Thymine DNA Glycosylase Represses Myocardin-induced Smooth Muscle Cell Differentiation by Competing with Serum Response Factor for Myocardin Binding*

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Myocardin is a serum response factor (SRF) co-activator that regulates transcription of many smooth muscle-specific genes and is essential for development of vascular smooth muscle. We used a yeast two-hybrid screen, with myocardin as bait in a search for factors that regulate myocardin transcriptional activity. From this screen, thymine DNA glycosylase (TDG) was identified as a myocardin-associated protein. TDG was originally identified as an enzyme involved in base excision repair of T:G mismatches caused by spontaneous deamination of methylated cytosines. However, TDG has also been shown to act as a transcriptional co-activator or co-repressor. The interaction between TDG and myocardin was confirmed *in vitro* by glutathione *S*-transferase pull down and *in vivo* by co-immunoprecipitation assays. We found that TDG abrogates myocardin induced expression of smooth muscle-specific genes and represses the trans-activation of the promoters of myocardin of these genes. Overexpression of TDG in SMCs down-regulated smooth muscle marker expression. Conversely, depletion of endogenous TDG in SMCs increased *smooth muscle-specific myosin heavy chain* (*SM MHC*) and *Telokin* gene expression. Glutathione *S*-transferase pull-down assays demonstrated that TDG binds to a region of myocardin that includes the SRF binding domain. Furthermore, TDG was found to compete with SRF for binding to myocardin *in vitro* and *in vivo*, suggesting that TDG can inhibit expression of smooth muscle-specific genes, at least in part, through disrupting SRF/myocardin interactions. Finally, we demonstrated that the glycosylase activity of TDG is not required for its inhibitory effects on myocardin function. This study reveals a previously unsuspected role for the repair enzyme TDG as a repressor of smooth muscle differentiation via competing with SRF for binding to myocardin.

Smooth muscle cells (SMCs)[2] are important contractile components of cardiovascular, respiratory, genitourinary, and digestive systems. Fully differentiated or mature SMCs proliferate at an extremely low rate and are almost completely geared for contraction. Differentiated SMCs are characterized by the presence of a unique repertoire of contractile and regulatory proteins such as smooth muscle α- and γ-actin, smooth muscle myosin heavy chain (SM MHC), h-caldesmon, calponin, SM22α, and telokin. The expression of these proteins is markedly attenuated during the de-differentiation and proliferation of smooth muscle that occurs under many pathological conditions (1–4). The mechanisms that result in down-regulation of contractile proteins during phenotypic modulation of smooth muscle are poorly understood. Studies indicate that phenotypic modulation results from both down-regulation of genes required for activating smooth muscle-specific genes and increased expression of transcriptional repressors that facilitate attenuation of these genes (5).

SRF plays a central role in the expression of many different smooth muscle-specific genes including the *SM MHC*, *smooth muscle α-* and *γ-actin*, *SM22α*, *Calponin*, and *Telokin* genes (1). SRF is an evolutionarily conserved MADS (MDM1, agamous, deficiens, SRF) domain-containing protein that is required for specification of smooth, cardiac, and skeletal muscle lineages (2). Although SRF expression is greatest in muscle tissues, it is expressed in all tissues (3). 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 the conserved MADS 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 (4). Although several proteins have been identified that can modify myocardin function (5–7) how myocardin activity is regulated is still poorly understood. We thus performed a yeast two-hybrid screen, using

[2] The abbreviations used are: SMC, smooth muscle cell; CREB, cAMP-response element-binding protein; TK, thymidine kinase; MHC, myosin heavy chain; TDG, thymine DNA glycosylase; ER, estrogen receptor; TTF1, thyroid transcription factor 1; HA, hemagglutinin; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; qRT, quantitative real-time; RT, reverse transcriptase; siRNA, small interfering RNA; RPLPO, ribosomal phosphoprotein PO; SRF, serum response factor; CBP, CREB binding protein; RAR, retinoic acid receptor.

myocardin as bait, to identify proteins that may regulate myocardin function. This screen identified a DNA mismatch repair enzyme, TDG (thymine DNA glycosylase) as a myocardin-binding protein. TDG is a member of mismatch-directed uracil-DNA glycosylases (8, 9). It is widely expressed in multiple organs including SMC-rich tissues (10, 11). TDG has multiple functions that link DNA repair, the control of epigenetic DNA modification, and the regulation of gene expression (8, 9). *In vitro* assays demonstrated it is able to hydrolyze thymine and uracil from G:T and G:U mismatch pairs, implying a specific biological role in base excision repair of deamination-induced Cys → Thr mutations. Further studies have shown TDG also plays a role in the active removal of 5-meC from methylated CpG dinucleotides in DNA, thus implicating TDG in regulating epigenetic DNA modifications (12). In addition, TDG has been shown to act as either a co-activator or co-repressor of a variety of genes. For example, the physical association of retinoid or estrogen receptors (ER) with TDG results in transcriptional activation of reporter genes; and, at least for the ER, such transcriptional co-activation does not require a functional glycosylase catalytic domain (10, 13). On another hand, TDG was found to repress thyroid transcription factor 1 (TTF1)-activated transcription in thyroid and non-thyroid cells in transient co-transfection experiments (14). In the current study we show that the physical interaction between TDG and myocardin disrupts myocardin-SRF complexes and thereby attenuates smooth muscle differentiation.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—A fragment of the mouse myocardin cDNA encoding the N-terminal 585 amino acids was cloned into the bait vector pAS2-1 (Promega) by a standard PCR-based cloning strategy. A pretransformed mouse embryonic 17-day library was purchased from Promega. Screening of the library was performed essentially following instructions for the Matchmaker system (Promega) and has been previously described (15). Plasmid DNA was recovered from positive yeast clones and sequenced to identify the inserts. Three clones corresponding to the full-length open reading frame of *TDG* (NM011561) were isolated and characterized further.

Mammalian Expression and Reporter Gene Assays—The coding region of mouse *TDG* cDNA (encoding amino acids 1–397) was amplified from a yeast plasmid clone by PCR and ligated to pcDNA3.1His (Invitrogen), resulting in the expression of a fusion protein with N-terminal His$_6$ and Omni epitope tags. For generation of TDG adenovirus, the mouse TDG coding sequence was cloned into a modified pShuttle vector (Clontech) encoding an N-terminal HA tag. The mouse *Myocardin* pcDNA3.1-myc/his vector (cardiac-enriched isoform that is also expressed in smooth muscle) was kindly provided by Dr. Eric N. Olson (Southwestern Medical Center, Dallas, TX). The smooth muscle *Myocardin* isoform was generated by PCR and cloned into Omni-tagged pShuttle vector (BD Biosciences) and then the expression cassette was transferred into Adenoviral DNA to produce adenovirus as described previously (16). An expression plasmid encoding a myocardin leucine zipper mutant was described previously (17). The mouse *MRTF-B* expression plasmid was provided by Dr. Michael Parmacek (University of Pennsylvania, Philadelphia, PA). The mouse mammalian expression plasmid for *MRTF-A* was described in our previous report (18). All promoter reporter genes were constructed by cloning fragments of promoters into the pGL$_2$B or pGL$_3$B luciferase vectors (Promega, Madison, WI). The mouse *Telokin* promoter-luciferase reporter gene used includes nucleotides −190 to +181 (T370) of the mouse *Telokin* gene and rabbit *telokin* gene promoter T400 including nucleotides −256 to +147 was described previously (19, 20). The *SM22α*-luciferase reporter gene includes nucleotides −475 to +61 of mouse SM22α (21, 22). The *SM α-actin* promoter fragment extends from nucleotide −2,555 to +2,813 (23) and the *SM MHC* promoter from −4,200 to +11,600 (24), these latter plasmids were generously provided by Dr. Gary Owens. Plasmids were sequenced to verify the integrity of the insert. Transfection was carried out with FuGENE6 transfection reagent (Roche) as previously described (25). The level of promoter activity was evaluated by measurement of the firefly luciferase activity relative to the internal control TK-*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 per 60-mm dish overnight. Cells were transfected with the Omni-TDG expression plasmid for 24 h and then fixed, permeabilized, and incubated with polyclonal anti-Omni antibody (1:500, Invitrogen), followed by Texas Red-conjugated anti-rabbit IgG (1:400) secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Cells were counterstained with Hoechst (1:5000) to visualize nuclei.

