CAVEOLIN-1 MEDIATED P53 ACTIVATION IN STRESS INDUCED PREMATURE SENESCENCE AND ITS ANTAGONISTIC PLEIOTROPIC IMPLICATIONS IN CANCER

by

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Caveolin-1 (Cav-1) is a membrane associated scaffolding protein that regulates a myriad of signaling molecules. It has been implicated as both a tumor suppressor and promoter. Here, we examine the protein's link to senescence and cancer, and identify a novel pathway through which Cav-1 mediates stress induced premature senescence (SIPS) through p53 activation. Oxidative stress triggers $p38^{MAPK}$, which activates the transcription factor Sp1. Sp1 binds to two GC-rich regions in the caveolin-1 promoter up-regulating the protein. Cav-1 binds to p53's negative regulator, MDM2, sequestering the E3 ligase to allow p53 to become active. p53 activates its downstream targets, such as $p21^{WAF/CIP1}$, which initiates SIPS. This pathway is dysfunctional in many cancers that have a downregulated Cav-1 gene. The effects of oxidative stress in Cav-1 null backgrounds were examined. Breast cancer cells that do not express Cav-1 cannot undergo oxidatively induced SIPS. However, upon re-expression of Cav-1, the SIPS phenotype is restored. Utilization of Cav-1 knockout mouse embryonic fibroblasts show that without Cav-1 to sequester MDM2, allowing for the upregulation of p53 leading to SIPS, cells continued to proliferate. These results distinguish Cav-1 as a molecular "senescence switch," because in its absence oxidative SIPS does not occur, but in its presence it does. This effect is also not specific to a particular cell type; data supports Cav-1 as a molecular switch in epithelial and fibroblast cell lines. Finally, senescence is known to have antagonistic pleiotropic effects on an organism. That is, cell senescence is beneficial for younger organisms, as it prevents the

proliferation of mutated genomes through growth arrest. However, an accumulation of senescent cells can lead to aging and become detrimental. Cav-1's role in the antagonistic pleiotropic effects of senescent fibroblasts on neoplastic epithelial cells is also explored. Data shows that senescence of fibroblasts depends upon Cav-1 sequestering MDM2, which activates p53 and induces SIPS. These fibroblasts can secrete factors that make it advantageous for NIH 3T3 Ras^{G12V} transformed fibroblasts and MDA-MB-231 breast cancer epithelial cells to proliferate *in vitro* and *in vivo*. Hence, we propose that the Cav-1 gene functions with antagonistic pleiotropy.

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PREFACE

This dissertation is dedicated to four very special people - my grandparents. Each impacts my life in wonderful ways and they have been such extraordinary examples of how to live one's life. The greatest gift they have given me is their time. I would like to thank them for not only teaching me priceless life lessons but also for living them out in front of me. They have each taught me something to bring me to where I am today.

For as long as I can remember, Grandma B has always urged her grandchildren to value their education and reputation - because the two are with you for a life time. She always tells us of the significance of family and the importance of cherishing them on good days and bad.

If anyone has shown me what it means to be patient and slow to anger it is Papa B. He has demonstrated to me what it means to have a work ethic – not only to finish a project but to do it well.

Grandma Murtaugh is generous to all of those around her. She has taught me to see past my own problems and has shown me what it means to be a true friend to others. She reminds me that there is always something worth laughing and smiling about in life.

No one has inspired me to use my imagination, dream big and never fear a new adventure more than Papa Joe. He always says to see the best in people. He has taught me to attend to how I treat those around me and to look for the person who might just need a smile.

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1.0 INTRODUCTION

In the United States of America and in most developed countries, the population is aging. The United States census of 2000 shows the 50-54 year age group has experienced the largest percentage of growth out of all of the 5-year age groups, followed by the 45-49 year old group (www.census.gov). These two groups consist of the "baby boomers," or those born following World War II. The aging population is a major concern of the healthcare system as aging has many social, psychological and physiological ramifications. Of relevance to this dissertation, is aging is linked to cancer; sixty percent of all cancers and 70% of mortalities related to the disease occur in those 65 years and older (Yancik and Ries 2000).

Aging and cancer are by definition opposites. Aging is the cumulative effect of a decrease in proliferation of cells whereas cancer is the abnormal increase in proliferation of cells. Likewise, these two conditions share many of the same signaling pathways. I am interested in studying signaling molecules aging and cancer have in common, observing how their regulation is controlled to gain insight into the mechanisms of how aging and cancer relate. This dissertation focuses on the Cav-1 protein and how it regulates aging at the cellular level, defined as senescence. Cav-1 is a protein that is related to both senescence and cancer and is therefore a critical link with potential for therapeutic purposes.

1.1 SENESCENCE

In the 1960's Leonard Hayflick discovered cells in culture were not immortal, but would stop dividing after a certain number of cell divisions (Hayflick 1965). This started to shed light on the mechanisms of the aging process. It was determined this replicative limit was partially due to DNA damage and telomere shorting. Extrapolation to the organismal level predicts limited cell division would lead to a non-renewal of tissues and thus aging. 'Senescence' is a term derived from the Latin *senex* meaning to advance in age. It is broadly used to describe the cellular growth arrest, which Hayflick first described. Cellular senescence is triggered through intrinsic (e.g. telomere shorting from replication, and metabolic reactive oxygen species (ROS) production) and extrinsic (e.g. chemical toxicity or irradiation) stimuli. Cellular senescence is characterized by changes towards larger and flatter cell morphology, cessation of DNA replication, positive staining for senescence associated β-galactosidase (Cho, Ryu et al. 2003) and expression of certain cellular proteins such as $p16^{INK4a}$, p53 and $p21^{WAF/CIP1}$, which lead to growth arrest (Collado and Serrano 2006).

Despite current dogma, there is still a debate as to whether senescence was an *in vitro* phenomena or whether it played a role in organismal aging. As reviewed by Chen et al. (Chen, Hales et al. 2007), many recent findings show a relationship between cellular senescence and aging. For instance, an animal's known longevity correlates with the *in vitro* replicative potential of cells derived from it (Stanley, Pye et al. 1975; Rohme 1981). Additional evidence over the past 30 years demonstrated increased replicative senescence *in vitro* from cells taken from patients with Werner syndrome or other prematurely aging donors (Martin, Sprague et al. 1970; Martin 1982; Fujiwara, Kano et al. 1985; Davis, Wyllie et al. 2007). *In vivo,* there is also an increase of senescent cells with age as noted in primate skin (Herbig, Ferreira et al. 2006;

Jeyapalan, Ferreira et al. 2007), human vascular tissue (Minamino, Miyauchi et al. 2004; Matthews, Gorenne et al. 2006; Minamino and Komuro 2007), and rodent and human kidneys (Melk, Kittikowit et al. 2003; Krishnamurthy, Torrice et al. 2004; Melk, Schmidt et al. 2004).

Recent data gives evidence of *in vivo* SIPS. For instance, nephropathies, postoperative stress, and inflammation cause premature senescence of renal cells in the kidney (Famulski and Halloran 2005). Additionally, inflammation of chronic wounds causes SIPS in fibroblasts (Harding, Moore et al. 2005), and Laser-Assisted *In Situ* Keratomileusis (LASIK) causes in vivo SIPS of the corneal keratocytes (Dawson, O'Brien et al. 2006). SIPS is triggered when oxidative stresses cause DNA damage, or when there is oncogenic activation, as with Ras^{G12V}, leading to too much proliferation in primary cell lines (Sebastian, Malik et al. 2005). Common sources of oxidative stresses include hydrogen peroxide, UV exposure, bleomycin, tert-butyl-hydroperoxide, ethanol, psoralen plus UVA or hyperoxia (Rodemann, Bayreuther et al. 1989; Toussaint, Houbion et al. 1992; Chen and Ames 1994; von Zglinicki, Saretzki et al. 1995; Robles and Adami 1998; Toussaint, Medrano et al. 2000; Frippiat, Chen et al. 2001; Dumont, Chainiaux et al. 2002; Borlon, Debacq-Chainiaux et al. 2007). The hyroxyl radical ($^{\bullet}OH$) generated from the reaction of hydrogen peroxide with transition metals induces DNA damage such as single stranded breaks (SSBs) (McDonald, Pan et al. 1993; Lee, Madden et al. 1996; Chen, Nishida et al. 2003) and double strand breaks (DSBs) (Iliakis, Pantelias et al. 1992; Dahm-Daphi, Sass et al. 2000). DNA breaks stimulate DNA repair mechanisms involving the p53 protein, which can activate SIPS.

SIPS is triggered by cell signaling pathways that stimulate cell cycle arrest. Normally, SIPS is initiated by an exogenous DNA stressor, which causes SSBs or DSBs in the DNA. DNA repair proteins such as poly ADP-ribose polymerase-1 (PARP1) recognize these breaks. PARP-

1 is a nuclear protein that post-translationally modifies histones and DNA repair proteins resulting in chromatin relaxation and the binding of other proteins (D'Amours, Desnoyers et al. 1999; Kim, Zhang et al. 2005; Beneke and Burkle 2007). With assistance from Rad17, a DNA damage signal is propagated by the 9-1-1 signal or Rad9-Rad1-Hus1. This heterotrimeric complex is similar to proliferating cell nuclear antigen (PCNA) which binds to damaged DNA (Kostrub, Knudsen et al. 1998; Venclovas and Thelen 2000; Burtelow, Roos-Mattjus et al. 2001; Shiomi, Shinozaki et al. 2002). In conjunction, the serine/threonine protein kinase Ataxia Telangiectasia Mutated (ATM) is activated and phosphorylates proteins, regulating cell cycle arrest. ATM autophosphorylates, resulting in dissociation from its inactive homodimer complexes (Bakkenist and Kastan 2003) and is free to phosphorylate G1 checkpoint proteins (Kastan and Lim 2000) such as p53, histone H2AX, CHK2, CHK1 and MDM2. ATM's regulation of these proteins is linked: ATM phosphorylates CHK2 on threonine 68, activating CHK2, which phosphorylates p53 on serine 20 (Ahn, Schwarz et al. 2000; Matsuoka, Rotman et al. 2000). This decreases the association between p53 and MDM2, which stabilizes p53 and increases its tumor suppressor capabilities. Additionally, ATM phosphorylates MDM2 on serine 395, which decreases its affinity for p53 (Maya, Balass et al. 2001). ATM can also directly act on p53 by phosphorylating serine 15 (Banin, Moyal et al. 1998; Canman, Lim et al. 1998) causing an increase in p53 activity. The increase in p53 activity from the above mechanisms causes p53 to increase transcription of its downstream target $p21^{WAF1/CIP1}$. As a cyclin dependent kinase inhibitor, $p21^{WAF1/CIP1}$ inhibits cyclinE/CDK2. This blocks the cell in G_1 by inhibiting entry into S phase (Kastan, Onyekwere et al. 1991). Additionally, the activation of $p16^{INKa}$ and CDC25A inhibit CDKs. The inhibited CDKs can no longer phorsphorylate the Rb protein. Therefore, hypophosphorylated Rb can bind to its downstream target transcription factor E2F

and inhibit the protein's activity. E2F up-regulates genes responsible for continuation throughout the cell cycle. With E2F activity blocked, the cell cannot proliferate and is growth arrested.

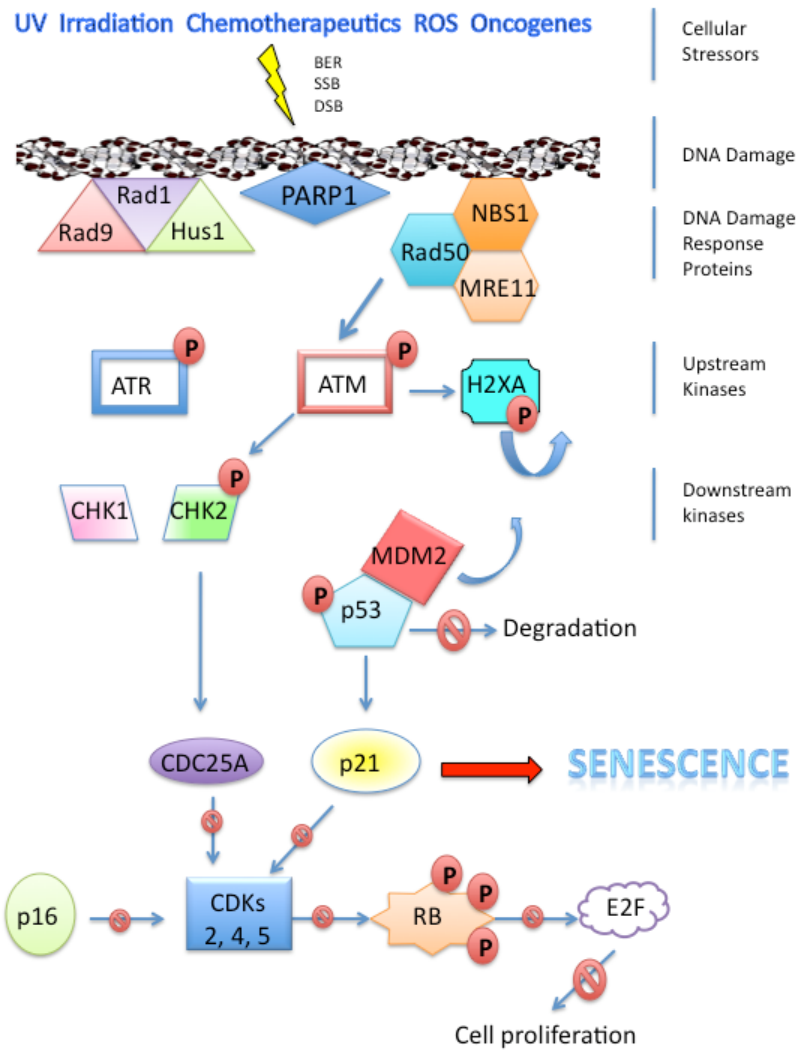


Figure 1. Signal transduction pathway leading to senescence. Cellular stressors lead to DNA damage resulting in BER, SSBs, and DSBs. This recruits DNA damage sensing proteins. Depending upon whether the DNA is repairable, the cell induces a signaling cascade resulting in transient growth arrest, apoptosis, or as illustrated here, senescence (Campisi and d'Adda di Fagagna 2007; Bertram and Hass 2008).

The field of biomedical science is now uncovering many more SIPS occurrences *in vivo*. LASIK eye surgery triggers senescence in keratocytes of the cornea (Dawson, O'Brien et al. 2006). Inflammatory factors of chronic wounds trigger SIPS in surrounding fibroblasts, which affect the ability of the wounds to heal (Harding, Moore et al. 2005). Senescence in kidney epithelial cells may affect organ transplantation success (Famulski and Halloran 2005). Treatments for certain diseases may cause early onset of age-related issues, as evidenced by the observation that PUVA treatment causes aged skin in psoriasis patients (Sator, Schmidt et al. 2002). In another example, antiretroviral thymidine analogues were linked to premature aging of HIV patients taking the drug (Caron, Auclairt et al. 2008). Knowing the mechanisms responsible for SIPS will aid in designing treatments to minimize its detrimental side effects. For instance, senescent cells are located at the sites of age-related diseases such as atherosclerotic lesions, skin ulcers, arthritic joints, benign and preneoplastic hyperproliferative lesions in prostate and liver (Campisi 2005). The mechanisms leading to cellular senescence *in vivo* will lay the foundation for improving public health in our ageing population as age-related diseases begin to increase.

It is critical to determine how SIPS is involved in organismal ageing as research has indicated senescent cells have positive and negative effects on an organism (Campisi 2005). The fact that senescent cells no longer undergo mitosis may indicate senescence is a tumor suppressor (Pelicci 2004; Schmitt 2007). However, senescent cells can alter their surrounding microenvironment to induce preneoplastic cells to become cancerous by secreting matrix metalloproteinases and cytokines (Liu and Hornsby 2007). Additionally, the age-induced accumulation of senescent cells disrupts normal tissue architecture and function (Campisi 2005), and senescent cells no longer proliferate causing tissues to lose the capability to adequately repair (Harding, Moore et al. 2005).

1.2 CAVEOLIN-1

1.2.1 Caveolae

Caveolae are membrane invaginations found in fibroblasts, adipocytes, endothelial cells, type I pneumocytes, epithelial cells, and smooth/striated muscle cells (Razani, Schlegel et al. 2000) (Capozza, Cohen et al. 2005) (Hagiwara, Nishina et al. 2002) (Razani, Woodman et al. 2002). Caveolae are characterized as 50-100 nm flask shaped microdomains at the plasma membrane and in plasmalemmal vesicles (Hagiwara, Nishina et al. 2002) (Razani, Wang et al. 2002). They are classified as a type of lipid raft containing cholesterol, sphingolipids and glycosyl phosphatidylinositol anchored proteins (Capozza, Cohen et al. 2005) (Razani, Woodman et al. 2002). Caveolae have numerous functions in the cell such as: cellular metabolism, vesicle trafficking, signal transduction, cholesterol homeostasis, endothelial transcytosis, calcium uptake and tumor suppression (Capozza, Cohen et al. 2005) (Hagiwara, Nishina et al. 2002) (Razani, Woodman et al. 2002). In muscle cells, the formation and opening of transverse T-tubules are associated with caveolae (Hagiwara, Nishina et al. 2002). The caveolin proteins are markers of caveolae (Williams, Medina et al. 2004) (Razani, Woodman et al. 2002).

1.2.2 Caveolin family of proteins

The three isoforms of caveolin are highly conserved across species with molecular weights averaging 18-24 kDa (Jain-Hua Chen 2004) (Razani, Woodman et al. 2002). Caveolins act as scaffolding proteins to compartmentalize signaling molecules within the cell (Razani, Schlegel et

al. 2000). The three products of the caveolin genes are Caveolin-1 (Cav-1), Caveolin-2 (Cav-2) and Caveolin -3 (Cav-3). Cav-1 and 2 are mapped to the 7q31.1 human chromosome and caveolin-3 is found on 3p25 (Williams, Medina et al. 2004). Cav-1 and 2 are co-expressed in many cell types and can form hetero-oligomers with each other, whereas Cav-3 is specific to muscle cells (Razani, Schlegel et al. 2000) (Hagiwara, Nishina et al. 2002). Thus far only arterial and bladder smooth muscle cells are known to co-express all three caveolin isoforms (Segal, Brett et al. 1999) (Woodman, Cheung et al. 2004). While the caveolin proteins make up caveolae, recent evidence indicates they are not sufficient for the organelle's formation. PTRF-Cavin associates with mature caveolae and is necessary to mobilize Cav-1 into immobile caveolae. Without PTRF-Cavin, Cav-1 still associates with lipid rafts on a flat plasma membrane surface (Chadda and Mayor 2008; Hill, Bastiani et al. 2008).

1.2.3 Cav-1 post-translational modifications

Cav-1 is a 22-kDa protein consisting of 178 amino acid residues. Two isoforms are known to exist in nature: α-caveolin (the full length protein), and β-caveolin (which contains residues 32-178) (Li, Seitz et al. 1996). Cav-1 is inserted into the plasma membrane by a hairpin intramembrane domain with the N and C terminals facing the cytosol (Goetz, Lajoie et al. 2008). The protein can undergo many post-translational modifications. Palmitoylation of cysteines 133, 143 and 156 is not necessary for the protein's localization to caveolae (Dietzen, Hastings et al. 1995), but is essential for cholesterol binding and transport complex formation (Uittenbogaard and Smart 2000). Tyrosine kinases Src, c-Abl and Fyn can phosphorylate tyrosine 14 of the Cav-1 protein (Li, Seitz et al. 1996; Sanguinetti, Cao et al. 2003; Sanguinetti and Mastick 2003). Exogenous cellular stressors activate Y14 phosphorylation on Cav-1 through a mechanism

involving $p38^{MAPK}$ and c-Src. Phosphorylated Cav-1 is localized to focal adhesions where various tyrosine kinase signaling takes place (Lee, Volonte et al. 2000; Volonte, Galbiati et al. 2000). Phosphorylated Cav-1 effects integrin-regulated membrane domain internalization (del Pozo, Balasubramanian et al. 2005), and epithelial growth factor mediated formation of caveolae in epithelial cells (Orlichenko, Huang et al. 2006). Phospho-Cav-1 is also a docking site for the Src Homology 2 (SH2) binding domain-containing proteins such as Growth Factor Receptor-Bound 7 (Grb7) (Lee, Volonte et al. 2000) and C-terminal Src Kinase (Csk). Additionally, tumor necrosis factor-alpha receptor-associated factor 2 (TRAF2)'s binding affinity for Cav-1 increases with Y14 phosphorylation (Cao, Courchesne et al. 2002). However, the mouse monoclonal anti-PY14 Cav-1 antibody used in many of the above studies recognizes phosphocaveolin-1 in immunoblot analyses, but the labeling obtained in immunostaining experiments is questionable because the antibody cross reacts with paxillin (Hill, Scherbakov et al. 2007). Phosphorylation at serine 80 allows caveolin-1 to become a soluble secreted protein (Schlegel, Arvan et al. 2001). Inhibition of protein kinase C causes decreased phosphorylation of serine 80 and increased sterol binding to Cav-1, which lead to a decrease in cellular sterol efflux (Fielding, Chau et al. 2004). Phosphorylation of Cav-1 is also associated with its tumor promoting capabilities as it causes interaction with growth stimulatory proteins (Williams and Lisanti 2005).

1.2.4 Cav-1 in non-caveolae environments

Cav-1 trafficking is not limited to embedding into the plasma membrane and incorporating into caveolae. Cav-1 also has cellular functions outside of the caveolae, as it is expressed in cells without caveolae and in non-caveolar regions (Head and Insel 2007). Recently it was found in

the cytoplasm and nucleus after oxidative stress (Chretien, Piront et al. 2008). Localization of Cav-1 is cell type specific: in skeletal muscle cells and keratinocytes it is located in the cytoplasm, in endocrine and exocrine cells it is found in secretory vesicles, and in epithelial cells it is found in mitochondria. This indicates novel pathways for Cav-1 to mediate lipid trafficking to multiple cellular compartments (Li, Liu et al. 2001). Cav-1 is also a soluble secreted protein as it is released by pancreatic explants and in the presence of gastrointestinal hormone stimulation it is secreted in pancreatic juice (Thomas, Krzykowski et al. 2004). Additionally, Cav-1 secretion by prostate cancer cells is correlated with proangiogenic effects, which may lead to the metastases of these tumors (Tahir, Yang et al. 2001; Tahir, Yang et al. 2008).

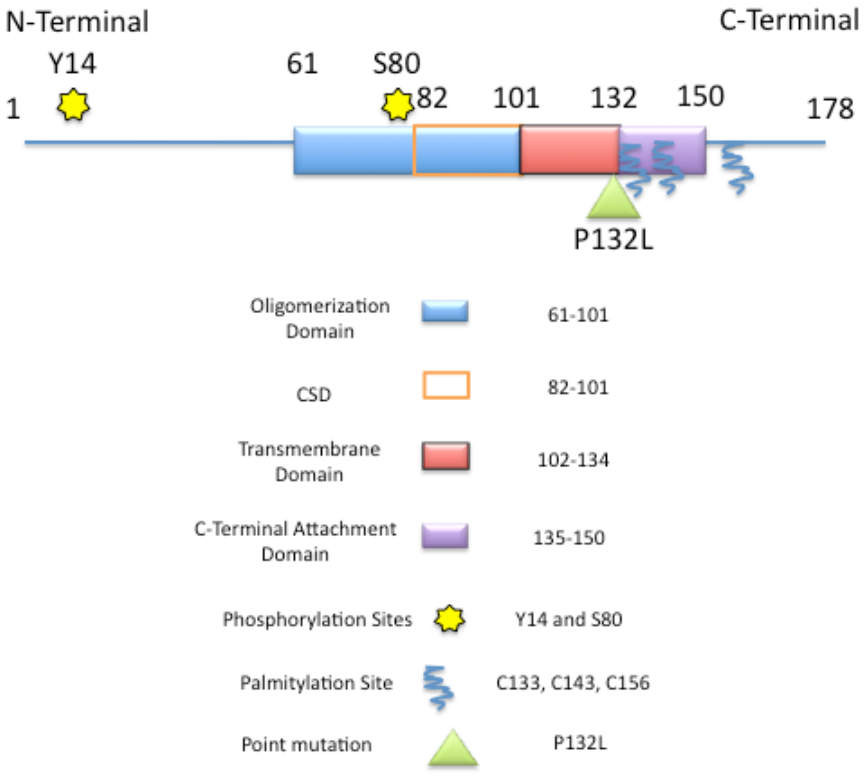


Figure 2. Schematic of the Cav-1 protein. The Cav-1 protein is a 22-kDa protein with 178 amino acid residues. The N and C terminals face the cytosol and a hairpin loop embeds the protein in the plasma membrane.

Palmitoylation of cysteines 133, 143 and 156 help to anchor the protein. Cav-1 has four major domains consisting of the 1) oligomerization domain (amino acids 61-101) which is needed to form monomers and heteromers with Caveolin-2, 2) the Caveolin Scaffolding Domain (CSD) binds to many signaling molecules, 3) the transmembrane domain (amino acids 102-134) which consists of the hairpin loop to associate the protein with the plasma membrane and 4) the C-terminal attachment domain (135-150). Post-translational phosphorylation can occur at tyrosine 14 and serine 80. Point mutation of proline 132 to leucine in the transmembrane domain has been identified in breast cancers in humans and causes a dominant negative protein (Razani, Woodman et al. 2002; Shatz and Liscovitch 2008).

1.2.5 Cav-1 and the signaling hypothesis

Cav-1 interacts with a number of diverse signaling molecules. Cav-1 can inhibit H-Ras (Song, Li et al. 1996), Src-family tyrosine kinases (Shenoy-Scaria, Dietzen et al. 1994; Li, Couet et al. 1996), G protein α subunits (Li, Okamoto et al. 1995; Schnitzer, McIntosh et al. 1995), endothelial nitric oxide synthase (Feron, Belhassen et al. 1996; Liu, Garcia-Cardena et al. 1996; Ju, Zou et al. 1997), epidermal growth factor receptor (Mineo, James et al. 1996; Couet, Sargiacomo et al. 1997; Segal, Brett et al. 1999), Neu (c-erbB2) (Engelman, Lee et al. 1998), protein kinase A (Razani and Lisanti 2001), protein kinase C platelet derived growth factor receptor (Smart, Foster et al. 1993; Yamamoto, Toya et al. 1999), tumor growth factor β receptor (Razani, Zhang et al. 2001), and neurotrophin receptor (Bilderback, Gazula et al. 1999). There are a few instances when binding to Cav-1 causes activation of a signaling molecule such as the estrogen and insulin receptors (Yamamoto, Toya et al. 1998; Schlegel, Wang et al. 2001). Furthermore, Cav-1 mediates cellular functions such as regulating endocytosis of α5β1 integrins and fibronectin (Shi and Sottile 2008). Many of the proteins interacting with Cav-1 bind to the stretch of amino acids referred to as the Caveolin Scaffolding Domain (CSD) (Couet, Li. et al.

1997). The CSD lies within amino acid resides 82-101: DGIWKASFTTFTVTKYWFYR. These findings have led to the caveolin signaling hypothesis, which states that caveolins serve as scaffolding domains to modulate cell signaling molecules (Lisanti, Scherer et al. 1994), as supported by caveolins organizing "preassembled signaling complexes" (Okamoto, Schlegel et al. 1998).

1.2.6 Cav-1 and tumor promotion

The Cav-1 protein is continually studied in the pathogenesis of malignancy. Many cancer cell lines have a high expression of the protein (Lavie, Fiucci et al. 1998; Nasu, Timme et al. 1998; Hurlstone, Reid et al. 1999; Davidson, Nesland et al. 2001). In particular, Cav-1 is over-expressed in prostate cancer and its levels are elevated in metastatic prostate cancer cell lines (Shatz and Liscovitch 2008). Additionally, Cav-1 has been linked to a predisposition of prostate cancer *in vivo* (Haeusler, Hoegel et al. 2005). Research using transgenic adenocarcinoma of mouse prostate (TRAMP) mice crossed to wildtype or knockout Cav-1 mice show in the absence of Cav-1, there is delayed onset of highly invasive prostate cancer and metastatic disease (Williams, Hassan et al. 2005). Numerous studies *in vitro* and *in vivo* show Cav-1 as a prognostic marker for the disease. Cav-1 is a predictor of biochemical failure defined by a prostate specific antigen [PSA]> 0.2 ng/mL (Goto, Nguyen et al. 2008). Additionally, high pre-operative levels of serum Cav-1 is a predictor for cancer reoccurrence (Tahir, Frolov et al. 2006), and Cav-1 secreted by prostate cancer cells, is taken up by endothelial cells where it triggers the proangiogenic phosphoinositide 3 kinase(PI3K)-protein kinase B (Akt)-endothelial nitric oxide synthase (eNOS) signaling pathway leading to tubulin formation, cell migration and nitric oxide production (Tahir, Yang et al. 2008). Additionally, over-expression of Cav-1 is associated with

prostate cancer and aggressive PSA reoccurrence in patients treated with radical prostatectomy (Karam, Lotan et al. 2007). Finally, tumor promoter, fatty acid synthase (FASN), is upregulated during prostate cancer progression and is linked to metastatic disease. Cav-1 expression is necessary to elevate levels of FASN in TRAMP mice (Di Vizio, Sotgia et al. 2007).

The upregulation of Cav-1 is most thoroughly documented in prostate cancer, but it occurs in other types of cancers as well. For instance, in ovarian carcinoma cells Cav-1 is localized to inactive chromatin and can bind to genes involved in proliferation (Sanna, Miotti et al. 2007). Additionally, in inflammatory breast cancer, a very aggressive form of the disease, Cav-1 is over-expressed and its gene promoter is hypomethylated (Van den Eynden, Van Laere et al. 2006). In patients with pleomorphic carcinoma of the lung, Cav-1 has high expression and is correlated with decreased disease-free and survival rates (Moon, Lee et al. 2005). In non-small cell lung cancer (NSCLC), Cav-1 expression is linked with tumor stage and lymph node metastasis (Yu, Wei et al. 2006). Furthermore, in NSCLC Cav-1's expression is associated with drug resistance and poor prognosis in patients treated with gemcitabine chemotherapy (Ho, Kuo et al. 2008).

1.2.7 Cav-1 as a tumor suppressor

Even though Cav-1 is linked to tumor promotion it is also hypothesized to be a tumor suppressor because the protein inhibits signaling molecules associated with cell proliferation. Cyclin D is a regulatory unit of cyclin dependent kinases (CDKs) necessary for progression through the cell cycle. Over-expression of Cav-1 causes transcriptional repression of cyclin-D (Hulit, Bash et al. 2000), and knocking out caveolin-1 causes upregulation of cyclin D1 levels (Williams, Cheung et al. 2003). Furthermore, fibroblasts transformed by oncogenes such as Ras^{G12V} and c-Abl or

the human papilloma virus have a downregulated Cav-1 gene (Koleske, Baltimore et al. 1995; Razani, Altschuler et al. 2000). When Cav-1 was re-expressed in these cells it rescued the cells from the transformed phenotype (Engelman, Wycoff et al. 1997; Razani, Altschuler et al. 2000). Recently, numerous oncogenes have been shown to down regulate Cav-1 including but not limited to: c-Myc, HPV E6, v-Abl, Bcr-Abl, H-Ras^{G12V}, v-Src, and Neu/Erb2, (reviewed by(Williams and Lisanti 2005).

The caveolin-1 gene is localized at chromosome 7q31.1, a region deleted in many human cancers where caveolin-1 is down-regulated (Razani, et al 2002 and van Deurs, et al. 2003). Continuing research illustrates a growing list of cancers in which Cav-1 is downregulated by promoter methylation, mutation, or yet to be discovered mechanisms. For instance, downregulation of Cav-1 has been reported for: osteoblast transformation and osteosarcoma progression (Cantiani, Manara et al. 2007), mucoepidermoid carcinoma (Shi, Chen et al. 2007), hepatocellular carcinoma (Hirasawa, Arai et al. 2006), invasive ductal carcinoma of the breast (Park, Kim et al. 2005), gastric carcinogenesis (Gao, Sun et al. 2005), small cell lung cancers (Wiechen, Diatchenko et al. 2001; Sunaga, Miyajima et al. 2004) human ovarian carcinoma (Wiechen, Diatchenko et al. 2001), and human colon tumors (Bender, Reymond et al. 2000). Cav-1's ties with cancer also extend to p53, which is known to be non-functioning in most cancers. Downregulation of p53 by viral oncoproteins also causes a decrease in Cav-1 expression. Additionally, p53 null mice $^{-/-}$ exhibit a decreased Cav-1 expression (Lee, Reimer et al. 1998; Razani, Altschuler et al. 2000).

