Molecular Mechanism of Src Regulation

by Csk, SHP-2, Cbp, and Caveolin-1

BY

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THESIS

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This Thesis is dedicated to: My Family, my Girlfriend, my Friends, and my Co-workers. Without all of their support, I certainly could not have completed this monumental accomplishment in my life.

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LIST OF ABBREVIATIONS

WT - Wild-type

KO - Knockout

SFK - Src Family Kinase

MF - Mouse Fibroblast Cell

HLMVEC - Human Lung Microvascular Endothelial Cell

RLMVEC - Rat Lung Microvascular Endothelial Cell

HEK - Human Embryonic Kidney Cell

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

Y14F - Tyrosine to Phenylalanine Mutant at Position 14 of Caveolin-1

Y14E - Tyrosine to Glutamic Acid Mutant at Position 14 of Caveolin-1

Y416F - Tyrosine to Phenylalanine Mutant at Position 416 of c-Src

Y527F - Tyrosine to Phenylalanine Mutant at Position 527 of c-Src

Y519F - Tyrosine to Phenylalanine Mutant at Position 519 of c-Src

Tyr - Tyrosine

Csk - C-Terminal Src Kinase

Cbp - Csk Binding Protein

Cav-1 - Caveolin-1

SHP-2 - SH2 Domain Containing Tyrosine Phosphatase 2

TNF-α - Tumor Necrosis Factor Alpha

TNFR - TNF Receptor

PAR-1 - Protease Activated Receptor 1

SH - Src Homology Domain

LIST OF ABBREVIATIONS (continued)

NF-kB - Nuclear Factor Kappa B

TRAF - TNF Receptor Associated Factor

RIPA - Radio Immunoprecipitation Assay

ODG - n-Octylglucoside

PMSF - Phenylmethylsulfonyl Fluoride

EDTA - Ethylenediaminetetraacetic Acid

BCA - Bicinchoninic Acid

SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TBS - Tris Buffered Saline

PBS - Phosphate Buffered Saline

HBSS - Hank's Balanced Salt Solution

GFP - Green Fluorescent Protein

CFP - Cyan Fluorescent Protein

YFP - Yellow Fluorescent Protein

FRET - Fluorescence Resonance Energy Transfer

VEGF - Vascular Endothelial Growth Factor

DTT - Dithiothreitol

GST - Glutathione-S-Transferase

siRNA - Small Interfering Ribonucleic Acid

cDNA - Complementary Deoxyribonucleic Acid

SUMMARY

Activation of the Src family kinases (SFKs) is associated with normal cellular homeostasis, and their activity is elevated in some pathological conditions such as cancer and acute inflammation. Despite the importance of SFK regulation, some aspects of the molecular mechanisms controlling these kinases remain largely unknown to date. The goal of the studies described herein were to further the current knowledge surrounding this mechanism, and were focused upon c-Src. First, we examined the negative regulation of c-Src kinase activity, which we postulated to be important for understanding the activation of c-Src under both normal and pathological conditions.

All SFKs share the same general structure, and two important tyrosine phosphorylation events have been associated with distinct levels of their kinase activity. Phosphorylation of an inhibitory tyrosine in the C-terminal tail of the molecules is associated with inhibition, and phosphorylation of another tyrosine in the activation loop of the kinase domain results in the full activity of the enzymes. For example, phosphorylated Tyr419 and dephosphorylated Tyr530 in human c-Src is thus thought to represent the fully active state. It has been demonstrated previously that the inhibitory residue of the Src family kinases is phosphorylated by another tyrosine kinase, Csk. Csk is intrinsically cytoplasmic, and thus requires membrane associated adapter proteins to localize to areas of c-Src activity. Caveolin-1 (Cav-1) and Csk Binding Protein (Cbp) can both serve as such adapters, and both are phosphorylated by c-Src. This creates a binding site for Csk, and a feedback inhibition loop to terminate c-Src signaling.

The studies described in this dissertation reveal a cooperation between Cav-1 and Cbp, which together function to protect against elevated or uncontrolled activation of c-

Src. This was demonstrated by the finding that the absence of both Cav-1 and Cbp in cells led to an elevated c-Src activity, which was not achieved by the absence of only one adapter alone. These two Csk adapters were also shown to be in a complex with each other, as well as Csk, under basal conditions in these studies. Additionally, the reduction of Cbp alone using siRNA in WT cells did not change the amount of Csk present in the complex. Under these reduced Cbp conditions, we observed increased Cav-1 phosphorylation, but c-Src activity remained the same as control cells. Examination of cells derived from Cav-1 null mice revealed an increased expression of Cbp, without any difference in c-Src activity when compared to cells from WT littermates. This occurred in these mice most likely because of the need to control c-Src, and its family of kinases, during embryonic development. Also, the expression of WT Cav-1 or a phospho-mimicking Y14E Cav-1 mutant in Cav-1 null cells reduced basal c-Src activity, a result that was not achieved by the expression of a phospho-defective Y14F Cav-1 mutant. We therefore conclude that inhibition of c-Src is an equilibrium, since it could be effected by increases or decreases in expression or phosphorylation of the Csk adapters.

To further explore this equilibrium in these studies, the activation of c-Src was examined. The idea was that if Csk phosphorylated the C-terminal inhibitory tyrosines, a tyrosine phosphatase may be dephosphorylating the same site, and was in equilibrium with Csk. Knockdown of SHP-2, one such phosphatase, in endothelial cells using siRNA led to reduced basal c-Src activity. Furthermore, the inhibitory C-terminal tyrosine phosphorylation of c-Src was elevated under these conditions. Interestingly, stimulation of c-Src activity by TNF-α was blocked, and the C-terminal tyrosine phosphorylation

level was not reduced. Therefore, SHP-2 was required for c-Src activation both basally, and by TNF-α, but could either be dephosphorylating the Csk adapters, or the c-Src C-terminal tail directly. However, the phosphorylation of Cav-1 at tyrosine 14 was also unchanged both basally, and after stimulation, as compared to control cells. Since the reduction of SHP-2 did not raise Cav-1 phosphorylation at its Csk binding site, we deduced that SHP-2 must be acting upon c-Src itself. This was indeed the case, as we observed an increase in c-Src Tyr530 phosphorylation both basally and upon stimulation with TNF-α in SHP-2 depleted cells. Thus, Csk and SHP-2 are in equilibrium, competing for the C-terminal tyrosine of c-Src, and most likely other family members as well. Changes in the activity of c-Src, also known as c-Src activation and inactivation, are thus dictated by perturbations and fluctuations in this equilibrium between Csk and SHP-2 at any given time. An increase in SHP-2 activity, either by expression or activation, would increase the active fraction of c-Src. An increase in Csk activity, either by expression (Csk or adapter) or activation would increase the inactive fraction of c-Src. From these data, we conclude that TNF-α increases Src activity by activating SHP-2, thereby shifting this equilibrium in favor of c-Src Tyr530 dephosphorylation.

I. INTRODUCTION

A. Background

Since the discovery of c-Src in 1976, the first proto-oncogene discovered, the mechanism of its regulation has been under intense investigation (Martin, 2001). Many groups of investigators all over the globe have provided insights into the complex and dynamic mechanism of c-Src activity regulation. With each step taken closer to solving the puzzle, more questions were raised, and more experimentation was warranted. This led to the discovery of a whole family of c-Src related enzymes, the Src family kinases (SFKs). The first major breakthrough occurred when the non-transforming cellular homolog (c-Src) of the Rous sarcoma virus transforming gene product (v-Src) was characterized. The difference between v-Src and c-Src was that the cellular form was about 10-fold less active (Coussens et. al., 1985). However, its regulation was not appreciated until mutagenesis and crystalization studies were carried out which determined that the C-terminal regulatory tyrosine, which was absent in v-Src, played an essential role in c-Src inactivation (Piwnica-Worms et. al., 1987; Xu et. al., 1997; Cowan-Jacob et. al., 2005). Further insight was gained when C-terminal Src Kinase (Csk), the enzyme responsible for SFK C-terminal tyrosine phosphorylation, was discovered in 1991 (Okada et. al., 1991).

An elegant feedback inhibition loop has been derived from numerous studies where phosphorylated c-Src substrates can recruit Csk to terminate c-Src signaling. Of the many c-Src substrates, and signaling pathways requiring SFK signaling, only a few Csk recruiting proteins (or Csk adapters) have been identified. There is strong evidence supporting the role of two membrane associated proteins as adapters for Csk to terminate

SFK signaling. These two proteins are Csk-Binding Protein (Cbp) (Kawabuchi et. al., 2000), also known as Phosphoprotein Associated with Glycosphingolipids-1 (PAG-1) (Brdicka et. al., 2000), and Caveolin-1 (Cav-1) (Cao et al., 2002; Lu et. al., 2006). Coincidentally, Cav-1 was one of the first phosphorylated targets of v-Src identified (Glenney and Zokas, 1989; Li et. al., 1996). Subsequent studies have demonstrated the involvement of at least one of these two adapters in the regulation of Integrin, PAR-1 receptor, T-Cells receptor, and Glutamate receptor signaling (Radel et. al., 2007; Lu et. al., 2006; Brdicka et. al., 2000; Khanna et. al., 2007).

B. Statement of Hypothesis

In this work, we tested the hypothesis that an equilibrium exists between phosphorylation (by Csk) and dephosphorylation (by SH2 domain containing tyrosine phosphatase-2, SHP-2) of the C-terminal regulatory tyrosine of c-Src, which reduces or increases c-Src activity level respectively. We hypothesized that this equilibrium requires: 1) the Csk adapters for recruiting Csk (and Csk per se) to terminate the activated c-Src and 2) that a tyrosine phosphatase (SHP-2) allows for dephosphorylation of the negative regulatory C-terminal tyrosine phosphorylation achieved by Csk.

C. Significance of the Study

The mechanism of c-Src (and SFK) regulation, and the ability to modulate the activity of the enzyme, has important and clinically significant applications. For instance, in many human cancers, SFKs are found be to overactive and transforming (Lynch et. al., 1993; Bjorge et. al., 2000; Campone et. al., 2011; Haura et. al., 2010).

Elevated SFK activity is associated with tumor growth and survival, and migration and metastasis, both of which are associated with worsened patient prognosis. In fact, SFK inhibitors are found in recent clinical trials for the treatment of breast cancer (Bosutinib, Campone et. al., 2011), and non-small cell lung cancer (Dasatinib, Haura et. al., 2010).

Aberrant SFK signaling is also implicated in inflammatory hyperpermeability syndromes and edema formation. Albumin transport across the endothelial layer of the blood vessel wall, as well as increases in fluid flux through the endothelium, has been demonstrated to involve c-Src (Minshall et. al., 2000; Mehta and Malik, 2006; Angelini et. al. 2006; Hu et. al. 2008; Sun et. al. 2009). Furthermore, acute lung injury and its more severe manifestation, acute respiratory distress syndrome, are thought to be driven in part by c-Src mediated increases in endothelial permeability, vascular injury, neutrophil accumulation, and edema formation (Severgnini et. al., 2005; Lee et. al., 2007; Hu et. al. 2008; Liu et. al., 2011). Thus, a better understanding of the molecular regulation of the SFK enzymes may someday be used clinically for diagnostic and prognostic determination, or the development of novel inhibitors, both of which will be beneficial to those who suffer from these pathologies associated with elevated SFK signaling.

II. LITERATURE REVIEW

A. Src Family Kinases

Many years have passed since the oncogenic transforming gene product of the Rous sarcoma virus, v-Src, was identified and discovered to be a constitutively active enzyme (Cvernilofsky, 1980). It belongs to a class of kinases, enzymes that add a phosphate group to amino acids, with v-Src having a target of tyrosines being described in 1980 (Hunter and Sefton, 1980). Later, the enzyme was found to be a hijacked component of normal cells (Shalloway et. al., 1981; Takeya and Hanafusa, 1982; Takeya and Hanafusa, 1983), and the c- (c- for cellular, v- for viral) in c-Src was coined. In the studies that followed, a whole family of related tyrosine kinases expressed in many cell types were discovered and characterized.

Src family tyrosine kinases (SFKs) are involved in many of the signaling mechanisms associated with G-protein-coupled receptors, integrins, receptor tyrosine kinases, T-cell receptors, and others (Thomas and Brugge, 1997). Of the nine family members, c-Src, Yes, and Fyn are expressed ubiquitously, with c-Fgr, Lck, Hck, Lyn, Blk, and Yrk being expressed primarily in lymphocytes (Chow and Veillette, 1995). SFKs all share a common general structural organization: an N-terminal

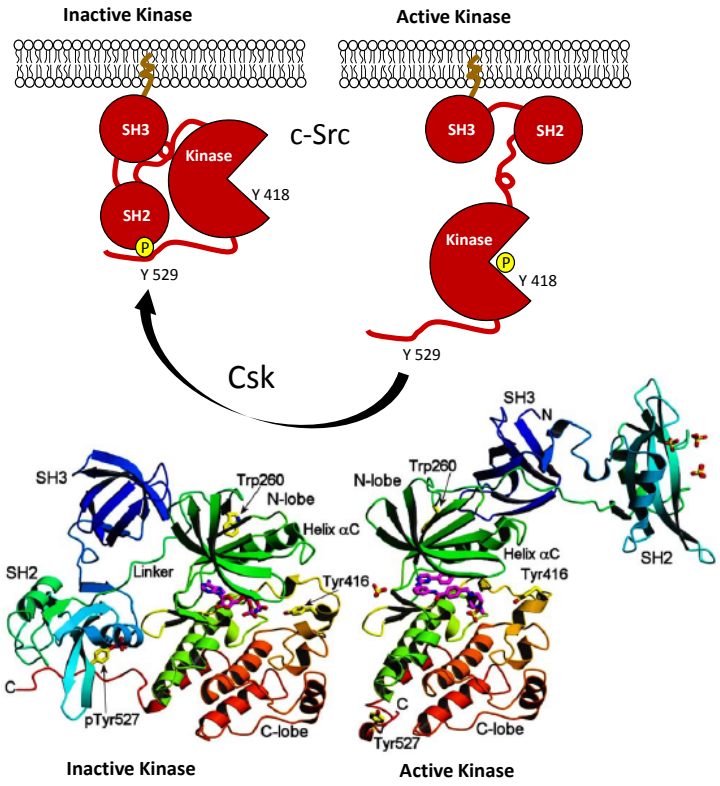

Figure 1: Conformations of c-Src. The inactive (or closed) form of the kinase is shown on the left, and active (or open) form on the right. The three-dimensional ribbon diagrams (from Cowan-Jacob et. al., 2005) are shown below each corresponding conformation. Phosphorylation of the C-terminal tyrosine of SFK's by Csk promotes the inactive conformation.

membrane association domain, a unique domain, a Src homology (SH) 3 domain, an SH2 domain, a catalytic kinase domain, and a C-terminal regulatory domain. The kinase domain contains a tyrosine residue, which is autophosphorylated when the enzyme is active. SH2 domains bind phosphotyrosine motifs, and SH3 domains bind polyproline motifs. In the inhibited state, the SH2 domain of c-Src is involved in an intramolecular interaction with a C-terminal regulatory domain phosphotyrosine (Xu et. al., 1997), locking the enzyme in an inactive or closed state (Figure 1). This conformation is further stabilized by the SH2 to kinase domain linker binding to the SH3 domain. Oncogenic activation in the case of v-Src results from the absence of this C-terminal regulatory domain (Martin, 2001).

Despite current knowledge of the inhibited closed state and the active open state, the specific mechanism of SFK activation remains unclear. For instance, mutation of tyrosines (Y) to phenylalanines (F) in the C-terminus, (Y519 and Y527 of chicken c-Src), and the active site (Y416 of chicken c-Src), was performed previously (Piwnica-Worms et. al., 1987). Both the Y527F mutant, and the double mutant Y519F/Y527F, had elevated kinase activities. The Y519F single mutant activity was not different than WT c-Src activity. Another single mutation, Y416F did not alter the kinase activity as compared to WT c-Src. However, the double mutant Y416F/Y527F was about half as active as the Y527F mutant alone. These mutagenesis studies demonstrated that the full activity level of c-Src requires phosphorylation of tyrosine 416, and dephosphorylation of tyrosine 527. Comparison of the three dimensional crystal structure of c-Src in active and inactive conformations has furthered these findings. In the active site of the inactive kinase, pinching of the two lobes of the kinase domain closes the active site, and helix αC

is displaced causing a rearrangement of the residues required for catalysis (Xu et. al., 1997). The activation loop which contains the unphosphorylated tyrosine 416 is not in a suitable position to allow for substrate docking (Cowan-Jacob et. al., 2005). The SH2 and SH3 domains are also blocked in the inactive conformation due to the intramolecular interactions described above (Cowan-Jacob et. al., 2005). Many studies have suggested that higher affinity SH2 and SH3 ligands can compete with these intramolecular interactions, but the results are not definitive since the affinities of these domains are determined by the overall molecular stability of c-Src.

