The Mechanism of Discoidin Domain Receptor 1 Mediated Vascular Calcification

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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University of Toronto

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ABSTRACT

Introduction: Activation of Runt Related Transcription Factor 2 (RUNX2) is required for transdifferentiation of Vascular Smooth Muscle Cells (VSMCs) into a calcifying osteoblast-like phenotype. Our lab showed that deletion of Discoidin Domain Receptor 1 (Ddr1), decreased atherosclerotic vascular calcification in the Ldlr−/− mouse.

Hypothesis: DDR1 regulates RUNX2 activity by affecting microtubule organization during VSMC mediated calcification.

Results: Ddr1−/− VSMCs show reduced RUNX2 activity when compared to Ddr1+/+ VSMCs. Addition of the microtubule-destabilizing agent nocodazole inhibited both RUNX2 activity and nuclear localization in Ddr1+/+ VSMCs. Addition of the microtubule-stabilizing agent taxol rescued RUNX2 nuclear localization in Ddr1−/− VSMCs. Despite this, Taxol was unable to rescue RUNX2 activity as it eliminated activity in both genotypes.

Conclusion: These findings indicate that under osteogenic conditions, Ddr1 deletion impedes the dynamic instability required for the maintenance of microtubule architecture. This prevents RUNX2 nuclear localization and transcriptional activation in VSMCs.
DEDICATIONS

“Affection without sentiment, authority without cruelty, discipline without aggression, humor without ridicule, sacrifice without obligation, companionship without possessiveness.”

- William E. Blatz

For my mother Lily and my father Jeremy, who truly embody everything it means to be great parents. Since the very beginning they poured their hearts and souls into me, and their only wish is to see me happy. I could not have asked for better parents. They have always been my biggest fans and most fervent supporters. It is to both of them that I dedicate my thesis.
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αSMA – Alpha Smooth Muscle Actin
ADP – Adenosine Diphosphosphate
ALP – Alkaline Phosphatase
AMP – Adenosine Monophosphate
APOE – Apolipoprotein E
ARP – Acidic Ribosomal Protein
ATP – Adenosine Triphosphate
ATF4 – Activated Transcription Factor 4
BMP2 – Bone Morphogenic Protein 2
BSA – Bovine Serum Albumin
CD40 – Clusters of Differentiation 40
cDNA – Complementary DNA
CKD – Chronic Kidney Disease
COL1A1 – Type 1 Collagen Alpha 1
COL1A2 – Type 1 Collagen Alpha 2
COX - Cyclooxygenase
Ct – Cycle Threshold
DDR – Discoidin Domain Receptor
DMSO – Dimethyl Sulfoxide
DNA – Deoxyribo Nucleic Acid
EC – Endothelial Cells
ESRD – End Stage Renal Disease
ER – Endoplasmic Reticulum
ERK- Extracellular Related Kinase
FAK – Focal Adhesion Kinase
FGFR1 – Fibroblast Growth Factor Receptor 1
GDP – Guanosine Diphosphate
GTP – Guanosine Triphosphate
HDAC – Histone Deacetylase
HIF1α – Hypoxia Inducible Factor 1 Alpha
IBSP – Integrin Binding Bone Sialoprotein
IGF-1 – Insulin-like Growth Factor 1
IL – Interleukin
LDL – Low Density Lipoprotein
LDLR – Low Density Lipoprotein Receptor
MΦ – Macrophage
MAP – Non Motor Microtubule Associated Protein
MAPK – Mitogen Activated Protein Kinase
MEKi – MEK Inhibition
MGP – Matrix GLA Protein
MMP – Metallomatrix Proteases
MTOC – Microtubule Organizing Center
NFκB – Nuclear Factor Kappa B
NO – Nitric Oxide
iNOS – Inflammatory Nitric Oxide Synthase
NPP1 - Neuroecto Pyrophosphatase 1
OCN – Osteocalcin
OCT4 – Octamer Binding Transcription Factor 4
ON - Osteonectin
OPN – Osteopontin
OSE – Osteoblast Sensitive Element
OxLDL – Oxidized Low Density Lipoprotein
P38i – P38 Inhibition
PBS – Phosphate Buffered Saline
PBST – Phosphate Buffered Saline Tween 20
PCR – Polymerase Chain Reaction
PFAv- Paraformaldehyde
Pi – Inorganic Phosphate
PIT – Sodium Dependant Phosphate Co-Transporter
PPi – Pyrophosphate
PTH – Parathyroid Hormone
qRT-PCR – Quantitative Real Time PCR
RBP – Retinoblastoma Protein
RNA – Ribo Nucleic Acid
RLU – Relative Luminescence Unit
ROS – Reactive Oxidative Species
RTK – Receptor Tyrosine Kinase
RUNX – Runt Related Transcription Factor
SHCA – Src Homology 2 Domain Containing A
SMED - Spondylo-Meta-Epiphysial Dysplasia
SOX9 – Sry Box 9
SSEA1 – Stage Specific Embryonic Antigen 1
STAT1- Signal Transducer and Activator of Transcription 1
TBST – Tris Buffered Saline Tween 20
TENC – Tenascin C
TGFβ – Transforming Growth Factor Beta
TNFα – Tumour Necrosis Factor Alpha
VSMC – Vascular Smooth Muscle Cell
VCAM – Vascular Cell Adhesion Molecule
VEGF – Vascular Endothelial Growth Factor
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INTRODUCTION

ATHEROSCLEROSIS

Atherosclerosis begins with dysfunction of the endothelial layer, which leads to the increased permeability of the endothelial cells (EC). This leads to the accumulation of low density lipoprotein (LDL) underneath the EC monolayer of the arterial wall, forming a fatty streak. This results in activation of the endothelial layer, which recruits monocytes. Upon egression, monocytes, differentiate into macrophages (Mφs). After the Mφs have entered the subendothelial space, they phagocytose low density lipoprotein (LDL) and oxidize LDL to produce Oxidized LDLs (oxLDLs). These cells thus become Mφ derived foam cells. In addition, they also promote Vascular Smooth Muscle Cells (VSMCs) to migrate and proliferate from the medial wall into the subendothelial space (Figure 1). During this process, Mφs and VSMCs will actively synthesize matrix proteins such as fibrillar collagens. These extracellular proteins serve many roles in atherosclerosis such as migratory lattices for the cells and also forming a fibrous cap which prevents plaque from rupturing.

As the lesion grows in size, the vessel lumen attempts to accommodate the bulging plaque through eccentric grow, if the lesion growth exceeds that of vessel growth, the vessel becomes increasingly stenosed or occluded at which point the blood can no longer perfuse tissues distal to the blockage. In a vulnerable plaque, matrix degrading enzymes along with the hemodynamic stresses from systole and diastole increasing the probability of rupture.

The probability of rupture is increased with the development of atherosclerotic or intimal calcification, which develops in advanced plaques. This process begins with the development of a necrotic core as result of dead cell accumulation which is susceptible to the
initiation of calcification\textsuperscript{11}. As calcification occurs, the plaque is destabilized from the increased focal stress points\textsuperscript{10}. Once rupture occurs, the clotting cascade initiates\textsuperscript{12}. The result is a thrombus overlaying the lesion and occluding the vessel\textsuperscript{12}. If the clot travels downstream it becomes an embolus which can block the blood supply to other tissues\textsuperscript{12}. Depending on the location of the block, this can either lead to an infarction, stroke or a myocardial infarction\textsuperscript{12}.

\textit{The Role of Collagens in Atherosclerosis:}

Collagens are expressed by both VSMCs and M\(\theta\)s during atherosclerosis and are the main protein constituent of atherosclerotic plaques, comprising \(\sim60\%\) of the total protein\textsuperscript{13}. These molecules are composed of three alpha helical chains containing Gly-X-Y repeats, where X and Y are usually proline or hydroxyproline residues\textsuperscript{14}. Type IV collagens line the basement membrane of ECs and VSMCs in normal vessels\textsuperscript{15}. Upon plaque development, the expression of Type IV collagen increases and it serves as an attachment and migration substrate for both M\(\theta\)s and VSMCs\textsuperscript{15}. Other collagens such as types I and III fibrillar collagens, provide tensile strength to the plaque\textsuperscript{15}. They are expressed in low amounts in the early plaque, but dramatically increase in expression driving the advanced stages of plaque development\textsuperscript{15}. Likewise Types V and VI collagens also increase in expression with plaque age, however their role within the plaque is largely unknown\textsuperscript{15}. Type VIII collagen is also highly upregulated during atherosclerotic development\textsuperscript{16}. This was later found to responsible for the growth and migration of VSMCS and thus is a critical component of the injury process\textsuperscript{16}.

The importance of collagens to atherosclerosis was first hinted in the diverse roles it played in VSMCs and M\(\theta\)s. Studies showed that inhibitors of collagen synthesis attenuate VSMC
migration, which indicated that VSMC required collagen for migration\textsuperscript{17}. Indeed migration assays which were later performed on VSMCs and M\(\theta\)s revealed that Types I\textsuperscript{18} and VIII\textsuperscript{19} collagens are able to induce migration. While adherence to collagens by VSMCs is required for migration, collagenase activity is also required for the process. Collagenases are required to release focal adhesion matrix contacts and allow cell translocation\textsuperscript{17}. Degraded type I collagen serves as a chemoattractant for migrating VSMCs and M\(\theta\)s\textsuperscript{20}. In addition, type I collagen also induces monocyte maturation. Experiments which plated monocytes on type I collagen increased cell spreading and increased expression of cell adhesion molecules\textsuperscript{21}. Together these studies underscore the importance of collagens, and in particular type I collagen in the biology of VSMCs and M\(\theta\)s, which are major cell types in atherosclerosis.

Type I collagen is also critical to the maintenance of the structural integrity of an atherosclerotic plaque. Type I collagen, along with type III collagen, form a fibrous cap which provides plaque rupture\textsuperscript{20}. As a result, plaque instability is induced by degrading type I collagen fibers at the shoulder regions of the atherosclerotic plaque\textsuperscript{22-24}. While type I collagen plays an essential role in stabilizing the plaque, studies on the type I collagenase MMP1 showed that its expression reduced atherosclerotic burden\textsuperscript{25}. Furthermore, type I collagen may also contribute to plaque rupture, as it likely plays a major role in atherosclerotic calcification.

**TYPE I COLLAGEN RECEPTORS:**

Cell signalling by type I collagen is mediated by cell surface receptors. There are two main families of type I collagen receptors, the integrins: \(\alpha 1, \alpha 2\) and \(\beta 1\) and the Discoidin Domain Receptors (DDRs).
Integrins:

Integrins are a family of glycoproteins which form heterodimers consisting of one α and one β subunit. In addition to binding type I collagen, integrins are also involved in assembling secreted type I collagen monomers into fibrils\textsuperscript{26}. Upon binding to type I collagen molecules, integrins are able to signal using intracellular signalling pathways. Since integrins do not contain any kinase activity, they will either interact with intracellular kinases such as SRC, Integrin Linked Kinases (ILK) or Focal Adhesion Kinase (FAK)\textsuperscript{27}. Crosstalk with Receptor Tyrosine Kinases (RTKs) is also another method integrins use to elicit signalling\textsuperscript{27}. Type I collagen signalling through integrins has been linked to many atherosclerotic processes such as mediating leukocyte adhesions, foam cell formation and smooth muscle cell proliferation\textsuperscript{28}.

Discoidin Domain Receptors:

In atherosclerosis, VSMCs and M\textregistered{s} express DDRs, a class of collagen receptors\textsuperscript{29}. DDRs are a group of RTKs characterized by an extracellular discoidin domain, which mediates binding to triple helical collagens\textsuperscript{30}. The discoidin domain was first sequenced on the protein discoidin 1, which is a lectin found on the slime mold \textit{Dictostelium Discoidium}\textsuperscript{31}. Since its first discovery, the discoidin domain has later been identified in blood coagulation factors V and VIII and also neuropilins 1 and 2 which are involved in axonal guidance and angiogenesis\textsuperscript{32}.

DDR\textregistered{s} were first discovered during a search for RTKs that were highly expressed in malignant cancer cell lines\textsuperscript{30}. To date two DDR genes have been identified in humans and mice, DDR1 and DDR2\textsuperscript{30}. DDR1 contains five splice variants which are widely expressed named a-e,
and an additional splice variant found in rat testes\(^\text{30}\) (**Figure 2**). Conversely, DDR2 has only one form\(^\text{30}\).

Although there is considerable overlap between the ligands of the DDRs, each receptor is able to bind its own repertoire of triple helical collagens. DDR1 binds types I-V and VIII collagens, while DDR2 binds types I-III and X collagen\(^\text{30}\).

**DDR Signalling:**

DDR1 phosphorylation leads to downstream signalling and activation of cellular pathways. The DDR proteins facilitate binding through an N terminal discoidin domain which is attached to the extracellular stalk domain, a transmembrane domain and a cytoplasmic tyrosine kinase domain at the C Terminus\(^\text{30}\). Upon binding triple helical collagen and dimerization, the intracellular kinase domains autotransphosphorylate each other and initiate signalling\(^\text{30}\). In comparison to most RTKs, DDR1 have delayed phosphorylation kinetics. Depending on the cell type, maximum phosphorylation can occur at an hour to several hours after initial stimulation with collagen\(^\text{33}\). This is a process that is dependent on cell adherence, as cells grown in suspension typically show peak phosphorylation at much earlier time point than cells grown on a monolayer\(^\text{34}\). Similarly, DDR2 receptor phosphorylation kinetics are also slow and occur in a process which is not well understood\(^\text{35}\).

Studies also show that DDR1 crosstalks with integrin, G-protein coupled receptors and WNT signalling pathways in order to fine tune specific cell responses to ligand activation\(^\text{36, 37}\). A recent proteomic study determined that DDR2 may cross talk with Insulin Receptor\(^\text{38}\). Ligand
activation of DDRs are also cell type dependent and is likely the result of differences in intracellular adaptors.

DDR1 has been shown to interact with several adaptor proteins such as Src homology 2 containing protein A (SHCA)\textsuperscript{39}, NCK2, and CAS which are recruited to DDR1 upon phosphorylation to facilitate signalling\textsuperscript{40}. However, not all adaptors are recruited upon DDR1 activation. Adaptors such as WW-domain containing protein 1\textsuperscript{41} and Protein phosphatase 1 regulatory subunit\textsuperscript{42} dissociate from DDR1 upon activation, followed by the activation of Mitogen Activated Protein Kinase (MAPK) ERK signalling pathways in HEK 293 cells upon stimulation with type IV collagen. DDR1 regulation of the MAPK Extracellular Related Kinase 1/2 (ERK1/2) has been identified with many DDR1 processes. DDR1 signalling is important in regulation of mouse embryonic stem cells in an undifferentiated state by type I collagen\textsuperscript{43}. In this study, the presence of DDR1 was responsible for maintaining the expression of NANOG, Octamer Binding Transcription Factor 4 (OCT4) and Stage Specific Embryonic Antigen 1 (SSEA-1). This process is dependent on DDR1 signalling through ERK1/2. DDR1 –ERK1/2 signalling is also required for the upregulation of MMP10 expression induced by type I collagen in human lung fibroblasts\textsuperscript{44}. Furthermore, our own laboratory discovered that DDR1 phosphorylates ERK1/2 upon stimulation with type I collagen in VSMCs\textsuperscript{18}. ERK1/2 has also been linked as a downstream component of DDR2 signalling in RUNX2 activation\textsuperscript{45}. In a study on mouse osteoblasts, the authors showed that DDR2 null cells showed decreased calcification, Ocn expression and RUNX2 activity\textsuperscript{46}. P38 another MAPK has also been linked to DDR1 signalling in various cell types. In mouse microglia, type I collagen induced DDR1 signalling through P38 is required for inflammatory induction of Clusters of Differentiation 40 (CD40), Cyclooxygenase 2 (COX2) and
MMP9. DDR1 is also important in the P38 induction of Nitric Oxide (NO) production in murine macrophages through the upregulation of Inflammatory Nitric Oxide Synthase (iNOS). The inflammatory role of DDR1-P38 signalling is further explored in a study demonstrating that it is crucial for the activation of Nuclear Factor Kappa B (NFκB) in mouse monocytes. As ERK and P38 signalling are fundamentally important to functioning of many different pathways in many different cell types, DDR1 signalling may contribute to a number of key processes in normal function and in pathology.