In keeping with its role as a potential tumor suppressor, Cav-1 is up-regulated during cell differentiation. There is high expression of the protein in terminally differentiated cells such as adipocytes, pneumocytes, chondrocytes and smooth muscle cells (Goetz, Lajoie et al. 2008).

The creation of Cav-1 knockout mice has also led to implications of Cav-1's tumor suppressor capabilities. The skin of the Cav-1 null$^{-/-}$ mice is more susceptible to tumors from carcinogenic treatment than the skin of wildtype mice is (Capozza, Williams et al. 2003). Embryonic fibroblasts derived from Cav-1 knockout mice are more sensitive to oncogenic transformation. Once transformed they over-express cyclin D1 and express hyperactive ERK 1/2, adding to their *in vivo* tumorigenesis potential (Williams, Lee et al. 2004). Interestingly, Cav-1 is known to inhibit ERK1/2 activation *in vitro* and *in vivo* (Engelman, Chu et al. 1998; Zhang, Razani et al. 2000).

Studies in mice also indicate that Cav-1 acts as a tumor suppressor. When the Cav-1 null mice are bred into mice with cancer-prone backgrounds, the resulting mice have much more aggressive tumors than those in the Cav-1 wildtype mice. This is illustrated by crossing Cav-1$^{-/-}$ mice with MMTV-PyMT (mouse mammary tumor virus-polyoma middle T antigen) mice. The mice were followed for 14 weeks and the study demonstrated mice with the Cav-1 null background had accelerated onset of mammary tumors, increased tumor burden and more metastatic lung cancer compared to those with a wildtype Cav-1 background (Williams, Medina et al. 2004).

1.2.8 Cav-1 and senescence

Senescence is tumor suppressing for cells undergoing permanent growth arrest. Cav-1, in keeping with its tumor suppressor properties, is involved in the senescence and aging process. Cav-1 plays a role in aging as the protein's expression increases in aged tissues (Cho, Ryu et al. 2003). While down regulation of Cav-1 is linked to cancer, there is also evidence that high levels of Cav-1 are linked to cell senescence. Over-expression of Cav-1 can arrest growth in fibroblasts

through a p53/p21 dependent mechanism (Galbiati, Volonte et al. 2001). The senescent phenotype is reversable by reducing Cav-1 expression with siRNA (Cho, Ryu et al. 2003). Likewise, mouse embryonic fibroblasts over-expressing Cav-1 have increased senescence associated-β-galactosidase activity, p53 activity and p21 expression. Oxidative stress, such as subcytotoxic doses of hydrogen peroxide or UV irradiation upregulates Cav-1 along with p53 and p21 resulting in SIPS (Volonte, Zhang et al. 2002). Thus, data from our laboratory and others distinguish the protein Cav-1 as a key mediator of SIPS in fibroblasts. How Cav-1 is mediating p53 activation and subsequent SIPS is not known.

1.2.9 Cav-1 and its dual roles as a tumor suppressor and promoter

There is solid evidence indicating Cav-1 acts as a tumor promoter and also a tumor suppressor. There are explanations for the protein's dual activities; post-translational modifications or mutations in the protein can disrupt Cav-1's tumor suppressing mechanisms and render it a tumor promoter (Williams and Lisanti 2005). First, Cav-1 phosphorylation on tyrosine 14 recruits c-Src/Grb7. Phosphorylated Tyr14 bound to Grb7 enriches anchorage independent growth and EGF stimulated cell migration. The residue is also constitutively active in v-Src and v-Abl transformed cells. Tyr14 can also be transiently phosphorylated by growth factor stimulation. Phosphorylated Cav-1 is localized to focal adhesions where much of the cell's tyrosine kinase cascades occur (Lee, Volonte et al. 2000; Lee, Volonte' et al. 2000; Lee, Woodman et al. 2001). Phosphorylation of a different residue, Ser80, converts Cav-1 to a soluble secreted form (Schlegel, Arvan et al. 2001; Liu, Rudick et al. 2002). Secretion of the protein may deplete the intracellular stores, thus decreasing the protein levels of the environment in which it can act as a tumor suppressor. Additionally, as the protein is secreted it may possess

different tumor promoting functions. For example, Cav-1 is secreted by androgen-insensitive human prostate cells and directly stimulates prostate and tumor cell growth/ survival while also exhibiting proangiogenic activities in prostate cancer (Tahir, Yang et al. 2001; Tahir, Yang et al. 2008). Lastly, a Pro132Leu mutation in the Cav-1 gene is in 16% of primary tumors examined for the mutation. When Cav-1 containing this mutation is expressed *in vitro*, it causes transformation of cells by acting as a dominant negative mutant (Hayashi, Matsuda et al. 2001; Lee, Park et al. 2002). Furthermore, screening of estrogen receptor alpha (ERα) positive breast cancer tumors for the mutation identified 19% as having the Pro132Leu mutation where ERα negative tumors did not. This study also found other mutations in the Cav-1 protein raising the occurrence of Cav-1 mutations in this tumor type to 35% (Li, Sotgia et al. 2006).

In an attempt to explain how Cav-1 is downregulated in some cancers and upregulated in others, Liscovitch hypothesizes there are changes in expression as transformation progresses (Shatz and Liscovitch 2008). Liscovitch observes Cav-1 expression is low in undifferentiated epithelial cells, allowing for proliferation, and the protein is upregulated upon differentiation where it can act as a tumor suppressor for the cell (Goetz, Lajoie et al. 2008). However, oncogenes can downregulate Cav-1 early in this transformation, causing excessive cellular proliferation resulting in hyperplasia and preneoplastic lesions. These Cav-1 null cells are more susceptible to chemotherapy and are thus present in low-grade cancers. Cellular stress is also known to upregulate Cav-1. Continuation of genetic changes allows the cancer cells to adapt and acquire metastatic abilities. Once this occurs, high levels of Cav-1 have a protective effect against chemotherapeutics and make cells resistant to apoptosis. The selective pressure of the chemotherapeutics drive the cellular population towards these more resistant cells, creating an invasive and multidrug resistant tumor (Liu, Oh et al. 1997; Ravid, Maor et al. 2006; Goetz,

Lajoie et al. 2008). This is one reason why aggressive cancers may exhibit high Cav-1 expression.

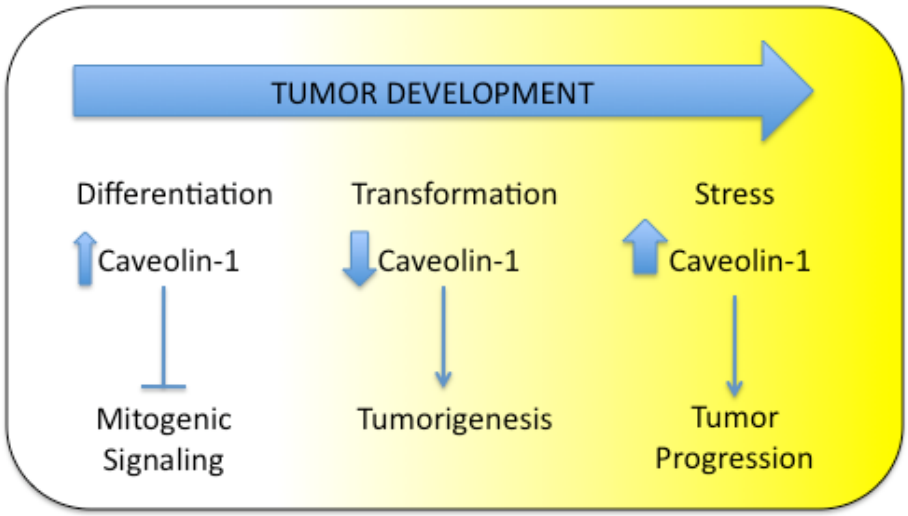


Figure 3. Schematic illustrating the changing Cav-1 expression in tumor development. Cav-1 is upregulated during differentiation, where it downregulates mitotic signaling and restrains tumor promotion. However, with time and progression of transformation, the protein is downregulated in proliferating cells and the average Cav-1 expression in a tumor decreases. Cellular stress upregulates Cav-1, and as stress selects for metastatic and drug resistant cells, the overall expression of Cav-1 in the tumor increases at later stages of the disease (Shatz and Liscovitch 2008).

2.0 MATERIALS AND METHODS

2.1 MATERIALS

Cell lines for this work, NIH 3T3 fibroblasts, WI-38 fibroblasts, MDA-MB231 (a gracious gift from Julie Eiseman), and Ras^{G12V} were purchased from ATCC (Manassas, VA). Mouse embryonic fibroblasts were derived from mice in our laboratory's breeding colony. Cellular media (DMEM and RPMI), along with components such as serum, antibiotics, glutamine and Cell Dissociation Buffer were manufactured by Gibco and purchased from Invitrogen. MEM was obtained from ATCC. Corning plates for tissue culture were the purchased from ThermoFisher Scientific. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). SA-β-Galactosidase Activity kit was purchased from Cell Signaling Technology (Danvers, MA). Chromatin Immunoprecipitation and TUNEL Apoptosis Detection kits were obtained from Upstate Cell Signaling Solutions (Temecula, CA). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), except for Ki67 which was purchased from Dako (Glostrup, Denmark). Kits for cloning were purchased from Qiagen (Valencia, CA). BA83 nitrocelluose membrane was purchased from Schleicher & Schuell (Keene, NH). ECL SuperSignal West Pico Chemilluminescent Substrate Kit detection kit was from Pierce (Rockford, IL). Slow-Fade anti-fade reagent was purchased from Molecular Probes, Inc. (Eugene, OR). 6-diamidino-2-phenylindole was purchased from Sigma-Aldrich and RNase was

obtained from Invitrogen. Sepharose A beads were purchased from GE Healthcare Life Sciences (Piscataway, NJ). Primers for PCR reacations were ordered from Invitrogen. The Cavtratin peptide was purchased from Tufts University Core Facility (Medford, MA). Nu/nu mice were purchased from Charles Rivers Laboratories.

2.2 TISSUE CULTURE

Normal tissue culturing conditions were used to grow cells for these experiments in Corning plates (Fisher Scientific) at 37°C and 5% CO_2. The following cell lines were utilized for the listed experiments. NIH 3T3 Fibroblasts and Ras^{G12V} transformed fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% donor bovine serum. Mouse embryonic fibroblasts (MEFs) and MCF7 cells were grown in DMEM plus 10% fetal bovine serum. WI-38 fetal lung fibroblasts were cultured in Minimum Essential Eagle's Medium (MEM) and 10% fetal bovine serum. MDA-MB-231 cells were grown in RPMI Medium 1640 and 10% fetal bovine serum. All of the above were supplemented with 2 mM of glutamine and 100 units of penicillin/ 100 μg streptomycin per mL**.** Human mammary epithelial cells (HMEC) were grown in Mammary Epithelial Basal Medium supplemented with 2 mM glutamine, bovine pituitary extracts (70 μg/mL), epidermal growth factor (20 ng/mL), hydrocortisone (5 μg/mL), insulin (10 μg/mL), gentamicin (50 μg/mL), and amphotericin (2.5 μg/mL).

2.3 HYDROGEN PEROXIDE TREATMENT

Cells were treated with hydrogen peroxide for 2 hours when dish confluency was between 50-70%. NIH 3T3 and Ras^{G12V} transformed fibroblasts, and MEFs were treated with 150 μM hydrogen peroxide diluted in media and HMEC, MCF7 and WI-38 cells were treated with 450 μM hydrogen peroxide diluted in media. After treatment, cells were washed twice with PBS and allowed to recover for the given amount of time in their respective culturing media.

2.4 CHROMATIN IMMUNOPRECIPITATION

NIH 3T3 cells were treated with H_2O_2 as stated above. Cells were crosslinked by adding 1% formaldehyde to the cell plate containing 8 ml of growth media for 10 minutes at 37°C. The crosslinking was quenched with the addition of 0.8 ml of 1.4 M glycine to each plate for 5 minutes at 4°C. Cell counts were approximately 5 x 10^6 cells per reaction. Cells were centrifuged at 600 x g for 10 minutes at 4°C, and the pellet was resuspended in 500 μL of cold Chromatin IP buffer (50 mM HEPES KOH pH 8, 1mM EDTA pH 8, 0.5 mM EGTA pH 8, 140 mM NaCl, 10% glycerol, 0.5% IGEPAL, 0.25% Triton X-100 and protease inhibitors). Cells were rotated at 4°C for 10 minutes and spun down at 600 x g at 4°C for 10 minutes. The pellet was resuspended in 500 μL of cold Wash Buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 0.5 mM EGTA pH 8, 200 mM NaCl, protease inhibitors). Cells were pelleted as above and resuspended in 500 μL cold RIPA buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 0.5 mM EGTA pH 8, 140 mM NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, and protease inhibitors) . The samples were sonicated in polypropylene tubes at an amplitude of 21%, 2 pulses on 1 pulse off,

for 10 seconds with 3 cycles (so the DNA was fragmented to ~250-1000 bp fragments) Samples were pelleted and the supernants are transferred to an eppendorf with the final volume brought to 500 µL in RIPA buffer + protease inhibitors. This is the ChIP lysate, and it is precleared with Upstate Protein A Sepharose beads plus conjugated salmon sperm DNA for 30 minutes at 4°C. Afterward, samples are spun down and the supernant saved. 10% of the sample (50 µL) is taken as input. The inputs were reverse crosslinked immediately by adding 950 µL ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, and 167 mM NaCl) with 40 µ 5M NaCl and incubated at 65°C for approximately 4 hours. ChIP lysate was combined with 70 µL of beads in addition to 3 ug of Sp1 antibody and rotated over night at 4°C. Beads were centrifuged at 600 x g and washed twice for 5 minutes with rotation at 4°C with cold RIPA buffer, twice with cold RIPA buffer + 500 mM EGTA pH 8, and once with LiCl Buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 0.5 mM EGTA pH 8, 250 mM LiCl, 1% Triton X-100, 1% Na-deoxycholate, and protease inhibitors). Complexes were eluted twice in 250 µL of fresh Elution Buffer (1% SDS and 0.1 M NaHCO3). Samples were reverse crosslinked by adding 20 µL of 5M NaCl and incubating at 65°C for approximately 4 hours. To both ChIP samples and inputs 10 µL of 0.5M EDTA, 20 µL of 1M Tris-HCl pH 6.5, and 2 µl of 10 mg/ml Proteinase K were added and incubated at 45°C for 1 hour. Inputs and sample DNA were recovered by using Qiagen PCR Purification kit (Valencia, CA) according to manufacturer's instructions and resuspended in 30 µL of H_2O. PCR was conducted using the following sequences for primers: sense (5' to 3') CAG GCT CTC AGC TCC CCG CCG, and antisense (5' to 3') GTA TAG AGG GGG GAA AGG CGC. Both ChIP samples and inputs, PCR reactions were prepared by adding 5 µL of sample DNA and 45 µL of a master mix containing 4 µL of sense and antisense primers, 27.5 µL H_2O, 4 µL dNTP, 5 µL Buffer and 0.5 µL Taq enzyme

(Takara Bio Inc., Japan). The PCR was run at 1) 94°C for 5 minutes 2) 94°C for 30 seconds 3) 58°C for 30 seconds 4) 72°C for 1 minute (steps 2-4 were repeated 39 times) and 5) 72°C for 7 minutes. 40 μL of ChIP PCR were added to 8 μL TAE 6X DNA loading dye, and run out on a 1.2% agarose gel with ethidium bromide. The gel was documented with a UV gel documentation device, Eagle Eye by Stratagene.

2.5 ADENOVIRUS INFECTION

Adenovirus carrying Cav-1 (Ad-Cav-1), GFP (Ad-GFP), and transactivator (Ad-tTA) were previously described (Zhang, Razani et al. 2000). Sub-confluent MCF7 cells and Human Mammary Epithelial Cells (HMEC) were co-infected with Ad-tTA (transactivator: 100 plaque-forming units/cell) and Ad-Cav-1 or Ad-GFP (500 plaque-forming units/cell) for 1 hour in serum-free DMEM, and washed with PBS. Cultures were grown in complete media for the indicated period of time. Cells were subjected to immunoblotting, 6-diamidino-2-phenylindole (DAPI) staining, or terminal deoxyribonucleo-tidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis.

2.6 IMMUNOBLOT

Cells were collected in boiling 1X sample buffer. Samples were separated by molecular weight on a SDS-PAGE 12.5% polyacryamide gel and transferred to a BA83 nitrocelluose membrane (Schleicher & Schuell, Keene, NH). Blots were blocked using 2% dried milk in TBST (10 mM

Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% Tween 20) for 45 minutes for monoclonal antibody treatment or 1% bovine serum albumin (BSA) and 2% dried milk in TBST for polyclonal antibody treatment. Blots were rinsed 3 times for 5 minutes each and incubated with primary antibody diluted in TBST for anywhere from 3 hours to overnight depending upon the affinity of the antibody. Blots were rinsed 3 times for 5 minutes and incubated with horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG diluted in the appropriate blocking buffer for 1 hour. Bound antibodies were detected using an ECL detection kit from Pierce: SuperSignal West Pico Chemilluminescent Substrate Kit (Rockford, IL).

2.7 SUCROSE DENSITY GRADIENT CENTRIFUGATION

Wi-38 cells were treated with 450 μM H_2O_2 for 2 hours and allowed to recover for 24 hours. Caveolae membranes were isolated by sucrose gradient centrifugation. Briefly, cells were collected in 2 ml of a MES buffer (0.01M MES pH 6.5, 0.15M NaCl and 1% (v/v) Triton X-100). Cells were homogenized using a loose fitting Dounce homogenizer and passing the glass tube 10 times. The homogenate was adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in MES buffer listed above and placed at the bottom of an ultracentrifuge tube. A 5-30% linear sucrose gradient was formed above the homogenate and centrifuged at 39,000 rpm for 16-20 h in a SW41 rotor (Beckman Instruments). From the top of each gradient, 1-ml gradient fractions were collected to yield a total of 12 fractions. Fractions 9-11 contained soluble proteins and fractions 4-6 contained insoluble proteins including Cav-1. Equal amounts of protein from each sample were subjected to immunoblot analysis.

2.8 IMMUNOFLUORESCENCE

Cells grown either on glass coverslips or culture dishes were washed 3 times in PBS/CM (a buffer containing 1X PBS with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$). Cells were fixed using 2% paraformaldehyde in PBS/CM for 30 minutes at room temperature. Cells were rinsed and permeabilized with 0.1% Triton X-100 and 0.2% BSA (bovine serum albumin) in PBS/CM (PBS/CM^{++}) for 10 minutes at room temperature. This was quenched with 50 mM NH_4Cl in PBS/CM for 10 minutes. Cells were washed once with PBS/CM^{++} and incubated with primary antibody diluted in same solution for 2 hours. Concentration was antibody dependent. Cells were washed 3X for 10 minutes each in PBS/CM^{++} and incubated with secondary antibody diluted in the same buffer for 1 hour. Secondary antibodies used in these studies were: lissamine rhodamine B sulfonyl chloride- or Cy3-conjugated goat anti-rabbit antibody (5 μg/ml) and/or fluorescein isothiocyanate-conjugated goat anti-mouse antibody (5 μg/ ml). Cells were washed three times in PBS/CM^{++} and rinsed once with PBS. Slides were mounted with slow-Fade anti-fade reagent (Molecular Probes, Inc., Eugene, OR) and observed using a Zeiss Confocal microscope or Olympus Confocal microscope.

2.9 4',6-DIAMIDINO-2-PHENYLINDOLE STAINING

Cells were grown on glass coverslips or just the culturing dish. Cells were washed 3 times in PBS/CM (a buffer containing 1X PBS with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$). The cells were fixed using 2% paraformaldehyde in PBS/CM for 30 minutes at room temperature. Cells were rinsed and permeabilized with 0.1% Triton X-100 and 0.2% BSA (bovine serum albumin) in

PBS/CM (PBS/CM^{++}) with 10 μg/ml RNase for 10 minutes at room temperature. This was quenched with 50 mM NH_4Cl in PBS/CM for 10 minutes, and cells were washed once with PBS/CM^{++}. Cells were incubated with 1 μg/ml 4', 6-diamidino-2-phenylindole (DAPI) in PBS/CM^{++} for 20 minutes. Cells were washed three times in PBS/CM^{++} and rinsed once with PBS. Slides were mounted with slow-Fade anti-fade reagent (Molecular Probes, Inc., Eugene, OR) and observed using a Zeiss Confocal microscope or Olympus Confocal microscope.

2.10 CO-IMMUNOPRECIPITATION

All steps were carried out at 4°C. Cells were washed twice in PBS and lysed in an IP buffer containing 10 mM Tris, pH 8.0, 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, 60 mM octyl glucoside and protease inhibitors. Samples were put on rotation for 45 minutes at 4°C. Soluble supernant was precleared with using protein A-Sepharose (20 μL; slurry, 1:1) at 4°C. Samples were centrifuged at 10,000 rpm for 10 minutes and supernant was taken and normalized for protein concentration. One tenth of the volume was taken as an aliquot for total input. The supernant was incubated over night with the particular antibody and protein A-Sepharose (30 μL; slurry, 1:1). Beads were washed in lysis buffer 4 times on rotation for 10 minutes at 4°C. The final wash was done with 2.5 mM of Tris-HCl pH 7.5. Beads were spun down at max speed for 1 minute and sample buffer was added. Results were resolved by immunoblot analysis on the samples and the inputs. For immunoprecipitations using MDM2, the following buffer was used: 50 mM Tris, pH 8.0, 0.15 M NaCl, 5 mM EDTA, 1% NP-40. Samples were not precleared and were incubated with anti-MDM2 IgGs conjugated to protein A Sepharose beads. Samples were treated the same as above.

2.11 MDM2 AND CAVEOLIN BINDING DOMAIN MUTANT MDM2 CONSTRUCTS

RNA was isolated from 30 mg of mouse lung using the RNeasy Mini Kit from Qiagen. cDNA was generated using Advantage RT-for-PCR Kit by Clontech according to manufacturer's instructions. Briefly, 1 μg of RNA was used and RT-PCR was conducted using 1 μL oligo $(dT)_{18}$. Using the cDNA as template, and using the following Mdm2 primers in a PCR reaction *MDM2* was amplified: 5' ccggccgaattcatgtgcaataccaacatgtct and 3'ccggccagatctttaattcagatcttcctcgctgatcagtttctgttcgcgccgttgaagtaagttagcacaat. The 3' primer includes a myc tag. The following components and the quantity of each were used in the PCR reaction: 5 μL $MgSO_4$, 5 μL DNA template, 5 μL Buffer 10X, 4 μL dNTPs, 5 μL 3'oligo primer, 5 μL 5' oligo primer, 24.5 μL H_2O, and 0.5 μL Taq enzyme. Invitrogen's Platinum Pfx DNA polymerase was used in all subsequent PCR reactions. The PCR program used was the following: 1) 94°C for 5 seconds 2) 94°C for 45 seconds 3) 58°C for 45 seconds 4) 72°C for 2 minutes 5) steps 2-4 are repeated 29 times 6) 72°C for 7 minutes and cooled to 4°C. PCR products were purified using the PCR Purification Kit by Qiagen. The pCAGGS vector and *Mdm2* oligos were cut using the EcoR1 and BGL2 restriction enzymes and gel purified using the QIAquick Extraction from Qiagen. The gene products were ligated into the pCAGGS vector overnight at 37°C, and transformed into DH5α *E. coli*. Briefly, the ligation reaction was added to the bacteria on ice for 30 minutes. The samples were heat shocked in a 37°C water bath for 30 seconds and placed back on ice for 2 minutes. 1 ml of LB was added to the samples along with ampicillin and they were incubated at 37°C for 1 hour. The culture was plated on ampicillin plates. Colonies were selected and the constructs were isolated using Qiagen Mini and Maxi Prep Kits according to manufacturer's instructions.

The Myc-tagged *Mdm2*construct was used as a template to make a mutated *Mdm2* (Φ→A *Mdm2*-Myc). The mutant *Mdm2* construct was made by point mutations. Mutant primers converted nucleotides corresponding to amino acids tyrosine 48, phenylalanine 55, tyrosine 56 and 60 to alanines. The following primers were used: *Mdm2* MUTANT 3'cataatagcctggccaatagcagctataatctctttcatagtggcagtgtc and *Mdm2* MUTANT 5'gacactgccactatgaagagattatagctgctattggccaggctattatg. Using the Myc- *Mdm2* construct as a template, the following primer combinations were used in 2 reactions: 1) Myc- *Mdm2* 5'+ *Mdm2*MUTANT 3'and 2) *Mdm2* MUTANT 5' and Myc- *Mdm2* 3'. Reactions 1 and 2 were combined to use as template for the final reaction with primers Myc- *Mdm2* 5' and Myc- *Mdm2* 3'. PCR product from this reaction was cloned into pCMV-HA using the same method as listed above for the Myc- *Mdm2* construct. The University of Pittsburgh Genomics and Proteomics Core Facility sequenced all constructs.

2.12 GST-FUSION CAVEOLIN SCAFFOLDING DOMAIN PULL-DOWN

GST-Cav-1 fusion proteins were synthesized with the full length, amino acids 42-101 and 82-101 of the caveolin-1 protein. The constructs were transformed into Escherichia coli (BL21 strain; Novagen Inc.). When cultures reached OD_{600}=0.3-0.6, cultures were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (Sigma Aldrich). The fusion proteins were solublized by using 100 μg/ml lysozyme, 5 mM DTT and 1.5% N-lauryl sarkosyl in STE buffer (150 mM NaCl, 7.52 mM Tris pH 8.0 and 3 mM EDTA pH 8.0) and a polytron was utilized to lyse the bacteria. The constructs were affinity purified by binding to glutathione-agarose beads washed in STE with 2.0% Triton X100 overnight at 4°C. Beads were washed in STET (150 mM NaCl, 7.52

mM Tris pH 8.0 and 3 mM EDTA pH 8.0, 1% Triton X-100 plus protease inhibitors) 3 times. To determine the concentration of GST-Cav-1 per 100 μL of packed bead volume, SDS-PAGE followed by coomassie staining was conducted.

The GST pulldown was conducted on 3T3 NIH fibroblasts cultured in 10 cm dishes. Cells were transfected with 30 μg of Wildtype *Mdm2* (WT- *Mdm2*) or mutant *Mdm2* (Φ→A *Mdm2*) DNA by the calcium phosphate method. 48 hours later cells were lysed in IP buffer(10 mM Tris-HCl pH 8.0, 150 mM $NaCl_2$, 5 mM EDTA, 1% Triton X100, 60 mM n-octyl glucoside, protease inhibitors). Cells were put on rotation for 45 minutes at 4°C, and the soluble fractions were collected. Lysates were precleared with glutathione-agarose beads for 1 hour. Samples were normalized based on protein concentration. Samples were incubated overnight with beads fused to GST alone, GST-full length Cav-1, GST-1-101 amino acids, or GST-81-101 amino acids at 4°C. Beads were washed 3 times in IP buffer, resuspended in sample buffer and subjected to SDS-PAGE.

2.13 CELL PERMEABLE CAVTRATIN PEPTIDE

Cavtratin is a cell-permeable peptide consisting of the caveolin scaffolding domain amino acids 82-101 (DGIWKASFTTFTVTKYWFYR) fused to the C-terminus of the internalization sequence of the Drosophila transcription factor antennapedia (AP) (RQIKIWFQNRRMKWKK), by Fmoc chemistry, purified and analyzed by reversed-phase high pressure liquid chromatography and mass spectrometry by the Tufts University Core Facility. Detection of the peptide was possible because of the biotin tag followed by an aminohexanoic acid spacer added to the amino terminus of the AP fusion peptide. The peptide was solublized in DMSO. WI-38

cells were treated with 4μM or 10 μM for 24 hours and subjected to immunoblot, immunoprecipitation, luciferase assay and SA-β-galactosidase staining.

2.14 P53 RESPONSIVE ELEMENT LUCIFERASE ASSAY WITH CAVTRATIN TREATMENT

NIH 3T3 fibroblasts were cultured in 6-well plates with 300,000 cells/well. Cells were transfected using calcium phosphate with the following DNA construct amounts: 3 μg of luciferase reporter fused to the p53 responsive element called p53 transactivator (pTA-p53) and pSV- β-galactosidase. After another 24 hours, cells were rinsed twice with 1X PBS and incubated with cavtratin (10μM) or the control peptide Antennapedia (AP) (10μM) in cellular media for 24 hours. A luciferase assay was conducted. Cells were washed twice in 1X PBS and lysed at 4°C in 500 μL of Extraction Buffer (1% w/v Trition-X-100, 1 mM DTT, GME Buffer: 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, dissolved in H_2O) and placed on a rocker at 4°C for 30 minutes. During this incubation, 300 μL of ATP Mix (GME Buffer, 100 mM K Phos, 1 M DTT, 200 mM ATP – kept at room temperature) is placed into cuvettes for luciferase assay and 600 μL of Z buffer (100 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) with 50 mM β-mercaptoethanol added before aliquoting into eppendorf tubes for the β-galactosidase assay. Each experimental sample was run in duplicate for β-galactosidase and luciferase assay. 100 μL of sample is added into each cuvette for the luciferase assay and 100 μL of sample into each eppendorf for the β-galactosidase assay. All are vortexed briefly to mix. Once this was completed, the luminometer cuvettes were read using a luminometer injecting 100 μl of luciferin substrate **(**Molecular Probes, Oregon) solution per tube (1mM luciferin substrate, 50 mM DTT).

For the β-galactosidase assay, 20 μL of CPRG (chlorophenol red-β-galactopyranoside, Boehringer Mannheim) was added into the eppendorf tubes. The reaction proceeded until there was a color difference between the blank and the samples (30 minutes- 2 or more hours). Reaction was stopped with the addition of 200 μL of 1 M Na_2CO_3. Absorbance was measured at λ= 574 nm. The p53 luciferase reporter activity was controlled for transfection efficiency and potential toxicity with the use of β-galactosidase activity. For each condition, at least three independent experiments were performed.

2.15 ISOLATION OF PRIMARY MOUSE EMBRYONIC FIBROBLASTS

At 13.5 days of gestation a pregnant mouse was sacrificed by isoflorane exposure and cervical dislocation. The uterus was removed and cleaned in sterile PBS. Embryos were isolated and removed from the embryonic sacs. The liver was discarded and the head used to genotype the embryos. The remaining embryo was passaged through a sterile 6 cc luer lock syringe with an 18-gauge needle with 3 ml of trypsin and placed in a 10 cm culturing dish. The embryo fragments were incubated at 37°C for 5 minutes. 17 ml of MEF media (Dulbecco Modified Eagle's Medium, 10% fetal bovine serum, glutamine and penicillin/streptomycin) were added to the dish and the sample was pipetted up and down to break up tissue fragments. The dish was incubated overnight. The next day large tissue fragments were removed, and the remaining annealed fibroblasts were cultured as normal.

2.16 PROTEOSOMAL INHIBITION OF CAV-1 NULL MEFS

Cav-1 null mouse embryonic fibroblasts were treated with 150 μM hydrogen peroxide diluted in media for 2 hours, washed twice in 1X PBS and allowed to recover in normal media for 18 hours. Cells were given 5 μM of proteasomal inhibitor MG132 dissolved in DMSO or the equivalent amount of DMSO for 6 hours. Cells were lysed and immunoblot analysis was done to determine p53 or p21 protein levels.

2.17 SENESCENCE-ASSOCIATED ß-GALACTOSIDASE STAINING

Cells were cultured under normal conditions and treated as stated. Cells were washed twice with PBS and fixed with 2% formaldehyde/0.2% glutaraldehyde at room temperature for 15 minutes. Cells were again washed twice with PBS and stained with Senescence Associated-β-Galactosidase stain (7.36 mM citric acid/sodium phosphate dibasic pH 6.0, 150 mM NaCl, 2 mM MgCl2, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-gal. overnight at 37°C. Plates were covered with 70% glycerol in PBS and stored at 4°C.

2.18 CO-CULTURING OF MEFS WITH RAS^{G12V} AND MDA-MB-231 EPITHELIAL CELLS

Three different populations of either wildtype or knockout MEFs were mixed and plated so on the day of the hydrogen peroxide treatment MEFs were approximately 50% confluent. For the

treatment, MEFs were exposed to 150 μM of hydrogen peroxide in the culturing media for 2 hours. Cells were washed with 1X PBS twice and complete media was replaced. Cells recovered for 4 days and were starved for 3 days in serum-free media for co-culturing. Ras^{G12V} and MDA-MB-231 cells were starved in their respective media for 3 days in concurrence with the MEF cells. Additionally, the MDA-MB-231 cells were supplemented with 5μg/ml of insulin when starved in serum free media. Cells were co-cultured by trypsinizing the Ras^{G12V} or MDA-MB-231 cells, neutralizing in complete media, and layering: 37,500 Ras^{G12V} cells on top of the 50% confluent starved 10 cm plate of MEFs or 37,500 MDA-MB-231 on top of the starved 6 cm plate of MEFs**.** Co-cultures were grown for 7 days, and stained with DAPI and Ki67 (Ras^{G12V} co-cultures) or DAPI and crystal violet (MDA-MB-231 co-cultures). For Ras^{G12V} co-cultures, results were quantified by counting the number of nuclei or Ki67 positive cells in 30 random fields per sample. For MDA-MB-231, results were quantified by counting the number of colonies in the DAPI stained dishes with greater than 20 cells, and the crystal violet quantification is found under section 2.21.

