A NOVEL NUCLEAR SRC AND P300 SIGNALING AXIS IN PANCREATIC CANCER

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ABSTRACT

Src is a non-receptor tyrosine kinase that has the distinction of being the first discovered oncogene. Its function at the cell membrane has been demonstrated in a wide array of cellular signaling pathways. Additionally, its role in promoting the oncogenic phenotype in a number of tumor types has been extensively researched, and its over activation is believed mainly to influence tumor migration and metastasis, as well as to a lesser extent survival and proliferation. Our lab has previously described a nuclear function of Src in which it associates with gene promoters and regulates their transcription. Indeed, nuclear localization of Src has long been observed, but its functional role has remained largely a mystery.

In this thesis the role and significance of nuclear Src in pancreatic cancer was characterized. In doing so, a novel mechanism was delineated by which Src in the nucleus modulates gene expression. Src functional nuclear activity was demonstrated by multiple independent approaches. Src association with p300 was also identified by mass spectrometry and validated by inverse co-immunoprecipitation. Putative Src target genes in the Panc-1 cell line were then defined by ChIP-array including HMGA2 and SMYD3. Small molecule inhibitors of Src and p300 resulted in a decrease in the transcription and protein expression of HMGA2 and SMYD3, indicating their regulation by Src and p300 enzymatic activity. Src and p300 inhibition both were found to regulate migration and invasion in Panc-1 cells, implicating their signaling in a major aspect of pancreatic cancer. Interestingly, these results were able to be replicated in a well-known series of mouse embryonic fibroblast (MEF) cell lines in which Src, and Src family kinase members Yes, and Fyn (SYF-/-) are knocked out or restored by over-expression of exogenous c-Src. The Src-dependent gene regulation and reliance on p300 was only observed in the Src-active (overexpressing) cell line.

Together, these results indicate a novel signaling modality of Src in pancreatic cancer or in cells over-expressing Src that reveal an epigenetic regulatory function in coordination with p300.
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CHAPTER I

INTRODUCTION

1.1 Pancreatic Cancer

1.1.1 Pathology

Pancreatic cancer represents one of the most lethal malignancies and the single most lethal common form of cancer [1]. Pancreatic cancer presents as either carcinoma of the exocrine or endocrine cells of the pancreas. Approximately 95% of pancreatic cancer is exocrine, 85% of which is ductal adenocarcinoma [1, 2]. As such, the term “pancreatic cancer” tends to be used synonymously with pancreatic ductal adenocarcinoma (PDAC). The five-year survival rate of ~6% is chiefly attributed to the aggressive nature of the disease and the late stage at which diagnosis occurs. This late diagnosis leads to most pancreatic cancer cases to be non-surgical, with resection only possible in roughly 15% of cases. Even in surgical cases, however, the five-year survival remains low at around 10% [3].

Pancreatic tumors are typically firm and sclerotic. The recruitment of stromal and immune cells produces such desmoplasia that a majority of the tumor mass is not even composed of neoplastic cells [4]. The extent of this desmoplastic reaction is so great that pancreatic tumors cannot recruit enough blood vessels and become poorly vascularized and hypoxic. The massive stromal influence and lack of adequate vascularization is a major obstacle to targeted and chemotherapy [5]. Strategies involving a modified hyaluronidase enzyme to digest the stroma have been employed to improve drug delivery, denoting a particularly important microenvironment-tumor interaction in pancreatic cancer [5, 6].

Pancreatic cancer derives from a progressive series of lesions called pancreatic intraepithelial neoplasia (PanIN) [4]. This nomenclature was defined and adopted in 2001 in order to standardize pathological grading of pancreatic tumors [7]. PanIN lesions range in grades from 1 to 3 and are accepted as the precursors to invasive carcinoma. PanIN-1 lesions can be detected in as many as 40% of adults without any malignant disease, whereas PanIN-3 lesions are detected in fewer than 5% of such pancreata [8]. Conversely, PanIN-3 lesions are detected in 30-50% of cases of invasive pancreatic carcinoma [8]. The linearity of the progression of PanIN lesions is a source of some debate. There is evidence that lesions of any grade can arise independently from the normal tissue and that early PanIN lesions are far more likely to undergo growth arrest than to progress further [9].
1.1.2 Risk Factors

There are few known preventable risk factors for pancreatic cancer and similarly few non-preventable risks, underscoring the lack of understanding of the disease. The major behavioral risks are tobacco use and obesity [2]. Cigarette smoking is by far the largest modifiable risk factor for pancreatic cancer, responsible for as many as one quarter of pancreatic cancer cases [10]. Smoking has a dose-dependent influence on risk; a moderate habit approximately doubles risk whereas heavy smokers are over three times as likely to develop pancreatic cancer [11]. Obesity is the other major known risk factor, though it only confers a roughly 20% increased risk of disease [12]. While obesity doesn’t directly add much risk, diabetes mellitus increases risk of pancreatic cancer by about twofold [13]. As diabetes is a metabolic disorder affecting pancreatic function, the connection between these diseases is unsurprising.

The non-preventable risk factors for pancreatic cancer are mainly age, chronic pancreatitis, family history, and genetic disorders [2]. Chronic pancreatitis is a strong risk factor, with a nearly threefold increased risk for patients previously diagnosed with chronic pancreatitis. The risk increases to thirteen times when the window between diagnoses of both diseases is limited to two years, indicating pancreatitis as a likely premalignant symptom of pancreatic cancer [14].

There are similarly few known genetic factors that predispose individuals to pancreatic cancer. As chronic pancreatitis is a major risk factor, so is hereditary pancreatitis. The disease is primarily caused by gain-of-function mutations in the pancreatic trypsinogen PRSS1 or loss of functions mutations in the trypsin inhibitor SPINK1. Individuals carrying germline mutations in these genes are at approximately fiftyfold higher risk of developing pancreatic cancer [15]. Another major genetic risk factor is an autosomal dominant disorder called Peutz-Jeghers syndrome. This disease has been linked to inactivating mutations of the tumor-suppressing kinase LKB1. Sufferers of Peutz-Jeghers syndrome have an increased risk for primarily breast cancer and assorted gastrointestinal cancers. The risk for development of pancreatic cancer in these individuals is approximately forty-fold higher than the normal risk [16]. The last major known genetic predisposition is familial atypical multiple mole and melanoma syndrome (FAMMM), which is typically caused by missense mutation of the CDKN2A gene. This gene, which independently codes the cell cycle regulators p14(ARF) and p16(INK4), is also frequently mutated in pancreatic cancer. This disorder primarily leads to melanomas but it also strongly predisposes affected individuals to pancreatic cancer at a roughly thirtyfold increased rate [17].
1.1.3 Molecular Progression

Pancreatic cancer progression as PanIN lesions follows a set of fairly well-conserved mutations between patients (Fig. 1.1). The earliest, and most defining, mutation that occurs in these lesions is an activating point mutation in codon 12 or 13 of the KRAS2 gene [18, 19]. Activating KRAS mutations are found in all grades of PanIN lesions and are present in upwards of 95% of cases of human pancreatic cancer [20]. The function of mutant KRAS in these hyperplastic lesions as well as in PDAC is to promote hyperproliferation and anabolic metabolism. In mice expressing a doxycycline-inducible mutant KRAS, withdrawal of KRAS expression leads to large scale cell death and tumor regression, indicating that KRAS activity is critical for tumor maintenance [21]. In agreement with these mouse models, depletion of KRAS has also shown to elicit a therapeutic response in subcutaneous and orthotopic xenograft models of human pancreatic cancer cells in mice [21]. The next common genetic alteration that occurs is an inactivation of the CDKN2A gene affecting INK4 function, which occurs in about a third of early PanIN lesions and becomes much more common as the disease state progresses [22]. The function of the tumor suppressor INK4A is to inhibit formation of the cyclin D1/CDK4 complex and induce G1 arrest [23]. In fact, mutant KRAS combined with deletion of INK4/ARF is enough to produce a faithful recapitulation of disease progression in a pancreas-specific murine model [24].

Once activation of KRAS and loss of INK4 expression have occurred, PanIN lesions accumulate further genetic alterations that serve to intensely drive malignant disease. The two most common targets of these late mutations are p53 and SMAD4 [1]. Inactivating mutations in p53 are pervasive through most malignancies and are present in nearly three quarters of pancreatic cancer cases [25]. The known tumor suppressor functions of wild type (WT) p53 involve regulation of cell cycle checkpoints and mediating cellular response to various stimuli including DNA damage and hypoxia [26]. Recent evidence has demonstrated a tumor-promoting role for mutant p53 beyond the loss of suppressor function. In pancreatic cancer, mutant p53 drives metastatic potential [27], which is the most influential tumor process leading to lethality in PDAC. The other common late inactivating mutation in pancreatic cancer is of SMAD4. SMAD4 is the protein product of the deleted in pancreatic carcinoma 4 (DPC4) gene, so it is of no surprise that its expression is lost in roughly half of human PDACs [28]. SMAD4 is a key regulator of TGF-β signaling, which is a potent anti-proliferative pathway, imparting a growth advantage to tumors harboring mutations in the gene [29].

In addition to genetic alterations, there are a number of deregulated molecular pathways that contribute to pancreatic cancer, including EGFR/PI3K/AKT [30], Src [31], Hedgehog [32], Stat3, and NF-κB [33]. As KRAS is strongly implicated in pancreatic cancer development, it is important
to note additional pathways that enhance Ras-induced transformation. Src overexpression and activation occurs in the earliest phases of pancreatic cancer development similarly to KRAS mutation [34]. Activation of Src kinase by genetic deletion of the negative regulatory kinase C-terminal Src Kinase (CSK) has been shown to dramatically potentiate the development of PDAC in mice harboring pancreatic-specific KRAS<sup>G12D</sup> mutation, whereas activation of Src alone by these means was insufficient to generate tumors [34]. As Src is known to regulate metastatic potential in a number of tumor types, including pancreatic cancer [35, 36], it is of interest that these early events are cooperative in the progression of this highly invasive and metastatic disease. In addition to the cooperation between Src and Ras pathways in pancreatic cancer, there are a number of other genes which are overexpressed and contribute to Ras-driven cancer. High Mobility Group A-T Hook 2 (HMGA2) is greatly overexpressed in pancreatic tumor tissue compared to normal tissue [37] and positively regulates the malignant phenotype of Ras-driven cancer [38]. SET and MYND Domain containing protein 3 (SMYD3) is a relatively novel lysine methyltransferase that is involved in oncogenic processes [39] and has been shown to be a key regulator in Ras-driven cancers [40]. Both HMGA2 and SMYD3 are associated with metastatic disease [41, 42], which is also a major hallmark of Src activation in human cancers.

1.1.4 Treatment

Pancreatic cancer presents as a group of non-specific symptoms, which frequently leads to diagnosis at late stages when the original tumor has already invaded other organ tissues [43]. Although resection is the preferred option for treatment, the advanced nature of the disease at diagnosis often precludes this option. Even so, survival projections for patients following resection is 17-27 months with a 5-year survival of 20% [44]. Surgical resection of PDAC is also associated with complications and a high morbidity rate [45]. For patients with lower grade localized or locally invasive tumors, neoadjuvant chemotherapy is an option that may shrink tumor margins to the point where surgery is an option [46]. Neoadjuvant therapy also provides a window of therapy in which existing metastases will present themselves, preventing patients from undergoing unnecessary major surgery [47]. The current front line chemotherapeutics for neoadjuvant therapy are 5-fluorouracil (5-FU) and gemcitabine, each exhibiting an overall survival of about 23 months in resected patients [48, 49].

For locally advanced non-surgical cases, patients generally undergo a combination of radiation and 5-FU or gemcitabine (chemoradiation). Radiation is delivered as external beam radiation therapy at a total dose of 50-54 Gy in combination with chemotherapy [50]. Median survival of these patients is approximately 9 months [50]. The toxicities of chemoradiation can be detrimental overall to therapy if they are severe enough to prevent additional rounds of therapy to maintain disease state. Additionally, metastases are commonly found shortly after
the completion of chemoradiation, which in retrospect render the approach moot [51]. Because of this, some patients undergo several rounds of chemotherapy first and are then restaged. If no metastases have presented, patients would then undergo more aggressive chemoradiation with a higher likelihood of a positive response [52]. Targeted therapies, including those against the epidermal growth factor receptor (EGFR) [53], vascular endothelial growth factor receptor (VEGFR) [54], and Src [55] have been tested in combination with gemcitabine with little benefit over chemotherapy alone. This lack of efficacy may be due in part to the dense desmoplastic reaction of these tumors restricting drug delivery [6]. Recently, a combination regimen of oxaliplatin, leucovorin, irinotecan, and 5-FU (designated FOLFIRINOX) has been reported to extend survival to over 11 months, a substantial improvement over standard therapies [56].

Ultimately, the best hope for adequate treatment for pancreatic cancer is the development of effective early detection strategies [57]. As such, a great deal of effort has been put forth into potential biomarkers and screening procedures. The ideal model for progress in this endeavor is the early diagnosis, particularly at the precancerous stage, via screening in colon cancer [58]. From a study involving sequencing of a number of primary patient tumors matched to metastases, it is estimated that the time between initiator mutation to development of advanced PanIN lesion is around a decade. Once the founder lesion has established, it will continue to advance for approximately five more years before it is capable of metastasis [59]. This time frame offers a reasonable screening window in which lesions or tumors can be treated if detection methods can be established. Intraductal papillary mucinous neoplasms (IPMNs) and PanIN lesions can be detected by endoscopic ultrasound (EUS) either directly [60] or by inference due to sclerotic regions [61]. Detection of early lesions by EUS can be followed up by endoscopic resection [60]. Another method of detection involves screening samples of pancreatic secretions for gene mutations. A recent biomarker of IPMN development is the presence of mutation in the GNAS gene in samples of pancreatic secretions from patients suffering IPMNs [62]. Techniques such as these could offer new hope to patients and clinicians seeking more viable treatment options by working with less advanced disease.

### 1.2 Src Kinase

#### 1.2.1 Discovery of vSrc

In 1910, Peyton Rous published the first paper describing a transmissible tumor phenotype in chickens [63]. Within a year, he showed that cell-free tumor isolates were sufficient to induce transformation in uninfected birds [64]. Rous noted that the discovery of a non-cellular agent that caused oncogenic transformation had "largely drawn the attention of cancer workers and has modified current theories of cancer origin"[64]. The Rous Sarcoma Virus was the focus of
much early cancer research over the next half-century. Once biochemical approaches gave rise to molecular biology, the gene product responsible for transformation was successfully cloned and sequenced by Duesberg and Hanafusa [65, 66]. This discovery marked the discovery of the first known oncogene and largely determined the direction of molecular cancer biology for at least the rest of the century. Three years later, Oppermann and Bishop found that the Src gene product was actually a viral homolog (vSrc) of a cellular protein (cSrc) [67]. This discovery would lead to a Nobel Prize and further cemented the theory of oncogene-induced transformation.

To add to the excitement for this newfound cellular proto-oncogene, it was soon determined that the c-Src gene product was a protein tyrosine kinase, the first such enzyme to be described [68].

1.2.2 Src Structure and Function

Src is composed of three well-conserved domains, the Src-homology domains 2 and 3 (SH2, SH3) and the tyrosine kinase (also called SH1) domain (Fig. 1.2A). There is also an N-terminal unique region of each Src kinase family member that facilitates differential signaling by the highly homologous SFKs. The major post-translational modifications of Src are phosphorylations at tyrosines 419 and 530, as well as initiator methionine cleavage and N-terminal myristoylation [69]. The Src SH2 domain is a modular protein domain found in many other signaling molecules and functions as a phosphotyrosine-interacting domain [70]. The SH3 domain is also present in other diverse proteins and is a protein-protein interaction domain that binds to proline-rich sequences [71]. The structure of Src serves as its principle regulatory mechanism. The N-terminal myristoylation as well as the first 14 amino acids function to target Src to the cytoplasmic membrane by hydrophobic and electrostatic interactions [72]. As there are numerous Src substrates at the cytoplasmic membrane,Src is further regulated by intramolecular binding of the SH3 domain to the linker region and of the SH2 domain to the phosphorylated Y530 to maintain an inactive conformation [73, 74]. Src activity is controlled mainly by the antagonistic activities of C-terminal Src Kinase (CSK) and various protein tyrosine phosphatases to modulate Y530 phosphorylation [69]. Once dephosphorylated, the SH2 domain disengages from Y530 and the kinase activity is elevated. This increase in kinase activity triggers autophosphorylation of Y419, leading to maximal kinase activity [75] (Fig. 1.2B).

The individual domains mediate protein-protein interactions as well as direct the specific association of Src with different cellular membranes. The first 14 amino acids are known to target Src to membranes and their fusion to pyruvate kinase (PK) causes the protein to be targeted to cytoplasmic granules. Conversely, fusion of amino acids 1-7 to PK yields diffuse cytoplasmic localization [76]. The SH3 domain mediates attachment to perinuclear membranes as well as specific attachment to the plasma membrane, while the SH2 domain is responsible
mainly for additional perinuclear targeting [76]. v-Src can also associate with a detergent-insoluble cytoskeletal matrix which is not the case with inactive Src [77]. This association is mediated by the SH2 domain in a phosphotyrosine dependent manner and is also regulated by the activity of the kinase domain [78]. Intriguingly, v-Src binding to the cytoskeletal matrix is decreased by an inactivating truncation of its kinase domain, while c-Src binding is greatly enhanced by a similar truncation [78]. The SH3 domain is also important in targeting activated Src to focal adhesions in a phosphatidylinositol (PI) 3-kinase-dependent manner [79].

Much additional work has focused on the transformative properties, rather than subcellular targeting, of various domain-deleted Src constructs. Deletions of portions of the SH3 or SH2 domains of c-Src promote activation of the kinase and are capable of inducing morphological transformation of chicken embryonic fibroblast (CEF) cells similar to that of v-Src [80]. Although this may seem to indicate that the Src-homology domains are dispensable for Src transformative function, their entire deletion severely hampers the ability of v-Src to transform CEFs [81]. The importance of the SH3 and SH2 domains is underscored by the fact that their insertion at the C-terminus of SH3/SH2 deleted v-Src can rescue its transformative capacity [81]. Furthermore, as demonstrated in a study by Hirai and Varmus, certain point mutations and short deletions within the SH3 and SH2 domains were shown to abrogate transformation of CEFs and mouse embryonic fibroblasts (MEFs), while others were able to preferentially retain their transformative capability in one host cell line over the other [82]. The SH3 and SH2 domains of v-Src and c-Src have obvious functional significance in the activity and localization of the proteins that differ depending on the cell type and stimulus involved.

### 1.2.3 Src Family Kinases

The Src family of kinases is made up of eleven structurally, and often functionally, similar proteins. These are Src, Yes, Fyn, Yrk, Blk, Fgr, Hck, Lck, Lyn, and the two Frk subfamily proteins, Frk/Rak and Iyk/Bsk [83]. The most highly expressed and homologous of these kinases are Src, Yes, and Fyn [84]. The other major kinases in this family are Hck, Fgr, Lck, and Lyn. These proteins share common structural features of an SH3 domain, an SH2 domain, and a tyrosine kinase domain [84]. Much work has been done to not only determine the oncogenic functions of SFKs, but also the normal functions, which is challenging due to the redundancy of the individual kinases.

