

# **Genetic and Chemical Genetic Tools for Examining Context-Dependent Protein- Protein Interactions**

by

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## **Dedication**

For my parents Omar and Aziza, sisters, and brother.

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## List of Abbreviations

<b>ABD</b>	Activator binding domains
<b>BPA</b>	p-benzoyl-l-phenylalanine
<b>CBP</b>	CREB-binding protein
<b>CLL</b>	Chronic lymphocytic leukemia
<b>DMSO</b>	Dimethyl sulfoxide
<b>EGF</b>	Epidermal growth factor
<b>EGFR</b>	Epidermal growth factor receptor
<b>FP</b>	Fluorescence polarization
<b>GAP</b>	GTPase activating protein
<b>GTP</b>	Guanosine-5'-triphosphate
<b>HAT</b>	Histone acetyltransferase
<b>IC<sub>50</sub></b>	Half-maximal inhibitory concentration
<b>IDP</b>	Intrinsically disordered protein
<b>KIX</b>	Kinase inducible domain interacting domain
<b>KO</b>	Knockout
<b>LC-MS</b>	Liquid chromatography mass spectrometry
<b>MAPK</b>	Mitogen activated protein kinase
<b>MLL</b>	Mixed Lineage Leukemia
<b>NMR</b>	Nuclear magnetic resonance
<b>PPI</b>	Protein-protein interaction
<b>qRT-PCR</b>	Real-time quantitative reverse transcription polymerase chain reaction
<b>RBD</b>	Ras binding domain
<b>SDM</b>	Site-directed mutagenesis
<b>SH2</b>	Src homology domain
<b>UAA</b>	Unnatural amino acid
<b>WT</b>	Wild type

## **Abstract**

A vast network of transient protein-protein interactions (PPIs) underlies many cellular processes such as homeostasis, development, and disease. Because these networks have evolved over time to a high degree of complexity, characterization of transient PPIs is key to elucidation of the structure-function relationship of this complex network. Due to the role that these many PPIs play in maintaining a healthy cellular environment, dysregulation is associated with several diseases, including cancer, cardiovascular defects, and neurological disorders. Thus, there is significant interest in the development of tools to characterize the transient PPIs that underlie these diseases.

In Chapter 2 of this dissertation we characterized the structure-function relationship of the transient PPIs of the mitogen activated protein kinase (MAPK) signaling pathway Ras-Raf. Ras is a small GTPase consisting of four isoforms that rapidly interact with the Ras binding domain (RBD) of the kinase Raf to promote signal transduction of the MAPK pathway. Characterization of this interaction is difficult due to the high degree of homology between isoforms and the fast rate of interaction between interacting partners. We have developed a photoaffinity probe that permits the covalent capture of interacting proteins through the site-specific genetic incorporation of an unnatural amino acid, benzoylphenylalanine (BPA), for quantitative detection of the transient PPIs *in vivo*. The development of this tool

was made possible through optimization of protein expression conditions in *E. coli* and incorporation sites. The photoreactive probe was then utilized to irreversibly capture active Ras. We then analyzed the crosslinked product by Western Blot and observed a molecular weight shift indicating the effectiveness of our probe. Overall, we demonstrate that BPA-photocrosslinking in RBD represents a feasible strategy for characterizing Ras in its native context and should illuminate points of therapeutic intervention of Ras-related disease in future efforts.

In Chapter 3 we continue our investigation of the structure and function of PPIs with a detailed analysis of the transcriptional coactivator-activator complexes CBP/p300 KIX-activator binding partners. CBP/p300 KIX is a highly conserved and intrinsically disordered coactivator involved in transcriptional regulation. The highly dynamic structure of KIX allows for the interaction of several transcriptional activators at the MLL or c-Myb binding sites. However, while there is extensive characterization of KIX-activator PPIs *in vitro*, there is little cellular data that demonstrates a role for the PPIs in a functional context. Here we used a uniquely selective natural product inhibitor of KIX-activator PPIs to demonstrate that these complexes play an important role in cell cycle regulation in the context of leukemia cells. Specifically, we have utilized the small molecule garcinolic acid to inhibit KIX-dependent transcription and its impact on cellular health. We evaluated its effect through phenotypic assays that analyzed viability and mechanistic assays that analyzed gene expression and DNA content. Inhibition of these PPIs with the

natural product garcinolic acid leads to down-regulation of key cyclins, induction of apoptosis, and G1 arrest. Taken together, these data indicate that the KIX-activator PPIs may be a potential candidate for therapeutic intervention.

# Chapter 1: Introduction

## A. Abstract

Protein-protein interactions (PPIs) are at the crux of a cell's ability to grow, proliferate, and survive. Cells depend on thousands of interactions to carry out distinct roles in order to maintain proper cellular health. Although these interactions occur in a tightly regulated environment, dysregulation does occur and is a significant contributor to disease. To target malfunctioning PPIs therapeutically, it is essential to understand the structure, function, and mechanisms of the relevant protein-protein interactions. As outlined in this Chapter, both elucidating and targeting PPIs that are transient in the cell has been especially challenging despite their critical roles in signal transduction and transcriptional regulation. Here I outline the current state of the field with regard to the structure, dynamics, and molecular recognition of transient PPIs and the major challenges with regard to targeting them using genetic and chemical genetic approaches.

## **B. Background**

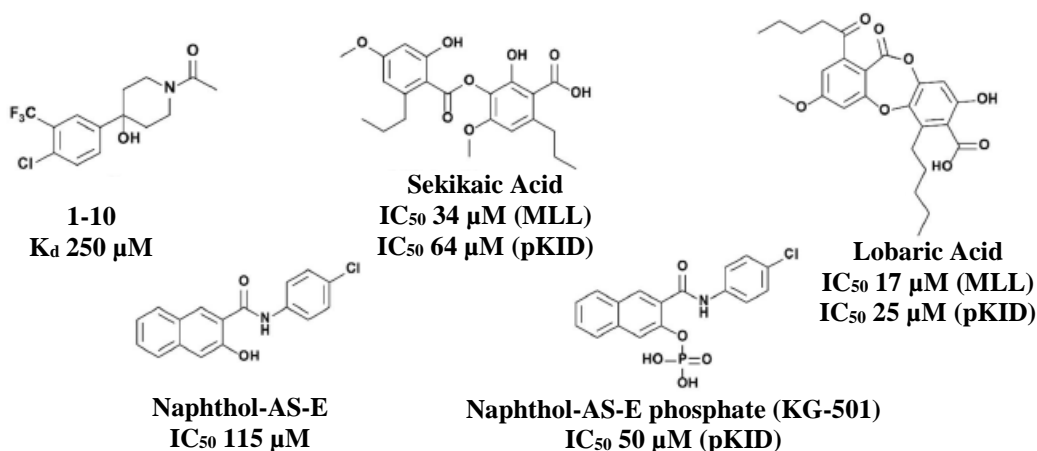
### **B.1 Targeting Transient Protein-Protein Interactions**

Protein-protein interactions (PPI) play a critical role in biological functions throughout the cell. They are particularly important, for example, in the formation of cellular machines such as signaling complexes, metabolic cycles, and the transcriptional machinery.<sup>1-3</sup> While some PPIs are easy to characterize due to their long lifetime, many interactions occur in a relatively short period time (milliseconds-seconds) (Table 1.1).<sup>4,5</sup> For example, the interaction between the activator CREB-Binding Protein (CBP) kinase inducible domain interacting domain (KIX) and activator binding partner mixed lineage leukemia (MLL) has a lifetime of approximately 3 ms, and a similar lifetime is observed in many other transcriptional machinery PPIs.<sup>5</sup> Transient interactions are vital to cellular processes such as signaling, cell cycling, and apoptosis.<sup>6</sup> Because of the key functional role that transient PPIs play, dysregulation is a frequent contributor of disease, making these PPIs promising targets for therapeutic intervention. However, transient PPIs are difficult to target due to their complex conformational dynamics, which plays a key role in some protein's ability to be promiscuous while maintaining a level of specificity.<sup>7-9</sup> This type of conformational disorder makes it difficult to inhibit these interactions, which will be discussed in detail in the *Characteristics of Protein-Protein Interactions* section. As a result, there are very few drugs that target these interactions and many are considered undruggable. This is primarily due to the instability of protein structure/surface that results in an absence of a binding site for native binding partners or small molecules to target.

Again, using KIX as an example, this intrinsically disordered protein (IDP) has been challenging to find inhibitors against due to the lack of well-defined binding pockets.<sup>10</sup> Specifically, there have only been a handful of CBP KIX inhibitors developed, including fragments **1-10**, the natural products Sekikaic acid and Lobaric acid, Naphthol-AS-E, and Naphthol-AS-E phosphate (KG-501)(Figure 1.1).<sup>11-13</sup> While each of these compounds have played a role in increasing our understand of the structure-function relationship of KIX, none of them are potent or selective enough to use as chemical probes or to target KIX therapeutically. For this reason, it is critical to expand our methodological toolbox for characterizing these challenging targets. So, a unique set of genetic and chemical genetic tools have been developed to elucidate the structure-function relationship of these transient PPIs, which will be discussed further in Chapters 2 & 3.

**Table 1.1: Lifetime and context of transient PPI complexes.**<sup>4,5</sup>

PPI Complex	Lifetime	Disease Association
CBP/p300 KIX:MLL	3 ms	Leukemia, Diabetes
Ras:Raf	50-100 ms	Cancer, Neurological Disorders, Heart Disease



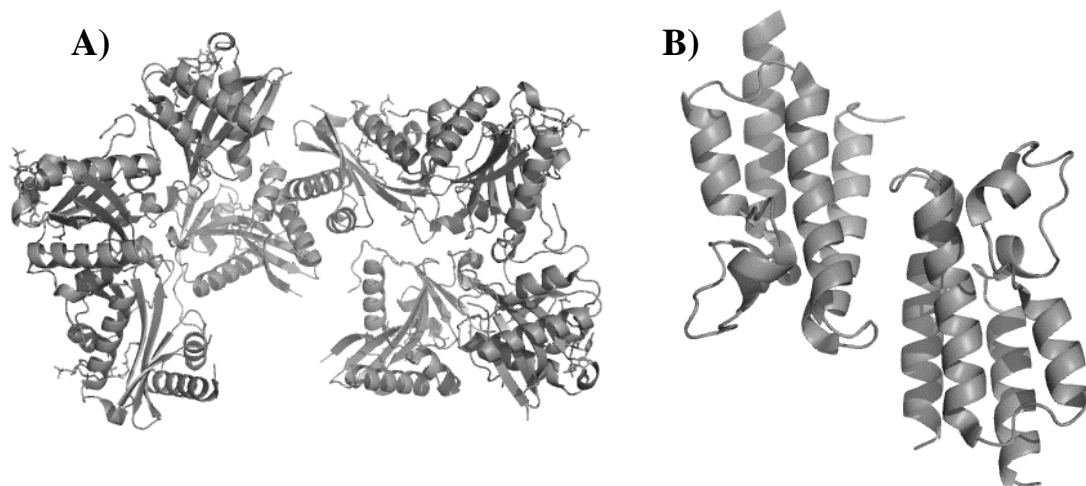
**Figure 1.1: Small molecule inhibitors of CBP KIX.** Structures of reported CBP KIX small molecule inhibitors.



## **B.2 Characteristics of Protein-Protein Interactions**

Underpinning the molecular recognition of PPIs are structure and dynamics of the constituent proteins. It is through these dynamics that PPIs can perform distinct biological functions. Key factors that underpin molecular recognition include the structural hierarchy of proteins, the PPI interface residues, and conformational dynamics of the individual binding partners and the PPI complexes.

The overall architecture and function of proteins and protein complexes is governed by primary, secondary, tertiary, and quaternary structural elements. These elements govern the general size, shape and fold of any given protein, which ultimately leads to molecular recognition of its binding partners. The primary structure of a protein is simply the constituent linear sequence of amino acids. Secondary structure occurs when inter-residue interactions lead to 3-dimensional folds such as an alpha-helix, beta-sheet, beta-loop, and loops and is directly related to the sequence of the protein. Tertiary structure is the higher-order arrangement of the secondary structural elements, which are stabilized by non-covalent forces. Quaternary structure, the highest level, is the spatial arrangement of multiple tertiary structures in a single protein. Proteins with multi-domains contain this type of quaternary structure, which typically suggests the protein has two or more distinct biological functions. One example of this is the histone acetyltransferase KAT2A (GCN5), which consist of a histone acetyltransferase (HAT) domain and bromodomain.<sup>14</sup> While the HAT domain functions as either a succinyltransferase or acetyltransferase for histones, the bromodomain is responsible for “reading” the acetylation marks. (Figure 1.2).<sup>15,16</sup>



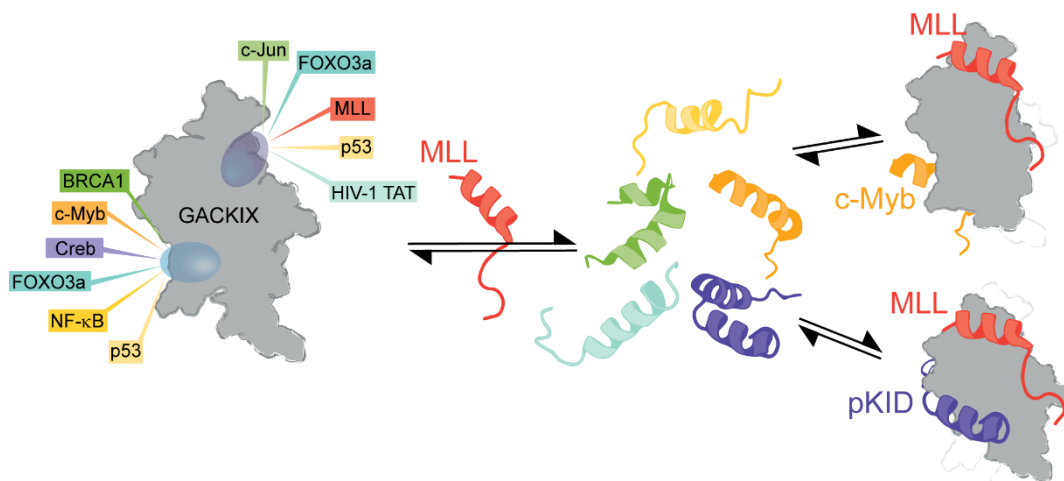
**Figure 1.2: Crystal structures of KAT2A HAT and bromodomain.** KAT2A is a multidomain protein with distinct biological functions: (A) HAT domain acetylates histones (B) bromodomain “reads” acetylation marks. PDB: 5TRL, 6J3P

The maintenance of a fully folded protein structure relies upon non-covalent electrostatic interactions through amino acids; the same noncovalent interactions are used for inter-protein binding events. These include residue-residue hydrogen bonds as well as hydrogen bonds between amino acids and water. Additionally, charge-charge interactions between Asp/Glu and Lys/Arg/His are also frequently observed.<sup>17,18</sup> In the context of transient PPIs, hydrophobic interactions often play an important role in the specificity and affinity of the complex by providing conformational flexibility to the interface. Nonpolar residues such as Ala, Leu, Ile, and Val are usually found at the interface of PPIs, enclosed away from the hydrophilic environment in which proteins typically reside.<sup>19</sup> These residues, known as dehydrons, play a critical role in the removal of water given that they are initially exposed to the water-soluble environment before interacting with a binding partner.<sup>20</sup> This is important for binding and stability of PPIs since desolvated hydrogen bonding is more favorable than those that are solvent exposed. An exception to this would be transmembrane proteins that are found in various membranes of the cell, which is made up of a thin lipid bilayer of polar molecules.<sup>21</sup> In this case, the hydrophilic residues are not

exposed to the hydrophobic environment.

Lastly, conformational dynamics enable a single protein to recognize many distinct binding partners, underpinning molecular recognition. As a result of disorder-to-order conformational changes, there is a substantial entropic cost that contributes significantly to the thermodynamics of the protein, which supports the molecular recognition of binding partners.<sup>22,23</sup> Additionally, cooperative binding of ligands is often observed in conformationally dynamic proteins. A representative of this is the KIX domain discussed earlier. KIX has two binding surfaces for interaction with transcriptional activators and more than 10 unique binding partners (Figure 1.3). It is the conformational plasticity of the motif that enables binding site remodeling to complement each unique binding partner.<sup>24</sup> A second role of the conformational dynamics is that the two binding sites are in allosteric communication. For example, when the activator MLL complexes with KIX, this leads to enhanced affinity for the CREB and cMyb binding partners.<sup>25</sup> It is likely that this is due to a decrease in conformational entropy that occurs upon MLL binding. This cooperativity plays a role in maintaining selective binding; the binary MLL•KIX complex selectively interacts with only two of the many potential binding partners. Lastly, KIX is only one domain in the multidomain protein CBP/p300 yet it is capable of interdomain communication with the HAT domain of CBP/p300. For example, when the activators Tax and CREB complexes with KIX, the coactivator recruits the HAT domain of CBP/p300 to promote Tax transactivation.<sup>26</sup> While these examples make clear the importance of dynamics in recognition and function, what is perhaps less clear is the challenge that dynamics pose for chemical tool discovery. Again, using the example of KIX, there is only a single crystal structure of this domain and as discussed

later in this chapter, discovering selective and potent small-molecule modulators by traditional routes has been filled with failure.<sup>27</sup> Thus, this motif, as is true of many other domains, lacks the chemical probes needed to dissect its role in healthy and diseased cellular processes.



**Figure 1.3: CBP/p300 KIX has two binding surfaces.** CBP/p300 KIX has an MLL and c-Myb binding site that interacts with more than 10 activator binding partners. The conformational plasticity of the domain enables the multipartner binding profile and also is key for selectivity. When MLL for example, binds the resulting binary complex selectively interacts with only two of the potential ligands for the second site due reduced conformational entropy.

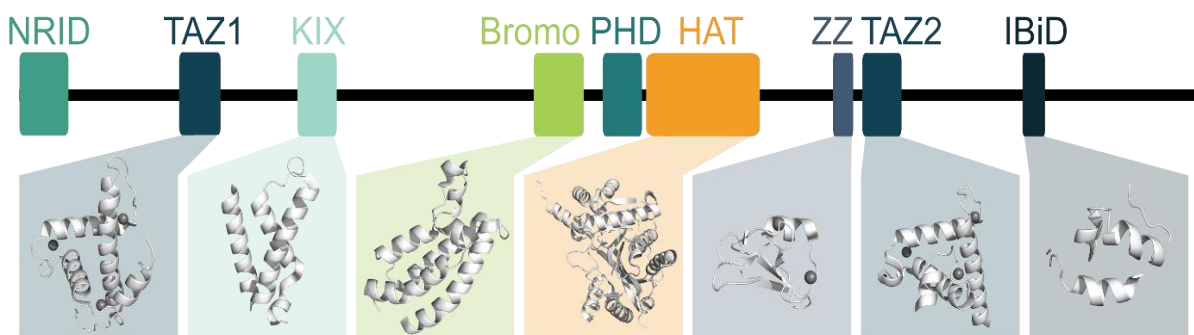
### B.3 Context-Dependent Protein-Protein Interactions: CBP/p300 and Ras

One often unrecognized advantage of targeting PPIs from a therapeutic standpoint is that many PPIs exist only in particular contexts, such as tissue type, subcellular compartment, or disease state. Coactivator-transcription factor complexes are excellent examples of this, consisting of many different heterodimer protein interactions that regulate gene expression. As mentioned previously, coactivator-transcription factor complexes rely on dynamic structural properties to interacting with proteins and elicit a functional response.<sup>28</sup> For example, CBP is a large multi- domain protein (265 kDa) encoded by the *CREBBP* gene that is involved in transcription coactivation and histone acetylation through its HAT domain.<sup>29</sup> CBP is a homolog of CBP-1 in *C. elegans* as well

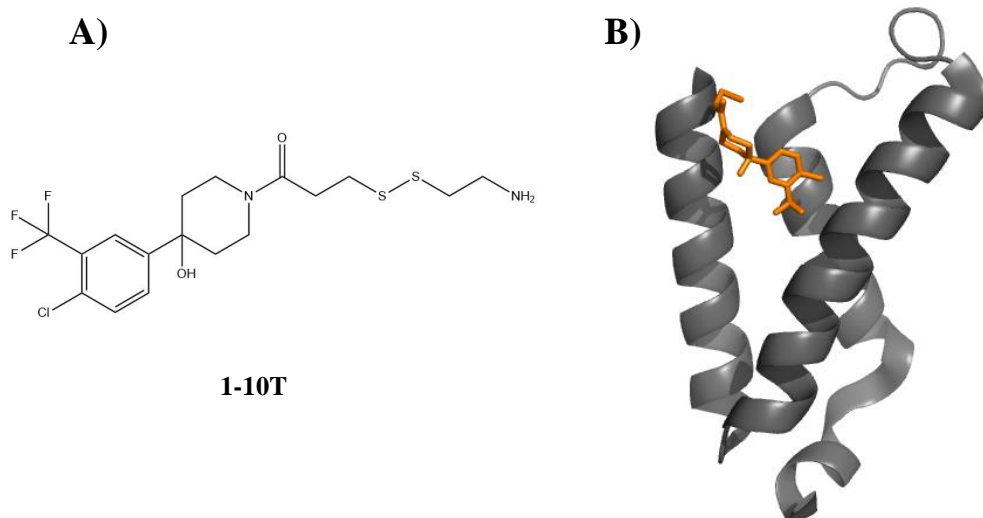
as a mammalian homolog p300.<sup>30-32</sup> While CBP and p300 share the same domains and have functional overlap, there is evidence that they are not interchangeable. For example, one study examined the impact on motor skill learning in mice when either CBP or p300 were mutated. It was observed that the CBP mutation lead to a deficit in motor skill learning while the same mutation in p300 exhibited normal motor skill learning.<sup>33</sup> This functional distinction becomes more apparent in biological systems where CBP/p300 is dysregulated, such as in prostate and lung cancer. For example, while CBP/p300 contributes to androgen-induced regulation of genes in prostate cancer cell C4-2B, 47% of those genes are dependent on p300 and only 0.3% on CBP.<sup>34</sup> One other example revealed the synergistic effect between CBP and cleavage protein CPSF4 in promoting tumor growth in lung cancer cells. Knockdown of CBP or inhibition its HAT activity induced programmed cell death and inhibited lung cancer cell growth.<sup>35</sup> Therefore, characterization of the unique interactions in these aberrant systems is critical for developing a therapeutic tool for intervention.