2.19 CONTRIBUTION OF EXTRACELLULAR MATRIX AND SECRETORY FACTORS IN CO-CULTURE PROLIFERATION

Wildtype and Cav-1 knockout MEFs were treated with oxidative stress and starved the same way as listed above under co-cultures in section 2.17. Ras^{G12V} were also starved as stated above. Before co-culturing, conditioned media was collected and saved from the MEFs. The MEF plates were washed twice with PBS and cells were removed by incubating in Cell Dissociation Buffer in PBS (Gibco) for 1-2 hours or until >90% of MEFs were removed from the dish. The

dishes were washed with PBS three times to remove any residual debris, cells, or dissociation buffer. Conditioned media was replaced onto the respective dishes along with 37,500 NIH 3T3 Ras^{G12V} transformed fibroblasts. Results were quantified as listed for Ras cells under Co-cultures section 2.19.

2.20 CRYSTAL VIOLET STAINING OF CO-CULTURES

MDA-MB-231 cells were subjected to crystal violet staining by incubating the cells with 0.1 gram per ml crystal violet in 70% ethanol for 2 minutes followed by extensive washes with PBS. Quantification of crystal violet staining was performed as followed: the image was preprocessed by cropping the central area of each plate, converting to the HSV color space, and finding connected regions of pixels with saturation greater than 0.2 on a [0, 1] scale. To reduce noise, only colonies with an area of > 32 pixels were counted ($\geq 0.3 mm^2$).

2.21 TUMORGENESIS ASSAY

Wildtype and Cav-1 knockout mouse embryonic fibroblasts were treated with 150 μM of hydrogen peroxide in the culturing media for 2 hours. Cells were rinsed twice with 1X PBS and allowed to recover for 7 days. The chart below shows the cell combinations, which were subcutaneously injected into the dorsal flap of nude (nu/nu) mice (5-6 weeks old from Charles Rivers Laboratories):

Table 1. Cell number and combinations injected into nude mice.

CELL COMBINATION	NUMBER OF CELLS
MDA-MB-231 ONLY	325,000
RAS ONLY	325,000
WT OR KO MEFS ONLY	1,000,000
MDA-MB-231 + KO MEFS	325,000 + 1,000,000
MDA-MB-231 + WT MEFS	325,000 + 1,000,000
RAS + KO MEFS	325,000 + 1,000,000
RAS +WT MEFS	325,000 + 1,000,000

All cells were resuspended into a 100 μL volume of PBS. Four weeks after the injection, tumors were excised and the x and y axes measured to determine tumor volume (size) according to the following formula: volume = 0.5 x width^2 x length. When only one population of cells was injected, two mice were injected in duplicate for a total of 4 tumor masses for each population of cells. When two populations were injected in combination, three mice were injected in duplicate totaling 6 measurable tumor masses for each combination. This animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

2.22 STATISTICAL ANALYSIS

Statistical analyses of data were conducted using Microsoft Excel software. The averages were determined using the AVERAGE function. Standard error of the mean was determined by

dividing the sample estimate of the standard deviation (computed with the STDEV function) by the square root of the number of values in the sample. The p-values were calculated using the Student's T-test.

3.0 OXIDATIVE STRESS INDUCES PREMATURE SENESCENCE BY STIMULATING CAVEOLIN-1 GENE TRANSCRIPTION THROUGH P38 MITOGEN-ACTIVATED PROTEIN KINASE/ SP1-MEDIATED ACTIVATION OF TWO GC-RICH PROMOTER ELEMENTS

3.1 INTRODUCTION

Cav-1 has been indicated to be a tumor suppressor. The protein has been shown to inhibit signaling molecules associated with cell proliferation, and it is down-regulated in many cancers. For example, the Cav-1 gene is hypermethylated in certain colon cancers (Mori, Cai et al. 2006). It is also downregulated in invasive ductal carcinoma of the breast (Park, Kim et al. 2005) and inactivated by downregulation or mutation in oral cancer (Han, Park et al. 2004). The caveolin-1 gene is localized at chromosome 7q31.1, a region deleted in many human cancers (Razani, et al 2002 and van Deurs, et al. 2003). Cav-1 is down-regulated in many human breast cancer cell lines and re-introduction of the protein into these cells result in growth inhibition and reduced colony formation in soft agar (Lee, Reimer et al. 1998). Down-regulation of Cav-1 in NIH 3T3 fibroblasts leads to cellular transformation (Koleske, Baltimore et al. 1995). In the 3T3 fibroblasts transformed by v-Abl or H-Ras^{G12V} the induction of Cav-1 abrogates anchorage-independent growth (Engelman, Wycoff et al. 1997).

While down regulation of Cav-1 is linked to cancer, there is also evidence connecting high levels of caveolin-1 with cellular senescence. Cav-1 over-expression can cause the growth arrest of mouse embryonic fibroblasts in G_0/G_1, reduce proliferation, and induce p53/p21 dependent SIPS (Galbiati, Volonte et al. 2001; Volonte, Zhang et al. 2002). Over-expression of Cav-1 is sufficient to induce premature senescence. Likewise, endogenous Cav-1 expression is up regulated in senescent cells. Oxidative SIPS is mediated by an up-regulation of Cav-1 (Volonte, et al. 2002). Cav-1 may play a role in aging because the protein's expression increases in aged tissues (Cho, et al. 2003). Additionally, the senescent phenotype is reversed by a reduction in Cav-1 expression by siRNA (Cho, Ryu et al. 2003).

Subcytotoxic levels of hydrogen peroxide treatment to cells generate SIPS with the characteristic phenotype of an enlarged morphology, cessation of DNA replication, and senescence associated-ß-galactosidase activity (Chen, Bartholomew et al. 1998; Frippiat, Chen et al. 2000; Frippiat, Dewelle et al. 2002). Work on the signaling pathway leading to SIPS shows $p38^{MAPK}$ is phosphorylated in IMR-90 hTERT cells after hydrogen peroxide treatment (Zdanov, Debacq-Chainiaux et al. 2006), and oncogenic induced SIPS occurs through activation of $p38^{MAPK}$ (Deng, Liao et al. 2004). Further research shows the initial cellular insult does not activate $p38^{MAPK}$, but activation of the kinase is through an unidentified cellular condition caused by the insult (Iwasa, Han et al. 2003). One of $p38^{MAPK}$'s downstream targets is the transcription factor Sp1 (D'Addario, Arora et al. 2006). Hydrogen peroxide increases phosphorylation of Sp1 (Chu and Ferro 2006). Additionally, multiple phosphorylations of Sp1 induces the transcription factor's binding to GC rich regions in DNA (Kadonaga, Carner et al. 1987; Jackson, MacDonald et al. 1990).

Subcytotoxic hydrogen peroxide treatment amplifies expression of Cav-1, which is followed by an escalation of p53 activity and SIPS (Volonte, Zhang et al. 2002). The oxidative stress signal transduction pathway leading to Cav-1 upregulation and SIPS is not well defined. Here we hypothesize $p38^{MAPK}$ may be a factor leading to Cav-1 up-regulation through Sp1. Additionally we explore SIPS in breast cancer epithelial cells. If a cancer cell retains wildtype p53 activity, it should be possible for these cells to undergo Cav-1 dependent senescence. We hypothesize MCF7 cells, a wildtype p53 but null Cav-1 breast cancer cell line, will not undergo senescence from oxidative stress.

3.2 RESULTS

3.2.1 Oxidative stress activates Sp1 which binds to GC-rich regions in the Cav-1 promoter

Increased Cav-1 protein expression from oxidative stress is necessary for SIPS in fibroblasts (Volonte, Zhang et al. 2002). The signaling events leading to Cav-1 upregulation are undefined. To identify oxidative stress response elements in Cav-1's gene promoter, deletion mutants of the promoter were fused to the luciferase gene. These constructs were transfected into NIH 3T3 fibroblasts and 24 hours later were treated with 150 μM H_2O_2 for 2 hours, rinsed and allowed to recover for 2 days in normal media. A luciferase activity assay identified 2 oxidative responsive elements between nucleotides -372 to -222 and -150 to -91 in the Cav-1 promoter. Analysis of these regions revealed GC-rich boxes. To determine whether these GC boxes were responsive to oxidative stress, the nucleotide sequences were fused to a luciferase gene. The constructs were

transfected into NIH 3T3 fibroblasts and were treated with hydrogen peroxide as listed above. A luciferase activity assay shows nucleotides -244/-22 and -124/-101 in the Cav-1 promoter responded to hydrogen peroxide (data not shown) (Dasari, Bartholomew et al. 2006).

Electromobility shift assays (EMSAs) were utilized to determine whether proteins would bind to the oxidative stress response elements found. Nuclear extracts from cells treated ± hydrogen peroxide were incubated with nucleotide oligos of the identified GC-rich oxidative stress response elements. Data from EMSAs reveal nucleotide sequences -244/-222 and -124/-101 from the Cav-1 promoter formed nucleoprotein complexes upon hydrogen peroxide treatment. Lysates from cells treated with hydrogen peroxide were incubated in the presence of unlabelled Sp1 consensus sequences. There was decreased formation of nucleoprotein complexes (data not shown) (Dasari, Bartholomew et al. 2006). A chromatin immunoprecipitation (ChIP) was done with the Sp1 antibody to determine whether Sp1 directly binds to the GC-boxes in the Cav-1 gene promoter after oxidative stress. NIH 3T3 cells were treated with or without hydrogen peroxide as described for the EMSA assay. Input was taken of the samples prior to ChIP. Results show increased binding of Sp1 to the Cav-1 promoter after oxidative stress compared to non-treated controls (Figure 4).

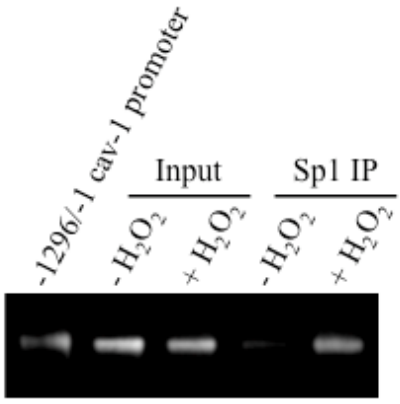


Figure 4. Sp1 binds directly to the Cav-1 gene promoter upon oxidative stress. Chromatin immunoprecipitation (ChIP) assay was done on chromatin derived from untreated or hydrogen peroxide–treated (150 μM for 2 hours) NIH 3T3 cells 48 hours after oxidative stress. Briefly, DNA was crosslinked with 1% formaldehyde and samples were normalized based on cell number. Sonication was used to produce DNA fragments approximately 250-1000 bp in length. ChIP lysate was precleared with Protein A Sepharose beads conjugated to salmon sperm DNA for 30 minutes at 4°C. Ten percent of the sample was taken for Input. ChIP lysate was incubated with the beads and 3 μg Sp1 antibody overnight. A control was done using a random antibody that did not bind to the promoter (data not shown). Samples were reverse crosslinked and PCR was done using primers amplifying the region of the caveolin-1 promoter containing the two GC-rich boxes. Amplification of input DNA from both untreated and hydrogen peroxide (H_2O_2)-treated cells was done before immunoprecipitation. A vector containing the entire caveolin-1 promoter sequence was used as a positive control for PCR. Results show Sp1 binding to the Cav-1 promoter after oxidative stress.

The Sp1 transcription factor is known to bind to GC-rich regions and mediate oxidative stress-induced gene transcription (Yang, Wilson-Van Patten et al. 2000; Ryu, Lee et al. 2003; Schafer, Cramer et al. 2003). In Figure 5, NIH 3T3 cells were treated with 150 μM hydrogen peroxide for 2 hours, rinsed and allowed to recover for 48 hours. Immunoblot analysis using anti-Sp1 shows the protein's expression increases from oxidative stress.

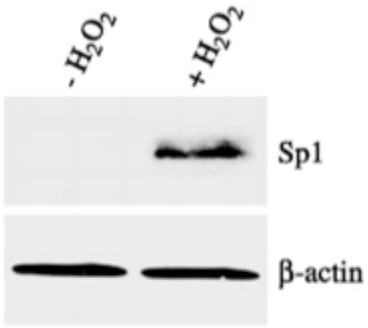


Figure 5. Sp1 expression increases in response to oxidative stress in fibroblasts. NIH 3T3 cells were treated with or without 150 μM hydrogen peroxide (H_2O_2) for 2 hours. After 48 hours, cells were collected and the expression of endogenous Sp1 was evaluated by immunoblotting analysis with an antibody probe specific for Sp1. Immunoblotting with anti-ß-actin IgGs was done to show equal loading.

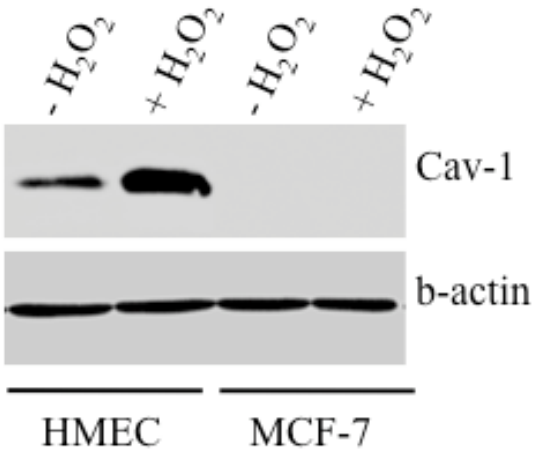
3.2.2 Contribution of $p38^{MAPK}$ to stress induced premature senescence

We showed that $p38^{MAPK}$ is activated by phosphorylation 48 hours after oxidative stress from hydrogen peroxide. NIH 3T3 fibroblasts were transfected with the GC-rich regions of the caveolin-1 promoter fused to a luciferase gene and treated with hydrogen peroxide in the presence or absence of the $p38^{MAPK}$ inhibitor SB203580. Results from a luciferase reporter assay show diminished activity at the oxidative stress response sites in the Cav-1 promoter when $p38^{MAPK}$ is inhibited. Likewise, Cav-1 and $p21^{WAF/CIP1}$ are not upregulated after oxidative stress when $p38^{MAPK}$ is inhibited by SB203580. Inhibition of $p38^{MAPK}$ also prevented SIPS as observed by senescence associated-ß-galactosidase staining activity (data not shown) (Dasari, Bartholomew et al. 2006).

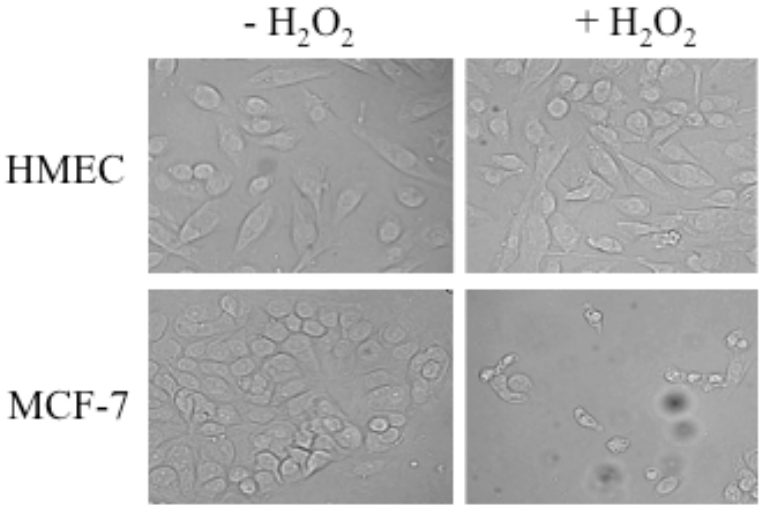
3.2.3 Breast cancer cells with a Cav-1 null background undergo apoptosis when treated with oxidative stress

MCF7 cells are human breast cancer derived epithelial cells with wildtype p53 expression (Olivier, Bautista et al. 1998). Cav-1 is downregulated in MCF7 cells compared to normal human mammary epithelial cells (HMEC) due to hypermethylation of CpG islands in the promoter sequence (Engelman, Zhang et al. 1999). Because of this background, the assumption was made that MCF7 cells should not be able to undergo SIPS when treated with hydrogen peroxide. It is hypothesized Cav-1 will be needed to rescue the SIPS phenotype after oxidative stress. Cav-1 expressing HMECs were utilized as a control and do not have the hypermethylation in the gene's promoter (Engelman, Zhang et al. 1999). HMEC and MCF7 cells were equally plated and treated with 450 μM hydrogen peroxide for 2 hours, rinsed twice with PBS and allowed to recover for 24 hours. Immunoblot analysis of normalized cellular lysate shows the MCF7 cells do not upregulate Cav-1, whereas the HMEC cells actually increase Cav-1 expression after oxidative stress (Figure 6A). Observation of cell morphology and cell number showed MCF7 cells were dying (Figure 6B). Twenty-four hours after treatment, MCF7 cells decreased in number by 60% based on total cell number from 10 random fields (Figure 6C). In contrast, HMEC cells did not decline in cell number and their morphology had no drastic change.

A.


- H_2O_2
+ H_2O_2
- H_2O_2
+ H_2O_2
Cav-1
b-actin
HMEC
MCF-7

B.


- H_2O_2
+ H_2O_2
HMEC
MCF-7

C.

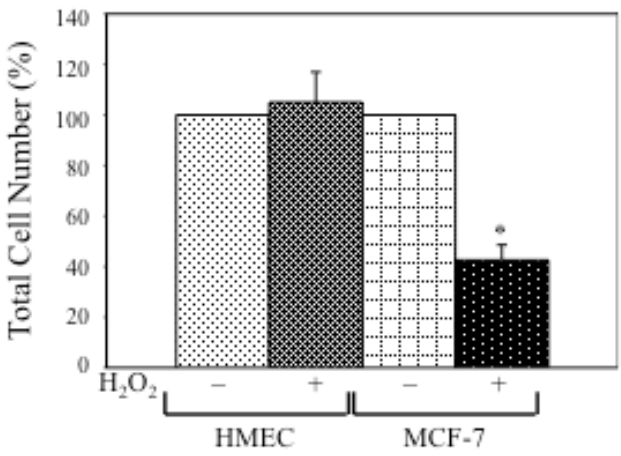

Total Cell Number (%)
140
120
100
80
60
40
20
0
H_2O_2
–
+
–
+
*
HMEC
MCF-7

Figure 6. MCF7 cells lacking Cav-1 expression do not survive oxidative stress. HMEC and MCF7 cells were treated with 450 μM of hydrogen peroxide for 2 hours, rinsed and allowed to recover in normal media for 24 hours. The cells were lysed, normalized based on protein concentration and subjected to SDS-PAGE. Immunoblot analysis using anti-Cav-1 shows expression and oxidative upregulation in the HMEC but not the MCF7 cells. Anti-β-actin shows equal loading (A). Representative field of HMEC and MCF7 cells treated as listed in A (B). Cells were treated as listed in (A) and 24 hours later, ten random fields were counted for cell number and expressed as a total cell number percentage (C). There was a 60% decrease in the number of MCF7 cells after oxidative stress. *Columns*, mean; SE. *, $P<0.005$

MCF7 cells treated with oxidative stress show diminished cell number (Figure 6). DAPI staining and Terminal Transferase dUTP Nick End Labeling (TUNEL) assays were conducted to determine whether these cells were going through programmed cell death. Results from DAPI staining show HMEC's nuclei do not condense, regardless of whether cells are treated with ± hydrogen peroxide. In contrast, MCF7 cells show marked condensation of cellular nuclei, a marker of apoptosis (Figure 7A). To further explore the possibility of apoptosis in these cells, a TUNEL assay was conducted. Results show treated and untreated HMEC and $-H_2O_2$ MCF7 cells did not exhibit TUNEL positive cells, as expected from cell number assays and DAPI staining. However, MCF7 cells treated with oxidative stress had an approximately 55-fold increase in TUNEL positive cells in comparison (Figure 7B).

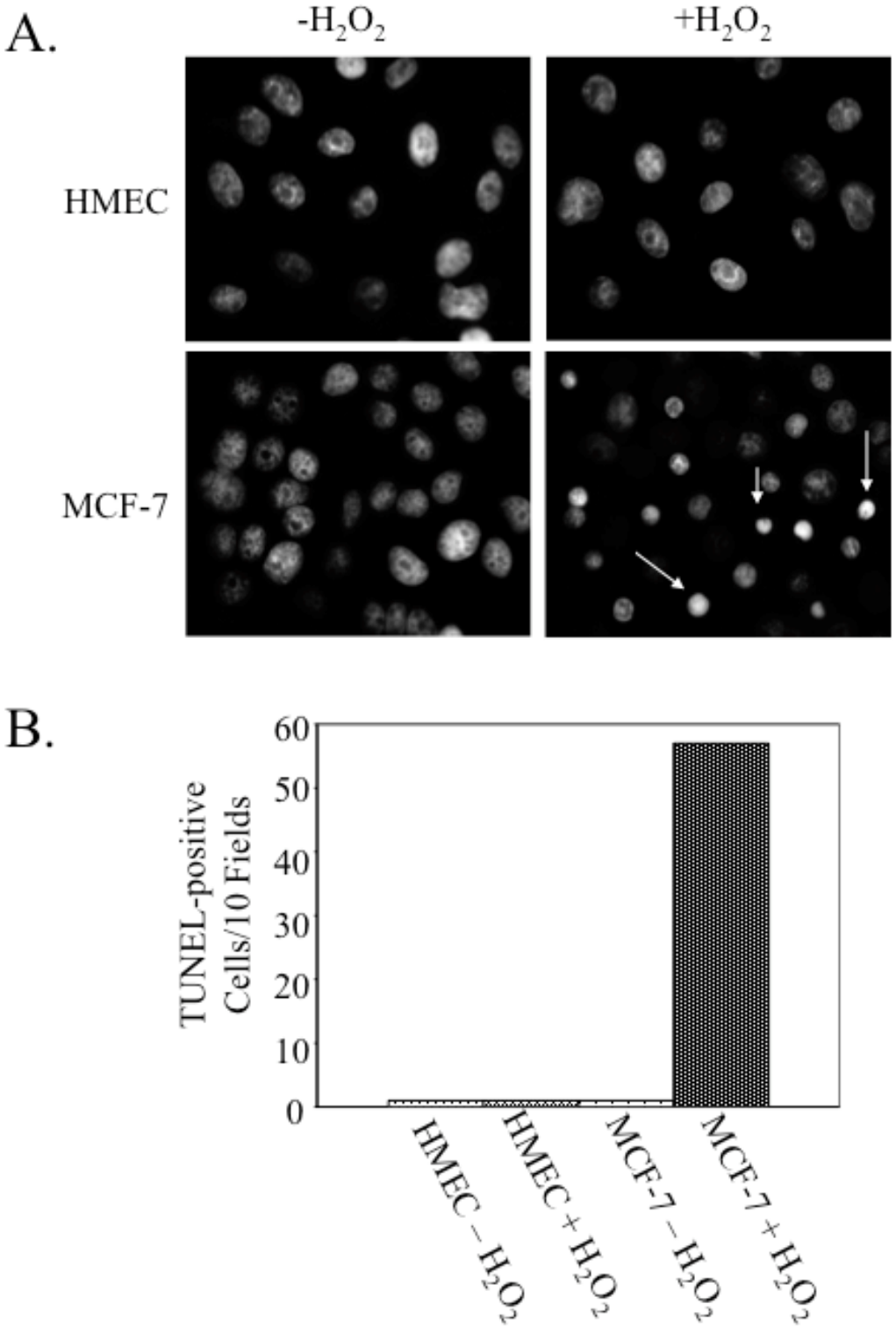

A.
-H2O2
+H2O2
HMEC
MCF-7
B.
TUNEL-positive Cells/10 Fields
60
50
40
30
20
10
0
HMEC – H2O2
HMEC + H2O2
MCF-7 – H2O2
MCF-7 + H2O2

Figure 7. Oxidative stress induces apoptosis in epithelial breast cancer cells. Human mammary epithelial cells and MCF-7 cells were treated with or without hydrogen peroxide (450 μmol/L) for 2 hours and allowed to recover for 24 hours. Nuclear morphology was examined by DAPI staining. Representative field shows HMEC nuclei do not change, whereas there is nuclei condensation in the MCF7 cells (A). Apoptotic cells were detected by TUNEL analysis. Cells were treated as listed in (A). TUNEL was conducted according to manufacturer's instructions (see Materials and Methods). The total number of TUNEL-positive cells was counted per 10 random fields (B).

Since the HMEC increase Cav-1 expression, we hypothesized these cells would undergo oxidatively stressed induced SIPS. SA-β-galactosidase staining of HMEC cells shows after hydrogen peroxide treatment, HMEC cells exhibit an 80% increase in cells stained positive for SA-β-galactosidase as compared to untreated cells. MCF7 cells ± hydrogen peroxide do not have any significant change in the amount of cells positive for SA-β-galactosidase (Figure 8).

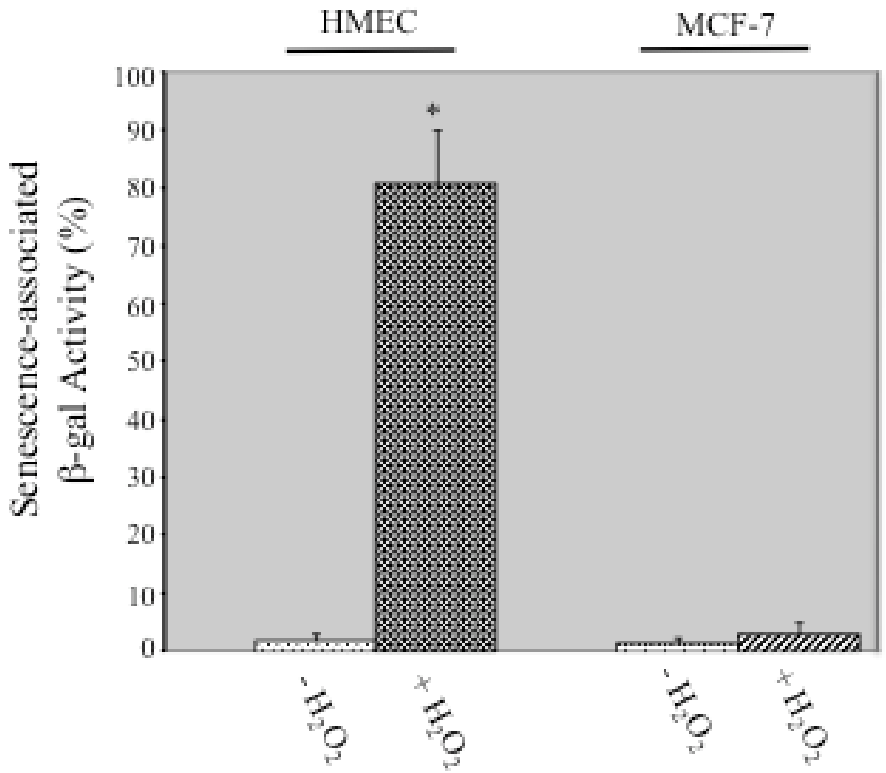


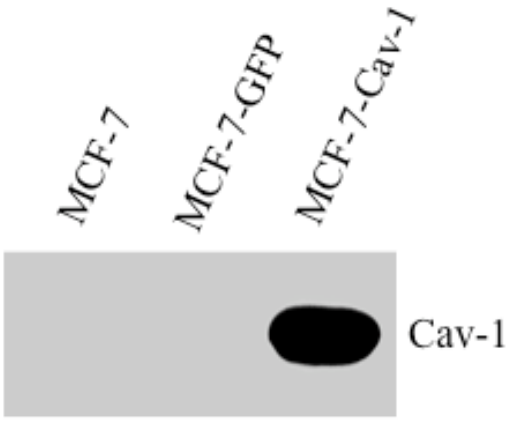
Figure 8. Oxidative stress induces SIPS in HMEC but not MCF7 cells. Cell lines were treated as in Figure 6. Untreated cells were used as a control. Cells were subjected to senescence-associated ß-galactosidase activity assay and observed under a BX50WI Olympus Optical light microscope at a magnification of x10. Values represent means ± SEM. **P*<0.001.

3.2.4 SIPS phenotype is restored by expression of Cav-1 in MCF7 cells

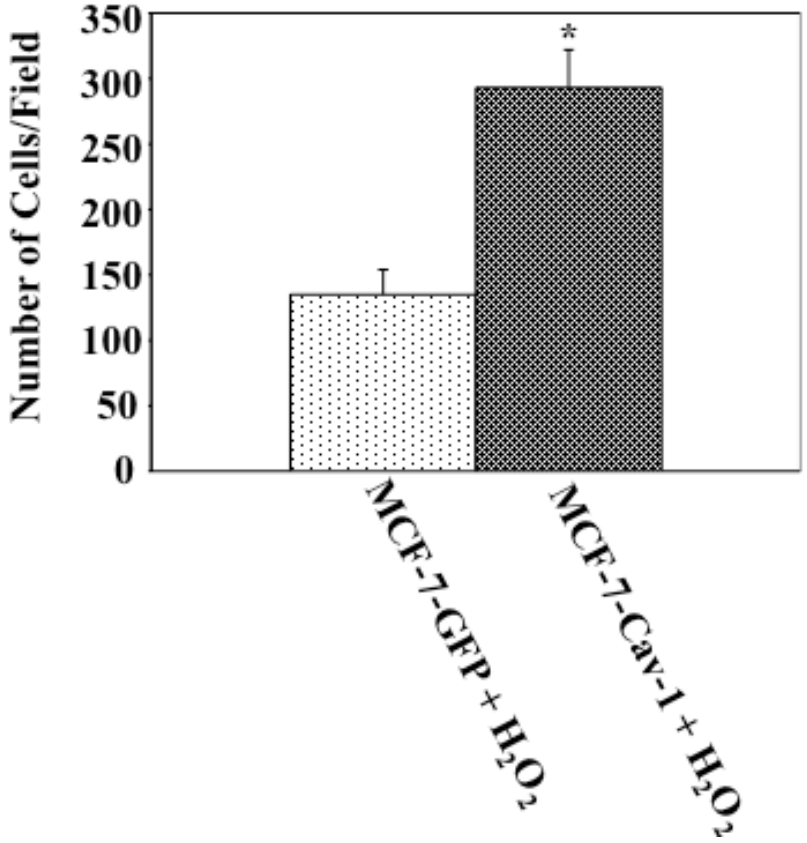
MCF7 cells contain a wildtype p53 gene. Thus, its conceivable that restoration of SIPS by p53 up-regulation in these breast cancer cells would be restored by Cav-1 re-expression. To test this hypothesis an adenovirus carrying the gene for Cav-1 (Ad-Cav-1) was utilized to infect MCF7

cells along with an adenovirus containing GFP (Ad-GFP) for a control. Cells were infected as described in the Materials and Methods section. An immunoblot shows 48 hours after infection, Ad-Cav-1 restored Cav-1 expression in MCF7 cells (Figure 9A). To determine whether this would allow MCF7 cells to undergo SIPS upon oxidative stress, cells were infected with Ad-GFP or Ad-Cav-1 and 24 hours later cells were treated with hydrogen peroxide and allowed to recover for 24 hours. Results show that the average number of cells in 10 random fields is over 2-fold greater in Ad-Cav-1 than Ad-GFP (Figure 9B). Likewise, DAPI staining of infected cells shows a greater number of condensed apoptotic nuclei in Ad-GFP than in Ad-Cav-1 (Figure 9C). This trend of Cav-1 re-expression protecting from apoptosis was also present when TUNEL assay was conducted. TUNEL positive cells decreased by 70% with the re-expression of Cav-1 as compared to Ad-GFP cells (Figure 9D). Since MCF7 cells were not undergoing apoptosis after Cav-1 re-expression, we wanted to determine whether Cav-1 could rescue the SIPS phenotype in these cells. Infected MCF7 cells were treated with oxidative stress and recovered for 7 days (during which time they underwent additional infections with virus). An assay for senescence associated-β-galactosidase staining shows that Ad-Cav-1 restores senescence in MCF7 cells. Ad-GFP MCF7 cells underwent apoptosis (Figure 9E).

