously reported a DNA mismatch repair enzyme, thymine DNA glycosylase, that binds to myocardin and attenuates myocardin function by disruption of myocardin/SRF interactions (11). In the current study we

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[3] The abbreviations used are: SMC, smooth muscle cell; IP, immunoprecipitation; qRT, quantitative real time; RT, reverse transcription; FHL2, four and one-half LIM domain-containing protein 2; SRF, serum response factor; GST, glutathione *S*-transferase; siRNA, small interfering RNA; MRTF, myocardin-related transcription factor; E, embryonic day.

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identified a HECT domain (for homologous to E6-AP carboxyl terminus domain) containing ubiquitin E3 ligase, UBR5 (ubiquitin-protein ligase E3 component n-recognin 5) as a myocardin-binding protein that can augment myocardin function. Human *UBR5* (previously known as EDD) mRNA is ubiquitously expressed (13) and UBR5 localizes in the nucleus or cytoplasm in different cell types (14). UBR5 appears to function as an E3 ubiquitin-protein ligase via its conserved carboxyl-terminal HECT domain. The HECT domain is ~350 amino acids in length, and previous studies have shown that the HECT domain of E6-AP is necessary and sufficient for ubiquitin thioester adduct formation (15). Furthermore, substitution of the conserved cysteine residue with alanine at amino acid 2768 prevented UBR5-mediated ubiquitin ligation (13). In addition to a HECT domain, the UBR5 protein contains a UBR1 zinc finger motif and ubiquitin-associated domain, each of which indicates involvement in ubiquitinylation pathways and protein-protein interactions. Near to the COOH-terminal HECT domain, UBR5 contains a poly(A)-binding protein domain, to bind with Paip2 and degrade it through ubiquitination (16). Recently studies have shown that UBR5 is involved in DNA repair (17, 18), cancer progression (19), and mouse yolk sac vasculature network development (20). UBR5 has also been identified as a co-activator of progesterone receptor and potentiated progestin-mediated gene transactivation independent of its E3 ligase activity (21). More importantly, *UBR5* null mouse embryos died by E10.5 and exhibited defective yolk sac vascularization (20), suggesting that UBR5 plays a critical role on vascular development. In the current study we identified UBR5 as a myocardin-binding protein and revealed an unexpected role for the ubiquitin E3 ligase UBR5 as an activator of smooth muscle differentiation through its ability to stabilize myocardin protein.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—A fragment of the mouse myocardin cDNA encoding the NH_2-terminal 585 amino acids was cloned into the bait vector pAS2-1 (Promega). A pre-transformed mouse embryonic 17-day library was purchased from Promega. A two-hybrid screen was carried out essentially as described previously (11). One clone spanning to the COOH-terminal HECT domain (2337–2799 amino acids) of *UBR5* (AY550908) was isolated and characterized further.

Mammalian Expression and Reporter Gene Assays—FLAG-tagged mammalian expression plasmids in pSG5 vector backbone for wild type UBR5 and E3 ligase-deficient mutant with cysteine 2768 mutated to alanine (C2768A) in the HECT domain were previously described (13). Omni epitope-tagged mammalian expression UBR5 plasmid was subcloned from UBR5 pSG5 plasmid to a modified pShuttle vector (Clontech) resulting in an Omni epitope tag fused to the UBR5 NH_2 terminus. A variety of UBR5 fragments, as described in Fig. 7, were amplified by PCR and fused to the SV40 nuclear location sequence LYPKKKRKGVEDQYK at the 3′ terminus of the coding sequence to force expression in cell nucleus. Those fragments were then ligated to pcDNA3.1His (Invitrogen), resulting in expression of a fusion protein with amino-terminal His_6 and Omni epitope tags. The Myc-tagged mammalian expression plasmids for myocardin, myocardin-related transcription factor (MRTF)-A, MRTF-B, and SRF were reported in our previous study (11). Adenovirus encoding cardiac myocardin isoform was described in the same report (11). For reporter assays, all promoter reporter genes were constructed by cloning fragments of promoters into the pGL_2B or pGL_3B luciferase vectors (Promega, Madison, WI) as described previously (11). Transfection was carried out with FuGENE 6 transfection reagent (Roche) as previously described (11, 22). The level of promoter activity was evaluated by measurement of the firefly luciferase activity relative to the internal control thymidine kinase-*Renilla* luciferase activity using the Dual Luciferase Assay System, essentially as described by the manufacturer (Promega). A minimum of six independent transfections were performed and all assays were replicated at least twice. Results are reported as the mean ± S.E.

Immunocytochemistry—Rat aortic A10 cells were grown on coverslips at 3×10^5 cells/60-mm dish overnight. Cells were transfected with the FLAG-tagged UBR5 HECT domain expression plasmid for 24 h and then fixed, permeabilized, and incubated with monoclonal anti-FLAG antibody (1:100, Sigma), followed by fluorescein isothiocyanate-conjugated anti-mouse IgG (1:400) secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Cells were counterstained with Hoechst (1:5000) to visualize nuclei.

Co-immunoprecipitation (IP)—For detecting UBR5 and myocardin interactions *in vivo*, A10 cells were transduced with adenovirus encoding myocardin as indicated in Fig. 1*B*. 48 h after transduction, nuclear protein was harvested and co-IP assays were performed using a nuclear complex co-IP kit as described by the manufacturer (Active Motif). 250 μg of nuclear protein extracts were incubated with 3 μg of anti-myocardin antibody (Santa Cruz, M16, goat), UBR5 (Santa Cruz, goat), or normal goat IgG controls in 500 μl of low salt IP buffer (Active Motif) overnight at 4 °C. 60 μl of TrueBlot™ anti-goat Ig IP Beads (eBioscience) were added to the mixture for an additional 1 h with rocking and then immobilized complexes were washed 6 times with the low salt IP buffer. The immunoprecipitated protein was mixed with 45 μl of 2× SDS sample buffer and analyzed to Western blotting.

GST Pull-down Assays—Fragments of mouse myocardin cDNAs were cloned into pGEX-4T vectors (Stratagene) to generate GST fusion proteins as described in our previous report (11). cDNA encoding the COOH-terminal end of the UBR5 HECT domain (2337–2799 amino acids) was cloned into pET28 vectors (Novagen) to generate T7 fusion proteins. These GST or T7 fusion proteins were produced in *Escherichia coli* BL21-star (Stratagene) cells and GST pulldown assays were carried out as reported in our previous study (11).

siRNA—Control siRNA or siRNA against UBR5 was designed and purchased from Dharmacon. The siRNA sequence for targeting endogenous mouse and rat UBR5 was 5′-GCAAATAGCATAAGAGCAA-3′. For testing the effects of smooth muscle gene expression by depletion of endogenous *UBR5*, control siRNA or UBR5 siRNA were transfected into A10 cells for 48 h and total RNA was harvested for quantitative real time (qRT)-PCR or protein was extracted for Western blotting as described below.

