NUCLEAR BEHAVIORS OF ERK1/2 SIGNALING

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DEDICATION

For all my Teachers

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by

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Abstract

The rat sarcoma (Ras)- rapidly accelerated fibrosarcoma (Raf)- mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) kinase (MEK)-ERK pathway is essential for proper development and homeostatic regulation in eukaryotic cells. Many pathway functions are carried out by ERK1 and ERK2 (ERK1/2), serine/threonine protein kinases that interact with a large number of substrates in several cell compartments. ERK1/2 are crucial for major cellular phenomena such as proliferation, differentiation, and programmed cell death. Despite a large body of knowledge about this pathway, a clear understanding of how specific signals elicit specific responses remains elusive. To dissect different modes of ERK1/2 regulation in the nucleus and on chromatin, I took a multifaceted approach to study nuclear ERK1/2 signaling. First, I investigated the direct DNA binding activity of ERK2 in response to phosphorylation and mutation of key residues based on characterization of ERK2 phosphorylated on T188, a residue proximal to the classically defined phosphorylation activation loop sites. To test the possibility that alternate phosphorylation inputs affect ERK2 in ways other than its kinase activity, I utilized phospho-mimetic mutants to assay oligonucleotide binding. I demonstrated that phosphorylation on the activation loop enhances ERK2 association with DNA and raised the notion that this understudied property of ERK2 dictates its interactions with DNA as well as substrates in the nucleus. Second, I investigated the regulatory relationship between ERK1/2 and CXXC finger protein 1 (Cfp1). Cfp1 is an epigenetic regulator that interacts with two major chromatin modifying complexes, and my studies clarified previously collected data to demonstrate that ERK2 and Cfp1 co-regulate target genes in a signal-dependent manner. Lastly, I sought to identify the extent of ERK1 and ERK2 interactions in the nucleus. To this end, I generated tools for a proximity biotinylation strategy that will not only identify transient and stable nuclear interactions of ERK1/2, but also address the long-standing question of whether ERK1 and ERK2 perform divergent functions. Through these approaches, I identified important areas of research to improve the current understanding of the nuclear behaviors of ERK1/2 signaling and discovered that ERK1/2 regulate gene expression through multiple modes of interaction with chromatin.

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LIST OF DEFINITIONS

BETA2/NeuroD1	β2/Neurogenic differentiation 1
ChIP	Chromatin immunoprecipitation
COMPASS	Complex of proteins associated with Set1
DMEM	Dulbecco's Modified Eagle's Media
DMSO	Dimethyl sulfoxide
DNMT1	DNA methyltransferase 1
EGF	Epidermal growth factor
ERK1/2	Extracellular signal-regulated protein kinase 1 and 2
ESC	Embryonic stem cell
EYA-1	Eyes absent homolog 1
FBS	Fetal bovine serum
GFP	Green fluorescent protein
GH1	Growth hormone 1
GST	Glutathione S-transferase
H3K4me3	Histone H3 lysine 4 trimethylation
HipA	High-persistence A (bacterial protein)
HipB	High-persistence B (bacterial protein)
Hog1p	High-osmolarity glycerol response protein
IEG	Immediate early gene
IGFBP2	Insulin-like growth factor binding protein 2
IPTG	Isopropyl β-d-1-thiogalactopyranoside
Kss1p	Kinase suppressor of Sst2 mutations
MAPK	Mitogen activated protein kinase
MAPKK (MAP2K)	Mitogen activated protein kinase kinase
MAPKKK (MAP3K)	Mitogen activated protein kinase kinase kinase
MBP	Myelin basic protein
MEK	MAPK/ERK kinase
mIns	Mouse Insulin
MKP3	MAP kinase phosphatase 3
MLL	Mixed lineage leukemia
NGF	Nerve growth factor
Nup153	Nucleoporin 153
PDX-1	Pancreatic and duodenal homeobox 1
Pol II	RNA polymerase II
PP2A	Protein Phosphatase 2
PTM	Post-translational modification
PTP1	Protein tyrosine phosphatase
qRT-PCR	Quantitative real time polymerase chain reaction
qRT-PCR	Quantitative real time polymerase chain reaction
SET	Su(var)3-9, Enhancer of zeste and Trithorax domain
Ulk1	Unc-51-like autophagy activating kinase 1

CHAPTER ONE

INTRODUCTION

Mitogen-activated Protein Kinase Signaling

The last several years have seen an increased awareness that protein kinases are more than phosphorylating enzymes; they are multifunctional molecules evolved to process critical biological information. Due to the prominent roles of MAPK pathways in both normal signal transmission and in a variety of disease states, examination of these core modules will continue to inform the general understanding of cellular signaling. For some time, it has been known that the MAPKs ERK1 and ERK2 interact with multiple factors in a multitude of ways to regulate cellular processes, including gene expression. Here, I have made an effort to use biochemical characterization to ascertain general properties of ERK1/2 signaling in the nucleus. My research was heavily guided by the extensive body of literature on ERK1/2 signaling as well as chromatin regulation and aimed to clarify the current understanding of the nuclear behaviors of the ERK1/2 pathway.

Background

Prototypical MAPK activation employs a three-kinase core module consisting of a MAPK kinase kinase (MAPKKK or MAP3K) that phosphorylates and activates a MAPK kinase (MAP2K, MEK, or MKK) that in turn phosphorylates and dramatically increases the activity of one or more MAPKs. Once activated, MAPKs can phosphorylate a wide array of

intracellular targets that include, but are not limited to, cytoskeletal elements, membrane transporters, nuclear pore proteins, transcription factors, as well as other protein kinases (**Figure 1-1**). Simplified models of the organization of MAPK cascades highlight the number of MAP2K-MAPK combinations a given MAP3K can regulate and further underscores putative nodes of cross talk (**Figure 1-2**).

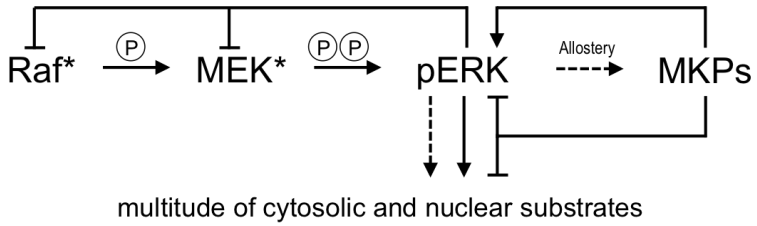

Figure 1-1 Selected intra-pathway inputs on the ERK1/2 signaling module.

Discovery of MAPKs

ERK1/2 were initially identified as mitogen-stimulated ~42kDa phosphoproteins in the late 1980s [1, 2]. Early studies demonstrated preparations of ERK1/2 retained the ability to phosphorylate the model substrates microtubule-associated protein 2 (MAP2) and myelin basic protein (MBP), and reactivate phosphatase-treated ribosomal protein S6 kinase (RSK or p90) [3-6].

In following years, the MAPK family was discovered to include three c-Jun N-terminal kinases (JNKs), four p38 isoforms, ERK3 isoforms, and ERK5 and ERK7 (**Figure 1-2**). The first JNK family members were independently identified as cycloheximide-activated MBP kinases and purified for their ability to interact with the N-terminus of the transcription factor c-Jun [7, 8]. p38α was determined to be an inflammatory cytokine-

stimulated tyrosine phosphoprotein, a target of an inhibitor of tumor necrosis factor α (TNFα) production, and a re-activating kinase for MAPK-activated protein kinase 2 (MAPKAP2 or MK2) [9-11]. PCR-based cloning strategies and a yeast two-hybrid screen identified additional JNK and p38 isoforms as well as ERKs 5 and 7 (reviewed by [12-16]; a summary of the cellular processes involving these MAPKs is shown in **Figure 1-2**, and detailed reviews [17-19] are recommended for further reading). Notably, ERK5 has become a recent target of therapeutic interest [20] because of its roles in cancer and structural differences from ERK1 and ERK2 [21, 22]. Although similar stimuli affect ERK1/2 and ERK5, particularly growth factors, activation of ERK5 is dependent on different upstream components such as cAMP response element (CRE) [21, 23]. Indeed, regulated cross talk and insulation are both hallmarks of most related MAPK pathways (**Figure 1-2**). Collectively, these findings established that prototypical three-kinase cascades culminating in activation of a MAPK are versatile signaling modules capable of integrating a multitude of inputs into a variety of responses.

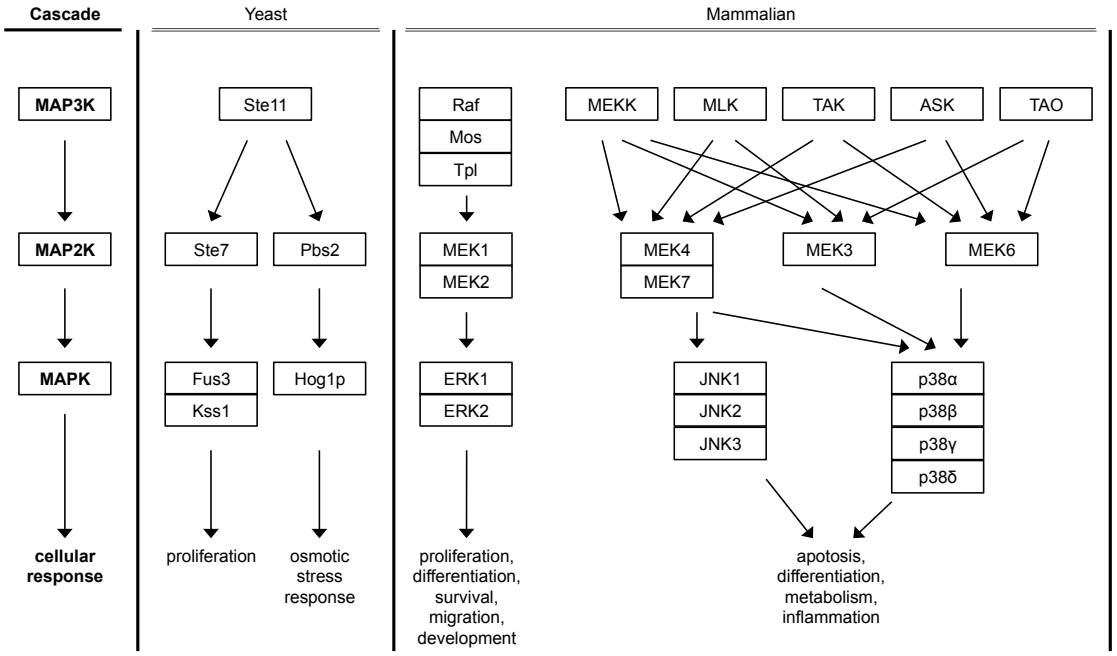

Figure 1-2 Selected MAPK pathways are subject to diverse upstream signaling and extensive cross-talk.

Upstream Regulation of ERK1/2

The observation that Ras and Raf mutations are prevalent in a variety of human tumors served as early evidence for ERK1/2 roles in proliferation and oncogenic growth [24]. Work from multiple laboratories led to the realization that Raf isoforms are effectors of Ras small GTP binding proteins and are upstream kinases for MAPK/ERK kinase 1 and 2 (MEK1/2), which in turn activate ERK1/2 via dual phosphorylation ([3, 25-29] and **Figure 1-1**). Consistent with these early reports, the capacity to drive tumorigenesis was further demonstrated by the ability of an activated mutant of MEK1 to transform cells and promote tumor growth in nude mice [30]. Subsequent work employing dominant interfering mutants, pharmacological inhibitors of MEK1/2, and disruptions in pathway component gene

expression all revealed ERK1/2 to be intimately involved in normal processes including embryogenesis, cell differentiation, glucose sensing, and synaptic plasticity [31-36]. The varied output of this pathway is well documented by numerous studies, but like other MAPKs, ERK1/2 primarily receive signals from a discrete set of upstream factors, which are discussed below.

MAP2Ks

MAP2Ks are dual-specificity kinases capable of phosphorylating both tyrosine and serine/threonine residues and serve as points of signal integration, largely through docking site-mediated protein-protein interactions and the activity of scaffolding proteins. In contrast to MAPKs that have a wide range of substrates, MAP2Ks are highly specific: they are primarily (but perhaps not exclusively) dedicated to phosphorylation of a small number of MAPK proteins (**Figure 1-2**).

The quintessential MAP2K protein MEK1 was initially purified as a ~45 kDa activator of ERK1/2, and DNA-based molecular techniques led to the subsequent identification of MEKs 2-7 (reviewed by Chen *et al* and Lewis *et al*) [12, 37-40]. While pharmacological inhibition of MEK1/2 is sufficient to effectively shut down ERK1/2 signaling, a recent report has suggested that MEK1/2 have an additional substrate: the proteotoxic stress response master regulator HSF1 [41]. In addition to uncovering a connection between MEK1/2 and regulation of proteomic stability, this report also identified opposing roles of ERK1/2 and MEK1/2 within the proteotoxic stress response, raising the possibility that ERK1/2 and MEK1/2 exert divergent influences on other cellular processes.

Somewhat supporting this notion, RNA-sequencing experiments in mouse embryonic stem cells (mESCs) under conditions of ERK1 depletion or pharmacological inhibition of MEK1/2 display different transcriptional profiles. However, these experiments are complicated by the continued presence of ERK2 [42]. Nevertheless, it remains to be seen if other MAP2Ks also have targets outside their respective three-kinase core cascades, and further how alternative substrates contribute to MAPK signaling regulation.

Raf Isoforms and Other MAP3Ks

The most readily identifiable feature of MAPK signaling is the three-kinase cascade consisting of a MAP3K, MAP2K and a MAPK comprising a module conserved from yeast to humans (**Figure 1-2**). One of the best-characterized mammalian MAP3Ks is Raf, originally identified as a retroviral oncogene notorious for its role in cancer promotion. Three isoforms, c-Raf (or Raf-1), B-Raf, and A-Raf, have been identified in mammals [43]. In addition to the core ~30 kDa kinase domain, Raf proteins contain an N-terminal regulatory region of roughly the same size that can directly bind Ras. Raf proteins specifically phosphorylate the MAP2Ks MEK1/2 and were initially thought to function tissue-specifically. Subsequent studies aided by the development of B-Raf inhibitors led to the understanding that Raf isoforms can dimerize to modulate signal transmission in a context-dependent manner [44, 45]. The unanticipated actions of these B-Raf inhibitors provoked more in-depth molecular analysis showing that dimerization can enhance Raf activity and that Raf heterodimers display distinct behaviors [46-48]. More recent work suggests that B-Raf dimerization is

subject to feedback control by ERK1/2, potentially explaining why Raf mutants unable to dimerize maintain inappropriately elevated levels of ERK1/2 signaling [49, 50].

Although there is no direct counterpart to Raf found in yeast, the highly conserved nature of MAPK signaling led researchers to look for homologs of MAP3Ks across organisms. This strategy led to the discovery of a mammalian homolog of the yeast MAP3K Ste11, named MEKK1. Despite being able to phosphorylate multiple MAP2Ks *in vitro*, numerous cell-based studies indicate MEKK1 predominantly coordinates downstream signaling through MEKs 4 and 7 and the JNK pathway [51-56]. Subsequent isolation of related cDNAs have led to the discovery of a family of enzymes (i.e. MEKKs 1-4; ASK1,2) that are reviewed by Raman *et al* and Johnson *et al* [19, 57]. As a group, these enzymes display broader substrate specificity than Raf and presumably regulate multiple MAPK pathways in a context-dependent manner.

Two more enzymes that function as MAP3Ks in the ERK1/2 pathway are Mos and Tpl2 (Cot), both originally identified as proteins able to transform cells, and both found to function in specialized situations to activate the core ERK1/2 cascade [58, 59]. Mos is expressed primarily in oocytes where translational recruitment of c-*mos* mRNA and subsequent MAPK pathway activation is critical for pronuclear formation, while Tpl2 is stabilized in response to lipopolysaccharide [60, 61]. Another MAP3K family, comprised of TAOs 1-3, serve as activators of the p38 signaling cascade and are homologs of the yeast protein Ste20 [19].

Properties of MAPK Cascades

A large body of work on yeast MAPK cascades and the mammalian ERK1/2, p38, and JNK signaling pathways has provided insight into the primary features of MAPK core modules. Efforts to understand how three-kinase cascades achieve such robust signaling have revealed multiple feedback mechanisms involving pathway effectors, scaffolds, and targets that serve to regulate MAPK signaling dynamics to achieve context-dependent outcomes. (For a detailed review on how signaling dynamics can shape cellular responses, refer to Purvis and Lahav [62].)

One major feature of ERK1/2 signaling is that two phosphorylation events on the activation loop are required for full activation, the first on a tyrosine residue and a second on a proximal threonine residue. In conjunction with scaffolding proteins that tether the three-kinase cascade together, the dual-specificity nature of MEK1/2 can behave either as a single switch via rapid double phosphorylation or exploited to generate a pool of monophosphorylated ERK1/2, thereby increasing the number of contexts that can ultimately achieve full pathway activation [4, 63-66].

Another emerging MAPK property is alternative phosphorylation of a threonine residue proximal to the canonical activation sites. In 2009 it was discovered that activated ERK2 can autophosphorylate upon dimerization to promote a distinct gene expression program in a model of cardiac hypertrophy. Subsequent biochemical characterization of a phosphomimetic mutant of the alternative phosphorylation site suggested divergent binding properties of ERK2 modified in that manner [67, 68]. Although it is far from clear as to how

different modes of phosphorylation contribute to pathway feedback regulation, these activities likely play some part in conferring network robustness.

Due to its interactions with nuclear pore proteins, free ERK1/2 can enter and exit the nucleus even in the absence of pathway activation [69, 70]. Activation of ERK1/2 can promote dimerization and perhaps nuclear localization [71], although there is evidence suggesting these outcomes may be independent of one another [72]. Recently, a small molecule able to interrupt dimer formation even when the pathway is activated has been identified, and interestingly, inhibition of dimerization seems to predominantly alter cytoplasmic ERK1/2 signaling by disrupting interactions between ERK dimers and scaffold proteins [73, 74]. More work is required to delineate how this drug affects the extensive nuclear activities of ERK1/2, especially given the potential role of dimerization in driving specific gene expression programs in an ERK1/2-dependent manner [67].

Once doubly phosphorylated, ERK1/2 have multiple substrates within the core-signaling pathway (**Figure 1-1**). As previously mentioned, ERK1/2 can phosphorylate Raf isoforms to inhibit re-activation by Ras and subsequent phosphorylation of MEK1/2, simultaneously buffering against inappropriate pathway activation and maintaining capacity for future responses [75-80]. In yeast it has been demonstrated that the p38 homolog Hog1p phosphorylates the kinase Ste20 (homologous to mammalian PAK) progressively over time, and the authors of a recent study implicated Ste20 in both negative and positive feedback loops due to its ability to act as a rheostatic effector of the MAPK [81]. Similarly, ERK1/2 are subject to modulation by several signaling molecules, indicating that interactions with other proteins are critical for normal pathway function.

Scaffolding Proteins

Although the importance of scaffold proteins has been appreciated for some time, recent advances in computer-aided modeling in combination with biochemistry is shedding light on how these proteins exert major influence on cascade function and strongly impact the activities and outputs of MAPK pathways [82]. One of the best examples of scaffold-mediated signal specificity comes from the yeast protein Ste5 in concert with the MAP3K Ste11, which is required to activate the downstream MAPKs Kss1 and Fus3 via Ste7 in response to pheromone [83-86]. However, in response to osmotic stress, the sensor protein Sho1 confers the ability of Pbs2, the MAP2K upstream of Hog1p, to form a stable interaction with Ste5 and Ste11, thus funneling distinct stimuli through common signaling components (**Figure 1-3**). More recently, in order to better understand the effects of scaffolds on pathway dynamics [87], a synthetic approach was taken to "rewire" Ste5 with highly modular PDZ domains [88]. Consistent with previous observations, the authors of this study found that scaffolds can act as logic gates by tethering core components together as well as modulate pathway output through the recruitment of protein phosphatases [87, 89, 90].

Mammalian signaling has similar complexities due to extensive crosstalk between upstream activators, and there have been many observations that a single MAP3K can regulate multiple MAPK cascades (**Figure 1-2**). However, because kinases themselves can act as binding hubs, tethering functions in mammalian MAPK cascades can be shared by both core pathway components as well as dedicated scaffolding proteins. Most interactions with MAPKs are mediated by two short sequence motifs: the docking (D) motif and the FXF

(DEF) motif, with one or more of these often present in scaffolds, activators, certain phosphatases, and many substrates [91, 92].