GST Pull-down Assays—Fragments of mouse *Myocardin* and *TDG* cDNAs were cloned into pGEX-4T vectors (Stratagene) to generate GST fusion proteins or cloned into pET28 vectors (Novagen) to generate T7 fusion proteins. These fusion proteins were produced in *Escherichia coli* BL21-star (Stratagene) cells. After 1 h of induction with 0.4 mM isopropyl β-D-thiogalactoside, cells were harvested by centrifugation, resuspended in PBS, 1% Triton containing protease inhibitors and then lysed by sonication. GST fusion protein lysates were clarified by centrifugation and incubated for 1 h with a 50% suspension of glutathione-agarose beads (Amersham Biosciences) in PBS. Washed fusion proteins bound to the beads were resuspended and incubated for 2 h with 500 μl of T7 fusion proteins in a total volume of 1 ml of binding buffer (PBS containing 1% Triton X-100, 1 mg/ml BSA + protease inhibitors). Beads were washed 3 times in 1 ml of washing buffer (PBS containing 1% Triton X-100) and then bound proteins eluted by heating at 95 °C for 5 min in SDS sample buffer. The eluted proteins were resolved on SDS-PAGE and proteins detected by Western blotting.

Co-immunoprecipitation—For detecting protein-protein interactions *in vivo*, COS cells were transduced with adenovirus encoding HA-tagged TDG and myc-tagged or Omni-tagged myocardin as indicated in figures. 24 h after transduction, nuclear protein was harvested from the COS cells. Co-immunoprecipitation assays were performed using a nuclear complex co-immunoprecipitation kit as described by the manufacturer (Active Motif). 250 μg of nuclear protein extracts were incu-

bated with 3 μg of anti-myc antibody (Invitrogen), anti-HA antibody (Covance), or appropriate IgG controls in 500 μl of low salt immunoprecipitation buffer (Active Motif) overnight at 4 °C. 60 μl of EZview protein A beads (Sigma) were added to the mixture for an additional 1 h with rocking and then immobilized complexes were washed 6 times with the low salt immunoprecipitation buffer. The immunoprecipitated protein was mixed with 45 μl of 2× SDS sample buffer and analyzed by Western blotting.

Adenovirus Construction and Cell Infection—Adenovirus constructs were made in the Adeno-X vectors essentially followed the manufacturer's instructions (BD Biosciences) as previously described (25). For adenoviral transduction, mouse colon primary SMCs were isolated from 4–6-week-old mice as previously described (18) and plated in 12-well plates at a density of 7×10^4 cells/well. The next day, cells were transduced with adenovirus encoding HA-tagged YFP-NLS (nuclear localized yellow fluorescent protein) or HA-tagged TDG in 10% growth media for 4 h at 37 °C. The adenovirus was then aspirated and replaced with 10% growth media. These conditions resulted in close to 100% infection of cells. 48 h following transduction total RNA were extracted from the transduced cells with TRIzol (Invitrogen).

Western Blotting—Western blot analysis was carried out essentially as described previously (17, 25, 27). 30 μg of protein were fractionated on 5 or 15% SDS-polyacrylamide gels, electrophoretically transferred to a nitrocellulose or PVDF membrane, and transfer verified by Ponceau S staining. The membranes were then probed with a series of antibodies. Antibodies used in this study were against: HA tag (Covance, 1:3,000), myc (Invitrogen, 1:5000), omni (Invitrogen, 1:3000), SRF (Santa Cruz, G20X, 1:10000), T7 (1:10000, Novagen), and vinculin (Santa Cruz, 1:5,000).

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 SuperScript first strand cDNA synthesis kit (Invitrogen). PCR was performed with 200 ng of cDNA and SYBR Green PCR master mix (ABgene) with respective gene-specific primers as previously reported (18). Primers sets for mouse *TDG* were designed as sense 5′-GTC TGT TCA TGT CGG GGC TGA GTG AG-3′ and antisense 5′-CTG CAG TTT CTG CAC CAG GAT GCG C-3′ and yielded a 182-bp product. Rat *telokin* PCR product was amplified using 5′-GAC ACC GCC TCA GTC CAA CCT CCG-3′ and 5′-GGC TTT TCC TCA GCA ACA GCC TCG-3′; rat *SM MHC* product was amplified using 5′-GCA GCT TCT ACA GGC AAA CCC AAT CC-3′ and 5′-GCA CCC ACA ATG TAA CCA GTG ACA TCA-3′; rat *TDG* PCR product was amplified using 5′-CCC TGG GCC CGG AAA-3′ and 5′-TCG CTC AAC CCA GAC ATG AA-3′; rat *Aortic carboxypeptidase-like protein* product was amplified with 5′-CGG CAC CAC AGC TAC AAG GA-3′ and 5′-TCC CCG GGG TTG TCT GAG AT-3′; rat *smoothelin B* was amplified with 5′-GGC TAC ACT CTC AAC AGC GAG AA-3′ and 5′-ACA TCG TTC ATG GAC TCC AGT CTC-3′. Mouse and rat *acidic ribosomal phosphoprotein PO (RPLPO)* PCR product was amplified using 5′-GGA CCC GAG AAG ACC TCC TT-3′ and 5′-TGC TGC
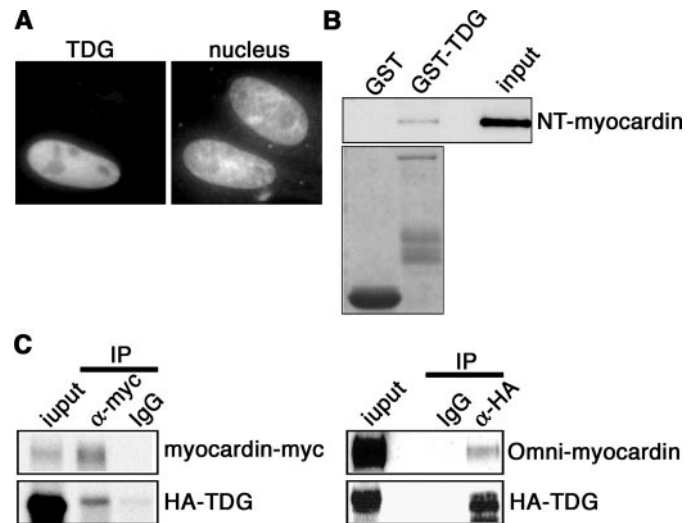

FIGURE 1. **TDG interacts with myocardin *in vitro* and *in vivo*.** *A*, TDG localizes to the nucleus in smooth muscle cells. A10 cells were plated on cover chips and transiently transfected with the mouse TDG expression plasmid. TDG was detected using a polyclonal anti-omni epitope tag (*left*). Cells were counterstained with Hoechst to detect nuclei (*right*). *B*, GST-TDG or GST alone were incubated with an N-terminal (*NT*) myocardin fragment (1–585 amino acids) expressed in bacteria. TDG-associated myocardin was detected by Western blotting using an antibody to the T7 epitope tag on myocardin. 10% of the sample was loaded in the input lane. *C*, myc-tagged or Omni-tagged myocardin and HA-tagged TDG adenovirus were transduced into COS cells. Subsequently nuclear extract was harvested and proteins were immunoprecipitated with myc, HA, or control IgG antibodies. The immunoprecipitated (*IP*) proteins were detected by Western blotting using anti-HA, anti-myocardin, or anti-Omni antibodies, as indicated at the *right* of the blot. 10% total extract was loaded as input.