Quantitative Real Time RT-PCR (qRT-PCR) Analysis—Total RNA was isolated with TRIzol reagent (Invitrogen). For qRT-PCR, 1 μg of RNA was utilized as a template for reverse transcription (RT) with random hexamer primers using the SuperScript first strand cDNA synthesis kit (Invitrogen). PCR was performed with 200 ng of cDNA and SYBR green PCR master mix (Applied Biosystems) with respective gene-specific primers as previously reported (11, 12). Primer sets for mouse and rat UBR5 were designed as sense 5′-CTG GGT ATT GAT AAT GAA GAT TCA GAA C-3′ and antisense 5′-TCC TTT CTA GCA TTT GGC TGC AAC A-3′. Quantification of the reaction product was carried out using an ABI7300 real time detection system. All samples were amplified in duplicate and every experiment was repeated independently 2 times. Relative gene expression was converted using the $2^{-\Delta\Delta C_t}$ method against the internal control *RPLP0* housekeeping gene.

Cycloheximide Chase Assays and Proteasome Inhibition Experiments—COS7 cells were seeded into a 6-well plate at a density of 1.4×10^5 cells/well. After an overnight culture, the cells were transfected with either myocardin alone or a combination of myocardin and UBR5 plasmids. Two days after transfection, the cells were treated with a final concentration of 50 μg/ml cycloheximide (Calbiochem) in 10% fetal bovine serum/Dulbecco's modified Eagle's medium for 2 or 6 h as indicated. Total proteins were collected at different time points and subjected to immunoblotting. For proteasome inhibition experiments, 36 h after transfection COS7 cells were treated with 5 μM MG132 (Calbiochem) or Me_2SO vehicle for 12 h. Protein was harvested with RIPA buffer and subjected to Western blotting as described below.

Western Blotting—Western blot analysis was carried out essentially as described previously (22–24). 30 μg of protein was fractionated on 5 or 15% SDS-polyacrylamide gels, electrophoretically transferred to a nitrocellulose membrane. The membrane was then probed with a series of antibodies. Antibodies used in this study were against: calponin (Sigma, C2687, 1:5,000), FLAG tag (Sigma, 1:2,000), Myc (Invitrogen, 1:5,000), myocardin (Santa Cruz, M16, 1:2,000), Omni (Invitrogen, 1:3,000), SM22α (Sigma, 1:5,000), SRF (Santa Cruz, G20X, 1:10,000), T7 (1:10,000, Novagen), and vinculin (Santa Cruz, 1:5,000).

Mouse Carotid Artery Ligation Model—All animal studies were approved by the Institutional Animal Care and Use Committee. Carotid artery ligation was performed on 3–4-month-old C57BL/6 mice as previously described (25). Briefly, the left common carotid artery was dissected and completely ligated near the carotid bifurcation. The right carotid artery served as an uninjured control. The right and left carotid arteries were harvested 5 and 10 days after injury and total RNA was extracted with RNAqueous-Micro kit (Ambion). cDNA was reverse transcribed and gene expression was measured by qRT-PCR as described above.

RESULTS

UBR5 Localizes to the Nucleus of Smooth Muscle Cells and Interacts with Myocardin in Vivo and in Vitro—As myocardin is a potent transcription factor that can convert most cells to SMC-like cells (26), we hypothesize that myocardin function is likely to be regulated through its association with other proteins in these non-SMCs. To identify myocardin regulatory proteins, the NH_2-terminal half of myocardin (amino-acids 1–585) was used as bait in a yeast two-hybrid screen of a 17-day embryonic mouse cDNA library. We identified the protein UBR5 (ubiquitin-protein ligase E3 component n-recognin 5). UBR5 has a total of 2799 amino acids and is a member of the HECT domain E3 ubiquitin ligase family of proteins and ubiquitously expressed. It also contains ubiquitin-associated domain, UBR1, poly(A)-binding protein, and HECT domains (21). We verified that the UBR5 protein predominantly localizes to the nuclei of smooth muscle cells (Fig. 1*A*) and confirmed the physical interaction between myocardin and UBR5 *in vivo* and *in vitro* (Fig. 1, *B–E*). To determine the subcellular distribution of UBR5, a UBR5-FLAG expression plasmid was transfected into rat aorta A10 SMCs and the protein expression was accessed by fluorescence microscopy. The data shown in Fig. 1*A* revealed that UBR5 localizes to the nucleus in vascular SMC. To investigate the UBR5 and myocardin interaction *in vivo*, A10 cells were transduced with myocardin adenovirus and myocardin (for immunoprecipitation of ectopic myocardin), or UBR5 antibodies (for immunoprecipitation of endogenous UBR5) were used to immunoprecipitate myocardin-UBR5 complexes. Western blotting of the precipitated complexes revealed that UBR5 complexes contained myocardin, and myocardin complexes contained UBR5 (Fig. 1*B*). The UBR5 clone obtained from our library screen encoded the COOH terminus of the molecule from amino acids 2337 to 2799 including only the HECT domain. To validate the UBR5/myocardin interaction *in vitro*, GST pulldown assays were performed and data from the experiments confirmed that the UBR5 HECT domain directly interacts with full-length myocardin (Fig. 1*D*) and the NH_2 terminus of myocardin (amino acids 1–585) but not the COOH terminus of myocardin (amino acids 586–935) (Fig. 1*A*). Moreover, UBR5 bound to the NTD domain within the amino-terminal half of myocardin (Fig. 1, *C* and *E*).

UBR5 Augments Myocardin-mediated Induction of Smooth Muscle-specific Genes and Promoter Activity—To determine the effects of UBR5 on the induction of endogenous smooth muscle-specific genes on myocardin, UBR5 and myocardin expression plasmids were co-transfected into 10T1/2 cells and endogenous *Telokin*, *SM22α*, and smooth muscle myosin heavy chain were detected by real time RT-PCR. The data demonstrated that UBR5 augmented the ability of myocardin to induce expression of endogenous smooth muscle-specific genes such as *Telokin*, *SM22α*, and smooth muscle myosin heavy chain in 10T1/2 cells in a dose-dependent manner. UBR5 also significantly augmented the induction of cardiac α-actin expression in myocardin (supplemental Fig. S1). Myocardin expression was not affected by co-transfection with UBR5 (Fig. 2*A*). UBR5 is widely expressed in cells in which myocardin has been shown to be able to induce expression of smooth muscle-specific genes. To determine whether endogenous UBR5 is required for myocardin-induced smooth muscle-specific gene expression, siRNA duplex targeted against UBR5 was used to knock down UBR5 expression in 10T1/2 cells. UBR5 siRNA was transected into 10T1/2 cells 24 h prior to transfection of the myocardin expression plasmid. 36 h later cells were harvested

