SMYD2 Modulates Lipid Peroxidation in C3H/10T1/2 Cells Through GPX4

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by

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Under the Direction of Dr. Chunying Li, Ph.D.

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ABSTRACT

Introduction: Oxidative stress induced vascular remodeling promotes development and progression of atherosclerosis. By scavenging excessive Lipid ROS, GPX4 reduces atherosclerotic plaque severity and VSMC sensitivity, whereas BRD4 promotes plaque progression by facilitating lipid uptake associated with atherosclerosis. This study aimed to explore smooth muscle cell (SMC)-specific role of SMYD2 in modulating oxidative stress in vitro through regulation of GPX4/BRD4

Methods: To evaluate the effect of ferroptosis on SMYD2 and BRD4, C3H/10T1/2 (10T1/2 cells) mouse embryonic fibroblasts were treated with Ferrostatin-1 (Potent and selective inhibitor of ferroptosis), and 1S,3R-RSL3 (Ferroptosis inducer), PAz-PC (oxLDL), in dose and time dependent manner. Next, to evaluate if SMYD2's mediated ferroptosis is dependent on BRD4, control and Smyd2-depelted 10T1/2 cells were treated with Ferrostatin-1/1S,3R-RSL3 and (+)-JQ1. Lipid hydroperoxides, and Glutathione assay along with Immunofluorescence C11-BODIPY581/591 staining was used to measure oxidative stress in the cellular in cultured cells. Primary VSMCs isolated from control and SMYD2 depleted mice and cultured C3H/10T1/2 cells were used for in vitro studies.

Results: Smyd2 depletion in C3H/10T1/2 and primary cells protected against oxidative stress by regulating GPX4 and BRD4 axis. Pharmacological inhibition (Ferrostatin-1, 2μM, 24hr) or activation (3R-RSL3, 0.8nM, 8hr) of GPX4 activity either by resulted in a significant upregulation and suppression of SMYD2 respectively. OxLDL (PAz-PC, 15ug, 48r) treatment resulted in elevated GPX4 expression in SMYD2-depleted cells compared to control, suggesting that SMYD2 deficiency mitigating ferroptosis by upregulating GPX4. Moreover, inhibiting BRD4 with (+)-JQ1 treatment

in C3H/10T1/2 cells, regardless of SMYD2 presence, led to an increase in ferroptosis. Immunofluorescence and C11-BODIPY581/591 staining of these cells revealed higher GPX4 expression and lower BRD4 expression in SMYD2-depleted cells compared to controls. MDA assay results from both cells and culture media corroborated these findings. qPCR analysis and the re-examination of our RNA-seq data demonstrated that either depletion of SMYD2, or (+)-JQ1 treatment significantly downregulated the expression of ferroptosis-associated genes, such as Hmox1, Acsl3, Nox2, GPX4, and Slc40a1. This suggests that SMYD2 may govern lipid peroxidation by modulating the expression of ferroptosis-associated genes regulated by BRD4.

Conclusion: SMYD2 depletion protects against the severity of oxidative stress by regulating the GPX4/BRD4 axis, suggesting its therapeutic potential against lipid peroxidation and cardiometabolic disease.

INDEX WORDS: Atherosclerosis, Calcification, C3H/10T1/2 Cells, SMYD2 (SET And MYND Domain Containing 2), GPX4 (Glutathione Peroxidase 4), ROS (Reactive Oxygen Species)

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VSMCs:	Vascular Smooth Muscle Cells
SMYD2:	SET and MYND Domain Containing 2
GPX4:	Glutathione Peroxidase 4
RUNX2:	Runt-related transcription factor 2
NBF:	Buffer Formalin solution
IHC:	Immunohistochemistry
IF:	Immunofluorescence
ARS:	Alizarin Red staining
ORO:	Oil Red O staining
C3H10T1/2:	10T1/2 cells (Mesenchymal stem cells)
RT-PCR:	Reverse transcription polymerase chain reaction

1. INTRODUCTION

Cardiovascular diseases are a group of disorders of the heart and blood vessels. Cells of the cardiovascular system include endothelial cells, vascular smooth muscle cells (VSMCs), immune cells such as lymphocytes and macrophages, and other types of cells. SET and MYND domain-containing protein 2 (SMYD2) is known to regulate pathophysiological processes in an organism, including being a therapeutic target against atherosclerosis. Its expression and function have been targeted for therapeutic purposes in treating cardiovascular disease. We have previously explored the molecular mechanism underlying SMYD2 expression and its functional significance in modulating VSMC phenotypic switch and neointima formation/intimal hyperplasia (Zhou 2023). Downregulation of SMYD2 was shown to promote VSMC phenotypic switching, and vice versa (Zhou 2023). Previous studies have shown that BRD4 inhibitor (+)-JQ1 suppresses BRD4 protein, thus decreasing GPX4 enzymatic activity (Geng 2023) . However, the functional role of SMYD2 in mediating oxidative stress remains unclear. SMYD2-mediated oxidative stress in VSMCs has never been reported before. We, therefore, investigated the activity of various Lipid Peroxidation molecules on the effects of 10T1/2 cells.

1.1 SMYD Family

Su(Var)3–9, Enhancer-of-zeste and Trithorax (SET) and Myeloid, Nervy, and DEAF-1 (MYND) domain-containing (SMYD) proteins are a family of enzymes that play vital roles in gene expression, muscle development, immune regulation, and cancer pathogenesis. SMYD proteins comprise of 5 members, namely, SMYD1, SMYD2, SMYD3, SMYD4, and SMYD5. All of these enzyme proteins methylate histone and non-histone substrates, and regulate diverse cellular functions in proliferation, migration, and apoptosis. SMYD1 functions within cancerous cells and

in striated muscle cells. SMYD2 has been previously studied in our laboratory to function in VSMCs and macrophages. More knowledge about this specific protein will be discussed in further detail throughout the thesis. SMYD3 plays roles in cancer cell proliferation. SMYD4 has been found to function as a methyltransferase that is upregulated in arthritis patients. SMYD5 does not have much prior studies done but is relevant in functioning within the immune system. Recently, studies from our laboratory revealed that SMYD5 attenuates mitochondrial functions in intestinal epithelial cells and promotes inflammatory bowel disease progression (Hou 2022). In all, the family of SMYD proteins play important roles in an assortment of system processes.

1.1.1 SMYD2

SMYD2 is a member of the SMYD family that is composed of a common domain structure with an N-terminal SET domain, MYND domain, and a C-terminal domain. SMYD2 is linked to cardiovascular pathology in which it reveals adverse effects on cardiac function (Miotto 2020). SMYD2 was previously studied in our laboratory to determine its role in VSMCs using the SMC-specific knockout mice in vivo. It has not been previously identified how SMYD2 mediates lipid peroxidation in 10T1/2 cells.

In apoptosis, SMYD2 plays a vital role during embryonic development. When SMYD2 is depleted in adult mouse hematopoietic stem cells (HSCs), apoptosis was upregulated. The very opposite occurred when SMYD2 was upregulated in the HSCs. From this, conclusions were made of the role that SMYD2 plays in being essential to maintaining homeostasis and a key factor in pathophysiological ailments such as heart failure. The multidimensional roles that SMYD2 plays in various biological processes, including embryonic development, viral infections, and cancerous diseases show its potential to be a vital therapeutic target (Skouta 2014). Exploring the role that

the protein plays in cardiometabolic diseases, through basic biomedical research, can contribute to the development of future hallmarks in treatments for various human diseases.