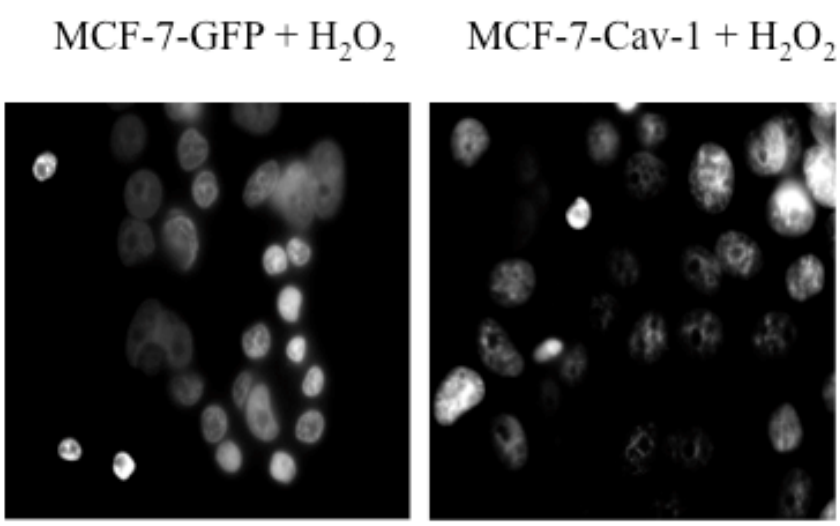
A.


MCF-7
MCF-7-GFP
MCF-7-Cav-1
Cav-1

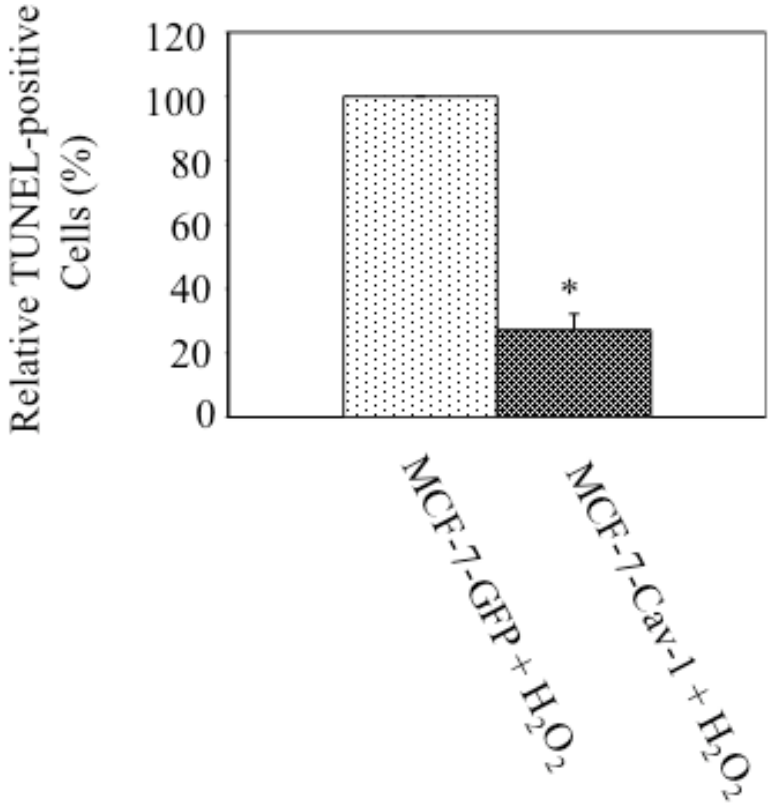
B.


Number of Cells/Field
350
300
250
200
150
100
50
0
*
MCF-7-GFP + H2O2
MCF-7-Cav-1 + H2O2

C.


MCF-7-GFP + H_2O_2
MCF-7-Cav-1 + H_2O_2

D.


Relative TUNEL-positive Cells (%)
120
100
80
60
40
20
0
*
MCF-7-GFP + H_2O_2
MCF-7-Cav-1 + H_2O_2

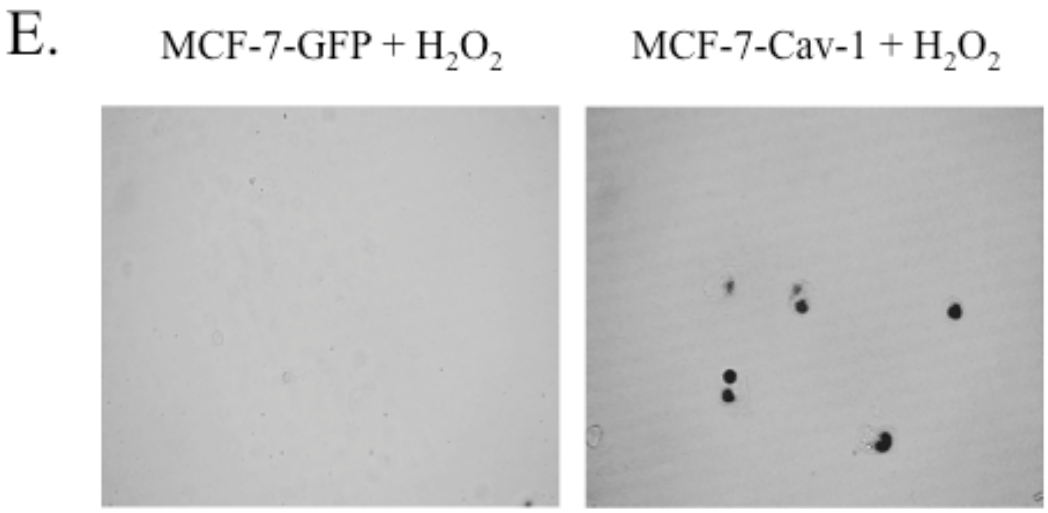


Figure 9. Re-expression of Cav-1 decreases apoptosis in MCF7 cells and induces SIPS. MCF-7 cells were infected with green fluorescent protein (GFP) or caveolin-1 using adenovirus. 24 hours later, cells were treated with 450 μM hydrogen peroxide for 2 hours and allowed to recover for 24 hours. An immunoblot for Cav-1 expression shows that infection increases the protein's expression (A). Counting of 10 random fields showed that Cav-1 expression increased total cell number after oxidative stress (B). Values represent means ± SEM. **P*<0.005. Nuclear morphology was examined by DAPI staining and a representative fields is shown in (C). Apoptotic cells were detected by TUNEL analysis. The total number of TUNEL-positive cells is expressed as a relative percentage. *Columns,* mean; *bars,* SE. *, $P < 0.005$ (D). MCF7 cells were infected with GFP or Cav-1 as in (A) and allowed to recover for 7 days. Cells were subjected to senescence-associated-β-galactosidase activity assay and observed under a BX50WI Olympus Optical light microscope at a magnification of x10. Representative field (E).

3.3 DISCUSSION

Aging is a multifaceted process associated with many physiological changes. A common hypothesis is the accumulation of somatic mutations, damage by ROS and the contribution of antagonistic pleiotropic genes add to aging. Current aging research indicates that cellular senescence plays a role in the aging process (Campisi 1996; Smith and Pereira-Smith 1996; Campisi 1997). This is supported by data showing senescent cells accumulate with age (Dimri, Lee et al. 1995; Mishima, Handa et al. 1999; Herbig, Ferreira et al. 2006; Jeyapalan, Ferreira et al. 2007). Interestingly, while senescence is a tumor suppressor mechanism (Campisi 2005) there is oddly an increased cancer risk with age. Therefore, it is imperative to explore the benefits of aging by examining cellular senescence and how it can protect against cancer.

The membrane scaffolding protein, Cav-1, is linked to aging and cancer, and is the target of our current studies to delineate the mechanisms of aging and cancer. Cav-1 expression is known to increase in aging tissues (Kawabe, Grant et al. 2001). Likewise, it is upregulated when cells are subjected to oxidative insult. Interestingly, Cav-1 is necessary for p53 and $p21^{WAF/CIP1}$ dependent SIPS (Volonte, Zhang et al. 2002). Cav-1 is also downregulated in numerous cancers (Williams and Lisanti 2005), and in particular, breast cancer (Xie, Zeng et al. 2003; Chen, Lin et al. 2004; Zhang, Shen et al. 2005).

While oxidative stress upregulates Cav-1, the signal transduction pathway leading to the protein's upregulation is not known. We discovered oxidative stress elements in the Cav-1 promoter respond to hydrogen peroxide treatment. These regions contain GC-rich boxes, which we hypothesized would be activated by Sp1 binding. Preceding this we found that oxidative stress caused $p38^{MAPK}$ activation, which was necessary for the upregulation of the Cav-1 gene. We propose that oxidative stress induces cellular changes, which phosphorylated $p38^{MAPK}$

resulting in its activation and subsequent activation of its downstream target Sp1. Sp1 binds to the GC-boxes in the Cav-1 promoter, upregulating the protein's expression resulting in SIPS (Figure 10). Recent data also provides another example of the involvement of $p38^{MAPK}$ in pathways that induce growth arrest in breast cancer cells. The growth factor activin depends on $p38^{MAPK}$ to induce growth arrest in breast cancer cell line T47D (Cocolakis, Lemay et al. 2001).

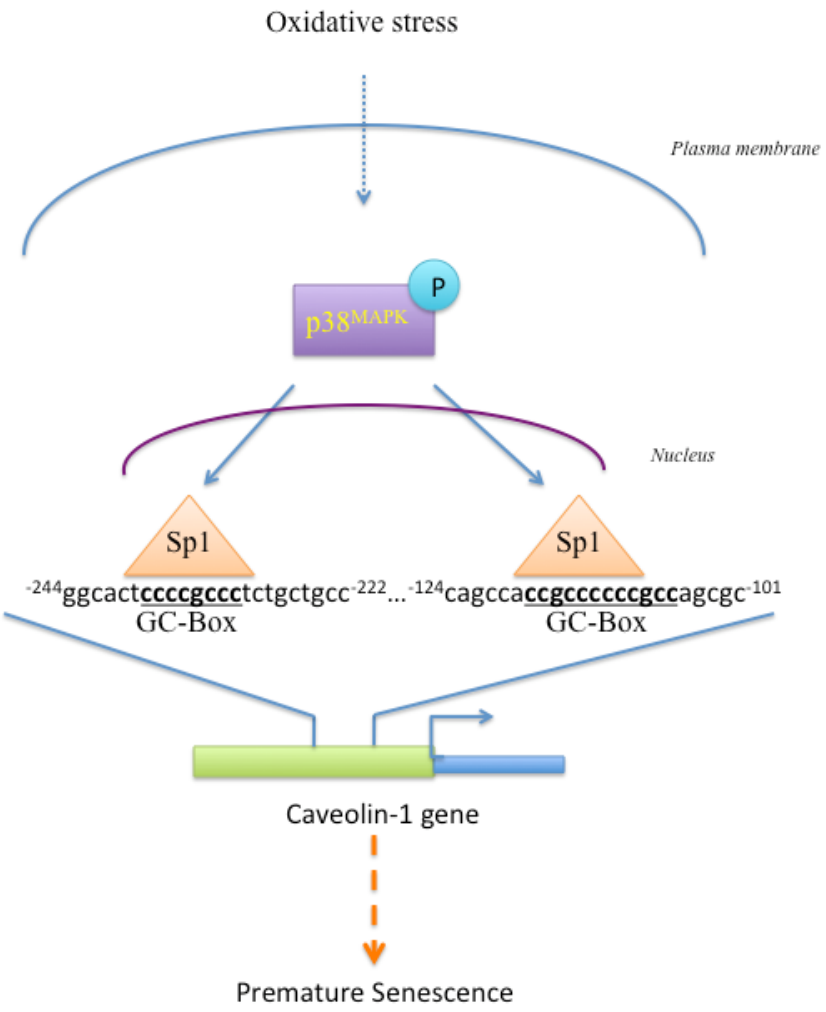


Figure 10. Schematic diagram of the Cav-1 gene promoter activated by oxidative stress. Oxidative stress induces cellular changes that upregulate $p38^{MAPK}$ by phosphorylation. The kinase activates transcription factor Sp1,

which binds to 2 GC-boxes in the Cav-1 promoter. Finally, the Cav-1 protein is upregulated and mediates stress induced premature senescence.

Transcription factors promote protective effects against ROS by regulating transcription of genes whose products manage cellular damage (Lezoualc'h and Behl 1998; Post, Holsboer et al. 1998; Scortegagna, Galdzicki et al. 1999). Here we show that Sp1 protects from ROS by binding to the Cav-1 promoter, leading to increased Cav-1 expression, which triggers SIPS. The Sp1 transcription factor, ubiquitously expressed in mammalian tissues, is a member of a family of DNA binding proteins that have 3 zinc finger motifs and bind to GC-rich DNA (Kadonaga, Carner et al. 1987; Courey, Holtzman et al. 1989). Work by another group has shown that Sp1 expression is related to Cav-1 and p53 expression. In MCF7 cells, exogenous expression of Cav-1 increased Sp1 levels in p53 expressing cells but not in p53 null MCF7 cells, thus linking the three proteins (Glait, Tencer et al. 2006). We have shown one mechanism of how Sp1 is involved in the induction of SIPS. However, Sp1 regulates other tumor suppressor genes as well. For example connexins, the protein component of gap junctions, are implicated in tumor suppression, as loss of communication via gap junctions appears to play a role in oncogenesis Sp1 binds to the Connexin 26 (Cx 26) GC-rich promoter and induces activation. Cx 26 is a structural component of gap junctions, and like Cav-1, is down regulated in breast cancer by methylation of its promoter, (Engelman, Zhang et al. 1999; Tan, Bianco et al. 2002).

Sp1 can also inhibit the transcription of certain genes. Senescence marker protein-30 (SMP30) enhances plasma membrane calcium pump activity and is protective against calcium ionophore-induced apoptosis (Jeong, Goo et al. 2008). Sp1 binds to its promoter and repress SMP30 (Supakar, Fujita et al. 2000) ((Rath, Pandey et al. 2008). Interestingly, SMP30

expression is shown to decrease in aging rat livers (Fujita, Shirasawa et al. 1998). This example also fits with our model of senescence, or aging, in which Sp1 is upregulated. A study on Sp1 and aging shows that with age there is an inverse correlation between Sp1 DNA-binding activity and total protein expression in old versus young rats (Ammendola, Mesuraca et al. 1994). This is intriguing in light of our results. It must be noted that premature senescence is a process that occurs in the young and old alike. Oxidative damage up-regulates Sp1 and causes cellular senescence. It is not known whether Sp1 activity remains elevated for an extended amount of time after oxidative stress. It is conceivable that Sp1 activates Cav-1 leading to senescence, but then the transcription factor's activity is not needed to maintain the senescence phenotype. Thus, in older tissues, where there would be an accumulation of senescent cells, there could be overall less Sp1 activity.

Our results indicate the breast cancer epithelial cell line, MCF7 does not undergo SIPS when exposed to hydrogen peroxide but alternatively undergoes apoptosis. This is in contrast to normal mammary epithelial cells (HMEC), which undergo SIPS. Even though MCF7 cells have a functional p53 protein, SIPS is still dependent upon Cav-1 expression. Cav-1 dependent SIPS is in multiple cell types (e.g. fibroblasts and epithelial cells), thus, illustrating Cav-1 is necessary for oxidatively induced SIPS. In the breast cancer Cav-1 null background, cells did not preferentially undergo SIPS, but instead undergo apoptosis. Therefore, we hypothesize that Cav-1 may function as a "senescence switch" in the presence of a cellular insult (Figure 11). When Cav-1 is present in a p53 wildtype background cells will undergo SIPS, and in the absence of Cav-1, cells will undergo alternative outcomes. Our results demonstrating Cav-1 induced senescence and tumor suppression in MCF-7 cells has been supported by evidence *in vivo* in

which Cav-1 expression in MCF-7 cells decreased invasion and migration (Wu, Wang et al. 2008), thus, suggesting Cav-1 acts as a tumor suppressor in these cells.

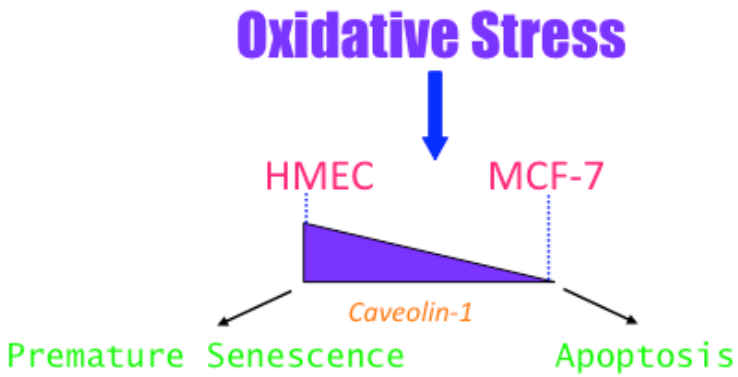


Figure 11. Schematic diagram of Cav-1 expression as a "senescence switch". Data from MCF7 and HMEC cells show that when epithelial cells express Cav-1, after oxidative stress the cells will undergo SIPS. However, cells in the Cav-1 deficient breast cancer cell line MCF7 preferentially undergo apoptosis.

From a therapeutic standpoint, these data shed light on new targets for anti-aging. The absence of Cav-1 in MCF7 cells is advantageous for a non-aging phenotype. Therefore, downregulation of the Cav-1 gene could possibly be a therapeutic target eliminating SIPS when it is deleterious to the organism. In addition to Cav-1, its upstream regulator, $p38^{MAPK}$ could be a target of anti-aging therapy as it is necessary for Cav-1 mediated SIPS. Inhibitors of $p38^{MAPK}$ could block SIPS in response to oxidative damage. However, this could cause proliferation of cells harboring genetic mutations that could be harmful to the organism.

Our research was done from the perspective that Cav-1 is downregulated in cancer and serves as a tumor suppressor. There are examples, even in breast cancers, where Cav-1 is upregulated (Pinilla, Honrado et al. 2006; Garcia, Dales et al. 2007). These are generally very

aggressive malignancies. It could be that mutations have occurred during the transformation of the cells, which allowed them to overcome SIPS irrespective of Cav-1 expression.

4.0 CAV-1 MEDIATES SIPS THROUGH P53 UP-REGULATION BY SEQUESTRATION OF MDM2

4.1 INTRODUCTION

Caveolae, plasma membrane organelles, are 50-100 nm flask-shaped invaginations in the plasma membrane. Caveolae are a subset of lipid rafts and are rich in cholesterol and sphingolipids making them Triton X-100 insoluble. The Cav-1 protein is a structural component of caveolae membranes (van Deurs, et al. 2003). Caveolae are present in a vast number of cells including fibroblasts, adipocytes, endothelial cells, type I pneumocytes, epithelial cells, smooth and striated muscle cells (Galbiati, et al, 2001). The physiological roles of this microdomain vary among different cell types and organ systems (Razani, et al 2002). For instance, caveolae function in endocytosis, transcytosis, cholesterol transport, homeostasis, positive and negative regulation of Ras-, NO-, G-protein coupled receptor-, and growth-factor mediated signaling (van Deurs, et al. 2003). Because of their low density and Triton X-100 insolubility, caveolae are easily isolated from the rest of the plasma membrane by treatment with mild nonionic detergents and sucrose ultracentrifugation. The Cav-1 protein is used as a selective marker for caveolae and verifies isolation of the caveolae microdomain from the rest of the plasma membrane (Razani, et al 2002).

Cellular localization of signaling molecules is mediated by scaffolding proteins (Faux and Scott 1996). Cav-1's cellular behavior is indicative of a scaffolding protein: Cav-1 forms multivalent homo-oligomers, most of the signaling molecules that interact with Cav-1 bind to the same site on the protein, and upon binding Cav-1 holds most signaling molecules in an inactive conformation (Li, Couet et al. 1996). The caveolin protein acts as a direct inhibitory scaffolding protein for numerous signaling molecules such as the G-protein α subunit, nitric oxide synthase (NOS), protein kinase C (PKC), and protein kinase A (PKA) (Garcia-Cardena, Oh et al. 1996; Li, Couet et al. 1996; Engelman, Zhang et al. 1998; Galbiati, Volonte et al. 1999; Razani, Rubin et al. 1999) There are a few exceptions to this, for example Cav-1 can stimulate the estrogen and insulin receptor signaling (Yamamoto, Toya et al. 1998; Schlegel, Wang et al. 2001). The majority of proteins seem to bind to the caveolin scaffolding domain (CSD), which spans residues 82-101 (Li et al. 1995) within the membrane-proximal region. Recently, work done with a cell permeable peptide of the CSD, deemed Cavtratin, shows that the minimal CSD can inhibit signaling molecules *in vitro* and *in vivo*. These studies add strength to the argument that Cav-1 is a scaffolding protein, as the CSD is sufficient to see results, which the full length Cav-1 protein would exhibit. For example, there is down-regulation of Cav-1 in atherosclerotic lesions, and treating porcine arteries with Cavtratin inhibited the activity of enzymes associated with plaque instability (Rodriguez-Feo, Hellings et al. 2008). Cav-1 null tumors exhibit hyper-permeability and increased eNOS activity. Treatment with Cavtratin inhibited eNOS and had antitumor effects (Gratton, Lin et al. 2003; Bauser-Heaton, Song et al. 2008). Additionally, Lewis lung carcinoma cells injected into Cav-1 knockout mice show increased tumor vascular permeability and higher tumor growth rates. Treatment of the knockout mice with Cavtratin attenuated tumor hyper-permeability and decreased tumor growth (Lin, Yu et al. 2007).

Furthermore, Cavtratin antagonizes the effects of siRNA knockdown of Cav-1 in brain microvascular endothelial cells (Song, Ge et al. 2007). Lastly, Cav-1 can mediate tumor suppressor effects. Use of the Cavtratin in metastatic PyMT mammary carcinoma cell line, MET, shows that Cavtratin is sufficient to inhibit invasion of these cells (Williams, Medina et al. 2004). The proteins that bind to the CSD share common amino acid motifs, deemed the caveolin binding domain (CBD). These sequences are variations of the following amino acid patterns ΦX ΦXXXX Φ, ΦXXXX ΦXX Φ or ΦXΦXXXXΦXXΦ, where X is any amino acid and Φ is an aromatic reside (Couet et al. 1997a).

Cav-1 inhibits signaling molecules associated with cell proliferation and is down-regulated in many cancers. This association is supported by the fact that the Cav-1 gene is localized at chromosome 7q31.1, a region deleted in many human cancers. These observations suggest Cav-1 acts as a tumor suppressor protein (Razani, et al 2002 and van Deurs, et al. 2003). Likewise, the Cav-1 gene is down-regulated in a myriad of cancers: breast adenocarcinoma, cervix carcinoma, lung small cell carcinoma, various mesenchyme sarcoma, ovary carcinoma, thyroid follicular carcinoma, vascular angiosarcoma (Williams and Lisanti 2005), osteosarcoma (Cantiani, Manara et al. 2007), and mucoepidermoid carcinoma (Shi, Chen et al. 2007).

While down regulation of Cav-1 is linked to cancer, high levels of Cav-1 are linked to cellular senescence. Data from our laboratory and others distinguish the protein Cav-1 as a key mediator of SIPS in fibroblasts. Senescent cells are characterized by growth arrest. Also, the cell morphology becomes larger and flatter, and expresses increased SA-β-Galactosidase activity (Cho, et al. 2003). Senescence is part of the natural aging process, and is a tumor-protection mechanism (Pelicci, 2004). Cav-1 induces growth arrest (Galbiati, Volonte et al. 2001) and evidence for Cav-1's growth inhibitory function is its overexpression causes transcriptional

repression of cyclin-D (Hulit, Bash et al. 2000) and knocking out Cav-1 causes upregulation of cyclin D1 levels (Williams, Cheung et al. 2003). It has been shown that overexpression of Cav-1 is sufficient to induce premature senescence in mouse embryonic fibroblasts. Likewise, endogenous Cav-1 expression is up-regulated in senescent cells (Volonte, Zhang et al. 2002), and the senescent phenotype is reversed by reducing Cav-1 expression with siRNA (Cho, Ryu et al. 2003). Cav-1 is implicated in the signaling pathway triggered by oxidative stress, which leads to SIPS (Dasari, Bartholomew et al. 2006). Oxidative stress induced premature senescence occurs through up-regulation of Cav-1. Additionally, Cav-1 has been linked to p53 activation and the accumulation of its downstream target p21 (Volonte, Zhang et al. 2002). Furthermore, Cav-1 may play a role in aging because the protein's expression increases in aged tissues (Cho, Ryu et al. 2003).

Taken together, these data lead to the hypothesis that Cav-1 and the proteins interacting with it have the potential to disclose the link between aging and cancer. Because aging and cell senescence seem to be a natural deterrent for cancer, knowing how aging is regulated and the proteins involved in the process will be important for developing effective cancer therapies.

p53 is a known tumor suppressor activated by cellular stresses such as DNA damage, oxidative stress and oncogenic signals. Once activated, p53 transcribes genes that allow the cell to go through apoptosis or growth arrest and senescence (Pelicci, 2004). p53 deters cells from passing on damaged DNA as the protein is the primary regulator of apoptosis, growth arrest and senescence. Under normal, non-stressed conditions, p53 levels are kept low by its short half-life (5-30 minutes) by its negative regulator, MDM2 (Levine 1997; Wu and Levine 1997). The importance of MDM2's regulation of p53 has been illustrated in knockout mouse models. In MDM2 knockouts, embryos die before E6.5. However, if MDM2 null mice are crossed with p53

knockouts, there is no lethality (Jones, Roe et al. 1995). Interestingly, MDM2 is a target gene of the p53 transcription factor (Barak, Juven et al. 1993; Chen, Marechal et al. 1993; Picksley and Lane 1993). The two proteins are involved in a negative autoregulatory feedback loop. MDM2 keeps p53 abundance low under non-stressful conditions and also limits biological responses after stress (Haupt, Maya et al. 1997; Kubbutat, Jones et al. 1997). The MDM2 protein is an E3 ubiquitin ligase and promotes p53 ubiquitination and degradation by nuclear and cytoplasmic 26S proteasomes (Bottger, Bottger et al. 1997; Haupt, Maya et al. 1997; Maki 1999; Shirangi, Zaika et al. 2002). Ubiquitination adds ubiquitin chains on lysine residues (Moll and Petrenko 2003). MDM2's E3 ligase activity is located in its C-terminal zinc binding RING finger domain. This domain transfers monoubiquitin tags to p53's C-terminal (Honda, Tanaka et al. 1997; Kubbutat, Ludwig et al. 1998; Fang, Jensen et al. 2000). Crystallographic data show that the p53 transactivation domain binds in the deep hydrophobic cleft of MDM2, deterring p53's interaction with transcriptional machinery. MDM2's N-terminus interacts with the α-helix of the p53 N-terminal transactivation domain (Kussie, Gorina et al. 1996), resulting in MDM2 repressing the transcriptional activity of p53 (Yang, et al. 2004(Oliner, Pietenpol et al. 1993). When p53 needs to be expressed in response to oncogenic signaling, MDM2 is targeted by Ataxia telangiectasia mutated (ATM) for phosphorylation. Modification by phosphorylation occurs in MDM2's p53 binding domain and central acid domain. These domains are thought to be required for p53 degradation, and thus blocking of these domains can lead to p53 activity (Hay and Meek 2000). Additional regulation of MDM2 is from p14 Alternative Reading Frame (ARF), which can bind to the RING finger domain of MDM2 and inhibit its E3 ligase activity (Honda and Yasuda 1999). Interestingly, over-expression of caveolin -1 activates p53 (Galbiati et al., 2001b).

Data in chapter 3 shows that hydrogen peroxide induced oxidative stress stimulates a signaling cascade that activates $p38^{MAPK}$ and its downstream target, the transcription factor Sp1. Sp1 binds to two GC-rich regions in the Cav-1 gene promoter and induces Cav-1 gene transcription. The up-regulation of Cav-1 is followed by p53 up-regulation and induction of SIPS (Dasari, Bartholomew et al. 2006). How Cav-1 increases p53 activity leading to SIPS is not known. We hypothesize that MDM2, the negative regulator of p53, is sequestered by Cav-1 upon oxidative stress, decreasing MDM2's ability to degrade p53, allowing the transcription factor to up-regulate cell cycle arrest genes and bring about SIPS.

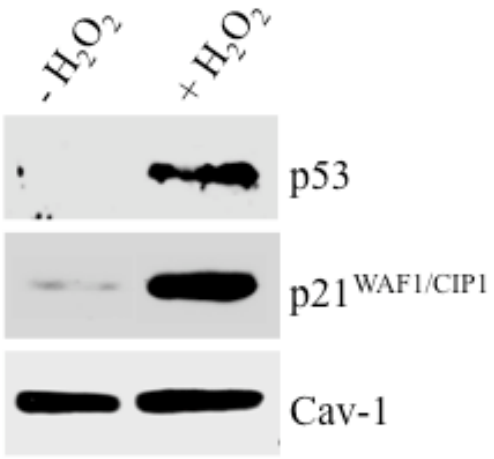
4.2 RESULTS

4.2.1 Characterization of Sequestration of MDM2 by Cav-1 After Oxidative Stress

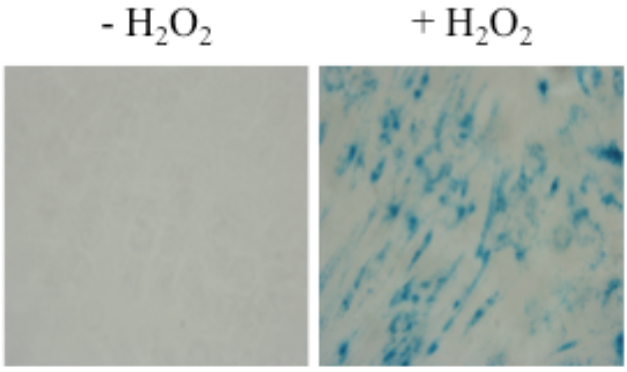
4.2.1.1 Oxidative stress from hydrogen peroxide induces SIPS

Subcytotoxic doses of hydrogen peroxide can induce SIPS in human and rodent fibroblasts. To show that these doses induce SIPS in the human cell line WI-38 fetal lung fibroblasts, the cells were treated with 450 μM of hydrogen peroxide for 2 hours, were washed twice in 1X PBS and allowed to recover for 24 hours. Immunoblotting shows that p53, p21 and Cav-1, all markers of SIPS, are up-regulated after hydrogen peroxide treatment (Figure 12A). Following the 7 days of treatment, Senescence Associated–ß-galactosidase (SA- ß-galactosidase) activity staining was conducted to detect senescent cells. Results in Figure 12B and C show that there is a 100-fold difference in SA- ß-galactosidase activity between treated and untreated groups.

A.


- H_2O_2
+ H_2O_2
p53
p21 WAF1/CIP1
Cav-1

B.


- H_2O_2
+ H_2O_2

C.

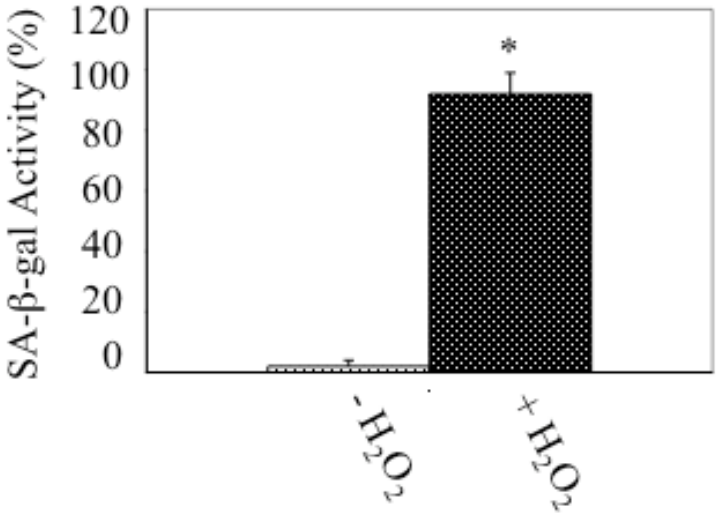

SA-β-gal Activity (%)
120
100
80
60
40
20
0
*
- H_2O_2
+ H_2O_2

Figure 12. Oxidative stress induces SIPS in fibroblasts. (A) Immunoblot analysis of proteins up-regulated in SIPS. WI-38 human fetal lung fibroblasts were treated with 450 μM of hydrogen peroxide (H_2O_2) for two hours, were washed twice in 1X PBS and recovered for 24 hours. Normalized cell lysates were subjected to SDS-PAGE and the resulting blots were probed with antibodies for p53, $p21^{WAF/CIP1}$ and Cav-1. (B) WI-38 human fetal lung fibroblasts were treated as listed in (A) and allowed to recover for 7 days. Cells were subjected to SA–ß-Galactosidase Activity assay according the manufacturer's instructions (Cell Signaling). Staining was analyzed using a BX50WI Olympus Optical light microscope at a magnification of 10X. A representative field is shown in (B). Quantification of the assay is shown in (C). Values represent means ± SEM. **P*<0.001.