Many tyrosine phosphatases have been implicated in dephosphorylation of the C-terminal tyrosine, such as PTP1B (Bjorge et. al., 2000) and RPTPα (Su et. al., 1999; Vacaresse et. al., 2008). However, these studies and conclusions are complicated by the fact that the tyrosine is masked by binding to its own SH2 domain, or they are based upon findings in abnormal cell types (i.e. cancer cells). The questions of how the C-terminal tail tyrosine becomes available for dephosphorylation as seen in the active conformation if it is bound to the SH2 domain, and how this occurs under conditions of normal cellular homeostasis, still remain to be solved.

B. C-terminal Src Kinase

Phosphorylation of the C-terminal regulatory tyrosine in SFKs is catalyzed by C-terminal Src kinase (Csk) (Okada et. al., 1991). Csk is required for normal development *in utero*, because Csk knockout mice die at embryonic day 9 or 10 (Imamoto and Soriano, 1993) due to neural tube defects. Cells derived from embryos prior to day 10 display elevated activities of c-Src and Fyn, about eleven-fold higher than WT cells

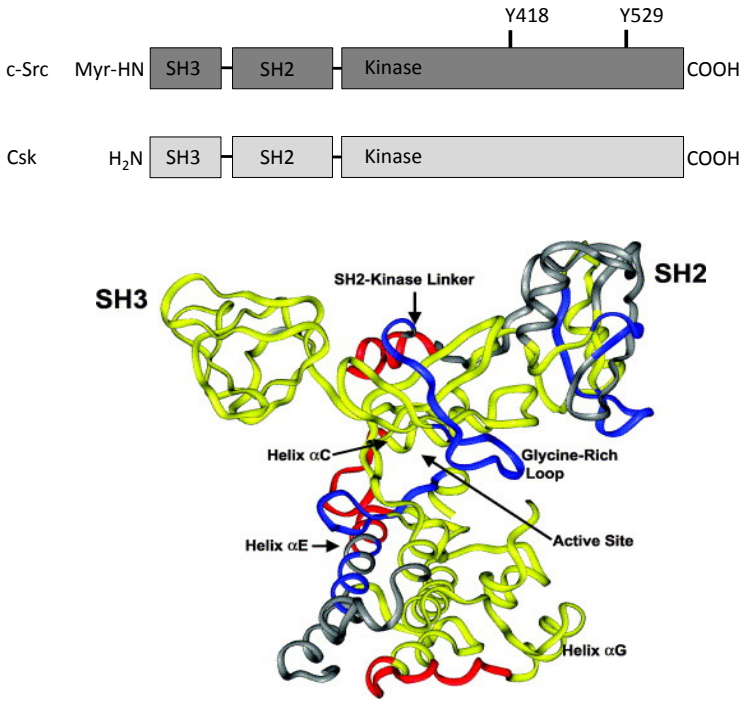

Figure 2: Structure of Csk. The linear domain maps of c-Src and Csk are shown above. Note the myristoylated N-terminus of c-Src and the C-terminal tyrosine, both of which are not present in Csk. The three-dimensional ribbon diagram of Csk (from Wong et. al., 2005) is below the linear maps. Engagement of the SH2 domain of Csk repositions the active site for enhanced catalysis.

derived at the same embryonic stage, with greatly reduced C-terminal phosphorylation. This phenotype of Csk knockout mice is demonstrative of the critical requirement of Csk expression during embryonic development, suggesting that control of the activity levels of the SFKs is required for normal mouse development.

The architecture of Csk (Figure 2) is similar to SFKs with one SH2 domain, one SH3 domain, and a kinase domain (Nada et. al., 1991). It is noteworthy that Csk lacks a regulatory C-terminal tyrosine, N-terminal myristoylation, and membrane association domain (Ogawa et. al., 2002; Wong et. al., 2005). So, although SFKs are membrane-associated and regulated by phosphorylation, Csk is intrinsically cytoplasmic (Howell and Cooper, 1994) and requires membrane adaptors to inhibit membrane-associated SFKs. Furthermore, the SH2 domain of Csk is critical for its localization and kinase activity. This fact was elegantly displayed in a study that mutated the SH2 domain of Csk, and in cells expressing this mutant, SFK activity could not be terminated because Csk could not translocate to the membrane where SFK's are localized (Howell and Cooper, 1994). The three dimensional crystal structure also revealed that the direct linkage between the SH2 domain and the n-lobe of the kinase domain is important for positioning the active site of Csk in the optimal position for catalysis (Ogawa et. al., 2002; Wong et. al., 2005). Therefore, the SH2 domain of Csk not only allows it to associate with the membrane, but it also stimulates its kinase activity.

One unusual feature of Csk is its specificity towards the SFKs. This was elegantly demonstrated by modeling of the crystal structure of the kinase domains of c-Src and Csk bound to each other (Levinson et. al., 2008). This structure revealed that the C-terminal lobe of the kinase domain of c-Src was required for Csk recognition and

binding. In fact, this portion of c-Src positioned the C-terminal tyrosine adjacent to the active site of Csk. Another study confirmed this phenomenon by mutagenesis of the SH2 domain and kinase n-lobe domain interface (Mikkola and Gahmberg, 2010). Mutations of Tyr116, Tyr133, Leu138, and Leu149 of Csk were unable to inactivate endogenous c-Src when expressed in HEK cells. This also explains why Csk is inefficient at phosphorylating tyrosine containing peptides *in vitro* (Sondhi et. al., 1998). In this study, the authors described Csk's activity towards a purified catalytically inactive Lck, with the correctly folded kinase domain, to be much more catalytically efficient as a substrate for Csk than the most efficient tyrosine-containing peptide. These studies illustrate that tertiary conformations of the SFKs contribute significantly to the specificity of Csk, and help explain why there are so few substrates for Csk.

C. Csk Binding Protein

Studies have identified and characterized a Csk adaptor in T-cells (Brdicka et. al., 2000) and rat brain (Kawabuchi et. al., 2000) known as Csk binding protein (Cbp), or phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG-1). The crystal structure of Cbp has not been solved to date. However, through primary amino acid sequence analysis, it can be extrapolated that Cbp has a short extracellular domain (~13 amino acids), a single transmembrane domain, and a long C-terminal cytoplasmic domain (Figure 3) containing ten tyrosines and eleven alpha helices (Takeuchi, 2006). It is also double palmitoylated on juxtamembrane cysteines allowing for lipid raft targeting (Kawabuchi et. al., 2000).

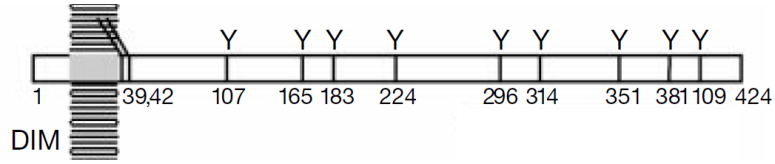

Figure 3: Cbp linear domain map. Cbp has a short N-terminal extracellular domain, a single transmembrane domain, and ten cytoplasmic tyrosines on its C-terminus. It is palmitoylated on two juxtamembrane cysteines, C39 and C42 (black lines) conferring lipid raft (aka detergent insoluble membrane, DIM) localization. (Modified from Kawabuchi et. al., 2000)

It has been demonstrated that phosphorylation of Cbp by SFKs takes place on tyrosine 314 (Yasuda et. al., 2002), and through mutagenesis studies, that this residue also facilitates the binding of Csk through its SH2 domain (Kawabuchi et. al., 2000). A FRET-based study using Cbp-YFP and CFP-Csk also demonstrated this binding in live cells. When SFK activity was stimulated, a FRET response was detected between CFP-Csk and Cbp-YFP, but not between CFP-Csk and the mutant CbpF314-YFP (Matsuoka et. al., 2004). Importantly, it was also demonstrated that Csk activity increases approximately 3-fold when bound to a peptide corresponding to the cytoplasmic region of Cbp containing phosphorylated tyrosine 314 as compared to the same unphosphorylated peptide (Takeuchi et. al., 2000; Wong et. al., 2005). Therefore, the interaction of Csk with Cbp helps to recruit the enzyme to the membrane and increase its activity, creating a feedback inhibition loop to terminate SFK signaling. In contrast to the embryonic lethality of Csk knockout mice, Cbp knockout mice are viable (Dobenecker et. al., 2005). In this study, it is noteworthy that Csk was still recruited to the membrane in cells derived from Cbp(-/-) mice, suggesting that there are additional membrane adaptors for Csk.

D. Caveolins

The Caveolin family of proteins has three members, Caveolin-1 (Cav-1), Caveolin-2 (Cav-2), and Caveolin-3 (Cav3), with molecular weights ranging from 18-24 kD (Gosens et. al. 2008). Cav-1 and Cav-2 are ubiquitously expressed, with high expression in endothelial cells and fibroblasts, and Cav-3 expression is limited to muscle cells (Okamoto et. al. 1998; Mercier et. al. 2009). Cav-1 has two isoforms, Cav-1α and Cav-1β, that result from an internal translation site that skips over the first 31 amino acids

in Cav-1α to make Cav-1β (Gosens et. al. 2008). Despite the absence of a crystal structure to date, amino acid sequence analysis has shown that all caveolins have the same general structural features, as well as the caveolin consensus sequence, FEDVIAEP. The cytoplasmic N- terminal domain contains an unordered region, followed by an alpha helical domain containing both the oligomerization domain (amino acids 61-101) and the caveolin scaffolding domain (82-101), with a central hydrophobic cholesterol binding hairpin (amino acids 102-134) in the midsection of the molecule, and a cytoplasmic C-terminal oligomer-oligomer interacting domain (Okamoto et. al. 1998; Gosens et. al. 2008; Mercier et. al. 2009). The oligomerization domain of Cav-1 allows it to form homo-oligomers with other Cav-1 molecules and hetero-oligomers with Cav-2 (Okamoto et. al. 1998; Quest et. al. 2008). These oligomers are thought to form rope-like structures (Figure 4) via the C-terminal oligomer-oligomer interacting domain, and that this caveolin oligomeric chain forms the intercellular coat of specialized lipid microdomains or lipid rafts called caveolae (Fernandez et. al. 2002; Lebbink et. al., 2010). Caveolae are cholesterol and glycosphingolipid rich areas of membrane marked by the presence of caveolins, and are found in most cell types. It has been demonstrated that caveolae can serve as endocytic vesicular structures (Parton et. al. 2007; Mercier et. al. 2009; Minshall et. al., 2000) and mobile signaling platforms (Okamoto et. al. 1998; Quest et. al. 2008; Mercier et. al. 2009).

Many signaling molecules are found in caveolae, and many of them directly interact with caveolins. Thus, caveolins have a prominent scaffolding function, and a special domain in Cav-1 has also been implicated as a Csk adapter. Cav-1 is

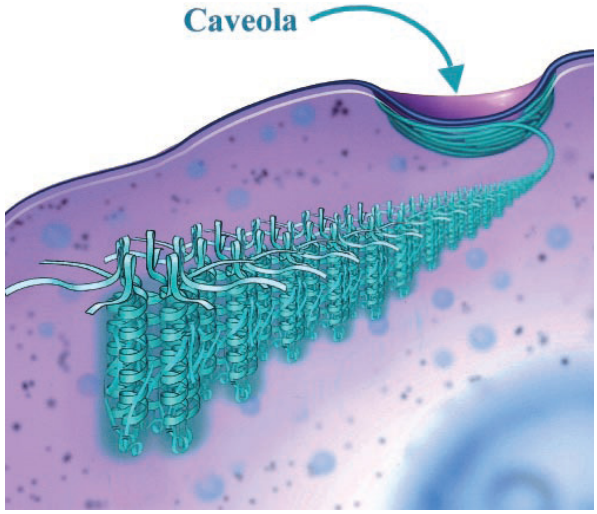

Figure 4: Caveolar coat model. Model of a Caveolin-1 oligomer-oligomer rope-like structure lining a caveola. (From Fernandez et. al., 2002)

phosphorylated on tyrosine 14 (Tyr14) by v-Src (Li et. al., 1996) and by c-Src (Aoki et. al., 1999; Lee et. al., 2000), which is believed to play a key role in the initiation of caveolae-mediated endocytosis (Minshall et. al., 2000; Shajahan et. al., 2004; Sverdlov et. al., 2007, 2009). Cav-1 also serves as an important membrane-associated scaffolding protein for endothelial nitric-oxide synthase (eNOS), G proteins, Ras, and SFKs (Okamoto et. al., 1998; Minshall et. al., 2000). This scaffolding function also includes the binding of Csk, as shown by a yeast two-hybrid screening (Cao et. al., 2002) and association of phospho-Cav-1 with Csk in the negative regulation of protease activated receptor-1 signaling (Lu et. al., 2006). Interestingly, Cbp has been shown to be localized to caveolae, and to be constitutively associated with Cav-1 (Jiang et. al., 2006).

E. SH2 Domain Containing Tyrosine Phosphatase Family

The SH2 domain containing tyrosine phosphatase (SHP-) family contains two members, SHP-1 and SHP-2. Whereas SHP-1 expression and its 3 isoforms are mainly restricted to hematopoietic cells and nuclei, SHP-2 is more ubiquitously expressed and has only one form (Poole et. al., 2005). SHP-1 has been shown to have a more negative or inhibitory signaling role, and SHP-2 a more positive or activating role (Qu, 2000; Poole et. al., 2005). SHP-1 knockout mice are viable, but display severe hematopoietic defects with chronic inflammation and autoimmune syndromes, whereas SHP-2 knockout mice are embryonic lethal. SHP-2 has been implicated in insulin, fibroblast growth factor, platelet-derived growth factor receptor, and tumor necrosis factor (TNF) signaling, all of which have been shown to activate SFK's (Thomas and Brugge, 1997; Poole et. al.,

2005; You et. al., 2001; Pincheira et. al., 2008). SHP-2 has also been implicated in the activation of SFK's by controlling the association of Csk with Cbp (Zhang et. al., 2004).

The regulation of SHP-2 is similar to SFK's because it has an open/active form and a closed/inactive form. These conformations are deduced from the solved three-dimensional crystal structure of SHP-2 (Figure 5), which revealed an architecture similar to SFKs and Csk despite its lack of an SH3 domain. SHP-2 has two SH2 domains in series that are referred to as the N- or C-terminal SH2 domains, a phosphatase domain, and a unique C-terminal tail (Hof et. al. 1998). The N-terminal SH2 domain interacts with the phosphatase domain through charge-charge interactions, and this is thought to be involved in repression of its activity by directly blocking the active site and thus promoting the closed/inactive form of the phosphatase (Hof et. al. 1998). Its regulation is also similar to that of Csk in that phosphotyrosine ligand binding to its SH2 domain stimulates SHP-2 phosphatase activity. When a phosphotyrosine ligand binds to the N-terminal SH2 domain, an allosteric conformation switch induces the open/active conformation and unblocking of the active site (Hof et. al. 1998; Poole et. al., 2005). The C-terminal SH2 domain is less active in contributing to the overall structure of SHP-2. Rather, it is thought to play a role in the regulation of the specificity of phosphotyrosine interactions and binding energy (Hof et. al., 1998). The C-terminal tail has also been implicated in the regulation of SHP-2, but its role is less clear. The tails of the family members contains tyrosines and serines that can be phosphorylated, sequences for membrane lipid raft targeting, and poly-proline motifs for SH3 domain binding (Poole et. al., 2005). Importantly, SHP-2 has been shown to lack both the lipid binding domain and

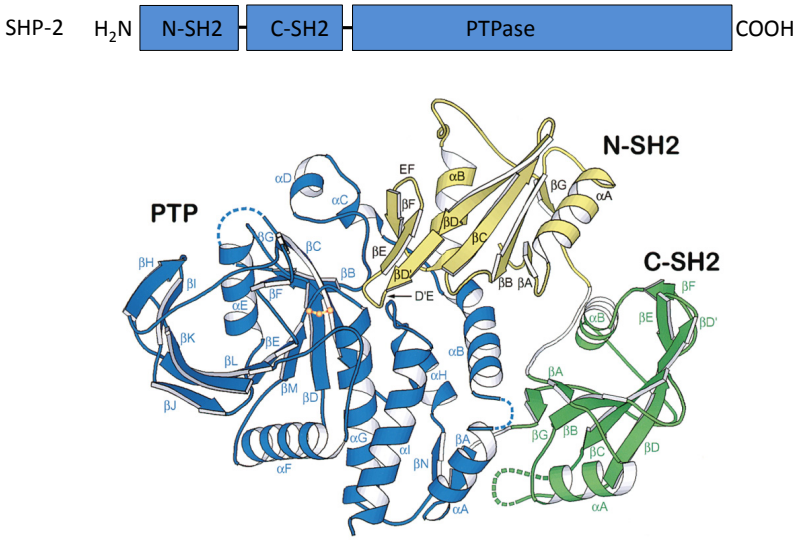

Figure 5: Structure of SHP-2. The linear domain map of SHP-2 is shown at top, and the three-dimensional ribbon structure (From Hof et. al., 1998) is at bottom. Note the two tandem SH2 domains, the N-SH2 and C-SH2, and a C-terminal phosphatase domain.

the nuclear localization signal in its C-terminal tail which are included in SHP-1's C-terminal tail (Poole et. al, 2005). The exact roles that these motifs play in regulating SHP- family tyrosine phosphatase targeting and activity remain an important and challenging problem for future investigations.