Many SFK functions have been described in immune signaling, the most prominent among them being Lyn, Fyn, and Hck [85]. The redundancy of SFKs is demonstrated by the fact that Fyn-deficient mice express thymocytes with defective T-cell receptor signaling, but still exhibit functional mature T lymphocytes [86]. When both Fyn and Lck are deleted, however, those
mice exhibit a severe defect in any T-cell development [87]. Similarly, genetic deletion of Blk, Fyn, and Lyn in the murine B-cell precursor lineage causes complete collapse of B-cell development in a pathway involving NF-κB signaling [88]. These types of redundancies have confounded efforts to study normal SFK signaling in genetic models.

As may be expected from their ubiquity and redundancy, deletion of any of the three major SFKs (Src, Yes, Fyn) produces a minimal phenotype in developed animals [89]. The deletion of Src was predicted to affect neurological or platelet development, as these are the tissues which express Src most highly. In fact, the major phenotype from Src deletion is osteoporosis due to defective osteoclast development [90]. These mice died within a few weeks of birth, due to a failure of the incisors to erupt and subsequent inability to eat solid food after weaning. Src-/−mice can be maintained on a soft food diet and display a greatly reduced fertility compared to normal mice [89]. Fyn-deficient mice do not display an overt phenotype, although they do exhibit an apparent defect in hippocampal development which impairs long-term potentiation (LTP) and learning [91]. This defect appears to be specific for LTP, as synaptic transmission and short-term synaptic plasticity are unaffected [91]. Fyn-deficient mice also exhibit slight molecular defects in keratinocyte differentiation, though this does not translate to any overt defect in skin development or function [92]. Both phenotypes are specific to Fyn-deficient mice. In the case of Yes, deletion produces no known phenotype in mice [93]. The lack of any observable phenotype in Yes-deficient mice prompted the authors to delve further into the potential for redundancy in SFK signaling. In their studies, breeding for mice deficient in Fyn/Src, Yes/Src, and Fyn/Yes produced considerable embryonic lethality in all instances [93]. These types of overt physiological phenotypes were expected from Src-deficient mice, and they highlight the ability of individual SFKs to compensate quite well for the lack of another. In cancer, SFKs are similarly overexpressed in an overlapping manner. Src and Yes are predominantly activated and overexpressed in epithelial carcinomas such as colon, breast, and pancreatic cancer [94]. Alternatively, Fgr and Lck are mainly associated with myeloid and T- and B-cell lymphocytic leukemias [95-97]. These tumor type-specific differences in kinase activation highlight the role of SFKs in cancer and at least partially mirror their regulatory roles in normal biology.

1.2.4 Src Membrane Signaling

Early studies on v-Src localization recognized that it was localized to the cytoplasmic membrane of transformed cells, particularly under membrane ruffles and at cell-cell junctions [98, 99]. These observations, coupled with the morphological transformation accompanying v-Src expression, led to hypotheses that v-Src was involved in cytoskeletal signaling. Studies using newly generated general phospho-tyrosine antibodies were able to identify a number of v-Src
substrates which localized to focal adhesions which included focal adhesion kinase (FAK), p130Cas, paxillin, and tensin [100, 101] (Fig. 1.3). Identification of v-Src substrates at sites of focal adhesions opened up new avenues for research on the function of Src in the regulation of these structures. In primary CEFs, a temperature-sensitive variant of v-Src was able to cause striking morphological change within 24 hours by disrupting focal adhesions to the point of cell detachment [102]. The regulation of focal adhesion turnover by Src in fibroblasts was further confirmed using less transformative strains of v-Src and showing that Src kinase activity was necessary for the enhancement of focal adhesion turnover during cell motility [103]. The importance of focal adhesion-associated Src was established by one of the early Src pioneers, Steven Martin, who showed that specific targeting of v-Src to focal adhesions is sufficient to cause transformation in CEFs [104]. While apparently demonstrating the functional localization of v-Src transformation, further analysis revealed that targeting activated Src to focal adhesions produces a greatly attenuated transformed phenotype compared to the non-targeted protein [105]. Together, these findings indicate that activity in additional subcellular locations is required for full transformation by v-Src.

Nonetheless, the functional association between c-Src and FAK in non-transformed cells is quite important. Integrin-mediated FAK activation and autophosphorylation creates a high affinity phosphotyrosine binding site for the Src SH2 domain [106]. FAK also contains a proline-rich sequence that corresponds to the Src SH3 consensus binding sequence and binds Src in vitro [107]. Src-null fibroblasts exhibit reduced spreading and adhesion, similar to FAK-null fibroblasts, and re-introduction of the deleted protein rescues this phenotype [108, 109]. Additionally, a negative regulatory form of FAK (called FRNK) causes the same reduction in cell adhesion [110], which is rescued by overexpression of c-Src and subsequent phosphorylation of paxillin [111]. These results confirm the importance of a Src/FAK complex in regulation of focal adhesions and cell attachment in normal cells. In addition to regulating adhesion and motility downstream of integrin signaling, Src and FAK also cooperate with integrins to induce mitogenic signaling through growth factor receptors including EGFR and PDGFR [112, 113], ephrin [114], and VEGFR [115]. These studies emphasize the important relationship between Src and FAK in normal cellular signaling as well as highlight their ability to cooperate in oncogenic signaling. Src also participates in signaling with receptor tyrosine kinases (RTKs) at the cell membrane as part of stimulus-induced signaling cascades (Fig. 1.3). Src is directly involved in the ligand-induced signaling pathways of PDGFR, EGFR, fibroblast growth factor receptor (FGFR), insulin-like growth factor-1 receptor (IGFIR), hepatocyte growth factor receptor (HGFR), and others [116-120]. Src signaling through RTKs generally involves autophosphorylation of cytoplasmic tyrosine residues on the receptor and recruitment of Src via its SH2 domain. One of the most well-studied RTKs that signals through Src is PDGFR [121]. Upon ligand stimulation, the PDGF
receptor undergoes phosphorylation of Tyr\textsuperscript{579}, which initiates binding to the Src SH2 domain and activates Src kinase activity, presumably due to disruption of the inhibitory intramolecular interactions [121, 122]. This association and activation of the kinase is critical for mitogenic PDGF stimulation, as evidenced by the fact that microinjection of kinase dead Src or neutralizing Src antibodies blocks PDGF-induced S-phase [123]. Src activation downstream of PDGF induces expression of Myc, and forced expression of Myc overcomes the requirement of Src for entry into S-phase [124]. The other best studied Src-associated RTK is EGFR. Src overexpression greatly potentiates EGF-stimulated mitogenesis in quiescent MEF cells while not altering other cellular signaling molecules such as cyclic adenosine monophosphate, diacylglycerol, or Ca\textsuperscript{2+} [125]. Additionally, expression of kinase dead or myristoylation-deficient c-Src mutants not only blocked the enhanced mitogenic effect of EGF, but also significantly attenuated it [126]. Src crosstalk with EGFR can also lead to induction of Ras. Zn\textsuperscript{2+} is known to induce EGFR and Ras signaling [127]. Studies in mouse fibroblasts show that Src kinase activity, but not EGFR kinase activity, is necessary for this activation of Ras [128]. C-terminal truncation of EGFR is still able to activate Ras in a Src-dependent manner, but mutation of Y845, a known Src substrate of EGFR [129] to phenylalanine, is sufficient to block Ras induction [128].

1.2.5 Src in the Nucleus

For as long as Src has been known to localize to the cytoplasmic membrane, it has also been observed in the nuclear compartment [130]. Early research on v-Src was fervent and unbiased as it was still the only oncogenic protein known (Ras would soon be confirmed as an oncogene in the early 1980s [131, 132]). In some of the first immunofluorescent studies to be performed using newly generated tumor bearing rabbit (TBR) antiserum against v-Src, Krueger, et al., identified a population of the protein localized to the nucleus and nuclear envelope of rat fibroblasts. This finding was confirmed by biochemical fractionation which showed that the majority of v-Src kinase activity was present in the nuclear fraction of the transformed rat cells, as opposed to a predominantly membrane-associated activity in transformed CEFs [130]. The same localization pattern was soon detected in virus-transformed goat cells as well [133]. Another early group which derived antibodies raised against v-Src protein purified from E. coli, as opposed to serum from rabbits harboring RSV-transformed sarcomas, was able to detect a nuclear population of v-Src in infected CEFs which was not detectable using TBR serum [134]. In the temperature sensitive model mentioned above, shifting to the permissive temperature induced not only translocation to focal adhesions, but also a nuclear co-localization of Src and FAK [102].

Mounting evidence for activity at focal adhesions drove Src research to the cell periphery where novel substrates and a functional significance had begun to develop [100, 101]. Even
still, indications of nuclear Src localization and activity continued to accumulate. In studies directed at understanding the role of tyrosine kinases in morphological development, Hanafusa and Krueger found an induction of nuclear Src activity during Ca^{2+}-stimulated differentiation of mouse and human keratinocytes [135]. In a series of immunolocalization experiments with c-Src overexpressing MEFs performed by David-Pfeuty and Shalloway, a cell cycle-specific nuclear translocation of Src was observed [136, 137]. Furthermore, a myristoylation-deficient mutant of c-Src translocated to the nucleus prematurely and was retained throughout the cell cycle [137]. Further immunofluorescence-based studies by David-Pfeuty recognized additional nuclear and nucleolar localization of Src in various human tumor cell lines [138]. In pursuit of nuclear Src function during mitosis, Shalloway identified an RNA-binding phosphoprotein that was named Src-associated in mitosis 68kD (Sam68) [139]. Sam68 was shown to bind Src through interactions with its SH2 and SH3 domains and to be tyrosine phosphorylated by Src while interacting during mitosis [139]. The SH3 interacting sites of Sam68 appear to be critical for functional binding, as their mutation or competitive inhibition by complementary peptides blocked Src phosphorylation of Sam68 [140]. Sam68 is a predominantly nuclear RNA-binding protein which is involved in cell cycle regulation and RNA splicing [141]. Unsurprisingly then, overexpression of activated Src has been shown in MEF cells to regulate RNA processing events [142]. Active Src affected pre-mRNA processing of TNF-β-responsive genes to a similar extent as the polyoma virus early region, an effect different from that of activated Ras, indicating a distinct role of Src in RNA processing [142].

Src is also known to bind another RNA-binding protein called heterogeneous nuclear ribonucleoprotein k (HNRNPK) [143]. HNRNPK is a highly abundant member of the mammalian heterogenous ribonucleoprotein particle, originally defined as a poly(C)-binding complex [144]. Similar to Sam68, it is a predominantly nuclear protein involved with pre-mRNA processing and nuclear export of mature mRNAs [145, 146]. HNRNPK is also recruited to areas of active transcription and involved in chromatin remodeling at those sites [147]. HNRNPK is a substrate of Src and its phosphorylation reverses HNRNPK-mediated mRNA silencing [148]. HNRNPK is also an activator of Src, presumably by engaging the autoinhibitory interactions as is observed with growth factor receptors [149]. Not only does exogenous HNRNPK expression induce Src activity, but it also induces strong nuclear localization that requires the Src-interacting proline-rich regions of HNRNPK [149]. Interestingly, Sam68 and HNRNPK interact and appear to have opposing functions in the regulation of Rev-response element-dependent during HIV infection [150]. The notion of Src function in the nucleus is generally dismissed in these reports, but the extensively studied Src regulation of Sam68 and HNRNPK-mediated events implores further study into the specific role of Src in these nuclear processes.
Recent evidence has further observed Src in the nuclear compartment in relation to a variety of processes, but lack of functional context for Src in the nucleus has curtailed any strong conclusions based on nuclear Src. Src has been shown to phosphorylate the inner nuclear membrane protein emerin and a related protein called LAP2β on multiple tyrosine residues in Her2-overexpressing cells [151]. A number of these phosphorylations were shown to increase the binding of emerin to BAF, a major chromatin remodeling complex involved in stem cell maintenance [151, 152]. A separate group also detected Src by mass spectrometry as part of a BAF-containing RUNX1 transcriptional complex [153]. In this system, Src was found to phosphorylate RUNX1 and prevent platelet production by megakaryocytes, thereby maintaining the undifferentiated state [153]. Another nuclear Src substrate is KRAB-associated protein 1 (KAP1), which is tyrosine phosphorylated at three tyrosine residues by Src. Src phosphorylation of these residues decreases the association of both KAP1 and heterochromatin protein 1 alpha (HP1α) with heterochromatin, an event that takes place during euchromatinization [154]. KAP1 is involved in a number of other genomic processes including transcription, DNA repair, and direction of histone modification [155]. With the growing number of Src nuclear substrates involved in modulation of gene expression and chromatin remodeling, the distinct possibility is raised that modification of chromatin architecture is a key function of Src. In fact, targeting of Src or SFKs to the nucleus induces wholesale changes in the genomic structure of MEFs and monkey kidney fibroblasts [156, 157]. These changes occur in response to growth factor or serum stimulation and represent genomic reorganization during mitogenic signaling [156]. These changes are abrogated by Src inhibition and are enhanced in SYF-/- cells by reintroduction of Src [156]. Together, this evidence strongly suggests that there are heretofore unknown mechanisms of Src modulation of gene expression by chromatin regulation and RNA processing within the nucleus.

Src also functions to regulate degradation of nuclear targets by phosphorylation. Src has been shown to bind and phosphorylate the von Hippel-Lindau (VHL) tumor suppressor in human embryonic kidney (HEK) and breast cancer cells, resulting in destabilization and degradation of VHL [158]. In this model, both proteins co-localized in the nucleus, and Src-mediated VHL degradation led to increased production of pro-angiogenic factors [158]. The same group identified another similar regulation of the inhibitor of growth 1 (ING1) tumor suppressor using HEK cells [159]. Src and ING1 again co-localize within the nucleus, and phosphorylation of ING1 mediates its degradation [158]. ING1 is a member of a major tumor-suppressing histone modifying complex [160], marking another nuclear target of Src involved in epigenetic regulation. Not all Src nuclear targets appear to promote oncogenic effects. Src has been shown to promote nuclear export and degradation of the NF-E3-related factor 2 (Nrf2) by phosphorylation under hypoxic and oxidative stress in the mouse hepatoma cell line Hepa-1.
Nrf2 is a cytoprotective transcription factor that mediates antioxidative gene expression, therefore Src-mediated degradation leads to increased cell death under such conditions [161]. It is curious that tumor cells would respond specifically to oxidative stress by degrading cytoprotective factors, but nonetheless this report describes a different type of function for nuclear Src.

Src is also known to participate extensively in steroid receptor signaling, presumably through interactions in the cytoplasm [162]. Src has been shown to interact directly with the progesterone receptor (PR), estrogen receptor (ER) α and β, and androgen receptor (AR) by its SH2 domain in a phosphorylated receptor-dependant manner [163, 164]. Kinase dead Src also blocks the mitogenic effects of hormone stimulation in various ER-expressing breast cancer cell lines [164]. Given the nuclear function and regulation of steroid receptors, it stands to reason that they may have functional interactions with Src within the nucleus. One study supporting this hypothesis showed that Src interacts with and phosphorylates the hepatocyte nuclear factor 4α (HNF4α) in human colon cancer [165]. This phosphorylation again leads to nuclear export and degradation of the receptor, a phenotype which is observed in colon cancers in vivo. Additionally, active Src correlates to histological loss of HNF4α expression in patient samples [165]. This group noted nuclear presence of active Src in their tissue microarray samples as well as their colon cancer cell lines, and pointed to an older study which also identified a strong nuclear presence of active Src in human colon cancer [166]. Activated nuclear Src has also been identified in patient samples of ER+ breast cancer [167]. Patients with nuclear localization of active Src prior to treatment with the ER antagonist Tamoxifen had a far higher survival rate compared to patients with non-nuclear Src [167]. This correlation raises the intriguing possibility that interaction between active Src and ER within the nucleus modulates critical ER survival signaling in breast cancer, the disruption of which causes tumor death. One group, while attempting to detect ER/Src dimers at the cell membrane, identified direct interaction of Src and ERα via proximity ligation assay (PLA) within the nuclei of ER+ breast cancer cell lines [168]. They also found that the pro-survival effects of ER overexpression were mediated by ER tyrosine phosphorylation and activation of Src [168].

Src also exhibits nuclear localization in fertilized oocytes [169]. Fertilization has been shown to activate SFKs in oocytes from a variety of different organisms [169-171]. This SH2-mediated activation of SFKs induces calcium signaling cascades and is required for proper activation of invertebrate eggs at fertilization [172]. While not required for calcium induction in vertebrates, SFKs do play important roles in the early cellular events following fertilization [173]. Activated Src localizes to the mitotic spindle during the first division of fertilized mouse oocytes [169]. Active Src is also localized within the early pronucleus and pronuclear membrane through to
the 2-cell stage [169]. In addition to nuclear localization of Src in oocytes, Src and phospholipase C γ (PLCγ) are co-recruited to sperm nuclei during fertilization [174]. Src and PLCγ are also co-recruited to the nucleus and early nuclear envelope of fertilized oocytes where they participate in proper nuclear envelope formation [174].

1.3 Histone Acetyltransferase p300

1.3.1 Structure and function of p300

Human p300 was identified originally as a binding partner necessary for transformation by the adenovirus E1A oncoprotein [175]. It was later determined by molecular cloning to contain multiple domains characteristic of a transcriptional coactivator, including a bromodomain-like region and two cysteine/histidine rich regions (CH) [176]. Just before the molecular characterization of p300, another protein of similar size was discovered to be associated with phosphorylated CREB during cyclic AMP signaling and named CREB-binding protein (CBP) [177]. CBP was soon discovered to be a transcriptional coactivator of CREB [178] as well as a highly (75%) homologous family member of p300 [179]. While there are functional differences between CBP and p300, they also overlap on a large number of functions. This section will predominantly focus on select interacting partners of p300, although seminal discoveries on interactions will be presented for the paralog in which they were discovered.

The first and third CH regions of p300 (CH1 and CH3) contain transcription adaptor putative zinc finger (TAZ) domains, called TAZ1 for the N-terminal domain and TAZ2 for the C-terminal domain [180] (Fig. 1.4). The TAZ1 domain functions in a variety of transactivational interactions, including interaction with the general transcription factor TATA-binding protein (TBP) [181]. In addition to general transactivation, the TAZ1 domain also acts as a transcriptional adaptor in hypoxic signaling [182]. Under hypoxic conditions, p300 is required to bring hypoxia inducible factor 1 α (HIF1α) together with the steroid receptor coactivator 1 (SRC-1) to induce hypoxic genes [182]. The kinase-inducible domain interacting (KIX) domain is just C-terminal to the TAZ1 domain and is the portion of p300 responsible for CREB interaction [183]. The KIX domain also recruits the hematopoietic transcription factor c-Myb, an association which is critical for c-Myb transactivational activity [184]. The CH3 region contains two zinc fingers, the ZZ-type zinc finger domain and the TAZ2 domain [185]. This region is the binding site for E1A and is capable of transactivating target genes in the presence of a binding-defective E1A mutant, but not the wild type protein [186]. The TAZ2 domain is similar in structure and function to the TAZ1 domain, although it interacts with a distinct set of partners. One such partner is the general transcription factor activator protein 1 (AP-1), which associates with the TAZ2 domain of p300 and induces expression of cyclin D1 [187]. This effect appears to be p300-specific, as CBP expression failed to induce cyclin D1 expression [187]. Both CH domains appear to recruit and
mediate the function of cJun and JunB, members of the AP-1 transcriptional complex [188]. The function of the ZZ domain is not well defined, although it has been determined that the ZZ domain is not involved in E1A binding [189]. The fourth zinc finger domain in p300, the plant homeodomain (PHD) finger, is similarly poorly understood. The PHD finger is situated near the bromodomain of p300, a structure mirrored in other bromodomain-containing proteins, and participates in binding to acetylated histones [190].

