Gravin Scaffolding Protein Mediates Signaling during Atherosclerosis

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Gravin Scaffolding Protein Mediates Signaling during Atherosclerosis

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Abstract

Atherosclerosis is an inflammatory response that occurs principally in the walls of arteries, contributing to cardiovascular mortality, which can lead to growth factors over-release. Gravin, an A-Kinase Anchoring Proteins (AKAP), targets Protein Kinase A (PKA), Protein Kinase C (PKC), and Ca^{2+}/Calmodulin-dependent Protein Kinase (CaMKII) and mediates intracellular signaling. This gravin coordinated kinase-dependent substrate phosphorylation leads to changes in intracellular Ca^{2+}, which is associated with cell proliferation and migration. Additionally, recent studies show that gravin can regulate lipid metabolism in the liver and affects cell proliferation and migration.

To study the role of gravin in atherosclerosis, five week-old wild-type (WT) and gravin-truncated (gravin-t/t) mice were subjected to high fat diet (HFD) or normal diet (ND) for 16-weeks. Cholesterol, triglyceride and VLDL level in serum were significantly decreased in gravin-t/t HFD compared to WT HFD mice. Furthermore, gravin-t/t (HFD) mice showed lower liver-to-body weight ratio as well as decreased lipid accumulation and decreased liver damage. In addition, gravin-t/t (HFD) mice showed lower genes expressions related to

cholesterol biosynthesis via less activation of the sterol-regulatory-element-binding protein 2 (SREBP2). We also observed less aortic lipid accumulation and lower blood pressure in gravin-t/t (HFD) mice.

VSMCs, play a crucial role in the progression of atherosclerosis. Gravin-t/t VSMCs showed decreased Ang II induced VSMCs migration and proliferation when compared to WT VSMCs. We also observed less migration in gravin-t/t VSMCs PDGF. Furthermore, gravin-t/t VSMCs exhibited decreased PKC activity and lower intracellular Ca2+ transients after either Ang II or PDGF treatment. These changes were accompanied by significant differences in PKC phosphorylation and PKC-dependent substrate phosphorylation, which involved ERK1/2 signaling pathway.

In conclusion, these findings indicated that the absence of gravin mediated signaling was able to decrease lipid metabolism and accumulation in the liver and lipid accumulation in the aorta as well as to lower the blood pressure in response to HFD. In addition, our findings indicated that in the absence of gravin mediated signaling, Ang II and PDGF induced VSMCs proliferation and migration were suppressed in gravin-t/t VSMCs. Taken together, our data

indicates that gravin is involved in the initiation and progression of atherosclerosis and/or vascular remodeling.

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ACE	angiotensin-converting enzyme
AKAP	A-kinase anchoring protein
ALT	alanine aminotransferase
Ang II	angiotensin II
AST	Aspartate aminotransferase
bFGF	basic fibroblast growth factor
cAMP	cyclic AMP
CHD	coronary heart disease
EGF	epidermal growth factor
ECM	extracellular matrix
FAS	fatty acid synthase
Gravin-t/t	gravin (truncated alleles; t/t)
HDL	high-density lipoprotein
HFD	high fat diet
HMGCR	HMG-CoA reductase
LDL	low-density lipoprotein
LDLR	low density lipoprotein receptor
LPL	lipoprotein lipase

MMP	matrix metallopeptidase
ND	normal diet
PDGF	platelet-derived growth factor
PKA	protein kinase A
PKB/Akt	protein kinase B
PKC	protein kinase C
SREBP-1c	sterol regulatory element binding protein-1c
SREBP-2	sterol regulatory element binding protein-2
TGF	transforming growth factor
VLDL	very-low density lipoprotein
VSMC	vascular smooth muscle cell

1 Introduction and statement of the problem

Atherosclerosis is a chronic inflammatory disease responsible for cardiovascular related mortality, which is one of the leading causes of death in the world (Cohen Tervaert, 2013). The development of atherosclerosis results from the contributions of atherogenic factors such as hyperlipidemia, hypertension, smoking, diabetes, and diets rich in fats (Lusis, 2000). Despite changes in lifestyle and the use of new pharmacologic therapies to lower plasma cholesterol concentrations, cardiovascular disease continues to be the principal cause of death all over the world. In pathological conditions, the retained lipoproteins in the vessel wall initiate the inflammatory response in the surrounding cells (Cohen Tervaert, 2013). Specifically, changes in the metabolism of lipoproteins including total cholesterol, low-density lipoprotein (LDL), very-low density lipoprotein (VLDL), triglyceride and high-density lipoprotein (HDL) are well recognized as important mediators in the pathogenesis of atherosclerosis (Lusis, 2000). These atherogenic factors subsequently cause endothelial injury/dysfunction, triggering cytokines and growth factors to be released by inflammatory cells, such as macrophage/monocyte, endothelial cells, and vascular smooth muscle cells (VSMCs) (Rafieian-Kopaei, Setorki, Doudi, Baradaran, & Nasri, 2014).

Vascular smooth muscle cells (VSMCs) are the major component of vessel walls and can control local blood pressure via contraction and relaxation. In atherosclerosis, the VSMC contributes to vessel wall inflammation, lipoprotein retention and stabilization of the plaque in the intima by the formation of a fibrous cap. The abnormal proliferation and migration of VSMC, in response to several growth factors, can contribute to the pathology of atherosclerosis (Dzau, Braun-Dullaeus, & Sedding, 2002). Angiotensin II (Ang II), the main peptide hormone of the renin–angiotensin system, has multiple effects on the control of cardiovascular homeostasis. Numerous studies have indicated that, during atherosclerosis, Ang II plays a critical role as a growth factor in VSMC proliferation and migration (Guo et al., 2012;Dong et al., 2012;Yin, Yan, & Berk, 2003).

VSMC proliferation, migration, apoptosis and extracellular matrix synthesis induce the formation of the atherosclerotic lesion, which can be regulated by matrix metalloproteinases (MMPs) (Newby, 2008). Once stimulated and activated, MMPs facilitate VSMCs migration by degrading extracellular matrix components and down-regulating their biosynthesis (Galis, Sukhova, Lark, & Libby, 1994; Chen et al., 2013). Although many insightful studies have identified key signaling components involved in atherosclerosis, our full

understanding of atherogenic factors leading to the development of atherosclerosis is still lacking. It is known that the elevation of circulating cholesterol and hypertension are two of the most important factors that induce and promote the internal damage of arterial walls (Russell Ross, 1999).

Gravin (AKAP12, also known as src-suppressed C kinase substrate (SSeCKS) is a member of the A-kinase anchoring proteins (AKAP) family of signalosome proteins. It can scaffold the substrates protein kinase A (PKA), protein kinase C (PKC), protein phosphatase 2B (PP2B) and β_2-adrenergic receptors, as well as other protein kinases, protein phosphatases, phosphodiesterases to specific intracellular locations resulting in localization of the binding complex (Wang, Tao, Shumay, & Malbon, 2006). Thus, gravin assembles intracellular signaling proteins into a specific complex within the cell, thereby enhancing signaling specificity as well as enhancing signaling efficiency (Diviani, Maric, Pérez López, Cavin, & Del Vescovo, 2013). Specifically, gravin scaffolds proteins in order to fine-tune cellular responses – *cellular proximity and protein effective concentrations* – by forming multi-component protein complexes (Wong & Scott, 2004). In the initiation and progression of atherosclerosis, the cytokine and growth factors (such as Ang II, EGF, PDGF, bFGF, TGF) released by inflammatory cells, endothelial cells and

VSMCs can cause downstream signaling substrate phosphorylation, which is associated with VSMCs proliferation, migration and extracellular matrix synthesis (Dzau et al., 2002; Berk, 2001). The growth factors converge on VSMCs and stimulate a number of common pathways, generating a complex level of signaling crosstalk. It activates VSMCs and triggers pathological vascular remodeling. These VSMC intracellular signaling pathways commonly involve phosphorylation mediated by PKA and Ca^{2+}- dependent PKC. It has already been shown that gravin (AKAP 12) regulates localization of PKA with its substrates complex involving Ca^{2+} and PKC (Sprague & Khalil, 2010). AKAPs regulate cAMP/PKA signaling events that control lipolysis, including regulation of Lipoprotein Lipase (LPL) expression via inhibition of translation, which is an enzyme that hydrolyzes triglycerides in lipoprotein (Ranganathan, Pokrovskaya, Ranganathan, & Kern, 2005; Rogne & Taskén, 2014). Some studies show gravin also mediates cellular lipid metabolism via inducing the activation of Sterol Regulatory Element Binding Proteins (SREBPs) in hepatocytes, which are transcription factors of several cholesterol-associated gene (Choi et al., 2008).

Our central hypothesis is that the absence of gravin mediated signaling, the cardiovascular system is protected against the development of

atherosclerosis, in part by, decreasing VSMC migration and proliferation, preventing vascular remodeling, along with a reduction of plaque formation and decreased cholesterol biosynthesis. Furthermore, we propose that by understanding gravin's role in mediated cell signaling pathways, will provide insights toward the development of novel targets for the prevention and treatment of atherosclerosis.

2 Literature Review

2.1 Prevalence

Atherosclerosis is a chronic and progressive inflammatory disease characterized by plaque formation in the vessel wall. Plaque buildup causes the arteries to narrow which limits the flow of oxygen-rich blood to the heart and other parts of the body. Atherosclerosis is the leading cause of cardiovascular related mortality, including heart attack and stroke (Cohen Tervaert, 2013). Coronary heart disease (CHD) is one of the clinical manifestations of atherosclerosis, which is the #1 killer in the U.S. In 2010, about every 1 minute 23 seconds one American died of CHD (Go et al., 2014).

Dyslipidemia, diabetes, smoking, family history, obesity, and hypertension are the major atherogenic factors. According to the National Institute of Health (NIH), the main treatment for atherosclerosis is lifestyle changes including diet control and physical exercise. Maintaining body weight, lowering the cholesterol level, glucose concentration, and reduced blood pressure are recommended treatments that can be taken that can be taken to reverse the effects of atherosclerosis (Robinson, Fox, Bullano, & Grandy, 2009). Despite

these therapies, atherosclerosis remains a leading health problem world-wide.

2.2 Hyperlipidemia

The pathological condition of atherosclerosis consists of two parts: an imbalance of lipid metabolism (lipid accumulation, oxidation and modification) and chronic inflammation of the arterial walls (Rafieian-Kopaei, Setorki, Doudi, Baradaran, & Nasri, 2014; Insull, 2009). Hyperlipidemia, one of the risk factor of atherosclerosis, is when high cholesterol and high triglycerides levels exist in the blood. Hyperlipidemia can be caused by a diet rich in fat, lack of exercise, smoking and so on. Except for free fatty acids, all other lipids are transported by lipoproteins, such as low density lipoprotein (LDL), very low density lipoprotein (VLDL) and high density lipoprotein (HDL). High fat diet (HFD) induced hyperlipidemia is a consequence of abnormalities in lipid and lipoprotein metabolism, which is related to alteration in transcription factors essential to regulate the gene expressions of enzymes involved in lipogensis and lipolysis in the liver (Lottenberg, Afonso, Lavrador, Machado, & Nakandakare, 2012).

In order to discover alternative treatments and prevention methods of

atherosclerosis and its risk of developing cardiovascular disease, an understanding of the metabolism of plasma lipid and lipoproteins is of great importance.

2.2.1 Lipoprotein Metabolism in Liver

There are three interconnected pathways involved in lipoprotein metabolism: first, the absorption of dietary fat (also called exogenous fat) in the small intestine, second, the transport of hepatic fat (also called endogenous fat) in the liver and, third, the process of reverse cholesterol transport. Dysfunction in any of the three pathways will promote the progression of atherosclerosis.

The liver is the major organ involved in lipid and lipoprotein metabolism. Elevated serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) indicate some form of liver inflammation, damage or injury in most cases. VLDL, a triglyceride rich lipoprotein, is synthesized and secreted from the liver. VLDL carries energy-rich triacylglycerol from the liver to the peripheral tissues, also along with free and esterified cholesterol. After removing triacylglycerol by lipoprotein lipase (LPL), VLDL is transformed to LDL in the liver. LDL transports cholesterol to the cells in the body. The

function of HDL is to deliver the excess cholesterol in cells in the body back to the liver. LDL and VLDL are considered the most important atherogenic factors, and HDL has the anti-atherogenic role (Wouters, Shiri-Sverdlov, van Gorp, van Bilsen, & Hofker, 2005; Rigotti et al., 1997).

2.2.2 Lipid Metabolism in the Liver

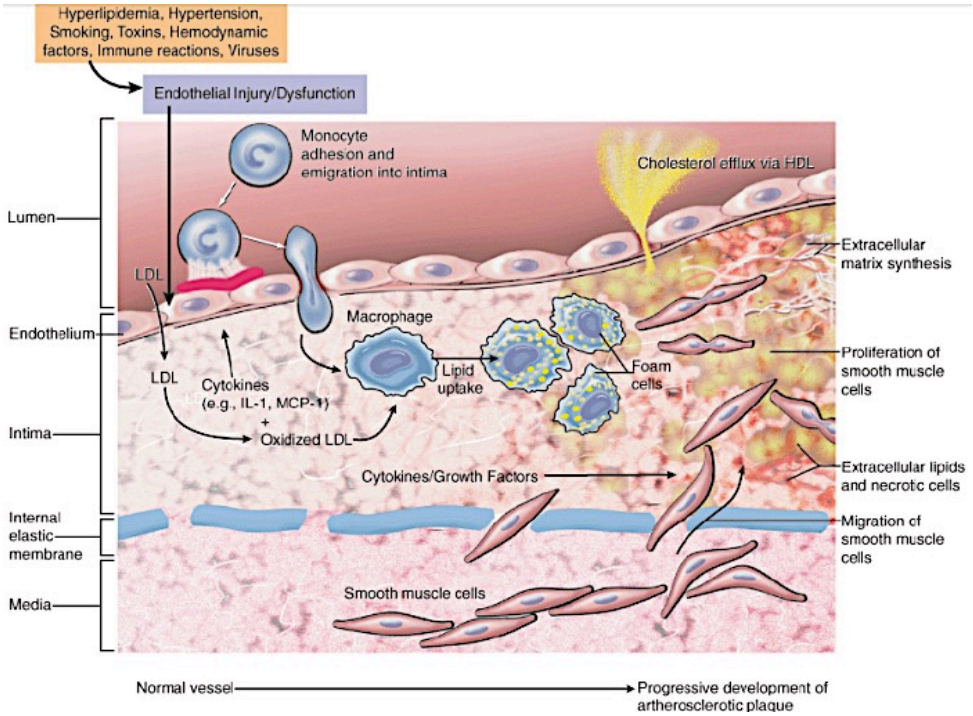
Lipid metabolism disorders induced by HFD can cause a massive accumulation of lipid in various tissues, especially in the liver, which is the major organ controlling lipid metabolism. A complex network of molecular mediators, including several proteins related to lipid and cholesterol biosynthesis regulates lipid metabolism. In the liver, SREBPs are the protein sensors that monitor sterol concentrations, and are the major transcription factors to regulate cholesterol, fatty acid, triglyceride biosynthesis and uptake (Brown & Goldstein, 1997; Goldstein, DeBose-Boyd, & Brown, 2006). In low concentrations of cholesterol in the cells, SREBPs form a complex with cleavage–activating protein SCAP and the complex together moves from ER to Golgi. After cleavage, active forms of SREBPs enter the nuclei and stimulate its target genes involved in cholesterol and fatty acid biosynthesis. In mammalian cells, SREBP-1c and SREBP-2 are two related isoforms of

SREBPs. SREBP-1c mainly stimulates the expression of genes related to fatty acid synthesis, such as fatty acid synthase (FAS), which catalyzes fatty acid synthesis. In contrast, the cholesterol synthesis is regulated by SREBP-2, which can target mRNAs for key cholesterogenic genes, including HMG-CoA reductase (HMGCR), which is a rate-limiting enzyme to produce cholesterol, and LDL receptor, which mediates the endocytosis of cholesterol-rich LDL (Brown & Goldstein, 2009). In the liver, the low-density lipoprotein receptor (LDLR) plays an important role in the clearance of cholesterol from the plasma to the liver by taking-up VLDL remnants and LDL (Dietschy, Turley, & Spady, 1993). The elevated hepatic cholesterol level can cause inhibition of HMGCR expression to suppress the *de novo* cholesterol biosynthesis (van der Wulp, Verkade, & Groen, 2013).

2.3 Initiation of Atherosclerosis

Atherogenetic factors (such as hyperlipidemia, hypertension, and smoking) cause endothelial dysfunction, consequently result in multiple processes including inflammation response, VSMCs activation, and plaque formation (Figure 1). An increase of LDL levels in the blood due to high fat diet results in elevated concentration of LDL in the arterial wall, where it gets trapped and

oxidized to be oxidized LDL (ox-LDL). After adhesion and migration into the intima in response to inflammatory signals, the circulating monocytes differentiate into the macrophages and consequently uptake the ox-LDL and become foam cells. Ox-LDL stimulates endothelial cells and macrophages to secrete several cytokines that cause the dysfunction of endothelium cells resulting in increased lipid permeability. Also, an increase of monocyte concentration in the intima where they differentiate into macrophages (Baldán et al., 2006;Rafieian-Kopaei et al., 2014).



Rafieian-Kopaei M et al. Int J Prev Med. 2014 Aug; 5(8): 927–946

Figure 1: Schematic diagram of cellular interactions in atherosclerosis.

2.3.1 Inflammatory Cytokines and Growth Factors

Inflammation begins with the dysfunction of endothelial cells, which lead to more monocyte adhesion and migration into intima (Insull, 2009). The cytokine and growth factors (such as Angiotensin II (Ang II), Epidermal Growth Factor (EGF), Platelet-derived Growth Factor (PDGF), Basic Fibroblast Growth Factor (bFGF), Transforming Growth Factor (TGF) released by inflammatory cells, endothelial cells and Vascular Smooth Muscle Cells (VSMCs) can cause downstream signaling substrate phosphorylation, which is associated with VSMC proliferation, migration and extracellular matrix (ECM) synthesis (Dzau, Braun-Dullaeus, & Sedding, 2002;Berk, 2001). The growth factors converge on VSMCs and stimulate a number of common pathways, generating a complex level of signaling crosstalk.

2.3.2 Vascular Smooth Muscle Cells (VSMCs) Activation

Atherosclerosis involves multiple processes including endothelial dysfunction, inflammation, VSMCs activation, and ECM modification. Early work, in 1973, identified VSMC proliferation and migration as an essential step in atherosclerosis (R Ross & Glomset, 1973). The inflammatory response,

including secretion of cytokine/growth factors, activates VSMCs and triggers pathological vascular remodeling. Depending on stimuli from the growth factors, VSMCs undergo a transition from a quiescent contractile phenotype to a proliferative synthetic phenotype, which can cause VSMCs migration and proliferation, ECM production and calcification (Lacolley, Regnault, Nicoletti, Li, & Michel, 2012).

VSMCs play an important role in all developmental stages of atherosclerosis associated with not only migration and proliferation, but also with secretion of pro-inflammatory mediators and apoptosis. VSMCs migrate from the medial layer of the arterial wall into intima, proliferate and secrete extracellular matrix proteins that form a fibrous plaque (Russell Ross, 1999). VSMCs synthesize several pro-inflammatory mediators, such as monocyte chemoattractant protein 1, vascular cell adhesion molecule, and extracellular matrix molecules essential for the retention of lipoproteins. These pro-inflammatory mediators may contribute to the development of the atherosclerosis (Dzau et al., 2002). In the advanced stage of atherosclerosis, leukocytes and lipids form a necrotic core with a thinning fibrous plaque rich of VSMCs. This fibrous plaque thinning may then undergo a process called atherosclerotic plaque rupture, which may be caused by VSMC apoptosis

(Newby, Libby, & Wal, 1999).

Those growth factors/ cytokines bind cell-surface receptors to stimulate VSMC migration, proliferation, apoptosis and ECM deposition through a process commonly referred to as intracellular signal transduction (Rudijanto, 2007). These VSMC intracellular signaling pathways commonly involve phosphorylation mediated by PKA, PKC and elevation of Ca^{2+}.

2.3.3 Angiotensin II (Ang II)

Angiotensin II (Ang II) is an octapeptide hormone with multiple effects on the cardiovascular system. As one of the local and systemic factors, Ang II plays important roles in vasoconstriction (hypertension), regulation of renal sodium absorption and plasma volume. It also acts as a growth factor in VSMC, which can regulate VSMC proliferation, migration and apoptosis, and induces extracellular matrix (ECM) deposition and expression of several pro-inflammatory cytokines (Touyz & Schiffrin, 2000). In 1982, Stephen Gunther et al. discovered that functional receptors in VSMCs have been activated in response to Ang II stimulation, which is associated with VSMC proliferation consistent with phenotypic alternation (Gunther, Alexander,

Atkinson, & Gimbrone, 1982). There are two major G protein-coupled Ang II receptor subtypes – Type 1 receptor (AT_1) and Type 2 receptor (AT_2). AT_1 receptors are predominantly expressed in VSMCs, which have been shown to be involved in cell proliferation and migration, endothelial dysfunction and atherosclerotic plaque formation, along with classical physiological action of Ang II (Kaschina & Unger, 2003). In contrast, it has been reported that the AT_2 receptor mediates major signaling pathways involved in vasodilation, anti-proliferation, apoptosis, differentiation and regeneration (Kaschina & Unger, 2003).

Ang II has been implicated to have a very important role in the development of atherosclerosis. In humans, increased plasma concentration of AngII has been implicated in atherogenesis (Alderman et al., 1991). Additionally, Ang II infusion augments the formation and progression of atherosclerosis lesions (Daugherty, Manning, & Cassis, 2000). Several studies have shown that pharmacological antagonism of Ang II synthesis by Angiotensin-Converting Enzyme (ACE) inhibitors as well as inhibition of the cellular effect of Ang II by AT_1 receptor blockers, have been shown to inhibit the progression of atherosclerosis (Lonn, 2002; Khan & Rahman, 2001; Nickenig, 2004).

2.3.4 Platelet-Derived Growth Factors (PDGF)

Platelet-derived growth factors (PDGFs) are known as a group of growth factors that can induce VSMC proliferation and migration, which constitute vascular remodeling, after injury, as well as undergoing atherogenesis (Chiou, Chen, & Wei, 2011; Louis & Zahradka, 2010). PDGFs consist of a family of homologous genes, PDGF-A, -B, -C and -D, which form homodimers to interact with their receptors PDGFR-α and PDGFR-β. During atherosclerosis, the over-release of PDGF is essential in the proliferation of VSMC activated in response to several atherogenic factors and other growth factors (Raines, 2004). In rat arterial injury models, PDGF induces migration of VSMCs from the media into the intima (Lewis, Olson, Raines, Reidy, & Jackson, 2001). In response to PDGF stimulation within minutes, it induces cell migration through PLC/PKC signaling pathway (Tallquist & Kazlauskas, 2004).

2.4 Animal Models of Atherosclerosis

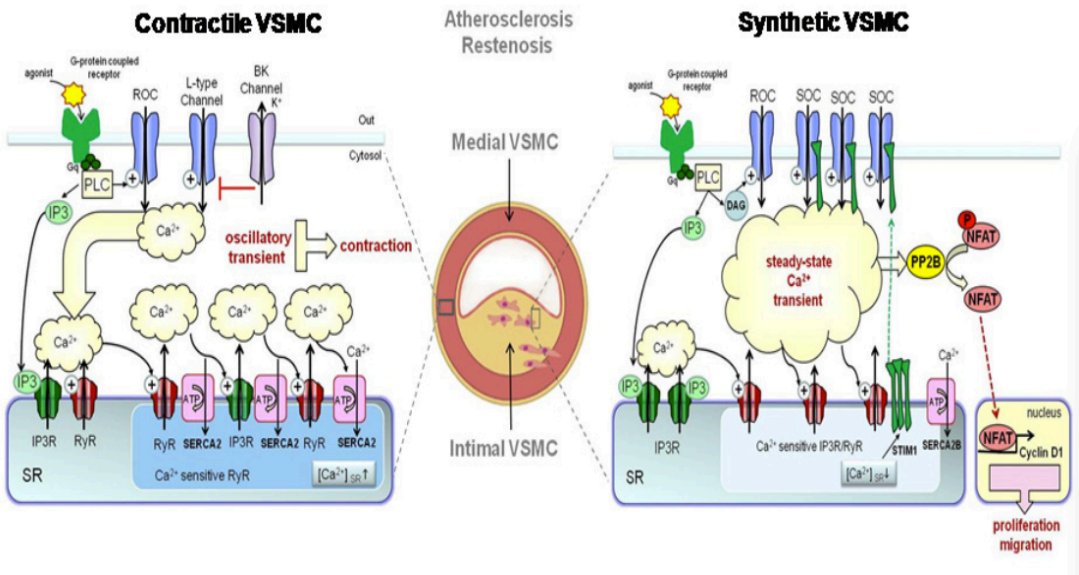
Animal models have been used to induce atherosclerosis for better understanding of the pathology of atherosclerosis in humans. Various animal species that have been used include both large (such as rabbits, pigs and

nonhuman primates) and small animals (such as mice). Since 1992, mice has been widely used as the animal model of atherosclerosis of choice, because mice are highly resistant to atherosclerosis, except for the C57BL/6 strain (Jawien, Nastalek, & Korbut, 2004). Genetic modified mouse models were subsequently developed. Of these genetic engineered mouse models, apolipoprotein E- knocked out (ApoE -/-) and LDL receptor- deficient (LDLR -/-) mouse models are the most commonly used to study the pathology of atherosclerosis (Getz & Reardon, 2012). Other than hyperlipidemia induced atherosclerosis, there are other models of atherosclerosis including pathology induced by arterial injury, carotid artery ligation and vascular graft (Xu, 2004).

2.5 Calcium

Calcium, as a second messenger, plays an important physiological role in cellular functions as diverse as contraction, metabolism, apoptosis, cell migration, proliferation and cell growth involved in initiation and procession of atherosclerosis. During atherosclerosis, in response to various cytokines, growth factors and inflammatory mediators, VSMCs progress from a quiescent contractile phenotype to a synthetic phenotype. The synthetic phenotype is

characterized by the VSMC becoming proliferative and migratory, along with a high level of extracellular matrix. When VSMCs switch to synthetic phenotype, contractile related functional proteins get down-regulated, such as voltage activated L-Type Calcium Channels (LTCC), sarcoplasmic reticulum (SR) Ryanodine Receptor (RyR) calcium release channel and the Sarco / Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) calcium pump SERCA2a, a “fast” calcium pump. In synthetic VSMC, agonist binding to G-Protein-Coupled Receptor (GPCRs) leads to increase cytosolic Ca^{2+} concentration that is consequently removed, by the SERCA2b calcium pump of the SR, a “slow” calcium pump. Subsequently, the opening of Receptor Operated Channel (ROC) and Store Operated Channel – Ca^{2+}-release activated Ca^{2+} channel (CRAC) result in extracellular Ca^{2+} influx. The resulting steady state Ca^{2+} transient is essential for triggering proliferation and migration related transcription factors -a Nuclear Factor of Activated T lymphocytes (NFAT) via phosphorylation of PP2B (Figure 2) (Lipskaia, Limon, Bobe, & Hajjar, 2012).



Lipskaia L et al. Intech. 2012. Chapter 2

Figure 2: Schematic representation of calcium cycling in contractile/synthetic VSMCs.

2.6 Protein Kinase C (PKC)

Protein kinase C (PKC) is the critical mediator in the signal transduction pathways interacting with molecules both upstream and downstream associated with the development of atherosclerosis. There are at least eleven isoforms that are divided into three subgroups based on the secondary messenger regulation: classical, novel and atypical. PKCs consist of classical PKCs: α, βI, βII and γ regulated by Ca^{2+}, diacylglycerol (DAG), phosphatidylserine, and phorbol esters; novel PKCs δ, ε, η, θ, and μ are

regulated by only DAG; atypical PKCs ξ and λ are unresponsive to DAG and calcium and are regulated by other lipid-derived second messengers (H.-C. Fan, Fernández-Hernando, & Lai, 2014).

Both PKCβ and PKCδ are the most potential therapeutic targets for atherosclerosis. The deficiency and blockage of PKCβ in ApoE-/- mice result in a significant reduction in the progression of atherosclerosis (Harja et al., 2009). PKCδ, through ERK1/2 activation, can also regulate VSMC DNA synthesis and cell proliferation during atherosclerosis (Ginnan, Pfleiderer, Pumiglia, & Singer, 2004). While atherogenesis is initiated by an accumulation of LDLs, removal of circulation LDLs via regulation of the hepatic LDL receptor (LDLR) is also an important therapeutic target of atherosclerosis. PKCβ and PKCξ may increase LDLR expression in the liver by promoting LDLR gene transcription and mRNA stability through regulating histone H3-Ser10 phosphorylation and the extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) signaling pathways (Vargas, Brewer, Rogers, & Wilson, 2008; Huang, Mishra, Batra, Dillon, & Mehta, 2004).

Vascular smooth muscle cell (VSMC) migration is involved in initiating neointima hyperplasia and ultimately, restenosis. Mechanistically, matrix

metalloproteinases (MMPs), particularly MMP-9, are known to be involved in VSMC migration and PKC can regulate MMPs expression (Ding et al., 2011). The release of Ang II in atherosclerosis can lead to intracellular Ca^{2+} elevation and PKC activation, which regulates activities of tyrosine kinases such as Src, focal adhesion kinase (FAK), and proline-rich tyrosine kinase 2 (Pyk2) (Yin et al., 2003).

