Specificity of molecular responses to ERK1/2 and MKK1/2 inhibitors in melanoma cells

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A thesis submitted to the Faculty
of the Graduate School in partial
fulfillment of the requirements
for the degree of Doctor of Philosophy

Department of Molecular, Cellular,
and Developmental Biology
2017

This thesis entitled:

Specificity of molecular responses to ERK1/2 and MKK1/2 inhibitors in melanoma cells

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the content and the form meet acceptable presentation standards of scholarly work in the
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Specificity of molecular responses to ERK1/2 and MKK1/2 inhibitors in melanoma cells

Thesis directed by Natalie G. Ahn

<u>**Abstract**</u>

Skin cancer is the most commonly diagnosed cancer in the U.S. and 75% of skin cancer related deaths are due to malignant melanoma, a cancer originating in melanin producing melanocytes. The RAF/MKK/ERK signaling cascade is constitutively activated in over 90% of melanomas and 52% of tumors contain BRAF V600E/K oncogenic driver mutations. Although small molecule inhibitors specifically targeting mutant BRAF V600E/K and the downstream kinases MKK1/2 have been successful in clinical settings, resistance invariably develops. In preclinical studies, inhibitors of ERK1/2 can overcome resistance to BRAF V600E/K and MKK1/2 inhibitors, making them promising alternative pathway inhibitors for the treatment of melanoma. However, the specificity of molecular responses to ERK1/2 inhibitors remains unknown. In this thesis, I use SILAC-based phosphoproteomics to quantify molecular responses to the clinically available MKK1/2 inhibitor, trametinib, and the ERK1/2 inhibitors, SCH772984, GDC0994 and Vertex-11e in WM239a human metastatic melanoma cells. I observed significant responses in approximately 5% of all phosphosites identified. Significantly regulated phosphosites showed a high degree of overlap between all inhibitors, suggesting that the pathway functions linearly with relatively little evidence for branchpoints that lead to bifurcation upstream of ERK1/2. I also observe phosphosites responsive to only one of four MKK1/2 or ERK1/2 inhibitors. For example, trametinib shows an ability to block activating phosphorylation sites on p38α MAPK, which are not shared by ERK1/2 inhibitors SCH772984 and GDC0994, the MKK1/2 inhibitor selumetinib, or the BRAF V600E/K inhibitor, vemurafenib. Trametinib

directly inhibits MKK6 in vitro, although with a IC_{50} 10-fold higher than its inhibition of p38α

MAPK in cells. This suggests the potential that the direct target of trametinib in cells is upstream

of MKK6. Further analyses of phosphoproteomics responses to two MKK1/2 inhibitors and two

ERK1/2 inhibitors identifies phosphorylation sites that can be classified as (i) known or novel

targets of BRAF-MKK-ERK signaling, (ii) potential branchpoints at MKK1/2 upstream of

ERK1/2, and (iii) off-targets of different inhibitors. My results show how information from

phosphoproteomics comparisons of multiple MKK1/2 and ERK1/2 inhibitors can be combined

to provide a deeper understanding of pathway specificity.

Acknowledgements

I would like to thank my advisor, Natalie Ahn for her mentoring and patience throughout my graduate school career. Her scientific passion and curiosity have been great motivation for me to pursue questions within my own project. She has also assembled an amazing group of lab members who have helped make this work possible. In particular, Dr. Scott Stuart has provided countless hours of training, scientific advice, troubleshooting, and friendship. A six pack of Milk Stout does not begin to express my appreciation, but it'll have to do. I would also like to thank Dr. Mary Katherine Tarrant for guidance on how to succeed as a graduate student and making our lab environment enjoyable. Jennifer Liddle has progressed through her Ph.D. alongside me and our conversations are always enlightening, although not always scientific.

Drs. Jeremy Balsbaugh and Thomas Lee of the JSCBB mass spectrometry facility taught me the fundamentals of proteomics, helped run my samples on the instruments, and tolerated my repeated questions. Drs. Will Old, Chris Ebmeier, and Zach Poss in the Old lab all helped maximize the quality of my phosphoproteomic data. Will explained statistical analysis methods which have been adopted by Andrew Kavran in our lab, and Chris juggled many different projects to optimize my protocol and run samples. The MCDB predoctoral class of 2010 including Lavan Khandan, Verity Johnson, Emily Pugach, Audrey Audetat, and Natalie Johnson made graduate school fun and I had a blast exploring Boulder and the rest of Colorado with you. I am grateful for my funding from MCDB, Natalie Ahn, and the NIH Creative Training in Molecular Biology Training Grant (5-T32 GM007135).

My thesis committee has provided vital feedback on this project and includes Will Old, Joaquin Espinosa, Dylan Taatjes, Xuedong Liu, and Tin Tin Su.

Pursuing and completing graduate school would not have been possible without the unconditional support of my family. My parents offered constant encouragement and enabled me to follow my own path. My brothers have given me perspective through their own graduate school experiences, and we never miss an opportunity to share a laugh at one another's expense. Finally, my fiancé Kate has been my best friend, my biggest ally, my adventure buddy, and my emotional support. I can't wait to see where this life will take us together.

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<u>**Chapter 1**</u>

Introduction

1.1 Cancer biology, mortality, and treatment

Cancer is a large group of related diseases characterized by uncontrolled proliferation of abnormal cells, which can include old and damaged cells that evade destruction as well as unnecessary growth of new cells. These abnormal cells can form solid masses in tissues called tumors and malignant tumors can break their normal boundaries and invade surrounding tissues. These cancerous cells can then spread to other tissues and organs, a process known as metastasis, which is responsible for the majority of cancer deaths. In 2016, approximately 1,685,210 new cases of cancer will be diagnosed in the U.S., and about 1 in every 4 deaths per year (595,690 Americans) will be due to cancer, making it the second leading cause of death in the U.S. (American Cancer Society, 2016). Worldwide, over 60% of new cases occur in Africa, Asia, and Central/South America, with 70% of cancer deaths occurring in these regions (Stewart, B. W., Wild, 2014). Consequently, cancer is both a massive financial burden as well as area of financial investment. Medical costs associated with cancer totaled $74.8 billion in the U.S. in 2013 (American Cancer Society, 2016). To combat this disease, the National Cancer Institute (NCI) received $5.1 billion in funding for 2016.

Cancer research in public institutions and private businesses (such as pharmaceutical companies) has led to new mechanism-based treatment strategies, which improve patient outcomes and build upon traditional approaches such as surgery, radiation, and cytotoxic chemotherapy. One of these mechanism-based strategies, immunotherapy, uses different approaches to activate the patient's immune system to recognize and attack the cancer. FDA-

approved immunotherapies are now first-line treatments for several cancer types and many more are being evaluated in ongoing clinical trials. Targeted therapies using cell-permeable small molecule inhibitors are a second mechanism-based treatment strategy developed over the past 20 years. These targeted therapeutics are a cornerstone of precision medicine which uses information about a patient's genetic and protein profile to diagnose and treat disease. Cancer is usually caused by genetic changes, often involving accelerated mutational rates. Over time, tumors accumulate a unique combination of mutations leading to disease progression. Some mutations known as driver mutations commonly promote cell survival and/or proliferation by affecting key regulators such as proto-oncogenes, tumor suppressor genes, and DNA repair genes. While some of these mutations are inherited, others are acquired somatic mutations called passenger mutations which in some cases enhance malignancy and in other cases have no effect on cells. Over the last decade, multi-disciplinary projects like The Cancer Genome Atlas (TCGA) have compiled genetic data from over 11,000 patients across 33 different cancer types (Tomczak, Czerwińska, & Wiznerowicz, 2015). These efforts have identified promising novel target genes in many different cancer types that have been used to develop effective targeted therapeutics.

1.2 Melanoma biology, disease progression, and genetic mutations

Skin cancer is the most commonly diagnosed cancer in the U.S. and begins in the epidermis. The epidermis consists of squamous keratinocytes and basal keratinocytes which make up 80% of tissue, melanocytes which produce skin pigments, Langerhans cells which provide immune defense, and neuronal Merkel cells which convey touch sensations. Squamous cell and basal cell carcinomas make up 99% of skin cell diagnoses and can almost always be

cured if treated early. In contrast, melanoma only accounts for 1% of skin cancer diagnoses but is responsible for 75% of skin cancer deaths (American Cancer Society, 2016). In 2012, 232,000 new cases of melanoma were diagnosed with 55,000 estimated deaths (Stewart, B. W., Wild, 2014). Melanoma involves malignant transformation of melanocytes to cancerous cells which typically occurs in the epidermis, but can also form in the eye (primarily uveal melanoma), mucosal tissue (head and neck, female genital tract, and anal/rectal), or unknown primary sites (only metastatic disease diagnosed) (Chang, Karnell, & Menck, 1998). Tumorigenesis is associated with familial history, multiple benign or atypical nevi (moles), previous melanoma, immunosuppression, sun sensitivity, and UV radiation exposure (Miller & Mihm, 2006). Accumulation of inherited genetic changes as well as molecular lesions arising from errors in cell division and DNA damage due to environmental exposures drive the progression of melanoma, from benign nevus to primary melanoma to metastatic melanoma (Figure 1.1). Before the implementation of gene sequencing to identify genetic changes in melanoma, it was common for oncologists to categorize melanoma into four distinct subtypes based on histology. These include nodular melanoma, superficial spreading melanoma, lentigo maligna, acral lentiginous melanoma, and demoplastic melanoma (Clark, From, Bernardino, & Mihm, 1969; McGovern et al., 1973; Scolyer, Long, & Thompson, 2011). However, the development of large-scale sequencing platforms and drugs that specifically target certain mutation profiles in patients has made genetic alterations a more effective way to categorize disease (Curtin et al., 2005). It is now common for a melanoma diagnosis to include both molecular markers as well as standardized pathological staging measuring thickness and other metrics of the primary tumor, metastatic nodes, and distant metastasis sites (Boland & Gershenwald, 2016). Almost all melanoma tumors diagnosed at early stages can be treated or cured by surgical resection, while

later stage cancer requires more aggressive therapy. Until recently, the chemotherapeutic drug, dacarbazine, and immune stimulating cytokine, interleukin 2 (IL2), were the only approved treatments for melanoma, however both had relatively little effect on overall patient survival (McArthur & Ribas, 2013).
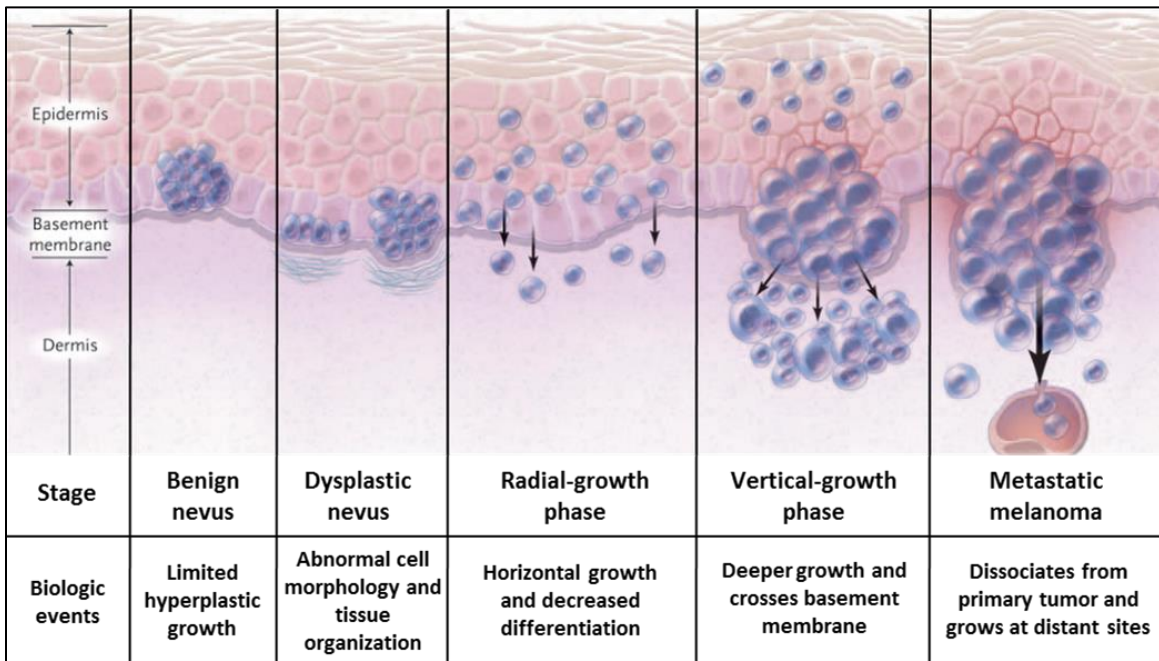
Figure 1.1: Pathological progression of melanoma. Biologic events are often accompanied by molecular lesions including BRAF mutation (benign nevus), CDKN2A and PTEN loss (dysplastic nevus), increased CD1 (radial-growth phase), and changes to cell adhesion in vertical growth phase and metastatic melanoma. Adapted from (Miller & Mihm, 2006).

Melanoma was among the first cancer genomes to be sequenced, along with leukemia (Ley et al., 2008; Mardis et al., 2009), breast cancer (Shah et al., 2009), and small-cell lung cancer (Pleasance, Stephens, et al., 2010), and researchers revealed the dramatic increase in mutation rate of cancerous cells compared to normal cells (Pleasance, Cheetham, et al., 2010). They catalogued all base substitutions, small insertions and deletions (indels), rearrangements, and copy number alterations in a metastatic melanoma cell line and subtracted the mutations found in a lymphoblastoid cell line derived from the same patient to generate a map of somatic

mutations across the genome. In total, 33,345 base substitutions were identified, and the majority of these (~24,000) were verified C>T mutations associated with UV light exposure (Pfeifer, You, & Besaratinia, 2005). Defective DNA repair pathways also contribute to the high mutation rate as seen in other cancers, although melanoma is unique in the high mutational load due to UV mutagenesis (Hodis et al., 2012). A major breakthrough in identifying the molecular alterations associated with malignant melanoma came in a 2002 study, which sequenced the coding sequence and intron-exon junctions of oncogenic candidate gene BRAF in a large human sample set covering a variety of cancers (H. Davies et al., 2002). They found the BRAF kinase mutated in 66% of malignant melanoma with a single amino acid substitution from valine to glutamic acid (V600E) making up the majority of mutations. Additional studies identified amino acid substitutions to arginine (V600R) or lysine (V600K) at lower frequencies (Klein et al., 2013). This BRAF V600 E/K mutation was sufficient to constitutively upregulate downstream signaling in mammalian cells, leading to cell proliferation and independence from upstream activation through the RAS proteins (H. Davies et al., 2002). The success of this study lead to increased interest in multi-center projects cataloging the genetic mutations in patients across many cancer types.

A recent interdisciplinary study performed by the TCGA characterized genetic changes across the entire genome in melanoma patients using whole exome sequencing, DNA copy-number profiling, mRNA sequencing, microRNA sequencing, DNA methylation profiling, protein expression profiling, and clinicopathological data on primary and/or metastatic melanomas in 331 patients (Akbani et al., 2015). The whole exome sequencing done on 318 paired tumor and germline normal genomic DNA samples identified BRAF (mutated in 52% of melanoma), NRAS (28% mutated), and NF1 (14% mutated) as the most commonly occurring

driver mutations in melanoma. Gain-of-function mutations at hot spot V600E/K and K601

residues make up 93% of the total mutations in BRAF and occur mutually exclusive to

oncogenic NRAS mutations. Mutations also occur in HRAS and KRAS, but at a much lower

frequency than NRAS. Loss of function mutations to the tumor suppressor GTPase-activating

protein (GAP), NF1, occur mutually exclusive to BRAF V600E/K and K601, but not NRAS

mutations. Additional oncogenes and tumor suppressors were mutated in a significant number of

the TCGA melanoma patients. These include TP53, ARID2, CDKN2A, PTEN, PPP6C, RAC1,

DDX3X, IDH1, MAP2K1, and RB1, many of which were also identified in a separate large-

scale comparison of somatic mutations in melanoma across multiple studies, in the COSMIC

database (Forbes et al., 2011) (Fig. 1.2). The three top driver mutations, BRAF V600E/K,

NRAS, and NF1, all result in activation of the canonical MAPK pathway, which is altered in

91% of TCGA melanoma samples.
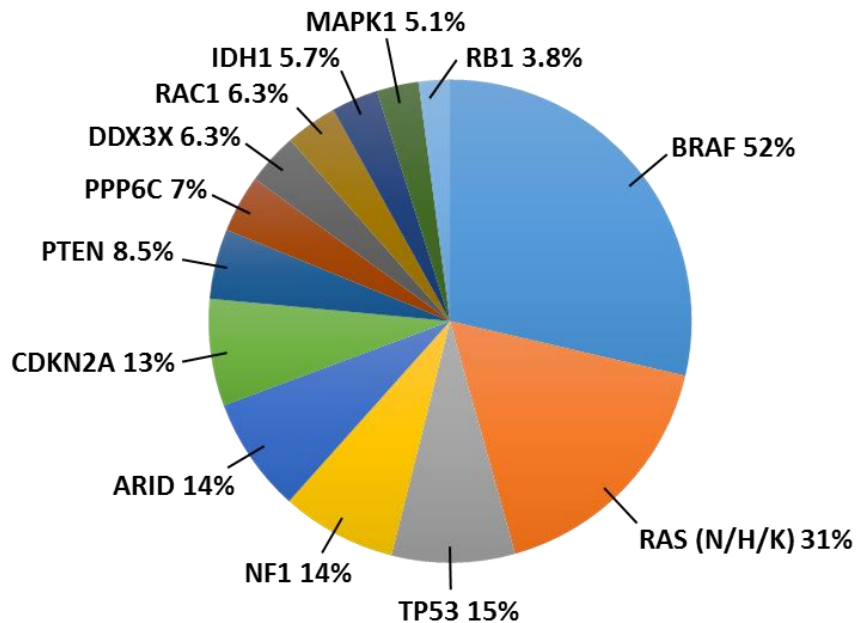
Figure 1.2: Somatic gene mutation frequency in melanoma. Top thirteen significantly
mutated genes passing statistical analysis in 331 patients. BRAF, RAS, NF1 are major
drivers of disease progression in melanoma. Figure adapted from (Akbani et al., 2015).

