Vascular Smooth Muscle LRP6 Limits Arteriosclerotic Calcification in Diabetic LDLR\(^{-/-}\) Mice by Restraining Noncanonical Wnt Signals

Su-Li Cheng,* Bindu Ramachandran,* Abraham Behrmann,* Jian-Su Shao, Megan Mead, Carolyn Smith, Karen Krchma, Yoanna Bello Arredondo, Attila Kovacs, Kapil Kapoor, Laurence M. Brill, Ranjan Perera, Bart O. Williams, Dwight A. Towler

**Rationale:** Wnt signaling regulates key aspects of diabetic vascular disease.

**Objective:** We generated SM22-Cre;LRP6(fl/fl);LDLR\(^{-/-}\) mice to determine contributions of Wnt coreceptor low-density lipoprotein receptor–related protein 6 (LRP6) in the vascular smooth muscle lineage of male low-density lipoprotein receptor–null mice, a background susceptible to diet (high-fat diet)–induced diabetic arteriosclerosis.

**Methods and Results:** As compared with LRP6(fl/fl);LDLR\(^{-/-}\) controls, SM22-Cre;LRP6(fl/fl);LDLR\(^{-/-}\) (LRP6-VKO) siblings exhibited increased aortic calcification on high-fat diet without changes in fasting glucose, lipids, or body composition. Pulse wave velocity (index of arterial stiffness) was also increased. Vascular calcification paralleled enhanced aortic osteochondrogenic programs and circulating osteopontin (OPN), a matricellular regulator of arteriosclerosis. Survey of ligands and Frizzled (Fzd) receptor profiles in LRP6-VKO revealed upregulation of canonical and noncanonical Wnts alongside Fzd10. Fzd10 stimulated noncanonical signaling and OPN promoter activity via an upstream stimulatory factor (USF)–activated cognate inhibited by LRP6. RNA interference revealed that USF1 but not USF2 supports OPN expression in LRP6-VKO vascular smooth muscle lineage, and immunoprecipitation confirmed increased USF1 association with OPN chromatin. ML141, an antagonist of cdc42/Rac1 noncanonical signaling, inhibited USF1 activation, osteochondrogenic programs, alkaline phosphatase, and vascular smooth muscle lineage calcification. Mass spectrometry identified LRP6 binding to protein arginine methyltransferase (PRMT)-1, and nuclear asymmetrical dimethylarginine modification was increased with LRP6-VKO. RNA interference demonstrated that PRMT1 inhibits OPN and TNAp, whereas PRMT4 supports expression. USF1 complexes containing the histone H3 asymmetrically dimethylated on Arg-17 signature of PRMT4 are increased with LRP6-VKO. Jmjdc6, a demethylase downregulated with LRP6 deficiency, inhibits OPN and TNAp expression, USF1: histone H3 asymmetrically dimethylated on Arg-17 complex formation, and transactivation.

**Conclusions:** LRP6 restrains vascular smooth muscle lineage noncanonical signals that promote osteochondrogenic differentiation, mediated in part via USF1- and arginine methylation–dependent relays. (Circ Res. 2015;117:142-156. DOI: 10.1161/CIRCRESAHA.117.306712.)

**Key Words:** arteriosclerosis ■ LRP6 ■ signal transduction ■ type 2 diabetes mellitus ■ USF1 ■ vascular calcification ■ Wnt

Hyperglycemia, hyperlipidemia, and uremia accelerate vascular aging, compromising arterial function necessary for normal blood flow, metabolism, and tissue homeostasis.\(^1\) Along with hypertension, these dysmetabolic states induce arteriosclerotic stiffening, thereby reducing vascular compliance that underlies Windkessel physiology—elasticity of conduit vessels that ensures smooth distal tissue perfusion throughout the cardiac cycle.\(^2\) Atherosclerotic burden, mural thickening and fibrosis, medial calcification, elastic fragmentation, nonenzymatic matrix crosslinking, and endothelial dysfunction are features of arteriosclerotic aging. Multiple laboratories have now identified bone morphogenetic proteins and Wnts—polypeptides that convey paracrine cues during skeletal morphogenesis—as pathogenic signals...
in arteriosclerotic calcification. In studies of low density lipoprotein receptor (LDLR)−/− mice fed high-fat diabetogenic diets (HFD) typical of western societies, we identified osteogenic Msx-Wnt signaling cascades. Expression of the osteoblast transcription factor Msx2 is increased in SM22-Cre;LRP6(fl/fl);LDLR−/− mice and thereby restrains mesenchymal expression of osteogenic programs in male LDLR−/− mice fed HFD, mediated in part via upstream stimulatory factor (USF)1– and protein arginine methylation–dependent relays.

To better understand the role of the vascular LRP6 Wnt receptor in arteriosclerosis, we used the SM22-Cre transgene22 to delete VSM LRP621 in LDLR−/− mice. We discover that LRP6 restraints noncanonical signals that drive VSM osteochondrogenic programs in male LDLR−/− mice fed HFD, mediated in part via upstream stimulatory factor (USF)1– and protein arginine methylation–dependent relays.

Methods

See online-only Data Supplement.

Results

Conditional Deletion of LRP6 in VSM Accentuates Aortic Calcification in LDLR−/− Mice Fed HFD, Increases Vessel Stiffening and Promotes Ectopic Arterial Expression of the Osteochondrogenic Phenotype

LRP6 is expressed in the arterial vasculature,23 primarily in mural VSM and fibrous caps of atherosclerotic lesions in the aortic sinus of LDLR−/− mice fed HFD (Figure 1A and 1B; Online Figures I–II). To better understand the role for LRP6 in the biology of arteriosclerotic calcification, we generated SM22-Cre;LRP6(fl/fl);LDLR−/− mice, conditionally depleting LRP6 in the VSM lineage in male LDLR-null mice, a background susceptible to HFD-induced diabetes mellitus and arteriosclerotic calcification.23 As shown in Figure 1B, after a 3 month challenge with HFD, arterial calcification was increased in SM22-Cre;LRP6(fl/fl);LDLR−/− mice as compared with LRP6(fl/fl);LDLR−/− sibling controls, with calcium deposition observed in both medial and atherosclerotic venues (Online Figure III). Chow-fed animals exhibited much lower aortic calcium levels that did not differ between genotypes (Figure 1B). Aortic stiffness was increased in SM22-Cre;LRP6(fl/fl);LDLR−/− mice and by HFD as determined by echocardiography4 (Figure 1C; reduced aortic arch distensibility); insulin resistance was diet-dependent but independent of genotype (Figure 1D). RT-qPCR analysis revealed reductions in aortic LRP6 mRNA in SM22-Cre;LRP6(fl/fl);LDLR−/− mice, with concomitant increases in LRP4 and markers of osteochondrogenic programming (Figure 1E). Axin2—a target of canonical β-catenin15—was diminished in aortas deficient for LRP6, whereas Klf5—a target of noncanonical Wnt signaling like OPN5,8—was upregulated along with the progenitor marker Sca17(Figure 1E). Although clear trends
Figure 1. Conditional deletion of low-density lipoprotein receptor-related protein 6 (LRP6) in vascular smooth muscle lineage (VSM) increases osteochondrogenic calcification and arterial stiffening in LDLR−/− mice fed high-fat diabetogenic diets (HFD). A, Immunohistochemistry reveals expression of LRP6 in VSM of aortic tunica media (arrows) and atherosclerotic caps (arrowheads) of the aortic sinus. Asterisks overlay atheroma. Scale bar=50 μm. Coronary artery VSM also expresses LRP6 (Online Figures I and II). B, On HFD, aortic calcium content is increased in aortas of SM22-Cre;LRP6(fl/fl);LDLR−/− mice as compared with LRP6(fl/fl);LDLR−/− on HFD or chow-fed controls (11–15 weeks of age). All HFD animals were 17 to 20 weeks of age at aortic analysis. Numbers of animals in each group were between 12 and 14 as indicated. ANOVA \( P < 0.0001 \). a, \( P < 0.001 \) vs chow-fed controls; b, \( P < 0.01 \) vs Cre-negative mice on HFD by Holm-Sidak’s post hoc testing corrected for multiple comparisons. C, Doppler echocardiography reveals increased vascular stiffness in SM22-Cre;LRP6(fl/fl);LDLR−/− mice, reflected in reduced distensibility (% change in aortic arch diameter from diastole to systole). ANOVA \( P < 0.0001 \). ***\( P < 0.001 \) vs LRP6(fl/fl);LDLR−/− control; **\( P < 0.01 \) between chow-fed SM22-Cre;LRP6(fl/fl);LDLR−/− animals. No other differences were significant. D, HFD-induced insulin resistance did not differ between genotypes. One-way ANOVA \( P < 0.001 \); significant differences between the groups as indicated by Holm-Sidak post hoc test (see also Online Figures XXII and XXIII). a, \( P < 0.05 \) vs chow-fed of either genotype; b, \( P < 0.05 \) vs chow-fed Cre-negative animals; c, \( P < 0.01 \) between chow-fed SM22-Cre;LRP6(fl/fl);LDLR−/− animals. No other differences were significant. E, The aortic expression of osteochondrogenic genes is concomitantly upregulated with reductions in VSM LRP6 expression. *\( P < 0.05 \) vs LRP6(fl/fl);LDLR−/− control; **\( P < 0.01 \) vs control; (Continued)
for increased aortic osteochondrogenic programming were noted in chow-fed mice with VSM LRP6 deficiency, these differences did not reach significance (Online Figure IV) in the absence of HFD. However, the increases in osteochondrogenic programs were robustly elaborated in primary aortic VSM cultures from SM22-Cre;LRP6(fl/fl);LDLR−/− mice (Figure 1F; TNAP, OCN, OPN) and associated with increased mineralization in vitro (vide infra). Aspects of the contractile VSM program were diminished, indicated by downregulation of Myh11 and myocardin (Figure 1F, and not shown). Plasma levels of osteopontin (OPN) were also increased in SM22-Cre;LRP6(fl/fl);LDLR−/− mice versus LRP6(fl/fl);LDLR−/− controls after HFD challenge (Figure 1G). Measurement of medial thickness in the ascending aorta sinus revealed no significant increases in HFD-fed animals with reduced VSM LRP6, although pulse wave velocity was increased (Online Figure V). Aortic proliferation indices did not differ between genotypes; however, cultured VSM from SM22-Cre;LRP6(fl/fl);LDLR−/− mice exhibited 10% greater bromodeoxyuridine incorporation (Online Figure VI–VIII). Aortic lumen diameter of animal on HFD did not differ between genotypes as quantified by echocardiography (Online Figure IX); however, a nonsignificant trend (P=0.1) for increased Mac2(+) atheroma area in the sinus was observed along with significantly increased thoracic aortic F4/80 and IL12A macrophage expression (Online Figures X and XI). Importantly, differences in aortic calcification and stiffness between genotypes arose in the absence of differences in HFD-induced changes in fasting blood glucose, lipids, insulin resistance, or body composition (Online Figures XII and XIII). Thus, absence of VSM LRP6 increases aortic calcification and vascular stiffness in diabetic LDLR−/− mice and enhances vascular elaboration of an osteochondrogenic gene program.