Aside from transactivation domains, p300 also contains domains responsible for coactivator activity. The N-terminus of p300 encompasses two LXXLL domains which bind nuclear receptors [185]. The N-terminus of p300 was first shown to be involved in signaling through the retinoic acid receptor, indicating its role as a nuclear receptor coactivator [191]. Shortly after, p300 was shown to cooperate in ER signaling [192] as well as AR signaling [193], indicating its fundamental role in nuclear receptor signaling. In addition to binding nuclear receptors at its N-terminus, p300 acts as a transactivational scaffold in hormone signaling by also binding coactivators such as the steroid receptor coactivator 1 (SRC-1) [194]. This interaction occurs at the C-terminal Q/P rich region and is essential for coactivation of nuclear receptors [194]. In addition to binding SRC-1, the Q/P-rich region mediates binding to and regulation of a homologous coactivator protein p/CIP [195]. Due to its extensive involvement in nuclear receptor signaling, p300 is considered to be a critical member of hormone activation pathways [196].

There also exists a major functional intersection between p300 and p53. Early research on p300 showed that it associates with both WT and mutant p53 and is recruited to p53-dependent promoters [197]. Mutation of p53 binding sites on p300 abrogates its enhancing effect on p53 transcription as well as negates the ability of p53 to induce G1 arrest [197]. In fact, the blockage of p53 activation by p300 is one of the critical molecular events in E1A-mediated transformation [198]. These effects are at least in part mediated by p300 acetylation of p53, which promotes its DNA-binding activity [199]. This acetylation appears to promote stability of p53 and is induced by p53-inducing stimuli and inhibited by MDM2 [200]. Recent studies have further implied the importance of p300 in p53 signaling by showing that there are four high affinity p53 binding sites within p300 which promote p53 tetramer formation [201]. In fact, this oligomerization of p53 is critical for its acetylation by p300 [202]. The significant overlap of p300 and p53 signaling prompted the early hypothesis that p300 acts as a tumor suppressor. While this may be the case in some contexts, there is also considerable evidence that p300 participates in tumorigenic signaling [203, 204]. Mutations in p53 are extremely common in cancer and represent both loss-of-function of tumor suppressor activity as well as gain-of-function in tumorigenesis [205]. Mutant p53 binds efficiently to p300 [197, 206], and has been
shown to participate in oncogenic signaling with p300 [207]. This interaction of p300 with mutant p53 could define a major condition under which p300 does not have tumor suppressor function.

Aside from the protein-protein interactions made by p300 that influence gene regulation, p300 itself can regulate chromatin dynamics. The bromodomain of p300 has been shown to mediate its time-dependent binding to histones, particularly histone H3 [208]. However, in this study, the bromodomain alone could bind to naked histones. The entire protein was required to facilitate the interaction [208]. This is likely due to the fact that the bromodomain preferentially binds acetylated histones rather than the unmodified protein [209]. It was hypothesized that induction of histone binding by the full protein was due to endogenous acetyltransferase activity. Indeed, p300 also acts as a histone acetyltransferase [210]. The acetyltransferase domain of p300 can acetylate all four core histone proteins and is structurally novel compared to many other known histone acetyltransferases [210, 211].

There are a number of cytosolic signaling molecules which translocate to the nucleus and phosphorylate p300 on serine residues which regulate its enzymatic activity. Erk, a major kinase implicated in PDAC, is known to phosphorylate and stimulate the HAT activity [212]. This report also found that the residues phosphorylated by ERK are critical for the mitogen-responsive stimulation of p300 [212]. AKT, another serine/threonine kinase which plays a major role in PDAC, is also known to phosphorylate C-terminal serine residues of p300 [213]. This phosphorylation again stimulates the HAT activity of p300 and is essential for its response to TNF-α stimulation [213]. ERK and AKT signaling are downstream of known Src-interacting pathways (Fig. 1.5). Their functions here can either be viewed as proof of concept that nuclear kinases can regulate p300 activation or as evidence that any Src signaling to p300 is occurring through these pathways.

**1.3.2 p300 in Development**

p300 plays a key role in embryogenesis as well as a number of other developmental processes, as evidenced by the fact that p300-null mice are embryonic lethal [214]. This is due to multiple failures including improper neural tube closure and impaired cellular proliferation [214]. A similar phenotype is observed in mice expressing truncated CBP, including improper neural tube closure and embryonic lethality [215]. Loss of CBP in drosophila leads to defects in mesodermal gene expression and development and loss of head and thorax structures, although it does not have any effect on homeotic gene expression [216]. Additionally, CBP haploinsufficiency has been observed in humans and causes a syndrome called Rubinstein-Taybi syndrome (RTS) [217]. RTS is characterized by malformation of the head and hand as well
as severe mental retardation [218], demonstrating the key role of CBP/p300 in neurological development.

On top of general embryological development, p300 controls the development of specific cell lineages. Chiefly among these processes is hematopoiesis [219]. Myb is a major transcription factor involved in hematopoietic self-renewal [220] and recruits p300 as a coactivator [221]. The transcriptional activity of the Myb-containing coactivator complex is further potentiated by p300-dependent recruitment of NF-M [222]. GATA-1, another key transcription factor in hematopoietic proliferation and differentiation, is also regulated by p300 [223]. The two proteins bind in vitro and E1A expression prevents induction of GATA-1 regulated genes [223]. As is a common motif with p300-regulated proteins, p300 can acetylate GATA-1 to further modulate its activity [224]. The CCAAT-box/enhancer binding protein β (C/EBPβ) is known both to be a major binding partner of p300 as well as a major regulator of macrophage and granulocytic development [225]. It has been shown that p300 acts as a coactivator for C/EBPβ during myelomelanocytic differentiation and that this interaction promotes synergistic activity between C/EBPβ and Myb [226].

Another major lineage-specific function of p300 is in myogenesis. The first indication that this process is p300-dependent actually came before p300 was identified, when it was discovered that E1A blocked myogenesis and myogenic gene transcription [227]. Specifically, E1A blocks transactivation of two of the four central myogenic transcription factors, myf-5 and myo-D [228, 229]. Transcriptional activation by Myo-D has been shown to be potentiated by p300 expression and blocked by microinjection of p300 antibodies [230]. The two proteins interact in vitro, but only when DNA fragments containing two Myo-D binding sites are present [230]. Myo-D is also acetylated by p300 and an unrelated HAT, a modification which promotes DNA-binding activity [231]. Genetic analysis of murine myogenesis has implicated p300 specifically, but not CBP, as a required cofactor for myf-5 and myo-D activation and proper myogenic differentiation [232].

1.3.3 p300 in Cancer

The role of p300 signaling in cancer is a subject of debate. There is considerable evidence surrounding loss of p300 and increased incidence of certain kinds of cancer, implying a role as a tumor suppressor protein [204]. In humans, haploinsufficiency of CBP (but almost never p300) leads to RTS as described previously. Sufferers of RTS are at increased risk of neural crest cell-derived tumors [233] as well as lymphocytic leukemia [234], cells from processes which are severely impacted by CBP/p300 genetic deletion in model organisms as well as humans. Mice which are heterozygous for CBP and (to a lesser extent) p300 are viable and generally healthy.
The CBP heterozygous mice exhibit some defects in hematopoiesis as well as increased incidence of hematologic malignancies in older animals. Interestingly, the tumors in the CBP heterozygous mice underwent loss-of-heterozygosity for the other allele, further suggesting the tumor suppressor function of CBP [235]. Given their considerable cooperation with p53, CBP and p300 were hypothesized to be classical tumor suppressors. Contrary to this evidence, however, mutations in CBP and p300 genes in cancer are quite rare. One group identified p300 mutations in two out of 29 colorectal and gastric carcinomas, a rate of less than 7% [236]. Another group identified two instances of p300 inactivating mutations in primary epithelial tumors and four more in cell lines out of a total of 196 samples [237]. The same group followed up with analysis of CBP status in a similar number of samples, finding no inactivating mutations in primary tumor samples [238].

Contrarily, the known roles of p300 in cellular proliferation and its interaction with the previously mentioned oncogenic transcription factors HIF1α, Jun, myb, and others, indicate a potential role in pro-oncogenic signaling. Indeed, p300 and myb interaction is essential for the transforming activity of myb and for transformation by other leukemic oncogenes including AML1-ETO, MLL-ENL, and MLL-AF9 [239]. p300 also participates in non-homologous end joining along with the SWI/SNF chromatin remodeling complex in lung cancer. In pancreatic cancer with active KRAS, p300 promotes expression of the proliferative kinase GSK3β [240]. Signaling via p300 has been implicated in migration and invasion in prostate cancer, presumably through its function as an AR coactivator [241]. p300 has also been recently implicated in chemokine-directed migration and metastasis and breast cancer [242]. In this study, CCL21 stimulation released a blockage of p300 acetyltransferase activity, promoting epigenetic changes that allowed other transcriptional regulators to be recruited, culminating in a noncanonical Hedgehog/GLI2 transcriptional program that induced migration [242]. These complex changes are common in epigenetic signaling, such as those observed in the Src-induced epigenetic switch from normal to transformed breast epithelial cells [243]. Inhibition of p300 by small molecule or siRNA has also proved to inhibit survival and increase chemosensitivity of a number of epithelial tumor types including prostate, melanoma, breast, and lung cancer cells [241, 244-246].

Given the molecular evidence, many early hypotheses that p300 acts as a tumor suppressor have been modified. It is possible that p300 participates in tumor suppressor or oncogenic signaling in a highly context-dependent manner, with a broader implication in tumorigenesis [204]. These contradictions also underscore the lack of understanding of how p300 signaling contributes to cancer.
Figure 1.1. Morphological and genetic progression of PDAC precursor lesions. KRAS mutations occur early in PanIN progression and are found in the first morphologically aberrant lesions. With PanIN-1 lesions, the epithelium becomes more columnar and papillary compared to the normal epithelial layer. Nuclei are still basal in these lesions. Later mutations, such as those of the tumor suppressor CDKN2A gene, occur with PanIN-2 lesions. These lesions are characteristically papillary and contain some degree of nuclear abnormality. PanIN-3 lesions exhibit the late mutations such as those inactivating p53 and SMAD4. The nuclei of these cells are highly abnormal and cribriiforming, a budding appearance of the epithelium, is a clear indicator of PanIN-3 lesions. Adapted from [4].
Figure 1.2. SFK structural organization and intramolecular regulation. A, The modular layout shared by the SFKs. The N-terminal unique domain is the most variable region amongst SFKs and aids in their ability to perform different functions. The SH3 domain is a modular domain which binds to proline-rich sequences. The SH2 domain specifically binds targets when they are phosphorylated on a critical tyrosine residue. The SH1, or kinase, domain is the enzymatically active portion responsible for tyrosine phosphorylation. The N-terminal myristoyl group aids in membrane targeting. SFKs also contain two phosphorylated tyrosine residues, one within the kinase and one at the C-terminus. B, The structure of the inactive SFK involves intramolecular interactions of the SH3 domain and the SH2-kinase linker region as well as those of the SH2 domain and the inhibitory pY527. The kinase becomes activated when Y527 becomes de-phosphorylated or when the SH3 and SH2 domains become engaged by other interactions. This conformation leads to phosphorylation of Y416 and maximal kinase activation.
Figure 1.3. Canonical Src membrane signaling. At the cell membrane, Src is involved in growth factor receptor signaling as well as adhesion signaling. Activation of growth factor receptors recruits Src where it can act by either phosphorylating docking tyrosine residues on the receptor or downstream targets directly. Src can directly phosphorylate and stimulate PI3K and Stat3 in this way. Membrane-bound Src can also activate the Ras pathway. Src also associates with focal adhesions where it phosphorylates FAK as well as other focal adhesion molecules. Both Src and FAK can phosphorylate p130Cas and initiate further downstream signaling. These signals lead to enhanced proliferation and migration.
Figure 1.4. Structural organization of p300 and interacting proteins. p300 is a large protein of over 2,400 amino acids. It contains a number of modular domains as well as conserved sequences for protein-protein interactions. The N-terminus contains two LXXLL domains for binding to nuclear receptors. The four zinc finger domains are the Taz1 domain (CH1), the PHD (CH2), and the ZZ and Taz2 domains (CH3). All of these domains mediate specific protein interactions. The Kix domain is a transactivational domain which binds a number of transcription factors. The bromodomain is responsible for binding to acetylated histones. The HAT domain is responsible for further acetylation of histones and other targets. The Q/P disordered region at the C-terminus is known to bind coactivator proteins. Selected interacting partners with p300 have been listed above the region with which they interact.
Figure 1.5. Src-responsive pathways which activate p300. Src is known to activate the Ras pathway through phosphorylation of Raf as well as by other mechanisms. The Ras pathway leads to ERK phosphorylation which is known to phosphorylate a critical serine residue on p300 that regulates its acetyltransferase activity. Src can also directly activate the p85 subunit of PI3K. PI3K stimulates AKT activity by recruitment to the cell membrane. Activated AKT then phosphorylates mTOR as well as p300 at a critical serine residue. Src-specific activity is represented by blue arrows, while other activation steps are indicated by black arrows.
CHAPTER II

SIGNIFICANCE

Pancreatic cancer is an extremely lethal malignancy for which there is almost no effective treatment. The major mutational events of pancreatic cancer progression are fairly well-defined, but this knowledge has led to no real advances in specific targeted therapy. Underscoring the lack of specific therapy is the fact that there is little understanding of the molecular events that contribute to the pancreatic cancer phenotype. Studies in our laboratory have identified a nuclear presence of activated Src in pancreatic cancer, raising questions about the potential functional significance of this localization. Our central focus was to define the role that nuclear Src plays by identification of nuclear Src protein-protein and protein-gene regulatory networks in pancreatic cancer. In the present studies, we define a novel interaction of Src and p300 as well as a novel set of genes regulated by this complex. We also show that Src and p300 function to promote migratory and invasive properties of pancreatic cancer cells. Considering the migratory and invasive nature of pancreatic cancer, the most appealing therapeutic approaches would combine cytostatic or cytotoxic drugs as well as anti-metastatic therapies. This Src/p300 axis signifies a novel target for therapeutic approaches to treat PDAC.
CHAPTER III

MATERIALS AND METHODS

3.1 Cell culture

The human pancreatic ductal adenocarcinoma cell line PANC-1 was purchased from the American Type Culture Collection (ATCC, Manassas, VA) (CRL-1469). These cells were originally isolated from a 56-year old Caucasian male patient with metastatic PDAC. These cells harbor mutant p53, an activating KRAS mutation, and homozygous deletion of p16. SYF+/+ cells are a wild-type (MEF) cell line immortalized with the SV40 large T antigen. SYF-/-(ATCC CRL-2459) cells are identical but for homozygous deletion of the Src, Yes, and Fyn genes. SYF-Src cells are created from the knockout SYF-/background and stably overexpress wild-type c-Src. All cells were cultured in high glucose DMEM containing 10% fetal bovine serum (FBS) and 100U/mL of penicillin and streptomycin (GIBCO) at 37°C in a humidified incubator at 5% CO₂ in air. Cells were passaged by rinsing with PBS and incubating with 0.25% trypsin-1mM EDTA for 1 minute at 37°C. Trypsin was neutralized by adding culture media containing FBS and cells were resuspended by pipetting. Cells were split 1:4 (Panc-1) or 1:8 (SYF+/+, SYF-/-, SYF-Src).

3.2 Nuclear fractionation

Cells were washed twice in ice-cold PBS then swelled in ice-cold hypotonic buffer (10mM HEPES pH 7.9, 1.5mM MgCl₂, 10mM KCl) supplemented with NaF (20mM), Na₄P₂O₇ (1mM), aprotinin (2ug/mL), leupeptin (1ug/mL), PMSF (2mM), and Na₂VO₄ (1mM). After swelling, hypotonic buffer was removed and cells were lysed in hypotonic buffer containing 0.2% NP-40 to selectively solubilize the plasma membrane. Nuclei were pelleted in a microcentrifuge at 4°C for five minutes at 800g. Nuclear pellet was washed once with hypotonic buffer + NP-40 to remove any cytoplasmic contaminants and spun down again. Washed nuclear pellets were lysed either in RIPA buffer (25mM Tris pH 7.5, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) or IP Lysis buffer (25mM Tris pH 7.4, 50mM NaCl, 0.75% NP-40, 0.75% CHAPS, 1mM EDTA, 5% glycerol), each supplemented with protease and phosphatase inhibitors aprotinin (2ug/mL), leupeptin (1ug/mL), PMSF (2mM), and Na₂VO₄ (1mM).

3.3 Subnuclear fractionation

Nuclear pellet was isolated as described above. Next, further fractionation was performed as previously described (matunis ref 2006) with slight variations. First, nuclei were resuspended in
0.5mL lysis buffer (0.1mM MgCl₂, 1mM DTT, 10µg/mL RNase A, 10µg/mL DNase I, and protease and phosphatase inhibitors as above) and transferred into a 15mL centrifuge tube. Next, 2mL extraction buffer (10% sucrose, 20mM triethanolamine pH 8.5, 0.1mM MgCl₂, 1mM DTT, and protease and phosphatase inhibitors) was added dropwise while gently vortexing. Nuclei were incubated at room temperature for 30 minutes to digest RNA and accessible chromatin. Next, 7mL of ice-cold sucrose cushion buffer (30% sucrose, 20mM triethanolamine pH 7.5, 0.1mM MgCl₂, 1mM DTT, and protease and phosphatase inhibitors) was underlayed and the tube was centrifuged in a tabletop centrifuge at 750g for ten minutes. The upper layer supernatant was removed and labeled as "DNase/RNase fraction" and the underlayment was removed. Next, the pellet was resuspended in 0.5mL extraction buffer (10% sucrose, 20mM triethanolamine pH 7.5, 0.1mM MgCl₂, 1mM DTT, and protease and phosphatase inhibitors) and an additional 0.25mL extraction buffer containing 0.3mg/mL heparin was added dropwise while gently vortexing. Again, 7mL sucrose cushion buffer was underlayed and centrifuged at 750g for 15 minutes. The supernatant was labeled as "Heparin extract" and the pellet was lysed in RIPA buffer and labeled "Nuclear envelope."

### 3.4 Immunoblotting

Cells were washed twice in ice-cold PBS then harvested with cold lysis buffer. Whole cell lysates were harvested using RIPA. Nuclear and cytosolic lysates were harvested in the above mentioned buffers. Samples were boiled in 2X SDS sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue) and run on SDS-PAGE gels cast in the Bio-Rad Mini-Protean system. Gels were then transferred onto nitrocellulose membranes (Bio-Rad). The following primary antibodies were used to probe membranes in these studies: Src (B-12), GAPDH, Tubulin, Grim19, (Santa Cruz Biotechnology, Santa Cruz, CA), Src (36D10), pY-416 Src, HMGA2 (D1A7), SMYD3 (D2Q4V), Lamin A/C (#2032), Histone H3 (D1H2), p-Tyr-100 (Cell Signaling Technology, Danvers, MA), p300 (NB100-507) (Novus Biologicals, Littleton, CO). Secondary antibodies used were Donkey-anti-Rabbit 800CW and Goat-anti-Mouse 680LT (Licor, Lincoln NE). Membranes were scanned using the Licor Odyssey CLx Infrared Imaging Scanner.