JNK-interacting protein (JIP) scaffolds, which organize JNK and sometimes p38 MAPK pathways, display some functional parallels to yeast Ste5. In a slight departure from the yeast situation, several MAP3Ks contain docking sites that promote stable interactions with specific MAPKs, such as the case with MEKK1 being able to bind tightly to JNKs through a docking motif [93]. Indeed, the scaffolding functions of kinases cannot be overstated, especially since some dedicated scaffolds are pseudokinases whose tethering functions were likely selected over enzymatic ones during the course of evolution [87]. One of the best-known examples of this is the scaffold kinase suppressor of Ras (KSR), which was discovered in the sevenless eye development pathway in *D. melanogaster* and in the vulval induction pathway of *C. elegans* [94, 95]. Although KSR proteins are members of the Raf family, they lack the essential ATP-binding lysine residue required for functional kinase activity. In mammals, KSR1 and KSR2 bind ERK1/2, MEK1/2, and can allosterically activate Raf [48]. Other proteins that influence assembly and activation of MAPK pathways include Sur8, CNK, MP-1 and IMP [92, 96-98].

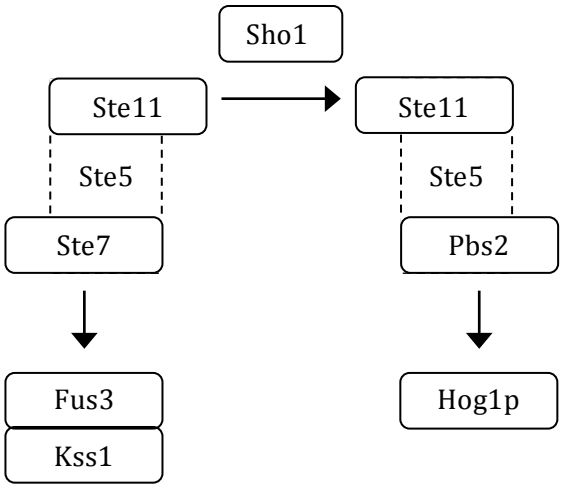

Figure 1-3 MAPK signaling is modulated by scaffolding proteins.

Tyrosine Kinase Receptor Activation of ERK1/2

ERK1/2 are activated by a wide variety of stimuli that act through multiple cell surface receptors, and of these receptor tyrosine kinase (RTK) pathways are among the best delineated [17, 19, 39]. Extracellular ligand binding increases RTK activity and generates multiple phosphotyrosine motifs on RTKs and their intracellular binding partners. These motifs are recognized by SH2 domains on a variety of factors including the adaptor proteins Shc and Grb2, which also contain proline-rich regions that can interact with the SH3 domain of the Ras guanine nucleotide exchange factor (GEF) son of sevenless (SOS). Upon association with the receptor-adaptor protein complex, SOS stimulates the exchange of GDP for GTP on Ras, promoting its interactions with a number of downstream effectors that include Raf. The direct interaction of Raf isoforms with Ras localizes these MAP3Ks to the plasma membrane and maintains proximity to non-receptor kinases such as Src family

members, p21-activated kinases (PAKs), and protein kinase C (PKC) isoforms, which in turn are able to phosphorylate Raf isoforms to alter their activity towards particular substrates or enhance interactions with other signaling factors [99, 100]. As discussed above, activated Raf isoforms can stimulate the MEK1/2-ERK1/2 pathway, thus forming the core MAPK module. Many ligands and other cellular cues also activate these pathways using similar and additional regulatory mechanisms [67, 101-106].

ERK1/2 Signaling in Chromatin Regulation and Gene Expression

Many MAPK substrates are transcription factors that participate in extensive signaling networks in the nucleus [107], where the genome is compacted into chromatin. More than just packaged DNA, chromatin provides eukaryotic cells a way to regulate access of transcriptional machinery to the underlying DNA template through chromatin modifications [108]. In addition to phosphorylating transcription factors, MAPKs can also target chromatin modifying complexes such as histone acetyl transferases (HATs), deacetylases (HDACs), and other remodeling complexes [107]. Chromatin immunoprecipitation (ChIP) studies have revealed MAP kinases also associate directly with target genes in yeast [109, 110] and in mammalian cells [34, 111-113].

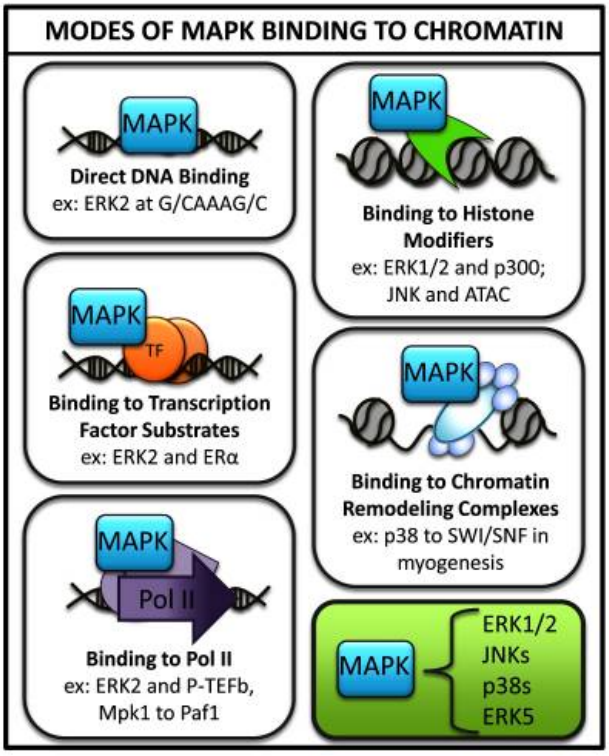

Figure 1-4 MAPK signaling in the nucleus. MAPKs affect gene expression through a variety of interactions and mechanisms. Adapted from Klein et al. 2013.

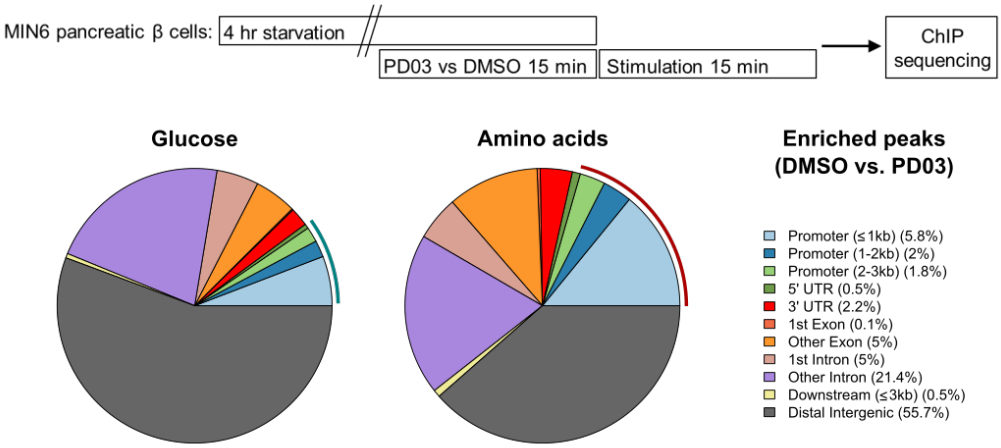

Figure 1-5 ChIP-sequencing studies demonstrate broad ERK1/2 association with chromatin. Mouse pancreatic β-cells (MIN6) were nutrient-starved and stimulated with either glucose or amino acids in the presence or absence of MEK1/2 inhibitor (PD0325901).

ERK1/2 are abundant in the nucleus [114] and associate with chromatin multiple ways [107], thus providing a signaling link from extracellular ligand-binding events to regulation of gene expression (**Figure 1-4**). Indeed, even upstream components of the Raf-MEK-ERK pathway are recruited to target genes [115], suggesting there are specific nuclear modes of pathway activation. In mouse pancreatic β-cells, ERK1/2 display broad association with distal intergenic regions and gene promoters (**Figure 1-5**) and are known to directly regulate the insulin gene in response to glucose [34, 116]. In mESCs, ERK2 has been shown to occupy polycomb-regulated promoters at developmentally poised genes to promote lineage commitment [113]. In this system, evidence suggests that at some genes ERK2 can phosphorylate RNA polymerase II (Pol II) in lieu of the general transcription factor typically required to promote transcription initiation [113]. Conversely, in human embryonic stem cells (hESCs), ERK2 has been shown to co-occupy target promoters with the E26 transformation specific (Ets) family transcription factor Elk-1 to regulate self-renewal [111]. The diversity of regulatory capacity reported for ERK1/2 signaling indicates these kinases are well-adapted for complex signaling environments.

However, many questions remain regarding the nature of ERK1/2-chromatin interactions. What properties of ERK1/2 dictate their association with DNA? How do ERK1/2 influence substrate activity to affect gene expression outcomes? And what factors do ERK1/2 interact with the nucleus? My work has attempted to address each of these questions with the purpose of better understanding the nuclear behaviors of ERK1/2 signaling.

CHAPTER TWO

MODIFICATION OF THE ERK2 ACTIVATION LOOP ALTERS OLIGONUCLEOTIDE BINDING

Introduction

The mitogen-activated protein kinases (MAPKs) ERK1/2 mediate changes in cellular behavior by catalyzing the phosphorylation of hundreds of substrates throughout the cell in a context-specific manner [19, 117]. Substrates include plasma membrane receptors, ion transporters, cytoskeletal proteins, a variety of enzymes, and transcription factors [118, 119]. These kinases are activated by a broad range of cellular stimuli through a three-protein kinase cascade. Activation requires sequential phosphorylation on activation loop residues by the MAPK kinases (MAP2Ks) MEK1/2, first on Y185 (rat ERK2 numbering) and then on T183, to yield doubly phosphorylated ERK1/2 (pERK2) with a k_{cat} increased as much as 50,000-fold [120].

Elevated circulating glucose activates ERK1/2 in pancreatic β cells and increases association of glucose-regulated factors, including the kinases themselves, with the insulin gene promoter [34, 116]. Chromatin immunoprecipitation (ChIP) experiments indicate that ERK1/2 activity is required to recruit and/or retain these factors on the insulin promoter in response to increased glucose. In these experiments, we found that inhibition of ERK1/2 activation with MEK1/2 inhibitors decreased association of ERK2 with chromatin [34]. Several glucose-regulated transcription factors that induce insulin gene transcription are ERK1/2 substrates, including BETA2/NeuroD1, the basic helix-loop-helix protein partner,

E47, and PDX-1 [121]. That all of these factors converge on a gene promoter suggests that ERK1/2 may phosphorylate these transcription factors directly on chromatin.

MAPKs can bind DNA directly in the absence of associated protein factors. In 1998, Bardwell, Thorner, and colleagues reported the direct binding of the yeast Kss1p MAPK to DNA [122]. In 2009 Hu et al reported the direct association of ERK2 with oligonucleotides and characterized a preferred binding site of G/GAAAG/C [112], similar to the consensus identified for Kss1p. Authors of both of these studies concluded that MAPK binding resulted in transcriptional inhibition. Hu et al. provided evidence that unphosphorylated ERK2 inhibits gene transcription by preventing the interaction of C/EBP-β with promoters of interferon-regulated genes. Direct binding of ERK2 to DNA was also recently demonstrated using a similar oligonucleotide in a study of ERK1/2 on developmental promoters [113].

Several MAPK family members have also been studied in the context of cardiac hypertrophy [67, 123-125]. Distinct functions of ERK2 in cardiac hypertrophy from those in normal cardiac function have recently been attributed to changes in kinase function resulting from its phosphorylation on a distinct site, T188, which lies near the well-described sites of activating phosphorylation, Y185 and T183 [67]. Phosphorylation of T188 was found following stimulation of cells through a receptor tyrosine kinase and a G protein-coupled receptor. This combination of stimuli is thought to elicit a series of protein-protein interactions that promote autophosphorylation of activated ERK2 on Thr188 and activation of specific transcription factors in the nucleus.

To examine effects of phosphorylation on binding of ERK2 to DNA, ERK2 was expressed in bacteria and analyzed by mass spectrometry to detect previously unrecognized

modifications. These studies identified fractions of ERK2 that were phosphorylated on T188. I tested oligonucleotide binding of recombinant ERK2 *in vitro* with and without phosphorylation on the activating sites. On the basis of characterization of these ERK2 preparations and phosphomimetic mutants, I showed that ERK2 activation enhances its ability to bind oligonucleotides and suggests that phosphorylation of T188, although inactivating, also enhances interactions with oligonucleotides. These studies lend support to the idea that phosphorylation of ERK2, on the known regulatory sites and on T188, influence the output of the ERK1/2 MAPK pathway.

Methods and Materials

Expression and Purification of Recombinant Rat ERK2 Protein

Expression of recombinant ERK2 was generally as described previously [126]. In detail, Origami competent cells were transformed with NpT7 plasmids containing the rat ERK2 construct and grown in Terrific Broth (TB) at 30°C until cultures reached an OD_{600} of 0.55 – 0.65. Cultures were induced with 0.5 mM isopropyl thiogalactopyranoside (IPTG) and grown for 14-16 h at 30°C. Cells were harvested in a sonication buffer of 50 mM $NaHPO_4$ pH 8.0, 300 mM NaCl, with protease inhibitors (0.4 μg/mL pepstatin A, 0.4 μg/mL leupeptin, 4 μg/mL Na-p-tosyl-L-arginine methyl ester, 4 μg/mL Na-p-tosyl-L-lysine chloromethyl ketone hydrochloride, 4 μg/mL Na-Benzoyl-L-arginine methyl ester, 4 μg/mL soybean trypsin inhibitor) and pellets were flash frozen in liquid nitrogen and stored at -80°C until purification. Pellets were thawed and lysozyme was added on ice. Following sonication, the lysate was centrifuged in a Ti-45 rotor at 105,000xg, for 1 h at 4°C. Clarified lysate was mixed with nickel resin for 2 h at 4°C that was washed three times with ice-cold sonication buffer containing protease inhibitors and 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4, 150 mM NaCl, 20 mM imidazole, pH 7.5, prior to addition to clarified lysate. Beads were washed with 5 ml 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 8.0, 300 mM NaCl, 50 mM imidazole before elution. Proteins were eluted with 5 ml 50 mM HEPES pH 8.0, 300 mM NaCl, 250 mM imidazole and dialyzed into either 20 mM NaCl for subsequent ion exchange chromatography or into 20 mM HEPES pH 7.4, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 10% glycerol, flash

frozen, and stored at -80°C. Purified proteins exchanged into 20 mM NaCl were applied to a MonoQ column and eluted with a linear gradient of 20-1000 mM NaCl, 7.5-column volumes, at a flow rate of 0.4 ml/min. Pooled and individual fractions were subjected to mass spectroscopy or dialyzed into 20 mM HEPES pH 7.4, 1 mM EGTA, 1 mM DTT, 10% glycerol, flash frozen, and stored at -80°C.

In vitro Kinase Assays

ERK2 and mutants (3 μM) were activated with 40-50 nM constitutively active MEK1 (MEK1R4F) and 100 μM ATP ([γ^{32}P-ATP] 200-300 cpm/pmol) in 10 mM Tris, 15 mM $MgCl_2$, 1 mM DTT, 1 mM benzamidine for 30 min at 30°C. ERK2 (0.3 μM) activity was measured with 50 μg/ml myelin basic protein (MBP) and other substrates for 10 min under conditions above. Following gel electrophoresis and autoradiography, protein bands were excised and analyzed by scintillation counting. Reactions with peptide substrates were spotted onto P81 paper and washed three times with 75 mM H3PO4 and then acetone, dried overnight, and counted as above.

Electrophoretic Mobility Shift Assays (EMSAs)

EMSAs were performed with both radioactively labeled oligonucleotides and a 5' labeled IRDye 700-tagged oligonucleotide (Integrated DNA Technologies) mapping to the rat insulin promoter. Briefly, oligonucleotides were incubated with purified ERK2 and mutants then resolved by non-denaturing polyacrylamide gel electrophoresis and imaged on a LI-COR Odyssey system or by exposure to film. Initial DNA binding reactions were

assembled by incubating ERK2 (2 μg) in 10 mM HEPES pH 7.4, 1 mM DTT, 1 mM benzamidine, 15 mM $MgCl_2$, 0.5 μg/ml poly dI:dC and fluorescently labeled oligonucleotide for 30 min at 30°C followed by an overnight incubation at 4°C. Reactions were separated on a 5% non-denaturing gel (5% acrylamide, 50 mM Tris pH 8.0, 190 mM glycine, 1.875 mM ethylenediaminetetraacetic acid (EDTA), 15 mM $MgCl_2$, and 5% glycerol) at 4°C in 10 mM Tris pH 8.0, 400 mM glycine and imaged on a LI-COR Odyssey imager. For titration experiments, reactions were for 30 min at room temperature then 16 h at 4°C, but without benzamidine or poly dI:dC.

Mass Spectrometry

Mass spectrometry was performed on ERK2 fractionated on MonoQ. Extracted peptides were analyzed by a nano-LC/MS/MS system with an Ultimate 3000 HPLC (Thermo-Dionex) connected to an Orbitrap Elite mass spectrometer (Thermo Scientific) via an Easy-Spray ion source (Thermo Scientific). Peptides were separated on ES800 Easy-Spray column (75 μm inner diameter, 15 cm length, 3 μm C18 beads; Thermo Scientific) at a flow rate of 300 nl/min with a 35 min linear gradient of 2–24% mobile phase B (mobile phase A: 2% acetonitrile, 0.1% formic acid; mobile phase B: 98% acetonitrile, 0.1% formic acid). Thermo Scientific Orbitrap Elite mass spectrometer was operated in positive nano-electrospray mode. MS data were acquired in both profile and data dependent modes. The resolution of the survey scan was set at 60k at m/z 400 with a target value of 1´106 ions. The m/z range for MS scans was 300–1600. The isolation window for MS/MS fragmentation was set to 1.9 and the top two most abundant ions were selected for product ion analysis. Ion trap

enhanced scan rate was used for the MS/MS data acquisition with decision tree procedure activated. Dynamic exclusion was 12 s and early expiration was enabled. Xcalibur RAW files were converted to peak list files in mgf format using Mascot Distiller (version 2.4.3.3). Database search was performed using Mascot Daemon (2.4.0) against a house-built database containing NCBI human sequences and Tag+Erk2 sequence.

Results

Purification of Recombinant ERK2 and Analysis by Mass Spectrometry

A ribbon diagram of rat ERK2 is shown in **Figure 2-1A**. Recombinant 6xHis-ERK2 was expressed and purified on nickel resin with little modification to previous protocols, yielding ERK2 with an estimated purity of 65%. Protein was further purified on MonoQ. Previous purifications of recombinant ERK2 did not yield an elution pattern comprised of several peaks, raising the question of whether higher expression of ERK2 could lead to mis-folded or otherwise modified protein. Equal amounts of protein from each fraction were assayed for kinase activity (**Figure 2-1B**). Activity was detected near the tail of the elution peaks (not shown) with more total activity at the end of the first peak than at the end of the second, similar to what was described previously by Prowse and Lew [127]. Pooled fractions were analyzed by mass spectrometry. Multiple phosphorylated species were detected, including phosphorylated Y185 (pY185), phosphorylated T183 (pT183), and phosphorylated T188 (pT188) (**Figure 2-1A**), as shown by the spectra (**Figure 2-1C–E**). Notably, the observed modifications were sub-stoichiometric. I confirmed that the elution profile of ERK2 purified on MonoQ exhibited multiple peaks (**Figure 2-2**).

To determine if phosphorylation of T188 was influencing the ion exchange elution profile, mass spectrometry was performed on fractions from another MonoQ separation. Many fractions contained pY185 and pT183 (not shown), but the low yield of pT188 indicated that only some of the ERK2 in each fraction was phosphorylated on this site and indicated that this modification is not the source of the aberrant elution profile. Further

purifications using more shallow NaCl gradients coupled to mass spectrometry analysis may resolve this issue in the future.

