The effect of histone deacetylase inhibitors on SRC and BCL2L1 gene expression and a potential role for phosphatases in their transcriptional repression

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ABSTRACT

Histone Deacetylase Inhibitors (HDACi) are a new class of chemotherapeutics which have shown promise in pre-clinical and clinical settings. HDACi have been shown to act by re-programming gene expression, with the transcription of some genes such as $p21^{WAF1}$ being activated, while others like SRC and BCL2L1 are repressed. The mechanism behind HDACi gene expression changes remains unknown; although it has been shown to involve a direct interaction with gene promoters.

Using a quantitative qRT-PCR approach, the effect of various HDACi on the transcription of $p21^{WAF1}$, SRC and BCL2L1 was examined. TSA and apicidin led to an up regulation of $p21^{WAF1}$ mRNA levels while c-Src and Bcl-x_L mRNA levels were downregulated. Short c-Src mRNA transcripts were unaffected following TSA and apicidin treatments, despite the full length transcripts being repressed. Repression of full length c-Src and Bcl-x_L mRNA transcripts was not seen following treatment with MS-275 and MGCD0103, although $p21^{WAF1}$ mRNA expression was induced. ChIP experiments revealed that following HDACi treatment, histone acetylation levels and RNA Polymerase II occupancy increased in the promoter regions of both the SRC and BCL2L1 genes. RNA Polymerase II occupancy lasted less than 15 minutes in the 3' regions of the gene following treatment with apicidin and TSA, but was more long-term following MS-275 and MGCD0103 treatment. The protein phosphatase inhibitor Calyculin A completely blocked HDACi mediated repression of c-Src and Bcl-x_L mRNA, suggesting a role for protein phosphatases in the mechanism behind HDACi.

It is therefore hypothesized that HDACi work through at least two different mechanisms. Whether or not an HDACi leads to gene repression depends on its ability to disrupt an HDAC/protein phosphatase complex and not on their HDAC specificities. The disruption of the complex leads to the release of an active protein phosphatase. The released phosphatase can then presumably act on various factors changing a gene from an active to paused state, possibly through promoter proximal pausing. HDACi unable to disrupt this complex are unable to induce gene repression. Collectively, these studies highlight not only the complexity of HDACi mediated effects within the cell, but also present a new explanation behind HDACi mediated gene repression.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BRCA1	Breast Cancer Susceptibility Gene 1
ChIP	Chromatin Immunoprecipitation
CKD9	Cyclin Dependent Kinase 9
Co-REST	Co-Repressor to RE1 Silencing Transcription Factor
CTD	Carboxyl-Terminal Domain
DMEM	Dulbecco's Modified Eagle's Medium
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DSIF	DRB Sensitivity Inducing Factor
EDTA	Ethylenediaminetetraacetic Acid
ELL	Eleven-Nineteen Lysine-Rich in Leukemia
FACT	Facilitate Chromatin Transcription
FBS	Fetal Bovine Serum
H3K36 Me_3	Lysine 36 on Histone 3 Trimethylation
H3K4 Me_3	Lysine 4 on Histone 3 Trimethylation
H3K9Me_3	Histone 3 Lysine 9 Trimethylation
H3K9Me_2	Histone 3 Lysine 9 Dimethylation
HAT	Histone Acetyl Transferase
Hda1	Histone Deacetylase 1
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase Inhibitors
HEAT	Huntingtin Elongation Factor 3 Protein Phosphatase 2A
INO80	Inositol Biosynthesis 80
ISWI	Imitation Switch
mRNA	Messenger RNA
NAD+	Nicotinamide Adenine Dinucleotide
N-Cor	Nuclear Receptor Co-Repressor
NELF	Negative Elongation Factor
NURD/Mi-2/CHD	Nucleosome Remodeling Histone Deacetylase/Chromodomain3

	for Mi-2alpha/chromodomain
p-Akt	Ser 473 Phosphorylation of Akt
PBS	Phosphate Buffered Saline
PCAF	p300 CREB Binding Protein Associated Factor
PCR	Polymerase Chain Reaction
PIC	Pre Initiation Complex
PP	Protein Phosphatase
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
PPA	Aspartate Based Phosphatases
PPM	Metal Dependent Protein Phosphatases
PPP	Phosphoprotein Phosphatases
PTEF-b	Positive Elongation Factor b
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
Rb	Retinoblastoma
RNA Pol II	RNA Polymerase II
Rpd3	Reduced Potassium Dependency 3
Sin3	Switch Independent 3
sir2	Silent Information Regulator 2
SMRT	Silencing Mediator for Retinoic Acid and Thyroid Hormone Receptor
SWI/SNF	Switching/Sucrose Non Fermenting
SWR1	SWI/SNF Related Factor 1
TAF1	TATA Binding Protein Associated Factor 1
TSA	Trichostatin A

1. REVIEW OF THE LITERATURE

1.1 Introduction

Histone deacetylase inhibitors (HDACi) are relatively new a class of chemotherapeutic agents which have shown considerable promise in both pre-clinical and clinical settings. Not only are HDACi able to induce cell cycle arrest, differentiation, and apoptosis, but they do so with a selectivity towards cancerous cells (Rosato *et al.,* 2005; Bolden *et al.,* 2006; Minucci and Pelicci, 2006; Dokmanovic *et al.,* 2007; Glaser *et al.,* 2007; Xu *et al.,* 2007; Marks and Xu, 2009). Initially the mechanism was assumed to involve HDACi shifting the balance of histone acetylation-deacetylation towards a state of hyperacetylation; leading to an overall increase in gene expression (Ma *et al.,* 2009). However, recent studies have shown that not only do HDACi increase gene expression, but also cause selective gene repression (Bolden *et al.,* 2006; Dokmanovic *et al.,* 2007; Marks and Xu, 2009). The mechanism of this selective gene repression is unknown, but may involve protein phosphorylation (Galasinski *et al.,* 2002**;** Brush *et al.,* 2004; Gregoretti *et al.,* 2004; Chen *et al.,* 2005; Gou *et al.,* 2007). Therefore the aim of this thesis is to further investigate the mechanism behind HDACi mediated selective gene repression by examining the SRC and BCL2L1 genes; both of which are repressed following treatment with this class of chemotherapeutics. The following literature review will summarize the current knowledge on chromatin modifications, transcriptional regulation, as well as introduce the human SRC and BCL2L1 genes.

1.2 Chromatin Modifications

The eukaryotic genome is regulated through a series of tightly controlled steps allowing for the regulation of cellular function. The nucleosome is one of the key structures involved. The nucleosome consists of 147 base pairs of DNA wrapped around an octamer of histones, containing two copies each of histones H2A, H2B, H3 and H4 (Arents *et al.,* 1991; Campos and Reinberg, 2009; Sivolob *et al.,* 2009). Each nucleosome is separated from the next by 10-60 base pairs of DNA which interact with histone H1 (Hansen *et al.,* 1998; Tolkunov *et al.,* 2010). The interaction of the nucleosomes and DNA creates the characteristic "beads on a string" fiber which is 10 nm in diameter (McBryant *et al.,* 2006; Fussner *et al.,* 2011). This

fiber undergoes further modifications and compaction to form a chromosome (Daban 2003; Fussner *et al.,* 2011).

Carboxyl and amino terminal domains of each histone within the nucleosome are seen as protruding tails (Campos and Reinberg, 2009). The protruding tails are around 40 amino acids in length and are subject to numerous post translational modification including lysine acetylation, methylation, ubiquitynation, SUMOylation, arginine methylation, and serine/threonine phosphorylation (Gelato and Fischle, 2008; Campos and Reinberg, 2009). These modifications allow for many protein-protein interactions, and determine the level of chromatin condensation which can vary from highly condensed heterochromatin to uncondensed euchromatin. Euchromatin tends to be gene rich and transcriptionally active whereas heterochromatin is generally transcriptionally silent, rich in repetitive sequences, and necessary for the formation of chromosomal structures such as the centromere and telomeres (Santos-Rosa and Caldas, 2005; Campos and Reinberg, 2009). Therefore the type of modifications present and the subsequent level of chromatin condensation, in a given region of the genome, will in part determine the level of gene transcription that is possible.

Post translational modifications to the chromatin occurs in many ways including intrinsic, extrinsic, and effector mediated modifications (Gelato and Fischle, 2008; Campos and Reinberg, 2009). Intrinsic effects include variations such as the incorporation of a variant histone protein. This type of modification directly alters the properties of the nucleosome, including the number of DNA contacts, the size of the nucleosome, and its stability (Ruthenburg *et al.,* 2007; Whitehouse *et al.,* 2009). Extrinsic modifications involve enzyme-mediated remodeling of chromatin structure, and are one of the major methods by which euchromatin can be converted into heterochromatin. Remodeling enzymes responsible for extrinsic modifications include the switching/sucrose non fermenting (SWI/SNF) family, the imitation switch (ISWI) family, the inositol biosynthesis 80 containing (INO80) family, the SWI/SNF related factor (SWR1) family, and the nucleosome remodeling histone deacetylase/chromodomain for Mi2alpha/chromodomain (NURD/Mi-2/CHD) family (Campos and Reinberg, 2009). These enzymes can change the location of the nucleosome along the DNA, as well as allow access for various transcription factors (Oki *et al.,* 2004; Portela *et al.,* 2010). The third method of nucleosome modification, effector mediated, involves

modifications to the histone tails, and are classified as epigenetic modifications (Klose and Bird, 2006; Ballestar, 2011).

Epigenetic modifications have been shown to facilitate the binding of nucleosome remodeling factors, many of which contain bromodomains and chromodomains binding to acetylated and methylated lysine residues respectively, as well as creating binding sites for various proteins (Gelato and Fischle, 2008; Portela *et al.,* 2010). Common modifications include acetylation, methylation, ubiquitination, and SUMOylation of lysine residues, arginine methylation, serine and threonine phosphorylation, glutamate ADP-ribosylation, and proline isomerization (Gelato and Fischle, 2008; Bannister *et al.,* 2011). The various modifications to the histone tails leads to a change in the net charge of the nucleosome, altering the electrostatic interactions and therefore the folding of the chromatin (Gelato and Fischle, 2008; Bannister *et al.,* 2011). The integration of all modifications present in a given region of the genome will determine whether or not the chromatin is in a transcriptionally active or inactive state, an idea termed the histone code (Strahl and Allis, 2000; Bannister *et al.,* 2011). One form of epigenetic modification, histone acetylation, has been shown to not only alter the interactions between histones and the associated DNA, but also the interactions of many non histone proteins. Acetylation is now thought to rival phosphorylation as a major means to regulate cellular events (Gelato and Fischle, 2008; Bannister *et al.,* 2011).

1.2.1 Histone Acetylation

Allfrey and colleagues first discovered in 1964 that the reversible addition of an acetyl group to lysine residues was important to both local and global gene expression (Allfrey *et al*., 1964; Dekker and Haisma, 2009). Since this initial discovery interest has grown substantially in determining how this epigenetic modification plays such an important role in gene expression. The first histone acetyl transferase (HAT) was discovered in 1995 (Kleff *et al.,* 1995; Yang and Seto, 2007) with histone deacetylases (HDAC) first reported in 1996 (Taunton *et al.,* 1996).

1.2.1.2 Histone Acetyl Transferases

The addition of an acetyl group from acetyl CoA decreases the positive charge of the histone, decreasing the interaction with the DNA backbone, and therefore, allowing increased access to various transcription factors. In addition to the role in chromatin modification, HATs have also been shown to play a role in post-transcriptional protein modification, pre-mRNA processing, mRNA stability, protein stability through increasing proteasomal degradation, regulation of folding mechanisms in the endoplasmic reticulum, cell cycle control, and cell metabolism (Kouzarides, 2000; Sprange *et al*., 2009). Acetylation of non histone proteins has also been shown to influence protein-protein interactions (Buchwald *et al.,* 2009). Therefore the acetylome now includes not only modifications to chromatin, but also modifications to many cellular proteins, and rivals phosphorylation for its role in cell regulation (Minucci and Pelicci, 2006; Choudhary *et al.,* 2009; Norris *et al.,* 2009). HATs are subdivided into five different families the GCN5, MYST, p300/CBP, nuclear receptor co-activators, and general transcription factor family (Table 1.1). The overall level of acetylation in the cell is determined by the balance between the activity levels of HATs and HDACs.

1.2.1.3 Histone Deacetylases

Like HATs, HDACs plays a role in epigenetic modification of the histone tail, as well as various non-histone protein substrates. Eighteen HDACs have been identified in the human genome, which are classified into four families based upon their homology to the yeast HDACs reduced potassium dependency (Rpd3), histone Deacetylase 1 (Hda1), and silent information regulator 2 (sir2) (Table 1.2**)** (Sengupta and Seto, 2004; Smith, 2007; Buchwald *et al.,* 2009). The different families of HDAC show selectivity with respect to sub-cellular localization, substrates, and tissue dependent expression.

Although divided into different groups based upon their homology to yeast HDACs all of these enzymes remove acetyl groups from lysine residues on their target substrates. When acting on the histone tail, the removal of an acetyl group increases the positive charge of the histone allowing for stronger interactions with the DNA backbone. The subsequent tighter

Table 1.1 Histone Acetyl Transferase Families

Family	Members	Function
GCN5	GCN5, hGCN5, PCAF	Transcription Initiation
MYST	MOZ, Ybf2/Sas3, Sas2, Tip60	Catalytic subunits found in large protein complexes
p300/CBP	p300, CBP	Associate with proteins involved in transcription regulation and tumor suppression
Nuclear receptor co-activator	Various proteins shown to contain intrinsic HAT activity but share low sequence similarity	
General Transcription Factors		

(Sterner and Berger, 2000; Schrump, 2009; Sprange *et al.*, 2009; Sapountzi *et al.*, 2011)

Table 1.2 Classes of Histone Deacetylases

Class	Members	Related Yeast HDAC	Tissue Location	Cell Location
Class I	1, 2, 3, 8	Rpd3	All tissue types	Nucleus
Class IIa	4, 5, 7, 9	hda1	Heart, brain, skeletal muscle	Nucleus and Cytoplasm
Class IIb	6, 10	hda1	Heart, brain, skeletal muscle	6 – Cytoplasm
				10- Nucleus
Class III	Sirt 1-7	Sir2	All tissue types	Nucleus and Mitochondria
Class IV	11	Unknown	Unknown	Unknown

(de Rujiter *et al.*, 2003; Holbert and Marmorstein, 2005; Lin *et al.*, 2006; Witt *et al.*, 2009; Peserico *et al.*, 2011)

wrapping around the histone, decreases the accessibility of DNA to transcription factors, leading to a decrease in gene transcription (Bolden *et al.*, 2006; Bannister *et al.*, 2011).

The role of the HDAC family members has recently been expanded and now includes modifications to the histone tail, as well as post translational modifications of various proteins as a method to regulate protein activity. More than 1700 different protein targets have now been identified containing over 3600 acetylation sites (De Ruijter *et al.*, 2003; Choudhary *et al.*, 2009; Bradner *et al.*, 2010). Protein targets include transcription factors, transcriptional co-regulators, α-tubulin, acetyl-coA synthetase, Hsp90, MyoD, p53, and E2F (Kouzarides, 2000; Sprange *et al.*, 2009). The change in acetylation of proteins can lead to an increase or decrease in the proteins activity, change the subcellular localization, facilitate binding to other proteins, or can modify other post translational modifications such as ubiquitination and phosphorylation (Buchwald *et al.*, 2009). Due to the number of targets now identified as HDAC substrates, this class of enzymes may be more appropriately named lysine deacetylases (Kouzarides, 2000; Sprange *et al.*, 2009).

Class I HDACs include HDAC 1, HDAC 2, HDAC 3, and HDAC 8 and are homologus to the yeast protein Rpd3 (Witt *et al.*, 2009). They are ubiquitously expressed throughout the body and are present mainly within the nucleus of the cell (Witt *et al.*, 2009). Enzymes in this class are characterized by their N-terminal catalytic domain which is utilized for the removal of acetyl groups through a two histidine-asparagine charge relay system co-ordinated by a zinc ion (de Rujiter *et al.*, 2003; Holbert and Marmorstein, 2005; Lin *et al.*, 2006; Peserico *et al.*, 2011). Members of this family, particularly HDAC1 and HDAC2, are commonly found active only when incorporated into a multi protein complex (de Rujiter *et al.*, 2003). HDAC1 and HDAC2 are associated with the switch independent 3 (Sin3), nucleosome remodeling histone deacetylase (NuRD), and co-repressor to RE1 silencing transcription factor (Co-REST) complexes (Yang and Seto, 2003; Peserico *et al.*, 2011).

HDAC3 is somewhat distinct from the other members of Class I, in that it not only contains a nuclear localization signal, but also an export signal, allowing it to shuttle between the nucleus and cytoplasm (de Rujiter *et al.*, 2003). HDAC3 has been shown to belong to various complexes including the silencing mediator for retinoic acid, thyroid hormone receptor (SMRT), nuclear receptor co-repressor complexes (N-CoR), and interacts with HDAC's from other families including HDAC 4, 5, and 7 (Li *et al.*, 2000; Gregoretti *et al.*, 2004). HDAC8,

the final and newest member of the Class I family, has not been identified in any complexes as to date (Yang and Seto, 2003; Somoza *et al.,* 2004).

Class II HDACs include HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 and are homologous to the yeast protein hda1. This class is commonly divided into two subclasses, class IIa including HDACs 4, 5, 7, and 9 and class IIb containing HDAC 6 and 10 (Gregoretti *et al.,* 2004; Bolden *et al.,* 2006; Parra *et al.,* 2010). Expression of members in this class is more tissue specific than was seen with class I HDACs, with HDAC 4, 5, and 9 being observed mainly within the heart, brain and skeletal muscle. HDAC 7 is found within the heart and lung, HDAC 6 in the testes, and HDAC 10 within the liver, kidney, and spleen (Verdin *et al.,* 2003; Chang *et al.,* 2004).

HDACs belonging to the IIa family contain a single C-terminal catalytic domain, along with nuclear localization and nuclear export signals, while the N terminal domain contains various protein interacting domains (Verdin *et al.,* 2003; Yang *et al.,* 2005; Martin *et al.,* 2007). Therefore, these HDACs are found within the nucleus and cytoplasm of their respective tissues, with their location being determined by the proteins with which they interact (de Rujiter *et al.,* 2003; Martin *et al.,* 2007). In contrast to class IIa, class IIb HDACs contain two separate catalytic domains (Gregoretti *et al.,* 2004; Yang *et al.,* 2005). HDAC6 associates with the microtubule network and therefore is found mostly within the cytoplasm (Verdin *et al.,* 2003). The second class IIb member, HDAC10, resides mainly within the nucleus and is known to associate with HDAC3 and the SMRT complex (Gregoretti *et al.,* 2004; Yang *et al.,* 2005).

Class III HDACs are both structurally and mechanistically very different from the previous two classes. Class III HDAC are dependent on nicotinamide adenine dinucleotide (NAD+) for their deacetylase activity, instead of the charge relay system utilized by class I and II HDACs (Greiss and Gartner, 2009). This class of enzymes works through a highly conserved catalytic core comprised of 250 amino acids (Greiss and Gartner, 2009). The catalytic core is comprised of 2 domains, an NAD+ binding fold domain, and a zinc binding domain located within a hydrophobic channel. In contrast to class I and II HDACs, class III HDACs use the zinc ion in a structural role rather than as a catalytic center. The class III enzymes transfer the acetyl group from a lysine residue to the ribose of the NAD+ group (Greiss and Gartner, 2009).

Class III HDACs are named Sirtuins (Sirt) one through seven; with Sirt 1, 6, and 7 being found mainly within the nucleus, Sirt 3, 4, 5 within the mitochondria, and Sirt2 in the cytoplasm (Greiss and Gartner, 2009). Sirt 1 is the member of this family that is best characterized to date, and has been shown to interact with various histone and non-histone proteins including p53 and NF-κB (Lou *et al.,* 2001; Yeung *et al.,* 2004; Dai *et al.,* 2008). Class IV HDAC contains one member, HDAC11, of which very little is known.

HDACs have been found to have aberrant activity in various diseases including neurodegeneration, cardiovascular disorders, inflammatory lung diseases, as well as a large proportion of human malignancies including hematological and solid tumors (Dokmanovic *et al.,* 2007; Sprange *et al.,* 2009). Although mutations within HDAC genes are rare, HDACs often show altered expression and aberrant recruitment. Over expression has been seen with HDAC1, HDAC2, HDAC4, HDAC6, and SIRT7 in cancers of the colon, breast, prostate, and thyroid (Bolden *et al.,* 2006; Mashall *et al.,* 2010; Aldana-Masangkay *et al.,* 2011; Park *et al.,* 2011). The altered activity of HDACs will affect cell signaling and gene expression, and has been linked to not only the progression, but also the development of various cancers (Wang *et al.,* 2009; Buchwald *et al.,* 2009). Based on the role HDACs play in a variety of cancers, drugs inhibiting their activity have become of great interest for their potential inin treating a wide variety of cancers.

1.2.1.3.1 Histone Deacetylase Inhibitors

Histone deacetylase inhibitors (HDACi) are a relatively new group of chemotherapeutic agents, which aim to reverse the gene silencing observed in various cancers by altering the epigenetic landscape (Ma *et al.,* 2009). Many different HDACi have been identified ranging from natural products such as the fatty acid butyrate and the bacteria metabolite Trichostatin A (TSA), to newer, synthetic inhibitors. The first HDACi approved for clinical use, suberoylanilide hydroxaimc acid (SAHA) or Vorinostat (Zolina), entered the clinic in 2006 for the treatment of cuteanous T-cell lymphoma (Epping and Bernards, 2009).

HDACi are grouped into one of four classes based upon their structure with functional groups including hydroxamic acids, benzamides, cyclic tetrapeptides, and aliphatic acids (Table 1.3) (Drummond *et al.,* 2005; Chavan *et al.,* 2010). Despite their different classifications, in

general, all HDACi contain three conserved structural groups: a zinc binding moiety, a capping group, and a straight chain alkyl, vinyl or aryl linker group connecting the zinc binding and capping groups (Marks and Xu, 2009). Crystal structures of HDACi bound to various HDACs have revealed that all three regions interact with the HDAC active site (Marks and Xu, 2009).

Through the alteration of the pattern of acetylation HDACi have been shown to alter not only the level of gene expression, but to also lead to an induction of extrinsic and intrinsic apoptosis pathways, affect cell cycle progression, differentiation, cell division, DNA repair, down regulate growth factors, induce oxidative stress, autophagy, and angiogenesis (Rosato *et al.,* 2005; Bolden *et al.,* 2006; Minucci and Pelicci, 2006; Dokmanovic *et al.,* 2007; Glaser *et al.,* 2007; Xu *et al.,* 2007; Marks and Xu, 2009). The mechanisms of how HDACi lead to all of the above effects are complex and not fully understood. However, HDACi have been shown to induce cell cycle arrest through the up regulation of the $p21^{WAF1}$, p27, and p57 proteins, which are all members of the cip/kip family of cyclin-dependent kinase inhibitors (Denicourt *et al.,* 2004). HDACi have also been shown to regulate the cell cycle through the down regulation of various cyclin proteins including Cyclin D1 and A (Smith and Workman, 2009). The HDACi mediated induction of apoptosis is thought to occur through up regulation of the pro-apoptotic factors Bid and Bim, and the down regulation of anti-apoptotic factors such as Bcl-$x_{L.}$ Hypo-acetylation of proteins such as E2F is also thought to play a role (Peart *et al.,* 2005). Apoptosis is also thought to result from an increase in caspase cleavage due to altered regulation of caspases 3 and 6 following HDACi treatments (Peart *et al.,* 2005).

While HDACi may be expected to have a global effect on transcription, microarray studies have shown that only between 2% and 20% of all expressed genes are affected following HDACi treatments (Bolden *et al.,* 2006; Dokmanovic *et al.,* 2007; Marks and Xu, 2009). Of the genes affected, approximately equal numbers were found to have their expression increased as were repressed. This suggests that not only are HDACi able to target specific genes, but they can lead to differential effects of the genes targeted. The mechanism behind such differential effects of HDACi is not well understood, but Marks and Xu have shown that the change in gene expression is a direct effect of the HDACi treatments (Marks and Xu, 2009). Determining the exact mechanism of action is important, as these agents are in clinical and pre-clinical use for various cancers.

Cancerous cells have been shown to contain a multitude of genetic defects that lead to the disruption of normal cellular function. In general, genes involved with the negative regulation of the cell cycle and tumor suppressor genes are repressed or silenced. Whereas genes involved with the positive regulation of the cell cycle or cell survival, are over expressed (Croce, 2008). These changes in gene expression allow for the continued proliferation of cancerous cells as well as their escape from apoptosis. HDACi through their differential effects on gene expression, provide a possible mechanism to allow both the re-expression of previously silenced genes, and also the repression of over expressed genes. In support of this, the SRC and BCL2L1 genes have been found to be over expressed in a wide variety of cancers and are both repressed following treatments with various different HDACi. In contrast $p21^{WAF1}$, a negative regulator of the cell cycle, is induced following HDACi treatment (Yeatman, 2004; Dehm and Bonham, 2004; Rada-Iglesias *et al.,* 2007; Sillars-Hardebol *et al.,* 2011). The success of the HDACi Vorinostat (Zolina, SAHA) has led to a great deal of interest in the development of new HDACi. Currently there are at least 16 different HDACi in clinical trials, which are being tested both as mono-therapies as well as in conjunction with other approved chemotherapeutics (Bolden e*t al.,* 2006; Glaser, 2007; Xu *et al.,* 2007; Carew e*t al.,* 2008; Ma *et al.,* 2009). Four representative HDACi, TSA, apicidin, MS-275, and MGCD0103 are discussed in greater detail below (Table 1.3).

TSA (Figure 1.1 A) is a pan specific HDACi acting on all class I and II HDAC. It is an antifungal derived from *Streptomyces hygroscopious* (Tsiji *et al.,* 1976; Yoshida *et al.,* 1990) and is one of the founding members of the hydroxamic acid class of HDACi. TSA is very similar in structure to Vorinostat (Zolina, SAHA), which is currently in clinical use. TSA has been shown to interact with the zinc ion at the bottom of the HDAC catalytic pocket thereby blocking the function of both class I and class II HDAC at nanomolar concentrations (Somoza *et al.,* 2004). It has been shown to inhibit G1 and G2 phases of the cell cycle and was shown to not only reduce or prevent tumorgenesis, but also to prevent metastasis (Alienberg and Silverman, 2002). TSA has a high level of cellular toxicity and therefore has never entered clinical trials (Lin *et al.,* 2006).

Apicidin (Figure 1.1 B) is a class I specific HDAC inhibitor with high specificity towards HDAC 2 and 3 (Witt *et al.,* 2009). It is a fungal metabolite produced from *Fusarium* species, with a cyclic tetrapeptide structure (Darkin-Rattray *et al.,* 1996). Apicidin has been

Table 1.3 Representative Histone Deacetylase Inhibitors

Drug	Class	HDACs Affected
TSA	Hydroxamic Acid	Class I and II
Apicidin	Cyclic Tetrapeptide	HDAC 2 and 3
MS-275	Synthetic Aminophenyl Benzamide	HDAC 1
MGCD0103	Synthetic Aminophenyl Benzamide	HDAC 1 and 2

(Somoza et al., 2004; Hess-Stump et al., 2007; Zhou et al., 2008; Witt et al., 2009; Prince et al., 2009)

shown to have anti-proliferative activity against a broad spectrum of cancerous cell lines, and induces apoptosis through a cytochrome c dependent pathway (Kwon *et al.,* 2002). It has also been shown to increase acetylation at H4 as well as inducing cell cycle arrest at the G1 stage of the cell cycle (Kwon *et al.,* 2002). Increased expression of $p21^{WAF1}$, gelsolin, the FAS ligand, as well as Bax are seen following apicidin treatment (Kwon *et al.,* 2002). Activity is seen at nanomolar to low micromolar concentrations (Smith and Workman, 2009). Apicidin has not yet entered into clinical trials, and is currently still in the preclinical stage of development (Smith and Workman, 2009).

MS-275 (Entinostat) (Figure 1.1 C) is another class I specific HDACi, and is one of the synthetic aminophenyl benzamide inhibitors (Hess-Stumpp *et al.,* 2007). MS-275 has been shown to have a high affinity specifically towards HDAC1 (Witt *et al.,* 2009). MS-275 has a long retention time within the cell and is able to induce cell cycle arrest at the G1 phase of the cell cycle at micromolar concentrations (Saito *et al.,* 1998; Minucci and Pelicci, 2006; Garber, 2007). MS-275 also induces apoptosis in a dose-dependent manner through the up regulation of $p21^{WAF1}$ and TRAIL receptors (Hess-Stumpp *et al.,* 2007). MS-275 is currently in phase I and II clinical trials and has been reported to inhibit tumor growth by up to seventy percent in some cases (Hess-Stumpp *et al.,* 2007). Originally being tested for acute myeloid leukemia treatment, MS-275 is now being tested against a variety of tumor types including prostate,

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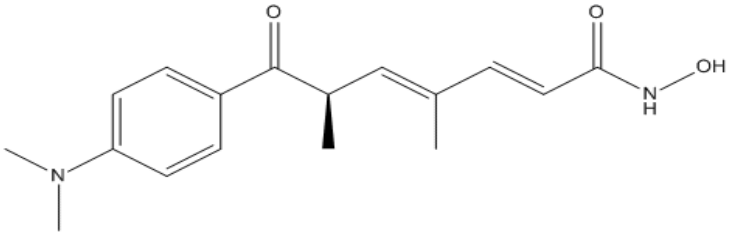

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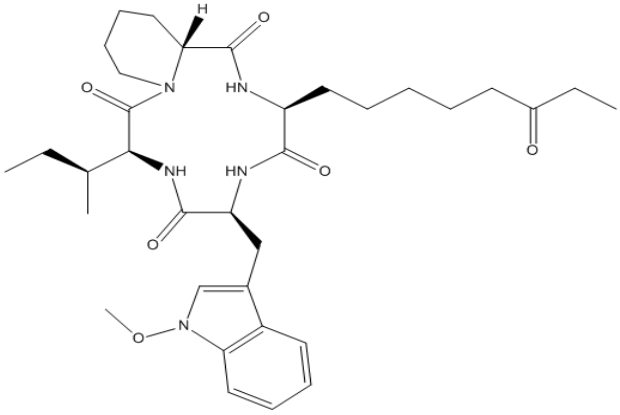

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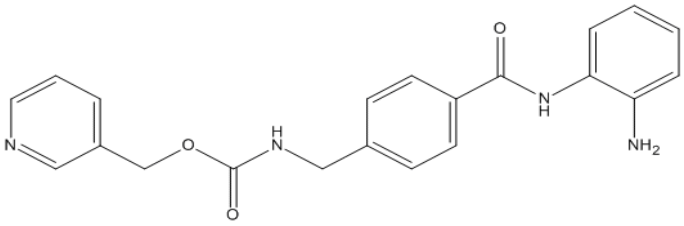

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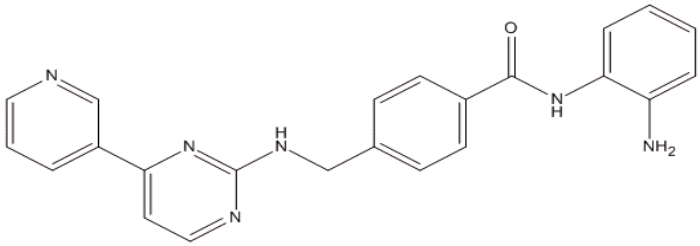


Figure 1.1: Chemical structure of histone deacetylase inhibitors. Structures of (A) Trichostatin A, a pan specific inhibitor belonging to the hydroxamic acid class (B) apicidin an HDAC 2 and 3 inhibitor in the cyclic tetrapeptide class (C) MS-275 a HDAC 1 inhibitor in the synthetic inhibitor class and (D) MGCD0103 a synthetic histone deacetylase inhibitor targeting HDAC 1 and 2.

breast, colon, solid pediatric tumors, and refractory and relapsed Hodgkin's lymphoma (Hess-Stumpp *et al.,* 2007; Sabnis *et al.,* 2011).