**Wnt Ligands and Fzd Receptors Capable of Activating Noncanonical Signals Are Upregulated in Aortic Tissues of SM22-Cre;LRP6(fl/fl);LDLR−/− Mice on HFD**

Skeletal biomineralization occurs via the overlapping yet distinct mechanisms of membranous (type 1 collagen–oriented) and endochondral (type 10 collagen–oriented) ossification. Canonical Wnt signals promote initiation of the former and inhibit bone resorption, whereas noncanonical Wnt signals promote mature tissue calcification via both mechanisms. LRP6 can restrain noncanonical signals in part by sequestering certain Fzd coreceptors. To assess whether paracrine relays capable of noncanonical signaling were altered in aortic tissues with LRP6 deficiency, we surveyed the expression of Wnt ligands and Fzd coreceptors. Array analysis of aortic RNA from mice on HFD for 3 months (n=5/genotype) revealed upregulation of genes encoding multiple Wnt ligands and Fzd10 (Online Figure XIV). This was confirmed by RT-qPCR; as compared with LRP6(fl/fl);LDLR−/− controls, SM22-Cre;LRP6(fl/fl);LDLR−/− mice on HFD exhibited elevated levels of Wnt7b, Wnt4, Wnt10a, Wnt3a, and Fzd10 (Figure 2A and 2B). Increases in aortic mRNAs observed in vivo required HFD challenge (Online Figure XV). However, significant upregulation of ligands and Fzd10 expression was observed in primary VSM cultures (Online Figure XVI; and vide infra). Wnt5a—the abundant noncanonical agonist—remained unchanged. Immunohistochemistry and western blot analysis of primary VSM cultures confirmed upregulation of Wnt7b (Figure 2C), Wnt10a (Figure 2D), and Fzd10 protein with downregulation of LRP6 (Figure 2E). Of note, although Wnt3a and Wnt10a are reported to elicit only canonical signals, Wnt4 and Wnt7b are capable of supporting both canonical and noncanonical pathways. Thus, loss of VSM LRP6 leads to the upregulation of Wnt ligands capable of supporting canonical and noncanonical signaling.

**Fzd10 Activates Noncanonical Signaling That Is Inhibited by LRP6 Expression and Promotes OPN Transcription via USF Protein–DNA Interactions**

Fzd10 activation in sarcoma elicits noncanonical signals similar to Fzd9 in bone. To confirm and extend this, we transiently cotransfected Fzd10 expression vectors with nuclear factor of activated T cells–luciferase reporter (NFAT-LUC) and lymphoid enhancing factor-LUC reporters that register noncanonical and canonical signals, respectively, as indicated. ANOVA P=0.022, *P<0.05 vs all others by Fisher’s LSD post hoc test, and P<0.05 vs Cre-minus control on HFD after correction for multiple comparisons. LDLR indicates low-density lipoprotein receptor; and LRP6(fl/fl), LRP6 gene floxed.
This indicated that Fzd10 conveys responsiveness to a noncanonical agonist elaborated by SM22-Cre;LRP6(fl/fl);LDLR−/− VSM. 

OPN is an endogenous noncanonical Wnt target and osteochondrogenic gene active during membranous and endochondral bone formation and is upregulated in SM22-Cre;LRP6(fl/fl);LDLR−/− VSM. RNA interference (RNAi) targeting Fzd10 but not Fzd9 reduces OPN gene expression in SM22-Cre;LRP6(fl/fl);LDLR−/− VSM (Figure 4A). As with NFAT-LUC, Fzd10 upregulates OPN promoter activity and is inhibited by LRP6 expression (Figure 4B). Unlike Fzd10, Fzd7 and Fzd1 are inactive.
in this assay (Online Figure XVII and not shown). Corepression of either Wnt7b or Wnt5a enhanced Fzd10 activation, while Wnt11 and Wnt4 did not (Online Figure XVIII).

OPN promoter mapping identified that the glucose-responsive USF cognate at −80 to −72 relative to the transcriptional start site is required for Fzd10 induction (left). By contrast, LRP6 activates canonical T cell factor (TCF)/lymphoid enhancing factor (LEF) signaling via pathways inhibited by Fzd10 (right). ANOVA P<0.0001 with post hoc Holm–Sidak testing. N=3 per group. B, ML141, a cdc42 antagonist, inhibits Fzd9 and Fzd10 induction of NFAT-LUC. Fzd1, Fzd2, and Fzd6 were inactive (not shown). ANOVA P<0.0001 with post hoc Holm–Sidak testing. N=6 per group. C, Constitutively active cdc42(Q61L) stimulation of noncanonical NFAT signaling is enhanced by Fzd10 and inhibited by LRP6. ANOVA P<0.0001 with post hoc Holm–Sidak testing. N=3 to 9 per group. D, HEK cells were transfected with either pCMV vector+NFAT-LUC or pCMV-Fzd10+NFAT-LUC, then parachuted for coculture (Co-Cx) onto lawns of either LRP6(fl/fl);LDLR−/− vascular smooth muscle lineage (VSM) or SM22-Cre;LRP6(fl/fl);LDLR−/− VSM. Note that SM22-Cre;LRP6(fl/fl);LDLR−/− cells supported Fzd10-dependent activation of NFAT-LUC signaling, indicating enhanced elaboration of a noncanonical agonist with VSM LRP6 deficiency. ANOVA P<0.0001, with post hoc Holm–Sidak testing. N=6 per group. CMV indicates cytomegalovirus promoter/enhancer vector; Fzd, frizzled receptor; LDLR, low-density lipoprotein receptor; LRP6(fl/fl), LRP6 gene floxed; LUC, luciferase reporter; NFAT, nuclear factor of activated T cells–luciferase reporter; and Wnt, Wingless/int-1 family member.
Moreover, ML141 reduced OPN elevation in LRP6-deficient VSM (Figure 4H); by contrast, the selective Rac1 inhibitor EHT1864 exerted little if any effect on OPN expression (Figure 4H). Thus, Fzd10- and USF1-activation of the OPN promoter is inhibited by LRP6, with LRP6 actions phenocopied by ML141 treatment.
LRP6 Associates With PRMT1 and SM22-Cre;LRP6(fl/fl);LDLR−/− Aortic VSM Exhibits Increased Nuclear Protein Asymmetrical Dimethylarginine Accumulation

To better understand the mechanisms whereby LRP6 regulates signaling, we expressed FLAG-epitope tagged LRP6 in HEK cells, immunoprecipitated FLAG-containing complexes under nondenaturing conditions, and began to characterize the LRP6 interactome by mass spectrometry (to be presented elsewhere). As compared with control cells, immune complexes from LRP6-FLAG expressing cells coprecipitated when coexpressed in human embryonic kidney (HEK) cells. B left. Protein asymmetrical dimethylarginine (ADMA) profiles are perturbed in LRP6-deficient VSM. Although a few ADMA proteins are downregulated (white arrows), the majority of those visualized are increased in SM22-Cre;LRP6(fl/fl);LDLR−/− nuclear fraction (black arrows). Right. Although Cre-mediated downregulation of LRP6 protein in the membrane fraction was readily detected (upper right), the broad distribution of PRMT1 exhibited little if any change. v2, PRMT1 variant 2 possessing the nuclear export signal (NES). v1, PRMT1 variant 1 lacking the NES. C and D, Digital image analysis confirmed significant 2- to 3-fold increases in total nuclear ADMA protein accumulation in LRP6-deficient VSM. N=3 per group. *P<0.05 by Student’s t test. Fzd indicates frizzled receptor; LDLR, low-density lipoprotein receptor; LRP6(fl/fl), LRP6 gene floxed; LUC, luciferase reporter; NFAT, nuclear factor of activated T cells–luciferase reporter; and PRMT, protein arginine methyltransferase.

Figure 5. Low-density lipoprotein receptor–related protein 6 (LRP6) forms a complex with PRMT1v1, and cellular protein ADMA accumulation is perturbed in LRP6-deficient vascular smooth muscle lineage (VSM). A, PRMT1v1 and Flag-tagged LRP6 are coprecipitated when coexpressed in human embryonic kidney (HEK) cells. B left. Protein asymmetrical dimethylarginine (ADMA) profiles are perturbed in LRP6-deficient VSM. Although a few ADMA proteins are downregulated (white arrows), the majority of those visualized are increased in SM22-Cre;LRP6(fl/fl);LDLR−/− nuclear fraction (black arrows). Right. Although Cre-mediated downregulation of LRP6 protein in the membrane fraction was readily detected (upper right), the broad distribution of PRMT1 exhibited little if any change. v2, PRMT1 variant 2 possessing the nuclear export signal (NES). v1, PRMT1 variant 1 lacking the NES. C and D, Digital image analysis confirmed significant 2- to 3-fold increases in total nuclear ADMA protein accumulation in LRP6-deficient VSM. N=3 per group. *P<0.05 by Student’s t test. Fzd indicates frizzled receptor; LDLR, low-density lipoprotein receptor; LRP6(fl/fl), LRP6 gene floxed; LUC, luciferase reporter; NFAT, nuclear factor of activated T cells–luciferase reporter; and PRMT, protein arginine methyltransferase.
that PRMT1v1 is present in cytoplasmic, membrane, and nuclear fractions of VSM by western blot analysis and immunohistochemistry (Figure 5B; Online Figure XXII). PRMT1v2, possessing a nuclear export sequence, is only found in the non-nuclear fractions (Figure 5B). These observations prompted assessment of the cellular ADMA profile by western blot. As shown in Figure 5B, protein ADMA modification profiles were altered in the cytoplasmic, membrane, and nuclear fractions of SM22-Cre;LRP6(fl/fl);LDLR−/− VSM; the greatest changes observed were increases in a subset of ADMA-modified nuclear proteins (Figure 5B).

Independent assessment of replicates demonstrated ≈2- to 3-fold increases in specific nuclear ADMA-modified proteins (Figures 5C and 5D). PRMT1 and PRMT4 are the 2 major PRMTs responsible for nuclear protein ADMA modification. Therefore, we examined the consequence of RNA interference–mediated knockdown of PRMT1 and PRMT4 in LRP6-deficient vascular smooth muscle lineage (VSM). A, PRMT1 siRNA increases OPN gene expression. ANOVA $P<0.0001$, with post hoc testing corrected for multiple comparisons. N=4 per group. B, RNAi targeting PRMT4 almost completely inhibits induction of OPN with LRP6 deficiency. ANOVA $P<0.0001$, N=4 per group. C and D, LRP6 deficiency increases upstream stimulatory factor 1 (USF1) nuclear protein accumulation without altering PRMT4: N=3 per group. **$P<0.01$ by Student’s t test. PRMT indicates protein arginine methyltransferase.