CBP contains several globular domains that interact with a wide array of transcriptional factors and is involved in the regulation of more than 16,000 genes.<sup>36</sup> CBP is made up of a TAZ1, TAZ2, KIX, HAT, NCBD, ZZ, BRD, and CH2 domains (Figure 1.4).<sup>37-43</sup> Due to the domains being either intrinsically disordered or partially ordered it has been challenging to determine the structure of each individual domain as well as the structure of full-length CBP. Nevertheless, many of the structures have been determined when introducing biological binding partners of each domain, such as KIX binding partners CREB, FOXO3a, and c-Myb.<sup>44-46</sup> In particular, the KIX domain has been difficult to characterize due to its intrinsically disordered nature. Yet, our lab has been able to

characterize the KIX domain through stabilization with a small molecule, 1-10.<sup>27</sup> This has led to the computational and experimental elucidation of the conformational dynamics of KIX, which has expanded our molecular knowledge of the protein. Specifically, 1-10 tethered to KIX L664C led to a  $\geq 20\%$  increase in melting temperature of KIX and the crystallization of KIX:1-10 complex to a 2.0 Å resolution, which was the first crystal structure of the domain (Figure 1.5).<sup>27,47</sup> From this stabilization, we were able to characterize specific residues (Ile611, Leu628, Leu607, Val635, and Tyr63) within the MLL binding site in which 1-10 occupied, with two of them being previously reported.<sup>48,49</sup> Additionally, a flexible loop region between helices  $\alpha_1$  and  $\alpha_2$  were determined to play a critical in the ability of KIX to bind a diverse group of activator binding proteins. 1-10 was instrumental in increasing our understanding of the structure dynamics of KIX. As a result, it opened the door for additional opportunities to further explore the structure-function relationship of KIX, especially in regulating transcription. This molecular insight is critical for elucidating the coactivator's role promoting disease such as elucidating KIX's role in supporting the survival of leukemia cells.



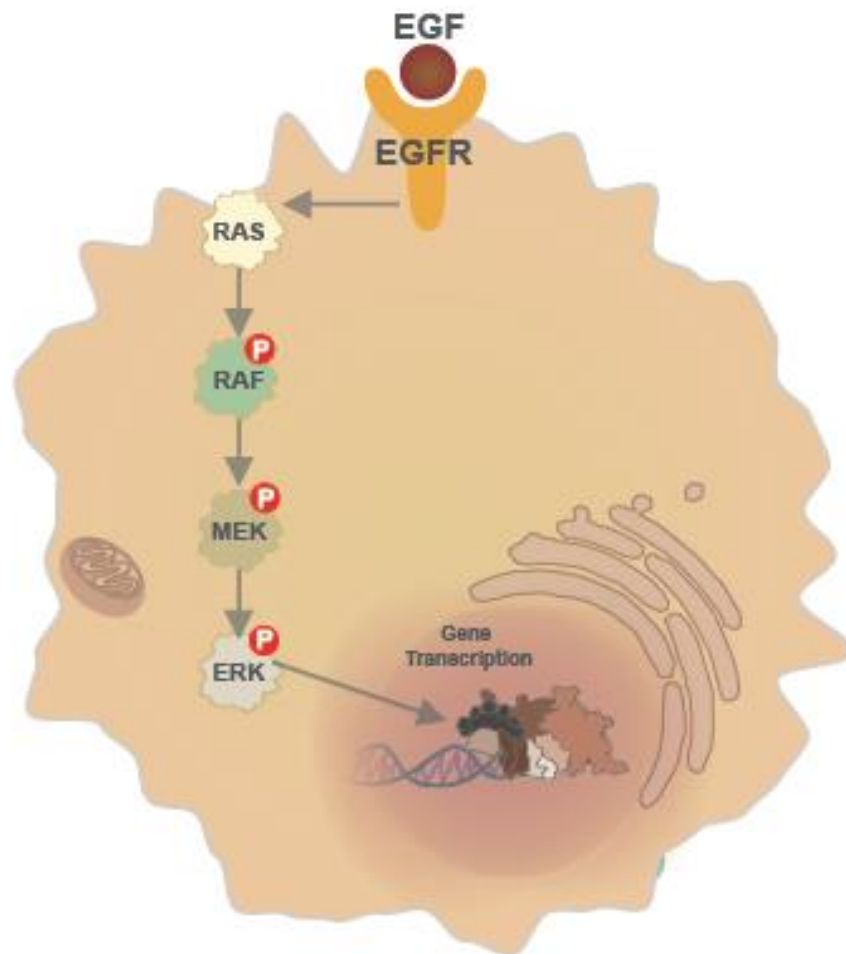
**Figure 1.4: CBP/p300 domains.** Domains that comprise the CBP/p300 coactivator. The Taz1, KIX, TAZ2, ZZ and IBiD motifs are responsible for forming complexes with DNA-bound transcription factors. The HAT and Bromo domains regulate lysine acetylation.



**Figure 1.5: 1-10T tethered to CBP/p300 KIX.** (A) Small molecule fragment 1-10 with disulfide linkage for tethering experiments. (B) Crystal structure of 1-10T tethered to KIX L644C.

Signaling cascades are another place in which context-dependent and dynamic PPIs play a critical role. The MAPK pathway regulates cell growth, proliferation, and apoptosis, all of which are critical processes in determining cell fate.<sup>50</sup> The MAPK signaling pathway consist of many kinases that facilitate signaling transduction for promoting gene transcription. Signaling typically is initiated upon binding of epidermal growth factor (EGF) to the transmembrane protein epidermal growth factor receptor (EGFR).<sup>51</sup> Small GTPase Ras is then recruited to the receptor via palmitoylation to initiate a cascade of phosphorylation, which is critical for signal transduction.<sup>52</sup> Ras activation leads to the phosphorylation of kinases Raf, Mek, and Erk.<sup>53</sup> Erk localizes to the nucleus to facilitate transcription of target genes of the pathway (Figure 1.6).<sup>54</sup> Many of these proteins have also been implicated in the localization and regulation of other signaling pathways. As a result of these multiple-functions, these proteins are implicated in the development of several diseases, such as cancer, heart disease, and neurological

disorders.<sup>47,55,56</sup> One such example is the mutation and overexpression of Ras, which is responsible for promoting growth activity through phosphorylation of Raf. Through this central regulatory interaction of the MAPK pathway, healthy cells can develop into various types of cancer, such as pancreatic, lung, and colon cancer.<sup>57-60</sup>



**Figure 1.6: MAPK Signaling Pathway.** The MAPK signaling cascade is initiated at EGFR upon epidermal growth factor engagement. From there, Ras is activated and leads to downstream phosphorylation of Raf, Mek, and Erk, which localizes to the nucleus to promote transcription.



Ras regulates the MAPK signaling pathway through catalysis of guanosine-5'-triphosphate (GTP), with assistance from GTPase activating protein (GAP).<sup>61</sup> Although Ras can catalyze GTP independently, the rate the hydrolysis reaction is significantly increased through its interaction with GAP.<sup>62</sup> GAP harbors a highly conserved “arginine finger” that mediates cleavage of GTP upon entering the binding pocket.<sup>63</sup> Arginine stabilizes glutamine-61 in Ras to position a water molecule for a nucleophilic attack. Through this stabilization and catalysis of RasGTP, Raf is activated via phosphorylation and promotes downstream signal transduction of the MAPK pathway.<sup>64</sup> Ras isoform often undergoes mutations that causes this catalytic process to be constitutively active and leads to the various forms of cancers mentioned above.<sup>57</sup> While there is evidence that these different Ras-Raf complexes play distinct roles in cancer, it has not been possible to dissect the them. For example, K-Ras mutations are commonly present in prostate cancer, but it remains unclear the contribution between K-Ras isoforms K-Ras4A and K-Ras4B.<sup>58,65</sup> This dysregulation of the MAPK signaling cascade presents an opportunity to further understand the impact of Ras mutations in the promotion of cancer.

## **C. Genetic Methods for Studying Protein-Protein Interactions**

### **C.1 Overview of Current Genetic Methods for Examining Protein-Protein Interactions**

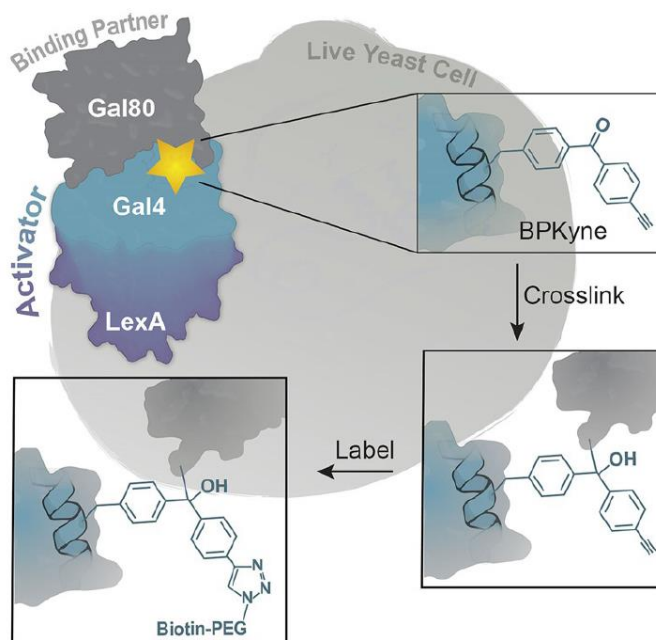
The characterization of PPIs is an essential step in improving our understanding of the structure-function relationship of transient interactions, especially in disease states. Specifically, important questions include the identification and examination of unknown binding partners, the nature of the disease milieu in which PPIs occurs, and key amino acid residues responsible propagating cell aberration. For example, cancer

involves a spectrum of PPI abnormalities at both the genetic and protein level, such as translocations, overexpression or loss of expression. By elucidating the interactions that are dysregulated in disease, we are better equipped to treat these aberrant cells. One way to accomplish this characterization is by altering the genetic code from which the interacting proteins were synthesized. These alterations facilitate the examination of context-dependent protein interactions for both *in vitro* and *in vivo* systems, especially of transient PPIs. Conventional methods such as affinity purification, co-immunoprecipitation, and yeast two hybrid are not effective for characterization of transient and/or conformationally dynamic PPIs. For example, while GST-Raf-RBD is the conventional method for active Ras isolation, this pulldown method lacks the ability to stabilize the transient interaction risking loss of the complex before an opportunity to characterize.<sup>66</sup> As a result, many of these conventional methods have been used in combination with other techniques such as unnatural amino acid (UAA) incorporation to effectively stabilize and analyze transient PPIs. This sort of combination can be used to illuminate a wide array of structure-function relationships between transient PPIs in ways that conventional methods cannot achieve. In the next section, a few of these cases will be discussed.

## **C.2 Genetic Methods for Characterizing Protein-Protein Interactions**

One example of a recently developed genetic method is the use of non-canonical amino acids for genetic incorporation using *E. coli* tyrosyl tRNA/synthetase pair (tRNA<sup>Tyr</sup>[CUA-TyrRS]).<sup>67</sup> This system has led to the development and expansion of the UAA tool kit for characterizing PPIs. In addition to the tRNA synthetase pair, UAA

system relies on site-directed mutagenesis for directing the site of incorporation. Specifically, mutating a codon of interest to an amber stop codon allows the *E. coli* based tRNA system to selectively recognize unnatural amino acids such as aryl azides, diazirines, or benzophenones. Alteration of native residues to non-canonical residues permits stabilization of transient interactions such as transcriptional coactivators through photoreactive crosslinking. For example, aryl azide-functionalized amino acids are desirable for performing selective labeling of protein complexes in the cell and diazirines are attractive for their ease of activation while having better photostability.<sup>68,69</sup> This presents an opportunity to thoroughly characterize transient PPIs. Our lab has successfully designed a bifunctional UAA for crosslinking and isolating PPIs in yeast. By incorporating the UAA BPKyne in yeast protein Gal4, our lab has been able to not only crosslink the Gal80-Gal4 protein complex, but also isolate the interaction through treatment of the alkyne handle on BPKyne with biotin-azide probe.<sup>70</sup>



**Figure 1.7: Incorporation of BPKyne in Gal4.** Unnatural amino acid BPKyne is incorporated in yeast protein Gal4 to covalently capture and isolate Gal80.

UAA incorporation has also been successfully implemented in human biological systems for characterization of PPIs. One such example is the incorporation of *p*-benzoyl-L-phenylalanine (*p*Bpa) into Grb2's Src homology 2 (SH2) domain to examine its interacting proteins.<sup>71</sup> Site of incorporation of *p*Bpa was determined based on the solution structure of Grb2 with a peptide inhibitor.<sup>72</sup> Once incorporated, Grb2 interacting partners were photocrosslinked upon exposure to 365 nm light. The EGFR is a known interacting partner of Grb2, which was used as a proof of concept.<sup>73</sup> Grb2 and EGFR were successfully photocrosslinked when *p*Bpa was incorporated at different positions within the SH2 domain. Overall, this suggest that UAAs can be applied to mammalian systems for examining PPIs in a site-specific manner.

In addition to SDM and UAA incorporation, PPIs have also been examined through employing the transfection of gene products into mammalian cells. Rather than altering the native genetic material, transfections are a temporary introduction of genetic material, leading to overexpression of the gene being introduced, which is reduced significantly after passaging the cells. This method can be especially useful when building a model system to mimic that of a cancer cell. One example is the transfection of K-Ras mutant G12V, which is often observed in pancreatic cancer cells, and T35S in SW48 cells. Transfection of this double mutant permitted the analysis on downstream signaling kinase Erk1/2 and H3K9 acetylation in colorectal cancer.<sup>74</sup> This revealed that K-Ras and Erk was a critical regulatory complex for H3K9 acetylation.

While the genetic methods mentioned above for evaluating PPIs in structure and function have been significantly effective in characterizing transient and context-dependent interactions, there are a number of caveats to these methods that prevent a more

complex understanding of PPIs. Generally, many of the genetic approaches discussed above do not possess the capability to be conditional (or activated on command). Further, cells can adapt to mutations, they may not faithfully recapitulate endogenous interactions in the case of UAAs or led to unwanted stress events, which both diminish the desired outcome of the approach. Chemical genetic methods help to alleviate some these caveats faced by genetic approaches.

## **D. Chemical Genetic Methods for Studying Protein-Protein Interactions**

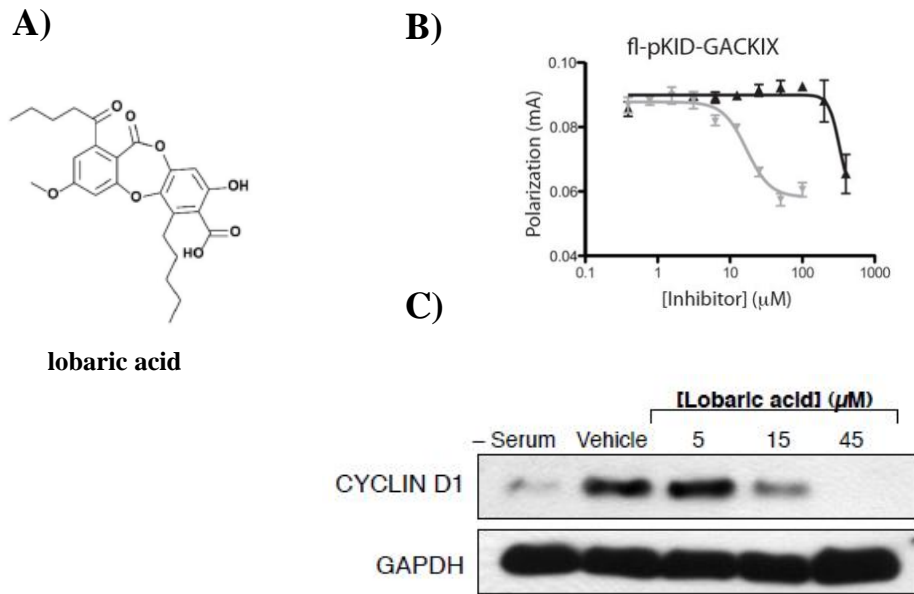
### **D.1 Overview of Current Genetic Methods for Examining Protein-Protein Interactions**

In addition to characterizing PPIs by genetic methods, a chemical genetic approach also permits analysis of a wide array of protein interactions. This approach can be made conditional and easily accessible, unlike the genetic approach, due to the ability to dose cells with compounds and wash away immediately.<sup>75</sup> Phenotypic and mechanistic analysis may be conducted shortly after dosing cells with compound without altering the genetic code. To this end, chemical genetic methods have played a significant role in elucidating the structure-function relationship of proteins and their interacting partners, which has transformed our ability to effectively treat many diseases. Chemical genetic approaches often begin with a library screen to identify a profile of lead compounds for a target of interest.<sup>76</sup> The approach can also utilize peptides or natural products synthesized by enzymes.

## **D.2 Chemical Genetic Methods for Characterizing Protein-Protein Interaction**

Small molecule PPI inhibitors are often discovered through a chemical library screen against the target of interest. These small molecules can typically be used as chemical probes that may be further developed. For example, chronic lymphocytic leukemia (CLL) is a cancer of the white blood cells and the most common type of leukemia in adults; so, developing small molecules that can inhibit its growth has significant impacts. Antiapoptotic protein BCL2 is vital to the survival of CLL cells making them resistant to apoptosis. Through a chemical compound screen, small molecule Venetoclax was found to be a selective inhibitor of BCL2. Venetoclax treatment of CLL cells led to the induction of apoptosis and ultimately became the first FDA approved PPI inhibitor.<sup>77</sup>

One other example of chemical library screens being utilized to identify PPI inhibitors involves the KIX domain of CBP/p300. As mentioned previously, KIX is an intrinsically disordered protein that has been challenging to target with small molecules until our lab began examining the protein and its activator interacting partners. Through a high-throughput fluorescence polarization (FP) assay, a chemical library screen of 15,000 natural product compounds was performed with 64 active compounds identified as inhibiting KIX and its interacting partner MLL. Further examination revealed that the natural product lobaric acid dose dependently downregulated Cyclin D1, an activator gene target of c-Jun (Figure 1.8).<sup>11</sup> Now, using lobaric acid as a chemical probe, KIX and its various interacting partners may be further characterized. Additionally, it presents an opportunity for the utility of other natural products to be used as chemical probes in interrogating transient coactivator-activator complexes and act as drug leads.



**Figure 1.8: Natural product lobaric acid blocking KIX-dependent transcription.** (A) Natural product lobaric acid was discovered from a chemical library screen against CBP/p300 KIX. (B) The small molecule inhibits MLL from binding to KIX as shown by lobaric acid (grey) and lecanoric acid (black) and (C) dose dependently downregulations Cyclin D1 protein expression in HeLa cells.

Current chemical genetic methods are successful for evaluating PPI structure and function with well-defined binding sites within proteins; however, there are limitations to this approach with proteins surfaces that are dynamic and traditionally thought to be “undruggable”. Unfortunately, this often results in screening for compounds that are not selective and/or potent enough to permit rigorous analysis of the target protein. One such example is the inhibitor C646, which is neither potent nor selective for CBP/p300 HAT domain yet has been widely used in examining histone acetyltransferase activity.<sup>78</sup> Alternatively, chemical libraries may contain compounds that bind with a relatively high affinity to a specific in protein with a well-defined binding site, but remain non-selective due to significant overlap amongst proteins with comparable binding motifs.

## E. Dissertation Summary

The overarching goal of my research has been to characterize transient and context-dependent PPIs. Specifically, examination of upstream and downstream regulatory machinery for cellular processes has been successfully demonstrated using both genetic and chemical approaches. In Chapter 2, I discuss the development of a biochemical probe derived from Raf for covalently capturing active Ras using a photocrosslinkable UAA. Examining this interaction is particularly difficult due to Ras remaining active for <100 ms. We have constructed a novel biochemical tool using in combination site-directed mutagenesis, UAA incorporation, and *E. coli* expression. We have validated our probe by mimicking normal and abnormal cellular environments. Lastly, we have created an opportunity for the analysis of Ras isoform's distinct context-dependent contribution in signaling pathways. Chapter 3 further explores transient interactions at the transcriptional level. As mentioned previously, characterizing KIX has been difficult due to its disordered conformational dynamics. We have discovered and characterized *in vitro* the CBP/p300 KIX inhibitor garcinolic acid. Using garcinolic acid, I have characterized CBP/p300 KIX-dependent transcription and found that the coactivator regulates cell cycle and cell viability. This work is significant to the field of chemical biology, as it expands the chemical biologist's toolkit for examining transient PPIs and understanding of intrinsically disordered transcriptional coactivators.