CGT TGT CAA ACA CC-3′ as internal control; quantification of the reaction product was carried out using the ABI9700 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 $2^{-\Delta\Delta Ct}$ method against the internal control RPLPO housekeeping gene.

siRNA—Control siRNA or siRNA against TDG was designed and purchased from Dharmacon. The siRNA sequence for targeting endogenous mouse and rat TDG was 5′-GCAAG-GATCTGTCTAGTAA-3′. For determining the siRNA depletion efficacy expression plasmids encoding mouse TDG were transfected into 10T1/2 cells with FuGENE6 (Roche) for 12 h. Subsequently cells were transfected either with control siRNA or TDG siRNA duplex using Lipofectamine transfection reagent (Invitrogen) following the manufacturer's protocol. 24 h later, protein was harvested and subjected to Western blotting. For testing the effects of smooth muscle gene expression by depletion of endogenous TDG, control siRNA or TDG siRNA was transfected into A10 cells for 24 or 48 h and total RNA was harvested for qRT-PCR as described above.

Quantitative Chromatin Immunoprecipitation Assays—10T1/2 cells were cultured in 10% growth media and infected with adenovirus encoding omni-tagged myocardin, HA-tagged YFP or TDG, for 36 h. After a PBS wash, cells were fixed with 10 ml of PBS supplemented with 270 μl of 37% formaldehyde for 15 min at room temperature. Fixed cells were harvested for chromatin immunoprecipitation essentially as described by the manufacturer (Upstate) with minor modifications. Cross-linked chromatin was immunoprecipitated with 6 μg of anti-
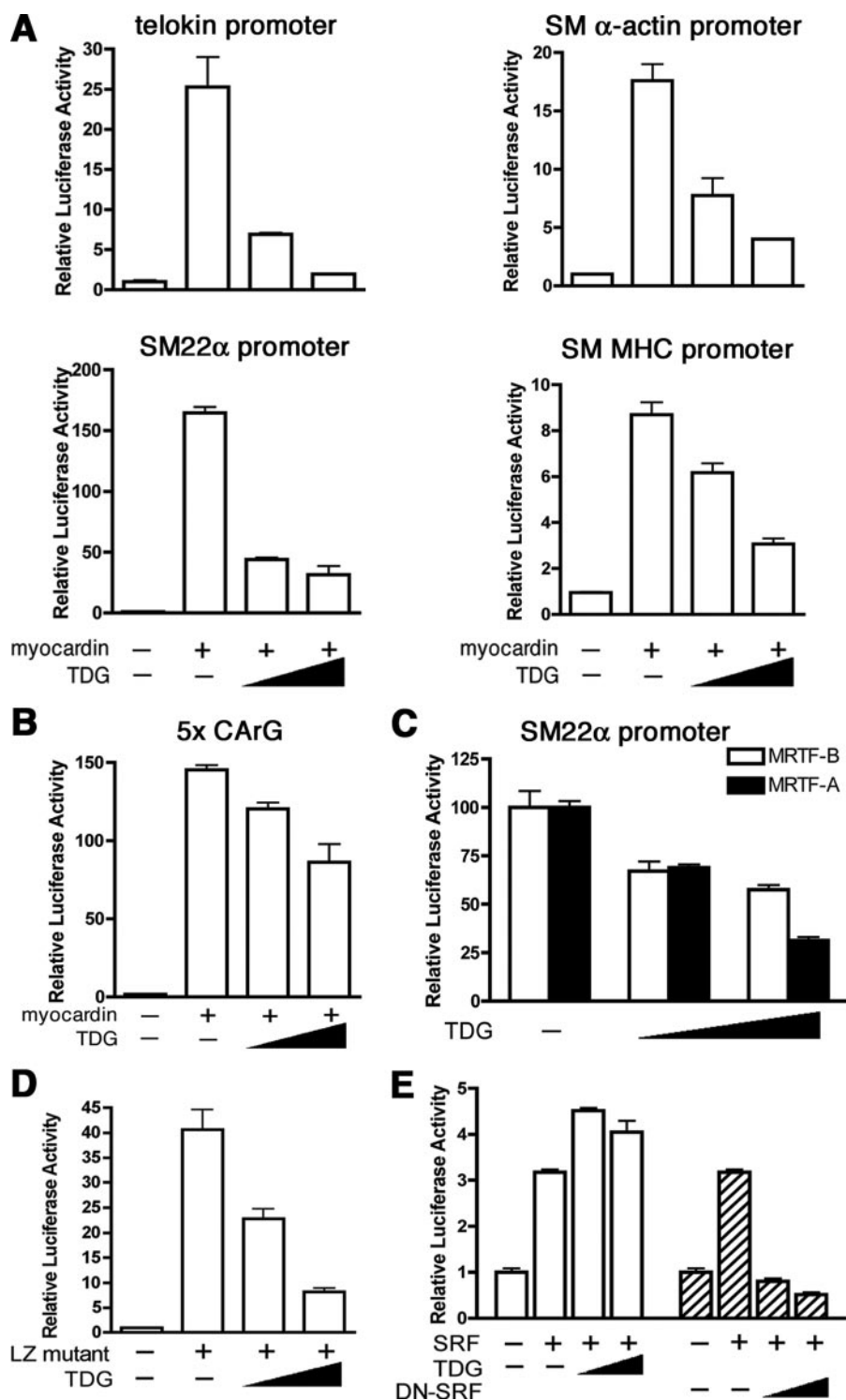
FIGURE 2. **TDG represses the myocardin and MRTF-induced activation of smooth muscle-specific promoters.** *A,* luciferase reporter genes were transfected into 10T1/2 cells together with a constant amount of myocardin and increasing amounts of TDG expression plasmids. Values are presented as relative luciferase activity compared with empty expression vector (set to 1) and are the mean ± S.E. of 6 samples. *B,* luciferase activity was assayed with a 5 CArG box-containing reporter (Stratagene) as described in *A. C,* an *SM22α* promoter reporter gene was transfected into 10T1/2 cells together with MRTF-A or MRTF-B in the presence of increasing amounts of TDG expression plasmids. All data are normalized to the activation produced by MRTF-A or MRTF-B alone (set to 100). *D,* an *SM22α* promoter reporter gene was transfected into 10T1/2 cells together with a myocardin LZ mutant in the absence or presence of increasing amounts of TDG expression plasmids. 24 h later luciferase activity was measured. All data are expressed relative to the basal activity of *SM22α* promoter (set to 1). *n* = 6. *E,* an SRF expression plasmid was co-transfected with increasing amounts of either DN-SRF (amino acids 1–222) or TDG expression vector together with a *Telokin* reporter gene into 10T1/2 cells and luciferase activity was measured. An arbitrary value of 1 assigned to the activity of *Telokin* promoter alone. *n* = 6.

omni antibody (Santa Cruz) and bound to single-stranded DNA/protein A-agarose beads (Sigma). The precipitated DNA was then purified and amplified by real time PCR for quantification of the target sequences using SYBR Green PCR master mix (ABgene) with SM22α and SM MHC gene-specific primers as described previously (18).