1.2 Atherosclerosis

Atherosclerosis is a type of arteriosclerosis, i.e., hardening and narrowing of the arteries caused by the buildup of fatty substances (cholesterol, fat, calcium, and other materials) called plaques in the inner lining of an artery. Plaques may rupture causing acute occlusion of the artery by clotting. The mechanism to this disease in the arteries begins with normal arteries containing predisposed sites that will tolerate easier initiations of focal injury within the macrophages, lipids, smooth muscle cells, or matrix. Once the plaque formation begins, the lumen attempts to remodel itself. Upon immense intrusion of plaque buildup to the arterial walls, calcification can lead to rupture of the wall, thus causing progression of further clinical disease. The culmination of these events is characterized by integral components, including VSMCs, macrophages, dendritic cells, lymphocytes, mast cells, endothelial cells (ECs), and T lymphocytes. This particular type of cell set of cells play a large role in vascular pathology. A key factor in atherogenesis, NRF2 promotes cell survival through improvement of the stress state in the body. NRF2 decreases the onset of lipid peroxidation, through maintaining cell homeostasis, thus allowing the optimal formation of glutathione and GPX4.

1.2.1 Reactive Oxygen Species (ROS) and Lipid Peroxidation

Reactive oxygen species (ROS) is a pathway of signaling molecules that contribute of a normal attribute of aerobic life. Its name, given by the reactive atom oxygen, encompasses a magnitude of specific targets. Key species that are involved in the mechanism are hydrogen peroxide (H_2O_2) and a superoxide anion radial (O_2-).

Accumulation of lipid hydroperoxides can lead to degenerative diseases. This can either occur through autoxidation or processes catalyzed by iron-dependent lipoxygenases (Miotto 2020). At low and regulated concentrations, the two redox regulators in ROS generation subsidize protein activity, localization, and interactions. When promoted in high levels, ROS contributes significantly to vascular pathobiology including, atherosclerosis, intimal lesions and thickening, plaque calcification and metabolic disease. Additionally, onset and progression of subclinical non-atherosclerotic or atherosclerotic events by ROS is marked by EC activation, increased vascular permeability, impairment of glycocalyx leading to SMCs-like cells within the intima.

1.3 Cell Death: Ferroptosis

Various types of cell death have been documented in the literature and have their own distinct mechanisms based on signals received. These include apoptosis, autophagy, ferroptosis, and necroptosis. Self-induced death, apoptosis, is a coordinated form of programmed cell death that the cell induces onto itself. Autophagy is a form of cell death that is a natural stage of some organisms, in which the cell ultimately eats itself.

The primary point of discussion is a form of cell death that occurs due to upregulated iron mediation to the cell membrane, ferroptosis. A non-apoptotic form of cell death, ferroptosis occurs when a profuse amount iron collects in the cellular components through transferrin receptor 1. The iron is converted to ferritin. Reactive oxygen species occurs, prompting a strain of the glutathione antioxidant mechanism. This leaves lipid peroxides left free of conversion to lipid alcohols in order to safely pass through the lipid bilayer. The enzymatic reduction of phospholipid hydroperoxides absence or limited quantities progress the rate of the ferroptosis reaction. The rate lipid peroxides destruct the cell membrane, injuring the ferroportin, a

transmembrane protein that transports iron from the inside of a cell to the outside of the cell, and thus leading to cell death.

Many genes are involved in regulating ferroptosis, consisting of some positive regulators and some negative regulators. The positive regulators of ferroptosis include NADPH oxidase (NOX), Transferrin receptor protein 1 (TfR1), Acyl-CoA synthase long-chain family member 4 (ACSL4), Cellular tumor antigen p53 (p53), cysteinyl-tRNA synthase (CARS), Mitogen-activated protein kinase 1 (MAPK), Nuclear receptor coactivator 4 (NCOA4), 15-lipoxygenases (15LO), Phosphatidylethanolamine-binding protein 1 (PEBP1), and Heme oxygenase 1 (Hmox1). The negative regulators of ferroptosis are Glutathione peroxidase 4 (GPX4), Cystine/glutamate antiporter solute carrier family 7 member 11 (SLC7A11), Heat shock protein beta-1 (HSPB1), Nuclear factor erythroid 2-related factor 2 (NRF2), and ferroptosis suppressor protein (FSP1).

1.3.1 GPX4

Endogenous peroxides have been previously found to play a wide variety of roles within atherogenic injury to vascular cells, induction of VSMC proliferation, interaction with inflammatory and endothelial cells, and most relevant to this study, the contribution the peroxides play in the pathogenesis of atherosclerosis (Rubio-Tomás 2021). Peroxides such as hydrogen peroxide and lipid hydroperoxides serve to carry out two very important mechanistic steps: increasing the sensitivity of vascular cells and oxidizing low-density lipoproteins (LDL) and lipid components.

Glutathione peroxidase-4 (GPX4) is a phospholipid hydroperoxide glutathione peroxidase, one of the main antioxidant mediators in the body, that protects against the degradation of cells by oxidants and cytokines. During ferroptosis, GPX4 works to eliminate lipid peroxides and

maintain cellular redox balance. It converts lipid peroxides into lipid alcohols, giving the newly formed molecule a safe pass through the cellular membrane. Without this key component during the cycle, membrane permeability is loss at the cell wall due to unchecked lipid peroxide leading to membrane destruction. Thus, overexpression of GPX4 inhibits the development of atherosclerosis through suppression of lipid peroxidation and impeding on the sensitivity of vascular cells to oxidized lipids. The finding of a decreasing amount of atherosclerotic lesions when GPX4 is present (overexpressed) suggest a promising therapeutic for the disease.

1.3.2 Ferrostatin 1

An inhibitor of ferroptosis, ferrostatin-1 (Ferro-1), is a synthetic compound that functions to inhibit lipid peroxidation without being consumed during its process. Found through screening of small molecule cell assays inducing ferroptosis, ferro-1 was seen to suppress the accumulation of lipid hydroperoxides (Feil 2014). As an arylamine, it does a thorough job in radical trapping antioxidants. From this, it is able to prevent cell death when GPX4 is inhibited.

1.3.3 RSL-3

RAS-selective lethal 3 (RSL3), a ferroptosis activator and inducer, is a synthetic compound that is dependent on the activity of GPX4. In the absence of GPX4, it was found that the ferroptosis-inducing effect of RSL3 was significantly decreased or even lost, as was the inception of cell death. In previous *in vitro* studies, RSL3 was founded to nurture ferroptotic cell death through the promotion of ROS accumulation and increase of lipid ion pool levels. When tested on colorectal cancer cells, transferrin levels were observed in large amounts when treated with RSL3. A decreased expression of GPX4 was also noted within the same cells, indicative of the

onset of iron-dependent cell death. This suggests that RSL3 interferes with the ferroptosis inhibition factor GPX4.

1.3.4 BRD4

Bromodomain-containing protein 4 (BRD4) is an essential epigenetic regulator associated with ferroptosis. Known for its function as a tumor driver, BRD4 has been reported to also be a therapeutic target in cardiovascular disease. BRD4 belongs to a family of proteins comprising of BRD2, BRD3, BRD4, and BRDt. Studies have shown that BRD4 inhibition suppressed erastin-induced ferroptotic cell death. This is elicited by disruption of mitochondrial maintenance and suppression of lipid ROS formation.

