SUPPLEMENT

Because of the apparent absence of anti-Smad3 and anti-Smad4 antibody-sensitive complexes assembled by SM22[-215/-182] (**Figure 6B**, lanes 4 – 9) we wished to confirm the activity of the immunoreagents used in these gel shift assays. Therefore, we assessed the activities of these antibodies on complexes binding SM22[-18/+18], a fragment encompassing the developmentally important Smad3 binding element recently described(1, 2). We first took advantage of the fact that R-Smads can bind to DNA cognates in vitro either as monomers (3)or homotrimers (4) as well as heterotrimers(5). Commercially available recombinant purified GST-Smad3 (glutathione S-transferase - Smad3 fusion protein) forms 3 complexes with SM22[-18/+18] (**Supplement Figure S1,** lane 2, complexes B, D, and E). Addition of anti-Smad2/3 antibody – the reagent that disrupts complex formation on the novel SM22[-215/-182] element – did not disrupt the Smad3 complexes; however it was permissive for the formation of two new rapidly migrating complexes (**Supplement Figure S1**, lane 3, complexes A and C). By contrast, addition of the anti-Smad3 antibody inhibited formation of complexes B, D, and E (black arrowheads) and yielded a lower-mobility "supershifted" band (**Supplement Figure S1**, lane 4, fine arrow). This same reagent did not disrupt complex formation on the novel SM22[-215/-182] element **Figure 6B**, lanes 4 and 5). Using extracts obtained from TGFβ-treated C3H10T1/2 cells, multiple migrating complexes binding SM22[-18/+18] were also identified (**Supplement Figure S2**, lane 2 vs. lane 1) as previously described(1, 2). Two of these complexes migrated very close to one another (complexes A and B). Addition of the anti-Smad2/3 antibody diminished complex B formation (**Supplement Figure S2**, lane 3, black arrowhead) and anti-Smad4 antibody yielded two super-shifted complexes (**Supplement Figure S2**, lane 5, fine arrows). Complex B assembly on the SM22[-18/+18] Smad site was also slightly reduced by anti-Smad4 (**Supplement Figure S2**, lane 5, black arrowhead) – even though no anti-Smad4 sensitive complexes were detectable on the novel SM22[-215/-182] element (**Figure 6A**, lanes 7 – 9). .Complex E was unaffected by

any of the antibodies (**Supplement Figure S2**). Moreover, none of the antibodies used interacted directed with DNA probes (See manuscript **Figures 6A and 6B**, lanes 3, 6, and 9). Thus, functionally important DNA-protein complexes containing β-catenin, TCF7, and Smad2 bind the CAGAG motif at nucleotides -203 to -199 in the *SM22α* promoter. Smad3- and Smad4-containing were not detected in the CAGAG DNA-protein binding complex assembled by SM22[-215/-182].

SUPPLEMENT FIGURE LEGENDS

Figure S1: Validation of anti-Smad3 antibody. The radiolabeled SM22α promoter fragment -18 to + 18, encompassing the Smad3-binding exon 1 SBE(1, 2) was used in gel shift assay with recombinant purified GST-Smad3. Note that the 3 GST-Smad3 complexes visualized (B, C, D; lane 2) are inhibited by the anti-Smad3 antibody, with formation of a prominent and stable "supershift" complex (lane 4). The anti-Smad2/3 antibody enhanced the formation of a stable, rapidly migrating complex (lane 3, complex A), likely representing the monomer.

Figure S2: Validation of anti-Smad4 antibody. Extracts from TGFβ treated cells also possess SM22[-18/+18] binding activities (lanes 1 and 2). Anti-Smad4 (lane 5) either eliminated or reduced complex 4 formation (arrowhead). Anti-Smad4 also yielded stable supershifted complexes (fine arrows).

Figure S3. Regulation of *SM22α* gene expression by Wtn3a: A working model: Signals provided by Wnt3a upregulate *SM22α* gene transcription by activating a TCF7:Smad2:β-catenin ternary complex assembled by the promoter region -213 to -192, dependent upon the CAGAG motif at -203 to -199. Based upon co-expression studies, Smad2Δexon3, not Smad2(FL),

mediates transactivation. In other contexts, Smad3 supports activity of *SM22α* transcription via other important regulatory elements, including the exonic SBE(1, 2). TGFβ also enhances transcription via these motifs -- and via other elements that mediate Kruppel-like factor 4 repression (6, 7). As a large transcriptional co-adapter(8-10), β-catenin potentially mediates Wnt3a-dependent coupling between the novel CAGAG element and other motifs (SBE, CArG boxes, TCE or TGFβ-control element) that support *SM22α* transcription during SMC development and disease (11, 12). Functional relationships between β-catenin, SRF, and the co-adapter myocardin -- the master regulator of the SMC phenotype (13, 14) -- have yet to be studied.