2.7 Protein Kinase A (PKA)

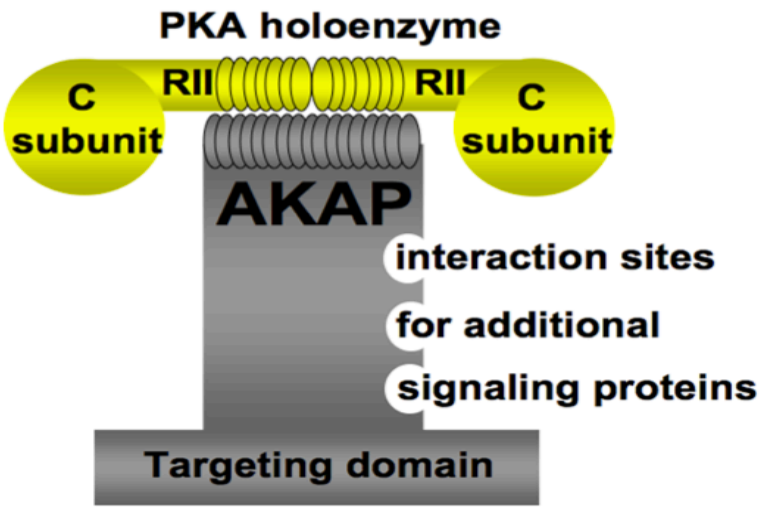
Protein kinase A (PKA), also known as cyclic AMP (cAMP) dependent protein kinase, is a threonine/serine kinase that phosphorylates a wide range of proteins. PKA is a heterotetramer consisting of two regulatory (R) subunits and two catalytic (C). Each regulatory subunit is capable of binding two cyclic monophosphate (cAMP) molecules. When cytosolic cAMP concentration increases, the regulatory units activate via binding to cAMP and release the catalytic subunits. The unbound catalytic subunits scatter to phosphorylate PKA's substrates (Skalhegg & Tasken, 2000). It is reported that phosphorylation of Thr 197 of PKA catalytic subunit is an indicator of PKA activity (Shen et al., 2004).

PKA substrate phosphorylation is required for many cellular processes, including metabolism, contraction, cell proliferation and migration (Walsh & Van Patten, 1994). The increased phosphorylation of these substrates, and other related proteins mediated by PKA, induce the increase of Ca^{2+} current, which also can lead to activation of proliferation and migration related transcription factors.

2.8 A Kinase Anchoring Proteins (AKAPs)

A-Kinase Anchoring Proteins (AKAPs) is a family of scaffolding proteins first identified as mediators, which target PKA to specific subcellular compartments to coordinate cell signaling, even with different structures (Langeberg & Scott, 2005). In addition, AKAPs also bind to other signaling molecules, including other protein kinases, protein phosphatases, phosphodiesterases, adenylyl cyclases and small G proteins. AKAPs function to tether these binding signaling complexes to discreet intracellular locations (Figure 3). Currently, more than 50 AKAPs are identified in several organisms (Carnegie, Means, & Scott, 2010). Based on distinct components of signaling molecules bound to each AKAP, AKAPs sequester discrete sets of signaling molecules to particular regions of the cell in order to cause specificity and

diversity of local cellular signaling dynamics. Recent studies show that some of the AKAPs found in the heart, including mAKAP, AKAP-Lbc, AKAP15 and gravin (AKAP12). Thus, AKAPs are central mediators of cross talk and integration of cAMP/PKA signaling with other signaling pathways. The most commonly known result is that the activation of PKA can regulate cardiac function (Mauban, Donnell, Warrier, Manni, & Bond, 2009).



Carnegie GK et al. IUBMB Life 2009 April ; 61(4): 394–406. doi:10.1002/iub.168.

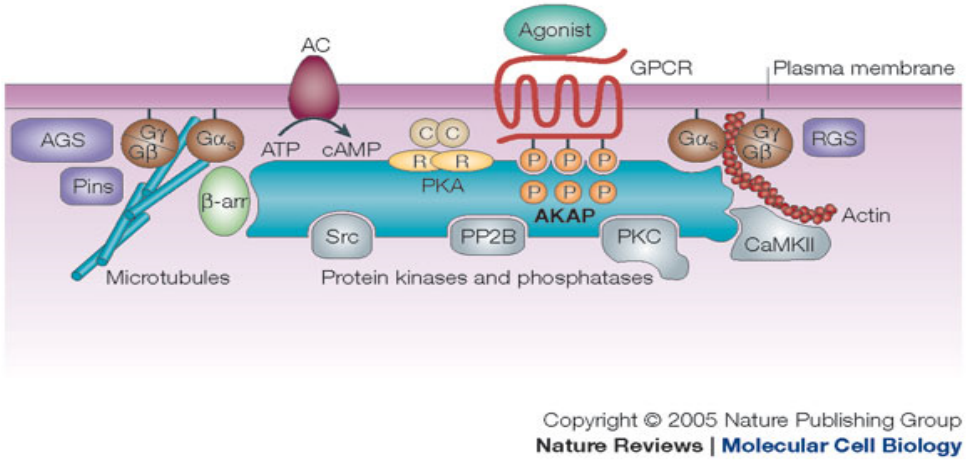
Figure 3: Structure and properties of AKAPs

2.9 Gravin

Gravin is a 250 kD human A kinase anchoring protein (AKAP) expressed in multiple tissues (heart, bladder, brain, lungs etc.) that has been identified in

other species as AKAP12, AKAP250 and Src suppressed C kinase substrate (G. Fan, Shumay, Wang, & Malbon, 2001). Gravin localizes PKA to the β2-adrenergic receptor (β_2-AR) in order to facilitate PKA's phosphorylation of substrates. In addition to anchoring PKA, gravin also has binding sites for protein phosphatase 2B, PKC and other signaling and scaffolding proteins (G. Fan et al., 2001) (Figure 4). Current major studies about gravin are investigating its role in in various forms of cancer. Indeed, gravin-null mice has shown increased ontogenesis as gravin suppresses cell cycle progression and metastasis in cancer (Akakura, Bouchard, Bshara, Morrison, & Gelman, 2011; Akakura, Huang, Nelson, Foster, & Gelman, 2008). These studies have shown that gravin plays an important role in regulating cell migration as well as cell proliferation. Gravin functions not only to assist in facilitating PKA's action, but also to regulate the desensitization / resensitization cycle of the β_2-AR (M Shih, Lin, Scott, Wang, & Malbon, 1999). Desensitization of β_2-AR obstructs its ability to respond to agonist stimulation, resulting in reduced contractility (Katz & Lorell, 2012; Dorn & Molkentin, 2004). Studies have shown that the ablation of gravin gene expression disrupts the association of GRK2 and β-arrestin with the β_2-AR (Tong, Bernstein, Murphy, & Steenbergen, 2005; Lin, Wang, & Malbon, 2000). Additionally, gravin scaffolds proteins, such as PKA and PKC, that can, under specific conditions, phosphorylate and desensitize the β_2-AR

(G. Fan et al., 2001). In our laboratory, we have shown in gravin knockout mice that (a) baseline cardiac contractility is increased, (b) contractility in response to acute β-AR stimulation is augmented, (c) chronic catecholamine stimulation results in normalization of β-AR stimulation through PKA signaling and (d) this restoration of β-AR signaling is responsible for the increased cardiac function in vivo.



Malbon CC. Nat Rev Mol Cell Biol. 2005 Sep; 6(9):689-701

Figure 4: Role of scaffold molecules in G-protein-signaling-complex formation and spatial localization

2.9.1 Gravin and Atherosclerosis

It has already been shown that disruption of the PKA/ AKAP complex can inhibit VSMC migration (Raymond, Carter, Ward, & Maurice, 2009).

Additionally, gravin (AKAP 12) is involved in phosphorylation of PKC and PKC substrates leading to a change in intracellular Ca^{2+}, which is associated with cellular function such as cell proliferation and migration (Langeberg & Scott, 2005). Activation of PKC isoforms by growth factor can regulate the expression of MMPs and VSMCs migration while selective PKC isoform inhibitors suppressed growth factor-induced MMP expression, activity and cell migration (Ding et al., 2011). It has been shown that Ang II can promote VSMC function like cell growth, migration and inflammation through activation of the AT_1R. Signaling cascades initiated by the AT_1R require activation of tyrosine kinases bound to the AT_1R (JAK2), transactivation of receptor tyrosine kinases (Src, Fyn, Yes, Pyk2, and FAK) and indirect activation downstream of other signaling cascades (i.e. calcium and/or PKC) (Yin et al., 2003). Based on these observations, the absence of gravin mediated signaling may reduce PKC activity by reducing phosphorylation of PKC dependent Src kinase, Pyk2 and FAK, as well as calcium homeostasis in response to atherogenic factors stimulation, which is related to decreased VSMC migration and MMPs expression (in particular MMP-9 expression). Some studies showed that gravin can also mediate the cellular lipid metabolism via induction of the activation of SREBPs, which are transcription factors of several cholesterol-associated gene (Choi et al., 2008; Moon et al., 2012).

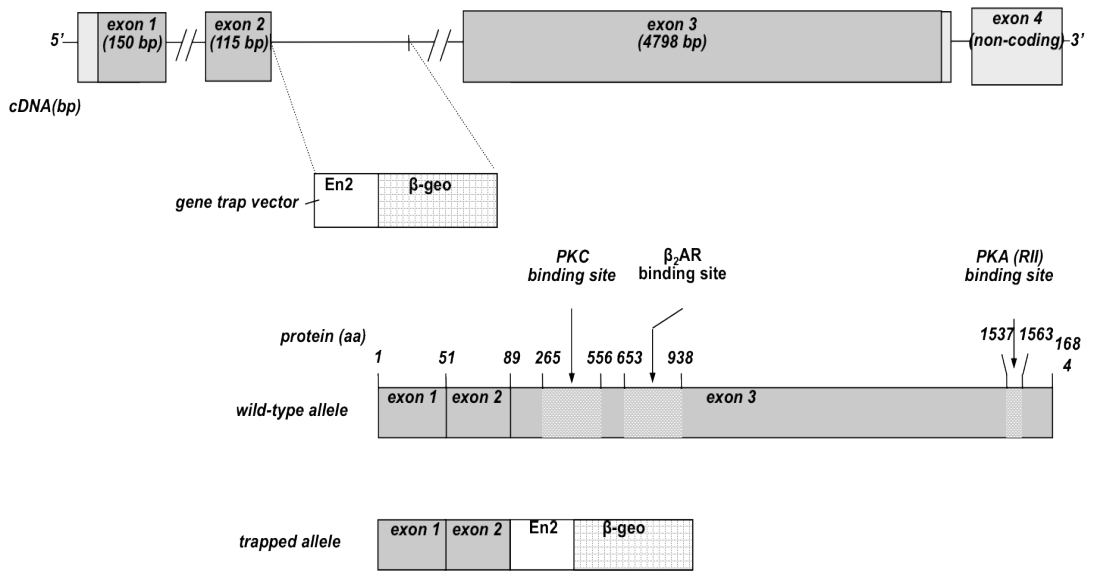
3 Methods and Materials

3.1 Animals and Diet

Gravin mutant mice were produced using gene trap technology to ablate the Akap12 (gravin) gene (NM_031185) (Figure 5) (Guillory et al., 2013). Homozygous mice lacking a functional gravin protein (designated gravin-t/t; where t refers to truncation) do not express the critical region- exon 3, which encodes the binding sites for β_2-AR, PKA or PKC binding (Guillory et al., 2013). Wild-type (WT) mice and homozygous mutant gravin mice (gravin-t/t) were bred on the C57BL/6J background. Gene expression was characterized by polymerase chain reaction (PCR) and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Protein expression was characterized by Western blot analysis. Both gene and protein expression experiments were used to confirm the absence or near absence of expression. Male WT and gravin-t/t mice were maintained at room temperature (22°C) with a 12-hour light/dark cycle and on a standard rodent chow until 5 weeks of age. Mice were randomly placed into four different groups: WT normal diet (ND) treated mice, gravin-t/t ND-treated mice, WT high fat diet (HFD) treated mice and gravin-t/t HFD-treated mice. The mice were then fed a Normal Diet (ND) (5053, Rodent

Diet 20, PicoLab) or a High Fat Diet (HFD) (TD88137, Teklad, Harlan Laboratories) for 16 additional weeks. At the end of the 16 weeks of specialized diet, the animals were sacrificed and the tissues were used for analysis.

All procedures involving experimental animals have been approved by the *Institutional Animal Care and Use Committee (IACUC)* and ethics committee at the University of Houston (UH; #UH-ACP-14-023), which are in accordance to the National Institutes of Health (NIH) guidelines. Animal care was provided for in AAALAC accredited animal barrier facilities at UH and have, therefore, been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.



Guillory, A. N. *et al.* PLoS One. 2013 Sep 18;8(9):e74784.

Figure 5: Disruption of the Akap12 (gravin) gene and the expression of the gravin in WT and gravin-t/t mice.

The strategy to ablate the Akap12 (gravin) gene (NM_031185) was achieved by using gene trap technology. The ES cell line (XE450), obtained from BayGenomics, contains genetically engineered retroviral gene trap. The gene trap vector (gene trap vector, En2: β-gal) integrated into the chromosome within the Akap12 gene, immediately following exon-2.

3.2 Western Blot Analysis for Protein in Tissues

Protein expression was performed by Western blot as previously described (McConnell et al., 2009). The tissues were homogenized using a

RIPA buffer containing 50 mM Tris Base pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol, and 0.25% Na-deoxycholate. To prepare nuclear and cytosolic extracts of liver for western blot analysis, approximately 0.1 g of frozen liver was homogenized as described previously (Ghose et al., 2011). Briefly, 0.1g frozen liver was homogenized by homogenizer in a cold hypotonic buffer containing 10 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and Protease Inhibitors (100 μl/ 10mL buffer) (Cat # 80053-846, VWR). Cytosolic fractions were isolated by centrifugation for 5 min at 5000 rpm at 4 °C. Nuclear fractions were then incubated with a lysis buffer containing 140 mM NaCl, 2 mM EDTA, 1% NP-40, 50 mM of Tris–HCl and protease inhibitor cocktail (100 μl/ 10ml buffer) (Cat# 80053-846, VWR). After incubation on ice, nuclear extracts were collected after centrifugation at 13,000 rpm for 5 min at 4 °C.

Protein homogenates were resolved by SDS-PAGE (4–12%) gradient gels (25 µg per well) and then transferred to polyvinylidinedifluoride (PVDF) membranes for immunoblots analysis using primary antibodies to AKAP250/gravin (AKAP12) (Cat# sc-33578; Santa Cruz), SREBP-2 (Cat# PA1-338; Thermo Scientific) and GAPDH (Cat# 2118S; Cell signaling). Blots were then incubated overnight at 4˚C with primary antibodies. The blots were

washed with TBS containing 0.1% Tween 20 (TBST), and then probed with the appropriate HRP-conjugated secondary antibodies (anti-mouse, Cat# 7076; anti-rabbit, Cat# 7074; Cell Signaling) for 1 hour. The signal was detected by using the SuperSignal West Pico Chemiluminescent Substrate (Cat# 34078; Thermo Scientific). Densitometric analyses of the immunoblots were performed via ImageJ Data Acquisition Software (National Institute of Health (NIH), Bethesda, MD). The anti-protein antibody signal was normalized to the GAPDH protein antibody signal. In some cases, blots were stripped and reprobed with other antibodies.

3.3 Protein Estimation

Protein concentrations in the cell's lysate were determined using a bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific Inc, IL) containing protein assay reagent A and reagent B according to the manufacturer's instructions. The optical density of the samples was measured at a wavelength of 562 nm using an Eppendorf NanoDrop spectrophotometer. Bovine serum albumin was used as a standard.

3.4 Blood Lipid Panel Measurements

Plasma lipid levels from serum samples were collected for analysis of the lipoprotein subtypes. Blood collection was obtained using the mandibulofacial artery blood collection method and cardiac puncture (Parasuraman, Raveendran, & Kesavan, 2010; Golde, Gollobin, & Rodriguez, 2005). The blood was collected in Microtainer No Additive Tubes (Cat# 365957; BD) and kept at room temperature for 30 to 45 mins. Then the serum samples were collected in another tube after centrifugation at 3000-5000 rpm for 10 minutes, according to the manufacturers' instructions. Total cholesterol, triglyceride, very low-density lipoprotein (VLDL), high-density lipoprotein (HDL), low-density lipoprotein (LDL), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured by using Cobas Integra 400 Plus (Roche; Baylor College of Medicine Core Facility) and Piccolo Express Chemistry Analyzer (Roche; University of Houston College of Pharmacy; Dr. Vincent Tam's Laboratory) using the Piccolo® Lipid Panel Plus reagent (Abbott), according to the manufacturers' instructions.

3.5 Oil Red O Staining

Whole aortas and livers were isolated and stained with enface Oil Red O solution, as described (Maganto-Garcia, Tarrio, & Lichtman, 2012). The fresh dissected livers were covered in OCT for 10-20 minutes in a labeled cryomold surrounding with dry ice. The cryostat sections (10 μm) of liver were used for Oil Red O staining. The entire en-face aorta and aortic arch were isolated and stained for atherosclerosis lesions by Oil Red O staining. In brief, the aortic tree from the aortic root to the abdominal aorta was dissected from the mice. The aorta was mechanically cleaned of adventitia. Aortas were then immersed in an Oil Red O solution (0.5% solubilized in 60% isopropanol) and subsequently differentiated in 85% propylene glycol. The complete stained vessel was photographed and the images digitized by blind observation. The bright field images of the aortas were taken using the Nikon Eclipse Ti microscope. Quantification of the atherosclerosis lesion (red) was measured via Image-Pro Plus (Media Cybernetics, Inc). The positive percentage of the areas of atherosclerotic lesion was calculated as the sum of red stained area divided by the sum of the whole aorta area (Koupenova et al., 2012).

3.6 Histological and Immunohistochemical Analysis

Tissues (whole aorta and liver) were harvested from WT and gravin-t/t

mice and then fixed in 10% formalin. In separate animal experiments, regions of thoracic and abdominal aortas were paraffin-embedded. The cross-sections (5 μm) were immunoblotted as previously described using antibodies for MMP9 (Cat# 3852; Cell Signaling) and F4/80 (Cat# MCA497BT; AbD Serotec). After deparaffinization and hydration, sections were blocked for endogenous peroxidase for 30 minutes using 3% H_2O_2 in deionized water and then washed with phosphate-buffered saline (PBS). The sections were incubated in a staining vessel containing pre-heated sodium citrate buffer (10 mM citric acid, pH 6.0) at 95-100°Cfor 10 minutes. After cooling for 20 minutes, the sections were rinsed with PBS. The tissue sections were then blocked with diluted normal blocking serum for 20 minutes. Aortic sections were then incubated overnight at 4˚C with primary antibodies. The sections were washed with PBS containing 0.1% Tween 20 (PBST), and then probed with the appropriate Biotinylated-conjugated secondary antibodies (anti-mouse, Cat# BA 9200; anti-rabbit, Cat# BA 1000; Vector laboratories). The VECTASTAIN® Elite ABC Reagent (Cat# PK-6100; Vector laboratories) and ImmPACT DAB Peroxidase Substrates (Cat# SK-4105; Vector laboratories) were used to detect the protein signal, according to manufacturers' instructions.

For morphological analysis, Liver cryostat sections (10 μm) were stained

with Hematoxylin and Eosin (H&E) solution (Mehlem, Hagberg, & Muhl, 2013). The images were then visualized using the BX41 Olympus microscope and along with a digital camera (Spot Insight 2, Diagnostic Instruments, Inc.).

3.7 Reverse Transcription Quantitative Polymerase Chain Reaction

Gene expression was quantified with Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR). RNA was isolated from tissue using RNeasy mini kit (Qiagen, U.S.A.) according to the manufacturer's instructions. The RNA purity and concentration were then assessed by a spectrophotometer. First strand cDNA was synthesized using the Invitrogen Superscript III Kit (Invitrogen, U.S.A.) according to the manufacturer's instructions. The mRNA levels were quantified with SYBR Green (Cat# 4368577; Applied Biosystems) using an ABI 7900 Sequence Detection System (StepOnePlus, Applied Biosystems). Each gene has triplicate reactions to get the mean Ct (cycle threshold) value. And the value was normalized against the mean expression (Ct value) of the housekeeping gene encoding18s RNA. Primers were used at 2.5 μM. The primers used were used at 5μM and are listed in *Table 1*.

Table 1: List of the qRT-PCR Primers.

Gene	Primer	Sequence
3’ end of exon 3 of gravin	Forward	5’-GCCAGTGAAGAACATGAGCA-3'
	Reverse	5'-TGCAATCTGCTTTGTCTTGG-3'
5’ end of exon 3 of gravin	Forward	5‘-GAGCAGGAGACCACCAAGAG-3'
	Reverse	5'-TTCTCCATCTTTGGCTGCTT-3'
FAS	Forward	5’-GGAGGTGGTGATAGCCGGTAT-3’
	Reverse	5’-TGGGTAATCCATAGAGCCCAG-3’
LPL	Forward	5’-TTGCCCTAAGGACCCCTGAA-3’
	Reverse	5’-ACAGAGTCTGCTAATCCAGGAAT-3’
SREBP-1c	Forward	5’-GATGTGCGAACTGGACA-3’
	Reverse	5’-CATAGGGGGCGTCAAACAG-3’
HMGCR	Forward	5’-GGCATTTGACAGCACTAGCA-3’
	Reverse	5’-CTTTGCATGCTCCTTGAACA-3’
LDLR	Forward	5’-CTCGCTGGTGACTGAAAACA-3’
	Reverse	5’-CAAAGGAAGACGAGGAGCAC-3’
SREBP-2	Forward	5’-AAGTCTGGCGTTCTGAGGAA-3’
	Reverse	5’-CACAAAGACGCTCAGGACAA-3’

Gene	Primer	Sequence
exon 1 of gravin	Forward	5‘-ATGGGTGCAGGCAGTTCC-3'
	Reverse	5'-CGGGATCTCCAGCTGCTC-3'
exon 2 of gravin	Forward	5’-CTCCCACAGAAGAATGGTCAG-3'
	Reverse	5'-GACTTCTTCCTCTTGCCCATC-3'
exon 3A of gravin	Forward	5‘-GAGCAGGAGACCACCAAGAG-3'
	Reverse	5'-TTCTCCATCTTTGGCTGCTT-3'
exon 3B of gravin	Forward	5’-TGGGCATCCTTCAAAAAGATG-3’
	Reverse	5’-CCTTAAGCTCTTCTTCCTTGT-3’
exon 3C of gravin	Forward	5’-GCCAGTGAAGAACATGAGCA-3'
	Reverse	5'-TGCAATCTGCTTTGTCTTGG-3'
18s RNA	Forward	5’-TCAAGAACGAAAGTCGGAGG-3’
	Reverse	5’-GGACATCTAAGGGCATCA C-3’

3.8 Blood Pressure Measurements

A mouse pressure-volume conductance catheter (PVR-1045; Millar Instruments) was inserted into the left carotid artery to measure systolic and diastolic blood pressures, mean arterial pressure and heart rate as previously described (Pacher, Nagayama, Mukhopadhyay, Bátkai, & Kass, 2008). Data

collection and analysis were performed using Labchart Pro (AdInstruments), according to the manufacturer's instruction.

3.9 Vascular Smooth Muscle Cell Isolation and Culture

Vascular smooth muscle cell isolation was performed similar to previous studies (Langenickel, Olive, & Boehm, 2008). Thoracic arteries were removed from 9-12 week old male WT and gravin-t/t mice and placed in cell culture dishes containing ice-cold PBS. Fat, adventitia and venous structures were removed by blunt dissection under a stereo microscope and the cleaned thoracic arteries were cut into small pieces and transferred into 3.5 cm cell culture dishes (BD) containing 1ml fresh Dulbecco's modified Eagle's medium (free of enzymes) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (M. A. Bioproducts). Explants were incubated at 37 °C in 5% CO_2 for 7-10 days and outgrown VSMCs were passaged by treatment with 0.25 x trypsin. Experiments were conducted on VSMCs that formed a confluent monolayer at passages 5-8 and were serum-starved by incubation in DMEM supplemented with 0.1% FBS for 72 hours and released by adding FBS to a final concentration of 20%.

3.10 Western Blot Analysis for Protein in Cell Lysates

Cells were incubated in cold lysis buffer (Cat# 7722, Cell signaling) and protease inhibitor cocktail (100 μl / 10ml buffer) (Cat# 80053-846,VWR) and extracts were cleared by centrifugation at 16,000 x *g* for 10 min. Protein concentration was measured using the BCA protein assay (Pierce) and were subsequently loaded onto gels with equal protein (25 μg/lane) followed by western blot analysis, using primary antibodies for total-p44/42 MAPK (ERK1/2) (Cat# 9107,Cell Signaling) and phospho-p44/42 MAPK (ERK1/2) (Cat# 9106, Cell Signaling), phospho-PKA substrates (Cat# 9621, Cell Signaling), phosphor (ser) PKC substrates (Cat# 2261, Cell Signaling), MMP-9 (Cat# 3852, Cell Signaling), total-PKCα/β II and phospho-PKCα/β II (Thr 638/641) (Cat# 9375, Cell Signaling), total- PKA C-α (Cat# 4782, Cell Signaling) and phospho-PKA C (Thr197) (Cat# 5661, Cell Signaling), AT_1R (Cat# sc-1173; Santa Cruz). The signal was detected using SuperSignal West Pico Chemiluminescent Substrate (Cat#34078; Thermo Scientific). Densitometric analyses of the immunoblots were performed via ImageJ Data Acquisition Software (National Institute of Health (NIH), Bethesda, MD). The anti-protein antibody signal was normalized to the GAPDH protein antibody signal. In some cases, blots were stripped and reprobed with other antibodies.

3.11 Measurement of Intracellular Calcium Using Fluo-8

Vascular smooth muscle cells (VSMCs) were grown in a DMEM medium (Gibco) containing 20% FBS, harvested with trypsin and plated into 12-well plates with crystal bottom (BD-Biocoat poly-lysine) at 3×10^5 cells per well. The VSMCs were starved with serum-free DMEM for 24 hours. Then, the cells were incubated with 4 μM of the calcium fluorescence indicator fluo-8 AM (AAT Bioquest, Inc), dissolved in DMEM supplemented with 2.5 mM Probenecid Acid (AAT Bioquest, Inc.) and Pluronic F-127 at room temperature or 37 °C, for 15 min. The cells in the plate were washed three times with a calcium indicator-free buffer (DMEM supplemented with 2.5 mM Probenecid Acid and 1% Pluronic F-68) to remove excess probes. The experiment was run at 490 nm / 514 nm (excitation/emission) using a Nikon Ti-S eclipse microscope (40X objective). After 10 seconds recording at baseline, calcium signaling for each well of cells was individually recorded in the presence and absence of 100 nM angiotensin II (Ang II) or 10 ng/ml PDGF.

3.12 In Vitro VSMCs Migration Following Scratch Wound Assays

Migration of mouse aortic VSMCs was investigated through the use of a standard *in vitro* Scratch wound assay. VSMCs were placed in 6-well cell culture plates at 2.5 x 10^4 cells per well in DMEM with 20% FBS in a humidified atmosphere of 5% CO_2 at 37 °C. When the cells were confluent, the cells monolayer were starved in DMEM with 0.1% FBS, then 24 hours later, the confluent cells were scratched with a sterile rubber policeman to create cell-free zones and allowed to migrate to the zones. Cells were then cultured with or without the treatments of growth factors. Angiotensin II (Ang II) or platelet-derived growth factor (PDGF) was added to the culture medium at final concentrations of 100 nM and 10 ng/ml, respectively. A reference point was created on the bottom of the plate each time in the field of the wound using direct microscopic visualization. This procedure allowed photographing the same spot each time. The remaining cell-free area was determined via microphotography performed immediately after scratching the wound as well as 24 hours after treatment. The cells were subsequently washed with PBS to remove unattached cells and incubated in DMEM with 0.1% FBS with or without Ang II (10 μM) and with or without PDGF (10 ng/ml) at 37°C. Three

random fields of view were photographed at 40x magnification using a phase contrast microscope (Nikon Instruments, Inc.) at baseline (immediately after creating the zones) and at 24 h after wounding.

3.13 VSMCs Proliferation by MTS Assays

VSMCs proliferation was determined by using MTS assays (Cell Titer 96, Promega). Briefly, the VSMCs were placed in 96-well culture plates at 2.5×10^3 cells/well at 37 °C in 5% CO_2. After 24 hours, the confluent cells monolayer were incubated in DMEM with 0.1% FBS. After another 24 hours, the cells were incubated in DMEM with 0.1% FBS with or without Ang II (10 nM) and with or without PDGF (10 ng/ml) at 37 °C and allowed to proliferate for 1, 3 or 5 days, followed by incubation with MTS reagent for 2 hours. Then, aliquots were pipetted into 96-well plates according to the supplier's protocol. The absorbance of each well was measured at 490 nm using a microplate reader (Model 680, Bio Rad).