1.3.5 JQ-1

JQ1, inhibitor of BRD4 is used to inhibit BET family proteins and block various ferroptosis-resistance proteins, including GPX4 and SLC7A11. In a prior nude mouse model *in vivo* experiment, ferroptosis was induced though JQ1 treatment and BRD4 knockdown. This experiment was an indicator that JQ1 contributed to ferroptosis induction, while BRD4 acted as an inhibitor (Lo Sasso 2016). Additionally, it was found that JQ1 increased iron levels during ferroptosis via ferritinophagy. When treated with JQ1, cells showed downregulation of various ferroptosis-associated genes, including GPX4, SLC7A11, and SLC3A2. In this case, the story ties together that when JQ1 is present, ferroptosis is more susceptible to occur due to the regulation that the inhibitor has on BRD4 by epigenetic repression.

1.4 Research Objectives and Knowledge Gap

The role of Lipid Peroxidation molecules in cardiovascular diseases, namely atherosclerosis, have shown to play various roles in the effects of the condition. In that, a

comprehensive understanding of the interplay of each particular molecule continues to lack in the literature. By providing a background on these relevant players, this study will attest to the crucial roles of the molecules in disease progressions and regressions.

Furthermore, the ability for SMYD2 to mediate oxidative stress in 10T1/2 cells remain unclear. SMYD2-mediated VSMC oxidative stress has never been reported before. This thesis aims to contribute to ongoing explorations of therapeutic strategies for cardiovascular diseases by the activity of various Lipid Peroxidation molecules on the effects of 10T1/2 cells.

2. MATERIALS AND METHODS

To evaluate the effect of ferroptosis on SMYD2 and BRD4, C3H/10T1/2 (10T1/2 cells) mouse embryonic fibroblasts were treated with Ferrostatin-1 (Potent and selective inhibitor of ferroptosis), and 1S,3R-RSL3 (Ferroptosis inducer), PAz-PC (oxLDL), in dose and time dependent manner. Next, to evaluate if SMYD2's mediated ferroptosis is dependent on BRD4, control and Smyd2-depelted 10T1/2 cells were treated with Ferrostatin-1/1S,3R-RSL3 and (+)-JQ1. Lipid hydroperoxides, and Glutathione assay along with Immunofluorescence C11-BODIPY581/591 staining was used to measure oxidative stress in cultured cells. Primary VSMCs isolated from control and SMYD2 depleted mice and cultured C3H/10T1/2 cells were used for in vitro studies.

All animal experiments were conducted in certified animal biosafety lavel-2 (ABSL-2) at Georgia State University (GSU). The protocol was approved by GSU IACUC (Protocol number A21026).

2.1 Staining Techniques

2.1.1 Alizerin Red Staining of Western Diet-Fed Mice and Cultured 10T1/2 Cells

Alizarin Red solution used in this study was prepared within our laboratory. To create this solution, 137 mg of Alizarin Red powder (Sigma-Aldrich, A5533-25G) was carefully dissolved in 10 ml of distilled deionized water (ddH2O), yielding a final concentration of 40 mM. It is crucial to adjust the pH of the solution using 10% ammonium hydroxide, as the pH level significantly influences the staining strength.

Slides, which had been prepared according to the procedures detailed in section 2.2.1 covering formalin-fixed tissue processing and sectioning, were employed for conducting Alizarin Red staining using Sigma-Aldrich's Cat. No. TMS-008-C. To provide a succinct overview of this staining protocol, the following steps were meticulously carried out –

Firstly, the slides were subjected to deparaffinization and hydration in distilled deionized water (ddH2O), as outlined in Table 2.6. Subsequently, the slides were incubated with Alizarin Red solution for a duration of 5-15 minutes, during which the characteristic red- orange stain was observed under a microscope.

Excess dye was gently shaken off, and the slides were then dehydrated successively in acetone, acetone: xylene (1:1), and finally in xylene. Following this, the slides were mounted using toluene, following the specifications listed in table 2.6. The prepared slides were left at room temperature for drying, and images were captured after a period of 24 to 48 hours to facilitate comprehensive analysis.

For the purpose of identifying calcification in cultured cells, a variety of cell types, including 10T1/2 cells, primary cells derived from vascular smooth muscle (VSMC) and

bone marrow-derived macrophages (BMDM), as well as RAW 264.7 cells, were employed in the Alizarin Red staining procedure. These cells were cultivated under different experimental conditions, and upon reaching specified time points, the following staining process was meticulously carried out –

Initially, the cells were washed with PBS three times to ensure thorough removal of any residues. Any remaining PBS was aspirated out to prevent interference with the subsequent steps. Subsequently, NBF (Neutral Buffered Formalin) was added (500ul for 24-well plates, 200 ul for 48-well plates), and the plates were incubated at room temperature for 30 minutes. Following this incubation, the NBF was discarded, adhering to research safety guidelines, and the cells underwent a three-time wash with distilled deionized water (ddH2O). Any trace amounts of ddH2O were also aspirated out to avoid interference with the Alizarin Red solution.

Next, 200 ul of Alizarin Red solution (Sigma-Aldrich, Cat. No. TMS-008-C) was added, and the cells were incubated for 20-30 minutes at room temperature. It's worth noting that the Alizarin Red solution can be reused and should be stored at 4 degrees Celsius for future experiments. The cells were further washed with ddH2O until a bright orange-red color was observable. To eliminate excess water, the plates were inverted, tapped, or placed under a Kim-wipe. Finally, the plates were stored at room temperature, and images were captured using an AmScope Stereo zoom microscope (SM-4TPZ-144-18M3).

2.3.4 Immunofluorescence Staining of *In Vitro* Culture Cells

For the Immunofluorescence (IF) analysis, cells cultivated in their respective culture media were employed. The procedure was conducted as follows –

Initially, the cells were washed thoroughly with PBS three times, after which they were fixed using 4% formalin for 20 minutes at room temperature. Subsequently, the cells were subjected to another round of PBS washing (three times). Following this, the cells were blocked with a solution comprising 5% goat serum in PBSt (0.1% Triton X 100) for 1 hour at room temperature. The coverslips, housing the treated cells, were then carefully removed from the 6-well plate and placed in a humidifier chamber. Within this chamber, they were incubated with the respective primary antibodies (dilution and antibody details provided below) overnight at 4 degrees Celsius. On the following day, the coverslips were taken out and transferred to a 6-well plate, where they underwent three washes with PBSt, each lasting 5 minutes. Subsequently, the coverslips were incubated in secondary antibodies (fluorescent HRP conjugated), prepared in PBSt, for 1 hour in the absence of light. Following this incubation, the cells were washed thrice with PBSt. The coverslips were then subjected to staining with diamidino-2-phenylindole (DAPI) at a 1:10,000 dilution (Thermo ScientificTM 62248, 1 mg/ml) for 5 minutes. Immediately after DAPI staining, the coverslips were washed three times using PBSt and were then mounted using mounting medium (InvitrogenTM ProLongTM Gold Antifade Mounting, Catalog No. P36931).