F. Tumor Necrosis Factor

Tumor necrosis factor alpha (TNF-α), is a peptide signaling molecule involved in many cellular responses. TNF-α has been demonstrated to induce cell proliferation, inflammatory responses, and necrosis or apoptosis (MacEwan, 2002). This variety of responses mediated in cells exposed to TNF is due to the complex and variable signaling mechanisms within specific cell types. Work from many laboratories has provided insights in to the complexity of TNF-α responses, however the reason why one response occurs over another in a given cell type is still unclear. TNF-α can exist as either a membrane bound 26 kD transmembrane protein, or as a freely circulating 17 kD protein which results from cleavage of the membrane bound form (MacEwan, 2002). The three dimensional crystal structure has been solved, revealing that TNF-α exists as a cone shaped homo-trimer (Jones et. al., 1989; Eck and Sprang, 1989).

Both the soluble and membrane bound forms of TNF can engage TNF receptors, of which there are two isoforms, TNFR1 (also known as p55TNFR, 55kD) and TNFR2 (also known as p75TNFR, 75kD). Both are single transmembrane spanning proteins with extracellular N-termini, and intracellular C-termini. TNFR1 and TNFR2 only share 28% homology, mostly in their four extracellular cysteine rich motifs, with no intracellular homology (Barbara et. al. 1996; MacEwan 2002). TNFR1 is ubiquitously expressed,

whereas TNFR2 is primarily found in immune cells (Li and Lyn, 2008). Downstream signaling from both receptors, as stated above, is not fully understood, but can be mediated in part by the association of numerous TNF Receptor Associated Factors (TRAF's). Numerous studies have identified many pathways that are activated downstream of these receptors such as: nuclear factor-kappa B (NF-kB) and activator protein-1 (AP-1) transcription factor activation, caspase cascade induction, ceramide production, lipid signaling, heterotrimeric and small G-protein activation, phosphatase activation, and kinase activation (MacEwan 2002; Li and Lin, 2008). Unraveling the maze that is TNF signaling will be a complicated and important task for future investigators.

In the case of tyrosine kinase activation, it has been demonstrated that tyrosine phosphorylation downstream of TNF receptors is necessary for its cytotoxicity, NF-kB activation, and expression of endothelial adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Mishra et. al., 1994; Weber et. al., 1995; Natarajan et. al., 1998; Pincheira et. al., 2008). These studies employed various tyrosine kinase inhibitors such as genistein, quercetin, or PP2 to demonstrate the requirement of tyrosine kinases in the effects of TNF-α. In the case of NF-kB activation, it was demonstrated that the tyrosine kinase inhibitor genistein blocked the degradation of IkBα, an inhibitor of NF-kB nuclear translocation (Natarajan et. al., 1998). In addition to tyrosine kinase inhibitor experiments, another study demonstrated that a dominant negative mutant of c-Src blocked NF-kB activation and ICAM-1 expression, and indicated that c-Src is responsible for inhibitor of kappa-B kinase B (IKKB) phosphorylation after TNF-α stimulation (Huang et. al., 2003). Indeed,

association of c-Src with TNFR1 has been recently demonstrated, and this association is increased by TNF-α administration (Pincheira et. al., 2008). TNFR1 has also been demonstrated to be localized in caveolae (Ko et. al. 1999; D'Alessio et. al. 2010) and is associated with Cav-1 in immunoprecipitation and western blot analysis (D'Alessio et. al. 2005). Cav-1 is a known binding partner of c-Src which has been shown to localize to caveolae (Li et. al., 1996; Minshall et. al., 2000). Additionally, TRAF-2 has been implicated as a binding partner for the Cav-1 N-terminus, which may further facilitate TNF-α signaling and c-Src activation in caveolae by TNF-α (Cao et. al., 2002).

Interestingly, tyrosine phosphatase activity has also been implicated in TNF-α signaling (Mishra et. al. 1994; Menon et. al., 1995; Singh and Aggarwal, 1995; Natarajan et. al. 1997; Darnay and Aggarwal et. al., 1997; Dhawan et. al., 1997; Guo et. al., 2000). Subsequent studies have identified the involvement of SHP-2 in TNF-α signaling using SHP-2 knockout mouse fibroblasts (You et. al. 2001) and by overexpression of a catalytically inactive mutant (Lerner-Marmarosh et. al., 2003). These studies also demonstrated that SHP-2 activity is increased by TNF-α stimulation using a SHP-2 immunoprecipitation and *in vitro* phosphatase activity assay with a radiolabeled substrate peptide. The fact that both tyrosine phosphatases and tyrosine kinases are involved in TNF-α mediated signaling is intriguing.

G. SFKs and Cancer

Increased SFK activity has been described in many forms of cancer, including leukemia, breast, lung, and colon cancer (Lynch et. al., 1993; Bjorge et. al., 2000; Campone et. al., 2011; Haura et. al., 2010). For instance, c-Src activity in 13 of 21

primary human colon carcinomas was demonstrated to be five to seven times higher than normal colon tissue (Cartwright et. al., 1989). Also, in cells derived from human colon cancer biopsies, Csk overexpression reduced invasiveness and SFK activity (Rengifo-Cam et. al., 2004). On the other hand, dominant-negative Csk expression increased the invasiveness and migration of these cells. Consistent with this finding, it was demonstrated that in a mouse model of colon cancer, Csk overexpression was associated with suppression of metastasis and invasiveness in vivo (Nakagawa et. al., 2000). Furthermore, it has been demonstrated that reduced localization of Csk at the membrane may be an underlying cause of the pathologic increase in c-Src activity in human colon cancer (Sirvent et. al., 2010). This study concluded that the mislocalization of Csk in the most highly metastatic cells was a direct result of a reduction in the expression of Cav-1 and Cbp. It is noteworthy that when the reduced Csk adapter protein expression in the highly metastatic cells was rescued by increasing Cbp expression, c-Src activity decreased, and the invasiveness of these cells was also reduced (Sirvent et. al., 2010). Because of these findings, SFK inhibitors are currently being investigated in clinical trials as cancer therapeutics. Some examples of the molecules in clinical trials are: Bosutinib for breast cancer (Campone et. al., 2011), and Dasatinib for non-small cell lung cancer (Haura et. al., 2010).

III. MATERIALS AND METHODS

*Portions of the text were reprinted from: "Place AT et. al.: Cooperative Role of Caveolin-1 and Cbp in Csk-Mediated Inactivation of c-Src. Molecular Pharmacology 80:665-672, October 2011, with permission of the American Society for Pharmacology and Experimental Therapeutics.

A. Cells and Reagents

WT and Cav-1(-/-) mouse lung fibroblasts (MFs) were isolated from 4- to 6-week-old C57BL6 and Cav-1(-/-) mice (The Jackson Laboratory, Bar Harbor, ME). Lung lobes were perfused with RPMI medium and homogenized in RIPA buffer supplemented as described below for Western blot analysis or digested in collagenase type 1 solution (both from Sigma-Aldrich, St. Louis, MO) for MF isolation. Cells were collected and cultured in 10% fetal bovine serum containing Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Subcultures with fibroblast-like morphology were selected and maintained in 5% CO2/95% room air in a water-jacketed 37°C incubator. RIPA buffer (Boston Bioproducts, Ashland, MA) was supplemented with protease inhibitor cocktail, 200 mM PMSF, 1 mM EDTA, 1 mM NaF, and 1 mM Na3VO4 (all from Sigma-Aldrich). *n*-Octylglucoside buffer (ODG, 2%) (Research Products International, Mt. Prospect, IL) was supplemented the same as RIPA buffer. Total protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). Antibodies for c-Src (polyclonal), Csk, SHP-2, and glyceraldehyde-3-phosphate dehydrogenase were from

Santa Cruz Biotechnology (Santa Cruz, CA); β-actin, Cav-1, and p-Cav-1 Tyr14 antibodies were from BD Biosciences (San Jose, CA); c-Src (monoclonal), p-Src Tyr418, and p-Src Tyr529 were from Cell Signaling Technology (Danvers, MA); and Cbp and Na+/K+ ATPase-α1 were from Abcam Inc. (Cambridge, MA).

B. Western Blotting

Cells were lysed in RIPA or ODG buffer supplemented as described above, sonicated briefly, and cleared for 5 min at 13,200 rpm at 4°C in an Eppendorf 5415R microcentrifuge (Eppendorf North America, New York, NY). Lysates were boiled in lysis buffer plus 6X Laemmli sample buffer (Boston Bioproducts) and dithiothreitol (final concentration, 30 mM; Sigma-Aldrich) for 5 min before SDS-PAGE with equal quantities of protein loaded in each lane. Protein concentration of lysates was determined by a BCA protein assay kit (Pierce). After SDS-PAGE separation, proteins were blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA) and blocked with 5% blotting grade nonfat dry milk (Bio-Rad) in Tris-buffered saline with 0.05% Tween 20 (Sigma-Aldrich) (TBST) for 1 h. The membranes were then probed with primary antibodies in blocking buffer rocking overnight at 4°C. After three washes in TBST, secondary species-specific horseradish peroxidase-conjugated antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were then incubated for 1 h at room temperature in blocking buffer. After three more washes with TBST, enhanced chemiluminescence substrate (Pierce) was then used to visualize the bands on HyBlot CL film (Denville, South Plainfield, NJ).

C. Fluorescent Imaging

Cells were seeded on glass coverslips and serum-deprived for at least 2 h before fixation for 20 min with 4% paraformaldehyde in Hanks' balanced salt solution with Ca2+ and Mg2+ (HBSS+/+; Invitrogen). Cells were then permeabilized for 30 min with buffer containing 5% goat serum, 0.2% bovine serum albumin, 0.01% NaN3, and 0.1% Triton X-100 (all from Sigma-Aldrich), washed briefly with HBSS+/+, and incubated overnight at 4°C with appropriate antibodies in the same buffer used for permeabilization. After three more washes with HBSS+/+, Alexa 488-labeled secondary goat anti rabbit IgG antibodies (Invitrogen) were then added and incubated for 2 h at room temperature. Coverslips were washed again three times with HBSS+/+ and mounted to glass slides with ProLong Gold antifade mounting reagent with 4,6-diamidino-2-phenylindole (Invitrogen). Images of cells were captured on a Zeiss LSM 510 META confocal microscope (Carl Zeiss Inc., Thornwood, NY), as described previously (Minshall et al., 2000).

D. Fluorescence Resonance Energy Transfer (FRET)

HEK cells were transfected with 8 μg of the Src activity reporter cDNA (SrcAR 2.9, from Wang et. al., 2005) using 15 μl of Lipofectamine 2000 (Invitrogen) in 35 mm glass bottom dishes (MatTek, Ashland, MA, USA) for 24 hrs. Both before and after stimulation with 20ng/ml TNF-α, cells expressing the sensor were imaged every 30 seconds with a Zeiss LSM-510 META confocal microscope. Cells were excited with the 458 nm laser, and emission spectra spanning from 462.9 to 602 nm was collected in 10.7 nm bandwidths with the pinhole set at 1.66 Airy units using the META scanhead. The

spectra of pure CFP and YFP emission signatures were used as a reference for the Zeiss AIM linear unmixing algorithm to separate the fluorescence contributions of CFP and YFP (on a pixel by pixel basis) in the images. Regions of interest (ROI) were examined by quantification of the donor and acceptor emission intensities, which were averaged and plotted as a ratio of CFP/YFP (the donor over the acceptor) to indicate changes in FRET. By design of the sensor, an increase in the CFP/YFP ratio (a decrease in FRET) was indicative of an increase in c-Src activity.

E. Cytosol and Membrane Fractionation

Cells were serum-deprived for indicated times (2 hours for MFs and 18 hours for HLMVECs) and then scraped in 50 mM Tris-HCl, pH 7.5, supplemented with protease inhibitor cocktail, 1 mM Na3VO4, 1 mM NaF, 1 mM EDTA, and 1 mM PMSF (Sigma-Aldrich). The scraped cells were then centrifuged at 100,000*g* at 4°C for 1 hour. The supernatant (cytosolic fraction) was collected, and the pellet (membranous fraction) was resuspended in RIPA buffer and sonicated. Fractions were boiled for 5 minutes at 100 degrees C in the presence of 1X Laemmli sample buffer (Boston Bioproducts) and 30mM DTT (Sigma-Aldrich) and subsequently frozen. Aliquots of each lysate were frozen as well and used for total protein quantification. Protein concentration was measured by a BCA assay (Pierce), and equal amounts of protein (10-20 μg) were loaded per lane onto 10% SDS-PAGE gels for western blotting. Sodium/potassium ATPase-α1 subunit was used as a positive control marker and loading control for the membrane fraction (MFs).

F. Immunoprecipitation

MFs or HLMVECs were seeded at 125,000 cells/well in a six-well plate and serum-deprived the following day for 2 hours (MFs) or 18 hours (HLMVECs, when confluent). Cells were lysed in ODG buffer without sonication. Lysates (two wells per treatment) were then added to magnetic sheep anti-mouse-IgG coated Dynabeads (Invitrogen) that were preincubated for 30 min at 4°C with nonspecific mouse IgG (as a negative control) or anti-Cav-1 monoclonal antibodies (both from BD Biosciences). After a 1-h rotating incubation at 4°C, the lysates were placed on a magnetic particle concentrator (Invitrogen) and washed with cold (4°C) phosphate-buffered saline without Ca2+ and Mg2+ (PBS -/-) supplemented with protease inhibitor cocktail and NaVO4 as described above. After three washes, 6X Laemmli sample buffer was added (1X final, Boston Bioproducts) with ODG lysis buffer and DTT (final concentration, 30 mM, Sigma-Aldrich) and boiled for 5 min before loading onto 10 % SDS-PAGE gels for western blotting.

G. Cav-1 Mutant Transfection

Cav-1**(-/-)**MFs were seeded at 125,000 cells/well in a six-well plate and treated the following day with Cav-1 WT, Y14F, or Y14E mutant cDNA in pEGFP-N1 (Clontech) vector, or empty vector as a control, using 10 µl of Lipofectamine 2000 (Invitrogen) per well. 24 hours after transfection, the cells were serum-deprived for one hour and placed on ice for lysis with RIPA buffer (Boston Bioproducts) supplemented as described above in reagents. Lysates were boiled for 5 minutes at 100 degrees C in the presence of 1X Laemmli sample buffer (Boston Bioproducts) and 30mM DTT (Sigma-

Aldrich) and subsequently frozen. Aliquots of each lysate were frozen as well and used for total protein quantification. Quantities of total protein were determined with a BCA assay (Pierce) and equal amounts (10-20 μg) were loaded per well onto 10% SDS-PAGE gels for western blotting.