1.3 MAPK pathway activation, targeted inhibition, and drug resistance in melanoma

The mitogen-activated protein kinase (MAPK) pathway is a signaling cascade composed of several growth factor receptors, the small GTPase RAS, and downstream kinases, A,B,C-RAF, MKK1/2, and ERK1/2, which elicits a cellular response to extracellular stimuli such as hormones and growth factors through receptor tyrosine kinases (RTKs). The discovery of this pathway in the late 1980s and early 1990s followed a strategy of "working backwards", similar to characterization of the glycogen phosphorylase kinase signaling cascade by Krebs and Fischer. The strategy started by characterization of kinase activity towards ribosomal protein S6, later shown to represent p90 ribosomal S6 kinase (RSK), in response to cell stimulation with insulin, as well as EGF, PDGF, and phorbol ester (Ahn, 1993). The S6 kinase activity could be activated by a serine/threonine specific kinase which phosphorylated microtubule associated protein-2 (MAP2) or myelin basic protein (MBP). The kinase was later renamed mitogen-activated protein (MAP) kinase, as well as extracellular-signal related kinase (ERK). It integrates upstream signals from different cell stimuli and transmits this message by phosphorylating more than 150 downstream targets in both the nucleus and cytoplasm (Carlson et al., 2011; Yoon & Seger, 2006). Tracking the upstream control of ERK led to the discovery and characterization of the dual specificity MAP kinase kinases (MKK1/2), also known as MAP/ERK kinase (MEK1/2), which phosphorylate ERK1/2 at pThr and pTyr regulatory phosphorylation sites within its activation loop sequence, Thr-Glu-Tyr. MKKs are in turn phosphorylated by upstream serine/threonine kinases named A-RAF, BRAF and CRAF (aka RAF1). The canonical MAPK pathway thus describes a linear cascade starting with the phosphorylation and activation of MKK by A,B or C-RAF, and followed by the phosphorylation and activation of ERK1/2 by MKK1/2 (Katz, Amit, & Yarden, 2007). The pathway is constitutively activated in nearly all cancer types,

with oncogenic BRAF V600E/K mutations prevalent in melanoma, colorectal, thyroid, and

ovarian cancers (Burotto, Chiou, Lee, & Kohn, 2014). These cells often depend on this sustained

MAPK activation for survival and upregulation of this pathway leads to cell survival,

proliferation, and disease progression (Fig. 1.3A,B).
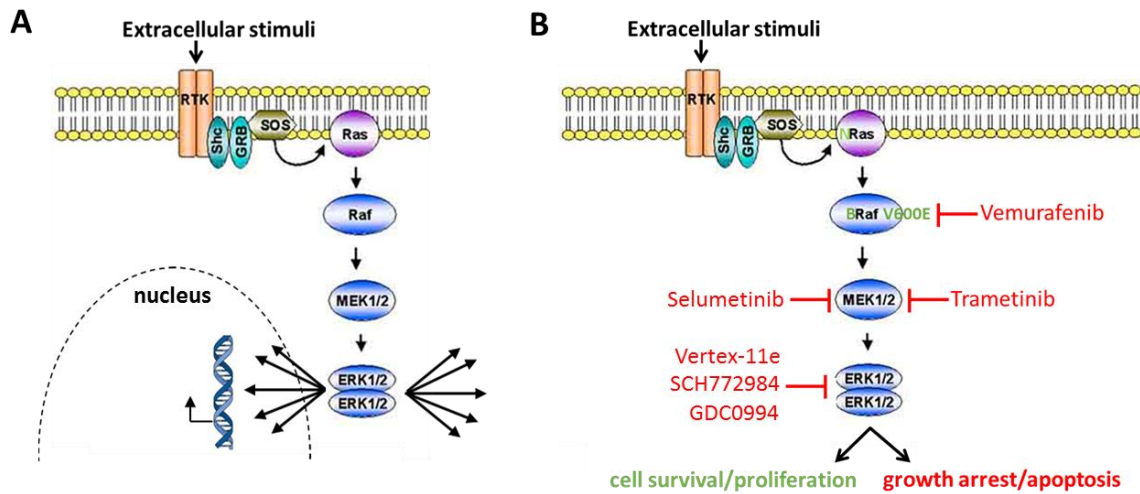
Figure 1.3: MAPK-ERK signaling cascade, oncogenic activation, and inhibition. A, extracellular signals such as growth factors and hormones elicit a downstream response by signaling through the RAF-MKK-ERK kinase cascade. B, 93% of melanoma has activation of the MAPK pathway, primarily through mutations in NRAS (28%) and BRAF V600E/K (52%) leading to cell survival and proliferation. Targeted therapeutics that inhibit the BRAF V600E/K, MKK1/2, and ERK1/2 kinases have been developed to inhibit this pathway in melanoma. Inhibitors characterized in this thesis are listed.

If diagnosed early, primary melanomas can be cured by clinical resection in 80% of

cases, but metastatic melanomas are often resistant to traditional chemotherapy and radiotherapy

due to the inherently high level of apoptosis resistance in the progenitor melanocyte cells (Gray-

Schopfer, Wellbrock, & Marais, 2007). Immunotherapy has emerged as a promising treatment

strategy for metastatic melanoma with FDA approval of four drugs in the last five years.

Monoclonal antibodies against cytotoxic T-lymphocyte antigen 4 (CTLA-4) (ipilimumab 2011)

and programmed cell death protein 1 (PD-1, nivolumab/pembrolizumab 2014) work by blocking

CTLA-4 and PD-1 checkpoint inhibitors, thus re-activating the ability of the immune system to

attack cancer cells. A fourth immunotherapy currently used to treat melanoma is the oncolytic viral drug, talimogene laherparepvec (TVEC) which when injected into tumors, causes tumor cell lysis and release of antigens, eliciting an immune response (Franklin, Livingstone, Roesch, Schilling, & Schadendorf, 2016). These immunotherapy drugs are currently used in combination as first-line therapy in metastatic melanoma patients lacking mutant BRAF V600E/K (Coit et al., 2016). While the patient response is generally durable, long-term survival occurs in only ~50% of patients.

Like immunotherapy, the development of drugs that specifically target the MAPK pathway has led to a momentous shift in the treatment of melanoma, by successfully inducing cell death in melanomas containing the BRAF V600E/K mutation. Following the discovery of driver mutations in BRAF V600E/K (H. Davies et al., 2002), several ATP-competitive inhibitors specific for oncogenic BRAF mutated at V600E/K were developed and showed promise in both *in vitro* and animal models (Koo et al., 2002; Weinstein & Joe, 2008), followed by clinical trials. These inhibitors showed dramatic results in patients because they show a significant preference for tumor cells containing the BRAF V600E/K mutation but not cells containing wild type BRAF, allowing effective monotherapy without the toxic effects of targeting BRAF in all tissues. Thus, the FDA has now approved two inhibitors of mutant BRAF V600E/K, vemurafenib (Chapman et al., 2011) in August 2011 and dabrafenib (Hauschild et al., 2012) in May 2013. These are approved for use as single-agent therapies, with several improved "second generation" BRAF V600E/K inhibitors currently in clinical trials (Le, Blomain, Rodeck, & Aplin, 2013; Uehling & Harris, 2015). Other inhibitors target MKK1/2, and Trametinib (aka JTP-74057, GSK1120212) (Gilmartin et al., 2011; Yamaguchi, Kakefuda, Tajima, Sowa, & Sakai, 2011), an allosteric non-ATP competitive inhibitor of MKK1/2, was also approved by the

FDA for monotherapy use in melanomas with mutant BRAF V600E/K, in May 2013. MKK1/2 inhibitor Cobimetinib (Signorelli & Shah Gandhi, 2016) was approved for use in combination with vemurafenib in November 2015.

During dose escalation studies in phase 1 clinical trials for vemurafenib, it was determined that greater than 80% inhibition of phosphorylated ERK1/2 (pERK) was necessary to achieve significant tumor regressions in patients (Bollag et al., 2010). Because this level of pERK inhibition can be difficult to reach with monotherapy targeting the MAPK pathway, clinical trials combining BRAF V600E/K and MKK1/2 inhibitors were conducted with improved patient response compared to BRAF V600E/K inhibitor alone (Flaherty, Infante, et al., 2012; Hartsough, Shao, & Aplin, 2014). The median progression-free survival in the combination group was 9.4 months and 5.8 months in the monotherapy group, and the rate of complete or partial response with combination therapy was 76% with combination and 54% with monotherapy. This lead to FDA approval of combination therapies that target both mutant BRAF V600E/K and MKK1/2, including dabrafenib + trametinib (GlaxoSmithKline, January 2014) as well as vemurafenib + cobimetinib (Genentech, November 2015) (Signorelli & Shah Gandhi, 2016; Wu, Nielsen, & Clausen, 2015). Combination therapy using dabrafenib+trametinib or vemurafenib+cobimetinib is currently the preferred first-line therapy in patients with oncogenic mutant BRAF V600E/K (Coit et al., 2016) and have demonstrated very promising results in stage III clinical trials. For the comparison of vemurafenib+cobimetinib and vemurafenib alone, respectively 70% and 50% of patients responded to treatment, with 16% and 11% of patients showing a complete response. The median progression-free survival was respectively 12.2 months and 7.2 months, and the median overall survival was respectively 22.3 months and 17.4

months (Ascierto et al., 2016). Similar responses in clinical trials were seen with the dabrafenib+trametinib combination (Smalley & Sondak, 2015).

Although promising, the tumor regression and progression-free survival of patients typically lasted only one year before resistance to these inhibitors invariably developed, even with combination therapy (Flaherty, Infante, et al., 2012). In both patients and pre-clinical studies of resistance to mono or combination therapy, greater than 70% of cases showed reactivation of the MAPK signaling cascade through a variety of mechanisms (Fedorenko, Gibney, Sondak, & Smalley, 2015). These include genetic causes such as BRAF amplification, MITF amplification, MKK mutations, NRAS mutations, loss of NF1, and PTEN loss leading to increased PI3K pathway activity, as well as non-genetic causes such as BRAF splice-site mutants, activation of the COT kinase, and activation of EGFR (Nazarian et al., 2010; Poulikakos et al., 2011; Shi et al., 2014; Van Allen et al., 2014; Villanueva et al., 2010; Wagle et al., 2014). In addition to the clinical success of MAPK inhibitors, great progress in understanding the underlying cell mechanisms involved in cell death, combination therapy, and resistance has been made in preclinical studies using MAPK inhibitors that failed to obtain FDA approval (Carvajal et al., 2014; Kirkwood et al., 2012; Robert et al., 2013). The MKK1/2 inhibitor selumetinib (*aka* AZD6244) (Yeh et al., 2007) is one such inhibitor used by many labs, including ours, to model MAPK inhibition in preclinical cancer models (B. R. Davies et al., 2007; Ku et al., 2015; Rebecca et al., 2014).

It is clear that targeting the MAPK pathway using combinations of BRAF V600E/K and MKK1/2 inhibitors has failed to overcome resistance mechanisms leading to the re-activation of ERK1/2. For this reason, ERK1/2-specific inhibitors are now being developed, which in preclinical settings show promising ability to overcome acquired resistance (Morris et al., 2013).

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Additionally, ERK1/2 inhibitors may build on the success seen in targeting multiple points upstream in the MAPK pathway and disrupt negative feedback loops that potentially lead to MAPK reactivation (Samatar & Poulikakos, 2014). Clinical trials using ERK1/2 inhibitors (Uehling & Harris, 2015) can be found on the clinicaltrials.gov database, and there is encouraging evidence that ERK1/2 inhibition can be used as a second-line clinical therapy following acquired resistance to BRAF V600E/K inhibitors (Krepler et al., 2016). Several ERK1/2 inhibitors with different conformational selectivity are being investigated by our lab (Rudolph, Xiao, Pardi, & Ahn, 2015) including SCH772984 (Morris et al., 2013), Vertex-11e (Aronov et al., 2009), and GDC0994 (Robarge et al., 2014) (Fig. 1.3,1.4). The half-life of each drug is listed in Figure 1.4, and the half-life for ERK1 (68 h) and ERK2 (53 h) was determined by proteomic analysis (Schwanhäusser et al., 2011). Prospective preclinical resistance modeling using chronic dosing of SCH772984 in KRAS mutant HCT-116 cells has shown ERK mutations leading to resistance (Jha et al., 2016), but it remains to be seen if resistance develops in BRAF V600E/K mutant cells or patients.
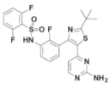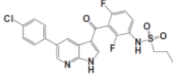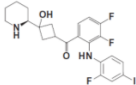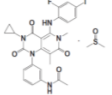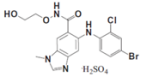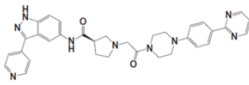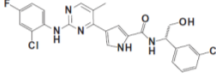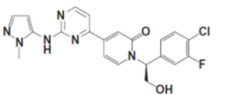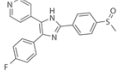
Drug	kinase target	FDA approval	Structure	IC$_{50}$	Half-life
Dabrafenib (tafinlar)	BRAF^{V600E}	approved as mono and combo therapy in V600 mutant melanoma		0.8nM	8 hours
Vemurafenib (Plx4032)	BRAF^{V600E}	approved as mono and combo therapy in V600 mutant melanoma		31nM	2.1 days
Cobimetinib	MKK1/2	approved as combo therapy in V600 mutant melanoma		4.2nM	2.2 days
Trametinib (mekinist)	MKK1/2	approved as mono and combo therapy in V600 mutant melanoma		1.8nM	4 days
Selumetinib (Azd6244)	MKK1/2	currently in clinical trials		14nM	5-8 hours
Sch772984	ERK1/2	modified form in clinical trials		4nM	
Vertex-11e	ERK1/2	preclinical testing only		<2nM	
Gdc-0994	ERK1/2	currently in clinical trials		1.1nM	1 day
SB203580	p38	currently in clinical trials		40nM	1 day

Figure 1.4: Representative kinase inhibitors targeting mutant BRAF V600E/K, MKK1/2, and ERK1/2. Several inhibitors are approved for use as monotherapy although combination therapies are currently front line treatment in metastatic melanomas with BRAF V600E/K mutations. IC$_{50}$ values are specific for the targeted kinase.

1.4 Other MAPK pathways and osmotic stress activation of p38 MAPK

In addition to the RAF-MKK-ERK pathway, there are three additional MAPK signaling cascades which also have a three-tiered kinase structure with sequential kinase activation by phosphorylation. In each case, the terminal MAP kinases (MAPKs) transmit the pathway signal to many downstream substrates. These MAPKs are evolutionarily conserved and preferentially phosphorylate serine and threonine residues followed immediately by proline, with specificity also determined by substrate binding to a separate docking domain binding site in the MAP kinase (Tanoue & Nishida, 2003). Activation of the MAPKs by dual phosphorylation at Thr and

Tyr residues within the activation loop is catalyzed by specific MAP kinase kinases (MKKs).

MKKs are in turn phosphorylated by several MAPK kinase kinases (MKKKs) which are

activated in response to many stimuli (Johnson & Lapadat, 2002) (Fig. 1.5). In general, the

ERK1/2 pathway is activated by cell stimuli such as cytokines and growth factors, and controls

cell proliferation, differentiation, survival, and migration. The p38 MAP kinase and c-Jun

amino-terminal kinase (JNK) pathways are activated by environmental stress and mediate stress

responses by regulating transcription. The ERK5 pathway responds to growth factors as well as

cellular stress and is the least well characterized (Drew, Burow, & Beckman, 2012).
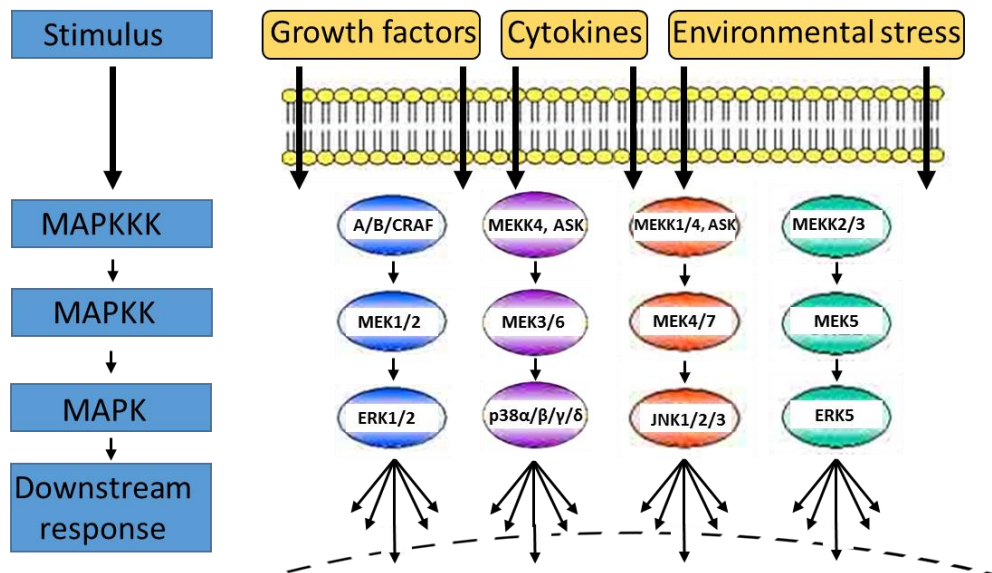
Figure 1.5: ERK, p38, JNK, and ERK5 MAPK signaling cascades. The four mitogen-activated protein kinase signaling cascades regulate many physiological processes through phosphorylation of substrates downstream of MAPKs and respond to a variety of extracellular stimuli. Figure adapted from (Roberts & Der, 2007)

The serine-threonine kinase, p38α MAPK, is part of the MKKK-MKK3/6-p38α/β/γ/δ

MAPK signaling pathway which is distinct from RAF-MKK-ERK and MKKK-MKK-JNK

pathways. Stimulation of this pathway by cytokines and environmental stress has been shown to

result in cell proliferation and cytokine production as well as apoptosis and cell death (Zarubin,

T. & Han, 2005). Although the p38 MAPK pathway is similar in its organization to the ERK

pathway, targeted kinase inhibitors directed at mutant BRAF V600E/K or MKK1/2 are very

specific for ERK1/2 signaling (Uitdehaag et al., 2014; Yamaguchi et al., 2011). Up to now, they

have not been previously shown to target components of the p38 pathway for inhibition.