### 3.5 Nuclear kinase assay

Cell nuclei were isolated as previously described except without the addition of Na₃VO₄. During the first wash of nuclei in cytosolic lysis buffer, equal volumes were split amongst four tubes and spun down to yield equal numbers of purified nuclei. The nuclei in these tubes were resuspended in ice-cold Src kinase assay buffer (50mM Tris pH7.5, 10mM MgCl₂, 2.5mM MgCl₂, 1mM ATP, 10mM DTT) which was supplemented with either 1mM Na₃VO₄, 100nM dasatinib, or
both. These reaction mixtures were then incubated at 37°C for 30 minutes to allow for kinase activity. After incubation, the nuclei were lysed using 5X RIPA buffer with protease and phosphatase inhibitors. Protein was quantified and equal amounts were prepared for SDS PAGE. Membranes were probed with general phosphotyrosine antibodies to determine the activity of nuclear tyrosine kinases under each condition.

### 3.6 Immunoprecipitation

Nuclear lysate for immunoprecipitation was prepared via nuclear fractionation and lysis in IP Lysis buffer. While nuclei are incubating in lysis buffer, 20µL protein A/G agarose plus (Santa Cruz Biotechnology) was pre-equilibrated in IP Lysis buffer. The equilibrated beads were spun down in a microcentrifuge at 4°C for three minutes at 400g and then added to the nuclear lysate and incubated on a rocker at 4°C for one hour to pre-clear. Lysates were then spun down at 400g and the cleared supernatant was transferred to a new tube on ice. Next, equal volumes were split amongst separate tubes for control or specific immunoprecipitations. Additionally, 2-5% of the specific IP volume was collected in a separate tube to be used as input. Next, 2-4µg of the antibodies were added to their respective tubes and incubated on a rocker at 4°C overnight. After binding to antibodies, another 20µL of beads were pre-equilibrated in IP Lysis buffer and added to the lysates for one hour while rocking to bind the antibody-target complexes. The tubes were spun down at 4°C for three minutes at 400g and the supernatant was removed. The bound beads were washed twice in IP Wash buffer (Tris pH 7.4, 150mM NaCl, 1% NP-40, 1mM EDTA, 5% glycerol, protease and phosphatase inhibitors). Once washed, the beads were resuspended in 2X SDS Loading buffer, boiled for five minutes, and processed for immunoblotting. The immunoprecipitation antibodies used in these studies were c-Src (B-12, Santa Cruz), p300 (NB100-507, Novus), and normal mouse IgG (sc-2025, Santa Cruz).

### 3.7 Chromatin immunoprecipitation

After treatment, cells were removed from the incubator and culture medium was removed by vacuum and replaced with a volume of PBS. Next, 37% formaldehyde was added to a final concentration of 1% and incubated on a rocker at room temperature for ten minutes to crosslink protein-chromatin interactions. After crosslinking, glycine was added to a final concentration of 125mM to quench the remaining formaldehyde and stop the reaction. Quenching took place at room temperature for 15 minutes. Cells were then washed once in PBS, covered with enough PBS to coat the dish, and scraped into a 15mL conical tube. Cells were spun down at 450g for five minutes at 4°C and the pellet was washed twice more in ice-cold PBS. Next, cells were lysed and spun down a total of three times in ChIP Lysis Buffer.
(10mM Tris pH 7.5, 10mM NaCl, 3mM MgCl₂, 0.5% NP-40, 2mM PMSF [added fresh]). The nuclear pellet was then resuspended in Pre-IP Dilution Buffer (10mM Tris pH 7.5, 10mM NaCl, 3mM MgCl₂, 1mM CaCl₂, 4% NP-40, 2mM PMSF [added fresh]). Components were added to the indicated final concentrations: SDS (0.8%), NaCl (160mM), as well as protease and phosphatase inhibitors. The resuspended samples were then sonicated with a probe tip sonicator until the chromatin was sheared to an average size of 500bp. The samples were cleared of debris by centrifugation and the supernatant was diluted with 5 volumes of IP Dilution Buffer (20mM Tris pH 8.0, 2mM EDTA, 1% Triton X-100, 150mM NaCl, and protease and phosphatase inhibitors) and split amongst different tubes for immunoprecipitation as described above. After the antibody-bead complexes were spun down, the beads were transferred into 1.5mL microcentrifuge tubes and washed twice at room temperature with IP Dilution Buffer. They were then washed once with ChIP Wash 2 (20mM Tris pH 8.0, 2mM EDTA, 1% Triton X-100, 0.1% SDS, 500mM NaCl, and protease and phosphatase inhibitors) and once more in ChIP Wash 3 (10mM Tris pH 8.0, 1mM EDTA, 25mM LiCl, 0.5% NP-40, 0.5% Deoxycholate). Beads were then buffer exchanged by washing twice in TE buffer (10mM Tris pH 8, 1mM EDTA). Beads were then eluted by 30 minute incubation at 65°C with Elution Buffer (25mM Tris pH 7.5, 10mM EDTA, 0.5% SDS), spun down and removed into a new tube. The beads were then eluted a second time by adding the same volume of Elution Buffer and vortexed briefly. The resulting supernatant was then reversed of crosslinks and digested of protein by addition of proteinase K (1µg/µL final) and incubation at 55°C overnight. De-crosslinked samples were then purified with ChIP DNA Clean and Concentrator columns (Zymo Research, Irvine, CA). Eluted samples were then analyzed by qPCR. Specific IP and IgG were normalized by ΔΔCt (as validated by >90% PCR efficiency) to the individual sample inputs to determine relative enrichment for each antibody and expressed as such. Samples were run in triplicate. Antibodies used in these studies were anti-Src (B-12), p300 (C20X), normal mouse IgG (sc-2025), and normal rabbit IgG (sc-2027).

3.8 ChIP-chip

Panc-1 cells were seeded in two 15-cm dishes per IP in duplicate. Cells were treated for three hours with either DMSO or dasatinib (100nM). DMSO-treated plates were used for control IgG and Src IPs while the dasatinib-treated plates were used for Src IP. Technical duplicates were ultimately pooled onto one chip and an additional set of biological replicates was performed to achieve n=4 for each experimental condition. Cells were processed for ChIP as described above up until reversal of crosslinks and purification of DNA. Protocol is derived from Affymetrix Chromatin Immunoprecipitation Assay Protocol. Purified DNA was subjected to two rounds of manual linear amplification with adapter-linked random nonamer primers (GTTTCCAGTCACGGTCT(N)₉) using Sequenase (Affymetrix, Santa Clara, CA) reagents. After
linear amplification, samples were exponentially amplified using Taq polymerase for 30 cycles with 20% dUTP content in order to incorporate uracil into the fragments. Amplified DNA was sent to the University of Hawaii Cancer Center Genomics Core Facility for uracil DNA Glycosylase fragmentation and labeling, and hybridization to Human Promoter 1.0r Microarrays. Once the arrays were hybridized and scanned, CEL files containing raw intensity values were provided for analysis. CEL files corresponding to each array (two control, two untreated, two treated) were converted to TAG files in the Affymetrix Tiling Analysis software suite. TAG files represented the comparison of the specific IPs vs. the control IgG using quartile normalization with a target intensity of 250. The data were expressed with a Log$_2$ signal scale and a -10Log$_{10}$ p-value scale. Analysis of TAG files was performed as a one-sided upper perfect match only using a bandwidth of 250. The p-value threshold was set to 23 (p=.005) with a maximum gap of 100bp and a minimum run of 200bp in order to exclude nonspecific hits from individual probes with artificially high signal. The analysis generated CHP files which could be viewed on the Affymetrix Integrated Genome Browser represented as p-value of enrichment of the specific IP vs the control IgG for each probe. The CHP files were further subjected to interval and probe analysis to generate a list of reference numbers corresponding to hits based on the probe enrichment and tiling library.

### 3.9 Immunofluorescence

Cells were seeded onto glass coverslips (thickness #1.5) in individual wells of a 24-well plate and allowed to attach overnight. Once ready for processing, cells were washed two times for one minute each with PBS. After washing, cells were fixed with 4% paraformaldehyde in PBS for ten minutes at room temperature. After fixation, cells were permeabilized by ten minute incubation with PBS+.025% Triton X-100. Cells were then washed twice for five minutes each with PBS-T (PBS + 0.1% Tween-20) and blocked for 30 minutes with PBS-T + 0.2% BSA. After blocking, antibodies were then diluted to appropriate concentrations in PBS-T and incubated with cells for one hour at room temperature. Each experiment also contained a sample in which no primary antibodies were added to determine non-specific background staining. After incubation with primary, cells were washed twice for five minutes each in PBS-T and then incubated with secondary antibodies for one hour (1:500 dilution for all secondaries). After secondary incubation, cells were washed three times for seven minutes each in PBS-T and mounted onto microscope slides with Fluoromount-G +DAPI (SouthernBiotech) and imaged on the Leica TCS-SP5 microscope system. Primary antibodies used in these studies were anti-Src (36D10), p418Src (NB100-92633), and p300 (NB100-507). Secondary antibodies were Alexa 488 goat-anti-rabbit and Alexa 594 goat-anti-mouse (Molecular Probes).
3.10 Scratch assay

Cells were first seeded in 12-well plates at a density to which they would reach confluency the following day and allowed to attach for 24 hours. Once confluent, a scratch was created in the cell monolayer using a 2-200 microliter pipette tip in each well. Once scratched, cells were washed once in culture medium to remove floating cells from the wounded area. Wash medium was replaced with medium containing either DMSO, 100nM dasatinib, 20µM C646, or 100nM dasatinib + 20µM C646. Plates were marked across the center of each well with a fine-tipped marker to indicate the field to be imaged. Pictures were then taken on a Zeiss Axiovert microscope with phase contrast for the zero hour time point. Cells were allowed to migrate into the scratch for the indicated period of time (generally 13 hours) and pictures were taken again at the same field. Cells were further allowed to migrate up until 24 hours to determine further effects of the inhibitors on the migratory capability of the cells. Pictures were then exported into MS Paint at equal resolutions and lines parallel to the cell fronts were drawn. A perpendicular line was then drawn to connect the cell fronts so that scratch width could be determined by pixel length. In the second and third time points, the scratch width was compared to the zero hour image to determine the distance migrated in that well. Each condition was performed in triplicate and results were depicted as SEM.

3.11 RNA isolation and reverse transcription

Total RNA was isolated from cells using the PureLink RNA Mini kit (Ambion, Foster City, CA). Cells were seeded in six-well plates and allowed to attach overnight. After treatment, culture medium was removed and cells were lysed in Lysis Buffer with fresh 2-mercaptoethanol and vortexed until well dispersed. Cells were then homogenized by passing the lysate through a 2-200 microliter pipette tip 12 times. After homogenization, one volume of 70% ethanol was added and lysate was centrifuged in the provided spin cartridge. RNA was washed twice with the provided buffers and eluted using RNase-free water. RNA concentration was determined spectrophotometrically with a NanoDrop. After RNA isolation, reverse transcription was carried out using the qScript cDNA Synthesis kit (Quanta Biosciences, Gaithersburg, MD). The qScript kit utilizes both oligo(dT) primers as well as random primers to reduce RT target bias. RNA was added at 1µg per reaction in a total volume of 20µL. The reaction conditions of the thermal cycler were as follows: 22°C for five minutes, 42°C for 30 minutes, and 85°C for five minutes. Newly generated cDNA was diluted 1:5 and analyzed by qPCR. Specific targets were normalized to the geometric mean of the β-actin and β2 microglobulin Ct values and calculated by ΔΔCt as validated by >90% PCR efficiency for all primer sets.
3.12 qPCR

Quantitative PCR was performed on a StepOnePlus thermocycler (Applied Biosystems Incorporated, Carlsbad, CA) using SYBR Select qPCR master mix (ABI). Reactions were carried out in 96-well optical MicroAmp Fast plates (ABI) with a total reaction volume of 10µL per well. All primer sets were designed using the Primer3 software to perform under the same cycling conditions in order to increase throughput of the assay. All primers were added at a concentration of 400nM and their robustness was determined by having a >90% PCR efficiency, a single peak on melt curve analysis, and a single band at the predicted DNA size on an agarose gel. Runs were performed using the Fast parameters and the conditions were four minutes of 95°C to activate the hot start polymerase, then three seconds at 95°C for denaturation and 30 seconds at 57°C for annealing and extension for 40 cycles. Fluorescent intensity was measured at the end of the extension phase for each cycle.

Primers used for cDNA qPCR were as follows:

ACTB: 5'-TGCCGACAGGATGCAGAAG-3'; 5'-GCCGATCCACACGGAGTACT-3'
B2M: 5'-TACATGTCTCGATCCCCACTTAACAT-3'; 5'-AGCGTACTCCAAAGATTTCAGGT-3'
HMGA2: 5'-CAGCCGTCCACATCGCGCCAG-3'; CTTGCGAGGATGTCCTTTCCAG-3'
SMYD3: 5'-TGTTCGACTGTATGGGCTTTT-3'; 5'-TTTGCAGCTTTTAAAGGCATTAG-3'

Primers used for ChIP were as follows:

Human ACTB: 5'-GGACATCCTTGGCCACTGA-3'; 5'-GGTGCTCTGAAAGCTGGCAAG-3'
Human HMGA2: 5'-TGTCAATGGAATGCAATATGAGAA-3'; 5'-TGCCATACAGGATGAGAAGC-3'
Human SMYD3: 5'-GGACATCCTTGGCCACTGA-3'; 5'-GGTGCTCTGAAAGCTGGCAAG-3'
Mouse HMGA2: 5'-CTTGCTGTCTGCGGAATCTCAC-3'; 5'-AAGATGACCTTTGCCGCACTC-3'

3.13 CyQuant proliferation assay:

Cells were plated at a density of 2,000-3,000 per well in a 96-well plate in triplicate wells and allowed to attach overnight. Medium containing a 10X concentrate of the treatment to be used was added to a final concentration of 1X. Cells were incubated in the cell culture incubator for two to three days depending on the cell type, and then removed from the incubator. CyQuant dye (Molecular Probes, Eugene, OR) was then diluted into Hank's buffered saline solution as per the manufacturer’s instructions. The cell culture medium was vacuumed off of each well of the 96-well plate and the diluted CyQuant was added to each well containing cells as well as three
that did not. The plate was immediately taken to the EnVision plate reader (Perkin Elmer, Waltham, MA) and read at a wavelength of 495nm. The background signal from the wells containing no cells was subtracted from the signal of the assay wells and the resulting signals were all compared to a DMSO control condition. A second, untreated, control was used to verify that the concentration of DMSO in the assay did not affect cell proliferation.

3.14 Soft agar colony formation assay:

Aliquots of sterile 2X agarose at concentrations of 1% and 0.7% were incubated at 70°C to fully melt for 30 minutes. Once melted, the higher concentration of agarose was mixed at a 1:1 ratio with 2X culture medium to yield 1X, 0.5% agarose medium. This agarose-containing medium was added gently to the wells of a six-well plate at a rate of 1mL/well and placed in the 4°C refrigerator for 10 minutes to solidify. This constituted the basal layer for the assay. During solidification of the basal layer, the lower concentration tubes of agarose were removed and mixed with 2X culture medium to yield 1X, 0.35% agarose medium. During this time the cells to be assayed were also trypsinized and counted by trypan blue counting using a hemocytometer. Next, 1,000 cells/mL were then suspended evenly in the 0.35% agarose medium, which was then gently pipetted in triplicate onto the top of the basal layer at a rate of 1mL/well. This upper layer constituted the growth layer. The plates were then placed in the 4°C refrigerator for 5 minutes to solidify. During this time, 1X culture medium was prepared which contained the indicated concentration of inhibitor. After solidification, the inhibitor-containing medium was gently overlaid onto the top layer at a rate of 2mL/well. Once the medium was added, the cells were allowed to grow for 18 days. After colonies had formed, the wells were stained for three hours by addition of 150µL of 0.005% crystal violet to the medium overlay. Colonies were imaged by the Coomassie setting on a FluorChem E gel imaging system (ProteinSimple, San Jose, CA). Images were quantified using the AlphaView software package (ProteinSimple) and absolute colony numbers were recorded.

3.15 Zymography

Equal numbers of cells were seeded onto six-well plates and allowed to attach overnight. Culture medium was then replaced with DMEM containing 1% FBS and supplemented with the indicated concentrations of inhibitors. Cells were allowed to grow for two days with inhibitors to generate conditioned medium. Equal volumes of conditioned medium were then harvested and mixed with SDS loading buffer without β-ME and not boiled. The samples were then loaded onto an 8% polyacrylamide gel cast containing gelatin at a final concentration of 1mg/mL. The gel was then washed in renaturation buffer (2.5% Triton-X100 in 50 mM tris pH 7.4, 5mM CaCl₂,
1μM ZnCl$_2$) at room temperature for one hour. The gel was then incubated with gentle rocking at 37°C overnight in renaturation buffer without Triton to facilitate digestion of the gelatin. After overnight incubation, the gel was stained with Coomassie brilliant blue dye for one hour. The gel was next de-stained with a solution of 10% acetic acid and 30% methanol in water for 3 hours at room temperature. The gel was imaged using the FluorChem E (ProteinSimple) with the Coomassie setting. The negative staining represented digestion by active MMPs, therefore the extent of the negative stain at each appropriate molecular weight was interpreted as MMP2 or MMP9 activity.

### 3.16 Invasion assay

BioCoat Matrigel Invasion Chamber inserts were first allowed to re-hydrate in culture medium for two hours at 37°C inside the tissue culture incubator. Next, culture medium containing 10% FBS was added to the lower portion of the invasion well. Cells were then trypsinized, counted, and seeded into the upper portion at a rate of 20,000 cells/well. This upper medium contained 1% FBS and was supplemented with the indicated concentration of inhibitor. Cells were allowed to invade through the matrigel at 37°C inside the tissue culture incubator for 18 hours. Inserts were then removed and the upper portion was swabbed clean with a cotton swab to remove the non-invasive cells. The inserts were then fixed in 100% methanol at room temperature for 10 minutes and stained in 0.005% crystal violet for one hour. The stained inserts were then washed for five minutes in 10% methanol to de-stain the background. Inserts were imaged using a Canon EOS 6D digital SLR camera (Canon, Melville, NY) mounted in the eyepiece of an inverted tissue culture microscope. Stained cells representing those which had invaded through the matrigel and migrated through the pores in the insert membrane were counted manually and absolute numbers of invaded cells were recorded.

### 3.17 Statistical analysis

Data are depicted as the mean ± SEM. Statistical significance was determined using 1-way ANOVA followed by student’s t-test. Significant p-values are labeled where indicated in the figures.
CHAPTER IV

RESULTS

4.1 Specific Aim 1: Investigate localization and interacting partners of nuclear Src

Rationale:
Previous studies by our lab have identified active Src in the nuclei of pancreatic cancer cells. As other instances of active nuclear or perinuclear Src have been reported in addition to ours, we wanted to describe the particular localization of Src in our cell line models. We have also previously identified some putative gene regulation by Src in the nucleus. Src has no conserved or experimentally demonstrated DNA-interacting or transactivational domains, meaning that any regulation of target genes is likely mediated through other proteins. In this section, we identify p300 as a Src-interacting protein and demonstrate the specific localization of Src and p300 in the nucleus.