**DDR Function in Smooth Muscle Cells**

Our laboratory discovered that, denudation of DDR deficient mice showed reduced intimal thickening compared to WT mice. This suggests an important role of DDR1 in vascular injury. Later it was found that both DDR1 and DDR2 are present in the atherosclerotic plaques of humans and primates. These studies implied an important role of DDR1 in VSMCs. Indeed, VSMCs were later found to express a high level of DDR1 *in vivo* and *in vitro*. In DDR1 KO VSMCs, migration, proliferation and adhesion were significantly decreased. In addition, KO VSMCs also showed decreased expression of proatherogenic MMPs 2 and 9. These deficiencies in the DDR1 KO cells were later found to be rescued with DDR1 transfection. These studies all suggested that DDR1 played an important role in VSMCs during atherosclerosis.

Studies on DDR2 in VSMCs by others have demonstrated that hypoxia induces the P38 signalling pathway to promote MYC and Myc Associated factor X binding to DDR2 promoter. The process increases the expression of DDR2 and increases the migration of VSMCs. Another study furthering the understanding of this mechanism showed that TNFα also contributes to
DDR2 upregulation during VSMC migration\textsuperscript{54}. The process by which DDR2 facilitates migration in VSMCs is revealed to be dependent on MMP2 activity. In support of DDR2s migratory role, another study also showed that DDR2 functions to reduce Focal Adhesion Kinase activity in VSMCs\textsuperscript{55}. Despite these identified migratory roles of DDR2, similar studies carried out to investigate the role of DDR2 in VSMCs by our laboratory showed that deletion of Ddr2 did not affect cell migration, proliferation or migration in VSMCs\textsuperscript{56}. These studies may reflect the differences of the model system used to study DDR2 function. Our lab studied DDR2 function through the use of carotid VSMCs from DDR2 KO mice, while the mentioned studied used VSMCs from rat aorta. Furthermore, the aforementioned studies performed migratory experiments in hypoxia whilst our study was carried out under normoxia.

**DDR1 in Atherosclerosis and Calcification:**

Studies by our laboratory have demonstrated that DDR1 has important and different functions in VSMCs and monocytes in atherosclerosis. DDR1 expression and function was investigated in the Ldlr\textsuperscript{-/-} model of atherosclerosis \textsuperscript{57}. Ddr1\textsuperscript{-/-}; Ldlr\textsuperscript{-/-} mice exhibited smaller plaques that were rich in fibrous proteins such as fibrillar collagen and elastin compared with Ddr1\textsuperscript{+/+}; Ldlr\textsuperscript{-/-} mice \textsuperscript{29}. These studies also revealed that the increase in fibrous proteins was due to increased synthesis of matrix molecules such as type I collagen by VSMCs. It was revealed that Ddr1\textsuperscript{-/-}; Ldlr\textsuperscript{-/-} mice had decreased decrease in the expression of Monocyte Chemotactic Protein 1 (MCP-1) and Vascular Cell Adhesion Molecule 1 (VCAM-1). Furthermore, this study also indicated that Ddr1\textsuperscript{-/-}; Ldlr\textsuperscript{-/-} M\textsuperscript{0}s showed decreased mRNA expression of MMP2, 8, 13 and 14. These results suggested that DDR1 deficiency in M\textsuperscript{0}s prevented plaque invasion.
A later study by our laboratory confirmed that MØs deficient in DDR1 have reduced invasion into the plaque as a result of decreased attachment to type IV collagen and response to Monocyte Chemoattractant Protein 1 (MCP-1)\textsuperscript{58}. Overall these experiments showed that MØs deficient in DDR1 reduced atherogenesis. While the function of DDR1 indicates that it functions to increase attachment and plaque invasion in MØs, studies carried out by our lab demonstrate other functions in VSMCs. A later study carried out by our lab demonstrated that DDR1 deficiency in the vessel increases both matrix production and plaque size\textsuperscript{29}. This is presumed to be carried out by VSMCs which show increased synthesis of procollagen I mRNA and also had a net increase of MMP and net decrease of TIMP mRNA production in the DDR1 deficient VSMCs, which allowed for increased matrix production and remodelling in the plaque. Taken together these studies indicate that DDR1 plays important roles in modulating the infiltration and inflammation of MØs and suppressing matrix remodelling and synthesis in VSMCs.

Another hallmark of atherosclerosis which was discovered in the course of studies on DDR1 in atherosclerosis showed that $Ddr1^{+/+}; Ldlr^{-/-}$ mice had indications of increased markers of chondrogenic differentiation, Sry Box 9 (SOX9) and type X collagen, along with increased propensity to calcification of the atherosclerotic plaques. By contrast, $Ddr1^{-/-}; Ldlr^{-/-}$ mice showed decreased plaque calcification\textsuperscript{59}. Together, these studies indicated that DDR1 plays an important functional role in atherogenesis and the development of atherosclerotic calcification.
DDR2 in Bone Mineralization and Arthritis:

DDR2 has been implicated in several studies related to bone development and osteoarthritis. The role of DDR2 in bone biology was first hinted when a screen of RTKs of human osteoarthritis chondrocytes which showed an upregulation of the DDR2 mRNA transcript when compared to un-diseased chondrocytes\(^6^0\). Indeed later studies showed that DDR2 expression is increased in the cartilage joints of the collagen type IX and XI deficient mouse models of osteoarthritis\(^6^1\). It was also found in these studies that DDR2 when bound to type II collagen acted to induce the expression of MMP13 in these chondrocytes, which is hypothesized to contribute to the mechanism of osteoarthritis\(^6^2\). Further evidence demonstrating the importance of DDR2 in chondrocyte biology is revealed that upon stimulation with type II collagen, DDR2 was able to upregulate the mRNA expression of the proinflammatory cytokine IL-6\(^6^0\). This process was dependant on DDR2 signalling through ERK, P38 and Nuclear Factor κ B (NFκB).

The consequence of DDR2 mutation was also identified with a clinical manifestation in calcification abnormalities. Spondylo-meta-epiphyseal dysplasia (SMED) is a autosomal recessive disease characterized by short limbs and a host of skeletal abnormalities. This disease was recently mapped to mutations within the Ddr2 gene which affected the tyrosine kinase domain of the receptor\(^6^3\). Although the mechanisms have not been identified, it is likely that these mutations affect DDR2 signalling. The role of DDR2 in calcification was solidified in a study demonstrating that DDR2 signals to activate RUNX2 through ERK signalling in chondrocytes\(^4^5\). Together, these studies indicate an important role for DDR2 in calcification. Although DDR1 has
not been studied as extensively in the context of calcification, the similarities between receptors suggest a probable role of DDR1 in calcification.

**VASCULAR CALCIFICATION**

*Types of Vascular Calcification:*

There are three main types of vascular calcification: 1) intimal calcification which is seen in patients with atherosclerosis, 2) medial calcification, also known as Monckeberg’s arteriosclerosis and 3) calciphylaxis, a systemic calcification of the vascular system. Although these are separate and distinct forms of vascular calcification, there is frequent overlap of these diseases in pathology where several forms of calcification can be found together\(^{64}\). Such is the case when medial calcification occurs with atherosclerotic calcification.

Atherosclerotic calcification is a feature of advanced atherosclerotic plaques, and manifests as calcification in the intimal layer\(^{65}\). Although there is limited evidence showing that calcification may occur as a result of circulating progenitors with a calcifying phenotype, most evidence suggests that mineralization in atherosclerosis is affected by two major cell types, VSMCs\(^{66}\) and M\(\Phi\)s\(^{67}\). Although both cell types are found adjacent to the calcified region, there is no evidence that M\(\Phi\)s transdifferentiate into an osteoblast phenotype under these conditions. VSMCs are the main candidate for driving mineralization as they are phenotypically plastic and derive from the same mesenchymal precursor cells as osteoblasts\(^{68, 69}\). Evidence suggests that the expression of RUNX2 promotes VSMC conversion to an osteochondrogenic phenotype\(^{70}\). In mature atherosclerotic calcifications, the tissue can appear as bone\(^{71}\). In extreme cases, this tissue is complete with vascularisation and a hematopoietically active marrow\(^{71}\).
Medial calcification of the arteries can happen as an entity by itself, or as a result of intimal calcification in advanced atherosclerosis\textsuperscript{72}. When medial calcification occurs in association with atherosclerosis, it usually lies in the medial layer adjacent to the atherosclerotic calcification. Histologically, hydroxyl apatite crystals depositions occur along the internal elastic lamina. While the mechanism of medial calcification is not as well defined as in atherosclerotic calcification, current work suggests that RUNX2 plays a role. The consequences of the medial calcification result in the hardening of the arterial wall known as arteriosclerosis\textsuperscript{10}. As a result, pulse pressure is increased and therefore chronic stress can further exacerbate kidney failure in addition to heart failure\textsuperscript{10}.

Calciphylaxis is another form of vascular calcification that occurs in the medial layer. Similar to medial calcification, calciphylaxis may also arise as result of End Stage Renal Disease (ESRD)\textsuperscript{10}. In addition to ESRD, calciphylaxis may also arise from non-uremic disease conditions such as hyperthyroidism, liver cirrhosis, inflammatory diseases such as rheumatoid arthritis and Crohn’s disease or as a secondary complication of chemotherapy\textsuperscript{10}. Despite their similarities, calciphylaxis and medial calcification represent vastly different pathologies. Calciphylaxis refers to the systemic calcification of all blood vessels at the medial layer and non-bone extravascular tissues, whereas medial calcification occurs primarily in the major arteries\textsuperscript{10}. As the calcification is systemic, this leads to poor perfusion of the tissues resulting in tissue necrosis\textsuperscript{10}. Also, the hardened blood vessels which can also lead to thrombosis and increased blood pressure, which ultimately results as increased pressure on cardiac pulmonary system and ultimately heart failure.
Models of Vascular Calcification:

Calcification is studied using models which emulate the pathophysiologic perturbations which occur that ultimately lead to calcification. The factors which result in calcification stem from a hormonal imbalance such as high levels of Parathyroid Hormone (PTH), hyperphosphatemia, loss of calcification inhibitors, drug usage and as a secondary complication of a disease; because of this most models of calcification perturb either one or more of these factors. In vivo studies have largely been carried out in rabbit, rat and mice, while in vitro studies have been carried out on VSMCs, and organ cultures of varying species origin\textsuperscript{73}. As it is relatively easy to genetically manipulate a mouse, various genes which inhibit calcification such as Fetuin A\textsuperscript{74}, Matrix GLA Protein (MGP)\textsuperscript{75} or Osteopontin (OPN)\textsuperscript{76} have been knocked out in order to produce models of calcification. However, because some of these genes are expressed in most tissues, extraskeletal mineralization is not limited to vascular tissue, but is extensive throughout the soft tissues of the animal. This can be addressed by using a smooth muscle cell specific gene such as SM22α to drive the cre lox recombinase system\textsuperscript{77}. Genes with high penetrance such as Feutin A KO animals can produce vascular calcification without further manipulation\textsuperscript{78}. However animal models making use of genes with lower penetrance such as OPN knockout mice, need to be combined with other perturbations in order to produce a vascular calcification model\textsuperscript{78}.

A commonly used disease model to study calcification is atherosclerosis. Most atherosclerotic calcification studies are performed by feeding animals a high fat and cholesterol diet to develop atherosclerosis. As such there have been many studies on atherosclerosis using this model with rabbits, rats, and mice. Mice however, are inherently resistant to developing
atherosclerosis even with an atherogenic diet \(^79\). As a result, an atherogenic diet is accompanied with genetic manipulation deleting either the LDL receptor or Apolipoprotein E (APOE), by deleting the LDL receptor or APOE you are able to increase the circulating levels of cholesterol to promote atherosclerosis\(^79\). As the animals develop atherosclerosis and the complications that come with it, calcific deposition will occur in the intima of the plaque. In addition, various factors can also be studied while the animals are undergoing calcification. In mice, genes can be manipulated to study their affects on atherosclerotic calcification.

Medial calcification commonly occurs in association with ESRD, because this is the case, animals models of Chronic Kidney Disease (CKD) combined with a high phosphate diet are commonly used to study medial calcification. Renal failure is most commonly induced by the 5/6 nephrectomy, which involves the surgical removal of one kidney along with the partial ablation of the remaining kidney either by partial nephrectomy, posterior or anterior renal artery ligation or by electrocauterization on the cortex of the remaining kidney\(^80\). As a result of the reduced nephron number combined with the high phosphate diet, the animals will develop severe hyperphosphatemia\(^80\). Thus, the resulting process promotes calcification of the arteries. Due to the complexity of this surgery it is usually done in larger animals such as rabbits and rats, but there has been some success using this technique with mice\(^81\).

The effects of high phosphate can also be studied in VSMCs\(^82\) and aortic ring sections\(^83\) in tissue culture. Prior to studies on calcification of vascular tissues these cultures were used to stimulate mineralization in long bones, calvaria, and cells of the osteoblast lineage\(^84\). The major benefit of this technique is the relatively fast endpoint in which you can acquire data. These cultures typically involve supplementation of phosphate either in the inorganic form as Pi\(^85\) or
as β Glycerol Phosphate\textsuperscript{86}. In addition the process can be accelerated with supplementation of ascorbic acid to induce matrix synthesis to provide the calcifying cells with an osteoid\textsuperscript{87}. Also dexamethsone can be added which helps to inhibit the VSMC proliferation and promote the ossifying phenotype\textsuperscript{88}. As a result, in vitro assays provide the potential to study the effects of cytokines, various ligands, extracellular matrix and other extracellular forces such as shear and tension which can provide mechanistic cues for vascular calcification.

\textit{Mechanisms of Vascular Calcification:}

The mechanism for vascular calcification was long thought to be the passive deposition of hydroxy apatite crystals in the blood vessel walls; while this may hold true for calciphylaxis, more recent evidence shows that both atherosclerotic and medial calcification is an active cellular osteogenic process\textsuperscript{10}. This process is regulated by the osteochondrogenic transcription factor RUNX2 and regulation of its activity\textsuperscript{89}.