MGCD0103 (Mocetinostat) is a synthetic aminophenyl benzamide inhibitor (Figure 1.1 D). It is a class one specific inhibitor with a high affinity for HDAC 1 and HDAC 2 (Prince *et al.,* 2009; Zhou *et al.,* 2008). MGCD0103 has been shown to not only induce expression of $p21^{WAF1}$ as is commonly seen with HDACi, but induces histone hyper-acetylation as well as cell cycle arrest, all in a dose dependent manner (Fournel *et al.,* 2008; Zhou *et al.,* 2008). MGCD0103 is currently in phase II clinical trials for a variety of hematological malignancies including relapsed cases of Hodgkin's lymphoma (Zhou *et al.,* 2008; Younes *et al.,* 2011). The most common side effect reported requiring discontinuation of therapy is neutropenia (Younes *et al.,* 2011).

1.2.1.3.2 Histone Deacetylase Inhibitors and their Relationship to Protein Phosphatases

A review of the literature provides abundant evidence that HDACs are commonly found as part of protein complexes (Minucci and Pelicci, 2006). Research has revealed that these complexes often include various protein phosphatases (PP) (Galasinski *et al.,* 2002**;** Brush *et al.,* 2004; Gregoretti *et al.,* 2004; Chen *et al.,* 2005; Gou *et al.,* 2007). The association between HDAC and PP has been suggested by some as a method to coordinate the activity of the two enzymes to a common substrate (Brush *et al.,* 2004). As such, an understanding of the major PP involved in these complexes is necessary as this could provide clues to the possible targets.

PP are classified according to their target residue, either a tyrosine or serine/threonine phosphatase (Shi, 2009). Serine/threonine phosphatases are further subdivided into three different classes, the phosphoprotein phosphatases (PPP), metal-dependent protein phosphatases (PPM), and aspartate based phosphatases (PPA) with the major PP members of the PPP family. The family of PPP consists of protein phosphatases PP1, PP2A, PP2B, PP4, PP5, PP6, and PP7 (Shi, 2009). PP1 and PP2A play major roles in the regulation of cellular function.

Protein phosphatase 1 is ubiquitously expressed throughout the cell and has been shown to be involved in a wide variety of cellular processes including meiosis, cell division, apoptosis,

protein synthesis, cell metabolism, cytoskeleton reorganization, and the regulation of membrane channels and receptors (Cohen, 2002; Ceulemans and Bollen, 2004; Bollen *et al.*, 2010; Fardilha *et al.*, 2010). An active enzyme consists of the catalytic subunit bound to one of more than one hundred regulatory subunits which have been identified (Cohen, 2002; Bollen *et al.*, 2010). The regulatory subunit targets the catalytic unit of PP1 to a specific sub cellular compartment, as well as modulating substrate specificity (Shi, 2009).

Protein phosphatase 2A has been shown to play an important role in cell development, proliferation, cell death, cell mobility, cytoskeleton dynamics, and control of the cell cycle (Janssens and Goris, 2001; Martin *et al.*, 2010). PP2A is found in high quantities in the cell, accounting for one percent of total cellular proteins (Shi, 2009). The structure of PP2A is more complex than that of PP1, containing a catalytic core as well as a scaffold subunit (Shi, 2009; Martin *et al.*, 2010). Both the scaffold and catalytic subunits have 2 different isoforms, alpha and beta. The scaffold subunit consists of 15 tandem Huntingtin Elongation Factor 3 Protein Phosphatase 2A (HEAT) repeats, creating a horseshoe like shape and allowing for recognition of the catalytic subunit (Groves *et al.*, 1999; Martin *et al.*, 2010). Like PP1, the PP2A catalytic subunit interacts with various different regulatory subunits found within the cell.

Protein phosphatases have widespread effects within the cell, affecting many cellular processes. Many PP preferentially target nuclear proteins which is not surprising as phosphorylation is thought of as the dominant method of regulation within the nucleus (Moorhead *et al.*, 2007). RNA interference and PP inhibitor studies have revealed that PP play a role in many nuclear processes including DNA replication, DNA repair, chromosome condensation, ribosome biogenesis, chromatin remodelling, as well as affecting various signal transduction pathways (Bollen and Beullens, 2002; Shi *et al.*, 2006; Moorhead *et al.*, 2007). For example, PP control the level of chromatin condensation by modifying the serine 10 and 28 residues on histone 3 (H3Ser10 and H3Ser28) (Gurley *et al.*, 1978; Goto *et al.*, 1999; Kinney *et al.*, 2008). The H3Ser10 modification has been shown to be important not only in cell division (chromatin condensation), but also in gene transcription (chromatin de-condensation) (Prigent and Dimitrov 2003). H3Ser10 phosphorylation status only affects a subset of genes in the immediate vicinity of H3, and has also been shown to be highly dependent on additional modifications in the surrounding area (Prigent and Dimitrov 2003). For example, when Lysine 9 is not methylated there is an increase in H3Ser10 phosphorylation, but when Lysine 9

becomes di-methylated H3Ser10 phosphorylation decreases (Rea *et al.,* 2000). Genes shown to be regulated by H3Ser10 phosphorylation include c-fos and c-jun, both of which are early response genes involved in responding to stimuli from cytokines, growth factors, stress, as well as bacterial or viral infections (Barratt *et al.,* 1994; Johansen and Johansen, 2006).

In addition to PP playing an important in role in chromatin condensation and gene expression, they have also been shown to be involved in various protein complexes. Brush and colleagues have shown that PP1 is readily found in complex with HATs, CREB-binding protein, and HDAC1 and HDAC2 (Brush *et al.,* 2004). Complexes may also form between the CREB-binding protein, PP2A and PP4, as both have been found to immunoprecipite with HATs (Canettieri *et al.,* 2003; Brush *et al.,* 2004). Complexes have also been found to occur directly between HDAC6 and PP1 during the regulation of microtubule function (Liao *et al.,* 1998; Bollen *et al.,* 2010; Brush *et al.,* 2004). When the function of HDAC6 is disrupted by pharmacological inhibitors or mutagenesis it leads to a disruption of the complex (Brush *et al.,* 2004). For example, TSA disrupts the HDAC6:PP1 complex leading to the release of an active PP1, which then associates with the Akt protein (Chen *et al.,* 2005). This finding suggests that HDACi are not only able to disrupt PP complexes but also lead to the release of a free PP which affects cellular function.

Akt is a serine/threonine protein kinase which plays multiple regulatory roles in the cell including processes such as glucose metabolism, cell proliferation, and apoptosis (Brodeur, 2010). Akt is activated through the phosphoinositide 3' kinase signaling pathway, which leads to phosphorylation of Akt at Ser 473 and Thr 308. These two modifications are necessary for the full activation of the Akt protein (Duronio, 2008; Woodgett, 2005; Brodeur, 2010). Akt has been shown to be activated in a wide variety of cancerous cells (Woodgett, 2005; Brodeur, 2010). The increased activation allows substrates such as Bad and Foxo, both pro apoptotic proteins to be phosphorylated and inactivated. This is one method by which cancer cells can avoid cell arrest and apoptosis (Woodgett, 2005; Brodeur, 2010). Therefore the HDACi mediated disruption of complexes, such as the HDAC6:PP1 complex described above provides a possible mechanism to remove the block on apoptosis that is commonly seen in cancerous cells. Similar effects could occur with cellular regulators other than Akt, further adding to the complexity of the HDACi (Duronio, 2008).

1.3 Regulation of Eukaryotic Transcription

The progression of gene transcription is a complex and highly regulated process. Transcription is generally broken down into four different phases; the pre-initiation complex, initiation, elongation and finally termination (Table 1.4 Figure 1.2). This process begins with the ordered binding of various factors to a core promoter and ultimately leads to the production of a mature messenger RNA (mRNA). Due to the importance of this process in the overall function of the cell, many regulatory points are incorporated into the process to ensure it occurs without error. The steps involved in the production and maturation of mRNA are examined in the following sections.

The first step of transcription involves the recruitment and assembly of the pre-initiation complex (PIC) to the core promoter element of a gene. Prior to the PIC assembly, chromatin remodeling must occur for the core promoter to be accessible to the PIC. The PIC is composed of six general transcription factors, TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH, as well as RNA Polymerase II (RNA Pol II) (Boeger *et al.,* 2005; Guermah *et al.,* 2009). A second complex, known as mediator, is also required and functions as an RNA Pol II activator (Szutorisz *et al.,* 2005; Conaway *et al.,* 2011). The mediator complex communicates regulatory signals from enhancers and proximal promoter elements to the PIC, allowing the rate of RNA Pol II mediated transcription to be increased or decreased (Kornberg, 2005; Conaway *et al.,* 2011). Currently there are two different models to describe how the PIC is assembled, the preassembly and the sequential assembly model. The preassembly model proposes that TFIID and TFIIA are normally associated with the core promoter elements, and when transcription is required, a large complex containing RNA Pol II, TFIIB, TFIIE, TFIIF, and TFIIH is recruited by TF11D/TF11A positioning the PIC at the core promoter (Ossipow *et al.,* 1995; Lemon and Tjian, 2000; Bing *et al.,* 2007).

The sequential assembly model proposes that the general transcription factors are all recruited to the core promoter element in a specific manner (Lemon and Tjian 2000); for example TFIID binds to the TATA box/Inr element, and only once it has bound will the recruitment of the other general transcription factors occur. The order of recruitment is proposed to be: TFIIA, TFIIB, RNA Pol II/TFIIF, TFIIE and TFIIH and only when all components are present will transcription begin (Lemon and Tjian 2000). Although neither model has been proven or disproven, the sequential model has gathered the most support and is

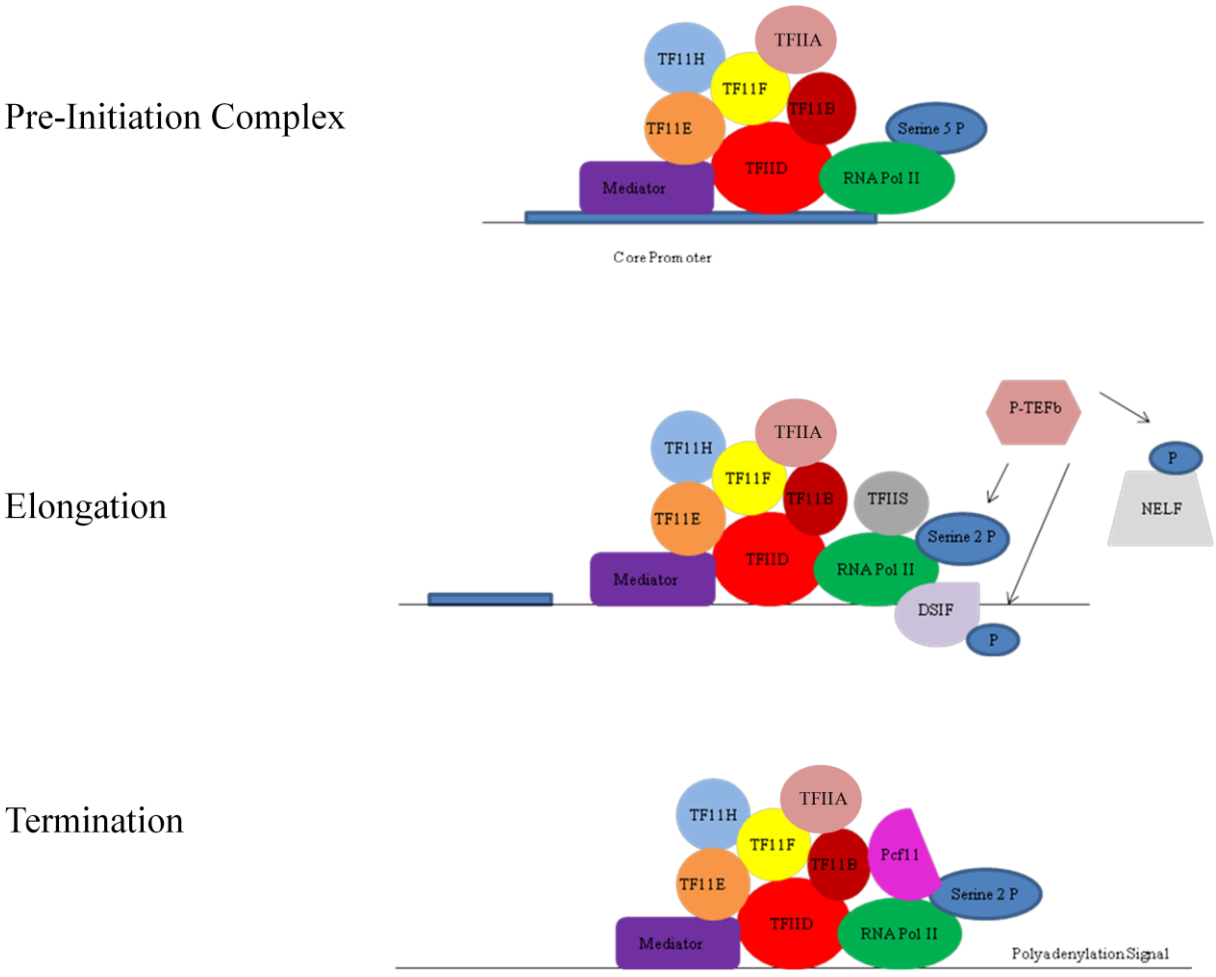


Figure 1.2: Phases of Eukaryotic Transcription. The pre-initiation complex assembles on a core promoter element and moves into productive elongation. Moving away from the core promoter, the elongating complex is stabilized by factors such as DSIF when present in a phosphorylated form. When a poly-adenylation signal is reached the Pcf11 protein binds and tethers the Poly A machinery to the transcription complex. The synthesis of the Poly A signal is thought to lead to the dissociation of positive elongation factors subsequently leading to the release of RNA Pol II and transcription termination occurs.

the favored theory at the current time.

Although the exact method of PIC assembly is not fully understood, TFIID is the general transcription factor responsible for the actual binding to the core promoter. TFIID is a large transcription factor, containing multiple subunits and is composed of a TATA binding protein as well as 13 TATA binding protein associated factors (Thomas and Chiang, 2006; Papai *et al.,* 2011). The subunit which contains the TATA binding protein recognizes and directly interacts with TATA boxes found in core promoters (Woychik and Hampsey, 2002; Papai *et al.,* 2011). If a promoter lacks a TATA box, the TATA binding protein associated factors 1 and 2 function in the identification of Initiator (Inr) elements, while TATA binding protein associated factors 6 through 9 recognize downstream promoter elements (Chalkley and Verrijzer, 1999; Papai *et al.,* 2011).

TATA binding protein associated factor 1 (TAF1) has been shown to be a crucial part of the TFIID complex, responsible for binding the core promoter element, but also functioning as a co-activator of transcription (Thomas and Chiang, 2006). TAF1 is capable of interacting with activator proteins including jun, Rb, MDM2, cyclin D, as well as other general transcription factors such as TFIIA, TFIIE, and TFIIF (Wassarman and Sauer, 2001). TAF1 also contains two bromodomains which are used to bind to acetylated H3K14, H4K16, H4K12, and H4K8 (Wasarman and Sauer, 2001). Through all of the above interactions, TAF1 can modify histones as well as link the RNA Pol II complex to the core promoter by interacting with its component proteins.

TFIIB has also been shown to be an essential component of the PIC. The C terminal of TFIIB interacts with sequences both up- and down-stream of the TATA box (Woychik and Hampsey, 2002; Deng *et al.,* 2007). This interaction stabilizes the interaction with the TATA box as well as creates a binding site within the N-terminal zinc binding domain of TFIIB, for RNA Pol II (Bushnell *et al.,* 2004; Deng *et al.,* 2007). TFIIB is also involved in RNA Pol II's identification of the transcription start site and entry of downstream DNA into the catalytic site (Thomas and Chiang, 2006; Deng *et al.,* 2007). The other general transcription factors involved in the PIC each play a distinct role. TFIIF functions in the recruitment of RNA Pol II to complexes already containing the TFIIB transcription factor, as well as interacting with DNA sequences on both sides of a TATA box, thereby aiding in the stabilization of the complex (Woychick and Hampsey, 2002; Bernecky *et al.,* 2011). TFIIE interacts with the

promoter, TFIIB, RNA Pol II as well as TFIIF, and functions to recruit TFIIH to the PIC (Thomas and Chiang, 2006). TFIIH is the largest general transcription factor, being comprised of ten different subunits which include the XBP and XPD subunits. These subunits act as ATPase DNA helicases (Svejstrup *et al.,* 1996; Conaway *et al.,* 2000). This helicase activity is required for the unwinding of DNA 11-15 base pairs at a time around the transcription start site, and allows for the formation of an open complex (Svejstrup *et al.,* 1996; Saunders *et al.,* 2006). The single stranded DNA in the open complex then enters the active site of RNA Pol II while the second non-template strand is bound by TFIIF (Bushnell *et al.,* 2004).

In order for transcription to continue, the PIC must leave the initiation phase in the promoter region, and move downstream to function in elongation. As previous stated, after the assembly of the PIC, TFIIH activity unwinds the double stranded DNA allowing a single stranded template to enter the active site of RNA Pol II (Saunders *et al.,* 2006; Nechaev *et al.,* 2011). The entry of the single stranded template is one of many factors that converts the PIC into a complex capable of transcription. For transcription to occur, RNA Pol II must move away from the core promoter and move further downstream. Whether or not RNA Pol II is able to escape the promoter region is dependent on the general transcription factor TFIIB. In the early elongating complex the short transcripts that are produced are stabilized by interaction of TFIIB with the active site of RNA Pol II. This interaction occurs through the RNA Pol II exit channel. Therefore, for continued RNA synthesis to occur TFIIB must be forced out of the exit channel allowing the RNA transcript to leave the active site of RNA Pol II (Bushnell *et al.,* 2004; Nechaev *et al.,* 2011). If the transcript is unable to force TFIIB from the exit channel, transcription will be aborted and a new round of transcription initiation will occur. If the transcript is able to force TFIIB from the exit channel transcription will continue allowing the RNA Pol II complex to escape the promoter, and conversion to an elongating complex can occur (Bushnell *et al.,* 2004; Nechaev *et al.,* 2011). It is not until a PIC has escaped the promoter that a second PIC can be generated at the promoter (Sims *et al.,* 2004; Nechaev *et al.,* 2011).

The phosphorylation state of RNA Pol IIs carboxyl-terminal domain (CTD) plays a major role in determining the phase of transcription (Saunders *et al.,* 2006; Nechaev *et al.,* 2011). Phosphorylation of serine 5 of the CTD by the cyclin dependent kinase 7 subunit of TFIIH is associated with transcription initiation and the presence of RNA Pol II at a promoter

(Conaway *et al.,* 2000; Svejstrup, 2004; Phatnani and Greenleaf, 2006; Nechaev *et al.,* 2011). Therefore, this modification is found at high levels at the 5' end of genes. In contrast to serine 5, serine 2 CTD phosphorylation is found at high levels in the 3' regions of genes and has been shown to be associated with productive elongation (Svejstrup, 2004; Phatnani and Greenleaf, 2006). Serine 2 is phosphorylated by positive transcription factor b (P-TEFb). P-TEFb is a protein complex containing cyclin dependent kinase 9 (CDK9) as well as cyclin T (Peng *et al.,* 1998; Peterlin and Price, 2006; Zhou and Yik, 2006). These different residues are thought to mediate protein complex interactions with RNA Pol II at the different stages of transcription (Svejstrup, 2004; Buratowski, 2009). Serine 5 phosphorylation has been shown to recruit capping enzymes such as guanylyltransferase, while serine 2 phosphorylation is involved in recruiting polyadenylation factors (Ahn *et al.,* 2004).

The early elongating complex is very unstable until the first 30 nucleotides have been synthesized, and is prone to pausing or arrest (Saunders *et al.,* 2006; Nechaev *et al.,* 2011). Therefore, whether or not the early elongating complex continues into productive elongation is dependent on various elongation factors preventing a pause or arrest. Stabilization of the complex is reliant on the elongation factors 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) sensitivity inducing factor (DSIF) (Sims *et al.,* 2004; Nechaev *et al.,* 2011). Through the interaction of DSIF with the negative elongation factor (NELF) an early pause occurs between the + 20 and +100 range, allowing for capping of the emerging RNA. This early pause also acts as a checkpoint for the complex before it enters into productive elongation (Yamaguchi *et al.,* 1999; Sims *et al.,* 2004; Saunders *et al.,* 2006; Nechaev *et al.,* 2011). To release the elongating complex from this check point, both NELF and DSIF become phosphorylated by P-TEFb. The phosphorylation of NELF leads to its dissociation from DSIF and the elongating complex, which removes the inhibition. The phosphorylation of DSIF allows it to act as a positive elongation factor promoting productive elongation (Sims *et al.,* 2004; Nechaev *et al.,* 2011). P-TEFb has also been shown to phosphorylate the serine 2 residue of RNA Pol IIs CTD domain, increasing the stability of the elongation complex (Hirose and Manley, 2000; Sims *et al.,* 2004; Zhuoyu *et al.,* 2008; Brookes *et al.,* 2009). An additional elongation factor, TFIIS, promotes productive elongation by realigning the active site of RNA Pol II with the correct 3'OH of the RNA transcript (Sims *et al.,* 2004; Nechaev *et al.,* 2011). The rate of productive elongation is controlled by many factors including TFIIS, TFIIF, as well

as proteins belonging to the eleven-nineteen lysine-rich in leukemia (ELL) and elongin families (Shilatifard *et al.*, 2003; Nechaev *et al.*, 2011). In addition, through interaction with the H2A-H2B dimers creating nucleosomal destabilization, FACT (facilitates chromatin transcription) facilitates RNA Pol II movement through chromatin, thereby allowing productive elongation to progress (Reinberg and Sins, 2006).

When RNA Pol II reaches the end of the DNA template transcription termination must occur. Unlike transcription initiation, termination is not due to a precise consensus sequence, but is associated with the polyadenylation of the produced mRNA (Gromak *et al.*, 2006). As previously stated, the different modifications to the CTD domain of RNA Pol II, in different locations along the gene, allows for the recruitment of various protein complexes. Phosphorylation of serine 2 on the CTD domain of RNA Pol II allows a key polyadenylation factor, Pcf11, to bind and tether the rest of the polyA machinery (Buratowski, 2009). Therefore the presence of serine 2 phosphorylation near the 3' region of the gene is key for transcription termination.

Two different models have been proposed to explain the relationship between the synthesis of the poly-adenylation signal and transcription termination. The anti-terminator model explains this relationship through the synthesis of the polyadenylation signal leading to a change in RNA Pol II associated factors, and the dissociation of positive elongation factors (Proudfoot *et al.*, 2002; Tollervey, 2004; Grohmann *et al.*, 2010). This change effectively stops the activity of RNA Pol II leading to its release. The second proposed model, the torpedo model, suggests that the cleavage of the mRNA leads to the production of a new uncapped RNA. The degradation of this new RNA by various exonucleases leads to RNA Pol II dissociation (Proudfoot *et al.*, 2002; Kim *et al.*, 2004; Tollervey, 2004).

1.3.1 Importance of Promoter Proximal Pausing

As previously described, gene transcription occurs in various phases, assembly of the PIC, initiation, elongation, and termination. The recruitment and formation of the PIC is traditionally thought of as the rate limiting step of transcription, and therefore determines the level of gene expression (Fujita and Schlegel, 2010). Recently, this assumption has been called into question, as genome wide studies have shown that regardless of the activation state of a

gene, the PIC as well as RNA Pol II are present on the core promoter, suggesting that PIC formation is not the rate limiting step (Kim *et al.*, 2005; Guenther *et al.*, 2007; Brookes *et al.*, 2009).

A second proposed mechanism to control the rate of transcription, promoter proximal pausing, has been described (Brookes *et al.*, 2009). Promoter proximal pausing has been defined as a state where RNA Pol II is transcriptionally active, but becomes stalled by negative elongation factors (Brookes *et al.*, 2009). Promoter proximal pausing has been described at multiple genes including Hsp70 and c-Myc (Krumm *et al.*, 1995; Core and Lis, 2008). At the c-Myc gene, transcript levels have been shown to decrease despite an unchanged level of transcriptional initiation (Krumm *et al.*, 1995). Originally it was thought that promoter proximal pausing only occurred at rapidly induced genes such as c-Myc, c-Fos, Jun-B, as well as estrogen receptor target genes which are induced by external stimuli (Krumm *et al.*, 1995; Uptain *et al.*, 1997; Orphanides and Reinberg, 2002; Kinnis *et al.*, 2009). These rapidly induced genes were found to be held in a paused state, allowing for rapid induction of expression (Fujita and Schlegel, 2010). Due to the small number of genes originally found to be regulated in this manner, it was thought that a specific DNA sequence, such as a stem-loop close to a poly-U rich region, was responsible for initiating the promoter proximal pausing; a system very similar to that seen in prokaryotes (Bentley and Groudine, 1988). It is now thought, that like control over transcriptional initiation, promoter proximal pausing is regulated by the binding of specific transcription factors (Fujita and Schlegel, 2010).

It is important to note that promoter proximal pausing is distinct from transcriptional arrest. In transcriptional arrest there is a decrease in RNA Pol II occupancy and short transcripts are recycled. In promoter proximal pausing, RNA Pol II remains bound to the promoter region poised to resume transcription, but is undetectable farther downstream (Adelman *et al.*, 2009; Fujita and Schlegel, 2010). The transcripts produced during promoter proximal pausing are not recycled, but elongated when transcription re-commences. The elongation of the previously started transcripts supports the idea that initiation of RNA Pol II has occurred, but that it is unable to transcribe the full gene (Fujita and Schlegel, 2010).

In the search to determine the mechanism behind RNA Pol II switching from an active transcribing state to a paused state several negative elongation factors were identified (Gilmour, 2009). Among these DSIF and NELF appear particularly important with respect to promoter

proximal pausing. Two proteins make up the DSIF complex, Spt4 and Spt5 (Zhu *et al.*, 2007). DSIF has been shown to exert both negative and positive effects on elongation due to Spt5 directly binding to RNA Pol II (Hartzog *et al.*, 1998; Wada *et al.*, 1998; Yamaguchi *et al.*, 1999). When both DSIF and NELF are present, DSIF acts in a negative manner leading to promoter proximal pausing. In the absence of NELF, DSIF acts in a positive manner on RNA Pol II (Zhu *et al.*, 2007). It is thought that the phosphorylation status of the C-terminal repeat domain of Spt5 determines whether DSIF acts in a positive or negative manner (Andrulis *et al.*, 2000; Invanov *et al.*, 2000; Mason and Struhl, 2005; Aida *et al.*, 2006; Yamada *et al.*, 2006). DSIF has also been shown to associate with a number of other transcription regulatory factors such as TFIIF, TFIIS, and FACT suggesting it may play a role in other steps of transcription as well (Andrulis *et al.*, 2002; Pei and Shuman, 2002; Lindstrom *et al.*, 2003; Mandal *et al.*, 2004).

Like DSIF, NELF is also a protein complex consisting of four different subunits: A, B, C/D, and E (Fujita and Schlegel, 2010). The NELF-C and NELF-D subunits are produced from the same mRNA but use different transcription start sites and therefore are redundant in function (Narita *et al.*, 2003; Gilchist *et al.*, 2008). Mutational studies have suggested that the E subunit of NELF also interacts with the nascent RNA, creating a rigid body that restricts the movement of RNA Pol II, leading to the paused state (Fujita and Schlegel, 2010). The NELF complex has been shown to be capable of stalling RNA Pol II only when it is associated with the DSIF complex (Fujita and Schlegel, 2010). When NELF and DSIF are together, RNA Pol II pauses between the +20 to +100 nucleotide range (Yamaguchi *et al.*, 1999; Sims *et al.*, 2004; Saunders *et al.*, 2006; Nechaev *et al.*, 2011). This suggests that DSIF, NELF and RNA Pol II associate upstream of the promoter.

Reverting RNA Pol II from a paused to active state requires phosphorylation. P-TEFb interacts with proteins which are key in promoter proximal pausing and, therefore, it may be involved in re-activating RNA Pol II. In support of the involvement of P-TEFb, phosphorylation of DSIF has been shown in yeast to not only eliminate its negative function and relieve its inhibitory effect on RNA Pol II, but also to convert DSIF into an activating elongation factor (Wade *et al.*, 1998; Ivanov *et al.*, 2000). The phosphorylation of NELF by P-TEFb has also been shown to support the continuation of productive elongation by causing a complete dissociation from the proximal promoter region (Adelman *et al.*, 2009). Binding to

DSIF and RNA Pol II only occurs when NELF is in the un-phosphorylated form (Adelman *et al.,* 2009). The current model by which RNA Pol II is released from its paused state is therefore thought to involve the recruitment of P-TEFb to the RNA Pol II complex, where it phosphorylates both DSIF and NELF, leading to a functional change of DSIF and the detachment of NELF (Fujita and Schlegel, 2010) (Figure 1.3).

Many other factors have been suggested to play a role in releasing RNA Pol II from a paused state. For example the transcription factor TFIIS has been shown to be recruited to the phosphorylated DSIF/NELF complex (Palangat *et al.,* 2005; Fujita and Schlegel, 2010). Once recruited, TFIIS promotes cleavage of any inverted transcripts produced from RNA Pol II backtracking while in a paused state. The cleavage of the transcripts promotes productive elongation (Wind and Reines, 2000; Cheung *et al.,* 2011). Other factors such as FACT, and heat shock genes, have been shown to also play a role in releasing RNA Pol II in *Drosophila* (Kaplan *et al.,* 2000; Saunders *et al.,* 2003; Ni *et al.,* 2008). Data in *Drosophila* has also indicated that genes which exhibit promoter proximal pausing may contain common elements such as Initiator element (Inr), downstream promoter element (DPE) recognized by TFIIB, as well as a GAGA factor binding sequence (Hendrix *et al.,* 2008; Lee *et al,* 2008).