Hypothesis:
We hypothesize that Src is localized to chromatin-containing portions of the nucleus and associates with proteins capable of mediating gene regulatory effects.

4.1.1 Identify binding partners of Src in the nucleus
We hypothesized that Src would mediate its effects in the nucleus through modulation of binding partners, similarly to the mechanism of Src activity at the membrane. Unfortunately, strong literature evidence of Src interactions in the nucleus is almost nonexistent. In order to generate a list of putative interactions, we used Panc-1 nuclear extracts and performed immunoprecipitation coupled to mass spectrometry analysis. We then compared the specific proteins identified in the Src IP to those which were absent in the pre-immune IgG control. The list of hits is represented in Table 4.1 in the order of most the specific peptides identified to the least. All proteins identified have reported nuclear or perinuclear localization, strengthening the assertion that these samples came from a clean nuclear prep. The first protein identified was USO1, a general vesicular trafficking protein that is centrally involved in transport from the endoplasmic reticulum to the golgi [247]. The next hit on the list is the other protein of interest,
p300. This protein is a major regulator of gene expression through a host of different mechanisms and was therefore an especially intriguing candidate for further validation. The next protein identified was glutathione-S-transferase π (GSTP1), which is involved in drug resistance and cytoprotection primarily in prostate cancer [248]. Heat shock protein-like 70 (HSPA1L) is a chaperone protein of the HSP70 family whose function has not been clearly identified. It is, however, involved in spermatogenesis which is also a Src-influenced developmental process [249]. The next protein is a guanine exchange factor called ARHGEF7, also known as β-PIX. β-PIX is involved in Rho/Cdc 42 signaling in trafficking [250] as well as in membrane ruffling [251]. It also enhances nuclear activation of p38 to stimulate cell migration [251]. The final protein is ALY/REF, an RNA-binding protein that is a central player in the mammalian transcription coupled to export (TREX) pathway [252]. In addition to promoting nuclear export of spliced mRNAs, ALY/REF can also activate transcription of genes [253]. These RNA-binding and gene modulation properties make ALY/REF an interesting binding partner due to the functional similarity with the early identified nuclear Src interacting proteins. These hits represent a novel reservoir of potential nuclear Src interactions and therefore insights into the function of nuclear Src.

4.1.2 Visualize Src and p300 immunolocalization in Panc-1 cells

Our Panc-1 cell line behaved similarly to cells commonly reported as Panc-1, which have a much lower doubling time [254]. We re-validated our Panc-1 cells for the nuclear localization of Src as well as for localization of p300 in these cells. Using immunofluorescent staining and confocal microscopy, we detected a localization pattern similar to that previously reported by us and others. Specifically, Src was localized diffusely throughout the cell with increased localization at cell-cell contact points. Src was localized also to perinuclear locations as well as within the nucleus itself as demonstrated by signal overlap with DAPI (Fig. 4.1A). This signal was validated as being specific by being far brighter compared to samples prepared identically except for the omission of primary anti-Src antibody. In the case of p300, a distinct nuclear localization was observed, which is the expected subcellular distribution. Notably, the p300 signal is absent from the outermost perimeter of the nuclear rim as determined by the edge of the DAPI signal, whereas Src is indeed present at this location. This indicates that p300 is likely not associated with the nuclear envelope and nuclear periphery in these cells, but Src likely is. Furthermore, Src and p300 show co-localization within the nucleus, indicating a spatial potential for interaction.
4.1.3 Investigate Src and p300 subcellular localization by fractionation

The immunofluorescent imaging of Src and p300 clearly shows gross localization, but the specific functional compartments are still not clear. Furthermore, the possibility exists that the signal seen is non-specific and the protein does not reside in that location. To address this, we performed a subcellular technique developed in Günter Blobel's lab for studying nuclear envelope proteins [255]. Each lane contains equal total protein for comparison of enrichment in each fraction (Fig 4.1B). The cytoplasmic fraction contains the cytosol as well as soluble membranous proteins, as there is NP-40 in the extraction buffer. This fraction contains relatively low total or active Src, and no detectable p300. The enrichment of GAPDH in this fraction verifies that it indeed contains soluble cytosolic proteins. Soluble tubulin also resides in the cytosol, so its presence in this fraction is expected. Grim19 is a mitochondrial protein that participates in the regulation of the electron transport chain [256]. Grim19 in this fraction indicates that the mitochondrial membrane is solubilized by the cytosolic extraction buffer. Calnexin, histone H3, and lamin A/C are almost undetectable in this fraction, indicating minimal contamination of endoplasmic reticulum or nuclear components. The next fraction (Insoluble Cytoplasmic) is derived from the insoluble cellular material that was not pelleted at low g force, but was able to be pelleted at high g force. Activated Src is detected in this fraction, a localization that is well known for Src [257], while p300 is again undetectable. The insoluble cytoplasmic matrix contains cytoskeletal proteins. This accounts for the tubulin levels observed in this fraction. Additionally, the presence of calnexin indicates that a large proportion of the endoplasmic reticulum is localized to this fraction. Because the endoplasmic reticulum is an organelle whose membranes are contiguous with the nuclear envelope, its separation from the nuclear fractions is a favorable result. This fraction is clear again of histone H3 and lamin A/C, verifying no nuclear contamination. The next fraction is the soluble protein from DNase/RNase digested nuclei. This fraction represents largely RNA- and chromatin-bound proteins, and specifically proteins associated with open chromatin that is accessible to DNase digestion. Here we see that p300 is highly enriched, along with active and total Src protein. The p300 association with active chromatin is expected, while the presence of active Src in this fraction confirms that Src is present and active within the nuclei of these cells. We also see that there is no cytoplasmic contamination of this fraction by the fact that GAPDH and tubulin are no longer detected. Histone H3, as one of the core subunits of nucleosomes, demonstrates the presence of chromatin in this fraction. Calnexin and lamin A/C are both almost undetectable, indicating that there is minimal endoplasmic reticulum contamination and nuclear envelope contamination in this fraction. Their absence here also confirms that Src is specifically enriched in the nucleus, not only associated with perinuclear membranous structures. The nuclear envelope fraction harbors very little p300 but a substantial amount of total and active Src,
which confirms the previous immunofluorescence data. This fraction is again free of cytosolic contamination but contains a higher level of endoplasmic reticulum contamination than the previous fraction, likely due to the fact that this organelle is structurally linked to the nuclear envelope. There is also chromatin associated with this fraction as demonstrated by histone H3. This is likely inactive heterochromatin, as strong association with the nuclear lamina is a major known mechanism of gene silencing [258]. The final fraction is the soluble heparin-extracted supernatant obtained after the nuclei are digested by DNase/RNase. Heparin binds strongly to DNA-binding proteins and is able to strip them away, serving the purpose of removing heterochromatin from the DNase/RNase digested nuclei in order to obtain a clean nuclear envelope fraction. In this fraction, we see p300 as well as total and active Src, implying that both proteins are involved in regulation of genomic architecture aside from simple gene activation. This fraction is clear of almost all other subcellular marker proteins aside from a large enrichment of histone H3.

4.1.4 Verification of Src activity in the nucleus

We next wanted to show that Src was functionally active within the nucleus. As no specifically nuclear substrates of Src have been identified, we were compelled to observe Src-mediated changes in general nuclear tyrosine phosphorylation. This presents a challenge, however, as Src substrates outside the nucleus are well-known to translocate and accumulate within the nucleus [259]. To overcome this issue, we decided to use purified nuclei as an incubator for an in vitro kinase assay. We isolated nuclei in the absence of the phosphatase inhibitor Na$_3$VO$_4$ and then resuspended nuclei in Src kinase assay buffer. The isolated nuclei were incubated at 37°C and then global phosphotyrosine was detected by Western blotting. The first lane shows that without Na$_3$VO$_4$, minimal net induction of phosphorylation was achieved. When Na$_3$VO$_4$ was added to the reaction mixture, a robust induction of phosphotyrosine was detected, demonstrating the presence of active tyrosine kinases within the nucleus (Fig 4.1C). This in itself is an under studied concept, but the result does not prove that Src in the nucleus has activity. To address this, we performed the assay with the additional condition of the Src inhibitor dasatinib. Again, in the lane without Na$_3$VO$_4$, there was no net increase in phosphotyrosine; however, the addition of the phosphatase inhibitor resulted in another robust induction of phosphotyrosine even in the presence of dasatinib (Fig 4.1C). Of critical interest is the fact that not all phosphotyrosine bands were induced in the dasatinib-treated samples. This result suggests two key points. First, that Src has specific kinase activity and substrates within the isolated nuclei of these cells, and second, that some nuclear tyrosine phosphorylation is mediated by kinases other than Src. This second point is largely understudied in the literature and represents a largely unknown mechanism of nuclear regulation in metazoans.
4.1.5 Verify IP-MS by co-IP

We employed mass spectrometry to identify potential Src interactions within the nucleus, but this technique itself does not confirm interaction. In order to validate the interaction between Src and p300, we employed co-immunoprecipitation of each protein. We first immunoprecipitated Src from non-denaturing nuclear lysates and probed for the specific presence of Src as well as the presence of bound p300 (Fig. 4.1D). The position of the Src band was determined by running a set percentage of input lysate. We detected a high level of Src under these conditions when compared to the pre-immune IgG control lane, indicating a robust native IP. We also probed for p300 and detected a band specifically in the Src IP lane, verifying the interaction we observed in the IP-MS experiment. In order to confirm that association of p300 and Src is not an artifact of the antibody used, we performed the inverse co-IP of p300 for Src. When immunoprecipitating with anti-p300 antibody, we detected a lower, although still substantial, amount of the target protein by Western blotting. We were also able to detect a band corresponding to Src in the specific IP but not the control. The interaction varies in strength though it appears to not be particularly strong. However, detection with any co-IP of endogenously expressed protein can often be difficult.

4.1.6 Characterize SYF cell lines

The mouse SYF cell lines are frequently used to study Src signaling and biology. We acquired the three MEF cell lines: SYF+/+ (normal MEF cells), SYF-/-(knockout of Src, Yes, and Fyn), and SYF-Src (SYF-/- cells stably overexpressing WT c-Src) as a generous gift from Leda Raptis (Queen's University, Kingston, ON). Previous reports have indicated that overexpression of c-Src in MEF cells induces activation of the kinase and modest ability to grow in soft agar (That shalloway ref). We wanted to verify this phenotypic change in our cell lines, so we first compared them to transformed v-Src MEFs in a soft agar colony formation assay. The v-Src transformed cells formed many colonies in soft agar as they are well known to do, indicating their loss of anchorage dependant growth. The SYF+/+ cells likewise performed as expected and failed to form any colonies in soft agar. This dependence on attachment for growth is characteristic of their normal and untransformed phenotype. The SYF-Src cells, on the other hand, formed about a third of the number of colonies as the v-Src transformed cells, most of which are also smaller in size (Fig. 4.2A). This enhanced growth phenotype does not represent full morphological transformation, but does show that the SYF-Src cells have altered signaling compared to the WT cells. When comparing the cells by Western blot, we can see that the SYF+/+ cells do not harbor appreciable levels of activated Src, whereas the SYF-Src cells express it at a level very similar to that of the v-Src cells. The total Src level is much higher in the SYF-Src cells, confirming its overexpression, while the levels in the v-Src detected by this antibody are close to that of the
WT cells (Fig. 4.2B). This is because most contemporary Src antibodies recognize an epitope in the C-terminus where c-Src and v-Src protein sequences differ considerably.

4.1.7 Investigate immunofluorescent localization of Src in MEF lines

Once we identified altered Src signaling in the SYF-Src cells, we were curious to see if total and active Src was also present in the nuclei of these cells. To assess this, we performed immunofluorescence and confocal microscopy as above. We wanted to compare the SYF-Src cells to the SYF+/+ cells because they present an opportunity to distinguish between normal cells and those with dysregulated Src signaling. We saw increased staining for Src in the SYF-Src cells as would be expected for cells overexpressing the target protein. We also detected staining for Src in the nuclei of both the SYF-Src and SYF+/+ cells as determined by overlap with DAPI (Fig. 4.3A). We reasoned that if the total protein can be localized to the nucleus under normal and hyperactivated conditions, perhaps the active form of the protein would offer some sort of differential expression. To address this question, we performed immunofluorescent staining for pY419-Src, the modification indicating kinase activity. Indeed, the SYF-Src cells expressed active Src in the nucleus while the SYF+/+ cells do not exhibit any such staining (Fig. 4.3B). The fact that the epitope is detected in the nuclei of cells with active Src while it remains undetected in the nuclei of cells without active Src demonstrates that the signal is not a nonspecific antibody interaction. It also establishes a differential molecular phenotype in cells exhibiting different signaling and growth patterns.

4.1.8 Compare SYF cell lines by subcellular fractionation

We wanted to further verify Src nuclear localization in the SYF cell lines as with Panc-1, so we performed the previously mentioned subcellular fractionation technique to separate the cellular compartments (Fig. 4.3C). The SYF+/+ cells contain a detectable amount of active Src in the insoluble cytoplasmic fraction, while the SYF-Src cells are strongly enriched in this fraction for active and total Src. The soluble cytoplasm of the SYF+/+ cells does not contain detectable levels of total or active Src, similar to the Panc-1 cells. Interestingly, the SYF-Src cells contain total and active Src in the soluble cytoplasmic portion, possibly due to the sheer extent of overexpression. Critically, while Src activity was detected in the insoluble cytoplasmic fraction of the SYF+/+ cells, it was not detected in the DNase/RNase fraction. Total Src protein was also only barely detectable. Contrarily, this fraction was heavily enriched for both active and total Src in the SYF-Src cells. This result confirms the immunofluorescent data and additionally verifies that active Src is present in chromatin-containing subcellular fractions in the SYF-Src cell line. Finally, in this instance, we lysed the crude digested nuclear pellet with RIPA instead of
first extracting with heparin, so the last lane represents both the nuclear envelope and the heparin extract fractions. In this lane, we see that again the SYF-Src cells are specifically enriched for total and active Src compared to the SYF+/+ cells. In both cell lines, p300 is localized to the nuclear fractions, which agrees with the localization in Panc-1 and in the reported literature. GAPDH was used as a cytosolic marker, HDAC1 as a nuclear marker, and lamin A/C as a nuclear envelope marker. Each of these marker proteins were localized to their expected subcellular localizations, indicating minimal contamination between cellular compartments.

### 4.1.9 Examine nuclear Src kinase activity in the SYF cell lines

Our identification of specific Src activity in Panc-1 nuclei prompted us to investigate the same property in the SYF model. We examined SYF-Src cells due to their exhibition of hyperactive Src as well as SYF+/+ cells to determine the normal activity of Src in the nucleus. The SYF-Src cells express a high basal level of tyrosine phosphorylation as well as a robust induction by addition of Na$_3$VO$_4$. Conversely to the Panc-1 cells, the SYF-Src phosphotyrosine induction was almost completely blocked by addition of dasatinib. Only a small number or phosphotyrosine bands were induced to the same degree as in the DMSO Na$_3$VO$_4$ control (Fig. 4.4A). In the SYF+/+ cells, there was a far lower level of basal tyrosine phosphorylation. Na$_3$VO$_4$ induced a robust increase in tyrosine phosphorylation in both the DMSO- and dasatinib-treated conditions (Fig. 4.4A). In fact, there were no detectable changes between these conditions, indicating that Src harbors no activity in the nuclei of these cells in vitro.

### 4.1.10 Investigate Src-p300 interaction in SYF cell lines

Considering the interaction between Src and p300 in Panc-1 cells harboring dysregulated Src, we hypothesized that the interaction would occur specifically in the MEFs that also exhibit dysregulated Src signaling. To address this, we performed co-immunoprecipitation on non-denaturing nuclear lysates. In the SYF+/+ cells, we used anti-p300 antibody for IP to demonstrate expression and to detect the band by Western (Fig. 4.4B). This lane showed a specific band at the molecular weight of p300 that is not present in the pre-immune IgG control lane. To detect whether Src associates with p300 in these cells, we used the anti-Src antibody for IP and probed p300 as well. There was no detectable interaction between Src and p300 in these cells as evidenced by the lack of a band at the p300 molecular weight. To further interrogate our hypothesis, we also performed co-IP for Src in SYF-Src cells. In these cells, we were able to detect a band in the specific IP compared to the pre-immune IgG corresponding to p300. This indicates that the Src-p300 interaction is specifically occurring in the cells harboring
active Src compared to cells of the same background that express endogenous and low levels of active Src.
4.2 Specific Aim 2: Examine gene regulation potential of nuclear Src

Rationale:
Previously our lab has identified gene promoter association and regulation by nuclear Src in pancreatic cancer by looking for known Stat3-regulated genes. This approach yielded only one gene, c-Myc, whose regulation is rather complex. We wanted to use a more genomic scale and blind approach to identify novel genes which nuclear Src may be regulating in order to get a clearer picture of its role in these cells. We also understand that protein-genome interactions can regulate genes and pathways that are megabases away. With that in mind, we reasoned that a focus on Src interactions with gene promoters would likely give the most straightforward genes regulated. In this section, we describe a set of nuclear Src-regulated genes and the concurrent regulation by p300.

Hypothesis:
We hypothesize that Src is associated with specific gene promoters and, in cooperation with p300, regulates their transcription.

4.2.1 Define putative nuclear Src-regulated genes by ChIP-chip
We utilized ChIP coupled to human promoter microarray to generate a pool of genes whose promoters with which Src is associated. To address this, we performed ChIP using anti-Src antibody or pre-immune IgG as control. As our goal was to identify genes for which Src association correlated with gene regulation, we also performed ChIP for Src under the condition of Src inhibition by dasatinib. Our expectation was that if Src association was the driver for transcriptional regulation, then Src disassociation with the gene promoter after inhibition would provide strong candidate genes for further study. We performed multiple sets of analysis for the data including comparing the untreated Src ChIP to the control IgG as well as the untreated compared to treated Src ChIPs. While the comparison of Src to IgG yielded a considerable number of genes, the comparison of treated vs. untreated yielded no such results. This seemingly negative result encompasses the information that at least a full set of technical and biological replicates using the same antibody generates the same set of enriched probes. The list of genes generated when comparing Src to IgG at a probe p-value of ≤0.005 and a read >150bp was approximately 325 genes. As this is still far too many to analyze thoroughly, we further selected genes that meet the criteria of being (1) of known function and (2) of relevance.
to pancreatic cancer. While these genes did not meet either criteria, there were over 15 different olfactory receptor gene family members in the list. This either represents a major function of nuclear Src or a product of significant noise within the Src-associated genomic material. There were 34 genes which met both criteria, many of which that shared similar functional significance (Table 4.2). There were a number of interesting hits that were not followed up on including polyhomeotic, SMARCE1, CHD4, and ASH1L. These are all components of major chromatin remodeling complexes in eukaryotes and represent a potential large-scale signaling axis of nuclear Src in pancreatic cancer. For our purposes, their characterization would have been too technically demanding, but they are an attractive set of genes for future study. Once we narrowed the list down to 34, we focused on pro-migratory genes. Our reasoning was that much of the known function of Src involves migration, therefore its nuclear regulation may also involve pro-migratory genes. To that end, we identified HMGA2 and SMYD3, two genes that are heavily involved in metastatic and migratory potential in a number of carcinomas including pancreatic cancer. Their probe enrichment profile is shown in Fig. 4.5A and B.