Conserved residues in the ERK2 catalytic and activation loops, including T188, create the P+1 pocket, a binding site for the residue of protein substrates that is on the C-terminal side of the phosphorylation site (P+1 residue). Alignments of protein kinase sequences indicate that threonine is highly conserved in this position (**Figure 2-3**). In 2009, phosphorylation of ERK2 on T188 was identified from phosphopeptide maps that revealed a unique peptide from ERK2 incubated with heterotrimeric G protein βγ subunits compared to ERK2 incubated only with upstream kinase cascade components [67]. The novel site in the phosphopeptide was identified by mutagenesis as pT188 and was suggested to arise by autophosphorylation. Using a pT188-specific antibody [67], this phospho form was detected in multiple MonoQ fractions and in a pooled preparation of wild-type protein but not in fractions of the inactive ERK2 mutant (K52R) (**Figure 2-4A**). Notably, treatment with the MAPK phosphatase MKP3 did not dephosphorylate pT188 despite removing phosphate from the activating sites, as determined by immunoblotting with an antibody specific for ERK2 phosphorylated on T183 and Y185 (active or pERK2) and an antibody that recognizes total ERK2 (independent of its phosphorylation state) (**Figure 2-4B**). Mutation of T188 to alanine inactivates ERK2 [128], and mutation of the comparable residue in some other protein kinases is inactivating, as well [129, 130], leading us to hypothesize that ERK2 phosphorylated on T188 is inactive. This ERK2 phospho form was probably not detected earlier because it is a minor component in bacterial preparations, consistent with our finding

that pT188-containing fractions still harbor kinase activity toward MBP upon activation by constitutively active MEK1 (MEK1-R4F) (**Figure 2-4C**).

To model how autophosphorylation of T188 might occur, molecular dynamics was performed by Dr. Adrian Turjanski with the expectation that the target residues must come within 4 Å of the γ-phosphate of bound ATP [68]. Structural studies of ERK2 indicate that the T188 hydroxyl group is within 6 Å of the γ-phosphate group, and umbrella sampling reveals ERK2 phosphorylated on Y185 is energetically stable with the distance reduced to 4 Å. Molecular modeling suggests that the ERK2 activation loop can sustain triple phosphorylation events and also that autophosphorylation of ERK2 on T188 is most likely to occur after phosphorylation of ERK2 on Y185 but prior to its phosphorylation on T183 [68].

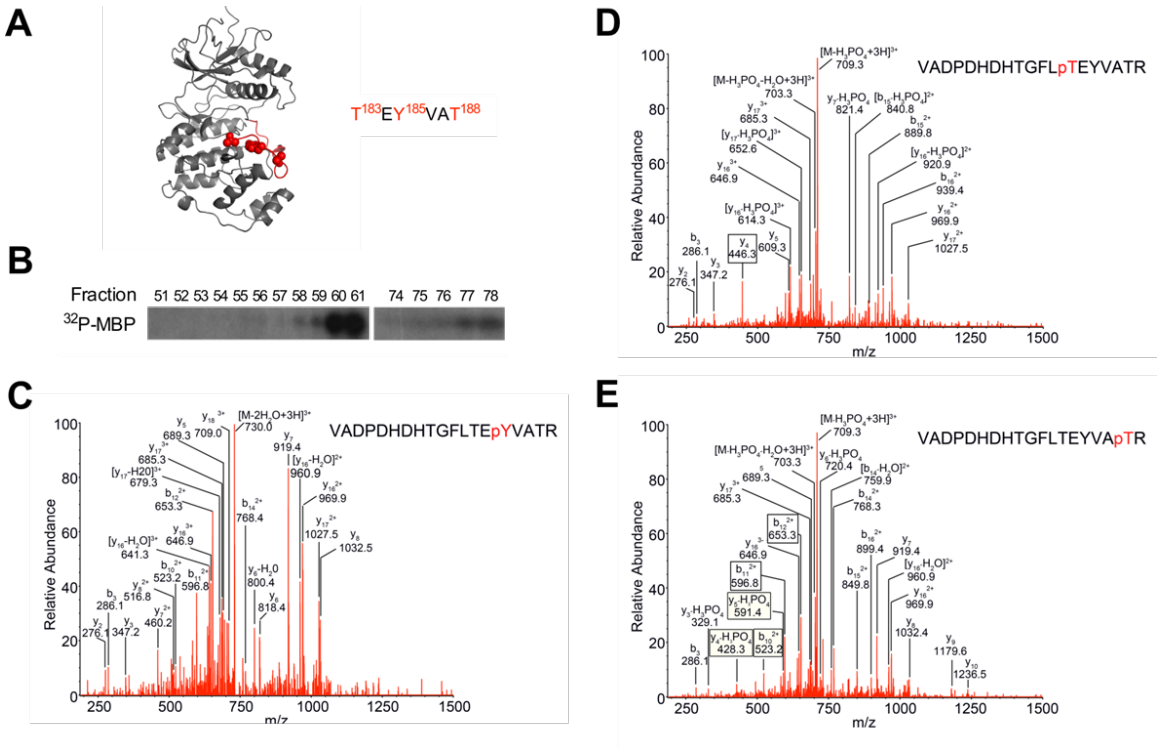

Figure 2-1. Anion exchange chromatography of rat ERK2 and subsequent mass spectrometry (MS) analysis reveals multiple phosphorylation and activity states. (A) Low-activity structure of ERK2 with activation loop phosphorylation sites indicated in red [PDB: 1ERK]. (B) Nickel purified 6XHis-ERK2 was purified further on MonoQ. Individual fractions were assayed for kinase activity. (C-E) Multiple phosphoforms were identified by MS of pooled fractions. Representative of three experiments. Three residues were found to be phosphorylated: Y185 (D), T183 (E), and T188 (F). Experiment in panel B performed by AMC, panels C-E by YL. Adapted from McReynolds and Karra, et al. 2016.

Activities of ERK2 and Mutants

ERK2 fractions containing pT188 can be phosphorylated and activated by constitutively active MEK1 (MEK1R4F) like the unphosphorylated protein. Because ERK2 pT188 was a minor component of each fraction, I could not determine unequivocally whether ERK2 pT188 was active. This form proved to be difficult to purify because of the heterogeneous mixture of trace phosphorylations. Instead, I expressed the ERK2 mutants,

T188D and T188E, as phosphomimetic substitutions. The expression and solubility of ERK2 T188D and ERK2 T188E were both similar to those of wild-type ERK2 (**Figure 2-5**). The protein kinase activities of wild-type ERK2, T188D, and T188E toward the model substrate MBP were compared and demonstrated that wild-type ERK2 had readily detectable activity, but ERK2 T188D and T188E did not [68]. To test whether this was a consequence of misfolding, these proteins were phosphorylated by MEK1R4F in vitro. MEK1 binds at least two regions on ERK2: the common docking (CD) loop and the MAPK insert [91, 131, 132], the latter of which is required for ERK activation by MEK. The T188 mutants were both phosphorylated roughly as well as wild-type ERK2, while the inactive ERK2 mutant Y185F (inactive because it lacks the initial MEK phosphorylation site [65]) was not [68]. Immunoblotting with the active ERK2 antibody shows that phosphorylation of ERK2 and T188 mutants was comparable, indicating that the T188 mutants were well folded.

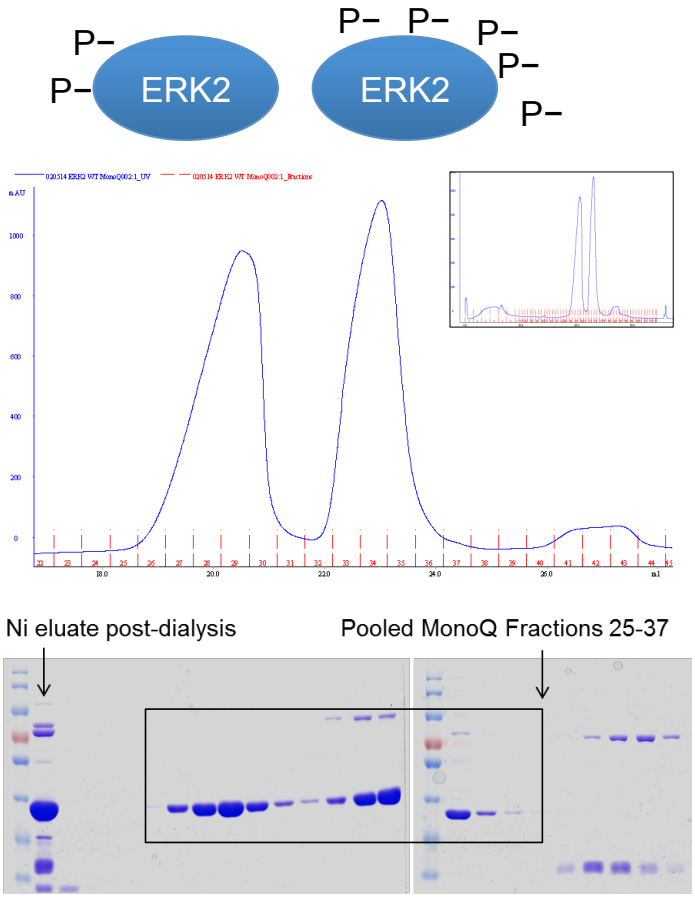

Figure 2-2. Anion exchange chromatography of rat ERK2 suggests multiple phosphorylation sites. Recombinant wildtype 6xHis-ERK2 was purified from bacteria with nickel resin and further separated by MonoQ. UV absorbance elution profile is shown, as well as Coomassie-stained fractions separated by 10% SDS-PAGE.

ERK2 Binds Oligonucleotides Derived from Several Gene Promoters

A range of experiments has identified multiple genes with which ERK1/2 may associate [34, 112, 113, 115, 133]. A subset of genes that ERK2 binds, as confirmed by chromatin immunoprecipitation (ChIP), include growth hormone (GH), unc-like kinase 1 (Ulk1), insulin-like growth factor binding protein 2 (IGFBP2), chromatin regulating lysine

demethylase 2a (KDM2a-2), and the phosphatase eyes absent 1 (EYA-1) [68]. Inhibition of pathway activation by the MEK inhibitor PD0325901 decreased the association of ERK2 with each of these genes in the ChIP assays [68]. Oligonucleotides were synthesized derived from the ERK2 binding peak of these genes and used for electrophoretic mobility shift assays (EMSAs) [68]. The oligonucleotides each contained the ERK2 binding consensus G/CAAAG/C, except for that from KDM2a. Oligonucleotides derived from ULK1 and EYA1 gene promoters each bound active ERK2. Less binding was observed to an equivalent amount of ERK2 T183E or Y185F. The EYA-1 oligonucleotide bound almost equally well to Y185F ERK2. In contrast, the oligonucleotide from KDM2a, which lacks the G/CAAAG/C consensus, bound less ERK2 than other oligonucleotides tested. Importantly, phosphorylated ERK2 (pERK2) bound DNA more robustly than ERK2 that has not been activated with MEK1R4F (**Figure 2-6**).

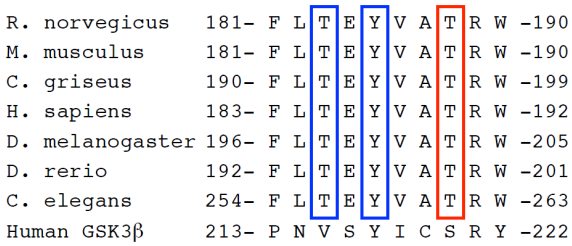

Figure 2-3. Multiple alignment of ERK2 across species. Threonine at position 188 by rat ERK2 numbering is conserved across species. Adapted from McReynolds and Karra, et al. 2016.

ERK2 and Activation Loop Mutants Bind Directly to an Insulin Gene Promoter Sequence

The Cobb Laboratory has previously shown that ERK1/2 bind to the insulin gene promoter in pancreatic β cells and that the interaction is strongly enhanced if the kinases are

active [34, 116]. 6xHis-ERK2 was co-expressed with MEK1R4F to generate quantities of active ERK2 phosphorylated on Y185 and T183 to compare to ERK2 and mutants. Synthetic oligonucleotides representing 47 and 19 bp sequences from the mouse insulin gene promoter that contain the sequence G/GAAAG/C, previously identified as a consensus recognized by ERK2 and the yeast MAPK Kss1p [112, 122], were used to determine binding [68]. To assess better the relative DNA binding ability of ERK2 upon perturbation of its activation loop, I employed a fluorescently labeled version of the 19 bp insulin promoter sequence in gel shift assays over a range of protein concentrations (**Figure 2-6**). Wild-type ERK2 binds the probe at the highest concentrations tested (4 μM). Activated ERK2 and activation loop mutants, e.g., T188D, shift the probe at lower concentrations. This suggests that activation of ERK2 enhances its ability to bind DNA. Interestingly, eliminating a potential hydrogen bond by mutating T188 to the nearly isosteric amino acid valine also alters the DNA binding activity of ERK2 relative to that of the wild-type protein, highlighting the sensitivity of this residue in ERK2–DNA interactions (**Figure 2-6**).

It should be pointed out that some oligonucleotides did not bind ERK2 whether activated or not (data not shown). Furthermore, whether different phosphorylation states dictate specific DNA-binding preferences also remains to be tested.

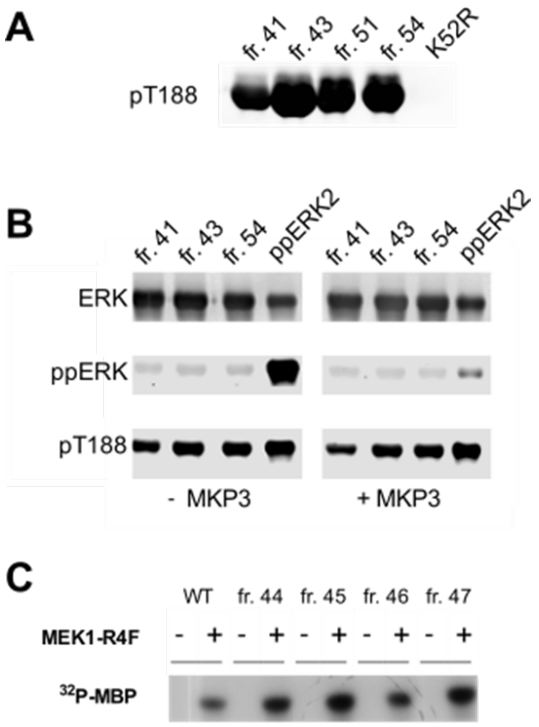

Figure 2-4. Phosphorylated T188 (pT188) is present in bacterial preparations of rat ERK2. (A) Immunoblot of MonoQ fractions of purified wildtype ERK2 and a preparation of a kinase-dead ERK2 (K52R) with a pT188-selective antibody. (B) Selected fractions were immunoblotted with antibodies that discriminate ERK2 independent of its phosphorylation state, activated ERK2 (ppERK2), and pT188 selectively. MAP kinase phosphatase 3 (MKP3) is able to dephosphorylate pY185 and pT183 but not pT188. (C) MEK1R4F recognizes and phosphorylates eluted fractions containing pT188, thereby conferring ERK kinase activity on MBP. Experiments performed by SS. Adapted from McReynolds and Karra, et al. 2016.

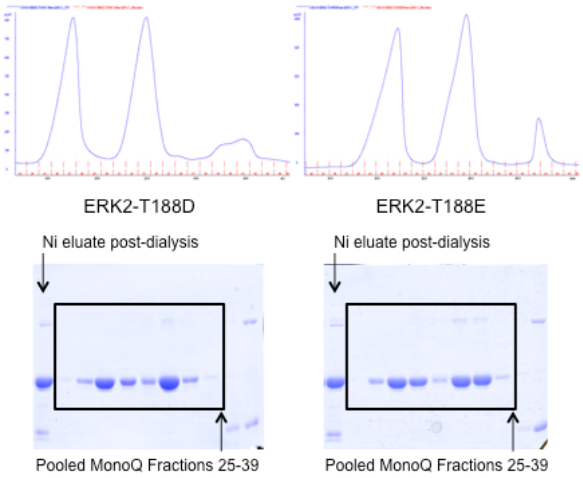

Figure 2-5. Anion exchange chromatography of rat ERK2 mutants. Recombinant ERK2 mutants were purified from bacteria as in Figure 2-2.

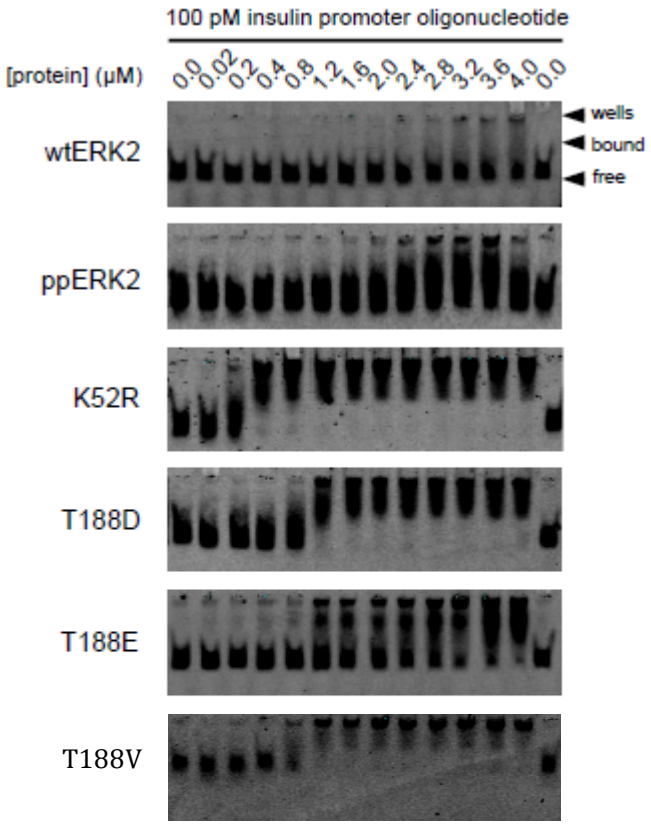

Figure 2-6. ERK2 and mutants directly bind DNA *in vitro*. Electrophoretic migration shift assays (EMSAs) for ERK2, activated ERK2, and ERK2 mutants with fluorescently labeled oligonucleotide that maps to the insulin promoter and contains the motif GAAAC. Modified from McReynolds and Karra et al. 2016.

Discussion

The activation loop of ERK2 makes critical contacts with the MAPK insert that are destabilized upon activating phosphorylation, thereby altering the accessibility of the insert. The greater accessibility of the MAPK insert accounts for the increased binding of active ERK2 to FXF-containing docking motifs present on many of its substrates [134]. The MAPK insert region of ERK2 has also been implicated by mutagenesis as the portion of ERK2 that contacts the DNA consensus sequence [112]. Hu et al. suggested that unphosphorylated ERK2 binds the G/CAAAG/C consensus. I also found that ERK2 binds this consensus and that phosphorylated ERK2 binds more strongly (**Figure 2-6**). Intriguingly, direct interactions of DNA with prokaryotic kinases have also been described, such as the bacterial persistence factor HipA, which possesses a eukaryotic kinase fold [135]. In complex with the DNA binding neutralizing protein HipB, HipA contacts DNA through a region of the molecule similar to the MAPK insert in ERK2. The MAPK insert is present in members of the CGMC branch of the kinome, including GSK3, cyclin-dependent kinases, and yeast Kss1p, all of which are known to associate with chromatin and regulate gene expression [122, 136, 137]. Moreover, it is conceivable that multiple members of this kinase cohort with positively charged insert regions can associate with DNA through this structural element.

Alignments of eukaryotic protein kinases reveal a highly conserved threonine at the position comparable to T188 in ERK2 (**Figure 2-3**), which forms part of the P+1 pocket [138]. The flexibility of the P+1 pocket contributes to substrate binding, positioning, and release. T188 is part of a hydrogen-bonding network within the ERK2 core, as elaborated

upon by Emrick et al [128]. Mutation of this threonine in ERK2 and the comparable residue in other protein kinases has been previously shown to yield inactive enzymes [128, 130], consistent with the findings here. A logical assumption is that this residue is not regulated by phosphorylation. Nevertheless, in the kinase MARK1, phosphorylation of the comparable residue (S212) was detected in brain lysates using a phospho-specific antibody, similar to the observation of ERK2 pT188 in a cardiac hypertrophy model [67, 130].

