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Investigations into the Interaction between Src Kinase and Wnt Signaling

by

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ABSTRACT

In this study, I attempted to elucidate a possible interaction between Src kinase and canonical Wnt signaling, both of which have been shown to contribute to cancer. I hypothesized that Src kinase could induce canonical Wnt signaling through the phosphorylation of β-catenin and subsequent nuclear localization and activation of Wnt target gene transcription. Using immunofluorescence microscopy I observed that β-catenin possessed a peripheral and juxta-nuclear localization in v-Src containing Rous sarcoma virus infected chicken fibroblasts, compared to a diffuse and punctate distribution in uninfected cells. In human colon adenocarcinoma SW480 cells biochemical methods did not show a dramatic difference in the nuclear levels or protein stability of β-catenin induced by the activated mutant SrcY530F. However, in tsLA29 Rat-1 cells incubated at the permissive temperature the half-life of β-catenin was shortened compared to cells incubated at the non-permissive temperature.

I also tested the ability of Src to regulate canonical Wnt pathway mediated transcription using real-time PCR and luciferase reporters. Transient transfection of wt and mutant Src kinases did not alter the transcription of the Wnt target genes *FGF18*, *CCND1* or *MYC* in SW480 cells relative to empty vector pCI transfected cells. However, the activated mutant SrcY530F was able to downregulate the β-catenin responsive Wnt luciferase reporter Super8XTopFlash. To identify the mechanism of reporter downregulation, I mutated β-catenin, used pharmacological inhibitors and siRNA to inhibit GSK3β, and the calcium chelator BAPTA-AM, I was not able to reverse the effect of Src on reporter activity. However, activated SrcY530F was shown to downregulate 7 out of 8

different luciferase reporters not predicted to be regulated by Src, including the empty vector backbone pTA-Luc and the constitutively activated luciferase reporter plasmid pGL3-Control.

Taken together, these data strongly suggest that the downregulation of Wnt luciferase reporter activity by SrcY530F is an anomalous result, and cautions against the use of luciferase reporters as a measure of the effects of Src on transcription. Furthermore, the results of this study do not prove or disprove my hypothesis.

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LIST OF SYMBOLS & ABBREVIATIONS

APC	Adenomatous Polyposis Coli
ASV	avian sarcoma virus
ATCC	American type tissue collection
ATP	adenosine triphosphate
BAPTA-AM	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester
β-Gal	β-galactosidase
β-ME	2-Mercaptoethanol
BSA	bovine serum albumin
BTK	Bruton's tyrosine kinase
CCLR	cell culture lysis reagent
CDC2	cell division cycle 2
CEF	chicken embryo fibroblasts
cm	centimetre
CR	cytokine receptor
Crk	Csk homologous kinase
Csk	C-terminal Src kianse
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DMEM	Dulbeco's modified Eagles medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EMSA	electromobility shif assay
FAK	focal adhesion kinase
FCS	fetal calf serum

FGFR	fibroblast growth factor receptor
FSV	Fujinami sarcoma virus
GFR	growth factor receptor
GPCR	G-protein coupled receptor
Grb2	growth factor receptor-bound protein 2
HER-1	human epidermal growth factor receptor 1
HER-2	human epidermal growth factor receptor 2
JAK	Janus Kinsase
KDa	kilodalton
L	litre
LF2000	Lipofectamine 2000
LiCl	lithium chloride
mA	milliampere
MAPK	mitogen-activated protein kinase
mg	milligrams
µg	microgram
µl	microlitre
mM	millimolar
µM	micromolar
MMTV	mouse mammary tumour virus
NP-40	Nonidet P40
NTF	normal transformed medium
ONPG	ortho-nitrophenyl-β-D-galactopyranoside
PBS	phosphate buffered saline
PDGFR	platelet derived growth factor receptor
phCMV-CLUC	phCMV C-terminal luciferase
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PTP	protein tyrosine phosphatase
r.p.m.	revolutions per minute

RNA	ribonucleic acid
r.t.PCR	reverse-transcription polymerase chain reaction
RSV	Rous sarcoma virus
SDS-PAGE	sodium dodecyl sulphate polyacrylamide electrophoresis
SH1 domain	Src Homology domain 1
SH2	Src homology domain 2
SH3	Src homology domain 3
SH4	Src homology domain 4
SHP	Src Homology domain containing phosphatase
siRNA	short-interfering RNA, or silencing RNA
SRA	Schmidt-Ruppin strain A transformed chicken embryo fibroblasts
STAT	signal transducer and activator of transcription
TBE	tris buffered EDTA
TBS-T	tris buffered saline - tween 20
TEMED	N, N, N',N'-tetramethylethylenediamine
Tg	thapsigargin
Tris	tris(hydroxymethyl)amino methane
tsLA29 Rat-1	temperature-sensitive LA29 v-Src transformed Rat-1 cells
UTP	uridine-5'-triphosphate
UV	ultraviolet
V	volts
Wt	wild type
ψ	hydrophobic amino acid

SECTION I

INTRODUCTION & METHODS

CHAPTER ONE: INTRODUCTION

1.1 Historical Overview – The Importance of Src Kinase

The human non-receptor tyrosine kinase c-Src is an enzyme that is part of a larger family of tyrosine specific kinases found throughout metazoan phylogeny (1-4). In addition to Src, the vertebrate Src family members include Fyn, Yes, Lyn, Hck, Lck, Fgr, Blk, and Yrk (5). According to Brown and Cooper (1996), at least one Src family member is expressed in every cell type examined from complex animals (5). Many studies have revealed the complex nature of its regulation and function. Its unregulated function has also been shown to contribute to various disease states, with a particular contribution to cancer. Therefore, these studies suggest that Src and Src family kinases make a significant contribution to cellular biology.

Much of what is known today about the cellular properties of c-Src has come from a close study of its viral counterpart, v-Src. Almost 100 years ago, Peyton Rous described the transmission of neoplasms and malignant growth using cell free extracts derived from bacteria-free filtrate ((4) from Readings in Tumor Virology, 1983). This was a landmark study, not only because it was the first description of a tumour inducing virus, but also because our understanding of intracellular signaling has been derived through the subsequent detailed analysis of Src kinase.

The use of various Rous sarcoma virus transformation mutants was key to the identification of the *Src* gene. The first of these studies credited for identifying the *v-Src* gene (viral *Src*), a temperature sensitive mutant was isolated which incapacitated both the growth and transforming abilities of the virus (6). Subsequently, Martin (1970) identified a

mutant that was only defective for its transforming potential and not for its growth potential (7). That is, the mutation allowed for the reversible inactivation of v-Src kinase activity and therefore its ability to transform infected cells, while the infectivity of the Rous sarcoma virus was not impeded. Importantly, Kawai and Hanafusa (1971) soon isolated another temperature sensitive mutant, Ts-68 (later ts NY68), by treating cells with the chemotherapeutic agent 5-flurouracil (8). Ts-68 was 'converted' much more quickly than the previously identified mutants and, using puromycin and cyclohexamide, the authors demonstrated that the transforming ability of the virus was due to a non-structural protein product (v-Src) of the viral genome. Finally, Biggs *et al.* (1973) used temperature sensitive mutants to not only reversibly transform cells, but also to induce tumours and provide further evidence that these two properties were connected (9).

The identification and description of other types of v-Src mutants was also very important to our understanding of c-Src. A thorough analysis of transformation revertants in a two part study by Varmus's laboratory (10,11) identified several revertant avian sarcoma virus (ASV)-transformed rat1 cell lines. In these studies, the authors were able to show not only that the reversion process was most likely due to specific point mutations, but also that the transforming ability of Src may be due to host-cell specific components as demonstrated by the altered phenotype of a rescued revertant in chicken cell lines. While various types of v-Src mutants had been identified, an important study by Anderson *et al.* (1981) utilized unmutagenized partial mutants (12). This study described the isolation of v-Src mutants with various combinations of transformation phenotypes. Upon the characterization of these mutants, the authors insightfully suggested that the Src protein interacts with numerous down-stream proteins to achieve the complete transformation

phenotype. Taken together, these studies were fundamental to our understanding of protein-protein interaction (i.e. via the Src homology, or SH2 & SH3 domains) and therefore signal transduction.

With the identification of v-Src kinase as the cause of the Rous sarcoma virus induced fibroblast transformation, several studies by Erikson's group quickly characterized this novel phosphortransferase (13-15). Erikson's group initially identified a 60,000 MW peptide using serum from Schmidt-Ruppin Avian Sarcoma Virus induced newborn rabbit virus to immunoprecipitate lysate from transformed chicken cells. Subsequent analysis by two studies, Colette and Erikson (1978), and Levinson *et al*. (1978), confirmed that the *src* gene product possessed protein kinase activity (16,17). Soon after this characterization, Hunter and Sefton (1980) demonstrated that the *src* gene product was not only responsible for transformation, but also possessed a tyrosine kinase activity (18). In fact, the $pp60^{src}$ kinase was the first tyrosine kinase activity to be demonstrated. Importantly, the epidermal growth factor receptor (EGFR) was also found to be a tyrosine kinase (19) and together with Src kinase are now know to be the earliest identified kinases involved in cell growth and differentiation (20).

In 1976, the cellular origin of the v-Src gene was identified (21). The strategy in this study involved using ASV based probes required for transformation to screen the DNA of various normal avian species including chicken, duck, quail, turkey and emu. The authors correctly suggested that the transforming gene found in the ASV genome was derived from chicken or a closely related relative. Later studies characterized in detail the full length genomic sequence of chicken Src, confirming the cellular origin of v-Src (22,23). From this observation, the idea of the proto-oncogene was born (20).

In short, characterization of the non-receptor tyrosine kinase Src has provided invaluable knowledge to our understanding of intracellular signaling, proliferation and consequently cancer biology. Since these early observations, much more about the function of Src and Src family kinases has been elucidated. These functions arise from both its non-kinase and kinase elements and include involvement in a wide variety of cellular processes including proliferation, apoptosis, and motility. It is clear then that continued research into its regulation and function will surely provide novel insights into fundamental cellular regulation and deregulation in the diseased state.

1.2 Src Kinase - Structure

The regulation of Src kinase is of great importance to the understanding of its overall contribution to cellular biology. The interaction of Src kinase with various growth factor receptors, cytokine receptor and G-protein coupled receptors through allosteric changes play an important role in the regulation of Src kinase activity and is well reviewed elsewhere (5,24). A second mechanism, not mutually exclusive to the allosteric mechanism of regulation, is through the phosphorylation state of its c-terminal tyrosine. Within human c-Src this residue is Y530, and its contribution to Src activity will be discussed in greater detail below. Two non-receptor tyrosine kinases have been shown to target this residue on Src and result in downregulation of the enzyme. These are Csk (C-terminal Src kinase, the predominant kinase targeting Y530) and CHK (Csk-homologous kinase). Interestingly, CHK has been shown to inhibit Src activity by a dual mechanism that includes phosphorylation of Y530 and also a non-phosphorylation mechanism that requires only interaction (25). Similarly, several phosphatases have also been shown to target this residue

for de-phosphorylation and subsequent activation of Src kinase activity. Some of these include PTP-α (protein tyrosine phosphatase-α), PTPl, PTP1B, SHP-1 (Src Homology 2 domain containing phosphatase-1, also known as PTP1C) and SHP-2. Other phosphatases which may regulate the phosphorylation state of SrcY530 have been recently identified; however, a more thorough review of the major mechanisms of Src regulation can be found in several reviews (5,24,26,27). Taken together, these studies highlight the importance of the state of tyrosine-530 phosphorylation in the regulation of Src kinase activity. The remainder of this review will only briefly describe regulation of Src activity as it may relate to its targets and hence its potential functions.

The human and chicken forms of c-Src are highly similar. Both the normal chicken c-Src and v-Src have been cloned and characterized, and comparisons between them show a high degree of similarity (22,23,28-30). Similarly, the human normal c-Src has also been cloned and characterized (12,31). These studies have shown that the normal chicken Src kinase contains 533 amino acids, while human c-Src contains 536 amino acids. A sequence comparison of normal human and chicken Src shows an 85% identity (NCBI Blast sequence alignment of accession no. NM_005417 (human) and accession number V00402 J00844 (chicken)). Therefore, although the following description of c-Src structure and function has been derived from studies of the chicken orthologue, the observations can be applied to human c-Src with important differences being highlighted when required.

Having characterized and cloned the cellular *src* gene, as well as identifying the tyrosine kinase enzymatic activity, investigators focused on a detailed analysis of both its protein structure and precise biological functions. The contribution of Src kinase to tumourigenesis was self evident, although many of its targets and the details of just how

these interactions would promote transformation were yet to be characterized. As previously mentioned, early studies suggested that the non-kinase and kinase domain played a role in Src's ability to transform cells. This was shown to be correct, as the structure of Src kinase was identified and the roles of individual regions of Src in various biological functions were characterized.

The structural domains of c-Src kinase have been well reviewed (5). Its structure can be described as having 4 domains (known as the Src-Homology domains, or SH1 to SH4) which are common to all Src family members, as well as a unique and tail domain (5). Each domain contains various elements critical to the regulation of kinase activity and/or its biological function (Figure 1.1).

1.2.1 Kinase Domain

The kinase domain, (or SH1) of human c-Src lies approximately between residues 263-524 and catalyses the transfer of the γ-phosphate from ATP to tyrosine residues in target proteins. The kinase activity of Src significantly contributes to the transforming activity of v-Src first identified in the Rous Sarcoma Virus.

Many studies had already demonstrated the functional importance of phosphorylation events in various biological processes (16,32). This was the basis for the investigation by Collett and Erikson into Src's potential enzymatic activity as a mechanism of its transforming activity (16). In an independent study by Levinson *et al*. (1978), immunoprecipitation of v-Src or temperature sensitive mutant v-Src (NY68) from infected chicken and hamster cells provided further evidence for the kinase activity of Src (17).

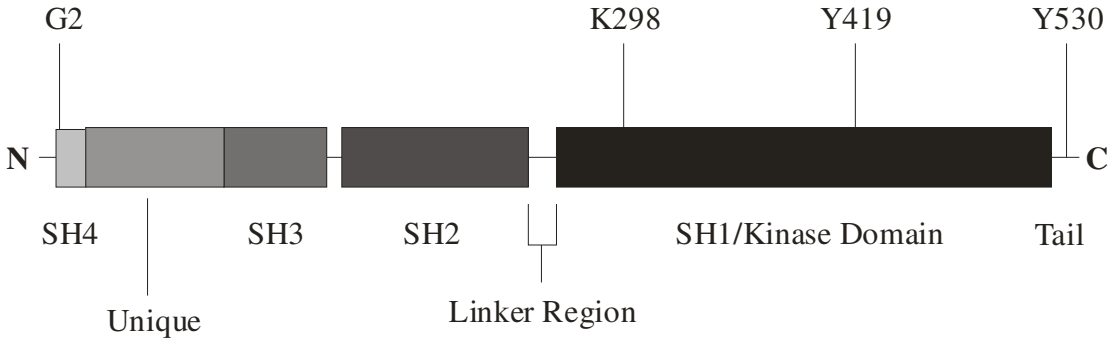


Figure 1.1. Schematic of human c-Src kinase, illustrating functional domains and key residues. Src homology (SH) domains 1-4. G2, glycine 2 residue site of myristoylation. Lysine 298 (K298), important for co-ordination of ATP. Tyrosine 419 (Y419), site of autophosphorylation, indicative of activation. Tyrosine 530 (Y530), critical for regulation of catalytic activity. N, amino-terminus. C, carboxyl-terminus.

Although the endogenous targets had not been identified (their experiments showed the phosphorylation of immunoglobulin molecules) these authors also suggested that the transforming potential of Src was related to its kinase activity.

As previously mentioned, the enzymatic activity of Src kinase was the earliest known tyrosine phosphorylation event (20). This discovery has been described as serendipitous (20). In their efforts to further characterize the transforming ability of polyoma virus, Eckhart *et al*. showed that immunoprecipitates from polyoma virus infected cells contain a tyrosine phosphorylating activity in the 60 KDa molecular weight range (33). Interestingly, the resolution of phosphortyrosine from phosphorthreonine by electrophoresis was a consequence of not preparing the proper buffer at pH 1.9 (20). After observing the phosphorylation of tyrosine residues associated with polyomavirus infected lysates, Hunter and Sefton (1980) proceeded to ask whether or not the protein product of *src* could also catalyse the phosphorylation of tyrosine (18). Indeed, their intuition led them to describe the first account of tyrosine phosphorylation by Src kinase on the immunoglobulin heavy chain (18). Interestingly, several studies soon described the interaction between the polyoma virus middle-T antigen and Src. These studies showed that it was the kinase activity of Src that phosphorylated the middle T antigen and that the association between the enhanced specific activity of Src (34,35).

Phosphorylation within the kinase domain of Src on tyrosine 419 may be important for the full activation of c-Src's kinase activity. Soon after the identification that Src kinase targeted tyrosine residues, it was also observed that both v-Src and c-Src were themselves phosphorylated (36). In this study the authors demonstrate that while *in vitro* tyrosine phosphorylation of v-Src and chicken c-Src targeted residue 416 (Y419 on human c-Src),

their assays revealed a different tyrosine phosphorylation profile (36). Only later was it revealed that v-Src lacked the negative regulatory carboxy terminal tyrosine found in c-Src (Y527, see below). Mutational analysis using v-SrcY416F has revealed lowered tumourigenic potential when cells transformed by v-Src or mutant v-SrcY416F were injected into mice even though the kinase activity is relatively unchanged (37-39). Additional studies on c-Src mutants Y416F and Y527F, and the phosphatase inhibitor orthovanadate show a similar pattern whereby phosphorylation of residue Y416 of c-Src can contribute to both the kinase activity and transforming potential (32,40,41). The data from several studies also strongly suggests that the phosphorylation at Y416 of c-Src is in *trans* (5,42,43). Taken together these data strongly suggest that the phosphorylation of chicken c-Src Y416 is required for full activation and that this occurs in *trans*.

It should be noted that this particular mode of regulation has been less extensively studied in humans. While many studies have utilized the phosphorylation state of Y419 of human c-Src as an indicator of activation (eg. (44)), Zhu *et al.* (2009) show that the higher specific activity of Src in several colon cell lines is more closely related to the phosphorylation state of Y530 and not Y419 (45). Interestingly, in a study by Sen *et al.* (2009) it was demonstrated using kinase assays that the phosphorylation of JAK (commonly used as an indicator of its activation) did not correlate with its activation (44). Thus, in any given system it is necessary to demonstrate the direct relationship between phospho-Y419 with Src kinase activation.

1.2.2 Tail

The tail region of human c-Src kinase is located at the carboxy terminus of the enzyme, including residues 525 to 536 (approx.) (5,46). The importance of this tail region was recognized early on in the characterization of *src* where Smart *et al*. (1981) observed that while v-Src and chicken c-Src were both phosphorylated in a similar manner *in vitro* (at tyrosine 416), their *in viv*o tyrosine phosphorylation sites differed (36). The subsequent characterization of the v-Src gene by Takeya and Hanafusa (1983) compared the amino acid sequence of carboxy terminal region of v-Src with c-Src and revealed the missing regulatory tyrosine found on c-Src and not v-Src, although it was not recognized as such at the time (23). In a study of c-Src by Courtneidge (1985), the phosphorylation state of Src kinase was shown to be important for its regulation (35). In this study Courtneidge utilized the polyoma middle T antigen and the phosphatase inhibitor orthovanadate in kinetic studies to confirm previous observations that Src kinase plays an important role in the transforming abilities of the polyoma virus, and that phosphorylation of Src played a negative regulatory role, unlike v-Src where phosphorylation of tyrosine was activating. This was the first description of tyrosine phosphorylation as a negative regulatory event. Elsewhere, in a detailed tryptic and chymotryptic two-dimentional phosphorpeptide analysis of chicken c-Src and v-Src, and in comparison with previous data, Cooper *et al*. (1986) demonstrated that the carboxy terminal phosphorylation occurred on Y527 (47). The authors correctly hypothesized that the increased kinase activity and transforming ability of v-Src is a result of a loss of this crucial tyrosine residue.

Many studies have demonstrated the importance of the carboxy terminal tyrosine (Y527 in chicken, Y530 in human) to enzymatic activation and cellular transformation

(32,48-50). In these studies, various strategies were utilized to measure the activity of Src kinase including *in vitro* and *in vivo* kinase assays, as well as focus forming and transformation assays performed on transfected or virally infected NIH 3T3 cells. The investigators utilized c-terminal specific antibodies, point mutations and Amber mutants to assess the contribution of various carboxy-terminal tyrosines. In the end, it was realized that the carboxy-terminal tail Y530 (Y527 in chicken) played a major role in the regulation of kinase activity.

1.2.3 SH2 Domain

The SH2 domain lies approximately between residues 150 and 248, and confers phospho-tyrosine specific protein interactions (46,51). In a study to further characterize the Fujinami Sarcoma Virus (FSV), mutational analysis of the transforming viral protein (v-Fps) product was performed (52). The authors identified a mutant with an amino-terminus insertion which did not hinder its tyrosine kinase activity yet was unable to transform rat-2 cells, as did wild-type v-Fps from FSV in the same cell type. In a subsequent study this mutant was further characterized and compared to all other known non-receptor tyrosine kinases (NRTK) at the time (51). The authors described this highly conserved region as the SH2 domain, which is noncatalytic but able to alter catalytic function through interaction with other cytoplasmic constituents (ie. through protein-protein interactions). The importance of the SH2 domain was further highlighted by the identification of a single residue deletion in the v-Src mutant v-*src*-L at F142, which limited its transformation potential to chicken cells (53). In another study characterizing the SH2 domain of the *v-fps* oncogene, it was proposed that a common function of SH2 regions is the interaction with

the kinase domains of the enzymes on which they are found, and, as a consequence, play an important role in regulating enzyme activity (51).

In addition to its role in mediating Src kinase-substrate interactions, the SH2 domain was further implicated in the regulation of Src kinase activity. Data from several studies suggested that the SH2 domain could interact with the tyrosine phosphorylated carboxy terminus of Src, and contribute to the regulation of its kinase acitivity. For example, an SH2 containing transforming viral protein, which does not possess kinase activity (p47gag-crk), was shown to bind to v-Src and binding was significantly decreased following treatment with potato acid phosphatase (54). Phosphorylated and non-phosphorylated peptides engineered to mimic the terminal 13 amino acids of c-Src were used in binding studies and showed that mutant chicken c-SrcY527F and v-Src bound to the phospho-peptide, but not when the SH2 domain was deleted (55). Superti-Furga *et al*. (1993) also utilized peptides in their analysis of the contribution of Csk (the major kinase which targets the c-terminal tyrosine,Y530 (see (5,56)) to the regulation of c-Src by the SH2 domain (56). Koch *et al*. (1992) utilized bacterially expressed v-Src SH2 peptides to pull down phosphortyrosine proteins from v-Src transformed rat cells, and showed that the SH2 domains from Ras and Crk also bind the same phosphorpeptides, demonstrating the importance of SH2 domains for protein-protein interaction (57). By introducing a variety of point mutations in c-Src, Hirai and Varmus revealed that even a single critical residue in the SH2 domain could activate the enzyme to a level similar to mutant SrcY527F (58). Crystal structures of Src SH2 and various peptides also demonstrated both the phosphortyrosine-SH2 interaction, and the Src SH2 possesses an optimal affinity for the sequence pYEEI rather than the pYQPG sequence found at Y527 of Src (59-61). This

seemingly unexpected result was clarified by Songyang *et al*. (1995) when the authors showed that the residues both amino- and carboxy- to the Y530 contributed to optimal binding (62). For human c-Src, the optimal peptide sequence was found to be AEEEIYGEFEAKKKK. Taken together, all of these data strongly suggested a model of regulation whereby tyrosine phosphorylation of Y527 (Y530 human Src) and interaction with the SH2 domain induces a conformation change which limits access to the kinase (or SH1) domain (5,48-50). This model was confirmed when the crystal structure of an inactive large Src fragment was described (63).

1.2.4 SH3 Domain

The SH3 domain lies approximately between residues 88 to 142 and provides important functionality to the Src protein (46,64,65). This region was shown to be conserved amongst many non-receptor tyrosine kinase, and was extensively studied for its similarity to the oncoprotein v-Crk and phosphorlipase C gamma (PLCγ) (66,67). Various studies showed that this Src homology 3 domain is also found in many other proteins and is important for protein-protein interactions (64,65,68). Deletion and some point mutations of specific residues in the Src SH3 domain were also found to be important to Src's kinase and transformation potential, having a negative regulatory effect (58). This study also demonstrated that the regulation of Src activity by its various functional domains is complex, and is one that encompasses not only the tail and SH2 domains, but also interaction between the SH3 domains and the linker region between the SH2 and the kinase (SH1) domain, a model confirmed by Xu *et al*. (1997) (63,65). Furthermore, although the SH3 domain was shown to bind to proline rich sequences (consensus sequence

XPXXPPPΨXP, (69)), the close association of the Src SH3 to the linker region which does not possess any proline residues emphasizes this complexity (63,69).

1.2.5 SH4 & Unique Domains

The amino acids at the extreme amino-terminus of c-Src have been described as the SH4 domain and are important for membrane localization (17,70). This membrane association is accomplished by the myristylation of Gly-2 during translation and is a permanent modification requiring, at minimum, the first seven residues, but optimally including several lysines within the first 10-15 residues (70-76). Unlike other Src family members, Src (and Blk) is not palmitylated, suggesting that it possess different cell/tissue specific functions (70). Membrane localization is also to non-plasma membrane sites including endosomes and the endoplasmic reticulum (5,77,78). Src has also been identified as possessing a peri-nuclear membrane localization and demonstrates a re-distribution throughout mitosis (79,80). Although all membrane bound Src protein is myristoylated, not all myristoylated Src is bound to membrane, as has been shown by the cytosolic and membrane localization of v-Src (17,73). A cytosolic localization for Src has also been described, but was attributed to its being in transit from its origin of synthesis to the plasma membrane, and/or in transit from the plasma membrane to the cytosol as part of a cycling process (5,79,81). Membrane localization is required for the transformation properties but not the kinase activity of v-Src in chicken cells, highlighting the importance of localization for Src's function (82).

Following the initial amino acids involved in myristylation and membrane localization is a stretch of approximately 80 residues know as the unique region. This

region is so named due to the observation that these residues do not share a consensus sequence across the Src family members. The function of this region has not been well defined, but it does contain a number of non-tyrosine phosphorylation targets. For example, early studies showed that both protein kinase C (PKC) and protein kinase A can phosphorylate chicken c-Src on Ser 12, 17 and 48 respectively (37,83-85). A study by Cross and Hanfusa suggests that S17 phosphorylation does not affect kinase activity or transformation potential (37). In addition, Shenoy *et al.* (1989) showed that Src is also phosphorylated at Thr 34, Thr 46 and Ser 72 by CDC2 and that this may contribute to changes in cellular architecture during mitosis (86). Overall, the precise role of Src phosphorylation within the unique region is not definitive but these data suggest that this event may contribute to cell cycle progression.

1.3 Src Kinase – Biological Function

The identification of Src kinase as the sole agent responsible for tumours in chickens induced by the ASV/RSV revealed its potential role in the regulation of cellular proliferation. Further characterization of *src* and its protein product revealed not only the complex nature of its regulation but also gave hints as to its potentially wide reaching biological roles through its various protein-protein interaction domains (SH2 & SH3). Furthermore, many Src targets have been identified including those that are directly phosphorylated by (or for which there is good evidence of) and those that are found in v-Src transformed cells (5,87).

Src kinase is a non-receptor tyrosine kinase that can mediate multiple pathways down-stream of receptors. A general model for Src function is illustrated in Figure 1.2. In

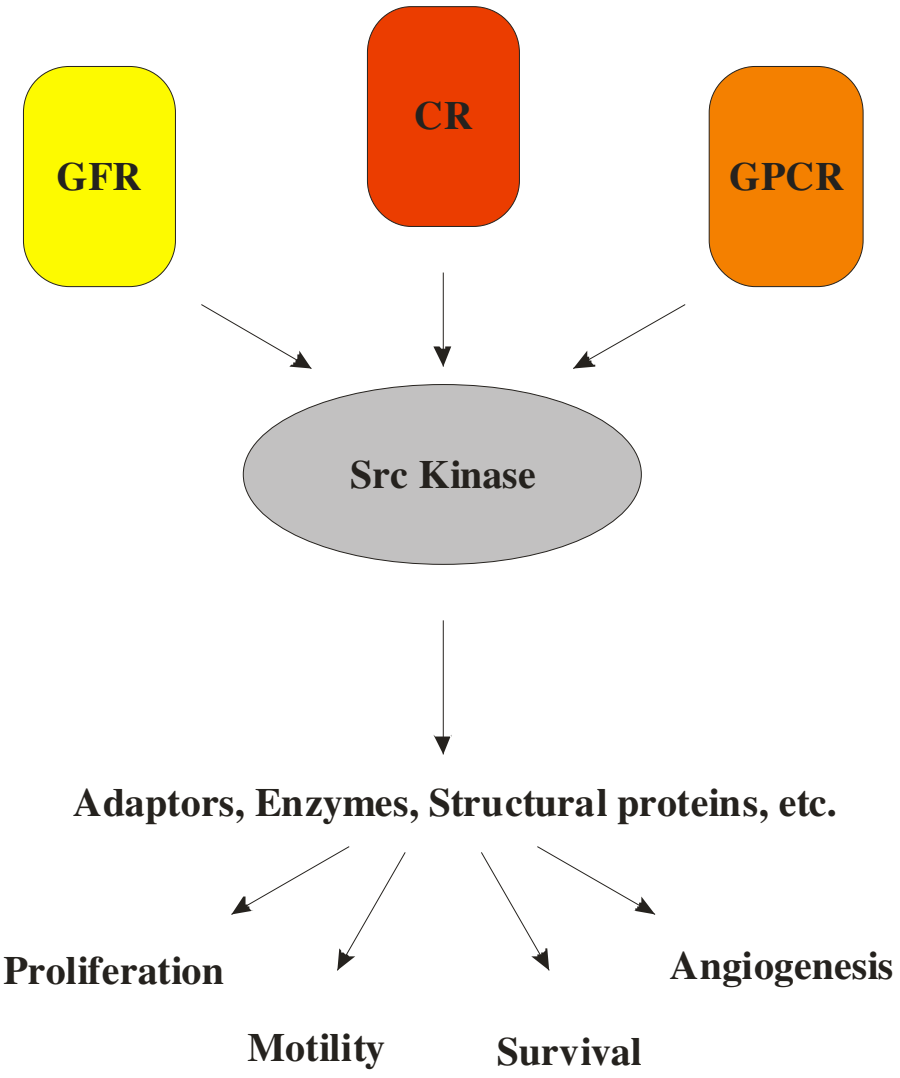


Figure 1.2. Schematic of Src Function. Simplified schematic of how Src kinase functions as a part of different signaling pathways that are initiated at growth factor receptors (GFR), cytokine receptors (CR) and G-protein coupled receptors (GPCR). Activated Src kinase interacts with and phosphorylates many intracellular proteins (enzymes and adaptor molecules) leading to downstream activation of additional signaling intermediates and ultimately a change in transcriptional activation. The cellular effects of Src activity are wide ranging and include proliferation, motility, survival and angiogenesis.

this model, ligand activation of receptors recruits Src kinase and activates its activity. This in turn results in the phosphorylation and activation of other proteins or enzymes ultimately resulting in the regulation of various biological functions including proliferation, motility, survival and angiogenesis.

Src kinase interacts with and is activated by several growth factor receptors and cytokine receptors (5,87). For example, stimulation of the platelet derived growth factor receptor (PDGFR) results in the phosphorylation and concomitant activation of Src (88). Upon the identification and initial characterization of the Src protein, it was clear that the SH2 domain, common in many other non-receptor tyrosin kinases, allowed it to interact with the phosphorylated tyrosines of activated growth factor receptors (89). Some of these receptors include the epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR) and colony stimulating factor-1 receptor (87,89,90). Because it is known that growth factor receptors initiate many biological responses including growth and differentiation, their interaction and activation of Src kinase strongly suggests an important contribution to these processes (91,92). This was demonstrated using antibodies against Src by several studies where Src kinase was shown to be important for mitogenic signaling and DNA synthesis induced by PDGF (93,94).

Src kinase is also important for mediating the effects of G-protein coupled receptors (GPCRs) (87,95). For example, Src was shown to mediate the activation of MAPK (mitogen activated protein kinase) by the $G_{\beta\gamma}$ subunit of G-proteins upon the stimulation of cells with LPA (lysophosphatidic acid, a potent stimulator of mitogenesis in fibroblasts) (96). Activation by GPCRs may occur by indirect or direct association with the

receptor, and may also involve cross talk with other receptor types such as growth factor receptors. For example, the purinergic receptor $P2Y_2R$, is a GPCR contains several SH3 domains which facilitate its interaction directly with Src kinase. When stimulated by UTP or ATP (uridine-, or adenosine-5'-triphosphate), the $P2Y_2R$ induced Src kinase activation, which was followed by phosphorylation and activation of EGFR in human astrocytoma cells (97). Activation of the EGFR seemed to require the formation of a large multiprotein complex, as measured by co-localization using immunofluorescence (97). Several other GPCRs have been linked to Src, including the lysophosphatidic acid (LPA) and the β2-Adrenergic (β_2AR) receptors (87,95). Since GPCRs have been implicated in a variety of biological functions, including growth, proliferation and differentiation, Src is also an important player in these processes (98).

Src has also been implicated in the down-stream signaling of other receptors and channels (87). The predominant Src family members coupled to cytokines are Fyn, Lck and Hck but Src is activated by or contributes to downstream signaling of the erythropoietin and IL-11 receptors (87,99-101). Activation of Src-specific activity in PC12 cells and human keratinocytes has been observed upon activation of Ca^{2+} channels, thus implicating Src in neurite extension and keratinocye differentiation (102,103). Src is also associated with the NMDA receptor and can affect its gating, a function that involves the unique region of Src (104,105).

As can be seen in the simplified model of Src kinase function (Figure 1.2), ligand activation of receptors activates Src directly and indirectly. This leads to the down-stream interaction and phosphorylation of other signaling molecules. A broad range of intracellular proteins have been shown to be direct targets of Src, or at least phosphorylated as a

consequence of Src activity. The large majority of these targets have been identified by comparing their phosphorylation state from untransformed cells with their phosphorylation state in cells transformed by RSV, and has been well reviewed (5,87). Many of these targets were identified in complexes with the growth factors described above. Some of the enzymatic targets include focal-adhesion kinase (FAK), PLCγ and PI3K (106-108). Some cytoskeletal targets include tensin, vinculin and talin (109-111). Importantly, Src also targets adaptor proteins and other proteins such as RasGAP and β-catenin (112-116). The extensive list of 'targets' reveals the diverse roles which Src plays not only simply in signaling, but also various biological processes.

An example of how certain phosphorylation targets of Src implicate the enzyme in the context of a biological process, is its phosphorylation of FAK (focal adhesion kinase) and regulation of the formation of focal adhesions (87,113,116-118). Focal adhesions are regions of the cellular membrane at which integrin receptors mediate the interaction between intracellular cytoskeletal elements (such as actin, vinculin, tensin and talin) and the extracellular matrix (ECM). In a simplified model of signaling at focal adhesions, the engagement of integrins induces FAK phosphorylation, which is followed by complex formation and the activation of Src. Src can then phosphorylate multiple sites on FAK, and together as a complex (FAK-Src) target other adaptor proteins (which contain SH2 and SH3 domains) for phosphorylation. Some evidence suggests that integrins can directly activate Src without FAK (119). In either case, the cycle of formation and disruption of focal adhesions is an important aspect of cell adhesion and motility and cell morphology, and the ability of Src to phosphorylate and regulate FAK implicates Src in these functions

(120-122). Therefore, many Src targets are involved in biological processes that, taken together, also implicate Src in various aspects of cancer.

The activation of Src and its subsequent activation at focal adhesions exemplifies the principal mechanism by which Src influences different signaling pathways involved in different biological processes. As described above, Src activity at focal adhesions results in phosphorylation of a host of adaptor molecules which in-turn promotes the disassembly and turnover of cytoskeletal-focal adhesion complexes. This mechanism is similar to its effects on catenins whereby Src phosphorylation of these proteins destabilizes adherens junctions, which are mediated by E-cadherin (see Figures 1.3 & section 3.4).

Another mechanism by which Src affects signaling pathways is exemplified by its activation of STAT3. STATs (signal transducers and activators of transcription) are a family of transcription factors that homo- or heterodimerize upon JAK tyrosine kinase phosphorylation in response to interferon stimulation of cytokine receptors (reviewed in (123)). STAT3 has been shown to interact with, and is phosphorylated by, Src where it then translocates into the nucleus and activates transcription (124-126). Interestingly, in a mechanism that involves STAT3, Src has been shown to increase the transcription of VEGF (vascular endothelial growth factor), an important peptide tha promotes proliferation, migration and cell survival (127).

Src kinase can also mediate intracellular signaling through its phosphorylation of the adaptor molecule Shc (Src homology and collagen). Upon the identification of the tyrosine phosphorylation of Shc in v-Src transformed cells, further investigation demonstrated the importance of this event for downstream signaling of EGFR (epidermal growth factor receptor), FGFR-1 (fibroblast growth factor receptor 1) and c-kit receptors

(a.k.a. stem cell factor receptor) leading to the formation of Grb2-Sos-Shc complexes and subsequent activation of MAP kinase (128-131). This pathway plays an important role in proliferation. Taken together, these studies demonstrate the important role Src plays in mediating signal transduction from a diverse range of receptors. The ability of Src to transduce signaling through the activation of a large variety of signaling intermediates, ranging from enzymes and adaptor proteins to transcription factors, which regulate a large variety of cellular biological functions including proliferation, migration, cell survival and angiogenesis, further emphasizes the important role Src plays in cellular function (see Figure 1.2).

Given Src's contribution to a diverse variety of cellular functions, the results of knockout mouse models were somewhat surprising. When $Src^{-/+}$ heterozygotes were bred, Soriano *et al*. (1991) found that the $Src^{-/-}$ mice were viable at birth but grew slower and were 30-50% lower weight than control littermates (132). The most noticeable defect in these mice was the absence of incisors, described as failing to erupt, and survival could be prolonged depending on the feeding protocol. The only other abnormalities occurred in the bones and teeth, which were described as classical examples of osteopetrosis. It was known that the cause of osteopetrosis was a decrease in osteoclast function, and the authors suggested that the observed osteopetrosis in $Src^{-/-}$ mice was casued by a deficient microenvironment, or defective osteocalst function, since osteoclasts were still present. In a subsequent study by Boyce *et al*. (1992), it was demonstrated that the osteoclasts from Src-/- mice do not form ruffled borders and therefore do not resorb bone normally (133). For an enzyme that could potentially regulate so many biological processes it was surprising that the knockout phenotype seemed relatively inert. It was hypothesized that due to the

presence of other Src family kinases the absence or non-function of a single member could be tolerated. This hypothesis was validated when double knockout mice (for *src* and *yes* or *src* and *fyn*) and triple knockout mice (*src*, *yes* and *fyn*) were bred and shown to die shortly after birth (3 weeks) or be embryonic lethal (E9.5), respectively (132,134,135).

1.4 Src & Cancer

The contribution and implication of Src kinase to tumourigenesis is well documented and has been a defining characteristic of the enzyme since its first description. Src phosphorylates and regulates many targets that are also involved in many aspects of cancer (see above). The role of Src in cancer has been predominantly studied in colon and breast cancers. For example, Bolen *et al*. (1987) assessed the kinase activity from numerous tumour cell lines, and compared Src kinase activity from colon tumour-derived tissue with that of adjacent normal mucosa and found that the elevated activity was due to eleveated specific activity and not increase levels of Src protein (136).

In colon cancers, the contribution of Src appears to be at later stages. For example, in a study by Talamonti *et al*. (1993) the neoplasias from a number patients were screened for Src activation and protein levels, as measured by auto- or enolase phosphorylation (137). Compared to normal adjacent mucosa, small polyps did not show an increase in either kinase activity or protein levels. However, in larger benign villous adenomas, Src activity showed a significant increase, but not protein levels. In patients with primary colonic tumours, Src kinase activity was found to be significantly increased with a moderate increase in Src protein levels. Importantly, the largest changes in both kinase activity and protein levels were oberserved in samples from liver metastases when primary

and metastatic samples obtained from the same patients were compared. Other studies have also highlighted the important correlation between increased specific activity and elevated protein levels of Src (138-140). There is some evidence, however, that Src may also contribute to the early stages of colon cancer progression, where a smaller patient group was screened for Src specific activity and protein levels from adenomatous and metastatic tissue (141). Using an antibody targeting activated Src, Sakai *et al*. (1998) screened various adenomas and other colon tumour samples and concluded that Src activation is an early event in colon carcinogenesis (142). Nonetheless, there is clear evidence that Src can contribute to all stages of the malignant process and has been shown to be an independent poor clinical prognosticator (143).