Human Coronary SMC Culture—Primary human coronary artery SMCs (passages 3–8; Cell Applications, Inc.) were plated in SMC growth medium (MCDB131 containing 5% fetal calf serum, 2 ng/ml human basic fibroblast growth factor, 5 mg/ml human insulin, and 0.5 ng/ml human epidermal growth factor) for 24 h. Cells were incubated in serum-free MCDB131 medium to induce smooth muscle genes for 0, 2, and 8 days. Where indicated, growth medium was added back to serum-depleted cells to induce proliferation for 8 or 24 h. Total RNA was extracted at the time points indicated using TRIzol reagent. qRT-PCR was performed using primers to TDG and SM-specific genes as described above. Sequences for human TDG primers were designed as sense: 5′-TCA GTG AGG TCC AGC TGA ACC ATA TG-3′ and antisense: 5′-TTC CAT TAA ACA CTG CTA TTC GTG GCT G-3′.

RESULTS

TDG Localizes to the Nucleus of Smooth Muscle Cells and Interacts with Myocardin in Vitro and in Vivo—Myocardin is a potent transcription factor important for smooth muscle development (28). However, the molecular mechanisms that regulate myocardin activity are not fully explored. We hypothesize that myocardin function is likely to be regulated through its association with other proteins. To identify myocardin regulatory proteins, the N-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. From this screen three independent clones
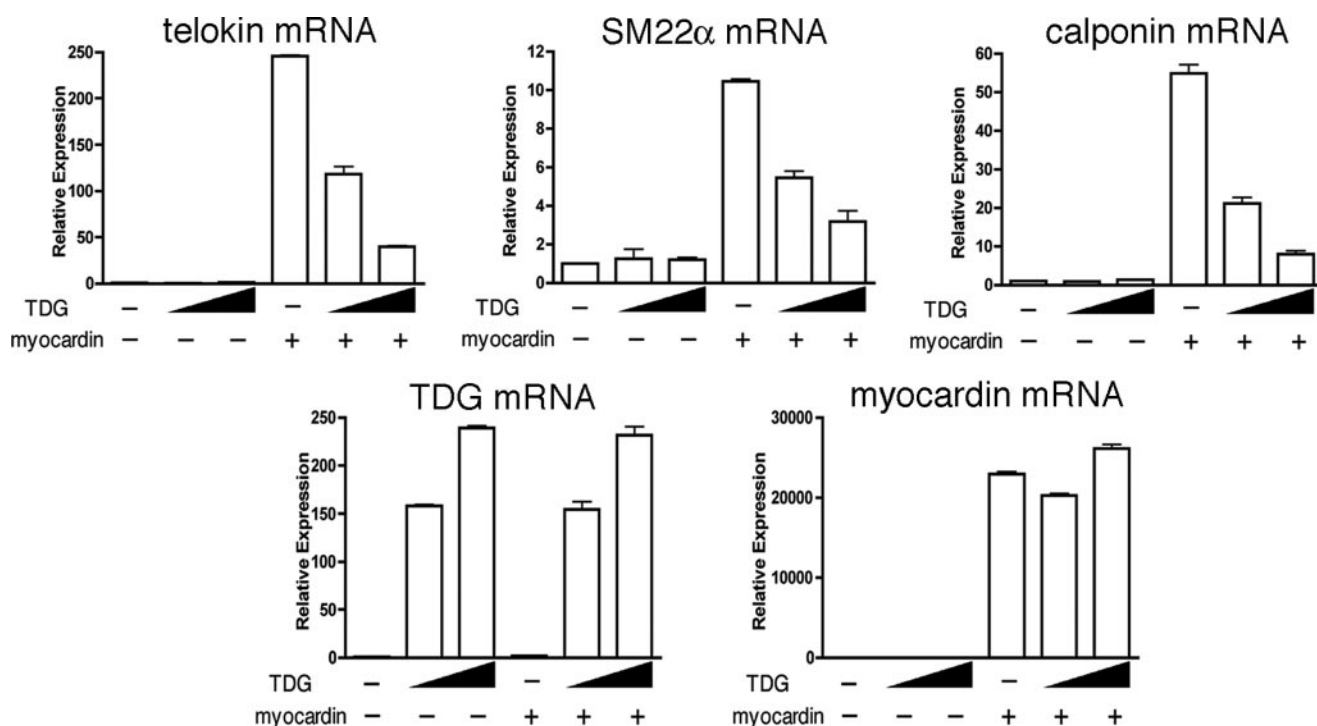
FIGURE 3. **TDG blocks the induction of endogenous smooth muscle-specific genes by myocardin.** Mouse myocardin expression vector, together with mouse TDG or empty plasmid pcDNA3.1 were transfected into 10T1/2 cells. 24 h post-transfection total RNA was harvested from cells and qRT-PCR was performed to examine expression of endogenous smooth muscle-specific genes. The ectopic expression of TDG and myocardin was also measured. Transcript levels was first normalized to *acidic ribosomal phosphoprotein* internal loading (*RPLPO*) 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 = $2^{-\Delta\Delta Ct}$ and $\Delta\Delta C_t = (C_{t\ experimental} - C_{t\ RPLPO}) - (C_{t\ control} - C_{t\ RPLPO})$. Data presented are the mean $\pm$ S.E. of 4 samples obtained from two independent experiments.

were identified that encoded TDG (GenBank™ accession number NM011561). We verified that TDG protein predominantly localizes to the nuclei of smooth muscle cells (Fig. 1*A*) and confirmed the physical interaction between myocardin and TDG *in vitro* and *in vivo*. Consistent with the yeast two-hybrid screen, GST pull down assays demonstrated that the N-terminal half of myocardin (amino acids 1–585), expressed in bacteria, specifically binds to a GST-TDG fusion protein (Fig. 1*B*). To investigate the TDG and myocardin interaction *in vivo*, COS cells were transduced with cardiac or smooth muscle isoforms of myocardin (myc-tagged or Omni-tagged, respectively) and TDG (HA-tagged) adenovirus and anti-myc or anti-HA antibodies were used to immunoprecipitate myocardin-TDG complexes. Western blotting of the precipitated complexes revealed that TDG bound to both myocardin isoforms within cells (Fig. 1*C*).

TDG Suppresses Myocardin- and Myocardin-related Transcription Factor-induced Transactivation of Smooth Muscle-specific Promoters—Data described above demonstrated that TDG localizes in nuclei of smooth muscle cells and physically binds to myocardin *in vitro* and *in vivo*. To determine whether TDG functionally interacts with myocardin, myocardin was co-transfected together with telokin, SM22α, SM α-actin, and SM MHC promoter-luciferase reporter genes with or without TDG expression plasmid into 10T1/2 cells and the subsequent affects on promoter activity determined. Data from these experiments revealed that TDG repressed the myocardin-mediated activation of each of these promoters in a dose-dependent manner

(Fig. 2*A*). TDG alone had no effect on the basal activity of these reporters in these non-muscle cells (data not shown). TDG also repressed the myocardin-induced activation of a reporter plasmid containing 5 copies of a consensus CArG box (Fig. 2*B*). These data demonstrate that the CArG box motif is sufficient for TDG-mediated repression of the myocardin-stimulated promoter activity. Furthermore, TDG attenuated the transactivation of the SM22α promoter induced by myocardin-related transcription factors, MRTF-A and MRTF-B (Fig. 2*C*). TDG also attenuated transactivation induced by a dimerization defective myocardin, indicating the TDG is unlikely to inhibit myocardin through interfering with its dimerization (Fig. 2*D*). The inhibitory effects of TDG on myocardin and the MRTFs are specific, as TDG was not able to affect SRF-induced transactivation of the telokin promoter, whereas an SRF mutant that lacked the C-terminal transactivation domain decreases the activation in a dose dependent fashion (Fig. 2*E*).