However, a phosphoproteomic screen which I conducted revealed that the MKK1/2 inhibitor,

trametinib, specifically inhibits p38α MAPK phosphorylation. This intriguing result led to

biochemical examination of the p38 pathway to validate this result, which I describe in Chapter

2.

The first member of the p38 pathway was identified by four independent groups in 1994

(Freshney et al., 1994; J Han, Lee, Bibbs, & Ulevitch, 1994; Lee et al., n.d.; Rouse et al., 1994).

This isoform became known as p38α (aka MAPK14 or SAPK2a) and is the most well-

characterized. Identification of p38β (MAPK11), p38γ (MAPK12), and p38δ (MAPK13)

followed shortly thereafter. These four isoforms are 60% identical in the amino acid sequence

and 40-45% identical to other MAP-kinases identified (Jiang et al., 1997). Expression of p38α is

high across all cell types tested while the other three isoform levels vary based on cell type

(Cuadrado & Nebreda, 2010) and only p38α and p38β isoforms respond to the widely used small

molecule inhibitor, SB203580 (Goedert, Cuenda, Craxton, Jakes, & Cohen, 1997; Lali, Hunt,

Turner, & Foxwell, 2000). All p38 isoforms have a Thr-Gly-Tyr (TGY) phosphorylation motif in

their activation loop. Dual phosphorylation of the Thr and Tyr residues in this motif is directly

catalyzed by the kinases, MKK3/6, and results in activation (Cohen, 1997). MKK3/6 are

activated by a group of MKKKs which include upstream Rho GTPase binding proteins,

including MLKs, ASK1, TAK1, MEKK3/4, and Rac1. These are activated by environmental

stresses and inflammatory cytokines but not typically by growth factors (Cuenda & Rousseau,

2007). The p38 MAPK pathway, and p38α in particular, has been shown in some systems to have a role as a tumor suppressor and in other systems to have an oncogenic role, in a variety of cancer models including cultured cells, animals, and patients. Tumor suppressor behavior involves negative regulation of cell cycle progression through checkpoint controls and apoptotic regulation, while oncogenic behavior involves promotion of cell invasion, inflammation, and angiogenesis. Thus, p38 can act as a tumor suppressor or an oncogene, depending on the context of the experiment and variables such as cell type, mutational profile, and method of pathway stimulation or inhibition (Cuenda & Rousseau, 2007; Wagner & Nebreda, 2009). Several clinical trials using p38 inhibitors have been initiated but have been unable to pass phase I due to toxicity, which may be ascribed to p38 inhibition or to off-target effects.

1.5 Phosphoproteomic analysis of molecular responses to MAPK pathway inhibitors

Reversible phosphorylation of substrates are critical post translational modifications (PTMs) that regulate processes downstream of the MAPK pathway. Proteomics has been used to characterize targets of the ERK pathway in many ways, including two dimensional gel electrophoresis (Kosako et al., 2009; Lewis et al., 2000), ERK analog sensitive mutants utilizing a radiolabeled ATP analog (Carlson et al., 2011; Eblen et al., 2003), and -79 Da negative precursor ion MS scanning which identifies phosphopeptides by their loss of PO_3^- (Old et al., 2009). However, the overlap of substrate identification between different studies has been poor, likely due to low sampling or variation between cell types (Courcelles et al., 2013). Bottom-up phosphoproteomics using a complex mixture of digested proteins extracted from cells coupled with stable isotope labeling of amino acids in cell culture (SILAC) (Mann, 2006) has emerged as

a powerful technique to quantify changes in phosphorylation on a deeper, more global scale (Galan et al., 2014; Pan, Olsen, Daub, & Mann, 2009).

Our lab previously used a SILAC-based approach to identify shared and unique targets of the BRAF V600E/K inhibitor, vemurafenib, and the MKK1/2 inhibitor, selumetinib (Stuart et al., 2015). A metastatic melanoma cell line was triply labeled with media supplemented with arginine and lysine containing different combinations of isotopic labeling with ^{13}C and ^{15}N (Fig. 1.6). During protein digestion, trypsin preferentially cleaves on the carboxyl side of arginine or lysine, and when the resulting peptides are analyzed on a mass spectrometer, the masses of identical peptides will shift depending on the light/medium/heavy isotopic labeling. This allows for quantification of changes in peptide abundance in a mixtures of lysates from cells under three different treatments. Titanium oxide was used to enrich phosphopeptides from the admixtures, which enabled a direct comparison of changes in protein phosphorylation in response to the BRAF V600E/K and MKK1/2 inhibitors. Notably, very few targets were specific to only one drug, thus the high degree of overlap was consistent with a linear model describing signaling from BRAF to MKK1/2. This result also indicated that BRAF V600E/K and MKK1/2 inhibitors when used in combination lead to an additive effect on phosphoproteins, instead of targets unique to one but not the other drug. This suggested that the clinical success of the drug combination is likely due to additive and more complete inhibition of pERK compared to either drug alone, and not due to synergy between targets uniquely regulated by each drug.
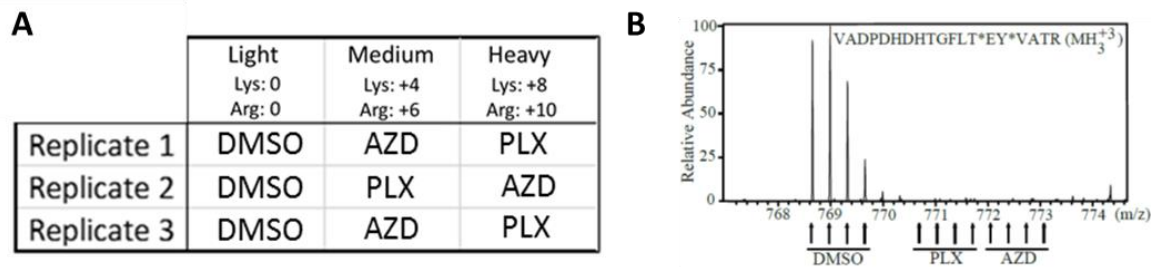
Figure 1.6: SILAC labeling allows quantification of changes in phosphopeptide levels in response to inhibitor. A, label-swapping design of SILAC experiment with triplicate biological replicates, described by Stuart et al., 2015. The labeling code +4/+6/+8/+10 indicates Lys and Arg mass increases in Daltons due to isotopic labeling. Metabolic labeling of cells yields proteins incorporating these labeled amino acids, which generate peptides with increased masses depending on their Lys and Arg composition. All treatments used the metastatic melanoma cell line, WM239a, adding 10 µM drug for 2 h. AZD=AZD6244 (selumetinib), PLX=PLX4032 (vemurafenib). B, mass spectrum of labeled peptide from ERK2, with dual phosphorylation at the TEY regulatory phosphorylation sites, show a dramatic decrease in response to kinase inhibitor drugs. Adapted from (Stuart et al., 2015).

1.6 Thesis overview

There is a high degree of specificity in the MAPK pathway (Hindley & Kolch, 2002; Matallanas et al., 2011; Roskoski, 2012; Yoon & Seger, 2006), and our lab has used phosphoproteomics to demonstrate a remarkable degree of overlap in molecular changes induced by inhibitors of BRAF V600E/K and MKK1/2 in melanoma cells (Stuart et al., 2015). Using subsaturating concentrations, it was found that inhibitors suppress phosphorylation events in an additive manner, suggesting that the major effects of combination treatment are due to additive effects on signaling responses between inhibitors. The question that is unsolved is: how do molecular responses to ERK1/2 inhibitors compare against those of BRAF V600E/K and MKK1/2 inhibitors? Although a recent study of KRAS mutant colorectal cancer cells reported high overlap between responses to MKK1/2 or ERK1/2 inhibitors (Gnad et al., 2016), the degree of overlap in BRAF V600E/K mutant melanoma cells remains unknown. In Chapter 2, I compare the ERK1/2 inhibitor, SCH772984, which was shown to effectively overcome acquired

resistance to BRAF V600E/K and MKK1/2 inhibitors (Morris et al., 2013), to the FDA-approved

MKK1/2 inhibitor, trametinib, in mutant BRAF V600D melanoma cells. Characterizing these

inhibitory effects will directly inform the results of ongoing clinical trials using these inhibitors.

Afterwards, I compare the phosphoproteomics responses to two MKK1/2 inhibitors, trametinib

and selumetinib, and demonstrate a novel off-target of trametinib. Using biochemical assays, I

confirm that trametinib inhibition of MKK6 at high doses leads to dephosphorylation of the

downstream effector, p38α MAPK, and speculate that this may help explain the higher drug

efficacy that has been observed with trametinib. Finally, I perform a comparative

phosphoproteomic screen of three separate ERK1/2 inhibitors (SCH772984, GDC0994, and

Vertex-11e) which show different binding properties to ERK2, and compare their specificity

with respect to known ERK1/2 substrates, regulatory phosphosites, and molecular pathway

enrichment. In Chapter 3, I summarize the significance of this study as well as future directions

for a more complete understanding of MAPK inhibition using phosphoproteomics.

Chapter 2

Selective phosphoproteome responses to MKK1/2 and ERK1/2 inhibitors in human melanoma cells

2.1 Abstract

The RAF/MKK/ERK signaling cascade is constitutively activated by oncogenic BRAF V600E/K in melanomas and other cancer types. Although small molecules which inhibit BRAF V600E/K and MKK1/2 have been successful in clinical settings, resistance invariably develops. In preclinical studies, high affinity inhibitors of ERK1/2 block the viability of melanoma cells which are otherwise resistant to BRAF V600E/K and MKK1/2 inhibitors. Thus, ERK1/2 inhibitors are promising as alternative drugs for pathway inhibition. But still unknown is how molecular responses compare between these new ERK1/2 inhibitors and the targeted therapeutics in clinical use. Previously, our lab has shown near complete overlap in the phosphoproteomic changes induced by BRAF V600E/K and MKK1/2 inhibitors. Here, I employ quantitative SILAC-based phosphoproteomics to measure the degree of overlap in molecular responses to the MKK1/2 inhibitor, trametinib, and the ERK1/2 inhibitor, SCH772984, in human metastatic melanoma cells. Biological triplicate experiments reproducibly quantified 8,577 phosphosites, 400 of which were altered significantly in response to either drug. Only 18 phosphosites decreased in response to SCH772984 or trametinib alone. Nevertheless, trametinib shows an ability to block activating phosphorylation sites on p38α MAPK, which is not shared by the ERK1/2 inhibitor, SCH772984, the MKK1/2 inhibitor, selumetinib, or the BRAF V600E/K inhibitor, vemurafenib. The inhibition can be traced to MKK6, suggesting that MKK6-p38α signaling is a novel off-target for trametinib. This occurs at concentrations above those achieved in clinical studies but within the range commonly used in preclinical studies. Additionally,

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comparison of phosphoproteome changes show a high degree of overlap in responses to the

ERK1/2 inhibitors, SCH772984 and GDC0994. Together with previous studies comparing

BRAF V600E/K and MKK1/2 inhibitors (Stuart et al. 2015), my findings support linearity in

signal transduction through the MAPK pathway, with little evidence for bifurcation in signaling

from BRAF V600E/K or MKK1/2 upstream of ERK1/2, and few differences in molecular

responses to inhibitors of MKK1/2 and ERK1/2.

2.2 Significance of study

I conducted comparative phosphoproteomics of the ERK1/2 inhibitor, SCH772984, and

the MKK1/2 inhibitor, trametinib, in melanoma cells and found almost complete overlap among

the phosphosites that significantly changed in response to drug, indicating pathway linearity

extends from BRAF V600E/K and MKK1/2 to ERK1/2. Among the small number of unique

targets inhibited by trametinib, the canonical TGY activation motif of p38α is significantly

inhibited by trametinib but not other BRAF V600E/K, MKK1/2, or ERK1/2 inhibitors tested.

MKK6 is identified as the direct target of trametinib, leading to decreased p38α activation.

Inhibition of p38α MAPK activation is shown to cooperate with MKK1/2 inhibitors that do not

display an off-target effect on this kinase and thus inhibition of p38α MAPK may augment

effects in preclinical studies using trametinib at high concentrations.

2.3 Introduction

The MAPK signaling cascade (RAF-MKK-ERK) is constitutively activated in many

cancer types, where sustained activity drives malignancy of melanoma, colorectal, thyroid, and

ovarian cancers (Burotto et al., 2014). Upregulation of this pathway leads to cell survival,

proliferation, and disease progression in malignant melanomas, where as many as 50% of cases display activating mutations in BRAF V600E/K, and 20% have mutations in NRAS (Flaherty, Hodi, & Fisher, 2012). Therapeutics that specifically target key protein kinases in this pathway have been successful in clinical as well as preclinical settings. To date, two MKK1/2 inhibitors (trametinib and cobimetinib) and two BRAF V600E/K inhibitors (vemurafenib and dabrafenib) (Signorelli & Shah Gandhi, 2016; Wu et al., 2015) have received FDA approval, as single agent or combination drug therapies. These inhibitors elicit dramatic responses in patients, and combination treatments using BRAF V600E/K and MKK1/2 inhibitors are now frontline therapies for treating metastatic melanomas harboring oncogenic BRAF V600E/K mutations. However, resistance regularly develops through mechanisms that activate MAPK signaling, even in the presence of drug (Van Allen et al., 2014). Development of ERK1/2 inhibitors has emerged as a promising strategy to combat this resistance and several are in early stage clinical trials (Morris et al., 2013). Addition of ERK1/2 inhibitors to the treatment strategy may provide an effective way to extend the average progression-free survival time for patients. Therefore, understanding the molecular responses to ERK1/2 inhibitors and comparing them to clinically used drugs would be important for maximizing their effectiveness.

Previous studies comparing molecular responses to inhibitors of BRAF V600E/K and MKK1/2 by phosphoproteomics overlapped strongly with only a small number of differences (Stuart et al., 2015). This suggests that MAPK pathway signaling at the level of BRAF V600E/K and MKK1/2 works in a predominantly linear manner, with little evidence for targets bifurcating upstream of MKK1/2. Consistent with this finding, mixtures of inhibitors at subsaturating concentrations induced responses that were largely additive (Stuart et al., 2015). This suggested that combinations of BRAF V600E/K and MKK1/2 inhibitors are more effective due to their

additive effects on ERK1/2 inhibition, and that incomplete ERK1/2 inhibition at maximally tolerated doses may limit the efficacy of single drug therapies.

However, an unanswered question is the degree to which promising ERK1/2 inhibitors target the same responses as clinically used MKK1/2 inhibitors. Here I compare phosphorylation responses to ERK1/2 inhibitors SCH772984 (SCH) and GDC0994 (GDC) against the MKK1/2 inhibitor, trametinib (TRA) in human metastatic melanoma cells. The results show strong agreement between phosphorylation responses to SCH772984, GDC0994 and trametinib, revealing pathway linearity at the level of MKK1/2 and ERK1/2. However, trametinib selectively inhibits activating phosphorylation sites in p38α MAPK, Thr180 and Tyr182. This is due to the ability of trametinib to inhibit MKK6, within a concentration range commonly used in preclinical studies (Ki = 1 µM). The MKK1/2 inhibitor, selumetinib, has no effect towards MKK6 or p38α MAPK, therefore, inhibition by trametinib can be attributed to an off-target effect unique to this compound. All four inhibitors of MKK1/2 and ERK1/2 alter phosphorylation of known MAPK pathway targets, such as S642 on RAF1 (CRAF) which is a negative feedback regulator of MKK activity (Dougherty et al., 2005) and T526 on transcription factor and ERF which controls activity (Sgouras et al., 1995), as well as novel targets. One example is S22 on NCBP1, a known regulatory site targeted by RPS6KB1 downstream of mTOR (Wilson, Wu, & Cerione, 2000), a finding which adds support to the crosstalk model between RAF-MKK-ERK and PI3K signaling networks (Mendoza, Er, & Blenis, 2011). None of the phosphorylation sites that are uniquely altered by any ERK1/2 or MKK1/2 inhibitor are shared with another inhibitor of the same kinase. Taken together, my findings show that MAPK signaling is predominantly linear, with few if any points of bifurcation upstream of ERK1/2. This

establishes a basis for identifying off-target effects induced by future drug candidates developed towards kinases in this pathway.

2.4. Materials and methods

Cell Culture

The metastatic melanoma cell line WM239a was a gift of Dr. Meenhard Herlyn, Wistar Institute, Philadelphia PA. Cells were SILAC-labeled using SILAC RPMI media (Thermo Fisher Scientific 89984) supplemented with heavy, medium, or light isotopically-labeled arginine (40 µg/mL) and lysine (200 µg/mL) (Cambridge Isotope Laboratories), 10% (v/v) dialyzed FBS (Life Technologies 88440), penicillin (100 µg/mL, Gibco), and streptomycin (100 µg/mL, Gibco). Non-SILAC experiments used cells cultured in Gibco RPMI 1640 media (2400-089) and 10% dialyzed FBS, with or without penicillin-streptomycin. Cells were incubated in a humidified chamber maintained at 37°C with 5% CO_2.

SILAC-labelled phosphopeptide sample preparation

WM239a cells were grown in heavy (H), medium (M) or light (L) SILAC RPMI. Cells in each label were grown in 6 x 15 cm dishes, then then trypsinized, combined, counted, and plated into three dishes at 100% confluence (~35 x 10^6 cells/dish). For each experiment, one dish with cells labeled H, M or L was treated with drug (10 µM) or DMSO carrier for 2 h. Cells in each dish were washed quickly with PBS, then lysed by adding 0.7 mL of 4% (w/v) SDS, 100 mM dithiothreitol, 100 mM Tris pH 7.6 (SDT buffer), and harvested by scraping.

Cell lysates were then sonicated for 30 s with a microtip sonicator and H, M and L samples were combined from each replicate, brought up to 30 mL in urea buffer (8 M urea, 0.1 M Tris pH 8.5) and processed using a modified filter-aided sample preparation (FASP) method

to remove SDS (Wiśniewski, Zougman, Nagaraj, Mann, & Wiśniewski, 2009). The 30 mL sample volume was divided into two Amicon Ultra-15 10K (Millipore) filter units and centrifuged at 4,000 x g for 25 min. Filter-bound samples were washed in 5 mL urea buffer and carbamidomethylated with 5 mL iodoacetamide (50 mM) in urea buffer. After centrifugation and three washes with 5 mL urea buffer, samples were washed with 5 mL ammonium bicarbonate (50 mM) to reduce the urea concentration and incubated with 2% (w/w) sequencing grade modified trypsin (Promega) overnight at 37 °C to digest labeled proteins into peptides. Filter units were centrifuged and washed with 3 mL HPLC grade H_2O. The flow-through was combined for each replicate and acidified to pH 2-3 using 98% formic acid, measured with pH strips.