Src kinase has also been shown to contribute to breast cancer. In an early study of its involvement in 21 different sarcomas and mammary carcinomas, approximately 30% of the samples exhibited increased Src kinase activity compared to normal tissues and fibroblasts (144). In a screen comparing tissue samples from human breast tumours with normal breast tissue, Src kinase activity was increased between 4 and 20 fold, with a concomitant increase in Src protein levels (145). Biscardi *et al*. (1998) also showed demonstrated an association between HER-1 and Src kinase in the majority of human breast tumour cell lines, and that some of these cell lines displayed increased tumourigenicity in mice (146). In yet another study using fibroblasts engineered to overexpress an EGFR-HER-2 chimera, Src anti-sense RNA was able to induce growth arrest, apoptosis and reduce colony formation in soft agar, an indicator of transformation (147). These studies are important since the overexpression of HER-2/Neu (or Erb-2) is an important predictor of survival and time to relapse in breast cancer patients (148). It has

also been shown using a human breast cancer cell line transplanted into an animal model, that increased Src activity is correlated with bone metastasis (149).

Src and Src family kinases have been implicated in other cancers. These include, melanomas, ovarian, head and neck, bladder, lung, brain and neuroendocrine system, and blood. Interestingly, in human cancers there is little evidence of activating mutations of Src similar to those found in v-Src even while an increase in Src activity has been shown in many tumour cell lines (27,150,151). There is evidence for increased transcriptional regulation of Src, consistent with the previous data showing elevated levels in various colon tumour samples and cell lines (152), but the observed increase in specific activity is, in most cases, a consequence of altered regulatory mechanisms including activation by upstream receptor tyrosine kinases and integrins, or other regulatory molecues such as Csk (5,27,151). Upon increased activation, Src is able to promote the various aspects of tumour biology, including proliferation, motility and angiogenesis as outlined above.

Given its contribution to cancer, it is not a surprise that many pharmacological inhibitors of Src are being developed or have been approved to treat various types of cancer. For example, dasatinib is a compound that has been shown to inhibit several tyrosine kinases including the PDGFR, Bcr-Abl as well as Src. It has been approved for use by the USDA, and has been used to treat patients with chronic myelogenous leukemia and acute lymphoblastic leukemia (153). Another inhibitor SKI-606 (or Bosutinib), has been shown in a breast cancer cell line, to reduce proliferation, migration and invasion. In animal studies, this compound was also effective in blocking metastasis, proliferation and angiogenesis (154). Therefore, the development of new therapeutics that target Src (and

other tyrosine kinases) will enable clinicians to more effectively treat patients with cancers where Src is a contributing factor.

1.5 Wnt Signalling - Biological Relevance

Another important topic of research in cancer biology is the Wnt signaling pathway. The name 'Wnt' is an amalgamation of the names for *wingless* and *int-1*, genes first discovered independently in *Drosophila* studies and mouse studies, respectively, and then found to be the same gene (155-157). Wnt ligands are a group of extracellular glycoproteins found in a diverse group of organisms from sea anemonae to humans, and are important throughout development. They are characterized by a conserved sequence of 21 regularly spaced cystein residues and are secreted in a highly regulated manner, regulating processes as diverse as embryonic axis development and patterning, and proliferation in adult tissues. For example, in *Drosophila* besides playing an important role in wing and haltere development, mutations of different canonical Wnt genes results in segment polarity defects (158-160). In *C. elegans*, Wnt proteins contribute to establishing embryonic polarity through the promotion of asymmetric cell division (161-164). In mouse and chicken models Wnt-7A has been shown to contribute to limb development through regulation of dorsoventral and anteroposterior axis patterning (165,166). In *Xenopus* model systems, β-catenin is crucial for primary axis specification through its dorsalizing effects (167). In mouse models, another Wnt signaling component called Axin also been shown to play an important role in primary axis formation (168). Wnt signaling promotes proliferation of human crypt cells and in murine neurodevelopment (169). For example, Wnt1 or β-catenin knockout mice show similar losses of midbrain and other severe brain

malformations (170,171). In a study by Kalani *et al*. (2008) Wnt signaling was shown to directly influence neural stem cell renewal (171,172). Taken together these data demonstrate the importance of Wnt signaling throughout development, and it is therefore not a surprise that mutations of various signaling components are found in various abnormal and disease conditions such as cancer.

The current model of the canonical or Wnt/β-catenin signaling pathway can be understood through the key mediator protein β-catenin, the mammalian homologue of the *Drosophila* armadillo protein. β-Catenin is approximately 780 a.a. in length. The overall structure of the protein consists of three domains. The amino terminus consists of several Ser and Thr residues important for its regulation (see pg 30), and mutations in this region have been found in various cancers. The central regions consists of twelve 42 amino acid imperfect repeats that mediate protein-proteinn interaction (173,174). The carboxyl terminus contains the transactivation domain, reponsible for recruiting transcription factors (175). Initially, β-catenin was identified as a structural component within the cell, where it mediates the interaction between the cytoskeleton via α-catenin and the intracellular domain of E-cadherin (176,177). As a complex with other catenin molecules, such as plakoglobin and p120-catenin, α- and β-catenin are important intracellular components of adherens junctions, which mediate intercellular interactions. Subsequently, β-catenin was also shown to mediate Wnt signaling. In a *Drosophila* genetic epistasis experiment looking for mediators of *wingless* (wg or Wnt-1), both *dishevelled* and *armadillo* genes were shown to act downstream (178). In both *Xenopus* and zebrafish model systems, overexpression of β-catenin by RNA injection can induce a secondary body axis in a manner similar to the

injection of embryos with Wnt-1 or Wnt-8 (179-181). Several other studies have also shown that β-catenin co-localizes and interacts with Tcf-3 and Lef-1 in the nucleus in a *Xenopus* and mammalian cell culture model, respectively (182-184). Therefore, the translocation of β-catenin to the nucleus, and its subsequent interaction with Tcf/Lef family proteins is the mechanism by which Wnt signaling activates transcription (Figure 1.3).

When the canonical Wnt pathway is inactive, β-catenin can be found at the inner region of the plasma membrane where it mediates the interaction between the intracellular domain of E-cadherin complexes and the cytoskeleton via α-catenin. It is the central 550 a.a. region of β-catenin which mediates this and other protein-protein interactions (see above). Ubiquitin mediated degradation of β-catenin is also active when there is no Wnt activation and requires at minimum an interaction of the arm repeat/central domain with glycogen-synthase kinase 3-β (GSK3β), casein kinase 1-α (CK1α), Axin and the adenomatous polyposis coli protein (APC). Formation of a complex between these proteins and β-catenin induces the sequential phosphorylation of residues Ser-45 by CK1α (priming phosphorylation) and S33, S37 and T41 by GSK3β (185-187). This targets β-catenin for degradation mediated by β-TrCP, an E3 ubiquitin ligase/F-box protein. When Wnt signaling is activated through the interaction of Wnt protein ligands with Frizzled (Fz) family recpetors and LRP5/6 co-receptors, formation of the degradation complex is inhibited by the interaction of Disheveled (DVL) with Axin, and phosphorylation by CK1α and GSK3β does not take place (188-190). This results in stabilization of cytosolic β-catenin levels. β-catenin is then free to enter the nucleus where it interacts with the Tcf/Lef family of DNA binding proteins. Interaction between β-catenin and Tcf/Lef proteins

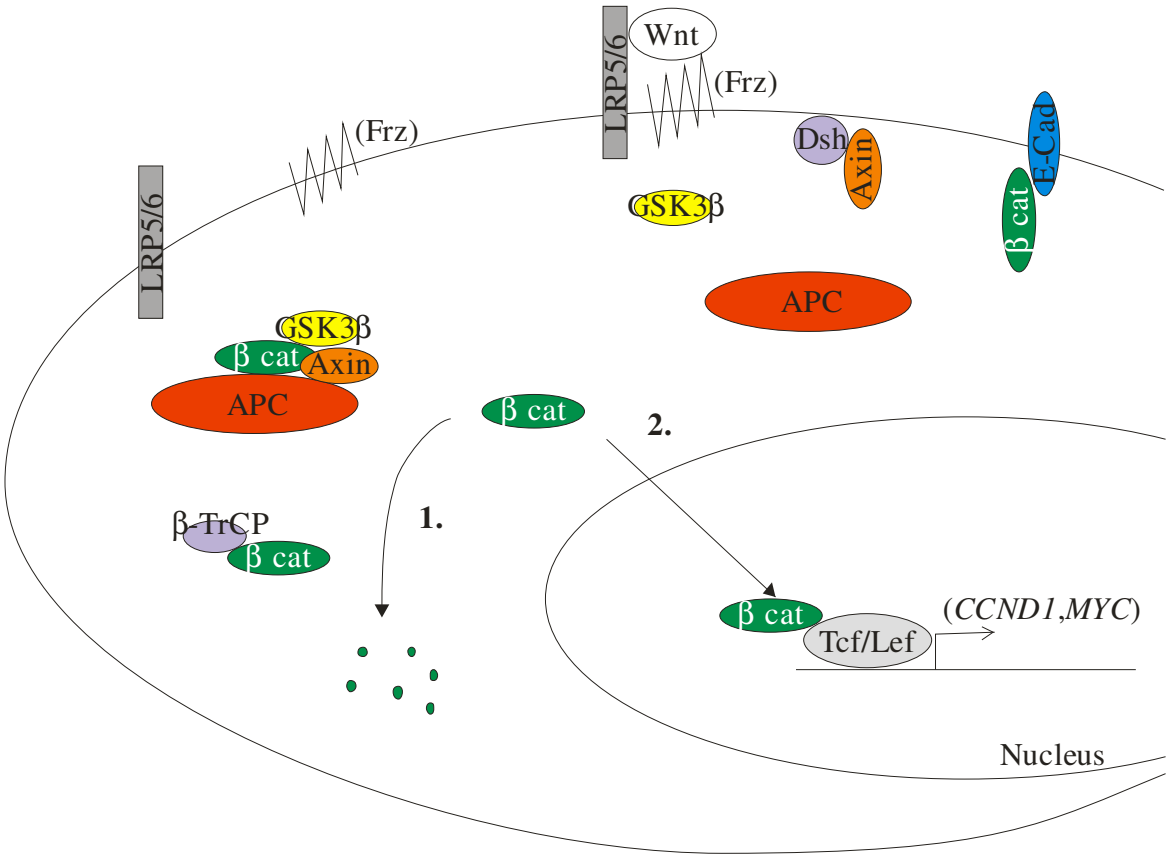


Figure 1.3. The Wnt/β-catenin signaling pathway. β-catenin (**β-cat**) is a structural component of adherens junctions where it bridges the cytoplasmic tail of E-cadherin (**E-Cad**) to the cytoskeleton via α-catenin (not shown). β-catenin also plays an essential role as the principal transducer of this particular Wnt pathway. Without Wnt ligand (**1**.) engagement, β-catenin is sequentially phosphorylated first by CK1α (not indicated) and then by **GSK3**β which requires the formation of a multi-protein complex including **APC** and **AXIN**. β-catenin is then targeted for ubiquitin mediated proteasome degradation via **β-TrCP**. Upon the engagement of certain Wnt molecules (eg.Wnt-1, Wnt-8) with certain Frizzled (**Frz**) family receptors and the **LRP5/6** co-receptors cytosolic levels of β-catenin are stabilized through a mechanism that involves Dishevelled (**Dsh**) and an inhibition of the multi-complex formation (**2.**). β-catenin can then enter the nucleus and interact with the **Tcf/Lef** family of co-transcription factors and promote transcription of various genes, including the cyclin D1 (***CCND1***) and myc (***MYC***) genes.

promotes transcriptional activation through the carboxy terminal transactivation domain of β-catenin. Formation of this transcriptional complex requires BCL9/Legless and Pygopus (191-193).

As a principal pathway intermediate, β-catenin is an important target for regulation. Besides its interaction with APC and the other degradation complex components, various regulatory proteins have been shown to target β-catenin as a mechanism of pathway regulation. For example, the tumour suppressors p53 and WTX (the gene product mutated in 30% of patients with Wilms' tumour) have both been shown to downregulate β-catenin levels via the proteasome degradation pathway (194,195). The MAP kinase-related Nemo-like kinase (NLK) also downregulates Wnt/β-catenin signaling by phosphorylating Lef/TCF proteins and preventing their interaction with β-catenin (196,197). Furthermore, the POZ motif containing protein Kaiso has been shown to compete with β-catenin for interation with Tcf/Lef DNA binding proteins and Wnt target gene promotor regions to antagonize Wnt/β-catenin signaling (198,199). In a recent study using a combinatorial screening strategy, BTK (Bruton's tyrosine kinase) was shown to negatively regulate canonical Wnt signaling by phosphorylation and the stabilization of a component of the transcriptional machinery recruited by β-catenin (200). These examples are only a small sample of the many studies which have demonstrated that β-catenin is a major target of canonical Wnt pathway regulation. Similarly, many studies have shown that the canonical Wnt pathway can be regulated at multiple levels and is well reviewed elsewhere (176,201-203).

1.6 Wnt & Cancer

Wnt signaling plays an important role in human cancers, and many components of the pathway have been identified as either oncogenes or tumour suppressors. The earliest identified oncogene component of the pathway was Wnt-1. The genes were identified independently. In *Drosophila* Wg was first observed to affect wing and haltere development, and in mouse it was identified as the preferred integration site of the mouse mammary tumour virus (MMTV) (155,156,204-207). Although not normally expressed in mammary tissue, *Wnt1* transgenic mice (MMTV-*Wnt-1*) have demonstrated its potential contribution to cancer. In this particular mouse model, tissue specific exogenous expression of Wnt1 in the mammary gland was accomplished by incorporating the MMTV-LTR (mouse mammary tumor virus-Long terminal repeat region) into the Wnt1 transgene (208). Mice (both males and females) that harbour this transgene exhibit grossly hyperplastic mammary glands, which lead to the development of mammary adenocarcinomas and eventual death by 1 year (208).

Another mouse model that demonstrates canonical Wnt signaling in cancer is the Min (for multiple intestinal neoplasia) mouse model. This mouse lineage was identified in a study which sought to identify germline mutations that result in a susceptibility to tumor formation (209). These mice were first identified through a heritable (autosomal dominant) circling behaviour. Further observation of these mice revealed that they developed anemia secondary to the growth of intestinal polyps in both the small and large intestines. Analysis of theses tumors showed that they were predominantly benign adenomas, with some localized invasion and small areas of carcinoma *in situ*, but no metastasis. In a subsequent study, the Min mutation was identified as a nonsense mutation in the *APC* gene (210).

Previous to this, other studies already showed that the *APC* gene was mutated in sporadic colon cancers and in patients with familial adenomatous polyposis (FAP), which was known to be an autosomal dominant heritable condition predisposing patients to colorectal cancer (211-213). Although the Wnt signaling pathway had not been completely characterized, the demonstration that APC interacted with β-catenin was crucial to understanding how APC could contribute to colorectal cancers (214,215). These studies suggested that the interaction between APC with β-catenin plays an important role in tumourigenesis since the observed interaction was defective in several colon cancer cell lines containing mutant APC protein, including SW480, HCT116 and DLD-1 cell lines. Importantly, subsequent to these studies it was shown that expression of wild-type APC in SW480 colon cancer cells (which lack functional APC) leads to the downregulation of protein levels of β-catenin, by enhancing the rate of degradation (216).

As mentioned above, the involvement of the APC in cancer had already been established, and has been shown to be mutated in various types of cancer but especially in the context of Familial Adenomatous Polyposis (FAP) and Hereditary Nonpolyposis Colorectal Cancer (HNPCC) (217). The gene for APC was isolated from patients with FAP, and from those with sporadic colorectal carcinoma, revealing various truncation mutations and a requirement for mutations in both alleles as a prerequisite for the development of ademato us polyposis (211,218,219). In a study by Powell *et al* (1993), 51 out of 62 patients with FAP were found to possess a truncated APC protein (220). Of the remaining 11 FAP patients, 3 possessed significantly lower expression levels of one APC gene. Overall, this represents 87% of the FAP patients as possessing a germline mutation in the APC gene. Interestingly, a study by Ishhi et al. found that 96% of the APC mutations were truncating

mutations in patients with FAP (221). Patients with hereditary nonpolyposis colorectal cancer HNPCC also show a high incidence of mutation in the APC gene. For example, various studies have shown that between 39% (12 out of 31) and 58% (11 out of 19) of tumours from patients with HNPCC harbour a putation in APC (222,223). In sporadic cancers, a study by Jen *et al*. showed that 10 out of 12 adenomas from different patients possessed a mutation in the APC gene, while a study by Smith *et al*. (1993) showed that 24 out of 32 cell lines tested (22 of which were derived from sporadic carcinomas) containted a truncated APC protein (224,225). Taken together, these studies further demonstrate the important contribution that APC makes to colon cancer.

Activating mutations of β-catenin have also been identified in various cancers and mutations were predominantly localized to the amino-terminal phosphorylation domain, important for negative regulation by GSK3β (214,215,226). It has been estimated that mutations in β-catenin are found in approximately 1% of sporadic colorectral cancers, and may play a more important role in contributing to HNPCC (227). In particular, mutations in β-catenin appear to be common to endometrioid ovarian cancers (227). The identification of Bcl9 (B cell lymphoma 9 gene product) as a positive regulator of the Wnt pathway (where it acts both as an adaptor and transcriptional coactivator through its interaction with β-catenin) also suggested a possible role in B cell lymphomas (191). A comprehensive list of β-catenin mutations and the cancers they were derived from has been compiled from the literature and reveals a diverse list that includes colorectal, hepatocellular, melanoma and prostatic cancers (228).

Other Wnt signaling components described as tumour-suppressors include Axin 1 and Axin 2. Both of these proteins have been shown to down-regulate Wnt signaling, and importantly, have been shown to be mutated in medulloblastomas in the case of Axin1, and predispose patients with familial tooth agenesis to colorectal cancer (229,230). Finally, Tcf1 has also been shown to contribute to the presence of adenomas in the gut and mammary gland of mice that are mutant for its gene (231). In this study, it was shown that not only was Tcf1 a target of Wnt signaling, but that when mutant APC alleles were introduced into $Tcf^{-/-}$ mice, an even greater number of adenomas were observed. Therefore, given its extensive contribution throughout development, it is not suprising that defective unregulated Wnt signaling is a major contributor to multiple types of cancer.

1.7 Src Kinase & Wnt Signaling

The key modulator of the canonical Wnt pathway, β-catenin, is a target of Src kinase. Early studies identified a host of tyrosine phosphorylated proteins interacting with the intracellular domain of E-cadherin in v-Src transformed cells (114-116). Src has also been shown to interact with and phosphorylate β-catenin on tyrosine residues 86 and 654 (232). The findings from these studies suggest that Src may play a role in destabilizing cell-cell interaction in fibroblasts, although the work by Reynolds *et al*. (1994) using v-Src transformed Madin-Darby canine kidney cell line did not come to the same conclusion (116).

The presence of β-catenin in the cell has been described in the context of two pools, one in association with E-cadherin at adhesion junctions and a second free, or cytoplasmic, pool that is degraded when Wnt signaling is not activated (233). In that study, the author

assessed the role of v-Src on these two pools. The author's findings in mouse fibroblasts suggested that although tyrosine phosphorylation of β-catenin did occur, there was no detectable change in the free-pool level of β-catenin or in its interaction with E-cadherin (233). In contrast to these results, Haraguchi *et al*. (2004) found that v-Src did promote Wnt signaling when cells were transfected with a stabilizing mutant of β-catenin, as measured by a luciferase based Wnt reporter system (234). Another study utilizing constitutively activated Src in fibroblasts showed that Src upregulated the Wnt target gene *CCND1* (cyclin D1) in a mechanism that increased nuclear accumulation and transcriptional upregulation of β-catenin (235). Their data suggested a mechanism that upregulates translation through Ras/Raf/ERK mediated phosphorylation of eukaryotic initiation factor 4E (EIF-4E) and through PI3K/AKT phosphorylation of its inhibitor 4E-BP1 (235). Furthermore, a study by Rivat *et al*. (2003) demonstrate a link between Src Kinase and Wnt signaling by presenting data to suggest that Src promotes invasion through the upregulation of matrilysin by promoting the interaction between LEF-1 and AP-1 at the matrilysin promotor in a β-catenin independent mechanism (236).

There is evidence that the Src kinase and Wnt signaling pathway interact in other organisms. For example, in *C. elegans* one of two Src homologues, SRC-1, together with another tyrosine kinase required for asymmetric division (MES-1), work in conjunction with Wnt signaling to specify cell fate of the EMS cell after the 3rd division (237). In this process Wnt and SRC-1/MES-1 activities are required for complete rotation of the centrosome-nuclear complex, which determines the axis of cell division resulting in endoderm and mesoderm specification. Data in support of this can be seen when complete

mutant phenotypes occur (embryos lacking endoderm) only in embryos mutant for both Wnt components and *src-1* or *mes-1*, whereas partial mutant phenotypes were observed for single mutants. Recently, evidence by Zhang *et al*. (2008) suggest that the molecular motor component dynactin was a possible mediator of the Wnt and Src effect on spindle orientation in EMS (238). Interestingly, in *Drosophila* the homologous Src proteins Src64B and Src42A were found to interact with and phosphorylate Derailed/RYK receptors (239). This study suggests that the *Drosophila* Src homologs act downstream of RYK/Derailed receptors upon Wnt5 activation which does not require Tcf/Lef-mediated transcription in effecting embryonic development of the central nervous system (239). Therefore, these data suggest that Src may interact with the Wnt signaling pathway in other organisms.

The purpose of this investigation was to elucidate the possible cross-talk between Src kinase and canonical Wnt signaling in the context of cancer. Both Src kinase and Wnt signaling contribute to cancers of the colon and breast. Both Src kinase and Wnt signaling can upregulate transcription of common genes that are also important to the progression of cancer such as Myc and cyclin D1 (240,241). Based on these observations, and the results of the studies described above, I hypothesised that Src may promote canonical Wnt signaling which inturn contributes to cancer.

CHAPTER 2: MATERIALS & METHODS

2.1 Cells and Conditions

Normal chicken embryo fibroblasts (CEF) were obtained from liquid nitrogen frozen stocks, prepared in our laboratory as previously described from 10-day embryos (242). Cells were thawed in normal CEF media (Dulbeco's Modified Eagles Medium (DMEM) supplemented with 10% tryptose phosphate broth, 0.6% calf serum, 1 X penicillin-streptomycin (pen-strep)), and passaged 1-2 X after reaching 90% confluency in 100 mm tissue culture plates.

Schmidt-Ruppin A strain v-Src transformed chicken embryo fibroblasts (SRA) were made from normal CEF cells infected with wild-type (wt) Schmidt-Ruppin A strain virus originally obtained from H. Hanafusa through W.S. Robinson and H. Robinson. To ensure that SRA cells remained fully transformed and did not 'revert' to a less transformed phenotype over time, the cells were periodically inspected for qualitative measures including a rounded morphology, rapid rate of growth as measured by time taken to reach confluency and a high metabolic rate as assessed by acidification of the culture media. When the cells did not appear to meet these criteria fresh normal CEFs were added to transformed cells. SRA cells were maintained in DMEM supplemented with 10% tryptose phosphate broth, 5% newborn calf serum (NBCS), 0.6% DMSO and 1 x pen-strep (NTF media).

tsLA29Rat-1 fibroblasts were obtained from Dr. John A. Wyke and maintained at the permissive temperature (35^0C) in NTF media. When cells were shifted to non-permissive temperatures (39.5-40^0C), the media contained 0.2% NBCS instead of 5% in

NTF media as previously described (243). Before cells were shifted to either permissive or non-permissive temperatures, cells were washed 2 x in PBS and 1 x in NTF media supplemented with 5% or 0.2% NBCS depending on the required conditions. When cells were shifted to non-permissive conditions, this was done using Forma Scientific Water-Jacketed incubators or continuous airflow incubators when cells were utilized in pulse-chase experiments (see below).

SW480 and **DLD-1** cells were originally purchased from ATCC and maintained in DMEM supplemented with 10 % fetal bovine serum (GIBCO) and 1% penicillin-streptomycin. Rat-1 cells were obtained from the Cell Bank, SACRI Antibody Services at the University of Calgary and maintained in the same media. Similarly, the following cells lines were maintained in DMEM supplemented with 10% FCS (Fetal Calf Serum) and 1X pen-strep: SK-BR-3, BT483, Hs 578T, MDA-MB435s and MDA-MB468. MCF10A cells were maintained in 1:1 DMEM and HAM's F-12 medium, supplemented with 5% Horse Serum, 1 X pen-strep, 0. 5 μg/ml cortisol, 0.02 μg/ml EGF (Epidermal Growth Factor), 0.1 μg/ml cholera enterotoxin, 0.01 mg/ml insulin and 2 mM L-Glutamine.

All cells were normally maintained in standard tissue culture incubators kept at 37^0C and at a CO_2 concentration of 5%.

2.2 Microscopy and Indirect Immunofluorescence

Cells designated for microscopic analysis were seeded at approximately 80% confluency on sterile glass coverslips placed in 35 mm tissue culture dishes in complete medium and incubated overnight. Coverslips were then washed 2X, 5-10 minutes each wash, with PBS, followed by incubation in filtered 3.7% paraformaldehyde fixing solution

(made fresh or pre-made and frozen at -20^{0}C for no more than a week) for 20 minutes on a Bellydancer shaker. After fixing, coverslips/cells were washed 3X, 5-10 minutes each wash, with PBS, before permeabilization with a 0.1% NP-40 or 0.5% Triton X-100 (diluted in PBS) solution for 5 minutes at room temperature on the Bellydancer. Alternatively, samples were fixed and permeabilized with ice-cold methanol (95-100%). Cells were not treated with 0.1% NP-40 solution if fixed with ice-cold methanol. After permeabilization, cells were washed 2X with PBS and then incubated with primary antibody (rabbit anti-β-catenin, Santa Cruz Biotechnology Inc. sc-7199) dilution (1:200) at 37^{0}C for 1 hour. Cells were then washed again 2X with PBS and subsequently incubated with a secondary antibody dilution (1:100 dilution, donkey anti-rabbit Rhodamine Red-X, Jackson ImmunoResearch Laboratories, No. 711-295-152) for 1 hour at 37^{0}C. Subsequently, cells were incubated with DAPI (1:1000 in PBS, Sigma-Aldrich D9642) for 10 minutes at room-temperature in the dark on the Bellydancer, with 2X PBS washes before and after DAPI incubation. Coverslips were then mounted onto microscope slides using Mowiol mounting medium.

2.3 Transient Transfections

Transient transfections of all cells utilized Lipofectamine 2000 reagent (LF2000) and the manufacturers recommended protocol. Cells were seeded using complete media without antibiotics 12 -24 hours before transfection at a density of 1.0-1.5 x 10^6 cells per 6 well plate, or scaled up to 5.6-8.4 cells per 10 cm plate. All cells prepared for luciferase assays were seeded using 6-well plates. The following day, a plasmid DNA/DMEM dilution was prepared at a ratio of 1 μg total DNA: 55.6 μl DMEM (1-4 μg total DNA/250

µl DMEM was combined with a LF2000/DMEM dilution (1 µl LF2000: 25 µl DMEM) at a final DNA/LF2000 ratio of 1:1. LF2000/DMEM dilutions were incubated at room-temperature for 4-5 minutes after gentle vortexing, before being combined to DNA/DMEM dilution. The total amount of DNA per well never exceeded 4.5 µg, and equal amounts of DNA per well were used when co-transfection of more than one type of DNA was performed unless stated otherwise. DNA/LF2000 dilutions were gently mixed using the vortex and incubated at room-temperature for a minimum of 20 minutes. Before the addition of DNA/LF2000 mixtures, cells were washed 1x with DMEM, 2 ml of DMEM was added per well of the 6-well plate. DNA/LF2000 mixtures were added directly into wells and mixed gently. Cells were then incubated at 37^0C for 5 hours, after which media was removed and fresh 10% FBS/DMEM without antibiotics was added. At this point, the various pharmacological inhibitors were added, as required (see results). Cells were then returned to incubators for an additional 19-21 hours until harvesting.

2.4 Cell Lysis

Cells were normally harvested with 3 types of lysis buffer. When lysates were prepared for Western blotting only, RIPA buffer was used (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholic acid (DOC), 0.1% SDS, 50 mM Tris pH8.0), supplemented with a cocktail of inhibitors (10 µg/ml Aprotinin, 50 µg/ml Leupeptin, 7.5 mg/ml PNPP and 1 mM Na_3VO_4). NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40 50 mM Tris pH 8.0 and supplemented with the same inhibitor cocktail as for RIPA) was prepared for immunoprecipitation followed by Western blot analysis. Cell Culture Lysis Reagent (CCLR)(Promega) (25 mM Tris pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-

N,N,N',N'-tetraacetic acid, 10% glycerol and 1% Triton X-100) was purchased commercially and used specifically for harvesting cell lysate for subsequent luciferase assays performed on the Monolight 2000 in the laboratory of Dr. Brent Winston, University of Calgary.

2.5 Luciferase & β-Galactosidase Assays

Two different protocols were utilized when performing luciferase assays. For TopFlash experiments, co-transfection of reporter plasmids (TopFlash or FopFlash) with mutant Src kinases included transfection with the β-galactosidase (β-gal) containing plasmid, pCH110 (Amersham Pharmacia Biotech, prod. No. 27-4508). After a 24 hour transfection period, cells were harvested by washing 2 x with PBS and lysed by the addition of 200 µl of 1 x CCLR to each well and scraping into 1.5 ml microfuge tubes, which were kept on ice. After all the cells were harvested, tubes were vortexed for 15 seconds and centrifuged at maximum (14,000) r.p.m. for 12 minutes at 4^0C in an Eppendorf microcentrifuge 5417R. Lysates (supernatant) were then transferred to clean microfuge tubes and stored at -80^0C or used immediately for β-gal and luciferase assays.

β-gal assays were performed essentially following the Invitrogen β-Gal Assay Kit protocol using reagents formulated in the laboratory. Three different sample volumes (1, 5 and 10 µl) were taken for each sample and equilibrated to a final volume of 30 µl of distilled, deionized water. To each sample, 70 µl of ortho-nitrophenyl-β-D-galactopyranoside (ONPG) and 200 µl of 1 x Cleavage Buffer (60 mM Na_2HPO_4-$7H_2O$, 40 mM NaH_2PO_4-H_2O, 10 mM KCl, 1 mM $MgSO_4$-$7H_2O$, pH 7.0 and 0.54 µl β-ME) was

added, mixed and briefly centrifuged. Samples were then incubated in a 37^0C water bath for approximately 30 minutes or more. Reactions were then stopped by the addition of 500 µl Stop Buffer per reaction (1 M Na_2CaCO_3). Absorbance of samples at 420 nm were then read using a spectrophotometer against a blank containing ONPG and Cleavage Buffer without lysate. After determining the protein concentration of each sample by the Bradford protein assay method (using Bio-Rad Protein Assay Dye Reagent concentrate #500-0006EDU), the specific activity of β-gal was calculated for each sample by the following formulas:

$$\text{Specific Activity} = \text{nmoles of ONPG hydrolyzed/t(min)/mg protein}$$

$$\text{nmoles of ONPH hydrolyzed} = (OD_{420})(8 \times 10^5 \text{ nl})/4500 \text{ nl/nmoles-cm})(1 \text{ cm})$$

The sample with the greatest specific activity was then used to calculate a 'normalization factor' which was subsequently used to normalize luciferase readings. 2-20 µl of each sample was combined with 100 µl of Luciferase Assay Reagent (Promega, #E1500) in Falcon 352052 12 x 75mm round-bottom tubes and gently mixed immediately prior to analysis using the Monolight 2000.

A second method of performing luciferase assays was to normalize total protein concentrations. This was done due to the observation that activated SrcY530F appeared to down-regulate β-galactosidase activity compared to samples transfected with empty vector pCI. Cells were transfected in a similar manner except without pCH110/β-Gal plasmid. Cells were then harvested in a similar manner and Bradford assays were performed for each

sample, after which protein concentrations were normalized, usually at a concentration of 1μg/μl. Readings were performed on the Monolight 2000 as described above. All luciferase assays performed after Figure 4.2.3 were carried out in this manner.

2.6 SDS-PAGE and Western Blotting

SDS-PAGE was carried out essentially according to the protocol described by Laemmli (244). Whole cell lysates were prepared using either RIPA, NP-40 or CCLR lysis buffer prepared in the same manner as described above for luciferase assays. After equal amounts of protein were prepared in a solution with Laemmli Sample Buffer (200 mM Tris pH 6.8, 20% glycerol, 4% β-ME, 0.01% bromophenol blue) samples were boiled for a minimum of 5 minutes, in preparation for SDS-PAGE. Denatured samples were then loaded on to polyacrylamide gels using gel loading tips and run through the stacking gel for 10-15 minutes at 100 V and then through the separating gel at 155-160 V until the lowest molecular weight pre-stained protein markers were observed to run off of gel. Gels were immersed in Running Buffer during electrophoresis (0.3% Tris (w/v), 1.44% glycine (w/v), 0.1% sodium dodecyl sulphate (w/v)).

Separated proteins were then transferred onto Protran pure nitrocellulose transfer and immobilization membranes (Schleicher and Schuell) using Bio-Rad Mini Format 1-D Electrophoresis transfer apparatus. Polyacrylamide gels were initially equilibrated in Transfer Buffer (0.303% (w/v) Tris, 1.44% (w/v) glycine, 20% (v/v) methanol and 0.01% SDS (w/v)) for 5 minutes before assembly into protein transfer apparatus, after which air bubbles were removed. Peptides were then transferred onto nitrocellulose membranes at 21-25V overnight at 4^0C.

2.7 Immunostaining of Immobilized Proteins

Proteins transferred onto nitrocellulose membranes were detected in the following manner. Membranes were initially immersed for 1 hour at room temperature with gentle agitation (Bellydancer shaker) in TBS-T (tris buffered saline; 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Tween-20, in distilled deionized H_2O) supplemented with 1% BSA (bovine serum albumin) when probing for phospho-tyrosine containing proteins (i.e. when using anti-phospho-tyrosine antibody 4G10) or 5% non-fat dry milk powder. After blocking, membranes were washed 1-2X in TBS-T, then incubated with the primary antibody diluted in TBS-T for 2 hours at room-temperature with gentle agitation. Membranes were then washed 3 X for 5 minutes per wash at room-temperature using TBS-T before immersion in the secondary antibody for 2 hours at room-temperature with gentle agitation. Secondary antibodies were also diluted in TBS-T at a ratio of 1:5,000 to 1:10,000. Incubation in secondary antibody was followed by washing of membranes 3 X with TBS-T. Detection of protein bands was usually performed immediately following washes by removing excess TBS-T and by the addition of ECL Western Blotting Detection solution (Amersham) or ECL Plus Western Blotting Detection solution (Amersham). When film was used to visualize peptides (using Kodak Biomax XAR), ECL Western Blotting Detection solution was used and films were fixed and developed either by hand or using an automated film devloper. ECL Plus Western Blotting Detection reagents were used when protein bands were visualized and/or quantified (band densitometry) using the Storm gel and blot imaging system and ImageQuant software (GE Healthcare).

2.8 Pulse-Chase/Metabolic labeling

Pulse-chase analysis was performed using tsLA29 transformed Rat-1 cells (see above) seeded in 6-well plates at approximately 1.0 X 10^6 cells per well. tsLA29 Rat-1 cells maintained at 35^0C (permissive temperature) were washed two times with and incubated in methionine- and cysteine-free DMEM supplemented with 0.584 mg/ml L-glutamine (Life Technologies No. 21013, now Invitrogen Cat. No. 21013-024) for 20 minutes prior to metabolic labelling. Cells were then incubated with the same media used to wash cells but additionally supplemented with 50 μCi [^{35}S]methionine/cysteine per ml (TRAN^{35}S-LABEL, formerly ICN Biomedical Inc. Cat no. 51006, now MP Biomedicals cat. No. 51006) for three hours. Cells were then quickly washed once with complete media (ie. 5% NBCS) then incubated in complete media supplemented with methionine and cysteine (final concentration 300 mg/L methionine and 630 mg/L cysteine). Subsequently, one set of cells (0 hours) were washed 2 X with PBS then harvested using NP-40 lysis buffer, centrifuged and supernatants frozen at -80^0C. The remaining cells were either maintained at permissive temperature (35^0C) or incubated at the non-permissive temperature, 39.5^0C in a forced-air incubator. Cells were then harvested using NP-40 lysis buffer at the indicated times (Figure 3.7; 2, 4, 8, 16 or 24 hours), centrifuged at 4^0C using the 14,000 r.p.m. on the table-top refrigerated microfuge for 12 minutes (see above).

The protein concentration of each sample was calculated and 500 μg of total protein was used for immunoprecipitation. 2 μg of anti-β-catenin antibody (Santa Cruz, sc-7199 polyclonal rabbit) was incubated with the extracts for 2 hours on ice. The antibody-antigen complexes were collected by incubation with agarose Protein-A beads (Bio Rad or Invitrogen) for a minimum of 2 hours (with rotation at 4^0C), but usually over-night with

rotation at 4^0C. Samples were then centrifuged at approximately 10,000g at 4^0C for 15-30 seconds. The supernatant was removed and replaced with Laemmli sample buffer (final volume 50 µl). Samples were then boiled and loaded onto large 8% SDS-polyacrylamide gels for electrophoresis and run at 65 V overnight.

The gel was fixed in a solution containing 7% acetic acid and 40% methanol for 1 hour at room temperature with gentle agitation (Bellydancer), followed by immersing gels in En3hance (PerkinElmer No. 6NE9701) solution for another hour at room temperature and gentle agitation. Gels were then immersed in a 10% glycerol solution for 1-2 hours at 4^0C with gentle agitation, followed by drying using a Bio Rad 483 Slab Dryer set to 80^0C for 3 hours. Kodak BioMax MR film was exposed to dried gels for 2 days at 4^0C. Films were fixed and developed manually, or automatically.

2.9 Immunoprecipitation

Samples used for immunoprecipitation experiments were equalized to a minimum of 500 µg/µl or higher with lysis buffer containing inhibitors depending on estimated abundance in any given cell line or experiment. Equilized lysates were then incubated with 1-5 µg of antibody, gently vortexed for 1-3 seconds and placed in ice for a minimum of 1 hour. A 15-20 % slurry of washed Protein A-agarose or Protein G-agarose beads in lysis buffer was then added to each sample-antibody solution for a total volume of 600 µl. These samples were then rotated for a minimum of 2 hours, or overnight at 4^0C. Samples were then centrifuged at 10,000g for 15-30 seconds at 4^0C. Supernatants were aspirated using a 23-guage needle. Beads were then washed 3X with cold PBS and 1X lysis buffer, after

which 10-15 µl of 2X Laemmli sample buffer was added to each tube followed by the addition of 20-30 µl of 1X Laemmli sample buffer for a final volume of 50 µl. Boiled samples were subjected to SDS-PAGE as described above.

2.10 Nuclear & Cytoplasmic/Membrane Preparation

SW480 cells were transfected as described above and then prepared for nuclear and cytoplasmic/membrane separation in the following manner. First, cells were washed 2 X with cold PBS then incubated for 3 minutes at 37^0C in a trypsin/EDTA solution. Trypsin/ EDTA solution was then gently aspirated away and cold PBS added to each plate. Cells which did not detach from plate surface after the addition of PBS were gently scraped off using a rubber-policeman. All the detached cells were collected into 15 ml Falcon tubes and kept on ice. The cells were pelleted by centrifugation at 1000-2000 rpm for 8 minutes at 4^0C on a Beckman Coulter Counter. The supernatant from each sample was removed and 200 ml of lysis buffer added (10 mM HEPES pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.5% NP-40, distilled deionized H_2O). Cells were mixed by flicking (not using pipetteman) and carefully transferred to 1.5 ml microfuge tubes, then kept on ice for 5 minutes. Tubes were then centrifuged at 1200g for 5 minutes at 4^0C. Supernatants (containing the cytosol and membrane fractions) were carefully removed and frozen at -80^0C. Pellets (containing nuclei) were washed 4 times by adding 200 µl of lysis buffer without NP-40, gently mixing, followed by centrifugation at 1200g for 5 minutes at 4^0C. Supernatants were collected and stored together with initial sample of supernatant. Pellets were then resuspended by gentle mixing/flicking in a nuclear suspension buffer (25 mM Tris HCl pH 8.0, 400 mM KCl, 1 mM DTT, 1mM PMSF, 20% glycerol and distilled

deionized H_2O). Nuclear samples were then subjected to 3 freeze-thaw cycles using liquid nitrogen (15 seconds) and boiling water. Thawed samples were then centrifuged once more at 7000g for 12 minutes at 4^0C. Supernatants were immediately collected and stored at -80^0C. Small 5-10 ml samples of both cytoplasmic/membrane and nuclear fractions were kept on ice for Bradford protein concentration analysis.