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## **Chapter 2: Photocrosslinking Small GTPase Ras Using Unnatural Amino Acid Incorporated Ras Binding Domain**

### **A. Abstract**

The mitogen activated protein kinase (MAPK) pathway plays an essential role in regulating cell growth, proliferation, and apoptosis. Although the MAPK pathway has been extensively studied, it remains unclear how MAPK proteins are dysregulated during cancer development. One such example is the oncogenic small GTPase Ras, which plays an essential role in promoting MAPK signaling through activation of Raf. Raf is the regulatory link between Ras and downstream signaling molecules that ultimately promote transcription. Dysregulation of Ras has been reported to promote transformation in different epithelial cancer cell lines; however, the distinct role of each of the four Ras isoforms is unclear. Here we develop a technique for the incorporation of an unnatural amino acid into the Ras binding domain (RBD) of Raf for covalent capture of the complexes. This permits analysis of Ras isoforms by stabilization of the Ras-RBD complex. In the future, this will enable the quantitative measurement of *in vivo* concentration of each active Ras isoform bound to Raf.

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The research described in chapter 2 was performed in partnership with Dr. Rachel Pricer. Dr. Sarah Haynes performed the mass spectrometry experiments.



## **B. Background**

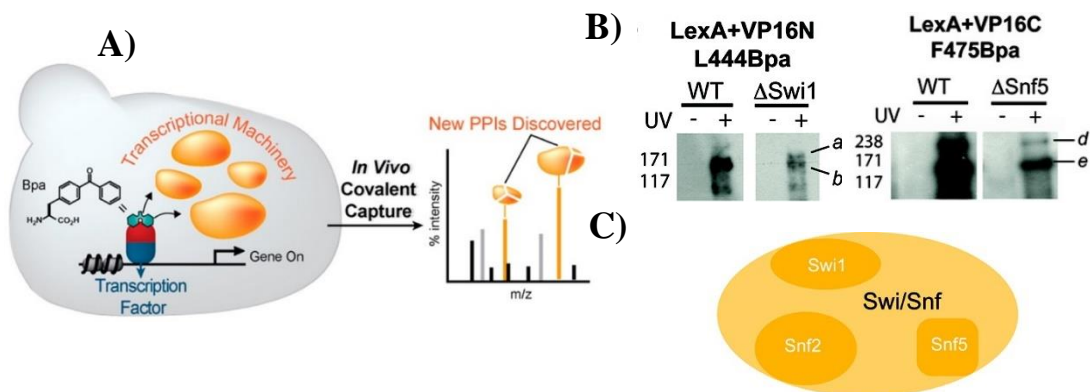
The MAPK pathway regulates cell growth, proliferation, and apoptosis, all of which are critical processes in determining cell fate.<sup>1-3</sup> The structure and function of the key signaling proteins of the MAPK pathway have been well-characterized; however, it is not understood how this signaling cascade is upregulated during early cancer initiation stages as well as in progression. One such example is Ras, which interacts with and plays an essential role activating downstream kinase Raf.<sup>4</sup> Due to the fast rate of signal transduction from Ras to Raf, it has been challenging to capture the distinct contribution of each isoform. Since the lifetime of the Ras-Raf interaction is on the order of 50-100 milliseconds, it is challenging to effectively capture this transient interaction with current pulldown.<sup>5,6</sup> Mutation of Ras isoforms has been linked to the development of many different cancer types, including colon, pancreas, and lung (Table 2.1).<sup>7-10</sup> Due to its vital role, elucidation of these individual isoforms is essential to specifically targeting and preventing their negative impact. For example, K-Ras mutations are found in over 60% of pancreatic cancers. H-Ras mutations are found in about 10% of bladder cancer. N-Ras mutations are commonly found in melanomas.<sup>7</sup> In addition to Ras mutations, there are also reports of overexpression of Ras in many cancer types.<sup>11,12</sup>

**Table 2.1: Prevalence of Ras isoforms in cancer.**

Percentages are based on tumor samples collected from each tissue type that harbored Ras mutants

<b>Primary Tissue</b>	<b>% H-Ras</b>	<b>% K-Ras</b>	<b>% N-Ras</b>
<b>Adrenal Gland</b>	<1%	<1%	4%
<b>Autonomic Ganglia</b>	0%	3%	7%
<b>Biliary Tract</b>	0%	31%	1%
<b>Bone</b>	2%	1%	0%
<b>Breast</b>	<1%	4%	2%
<b>Central Nervous System</b>	0%	<1%	<1%
<b>Cervix</b>	9%	7%	2%
<b>Endometrium</b>	1%	14%	<1%
<b>Haematopoietic/Lymphoid</b>	<1%	5%	10%
<b>Kidney</b>	<1%	<1%	<1%
<b>Large Intestine</b>	<1%	33%	3%
<b>Liver</b>	0%	5%	3%
<b>Lung</b>	<1%	17%	1%
<b>Oesophagus</b>	1%	4%	0%
<b>Ovary</b>	0%	14%	5%
<b>Pancreas</b>	0%	61%	2%
<b>Prostate</b>	6%	8%	2%
<b>Salivary Gland</b>	15%	3%	0%
<b>Skin</b>	6%	3%	18%
<b>Small Intestine</b>	0%	20%	0%
<b>Stomach</b>	4%	6%	2%
<b>Testis</b>	4%	4%	3%
<b>Thyroid</b>	3%	3%	8%
<b>Urinary Tract</b>	11%	5%	2%

To address the gaps in knowledge regarding Ras isoforms, we sought to develop a chemical tool to link active Ras (GTP-bound Ras) with Raf-Ras binding domain (RBD) to evaluate individual Ras isoforms.<sup>13-15</sup> We hypothesized that photocrosslinking active Ras utilizing an unnatural amino acid would permit quantitative analysis of active Ras isoforms present in the cell. Previously, our lab has utilized unnatural amino acids in yeast to successfully capture and identify novel and transient interacting partners of transcriptional machinery protein complex.<sup>16</sup> For example, we genetically incorporated *p*-benzoyl-l-phenylalanine (pBpa) into *S. cerevisiae* transcriptional coactivator VP16. VP16 forms transient multiprotein interactions with binding partners such as the chromatin-modifying complex Swi/Snf. Through *in vivo* photocrosslinking, novel VP16 binding partners ATPase Snf2 and Snf5 were discovered (Figure 2.1). This study presents an opportunity for leveraging unnatural amino acid such as pBpa for capturing transient protein complexes. By genetically incorporating pBpa into RBD, we can distinguish between Ras isoforms and begin to elucidate the influence of each, particularly in cancer with Ras dysregulation. Herein we report the development of a photoreactive probe by incorporating unnatural amino acid pBpa in Raf RBD to covalently capture active Ras.



**Figure 2.1: Genetic incorporation of pBpa for capturing PPIs.** (A) Schematic of pBpa being genetically incorporated in a coactivator that is responsible for recruiting transcriptional machinery. (B) Data showing VP16 L444Bpa and F475Bpa is capable of covalently linking novel binding partners. (C) Expected model of protein complex based on data.

## **C. Results and Discussion**

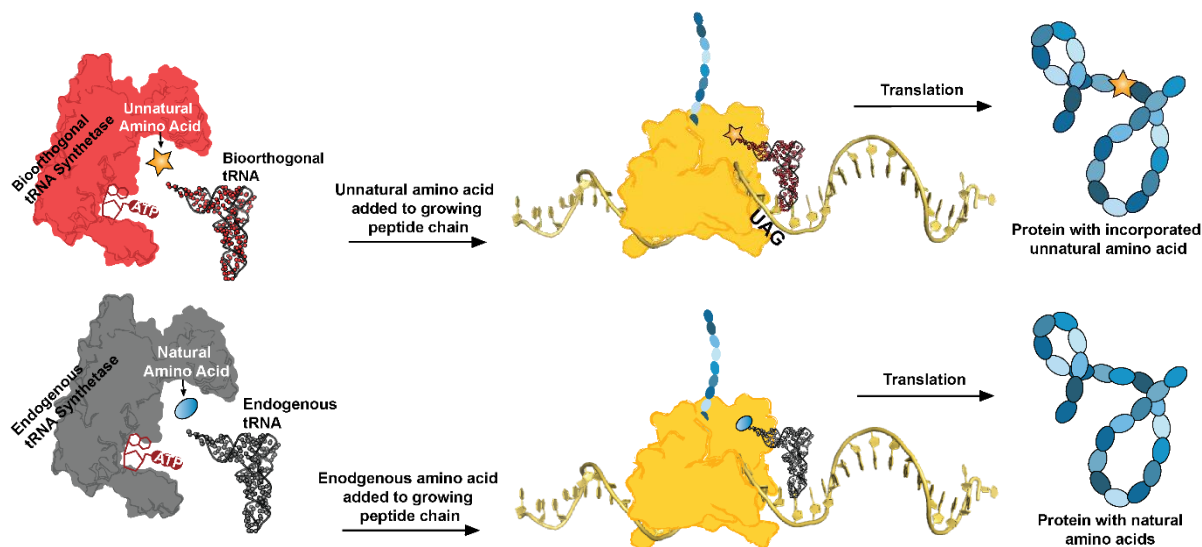
The major goal of this study was to be able to successfully quantify the binding of Ras isoforms to Raf through covalent capture with pBpa, which can be expanded to examine other small GTPases in the future. Our strategy utilizes pBpa incorporated in Raf RBD to covalently capture and quantify activated Ras isoforms in 293T cells. Dysregulation of Ras can occur through overexpression of wildtype Ras or through mutations such as G12V that cause the protein to be constitutively active.<sup>17,18</sup> In contrast, mutations such as S17N block GTP loading on Ras and ultimately MAPK signal transduction.<sup>19,20</sup> So, to test the fidelity of our photoreactive probe, we utilized several variants of H-Ras gene products: wildtype (WT), G12V (constitutively active), and S17N (dominant negative) to mimic aberrant cellular environments. We used an *E. coli* expression system to transform plasmids containing our tRNA synthetase and RBD with the necessary amber stop codon to grow and purify recombinant photocrosslinkable RBD probe. The photoreactive RBD probe was then used to covalently capture active Ras isoforms from mammalian cells. The successful capture of the variants was observed by Western Blot. Also, recombinant Ras isoforms H-Ras, N-Ras, K-Ras4A, and K-Ras4B were purified and analyzed by LC-MS.

### **C.1 Genetic Incorporation of pBpa into Raf Ras Binding Domain**

Although unnatural amino acids have been incorporated in various biological systems, there is considerable variation in incorporation efficiency based on protein and position. First, we assessed optimal positions in the Raf-RBD sequence for UAA incorporation. Based on structural data of the Ras-Raf protein interaction, we were able

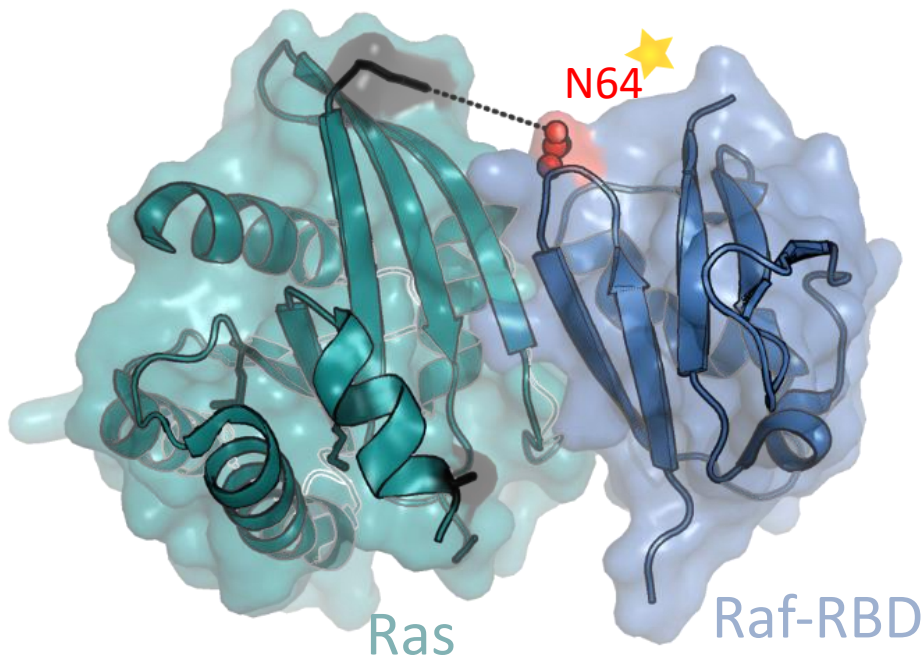
to identify two potential sites of UAA incorporation. Incorporation sites were chosen based on the reactive properties of pBpa, position of critical residues in the RBD binding interface, proximity of RBD residue sites to the Ras binding interface.

Next, we optimized an *E. coli* expression system for genetic incorporation of pBpa in RBD. We established the required plasmids for incorporation of unnatural amino acids. We used the expression system of *Methanococcus jannaschii*, which harbors an tRNA and its aminoacyl-tRNA synthetase that have been evolved to recognize UAAs and are unable to recognize natural amino acids (Figure 2.2).<sup>21</sup> This limits the ability of *E. coli* to incorporate other amino acids besides UAA into the amber stop codon position. In addition to the tRNA/t-RNA synthetase pair plasmid, we also used a pRARE plasmid transformed in BL21-AI for efficient protein expression. Lastly, we incorporated a GST-RBD plasmid; which has been shown to be useful for active Ras pulldown experiments.<sup>22,23</sup>

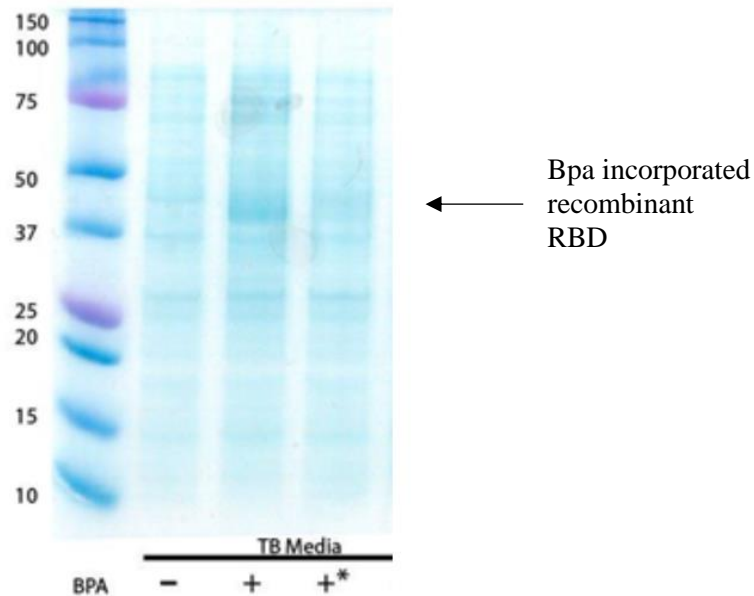


**Figure 2.2: UAA incorporation expression system.** UAA *E. coli* expression systems require an orthogonal tRNA synthetase/tRNA pair to recognize UAAs. This orthogonal systems works in the native system because endogenous tRNA synthetase/tRNA pair cannot recognize UAAs.

We initially mutated the N64 residue with an amber stop codon for incorporation of pBpa due to its close proximity (9.4 Å ) to an adjacent residue on the Ras interface (Figure 2.3). However, expression conditions required further optimization due to the lack of expression of full length RBD probe suggesting pBpa was not being incorporated. Next, we tested variations of the induction protocol to optimize protein expression with incorporation of pBpa. Initially we tested media conditions previously reported on in the literature, minimal and auto-induction media, but expression yield was minimal. Terrific Broth (TB) yielded the highest expression of the three when induced with 0.5mM IPTG and 0.02% arabinose. Additionally, the period of adding the UAA was inconsistent across the literature, before or during induction.<sup>24-26</sup> We observed that adding pBpa during the inoculation period was best for optimal expression of the GST-RBD (Figure 2.4).



**Figure 2.3: pBpa incorporated in Raf-RBD N64.** pBpa is genetically incorporated in Raf-RBD at the N64 position. This position is 9.4 Å from the nearest expected covalent binding partner of pBpa.

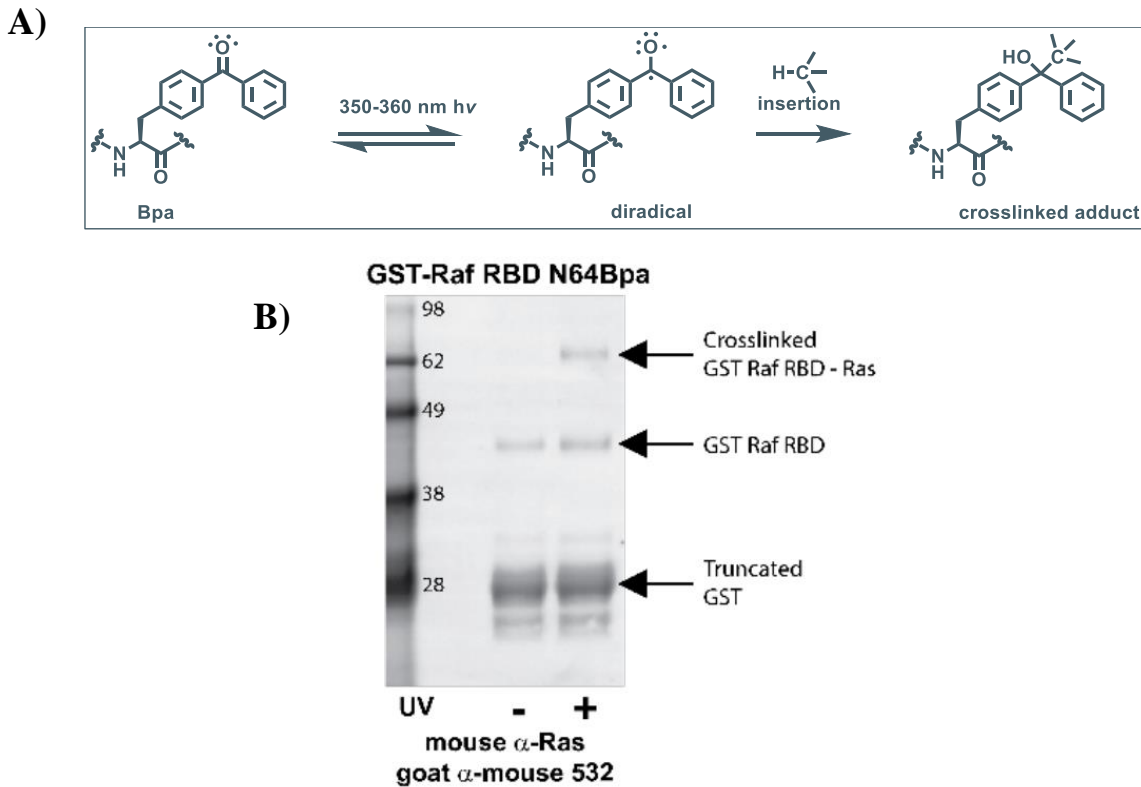


**Figure 2.4: Induction test with pBpa in TB media.** RBD N64Bpa induction was tested with pBpa by adding the UAA before (+\*) or at induction (+). Recombinant RBD-N64Bpa expressed best when pBpa is added prior to induction (+\*).