TDG Abrogates Myocardin-induced Expression of Smooth Muscle-specific Genes—Data presented above show that TDG suppressed myocardin-induced trans-activation of smooth muscle-specific promoters. We next determined the effects of TDG on the induction of endogenous smooth muscle-specific genes by myocardin in fibroblast cells. Myocardin or empty pcDNA plasmids were transfected into 10T1/2 cells together TDG expression plasmids and 24 h after transfection, RNA was harvested from these cells and subjected to quantitative RT-PCR (Fig. 3). Consistent with previous reports (17, 29, 30), ectopic expression of myocardin in 10T1/2 cells resulted in a
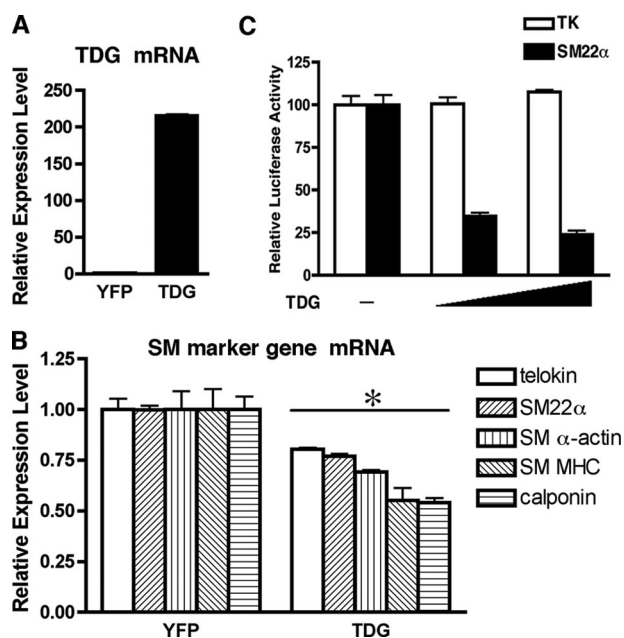
FIGURE 4. **TDG down-regulates smooth muscle-specific gene expression in primary SMCs.** *A* and *B*, primary SMCs were prepared from the colon of 4–6-week-old mice and transduced with adenovirus encoding TDG or YFP as indicated. 48 h following transduction mRNA was isolated and analyzed by qRT-PCR. *, $p < 0.05$. *C*, A10 smooth muscle cells were transiently co-transfected with TDG and either SM22α or minimal TK promoter-luciferase reporter genes. Promoter activity is normalized to a *Renilla* luciferase internal control and expressed relative to vector control transfections (set to 100). Data were presented as mean ± S.E. of 6 samples.

FIGURE 5. **Knocking down endogenous TDG results in increased telokin and SM MHC mRNA expression in A10 smooth muscle cells.** *A*, 10T1/2 cells were transfected with omni-tagged mouse TDG followed by either 100 pmol of an RNA duplex directed against TDG (sequence targets rat and mouse; Dharmacon) or scrambled control RNA duplex (Dharmacon). Western blotting was used to detect TDG expression (indicated by an *arrow*). Vinculin served as a loading control. *B–F*, A10 cells were transfected with either control siRNA or TDG siRNA. 24 h later total RNA was harvested, and *telokin* and *SM MHC* gene expression were analyzed by qRT-PCR. *Aortic carboxypeptidase-like protein* (*ACLP*) and *smoothelin B* gene expression were analyzed after a 48-h siRNA transfection. Data represent 4 samples from two independent experiments. *, $p < 0.05$.

significant induction of endogenous telokin, calponin, and SM22α genes (Fig. 3). Co-transfection of TDG with myocardin attenuated the myocardin-induced expression of telokin, calponin, and SM22α in a dose-dependent manner without affecting ectopic myocardin expression (Fig. 3).

Overexpression of TDG Down-regulates Smooth Muscle-specific Gene Expression and Promoter Activity in Smooth Muscle Cells—Data presented above demonstrate that TDG abrogates the myocardin ability to transactivate smooth muscle-specific genes. To further explore the role of TDG in the expression of endogenous smooth muscle-specific genes, we transduced mouse colon primary smooth muscle cells with TDG adenovirus. All of smooth muscle-specific genes examined, including *Telokin, SM22α, SM α-actin, Calponin,* and *SM MHC* were significantly down-regulated 25–50% following TDG overexpression (Fig. 4*B*). To test whether TDG is able to affect the basal promoter activity of smooth muscle-specific genes in SMCs, A10 cells were transiently co-transfected with increasing amounts of TDG expression vector and either SM22α or minimal thymidine kinase (TK) promoter luciferase reporter genes. These experiments demonstrated that overexpression of TDG resulted in a dose-dependent inhibition of the SM22α promoter without affecting a housekeeping TK promoter in A10 vascular smooth muscle cells (Fig. 4*C*).

Depletion of Endogenous TDG in Smooth Muscle Cells Results in Up-regulation of Telokin and SM MHC Gene Expression—Thus far the data presented suggest that TDG can inhibit smooth muscle-specific gene expression when TDG is expressed at high levels. To determine the role of endogenous TDG in regulating SM-specific gene expression, endogenous
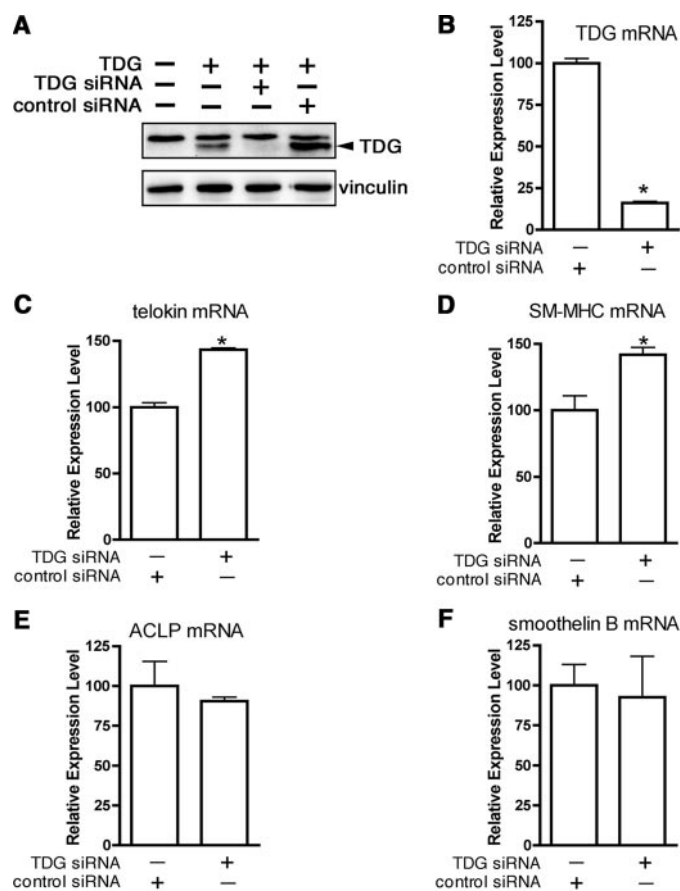
TDG levels were depleted using siRNA. siRNA specific for TDG was able to almost completely ablate expression of exogenous TDG in 10T1/2 cells (Fig. 5*A*), and to decrease endogenous *TDG* by 80% (Fig. 5*B*) in A10 smooth muscle cells. The control levels of TDG expression in A10 smooth muscle cells was relatively high being comparable with that of RPLPO (data not shown). Depletion of TDG from A10 smooth muscle cells resulted in a significant 1.5-fold increase in *telokin* and *SM MHC* mRNA expression (Fig. 5, *C* and *D*), without affecting expression of the non-CarG-dependent smooth muscle markers, *aortic carboxypeptidase-like protein* and *smoothelin B* (Fig. 5, *E* and *F*). Similar results of telokin and SM MHC were seen either 24 or 48 h following transfection of the TDG siRNA (only data from 24 h are shown in Fig. 5).