2.4 Western Blot (Immunoblotting)

To prepare, RIPA buffer was made from an allocated stock solution of NaCl (5 M), 0.5 M EDTA, pH 8.0 (0.5 M), 1 M Tris, pH 8.0 (1 M), NP-40 (IGEPAL CA-630), 10% sodium deoxycholate, 10% SDS. From the stock solution**,** NaCl (3ml, that is 150 mM of final concentration), EDTA, pH 8.0 (1ml, that is 5 mM of final concentration), 1 M Tris, pH 8.0 (5 ml, that is 50 mM of final concentration), NP-40 (IGEPAL CA-630) (1 ml, that is 1 % of

final concentration), 10% sodium deoxycholate (5 ml, that is 0.5% of final concentration), 10% SDS (1 ml, that is 0.1% of final concentration), and remaining water up to 100 ml were added together.

Western blot analysis was performed according to standard procedures using lysates from tissues or cells as described previously. In brief, tissues or cells were solubilized in RIPA or M-PER mammalian protein extraction reagent (78501; Thermo Scientific, Waltham, MA) supplemented with protease inhibitor cocktail (P8340; Sigma). After the protein concentration was measured using the Bradford method, the same amounts of total proteins for each sample or treatment were separated by sodium dodecyl sulfate– polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (#1620177; Bio-Rad, Hercules, CA). The membranes were blocked with 5% goat serum at room temperature for 1 hour before the addition of primary antibodies (table 2.8) for overnight incubation at 4°C. The following day, the membranes were incubated with secondary antibodies for 1 hour at room temperature. The enhanced chemiluminescent kit was used for development, and the images were captured using the Amersham Imager 600 RGB system (GE Health Care, Chicago, IL).

2.5 Co-Immunoprecipitation (CO-Op)

Immunoprecipitation assay was used for detecting the methylation status of p65. Briefly, C3H/10T1/2, (Clone 8 - CCL-226) cells with or without *Smyd2* were treated with Pi or Bay 598 (*Smyd2* inhibitor). Forty-eight hours after treatments, cells were lysed with RIPA/ M-PER mammalian protein extraction reagent supplemented with protease inhibitor cocktail, and the cleared lysates were incubated with specific antibodies (table 2.8), and magnetic beads (M8823; Sigma) at 4°C overnight. After washing, the bound

proteins were eluted by boiling in Laemmli sample buffer. Immunoblot analysis was conducted to detect the mono-methylated lysine (16479; Cell Signaling Technology), dimethylated lysine (14117; Cell Signaling Technology), and trimethylated lysine (14680; Cell Signaling Technology), as described earlier.

2.6 Gene Expression Analysis (PCR)

Freshly collected aortic tissue and in-vitro cultured primary cells were subjected to RNA isolation using Qiagen Kit (RNeasy Mini Kit, Cat. No. 74104). Briefly, collected aortic tissue (section between the aortic root containing Brachiocephalic artery (BCA), Thoracic aorta) were subjected to mixture of RLT and 2-Mercaptoethanol (also β-mercaptoethanol, BME, 2BME, 2-ME or β-met) (BME). Final elution was performed using Nuclease free water, and total RNA content was quantified using Nanodrop (NanoDrop 2000c, Thermo Fisher). cDNA library was prepared using 2 ug of total RNA following company recommendation (iScriptTM cDNA Synthesis Kit, Biorad, Cat #1708890 or Denville cDNA Synthesis Master Mix, Denville, Cat # 1005984). In brief, 2ug of Total RNA was diluted with nuclease free water upto 15 µl, followed by adding 4 µl and 1 µl of 5x iScript Reaction Mix and iScript Reverse Transcriptase respectively. Incubate the mixture in thermocycler to undergo multiple steps Priming (5 min at 25^{o}C), Reverse transcription (20 min at 46oC), RT inactivation (1 min at 95oC) and hold at 4oC. Synthesis cDNA was diluted to 3.33 ng/µl using nuclease free water, and Real-time PCR (Quant Studio, Fast 7500) was performed on either Aortic tissue, or VSMCs primary cells and RAW cells using SYBR-green reagent (5X, Fast SYBRTM Green Master Mix, Thermo Fisher Scientific Inc. Cat # A25742.

Expression of calcification associated transcription factor, mineralization inhibitor, mineralization facilitator, ferroptosis signaling genes, inflammatory/anti-inflammatory markers and Notch pathways genes were evaluated and normalized with cyclophilin (*Ppia*), and GAPDH. Table 2.9 Primers used for gene expression studies.

3. RESULTS

SMYD proteins have multidimensional functions and roles within biological science as we are learning more about today. Research remains limited on the roles and effects that SMYD2 has on lipid peroxidation and oxidative stress. The basis of my thesis research delved to tie loose ends on the abilities of SMYD2 to control atherosclerotic plaque formation by modulating oxidative stress through the GPX4/NRF2/HO-1 axis.

3.1 Hmox1 and GPX4 are Upregulated in VSMCs from SMYD2$^{\Delta SMC}$ Mice

Ferrostatin-1, RSL-3, and JQ-1 compounds have been reported to regulate the ferroptosis pathway. Analysis of our bulk-RNA sequencing data in VSMCs obtained from control and SMYD2 depleted mice indicate significant changes of ferroptosis associated genes (TRF, Hmox1, Acsl3, Cybb NOX2, Acsl4, and GPX4) in primary VSMCs. Fragments Per Kilobase of transcript per Million mapped reads (FPKM) declaimed upregulations of ferroptosis associated genes including Trf, Hmox1, Acsl3, Cyb (NOX2), Acsl4, and GPX4 in VSMCs with SMYD2 deletion. Of note, a few transcripts were shown to be significantly reduced, including Slc40a1 and Acsl6.

3.2 Immunofluorescence C11-BODIPY$^{581/591}$ Staining

An immunofluorescence assay was performed for the confirmation of oxidative stress presence in erastin and ferrostatin-1 treated control and SMYD2-KO cells. We chose this particular staining to check if SMYD2 mediated lipid peroxidation oxidative stress in 10T1/2 cells.

Since erastin and ferrostatin-1 have been shown to modulate ferroptosis, this staining was of importance to view lipid peroxidation levels present in the cells. The results showed decreased lipid peroxidation presence in SMYD2-KO cells that were induced by erastin treatment.

3.3 C3H10T1/2 Cell Cultures of Primary VSMC Isolated Control and SMYD2 Depleted Mice

Preliminary findings of an FPKM that compares gene expression in WT and KO mice were obtained. Shown in this graph was the increased gene expressions of Hmox1 and GPX4 in VSMCs isolated from SMYD2-KO mice.

In WT control cells, western blots were performed to view how control cells would react when treated with ferrostatin-1 and RSL-3. Ferrostatin-1 is one of the first synthetic radical-trapping antioxidants reported to block ferroptosis. RSL-3 is a pharmalogical inhibitor of the protein coding gene for GPX4. The expectations were that when treated with RSL-3, an elicited decrease in GPX4 expression would be present. When the cells were treated with ferrostatin-1, we presumed a decrease in GPX4 expression would present itself. Although the study was confirmed with notable depletion of GPX4 expression when treated with RSL-3, a notable change was unable to be identified in ferrostatin-1 treated cells in our studies, though we speculate that repeated experiments may have resulted in more positive confirmations.