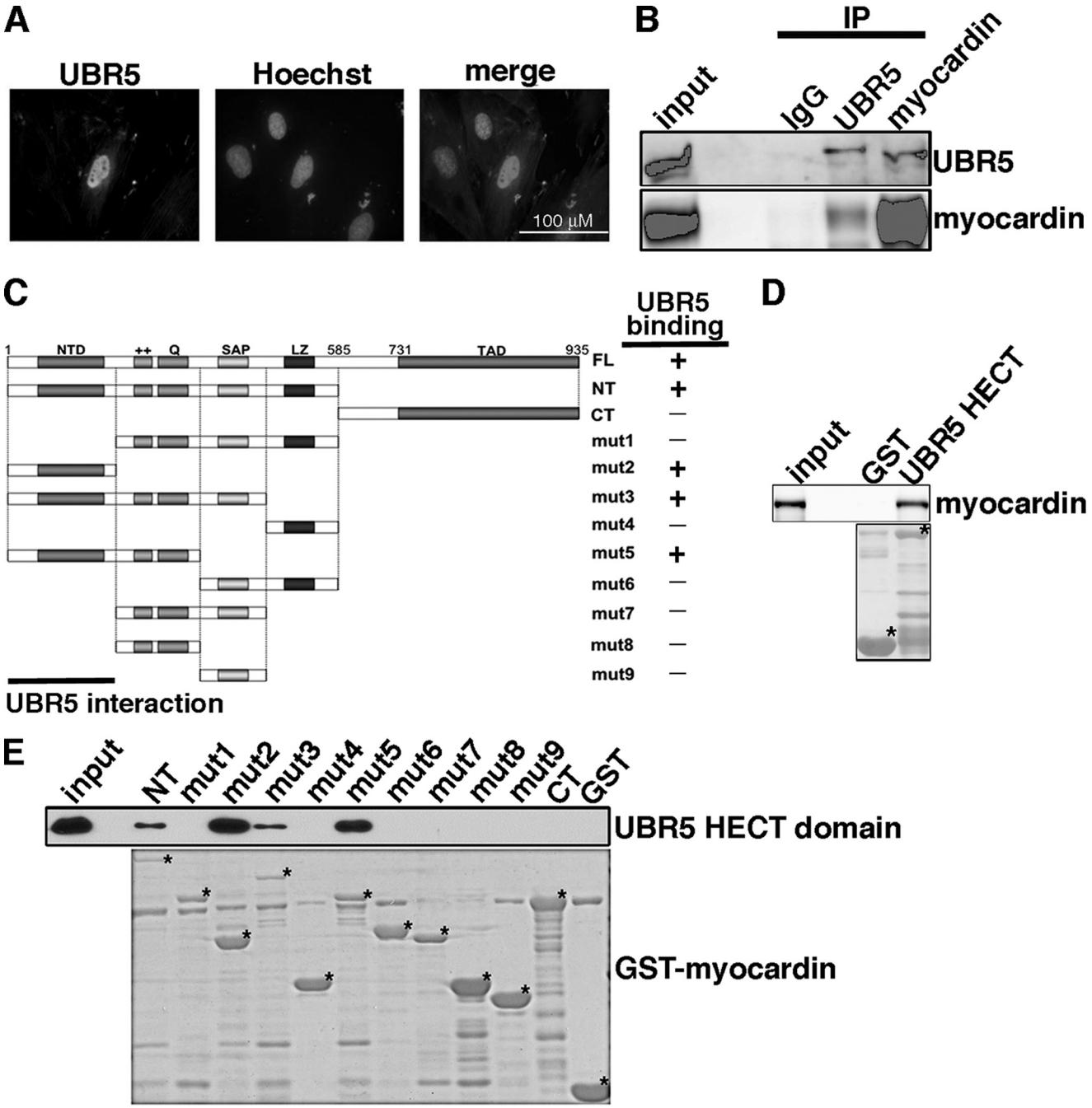

FIGURE 1. **UBR5 interacts with myocardin *in vitro* and *in vivo*.** *A*, UBR5 localizes to the nucleus in smooth muscle cells. A10 cells were plated on cover chips and transiently transfected with the FLAG-tagged UBR5 expression plasmid. UBR5 was detected using a monoclonal anti-FLAG epitope tag (*left*, *green*). Cells were counterstained with Hoechst to detect nuclei (*middle*, *blue*). An overlay image is presented on the *right* (*merge*). *B*, UBR5 binds to myocardin *in vivo*. Adenovirus encoding full-length cardiac myocardin was transduced into A10 SMCs. 48 h later nuclear extract was harvested and proteins were immunoprecipitated with myocardin, UBR5, or control IgG antibodies. The immunoprecipitated proteins were detected by Western blotting using anti-myocardin or anti-UBR5 antibodies, as indicated at the *right* of the blot. 10% total extract was loaded as input. *C*, schematic representation of myocardin indicating the GST fusion proteins analyzed. *NTD*, N-terminal domain; ++, basic domain; *Q*, poly(Q) domain; *LZ*, leuzine zipper domain; *TAD*, transcriptional activation domain; *mut*, mutant. *D*, UBR5 HECT domain binds to the full-length myocardin. Bacterial expressed full-length myocardin was incubated with the GST-UBR5 HECT domain fusion protein. Western blotting was performed to detect the myocardin fusion proteins that bound the UBR5 HECT domain (*upper panel*). The *lower panel* in *D* indicates expression of the GST fusion protein of the UBR5 HECT domain as detected by Ponceau S staining. UBR5 was found to bind to full-length myocardin. *E*, the UBR5 HECT domain binds to the myocardin NH_2-terminal (*NT*) domain. Bacterial expressed UBR5 HECT domain was incubated with the series of GST-myocardin fusion proteins indicated in *C*. Western blotting was performed to detect the GST-myocardin fusion proteins that bound the UBR5 HECT domain (*upper panel*). The *lower panel* in *D* indicates the expression of the GST fusion proteins as detected by Ponceau S staining. * indicates the GST fusion protein. UBR5 was found to bind to the myocardin NH_2-terminal domain as summarized in *C*.

with TRIzol and qRT-PCR was carried out to examine the myocardin-mediated induction of smooth muscle-specific genes. Data from this experiment reveals that knocking down endogenous UBR5 attenuated myocardin-induced expression of SM22α and calponin by 50%. Silencing endogenous UBR5 did not change ectopic myocardin mRNA expression (Fig. 2*B*). We