Figure 6. RNAi targeting PRMT4 reduces, whereas PRMT1 small interfering RNA (siRNA) increases, osteopontin (OPN) gene induction in low-density lipoprotein receptor–related protein 6 (LRP6)-deficient vascular smooth muscle lineage (VSM). A, PRMT1 siRNA increases OPN gene expression. ANOVA $P<0.0001$, with post hoc testing corrected for multiple comparisons. N=4 per group. B, RNAi targeting PRMT4 almost completely inhibits induction of OPN with LRP6 deficiency. ANOVA $P<0.0001$, N=4 per group. C and D, LRP6 deficiency increases upstream stimulatory factor 1 (USF1) nuclear protein accumulation without altering PRMT4: N=3 per group. **$P<0.01$ by Student’s t test. PRMT indicates protein arginine methyltransferase.

Because nuclear ADMA accumulation was altered in the absence of increased nuclear PRMT4, we examined the expression of Jmjd645,46 and PADI447, genes encoding broad spectrum nuclear arginine demethylase and deimase activities, respectively, that remove the arginine N-methyl signature. As shown in Figure 7A, the levels of both Jmjd6 and PADI4 mRNAs were reduced in SM22-Cre;LRP6(fl/fl);LDLR−/− mice, with concomitant upregulation of OPN and nuclear ADMA protein accumulation. However, only small interfering RNA targeting Jmjd6 increased OPN and TnA gene expression in control VSM cultures (Figure 7B; Online Figure XXIII); PADI4 small interfering RNA reduced mRNA accumulation for select osteochondrogenic genes (Figure 7C). Moreover, Junonji domain containing 6 arginine demethylase (Jmjd6) expression inhibited USF1 activation of the OPN promoter (Figure 7D), whereas peptidyl arginine deiminase 4 did not (Online Figure XXIV).

In HEK cells, USF1 is not ADMA modified, but a low-molecular weight ADMA protein coimmunoprecipitated...
with FLAG-tagged USF1 (Online Figure XXV). The size of this ADMA protein, 17 kDa, suggested it might be histone H3. Western blot analysis confirmed H3 coprecipitation with USF1, revealed the presence of the PRMT4-specific histone H3 asymmetrically dimethylated on Arg-17 (H3R17Me2a) signature, and demonstrated USF1-associated H3R17Me2a was reduced by coexpression of Jmjd6 (Figure 7E and 7F).

Furthermore, USF1 complexes immunoprecipitated from LRP6-deficient VSM cultures contain increased histone H3 with the H3R17Me2a signature (Figure 7G). Thus, Jmjd6 inhibits the osteochondrogenic phenotype in VSM, antagonizes USF1-dependent transcriptional activation of OPN gene expression, and reduces PRMT4-dependent histone signatures in USF1 protein complexes.

Figure 7. The broad specificity arginine demethylase Jmjd6 is reduced in low-density lipoprotein receptor–related protein 6 (LRP6)–deficient vascular smooth muscle lineage (VSM), restraints VSM osteochondrogenic gene expression, and inhibits upstream stimulatory factor 1 (USF1)–dependent osteopontin (OPN) transcription. A, Although OPN is increased with LRP6 deficiency in VSM, Jmjd6 and PADI4 are significantly downregulated. The relative level of PADI4 is ≈100-fold less than Jmjd6. N=4 per group. *P<0.05; ***P<0.001 vs Cre-negative control by Student’s 2-tailed t test. B and C, Jmjd6 small interfering RNA (siRNA) significantly upregulates OPN and TNAP expression in VSM, whereas PADI4 siRNA does not. N=4 per group. **P<0.01; ***P<0.001 vs control siRNA by Student’s 2-tailed t test. D, Jmjd6 inhibits USF1 activation of the OPN promoter in transient transfection assays. ANOVA P<0.001. ***P<0.001 vs all other treatments by Holm–Sidak post hoc test. N=4 per group. E and F, H3 coprecipitates with Flag-tagged USF1. Co-expression of Jmjd6 significantly reduces the PRMT4 histone H3 asymmetrically dimethylated on Arg-17 (H3R17Me2a) signature on histone H3 coprecipitating with USF1. N=4 per group. *P<0.01 by Student’s 2-tailed t test. G, USF1 immunoprecipitates from LRP6-deficient VSM cultures contain histone H3 bearing the PRMT4 signature H3R17Me2a. Jmjd6 indicates Jumonji domain containing 6 arginine; PADI4, peptidyl arginine deiminase; and PRMT, protein arginine methyltransferase.
Figure 8. The cdc42 antagonist ML141 downregulates upstream stimulatory factor 1 (USF1) protein accumulation and osteochondrogenic mineralization of low-density lipoprotein receptor–related protein 6 (LRP6)–deficient vascular smooth muscle lineage (VSM) without globally altering asymmetrical dimethylarginine (ADMA) protein profiles. A, The increased VSM nuclear USF1 protein levels arising from LRP6 deficiency is reduced by ML141 treatment. Although select nuclear ADMA proteins were reduced, the global ADMA profile was largely unaffected (Online Figure XXVI and data not shown), ANOVA $P<0.015$, $P<0.01$ from others (Holm–Sidak). N=3 to 4 per group as indicated. B and C, Like osteopontin (OPN), the increases in TNAP and Col10A1 arising from LRP6 deficiency are inhibited by ML141 but not by EHT1864. $P<0.05$ vs vehicle-treated LRP6(fl/fl);LDLR $−/−$ control. $P<0.05$ vs vehicle-treated LRP6(fl/fl);LDLR $−/−$ control by post hoc testing. N=4 per group. D, TNAP enzyme activity is induced with LRP6 deficiency and inhibited by ML141. ANOVA $P<0.001$. a, $P<0.01$ vs vehicle-treated LRP6(fl/fl);LDLR $−/−$ control; b, $P<0.001$ vs ML141-treated LRP6(fl/fl);LDLR $−/−$ VSM by the Holm–Sidak’s multiple comparisons test. E and F, Alizarin red staining demonstrates that the increased calcification arising in SM22-Cre;LRP6(fl/fl);LDLR $−/−$ VSM is reversed by ML141. $P<0.05$ vs vehicle SM22-Cre;LRP6(fl/fl);LDLR $−/−$ VSM. N=3 per group. ANOVA $P=0.011$ with post hoc Holm–Sidak’s testing. G, Working model. See Online Figure XXX for details. LDLR indicates low-density lipoprotein receptor; LRP6(fl/fl), LRP6 gene floxed; LUC, luciferase reporter; NFAT, nuclear factor of activated T cells–luciferase reporter; PRMT, protein arginine methyltransferase; and TNAP, tissue nonspecific alkaline phosphatase.
ML141 Inhibits USF1 Protein Accumulation, Osteochondrogenic Gene Expression, TNAP Upregulation, and Calcification in VSM Cultures From SM22-Cre;LRP6(fl/fl);LDLR−/− Mice

ML141 treatment downregulated a subset of nuclear ADMA proteins upregulated with LRP6 deficiency (Online Figure XXVI)—and reversed the nuclear USF1 protein accumulation and OPN chromatin association arising in SM22-Cre;LRP6(fl/fl);LDLR−/− VSM (Figure 8A; Online Figure XXVII and XXVIII). Because ML141 inhibited Fzd10 activation of noncanonical Wnt signaling and USF1-dependent OPN expression (vide supra), we assessed the impact of ML141 on osteochondrogenic programs upregulated by LRP6 deficiency. Like OPN, TNAP and Col10A1 were reduced by ML141 (Figure 8B and 8C). Interestingly, Wnt ligand genes including Wnt4, Wnt5a, and Wnt5b were concomitantly downregulated by ML141 in LRP6-deficient VSM, whereas Jmjd6 was unaffected (Online Figure XXIX). TNAP enzyme activity—necessary for OPN dephosphorylation and osteochondrogenic mineralization—was upregulated in SM22-Cre;LRP6(fl/fl);LDLR−/− VSM (Figure 8A; Online Figure XXVII and XXVIII). Because ML141 inhibited Fzd10 activation of noncanonical Wnt signaling and USF1-dependent OPN expression (vide supra), we assessed the impact of ML141 on osteochondrogenic programs upregulated by LRP6 deficiency. Like OPN, TNAP and Col10A1 were reduced by ML141 (Figure 8B and 8C). Interestingly, Wnt ligand genes including Wnt4, Wnt5a, and Wnt5b were concomitantly downregulated by ML141 in LRP6-deficient VSM, whereas Jmjd6 was unaffected (Online Figure XXIX). TNAP enzyme activity—necessary for OPN dephosphorylation and osteochondrogenic mineralization—was upregulated in SM22-Cre;LRP6(fl/fl);LDLR−/− VSM and inhibited by ML141 treatment (Figure 8D). Consistent with this, calcium deposition quantified by Alizarin red staining was significantly increased in SM22-Cre;LRP6(fl/fl);LDLR−/− VSM and inhibited by ML141 treatment (Figure 8E and 8F). Thus, LRP6 restrains vascular noncanonical Wnt signals sensitive to ML141 that promote osteochondrogenic responses, mediated in part via USF1- and arginine methylation–dependent relays (Figure 8G).

Discussion

There are 3 principal findings of this study that help advance our understanding of arteriosclerotic calcification. First, LRP6 signaling plays a cell-autonomous role in regulating the osteochondrogenic response within the VSM lineage. The VSM functions of LRP6 in arteriosclerotic calcification have not been previously characterized. Surprisingly, in addition to conveying canonical signals, LRP6 restrains noncanonical signals that reinforce osteochondrogenic transdifferentiation and mineralization of VSM.41 Based on lineage tracing, Speer estimated that ≈80% of mineralizing cells in the vessel wall arise from this process; the remaining 20% reflect lineage allocation of regional or circulating osteoprogenitors.89 Elegant studies by Mani first identified the private mutation LRP6(R611C) as causing a precocious atherosclerosis–osteoporosis syndrome,18,22 and hypomorphic function may also extend to reduced inhibition of noncanonical signals.18 Second, USF1 emerges as a novel mediator of noncanonical Wnt signaling alongside the Jun/ATF and Cae4+/NFAT pathways. The role of USF1 as relevant to the β-globin locus control region,50 glucose signaling,41 and lipid homeostasis81 is well appreciated, and the genetic link between USF1 and combined hyperlipidemia has been established in parallel with the identification of USF1 within atherosclerotic plaques.51 As in bone and cartilage,29,56 cdc42-modulated NFAT signaling will also be an important component of the VSM osteochondrogenic response during arteriosclerotic calcification along with Runt-related transcription factor 2 and USF1. Given that ML141 inhibits VSM biomineralization—and cdc42’s role in vascular inflammation52 and endochondral ossification51—we speculate that modulators of the cdc42/Rac1 family may prove useful in treating arteriosclerosis. Third, discovery that LRP6 functionally and physically interacts with the protein arginine methylation cascade reveals a new dimension in LRP biology. Our data indicate that non-canonical PRMT1 associated with LRP6 is part of a pathway suppressing noncanonical signals, whereas nuclear PRMT4 supports the osteochondrogenic phenotype in collaboration with osteogenic transcription factors.29 Others have noted that nuclear PRMT4 (coactivator-associated arginine methyltransferase 1) forms a complex with Jmjd641. Whether nuclear versus non-nuclear pools of PRMT1 differentially impact LRP6 signaling remains to be studied.