3.14 PKA and PKC Activity

PKA and PKC activity were determined via a non-radioactive

immunoassay kit (Cat# ADI-EKS-390A and Cat# ADI-EKS-420A; Enzo LifeSciences) according to the manufacturer's instructions. PKA and PKC activity were determined via a non-radioactive enzyme immunoassay according to the manufacturer's instructions. The kinase activity assay is based on enzyme-linked immune-absorbent assay (ELISA) that used a synthetic peptide as the substrate and a polyclonal antibody that recognized the phosphorylated form of the substrate. The substrate was pre-coated onto wells of a 96- well plate. The samples to be assayed (30 µls) were added followed by the addition of ATP to initiate the reaction. After the addition of the polyclonal antibody and the peroxidase conjugated secondary antibody, the assay was developed with tetramethylbenzidine substrate (TMB). The color development is proportional to the phosphotransferase kinase activity. Color development was stopped by addition of acidic stop solution and the absorbance was measured at 450nm using a micro plate reader. Each experiment was performed in quadruple. Relative PKA and PKC activity were normalized to milligrams of protein.

3.15 Statistical Analysis

Data were processed using Microsoft Excel and GraphPad Prism 5.0. All

values are present as mean ± S.E.M. Comparisons between the two groups were determined using unpaired 2-tailed Student's *t* test. Analysis was performed using one-way ANOVA, followed by a Tukey's Multiple Comparison Test when multiple groups were compared. P values of less than 0.05 were considered significant.

4 Gravin-t/t Mice Shows Resistant to Hyperlipidemia and Atherosclerosis

4.1 Characterization of Gravin-t/t Mice in Response to High Fat Diet

To characterize the effect of gravin truncated (gravin-t/t) mice treated with HFD, male WT, gravin-t/t were fed with either a ND (total fat: 4.7% by weight; 14% kcal from fat) or a HFD (total fat: 21% by weight; 42% kcal from fat) for 16 continuous weeks, starting at 5 weeks of age until 21 weeks of age. Body weight, food consumption and water intake were measured throughout the 16 weeks administration of continuous ND and HFD treatment. As expected, with a diet high in calories derived from fats, the body weight of both WT and gravin-t/t mice increased when treated with the HFD (Figure 6; lower panel). Interestingly, the increase in body weight in response to the HFD was significantly less in gravin-t/t versus WT mice, starting at 2 weeks and continuing for the duration of the 16 weeks of the HFD-treatment (116% increase in body weight in WT mice versus 93% increase in body weight in gravin-t/t mice). There was no difference between WT and gravin-t/t mice fed on the ND. A whole animal image showing the difference in body weight is

shown in Figure 6 (upper panel). Although food consumption for both WT and gravin-t/t mice was significantly more in HFD-treated mice versus ND-treated mice, respectively, there was no significant difference in the amount of food consumed per body weight between WT and gravin-t/t mice on either diet (Figure 7; upper panel). In addition, there was no significant difference in water consumption for either the WT and gravin-t/t mice or between the ND and HFD-treatment (Figure 7; lower panel).

To determine the effects of a diet high in fat on key organs involved in lipid metabolism and cardiac circulation, the liver-weight to body-weight (LW / BW) ratio and the heart-weight to body-weight (HW / BW) ratio were measured in WT and gravin-t/t mice on either the ND or HFD. The LW / BW ratio was significantly greater in WT HFD-treated versus WT ND-treated mice whereas there was no difference in gravin-t/t HFD-treated versus gravin-t/t ND-treated mice (Figure 8). Furthermore, the LW / BW ratio was significantly reduced in gravin-t/t HFD versus WT HFD-treated mice ($p=0.0251$). Although there was no difference in the HW / BW ratio in WT and gravin-t/t ND-treated mice, there was an observed increase in the HW / BW ratio in the gravin-t/t versus WT HFD-treated mice (Figure 8). These data indicate that mice lacking functional gravin protein have significantly reduced liver weight but slightly increased

heart weight in response to HFD.

4.2 Gravin-t/t Mice Protects Against Hypertension

Diets rich in fat have been shown to be involved in hypertension, and this elevation in blood pressure is known to be associated with the development of atherosclerosis (Wilde, Massey, & Walker, 2000). To examine the effect of mice lacking functional gravin protein on blood pressure, a mouse pressure-volume conductance catheter was inserted into the right carotid artery of WT and gravin-t/t mice that were treated with either ND or HFD for 16 weeks. The heart rate was also measured and was derived from the electrocardiogram recording. As expected, we observed an increase in systolic and diastolic blood pressures as well as the mean arterial pressure in WT HFD-treated mice as compared to WT ND-treated mice (Figure 9). Interestingly, however, there was a significant decrease in systolic pressure (Figure 9A), diastolic pressure (Figure 9B) and mean arterial pressure (Figure 9C) in gravin-t/t HFD-treated mice versus WT HFD-treated mice. There were no significant differences in either the blood pressure or the arterial mean pressure between WT and gravin-t/t ND-treated mice and also no significant difference between gravin-t/t ND-treated and gravin-t/t HFD-treated mice

(Figure 9A-C). Additionally, there were no significant differences in the heart rate between WT and gravin-t/t, either treated with ND or HFD (Figure 9D). These results indicate that mice lacking functional gravin protein have significantly reduced blood pressure in response to HFD.

4.3 Gravin-t/t Mice Protects Against Atherosclerosis

We previously reported (Guillory et al., 2013) that gravin gene (*Akap12*) transcription was significantly reduced (approx. 90%) and that less than 10% of the gravin protein was expressed in gravin-t/t hearts versus WT hearts; mice generated using gene trap technology, that uses genetically engineered retroviruses which had integrated into the chromosome within the *Akap12* gene, permitting the generation of global gravin truncated mice (designate gravin-t/t; *where t refers to truncated alleles*). In this study, it has now determined the gravin gene and protein expression in aortas isolated from WT and gravin-t/t mice. Gravin gene expression was quantified by reverse transcription quantitative PCR (RT-qPCR) using SYBR Green and specific forward and reverse primers for exon 3 (two regions of exon 3; 3'-end of exon 3 and 5'-end of exon 3). Exon 3 of gravin encodes for approximately 95% of the *Akap12* gene and this region of the gravin protein also contains the binding

domains for PKA, PKC, β_2-AR and various other signaling proteins (Meiling Shih, Lin, Scott, Wang, & Malbon, 1999). Figure 10A (*upper panel*) shows that the PCR products of exon 3 of gravin was absent in gravin-t/t aortic tissue and that the RT-qPCR quantitative gravin gene expression in the aorta was significantly reduced by 85% (3'-end of exon 3) and 86% (5'-end of exon 3) (*lower panel*). As a control to show equivalent gene expression and cDNA amplification, 18s ribosomal RNA (rRNA) was quantified and used as an internal reference gene to normalize the other gene expression in aortas isolated from WT and gravin-t/t mice. Figure 10B shows that the gravin protein was expressed in WT aortic tissue (MW = 250 kDa; monomer), as recognized by the gravin antibody specific to the carboxyl-terminal amino acids (encoded by exon 3), but was absent in gravin-t/t aortic tissue. GAPDH (MW = 37 kDa) was used as a control housekeeping protein to normalize equal aortic protein loading between WT and gravin-t/t.

Diets high in fats are also directly associated to aortic atherosclerosis where excessive amounts of extracellular lipids, along with migrated and proliferated VSMCs and extracellular matrix, form plaques on the aortic luminal wall followed by calcification, hardening of the aorta and, eventually, restriction of blood flow(Lusis, 2000). Together, these events lead to hypertension and

potentially the basis of a heart attack or stroke due to plaque rupture (Robinson et al., 2009). In order to characterize the development of atherosclerosis in mice lacking functional gravin, whole aortas were isolated and stained with enface Oil Red O solution and regions of abdominal aortas were sectioned and stained for MMP9 or F4/80 expression using WT and gravin-t/t mice treated with either ND or HFD for 16 weeks. Figure 11 (*upper panel*) shows the Oil Red O staining of the enfaced aortas from the ND and HFD-treated WT and gravin-t/t mice, identifying atherosclerotic lesions. These aortic lesions were then quantified, as shown in Figure 11 (*lower panel*). The expected increase in the observed aortic lesions in the WT HFD-treated versus the WT ND-treated mice was significantly reduced in the aortas isolated from gravin-t/t HFD-treated mice ($p = 0.0073$); while there was no significant difference between WT ND and gravin-t/t ND. To confirm the observed reduced aortic lesions in gravin-t/t HFD-treated mice versus WT HFD-treated mice, an immunohistochemical analysis of the abdominal aorta was performed using the known atherosclerotic marker, matrix metallopeptidase 9 (MMP-9) and using the monocyte / macrophage specific surface marker, F4/80. Decreased atherosclerosis formation in the abdominal aortas isolated from gravin-t/t HFD treated mice was confirmed, as shown by decreased MMP-9 aortic expression (Figure 12A-B) and decreased F4/80 aortic expression

(Figure 12C-D) in the gravin-t/t HFD-treated mice. These results indicate that mice lacking functional gravin protein have significantly less plaque formation and inflammatory response in response to HFD.

4.4 Loss of Functional Gravin Reduces the Serum Lipid Profile

Since the development of atherosclerosis and the presence of aortic plaques is associated with the dysregulation of serum lipids, we measured the serum levels of cholesterol, triglycerides, very-low density lipoprotein (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) in WT and gravin-t/t mice following 16 weeks treatment of either ND or HFD (Assini et al., 2013). Blood was collected from the mandibulofacial artery and the serum lipid levels were measured using the Cobas Integra 400 Plus and Piccolo Express Chemistry Analyzer. As expected, an increase in serum cholesterol was observed in WT HFD-treated mice as compared to WT ND-treated mice, as well as increase in cholesterol in gravin-t/t HFD-treated mice (Figure 13A). However, this increase in serum cholesterol observed in the gravin-t/t HFD-treated mice was significantly decreased as compared to the WT HFD-treated mice (Figure 13A), while there is no significant difference

between WT ND-treated mice and gravin-t/t ND-treated mice. Also as expected, serum triglyceride levels were increased in WT HFD-treated mice as compared to WT ND-treated mice; however, the triglyceride levels in gravin-t/t HFD-treated mice were significantly lower as compared to the WT HFD-treated mice (Figure 13B) while there is no significant difference between WT ND-treated mice and gravin-t/t ND-treated mice. Similarly, serum VLDL levels in gravin-t/t HFD-treated mice were significantly lower as compared to the WT HFD-treated mice (Figure 13C). Interestingly, HDL and LDL serum levels were similarly increased in both WT HFD-treated and gravin-t/t HFD-treated mice; however, there were no significant differences in either HDL or LDL levels between WT and gravin-t/t ND-treated and also no significant differences in WT and gravin-t/t HFD-treated mice (Figure 13D-E). These results indicate that mice lacking functional gravin protein have significantly reduced cholesterol, triglycerides and VLDL levels, but no differences in HDL and LDL levels in response to HFD.

4.5 Down-regulates Expression of Hepatic Genes Involved in Lipid and Cholesterol Metabolism and thus Affecting Liver Lipid Accumulation

Since gravin-t/t mice had an altered serum lipid profile, and the liver is a major organ involved in lipid metabolism, we therefore then determined that gravin was indeed expressed in the liver of WT mice and also the absence of gravin expression in liver of gravin-t/t mice. Thus, in order to confirm the presence of gravin expression in the liver, studies were first performed to measure gravin gene expression by RT-qPCR as described above. Figure 14A (*upper panel*) shows that gravin is indeed expressed in the liver of WT mice and that the expression of gravin was reduced in the liver of gravin-t/t mice. Using RT-qPCR, gravin gene expression in the liver was found to significantly reduce by 64% (3'-end of exon 3) and 78% (5'-end of exon 3) in gravin-t/t liver tissue (Figure 14A; *lower panel*). To normalize gene expression and cDNA amplification, 18s rRNA was quantified and used as an internal reference gene between livers isolated from WT and gravin-t/t mice. Figure 14B shows that gravin was also expressed in WT liver tissue (MW = 250 kDa; monomer), but was absent in gravin-t/t liver tissue. GAPDH (MW = 37 kDa) was used as a control housekeeping protein to confirmed equal liver protein loading between WT and gravin-t/t.

Gravin also has been reported to be expressed in various tissues isolated from mice, rat and human tissues (Streb, Kitchen, Gelman, & Miano, 2004).

Therefore, to determine the expression of gravin in other tissues, we measured gravin gene (Figure 15; *upper panel*) and gravin protein (Figure 15; *lower panel*) expression in the heart, liver, brain, skeletal muscle, testes, lungs, kidney and pancreas. Gravin gene expression was also found to be significantly reduced or absent in the isolated tissue analyzed from gravin-t/t mice (Figure 15;*upper panel*). Although the gravin gene is expressed in each of the tissues analyzed from WT mice, its expression was observed to be lower in the liver. We then determined whether the gravin protein expression correlated to its gene expression. The gravin antibody recognizes both the dimeric (MW = 500 kDa) and the monomeric (MW = 250 kDa) form of the protein where, under certain conditions, gravin can form a dimeric form when analyzed using a denaturing SDS-PAGE gel(Gao, Wang, & Malbon, 2011). In the WT tissue analyzed, the gravin antibody recognized both the dimeric form of gravin (in the heart, brain, skeletal muscle, testis, lung and pancreas) and the momeric form of gravin (in each of the tissues analyzed) (Figure 15; *lower panel*). Similar to gravin gene expression, we also observed a broad range in gravin protein expression levels in these other tissues isolated from WT mice. Specifically, gravin expression in WT mice was expressed at highest levels in testis, brain, lungs and heart. Also, gravin protein expression in gravin-t/t mice was found to be absent or significantly reduced in heart, liver, skeletal muscle,

kidney, lungs and pancreas. Interestingly, in brain and testis, where the gravin protein is known to be highly expressed (Streb et al., 2004) the gravin protein expression in the brain was similar between WT and gravin-t/t mice, whereas, in the testis, gravin protein expression was reduce by more than 50% in gravin-t/t as compared to WT. GAPDH (MW = 37 kDa) was used as a control housekeeping protein to confirm equal aortic protein loading between WT and gravin-t/t.

Because the liver is a major organ involved in lipid metabolism and the absence of gravin in the liver is associated with reduced serum cholesterol, triglycerides and VLDL, it was then determined whether the accumulation of fat in the liver was altered in WT and gravin-t/t mice following 16 weeks of treatment of either ND or HFD. First, significantly less lipid accumulation in the liver of gravin-t/t HFD-treated mice was observed as compared to WT HFD-treated mice, shown in both the H&E stained liver sections (Figure 16A-B) and the Oil Red O stained (counterstained with Hematoxylin) liver sections (Figure 16C-D). There was no significant difference in the levels of lipid accumulation in liver sections between WT and gravin-t/t ND-treated mice (Figure 16C-D). Also, the livers from gravin-t/t mice were protected from liver damage, as shown in H&E staining (Figure 16A-B). Second, to determine

whether this observation of increased lipid accumulation in WT HFD but not gravin-t/t HFD-treated mice was associated with liver injury, we quantified the two enzymes found in blood serum, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), that are known to be associated with liver damage or disease. As expected, we observed an increase in both ALT (Figure 17A) and AST (Figure 17B) serum levels in WT HFD-treated mice as compared to WT ND-treated mice. In contrast, there was no significant increase in either ALT or AST levels in gravin-t/t HFD-treated versus gravin-t/t ND-treated mice (Figure 17A-B); where these enzyme levels were significantly reduced in gravin-t/t HFD versus WT HFD-treated mice. Together, we observed that mice lacking gravin expression in the liver were associated with significantly decreased lipid accumulation and less damage in the liver as well as significantly decrease enzymes in the serum associated with liver damage in response to HFD.

Finally, to determine whether altered serum lipid metabolism, liver lipid accumulation and liver damage were associated with altered liver lipogenesis in gravin-t/t mice, it was then determined whether critical hepatic receptors and enzymes involved in lipid and cholesterol metabolism were altered by RT-qPCR in WT and gravin-t/t mice following 16 weeks treatment of either ND

or HFD.A significant increase in fatty acid synthase (FAS) gene expression, the enzyme that catalyzes fatty acid synthesis, was observed in WT HFD-treated mice as compared to WT ND-treated mice. However, FAS expression was significantly decreased in gravin-t/t HFD-treated versus WT HFD-treated mice (2.2 fold; $p<0.0001$) (Figure 18A). In contrast, there was no significant difference in the expression of lipoprotein lipase (LPL), the enzyme that hydrolyzes triglycerides into lipoproteins, between WT and gravin-t/t mice with either ND or HFD-treatment (Figure 18B) even though trends toward increased expression in HFD-treated mice were observed. In regard to genes involved in cholesterol biosynthesis in the liver, 3-hydroxy-3-methyl-glutaryl-CoA reductase or HMG-CoA reductase (HMGCR), the rate-controlling enzyme of the mevalonate pathway that produces cholesterol was significantly increased in WT HFD-treated mice as compared to WT ND-treated mice. In contrast, HMGCR expression was not affected in gravin-t/t mice in response to the HFD versus ND treatment (down-regulated by 4.28 fold versus WT HFD-treatment; $p=0.0003$) (Figure 18C). Similar to HMGCR expression, the expression of low density lipoprotein receptor (LDLR), which mediates endocytosis of cholesterol-rich LDL, was down-regulated in the liver by 0.98 fold ($p<0.0001$) in gravin-t/t HFD-treated versus WT HFD-treated mice (Figure 18D). Together, these results indicate that mice

lacking functional gravin protein have significantly reduced hepatic gene expression involved in lipid and cholesterol metabolism in response to HFD.

4.6 Gravin Dependent Regulation of Lipid Metabolism is Associated with SREBP-2

To account for these differences in liver damage and lipid accumulation, accompanied by changes in circulating lipid levels, aortic plaque formation, blood pressure and body weight, it was hypothesized that transcription factors in the liver regulating lipid metabolism was decreased in the absence of gravin mediated signaling. The Sterol Regulatory Element-Binding Proteins (SREBPs) are the transcription factors that regulate cholesterol and fatty acid biosynthesis; thus, a decrease in SREBPs expression could account for the decreased FAS, HMGCR and LDLR gene expression. Therefore, the expression of SREBPs by RT-qPCR in WT and gravin-t/t mice following 16 weeks treatment of either ND or HFD was measured. The expression of SREBP-1c, the SREBP isoform that regulates *de novo* lipogenesis, was not different between WT and gravin-t/t mice in either ND or HFD-treated mice (Figure 19A). Thus, this correlates with the no changes observed in LPL gene expression. However, it was found that the expression of SREBP-2, the

SREBP isoform that regulates the genes involved in cholesterol metabolism, was significantly reduced in gravin-t/t versus WT mice (down-regulated by 0.67 fold versus WT ND-treatment; $p=0.0024$) (Figure 19B). The decreased liver SREBP-2 gene expression of gravin-t/t mice was then further investigated to determine this correlated to a decrease in the SREBP-2 protein expression in liver of the ND and HFD-treated mice. Interestingly, it was found that less biologically SREBP-2 precursor was cleaved and biologically activated as nuclear SREBP-2n in gravin-t/t HFD versus WT HFD-treated mice (down-regulated by 0.49 fold versus WT HFD-treatment; $p<0.0001$) (Figure 20). Thus, this gravin-dependent regulation of lipid metabolism is likely to be mediated by SREBP-2.

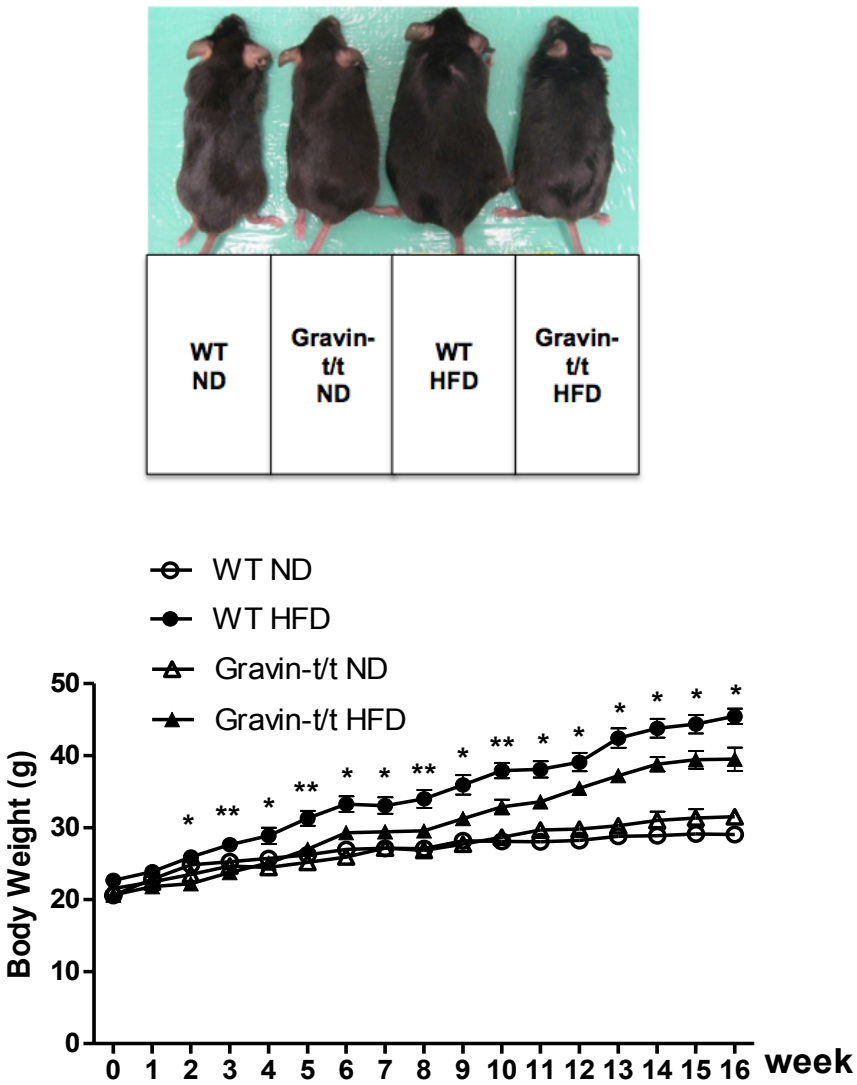


Figure 6: Gravin-t/t mice responses to ND and HFD. Body weight of WT and gravin-t/t mice on the ND and the HFD was measured from 0 to 16 weeks. Gravin-t/t mice showed significant reduction in body weight since week 2 as compared to WT on the HFD. No significant difference in body weight was observed between gravin-t/t and WT on the ND from week 0 to 16. The *upper panel* is the representative image for all the mice. The *lower panel* shows the quantification analysis for body weight. The asterisks indicate the comparison between WT and gravin-t/t with HFD. Data are expressed as the mean ± S.E.M.; n=6; *p-value < 0.05; **p-value < 0.01.

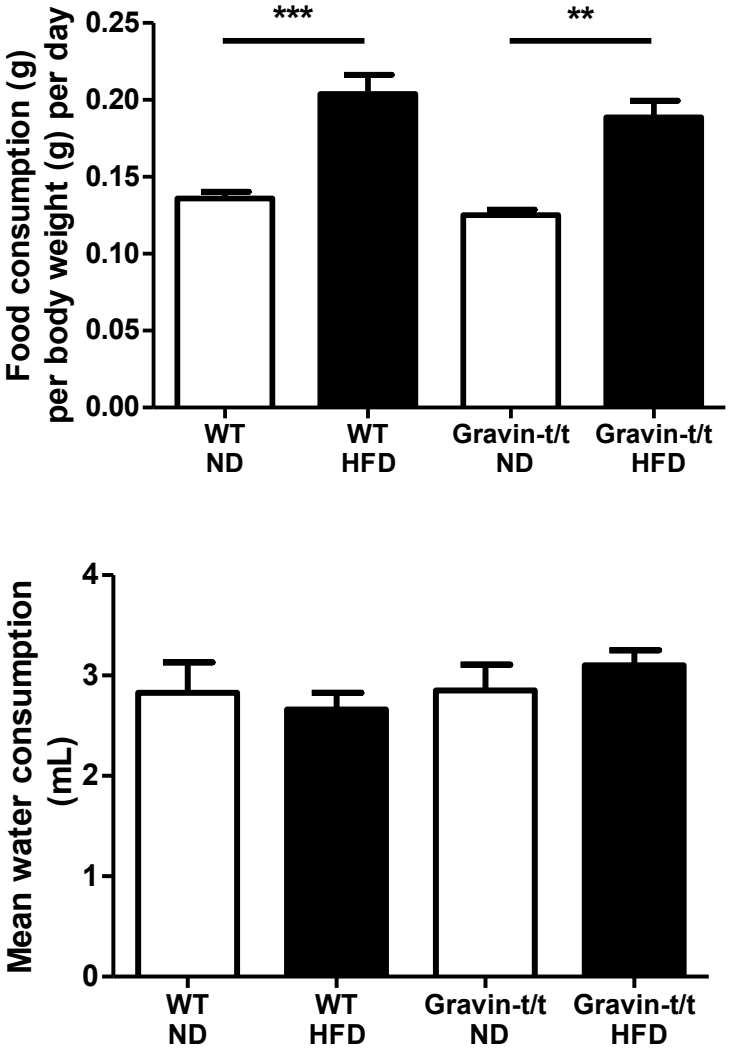


Figure 7: Food and water consumption. No significant differences in food consumption and water consumption were observed between WT and gravin-t/t on the HFD during the course of 16 weeks. Data are expressed as the mean ± S.E.M.; n=13; **p-value < 0.01; ***p-value <0.001.

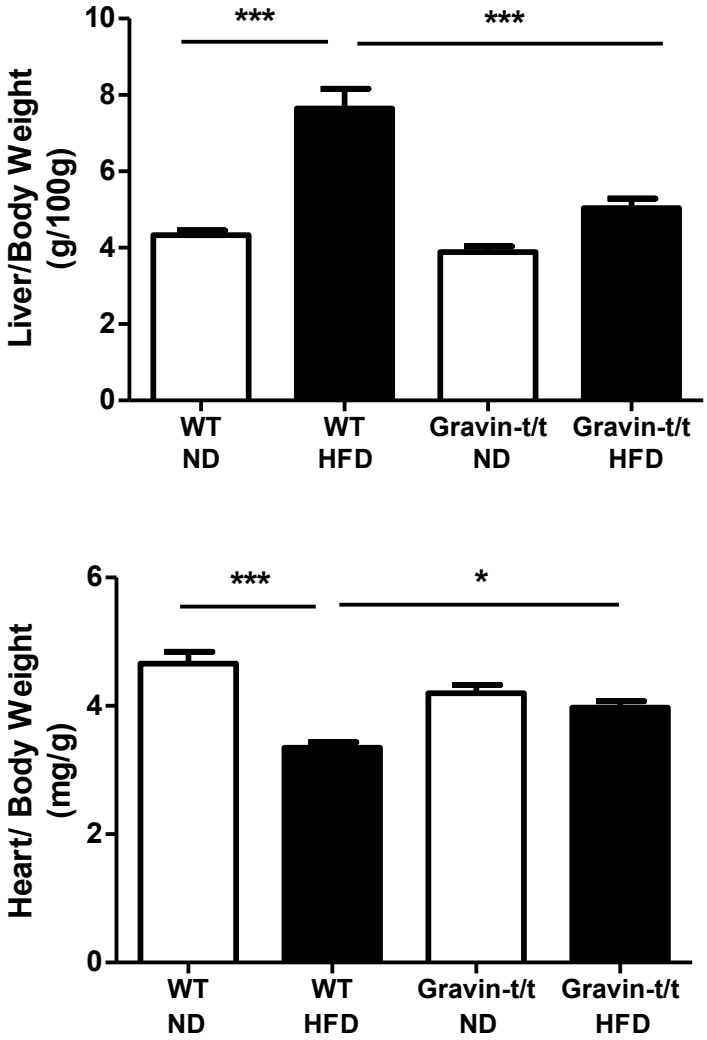


Figure 8: Liver/body weight ratio and Heart/body weight ratio. Liver weight / body weight ratio and heart weight / body weight ratio of WT and gravin-t/t mice were measured at 16-weeks of age. Results are presented as mean ± SEM; n=7-11; *p-value < 0.05; ***p-value < 0.001.