**4.2.2 Investigate the binding of Src and p300 at the HMGA2 and SMYD3 promoters**

We wanted first to validate our ChIP-chip results by performing standard ChIP and quantifying target enrichment by qPCR. To that end, we first tested for Src association with the two gene promoters compared to pre-immune IgG. To control for non-specific binding, we also tested for Src enrichment of the non-related gene promoter of actin-β (ACTB). When compared to the IgG control, both HMGA2 and SMYD3 promoter sequences were enriched in the Src ChIP samples by approximately sixfold. (Fig. 4.6A) By contrast, the signal from the Src ChIP sample was equivalent to that of the IgG sample when assaying for ACTB enrichment. This indicates that the Src association with HMGA2 and SMYD3 promoters is specific within the assay as well as specific association with target genes as opposed to a general DNA-binding event. We wanted to further validate the results obtained from the ChIP-chip by treating the cells with dasatinib. When comparing the dasatinib-treated samples to the untreated control, there is no observed decrease in promoter enrichment for either gene. Both the treated and untreated Src samples are also enriched at levels about fivefold higher than the control IgG (Fig. 4.6B). These results verify the lack of signal change in the ChIP-chip between treated and untreated samples. They also indicate that any gene regulation is likely mediated by the kinase activity of Src rather than the association of the protein itself. The essential line of reasoning, then, is to investigate whether p300 is also associated with these gene promoters and whether Src inhibition affects the localization. When comparing p300 to IgG ChIP for both HMGA2 and SMYD3, the promoter enrichment is approximately tenfold higher, showing that p300 is indeed associated with the same gene promoters as Src in these cells (Fig. 4.6C). In addition, Src inhibition does not reduce
the level of p300 at these gene promoters and, if anything, increases it. This increase does not appear to be enough to be physiologically significant though. The fact that these proteins not only physically associate, but also both associate with promoters obtained through unbiased screening, suggests that they are involved in the functional regulation of those genes in cells.

4.2.3 Analyze transcriptional regulation of HMGA2 and SMYD3 by Src and p300

Given the co-association of Src and p300 in the nucleus and their association with the promoter regions of HMGA2 and SMYD3, we wanted to determine whether they serve to regulate transcription of the target genes. To do so, we used the Src inhibitor dasatinib and the novel p300 histone acetyltransferase inhibitor C646 [260] and analyzed mRNA transcript levels of HMGA2 and SMYD3. We treated cells for short time points of one hour and three hours in an attempt to capture primary transcript regulation by Src and p300 as opposed to secondary events through other signaling cascades. At one hour, Src inhibition has little if any effect on HMGA2 or SMYD3 mRNA levels. By three hours, the levels of both transcripts are reduced to about 50% of the control (Fig. 4.7A). Inhibition of p300 for one hour decreases transcript levels by about 25-30%, but three hour inhibition reduces HMGA2 and SMYD3 transcript levels to about 50% (Fig. 4.7A). The modest change of mRNA transcripts at 1 hour for either inhibitor is expected, as the rate of mRNA turnover would have to be extremely fast to see a large change even with complete termination of de novo transcription. Given the similar decrease for both inhibitors at three hours, it is most likely that they are influencing direct transcriptional regulation of these mRNAs, although change in regulation of miRNA expression cannot be ruled out even under these kinetics [261]. The spatial association of the proteins with the gene promoter, however, points toward either regulation of transcription or of mRNA extension or processing as a probable mechanism of mRNA transcript decrease.

4.2.4 Determine HMGA2 and SMYD3 protein expression following Src or p300 inhibition

While a change in target gene transcript levels after inhibition of Src or p300 demonstrates their regulation of gene message, the more functionally relevant question is whether the protein expression level changes. To address this question, we performed Western blotting against the target proteins 24 hours after inhibiting Src and p300. As shown in Fig. 4.7B, both lanes that received dasatinib exhibit reduced active Src as represented by pY419 level. The p300 inhibitor C646 also has no effect on Src activity, which negates the potential that p300 is regulating Src activity. SMYD3 and HMGA2 levels are both reduced again by about half under Src and p300 inhibition individually, showing that the decrease in mRNA translates to a
decrease in protein expression. Furthermore, the combination of Src inhibition with p300 inhibition does not produce synergistic or even additive decrease in SMYD3 or HMGA2 protein expression. This data suggests that the regulation of these gene promoters by Src and p300 is convergent, as gene regulation through different mechanisms by these proteins would be expected to produce at least some additive effect under dual inhibition. Given again the interaction between the proteins and their associations at the same promoter regions, this hypothesis is in accordance with the observed data.

4.2.5 Assess p300-regulating pathways in response to Src or p300 inhibition

It is known that p300 histone acetyltransferase activity is regulated by phosphorylation from ERK [212] and PI3K/AKT signaling [213]. This raises the possibility that Src inhibition is actually modulating p300 activity through influence on ERK or PI3K pathways (Fig. 1.5). As such, it is possible that the p300 inhibitor may in some way influence those signaling pathways as well, considering the small body of literature surrounding it. To address this issue, we treated cells with dasatinib or C646 for three hours, as this amount of time was sufficient to produce an effect of HMGA2 and SMYD3 transcript levels, then probed for activity status of ERK and PI3K pathway members (Fig. 4.7C). We observed no change in the activation status of ERK as evidenced by p-T202/Y204 ERK1/2 expression in the dasatinib- and C646-treated lanes compared to the DMSO control. Likewise, no change in AKT activity as represented by p-S473 expression was detected between the dasatinib- or C646-treated lanes compared to DMSO. We also probed for pS-2448 mTOR, a substrate of the PI3K/AKT pathway [262], to further demonstrate a lack of effect on PI3K/AKT signaling by dasatinib or C646. We observed again no change in this phosphorylation site with either inhibitor compared to the control. These results confirm that the effects observed on Src/p300 control of HMGA2 and SMYD3 are not being mediated through known tangential pathways.

4.2.6 Determine whether Src activity correlates to HMGA2 or SMYD3 promoter association in SYF model

As we were able to define a novel interaction of Src and p300 as well as their co-regulation of specific genes in Panc-1 cells, we asked whether the similarities in Src activation and localization observed in the SYF cells correlated similarly to Src association with target gene promoters. We were unable to detect SMYD3 expression in any of the three SYF cell lines (data not shown). They did express HMGA2, however, so we decided to focus on that promoter in our studies. We then performed ChIP using anti-Src and pre-immune IgG antibodies in the SYF-Src cells, which harbor active Src and exhibit an association between Src and p300. When comparing HMGA2
promoter enrichment of the Src ChIP to the IgG ChIP, we detected a roughly sevenfold increase in signal (Fig. 4.8A). This result, however, does not rule out off-target antibody interaction with a protein associated with the HMGA2 promoter. To control for this, we performed the same experiment in parallel using SYF+/+ cells. These cells are derived from the same background as SYF-Src and express endogenous levels of wild-type Src, so non-specific binding or normal cell promoter association would be detected using these cells. As seen in Fig. 4.8A, the Src ChIP shows no enrichment over the control IgG at the HMGA2 promoter, indicating that Src association with the HMGA2 promoter in MEF cells corresponds to its activity status in the whole cell as well as the nucleus. The emulation of the SYF-Src but not the SYF+/+ cells in the molecular phenotypes observed in the Panc-1 cells provides a fertile model in which to study the signaling implications of active nuclear Src.

4.2.7 Investigate the regulation of HMGA2 by Src in the SYF model

The molecular similarities between the SYF-Src cells and Panc-1 cells brings about the question as to whether dysregulated Src is also involved in HMGA2 expression in the SYF cells. To answer this, we performed Western blotting on SYF-Src, SYF+/+, and SYF/- cells after dasatinib treatment for either eight or 24 hours (Fig. 4.8B). The DMSO-treated control lane for each cell line confirms that Src is only activated in the SYF-Src cells. The SYF+/+ cells have low background Src activity and the SYF/- cells do not express the protein at all and therefore, do not harbor any Src activity. dasatinib treatment for eight and 24 hours produces a robust inhibition of Src in the SYF-Src cells while having no detectable effect in the cells that do not harbor Src activity. HMGA2 expression is detected in the DMSO-treated samples for all three embryonic cell lines, which is expected given the fact that HMGA2 is expressed in fetal as opposed to adult tissues [263]. Src inhibition in SYF-Src cells causes a decrease in HMGA2 protein level by 8 hours and an even greater reduction by 24 hours. Critically, though, Src inhibition has no effect on HMGA2 expression at eight or 24 hours in the SYF+/+ or -/- cells which do not contain active Src. This result is both reflective of expectations and puzzling. While it is difficult to explain fully, it appears that stable Src overexpression and hyperactivation in the MEF background shifts control of HMGA2 expression to Src activity from the WT regulatory circuits. In any case, the fact that Src regulation of HMGA2 is predicated on activation state in the Panc-1 and MEF cell lines implicates hyperactivation of nuclear Src in a potentially conserved signaling pathway.
**4.2.8 Evaluate the role of p300 in HMGA2 regulation in SYF cells**

Our evidence suggests that Src hyperactivation leads to a Src/p300 signaling axis that is capable of contributing to transcriptional regulation. Because Src activation in the SYF model leads to interaction with p300 and binding to and regulation of the HMGA2 gene, we sought to test whether p300 similarly regulates nuclear Src target gene expression as it does in Panc-1 cells. We treated SYF-Src and SYF+/+ cells for 24 hours with dasatinib and C646, and performed Western blotting for HMGA2 expression (Fig. 4.9). Dasatinib treatment again robustly inhibited Src activity in the SYF-Src cells while having no observable effect on the SYF+/+ cells which do not express basal Src activity. In SYF-Src but not SYF+/+ cells, dasatinib treatment lead to the expression of HMGA2 protein. Similarly, p300 inhibition decreased HMGA2 in SYF-Src cells while having no such effect in the SYF+/+ cells. Interestingly, the combination did not produce an additive effect in the SYF-Src cells as was observed in the Panc-1 cells, while again having no effect in the SYF+/+ cells. This result illustrates the power of the ability to compare hyperactivated Src signaling to normal signaling in the SYF model. Not only does Src activation correlate to its association with and regulation of HMGA2, but it also aberrantly implicates p300 in the regulation as well. These observations further strengthen the hypothesis of a nuclear Src/p300 signaling axis.
4.3 Specific aim 3: Interrogate the biological implications of the Src/p300 signaling axis

Rationale:
The results from our line of study have demonstrated a nuclear signaling axis between Src and p300 in cells where Src signaling is dysregulated. These molecules act in concert to drive expression of the pro-migratory genes HMGA2 and SMYD3 in Panc-1 cells and HMGA2 in SYF-Src cells. Based on the known involvement of Src in migration and metastasis, we hypothesized that the Src/p300 signaling axis may also exert control over those cellular processes. We also wanted to determine whether the Src/p300 axis exerted any control over other physiological processes such as cell viability or colony formation. Lastly, we were curious as to whether physiological stimuli could induce Src nuclear activation. Other reports suggested that conditions such as calcium stimulation may have an effect. The PDAC microenvironment is also hypoxic and hypoglycemic, so we investigated stimuli related to those conditions as well.

Hypothesis:
Nuclear Src/p300 signaling drives a series of biological phenotypes including those related to migration and metastasis.

4.3.1 Seek biological stimuli that induce nuclear Src activation
Our previous studies have all taken place under baseline conditions. While there are merits to describing gene regulation by Src and p300 without any exogenous input, we reasoned that the ability to induce the phenotype would yield additional insight into the mechanisms of Src/p300 signaling. We decided to use SYF+/+ cells to determine whether any induction could be a part of normal biology and further give insight into nuclear Src function as well as Panc-1 cells to glean information about nuclear Src in pancreatic cancer. As the pancreatic cancer microenvironment is poorly vascularized, it is also glucose-deprived. We grew cells in either regular glucose (4.5mM) or low glucose (1mM) medium for 24 hours and performed nuclear fractionation to detect levels of active Src in the nuclei of SYF+/+ cells and Panc-1 cells. Neither cell line underwent Src activation or nuclear translocation under hypoglycemic conditions, and HMGA2 expression was unchanged (Fig. 4.10A). Considering the PDAC microenvironment again, hypoxia is a hallmark of pancreatic tumors. We grew cells in hypoxia bags for 24 or 48 hours to see if Src nuclear translocation might be a response to low oxygen availability. Again, Src activity and nuclear expression was not induced in either cell line at either hypoxic time point. It is of
interest to note that the Panc-1 cells grew happily under hypoxia, likely due to their PDAC origins, while the SYF+/+ cells underwent considerable cell death. Under both hypoxic conditions as well as hypoglycemia, the normally nuclear HMGA2 protein was readily detectable in the cytosol for the SYF+/+ but not the Panc-1 cells (Fig. 4.10B). This could represent a loss of nuclear envelope integrity and nuclear contamination of the cytosolic fraction under stressful conditions, or potentially a novel function of HMGA2, as its presence in the cytosol is not reported in the literature. The inflammatory tumor microenvironment also tends to promote oxidative stress of the cancer cells. We used hydrogen peroxide (H$_2$O$_2$) at concentrations of 0.1mM, 0.3mM, and 1mM for 30 minutes to see whether nuclear Src could be a response to oxidative damage. None of the conditions stimulated nuclear translocation or activation of Src in either cell line and HMGA2 expression remained steady as well (Fig. 4.10C).

Finally, it was reported that Src becomes activated and translocates into the nuclei of mouse keratinocytes during Ca$^{2+}$-stimulated differentiation [135]. To test this, we stimulated SYF+/+ cells with 3mM or 5mM extracellular Ca$^{2+}$ for three hours and isolated the nuclei. Again, there was no induction or nuclear translocation of Src under these conditions (Fig. 4.10D). While none of these stimuli induced activity of nuclear Src in either cell line, identification of conditions under which nuclear Src activity is provoked would be of great benefit to the study of this phenomenon.

### 4.3.2 Determine effects of Src and p300 inhibition on Panc-1 cell growth

We have established a set of genes regulated by Src and p300, but it is of importance to determine a biological phenotype associated with their inhibition. Cell growth phenotypes are representative of a number of biological processes, providing a simple approach to test gross changes. We first examined the effects of Src and p300 inhibition on proliferation of Panc-1 cells, as they are known to be highly refractory to most treatments. We treated cells for 72 hours and quantified cell number by CyQuant. Dasatinib treatment gave a roughly 10-15% reduction in proliferation compared to the control. This difference, if even there is one, is certainly not large enough to indicate a therapeutic use. Similarly, 10 and 20µM treatments of C646 produced negligible growth reductions in the 10-20% range. We reasoned that the combination of these drugs may overcome some of the Panc-1 resistance to therapy, so we combined dasatinib treatment with both concentrations of C646. Both combinations produced effects almost identical to the minor reductions observed in the single inhibitor treatments (Fig. 4.11A). While these results do not indicate a cytotoxic potential of Src or p300 inhibition in pancreatic cancer, they do agree with the previous results that Src and p300 signaling appear to be convergent in Panc-1 cells. Additionally, we can confirm from these studies that loss of viability does not contribute to the phenotypes observed in any of the other 2-D experiments.
using Panc-1 cells. While much research is performed in 2-D models, cancer exists in vivo as a 3-D entity. We therefore also assayed the ability of dasatinib and C646 to inhibit Panc-1 3-D growth in soft agar. Both dasatinib and C646 reduced the ability of Panc-1 cells to grow in soft agar by about 40% (Fig. 4.11B). This is encouraging for therapeutic value because it indicates that, while Src and p300 are not involved in cell proliferation and survival, they are contributing to the ability of the cells to grow under anchorage-independent conditions. Also, combination of dasatinib and C646 potentiated the effect by a small amount to about 50% inhibition. It is certainly feasible that 3-D signaling patterns are altered enough to allow combined inhibition to show for the first time in our studies any additive effect. The small benefit observed from dual inhibition, however, still supports the hypothesis that Src and p300 are working in concert in Panc-1 cells.

4.3.3 Investigate the effects of Src and p300 inhibition in the SYF model

We have utilized the three SYF cell lines to model normal Src signaling (SYF+/+), hyperactive Src signaling (SYF-Src), and genetic deletion of Src signaling (SYF-/-). While we have characterized molecular phenotypes of Src and p300 inhibition with these cells, we have not demonstrated any physiological effects. Here we once again utilized the CyQuant assay to quantify cell number, however, our end point time was 48 hours due to the incredibly fast doubling time of these MEF cells (Fig. 4.11C). We were first interested in the effects of dasatinib and C646 in the SYF-Src cells due to their dysregulation of Src signaling as well as their induced involvement of p300. Inhibition of Src and p300 affected cell morphology as well as proliferation by 25-40%. The combination did not produce a large additive effect, generating about a 50% decrease in proliferation in this assay. These results indicate a sensitivity of MEF cells with dysregulated Src signaling to Src and p300 inhibition. The SYF+/+ cells underwent minimal inhibition of proliferation when treated with dasatinib or C646, both yielding about a 15% decrease. The combination reduced proliferation by about 25% as well. It should be noted that the morphology was never altered and that these inhibitors seemed to have only a slight effect on proliferative ability as opposed to key cellular signaling events. The SYF-/- cells were essentially unaffected by either single inhibitor or combination inhibitor treatment. The differential sensitivities of each cell line in this assay are interesting, particularly with regard to p300 inhibition. The cells expressing endogenous Src are only slightly effected by p300 inhibition while the cells that do not express any Src are completely unaffected by the same treatment. The cells with hyperactivated Src are quite sensitive to p300 inhibition, supporting the idea that Src activation initiates a Src/p300 signaling axis.
4.3.4 Examine the role of Src and p300 in cell migration

Our data indicate a functional role for Src and p300 in gene regulation and general cell growth processes in Panc-1 and SYF cell lines. Given the known function of Src in migration, we questioned whether dysregulation of Src also implicates p300 in migratory potential. We first analyzed Panc-1 migration via scratch assay in the presence of dasatinib, C646, or both. It is important to note that none of these conditions affected Panc-1 viability or proliferation in 2-D culture, meaning any effect observed is specific to the process of migration. We photographed and quantified migration at 13 and 24 hours for Panc-1 cells. The DMSO treated scratch was nearly closed by 13 hours and fully closed by 24 hours. Inhibition of Src strongly inhibits migration at both time points (Fig. 4.12 A, B). This is expected and has been reported a number of times in the literature [264-266]. Interestingly, p300 inhibition reduces migration to an almost identical extent at both time points. The role of p300 in pancreatic cancer is extremely poorly defined and has never been implicated in migration in this tumor type. Additionally, combined inhibition of Src and p300 does not produce additive effects on cell migration at either time point. This result is in full agreement with all of our other 2-D studies involving Src and p300 dual inhibition in that their signaling appears to be convergent in each process assayed. These results illustrate the intriguing and novel potential of Src/p300 dual nuclear regulation of migratory capacity.