PRMT4 plays an important role in endochondral bone formation82 and regulation of estrogen receptor methylation41 in concert with Jmjd6. Because USF1 lacks overt ADMA modification, we anticipate that proteinaceous partners of USF1 will be directed for ADMA modification. Our data indicate that histone H3—bearing the H3R17Me2a signature of PRMT4—is one component of the protein complexes associated with USF1. It remains to be determined whether affinity of USF1 for chromatin is enhanced by H3R17Me2a. USF1 dimerizes with other basic helix-loop-helix and leucine zipper transcription factors to create unique DNA binding specificities.38 Furthermore, USF1 recruits nuclear PRMT1 to the β-globin locus control region to preserve euchromatin structure via histone H4 asymmetrically dimethylated on Arg-3 formation.50 It will be important to elucidate the USF1 interactome and its regulation by noncanonical signals.

Fzd9 and Fzd10 are closely related family members that convey noncanonical Wnt signals.31,35 Upregulation of noncanonical Fzd10 signaling participates in the pathobiology of synovial sarcoma.35 Conversely, Fzd9-null mice exhibit reduced endochondral bone formation during fracture repair, arising from deficiency in noncanonical signals necessary for osteoblast maturation and mineralization.31 It remains to be determined the relative extent to which canonical versus noncanonical Wnt signals contribute to arterial calcification in the LDLR−/− model. As noted, osteogenic lineage allocation is promoted by Msx and canonical Wnt signals52—but these signals need to be downregulated to permit osteoblast maturation.59 This sequence is emerging as important for the osteogenic programming of vascular progenitors.9,49 VSM transdifferentiation is responsible for a majority of the vascular osteochondrogenic cell load.49 We propose that upregulation of noncanonical Fzd activity with LRP6 deficiency enhances VSM osteochondrogenic transdifferentiation in response to metabolic stress.

Our study has limitations. The composition of the LRP6 complex that negatively regulates noncanonical Fzd relays remains to be determined. It is interesting to note that PRMT1 modifies Ras-GTPase activating protein SH3 domain binding protein family members.56 Because Ras-GTPase activating protein SH3 domain binding protein 1 localizes
GTGase activating complexes, homologs might be involved in negative regulation of cdc42. However, VSM LRP6 likely orchestrates protein–protein interactions between multiple regulatory components of the noncanonical pathway. Furthermore, LRP6 forms heterodimers with LRP5. Given that LRP5 activity drives proscerolic canonical Wnt signaling in valve calcification, it remains probable that heterodimeric interactions between specific LRPs and Fzds finely tune signaling via the canonical and noncanonical pathways. LRPs/6 heterodimers confer selectivity for Wnt ligand activation; this combinatorial complexity indicates that changes in levels of a specific LRP receptor will impact canonical vs. noncanonical Wnt signaling as a function of the prevailing Wnt ligand milieu while directly modulating noncanonical tone. Even though we targeted LRP6 expression in the VSM lineage, secondary alterations in the monococyte/macrophage lineage may contribute to arteriosclerosis. Innovative studies published recently have identified that a subset of inflammatory macrophages arise from the VSM lineage (reviewed in Fisher and Miano and Swirski and Nahrendorf). Future studies will assess whether VSM LRP6 orchestrates phenotypic modulation and myeloid cell differentiation, recruitment, and function in vascular lesions in response to metabolic stressors. As in bone stage, specific roles for canonical and noncanonical Wnts are emerging in the regulatory sequence that drives arterial osteochondrogenic programming. The evolving models of LRP-dependent vascular disease have yet to fully address this sequence. In vivo quantitative measures of ligand expression and signal activation are needed to temporally and spatially resolve the contributions of specific Wnt ligand–receptor engagement to disease biology. Finally, Runt-related transcription factor 2—the master osteochondrogenic transcriptional regulator—is post-transcriptionally activated in VSM. How USF1 supports programs enabled by Runt-related transcription factor 2 remains to be determined. USF1 increases with osteoblast differentiation, and USF mechanisms encompass the maintenance of chromatin structure necessary for tissue-specific enhancer function. Nevertheless, the discovery that ML141 downregulates noncanonical sclerotic programs restrained by LRP6 signaling indicates that strategies targeting the cdc42-related GTPases can function as LRP6 mimetics—and might mitigate vascular disease in patients afflicted with diabetes mellitus and dyslipidemia.

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D.A. Towler consulted for Daiichi-Sankyo. B.O. Williams consulted for Amgen, and received grant support from Genentech. The other authors report no conflicts.

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What Is Known?

- Paracrine Wnt signaling controls bone mineralization, mediated in part by low-density lipoprotein receptor–related protein (LRP) family heterodimers with frizzled (Fzd) coreceptors.
- Rare hypomorphic mutations in human LRP6 canonical signaling proteins cause autosomal dominant osteoporosis and early cardiometabolic disease.
- Multiple Wnt ligands are expressed in vessels undergoing calcification, suggesting that Wnt signaling plays an important role in arteriosclerosis.

What New Information Does This Article Contribute?

- Deletion of vascular smooth muscle lineage (VSM) LRP6 worsens arteriosclerotic disease in a model of diet-induced insulin-resistant diabetes mellitus, dyslipidemia, and cardiovascular calcification.
- Loss of VSM LRP6 enhances Wnt ligand expression and osteochondrogenic signaling via noncanonical Fzd coreceptors, mediated in part by upstream stimulatory factor 1 and protein arginine methyltransferase relays.
- Like LRP6, the cdc42/rac1 G-protein antagonist ML141 inhibits noncanonical Wnt signals that drive VSM mineralization, indicating that certain LRP6 mimetics may prove useful in the treatment of arteriosclerotic calcification.

In this study, we demonstrate that the Wnt coreceptor LRP6 plays a rate-limiting role in restraining VSM noncanonical Wnt signals that drive arteriosclerotic calcification and vascular stiffening with insulin-resistant hyperglycemia and hyperlipidemia. Vascular Wnt ligands, Fzd10-dependent noncanonical signals, and osteochondrogenic mineralization programs are upregulated with reduction in VSM LRP6. We show that protein arginine methyltransferases and the Jumonji domain containing 6 arginine demethylase are novel components of LRP6-regulated relays that control VSM drift to the osteochondrogenic phenotype. The transcription factor upstream stimulatory factor 1 is identified as a new component of the VSM noncanonical response, regulated at the level of nuclear chromatin complex formation by LRP6, ML141, and Jumonji domain containing 6 arginine. These results reveal novel dimensions of LRP6 vascular biology that provide insights useful for crafting new approaches to treat arteriosclerotic disease.
Vascular Smooth Muscle LRP6 Limits Arteriosclerotic Calcification in Diabetic LDLR<sup>−/−</sup> Mice by Restraining Noncanonical Wnt Signals
Su-Li Cheng, Bindu Ramachandran, Abraham Behrmann, Jian-Su Shao, Megan Mead, Carolyn Smith, Karen Krchma, Yoanna Bello Arredondo, Attila Kovacs, Kapil Kapoor, Laurence M. Brill, Ranjan Perera, Bart O. Williams and Dwight A. Towler

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Supplement Figure I: Aortic sinus VSM expression of LRP6 is reduced in SM22-Cre;LRP6(fl/fl);LDLR-/- mice. Indirect immunofluorescence staining for LRP6 reveals protein accumulation in the aortic VSM layers of LRP6(fl/fl);LDLR-/- mice. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole). Scale bar = 100 microns. While certainly still detectable, LRP staining intensity is reduced in SM22-Cre;LRP6(fl/fl);LDLR-/- mice. Western blot analysis of cultured VSM from SM22-Cre;LRP6(fl/fl);LDLR-/- mice established reduced LRP6 protein accumulation (see text).
**Supplement Figure II: Arterial VSM expression of LRP6.** Indirect immunofluorescence staining for LRP6 reveals protein accumulation in the VSM layers of the aortic wall and the coronary artery of LDLR/- mice. Scale bar = 200 microns. Nuclei were counterstained with DAPI (4’,6-diamidino-2-phenylindole).
Supplemental Material:

"Vascular Smooth Muscle LRP6 Limits Arteriosclerotic Calcification in Diabetic LDLR-/- Mice By Restraining Noncanonical Wnt Signals"

Supplement Figure III: Medial and atherosclerotic calcification in conduit arteries of LRP6(fl/fl);LDLR-/- and SM22-Cre;LRP6(fl/fl);LDLR-/- mice. As previously detailed, the earliest Alizarin red S –stained calcification in male LDLR-/- mice fed HFD occurs as a patchy involvement of the tunica media, seen in the coronary arteries (upper panel) as well as in the aortic media. Lower panel, with more extensive dietary challenge, atherosclerotic lesions in the aortic sinus and the thoracic aorta (lower panel, white arrows) become calcified in addition to the arterial media (black arrow). Scale bar = 50 microns.
Supplement Figure IV: Significant differences in aortic osteochondrogenic gene programs require HFD challenge. While non-significant trends for increased Col10A1, KLF5, OCN and MMP13 are observed in SM22-Cre;LRP6(fl/fl);LDLR-/- animals on rodent chow diets, robust differences require HFD challenge (see Figure 1). N = 3 to 4 per group as indicated in the legend. Statistical testing was performed using the Student’s 2-tailed t-test for each gene.
**Supplemental Material: "Vascular Smooth Muscle LRP6 Limits Arteriosclerotic Calcification in Diabetic LDLR-/ Mice By Restraining Noncanonical Wnt Signals"**