Figure S4. Relative level of Wnt1, Wnt3a, and Wnt5a gene expression in quiescent aortic myofibroblasts. RNA was extracted from aortic myofibroblasts treated with either vehicle, Wnt3a (15 ng /ml), or Wnt3a (15 ng/ml) + TGFβ1 (5 ng/ml) in combination for 3 days as indicated. Commercially purchased TaqMan assays were used to quantify the relative levels of Wnt1, Wnt3a, and Wnt5a mRNA accumulation (GAPD normalized) as described in the methods of the main text. Data are expressed the mean +/- s.e.m. percent of vehicle –treated Wnt1 level. Because Wnt5a mRNA expression is much greater than that of either Wnt1 or Wnt3a, Wnt5a percentages have been multiplied by 0.01 as indicated (Wnt5a x 0.01) for graphic presentation Baseline vehicle-treated values for Wnt5a message are ~600-fold greater than that of Wnt3a in quiescent myofibroblasts.

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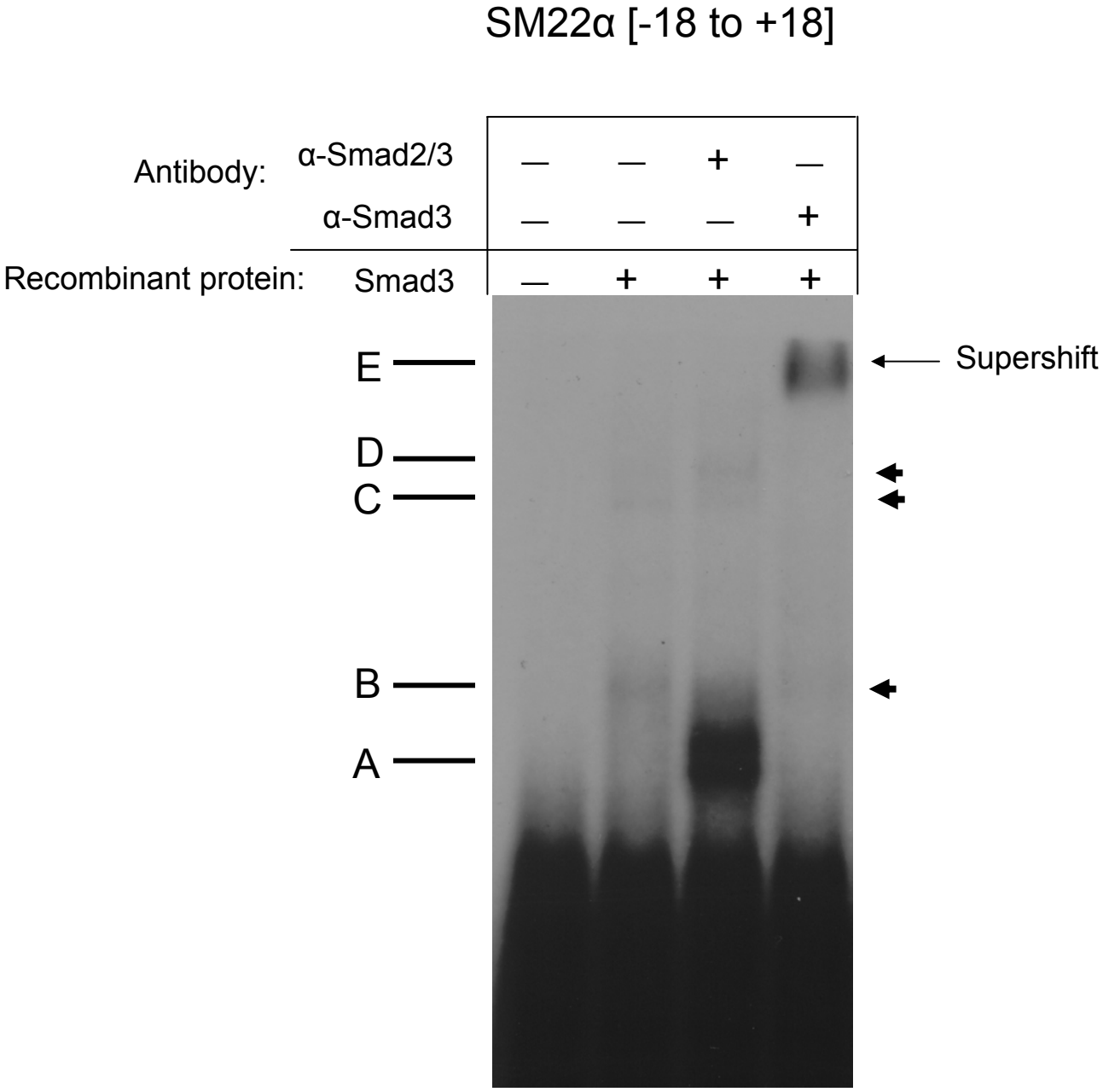
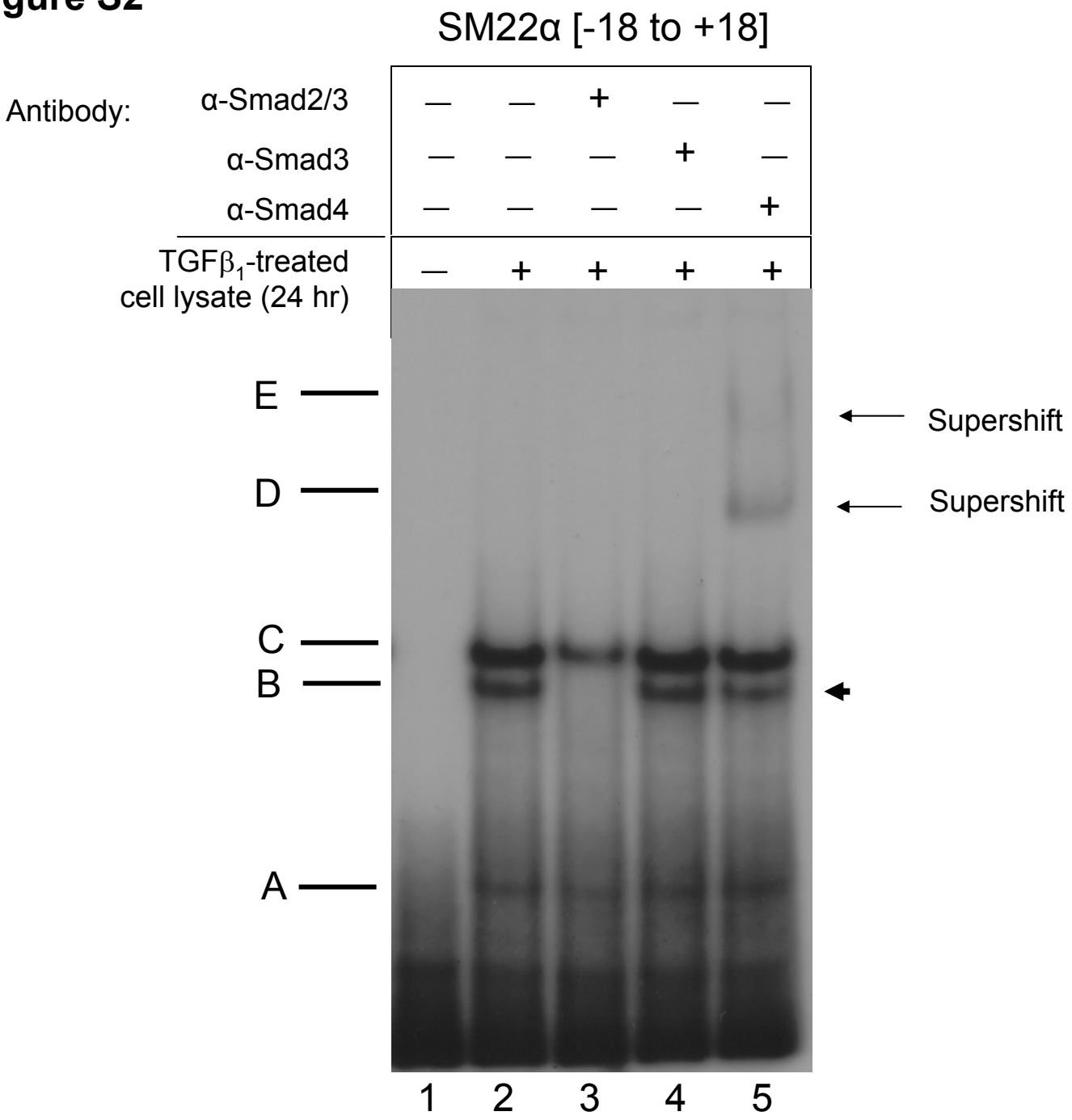
Supplement Figure S1