Mapping Myocardin Domains That Bind to TDG—Data described above demonstrate that TDG negatively regulates smooth muscle-specific gene expression. To understand the mechanism by which TDG represses myocardin function, we performed GST pull down assays to map the myocardin domains that bind to TDG. As shown in Fig. 6*A*, TDG specifi-
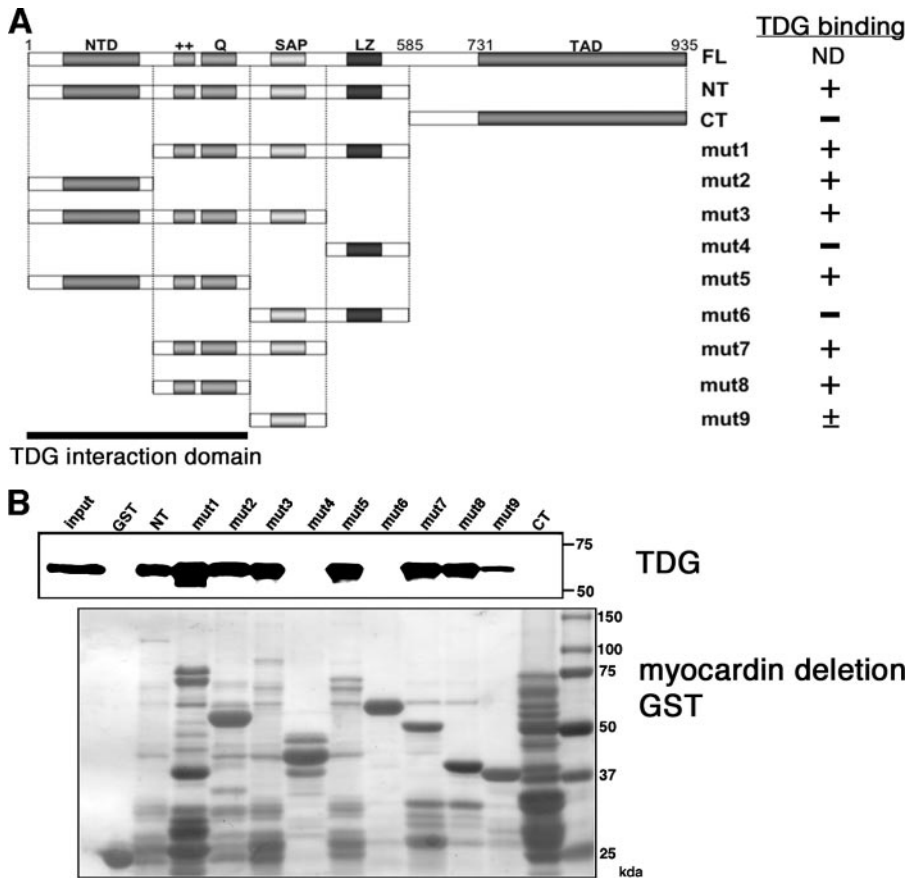
FIGURE 6. **Characterization of myocardin domains that bind TDG.** *A,* schematic representation of myocardin indicating the GST fusion proteins analyzed. *NTD,* N-terminal domain; *++,* basic domain; *Q,* poly(Q) domain; *LZ,* leucine zipper domain; *TAD,* transcriptional activation domain; *ND,* not determined; *mut,* mutant. *B,* bacterial expressed TDG was incubated with the series of GST-myocardin fusion proteins indicated in *panel A.* Western blotting was performed to detect the GST-myocardin bound TDG (*upper panel*). The *lower panel* in *B* indicates the expression of the GST fusion proteins as detected by a Ponceau S staining. TDG was found to bind to myocardin NTD (N-terminal domain) and basic (++), poly(Q) domain as summarized in *panel A.*

cally binds to two portions of myocardin, the N-terminal domain and the region that includes the basic domain and poly(Q) domain. TDG also binds weakly to the myocardin SAP domain (Fig. 6*A*).

TDG Competes with SRF for Binding to Myocardin—As both TDG and SRF bind to an overlapping region of myocardin (the poly(Q)/basic region) we next determined if TDG competes with SRF for binding to myocardin. Using GST pull-down assays we found that SRF binding to myocardin was diminished in the presence of TDG and conversely TDG binding to myocardin could be decreased by increasing amounts of SRF (Fig. 7*A*). Moreover, the co-immunoprecipitation of myocardin and SRF was significantly decreased in the presence of TDG in a dose-dependent fashion, suggesting that TDG disrupts SRF-myocardin complex formation *in vivo* (Fig. 7*B*). To confirm that TDG can disrupt myocardin binding to SRF within intact chromatin we performed quantitative chromatin immunoprecipitation assays. Results from this analysis showed that TDG significantly attenuated myocardin binding to the *SM22α* and *SM MHC* promoters (Fig. 7, *C* and *D*). Taken together, these data suggest that the TDG suppression of smooth muscle-specific gene expression occurs

through disrupting SRF/myocardin interactions thus abrogating myocardin binding to promoters of smooth muscle-specific genes.

TDG Glycosylase Activity Is Dispensible for Repression of Myocardin Function—Previous studies have shown that substitution of the catalytic site asparagine with alanine (N140A) in TDG resulted in a glycosylase-deficient enzyme that bound mismatched substrates but was unable to catalyze base removal (31). To test whether TDG glycosylase activity is required to inhibit myocardin activity, a catalytically inactive mutant TDG (N140A) was generated by site-directed mutagenesis. This mutant was then co-transfected together with myocardin into 10T1/2 cells, and the myogenic activity of myocardin was determined by reporter gene assays or qRT-PCR. Data from these experiments demonstrated that the TDG N140A mutant attenuated the activation of *Telokin* and *SM22α* reporter genes of myocardin and abrogated the induction of endogenous telokin and SM22α of myocardin as efficiently as the wild type TDG (Fig. 8, *A* and *B*). These data suggest that the DNA glycosylase activity of TDG is not required to inhibit myocardin.