As isolated from bacteria, ERK2 is largely unphosphorylated but contains a heterogeneous background of phosphorylation states, including detectable phosphorylation on the well-known activating sites, Y185 and T183. Surprisingly, ERK2 phosphorylated on T188 was also found in this heterogeneous array. ERK2 is capable of autophosphorylation not only on Y185 [139] but also, at least under certain circumstances, on T183 and T188. The prediction from molecular dynamics is that the ERK2 activation loop can support all three phosphorylations with ERK2 T188 autophosphorylation following that on Y185, paralleling the experimentally derived sequence of events proposed by Lorenz et al [67, 68]. If the MAPK insert is indeed the site of DNA binding, modification of T188 may also facilitate the DNA accessibility of ERK2 on this region.

The physiological relevance of phosphorylation of T188 is not known apart from its proposed role in cardiac function, but further work has suggested that preventing phosphorylation of ERK2 on T188 by mutagenesis reduces pathological cardiac hypertrophy [140]. The inability of ERK2 T188 phosphomimetic mutants to phosphorylate the various substrates that we tested suggests that the substrate recognition groove, as well as critical contacts in the active site, are altered, rendering the enzyme inactive. However, although this

work suggests that perturbation of T188 results in inactivity toward known substrates, it remains possible that phosphorylation of T188 confers on ERK2 the ability to phosphorylate distinct substrates. A precedent for this within the MAPK family comes from the work of Ashwell and colleagues, who have demonstrated that an alternative form of activated p38 has distinct substrate specificity in T cells [141]. This alternative state is induced by a newly identified C-terminal phosphorylation event that causes refolding of the protein substrate-binding region.

ERK2 has a variety of functions in regulating transcription, many of which may be aided by the direct association of ERK2 with genes. ERK2 is in excess of most transcriptional regulators at a cellular concentration estimated to be greater than 100 nM (~800 nM in a recent paper on HeLa [114]), and perhaps much higher on the basis of recently reported concentrations of C-Raf and MEK1 in the micromolar range [142]. Given the polyelectrolytic nature of DNA, low-affinity interactions between an exposed duplex and proteins are primarily driven by electrostatics. Nevertheless, beyond the generalized affinity of a nuclear regulator such as ERK2 for chromatin, phosphorylation events can drive conformations toward more specific interactions, similar to what has been observed in prokaryotic systems, as discussed by von Hippel [143]. The consensus binding site may offer a relatively low-affinity depot of ERK2 to regulate nearby chromatin-bound factors, many of which are capable of generating higher-affinity sites on chromatin through scaffolds and substrate proteins [144].

ERK1/2 promoter residency is not limited to the insulin gene promoter [34]. ERK1/2 and multiple upstream kinases have been studied on the Egr1 promoter [115], and

widespread MAPK promoter binding has been suggested through several studies in yeast and mammalian cells [145] Extensive interactions of Hog1p, a yeast p38 MAPK, with chromatin have been documented [110, 146], and more recently, ERK1/2 were shown to be recruited to estrogen response elements along with estrogen receptor A [147]. ERK1/2 have also been found to occupy promoters of numerous genes in stem cells [111]. Given the prominence of phosphorylation in controlling gene expression [148], the malleable DNA binding ability of ERK2 may be critical for modulating the signaling output of this pathway. ERK2 phosphorylation states with different effects on DNA binding indicate a necessary focus of future work in this area, especially as it pertains to the promotion of disease. The possibility of widespread ERK1/2–DNA binding and potential regulation of gene expression or alternative signaling has implications for both kinase biology and chromatin regulation.

CHAPTER THREE

ERK2 PHOSPHORYLATES THE EPIGENETIC REGULATOR CXXC-FINGER PROTEIN 1 (CFP1)

Introduction

The extracellular signal-regulated kinases 1 and 2 (ERK1/2) function as key relays to couple extracellular signals with appropriate intracellular responses [19]. These important signaling molecules are critical for large-scale cellular processes such as proliferation, differentiation, and programmed cell death, and as such have been heavily scrutinized for their varied roles in disease progression. The physiological and pathological consequences of ERK1/2 phosphorylation on many individual substrates in several cell compartments have been documented, highlighting their far-reaching cellular functions and importance for homeostatic control [69, 119, 121, 149]. ERK1/2 are the branching kinases of a well-conserved MAPK module that modulates gene expression through extensive interactions in the nucleus [107], where chromatin modifying enzymes regulate access to genetic information. Across species ranging from yeast to humans, trimethylation of histone H3 lysine 4 (H3K4me3) is strongly associated with actively transcribed gene promoters [150-153]. Several studies have shown that H3K4me3 is induced by various stimuli to support transcriptional responses. Among these, MAPKs have been implicated in dynamic signal-driven histone modification, including deposition of H3K4me3, in several cell systems [111, 154-156].

CXXC-finger protein 1 (Cfp1) is a conserved component of the Set1A and Set1B Histone 3 Lysine 4 (H3K4) methylation complexes and is required for maintaining specific H3K4me3 patterns in development [157-159]. Cfp1 specifically binds to unmethylated DNA and can recognize unmodified di-nucleotides even in methylation-rich environments [160, 161]. Interestingly, Cfp1 also interacts with DNA methyltransferase 1 (DNMT1), the epigenetic factor primarily responsible for maintenance of DNA 5-methylcytosine patterns established by the *de novo* methyltransferases DNMT3a and DNMT3b [162]. DNMT1 is the most abundant DNA methyltransferase in adult cells [163] and displays a distinct preference for associating with hemimethylated DNA over hypo- or hypermethylated DNA. ERK1/2 are known to regulate DNMT1 [164-166], suggesting a conserved and multifaceted relationship between MAPK signaling and chromatin regulation. Significantly, deletion of Cfp1 in mouse embryonic stem cells (mESCs) results in a dramatic depletion of DNMT1 protein and resultant genomic DNA methylation [167].

A mechanistic rationale for interaction between Set1A/B, Cfp1, and DNMT1 has yet to be uncovered. DNMT1 and Cfp1 contain redundant DNA targeting domains [168, 169], but unlike Set1A/B, a dependence on Cfp1 for genomic targeting of DNMT1 has not been directly demonstrated. Moreover, very little is understood about how epigenetic inputs from DNA methylation and histone methylation are integrated into gene expression outcomes in a signal-dependent manner (**Figure 3-1**). One likely possibility is that interactions are restricted to a subset of targeted genes or responsive to specific signaling pathways. To gain insight as to how ERK1/2 influence gene expression, I followed up on the identification of Cfp1 as an important signaling molecule. ERK1/2 signaling and Cfp1 are both known to

play extensive roles in development, but less is known about Cfp1 in the regulation of acute responses to extracellular cues [160, 170]. Here, I show evidence suggesting that Cfp1 is required for signal-dependent expression of ERK1/2 target genes and establish a functional relationship between MAPK signaling and chromatin dynamics.

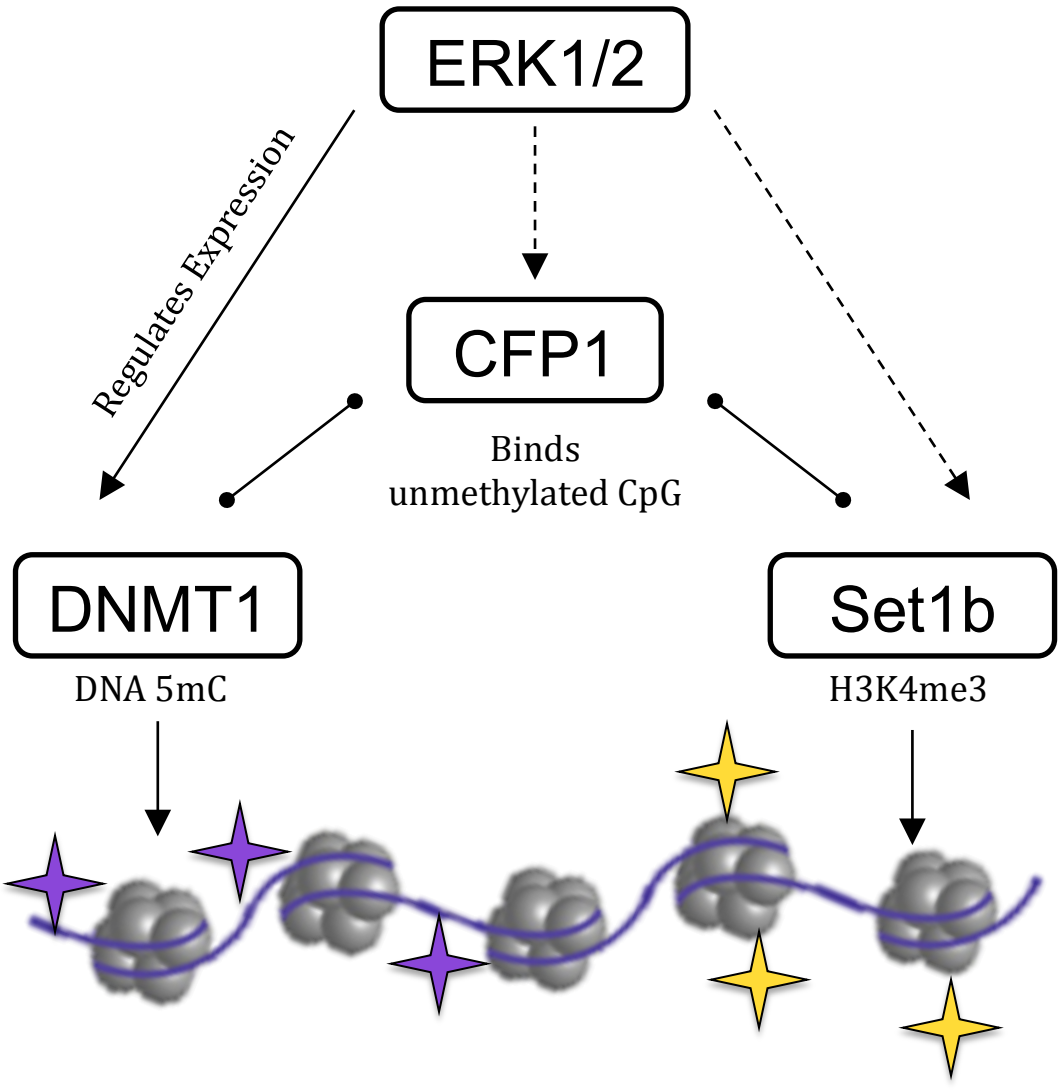

Figure 3-1. ERK1/2 signaling regulation of two epigenetic processes through interaction with Cfp1.

Methods and Materials

Immunoblotting and LiCor Imaging

Lysates were segregated by sodium dodecyl sulfate (SDS) -PAGE and transferred to nitrocellulose membrane (Millipore). Membranes were blocked with LiCor blocking buffer and incubated with the indicated antibodies. Fluorescent-labeled secondary antibodies were used in conjunction with the LI-COR Odyssey dual-color system, and band quantitation was performed using Image Studio software. Western blot analysis was performed using the following primary antibodies: rabbit anti-Cfp1 (Bethyl Laboratories); rabbit anti-ERK1/2 (produced in-house [171]); mouse anti-pERK1/2 (T185/Y187), mouse anti-Flag M2 (Sigma); mouse anti-Histone 3, rabbit anti-H3K4 trimethyl (Active Motif).

Cell Culture, Transfections and siRNA Treatments

HeLa cells were obtained from the ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v fetal bovine serum (Sigma) and 2 mM L-glutamine. Human Cfp1 cDNA was subcloned into p3xFLAG-CMV-7.1 (Sigma E4023). Point mutations were generated using standard site-directed mutagenesis and all constructs were Sanger-sequenced to verify the lack of spurious mutations. Duplex siRNA oligonucleotides targeting human Cfp1 (s26937) or a non-targeting control (4390843) were acquired from Life Technologies. Oligonucleotides were reverse transfected into HeLa cells at 20 nM for 72 hr with RNAiMax (Life Technologies).

Cell Harvest, Mononucleosome Preparation and Immunoprecipitation

For immunoblotting, cells were lysed with RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 80 mM beta-glycerophosphate, 100 mM NaF and 2 μM Na_3VO_4) with protease inhibitors, and sonicated.

Mononucleosomes were prepared by resuspending chromatin pellets with Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT with protease inhibitors) containing 1 mM $CaCl_2$, then digested at room temperature with micrococcal nuclease (Thermo Scientific). Digestions were stopped by adding a final concentration of 1 mM EGTA. Undigested chromatin was pelleted by sedimentation at 4000 rpm for 5 min. Supernatants were used for immunoprecipitation with the indicated antibodies as previously reported [80].

Nuclear Fractionation

For Flag-ERK2 immunoprecipitations, cells were transfected with p3xFLAG-CMV-ERK2 [131] for 48 hours with Lipofectamine 2000. Cells were rinsed with phosphate-buffered saline (PBS) and resuspended at 10^7 cells/mL in 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT. Triton X-100 was added to a final concentration of 0.1% and cells were incubated on ice for 5 min. Crude nuclear fractions were pelleted in a microfuge at 3500 rpm for 5 min. For further isolation of chromatin, nuclear pellets were washed once in the buffer above without Triton then resuspended in 3 mM EDTA, 0.2 mM EGTA, 1 mM DTT for 15 min on ice with periodic vortexing. Chromatin was pelleted in a microfuge at 4000 rpm for 5 min.

Acid-extraction of Histones

Cells were harvested in ice cold 1X PBS and resuspended in cold hypotonic lysis buffer (10 mM Tris pH 8.0, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT) prior to acid extractions of histones as described [172]. Total histone content was measured after separation by 15% SDS-PAGE and Coomassie staining with protein concentration standards.

Recombinant Protein Expression and Purification

6xHis-tagged ERK2 protein was expressed and activated as in Chapter 2. Human Cfp1 and truncation constructs were subcloned into the pGEX-6P-1 vector encoding an N-terminal glutathione S-transferase (GST) tag (GE Healthcare) prior to expression in Origami cells. Fusion proteins were induced overnight at 20°C with 0.4 mM isopropyl-1-thio-b-D-galactopyranoside (IPTG). Cells were lysed in buffer containing 20 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM DTT with protease inhibitors (as described in Chapter 2). Pellets were lysed and clarified, and GST-tagged proteins were bound to glutathione resin (Pierce). Protein-bound resin was washed three times with lysis buffer and bound protein was eluted with 20 mM glutathione. GST tags were cleaved with PreScission protease per the manufacturer's instructions (GE Healthcare). Isolated Cfp1 fragments were dialyzed against 25% glycerol, 150 mM NaCl, 10 μM $ZnCl_2$, 1 mM EDTA and 1 mM DTT.

DNA Methylation Assay

Genomic DNA was isolated with *Quick*-DNA™ Miniprep Plus Kit (Zymo Research) and global 5mC content was measured by MethylFlash Methylated DNA 5-mC Quantification Kit (Epigentek) according to manufacturer's instructions.

Microarray and qRT-PCR

Total RNA was prepared from HeLa cells using the Pure-link RNA Mini Kit (Life Technologies) according to the manufacturer's instructions. Briefly, RNA was checked for concentration and quality using an Agilent 2100 Bioanalyzer, then cRNA was synthesized and labeled prior to hybridization to an Affymetrix Human Transcriptiome 2.0 Array chip and detection. Raw data was analyzed using Affymetrix Transcriptome Analysis Console 2.0 software.

Cellular RNA was isolated with TRI reagent (Applied Biosystems) and reverse transcribed using the iScript cDNA synthesis kit (BioRad). qRT-PCR was performed with SYBR Green Supermix (BioRad) and fluorescence was measured using a quantitative real-time thermocycler (BioRad). Relative changes in gene expression were calculated by $2^{-\Delta Ct}$, with *actin* as internal expression control.

Results

ERK1/2 and Cfp1 Interact in Cells and in vitro

To discover novel ERK2 substrates and binding partners, a yeast-two hybrid screen designed to identify interacting factors based on the kinase activation state of ERK2 was employed as previously described [173, 174]. Two yeast strains were assayed in parallel, one bearing a construct expressing ERK2 alone, and another with ERK2 co-expressed with a constitutively active form of the upstream activating kinase, MEK1 (MEK1R4F) [30, 173]. A mouse neonatal cDNA library was used as prey, and among putative ERK2-interacting proteins a fragment of Cfp1 encompassing the C-terminal residues 373-660 was identified with an interaction score that indicated a 2.5-fold preference for activated ERK2 (pERK2) (**Figure 3-2A**). Subsequent experiments and mass spectrometry analysis confirmed Cfp1 is an ERK2 substrate *in vitro*.

Because ERK1/2 and Cfp1 each interact with critical chromatin modifying complexes ([175] and **Figure 3-2B**), two alternative approaches were taken to validate the interaction between CFP1 and ERK2 in human cells. First, cells were briefly cross-linked with formaldehyde prior to immunoprecipitation with antibodies targeting ERK1/2, histone H3, H3K4me3, and rabbit IgG as a negative control (data not shown). Second, mononucleosomes were isolated from HeLa cells expressing Flag-ERK2, immunoprecipitated with resin-conjugated monoclonal Flag antibody, and blotted for endogenous Cfp1, thus confirming that ERK2 associates with Cfp1 on chromatin (**Figure 3-2C**). Subsequent immunoprecipitation experiments with antibodies that directly target ERK2

indicate this interaction is maintained by endogenous ERK2 and Cfp1 on mononucleosomes (**Figure 3-2D**).

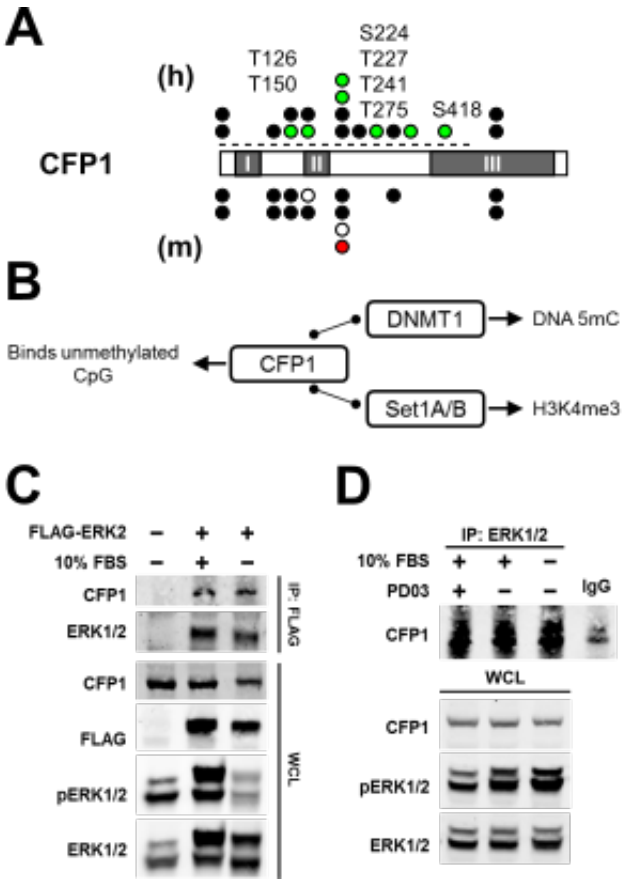

Figure 3-2. ERK1/2 interact with Cfp1 in cells and *in vitro*. (A) Cfp1 contains multiple phosphorylation sites identified by mass spectrometry and strongly predicted by previous reports and/or primary sequence. Black circles indicate all sites identified and banked on phosphosite.org [42], white circles indicate identified sites on S/T-P sequences, red circles indicate identified sites on P-x-S/T-P sequences, and green circles indicate sites identified by mass spectrometry (MS) with sites labeled by position above. Human (h) and mouse (m) Cfp1 are highly conserved and both contain I. plant homeobox domain (PHD), II. CXXC domain, and III. coiled coiled (CC) domain. Dashed line indicates portion of (h) Cfp1 subjected to MS analysis and dotted line indicates portion of (m) Cfp1 expressed for yeast two-hybrid screen. (B) Cfp1 interacts with several major epigenetic regulators, including DNMT1 and Set1A/B. (C) Immunoprecipitation from micrococcal nuclease-digested chromatin from HeLa cells expressing Flag-ERK2. (D) HeLa cells were treated and processed as in C. but immunoprecipitated with antibody recognizing endogenous ERK1/2. Work shown in panels C and D performed by AMK.