During atherosclerotic calcification, the VSMCs respond to many different stimuli which can promote osteochondrogenic differentiation. Oxidized LDL particles (OxLDL) which are in abundance within the atherosclerotic plaque are known to activate Toll Like Receptor 2 to activate the osteogenic process\textsuperscript{90}. In addition, because atherosclerosis is an inflammatory vascular disease, many inflammatory cells such as macrophages and other leukocytes which secrete cytokines such as IL6\textsuperscript{91} and TGFβ\textsuperscript{92} are a part of the inflammatory process. These molecules have shown to elevate the activity of RUNX2 and ultimately calcification. Other factors such as cell death from apoptosis and necrosis also contribute to calcification. Upon death these cells leave behind apoptotic bodies, which can provide the seeding points for
amorphous calcium phosphate deposition\textsuperscript{11}. Not only do amorphous crystals provide the primary seed sites for apatite crystals, but the crystals themselves are able to promote osteochondrogenic transdifferentiation through an unknown process\textsuperscript{93}. Furthermore, physical factors such as shear stress are able to regulate calcification as both regions of low shear and low shear \textit{in vitro} environments promote osteogenic conversion in cells\textsuperscript{94}. Ultimately these factors result in VSMCs transdifferentiation into an osteochondrogenic phenotype. These cells which are the precursors to chondrocyte and osteoblast-like cells synthesize a matrix which is permissive to calcification called the osteoid\textsuperscript{95}. This matrix is rich with type I collagen and calcium binding proteins such as Osteocalcin (OCN), Osteonectin (ON) and OPN\textsuperscript{96}. At later stages it is hypothesized to mirror endochondral ossification, in which the chondrogenic-like cells die while the osteoblast-like cells predominate\textsuperscript{97}. The osteoblast-like cells cause mineralization of the osteoid by secretion of calcium phosphate containing matrix vesicles\textsuperscript{98}. During this period, osteoblast-like cells produce proteins such as ALP\textsuperscript{99} and PiT-1\textsuperscript{100} in order to increase intracellular Pi levels and mineralize the matrix. Initially this matrix is largely amorphous calcium phosphate, over time the amorphous calcium phosphate is replaced with hydroxyl apatite and later develops into bone-like tissue\textsuperscript{101}.

In contrast to atherosclerotic plaque calcification, medial calcification largely stems as a secondary complication from a pathological state. These states include atherosclerosis, parathyroidism, diabetes mellitus and hyperphosphatemia from ESRD caused by CKD\textsuperscript{10}. The most common cause of medial calcification is CKD\textsuperscript{102}. In CKD, the function of the kidney is compromised and it is unable to secrete phosphate leading to elevation of phosphate concentration in the serum, the resulting hyperphosphatemia leads to medial calcification\textsuperscript{103}. 
While it was previously thought that medial calcification occurred through an osteogenic independent process, recent studies by Giachelli and colleagues have demonstrated that activation of the RUNX2 osteogenic gene program occurs in medial calcification81.

Medial calcification can also arise as a result of deficiency of tissue inhibitors of calcification. Genetic deletion of tissue inhibitors of calcification such as Neuroecto Pyrophosphatase 1 (NPP1)104, and MGP105 results in ectopic calcification in both humans and mice. Furthermore, studies show that deficiency of circulating inhibitors such as Feutin A74 and Klotho106 also promote medial calcification. The use of drugs which prevent inhibiting function may also be the cause of medial calcification. Warfarin is a drug that is commonly used to treat anticoagulation in patients with cardiovascular problems. It works by preventing the γ carboxylation of several clotting factors. As MGP also requires γ carboxylation, this process is hindered as well107.

Although atherosclerotic and medial calcifications are initiated through separate pathways, both ultimately converge upon the expression of RUNX2. With respect to medial calcification, whether RUNX2 contributes to the calcification process or is the result of activation by calcium phosphate depositions is unclear. As in medial calcification, calcium deposition occurs in association with degraded elastin fibers in the internal elastic lamina, and not reminiscent of the osteoid synthesis in atherosclerotic calcification108. However, in atherosclerotic calcification, studies have demonstrated that the activation of RUNX2 is indispensible for osteochondrogenic differentiation109.
The Role of Phosphate in Vascular Calcification:

Hyperphosphatemia leads to medial calcification and calciphylaxis and can exacerbate atherosclerotic calcification\textsuperscript{10}. Previously, it was thought that hyperphosphatemia causes calcification primarily due to the increased inorganic phosphate (Pi) available for calcium phosphate formation. While this is the case for calciphylaxis, later studies, revealed a crucial role for Pi\textsuperscript{110}. Furthermore, culturing VSMCs in high Pi resulted in a VSMC phenotypic change to an osteoblastic/chondrogenic phenotype which was accompanied by the expression of RUNX2 and markers of calcification such as OCN, OPN and Alkaline Phosphatase (ALP)\textsuperscript{69}. Despite this, the precise mechanism by which Pi is able to activate the osteogenic gene program is unknown. Current evidence demonstrates that the propensity of vascular calcification is dependent upon the careful balance of Pi and pyrophosphate (PPI)\textsuperscript{111} (Figure 3).

PPI is an inhibitor of calcification that acts by inhibiting the nucleation and propagation of hydroxyapatite crystals\textsuperscript{111}. The intracellular levels of PPI in VSMCs are determined largely by the expression of NPP1\textsuperscript{112}. The primary function of NPPs is to catabolise ATP to produce PPI\textsuperscript{104}. In healthy vascular tissue NPP1 is highly expressed and its levels are reduced during vascular calcification\textsuperscript{113}. In VSMCs, NPP1 is the major protein that carries out the reaction converting ATP to AMP and PPI\textsuperscript{114}. NPP1 deficiencies in mice have manifested themselves in severe soft tissue calcification, arterial calcification and spontaneous aortic calcification\textsuperscript{104}. In humans, NPP1 deficiency has been linked to infantile medial arterial calcification\textsuperscript{115}. As NPP1 competes with Alkaline Phosphatase (ALP) for the ATP substrate, it was thought that NPP1 prevented calcification by competing for ATP. However, studies showed that PPI alone was able to prevent nucleation of calcium phosphate crystals in cell culture\textsuperscript{116}. These studies revealed that balance...
of PPi and Pi levels are important in ultimately regulating calcification and not substrate competition between NPP1 and ALP. Furthermore, the PPi which is produced by NPP1 can be hydrolyzed into two free Pi molecules by ALP, negating the competition hypothesis\textsuperscript{117}. In addition to ATP and PPi, ALP is also able to generate free Pi by the hydrolysis of ADP, AMP\textsuperscript{118} and phosphate groups on phosphoproteins\textsuperscript{119}. Unsurprisingly, ALP has been observed in many studies to be indispensable in VSMC mediated calcification\textsuperscript{120, 121}. ALP in normal physiology is required for osteoblasts to create bone tissue\textsuperscript{122}. High serum concentration of ALP is often indicative of rapid bone development, such as in childhood\textsuperscript{123}. ALP deficiency in human osteoblasts causes hypophosphatasia often with fatal results\textsuperscript{124}. If a patient with ALP deficiency survives to early childhood, they will have a severe form of rickets and early loss of deciduous teeth.

Another major determinant of intracellular Pi levels is the transport of Pi into the VSMC. Intracellular phosphate transport is mediated primarily by the family of Sodium Dependant Phosphate Co-Transporters (PiTs)\textsuperscript{125}. Type I and type II PiTs are expressed primarily in the kidney and intestinal epithelium\textsuperscript{126}, while type III PiTs are less tissue specific and play a major role in Pi transport within VSMCs\textsuperscript{127}. There are two transporters within the type III class; they are PiT-1 and PiT-2\textsuperscript{128}. PiT-1 is highly expressed in VSMCs, but PiT-2 is expressed to a far lesser degree\textsuperscript{128}. The importance of PiT-1 to vascular calcification was first revealed when it was discovered that PiT-1 is necessary for VSMC to osteochondrogenic conversion in high Pi treatment\textsuperscript{129}.

Together these studies on NPP1, ALP and PiT-1 underscore the importance of Pi in VSMC mediated calcification (Figure 3).
RUNX2

RUNX2 Structure and Function:

The Runx2 gene contains two promoters which give rise to two isoforms, named type I and type II. These isoforms contain an identical DNA binding domain, an α-helical structure for microtubule binding and translocation and a nuclear targeting matrix (NTM). Both RUNX2 isoforms also contain a 200 amino acid sequence called a PEST domain (Figure 4). The PEST domain is destabilizing and is hypothesized to facilitate degradation through either the proteosome or calpain pathways.

These isoforms differ in the N-terminus. The type I N-terminus contains the MRIPV amino acid sequence. In type II RUNX2, the N-terminus contains the MASNS amino acid sequence instead. How the N-terminus affects isoform differences is unknown. During early calcification the type I isoform is favoured, but as calcification progresses, type II expression predominates. It is not known how or why this process occurs, but both are important for bone development.

RUNX2 functions in the ossification process by committing mesenchymal stem cells to become osteochondrogenic cells. Although RUNX2 can bind DNA as a monomer, RUNX2 must bind DNA with Osterix in a heterodimeric complex for osteoblast differentiation. It can also function in the chondrocyte lineage pathway, bound with SOX 9 instead. The importance of RUNX2 in bone development can be seen as Runx2 null mice are embryonic lethal due to a failure in skeletal tissue ossification and Runx2+/- mice develop cranial and collarbone malformations termed cluedocranial dysplasia. Although RUNX2 is indispensable for calcification, overexpression of RUNX2 in mice did not show the opposite phenotype of RUNX2
deficiency. While the number of osteoblasts increased in RUNX2 transgenic animals, osteoid development and proper mineralization was impaired resulting in osteopenia\textsuperscript{141}. From these studies it can be concluded that precise regulation RUNX2 activity is required for osteoblasts and chondrocytes during bone development.

**RUNX2 Transcriptional Targets:**

RUNX2 binds to a DNA consensus sequence 5’CCACA3’ called Osteoblast Sensitive Elements (OSE)\textsuperscript{142}, in the promoter regions of target genes, such as Type I Collagen alpha 1 (Col1a1)\textsuperscript{143}, Type I Collagen alpha 2 (Col1a2)\textsuperscript{143}, Opn\textsuperscript{144}, Integrin Binding Bone Sialoprotein (Ibsp)\textsuperscript{145}, Ocn\textsuperscript{143} and Vascular Endothelial Growth Factor (Vegf)\textsuperscript{146}. As the binding of OSE by RUNX2 represents a clear indication of RUNX2 activity, OSEs have been used in promoter reporter constructs to demonstrate RUNX2 activity\textsuperscript{147}. Most of the identified genes which are activated by RUNX2 are major constituents of the bone osteoid or play functional roles in calcification. For example, type I collagen is an important structural component of the bone osteoid\textsuperscript{148}. Additionally, type I collagen is also ligand for pro-osteogenic signalling\textsuperscript{149}. Other gene products such as OPN, IBSP and OCN contain calcium phosphate binding sites, are hypothesized for facilitating calcium phosphate nucleation and provide the physical means for osteoclasts to attach and remodel bone\textsuperscript{150}. Another RUNX2 target, VEGF is promotes angiogenesis, which is important for the vascularisation of bone\textsuperscript{151}. As such, many of the mRNAs of these RUNX2 target genes have been used as a measure of its activity in studies on osteogenic development and in vascular calcification. However it is also important to note that the expressions of these genes are likely influenced as well by other transcriptional activators. Vegf mRNA expression for
example can be induced by Hypoxia Inducible Factor 1 alpha (HIF1α), which is RUNX2 independent152.

*Regulation of RUNX2:*

RUNX2 is regulated by transcription of the gene to produce mRNA. Bone Morphogenic Protein 2 (BMP2), which is an important pathway to osteogenic development, regulates the transcription of Runx2 mRNA153. Other molecules which regulate the expression of Runx2 mRNA include Retinoblastoma Protein (RBP)154, and Insulin-like Growth Factor 1 (IGF1)155.

In addition to regulating the expression levels of RUNX2, the activity of RUNX2 is also regulated by many signalling pathways. Signalling pathways which have been identified to positively regulate RUNX2 activity include BMP2, Fibroblast Growth Factor Receptor 1 (FGFR1), Interleukin 1 μ (IL-1β) and Transforming Growth Factor β (TGFβ)156. These pathways ultimately lead to the phosphorylation and activation of RUNX2. Regulation of RUNX2 activity occurs through phosphorylation of serine sites, the identified sites all follow the sequence of proline-serine-theronine (*Figure 5*). Recent work showed that RUNX2 contains three clearly identified phosphoserine sites (S14, S104 and S451), which have inhibitory and stimulatory effects, both S104 and S451 are inhibitory while S14 is stimulatory70 (*Figure 5*). While many kinases have been identified as crucial to RUNX2 phosphorylation, only the MAPKs, ERK1/2 and P38 have been identified as direct kinases for S14147. ERK1/2 and P38 have also been identified as direct RUNX2 binding partners147. Furthermore, ERK1/2 and P38 have also been identified as crucial MAPKs for the maturation of osteoblasts further solidifying their roles in calcification82. While the exact subcellular location where phosphorylation of RUNX2 occurs has not been studied,
P38 and ERK1/2 typically phosphorylate transcription factors after they have entered the nucleus.

To elicit its effects on calcification, RUNX2 is directed to the nucleus from the cytoplasm. From there RUNX2 is able to bind to DNA elements and induce gene expression. To enter the nucleus RUNX2 interacts with the microtubule network via its N terminus for transport. Upon entry to the nucleus, RUNX2 activate genes in the osteogenic process. These processes demonstrate the complexity and redundancy of mechanisms by which RUNX2 activity can be regulated.

**RUNX2 in Vascular Calcification:**

RUNX2 is required for osteogenic modulated vascular calcification, and osteochondrogenic transdifferentiation in VSMCs. Many of the factors which activate RUNX2 in osteochondrogenic development in bone tissue also activate RUNX2 in VSMCs. In most vascular calcification studies, RUNX2 activity is detected by phosphorylated serine residues, promoter-report construct or transcriptional activation of RUNX2 targets. Such targets include Col1a1, Col1a2, Opn, and Ocn. As the vascularisation of advanced vascular calcification, Vegf has become the marker for late stage vascular calcification. Although RUNX2 activation in VSMCs promotes the expression of osteochondrogenic genes and ossification, transdifferentiated VSMCs still maintain many smooth muscle markers such as αSMA, SM22α, myocardin and smooth muscle myosin heavy chain.
MICROTUBULES

As previously discussed, microtubules are key components which regulate the translocation of RUNX2 into the nucleus. Because the association between RUNX2 and microtubules is a relatively novel finding and sparsely defined, this latter part of the review will be dedicated to fully exploring microtubules.

Structure and Organization of Microtubules:

Microtubules along with actin and intermediate filaments are a part of the eukaryotic cellular cytoskeleton. Microtubules are typically organized at the Microtubule Organizing Center (MTOC), which is composed of γ-tubulin, and situated at the centriole\textsuperscript{159}. Typically, nucleation of microtubules occurs at the MTOC. However in cells which lack MTOC, microtubules nucleate at discrete points in the cytoplasm through a poorly understood process\textsuperscript{159}.

The microtubule network is made up of repeating subunits of α and β-tubulin dimers, and forms an imperfect helical orientation of 13 tubulin dimers per turn (Figure 6). Overall these form the microtubule structure which is a hollow cylinder. This cylinder has a diameter of 25 nm and can grow up to 25 µm in length\textsuperscript{160}. Microtubule filaments have polarity with + or − ends which have β-tubulin or α-tubulin exposed respectively\textsuperscript{160}. This orientation is essential for the biological function of microtubules. The orientation of microtubules is such that subunits are added from the + end\textsuperscript{160}. Typically, the + end refers to the end at the MTOC and the − end refers to the end extending to the cell periphery. This orientation is particularly important for the motor proteins dyenin and kinesin which shuttle cargo from + to − and − to + respectively.
Microtubules polymerize when α and β-tubulin dimers are bound to GTP to form a stabilized GTP cap at the – end (Figure 6)\textsuperscript{161}. Hydrolysis of GTP to GDP within β-tubulin, promotes a bent conformation of the proteins hence promoting depolymerisation\textsuperscript{161}. This active assembly and disassembly of microtubules is termed dynamic instability.