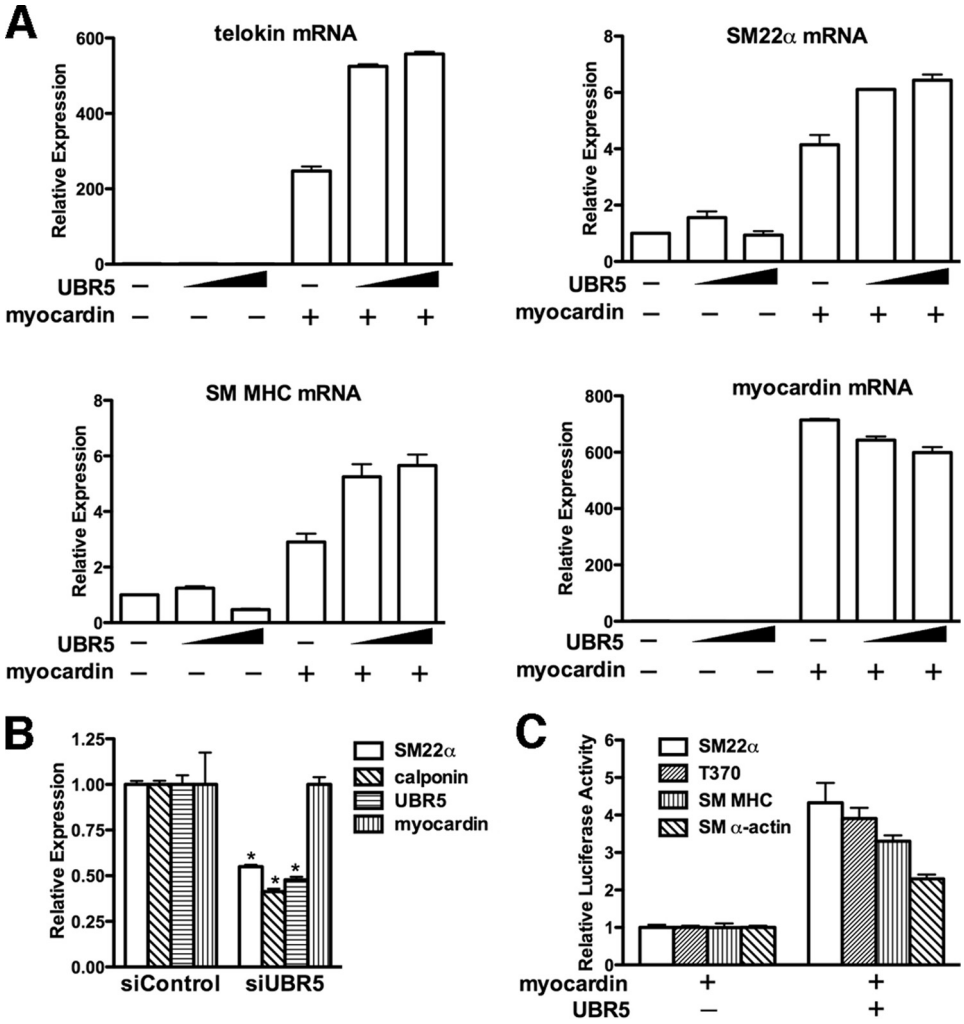

FIGURE 2. **UBR5 augments the promyogenic function of myocardin.** *A*, mouse myocardin expression vector, together with increasing amounts of UBR5 expression vector or empty plasmid pcDNA3.1 were transfected into 10T1/2 cells. 24 h post-transfection total RNA was harvested from cells and qRT-PCR was performed to examine expression of endogenous smooth muscle (*SM*)-specific genes. The ectopic expression of myocardin was also measured. Transcript levels was first normalized to acidic ribosomal phosphoprotein (*RPLP0*) internal loading control and then normalized to their respective vector-transfected control group. The $\Delta\Delta C_t$ method was used to calculate the relative quantity values of gene expression levels; relative expression $= 2^{-\Delta\Delta C_t}$ and $\Delta\Delta C_t = (C_{t,\text{experimental}} - C_{t,\text{RPLP0}}) - (C_{t,\text{control}} - C_{t,\text{RPLP0}})$. Data presented are the mean ± S.E. of 4 samples obtained from two independent experiments. *B*, 10T1/2 cells were transfected with either 100 pmol of an RNA duplex directed against *UBR5* (sequence targets rat and mouse; Dharmacon) or scrambled control RNA duplex (Dharmacon). 24 h later they were transfected with myocardin expression plasmid. Cells were harvested with TRIzol for qRT-PCR analysis of smooth muscle gene expression as described in *A*. Knocking down endogenous UBR5 attenuated myocardin-induced smooth muscle gene expression but had no effect on ectopic myocardin mRNA expression. Data are presented as mean ± S.D. from 4 samples. *, $p < 0.05$, Student's *t* test. *C*, luciferase reporter genes and myocardin expression plasmids were transfected into 10T1/2 cells with or without UBR5 expression plasmids. Values are presented as relative luciferase activity compared with myocardin-induced activation (set to 1) and are the mean ± S.E. of 6 samples from two independent experiments. Co-transfection with UBR5 significantly increases myocardin-induced transactivation of each of the smooth muscle gene reporters examined. *T370*, mouse *Telokin* gene reporter containing a 370-bp length promoter fragment. *SM-MHC*, smooth muscle myosin heavy chain.

next sought to determine the functional effects of UBR5 on myocardin-mediated transactivation of smooth muscle promoters. As shown in Fig. 2*C*, UBR5 can significantly increase the ability of myocardin to activate a number of smooth muscle-specific promoters from 2- to 4-fold.

Depletion of Endogenous UBR5 Decreases Smooth Muscle Gene Expression and Promoter Activity in SMCs—To determine the role of endogenous *UBR5* in regulating SM-specific gene expression, endogenous UBR5 levels were depleted in smooth muscle cells using siRNA. siRNA specific for *UBR5* was able to decrease endogenous *UBR5* by 70% (Fig. 3*A*) at the mRNA level and by 50% at the protein level (Fig. 3*B*) in A10 smooth muscle cells. Depletion of UBR5 from A10 smooth muscle cells resulted in a significant 40-50% decrease in calponin and *SM22α* mRNA expression (Fig. 3*A*), without affecting expression of the non-CArG-dependent smooth muscle marker *smoothelin B* (Fig. 3*A*). Consistent with this, down-regulation of SM22α and calponin protein was observed after knocking down endogenous UBR5 in A10 cells (Fig. 3*B*). Similarly depletion of endogenous UBR5 from A10 cells decreased the activity of *SM22α* and *SM α-actin* reporter genes by 40% without affecting control thymidine kinase promoter activity (Fig. 3*C*).

UBR5 Stabilizes Myocardin Protein—A recent report demonstrated that UBR5 can enhance expression of its binding partner APC by stabilizing APC protein without altering APC mRNA levels (14). Furthermore, myocardin factors are known to be regulated by proteasome-mediated degradation and FHL2 can bind to myocardin and stabilize myocardin protein (27). Given that UBR5 is able to augment the promyogenic function of myocardin (Fig. 2), we tested whether myocardin protein expression can be altered by UBR5. We transfected myocardin expression plasmid alone or together with increasing amounts of UBR5 expression plasmids into COS7 cells and analyzed the myocardin protein expression by Western blotting. Data from this experiment demonstrated that myocardin protein levels were significantly increased upon co-expression of UBR5 in a dose-dependent manner (Fig. 4*A*). To eliminate the possibility that up-regulation of myocardin protein expression was an artificial result by plasmid co-transfection, we next tested the effects of endogenous UBR5 on myocardin expression. As antibodies to detect endogenous myocardin levels by Western blot are not available, we transfected the myocardin expression plasmid into COS7 cells or infected rat aortic primary SMCs with myocardin adenovirus followed by knock down of endogenous UBR5 with siRNA. Depletion of endogenous UBR5 in COS7 cells results in