## C.2 Covalent Capture of H-Ras Variants with pBpa incorporated Ras binding domain

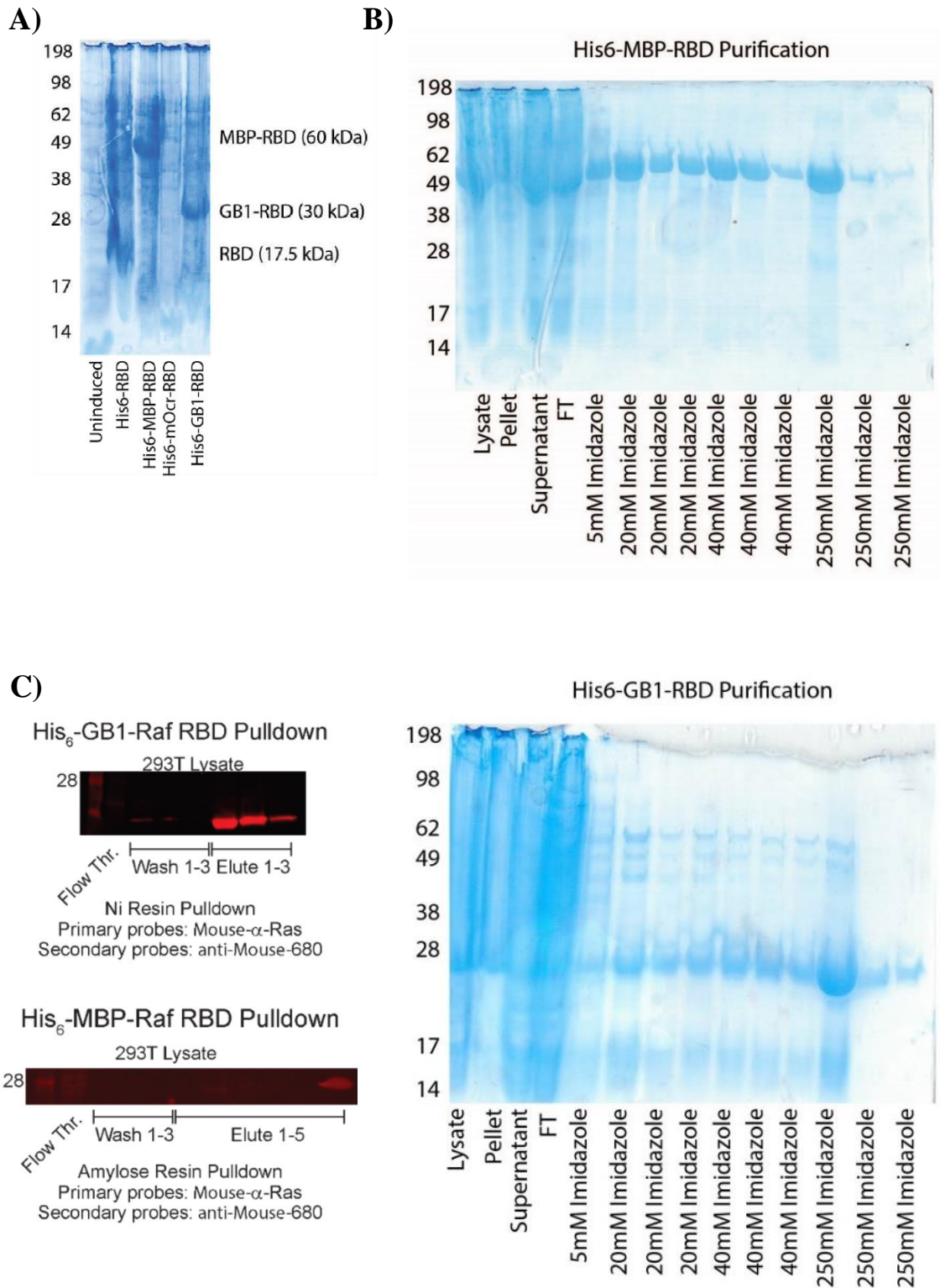
Next, we conducted crosslinking experiments with GST-RBD N64pBpa with 293T cell lysates that were transfected with H-Ras variants previously discussed. Crosslinking experiments with these variants allow us to validate if our probe functions as expected. We hypothesized that GST-RBD N64pBpa will capture more active Ras from 293T cells transfected with constitutively active H-RasG12V and a minimal amount of active Ras from 293T cells transfected with dominant negative H-RasS17N. 293T cells transfected with H-Ras WT is expected to capture active Ras at a rate that is between H-RasG12V and H-RasS17N samples. Using GST-RBD N64pBpa, we covalently captured active H-Ras with pBpa upon exposure to 365 nm light as observed by Western blot (Figure 2.5).<sup>27</sup> Although this was a promising result, the Western blot also revealed presence of a truncated variant of the biochemical probe. This poses a challenge for

purification of our probe due to the combination of GST forming dimers and the amber stop codon for UAA incorporation, which leads to purification of heterodimers of full-length probe.<sup>28,29</sup> Therefore, we needed to identify other purification tags that do not dimerize and position a His<sub>6</sub> tag on the C-terminus to ensure uniform purification of full length RBD. We evaluated three different purification tags, Mocr, GB1, and MBP to improve protein yield.<sup>30-32</sup> Although GST is traditionally used with RBD, we found that GB1 produced both better expression of our probe and more effective pulldown of Ras when compared with GST and MBP tags (Figure 2.6). Additionally, the primary antibody for Ras non-specifically complexed to GST/GST-RBD; so, we also altered our choice of primary antibody to specifically observe Ras and RBD independently. In total, we successfully incorporated pBpa into GB1-RBD without truncated variants of the protein.



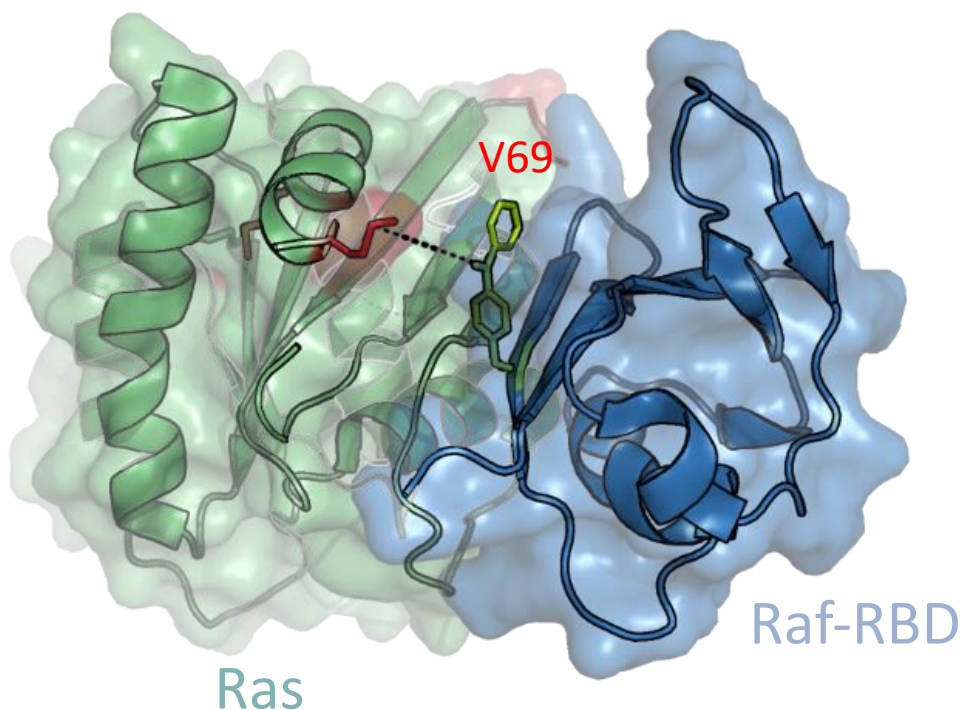
**Figure 2.5: Photocrosslinking of Ras with GST-RBD N64Bpa.** (A) Photoreactive UAA pBpa excited at 350-365 nm wavelength forms a diradical that crosslinks to adjacent amino acids. (B) A pulldown experiment from 293T cells show GST-RBD N64Bpa photocrosslinks active Ras. Truncated GST-RBD is also observed.



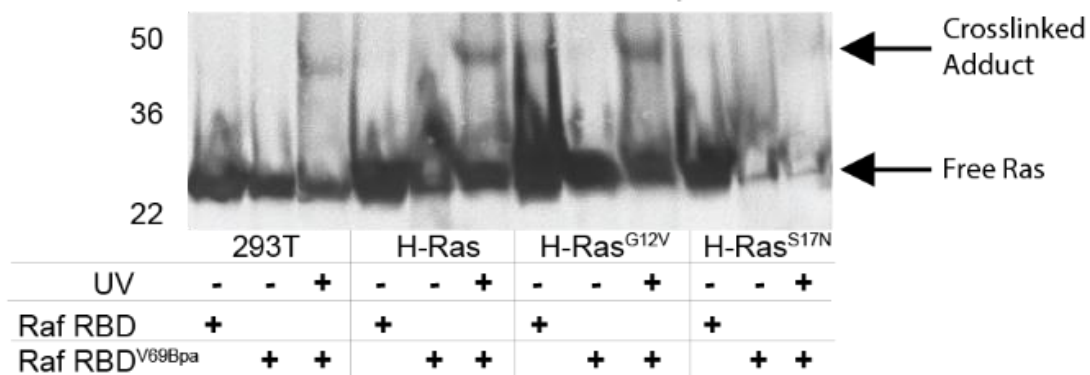


**Figure 2.6: Optimization of Raf-RBD purification.** (A) Small scale expression test of purification tags GB1, Mocr, and MBP shows GB1 and MBP express the best. (B) Large scale expression test shows GB1 and MBP both express and purify His<sub>6</sub>-Raf-RBD. (C) His<sub>6</sub>-GB1-Raf-RBD is best for active Ras pulldown from 293T cell lysate.

To further optimize chemical capture of active Ras, we altered the position of pBpa incorporation based on both proximity and surrounding residues of Raf to a GB1-RBD V69pBpa variant (Figure 2.7). To examine the effectiveness of RBD V69pBpa in capturing active Ras, we transfected 293T cells with various Flag-H-Ras plasmids, namely WT, G12V, and S17N. A flag tag is positioned at the N-terminus of the protein to facilitate isolation of the complex. Together, we were able to observe the effectiveness of GB1-RBD V69pBpa in photocrosslinking H-Ras, with G12V and S17N serving as positive and negative controls, respectively. As expected, 293T cell lysate transfected with Flag-H-RasG12V exhibited the highest yield of crosslinked GB1-RBD V69pBpa and the biochemical probe crosslinked a minimal amount of H-Ras from Flag-H-RasS17N 293T transfected cells. H-Ras crosslink yield from wildtype 293T and Flag-H-Ras transfected 293T cells was between both H-RasG12V and H-RasS17N (Figure 2.8).



**Figure 2.7: pBpa incorporated in Raf-RBD V69.** pBpa is genetically incorporated in Raf-RBD at the V69 position.

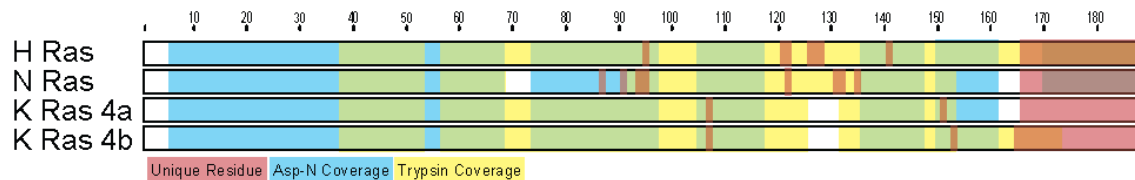


**Figure 2.8: Photocrosslinking of Ras variants with GB1-RBD V69Bpa.** GB1-RBD V69Bpa successfully crosslinks active H-Ras from 293T lysate samples. Samples were taken directly from cell lysate (not a pull-down). H-Ras<sup>G12V</sup> yielded the most crosslinked Ras adduct. H-Ras<sup>S17N</sup> yielded the least crosslinked Ras adduct.

### C.3 Ras Isoform LC-MS Analysis

Finally, we evaluated the four major Ras isoforms by LC-MS to establish references for additional experimentation with the biochemical probe as well as test if the isoforms could be distinguished from each other. Based on a sequence analysis of the four Ras isoforms, we expected to hydrolyze unique fragments from an Asp-N and trypsin digestion. To accomplish this, we purified recombinant Ras isoforms H-Ras, N-Ras, K-Ras4A and K-Ras4B, which were gifted to us from the Fierke Lab (University of Michigan), from *E. coli* cultures and subjected them to Asp-N and trypsin digestion conditions. Trypsin catalyzes the hydrolysis of peptide bonds on the carboxyl terminal side of arginine and lysine residues. Asp-N catalyzes the hydrolysis of peptide bonds on the amino side of aspartic acid residues. The goal of the lysing conditions was to have as many unique protein fragments as possible for distinguishing individual isoforms. As expected, proteases Asp-N and trypsin resulted in unique Ras fragments across all 4

isoforms (Figure 2.9). In the future, LC-MS will be used to quantitate the pull-downs of each isoform.



**Figure 2.9: Protease digestion of Ras isoforms.** Asp-N and trypsin protease digestion of Ras isoforms H-Ras, N-Ras, K-Ras4A, and K-Ras4B produces unique protein fragments that can be used to distinguish each isoform.

## D. Conclusion

The MAPK pathway regulates the cellular growth, proliferation, and survival through a series of proteins involved in signal transduction. The Ras-Raf protein-protein interaction is a critical point in the signaling cascade and has been implicated in initiation and progression of cancer. Though Ras mutations are well known to be linked to cancer, prior to this work there has been no method to distinguish Ras isoforms and elucidate the contribution to each to the mechanism of the specific disease.

Using genetic incorporation of UAAs, we demonstrated that Raf RBD can be utilized as a chemical probe for covalently capturing Ras isoforms. Traditionally, Raf RBD served as a tool for performing pulldown experiments for active Ras. Due to the temporary nature of protein complex pulldowns, it is not reliable for effective quantification of transient interacting partners. Since this covalent capture makes the interaction permanent, we were able to detect the crosslinked complex by Western blot. This also may eliminate the need to conduct a co-pulldown to observe complexed product due to the molecular weight shift. Additionally, Ras isoforms may be distinguished upon

capture through protease digestion and LC-MS analysis. Digestion with either Asp-N or Trypsin permits the analysis of distinct Ras isoforms as unique fragments are generated for each isoform. Together, there is the potential to distinguish amongst Ras isoform H-Ras, N-Ras, K-Ras4A, and K-Ras4B through covalent capture and proteolysis. Furthermore, this method may serve as framework for examining other small GTPases using the domains of their unique interacting partners.

## E. Materials and Methods

### Construction of plasmids

#### pGB1+Raf-RBD-His6

A high copy plasmid expressing Raf-RBD (residues 2-149 of Raf-1) + His6 was created via ligation of the fusion gene into a pGB1(Protein Core in the Center for Structural Biology (Life Science Institute, University of Michigan) via LIC. Primers 5'-TACTTCCAATCCAATGCAGAGCACATTCAGGGTGC-3' and 5'-TTATCCACTTCCAATGTTAGTCGCGATGAATTCCC-3' were used to amplify Raf-RBD from a codon optimized gene fragment purchased from IDT. Site directed mutagenesis (SDM) was used to remove the His tag from the N-terminus with the primers 5'-CTTTAAGAAGGAGATATACATATGTCTTCTGGTGTAGATCTGCAGTAC-3' and 5'-GTACTGCAGATCTACACCAGAAGACATATGTATATCTCCTTCTTAAAG-3'.

The His tag was then attached to the C-terminus with the primers 5'-CTGGGAATTCATCGCGACCACCATCATCATCATTAACATTGGAAGTGGGATAACGG-3' and 5'-CCGTTATCCCCTTCCAATGTTAATGATGATGATGATGGTGGTCGCGATGAATTCCCAG-3'. The His tag was used to aid in the pulldown of only full-length protein.

#### pGB1+Raf-RBD-His6 TAG Mutants

Site-directed mutagenesis was used to genetically alter native amino acid codons with a TAG codon within the Raf-RBD sequence. PCR primers were designed to have approximately 15 bases of homology on either side of the TAG mutation and mutagenesis reactions were carried out using manufacturer recommended conditions (Qiagen).

### **Expression and purification of pBpa incorporated Raf-RBD**

A 30 mL of BL21ai (transformed with pRARECDF vector from Protein Core in the Center for Structural Biology, Life Sciences Institute, University of Michigan) starter culture in LB media with ampicillin, streptomycin, and chloramphenicol was inoculated and grown overnight at 37°C. The starter culture was used to inoculate 1 L of Terrific Broth culture containing the same antibiotics and 1 mM pBpa (Chem-Impex International). This was grown to an OD600 of 0.8 and chilled at 22°C for 1 hour. Next, induction was carried out with 0.5 mM IPTG and 0.2% arabinose. After growth overnight, cells were collected by centrifugation for 20 minutes at 6,500 x g and 4°C. The cell pellet was dissolved in 50 mM NaPO<sub>4</sub> at pH 8, 300 mM NaCl, and 10% glycerol and with a Roche cOmplete, EDTA-free protease inhibitor tablet. Lysis was done by sonication for 4 minutes with 3 second pulses. To remove insoluble cell debris, lysate was centrifuged at 12,000 x g and 4°C for 30 minutes. The supernatant was incubated with 1 mL of Ni-NTA agarose resin (Qiagen) for 2 hours at 4°C. A purification column was used to wash the resin with 10 mL of lysis buffer with 5 mM imidazole. An imidazole gradient was used to elute protein beginning with 10 mL of 20 mM imidazole, three 5mL fractions of 40 mM imidazole, and three 5 mL fractions of 250 mM imidazole for elution. To remove imidazole, protein as dialyzed into NaPO<sub>4</sub> at pH 8, 150 mM NaCl, and 5% glycerol overnight. Concentration was done using Amicon Ultra PL-10 (10,000 MWCO).

### **Cell lines**

293T cells were obtained from ATCC. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) with 10% fetal bovine serum in 10 mm culture dish at 37°C and 5% CO<sub>2</sub>. Transfection of H-Ras and H-Ras mutants were conducted

using the FuGENE kit and protocol from Promega.

### **Covalent chemical capture and Pulldown of Crosslinked Adducts.**

Mammalian cells overexpressing H-Ras WT or H-Ras mutants were resuspended in 100  $\mu$ L of lysis buffer (25 mM Tris HCl, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% NP-40, 5% glycerol). Cells were sonicated at 10% amplitude for 20 seconds. 100  $\mu$ g of GST-Raf-RBD or GB1-Raf-RBD-His<sub>6</sub> mutants was added and incubate for 20 minutes at 4°C. +UV samples were then irradiated for 20 minutes on ice at 365 nm while -UV samples were maintained on ice on bench for this time. Half (by volume) of the samples were removed to run on the gel without a pulldown of any kind. Pulldown samples were incubated with 40  $\mu$ L of Dynabeads magnetic Ni beads for 2 hours at 4°C. Dynabeads were pre-washed and equilibrated in 3 x 1 mL of lysis buffer. Samples were then washed with 3 x 100  $\mu$ L lysis buffer + 20 mM imidazole and eluted with 3 x 50  $\mu$ L of lysis buffer + 250 mM imidazole with 5 minutes of incubation for each wash and elution.

### **Western Blots**

Samples were denatured by heating at 95°C for 10 minutes in Laemmli 2x Buffer (BioRad) containing 250 mM DTT and run on a 5-15% Bis-Tris gel (BioRad). The separated proteins were then transferred to PVDF membrane and probed using Western Blot analysis with a 1:1000 dilution of rabbit-anti-His antibody (Abcam) and mouse-anti-Ras (Millipore) in Odyssey Blocking Buffer (Lycore) followed by a 1:10,000 dilution of donkey-anti-mouse IRDye 680LT and donkey-anti-rabbit IRDye 800CW. Imaging was conducted on an Azure C600.



## **Preparation of Ras Isoforms**

Using the pETM11-Ras plasmids given by the Fierke Lab, recombinant protein was grown in BL21-AI cells and Terrific Broth rich media. Cells were grown at 37°C until an OD600 of 0.8. Cells were induced with 1 mM IPTG and 0.1% arabinose and incubated overnight at 18°C. Cells were harvested via centrifugation for 20 minutes at 6,500 x g and 4°C. To lyse cells, the pellet was dissolved in 10 mM Tris/HCl at pH 7.6, 0.1 mM GDP, 20mM sodium citrate, 50 mM KCl, and 5 mM MgCl<sub>2</sub> and 2 mM β-mercaptoethanol with a Roche cOmplete, EDTA-free protease inhibitor tablet. Lysis was done by passing the dissolved pellet through an Avestin EmulsiFlex-C3 high-pressure homogenizer at 10,000-15,000 psi. Homogenization was used to aid in the isolation of inclusion bodies by giving a more uniform lysis. Lysate was centrifuged at 12,000 x g and 4°C for 30 minutes and supernatant was incubated with 1 mL of Ni-NTA agarose resin (Qiagen) for 3 hours at 4°C. The pellet was extracted a second time with 10 mM Tris/HCl at pH 7.6, 0.1 mM GDP, 20 mM sodium citrate, 50mM KCl, and 5 mM MgCl<sub>2</sub> and 2 mM β-mercaptoethanol for 1 hour at 4°C with shaking and then re-centrifuged as before. Using a column, the resin was washed with 10 mL of lysis buffer with 5 mM imidazole. An imidazole gradient was used to elute protein beginning with 10 mL of 20 mM imidazole, 3 x 5mL fractions of 40 mM imidazole, and 3 x 5 mL fractions of 250 mM imidazole for elution. To remove imidazole, protein was dialyzed into 50 mM tris-citrate at pH 6.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.01 mM GDP and 10 mM β-mercaptoethanol overnight. Concentration was done using Amicon Ultra PL-10 (10,000 MWCO).

## **Protease Digestion**

Protease digestions were prepared according to Promega manufacturer instructions. Approximately 8 µg of protein was incubated at 37°C for 1 hour with 7.5 M urea. It was then incubated for 20 minutes at 60°C with 15 mM DTT and 15 minutes in the dark at room temperature with 60 mM iodoacetamide. The final mixture was diluted with 100 mM tris buffer at pH 8 and 1 mM CaCl<sub>2</sub>. Protease (2 µL:2 µg) was added and digested overnight at 37°C. Peptides were desalted with an Oasis 96-well plate (Waters Corporation) on a vacuum manifold. 70% acetonitrile was used to elute peptides. After transfer, a Thermo Savant Vacuum Concentrator was used to remove solvent at 45°C.

## **LC-MS Ras Isoform Identification**

Protein digests were prepared in 3% acetonitrile, 0.1% formic acid and 10 fmol/µL alcohol dehydrogenase (ADH, Waters Corporation) in water. In triplicate, peptides were separated using a one-dimensional Waters Nano Acquity UPLC system fitted with a 5 µm C18 (180 µm x 20 mm) trap column and a 1.8 µm HSS T3 analytical column (75 µm x 250 mm). Digested peptides were loaded onto the trap column over 3 minutes, followed by analytical separation over a 110 minute gradient (3% acetonitrile to 40% acetonitrile over 92 minutes). Peptides were analyzed in positive mode on a Synapt G2-S HDMS traveling wave ion mobility time-of-flight mass spectrometer (Waters Corporation). The nanoESI source was set to 70°C and a 3kV potential was applied. Data-independent acquisition was performed as described previously. Raw data were analyzed with ProteinLynx Global SERVER version 3.0.2 (PLGS, Waters Corporation), searching against a database of the human proteome (downloaded from UniProt on November 9, 2015). The following criteria were applied to perform the

search, in addition to the appropriate enzyme specificity: (i) one missed cleavage permitted, (ii) carbamidomethyl cysteine as a fixed modification and methionine oxidation as a variable modification, (iii) minimum of two identified fragment ions per peptide and minimum of five fragments per protein, and (iv) at least two identified peptides per protein. The false discovery rate (FDR) for peptide and protein identification was set at 1% using a reversed database. Any identified peptides with a calculated mass error greater than 10 ppm were not considered.