H. Cbp siRNA

MFs were seeded at 125,000 cells/well in a six-well plate and treated the following day with varying concentrations of scrambled (S) or Cbp siRNA. The siRNA was delivered using 6 to 8 μl of Dharmafect-1 per well according to the manufacturer's protocol. Both siRNA duplexes and Dharmafect-1 were from Dharmacon RNA Technologies (Lafayette, CO). The amount of scrambled control siRNA transfected was equal to the highest concentration of specific siRNA targeting mouse Cbp that was used in each experiment. Two or three days after transfection, cells were serum-deprived (2–4 hours) and subsequently lysed in RIPA buffer on ice, sonicated, cleared for 5 min at 13,200 rpm at 4°C. Lysates were then boiled at 100 degrees C for 5 minutes in the presence of 1X Laemmli SDS sample buffer (Boston Bioproducts) and 30 mM DTT (Sigma-Aldrich) and subsequently frozen for use later in western blotting, with unboiled aliquots frozen as well for protein quantification. Total protein quantities were determined with a BCA assay (Pierce) and equal amounts (10-20 μg) were loaded per well onto 10% SDS-PAGE gels for western blotting. The siRNA sequences were as follows: 5'-AAGCCATACAGACTCTAAACA-3'targeting mouse and rat Cbp, and 5'-AAGC**G**ATACAGACTCT**C**AACA-3' targeting human Cbp, which was used as the scrambled control in the mouse cells as described previously (Jiang et. al., 2006).

I. SHP-2 siRNA

Confluent HLMVEC monolayers seeded in 6 well plates were transfected with SHP-2 siRNA (Santa Cruz Biotechnology) using 5 µl of Dharmafect-1 (Dharmacon RNA Technologies) per well according to the manufacturer's protocol in complete VascuLife VEGF-Mv medium (Lifeline Cell Technology, Walkersville, MD). After 48 hours post transfection, cells were serum-deprived in basal media (Lifeline Cell Technology) for 18 hours. Following serum deprivation, cells were treated with recombinant human TNF-α (Calbiochem, LaJolla, CA) at 20 ng/ml for the times indicated. After stimulation, the cells were placed on ice and lysed in RIPA buffer supplemented as described above under Cells and Reagents. Lysates were then boiled at 100 degrees C for 5 minutes in the presence of 1X Laemmli SDS sample buffer (Boston Bioproducts) and 30 mM DTT (Sigma-Aldrich) and subsequently frozen. Aliquots of each lysate were frozen as well and used for total protein quantification. Total protein quantities were determined with a BCA assay (Pierce) and equal amounts (10-20 µg) were loaded per well onto 10% SDS-PAGE gels for western blotting.

J. *In Vitro* Kinase Assay

The tyrosine kinase assay was performed according to the manufacturer's instructions (Millipore, Billerica, MA). In brief, c-Src was immunoprecipitated in RIPA buffer lysates from 2 hour serum-deprived 10-cm dishes using goat anti-mouse Dynabeads (Invitrogen) coated with c-Src monoclonal antibodies (37.5 µg/IP; Cell Signaling Technology) for 1 hour at 4°C, washed two times with Tris buffered saline, and

once with reaction buffer. Immune complexes were then incubated with reaction buffer and biotinylated substrate peptide for 1 hour at 37°C. The reaction was stopped by heating to 95 degrees C for 5 minutes and added to a well of a streptavidin coated ELISA plate for binding and washing with Tris buffered saline. Phosphorylation of the substrate peptide was detected using a horseradish peroxidase-conjugated anti-phosphotyrosine (4G10) antibody and TMB substrate, read at 450 nm. Phosphorylated control peptide was added to separate wells to generate the standard curve for each ELISA assay, and phosphorylated substrate peptide (in nanograms per hour) was determined using the extrapolated linear equation of the standard curve. Units (U) of immunoprecipitated c-Src were determined by densitometry of western blots of each well on the ELISA plate. The highest density bands were considered 1 U, and the other lanes were normalized to this value.

K. ***In Vitro*** **Peptide Pull-Down Assay**

Streptavidin coated magnetic Dynabeads (Invitrogen) were washed three times with pull-down buffer (20 mM HEPES, 150 mM NaCl, and 10mg/ml BSA) using a magnetic particle concentrator (Invitrogen). 10 µg of each biotinylated Cav-1 N-term peptide (synthesized by Genscript, Piscataway, NJ) was diluted in pull-down buffer and added to a separate tube containing 90 µl of the beads for a 15 min coating incubation (200 µl total volume) at room temperature. The beads were again washed with pull-down buffer three times, before addition of 250 ng purified GST-tagged Csk (Calbiochem) to each tube for 15 min at room temperature (200 µl total volume). Following the incubation, the beads were again washed three times with pull-down buffer, and boiled

for 5 min at 100 degrees C in the presence of RIPA buffer, SDS sample buffer (both from Boston Bioproducts), and 30 mM DTT (Sigma-Aldrich). The entire contents of each pull-down reaction was loaded into a lane of a 10% SDS-PAGE gel and western blotted. Csk-GST alone diluted in RIPA buffer, 1X SDS sample buffer, and DTT was loaded into the first lane (next to the ladder) of the same gel as a positive control. The membranes were probed with a Csk polyclonal antibody (Santa Cruz Biotechnology) or horseradish peroxidase-conjugated streptavidin (Cayman Chemicals, Ann Arbor, MI) to detect the biotinylated peptides.

L. Densitometry and Statistics

Densitometry of scanned protein bands was performed using ImageJ software (http://rsbweb.nih.gov/ij/). All changes in protein phosphorylation or expression were normalized to loading controls blotted onto the same membrane. Statistical significance was determined by Student's *t* test or ANOVA, with $P < 0.05$ considered significant.

IV. RESULTS

*Portions of the text and Figures 6, 7, 8, 9, 10, 11, 13, 14, and 15 were reprinted from: "Place AT et. al.: Cooperative Role of Caveolin-1 and Cbp in Csk-Mediated Inactivation of c-Src. Molecular Pharmacology 80:665-672, October 2011, with permission of the American Society for Pharmacology and Experimental Therapeutics.

A. Cbp Expression is Increased in Cav-1(-/-) Fibroblasts and Lungs

We hypothesized that Cav-1 functions as a negative regulator of c-Src and that Cav-1(-/-) mice should have elevated c-Src activity compared with WT controls. To examine this, lungs from Cav-1(-/-) mice and WT age- and strain-matched control mice were excised from 4- to 6-week old mice, perfused, and digested with Collagenase type I. MFs were cultured as described under Materials and Methods. Whole lungs from these mice were also excised, perfused, and homogenized in RIPA buffer for whole lung protein analysis. We were surprised to find that despite total eradication of Cav-1 protein in MFs, c-Src activity remained equal to WT control MFs under serum-deprived conditions, and there was no difference in total c-Src expression (Figure 6A, 6B, and 7A). Src activity was measured by examining the phosphorylation state of c-Src Tyr418. There was no statistically significant difference between the two genotypes (Fig. 6B). The phosphorylation state of the Tyr529 residue of c-Src was also examined and we found no difference between the two cell lines as well (Figure 6B). These results were validated by using immunoprecipitated c-Src from WT and Cav-1(-/-) MFs in an *in vitro* kinase

reaction. Using standard curves of phosphorylated control peptide to determine the phosphorylated substrate (in nanograms), and normalization of differences in immunoprecipitated c-Src in the reactions to determine the units (nanograms per unit; see *Materials and Methods*), we found basal c-Src activity to be 0.343 +/- 0.06 ng/U/h in the WT MFs and 0.285 +/- 0.14 ng/U/h in the Cav-1(-/-) MFs (Fig. 6A). These results were not statistically different and confirmed that the measure of Tyr418 phosphorylation by western blotting indeed reflects c-Src activity in our studies.

The primary difference observed in our study was that the level of expression of Cbp in the Cav-1(-/-) MFs and lungs was approximately two times higher than in WT MFs and lungs (Figure 7A, 7B, and 8). This increase in Cbp expression was further confirmed by immunofluorescent staining of Cbp in the MFs. Confocal micrographs captured using identical detector settings confirmed increased Cbp expression in Cav-1(-/-) MFs. These micrographs also revealed that both Cbp and Csk were similarly localized to the plasma membrane in WT and Cav-1(-/-) MFs (Figure 8). These results demonstrate that Cbp expression is about 2-fold higher in Cav-1(-/-) mouse lungs and MFs compared with WT litter mates and that Cbp and Csk are localized correctly. Furthermore, c-Src activity in Cav-1(-/-) MFs was the same as that in WT control cells, suggesting that the increase in Cbp expression compensates for the absence of Cav-1 to negatively regulate c-Src activity.

B. Csk Expression and Subcellular Localization is Similar in Cav-1(-/-) and WT MFs

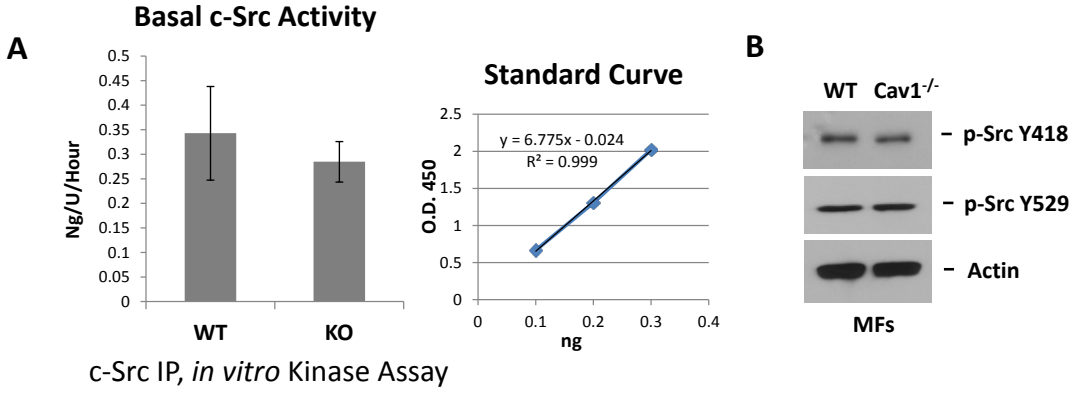

Figure 6: c-Src activity is unaffected by Cav-1 knockout. **A**, In vitro kinase assays of immunoprecipitated c-Src from confluent 2 hour serum-deprived Cav-1(-/-) and WT MFs were normalized for differences in immunoprecipitated enzyme and are shown as the mean +/- S.E. (n=5). **B**, Cav-1(-/-) and WT MFs were grown to confluence and serum-deprived for 2 hours before lysis as described under *Materials and Methods*. Phosphorylation of c-Src Tyr418 and Tyr529 were equal.

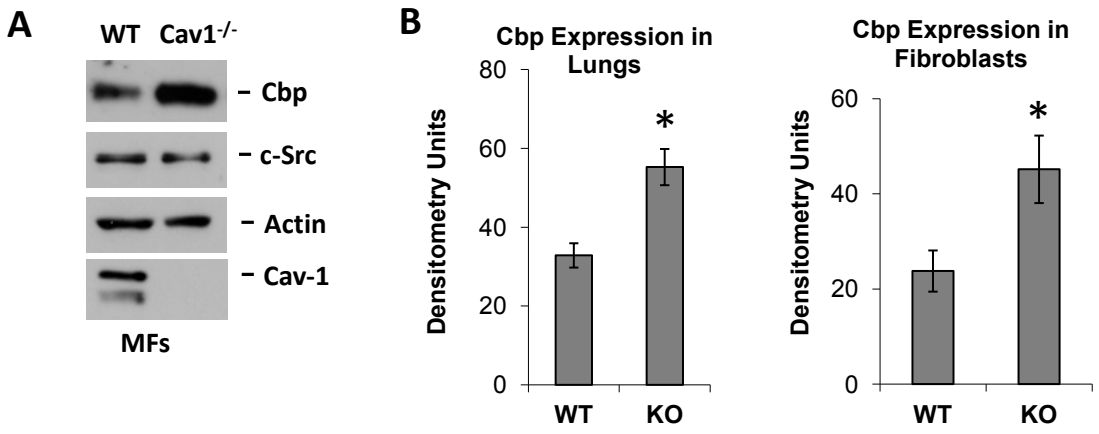

Figure 7: Cbp expression is increased in Cav-1(-/-) lungs and cultured fibroblasts. **A,** Confluent, 2 hour serum deprived Cav-1(-/-) and WT MF lysates were prepared as described under *Materials and Methods*, and Cbp expression was determined by western blot. **B,** Quantification by densitometry of three independent experiments from total lung homogenates and MF lysates are shown (mean +/- S.E.). Cbp expression increased approximately 2-fold in Cav-1(-/-) MFs and lungs. * = $P < 0.05$ versus WT (n=3)

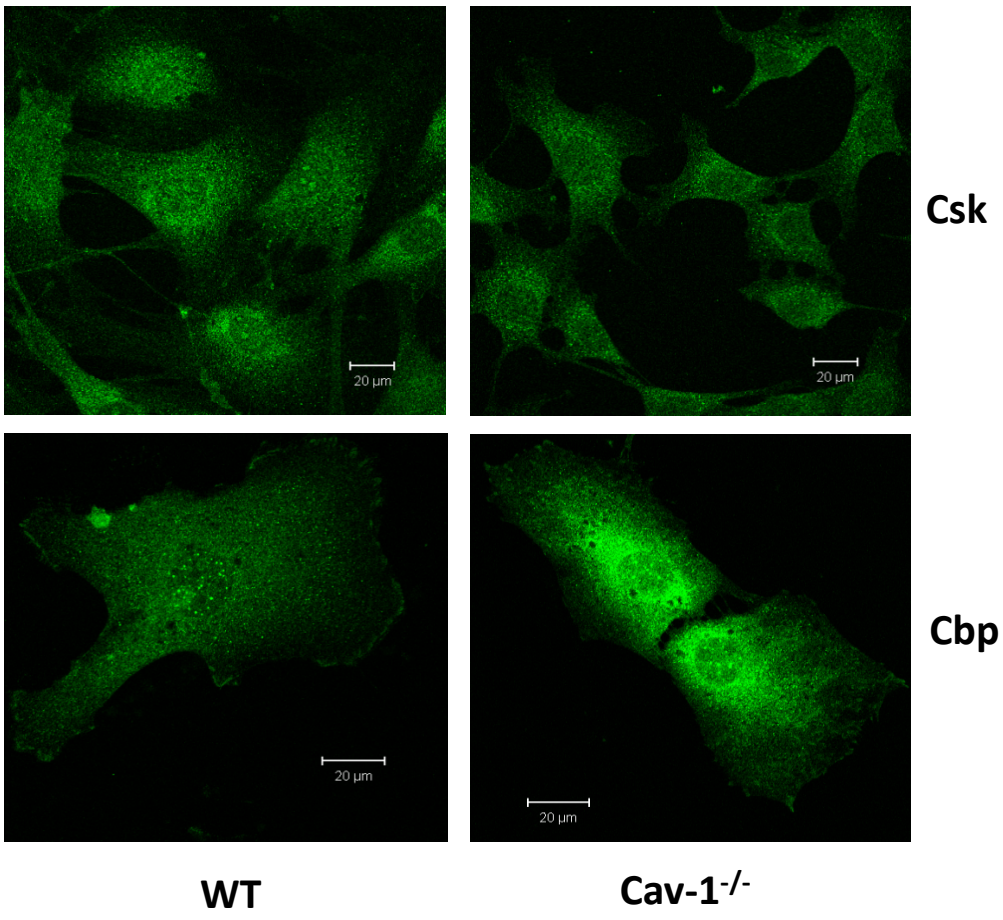

Figure 8: Cbp and Csk immunostaining. The MFs were immunostained for Csk and Cbp and imaged by confocal microscopy. The confocal images confirm increased expression of Cbp in Cav-1(-/-) MFs and Cbp localization in membrane-associated structures in both cell types. Both Csk expression and localization were similar in the two cell types. Scale bar, 20 µm.

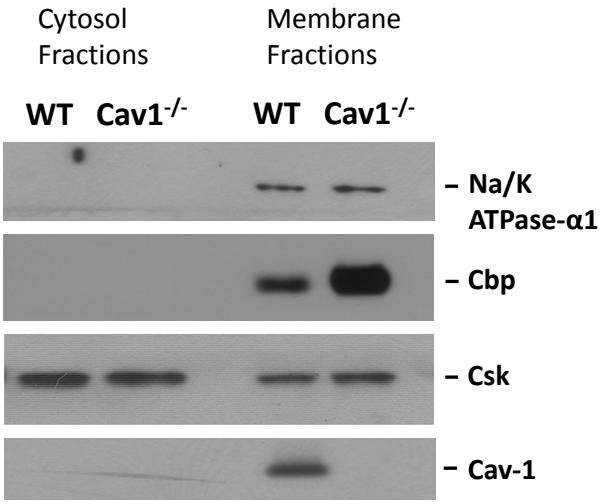

Figure 9: Csk subcellular localization. WT and Cav-1(-/-) MFs were grown to confluence and serum-deprived for 3 hours. Cytosol and membrane fractions, prepared as described under *Materials and Methods*, contained equivalent amounts of Csk despite total loss of Cav-1 expression. Cbp levels were greater in the Cav-1(-/-) MFs and present in the membrane fraction only. Na/K ATPase-α1 subunit was used as a membrane loading control.