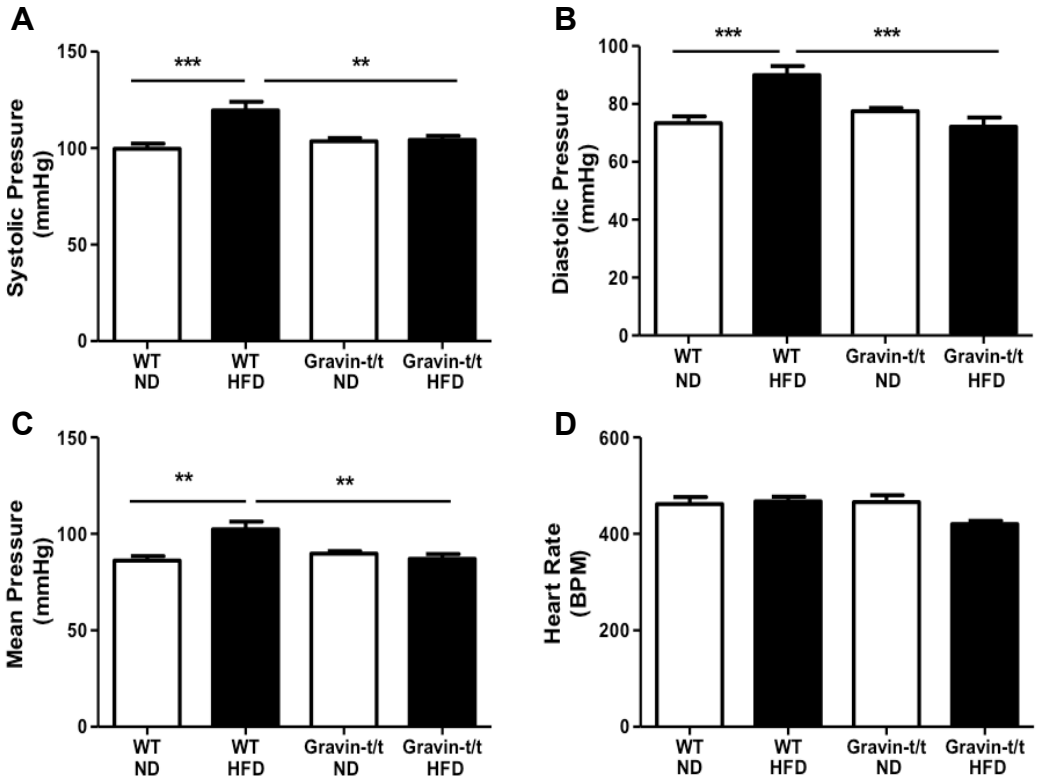
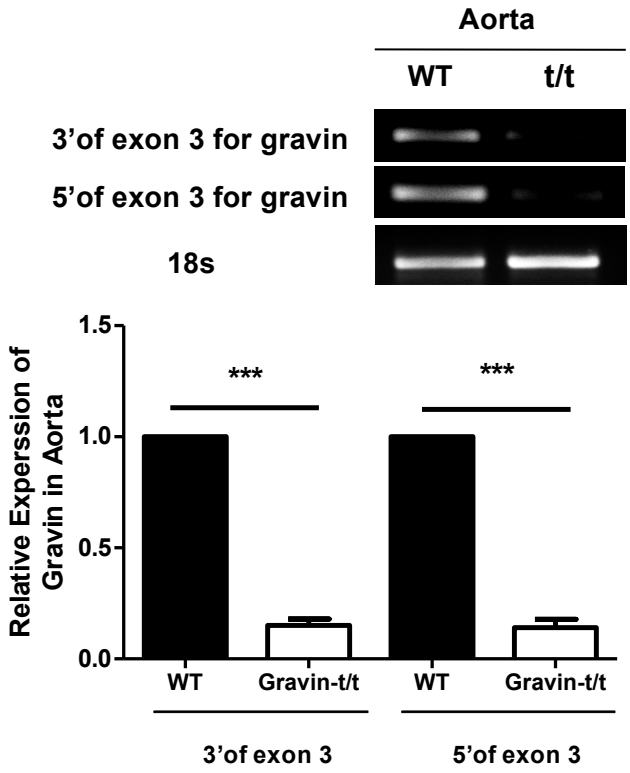


Figure 9: Gravin-t/t mice show decreased blood pressure in response to HFD. Gravin-t/t HFD-treated mice showed reduced **(A)** systolic blood pressure, **(B)** diastolic blood pressure and **(C)** mean blood pressure as compared to WT when measured at 16-weeks. No difference in the **(D)** heart rate was observed. Results are presented as mean ± SEM; n=7-9; *p-value < 0.05; **p-value < 0.01, ***p-value < 0.001.

A


Aorta
WT
t/t
3'of exon 3 for gravin
5'of exon 3 for gravin
18s
Relative Experssion of Gravin in Aorta
1.5
1.0
0.5
0.0
***
***
WT
Gravin-t/t
WT
Gravin-t/t
3'of exon 3
5'of exon 3

B

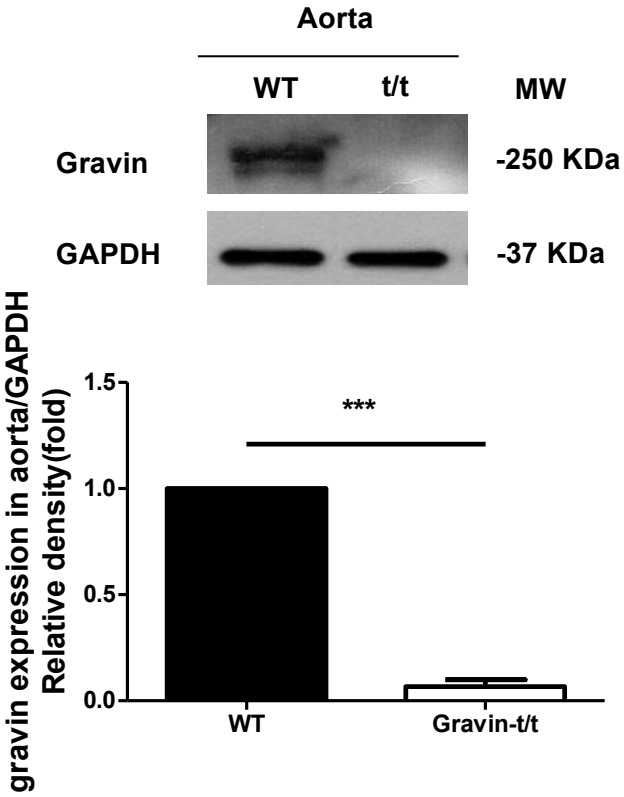

Aorta
WT
t/t
MW
Gravin
-250 KDa
GAPDH
-37 KDa
gravin expression in aorta/GAPDH Relative density(fold)
1.5
1.0
0.5
0.0
***
WT
Gravin-t/t

Figure 10: Gravin expression in aorta. (A) Gravin mRNA expression was quantified in the aorta by RT-qPCR from WT and gravin-t/t mice. Gravin specific primers amplified regions of exon 3 that were either 3' or 5' of the exon. The aorta from gravin-t/t mice showed significantly decreased gravin mRNA expression as compared to WT mice. Results are presented as the mean ± SEM; n = 4; ***p-value = 0.0006. **(B)** Gravin protein expression was measured in the aorta by Western Blot from WT and gravin-t/t mice where the gravin antibody recognized momeric (250 KDa) forms of the gravin protein. The aorta from gravin-t/t mice showed significantly decreased gravin protein expression as compared to WT mice, consistent with the gravin mRNA aorta expression results. The lower panel showed the statistic quantification. Results are presented as the mean ± SEM; n = 3; ***p-value < 0.001.

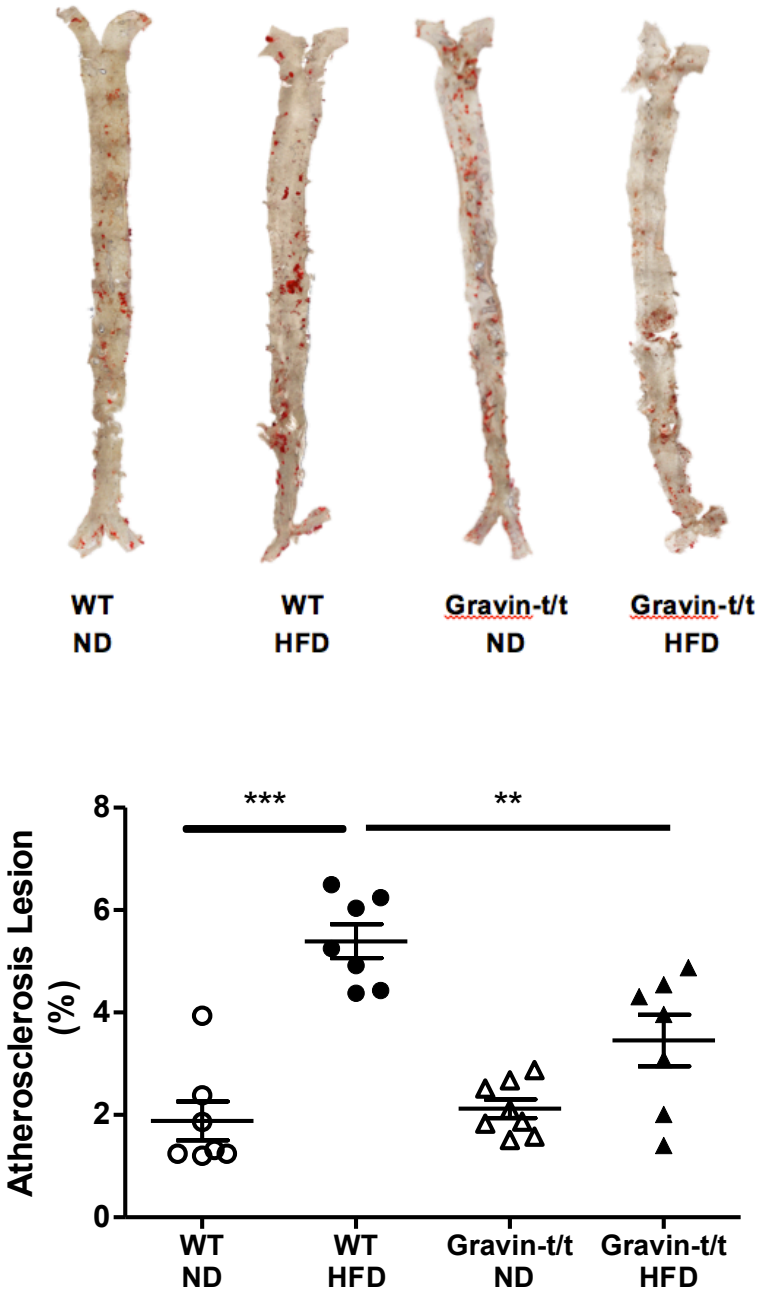


Figure 11: Lipid lesion in the aorta. Dissected whole aortas were stained enfaced with Oil Red O solution, isolated from WT and gravin-t/t mice following treatment with either ND or HFD for 16-weeks (*upper panel)* and then quantified for atherosclerotic plaque formation by Image-Pro Plus (*lower panel*). Representative whole aortas images showing atherosclerotic plaques are shown (upper panel), n=7-8**;** **p-value < 0.01, ***p-value < 0.001.

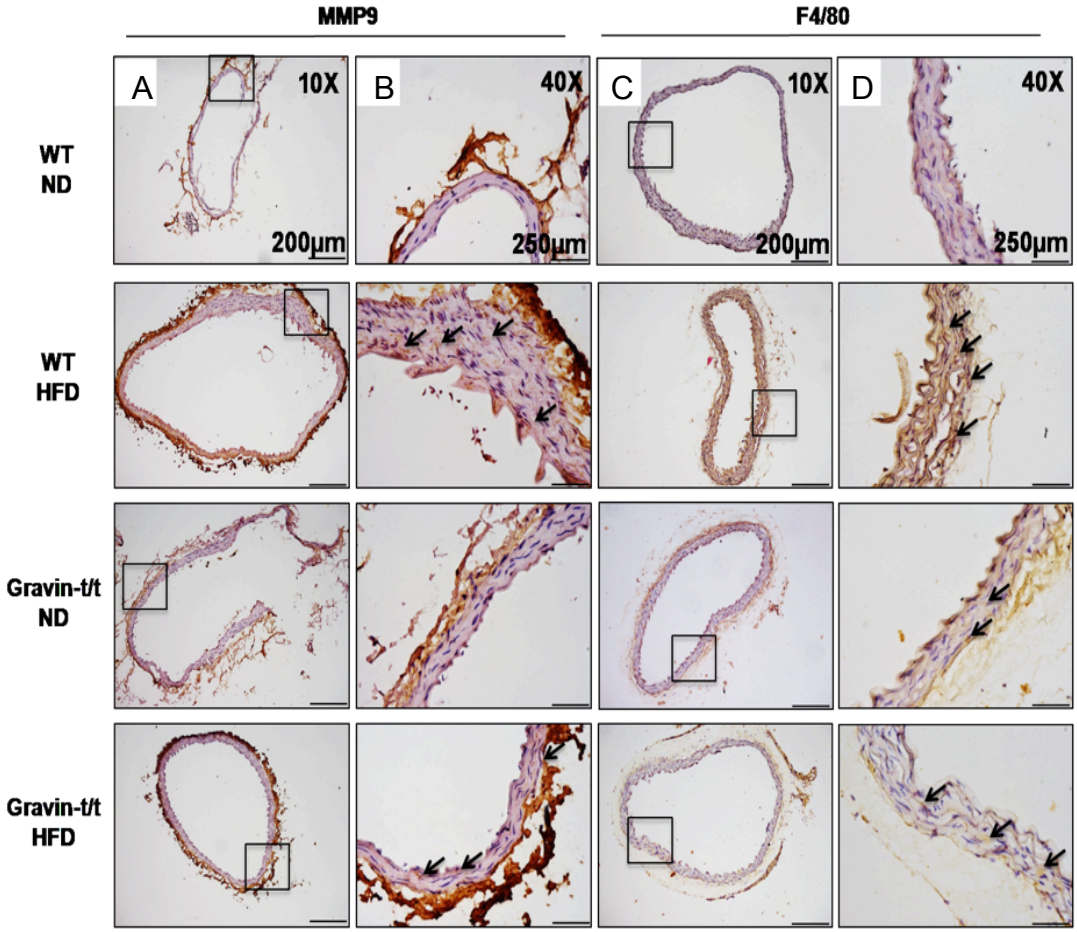


Figure 12: Immunohistochemical analysis of aortic section of WT and gravin-t/t mice. Cross-sections of paraffin-embedded thoracic and abdominal aortas stained with antibodies to MMP-9 (**A** and **B**) and F4/80 (**C** and **D**). Images were measured by Nikon microscopy (Bright field, 10*x* (**A** and **C**) & 40*x* (**B** and **D**).Gravin-t/t HFD-treated mice showed lower aortic MMP-9 and aortic F4/80 expression as compared to WT HFD-treated mice. Boxed areas shown in **A** and **C** panels are shown in higher magnification in panels **B** and **D**, respectively. Arrows indicate the expression of the protein of interest.

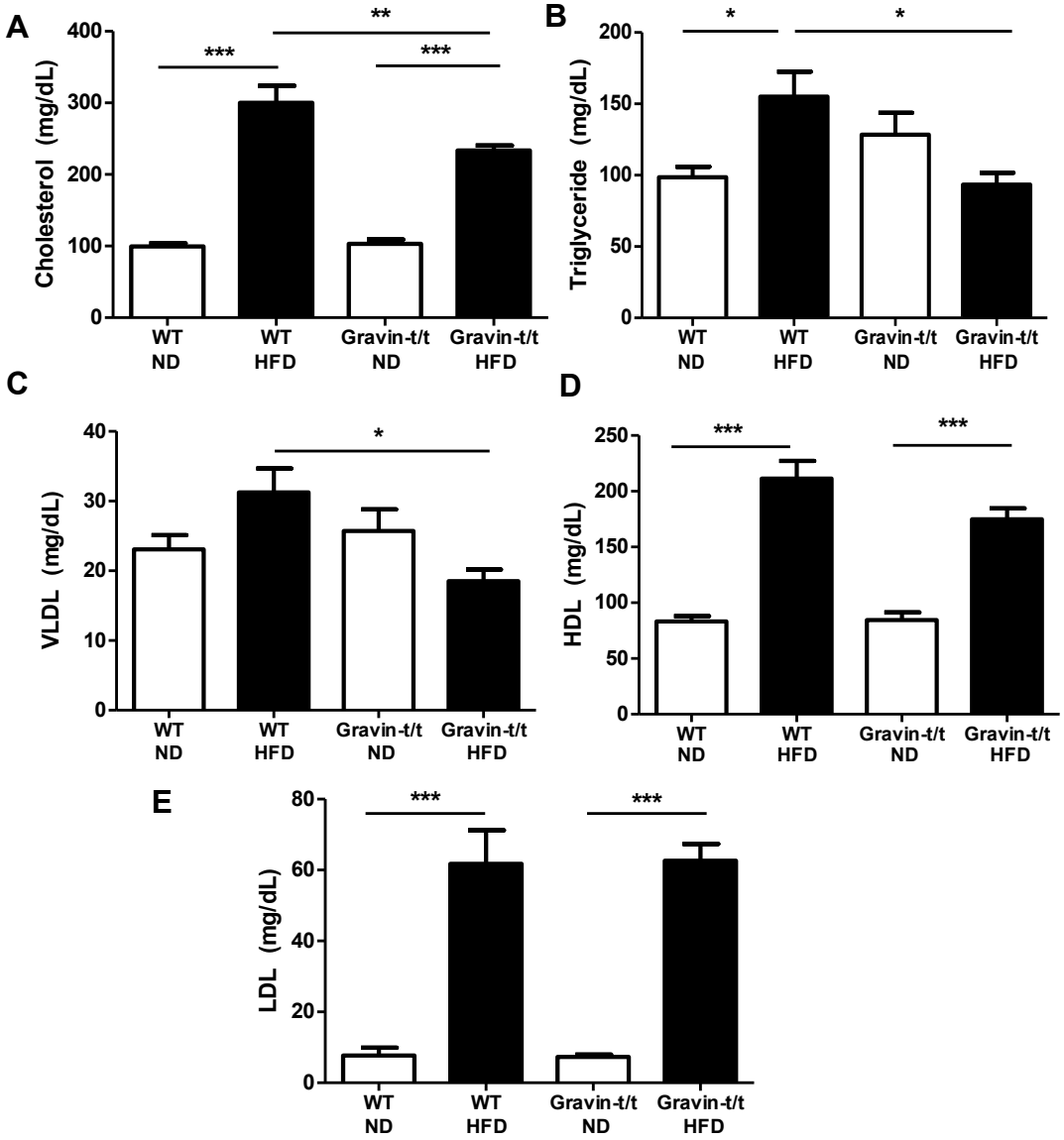


Figure 13: Gravin-t/t mice show plasma lipid differences in response to HFD. Plasma lipid levels were measured from WT and gravin-t/t mice following 16-weeks of treatment of either the ND or the HFD. The plasma level of **(A)** cholesterol (n=5-7), **(B)** triglyceride (n=5), and **(C)** VLDL (n=4-6) were significantly lower in gravin-t/t HFD-treated mice as compared to WT HFD-treated mice. The plasma level of **(D)** HDL (n=5-7) and **(E)** LDL (n=4-7) were not significantly different between WT HFD-treated and gravin-t/t HFD-treated mice. Results are presented as the mean ± SEM; n=11-14; *p-value < 0.05. **p-value< 0.01; ***p-value < 0.001.

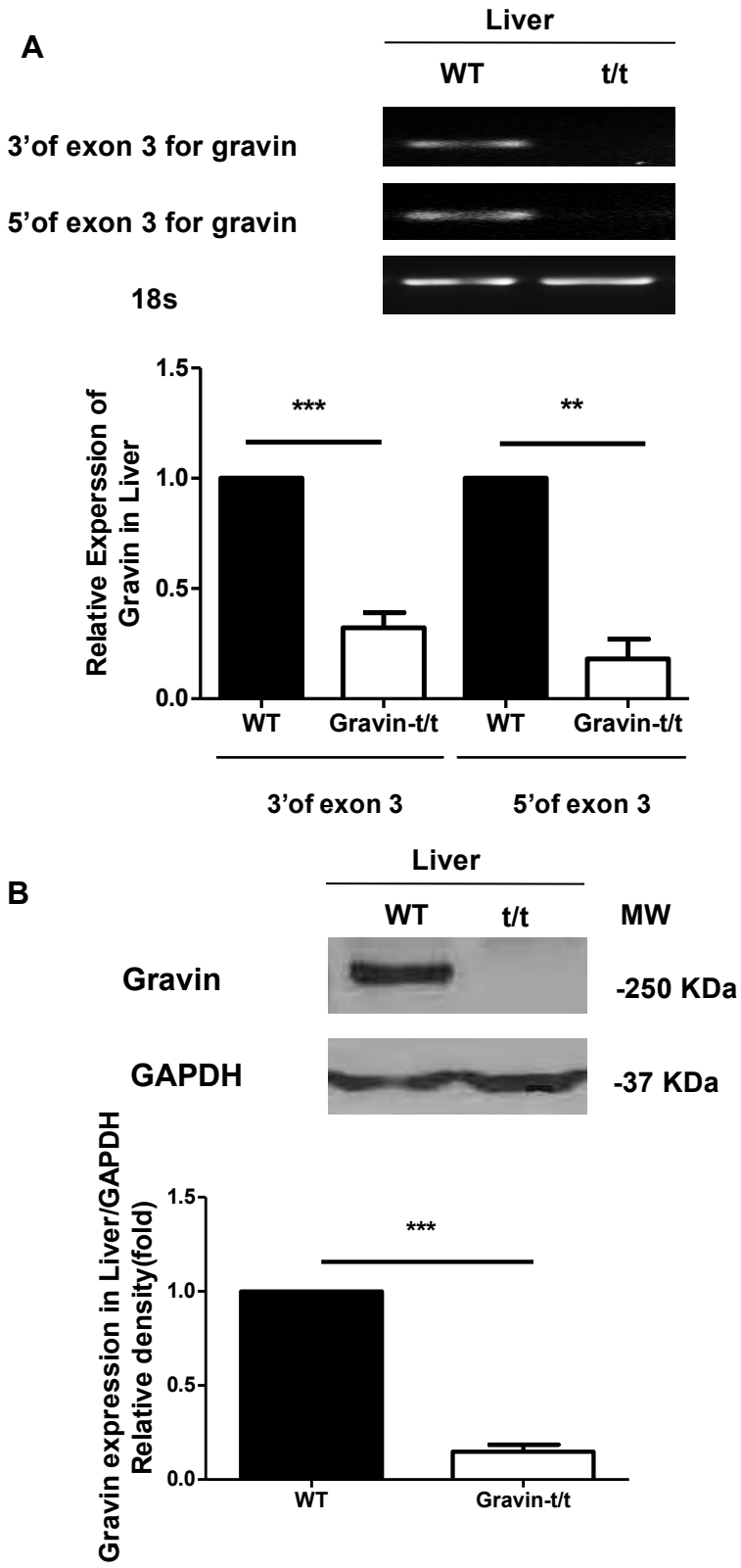
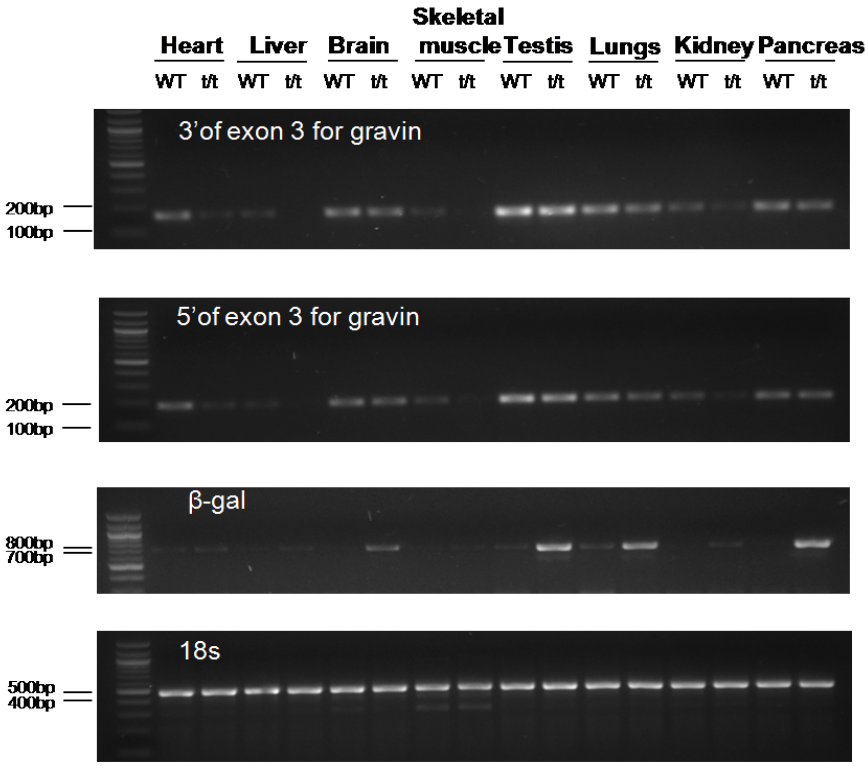

A
Liver
WT
t/t
3'of exon 3 for gravin
5'of exon 3 for gravin
18s
Relative Experssion of Gravin in Liver
1.5
1.0
0.5
0.0
***
**
WT
Gravin-t/t
WT
Gravin-t/t
3'of exon 3
5'of exon 3
B
Liver
WT
t/t
MW
Gravin
-250 KDa
GAPDH
-37 KDa
Gravin expression in Liver/GAPDH Relative density(fold)
1.5
1.0
0.5
0.0
***
WT
Gravin-t/t

Figure 14: Gravin expression in the liver. (A) Gravin mRNA expression was quantified in the liver by RT-qPCR from WT and gravin-t/t mice. The liver from gravin-t/t mice showed significantly decreased gravin mRNA expression as compared to WT mice. Results are presented as the mean ± SEM; n = 6; ***p-value = 0.0006; **p-value = 0.0063. n=4. **(B)** Gravin protein expression was measured in the liver by Western Blot from WT and gravin-t/t mice. The liver from gravin-t/t mice showed significantly decreased gravin protein expression as compared to WT mice, consistent with the gravin mRNA liver expression results. The lower panel showed the statistic quantification. Results are presented as the mean ± SEM; n = 3; ***p-value < 0.001.

A


Heart
Liver
Brain
Skeletal muscle
Testis
Lungs
Kidney
Pancreas
WT t/t WT t/t WT t/t WT t/t WT t/t WT t/t WT t/t WT t/t
3'of exon 3 for gravin
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5'of exon 3 for gravin
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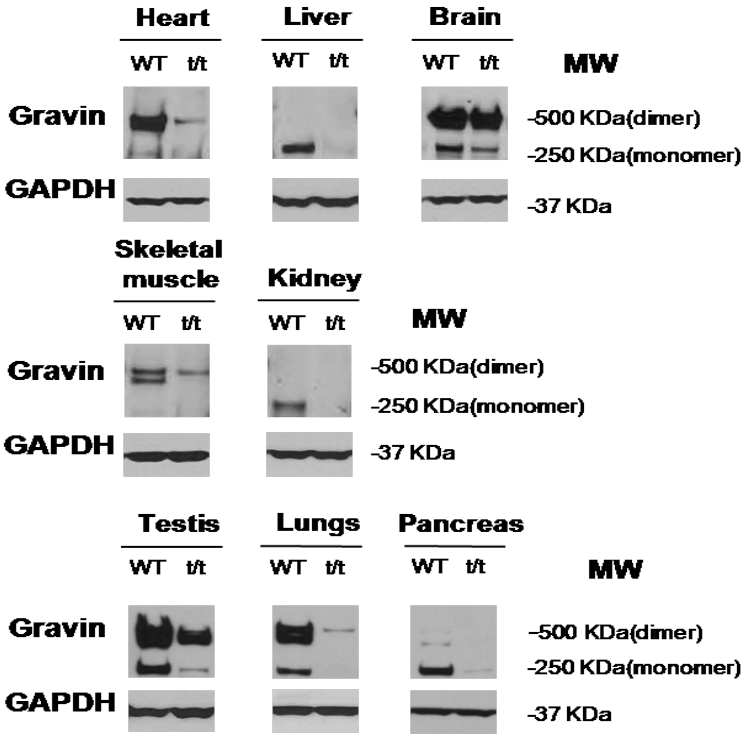
800bp
700bp
18s
500bp
400bp

B


Heart
Liver
Brain
WT t/t WT t/t WT t/t
MW
Gravin
GAPDH
-500 KDa(dimer)
-250 KDa(monomer)
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Kidney
WT t/t WT t/t
MW
Gravin
GAPDH
-500 KDa(dimer)
-250 KDa(monomer)
-37 KDa
Testis
Lungs
Pancreas
WT t/t WT t/t WT t/t
MW
Gravin
GAPDH
-500 KDa(dimer)
-250 KDa(monomer)
-37 KDa

Figure 15: Gravin gene and protein expression isolated from various tissues from WT and gravin-t/t mice. (A) Gravin mRNA expression was quantified in the heart, liver, brain, skeletal muscle, testis, lungs, kidneys and pancreas by RT-qPCR from WT and gravin-t/t mice. Gravin specific primers amplified regions of exon 3 that were either 3' or 5' of the exon. Gene trap technology was used to generate the gravin-t/t mice by using the vector contained the splice-acceptor sequence upstream of the reporter genes, which is a fusion of β- galactosidase (β-gal) and neomycin phosphotransferase II (β-geo). The mutation produced the transcription of exons upstream to the insertion along with the β-geo marker, thus disrupting the Akap12 gene.18s, a housekeeping gene, was used as internal control for gene expression by PCR. **(B)** Gravin protein expression was measured in the heart, liver, brain, skeletal muscle, testes, lungs, kidney and pancreas by Western Blot from WT and gravin-t/t mice. The gravin antibody recognizes both the dimeric (500 KDa) and momeric (250 KDa) forms of the gravin protein.