Further on, GPX4 being the rate limiting enzyme of lipid peroxidation, we evaluated the expression of GPX4 in control and SMYD2 depleted cells. Results from qPCR analysis indicated that modulation of 10T cells with ferrostatin-1, RSL-3, or JQ-1 suggests. Conclusions gathered showed the immense importance of the presence of SMYD2 in order to evaluate notable results in any of the selected genes being used to provoke GPX4 expression.

This led to further studies conducting the various expressions of SMYD2, GPX4, and HO-1 in SMYD2 WT and SMYD2-KO cells when treated with RSL-3, ferro-1, and JQ-1. When cells are treated with RSL-3, there is a significant downregulation of GPX4 expression. Although literature findings suggest upregulation of GPX4 in the presence of Ferrostatin-1 treatment, no noteworthy findings were conveyed in this study. Some discrepancies in my collected data arose when treating cells with RSL-3 and ferro-1 to elicit sound responses to HO-1. Lastly, JQ-1 treatment showed a suppression of GPX4 expression in cells. This, in turn, would create a promoting effect on ferroptosis, and contribute to cell death.

3.4 *In Vitro* Insights of SMYD2 Mediation of C3H/10T1/2 Cells

Primary VSMCs isolated from control and knockout SMYD2 mice and 10T ½ cells (C3H10T1/2) were treated with and without cholesterol PAz-PC. PAz-PC is a predominant oxLDL species that has been shown of being an important structural determinant of oxLDL levels. My focus was to use an appropriate concentration of inorganic phosphate (Pi) for further experimentation. Standard pathophysiological concentration of Pi is approximately 1.3 mM or 1.3 mmol/L. A concentration dependent experiment was used to identify ideal concentration required for the cells.

Primary VSMCs that were isolated of control and SMYD2 knockout mice were then grown at 70-80% confluency, given inorganic phosphate (Pi) treatment (1, 2.5, and 5 mmol/L) in serum free medium (SFM = Dulbecco's Modified Eagle Medium + 0.5% fetal bovine serum + 1% P/S) for 5-7 days. Media were changed every 3 days. AR-staining of the primary cells depicted that the inorganic phosphate treatment significantly increased calcification of VSMCs. On the other hand,

the staining revealed SMYD2 depleted VSMCs lacked AR^{+ve} positive staining. Experiments were performed in 10T1/2 cells with both AR and VK staining for confirmation.

In order to confirm *in vitro* data, *ex vivo* aortic ring studies were completed using the aortic tissues obtained from control and SMYD2 knockout mice. Tissue rings (1 mm) were cut and incubated in inorganic phosphate for 14 days. Estimation of Ca2+ measures was obtained in the aortic ring tissues. When control and Pi-treated aortic tissues were compared to SMYD2 depleted aortic tissue, a significant accumulation of Ca2+ was found in the treated tissue versus a noteworthy reduction of Ca2+ in the SMYD2 depleted tissue. This suggested that SMYD2 in VSMCs participate in vascular calcification.

Atherosclerosis is fundamentally correlated with increased levels of cholesterol in circulation. Using an optimum concentration of inorganic phosphate, cholesterol was added in dosage dependent manner (5, 15, 25 ug/ml). Results showed that the combination of cholesterol and increased inorganic phosphate promote calcification in control cells compared to SMYD2 knockout cells. These findings suggests that SMYD2 promotes VSMC calcification. Additionally, the depletion of SMYD2 may protect against severity of calcifications. Findings of our experiments are in agreeance with previously established literature norms that inorganic phosphate can induce significant calcification in VSMCs.

3.5 SMYD2 Deficiency Mitigates Atherosclerotic Plaque Formation by Modulating Oxidative Stress

Accumulated oxidized lipids in arterial walls drastically play a role in atherosclerosis and is modulated by GPX4, a hydroperoxide scavenger. This molecule works to effectively remove oxidative lipids such as fatty free acids, cholesterols, and phospholipids. Through two key

mechanisms, endogenous peroxides are involved in atherosclerosis. Hydrogen peroxide (H_2O_2) and lipid hydroperoxides function to induce oxidative changes in lipoproteins and LDL and increase the sensitivity of vascular cells to oxidized LDL and lipid components. Hydrogen peroxides released from vascular cells are able to oxidize LDL *in vitro*. Moreover, recent studies have demonstrated the reduction of atherosclerotic lesions and oxidized lipid levels in ApoE-/- mice upon overexpressing a hydrogen peroxide scavenger, catalase. This proposes a hopeful treatment strategy for atherosclerosis.

We aimed to explore if SMYD2 deficiency can mitigate the severity of atherosclerotic plaque formation by modulating oxidative stress through the GPX4/NRF2/HO-1 axis. To begin this assessment, we firstly reanalyzed the bulk RNA-seq data obtained from our previous study comparing SMYD2 flox to SMYD2 depleted VSMCs. What was found was that fragment per kilobase of transcript per million mapped reads (FPKM) were notably upregulated for ferroptosis associated genes, including Trf, Hmox1 (HO-1), Acsl3, Cybb (NOX2), ACSL4, and GPX4, in SMYD2 depleted VSMCs. On the other hand, there were two genes that showed to be significantly downregulated, including Slc40a1 and Acsl6.

For further confirmation, an immunofluorescence assay was performed on gene expression. Compared to control mice, SMYD2$^{\Delta SMC}$ ApoE$^{-/-}$ knockout mice displayed a prominent increase in expression of NOX2, GPX4, NRF2, and HO-1 in VSMCs. The increase in expression was primarily located in the medical layer of the vasculature.

To add on, a Thiobarbituric Acid Reactive Substances (TBARS) assay was performed to screen for lipid peroxidation. Higher TBARS levels are indicative of lipids with greater unsaturation. Measurements were monitored by Malondialdehyde (MDA), one of the final

products of polyunsaturated fatty acids peroxidation in the cells. Mice were fed with Western Diet for 26 weeks, and TBARS assay showed that the SMYD2 depleted plasma and tissues demonstrated decrease in MDA concentration.

Western blot analysis was performed on aortic tissue lysates that were taken from mice fed a Western Diet (WD). In this approach, results confirmed increased expression of GPX4 upon SMYD2 deletion. A notable increase in HO-1 expression was also observed. This protein catalyzes the breakdown of heme into biliverdin, carbon monoxide, and free iron, which each have key functions in exerting potent anti-oxidative stress and anti-inflammatory roles in atherosclerosis. Our results denote that loss of SMYD2 considerably increased the expressions of both GPX4 and HO-1 in aortic tissue, indicating a protection against the progression of atherosclerosis.

In another approach*, in vitro* studies using C3H/10T1/2 signified that SMYD2 deficiency protect against oxidative stress through reduced ER stress or increased expression of HO-1 and GPX4. Thapsigargin (TG, 0.25 μM/4hr) was used to induce ER stress in WT and SMYD2 depleted C3H/10T1/2 cells, followed by examination of the expression of IRE1α by western-blot. As anticipated, TG greatly induces expression of IRE1α (induction of ER stress) in control cells, compared to SMYD2 KO 10T1/2 cells . This finding was also corroborated by genetic manipulation of SMYD2 (via loss/gain of SMYD2 function) in WT VSMCs. Interestingly, ER stress inducer (TG) activated IRE1α, but did not alter GPX4 and HO-1 expression. Moreover, the SMYD2 KO 10T1/2 cells also showed significant increases in HO-1 expression at basal level (in the absence of TG).