We then wanted to return to the SYF model to see whether the regulation of migration by p300 was also recapitulated in the SYF-Src cells, as well as to compare hyperactivated Src model cells to normal ones. For the SYF-Src cells, we quantified migration at ten and 24 hours. The DMSO treated scratch had again almost closed by ten hours and completely closed by 24 (Fig. 4.12A). Unsurprisingly, Src inhibition completely abolished cell migration at ten and 24 hour time points. It is important to point out the effect of dasatinib on the viability and proliferation of these cells, which makes it difficult to rule out the anti-migratory effect as being a function of reduced viability. We can make the argument, however, that the reduction in cell number at 48 hours (Fig. 4.11C) is so much less dramatic than the effect on migration that cell viability can't be the only factor influencing this result. We were pleased to find that p300 inhibition had a similarly impressive effect on the migration of these cells, blocking almost all movement at both time points. This condition again affected the viability of these cells; however, this effect was not nearly as dramatic as the effect on migration. As expected, the combination similarly blocked all cell mobility. These results are striking and highlight the strong implication of p300 in cell migration when Src signaling is dysregulated. We then investigated the effects of Src and p300 inhibition on the migration of the normal SYF+/+ cells. These cells did not display significant proliferative sensitivity to the inhibitors used, therefore inhibition of migration in this assay can be interpreted as being a direct effect of the inhibitor. The migration of these cells
was quantified at 14 and 24 hours (Fig. 4.12B). The DMSO-treated scratch closed rapidly as with the other cells used, while the dasatinib-treated cells were restricted in their migration. As migration is a normal function of Src, it is expected that the Src inhibitor would have an effect on the migration of these cells. Inhibition of p300, however, had no effect whatsoever on the migration of these cells. This demonstrates that p300 regulation of migration is not a normal function in MEFs but it is induced in cells with dysregulated Src signaling. The combination of inhibitors expectedly produced the same effect on migration as dasatinib alone. Finally, the SYF-/- cells were interrogated for their migratory response to Src and p300 inhibition. At 14 and 24 hours, Src inhibition had no significant effect on the migration of these cells (Fig. 4.12B). This result confirms the specificity of dasatinib in that the cells that do not express the drug target are unaffected by the inhibitor at the concentrations used. It is also interesting to note that the SYF-/- cell line is equally migratory as the WT MEFs, indicating that these cells have developed the ability to migrate independent of SFKs (Fig. 4.12B). Inhibition of p300 has no effect either, similarly to the SYF+/- cells, further demonstrating that p300 is not involved in normal migration of MEF cells. Dual inhibition expectedly has no effect on migration either. This result tells us that the combination of inhibitors does not produce general toxicity or non-specific effects at the concentrations used.

4.3.5 Investigate the role of Src and p300 in invasive processes

A major aspect of pancreatic cancer is its metastatic and invasive properties. Most of the mortality from PDAC is due to metastases, and therapies which can prevent this aspect of the disease may prove to be quite valuable in its treatment. To that end, we used gelatin zymography to test the activity of matrix metalloproteinases (MMPs) secreted into the culture medium. We treated Panc-1 cells with dasatinib, C646, or a combination of the two, for two days and harvested the conditioned medium. The inhibition of Src and p300 both produced a small decrease in active MMP9 as represented by degradation in the gel at approximately 84 kDa (Fig. 4.13A). The combination produced a similarly small effect, although the activity of MMP9 from these cells is quite low. We also observed changes in active MMP2, as demonstrated by degradation of the gel at approximately 64kDa. In this case, Src inhibition produced an extremely weak reduction in active MMP2, whereas p300 inhibition affected it more robustly (Fig. 4.13A). The combination did not potentiate the effect, however. These enzymes are critical for the invasion of the tumor into new tissues. We also used a matrigel-coated boyden chamber assay to assess the effect on invasive potential of Src or p300 inhibition. In the DMSO-treated wells, about 1,300 cells were able to degrade the upper matrix and migrate through the membrane to the lower chamber. Dasatinib treatment reduced this invasive potential by about half while C646 treatment reduced it by about the same amount (Fig. 4.13B). In this 3-D functional assay, the combination of inhibitors was additive, reducing
the invasive potential of these cells by about 75% compared to control. The combinatorial effect seen in 3-D is encouraging for therapeutic purposes because these conditions more closely resemble the *in vivo* environment as well as assess the critical aspects of PDAC.
<table>
<thead>
<tr>
<th>Specific Peptides</th>
<th>Total Peptides</th>
<th>Protein Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
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<td>17</td>
<td>USO1</td>
<td>Vesicular trafficking</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>p300</td>
<td>Coactivator and histone acetyltransferase</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>GSTP1</td>
<td>Regulator of redox</td>
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<td>2</td>
<td>18</td>
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**Table 4.1.** IP-MS table of proteins associated with nuclear Src in pancreatic cancer cells.
Protein lysates from isolated nuclei were subjected to IP-MS using Src and pre-immune IgG antibodies. Peptides in this table represent hits in which at least two specific peptides were present in the Src IP and absent in the IgG IP. Protein function represents a brief description of known protein activity.
Figure 4.1. Active functional Src is present in the nuclei of Panc-1 cells and is associated with p300. A, Cells were doubly immunostained with the indicated primary antibodies and visualized with Alexa488 (green) and Alexa594 (red) and mounted with Fluoromount G + DAPI (blue). Coverslips were imaged on a Leica TCS-SP5 confocal microscope. Co-localization is represented in yellow. B, Cells were subjected to subcellular fractionation and various extraction conditions to separate the indicated fractions. The indicated proteins were detected by immunoblotting with specific antibodies. C, Isolated nuclei were resuspended in kinase assay buffer and incubated 37°C and samples were lysed with RIPA buffer and probed for phosphotyrosine. Nuclei were incubated with 1mM Na$_3$VO$_4$ and 100nM dasatinib as indicated. D, Nuclear protein was harvested and incubated with indicated primary antibodies overnight. Antibody-protein complexes were precipitated and washed and run alongside the indicated amount of lysate as input. Interacting proteins were analyzed by Western. Similar results were detected in at least 4 independent experiments.
Figure 4.2. SYF-Src cells exhibit increased activity of Src kinase activity. A, Soft agar colony formation assay with SYF+/+, SYF-Src, and NIH3T3-vSrc cells. Equal cells were plated in all plates and grown in soft agar for the same duration before staining colonies with 0.005% crystal violet. B, Immunoblotting of SYF+/+, SYF-Src, and NIH3T3-vSrc cells depicting Src expression, Src kinase activity, and GAPDH as a loading control. The Src antibody used (SCBT) recognizes a C-terminal epitope of Src and therefore does not detect vSrc protein. These results were reproduced in multiple independent experiments.
Figure 4.3. **Active Src is specifically present in the nuclei of SYF-Src cells.** A, SYF+/+ and SYF-Src cells were fixed and immunostained to determine Src localization and visualized by Alexa488 (green) and DAPI (blue). Merged images show nuclear localization. B, SYF+/+ and SYF-Src cells were fixed and immunostained for pY-416 Src denoting activity of the kinase. Selected cells were imaged under a 4X zoom feature to demonstrate nuclear localization of active Src. Both cell lines were imaged under the same microscope settings and the images presented are representative of multiple image fields taken over at least two biological replicates. C, SYF+/+ and SYF-Src cells were subjected to subcellular localization and samples were probed by Western. GAPDH acts a cytosolic marker, HDAC1 as a nuclear marker, and lamin A/C as a nuclear envelope marker. The crude nuclear envelope was not extracted with heparin in these experiments.
Figure 4.4. Src is active in the nuclei of SYF-Src cells and associates with p300. A, Isolated nuclei from SYF-Src and SYF+/+ cells were resuspended in kinase buffer and incubated at 37°C to induce tyrosine phosphorylation. Nuclei were incubated with 1mM Na₃VO₄ and 100nM dasatinib as indicated. Samples were extracted with RIPA buffer and equal protein was loaded for SDS-PAGE. Membranes were probed with p-TYR-100 antibody to detect general phosphotyrosine levels. B, Nuclear extracts of SYF+/+ and SYF-Src cells were incubated with the indicated IP antibodies and complexes were precipitated, washed, and subjected to Western blotting for p300. The p300 IP for SYF+/+ depicts the positive p300 band in those cells. These results are representative of at least two independent experiments.
<table>
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<td>SMYD3</td>
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Table 4.2. Putative hits from Src ChIP-chip. ChIP-chip was performed for Src or IgG from Panc-1 nuclear extracts. The human promoter 1.0R array was used for hybridization and promoters with enrichment values of $p \leq 0.005$ spanning at least 150 base pairs were considered hits. These hits were narrowed down to those proteins with known function in pancreatic cancer and separated into general categories. Hits marked for further validation are highlighted in red.
Figure 4.5. HMGA2 and SMYD3 promoter probe signal analysis. A, HMGA2 gene image from the 2006 human genome assembly from the UCSC genome browser covering the region analyzed in the ChIP-chip data. The genomic image was superimposed above the analyzed probe sets from our ChIP-chip to provide context of the probe hits. The X-axis indicates genomic position and the Y-axis denotes the $-2\log_2$ of the enrichment p-value for each probe. The threshold line marks our threshold as $-2\log_2(23)$, or $p \leq 0.005$. Blue lines below the probe enrichment peaks indicate the regions that meet the threshold limit for at least 150 base pairs. B, Same as above for the SMYD3 gene. All results are derived from duplicate biological replicates each containing technical duplicates ($n=4$).
Figure 4.6. Src and p300 associate with the HMGA2 and SMYD3 promoters in Panc-1 cells. A, Panc-1 cells were crosslinked and prepared for ChIP with Src or IgG. Final ChIP samples were analyzed by qPCR using primers designed to amplify the target gene promoter region. Specific signal for each antibody was normalized to a 1% input sample from the same condition. Normalized IgG signal was then set as 1 and specific signals were compared to IgG. B, Panc-1 cells were treated for three hours with 100nM Dasatinib or DMSO and processed for ChIP. HMGA2 and SMYD3 promoter occupancy was analyzed and Src samples were normalized to IgG. C, Panc-1 cells were treated for three hours with 100nM dasatinib or DMSO and processed for p300 ChIP. Specific signal was compared to IgG. All data represents the mean and SEM of at least three independent experiments.
Figure 4.7. Src and p300 inhibition regulate HMGA2 and SMYD3 independent of PI3K/ERK signaling. A, Panc-1 cells were treated with 100nM dasatinib, 20µM C646, or DMSO for the indicated amount of time. After treatment, total RNA was collected and cDNA was generated and analyzed by qPCR. HMGA2 and SMYD3 mRNA levels were normalized to the geometric mean of ACTB and β2M housekeeping genes as loading control. Data represents the mean and SEM of three independent experiments. Statistically significant changes are marked, ** p<.05. B, Panc-1 cells were treated with dasatinib and C646 at the indicated concentrations for 24 hours and then whole cell lysates were subjected to Western blotting. GAPDH served as a loading control and results are representative of at least three independent experiments. C, Panc-1 cells were treated with dasatinib and C646 for the amount of time required to detect mRNA changes (three hours) and then whole cell lysates were harvested. Samples were probed with specific antibodies for the indicated phospho-epitopes of mTOR, AKT, and Erk. These total proteins as well as GAPDH serve as loading control.
Figure 4.8. Src is associated with the HMGA2 promoter and regulates its expression specifically in SYF-Src MEFs. A, An equal number of SYF+/+ and SYF-Src cells were prepared for ChIP with the indicated antibodies. Each antibody signal was normalized to the corresponding 1% input sample and IgG signal was set to 1. Primers for qPCR were synthesized against the mouse HMGA2 promoter region corresponding to the same region analyzed in the human promoter. Data represents mean and SEM of three independent experiments. B, Equal numbers of SYF-Src, SYF+/+, and SYF-/- cells were treated with 100nM dasatinib for the indicated amount of time. Whole cell lysates were subjected to Western blotting with specific antibodies with GAPDH serving as a loading control. Results are representative of at least three independent experiments.
Figure 4.9. Src and p300 specifically regulate HMGA2 in SYF-Src cells. Equal numbers of SYF-Src and SYF+/+ cells were treated with dasatinib or C646 as indicated for 24 hours and whole cell lysates were collected. Samples were subjected to Western blotting with specific antibodies. GAPDH is used as a loading control. Results are representative of three independent experiments.
Figure 4.10. Src nuclear activation is not mediated by hypoxia, hypoglycemia, oxidative stress, or calcium. A, SYF+/+ and Panc-1 cells were cultured in DMEM containing 4.5mM (high glucose)
or 1mM (low glucose) for 24 hours to induce hypoglycemic signaling pathways. Cells were then separated into cytosolic and nuclear fractions for analysis. Samples were subjected to Western blotting with specific antibodies. GAPDH served as a cytoplasmic loading control and a maker of uncontaminated nuclear fractions. B, SYF+/+ and Panc-1 cells were cultured in hypoxia bags for 24 or 48 hours to induce the hypoxic response and then separated into cytosolic and nuclear fractions as above. Samples were subjected to Western blotting using the same antibodies. C, SYF+/+ and Panc-1 cells were treated with the indicated concentrations of hydrogen peroxide (H$_2$O$_2$) for 30 minutes to induce oxidative stress. Cells were then separated into cytosolic and nuclear fractions and samples were subjected to Western blotting as above. D, SYF+/+ cells were cultured in DMEM with extracellular Ca$^{2+}$ concentrations of 1.8mM, 3mM, and 5mM for three hours to induce Ca$^{2+}$-responsive signaling. Cells were then separated into cytosolic and nuclear fractions and samples were subjected to Western blotting as above.
Figure 4.11. Effects of Src and p300 inhibition on viability and soft agar colony formation. A, Equal numbers of Panc-1 cells were seeded into 96-well plates and treated with 100nM dasatinib and the indicated concentrations of C646 for 72 hours. Cell number was then quantified using CyQuant reagent and cell numbers were normalized to DMSO. B, Equal numbers of Panc-1 cells were seeded in the top layer of a soft agar overlay in 6-well plates. Medium containing 100nM dasatinib or 20µM C646 was added after solidification and cells were allowed to grow for three weeks. Wells were then stained with 0.005% crystal violet and colonies were counted using AlphaView software. C, Equal numbers of SYF-Src, SYF+/+, and SYF-/- cells were seeded into 96-well plates and treated with 100nM dasatinib or 20µM C646 as indicated for 48 hours. Cell number was then quantified using CyQuant reagent and the cells numbers were normalized to the DMSO value for each cell line. All data above represents the mean and SEM of three independent experiments performed in triplicate.
Figure 4.12. Src and p300 regulate migration of Src-active cell lines. A, Panc-1, SYF-Src, SYF+/+, and SYF−/− cells were grown to confluency in 6-well plates and then scratched with a pipette tip. Cells were washed once with DMEM to remove floating cells and then medium containing 100nM dasatinib or 20µM C646 was added as indicated to the plates. Initial images were taken and follow-up images were taken at the same position at the indicated time point. Images shown are representative of multiple experiments. B, Quantification of the images in A. Initial scratch width was measured in each zero hour image and then the widths of the corresponding scratches were measured for each time point. The distance migrated was determined as the difference between the width at the indicated time point and the width at the corresponding zero hour. Due to variability in scratch width in repeats, data is presented as mean and SEM of one experiment performed in triplicate and is representative of three independent experiments.
Figure 4.13. Src and p300 regulate invasive properties of Panc-1 cells. A, Zymography of conditioned medium to determine activity of secreted MMPs. An equal number of cells were grown for two days in medium containing 100nM dasatinib or 20μM C646 as indicated and medium was collected and run on a gelatin-containing polyacrylamide gel. Gels were incubated at 37°C to allow digestion of gelatin and gel was stained with Coomassie dye. Negative staining indicates MMP activity and molecular weight discriminates the specific MMP. These results are representative of three individual experiments. B, Equal numbers of cells were plated onto BD Biocoat Matrigel Chamber inserts with serum-free medium in the upper half and serum-containing medium in the lower half. The medium in the upper half contained 100nM dasatinib or 20μM C646 as indicated and cells were allowed to invade through the matrigel and migrate through the pores in the membrane for 18 hours. Cells on the lower side of the membrane representing invading cells were stained with 0.005% crystal violet and counted. Results shown represent the mean and SEM of three independent experiments.
CHAPTER V

DISCUSSION

5.1  
Src is functionally localized to the nucleus in cells harboring hyperactive Src

Src has been observed to have localization throughout the cell including at focal adhesions, with perinuclear structures, and within the nuclear compartment itself. Src signaling at the membrane has been thoroughly described and most of its total function has been ascribed to that location. Much of the reason for this is that the first Src substrates to be defined were localized to focal adhesions [100, 101], as well as the fact that vSrc which is targeted to focal adhesions still maintains some transformative capacity [104]. While these are both invaluable approaches that have yielded significant findings, their inability to describe non-membrane signaling functions of Src should be questioned. The advent of general phosphotyrosine antibodies was a major breakthrough in the study of tyrosine kinases, but the assumption that they have little antigenic bias has been recently challenged [267]. The three most common phosphotyrosine antibodies, 4G10, pY20, and P-TYR-100 diverge almost as much as they overlap in antigenic sequence preference [267]. This means that targets identified by general phosphotyrosine blotting or phosphoproteomics (which requires affinity purification of pY-containing peptides) are likely to be genuine, however the absence of a target cannot be interpreted as its lack of tyrosine phosphorylation. Taken together with the fact that cytosolic proteins constitute an overwhelmingly large percentage of total cellular proteins, it is conceivable that the focal adhesion substrates are simply more easily identified and therefore presumed to be the fundamental targets of Src. In addition, while focal adhesion-targeted vSrc can induce some morphological transformation, it is far less transformative than WT vSrc [104]. This indicates a role of Src in transformation both at the focal adhesions based on the induction of some transformation, as well as a role outside of focal adhesions based on the lack of strong transformation when the kinase is not present in other locations. Our lab has previously identified Src presence in the nuclei of pancreatic cancer cells and ascribed some functional significance to its nuclear localization [268].

We sought to provide further insight into this under-studied subcellular localization of Src by identifying Src-interacting partners in purified nuclear extracts. Our mass spectrometry-based approach yielded a number of intriguing potential binding partners for Src. Of the hits that were not pursued, the most interesting with potential functional interaction were USO1 and ARHGEF7. USO1 is known to be a critical endoplasmic reticulum to golgi transport molecule
The endoplasmic reticulum is a structure contiguous with the nuclear envelope and, by our preparation method, at least partially purifies with the nuclear pellet and its constituent proteins, thereby partially present in our co-IP study. The potential for Src regulation of endoplasmic reticulum to golgi is interesting because this trafficking is increased in cancer and its inhibition can induce cell death [269, 270]. The next hit with promise is ARHGEF7, a GEF for Rac1 [271]. This is of particular interest because activation of Src is well known to activate Rac1 signaling, partially through interaction with the GEFs Tiam1 and Vav2 [272]. Src is also known to regulate vesicular trafficking with RhoD, although Src activity appears to inhibit movement of endosomes in this study [273]. ARHGEF7 is also involved in regulation of vesicular trafficking from the trans golgi network [274]. Given the known functions of these interacting proteins, the potential exists that Src is involved in a vesicular trafficking pathway that may extend from the endoplasmic reticulum to the trans golgi network. These functions, however, were outside the scope of our work and we did not pursue them. The last hit of interest was ALY/REF which, as mentioned previously, functions similarly to other known RNA-binding Src-associated proteins. ALY/REF is a major RNA export protein that is highly involved in export of spliced mRNAs from specialized transcriptional regions [275]. It is plausible that a Src-containing epigenetic regulatory complex would also function within specific chromatin regions within the nucleus to perform specialized gene regulatory roles. In fact, ALY/REF is known to engage with RUNX1 in critical gene regulation in T-lymphocytes [276], the same transcription factor with which Src associates in those cells [153].