Contrary to the data in *Drosophila,* it has been suggested that all genes may in fact undergo promoter proximal pausing. This state may function as a check point in early transcription and may be a necessary step (Core and Lis, 2008). Genome wide studies support this idea and have shown that there is a high occupancy of RNA Pol II on the 5' end of mammalian and *Drosophila* genes (Kim *et al.,* 2005; Guenther *et al.,* 2007; Muse *et al.,* 2007; Zeitlinger *et al.,* 2007). In a developmental sense, promoter proximal pausing would facilitate RNA Pol II switching from a poised to an active state very rapidly, and would allow rapid onset of gene expression (Price, 2008). It has also been suggested that promoter proximal pausing may facilitate the assembly of RNA-processing factors and allow the coupling of transcription and mRNA processing (Glover-Cutter *et al.,* 2008; Moore and Proudfoot, 2009). In support of this, DSIF and the phosphorylated serine 5 residue in the CTD of RNA Pol II, both bind to mRNA capping enzymes to stimulate the capping of mRNA (McCracken *et al.,* 1997; Mandal *et al.,* 2004; Ghosh, *et al.,* 2011). The phosphorylated serine 2 residue on the CTD of RNA

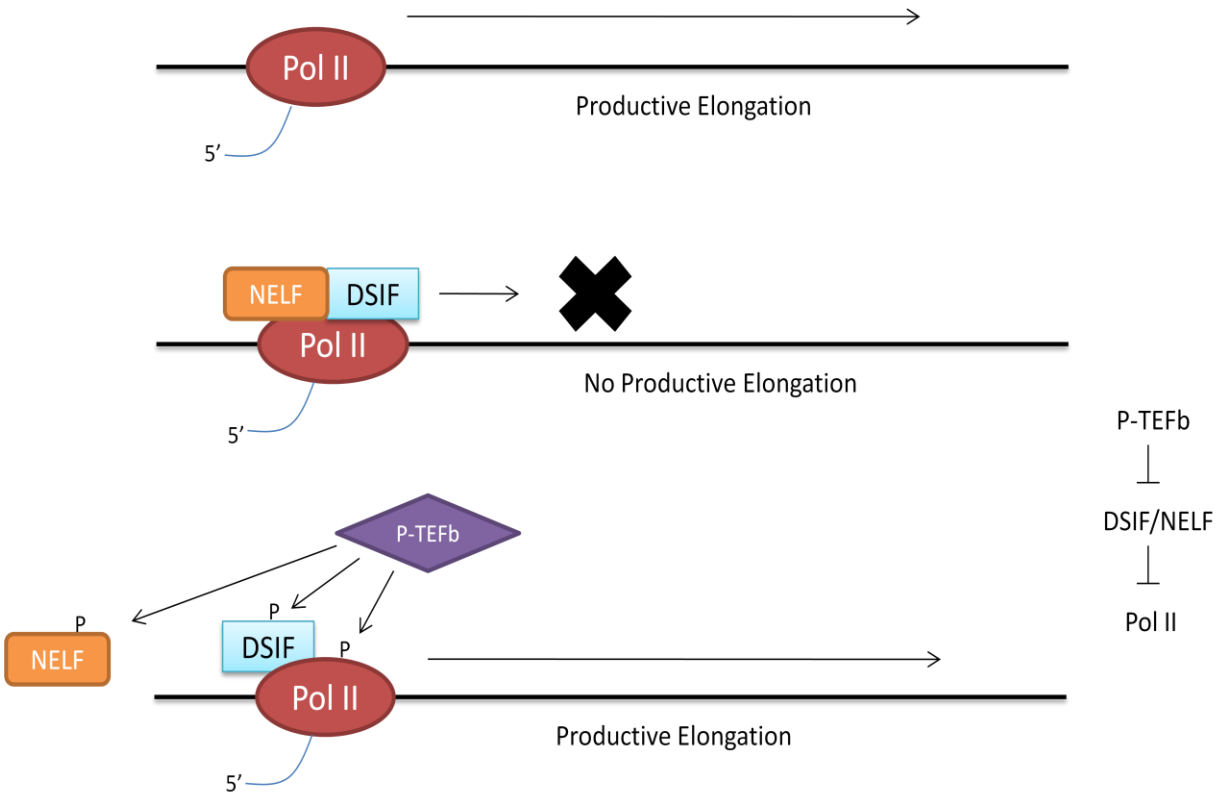


Figure 1.3: Proposed mechanism for promoter proximal pausing. DSIF and NELF complexes bind to RNA Pol II creating a paused state, preventing productive elongation. When active P-TEFb phosphorylates both NELF and DSIF, NELF dissociates from RN Pol II and DSIF acts in a positive manner on RNA Pol II allowing productive elongation to occur. Adapted from Yamaguchi *et al.*, 1999.

Pol II has also been shown to lead to the recruitment of splicing factor as well as 3' end processing factors (Komarnitsky *et al.*, 2000; Ahn *et al.*, 2004; Egloff *et al.*, 2008). Promoter proximal pausing has also been suggested to be a mechanism to obtain transcripts from bi-directional promoters, as the paused RNA Pol II has been shown to backtrack and produced inverted transcripts (Core and Lis, 2008; Seila *et al.*, 2008). The current accepted marker for promoter proximal pausing is the presence of a high level of serine 5 phosphorylation (RNA Pol II initiation), in conjunction with low serine 2 phosphorylation (productive elongation) (Adelman *et al.*, 2009).

1.4 SRC

SRC encodes the protein c-Src, a 60 kDa non-receptor tyrosine kinase and is the founding member of the SRC kinase (SFK) proteins. It is a homologue of the *v-src* gene from the Rous Sarcoma Virus (Rous, 1911). Other members of the SFK include c-YES, FYN, LYN, LCK, HCK, FGR, and BLK (Thomas and Brugge, 1997; Ingley, 2008; Aleshin *et al.*, 2010). Proteins in this family range in size from 52-62 kDa, and contain six conserved domains, the N-terminal SRC homology 4 (SH4) domain, the SRC homology 3 (SH3) domain, SRC homology 2 (SH2) domain, SRC homology 1 (SH1) or kinase domain, a unique domain, and a C terminal regulatory domain (Boggon and Eck, 2004; Ingley, 2008; Aleshin *et al.*, 2010). The SH4 domain includes fourteen amino acids and is the location of a myristic or palmitic acid co-translational modification which facilitates SFK localization to the plasma membrane (Biscardi *et al.*, 1999). Both the fifty amino acid SH2, and one hundred amino acid SH3 domains allow for various protein-protein interactions, through the pY and P-X-X-P consensus sequences respectively (Thomas and Bruge, 1997; Alvarez *et al.*, 2006; Ingley, 2008). The SH1 domain is a highly conserved two hundred and fifty amino acid catalytic domain (Brown and Cooper, 1996; Ingley, 2008). The unique domain contains 40-70 amino acids depending on the protein in question, and acts to mediate protein-protein interactions between members of the SFK family (Brown and Cooper, 1996; Ingley, 2008). The C terminal regulatory domain contains an auto-regulatory inhibitory phosphorylation site, which allows for negative regulation of the SFK family members (Boggon and Eck, 2004; Ingley, 2008).

Activity of the SFK family members is determined by the level of phosphorylation as well as by intramolecular interactions (Frame, 2002; Ingley, 2008). The c-Src protein is inactivated following the addition of a phosphate group on Y527, promoting intramolecular interactions between the pY527 and the SH2 domain. This also positions a stretch of the c-Src protein containing proline rich sequences to bind to the SH3 domain, which leads to a closed, inactive, conformation of the protein (Okada and Nakagawa, 1989; Ayrapetov, 2006). To convert c-Src to an open and active conformation Y527 is dephosphorylated by various phosphatases such as protein tyrosine phosphatase 1B (Bjorge *et al.*, 2000; Roskoski, 2005). This dephosphorylation event disrupts the binding between Y527 and the SH2 domain. The resultant conformational change prevents binding to the SH3 domain, creating an open and active c-Src protein (Bjorge *et al.*, 2000; Roskoski, 2005).

c-Src plays an important role in many cell signaling pathways. It has been shown to simultaneously activate various pathways that are involved in the cell cycle as well as cytoskeleton organization (Malek *et al.,* 2002; Kim *et al.,* 2009). c-Src has also been linked to the development of many cancers (Malek *et al.,* 2002; Kim *et al.,* 2009). Over-expression or increased activity of c-Src has been linked to cellular transformation, tumor progression and metastasis (Biscardi *et al.,* 1999; Kim *et al.,* 2009). High kinase activity has been reported in various forms of cancer such as colon, breast, as well as occasionally in cancers of the pancreas, lung, brain, ovary, and bladder (Summy and Gallick, 2003; Zhang *et al.*, 2009).

1.4.1 SRC Gene Regulation

SRC is located on chromosome 20q12.3 and contains 14 exons (Figure 1.4). Exon 1A, 1α, 1B, and 1C code for the 5' region of c-Src mRNA, while exons 2-12 code for the c-Src protein and the 3' regions. SRC expression is regulated by two promoters, SRC1α and SRC1A, which are separated by 1 kb (Dehm *et al.*, 2004). Both promoters produce transcripts which are spliced onto the 1B exon and, therefore, produce almost identical transcripts which will only vary in their extreme 5' ends (Figure 1.4). SRC1A is a housekeeping like promoter with high

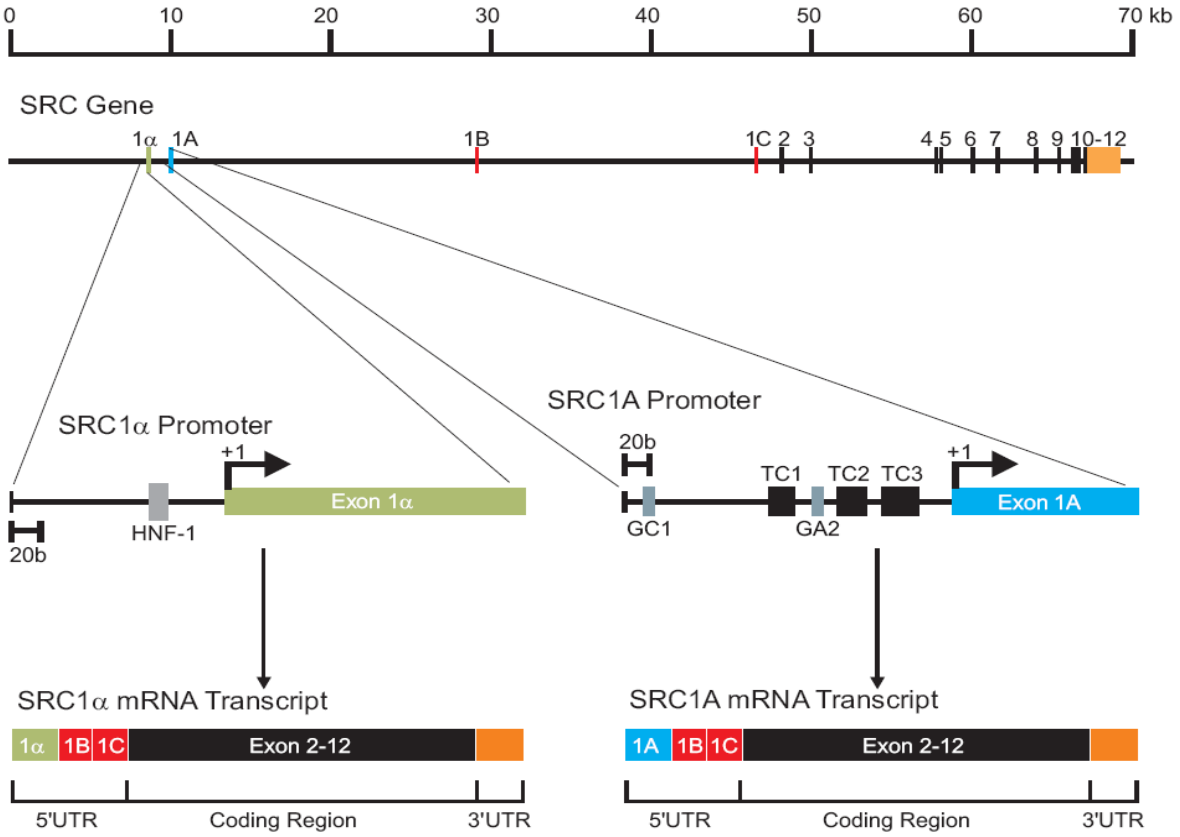


Figure 1.4: The SRC gene. SRC expression is regulated by two distinct promoters, SRC1α and SRC1A. The SRC1α promoter is dependent on HNF-1 factor for transcription while the SRC1A promoter is regulated by the Sp family of transcription factors. Identical transcripts are produced from each promoter varying only at the extreme 5' region. (Bonham and Fujita, 1997, Bonham et al., 2000, Ritchie et al., 2000 and Dehm et al., 2004)

GC content and is regulated by the Sp family of transcription factors (Dehm *et al.*, 2004). The SRC1α promoter is more tissue specific and is expressed primarily in the stomach, prostate, liver, kidney, and pancreas (Bonham *et al.*, 2000). SRC1α is regulated by Hepatocyte Nuclear Factor-1 (HNF-1) (Dehm *et al.*, 2004). Although very different, both promoters utilize an Initiator element (Inr) and lack a TATA box (Dehm *et al.*, 2004). Both the SRC1α and SRC1A promoters have been shown to be directly repressed by various HDACi (Kostyniuk *et al.*, 2002).

1.4.2 SRC and Cancer

The best example of c-Src over expression in cancer is in cancers of the colon and breast. Approximately 80% of all colorectal cancers show increased c-Src expression (Lieu and Kopetz, 2010) with up to 40-fold increases in c-Src activity observed (Rosen *et al.*, 1986). c-Src activity has been shown to be an independent indicator for poor clinical prognosis in patients with colon cancer (Lieu and Lopetz, 2010). This correlation is due to the fact that c-Src over expression in colon cancers influences cell mobility and the spread of cells across the basement membrane (Brunton *et al.*, 1997; Jones *et al.*, 2002).

c-Src expression has also been found to be increased in various cancers of the breast with levels ranging from 4 to 30 fold above that seen in normal breast tissue (Verbeek *et al.*, 1996). c-Src expression in breast cancer has also been shown to positively correlate with lymph node metastasis, disease recurrence and poor disease-free survival (Fleming *et al.*, 2004; Myers *et al.*, 2004). In addition, the level of c-Src expression has consistently been shown to be a predictor of breast cancer recurrence following treatment (Redmond *et al.*, 2009). Like in colon cancer, c-Src expression has been related to the rate of growth and invasion (Xu *et al.*, 2009). The level of c-Src expression has also been shown to relate to the level of expression of estrogen responsive genes (Tai *et al.*, 2000).

Based upon the important role c-Src appears to play in various cancers, as well as results from antisense and inhibitor experiments showing a decrease in c-Src activity leads to decreased tumor growth, it has been suggested that c-Src represents a therapeutic target (Staley *et al.*, 1997; Homsi *et al.*, 2009; Purnell *et al.*, 2009; Zhang *et al.*, 2009). SRC expression is directly repressed by HDACi treatments (Kostyniuk *et al.*, 2002) suggesting that HDACi may

provide an effective treatment for cancers over expressing SRC. Using a semi-quantitative ChIP approach, HDACi treatment was found to increase acetylation at both of the SRC promoters despite overall SRC expression decreasing (Ellis, 2007). RNA Pol II was also lost from the 3' region of the SRC gene while it remained at the SRC promoters, suggesting that a block in productive elongation may be the mechanism of the repression seen following HDACi treatment (Ellis, 2007). Further support for a mechanism of this variety is provided by the finding of the lysine 4 residue on histone 3 trimethylation ($H3K4Me_3$), a marker for transcriptional activation, remains unaffected by HDACi treatment. In contrast, lysine 36 on histone 3 trimethylation ($H3K36Me_3$), a marker for transcriptional elongation, is rapidly lost in the 3' region of the SRC gene following HDACi treatment (Ellis, 2007). Previous semi-quantitative work in the Bonham lab has shown that short SRC transcripts, of 100 nucleotides in length are also unaffected by HDACi treatments (Bonham, personal communication). This suggests that full length transcripts are rapidly down regulated by HDACi, but the production of short transcripts from the SRC promoters is unaffected. This again further supports the idea that there may be a block in productive elongation occurring following HDACi treatment.

1.5 BCL2L1

Bcl-x_L is an anti-apoptotic protein belonging to the B cell lymphoma 2 (Bcl-2) family of proteins, which has also been reported to be down regulated following HDACi treatment (Adams and Cory, 1998; Zhou *et al.*, 2010). At least fifteen different family members have been identified in mammalian cells (Chao and Korsmeyer, 1998; Zhou *et al.*, 2010). Members of this family possess at least one of four conserved domains known as the Bcl-2 homology domain (BH) 1 through 4 (Adams and Cory, 1998; Zhou *et al.*, 2010). Anti-apoptotic members of the family contain BH1 and BH2 domains, while pro-apoptotic family members may or may not contain these domains (Adams and Cory, 1998; Zhou *et al.*, 2010). Pro-apoptotic members are broken down into two different sub-groups, the MTD and BH3 groups. The MTD group, has high similarity to the Bcl-x_L protein, and includes Bax, Bak, and Bok, all of which contain a BH1, BH2, and BH3 domains (Adams and Cory, 1998; Zhou *et al.*, 2010). The BH3 sub-group of pro-apoptotic proteins, includes Bik, and Blk, which only contain short (9-16 residues) BH3 domains (Adams and Cory, 1998; Shamas-Din *et al.*, 2011).

Both pro- and anti-apoptotic proteins in this family are capable of forming heterodimers with other family members to inhibit their activity (Oltval *et al.,* 1993; Zhou *et al.,* 2010). The formation of heterodimers has been shown to be essential for the pro-apoptotic activities of the BH3 domain members; while heterodimerization is not required for activity of the MTD or anti-apoptotic family members (Cheng *et al.,* 1996; Kelekar and Thompson. 1998; Zhou *et al.,* 2010; Shamas-Din *et al.,* 2011).

The Bcl-x_L protein is found on the cytosolic side of the mitochondrial, endoplasmic reticulum, and nuclear envelope membranes (Green and Reed, 1998; Zamzami *et al.,* 1998; Bogner *et al.,* 2010). Bcl-x_L contains a C-terminal hydrophobic tail which facilitates this membrane localization. It is proposed that at the membrane Bcl-x_L acts to detect damage in the sub-cellular organelles (Green and Reed, 1998; Zamzami *et al.,* 1998; Leber *et al.,* 2010). In the absence of damage, Bcl-x_L forms heterodimers with various pro-apoptotic proteins, inhibiting their activities. Bcl-x_L has also been shown to play a role in preventing Apaf-1 associating with pro-caspase 9, thereby preventing caspase activation (Pan *et al.,* 1998; Bogner *et al.,* 2010; Shamas-Din *et al.,* 2011).

1.5.1 BCL2L1 Gene Regulation

The gene for the Bcl-x_L protein, BCL2L1, is located on chromosome 20q11.21 and codes for three different proteins. The BCL2L1 gene is driven by a GC rich promoter approximately 3.8 kb in length. It is a TATA-less promoter, but contains an Inr element, as well as binding sites for various transcription factors including Sp1, Ap1, NF-κB, and STATs (Fujio *et al.,* 1997; Adams and Cory, 1998; Chen *et al.,* 2000). The three splice variants produced from the BCL2L1 gene consist of the full transcripts Bcl-x_L and two truncated versions named Bcl-x_S, and Bcl-x_β (Figure 1.5) (Huang, 2000).

The Bcl-x_β protein is a 217 amino acid protein and is the product of an unspliced BCL2L1 transcript (Gonzalez-Garcia *et al.,* 1994). It is identical to the Bcl-x_L protein, lacking

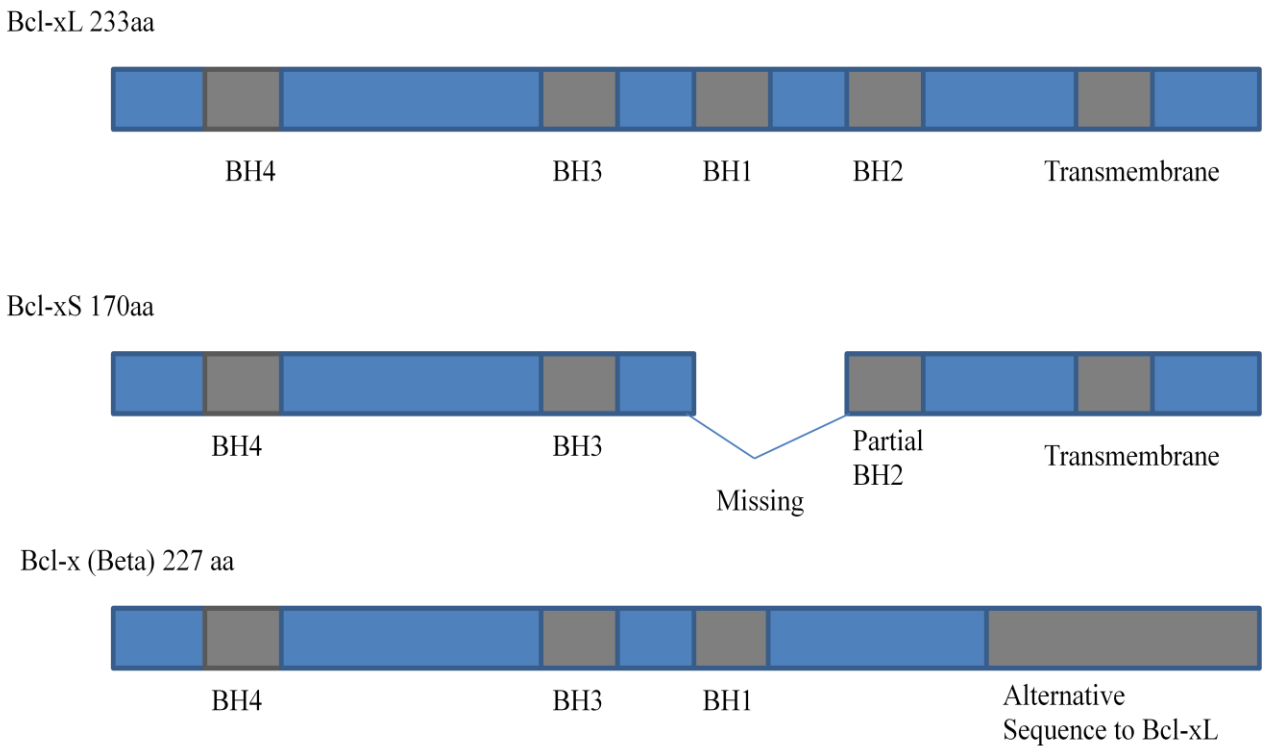


Figure 1.5: Structure of mRNA splice variants produced from the BCL2L1 gene. Three splice variants are produced from the BCL2L1 gene including Bcl-x_L and Bcl-x_β, which differ only in the 5' end of the transcript, both of which act in an anti-apoptotic manner. Bcl-x_S, the smallest variant acts in a pro-apoptotic manner due to differential splicing which eliminates the conserved anti-apoptotic domains.

only the C-terminal hydrophobic tail (Gonzalez-Garcia *et al.,* 1994). It is localized to the cytosol and has anti-apoptotic functions (Gonzalez-Garcia *et al.,* 1994). Bcl-x_S is a 19.5 kDa protein that acts as a pro-apoptotic factor. Due to differential splicing of the BCL2L1 gene, Bcl-x_S lacks 63 amino acids, which contain the conserved BH1 and BH2 domains (Lindenboim e*t al.,* 2000). The lack of these two domains is thought to account for the difference in function between the Bcl-x_L and Bcl-x_S proteins (Lindenboim e*t al.,* 2000). Bcl-x_S has been shown to lead to the induction of apoptosis through a caspase-dependent mechanism which is inhibited by Bcl-2 (Lindenboim e*t al.,* 2000).

Bcl-x_L, a 233 amino acid 27 kDa protein, contains all of the BH domains conserved in the Bcl-2 family. Bcl-x_L has a conserved hydrophobic surface pocket formed by the BH1, BH2, and BH3 domains. This pocket has been shown through mutational studies to be essential for Bcl-x_L binding to pro-apoptotic factors (Kim, 2005). Bcl-x_L has a C-terminal hydrophobic tail, which allows it to localize to various membranes (Shiraiwa *et al.,* 1996; Leber *et al.,* 2010; Zhou *et al.,* 2010). Heterodimers are formed with pro-apoptotic factors, including the Bax/Bak proteins at the mitochondrial membrane, preventing them from releasing cytochrome C (Boise *et al.,* 1993; Leber *et al.,* 2010; Zhou *et al.,* 2010). It has been recently demonstrated that the expression of Bcl-x_L, like c-Src, is repressed following treatments with HDACi (Rada-Iglesias *et al.,* 2007).

1.6 $p21^{WAF1}$

The CKDN1a gene, which encodes the $p21^{WAF1}$ protein, is the classic example of a gene up-regulated following HDACi treatment (Richon *et al.,* 2000). $p21^{WAF1}$ is a cyclin dependent kinase inhibitor belonging to the Cip/Kip family of proteins, and has been shown to interact with Cdk2, Cdk4, and Cdk6 (Harper *et al.,* 1995; Coqueret, 2003). The ordered activation of cyclin/Cdk complexes allows for progression through the cell cycle. $p21^{WAF1}$ blocks the progression from the G1 stage to the synthesis stage of the cycle (Satyanarayana *et al.,* 2007). In the G1 phase, mitogenic signals lead to the up regulation of D type cyclins including D1, D2, and D3. These cyclins are free to interact with either Cdk4 or Cdk6 to create a cyclin D-Cdk complex. Active complexes are transported into the nucleus and activate Cdk-activating kinases (Sherr and Roberts, 1999; Coqueret, 2003). The activated complexes also

lead to the activation of cyclin E-Cdk2 complexes, which in turn lead to the phosphorylation of the Retinoblastoma (Rb) protein. Phosphorylation of Rb disrupts the normal interaction between Rb and the E2F transcription factor, leading to its release. The freed E2F activates target genes which will then continue to move the cell through the cell cycle (Sherr and Roberts, 2004). $p21^{WAF1}$ inhibition of the Cdk kinases prevents the activation of cyclin E-Cdk2 and cyclin A-Cdk2 complexes thereby preventing Rb phosphorylation and the release of E2F.

$p21^{WAF1}$ expression has been found to be up-regulated by the expression of the tumor suppressor gene p53 (el-Deiry *et al.,* 1993; Conqueret, 2003; Brown *et al.,* 2007). Although $p21^{WAF1}$ itself is rarely found to be mutated in human malignancies, its regulator p53 has had over 21,000 different mutations identified (Hollstein, 1991; Soussi and Lozano 2005). Most of these mutations are located within the DNA binding domain of the protein, thereby preventing its activating properties (Shiohara *et al.,* 1994; Liu *et al.,* 2006). Therefore, any tumors containing mutations in p53 may have compromised $p21^{WAF1}$ expression and decreased ability to arrest the cell cycle. $p21^{WAF1}$ can also be activated in a p53-independent manner including activation through the breast cancer gene 1 (BRCA1) and Sp binding elements (Gartel and Tyner, 1999; Promkain *et al.,* 2009).

HDACi have been shown to up-regulate the expression of $p21^{WAF1}$ through the Sp1-3 and Sp1-4 sites located within the $p21^{WAF1}$ promoter (Nakano *et al.,* 1997; Ocker *et al.,* 2007). Through this up regulation HDACi can lead to an arrest of the cell cycle, indicative of chemotherapeutic potential. Up regulation of $p21^{WAF1}$ is seen as a requirement for the HDACi cell cycle arrest, differentiation, and apoptosis effects (Archer *et al.,* 1998; Ocker *et al.,* 2007). Due to the important role that $p21^{WAF1}$ up regulation appears to have in the HDACi mechanism of action it is often used as a marker for HDACi activity within the cell.

2. SPECIFIC AIMS AND HYPOTHESIS

Hypothesis: Previous semi quantitative data from the Bonham lab revealed that full length Src and Bcl-xL gene expression is repressed following HDACi treatment, while short SRC transcripts remained unaffected. The above repression was seen despite increased promoter histone acetylation at both gene loci. Based upon these findings it was proposed that the above gene repression may require the inhibition of a certain HDAC. Therefore using a variety of HDACi and qRT-PCR the level of mRNA expression was examined. However through the course of investigations in this thesis evidence suggested that the HDACi mediated mRNA repression may involve activity of a phosphatase. To further explore the involvement of a phosphatase, the effect of phosphatase inhibitors along with HDACi was examined. It is hypothesized that genes such as SRC and BCL2L1 are repressed by HDACi through a mechanism involving promoter proximal pausing. Other genes such as $p21^{WAF1}$ are induced due to changes in histone acetylation following HDACi treatment. As promoter proximal pausing is known to be regulated through phosphorylation, the mechanism of repression may involve PP activity, with a PP being released from an HDAC/PP complex following HDACi treatment.

Specific Aims

1) Determine the effect of various class I specific HDACi on SRC, BCL2L1, and $p21^{WAF1}$ expression
2) Determine the effect of various class I specific HDACi on SRC and BCL2L1 promoter histone acetylation and RNA Pol II occupancy
3) Determine the effect of Phosphatase Inhibitors on SRC Expression and RNA Pol II occupancy

3. MATERIALS AND METHODS

3.1 Reagents, Equipment, Software and Distributors

All of the reagents and commercially available kits used are listed in Tables 3.1 and 3.2. Equipment that has been used, as well as company of origin, is included in Tables 3.3 and 3.4. All cell lines used are listed in Table 3.5, while all DNA primers used were purchased from Invitrogen (Burlington, ON, Canada) and are listed in Table 3.6. The location of primer binding is shown in Figure 3.0.