Further, we evaluated ferroptosis mediated oxidative ER stress, finding that it aligned with our bulk RNA-seq findings. WT cells were treated with ferroptosis inducer/inhibitor molecules, including ferrostatin-1 (2 uM/24 hr), RSL-3 (0.8 mM/24 hr), and a modified form of

oxLDL (Paz-PC, 15 ug/48 hr). The results showed that ferrostatin-1 and ox-LDL notably upregulated IRE1α expression. On the other hand, RSL-3 did not show expression changes. In addition, a significant reduction in IRE1α levels with increased expression of HO-1 and GPX4 were observed in SMYD2 knockout group compared to control group. Additional proteins were evaluated, but they were beyond the scope of this thesis. NRF2, a master transcription factor known to mitigate oxidative stress in cellular response and interaction with HO-1, help neutralize oxidative stress. A co-immunoprecipitation assay was performed to examine if SMYD2 or its methyltransferase activity participate in NRF2 interaction. Interestingly, pharmacological inhibition of SMYD2 methyltransferase activity increased the expression of NRF2. On the other hand, expression of HO-1 was reduced, suggesting that methyltransferase activity is crucial for the interaction of HO-1 and NRF2. In all, the genetic inhibition of SMYD2 increased NRF2 (in-vivo) and HO-1 (in-vitro and in-vivo). The loss of SMYD2 function may activate a non-canonical pathway to mediate oxidative stress. It's worthy to note that SMYD2 shows context and cell specific function as shown in our previous and current work. Depletion of SMYD2 in VSMCs results in an increase in ferroptosis related genes.

4. CONCLUSION

Atherosclerosis is a chronic progressive inflammatory disease that is onset by multiple factors including OxLDL, cholesterol, diabetes, smoking, etc. Atherosclerosis involves multiple cell types such as endothelial cells, VSMCs, fibroblast and immune cells (i.e., macrophages, lymphocytes). Signaling and synchronization of these cell types are key factors in vascular pathogenesis. Important risk factors include smoking, hypertension, obesity, diabetes, hyperlipidemia, family history, coagulation disorders, and regions of turbulent flow. For the

current study, I aimed to study the novel functions of SMYD2 in vascular diseases and cell death. Specifically, my studies revolved around atherosclerosis and ferroptosis using two different mice models (control and SMYD2 KO mice) targeting two very important cell types i.e., VSMCs and macrophages, which play vital roles in atherosclerosis.

The onset of atherosclerosis, a chronic inflammatory ailment, is instigated by the accumulation of lipids within the arterial wall, facilitated by the presence of circulating lipoprotein particles, macrophages, and cholesterol, this interplay results in atherosclerotic plaque formation within the intimal layer of the arteries. Notably, owing to an advantage over other disease models, $ApoE^{-/-}$ mice display all characteristic features of atherosclerosis including early establishment of fatty streaks, distinctive lesion distribution its progression and maturation results in atherosclerotic plaque. Inclusion of Western-type diet significantly expedites the progression of lesions across all stages, spanning from the formation of foam cell lesions to the eventual development of fibrous plaques. Intriguingly, lesions in mice fed the Western-type diet are characterized by a higher lipid content compared to those observed in mice on a chow diet.

Owing to the advantage of screening different stages of atherosclerosis we developed Vascular Smooth Muscle cells specific SMYD2 (VSMC-SMYD2) knockout mice on ApoE-/- background (Inhouse breeding). Knockout of SMYD2 were confirmed using western blot and qPCR before initiating the experiments. As this is the first of its kind of study, we collected all physiological parameters, and measured various parameters. In parallel we also performed re-analysis of GEO datasets. $Ldlr^{-/-}$ mice dataset from GSE24342 indicates that SMYD family proteins are ubiquitously distributed in aortic or macrophages. Compared to macrophages, aorta express

higher level of SMYD, however, results also warrant further investigation if SMYD protein expression pattern in EC, VSMC and adventitia cells resembles that of total aortic SMYD expression, and how SMYD contributes to severity of atherosclerosis. Increased expression of *SMYD2* in aortic tissue further corelates with increased severity of atheroma formation, in human tissue samples. Further, analysis of GSE41571 indicate that SMYD2 is present in both stable and unstable plaque with no significance among the groups. Furthermore, GEO2R results were confirmed by Immunohistochemistry (IHC) staining of human tissue samples, concluding that SMYD2 expression increase in human atherosclerotic plaque. Because of the cell specific variation in SMYD2 function in atherosclerosis, additional results are discussed below.

4.1 SMYD2 Modulates Oxidative Stress Lipid Peroxidation in C3H/10T1/2 Cells Through GPX4

Atherosclerosis is characterized by endothelial dysfunction, fibrocellular plaque, ulceration, and calcification. SMYD2 is known to regulate pathophysiological processes in an organism, including that of being a therapeutic target against atherosclerosis. Ferroptosis is a form of cell death that is dependent on the works of GPX4. This major lipid repair enzyme plays a critical role in the conversion of lipid peroxides to lipid alcohols. In agreement with the notion that GPX4 is downregulated in atherosclerosis, we evaluated if *Smyd2* can have a regulatory role in GPX4 function. Through in vitro studies, a conclusion was drawn, in that the suppression of SMYD2 upregulated the GPX4/BRD4 axis. Although the study was confirmed with notable depletion of GPX4 expression when treated with RSL-3, a notable change was unable to identified in ferrostatin-1 during our studies, though we are confident that repeated experiments may have resulted in more positive confirmations. In turn, this protects against oxidative stress and reduces the onset of ferroptosis.

Indeed, our bulk RNA-seq data indicated that VSMC specific Smyd2 in deletion resulted in significant increase in Gpx4, Trf, Hmox1 (HO-1), and Ascl4.However, when depleted in VSMC on top of global ApoE knockout mice (SMYD2$^{\Delta SMC}$ ApoE$^{-/-}$), there was significant reduction in Hmox1, and Nox2, while expression of Gpx4, Slc40a1 and Acsl4 did not change, as was in single knockout mice tissues. Interestingly, western blot analysis and immunofluorescence staining of WD fed aortic tissue (26W) showed an opposite trend marked by increased expression of GPX4, HO1, NOX2 and NRF2 in SMYD2$^{\Delta SMC}$ ApoE$^{-/-}$ mice. Subsequently, tissue and plasma analysis of control and SMYD2$^{\Delta SMC}$ ApoE$^{-/-}$ mice showed significant increase in antioxidant MDA to protect against oxidative stress in SMYD2$^{\Delta SMC}$ ApoE$^{-/-}$ mice. Immunofluorescence staining indicates that increased expression of NOX2/NRF2 and HO1 is primarily localized in the vasculature. Collectively, these results agree that secreted antioxidant enzymes such as GPX4, from VSMCs may confer protection against vascular pathobiology and severity of atherosclerosis.