Oasis HLB sorbent cartridges (150 mg, Waters) were used to desalt samples using 4 mL of 65% (v/v) acetonitrile, 1% (v/v) trifluoroacetic acid (TFA) to elute. TFA was added to bring the final concentration to 2% (v/v), and the protein concentration was determined using the DC protein assay (Bio-Rad). Thirty micrograms of each sample was removed for total protein measurement by mass spectrometry and L-glutamate (Sigma) was added to the remainder to a final concentration of 140 mM. Titanium dioxide beads (5 µm, GL Sciences) were equilibrated with 1 mL washes of eluting buffer 1 [20% (v/v) acetonitrile, 1% (w/v) ammonium hydroxide], wash buffer 1 [65% (v/v) acetonitrile, 0.5% (v/v) TFA], and loading buffer [65% (w/v) acetonitrile, 2% (v/v) TFA, 140 mM glutamic acid]. 20 mg of titanium beads in loading buffer were then added to aliquots of 2 mg protein for each sample, and rotated for 15 min at room temperature to bind phosphopeptides. Sample aliquots with titanium beads were centrifuged and washed with 1 mL loading buffer, 1 mL wash buffer 1, and two times with 1 mL wash buffer 2[65% (v/v) acetonitrile, 1.0% (v/v) TFA] before being resuspended in 200 µL eluting buffer 1.

This volume was added to C8 stagetips (Proxeon), eluted with a 1 mL syringe followed by 400 µL of 65% acetonitrile, 1% ammonium hydroxide and lyophilized overnight.

Lyophilized samples enriched for phosphopeptides were resuspended in 65 µL Buffer A (16.7 mM ammonium formate, 70% acetonitrile, pH 2.2) and sonicated in a water bath for 4 x 30 s pulses before being centrifuged for 1 min at 14,000 x g. The sample was then injected into a 50 µL sample loop using an Agilent 1100 HPLC with a 100 x 4.6 mm 5 µm polyWAX LP column (PolyLC) separated using an ERLIC gradient (Zarei, Sprenger, Gretzmeier, & Dengjel, 2013). The gradient was 0-5 min: 100% Buffer A, 5-15 min: increasing linear gradient to 100% Buffer B (16.7 mM ammonium formate, 10% acetonitrile, pH 2.2), 15-20 min increasing linear gradient to 100% Buffer C (1% TFA, 10% acetonitrile), 20-24 min: 100% Buffer C. One minute fractions were collected at a flow rate of 1 mL/min and immediately frozen in liquid nitrogen before being speedvac evaporated and resuspended in 0.1% formic acid, 5% acetonitrile.

Total protein samples (30 µg) were speedvac evaporated and resuspended in 17 µL of 1% TFA pH 2.5, 5% (v/v) acetonitrile. High pH reverse-phase fractionation was performed off-line using a Waters M-class Acquity UPLC, loading 15 µL of each digest onto a hand-packed C18 column (1.8 µm 120Å UChrom C18, 0.5 mm i.d./0.793 mm o.d. X 200 mm) equilibrated in Buffer A (10 mM ammonium formate pH 10) and eluted at 15 µL/min for 170 min with a linear gradient of increasing Buffer B (10 mM ammonium formate pH 10, 80% acetonitrile). Twelve fractions per replicate were collected.

LC-MS/MS

For SILAC experiments comparing DMSO-trametinib-SCH772984, phosphopeptide fractions were loaded onto a Waters Acquity UPLC M-class Peptide BEH C18 analytical column (1.7 µm, 130Å, 75 µm i.d., 250 mm). LC-MS/MS was run at 300 nL/min using either a Thermo

nLC100 or a Waters M-class Acquity UPLC. Peptides were eluted from Buffer A (0.1% formic

acid in water) into Buffer B (0.1% formic acid, 100% acetonitrile) using a gradient from 3%-

85% acetonitrile in 130 min (3% B for 0-5 min, 3%-20% B for 5-105 min, 20%-32% B for 105-

125 min, 32%-85% B for 125-126 min, 85% B for 126-130 min). MS/MS was performed on an

Orbitrap Fusion mass spectrometer with MS1 120,000 resolution scanning between 380-1500

m/z, 2 x 10^5 AGC, 50 ms injection time, 20 s dynamic exclusion and data dependent mode top

speed on the most intense ions. MS2 was performed using 1.6 m/z isolation with quadrupole,

35% HCD collision energy, 1 μscan, centroid in ion trap (1.0 x 10^4 AGC, 35 ms fill time) or

orbitrap (30,000 resolution, 5.0 x 10^4 AGC, 60 ms fill time). Total protein samples were

analyzed by MS/MS using the same method and ion trap isolation. For the DMSO-SCH772984-

GDC0994 and DMSO-SCH772984-Vertex-11e SILAC experiments, LC-MS/MS analysis was

performed using Waters Acquity UPLC and LTQ Orbitrap Velos instrumentation as previously

described (Stuart et al., 2015).

Data and statistical analysis

Raw MS files for phosphopeptide and total peptide fractions were searched together

using MaxQuant (Cox & Mann, 2008) software and processed with Perseus software as

described (Stuart et al., 2015). MaxQuant identifies common contaminants and peptides

matching to a target-decoy database containing reversed versions of each peptide in a protein

database FASTA file. The DMSO-trametinib-SCH772984 dataset was searched using MaxQuant

v1.5.4.2 and further analyzed using Perseus v1.5.4.2. The DMSO-SCH772984-GDC0994 and

previously published DMSO-selumetinib-vemurafenib datasets were searched using MaxQuant

v1.4.1.2 and further analyzed using Perseus v1.4.1.3. Searches used the Uniprot human proteome

reference (05-11-2016 release for DMSO-trametinib-SCH772984; 08-21-2015 release for DMSO-SCH772984-GDC0994; 01-27-2014 release for DMSO-selumetinib-vemurafenib).

Greater than 1.8 fold changes ($\log_2 \pm 0.840$) was used as significance threshold for SILAC ratios of phosphopeptides, corresponding to FDR ≤ 0.01 as determined from control replicate experiments of WM239a cells treated with DMSO (Stuart et al., 2015). To identify high-confidence phosphosites and control for variability among replicates, an empirical Bayesian analysis using Bioconductor-Limma software (Ritchie et al., 2015) was used to calculate adjusted p-values controlling the false discovery rate (q-value) (Margolin et al., 2009; Poss et al., 2016). Limma estimated the $\log_2$(drug:DMSO) ratios of phosphosites from two or three replicates, using phosphosite-wise linear models.

Immunoblotting

After treating cells with DMSO or drug for 2 h, hyperosmotic stress was induced by adding a solution of 5 M NaCl + 0.4 M KCl to a final concentration of 181 mM NaCl and 12.5 mM KCl, yielding media with total osmolality of 500 mOsm/L. Cells were harvested in RIPA lysis buffer supplemented with cOmplete protease inhibitor and Phos-Stop phosphatase inhibitor cocktails (Roche), and lysate protein concentrations were determined using the BioRad DC protein assay. Immunoprecipitation of p38α MAPK was carried out by incubating 250 µg cell lysate with 1 µg anti-p38α antibody overnight at 4°C, followed by 20 µL Dynabeads Protein G (Novex) for 1 h at 4°C. Beads were collected magnetically, washed with cold phosphate-buffered saline (PBS) and incubated in Laemmli sample buffer for 10 min at 95°C. All antibodies used were from Cell Signaling Technology and included anti-phospho-p38α (#9218), anti-p38α (#4511), anti-β-tubulin (#2146), anti-phospho-ERK1/2 (#4370), anti-MKK6 (#8550), anti-phospho-p90RSK (#9335), anti-BIM (#2819), and anti-cleaved PARP (#5625). Protein samples

were separated on 7.5% or 10% SDS-PAGE and transferred to PVDF membranes (Millipore-SQ), which were incubated with primary antibody overnight at 4°C and secondary antibody for 1 h at room temperature. Pierce ECL2 substrate was used for immunoblot development using X-ray film and/or fluorescence imaging (Typhoon, GE Healthcare).

In vitro kinase assays

Plasmids for expression of Flag-MKK6 and Flag-MKK3 in mammalian cells were generated by Roger Davis' laboratory (Dérijard et al., 1995; Enslen, Brancho, & Davis, 2000; Raingeaud, Whitmarsh, Barrett, Dérijard, & Davis, 1996) and obtained from Addgene: Flag-MKK6 (#13517), Flag-MKK6-S207E/T211E (#13518), Flag-MKK6-K82A (#13519), Flag-MKK3-S189E/T193E (#14670), Flag-MKK3-S189A/T193A (#14669), and Flag-MKK3b-S218E/S222E (#50449). Plasmids were purified using the PureLink HiPure maxiprep kit, and cells were transfected by electroporation using the NEON transfection system both according to manufacturers' protocol (Thermo Fisher Scientific). 1 x 10^6 cells were transfected with 5 µg cDNA using 1,200 V x 2 pulses X 20 ms/pulse, then incubated for 72 h, followed by lysis in RIPA buffer. Flag-tagged proteins were purified using anti-Flag M2 affinity gel resin (Sigma-Aldrich) and incubated with 300 µg lysates overnight at 4°C. Beads were washed in cold Tris-buffered saline (TBS), resuspended in reaction buffer containing 10 mM $MgCl_2$, 1 mM ATP, 50 mM Tris-HCl pH 7.2, 50 mM NaCl and 1 mM dithiothreitol, and then incubated with DMSO or drug at room temperature for 15 min. Reactions were initiated by adding 1 µg of unphosphorylated His_6-p38α MAPK, expressed in *E. coli* and purified by Ni-NTA and MonoQ chromatography as described (Sours, Xiao, & Ahn, 2014). Reactions were incubated for 2 min at 30°C, then quenched by adding Laemmli sample buffer followed by heating for 10 min at 95°C. Proteins were separated by SDS-PAGE followed by immunoblotting for phospho-p38α MAPK.

Reaction time and kinase levels were chosen within ranges of linear phosphorylation of p38α

MAPK.

The Thermo Fisher Scientific SelectScreen service was used to generate a 10-point dose response curve for trametinib (0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 and 30 µM) against MKK6 activity. The cascade format of this assay uses *in vitro* phosphorylation of inactive p38γ substrate by MKK6 to then phosphorylate a proprietary fluorophore-conjugated peptide substrate (Z'-LYTE) in the primary reaction. In the secondary reaction, site-specific proteolytic cleavage disrupts the Förster resonance energy transfer (FRET) of donor (coumarin) and acceptor (fluorescein) fluorophores coupled to unphosphorylated Z'LYTE peptide, leading to a decrease in FRET. The emission ratio (coumarin emission 445 nm/fluorescein emission 520 nm) remains high if the Z'LYTE peptide remains unphosphorylated. Maximum and minimum emission ratios were established using a synthetically phosphorylated p38γ peptide and a control reaction containing no ATP. Inhibition by trametinib was compared to a known inhibitor of MKK6, staurosporine, in a separate 10-point titration activity assay.

Cell viability assay

WM239a cells were seeded into 96-well dishes at 5,000 cells per well and allowed to adhere for 4 h before treating with DMSO or varying concentrations of drug. After 72 h, cellular ATP was quantified using the CellTiter Glo 2.0 Assay (Promega) according to the manufacturer's protocol. Luminescence was recorded using a Biotek Synergy H1 plate reader and curve-fitting was done using Origin software.

Cell death measurements

WM239a cells were seeded into 6 cm dishes at 750,000 cells per dish, and allowed to adhere overnight before treating with DMSO or with 10 µM selumetinib, Vertex-11e, or

SCH772984. After 48 h, apoptosis and necrosis were quantified using the annexin V-FITC PI assay (Thermo Fisher Scientific) using a FacScan flow cytometer (Beckman Coulter) according to the manufacturer's protocol. Recombinant annexin V conjugated to fluorescein (FITC) binds phosphatidylserine (PS) which translocates to the outer plasma membrane during apoptosis, and marks cells with green fluorescence. Propidium iodide (PI) is a DNA intercalating red-fluorescent molecule that only passes the plasma membrane of cells that have been permeabilized during late-stage cell death. The percentage of total cells staining positive for these markers is compared between drug treatments.

Kinase assays

The Human Phospho-Kinase Array (R & D Systems) was used to profile the relative levels of protein phosphorylation of 43 kinases simultaneously, according to the manufacturer's protocol. Following 4 h treatment with DMSO or 10 µM drug, cells were harvested in lysis buffer and incubated overnight with a nitrocellulose membrane containing capture antibodies for each phosphorylation site bound to the membrane in duplicate. After washing to remove unbound proteins, the arrays were incubated with a mixture of biotinylated detection antibodies which produce a chemiluminescent reaction when streptavidin-conjugated HRP and substrate are added. The signal produced at each spot corresponds to the amount of phosphorylated protein bound, and was quantified using a Typhoon fluorescence image scanner and ImageQuant software (GE Healthcare).

2.5 Results

I first compared molecular responses to the MKK1/2 inhibitor, trametinib, and the ERK inhibitor, SCH772984, using phosphoproteomics to screen WM239a human metastatic

melanoma cells. Phosphorylation changes were quantified in triple SILAC-labeled cells, treated

with DMSO 10 µM trametinib, or 10 µM SCH772984 for 2 h followed by cell lysis and

trypsinization using a modified FASP protocol, batch enrichment of phosphopeptides with TiO_2

resin, and fractionation by ERLIC chromatography as described previously (Stuart et al., 2015;

Wiśniewski et al., 2009; Zarei et al., 2013) (Fig. 2.1A). The WM239a metastatic melanoma cell

line harbors a V600D BRAF mutation leading to constitutive upregulation of the MAPK

pathway. A two-hour time point allows us to observe downstream effects of kinase inhibition

before directed protein degradation of other regulated proteins occurs. This cell line and time

point also allow a direct comparison to the previous SILAC phosphoproteomic experiment using

MKK1/2 inhibitor AZD6244 published by Scott Stuart in our lab (Stuart et al., 2015).

Experiments were conducted in triplicates, varying the isotopic labeling for each condition (Fig.

2.1B). In total, SILAC ratios could be quantified for 12,924 unique phosphosites on 3,851

proteins corresponding to class I phosphosites (localization probability >0.75, delta score >5). Of

these, 8,577 class I phosphosites could be quantified in two or more replicate experiments (Fig.

2.1C). ERLIC fractionation separated phosphopeptides across 24 fractions, allowing a higher

number of identifications in complex samples (Fig. 2.2A,B), with the vast majority of

phosphopeptides being singly phosphorylated (Fig. 2.2C).
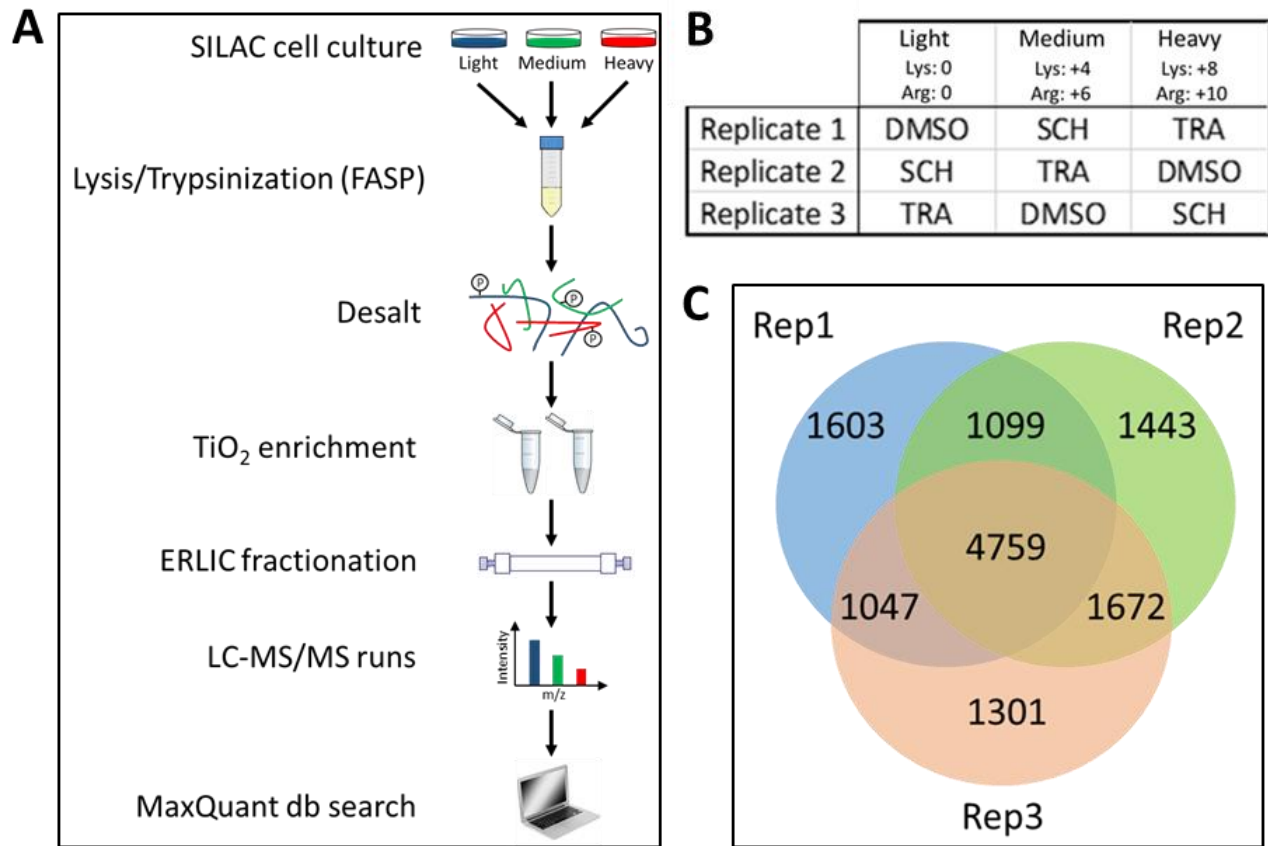
Figure 2.1: SILAC experiment design. A, Experimental workflow and mass spectrum of isotopically labeled peptides. B, Isotope labeling design and label-swap of replicates. WM239a cells were treated 2 h with DMSO or 10 µM SCH772984 (SCH) or 10 µM trametinib (TRA). C, Overlap among replicates in 12,924 phosphosites that could be quantified.
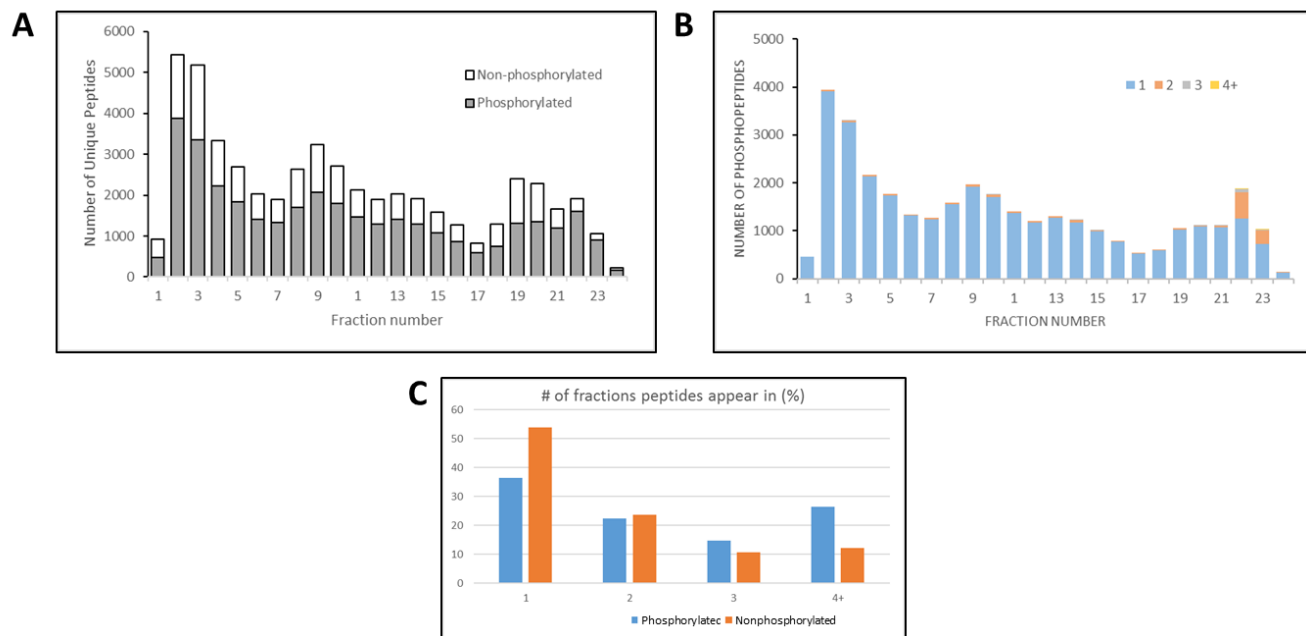
Figure 2.2. Performance of the ERLIC fractionation method. A, Unique phosphorylated and non-phosphorylated peptides in each ERLIC fraction, identified in three replicates. B, Number of singly and multiply-phosphorylated peptides in each fraction identified in three replicates. C, Number of fractions in which each phosphorylated and non-phosphorylated peptide can be identified, among three replicate experiments.