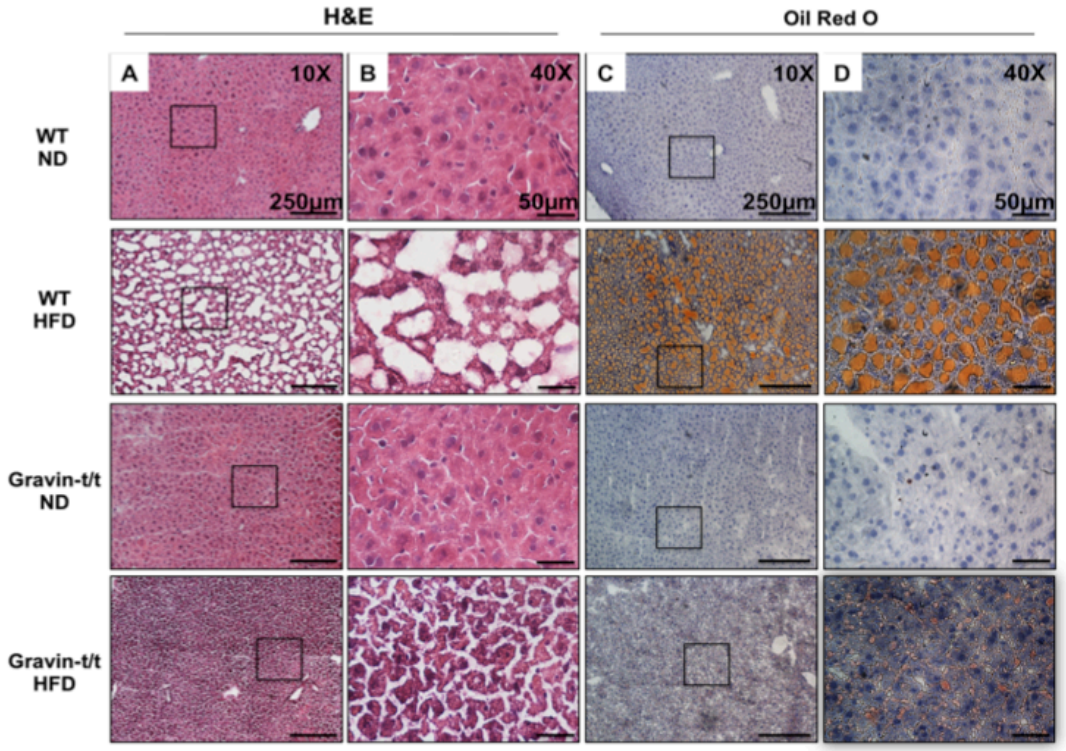

Figure 16: Lipid accumulation in the liver. H&E staining and (**A** and **B**) Oil Red O staining (counterstained with Hematoxylin) (**C** and **D**) were performed on liver frozen sections from WT and gravin-t/t mice following treatment with either ND or HFD for 16-weeks. Images were measured by Nikon microscopy (Bright field, 10*x* (**A** and **C**) & 40*x* (**B** and **D**)). Boxed areas shown in **A** and **C** panels are shown in higher magnification in panels **B** and **D**, respectively.

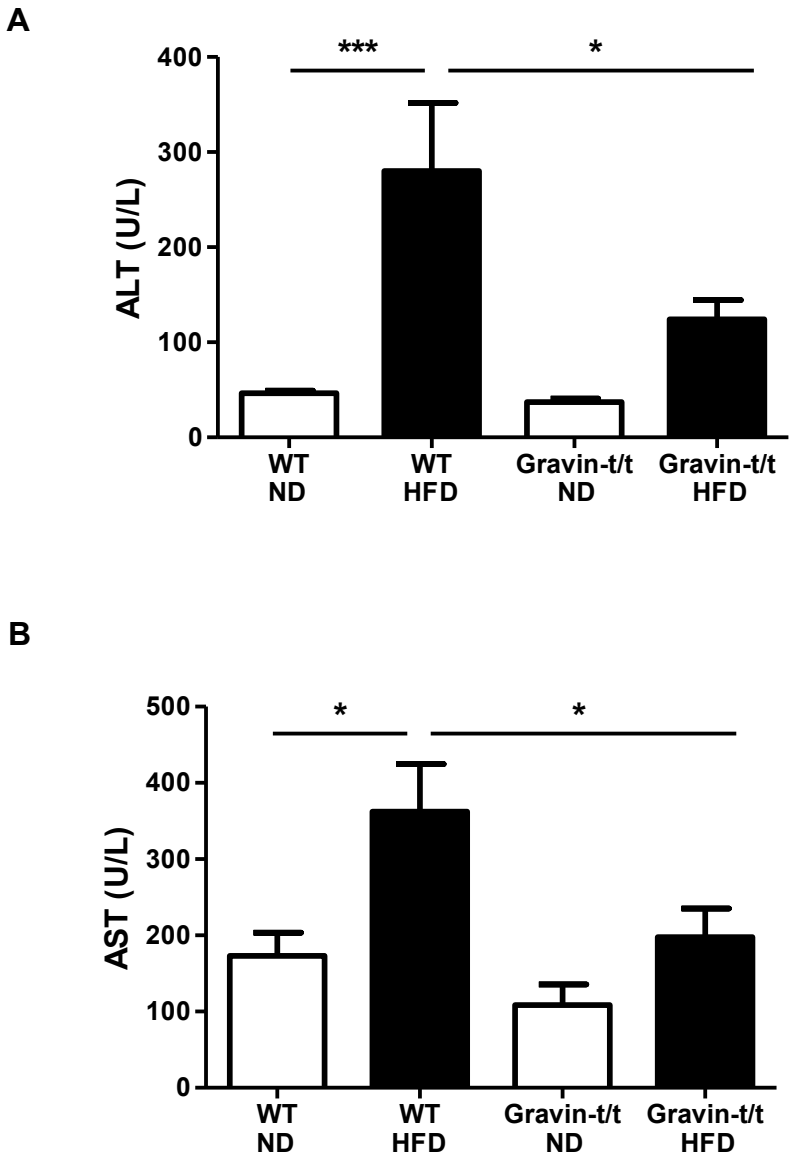

Figure 17: ALT and AST serum level. (A) Serum ALT and **(B)** serum AST were significantly lower in gravin-t/t HFD-treated mice as compared to WT HFD-treated mice, showing greater liver damage and more lipid accumulation in gravin-t/t HFD-treated mice as compared to WT HFD-treated mice. Results are presented as the mean ± SEM; n = 7-8; *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

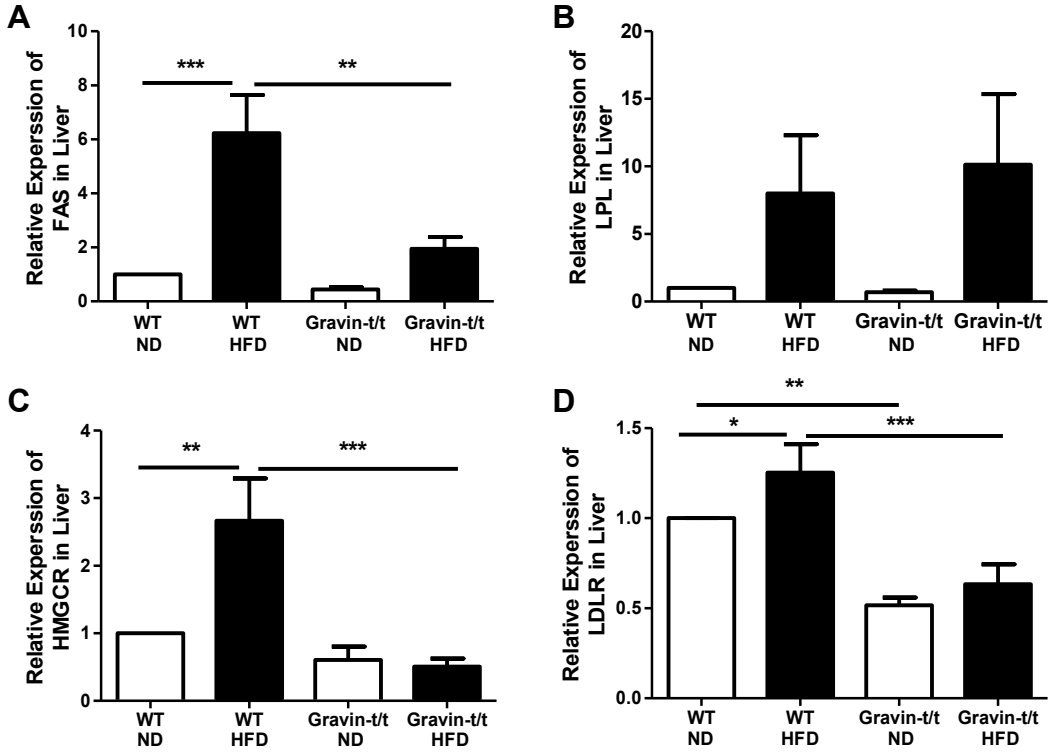

Figure 18: Gravin-t/t mice show reduced hepatic gene expression involved in lipid and cholesterol metabolism in response to HFD. The mRNA expression of genes involved in lipid and cholesterol metabolism was quantified in the liver by RT-qPCR from WT and gravin-t/t mice. The mRNA expression of genes related to lipid metabolism includes: **(A)** FAS, and **(B)** LPL. The mRNA expression of genes related to cholesterol metabolism includes: **(C)** HMGCR, and **(D)** LDLR. Gravin-t/t HFD-treated mice showed significantly lower liver expression of **(A)** FAS, **(C)** HMGCR, and **(D)** LDLR as compared to WT HFD-treated mice. Results are presented as the mean ± SEM; n = 5-7; *p-value < 0.05; **p-value < 0.01, ***p-value < 0.001.

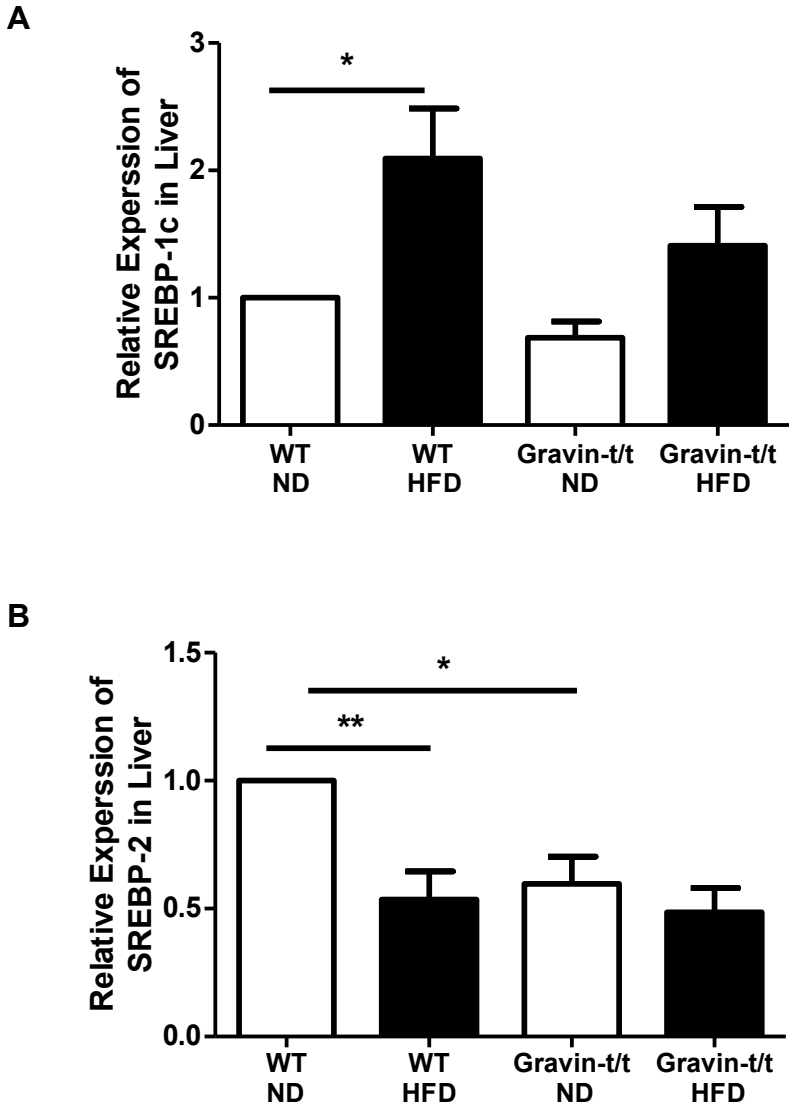

Figure 19: Gravin-t/t mice show less activation of SREBP-2 involved in cholesterol metabolism in response to HFD. The mRNA expression of **(A)** SREBP-1c and **(B)** SREBP-2 was quantified in the liver by RT-qPCR from WT and gravin-t/t mice. The expression of SREBP-2 was significantly reduced in gravin-t/t versus WT mice with ND. Results are presented as the mean ± SEM; n = 6; *p-value< 0.05; **p-value < 0.01; ***p-value < 0.0001.

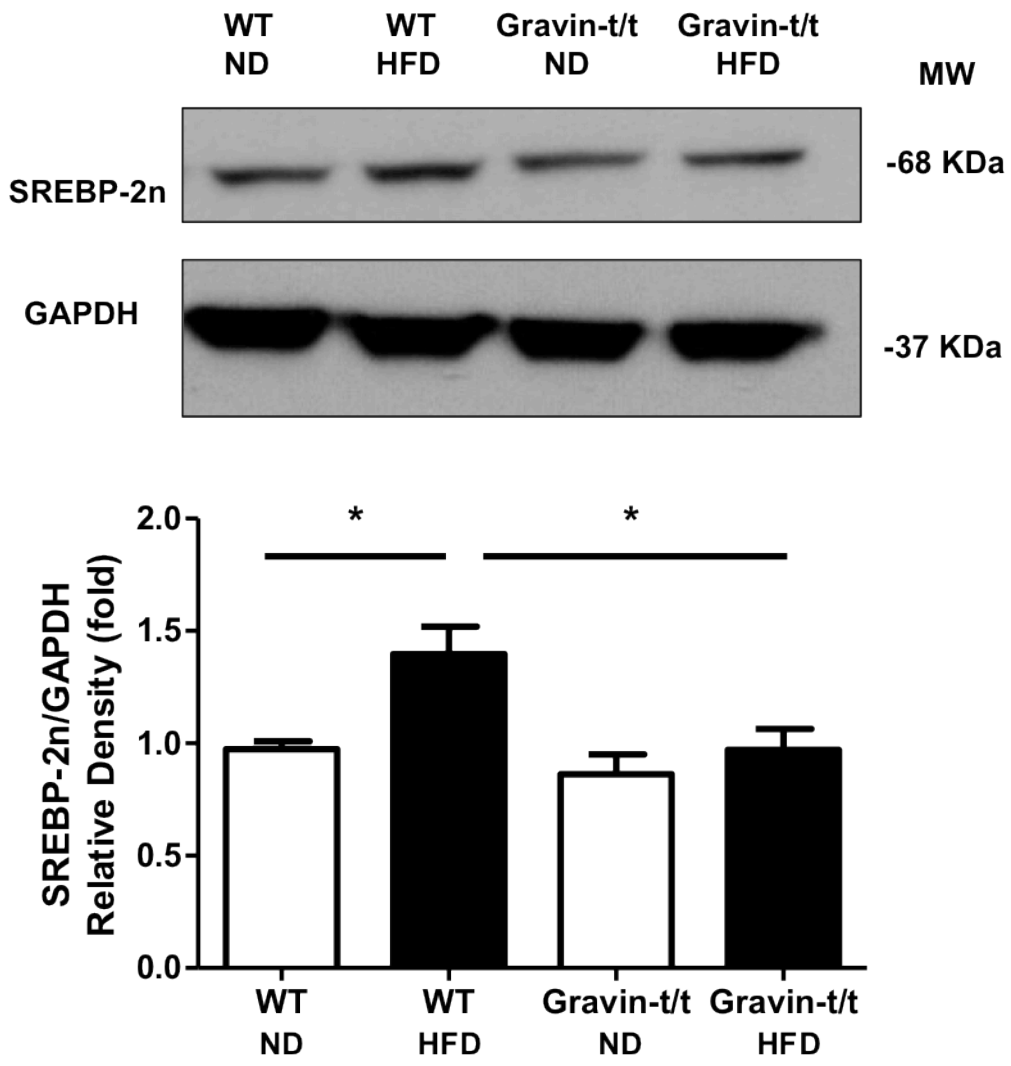

Figure 20: Activation of SREBP-2 in the liver. Expression of the active form of SREBP-2 in the liver homogenized lysates, SREBP-2n, was significantly lower in gravin-t/t mice, as compared with WT mice, either with or without HFD-treatment. Results are presented as the mean ± SEM; n = 5-6; *p-value< 0.05.

5 Characterization of the Role of Gravin in VSMCs in Response to Ang II

5.1 Expression of the Gravin Gene and Protein in WT and Gravin-t/t VSMCs

To assess the potential function of gravin on VSCMs, the RNA and proteins from VSMCs of thoracic arteries of WT and gravin-t/t mice were isolated. As shown in Figure 21A, the expression of the gravin gene was absent in VSMCs isolated from gravin-t/t mice. The data from RT-qPCR (Figure 21A, lower panel) showed that exon 3 was significantly reduced (≈70%) when quantified at three different regions within the exon (exon 3A, WT: 1.00, gravin-t/t: 0.2926 ± 0.06542; exon 3B, WT: 1.00, gravin-t/t: 0.2671 ± 0.05328; exon 3C, WT: 1.00, gravin-t/t: 0.2935 ± 0.08117; n= 3; p value< 0.0001). Exon 1 and 2 of the mutant allele were expressed at levels comparable to WT levels (exon 1, WT: 1.00, gravin-t/t: 1.0040 ± 0.09517; exon 2, WT: 1.00, gravin-t/t: 1.0190 ± 0.07529; n=3; p value< 0.0001). The RT-qPCR data was consistent with the protein expression data of WT non-functional gravin in gravin-t/t VSMCs isolated (Figure 21B).

5.2 Migration and Proliferation of VSMCs after Ang II Stimulation

In response to various environmental stimuli, including growth factors, cytokine and various inflammatory mediators, VSMC can switch to a non-contractile, proliferative and migratory phenotype after vessel injury (e.g., during pathology of atherosclerosis) (House, Potier, Bisaillon, Singer, & Trebak, 2008). Therefore, VSMC migration and proliferation are regulated by various growth factors and cytokines, such as Ang II. Understanding the potential mechanisms governing VSMC migration and proliferation may provide new perspectives to inhibit this inflammatory process. In order to examine the effect of gravin on the migration and proliferation of VSMC stimulated by Ang II, VSMCs from WT and gravin-t/t mice were isolated. The Scratch Wound test revealed less VSMC migration after 24 hours of Ang II stimulation (Figure 22A) in gravin-t/t VSMCs compared with WT VSMCs. Also, gravin-t/t VSMC showed less proliferation after 3 day (by $\approx$8.3%, n=3, p value<0.0001) and 5 days (by ≈12.4%, n=3, p value<0.0001) (Figure 22B) of Ang II stimulation while there was no significant difference between WT and gravin-t/t on day 1 (n=3) as determined by the MTS colorimetric assays. These findings further demonstrate that the disruption of the gravin gene affects VSMC cell function,

vascular remodeling and neointima formation in response to various stimulations during atherosclerosis.

5.3 Decreased Amplitude of Ang II Induced Intracellular Ca^{2+} Transient in Gravin-t/t

As previously discussed, calcium plays as a second messenger involved in cellular functions such as cell proliferation and migration associated with the initiation and procession of atherosclerosis. Others have reported that disruption of the PKA/AKAP complex can inhibit VSMC migration (Raymond et al., 2009). Additionally, AKAPs are involved in the phosphorylation of PKC and PKC substrates, leading to a change in intracellular Ca^{2+}, which is associated with cellular function such as cell proliferation and migration (Langeberg & Scott, 2005). It was hypothesized that the decrease in PKA and PKC activities and the decrease in calcium signaling contributed to decreased VSMC migration and proliferation in gravin-t/t VSMCs. To further determine whether the absence of functional gravin-mediated signaling has any effect on activities of PKA, PKC and intracellular Ca^{2+} transients, this research first examined the Ca^{2+} transit after stimulated with Ang II. As shown in Figure 25, as expected, WT and gravin-t/t VSMCs displayed the Ca^{2+} transient amplitude (peak) in

response to Ang II stimulation (Figure 23). There was significant decrease in peak Ca^{2+} transient in gravin-t/t VSMCs versus that of WT while no significant differences were observed between the baseline in WT and gravin-t/t (Figure 23): WT baseline: 390.7 ± 3.361, t/t baseline: 375.3 ± 1.190, WT peak: 971.9 ± 6.788, t/t peak: 537.9 ± 28.49, n=9; p value<0.0001. The graph in Figure 23 shows it is possible that gravin-t/t VSMCs display a lower Intracellular Ca^{2+} content.

5.4 Decreased Activities of PKA and PKC in Gravin-t/t Mice in Response to Ang II

In order to determine how the absence of functional gravin affects PKA and PKC signaling pathways, the level of PKA and PKC activities and dependent substrate phosphorylation in the VSMCs of WT and gravin-t/t mice was examined. WT VSMCs displayed a significant increase in PKA activities after Ang II stimulation when compared with WT VSMCs control while gravin-t/t VSMCs showed no significant difference in PKA activities either with Ang II stimulation. In order to determine how the absence of functional gravin affects PKA signaling pathways, the level of PKA- dependent substrate phosphorylation in the VSMCs of WT and gravin-t/t mice was examined. As

expected, WT VSMCs showed increased PKA substrate phosphorylation, while a decreased PKA-dependent substrate phosphorylation was observed in the gravin-t/t VSMCs induced by Ang II as compared to WT VSMCs (Figure 24A).

It has been reported that gravin binding to PKC can inhibit PKC kinase activity (L.-W. Guo, Gao, Rothschild, Su, & Gelman, 2011a). As expected, gravin-t/t VSMCs displayed increased PKC basal activity compared to WT VSMCs control. Interestingly, a significant decrease in PKC activities in response to Ang II in gravin-t/t VSMCs was observed when compared with gravin-t/t VSMCs control while there was an increase of PKC activity after Ang II stimulation in WT VSMCs (Figure 25A). WT VSMCs showed increased PKC substrate phosphorylation, which wasn't affected by calcium chelator Bapta-AM (Figure 25B). In addition, different PKC substrates have been phosphorylated, which had been suppressed by the treatment of calcium chelator Bapta-AM. These findings further confirmed that disruption of the PKA/gravin interaction affect PKA/PKC activity levels as well as their downstream signaling in response to Ang II treatment. The data from Ca^{2+} chelator Bapta-AM confirmed that Ca^{2+} is involved in gravin mediated PKC signaling pathway in response to Ang II treatment. Similar observations

(Schott & Grove, 2013; Cullen, 2003) were seen also in several studies showed that the involvement of PKA and PKC in Ca^{2+} signaling consistent with those data in this study.

5.5 A Decrease in Phosphorylation of ERK1/2 and the Expression of MMP-9 in Gravin-t/t VSMCs Stimulated with Ang II

Activation of PKC via a growth factor can regulate MMP expression and VSMC migration; while selective PKC inhibitors suppressed growth factor-induced MMP expression, activity and cell migration (Ding et al., 2011). To assess the effects of Ang II on matrix synthesis, MMP9 was used as a marker for matrix alteration. It has been shown that MMP9 is associated with cell migration. Therefore, the expression of MMP9 was examined a marker of atherosclerosis and Ang II induced-ERK1/2 phosphorylation following disruption of the gravin gene in gravin-t/t VSMCs. As seen in Figure 26, the data showed decreased Ang II-induced ERK1/2 phosphorylation (Figure 26A) and decreased MMP9 expression (Figure 26B) in gravin-t/t VSMCs when compared with WT VSMCs. There is no difference in Angiotensin type 1 receptor expression (AT_1R) (Figure 26C) between WT and gravin-t/t VSMCs

with or without PDGF treatment. Together, our results revealed that gravin is a modulator in Ang II receptor signaling involving in PKA, PKC and calcium signaling, indicating that gravin-t/t suppresses the initiation and progression of atherosclerosis.

5.6 Effects of Intracellular Calcium on Ang II Induced ERK1/2 Phosphorylation in WT and Gravin-t/t VSMCs

We observed a decrease in PKA and PKC activities as well as Ca^{2+} transients in gravin-t/t VSMCs stimulated with Ang II. This data revealed that gravin is indeed involved in Ang II receptor signaling via PKA, PKC and calcium signaling in VSMCs. Therefore, it was then further examined whether disruption of the PKA and/gravin interaction could also contribute to changes in ERK1/2 activity. As shown in Figure 27, the western blot data demonstrated that Ca^{2+}chelator Bapta-AM significantly decreased Ang II-induced ERK1/2 phosphorylation in gravin-t/t VSMCs (n=4, p value=0.0013). Thus, gravin-t/t and WT VSMCs exhibited contrasting roles in Bapta-AM-mediated mitogen activated protein kinase (MAPK) stimulated by Ang II, as evident by this change in Ang II induced ERK1/2 phosphorylation. The possibility is that in gravin-t/t VSMCs the decreased level of PKA phosphorylation and Ca^{2+}

signaling would reach the threshold of effective interaction with the compensatory change necessary for the inhibition of MAPK activity induced by Ang II.

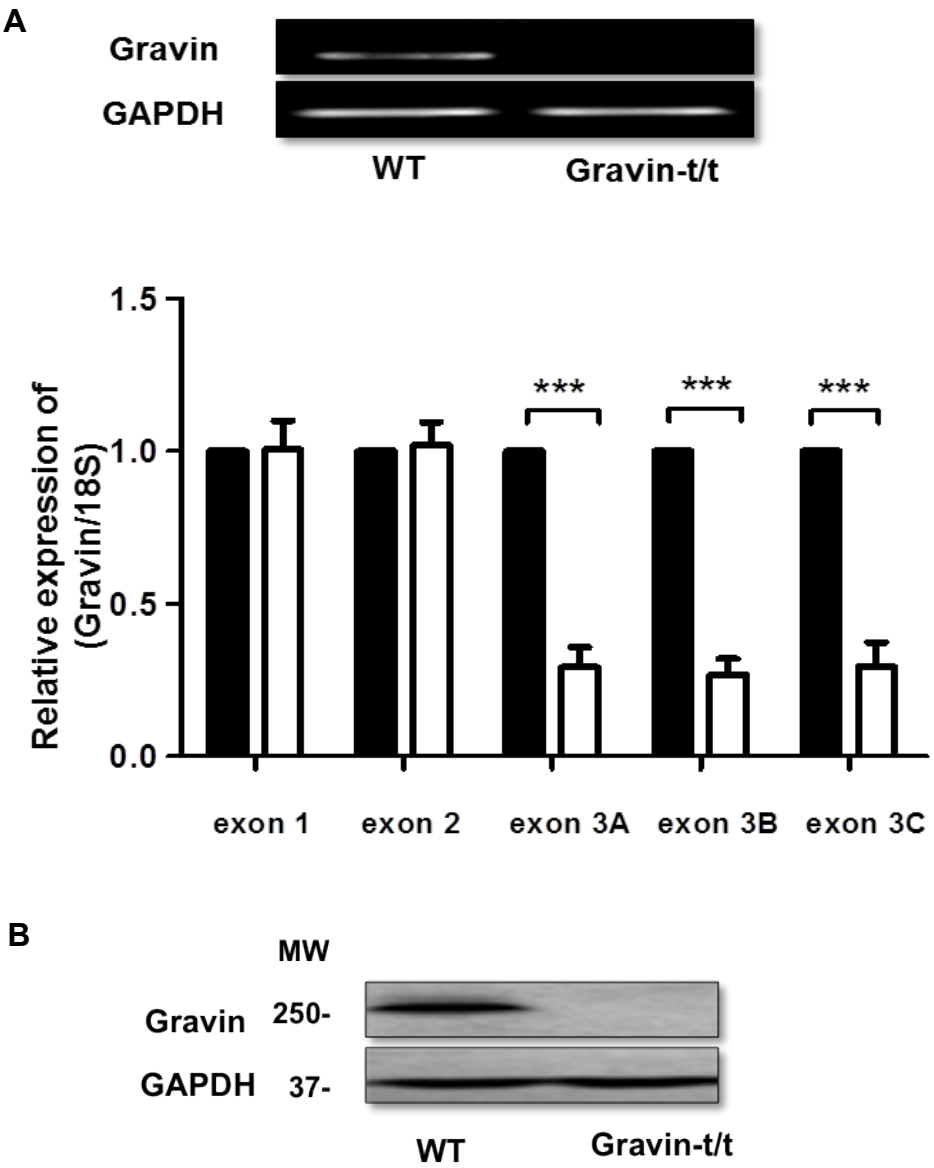

Figure 21: Targeted disruption of the mouse gravin (AKAP12) gene and the expression of gravin in Wild Type (WT) and gravin-t/t VSMCs (A) Targeted disruption of gravin (AKAP12) gene expression: The mRNA of gravin in VSMCs isolated from WT and the gravin-t/t mice was determined using qRT-PCR, loaded onto gel (upper panel) and quantified (lower panel). Results are presented as the mean ± SEM; n=3; ***, p value<0.0001 **(B)** The protein expression of gravin in VSMCs isolated from WT and the gravin-t/t mice was determined by western blot.