2.11 Gel Shift/Electromobility Shift Assay (EMSA)

A non-radioactive method of detecting changes in oligonucleotide duplex-protein complex interaction was used to measure changes at the transcriptional complex of the Tcf/Lef consensus sequence as defined by Korinek *et al*. (245). The Pierce/Thermo Scientific LightShift Chemiluminescent EMSA kit (No. 20148) was used in conjunction with biotin end-labelled DNA duplex probes synthesized at the University of Calgary University Core DNA (UCDNA lab) Services laboratory. The sequences used for the DNA probes (taken from the published Super8XTopFlash sequence (246)) were: 5'-GCTCTTACGCGAGATCAAAGGGGGTAAGATCAAAGGGGGTAAGATCAAA-GGGGGTA-3' and 5'-TACCCCCTTTGATCTTACCCCCTTTGATCTTACCCCCTTT-GATCTCGCGTAAGAGC-3'. Two sets of these complementary DNA sequences were synthesized, one set was 5' biotin labeled and the other left unmodified. To anneal complimentary strands, oligonucleotides were resuspended at equal concentrations in annealing buffer (10 mM Tris pH 7.5, 1 mM EDTA, 50 mM NaCl). Equal volumes of resuspended oligonucleotides were then combined and gently mixed in 1.5 ml microfuge tubes and placed in a heat block set to 95^0C. After 5 minutes, tubes were cooled slowly to

room temperature by removing the heat block. Annealed probes were stored at 4^0C or -20^0C.

A native 10% polyacrylamide gel (10% polyacrylamide (ProtoGel (30%), 37.5:1 Acrylamide to Bis Acrylamide, National Diagnostics), 0.5X tris-boric acid-EDTA (TBE), 0.14% ammonium per sulphate (APS), 0.35% tetramethylethylenediamine (TEMED), distilled deionized H_2O) was pre-run in 0.5 X TBE at 100 V. Incubation of nuclear fractions from either kinase inactive mutant SrcK298M or activated mutant SrcY530F with DNA probes was carried out in the following manner. For each nuclear fraction sample, 3 X 20 µl reactions were carried out. Each reaction contained one of the following: labeled probe only (4 pmol); labeled probe (4 pmol) plus nuclear fraction (10.6 µg); or, labeled probe (4 pmol) plus nuclear fraction (10.6 µg) plus excess unlabeled DNA probe (20 fmol), in reaction buffer (1 X Binding Buffer (Pierce No. 30148A), 50 ng/µl poly (dI·dC), 4% glycerol (v/v), 1 mM MgCl, 0.5 mM EDTA). Binding reactions were incubated at room temperature for 20 minutes. Following this incubation, 5 µl of 5 X Loading Buffer was added to each reaction and gently mixed by pipetting up and down. Samples were then loaded and electrophoresed (using 0.5 X TBE buffer) through the pre-run native polyacrylamide gels until the dye front reached approximately 2/3 down the length of the gel. DNA probe-protein complexes were then transferred onto positively charged nylon membranes using standard mini Bio Rad electrophoresis transfer apparatus in 0.5 X TBE buffer pre-cooled to 10^0C. Transfers were performed at room temperature for 30 minutes and 380 mA. Membranes were then placed on paper towels to remove excess buffer without completely drying membranes. Complexes were then cross-linked to nylon

membranes by placing membranes, face-up, under a hand-held UV illuminator for 10 minutes at a distance of approximately 0.5 cm.

To detect the transferred DNA probe-protein complexes, membranes were incubated in Blocking Buffer (Pierce) for 15 minutes at room temperature with gentle shaking. Membranes were then incubated in a Stabilized Streptavidin-Horse Radish Peroxidase Conjugate (Pierce)-Blocking Buffer solution for 15 minutes with gentle shaking. Membranes were then washed 6 X in a Wash Buffer dilution (Pierce) for 5 minutes each wash using fresh Wash Buffer dilution each wash. Membranes were then transferred to a fresh container and incubated in Substrate Equilibration Buffer for 5 minutes at room temperature with gently shaking. Following this incubation, membranes were removed and excess liquid adsorbed using a paper towel. Membranes were the placed on cellophane wrap and Substrate Working Solution (Luminol/Enhancer Solution combined with Stable Peroxide Solution, Pierce) was added. Incubations were performed in the dark for 5 minutes at room temperature without shaking. Excess liquid was removed before exposure to Kodak Biomax XAR film.

2.12 Site-Directed Mutagenesis

Mutant β-catenins were engineered following the Stratagene QuikChange Site-Directed Mutagenesis methodology. Wild-type β-catenin (human) was obtained from Open Biosystems (cat. no. MHS1010-9205712), and used to generate tyrosine to phenylalanine single mutants Y86F, Y142F and Y654F. Mutant oligonucleotide primers were then used again on single mutants to generate double mutants (Y86, 142F, Y86, 654F and Y142, 654F). The same mutant oligonucleotide primers were then used in conjunction with the

double mutants to generate the triple mutant β-catY86,142,654F. The three pairs of complementary mutant oligonucleotides were synthesized and purified at the UCDNA Services facility at the University of Calgary with the following sequences: for Y86F,

5'-CAAGTAGCTGATATTGATGGACAGTTTGCAATGACTCGA-3'

and,

5'-TCGAGTCATTGCAAACTGTCCATCAATATCAGCTACTTG-3';

for Y142F,

5'-CTGAAACATGCAGTTGTAAACTTGATTAACTTTCAAGATGATGCAGAA-3'

and,

5'-TTCTGCATCATCTTGAAAGTTAATCAAGTTTACAACTGCATGTTTCAG-3';

for Y654F,

5'-ATGAAGGTGTGGCGACATTTGCAGCTGCTGTTTT-3'

and,

5'-AAACAGCAGCTGCAAATGTCGCCACACCTTCAT-3'.

All primers were designed based on the guidelines described in the QuikChange Site-Directed Mutagenesis instruction manual with the assistance of NetPrimer primer analysis software (available free online at, http://www.premierbiosoft.com/netprimer/ index.html). The cycling parameters used on a thermocycler were the same as described in the QuikChange instruction manual with the following modifications: 15 cycles; and, a 10 minutes final incubation at 72^0C following the 15 cycles. All of the enzymes used in the mutagenesis were purchased from Stratagene. The 10X PCR reaction buffer was made following the recipe described in the QuikChange instruction manual (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl pH 8.8, 10 mM MgSO4, 1% Triton X-100, 1 mg/ml

nuclease free bovine serum albumin (BSA)). Cloning was carried out using either laboratory stocks of competent DH5α bacteria, or Subcloning efficiency DH5α Competent cells (Invitrogen cat. no. 18265-017), or MAX Efficiency DH5α competent cells (Invitrogen cat. no. 18258-012). The empty vector pCMV-SPORT 6 was synthesized by removing wild-type βcatenin from its backbone by performing a double restriction endonuclease digest (ApaI and EcoRI) followed by sample purification using the QIAquick PCR Purification Kit (cat. no. 28104). Digested plasmids with sticky-ends were then filled using DNA polymerase I, large fragment (Invitrogen cat. no. 18012021). Blunt-ended oligos were then subjected to agarose gel electrophoresis and the large fragments were purified from gels using the QIAquick Gel Extraction Kit (cat. no. 28704). Large blunt ended fragments representing the linearized empty vector pCMV-SPORT 6 were then ligated overnight using T4 DNA Ligase (Invitrogen cat. no. 15224017). Vectors were then transformed into MAX Efficiency DH5α (Invitrogen) competent cells and grown in standard LB-agar plates inoculated with ampicilin (100 μg/ml). Plasmids were purified from large LB cultures using the Qiagen EndoFree Plasmid Maxi purification kit (cat. no. 12362).

2.13 siRNA

Treatment of SW480 cells with siRNA oligos obtained commercially was performed according to the manufacturer's protocols. Cells were seeded at approximately 50% confluency and treated with siRNA or non-targeting control duplex RNA the next day. Following another 24 hours, cells were transfected with mutant Src kinases, or controls, as

described above. Cells were then harvested following another 24 hours and assays for protein level was conducted. Anti-β-catenin and anti-PLCγ1 & -2 siRNA was purchased from Dharmacon and transfected using Oligofectamine (Invitrogen). siRNA targeting GSK3β was obtained from Bio Rad (10 pmol per reaction of SiLentMer Dicer-Substrate siRNA Duplex cat. no. 179.0159) and transfected using 5 µl per reaction LipofectAmine 2000 (Invitrogen).

2.14 Multiplex r.t.PCR

RNA from transiently transfected (with siRNA or DNA or both) SW480 cells was harvested using the Qiagen RNeasy Mini kit (cat. no. 74104). Primer sequences for FGF18 and GAPDH were taken from Shimokawa T. *et al*. (2003) and are as follows: for FGF18 5'-GGACATGTGCAGGCTGGGCTA-3'; and, 5'-GTAGAATTCCGTCTCCTTGCCC-TT. For GAPDH, the sequences were 5'-ACAACAGCCTCAAGATCATCAG-3'; and 5'-GGTCCACCACTGACACGTTG-3' (247). Primers were synthesized at the UCDNA Services facility at the University of Calgary. RT-PCR was carried out using the Qiagen OneStep RT-PCR kit in conjunction with the primers synthesized as described. 28 cycles were performed on a thermal cycler with the following step times and temperatures: 30 seconds at 94^0C, 30 seconds at 60^0C and 60 seconds at 72^0C. A final extension step of 60 seconds at 72^0C was performed before samples were frozen at -20^0C. PCR products were loaded onto 1.5% agarose gels, electrophoresed and photographed over a UV light box.

2.15 Real-Time PCR

All real-time PCR assays were carried out using the Applied Biosystems 7900HT Fast Real-Time PCR System in the laboratory of Dr. Jennifer Cobb at the University of Calgary. Total RNA from transiently transfected SW480 cells (using pCI, SrcK298M or SrcY530F done in triplicate) was harvested using the RNeasy Mini Kit (Qiagen). In conjunction with target gene analysis, glyceraldehyde-3-phosphate (GAPDH) gene expression was also measured as an endogenous control. Two different chemistries were used to perform real-time analysis, including SYBR Green I dye chemistry and TaqMan probe-based chemistry.

Expression of FGF18 was analysed using SYBR Green I dye chemistry. Primers used in this assay were identical to those used in the multiplex r.t.PCR (Figure 4.4.1, B and C). PCR reactions were performed using RNA from each transfection reaction in conjunction with the QuantiFast SYBR Green RT-PCR kit (Qiagen cat. no. 204154) following the manufacturer's protocol. RNA from each transfection reaction was equilibrated to 100 ng/µl. A portion of one RNA sample from pCI transfected cells was diluted to create a standard curve for FGF18 and GAPDH target genes as follows: 1, 0.5, 0.25, 0.125 and 0.0625. For the real-time PCR reaction, samples were diluted to 0.25. All sample reactions and standards for FGF18 and GAPDH were loaded onto the same plate (MicroAmp Fast Optical 96-well Reaction plates, part no. 4346906). Temperature and cycle settings were programmed according to the Qiagen Supplementary Protocol for the ABI PRISM 7900 using the QuantiFast SYBR Green RT-PCR kit. Mean relative amounts of FGF18 in mutant Src transfected cells were then calculated based on the standard curve (Log Total RNA vs C_t value) and normalized against the mean relative amount of GAPDH

from the same samples and calculated in the same way. Mean relative FGF18 expression levels in mutant Src transfected cells were then compared to the mean relative FGF18 expression levels in empty vector pCI transfected cells and plotted as Relative Mean Fold Activation (Figure 4.4.1, C).

Real-time analysis of CTNNB1 (β-catenin) expression and the Wnt target genes, CCND1 (cyclin D1) and MYC was performed using TaqMan primer-probe chemistry (cat. no's. Hs00170025_m1, Hs00277039_m1, and Hs00153408_m1, respectively). RNA samples used in this assay were identical to those used in the FGF18 real-time analysis except samples were not diluted from 100 ng/μl to 0.25 ng/μl. Analysis of data was performed by the RQ Manager 1.2 software within the 7900 HT system.

2.16 Plasmids

All of the plasmids used in this study were transformed into competent DH5α bacterial cells by standard protocols. Cultures were then grown in Luria-Bertani medium (LB) with ampicillin (100 μg/ml) antibiotics or kanamycin (50 μg/ml). Plasmids were then purified using a Qiagen Endo-Free Plasmid Maxi kit, following the manufacturer's protocol. All circle maps were obtained from manufacturers' literature.

2.16.1 pCI

The pCI plasmid vector (Promega, Cat. No. E1731; full name pCI Mammalian Expression Vector) was used as an empty vector control for transfection experiments when wild-type (wt) or mutant Src kinases were being used. All of the wt or Src kinase mutants used in this study were constructed by previous laboratory personel using this plasmid

vector backbone. The wtSrc and mutant Src plasmids were prepared by other laboratory personel, and were inserted into the EcoRI-(blunt) and MluI restriction enzyme cleavage sites within the multiple cloning site (MCS) of the pCI vector (Figure 2.1, A, page 63). This plasmid vector does not contain a luciferase gene.

2.16.2 pCMV-SPORT 6

The pCMV-SPORT 6 plasmid vector was used as an empty vector control in experiments where wt- or mutant *CTNNB1*'s were being transfected into cells. cDNA containing wt-β-catenin was purchased from Open Biosystems and the empty vector pCMV-SPORT 6 was made by removing wt-β-catenin (Kpn I-(blunt)-Mlu I) (Figure 2.1, B, page 63). This plasmid vector does not contain a luciferase gene.

2.16.3 TopFlash & FopFlash

The Wnt signaling (β-catenin responsive) luciferase reporter TopFlash and its negative control FopFlash were obtained from Randal Moon.(University of Washington). TopFlash contains 3 copies of the optimal Tcf motif (CCTTTGATC) upstream a minimal *c-Fos* promoter driving luciferase, while FopFlash contains 3 copies of a mutant sequence (CCTTTGGCC) (245). I was unable to obtain further detailed (sequence) information from the original creators of the reporters beyond what was provided in Korinek, 1997.

2.16.4 Super8XTopFlash, Super8XFopFlash & pTA-Luc

Super8XTopFlash and its negative control Super8XFopFlash was obtained from Randall Moon (University of Washington) and were engineered based on the TopFlash & FopFlash Wnt luciferase reporters (246). These reporters contain 8 copies of the Tcf/Lef binding motif (AGATCAAAGG) with the sequence, GGGTA, used as a spacer between each repeat of the optimal motif. The mutant sequence found in Super8XFopFlash is AGGCCAAAGG. The same sequence used as a spacer in Super8XTopFlash was also used in its negative control. These binding motifs were cloned into the Mlu I site of Clontech's luciferase vector plasmid pTA-Luc (Figure 2.2, A, page 64). The multiple cloning site (MCS) is located upstream of the minimal promter "TA", the herpes simplex virus thymidine kinase promoter TATA box.

2.16.5 Pathway Profiling System 4

The Pathway Profiling system 4 used in this study was purchased from Clontech, and contains 5 luciferase plasmid constructs using the pTA-Luc backbone vector (Figure 2.2, A, page 64). This luciferase reporter kit was designed to survey various signaling pathways which are involved with the cell cycle, and measure transcriptional activity associated with the E2F-family of transcription factors (pE2F-TA-Luc), Myc (pMyc-TA-Luc), p53 (pp53-TA-Luc) and pRb (pRb-TA-Luc). This pathway profiling system also includes the empty vector plasmid control (pTA-Luc) which exhibits low constitutive activity.

2.16.6 pGL3-Control

The pGL3-Control plasmid vector was purchased from Promega (Cat. No. 1741). According to the manufacturer, this plasmid contains SV40 virus promoter and enhancer elements that promote the strong expression of a modified luciferase gene (*luc*+) in mammalian cells (Figure 2.2, B, page 64).

2.16.7 Canonical pE2F4B-Luc

The E2F4B-Luc luciferase reporter obtained from the Erick Morris of the Dyson lab (Massachusetts General Hospital, Charlestown Ma, USA) was constructed using the pGL3 series of vector plasmid, pGL3-Basic (Figure 2.3, page 65). This plasmid is identical to pGL3-Control, but does not contain the SV40 promoter and enhancer elements. The pE2F4B-Luc reporter contains 4X E2F family binding sites and the E1B TATA minimal promoter, cloned into the SmaI and BglII sites of pGL3-Control.

2.16.8 pCH110

The pCH110 plasmid vector expresses β-galactosidase/lacZ under the control of the SV40 early promoter (Figure 2.4, page 66). This reporter was purchased from Amersham-Pharmacia Biotech (Product No. 27-4508).

2.16.9 pEGFP-C1

The pEGFP-C1 plasmid vector expresses a red-shifted variant of wild-type green-fluorescent protein (GFP), optimized for brighter fluorescence and higher expression in

mammalian cells, according to the manufacturer, Clontech (Cat. No. 6084-1). This plasmid vector expresses the modified GFP under the control of the human cytomegalovirus immediate early promoter, and contains a kanamycin/neomycin antibiotics resistance gene (Kan^r/Neo^r) (Figure 2.5, page 67).

2.16.10 phCMV-CLUC

The phCMV-CLUC (Figure 2.2, C, page 64) constitutively activated luciferase reporter plasmid was purchased from Genlantis (Cat. No. P003500, Fusion Stable Reporters). According to the manufacturer, this plasmid contains a CMV-intron sequence which has been optimized for significantly higher constitutive expression levels of luciferase, compared to other mammalian expression vectors.

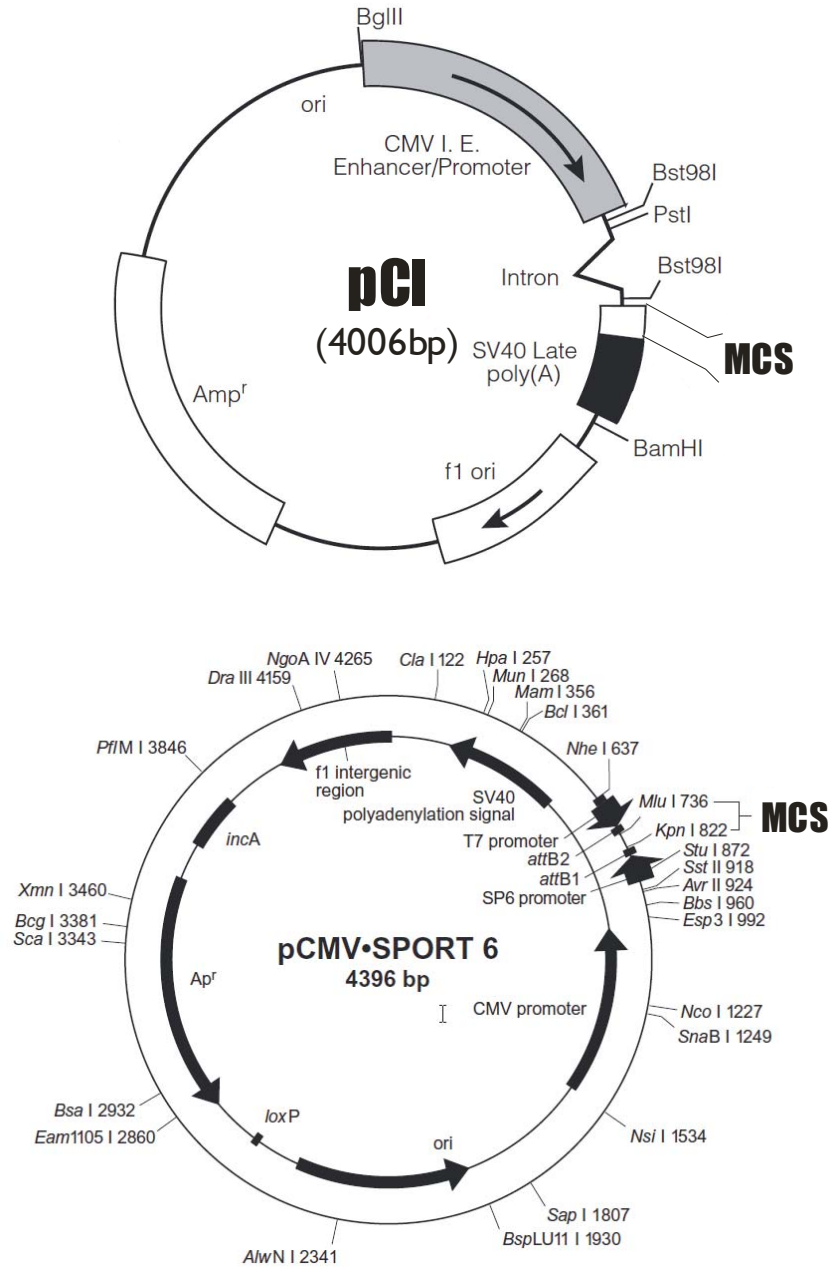


Figure 2.1 Circle maps of the plasmid vector backbones for wt and Src mutants, and wt and mutant β-catenins. **A**, pCI (Promega), backbone for wt- and mutant Src Kinases. **B**, pCMV-SPORT 6 (Invitrogen), backbone for wt- and mutant β-catenins. MCS, multiple cloning site. Amp^r & Ap^r, β-lactamase ampicilin resistance gene. Arrows indicate direction of transcription. Images taken from Promega and Invitrogen technical manuals.

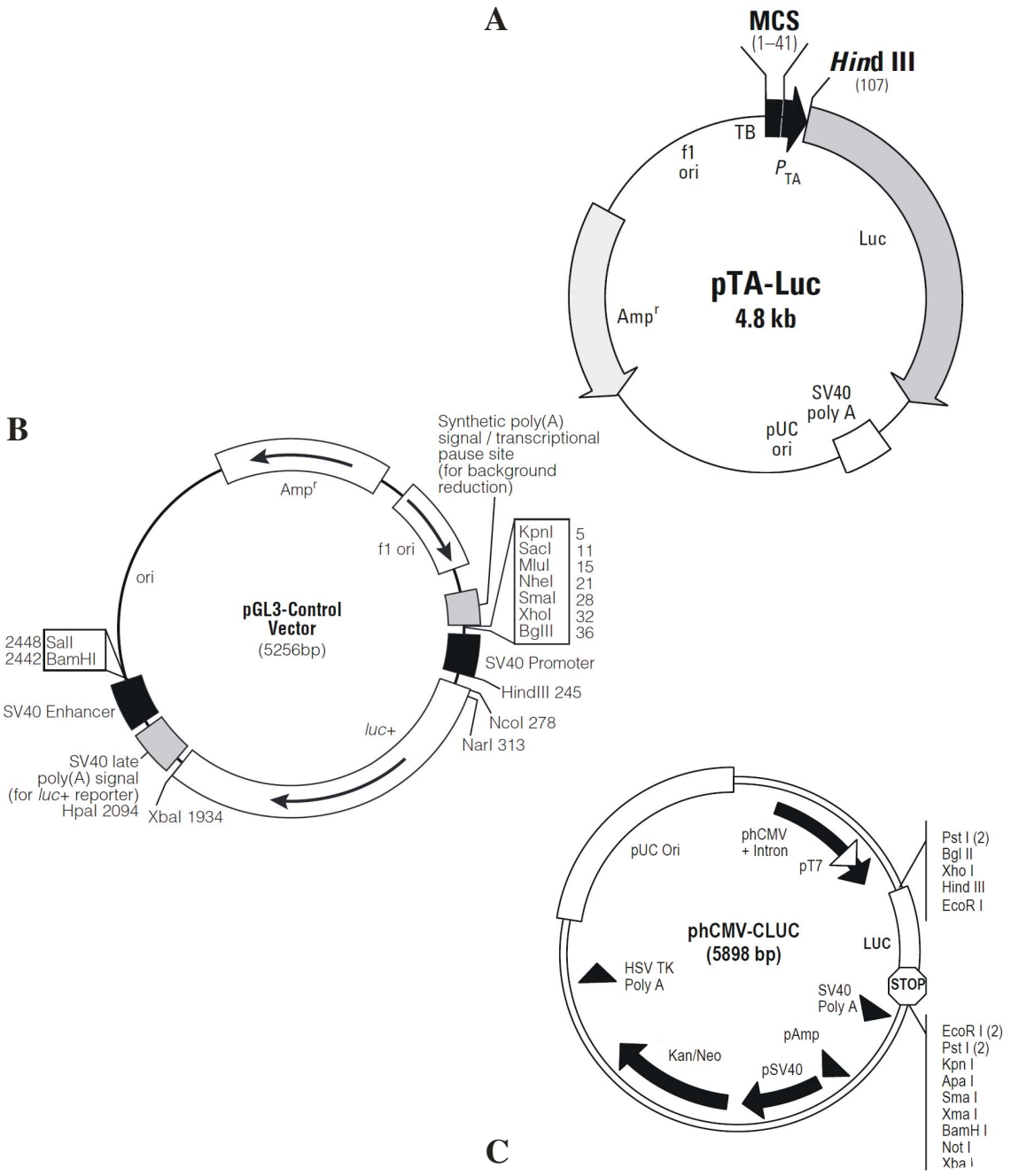


Figure 2.2 Luciferase containing plasmid vector circle maps. **A**, pTA-Luc (Clontech). **B**, pGL3-Control (Promega). *luc*+ indicates a modified luciferase gene. **C**, pCMV-CLUC (Genlantis). Both pGL3-Control and pCMV-CLUC possess high constitutive activaty. MCS, multiple cloning site. Amp^r, β-lactamase ampilcilin resistance gene. Kan/Neo, kanamycin/neomycin resistance gene. Arrows indicate direction of transcription. All images taken from the manufacturers technical bulletins/user manuals.

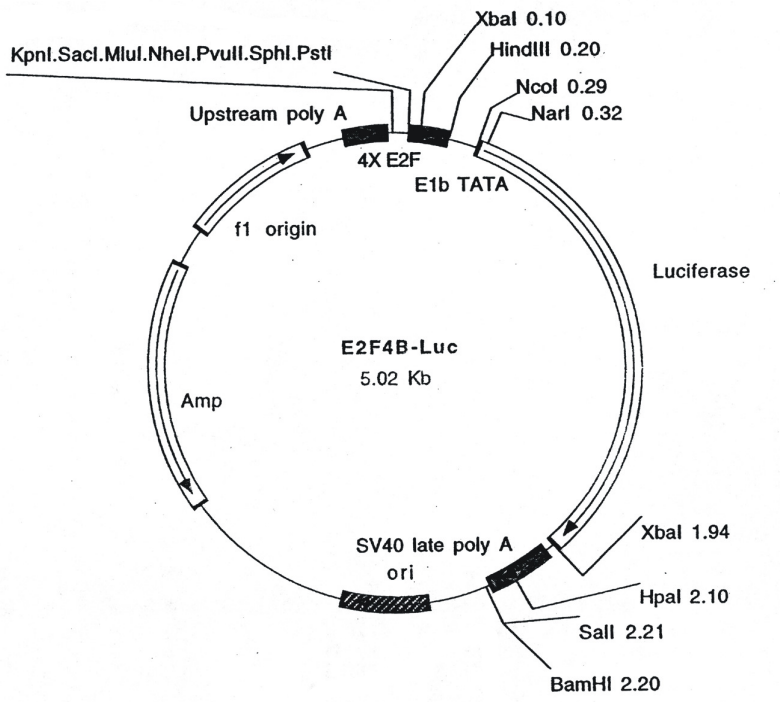


Figure 2.3 Canonical E2F luciferase reporter plasmid E2F4B-Luc circle map. Image provided by Eric Morris.

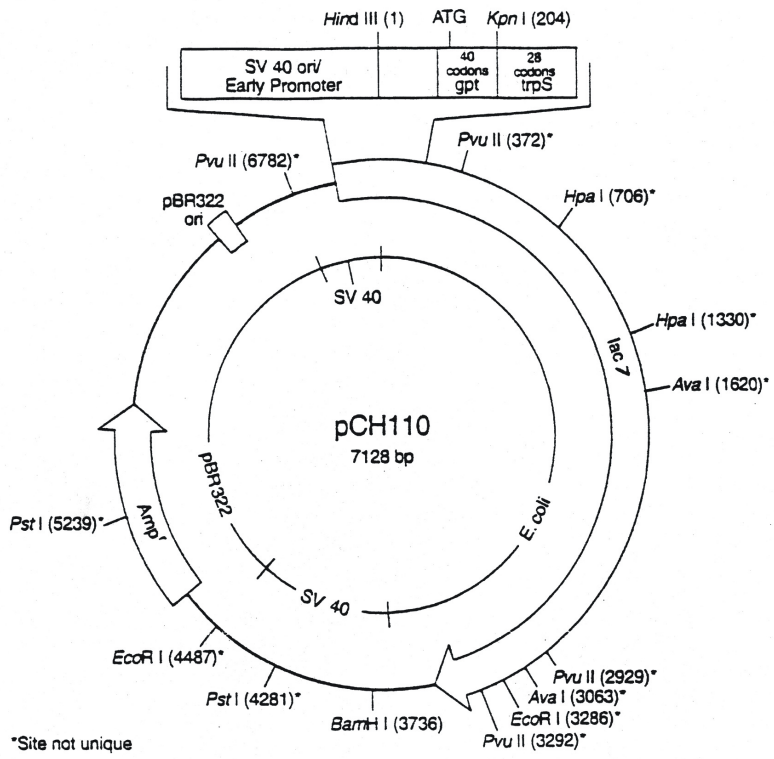


Figure 2.4 β-Galactosidase/lac Z expression vector pCH110 circle map. Image provided by Amersham-Pharmacia Biotech.

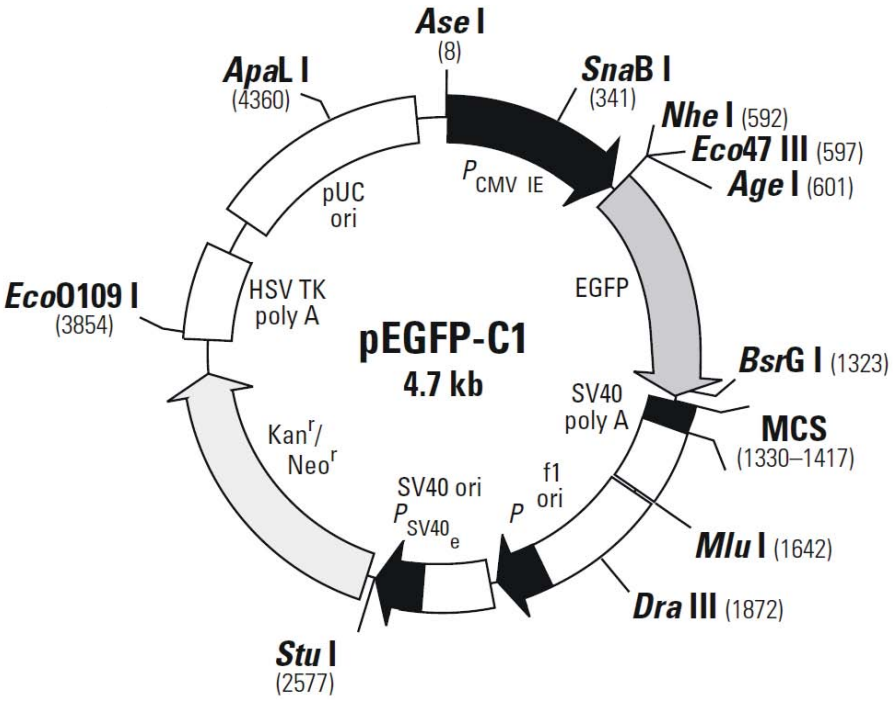


Figure 2.5 Green-Fluorescent protein expression vector pEGFP-C1 circle map.

SECTION II

RESULTS

CHAPTER 3: β-CATENIN AS A TARGET OF SRC

3.1 Hypothesis & Rationale

We hypothesized that Src kinase can phosphorylate, stabilize the protein levels of, and induce the nuclear localization of β-catenin. This idea is based on the observation that Src can interact with, phosphorylate and alter the ability of β-catenin to complex with proteins at the intracellular domain of adherens junctions (114-116,232). In order to test this hypothesis, the intracellular localization, tyrosine phosphorylation and protein stability of β-catenin was assessed upon the activation or overexpression of Src in various cell types.

3.2 Results

3.2.1: Localization of β-Catenin in Chicken Cells

Normal chicken embryo fibroblasts (CEFs) and Schmidt-Ruppin A strain Rous sarcoma virus infected chicken fibroblasts (SRAs) were used to visualize the potential changes in β-catenin localization associated with Src kinase activity. It has been shown that infection of chicken cells with the Schmidt-Ruppin A strain of the Rous sarcoma virus leads to cellular transformation of fibroblasts, and that this property is due to v-Src (6-9,248). Therefore, the distribution of β-catenin was compared in CEF and SRA cells by indirect immunofluorescence microscopy using a commercial polyclonal β-catenin antibody (Santa Cruz Biotech. Inc. SC7199).

Figure 3.2.1 shows that infection of CEFs with the Schmidt-Ruppin A strain of Rous sarcoma virus changes the overall morphology of the fibroblast from one that is

flattened to one that is rounded, when prepared using the same protocol (compare A and B). In these cells the distribution of β-catenin also differed (compare C and D). In normal CEFs, the distribution of β-catenin was punctate throughout the cell, excluding the nucleus (Figure 3.2.1, C). In SRA cells, the most prominent staining for β-catenin was found towards the cell periphery, with a smaller amount at a juxta-nuclear localization (Figure 3.2.1, D). Quantification of the number of cells with juxta-nuclear β-catenin shows a large difference between normal CEFs and SRA cells (Table 3.2.2).

3.3 Tyrosine Phosphorylation of β-catenin

To confirm that Src kinase was able to tyrosine phosphorylate β-catenin, I used several different cell types. These cell types include the normal chicken embryo fibroblasts and SRA cells used in Figure 3.2.1, tsLA29 infected Rat-1 (fibroblasts) cells, and the human colon adenocarcinoma cell line SW480 (ATCC CCL-228). Lysates from these cell lines were immunoprecipitated with the β-catenin antibody (SC7199) used previously (Figure 3.2.1) followed by Western blot analysis using the phospho-tyrosine antibody 4G10.

The lysates from CEF and SRA cells were initially tested for the presence of phosphorylated β-catenin, and subsequently used as controls as it was previously shown that compared to CEF cells, SRA cells contain higher levels of tyrosine phosphorylated β-catenin (114). Figure 3.3.1, A shows that β-catenin is dramatically tyrosine phosphorylated

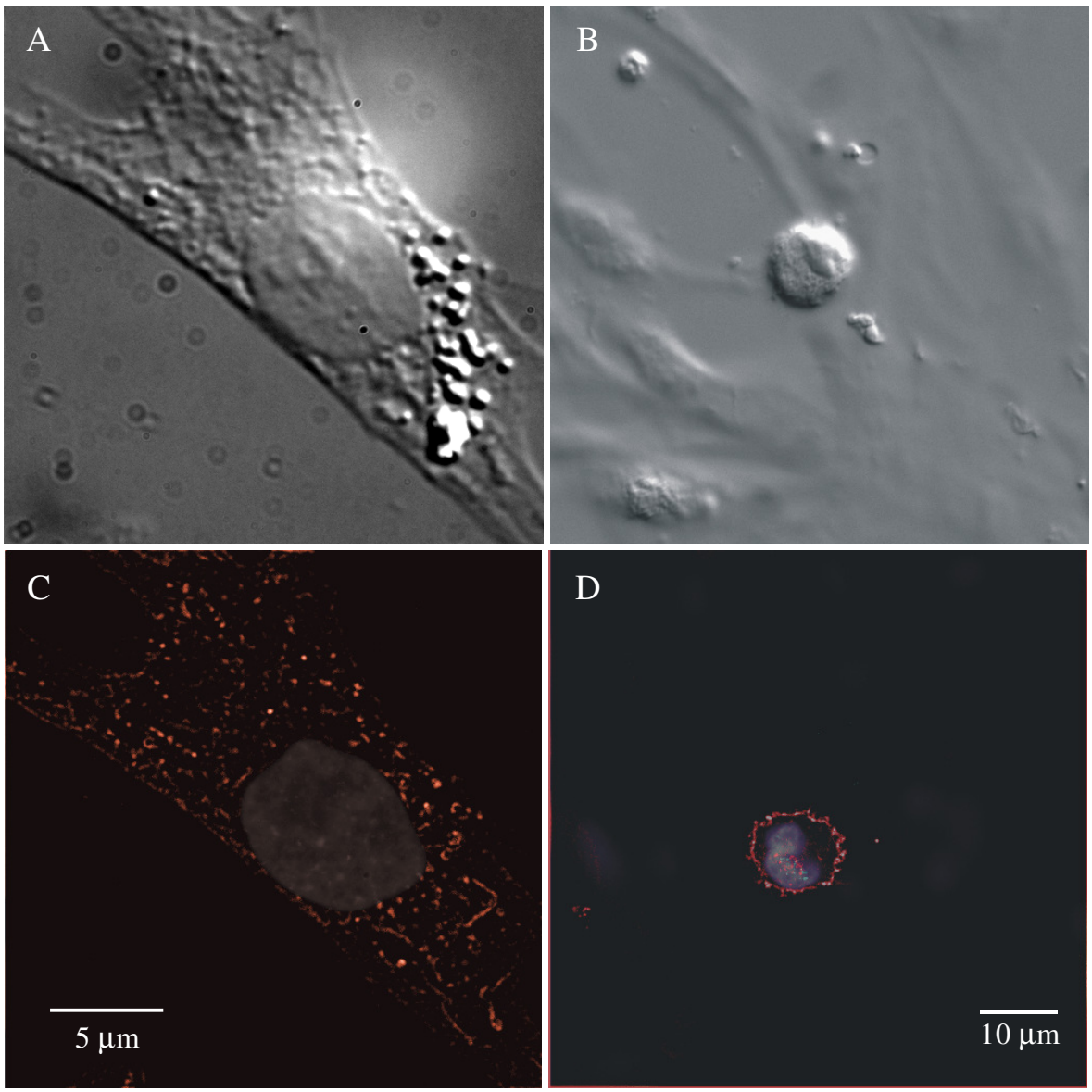


Figure 3.2.1 Altered distribution of β-catenin in Schmidt-Ruppin A strain Rous sarcoma virus infected chicken embryo fibroblasts (SRA). A, Differntial interference contrast (DIC) microscopy image of uninfected normal chicken embryo fibroblasts (CEF). **B**, DIC image of SRA cells. **C**, CEF cells stained for β-catenin (red) and nucleus (DAPI). **D**, SRA cells stained for β-catenin (red) and nucleus (DAPI, blue).

No. of CEFs	No. of CEFs with Juxta-Nuclear Staining	No. of SR-As	No. of SR-A with Juxta-Nuclear Staining
77	2	49	9
103	2	17	6
102	1	22	8
108	5	19	3
93	1	22	3
Total: 483	11	129	29
Percentage of Total	2.3%		22.5%

Table 3.2.2 Quantification of CEF and SRA cells with juxta-nuclear localization of β-catenin (see Figure 3.2.1). CEFs (normal chicken embryo fibroblasts) or SRA cells (Schmidt-Ruppin A strain Rous sarcoma virus infected CEFs) were counted in 5 different fields-of-view, and the number of cells with juxta-nuclear β-catenin was recorded.