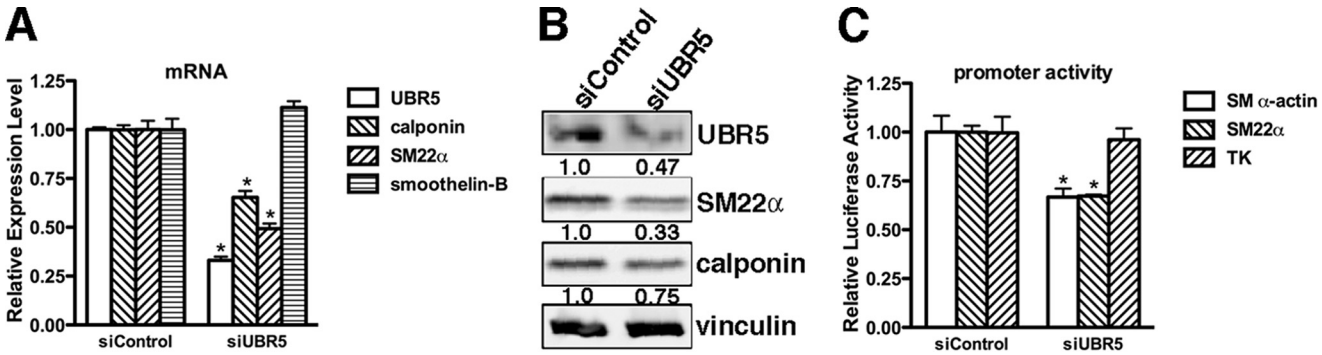

FIGURE 3. **Knocking down endogenous UBR5 attenuates smooth muscle-specific gene expression and promoter activity in A10 smooth muscle cells.** *A* and *B*, A10 cells were transfected with either control siRNA or UBR5 siRNA duplex. 48 h later total RNA was harvested, SM22α and calponin gene expression were analyzed by qRT-PCR (*A*) or Western blot (*B*). Vinculin served as a loading control. *Numbers below* each lane indicate relative expression after normalized to vinculin. Data are shown as mean ± S.D. from four samples. *, $p < 0.05$. *C*, A10 cells were transfected with either control siRNA (*siControl*) or UBR5 siRNA (*siUBR5*) for 12 h and then transfected with smooth muscle gene reporters or minimal thymidine kinase (*TK*) promoter-luciferase reporter gene. 24 h later promoter activity was measured by dual luciferase assay. Reporter activity is normalized to a *Renilla* luciferase internal control and expressed relative to siRNA control transfections (set to 1). Data were presented as mean ± S.E. of 6 samples from two independent experiments. Silencing endogenous *UBR5* attenuated smooth muscle gene *SM α-actin* and *SM22α* promoter activity but had no effect on control TK promoter. *, $p < 0.05$, Student's *t* test.

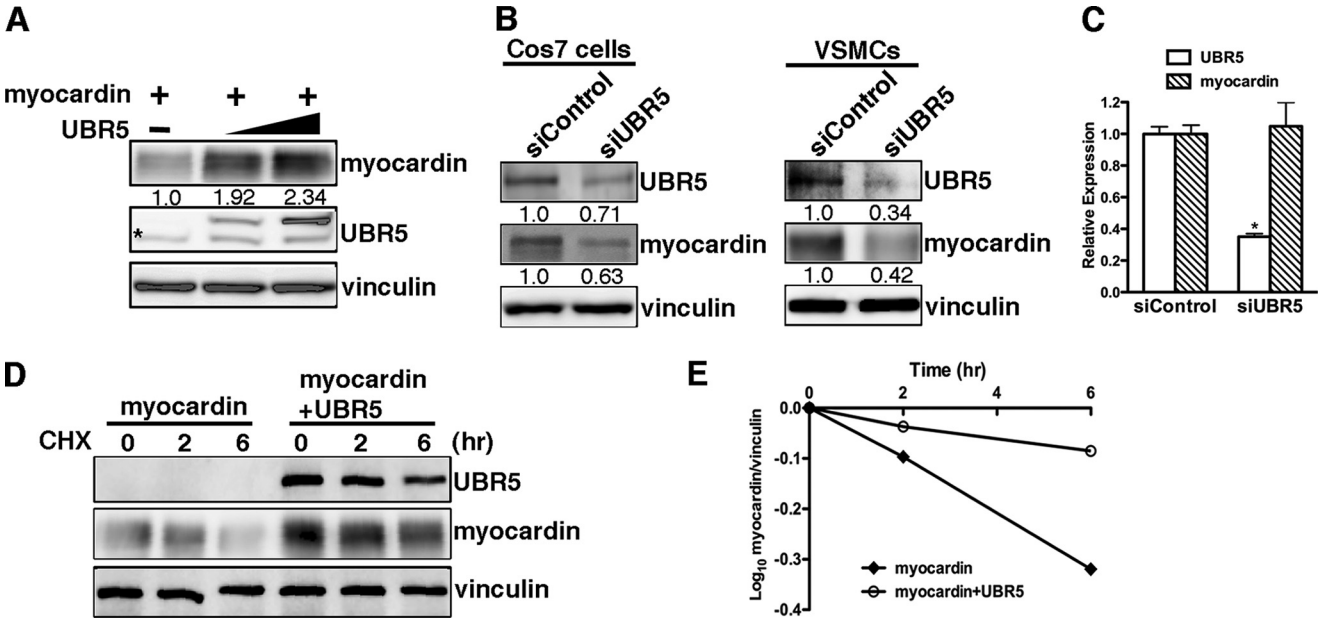

FIGURE 4. **UBR5 increases myocardin protein expression and extends myocardin protein half-life.** *A*, COS7 cells were transfected with myocardin expression plasmid alone or with increasing amounts of UBR5 expression plasmid. 36 h later cells were harvested for Western blot analysis of myocardin and UBR5 protein expression. Vinculin served as a loading control. *Numbers below* each lane indicate relative expression of myocardin normalized to vinculin. * indicates a nonspecific signal in anti-UBR5 blot. *B*, COS7 cells or rat aortic primary vascular SMCs (*VSMCs*, passage 2) were transfected with control siRNA or UBR5 siRNA duplex. 16 h later they were transfected (for COS7 cells) or infected (for VSMCs) with myocardin expression plasmid or adenovirus, respectively. 24 h later Western blotting was used to detect the protein expression of exogenous myocardin. *Numbers below* each lane indicate relative expression of UBR5 or myocardin normalized to vinculin. These results show that knocking down endogenous UBR5 decreased myocardin protein expression in non-SMCs and SMCs. *C*, A10 aortic SMCs were transfected with control siRNA or UBR5 siRNA duplex for 48 h. Total RNA was extracted and qRT-PCR was performed to measure endogenous UBR5 and myocardin mRNA expression. Data are presented as mean ± S.D. from 4 samples. *, $p < 0.05$. *D* and *E*, COS7 cells were transfected with UBR5 and myocardin expression constructs. 2 days later the transfected cells were treated with cycloheximide (*CHX*), an inhibitor of protein translation for 2 or 6 h, as indicated. Cells were harvested for Western blot to detect myocardin protein expression. Myocardin and loading control vinculin band intensity at different time points was quantified using densitometry and was plotted as $\log_{10}$ myocardin/vinculin as shown in *E*. Co-expression with UBR5 significantly increased the half-life of myocardin protein.

a significant decrease of myocardin protein expression as well as in rat vascular aortic SMCs (Fig. 4*B*). In contrast, knocking down endogenous UBR5 in A10 smooth muscle cells did not affect myocardin mRNA expression (Fig. 4*C*). Similarly knocking down UBR5 did not affect exogenous *Myocardin* mRNA expression (Fig. 2, *A* and *B*). Together these data suggest that UBR5 increases myocardin protein levels without affecting levels of mRNA.