4.2.1.2 MDM2 translocates to detergent resistant membranes after oxidative stress

Caveolae are membrane invaginations that are a subclass of lipid rafts. These lipid rafts are cholesterol and spingolipid rich areas that contain polymerized caveolin proteins. Caveolins are membrane bound proteins that are palmitoylated and bind cholesterol (Dietzen, Hastings et al. 1995; Murata, Peranen et al. 1995; Li, Song et al. 1996; Smart, Ying et al. 1996). Therefore by isolating the caveolae, it is possible to isolate proteins that may be associated with caveolae and caveolins. Caveolae are part of the class of detergent resistant membranes (DRMs), which will float to low densities in sucrose gradients. Therefore, sucrose gradients are used to sort membranes by size or density and identify proteins with lipid raft association (Simons and Ikonen 1997; Brown and London 1998; Hooper 1999; Simons and Toomre 2000). Caveolins are established protein markers of caveolae and can thus be used to determine isolation of the organelle. Cav-1 and its associated proteins are Triton X-100 detergent resistant. Therefore, in a sucrose gradient Cav-1, Flotillin-1 and other lipid raft associated proteins float in detergent insoluble fractions. This is normally at the interface between 5-30% sucrose. In a 12-fraction sucrose gradient, this would correspond to fractions 4, 5, and 6. If enough protein is present and the gradient has run properly, the lipid raft fraction also has a visible ring of insoluble proteins.

A sucrose gradient was employed to determine whether oxidative stress caused movement of MDM2 into caveolae fractions from soluble fractions. Results in Figure 13 show that gradient separation was successful, because ± hydrogen peroxide both have Cav-1 in the insoluble fractions 4-6. Additionally, p53 is known to increase with oxidative stress (Volonte, Zhang et al. 2002). The immunoblot for p53 shows that under normal conditions p53 expression is undetectable. However, there is a marked increase in the protein's expression with oxidative stress, demonstrating that the hydrogen peroxide treatment activated distress signaling in these cells. p53 is found in soluble fractions where nuclear proteins and other non-raft associated proteins reside. There is an increase in MDM2 expression after hydrogen peroxide treatment attributed to the increase in p53. (p53 can induce MDM2 expression as part of its negative feedback loop for eliminating itself.) Figure 13 shows that under normal conditions, MDM2 is localized in soluble fractions (9, 10, and 11) along with p53. However, after oxidative stress, MDM2 translocates to insoluble fractions (4, 5, and 6) where Cav-1 and lipid rafts are localized. After hydrogen peroxide treatment MDM2 is found in fractions where it can no longer localize with p53.

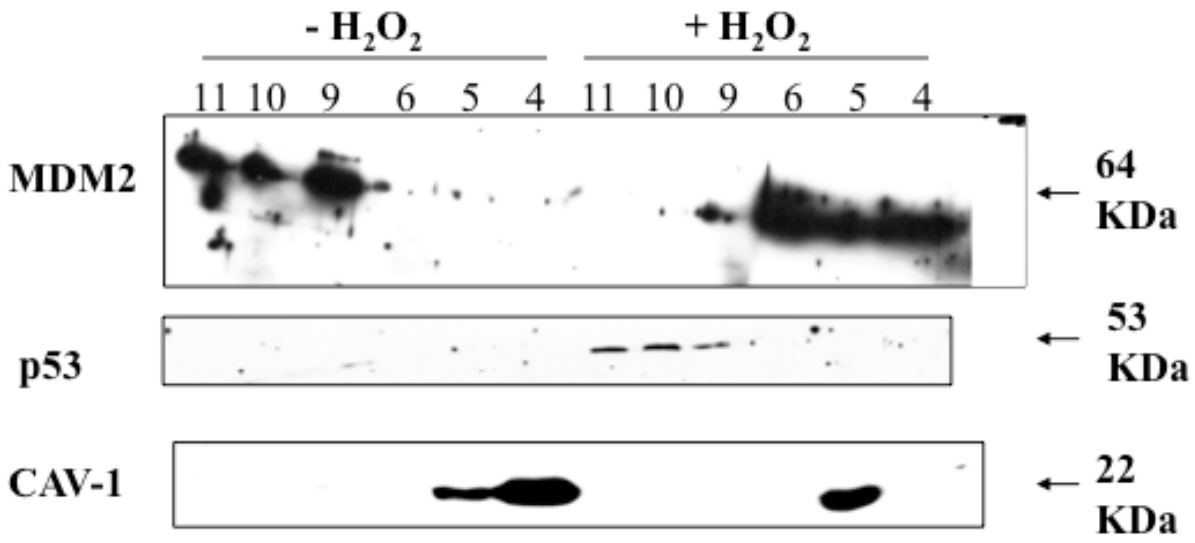


Figure 13. MDM2 translocates to caveolae-associated fractions of a sucrose density gradient upon hydrogen peroxide treatment. WI-38 fetal lung fibroblasts were treated ± 450 μM hydrogen peroxide (H_2O_2) for 2 hours, washed twice with 1X PBS and allowed to recover for 24 hours in regular growth media. Samples were subjected to sucrose density centrifugation by layering 40-5% sucrose and centrifuging for 16 hours. 12 fractions were collected from the top of gradient with the first one considered fraction one. Non-caveolae (fractions 9-11) and caveolae (fractions 4-6) fractions were analyzed using SDS-PAGE immunoblot analysis with antibodies for anti-MDM2, anti-p53 and anti-Cav-1. Data is representative of three independent experiments.

4.2.1.3 MDM2 localizes with Cav-1 after hydrogen peroxide induced oxidative stress

Sucrose gradient centrifugation showed that MDM2 translocates into Cav-1 associated lipid rafts after oxidative stress. Immunofluorescence microscopy was conducted to directly assess the location of MDM2 before and after oxidative stress. WI-38 fetal lung fibroblasts were treated ± hydrogen peroxide and 48 hours later staining for MDM2, p53, and Cav-1. Results from Figure 14A and Figure 14B show oxidative stress induced increases in Cav-1 and p53 protein expression, which agree with previous observations (Volonte, Zhang et al. 2002). In untreated cells, MDM2 co-localizes with p53 in the nucleus (Figure 14B). However, upon treatment, p53

intensity in the nucleus increases and MDM2 signal translocates to the cytoplasm and membrane of the cells. Results from Figure 14 also show that MDM2 re-localization causes a change in its compartmentalization with p53; it is no longer predominantly co-localized with p53. Additionally, in Figure 14A we see that with hydrogen peroxide induced oxidative stress, Cav-1 expression increases and, like MDM2, is also localized to the membrane and the cytoplasm. Following hydrogen peroxide treatment, there is movement of MDM2 from the nucleus to the membrane and cytoplasm where it is co-localized with Cav-1.

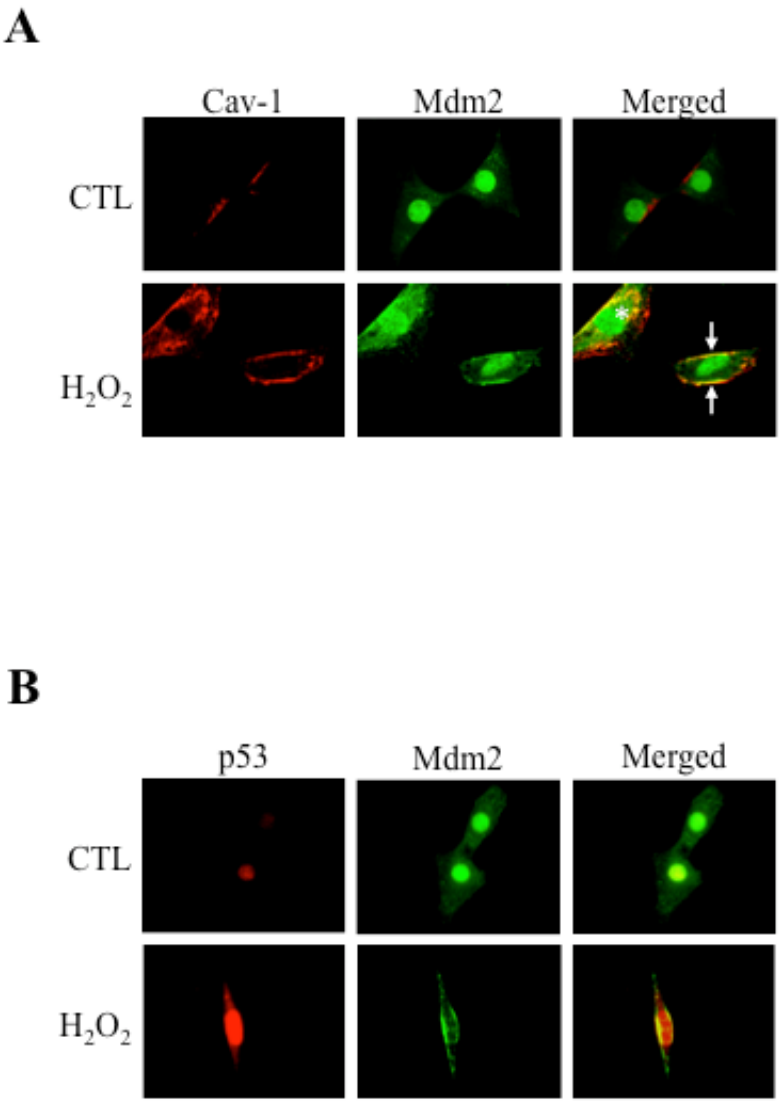


Figure 14. MDM2 co-localizes with Cav-1 after hydrogen peroxide induced oxidative stress. Wi-38 fetal lung fibroblasts were cultured on glass coverslips, treated with ± 450 µM hydrogen peroxide (H_2O_2) for 2 hours, washed twice with 1X PBS and allowed to recover for 24 hours in conditioned media. Cells were subjected to immunohistochemistry 48 hours after treatment. Cells were fixed with formaldehyde, permeabilized with Triton X-100 and co-stained with MDM2 and Cav-1 antibodies (A) or MDM2 and p53 (B) and visualized with fluorescent secondary antibodies. Control cells were not treated (CTL). Arrows show areas of co-localization. The asterisk

shows an area where MDM2 and Cav-1 co-localize in the cytoplasm. Images were taken on a Zeiss confocal microscope.

4.2.1.4 MDM2 plasmid construction

In order to detect protein-protein interactions more readily, a c-Myc tagged construct of murine MDM2 was created (WT-MDM2-cMyc). Reverse transcription of mRNA from mouse lung tissue resulted in the corresponding cDNA. Using common cloning techniques (see Methods for details), MDM2 was ligated into the pCAGGS vector. Figure 15 shows the protein is expressed 48 hours after transfection in NIH 3T3 fibroblasts. The same cloning technique was utilized to make point mutations in MDM2 (Φ→A-MDM2-cMyc) (see section 4.2.1.10). The mutant protein is also expressed in NIH 3T3 fibroblasts 48 hours after transfection. Both constructs were the appropriate molecular weight for the MDM2 protein.

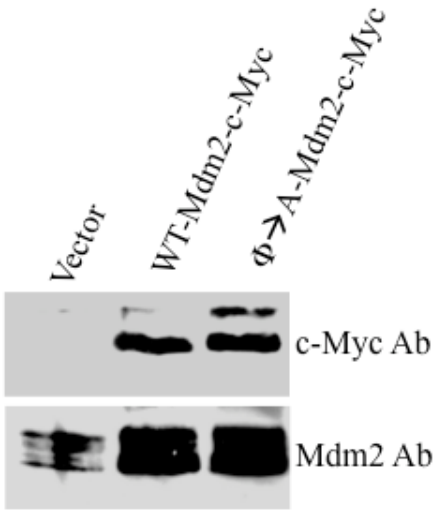


Figure 15. Expression of c-Myc tagged MDM2. The mouse MDM2 gene was cloned from murine lung tissue. Using common cloning techniques, it was c-Myc tagged and ligated into the pCAGGS vector. The sizes of the PCR products were consistent with the size of the gene (data not shown). NIH 3T3 fibroblasts were transfected by

calcium phosphate. Cells were lysed in boiling sample buffer 48 hours after transfection, normalized for protein concentration and subjected to SDS-PAGE and subsequent immunoblotting. Anti-MDM2 and anti-c-Myc antibodies show that wildtype MDM2 and the mutant form are equally expressed.

4.2.1.5 MDM2 and Cav-1 associate with one another after hydrogen peroxide treatment

Immunofluorescence experiments show that upon hydrogen peroxide treatment, MDM2 and Cav-1 co-localize together at the plasma membrane. To see whether MDM2 and Cav-1 associate with one another under oxidative stress, as suggested by the immunohistochemistry experiments, a co-immunoprecipitation was conducted. NIH 3T3 cells were transfected with 15 μg of each of Cav-1 and cMyc-MDM2 constructs. After 24 hours, the cells were treated with 150 mM hydrogen peroxide for 24 hours, whereas the control group was not treated with hydrogen peroxide. Normalized cellular extracts were subjected to immunoprecipitation using a cMyc monoclonal antibody to detect MDM2-c-Myc. Resolution of the results was determined by immunoblot analysis for the proteins of interest. Data shows that there is hardly any interaction between MDM2 and Cav-1 before oxidative stress. However, after hydrogen peroxide treatment there is a strong association between the two proteins. Immunoblotting of total cellular extract shows normalization of samples was achieved (Figure 16).

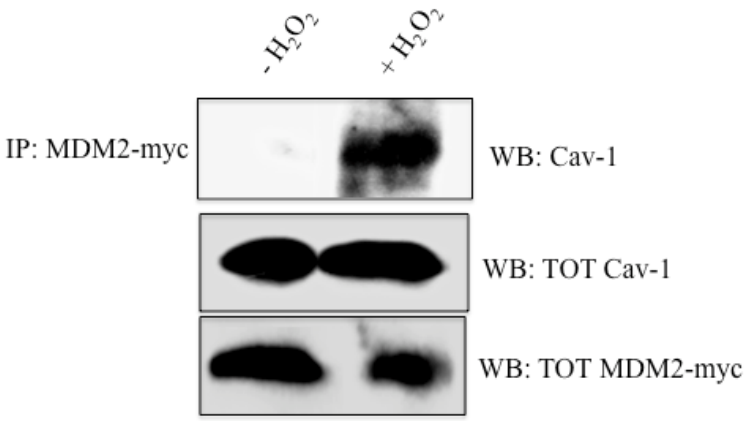


Figure 16. Hydrogen peroxide triggers association between MDM2 and Cav-1. NIH 3T3 cells were co-transfected with 15 μg of caveolin-1 and MDM2-c-Myc constructs. After 24 hours, the cells were treated with 150 mM hydrogen peroxide (H_2O_2) for 2 hours, whereas the control group was untreated. Normalized cellular extracts were subjected to immunoprecipitation using a cMyc monoclonal antibody. (cMyc was appropriate because the MDM2 construct has a Myc tag.) Immunoblot analysis anti-Cav-1 shows that the H_2O_2 treatment resulted in more interaction between the two proteins than the control group.

4.2.1.6 Caveolin scaffolding domain is the minimal sequence necessary to bind MDM2

Since the immunoprecipitation experiments show MDM2-Cav-1 association, we wanted to determine which amino acids on Cav-1 were necessary for the interaction. A GST pull-down was conducted using GST-fusion proteins. Fusion proteins were made by fusing glutathione transferase (GST) with the full length Cav-1 protein, a shorter fragment of Cav-1 (amino acids 1-101), and with the minimal Cav-1 scaffolding domain (amino acids 82-101). The fusion proteins were affinity purified by binding to glutathione agarose beads. Figure 17A shows that the purified fusion proteins that were expressed have the correct predicted molecular weights. The fusion proteins were incubated with cellular extract in which MDM2 was over-expressed. Denaturing the beads and conducting an immunoblot for MDM2 showed Cav-1 association.

Figure 17B, depicting the GST pulldown assay, shows MDM2 can bind to all of the GST-Cav-1 fusions, and the minimal amino acid sequence necessary is amino acids 82-101. This sufficient stretch of amino acids in Cav-1 corresponds to the caveolin scaffolding domain (CSD). The control of GST only (not fused to Cav-1) did not show any association with MDM2. This demonstrates absence of non-specific binding.

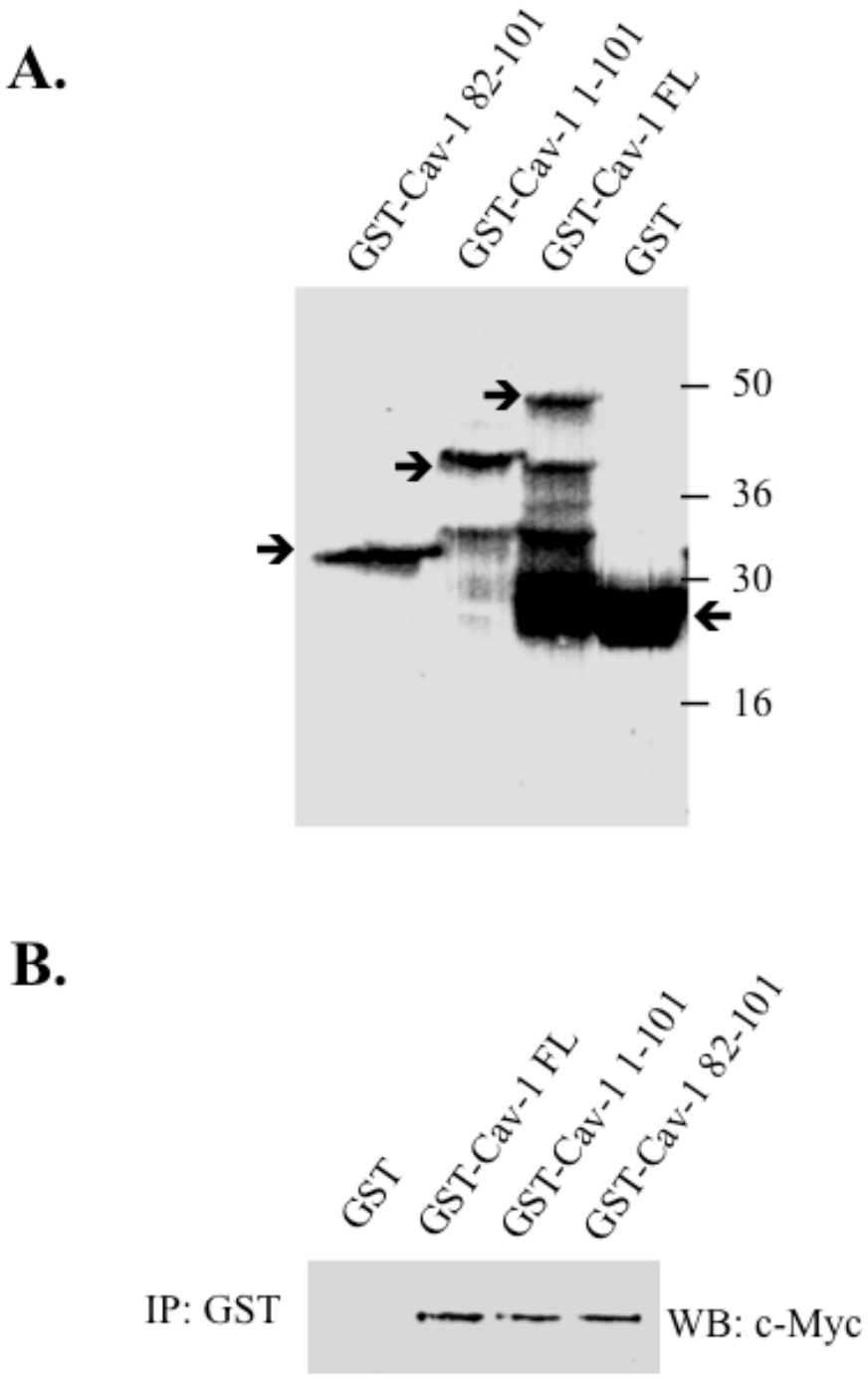


Figure 17. MDM2 binds to the Caveolin Scaffolding Domain. GST-Cav-1 fusion proteins containing full length Cav-1, amino acids 1-101, or only the CSD (amino acids 82-101) were affinity purified, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. Ponceau staining shows the correct size of the fusion proteins created highlighted by arrows (A). GST alone or GST- Cav-1 deletion mutants were conjugated to glutathione-agarose

beads and incubated overnight with cell lysate expressing exogenous MDM2-c-Myc. Eluted proteins from the GST pull-down were resolved by SDS-PAGE and immunoblotted for c-Myc (B) to detect MDM2 binding.

4.2.1.7 MDM2 contains a putative Caveolin Binding Domain (CBD)

Many signaling molecules that bind to the CSD contain a CBD (Couet, Li. et al. 1997). A CBD is normally defined to be a sequence motif with aromatic amino acids separated by a variable amount of amino acids. Since MDM2 associates with Cav-1, a search of its amino acid sequence was conducted to determine whether it contains a putative CBD. The amino acid sequences of human and mouse MDM2 contain a putative CBD. Figure 18A shows potential CBD motifs that have been verified to be in other signaling molecules (Couet, Li. et al. 1997). Evaluation of MDM2's amino acid sequence has shown that the protein contains one of these motifs located between amino acids 48-60: Y(6X)FY(3X)Y. Interestingly, this putative CBD is located within one of the 2 determined p53 binding domains on MDM2, which stretches from amino acids 23-108 in the MDM2 protein (Figure 18B).

A.

Mdm2: $_{48}\mathbf{Y(X_6)\ FY(X_3)Y}_{60}$

$\Phi(X_2)\Phi\Phi(X_3)\Phi$
$\Phi(X_3)\Phi\Phi(X_2)\Phi$
$\Phi(X_2)\Phi\Phi(X_1)\Phi$
$\Phi(X_1)\Phi\Phi(X_2)\Phi$
$\Phi(X_2)\Phi(X_2)\Phi\Phi(X_3)\Phi$
$\Phi(X_3)\Phi$
$\Phi(X_3)\Phi\Phi$
$\Phi(X_1)\Phi\Phi$
$\Phi(X_5)\Phi$
$\Phi(X_4)\Phi$
$\Phi(X_2)\Phi$
$\Phi(X_1)\Phi$

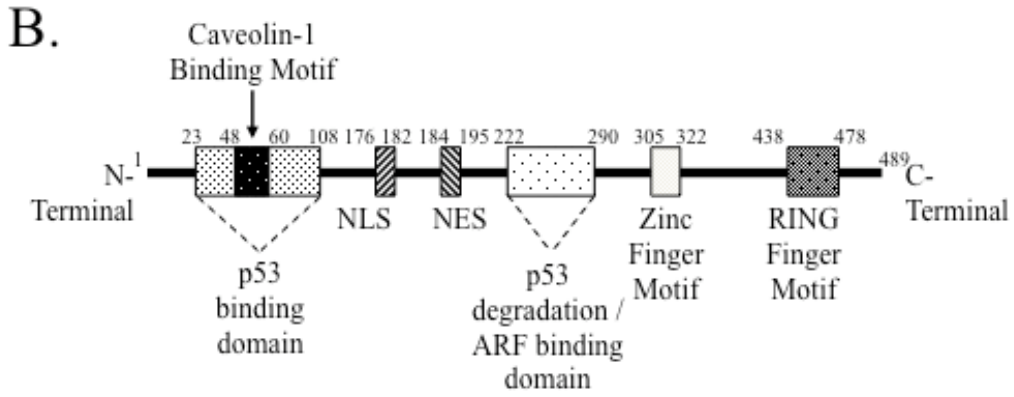


Figure 18. Putative Caveolin Binding Domain in the amino acid sequence of MDM2. MDM2 has a putative CBD located between amino acids 48-60. This is an area with aromatic resides, which corresponds to other CBD motifs found in other signaling molecules that bind to Cav-1. A list of other previously identified peptide ligands from screening phage-display libraries using a GST fusion protein containing the CSD is depicted. Φ = aromatic residue (W, Y or F) and X= any amino acid (A). A schematic of the MDM2 protein shows the CBD (residues 48-60) is located within the p53 binding domain (residues 23-108) of MDM2 (B).

4.2.1.8 Amino acids 48-60 on MDM2 are necessary for interaction with Cav-1

The previously listed experiments determined that the CSD was sufficient to interact with the MDM2 protein and stimulate SIPS. However, it is not known whether the putative CBD on MDM2 is necessary for the interaction with Cav-1. To test the hypothesis that the putative CBD in MDM2 stretches from the aromatic region of MDM2 between amino acids 48-60, a mutant MDM2 protein was made by PCR point mutation of specific nucleotides changing what would be aromatic residues to alanines. The mutant was expressed at the same level as the wildtype protein in NIH 3T3 cells (Figure 15). To determine whether the mutated Φ→A MDM2-c-Myc had decreased interaction with Cav-1, a GST pull-down using GST fused to the caveolin

scaffolding domain CSD was conducted. Results in Figure 19 show that when equal amounts of wildtype and mutant Φ→A MDM2 are expressed, the mutant ceases to bind to the CSD.

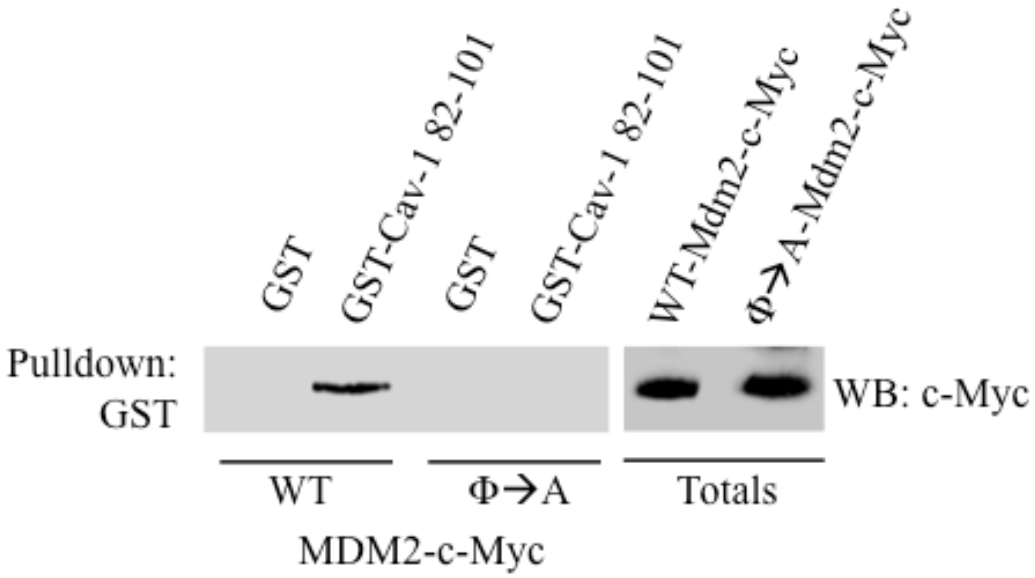


Figure 19. MDM2 with a mutated CBD does not associate with the CSD. Mutant MDM2 was constructed from the wildtype MDM2 template by PCR point mutation. A GST-CSD fusion protein was utilized in a GST pull-down assay. GST alone or fused GST-CSD was conjugated to glutathione sepharose beads. NIH 3T3 fibroblasts were transfected with 30 μg of wildtype (WT) or mutant (Φ→A) MDM2. Twenty-four hours later the cellular extracts were normalized based on MDM2's expression. Cellular extracts were incubated with the GST fusion beads overnight and eluted proteins were resolved by SDS-PAGE and immunoblotting for anti-c-Myc to detect MDM2.

4.2.1.9 Cav-1 can compete with p53 for MDM2 binding

Figure 19 demonstrates MDM2 contains a CBD. This domain is situated within amino acid residues 48-60. Interestingly, this falls within one of p53's binding domains on MDM2 (amino acid residues 23-108). Since the CBD is located within the p53 binding domain, it is

hypothesized that Cav-1 should compete with p53 for MDM2 binding. To test this hypothesis a competition assay between p53 and Cav-1 was conducted for MDM2 binding. NIH 3T3 fibroblasts were transfected with DNA by calcium phosphate in the following combinations:

p53	10 µg	10 µg	10 µg
MDM2	10 µg	10 µg	10 µg
Cav-1	0 µg	1 µg	5 µg
pCAGGS Vector	10 µg	9 µg	5 µg

Forty-eight hours later, an immunoprecipitation was conducted using c-Myc to pulldown MDM2-c-Myc associated proteins. Results were resolved by immunoblot analysis of Cav-1 and p53.

Figure 20 depicts total normalized cell lysate showing that the amount of Cav-1 DNA transfected was proportional to the protein's expression. Additionally, with increased Cav-1 expression, there is increased p53 expression in the normalized total cellular extract, even though equal amounts were transfected in each sample (see totals). This is to be expected based on previous findings that Cav-1 overexpression increases p53 levels (Galbiati, Volonte et al. 2001). Results show that with minimal Cav-1 present, MDM2 has a strong association with p53. As the amount of Cav-1 is increased there is disassociation between MDM2 and p53, with increasing association between MDM2 and Cav-1 (Figure 20).

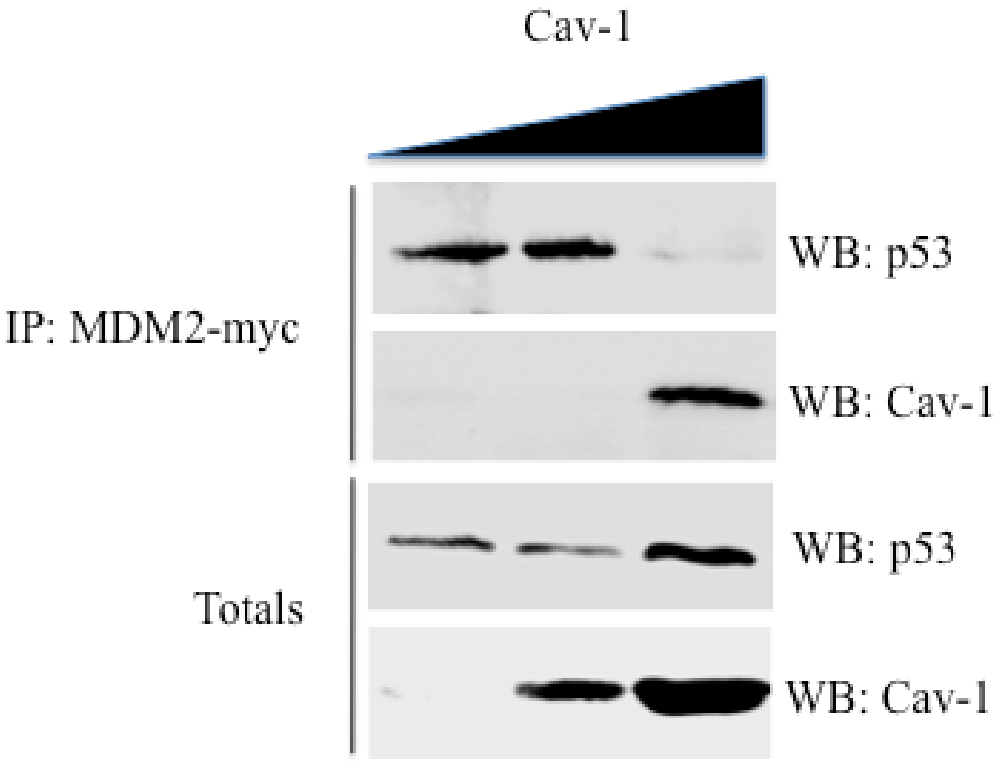
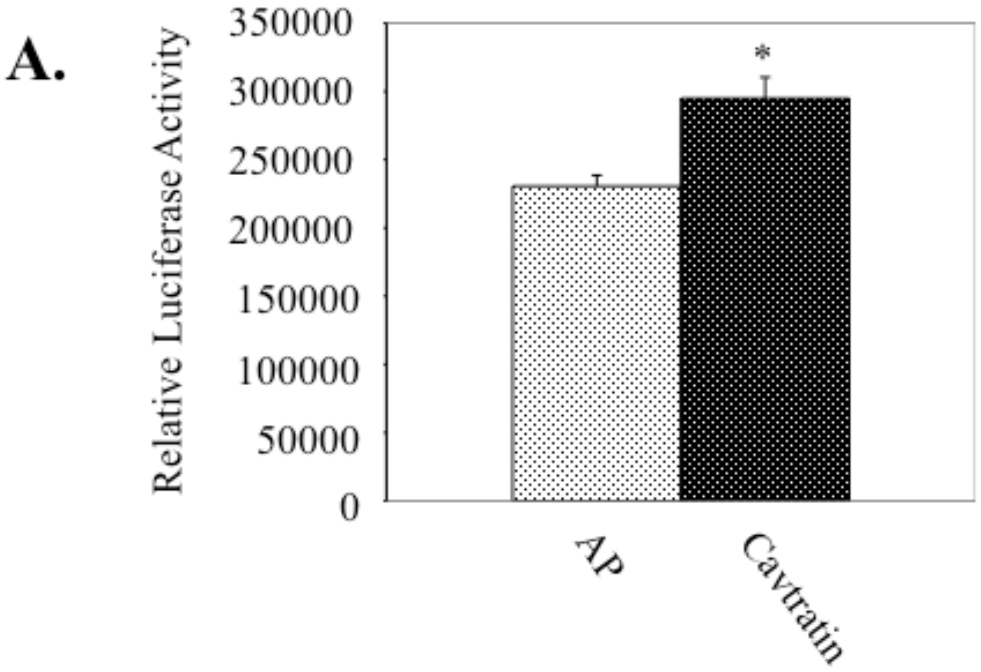


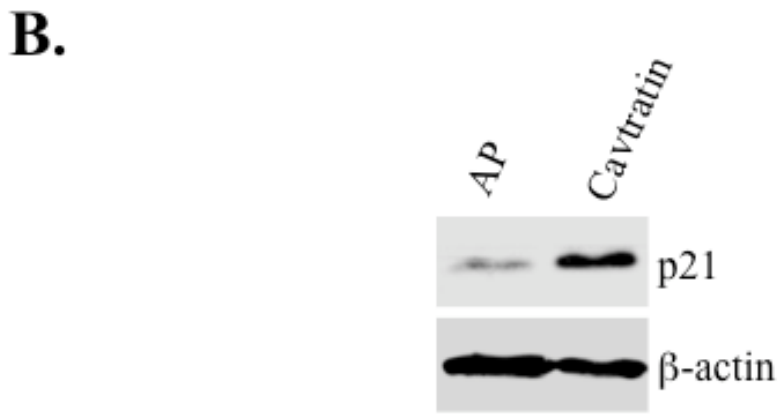
Figure 20. Cav-1 and p53 compete for the binding of MDM2. NIH 3T3 fibroblasts were transfected using a standard calcium phosphate method with equal amounts of p53 and MDM2-Myc constructs. Additionally, increasing amounts of the Cav-1 construct were also transfected. Forty-eight hours after transfection, an immunoprecipitation using anti-c-Myc was done to see if the interaction between p53 and MDM2 would vary in the presence of different amounts of Cav-1. An immunoblot was done with anti-p53 and anti-Cav-1 to detect p53 binding to MDM2. Results show that Cav-1 competes for binding of MDM2 with p53. Total Cav-1 and p53 expression are shown in lower panels.