A threshold of 1.8-fold ($\log_2 \pm 0.84$) was used to identify significant changes in phosphosite abundance, based on control experiments of WM239a cells previously reported by our lab (Stuart et al., 2015) (Table 2.1). After filtering with this threshold, 564 class I phosphosites were found to be altered significantly by one or both inhibitors in multiple experiments. As an additional filter for significant changes, an empirical Bayesian analysis using the Limma statistical package was employed to calculate adjusted p-values for each phosphosite (Ritchie et al., 2015). We focused our remaining analysis on the 400 phosphosites that exhibited $\log_2$(drug:DMSO ratios) less than -0.84 or greater than +0.84, with an adjusted p-value ≤ 0.05 (Fig. 2.3A-C).

Table 2.1. Comparison of phosphosites identified in each triple-labeled replicate comparing trametinib and SCH772984. Number of phosphosites quantified after removing reverse protein database hits and contaminants and expanding the phosphopeptide results to separate phosphosites that were identified in multiply phosphorylated peptides. Ratios were considered significant when $|\log_2$ ratio$| \geq \pm 0.84$ determined by empirical Bayes-generated $\log_2$(combined ratio).

Replicate	Significance	TRA/DMSO	% Total	SCH/DMSO	% Total	SCH/TRA	% Total
1 (8517 sites)	Decreased	447	5.2	490	5.8	149	1.7
	Increased	185	2.2	85	1.0	55	0.6
2 (8989 sites)	Decreased	473	5.3	489	5.4	76	0.8
	Increased	101	1.1	92	1.0	64	0.7
3 (8788 sites)	Decreased	535	6.1	473	5.4	96	1.1
	Increased	104	1.2	139	1.6	181	2.1
Avg (8577 sites)	Decreased	422	4.9	415	4.8	33	0.4
	Increased	43	0.5	73	0.9	33	0.4
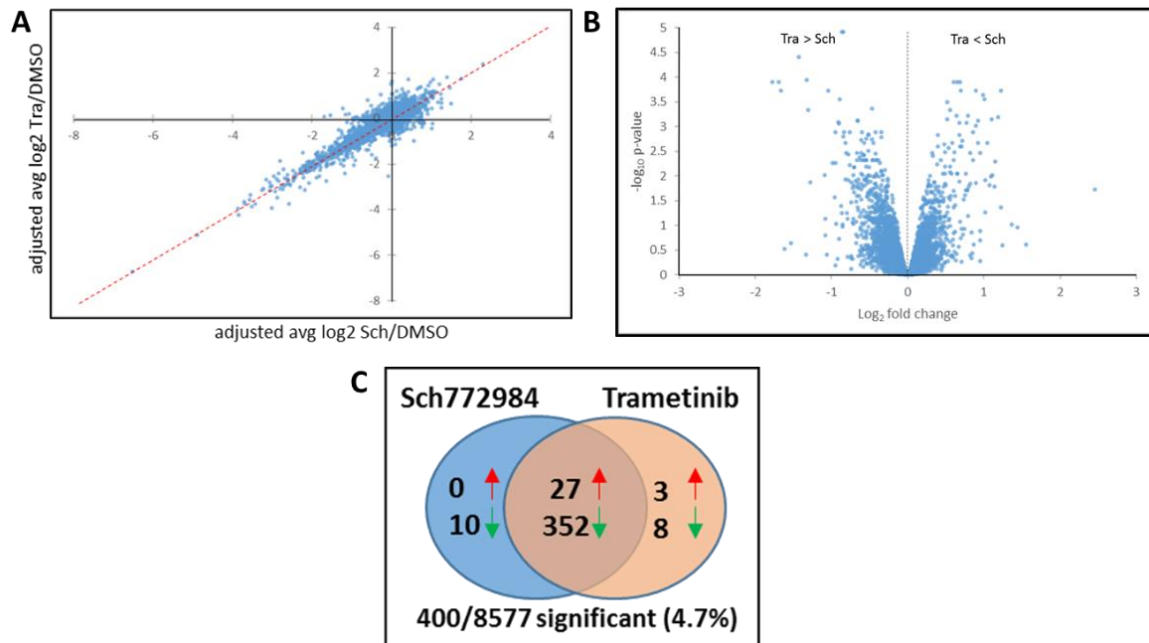
Figure 2.3: SILAC labeled phosphoproteomics comparison of trametinib and SCH772984. A, Log_2-transformed plot showing 8,577 phosphosites changing reproducibly in at least two replicates, in response to drug. B, Volcano plot showing confidence (adjusted p-value) vs fold change in response to SCH772984 or trametinib. Significant changes for phosphosites with $|log_2(\text{combined ratio})| \geq 0.84$, and adjusted p-value >0.05. C, Counts of significantly altered phosphosites with $|log_2(\text{combined ratio})| \geq 0.84$, and adjusted p-value >0.05.

As expected, the regulatory phosphorylation sites on ERK1 and ERK2, which are the primary substrates of MKK1/2, were among those most significantly inhibited by trametinib. The same sites were also inhibited by SCH772984, reflecting the ability of this molecule to induce disorder in the activation loop of ERK1/2 and interfere with its phosphorylation by MKK1/2 (Chaikuad et al., 2014). We found that the majority of phosphosites responsive to trametinib were similarly responsive to SCH772984 (Fig. 2.3A,C), indicating their regulation downstream of ERK1/2. In contrast, only 11 phosphosites responded to trametinib but not SCH772984, of which 8 decreased and 3 increased with drug (Fig. 2.3C, Table 2.2). The strongest effects unique to trametinib were seen in the regulatory phosphorylation sites in MKK1 and MKK2 (Table 2.2,

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gene names MAP2K1, MAP2K2), reflecting interference of the drug-bound MKK1/2 phosphorylation by BRAF V600E/K (Gilmartin et al., 2011). Likewise, only 10 phosphosites responded to SCH772984 but not trametinib, all inhibited by drug (Fig. 2.3C, Table 2.2).

Table 2.2. Comparison of phosphosites uniquely responsive to trametinib or SCH772984. [a] $\log_2$ values of quantified ratios in individual SILAC replicates. [b] Empirical Bayes-generated $\log_2$(combined ratio) and adjusted p-value. [=] Phosphosites above the double line are significantly decreased, and below the double line are significantly increased, in response to drug.

Unique phosphosite changes in response to SCH772984

Uniprot ID	Gene	Position	9 aa window	SCH772984/DMSO[a]			Trametinib/DMSO[a]			SCH/TRA[b]	
				Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Combined	adj p-value
Q9BXS5	AP1M1	223	LFDNTGRGK		-1.598	-1.815		0.214	-0.002	-1.777	<0.001
Q03252	LMNB2	419	SRATSSSSG	-1.256	-0.981	-1.235	0.376	0.107	0.014	-1.324	<0.001
Q8IXT5	RBM12B	562	FRHSSEDFR	-0.886	-0.957	-0.794	0.346	0.008	-0.194	-0.910	0.001
P38159	RBMX	88	ATKPSFESG	-1.045	-0.973	-0.850	0.044	-0.329	0.114	-0.890	<0.001
Q9NWH9	SLTM	550	KKRISSKSP		-1.094	-0.799		-0.242	0.212	-0.987	0.005
Q9NWH9	SLTM	553	ISSKSPGHM		-1.094	-0.733		-0.242	0.103	-0.945	0.005
Q8IYB3	SRRM1	234	VKEPSVQEA		-0.829	-0.923		0.008	0.075	-0.945	0.001
Q8N1F8	STK11IP	481	APRPSPPQE	-1.297	-0.963		0.579	-0.081		-1.273	0.013
Q8NI27	THOC2	1285	KKEKTPATT	-2.041	-1.231	-1.442	0.192	0.219	0.086	-1.688	<0.001
Q9H3N1	TMX1	270	IRQRSLGPS	-1.447	-1.396	-0.855	0.634	0.424	0.308	-1.662	<0.001

Unique phosphosite changes in response to Trametinib

Uniprot ID	Gene	Position	9 aa window	SCH772984/DMSO[a]			Trametinib/DMSO[a]			SCH/TRA[b]	
				Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Combined	adj p-value
Q99704	DOK1	269	LRADSHEGE	0.057	-0.223	0.146	-0.849	-1.540	-1.176	1.186	0.001
Q68DA7	FMN1	243	DIPKTPDTD	-0.198	-0.730	-0.606	-1.285	-1.522	-1.933	1.099	0.001
P36507	MAP2K2; MAP2K1	226; 222	SMANSFVGT	-0.334	0.169		-3.471	-1.631		2.451	0.019
Q16539	MAPK14	180	DDEMTGYVA	-0.028	0.080	0.377	-0.958	-1.135	-1.413	1.223	<0.001
Q16539	MAPK14	182	EMTGYVATR	0.004	0.103	0.202	-0.850	-1.183	-0.728	1.012	<0.001
Q86UE4	MTDH	298	SSQISAGEE	-0.095	-0.130	0.117	-0.826	-1.093	-1.042	0.985	<0.001
Q15154	PCM1	93	MSQMSVPEQ	0.000	-0.176	0.750	-0.533	-1.136	-0.958	1.074	0.015
Q5JSZ5	PRRC2B	480	FRQQSIEDK		-0.020	-0.080		-0.775	-1.196	0.947	0.003
Q8TDZ2	MICAL1	1057	ERRLSELAL		0.036	-0.177		0.967	1.349	-1.089	0.010
Q9BXB5	OSBPL10	30	SAGSSPSCS	0.416	0.018	0.348	1.494	1.238	1.191	-1.040	<0.001
Q8IY26	PPAPDC2	70	HRRGSFPLA	0.749	0.316	0.122	1.741	1.621	1.852	-1.304	<0.001

I also examined non-TiO$_2$-enriched proteolytic digests by 2D LC-MS/MS, in order to assess responses to drug at the protein level. Few significant changes were seen in protein abundances after 2 h of drug treatment, and none accounted for any of the phosphorylation sites that changed significantly in response to trametinib or SCH772984. Overall, the results show strong overlap in phosphorylation sites which respond to MKK1/2 and ERK1/2 inhibitors at the level of posttranslational modifications rather than protein synthesis or degradation. Such behavior is similar to our lab's previous phosphoproteomics comparison of the BRAF V600E/K inhibitor, vemurafenib, and the MKK1/2 inhibitor, selumetinib, and reveals linearity in signaling through the MAPK pathway downstream of oncogenic BRAF V600E/K (Stuart et al., 2015). Examples of targets shared by both inhibitors included known ERK1/2 substrates, neuroblast differentiation-associated protein Thr5824 (AHNAK), which has 7 sites significantly decreased and 2 sites significantly increased in response to inhibitor, and cortactin (CTTN), where Ser405 and Ser418, which are known regulatory sites controlling protein degradation and ubiquitination were significantly decreased by inhibitor.

Among the targets that responded only to trametinib were the regulatory phosphorylation sites, T180 and Y182, which control the activity of p38α MAPK (gene name MAPK14) (Table 2.2). Together with MKK1/2, these were the only phosphorylation sites on protein kinases that differentially responded to only one and not both drugs (Table 2.2). Phosphorylation of both T180 and Y182 in p38α was decreased by ~2-fold with trametinib [log$_2$(trametinib/DMSO) ≈ - 1.0], but by less than 8% with SCH772984 [log$_2$(SCH772984/DMSO) < + 0.1]. There are four isoforms of p38 MAPKs (α, β, γ, δ) with p38α MAPK being the most characterized and most consistently expressed across cell types (Cuenda & Rousseau, 2007). RNA-seq analysis showed that the alpha isoform of p38 is the most highly expressed in WM239a cells (Zarubin, T. & Han,

2005, data not shown), and was the only isoform observed in our proteomics dataset. The results suggest that p38α MAPK is inhibited by trametinib.

In order to examine if inhibition of p38α phosphorylation is common to other MKK1/2 inhibitors, we compared our trametinib dataset to a previous SILAC dataset examining phosphoproteomics responses to the MKK1/2 inhibitor, selumetinib (AZD6244) (Stuart et al., 2015). In total, 6,495 reproducible phosphosites were quantified in both experiments (Fig. 2.4A,B).
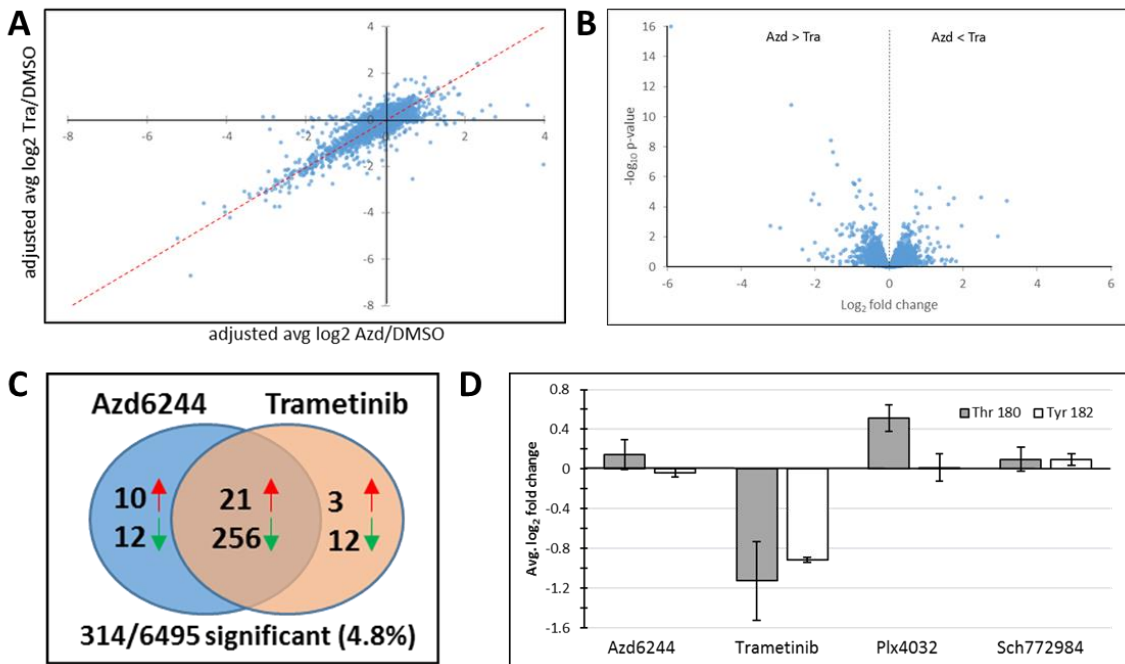
Figure 2.4: SILAC labeled phosphoproteomics comparison of trametinib and selumetinib (AZD6244). A, WM239a cells were treated 2 h with 10 µM drug, and phosphosite responses were examined in a log$_2$ transformed plot. The results compared 6,495 phosphosites reproducibly quantified in at least two replicates. B, Volcano plot showing confidence (adjusted p-value) vs fold change in response to trametinib or selumetinib (AZD6244). Significant changes with |log$_2$(combined ratio)| $\geq$ 0.84, and adjusted p-value >0.05. C, Counts with significant changes in |log$_2$(combined ratio)| $\geq$ 0.84, and adjusted p-value >0.05. D, Log$_2$(combined ratios) of canonical activation sites on p38α MAPK, comparing effects of BRAF V600E/K, MKK1/2, and ERK1/2 inhibitors.