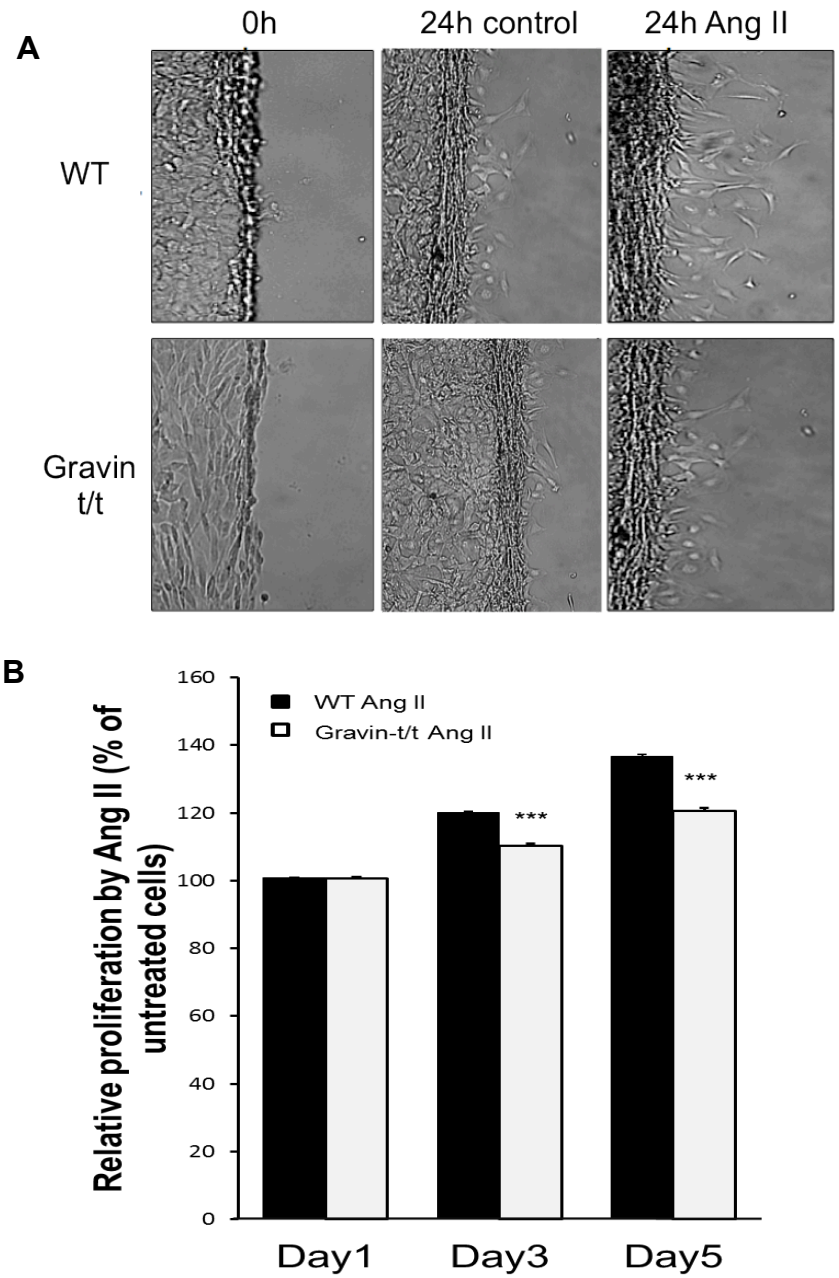

Figure 22: Migration and proliferation of VSMCs isolated from WT and gravin-t/t mice, in presence or absence of Ang II (100 nM) stimulation. (A) A scratch wound assay was used to determine VSMCs migration isolated from WT and gravin-t/t mice following 24 hours in presence or absence of Ang II (100 nM) stimulation. **(B)** VSMCs proliferation with or without stimulation of Ang II was determined by using the MTS colorimetric assays on day 1, 3 and 5 after the stimulation. Results are presented as the mean ± SEM; n=3; ***, p value<0.0001

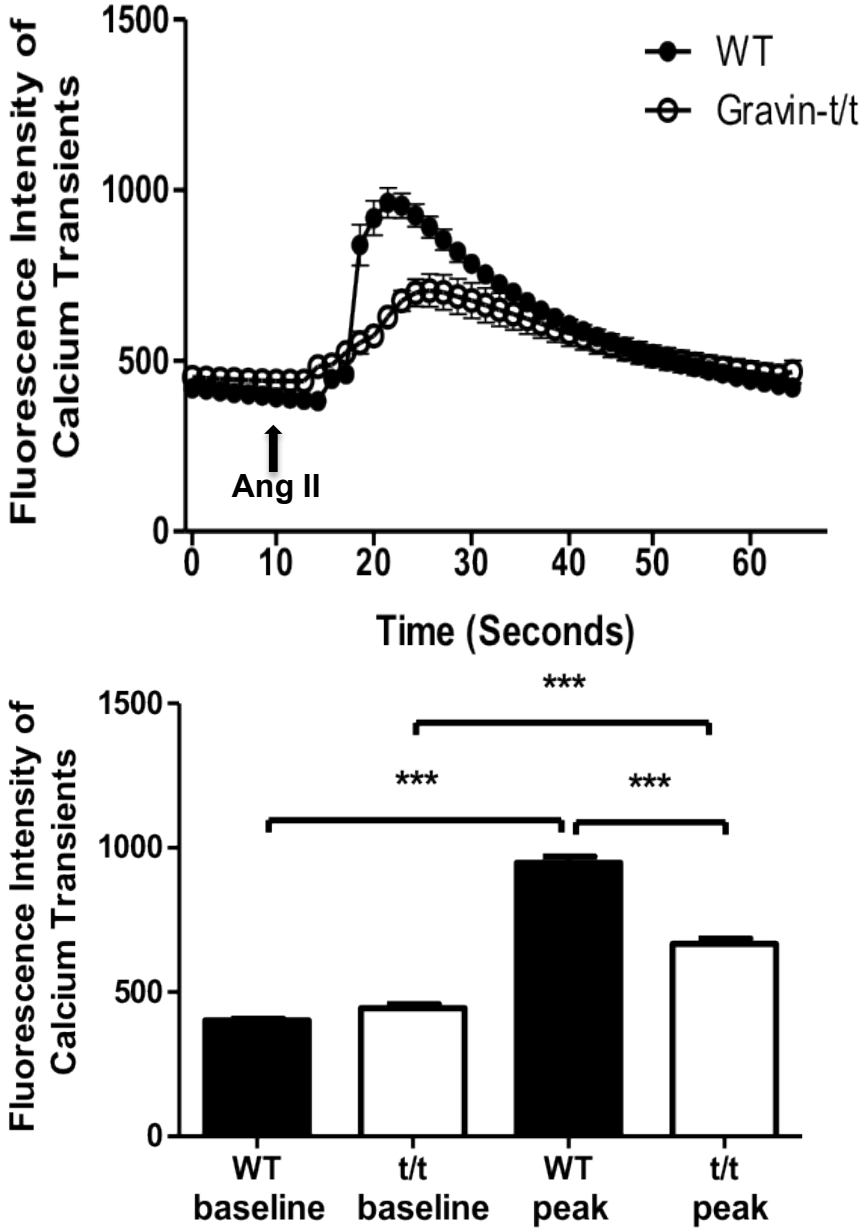

Figure 23: Ca^{2+} transients in response to Ang II. Ca^{2+} transient tracing in aortic VSMC isolated from WT and gravin-t/t mice before and after Ang II (100 nM) stimulation (upper panel) (n=9). Results are presented as the mean ± SEM. Quantitative analysis of fluorescence intensity of calcium transients (lower panel) demonstrated that absence of gravin-mediated signaling inhibited Ang II induced calcium release. Results are presented as the mean ± SEM; n =9, ***, p value<0.0001.

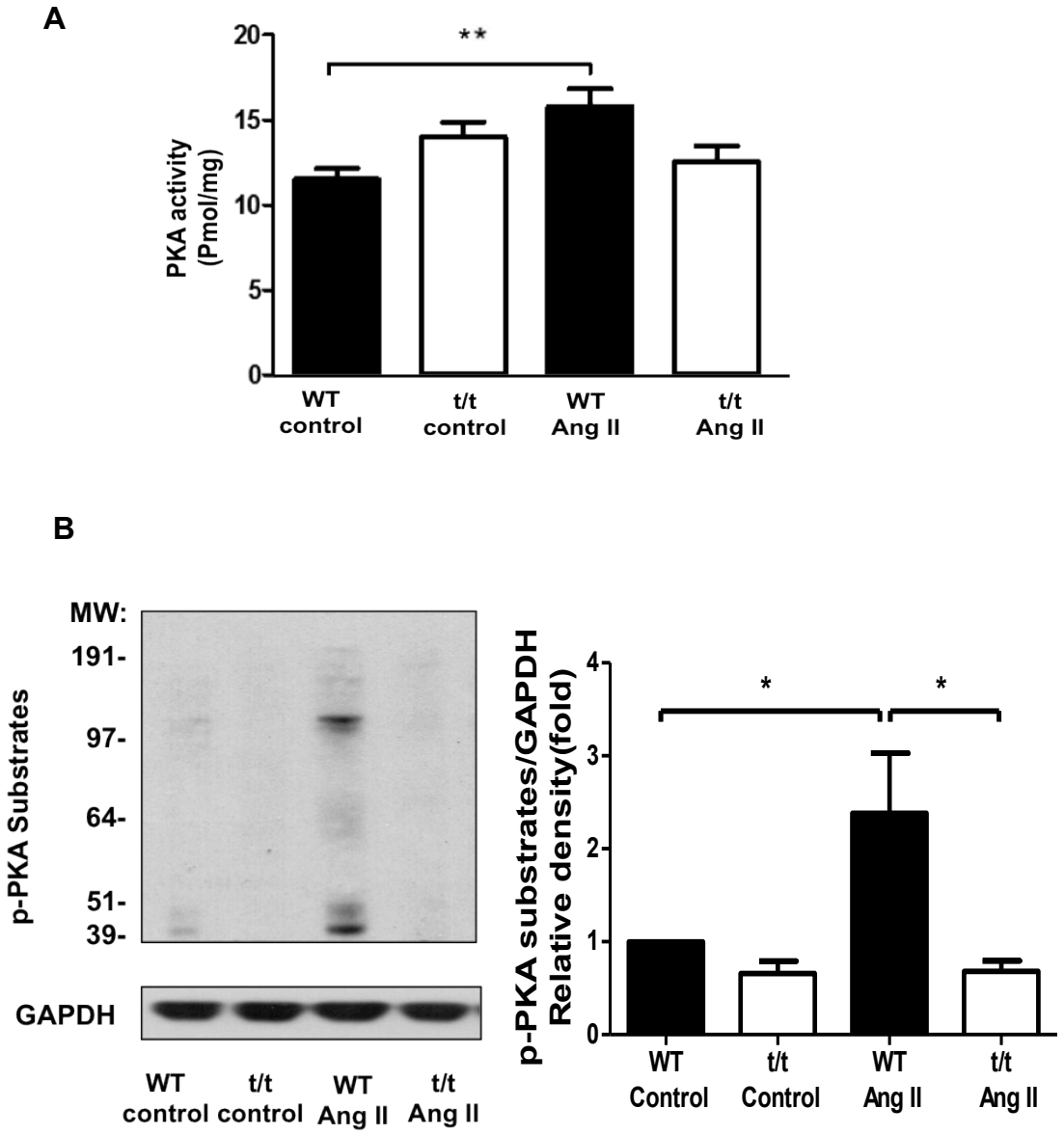

Figure 24: PKA activity and phosphorylation of PKA substrates

(A) Decreased PKA (n=5-7) activity induced by Ang II in gravin-t/t VSMCs, compared to WT was determined by enzyme immunoassay kits (Enzo Life Sciences). Results are presented as the mean ± SEM; n =5-7, **, p value =0.0054; **(B)** VSMCs from the gravin-t/t mice have decreased PKA-dependent substrate phosphorylation induced by Ang II (100 nM) when compared with VSMCs from WT mice. n=4-5; ; *p-value <0.05.

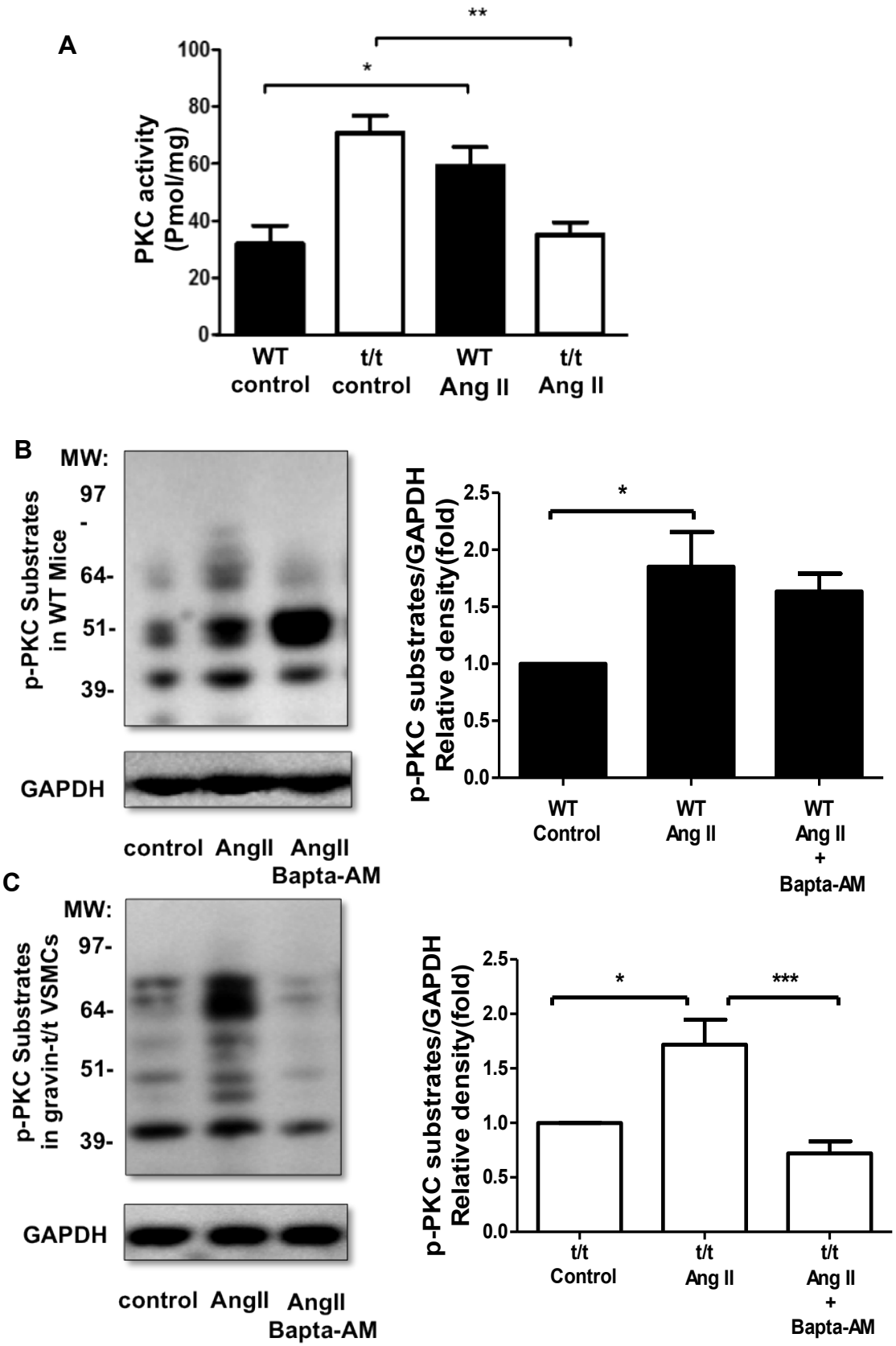
A
PKC activity (Pmol/mg)
100
80
60
40
20
0
*
**
WT control
t/t control
WT Ang II
t/t Ang II
B
MW:
97
64-
51-
39-
p-PKC Substrates in WT Mice
GAPDH
control
AngII
AngII Bapta-AM
p-PKC substrates/GAPDH Relative density(fold)
2.5
2.0
1.5
1.0
0.5
0.0
*
WT Control
WT Ang II
WT Ang II + Bapta-AM
C
MW:
97-
64-
51-
39-
p-PKC Substrates in gravin-t/t VSMCs
GAPDH
control
AngII
AngII Bapta-AM
p-PKC substrates/GAPDH Relative density(fold)
2.5
2.0
1.5
1.0
0.5
0.0
*
***
t/t Control
t/t Ang II
t/t Ang II + Bapta-AM

Figure 25: PKC activity and phosphorylation of PKC substrates. (A) Decreased PKC activity induced by Ang II in gravin-t/t VSMCs, compared to WT was determined by enzyme immunoassay kits (Enzo Life Sciences). Results are presented as the mean ± SEM; n =4, p value=0.0011. **(B)** VSMCs from the gravin-t/t mice have different PKC-dependent substrates phosphorylation induced by Ang II (100 nM) when compared with VSMCs from WT mice, which has been suppressed by the calcium chelator Bata-AM. n=4-6 ; *p-value <0.05; **p-value <0.01, ***p-value <0.001.

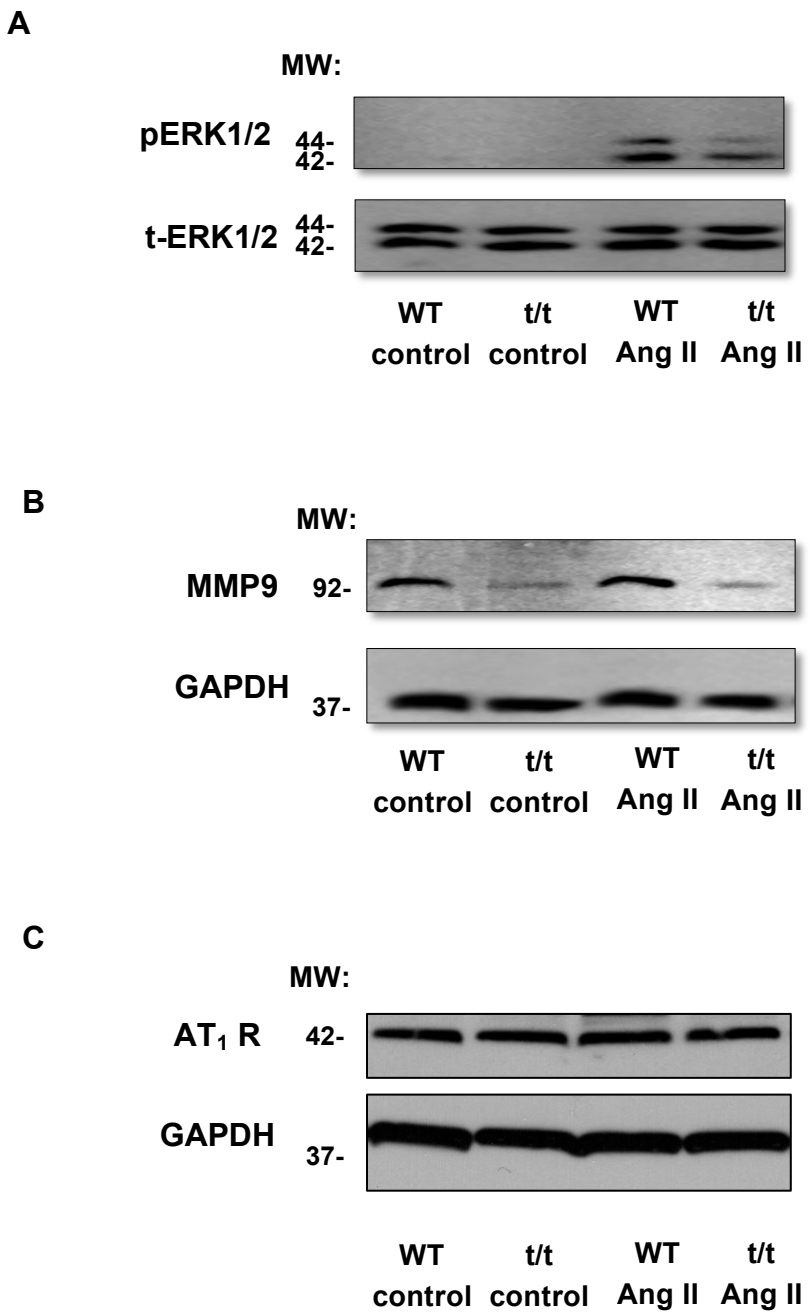

Figure 26: Phosphorylation of ERK1/2 and expression of MMP. (A) Representative western blots of p-ERK1/2, **(B)** MMP-9, a atherosclerotic marker, and **(C)** AT_1 receptor in WT and gravin-t/t VSMCs, from the mice following with or without Ang II (100 nM).Gravin-t/t mice showed decreased ERK1/2 phosphorylation and MMP9 expression but no difference in AT_1 receptor expression induced by Ang II.

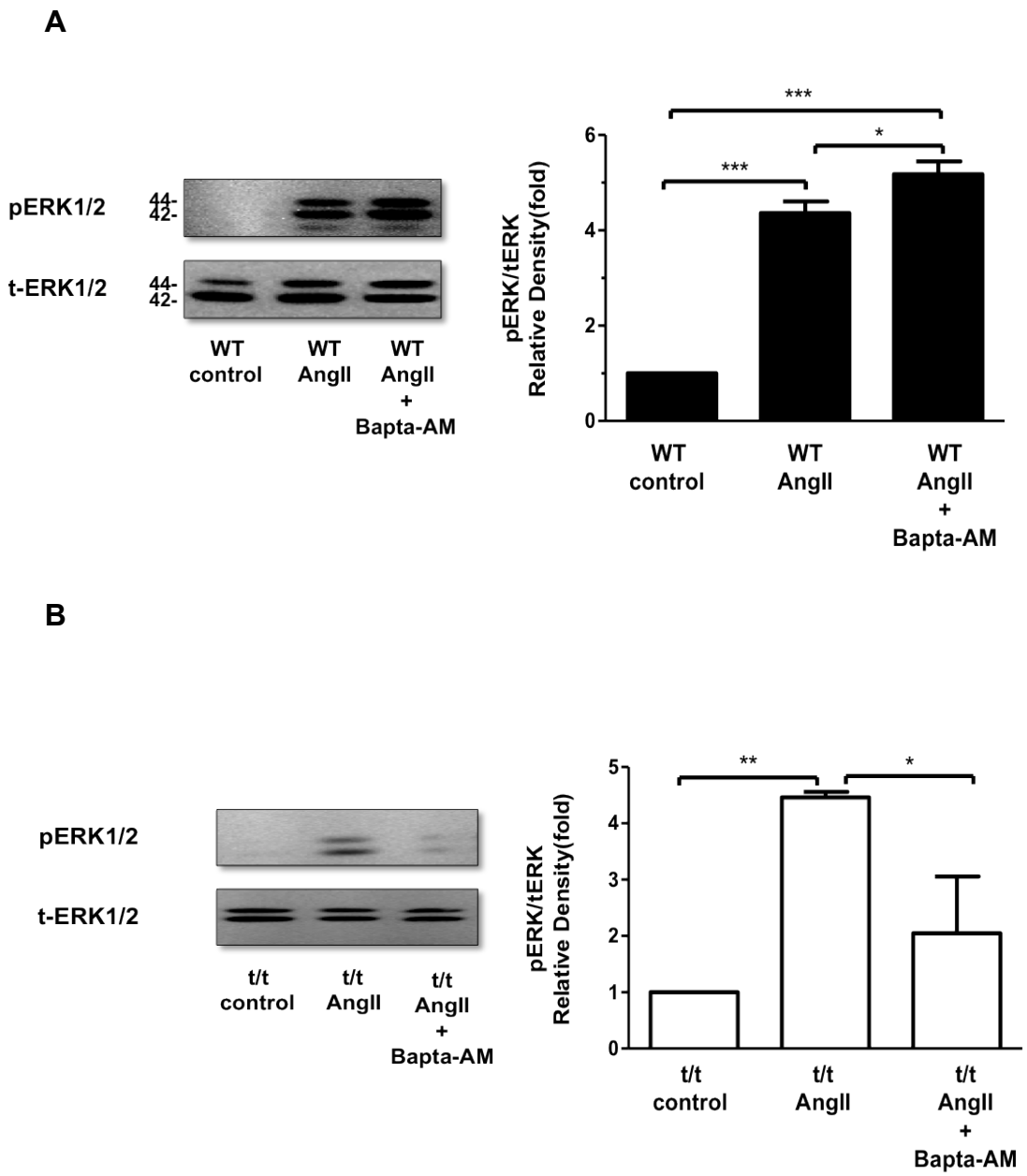

Figure 27: Effects of intracellular calcium on ERK1/2 phosphorylation in VSMCs from WT and gravin-t/t mice, stimulated by Ang II. In contrast to WT mice **(A)**, the calcium chelator Bapta-AM significantly decreased Ang II-induced ERK1/2 phosphorylation in gravin-t/t mice **(B)**. The *left panel* showed the statistical analysis of the western blots. Results are presented as the mean ± SEM; n= 4; *, p<0.01.

6 Characterization of the role of gravin in VSMCs in Response to PDGF

6.1 Migration and Proliferation of VSMCs after PDGF Stimulation

We observed that the absence of gravin mediated signaling prevents Ang II induced VSMC migration and proliferation. The next step is to test whether gravin plays a similar role in mediating signaling involved in various other growth factors stimulation during atherosclerosis. Under pathological conditions, the expression of PDGF is induced in vascular cell and inflammatory cells (Raines, 2004). As previously described, VSMC migration and proliferation are regulated by various growth factors including PDGF. In order to test the effect of gravin on the migration and proliferation of VSMC induced by PDGF, isolated VSMCs were cultured and then treated by PDGF (10ng/ml) for 15 mins. As expected, PDGF treatment induced cell migration in both WT and gravin-t/t VSMCs (Figure 28A). However, gravin-t/t VSMCs showed less cell migration after PDGF treatment for 24 hours as compared to WT VSMCs, as detected by the Scratch Wound Test (Figure 28A). Unlike the Ang II stimulation, gravin-t/t VSMC showed more cell proliferation after 3 day

and 5 days (Figure 28B) in response to PDGF treatment while there was no significant difference between WT and gravin-t/t on Day 1 (n=3), as determined by the MTS colorimetric assays. These findings further demonstrate that the disruption of the gravin gene affects VSMC cell function differently in response to different growth factors during atherosclerosis.

6.2 Decreased Amplitude of PDGF Induced Intracellular Ca^{2+} Transient in Gravin-t/t VSMCs

To further determine whether the absence of functional gravin-mediated signaling has any effect on activities of PKA, PKC and intracellular Ca^{2+} transients, we then examined the Ca^{2+} transients in response to 10 ng/ml PDGF stimulation. As shown in Figure 29, this PDGF mediated peak Ca^{2+} amplitude response in WT VSMCs was similar to the Ang II mediated response in WT VSMCs, as previously described. However, there was no significant difference in calcium transient in gravin-t/t VSMCs before or after PDGF stimulation. Furthermore, there was no significant difference between baseline Ca^{2+} in WT and gravin-t/t (Figure 29) as follows: WT baseline: 422.6 ± 25.7, gravin-t/t baseline: 402.1 ± 12.1, WT peak: 584.7 ± 55.48, gravin-t/t peak: 427.2 ± 12.78, n=7-8; p value<0.0001. Overall, we observed decreased

intracellular in gravin-t/t VSMCs versus WT VSMCs in response to PDGF stimulation (Figure 29B).

6.3 Decreased Activity of PKC, and PKA- and PKC-Dependent Substrate Phosphorylation in Gravin-t/t VSMCs in Response to PDGF

In order to determine how the absence of functional gravin affects PKA and PKC signaling pathways in response to PDGF, we tested the level of PKA and PKC activities. Both WT VSMCs and gravin-t/t displayed no significant increase in phosphorylation of PKA catalytic subunit either in the presence or absence of PDGF stimulation. Furthermore, treatment with the calcium chelator Bapta-AM did not affect the phosphorylation of the PKA catalytic subunit (Figure 30). Even though no significant in the phosphorylation of PKA catalytic subunit were observed, PKA- dependent substrate phosphorylation may still show significant differences in WT and gravin-t/t VSMCs. Therefore, the levels of PKA- dependent substrate phosphorylation in WT and gravin-t/t VSMCs was examined. As expected, WT VSMCs showed increased PKA substrate phosphorylation in response to PDGF (Figure 31). This increased PKA –dependent substrates phosphorylation was not inhibited by the

treatment of calcium chelator Bapta-AM. Furthermore, a decrease in PKA-dependent substrate phosphorylation was observed in PDGF stimulated gravin-t/t VSMCs as compared to WT VSMCs. In gravin-t/t VSMCs, there was no significant change before and after PDGF treatment, even with or without the pretreatment of calcium chelator Bapta-AM (Figure 31).