4.2.1.10 The Caveolin Scaffolding Domain is sufficient to increase p53 activity leading to senescence

Increased Cav-1 expression is correlated with SIPS. Overexpression of Cav-1 is known to induce growth arrest (Galbiati, Volonte et al. 2001; Kawabe, Grant et al. 2001) and amplified expression of Cav-1 is found in aging tissues (Kawabe, Grant et al. 2001). It is not known, however, whether it is the protein's presence or the caveolae organelle that is responsible for mediating SIPS. Our previous experiments indicate that it is Cav-1's binding to MDM2 that mediates the senescence effect after oxidative stress. Cavtratin was utilized to distinguish whether the caveolae organelle, the full-length Cav-1 protein, or the CSD are necessary to induce SIPS. Cavtratin is a cell-permeable peptide consisting of the CSD (CSDDGIWKASFTTFTVTKYWFYR) fused to the C-terminus of the internalization sequence of the Drosophila transcription factor antennapedia (AP) by Fmoc chemistry, purified and analyzed by reversed-phase HPLC and mass spectrometry by the Tufts University Core Facility. Detection of the peptide is made possible by a biotin tag followed by an aminohexanoic acid spacer added to the amino terminus of the AP fusion peptide. Cavtratin can mimic the inhibitory effects of Cav-1 on signaling molecules. Cavtratin does not form caveolae as it cannot oligomerize with the full-length protein. Therefore, it is suitable to determine whether the CSD is sufficient to induce the SIPS effects of Cav-1. It was hypothesized that the CSD would upregulate p53 activity based on the results that it decreased interaction between p53 and its negative regulator MDM2 leading to p53's stability. A luciferase assay utilizing the p53 responsive element was conducted to determine whether the CSD or the caveolae organelle is responsible for the upregulation of p53. NIH 3T3 murine fibroblasts were transfected with the luciferase p53 responsive element and ß-galactosidase for sample normalization. The next day

samples were treated with 10 μM of cavtratin or AP for 24 hours. Results from the luciferase assay demonstrate that CSD upregulates p53 1.3 times more than AP alone does (Figure 21A).





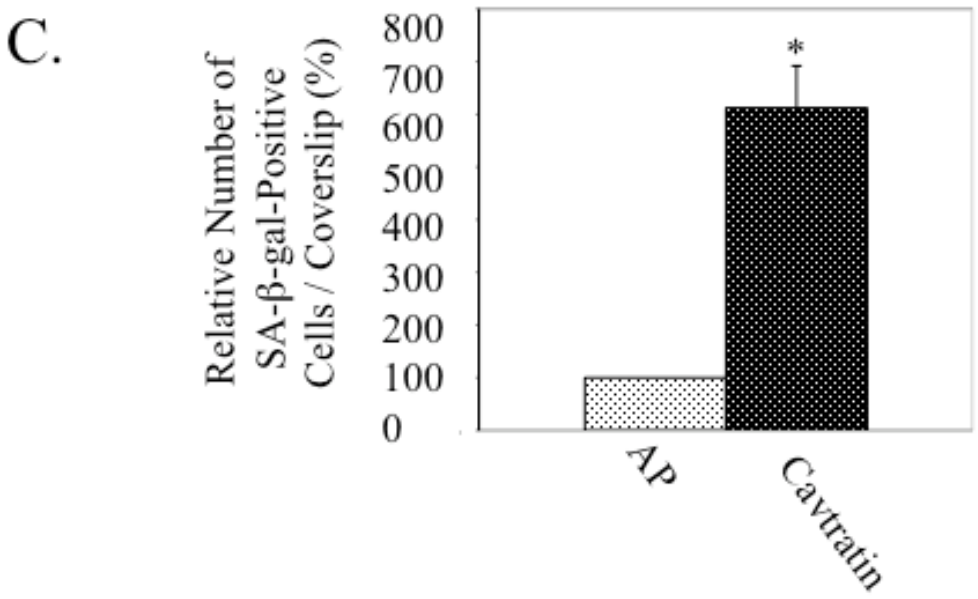


Figure 21. The CSD is sufficient to upregulate SIPS. A luciferase assay measuring the activity of the p53 responsive element upon treatment of 10 μM Cavtratin or AP for 24 hours was conducted. NIH 3T3 murine fibroblasts were transfected with the 3 μg of both the p53 responsive element along with β-galactosidase to normalize all samples. After 24 hours, cells were treated with 10 μM Cavtratin or AP for an additional 24 hours and the luciferase activity was determined. Values represent means ± SEM. **P*<0.01 (A). WI-38 human fibroblasts were treated with 10 μM Cavtratin or AP for 24 hours. An immunoblot assay was conducted to determine the expression of $p21^{WAF/CIP1}$, a cyclin dependent kinase inhibitor. Immunoblotting with anti-ß-actin IgGs show cell extract normalization (B). WI-38 cells were treated with 10 μM Cavtratin or AP and stained for Senescence Associated-β-Galactosidase activity after 7 days. Cells were observed under a BX50WI Olympus Optical light microscope at a magnification of 10X. Since AP treated cells continue to grow, samples were normalized based on cell number. Quantification of the SA-β-Galactosidase positive cells is shown (C). Values represent means ± SEM. **P*<0.001.

This increased p53 activity led to up-regulation of downstream factors. It was found that $p21^{WAF/CIP1}$, p53's downstream target, was upregulated 24 hours after cavtratin treatment (Figure 21B). If the CSD is sufficient to block interaction between p53 and MDM2, then it should increase senescence. Using SA β-galactosidase activity assay, it was also found that 10 μM cavtratin treatment is sufficient to induce premature senescence in WI-38 fibroblasts after 7 days (Figure 21C). After adjusting for total cell number (non-senescent cells continue to proliferate) the cavtratin treated group contained four times more senescent cells than the AP only group did.

4.2.2 SIPS is Dependent Upon Cav-1

4.2.2.1 Cav-1 null mouse embryonic fibroblasts do not undergo SIPS after oxidative stress

Increased expression of Cav-1 induces SIPS (Volonte, Zhang et al. 2002) and our studies show that the CSD stimulates SIPS. However, it is not known whether SIPS triggered by oxidative stress is dependent upon Cav-1 expression in fibroblasts. To determine if Cav-1 is necessary for SIPS, a null Cav-1 mouse embryonic fibroblast (MEFs) model was utilized. MEFs were derived from Cav-1 wildtype (WT) and Cav-1 knockout (KO) mice. The cells were cultured at the youngest passages possible (p3-5) and treated with 150 μM hydrogen peroxide for 2 hours and allowed to recover for a time course of 3, 5, 6, 8 or 10 days before SA-β-Galactosidase activity was quantified (Figure 22A). Data was quantified by counting the number of SA-β-Galactosidase positive cells and adjusting for cell number between the samples, as the KO MEFs continue to grow even after treatment with oxidative stress. This was done by multiplying the ratio of the number of total WT and KO cells to the number of WT positive cells, to get the number of positive cells if the cell number were equal. Even though the same numbers of WT and KO cells

were treated with hydrogen peroxide, data from Figure 22 shows that the knockout cells continued to proliferate and the wildtype did not. Beginning 5 days after hydrogen peroxide treatment, there is a divergence in the amount of senescent cells between WT and Cav-1 KO MEFs. By 10 days after treatment the difference is greatest with 10 times more senescent cells in the wildtype than in the knockout.

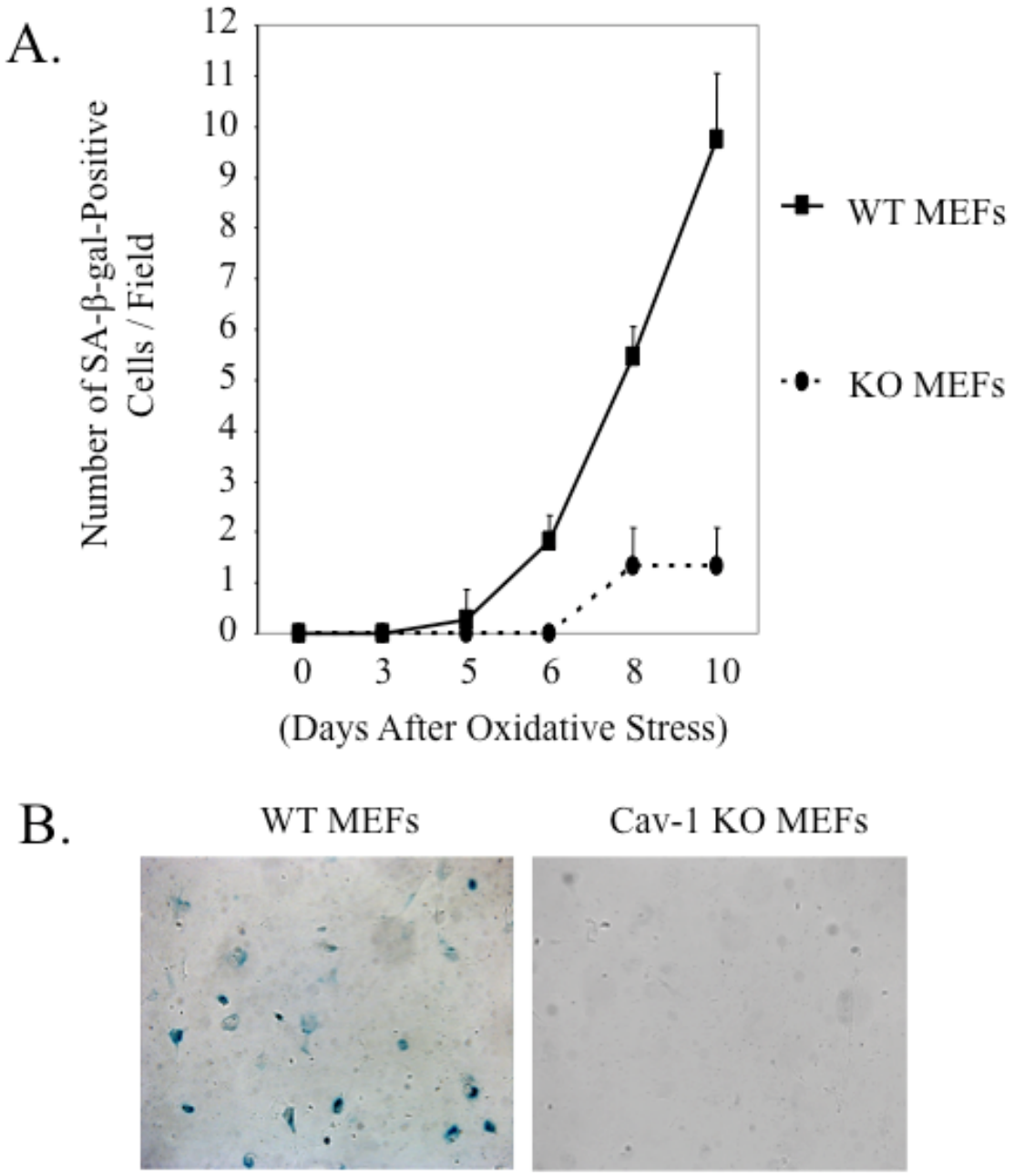


Figure 22. Cav-1 knockout mouse embryonic fibroblasts do not undergo SIPS upon oxidative stress treatment. MEFs were derived from C57B pregnant mice. Embryos were screened for genotype, and fibroblasts were derived and cultured. (A) Wildtype (WT MEFs) and Cav-1 knockout (CAV-1 KO MEFs) were treated with 150 μM of hydrogen peroxide for 2 hours, rinsed twice with 1X PBS and allowed to recover for 0, 3, 5, 6, 8 or 10 days before SA-ß-Galactosidase activity staining was conducted according to manufacturer's instructions. Cells were observed under a BX50WI Olympus Optical light microscope at a magnification of 10X. Quantification of the

SA-ß-Galactosidase activity assay is shown in (A). A representative field from 5 days after oxidative stress is shown in (B).

4.2.2.2 p53 expression is decreased in hydrogen peroxide treated Cav-1 null MEFs

It was hypothesized that the decreased senescent phenotype in the Cavoelin-1 KO MEFs may be due to the absence of senescence inducing proteins. Because p53 is increased in the presence of Cav-1, its expression was analyzed in the absence of Cav-1. Immunoblotting was conducted to determine the expression of p53 and p21 after oxidative stress in WT and Cav-1 KO MEFs. In Figure 23A, results show that after 48 hours the expression of p53 increased in the WT, but not in the KO MEFs. Also at this same time point, p53's downstream target p21 increased more in the WT than in the KO.

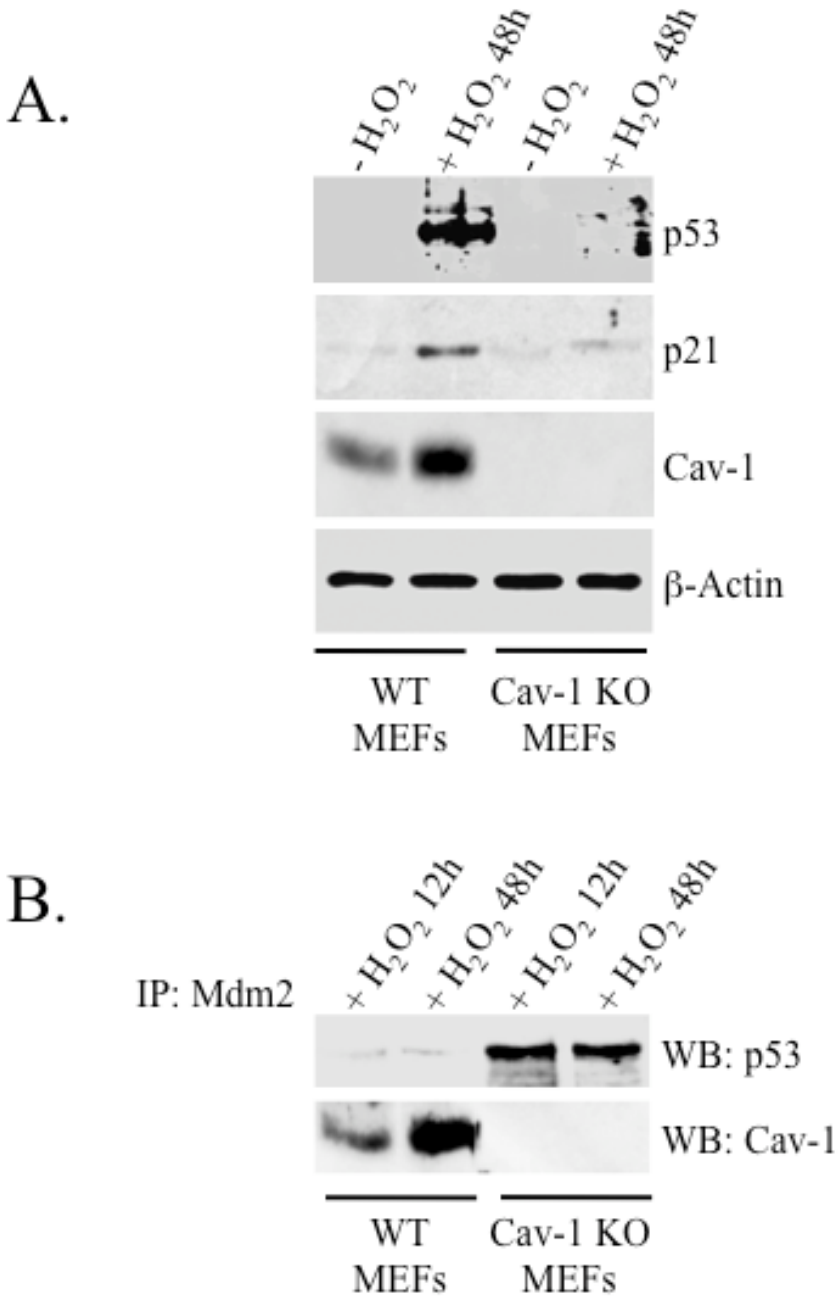


Figure 23. Without sequestration of MDM2 by Cav-1 the expression of p21 and p53 decreases. WT and Cav-1 KO MEFs were treated with 150 μM of hydrogen peroxide (H_2O_2) for 2 hours, rinsed twice with 1X PBS and allowed to recover for 48 hours in regular media. An immunoblot analysis of Cav-1, p53 and $p21^{WAF/CIP1}$ expression shows that the proteins are upregulated 48 hours after oxidative stress in the WT MEFs. Immunoblotting with anti-ß-actin showed equal loading (A). An immunoprecipitation was conducted on samples 12 and 48 hours after oxidative stress. Anti-MDM2 conjugated Sepharose A beads were mixed with cellular extract overnight. SDS-

PAGE was done to resolve the proteins and anti-p53 and anti-Cav-1 IgGs were used to determine association with MDM2 (B).

4.2.2.3 Decreased p53 associated with MDM2 in Cav-1 null MEFs

It is hypothesized that in Cav-1 knockouts, the decreased p53 and subsequent p21 expression is because Cav-1 is not present to sequester MDM2. Thus, MDM2 will remain associated with p53, leading to the transcription factor's degradation and inhibition of SIPS. To test this hypothesis, 12 and 48 hours after oxidative stress, an immunoprecipitation was conducted to determine the interaction between p53, MDM2 and Cav-1 in WT and Cav-1 KO MEFs. Sepharose A beads conjugated to MDM2 IgGs were utilized to bind to endogenous protein. The untreated WT MEFs show that there is very little expression of p53 and therefore little binding between the two proteins. This is expected as previous experiments show untreated control cells have very little p53 expression. Results show that after hydrogen peroxide treatment, WT MEFs exhibit only slight interaction between p53 and MDM2 even though there is upregulation of p53 in contrast to the Cav-1 null MEFs (Figure 23A and B). Without Cav-1 present to block their interaction, MDM2 and p53 have a stable interaction in the KO MEFs even 48 hours after hydrogen peroxide treatment. Moreover, because of the association between MDM2 and p53, there would be less p53 expression in the KO than in the WT (Figure 23A). Additionally, in the WT, after oxidative stress, there is increased association between Cav-1 and MDM2, which is inversely proportional to the interaction between p53 and MDM2 (Figure 23B).

4.2.2.4 p53 reduction in Cav-1 knockout MEFs is due to proteasomal degradation

Evidence from immunoprecipitations on Cav-1 knockout MEFs indicates that the continued association between MDM2 and p53 after oxidative stress leads to p53's subsequent proteosomal

degradation by MDM2's ubiquitination. To determine whether the decreased p53 expression occurs at the transcriptional level or the protein level, the proteasomal inhibitor MG132 was utilized. Eighteen hours after hydrogen peroxide treatment, cells were treated with MG132 for 6 hours and lysed. Immunoblot analysis of normalized cellular lysates shows that there is an accumulation of p53 and p21 as compared to the vehicle alone (Figure 24).

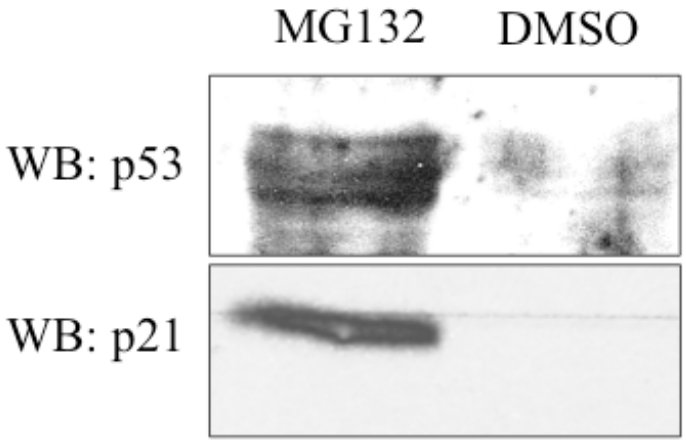


Figure 24. p53 and p21 in Cav-1 null MEFs undergo proteasomal degradation. Cav-1 KO MEFs were treated with 150 μM of hydrogen peroxide, washed twice with 1X PBS and allowed to recover for 18 hours in normal media. The MEFS were treated with 5 μM of the proteosomal inhibitor MG132 or the equivalent of vehicle DMSO for 6 hours. Cells were lysed and an immunoblot analysis was done to determine the expression of proteins p53 and p21.

4.3 DISCUSSION

The tumor suppressor protein p53 has been implicated as a keeper of the genome. Well known for its role as a deterrent for cancer, it is now becoming a major player in organismal longevity

as well. The transcription factor works to reduce somatic mutations and deter the proliferation of transformed cells. This highlights its role as a decision maker in cellular senescence, organismal aging and longevity (Campisi 2003; Vijg and Suh 2005; Kuningas, Mooijaart et al. 2008). Once p53 is activated by DNA damage it can direct a cell to one of 4 decisions to effect its fate and potentially the fitness of the organism: 1) transient arrest to try to fix the DNA damage. If repair is done properly there is no deleterious effect to the organism, however, improper repair can cause mutations in the DNA leading to cancer. 2) Apoptosis, which eventually leads to decreased tissue integrity from cellular depletion and to aging. 3) Senescence, which can decrease tissue renewal and alter tissue microenvironment leading to aging. 4) Or defective p53 activity leads to its failure as a tumor suppressor resulting in somatic mutations that can potentiate cancer (Rodier, Campisi et al. 2007). Therefore, p53 has been implicated in organismal aging (Tyner, Venkatachalam et al. 2002; Maier, Gluba et al. 2004). These findings and others show that the loss of p53 increases carcinogenesis risk (Christensen and Wallace 1976; Malkin, Li et al. 1990; Donehower, Harvey et al. 1992; Frebourg, Kassel et al. 1992) and establish that p53 links aging and cancer.

p53 half-life is mediated by the E3 ubiquitin ligase MDM2. MDM2 binds and inhibits p53 activation by tagging the protein for ubiquitin-mediated degradation (Momand, Zambetti et al. 1992; Haupt, Maya et al. 1997; Honda, Tanaka et al. 1997; Kubbutat, Jones et al. 1997; Midgley and Lane 1997; Giaccia and Kastan 1998). A recent report shows Nutlin-3A, a small molecular antagonist of MDM2, can promote senescence. In the presence of functional p53, Nutlin-3A can bind in the p53 binding pocket of MDM2, blocking its interaction with p53 and inducing senescence in human and primary murine fibroblasts, transformed fibroblasts, and fibrosarcoma cell lines (Efeyan, Ortega-Molina et al. 2007; Kumamoto, Spillare et al. 2008).

This work illustrates that p53-dependent senescence requires a blockage in the interaction of p53 and MDM2. Until now it was not known whether, with a synthetic inhibitor not present, what could trigger the break in interaction of p53 and MDM2 under normal cellular circumstances. We present Cav-1 as a mediator of cellular senescence by sequestering MDM2 after oxidative stress to allow for p53 upregulation and subsequent senescence.

Previous work implicates Cav-1 as having a role in p53-mediated senescence. Cav-1 arrests cells in the $G_{(0)}/G_{(1)}$ phase of the cell cycle. Transgenic mice over-expressing Cav-1 have primary cells with a reduced DNA replication rate and cell proliferation. Furthermore, the over-expression of Cav-1 can induce p53 activity (Galbiati, Volonte et al. 2001), and overexpression of Cav-1 in mouse embryonic fibroblasts induces senescence (Volonte, Zhang et al. 2002). Oxidative stress such as UV irradiation or hydrogen peroxide treatment actually upregulates Cav-1 and p53 leading to stress induced premature senescence. Our investigations show that upon oxidative stress $p38^{MAPK}$ is activated and stimulates Sp1, which binds to the Cav-1 gene promoter at 2 GC-rich regions. This up regulates Cav-1 and there is a subsequent increase in p53 activity leading to SIPS (Dasari, Bartholomew et al. 2006). This study focused on MDM2 and whether it was the link between caveolin-1 and p53 activi because of the aforementioned Nutlin 3A studies and MDM2 containing a CBD.

Our results show that after oxidative stress MDM2 translocates to caveolar fractions, and is sequestered by Cav-1. This re-compartmentalization from the nucleus to the cytosol and plasma membrane where Cav-1 is located breaks the interaction between MDM2 and p53. Without p53 being degraded by MDM2, it can activate is downstream target p21 and induce SIPS (Figure 25).

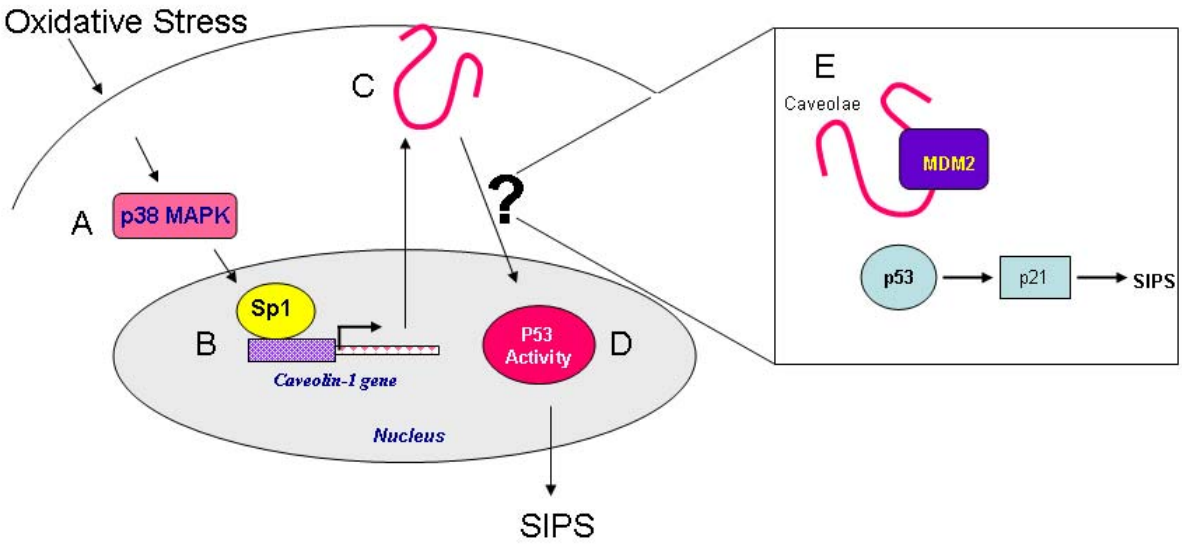


Figure 25. The proposed mechanism whereby Cav-1 mediated activation of p53 causes SIPS. The previous chapter's results show that $p38^{MAPK}$ is phosphorylated and activated by oxidative stress (A). This leads to the binding of Sp1 to GC-rich boxes in the caveolin-1 gene promoter (B), and results in the upregulation of Cav-1 (C). As previously demonstrated by our laboratory, the upregulation of Cav-1 is correlated with the activation of p53 and SIPS. However, the mechanism by which Cav-1 influences p53 was still unknown (?). Data from this chapter supports the hypothesis that MDM2 is sequestered by Cav-1, which allows p53 to become activated, thereby regulating downstream effectors such as p21, which induces SIPS (E).

Our results show that MDM2 contains a CBD, which is necessary for its interaction with Cav-1. Unfortunately, experiments to determine whether the mutated CBD in MDM2 was sufficient to abrogate SIPS were not possible. Brown et al. shows that the *Mdm2* gene expressed in non-neoplastic cells induces cell cycle arrest (Brown, Thomas et al. 1998). Therefore, we could not stably express our mutant MDM2 to determine its effects on SIPS. Interestingly though, MDM2's CBD is located in the p53 binding domain of MDM2. This sets Cav-1 up to be a competitor for binding between MDM2 and p53. Hence, when MDM2 is sequestered to Cav-

1, it can no longer interact with p53 allowing for increased activity of the transcription factor. Since MDM2 is an E3 ubiquitin ligase, it was a possibility that by its sequestration in the caveolae, it could interact with proteins residing there that would inhibit senescence. Thus by tagging these proteins for degradation, it is positively influencing the SIPS phenotype. To determine whether the caveolae organelle was necessary for the sequestration of MDM2 leading to SIPS, the cell permeable CSD, Cavtratin, was utilized. We show that cavtratin is sufficient to induce senescence by decreasing interaction between p53 and MDM2 and by increasing p53 activity leading to senescence. Thus, only the CSD, which is the minimal sequence necessary to bind MDM2, and not the caveolae, is needed. Our data with cavtratin mediating SIPS complements previous work, which shows that cavtratin, is sufficient to abrogate invasion of metastatic PyMT mammary carcinoma cell line Met-1 (Williams, Medina et al. 2004). It is an example of how Cav-1 can act as a tumor suppressor.

The work in Cav-1 null MEFs confirms the hypothesized mechanism that Cav-1 mediates senescence by sequestering MDM2 to allow for increased p53 activity (Figure 26). Additionally, it shows that in the absence of Cav-1, p53 is degraded and thus unable to initiate the signaling pathway to induce senescence. This data is in agreement with previous findings in the field. Cho et al. showed that by knocking down Cav-1 expression with siRNA, the SIPS phenotype could be reversed as evidenced by: DNA synthesis, re-entry of senescent cells into cell cycle upon epidermal growth factor stimulation, and decreases in p53 and p21 (Cho, Ryu et al. 2003). Our studies in a Cav-1 null background shed light on how cancer cells can overcome senescence in their transformation. It is estimated that 50% of all malignancies have a mutated p53 gene (Toledo and Wahl 2006). Malignancies with functioning p53 may have the problem of p53 instability from its regulators ARF/MDM2. (Sharpless 2005; Vousden and Prives 2005).

Additionally, many cancers exhibit downregulated Cav-1 (Williams and Lisanti 2005). From our results we speculate that cancer cells having wildtype p53 and downregulated Cav-1 cannot senesce because p53 is being inactivated by MDM2. Our work with MCF7 cells shows that hydrogen peroxide treatment does not induce SIPS. When Cav-1 is re-introduced by adenovirus infection, the SIPS phenotype is restored supporting the claim of Cav-1 being a tumor suppressor.