**Supplement Figure V:** Average ascending aortic wall thickness did not significantly differ LRP6(fl/fl);LDLR-/- and SM22-Cre;LRP6(fl/fl);LDLR-/- mice on HFD. Male animal cohorts were fed high fat diabetogenic diets for 3 months, and 5 micron paraffin sections of ascending aorta encompassing the aortic sinus to the region just proximal to the innominate were stained with picrosirius red. The average aortic wall thickness was measured in n = 7 animals of each genotype, including and excluding atheroma as indicated. No differences in average wall thickness were observed (Student’s 2 tailed t-test, n = 7 per group as indicated). Scale bar = 200 microns. Bottom panel, aortic PWV, another index of arterial stiffness, was increased in SM22-Cre;LRP6(fl/fl);LDLR-/- on HFD. N = 6 per group as indicated in the X-axis labels. Significance was assessed by Student’s 2-tail t-test.
**Figure VI:** Aorta and aortic valve indices of cellular proliferation were not significantly increased in SM22-Cre;LRP6(fl/fl);LDLR-/- mice fed HFD. Upper panel, RT-qPCR for aortic *cyclin D1*, one marker of VSM cellular proliferation\(^1\). N = 4 to 7 per group as indicated in the X-axis labels. Lower panel, microdissection of aortic valve leaflets and RT-qPCR analysis also revealed no increases in *PCNA* or *cyclin D1*, even though *LRP6* mRNA was reduced and *Wnt7b* increased by SM22-Cre mediated targeting (see below). N=4 per group as indicated in the legends. *, p < 0.05, **, p < 0.01 by Student's t-test.
Figure VII: Aortic sinus BrdU incorporation did not significantly differ between LRP6(fl/fl);LDLR-/- and SM22-Cre:LRP6(fl/fl);LDLR-/- mice fed HFD. Male cohorts of the indicated genotype were challenged with HFD for 2 months then injected intraperitoneally with 100 ul of 10 mg/ml bromodeoxyuridine. The next day animals were sacrificed and paraffin-embedded sections prepared of aorta and intestine (positive labeling control) for immunolocalization of BrdU using the BrdU In-Situ Detection Kit (BD Pharmingen). Data are presented as the percentage of aortic media, aortic valve, and aortic sinus atheroma nuclei incorporating BrdU. N=3 to 4 per genotype as indicated in the X-axis labels. No significant difference is detected by the Mann-Whitney U-test.
Supplement Figure VIII: Primary cultures of VSM prepared from SM22-Cre;LRP6(fl/fl);LDLR−/− mice exhibit a small but significant increase in basal bromodeoxyuridine (BrdU) incorporation, an index of DNA synthesis\(^1\). *** p < 0.001 vs. Cre-negative control by Student’s 2-tail t-test. N = 24 per genotype as indicated in the X-axis labels.
Supplement Figure XIX: Average aortic arch lumen diameter as assessed by transthoracic echocardiography did not differ between LRP6(fl/fl);LDLR-/- and SM22-Cre;LRP6(fl/fl);LDLR-/- mice. N=6 per genotype as indicated in the legend. No significant differences by Student’s 2-tail t-test between Cre-minus and Cre-plus groups.
Supplemental Material:
“Vascular Smooth Muscle LRP6 Limits Arteriosclerotic Calcification in Diabetic LDLR/- Mice By Restraining Noncanonical Wnt Signals”

**Mac2 Staining**

*SM22-Cre;LRP6(fl/fl);LDLR-/*

Supplement Figure X: A non-significant trend for increased aortic sinus Mac2- positive macrophage area is observed in *SM22-Cre;LRP6(fl/fl);LDLR-/-* mice. Male animal cohorts were fed high fat diabetogenic diets for 3 months, and 5 micron frozen sections of ascending aorta encompassing the aortic sinus were stained with the macrophage marker Mac2$^{3,4}$, and counterstained for nuclei with DAPI. The average aortic sinus atheroma area was measured in n =3 animals of each genotype as indicated in the X-axis labels. Non-significant trend of p = 0.1 using Student’s 2-tail t-test. Scale bar = 100 microns.
Supplement Figure XI: Messenger RNAs encoding macrophage markers F4/80 and IL12A are increased in aortas of SM22-Cre;LRP6(fl/fl);LDLR-/- mice fed HFD. Increases in aortic sinus Mac2 staining (Figure S10) were paralleled by increased thoracic aorta F4/80 and IL12A mRNA accumulation in SM22-Cre;LRP6(fl/fl);LDLR-/- the western diet for 3-4 months. No differences were observed in IL6 mRNA levels *, p ≤ 0.05 vs. LRP6(fl/fl);LDLR-/- control; **, p ≤ 0.01 vs. LRP6(fl/fl);LDLR-/- control in Student’s 2 -tail t-test. N = 4 per genotype.
**Supplemental Material:**

“Vascular Smooth Muscle LRP6 Limits Arteriosclerotic Calcification in Diabetic LDLR-/ Mice By Restraining Noncanonical Wnt Signals”

**Supplement Figure XII:** Fasting plasma glucose, triglycerides and cholesterol did not differ between LRP6(fl/fl);LDLR-/ and SM22-Cre;LRP6(fl/fl);LDLR-/ mice following 3 months of HFD feeding. Upper left panel, insulin resistance as quantified by validated HOMA-IR methods\(^7,8\) was induced by HFD feeding and did not differ between genotypes. N = 7 to 13 per genotype indicated (viz., n = 7 for chow for each genotype; n = 12 for Cre-minus HFD, n = 13 for Cre-plus HFD). One way ANOVA p < 0.001; significant differences between the groups as indicated by Holm-Sidak post-hoc test adjusting for multiple comparisons. a, p < 0.05 vs. chow fed of either genotype; b, p < 0.05 vs. chow fed Cre-negative animals; c, p < 0.01 between chow fed SM22-Cre;LRP6(fl/fl);LDLR-/ animals. No other differences were significant. Upper right, HFD-induced weight gain did not differ between genotypes. N = 4 to 10 per group as indicated in the X-axis labels, and data were derived from animals just prior to echocardiographic analysis. ANOVA p < 0.0001. *** p < 0.001 vs. rodent chow diet controls by Holm-Sidak post-hoc testing. No other comparisons were significantly different. Lower panel, n = 7/genotype; no difference by Student’s 2-tail t test between genotypes for each metabolic parameter. Dashed lines indicate fasting basal values in chow-fed floxed / male LDLR-/ mice for glucose (289 +/- 25 mg/dL; n = 10), triglycerides (89 +/- 7 mg/dL; n = 10) and cholesterol (179 +/- 17 mg/dL; n = 10). Fasting blood glucose levels, triglyceride levels, and cholesterol levels do not significantly differ between 2-3 month old chow fed LRP6(fl/fl);LDLR-/ and SM22-Cre;LRP6(fl/fl);LDLR-/ male cohorts (Student’s 2-tail t test). Diet-induced obesity and insulin-resistant diabetes has been previous established in the male LDLR receptor-deficient mouse.\(^9,10\) However, note that necropsy following
immediate exsanguination via cardiac puncture under acute anesthesia gives rise to somewhat higher fasting glucose levels (above, and our ref. 10) vs. those reported by LeBoeuf9.

Supplement Figure XIII: Body composition as assessed by dual electron X-ray absorptiometry did not differ between LRP6(fl/fl);LDLR/- and SM22-Cre;LRP6(fl/fl);LDLR/- mice following 3 months of HFD feeding. Differences between genotypes were not significant as assessed by Student’s 2-tail t-test. BMD, areal bone mineral density. BMC, bone mineral content. N = 10 to 11 animals per group as indicated in the legend. Note that diet-induced obesity, increased body fat, and diabetes has been previous established in the male LDLR receptor-deficient mouse9,10.
Supplement Figure XIV: Gene array / heat map analysis indicates increased expression of multiple Wnt genes and Fzd10 in aortic RNA from SM22-Cre;LRP6(fl/fl);LDLR-/ mice as compared to LRP6(fl/fl);LDLR-/ controls. Mice of the indicated genotype (n = 5/group) were challenged with HFD for 3 months and total aortic RNA isolated and analyzed as described in Materials and Methods.
Supplement Figure XV: HFD challenge is necessary to elicit the marked induction of Wnt3a, Wnt4, Wnt7b, Wnt10a, and Fzd10 in SM22-Cre;LRP6(fl/fl);LDLR-/- aortas. N = 3 to 7 animals per group as indicated in the legend. Statistical testing was performed using the Student’s 2-tail t-test for the indicated genes, comparing Cre-minus vs. Cre-plus cohorts.
**Supplemental Material:**

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**Relative Fzd Expression (% 18S)**

![Bar graph showing relative Fzd expression.](image)