As expected, WT VSMCs stimulated with PDGF showed increased phosphorylation of PKC as compared to unstimulated WT VSMCs control. In contrast, there was no significant difference in the phosphorylation of PKC in gravin-t/t VSMCs, either in the presence or absence of PDGF stimulation. However, pretreatment with the calcium chelator Bapta-AM prevented the PDGF induced increased phosphorylation of PKC in WT VSMCs. Pretreatment of Bapta-AM had not effect on the phosphorylation of PKC in gravin-t/t VSMCs (Figure 32). Next, we determined the effect of PDGF stimulation on PKC-dependent substrate phosphorylation in WT and gravin-t/t VSMCs. In Figure 33, WT VSMCs showed increased PKC-dependent substrate phosphorylation in response to PDGF stimulation; where this increase was not inhibited by the calcium chelator Bapta-AM. In contrast, PDGF stimulated gravin-t/t VSMCs showed no difference in PKC-dependent substrate phosphorylation as compared to unstimulated gravin-t/t VSMCs and where this

substrate phosphorylation was unaffected by pretreatment with the calcium chelator Bapta-AM (Figure 33). These findings further confirmed that disruption of the gravin mediated signaling affects PKC activity (as determined by the phosphorlation of PKC) as well as their downstream signaling in response to PDGF treatment. Furthermore, our results suggests that Ca^{2+} is involved in gravin mediating both PKA and PKC signaling pathways, in response to PDGF treatment.

6.4 Effects of Intracellular Calcium on PDGF-induced ERK1/2 Phosphorylation in WT and Gravin-t/t VSMCs

To determine the effect of PDGF on PKC-dependent substrate phosphorylation in WT versus gravin-t/t VSMCs, we measured PDGF induced ERK1/2 phosphorylation. Stimulation with PDGF induced a significant increase in ERK1/2 phosphorylation in WT VSMCs versus unstimulated WT VSMCs (Figure 34). However, the PDGF induced increase in ERK1/2 phosphorylation in gravin-t/t VSMCs was significantly less as compared to WT VSMCs (Figure 34). Furthermore, the calcium chelator Bapta-AM decreased the PDGF induced ERK1/2 phosphorylation observed in WT VSMCs, however, Bapta-AM had no effect in gravin-t/t VSMCs (Figure 34). Taken together, our

results indicate that gravin modulates PDGF receptor mediated signaling via the PKC / ERK1/2 signaling pathway. Thus, PDGF mediated and calcium dependent cell migration is suppressed in gravin-t/t VSMCs. Therefore, the absence of gravin may potentially inhibit the initiation and progression of atherosclerosis.

6.5 Effects of Intracellular Calcium on PDGF-induced Akt Phosphorylation in WT and Gravin-t/t VSMCs

PDGF is a key survival factor due to its anti-apoptosis properties (Romashkova & Makarov, 1999) and can also promote cell growth and proliferation through (Monje, Marinissen, & Gutkind, 2003). The mechanisms of PDGF induced cell proliferation and transformation are intrinsically linked to the process of apoptosis; where the default of proliferating cells is to undergo apoptosis unless specific survival signals are provided. We have shown that a decrease in PKA- and PKC-dependent substrate phosphorylation as well as a decreased Ca^{2+} transient in PDGF stimulated gravin-t/t VSMCs. This indicates that gravin is involved in PDGF signaling via calcium dependent PKA and PKC signaling pathways. It is known that through protein kinase B (PKB) / Akt signaling, NF-kB can be activated by PDGF stimulation. This NF-kB activation

can then induce cell proliferation and inhibit apoptosis (Romashkova & Makarov, 1999). Furthermore, it has been shown that PKC can regulate the activation of Akt to modify the cell apoptosis (L. Li, Sampat, Hu, Zakari, & Yuspa, 2006). Therefore, we examined whether disruption of the PKC/gravin interaction also contributed to Akt activity. No significant difference (Figure 35) in PDGF stimulated Akt phosphorylation was observed between WT and gravin-t/t VSMCs. Furthermore, pretreatment with the calcium chelator Bapta-AM showed no difference in PDGF stimulated Akt phosphorylation (Figure 35). Therefore, we conclude that the observed differences in PDGF stimulated cell proliferation in WT versus gravin-t/t VSMCs is not mediated through the PKB / Akt signaling pathway.

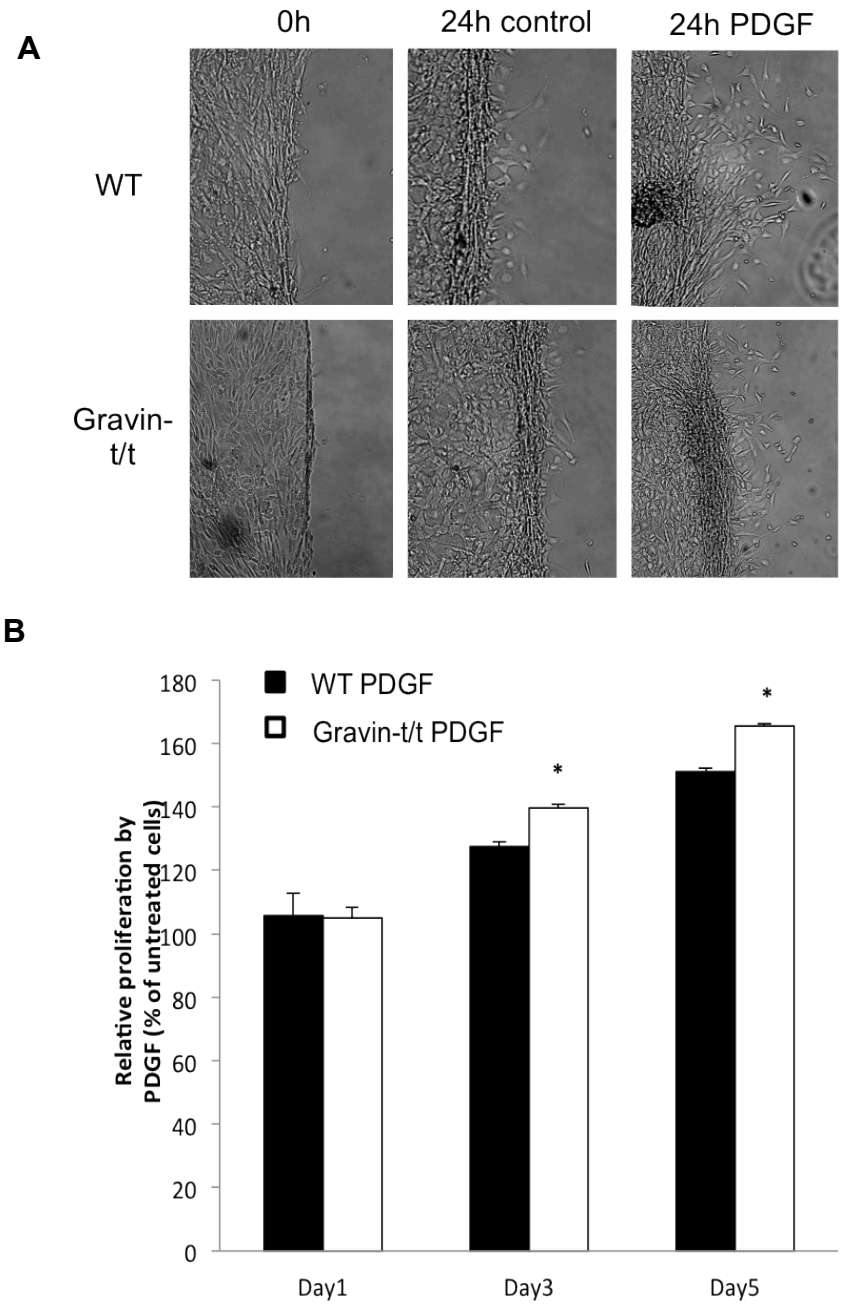

Figure 28: Migration and proliferation of VSMCs isolated from WT and gravin-t/t mice, in the presence or absence of PDGF (10 ng/ml) stimulation. (A) A scratch wound assay was used to determine VSMCs migration isolated from WT and gravin-t/t mice following 24 hours with or without PDGF (10 ng/ml) stimulation. **(B)** VSMCs proliferation in the presence or absence of PDGF was determined by using the MTS colorimetric assays on day 1, 3 and 5 after the stimulation. Results are presented as the mean ± SEM; n=3; *, p value< 0.005.

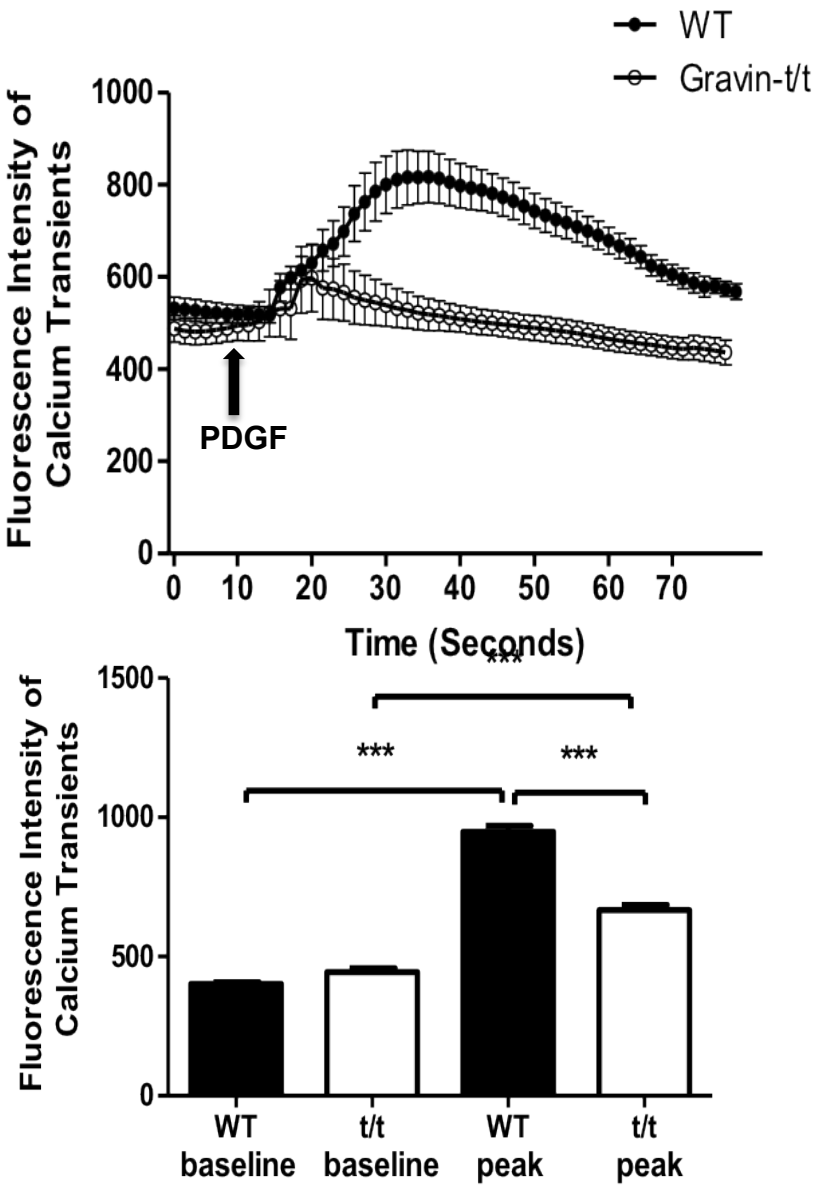

Figure 29: Ca^{2+} transients in response to PDGF. Ca^{2+} transients tracing in aortic VSMC isolated from WT and gravin-t/t mice before (10 second) and after PDGF (10 ng/ml) stimulation (*upper panel*) (n=9). Results are presented as the mean ± SEM. Quantitative analysis of fluorescence intensity of calcium transients (*lower panel*) demonstrated that absence of gravin-mediated signaling inhibited PDGF induced calcium release. Results are presented as the mean ± SEM; n=9-10, ***, p value<0.001.

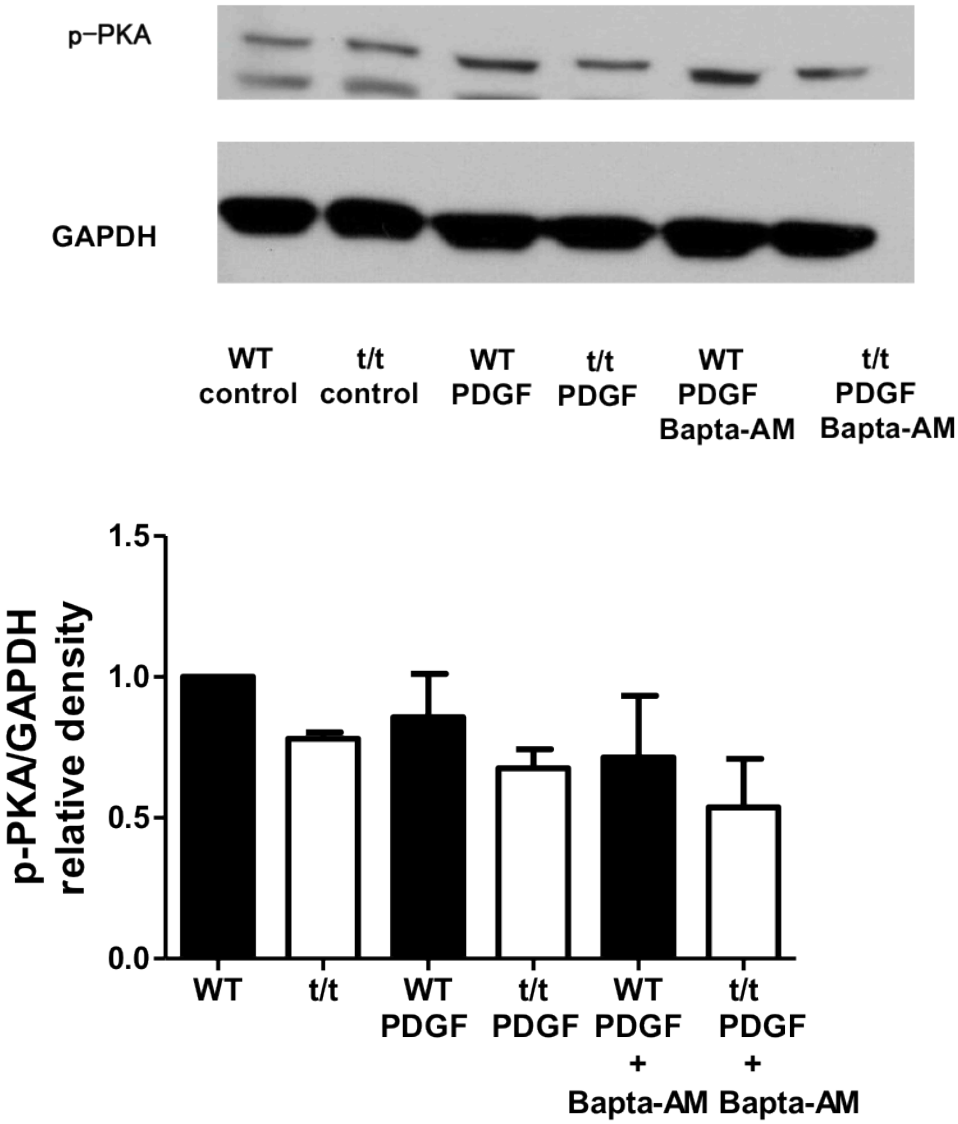

Figure 30: PKA activity. Phosphorylation of PKA catalytic subunit (n=4) indicating the PKA activity, were determined via western blot. There is no significant difference between the WT VSMC and t/t VSMC either with PDGF stimulation or Bapta-AM stimulation. The *lower panel* showed the statistical analysis of the Western blots. Results are presented as the mean ± SEM, n=4.

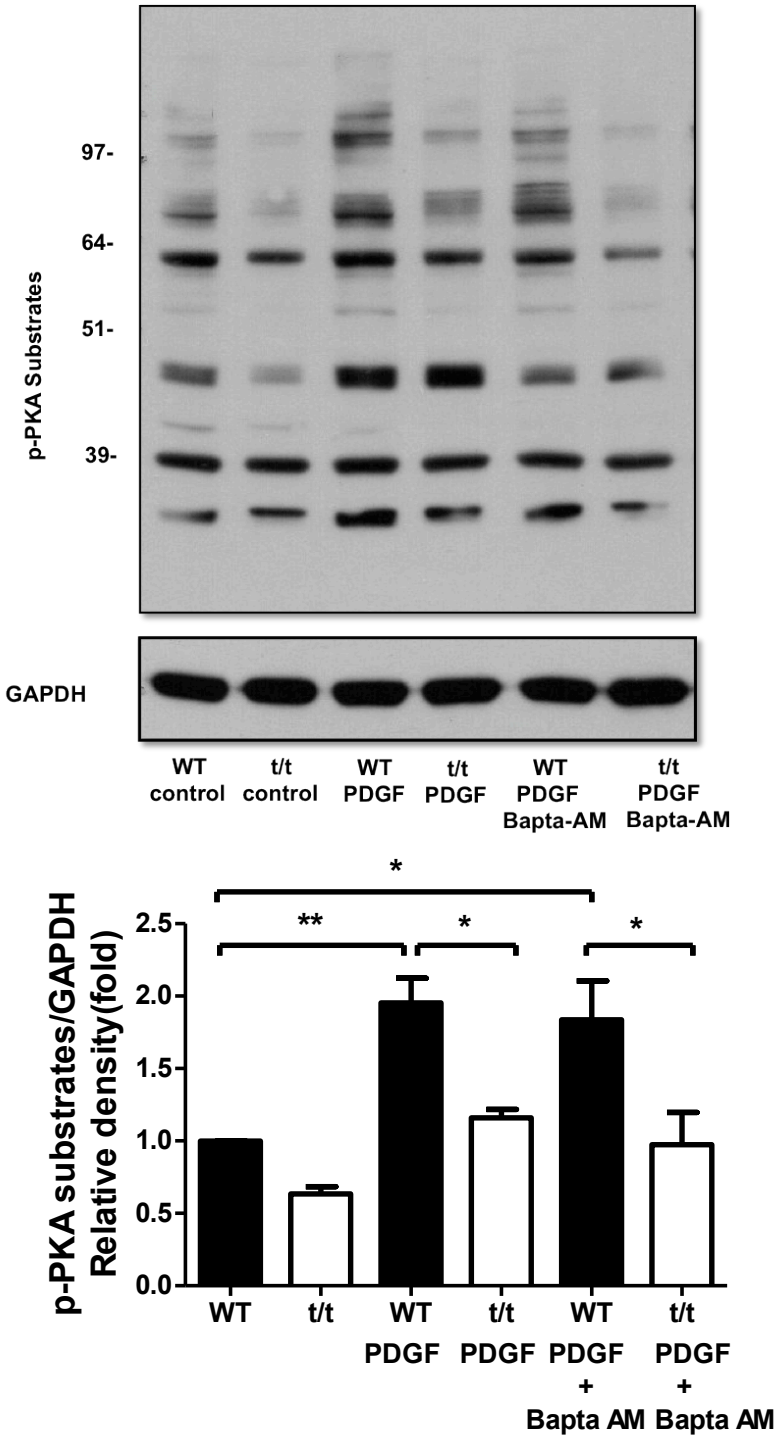

Figure 31: Phosphorylation of PKA substrates, VSMCs from the gravin-t/t mice have decreased PKA-dependent substrate phosphorylation induced by PDGF (10 ng/ml) when compared with VSMCs from WT mice and also has been inhibited by Bapta-AM. n=4-6, ; *p-value < 0.05; **p-value < 0.01, ***p-value < 0.001.

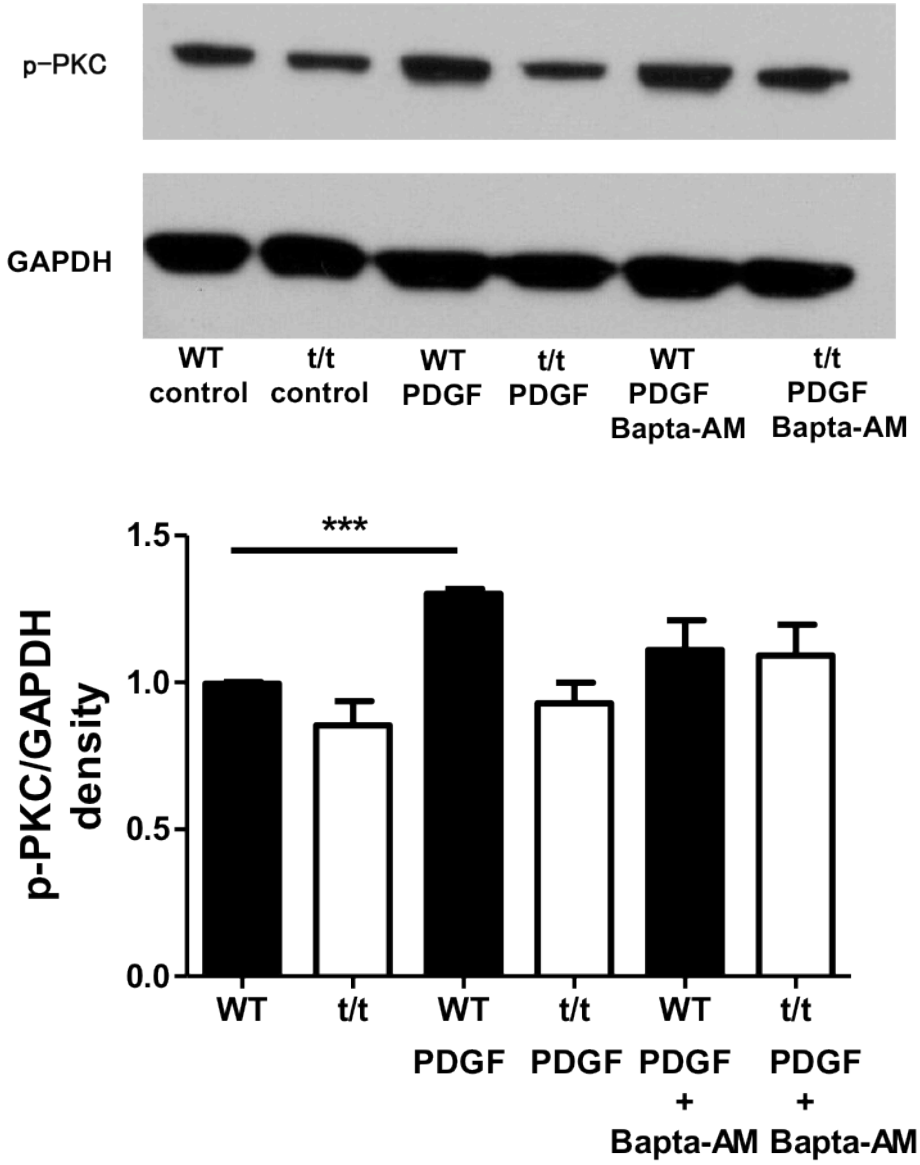

Figure 32: PKC activity. Phosphorylation of PKC (n=4), indicating the PKC activity, was determined by western blot. There is no significant difference between the WT VSMC and gravin-t/t VSMC either with PDGF stimulation or Bapta-AM stimulation. The *lower panel* shows the statistical analysis of the western blots. Results are presented as the mean ± SEM, n=4; ***p-value<0.001.

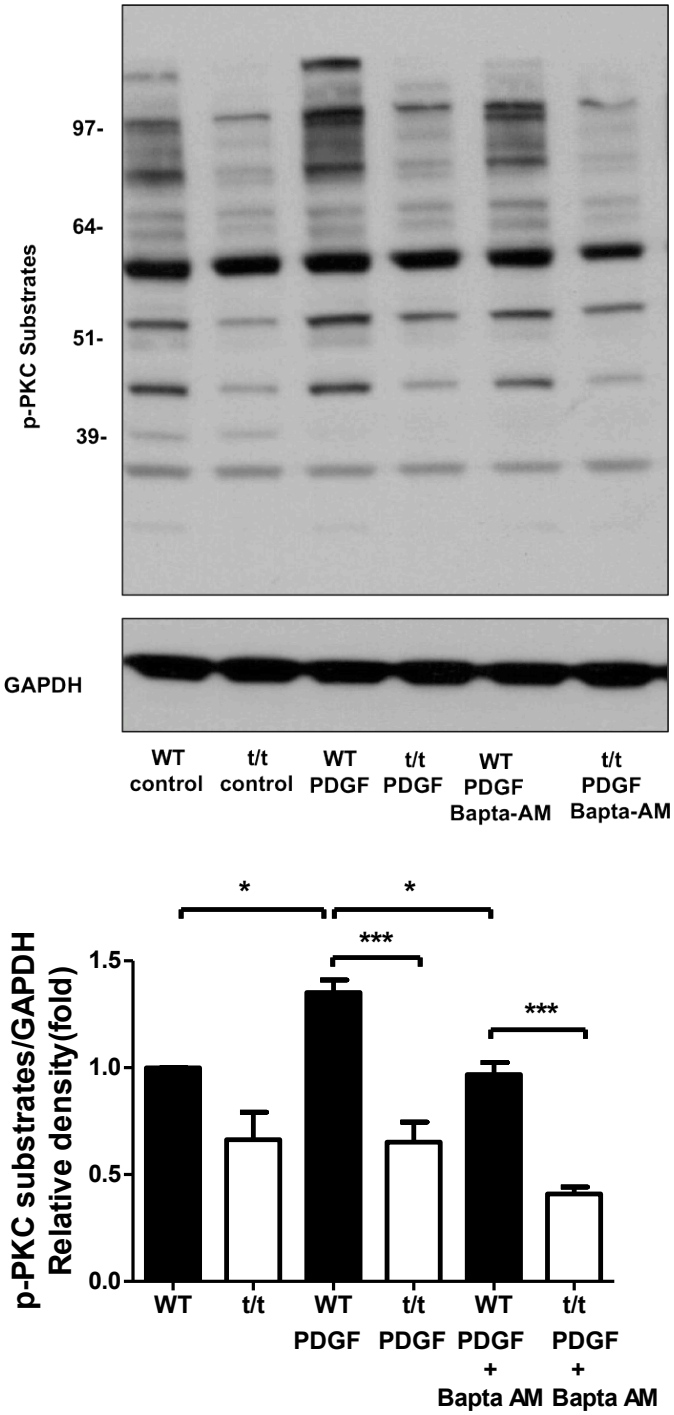

Figure 33: Phosphorylation of PKC substrates. VSMCs from the gravin-t/t mice have shown different PKC-dependent substrate phosphorylation induced by PDGF (10 ng/ml) when compared with VSMCs from WT mice and also has been inhibited by Bapta-AM. n=4-6; *p-value<0.05; ***p-value<0.001.

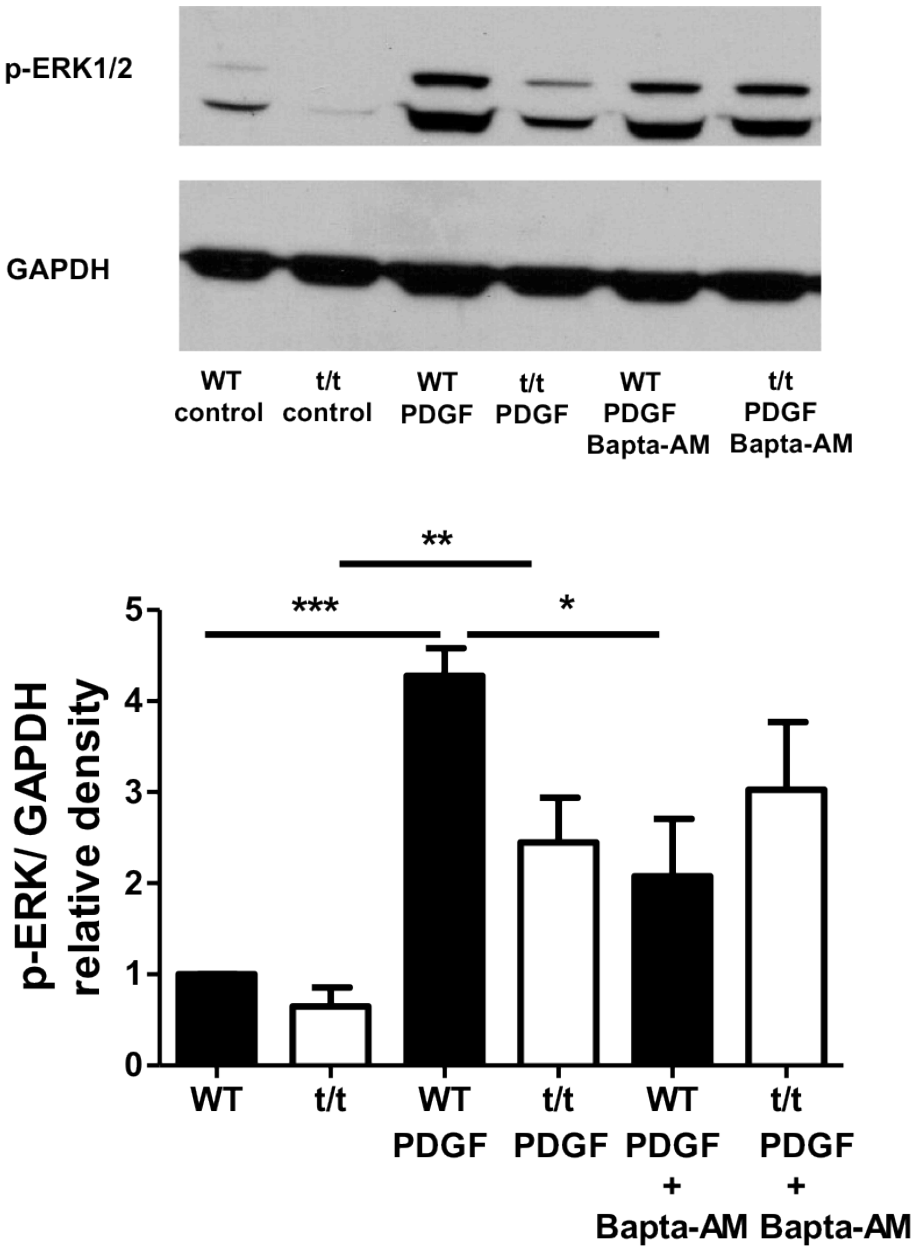

Figure 34: Effects of intracellular calcium on ERK1/2 phosphorylation in VSMCs from WT and gravin-t/t mice, induced by PDGF. In contrast to WT mice, the calcium chelator Bapta-AM significantly decreased PDGF-induced ERK1/2 phosphorylation in gravin-t/t mice. The *lower panel* showed the statistical analysis of the western blots. Results are presented as the mean ± SEM; n= 4; *, $p<0.01$; ***, $p<0.001$.