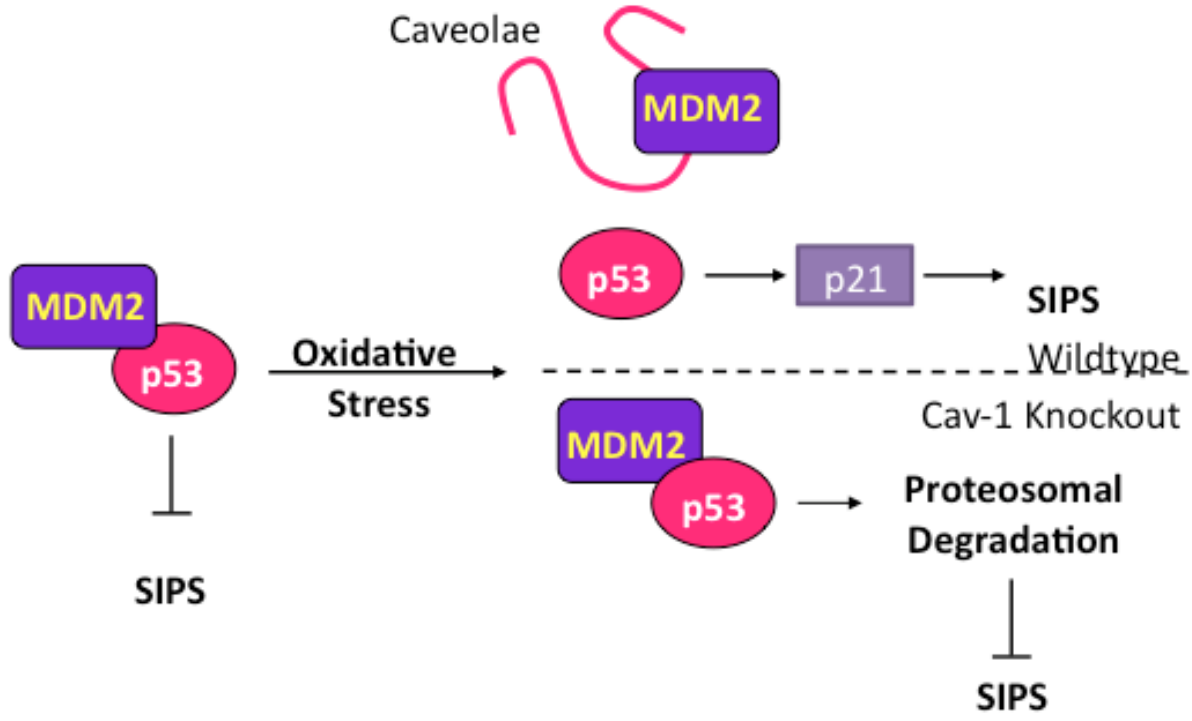


Figure 26. Schematic diagram recapitulating oxidant-induced Cav-1 dependent activation of the p53/p21$^{WAF/CIP1}$ pathway and initiation of SIPS in fibroblasts. Using MEFs, we show hydrogen peroxide treatment induces oxidative stress triggering the p53 pathway leading to SIPS by Cav-1 sequestering p53's negative regulator, MDM2. This allows for decreased degradation of p53 allowing it to activate its downstream target p21 and induces SIPS. In the absence of Cav-1, even after oxidative stress, there is still great association between p53 and MDM2 leading to p53's degradation and inhibition of SIPS in these fibroblasts.

5.0 THE ANTAGONIST PLEIOTROPIC EFFECTS OF CAVEOLIN-1

5.1 INTRODUCTION

In 1957 George Williams published the idea of the antagonistic pleiotrophy theory of aging. He believed that some genes have a pluralistic effect of phenotypes, which could affect the organism differently depending upon the age of the organism. For instance, a gene can have a positive effect by being protective early in life increasing the health and fitness of the organism, but it may become negative over time leading to aging and organismal death (Williams 1957). More recently, data has emerged establishing a link between aging and cancer. Blagosklonny et al. points out two pieces of evidence demonstrating that systemic aging is linked to increased cancer: 1) Studies that show caloric restriction increases life expectancy and decreases the incidence of cancer (Klurfeld, Weber et al. 1987; Grasl-Kraupp, Bursch et al. 1994; Muskhelishvili, Hart et al. 1995; Hursting, Lavigne et al. 2003). And 2) the obvious observation that centenarians have a very low incidence of cancer as well as animal studies that show long-lived mice have decreased cancer compared to shorter lived mice (Anisimov, Zabezhinski et al. 2001; Ikeno, Bronson et al. 2003; Caruso, Lio et al. 2004; Blagosklonny and Campisi 2008).

The senescence field suggests SIPS is a form of antagonistic pleiotropy. Cells responding to damage can undergo senescence and thus avoid abnormal proliferation, which would have deleterious effects to the organism. When the organism is young, this has a positive

effect allowing for reproduction and a high level of fitness. However, with time, an accumulation of senescent cells becomes harmful as they change the tissue microenvironment, and inhibit adequate repair. Interestingly, senescent fibroblasts cause the proliferation of transformed epithelial cells (Krtolica, Parrinello et al. 2001; Parrinello, Coppe et al. 2005; Liu and Hornsby 2007; Coppe, Boysen et al. 2008). Judith Campisi has coined the phrase "Good citizens and bad neighbors" to describe senescent fibroblasts. She reasons that whatever cellular insult causes a fibroblast to senesce will also cause mutations in surrounding cells such as epithelial cells. For a time, senescence has positive effect on the fibroblast stopping it from passing on its damaged or mutated genome. However, the prematurely senescent fibroblast will start to secrete growth factors, cytokines, extracellular matrix and matrix metalloproteases that disrupt the surrounding microenvironment (Campisi 1997; Rinehart and Torti 1997; Kang, Chen et al. 2008). The changing stroma can cause pre-neoplastic and transformed epithelial cells to become neoplastic and prime the environment for their growth advantage (Campisi 2005). Evidence of senescence having antagonistic pleiotropic effects is that there is an increased cancer risk with age. Statistics show that 60% of cancers and 70% of cancer mortalities occur in individuals 65 and older (Yancik and Ries 2000). Additionally, the majority of age-related cancers arise from epithelial cells (DePinho 2000).

Recent data shows that prematurely senescent fibroblasts induce proliferation of breast cancer cells *in vitro* and *in vivo* (Krtolica, Parrinello et al. 2001; Liu and Hornsby 2007). However, there is still much to be known about how senescent cells regulate the antagonistic pleiotropic effect on neoplastic epithelial cells. Since we have shown that Cav-1 is necessary to induce p53 dependent SIPS, we hypothesize that it will play an integral role in the antagonistic pleiotropic effects of senescent fibroblasts on epithelial cells. To study the effects of Cav-1 on

the antagonistic pleiotropic effects of senescent fibroblasts, we utilized wildtype (WT MEFs) and Cav-1 null mouse embryonic fibroblasts (KO MEFs). We hypothesize that without Cav-1, MEFs treated with oxidative stress will not stimulate the growth of transformed cells.

5.2 RESULTS

5.2.1 NIH Ras^{G12V} transformed fibroblasts undergo SIPS when Cav-1 is re-expressed

To determine the role of Cav-1 in antagonistic pleiotropy, we first wanted to determine how the presence of Cav-1 would effect cancer cells and whether its expression in senescent fibroblasts would effect the growth of cancer cells. NIH Ras^{G12V} are a transformed fibroblast cell line known to have a downregulated Cav-1 expression (Engelman, Wycoff et al. 1997). When Cav-1 was re-expressed in NIH Ras^{G12V} by transfection with a selection vector pCB7 for hygromycin, the transformed fibroblasts underwent senescence (Figure 27). Results from quantification of the number of SA-β-galactosidase positive cells show approximately a 5-fold increase over vector only expressing cells.

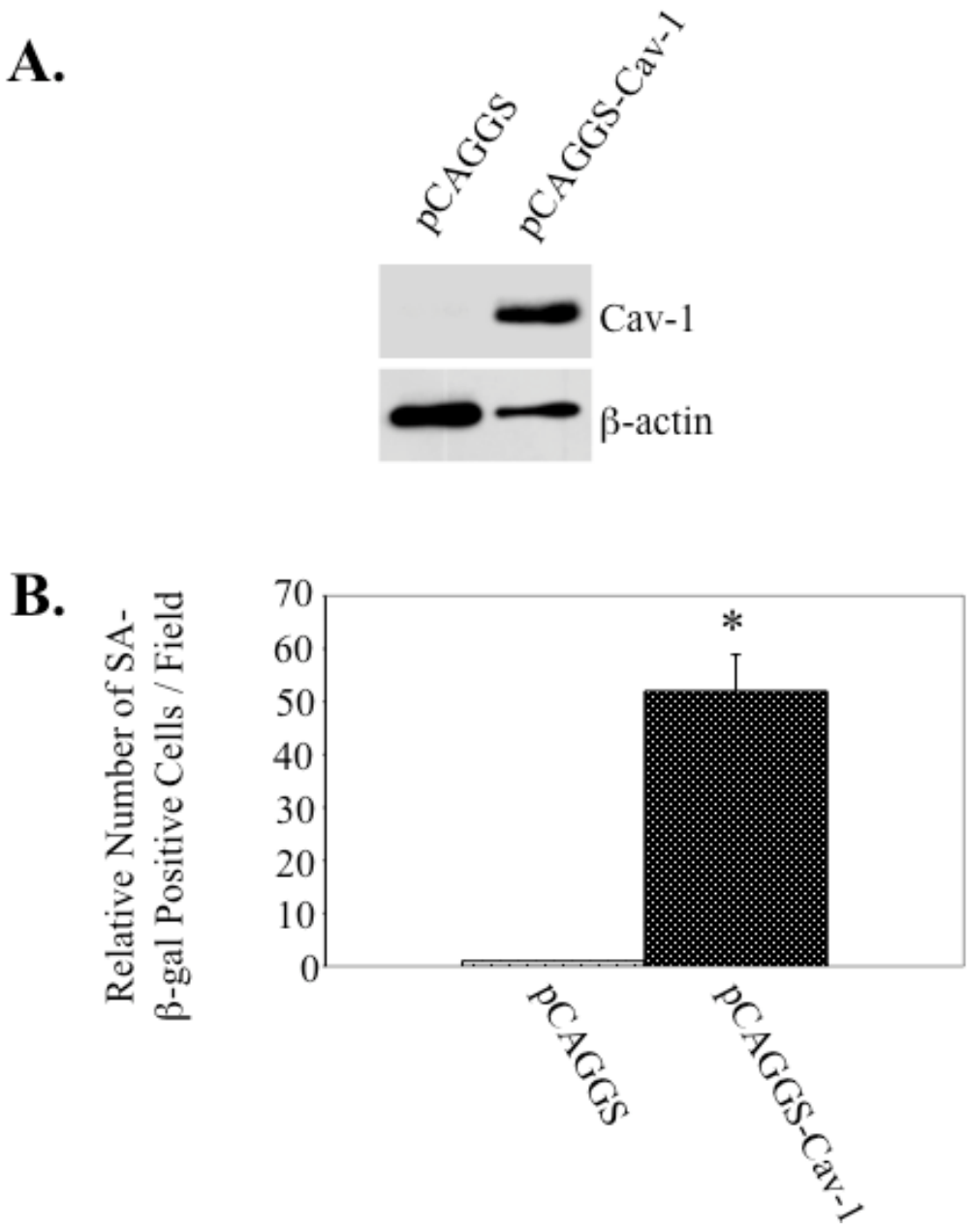


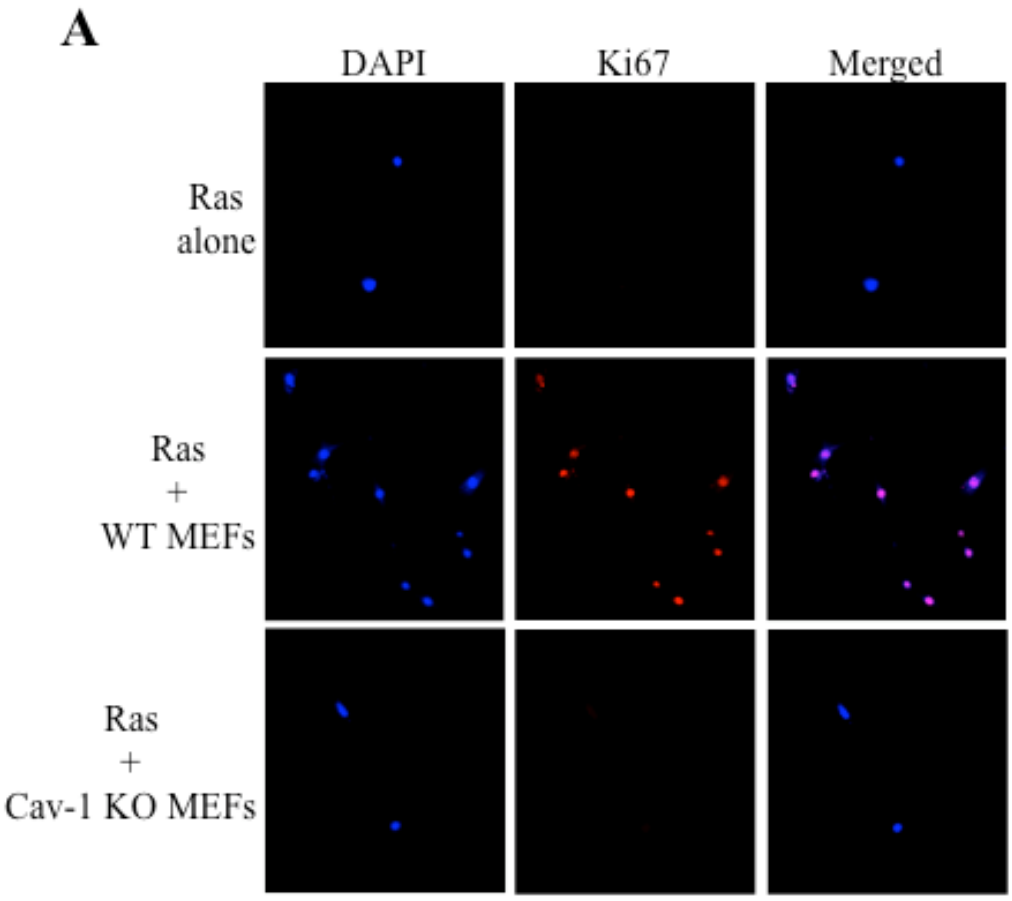
Figure 27. Re-expression of Cav-1 in NIH 3T3 Ras^{G12V} rescues the SIPS phenotype. (A) Cav-1 and pCB7, a vector carrying hygromycin resistance, were transfected by calcium phosphate into NIH 3T3 Ras^{G12V} cells. pCAGGS (vector alone) was transfected as a control. After 48 hours transfected cells were subjected to immunoblot analysis using anti-Cav-1 antibodies to detect the protein's expression. Anti-β-actin IgGs were used in the immunoblot analysis to determine equal loading of samples (A). In addition, cells were cultured in media containing 200μg/mL hygromycin for 14 days to select for Cav-1 or pCAGGS expressing cells. Cells were

subjected to the senescence associated-β̃galactosidase activity assay. Cells were observed with a BX50WI Olympus Optical light microscope at a magnification of 10x. Quantification of the SA-β-galactosidase activity assay is shown. Values represent means ± SEM. $*P<0.001$.

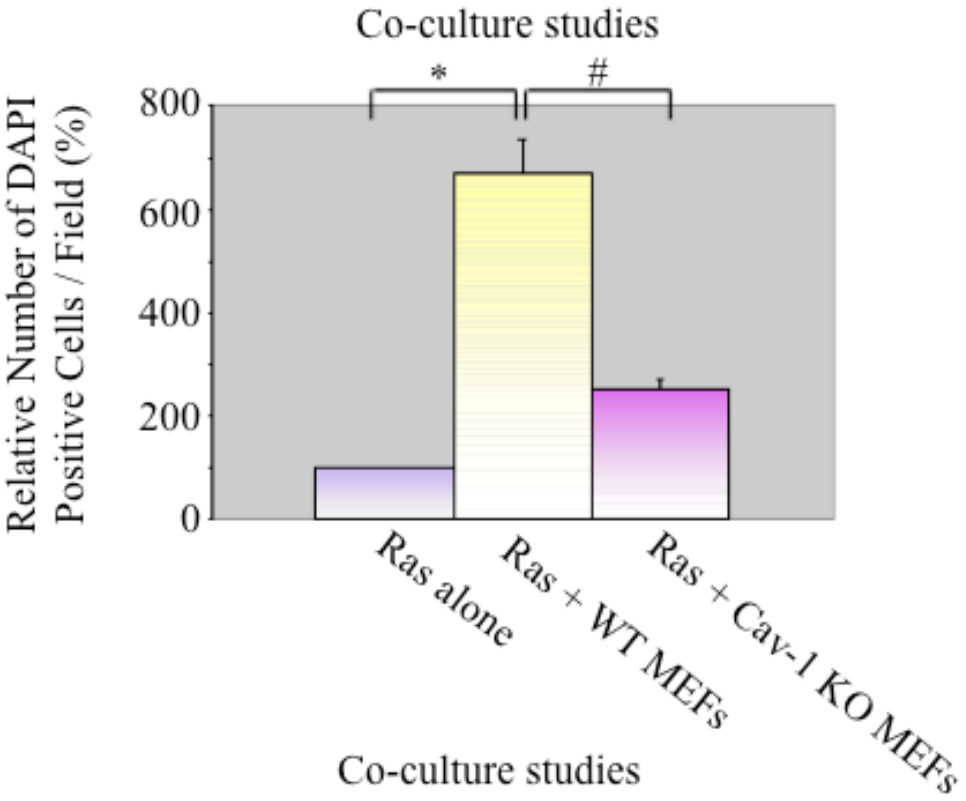
5.2.2 Cav-1 expressing senescent MEFs induce proliferation of NIH 3T3 Ras^{G12V} transformed fibroblasts *in vitro*

Previous experiments from section 4.2.2, determined that Cav-1 is necessary for MEFs to undergo SIPS. Thus, these wildtype and knockout Cav-1 cell lines were used to determine whether Cav-1 mediated SIPS is necessary to induce proliferation of transformed fibroblasts. Wildtype and Cav-1 knockout MEFs were treated with 150 μM hydrogen peroxide for 2 hours and recovered for 4 days. MEFs along with Ras^{G12V} cells were serum starved for 3 days (thus, all cells were growth arrested) and co-cultured together. 7 days later cells were stained with DAPI to quantify the number of Ras^{G12V} cells. Observation of light phase microscopy and UV settings show that MEFs have larger nuclei than Ras^{G12V} cells and are distinguished accordingly (data not shown). Interphase marker, Ki67 was used to quantify the number of proliferating Ras^{G12V} cells. Quantification was conducted by counting the number of Ras^{G12V} nuclei by DAPI staining and positive Ki67 cells in 30 random fields and are reported in the relative number of DAPI positive Ras^{G12V} cells per field. Results show that compared to Ras^{G12V} cells cultured alone, there is a 2-fold increase in Ras^{G12V} cell number cultured with KO MEFs. For Ras^{G12V} cells cultured with WT MEFs there is a 6.5 fold increase in Ras^{G12V} number compared to Ras^{G12V} alone and a 2.6-fold increase above Ras^{G12V} cells co-cultured with Cav-1 null MEFs (Figure 28B). As an alternative proliferative measurement, Ki67 positive Ras^{G12V} cells were quantified in the same way. Neither WT nor Cav-1 KO MEFs stained positive for Ki67 (data not shown) as they were

growth arrested from serum starvation. Results show Ras^{G12V} cells cultured with senescent WT MEFs exhibit a 15-fold increase in the amount of Ki67 positive cells per field compared to Ras^{G12V} only. In contrast, Ras^{G12V} cells cultured with Cav-1 KO MEFs only had a 2-fold increase in Ki67 positive cells compared to Ras^{G12V} alone. Comparison between WT and Cav-1 KO co-cultures shows a 7.5 fold difference in Ki67 positive cells between these two populations (Figure 28C).


A
DAPI
Ki67
Merged
Ras alone
Ras + WT MEFs
Ras + Cav-1 KO MEFs

B.


Co-culture studies
*
#
Relative Number of DAPI Positive Cells / Field (%)
800
600
400
200
0
Ras alone
Ras + WT MEFs
Ras + Cav-1 KO MEFs

C.

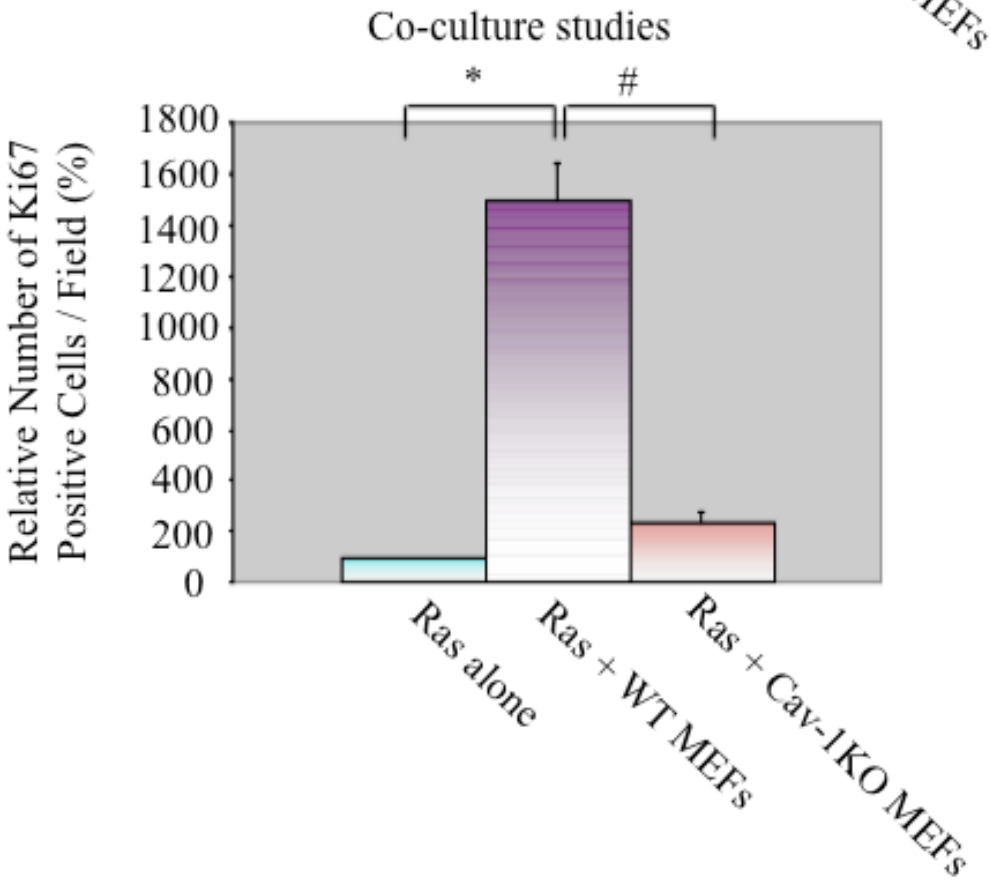

Co-culture studies
*
#
Relative Number of Ki67 Positive Cells / Field (%)
1800
1600
1400
1200
1000
800
600
400
200
0
Ras alone
Ras + WT MEFs
Ras + Cav-1KO MEFs

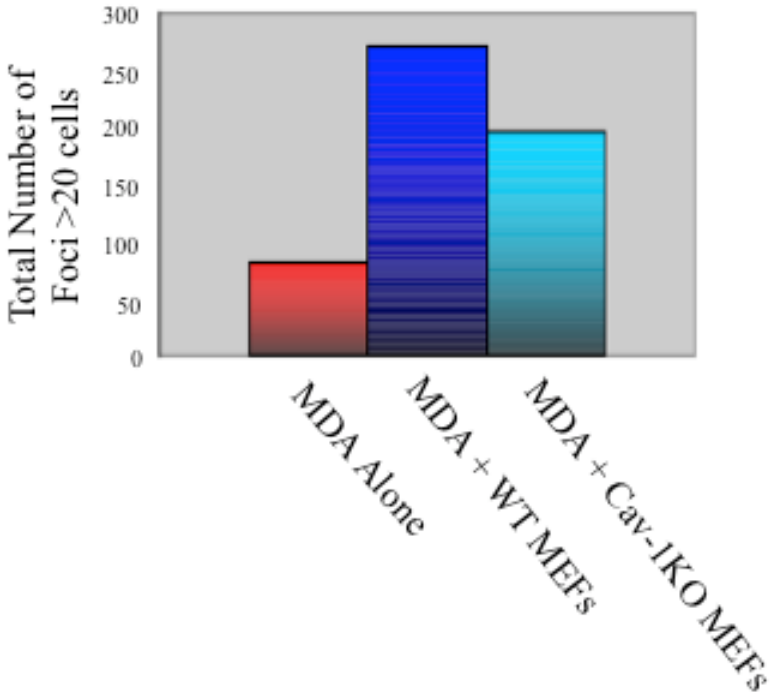
Figure 28. Proliferation of transformed Ras^{G12V} fibroblasts is dependent upon Cav-1 expressing senescent fibroblasts. Wildtype (WT MEFs) and Cav-1 knockout (KO MEFs) mouse embryonic fibroblasts were normalized for cell number and plated into 10 cm dishes. When cultures were approximately 50% confluent, MEFs were treated with 150 mM hydrogen peroxide for 2 hours, washed twice with 1X PBS and recovered for 4 days in normal media. The MEFs along with Ras^{G12V} cells were serum starved for 3 days and co-cultured together by plating 37,500 Ras^{G12V} cells onto the MEF lawns. Immunofluorescence was carried out by co-staining cultures with DAPI and Ki67. Fluorescent secondary antibodies detected the Ki67. A representation of the immunofluorescent staining is depicted in (A). Quantification was conducted by counting the total number of Ras^{G12V} nuclei by DAPI staining, and positive Ki67 cells in 30 random fields (B and C). Results are representative of 3 independent experiments and the values represent ± SEM $^{*,\#}P<0.001$.

5.2.3 Cav-1 expressing senescent fibroblasts stimulate growth of MDA-MB-231 breast cancer cells *in vitro*

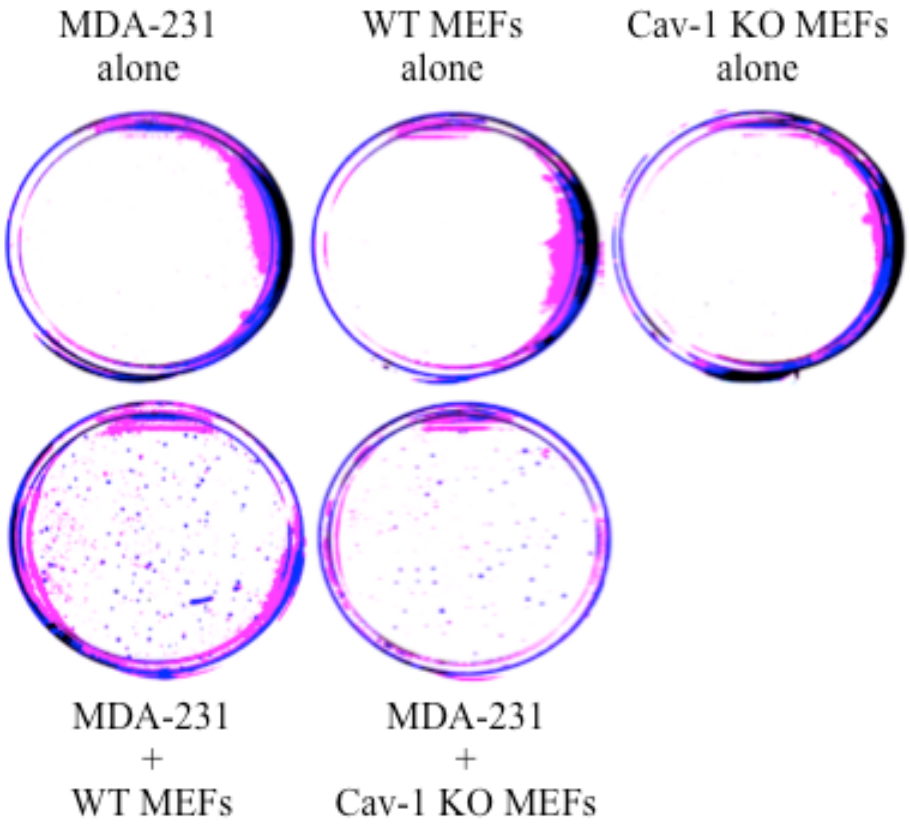
MDA-MB-231 is a breast cancer cell line known to be stimulated by senescent fibroblasts (Krtolica and Campisi 2003; Liu and Hornsby 2007). Co-culturing MDA-MB-231 cells with growth arrested Cav-1 deficient fibroblasts was done to determine whether the senescent fibroblasts' antagonistic pleiotropic effect is dependent upon Cav-1. Wildtype and Cav-1 knockout mouse embryonic fibroblasts (MEFs) were treated the same way as in Figure 28. After 3 days of serum starvation MEFs and MDA-MB-231 cells were co-cultured together. Cells were co-cultured by adding MDA-MB-231 cells to MEFs and allowing growth for 7 days. DAPI staining was conducted to determine foci formation and quantification of MDA-MB-231 colonies. Figure 29A shows the number of foci with > 20 cells per foci. Results show that there is a 28% decrease in foci in breast cancer cells cultured with Cav-1 knockout MEFs compared to those co-cultured with WT MEFs. Co-cultures were also stained with crystal violet to detect larger colonies of MDA-MB-231 cells (Figure 29B). Results were obtained by counting the

number of colonies ≥0.3mm^2 per cm^2. Crystal violet staining shows there is an ~ 250% reduction in foci with MDA-MB-231 cells cultured with Cav-1 null MEFs compared to WT. The MDA-MB-231 cells alone, WT MEFs alone, and Cav-1 KO MEFs alone did not produce colonies (Figure 29C).

A.


Total Number of Foci >20 cells
300
250
200
150
100
50
0
MDA Alone
MDA + WT MEFs
MDA + Cav-1KO MEFs

B.


MDA-231 alone
WT MEFs alone
Cav-1 KO MEFs alone
MDA-231 + WT MEFs
MDA-231 + Cav-1 KO MEFs

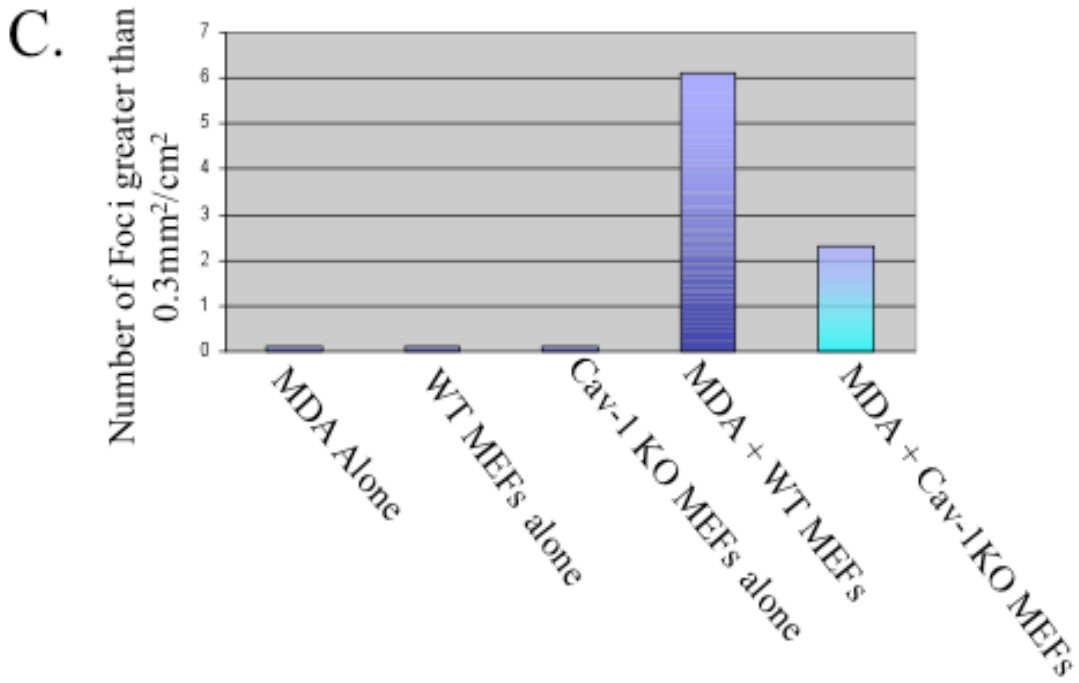


Figure 29. Breast cancer cell proliferation is stimulated by Cav-1 expressing senescent fibroblasts. Wildtype (WT MEFs) and Cav-1 knockout (KO MEFs) mouse embryonic fibroblasts were normalized for cell number and plated into 6 or 10 cm dishes. When cultures were approximately 50% confluent, MEFs were treated with 150 mM hydrogen peroxide for 2 hours, washed twice with 1X PBS and recovered for 4 days in normal media. The MEFs were serum starved for 3 days as well as the MDA-MB-231 which were supplemented with 5µg/mL insulin. MBA-MB-231 cells and MEFs were co-cultured together by plating 37,500 (for 6 cm dish) or 112,500 (for a 10 cm dish) MDA-MB-231 cells onto the MEF lawns. The cultures in 6 cm dishes were stained with DAPI and the number of MDA-MB-231 colonies containing ≥ 20 cells on the dish was quantified. (A). Co-cultures in 10 cm dishes were stained with crystal violet (0.1 gram per ml crystal violet in 70% ethanol) for 2 minutes followed by extensive washing with 1X PBS (B). Quantification of crystal violet staining was done by Matlab software which preprocessed the image by cropping the central area of each plate, converting to the HSV color space, and finding connected regions of pixels with saturation greater than 0.2 on a [0, 1] scale. Only colonies with area greater than 32 pixels, which corresponds to $\geq 0.3mm^2$ were counted (C).