\textit{Regulation of Microtubule Dynamics:}

The stability of microtubules can be regulated by interactions with other proteins such as motor proteins and non motor microtubule associated proteins. These proteins may or may not influence the GTPase activity of β-tubulin. Motor proteins which affect the stability of microtubule are the kinesins. Kinesins from the kinesin 13 family play major roles in regulating microtubules. Their primary function is to catalyze the intrinsic property of microtubules to depolymerize\textsuperscript{162}. They are able to accomplish this by binding to the curved ends of microtubule filaments to promote instability in an ATP dependant process\textsuperscript{163}.

Microtubules dynamics are also regulated by post transcriptional modifications which are catalyzed by various microtubule associated proteins. The major ones include detyrosination, Δ2-tubulin generation and acetylation. Detyrosination of α-tubulin refers to the removal of the terminal Tyr residue at the C-Terminus of α-tubulin, thus rendering Glu the new terminal residue\textsuperscript{164}. This process is mediated by an as of yet unidentified detyrosinase. Detyrosinated α-tubulin favours the polymerized state in microtubules. However this process is reversible, as Tubulin Tyr ligase can re-add Tyr to the Glu residue\textsuperscript{164}. In order to make α-tubulin permanently favour polymerization, detyrosinated α-tubulin is processed by cytosolic carboxypeptidase. This removes the Glu residue in α-tubulin giving rise to Δ2-tubulin\textsuperscript{164}.
Detyrosinated tubulin and Δ2-tubulin are commonly found in terminally differentiated neuronal cells where the dynamic cycling of microtubules is not required\textsuperscript{164-166}.

Another microtubule modification that is associated with stability is the acetylation of α-tubulin. Acetylation is a process where an acetyl group is conjugated to K40 on α-tubulin by a currently unknown acetyl transferase. HDAC6 is the only deacetylase known to regulate α-tubulin acetylation\textsuperscript{167}. Evidence to support this comes from HDAC6 overexpression which resulted in a global decrease in tubulin acetylation and increased susceptibility to destabilizing agents such as nocodazole\textsuperscript{168}. It is hypothesized that acetylation of K40 site induces a conformational change, which affects the α-tubulin-β-tubulin dimer binding to prevent polymerization of microtubules\textsuperscript{164}. The regulation of microtubule polymerization is crucial for biological function.

\textit{Microtubules and Nuclear Transport:}

Though it was previously thought that nuclear localization occurred via Brownian motion, recent work shows that nuclear translocation for a variety of different molecules requires the use of microtubules. For examples signal transduction kinases such as P38 use the microtubule network in order to translocate into the nucleus\textsuperscript{169}. Recent work on steroid hormone receptors has demonstrated that they also require microtubules for nuclear transport\textsuperscript{170}.

Microtubules are also required for the translocation for transcription factors including RUNX2\textsuperscript{131} and HIF1α\textsuperscript{171}. RUNX2 is only able to bind to polymerized microtubules, but not monomeric tubulin\textsuperscript{131}. The study also showed that RUNX2 associates with the microtubule
network via the N terminal amino acid residues 1 – 361. However, it is not known whether RUNX2 associates directly with the microtubule network or through a protein complex. A study on HIF1α and steroid hormone receptor translocation to the nucleus by microtubules showed that both molecules bind to Dynamitin, which is a part of the dynein activator complex\textsuperscript{170, 171}. This complex is able to bind both dynein and kinesin and thus is used for bidirectional transport of organelles and vesicles on microtubules within the cell\textsuperscript{172}. RUNX2 may also bind to Dynamitin for transport.

Interestingly, although transcription factors require microtubules, purely polymerizing microtubules is not sufficient for nuclear translocation for most transcription factors\textsuperscript{131, 171}. For most nuclear transcription factors, a state of dynamic instability is required for nuclear translocation. Why dynamic instability is required for the translocation of proteins is not known.

Mitochondrial translocation also requires dynamic instability for translocation within the cell. Mitochondria interact with positive end proteins which latch on and carry them into the microtubule network\textsuperscript{160}. A similar latching process may be required for transcription factors as well. Upon arrival at the nuclear pore complex, the protein is recognized and translocated within by a poorly understood process.

In mesenchymal stem cells, DDR1 is crucial for forming dendritic extensions by microtubules\textsuperscript{173}. As transcription factors such as RUNX2, HIF1α and signalling molecules such as ERK, P38 and STATs all require microtubule translocation; this might represent a master regulatory network for controlling nuclear activity of transcription factors by DDR1.
RATIONALE AND HYPOTHESIS

Type I collagen has been associated with both osteochondrogenic differentiation and vascular calcification\textsuperscript{149, 174}. The alpha 1 integrin receptor has been implicated in regulating collagen induced osteochondrogenic differentiation in bone, however, the receptor by which type I collagen elicits its osteochondrogenic effects in VSMCs has not been identified. In a published study, our group observed that $Ddr1^{-/-}$; $Ldlr^{-/-}$ mice had reduced atherosclerotic calcification\textsuperscript{59}. As type I collagen is a ligand for DDR1, this suggested a novel mechanism by which type I collagen can promote osteochondrogenic transdifferentiation. Our laboratory also reported that $Ddr1^{-/-}$ VSMCs showed attenuated calcifying potential, with some indication of decreased osteochondrogenic transdifferentiation, such as decreased ALP activity\textsuperscript{59}. This led me to perform a screen on $Ddr1^{+/+}$ and $Ddr1^{-/-}$ VSMCs to identify genes which might be involved in osteochondrogenic transdifferentiation. My screens revealed that $\text{Ocn}$, showed a significant reduction in $Ddr1^{-/-}$ cells. This result led me to investigate the mechanism by which DDR1 influenced Ocn expression. $\text{Ocn}$ is a target gene of the osteochondrogenic transcription factor RUNX2, and RUNX2 activity is regulated by MAPKs such as ERK1/2 and P38\textsuperscript{147, 175-177}. DDR1 has been shown to signal through those MAPKs\textsuperscript{18, 43, 44, 48}. As this represented a possible link between DDR1 and RUNX2 I hypothesized that: DDR1 activates RUNX2 by signalling through MAPKs in VSMCs during calcification. However later in the course of my studies I discovered that the absence of DDR1 diminished RUNX2 activity at early stage calcification in spite of no DDR1 dependant differences in ERK1/2 and P38 signalling at that time point. In addition, P38 inhibition reduced Ocn expression and RUNX2 activity in DDR1 expressing cells. My studies also revealed that $Ddr1^{-/-}$ VSMCs show reduced RUNX2 and phosphoP38 nuclear localization.
Furthermore, close observations of my $Ddr1^{-/-}$ VSMCs showed increased cell spreading which was absent in $Ddr1^{+/+}$ VSMCs. As nuclear translocation of RUNX2 and phosphoP38 and maintenance of cell morphology require a microtubule network$^{131,171}$, I postulated that there were differences in microtubule structure between the $Ddr1^{+/+}$ and $Ddr1^{-/-}$ VSMCs. Supporting this, an article published by Lund et. al. showed that mesenchymal stem cells sense type I collagen using a DDR1 stabilized microtubule network$^{173}$. A prior study also demonstrates that RUNX2 requires a microtubule network for nuclear translocation$^{131}$. Together these studies suggest that DDR1 may able to regulate the translocation and thus the activity of RUNX2. Thus I later hypothesized that: **DDR1 regulates RUNX2 activity by microtubule organization in VSMCs during calcification.**

**MATERIALS AND METHODS**

**Chemicals and Reagents**

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

**Smooth Muscle Cell Isolation and Culturing**

Mouse VSMCs were isolated using elastase type III digest from $Ddr1^{+/+}$ and $Ddr1^{-/-}$ C57BL6 mice carotid arteries as described in Hou et. al.$^{50}$ and propagated in a medium composed of high glucose Dulbecco’s modified Eagle’s Medium (Gibco) and supplemented with 10% heat inactivated fetal bovine serum, 1% antibiotic-antimyocytic (culturing media) at 37°C
with 5% CO₂ in 75 cm² culture flasks. Cells were passaged upon growth to approximately 90% confluency. To passage the cells, culture media was aspirated and the cells were washed 3 times using Dulbecco’s phosphate buffered saline with MgCl₂ and CaCl₂ (PBS). After washing, the cells were passaged through enzymatic digest by incubating the cells with 2mL of 0.02% trypsin for 1 min at 37°C to detach cells from the culture flask. The cell suspension was removed and placed in 50 mL falcon tubes (BD Falcon) containing culture media that has been warmed to 37°C. This cell suspension was centrifuged at 1,000 rpm for 5 mins. The supernatant was aspirated and the cell pellet was redistributed into 3 new culture flasks, each containing 10 mL of culture media. The periodicity by which the cells were passaged was approximately 4 days after each passage. Experiments were performed from these isolated VSMCs from passage 6 through to passage 10.

**In Vitro Calcification Assay**

VSMCs from passages 6 to 10 were seeded at 1563 cells/cm² and cultured in culturing media. After 72 hours the culturing media was washed out using PBS and replaced with media consisting of high glucose Dulbecco’s modified Eagle’s Medium (Gibco) supplemented with 3% heat inactivated fetal bovine serum (Hyclone), 1% antibiotic-antimycotic (Penicillin-Streptomycin/Amphotericin B) (Gibco) and 2.4 mM Pi (osteogenic media). In parallel experiments involving MAPK pathway inhibition, 10 mM stocks of PD098059 (MEK inhibitor) and SB203580 (P38 inhibitor) were made in Dimethyl Sulfoxide (DMSO) and diluted at 1:1,000 to create a final concentration of 10 µM within the osteogenic media. Controls to inhibitor experiments were prepared by adding DMSO at a 1:1,000 dilution in the media. In experiments
involving microtubule destabilization, 10 mM stock of nocodazole was made in DMSO and diluted at 1:1,000 to create a final concentration of 10 µM. To stabilize microtubules, a 10 mM stock of taxol was made in DMSO and diluted at 1:1,000 to create a final concentration of 10 µM. These cells were incubated for 0, 2, 4 or 12 days after the addition of the osteogenic media and then used for calcium quantification, RT-PCR, immunoblotting, immunohistochemistry and luciferase activity assays.

**Calcium Quantification**

The calcium content of calcified VSMCs was determined using the o-cresophthalein complexone colorimetric assay (Clinotech Diagnostics). The assay was performed according to manufacturer protocols. Initially, the assayed cells were incubated with 500 µL of 0.6 N HCl per well in a 6 well culture plate (BD Falcon) at 4°C overnight. The HCl was added to release the calcium phosphate crystals. The resultant HCl solution was diluted 50 fold with 0.6N HCl. Following this, the diluted sample was added in triplicate, 10 µL each in a clear 96 well plate (BD Falcon). To quantify the amount of calcium within each sample, a standard curve generated using the calcium standard solution with O-cresophthalein contained within the assay kit ranging from 0 to 7.5 mg/ml. 100 µL of the prepared calcium quantification reagent was pipetted into each well containing experimental and standard curve samples and incubated for 5 mins at room temperature. O-cresophthalein functions by binding calcium within the solution and forms a purple coloured complex. As a result, the amount of calcium was determined by measuring intensity of purple colour at 540 nm. The measured values were calculated by interpolating the absorbance values to the calculated standard curve formula.
Measured calcium content was normalized to total cellular protein. Protein content was measured using a microBCA assay according to manufacturer protocols (Pierce). To obtain total cellular protein, decalcified cells were washed 3 times with PBS and incubated with 0.1 N NaOH at room temperature for 1 hour to solubilise the protein. To prepare the protein samples collected in NaOH for quantification, the samples were diluted 1:200 with 0.1 N NaOH. To correlate absorbance values to protein concentration a BSA standard ranging from 0 to 40 µg/mL was prepared according to manufacturer protocol by diluting the supplied BSA stock with 0.1 NaOH. The BSA reaction was performed by pipetting 100 µL samples and the prepared BSA standard in triplicate on a 96 well plate. To visualize the protein content with in each sample 100 µL of prepared BCA reagent was pipetted into each of the sample and standard containing wells and incubated for 1.5 hours at 37°C. In a BCA reaction Cu²⁺ ions are reduced by peptide bonds to Cu⁺ in a concentration dependant manner. The Cu⁺ is bound by bicinchronic acid to a form a purple complex, which absorbs light at 562 nm. The measured values were calculated by interpolating the absorbance values to the calculated standard curve formula.

In addition, calcium content was also normalized to total cellular DNA using the Fluoreporter dsDNA quantification kit (Molecular Probes). Following calcium extraction with 0.6N HCl and washed with PBS, cells are incubated with 500 µL of ddH₂O per well and incubated at -80°C for 1 hour. Following this the samples were thawed and incubated at 37°C for 1 hour. To quantify the amount of DNA a standard curve ranging from 0 to 10 ng/µL was prepared according to manufacture protocol using the supplied salmon sperm DNA stock. Each of the samples and DNA standards were pipetted in triplicate on a black walled 96 well plate (BD falcon). To visualize the DNA content 100 µL of Hoechst TNE buffer was pipette into each well
containing a sample or standard and incubated at room temperature for 5 mins. The Hoechst dye within the solution is able to bind dsDNA and thus providing a means to visualize the DNA. After incubation, the plate was read on a HTS fluorescent microplate reader (PherSTAR) by setting excitation and emission filters at 360 nm and 460 nm respectively. The measured values were calculated by interpolating the absorbance values to the calculated standard curve formula.

**Reverse Transcription and Quantitative Real Time-PCR**

RNA was isolated from VSMCs using the RNeasy RNA isolation kit (Qiagen). RNA was later quantified using the NanoDrop 2000c (Thermo Scientific). To prepare the RNA for reverse transcription, 1 μg of RNA was treated with DNase I (Fermentas) for 30 mins at 37°C in supplied buffer (10 mM Tris-HCl pH 7.5, 2.5 mM MgCl₂ and 0.1 mM CaCl₂) to remove genomic DNA. DNase I was inactivated by the addition of 1 μg of EDTA and heat inactivation for 10 mins at 65°C. This solution was reverse transcribed into cDNA using the Superscript First Strand Synthesis Kit (Invitrogen) with 100 ng of random hexamers, 200 nM dNTP, 10 mM DTT, 40 u of RNaseOUT and 50 u of Superscript II in a buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM MgCl₂. qRT-PCR reactions were performed using SYBR advantage qRT-PCR premix kit (Clonetech) on the Quantitative Real Time (qRT)-PCR using the ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems). Individual reactions were performed in 10 μL with 4 ng of cDNA and 450 nM of the following forward and reverse primers DDR1 (FWD 5’-CTGCTCTTTACTGAAGGCTC-3’, REV 5’-CAGGCCATAGCGGCACTTG-3’), PIT-1 (FWD 5’-GCGCCCTTCCGGGCTTT-3’, REV 5’ GTCTCCGGCCGCGCCGGCTTGCCGC-3’), NPP1 (FWD 5’-
CTCGGTTGAGACCCACTGATG-3', REV 5'-GCTCCGGCAAGAAAGATT-3'), ALP (FWD 5'-AGACACAAGCATCCTCCACTAT-3', REV 5'-CACCATTCTCGGAGACG-3'), RUNX2 (FWD 5'-GATGCTCTTGTTCTTTCCAGG-3', REV 5'-CTCCAGCATTTCATGCTAGT-3'), ATF4 (FWD 5'-TTGACCACGTTGGATGACAC-3', REV 5'-CAGAGATATCAACTTCACTGCCTA-3'), OCN (FWD 5'-GATGCTCTGTTTCTTTCTTTCAGG-3', REV 5'-CTCCAGCATTTCATGCTAGT-3'), TENC (FWD 5'-ACCGCAGAGAAGAATTTTGG-3', REV 5'-TCCCCATGGTCTTGTAGGTC-3') and Acidic Ribsomal Protein (ARP) (FWD 5'-AGACCTCCTTCTCCAGGCTT-3'), (REV 5'-CCCACCTTGCTCCAGTCTTTATC-3'). The conditions for the PCR reaction were 95°C for 10 mins followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. Following PCR, a dissociation step was performed by heating the samples to 95°C for 15 secs and then cooling them to 60°C for 15 secs and then heating again to 95°C for 15 secs. The data collected was used to generate the dissociation curve from which the cycle threshold (Ct) was determined using SDS 2.3 software (Applied Biosystems). To determine relative expression levels the comparative Ct method \(2^{-\Delta\Delta C_t}\) was used as previously described\(^59\).