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## **Chapter 3: Inhibition of CBP/p300 KIX-Dependent Transcription with the Natural Product Garcinolic Acid**

### **A. Abstract**

Homologues CREB-binding protein (CBP) and p300 serve as master regulators of gene transcription and are required for proper cell development, cell growth, and DNA repair. The KIX domain is conserved between both CBP and p300 and mediates interactions with transcription factors to facilitate cellular functions. Many of these KIX-dependent transcription factors such as CREB, c-Jun, and MLL have been implicated in cancer cell growth as the expression of their respective gene targets are dysregulated. However, due to the absence of selective small molecule probes for CBP/p300 KIX, there have been limited chemical genetic studies to understand if these homologues have distinct functions due to specific KIX transcription factor interactions. In this work, here we have utilized a newly discovered selective chemical probe, garcinolic acid, as a tool to characterize the structure and function relationship of CBP/p300 KIX and its interacting partners in leukemic cells. These studies show that CBP/p300 KIX-dependent transcription regulates cell cycle and viability in chronic myeloid leukemia and breast cancer.

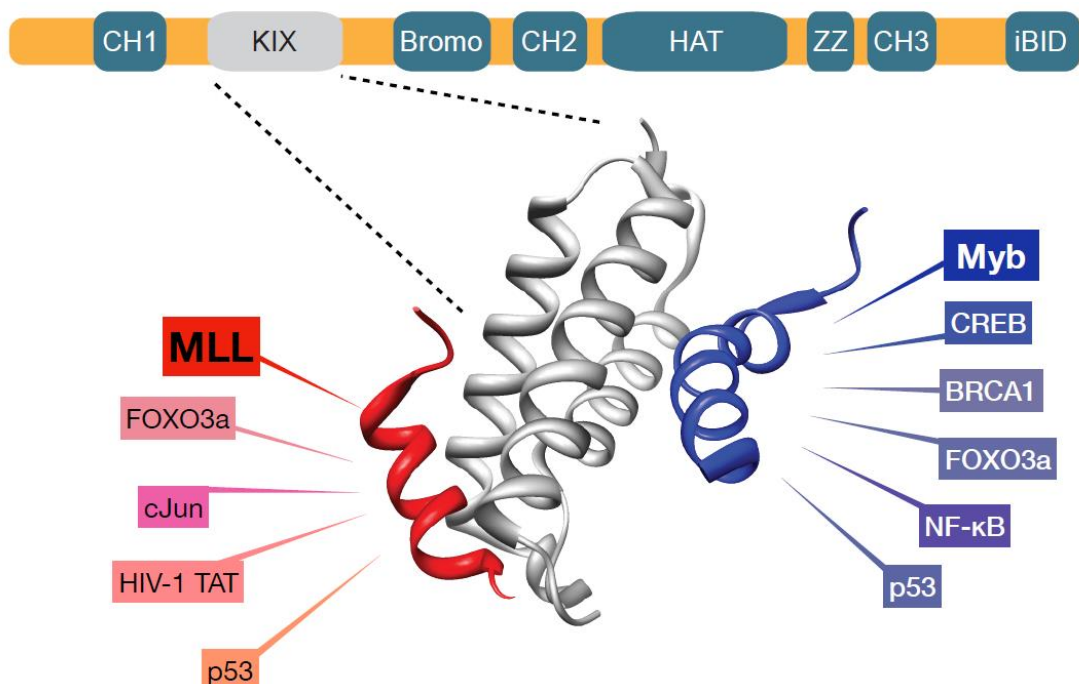
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The research described in chapter 3 is a collaborative effort. Dr. Meghan Breen conducted the chemical library screen with Steve Vander Roest of the Life Science Institute's Center for Chemical Genomics and characterized garcinolic acid. Dr. Stephen Joy conducted the qRT-PCR experiments in MV4-11 cells. I conducted cell viability, flow cytometry, and qRT-PCR experiments in MCF7, MDA-MB-231, and Hap1 cell lines. I calculated p-values for all statistical analysis.

## **B. Background**

Transcription plays a critical role in maintaining a healthy cellular environment by regulating gene expression levels. Transcriptional dysregulation is thus linked to many different diseases. At the crux of this dysregulation are a class of proteins called coactivators, which bind to many different activator proteins through activator binding domains (ABDs). Two such examples are the homologous coactivators CREB-binding protein (CBP) and p300. CBP/p300 are large multi-domain proteins involved in transcription coactivation through their ABD named kinase-inducible domain interacting domain (KIX) domain.<sup>1-4</sup> The KIX domain plays a critical role in regulating transcription through the recruit of several transcriptional activator binding partners (Figure 3.1).<sup>5-7</sup> Many of the activator interacting proteins have been implicated in the promotion of cancer growth through its ability to target a specific set of target gene promoter regions.<sup>8-11</sup> Thus, it is of interest to understand the role of KIX in cancer progression for clinical intervention. Some of these activator proteins directly regulate the expression of cell cycle genes. For example, c-Jun and CREB are activator proteins that are responsible for regulating expression of Cyclin D1 and Cyclin A2 through distinct gene promoter sequence, such as the CRE promoter motif.<sup>12,13</sup> KIX mediates binding to many different partners through its intrinsically disordered domain.<sup>14,15</sup> Therefore, it has been challenging to selectively target CBP/p300 KIX.





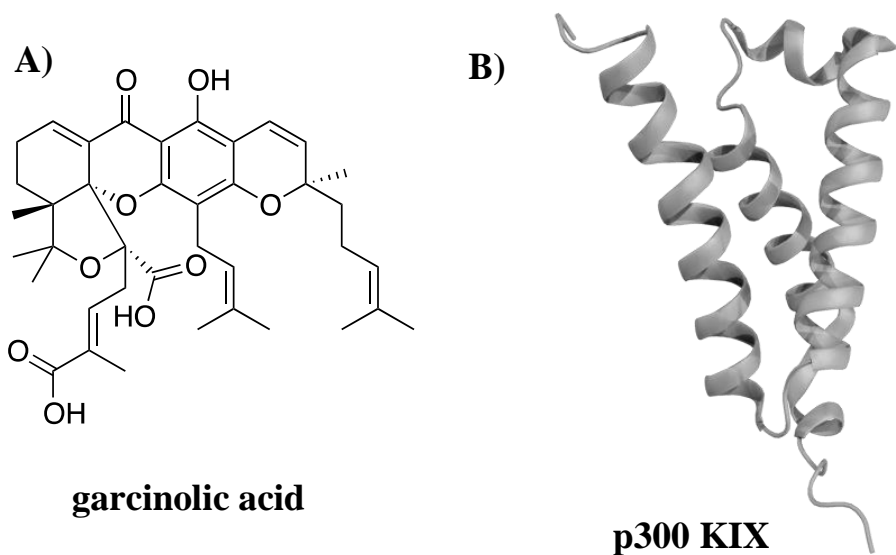
**Figure 3.1: CBP/p300 KIX activator binding partners.** CBP/p300 KIX domain is able to bind more than 10 activator proteins due to its conformational plasticity and orthosteric/allosteric communication. PDB: 2AGH

We hypothesized that a selective chemical probe could be used to elucidate the functional role of CBP/p300 KIX in regulating the cell cycle. CBP/p300 KIX has been reported to be involved in cell cycle regulation via its interaction with activator protein partners. For example, direct inhibition of CBP/p300 KIX leads to the downregulation of cyclin gene expression in lung adenocarcinoma cells.<sup>16</sup> It has been particularly challenging to develop selective probes to assess the functional differences between CBP and p300. Many of the current tools do not exhibit much selectivity for CBP/p300.<sup>17,18</sup> Our lab has discovered a novel natural product, namely garcinolic acid, that selectively targets the KIX domain of CBP over p300. Herein we report the examination of the role CBP/p300 KIX plays in cell cycle regulation by interrogating the interaction partners with garcinolic acid. Furthermore, we demonstrate the utility of garcinolic acid as a chemical

probe for interrogating CBP/p300 KIX and a promising starting point for future drug development.

### C. Results and Discussion

The major goals of our study were to elucidate the role of KIX-dependent transcriptional PPIs in regulating the cell cycle. Since KIX as well as its activator interacting partners have been implicated in several diseases, targeting the KIX domain to prevent these critical interactions could have significant therapeutic relevance. Previously, our lab has conducted a chemical compound screen against the KIX and identified the natural product garcinolic acid as a potential hit against the KIX domain. Further *in vitro* analysis suggested that garcinolic acid selectively targets CBP/p300 KIX over other protein's KIX motifs with low micromolar affinity (Figure 3.2).

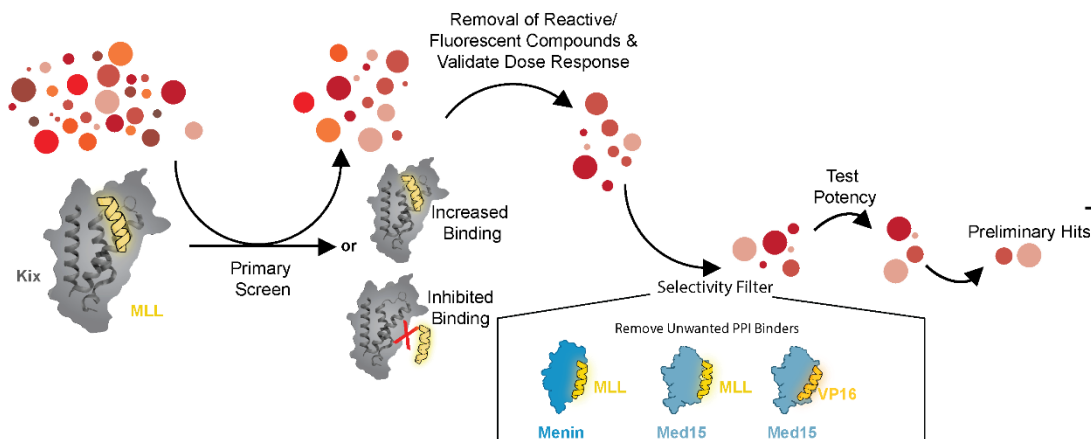


**Figure 3.2: Natural product garcinolic acid binds to KIX.** (A) Natural product garcinolic acid. (B) CBP/p300 KIX structure.

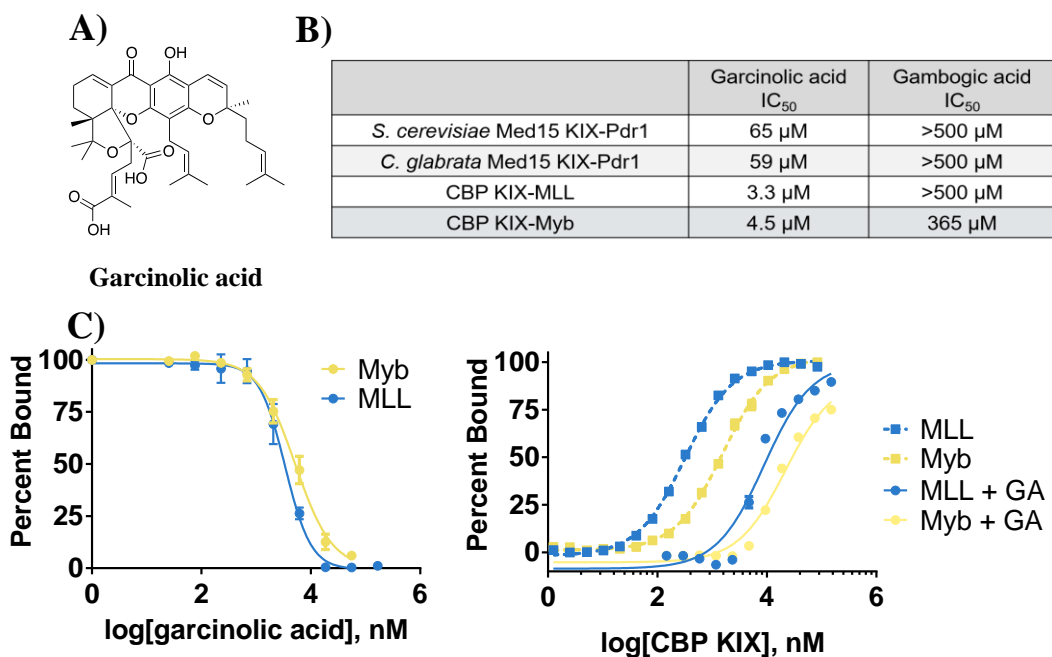
Our strategy takes advantage of three chronic myeloid leukemia cell lines, Hap1 WT, Hap1 CBP knockout and p300 knockout (KO). These cell lines are dependent on BCR-ABL protein, which regulate c-Jun gene target expression, and activator-binding partners of the KIX co-activator and thus provide an excellent milieu in which to examine the mechanistic and phenotypic impact of blocking KIX-dependent transcriptional processes.<sup>19-21</sup> This approach has the advantage of being able to selectively interrogate the role of CBP/p300 KIX without impacting other protein's KIX motifs.

### **C.1 *In vitro* Analysis Characterization of Garcinolic Acid and CBP/p300 KIX**

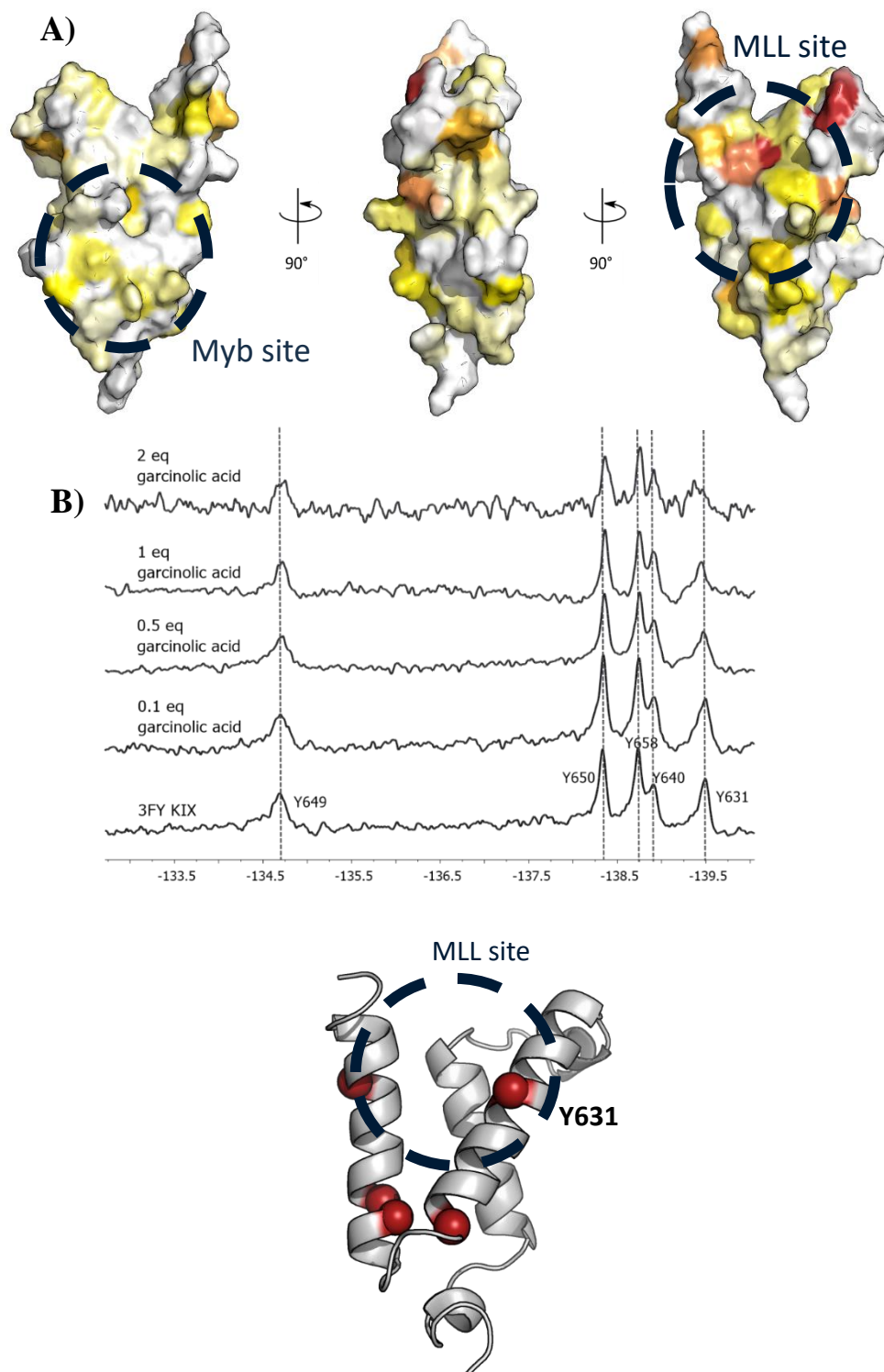
To identify potential lead compounds that inhibit the KIX motif, we screened 5000 compounds against Med15 KIX motifs from both *S. cerevisiae* and *C. glabrata* followed by a counter screen against other coactivator-activator complexes, namely CBP and p300 KIX (Figure 3.3).<sup>22</sup> From this, we discovered the natural product garcinolic acid as potent inhibitor of both CBP KIX and, to a lesser extent, p300 KIX. CBP/p300 KIX motifs harbor two binding sites essential for transcription regulation, namely the cMyb and MLL binding sites.<sup>23,24</sup> By using peptides of activator protein interacting partners Myb and MLL, it was observed that garcinolic has a  $K_i$  of 3.3  $\mu\text{M}$  and 4.5  $\mu\text{M}$  for MLL and Myb, respectively (Figure 3.4). Further NMR studies revealed that garcinolic acid specifically binds at the MLL binding site and subsequently perturbs the c-Myb binding site (Figure 3.5). Thus, it has a mixed orthosteric/allosteric mechanism.



**Figure 3.3: Schematic of chemical library screen against Med15 KIX.** A chemical library screen was conducted against Med15 KIX.



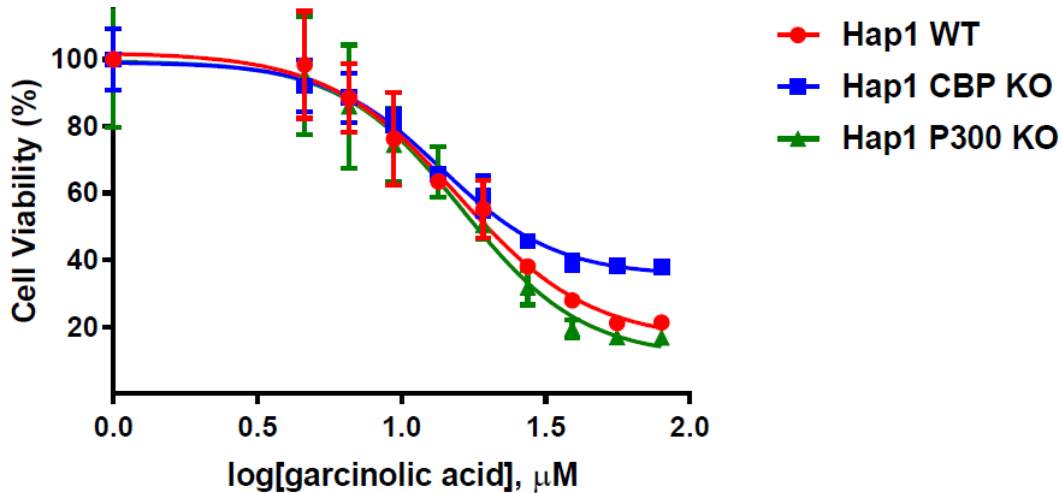
**Figure 3.4: Garcinolic acid inhibits KIX binding partners.** (A) Structure of garcinolic acid. (B) Table of half-maximal inhibitory concentrations of garcinolic acid:KIX motif binding partners. (C) garcinolic acid inhibits binding of peptides of coactivator binding partners *in vitro*. Data provided from Dr. Meghan Breen.