5.2.4 Secreted factors mediate the ability of Cav-1 expressing senescent MEFs to stimulate the proliferation of NIH 3T3 Ras^{G12V}-transformed fibroblasts

It has been shown that secreted factors, such as cytokines and matrix metalloproteinases, from stress induced senescent cells can change the stroma and microenvironment of a cell. Additionally, these factors can induce the growth of neoplastic epithelial cells (Krtolica and Campisi 2003; Liu and Hornsby 2007; Coppe, Boysen et al. 2008). Based on the co-culturing experiments, it was determined that wildtype senescent fibroblasts can induce proliferation of transformed epithelial and transformed fibroblast cells. It is hypothesized that Cav-1 wildtype senescent cells may have a different secretion profile compared to Cav-1 null cells. To test this hypothesis, wildtype and Cav-1 knockout mouse embryonic fibroblasts (MEFs) were treated with oxidative stress and recovered for 4 days. The treated MEFs were serum starved for 3 days along with Ras^{G12V} cells. Before co-culturing, conditioned media from MEFs was collected and MEFs were removed from culturing dishes by calcium chelation. This did not disrupt the extracellular matrix (insoluble secreted factors). Conditioned media was replaced back into its respective dishes and Ras^{G12V} cells were added to the plates. Cells were permitted to grow for 7 days and DAPI and Ki67 staining were conducted to quantify Ras cell proliferation due to secreted factors from the MEFs. DAPI staining shows secreted factors from senescent Cav-1 expressing MEFs cause a 5-fold increase in the proliferation of Ras cells (Figure 30A). This is decreased 2.5 fold in Cav-1 null MEFs. Likewise, Ki67 staining shows an 8-fold increase in Ras cells entering mitosis when cultured in factors from WT MEFs compared to Ras^{G12V} cells in serum free media. When comparing Ras^{G12V} cell growth from WT versus KO secreted factors there is a 5.3-fold decrease in mitotic Ras^{G12V} cells from KO cultures (Figure 30B).

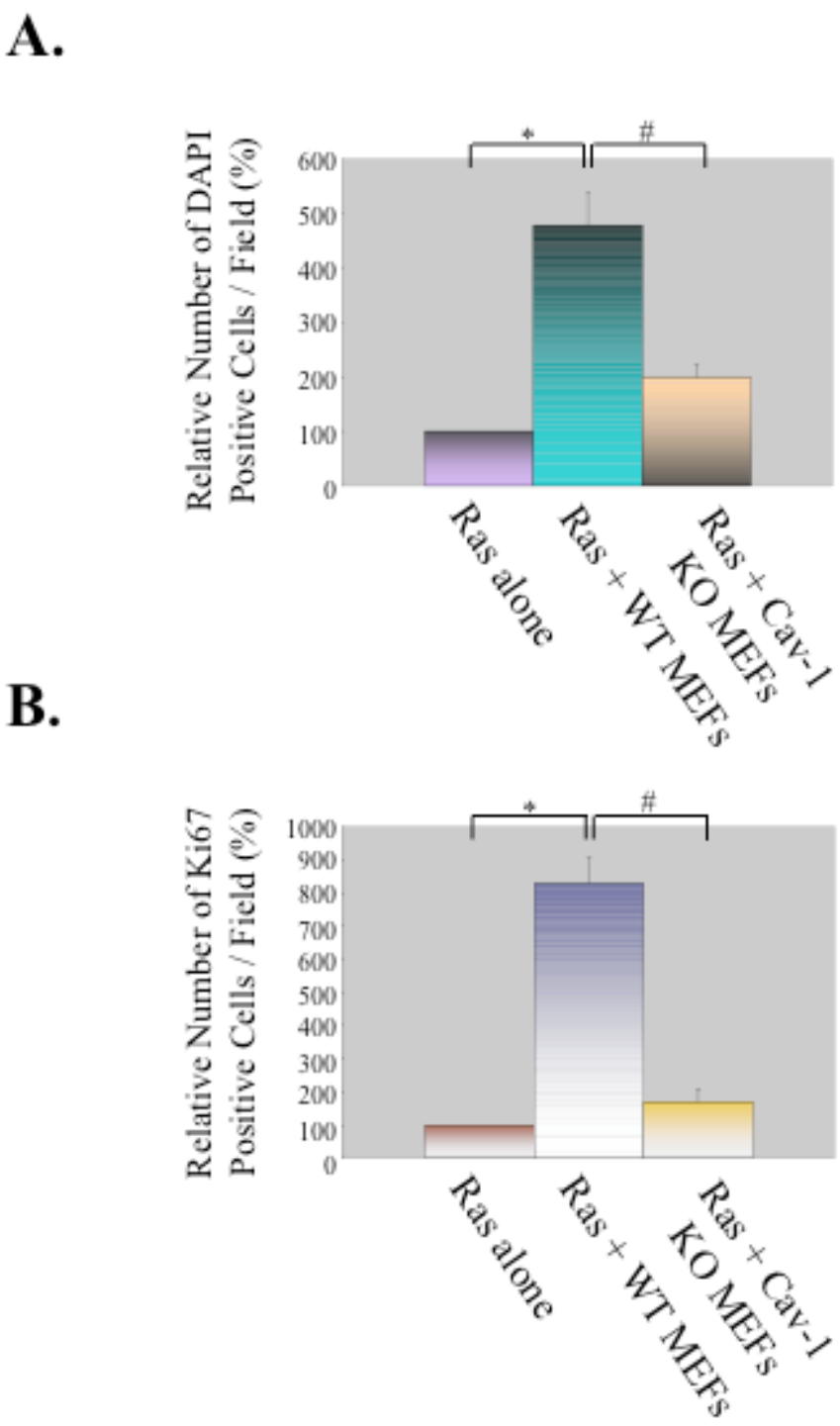


Figure 30. Cav-1 expressing fibroblasts have a secretion profile that stimulates the growth of NIH 3T3 Ras^{G12V} transformed fibroblasts. Wildtype (WT MEFs) and Cav-1 knockout (KO MEFs) mouse embryonic fibroblasts were normalized for cell number and plated into 10 cm dishes. When cultures were approximately 50%

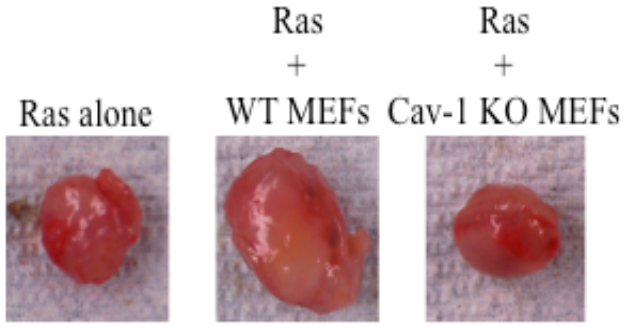
confluent, MEFs were treated with 150 mM hydrogen peroxide for 2 hours, washed twice with 1X PBS and recovered for 4 days in normal media. The MEFs along with Ras^{G12V}cells were serum starved for 3 days. Media was collected off of the MEF cultures and saved while the cells were removed by mild calcium chelation from Cell Dissociation Buffer in PBS (Gibco). Media was replaced back onto the respective plates and 37,000 Ras^{G12V}cells were cultured in the conditioned media and the extracellular matrix from the MEFs. To determine proliferation, seven days later cells were double stained with DAPI (A) and Ki67 (B). Cells were quantified by counting the number of nuclei and Ki67 positive cells in 30 random fields from 2 independent experiments. Values represent means ± SEM $^{*,\#}P<0.001$.

5.2.5 The antagonistic pleiotropic effect of senescent fibroblasts on the proliferation of neoplastic cells *in vivo* is dependent upon Cav-1

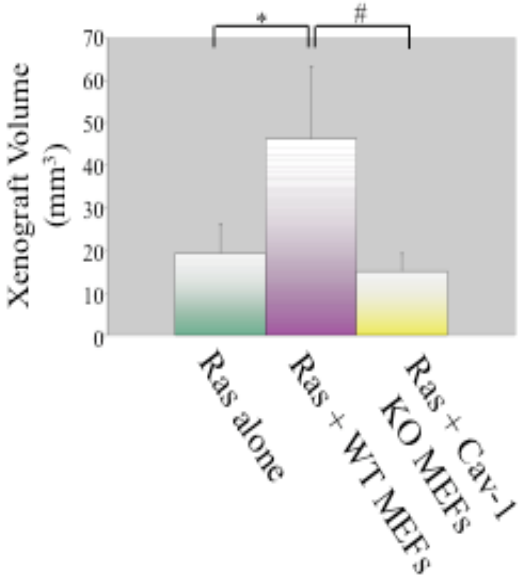
In vitro co-culturing of senescent Cav-1 wildtype and Cav-1 null fibroblasts demonstrated that Cav-1 expression was necessary for the antagonistic pleiotropic effect of senescent fibroblasts on neoplastic cells. Co-cultures using oxidatively stressed and growth arrested Cav-1 knockout cells did not significantly induce proliferation of Ras^{G12V} fibroblasts or breast cancer cells MDA-MB-231. Additionally, the soluble and insoluble factors secreted by the Cav-1 knockouts were not sufficient to generate growth of the Ras^{G12V} cells. In contrast, Cav-1 WT fibroblasts that have undergone SIPS stimulate the growth of Ras^{G12V} and MDA-MB-231 *in vitro*. Likewise, secretion factor studies show that WT MEFs have a secretory profile that supports the growth of transformed cells. This data supports the hypothesis that Cav-1 is necessary for the antagonistic pleiotropic effect of SIPS cells causing proliferation of neoplastic cells. However, it is not known whether Cav-1 is necessary for the antagonistic pleiotropic effect of SIPS cells producing tumorigenesis *in vivo.*

To test whether Cav-1 is necessary for SIPS cells inducing tumorigenesis *in vivo* a tumorigenesis assay was done in nude mice. Cav-1 WT and KO MEFs were treated with hydrogen peroxide and allowed to recover for 7 days. The MEFs were mixed with Ras^{G12V} or MDA-MB-231 cells and injected into 5-week old nu/nu mice. After 4 weeks tumors were excised and the volume was determined. Results show that mice injected either WT or KO MEFs alone did not form tumors (data not shown). However, data from mice injected with MEFs and Ras^{G12V} show that Ras^{G12V} alone had a tumor size of 18 mm^3 whereas Ras^{G12V} + WT MEFs had 47 mm^3 and Ras^{G12V} + KO MEFs exhibited a tumor volume of 16 mm^3. Thus, Cav-1 expressing senescent fibroblasts stimulated tumor growth of Ras^{G12V} cells 2.25-fold more than Ras alone and 3 fold more than Cav-1 knockout MEFs (Figure 31A and B). The same procedure was carried out for tumorigenesis assays with MDA-MB-231 epithelial breast cancer cells. MDA-MB-231 cells injected alone showed a tumor volume of 0.5 mm^3. When these epithelial cells were mixed with hydrogen peroxide treated WT MEFs, the tumor volume increased to 7 mm^3, and with treated KO MEFs it was found to be 2 mm^3. Hence, tumors extracted from MDA-MB-231 cells co-injected with wildtype MEFs were 14-fold larger than that of MDA-MB-231 alone and 3.5-fold larger than that of epithelial cells injected along with Cav-1 knockout MEFs (Figure 31C and D).

A


Ras alone
Ras + WT MEFs
Ras + Cav-1 KO MEFs

B


Xenograft Volume (mm³)
0
10
20
30
40
50
60
70
*
#
Ras alone
Ras + WT MEFs
Ras + Cav-1 KO MEFs

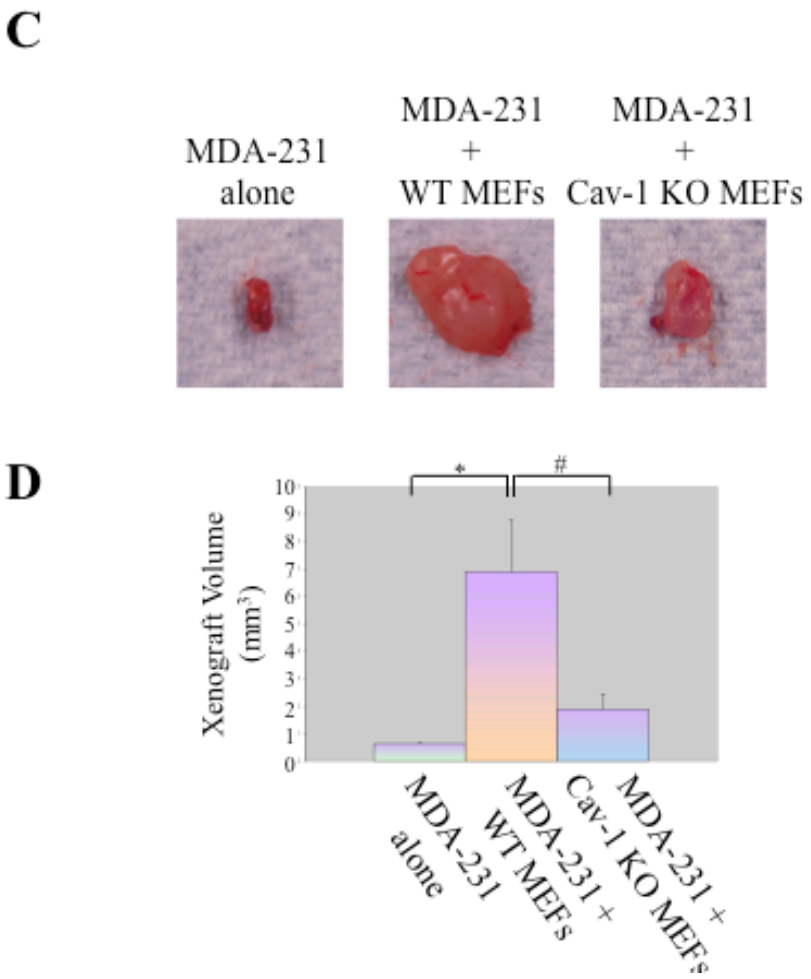


Figure 31. Antagonistic pleiotropic effect of senescent fibroblasts is dependent upon Cav-1 *in vivo*. Wildtype (WT) and Cav-1 knockout (KO) MEFs were treated with oxidative stress by exposure to 150 μM of hydrogen peroxide in the culturing media for 2 hours. Cells were rinsed twice with 1X PBS and allowed to recover for 7 days. The following groups were in subcutaneously injected into the dorsal flap of nude (nu/nu) mice (5-6 weeks old from Charles Rivers Laboratories): 3.25 x10^5 Ras^{G12V} cells only, 3.25 x10^5 MDA-MB-231 cells only, 1 x 10^6 WT or KO MEFs only, 3.25 x10^5 Ras^{G12V} cells plus 1 x 10^6 WT or KO MEFs, and 3.25 x 10^5MDA-MB-231 cells plus 1 x 10^6 WT or KO MEFs. All cells were resuspended into a 100 μL volume. Four weeks after the injection, tumors were excised and the x and y axes measured to determine tumor volume (size) according to the following formula: volume = 0.5 x width^2 x length. Representative images are shown in (A) and (C), quantification of tumor size is shown in (B) and (D). Values in (B) represent means ± SEM. *, #P<0.01. Values in (D) represent means ± SEM. *, #P<0.001.

5.3 DISCUSSION

Our studies identify Cav-1 as a novel gene fitting the evolutionary theory of antagonistic pleiotropy as it pertains to aging. Cav-1 is a double-edged sword when it comes to aging and cancer: when expressed in cancer cells it acts as a tumor suppressor as evidenced its expression in Ras^{G12V} inducing SIPS. On the other hand, Cav-1 is needed for premature senescence of fibroblasts, which can induce the growth of neoplastic cells. This work supports the hypothesis that Cav-1 is involved in senescent fibroblasts exhibiting antagonistic pleiotropy on neoplastic cells. *In vitro* and *in vivo* studies show that Cav-1 expression is necessary for senescent fibroblasts and for their ability to stimulate hyperproliferation and tumorigenesis. Fibroblasts were treated with hydrogen peroxide and all cells were starved in serum free media. This was done to i) avoid influences of growth factors contained in the media, ii) arrest the Cav-1 null MEFs which would continue to grow after oxidative stress, and iii) arrest the transformed cells to observe growth caused by co-culturing. In co-cultures and tumors using Cav-1 knockout MEFs, growth arrest of fibroblasts is not sufficient to cause hyperproliferation of transformed cells.

Cav-1 is mediating the antagonistic pleiotropic effect of senescent fibroblasts. Fibroblasts must be senescent as growth arrest is not sufficient for senescence dependent antagonistic pleiotropy in the Cav-1 knockout MEFs. Ras^{G12V} and MDA-MB-231 cells co-cultured with KO cells had *in vivo* tumor growth 3-fold and 14-fold less than wildtype. Data in Chapter 4 support that Cav-1 is necessary for SIPS triggered by oxidative stress in fibroblasts. Upon oxidative stress from hydrogen peroxide treatment, Cav-1 sequesters p53's negative regulator, MDM2, which allows p53 to become active stimulating signaling pathways leading to SIPS. Thus, Cav-1 is necessary to potentiate the SIPS cell program.

Senescent cells possess an altered secretory profile. Interestingly, the prematurely senescent fibroblast secretion profile is similar to the proliferative cancer associated fibroblasts and how they stimulate growth of transformed epithelial cells (Fusenig and Boukamp 1998; Olumi, Grossfeld et al. 1999; Mercier, Casimiro et al. 2008). The reason senescent fibroblasts express this phenotype may be to sustain the tissue and give a growth advantage to the non-senescent fibroblasts in the area. However, an accumulation of senescent fibroblasts with age also illustrates the antagonistic pleiotropy of the prematurely senescent cells as it promotes the growth of mutated epithelial cells (Campisi 2003). Senescent fibroblasts are found to secrete factors related to proliferation and changing the microenvironment such as: VEGF, (Coppe, Kauser et al. 2006), matrix metalloproteases (Dilley, Bowden et al. 2003; Liu and Hornsby 2007), chemokines (Acosta, O'Loghlen et al. 2008), TGF-ß1 (Frippiat, Chen et al. 2001), fibronectin (Chainiaux, Magalhaes et al. 2002) and IGFBP4 (Coppe, Boysen et al. 2008). Secretion studies show that the factors necessary to cause hyperproliferation are exclusive to Cav-1 expressing senescent fibroblasts. Cav-1 is necessary to trigger the senescent phenotype, which initiates the different expression profile. Whether Cav-1 has additional influence has yet to be elucidated.

Cancer associated fibroblasts (CAFs) have recently been shown to exhibit decreased Cav-1 expression in breast cancer. These data initially appear to be in opposition to our findings, which say an upregulation of Cav-1 followed by SIPS can induce proliferation of breast cancer cells. However, in this study the CAFs were isolated by their proliferation capabilities (Mercier, Casimiro et al. 2008), which completely excluded senescent fibroblasts and only focuses on non-senescent proliferating fibroblasts. Thus, the contribution of senescent fibroblasts in breast cancer tumors is still unknown. From our *in vivo* experiments it would seem that senescent

fibroblasts are involved early in tumorgenesis, as neoplastic cells dilute the senescent cells very quickly. Therefore, the senescent cells and the factors they secrete may be needed at the onset of tumor growth to give the neoplastic cells a growth advantage.

Much work on antagonistic pleiotropy of senescent fibroblasts has been done *in vitro*, but there is also evidence of this *in vivo*. The disease model of psoriasis is an illustration of antagonistic pleiotropy in the realm of premature senescence. PUVA treatment is used to treat a wide variety of skin ailments including psoriasis (Parrish, Fitzpatrick et al. 1974; Wolff, Gschnait et al. 1977; Ma, Wlaschek et al. 2002). The therapy utilizes the photosensitizer 8-methoxypsoralen and UVA (320—400 nm) irradiation to induce the formation of ROS (such as singlet oxygen, superoxide anion and hydrogen peroxide), which leads to oxidative stress and DNA damage (Pathak and Joshi 1983; Gasparro, Felli et al. 1997; Gasparro, Liao et al. 1998). Among the list of side effects for PUVA treatment is premature aging of the skin similar to other types of photodamage. For example, PUVA treatment causes thinning of the skin in psoriasis patients compared to age matched controls (Sator, Schmidt et al. 2002). In concurrence with these findings, PUVA induces SIPS in human dermal fibroblasts (Herrmann, Brenneisen et al. 1998; Hovest, Bruggenolte et al. 2006) evidenced by upregulated matrix metalloproteases, decreased cumulative population doublings, a large and squamous morphology and positive SA-β-galactosidase activity – all hallmarks of SIPS. Additionally, PUVA-stimulated SIPS is mediated in part by hydrogen peroxide generation (Herrmann, Brenneisen et al. 1998), a reactive signaling mediator, which is regularly used to induce SIPS. Gene expression profiles of PUVA treatment on human fibroblasts display altered expression of genes involved in growth arrest, stress response, matrix metalloproteases and senescence (Borlon, Debacq-Chainiaux et al. 2007).

PUVA treated psoriasis patients have high incidences of epithelial skin carcinomas (Stern and Lunder 1998). However, none of the tumors are derived from fibroblasts (Ma, Hommel et al. 2003). It is hypothesized that this is antagonistic pleiotropy (Campisi 2005). It could be that upon PUVA treatment fibroblasts undergo SIPS, and overtime the MMPs and cytokines they secrete cause preneoplastic cells in the surrounding environment to become carcinogenic. While the psoriatic plaque environment is seemingly prime to induce carcinogenesis, lesions do not generate in the plaque but in adjacent areas. The keratinocytes associated with the plaques are senescent and tumor resistant. Thus, this medical paradox is explained by senescent cells having a tumor suppressor mechanism for themselves while activating growth of neighboring cells (Nickoloff 2001; Nickoloff 2004).

In conclusion we have found a novel mechanism in which Cav-1 plays two seemingly opposite roles when it comes to aging and cancer. When Cav-1 is re-expressed in cancer cells where the gene is down-regulated, Cav-1 shows tumor suppressor properties by triggering a "senescent switch" which ceases the growth of the cell. In contrast, fibroblasts that undergo Cav-1 dependent SIPS from an oxidative stressor, can cause the proliferation of mutated epithelial cells. Thus, Cav-1 acts as a tumor promoter to adjacent cells (Figure 32). Our work determines that Cav-1 is necessary to induce SIPS by oxidative stress in fibroblasts, as supported by the unique mechanism that upon oxidative stress Cav-1 sequesters MDM2, thereby allowing for p53 activation and SIPS. While this is a tumor suppressing mechanism for the fibroblast, the Cav-1 dependent senescence can cause antagonistic pleiotropy and induce proliferation of initiated epithelial cells.

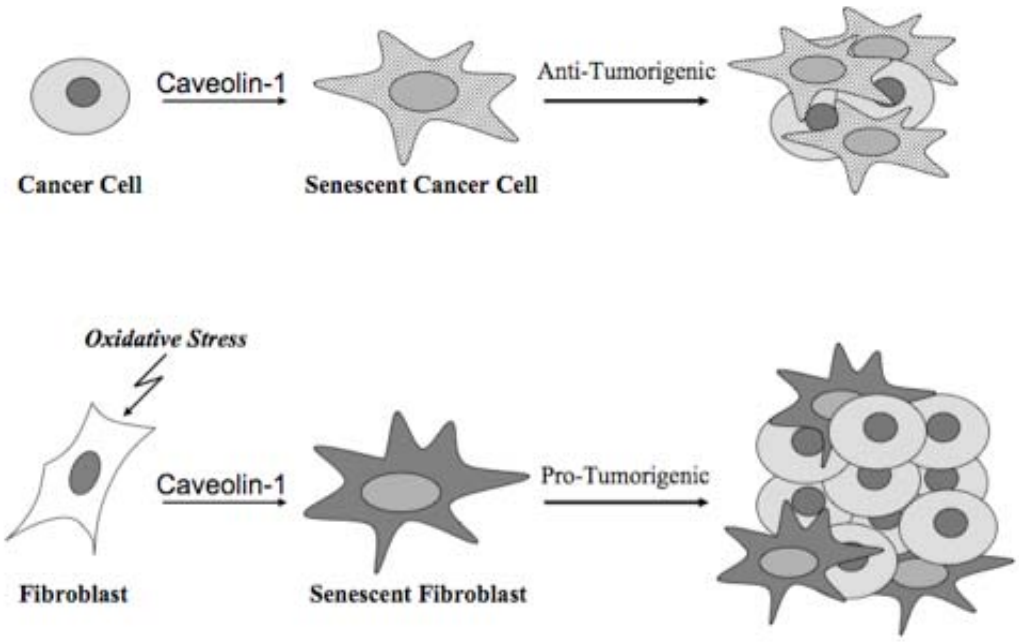


Figure 32. Schematic diagram of the diverse roles of Cav-1, illustrating how Cav-1 demonstrates tumor suppressor activities. On the single cell level, Cav-1 can cause senescence in transformed cells. It is also needed by fibroblasts damaged by oxidative stress to undergo senescence. Both of these instances cause diminished growth in the individual cell. On the tissue level, an accumulation of senescent cells can lead to tumor promotion. The change in the secretory profile of senescent cells contributes to changes in the microenvironment that have pro-tumorigenic effects on mutated cells in the surrounding area. Since oxidative stress induced SIPS is dependent upon Cav-1 the downstream effect of antagonistic pleiotropy is also dependent upon the protein.

6.0 CONCLUSION

We have found that oxidative stress triggers a Cav-1 mediated signaling pathway, which induces SIPS. Upon oxidative stress cellular changes activate $p38^{MAPK}$, resulting in activation of the transcription factor Sp1. Sp1 binds to 2 GC-boxes in the Cav-1 promoter upregulating the protein. Cav-1 sequesters MDM2, a negative regulator of p53, so that p53 degradation is halted and it is active to up-regulate its downstream target $p21^{WAF/CIP1}$ leading to SIPS. Additionally, Cav-1 exhibits tumor suppressor activities as it works as a senescence switch. Interestingly, Cav-1 functions to induce cancer by its antagonistic pleiotropic effect in senescent fibroblasts secreting factors, which lead to proliferation of neighboring neoplastic cells.

Biomedical science is uncovering many SIPS occurrences *in vivo*. LASIK eye surgery triggers senescence in keratocytes of the cornea (Dawson, O'Brien et al. 2006). Psoriatic plaques contain senescent keratinocytes which induce lesions adjacent to the plaques (Nickoloff 2004). Inflammatory factors of chronic wounds trigger SIPS in surrounding fibroblasts affecting the ability of the wounds to heal (Harding, Moore et al. 2005), and senescence in kidney epithelial cells may affect organ transplantation success (Famulski and Halloran 2005). Current understanding of the SIPS phenomena does not explain what is occurring at the cellular level in these examples nor how to minimize the potential detrimental side effects of SIPS. Senescent cells are located at the sites of age-related diseases such as atherosclerotic lesions, skin ulcers, arthritic joints, and both benign and preneoplastic hyperproliferative lesions in prostrate and liver

(Campisi 2005). As the age of our population increases age-related diseases, revealing the mechanisms leading to cellular senescence *in vivo* becomes increasingly necessary for improving public health.

Understanding SIPS and aging is significant for improving health. First, SIPS is a tumor suppressor for the cells that undergo growth arrest, which is a positive effect from a carcinogenic standpoint. However, SIPS cells can induce neighboring preneoplastic cells to become tumorigenic, which is deleterious. Since many medical treatments actually induce SIPS (LASIK, psoralen plus UVA, and chemotherapy for malignancies) research on the underlying mechanism of how SIPS occurs will provide information on preventing SIPS from occurring when it could be detrimental to the patient. Because SIPS is exogenously triggered, its reasoned that SIPS is a drug-responsive cellular program, which can be exploited for therapeutic treatments (Nickoloff 2004). Understanding the mechanisms of these phenomenon will aid in the creation of better interventions and therapies to slow ageing due to oxidative damage, and will allow the manipulation of the system by encouraging aging when it would be beneficial, (e.g. as a tumor suppressing mechanism).

The data herein identify the protein Cav-1 as a key therapeutic target for aging and cancer. Managing Cav-1 expression is a delicate balancing act (Figure 33). Cav-1 can have tumorigenic and tumor suppressor activities depending on the cell type and environment, demonstrating that it is clearly an antagonistic pleiotropic gene. Decreasing Cav-1 in cells would have an anti-aging effect, as cells would not respond to oxidative stress with SIPS and would either continue proliferation, or undergo apoptosis. However, in transformed cells, increasing Cav-1 would have the beneficial effect of inducing growth arrest and SIPS to slow the growth of

the neoplasm. The CSD would be sufficient to induce SIPS and further work using the Cavtratin peptide could determine whether the CSD could induce SIPS in tumor cells *in vivo*.

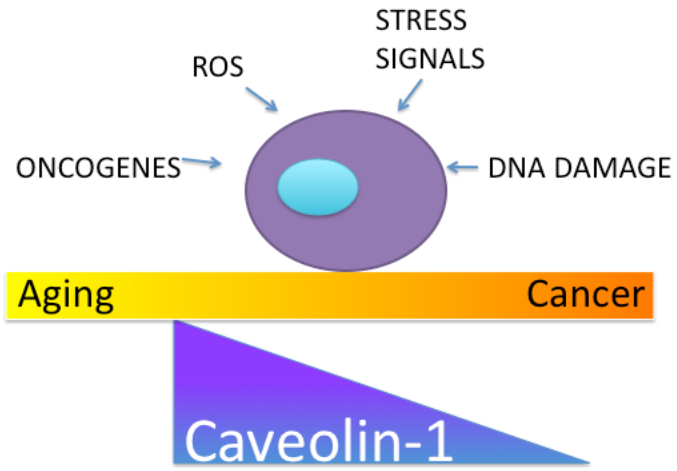


Figure 33. Schematic depicting the balancing act of managing Cav-1 as a potential therapeutic target in aging and cancer. Exogenous signals cause DNA damage, which cause cells to either senesce or become cancerous. The expression of Cav-1 in these cells is a delicate balancing act. Cav-1 exhibits tumor suppressor properties, which is a contributor to the aging phenotype. However, the absence of Cav-1 can also have deleterious effects, as it is downregulated in many tumors. Cav-1 is now identified as an antagonistic pleiotropic gene. Therefore, the system is complicated by the fact in some instances Cav-1 has positive attributes where it mediates senescence to allow the organism to avoid cancer. However, senescent fibroblasts can induce the proliferation of surrounding mutated epithelial cells giving them the push they need to proliferate.

In terms of treating cancer, the identification of Cav-1 as a mediator of antagonistic pleiotropy pertaining to SIPS, sheds light on a new way to target therapeutics. Fibroblasts that contain Cav-1 respond to cellular damage by undergoing SIPS, which is a tumor suppressing mechanism for these cells. However, the detrimental effect these fibroblasts have on their microenvironment must be addressed. Cav-1 is necessary for cells to undergo tumor suppressing SIPS, however, mutated epithelial cells in the area will respond to the secreted MMPs,

chemokines, and growth factors by proliferating. Therefore, Cav-1 should not be targeted in cancer, as it is performing its tumor suppressing duty, but the secretion profile it creates in the senescent cells should be examined. If there are ways of inhibiting these factors (e.g. by inhibitory antibodies or small molecules) it is plausible to allow Cav-1's tumor suppressing properties and curtail its antagonistic pleiotropic effects.

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