Table 3.1 List of Reagents and Distributors

Reagent	Distributor
Actinomycin D	Sigma-Aldrich Co.
Agarose	EMD Chemicals Inc.
Akt Antibody	Abcam
apicidin	Sigma-Aldrich Co.
Aps	EMD Chemicals Inc.
ß-mercaptoethanol	EMD Chemicals Inc.
Bis-acrylamide	Biorad
Cyclohexamide	Sigma-Aldrich Co.
DMEM	Invitrogen Gibco- Cell Culture Systems
DMSO	Sigma-Aldrich Co.
dNTPs	New England Bio Labs Ltd.
Ethanol	EMD Chemicals
Ethidium Bromide	VWR
Fetal Bovine Serum	HyClone
Formaldehyde	EMD Chemicals
Glycine	EMD Chemicals
H3K4 Acetylation Antibody	Millipore
HDAC1 Antibody	Santa Cruz Biotechnology
HDAC2 Antibody	Santa Cruz Biotechnology
HDAC3 Antibody	Santa Cruz Biotechnology
HDAC8 Antibody	Santa Cruz Biotechnology
Insulin	Invitrogen Gibco- Cell Culture Systems

MGCD0103	Selleck Chemicals
$MgCl_2$	Qiagen
MS-275	Selleck Chemicals
Nitrocellulose Membrane	Sigma-Aldrich Co.
O'GeneRuler 50bp DNA ladder	Fermentas Canada Inc
Odyssey Blocking Buffer	LICOR Technologies
PageRuler Plus Pre-stained Protein Ladder	Fermentas Canada Inc.
Penicillin/ Streptomycin	Invitrogen Gibco- Cell Culture Systems
pSer476-Akt Antibody	Cell Signaling
Anti-rabbit Secondary Antibody	LICOR Technologies
RPMI	Invitrogen Gibco- Cell Culture Systems
RNA Polymerase II Antibody	Millipore
Sybr Green	Biorad Laboratories Ltd.
Taq polymerase	Qiagen
Taq Buffer	Qiagen
TEMED	EMD Chemicals
Trypsin-EDTA 1x	Invitrogen Gibco- Cell Culture Systems
TSA	Sigma-Aldrich Co.
Tween-20	EMD Chemicals

Table 3.2 Commercially Available Kits and Distributor

Commercial Kit	Distributor
cDNA Synthesis Kit	Biorad
Epigentek ChIP Kit	Epigentek
RNeasy Plus Mini Kit	Qiagen
Total Protein Kit, Micro Lowry	Sigma-Aldrich Inc

Table 3.3 Equipment and Distributors

Equipment	Distributor
Biofuge 13 Microcentrifuge	Thermo Electron Corporation- Heraeus
Bio-Rad IQ5 Real Time Detection System	Bio-Rad Laboratories Ltd
Bio-Rad IQ5 Image Software	Bio-Rad Laboratories Ltd
Branson Sonifier 450	Branson
CO_2 Incubator 3326	Forma Scientific, Inc.
Coulter Counter ZM	Coulter Electronics Ltd.
Gel Doc 2000	Bio-Rad Laboratories Ltd.
Gene Amp PCR System 2700	Applied Biosystems Canada
LICOR Odyssey	LICOR Technologies
Mac Vector 7.2.3	Accelrys Inc.
OWL Transfer Apparatus	Thermo Scientific
Protein Electrophoresis System	Bio-Rad Laboratories Ltd
Quantity One Software Version 4	Bio-Rad Laboratories Ltd
SmartSpec3000 Spectrophotometer	Bio-Rad Laboratories Ltd.
Sorvall RT6000D	Du Pont Canada Ltd.
Step One Plus Real Time PCR Machine	Applied Biosystems Canada
Step One Plus Software	Applied Biosystems Canada

Table 3.4 Distributor Address

Distributor Name	Address
Abcam	Abcam, Cambridge, MA. USA
Accerylrys Inc.	Accerlrys Inc., San Diego, CA, USA
Applied Biosystems Canada	Applied Biosystems Canada, Streetsville, ON, Canada
ATCC	American Type Culture Collection, Manassas, VA, USA
Bio-Rad Laboratories Ltd	Bio-Rad Laboratories Ltd, Mississauga, ON, Canada
Branson	Branson, Danbury, CT, USA
Cell Signaling	Cell Signaling, Danvers, MA. USA

Coulter Electronics Ltd	Coulter Electronics Ltd. Bath, UK
Du Pont Canada Ltd	Du Pont Canada Ltd, Mississauga, ON, Canada
EMD Chemicals	EMD Chemicals, Gibbstown, NJ, USA
Epigentek	Epigentek, Brooklyn, NY, USA
Fermentas Canada Inc.	Fermentas Canada Inc., Burlington ON Canada
Invitrogen Canada Inc	Invitrogen Canada Inc., Burlington, ON, Canada
LICOR Technologies	LICOR Technologies, Lincoln, NB, USA
Millipore	Millipore, Billerica, MA, USA
Qiagen Inc	Qiagen Inc., Mississauga, ON, Canada
Santa Cruz Biotechnology Inc.	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA
Sigma-Aldrich Co.	Sigma-Aldrich Co., Oakville, ON, Canada
Thermo Scientific	Thermo Scientific, Rochester, NY, USA
VWR	VWR, Mississauga, ON, Canada

Table 3.5 Cell Lines and Distributor

Cell Line	Tissue Type	Distributor
Colo201	Colorectal Adenocarcinoma	ATCC
HepG2	Hepatocellular Carcinoma	ATCC
HT29	Colorectal Adenocarcinoma	ATCC
SW480	Colorectal Adenocarcinoma	ATCC
T47D	Colorectal Adenocarcinoma	ATCC

Table 3.6 Primer Sets

Primer Name	Sequence	Annealing
Bcl-x_L 3' region Fwd	TCGGGCCAGACACTGACCATCCACT	57°C
Bcl-x_L 3' region Rev	GAACTGCACTTTCACCTTCACA	57°C
Bcl-x_L Fwd	GCAGGTATTGGTGAGTCGGATCGC	57.5°C
Bcl-x_L Rev	CACAAAAGTATCCCAGCCGCCG	57.5°C
Bcl-x_L Promoter Fwd	CGAGCAGTCAGCCAGGTAG	57°C
Bcl-x_L Promoter Rev	GACGGCGAAGGCTCCTATTG	57°C
$p21^{WAF1}$ Fwd	GCCTGCCGCCGCCTCTTC	55°C
$p21^{WAF1}$ Rev	GCCGCCTGCCTCCTCCCAACTC	55°C
RPL13A Fwd	CAAGGTGTTTGACGGCATCC	55°C
RPL13A Rev	GCTTTCTCTTTCCTCTTCTCCTCC	55°C
Src +10 Fwd	GCGGCCATTTCACCAGCC	55°C
Src +100 Rev	GCGGTGATAAACTGAGGCTAG	55°C
Src 3' region Fwd	TCAAACCCTGCCCTCCTTAGAC	57°C
Src 3' region Rev	CATCACCCACAAGCCGATTG	57°C
Src Fwd	CAGAGGAGCCCATTTACATCGTC	55°C
Src Rev	CCCTTGAGAAAGTCCAGCAAACTC	55°C
Src1A Fwd	AGGCGGATCTGGGGCGTAG	57°C
Src1A Rev	ATTCCGGGCCGGGAGAGAC	57°C
Src1α Fwd	GACAAGTCGATCAGCTTCC	57°C
Src1α Rev	GCAAGTAGGTAAGGGCCAG	57°C

A

B

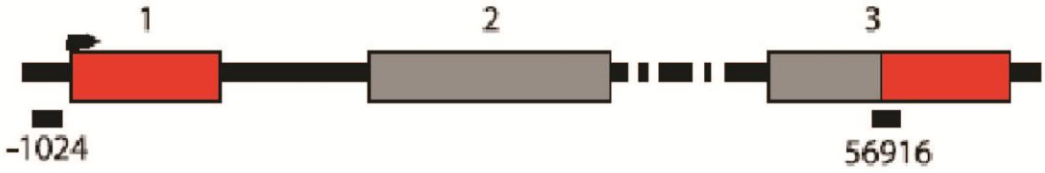


Figure 3.0: Location of Primer Binding in the Promoter and 3' Region of the (A) SRC and (B) BCL2L1 genes. All primers were designed using the MacVector software program, and were designed to span introns when possible.

3.2 Culture of Cell Lines

3.2.1 Culture and Maintenance of Cell Lines

All cell lines used in this study were obtained from American Type Culture Collection (ATCC). Cell media was purchased from Invitrogen-Gibco Cell Culture Systems. HT29 and SW480 colorectal adenocarcinoma cells, and HepG2, a heptocellular carcinoma cell line, were cultured in Dulbeccos's Modified Eagle's Medium (DMEM). Colo201 a colorectal adenocarinoma cell line was cultured in RPMI-1640 medium. T47D, a breast cancer cell line was cultured in RPMI-1640 plus insulin medium. All media was supplemented with 10% fetal bovine serum (FBS) as well as 1% penicillin-streptomycin. Cells were grown in a CO_2 incubator with 5% carbon dioxide atmosphere at 37°C.

3.2.2 Histone Deacetylase Inhibitors and Protein Phosphatase Inhibitor Treatments

Prior to experiments, cells were grown to 60-70% confluency in 10 cm dishes. When cells reached the required confluency the medium was removed by aspiration and the cells were washed with 5 mL of phosphate buffered saline (PBS). For adherent cells PBS was removed by aspiration and 10mL of new media containing the appropriate inhibitor was added. Semi-adherent cells were placed into a 15 ml conical tube with their original medium and centrifuged in a Sorvall RT6000D centrifuge at 1500 rpm x 5 minutes. The medium was then removed by aspiration, and cells were re-suspended in PBS. Cells were again centrifuged with the PBS being removed and medium containing an inhibitor added.

Trichostatin A and MGCD0103 were used at a final concentration of 1 μM. Apicidin and MS-275 were used at a final concentration of 2 μM. Final drug concentrations were obtained from dilutions of a stock solution created using DMSO. DMSO only controls were also used initially to rule out any affect DMSO may be having on the genes of interest. Drug concentrations were chosen based upon literature values that lead to an induction of $p21^{WAF1}$ expression (Yoshida *et al.,* 1990; Han *et al.,* 2000; Siavoshian *et al.,* 2000; Kwon *et al.,* 2002; Rosato *et al.,* 2003; Zhang *et al.,* 2007; Fournel *et al.,* 2008; Duglio *et al.,* 2010).

Cells were incubated in the inhibitor containing media for the indicated time periods. For adherent cells (HepG2, SW480, T47D and HT29) media was removed by aspiration and

cells washed with PBS. The PBS was removed by aspiration and cells were removed from the plate by treatment with 1 x Trypsin-EDTA for 5 minutes at 37°C. Cells were collected in 3 mL of media and placed in a 15 mL conical tube. For semi-adherent cells (Colo201), the media and cells were placed directly into a 15 mL conical tube without washing. Semi-adherent and adherent cells were centrifuged in a Sorvall RT6000D centrifuge at 1500 rpm x 5 minutes to pellet the cells. Following removal of the supernatant cells were then placed in a -80°C freezer for future use.

Experiments with HDACi and phosphatase inhibitors were performed with cells grown to 60-70% confluency. Cell media was removed by aspiration and cells were washed in 10 mL of PBS. Following aspiration of the PBS new media containing an HDACi was added as described above. Thirty minutes after the addition of HDACi select plates had one of the phosphatase inhibitors (Calyculin A or Fostriecin) added to give a final concentration of 50nM (Ishihara *et al.* 1989; Zhang *et al.,* 2005). Cells were then incubated at 37°C, 5% CO_2 for varying time periods.

At each time point media from adherent cells was removed by aspiration and cells were washed with PBS. Following aspiration of the PBS, cells were then trypsinized with 1 x Trypsin-EDTA for 5 minutes at 37°C. Cells were re-suspended with 3 mL of media and placed in a 15 mL conical tube. Following centrifugation at 1500 rpm for 5 minutes and removal of the supernatant, cells were placed in a -80°C freezer for future use. Semi adherent cells were placed along with their media into a 15 mL conical tube and cells were pelleted by centrifugation. Cells pellets were stored in a -80°C freezer after appropriate washing as described above.

3.3 General Molecular Biology Techniques

3.3.1 Polymerase Chain Reaction

Primer sets were designed for the DNA fragments of interest using MacVector. Primers were designed to span intronic sequences to avoid amplification of genomic DNA. The DNA of interest was amplified using a mixture containing 1 µL of cDNA, 5 µL 10x reaction buffer, 3 µL 25 mM $MgCl_2$, 66 ng of each primer, 2 µL of each 10 mM dNTP, and 0.2 µL of Taq

Polymerase. The Polymerase Chain Reaction (PCR) was completed in a GeneAmp PCR System 2700. The DNA template strands were first denatured at 95°C for 5 minutes followed by 30 cycles of 30 seconds at 95°C for denaturation, 30 seconds at 55°C for primer annealing, and finally 30 seconds at 72°C for extension. Following the completion of the 30 cycles a final extension step was completed at 72°C for 10 minutes. Agarose gel electrophoresis was completed on all PCR products to ensure specific amplification and to rule out any contamination.

3.3.2 Agarose Gel Electrophoresis

DNA that was to be loaded in the agarose gel was mixed with a DNA gel loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, and 30% (v/v) glycerol). A 2% agarose gel was prepared in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) with 1 µL of ethidium bromide added. Gel electrophoresis was completed in a Horizontal Gel Electrophoresis System with the TAE buffer at 100 Volts. DNA was visualized using a Gel Doc 2000 Gel Documentation System.

3.4 RNA Isolation from Cultured Cells

RNA isolation was completed using an RNeasy Plus Kit obtained from Qiagen. Cell pellets were thawed and lysed with RLT lysis buffer (guanidine thiocyanate) containing β-mercaptoethanol. Following cell lysis the cells were passed five times through a 4 gauge needle in order to homogenize the sample. The homogenized cell lysate was then passed through a Qiagen DNA column to remove any genomic DNA that was present. Samples were then added to the RNeasy Plus columns and manufactures instructions were followed for the purification of the sample RNA. RNA quality was determined by the A260/A280 ratio using a SmartSpec 3000 Spectrophotometer. RNA was determined to be pure when the A260/280 ratio was between 1.8 and 2.0 (Barbas *et al.*, 2007). RNA concentration was determined using the Beer-Lambert law.

3.5 Production of Complementary DNA

Complementary DNA was produced using a cDNA Synthesis Kit acquired from BioRad Laboratories Inc. One µg of the RNA collected using the RNeasy Plus Kit as described above was added in addition to reverse transcriptase, 5x iscript master mix (dNTPs, randomly generated primers, and buffer) and RNase free water according to manufacturer's instructions. Total sample volume was 20 µL. The production of the cDNA was completed in a GeneAmp PCR System 2700 following the program: 25°C for 5 min, followed by 30 min at 42°C, 5 min at 85°C. Complementary DNA which was produced was stored in a -20°C freezer until required.

3.6 Chromatin Immunoprecipitation Assay

Chromatin Immunoprecipitation Assays were completed using an Epigentek Kit. Cells were grown until there was a minimum of one million cells per 10 cm plate as determined by a Coulter Counter ZM. Upon reaching a sufficient confluency, cells were treated with one of the HDACi for 0, 0.25, 0.5, 1, 3, or 6 hours. After incubation cells were washed with 10 mL of PBS and pelleted by centrifugation at 1500 rpm for 8 minutes. Cells were then re-suspended in their respective media containing 1% formaldehyde and left to incubate for 10 minutes. Following the 10 minute incubation, 1 mL of 1.25M glycine was added and the cells were once again pelleted by centrifugation. Cells were washed once in cold PBS and pelleted by centrifugation. The supernatant was removed and cell pellets were stored in a -80°C freezer until the following day.

Cells were re-suspended in the supplied lysis buffer with protease inhibitor cocktail and left to incubate on ice for 10 minutes. The suspension was then sonicated using a Branson Sonifier 450 for 3 x 30 seconds (60% duty cycle and output three). DNA fragments were confirmed to be between 200 and 1000 base pairs in length by running a 5 µL sample on an agarose gel. Following sonication, cell debris was pelleted by centrifugation at 14 000 rpm for 10 minutes. The supernatant was collected and diluted in a 1:1 ratio with the supplied ChIP dilution buffer. 5 µL of this solution was then removed and used as the input DNA.

Following incubation of the provided strip wells with either 4 µg of anti-RNA Polymerase II or anti-acetylated H3K4 antibody, the wells were washed with TE buffer, and 100 µL of the fragmented DNA was added to each well and left to incubate for 90 minutes on an orbital shaker (100 rpm). Normal mouse IgG is included within the Epigentek Kit and was used as a negative control. GAPDH gene promoter primers are also included and are used as a positive control. GAPDH was chosen as it is an actively transcribed gene in most mammalian cells. At the completion of the incubation period on the orbital shaker, all wells were washed with TE buffer and 1 µL of proteinase K was then added to each well and left to incubate for 15 minutes in a 65°C water bath. Reverse buffer was added and the wells were left to incubate for 90 minutes in the 65°C water bath. DNA was then eluted from the strip wells by centrifugation with the included binding and elution buffers. The collected DNA was stored at -20°C for future use.

3.7 Real Time Polymerase Chain Reaction

Total RNA was collected from cell lines of interest. The quality of the collected RNA was determined by A260/A280 absorbance ratio as previously described in section 3.4. cDNA was subsequently produced, the details of which may be found in section 3.5. ChIP DNA was also used, and details of the procedure can be found in section 3.6. For all real time polymerase chain reactions (qRT-PCR) the DNA/cDNA of interest was combined with Sybr Green master mix purchased from BioRad Laboratories Inc. The genes of interest were amplified using primers previously designed in our lab using the MacVector software program (Ellis, 2007). Products of all primers were visualized using agarose gel electrophoresis.

qRT-PCR reactions were carried out using the iQ5 Multi-colour real-time PCR Detection system, a fluorescence based detection system (BioRad Laboratories Inc.). Prior to qRT-PCR experiments being completed primer efficiencies were tested to ensure equal amplification between the primer sets. Manufactures' instructions were followed for setting up the reactions and all samples were run in triplicate. Samples had a final volume of 20 µL which contained either 0.6 µL ChIP DNA or 1 µL cDNA, 66 ng of each primer as well as 10 µL of SYBR Green Supermix with the remainder of the volume constituted by water. In addition to the genes of interest, RPL13A, a 60s ribosomal protein, was also examined. Previous

experiment in our lab have shown that RPL13A is unaffected by HDACi treatment, and therefore was used as an internal control in all experiments (Ellis, 2007). The PCR cycling conditions included an initial denaturation of 95°C for 2 minutes followed by 45 cycles of 95°C for 15 seconds, annealing temperature for 20 seconds, and 72°C for 25 seconds. A final extension time of 3 minutes at 65°C was performed at the end of each reaction. Following each reaction, a melt curve analysis was done to ensure amplification specificity. Data was analyzed by the ΔΔCT method (Livak and Schmittgen, 2001).

3.8 Western Blot

3.8.1 Lowry Method

Cells were harvested in 1 mL of 65°C SDS sample buffer containing 10% (v/v) glyercol, 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 65 mM Tris-HCl (pH 7.0) and 0.05% (w/v) bromophenol blue. Twenty five μL of each sample was then used along with a Lowry kit purchased from Sigma-Aldrich. The absorbance of each sample was determined at 750 nm using a spectrophotometer. Protein concentration was determined by comparing the absorbance of the samples to a standard curve.

3.8.2 Western Procedure

Samples collected were run on a 10% SDS-polyacrylamide resolving gel which contained 10% (w/v) acrylamide: bis-acrylamide (29.2%: 0.8%), 375 mM Tri-HCl pH 8.0, 0.1% (v/v) SDS, 0.1% (w/v) ammonium persulfate and 0.4% (w/v) N,N,N',N'-tetramethylethylenediamine (TEMED) (Sambrook *et al.*,1989). After the gel had polymerized a 5% (w/v) acrylamide stacking gel (29.2% acrylamide: 0.8% bis acrylamide, 130 mM Tris-HCl, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate and 0.4% (w/v) TEMED) was added on top of the 10% acrylamide gel (Sanbrook *et al.,* 1989). Samples containing 30ug of protein were added to the gel and resolved by SDS-page using an SDS running buffer (25 mM Tris-Hcl, 200 mM glycine, 0.1% (w/v) SDS). Electrophoresis was carried out for 90 min at 160 volts.

Following electrophoresis, gels were washed in transfer buffer (10 mM Tris, 15 mM NaCl, 0.5%(v/v) TWEEN-20) for fifteen minutes. Proteins were then transferred by electroblotting to a nitrocellulose membrane. Membranes were washed twice with PBS and then blocked for one hour at room temperature in Odyssey blocking buffer. After blocking membranes were incubated in either anti-Akt rabbit monoclonal antibody, or anti-pSer473Akt rabbit monoclonal antibody, at a 1: 15,000 dilution for one hour at room temperature. Membranes were washed in PBS containing TWEEN-20, and incubated in IR680 labeled anti-rabbit secondary antibodies at a 1: 15,000 dilution in Odyssey blocking buffer. Membranes were visualized using fluorescence in a LICOR Odyssey visualization system

4. RESULTS

4.1 Effect of Class Specific Histone Deacetylase Inhibitors on c-Src, Bcl-x_L, and $p21^{WAF1}$ mRNA Expression

4.1.1 Response of c-Src, Bcl-x_L, and $p21^{WAF1}$ mRNA to TSA and apicidin

To determine whether repression of the examined mRNA was dependent on inhibition of a particular HDAC a variety of HDACi with varying specificities were examined to determine their effect on c-Src, Bcl-x_L and $p21^{WAF1}$ mRNA expression. The expression of $p21^{WAF1}$ mRNA was used as a marker for HDACi activity as it has been found to be universally upregulated by HDACi (Lu *et al.,* 2004). Four different HDACi were surveyed over a 12 hour time course in the following cancerous cell lines: SW480, Colo201, and HT29, colorectal adenocarcinoma cell lines; HepG2, a hepatocellular carcinoma cell line, and T47D a breast cancer cell line (Figures 4.1, 4.2, 4.3). A 12 hour time course was used, as previous research in the lab has shown that c-Src mRNA expression is consistently repressed within this time frame. Four independent experiments were completed for each HDACi and cell line. As the results of the independent experiments were highly consistent representative data is shown in this thesis.

TSA and apicidin, pan specific and HDAC2/HDAC3 specific inhibitors respectively, were found to repress c-Src mRNA expression in all cell lines tested (Figure 4.0, 4.1). In Colo201, HepG2, and T47D cells, a transient induction of c-Src mRNA expression was observed followed by subsequent repression. $p21^{WAF1}$ mRNA expression was found to increase following treatment with both TSA and apicidin, in all five cell lines tested, with the level of induction ranging from 10 to 40 fold of the control levels (Figure 4.2). Based upon the results of the c-Src mRNA expression and $p21^{WAF1}$ induction (Figure 4.2), the Colo201 and T47D cell lines were chosen to examine the expression of Bcl-$x_{L,}$ as both cell lines showed consistent induction of $p21^{WAF1}$ and repression of c-Src mRNA expression. Expression of Bcl-x_L mRNA was repressed following treatment with both TSA and apicidin (Figure 4.3). In the Colo201 cell line a transient increase in Bcl-x_L mRNA expression was seen following treatment with TSA, while a transient increase was seen following treatments with both apicidin and TSA in the T47D cell line. Although slight differences were present in individual cell lines, in general, TSA and apicidin act in a similar manner with respect to gene expression in each cell line.

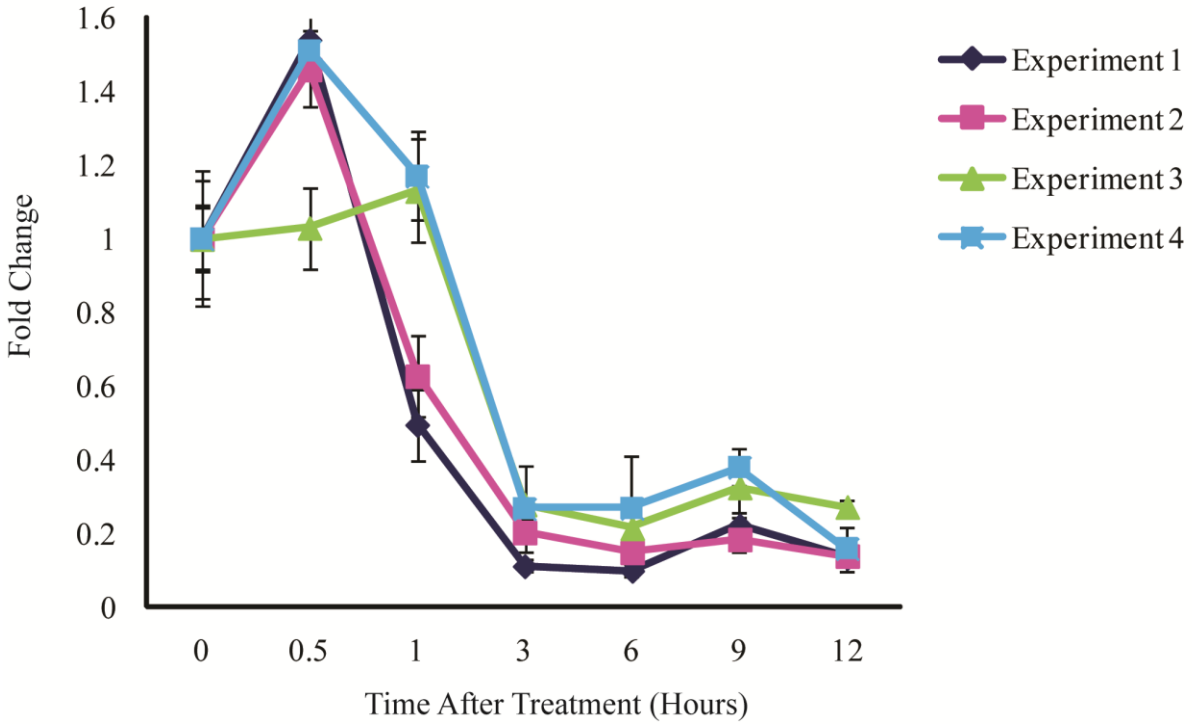


Figure 4.0: c-Src mRNA expression following treatment with TSA. Colo201 cells were treated with 1 µM TSA for the indicated time periods. Following RNA isolation mRNA levels were determined using qRT-PCR. Four independent experiments were completed. Error bars represent standard deviation of three different replicates completed in each experiment.

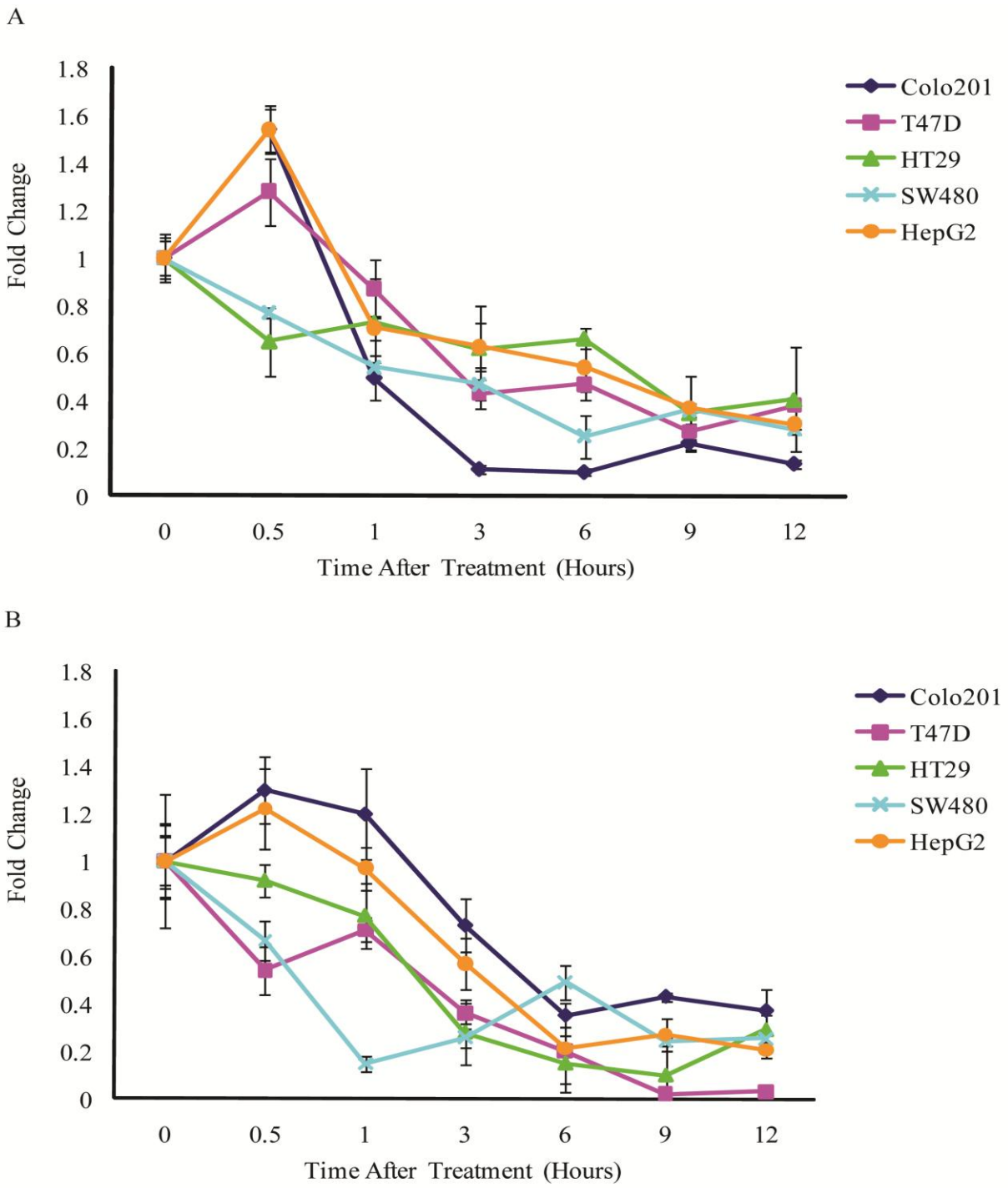


Figure 4.1: c-Src mRNA expression following treatment with TSA or apicidin. Colo201, T47D, HT29, SW480, and HepG2 cells were treated with either (A) 1 µM TSA or (B) 2 µM apicidin for the indicated time periods. Following RNA isolation mRNA levels were determined using qRT-PCR. Data representative of at least four independent experiments. Error bars represent standard deviation of at least three different replicates.

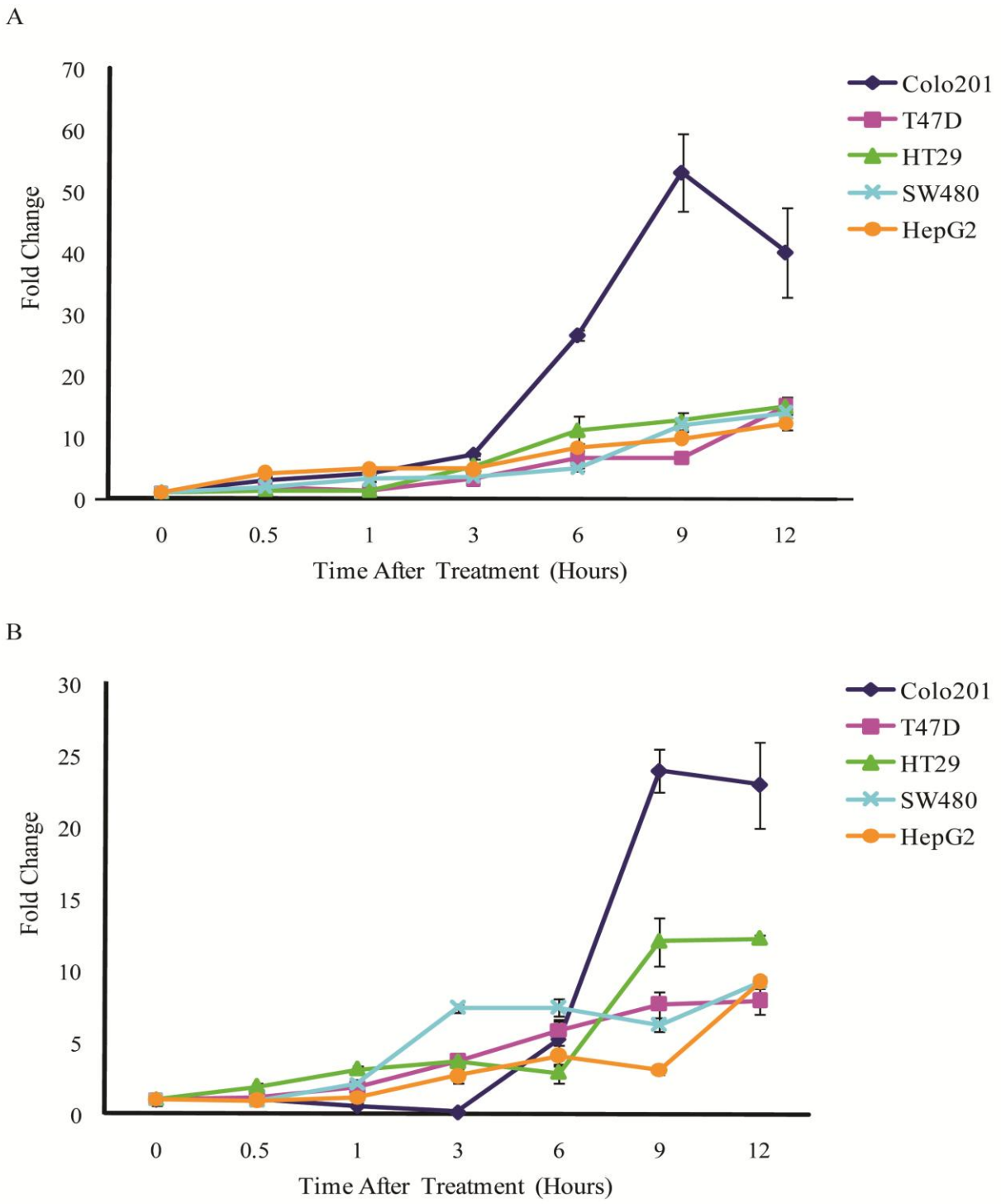


Figure 4.2: $p21^{WAF1}$ mRNA expression following treatment with TSA or apicidin. Colo201, T47D, HT29, SW480 and HepG2 cells were treated with either (A) 1µM TSA or (B) 2µM apicidin for the indicated time periods. Following RNA isolation mRNA levels were determined using qRT-PCR. Data representative of at least four independent experiments. Error bars represent standard deviation of at least three different replicates.