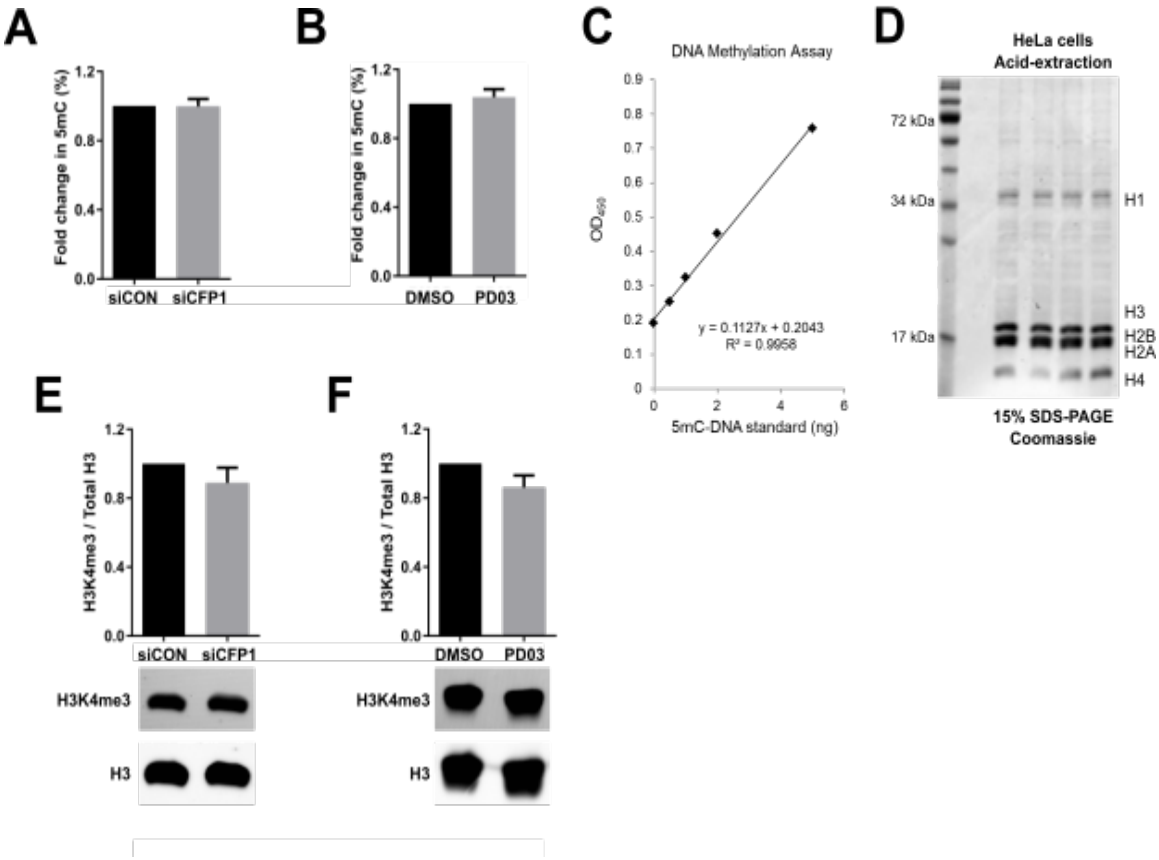

Figure 3-3. Cfp1 depletion and ERK1/2 signaling inhibition do not affect global 5mC or H4K4me3 chromatin modifications. (A) Genomic 5mC content was measured for HeLa cells treated with siRNA directed at Cfp1 for 72 hr by ELISA. Results are representative of 3 independent experiments. (B) Global 5mC content was measured for HeLa cells treated with 500 nM PD0325901 (MEK1/2 inhibitor) or DMSO for 24 hr as in A. (C) Representative standard curve for DNA methylation assays. (D) Representative histone acid extraction from HeLa cells. (E) Acid-extracted histones from HeLa cells treated as in A. and immunoblotted for total histone H3 and H3K4me3. Results from 3 independent experiments are expressed as H3K4me3 signal relative to total histone H3. (F) Acid-extracted histones from HeLa cells treated with 500 nM PD0325901 (MEK1/2 inhibitor) or DMSO for 24 hr were measured for H3K4me3 as in C.

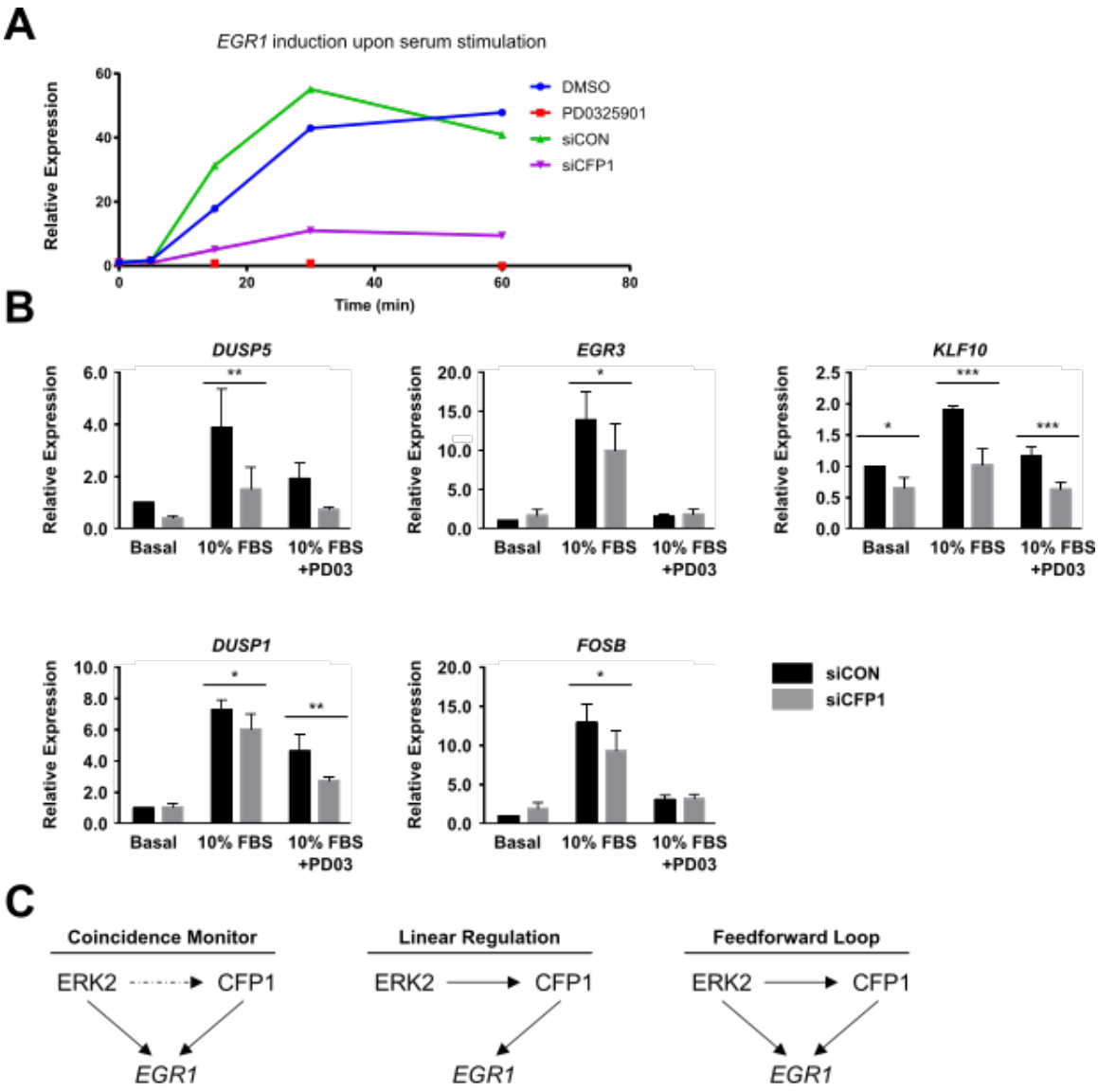

Figure 3-4 ERK1/2 signaling and Cfp1 contribute differentially to serum-responsive regulation of target genes. (A) EGR1 mRNA induction measured by qPCR upon MEK1/2 inhibition (500 nM PD0325901) or Cfp1 depletion (siCFP1). (B) HeLa cells were subjected to 72 hrs of siRNA treatment with either control (black bars) or Cxcc1-targeted (grey bars) oligonucleotides. Cells were serum-starved for 4 hrs prior to pretreatment with 500 nM PD0325901 or vehicle control and subsequently stimulated with 10% FBS for 30 min. Total mRNA was extracted from cells, transcribed to cDNA and qRT-PCR was performed for the indicated target mRNAs. All changes were normalized to the siControl basal condition. Error bars represent standard deviation of three separate experiments. Statistical significance was assessed by Student's two-tailed t-test: * = $p<0.05$; ** = $p<0.005$. (C) Models of non-mutually exclusive modes of regulation of serum responsive genes by ERK1/2 and Cfp1. Work in panel B performed by AMK.

Cfp1 and ERK1/2 Signaling in Global Chromatin Modification Regulation

Due to the known roles of Cfp1 in regulation of DNA methylation (5mC) and histone H3K4me3 deposition we investigated the effects of either Cfp1 depletion or ERK1/2 signaling inhibition on global levels of these epigenetic marks. Genomic 5mC content of cells treated with siRNA directed at Cfp1 (**Figure 3-3A**) or the MEK1/2 inhibitor PD0325901 (**Figure 3-3B**) was not altered compared to control treatments when measured by a 5mC-specific ELISA employing a standard curve (**Figure 3-3C**).

Previous measurements of global H3K4me3 levels were taken from whole cell lysates, in which a roughly 20% drop in relative H3K4me3 upon Cfp1 depletion was observed (not shown). Although somewhat consistent with previous studies conducted in mouse embryonic stem cells (mESCs), where both Cfp1 and ERK1/2 signaling are required for normal development, this result was unexpected because of inherent differences between stem cell regulation and regulation of an acute response. For the latter, the reasonable expectation is that rapid responses do not require changes in chromatin modification detectable on a global scale. To address this issue, I examined literature on the topic and modified my experimental strategy. Because the association of core histone H3 and variants with chromatin is a regulated process [176], I decided to examine histones acid-extracted from nuclear fractions (**Figure 3-3D**). In contrast to more crude preparations of histone proteins, I observed that nuclear H3K4me3 is not perturbed upon knockdown of Cfp1 (**Figure 3-3E**) or inhibition of ERK1/2 signaling (**Figure 3-3F**). Whether ERK1/2 or Cfp1 influence histone H3 turnover or regulation of histone variant deposition remains to be tested [176].

Cfp1 Depletion or ERK1/2 Signaling Inhibition Blunts Target Gene Induction

Cfp1 has also been shown to regulate rapid target gene-induction in B-cells [160], prompting me and others to test whether Cfp1 is required for an ERK1/2-mediated immediate early gene (IEG) response in HeLa cells. Indeed, microarray analysis of RNA purified from cells with depleted Cfp1 under serum-starved and serum-stimulated conditions revealed blunted expression of several genes, including *EGR1* (**Figure 3-4** and data not shown). To further characterize this defect in signaling, I performed an extended time course experiment and demonstrated that cells from which Cfp1 was depleted or treated with PD0325901 display markedly similar inabilities to activate *EGR1* **(Figure 3-4A**). In contrast, other serum-responsive genes did not demonstrate mutually overlapping induction profiles when comparing depletion of Cfp1 and ERK1/2 signaling inhibition (**Fig. 3-4B**). *KLF10*, for instance, is hampered under both basal and stimulated conditions when Cfp1 is depleted, while the reduction in *DUSP1* induction is exacerbated upon both Cfp1 knockdown and ERK1/2 signaling inhibition. Similar to *EGR1*, the prototypical IEGs *EGR3 and FOSB*, as well as *DUSP5*, are compromised by both treatments, albeit to differing degrees. Diverse signaling of ERK1/2 and Cfp1 at target genes was not entirely unexpected and suggests multiple modes of co-regulation (**Fig 3-4C**).

Predicted ERK2 Phosphorylation of Set1B Is Confirmed in vitro.

Due to the centrality of Cfp1 in multiple modes of chromatin regulation, its role as an ERK2 substrate has clear implications for how Cfp1, as well as ERK1/2 signaling, are able to

respond acutely to a variety of cues. Therefore, I postulated that ERK1/2-Cfp1 co-regulation of target genes is likely to involve other catalytic factors that interact with Cfp1. Comparisons of publicly available mass spectrometry data of DNMT1, Set1A, and Set1B reveal that all three contain multiple putative MAPK-targeted sites [177]. In particular, Set1B displays the highest enrichment of prototypical MAPK recognition motifs S/T-P or P-X-S/T-P (**Figure 3-5A**). Finally, in vitro kinase assays with recombinant Set1B (either with or without an intact GST affinity tag) confirm that both activated ERK1 (pERK1) and ERK2 can phosphorylate Set1B (**Figure 3-5B**).

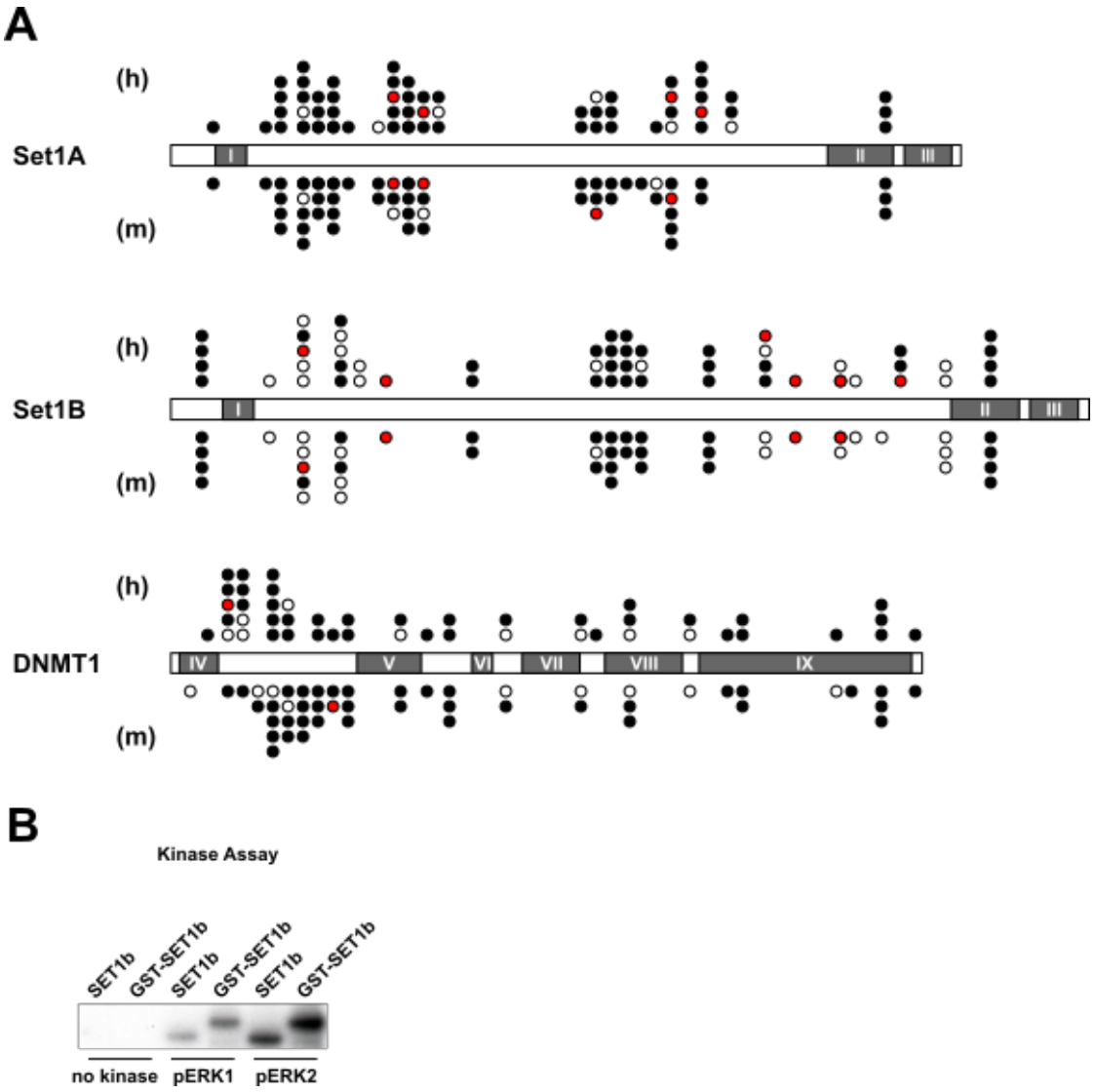

Figure 3-5. Set1B, a CFP1-interacting protein, is also an ERK2 substrate. (A) Set1A, Set1B, and DNMT1 contain multiple phosphorylation sites. Black circles indicate all sites identified and banked on phosphosite.org [42], white circles indicate identified sites on S/T-P sequences, and red circles indicate identified sites on P-x-S/T-P sequences. I. RNA recognition motifs, II. N-SET domains, and III. SET domains. DNMT1 contains IV. DMAP-binding domain, V. cytosine specific DNA methyltransferase replication foci domain, VI. CXXC zinc-finger domain, VII and VIII. Bromo-adjacent homology domains, and IX. DNA methyltransferase catalytic domain. (B) Radiolabeled kinase assay with recombinant Set1b with or without a GST tag as substrate and activated ERK1 or ERK2 (pERK1 and pERK2, respectively), performed by SE.

Discussion

The extracellular signal response kinases 1 and 2 (ERK1/2) can both mediate large-scale cellular dynamics and maintain homeostatic control by rapid response to upstream signals. Many of these processes require regulated changes in gene expression, consistent with the abundance of evidence showing ERK1/2 form extensive interactions with chromatin and associated factors (reviewed in [107]). To better understand how ERK1/2 signaling influences genetic output, we sought to identify novel ERK2-interaction partners that are central to chromatin regulation. To this end, we utilized a yeast-two hybrid approach that employed selective activation of ERK2 to compare relative affinities for binding partners between phosphorylated and unphosphorylated states [30, 173, 174]. By this method we identified the epigenetic regulator CXXC finger protein 1 (Cfp1) as a factor that interacts with ERK2 in eukaryotic cells.

There is a wealth of literature on Cfp1 in a developmental signaling context, where it is known to be important for both DNA methylation and H3K4me3 deposition [175]. Cfp1 has been most widely studied in mESCs, where ERK1/2 signaling is required for differentiation (in contrast to human ESCs, where ERK1/2 are critical for self-renewal) [178, 179]. ESCs derived from Cfp1-knockout mice self-renewed but could not differentiate following removal of the cytokine leukemia inhibitory factor (LIF) from culture medium [167, 180]. These cells exhibited a 70% global deficit in DNA methylation, with detectable hypomethylation at satellite repeats, stably methylated gene promoters and imprinted genes. Subsequent experiments suggested impairment of maintenance, but not *de novo* methyltransferase activity [167, 180]. Although both ERK1/2 signaling and Cfp1 are known

to regulate DNMT1 expression [164-166, 181], depletion of Cfp1 or inhibition of ERK1/2 signaling did not result in a global reduction of 5mC in HeLa cells, an immortalized cell line that relies on intact ERK1/2 signaling for sustained hyperproliferation.

In addition to interacting with DNMT1 and regulating DNA methylation, Cfp1 is also a member of two histone methyltransferase complexes, Set1A and Set1B. Both of these complexes mediate H3K4me3 at target genes and associate with largely non-overlapping regions of chromatin [182]. Notably, chromatin immunoprecipitation coupled to deep sequencing (ChIP-seq) studies indicate extensive colocalization of ERK1/2 and H3K4me3 [111]. Loss of Cfp1 in mESCs resulted in aberrant placement of H3K4me3 to chromatin regions outside promoters, indicating Cfp1 is essential for proper targeting of Set1A/B complexes. Previous work has shown that Set1A and Set1B target distinct regions of the genome and are subject to different upstream signals, suggesting that Cfp1 regulation is highly context-dependent [182, 183]. Supporting this notion, Cfp1 has also been shown to mediate rapid H3K4me3 enrichment in a gene-induction response in B-cells [160], however, no such role for Set1b has been reported to date. This work demonstrates that both Cfp1 and Set1b are ERK2 substrates and suggests that interactions with these factors modulate ERK1/2 signaling functions in the nucleus. Much like their varied roles in regulation of cytosolic substrates, ERK1/2 signaling in the nucleus is abundant if not somewhat cryptic.. As previously discussed, ERK1/2 have evolved divergent roles in development between humans and mice, suggesting adaptability is inherent to ERK1/2 biochemical activity. In addition to varied roles in development, ERK1/2 are required for a diversity of signal-dependent

responses, further suggesting that vulnerability to misregulation is a consequence of its versatility.