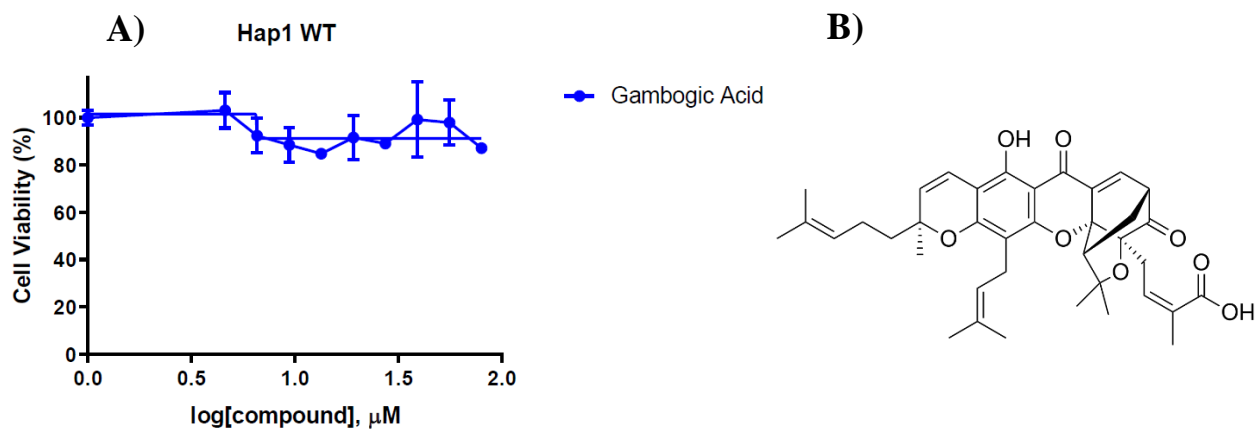
**Figure 3.5: NMR studies of KIX and garcinolic acid.** (A) HSQC NMR demonstrates that garcinolic acid inhibits KIX at the MLL site (red) and allosterically inhibits the c-Myb binding site (yellow). (B) ProF NMR reveals garcinolic acid solely perturbs residue Y631. Data provided from Dr. Matthew Beyersdorf

## C.2 Phenotypic Examination of KIX-dependent Transcription

To test phenotypic effects of blocking KIX transcriptional activity in Hap1 cell lines, we first conducted a cell proliferation assay to examine its impact of cell growth and proliferation. We treated Hap1 WT, Hap1 CBP KO, & Hap1 p300 KO cells with various concentrations of garcinolic acid over 24h. While we observed comparable half-maximal inhibitory concentrations ( $IC_{50}$ ) for each line, the overall effectiveness in inducing cell death varies by ~20% between Hap1 WT and Hap1 p300 KO cell lines and Hap1 CBP KO. This suggests that inhibiting CBP may elicit a greater effect on cell viability than p300 (Figure 3.6). Similar experiments were conducted with a garcinolic acid derivative that does not bind to the KIX motif, gambogic acid, which had no effect on Hap1 WT cell viability (Figure 3.7). Together, this further suggests that it is the inhibition of CBP KIX that leads to 20% more cell death at a concentration of up to 80  $\mu$ M.



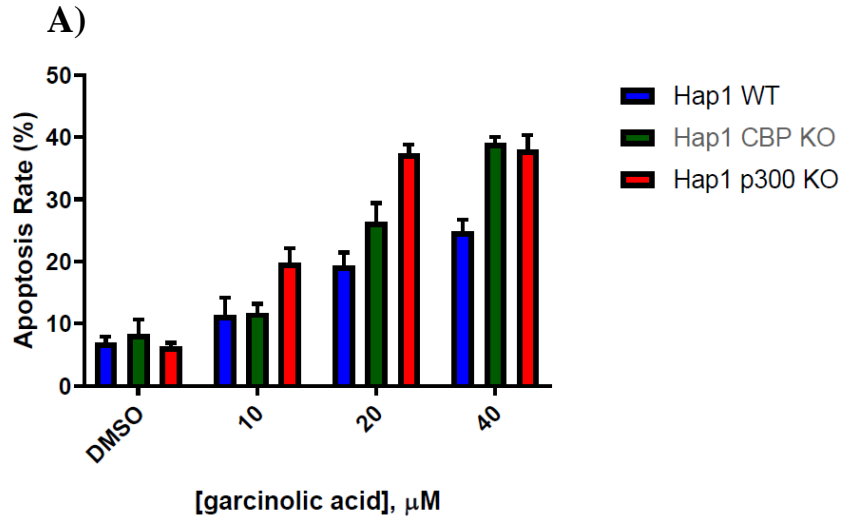
**Figure 3.6: Garcinolic acid impacts Hap1 cell viability.** Cell viability of Hap1 WT ( $IC_{50}$  16  $\mu$ M), Hap1 CBP KO ( $IC_{50}$  14  $\mu$ M), and Hap1 p300 KO ( $IC_{50}$  16  $\mu$ M) were all performed in 24 h with a WST-1 assay. n=3



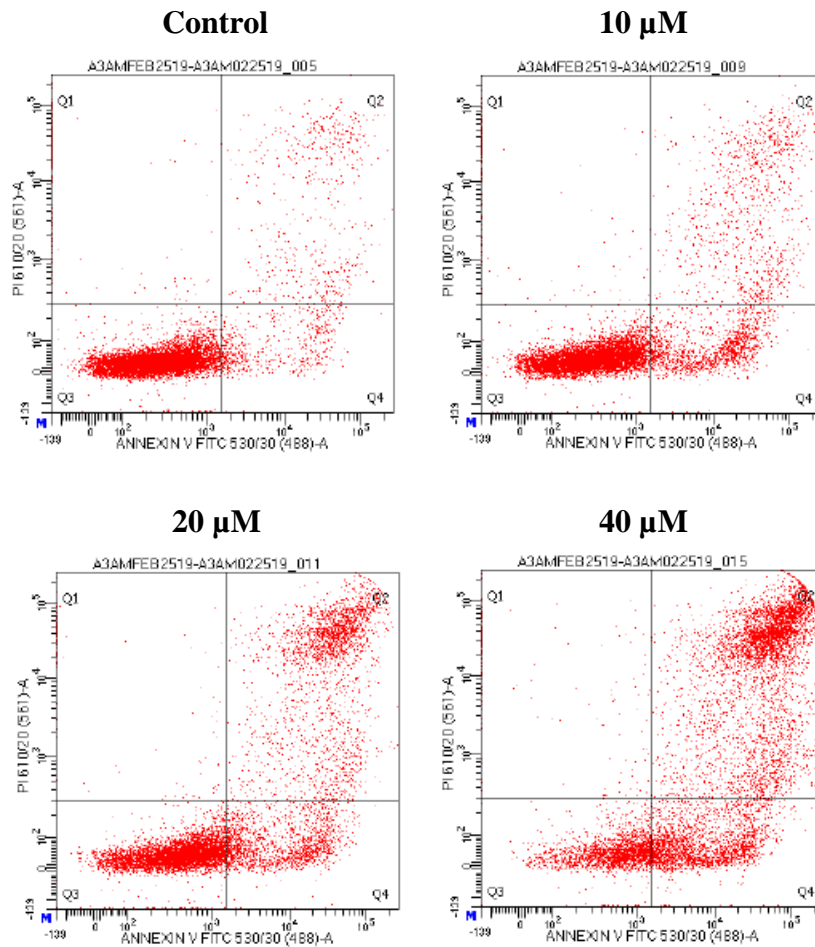
**Figure 3.7: Gambogic acid has no effect on Hap1 cell viability.** (A) Garcinolic acid derivative gambogic acid does not impact cell viability of Hap1 WT cells as revealed by a WST-1 assay. (B) Chemical structure of gambogic acid. n=3

We further analyzed the ability of garcinolic acid to induce programmed cell death using flow cytometry. Previous studies have shown that inhibiting CBP KIX may induce a stress response leading to cell death.<sup>16</sup> So, we next examined the impact of blocking CBP/p300 KIX-dependent transcription on inducing apoptosis. Apoptotic cell death was induced across all three cell lines as observed by the apoptotic marker, Annexin-V. Hap1 CBP and Hap1 p300 knockout cell lines were more susceptible to programmed cell death after treatment with garcinolic acid than were Hap1 WT cells. Hap1 p300 KO exhibited a higher apoptosis rate than Hap1 CBP KO until 40  $\mu\text{M}$ , which resulted in approximately 40% cell death, with both increasing in apoptosis in a dose dependent manner (Figure 3.8). Over the concentration range of 10 to 40  $\mu\text{M}$ , Hap1 WT had the lowest apoptosis rate. Overall, we observed that garcinolic acid, a small molecule inhibitor of KIX-dependent transcription, negatively impacts cell viability to various degrees across Hap1 WT, Hap1 CBP KO, and Hap1 p300 cell lines. In total, this suggest that both CBP and p300 KIX may regulate cell survival mechanisms in chronic myeloid leukemia. Further analysis of the apoptotic regulatory system is required to elucidate the role of

Hap1 CBP and p300.

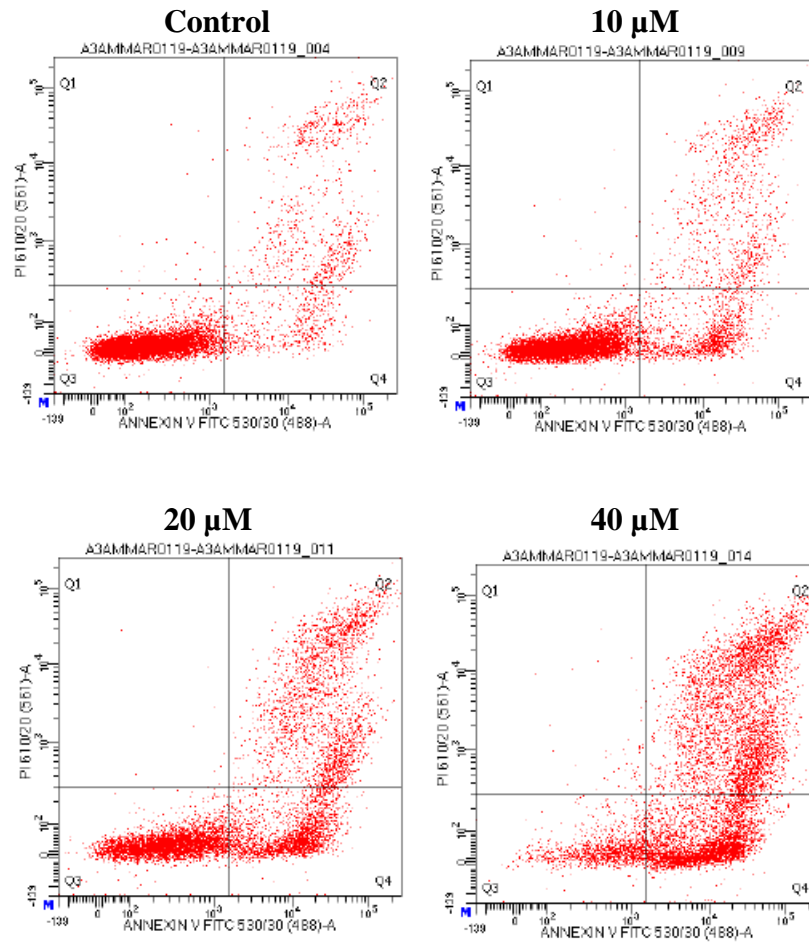


**B)**  
**Hap1 WT**

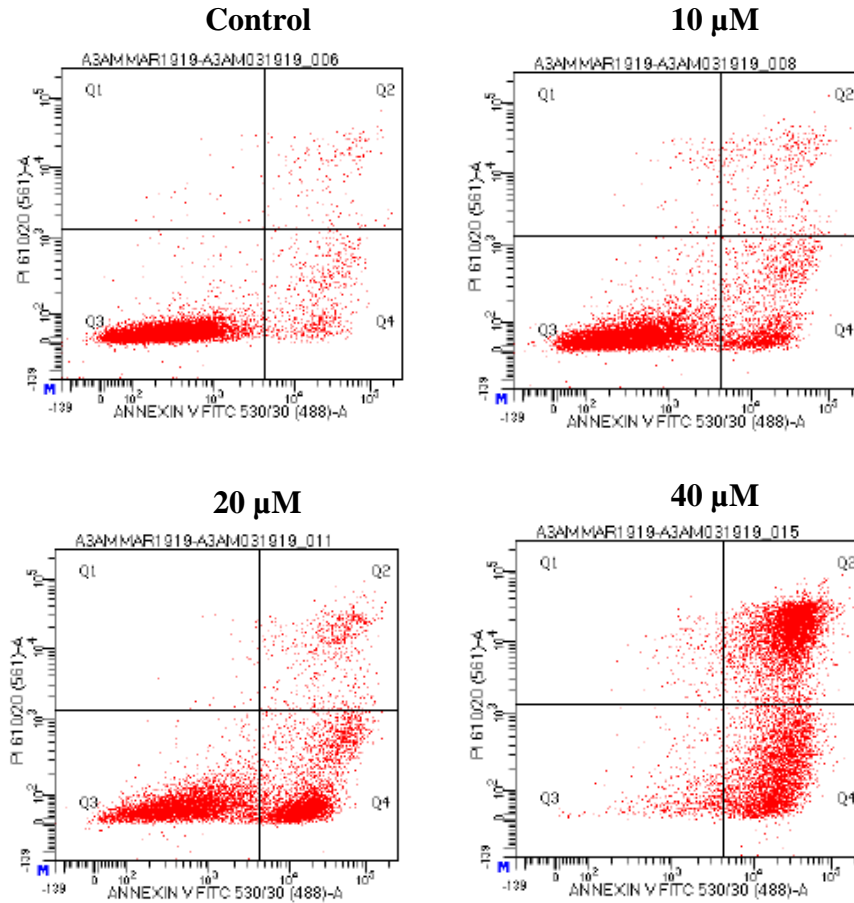




# Hap1 CBP KO



## Hap1 p300 KO

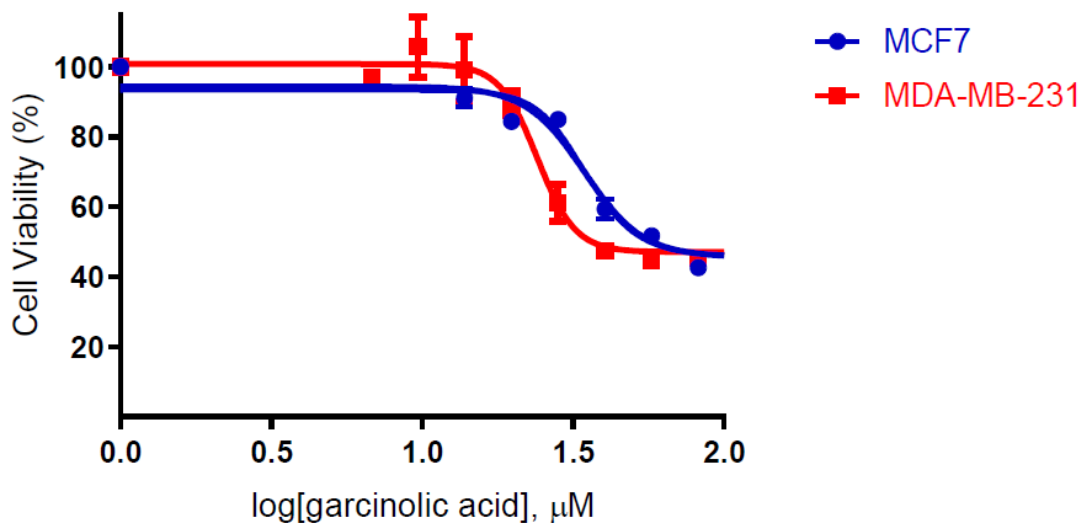


C)

	Hap1 Cell Lines p-value		
[garcinolic acid], $\mu\text{M}$	Hap1 WT	Hap1 CBP KO	Hap1 p300 KO
<b>0:10</b>	0.0530	0.1621	<0.0001 (***)
<b>0:20</b>	<0.0001 (***)	<0.0001 (***)	<0.0001 (***)
<b>0:40</b>	<0.0001 (***)	<0.0001 (***)	<0.0001 (***)

**Figure 3.8: Garcinolic acid induces Hap1 apoptosis.** (A) Apoptosis rate of Hap1 WT, Hap1 CBP KO, and Hap1 p300 KO after treatment with various concentrations of garcinolic acid. (B) Histograms of Hap1 cell lines treated with 0.25% DMSO, 10  $\mu\text{M}$ , 20  $\mu\text{M}$ , and 40  $\mu\text{M}$  garcinolic acid for 24 h. Data in (A) is derived from Q4 in (B) from each cell line. (C) Statistical analysis (p-value) of Hap1 cell lines treated with garcinolic acid. n=3

CREB, CBP/p300 activator binding partner, is well-known to be overexpressed and/or overactivated in some breast cancers.<sup>25,26</sup> To this end, we further examined the impact of blocking KIX-dependent transcription in breast cancer. Additional cell viability experiments were conducted in non-invasive and metastatic breast cancer cell lines MCF7 and MDA-MB-231, respectively.<sup>27,28</sup> Here we observed that in both cell lines garcinolic acid caused 50% cell death at a concentration of up to 80  $\mu\text{M}$  (Figure 3.9). This further suggest that blocking KIX-dependent transcription elicits negative phenotypic changes in cancer cells. This may be further validated through rescue experiments involving the expression of the CBP and p300 in Hap1 CBP KO and Hap1 p300 KO cell lines respectively.

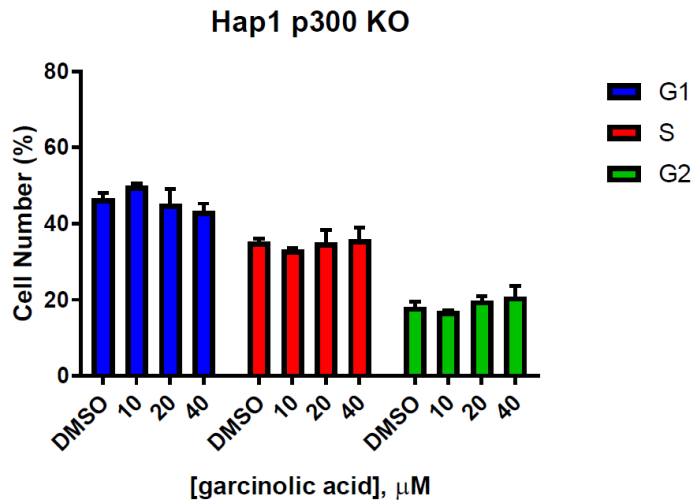
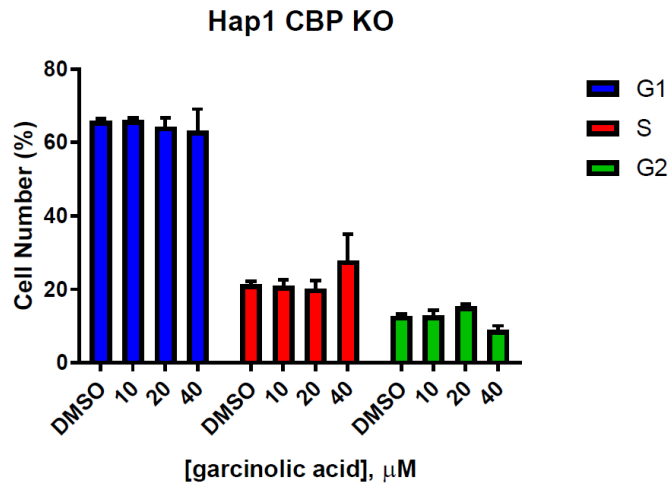
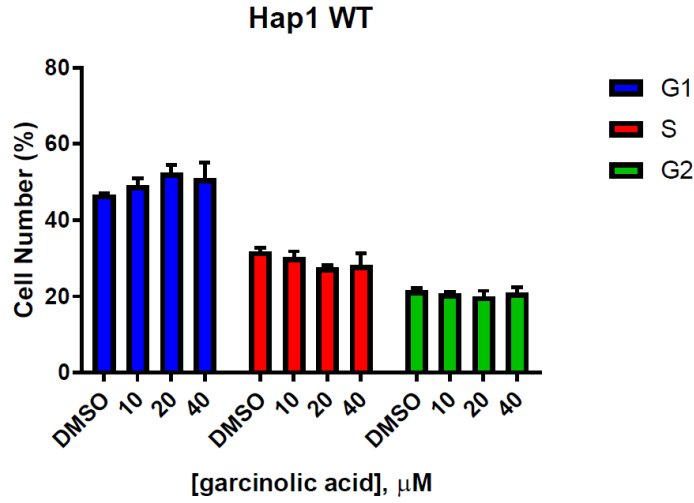


**Figure 3.9: Garcinolic acid impacts breast cancer cell viability.** Cell viability of MCF7 ( $\text{IC}_{50}$  34  $\mu\text{M}$ ) and MDA-MB-231 ( $\text{IC}_{50}$  24  $\mu\text{M}$ ) breast cancer cell lines were performed in 24 h with WST-1 assay. n=3

### **C.3 Mechanistic Examination of KIX-dependent Transcription**

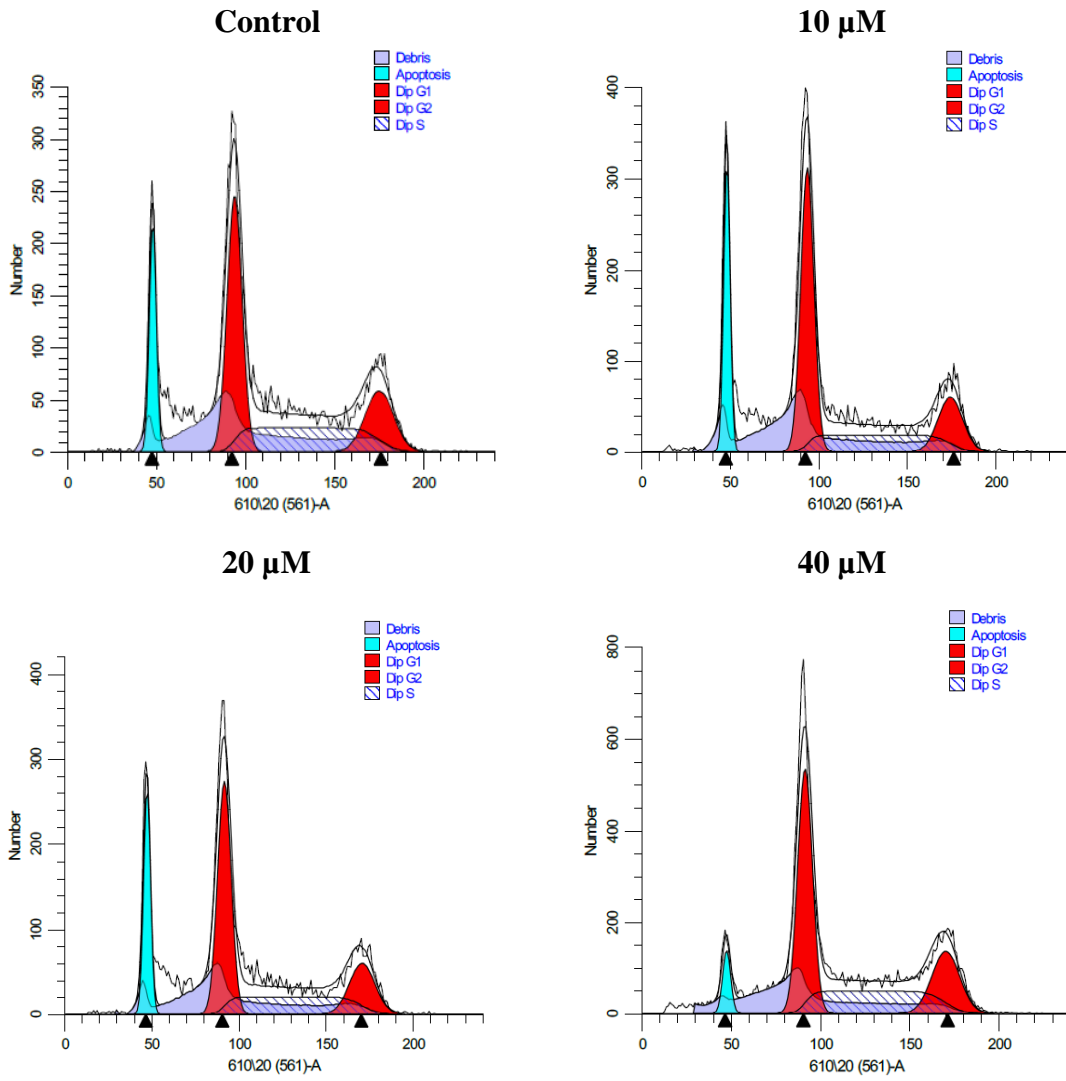
We then assessed the mechanistic impact on Hap1 cell lines upon treatment with garcinolic acid. In order to test the effects of garcinolic acid on cell cycle regulation, we conducted a cell cycle assay using propidium iodide and flow cytometry. Hap1 WT, CBP KO, and p300 KO cells were treated with garcinolic acid for 12h. Cells were then washed, fixed, and treated with propidium iodide for 30 min. DNA content of each cell analyzed using flow cytometry. Hap1 WT cells treated with garcinolic acid were arrested at the G1 phase of the cell cycle (Figure 3.10). There were no significant dose dependent changes observed in any phase of the cell cycle in Hap1 p300 KO cells. There were no dose dependent changes in any of the cell cycle phases of Hap1 CBP KO except for at 40  $\mu$ M, which we observed an increase of approximately 10% in the S phase with G2 phase compensating for much this phase shift. This could be due to garcinolic having a toxic effect on the cell than directly blocking KIX-dependent transcription. Overall, this suggest that both CBP and p300 are essential for cell cycle regulation through the KIX domain. Further regulatory analysis of the cell cycle pathway such as evaluation of cyclin proteins may elucidate CBP/p300 KIX's role in the pathway.