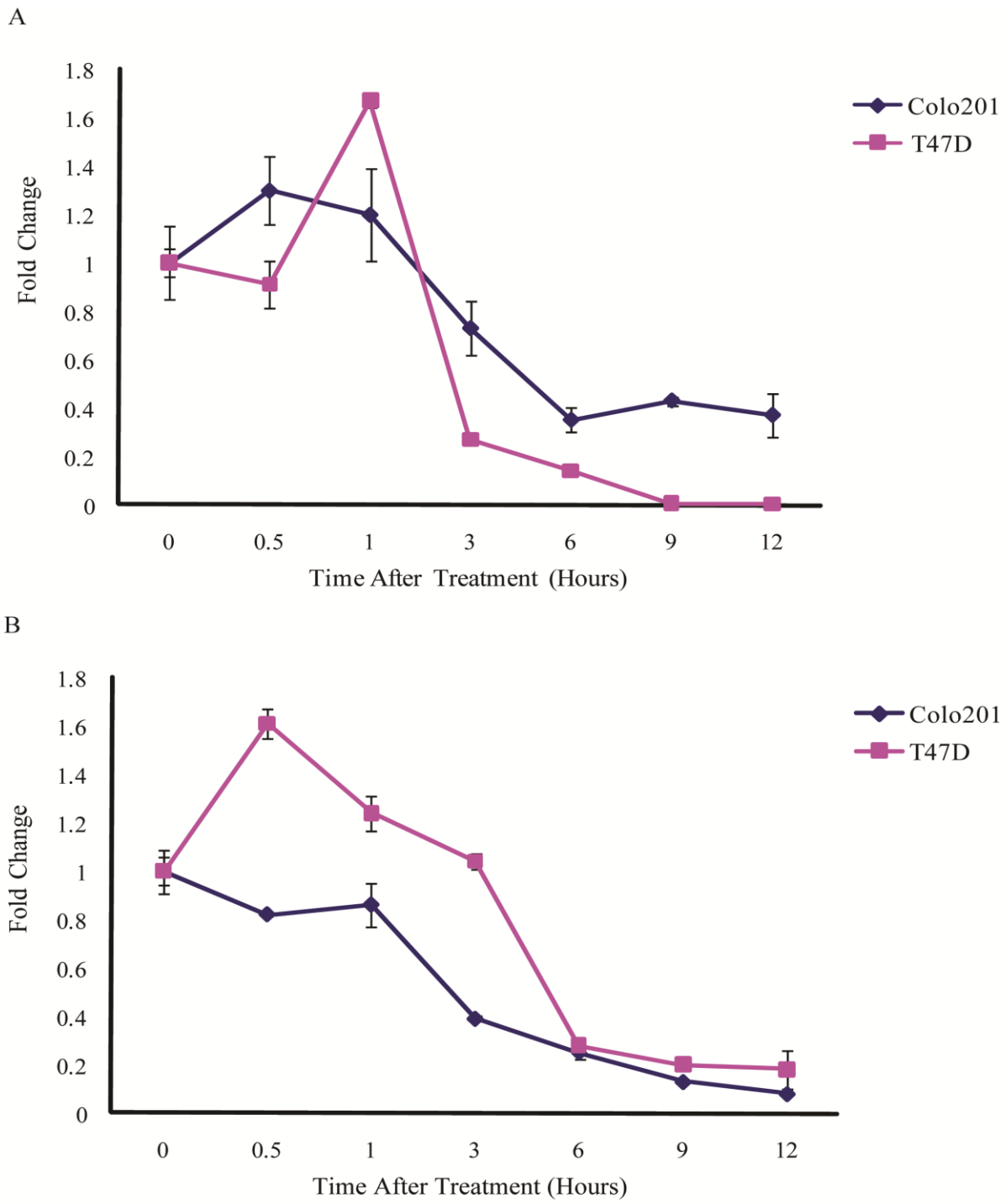


Figure 4.3: Bcl-x_L mRNA expression following treatment with TSA or apicidin. Colo201 and T47D cells were treated with either (A) 1 µM TSA or (B) 2 µM apicidin for the indicated time periods. Following RNA isolation mRNA levels were determined using qRT-PCR. Data representative of at least four independent experiments. Error bars represent standard deviation of at least three different replicates.

4.1.2 Response of c-Src and Bcl-x_L mRNA to MS-275 and MGCD0103

Colo201 and T47D cells were also used to determine the effect of two synthetic benzamide HDACi on c-Src, Bcl-x_L, and $p21^{WAF1}$ mRNA expression (Figure 4.4). MS-275 has been shown to inhibit HDAC1 while MGCD0103 targets both HDAC 1 and HDAC 2 (Zhou *et al.*, 2008; Prince *et al.*, 2009; Witt *et al.*, 2009). Interestingly, it was determined that neither MS-275 nor MGCD0103 were capable of repressing c-Src or Bcl-x_L mRNA expression. Following treatments with MS-275 c-Src mRNA levels were relatively unaffected in the Colo201 cell line, but were induced in the T47D line (Figure 4.4). Bcl-x_L mRNA expression remained unaffected or slightly induced in both cell lines following treatment with MS-275 (Figure 4.4). In contrast, treatment with MGCD0103 led to an induction of Bcl-x_L mRNA while c-Src mRNA expression remained unchanged in both cell lines (Figure 4.4).

The level of $p21^{WAF1}$ mRNA expression increased following treatment with MS-275 and MGCD0103 but only to 3.5-4.0 fold of control levels (Figure 4.5). This induction is rather low when compared to the level of induction seen with the TSA and apicidin treatments. This raised the question of whether or not a higher drug concentration was required to lead to the repression of c-Src and Bcl-x_L, and a subsequent larger induction of $p21^{WAF1}$ mRNA. To determine if this was in fact the case Colo201 cells were treated with a higher concentration of both MS-275 and MGCD0103 for 6 hours (Figure 4.6). Longer incubation times were not possible due to the onset of cellular toxicity at the higher concentrations. The increased concentration of both HDACi led to a larger induction of $p21^{WAF1}$ mRNA expression levels; however neither c-Src nor Bcl-x_L mRNA levels were repressed (Figure 4.6). Thus following treatment with benzamides HDACi c-Src and Bcl-x_L mRNA expression remained unaffected or induced, while $p21^{waf1}$ mRNA expression was induced.

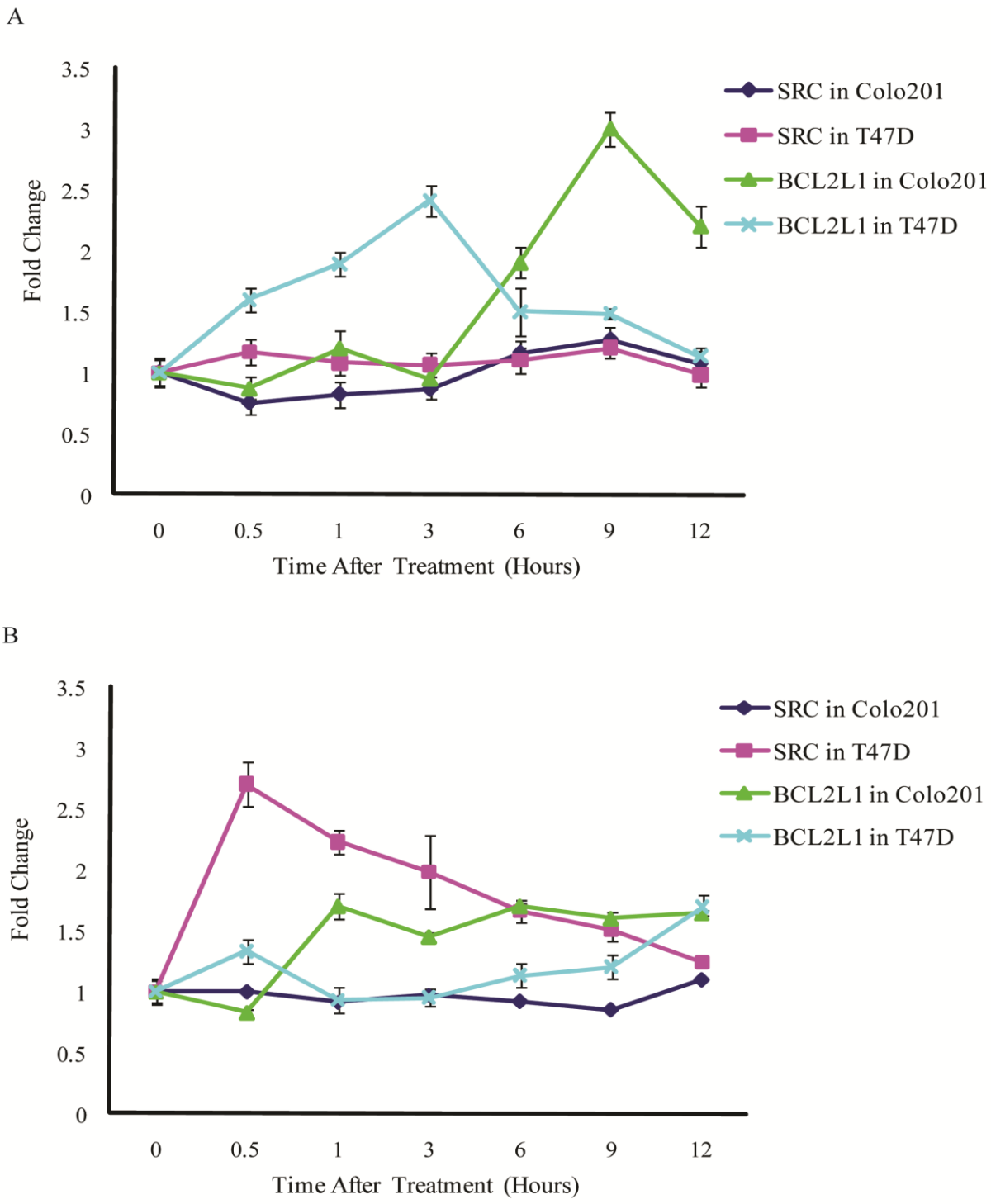


Figure 4.4: Expression of c-Src and Bcl-x_L mRNA following treatment with MS-275 or MGCD0103. Colo201 and T47D cells were treated with either (A) 1 µM MGCD0103 or (B) 2 µM MS-275 for the indicated time periods. Following RNA isolation mRNA levels were determined using qRT-PCR. Data representative of at least two four independent experiments. Error bars represent standard deviation of at least three different replicates.

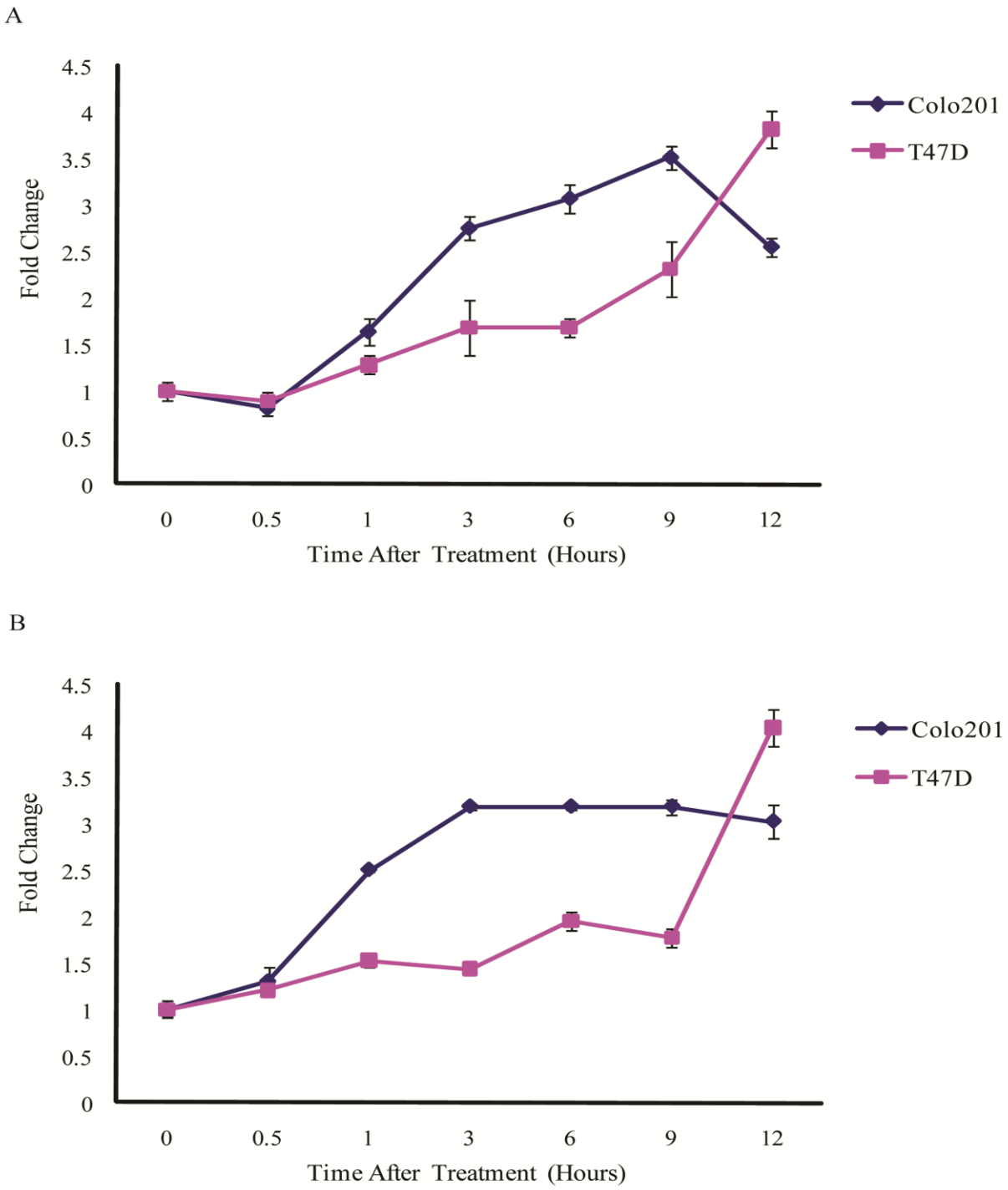


Figure 4.5: $p21^{WAF1}$ mRNA expression following treatment with MS-275 and MGCD0103. Colo201 and T47D cells were treated with either (A) 1 µM MGCD0103 or (B) 2 µM MS-275 for the indicated time periods. Following RNA isolation mRNA levels were determined using qRT-PCR. Data representative of at least four independent experiments. Error bars represent standard deviation of at least three different replicates.

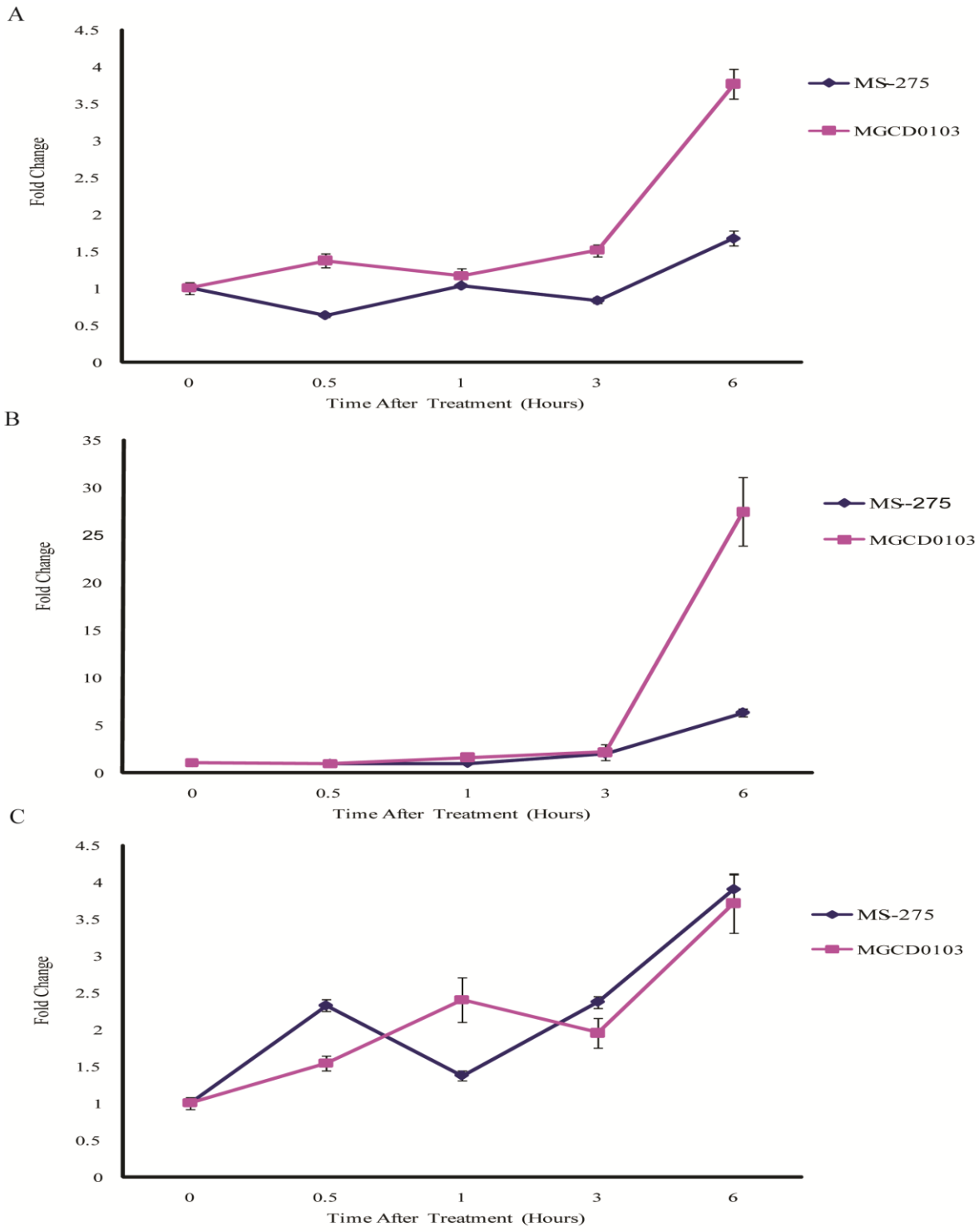


Figure 4.6: Expression of (A) c-Src, (B) $p21^{WAF1,}$ and (C) Bcl-x_L mRNA following treatment with high dose MS-275 and MGCD0103. Cells were treated with either 6 µM of MGCD0103 or MS-275 for the indicated time periods. Following RNA isolation mRNA levels were determined using qRT-PCR. Data representative of at least four independent experiments. Error bars represent standard deviation of at least three different replicates.

4.2 Effect of Histone Deacetylase Inhibitors on Histone Acetylation, RNA Polymerase II Occupancy, and Akt Phosphorylation

4.2.1 Effect of Histone Deacetylase Inhibitors on Histone Acetylation at the SRC and BCL2L1 Promoters

The effect of HDACi on histone acetylation, at the SRC and BCL2L1 promoters, was examined by ChIP analysis in two representative cell lines to confirm whether changes in acetylation were occurring. An increase in histone acetylation should be observed if HDACs are being inhibited by the tested HDACi. If this is true, it would suggest that TSA and apicidin work through a much more complicated mechanism of action, as they would be capable of gene repression while increasing histone acetylation; a state normally associated with increase gene expression.

In the Colo201 cell line, promoter acetylation was found to increase at the BCL2L1 and SRC promoters following treatment with all four HDACi (Figure 4.7, 4.8). Despite leading to both c-Src and Bcl-x_L mRNA repression, treatment with TSA and apicidin led to an equal, if not larger increase in promoter histone acetylation than the two synthetic benzamides inhibitors. Comparing the two SRC promoters with the BCL2L1 promoter, the level of increase in the histone acetylation was much lower at BCL2L1. It is also interesting to note that at all promoters tested in the Colo201 cell line, MGCD0103 led to the highest levels of promoter histone acetylation at the 6 hour time point, while MS-275 had the lowest.

Similar trends in promoter histone acetylation were also seen in the T47D cell line (Figure 4.9, 4.10). Following treatment with all four HDACi, promoter histone acetylation was found to increase at both SRC and BCL2L1 to varying degrees. Based on the above findings all four HDACi treatments increase promoter histone acetylation. Therefore the mechanism of repression following TSA and apicidin treatments appears to be more complicated that a simple change in acetylation status or inhibition of a single HDAC. To further examine the possible mechanism behind this repression, the level of RNA Pol II occupancy was next examined.

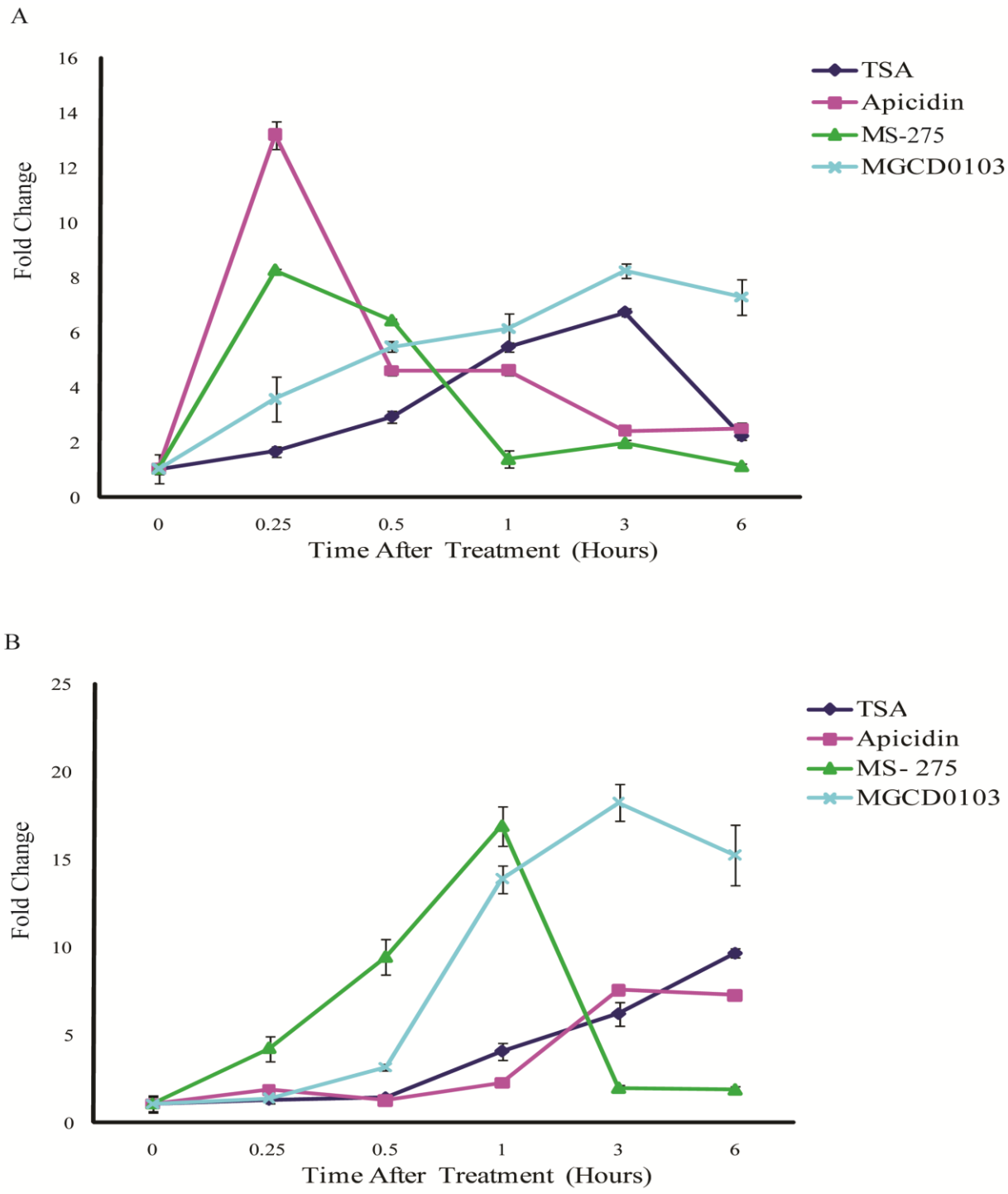


Figure 4.7: Histone acetylation at the (A) SRC1α and (B) SRC1A promoters in the Colo201 cell line. Colo201 cells were treated with 1 µM of TSA or MGCD0103 or 2 µM apicidin or MS-275 for the indicated time periods. Following cell collection Chromatin Immunoprecipitation Assays were completed using an anti-acetyl H3 antibody. qRT-PCR was completed for the various regions of the gene. Data representative of at least two independent experiments. Error bars represent standard deviation of at least three different replicates.

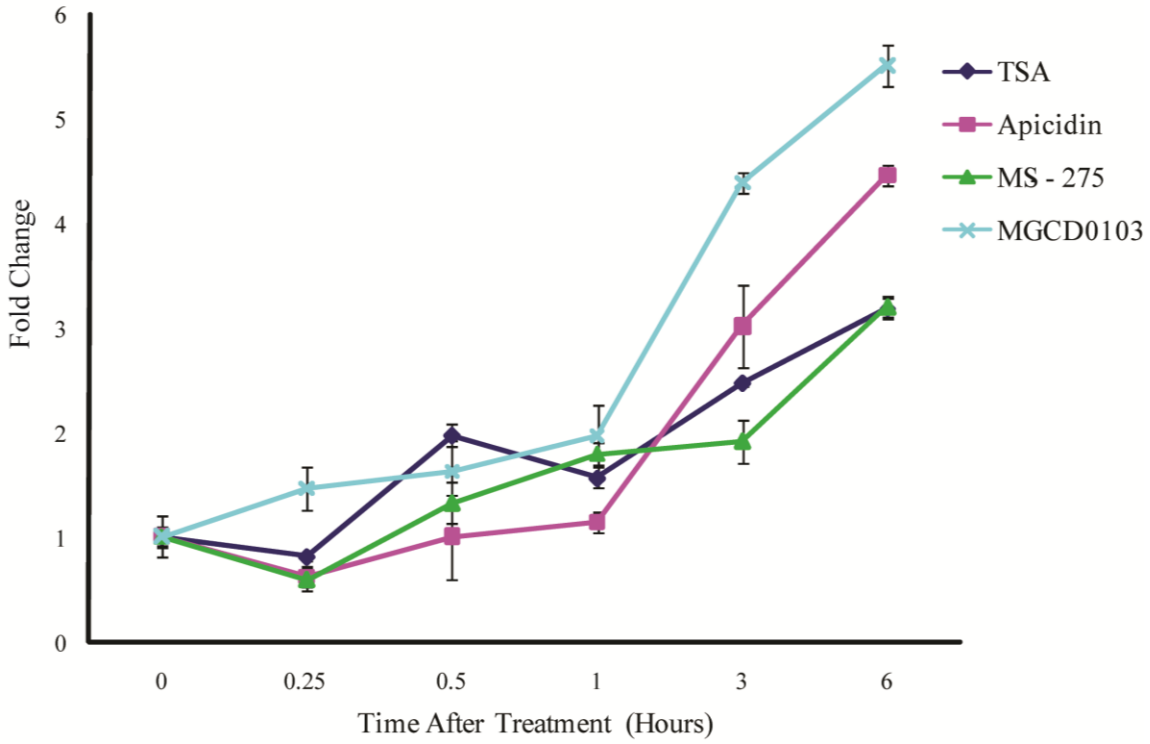


Figure 4.8: Histone acetylation at the BCL2L1 promoter in the Colo201 cell line. Colo201 cells were treated with 1 µM of TSA or MGCD0103 or 2 µM apicidin or MS-275 for the indicated time periods. Following cell collection Chromatin Immunoprecipitation Assays were completed using an anti-acetyl H3 antibody. qRT-PCR was completed for the indicated region of the gene. Data representative of at least two independent experiments. Error bars represent standard deviation of at least three replicates.

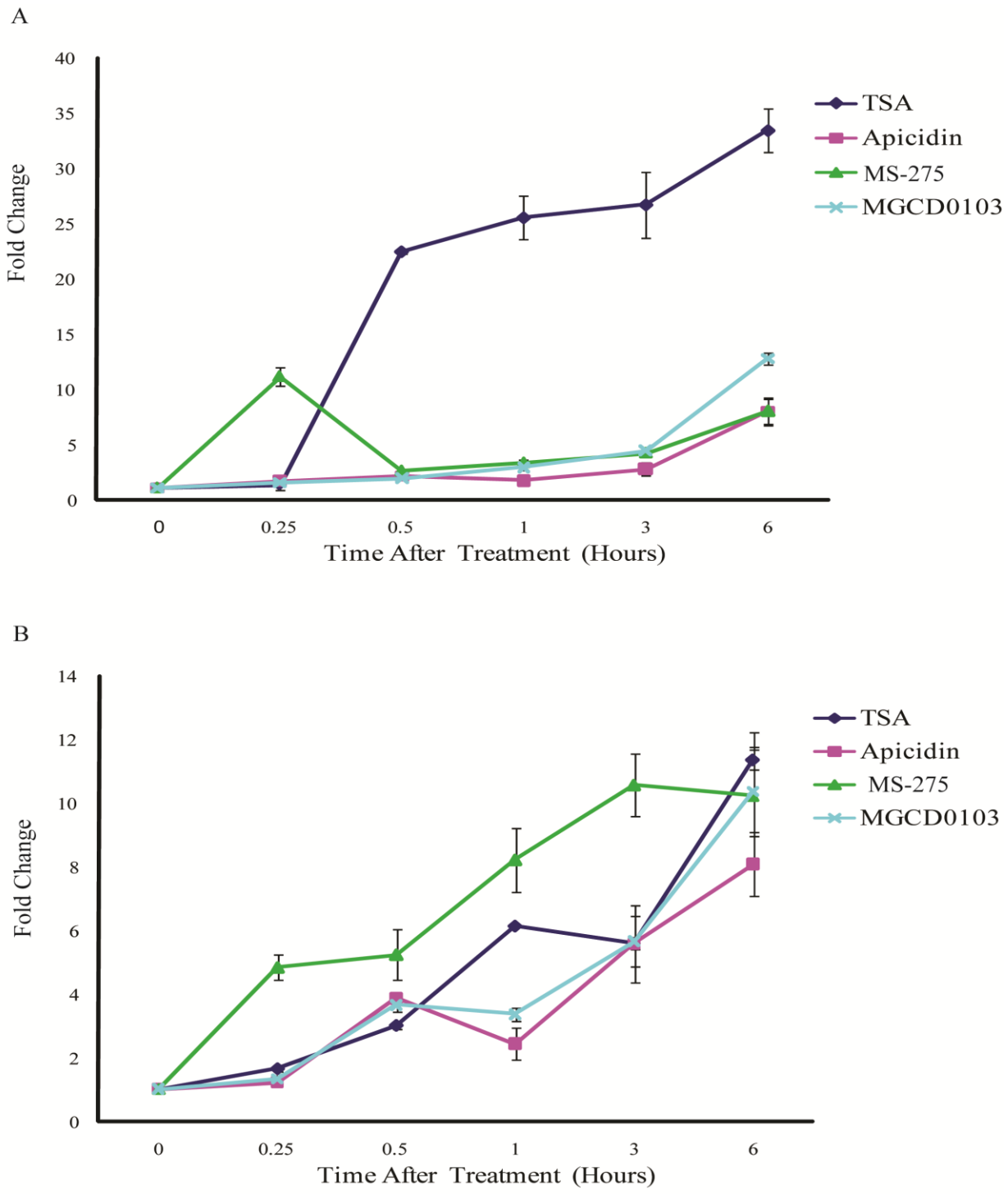


Figure 4.9: Histone acetylation at the (A) Src1α and (B) SRC1A promoters in the T47D cell line. T47D cells were treated with 1 µM of TSA or MGCD0103 or 2 µM of MS-275 or apicidin for the indicated time periods. Following cell collection Chromatin Immunoprecipitation Assays were completed using an anti-acetyl H3 antibody. qRT-PCR was completed for the various regions of the gene. Data representative of at least two independent experiments. Error bars represent standard deviation of at least three different replicates.