Of those phosphorylation sites that could be quantified in each of the two datasets, strong overlap was observed in those responsive to both trametinib and selumetinib (Fig. 2.4A,C). Of these, 256 were significantly inhibited in response to both inhibitors while 12 were uniquely inhibited by trametinib but not selumetinib, and 12 were uniquely inhibited by selumetinib but not trametinib (Fig 2.4C, Table 2.3). Among the phosphosites inhibited by trametinib but not selumetinib, Ser269 on docking protein 1 (DOK1) and Ser298 on metadherin (MTDH) both appear to be novel off-targets of trametinib. In a phosphoproteomics SILAC screen of HeLa cells stimulated with EGF with or without MKK1/2 inhibitor, U0126, both phosphosites were elevated rather than inhibited by U0126. They also detected no change in T180 and Y182 on p38α MAPK, in response to U0126 (Pan et al., 2009). In the present study, neither T180 nor Y182 in p38α MAPK changed significantly in response to selumetinib (Fig. 2.4D) and our lab's previous dataset also showed no effect at these two sites, in response to the BRAF V600E/K inhibitor, vemurafenib (Stuart et al., 2015). Thus, the ability of MKK1/2 inhibitor to inhibit p38α MAPK phosphorylation appears to be specific for trametinib.

Table 2.3. Comparison of phosphosites that change uniquely in response to trametinib or selumetinib (AZD6244). [a] log$_2$ (ratio) values measured individually in three replicate SILAC experiments. [b] Empirical Bayes-generated log$_2$(combined ratio) and adjusted p-value. [=] Phosphosites above the double line were significantly decreased, and below the double line were significantly increased, in response to drug.

Unique phosphosite changes in response to Trametinib

Uniprot ID	Gene	Position	9 aa window	Trametinib/DMSO[a]			AZD6244/DMSO[a]			TRA/AZD[b]	
				Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Combined	adj p-value
P35611	ADD1	12	AVVTSPPPT		-0.988	-0.920	-0.473	-0.284	0.559	-0.889	0.037
Q96RK0	CIC	1397	KRKNSTDLD	-2.094	-1.896	-1.788	4.001	3.876	4.094	-5.903	<0.001
Q99704	DOK1	269	LRADSHEGE	-0.849	-1.540	-1.176	0.267	-0.170	-0.424	-1.063	0.001
Q16828	DUSP6	178	SDSSSDIES	-1.276	-1.235	-1.205	0.276	0.381	0.122	-1.581	<0.001
P42566	EPS15	796	NKLDSPDPF	-1.322	-1.230	-1.495	-0.432	-0.441	-0.325	-0.966	<0.001
P05412	JUN	62	SDLLTSPDV	-1.853	-1.770		-0.848	-0.427	-0.402	-1.269	0.007
P17535	JUN; JUND	73; 100	LKLASPELE	-1.755	-1.581	-1.687	-0.836	-0.650	-0.626	-0.936	<0.001
P36507	MAP2K2; MAP2K1	226; 222	SMANSFVGT	-3.471	-1.631		0.578	0.802	0.702	-3.219	0.002
Q16539	MAPK14	180	DDEMTGYVA	-0.958	-1.135	-1.413		0.006	0.304	-1.269	0.002
Q16539	MAPK14	182	EMTGYVATR	-0.850	-1.183	-0.728	-0.091	0.043	-0.063	-0.877	<0.001
Q86UE4	MTDH	298	SSQISAGEE	-0.826	-1.093	-1.042		-0.048	-0.239	-0.870	0.003
P04920	SLC4A2	144	LTQPSPVST	-1.251	-0.861	-0.793	1.583		0.663	-2.101	<0.001
Q9BWH2	FUNDC2	151	KIRKSNQIP	1.313	0.975	0.791	0.099	-0.051	0.043	1.024	0.002
Q8IY26	PPAPDC2	70	HRRGSFPLA	1.741	1.621	1.852	-0.004	0.850	1.102	1.064	0.011
O14828	SCAMP3	32	QHRPSRQYA		1.010	0.853	-0.268	-0.038	-0.805	1.305	0.003

Unique phosphosite changes in response to AZD6244

Uniprot ID	Gene	Position	9 aa window	Trametinib/DMSO[a]			AZD6244/DMSO[a]			TRA/AZD[b]	
				Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Combined	adj p-value
Q09666	AHNAK	210	IRLPSGSGA	-0.244	-0.522	-0.475	-1.422	-1.316	-1.219	0.878	<0.001
Q68DQ2	CRYBG3	1280	KPCVSPTVG	-0.644	-0.538		-2.396		-2.001	1.597	<0.001
P21333	FLNA	1084	GSAGSPARF	0.066	-0.154	0.325	-1.980	-1.395		1.759	<0.001
Q4G0J3	LARP7	299	RKRSSSEDA	0.362	-1.093	0.183	-0.792	-1.783	-1.872	1.312	0.022
P10636	MAPT	579	SKIGSTENL		0.146	0.229	-3.982	-1.655		2.942	0.009
P30414	NKTR	1146	VEETSPLGN	0.085	0.129	-0.116	-1.383	-0.956	-1.579	1.358	<0.001
Q16875	PFKFB3	461	MRRNSVTPL	-0.316	-0.353	-0.750	-1.120		-3.068	1.604	0.047
Q14160	SCRIB	1223	NSLESISSI	-0.471	-0.177	-0.569	-2.747	-3.635	-2.355	2.480	<0.001
Q9ULF5	SLC39A10	540	TEESTIGRK	0.374		-0.134		-2.253	-1.517	1.964	0.002
Q8IYB3	SRRM1	756	SVSGSPEPA	0.194	0.127	0.188	-0.560	-0.420	-2.380	1.376	0.043
Q8WVM7	STAG1	1062	DDRMSVNSG	-0.058	0.316		-3.489	-2.651		3.191	<0.001
Q9BRZ2	TRIM56	471	LKSISREPS	0.342	0.325	0.221		-0.709	-0.954	1.093	<0.001
Q09666	AHNAK	5782	HRSNSFSDE	0.171	0.580	0.487	1.378	1.410	1.321	-0.939	<0.001
Q96RK0	CIC	1409	EDPTSPKRK	-0.685	2.195	0.755	2.993	3.729	4.060	-2.946	0.003
Q99543	DNAJC2	49	NASASFQEL	-0.216	-0.154	-0.303	1.148	1.211	1.499	-1.529	<0.001
O60841	EIF5B	186	SSGESGDES		0.180	0.128	1.914	1.577	1.113	-1.327	0.007
O60841	EIF5B	182	SRINSSGES	-0.240		-0.064	2.157	1.587		-1.892	<0.001
Q12789	GTF3C1	1068	TDQGSDEEG	-0.312	0.682	0.282	1.218	1.012	1.710	-1.009	0.003
Q9Y4B5	MTCL1	1417	KEDVTPPLS	0.544		0.760	1.897	2.224	3.836	-2.009	0.022
O43765	SGTA	303	IRSRTPSAS	0.059	-0.595	0.254	0.680	1.046	1.028	-1.000	0.002
O95359	TACC2	2317	NTPASPPRS	1.025	-0.054	0.218	2.644	2.438	2.242	-2.037	<0.001
O60343	TBC1D4	591	GSVDSFERS	0.224	0.237	-0.089	2.618	3.050	2.694	-2.639	<0.001

6495 phosphosites identified in 2 or 3 replicates

Significance	TRA/DMSO	% Total	AZD/DMSO	% Total	TRA/AZD	% Total
Decreased	335	5.2	319	4.9	64	1.0
Increased	51	0.8	64	1.0	47	0.7

Conceivably, phosphosites that were inhibited by trametinib but not selumetinib might reflect the trametinib-specific inhibition of p38α MAPK. This was examined by searching literature sources for known substrates of p38 signaling (Cuadrado & Nebreda, 2010; Cuenda & Rousseau, 2007; Trempolec, Dave-Coll, & Nebreda, 2013). Of 103 proteins found to be substrates of p38 MAPK, 32 were represented in both trametinib and selumetinib datasets. However, only one protein, c-Jun, showed a phosphorylation site that was inhibited more by trametinib than selumetinib. c-Jun is also known substrate of ERK1/2 as well as JNK, and indeed, a partial inhibition by selumetinib could be observed. Therefore, only p38α MAPK-Thr180 and Tyr182 were useful as reporters of the selective effect of trametinib on this kinase.

We next carried out biochemical analyses to ask if p38α MAPK phosphorylation is indeed inhibited by trametinib. Western blots probed with anti-phospho-p38 antibodies showed nearly undetectable basal levels of phosphorylated p38 in whole cell lysates (not shown). These antibodies also could not distinguish different isoforms of p38 MAPK. Therefore, isoform-specific antibodies were used to concentrate p38α MAPK by immunoprecipitation from cell lysates, followed by Western blotting with the anti-phospho-p38 antibody. The results showed significant inhibition of basal Thr180 and Tyr182 phosphorylation in p38α MAPK, after treating cells for 2 h with trametinib, but not DMSO or SCH772984 (Fig. 2.5A).
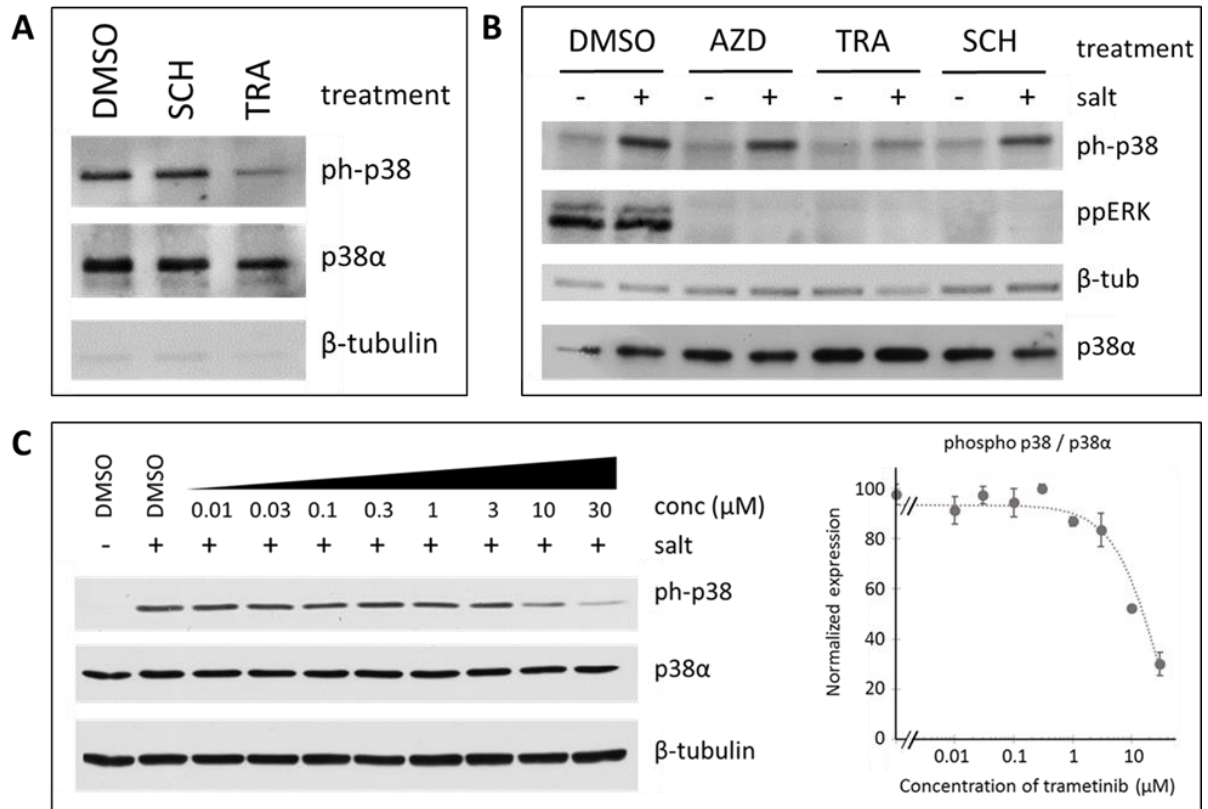
Figure 2.5. Analysis of phosphorylated p38 MAPK in WM239a cells by Western blotting. A, Immunoprecipitation of p38α MAPK following 2 h treatment with DMSO or 10 µM SCH772984 (SCH) or 10 µM trametinib (TRA), followed by Western blotting of phosphorylated p38 MAPK. B, Osmotic stress stimulates p38 phosphorylation. Cells were treated for 30 min with DMSO or 10 µM drug (selumetinib=AZD), then stimulated without or with osmotic stress for 2 h, by raising osmolality of media from 300 mOsm to 500 mOsm. Cells were harvested and lysates examined by Western blotting for phosphorylated p38 MAPK. C, Biological triplicate dose response of trametinib at concentrations 0.01-30 µM (left to right). Cells were treated for 30 min with DMSO or 10 µM drug, then stimulated without or with osmotic stress for 2 h. Cells were harvested and lysates examined by Western blotting for phosphorylated p38 MAPK. Quantification of ph-p38 normalized to p38α using ImageJ quantification on biological triplicate experiments.

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Trametinib inhibited the phospho-p38 MAPK signal by approximately 50%, comparable to the estimated decrease quantified by phosphoproteomics (Fig. 2.4D). In order to examine the effect of different MKK1/2 and ERK1/2 inhibitors on p38α phosphorylation following stress pathway activation, we pretreated cells with selumetinib, trametinib, and SCH772984 for 2 h, and then added NaCl+KCl to induce hyperosmotic stress which has been shown to activate p38α (Mavrogonatou & Kletsas, 2009, 2012). Salt-stimulation induced a substantial phosphorylated p38α signal in Western blots of cell lysates, in a manner that was inhibited by trametinib (~50%) but not selumetinib or SCH772984 (Fig. 2.5B). We also carried out a dose response experiment, and observed substantial inhibition of salt-induced p38 phosphorylation at concentrations above 10 μM trametinib, with estimated IC_{50} of 10 μM (Fig. 2.5C). Thus, complementary biochemical strategies using Western blotting and phosphoproteomics confirm that trametinib inhibits p38α MAPK phosphorylation. Given that the response is unique to trametinib and not observed with SCH772984 or selumetinib, the results suggest a bona-fide off-target effect of trametinib as an inhibitor of p38α phosphorylation, occurring at ~10 μM concentrations commonly used in preclinical studies (Yamaguchi et al., 2011) and ~300-fold higher than the maximal plasma concentration observed clinically (36 nM) (Infante et al., 2012).

The specificity of trametinib for MKK1/2 suggested that it might also target MKK3 and/or MKK6, which following hyperosmotic stress are known to phosphorylate and activate p38α (Zarubin, T. & Han, 2005) (Fig. 2.5B). However, previous reports have shown weak or variable inhibition of MKK3 or MKK6 activity by trametinib. An *in vitro* ELISA assay panel from Yamaguchi et al. 2011, on 99 kinases showed the activity of MKK6 decreased 30% with 10 μM trametinib, while the target kinase MKK1 decreased 72%. Very few other kinase activity levels changed significantly, indicating high specificity of trametinib including: ERK1 (-14%),