To directly measure the effects of UBR5 on myocardin protein stability, we treated cells with the protein synthesis inhibitor cycloheximide and measured myocardin protein by Western blots 2 and 6 h following treatment. As shown in Fig. 4*D*, expression of ectopic myocardin decreased dramatically between 2 and 6 h. This decrease was significantly attenuated by co-expression of UBR5 (Fig. 4, *D* and *E*). As reported previously myocardin expression was dramatically increased by proteasome inhibition with MG132 (27) (supplemental Fig. S2). Moreover, UBR5 had no effect on myocardin protein levels in the presence of MG132 (supplemental Fig. S2).

The Effects of UBR5 on Myocardin Are Independent on Its E3 Ligase Activity—Previous published results (21) have shown that UBR5 activation of the progesterone receptor is independent on its E3 ligase function. To test the role of UBR5 E3 ligase activity in myocardin expression and activation, UBR5 E3 ligase-defective mutant C2768A (13) was generated and analyzed. This mutant was found to be able to increase myocardin protein expression (Fig. 5*A*) and activate smooth muscle-specific genes (Fig. 5, *B* and *C*) in an indistinguishable manner from the wild type UBR5. These data suggest that the ability of UBR5 to enhance *Myocardin* expression and function is independent of its E3 ligase activity.

The Activation Effects of UBR5 Are Specific to Myocardin Family Proteins—We next tested whether UBR5 can specifically activate other myocardin family transcription factors including MRTF-A and MRTF-B. Because we have had little success in measuring MRTF-A or MRTF-B protein levels with the available antibodies, we co-transfected UBR5 along with Myc-tagged MRTF-A or MRTF-B expression plasmids into COS7 cells. We also transfected UBR5 expression plasmid into COS7 alone to measure changes in endogenous SRF expression. As shown in Fig. 6*A*, overexpression of UBR5 had no effect on the expression level of the endogenous SRF protein but led to enhanced levels of exogenous MRTF-A and MRTF-B proteins (Fig. 6*A*). Consistent with these data, UBR5 failed to augment SRF-mediated transactivation of an *SM22α* gene reporter in 10T1/2 cells, whereas it augmented the transactivation mediated by MRTF-A and MRTF-B (Fig. 6*B*).

UBR5 HECT and UBR1 Domains Are Required to Enhance Myocardin Protein Expression and Transactivation—The UBR5 HECT domain alone is sufficient to bind to myocardin (Fig. 1, *C* and *D*), however, this domain alone (mut1) is insufficient to stabilize the myocardin protein or augment its transactivation of a reporter gene (Fig. 7, *B* and *C*). This is not due to mis-localization of the HECT domain as this domain (amino acids 2337–2799 of UBR5) was found to dominantly localize in the nucleus (data not shown). This suggests that the UBR5 HECT domain must cooperate with other domains to enhance myocardin expression and function. To map UBR5 functional domains we generated a variety of UBR5 deletion mutants that included an SV40 nuclear location sequence at their COOH termini to force them express in nucleus (Fig. 7*A*). These mutants were co-transfected with myocardin into COS7 cells and protein expression was analyzed 48 h later. Data from this experiment demonstrated that both HECT and UBR1 domains of UBR5 (mutant 3) are required to increase the myocardin protein expression in a similar manner to the full-length UBR5 (1.75- and 2.05-fold, respectively). Consistent with this, the fragment of UBR5 containing HECT and UBR1 domains was also able to enhance myocardin-induced activation of an *SM22α* reporter to levels comparable with those seen with wild type UBR5 (Fig. 7*C*). In contrast, the HECT domain alone or together with either ubiquitin-associated domain or poly(A)-binding protein-C domains did not increase myocardin protein levels or activate the *SM22α* reporter gene. As predicted the UBR5 mutant lacking the HECT domain (mut5) was also not able to increase the myocardin protein or activate the *SM22α* reporter gene. Together, these data suggest that although UBR5 interacts with myocardin through its HECT domain, the HECT domain cooperates with the UBR1 domain to increase myocardin stability and function.

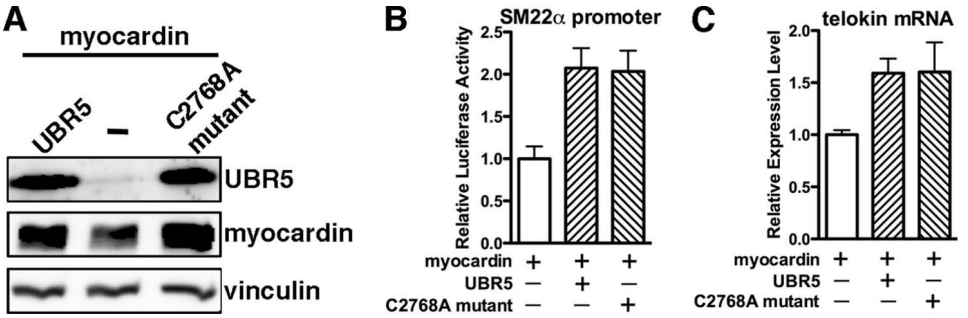

FIGURE 5. **UBR5 E3 ligase-deficient mutant mimics wild type UBR5 in increasing myocardin protein expression and promyogenic activity.** *A*, myocardin expression plasmid was co-transfected together with wild type UBR5 or E3 ligase defective mutant UBR5 (C2768A) expression plasmids in COS7 cells. After 48 h cells were harvested for Western blot analysis with the indicated antibodies. *B*, an SM22α promoter reporter was transfected into 10T1/2 cells together with UBR5 wild type or C2768A mutant expression plasmids and luciferase activity was measured. *C*, a myocardin expression plasmid was transfected into 10T1/2 cells alone or together with UBR5 or UBR5 C2768A mutant expression plasmids. 24 h following transfection, RNA was isolated and analyzed by real time RT-PCR with telokin-specific gene primers as indicated. All data are normalized to the activation produced by myocardin (set to 1). $n = 4$.