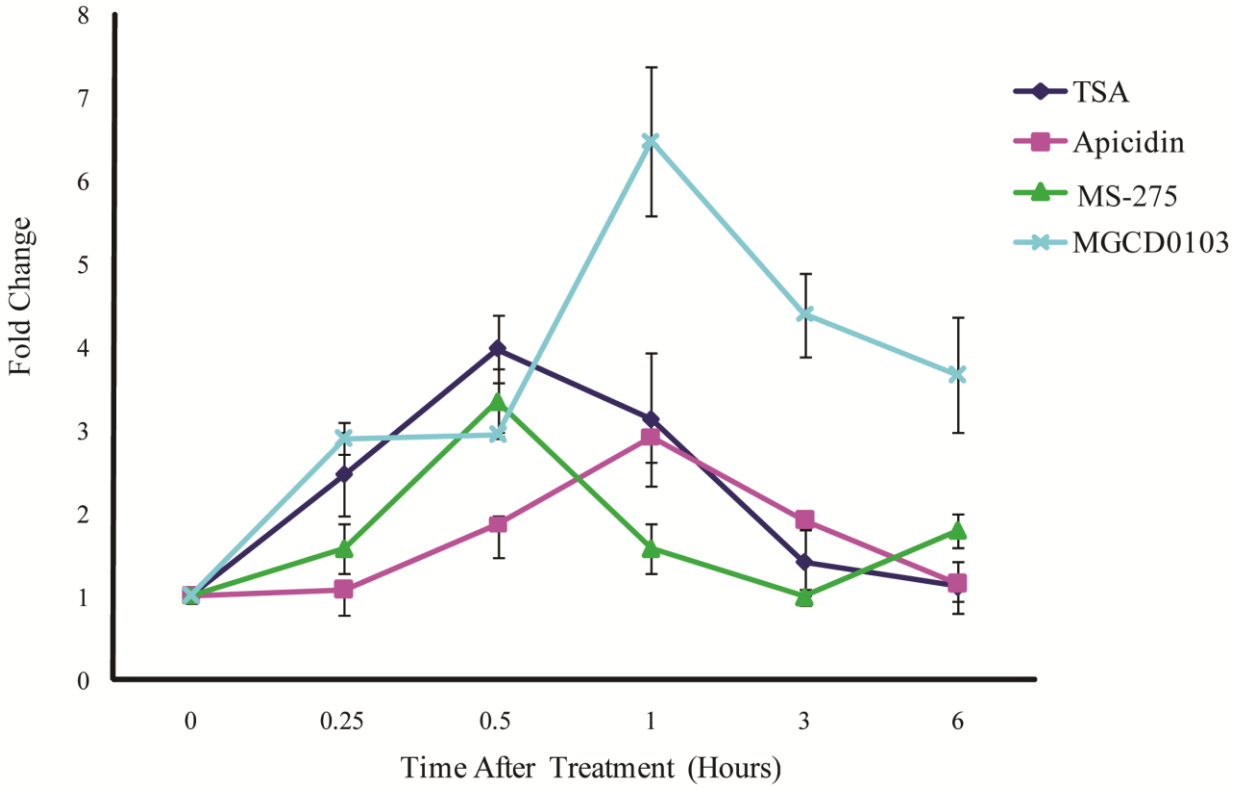


Figure 4.10: Histone acetylation at the BCL2L1 promoter in the T47D cell line. T47D cells were treated with 1 µM of TSA or MGCD0103 or 2 µM apicidin or MS-275 for the indicated time periods. Following cell collection Chromatin Immunoprecipitation Assays were completed using an anti-acetyl H3 antibody. qRT-PCR was completed for the various regions of the gene. Data representative of at least two independent experiments. Error bars represent standard deviation of at least three different experiments.

4.2.2 Effect of TSA and MS-275 on RNA Polymerase II Occupancy at the Promoter and 3' Regions of the SRC and BCL2L1 Genes

RNA Pol II occupancy was determined by ChIP analysis at the promoter and 3' regions of the SRC and BCL2L1 genes in two representative cell lines (Figure 4.11, 4.12). TSA and MS-275 were used as they had opposing effects on c-Src and Bcl-x_L mRNA expression. In Colo201 cells RNA Pol II occupancy was found to increase at both the SRC1α and SRC1A promoters following treatment with both TSA and MS-275 (Figure 4.11 A-B). In stark contrast the occupancy of RNA Pol II was found to decrease dramatically in the 3' region of the gene following treatment with TSA, but did not decrease following MS-275 treatment. It is particularly interesting that this decrease in occupancy following TSA treatment occurred within fifteen minutes. Very similar results were obtained when examining the BCL2L1 gene: RNA Pol II occupancy increased in the promoter region following treatment with MS-275 (Figure 4.12 B). However, following treatment with TSA there was an initial decrease in RNA Pol II occupancy which returned to the control level 3 hours after the addition of the HDACi (Figure 4.12A). When examining the 3' region of the gene, treatment with TSA caused a rapid decrease in RNA Pol II occupancy while occupancy remained stable or increased slightly following MS-275 treatment. Like the decrease seen at the SRC gene following treatment with TSA, the loss of RNA Pol II occupancy in the 3' region of the BCL2L1 gene occurred within 15 minutes of treatment (Figure 4.12A).

The level of RNA Pol II occupancy was also examined at the SRC and BCL2L1 3' regions in the T47D cell line (Figure 4.13). Again, similar to the Colo201 cell line, RNA Pol II occupancy was found to decrease at the 3' region of both SRC and BCL2L1 within 15 minutes of TSA treatment, while it increased following treatment with MS-275 (Figure 4.13). In contrast, TSA treatment has been shown to increase the occupancy at the promoter regions of both genes in the T47D cell line (Bonham, personal communication).

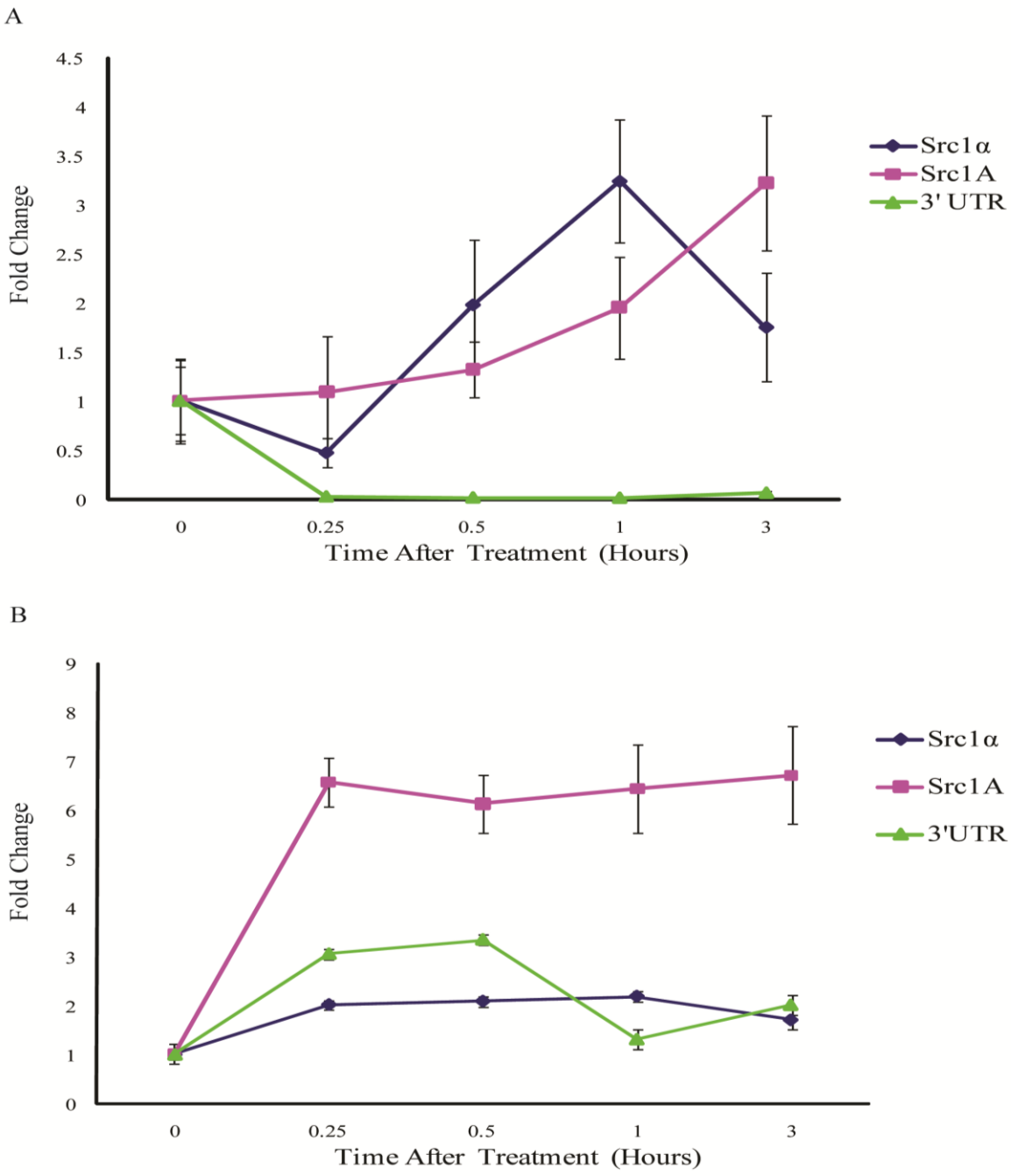


Figure 4.11: RNA Polymerase II occupancy at the SRC1α, SRC1A promoters, and 3' region following treatment with (A) TSA and (B) MS-275. Colo201 Cells were treated with either 1 µM of TSA or 2 µM MS-275 for the indicated time periods. Following cell collection Chromatin Immunoprecipitation assays were completed using at anti-RNA Polymerase II antibody. qRT-PCR was completed for the various regions of the gene. Data representative of at least three independent experiments. Error bars represent standard deviation of at least three different replicates.

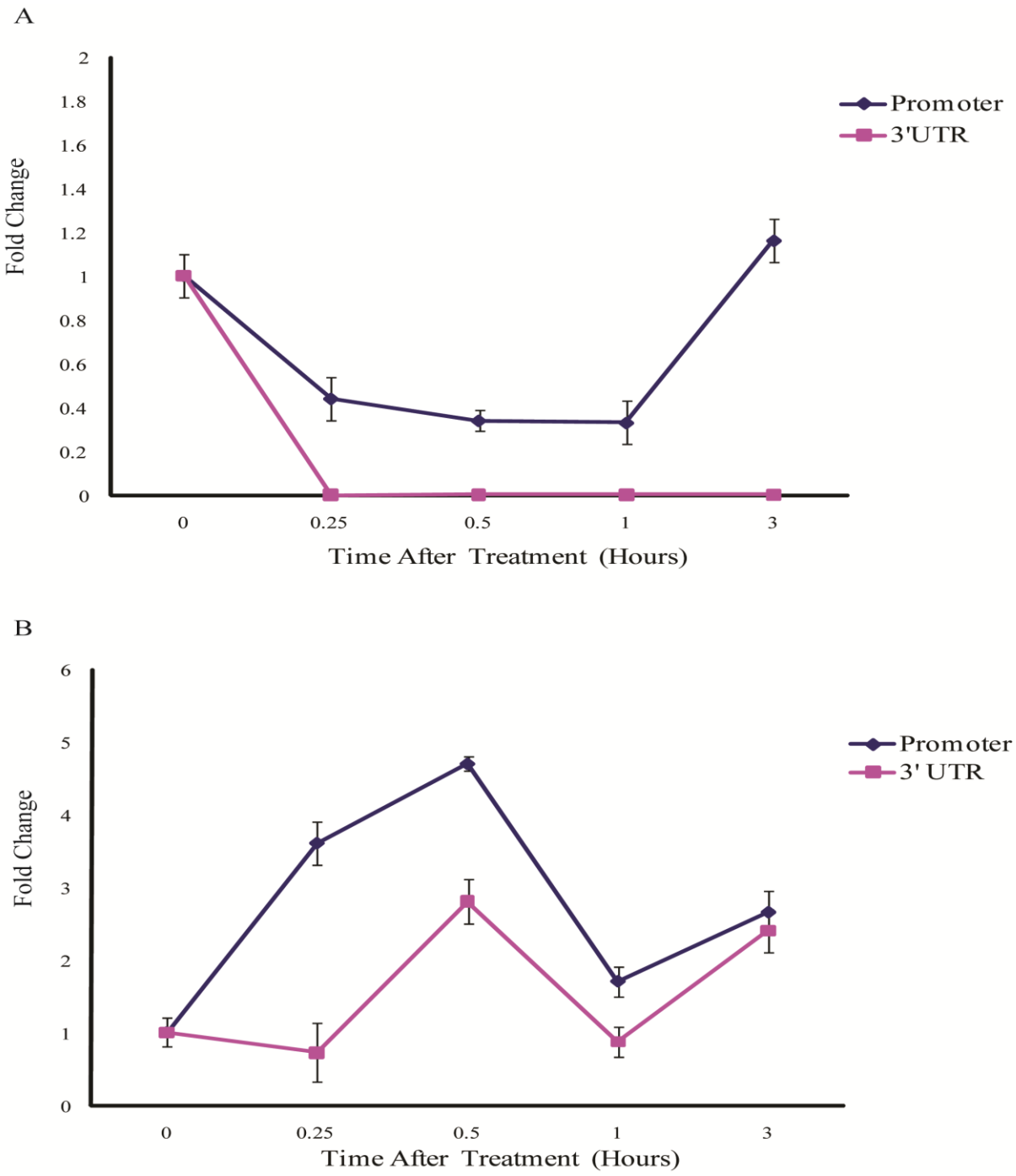


Figure 4.12: RNA Polymerase II occupancy at the BCL2L1 promoter, and 3' following treatment with (A) TSA and (B) MS-275. Colo201 cells were treated with either 1 µM TSA or 2 µM MS-275 for the indicated time periods. Following cell collection Chromatin Immunoprecipitation assays were completed using anti-RNA Polymerase II antibody. qRT-PCR was completed for the various regions of the gene. Data representative of at least three independent experiments. Error bars represent standard deviation of at least three different replicates.

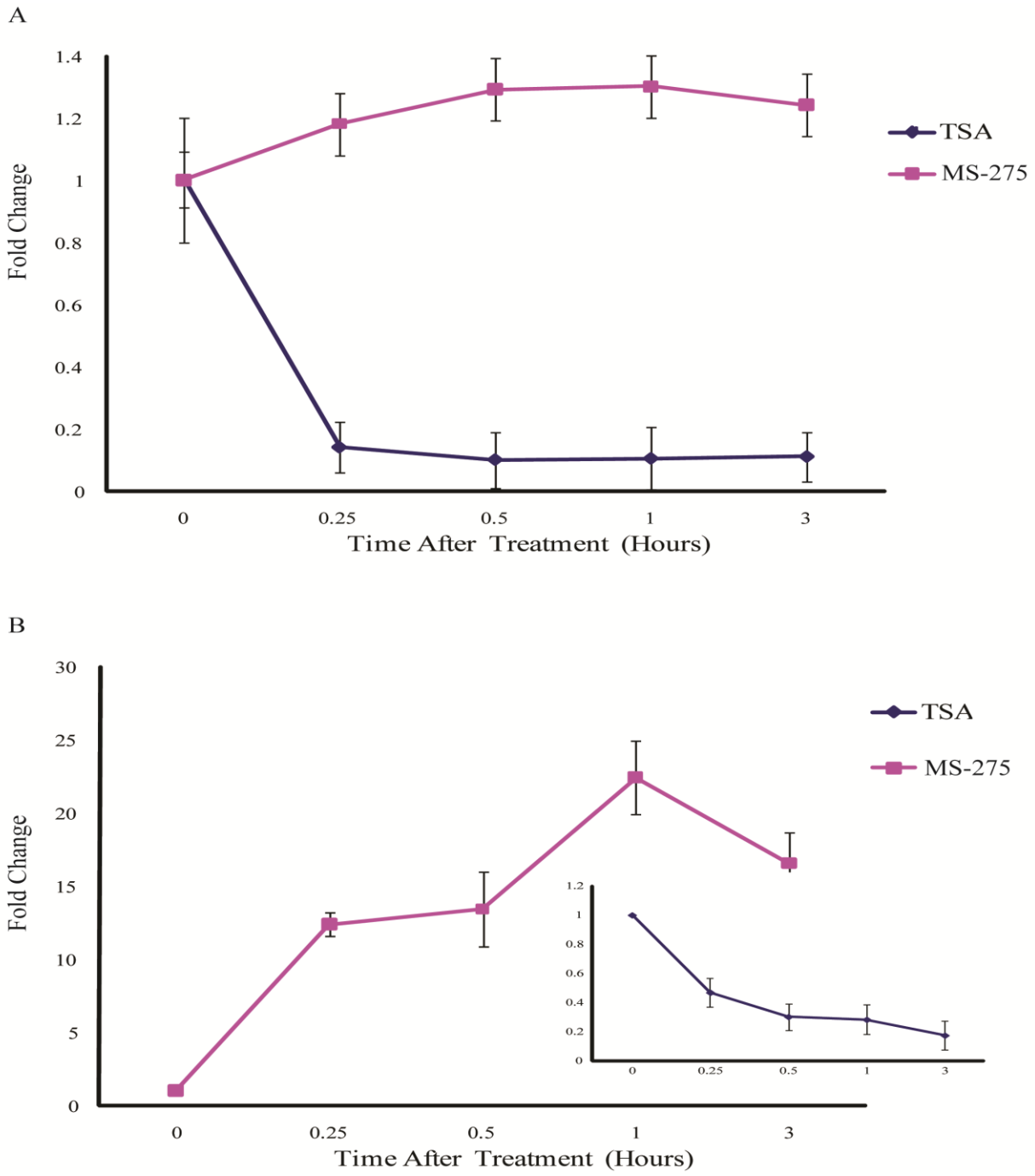


Figure 4.13: RNA Polymerase II occupancy at the 3' region of the (A) BCL2L1 and (B) SRC genes following treatment with TSA and MS-275 in the T47D cell line. T47D cells were treated with either 1 µM TSA or 2 µM MS-275 for the indicated time periods. Upon cell collection Chromatin Immunoprecipitation assays were completed using an anti- RNA Pol II antibody. qRT-PCR was completed for the various genes. Data representative of at least two independent experiments. Error bars represent standard deviation of at least three different replicates.

4.2.3 Short c-Src mRNA Transcripts Unaffected by Histone Deacetylase Inhibitor Treatments

Results from the RNA Pol II occupancy experiments suggest that the HDACi mediated repression may involve either transcriptional arrest or a block in productive elongation, as RNA Pol II remained at the promoters but was lost in the 3' regions of the genes. If a transcriptional arrest is occurring, short mRNA transcripts should decrease following HDACi treatment, but if the mechanism involves a block in productive elongation such as promoter proximal pausing, short mRNA transcripts will be continually produced, and therefore should not decrease following HDACi treatments. Therefore production of 100 nucleotide long transcripts from the SRC1α promoter was examined by qRT-PCR following treatment with all four HDACi (Figure 4.14, 4.15). Short transcript expression was examined using the same input DNA used for the full length mRNA expression experiments. 100 nucleotides was chosen for the length of the short transcripts as DSIF and NELF pause the early elongating complex in this region during promoter proximal pausing (Yamaguchi *et al.,* 1999; Sims *et al.,* 2004; Saunders *et al.,* 2006; Nechaev *et al.,* 2011). Following treatment with TSA in the Colo201 cell line the level of short c-Src mRNA transcripts was found to be unaffected (Figure 4.14). Treatment with apicidin, MS-275, and MGCD0103 all led to a small increase in the level of transcript production (Figure 4.14). The level of short transcript production was also examined in the SW480, HT29, and HepG2 cell lines following TSA or apicidin treatment (Figure 4.15). Levels were again found to remain very similar to that of the control with the exception of an increase in transcript production in the HepG2 cell line following apicidin treatment (Figure 4.15B). Processes such as transcriptional arrest and productive elongation, which may account for the observed gene repression, are both known to be regulated through RNA Pol II phosphorylation. Therefore although the inhibition of a certain HDAC may be involved in the observed repression, alternative mechanisms including the role of phosphorylation was examined. A possible role for phosphorylation is supported by the findings of HDACs commonly co-precipitating with various phosphotases, as well as the research completed by Chen and colleagues showing that TSA but not MS-275 leads to the release of an active PP (Chen *et al.,* 2005).

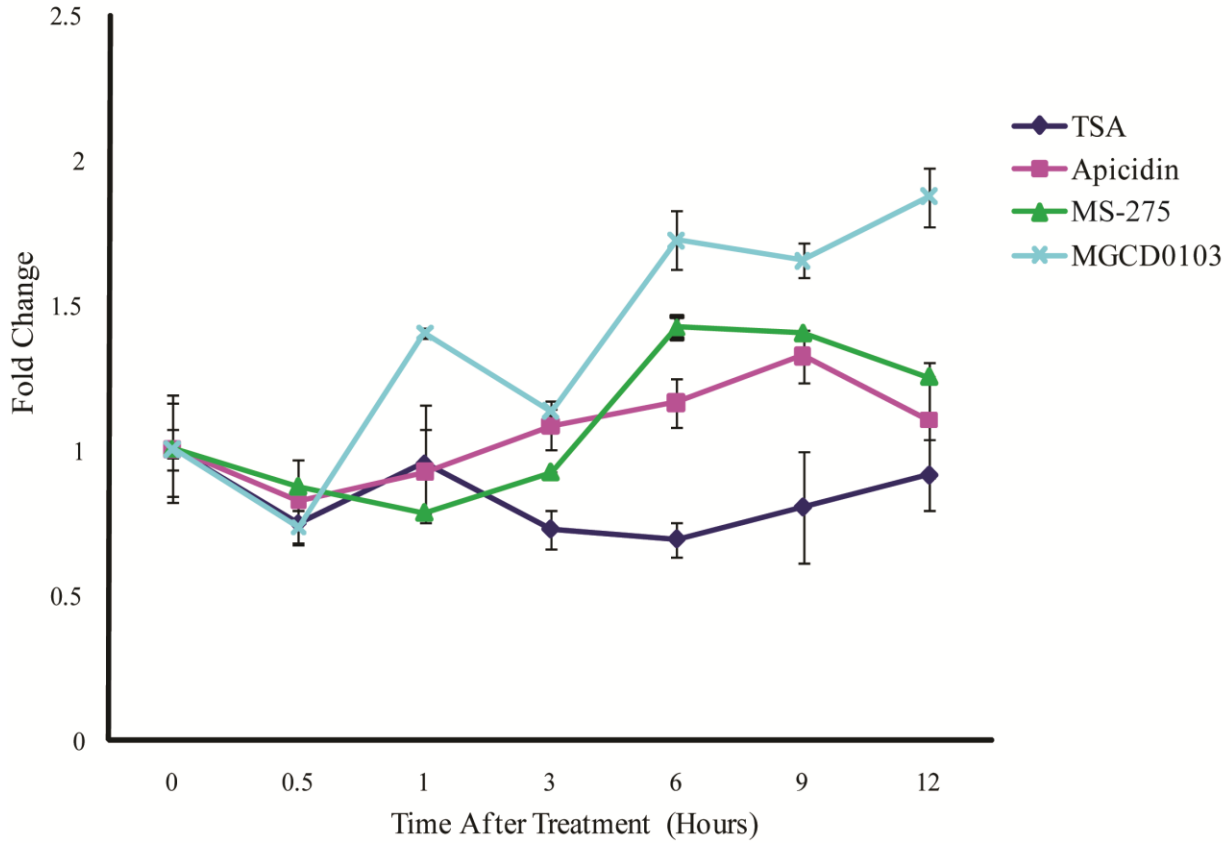


Figure 4.14: Short c-Src mRNA transcripts following treatment with various HDACi in the Colo201 cell line. Colo201 cells were treated with 1 µM of TSA or MGCD0103 or 2 µM apicidin or MS-275 for the indicated time periods. Following RNA isolation qRT-PCR was completed for the c-Src mRNA transcript. Data representative of at least four independent experiments. Error bars represent standard deviation of at least three replicates.

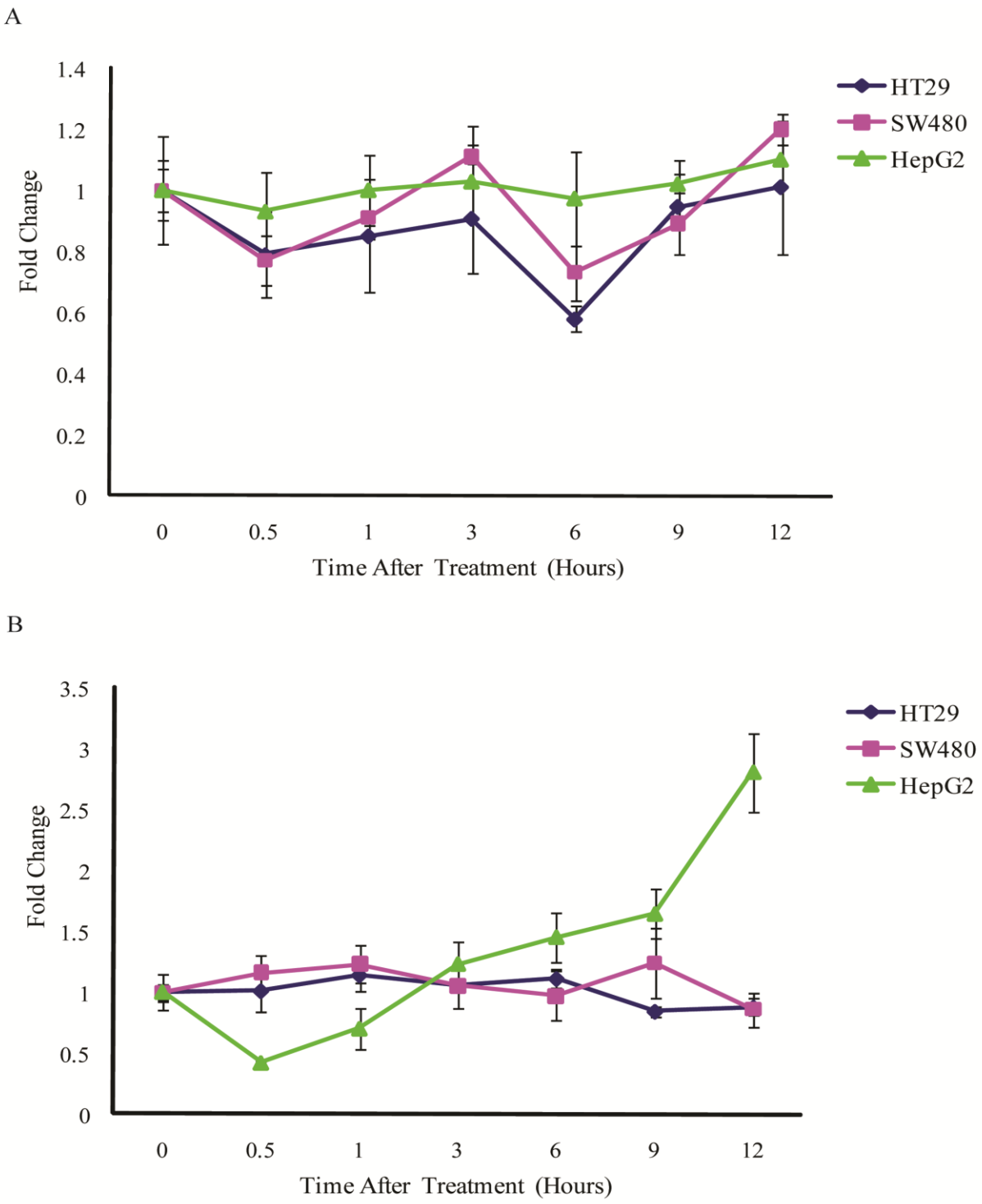


Figure 4.15: Short c-Src mRNA transcripts following treatment with TSA and apicidin in the SW480, HT29, and HepG2 cell lines. Cells were treated with either (A) 1µM of TSA or (B) 2 µM apicidin for the indicated time periods. Following RNA isolation qRT-PCR was completed for c-Src mRNA. Data representative of at least four independent experiments. Error bars represent standard deviation of at least three replicates.

4.2.4 Effect of Calyculin A and Fostriecin on c-Src and Bcl-x_L mRNA Expression

To determine whether or not a protein phosphatase was involved in the repression following TSA/apicidin treatments, the effect of the protein phosphatase inhibitor, Calyculin A was examined. The effect of Calyculin A, a PP1 and PP2A inhibitor was examined on c-Src and Bcl-x_L mRNA transcription alone, as well as in combination with TSA and apicidin treatment (Figure 4.16, 4.17).

The addition of Calyculin A to Colo201 cells treated with either TSA or apicidin led to a complete block in the previously observed repression of c-Src and Bcl-x_L mRNA. Further, an induction of mRNA was seen following treatment with the TSA/Calyculin A combination (Figure 4.16). The block in repression occurred within half an hour of the Calyculin A addition. Treatment of the Colo201 cells with Calyculin A alone also led to an induction of the c-Src and Bcl-x_L mRNA levels, but at later time points than seen with the TSA/Calyculin A combination. The effect of Calyculin A was also examined in the T47D cell line. The combination of the HDACi/Calyculin A treatment was extremely toxic to the T47D cells and studies could only be completed for a one hour period. Despite having a shorter time course study the trend seen in the T47D cell line does mirror that seen in the Colo201 with a lower level of induction being observed. Calyculin A appeared to be blocking the repression previously seen with both TSA and apicidin in both the Colo201 and T47D cell lines (Figure 4.16, 4.17). At the final time point, one hour, the expression of both c-Src and Bcl-x_L mRNA was back to that of control levels. Treatments involving only Calyculin A led to an increase in expression of both c-Src and Bcl-x_L mRNA (Figure 4.16, 4.17). The effects seen with TSA and apicidin were again compared to the effects of MS-275 and MGCD0103, which did not repress c-Src or Bcl-x_L mRNA, in the presence of Calyculin A in the Colo201 cell line (Figure 4.18). Following the addition of Calyculin A the level of c-Src and Bcl-x_L mRNA increased to a higher level that seen with the HDACi treatments alone, but lower than the treatment with only Calyculin A.

A second PP inhibitor, Fostriecin, was also examined to determine if it had a similar effect to what was seen with Calyculin A. Fostriecin is reported to be specific against PP2A and PP4 (Honkanen and Golden, 2002). As before the effect of Fostriecin on TSA and apicidin responses in the Colo201 and T47D cell line was examined (Figure 4.19, 4.20). In Colo201 cells Fostriecin was found to also block the repression of the c-Src and Bcl-xL mRNA

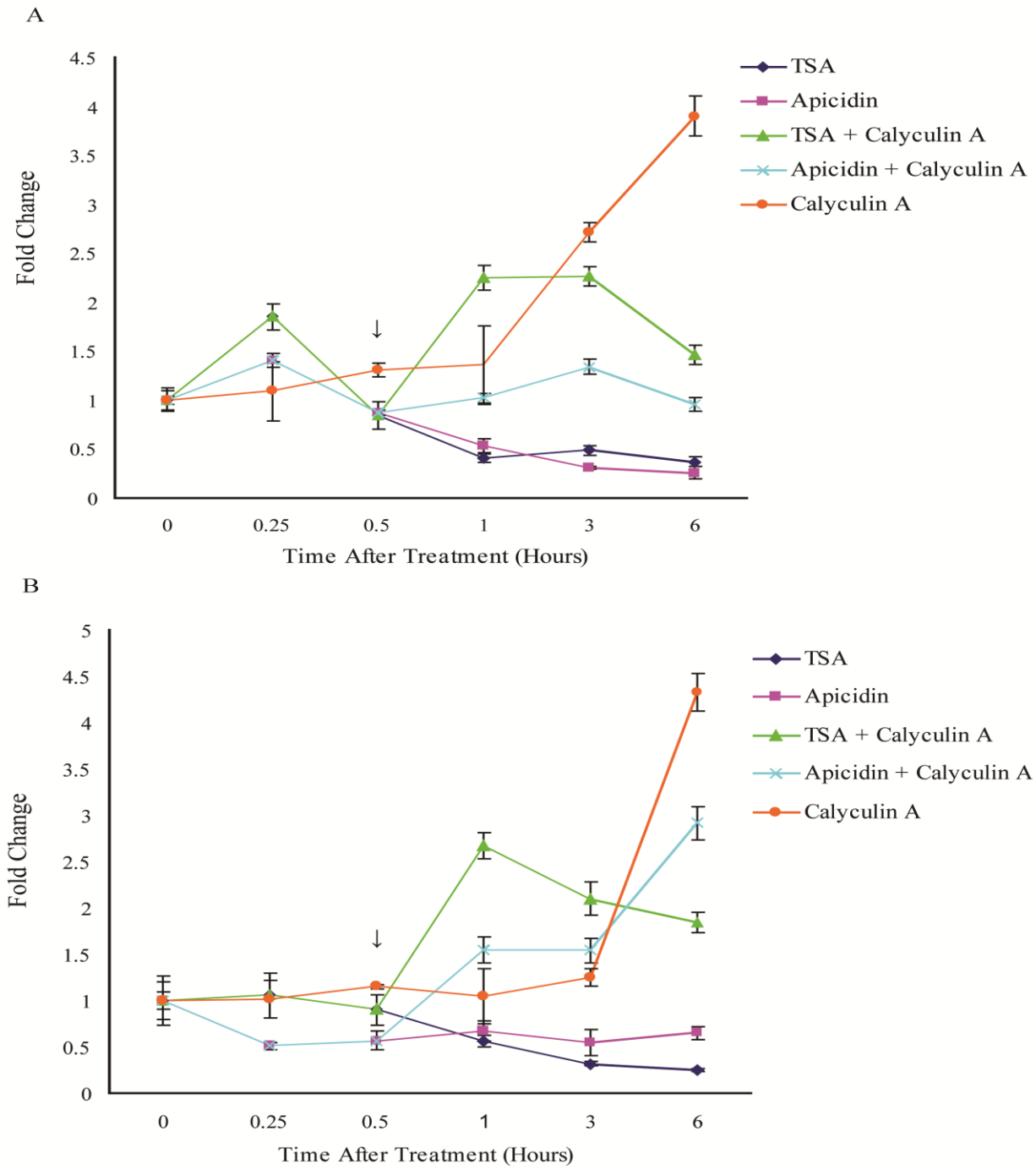


Figure 4.16: Expression of (A) c-Src and (B) Bcl-xL mRNA following treatment with TSA, apicidin, and Calyculin A in the Colo201 cell line. Colo201 cells were treated with either 1 µM TSA or 2 µM apicidin for the indicated time periods. Calyculin A was introduced to some of the cells at the half hour time point shown by the arrow at a concentration of 50 nM. Following RNA isolation qRT-PCR was completed. Data representative of at least two independent experiments. Error bars represent standard deviation of at least three different replicates.