**Supplement Figure XVI:** Fzd10 but not Fzd9 gene expression in upregulated in primary aortic VSM from SM22-Cre;LRP6(fl/fl);LDLR-/ mice as compared to LRP6(fl/fl);LDLR-/ controls. Total RNA from primary VSM cultures was analyzed for Fzd10 and Fzd9 by RT-qPCR as described in Methods (n = 3 independent replicates). ***, p < 0.001** vs. LRP6(fl/fl);LDLR-/ control in Student’s 2-tail t-test for the indicated gene.
Supplement Figure XVII: Fzd10 but not Fzd7 upregulates osteopontin (OPN) promoter activity (firefly luciferase reporter) in transient transfection assays. Fzd10, Fzd7, OPN-LUC activity measure when co-transfected with pCMV-Fzd10 or pCMV-Fzd7 expression vectors in HEK cells (n = 3 independent replicates). RLU, relative light units, TK, thymidine kinase promoter (Renilla reporter) used to control for transfection efficiency. pCMV, empty expression vector. ANOVA p < 0.0001, *** p < 0.001 vs. pCMV vector control and Fzd7 in Holm-Sidak post-hoc testing.
Supplement Figure XVIII: Wnt7b and Wnt5a, but not Wnt11 or Wnt4, augment Fzd10 activation of the osteopontin promoter. pCMV expression vectors for Fzd10 and the indicated Wnts were transiently co-transfected with the 135 OPNLUC (osteopontin promoter luciferase reporter) construct as indicated in HEK cells (n= 3 independent replicates for Fzd10 combinations with Wnt ligands, n = 6 independent replicates for pCMV and Fzd10). ANOVA p < 0.0001. Note that while Wnt7b and Wnt5a increase Fzd10 activation of OPNLUC, Wnt11 has little effect and Wnt4 inhibits Fzd10 activity in this assay. *, p < 0.05 vs. Fzd10 in post-hoc testing corrected for multiple comparisons. OPNLUC activity with Fzd10+Wnt4 was not different from the pCMV control.
Supplement Figure XIX: **USF1** and **USF2** are successfully knockdown by siRNA in transfected primary cultures of aortic VSM. RT-qPCR analysis, n = 4 independent transfections per siRNA per genotype. ANOVA p < 0.0001, performed individually for USF1 and USF2 message levels. ***, p < 0.01** by post-hoc testing corrected for multiple comparisons vs. corresponding control siRNA. **, p < 0.01** vs. corresponding control siRNA; ****, p < 0.001 vs. corresponding control siRNA.
**Supplement Figure XX:** The cdc42 antagonist ML141 inhibits osteopontin (OPN) promoter – luciferase reporter activation by Fzd10. OPN-LUC activity was measured following treatment of HEK cells co-transfected with pCMV-Fzd10 or pCMV vectors (n = 3 independent replicates) with either DMSO vehicle or the cdc42 antagonist ML141 as indicated. RLU, relative light units, TK, thymidine kinase promoter (Renilla reporter) used to control for transfection efficiency. pCMV, empty expression vector. ANOVA \( p < 0.0001 \) ***; \( p < 0.001 \) vs. pCMV vector all ML141 treated samples by Holm-Sidak post-hoc testing.
Supplement Figure XXI: The constitutively active Cdc42 variant Cdc42(Q61L) activates the proximal osteopontin (OPN) promoter. Note that co-transfection of constitutively active Cdc42(Q61L) alone maximally activates proximal OPN promoter activity in HEK293T cells. ANOVA p < 0.0001, with p < 0.001 significant increase in OPNLUC activity with all conditions co-expressing Cdc42(Q61L) in post-hoc testing corrected for multiple comparisons. N = 3 per group.
Supplement Figure XXII: PRMT1 exists in nuclear and non-nuclear compartments in primary cultures of aortic VSM. Indirect immunofluorescence staining for PRMT1 reveals protein accumulation both nuclear and non-nuclear compartments of aortic VSM. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole).
Supplement Figure XXIII: Specificity of USF1 and Jmjd6 siRNAs for reductions in USF1 and Jmjd6 proteins as assessed by western blot. Data presented are western blots from 4 independent VSM transfections per siRNA as indicated. Note that Jmjd6 siRNA did not alter USF1 protein levels. In primary VSM cultures, we consistently visualized immunoreactive Jmjd6 as a doublet of Mr (apparent) at 50 kDa and 56 kDa.
Supplement Figure XXIV: PADI4 does not inhibit USF1 activation of the osteopontin (OPN) promoter in transient transfection assays. USF1 and PADI4 expression vectors were co-transfected with OPNLUC (OPN promoter-luciferase reporter) in HEK cells (n = 4 independent replicates). TK Renilla was included to control for transfection efficiency. ANOVA p < 0.0001. Of note, PADI4 did not inhibit but significantly increased USF1 activation of OPNLUC (p < 0.01 in Holm-Sidak’s multiple comparisons post-hoc testing). *** p < 0.001 vs. pCMV vector control in post-hoc testing.
Supplement Figure XXV: USF1 is not ADMA modified, but associates with a 17 kDa ADMA modified protein in HEK cells. FLAG-tagged USF1 was transiently transfected into HEK cells, immunoprecipitated as indicated, and probed by western blot for either the FLAG epitope or ADMA as indicated. While no ADMA modification of USF1 was observed, a 17kDa protein co-precipitated with FLAG-tagged USF1.
Supplement Figure XXVI: The cdc42 inhibitor ML141 mitigates the upregulation of ADMA protein accumulation in response to VSM LRP6 deficiency. Primary VSM cultures were treated with 10 uM ML141 as indicated for 20 hours and nuclear extracts analyzed for ADMA protein modification by western blot. While a number of ADMA modified proteins were decreased by ML141, the global pattern observed with LRP6 deficiency was not perturbed. Data shown are from three independent cell cultures, and have been replicated twice. ML141 had no impact upon non-nuclear ADMA protein profiles (data not shown). ADMA, asymmetric dimethylarginine.
Supplement Figure XXVII: The cdc42 inhibitor ML14 inhibits the upregulation of nuclear USF1 protein in LRP6-deficient cultured VSM. Primary VSM cultures were treated with 10 uM ML141 as indicated for 20 hours and nuclear extracts analyzed for USF1 and histone H3 as indicated by western blot.
Supplement Figure XXVIII: The cdc42 inhibitor ML14 inhibits the upregulation of USF1 association with OPN chromatin LRP6-deficient cultured VSM. Primary VSM cultures were treated with 10 μM ML141 as indicated for 20 hours and chromatin precipitation (ChIP) assays performed to quantify USF1 association with the OPN promoter. N = 4 per group. ANOVA p = 0.0006. *, p < 0.05 vs. vehicle treated Cre-negative control cells; **, p < 0.01 vs. vehicle-treated Cre-positive cells in post-hoc testing corrected for multiple comparisons.
Supplement Figure XXIX: ML141 treatment significantly down-regulates canonical and non-canonical Wnt ligand expression in SM22-Cre;LRP6(fl/fl);LDLR-/- VSM without altering Jmjd6. n = 4 per treatment and genotype *, p < 0.05; **, p < 0.01; and ***, p < 0.001 vs. vehicle control by Student’s 2-tail t-test.
**Supplement Figure XXX**: Working model for VSM LRP6 actions in arteriosclerotic calcification via non-canonical and canonical Wnt signaling. As a co-receptor for canonical Fzd receptors, LRP6 supports osteogenic programming of mesenchymal progenitors such as bone marrow stromal cells, C3H10T1/2 cells and myofibroblasts. However, LRP6 also restrains non-canonical Fzd signaling cascades that contribute to the osteogenic and osteochondrogenic differentiation of VSM. As such, LRP6 regulation may play uniquely important roles during the initiation and progression phases of arteriosclerotic disease. Strategies targeting the advancing osteochondrogenic programs driven by non-canonical Wnt signaling hold promise for greatest impact on arteriosclerotic disease progression once initiated; this notion has yet to be tested. USF1 and protein arginine methylation regulation emerge as new components of the non-canonical Wnt signaling cascade controlling osteopontin (OPN) expression and other features of the osteochondrogenic phenotype. The interactions between USF1 and the other transcriptional regulators (Runx2, Sox9, Msx, Smads, Slug, NFAT, Osx, FOXO1) vital to the initiation and progression of osteochondrogenic vascular mineralization have yet to be established. See text for details.
SUPPLEMENTAL MATERIAL:
“Vascular Smooth Muscle LRP6 Limits Arteriosclerotic Calcification in Diabetic LDLR-/ Mice By Restraining Noncanonical Wnt Signals “

CITATIONS FOR SUPPLEMENTAL DATA


SUPPLEMENTAL METHODS:
“Vascular Smooth Muscle LRP6 Limits Arteriosclerotic Calcification in Diabetic LDLR-/- Mice By Restraining Noncanonical Wnt Signals”

METHODS SUPPLEMENT

Generation and biochemical characterization of SM22-Cre;LRP6(fl/fl);LDLR<sup>−/−</sup> mice
All procedures for handling mice were approved by either the Washington University or Sanford-Burnham Institutional Animal Care and Use Committees. LDLR<sup>−/−</sup>B6.129S7-Ldlr<sup>Tm1Her/J</sup> and SM22-CreTg(Tagln-cre)<sup>1Her/J</sup> mice were obtained from The Jackson Laboratory. LRP6(fl/fl) mice were generated as detailed<sup>3</sup> and were bred onto the LDLR<sup>−/−</sup> background. All experimental animals (SM22-Cre;LRP6(fl/fl);LDLR<sup>−/−</sup> and LRP6(fl/fl);LDLR<sup>−/−</sup> controls) were siblings bred onto the C57Bl/6 background. At 5–8 weeks of age, animals were weighed, and male sibling cohorts of LRP6(fl/fl);LDLR<sup>−/−</sup> and SM22-Cre;LRP6(fl/fl);LDLR<sup>−/−</sup> mice with equivalent starting weights were challenged with high-fat Western diet (HFD) (TD88137; Teklad Lab Animal Diets; Harlan) for 2-3 months. As previously detailed<sup>4</sup>, high fat diet feed in the male LDLR<sup>−/−</sup> mouse induces insulin-resistant diabetes<sup>4-8</sup>. Comparison was made to male mice of the same genotypes fed chow diet (Teklad Rodent Diet 2016). At the end of the dietary challenge, thoracic aortas were harvested, weighed, and extracted for calcium, collagen, or total RNA, implementing methods we’ve previously detailed<sup>5, 6, 9</sup> and outlined below. Arterial blood (obtained by cardiac puncture) was biochemically assessed precisely as we’ve previously described<sup>5-7, 10, 11</sup> for fasting cholesterol (Thermo Scientific #2350-400H), glucose (Thermo Scientific #1524-400H), triglycerides (Sigma #TR0100), and insulin (Crystal Chem #90080) were performed using commercially available assay kits, following the manufacturers’ instructions. For assessment of aortic BrdU incorporation<sup>12</sup> cohorts of mice as indicated were injected intraperitoneally with 1 mg of BrdU<sup>12</sup>, sacrificed 24 hours later, and formalin-fixed paraffin-embedded sections of aorta and intestine (positive labeling control) stained for incorporation using the BrdU In situ Detection System (BD Pharmingen, #550803) following the manufacturer’s instructions essentially as described<sup>12</sup>. Insulin resistance was determined using the HOMA-IR index as has been previously validated by Wasserman and colleagues in four murine genetic backgrounds including C57Bl/6<sup>13</sup>.

Assessment of aortic stiffness by aortic pulse wave velocity
For echocardiography studies, cohorts of male LRP6(fl/fl);LDLR<sup>−/−</sup> and SM22-Cre;LRP6(fl/fl);LDLR<sup>−/−</sup> mice (n=6, each group) were challenged with HFD for 3 months. Aortic arch pulse wave velocity was then determined using a modification of the foot-to-foot transit time method of Hartley et al.<sup>14</sup> as we’ve previously detailed<sup>5, 11</sup>. Briefly, non-invasive ultrasound examination of the vascular system was performed using a Vevo 2100 Ultrasound System (VisualSonics Inc, Toronto, Ontario, Canada). Mice were anesthetized with continuous inhalation of 1.5% gaseous Isoflurane administered through customized nose cone, secured on an imaging platform in supine position while monitoring physiologic parameters including heart rate, respiratory rate, and core body temperature. Ultrasound studies were performed using a 30 MHz transducer. The ascending aorta, the aortic arch and the proximal portion of the descending aorta were
imaged in one 2-D imaging plane from the right superior parasternal view. The pulse wave Doppler sample volume was placed first near the aortic valve to record blood flow velocity in the proximal aorta, then promptly it was moved to the visualized portion of the descending aorta without changing the imaging plane in order to record blood flow velocity in the descending aorta. The curvilinear distance (D) between the proximal and distal points of the aortic velocity interrogation were measured using the exact coordinates of the Doppler sample volumes. The time delay (T2-T1) between the onset of flow velocity in the distal (T2) and proximal portions (T1) of the aorta was measured relative to the simultaneously recorded ECG signal. Pulse wave velocity was calculated by the formula 

$$ \text{Pulse wave velocity} = \frac{D}{T2 - T1} $$

### Aortic calcium measurement

Aortic calcium content was measured essentially as previously detailed\(^5\)\(^,\)\(^11\). Briefly, dissected aortae were rinsed in water and pressed against Kimwipes 5 - 7 times with wetting in between to remove as much blood as possible. After measuring the wet weights, each aorta was cut into 3-4 pieces and transferred to a 1.7 ml microfuge tube. The tubes were incubated at 70°C for 30 min with the lid open followed by additional drying in a Speed Vac at 70°C for 3 h. The dry weight of each aorta was measured. Each dried aorta pellet was extracted with 100 µl of 10% formic acid in a 37°C water bath for 20 h. Two control tubes containing 100 µl of 10% formic acid each were also included as Blanks. After acid extraction, the tubes were spun twice for 5 min each in a microfuge. Supernatants were collected and frozen at -20°C until assay. On the day of analysis, the samples were thawed and spun for 5 min again at 14,000 rpm. Supernatants were analyzed for calcium concentration according to the protocol provided by manufacturer (Calcium Reagent Set, Pointe Scientific, Inc) and normalized to aorta dry weight.