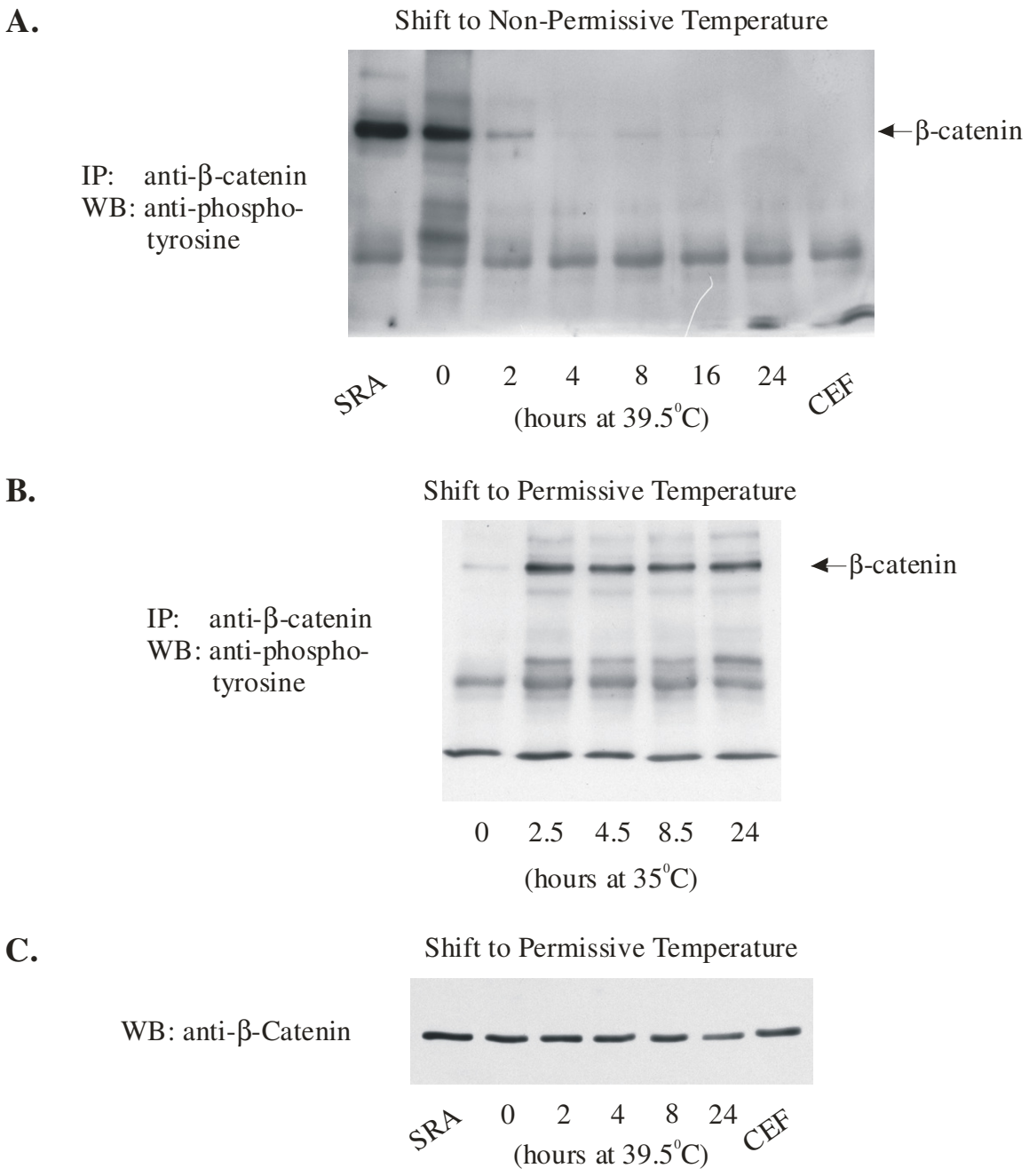


Figure 3.3.1 The phosphorylation state of β-catenin in tsLA29 Rat-1 cells corresponds to the incubation temperature. A, Western blot for phospho-tyrosine on β-catenin immunoprecipitated from tsLA29 Rat-1 cells shifted to non-permissive temperature for the indicated times, and from v-Src transformed (SRA) and normal chicken embryo fibroblasts (CEF). **B,** Similar to A, except tsLA29 cells were kept at non-permissive temperature for 2 days before activation of v-Src by temperature shift to permissive temperature for the indicated times. **C,** Western blot for protein levels of β-catenin from cells treated as in B.

in SRA cells compared to β-catenin from CEF cells (compare SRA and CEF).

tsLA29 infected Rat-1 cells (tsLA29 Rat-1) were also used as a model to regulate the activity of v-Src, and to assess the ability of v-Src to phosphorylate β-catenin. The tsLA29 mutant Rous sarcoma virus was one of several temperature sensitive mutants that were isolated in a study to elucidate the various functional domains of v-Src (249). It has been shown that the mutant v-Src in Rat-1 cells is activated at the permissive temperature (35^0C) and inactivated at the non-permissive temperature (39.5-40^0C) (243). A single point mutation (P507) was found to be responsible for its temperature sensitive properities (250).

When tsLA29 Rat-1 infected cells that were initially incubated at the permissive temperature were shifted to the non-permissive temperature, β-catenin tyrosine phosphorylation was redueced within 2 hours (Figure 3.3.1, A). Conversely, in tsLA29 Rat-1 cells incubated at the non-permissive temperature, β-catenin was tyrosine phosphorylated at very low levels until shifted to the permissive temperature where β-catenin was tyrosine phosphorylated at 2 hours (Figure 3.3.1, B). A Western blot analysis of whole cell lysates from the experiment in Figure 3.3.1, A was performed and showed that the total protein levels of β-catenin were not dramatically altered by incubation of ts-LA29 Rat-1 cells at either the permissive or non-permissive temperature under the conditions tested (ie. 24 hour experiment). Although it was observed that at the 24 hour time-point the band was less intense in this particular example, subsequent experiments comparing protein levels of

β-catenin after 24 hours at the permissive temperature did not reveal a substantial difference.

The ability of Src to phosphorylate β-catenin was also tested in human SW480 colon adenocarcinoma cells (Figure 3.3.2). SW480 cells contain high levels of β-catenin due to a truncated APC (Figure 1.3) and according to Dehm *et al*. (2004) they express very low levels of endogenous Src protein, although Src RNA was not detected in an earlier study (152,251). 4G10 antibody was again used to probe a Western blot prepared using immunoprecipitated β-catenin from one of the following treatments: untransfected SW480 cells; SW480 cells transiently transfected with the empty plasmid vector pCI; pCI containing the kinase inactive mutant SrcdK298M, or pCI containing the kinase active mutant SrcY530F. In this tyrosine phosphorylated β-catenin was only clearly observed in lysates from SW480 cells transiently transfected with the activated mutant SrcY530F. A Western blot of the whole cell lysates of control and mutant Src transfected cells using the monoclonal Src antibody mAb327 confirmed the expression of mutant Src in SW480 cells (Figure 3.3.2, B).

Athough we were not able to detect a direct interaction between β-catenin and Src (or v-Src) by co-immunoprecipitation, this has been shown *in vitro* (232). Taken together, these results demonstrate that in the presence of activated Src kinase, β-catenin is tyrosine phosphorylated.

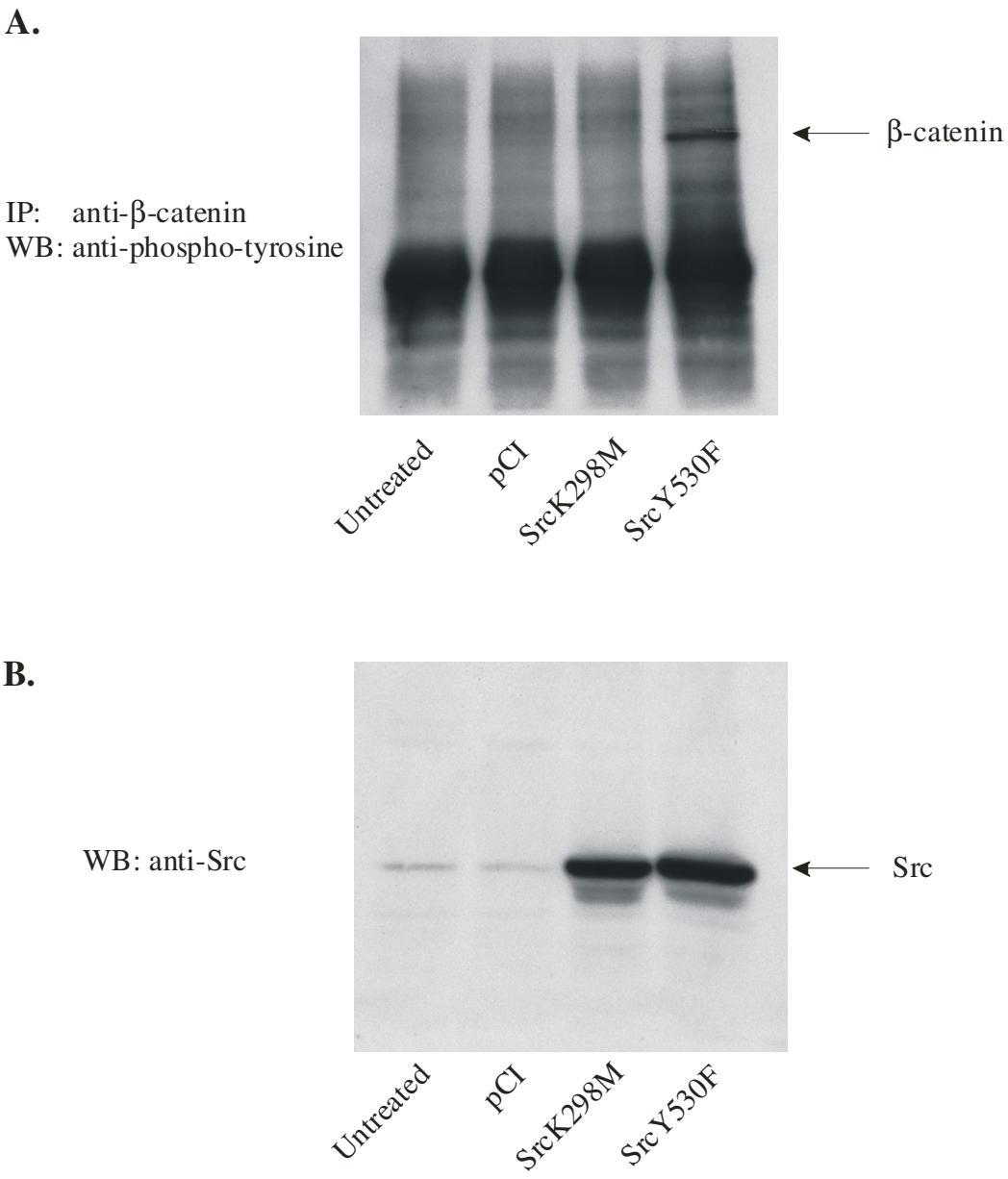


Figure 3.3.2. Phosphorylation of β-catenin in SW480 cells is detectable when cells are transiently transfected with kinase active mutant SrcY530F. Transient transfection of SW480 cells with mutant Src kinases, and controls as indicated, were performed as in previous experiments but scaled up for 10 cm plates (see methods). Cells were then harvested using NP-40 lysis buffer and immune-precipitated (**A**) with anti-β-catenin antibody (Sant Cruz SC-7199, polyclonal rabbit) followed by Western blot analysis using anti-phospho-tyrosine antibody (4G10). **B**, Whole cell lysates were also subjected to direct Western blot analysis and staining for Src expression using anti-Src antibody Mab327.

3.4 Results: β-Catenin Stability

In this section I tested the ability of Src kinase to alter the protein levels of β-catenin. Two cell types were used for this analysis, tsLA29 Rat-1 cells used to regulate v-Src acitivity by temperature shift, and SW480 cells where activated mutant SrcY530F was transiently transfected.

3.4.1 Half-Life of β-Catenin in tsLA29 Rat-1 Cells

tsLA29 Rat-1 cells were used to manipulate mutant v-Src kinase activity and assess its potential effects on β-catenin protein levels in a pulse-chase assay. tsLA29 Rat-1 cells were grown at the permissive temperature (35^0C) and then split into 2 groups, one group of cells to be maintained at the permissive temperature and a second group to be incubated at the non-permissive temperature 39.5-40^0C. Prior to the temperature shift, all cells were pulsed with S^{35} for 3 hours, then chased and harvested at 2 hour intervals (see methods). Densitometry was performed on the scanned image of the autoradiogram of immunoprecipitated β-catenin. The bands that were analysed corresponded to the molecular weight of β-catenin (approximately 94 KDa). A corresponding band was not observed in the lane probed with non-immune immunoglobulin G (IgG), providing greater confidence that the measured band was in fact β-catenin (Figure 3.4.1, A). The results of the densitometry on the scanned image of the autoradiogram using ImageQuant software are shown in Figure 3.4.1, B. The data show a lower amount of radiolabelled β-catenin at earlier time points (2 and 4 hours) when cells were incubated at the permissive temperature

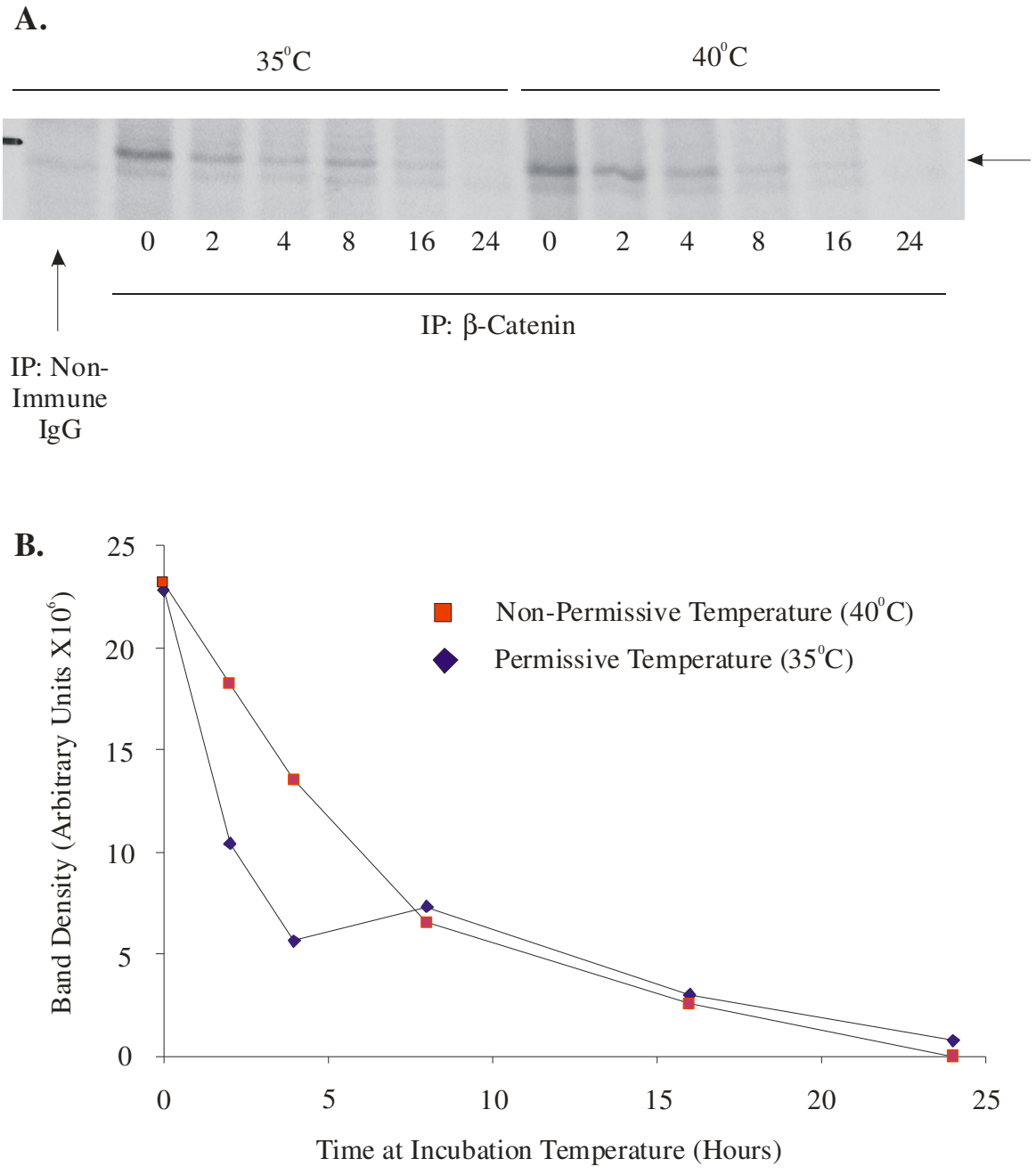


Figure 3.4.1 The half-life of β-catenin in tsLA29Rat-1 cells is not identical when cells are incubated at the permissive or non-permissive temperature. Cells were maintained and seeded at permissive temperature and pulsed with S^{35} Met/Cys for 3 hours prior to starting the experiment. Cells were then chased with media containing 10X non-radioactive Met/Cys, shifted to to 35°C and harvested at the indicated times (hours). Arrow indicates β-catenin. **A**, Autoradiogram of immunoprecipitated β-catenin. **B**, Image-Quant densitometry analysis of bands in A.

(35^0C), compared to when cells are incubated at the non-permissive temperature (40^0C). At later time points, the levels of radiolabelled β-catenin were very similar (from 7.5 hours to 24 hours). An increase in band intensity was observed in the lane loaded with sample from the 8 hour time point compared to the 4 hour time point, suggesting increased incorporation of radioactivty. This was most likely an anomalous result, reflecting possible loading errors, as at later time points the levels of β-catenin were very similar. Due to time constraints, this experiment was not repeated.

3.4.2 β-Catenin Stability in SW480 Cells

To test the possible changes in protein stability due to the presence of Src in the context of human cells, we used the colon adenocarcinoma cell line SW480. A time-course analysis of β-catenin levels in SW480 cells transiently transfected with either empty vector control pCI, or activated mutant SrcY530F was carried out (Figure 3.4.2). Cells were transfected and then harvested every two hours. Western blot analysis of whole-cell lysates were probed for β-catenin (A) or Src (B). Figure 3.4.2, A (SW480 vs. pCI) shows that the levels β-catenin did not dramatically change, based on the relatively similar band intensities at the indicated time points. Also, Figure 3.4.2, B demonstrates the increasing levels of SrcY530F overexpression within the first 6 hours of the experiment. This data shows that the transient transfection of activated SrcY530F into SW480 cells does not dramatically increase or decrease the total protein levels of β-catenin.

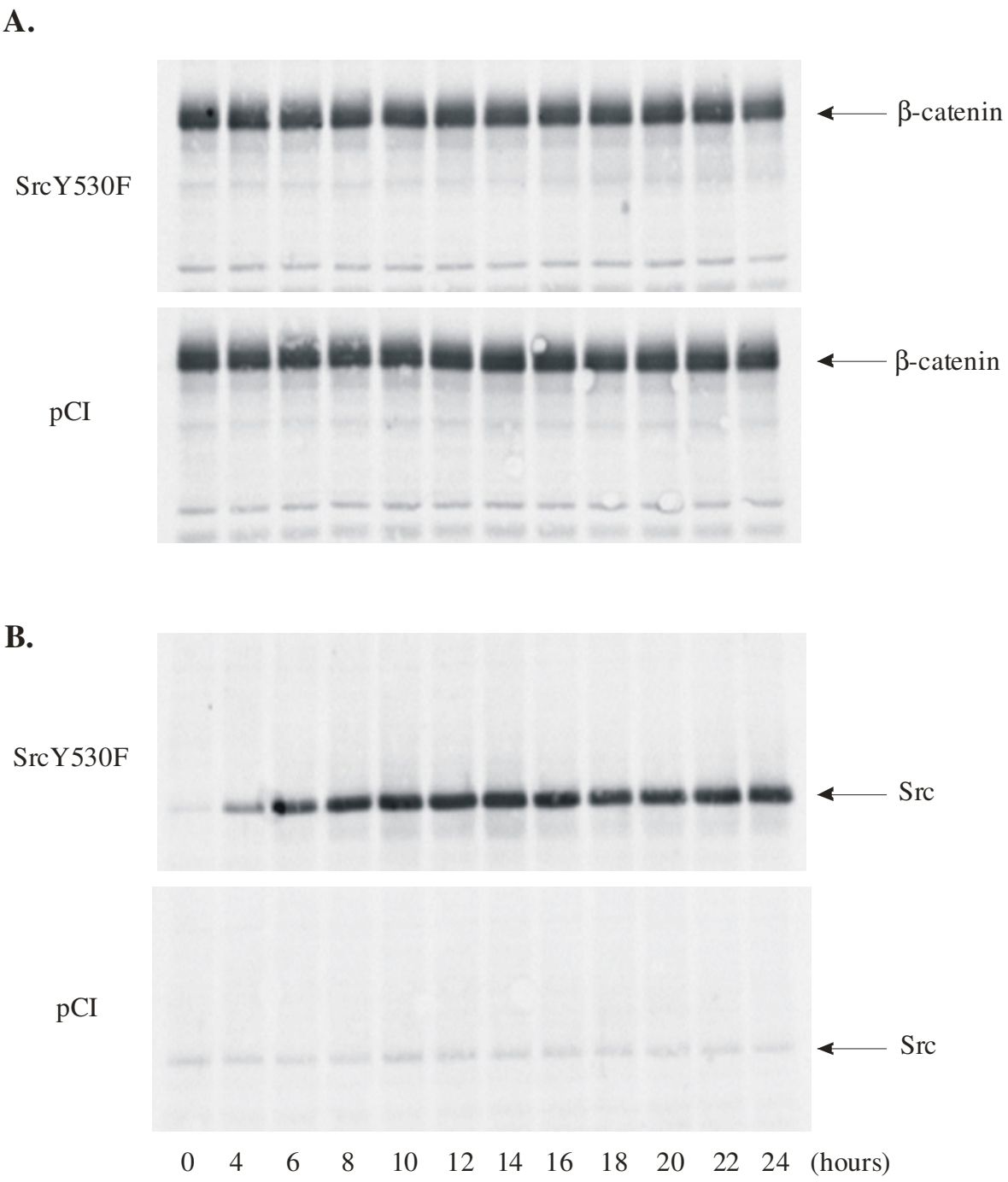


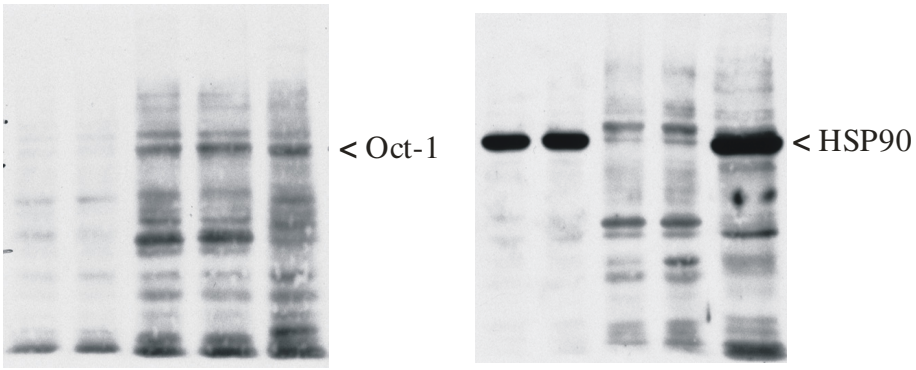
Figure 3.4.2. Total β-catenin protein levels are not altered by activated mutant SrcY530F in SW480 cells. Whole cell lysates from SW480 cells transiently transfected with activated mutant SrcY530F or control pCI harvested every two hours after transfection over a 24 hour period. **A**. Western blot for β-catenin. **B**. Western blot for Src (MAb327 antibody) using the same cell lysates as in A.

3.4.3 Nuclear β-Catenin

An alternative method was used to test the possibility that Src kinase activity induces nuclear β-catenin. This would not be shown by looking at total protein levels and therefore nuclear and cytosolic/membrane fractions were prepared from SW480 cells to measure the potential changes in β-catenin due to Src. Two proteins were used to measure the amount of contamination between cytoplasmic/membrane and nuclear fractions, Hsp90 and Oct-1. Hsp90 is a chaperone protein that functions to prevent non-specific aggregation of proteins and also functions to induce changes in proteins necessary for their subsequent activation and stabilization (252-254). Its localization has been shown to be cytoplasmic (255-257). The nuclear marker used was the ubiquitous transcription factor octamer-motif binding protein, Oct-1, which specifically recognizes the sequence ATGCAAAT (258). This POU domain containing transcription factor contributes to the regulation of various genes including histones, small nuclear RNAs and light and heavy chain immunoglobulins (258,259). The presence of Oct-1 has also been used as a nuclear marker (260).

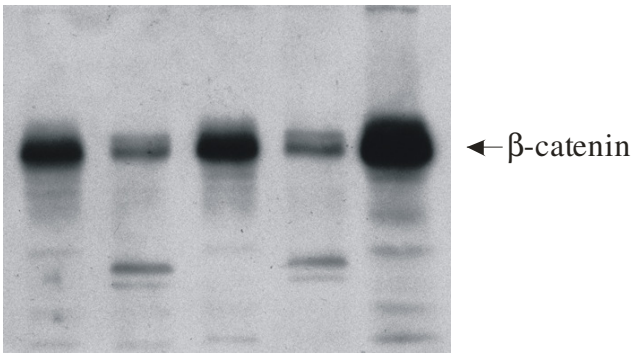
Figure 3.4.3, A shows that SW480 cells were effectively separated into cytoplasmic and nuclear fractions, as measured by the presence of Oct-1 (nuclear marker) or HSP90 (cytosolic/membrane marker) with a small amount of contamination. Equal amounts of lysate from cytosolic/membrane and nuclear fractions were separated by SDS-PAGE followed by Western blot analysis using the same β-catenin antibody used in previous experiments. Based on the knowledge that SW480 cells contain abundant levels of β-catenin, whole cell lysates from SW480 cells was loaded as a control for the identification

A.



SrcK298M	+	-	+	-	-	+	-	+	-	-
SrcY530F	-	+	-	+	-	-	+	-	+	-
Cyto. Fraction	+	+	-	-	-	+	+	-	-	-
Nuclear Fraction	-	-	+	+	-	-	-	+	+	-
Jurkat Control	-	-	-	-	+	-	-	-	-	-
HeLa Control	-	-	-	-	-	-	-	-	-	+

B.



SrcK298M	+	+	-	-	-
SrcY530F	-	-	+	+	-
Cytoplasmic Fraction	+	-	+	-	-
Nuclear Fraction	-	+	-	+	-
SW480 WCL Control	-	-	-	-	+

Figure 3.4.3 Kinase active mutant SrcY530F does not substantially alter nuclear levels of β-catenin in SW480 cells compared to kinase inactive SrcK298M. A. Transiently transfected SW480 cells were separated into cytoplasmic and nuclear fractions as indicated by Oct-1 (nuclear marker) or HSP90 (cytoplasmic marker) levels. Control lysates for Oct-1 (Jurkat nuclear extract) and HSP90 (HeLa whole cell lysate) were obtained from Santa Cruz Biotechnology Inc. **B.** Western blot for β-catenin from cytoplasmic and nuclear fractions of SrcK298M and SrcY530F (as indicated). SW480 cells with endogenously high levels of total β-catenin used as control for β-catenin.

of β-catenin. The blot shows a slightly darker β-catenin band in nuclear fractions from SW480 cells transfected with activated SrcY530F, compared to nuclear fractions from SW480 cells from kinase inactive SrcK298M transfected cells (Figure 3.4.3, B). However, repeated trials and statistical analysis of the resulting data are necessary in order for a more meaningful interpretation.

These data show that in tsLA29 Rat-1 cells the stability of β-catenin is not substantially affected after 24 hours due to the activation of mutant v-Src. Similarly, in SW480 cells the total protein levels of β-catenin are not altered by transient transfection of activated SrcY530F over a 24 hour period. In addition, the transient transfection of SrcY530F into SW480 cells did not dramatically alter the nuclear levels of β-catenin compared to SrcK298M transfected cells.

CHAPTER 4: TRANSCRIPTIONAL REGULATION OF WNT SIGNALING

4.1 Hypothesis & Rationale:

Canonical Wnt signaling regulates a number of genes required throughout development, and changes in the activation of Wnt target genes is a fundamental mechanism by which Wnt signaling affects change. Therefore, we hypothesized that Src can modulate the transcriptional potential of Wnt signaling. To measure transcriptional changes in Wnt signaling, I used two sets of luciferase based Wnt reporters, and analysed changes in the transcription of previously identified Wnt target genes was conducted.

4.2 Results

4.2.1 SrcY530F Downregulates Wnt Luciferase Reporter Activity

The first Wnt luciferase reporters tested were TopFlash and its negative control reporter FopFlash. These were obtained from the Randall Moon (University of Washington), but designed and engineered in the laboratory of Hans Clevers (Utrect, Netherlands) (245). TopFlash contains 3 repeats of the optimal Tcf DNA binding motif with the sequence CCTTTGATC upstream of the luciferase gene, while the negative control reporter FopFlash contains the sequence CCTTTGGCC (245). When cells possess active Wnt signaling, functional luciferase is expressed. Analysis of luciferase activity from harvested lysates can then be used as a measure of transcriptional regulation (see methods). Thus, the amount of active Wnt signaling can then be measured by the relative amount of luciferase activity.

To measure the potential for Src to regulate Wnt signaling, tsLA29 Rat-1 cells and control Rat-1 fibroblasts were transiently transfected with either TopFlash or FopFlash and β-galactosidase, which was used as an internal control for transfection efficiency. Cells were then harvested after incubation at the permissive temperature (35^0C) or the non-permissive temperature ($39.5\text{-}40^0$C) according to the schedule described in Figure 4.2.1. The data show that the luciferase activity from the negative control reporter FopFlash was greater than the luciferase activity in cells transfected with TopFlash. A similar result was observed upon repeating the experiment (data not shown). This data suggested that background activity was too high, and is likely a cell-line specific effect. To ensure that the Top- and FopFlash luciferase reporter system was functioning as previously described, they were transiently transfected into SW480 colorectal adenocarcinoma cells. As previously mentioned, this cell line contains a truncated non-functional APC and exhibits constitutive Wnt signaling, and the ability of transiently transfected wt APC to inhibit reporter activity in these cells demonstrates the usefulness of this reporter system (245). Figure 4.2.2 (pCI) shows that Topflash activity is substantially higher than FopFlash activity by approximately 13 fold (TopFlash/FopFlash). Therefore, the Top- and FopFlash reporters do function as previously shown.

When the kinase inactive mutant SrcK298M or the kinase active mutant SrcY530F were transiently co-transfected into SW480 cells, TopFlash reporter activity was decreased compared to when cells were transfected with empty vector (pCI). Lysates from kinase active (SrcY530F) showed the greatest decrease in luciferase activity (Figure 4.2.2).

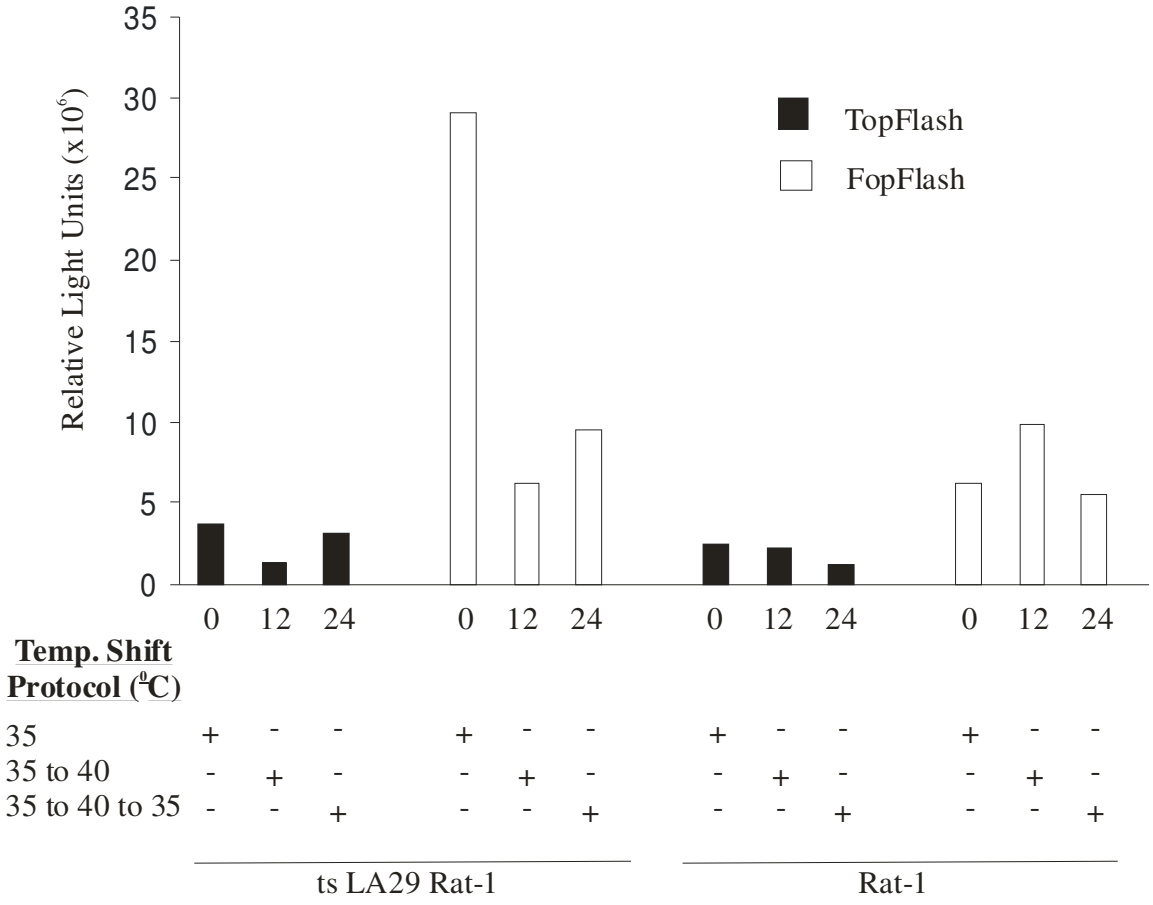


Figure 4.2.1. Noticeably high background levels of luciferase activity in temperature shifted tsLA29 Rat-1 cells. Temperature sensitive mutant v-Src tsLA29 Rat-1 cells or control Rat-1 cells were transiently transfected with the canonical Wnt signaling pathway reporters TopFlash or the negative control FopFlash. After a 24 hour transfection period at 35^{0}C (permissive temperature), cells were harvested (0) with Cell Culture Lysis Reagent (CCLR, Promega) or shifted to the non-permissive temperature 39.5-40^{0}C for 12 hours and harvested (12), or incubated back at 35^{0}C for an additional 12 hours (24) followed by cell lysing. At the end of the experiment, all cells were prepared for luciferase assays as described (see Methods, Luciferase Assays with β-Gal).

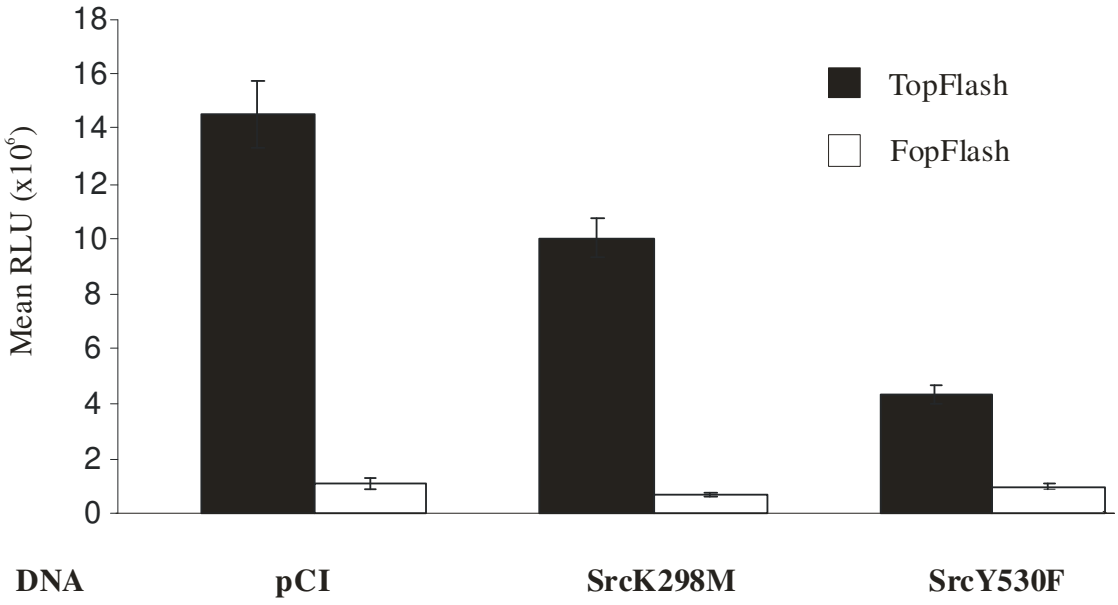


Figure 4.2.2 TopFlash luciferase activity in SW480 colon adenocarcinoma cells is downregulated by mutant Src kinases. Luciferase readings were normalized to β-gal activity by co-transfection of β-gal plasmid, pCH110 (see methods) with Src mutants. Data represents mean luciferase activity from triplicate samples of Topflash or Fopflash and mutant Src kinase transfected SW480 cells. RLU, relative light units. Error bars indicate standard deviation.

4.2.2 SrcY530F Downregulates Super8XTopFlash Activity

A second Wnt signaling luciferase reporter was obtained from the Moon laboratory, and used in SW480 cells to measure the effect of Src kinase on Wnt signaling in order to confirm the observations seen with the TopFlash reporter. This second set of luciferase reporters, Super8XTopFlash and Super8XFopFlash, were designed based on the original TopFlash reporter, but contains 8 Tcf/Lef binding sites (bold) joined by a short linker oligo (**AGATCAAAGG**GGGTA), and exhibits a several hundred fold difference in luciferase activity between Super8XTopFlash and Super8XFopFlash (the negative control) activity (246). The optimal binding sites were inserted in the pTA-Luc plasmid vector backbone (Figure 2.2, A). We tested this reporter pair in both SW480 cells and DLD-1 cells, both of which have been used as positive control cell lines for canonical Wnt signaling due to non-functional APC molecules which result in particularly high levels of β-catenin (245). Figure 4.2.3 A and B (pCI) shows that Super8XTopFlash activity exhibits a several-hundred fold activation above the negative control Super8XFopFlash activity in these cells as expected. It was also observed that in both cell lines transient transfection of activated mutant SrcY530F was able to downregulate reporter luciferase activity, whereas luciferase activity was also downregulated by transient transfection of SrcK298M in DLD-1 cells but not SW480 cells (Figure 4.2.3, A and B).

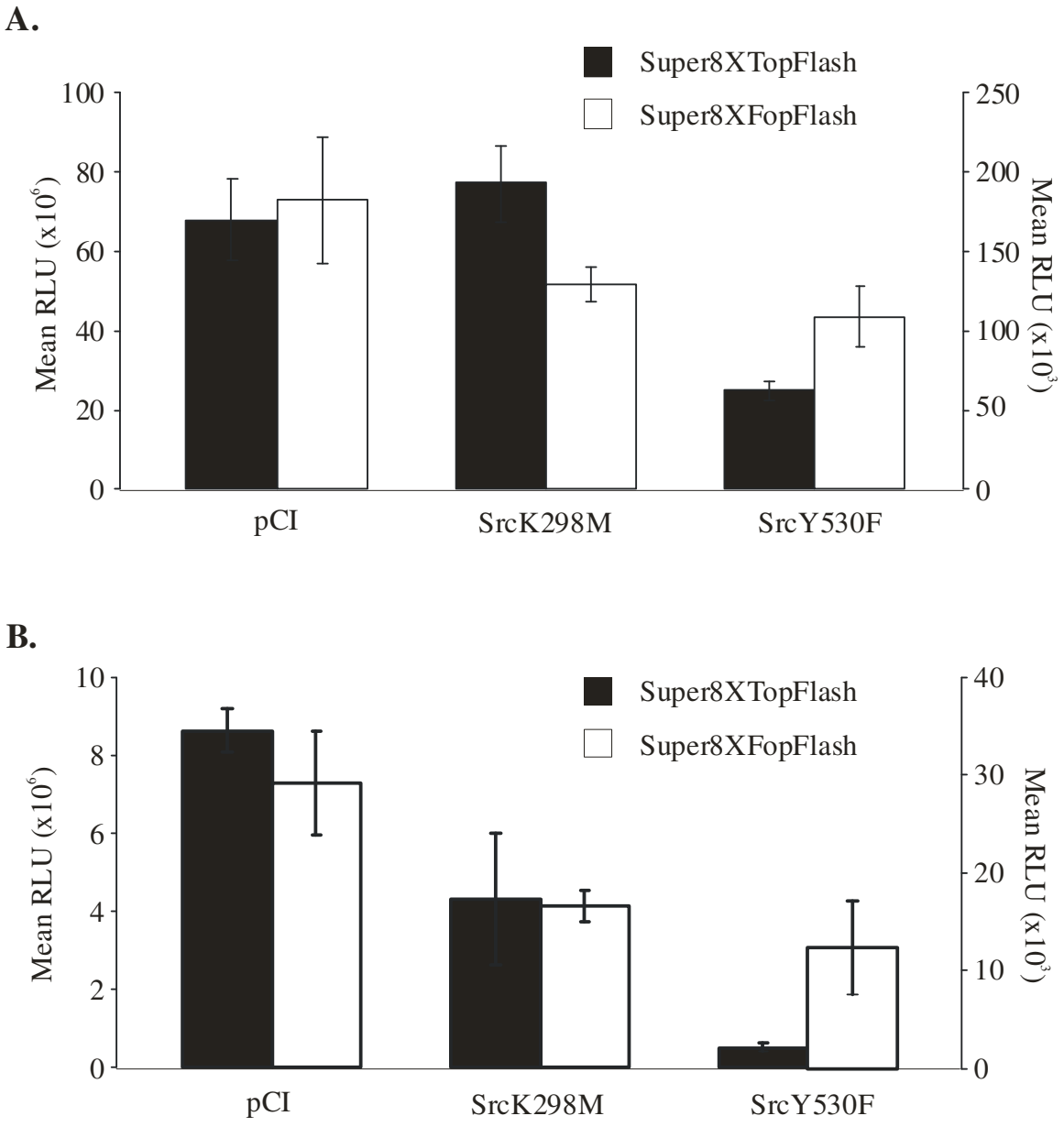


Figure 4.2.3 Activated mutant SrcY530F can downregulate Super8XTopFlash activity in SW480 and DLD-1 cells. A. SW480 cells transiently transfected with equal amounts (1 μg each) of reporter (Super8XTopFlash or Super8XFopFlash) and Src mutant (SrcK298M or SrcY530). **B.** DLD-1 cells transfected as in A. Data represents mean luciferase activity from 3 individually transfected wells. RLU, relative light units. Error represents standard deviation. Scale bars for Super8XTopFlash on left. Scale bars for Super8XFopFlash on right.