A)

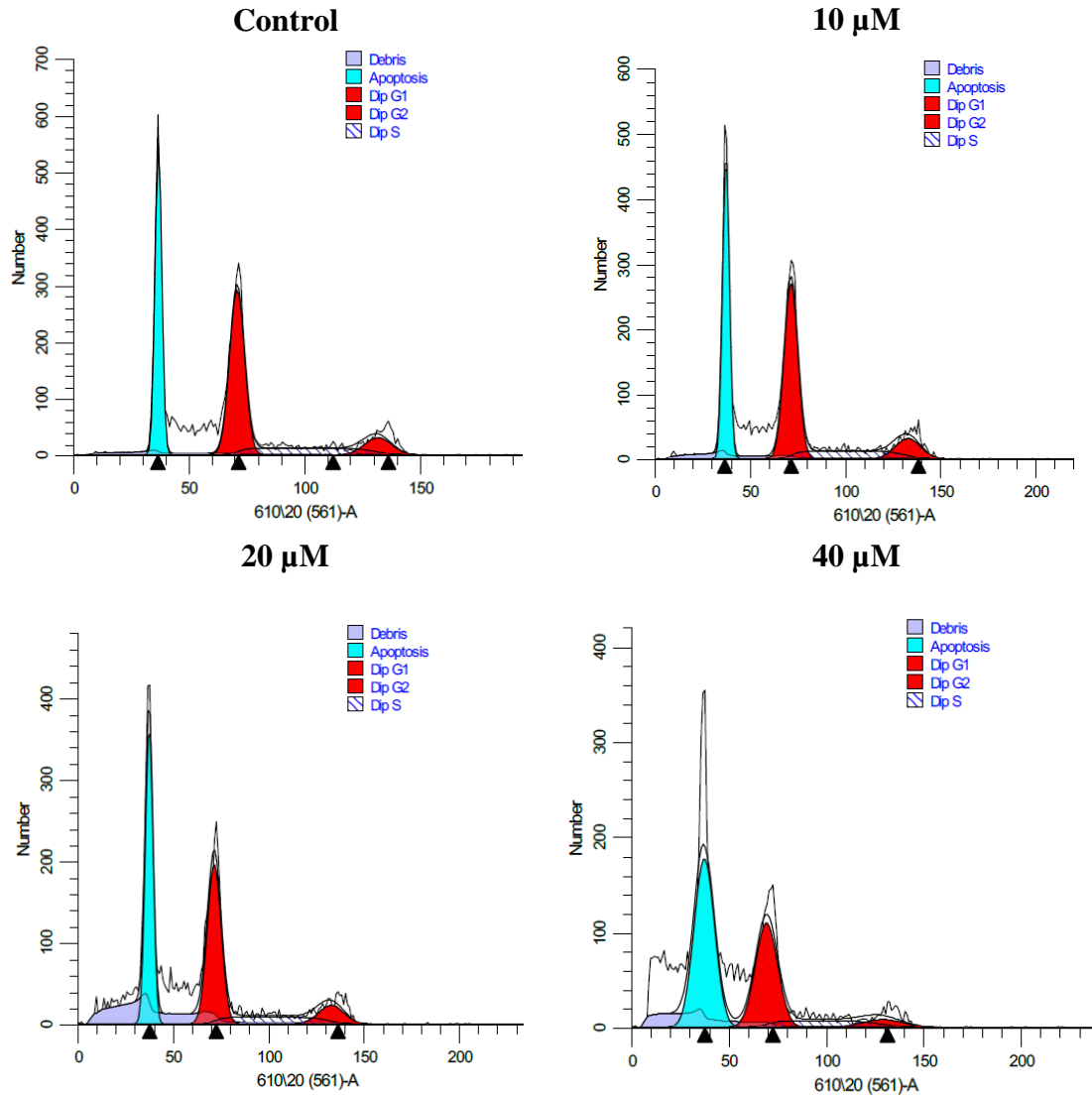


**B)**

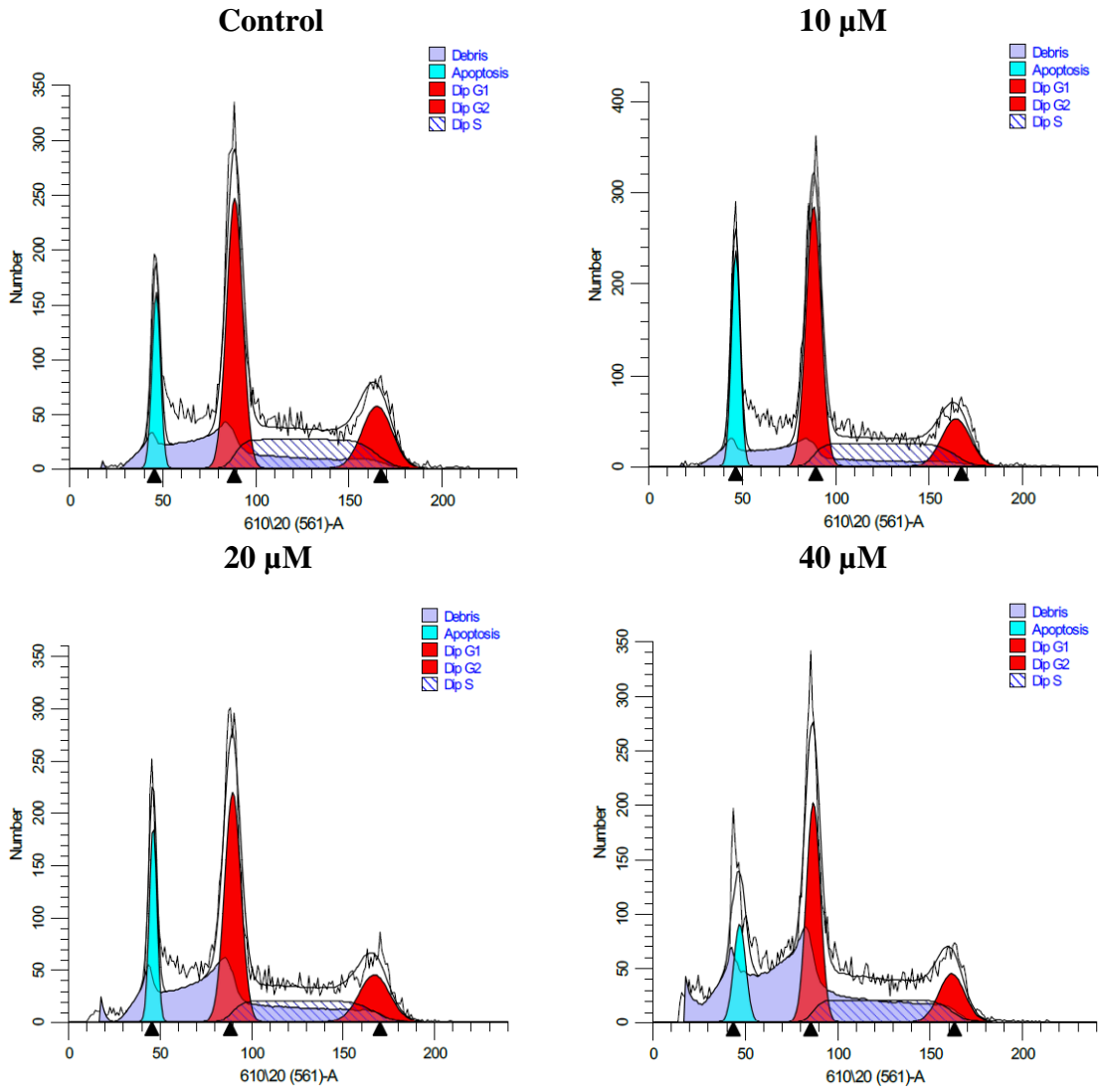
### Hap1 WT



# Hap1 CBP KO



# Hap1 p300 KO





C)

	<b>Hap1 WT p-value</b>		
<b>[garcinolic acid], <math>\mu\text{M}</math></b>	G1 p-value	S p-value	G2 p-value
<b>0:10</b>	0.4608	0.7519	0.9604
<b>0:20</b>	0.0066 (**)	0.0515	0.8012
<b>0:40</b>	0.0556	0.1134	0.9843

	<b>Hap1 CBP KO p-value</b>		
<b>[garcinolic acid], <math>\mu\text{M}</math></b>	G1 p-value	S p-value	G2 p-value
<b>0:10</b>	0.9999	0.9993	>0.9999
<b>0:20</b>	0.9132	0.9702	0.6953
<b>0:40</b>	0.6500	0.0546	0.4260

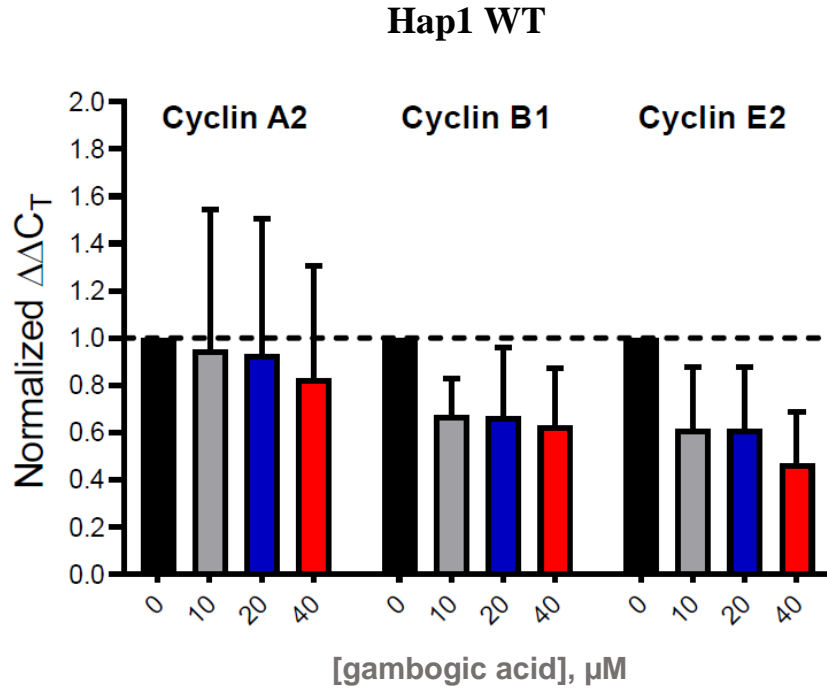
	<b>Hap1 p300 KO p-value</b>		
<b>[garcinolic acid], <math>\mu\text{M}</math></b>	G1 p-value	S p-value	G2 p-value
<b>0:10</b>	0.2624	0.6137	0.9159
<b>0:20</b>	0.8307	0.9995	0.7738
<b>0:40</b>	0.2737	0.9880	0.4346

**Figure 3.10: Impact of garcinolic acid on Hap1 cell cycle.** (A) Cell cycle phase (G1, S, G2) shifts of Hap1 WT, Hap1 CBP KO, and Hap1 p300 KO cells upon treatment with garcinolic acid. (B) Histograms of Hap1 cell lines treated with 0.25% DMSO, 10  $\mu\text{M}$ , 20  $\mu\text{M}$ , and 40  $\mu\text{M}$  garcinolic acid for 12 h. (C) Statistical analysis (p-value) of Hap1 cell lines treated with garcinolic acid. n=3

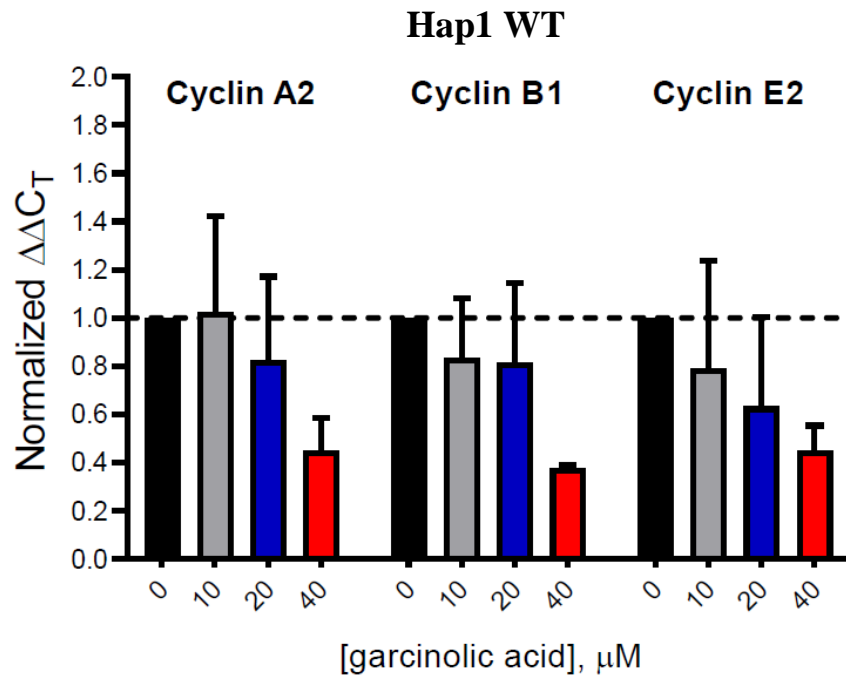
We next examined the impact on cell cycle gene expression. CBP/p300 has been reported to regulate cell cycle progression.<sup>29,30</sup> Specifically, Cyclin A2, B1, and E2 have been reported to be responsible for regulating cell cycle progression from G1 to S phase,<sup>31-33</sup> and previous studies suggest that these cyclin proteins are regulated by CBP/p300 KIX.<sup>16</sup> To this end, we examined the role of KIX in regulating cell cycle through these cyclin genes. We treated Hap1 WT, CBP KO, and p300 KO cells with garcinolic acid for 8hrs, which is required to observe direct effects from dosing cells with garcinolic acid and extracted mRNA from samples to analyze gene expression changes by qRT-PCR. Using RPL19 as our housekeeping gene and normalizing to DMSO as a control, we were able to observe the impact of dose dependent gene expression level changes. Cyclin A2, B1, and E2 gene expression levels decreased dose dependently and each less than 50% at 40  $\mu$ M in Hap1 WT cells (Figure 3.11). Additionally, Hap1 WT cells treated with gambogic acid did exhibit dose dependent downregulation. We observed the most impact at 20  $\mu$ M with ~40% downregulation of Cyclin B1 and E2 and ~50% downregulation of Cyclin A2 in Hap1 p300 KO cells. In total, this suggest that CBP KIX may regulate the cell cycle through Cyclin A2, B1, and E2 and p300 may have minimal a role in regulating the expression of those same genes. Further examination of KIX-dependent transcription was conducted in MV4-11 cells, which are acute monocytic leukemia (AML). MV4-11 cells, like other AML subtypes, harbor an MLL translocation. This may play a role in KIX-dependent transcription as MLL is an activator binding partner of KIX.<sup>34,35</sup> We observed dose dependent downregulation of Cyclin A2, B1, and E2 in MV4-11 cells when treated with increasing concentrations of garcinolic acid (Figure 3.12). Further examination is needed to fully understand the distinct roles of CBP and

p300 in regulating the cell cycle.

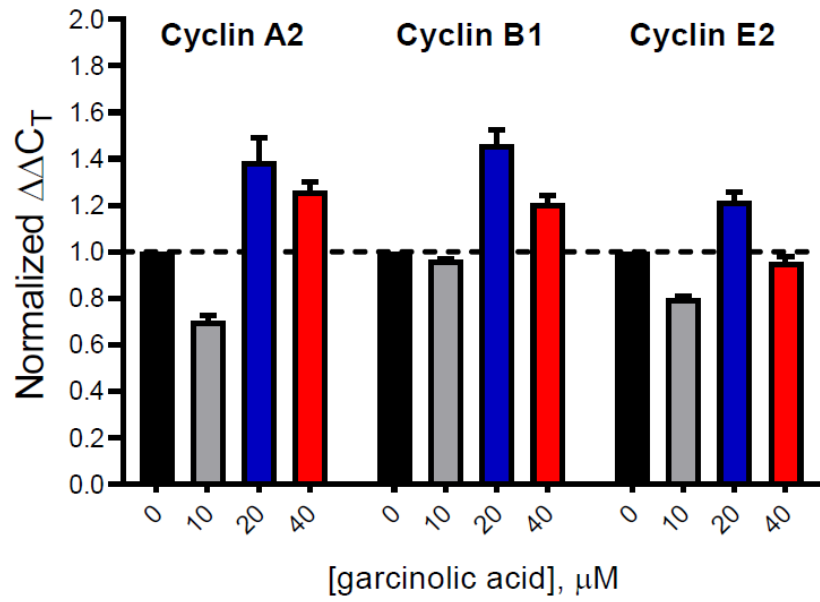
A)



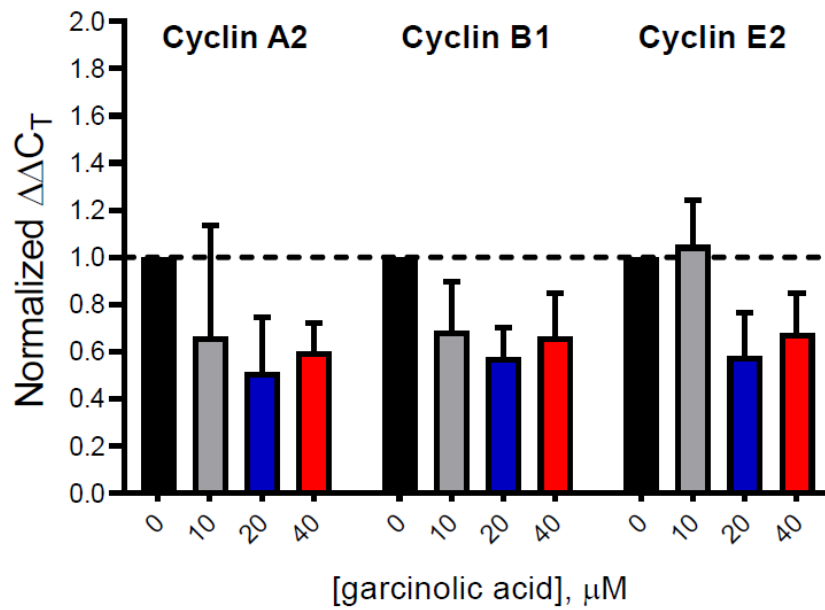
B)



### Hap1 CBP KO



### Hap1 p300 KO



C)