### Aorta collagen measurement

Aortic calcium content was measured essentially as previously detailed\(^6\). Briefly, the aorta pellets obtained following formic acid extraction described above for calcium measurement were digested with sterile-filtered pepsin (10 mg/ml in 0.5 M acetic acid) at room temperature for 5 days on a Nutator. Samples were microcentrifuged at top speed for 10 min. Supernatants were used for collagen analysis as described previously\(^6\) using the Sirius red dye binding assay (Sircol Soluble Collagen Assay, Biocolor Life Sciences, US supplier Accurate Chemical and Scientific, Westbury, NY, cat # CLR S1000). The collagen content in each aorta was normalized to aorta dry weight.

### Isolation of aortic primary cells

Aortic primary cells were isolated from Tgln-Cre;LRP6(f/f); LDLR-/- and LRP6(f/f);LDLR-/- mice as previously described\(^6\). Briefly, aorta was dissected from diaphragm to heart and subjected to twice 1 h digestion with type 1 collagenase (Worthington Biochemical Corporation, cat. # LS004149, 1 mg/ml), DNase I (Sigma-Aldrich, D5025, 60 U/ml), and hyaluronidase (Sigma-Aldrich, H3506, 0.5 mg/ml) in DMEM with 2X P/S and fungizone
SUPPLEMENTAL METHODS:
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(2.5 µg/ml). Cells released from these two digests were combined (Fraction 1). The remainder of the aorta was further digested with the same enzymes plus elastase (Worthington Biochemical Corporation, cat. # LS002279, 0.8 mg/ml) for another hour (Fraction 2). Expression of basal and the Cre-mitigated endogenous LRP6 mRNA levels were virtually identical in adherent cell cultures arising from two timed digests although the SM22-Cre transgene was expressed about 2.5 – 3 fold greater in the latter. For experiments, pools of cells isolated from 8 - 10 male animals per genotype were expanded with passage on collagen in phenol-red free media as described previously and then used between passages 14-20 for biochemical and molecular studies performed in parallel, ensuring that expression of SM22, LRP6, OPN, Wnt4 and Wnt7b genes phenocopied adherent passage 1 cultures. Results were confirmed in at least two independent primary culture preparations (range 2 – 6), with 3 to 4 replicates per condition as indicated.

Alizarin red S assessment of aortic primary cell culture and tissue calcification.
Aortic primary VSM cells were cultured on a type I collagen-coated 12 well plates (100,000 cells/well, 3 wells/genotype). One to two days after seeding, cells were treated with β-glycerophosphate (5 mM) and ascorbic acid (50 µg/ml) with or without ML141 (10 µM) in DMEM growth medium every 2-3 days for 11 days. Cells were washed with 1X TBS (20 mM Tris HCl, 1.5 M NaCl, pH7.5) 3 times followed by fixation with 4% paraformaldehyde in 1X TBS for 4 min and washed again 3 times with 1X TBS. Mineralized matrix was detected by Alizarin red S staining as described previously images (6-9 images/well) were captured by Nikon Eclipse Ti microscope. Calcium deposition in each replicate well as determined by Alizarin red S staining was quantified by spectrophotometry after 10% acetic acid solubilization of precipitated dye as described by Gregory et al. Alizarin red S staining of coronary and aortic calcification was carried out precisely as we’ve previously detailed.

Alkaline phosphatase activity analysis and BrdU incorporation assays in aortic primary cell cultures
Aortic primary VSM were cultured on type I collagen-coated 12 well plates and treated with either vehicle (DMSO) or 10 µM ML141 in vehicle as indicated. To measure alkaline phosphatase enzyme activity biochemically, treated cells were washed twice with 1X TBS and extracted with 10 mM Tris HCl, pH 8.0, containing 0.5 mM MgCl₂ and 0.1% Triton X-100 (0.5 ml/well). Extracts together with cell residues were scrapped into sonication tubes and sonicated for 3 cycles of 20 seconds with 90 sec rest in between using Diagenode Bioruptor Pico Sonication System. Alkaline phosphatase activity in the sonicates were measured using p-nitrophenyl phosphate as substrate as described previously. The protein concentrate in the sonicates were measured using Pierce BCA Protein Assay Kit (Thermo Scientifics, Prod # 23227). Alkaline phosphatase activity in each cell extract was expressed as nmol of para-nitrophenol produced/min/mg cellular protein. BrdU incorporation was analyzed under serum free conditions using primary
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VSM cultured in 96 well cluster plates, implementing the BrdU Cell Proliferation Assay Kit #6813 from Cell Signaling Technologies as per the manufacturer’s instructions.

**RNA analysis**

Total RNA of aortic primary cells were isolated using RNAeasy kit (Qiagen), whereas total RNA of whole aorta was isolated using RNAeasy Lipid Tissue Mini kit after homogenization with Rotorstator in QIAzol Lysis Reagent and chloroform extraction as previously described\(^5,6,11,17\). To quantify mRNA levels, real-time fluorescence RT-PCR was performed as previously described using commercially available, inventoried Taqman Probes that provided optimal coverage (Applied Biosystems, Foster City, CA). Relative mRNA levels were normalized to 18S ribosomal RNA. For array analysis of total aortic RNA, the purity and integrity of the total RNA was measured on RNA Nano chip (Agilent Technologies) using Eukaryote Total RNA Nano series protocol. Next, total RNA was subjected to single round of linear IVT-amplification and labeled with Cy3-labeled CTP using One-Color Low Input Quick Amp Labeling Kit (Ambion). The resulting Cy3 dye incorporated amplified RNA (aRNA) was quantified using ND-1000 spectrophotometer (Nano Drop Technologies) and 600ng of labeled aRNA was hybridized onto Agilent’s 8x60k format Mouse LINC RNA array (Agilent Technologies) encompassing both coding and long noncoding gene probes. After hybridization, the arrays were washed and processed by using Gene Expression Wash Pack (Agilent Technologies) according to manufacturer’s protocol. Finally, the arrays were scanned using the Agilent C Scanner. The intensities of the scanned fluorescence images were extracted with Agilent Feature Extraction software version 10.7.3.1.

**Plasmids, siRNA, and transfection**

Our murine OPNLUC (osteonectin promoter – luciferase reporter) constructs have been detailed described\(^18\); all constructs contain 79 nucleotides of the 5'- untranslated region. NFAT- LUC reporter was obtained from Stratagene. TK-Renilla and SV40-Renilla were obtained from Promega. Expression plasmids listed in Supplement Table I were generated by using In-Fusion HD Cloning kit (cat. # 639692) according to the protocol provided by Clontech (PT5162-1). The remaining plasmids were purchased either from Origene (TruORF Gold) or from Addgene. For plasmid transfection, HEK293T cells were seeded the day before at 65,000 cells/well in 12 well culture plates and transient transfections and reporter assays carried out precisely as previously detailed\(^6,18\), using Qiagen Superfect. All transfections were performed in triplicates and repeated at least once. HEK293 cells were used as a cell reporter system\(^19\) that is very sensitive to both canonical and noncanonical Wnt signaling\(^19\). For siRNA transfection, aortic primary cells were seeded at 100,000 cells/well in type I collagen-coated 12 well culture plates the day before transfection. Control or siRNA (Listed in Supplement Table II) at 50 nM was incubated with Lipofectamine RNAiMAX reagent (3 uL/well, Invitrogen, cat. #13778-
150) in Opti-MEM for 20 min. During incubation, cells were washed once with DMEM medium and fresh DMEM growth medium containing 10% FBS and penicillin / streptomycin was added (0.9 ml/well). At the end of incubation, siRNA mixture (103 uL/well) was added in triplicate to cells. Cells were harvested for RNA isolation 3 days later.

**Mass spectrometry of LRP6-associated proteins**

Analysis was carried out essentially as described. Flag-tagged LRP6 was expressed in HEK293T cells either in the presence or absence of constitutively active PTH/PTHrP receptor in independent duplicate replicates. Anti-FLAG immunoprecipitates were also compared to cultured HEK293T cells lacking the expression. Immunoprecipitates were then analyzed by LC-MS/MS essentially as previously detailed. Briefly, protein samples were reduced, alkylated, digested with trypsin, using Filter-Aided Sample Preparation (FASP) as described previously (Wisniewski, J.R. et al., 2009, Nature Methods V 6, p. 359-62). Phosphopeptides were enriched as described previously. Enriched phosphopeptides and the non-binding plus wash fractions were analyzed. Peptides were desalted, re-suspended in 0.1% formic acid / 5% acetonitrile, and raw MS/MS data generated using a Thermo Fisher Scientific LTQ Orbitrap Velos Pro mass spectrometer equipped with electron transfer dissociation (ETD). Data base searching was carried out against a concatenated forward-reverse human protein database (ipi.HUMAN.v.3.73) with Sorcerer-SEQUEST on Sorcerer Enterprise hardware/software (SageN Research, Inc., Milpitas, CA). Postsearch filtering was with ProteinProphet (trans-proteomic pipeline (TPP), Institute for Systems Biology). Differences in spectral count enrichment with expression of LRP6 expression alone were used to prioritize specificity of interaction, with confirmation by co-immunoprecipitation of co-expressed recombinant proteins (e.g., LRP6 and PRMT1). Other components of the regulated LRP6 interactome will presented elsewhere as functionally validated.

**Western blot analyses**

Western blot analyses were performed using either whole cell extracts or extracts subjected to subcellular fractionation as indicated. For whole cell extracts, cell layer were twice rinsed with 1X TBS and extracted with modified RIPA buffer containing 10 mM Tris HCl, pH8.0, 1 mM EDTA, 140 mM NaCl, 1% Triton X-100 and 0.5% NP40 plus cocktails of protease inhibitors and phosphatase inhibitors (Sigma). For subcellular fractions, confluent cells in 10 cm culture dishes were processed using Pierce Subcellular Protein Fractionation Kit for Cultured Cells (Cat # 78840) according to the manufacturer's protocol. Extracts and fractions containing equal amounts of protein were subjected to SDS-PAGE and Western blotting was performed as previously described, with immune complexes visualization by enhanced chemiluminescence. Gel band intensity was quantified as previously described using ImageJ (ImageJ64 for Mac OS X; National
Institutes of Health, Bethesda, MD). The antibodies utilized in Western blot analysis are listed in Supplement Table III.