**Immunoblotting**

Protein was isolated from smooth muscle cells incubated in osteogenic conditions for either 2 or 12 days using the cell lysis buffer (20 mM Tris-HCl, (pH 7.5), 150 mM NaCl, 1 mM Na\(_2\)EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM betaglycerophosphate, 1 mM Na\(_3\)VO\(_4\), 1 µg/ml leupeptin) (Cell Signalling Technology). Using the microBCA assay as described above, appropriate sample volumes of sample were added to 2X sample buffer (0.1 M Tris pH 6.8, 2% SDS, 20% glycerol, 0.4% bromophenol blue, 5% β-mercaptoethanol) and
boiled for 5 mins. Samples were loaded on and separated with a 10% TGX separating gel (Bio-Rad) and were subsequently transferred to a nitrocellulose membrane (Bio-Rad) at 100 V for 1 hour at 4°C. Membranes were incubated in blocking buffer (5% non-fat dry skim milk, in Tris Buffered Saline-Tween 20 (TBST)) for 1 hour at room temperature, followed by washing in TBST and incubated in the appropriate primary and secondary antibodies in 2.5% non-fat dry skim milk in TBST. The antibodies were diluted at the following concentrations: rabbit polyclonal anti-ERK1/2 (Cell Signalling Technology) (1:1000), rabbit polyclonal anti-pERK1/2 (Cell Signalling Technology) (1:250), rabbit polyclonal anti-P38 (Cell Signalling Technology) (1:250), rabbit polyclonal anti-pP38 (Cell Signalling Technology) (1:250), mouse monoclonal anti-RUNX2 (Abcam) (1:250), rabbit polyclonal anti-β actin (Abcam) (1:1000), HRP conjugated goat anti-rabbit (Cell Signalling Technology) (1:1000) and HRP conjugated horse anti-mouse (Cell Signalling Technology) (1:1,000). Membranes probed with antibodies was incubated with western blot chemiluminescence reagent Plus (Perkin Elmer), the membrane was stripped by incubating Restore Plus western blot stripping buffer (Pierce) for 30 mins at 37°C, Washed with TBST and re-probed for next protein of interest. Immunoblots were imaged using an Image Station 4000MM Pro (Kodak) and quantified using Image J software (NIH). To normalize, densitometry values obtained were divided by the β-actin loading control densitometry values. pERK1/2 and pP38 expression values were obtained by dividing the β-actin normalized values of the phospho forms to the β-actin normalized values of total forms.
Immunocytochemistry

VSMCs were grown using the calcification assay conditions on 8 chambered culture slides (BD Falcon) to visualize cells after immunocytochemistry staining. The cells were subsequently washed with PBS and fixed with 5% PFA (Paraformaldehyde) for 10 mins at room temperature followed by a PBS wash and permeablized with PBS containing 0.25% Triton X-100 for 10 mins. After permeabilization the cells were washed again and blocked with 1% BSA with PBS and 0.1% Tween (PBST). The cells were later incubated with mouse monoclonal anti-RUNX2 (Abcam) (1:100), rabbit polyclonal anti-pP38 (Cell Signalling Technology) (1:100) and rabbit polyclonal anti-α tubulin (Abcam) (1:100) in 1% BSA PBST overnight at 4°C. The primary antibody solution was decanted and the cells were washed with PBS after overnight incubation. Secondary anti-mouse Alexa 488 conjugated goat antibody (Invitrogen) (1:100), secondary anti-rabbit Alexa 488 conjugated donkey antibody (Invitrogen) (1:100), secondary anti-mouse Alexa 568 conjugated goat antibody (Invitrogen) (1:100), and secondary anti-rabbit Alexa 568 conjugated donkey antibody (Invitrogen) (1:100) in 1% BSA PBST were added and incubated for 1 hour at room temperature in the dark. Alternatively, VSMC membrane was visualized by incubating Alexa 647 Wheat Germ Agglutinin (WGA) (Invitrogen) (1:1,000) in 1% BSA PBST was added and incubated for 1 hour at room temperature in the dark.

Following secondary antibody or WGA incubation the cells were washed again with PBS and then incubated for 5 mins with Hoechst in PBS (1:1,000) to stain the nucleus. After Hoechst incubation the cells are washed with PBS and fixed with CC mount (Invitrogen) and sealed with a #1 thickness glass cover slide (VWR) and clear nail polish. The cells were visualized by confocal fluorescence microscopy using the Nikon AR1 laser scanning confocal microscope (Nikon).
**Immunofluorescence Quantification**

To quantify the extent of nuclear localization of RUNX2 in the immunofluorescence images, Image J was used to measure the intensity of the fluorescent signals. First using the free form tools, the cytoplasm of the cell was traced out, and the average intensity of the signal is measured and tabulated. Next, using free form tools the nucleus was also selected and the average intensity of the signal is measured and tabulated. The average intensity of the nucleus is then divided by the average intensity of the cytoplasm to obtain the nuclear:cytoplasmic ratio. For each experiment, one field of view was captured at 400X magnification from each of 3 separate wells. For each field of view 3 cells were chosen at random for quantification. The images were quantified in a fashion blinded to both genotype and treatment. The values obtained for each individual experiment were averaged and treated as one replicate. To obtain data for statistical analysis a total of 3 separate experiments were analyzed.

**Plasmid Preparation and Transformation**

The 6OSE2 Luc plasmid to assess RUNX2 activity was a kind gift from Dr. Renny Franceschi from University of Michigan Ann Arbor \(^{147}\). To prepare 6OSE2 plasmid for experiments, the plasmid was transformed into E-coli. To perform the transformation, a 100 μL aliquot of XL-1 Blue Competent E-Coli Cells (Strategene) was thawed on ice. After thawing, 1.7 μL of β-mercaptoethanol was added to thawed cells and swirled gently. The cells were allowed to incubate on ice for an additional 10 mins. To propagate the plasmid 20 ng of 6OSE2 Luc was added to the thawed cell mixture and incubated on ice for 30 mins. A heat pulse of 42°C was applied to the cell mixture for 45 secs. Following the heat pulse, the cells were incubated on ice
for 2 mins. 37°C Pre-warmed 0.9 mL of SOC media (Invitrogen) was added to the cell mixture. The cell containing SOC (Invitrogen) mixture was incubated at 37°C for 1 hour with shaking at 250 rpm. The transformed cells were plated on LB-ampicillin agar plates prepared from a LB stock containing 100 mg/mL of ampicillin to select for cells containing the plasmid. Cells were grown overnight at 37 °C to allow growth. After growth period a single colony was selected and subsequently cultured in a starter culture of 40 mL of LB-ampicillin at 37 °C with constant shaking at 100 rpm for 4 hours. After which, the starter culture was placed in a larger LB-ampicillin culture containing 1 L of media at 37 °C with constant shaking at 100 rpm overnight. Plasmid DNA was extracted from the overnight culture using a Maxi Prep DNA isolation kit (Invitrogen) according to manufacturer instructions. Isolated Plasmid DNA was quantified using the NanoDrop 2000c spectrophotometer (Thermo Scientific) for future preparations.

**Transfection**

VSMCs were grown in culturing media in 24 well plates (BD Falcon) for 2 days. Following the incubation, calcifying media was removed and cells were washed with PBS. For each well of cells the following mixture was prepared: 5 µg of 6OSE2 Luc plasmid was diluted in 100 µL of Opti-MEM (Gibo), then 0.5 µL of Plus Reagent (Invitrogen) was added to the mixture and allowed to incubate at room temperature for 15 mins. For each volume of the above prepared mixture, 2.5 µL of Lipofectamine LTX (Invitrogen) was added and incubated for 30 mins at room temperature. Following incubation, the subsequent mixture was added to 500 µL of antibiotic free DMEM with 10% FBS and cultured for an additional 24 hours to complete the transfection. After transfection, cells were cultured according to the calcification assay conditions.
Luciferase Activity Assay

Following calcification assay culturing, transfected cells were assayed for RUNX2 activity with reagents provided with the Dual Luciferase Assay Kit (Promega). Each well of cells was lysed by incubating 100 µL of passive lysis buffer (Promega) for 30 mins with gentle shaking. To measure the activity of the lysate samples, 20 µL of the obtained lysate was pre-dispensed in triplicate on to a black walled 96 well plate. The Luminat LB9507 luminometer (Berthold Technologies) added the following reagents automatically: First, 100 µL of Luciferase Assay Reagent II (Promega) to measure the activity of firefly luciferase. After a 2 second delay, Stop and Glo Reagent (Promega) was added to quench the firefly luciferase signal and provide the substrate for the renilla luciferase signal. Relative Luminescence Units (RLUs) were obtained by normalizing the firefly luciferase signal driven by the activity of RUNX2 to the renilla luciferase signal generated by the CMV promoter on each plasmid.

Statistical Analyses

Statistics were performed using Sigma Stat (SysStat Software). Data was presented as ± SEM. To test for statistical significance, in vitro calcification assays and densitometry on immunoblots were analyzed using a paired Student’s t-test. qRT-PCR, nuclear localization quantification and luciferase assays were analyzed using Two way ANOVA. Pairwise comparisons between groups were made following the Two way ANOVA using Tukey’s posthoc test. Statistical significance was set at P<0.05 for all tests.
RESULTS

**DDR1 Deficiency Attenuates Vascular Smooth Muscle Cell Calcification of Matrix**

To study the role of DDR1 in VSMC mediated calcification, I utilized a high phosphate osteogenic model of *in vitro* calcification adapted from Speer *et al.* After 12 days of culture, total calcium content in the extracellular matrix in *Ddr1*−/− VSMCs was reduced by 43% when compared with *Ddr1*+/+ VSMCs after normalizing to total protein level (Figure 7A). *Ddr1*−/− VSMCs showed a 38% reduction in calcium content compared to *Ddr1*+/+ VSMCs after normalizing to total DNA level (Figure 7B). It is clear that VSMC mediated calcification is diminished in the absence of DDR1.

**DDR1 Deficiency Alters Expression of Phosphate Handling Genes in Vascular Smooth Muscle Cells at an Early, but not at a Late Stage of Calcification**

Previous research has suggested that vascular calcification results from an imbalance of the Pi to PPI levels in VSMCs, which is in turn due to an imbalance of phosphate regulators. I therefore hypothesized that the blunted calcification observed in *Ddr1*−/− VSMCs was due to altered expression of genes involved in phosphate handling. The mRNA expression of various phosphate regulatory molecules was measured using qRT-PCR. I first looked at Pit-1 and Alp, which are involved in increasing intracellular phosphate levels. Pit-1 mRNA was increased in *Ddr1*−/− VSMCs after 2 days in osteogenic culture, but by 12 days Pit-1 mRNA expression was decreased and there were comparable levels in both genotypes (Figure 8A). I saw a significant decrease in the mRNA expression of Alp at 2 days in *Ddr1*−/− VSMCs but by 12 days, both *Ddr1*−/− and *Ddr1*+/+ VSMCs increased their expression and there were no differences when comparing
the two cell types (Figure 8B). This data shows that deficiency of DDR1 affects expression of Pit-1 and Alp at an early stage, but by late stage calcification the gene expression of Pit-1 and Alp mRNA in Ddr1/− VSMCs catches up to Ddr1+/+ VSMC levels.

Next I looked at mRNA levels of the pyrophosphatase, Npp1, which inhibits calcification by promoting the accumulation of PPI. I saw a significant decrease in Npp1 mRNA expression in Ddr1/− VSMCs at the 2 day time point, but expression levels in Ddr1/− and Ddr1+/+ VSMCs increased and there were no significant differences between the cell types at 12 days (Figure 8C). This indicated to me that loss of DDR1 affects the mRNA expression of npp1 in VSMCs during early stage calcification, but not at late stage calcification.

Together my results indicate that loss of DDR1 impacts the mRNA expression of Pit-1, Alp, Npp1 at an early point of calcification, but not at a late stage. This indicates that DDR1 delays the gene expression of phosphate handling genes in response to high phosphate. If these changes in mRNA expression carry forward to protein expression changes, they will likely impact phosphate handling, nucleation of calcium apatite and thus vascular calcification.

In my experiments I saw a decrease in Alp expression in Ddr1/− VSMCs. This could lead to decreased Pi production by these VSMCs and thus less calcium phosphate crystal deposition. I also observed a decrease of Npp1 expression in Ddr1/− VSMCs, which seems counterintuitive as these cells clearly show decreased calcification. However PPI produced by NPP1 can also be a substrate for ALP, producing two Pi molecules178. Therefore decreased NPP1 levels in Ddr1/− VSMCs may also lead indirectly to less vascular calcification. With respect to Pit-1 expression levels, Pit-1 mRNA expression is likely increased in the Ddr1/− VSMCs in response to the decrease in available Pi. Over time, these gene expression differences as a result of DDR1
absence are overcome. Nevertheless, the early expression of phosphate handling proteins may provide a quick start to calcium phosphate nucleation in $Ddr1^{+/+}$ VSMCs. Therefore while the $Ddr1^{-/-}$ VSMCs may eventually match the $Ddr1^{+/+}$ VSMCs in phosphate handling, they are unlikely to reach the same extent of matrix calcification.

**DDR1 Deficiency Increases the Expression of TenC at Late Stage Calcification, but not αSMA and Atf4**

During vascular calcification, there is VSMC transdifferentiation into an osteoblast-like phenotype with the gradual loss of the VSMC phenotype\(^69\). I wanted to determine whether loss of DDR1 attenuated VSMC phenotypic transition whereas DDR1 is permissive to transdifferentiation. To address this I first examined the protein levels of the smooth muscle cell marker Alpha Smooth Muscle Actin (αSMA). Immunoblotting to detect αSMA revealed no significant differences comparing $Ddr1^{-/-}$ and $Ddr1^{+/+}$ VSMCs at the 12 day timepoint (Figure 9A). This indicated to me that deficiency of DDR1 did not alter the expression of αSMA under osteogenic conditions.

During vascular calcification, VSMC proliferation is inhibited to promote osteochondrogenic transdifferentiation\(^179\). I therefore hypothesized that $Ddr1$ deletion may increase VSMC proliferation, thus preventing transdifferentiation. To determine this I examined two markers of smooth muscle proliferation, Activated Transcription Factor 4 (Atf4)\(^180\) and Tenascin C (TenC)\(^181\). qRT-PCR of $Ddr1^{-/-}$ and $Ddr1^{+/+}$ VSMCs showed no differences in the expression of Atf4 between the genotypes at either time point (Figure 9B). However there was
a decrease in the mRNA expression of Atf4 from 2 days to 12 days in both \( Ddr1^{-/-} \) and \( Ddr1^{+/+} \) VSMCs.