Table 1.1: Steps of Alizerin red (Calcium Stain) Staining of Aortic Tissues Obtained From Western Diet-Fed Mice

Steps	Reagent	Time
1	Xylene - I	5 minutes
2	Xylene - II	5 minutes
3	Xylene - III	5 minutes
4	100% alcohol - I	5 minutes
5	100% alcohol - II	5 minutes
6	95% alcohol	5 minutes
7	80% alcohol	5 minutes
8	70% alcohol	5 minutes
9	ddH2O	5 minutes
10	Alizarin red solution	5 minutes
11	Shake off excess dye	5 minutes
12	Acetone – I	5 minutes
13	Acetone – II	5 minutes
14	Acetone: Xylene	5 minutes
15	Xylene – I	5 minutes
16	Xylene – II	5 minutes
17	Xylene - III	5 minutes
18	Mounting	

Table 2.2: List of reagents used Immunofluorescence

Secondary antibody	Catalogue Number	Company	Dilution
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 555	A-21422	Invitrogen	1:400
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 555	A-11035	Invitrogen	1:400
Goat anti-Mouse IgG (H+L), SuperclonalTM Recombinant Secondary Antibody, Alexa Fluor™ 488	A28175	Invitrogen	1:400
Goat Anti-Rabbit Igg (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	A28175	Invitrogen	1:400

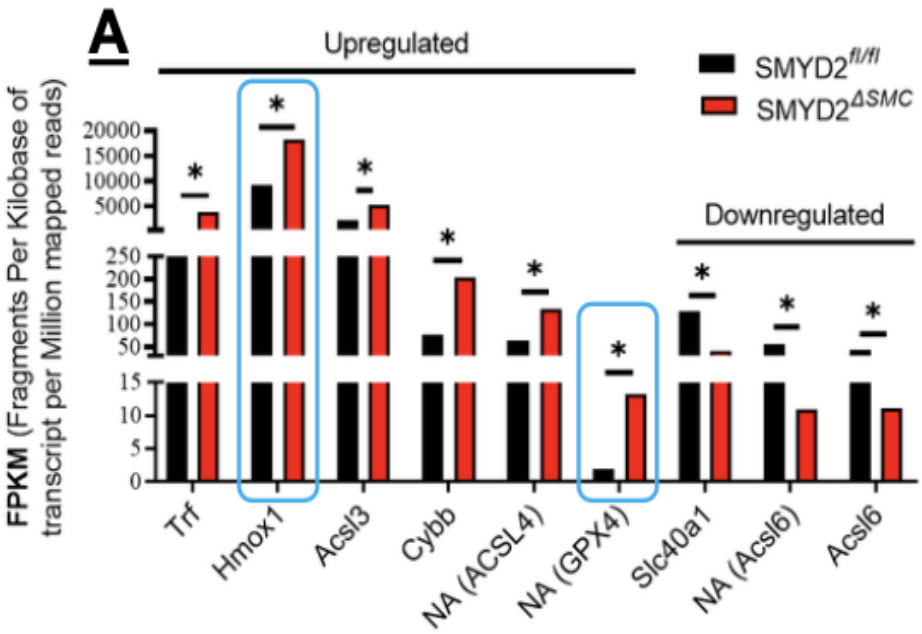

Figure 1.1: Gene expression is functionally active and upregulated in Hmox1 and GPX4 SMYD2-KO mice.
A) mRNA analysis of oxidative stress and lipid peroxidation associated genes in control and SMYD2 depleted VSMCs isolated from mice.

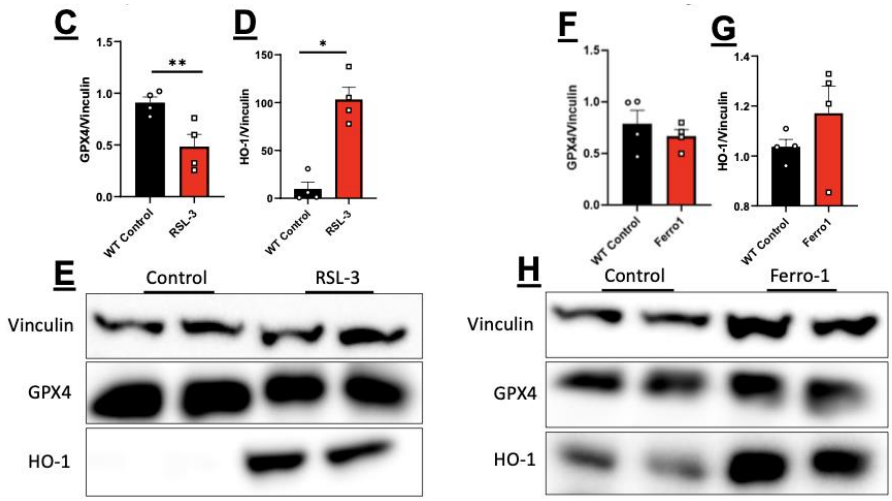

Figure 2.2: RSL-3 decreases GPX4 expression, while significantly increases HO-1 expression. No significant difference in expression of GPX4 or HO-1 upon Ferrostatin-1 treatment.
Note. C) GPX4 expression in control and RSL-3 treated cells D) HO-1 expression in control and RSL-3 treated cells E) Western blots of GPX4 and HO-1 in WT and SMYD2-deficient C3H/10T/1/2 cells treated with RSL-3 F) GPX4 expression in control and Ferrostatin-1 treated cells G) HO-1 expression in control and Ferrostatin-1 treated cells H) Western blots of GPX4 and HO-1 in WT and SMYD2-deficient C3H/10T/1/2 cells treated with Ferrostatin-1

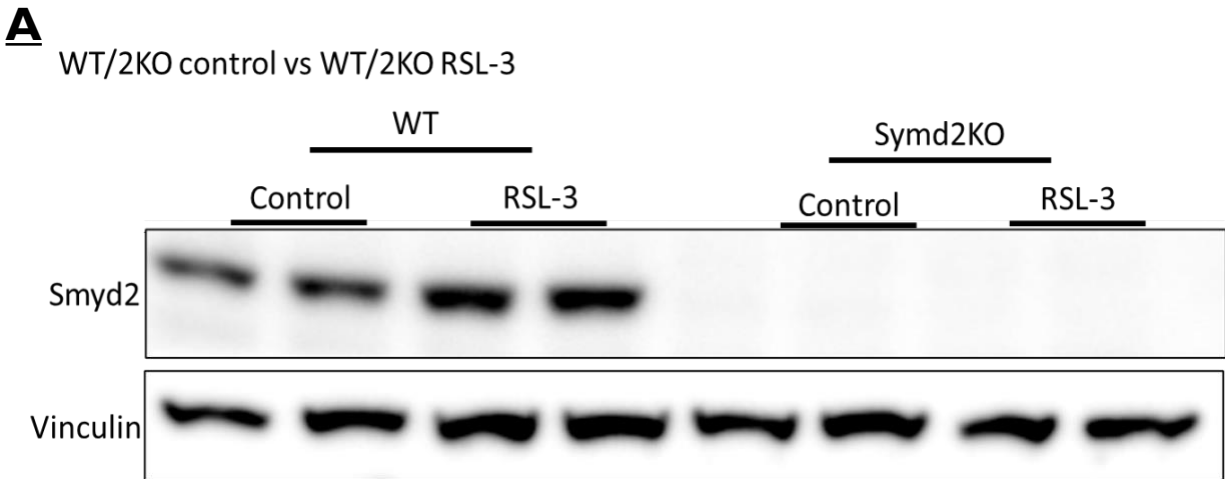

Figure 3.3: SMYD2 mediates protein expression.
A) Western blot analysis of SMYD2 expression in control and SMYD2 depleted cells treated with RSL-3 or control (vehicle-treated).