Co-Immunoprecipitation studies

To analyze the co-localization of LRP6 and PRMT1, HEK293T cells in 6 well plates were transfected with C-terminal Flag-tagged pCMV-hLRP6-FLAG and untagged pcDNA3-hPRMT1v1 plasmids as described above. 72 h after transfection, cells were washed with cold PBS twice followed by treatment with 2 mM DTBP cell permeable crosslinker (Pierce #20665) in PBS at room temperature for 2 h. After removal of the crosslinker solution, cells were incubated in 1X TBS containing protease and phosphatase inhibitors for another 2 h to quench crosslinking reaction. Cells were then harvested into microfuge tubes and cell pellets obtained by centrifugation at 5000 rpm for 1 min were extracted with 500 uL modified RIPA buffer/pellet as described above for 80 min at 4°C. After centrifugation at top speed for 10 min, supernatants were harvested and stored frozen at -80°C until immunoprecipitation. For immunoprecipitation, cell extracts were thawed on ice. Protein G Dynabeads were resuspended via vortexing. 50 µl of Protein G dynabeads were transfer to a 1.7 ml microfuge tube. The tubes were placed in a magnetic holder and liquid aspirated. After washing once with PBS-T (PBS with 0.02% Tween 20, 100 uL/tube), 200 uL of rabbit anti-Flag antibody (10 µg antibody) in PBS-T was added to each tube. Antibody was allowed to bind to Protein G dynabeads by rotating the tubes on a Nutator at room temperature for 20 min. At the end of incubation, the beads were washed twice with PBS-T. All but 40 µl (reserved for Input) of modified RIPA buffer cell extract was added to each tube. The solution was gently mixed via pipetting up and down a few times. Antigen-antibody interaction was allowed to proceed on a Nutator for 40 min at room temperature. After removal of the supernatant, the Dynabeads were washed 3 times with 200 uL PBS-T/tube. Protein G Dynabead-bound antigen was then extracted with 20 uL elution buffer (50 mM Glycine, pH2.8). Ten microliters of 5X Laemmli buffer containing β-mercaptoethanol (3%) and protein and phosphatase inhibitors and 20 uL of 100 mM DTT were also added. Twenty microliters of Elution buffer, 15 uL 5X Laemmli buffer with β-mercaptoethanol and inhibitors and 20 uL of 100 mM DTT were also added to each Input. Both immunoprecipitates (IP) and inputs were placed in a 95°C heating block for 1 h to allow the cleavage of DTBP crosslinker via DTT reduction. After cooling, IP and Inputs were applied to 15 x1.5 mm 4-12% Tris-Glycine gels (15 uL /lane). Western blot analysis was performed using anti-Flag and anti-PRMT1 antibodies. For analysis of USF1 and histone H3 complexes formation, primary VSM cultures from LRP6(fl/fl);LDLR-/- vs. SM22-Cre;LRP6(fl/fl);LDLR-/- cultures were processed as above following immunoprecipitation of endogenous USF1 and western blot analysis using anti- H3R17Me2a antibodies (see Supplement Table III for immunoreagents).

Immunohistochemistry and fluorescence imaging

Immunolocalization of aortic LRP6 was carried out on 5-micron fresh frozen tissue sections. Sections were washed with 1XTBS twice for 5 min each followed by fixation
with 4% paraformaldehyde in 1xTBS for 8 min at room temperature. Sections were further washed with 1XTBS three times and incubated in TBST (TBS with 0.1% Tween 20) for 15 minutes at room temperature to permeabilize the cells. After washing three times with 1XTBS, sections were blocked for 1 hour with 5% Normal Goat Serum (NGS) in 1X TBST. Incubation with primary antibodies [rabbit anti-LRP6 (custom-made by YenZyme, yz2136) 1:100] was carried out at 4 °C overnight. All antibodies were made up in TBST with 5% NGS. After incubation, sections were washed with 1XTBS three times followed by incubation with the secondary antibody [Alexa Fluor 594 goat anti-rabbit IgG (H+L) (Life Technologies, A-11037), 1:200] for 1 h. Sections were washed again with 1xTBS and mounted with ProLong Gold Antifade Reagent (Invitrogen #P-36931) containing 4’, 6-diamidino-2-phenylindole (DAPI) for nuclei staining. Fluorescent images were captured using Leica DM4000B and a DFC 420C Firewire camera, implementing Leica Application Suite Imaging Software to process the digital images as previously described6, 11, 15. Pseudocoloring and image overlay was carried out using ImageJ25 (ImageJ64 for Mac OS X; National Institutes of Health, Bethesda, MD). Image analysis for Mac2 staining was also performed using ImageJ.

Chromatin immunoprecipitation (ChiP) assays.
ChiP was carried out following our modification of the previously reported fast ChiP method of Nelson et al26, 27. Briefly, cells were cross-linked with 1.42% formaldehyde for 15 minutes at room temperature followed by quenching with 125mM Glycine for 5 minutes. They were then lysed with IP buffer (150mM NaCl, 50 mm Tris-Cl, pH 7.5, 5mM EDTA, 0.5% NP-40, 1%, Triton X-100) containing protease and phosphatase inhibitors on ice for 10 minutes. The nuclear pellet was washed once with IP buffer and re-suspended in the same buffer. Nuclear suspension was sonicated using the Diagenode Bioruptor Pico Sonication system. The sonicated nuclear suspension was pre-cleared with Protein A beads followed by immunoprecipitation with 1-3 ug of antibody at 4°C overnight. The mixture was then added to fresh Protein A bead slurry and the incubation continued for another hour. After centrifugation at 2000xg for a few seconds, the supernatant was saved for processing as “input” (see below) and the beads were washed six times with cold IP buffer without inhibitors. 10% Chelex beads (BioRad 142-1253) were added to the washed beads containing immunoprecipitates. The samples were boiled for 10 minutes followed by treatment with Proteinase K at 55°C for 30 minutes. After inactivation of proteinase K by heating at 95 C for 10 minutes, the beads were pelleted by centrifugation and the supernatant was transferred to a fresh tube, taking care not to transfer any beads. The beads were further extracted once with equal volume of water. After vortexing and centrifugation, the supernatant was pooled with the first aliquot and store at -20°C until qPCR analysis. Input DNA was also precipitated with ethanol in the presence of 0.2 ug/uL glycogen. Precipitated DNA was washed with 70 % ethanol and pellets air-dried. Pellets were dissolved in 10% Chelex 100 beads and continued to process along with the ChiP samples. Input and precipitated DNA was quantified using Sybr Green as previously described27 with a 7300 Real Time PCR
system, analyzing 1 pg of mouse genomic DNA assessed in parallel as a standard to normalize quantitation between each plate and experiment.

**Statistical analyses**
All statistical analyses were performed with 3–12 independent replicates per group as indicated. Statistical analyses were performed using GraphPad InStat version 3.06 software, implementing standard parametric or nonparametric methods (two-tailed testing) when indicated. Non-parametric Mann-Whitney U-test was implemented for assessment of *in vivo* BrdU labeling, Mac2 staining, and plasma OPN levels (not normally distributed). Graphic data are presented as mean ± SEM.
**Supplement Table I: Plasmid List**

<table>
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<tr>
<th>Gene</th>
<th>Vector</th>
<th>Cloning sites</th>
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<td>pcDNA3</td>
<td>HindIII/XhoI</td>
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<td>KpnI/BamHI</td>
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<td>pcDNA3</td>
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<td>HindIII/EcoRI</td>
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<td>hLRP6-Flag-C</td>
<td>pCMV-Flag-C</td>
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<td>pAc-GFP1-N3</td>
<td>BglII/KpnI</td>
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<td>hPRMT1, variant 1, no tag</td>
<td>pcDNA3</td>
<td>HindIII/XhoI</td>
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m: mouse  
h: human
Supplement Table II: siRNAs (purchased from GE Dharmacon ON-TARGETplus SMART pool)

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<td>D-001810-10-20</td>
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<td>L-040656-00</td>
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<tr>
<td>mouse USF2</td>
<td>L-065499-01</td>
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<td>mouse PRMT1</td>
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</tr>
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<td>mouse PRMT4 (Carm1)</td>
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<tr>
<td>mouse Jmjd6</td>
<td>L-058805-01</td>
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<tr>
<td>mouse Padi4</td>
<td>L-041055-01</td>
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**Supplement Table III: Antibody used in Western blot and immunoprecipitation**

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<th>Source</th>
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<td>Rabbit anti-dimethyl-arginine, asymmetric (ASYM24)</td>
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<td>Mouse anti-Flag antibody (M2)</td>
<td>Sigma-Aldrich #F3165</td>
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<tr>
<td>Rabbit anti-Fzd10 antibody</td>
<td>Acris #APO1204-pu-N</td>
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<tr>
<td>Rabbit monoclonal anti-LRP5 (D80F2)</td>
<td>Cell Signaling Technology #5731s</td>
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<tr>
<td>Rabbit anti-LRP6 (C47E12) western blot</td>
<td>Cell Signaling Technology #3395s</td>
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<tr>
<td>Rabbit monoclonal anti-Myc-Tag (71D10)</td>
<td>Cell Signaling Technology #2278s</td>
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<tr>
<td>Rabbit anti-PRMT1 (A33)</td>
<td>Cell Signaling Technology #2449s</td>
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<td>Santa Cruz sc-8983X</td>
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<td>Rat Anti-mouse Mac2 Mab clone M3/38</td>
<td>CedarLane/AccurateChemical ACL8942AP</td>
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<td>Rabbit anti-Wnt10a</td>
<td>Abcam ab97469</td>
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<tr>
<td>Rabbit anti-eIF2α</td>
<td>Santa Cruz sc-133227</td>
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<tr>
<td>Rabbit anti-histone H3</td>
<td>Abcam ab1791</td>
</tr>
<tr>
<td>Rabbit anti-H3R17Me2a</td>
<td>Active Motif #39709</td>
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<tr>
<td>Rabbit anti-PRMT4</td>
<td>Cell Signaling Technologies #3379s</td>
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<tr>
<td>Rabbit anti-Wnt7a/b antibody YZ4837</td>
<td>Yenzyne custom-made affinity-purified polyclonal antibody to the respective KLH-conjugated unique Wnt motif (Wnt7b and Wnt7a sequences, respectively): SRLROPTFLRIKQLRSYQKPMENH2 and SRNKRPTFLIKKPLSYRKPM David –NH2</td>
</tr>
</tbody>
</table>
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METHODS SUPPLEMENT CITATIONS


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