Following this, I examined the mRNA expression of TenC under the same osteogenic conditions and saw no differences between \( Ddr1^{-/-} \) and \( Ddr1^{+/+} \) VSMCs at 2 days in osteogenic culture. However, by 12 days the \( Ddr1^{-/-} \) VSMCs showed a significant increase in the expression of TenC (Figure 9C). Taken together these results imply that loss of DDR1 does not alter the expression of αSMA or Atf4. However, the loss of DDR1 does increase the expression of TenC at late stage calcification. In previous literature, it has been shown that TENC promotes VSMC proliferation through an autocrine and paracrine process\(^{181}\). As the calcification of VSMCs requires proliferation to be halted in order for transdifferentiation into an osteochondrogenic phenotype, the high expression of TenC mRNA in \( Ddr1^{-/-} \) VSMCs may result in increased TENC protein expression, thus restricting transdifferentiation by promoting proliferation.

**DDR1 Deficiency Results in Reduced RUNX2 Activity and Osteocalcin Expression in Vascular Smooth Muscle Cells**

The transcription factor RUNX2 mediates the transdifferentiation of VSMCs to an osteoblast-like phenotype\(^{69, 173}\). Therefore, I was interested in determining whether DDR1 regulates either RUNX2 expression or its activity. The mRNA expression of Runx2 increased after culturing in osteogenic media and peaked at 2 days in both \( Ddr1^{-/-} \) and \( Ddr1^{+/+} \) VSMCs(Figure 10A). By 12 days however the Runx2 mRNA diminished back to pre osteogenic culturing levels. Despite the upregulation in Runx2 mRNA expression with osteogenic culture, the levels of Runx2 mRNA in \( Ddr1^{-/-} \) and \( Ddr1^{+/+} \) VSMCs showed no differences at any time.
Immunoblotting revealed that RUNX2 protein expression increased from 2 days to 12 days in VSMCs. However, similar to mRNA levels there were no significant differences in RUNX2 protein expression between Ddr1−/− or Ddr1+/+ VSMCs at either the 2 day (Figure 10B), or the 12 day (Figure 10C) time points. Overall, this indicated to me that DDR1 did not change the expression of RUNX2.

To determine whether RUNX2 activity was altered between Ddr1−/− and Ddr1+/+ VSMCs, a luciferase activity assay was performed on cells that were transfected with 6OSE2-LUC construct. The 6OSE2-LUC construct measures RUNX2 activity using a firefly luciferase reporter. Ddr1−/− VSMCs showed a decrease in RUNX2 activity compared to Ddr1+/+ VSMCs even prior to the addition of osteogenic media (Figure 11A). The RUNX2 activity remained unchanged in Ddr1−/− VSMCs after 2 days of osteogenic treatment, whereas RUNX2 activity in Ddr1+/+ VSMCs drastically increased (Figure 11A). These experiments indicated that Ddr1 deletion prevented RUNX2 activation under osteogenic conditions.

Next, I wanted to determine whether the expression of Ocn, a gene regulated by RUNX2, would reflect the results observed with the activity assay. Ocn mRNA expression showed a decrease in expression in Ddr1−/− VMSCs at 0, 2 and 12 days in osteogenic culture when compared to Ddr1+/+ VSMCs (Figure 11B). In addition I saw an increase in Ocn expression in Ddr1+/+ VSMCs after osteogenic treatment. This increase was absent in Ddr1−/− VMSCs. Taken together these results suggest that VSMCs were undergoing osteogenic transdifferentiation and the absence of DDR1 attenuated the process.
P38 inhibition Reduces RUNX2 Activity and Osteocalcin mRNA Expression, but not RUNX2 Nuclear Localization in DDR1 Expressing Vascular Smooth Muscle Cells

The effective nuclear transport of RUNX2 is important in regulating its activity. To test whether DDR1 affects the nuclear transport of RUNX2 I immunostained for RUNX2 in Ddr1\(^{-/-}\) and Ddr1\(^{+/+}\) VSMCs in osteogenic culture. There was a significant decrease in RUNX2 nuclear localization in Ddr1\(^{-/-}\) VSMCs compared with Ddr1\(^{+/+}\) VSMCs at both 2 and 12 days (Figures 12A, B and C). Next, since ERK1/2 and P38 are activated downstream of DDR1 signalling and are activators of RUNX2 \(^5,9,17,18\), I determined whether inhibiting MEK or P38 would alter DDR1 dependant RUNX2 activity (Figure 13A) and Ocn mRNA expression. Treatment with MEKi did not alter RUNX2 activity or Ocn mRNA levels (Figure 13B) in Ddr1\(^{-/-}\) or Ddr1\(^{+/+}\) VSMCs compared to their respective DMSO controls. However, treatment with P38i significantly decreased RUNX2 activity in the Ddr1\(^{-/-}\) and Ddr1\(^{+/+}\) VSMCs (Figure 13A). P38i significantly reduced Ocn expression levels in Ddr1\(^{+/+}\) VSMCs, but did not alter levels in Ddr1\(^{-/-}\) VSMCs (Figure 13B). The differences in RUNX2 activity and Ocn mRNA expression were not due to change in Runx2 mRNA expression, as P38 inhibition treatment failed to alter RUNX2 mRNA (Figure 13C) This shows that P38 inhibition decreased RUNX2 activity and not expression of mRNA.

Next I determined whether the decrease in RUNX2 activity after P38 inhibition was due to altered RUNX2 nuclear localization. I found that neither MEKi (Figure 14B) nor P38i (Figure 14C) affected RUNX2 nuclear localization in Ddr1\(^{-/-}\) and Ddr1\(^{+/+}\) VSMCs. Thus, although P38 signalling may play a role in the activation of RUNX2, it does not affect the nuclear localization of RUNX2 under osteogenic conditions in VSMCs. This suggested to me that DDR1 regulates the nuclear localization of RUNX2 by another mechanism.
**DDR1 Deficiency Impairs phosphoP38 Nuclear Localization**

I wanted to understand ERK and P38 signalling in DDR1 modulation of osteogenically cultured VSMCs, because DDR1 was previously shown to activate ERK1/2 and P38\(^9,17,18\). To address this, I performed immunoblotting of ERK1/2 and P38 in \(Ddr1^{-/-}\) and \(Ddr1^{+/+}\) VSMC. There were no differences in ERK1/2 or P38 phosphorylation comparing \(Ddr1^{-/-}\) and \(Ddr1^{+/+}\) VSMCs after 2 days of culture (Figures 15A and 16A). However, by 12 days \(Ddr1^{-/-}\) VSMCs had significant decreases in ERK1/2 and P38 phosphorylation compared to \(Ddr1^{+/+}\) VSMCs (Figures 15B and 16B).

Although I did not see an alteration in P38 phosphorylation at 2 days between \(Ddr1^{-/-}\) and \(Ddr1^{+/+}\) VSMCs (Figure 15A), I saw a decrease in RUNX2 activity in \(Ddr1^{-/-}\) VSMCs compared to \(Ddr1^{+/+}\) VSMCs (Figure 11A). In addition, I saw that RUNX2 activity in \(Ddr1^{+/+}\) VSMCs was sensitive to P38 inhibition (Figure 13A). Therefore I hypothesized that in the absence of DDR1 the delivery of phosphoP38 to RUNX2 in the nucleus might be hindered. Indeed, phospho P38 failed to localize to the nucleus in \(Ddr1^{-/-}\) VSMCs, but was present in the nucleus of \(Ddr1^{+/+}\) VSMCs (Figure 17 A and B).

**DDR1 Regulates RUNX2 Nuclear Localization and Activity by Modulating a Dynamically Unstable Microtubule Network in Vascular Smooth Muscle Cells**

The loss of DDR1 disrupts both phosphoP38 and RUNX2 nuclear localization, Therefore, DDR1 likely regulates a mechanism common to both proteins. Previously, the expression of DDR1 was shown to regulate the microtubule network in mesenchymal stem cells\(^15\). As microtubules regulate the nuclear localization of phosphoP38 and RUNX2\(^131,182\), I wanted to
determine whether the microtubule network was disrupted in DDR1-deficient VSMCs in osteogenic culture. I first analyzed cell morphology by examining \( Ddr1^{-/-} \) and \( Ddr1^{+/+} \) VSMCs in osteogenic media using differential interference contrast microscopy. I saw that \( Ddr1^{+/+} \) VSMCs cultured in osteogenic media for 2 days had a more slender cell shape, compared to \( Ddr1^{-/-} \) VSMCs of the same cohort, which exhibited a more spread cell shape (Figure 18). Also, α-tubulin staining of \( Ddr1^{-/-} \) VSMCs after 2 (Figure 18B) and 12 days (Figure 18C) showed diffuse cytoplasmic staining, while \( Ddr1^{+/+} \) VSMCs showed strong staining of well organized microtubules. Since I observed differences in microtubule organization between \( Ddr1^{+/+} \) and \( Ddr1^{-/-} \) VSMCs, I next wanted to determine whether RUNX2 binding to the microtubule network was affected. Immunostaining revealed that RUNX2 shows relatively little co-localization with the diffuse cytoplasmic tubulin in \( Ddr1^{-/-} \) VSMCs, whereas RUNX2 strongly co-localizes with the well defined microtubules in the \( Ddr1^{+/+} \) VSMCs (Figure 19). This indicates that RUNX2 does not bind to diffuse cytoplasmic tubulin.

I next hypothesized that experimental disruption of microtubules to prevent microtubule polymerization would prevent the nuclear localization and activity of RUNX2 in \( Ddr1^{+/+} \) VSMCs. I chose to use nocodazole, as it binds to α and β-tubulin heterodimers to accelerate GTPase activity in β-tubulin to prevent polymerization into microtubules\(^{183}\). After depolymerisation of microtubules with nocodazole there was a significant reduction in nuclear localization of RUNX2 in the \( Ddr1^{+/+} \) VSMCs (Figure 20B ) compared to DMSO treated controls (Figure 20A). I then analyzed RUNX2 activity and Ocn mRNA expression in \( Ddr1^{-/-} \) and \( Ddr1^{+/+} \) VSMCs treated with nocodazole. Treatment with nocodazole did not affect RUNX2 activity in \( Ddr1^{-/-} \) VSMCs, but significantly decreased RUNX2 activity in \( Ddr1^{+/+} \) VSMCs (Figure 21A). This
indicated that DDR1 mediated RUNX2 activity is sensitive to microtubule depolymerization. However Ocn mRNA expression under nocodazole treatment did not follow that of RUNX2 activity. Instead nocodazole significantly increased Ocn mRNA expression in both DDR1⁻/⁻ and DDR1⁺/⁺ VSMCs (Figure 21B). Thus Ocn mRNA expression may be influenced by factors other than RUNX2 after microtubule destabilization.

I next hypothesized that stabilizing microtubules with the microtubule stabilizing agent would affect RUNX2 localization and activity in a DDR1 dependant manner in VSMCs. Taxol, the microtubule stabilizing agent binds a hydrophobic pocket in β-tubulin to promote polymerization in absence of GTP. The addition of taxol was able to rescue the nuclear localization of RUNX2 in DDR1⁻/⁻ VSMCs, (Figure 20C). I therefore hypothesized that RUNX2 activity and Ocn mRNA in DDR1⁻/⁻ VSMCs would be similarly rescued by the addition of taxol. However, treatment with taxol did not affect RUNX2 activity in DDR1⁻/⁻ VSMCs, but significantly decreased RUNX2 activity in DDR1⁺/⁺ VSMCs (Figure 21A). By contrast, taxol significantly increased Ocn mRNA expression in both DDR1⁻/⁻ and DDR1⁺/⁺ VSMCs (Figure 21B). These results suggest that Ocn mRNA expression is subject to other factors related to microtubule kinetics, which are independent of RUNX2 activity in VSMCs. I then wanted to demonstrate that treatments with nocodazole and taxol decreased RUNX2 activity by altering microtubule stability but not by decreasing RUNX2 expression. Runx2 mRNA levels showed that nocodazole or taxol treatment did not diminish levels, but rather increased expression (Figure 21C). Taken together the results suggest that DDR1 mediated RUNX2 activity by stabilizing microtubule translocation. Hindrance of RUNX2 activity by microtubule depolymerizing or stabilizing agents
indicates the DDR1 mediated activation of RUNX2 requires the dynamic instability of microtubules.

DISCUSSION

**DDR1 in matrix calcification**

In the current study, I examined the role of DDR1 in VSMC mediated calcification. To begin, I utilized an *in vitro* osteogenic model and examined the functional consequences of *Ddr1* deletion in VSMCs on matrix calcification. I found that *Ddr1* deletion blunts calcifying potential in VSMCs cultured in osteogenic media for 12 days. Previous work has linked type I collagen to VSMC transdifferentiation into an osteoblast-like calcifying phenotype. However, work on another type I collagen receptor, integrin β1 demonstrated that it functioned to prevent calcification instead. While the exact mechanism by which integrin β1 is able to inhibit calcification is not entirely clear, the pro- or anti-calcifying properties of type I collagen are likely to be receptor-specific. DDR1 represents a receptor through which type I collagen may be able to promote a calcifying phenotype. Although DDR1 interacts with type I collagen, it also interacts with types II-VI and VIII collagens, all of which are present in the atherosclerotic plaque. I therefore could not clearly identify which collagen type(s) interacted with DDR1 to promote osteochondrogenic differentiation in my study. Although it has not been implicated in VSMCs transdifferentiation or calcification, type II collagen is able to induce chondrogenesis in mesenchymal stem cells and adipocytes. Other studies, including a previous study from our laboratory, have demonstrated that Type II collagen is deposited
within calcified regions the atheroscleroslerotic plaque\textsuperscript{59, 188}. This represents another collagen which may regulate the osteochondrogenic transdifferentiation process in VSMCs. My study is the first to demonstrate that DDR1 is able to accelerate the calcifying potential of VSMCs and provides a receptor through which collagens may be able to regulate vascular calcification.

**Phosphate Handling in DDR1 Mediated Calcification**

I observed alterations in the expression of Pit-1, Alp and Npp1 in $Ddr1^{-/-}$ VSMCs during early but not late stage calcification. My results showed that during early stage calcification both Alp and Npp1 expression were decreased in the $Ddr1^{-/-}$ VSMCs while the expression of Pit-1 was increased. While the decrease and increase in Npp1 and Pit-1 expression in the $Ddr1^{-/-}$ VSMCs respectively did not seem to be in line with the decreased calcification observed, it is likely the decrease in Alp expression was able to overcome the Npp1 and Pit-1 expression levels to decrease calcification in the $Ddr1^{-/-}$ VSMCs. Furthermore, the increase in Pit-1 expression in the $Ddr1^{-/-}$ VSMCs maybe a compensatory mechanism in response to the decrease in Alp expression in order to increase intracellular Pi levels. Although at late stage calcification both $Ddr1^{+/+}$ and $Ddr1^{-/-}$ VSMCs had no significant changes in expression of these genes, the early changes in phosphate handling genes were likely sufficient to create the differences in calcification between $Ddr1^{+/+}$ and $Ddr1^{-/-}$ VSMCs.

I also observed in the course of this study, changes in the mRNA levels of Pit-1, Alp and Npp1 from early to late stage calcification. As Alp is a marker for osteogenic transdifferentiation it was not surprising to see its levels increase as calcification progresses as is reported in
literature\textsuperscript{121}. Conversely, Pit-1 mRNA expression was reduced as calcification progressed. While the change of expression in Pit-1 through the progression of calcification has not been described in literature, it is possible that there are sufficient intracellular levels of Pi at late stage calcification and hence the reduction in Pit-1 mRNA expression at that time point. Another possibility for the reduction of Pit-1 expression is to compensate for the increased Alp expression at that time.