ERK2 (+8%), JNK1 (+4%), JNK2 (-3%), JNK3 (-24%), p38α (-16%), p38β (+4%), p38γ (-3%), p38δ (+9%). A second study by Uitdehaag et al. 2014 measured the changes in activity of over 300 kinases in response to 1 µM trametinib using *in vitro* mobility shift assays and reports a 21% increase in MKK6, a 3% decrease in MKK3, a 99% decrease in MKK1, and a 97% decrease in MKK2 activities. Additional kinases measured include: ERK1 (+2%), ERK2 (+4%), JNK1 (+5%), JNK2 (+5%), JNK3 (+2%), p38α (+4%), p38β (-2%), p38γ (-1%), p38δ (+3%). Therefore, we tested MKK6 and MKK3b using direct kinase assays of each Flag-tagged kinase expressed in WM239a cells. Flag-MKK6 was expressed as wild-type (wt), constitutively active mutant (S207E/T211E), and catalytically inactive mutant (K82A) forms, each immunopurified from cell lysates using immobilized anti-Flag antibody. Kinases were then preincubated with Mg^{2+}-ATP and DMSO, 10 µM trametinib, or 10 µM selumetinib, followed by addition of purified unphosphorylated p38α to initiate *in vitro* kinase assays (Fig. 2.6A). Trametinib inhibited both wt and constitutively active MKK6, leading to significant reduction of the initial rate of p38α phosphorylation. In contrast, selumetinib had a minimal effect on MKK6 activity. As expected, the MKK6-S207E/T211E mutant exhibited much higher activity than MKK6-wt towards p38α, although it appeared to be comparably inhibited by trametinib (Fig. 2.6A) (Raingeaud et al., 1996). Similar approaches were used to examine the effects of each drug on a constitutively activated isoform of MKK3, MKK3b-S218E/T222E, which contains a N-terminal recognition domain and has higher specific activity towards p38α than MKK3-S189E/T193E (Enslen et al., 2000; Jiahuai Han, Wang, Jiang, Ulevitch, & Lin, 1997). *In vitro,* MKK3b showed activity towards p38α MAPK that was too low to observe inhibition by trametinib or selumetinib (Fig. 2.6B).
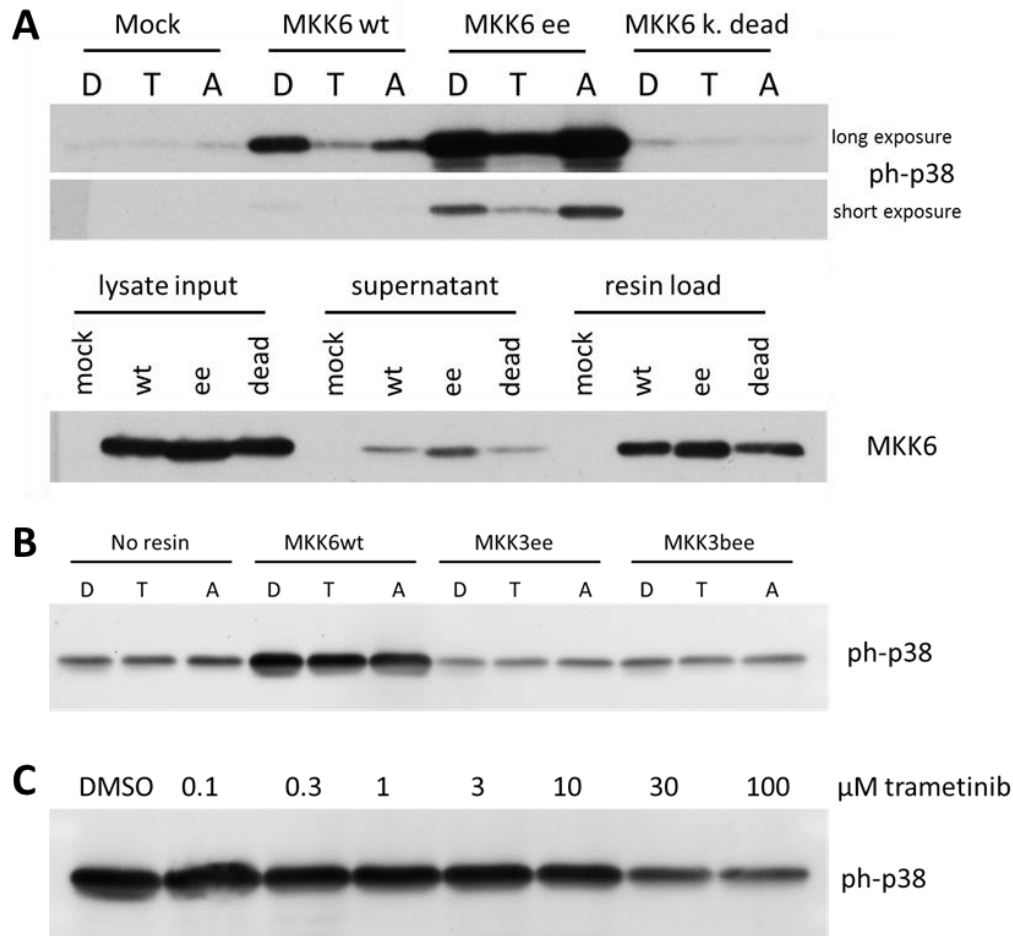
Figure 2.6. *In vitro* **kinase assay measurement of MKK6, MKK3 and MKK3b activity.** A, Flag-MKK6 was expressed in WM239a cells as wild type (wt), constitutively active (ee), or catalytically inactive (k.dead) mutants, then immunoprecipitated from lysates with immobilized anti-Flag antibody, and incubated for 15 min with DMSO (D), trametinib (T) and selumetinib (AZD6244) (A). *In vitro* kinase activity was measured by phosphorylation of recombinant unphosphorylated p38α MAPK. As expected, the activity of MKK6ee exceeded that of MKK6wt, but only trametinib inhibited MKK6 activity. B, *In vitro* kinase assays of constitutively active mutants of MKK6 (MKK6ee), MKK3 (MKK3ee) and MKK3b (MKK3bee), measuring phosphorylation of recombinant p38α by Western blotting with anti-phospho-p38 MAPK. The results show high activity of MKK6ee towards p38α substrate in a 10 min kinase reaction, but no activity of MKK3ee or MKK3b33 in 10 min reactions, in assays containing DMSO (D). Little effect of trametinib (T, 10 μM) or selumetinib (AZD6244) (A, 10 μM), is also seen. Thus, trametinib shows variable reproducibility with respect to MKK6ee inhibition. C, Kinase assays of MKK6ee activity measured by phosphorylation of p38α MAPK for 2 min, in the presence of varying concentrations of trametinib. The *in vitro* kinase assay reveals an apparent IC_{50} for trametinib of ≥ 10 μM.

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We then measured the dose response for *in vitro* inhibition of MKK6-S207E/T211E by varying concentrations of trametinib. Unexpectedly, the estimated IC_{50} was greater than 10 µM, for trametinib inhibition of MKK6-S207E/T211E using p38α MAPK as the substrate (Fig. 2.6C). This was surprising, because the sensitivity of MKK6 inhibition *in vitro* was much lower than the IC_{50} for inhibiting phospho-p38 MAPK in cells (Fig. 2.5C). In order to verify this result, the Scientific Select Screen service contracted with Thermo Corp. was used to generate a 10-point dose response curve of MKK6 inhibition by trametinib (0-30 µM), assayed by coupling its phosphorylation of p38γ MAPK to a proprietary fluorophore-conjugated p38 MAPK peptide substrate (Z'-LYTE) which undergoes changes in Förster resonance energy transfer (FRET) fluorescence signal upon phosphorylation. The results showed that similarly, $IC_{50} > 10$ µM for MKK6 inhibition (Fig. 2.7A), comparable to my *in vitro* kinase assay measurements of MKK6ee (Fig. 2.6C).
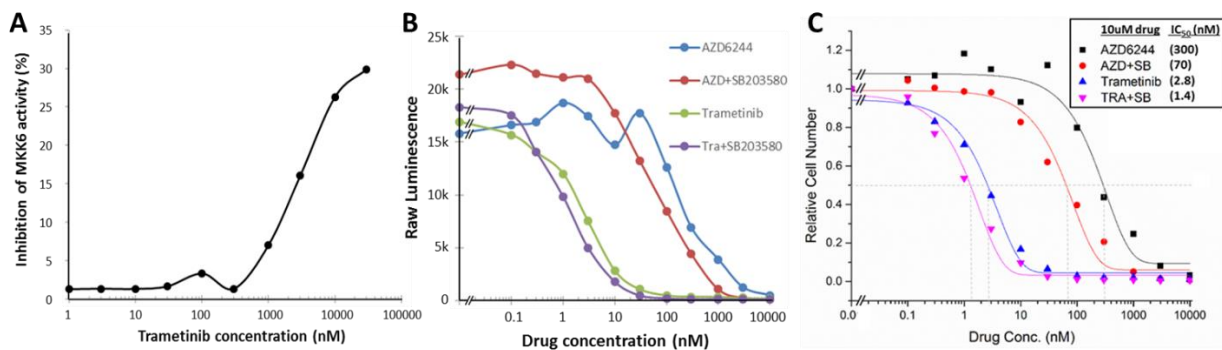
Figure 2.7. Effects of MKK1/2 inhibitors on p38 MAPK pathway effectors and signaling. A, Dose response of MKK6 activity measured by phosphorylation of p38γ MAPK, using FRET-based fluorescence measurements of p38γ MAPK activity using the Thermo SelectScreen assay. The assay shows 30% inhibition at the highest concentration of trametinib (10 µM), indicating IC_{50} consistent with measurements in 2.6C. C, CellTiter Glo raw intensity values used for C show a slight increase in cell viability with SB203580 alone C, CellTiter Glo viability assay shows that the p38 inhibitor, SB203580 (10 µM) shifts the IC_{50} for inhibition of cell viability with selumetinib (AZD6244) to 5-fold lower concentrations, but shifts the IC_{50} with trametinib by less than 2-fold.

Finally, we asked if the ability of trametinib to inhibit p38α might contribute to its effects on viability or growth of melanoma cells in a manner distinct from MKK1/2 inhibition. To address this, we added the p38α/β inhibitor, SB203580, to WM239a cells treated with varying concentrations of trametinib or selumetinib, measuring cell viability after 72 h. SB203580 alone had no inhibitory effect on cell viability, and shows a slight increase in cell viability (Fig. 2.7B). However, in combination with selumetinib, it shifted the dose response curve, reducing the IC_{50} for selumetinib by ~5-fold, from 300 nM in the absence of SB203580 to 60 nM in its presence (Fig. 2.7C). In contrast, SB203580 had minimal effect in combination with trametinib, shifting the IC_{50} for trametinib by less than 2-fold (Fig. 2.7C). The results are consistent with a model in which inhibiting p38 MAPK augments the ability of the MKK1/2 inhibitor, selumetinib, to suppress cell viability, but has little effect on trametinib due to the latter's off-target interference with p38α signaling. The results suggested that the off-target effect on MKK6, unique to trametinib, might contribute favorably to the properties of this anti-cancer drug.

Taken together, trametinib inhibits MKK6 *in vitro*, but in a concentration range that may be inconsistent with its ability to inhibit p38α MAPK in cells. The reason for this inconsistency is currently unknown. On one hand, it may reflect differences between the ability of trametinib to inhibit direct kinase assays *in vitro*, vs its effects on salt-induced pathway activation in cells. For example, the IC_{50} for p38 MAPK inhibition in cells may be abnormally low, if the compound is sequestered in cells to concentrations exceeding those added to the growth media. On the other hand, MKK3 and MKK6 may not be the relevant target of trametinib in cells, and the compound may target an enzyme located upstream of MKK3 and MKK6 to inhibit p38 MAPK in cells. For example, MEKK3 is a kinase activated by hyperosmotic stress, which functions immediately upstream to directly phosphorylate MKK3 and MKK6. WNK4 is another kinase which can be

directly activated by hyperosmotic stress and serves as an upstream activator of MEKK3 through

mechanisms that are not completely elucidated. Further studies are needed to determine if other

cellular target(s) of trametinib more fully explain its effects on inhibiting p38α MAPK. Such

studies could conceivably reveal new effectors of salt stress pathway signaling, using the

selective off-target response to trametinib as an assay for their identification.

Having identified an intriguing off-target effect specific to trametinib, we next

investigated potentially differential responses between two high affinity ERK1/2 inhibitors,

SCH772984 (Ki = 0.12 nM, (Rudolph et al., 2015)) and GDC0994 (Ki = 1.1 nM) (Robarge et al.,

2014). To do this, we used a triple SILAC phosphoproteomics screen to compare

phosphorylation responses to DMSO, SCH772984 and GDC0994. Triplicate experiments were

performed using different combinations of heavy, medium and light isotopically-labeled media.

In total, 6,893 class I phosphorylation sites were identified on 2,843 proteins, including 4,436

phosphosites quantified in at least two replicates (Fig. 2.8A-F, Table 2.4).

Figure 2.8: SILAC labeled phosphoproteomics comparison of SCH772984 and GDC0994. A, Log2-transformed plot showing 4,436 phosphosites observed reproducibly in at least two replicates. Strong correlations were seen between phosphosites changing in response to SCH772984 and GDC0994. B, Volcano plot showing confidence (adjusted p-value) vs fold change in response to SCH772984 or GDC0994. C, Overlap among replicates in 6,893 phosphosites quantified across three replicate experiments. D, Numbers of unique phosphorylated and non-phosphorylated peptides identified in each ERLIC fraction among three replicates. E, Number of singly phosphorylated peptides (blue) and multiply phosphorylated peptides (red, grey, yellow) eluting in each ERLIC fraction identified among three replicates. F, Number of ERLIC fractions in which each phosphorylated or non-phosphorylated peptide appear, among three replicates. G, Counts of phosphosites with significant changes, where |log2 (combined ratio)| ≥ 0.84, and adjusted p-value >0.05.

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Table 2.4. Comparison of phosphosites uniquely responsive to SCH772984 and GDC0994. [a] Log$_2$(ratio) values from individual SILAC replicates. [b] Empirical Bayes-generated log$_2$(combined ratio) and adjusted p-value. [=] Phosphosites above the double line are significantly decreased, and below the double line are significantly increased, in response to drug.

Unique phosphosite changes in response to SCH772984

Uniprot ID	Gene	Position	9 aa window	SCH772984/DMSO[a]			GDC0994/DMSO[a]			SCH/GDC[b]	
				Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Combined	adj p-value
P28482	MAPK1	187	FLTEYVATR	-3.206	-3.112	-3.057	-0.659	-0.732	-0.413	-2.470	<0.001
P27361	MAPK3	204	FLTEYVATR	-3.527	-3.640	-3.519	-0.607	-0.835	-0.670	-2.902	<0.001
P38159	RBMX	88	ATKPSFESG	-1.385	-0.920		0.139	-0.336		-1.087	0.015

Unique phosphosite changes in response to GDC0994

Uniprot ID	Gene	Position	9 aa window	SCH772984/DMSO[a]			GDC0994/DMSO[a]			SCH/GDC[b]	
				Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Combined	adj p-value
Q09666	AHNAK	210	IRLPSGSGA	-0.799	0.048	-0.927	-2.054	-1.150	-1.415	1.002	0.007
Q09666	AHNAK	216	SGAASPTGS	-0.750	0.048	-0.840	-2.014	-1.150	-1.259	0.972	0.008
Q68DQ2	CRYBG3	2902	GGRDTPGAK	0.063		0.015	-1.219		-1.271	1.337	<0.001
Q16555	DPYSL2	509	EVSVTPKTV	-0.070	0.566	0.298	-1.729	-1.751	-1.235	1.804	<0.001
Q14195	DPYSL3	509	DLTTTPKGG	0.287	0.068	-0.026	-1.089	-1.138	-1.721	1.490	<0.001
Q04637	EIF4G1	1209	RKAASLTED	-0.373	-0.427	0.418	-2.905	-3.068	-2.325	2.482	<0.001
P19419	ELK1	196	PPSGSRSTS	-0.257	-0.271	-0.763	-1.317	-1.170	-1.212	0.922	<0.001
Q9H840	GEMIN7	3	_MQTPVNI	-0.098	0.305	0.222	-1.164	-1.103	-1.010	1.230	<0.001
Q6Y7W6	GIGYF2	189	GGPTSVGRK		0.414	0.585		-1.225	-0.880	1.307	0.004
Q13098	GPS1	479	QGELTPANS	-0.070	0.265	-0.019	-0.795	-0.493	-2.517	1.353	0.029
P52272	HNRNPM	452	ERMGSGIER		0.076	0.116		-2.153	-2.448	2.384	<0.001
Q659C4	LARP1B	900	VPINSPRRN	0.342	0.292		-1.084	-1.063		1.403	<0.001
Q03252	LMNB2	17	ATPLSPTRL	-0.079	-0.063		-1.988	-0.806		1.349	0.037
Q96L50	LRR1	124	VSTLTPVKT	0.076	0.080		-0.965	-0.668		1.075	0.017
Q9Y608	LRRFIP2	320	TTPLSGNSS	0.385	0.310	0.105	-1.113	-1.175	-1.234	1.142	0.028
O14777	NDC80	69	GSRNSQLGI	-0.199	0.117		-0.680	-0.929		0.870	0.008
Q8NCF5	NFATC2IP	84	GPVASRDNS	0.270	0.137	-0.095	-0.693	-0.814	-1.053	0.961	<0.001
P06748	NPM1	254	KMQASIEKG	0.461	0.491	0.262	-1.010	-0.535	-0.922	1.342	<0.001
Q13131	PRKAA1	382	LVAETPRAR	-0.133	0.135		-1.198	-0.956		0.885	0.004
Q9NX01	TXNL4B	132	LIVQSPIDP	-0.118	0.098		-1.414	-1.123		1.171	0.001
Q09666	AHNAK	4564	VGIDTPDID		0.229	-0.203		0.747	1.266	-1.016	0.022
Q9UHR4	BAIAP2L1	261	TPQASPMIE	0.004	-0.110		1.083	0.733		-0.913	0.003
Q8WUZ0	BCL7C	126	SRPVSPAGP	-0.240	-0.412	-0.371	1.074	0.898	0.851	-1.178	<0.001
Q6PKG0	LARP1	766	TIARSLPTT	-0.346	0.393	-0.273	1.641	2.357	2.029	-2.062	<0.001
P19532	TFE3	548	LRAASDPLL	-0.093	0.230		1.098	1.086		-0.959	0.002

Table 2.5. Comparison of phosphosites identified in each triple-labeled SILAC replicate comparing SCH772984 and GDC0994. Number of phosphosites quantified after removing reverse protein database hits and contaminants and expanding the phosphopeptide table to separate phosphosites identified in multiply phosphorylated peptides. Changes were considered significant when their empirical Bayes-generated $|\log_2(\text{combined ratio})| \geq 0.84$.