4.3 Results

4.3.1 Luciferase Protein Levels

To confirm that transfection of activated mutant SrcY530F in SW480 cells was affecting the reporter expression and not luciferase enzymatic activity, the protein levels of luciferase in SW480 cells transiently transfected with mutant Src kinases and Super8XTopFlash were analysed (Figure 4.3.1, A). Lane 3 (activated mutant SrcY530F) shows a decreased level of luciferase signal compared to lanes 1 and 2 (pCI vector alone and kinase inactive mutant SrcK298M). When densitometry using ImageQuant software was performed on a scanned image of this film, the band density of luciferase in the SrcY530F lane was reduced to approximately 60% the band density of luciferase in the pCI lane (Figure 4.3.1, B). This data shows that the transient transfection of activated mutant SrcY530F into SW480 cells is negatively affecting protein levels of luciferase, which most likely accounts for the lower activity from harvested lysates.

4.3.2 Regulation of EGFP-C1 Expression

To confirm that Src was not inducing a non-specific generalized downregulation of co-transfected reporter plasmids, SW480 cells were transiently co-transfected with green fluorescent protein (EGFP-C1, figure 2.5 page 64) and either empty vector control (pCI) or activated mutant SrcY530F. Cells were harvested and analyzed by Western blotting using GFP antibody. When densitometry of protein bands was performed, no dramatic difference was observed between protein levels (as measured by band density) of GFP in cells transfected with pCI compared to cells transiently transfected with SrcY530F (Figure 4.3.2, B).

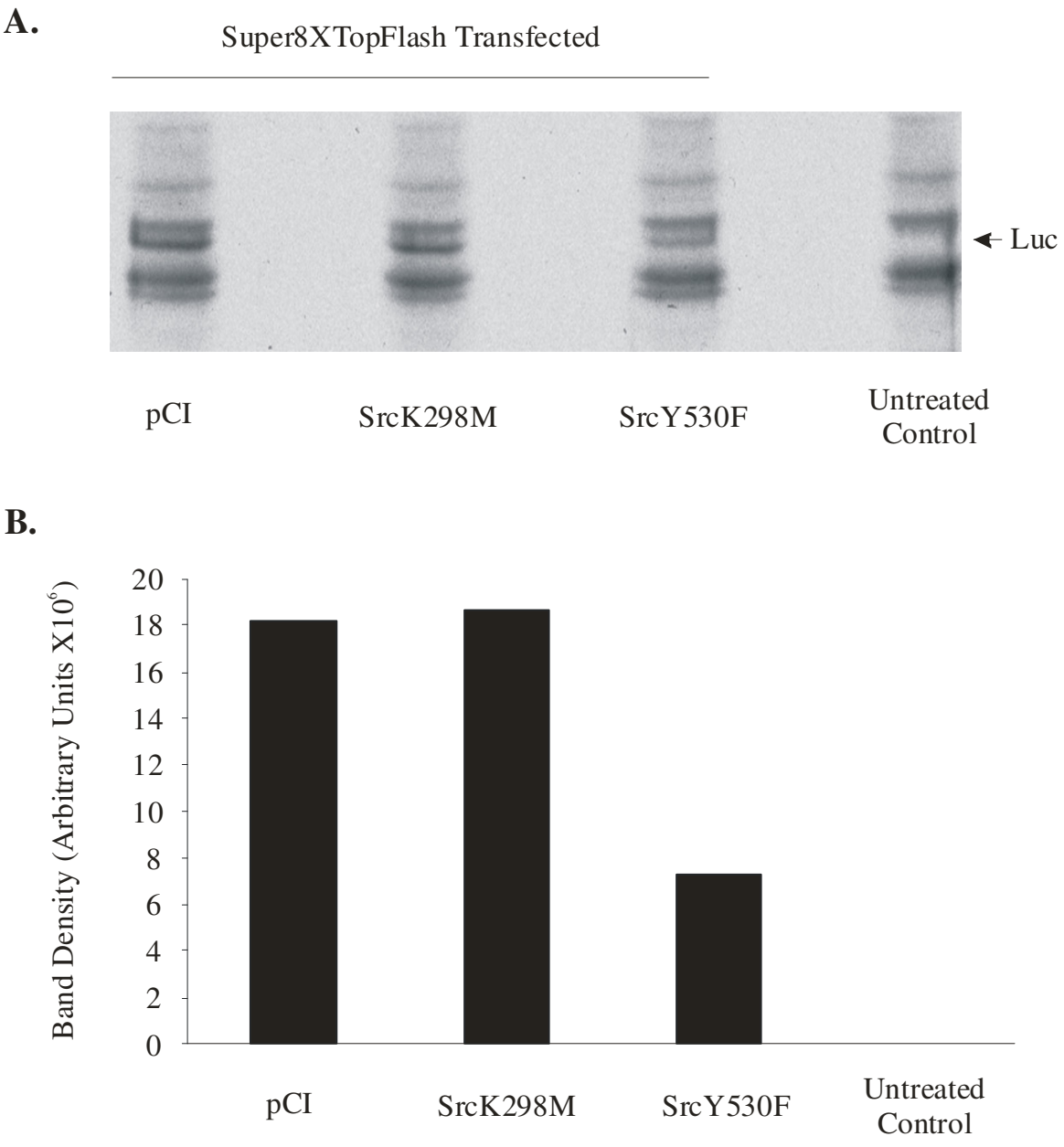


Figure 4.3.1 Luciferase protein levels are reduced in SW480 cells transiently transfected with kinase active mutant SrcY530F. SW480 cells were transiently transfected with Super8XTopFlash and mutant Src kinases or empty vector control pCI, then harvested using CCLR after 24 hours (see methods). Blots were incubated with Promega purified anti-luciferase goat antibody polyclonal antibody diluted to 1:1000. A, Western blot analysis for luciferase protein. B, Densitometry of band corresponding to molecular weight of luciferase, not present in untransfected lane .

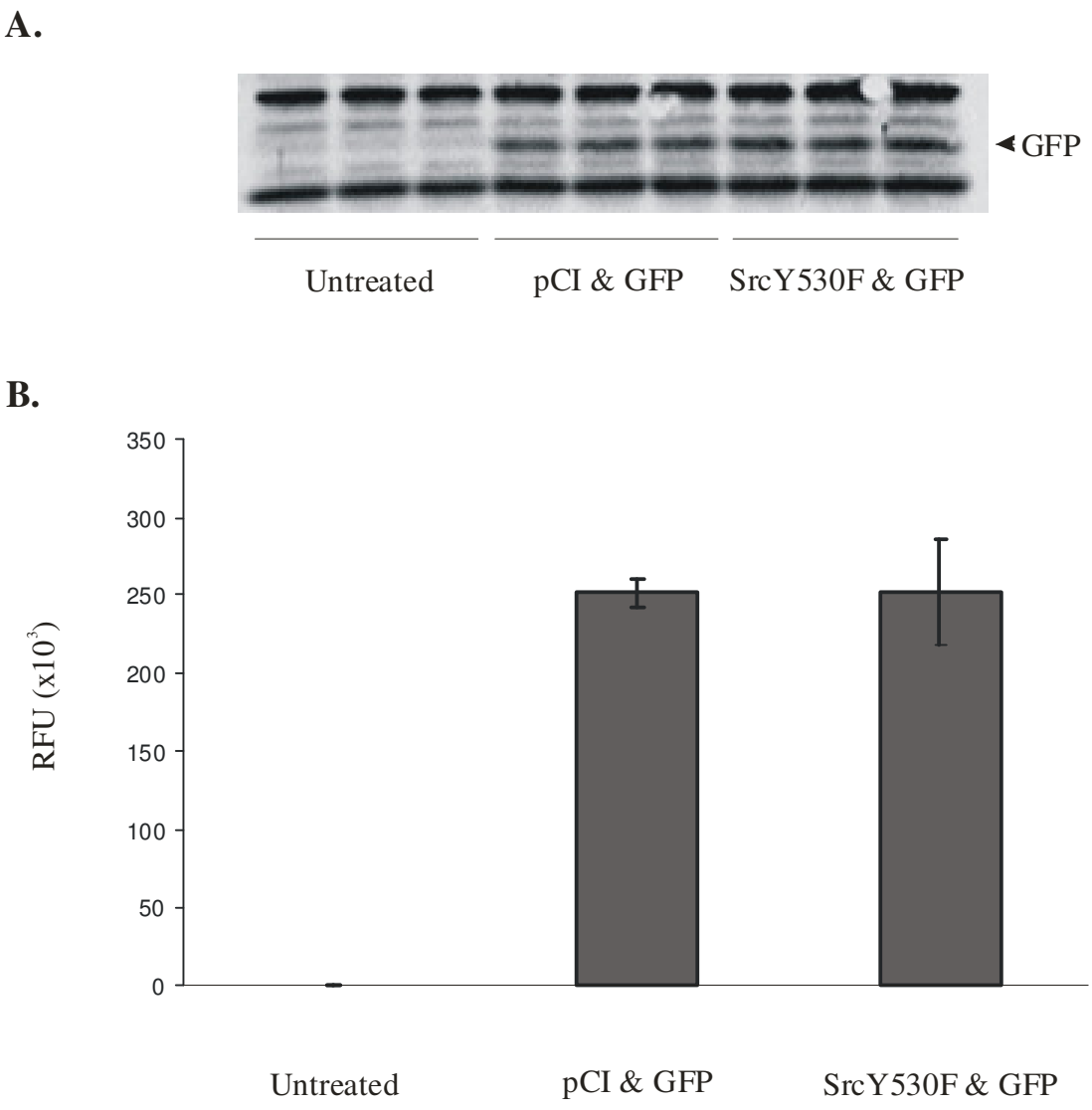


Figure 4.3.2 Transiently transfected activated mutant SrcY530F in SW480 cells does not downregulate the protein levels of green fluorescent protein. SW480 cells were untreated, or transiently co-transfected with equal amounts of empty vector, pCI, or activated mutant SrcY530F and the vector pEGFP-C1 (Clontech). Experiments were performed in triplicate and lysates were used for western blot analysis (**A**). Analysis of blot was performed using the Storm gel and blot imaging system, followed by densitometry using ImageQuant software. **B**, mean RFU from three measures of band density in A. RFU, relative fluorescence units, arbitrary.

4.4 Regulation of Endogenous Wnt Target Genes *FGF-18, CCND1 & MYC*

An alternative to using reporter plasmids as a means to measure regulation of Wnt mediated transcription is to analyse changes in the expression of previously identified Wnt gene targets. Three target genes were chosen, including *FGF18*, *CCND1* (cyclin D1) and *MYC*. Similar to *CCND1* and *MYC*, *FGF18* was chosen because it was shown to be a target of Wnt/β-catenin signaling in colorectal tumor cell lines (247). To confirm that *FGF18* expression could be used to measure Wnt signaling in SW480 cells transiently transfected with Src mutants, siRNA (Dharmacon) was first used to target β-catenin. Treatment of SW480 cells with β-catenin siRNA followed by transient transfection with mutant Src kinases resulted in effective downregulation of β-catenin levels (Figure 4.4.1, A). It was observed that the non-targeting siRNA was able to downregulate β-catenin levels compared to untreated control as well, although not to the same degree as β-catenin siRNA. This effect was not different in SrcK298M transfected cells compared to SrY530F transfected cells. Primers for *FGF18* and *GAPDH* DNA were synthesized based on the sequence described in Shimokawa *et al.* (2003), and multiplex rtPCR was performed using RNA harvested from SW480 cells transfected with SrcK298M or SrcY530F (247) (Figure 4.4.1, B). These data confirm that *FGF18* expression can be regulated by treatment SW480 cells with siRNA technology, and strongly suggests that *FGF18* is a target of Wnt/β-catenin signaling in SW480 cells. These primers were then used to perform real-time PCR on RNA harvested from SW480 cells transiently transfected with SrcK298M and SrcY530F. Relative quantitation was performed on the data, showing that activated SrcY530F does not alter the expression of *FGF18* in SW480 cells relative to pCI or the kinase inactive mutant

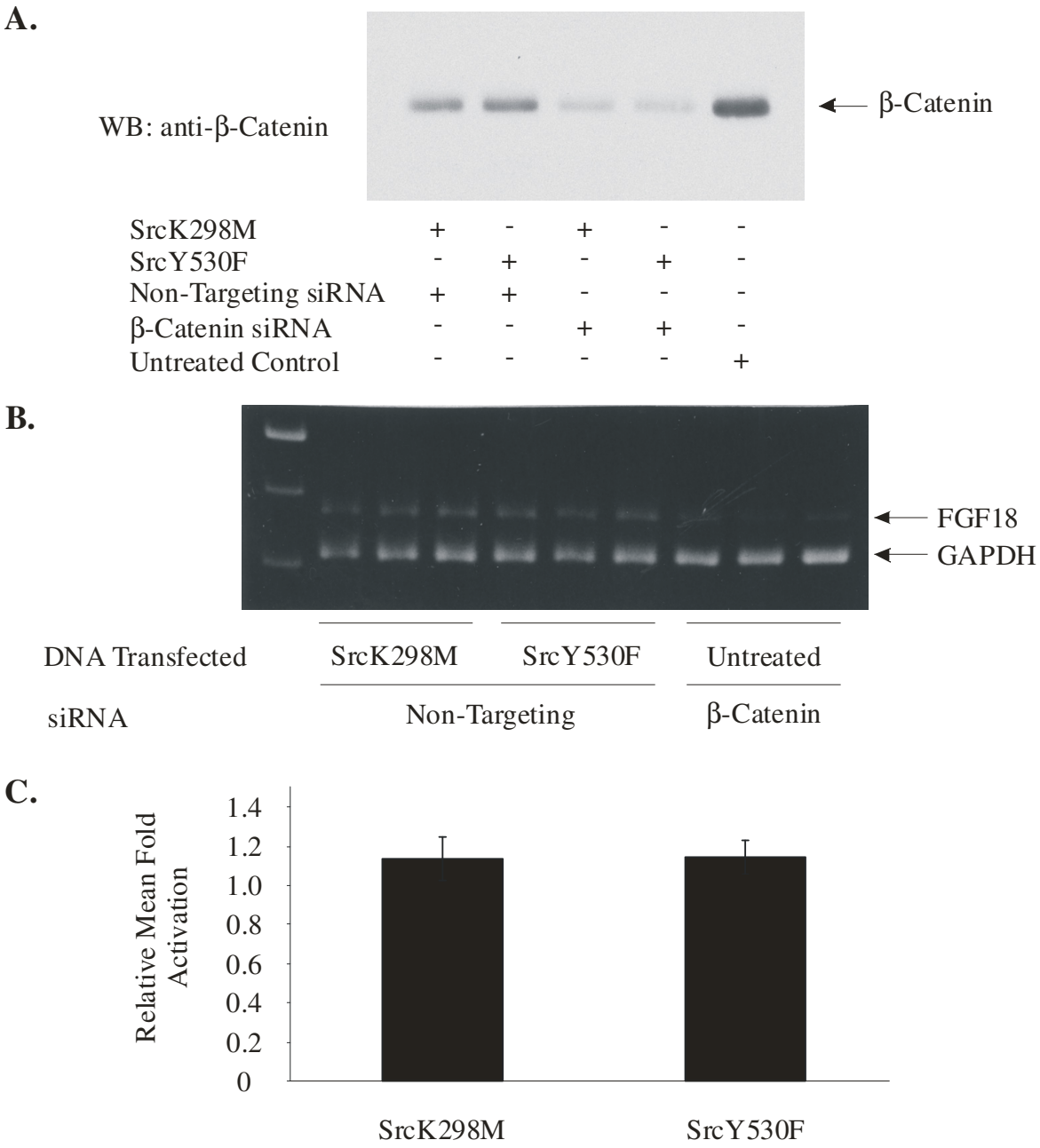


Figure 4.4.1 *FGF18* is a target of Wnt/β-catenin signaling but the relative expression of *FGF18* in SW480 cells is not affected by transiently transfection with kinase inactive mutant SrcK298M or kinase active mutant SrcY530F. A. Western blot analysis of lysates for β-catenin from SW480 cells treated with siRNA targeting β-catenin and transfected with mutant Src kinases as indicated. **B**. Multiplex PCR analysis of transcripts for FGF18 and GAPDH using RNA harvested from SW480 cells transfected as indicated and treated with β-catenin siRNA or non-targeting control. **C.** Relative quantitation of the Wnt target gene FGF18 levels by real-time r.t.PCR in SW480 cells transfected with mutant Src kinases. Values represent the relative amount of FGF18 transcript in mutant Src transfected cells compared to amount of FGF18 in empty vector transfected cells.

SrcK298M (Figure 4.4.1, C).

The cyclin D1 gene, *CCND1*, and the c-Myc gene, *MYC*, are associated with various tumour types and have also been shown to be upregulated by Wnt signaling (261-263). We therefore measured the ability of transiently transfected Src mutants to regulate the expression of these genes in SW480 cells. Real-time PCR analysis was again used to measure the relative expression in SW480 cells transfected and harvested in the same manner as in the previous experiments with FGF18. β-Catenin expression was also analysed. Primer pairs and TaqMan probe sets provided (in the case of *CTNNB1* and *CCND1*) by or purchased (in the case of MYC) from Applied Biosystems. Analysis of the data by the comparative C_t method shows that the activated mutant SrcY530F transiently transfected into SW480 cells does not downregulate any of the three genes, *CTNNB1*, *CCND1* and *MYC* relative to pCI transfected cells (Figure 4.4.2). As shown, none of the genes examined displayed a significant change relative to gene expression in control transfected SW480 cells, similar to what was observed with *FGF18*.

4.5 Src kinase and Changes at the Promotor Complex

Although there were no observable changes in the Wnt target genes tested, the possibility that Src could regulate other Wnt target genes could not be excluded. Based on the observations using the Wnt reporters (TopFlash and Super8XTopFlash), Src may be altering the components of the transcriptional complex which, in-turn, may lead to a downregulation of luciferase transcription. To test this hypothesis, SW480 cells were transiently transfected with either kinase inactive mutant SrcK298M or kinase active mutant SrcY530F and nuclear fractions were used for gel-shift/electromobility shift assays

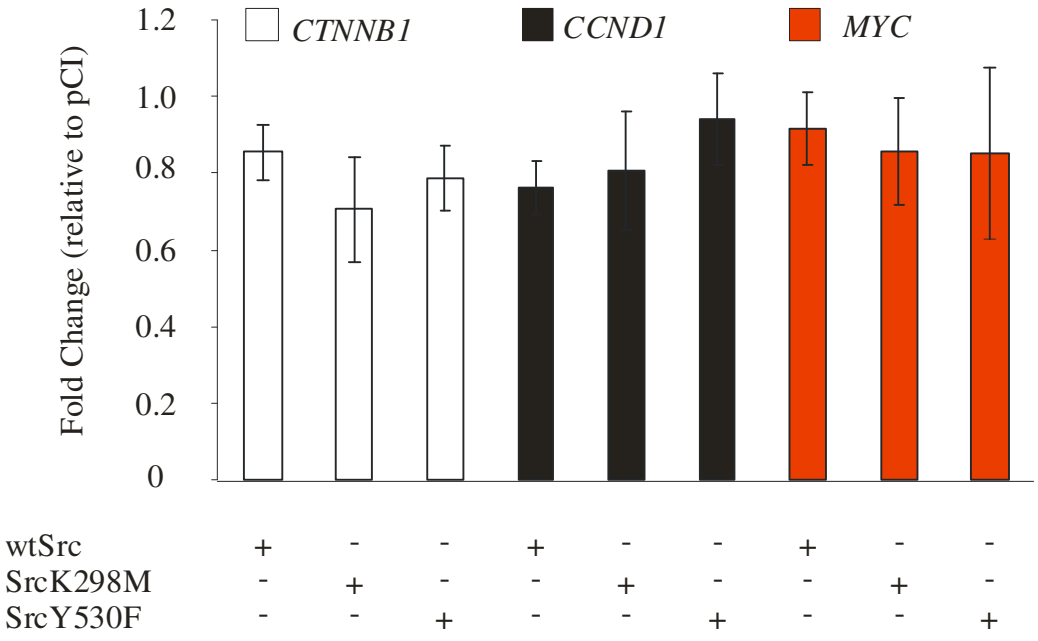


Figure 4.4.2 SW480 cells transiently transfected with wt or mutant Src kinases does not alter the expression of *CTNNB1*, or the Wnt target genes *CCND1* and *MYC* relative to controls. Data represents level of gene (as indicated) expression in cells transfected with wt or mutant Src kinases relative to levels of gene expression in SW480 cells transfected with empty vector pCI. Real-time PCR was performed using TaqMan primer pairs and probe sets and data analysed by the comparative C_t method using Applied Biosystems' SDS software.

(EMSA). Biotinylated sense and antisense oligos were designed based on the TopFlash Tcf/Lef consensus sequence (see methods) and synthesized at the University of Calgary DNA services facility. Figure 4.5, A shows that the nuclear fraction was prepared without significant cytosolic/membrane contamination. Nuclear fractions from cells transiently transfected with either inactive mutant SrcK298M or activated mutant SrcY530F were probed with a biotin labeled DNA duplex possessing the Tcf/Lef consensus sequence used in the TopFlash reporter (see methods). As shown in Figure 4.5, B there was no dramatic change in the banding pattern, although the bands in SrcY530F nuclear lysates appeared darker overall. One band (1b) appeared particularly darker than the corresponding band in SrcK298M (1a) nuclear lysates, while another band was observed in nuclear lysate from SrcK298M transfected cells (2a), but not in nuclear fractions from SrcY530F transfected cells (2b). When a 10 fold excess of unlabelled probe was added to a separate mixture of labeled probe and nuclear fraction (N.F.), some of the same bands were observed suggesting a small degree of non-specific binding. Nonetheless, this data does not demonstrate a dramatic change occurring at the transcriptional complex that would explain the effect of activated SrcY530F on the Super8XTopFlash reporter.

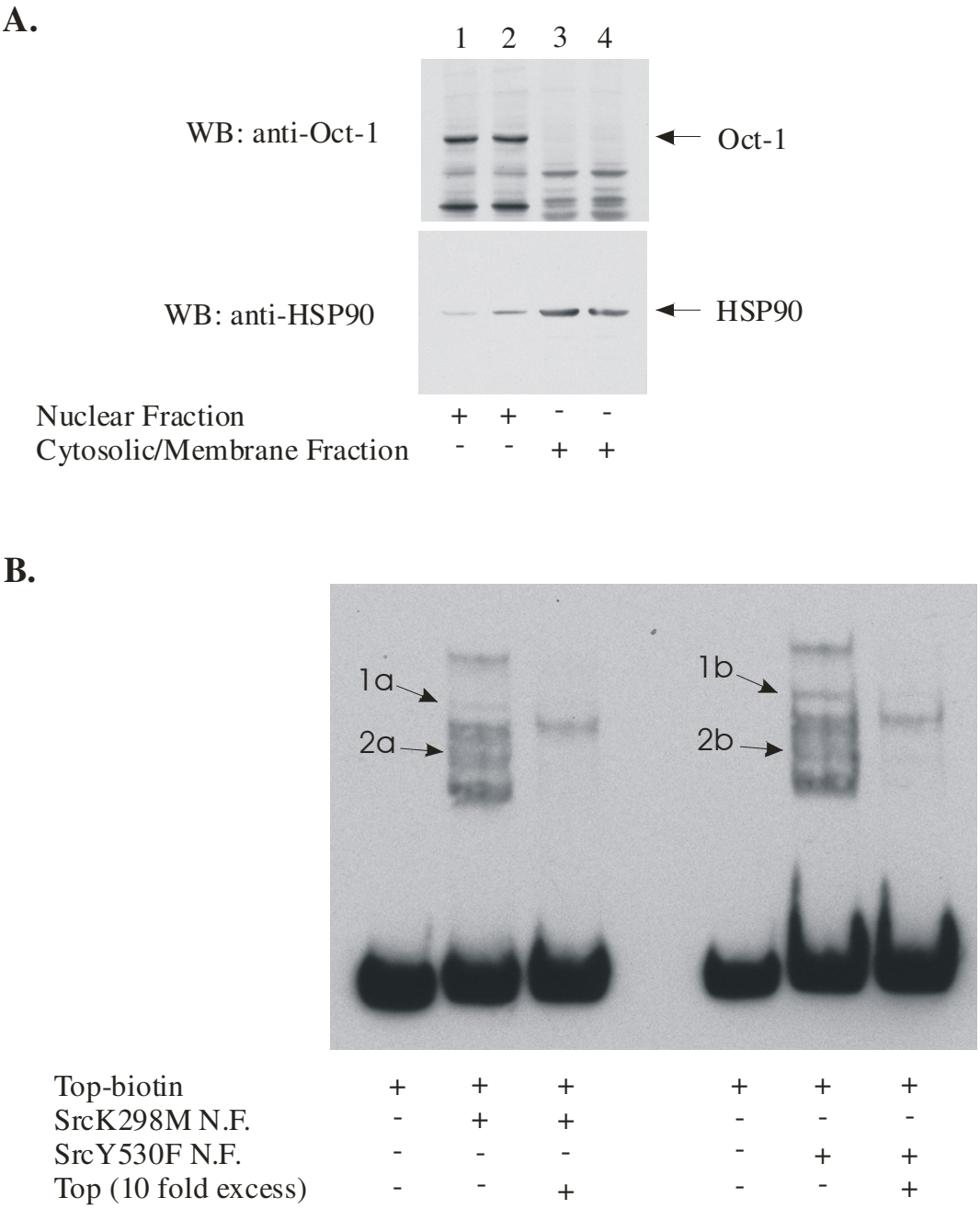


Figure 4.5 Kinase active mutant SrcY530F does not dramatically alter the composition of transcription complex molecules. A. Western blot for the nuclear marker Oct-1 (top) or the cytoplasmic marker HSP90 (bottom) from nuclear and cytoplasmic/membrane fractions. **B.** Biotin labelled Tcf/Lef consensus sequence duplex (Top-biotin) was subjected to non-denaturing electrophoresis alone, or after incubation with nuclear fractions (N.F.) from mutant Src (as indicated) transfected SW480 cells, or in combination with nuclear fractions and a 10 fold excess of unlabelled duplex. Arrows indicate bands that were particularly different intensities (1a vs 1b, 2a vs 2b).

CHAPTER 5: EVALUATING MECHANISMS OF REPORTER DOWNREGULATION BY SRC KINASE

5.1 Rationale

To identify the mechanism by which Src is downregulating the Wnt reporters, several approaches were undertaken. These include the use of β-catenin mutants, inhibition of GSK3β and analysis of non-canonical Wnt/Ca^{2+} pathways. Phosphorylation of β-catenin by Src leads weakens its interaction with E-cadherin and the adherens junction (114,115,232). Tyrosine phosphorylation of β-catenin may also weaken its interation with Tcf/Lef co-transcription factors, leading to a down-regulation of transcriptional activation. To test this possibility, mutant β-catenins which are not tyrosine phosphorylated were engineered.

5.2 Results

5.2.1 Wnt Reporter Response to Mutant β-Catenins.

In this section (5.2) I tested the hypothesis that tyrosine phosphorylation of β-catenin by Src kinase inhibits its ability to promote transcription. To test this hypothesis, β-catenins mutants were synthesized and co-transfected with activated mutant SrcY530F. Figure 3.3.2 shows that transient transfection of activated mutant SrcY530F is able to induce the tyrosine phosphorylation of β-catenin in SW480 cells. However, the high levels

of endogenous β-catenin in this cell line would not allow us to easily study the effects of mutant β-catenins.

In a Western blot screen of various mammary tumour cell lines, β-catenin was detected in all the cell lines tested but was substantially less abundant in SK-BR-3 cells (Figure 5.2.1, A). Therefore, this cell line was chosen for Super8XTopFlash experiments in combination with the β-catenin mutants. When wt β-catenin (Open Biosystems) was transiently transfected into SK-BR-3 cells, it was detectable by Western blot analysis following immunoprecipitation (Figure 5.2.1, B right panel, lanes 1 & 3). Similarly, phosphorylation of β-catenin was detected only when both activated SrcY530F and wt-β-catein was transfected into SK-BR-3 cells (Figure 5.2.1, B left). Consistent with these results, Super8XTopFlash luciferase activity was elevated above the control (empty vectors pCI & pCMV-SPORT6) when cells were overexpressing wt β-catenin (Figure 5.2.2, compare first and third columns). When the activated mutant SrcY530F was transiently transfected with wt β-catenin and Super8XTopFlash into SK-BR-3 cells, the luciferase activity of harvested lysates was decreased relative to the luciferase activity from lysates of SK-BR-3 cells transiently transfected with the empty vector pCI, wt β-catenin and Super8XTopflash (Figure 5.2.2, compare third and fourth columns). A small reduction in luciferase activity was also observed when SK-BR-3 cells were transfected with SrcY530F and Super8XTopFlash, compared to SK-BR-3 cells transfected with pCI, pCMV-SPORT6 and Super8XTopFlash (Figure 5.2.2, compare columns 1 & 2). Taken together these data suggest that the effect of SrcY530F on the Wnt reporter Super8XTopFlash in SW480 cells can be replicated in SK-BR-3 cells.

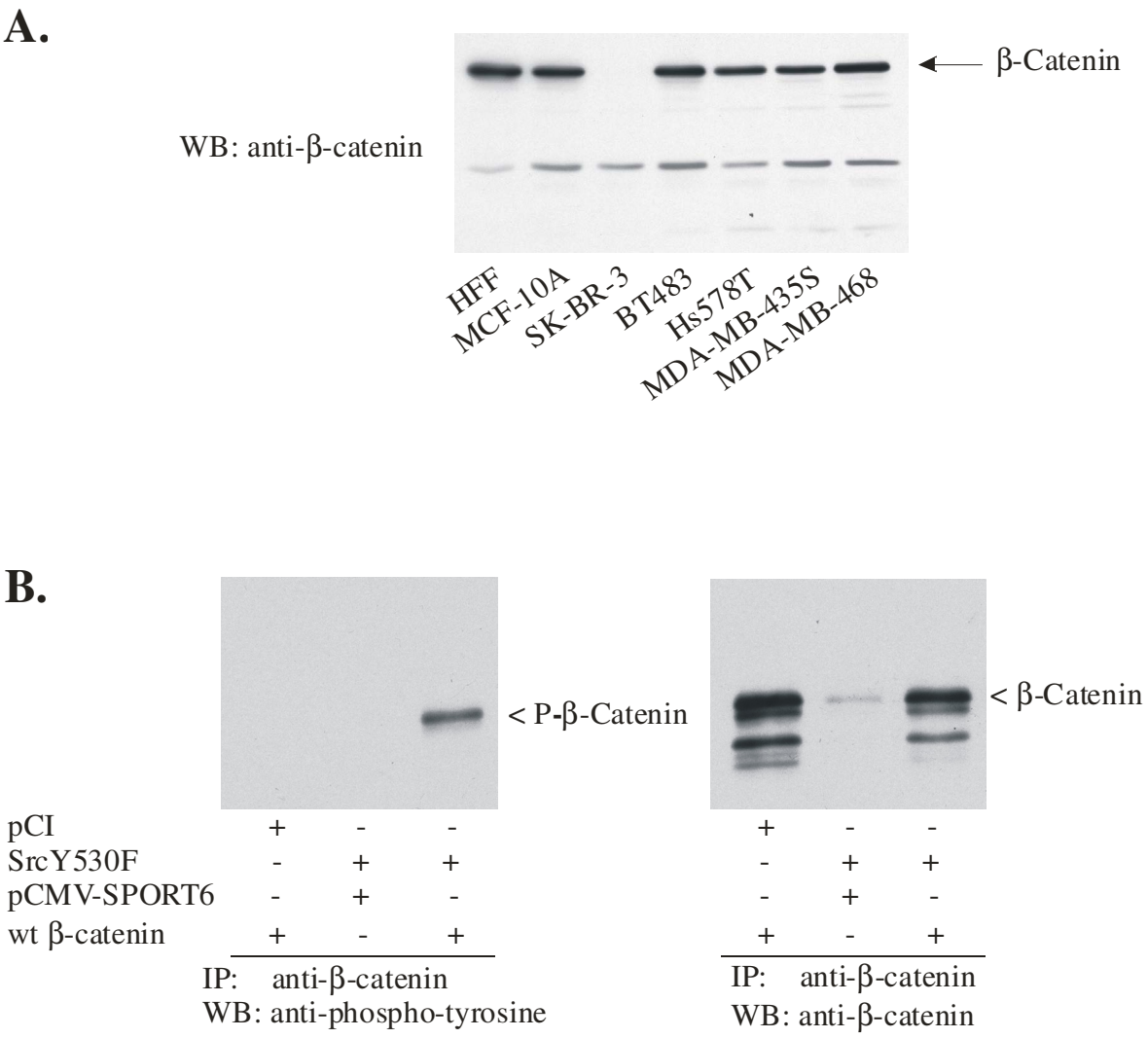


Figure 5.2.1 SK-BR-3 cells possess low levels of endogenous β-catenin. A. Western blot (WB) analysis of total β-catenin levels in various breast cancer cell lines. **B.** WB using anti-phospho-tyrosine antibody (4G10) of immunoprecipitated (IP) β-catenin (SC-7199) from SK-BR-3 cells transiently transfected with or without activated mutant SrcY530F and with or without wt β-catenin as indicated. **B.** (right), Western blot of β-catenin (SC-7199) immunoprecipitated (C19220-050, new 610153 BD Transduction Labs) from the same lysate as in left panel. Empty vectors pCI and pCMV-SPORT6.

To explore the potential of Src induced tyrosine phosphorylation of β-catenin as a mechanism to regulate Wnt signaling in SK-BR-3 cells, tyrosine to phenylalanine mutants were engineered and used for Super8XTopFlash reporter assays. Single and double β-catenin mutants (Y86F, Y654F and Y86,654F) were transiently co-transfected into SK-BR-3 cells along with activated mutant SrcY530F (Figure 5.2.3 A). Immunoprecipitation of lysates using β-catenin antibody (rabbit polyclonal) followed by immunoblotting for β-catenin using a second β-catenin antibody (mouse monoclonal), showed that all the mutant β-catenins were expressed to a similar degree (Figure 5.2.3, A bottom panel lanes 1, 3-5). A band corresponding to β-catenin was observed in the control lane, which was attributed to well-loading error. When immunoprecipitation of SK-BR-3 lysate harvested from cells transfected with the double mutant β-catenin (β-catY86,654F) was subjected to Western blot analysis and probed for tyrosine phosphorylation using the phosphortyrosine antibody 4G10, a band was detected suggesting that in SK-BR-3 cells one (or more) additional tyrosine(s) was being phosphorylated (Figure 5.2.3 A, top panel lane 5). A large number of bands at a lower molecular weight were observed below the band corresponding to β-catenin. These bands were presumed to be degradation products of β-catenin. This data suggests that in SK-BR-3 cells, the transient transfection of the activated mutant SrcY530F can induce the tyrosine phosphorylation of an additional tyrosine residue not previously identified as a Src target (or a tyrosine phosphorylation event induced by Src).

In a study by Brembeck *et al*. (2004), Y142 was identified as a target of phosphorylation upon HGF stimulation of the Met receptor tyrosine kinase activity (264). β-Catenin was also shown to be tyrosine phosphorylated on Y142 by Fyn, another Src

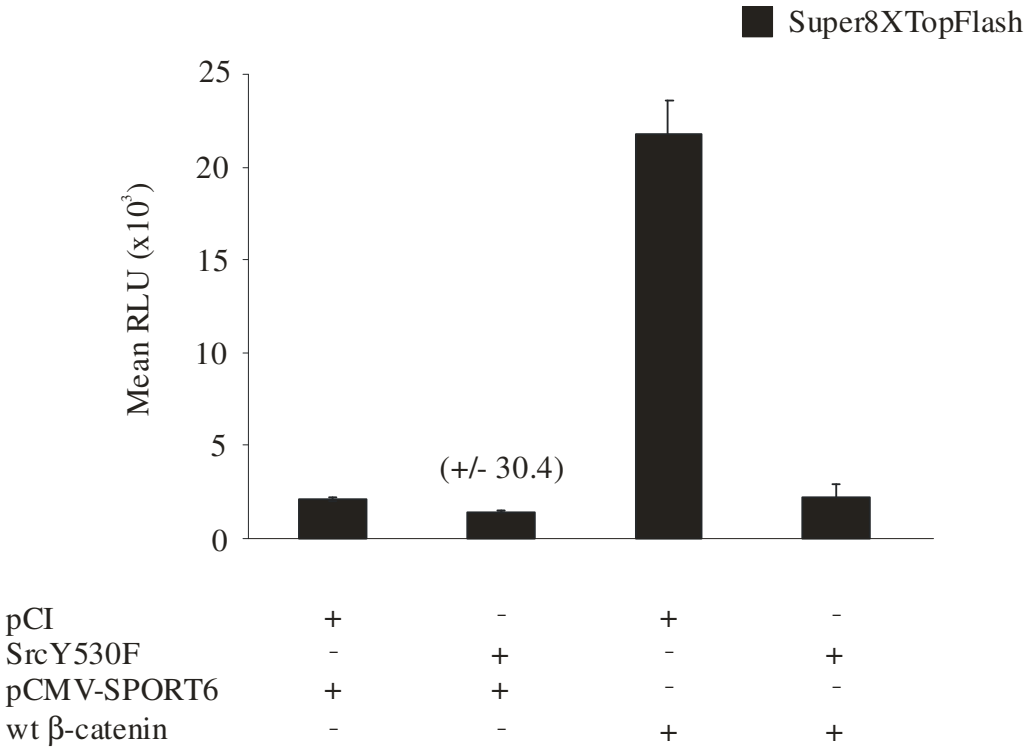


Figure 5.2.2 Super8XTopFlash activity in SK-BR-3 cells requires exogenous expression of wt β-catenin, and can be downregulated by the activated mutant SrcY530F. Empty vector control transfected cells (columns 1 & 2). wt β-catenin transfected SK-BR-3 cells with or without activated mutant SrcY530F as indicated. RLU, relative light units. Error represents standard deviation. Data represents mean RLU from transfections performed in triplicate.