Finally, the decrease in Npp1 mRNA levels over the course of vascular calcification has been described in literature. In most studies the decrease of NPP1 expression is the cause of vascular calcification\textsuperscript{104, 189}. As a result NPP1 expression decreases with the progression of calcification. While it has been determined this is the cause of vascular calcification, it is unknown whether this also occurs as a result of calcification. While my results may seem contradictory to current literature, it may also be possible that Npp1 increases in expression as a negative feedback mechanism to prevent calcification. Opn is an example of a gene which is associated with the development of vascular calcification and is commonly used as a marker of the osteochondrogenic phenotype\textsuperscript{93}. However it was discovered that OPN expression inhibited vascular calcification\textsuperscript{82, 190}. Together it appears that DDR1 alters the expression of Pit-1, Alp and NPP1 mRNA levels during calcifying conditions, which likely affects vascular calcification.

**Smooth Muscle Phenotype in DDR1 Mediated Calcification**

VSMCs are the primary cell type which transdifferentiates into an osteoblast like cell type to promote calcification of the vascular tissue\textsuperscript{69}. As the VSMCs transdifferentiates into a
osteoblast like cell, its smooth muscle characteristics are lost. I determined whether or not DDR1 affects the smooth muscle phenotype by examining the expression of smooth muscle cell markers. I determined in my studies that absence of DDR1 does not increase the expression of the smooth muscle marker αSMA. While it is generally accepted that VSMC markers decrease in expression as vascular calcification progresses, most documented studies show that they will continue to express VSMC markers. Another explanation would be that DDR1 has no affect on the VSMC phenotype during calcification. Despite this, I have not performed an exhaustive screen of all VSMC markers. Validated markers of the VSMC phenotype are SM22α, Myocardin and SM-MHC. Although useful as a gauge to determine VSMC phenotype, many studies have misidentified cells as being VSMC on the basis of an inadequate screen of markers. Furthermore, VSMCs have also been misidentified as other cell types due to a temporary loss of markers during injury or migration. Despite this, experimental evidence from Giachelli and colleagues showed that expression of VSMC markers does not impact osteochondrogenic pathways or hinder calcification. Thus, the expression level of VSMC markers likely has no impact the calcifying potential in my study.

I have also observed a significant increase in the expression of TenC mRNA, but no change in the expression of Atf4 in Ddr1−/− VSMCs. As TENC acts to promote VSMC proliferation, Ddr1−/− VSMCs might have higher rates of proliferation under my experimental conditions, however I did not measure proliferation in my studies ATF4 is also a marker of proliferation, but unlike TenC, I did not see any significant differences between Ddr1+/+ and Ddr1−/− VSMCs. Despite this, the increased TenC levels in Ddr1−/− VSMCs may be sufficient to promote a higher rate of proliferation in Ddr1−/− VSMCs. In line with my study, Curat et al.
previously reported that $Ddr1^{-/-}$ mesangial cells show higher rates of proliferation than $Ddr1^{+/+}$ cells\(^{196}\). As osteochondrogenic transdifferentiation occurs, VSMC proliferation needs to be suppressed\(^{179}\). This continued signal for proliferation in the $Ddr1^{-/-}$ VSMCs may serve to inhibit calcification in these VSMCs. Together; these results indicate that DDR1 does not alter VSMC phenotype under calcifying conditions, but may suppress VSMC proliferation to allow for osteochondrogenic conversion. However I have not yet measured proliferation.

During endochondral ossification, the osteoid is synthesized by chondrocytes, and is later calcified by osteoblasts\(^{97}\). While there is limited evidence that chondrocytes transdifferentiate into osteoblasts\(^{197}\), it is believed that osteoblasts stem largely from the common osteochondrogenic progenitor\(^{198}\). As VSMCs in atherosclerotic calcification frequently express both osteoblast and chondrocyte markers, the predominately held view is that atherosclerotic calcification mirrors endochondral ossification. Indeed, a previous study by our laboratory showed that chondrocyte markers such as SOX9, Type II and Type X collagens were expressed in atherosclerotic plaques\(^{59}\). In this present study I also observed that DDR1 deletion decreases osteoblast markers such as Ocn and Alp expression. However I did not screen for chondrocyte markers. During arterial calcification, it is not clear whether the expression of osteoblast and chondrocyte markers represents one homogenous population of cells performing both roles, or two separate populations of cells each with separate roles. Furthermore, prior work on ossification suggests that the chondrogenic phenotype antagonizes the osteoblast phenotype through the expression of SOX9\(^{199}\). Therefore, if DDR1 is able to alter the expression of SOX9, this represents a mechanism by which DDR1 can regulate the osteoblast phenotype and thus calcification. Despite the incomplete information regarding VSMC transdifferentiation during
Atherosclerotic calcification, it is clear that during atherosclerotic calcification VSMCs lose smooth muscle characteristics, and have transdifferentiated into cells which have characteristics of chondrocytes and or osteoblasts.

**DDR1 Mediated RUNX2 activation**

In this study I did not observe any DDR1 dependant differences of RUNX2 mRNA or protein expression. However I did see an increase in Runx2 mRNA expression which peaked at day 2 and declined back to baseline levels by day 12. RUNX2 protein levels increased steadily from 2 to 12 days. The increase in RUNX2 protein over the course of vascular calcification is well documented and is consistent with my findings\textsuperscript{109,157}. It is possible that Runx2 mRNA expression is decreased as a result of negative feedback from the RUNX2 protein that accumulates. It is noted that RUNX2 changes isoforms from the long lived type I to the rapidly degraded type II at late stage calcification in bone development\textsuperscript{134}. It would therefore be expected that Runx2 mRNA levels would be increased in order to replenish the rapidly degraded type II RUNX2 molecules at late stage calcification. However, this was not observed in my study. While the conversion of type I to type II RUNX2 has been established in bone development\textsuperscript{134}, this process may not occur in VSMCs undergoing osteochondrogenic transdifferentiation. Furthermore, the RUNX2 isoform switch may occur at a much later time point in VSMCs which this study has not analyzed.

There were no genotype-dependent differences in RUNX2 mRNA or protein levels, but I did see a significant decrease in RUNX2 activity in $Ddr1^{-/-}$ VSMCs. Paradoxically I saw that
RUNX2 activity was sensitive to P38i treatment despite not showing any differences in activated phosphoP38 levels at that stage of calcification. I attributed this to the regulation of RUNX2 by microtubules, which will be discussed in depth later. Even though P38 activation may not play a role in activating RUNX2 in a DDR1 dependant manner at this stage, it cannot be discounted as having an effect in DDR1 regulation of RUNX2. I observed a significant decrease in phosphoP38 levels at the 12 day time point. It is conceivable that while VSMCs are in culture they synthesized much more matrix by day 12 and hence there is an increase in DDR1 receptor activation by day 12 due to increased number of DDR1 ligands in the cell culture. As P38 signalling has been demonstrated as an important activator of RUNX2\textsuperscript{147,177}, it would likely alter RUNX2 activity at this stage. Unfortunately the effect of P38 inhibition on RUNX2 activity at late stage calcification could not be assessed due the sensitivity of Ddr1\textsuperscript{-/-} VSMCs to cell death under P38 inhibition.

I also found that inhibition of ERK1/2 signalling did not affect RUNX2 activity in VSMCs. Previous literature reported that ERK1/2 is an activator of RUNX2, and a even more potent activator of RUNX2 than P38\textsuperscript{147} in mesenchymal stem cells and osteoblasts. Taken together, my results demonstrate for the first time that DDR1 is able to regulate the activity RUNX2; however this is not dependent upon ERK1/2 or P38 activity at early stages of calcification.

**Microtubules in DDR1 Mediated RUNX2 activity**

Previously there have been no works linking DDR1 to the modulation of microtubules or the nuclear translocation of proteins. My study provides evidence that DDR1 is able to regulate
the microtubule network which controls RUNX2 nuclear localization and activity. As the regulation of vascular calcification by any component of the cytoskeleton has never been explored, my work on the regulation of RUNX2 by DDR1 via microtubules may represent a paradigm shift for further investigations on vascular calcification.

My study also showed that phosphoP38, another protein requiring microtubules for nuclear transport, also showed reduced nuclear localization in Ddr1⁻/⁻ VSMCs. While it was previously thought that most proteins translocate into the nucleus through Brownian motion, there is a growing body of literature that shows many proteins such as steroid hormone receptors, transcription factors, and signalling kinases which require the use of microtubules.

I have shown that RUNX2 co-localizes with the microtubule network in the Ddr1⁺/⁺ VSMCs, but not in Ddr1⁻/⁻ VSMCs. This is consistent with studies by Pockwinse et. al. who showed that RUNX2 binds to microtubules. Thus, it was not surprising to see that depolymerizing microtubules by treatment with nocodazole prevented both RUNX2 nuclear localization and activity in Ddr1⁻/⁻ VSMCs. Many previous studies have shown that either stabilizing or preventing the polymerization of microtubules alters transport of protein cargoes. In contrast, I found that stabilizing microtubules by treatment with taxol rescues RUNX2 nuclear localization in Ddr1⁻/⁻ VSMCs. This finding was consistent with the results of a study by Pockwinse et. al who showed that taxol treatment increased nuclear population of RUNX2 and decreased its cytoplasmic population. As microtubule facilitated nuclear transport is a relatively new area of investigation, there may be other proteins whose transport into the
nucleus depends solely on stabilization. Despite rescuing nuclear localization in Ddr1−/− VSMCs, the stabilization of microtubules by taxol was unable to restore RUNX2 activity. Since taxol acts to lock microtubules in a stable but static state, it prevents dynamic instability. Therefore my results suggest that activation of RUNX2 may require the dynamic instability of microtubules. Although we have not tested this, a possible explanation for this is that phosphoP38 requires dynamic instability as previously demonstrated. As RUNX2 phosphorylation by phosphoP38 likely occurs in the nucleus, restriction of phosphoP38 in the cytosol will effectively prevent RUNX2 activation. Another possibility is that the incubation time with taxol used in this assay was not sufficient to allow the newly translocated RUNX2 to act upon the reporter construct. Unfortunately, longer term treatments with taxol result in cell death and detachment so I am not able to assess the long term effects on RUNX2 activity and in vitro calcification.

In my studies I found that Ocn mRNA expression was increased with either taxol or nocodazole treatment in both genotypes. Thus Ocn expression was induced even in the absence of RUNX2 activation. A possible explanation lies with the differences between the reporter promoter construct I used and the Ocn promoter. Although both 6OSE2-LUC and the Ocn promoter contain six OSEs, the Ocn promoter may contain binding sites for other transcriptional elements. Although numerous pathways have been shown to activate Ocn expression, they all converge on RUNX2 activation. It is entirely possible that when manipulating microtubules, factors which have not yet been identified act on Ocn expression. Vegf is another gene which contains OSEs, it however also contains HIF1α Responsive Elements which are activated by HIF1α and entirely independent of RUNX2. Since the 6OSE2-Luc construct is only activated by RUNX2; its measured activity it is likely more accurate.
DDR1 regulation of microtubules, although poorly explored, it is not unprecedented\textsuperscript{131}. There have also been many documented cases where DDR1 is shown to regulate processes related to microtubule function. Cell spreading for example is a process that is regulated by a dynamic microtubule network\textsuperscript{206}. In this study, I observed \textit{Ddr1}\textsuperscript{−/−} VSMCs also showed more cell spreading than \textit{Ddr1}\textsuperscript{+/+} VSMCs. This finding is consistent with a previous study which showed that DDR1 suppressed the spreading of HEK293 cells \textsuperscript{207}. Studies on VSMCs have also hinted at DDR1 regulation of microtubules. Cell migration is a process that depends on microtubule action\textsuperscript{208}. As previously discussed in the introduction, \textit{Ddr1}\textsuperscript{−/−} VSMCs show significantly reduced cell migration\textsuperscript{52, 131}. Taken together, previous literature and my results suggest that DDR1 regulates dynamic instability in microtubules which is required for RUNX2 nuclear translocation and activity during VSMC calcification.

**Future Directions**

In the current study I identified a mechanism by which DDR1 is able to control the osteogenic process, determining that DDR1 mediate the nuclear translocation of RUNX2 via the microtubule network. Also of interest is whether kinase activity of DDR1 is required to modulate the microtubule network and RUNX2 nuclear translocation. Although there are many functions of DDR1 which require the phosphorylation, other functions such VSMC attachment to matrix and controlling the collective migration of cells does not require its kinase activity\textsuperscript{209}. Therefore it may be possible that DDR1 regulates microtubules through a kinase independent pathway. This can easily be tested by adding the RTK phosphorylation inhibitor, Imatinib in
VSMCs during osteogenic culture. Although Imatinib inhibits all RTKs, it most strongly inhibits the phosphorylation of DDR1\textsuperscript{210}. This method can be used to determine whether or not the translocation of RUNX2 requires the phosphorylation of DDR1. To further validate this mechanism, a kinase dead construct of DDR1 can be transfected into Ddr1\textsuperscript{-/-} VSMCs cultured in osteogenic conditions in order to determine if it affects RUNX2 translocation.

Work on osteogenic development shows that type I collagen is a pro-osteogenic molecule\textsuperscript{10, 93, 209}. While my work implies that DDR1 represents a functional link between type I collagen and RUNX2 activity, further work is need to determine this. Although DDR1 is a Type I collagen receptor, it is also able to bind other collagen ligands\textsuperscript{185}. Although studies have shown that VSMCs synthesize type I collagen \textit{in vitro}, VSMCs also synthesize other collagens which are DDR1 ligands\textsuperscript{185, 211}. In order to fully demonstrate which collagen is able to elicit the pro-osteogenic effects through DDR1, VSMCs can be cultured in osteogenic conditions with the addition of separate collagen ligands for DDR1. This will help determine which DDR1 ligand(s) if any affect the microtubule network which impacts RUNX2 translocation.

Furthermore, despite numerous lines of evidence suggesting that DDR1 impacts microtubule related functions such as cell migration, invasion and proliferation\textsuperscript{18, 29, 57, 58, 131, 212}, the mechanism by which DDR1 regulates microtubules, during osteogenic conditions or otherwise remains unknown. My study suggests that there is a difference in the dynamic instability between microtubules in Ddr1\textsuperscript{-/-} and Ddr1\textsuperscript{+/+} VSMCs. However, in my study I was only able to examine microtubule structure at the endpoints of calcification due to available materials and reagents. Further studies can be carried out to examine image the microtubule
network throughout the calcification process with Tubulin Tracker (Life Technologies) which is flurophore conjugated to a microtubule binding compound which does not hinder microtubule formation. This compound will allow me to determine microtubule growth rates and cycling as modulated by DDR1 through live cell microscopy. In addition, the dynamic instability of microtubules is regulated by two main factors, acetylation of tubulin and the binding of microtubule associated proteins to tubulin\textsuperscript{213}. Hypothetically DDR1 could regulate either or both of these factors to regulate dynamic instability of microtubules. This can further be investigated by using an acetyled tubulin antibody for immunofluoresce and immunoblotting in the cultured cells. Candidate microtubule associated proteins can also be identified through mass spectrometry.