Given the 2-fold increase in expression of Cbp but equal c-Src activity in Cav-1(-/-) MFs, we assessed Csk expression level and localization. Csk expression level was found to be the same in WT and Cav-1(-/-) MFs, and subcellular fractionation revealed that the same amount of Csk was present in both the membrane and cytosolic fractions from serum-deprived cells (Figure 9). Na+/K+ ATPase-α1 was used as a loading control for the membrane fraction, and Cbp localization was shown to be restricted to this compartment. These results demonstrate that Csk is still localized to the membrane to hold c-Src at its baseline level of activity in the absence of Cav-1, and that Csk expression was not affected by the absence of Cav-1. Furthermore, we speculated that Cbp and Cav-1 may be the only membrane associated Csk adapter proteins expressed in MFs, because the increase in Cbp expression in the absence of Cav-1 appeared to compensate for the lack of Cav-1 and enable recruitment of Csk to the membrane to the same extent as that observed in WT MFs.

C. Csk siRNA Increases Basal c-Src Activity

As described previously, Csk negatively regulates SFKs by phosphorylating the negative regulatory tyrosine in their C termini (Okada et. al., 1991; Imamoto and Soriano, 1993; Howell and Cooper, 1994; Brdicka et. al., 2000; Takeuchi et. al., 2000; Khanna et. al., 2007). To confirm that Csk inhibits c-Src to its basal state in our experimental system, we performed knockdown experiments using Csk siRNA in WT MFs. Csk levels were reduced by 30% compared with scrambled siRNA treated cells levels after 48 h. Basal activity of c-Src, as measured by Tyr418 phosphorylation levels after serum deprivation, was approximately 2-fold higher in the Csk siRNA treated cells compared

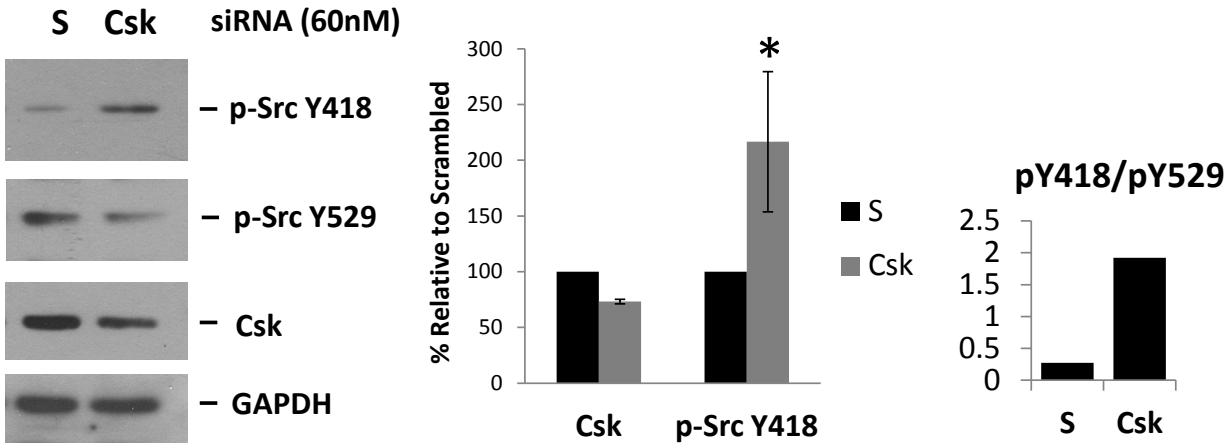

Figure 10: Csk negatively regulates basal c-Src activity. WT MFs were treated with 60 nM Csk siRNA for 48 h, serum-deprived for 2.5 hours, and lysed. S, 60 nM scrambled nontargeting siRNA. Csk level was reduced by approximately 30%, which increased c-Src Tyr418 phosphorylation (approximately 2-fold) and decreased c-Src Tyr529 phosphorylation. Bar graph represents three independent experiments (mean +/- S.E.) quantified using densitometry. * = $P < 0.05$ versus S (n= 3). The ratio of c-Src p-Tyr418/p-Tyr529 from a representative experiment is shown.

with the scrambled control siRNA treated cells (Figure 10). In addition, c-Src Tyr529 phosphorylation was diminished in MFs treated with Csk siRNA (Figure 10). These data indicate that inhibition of c-Src activity to basal levels after serum removal is dependent on Csk.

D. Cbp Depletion Increases Cav-1 Tyr14 Phosphorylation

To specifically explore the role of Cav-1 in the coordination of Csk, we reduced Cbp expression by siRNA transfection in WT MFs and measured c-Src activity. Knockdown of Cbp in WT MFs (using a specific siRNA that targets the mouse isoform at residues 1160–1180), compared with scrambled siRNA targeting the human isoform (residues 1163–1183), was very efficient and specific as described previously (Jiang et. al., 2006). We routinely achieved Cbp knockdown of more than 80% in these cells (Figure 11). However, to our surprise, we observed no increase in the phosphorylation of c-Src Tyr418 under basal, serum-deprived conditions in reprobes of the original blots used to detect Cbp expression (Figure 11). We did however, detect a 65% increase in the phosphorylation state of Cav-1 Tyr14, a known c-Src substrate (Li et. al., 1996; Aoki et. al., 1999; Lee et. al., 2000), under the same conditions without effecting total Cav-1 expression (Fig. 11). It should be noted that this is also the tyrosine that Csk previously was reported to bind to on Cav-1 (Cao et. al., 2002; Lu et. al., 2006). This increase in Cav-1 Tyr14 phosphorylation was statistically significant and detected both in WT MFs and rat lung microvascular endothelial cells (Figure 12). Total Cav-1 levels were unaffected by siRNA treatment in these cells as well (Figure 12).

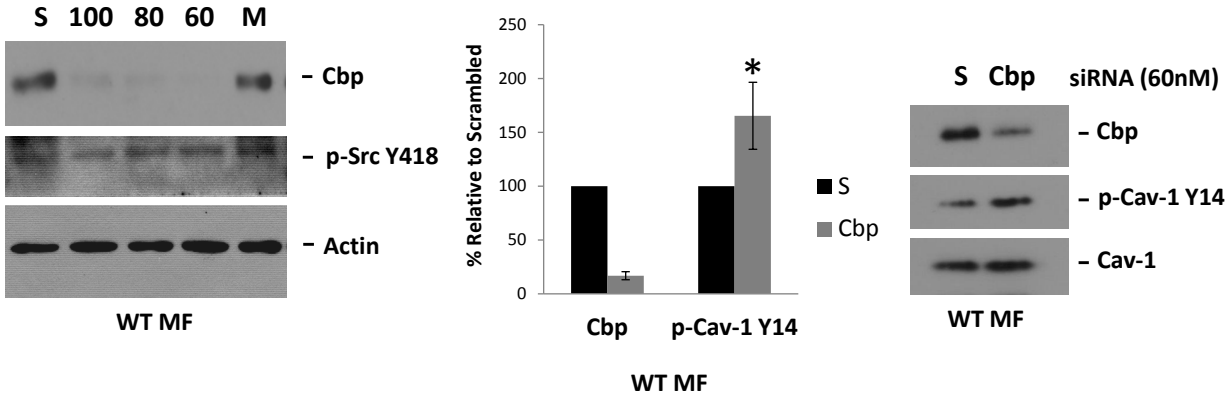

Figure 11: Cbp reduction does not increase c-Src activity in WT MFs. WT MFs were treated with Cbp siRNA for 72 hours as described under *Materials and Methods*. S represents scrambled siRNA (100 nM, directed against human Cbp), and 100, 80, and 60 is the siRNA concentration (nM) directed against rodent Cbp. Cells were serum deprived for 2 hours before lysis. Activity of c-Src (p-Tyr418) remained constant under the same conditions as seen in reprobed blots of the original Cbp blots. Under the same conditions, Cav-1 Tyr14 basal phosphorylation increased without effecting total Cav-1 expression. Bar graph displays four independent experiments (mean +/- S.E.) performed on the WT MFs indicating that Cbp levels were decreased by approximately 85% with 60 nM Cbp siRNA, and Cav-1 Tyr14 phosphorylation increased 65% after Cbp knockdown. * = $P < 0.05$ versus S (n=4).

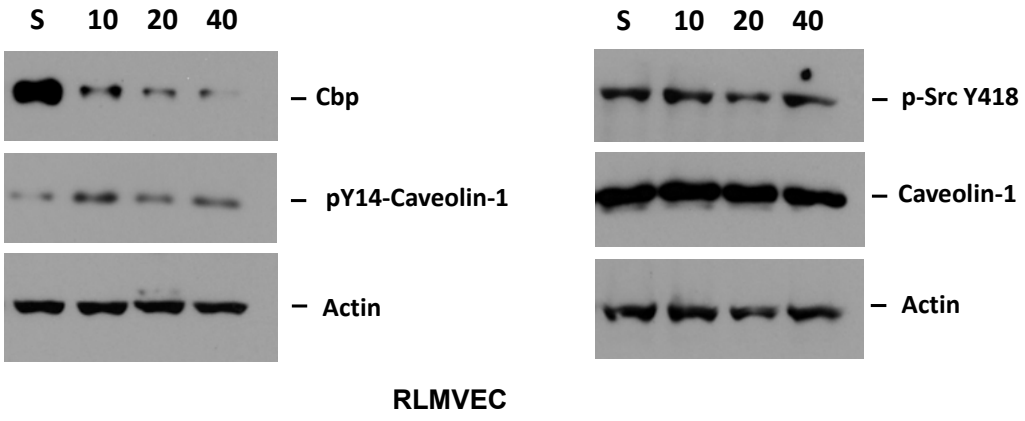

Figure 12: Cbp reduction does not increase c-Src activity in WT endothelial cells. Confluent RLMVECs were treated with Cbp siRNA for 72 hours as described under *Materials and Methods*. S represents scrambled siRNA (40 nM, directed against human Cbp), and 10, 20, and 40 is the siRNA concentration (nanomolar) directed against rodent Cbp. Cells were serum deprived for 2 hours before lysis. Activity of c-Src (p-Tyr418) remained constant, and Cav-1 Tyr14 basal phosphorylation increased.

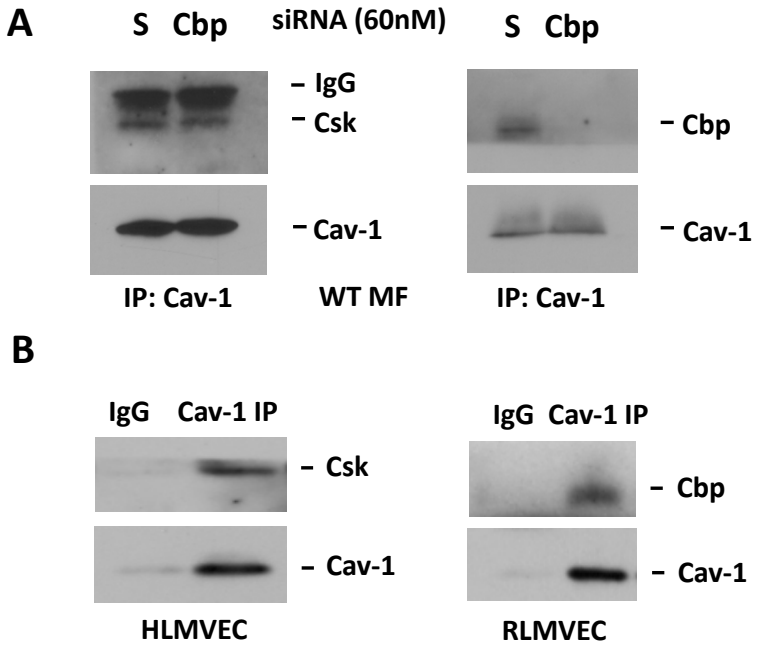

Figure 13: Cav-1, Csk, and Cbp basally associate in MFs and ECs. A, Cav-1 immunoprecipitates from serum-deprived WT MFs indicate basal association of Csk and Cbp. MFs treated with Cbp siRNA or scrambled siRNA as described in Fig. 8 displayed equivalent amounts of Cav-1 associated Csk, despite the loss of Cbp from the immunoprecipitated complex. **B,** Cav-1 immunoprecipitates from serum-deprived endothelial cells also display basal association of Cbp and Csk with Cav-1. These studies were repeated 3-times and equivalent results were observed.

E. Increases in Cav-1 Tyr14 Phosphorylation Facilitate Csk Association after Cbp Knockdown

Because Cbp knockdown in WT MFs increased Cav-1 Tyr14 phosphorylation levels without increasing c-Src Tyr418 phosphorylation, we tested the hypothesis that this increase was facilitating Csk binding and localization at the plasma membrane to inhibit c-Src. In Cav-1 immunoprecipitates of serum-deprived WT cells, we observed the presence of associated Csk and Cbp under normal conditions (Figure 13A and 13B). This revealed a complex of Csk and the two adapters present at the plasma membrane under basal conditions. It should be noted here that this is consistent with another group's finding that Cav-1 and Cbp were constitutively associated based upon immunoprecipitation analysis (Jiang et. al., 2006). We then reduced Cbp expression in the WT MFs with Cbp siRNA in the same Cav-1immunoprecipitation experiment, and we observed equivalent amounts of Csk associated with Cav-1 and no Cbp present in the complex (Figure 13A). This further suggests that the elevated Cav-1 Tyr14 phosphorylation state indeed was allowing for an appropriate amount of Csk coordination at the plasma membrane to maintain low basal c-Src activity.

F. Knockdown of Cbp in Cav-1(-/-) Cells Increases c-Src Basal Activity

Because c-Src activity did not increase in the absence of Cbp or Cav-1 alone, studies were carried out to investigate the basal c-Src activity during the reduction of both membrane adapters for Csk, i.e. Cbp and Cav-1. Cbp siRNA specific for the mouse isoform was transfected into Cav-1(-/-) MFs for 48 h followed by 3 hours of serum deprivation before lysis. As seen in Figure 14, Cbp expression was reduced by more than

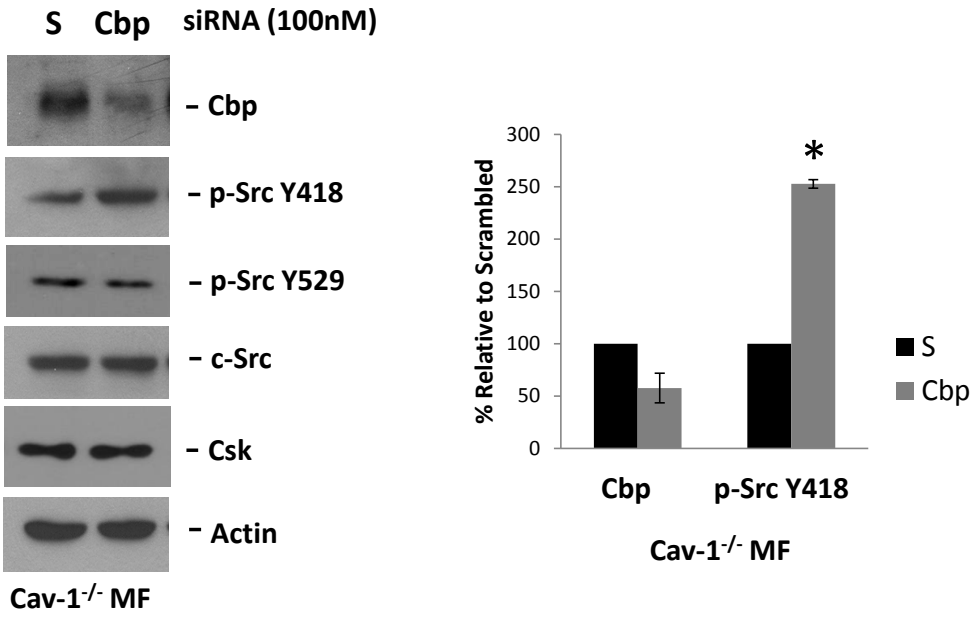

Figure 14: Cbp reduction increases c-Src activity in Cav-1(-/-) MFs. Cav-1(-/-) MFs were treated with Cbp siRNA or scrambled siRNA (both 100 nM) for 48 hours and then serum-deprived for 3 hours before lysis. Cbp siRNA reduced Cbp expression by more than 40%, and c-Src activity increased 2.5-fold over scrambled siRNA-treated cells. Bar graph of summarized data of Cbp expression and Src Tyr418 phosphorylation (mean +/- S.E.), indicates that Cbp knockdown in Cav-1(-/-) cells significantly increases c-Src activity. * = $P < 0.05$ versus S (n=3).