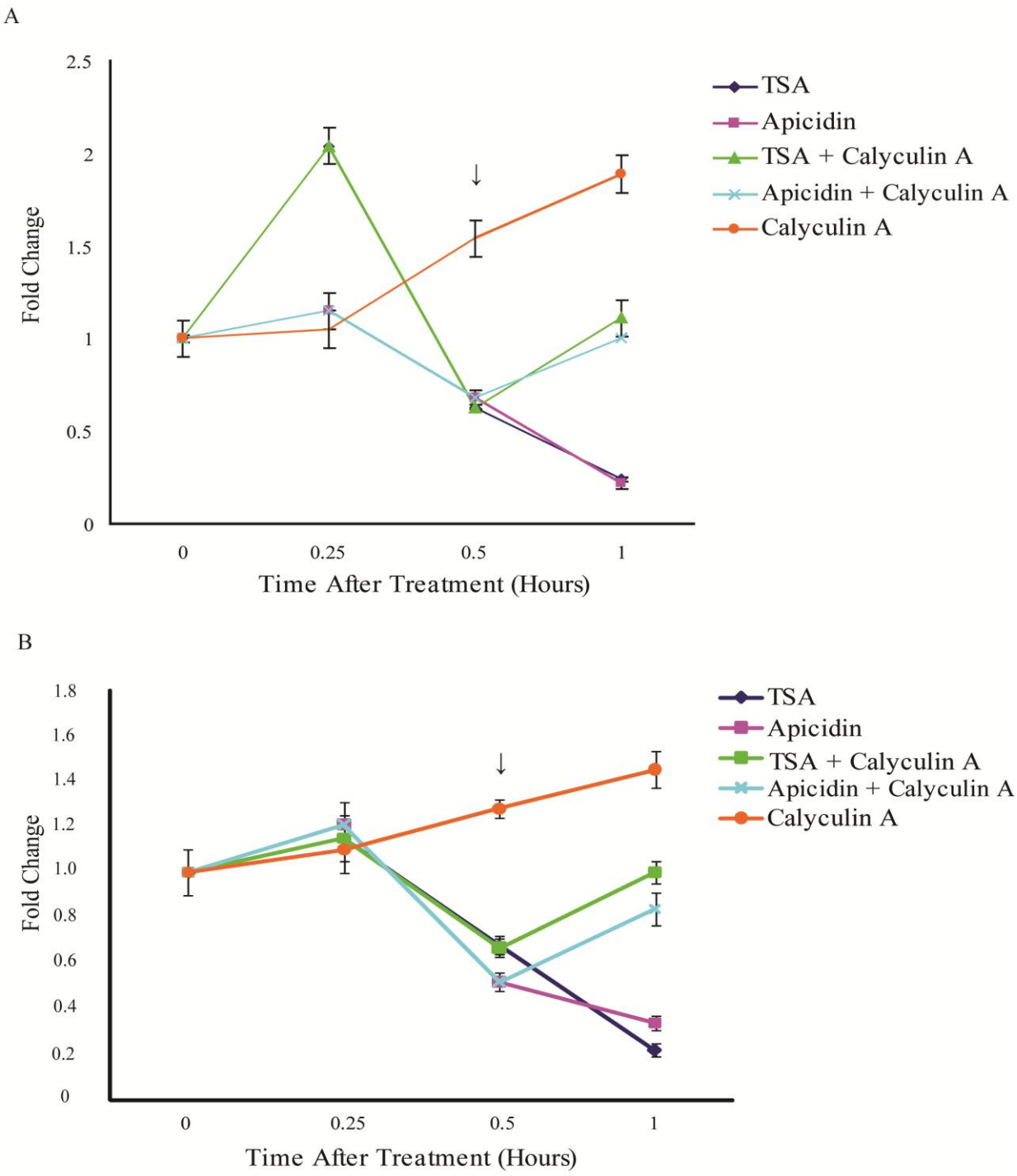


Figure 4.17: Expression of (A) c-Src and (B) Bcl-x_L mRNA following treatment with TSA, apicidin and Calyculin A in the T47D cell line. T47D cells were treated with either 1 µM TSA or 2 µM Apicidin for the indicated time points. Calyculin A was introduced to some of the cells at the half hour time point shown by the arrow at a concentration of 50 nM. Following RNA collection Real Time PCR was completed. Data representative of at least two independent experiments. Error bars represent standard deviation of at least three different replicates.

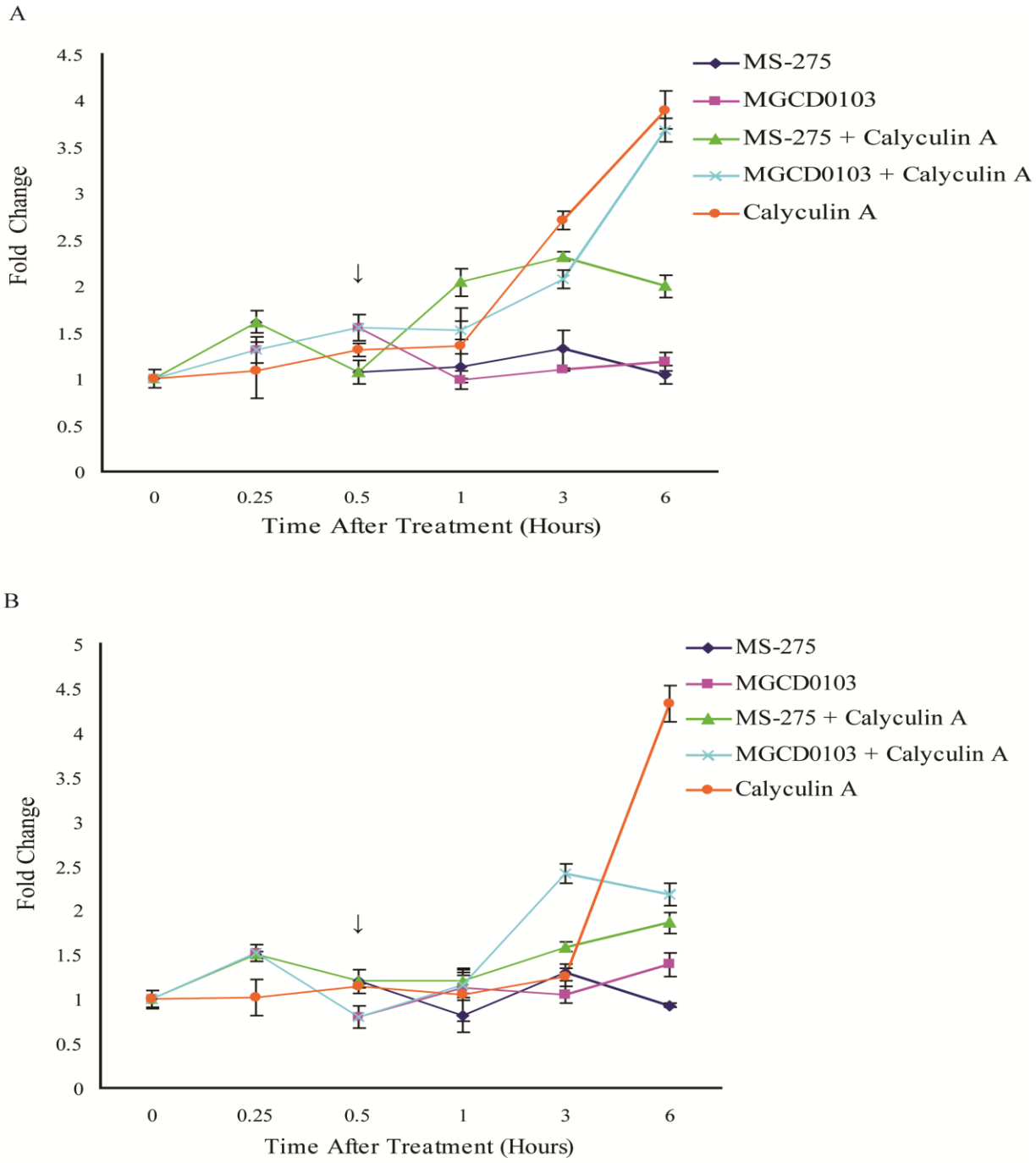


Figure 4.18: Expression of (A) SRC and (B) Bcl-x_L following treatments with MS-275, MGCD0103, and Calyculin A. Colo201 cells were treated with either 1 µM MGCD0103 or 2 µM MS-275 for the indicated time periods. Calyculin A was introduced to some of the cells at the half hour time point shown by the arrow at a concentration of 50 nM. Following RNA isolation qRT-PCR was completed. Data representative of at least two independent experiments. Error bars represent standard deviation of at least three different replicates.

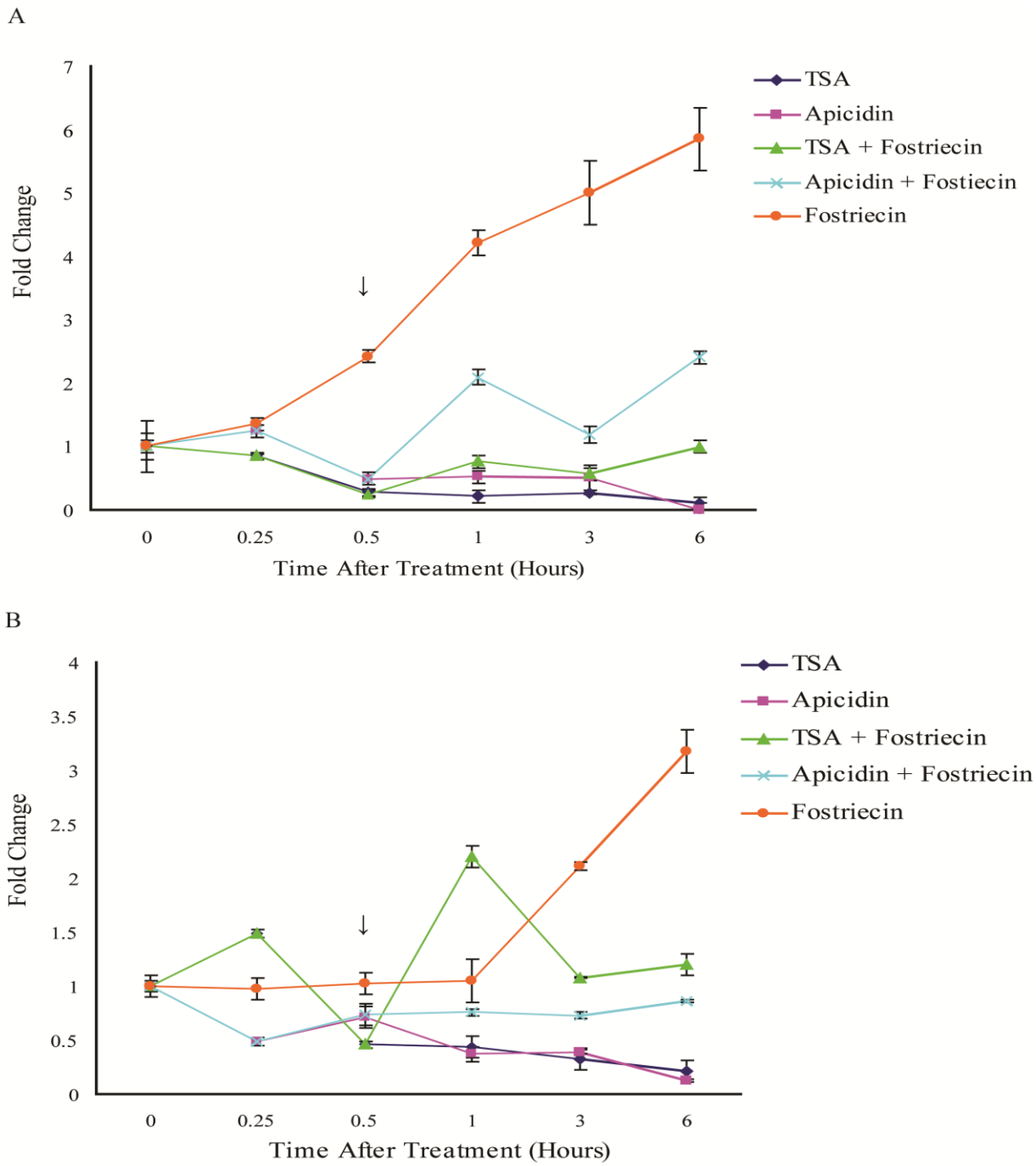


Figure 4.19: Expression of (A) c-Src and (B) Bcl-x_L mRNA following treatment with TSA, apicidin, and Fostriecin in Colo201 cell line. Colo201 cells were treated with either 1 µM TSA or 2 µM apicidin for the indicated time periods. Fostriecin was introduced to some of the cells at the half hour time point shown by the arrow at a concentration of 50 nM. Following RNA isolation qRT-PCR was completed. Data representative of at least two independent experiments. Error bars represent standard deviation of at least three different replicates.

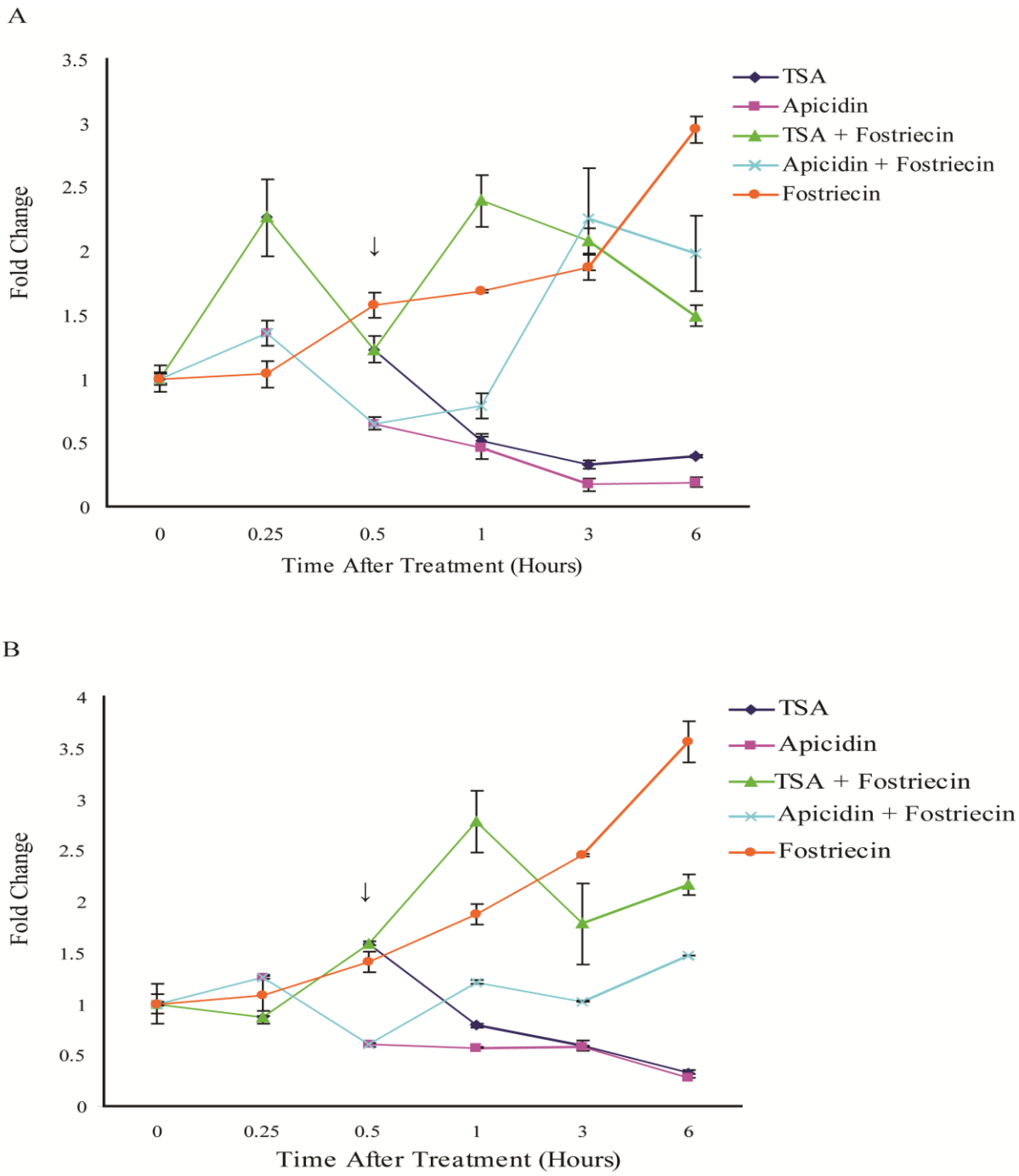


Figure 4.20: Expression of (A) c-Src and (B) Bcl-x_L mRNA following TSA, apicidin and Fostriecin in the T47D cell line. T47D cells were treated with either 1 µM TSA or 2 µM apicidin for the indicated time periods. Fostriecin was introduced to some of the cells at the half hour time point shown by the arrow at a concentration of 50 nM. Following RNA isolation qRT-PCR was completed. Data representative of at least two independent experiments. Error bars represent standard deviation of at least three different replicates.

following treatment with TSA and apicidin (Figure 4.19 A and B). Fostriecin not only blocked the previously observed repression, but like Calyculin A, actually led to an induction of both genes with certain HDACi treatments. Fostriecin treatments alone led to the induction of both the c-Src and Bcl-x_L mRNA. Results in the T47D cell line supported the data collected in the Colo201 cell line. Fostriecin was found to again block the repression normally observed with both TSA and apicidin (Figure 4.20 A and B). Fostriecin not only blocked the repression allowing expression levels to return to that of the control state, but also lead to their induction.

4.2.5 Effect of Calyculin A on RNA Polymerase II Occupancy at the SRC Gene

The effect of HDACi and Calyculin A on RNA Pol II occupancy was next examined at the SRC 3' region using a ChIP approach. Colo201 cells were treated with either MS-275 or TSA with Calyculin A being added half an hour after the addition of the HDACi. Following the addition of Calyculin A, to cells treated with either TSA or MS-275, the level of RNA Pol II occupancy increased in the 3' region of the SRC gene (Figure 4.21). The addition of Calyculin A reversed the loss of occupancy previously observed following TSA treatment, with occupancy returning close to that of the control (Figure 4.21). Treatments with MS- 275 alone did not lead to a loss of RNA Pol II occupancy in the 3' region, as previously observed, but upon the addition of Calyculin A the level of occupancy increased even further with levels reaching seven fold that of the control samples.

4.2.6 Effect of Calyculin A and Histone Deacetylase Inhibitors on Akt Phosphorylation

Results of studies presented in this thesis suggest that class I specific HDACi have differential effects on c-Src and Bcl-x_L mRNA expression. The mechanism behind the observed mRNA repression appears to be potentially related to promoter proximal pausing as supported by short c-Src mRNA transcripts remaining unaffected by HDACi treatments which lead to repression of the full length transcript. Further support for the involvement of promoter proximal pausing involves the finding of Calyculin A and Fostriecin blocking the previously observed repression. This finding is significant as promoter proximal pausing is known to be regulated through phosphorylation. The PP experiments are further supported by work

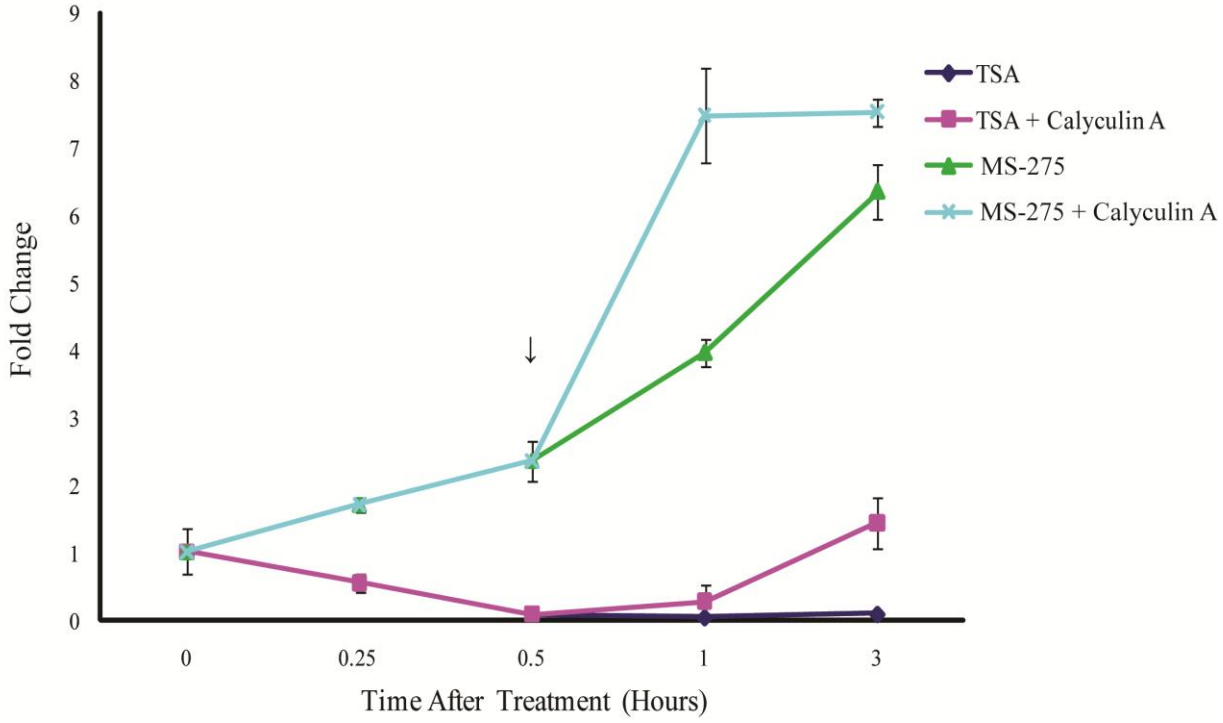


Figure 4.21: RNA Polymerase II occupancy at the SRC 3' region following addition of Calyculin A. Colo201 cells were treated with either 1 µM TSA or 2 µM MS-275 for the indicated time periods. Calyculin A was introduced to some of the cells at the half an hour time point shown by the arrow at a concentration of 50 nM. Following cell collection Chromatin Immunoprecipitation assays were completed using an anti-RNA Pol II antibody. Data representative of at least two independent experiments. Error bars represent standard deviation of at least three different replicates.

completed by Chen and colleagues which showed that a PP is released from a HDAC/PP complex following treatment with TSA, but not following treatment with MS-275 (Chen *et al.*, 2005). In these studies it was also reported that the PP released acts on the Akt protein within 48 hours of being liberated from the HDAC/PP complex (Chen *et al.*, 2005). Although the data presented here is, in general, consistent with that of Chen *et al.* (1995), the timing of the effects observed following putative release of PP is different. In the work completed by Chen and colleagues, the released PP acted on the Akt protein in 48 hours, while in studies presented in this thesis, affects of the PP can be seen as early as fifteen minutes. However the ultimate substrates examined are different. Therefore, the level of Akt phosphorylation was examined following various HDACi treatment as a method to monitor the activity of PP in the cell. The following Western blots examining the level of Akt phosphorylation were completed by an undergraduate student, Robert Laprairie, working under my supervision. The level of Akt phosphorylation was examined in the Colo201 cell line following treatment with all four of the HDACi, the two PP inhibitors, as well as HDACi and PP inhibitors in combination.

The level of Akt protein, assessed visually, was unchanged following treatment with all four of the HDACi tested (Figure 4.22 A-D). In contrast, the level of Ser 473 phosphorylation of Akt (p-Akt), a modification necessary for Akt activity, was found to decrease following treatment with TSA and apicidin (Figure 4.22 A-B). It is very interesting to note that the level of Ser473 decreases within fifteen minutes of HDACi treatment. This is the same time frame that was required for both TSA and apicidin to represses c-Src and Bcl-x_L mRNA expression (Figures 4.1, 4.3), as well as the time required for Calyculin A and Fostriecin to block the repression normally seen with TSA and apicidin (Figure 4.16, 4.17, 4.19, 4.20). Cells which were treated with MS-275 showed a slight decrease of p-Akt at the fifteen minute mark with levels returning to that of the control and increasing by one hour (Figure 4.22 C). Therefore following MS-275 treatment there is actually a transient increase in p-Akt levels. The level of p-Akt also increased following treatment with MGCD0103 (Figure 4.22 D). Unlike MS-275 there was no transient decrease in p-Akt levels.

Colo201 cells were also treated with Calyculin A and Fostriecin alone to determine what effect if any they may have on Akt phosphorylation. Calyculin A led to an increase in the level of p-Akt, while the level of Akt remained consistent across the time course (Figure 4.23 A). This suggests that either PP1 or PP2A alters the level of Akt phosphorylation.

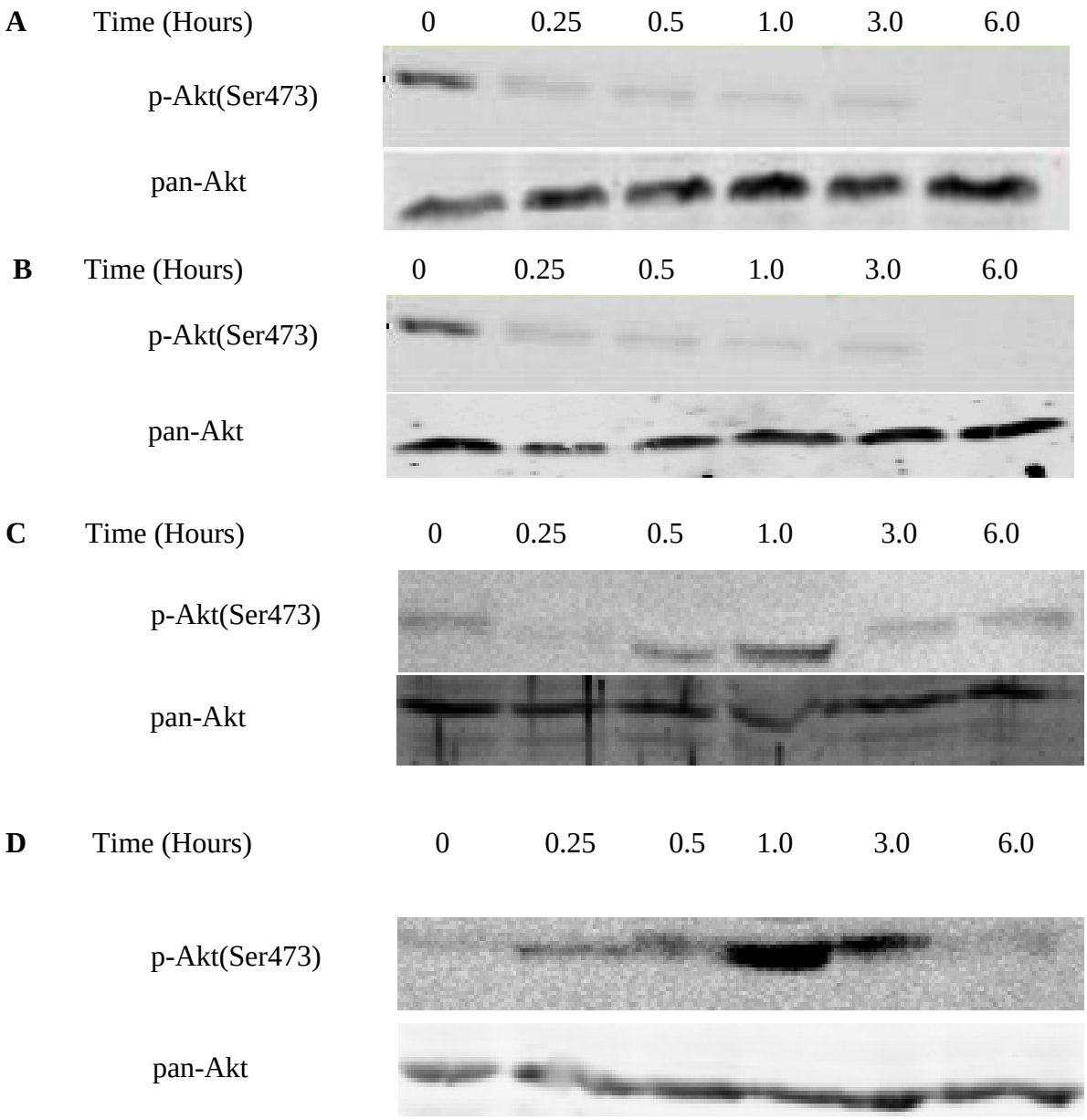
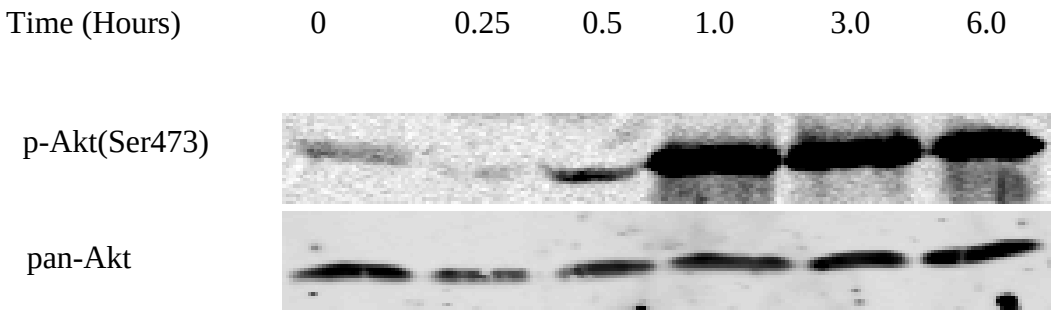


Figure 4.22: Akt Ser473 phosphorylation following treatment with (A) TSA (B) apicidin, (C) MS-275 and (D) MGCD0103. Colo201 cells were treated with either 1 μM of TSA or MGCD0103 or 2 μM apicidin or MS-275 for the indicated time points. Following cell collection Western blotting was performed using an anti-Akt and anti-phosphoSer473Akt antibodies. Data representative of two independent experiments.