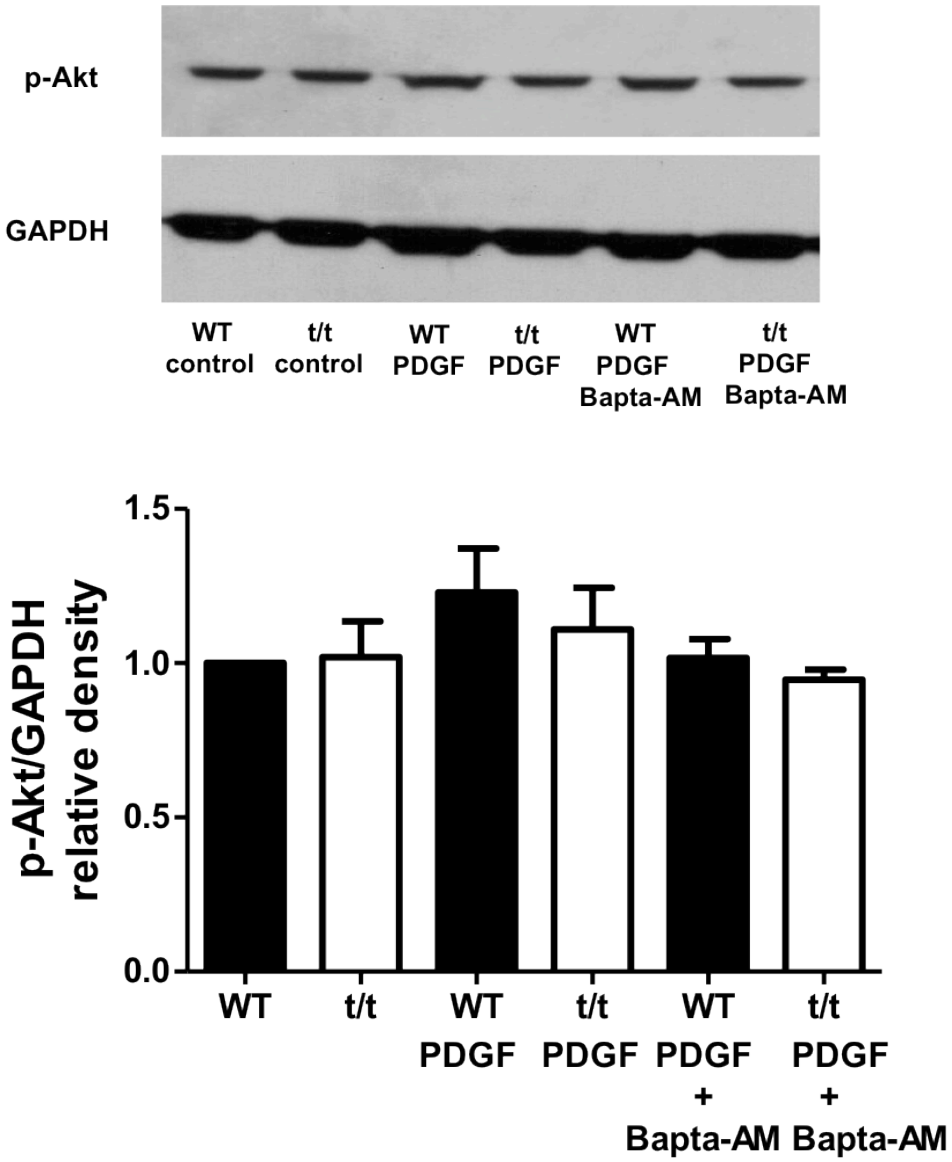

Figure 35: Effects of intracellular calcium on Akt phosphorylation in VSMCs from WT and gravin-t/t mice, induced by PDGF. There was no significant difference between the WT and gravin-t/t either with or without PDGF treatment, with no effect of calcium chelator Bapta-AM treatment. The *lower panel* showed the statistical analysis of the western blots. Results are presented as the mean ± SEM; n= 4.

7 Discussion

Gravin is thought to be a suppressor of tumor and metastasis progression (Bing Su, Bu, Engelberg, & Gelman, 2010). It has been shown that re-expression of gravin in tumor tissue suppresses oncogenic signaling pathways through its ability to scaffold its binding components, such as PKA, PKC, Src and CaMKII, which correlates with metastasis progression. Previous studies have shown that gravin can regulate the desensitization/resensitization cycle of β_2-AR, which is related to cardiac contractility (G. Fan et al., 2001). Recent studies show that gravin affects multiple processes including cell proliferation (I H Gelman, Lee, Tombler, Gordon, & Lin, 1998; Irwin H Gelman, 2010), migration (B Su et al., 2012) , apoptosis (X. Li et al., 2010) and matrix alteration in response to stimulation of growth factors in atherosclerosis. During atherosclerosis, after vascular injury, the inflammatory responses cause the release of growth factors and cytokine. These growth factors, such as angiotensin II (Ang II) and platelet-derived growth factor (PDGF), can mediate cell proliferation, migration, extracellular matrix synthesis as well as promote apoptosis of vascular smooth muscle cells (VSMCs) (Stoneman & Bennett, 2004;Newby, 2012). In this dissertation project, we investigated, for the first time, the function of gravin in atherosclerosis that was induced by a

high fat diet as well as in vascular remodeling that was in response to stimulation of various growth factors. In order to perform these studies, we used the gravin-t/t mouse model that was developed in our laboratory. Homozygous mutant gravin mice (gravin-t/t) lacks functional gravin protein that do not express the critical region of exon 3; the exon that encodes the binding sites for β_2-AR, PKA or PKC binding (Guillory et al., 2013). This project focuses on new different approach to study the pro-atherosclerotic signaling involving gravin, in mice as well as isolated VSMC.

7.1 The effect of HFD induced atherosclerosis in gravin-t/t mice

In current study, it was observed that the absence of gravin signaling would protect against the development and progression of atherosclerosis and hyperlipidemia induced by a high fat diet (HFD). Gravin-t/t mice on HFD showed less body weight (Figure 6), lower cholesterol, triglyceride, and VLDL levels (Figure 13) resulting in less lipid lesion in the aorta (Figure 11). In wild-type mice, HFD treatment causes atherosclerosis related phenotype including excessive gain in body weight, aortic plaque formation, altered lipid profiles and a fatty liver. The plaque formation can harden and narrow the

arteries and limit the blood flow to the heart and other parts of the body, possibly resulting in hypertension (Lusis, 2000; Rader & Daugherty, 2008a). Studies have observed a similar phenotype in C57BL6 mice fed with a HFD (Biddinger et al., 2005).

Activation of macrophage and increased expression of MMPs has been shown to contribute to the development of human atherosclerosis lesions (Galis et al., 1994). It has also been shown that gravin down-regulates the expression of MMP-9, a protein involved in endothelial cell migration via down-regulation of activator protein-1 (AP-1) transcriptional activity (S.-W. Lee et al., 2011). Gravin-t/t showed less MMP-9 expression on aortic sections and consequently reduced atherosclerotic vascular remodeling and plaque formation (Figure 12). A possible explanation for their distinct roles may be related to different substrates specificities in different tissues. It has been shown that gravin binds to PKC and that gravin also plays a central role in regulating several cell signaling pathways that are related to cell remodeling and apoptosis (L.-W. Guo et al., 2011a). PKC can regulate MMP-9 expression and VSMC cell migration (Ding et al., 2011). Therefore, we hypothesized that gravin may regulate expression of MMP-9 via PKC/Ca^{2+}/ERK signaling pathway. This signaling pathway then mediates the macrophage induced

atherosclerotic plaque formation. To test this hypothesis, we stimulated isolated VSMCs from gravin-t/t mice with several different growth factors that are known to be involved in progression of atherosclerosis in order to study gravin's role in this intracellular PKC/Ca^{2+}/ERK signaling pathway.

Lipid metabolism disorders induced by HFD can cause massive accumulation of lipids in various tissues, especially in the liver. The liver is the major organ controlling lipid metabolism. A complex network of molecular mediators regulates lipid metabolism, including several proteins related to lipid and cholesterol biosynthesis. Our study showed that the absence of gravin mediated signaling decreased the pathological effects of HFD in the liver. In particular, less liver damage and lipid accumulation were observed in the liver (Figure 16). In the liver, SREBPs are the proteins sensors that monitor sterol concentrations and are the primary transcription factors that regulate cholesterol, fatty acid, and triglyceride biosynthesis and uptake (Goldstein, DeBose-Boyd, & Brown, 2006; Brown & Goldstein, 2009). When cholesterol concentrations are low, SREBPs form a complex with the SREBP cleavage activating protein (Scap) and together moves from the ER to the Golgi. Following the cleavage of SREBP, active form of the protein stimulates the transcription of the genes essential for cholesterol biosynthesis

and transport (Goldstein et al., 2006). In mammalian cells, SREBP-1c and SREBP-2 are two related isoforms of SREBPs. SREBP-1c mainly stimulates the expression of genes related to fatty acid synthesis, such as FAS. In contrast, cholesterol synthesis is regulated by SREBP-2. SREBP-2 then targets mRNAs for key cholesterogenic genes, such as HMGCR and LDL receptor (Brown & Goldstein, 2009). Although there was no effect of gravin truncation on SREBP-1c expression, either in the presence or absence of HFD treatment, we did observe that gravin-t/t mice showed down-regulation of the mRNA expression of LDLR and SREBP-2. Interestingly, this decreased LDLR and SREBP-2 expression was observed, both in the absence and presence of the HFD, in gravin-t/t mice versus WT mice. Additionally, it has been reported that gravin participates in cholesterol metabolism via SREBP-2 activation and that overexpression of gravin activates SREBP-2 in a Scap-dependent manner (Choi et al., 2008). This is consistent with our results. Specifically, gravin-t/t mice showed lower mRNA level of SREBP-2 and less activation of SREBP-2 (mature form) along with downregulation of HMGCR and LDLR. Together, this led to lower cholesterol levels in circulation. We believe that gravin is involved in the escort process of SREBP-2 to Golgi in a Scap-dependent manner. Furthermore, we believe that this gravin dependent process induces the activation of SREBP-2 and consequently lowers the plasma lipid profile.

Another hypothesis is in absence of gravin mediated signaling, less activate form of SREBP-2 gets transported to the nuclei, which can regulate the expression of its downstream cholesterogenic gene targets (such as HMGCR and LDLR). Therefore, we propose a new insight into the therapy for hyperlipidemia and atherosclerosis – inhibition of gravin mediated signaling can decreased SREBP-2 expression leading to decreased SREBP-2 dependent cholesterol synthesis and thus, decreased serum lipid levels and ultimately vascular plaque formation.

7.2 The effect of growth factors (Ang II or PDGF) stimualtion in gravin-t/t VSMCs migration and proliferation

Atherosclerosis, a chronic inflammatory response in arterial walls, results from the contributions of atherogenic factors. These factors alter the vascular endothelium cells (EC), capable of triggering a cascade of events starting with escalated recruitment of leukocytes. Inflammatory cells and vascular cells release cytokines and growth factors, including Ang II and PDGF. These growth factors normally maintain a delicate balance in vascular homeostasis (Lusis, 2000; Rader & Daugherty, 2008). During atherogenesis, over-release of growth factors activate VSMC by stimulating their proliferation/migration and

synthesizing matrix components in intima, which eventually leads to vascular lesion. In advanced disease, fibroblasts and VSMCs with extracellular calcification give rise to fibrocalcific lesions. Also, atherogenic lesions are caused by extracellular and intramural calcification of arteries induced by hypercholesterolemia-promoted and MMP9-promoted calcium deposition (Rader & Daugherty, 2008; Demer & Tintut, 2008; Qin, Corriere, Matrisian, & Guzman, 2006).

Gravin binds PKA, PKC, Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) and β-ARs to coordinate signal transduction (Tao, Wang, & Malbon, 2003; Langeberg & Scott, 2005). A recent study has shown that cell function can be regulated by decreasing intracellular Ca^{2+} levels through growth factor receptor mediated signaling of PKA and PKC (Shobe, 2002). It was also previously reported that Ang II stimulation increases intracellular Ca^{2+} levels and activates PKC signaling (Freeman, Chisolm, & Tallant, 1995). Both increased levels in Ca^{2+} and PKC signaling promote cell migration and proliferation. In these present studies, isolated VSMCs from gravin-t/t mice showed less cell migration in response to both Ang II (Figure 22A) and PDGF (Figure 28A) and this response was correlated with decreased intracellular calcium transients (Figure 23 & 29) as well as decreased PKC activity and

PKC phosphorylation (Figure 25A & 32). Isolated VSMCs from gravin-t/t mice also displayed decreased PKA/PKC activity and PKA/PKC-dependent substrate phosphorylation following either Ang II or PDGF stimulation (Figure 24-25 & 30-31).

PKC, a binding partner of gravin, has been implicated in many important VSMC cellular processes, including apoptosis induced by Ang II (Yoshida, 2007). Activation of PKC is required for Ang II induced migration and hypertrophy of VSMCs (Nakashima et al., 2008). Additionally, after binding to the receptor, PDGF can also trigger PLC/PKC signaling to directly induce cell migration within minutes (Tallquist & Kazlauskas, 2004). It has been reported in the literature that gravin binds to PKC and that this PKC binding to gravin results in the suppression of PKC activation (Guo, Gao, Rothschild, Su, & Gelman, 2011). Here, our data showed that isolated gravin-t/t VSMCs had higher basal PKC activity and this was due to gravin's contact inhibition of PKC (Figure 25A). Interestingly, gravin-t/t showed higher basal PKC activity with similar PKC substrate phosphorylation as WT. The ELISA kit detects the phosphorylation of the coated substrate. The phospho-(Ser) PKC Substrate Antibody recognizes endogenous levels of many cellular proteins only when phosphorylated at serine residues surrounded by Arg or Lys at the -2 and +2

positions and a hydrophobic residue at the +1 position. That may make the PKC activity results uncorrelated with the PKC substrate phosphorylation results. It is also possible due to the spatial and dynamic properties of PKC signaling transduction in the cells. Scaffolding proteins plays important role not only in recruit PKC with other signaling transducer, but also direct the signaling complex into different subcellular compartments and regulate the transduction (Rosse et al., 2010).

Interestingly, PKC-dependent substrate phosphorylation in response to Ang II or PDGF treatment was altered in the absence of functional gravin, as compared to WT VSMCs (Figure 25B & 31). Specifically, one particular PKC-dependent substrate, ERK1/2 showed decreased phosphorylation in response to both Ang II and PDGF, in VSMCs isolated from gravin-t/t mice. This response is interesting since it has been reported that PKCổII is essential for ERK and JNK activation following post balloon injury (Yoshida, 2007). Furthermore, it is also known that Ang II induces phosphorylation of ERK1/2 and stimulates protein expression, which together contributes to vascular remodeling, ECM deposition and growth factors secretion (Touyz & Schiffrin, 2000). In addition, the activation of PKCβ, via the regulation of ERK1/2, contributes to cell migration and proliferation after vascular injury (Andrassy et

al., 2005). Upon activation, ERK1/2 alters transcriptional activity by phosphorylating and activating transcription factors related to cell migration and proliferation. While some PKC isoforms are calcium dependent and some are calcium independent, we used the calcium chelator Bapta-AM to determine the calcium dependency of the Ang II signaling response in gravin-t/t VSMCS. We found that Ang II induced ERK1/2 activation was inhibited by the calcium chelator Bapta-AM (Figure 27), which did not affect the ERK activation induced by PDGF. Furthermore, the Ca^{2+} chelator Bapta-AM inhibited total Ang II stimulated PKC-dependent phosphorylation of PKC substrates in gravin-t/t VSMCs, but did not inhibit the PDGF stimulated PKC-dependent substrates phosphorylation. This indicates that Ang II induced alternation of PKC signaling undergo calcium-dependent regulation in gravin-t/t VSMC while PDGF induced alterations of PKC signaling was calcium-independent.

PKC signaling activates numerous other targets, in addition to ERK1/2 activation. Additionally, PKC is also known to be involved in the regulation of MMP isoform expression in the vascular system. Specifically, activation of PKC increases MMP-2 and MMP-9 secretion. It has also been shown that resistin-induced VSMC migration was modulated by PKC activation partially

via increased MMP-2 and MMP-9 expressions (Ding et al., 2011). We demonstrated that MMP-9 expression in VSMCs was down-regulated as a compensatory response in the absence of functional gravin in response to Ang II stimulation (Figure 26).

Gravin-t/t VSMCs exhibit other important properties, including decreased expression of the putative protective signaling proteins of MMP-9 and ERK1/2, that are induced following Ang II stimulation. It is known that MAPK activation is required for cell cycle initiation (G0/G1), as well as cell cycle progression (Pages, Lenormand, Allemain, Chambard, & Meloche, 1993). Recent studies indicate that PLC / PKC signaling systems are particularly important during mid G1 instead of at the G0/G1 transition (Jones & Kazlauskas, 2000). The absence of functional gravin is accompanied by a subsequent down-regulation of calcium-dependent PKC signaling pathway involved in ERK1/2 activation and MMP-9 expression leading indirectly to normalized inhibition of cell cycle in response to Ang II.

Survival signal pathways have been implicated in the cytoprotective effect of gravin in ischemic heart disease, including PI-3K / Akt / Nrf2 (Lee et al.,

2006; Lee et al., 2006; Li et al. 2007), ERK1/2-p90RSK / p38MAPK / NFκB (M.-H. Li, Jang, Na, Cha, & Surh, 2007;Brouard et al., 2002), JAK-2 / STAT (Tongers et al., 2004). However, we did not observe any significant differences in Akt phosphorylation between WT and gravin-t/t VSMCs with or without PDGF stimulation (Figure 30).

Different subcellular localization of individual PKC isoforms indicates that isoform-specific functions of growth factors mediate VSMC signaling pathway. In our study, we did not rule out the specificity of PKC isoforms are involved in gravin mediated signaling. Thus, a possible explanation of the differing response on cell proliferation in response to Ang II and PDGF stimulation may be attributed to gravin's binding to different PKC isoforms in response to growth factor activation. Therefore, the absence of functional gravin is likely to decrease intracellular calcium as well as the activation of PKC isoforms, resulting in decreased VMSC proliferation and migration (Figure 22). Furthermore, our results indicate that decreased neointima formation and aortic wall lesion in gravin-t/t mice indicate the protective role of inhibiting gravin against atherosclerosis.

In accordance with our hypothesis, subsequent findings demonstrated that

gravin plays important role in mediating VSMC migration and proliferation during the formation of pathological lesion. We suggest blocking gravin protein expression as a potential approach to specifically reduce VSMC proliferation and migration, and thus to prevent atherogenesis.

We conclude that during atherogenesis, ablation of functional gravin may potentially protect against atherosclerosis, reduce lesion growth and promote lesion stability through multiple potential mechanisms (Figure 36). These mechanisms include: (1) inhibiting proliferation and migration of VSMC causing reduced aortic plaque and/or lesion formation; (2) limiting the production of proinflammatory cytokines and growth factors and protecting against cytotoxic inflammatory response; (3) reducing extracellular and intramural arterial calcification via a decrease of calcium deposits; and (4) decreasing total cholesterol and LDL by affecting lipid metabolism. In summary, our data imply a potential role of gravin during atherogenesis where by inhibiting gravin dependent signaling reduced excessive VSMC proliferation, VSMC migration and extracellular matrix synthesis through suppressing PKC activity, calcium transient, ERK1/2 activity. The phosphorylation of ERK1/2 and increase calcium transient induced by growth factors stimulation can activate the transcription factors (NFkB, NFAT), which regulate genes expression

related to cell migration and proliferation. For the lipid metabolism, we hypothesize that the absence of gravin mediated signaling in the liver, suppresses the cholesterol synthesis related gene expression due to inhibition of SREBP-2, leading to prevent HFD induced hyperlipidemia. Gravin-t/t showed less HFD induced hyperlipidemia, which causes less lipid accumulation in the liver to induce liver damage and less inflammation response in the aorta leading to less plaque formation.

This study was designed to develop our understanding of the central function and signaling transduction role of gravin in the initiation and progression of atherosclerosis. This study represents the first step to investigate the mechanisms and consequences of gravin-related signaling in response to growth factors (such as Ang II and PDGF) stimulation in atherosclerosis.

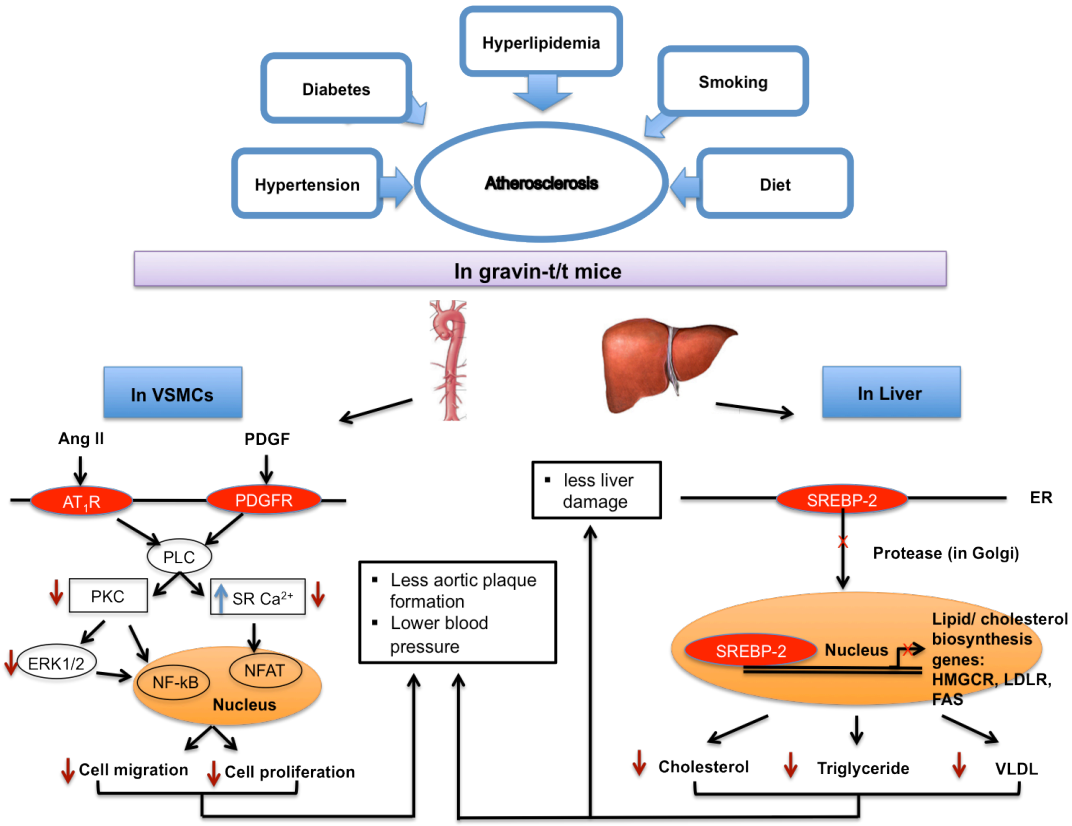

Figure 36: Schematic model of the signaling transduction in absence of gravin mediated signaling in VSMCs and in liver.

8 Limitations

In current *in vivo* study, we use dietary fat cholesterol to induce atherosclerosis in C57BL6 mice strain for 16 weeks. It has shown that C57BL/6J mouse strain has the capability to induce elevations in total cholesterol and mild atherosclerosis (Schreyer, Wilson, & LeBoeuf, 1998; Nishina, Wang, et al., 1993; Nishina, Lowe, et al., 1993). This is the first finding that the absence of gravin mediated signaling showed resistance to hyperlipidemia and atherosclerosis. Other atherosclerosis animal models, such as carotid artery ligation for arterial injury and transgenic mouse models (LDLR-/- and Apo E-/- mice), will be used in future studies to ascertain whether this resistance will still be maintained in gravin-t/t mice. However, it had been noted in the literature that no single animal model is a perfect model system to fully recapitulate the pathology the disease of atherosclerosis (Ye, Cheah, & Halliwell, 2013).

Further analysis of the vasculature of our gravin-t/t model as a model that is representative to human restenosis needs to be further investigated. During atherosclerosis, it is important to note that gravin might be involved in various

signaling processes, including Ang II mediated signaling, hypercholesterolemia and other atherogenic factors. In fact, our findings suggest that gravin is involved a complex network of multiple kinases. This complex network of kinases regulates VSMC proliferation, VSMC migration as well as lipid metabolism during the initiation ad progression of atherosclerosis. In addition, the reduced MMP-9 expression and decreased hypercholesterolemia, and decreased calcium deposits, all together limits arterial lesions and remodeling and thus limits the extent of atherosclerosis. Also, VSMCs apoptosis may retard the accumulation of lipids and the generation of foam cells (Stoneman & Bennett, 2004). These events together may prevent the initiation and progression of atherosclerosis.

9 Future Directions

Apo E-/- and gravin-t/t double knocked out will be generated in order to further ascertain the function of gravin in vascular lesion induced by high cholesterol western diets. As we know that lipid accumulation leads to the inflammatory response in the aorta. We observed that gravin is involved in LDL receptor expression in the liver. So, the next step is to test the cholesterol uptake in gravin-t/t VSMCs stimulated with ox-LDL or co-culture with macrophage and cholesterol compared with WT.

Atherogenic factors such as hypercholesterolemia, diabetes, hypertension, and heart failure result in increased release of angiotensin II, which acts on AT_1 receptors. In order to fully understand the role of gravin in response to Ang II stimulation during atherosclerosis, we will implant mini-osmotic pumps delivering Ang II or vehicle subcutaneously into male WT and gravin-t/t mice, mainly to detect the neointima formation.

Finally, environmental changes can induce physiological and pathological modification through alteration oF gene expression, which is regulated by DNA

methylation and histone modification during epigenetic processes (Burdge & Lillycrop, 2010). As we observed in gravin-t/t mice, several different classes of gene expression (MMP-9 and SREBP-2), which are induced by atherogenesis factors (growth factors or HFD), are suppressed. Thus, our data is suggestive that further testing is warranted in determine whether this modification involves DNA binding factors (co-activators and modified histone) and epigenetic chromatin remodeling by using Chromatin Immunoprecipitation (ChIP) assays.

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10 Summary and Conclusions

Gravin-t/t mice versus WT in response to high fat diet showed:

1. lower body weight and liver weight ratio with no significant difference in food and water consumption;
2. lower systolic and diastolic blood pressure with no significant difference in heart rate;
3. less liver lipid accumulation and damage with lower serum AST and ALT level;
4. lower total cholesterol, VLDL, and triglyceride with no significant difference in HDL and LDL;
5. less aortic plaque formation;
6. less MMP9 and F4/80 expression on the aortic sections,
7. reduced hepatic gene expression involved in lipid and cholesterol metabolism: lower relative mRNA level of HMG-CoA reductase (HMGCR) as well as lower level of fatty acid synthase (FAS) and low density lipoprotein receptor (LDLR), with no significant difference in the expression of lipoprotein lipase (LPL);
8. no significant difference of SREBP-1c mRNA expression in either ND or HFD-treated mice;

9. less SREBP-2 activation in the liver nuclei involved in cholesterol metabolism

Gravin-t/t VSMCs versus wild-type VSMCs in response to Ang II showed:

1. decreased cell migration and proliferation;
2. decreased Ca^{2+} transient;
3. decreased PKA activity and PKA-dependent substrate phosphorylation;
4. decreased PKC activity and significant differences in PKC-dependent substrate phosphorylation;
5. Ang II induced PKC substrate phosphorylation has been inhibited by calcium chelator Bapta-AM, which did not see in WT VSMCs;
6. lower expression of the atherosclerosis markers MMP9;
7. lower ERK1/2 phosphorylation;
8. Ang II induced ERK1/2 phosphorylation has been inhibited by calcium chelator Bapta-AM, which did not see in WT VSMCs;

Gravin-t/t VSMCs, versus wild-type VSMCs in response to PDGF showed:

1. decreased cell migration and increased proliferation;
2. decreased Ca^{2+} transit;

3. decreased PKA-dependent substrate phosphorylation with no significant difference in PKA phosphorylation;
4. decreased PKC activity and significant differences in PKC-dependent substrate phosphorylation;
5. decreased PDGF induced PKC substrate phosphorylation has been inhibited by calcium chelator Bapta-AM;
6. no significant difference in phosphorylation of Akt;
7. lower ERK1/2 phosphorylation;
8. no significant different in PDGF induced ERK1/2 phosphorylation after the treatment of calcium chelator Bapta-AM.

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