40% in these cells, which was associated with a 2.5-fold increase in c-Src activity compared with control siRNA-treated cells. This treatment had no effect on total c-Src or Csk expression levels (Figure 14). These data suggest that reduction of Cbp expression in cells lacking Cav-1 leads to an increase in basal c-Src activity because of reduced control by Csk, which was not perturbed when one or both of the adapters were present at appropriate levels. This further demonstrates the requirement of a certain level of expression of the membrane adapters for Csk to inhibit c-Src under basal conditions.

G. Expression of a Phosphodefective Cav-1 Tyr14 Mutant Fails to Reduce c-Src Activity

Because association of Csk with Cav-1 has been reported to follow phosphorylation of Cav-1 at tyrosine 14 (Cao et. al., 2002; Lu et. al., 2006), we explored the importance of this residue in the control of basal c-Src activity. We expressed WT Cav-1, a phospho-defective (Y14F) Cav-1 mutant, or a phosphomimicking (Y14E) Cav-1 mutant in Cav-1(-/-) MFs and measured basal c-Src activity after 1 hour of serum deprivation. Cells expressing the Y14F mutant failed to reduce basal c-Src activity below empty vector control levels. On the other hand, rescue with WT Cav-1 or Y14E Cav-1 expression reduced basal c-Src activity below control levels (Figure 15A and 15B). Total levels of Cbp, c-Src, and Csk were unaffected by the expression of these constructs (Figure 15A). The transfection of WT and Y14E Cav-1 lowered basal c-Src activity by approximately 35% compared with the Y14F-expressing cells (Figure 15B). This confirms the requirement of Cav-1 Tyr14 phosphorylation for control of c-Src basal activity and that this equilibrium can be modulated by increases or decreases in

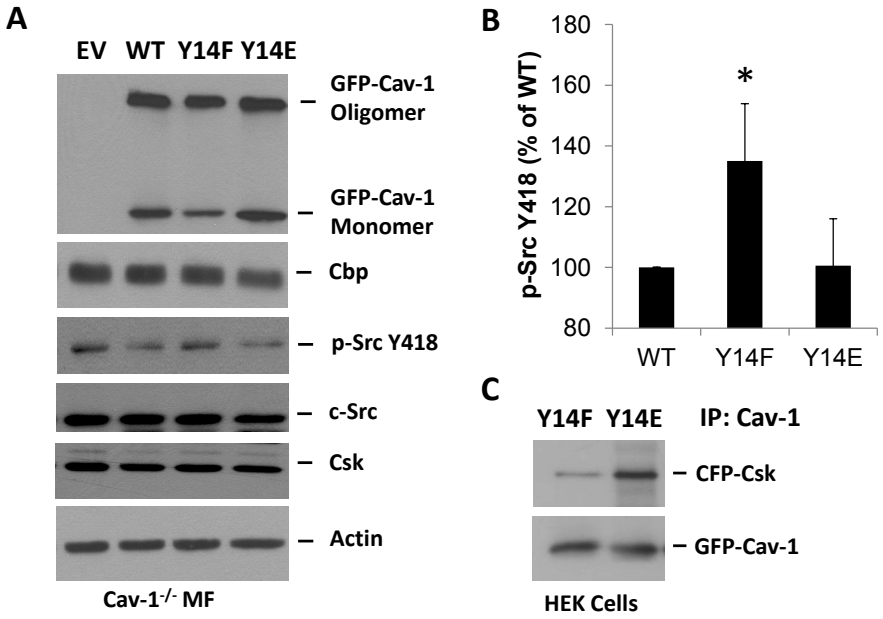

Figure 15: Importance of Cav-1 Tyr14 in Csk recruitment and c-Src inhibition. **A,** GFP-Cav-1 WT, GFP-Cav-1 Y14F (a phospho-defective mutant), or GFP-Cav-1 Y14E (a phosphomimicking mutant) were transiently transfected into Cav-1(-/-) MFs and then after 24 hours were serum deprived for 1 hour and lysed as described under *Materials and Methods*. Expression of the Cav-1 Y14F mutant failed to significantly lower basal c-Src activity compared with empty vector (EV)-transfected cells, whereas Cav-1 WT and Cav-1 Y14E expression significantly lowered c-Src activity. Total c-Src, Cbp, and Csk expression remained unchanged. **B,** Quantification of three independent experiments by densitometry normalized to loading controls (mean +/- S.E.) indicates that the Cav-1 Y14F mutant failed to lower basal c-Src activity (* = $P < 0.05$ versus Cav-1 WT). **C,** CFP-Csk and GFP-Cav-1 Y14F or GFP-Cav-1 Y14E were transiently co-transfected into HEK cells, lysed after 24 h, and Cav-1 was immunoprecipitated. CFP-Csk was associated with the immunoprecipitated GFP-Cav-1 Y14E but not with GFP-Cav-1 Y14F.

expression or phosphorylation state of Cav-1 Tyr14 because the expression of Cbp, c-Src, and Csk remained constant.

H. Phosphorylation Dependence of the Csk and Cav-1 Interaction

Other groups have reported that Cav-1 and Csk associate in a Csk SH2 domain-dependent manner when Cav-1 Tyr14 is phosphorylated (Cao et. al., 2002; Lu et. al., 2006). To confirm this interaction we expressed the Y14F and Y14E Cav-1 mutants, along with Csk-CFP, in HEK cells. After immunoprecipitation of Cav-1, we observed Csk binding to the Y14E mutant but not the Y14F mutant (Fig. 15C). Although we did see a faint band in the Y14F lane, this is most likely due to the small amount of endogenous Cav-1 found in HEK cells oligomerized with the expressed mutant and is therefore considered background signal.

We also designed a series of Cav-1 N-term peptides to use in a cell free binding assay (Figure 16). Starting at the N-terminus, we used a biotin tag for ease of pulldown. The biotin tag was followed by an antennapedia cell permeable peptide domain and a five glycine amino acid linker for flexibility and relief of steric constraints. Following the linker, we added the 9 amino acids of Cav-1α that surround Tyr14 (HLYTVPIRE). We had four different peptides synthesized (Genscript, Piscataway, NJ), using a different amino acid in the tyrosine position of the Cav-1α N-terminus. We used tyrosine (npY), phospho-tyrosine (pY), phenylalanine (F), and glutamic acid (E). The F and E peptides were modeled after the phospho-defective and phospho-mimicking Cav-1 mutants,

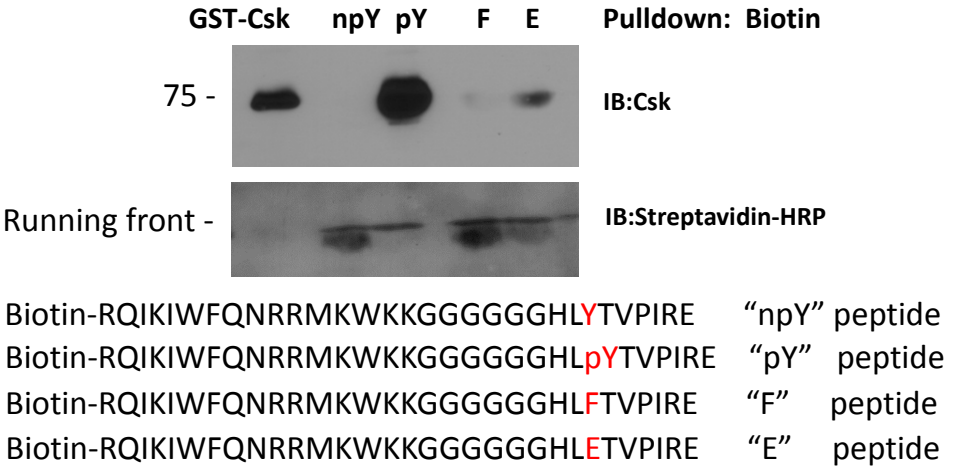

Figure 16: Cav-1 N-terminus peptides. Magnetic streptavidin coated Dynabeads were pre-incubated with the indicated peptide before addition of purified GST-tagged CSK. After washes with pull-down buffer, the beads were boiled in SDS sample buffer with DTT and loaded onto SDS-PAGE gels for western blotting. Membranes were probed with anti-Csk antibodies and streptavidin-HRP. The pY and E peptides pulled down Csk, while the F and npY peptides did not. The biotinylated peptides were detected at the running front of the gel because of their small size.

respectively, that we used in the transfection studies in Cav-1(-/-) MFs and HEK cells described above. We found that the pY and E peptides pulled down Csk, and the F and npY peptides did not (Figure 16). This result is consistent with results obtained in the immunoprecipitation experiments of the native proteins described above, and supports the mutant transfection experiments in HEK cells as well.

I. TNF-α Induces c-Src Activation

In order to provide further insight into the mechanism of SFK molecular regulation, we stimulated endothelial cells with TNF-α. Addition of 20 ng/ml recombinant human TNF-α to human lung microvascular endothelial cells (HLMVECs) induced c-Src activity within ten minutes, which was reduced to near basal levels at 60 minutes post agonist exposure (Figure 17). Cav-1 phosphorylation on Tyr14 also increased, and followed the same time course (Figure 17). As stated in the literature review, tyrosine phosphorylation events are important for the signaling outcomes of TNF-α, and we indeed verified the activation of c-Src after TNF-α stimulation in endothelial cells. We next employed a novel biosensor to examine the subcellular location of c-Src activation in cells. Using an expressed FRET based sensor composed of the SH2 domain of c-Src, a c-Src substrate peptide, YFP and CFP (Figure 18) expressed in HEK cells, we were able to measure c-Src activation and location. This intrinsically membrane targeted sensor is specific for c-Src, (Wang et. al., 2005), and has been well characterized by the laboratories of Roger Y. Tsien PhD and Shu Chien MD PhD, who created the sensor. In fact, this sensor is two and a half times more specific for c-Src than

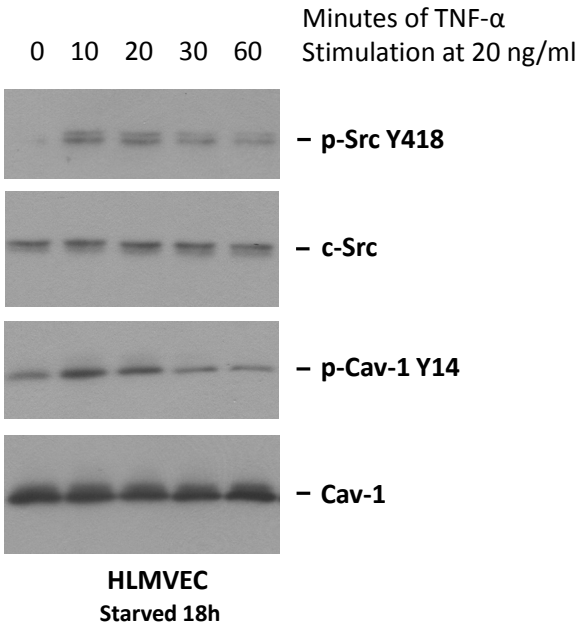

Figure 17: TNF-α stimulated c-Src time course. Confluent HLMVECs were serum-deprived overnight and stimulated with 20 ng/ml of TNF-α for the indicated time points before lysis as described in *Material and Methods*. Equal quantities of protein were loaded per well onto SDS-PAGE gels and western blotted. Activation of c-Src (p-Src Y418) occurred in less than 10 minutes, and returned to near baseline at 60 minutes. Cav-1 phosphorylation at Tyr14 followed the same time course.

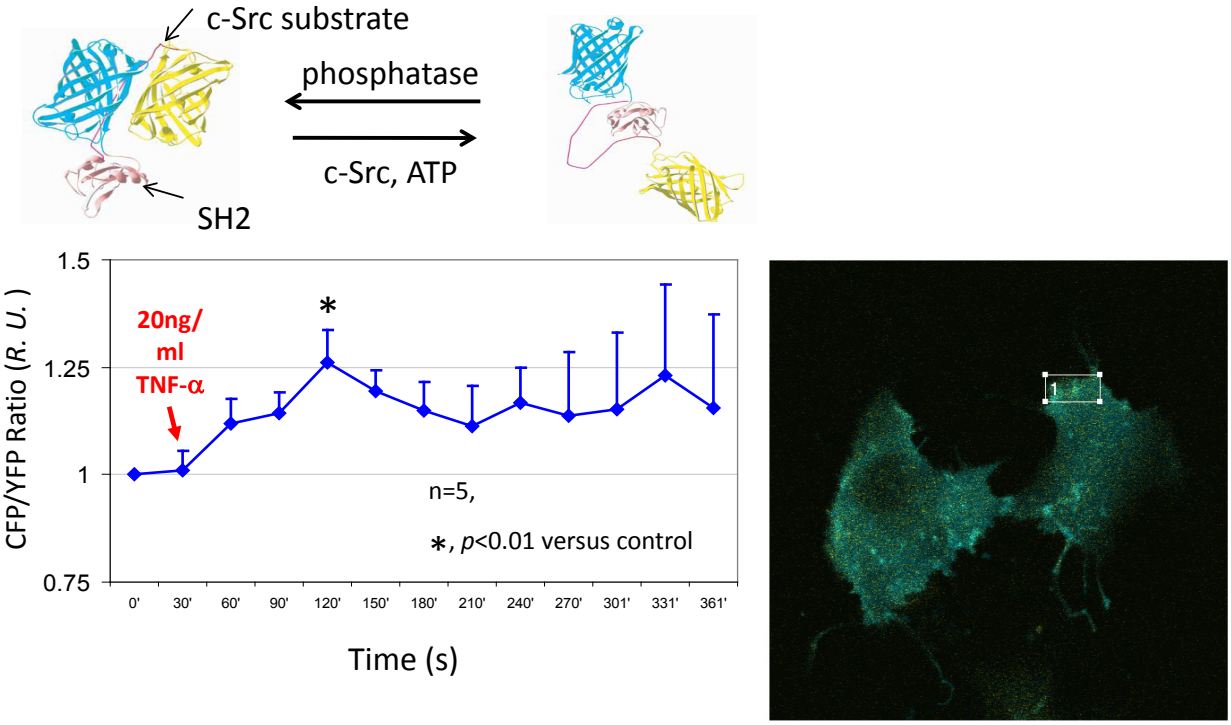

Figure 18: Location of TNF-α stimulated c-Src. HEK cells were transfected with SrcAR2.9 (c-Src activity reporter from: Wang et. al., 2005) and FRET signal was recorded before and after the addition of 20 ng/ml TNF-α at 30 second intervals. TNF-α significantly increased c-Src activity at 2 minutes. n=5, * = p<0.01 versus control

for Fyn, a closely related SFK (Wang et. al., 2005). After stimulation with 20ng/ml TNF-α, we measured a positive change in CFP/YFP ratio, which indicates a loss of FRET, in the HEK cells (Figure 18). The loss of FRET is associated with an increase in c-Src activity according to the design of this sensor. The change in FRET revealed that c-Src specifically was activated within two and a half minutes of exposure to TNF-α. Furthermore, the regions of interest that we analyzed were the plasma membrane at the periphery of the cells. This indicated that TNF-α-induced c-Src activation occurred early and just inside the cell membrane, suggesting that it is an early signal in TNF-α-mediated responses.