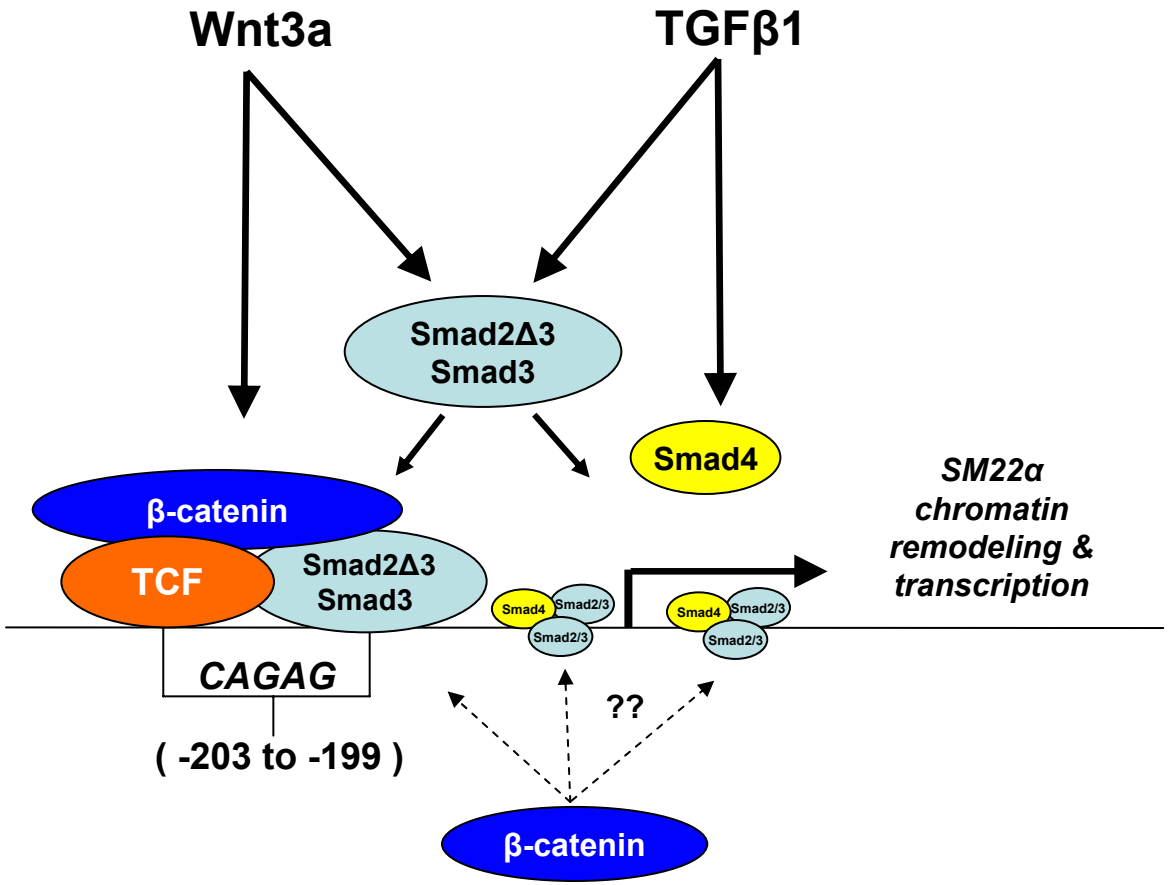


Supplement Figure S2



Supplement Figure S3

Regulation of SM22α Gene Expression By Wnt3a: A Working Model



Supplement Figure S4