As the effector kinases of the Ras-Raf-MEK-ERK pathway, ERK1/2 are crucial for normal cellular function and prone to driving disease states under conditions of inappropriate signaling. An improved understanding of how this pathway exerts influence on gene expression is key to the eventual development of more finely tuned treatment options. Here, I describe how identification of key substrates of the ERK1/2 pathway may clarify how one transduction relay is capable of a diverse array of outputs. Previous work identified the epigenetic regulator CXXC finger protein 1 (Cfp1) as an ERK2-interacting protein. Cfp1 is involved in multiple aspects of chromatin regulation, including histone methylation and DNA methylation. I showed overlapping roles for ERK1/2 and Cfp1 in regulation of immediate early gene induction. I underscored multiple modes of co-regulation and demonstrated that Cfp1 is required for an optimal signal-dependent response. Furthermore, I provide evidence that helps explain the finding that Set1b, a Cfp1-interacting histone methylase, is phosphorylated by ERK2 and may be regulated by Cfp1. My work builds on my previous studies and highlights ERK1/2 interactions with chromatin regulators that contribute to MAPK signaling diversity in the nucleus.

CHAPTER FOUR

DEVELOPING STRATEGIES FOR DETECTING ERK1/2 NUCLEAR INTERACTIONS

Introduction

My research into the DNA-binding properties of ERK2 and the relationship between ERK1/2 and Cfp1 highlights major gaps in knowledge, most pressing being the question of extent of ERK1/2 nuclear protein-protein interactions. Although ChIP studies and mass spectrometry approaches have yielded information on ERK1/2 behavior on chromatin, identification of protein complexes that ERK1/2 associate with in the nucleus is critical for a complete understanding of how signaling activity is targeted to specific genes. To this end, a proximity biotinylation approach coupled to lysate-based experiments and eventual mass spectrometry analysis may identify the full complement of ERK1/2 nuclear protein interactions [184].

The proximity biotinylation strategy employs conjugation of a protein of interest to a biotinylase mutated to be exceptionally promiscuous, hereafter referred to as BirA. Because BirA is a bacterial protein, it is largely orthogonal to eukaryotic signaling systems. Factors that interact with proteins tethered to BirA are highly likely to be biotinylated, thus providing a tractable method to detect novel interaction partners. The strength and specificity of the streptavidin-biotin interaction allows for identification of both sustained and transient binding events. Biotinylated species can be isolated with streptavidin resin from cells expressing chimeras of BirA and proteins of interest cultured in the presence of biotin. This approach is not without disadvantages and requires long-term treatment with biotin prior to

Western blot or mass spectrometry analysis. These requirements may prevent successful starvation/stimulation experiments, but use of an inducible promoter for chimera expression can mitigate these limitations.

Here, I describe the design and generation of target cell lines that express BirA-conjugated human ERK1 (hERK1) and ERK2 (hERK2) under control of a tetracycline-inducible promoter. I went on to test the robustness of chimera expression and whether ectopic proteins behave similarly to their endogenous counterparts. I strove to include controls to make future data analysis unambiguous and demonstrated that these cell lines are excellent tools for identifying novel nuclear protein interactions for hERK1 and hERK2.

Methods and Materials

Cell Line Generation

Flag-NLS-BirA, BirA-Flag-GFP, BirA-Flag-hERK1, hERK1-BirA-Flag, BirA-Flag-hERK2, and hERK2-BirA-Flag constructs were generated by Gateway cloning into pcDNA5-pDEST-FRT-TO (gift of Dr. Anne-Claude Gingras) and confirmed by sequencing. Stable cell lines were generated from Flp-In T-REx 293 cells (Thermo Fisher) through co-transfection with 2 μg pOG44, a plasmid encoding Flp recombinase, and 0.2 μg pcDNA5-pDEST plasmids bearing the constructs described above. Cell lines were maintained in DMEM with 10% FBS supplemented with 200 ug/mL hygromycin and treated with 50 uM biotin for indicated experiments. 1 ug/mL tetracycline (Sigma Aldrich) was used for induction of BirA-conjugated protein-expression. Cells were lysed and immunoblotted as

previously described in Chapter 3. Biotinylation was visualized on a LiCor Odyssey scanner with IRDye 680RD Streptavidin (Fisher Scientific).

Results

To characterize ERK1/2 interactions in the nucleus (as well as other cellular compartments), cell systems able to express chimeric proteins under the control of an inducible promoter were generated from an engineered human embryonic kidney (HEK293) cell line called Flp-In T-REx 293 (**Figure 4-1**). These cells contain a flippase recognition target (FRT) sequence at a transcriptionally active locus maintained through selection with Zeocin. These cells also constitutively express tetracycline (Tet) repressor protein through selection with Blasticidin. Using Gateway vectors expressing the bacterial biotinylase BirA with a Flag epitope (a generous gift from Dr. Anne-Claude Gingras), constructs for six target cell lines were cloned that included N- and C-tagged versions of hERK1 and hERK2. Even though the termini of well-folded ERK1/2 are proximal to one another, employment of two constructs for each protein of interest will eventually help filter out false positive nuclear interactions (or support alternative hypotheses, as discussed in Chapter 5). In addition to BirA-ERK chimeras, two control cell lines were generated: one expressing BirA-green fluorescent protein (GFP) and another expressing BirA conjugated to a nuclear localization signal (NLS) (**Figure 4-1**).

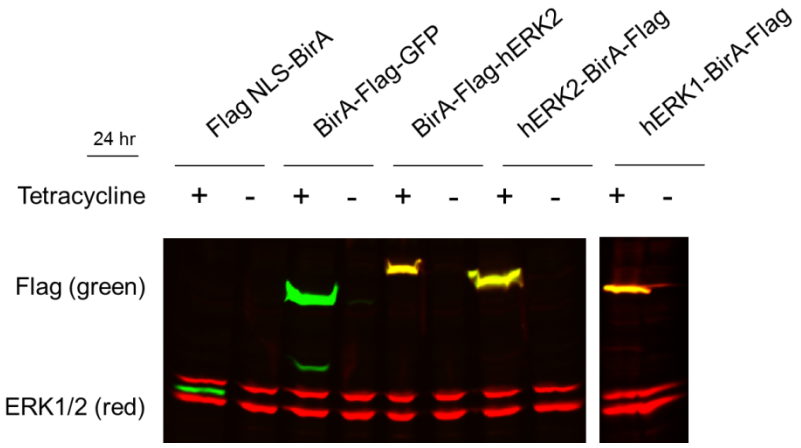

Figure 4-1. Generation of cell lines expressing ERK2 conjugated to a bacterial biotinylase under control of a tetracycline-inducible promoter. Flp-In T-REx 293 cells were used to establish cells lines with Tet-inducible expression of proteins or peptides conjugated to a bacterial biotinylase (BirA).

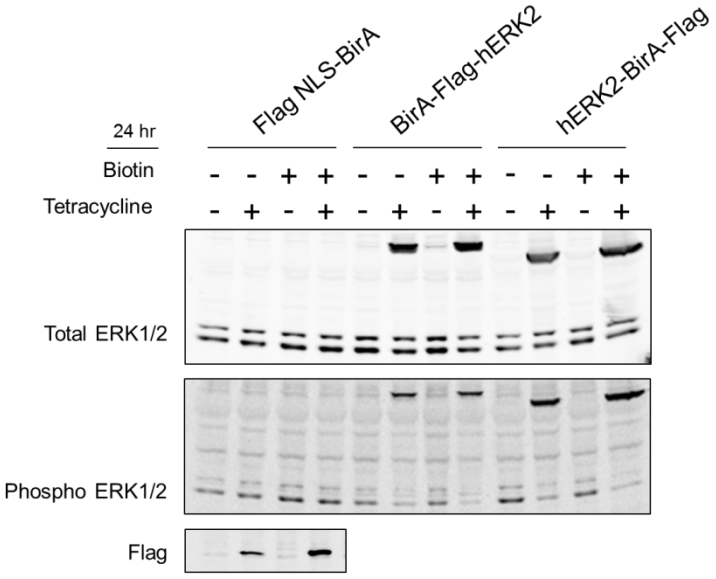

Figure 4-2. ERK2 conjugated to a bacterial biotinylase is phosphorylated in cells. Lysates from BirA-expressing cells were immunoblotted with antibodies that discriminate phosphorylated ERK1/2 (bottom) from total ERK1/2 protein (top). Flag signal is also shown to indicate ectopic expression occurs robustly in the presence of tetracycline.

As previously noted, MEK1/2-mediated dual phosphorylation of ERK1/2 in cells requires that substrate is well-folded. To evaluate whether exogenous ERK proteins were being expressed appropriately, cell lines expressing either BirA-NLS or BirA-ERK2 treated with tetracycline were lysed and blotted for total ERK1/2 and phosphorylated ERK1/2 protein (**Figure 4-2**). For both BirA conjugated ERK2 cell lines shown, exogenous ERK2 is phosphorylated on its activation loop and is recognized by a phospho-specific antibody. Future experiments with PD0325901 can confirm that endogenous MEK1/2 can function directly upstream of chimeric ERK constructs in these cells.

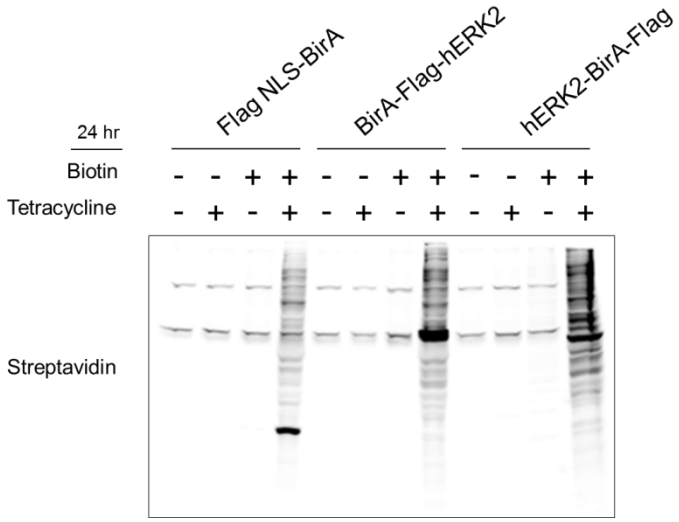

Figure 4-3. Induction of BirA expression results in increased biotinylation of cellular proteins. Biotinylated proteins are detectable in lysates from T-REx cell lines with antibody fluorescently labeled streptavidin. Importantly, multiple biotinylated species are observed in the combined presence of both 50 μM Biotin and 1 μg/mL tetracycline.

To assess the degree of background biotinylation under conditions of combined biotin and tetracycline treatment, fluorescently labeled streptavidin was used to visualize BirA

activity after 24 hours of induction (**Figure 4-3**). Future experiments using a candidate-based approach to immunoblot specific proteins known to interact with ERK1/2 will help to further validate this method.

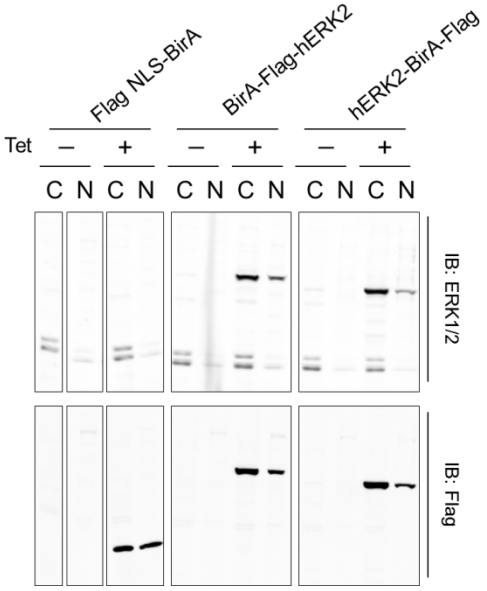

Figure 4-4. BirA conjugated to an NLS or ERK2 can enter the nucleus. Nuclear (N) and cytosolic (C) fractions were separated by SDS-PAGE and immunoblotted (IB) with antibodies recognizing total ERK1/2 (top) and Flag epitope (bottom). For the three cell lines shown, treatment with tetracycline resulted in ectopic expression of BirA constructs detectable in the nuclear fraction.

To confirm that BirA-ERK proteins and control constructs can enter the nucleus, cells were fractionated into nuclear and cytosolic portions and subjected to Western blot analysis with antibodies directed at either total ERK1/2 protein or the Flag epitope (**Figure 4-4**). For all chimera-expressing cell lines examined, exogenous proteins were detected in nuclear fraction upon treatment with tetracycline. Experiments to confirm these localization patterns by immunofluorescence microscopy are currently underway. Lastly, biotinylated proteins were precipitated from nuclear extracts using streptavidin-conjugated beads, separated by

SDS-PAGE, and visualized with fluorescently labeled streptavidin (**Figure 4-5**), indicating that protein recovery through this method is suitable for eventual mass spectrometry studies.

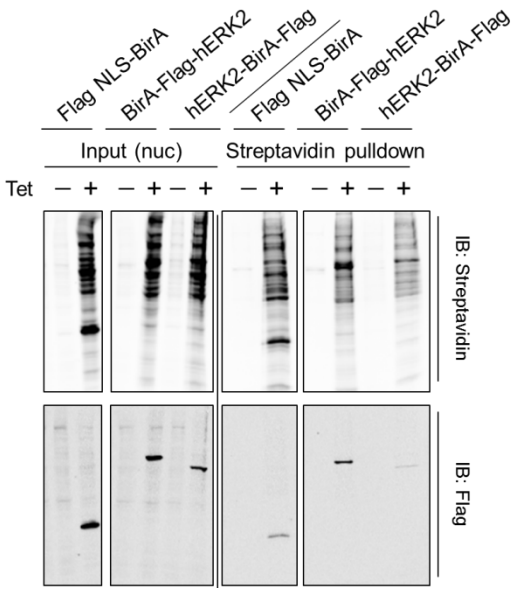

Figure 4-5. Induction of BirA expression results in increased biotinyltation of nuclear proteins. Nuclear extracts from BirA-expressing T-REx cell lines treated with biotin display multiple biotinylated species when induced with tetracycline. Precipitations from nuclear extract with streptavidin-conjugated beads were separated by SDS-PAGE and blotted with an antibody that recognizes streptavidin as well as the Flag epitope.

Discussion

As previously discussed, two limitations to this method are the length of time required for detectable biotinylation events and the reliance on overexpression of ERK proteins. Accordingly, the immediate future directions for this project are to determine optimal conditions for tetracycline concentration and duration of treatment that result in observable biotinylation of nuclear proteins as assessed by lysate-based experiments such as those described above. Testing different induction conditions can mitigate these shortcomings and guide future experiments. More to this point, experiments need to be performed that test the combined treatments of tetracycline, biotin, and the MEK1/2 inhibitor PD0325901.

After a closer determination of conditions, streptavidin-pulldowns from nuclear fractions of BirA-conjugated ERK-expressing cell lines can be prepared for mass spectrometry analysis. One reasonable expectation is that a large number of proteins will be identified, with many interactions representing background noise from the engineered cell systems. Inclusion of the BirA-GFP and BirA-NLS cell lines is therefore critical for the success of these studies, because it will help narrow down the number of putative positive hits. Moreover, use of four independent cell lines will also serve to corroborate identified interactions for ERK1/2.

Another important consideration is subsequent validation of ERK1 and ERK2 nuclear binding partners. By its nature, the proximity biotinylation method is sensitive to transient interactions as well as stable ones, but it cannot discriminate binding events from enzyme-substrate interactions. Traditional methods such as co-immunoprecipitation experiments can

be used to confirm binding interactions in cells and *in vitro*, and kinase assays with recombinant substrates will also be informative. However, because these experiments are not ideally suited to confirm transient interactions, other methods will need to be employed. With regards to ERK1/2 interactions in the nucleus, one strategy that has been successful in the past is co-immunoprecipitation studies done with enriched chromatin fractions (see Figure 3-1). With regards to the effects of specific nuclear interactions on ERK1/2 activity, I suggest *in vitro* kinase assays with purified recombinant protein identified by proximity biotinylation. Furthermore, I also suggest ERK1 and ERK2 *in vitro* kinase assays on typified substrate (e.g. myelin basic protein or ERKtide) in the presence or absence of factors identified by mass spectrometry in order to examine allosteric effects nuclear interactions may have on ERK kinase activity. Lastly, my previous work has demonstrated that ERK2 can form direct interactions with DNA, making it imperative to similarly test how interactions with other nuclear proteins affect ERK2-DNA binding behavior.

CHAPTER FIVE

FUTURE DIRECTIONS

Introduction

The work I have presented here highlights aspects of ERK1/2-chromatin interactions and calls attention to the need for a better understanding of nuclear ERK1/2 dynamics. Future research into how ERK1/2 affect gene expression should progress due to the indexing power afforded by next generation sequencing (NGS) technologies and mass spectrometry approaches that can detect changes to the phospho-proteome and a variety of protein-protein interactions. While these strategies represent the best path towards a better understanding of ERK1/2 signaling in the nucleus, I posit that much is also to be gained by examining the evolutionary trajectory of multiple pathway components including upstream factors and downstream targets. The success of these pathways, conserved from yeast to humans, is a testament to the fitness of their design, suggesting that an improved understanding of the pressures leading to their evolution and expansion can inform their signaling functions during the life of a cell.

I credit my training as a biochemist in the Cobb laboratory for leading me to the conclusion that a combination of computational and biochemical approaches will eventually identify mechanisms of ERK1/2-mediated gene regulation. Although the design of large-scale “omics” experiments that are both informative and reflective of real cellular events is challenging, these approaches will be critical for a general understanding of ERK1/2

signaling. Here, I have compiled suggestions for how the projects described in preceding chapters should proceed.

ERK1/2 Direct DNA Binding

One disadvantage of the EMSA approach is that it does not provide a true equilibrium measurement and is biased against complexes with fast off-rates. Because of the general weakness of the ERK2-DNA interaction, this must be addressed through use of different techniques. Fluorescence polarization (FP) is one way to move forward, especially because it also offers the advantage of being adaptable for screening. This is critical, because one major gap in knowledge of ERK2-DNA binding is whether ERK2 has preferences for specific DNA sequences. Two ways that this can be investigated are by mining published ERK2 ChIP-seq datasets and in-house datasets for candidates to test by FP (and F-EMSA), and through competition assays with unlabeled ("cold") probes. One distinct advantage of the latter approach is that there is technically no upper limit to how much cold oligonucleotide can be added, although chances of success would increase with a labeled probe bearing a sequence that ERK2 is known to strongly prefer. Furthermore FP assays do not preclude subsequent analysis by EMSA because they do not require destruction of sample.

FP can also be used to screen different ERK1 and ERK2 mutants, as well as ERK homologs from a panel of organisms, to assay ERK1/2 DNA binding affinity. More evidence is accumulating that ERK1/2 can process information from phosphorylation events outside of canonical activation (e.g. pT188 in promotion of cardiac hypertrophy). Data from

our lab and collaborators has identified several threonine and serine residues on ERK1 and ERK2 as putative phospho-acceptors. Having another readout in addition to kinase assays will be useful for a future understanding of how different post-translational modifications can alter ERK1/2 behaviors not only in terms of enzymatics, but also interactions. More to this point, I think that the recombinant protein prepared for these studies ideally should be free of affinity tag sequences to reduce the amount of lurking variable in experimental conditions.

After these methods identify a range of ERK1/2-oligonucleotide interactions, several approaches can be taken to better understand how ERK1/2-DNA binding is regulated. This project is in great need of more cell-based work, such as expression of ERK1/2 mutants and subsequent ChIP-qPCR assays, to understand the effects of phosphorylation on chromatin-association and specific positioning. Finally, the use of small molecule ERK inhibitors, such as one recently reported to inhibit ERK dimerization, can offer further information on mechanisms of ERK1/2-DNA binding through *in vitro* and cell-based experiments.