J. SHP-2 is Required for TNF-α-induced c-Src Activation

Many investigators and studies have implicated protein tyrosine phosphatases in TNF-α signaling as discussed above. However, the specific protein(s) involved have not been clearly identified. Conceptually, molecular regulation of an enzyme has two components, inactivation and activation. We can disregard protein synthesis and degradation for the purposes of this study since we did not observe any changes in total protein expression levels under our experimental time points and conditions. In an attempt to provide insight into the activation portion of molecular regulation, we examined the contribution of a tyrosine phosphatase, SHP-2, to the activation of c-Src after TNF-α addition to HLMVEC. We employed siRNA to reduce SHP-2 levels in endothelial cells, and routinely achieved knockdown of around 60% (Figure 19) with 100 nM siRNA. This reduced level of SHP-2 significantly affected c-Src activation both basally and after addition of TNF-α. We observed a decrease in basal c-Src pY419

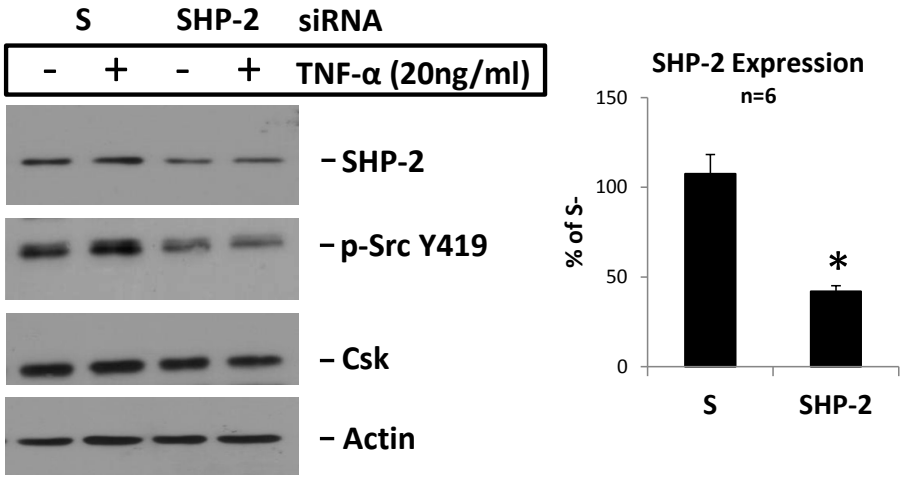

Figure 19: SHP-2 siRNA reduces c-Src activity. Confluent HLMVECs were treated for 48 hours with 100 nM SHP-2 siRNA, serum-deprived overnight, and lysed before or after stimulation with 20 ng/ml TNF-α for ten minutes. Total SHP-2 was reduced, whereas Csk expression remained constant. Activation of c-Src was reduced by the reduction of SHP-2. Stimulation of c-Src by TNF-α was also blocked by SHP-2 reduction. Black bar graph displays mean SHP-2 knockdown +/- S.E. (% of S-, n=6). * = $p < 0.05$ versus S-. S is scrambled control siRNA, SHP2 is siRNA specific for SHP-2.

after the reduction of SHP-2 expression (Figure 19 and 21). We also observed that c-Src pY419 was not increased following addition of TNF-α for 10 minutes in cells treated with SHP-2 siRNA, whereas in cells treated with the scrambled control siRNA (also 100 nM), we observed a 50% increase in phosphorylation of Y419 over baseline (Figure 19 and 21). Furthermore, we observed an increase in the basal phosphorylation state of c-Src Y530, which promotes the inactive conformation of c-Src (Figure 1, 20, and 21). The phosphorylation state of this residue remained elevated over basal even after the addition of TNF-α (Figure 20 and 21). The total levels of Csk and c-Src remained constant in the siRNA treated and TNF-α stimulated cells (Figure 19 and 20). This raised the possibility that SHP-2 was responsible for dephosphorylating Y530, allowing for c-Src activation.

K. Reduction of SHP-2 Expression Level Does Not Increase Cav-1 Tyr14 Phosphorylation

Another possible explanation for the reduction of c-Src basal activity, and inhibition of TNF-α-induced c-Src activation, following SHP-2 knockdown was that maybe Csk association with its adapters was being altered. Indeed, as it was demonstrated in earlier portions of this report, Csk requires association with its membrane adapters, Cbp and/or Cav-1, in order to repress c-Src activity (Figure 14). In order to address this, we examined the phosphorylation state of Cav-1 Tyr14 after knockdown of SHP-2, both at basal levels and after stimulation with 20 ng/ml TNF-α for 10 minutes. Following SHP-2 knockdown, we did not observe a statistically significant increase in either basal or stimulated Cav-1 phosphorylation at Tyr14 (Figure 20 and 21).

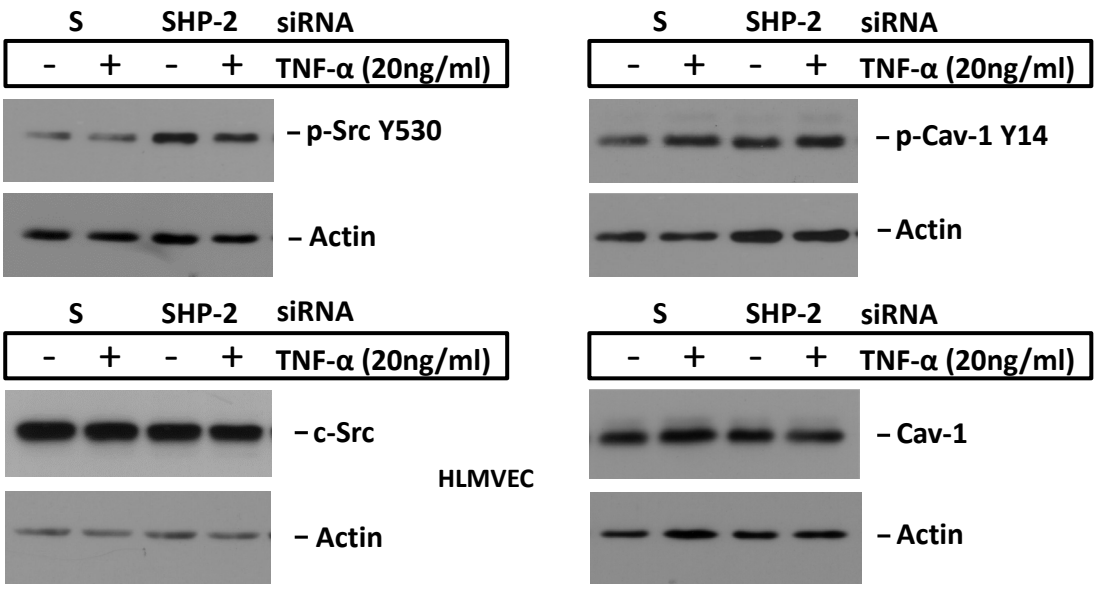

Figure 20: SHP-2 effects c-Src Tyr530 and not Cav-1 Tyr14. Confluent HLMVECs were treated as described in Fig. 19, and changes in c-Src pTyr 530 and Cav-1 pTyr14 were examined by western blot. Tyr530 phosphorylation of c-Src increased both basally, and upon TNF-α stimulation (20 ng/ml), whereas Cav-1 Tyr14 phosphorylation did not. S is scrambled control siRNA, SHP2 is siRNA specific for SHP-2.

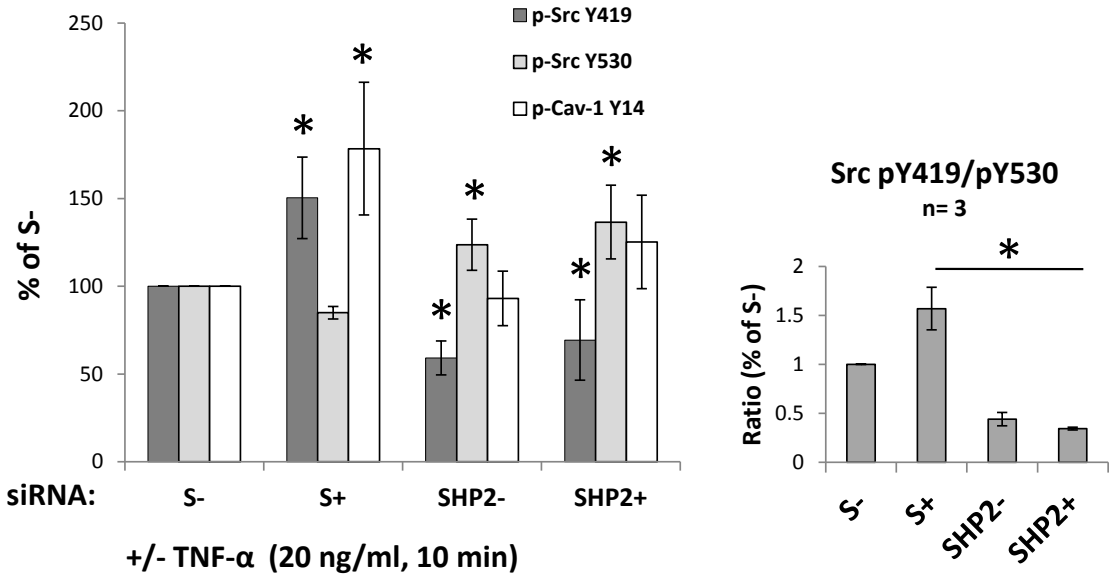

Figure 21: Summary of quantified SHP-2 siRNA data. The mean percent of control (S-) +/- S.E. is presented from at least three separate experiments. S is scrambled control siRNA, SHP2 is siRNA specific for SHP-2. Grey bar graph displays c-Src pTyr419/pTyr530 ratio +/- S.E. (n=3) * = $P < 0.05$ versus S-.

Total Cav-1 expression also remained unchanged by SHP-2 knockdown (Figure 20). In fact, Cav-1 Tyr14 phosphorylation in SHP-2 depleted cells remained similar to the basal levels observed in the scrambled siRNA treated cells in the absence of stimulation and did not increase upon TNF-α stimulation. In contrast, phosphorylation of Cav-1 Tyr14 increased in scrambled siRNA treated cells by about 60% in response to TNF-α, which was similar to the increase in c-Src activity observed in the same treatment group (Figure 20 and 21). Because total Cav-1 expression level and phosphorylation state were not elevated following SHP-2 knockdown, but c-Src C-terminal phosphorylation was increased, Tyr530 is thus the likely target for SHP-2.

L. Subcellular Localization of SHP-2 and Csk

Since both Csk and SHP-2 lack membrane association domains, in contrast to that identified in the SHP-1 C-terminal sequence, we examined the subcellular localization of both SHP-2 and Csk in endothelial cells. After preparation of general membrane and cytosolic fractions from serum-deprived HLMVECs, we observed a majority of both proteins in the cytosolic fractions as expected (Figure 22A). However, we also observed a small fraction of these proteins present in the membrane fractions as well (Figure 22A). The membrane fractions were also enriched in Cav-1 and VE-Cadherin. To further understand the mechanism for this membrane association of a cytoplasmic protein, we hypothesized that SHP-2, like Csk in the MFs and HLMVECs described earlier, may be associated with Cav-1 in endothelial cells. Indeed, in Cav-1 immunoprecipitations from serum-deprived HLMVEC, we observed a basal association of SHP-2 and Csk with Cav-1 (Figure 22B). Csk association with Cav-1 was described earlier in this report in

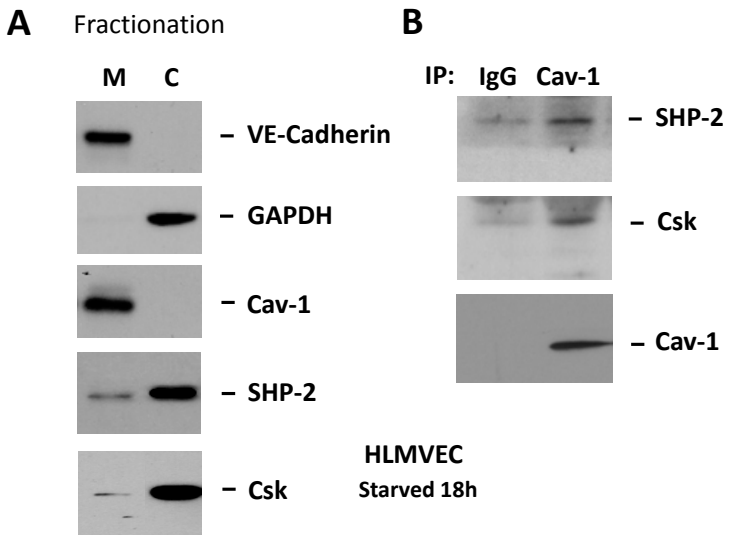

Figure 22: SHP-2 is membrane associated through interaction with Cav-1. A, Cytosolic and membrane fractions of confluent, overnight serum-deprived HLMVECs were prepared as described in *Materials and Methods*. SHP-2 subcellular localization was similar to that of Csk. Cav-1 and VE-Cadherin were strictly membrane localized and GAPDH was strictly found in the cytosolic fraction in these preparations. **B,** Cav-1 immunoprecipitates from the same conditions revealed a basal interaction of SHP-2 and Cav-1.

HLMVECs (Figure 13B), and was used as a positive control for these studies. This finding suggests that SHP-2 at least in part, like Csk, may use Cav-1 as an adapter or scaffold to localize to the membrane in order to perform the function of c-Src activation by dephosphorylating the C-terminal tyrosine of c-Src.

V. DISCUSSION

Constitutive activation of c-Src, as seen in v-Src, leads to cell proliferation, survival, cytoskeletal alteration, migration/invasiveness (Thomas and Brugge, 1997), and oncogenic transformation (Martin, 2001). Thus, tight control of SFKs by Csk is essential for normal cellular homeostasis. The importance of Csk as the central regulator of SFK activity is further evidenced by the fact that Csk knockout mice have neural tube malformation and other developmental defects, and die on day 9 or 10 of gestation (Imamoto and Soriano, 1993). The present study demonstrates that the Csk membrane adapter proteins Cav-1 and Cbp play cooperative roles in the coordination of c-Src inhibition by Csk. When Cbp is reduced transiently, Cav-1 phosphorylation increases to recruit Csk to the membrane where it can negatively regulate c-Src activity. On the other hand, when the Cav-1 gene is completely absent, Cbp expression increases to maintain Csk localization at the membrane to allow for c-Src inhibition. The removal of both adapter proteins, however, results in increased basal c-Src activity, similar to that seen during the reduction of Csk expression. This study also illustrates that c-Src activation is the result of a shift in the equilibrium between Csk and a tyrosine phosphatase, SHP-2.

Reduction of SHP-2 expression resulted in greater basal inhibition of c-Src activity, without effecting adapter protein phosphorylation or expression. The opposite result, greater c-Src basal activation, was observed when Csk expression was reduced. Furthermore, SHP-2 depletion prevented the activation of c-Src by TNF-α above basal levels, indicating that SHP-2 is required for activation of c-Src under this stimulus.

This study provides the first description of the cooperative role of Cav-1 and Cbp in the regulation of basal c-Src activity. The current observations reveal that cells respond to the loss of Cav-1 by increasing the expression level of Cbp. The increase in expression of Cbp in the Cav-1(-/-) mice may be a compensation that occurred during the development of these mice, probably before day 9 or 10 of gestation, to control SFK activity. This is likely because this is the point at which Csk(-/-) mice arrest (Imamoto and Soriano, 1993). Another mechanism for regulating c-Src activity in the Cav-1(-/-) mice would be to increase Csk expression or decrease c-Src expression. However, this was not the case because no change in expression of either Csk or c-Src was observed between WT and Cav-1(-/-) MFs. Not only did the expression level of Csk remain constant, but Csk localization also remained unchanged in these cells. An equivalent amount of Csk was present in the membrane and cytosolic fractions of the Cav-1(-/-) cells compared with WT cells under basal conditions. Increased Cbp expression therefore enabled appropriate Csk recruitment to the membrane to regulate c-Src. Furthermore, reduction of Cbp in Cav-1(-/-) cells increased basal c-Src activity, revealing that these two proteins may be the only membrane adapters for Csk in fibroblasts.

The observed cooperativity between Cav-1 and Cbp may explain why the single knockouts of Cav-1 (Murata et. al., 2007) or Cbp (Dobenecker et. al., 2005) are viable,

whereas Csk knockout mice are not (Imamoto and Soriano, 1993). Like Cav-1(-/-) mice, Cbp(-/-) mice proceed normally through development (Dobenecker et. al., 2005). The authors of this study concluded that Cbp is not essential for embryonic development or Csk compartmentalization, and that there may be other membrane adapters for Csk. In addition, there was no significant increase in overall tyrosine phosphorylation in T-cells from these mice. This study also found normal Csk, phospho-Fyn, and phospho-Lyn subcellular distribution in cells derived from Cbp(-/-) mice, which is similar to that observed in Cav-1(-/-) cells. Compensation for the lack of Cbp was confirmed by us in the present *in vitro* study by demonstrating that Cav-1 tyrosine 14 phosphorylation increased after a transient Cbp reduction of 80%, with no increase in c-Src activity. Consistent with this, Cav-1 immunoprecipitation experiments displayed equivalent amounts of Csk associated with Cav-1 under these reduced Cbp conditions. The increase in Cav-1 phosphorylation therefore served to coordinate Csk to maintain inhibition of basal c-Src activity despite reduced Cbp expression. It is noteworthy that a reduction of both adapters by knocking down Cbp in Cav-1(-/-) MFs led to elevated basal c-Src activity. Thus, in the absence of both adapters, elevated c-Src activity is the result of an inability of Csk to access and phosphorylate c-Src C-terminal tyrosine 529, which was still achieved when only one adapter (either Cav-1 or Cbp) was present. This observation is consistent with the finding that Csk SH2 domain mutants that are unable to localize to the plasma membrane do not inhibit c-Src (Howell and Cooper, 1994). Therefore, defective Csk targeting, whether by mutation or reduced expression of both the adapters or Csk itself, leads to an inability to return c-Src to its baseline activity.