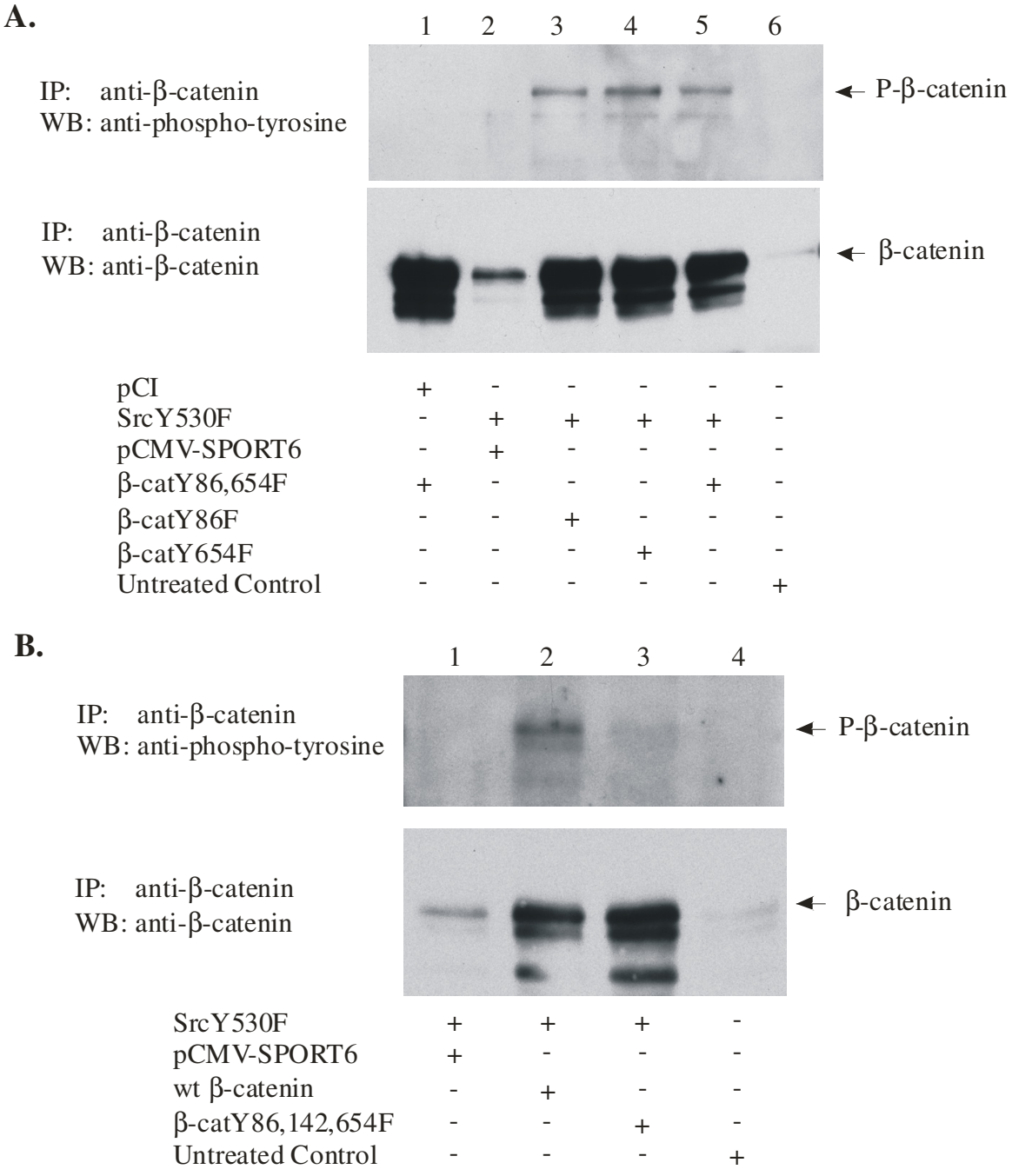


Figure 5.2.3 The activated mutant SrcY530F induces the tyrosine phosphorylation of β-catenin tyrosine residues 86, 142, 654 in SK-BR-3 cells. A. Western blot (WB), using phospho-tyrosine antibody 4G10, of immunoprecipitated (IP) β-catenin from SK-BR-3 cells transiently transfected with single (Y86F, Y654F) or double mutant (Y86,654F) β-catenin, and activated mutant SrcY530F (upper panel). IP and WB analysis of β-catenin from SK-BR-3 cells transiently transfected with mutant β-catenins (lower panel). **B.** IP and WB as indicated, from SK-BR-3 cells transiently transfected with activated mutant SrcY530F and either wt or triple mutant β-cateninY86,142,654F.

family tyrosine kinase (265). Therefore, a triple mutant was engineered encompassing all three Y-F mutations (Y86, 142, 654F). Western blot analysis, using the 4G10 antibody, of immunoprecipitated β-catenin from cells transiently transfected with the triple mutant β-catenin resulted in the detection of a faint band at the approximate molecular weight of β-catenin (Figure 5.2.3, B lane 3 top panel). This experiment was repeated three times, and I was not able to detect this faint band. Therefore, this is most likely an artifact or a result of loading error. The corresponding band from lysates of wt β-catenin transfected SK-BR-3 cells was much darker (Figure 5.2.3, B lane 2 top panel). This data demonstrated that mutation of tyrosine 142 strongly reduces the majority of β-catenin tyrosine phosphorylation induced by transient transfection of activated mutant SrcY530F compared to wt β-catenin in SK-BR-3 cells (compare Figure 5.2.3, B lanes 2 & 3).

The β-catenin mutants were then transiently transfected into SK-BR-3 cells to assess their potential to mediate the downregulation of Super8XTopFlash induced by activated mutant SrcY530F (Figrue 5.2.4). The triple mutant was first tested for its ability to activate the Super8XTopFlash Wnt reporter, and it could do so to a similar degree as wild-type (wt) β-catenin (Figure 5.2.4, A). When all the β-catenin mutants (single, double and triple) were individually co-transfected with activated mutant SrcY530F, there was no obvious reversal of Super8XTopFlash luciferase activity suppression (Figure 5.2.4, B).

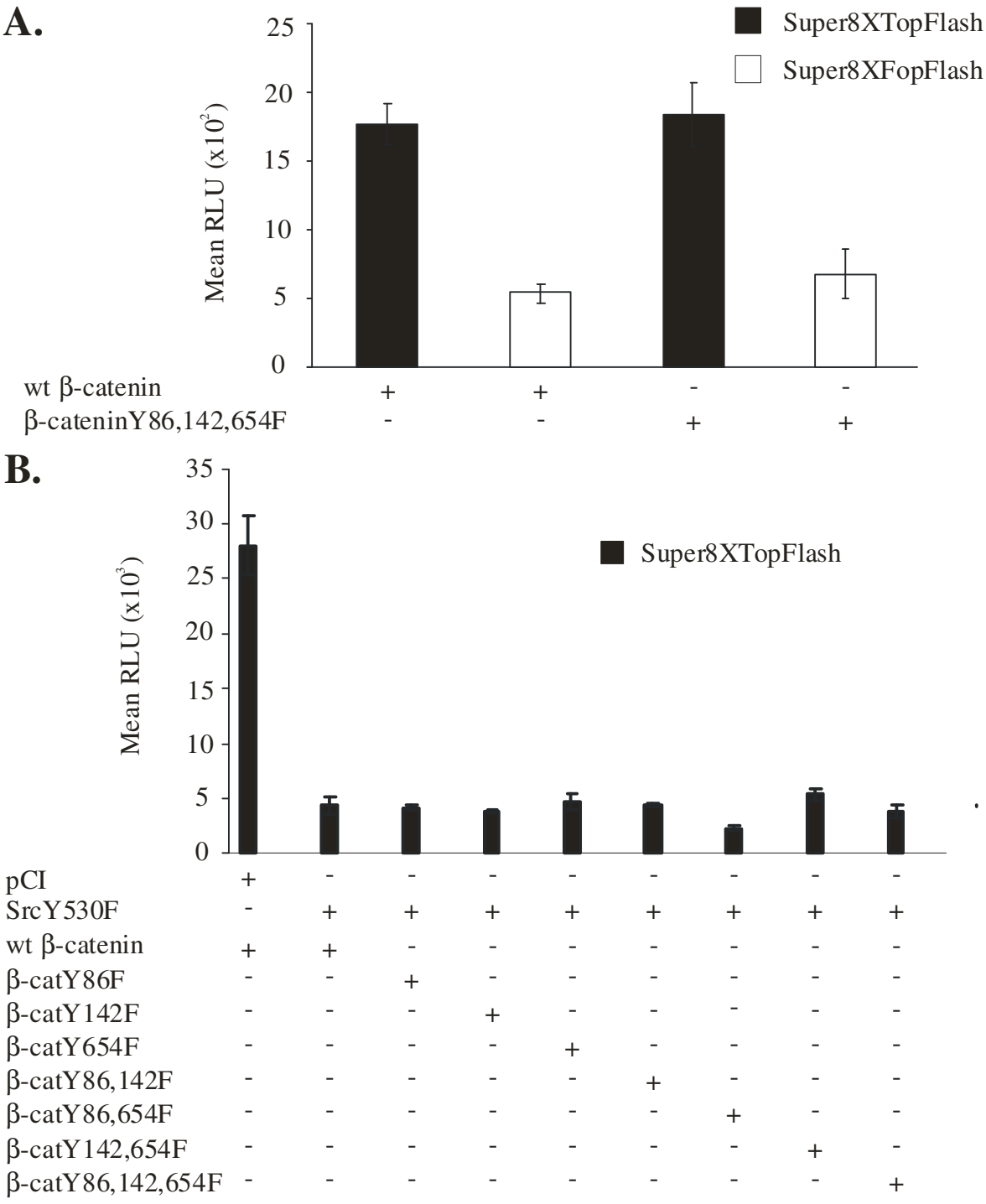


Figure 5.2.4 Tyrosine phosphorylation of β-catenin does not mediate the down-regulation of Super8XTopFlash activity by activated mutant SrcY530F. A. Triple mutant β-cateninY86,142,654F promotes activation of Super8XTopFlash similar to wt β-catenin. **B.** Super8XTopFlash activity in SK-BR-3 cells transiently co-transfected with mutant β-catenins and activated mutant SrcY530F. RLU, relative light units. Error represented by standard deviation.

5.3 Results: GSK3β Inhibition

In this section, the hypothesis that Src kinase may be activating GSK3β to downregulate the Super8XTopFlash reporter was tested. Along with CK1α, GSK3β phosphorylation of β-catenin is fundamental to its downregulation and the maintenance of the Wnt pathway in an inactive state (186,266). Inhibition of GSK3β by treatment with LiCl is a simple (although non-specific) and inexpensive method to assess its potential role in mediating the negative effect of Src on the Wnt reporter. When SK-BR-3 cells were treated with 10 mM LiCl, the luciferase activity of lysates from SrcY530F transfected cells was approximately 30% (3.3 fold decrease) of the luciferase activity from pCI transfected cells, whereas in NaCl control treated cells the luciferase activity of SrcY530F transfected cell lysates was approximately 12% (8 fold decrease) of the luciferase activity of pCI transfected cells (Figure 5.3.1, A). SW480 cells were then subjected to a similar treatment of LiCl (Figure 5.3.1, B). In SW480 cells treated with 10 mM LiCl, the luciferase activity from lysate of cells transiently transfected with the activated mutant SrcY530F was approximately 1.6 fold lower than the luciferase activity from pCI transfected cells (Figure 5.3.1, B last 2 columns). When cells were not treated with LiCl, the luciferase activity from activated mutant SrcY530F transfected cells was approximately 3.5 fold lower than the activity from pCI transfected cells (Figure 5.3.1 B, first 2 columns). This data suggested that treating SK-BR-3 cells or SW480 cells with 10mM LiCl diminishes the inhibition of Super8XTopFlash activity induced by activated SrcY530F.

To confirm that LiCl treatment of SW480 cells was inhibiting GSK3β, the phosphorylation state of Ser 9, which has been used as an indicator of inactivation, was

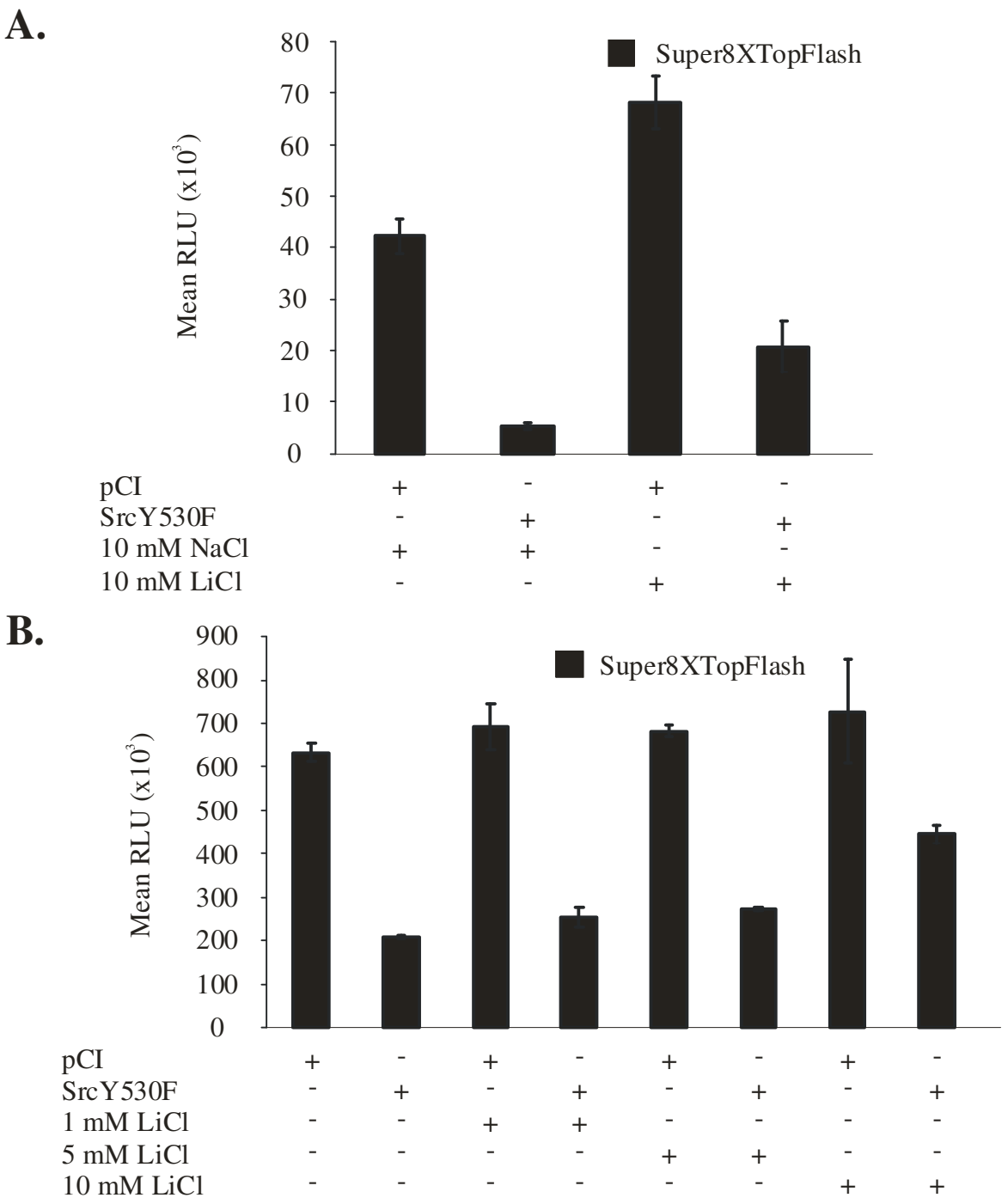


Figure 5.3.1 Lithium chloride partially reverses the downregulation of Super8X-TopFlash activity in SK-BR-3 cells and SW480 cells induced by activated mutant SrcY530F. A. SK-BR-3 cells transfected as indicated and treated with 10 mM LiCl for 19 hours prior to harvesting for luciferase assays. **B.** SW480 cells transfected as indicated and treated with various concentrations of LiCl (1, 5, 10 mM as indicated) for 19 hours prior to harvesting. RLU, relative light units. Error represents standard deviation. Data represents mean RLU value from three similarly transfected wells.

measured (267). Cell lysates from the previous experiment were analysed for the total protein levels of GSK3β, and phospho-Ser 9 levels of GSK3β. Western blot analysis of lysates using a GSK3β phospho-Ser 9 antibody (Cell Signaling) and a GSK3β antibody, did not show any noticeable changes in the intensity of the band at the approximate molecular weight of GSK3β (Figure 5.3.2, top and middle).

The protein levels of Src and its activation state was also analysed using the same lysates of SW480 cells treated with or without LiCl. Using the Src monoclonal antibody mAb327, Src was observed to be clearly present at higher levels than in transiently transfected with SrcY530F, compared to lysates from cells transfected with the empty vector pCI (Figure 5.3.3, A bottom panel). When a Western blot using the same lysates was probed with a Src phospho-tyrosine 416 antibody (used as an indicator of Src activity), a darker Src phospho-tyrosine 416 band was observed in the lane containing untreated SrcY530F cell lysate (Figure 5.3.3, A, lane 3). The corresponding band in lanes loaded with lysates from LiCl treated SW480 cells was noticeably lighter (Figure 5.3.3, A lanes 4-6). Densitometry of these bands using ImageQuant software shows a reduction of approximately 50% Figure 5.3.3, B). These data suggest that, either treatment of LiCl is not inhibiting GSK3β, or that phospho-Ser 9 is not a good indicator of GSK3β activity. These data also show that the reversal of SrcY530F induced Super8XTopFlash activity by treating cells with LiCl may be due to the inhibition of Src kinase activity, as measured by the phosphorylation state of Src tyrosine-419. In addition, as lithium is a non-specific inhibitor of many kinases (268), it may also be acting through other unknown agents.

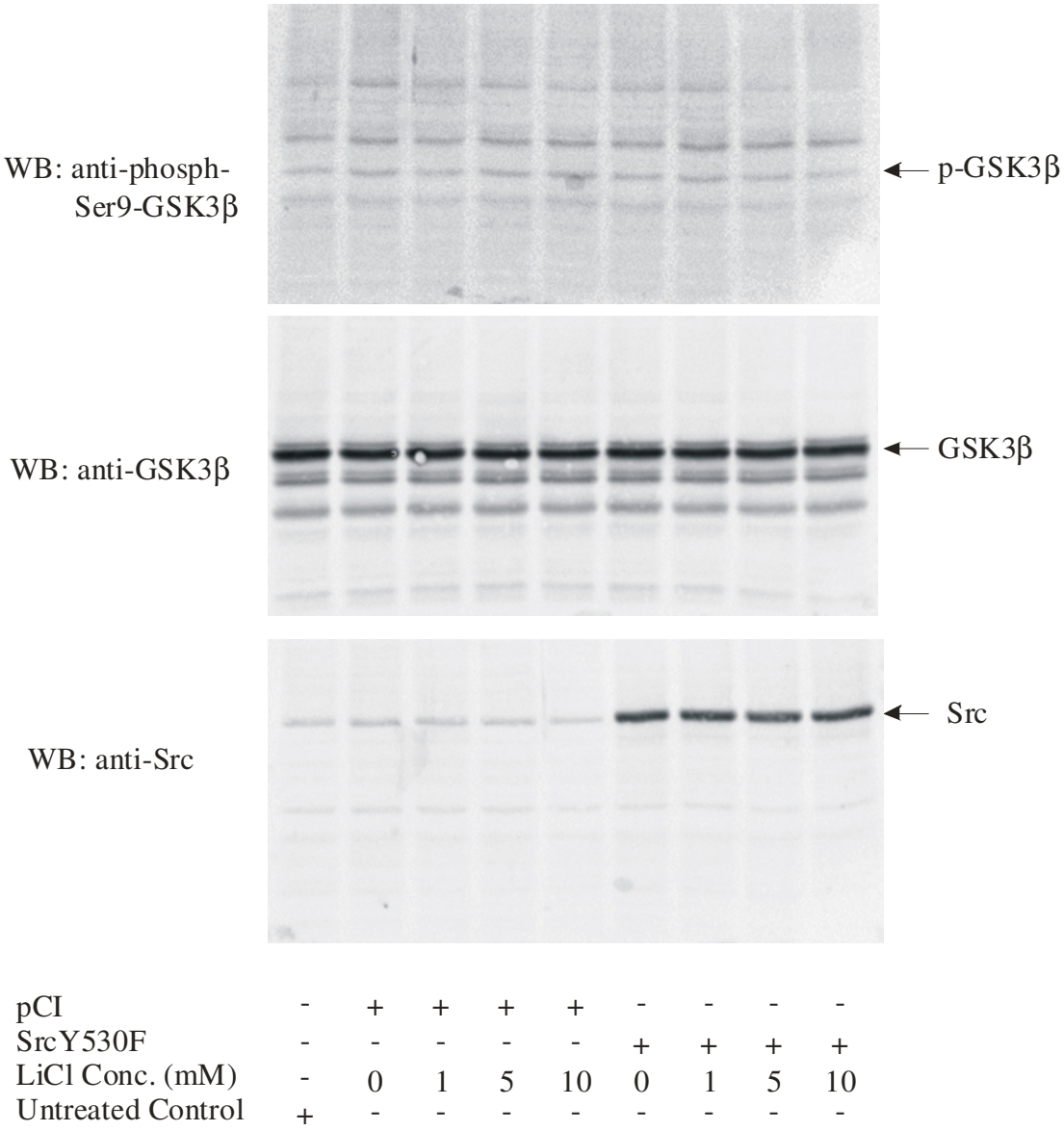


Figure 5.3.2 Lithium chloride treatment of SW480 cells does not affect the phosphorylation state of Ser 9 or total protein levels of GSK3β. (Top) Transiently transfected SW480 cells were treated with the indicated concentrations of LiCl and analysed by Western blot of whole cell lysates for changes in phospho-Ser9 (an indicator of GSK3β inhibition) of GSK3β (indicated by p-GSK3β). **(Middle),** The same lysates were used as in Top panel, but blotted for total GSK3β protein levels. **(Bottom)** Lysates from the same experiment were also use to confirm the expression of activated mutant SrcY530F.

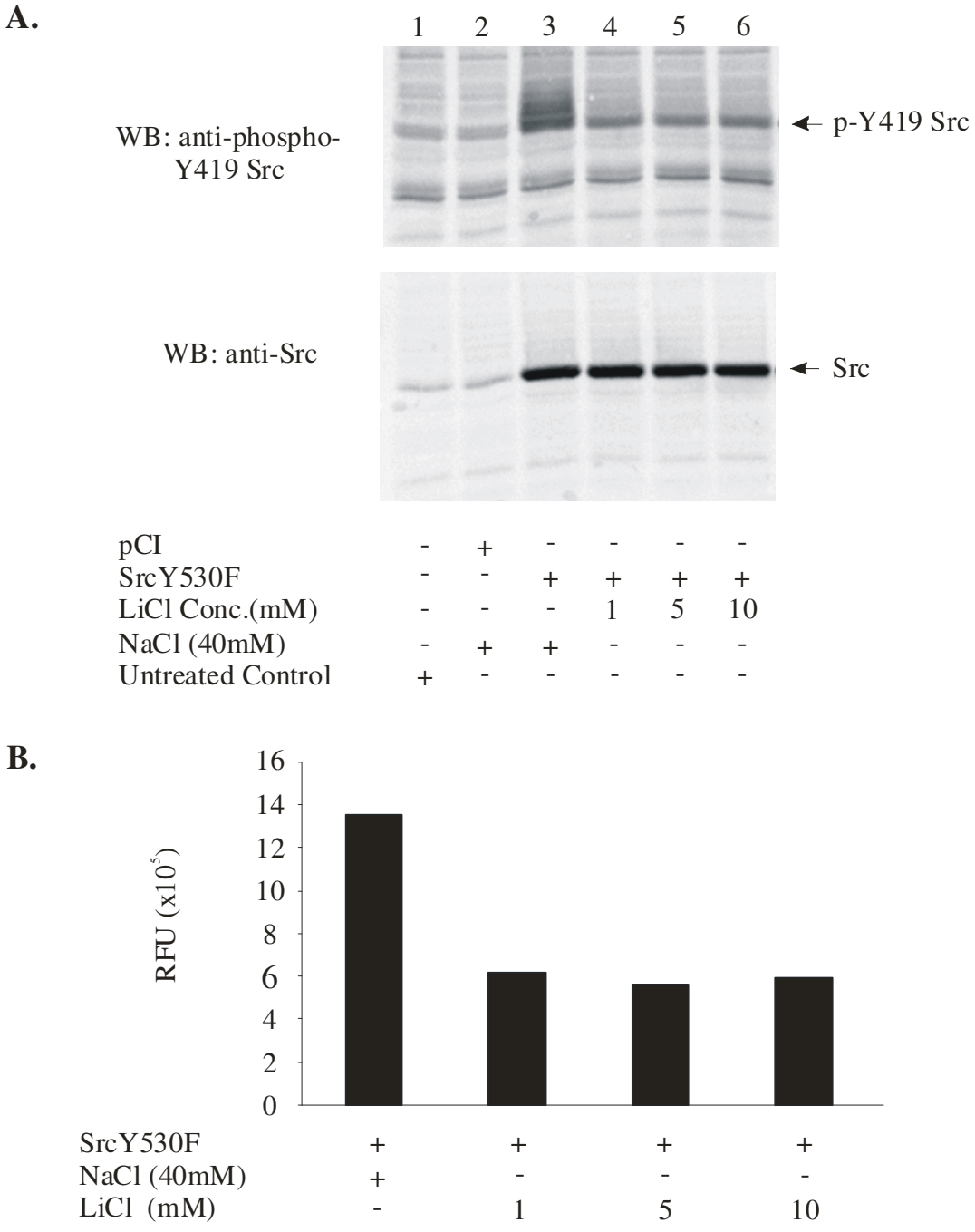


Figure 5.3.3 Lithium chloride treatment of SW480 cells decreases phosphorylation of SrcY419. A. Western blot analysis of SW480 whole cell lysated from cells transiently transfected with (or without) activated mutant SrcY53F and treated with 40 mM NaCl as control or various concentration of LiCl as indicated. **B.** ImageQuant densitometry of phospho-Y419 Src bands lanes 4 to 6 from A. RFU, relative fluorescence units, measuring band volume.

To minimize the pharmacological effects on Src, and to inhibit GSK3β more specifically, the pharmacological reagents Kenpaullone and CHIR99021 were tested. These compounds have been shown to target GSK3β with a relatively high degree of specificity (268,269). To measure the activity of GSK3β we chose an alternative antibody which targets phospho-GSK3β (and α) at Y216, where phosphorylation of this residue indicates activation (270).

We first tested the ability of Kenpaullone to inactivate GSK3β. SW480 cells were transiently transfected with the empty vector pCI or the activated mutant SrcY530F and treated with 10 μM Kenpaullone. As shown in Figure 5.3.4, treatment of transiently transfected cells (either pCI or SrcY530F) with Kenpaullone results in a noticeable decrease in the band density of phospho-tyrosine 216 of GSK3β (Figure 5.3.4, A lanes 5 & 6). This blot also shows a greater decrease in the staining intensity of a band corresponding to the molecular weight of GSK3α, which has also been shown to be detected by this antibody (269). Western blots probed using a commercial GSK3β antibody did not show a decrease in total GSK3β protein levels (Figure 5.3.4, B) in cells treated with or without Kenpaullone. Western blot analysis using the Src phospho-tyrosine 419 antibody, revealed a slightly darker band in SrcY530F transfected lysates (Figure 5.3.4, C lane 6) compared to control transfected cell lysates (Figure 5.3.4 C, lane 4). This data suggests that Kenpaullone inhibits GSK3β activity but not SrcY530F activity in SW480 cells. Therefore, Kenpaullone was used to treat cells transiently transfected with either pCI or SrcY530F and Super8XTopFlash (Figure 5.3.5).

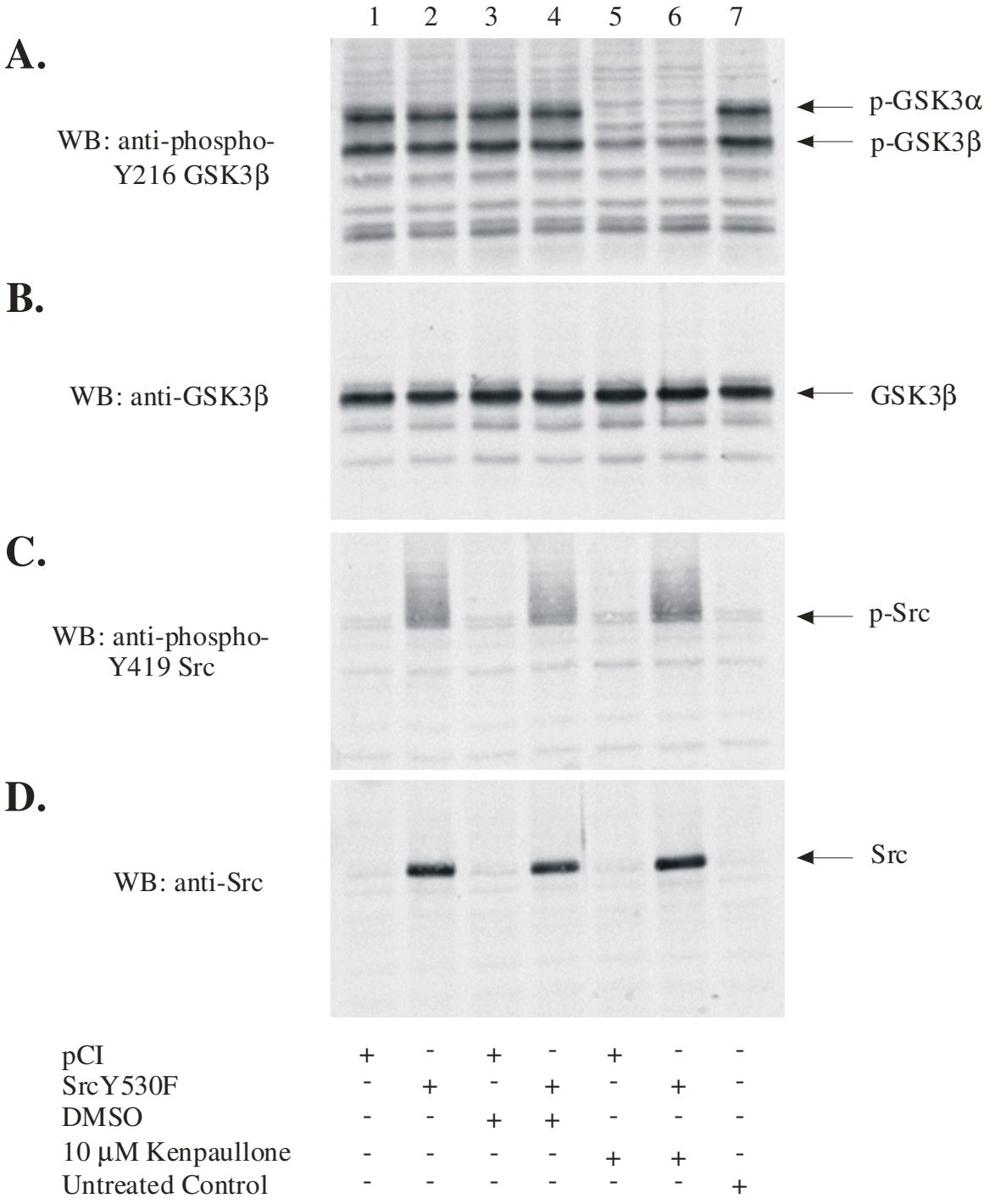


Figure 5.3.4 Inhibition of GSK3β activity in SW480 cells by Kenpaullone. Cells were transiently transfected with empty vector pCI or activated mutant SrcY530F and treated with 10 μM Kenpaullone or vehicle DMSO. Western blot of cells for phospho-Y216 GSK3β & GSK3α (**A**, p-GSK3β & p-GSK3α), total GSK3β (**B**), phospho-Y419 Src (**C**, p-Src) or total Src (**D**) as shown.

Similar to when cells were treated with LiCl, the treatment of cells with 10 μM Kenpaullone did not completely reverse the inhibition of Wnt reporter activity by activated mutant SrcY530F. A small but noticeable reversal of SrcY530F mediated reporter activity inhibition was observed (from 6.8 fold in untreated cells to 2.4 fold inhibition in Kenpaullone treated cells), and this could not be explained by an effect on Src activity (based on Src phospho-Y419) as was the case with LiCl (Figure 5.3.4, C).

A second GSK3β inhibitor (CHIR99021) was used to treat SW480 cells. In a comprehensive study of chemical inhibitors and their specificity, CHIR99021 showed a very similar (to Kenpaullone) level of inhibition of GSK3β activity (1% vs 3 % activity remaining after treatment, respectively), but was less specific for Src at 1 μM (268). The ability of CHIR99021 to inhibit GSK3β was tested in an identical manner as for Kenpaullone, except that additional concentrations were included (Figure 5.3.6). As shown in Figure 5.3.6 A, CHIR99021 treatment of cells effectively inhibited GSK3β as measured by the phosphorylation state of Y216. The most effective inhibition of GSK3β Y216 phosphorylation was observed using a concentration of 10 μM. As in the case of Kenpaullone, GSK3α was also inhibited at each concentration of CHIR99021 used (Figure 5.3.6 lanes 5, 6 and 7). Pharmacological treatment of cells with 10 μM CHIR99021 did not dramatically affect total protein levels of GSK3β (Figure 5.3.6, B), or the phosphorylation of SrcY419 (Figure 5.3.6, C). Total Src protein levels were also unchanged as measured by Western blot using mAb327 (Figure 5.3.6, D, lanes 5-7).

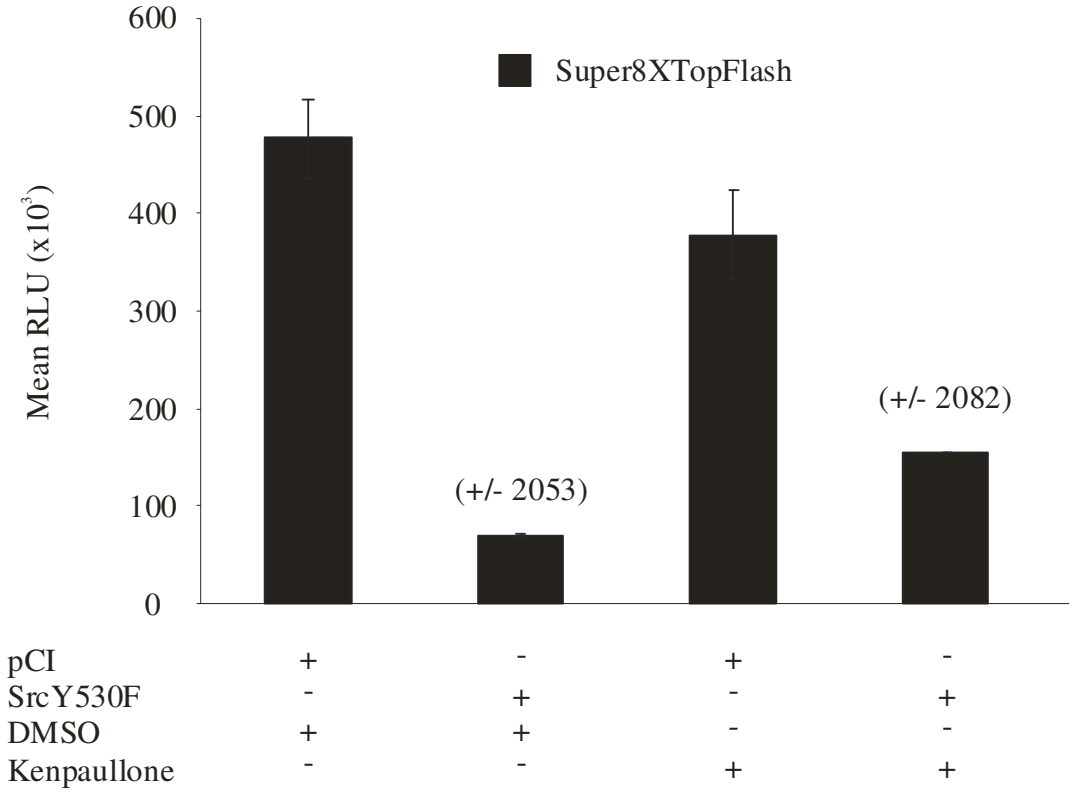


Figure 5.3.5 Treatment of SW480 cells with Kenpaullone does not fully reverse the downregulation of Super8XTopFlash activity induced by activated mutant SrcY530F. SW480 cells were transiently transfected as in previous experiments with either empty vector pCI or activated mutant SrcY530F. Cells were also treated with 10 μM Kenpaullone or DMSO vehicle control. As in previous experiments, Kenpaullone was added to fresh media after the initial 5 hour transfection incubation period. Cells were harvested following an additional 19 hours (see methods). RLU, relative light units. Error bars represent standard deviation. Error bars for Kenpaullone treated and SrcY530F transfected cells not shown, but represent 1.4% of mean RLU.

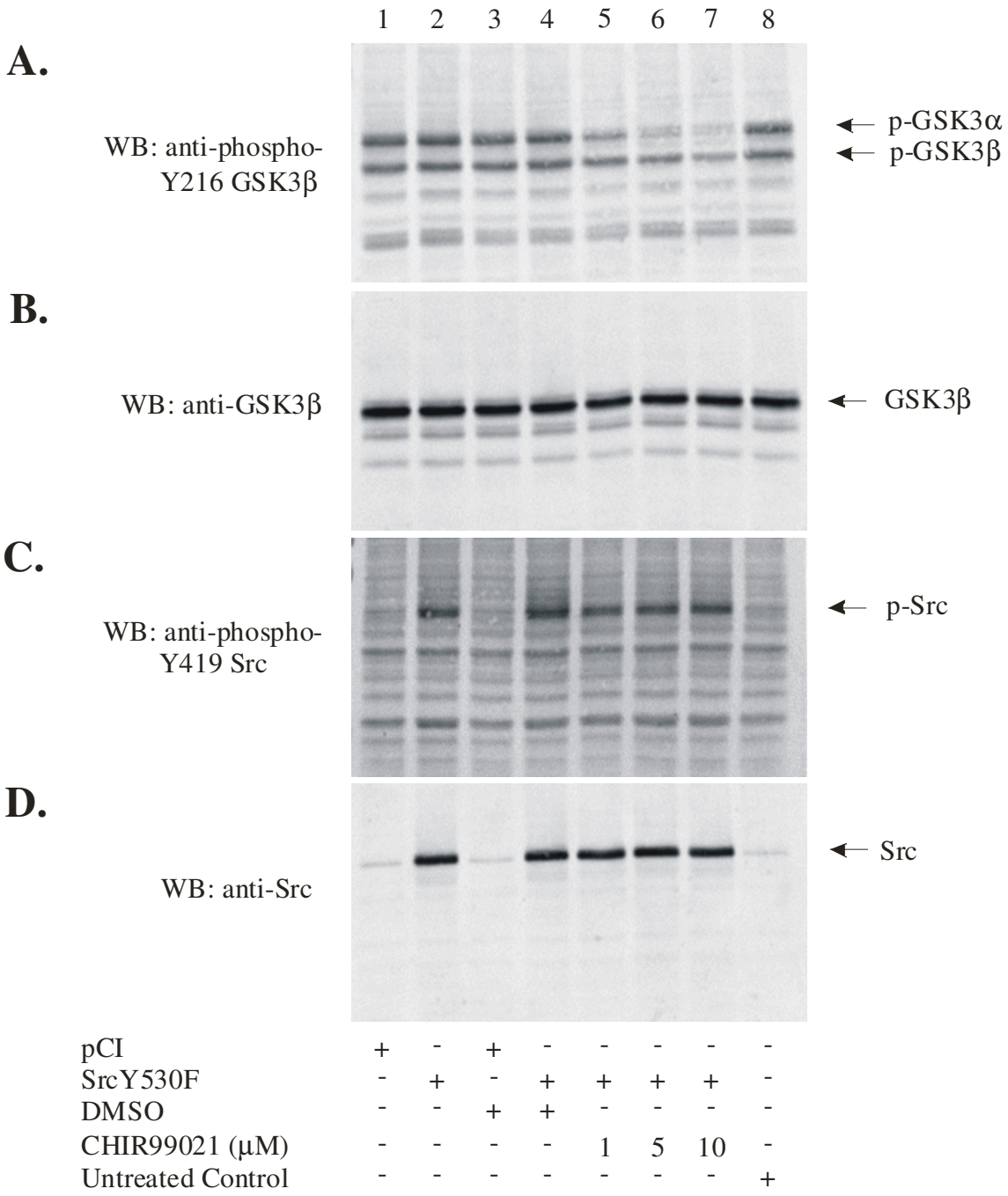


Figure 5.3.6 Inhibition of GSK3β activity by pharmcological compound CHIR99021. SW480 cells were transiently tansfected with empty vector pCI or activated mutant SrcY530F and treated with the GSK3β inhibitor CHIR99021 at various concentraitons as indicated. Lysates were Western blotted for phospho-Y216 on GSK3β (p-GSK3β) and on GSK3α (p-GSK3α) as an indicator of activation **(A)**. Total protein levels of GSK3β were also analysed **(B)**. Activation or inactivation of Src was also assessed as measured by phospho-Y419 levels **(C)**. Expression of activated mutant SrcY530F was confirmed by western blot analysis of lysates using the monoclonal anti-Src antibody MAb327 **(D)**.

Based on these results, CHIR99021 was used to inhibit GSK3β in SW480 cells co-transfected with activated SrcY530F and the Wnt reporter Super8XTopFlash. Figure 5.3.7 shows that inhibition of GSK3β by CHIR99021 induced a small but noticeable reversal of the effect of activated mutant SrcY530F on the Wnt luciferase reporter (compare Figure 5.3.7, compare fourth & seventh columns), which was similar what was observed with Kenpaullone.