It is interesting to note that under circumstances where there are large concentrations of the transcription factor in question, the requirement for microtubules in nuclear localization is uncoupled\textsuperscript{171, 214}. Since RUNX2 expression in both DDR1 expressing and \textit{Ddr1}\textsuperscript{−/−} VSMCs increases over time in osteogenic culture, it would be interesting to investigate whether microtubule requirement for nuclear localization is lost over time. This can also be determined by overexpressing RUNX2 in DDR1 expressing and \textit{Ddr1}\textsuperscript{−/−} VSMCs in osteogenic culture and determining if RUNX2 overexpression eliminates the need for DDR1 regulation of microtubules for nuclear translocation.
Conclusions

My study is the first to identify the mechanistic ability of DDR1 to regulate VSMC calcification. DDR1 is crucial in the regulation of osteochondrogenic transcriptional activity through its regulation of RUNX2 activity in VSMCs. I have demonstrated that DDR1 regulates the dynamic instability of microtubules in VSMCs. In absence of DDR1, microtubule polymerization is hindered and thus RUNX2 is unable to bind to un polymerized tubulin molecules, thus unable to enter the nucleus. I have also demonstrated that inducing microtubule polymerization in DDR1 KO VSMCs, rescues nuclear localization. Although the finding that polymerized microtubules results in RUNX2 nuclear translocation is not unprecedented\textsuperscript{131}, this study is the first to show that rescuing RUNX2 nuclear localization does not rescue activity. Although the mechanism has not been thoroughly investigated the cytoplasmic abundance of phosphoP38 during the absence of DDR1, hints that DDR1 regulation of microtubule has an effect on phosphoP38 localization as well. The effect of DDR1 regulation of microtubules in VSMCs does not seem to be limited to nuclear translocation alone. Cell spreading a common phenomenon in cells with hindered microtubule regulation\textsuperscript{215}, is also prevalent in the absence of DDR1 in VSMCs in my study. In conclusion my study demonstrates that DDR1 a novel collagen receptor regulates VSMC calcification through microtubule moderation of RUNX2 activity. The proposed model of DDR1 regulation of RUNX2 activity is illustrated (Figure 22).
Figure 1: Diagram of Normal Artery and Artery Affected by Atherosclerosis

This diagram depicts a normal artery (left) and an artery affected by Atherosclerosis. Diagram was taken from http://www.pharmaceutical-networking.com.
Figure 2: Diagram of DDR1 Isoforms in Humans and Mice

This diagram depicts the five different DDR1 isoforms in humans and mice. Disocoidin Domain (DD), Transmembrane (TM), Juxtamembrane (JM), Tyrosine Kinase (TK). Green represents 37 AA insert in the juxtamembrane region and red represents the six AA insert in the tyrosine kinase region.
**Figure 3: Diagram of Molecules Which Regulate Pi and PPI Levels**

This diagram depicts molecules which regulate Pi and PPI levels within a cell and how they ultimately give rise or inhibit calcification.
Figure 4: Diagram of RUNX2 Isoforms in Humans and Mice

This diagram depicts the two different RUNX2 isoforms in humans and mice. Amino Terminus (NT), RUNT DNA binding domain, Nuclear Translocation Matrix (NTM) and Destabilizing PEST domain. Red represents the region which contains the microtubule binding site.
Figure 5: Diagram of RUNX2 Stimulatory and Inhibitory Phosphoserine Residues

This diagram depicts the known stimulatory and inhibitory phosphoserine residues on RUNX2 and how they are activated by P38 and ERK1/2.
Figure 6: Diagram of Microtubule Dynamics

This diagram depicts the dynamic instability of microtubules. GTP bound tubulin promotes polymerization, while GDP bound tubulin promotes disassembly. Diagram taken from Nature Reviews Cancer 4, 253-265 (April 2004)
Figure 7: Ddr1⁻/⁻ VSMCs Show Decreased Calcifying Potential: (A) Calcium content of Ddr1⁺/⁺ (black bars) and Ddr1⁻/⁻ (gray bars) VSMC cultures measured by O-cresophathlein based colourimetric assay after 12 days and normalized to total cellular protein using a BCA colourimetic assay. (B) Calcium content of Ddr1⁺/⁺ (black bars) and Ddr1⁻/⁻ (gray bars) VSMC cultures measured by O-cresophathlein based colourimetric assay after 12 days and normalized to total cellular DNA using a Hoechst nuclear dye based assay. Data shown are means ± SEM; n=3
Figure 8: *Ddr1*<sup>−/−</sup> VSMCs Show Increased Pit-1 mRNA Expression and Decreased Alp and Npp1 mRNA Expression at Early but not Late Stage Calcification: (A) *Ddr1*<sup>+/+</sup> (black bars) and *Ddr1*<sup>−/−</sup> (gray bars) VSMC Pit-1 mRNA levels at 2 and 12 days measured using qRT-PCR. (B) *Ddr1*<sup>+/+</sup> (black bars) and *Ddr1*<sup>−/−</sup> (gray bars) VSMC Alp mRNA levels at 2 and 12 days measured using qRT-PCR. (C) *Ddr1*<sup>+/+</sup> (black bars) and *Ddr1*<sup>−/−</sup> (gray bars) VSMC Npp1 mRNA levels at 2 and 12 days measured using qRT-PCR. * indicates a significant difference between groups (P<0.05). Data shown are means ± SEM; n=3.
Figure 9: *Ddr1*+/− VSMC Show no Change in αSMA Protein and Atf4 mRNA Expression, but Show Increased TenC mRNA Expression at Late but Not Early Stage Calcification: (A) *Ddr1*+/+ (black bars) and *Ddr1*−/− (gray bars) VSMC αSMA protein levels at 12 days measured using immunoblotting. (B) *Ddr1*+/+ (black bars) and *Ddr1*−/− (gray bars) VSMC Atf4 mRNA levels at 2 and 12 days measured using qRT-PCR. (C) *Ddr1*+/+ (black bars) and *Ddr1*−/− (gray bars) VSMC TenC mRNA levels at 2 and 12 days measured using qRT-PCR. * indicates a significant difference between groups (P<0.05). Data shown are means ± SEM; n=3.
Figure 10: *Ddr1*⁻/⁻ VSMCs Show No Changes in RUNX2 Protein and mRNA Expression: (A) *Ddr1*⁺/⁺ (black bars) and *Ddr1*⁻/⁻ (gray bars) VSMC cultures from 2, 0 and 12 days was measured for Runx2 mRNA using qRT-PCR. (B) RUNX2 protein levels from *Ddr1*⁺/⁺ (black bars) and *Ddr1*⁻/⁻ (gray bars) VSMC measured with immunoblotting at 2 days. (C) RUNX2 protein levels from *Ddr1*⁺/⁺ (black bars) and *Ddr1*⁻/⁻ (gray bars) VSMC were measured with immunoblotting at 12 days. * indicates a significant difference between groups (P<0.05). Data shown are means ± SEM; n=3.
Figure 11: Ddr1⁻/⁻ VSMCs Show Reduced RUNX2 Activity and Ocn mRNA Expression: (A) RUNX2 activity of Ddr1⁺/⁺ (black bars) and Ddr1⁻/⁻ (gray bars) VSMCs was measured using a luciferase assay of 0 and 12 day cultures (B) mRNA levels of Ocn in Ddr1⁺/⁺ (black bars) and Ddr1⁻/⁻ (gray bars) VSMCs was measured using qRT-PCR 0, 2 and 12 day cultures. * indicates a significant difference between groups (P<0.05). Data shown are means ± SEM; n=3.
Figure 12: *Ddr1*<sup>−/−</sup> VSMCs Show Reduced RUNX2 Nuclear Localization: (A) *Ddr1*<sup>+/+</sup> and *Ddr1*<sup>−/−</sup> cells from 2 days were 4% PFA fixed and stained for RUNX2 (Green) Nuclei were stained with Hoechst (blue). Scale bars = 20 µm. (B) *Ddr1*<sup>+/+</sup> and *Ddr1*<sup>−/−</sup> cells from 12 days were 4% PFA fixed and stained for RUNX2 (Green) Nuclei were stained with Hoechst (blue). Scale bars = 20 µm. (C) Quantification of RUNX2 nuclear and cytoplasmic amounts from VSMC in 2 and 12 days under osteogenic conditions *Ddr1*<sup>+/+</sup> (black bars) and *Ddr1*<sup>−/−</sup> (gray bars). * indicates a significant difference between groups (P<0.05). Data shown are means ± SEM; n=3.
Figure 13: RUNX2 Activity and mRNA Levels of Osteocalcin are Sensitive to P38i in Ddr1+/+ VSMCs: (A) RUNX2 activity of Ddr1+/+ (black bars) and Ddr1−/− (gray bars) VSMC cultures treated with DMSO, 10 µM PD098059 (MEK inhibitor) or SB203580 (P38 inhibitor) measured using luciferase activity assay. (B) Ocn mRNA of Ddr1+/+ (black bars) and Ddr1−/− (gray bars) VSMC cultures treated with DMSO, 10 µM PD098059 (MEK inhibitor) or SB203580 (P38 inhibitor) measured using qRT-PCR. (C) Runx2 mRNA of Ddr1+/+ (black bars) and Ddr1−/− (gray bars) VSMC cultures treated with DMSO, 10 µM PD098059 (MEK inhibitor) or SB203580 (P38 inhibitor) measured using qRT-PCR. * indicates a significant difference between groups (P<0.05). Data shown are means ± SEM; n=3.
Figure 14: Nuclear Localization of RUNX2 in Ddr1+/+ and Ddr1−/− VSMCs Was Not Affected by MEKi or P38i: (A) Ddr1+/+ and Ddr1−/− cells treated with DMSO were 4% PFA fixed and stained for RUNX2 (Green) Nuclei were stained with Hoechst (blue). Scale bars = 20 μm. (B) Ddr1+/+ and Ddr1−/− cells treated with 10 μM PD098059 (MEK inhibitor) were 4% PFA fixed and stained for RUNX2 (Green) Nuclei were stained with Hoechst (blue). Scale bars = 20 μm. (C) Ddr1+/+ and Ddr1−/− cells treated with 10 μM SB203580 (P38 inhibitor) were 4% PFA fixed and stained for RUNX2 (Green) Nuclei were stained with Hoechst (blue). Scale bars = 20 μm. (D) Quantification of RUNX2 nuclear and cytoplasmic amounts of Ddr1+/+ (black bars) and Ddr1−/− (gray bars) VSMC.
cultures in 2 and 12 days under osteogenic conditions. * indicates a significant difference between groups (P<0.05). Data shown are means ± SEM; n=3.
Figure 15: Phospho ERK1/2 Signalling is Decreased in Ddr1−/− VSMCs at Late but Not Early Stage Calcification: (A) PhosphoERK1/2 protein levels from Ddr1+/+ (black bars) and Ddr1−/− (gray bars) VSMC measured with immunoblotting at 2 days. (B) PhosphoERK1/2 protein levels from Ddr1+/+ (black bars) and Ddr1−/− (gray bars) VSMC were measured with immunoblotting at 12 days. * indicates a significant difference between groups (P<0.05). Data shown are means ± SEM; n=3.
Figure 16: PhosphoP38 Signalling is Decreased in Ddr1^{-/-} VSMCs at Late but Not Early Stage Calcification: (A) PhosphoP38 protein levels from Ddr1^{+/+} (black bars) and Ddr1^{-/-} (gray bars) VSMC measured with immunoblotting at 2 days. (B) PhosphoP38 protein levels from Ddr1^{+/+} (black bars) and Ddr1^{-/-} (gray bars) VSMC were measured with immunoblotting at 12 days. * indicates a significant difference between groups (P<0.05). Data shown are means ± SEM; n=3.
Figure 17: *Ddr1*⁻/⁻ VSMCs Show Reduced PhosphoP38 Nuclear Localization: (A) *Ddr1*⁺/+ and *Ddr1*⁻/⁻ cells from 2 days were 4% PFA fixed and stained for phosphoP38 (Green) Nuclei were stained with Hoechst (blue). Scale bars = 20 µm. (B) *Ddr1*⁺/+ and *Ddr1*⁻/⁻ cells from 12 days were 4% PFA fixed and stained for phosphoP38 (Green) Nuclei were stained with Hoechst (blue). Scale bars = 20 µm. (C) Quantification of phosphoP38 nuclear and cytoplasmic amounts from VSMC in 2 and 12 days under osteogenic conditions *Ddr1*⁺/+ (black bars) and *Ddr1*⁻/⁻ (gray bars). * indicates a significant difference between groups (P<0.05). Data shown are means ± SEM; n=3.
Figure 18: *Ddr1*⁻/⁻ VSMCs Show Increased Cell Spreading and Reduced α Tubulin Polymerization: (A) *Ddr1*⁺/⁺ and *Ddr1*⁻/⁻ cells from 2 days were 4% PFA fixed and imaged using DIC microscopy. Scale bars = 20 μm. (B) *Ddr1*⁺/⁺ and *Ddr1*⁻/⁻ cells from 2 days were 4% PFA fixed and stained for α Tubulin (Green) Nuclei were stained with Hoechst (blue). Scale bars = 20 μm. (C) *Ddr1*⁺/⁺ and *Ddr1*⁻/⁻ cells from 12 days were 4% PFA fixed and stained for α Tubulin (Green) Nuclei were stained with Hoechst (blue). Scale bars = 20 μm.
Figure 19: \( Ddr1^{+/+} \) VSMCs Show Reduced RUNX2 Co-Localization With Microtubules: \( Ddr1^{+/+} \)

and \( Ddr1^{-/-} \) VSMC cultures in 2 days under osteogenic conditions (2.4 mM Pi) were 4% PFA fixed and stained for \( \alpha \) Tubulin (Green) and RUNX2 (Red). Scale bars = 40 \( \mu \)m.
Figure 20: Taxol Increased RUNX2 Nuclear Localization in Ddr1+/+ and Ddr1−/− VSMCs While Nocodazole, Prevented RUNX2 Nuclear Localization in Ddr1+/+ VSMCs: (A) Ddr1+/+ and Ddr1−/− cells treated with DMSO were 4% PFA fixed and stained for RUNX2 (Green) Nuclei were stained with Hoechst (blue). Scale bars = 20 µm. (B) Ddr1+/+ and Ddr1−/− cells treated with 10 µM nocodazole (microtubule destabilizing agent) were 4% PFA fixed and stained for RUNX2 (Green) Nuclei were stained with Hoechst (blue). Scale bars = 20 µm. (C) Ddr1+/+ and Ddr1−/− cells treated with 10 µM taxol (microtubule stabilizing agent) were 4% PFA fixed and stained for RUNX2 (Green) Nuclei were stained with Hoechst (blue). Scale bars = 20 µm. (D) Quantification
of RUNX2 nuclear and cytoplasmic amounts of $Ddr1^{+/+}$ (black bars) and $Ddr1^{-/-}$ (gray bars) VSMC cultures in 2 and 12 days under osteogenic conditions. * indicates a significant difference between groups (P<0.05). Data shown are means ± SEM; $n=3$. 
Figure 21: Nocodazole or Taxol Attenuated RUNX2 Activity in \textit{Ddr1}^{+/+} and \textit{Ddr1}^{-/-} VSMCs but Increased Ocn mRNA Expression: (A) RUNX2 activity of \textit{Ddr1}^{+/+} (black bars) and \textit{Ddr1}^{-/-} (gray bars) VSMC cultures treated with DMSO, 10 µM nocodazole or taxol measured using luciferase activity assay. (B) Ocn mRNA of \textit{Ddr1}^{+/+} (black bars) and \textit{Ddr1}^{-/-} (gray bars) VSMC cultures treated with DMSO, 10 µM nocodazole or taxol measured using qRT-PCR. (C) Runx2 mRNA of \textit{Ddr1}^{+/+} (black bars) and \textit{Ddr1}^{-/-} (gray bars) VSMC cultures treated with DMSO, 10 µM nocodazole or taxol measured using qRT-PCR. * indicates a significant difference between groups (P<0.05). Data shown are means ± SEM; n=3.
**Figure 22: Proposed model of DDR1 regulation of RUNX2 activity in VSMCs:** The schematic highlights the possible pathway by which DDR1 acts to regulate RUNX2 in VSMCs. DDR1 is essential for organizing a microtubule network for the nuclear localization of RUNX2.
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