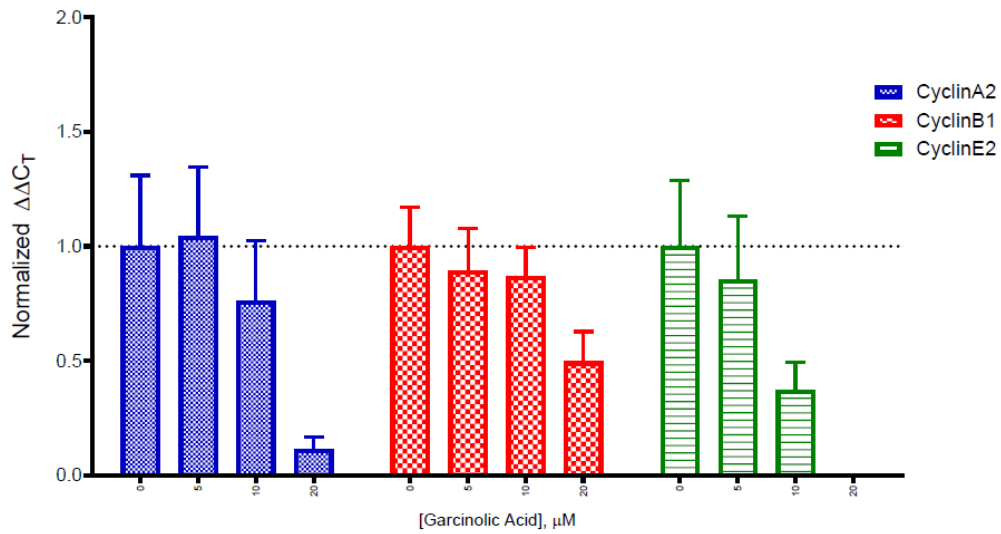
	<b>Hap1 WT</b>		
<b>[garcinolic acid], <math>\mu</math>M</b>	Cyclin A2 p-value	Cyclin B1 p-value	Cyclin E2 p-value
<b>0:10</b>	0.890830	0.179978	0.039191 (*)
<b>0:20</b>	0.113186	0.113186	0.000268 (***)
<b>0:40</b>	6.04e-07 (***)	5.22e-09 (***)	8.04e.07 (***)

	<b>Hap1 CBP KO</b>		
<b>[garcinolic acid], <math>\mu</math>M</b>	Cyclin A2 p-value	Cyclin B1 p-value	Cyclin E2 p-value
<b>0:10</b>	0.922264	0.377616	0.271128
<b>0:20</b>	0.347225	0.148341	0.373919
<b>0:40</b>	0.407348	0.518502	0.763411

	<b>Hap1 p300 KO</b>		
<b>[garcinolic acid], <math>\mu</math>M</b>	Cyclin A2 p-value	Cyclin B1 p-value	Cyclin E2 p-value
<b>0:10</b>	0.051767	0.209524	0.905337
<b>0:20</b>	0.004082 (*)	0.078362	0.069965
<b>0:40</b>	0.104197	0.177341	0.198437

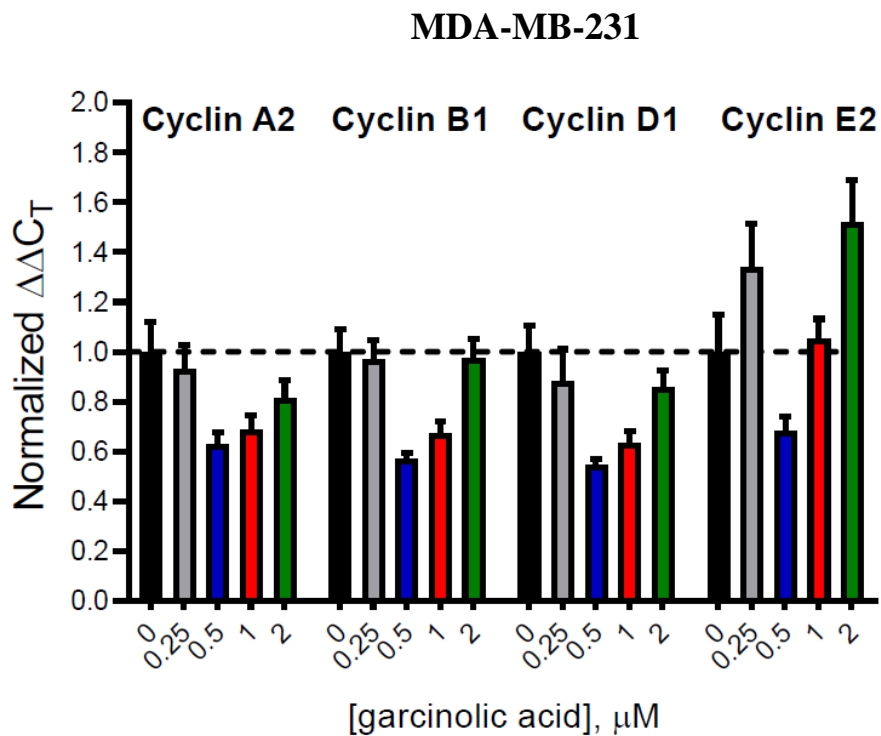
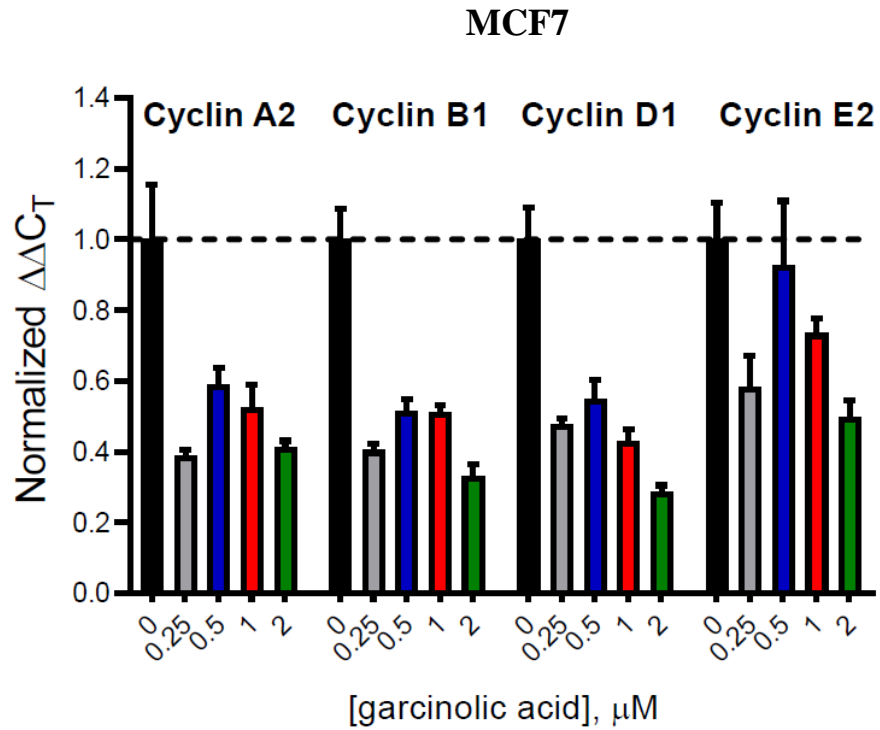
**Figure 3.11: Effect of garcinolic acid on Hap1 cyclin expression.** (A) Cyclin A2, B1, and E2 gene expression changes upon treatment of Hap1 WT cells with 0.25% and various gambogic acid concentrations for 8 h. (B) Cyclin A2, B1, and E2 gene expression changes upon treatment of Hap1 WT, Hap1 CBP KO, and Hap1 p300 KO cells with 0.25% DMSO and various garcinolic acid concentrations for 8h. (C) Statistical analysis (p-value) of Hap1 cell lines treated with garcinolic acid. n=3

## MV4-11



**Figure 3.12: Effect of garcinolic acid on MV4-11 cyclin expression.** Cyclin A2, B1, and E2 gene expression changes upon treatment of MV4-11 cells with DMSO, 5  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 20  $\mu\text{M}$  garcinolic acid for 8h. n=2 Data provided from Dr. Stephen Joy.

Additional gene expression studies were conducted in the non-invasive MCF7 breast cancer cell line and metastatic breast cancer line MDA-MB-231. Here we observed dose-dependent downregulation of Cyclin A2, B1, D1, and E2 in concentration range of 0.5  $\mu\text{M}$  to 2  $\mu\text{M}$  garcinolic acid in MCF7 cells (Figure 3.13). This further suggest the ability of KIX to regulate cell cycle through cyclin genes.



**Figure 3.13: Effect of garcinolic acid on breast cancer cyclin expression.** Cyclin A2, B1, and E2 gene expression changes upon treatment of MCF7 and MDA-MB-231 cells with various garcinolic acid concentrations for 4h. n=1 (error bars represent technical triplicates.)

## **D. Conclusion**

Coactivators CBP/p300 KIX maintain cellular homeostasis, growth, and proliferation through the regulation of gene expression. This regulation is facilitated through interactions with its many activator binding partners, and dysregulation of these essential PPIs is associated with the promotion of cancer. Though the functional role CBP/p300 KIX is well known, it is unclear how this network of PPIs contributes to cancer development through the cell cycle. Prior to this work there have been no effective strategies to examine the mechanistic and phenotypic role of KIX-dependent cell cycle regulation in chronic myeloid leukemia cell lines.

Using garcinolic acid as a chemical genetic probe, we have interrogated the KIX and activator binding partners interactions in chronic myeloid leukemia. We used garcinolic acid as a tool to block KIX-dependent transcription and examine cell viability, gene expression, and cell cycle regulation. Particularly, garcinolic acid causes apoptosis, where cell lines without CBP or p300 are the most vulnerable to cell death, which suggest CBP and p300 play a vital role in maintaining cellular health. Additionally, we observed that both CBP and p300 KIX inhibition impacts the regulation of the cell cycle. This is seen in both the gene expression profile of cell cycle genes and DNA content of cells. Overall, this suggest that both CBP and p300 KIX play a role in regulating the cell cycle, but the mechanism by which they regulate may be different based on their respective gene targets. Future studies will further interrogate the distinct roles of CBP and p300 KIX in regulated the cell cycle in chronic myeloid leukemia.



## **E. Materials and Methods**

### **Cell lines**

Hap1, Hap1 CBP KO, and Hap1 P300 KO were obtained from Horizon Discovery. MV4-11 cells were obtained from ATCC. Each cell line was grown in Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Assays with these cell lines were conducted in IMDM with 2.5% FBS. MCF7 and MDA-MB-231 cells were a gift from the Merajver lab. Both cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) with 10% FBS. Assays with these cell lines were conducted in DMEM with 2.5% FBS.

### **Evaluation of cytotoxicity**

For viability assays, cells were plated at a density of  $1.5 \times 10^4$ /well in a clear 96-well plate for 24hrs prior to drug treatment. Cells were either treated with compound dissolved in DMSO and serially diluted in culture media to achieve final concentration with DMSO less than 0.25% DMSO. Cells were cultured for 24 hours at 37°C at 5% CO<sub>2</sub>. The WST-1 cell proliferation assay kit (Sigma-Aldrich) was then used with 10 µL of reagent added to each well for 4hrs at 37°C. Plates were read for absorbance at 440 nm using a SpectraMax microplate reader. Drug concentrations were assayed in triplicate.

### **Evaluation of apoptosis and cell cycle via flow cytometry**

Apoptosis in cell culture was detected using the Annexin-V FITC staining kit (Sigma-Aldrich). Hap1 cell lines were plated in a 6-well plate at a density of  $8 \times 10^5$ /well for 24hrs and treated with various concentrations of compound. After 24 hours, cells were stained with Annexin-V FITC and propidium iodide (PI) (Life Technologies) for 10 min in the

dark and analyzed with a FACSAria III flow cytometer from BD Biosciences. Cell cycle was detected using PI staining kit (Life Technologies). Hap1 cell lines were plated in a 6-well plate at a density of  $8 \times 10^5$ /well for 12hrs and treated with various concentrations of compound. After 12 hours, cells were washed with 1X phosphate buffer saline, then washed 50% water and 50% ethanol at 4°C for 30 minutes. Samples were then stained with PI for 30 minutes and analyzed with a LSR Fortessa from BD Biosciences.

### Quantitative reverse transcription-PCR

Effect of garcinolic acid on expression level was assessed by qRT-PCR after 4hrs of incubation of compound with cells. Media was changed from 10% FBS in IMDM to 2.5% FBS in IMDM upon incubation with compound. RNA was isolated from samples using the Qiagen RNeasy kit. Real-time PCR reactions were performed with the SYBR green kit and the following primers:

**Table 3.1: qRT-PCR Primers.**

Gene	Primer Sequence
<b>RPL19</b>	Forward: 5'-ATGTATCACAGCCTGTACCTG-3' Reverse: 5'-TTCTTGGTCTCTCTTCCTCCTTG-3'
<b>Cyclin A2</b>	Forward: 5'-CCAAGAGGA CCAGGAGAATA-3' Reverse: 5'-CGGTGACATGCTCATCATT-3'
<b>Cyclin B1</b>	Forward: 5'-AGTCACCAGGAACTCGAAAA-3' Reverse: 5'-GTTACCAATGTCCCCAAGAG-3'
<b>Cyclin D1</b>	Forward: 5'-ACAAACAGATCATCCGCAAACAC-3' Reverse: 5'-TGTTGGGGCTCCTCAGGTTC-3'
<b>Cyclin E2</b>	Forward: 5'-AGAGAGGAGGTCACCAAGAAA-3' Reverse: 5'-CAGGCAAAGGTGAAGGATTA-3'

### Statistical Analysis

A two way ANOVA was conducted using GraphPad Prism to analyze the significance of the flow cytometry data. A linear mixed model that is categorical was conducted using RStudio to analyze the significance of the qRT-PCR data.

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## **Chapter 4: Conclusions and Future Directions**

### **A. Conclusions**

The characterization of protein-protein interactions (PPIs) is potentially a new strategy for developing novel therapeutics to treat disease. Historically, there has been much success in developing small molecules for stable PPIs, but there is still an urgent need for tools to examine transient interactions. This is particularly true for PPIs that occur in signal transduction and transcriptional activation. The Ras interaction with Raf has been demonstrated to be vital in initiating the mitogen activated protein kinase pathway (MAPK) signaling cascade, which leads to the maintenance of proper cellular health. As a result of this critical interaction, overexpression or overactivation of Ras lies at the crux of initiation and development of proliferative diseases such as cancer. An understanding could lead to new drug targets. We conducted an investigation for the development of genetic tools for interrogating this molecular interaction. Additionally, the CBP/p300 KIX interaction with activator binding proteins is at the center of the transcriptional regulatory complex. Through this vital PPI hub, there is an opportunity for several interacting proteins to be dysregulated and cause disease. Consequently, the reliance of activator binding proteins on the interaction with CBP/p300 KIX can be used to develop therapeutics that inhibit this molecular interaction. We conducted an investigation of the mechanistic and phenotypic impact of KIX-dependent transcriptional regulation using chemical genetic tools.

The results I have presented in this thesis detail the characterization of transient PPIs through utilization of genetic and chemical genetic techniques. We developed a Raf-based biochemical probe that can be used to covalently capture active Ras isoforms, providing a novel genetic tool for the capture and examination of the transient Ras-Raf interaction. We also discovered and utilized a natural product inhibitor of the CBP/p300 KIX activator binding protein interactions to elucidate KIX-dependent transcription in cancer.

### **A.1 Active Ras isoforms can be covalently captured and examined**

In Chapter 2, the traditional method for active Ras isolation pulldown was used as a platform to develop a novel construct for capturing and stabilizing Ras. We designed a plasmid construct for incorporating an unnatural amino acid (UAA) into Raf-Ras binding domain (RBD). We optimized an *E. coli* expression system for synthesizing recombinant protein with an amber stop codon and orthogonal tRNA/tRNA synthetase. We optimized purification of p-benzoyl-L-phenylalanine (pBpa) incorporated RBD using a GB1 purification tag for solubility and His<sub>6</sub> tag for isolation of full-length protein. We performed a proof-of-concept pulldown experiment using GB1-RBDV69pBpa-His<sub>6</sub> and H-Ras variants wildtype, G12V, and S17N transfected in 293T cells. We have validated that GB1-RBDV69pBpa-His<sub>6</sub> can covalently capture active Ras from cell lysate, which can be visualized directly from cell lysate due to the molecular



weight shift (without isolation of the complex). As a proof-of-concept, recombinant Ras isoforms H-Ras, N-Ras, K-Ras4A, K-Ras4B were digested with Asp-N and trypsin proteases. We identified unique Ras fragments by analyzing the digested Ras isoform products by LC-MS. Also, it is important to note that additional experiments need to be conducted to establish the probe as a practical tool. This includes an exhaustive evaluation of potential UAA incorporation sites in RBD for crosslinking active Ras. Also, an analysis of crosslinked product by LC-MS to further suggest our ability to distinguish Ras isoforms from crosslinked product is necessary. Once this has been completed, it strongly suggest that the probe can be utilized for other applications.

## **A.2 *In vivo* characterization of KIX-dependent transcription using a small molecule**

In Chapter 3, we elucidated the structure-function relationship of KIX-dependent transcription on cell cycle regulation and cell viability. Development of small molecule inhibitors targeting KIX is challenging due to intrinsically disordered conformational dynamics. We discovered an inhibitor of CBP/p300 KIX named garcinolic acid from a chemical compound screen. We demonstrated selectivity by screening against other KIX motifs *in vitro*. These efforts led to the utilization of garcinolic acid to characterize KIX-dependent transcription *in vivo*. We demonstrated efficacy in leukemia and breast cancer cells with garcinolic acid treatment leading to >50% cell death in all cells. A comparable study was conducted with a derivative of

garcinolic acid that does not bind to KIX named gambogic acid. Wildtype leukemia cells tolerate gambogic acid suggesting the selectivity of garcinolic acid. We further investigated the mechanism of leukemia cell death by performing an apoptosis assay. Our analysis revealed that leukemia cells lacking either CBP or p300 were more susceptible to apoptosis relative to wildtype.

We then used garcinolic acid to investigate the role of CBP/p300 KIX in regulating cell cycle. We demonstrated that garcinolic acid treatment led to G1 phase arrest of the cell cycle in Hap1 cells. The same treatment in Hap1 CBP and p300 KO cells had little to no effect on G1 phase. We further demonstrated that blocking KIX-dependent transcription with garcinolic acid resulted in dose dependent downregulation of cyclin proteins Cyclin A2, B1, and E2 in both Hap1 and MV4-11 leukemia cells and MCF7 breast cancer cells. Interestingly, we also observed downregulation of the same cyclin proteins in Hap1 p300 KO cells but not in Hap1 CBP KO cells. Although, both CBP and p300 KIX may play a role in regulating the cell cycle, there is some evidence of distinct roles between CBP and p300 KIX in regulating distinct cell cycle genes.

## **B. Future Directions**

### **B.1 Further optimization of photocrosslinkable GB1-RBD and Ras superfamily**

GB1-RBDV69pBpa-His<sub>6</sub> has successfully been used as a biochemical probe for covalently capturing small GTPase Ras. This novel biochemical tool can be further explored by utilizing it to isolate active Ras from various cancer cells and quantifying

the contribution of Ras isoforms under native and modeled conditions. Also, *in vivo* UAA incorporation can be applied to Raf kinase for a more effective examination of active Ras isoforms. This system can be used in any mammalian cell line/cell lysate. There is also an opportunity to alter the photoreactive crosslinker to optimize covalent capture of binding partners. Studies suggest that pBpa reacts with specific amino acid residues; so, this could limit its ability to effectively capture Ras. Examining other crosslinkers such as diazirines or azides could further optimize the probe for covalently capturing active Ras. In addition to optimization, similar biochemical probes can be predicated on the construct that we have designed. There exists a Ras superfamily of small GTPases that function similarly to Ras as a small GTP catalyst. Binding domains for the respective small GTPases can be used in a similar manner to Raf-RBD to develop biochemical probes that covalently capture active GTPases.

## **B.2 Future characterization of CBP/p300 KIX-dependent transcription**

Despite our recent success in elucidating the context-dependent role of KIX in maintaining cellular health and regulating the cell cycle, characterization of the structure-function relationship of KIX-dependent transcription requires further examination. Based on our observation of consistent dose-dependent downregulation of cyclin genes across cancer types, future studies can utilize techniques such as RNASeq to examine global gene expression changes upon blocking KIX-dependent transcription.

This will present an opportunity to not only understand the broader impacts of inhibiting CBP/p300 KIX, but also reveal other vital biological mechanisms that it regulates, especially in those exploited in disease states. Additionally, there is an opportunity to further develop and optimize the potency of garcinolic acid. While the inhibitor is capable of binding to KIX at low micromolar affinity, significantly higher concentrations are required to interrogate the CBP/p300 KIX and activator binding proteins interaction *in vivo*. To accomplish this, thorough characterization of garcinolic acid and KIX is required to understand the structure activity relationship between the small molecule and protein interaction. For instance, if we compare garcinolic acid to gambogic acid there are key differences that may play a role in garcinolic acid's ability to selectively bind to KIX. The chemical structure of gambogic acid consist of an additional bridge and ketone group while lack a carboxylic acid group observed in the structure of garcinolic acid. These distinct differences can be used as a starting point for optimizing garcinolic acid as a effective inhibitor of CBP/p300 KIX. Lastly, chemical proteomics is an important step in understanding the effect of garcinolic acid *in vivo* since it allows better understanding of small molecule-protein interactions. This in addition to optimization of the garcinolic as an inhibitor will improve both the selectivity and potency of the compound.