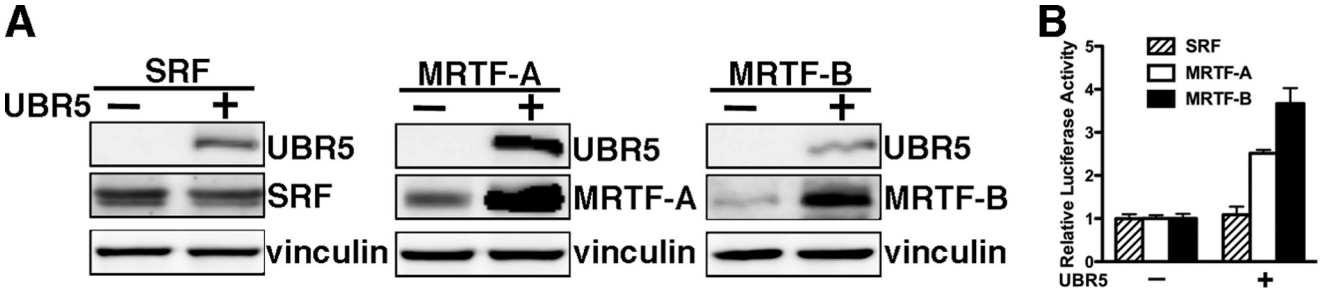

FIGURE 6. **UBR5 specifically increases expression of the myocardin family of proteins.** *A*, omni epitope-tagged UBR5 expression plasmid was transfected with or without Myc-tagged MRTF-A or MRTF-B expression plasmid into COS7 cells. 48 h later cells were harvested to detect endogenous SRF expression with anti-SRF antibody and ectopic MRTF-A and MRTF-B expression with anti-Myc antibody. The presence of UBR5 increased MRTF-A and MRTF-B expression without affecting SRF expression. *B*, a *Telokin* promoter reporter gene was co-transfected together with SRF, MRTF-A or MRTF-B expression plasmids with or without UBR5 and luciferase activity was measured. All data are normalized to the activation produced by MRTFs or SRF alone (set to 1). $n = 6$.

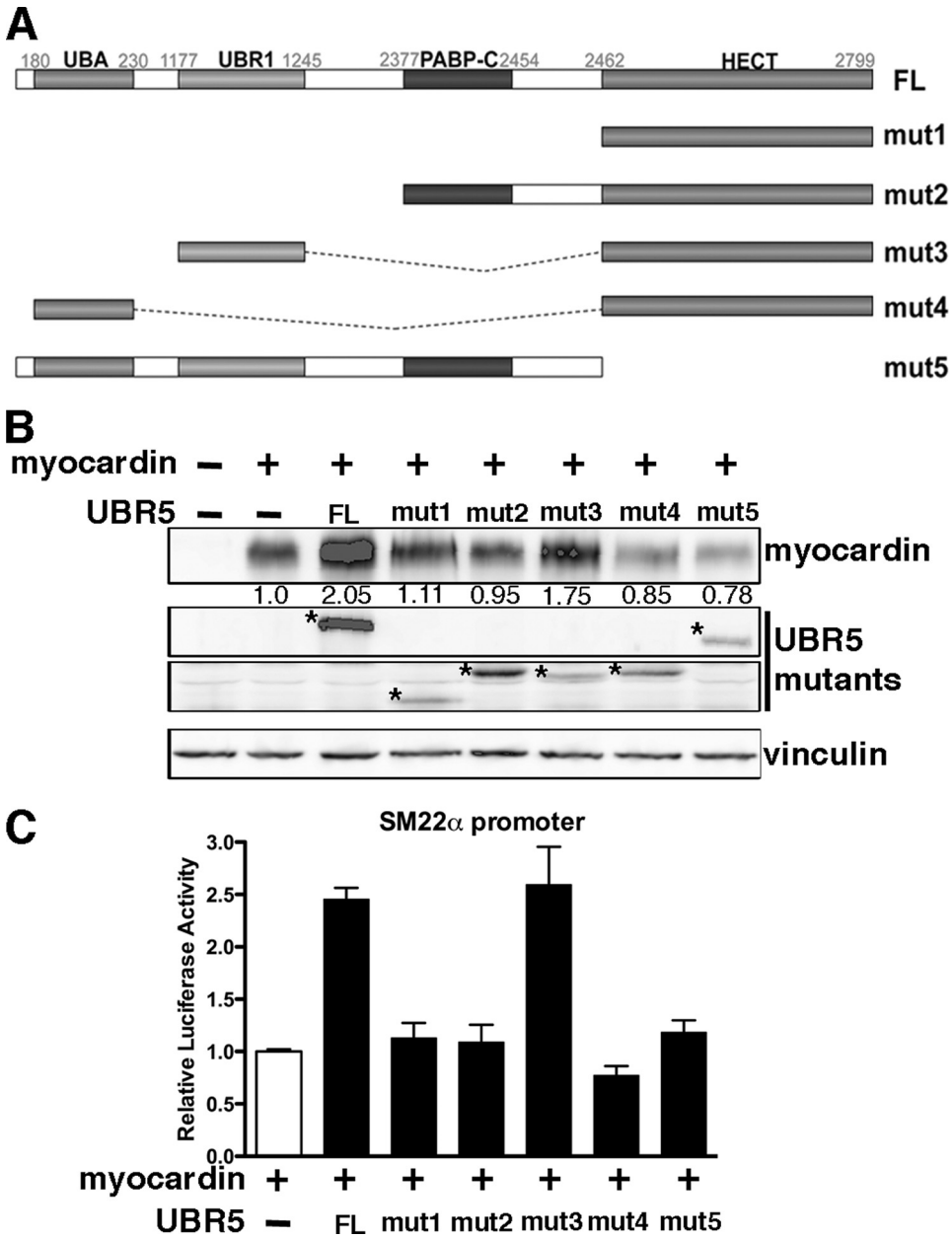

FIGURE 7. **Mapping UBR5 domains required to increase myocardin expression and activity.** *A*, schematic diagram showing the UBR5 mutants that were cloned into an omni epitope-tagged mammalian expression plasmid. *FL*, full-length; *mut*, mutant. *B*, myocardin expression plasmid was transfected into COS7 cells either alone or with the UBR5 expression plasmids shown in *A*. 48 h post-transfection cell lysates were harvested and subjected for Western blot analysis. Vinculin served as a loading control. *Numbers below* each lane indicate relative expression of myocardin normalized to vinculin. * indicates immuno signal of UBR5 mutants. Only the mutant that contained the UBR1 and HECT domains (*mut3*) increased myocardin expression comparable with full-length UBR5. *C*, *SM22α* reporter gene and myocardin expression plasmids were transfected into 10T1/2 cells with or without UBR5 mutant expression plasmids. Values are presented as relative luciferase activity compared with myocardin-induced activation (set to 1). The mutant that contained the UBR1 and HECT domains (*mut3*) increased myocardin-induced transactivation of the SM22α reporter similar to the full-length wild type UBR5.

The Expression of UBR5 and Myocardin Is Decreased following Arterial Injury—To examine whether UBR5 expression is altered in smooth muscle-related diseases, we examined its expression following vascular injury. Consistent with previous studies we observed a decrease in expression of myocardin and smooth muscle α-actin 5 and 10 days following ligation of the mouse carotid artery (Fig. 8) (28). This was also associated with a decrease in expression of *UBR5* mRNA.