To ensure that GSK3β activity was being downregulated more specifically, siRNA technology was used to knock-down this enzyme. Bio-Rad SilentMer Validated Dicer-Substrate siRNA duplex reagent targeting GSK3β was used according to the manufacturer's protocol. SW480 cells transiently transfected with either the empty vector pCI or activated mutant SrcY530F were pretreated with siRNA targeting GSK3β. Western blot analysis of GSK3β protein levels shows that pretreatment of SW480 cells with siRNA is effective (Figure 5.3.8 A, top panel, compare lanes 1 & 2 with 3 & 4). When GSK3β targeted siRNA was used to pre-treat SW480 cells transiently transfected with activated Src and Super8XTopFlash reporter, there was no significant change in the reporter response due to Src.

Taken together, the experiments targeting GSK3β through the use of pharmacological inhibitors (Kenpaullone and CHIR99021) and siRNA demonstrates that the repression of the Wnt reporter Super8XTopFlash by activated mutant SrcY530F does not act principally through GSK3β.

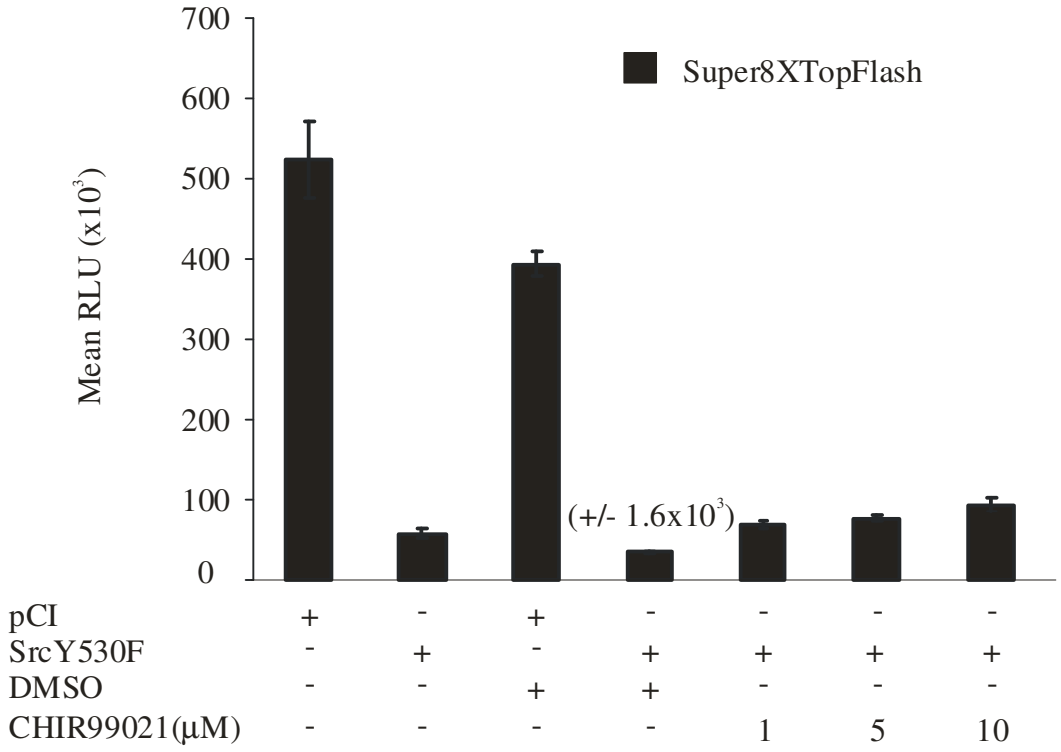


Figure 5.3.7 Treatment of SW480 cells with CHIR99021 does not dramatically reverse the downregulation of Super8XTopFlashh activity induced by SrcY530F. SW480 cells were transiently transfected with empty vector pCI or activated mutant SrcY530F and treated with various concentrations of CHIR99021 (as indicated) or vehicle control (dimethyl sulphoxide, DMSO). RLU,relative light units. Error represents standard deviation. Data represent mean RLU from triplicate transfections.

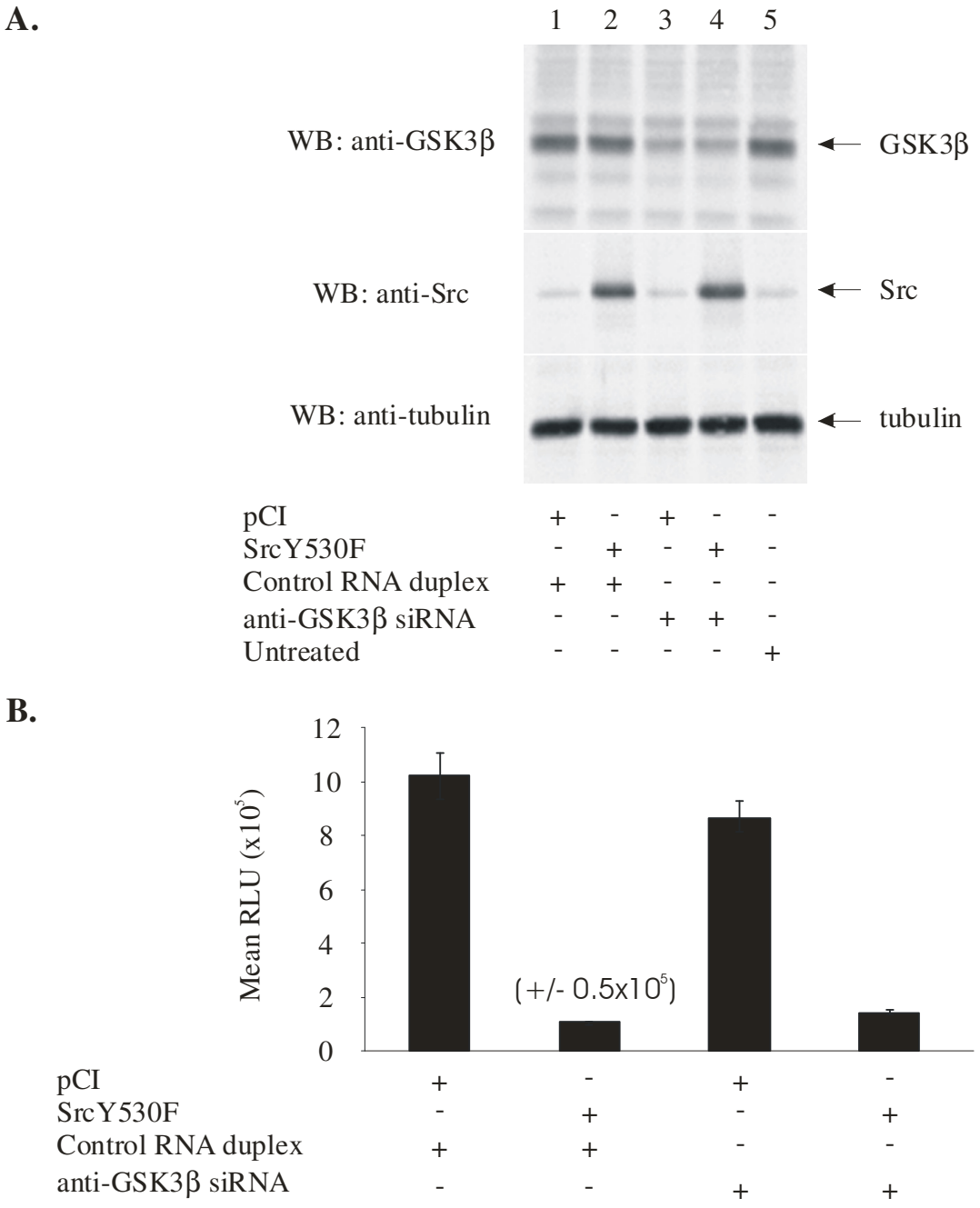


Figure 5.3.8 siRNA knock-down of GSK3β does not reverse the downregulation of Super8XTopFlash activity induced by activated mutant SrcY530F in SW480 cells. **A.** Western blot analysis of GSK3β protein levels in SW480 cells transfected as indicated and treated with either control RNA duplex or siRNA targeting GSK3β (anti-GSK3β siRNA). Control blots for Src and tubulin as indicated (loading control). **B.** Super8XTop-Flash luciferase activity in SW480 cells pretreated with siRNA and transiently transfected as in A. RLU, relative light units. Error represents standard deviation. Data represents mean RLU from triplicate transfections .

5.4 Hypothesis & Rationale: Wnt/Ca^{2+} Pathway & Src Kinase

It is now known that the Wnt family of extracellular ligands participate in a wide variety of developmental and proliferative processes (219). This broad range of functions is reflected in the number of Wnt ligands (of which there are 19 in humans) and its associated Frizzled family of receptors (of which there are 10 in humans) (203,271,272) and (http://www.stanford.edu/~rnusse/wntwindow.html). Early studies of Wnt signaling suggested the existence of two groups of Wnt ligands primarily defined by their ability to induce a secondary axis in *Xenopus* embryos and transform C57MG cells (Wnt1, -3 and 8, the canonical Wnts) and those that did not (Wnt4, -5a and -11, the non-canonical Wnts). Importantly, it was shown that some of the non-canonical Wnts (Wnt 11 and Wnt5a) could increase intracellular calcium in zebra fish models (273,274). Studies showed that Wnt5a can antagonize Wnt8 induced axis duplication and that this effect is mimicked by injection of the mammalian 5-HT1cR serotonin receptor, which in turn, is known to regulate intracellular calcium levels (273). Ectopic expression of Wnt5a can enhance intracellular calcium induced by Frz-2 receptor in zebrafish embryos (275). Furthermore, Wnt11 and Wnt5a have been shown to activate CamKII activity in *Xenopus* embryos, while Wnt5a has also been shown to induce the translocation of PKC to the plasma membrane, an effect that also involves Frz-2 (276,277). Therefore, calcium flux represents at least one downstream mediator of the non-canonical or Wnt/Ca^{2+} signaling pathway.

Src has also been associated with intracellular calcium flux. An early study using a temperature sensitive mutant v-Src in Rat-1 cells suggested that while changes in v-Src activity did not alter total Ca^{2+} levels, its increased activity did correlate with increased cytosolic calcium (278). Using another ts v-Src mutant (tsNY72), Spangler *et al*. (1989)

show that the induction the 9E3 cytokine gene by v-Src may be mediated by protein kinase C (PKC), as prolonged treatment with PMA (phorbol 12-myristate 13-acetate) which inhibits PKC, also blocked its transcription (279). In a study by Zang *et al*. (1995), v-Src was found to regulate some calcium-dependent PKC isoforms (280). Interestingly, a study by Hu *et al*. (1998) suggests that c-Src and FAK are directly associated with the L-type Ca^{2+} channel and are able to regulate Ca^{2+} currents induced by PDGF stimulation in rabbit colonic smooth muscle cells (281). In other organisms it was shown that Src and Fyn may be responsible for the fertilization induced calcium release from the endoplasmic reticulum in a mechanism involving their SH2 domains and inositol-trisphosphate production in starfish eggs (282). Therefore, there is evidence to suggest that Src may regulate intracellular calcium flux.

Based on these studies, I hypothesized that transient transfection of the activated mutant SrcY530F induces an increase in intracellular Ca^{2+} in a way that results in the downregulation of the Super8XtopFlash reporter.

5.5 Results: Treatment of Cells with BAPTA-AM

Based on these previous studies, I hypothesized that Src could induce an intracellular calcium flux that would antagonize canonical Wnt signaling. To test this model two molecules were examined - intracellular calcium and PLCγ, a well documented regulator of intracellular calcium (283,284). It was rationalized that if intracellular calcium could mediate non-canonical inhibition of canonical signaling, that Src may be inhibiting Wnt reporter activity by increasing intracellular calcium. To assess this as a possible mechanism BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-

acetoxymethyl ester), which is a membrane permeable calcium chelator that can deplete the cytosol, was used in reporter assays (285,286). 10 μM BAPTA-AM was used 4 hours prior to harvesting of SW480 cells co-transfected with mutant Src kinases and Super8XTopFlash (or Super8XFopFlash) reporter. This treatment did not reverse the inhibition of reporter activity by activated mutant SrcY530F (Figure 5.5.1, A). A second series of BAPTA-AM dosing regimes was tested to ensure that intracellular calcium was being completely chelated, although we did not test for this directly. This was done based on a previous study that followed the transcription of SERCA2b after various dosing protocols were tested (286). Figure 5.5.1,B suggests that increasing the number of BAPTA-AM doses further enhances the downregulation of Super8XTopFlash activity compared to treatment of SW480 cells with the vehicle control, dimethyl sulphoxide (DMSO) (Figure 5.5.1, B first two pairs). To test the possibility that the concentration of BAPTA-AM is ineffective, various increasing amounts of the drug were used (Figure 5.5.2). These results demonstrate that neither increasing the number of BAPTA-AM doses nor increasing the concentration is able to reverse the effect of Src on Super8XTopFlash activity in SW480 cells.

To be certain that increasing intracellular calcium is able to downregulate the Super8XTopFlash reporter, we treated cells with two different SERCA-specific inhibitors, thapsigargin and A23187. Treatment of cells with these compounds has been shown to increase cytosolic calcium while depleting intracellular stores (286-288). As shown in Figure 5.5.3, treatment of SW480 cells with A23187 (2 μM) or thapsigargin (Tg, 1 μM) for 8 hours prior to harvesting was able to decrease Super8XTopFlash activity (A and B, middle columns). When, cells were treated with two subsequent dose of BAPTA-AM (20 μM 2x at 8 hours and 3 hours prior to harvesting), the downregulation induced by either

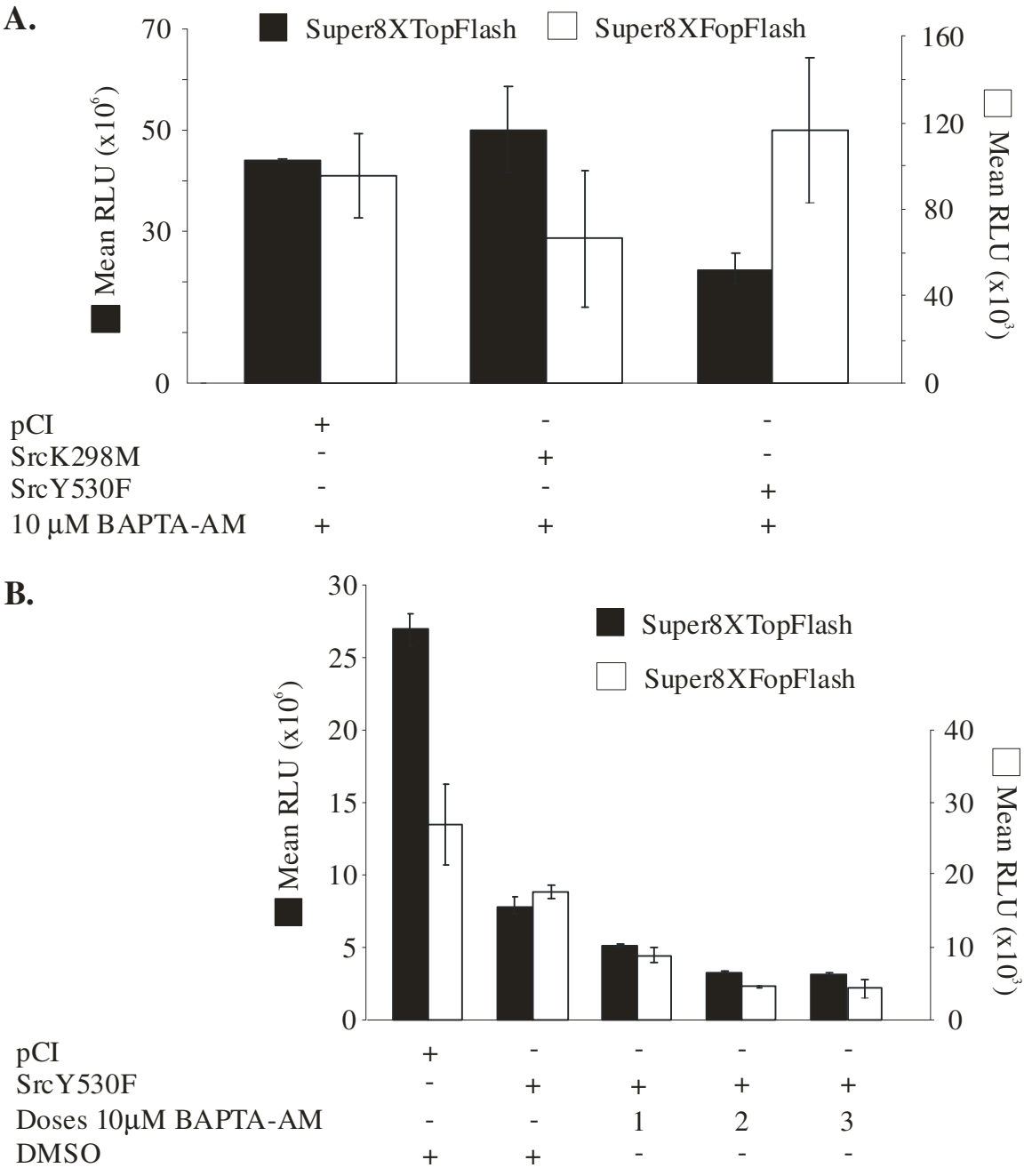


Figure 5.5.1. Treatment of SW480 cells with 10 µM BAPTA-AM does not reverse the downregulation of Super8XTopFlash activity by SrcY530F. A. Cells were treated with a single dose of BAPTA-AM 4 hours before harvesting of lysates. **B.** Cells were treated with one of the following dosing protocols: single dose, 3 hrs prior to harvesting; or, two doses at 6 hrs and 3 hrs prior to harvest; or, three doses at 6 hrs, 3 hrs and 1 hr prior to harvesting. RLU, relative light units. Error represents standard deviation. Scale bar for Super8XTopFlash provided on left side, and for Super8XFopFlash negative control shown on right side. Data represents mean RLU of triplicate transfections.

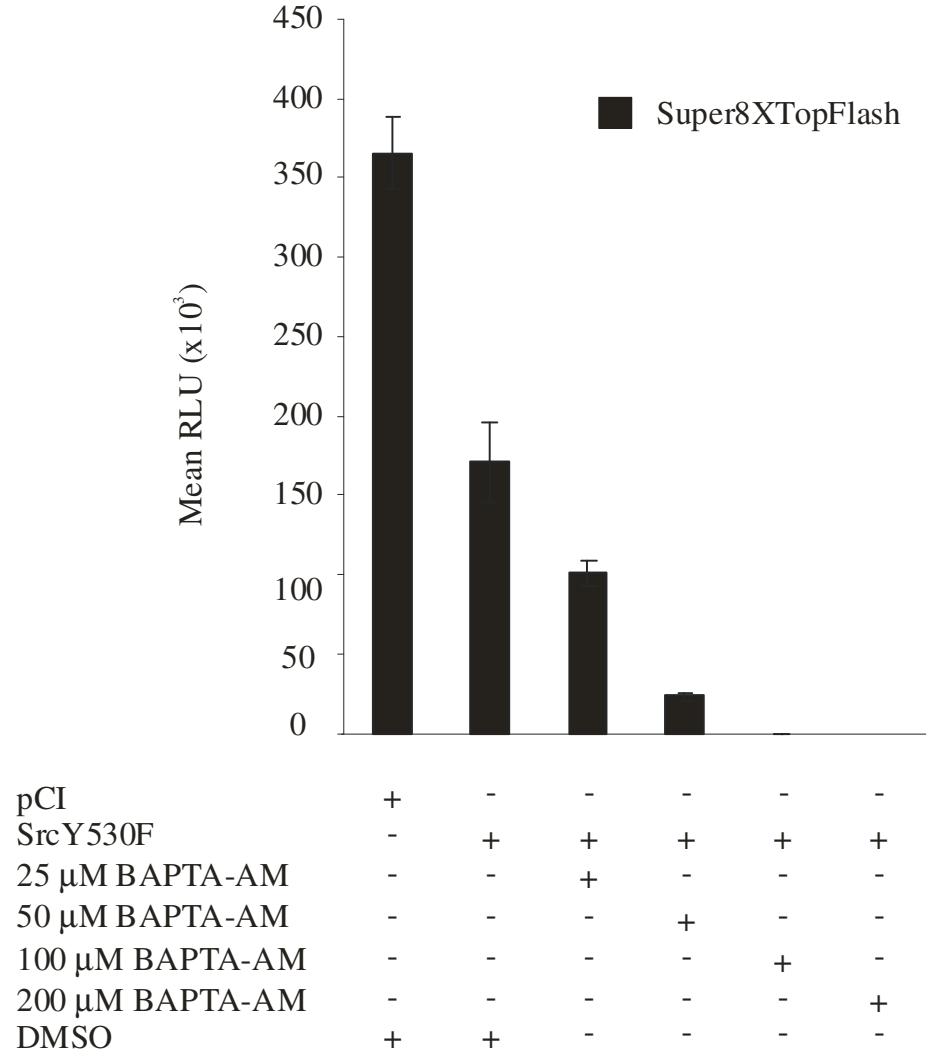


Figure 5.5.2 Increasing concentrations of BAPTA-AM further downregulates the luciferase activity from Super8XTopFlash in activated mutant transfected SW480 cells. SW480 cells were transiently transfected with activated mutant SrcY530F and treated with a single dose of BAPTA-AM with various increasing concentrations as indicated 3 hours before harvesting for subsequent luciferase assays. Note: at 200 µM luciferase readings (raw RLU) were below readings for untransfected control. Data represents mean RLU of triplicate transfetions.

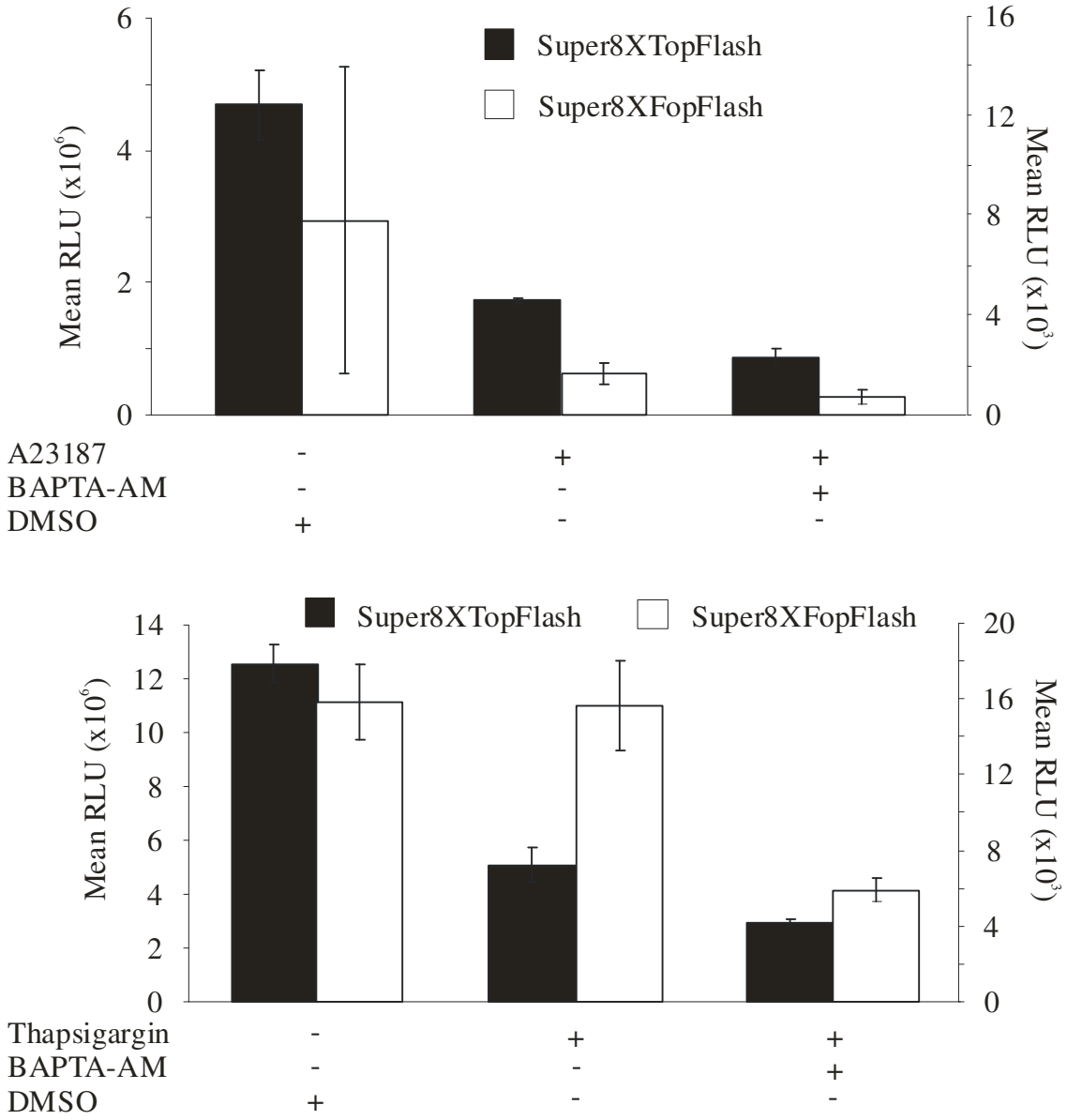


Figure 5.5.3 Treatment of SW480 cells with A23187 (A) or thapsigargin (Tg,B) in combination with BAPTA-AM downregulates Super8XTopFlash activity in SW480 cells. SW480 cells were transiently transfected with either Super8XTopFlash or Super8XFopFlash then treated with either A23187 alone for 8 hours prior to harvesting, or in combination with BAPTA-AM (20 μM) added twice at 8 hours and 3 hours prior to harvesting. Treatment of cells with thapsigargin (Tg) was done in an identical manner. RLU, relative light units. Error represents standard deviation. Data represent mean RLU of transfections performed in triplicate. Scale bar for Super8XTopFlash on left side, for Super8XFopFlash negative control on right.

A23187 or Tg, was further amplified. These data suggests that the luciferase enzymatic activity or expression from the reporter is being negatively affected by these particular compounds.

5.6 Background & Rationale: Targeting PLCγ

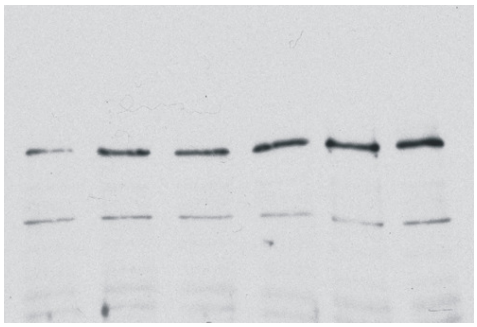
The second approach was to test the hypothesis that Src downregulates the Super8XTopFlash reporter by inducing a Ca^{2+} flux was to target phosphorlipase C (PLC). While there are several subtypes, PLC-β, -γ and δ, the PLC-γ subtype is regulated by receptor tyrosine kinases and is tyrosine phosphorylated in response to receptor activation (283,284). Activation of PLCγ results in the hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP_2) to inositol (1,4,5)-trisphosphate (Ins (1,4,5)P_3). Ins (1,4,5)P_3 ligand gated receptors on the endoplasmic reticulum are then activated resulting in the release of calcium into the cytosol (283). During the early characterization of Src kinase, PLC was identified as an enzyme that co-localized with Src to the intracellular domain of growth factor receptors and became phosphorylated upon ligand engagement (289,290). There is also evidence that both v-Src and c-Src associate with PLC-γ, and that activation of PLC-γ1 in a human endothelial/bladder carcinoma cell line (ECV304) requires upstream activation of Src (107,291). Both PLC-γ1 and -γ2 have been shown to be phosphorylated by c-Src in response to receptor activation (292). Therefore, since PLC-γ is a known regulator of intracellular calcium we hypothesized that Src may be inhibiting Wnt signaling through a PLC-γ/Ca^{2+} pathway.

To assess the potential role PLC-γ may play in the Src downregulation of Super8XTopFlash, we utilized a commercially obtained siRNA targeting PLC-γ1 and -γ2. We chose not to utilize the well documented PLC chemical inhibitor U73122 due to studies that show that it may potentially increase cytosolic calcium levels, and based on our experiments using chemical compounds in the previous experiment (293-295).

5.7 Results: Effect of PLCγ Knock-Down on Reporter Activity

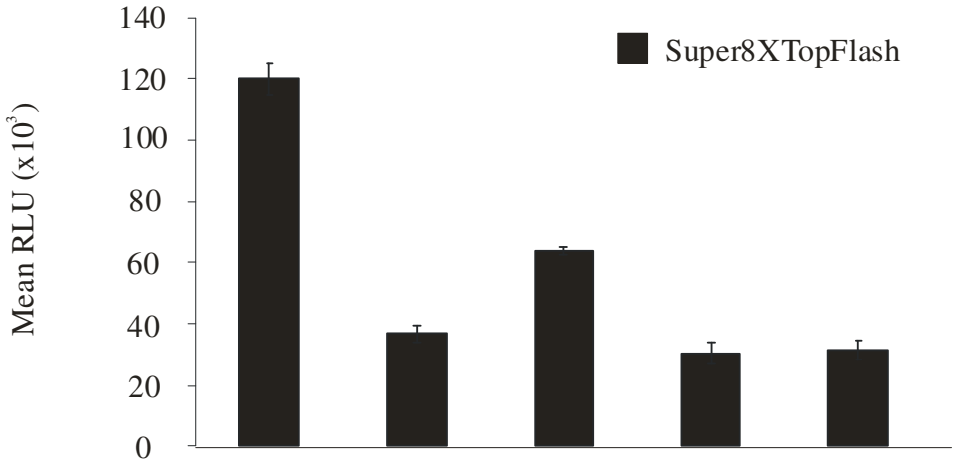
SMARTpool siRNA duplex solutions were purchased from Dharmacon which contain 4 pooled sets of duplexes targeting PLC-γ1 and -γ2. Scrambled control RNA duplex was obtained from our laboratory (designed by Dr. Jefferey Bjorge). Cells were pre-treated with siRNA before transfection with activated mutant SrcY530F and Super8XTopFlash reporter. As shown in Figure 5.7, treatment of SW480 cells with SMARTpool siRNA targeting PLC-γ1 effectively downregulates protein levels after 48 hours (A). Furthermore, siRNA pretreated SW480s continue to exhibit the downregulation of Super8XTopFlash activity induced by activated mutant SrcY530F (5.7, B). A noticeable decrease in reporter activity was also observed when cells were treated with control RNA duplex. Taken together, activated SrcY530F does not downregulate Super8XTopFlash reporter activity by a mechanism that involves PLC-γ1, and that it remains inconclusive as to whether or not Src is regulating intracellular calcium in these cells.

A.



PLC-γ1 siRNA	+	-	-	-	-	-
PLC-γ2 siRNA	-	+	-	-	-	-
Control RNA Duplex	-	-	+	-	-	-
SrcY530F	+	+	+	+	-	-
Super8XTopFlash	+	+	+	+	+	-
Untreated Control	-	-	-	-	-	+

B.



pCI	+	-	-	-	-
SrcY530F	-	+	+	+	+
PLCγ1 siRNA	-	-	-	+	-
PLCγ2 siRNA	-	-	-	-	+
Control RNA Duplex	-	-	+	-	-

Figure 5.7 PLC-γ1 and -γ2 siRNA treatment does not reverse the downregulation of Super8XTopFlash activity induced by SrcY530F in SW480 cells. A. Western blot analysis of PLC-γ1 protein levels in SW480 cells treated with siRNA targeting either PLC-γ1 (lane 1) or PLC-γ2 (lane 2). **B.** Super8XTopFlash activity in SW480 cells pre-treated with siRNA against PLC-γ1 or -γ2 as indicated. RLU, relative light units. Error bars represent standard deviation. Data represents mean RLU from triplicate transfections.

5.8 Hypothesis & Rationale: Targeting E2F1

E2F1 is a transcription factor that promotes proliferation but also promotes the upregulation of apoptotic genes. In a recent paper by Morris *et al*. (2008), E2F1 was found to be a negative regulator of Wnt/β-catenin signaling (296). Therefore, we hypothesized that E2F1 may potentially mediate the downregulation of Wnt signaling by Src.

5.9 Results: Src Downregulation of the Canonical E2F-Luciferase Reporter pE2F4B

As a first step to test this possibility, we assayed another luciferase based reporter pE2F4B (also called E2F4B-Luc, Figure 2.5, page 67) which was used by Morris *et al*. (2008) to measure the potential for Src to up-regulate E2F1, and thereby to potentially downregulate Wnt signaling (296). The canonical E2F-luciferase reporter (pE2F4B) was generously provided to us by Eric J. Morris (Harvard) and contains 4X E2F consensus binding sites. The Super8XTopFlash reporter responded as in previous experiments. The data also show that transfection of the activated mutant SrcY530F was able to downregulate the pE2F4B luciferase reporter in a manner similar to its downregulation of the Wnt reporter (Figure 5.9, A and B). Although the overall luciferase activity of Super8XTopFlash transfected cells was higher by approximately 400 fold (compare pCI columns from A and B), the relative changes were very similar upon transfection of cells with SrcY530F. Therefore, transient transfection of SrcY530F into SW480 cells downregulates both the Wnt reporter Super8XTopFlash and the canonical E2F-luciferase reporter pE2F4B in a similar manner.

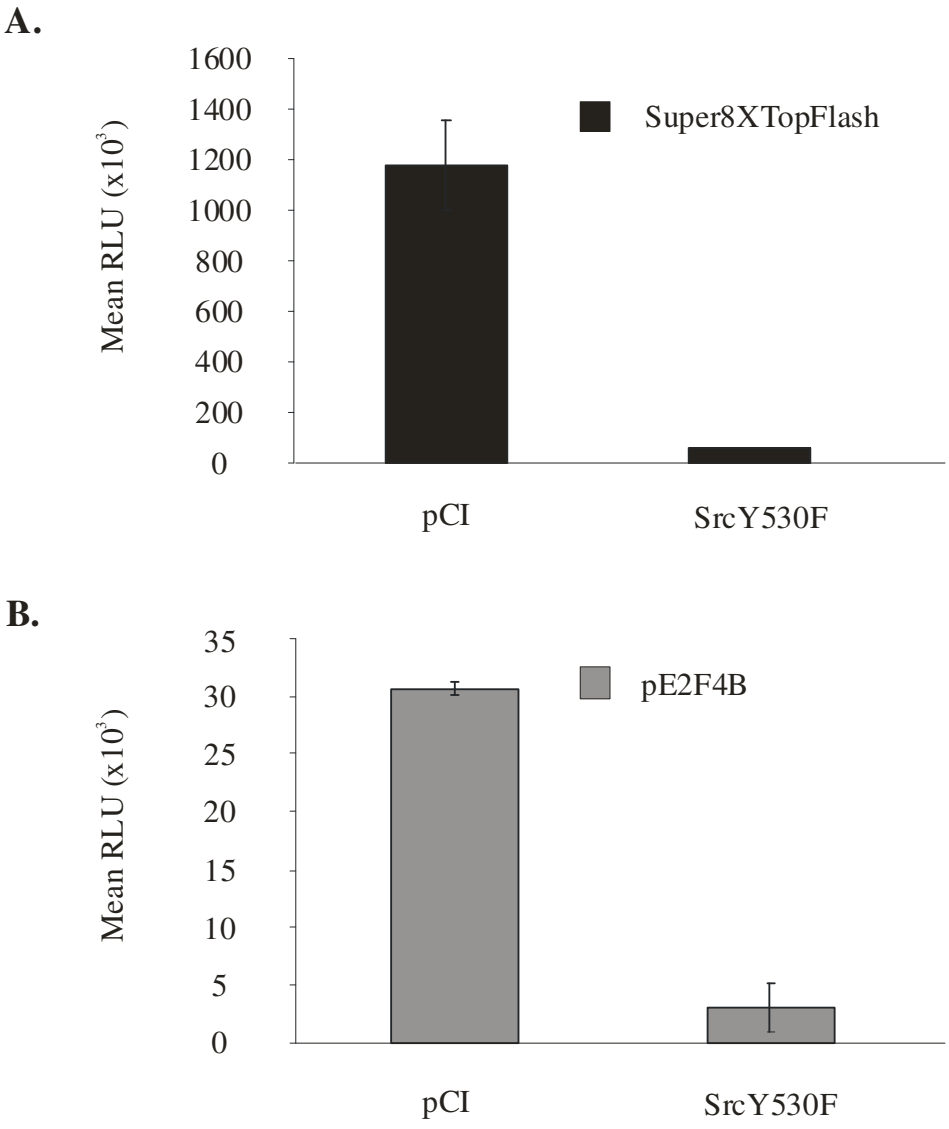


Figure 5.9 Activated mutant SrcY530F downregulates the canonical E2F-luciferase reporter pE2F4B. A. Super8XTopFlash activity in SW480 cells transfected with either empty vector pCI or activated mutant SrcY530F. **B.** Cells transfected as in A, except using the E2F-4B-Luc repoter. RLU, relative light units. Error represents standard deviation. Data shown as mean RLU of experiments done in triplicate. Error bars in SrcY530F (A) transfected cells not shown due to relatively small value and scale of figure (actual SD is approximately 3858.8 or 6.2% of mean).

CHAPTER 6: SRC KINASE REGULATION OF MULTIPLE REPORTER PLASMIDS

6.1 Rationale:

To identify the mechanism of reporter downregulation by Src kinase, several different control reporter plasmids that should not be regulated by Src were analysed. These included the Super8XTopFlash backbone pTA-Luc, pGL3-Control and phCMV-CLUC. Therefore, in this chapter I tested the hypothesis that Src was downregulating luciferase expression from the plasmid vector backbone. This was tested by co-transfecting activated SrcY530F with the individual luciferase plasmid vectors.

6.2 Results: Src Kinase Regulation of Cell Cycle Reporters

The empty vector backbone, pTA-Luc, was purchased from Clontech as a part of their Pathway Profiling Luciferase System 4. This kit contains luciferase reporter plasmids responsive to cell cycle signaling pathways that involve E2F transcription factors, pRb, p53 and Myc. This luciferase profiling kit also provided me a second method of testing the effect of SrcY530F on Myc transcriptional activity (and compare this data to the real-time data in chapter 4), as well as providing a second E2F-luciferase reporter (within the pTA-Luc vector backbone).