It has been suggested previously that the Cav-1 scaffolding domain (residues 82–101) can sequester c-Src and thus affect its activation (Okamoto et. al., 1998). Because no reduction in basal c-Src activity was observed when Y14F Cav-1 was expressed in Cav-1(-/-) cells, whereas c-Src activity was reduced upon WT Cav-1 and Y14E Cav-1 expression, Cav-1 Tyr14 phosphorylation seems to play the dominant role in the mechanism of c-Src inhibition by Cav-1. The Cav-1 scaffolding domain may therefore be necessary for correct localization of c-Src into caveolae but does not appear to mediate c-Src inhibition without tyrosine 14 phosphorylation of Cav-1 for Csk binding. Thus, the direct comparison of phosphodefective Y14F Cav-1 with phospho-mimicking Y14E Cav-1 and WT Cav-1 conducted here illustrates that Cav-1 Tyr14 phosphorylation is critical for c-Src inhibition through Csk coordination. It is also likely that the same importance of phosphorylation exists with Cbp tyrosine 314 which plays a similar role in Csk coordination for negative feedback regulation of c-Src, and since Cav-1 and Cbp are found in a complex with each other.

This study is also the first to provide insight into how c-Src may be activated, both downstream of the TNF receptor and under basal conditions. As shown by siRNA mediated transient reduction of SHP-2 expression levels, both basal and TNF-α stimulated c-Src activity was below the basal activity levels observed in the scrambled control siRNA treated cells. This was accomplished with a reduction in expression of SHP-2 by ~60%. The inhibitory C-terminal tyrosine of c-Src was hyperphosphorylated over scrambled control basal levels as well in the presence of reduced SHP-2. A possible mechanism of increased phosphorylation at the C-terminal tyrosine residue under both conditions would be the increased association of Csk at the membrane as a result of SHP-

2 knockdown. However, because Cav-1 phosphorylation and expression were not increased under any of these conditions, an increased membrane association of Csk seems unlikely. Rather, this data further suggests that under both basal and stimulated conditions, Csk and SHP-2 are in equilibrium, and have the same target tyrosine of c-Src. It should be noted here that upon discovery of Csk (Okada et. al., 1991), the authors also speculated towards this type of equilibrium of Csk with a tyrosine phosphatase in his discussion. Our model (Figure 24) of the hypothesized mechanism of c-Src activation and inactivation predicts that when SHP-2 activity is reduced (either by inhibition or reduced expression), Csk activity overcomes SHP-2 activity and results in the inactive form of c-Src dominating. On the other hand, if SHP-2 activity is increased (either by increased expression or activation), Csk activity level is overcome and the active form of c-Src dominates. This relationship can also be applied to Csk, as shown with the reduction of either Csk expression, or decreased expression of the Csk adapters, leading to more active c-Src. Exactly how this equilibrium is shifted is still not clear however. It was previously demonstrated that stimulation with TNF-α induces SHP-2 phosphatase activity in endothelial cells (Lerner-Marmarosh et. al., 2003). Whether this stimulation is mediated through recruitment of more SHP-2 molecules, or through a direct increase in SHP-2 catalytic activity (or both), remains to be determined.

In addition to the downstream signaling functions of c-Src activation by TNF-α discussed earlier, c-Src activation could also function as the mechanism for receptor internalization. As noted above, caveolae function as endocytic vesicular structures (Parton et. al. 2007; Mercier et. al. 2009; Minshall et. al., 2000). Previous studies have described the activation of c-Src, and subsequent phosphorylation of Cav-1 and dynamin-

2, to be a trigger for caveolae-mediated endocytosis (Minshall et. al., 2000; Shajahan et. al., 2004). Therefore, activation of c-Src following TNF-α ligation of its receptor could facilitate an attenuation of signaling to the cell to prevent prolonged activation of downstream effects such as NF-kB activation. It also could facilitate the recycling or removal of occupied receptors to prevent signaling saturation. In fact, internalization of the epidermal growth factor receptor (EGFR) and the transforming growth factor receptor (TGFR) by caveolae has been demonstrated previously (Di Guglielmo et. al., 2003; Parton and Simons, 2007). Furthermore, TNFR1 has been shown to be localized to caveolae (Ko et. al. 1999; D'Alessio et. al. 2010), and observed to be associated with Cav-1 and c-Src in immunoprecipitates (D'Alessio et. al. 2005; Pincheira et. al., 2008). If c-Src activation results in TNFR internalization or not, and what function the internalization via caveolae serves, has yet to be demonstrated however.

Additionally, we are the first to demonstrate an association between SHP-2 and Cav-1. This led us to speculate that since SHP-2 lacks its own intrinsic membrane lipid association domain (Poole et. al., 2005), it too may use membrane adapters through its SH2 domains. Cav-1 may be such an adapter in light of this association, especially since the present fractionation experiments displayed only a small amount of membrane associated but predominant cytoplasmic localization of SHP-2. It is also well known that Cav-1 has an extensive scaffolding function, and many signaling molecules reside in caveolae, including TNFR1 (Ko et. al. 1999; D'Alessio et. al. 2010). Further, since recent studies demonstrated that TNFR1 associates with c-Src and Cav-1 (D'Alessio et. al. 2005), Cav-1 may serve as a bridge to allow for activation of c-Src by SHP-2 following trimeric TNF-α induced clustering of TNFR's. It is also possible however, that there may

not be a direct interaction between Cav-1 and SHP-2, and intermediate molecules are required for this association. One such molecule may be Cbp, given its presence in this complex both in MFs and in RLMVECs. Another possibility is that SHP-2 may bind directly to TNFR1, which has also been demonstrated to bind to Cav-1 in other studies. *In vitro* peptide binding studies with highly purified SHP-2, like those included in this report with purified Csk, would be necessary to confirm a direct interaction. Thus, additional studies must be performed to confirm the requirement of membrane adapters for SHP-2 signaling.

In the present study, we examined the activity and phosphorylation state of c-Src, which is only one of the three ubiquitously expressed members of the SFKs (Chow and Veillette, 1995). It is likely that the Csk adapters Cbp and Cav-1 may have facilitated inactivation of the other SFK members in our study as well. There is data supporting the ability of Csk to phosphorylate other SFK members in the conserved C-terminal tyrosine other than c-Src (Chow and Veillette, 1995). It should also be noted that upon discovery of Csk (Okada et. al., 1991), Csk was shown to phosphorylate and repress the activity of the other ubiquitously expressed SFKs, Fyn and Lyn, and not just c-Src. Consistent with this, Cbp has also been implicated to be involved in the regulation of Fyn and Lyn by coordinating Csk (Brdicka et. al., 2000; Kawabuchi et. al., 2000; Ingley et. al., 2006). Thus, the membrane-localized Csk adapters Cav-1 and Cbp may also cooperatively participate in the negative regulation of these other SFKs as well. Furthermore, since there is significant homology of the C-terminal tails of SFKs, it is possible that the equilibrium between SHP-2 and Csk functions to regulate these other Src family members as well.

Serum deprivation has been used throughout this report to assess basal c-Src activity (i.e., in the absence of growth factor activation of c-Src). Cells were grown in the presence of serum, and then the serum was removed for indicated times before lysis. This protocol therefore allowed us to assess basal c-Src inactivation by Csk through C-terminal c-Src tyrosine phosphorylation, or to allow for examination of the signaling of a single specific stimulus (TNF-α), without the presence of the many growth factors found in serum. Increased basal c-Src activity was thus determined to be the result of an inability to inactivate c-Src through Csk-mediated C-terminal tyrosine phosphorylation of c-Src after the removal of serum. Reduced expression of Csk *per se,* or the membrane Csk adaptors Cbp and Cav-1, led to the sustained increase in basal c-Src activity that could not be reset, demonstrating that a certain level of either Cbp or Cav-1 (and Csk) is required for c-Src inhibition. As described in our proposed model (Figure 23), Cbp and Cav-1 recruit cytoplasmic Csk to the membrane where Csk mediates the inactivation of c-Src. If one adapter is absent or non functional, there is a compensatory increase in the expression or phosphorylation state of the other adapter to enable coordination of Csk and safeguard against sustained c-Src activation.

The finding that expression of WT or Y14E Cav-1 was sufficient to further reduce basal c-Src activity when expressed in Cav-1(-/-) MFs, whereas the phosphodefective Cav-1 mutant (Y14F) had no effect on c-Src activity and was unable to bind Csk, may be clinically important. This finding indicates that despite the approximately 2-fold increase in Cbp expression in these cells, the equilibrium of c-Src activation and inactivation can still be affected by both Csk expression level and Csk adapter protein expression level and/or phosphorylation state. Defective c-Src inactivation probably explains the increase

in c-Src activity noted in colon cancer (Rengifo-Cam et. al., 2004; Sirvent et. al., 2010), in which a decrease in Csk membrane localization was associated with increased metastasis and invasiveness. These studies also showed reduced adapter protein expression for both Cav-1 and Cbp in metastatic cells and tumors. Furthermore, Sirvent et. al. (2010) went on to demonstrate that rescued expression of Cbp in colorectal cancer cells reduced the invasiveness of these cells. Our study indicates that Cav-1 and Cbp are cooperative, and increases in their expression or phosphorylation favor repression of c-Src activity, suggesting that this may represent an important therapeutic mechanism or diagnostic tool for the treatment of colon cancer and other pathologies associated with sustained or elevated c-Src activity.

Finally, these studies, and the ability to modulate c-Src activity, may provide insight into the mechanism of sustained c-Src activation. This is similar to the case of v-Src whose activity is about ten-fold higher because of a lack of C-terminal regulation (Coussens et. al., 1985; Piwnica-Worms et. al., 1987; Martin, 2001). As observed in the studies of colon cancer described above, and other forms of cancer described earlier, chronically elevated c-Src activity may be a consequence of defective negative regulation. Either Csk itself is dysfunctional, or the adapters are, leading to a shift in equilibrium favoring active c-Src (Figure 24). Maybe by pushing the equilibrium back to favor inhibition of c-Src, such as by the rescue of adapter expression or the administration of cell permeable peptides to accomplish the same goal, it may be possible to overcome sustained c-Src activation. Another pathology, acute or uncontrolled inflammation such as that observed in sepsis or acute lung injury, may be a consequence of continuous agonist exposure driving sustained c-Src activation. It is known that

circulating levels of TNF-α are high during systemic inflammation and sepsis (Xuan et. al., 2001; Maitra et. al., 2004; Hartemink and Groeneveld, 2010) and that both inflammation and SFK's are involved in acute lung injury (Severgnini et. al., 2005; Lee et. al., 2007; Hu et. al. 2008). Therefore, sustained c-Src activation may be either receptor driven by continuous agonist exposure (as may be the case in chronic inflammation) or dysfunction of the regulatory machinery (as seen in some forms of cancer). Demonstration of the relevance of this idea in clinical syndromes may hold great therapeutic promise when combined with the principles displayed herein about the ability to negatively regulate c-Src activity.

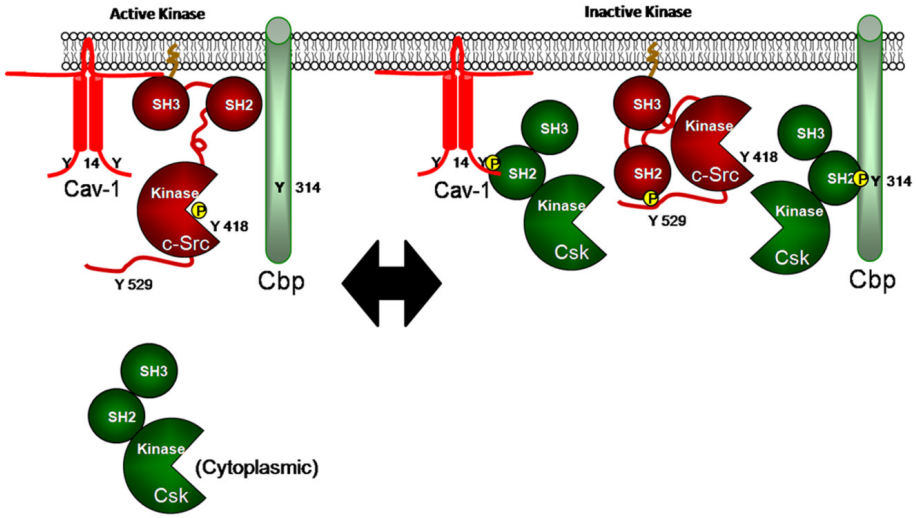

Figure 23: Proposed equilibrium model of c-Src activity regulation. Cbp and Cav-1 recruit cytoplasmic Csk to the membrane, where Csk mediates the inactivation of c-Src. If one adapter is absent or not functional, there is a compensatory increase in the other to enable coordination of Csk and negative regulation of c-Src activity. This model also predicts that when both Csk adapters are absent or nonfunctional, Csk is unable to localize to the membrane, resulting in sustained c-Src activation.

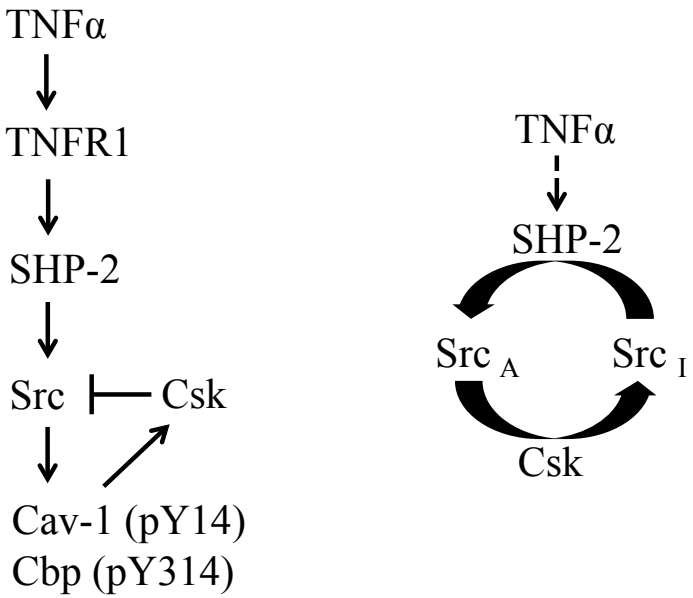

Figure 24: TNF-α stimulation of c-Src, and the Csk/SHP-2 equilibrium models. The proposed mechanism of c-Src stimulation by TNF-α is shown on the left. TNFR1 residing in caveolae and associating with c-Src, Cav-1, Cbp, and SHP-2, when engaged by TNF-α, stimulates SHP-2 activity leading to c-Src activation, followed by phosphorylation of the Csk adapters creating a feedback inhibition loop. An alternative view of this process is on the right describing the equilibrium between SHP-2 and Csk. If either proteins' activity is increased, it favors the conformation of c-Src as indicated by the arrow. For instance, under basal conditions, they are in a balanced equilibrium, whereas stimulation of SHP-2 activity can cause a shift towards the active conformation of c-Src, increasing c-Src activity. This model predicts that this shift in equilibrium may persist until SHP-2 is no longer activated by TNF-α.

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APPENDIX

September 30, 2011

Aaron T. Place
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Dear Mr. Place:

This is to grant you permission to include the following article in your thesis entitled "Molecular Mechanism of Src Regulation by Csk, SHP-2, Cbp and Caveolin-1," for the University of Illinois:

> Aaron T. Place, Zhenlong Chen, Farnaz R. Bakhshi, Guoquan Liu, John P. O'Bryan, and Richard D. Minshall, Cooperative Role of Caveolin-1 and C-Terminal Src Kinase Binding Protein in C-Terminal Src Kinase-Mediated Negative Regulation of c-Src, *Mol Pharmacol* October 2011 80:665-672

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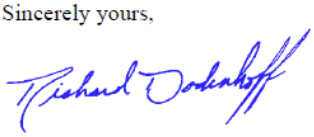

Richard Dodenhoff
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Education

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Minor in Biochemistry

Experience

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AHA Predoctoral Fellow 2009-2010
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Publications

Place AT, Chen Z, Liu G, Bakhshi FR, Minshall RD. Roles of SHP-2 and Csk in TNF-alpha-induced c-Src activation in endothelial cells. In preparation.

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