DISCUSSION

Myocardin is a cardiac and smooth muscle tissue-specific transcription factor that is required for vascular smooth muscle differentiation (6). In the current study we have identified a novel role of the E3 ligase UBR5 in promoting the myogenic activity of myocardin independent of the E3 ligase function of UBR5. We show that UBR5 can promote activation of smooth muscle-specific genes in smooth muscle cells by protecting myocardin from proteasomal degradation.

Mice lacking *Myocardin* have been show to die by embryonic day (E) 10.5 and appear to exhibit a lack of blood vessels in the yolk sac (29). In these mice vascular endothelial cell differentiation and organization were unaffected as determined by platelet endothelial cell adhesion molecule staining. Coincidently, the phenotype of *UBR5* knock-out mice is very similar to the phenotype of myocardin knock-out mice (20). *UBR5* null mouse embryos also died on E10.5 and showed defective yolk sac vascularization demonstrating a critical role for *UBR5* in vascular development. Although in this study the protein markers of smooth muscle differentiation were not examined, the vascularization defect was most likely due to defective smooth muscle cells as normal endothelial cell organization and differentiation were seen in *UBR5* null mice. These *in vivo* studies, together with our *in vitro* data, suggest that *UBR5* may be a critical regulator of myocardin activity during vascular smooth muscle development. The current study provides *in vitro* evidence that UBR5 effectively functions as a myocardin coactivator by inhibiting myocardin degradation. Lack of *UBR5* would thus destabilize myocardin protein, decrease myocardin binding to SRF and loss of myocardin function leading to the defects of smooth muscle differentiation that could account for vascular defects in *UBR5* null mice.

There are strong evidences that *UBR5* plays an important role in tumorigenesis by acting as a tumor suppressor (14). It has been reported that *UBR5* was one of the most frequently mutated loci of the 154 examined in microsatellite-unstable

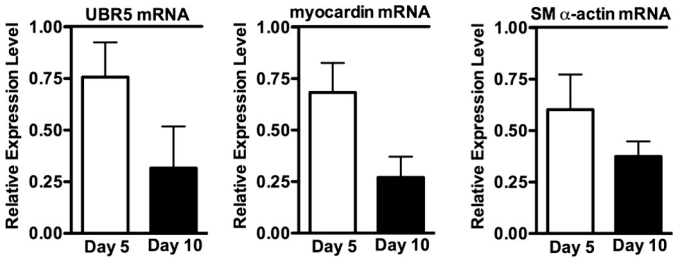

FIGURE 8. **UBR5 expression is decreased following carotid artery ligation injury.** Total RNA was extracted from mouse contralateral right and left ligated carotid arteries at post-ligation days 5 or 10 (see "Experimental Procedures" for details). Expression of UBR5, myocardin, and SM α-actin was analyzed by qRT-PCR. Data shown are the relative expression in injured samples to the control artery at each time point (set to 1) obtained from 4 animals at each time point.

gastric and colorectal cancers. Truncating mutations of UBR5 (resulting from coding region frameshift mutations) were also highly observed in gastric and colorectal cancers (30). Similarly myocardin has also been shown to act as a tumor suppressor (31). Several studies suggest that cancer and smooth muscle-related diseases such as atherosclerosis have several fundamental biological mechanisms in common (32). Abnormal proliferation of SMCs constitutes a key event in atherosclerosis, neointimal hyperplasia, and the response to vascular injury. For example, following vascular injury SMCs are highly proliferative and migratory with loss of expression of smooth muscle differentiation markers (33). This proliferation is also critical to early plaque development and plaques can therefore be viewed as benign smooth muscle cell tumors of the artery wall (34, 35). These findings together with our data showing that both UBR5 and myocardin expression are decreased in proliferating smooth muscle following arterial ligation (Fig. 8), suggest that changes in expression of these proteins may also play an important role in atherosclerotic plaque formation. It will be very important to assess UBR5 expression or mutation in the smooth muscle cells of atherosclerotic plaque in human patients as this may provide important clinical correlations to these basic and translational observations.

Previous studies have demonstrated myocardin expression can be tightly regulated by proteasome-mediated degradation (27, 36). For example, the E3 ligase COOH terminus of Hsc70-interacting protein has been found to interact with the COOH-terminal transactivation domain of myocardin and repress myocardin-dependent SMC gene expression by promoting degradation of myocardin protein. The COOH terminus of Hsc70-interacting protein functions to promote ubiquitination and degradation of myocardin by the proteasome in response to glycogen synthase kinase 3β-induced phosphorylation of myocardin. In contrast, the four and one-half LIM domain-containing protein 2 (FHL2) has been shown interact with the NH_2 terminus of all three myocardin factors and enhance myocardin and MRTF-A-dependent transactivation of smooth gene reporters due to an increased MRTF protein stability (27). Interestingly in our study UBR5 also interacts with the NH_2 terminus of myocardin and increases the protein stability of each of the MRTFs. It will be interesting to examine whether the NH_2-terminal region of the MRTFs contains ubiquitylation "hot" spots that are coupled to proteasome-mediated degradation that may be masked by UBR5 or FHL2 interactions. In addition to the ability of UBR5 to stabilize myocardin protein, we cannot rule out the possibility that UBR5 also behaves as a scaffold protein to recruit other coactivators into the myocardin complex leading to further increases in smooth muscle-specific gene expression.

Although UBR5 was originally identified as a HECT domain-containing E3 ligase our data clearly demonstrated that the ligase activity is not required for its ability to augment myocardin activity and expression as an UBR5 ligase-deficient mutant can effectively increase myocardin expression and function (see Fig. 5). Consistent with this, UBR5 ligase activity was also found to be dispensable for transcriptional co-activation of a progesterone receptor responsive reporter gene and potentiated progestin-mediated gene transactivation (21). Instead, we found that the UBR5 HECT domain must cooperate with the UBR1 domain to promote myocardin stability and activity. The molecular mechanisms underlying the requirement of the UBR1 and HECT domains for activation of myocardin function remain elusive at this time. Future studies are needed to test whether the UBR1 domain directly binds to myocardin. Although the HECT domain alone is sufficient to bind to myocardin this was not sufficient to stabilize myocardin protein (see Fig. 7). It has been postulated that the UBR5 UBR1 domain is likely to be involved in protein-protein interactions. In support of this, the UBR1 domain coincides with the type 1 site in UBR1 proteins, a binding site essential for substrate recognition (37). Thus it is possible that the UBR1 domain can recruit another protein to help stabilize the myocardin protein.

In summary, this study reveals an unexpected role for ubiquitin E3 ligase UBR5 as an activator of smooth muscle differentiation through its ability to prevent myocardin and MRTFs from proteasome-mediated degradation. In future studies it will be very important to determine the role of UBR5 in smooth muscle development *in vivo* by conditional ablation of UBR5 in smooth muscle cells and examining the subsequent effects on the development of smooth muscle-related diseases such as atherosclerosis.

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