When all of the reporter constructs were co-transfected in SW480 cells with either the empty vector or the activated mutant SrcY530F, all of the reporters were observed to be downregulated by transiently transfected SrcY530F, including the empty vector control (Figure 6.2.1). Figure 6.2.1, B highlights the inhibitoty response of luciferase vector

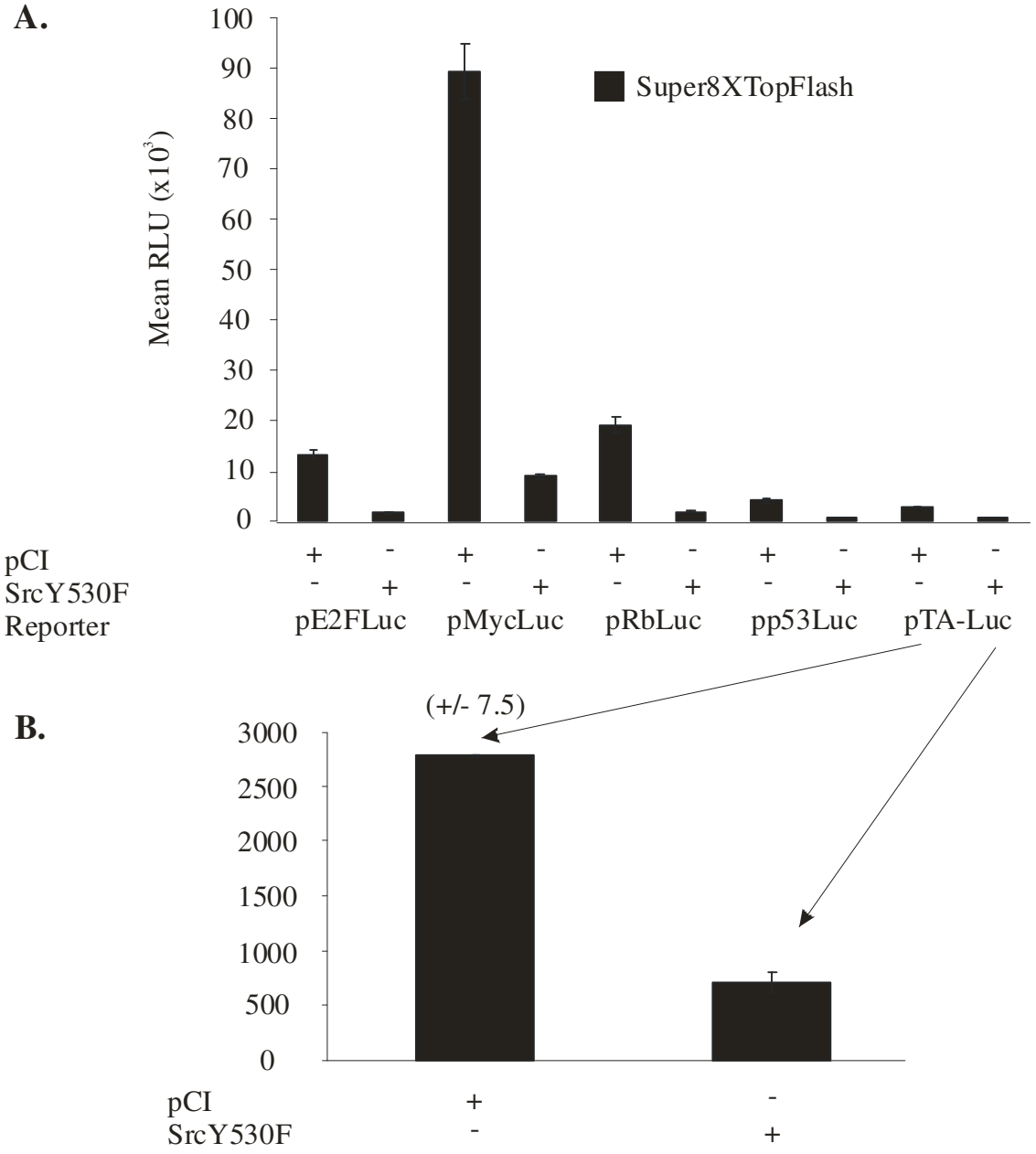


Figure 6.2.1 Activated mutant SrcY530F downregulates the luciferase activity of all Clontech Pathway Profiling Luciferase System 4 reporters in SW480 cells. These luciferase reporters contain cis-enhancer elements responsive to the indictated cell-cycle regulatory signaling molecules. The luciferase plasmid vector backbone is pTA-Luc (see Materials and Methods, Figure 2.2, A). **A.** Mean luciferase activity from SW480 cells co-transfected in triplicate with activated SrcY530F (or control pCI) and each of the 5 different vectors. **B.** Data for empty vector pTA-Luc luciferase readings from same experiment in A. RLU, relative light units.

pTA-Luc to transiently overexpressed SrcY530F. Of note, the mean values are significantly lower than compared to the other luciferase vectors in the profile. Importantly, the relative reduction, presumably due to SrcY530F, was very similar to the relative reduction observed both with Super8XTopFlash as well as pE2F-4B-Luc (see Figure 5.1). In order to determine whether or not the repression was a genuine effect on cell cycle regulators, wt and mutant Src transfected cells were assessed for their cell cycle distribution by flow cytometry. FACS analysis was carried out at the University of Calgary Flow Cytometry Core Facility, using propidium iodide staining (see methods). The data showed that neither wild-type nor mutant Src kinases (both kinase inactive K298M and active Y530F significantly altered the distribution of cells at the various stages of the cell cycle (Figure 6.3.2, A). The percentage of cells found to be non-viable (necrotic/apoptotic) was increased when transfected with any of the plasmid vectors compared to untreated cells (Figure 6.2.2, B). This data demonstrates that while transfection of the activated mutant SrcY530F downregulates all of the luciferase reporters from the Clontech cell-cycle Pathway Profiling System 4, it does not alter progression of SW480 cells through the cell-cycle.

6.3 Src Kinase Regulation of Constitutively Activated Luciferase Reporters

Activated mutant SrcY530F was co-transfected with two different control vectors that constitutively express luciferase. When SW480 cells were co-transfected with the pGL3-Control vector, luciferase activity was again downregulated relative to cells transfected with pCI (Figure 6.3, A). In contrast to this result, activated mutant Src was not able to downregulate the constitutively active reporter phCMV-CLUC (Genlantis)

(Figure 6.3, B). Lanes 4 to 6 represent lysate that was diluted 5 fold for luciferase assay in order to bring the activity within the same range as the Super8XTopFlash reporter activities. When this experiment was repeated using 0.2 X the amount of phCMV-CLUC plasmid compared to Super8XTopFlash, a similar result was observed where SrcY530F did not downregulate Super8XTopFlash activity (data not shown).

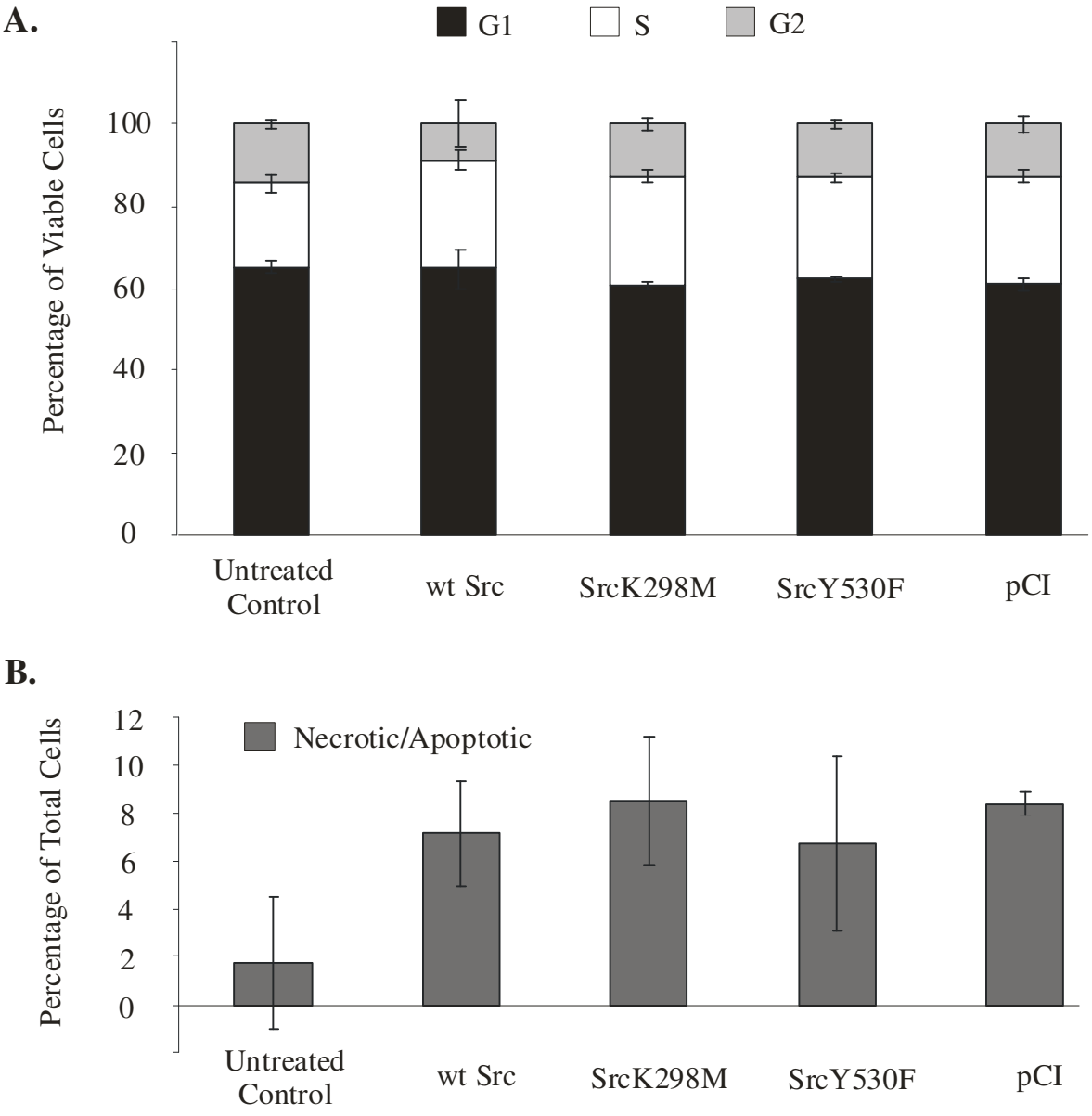


Figure 6.2.2 Transient expression of wt or mutant Src kinases does not alter the progression of SW480 cells through the cell cycle. A. FACS analysis of SW480 cells transiently transfected as indicated, expressed as a percentage of viable cells at a given cell cycle stage as determined using propidium iodide. **B.** Percentage of total number of cells identified as necrotic or apoptotic (i.e. non-viable).

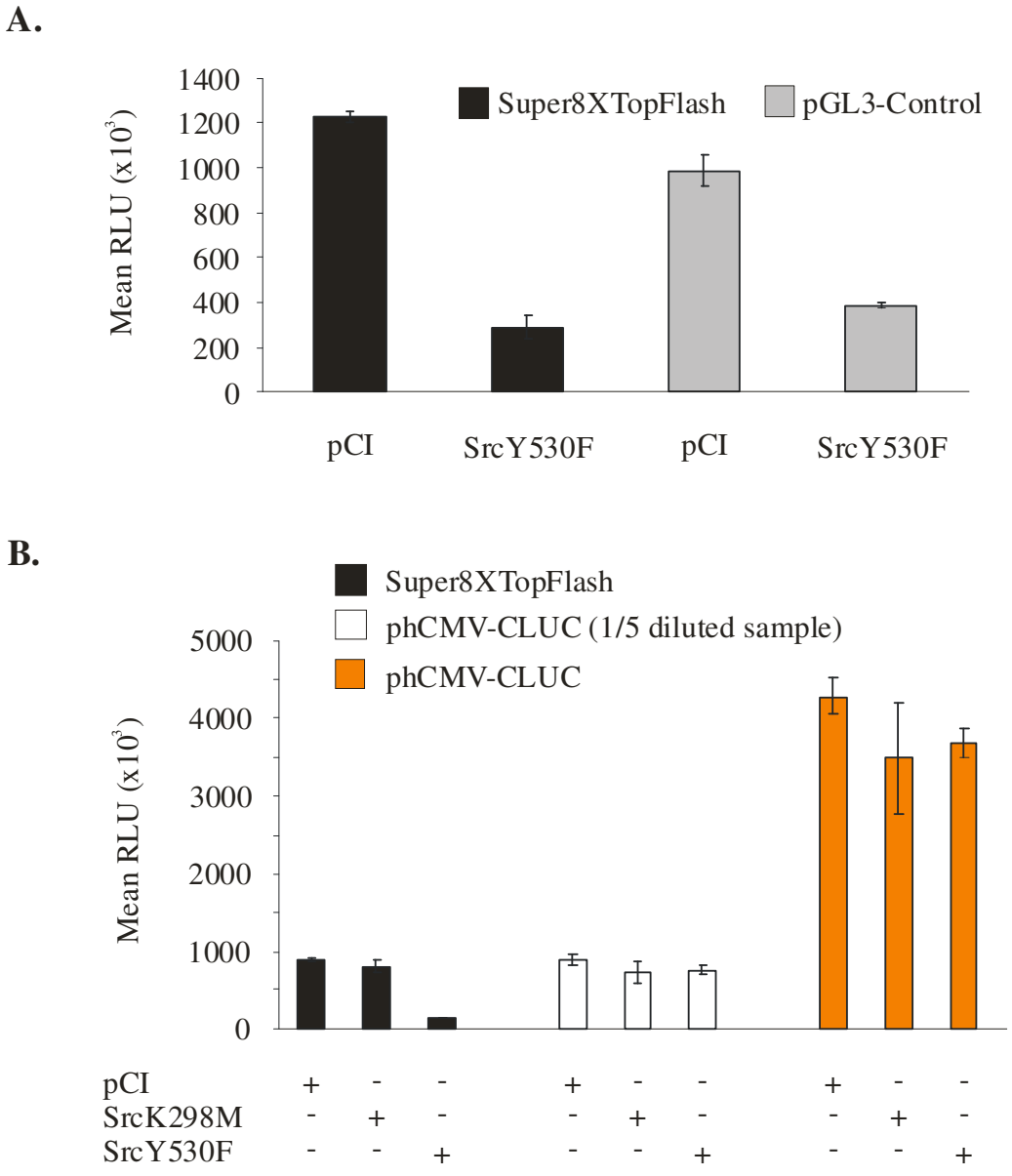


Figure 6.3 Src kinase differentially regulates two different constitutively activated luciferase reporters. A. SW480 cells transiently co-transfected with empty vector pCI or activated mutant SrcY530F and either Super8XTopFlash or pGL3-Control (Promega) that constitutively expresses luciferase. **B**. SW480 cells transfected as in A, except using the constitutively activated luciferase reporter plasmid phCMV-CLUC. Columns in orange represent data from phCMV-CLUC transfected SW480 cell lysate diluted 5 fold before using for luciferase assays. RLU, relative light units. Data represent mean RLU from transfections performed in triplicate.

SECTION III: DISCUSSION

CHAPTER 7: DISCUSSION

Src kinase and Wnt signaling both play important roles in cell biology. While Src is not critical for development, it is implicated in numerous signaling pathways that regulate a wide variety of cellular functions, which reveals its potential contribution to cancer. On the other hand, canonical Wnt signaling plays a crtical role at many stages throughout development and, consequently, it plays a critical role in the promotion of cancer, especially the inherited form of colon cancer FAP (familial adenomatous polyposis). In this study I attempted to test the hypothesis that there is an interaction between Src kinase and Wnt signaling pathways.

Initially, I tested the hypothesis that Src kinase could phosphorylate β-catenin and induce its nuclear localization. In turn, this would result in the transcriptional activation of Wnt target genes. I previously stated that this hypothesis was based on the observation that both Src kinase and Wnt signaling have been shown to upregulate some of the same genes (CCND1 and MYC), but they are also implicated in both breast and colon cancers. Other studies have shown that tyrosine phosphorylation of β-catenin was important for its transcriptional function. For example, Ilan *et al*. (1999) show that exogenous expression of platelet endothelial cell adhesion molecule (PECAM-1) can act as a reservoir for tyrosine phosphorylated β-catenin in EOMA and SW480 cells, and that stimulation of EOMA cells with vascular endothelial growth factor (VEGF) can induce this phosphorylation event. In a subsequent study by Zhang *et al*. (2001) it was shown that VEGF is also transcriptionally regulated by canonical Wnt signaling. These data not only show that tyrosine phosphorylation of β-catenin induces its transcriptional potential, but also demonstrates a

potential positive feedback mechanism in the case of VEGF. Interestingly, it was shown that a Src kinase family inhibitor (M475271) was able to prevent VEGF induced β-catenin tyrosine phosphorylation, and inhibited human umbilical vein endothelial cell (HUVEC) proliferation, migration and tube formation, although the authors acknowledged that other pathways and signaling molecules may have been involved but were not tested for their possible involvement (297). Another example of growth factor induced tyrosine phosphorylation of β-catenin is through the stimulation of cells with hepatocyte growth factor (HGF). Using normal rat primary hepatocyte cultures, Monga *et al*. (2002) showed that stimulation of cells with HGF (a.k.a. scatter factor) could induce the tyrosine phosphorylation of β-catenin and its subsequent nuclear localization. The interaction with the HGF receptor Met was shown to be direct, and the phosphorylation of β-catenin by Met could be antagonized through the exogenous expression of a dominant negative Met receptor. A third, and more recent, example of tyrosine phosphorylation induced nuclear localization of β-catenin was demonstrated through an association with the FMS-like tyrosine kinase-3 receptor (FLT3) (298). In this study, the authors showed using leukemic cell lines expressing wt or mutant FLT3, that there was a direct interation with β-catenin, and that stimulation of cells with FLT3 ligand could induce the tyrosine phosphorylation and nuclear accumulation of β-catenin. Finally, a study by Coluccia *et al*. (2007) , through the use of the Bcr-Abl inhibitor imatinib on Bcr-Abl^{+} chronic myelogenous leukemia (CML) cells, suggested that the tyrosine phosphorylation by β-catenin by Bcr-Abl promotes its stability and nuclear localization by preventing its interaction with GSK3β and subsequent serine/threonine phosphorylation. An event necessary for its degradation.

Therefore, these examples support a model whereby Src induced phosphorylation of β-catenin can induce its nuclear localization and transcriptional potential.

As previously mentioned, β-catenin is a substrate of Src and it plays an important role in maintaining the intracellular integrity of adherens junctions through its interaction with E-cadherin. Importantly, it is a critical mediator of canonical Wnt signaling. The first experiment was to test the potential for Src to induce the nuclear localization of β-catenin. To do this, the intracellular localization of β-catenin in normal chicken embryo fibroblasts (CEF) was compared to its localization in Rous sarcoma virus Schmidt-Ruppin A strain infected chicken fibroblasts (SRA). We observed that in normal CEFs, β-catenin appeared punctate and non-nuclear or non-peri-nuclear, confirming what others have also observed in fibroblasts (176,201). However, in SRA cells β-catenin was found to be predominantly at the cell periphery as well as at a juxta-nuclear localization. No co-localization with the nucleus was observed. If we assume that the changes are due to Src (or v-Src in these cells), there are several interpretations that could be made from this observation. First, the altered localization may be a mechanism by which β-catenin is prepared for nuclear entry, or by nature of its closer proximity to the nucleus it is more able to enter it. Alternatively, there may be no significance to the observed juxta-nuclear localization, which may represent the secondary effect of a more compact, transformed chicken fibroblast cell. However, it would be necessary to first establish that the change in β-catenin localization was not due simply to viral infection. This could be accomplished through the use of viral strains that either have the v-Src gene (e.g. SRA) or lack the v-Src gene, followed by infection of CEFs and

analysis of β-catenin localization. It would also be important to establish whether or not the effects on β-catenin are direct or indirect.

The intracellular localization of β-catenin was also analysed by subcellular fractionation of transiently transfected human colon adenocarcinoma SW480 cells (Figure 3.4.3). As previously mentioned, the data show that there was no dramatic difference between the nuclear and cytoplasmic/membrane levels of β-catenin in SW480 cells transiently transfected with kinase inactive SrcK298M or kinase active mutant SrcY530F. A slightly darker staining β-catenin band was observed in nuclear fractions from SrcY530F, but its significance could only be determined upon repeated trials and quantitative densitometric analysis. Overall, this data is consistent with the indirect immunofluorescence data in chicken cells, where no direct nuclear localization was observed in SRA cells. However, SW480 cells already contain high levels of total β-catenin, much of which can be found in the nucleus (216). This characteristic may prevent the identification of possible changes in the nuclear levels of β-catenin induced by Src, especially if they are subtle. The analysis of the subcellular distribution of phosphorylated β-catenin would have also been important to analyse, as this modification could be an alternative mechanism of regulating its localization (see above).

β-Catenin remained the focus of the initial experiments and therefore the ability of Src to phosphorylate (or at least induce the phosphorylation of) β-catenin was assessed in multiple cell types. As previously mentioned, tsLA29 Rat-1 cells express a temperature sensitive mutant form of v-Src and incubation of the cells at the appropriate temperature is a convenient way to both activate and inactivate v-Src kinase activity. When β-catenin was

immunoprecipitated from tsLA29 Rat-1 cells grown at either permissive or non-permissive temperatures, tyrosine phosphorylation was clearly dependent on the temperature at which they were incubated, as measured by Western Blot analysis using the anti-phosphortyrosine antibody 4G10. This strongly suggested that Src was inducing the tyrosine phosphorylation of β-catenin. However, to be more confident that this phosphorylation was due to v-Src activation not a temperature shift, it would be necessary to identify and/or use a mutant viral strain which lacks v-Src (or lacks the P507A mutantion that is responsible for its ts properties such as the SRA strain) in its genome and repeat the temperature shift experiments. Alternatively, if a direct interaction could be shown (e.g. co-immunoprecipitation of β-catenin and v-Src), this would also provide more direct evidence that v-Src is phosphorylating β-catenin. Similarly, tyrosine phosphorylated β-catenin was detected in SRA cells, but not in normal CEFs. In SW480 cells, tyrosine phosphorylated β-catenin was observed in lysates from cells transfected with activated SrcY530F, but not in empty vector pCI or kinase inactive SrcK298M transfected cell lysate. Attempts were unsuccessful to co-immunoprecipitate β-catenin and Src in SW480 cells, but an interaction between Src and β-catenin has been shown using GST-pull downs (232). Interestingly, there are not many examples in the literature of a direct interaction between Src and β-catenin. This suggests that either the interaction is extremely transient or, that it simply does not occur and that at least one intermediary kinase mediates this phosphorylation event. Taken together, however, the data strongly suggest that the tyrosine phosphorylation of β-catenin is, at minimum, induced by the presence of activated Src kinase.

The ability of Src to alter β-catenin stability in these cell lines was also assessed, as it is the stability of β-catenin that normally plays a crucial role in determining the activation state of Wnt signaling. In CEF and SRA cells, there was no observable difference in β-catenin levels when equal amounts of total protein were compared. Similarly, when the temperature sensitive mutant v-Src (tsLA29 infected Rat-1 cells) was activated after incubating cells at the permissive temperature, the total protein levels of β-catenin was not dramatically changed (Figure 3.3.1, C). In this particular example at the 24 time-point β-catenin levels may have decreased, when the experiment was repeated several times there was no noticeable difference. Subsequent pulse-chase analysis using tsLA29 Rat-1 cells also suggested that activated mutant v-Src does not dramatically affect the stability of β-catenin after 24 hours. Although the pulse-chase analysis showed a decrease in β-catenin stability within the first 8 hours when cells were incubated at the permissive temperature (i.e. when mutant v-Src was activated), the data also indicated that there was an increased incorporation of ^{32}P, and hence an increased β-catenin stability. This unlikely situation strongly suggests that there was a loading error, which could have been demonstrated by including a loading control (e.g. immunoprecipitation of β-catenin followed by analysis of autoradiogram). In addition, it would be necessary to repeat the experiment several times in order to confirm or refute the observed changes in β-catenin stability in tsLA29 Rat-1 cells.

The stability of β-catenin was also analysed by transient transfection of SW480 colon adenocarcinoma cells with activated mutant SrcY530F. The data showed that Src does not dramatically change the levels of β-catenin (increase or decrease) over the 24 hour period tested. The nuclear fractions of SW480 cells transfected with either kinase inactive

mutant SrcK298M or kinase active mutant SrcY530F were also compared, since the total β-catenin levels in these cells are very high and, it is the nuclear fraction of β-catenin that is important for activation of canonical Wnt pathway genes. Western blot analysis of these nuclear fractions did not show a dramatic difference in β-catenin levels, although a marginally darker staining β-catenin band was observed in SrcY530F transfected nuclear lysates indicating a very small increase in nuclear β-catenin due to Src. This data suggests that Src does not decrease nuclear levels of β-catenin, but the evidence also does not exclude the possibility that Src may significantly increase nuclear β-catenin levels. This is due to the observation that these cells already contain high levels of nuclear β-catenin, reflecting a nuclear saturation effect, a hypothesis which would require additional testing. Further analysis, which was not done, using these subcellular fractions to test for possible changes in the distribution of phosphorylated β-catenin may have revealed a relocalization due to Src induced phosphorylation.

The results obtained in chapter 3 find some consistency with what other studies have found. For example, the tyrosine phosphorylation of β-catenin has been shown to be associated with v-Src activity in v-Src transformed rat fibroblasts, and a direct interaction between v-Src and β-catenin, followed by phosphorylation of β-catenin has also been shown *in vitro* (115,232). However, other data in this chapter differ from what others have observed. These include changes in the localization of β-catenin, where it has been shown that treating DLD-1 and Ls174T cells (both colorectal carcinoma cell lines) with a Src family inhibitor (SKI-606) resulted in the re-localization of the nuclear fraction of β-catenin to the cytosol (299). The authors also concluded that treatment with the inhibitor promoted

the interaction with E-cadherin leading to its stabilization, presumably due to inhibition of Src activity. Importantly, the study did not demonstrate the degree of Src (or Src family) specific inhibition. Interestingly, this study also showed that the phosphorylation of β-catenin did not affect its stability, although a study by Kim and Lee (2001) using the phosphatase inhibitor pervanadate suggested that the phosphorylation state of β-catenin was correlated with its stability (300). In the present study, there was no obvious effect on β-catenin levels (total or nuclear) in the various cell types tested compared with pCI transfected cells. These differences may be a reflection of differences in the cell types used (i.e. fibroblasts vs. epithelial vs. adenocarcinoma cell line), and/or the uncharacterized non-specific effects of the pharmacological inhibitors used in the other studies. Furthermore, it is possible that the phosphorylation of β-catenin may have changed its subcellular distribution without affecting total or nuclear levels.

A possible explanation for the lack of observable change in *FGF18*, *CCND1* or *MYC*, is that Src does not regulate the canonical Wnt pathway in SW480 cells. This may be likely, as there was no dramatic difference in the banding pattern from gel-shift/EMSA asssays performed on nuclear fractions from kinase inactive mutant SrcK298M and kinase active mutant SrcY530F (Figure 4.5.1). Small and subtle changes were noted, although the experiment was not repeated due to time constraints. For example, the overall intensity of bands in the nuclear lysates of SrcY530F transfected cells was darker, but one band in particular was lighter than the corresponding band in SrcK298M transfected cells (Figure 4.5.1, B, 2b vs 2a). However, repeated trials of the EMSAs and further experiments would have to be conducted to be sure these differences were real. These might include isolating samples and using mass-spectometry to determine the possible composition of any

particular band. Other important control experiments would be to do “super-shift” experiments, utilizing β-catenin or Tcf/Lef antibodies in order to confirm that the DNA probes were behaving as expected.

The data generated in chapter 4 contradict one another and are not consistent with the literature. For example, based on the Topflash and Super8XTopFlash reporter data one might conclude that activated Src antagonizes Wnt signaling. However, the real-time PCR assays did not show changes in the expression of previously identified Wnt target genes in SW480 cells. Exogenous overexpression of mutant Src kinases did not upregulate the expression of *CCND1* and *MYC* genes in SW480 cells. These genes have been shown to be upregulated by Src (240,241,245,305,306). Therefore, the most likely explanation is that Src kinase does not regulate the transcriptional activity of canonical Wnt signaling. However, the analysis of a greater number of Wnt target genes would be required before any definitive statements could be made.

In chapter 5, I attempted to elucidate the mechanism by which activated SrcY530F could downregulate Super8XTopFlash luciferase expression. These experiments were carried out assuming that the effect of Src on the TopFlash and Super8XTopFlash reporters was a true reflection of the ability of Src to downregulate Wnt signaling. As such, two Wnt pathway components were targeted as potential mediators of this effect, including β-catenin and GSK3β.

β-catenin was analysed as a candidate mediator by mutation of the previously identified Src phosphorylation residues. In this mechanism, tyrosine phosphorylation of β-catenin would downregulate transcriptional activation by inhibiting interaction with Tcf/Lef

proteins, or by preventing its interaction with other transcription factors. A similar mode of action has been demonstrated for the MAP Kinase-related Nemo-like kinase (NLK), whereby NLK phosphorylates LEF-1/TCF to prevent its interaction with β-catenin (197). Similarly, tyrosine phosphorylation of β-catenin prevents its interaction with cadherins. The previously identified Src targets, tyrosine 86 and 654, were mutated to phenylalanine. In order to reduce the effects of endogenous β-catenin, I used a breast tumour cell line SK-BR-3, which possesses virtually undetectable endogenous levels. When mutant β-cateninY86,654F was expressed in SK-BR-3 cells with the activated mutant SrcY530F, I observed that tyrosine phosphorylation of β-catenin was not completely abolished. According to other studies, tyrosine 142 is also phosphorylated as a result of HGF stimulation or Fyn (a Src family kinase) (264,265). The triple mutant β-catenin Y86,142,654F was synthesized. This mutation essentially removed the tyrosine phosphorylation induced by SrcY530F. When expressed in SK-BR-3 cells, the triple mutant was able to promote reporter activity at levels similar to wt-β-catenin. Although this obersvation may strongly suggest that these particular residues may not play a role in transcriptional activation, it was rationalized that the amino acid residues that may be important for, or influence, negatively regulating β-catenin's transcriptional potential could be independent of influencing its transcriptional activating potential, which may be regulated by alternative residues. This idea is similar to regulation of GSK3β, where phosphorylation of Ser 9 is inactivating but phosphorylation of Tyr 216 is activating (267,270). Co-expression of the triple mutant β-catenin with activated SrcY530F, did not reverse the inhibitory effect of SrcY530F on Super8XTopFlash activity, suggesting that the

tyrosine phosphorylation of β-catenin does not mediate this effect. Interestingly, the authors who showed that Fyn phosphorylates β-catenin on tyrosine 142 suggest that in other cell systems other kinases may perform this task, which according to their model disrupts the interaction between β-catenin and α-catenin at adherens junctions. This may be an interesting model to test in the context of Src activation and its potential to regulate cell-cell interactions or motility; however, because the focus of this study was to elucidate a possible interaction between Src and Wnt signaling, this was not investigated any further.

An important negative regulator of canonical Wnt signaling is GSK3β,which was also tested as a potential mediator of the Src effect on Super8XTopFlash activity. GSK3β phosphorylates β-catenin in a complex with Axin and APC, but only after it is primed by CK1α phosphorylation. Therefore, we tested the possibility that GSK3β may mediate Src's effect on the Wnt luciferase reporter by treating cells with LiCl – commonly used to inhibit GSK3β. This treatment resulted in a partial reversal of the Src effect (i.e. from a 3.5 fold repression without LiCl, to a 1.6 fold repression with 10 mM LiCl treatment), but further testing strongly suggested it was due in part to the inhibition of Src kinase activity, due to the decreased phosphorylation state of Src tyrosine-419 (Figure 5.3.3, A). To more thoroughly investigate this initial observation several additional pharmacological reagents, which have been shown to inhibit GSK3β with a relatively high degree of specificity, were used to treat the transfected cells. These compounds included Kenpaullone and CHIR99021. In addition, siRNA was used to target GSK3β to ensure specificity. Although none of these treatments could reverse the effect of activated SrcY530F on Suepr8XTopFlash activity, there was a small reversal in the case of Kenpaullone treatment

(from a 6.8 fold repression by SrcY530F, to a 2.4 fold repression). Importantly, Src activity (as measured by the phosphorylation of Y419) was not lowered, and may have even been slightly increased (Figure 5.3.4, C). This partial reversal was the least evident in siRNA treated cells, which suggests that Src activity was being affected in such a way which was not detectable by the phosphorylation state of Y419. A similar phenomenon was shown by Sen *et al*. (2009) where the phosphorylation of JAK is normally associated with kinase activity (44). When the authors treated cells with the tyrosine kinase inhibitor dasatinib, they demonstrated a lack of JAK phosphorylation (normally indicative of inactivation), but when kinase assays were performed at later time points, kinase activity was clearly evident. Therefore, a similar situation may have occurred when treating cells with Kenpaullone where inhibiton of Src activity actually occurred but was not demonstrated through the phosphorylation state of Y419. As in the study by Sen *et al*. (2009), this could be tested by performing a kinase assay on Src (using Src optimal peptide as a substrate) following treatment of cells by the various pharmacological compounds.

It has been shown that non-canonical Wnt signaling can antagonize canonical signaling, and it was hypothesized that Src may be acting through this alternate pathway, or at least mobilizing intracellular calcium flux, followed by inhibition of canonical Wnt signaling. Mobilization of intracellular calcium is a key aspect to non-canonical Wnt signaling (307), and so to test this hypothesis, I attempted to chelate intracellular calcium using the membrane permeable pharmacological reagent BAPTA-AM. Several treatment protocols were tested but none were able to reverse the downregulation by SrcY530F of Super8XTopFlash activity. The potential for Ca^{2+} mediated inhibiton of Super8XTopFlash activity was then tested by treating cells with either A23187 or Thapsigargin (known to

increase intracellular Ca^{2+}) using concentrations based on the study by Wu *et al*. (2001) (286). Although, treatment with these compounds could decrease Super8XTopFlash activity, the viability of these cells was not tested, and may have accounted for the decrease in luciferase reporter acitivity. When SW480 cells were further treated with BAPTA-AM (with the hypothesis being that BAPTA-AM would chelate the intracellular calcium induced by treatment with A23187 or Thapsigargin and thereby reverse the Ca^{2+} mediated reporter downregulation), reporter activity was again further downregulated. Taken together, these data suggest that treatment of transfected SW480 cells using the multiple chemical compounds may have resulted in either decreased cell viability or decreased luciferase activity. A better approach to assessing the potential for Src to induce the non-canonical pathway would have been to first directly measure intracellular Ca^{2+} levels and thoroughly optimize the BAPTA-AM concentration to be used. Even so, it was unlikely that a Ca^{2+} flux was mediating the Src effect on Super8XTopFlash activity, as a similar concentration of BAPTA-AM (50 μM) that was used in Figure 5.5.2, was used previously to prevent the Ca^{2+} mediated degradation of β-catenin (via calpain) induced by a Gq pathway (308).

A potential explanation for the ability of Src to downregulate Super8XTopFlash activity emerged when E2F1 was analysed as a potential downstream mediator of the Src effect on Super8XTopFlash reporter activity. In a recent study by Morris *et al*. (2008), the transcription factor E2F1 was shown to inhibit canonical Wnt signaling (296). E2F can promote proliferation, but it can also activate the expression of pro-apoptotic genes in response to DNA damage. It is a target of the tumour suppressor pRb, and of the oncoprotein CDK8 (309). Based on these reports, I hypothesized that Src might activate

E2F1transcriptional activity, leading to canonical Wnt down-regulation as measured by Super8XTopFlash reporter activity. The E2F transcription factor family luciferase reporter pE2F4B-Luc was obtained from Erick Morris (Harvard) and co-transfected into SW480 cells along with activated mutant SrcY530F. The results suggested that Src also downregulates E2F family mediated transcription. There was little evidence of a bona fide interaction between Src kinase and E2F transcription factors. However, one study has shown a potential interaction between v-Src and E2F1 in chicken neuroretina cells, whereby the progression through G1 induced by infection of cells with another ts mutant v-Src (ts-NY68, Rous sarcoma virus infected) was blocked by transfection of cells with deletion mutant forms of chicken E2F1 (310). Nevertheless, given the lack of extensive documentation in the literature for a genuine interaction between these two well studied molecules, additional control experiments were carried out to further assess the validity of using Super8XTopFlash (and TopFlash) as measure of the potential for Src to regulate Wnt signaling.

To further explore the nature of luciferase reporter downregulation (both Super8XTopFlash and pE2F4B-Luc), several additional luciferase based reporters were tested in SW480 cells co-transfected with SrcY530F. The first set of reporters analysed in SW480 cells was the Clontech Pathway Profiling System 4, described by the manufacturer as profiling molecules that affect the cell cycle. This series of luciferase reporters was described as containing enhancer elements responsive to molecules and their associated pathways that affect the cell cycle. Importantly, this luciferase profiling system was designed using the pTA-Luc luciferase plasmid vector backbone used in the Super8XTopFlash Wnt reporter, and include the empty vector as a control. In addition, the

profiling system also included reporters for E2F and Myc, which could be used as way to validate some of the previous experiments in this study (i.e. allow for comparison with, a) the pE2F4B-luc reporter data, and b), the real-time PCR analysis of MYC gene expression data). The data from this experiment suggested that Src could downregulate all of the cell cycle regulators measured by the Pathway Profiling System 4, including E2F, Myc, p53 and Rb (Figure 6.2.1). Importantly, it was also observed that the transient transfection of the activated mutant SrcY530F into SW480 cell could downregulate the background expression of the empty vector pTA-Luc. This data strongly suggested that the downregulation of Super8XTopFlash (and pTopFlash) by SrcY530F was an artifact, and did not represent a *bona fide* effect on Wnt signaling. Subsequent FACS analysis demonstrated that Src was not affecting the cell-cycle. Further evidence that the effect of SrcY530F on Super8XTopFlash was likely an artifact, came from the observation that Src was also capable of downregulating luciferase expression from the constitutively activated reporter pGL3-Control (Figure 6.3).

A second constitutively activated luciferase reporter was tested. Unlike pGL3-Control, the phCMV-CLUC reporter was not downregulated by the activated mutant SrcY530F (Figure 6.3). Although this result is not consistant with the luciferase data obtained using all of the other luciferase reporters in this study, the overwhelming evidence strongly suggests that the downregulation of luciferase reporter activity induced by SrcY530F does not represent a true inhibition of canonical Wnt signaling. In fact, when compared to the real-time PCR data and the gel-shift assays where no dramatic changes were induced by SrcY530F, this would be a logical conclusion. One explanation for the inability of Src to downregulate luciferase expression from the phCMV-CLUC reporter

may be due to the strong expression driven by the CMV promoter. In chapter 4, SrcY530F was also not able to downregulate the expression of GFP driven by another CMV promoter. To test this hypothesis, the SV40 promoter from pGL3-Control could be used to replace the CMV promoter in the pEGFP-C1 plasmid, followed by co-transfection with SrcY530F in SW480 cells. The prediction from this experiment would be that SrcY530F would then be able to downregulate expression of GFP.

Another explanation for the observation that activated mutant SrcY530F could downregulate Super8XTopFlash may be that the kinase activity of Src is out-competing luciferase for ATP. This is not a likely explanation, however, since the decreased protein levels of luciferase were consistent with the decrease in luciferase activity induced by SrcY530F. The observed decrease in luciferase protein levels could also be explained by a specific inhibiton of luciferase translation, induced by Src. This could be shown by performing real-time PCR experiments on luciferase transcript in SW480 cells – an experiment that was attempted, but due to technical difficulties and time contstraints was not persued. Alternatively, if Src is negatively regulating another plasmid element (other than the promoter elements), a sequential deletion analysis of the various plasmid regions could be carried out to identify the regulatory/Src responsive plasmid element.

The observation that the activated mutant SrcY530F is able to downregulate Wnt signaling is in contradiction to a limited number of studies that have examined a potential connection between Src kinase and Wnt/β-catenin signaling. In fact, Haraguchi *et al.* (2004) showed using the Topflash and Fopflash reporters in SW480 and HEK298T cells that v-Src could significantly increase reporter activity (234). A study by Karni *et al.* (2005) also suggested that Src enhances the translation of β-catenin and that this results in

the nuclear localization and the transcriptional activation of Topflash and cyclin D1 (235). A more recent study by Coluccia *et al*. (2006) utilized a Src inhibitor to suggest that tyrosine phosphorylation of β-catenin by Src is a major regulator of growth and motility in two different colon cancer cell lines (299). These data, when compared to the results of this study, strongly suggest that Src does not antagonize Wnt/β-catenin signaling as suggested by the TopFlash and Super8XTopFlash assays. Interestingly, many of the earlier studies analyzing Src's relationship to β-catenin showed that it plays a role in affecting cell-cell interaction through disruption of the intracellular complex of adherens junctions mediated by E-cadherin. However, virtually no evidence was shown to suggest that Src could influence Wnt mediated signaling via β-catenin – a reasonable possibility given the understanding of how β-catenin was know to contribute to Wnt signaling at the time.

A re-examination of the possible interaction between Src kinase and Wnt signaling would require a different approach than those used in the present study. First, a broad analysis of multiple cells lines, including normal, non-transformed cell lines would be required in transient transfection assays using mutant Src kinases. Second, it would also be important to stimulate endogenous Src activity (for example using PDGF in PDGFR containing cells) to eliminate the possibility that results are an artifact of exogenous expression. Third, a more thorough examination of all Wnt target genes (eg microarray followed by quantitative PCR validation) as a readout would be more appropriate than an artificial reporter system.

Future studies investigating the potential interaction between Src kinase and Wnt signaling should also consider alternative Wnt activated pathways. In a recent review by

van Amerongen *et al.* (2008) the authors summarize the current understanding of Wnt and Wnt related pathways and suggest that due to the variety of Wnt ligand and receptor interations, it may not be valid to assign any single pathway a canonical designation (203). For example, Wnt signaling affecting planar cell polarity in *Drosophila* model systems reveal that although Frizzled receptors remain an important component, neither extracellular Wnt ligand nor downstream activation of β-catenin may be necessary (203). In addition, other Wnt pathways, in which signaling is initiated by Wnt ligands, do not require Frizzled family receptors such as the Ryk and Ror receptors (203,239). Interestingly, the *Drosophila* Src kinase orthologs SRC64B and SRC62A have been shown to act downstream of Wnt5/DRL signaling (Derailed, the *Drosophila* ortholog of RYK) required for axon guidance, but that the interaction between SRC62A and SRC64B with DRL does not affect TCF/LEF dependent transcription (239).

In conclusion, I was able to show that Src kinase could phosphorylate Y142 on β-catenin. The results of this study also cautions against the use of luciferase based reporters when assessing the effects of Src kinase on any given pathway. However, based on the overall data from this study, I was not able to support or disprove the hypothesis that Src kinase can promote canonical Wnt signaling.

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