ERK1/2 Signaling and Cfp1

The major gap in knowledge in the regulatory relationship between ERK1/2 and Cfp1 is that there is currently no attributable function for Cfp1 phosphorylation by ERK1/2. The first step to address this is to identify target genes occupied by both ERK1/2 and Cfp1. This will require extensive ChIP-sequencing studies under different starvation and stimulatory conditions and it would be prudent to try other ERK1/2 stimuli in addition to serum. Additionally, performance of these types of experiments in cells where either ERK1 or ERK2 have been knocked out by CRISPR methodology would be highly informative, because it

would likely be much more effective at reducing ERK1/2 signaling in the nucleus than pharmacological inhibition of MEK1/2 alone. Lastly, replacing wildtype Cfp1 with phosphorylation site mutants would be one way to define the functions of these sites in chromatin regulation.

Another, albeit far riskier, way to address the gap in knowledge of how Cfp1 is regulated by ERK1/2, is to shift to two different cell systems: mouse ESCs and human ESCs. Both of these cell systems require ERK1/2 signaling, but for opposing developmental outcomes, as noted above. The use of parallel systems has two immediate advantages. First, Cfp1 has been primarily characterized in a developmental context, with mESCs providing the background for the majority of reported studies. In this system, loss of Cfp1 results in major and tractable aberrations in H3K4me3 deposition and global DNA methylation, thus providing two functional readouts that can be tested in the context of ERK1/2 activation and inhibition. Notably, ERK1/2 signaling is required in mESC for self-renewal. Second, it would be interesting and informative to know if Cfp1 depletion leads to similar effects in hESCs, where ERK1/2 signaling is required for differentiation (and must be inhibited for self-renewal). These systems could be probed via CRISPR-Cas9 gene editing technology to express Cfp1 phosphorylation site mutants to measure effects on chromatin modifications. However, a shift to these types of settings will change the overall research question and move away from the issue of acute signal-dependent changes in Cfp1 activity as a function of ERK1/2 phosphorylation.

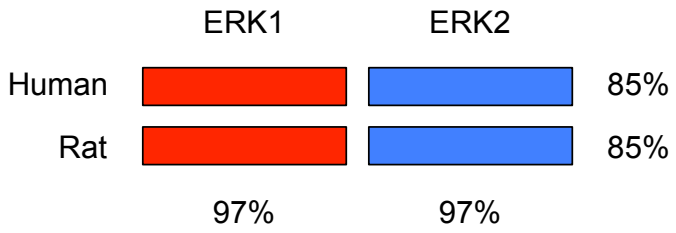

Figure 5-1. ERK1 and ERK2 are paralogs. Human and rat ERK1/2 are highly similar in amino acid sequence.

Proximity Biotinylation and Probing the Redundancy of ERK1 and ERK2

The immediate future directions for a proximity-biotinylation approach to identify ERK1/2-interacting partners in the nucleus have already been discussed in Chapter 4. What was not touched upon is that BioID also represents an opportunity to investigate the possibility that ERK1 and ERK2 have evolved different functions. While this hypothesis has been put forth by others in the past [185-187], the question of divergent function has not sufficiently been put to rest. Although initial BioID experiments will be conducted in engineered cell lines and employ overexpression of ERK proteins, this platform is uniquely suited to compare differences in protein-protein interactions between ERK1 and ERK2. Given the persistent lack of understanding how the ERK1/2 pathway operates in a highly context-dependent manner, one hypothesis in need of testing is that differences in ERK1 and ERK2 activity and protein-protein interactions underlie pathway versatility.

The evolution of MAPK signaling networks has been driven by gene duplication events that have led to the rise of paralogous pairs of kinases, such as in the case of ERK1 and ERK2. Although in some instances gene duplication can lead to stoichiometric expansion of cellular components (i.e. dosage effects), as in the case of histone proteins,

evolutionary pressures most often lead to purifying selection of one paralog over the other due to overly redundant function [188]. Conversely, both copies can be retained through the rise of divergent activity. In these cases, one paralog is typically expressed to a higher degree than the other, and it has been proposed that this is an evolutionary strategy to ensure fidelity of signaling through the more prevalent paralog while maintaining a 'backup' that can be exploited for new behaviors [189].

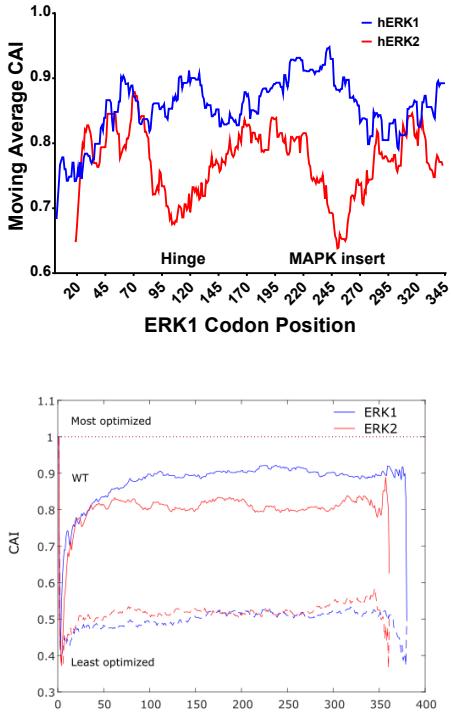

Figure 5-2. Moving average of codon adaptation indices (CAI) for ERK1 and ERK. Human-specific codon adaptation indices were calculated for ERK1 and ERK2 across a moving average window of 35 codons (above) and 100 codons (below). Corresponding domains of ERK1/2 are indicated, as well as codon adaptation index profiles for most- and least optimized nucleotide sequences for ERK1 and ERK2.

ERK1 and ERK2 share a high degree of amino acid sequence similarity between paralogs in the same organism, but display even higher degrees of similarity between homologs across organisms (**Figure 5-1**), suggesting fixed differences between them is beneficial to their function. Consistent with a report conducted in HeLa cells [114], publicly available expression data from different cell types, and the paralogous evolution paradigm, ERK1 is notably expressed less than ERK2 in mice and humans. Moreover, while there is limited evidence suggesting that ERK1 and ERK2 are regulated in different manners [190, 191], a hallmark of paralogous pairs is a high degree of redundancy. ERK1 and ERK2 certainly have largely overlapping functions, and this has been demonstrated recently by Meloche and colleagues using mouse whole body knockout (KO) models [185]. The authors were following up the puzzling observation that ERK1-KO mice are viable while ERK2-KO mice are not [192] and found that ectopic overexpression of ERK1 is sufficient to rescue ERK2 lethality [185]. Importantly, this does not preclude the notion that ERK1 and ERK2 contribute differently to the signaling pathway, and it has been shown in at least one context that ERK1 deletion alone leads to signaling defects [193].

Surprisingly, while the amino acid sequences and protein structures of ERK1 and ERK2 are very similar to one another, the genes encoding ERK1 and ERK2 differ markedly in their nucleotide sequences. Indeed, a recent examination of ERK1 and ERK2 across multiple organisms has shown that ERK1 is not expressed at all in some tetrapods while ERK2 expression has been lost in squamates [194]. Furthermore, the gene encoding ERK1 is much smaller than that encoding ERK2 and has been shown to evolve at a faster rate than ERK2, adding credence to the notion of neofunctionalization of ERK1 [188, 194]. Perhaps

even more intriguing is the observation that ERK1 and ERK2 employ remarkably different preference in codon usage (**Figure 5-2**). Although "silent" differences have been long thought of as mundane, there is growing evidence for unanticipated regulatory content in codon usage that can affect gene expression, translation, and even protein activity [195-197].

In light of these lines of evidence, I suggest that the proximity biotinylation approach is a good first step in investigating whether ERK1/2 signaling adaptability is rooted in non-redundant functions of ERK1 and ERK2. These studies can be tremendously aided through expression of codon-optimized and de-optimized versions of ERK1 and ERK2 and subsequent analysis through kinase assays and DNA-binding assays. If differences in measured behaviors are detected, investigations into co-transcriptional and co-translational regulation of ERK genes and proteins can be used to better understand the general functions of this important pathway.

Conclusion

Much like in different organisms, cell types, and cellular compartments, ERK1/2 signaling displays remarkable multi-functionality in the nucleus. Throughout the course of my research, my results were typically suggestive but rarely explanatory. However, once I learned to contextualize my findings with reports from the current literature, publicly available data, and evidence from my own work, I was able to successfully describe scientific observations in the form of published reports. Through this process, I learned how to better refine my research questions. In the future, I hope to improve my experimental focus in order to define a mechanism rather than study phenomena too broadly. Although nowhere near definitive, my work highlights several facets of ERK1/2 signaling in the nucleus and establishes recourse for continuing these projects, thus supporting the notion that the evolutionary success of the ERK1/2 pathway is rooted in its adaptability.

APPENDIX A
Featured Publications and Co-Author Attributions

CHAPTER ONE was modified from a proprietary review authored by Aroon S. Karra (ASK) and Melanie H. Cobb (MHC), entitled *MAP Kinase Pathways: Functions and Modulations*, TOCRIS Bioscience, 2016.

CHAPTER TWO was modified from McReynolds and Karra, et al. 2016 [68], a report written by ASK and MHC entitled *Phosphorylation or Mutation of the ERK2 Activation Loop Alters Oligonucleotide Binding*. Biochemistry 55(12):1909-1917, with contributions from Andrea C. McReynolds (ACM), Yan Li (YL), and Steve Stippec (SS).

CHAPTER THREE was modified from Karra and Klein, et al. 2017, a report in submission written by ASK and MHC entitled *ERK2 Phosphorylates the Epigenetic Regulator CXXC-finger protein 1 (Cfp1)*, with contributions from Aileen M. Klein (AMK) and Svetlana Earnest (SE).

CHAPTER FOUR contains experiments designed and executed by ASK with contributions from Kathleen McGlynn (KM) and Courtney N. Powell (CNP).

APPENDIX B was modified from Karra et al. 2017, a methods report in press at the Journal of Visualized Experiments (JoVE) authored by ASK with contributions from SS and MHC, entitled *Assaying the activity of protein kinases with radiolabeled ATP*.,J Vis Exp,

APPENDIX C was modified from Karra et al. 2015, a preview article entitled *A Kinase Divided*, Cancer Cell 28(2):145-147.

APPENDIX B
Methods Paper for Journal of Visualized Experiments

Title:
Assaying protein kinase activity with radiolabeled ATP

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Keywords:

Protein kinase; signal transduction; biochemical assay; radiolabeled assay

Short Abstract:

Protein kinases are highly evolved signaling enzymes and scaffolds that are critical for inter- and intracellular signal transduction. We present a protocol for measuring kinase activity through the use of radiolabeled adenosine triphosphate ([γ-^{32}P] ATP), a reliable method to aid in elucidation of cellular signaling regulation.

Long Abstract:

Protein kinases are able to govern large-scale cellular changes in response to complex arrays of stimuli, and much effort has been directed at uncovering allosteric details of their regulation. Kinases comprise signaling networks whose defects are often hallmarks of multiple forms of cancer and related diseases, making an assay platform amenable to manipulation of upstream regulatory factors and validation of reaction requirements critical in the search for improved therapeutics. Here, we describe a basic kinase assay that can be easily adapted to suit specific experimental questions including but not limited to testing the effects of biochemical and pharmacological agents, genetic manipulations such as mutation and deletion, as well as cell culture conditions and treatments to probe cell signaling mechanisms. This assay utilizes radiolabeled [γ-^{32}P] ATP, which allows for quantitative comparisons and clear visualization of results, and can be modified for use with immunoprecipitated or recombinant kinase, specific or typified substrates, all over a wide range of reaction conditions.

Introduction:

Protein kinases are sophisticated enzymes critical for the transmission of cellular signals into appropriate responses[1]. Given their roles in maintaining homeostasis and the prevention or promotion of disease states[2], biochemical methods for assessing kinase activity continue to be powerful tools for delineating the particulars of eukaryotic signaling[3]. Although strategies utilizing phospho-specific antibodies have been highly informative in

terms of measuring the effects of different treatment conditions on cell signaling status[4], a kinase assay allows for measuring the effects of different treatment conditions directly in terms of the enzymatic activity of a kinase of interest. While there are several options for similar assays that do not use radioactive materials[5], we continue to rely on this method for robust quantitation of results. There are two typical applications of this assay, both valuable for different reasons: the immunoprecipitated (IP) kinase assay (Figure 1) and the recombinant protein kinase assay (Figure 2).

The IP kinase assay is tremendously useful for identifying factors capable of activating specific protein kinases as well as gauging inhibitory treatment conditions. Briefly, an epitope-tagged kinase of interest is transfected into cultured eukaryotic cells, subjected to a variety of treatments, immunoprecipitated, and assayed for the ability to incorporate radiolabeled phosphate into a model substrate (e.g. myelin basic protein (MBP)). IP kinase assay can also be performed without resorting to overexpression, either by immunoprecipitation of endogenous protein or any number of genome-editing techniques. Because treatments are administered in culture, this method can detect stimulations transmitted through multiple upstream factors or parallel pathways by *in vitro* readout. One major advantage of this method is that it does not require prior knowledge of direct upstream or downstream factors or phosphorylation sites therein. Moreover, once specific substrates for a kinase of interest have been identified, the same kinase assay protocol can be used with recombinant components to measure specific activity towards natural substrates and identify specific phosphorylation sites when combined with mass spectrometry analysis. Lastly, this method can also be used to detect and measure autophosphorylation.

The protocol provided here assumes either an optimized protein purification scheme or transfection method for expressing an affinity-tagged kinase of interest in cultured cells. For more detailed expositions of transfection, lysis, immunoprecipitation, and protein purification protocols, we suggest referring to Cold Spring Harbor Protocols[6]. For more information regarding assay development and modification, please refer to Protein Phosphorylation: Selected Methods in Enzymology[7].

Protocol:

1. **Kinase purification resources and general immunoprecipitation pipeline**

Note: Kinases for use with this assay may be sourced from immunoprecipitates of cultured cells or by recombinant means such as affinity-tagged purification[6]. Below is a general protocol for immunoprecipitation that may have to be modified depending on the kinase of interest. Everything should be kept on ice when possible.

1.1. Add 2 µL of antibody to 200 µL of cell lysates and incubate at 4 °C for 1 h while rocking.

1.2. Wash protein A sepharose beads 2-3 times with lysis buffer by briefly spinning at 4 °C for 30 s to 1 min at 5000 x g (“touch spin”), removing supernatant with a pipette, and resuspending beads in buffer.

1.3. Add 30 µL of 50% slurry of protein A sepharose beads in lysis buffer to lysates; incubate at 4 °C for 1 hour while rocking.

1.4. Touch spin at 4 °C to pellet the beads and remove the supernatant.

1.5. Wash 3 times with 1 mL of bead wash buffer (1 M NaCl, 20 mM Tris pH 7.4).

1.6. Wash once with 1X kinase reaction buffer (10 mM HEPES pH 8.0, 10 mM $MgCl_2$).

1.7. Remove as much buffer as possible without removing beads.

2. **Initializing kinase reactions**

Note: For IP kinase assays, ~15 µL of unsuspended beads is a typical starting sample. For recombinant protein kinase assays, typical starting amounts range from 0.1-1.0 µg in 1-10 for 25-50 µL final reaction volumes. When using high amounts of kinase, it is useful to include a carrier protein such as BSA to aid in maintaining protein stability for the duration of the assay.

2.1. Adjust the volumes of samples to be equal so the same volume of reaction buffer can be added uniformly to all samples.

2.1.1. Prepare the 5X kinase reaction buffer given below:

50 mM HEPES pH 8.0

50 mM $MgCl_2$

50 mM Benzamidine (protease inhibitor)

50 mM DTT (reduction agent)

250 µM ATP (unlabeled, or "cold")

2.2. Keep kinase samples on ice in 1.5 mL tubes. Prepare the following reaction mixture in separate, chilled 1.5 mL tubes:

21 µL H_2O

6 µL 5X kinase reaction buffer

1 µL radiolabeled ("hot") ATP

2 µL substrate (~5 mg/mL)

Note: For recombinant kinase assays, volume may be adjusted to accommodate purified protein. Calculate such that final reaction volume is 30 µL. Use this recipe to prepare a master mix. Upon receipt of labeled ATP, dilute stock in H_2O to a specific activity of 0.01 mCi/µL prior to adding to reaction mixture.

2.3. To initialize the assay, add entire reaction mixture to kinase sample and incubate reaction at 30 °C for 5 min to 1 h depending on activity of kinase being assayed.

Note: When working with a kinase whose activity has not been previously assayed, perform a time course of kinase activity with 5 min intervals between time points.

3. Reaction termination and SDS-PAGE

3.1. Stop reaction by putting on ice and adding 7.5 µL 5X Laemli sample buffer [6].

3.2. Heat at 100 °C for 30 s to 2 min in heat block.

3.3. Touch spin and load 20 µL per well on 10-15% SDS-PAGE gel. Run gel long enough to separate kinase and substrate; make sure to keep gel apparatus shielded to limit exposure to ^{32}P.

4. **Gel staining and drying**

Caution: For all steps be sure to reduce personal exposure to ^{32}P by using radioactive shielding and wearing personal protective equipment. For more information on specific steps some helpful references are included.

4.1. Remove the gel from glass/alumina plates and place in 50 mL Coomassie stain (10% glacial acetic acid, 50% methanol, 0.25% R-250 dye) for 1 h on an orbital shaker set to 50 rpm. For this step use a container that is slightly larger than the gel itself.[8]

4.2. Move the gel from stain to fixing solution (10% glacial acetic acid, 20% methanol) in order to de-stain. Rock the gel in 600-700 mL of fixing solution overnight on orbital shaker at 50 rpm. Place pieces of foam or knotted laboratory wipes in the container with the gel to absorb the Coomassie dye [8,9].

4.3. Remove gel from fixing solution and soak in 200 mL methanol for 1-2 min with gentle agitation until gel turns milky white. This will help prevent cracking during the drying step.

4.4. Wet a 14 cm x 14 cm piece of qualitative filter paper with methanol and place onto slab gel vacuum dryer. Lay the gel down onto the filter paper front side facing up.

4.5. Cover the gel with plastic wrap carefully to avoid wrinkles and air bubbles. Run the dryer for 1.5 h at 80 ^{o}C. After the vacuum has been attained open the lid and roll out any air bubbles if needed with a soft rubber brayer.

5. Autoradiogram and scintillation counts

5.1. Remove dried gel and attach an autorad marker, phosphorescent ruler or dots to the filter paper on the side of the gel. If using dots make sure they are in an asymmetric pattern. This will align the gel with the film after exposure and development.

5.2. Use a Geiger counter to check the intensity of the signal. For weaker signals exposure at -70^{o} C to -80^{o} C with an intensifying screen will increase the film band density.

5.3. In a dark room put dried gel in a film cassette with film and an intensifying screen in the following order from top to bottom: gel, film, intensifying screen.

5.4. Attach the intensifying screen to the lid of the cassette and tape the gel in place to make this step easier. The intensifying screen emits light when irradiated by the beta particles from the ^{32}P. These light emissions penetrate the film more efficiently than the beta particles.

5.4.1. Make sure the wavelength emitted from the intensifying screen corresponds to a wavelength the film is most sensitive to.

5.4.2. Use a Geiger counter to determine how long to leave exposure for: if cpm count is ~100 or below, try overnight at -70^{o} C. If count is ~10000, start with a 1 h exposure and optimize from there.

5.5. At the conclusion of the exposure remove the film and develop in a medical/X-ray film processor. If the exposure was performed at -70 to -80^{o} C, either allow the

cassette to warm to room temperature to minimize condensation on the film or remove the film immediately before condensation forms [10].

5.6. Lay the film over the dried gel/filter paper and align the image of the marker/dots with the markers/dots on the dried gel. Mark on the film the bands corresponding to the protein standards. Label the protein standards and which lanes the reactions are for future reference.

5.7. Excise the bands from the gel that correspond to bands of interest on the film and place them in 7 mL scintillation vials. Add 4 mL of scintillation fluid and count with liquid scintillation counter. Confirm the counter is set to monitor the correct energy spectrum window for ^{32}P.

Note: Be sure to also excise the band corresponding to substrate from the no-kinase control lane. This will be used to calculate background radiation that will be subtracted from all sample scintillation counts.