TDG mRNA Decreases following Serum Withdrawal from SMCs, and Increases during SMC Proliferation—To examine the relationship between TDG and SMC differentiation and proliferation, proliferating human coronary artery SMCs were cultured in serum-free medium for up to 8 days to promote differentiation. After 8 days, growth medium was added back to stimulate proliferation. Total RNA was harvested from these differentiating or proliferating cells and analyzed by qRT-PCR (Fig. 9). These results show that following serum withdrawal, expression of TDG mRNA is down-regulated, whereas *SM MHC*, *CALPONIN*, and *TELOKIN* mRNA expression is increased. Conversely, reintroduction of growth medium to these differentiated SMCs resulted in down-regulation of SM-specific markers while increasing TDG expression. These data demonstrate that expression of TDG is inversely correlated with expression of smooth muscle-specific genes. Analysis of our raw qRT-PCR data revealed TDG mRNA levels are comparable with the levels of the RPLPO housekeeping gene (data not shown), as determined by similar C_T values using primer sets that have similar amplifying efficiencies, suggesting TDG has a high level expression in these SMCs.
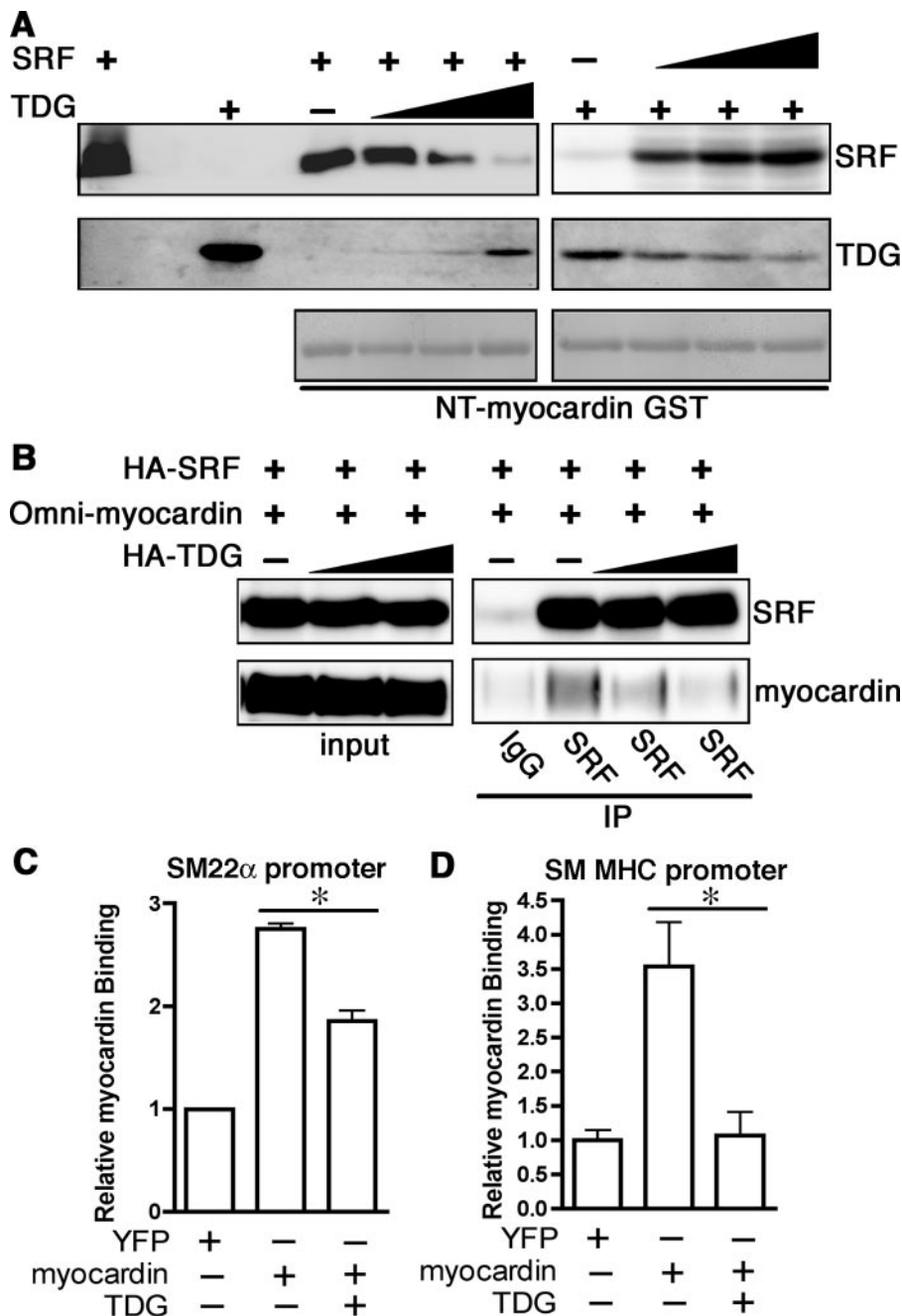
FIGURE 7. **TDG and SRF compete for binding to myocardin *in vitro* and TDG represses myocardin binding to the promoters of smooth muscle-specific genes.** *A*, GST pull-down assay using bacterial expressed SRF or TDG and GST-myocardin (1–585). *B*, COS cells were transduced with adenoviruses encoding HA-SRF (HA tag), Omni-myocardin, or HA-TDG as indicated. SRF was immunoprecipitated (*IP*) from nuclear extracts of the transduced cells and co-precipitating myocardin was detected by Western blotting. *C*, chromatin immunoprecipitation assays using 10T1/2 cells transduced with adenovirus encoding Omni-tagged myocardin, HA-tagged YFP, or TDG. Cross-linked chromatin was immunoprecipitated with anti-Omni antibody and the precipitated DNA was amplified by real time PCR using *SM22α* (*C*) and *SM MHC* (*D*) gene-specific primers. The increase in myocardin binding is indicated relative to YFP control transduced cells (set to 1). These data were calculated and normalized to input levels as follows: relative myocardin binding = $2^{-\Delta\Delta Ct}$, with $\Delta\Delta C_t = (C_{t,myocardin} - C_{t, input}) - (C_{t, YFP} - C_{t, input})$. Data shown are the mean ± S.E. of 4 samples obtained from two independent experiments. *, significantly different ($p < 0.05$).

DISCUSSION

Myocardin is a cardiac and smooth muscle tissue-specific transcription factor that is required for vascular smooth muscle differentiation (4). In the current study we have identified a novel role of the TDG DNA mismatch repair enzyme in atten-

uating myogenic activity of the myocardin. TDG appeared to have similar affects on both the long (cardiac enriched) and short (smooth muscle enriched) isoforms of myocardin. We show that TDG can suppress smooth muscle-specific gene expression in primary smooth muscle cells by preventing myocardin from binding to SRF at the promoters of smooth muscle-specific genes. Knocking down endogenous *TDG* levels in A10 vascular SMCs resulted in increased expression of SM MHC and *telokin*, demonstrating that TDG is playing a tonic role in attenuating myocardin activity in these partially de-differentiated cells. However, silencing TDG has a modest effect on increasing smooth muscle gene expression in A10 SMCs. We believe that the relatively modest effects of TDG knocking down on expression of smooth muscle genes reflects the relatively differentiated state of these A10 cells and thus the modest attenuation of expression of these genes by TDG under the conditions analyzed. It is also possible that other mismatch-directed uracil-DNA glycosylases or other inhibitory proteins are inhibiting myocardin function in the absence of TDG.