A



B

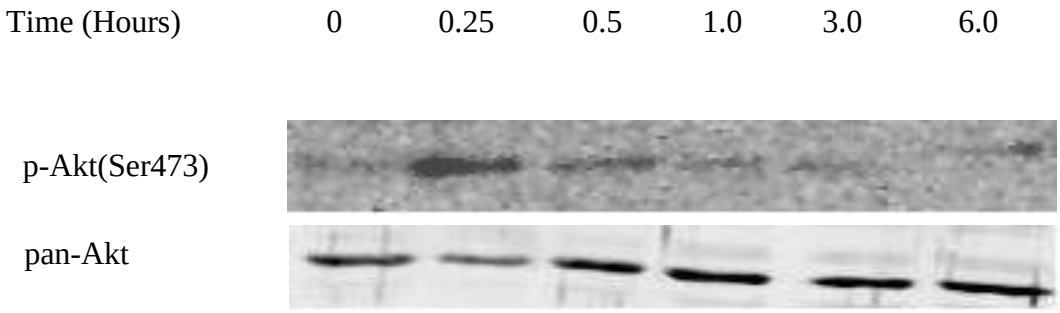
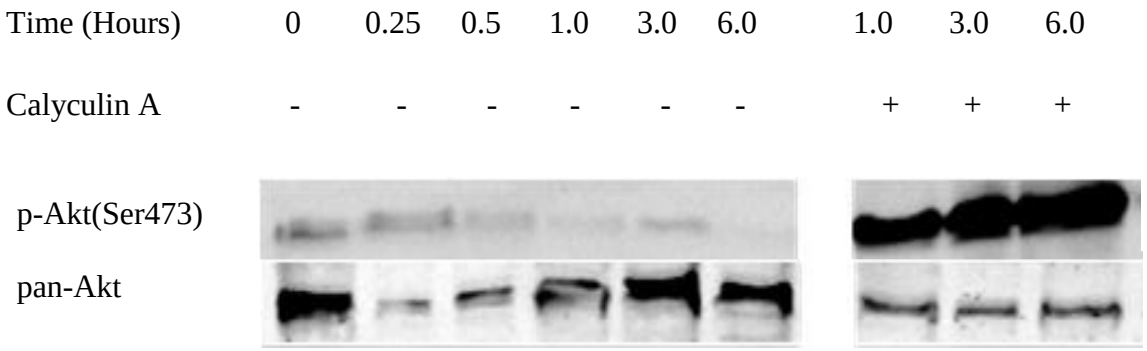


Figure 4.23: Akt Ser473 phosphorylation following treatment with (A) Calyculin A or (B) Fostriecin. Colo201 cells were treated with 50 nM of either Calyculin A or Fostriecin beginning at the 30 minutes time point. Following cell collection Western blotting was performed using an anti-Akt and anti-phosphoSer473Akt antibodies. Data representative of at least two independent experiments.

Surprisingly, Fostriecin alone had little effect on the level of Akt phosphorylation (Figure 4.23 B) suggesting that the PP inhibited by Fostriecin do not target Akt.

The effect of the TSA and apicidin in combination with the two PP inhibitors was then examined in the Colo201 cell line. Treatments with TSA and apicidin both led to a decrease in the p-Akt as previously seen, but following the addition of Calyculin A the level of phosphorylation increased markedly in both treatment groups (Figure 4.24 A and B). When Calyculin A was replaced with Fostriecin, although a decrease in the level of p-Akt was again observed with both TSA and apicidin alone, no increase in p-Akt was observed following the PP inhibitor addition (Figure 4.25A and B). Therefore although both PP inhibitors are capable of blocking c-Src and Bcl-x_L mRNA repression, it appears that only Calyculin A is capable of preventing the loss of Akt phosphorylation.

A



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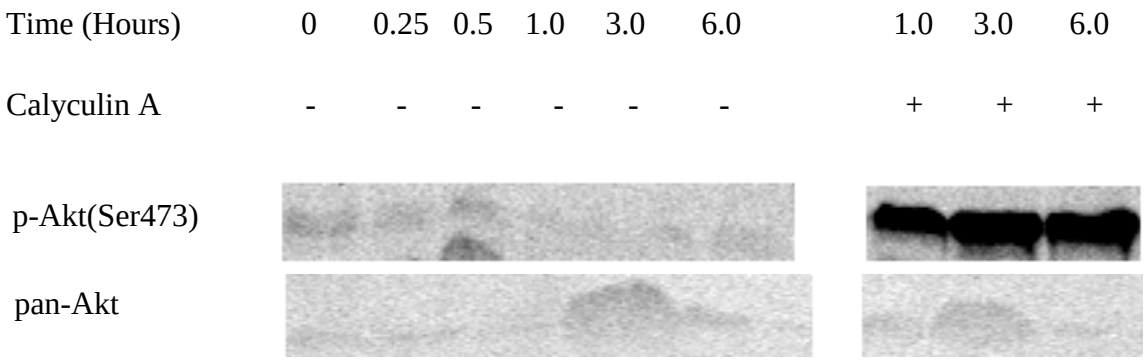
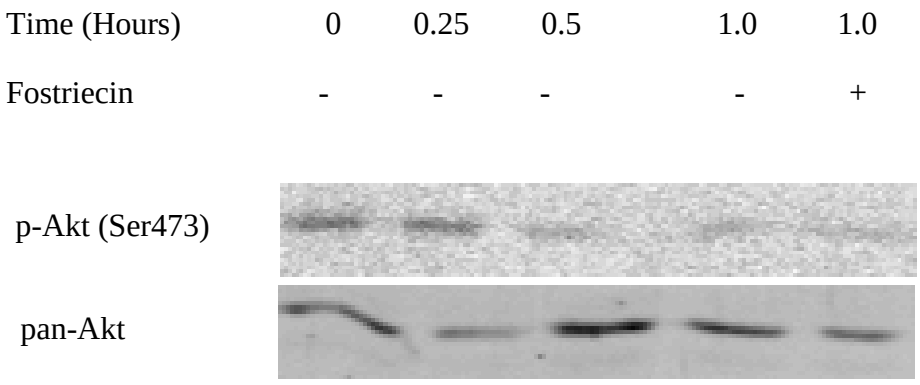


Figure 4.24: Akt Ser473 phosphorylation following treatment with TSA or apicidin with Calyculin A. Colo201 cells were treated with either 1 µM of (A) TSA or 2 µM (B) apicidin for the indicated time points. Calyculin A was added at the half an hour time point to some of the cells at a concentration of 50 nM. Following cell collection Western blotting was performed using an anti-Akt and anti-phosphoSer473Akt antibodies. Data representative of at least two independent experiments.

A



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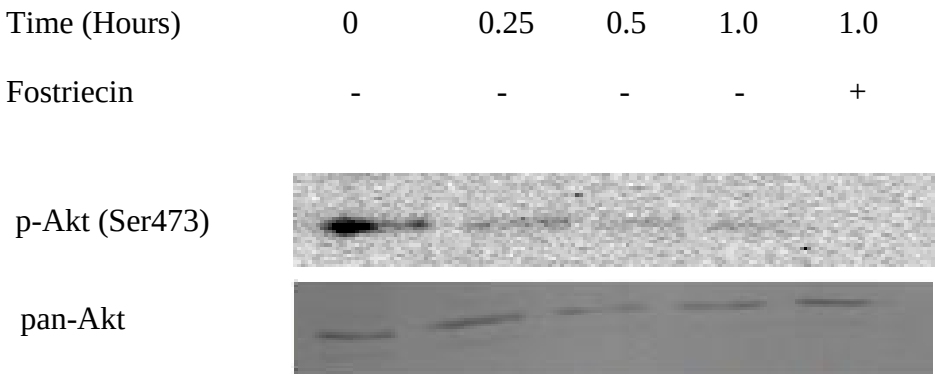

Figure 4.25: Akt Ser473 phosphorylation following treatment with TSA or apicidin and Fostriecin. Colo201 cells were treated with either 1 µM of (A) TSA or 2 µM (B) apicidin for the indicated time points. Fostriecin was added at the half an hour time point to some of the cells at a concentration of 50 nM. Following cell collection Western blotting was performed using an anti-Akt and anti-phosphoSer473Akt antibodies. Data representative of at least two independent experiments.

5. DISCUSSION

Previous work completed in the Bonham lab has shown that the repression of c-Src mRNA following HDACi treatment was a direct effect occurring at both of the SRC promoters (Kostyniuk *et al.,* 2002). In addition to the direct transcriptional repression, the Bonham lab had shown that histone acetylation as well as RNA Pol II occupancy increases in the promoter region following HDACi treatment, while RNA Pol II occupancy decreases in the distal regions of the gene (Ellis, 2007). In addition to examining the effect of HDACi on c-Src mRNA, the Bcl-x_L locus was also examined. Bcl-x_L has been shown throughout the literature to also be down regulated following HDACi treatment, and shares common promoter elements with SRC, both of which lack a TATA box but contain Inr elements (Fujio *et al.,* 1997; Adams and Cory, 1998; Chen *et al.,* 2000; Haiji *et al.,* 2008). In an attempt to determine whether the observed repression was dependent on inhibition of a particular HDAC, a variety of HDACi with varying class I specificities were examined.

5.1 Inhibition of HDAC3 may play a role in the Histone Deacetylase Inhibitor Mediated Repression of c-Src and Bcl-x_L mRNA

The mechanism by which HDACi act to reprogram gene expression is currently unknown. Microarray data has shown that between 2 and 20% of genes are affected by HDACi, and that of the genes affected an equal number are induced as are repressed (Bolden *et al.,* 2006; Dokmanovic *et al.,* 2007; Marks and Xu, 2009). Previous studies completed in the Bonham lab using HDACi and cyclohexamide, which blocks protein translation, have shown that the repression seen following TSA and apicidin treatment occurs through a direct effect on the SRC and BCL2L1 genes (Bonham, personal communication). Therefore whether there was selectivity for which HDAC must be inhibited for the observed repression to occur was examined.

The studies presented here suggest that HDAC3 may be playing an important role in the HDACi mediated repression of c-Src and Bcl-x_L mRNA. This conclusion is drawn from the fact that the HDACs inhibited by both TSA and apicidin, includes HDAC3, and both HDACi led to the repression of c-Src and Bcl-x_L mRNA.

In contrast, MS-275 and MGCD0103, which do not target HDAC3, were unable to repress mRNA levels. In addition to repressing the mRNA level of the genes examined, TSA and apicidin were also found to lead to a large induction of $p21^{WAF1}$ mRNA as was expected, indicating that HDAC inhibition is occurring. In comparison, MS-275 and MGCD0103 did not lead to the large induction of $p21^{WAF1}$ mRNA that is expected of HDAC inhibitors.

The lack of the expected induction of $p21^{WAF1}$ mRNA by MS-275 and MGCD0103 suggested minimal HDAC inhibition and that a larger dose of the two synthetic inhibitors may be required. Although a larger induction of $p21^{WAF1}$ mRNA was seen at higher concentrations, no repression was observed in either c-Src or Bcl-x_L mRNA. Therefore the lack of repression was not due to a dosage problem, but the two synthetic inhibitors were simply unable to repress c-Src and Bcl-x_L mRNA; lending further support to the hypothesis that these two inhibitors are unable to repress the examined genes due to their HDAC specificities.

When comparing the levels of induction and repression observed with the tested HDACi a large amount of variability was seen. This variability could be attributed to both cell line and gene variability. Each gene may in fact contain its own combination of HATs and HDACs in the promoter regions. The same can be said for the same gene when comparing cell line to cell line. Therefore, a varying combination of HATs and HDACs at the promoter region could account for the variability in the level of repression and induction observed throughout the above experiments. To further confirm the HDACi were functioning as expected within the cell, the level of histone acetylation following the four HDACi treatments was next examined.

5.2 Histone Deacetylase Inhibitors Lead to an Increase in Promoter Histone Acetylation

HDACi are generally thought to act by disrupting the balance of acetylation of histone and non histone targets in the cell leading to changes in gene expression. However work presented in this thesis has revealed a differential effect of some of these inhibitors on SRC and BCL2L1 expression. Therefore the effect of these drugs on SRC and BCL2L1 promoter histone acetylation was examined. Increased histone acetylation at the promoters of the SRC and BCL2L1 genes was seen following treatment with all four HDACi (Figures 4.7, 4.8, 4.9, 4.10), despite the fact the full length c-Src and Bcl-x_L mRNA transcripts were repressed by treatment with TSA and apicidin (Figures 4.1, 4.3). This indicates that despite the effect of HDACi on

gene expression, all HDACi do in fact disrupt the balance between HDAC and HATs, leading to the increased promoter histone acetylation observed. This lends support to the idea that the mechanism(s) behind HDACi actions in the cell are much more complex than altered acetylation levels, and further investigation into the mechanism behind this class of drugs is required.

5.3 Differential Effect of Histone Deacetylase Inhibitors on RNA Polymerase II Occupancy

While all HDACi examined were found to have a similar effect on promoter histone acetylation, only TSA and apicidin lead to a decrease in c-Src and Bcl-x_L mRNA expression. To differentiate the mechanism behind the different HDACi, the level of RNA Pol II occupancy was examined in the promoter regions of SRC and BCL2L1, as well as in the 3' regions of both genes. Results of the RNA Pol II occupancy studies revealed that levels remained stable or increased in the promoter regions of the genes examined (Figures 4.11, 4.12, 4.13). This finding, along with result of the histone acetylation studies, suggests that an increase in gene expression should be seen. Since this is not the case, the mechanism of repression must be occurring outside of the promoter region, as effects in this region favour transcription.

Further support for a mechanism of repression occurring outside the promoter region can be seen in the results of the RNA Pol II occupancy experiments looking at the 3' regions of the two genes. RNA Pol II occupancy remains in the promoter region, but is lost rapidly in the 3' regions, suggesting that somewhere between the promoter and the 3' region something is occurring leading to the loss of RNA Pol II, and subsequent mRNA repression. This concept is further supported by the finding of TSA leading to both a loss of RNA Pol II occupancy in the 3' regions as well as repression of c-Src and Bcl-x_L mRNA, while with MS-275, which was unable to repress c-Src and Bcl-x_L mRNA, no loss of RNA Pol II occupancy was observed in the 3' region. Thus, TSA and apicidin appear to be acting through an additional effect, which leads to the loss of downstream RNA Pol II occupancy and subsequent gene repression. MS-275 and MGCD0103 do not act through this additional effect, and therefore no gene repression is seen. The question then becomes how are TSA and apicidin leading to the loss of RNA Pol

II occupancy? Two possible causes of this loss include a block in productive elongation, preventing RNA Pol II from reaching the 3' region, and premature termination. Either one of these two situations would explain the maintained or increase RNA Pol II occupancy found in the promoter region, as well as the loss of occupancy in the 3' region. If the mechanism behind the loss of RNA Pol II occupancy involved premature termination, TSA and apicidin may be leading to the dissociation of RNA Pol II from the SRC and BCL2L1 genes. Premature termination has been shown to occur independent of the 3'poly A tail sequence, but only in yeast (Chakraborty *et al.*, 2002).

Based upon the lack of evidence outside of yeast supporting premature termination as the mechanism behind the RNA Pol II occupancy loss, the more likely explanation may involve a block in productive elongation. The finding of short c-Src mRNA transcripts being unaffected by HDACi treatment (Figures 4.14, 4.15) which lead to the repression of the full length mRNA (Figures 4.1, 4.3) lends further support to the mechanism involving a block in productive elongation. When RNA Pol II arrests, short transcripts that have been produced are rapidly degraded, but when there is a block in productive elongation, such as through promoter proximal pausing; short transcripts remain in the cell and are not degraded (Adelman *et al.*, 2009; Fujita and Schlegel, 2010). Promoter proximal pausing has been shown to occur at multiple gene loci in the human genome, especially that of early response genes, as a mechanism to allow for quick induction of gene expression (Fujita and Schlegel, 2010). It is plausible, that this system could also work in the reverse; taking an active gene and converting it back to a paused state. Further support for the involvement of promoter proximal pausing, comes from the fact that RNA Pol II is normally found to be stalled anywhere between the +20 and +100 range (Yamaguchi *et al.*, 1999; Sims *et al.*, 2004; Saunders *et al.*, 2006; Nechaev *et al.*, 2011). Therefore the continued production of short 100 nucleotide c-Src transcripts does fit with the model of SRC repression through promoter proximal pausing. Further support for a mechanism involving promoter proximal pausing can also be found in the results of the PP inhibitor experiments.

5.4 Repression of c-Src and Bcl-x_L mRNA Following Histone Deacetylase Inhibitor Treatment may be Controlled by Phosphorylation

Results of experiments completed in this thesis have not only revealed a much more complicated HDACi mediated mechanism of mRNA repression, but have also suggested that the mechanism may not be dependent on the HDAC inhibitors specificity. Experiments completed with PP inhibitors (Calyculin A and Fostriecin) suggest that the mechanism may involve phosphorylation, as the addition of the PP inhibitor was able to block the observed gene repression and loss of RNA Pol II occupancy in the 3' region of the genes (Figure 4.16, 4.17, 4.19, 4.20, 4.21). Work by Chen and colleagues (2005) has shown that TSA, but not MS-275, is able to disrupt an HDAC/PP complex leading to the release of a free PP. This finding as well as results from experiments in this thesis lends support to a new hypothesis involving TSA and apicidin leading to the disruption of an HDAC/PP complex leading to the release of a free PP. The released PP acts on various factors leading to a paused RNA Pol II possibly through promoter proximal pausing.

Factors important in promoter proximal pausing such as NELF and DSIF are known to be regulated through phosphorylation (Wade *et al.*, 1998; Ivanov *et al.*, 2000). Therefore, through the activation of the PP, NELF may become dephosphorylated, allowing it to bind to the promoter regions, where as in the phosphorylated state it cannot. The removal of a phosphate from DSIF has also been shown to switch DSIF from a positive elongation factor to a negative one (Zhu *et al.*, 2007). Therefore if a PP is released by certain HDACi treatments, it could act on regulatory factors such as DSIF and NELF, converting RNA Pol II to a paused state. The paused RNA Pol II would then only produce short transcripts and would not be able to move into full productive elongation. Therefore a decrease in RNA Pol II occupancy at the 3' region of affected genes would be observed. MS-275 and MGCD0103 are unable to disrupt the HDAC/PP complex and therefore RNA Pol II remains active. This new hypothesis also suggests that despite the four HDACi having overlapping specificities it is not their ability to inhibit a specific HDAC that is important, but their ability to disrupt an HDAC/PP complex. This new hypothesis also presents the idea that the gene repression may be reversible, and that phosphorylation and not acetylation may play a role in HDACi mediated mRNA repression.

To further determine the possible role of a PP, the level of Akt phosphorylation was examined as a marker of PP activity within the cell. Treatments with TSA and apicidin alone

both led to a decrease in the level of Ser 473 phosphorylation, supporting the idea of increased PP activity following the two treatments. The level of Ser473 phosphorylation was unaffected or even increased slightly after treatment with MS-275 and MGCD0103, consistent with only certain HDACi activating PP. Calyculin A alone, led to an increase in the level of Ser 473 phosphorylation, and also blocked the loss of Ser 473 phosphorylation when combined with TSA or apicidin (Figures 4.23, 4.24). Treatments with Fostriecin however, did not lead to an increase in Ser473 phosphorylation either alone (Figure 4.23) or in combination with either TSA or apicidin (Figure 4.25). This was unexpected since Fostriecin was able to block the repression of c-Src and Bcl-x_L mRNA following HDACi treatment (Figures 4.19, 4.20). Therefore, it may be that Fostriecin is capable of inhibiting the PP activated by TSA and apicidin treatment allowing it to block the repression seen at the SRC and BCL2L1 genes; but the phosphorylation of Akt at Ser473 is not Fostriecin sensitive. Both PP1 and PP2A are dependent on the recruitment of a regulatory subunit for their activity and subcellular location (Shi, 2009). Therefore it may be that the activated PP, be it PP1 or PP2A, recruits different regulatory subunits to act at the promoters of SRC and BCL2L1, than to target Akt dephosphorylation. In such a case, Calyculin A is capable of inhibiting both forms of the activated PP, while Fostriecin may not be capable of inhibiting the PP bound to the regulatory subunits associated with Akt dephosphorylation.

In summary, the following model for HDACi mediated repression of the SRC and BCL2L1 genes is proposed. All four HDACi act through binding to one or more HDAC, supported by the fact that promoter histone acetylation as well as RNA Pol II occupancy remain near control levels or increases following HDACi treatment. Based upon these findings an increase in the target gene expression would be expected, and is in fact seen with the MS-275 and MGCD0103 treatments. However, TSA and apicidin interact with and are able to disrupt a putative HDAC/PP complex. Binding of these inhibitors releases an active Calyculin A/Fostriecin sensitive PP, through disruption of the HDAC/PP complex. This mechanism is supported by the findings that PP inhibitors are capable of blocking the loss of RNA Pol II occupancy from the 3' regions of genes and the subsequent mRNA repression. The PP released by TSA and apicidin treatments, acts directly or indirectly through an unknown effect, proposed to involve promoter proximal pausing. If promoter proximal pausing is involved, the released PP may be acting on regulatory factors such as NELF and DSIF. Dephosphorylation

of NELF will allow it to bind in the promoter region of the genes involved and associated with DSIF. On dephosphorylation, DSIF acts as a negative elongation factor, where as in its phosphorylated form it acts as a positive elongation factor (Hirose and Manley, 2000; Sims *et al.,* 2004; Zhuoyu, *et al.,* 2008; Brookes *et al.,* 2009). These two factors together bring about promoter proximal pausing making RNA Pol II incapable of productive elongation, and mRNA repression occurs. Under such a situation PP released following TSA/apicidin treatment will facilitate SRC and BCL2L1 genes conversion from an active transcribing state to a paused state, capable only of producing short transcripts (Figure 5.1).

5.5 Scope and Significance

HDACi represent a new group of chemotherapeutic agents, which have been shown to regulate the level of gene expression. Through the regulation of gene expression, HDACi prevent tumour growth, as well as lead to the induction of cell differentiation and apoptosis. HDACi are now in clinical use and have been shown to be very promising agents. It is therefore increasingly important to determine their mechanism of action in the cell. It was previously believed that HDACi functioned simply by increasing the level of histone acetylation within the cell, leading to an overall increase in gene expression. This thesis provides evidence that certain HDACi act through an additional effect, not directly related to HDACi inhibition, which leads to the repression of c-Src and Bcl-x_L mRNA. This additional effect has been shown to involve a phosphorylation event, which is thought to occur by HDACi mediated disruption of an HDAC/PP complex. The released PP acts through an unknown downstream effect, thought to involve promoter proximal pausing, resulting in gene repression. With the use of PP inhibitors, this repression can be reversed.

The finding of only certain HDACi having the ability to repress genes such as SRC and BCL2L1 may become clinically relevant. When comparing drug such as apicidin to MS-275, both currently in clinical trials, both are able to up-regulate genes such as p21WAF1 making them valuable clinical tools in cancer treatment. However, the ability of apicidin to also down regulate gene expression could be of additional clinical value. Cancer of the colon and breast have both been shown to not only over express the SRC gene, but the level of over expression

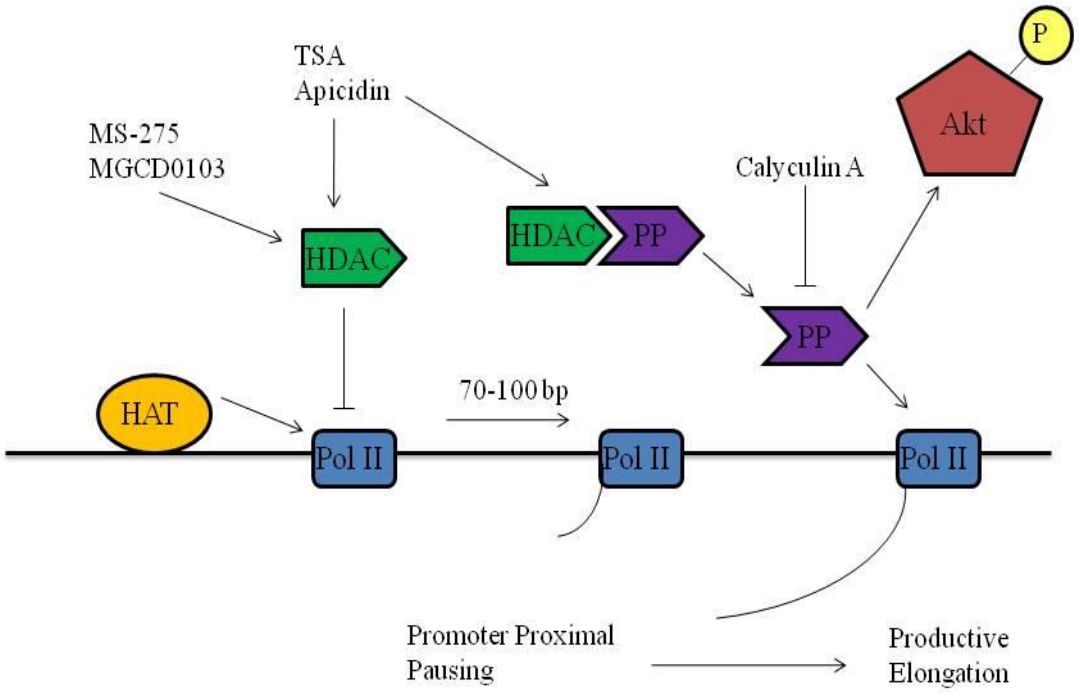

Figure 5.1: Proposed model of HDACi mediated repression at the SRC and BCL2L1 genes. TSA and apicidin have an additional effect which leads to the disruption of an HDAC/PP complex. This disruption leads to the release and activation of a phosphatase, which acts on RNA Polymerase II or some other factor leading to RNA Polymerase II becoming stalled on the genes. By blocking the active phosphatase with Calyculin A/Fostriecin RNA Polymerase II remains active and the repression is prevented rescuing expression of the genes.

has been shown to be an important prognostic indicator (Fleming *et al.*, 2004; Myers *et al.*, 2004; Lieu and Lopez, 2010). Therefore a drug such as apicidin may be of more value and lead to better clinical outcomes than MS-275, which has been shown to be unable to repress the SRC or BCL2L1 gene. The possible advantage that some HDACi may hold clinically over others makes research into the mechanism of action behind this additional effect even more crucial.

6. CONCLUSIONS AND FUTURE STUDIES

HDACi are a new group of chemotherapeutic agents which have received a great deal of attention due to their ability to stimulate cell cycle arrest, differentiation and apoptosis of cancerous cells, while leaving normal cells unaffected (Ma *et al.*, 2009). HDACi such as Vorinostat (Zolina, SAHA) have already entered the clinics, with many new inhibitors entering clinical trials being tested on their own or in combination with other approved treatments.

In this thesis, experiments have shown that only certain HDACi, including TSA and apicidin, are capable of decreasing expression of both the SRC and BCL2L1 genes in a variety of human tumor lines including colon, breast, and hepatic carcinoma cells. This repression has been previously shown to occur without new protein synthesis (Bonham, personal communication). Despite only certain HDACi being able to reduce gene expression, all four HDACi examined were shown to increase the acetylation of histones at the promoters of both the SRC and BCL2L1 genes. This suggests that all of the HDACi are inhibiting one or more HDAC, which is also supported by the increase of RNA Pol II occupancy at the promoter region of both of the genes. These finding suggest that, as previously proposed, an increase in gene expression should be seen. Such an effect was seen with MS-275 and MGCD0103 treatments.

The ability of certain HDACi to repress gene expression appears to be occurring through an additional effect. Based on the findings of histone acetylation and RNA Pol II occupancy in the promoter regions, it appears that these genes are originally active, but due to the second effect, the genes enter a paused state, allowing for gene repression to occur. This hypothesis is supported from the finding that despite RNA Pol II occupancy being maintained or increase in the promoter region, it is decreased in the 3' region following treatment with TSA/apicidin. Work with the phosphatase inhibitors Calyculin A and Fostriecin, appears to indicate that this additional action involves the activation of a phosphatase, and may be due to TSA/apicidin disrupting an HDAC/PP complex, facilitating the release and activation of a PP. Through an unknown direct or indirect effect, the released PP dephosphorylates key residues such as Ser2 and Ser5 on RNA Pol II. The PP may also act on transcription factors such as NELF or DSIF, allowing them to bind to target genes and stall RNA Pol II. Through one (or more) of these methods, the SRC and BCL2L1 genes are converted from an active to a paused state.

To further investigate this hypothesis Chromatin Immunoprecipitation assays could be performed to determine the presence of Ser2 and Ser 5 phosphorylation on the CTD domain of RNA Pol II before, as well as after, treatments with TSA and apicidin. These two modifications have been shown to be important for the transition of RNA Pol II from an initiation state to an active elongation state. Therefore identification of the presence or absence of these post translational modifications after TSA/apicidin treatment will help determine the mechanism of repression. siRNA studies could also be performed to determine which PP is necessary for the repression of SRC and BCL2L1 genes. siRNA studies should be first attempted with PP1 and PP2A which have been shown to be involved in HDAC/PP complexes. Theoretically when the necessary PP is knocked down, c-Src and Bcl-x_L mRNA expression should increase following TSA or apicidin treatments.

Chromatin Immunoprecipitation Assays should also be completed looking at the H3Ser10 residue. H3Ser10 phosphorylation has been shown to control the level of chromatin condensation and the level of gene transcription, and has been shown to be a target for both PP1 and PP2A (Gurley *et al.,* 1978; Goto *et al.,* 1999; Prigent and Dimitrov 2003; Kinney *et al.,* 2008). Therefore based on the role that phosphorylation appears to be playing in the HDACi mediated gene repression, this residue may help in determining the mechanism of repression.

To further investigate whether promoter proximal pausing is involved in this mechanism of repression, the presence or absence of NELF can be examined at both of the SRC and BCL2L1 promoters, both before and after treatment with TSA and apicidin. This could be determined using a Chromatin Immunoprecipitation approach. If in fact NELF is found to be present at the promoters after TSA/apicidin treatment but not before, it would suggest that promoter proximal pausing is involved, and that the activated PP may play a role in switching the genes to a paused state. The presence of NELF at a promoter is generally acceptable evidence that a gene is experiencing promoter proximal pausing. In further support of this idea the presence of DSIF in a phosphorylated or un-phosphorylated from could be determined using a Chromatin Immunoprecipitation approach before and after treatment with TSA and apicidin.

An attempt should also be made to try and determine the specificity of this mechanism of repression, to try and determine why it is seen at genes such as SRC and BCL2L1, but not

$p21^{WAF1}$. Promoter proximal pausing utilizes general transcription factors; therefore why only certain genes are targeted for promoter proximal pausing is of great interest. This specificity may in fact come from common elements within the promoter regions of the genes repressed. For example, both the SRC and BCL2L1 genes lack a TATA box, but contain an Initiator element. It has also been suggested that both promoter regions contain a GAGA element. Therefore, through the investigation of common promoter elements of repressed genes the mechanism of specificity may be elucidated.

The area of HDACi research has undergone a great deal of change in the past fifteen years and will continue to evolve due to their growing importance in the field of cancer. As HDACi gain momentum with more inhibitors being approved for clinical use determination of their mechanism of action becomes more and more important. Originally known for their anti-tumor effects by changing gene expression through histone hyperacetylation, this thesis reveals that HDACi are much more complex than originally thought. HDACi are anti-cancer compounds which act through multiple mechanisms to control gene regulation. These mechanisms ultimately work together to prevent tumor growth, as well as to facilitate cellular differentiation and apoptosis.

7. REFERENCES

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