6. **Analysis of results**

Note: The autoradiogram provides a qualitative visualization of results. For accurate quantitation, ^{32}P incorporation can be measured with a scintillation counter. Data are usually expressed in terms of relative activity, as shown in Figure 1B. As long as uniform conditions

are maintained for all samples, relative measurements of specific activity are sufficient to compare treatments.

6.1. When calculating absolute specific activity values, perform a rigorous optimization of all reaction conditions, including kinase, substrate, and cold ATP concentrations, in order to ensure a linear range of activity over a time course (e.g. 2 x [kinase] = 2 x specific activity). For kinases with high specific activity, perform assays with increased substrate concentration in case kinase activity is limited by amount of substrate.

6.2. Calculate the specific activity of kinase of interest in units of nanomoles phosphate transferred per minute per mg kinase.

6.2.1. Subtract blanks from the cpm in the counted bands.

6.2.2. Calculate the decay of hot ATP: $[(1/2)^{(t/t_{1/2})}]$ x 0.95 where *t* is the number of days since the reference date (should be provided by vendor), $t_{1/2}$ is the half-life of ^{32}P, which is 14.3 days, and 0.95 reflects the efficiency of the scintillation counter. Example: if using hot ATP that is 28 days old, the decay value should be 0.245.

6.2.3. Calculate the picomoles (pmol) of total ATP in the assay (usually this is equal to the amount of cold ATP in the reaction): assay volume (μL) x [cold ATP] (μM) = total ATP (pmols). Example: 30 μL x 50 μM = 1500 pmol

6.2.4. Calculate cpm/pmol ATP: [μL of hot ATP x (2.2×10^7) x decay factor]/ pmol of cold ATP.

Note: The value of 2.2×10^7 converts 0.01 mCi/μL of ATP to cpm.

6.2.5. Calculate the amount of kinase in the assay in mg. For both recombinant kinase and immunoprecipitated kinases, standard methods such as BCA assays are suitable to determine starting concentrations. Example: wtERK2 0.0002 μg/μL in a 50 μL kinase reaction is 0.01 μg, or 1×10^{-5} mg.

6.2.6. Calculate total cpm in assay. Assemble 30 μL reactions and run 20 μL from each reaction on a gel. Multiply the cpm counted (after blank subtraction) by a factor of total assay volume/ volume loaded. Continuing the above example, multiply all counts by 1.5, or 30/20.

6.2.7. Calculate the specific activity of the assayed kinase:

(Total cpm in assay)/(cpm/pmol ATP)/(assay time in minutes)/(amount of kinase in mg)

Note: Dividing the above value by 1000 yields the specific activity in nanomoles/minute/mg

6.3. Calculate how many mols of phosphate are incorporated into a substrate (as long as its molecular weight is known). This is used as a way to determine the number of phosphosites on a particular protein once the reaction has gone to completion.

[(Total cpm in assay)/(cpm/pmol ATP)]/(amount of substrate in pmols)

Representative Results:

WNK1 IP kinase assays.

Myc-tagged WNK1 was transfected into HEK293 cells and immunoprecipitated with an anti-Myc antibody [11]. The immunoprecipitate displayed kinase activity towards the model substrate MBP as well as toward itself (Figure 1A). WNK1 mutants were then tested for kinase activity towards MBP by the same method, this time employing GST-tagged constructs (Figure 1B). Analysis of mutant kinase behavior relative to wildtype revealed residues critical for optimal WNK1 activity.

HEK293 cells were exposed to a panel of pharmacological and biochemical treatments. Using an antibody raised against a WNK1 N-terminal peptide, immunoprecipitated endogenous WNK1 kinase activity was assayed using MBP as substrate. NaCl was demonstrated to be a regulator of WNK1 kinase activity.

ERK2 recombinant protein kinase assays.

Rat ERK2 bearing an N-terminal 6His tag was expressed in bacterial cells and affinity purified with nickel resin[12]. Protein was further purified by ion exchange chromatography on a MonoQ column, wherein several elution fractions were tested for kinase activity (Figure 2A). Because ERK2 requires dual phosphorylation by MEK to reach maximal kinase activity, ERK2 purified from bacteria in the absence of MEK is largely inactive. Therefore, in order to test fractions of recombinant ERK2 for activity towards MBP, a constitutively active form of MEK1 called MEK1R4F was included in kinase reactions. Notably, MEKR4F does not harbor high kinase activity on MBP (Figure 2).

Using an assay similar to the one shown in Figure 2A, an MBP kinase activity was used to measure activity of recombinant ERK2 mutants purified from bacterial cultures relative to wildtype protein (Figure 2B). Although ERK2 is able to phosphorylate MBP when stimulated by MEKR4F, mutation of either the catalytic lysine (K52R) or a threonine proximal to the canonical sites of dual phosphorylation (T188D and T188E) dramatically abrogates ERK2 kinase activity. ERK2-T188D and ERK2-T188E display marginal kinase activity toward a small, flexible peptide (Figure 2C), however, they are unable to robustly phosphorylate the known ERK2 substrates Nup153 and PDX1 (Figure 2D).

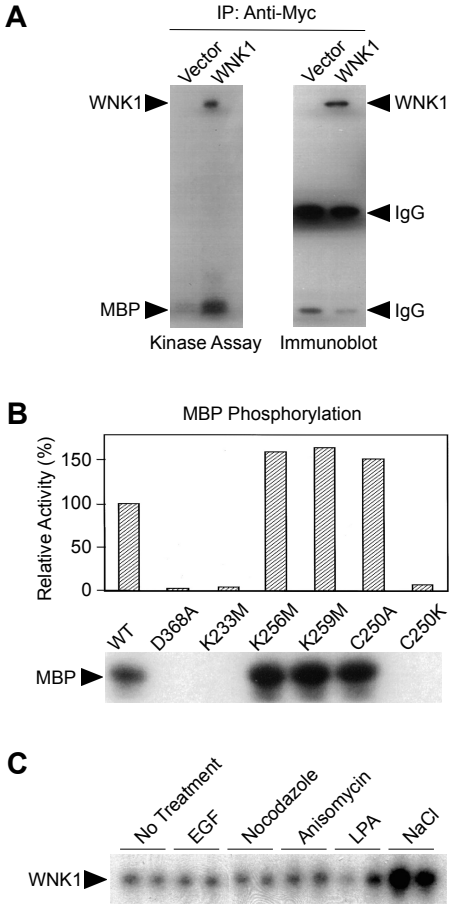

Figure 1 Immunoprecipitation kinase assays of WNK1. (A) HEK293 cells were transfected with either pCMV5-Myc without an insert or pCMV5-Myc-WNK1, tagged proteins were immunoprecipitated with the anti-Myc antibody followed by kinase assays using MBP as substrate. Autoradiography is shown on the left, and an immunoblot of the immunoprecipitates on the right. (B) Various GST-WNK1 mutant proteins were used in kinase assays with MBP as substrate; MBP phosphorylation is expressed as activity relative to wildtype WNK1. (C) Endogenous WNK1 was immunoprecipitated from HEK293 cells treated with various stimuli and assayed for autophosphorylation. This figure was modified from Xu et al, 2000.

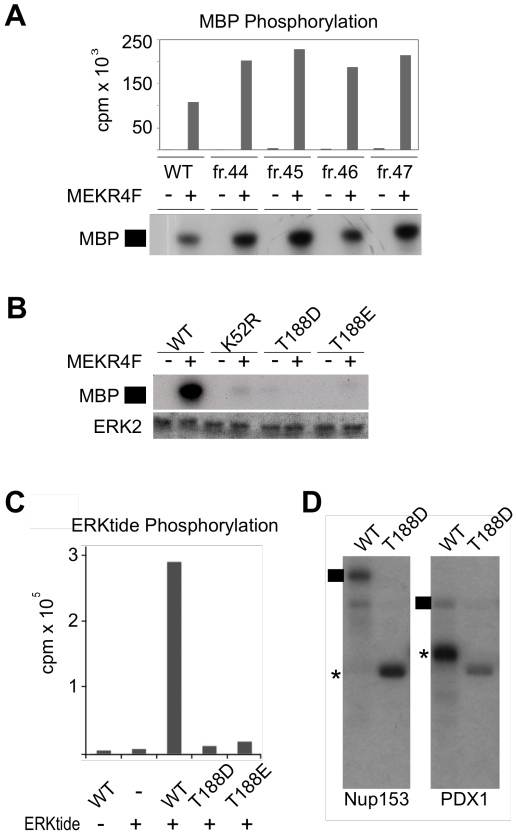

Figure 2 Recombinant protein kinase assays of ERK2. (A) Purified ERK2 fractions from a MonoQ anion exchange column were used in a kinase assay with MBP in the presence or absence of MEK1R4F, a constitutively active stimulator of ERK2 kinase activity. (B) ERK2 mutants were tested for kinase activity on MBP. (C) Activation loop mutants ERK2-T188D and ERK2-T188E display marginal kinase activity toward a small typified peptide substrate. (D) Comparison of ERK2 or T188D kinase activities following activation by MEK1R4F with known ERK2 substrates nucleoporin-153 (Nup153) and pancreatic and duodenal homeobox 1 (PDX1). Phosphorylated substrates are denoted by wedges and phosphorylated ERK2 and T188 by asterisks. Reprinted (and modified) with permission from McReynolds et al., 2016 (Copyright 2016 American Chemical Society).

Discussion:

Kinases are a diverse family of proteins that have evolved extensive functionality in numerous contexts, and kinase assays have been incredibly useful in studying several signaling proteins and have greatly contributed to our current understanding of cellular communication. Notably, the same basic assay was used in characterizing two disparate kinases despite major differences in structure and activity. WNK1 kinase contains an atypical catalytic pocket where the critical lysine has shifted to a unique position and is known to play roles in regulation of cation-chloride cotransporters through both kinase and scaffolding functions. The kinase activity of WNK1 is known to have a low turnover number, even on its best-characterized substrates, the protein kinases oxidative stress responsive 1 (OSR1) and SPS/STE20-related proline alanine-rich kinase (SPAK). In contrast, ERK2, along with the closely related kinase ERK1, displays robust kinase activity when activated *in vitro* and is known to phosphorylate over a hundred substrates in cells [13,14].

The most critical aspect of the assay is reaction assembly. In order to make accurate time point measurements, special care must be given to initializing kinase reactions. There are four basic requirements for a kinase assay to proceed: kinase, substrate, ATP, and a metal ion. For this protocol, which is primarily used for eukaryotic kinases, $MgCl_2$ is used as a source of magnesium. Both recombinant protein kinase and IP kinase preparations typically contain magnesium, rendering $MgCl_2$ insufficient as a reaction starter. Likewise, addition of unlabeled ("cold") ATP prior to the addition of labeled ("hot") ATP can initiate a reaction inappropriately early. We recommend preparing reactions using a concentrated kinase reaction buffer that facilitates simultaneous addition of $MgCl_2$, hot and cold ATP, and

substrate. In the case of measuring autophosphorylation activity, combined addition of magnesium and ATP can be used to initiate reactions. In all cases, samples should be prepared on ice and immediately transferred to a 30 °C water bath once reactions are assembled. It is also important to include a no-kinase control reaction to ascertain whether background signal will interfere with results. It may be necessary to troubleshoot various parameters of the assay, including time, temperature, and buffer content. The number of samples that can be processed at once is limited to the number of wells SDS-PAGE gels, which is high enough to effectively screen different conditions. As mentioned earlier in the protocol, we recommend beginning pilot experiments with a time course before moving on to testing other reaction conditions.

Although there is some distinction between IP kinase assays and recombinant protein kinase assays, the experimental paradigm common to both demonstrates how versatile this assay can be. Indeed, there are instances when features of both kinase assay types may be blended, as with studies on the mitogen-activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK) signaling pathway. In this pathway, ERK1/2 are activated by dual-phosphorylation by the upstream factor MAPK/ERK kinase 1 (MEK1). Previous studies have shown that while MEK1, as well as a constitutively active mutant termed MEK1R4F, is able to activate ERK1/2, it harbors very low activity towards MBP. Consequently, ERK1/2 purified from bacterial cells display limited kinase activity towards MBP unless treated with MEK1R4F, creating a robust platform for comparing the kinase activity of wildtype ERK1/2 to mutant constructs, as shown in Figure 2. Inclusion of immunoprecipitated components can

add even more nuance, highlighting the kinase assay as a highly adaptable method to probe the multifaceted nature of these important signaling molecules.

While some may find working with radioactive materials cumbersome, the quantitative tractability of the radiolabeled kinase assay is one of its major advantages. However, recent advancements in the fields of natural product chemistry, genomics, and mass spectrometry have created a demand for modified kinase assays with readouts more amenable to high throughput applications[15]. Because these assays take advantage of different labeling materials, compromises are made in terms of accuracy, but these can be overcome by using a radiolabeled assay to validate screen results. As our understanding of protein kinase signaling increases, it remains clear that techniques able to tease out the particulars of kinase regulatory functions will continue to aid in the development of tools and therapies.

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The authors have nothing to disclose.

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APPENDIX C
Short Preview for a Cancer Cell Article

Title:

A Kinase Divided

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Short Introduction:

In this issue of Cancer Cell, Herrero and colleagues identify an anti-tumorigenic small molecule that blocks ERK dimerization, but neither its catalytic activity nor its phosphorylation by MEK. These findings demonstrate that targeting protein dimerization could be a therapeutic avenue for inhibiting kinase signaling pathways associated with lower drug resistance.

Main Text:

The ERK/mitogen-activated protein kinase (MAPK) signaling pathway integrates a wide range of signals into major cellular programs such as proliferation, differentiation, or apoptosis. Canonical ERK signaling is initiated upon stimulation of the small GTPase Ras, which triggers a sequential three-kinase phosphorylation cascade through RAF, MEK, and ERK to enact focused and large-scale cellular changes. Half of all human malignancies display aberrations in the Ras-RAF-MEK-ERK pathway, revealing the

extent of its regulatory reach. To date, promethean effort has been expended identifying small molecule inhibitors of this pathway, because promising compounds are hampered by significant side effects and rapid development of drug resistance (Little et al., 2013).

Sometimes overlooked, kinases are more than phosphate-transferring enzymes. Many exhibit catalytic activity-independent protein-protein interactions that are important for context-appropriate signal regulation. In addition to phosphorylating substrates, kinases serve as binding partners for other signaling molecules. Indeed, the MAPK signaling scaffold protein KSR, a RAF homolog that lacks the catalytic lysine, tethers RAF, MEK, and ERK and can induce changes in RAF activity allosterically (Stewart et al., 1999, Rajakulendran et al., 2009). Allosteric regulation of kinases is not limited to scaffolds or pseudokinases, because RAF can regulate itself through dimerization (Lito et al., 2013). Regulated protein-protein interactions are critical for reducing entropic barriers to signaling. Although this makes them tempting for drug design, protein-protein interactions have long been considered challenging targets due, in part, to large molecular surfaces that must be disrupted by a small molecule (Whitty and Kumaravel, 2006). In this issue of Cancer Cell, Herrero et al. (2015)) succeeded in finding such a molecule and demonstrated that inhibition of ERK dimerization, but not catalytic activity, effectively re-routes tumorigenic signals toward programmed cell death (Figure 1).

Methods to distinguish different oligomeric forms of ERK are not readily adaptable to large-scale drug screening (Khokhlatchev et al., 1998, Casar et al., 2008). Thus, Herrero et al. (2015)) used native gel electrophoresis as a medium-throughput assay to discriminate ERK monomers from dimers in HEK293 cells. By screening a

library of 650 compounds pre-selected to have affinity for kinases, they identified DEL-22379 as a small molecule capable of inhibiting ERK dimerization without affecting EGF-stimulated ERK phosphorylation. The authors confirmed that DEL-22379 inhibited dimerization by gel filtration, analytical ultracentrifugation, co-immunoprecipitation, and proximity-ligation assays. The activity of the small molecule against a large kinase panel indicated little cross-reactivity. ERK kinase activity was unaffected by DEL-22379 in vitro, and monomeric ERK mutants were also active in vitro. Earlier studies reported that inhibition of cytoplasmic ERK signaling by blocking dimerization was due to impaired scaffolding, which was suggested to direct proliferative responses (Casar et al., 2008). It seems possible that cytoplasmic substrates not related to proliferative responses are still recognized by ERK monomers. Perhaps phosphorylation of these substrates also contributes to the shift toward growth suppression.

Because of the prevalence of dysregulated Ras-ERK signaling in human malignancies, the authors examined drug efficacy in the context of K- or N-Ras and BRAF mutations, in which high ERK activity drives cell proliferation and concomitant tumor progression (Herrero et al., 2015). Importantly, they demonstrate that DEL-22379 reduces tumor growth in diverse model systems more effectively than inhibitors of the catalytic activities of kinases in this cascade (Little et al., 2013). They conclude that, by blocking dimerization, DEL-22793 disrupts cytoplasmic signaling and promotes apoptosis over proliferation. Casar et al. (2008)) previously reported that ERK dimers are essential for activation of cytoplasmic, but not nuclear substrates, and that preventing ERK dimerization inhibited proliferation. In keeping with these earlier findings, Herrero

et al. (2015)) find that DEL-22379 increases phosphorylation of transcription factor substrates and, at the same time, phosphorylation of the ERK substrate protein kinase RSK, often in the cytoplasm, is decreased.

This study raises several pressing questions. Why is this inhibitor still effective under conditions that promote resistance to molecules targeting RAF, MEK, or ERK catalytic activities? A key mechanism underlying resistance to pathway inhibitors is the loss of ERK-mediated negative feedback exerted on multiple proteins upstream in the pathway and particularly on RAF itself (Lito et al., 2013). These feedback mechanisms include direct inhibitory phosphorylation, phospho-activation of negative regulators, and induction of inhibitory proteins. The absence of feedback creates inhibitor escape mechanisms, leading to strong ERK activation. Given the disturbance in dimeric ERK signaling caused by DEL-22379, one might anticipate that these feedback events, most of which occur in the cytoplasm, are also disabled. The current studies were not directed toward ERK feedback, but the answer is likely to provide more insight into the mechanisms of ERK pathway regulation. Perhaps greater understanding will come from determining how the compound binds ERK. Herrero et al. (2015)) present a model of the compound docked onto a groove partially overlapping with the ERK dimer interface (Khokhlatchev et al., 1998). This binding mode is clearly supported by mutagenesis of residues in the groove, yet DEL-22379 came from a library of molecules, most of which interact with protein kinase active sites. The allosteric potential of protein kinase cores in general, and ERK in particular, suggests that a molecule that partially overlaps the active site or other regulatory sites may influence protein-protein interactions (Goldsmith et al.,

2007, Taylor and Kornev, 2011). A high-resolution structure of DEL-22379 bound to ERK2 will clarify how it blocks ERK dimerization.

Whatever the mechanism, DEL-22379 will be a valuable research tool for revealing more about oligomerization dynamics in ERK signaling. Small molecules such as DEL-22379 may prove useful in targeting compartment-specific modes of kinase signaling, thus deepening our basic understanding of signaling and leading to exciting therapeutic avenues for blocking signaling pathways. Herrero et al. (2015)) now demonstrate the power of targeting ERK dimerization with a novel small molecule. The finding that DEL-22379 prevents tumor growth in mouse and patient-derived xenograft models provides evidence that targeting protein-protein interactions can be effective in vivo and foreshadows promise in developing similar compounds for clinical studies.

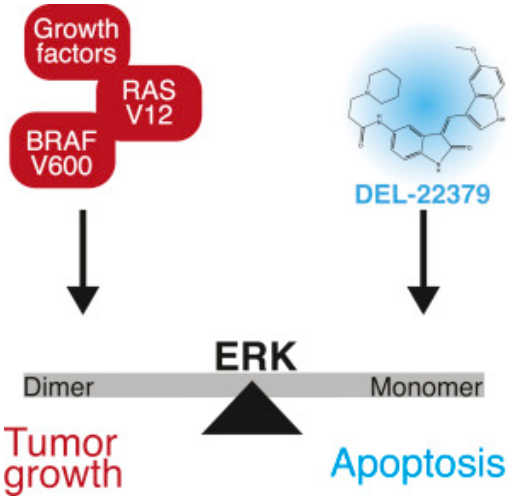

Figure 1 A Small Molecule Inhibitor of ERK Dimerization Can Tip the Balance of Its Downstream Signaling Output.

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