For our purposes, the most attractive hit was p300. Src transformation induces both morphological and transcriptional changes in various cell types [277, 278]. Also, a fairly recent major report has described the epigenetic changes induced in normal breast epithelial cells by transient Src activation [243]. In this report, the authors found that changes in microRNA, cytokine, and NF-κB signaling were responsible for the oncogenic transformation [243]. Src and p300 are already both known to stimulate NF-κB signaling [279, 280]. Description of a functional Src/p300 signaling pathway raises the distinct possibility that the epigenetic transformative events presented in that report are mediated through Src activation of p300. In any case, p300 is an exceptional candidate interacting protein to describe novel gene regulation by nuclear Src. We were able to detect Src and p300 together in the nuclei of Panc-1 cells by two distinct methodologies in order to exclude the possibility of a positive artifact from any one method. Our validation of the Src/p300 interaction by inverse co-IPs indicates that this novel interaction between the two proteins is genuine. Our next efforts in this area will be focused on mapping the regions of interaction between the two proteins in order to both gain functional insight into their interaction but also to provide an approach with which to dissect their convergent signaling in these cells. The SH3 domain of Src binds to proline-rich regions, while
p300 contains a Q/P-rich C-terminal region of about 500 amino acids, presenting a potential binding site for Src. There also exists phosphoproteomic data accessible through phosphosite.org that p300 is tyrosine phosphorylated on residues within the CH1 domain [281, 282] as well as the HAT domain [283, 284], which may be both substrates for Src as well as docking sites for SH2 domain interactions. These possibilities are currently being pursued. In addition to investigating Src phosphorylation of p300, we are also interested in defining the Src activity-specific phosphotyrosine bands we detected in our in vitro nuclear kinase assay to further define nuclear Src signaling events.

We were interested in comparing our results in Panc-1 cells to a normal cell line but we were unable to find a suitable normal pancreatic epithelial cell line. When we examined the commonly used human pancreatic ductal epithelial (HPDE) cell line, we observed very high Src activity and decided it would not be a suitable model for studying normal Src signaling. Upon research, we found that the HPDE cell line was immortalized using the human papillomavirus E6E7 antigen [285]. We also found that expression of the E7 protein induces activity of SFKs [286], thus confirming our initial evaluation. We then shifted our attention to the SYF cell line model, reasoning that a WT, null, and overexpression model would provide a platform to study aberrant Src signaling. We were pleased to find that the overexpression of Src in the SYF-Src cells induced the activity of the kinase as is reported in the literature [287, 288]. Our results confirm that the overexpression of Src in these cells activates the kinase activity to a degree which is comparable to that of vSrc but does not induce transformation like vSrc. These characteristics are more akin to those in human cancers, where Src activating mutations are almost completely absent, but overexpression and hyperactivation of the kinase activity are common [35]. In this model, total and activated Src are observed to a far greater extent in the SYF-Src cells both by immunofluorescence and fractionation compared to the SYF+/+ cells. This, along with the fact that Src-specific nuclear activity is high in the SYF-Src cells and insignificant in the SYF+/+ cells, firmly establishes a differential role for nuclear Src signaling in the two cell lines.

Because of these differences, Src association with p300 specifically in the cells harboring active Src is of considerable importance. Firstly, the fact that the Src/p300 interaction is maintained between human PDAC cells and MEFs signifies that this is potentially a conserved and highly novel functional interaction rather than an aberrant event unique to cancer cells. It is also specific to cells with active Src, indicating further that it is not a constitutive interaction but that it is rather brought about by activation of Src signaling. From these results, we propose a cooperative nuclear Src/p300 regulatory mechanism by which Src can exert its nuclear effects. To fully validate this hypothesis, it will be imperative to determine the mechanism by which Src
activity influences p300. Aside from specific mechanisms, however, studying the role of p300 in Src-mediated transformation would provide insight into their convergent function. Many proteins have been implicated as critical in Src-mediated transformation including Rac1 [272], Abl [289], and Stat3 [290]. The role of Ras in Src-mediated transformation has also been studied, although the findings are somewhat inconclusive [291]. In NIH3T3 cells, Ras activation is necessary for transformation by vSrc [292]. In CEFs and rat fibroblasts, however, Ras activation is dispensable [293]. Src activity also induces PI3K both directly and through Ras, and PI3K also contributes to Src-mediated transformation [291]. As these are all pathways which are already known to activate p300, interrogation of Src transformation p300-null MEFs could provide a simple link between the functionalities of these proteins.

It also is worth pointing out that, while the SYF-Src cells exhibit far more nuclear tyrosine kinase activity as may be expected, the SYF+/+ cells clearly express other active nuclear tyrosine kinases. Dedicated tyrosine kinases are a primarily metazoan feature [294], while one of the major model organisms for studying eukaryotic nuclear regulation is the non-metazoan S. cerevisiae. The growing body of evidence for a role of tyrosine kinase signaling in the nucleus suggests that this is an under-studied segment of nuclear regulation that has much more information yet to be gleaned. Additionally, while the nuclear Src substrates in the SYF-Src cells may seem like a promising set of leads to follow, the distinct possibility exists that they are artifacts of extreme protein overexpression and do not represent physiological targets.

5.2 Src and p300 cooperate to regulate gene expression

Our characterization of Src nuclear localization calls its function there into question. There are a plethora of diverse protein functions within the nucleus. Nuclear expression of a protein may implicate it in a variety of biological processes such as DNA repair [295], RNA-binding and processing [296], initiation of transcription [297], nuclear architecture [298], nuclear transport [299], histone modification [300], and a host of others. Obviously, the mere presence of a protein in the nucleus provides little to no information on its nuclear function. Because Src is a highly modular protein whose domains have been thoroughly investigated, we also know that Src does not contain any known DNA- or RNA-binding domains, transactivation domains, lysine acetyltransferase activity, methyltransferase activity, or even nuclear localization signals (NLS). In short, Src does not fit neatly within the known nuclear mold. One potentially interesting structural feature of Src is the fact that it contains 5 FG repeats, a pattern which would be expected to occur less than 1.5 times in a protein the size of Src. FG repeats are a common component of nuclear proteins, and particularly of membrane-bound nuclear proteins [301]. The number or FG repeats in a protein also positively correlates with its nuclear presence [302]. While this provides no functional information, it could provide some information as to how Src
can be localized to the nucleus without a canonical NLS. Although each of the nuclear biological processes are distinct, as they are in the closest contact with genomic structures, many of them tend to also converge on chromatin regulation. One of the earliest indications that nuclear proteins were this multifunctional was the evidence that nuclear lamins could participate in gene regulation and RNA polymerase activity [303]. This and other reports led to considerable debate about this uncharacteristic function of a protein family which was supposed to be a simple structural intermediate filament protein [304]. After considerable work, we now know that the nuclear lamina exerts considerable effects over chromatin structure and gene regulation [305]. Similarly, nucleoporins were originally discovered as the components of the nuclear pore complex [306], an enormous discovery in its own right. The function of the nuclear pore was then delineated as a selective gateway for nuclear ingress and egress [307, 308], and the role of nuclear pore subunit was ascribed to the nucleoporins. A decade later, the first evidence that nucleoporins might act as transcriptional regulators came from leukemia cells in the form of a transcription factor fusion protein [309]. We now know that nucleoporins play a critical role in metazoan gene regulation [310-312]. In our previous study [268] we identified some gene regulatory function by nuclear Src and our present findings indicate that it interacts with p300. Together, this evidence points strongly to the probability that Src exerts at least some of its effects through gene regulation.

Our previous efforts to derive gene targets of nuclear Src involved manually testing via known Stat3-regulated genes via ChIP. This approach was somewhat justified in that we were previously interested in Src/Stat3 transcriptional complexes, but only a single target gene was identified [268]. Our previous approach was focused on canonical Stat3 targets, of which there are many, but the number of Stat3 genomic binding sites in a cell with activated Stat3 numbers in the many thousands [313]. This obviously complicates any manual approach to identifying gene binding events cooperating with Stat3. Additionally, regulation of the gene we identified (Myc) is among the tightest and most complex of known genes [314-316], further complicating our ability to derive conclusions from its expression. To circumvent these issues, we decided to take a blind approach focusing on the more novel gene associations of Src. The three options we had available were ChIP-seq, ChIP- human genome chip, and ChIP-human genome promoter chip. We were interested in global binding events but, with the sheer data processing issue of ChIP-seq and the cost prohibitive expense of whole genome arrays, the human promoter arrays provided a reasonable balance between breadth of data and feasibility.

Our ChIP-chip findings demonstrate a significant amount of Src binding events with promoters. There is likely antibody-specific noise as well as some technical error within the data, but the number of promoter hits was sufficient to provide a pool of putative targets for further study.
In the present work, we focused on genes with known function in pancreatic cancer and preferably those with relevance to the known Src-mediated functions of migration and metastasis. We originally attempted to validate MACC1, USP17, CHI3L1, HMGA2, and SMYD3. We were unable to detect any expression of MACC1 or CHI3L1 mRNA, and the USP17 hits represented an irregularity in the tiling of the array in which long genomic repeat regions amplified experimental noise and gave a false positive. We were, however, able to validate Src and p300 binding to both HMGA2 and SMYD3. Also, because we did not observe Src association with the ACTB promoter we can confirm that these binding events are not due to non-specific DNA binding produced by the assay conditions. The fact that unbiased screening for Src-associated promoters correlates to p300-associated promoters in these cells where the two proteins interact is quite significant in itself and implies that the Src/p300 interaction translates to gene regulation. These genes are of recent interest and are known to be involved in metastasis of multiple types of cancer [40, 317-319].

It is interesting to note that a number of the ChIP-chip hits involve known chromatin-modifying proteins including polyhomeotic (PHC1), SMARCE1, CHD4, and ASH1L. SMARCE1 (also known as BAF57) is a component of the mammalian SWI/SNF complex [320], while PCH1 is a member of the polycomb group repressive complex 1 (PRC1) [321]. SWI/SNF is a major chromatin remodeling complex involved in a multitude of physiological processes including maintenance of stemness [322]. It also generally acts as a tumor-suppressing complex as evidenced by frequent mutation of its subunits in cancer [323], although high SMARCE1 expression is associated with cancer [324, 325]. The PRC complexes are similarly fundamental gene repressive complexes which are strongly implicated in cancer [326]. Interestingly, the signaling between the two complexes is highly overlapping and they play opposing roles in normal and tumor cell biology [327]. Additionally, ASH1L is a trithorax group protein, which represent another endogenous chromatin remodeling complex that counter-regulates PRC [328]. ASH1L is also implicated in cancer [329]. CHD4 represents the major subunit of a different chromatin remodeling complex implicated in cancer called the nucleosome remodeling and histone deacetylase (NuRD) complex [330, 331]. The NuRD complex has been shown to antagonize SWI/SNF [332] as well as facilitate PRC recruitment and activity [333]. The strong relation between these complexes could potentially implicate Src/p300 signaling in master regulation of major related epigenetic complex genes in pancreatic cancer, and also highlights the incredibly complex epigenetic regulatory networks of the cell [334].

Our finding that both Src and p300 inhibition downregulate HMGA2 and SMYD3 mRNA and protein expression implicates Src and p300 activity in regulation of the target genes. We did not observe a decrease in promoter occupancy of either Src or p300 when Src was inhibited,
meaning that their control of gene expression isn't mediated by simple association but more likely by enzymatic activity. We also critically do not see perturbation of ERK or the PI3K substrates mTOR and AKT when using Dasatinib and C646. These are known to be downstream of Src as well as activators of p300 signaling and represent a simple mechanism whereby canonical Src signaling might act upon p300 and negate our hypothesis. By demonstrating that changes in PI3K and ERK signaling are not influencing our results, we further strengthen our claims that nuclear Src and p300 are acting in concert to drive gene expression. In pursuing the SYF model, we were also able to establish a connection between Src activity and HMGA2 expression in those cells. Src association with the HMGA2 promoter in the SYF-Src cells but not the SYF+/+ cells mirrors the results of Src association with p300 in the same cells. This demonstrates another differential signaling pattern in the SYF cell lines and implies a conserved role for active nuclear Src. This differential association led to the hypothesis that Src also regulates HMGA2 expression specifically in the SYF-Src cell line. Our analysis of Src inhibition in these three cell lines confirmed this hypothesis in that Dasatinib reduced HMGA2 expression only in the SYF-Src cells but not the others. The fact that HMGA2 is at least as highly expressed in the SYF-/- and +/+ as it is in the SYF-Src cells indicates that Src is not required for HMGA2 expression and brings about the likelihood that Src does not exert any control of HMGA2 under normal conditions. HMGA2 is primarily an embryonic gene [335], so it is not surprising that it is expressed in all three MEF cell lines and not in an active Src-dependent manner. It is slightly surprising that Src overexpression and hyperactivation wrests control of this gene from its normal regulatory elements. We reason that because SYF-Src is a stable cell line, it would have been afforded enough time for signaling pathways to re-equilibrate around the hyperactivity of Src. Our observations that p300 is also involved in HMGA2 regulation only in the SYF-Src cells serves again to validate the hypothesis that active nuclear Src drives HMGA2 expression in conjunction with p300. The lack of additive effect of Src and p300 inhibition on HMGA2 expression in the SYF-Src cells indicates that their signaling is convergent here as it was observed to be in the Panc-1 cells. Altogether, these results suggest that active nuclear Src binds to p300 and induces its regulation of target genes such as HMGA2 in pancreatic cancer as well as MEF cells.

5.3 **Src and p300 are involved in migration, particularly when Src is activated**

Cellular migration is a complex phenomenon that requires modulation of the cytoskeleton as well as dynamic regulation of cell adhesion [336]. While these events can occur in the absence of new transcription [337], the induced expression of genes is often critical in migration [338]. As HMGA2 and SMYD3 are pro-migratory and invasive factors and PDAC is a highly invasive disease, we inquired as to whether the regulation of these genes would impair migration. Given
that much of what is known about Src function involves migration [339], we postulated that the involvement of p300 with active Src may also regulate migration in cancer. While the role of Src in migration is well established, the literature contains little evidence of p300 involvement in migration, and none involving migration in pancreatic cancer. Recently, the C. elegans CBP/p300 homolog has been implicated in the regulation of directionality during migration in a manner that also relies on the Src homolog src-1 [340]. There have also been recent advances in production of pharmacological inhibitors of p300 which have been shown to block migration of prostate cancer cells [241]. These cells express AR and it was presumed that the effects of p300 inhibition were mediated through AR signaling [241]. Another report found that natural products which inhibit p300 were able to decrease migration and metastasis in a breast cancer model [245]. These natural products have low potency and poorly defined specificity [341], calling into question the specific role of p300 in those studies. Lastly, a recent report has described p300 as part of an elaborate signaling complex which directs cell migration in response to CCL21 [242].

Our results show that inhibition of Src and p300 both strongly inhibit the migration of the Src-activated cell lines Panc-1 and SYF-Src. It is unsurprising that Src inhibits migration of these cells given what is reported about Src function, but p300-mediated migration in these cells is quite novel. As p300 subcellular localization is sometimes altered in cancer, it is important to note that these cells express p300 solely in the nucleus and that its regulatory action should then be taking place in that compartment. We also observed similarly reduced migratory capacity with both individual inhibitors, and no combinatorial effect over a single agent. While it is tempting to claim a convergent effect here, it is difficult to do so without additional controls. If the mechanism behind this convergent phenotype is truly because both proteins are promoting migration within the nucleus, then an inhibitor of non-nuclear migration should provide an additive effect. If perhaps a specific FAK or RSK inhibitor were able to provide additional inhibition on migration, a strong argument could be made for Src mediating its effects here in the nucleus. The effect of Src inhibition on the SYF+/+ MEFs was equally as strong as it was with the Src-active cells, which is to be expected based on the known functions of Src. The fact that p300 is in no way implicated in migration for these cells indicates that p300 does not control cellular migration under normal conditions. The possibility is also raised that these cells are utilizing Src in a more canonical fashion, whereas the cells with high basal Src activity are utilizing nuclear Src/p300 signaling. Finally, the complete lack of response by the SYF-/- cells firstly confirms that they do not rely on Src and that p300 is again not involved in normal migration. It also validates the specificity of the inhibitors, as non-specific inhibition of migratory proteins would be apparent in these or the SYF+/+ cells.
Invasion and metastasis are possibly the largest contributors to pancreatic cancer mortality [4]. Our findings indicate that Src and p300 are both implicated in 2-D migration, but the other metastatic properties such as matrix degradation and 3-D invasion are critical in evaluating cancer cell aggressiveness. The regulation of MMPs by p300 has been reported previously in prostate cancer and HeLa cell lines [241, 342], but our results show for the first time that p300 regulates the activity of MMP2 and MMP9 in pancreatic cancer cells. We were surprised at the lack of effect from Src inhibition because it has been shown to inhibit MMPs in many previous reports including in pancreatic cancer cells [343]. Our findings do, however, indicate that Src and p300 inhibition both decrease invasion of Panc-1 cells in a 3-D invasion assay. In this context, the combination of inhibitors does provide some additive effect. These results agree with the migration and zymography data which demonstrate that Src and p300 regulate invasive properties of Panc-1 cells. These results also confirm the novel finding that p300 is involved in the regulation of pro-invasive genes and invasive properties in pancreatic cancer cells.

5.4 Significance

Pancreatic cancer is a highly lethal and poorly understood malignancy which is exacerbated by late diagnosis and metastatic disease [4]. Many of the genetic alterations that occur in PDAC are known, but this has yet to lead to any meaningful improvement in targeted therapies. This is best evidenced by the fact that the largest improvement in survival in the past three decades came from advancement of chemotherapeutic combination in FOLFIRINOX [56]. In order to identify new targets for therapy, efforts have been redoubled into understanding the molecular aspects of PDAC [344]. Our studies indicate a novel role of Src in PDAC as well as a previously undefined relationship between p300 and pancreatic cancer. These findings may lead to additional therapeutic opportunities in PDAC. Src inhibitors have a number of side effects similar to those of chemotherapeutics [345], while p300 inhibitors have not been tested clinically and may have more tolerable side effects leading to a longer course of treatment. Our demonstration of Src/p300 convergence in PDAC suggests that Src and p300 inhibition would have a similar therapeutic effect.

To this end, Src inhibition has been pursued in advanced metastatic disease with little or no benefit to survival [55]. Src activity occurs early in PDAC progression [34], and in our studies Src inhibition has little effect on viability of the cells. In an advanced disease state, de-bulking is the most effective strategy [43] and may explain why targeted therapies are less effective than cytotoxic ones. Because of the advanced state of most PDAC diagnoses, considerable effort has been put forth in order to detect PDAC at a much earlier and more treatable state [346]. Based
on our findings, early detection of nascent carcinomas would warrant therapeutic intervention on Src/p300 signaling to prevent further tumor growth, migration, and metastasis.

In all, our findings describe a novel interaction of Src and p300 in the nuclei of pancreatic cancer cells. We also demonstrate the conserved nature of this interaction by detection of this interaction specifically in MEFs harboring hyperactivated c-Src. We also defined a novel set of Src- and p300-associated genes in Panc-1 cells and validated their regulation by small molecule inhibitors. We also characterized a similar regulation of HMGA2 specifically in Src-active MEFs, demonstrating a Src/p300 nuclear signaling axis under conditions of hyperactive Src signaling. We also showed that these pathways influence migration and metastasis in a Src activity-dependent manner using Panc-1 and SYF cells. Further studies are warranted to determine the extent of Src/p300 signaling in PDAC as well as other cancer types in which Src or p300 activity are implicated.
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