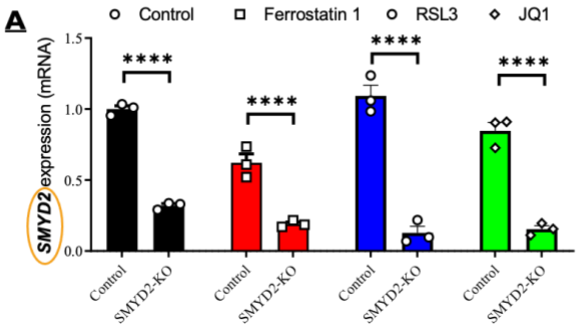

Figure 4.4: SMYD2 expression
A) qPCR graph of SMYD2 expression in response to various treatments.

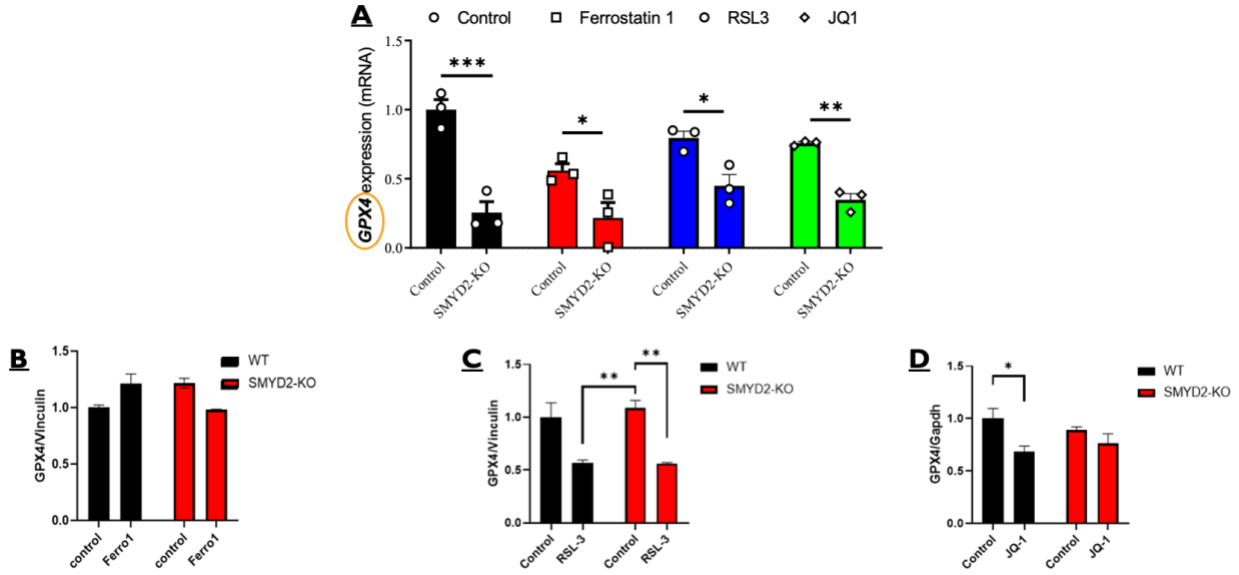

Figure 5.5: GPX4 expression

A) qPCR graph of GPX4 expression in response to various treatments. B) Western blot analysis of GPX4 expression when control and SMYD2 depleted cells are treated with ferrostatin-1 C) Western blot analysis of GPX4 expression when control and SMYD2 depleted cells are treated with RSL-3 D) Western blot analysis of GPX4 expression when control and SMYD2 depleted cells are treated with JQ-1

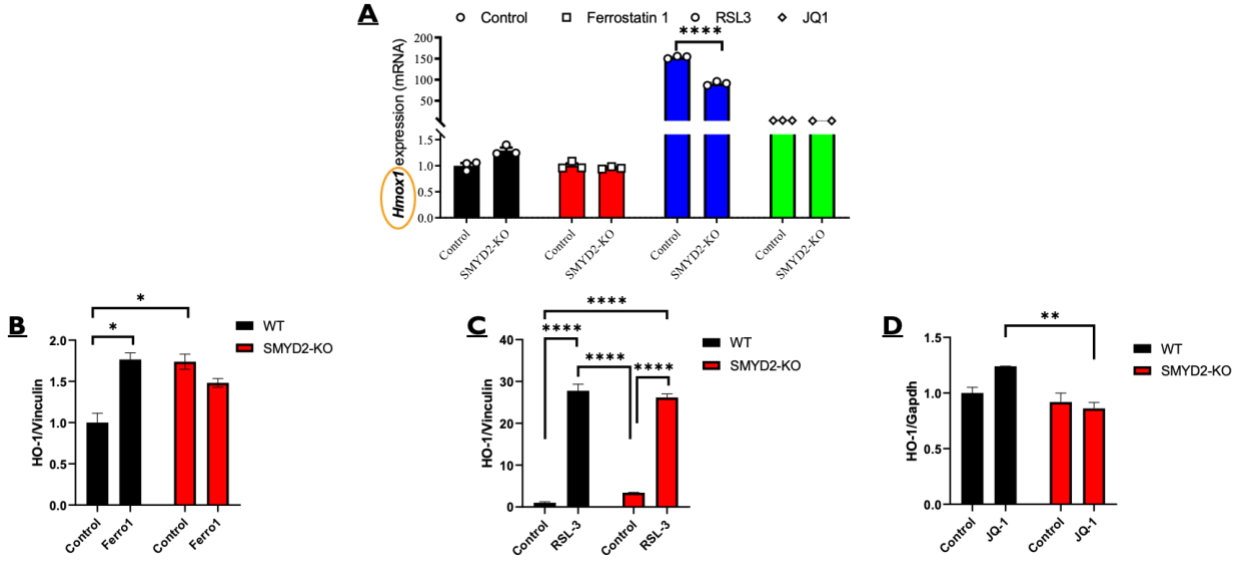

Figure 6.6: Hmox1 expression

A) qPCR graph of HO-1 expression in response to various treatments. B) Western blot analysis of HO-1 expression when control and SMYD2 depleted cells are treated with ferrostatin-1 C) Western blot analysis of HO-1 expression when control and SMYD2 depleted cells are treated with RSL-3 D) Western blot analysis of HO-1 expression when control and SMYD2 depleted cells are treated with JQ-1

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APPENDICES

Appendix A: Achievements:

"SMYD2 Modulates Lipid Peroxidation in C3H/10T1/2 Cells Through GPX4" accepted and presented as poster at ABRCMS 2023 in Phoenix, AZ on November 15-18, 2023

VITAE

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Poster Presentations:
Shaligram Sharma, Xiaoqing Guan, Yuning Hou, **Jada Harvey**, Pranav Lamba, Jacob Li, Arin Dorsey, Kotryna Diktonaite, Chunying Li. SMYD2 Deficiency Protects Against Atherosclerotic Plaque Formation through Modulation of Oxidative Stress. ***BCVS 2023***. Boston, MA. July 31–Aug 3, 2023

Jada Harvey, Nadia Ilse, Shaligram Sharma, Chunying Li. SMYD2 Modulates Lipid Peroxidation in C3H/10T1/2 Cells Through GPX4. **ABRCMS 2023.** Phoenix, AZ. November 13-18, 2023