Although TDG was originally identified as a repair enzyme for initiating correction of G:T or G:U mismatches in DNA, accumulating evidence have shown additional roles for TDG in regulating gene expression through modulating the activity of other transcription factors. For example, TDG functionally associates with several nuclear receptors including ER (10, 13), retinoic acid receptor (10), SRC1 (a p160 co-activator of ERα) (32), and CREB-binding protein and its paralog p300 (33) where TDG augments transcriptional activity of these receptors or transcriptional co-activators. It has been postulated that the recruitment of transcriptional co-activators of TDG such as p300 and CREB-binding protein accounts for its stimulatory effects on nuclear hormone receptors (33). However, TDG has also been reported to act as a repressor of TTF1, a member of the Nkx2 family of homeodomain proteins that is essential for the thy-
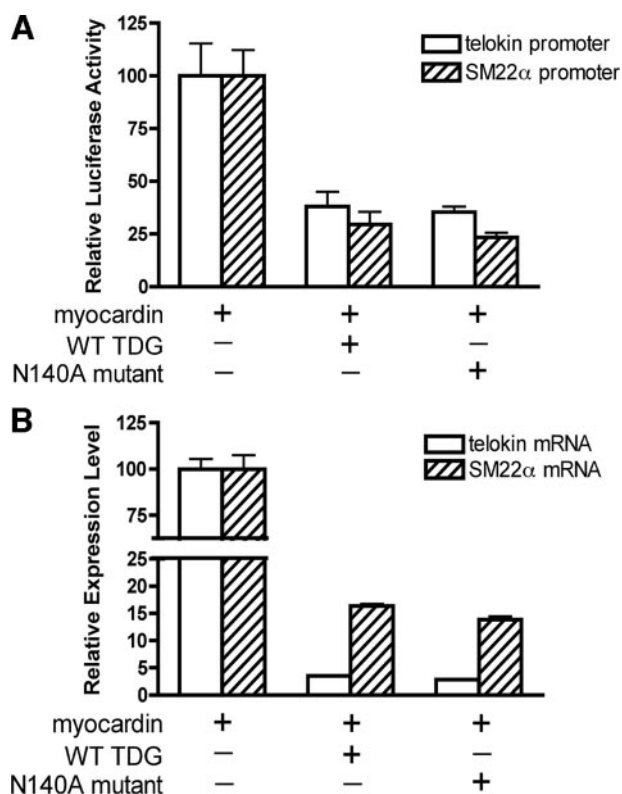
FIGURE 8. **A glycosylase-deficient mutant TDG (N140A) represses myocardin activity.** *A*, *SM22α* or *Telokin* reporter genes were co-transfected together with myocardin and either wild-type (*WT*) or glycosylase-deficient mutant TDG (N140A) expression plasmids into 10T1/2 cells. All data are normalized to the activation produced by myocardin alone (set to 100). $n = 6$. *B*, 10T1/2 cells were transfected with the indicated expression plasmids and changes in endogenous *Telokin* and *SM22α* mRNA were analyzed by qRT-PCR. All data are normalized to the induction by myocardin alone (set to 100). $n = 4$.

FIGURE 9. **TDG expression is negatively correlated with the expression level of SM-specific genes during SMC differentiation and proliferation.** Proliferating human coronary SMCs were cultured in serum-free medium for 0, 2, or 8 days and then growth medium was reintroduced for 8 or 24 h as indicated. Total RNA was harvested at each time point from these cells, and gene expression was examined by qRT-PCR as indicated. $n = 3$.

roid- and lung-specific gene expression (14). Although in this previous study the mechanism by which TDG repressed TTF1-activated transcription in thyroid and non-thyroid cells was not elucidated. In contrast, we have found that TDG represses myocardin function, at least in part, by disruption of myocardin-SRF complexes thus preventing myocardin binding to target promoters *in vivo*. It is unlikely that TDG recruits repressors such as histon deacetylases to myocardin-SRF complexes, because TDG-mediated repression on myocardin was not alleviated by the histone deacetylase inhibitor tricostatin A, and exogenous expression of p300 did not rescue the inhibitory effects of TDG on myocardin (data not shown). We also found that TDG can attenuate the activity of a dimerization defective myocardin (Fig. 2*D*) suggesting that inhibition of myocardin dimerization is not a likely inhibitory mechanism. Furthermore, repression of myocardin activity is independent on the glycosylase activity of TDG, a TDG catalytic deficient mutant (N140A) can effectively suppress myocardin function (Fig. 8). Indeed, the glycosylase function of TDG was also found to be dispensable for transcriptional co-activation of an ERα responsive reporter gene (13). Whether or not TDG-mediated repression of TTF1 requires its glycosylase activity has not been investigated. Taken together, these studies suggest that, besides its DNA base excision repair function,
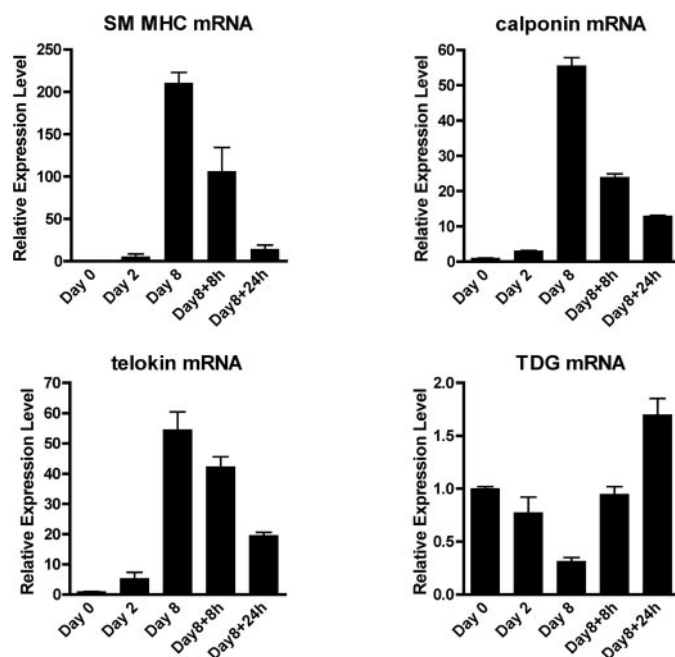
TDG also exerts important gene regulatory functions through its specific interactions with other transcription factors.

The ability of DNA repair factors like TDG to also regulate transcription could provide a possible mechanism to link these two processes to maintain the integrity of transcribed genes. For instance, previous studies have shown that proteins involved in nucleotide excision repair are components of the basal transcription factor TFIIH (34). The recruitment of DNA repair factors, which can also inhibit transcription, could provide a mechanism by which cells can avoid transcribing damaged DNA. Transcription factors, such as myocardin could thus play a role in region-specific DNA repair by sensing DNA damage in actively expressed areas of the genome, through their ability to recruit DNA repair enzymes such as TDG. Consistent with this hypothesis, a previous report showed that the TDG/CREB-binding protein and p300 complexes are competent for TDG-mediated excision repair (33). Additional studies will be required to determine whether myocardin/TDG interactions are required to help maintain the integrity of genes in smooth muscle cells. Previous studies have linked high levels of TDG expression with highly proliferative tissues during mouse embryonic development and in transformed tissues (35). We also found that TDG expression was greater in proliferating as compared with differentiated SMCs (Fig. 9). Similarly, a number of negative regulators of myocardin function, including Foxo4, HERP1, and KLF4 have been shown to be up-regulated in proliferating de-differentiated smooth muscle cells (5–7). Moreover, previous study has shown that overexpression of myocardin reduces cell growth (36) and inactivation of myocardin in fibroblasts has been shown to promote proliferation and oncogenesis (26). The high levels of TDG in proliferating cells

may reflect the increased need to repair DNA mismatches in these cells. Increased expression of TDG in proliferating smooth muscle cells may also provide an additional means for these cells to inhibit both the antiproliferative and promyogenic activities of myocardin.

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