Experiment	Significance	SCH/DMSO	% Total	GDC/DMSO	% Total	SCH/GDC	% Total
1 (4459 sites)	Decreased	366	8.2	428	9.6	73	1.6
	Increased	43	1.0	90	2.0	121	2.7
2 (5077 sites)	Decreased	316	6.2	368	7.2	41	0.8
	Increased	89	1.8	95	1.9	111	2.2
3 (4377 sites)	Decreased	305	7.0	325	7.4	126	2.9
	Increased	155	3.5	122	2.8	189	4.3
Avg (4436 sites)	Decreased	279	6.3	313	7.1	26	0.6
	Increased	34	0.8	52	1.2	60	1.4

Of these, 278 phosphosites were significantly responsive to ERK1/2 inhibitors, SCH772984 and GDC0994, the majority of which were inhibited by both compounds and also identified in the trametinib-SCH772984 SILAC experiment. These included known proteins known to be ERK1/2 substrates, such as RAF1, Ets2 repressor factor (ERF), and cortactin (CTTN) (Fig. 2.8G, Table 2.5). Three phosphosites decreased significantly only in response to SCH772984, and 20 phosphosites decreased only in response to GDC0994 (Fig. 2.8G, Table 2.4). Phosphorylation of the RBMX transcription factor was inhibited by SCH772984 and not GDC0994, and also inhibited by only SCH772984 in the trametinib-SCH772984 experiment (Table 2.2). The two remaining phosphorylation sites unique to SCH772984 were part of the canonical TEY activation loop motif on ERK1/2 (Tyr204 on ERK1 and Tyr187 on ERK2). This indicates that while both SCH772984 and GDC0994 inhibit ERK1/2 kinase activity, only SCH772984 also inhibits its phosphorylation by MKK1/2. This finding was validated by Western blotting of whole cell lysates from WM239a cells treated with MKK1/2 and ERK1/2

inhibitors using antibodies specific for the TEY activation motif (Fig. 2.9A). Although ERK1/2

inhibitors GDC0994 and Vertex-11e did not decrease levels of phosphorylated ERK1/2, both

were able to reduce phosphorylation of p90RSK (RSK1), an important signaling kinase

downstream of ERK1/2, and inhibition of ERK phosphorylation by SCH772984 has previously

been shown (Morris et al., 2013). GDC0994 and Vertex-11e also increased levels of the

apoptosis markers, BIM and cleaved PARP, after 48 h treatment with 10 µM inhibitor. An

apoptotic response to each inhibitor was confirmed using fluorescence based flow cytometry to

quantify recombinant annexin V-FITC bound to apoptotic cells by the externalization of

phosphatidylserine, and propidium iodide (PI) staining of cells with permeabilized plasma

membranes (Fig. 2.9B). The results demonstrate similar levels of cell death in response to

MKK1/2 inhibitor, selumetinib, and ERK1/2 inhibitor, SCH772984, at 48 h, although Vertex-

11e is less effective.
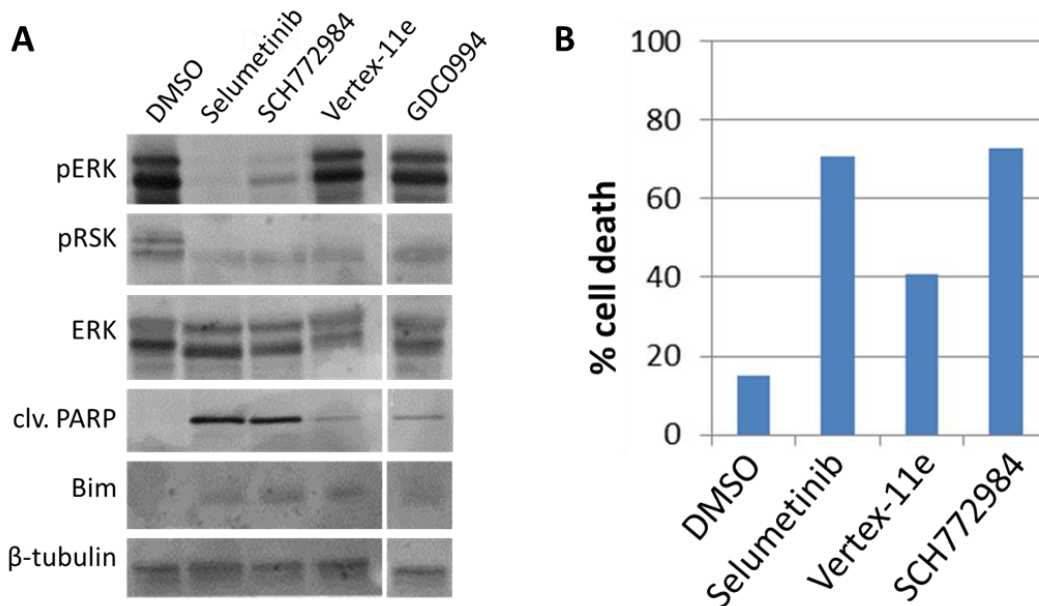
Figure 2.9: Apoptotic responses to MKK1/2 and ERK1/2 inhibitors in WM239a cells. A, Western blots of whole cell lysates harvested 48 h after treatment with 10 µM of each inhibitor. B, Annexin V-PI flow cytometry assay (Thermo Scientific) measuring apoptosis and cell permeabilization by flow cytometry after 48 h treatment with 10 µM of each inhibitor.

To explore whether the differences in phosphorylated TEY on ERK1/2 has an effect

on other downstream kinase substrates, the Human Phospho-Kinase Array (R & D Systems)

was used to simultaneously profile the relative levels of protein phosphorylation of 43

kinases and their substrates. Following 4 h treatment with 10 µM selumetinib (AZD6244),

Vertex-11e, or SCH772984, cell extracts were adsorbed onto membranes that were spotted

with capture antibodies for each kinase phosphorylation site, and then incubated overnight.

Processing and quantification was similar to a typical Western blotting procedure using a

Typhoon fluorescence image scanner and ImageQuant software (GE Healthcare) (Fig. 2.10

A). The results agreed with the individual phospho-ERK1/2 and phospho-RSK Western blots

in Fig. 2.9A and were grouped into categories based on response to inhibitor (Fig. 2.10B,

Appendix I). As expected, phosphorylation of some protein kinases showed no response to

MKK1/2 or ERK1/2 inhibitors (phospho-JNK1/2/3) while others were inhibited by all three

inhibitors (phospho-p70 S6 Kinase, phospho-STAT3). The kinases that responded uniquely

to certain inhibitors could be divided into those with phosphorylation sites that differed in

their responses to MKK1/2 inhibitor, selumetinib (AZD6244), and ERK1/2 inhibitors,

SCH772984 and Vertex-11e, and those with phosphorylation sites that responded specifically

to Vertex-11e. Interestingly, several kinase phosphorylation sites were increased in response

to Vertex-11e, including pTyr sites that suggest potential regulation of Tyrosine kinases and

phosphatases. However, we lack certainty that the different responses to MKK1/2 and

ERK1/2 inhibitors, and the increases in response to Vertex-11e, were necessarily

reproducible between replicate Western blots.
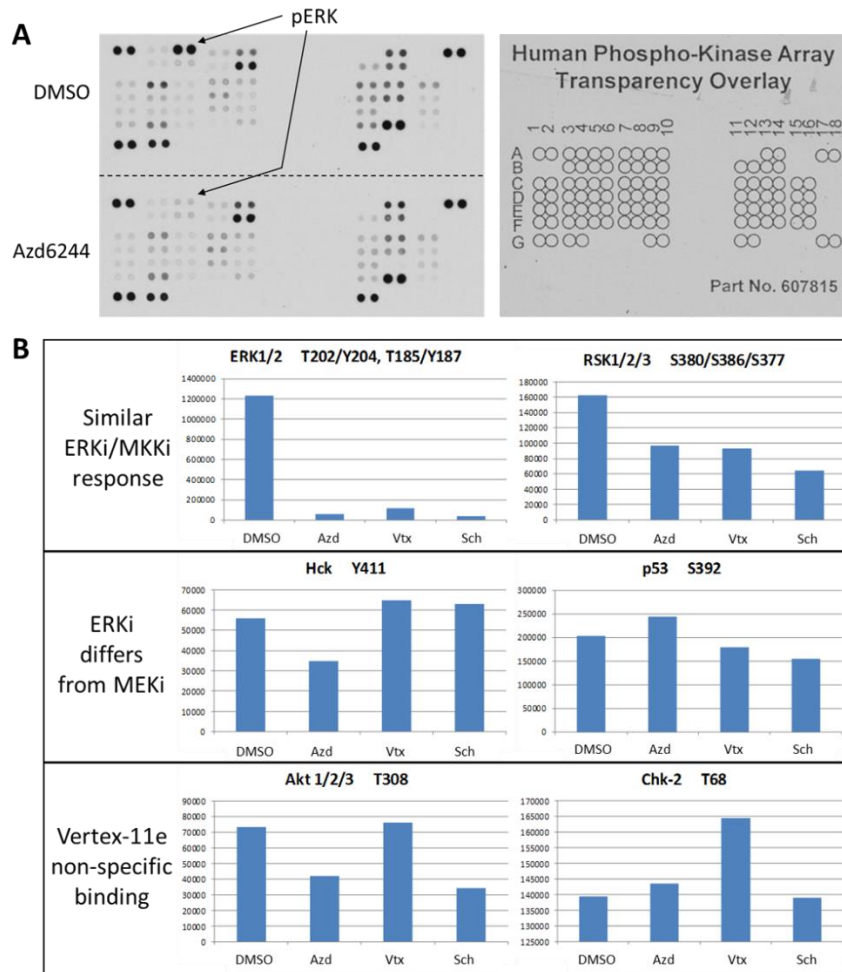
Figure 2.10: Human phospho-kinase antibody array. A, Changes in kinase phosphorylation in response to 10 µM treatment for 4 h. Decreased levels of pERK with MKK1/2 inhibitor selumetinib (AZD6244 - AZD) compared to DMSO control are indicated. The overlay is used as a key to look up the phosphorylated kinases identified in duplicate spots. B, Quantification of signal, measured as raw intensity of quantified Typhoon membrane scans using ImageQuant. Examples show groups that change in response to MKK1/2 inhibitor (AZD) or ERK1/2 inhibitor (SCH), as well as some that increase in response to Vertex-11e (VTX). Further studies are needed to validate these preliminary findings.

A previous study from our lab identified different binding affinities of Vertex-11e to ERK2 in its inactive, unphosphorylated form (Ki = 2.5 nM), and active, phosphorylated form (Ki = 0.34 nM) (Rudolph et al., 2015). Corresponding nuclear magnetic resonance (NMR) relaxation experiments further revealed that whereas the unphosphorylated ERK2 is restrained into a single conformer, phosphorylation reduces a thermodynamic barrier for forming a new conformer, allowing equilibrium interconversion between two conformers on a millisecond timescale. Vertex-11e binding shifts the equilibrium of phosphorylated ERK2 entirely to the new conformer. By contrast, unpublished studies show that SCH772984 shifts the equilibrium of phosphorylated ERK2 entirely to the conformer associated with unphosphorylated ERK2. Thus, Vertex-11e and SCH772984 both show properties of conformational selection, but differ in the conformers that they favor. The nature of these conformers is still unknown, however the result suggests that Vertex-11e and SCH772984 favor distinct binding modes. Therefore, I investigated if such differences in binding mode between Vertex-11e and SCH772984 might be reflected by any unique features of each inhibitor with respect to their cellular effects on protein phosphorylation.

Using triple-labeled SILAC phosphoproteomics, I compared phosphorylation responses in cells treated for 2 h with DMSO, 10 μM Vertex-11e, and 10 μM SCH772984, following procedures described above. Technical problems with data collection resulted in lower numbers of phosphopeptides than seen before, due to sample contaminants that blocked UPLC runs, such that LC-MS/MS data could be obtained only in ERLIC fractions #5-12. Nevertheless, in total, 2,701 class I phosphorylation sites were identified on 1,617 proteins, including 1,163 phosphosites quantified in at least two replicates (Fig 2.11 A,B, Table 2.6).
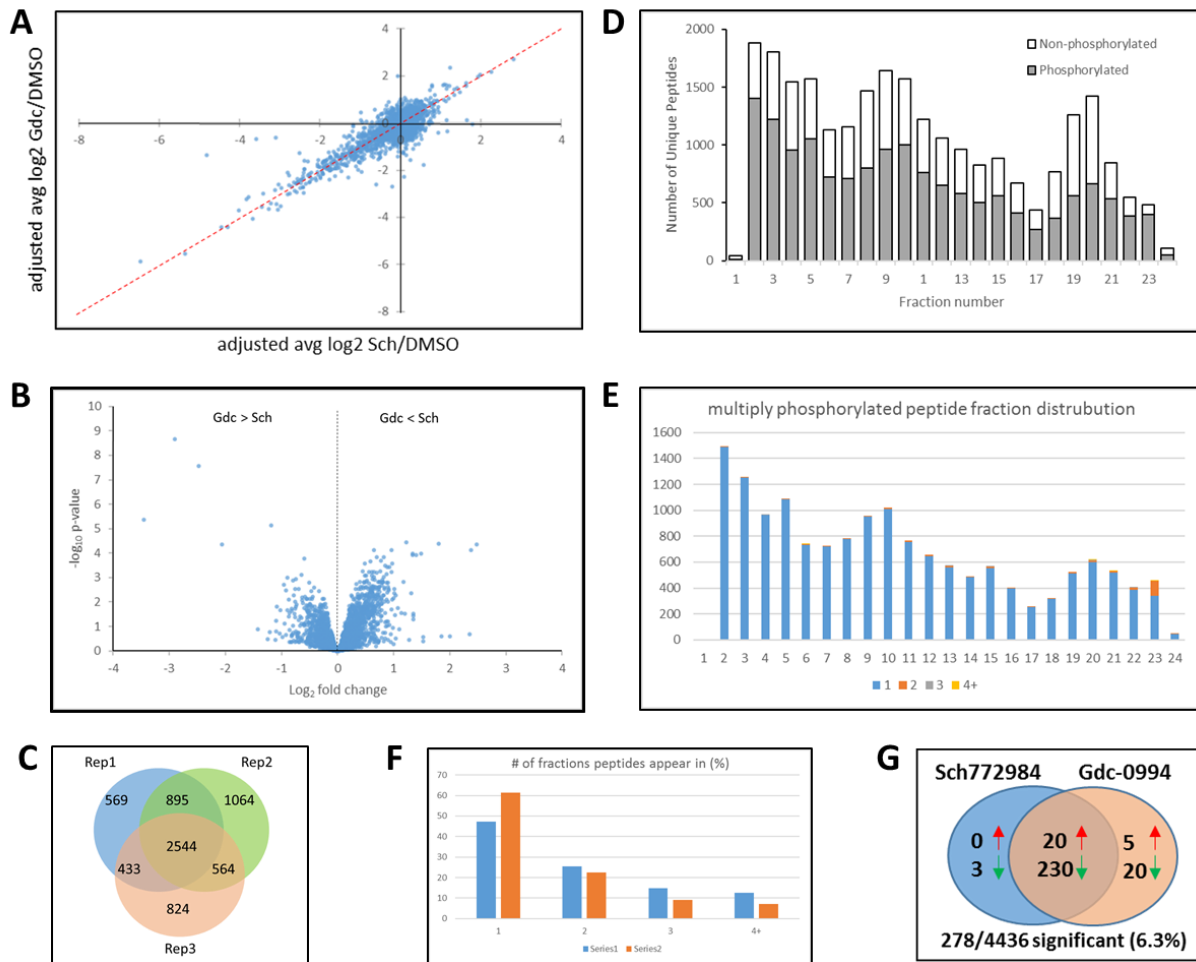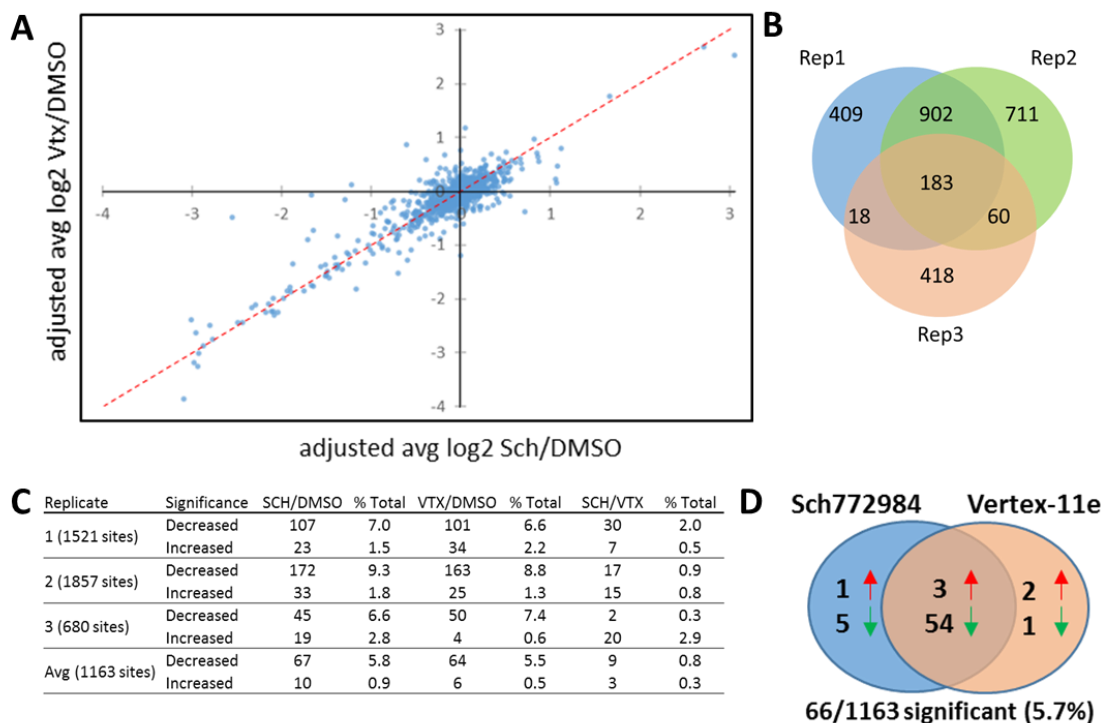
Figure 2.11: SILAC labeled phosphoproteomics comparison of SCH772984 and Vertex-11e. A, Log$_2$ transformed plot showing 1,163 phosphosites quantified in at least two replicate measurements of responses to treatment with 10 µM SCH772984 or 10 µM Vertex-11e each for 2 h. B, Overlap among three replicate experiments quantify 2,701 total phosphosites using SILAC. C, Number and percentages of phosphosites in each replicate showing a significant fold change in response to SCH772984 or Vertex-11e, where $|\log_2(drug/DMSO)| \geq 0.84$. D, Counts of significant changes seen in at least 2 replicates, with $|\log_2(combined\ ratio)| \geq 0.84$, and adjusted p-value >0.05.

I found that, like the previous SILAC comparisons, SCH772984 and Vertex-11e had almost complete overlap in the phosphosites that passed the statistical cutoff with adjusted p-value of <0.05. Only two were uniquely inhibited by SCH772984, and none were unique to Vertex-11e. One of the two unique sites was phospho-Tyr187 on ERK2 (MAPK1), which could be explained by the ability of SCH772984 to block the regulatory phosphorylation of ERK1/2 by MKK1/2. The corresponding site on ERK1 (MAPK3) was also uniquely inhibited, but variations in ratio between replicates yielded an adjusted p-value that was too high to consider. The other site was Ser270 on thioredoxin related transmembrane protein 1 (TMX1), has no

known function (Table 2.6). The results indicated that differences in conformational selection by these ERK1/2 inhibitors were not readily detectable by monitoring downstream drug targets in my experiments. Nevertheless, further phosphoproteomics studies which achieve higher sampling depth might be able to address this important question.

Table 2.6. Comparison of unique SCH7729834 and Vertex-11e significant phosphosites. [a] $\log_2$ values of quantified ratios in individual SILAC replicates. [b] empirical bayes generated $\log_2$ combined ratio and adjusted p-value. [=] phosphosites below double line are significantly increased in response to drug

Unique phosphosite changes in response to SCH772984

Uniprot ID	Gene	Position	9 aa window	SCH772984/DMSO[a]			Vertex-11e/DMSO[a]			SCH/VTX[b]	
				Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Combined	adj p-value
Q09666	AHNAK	210	IRLPSGSGA	-1.135	-1.449		-0.160	-0.144		-1.140	0.151
P28482	MAPK1	187	FLTEYVATR	-2.745	-2.182	-2.733	-0.740	-0.172	-0.545	-2.068	0.005
P27361	MAPK3	204	FLTEYVATR	-3.061	-1.901	0.031	-0.309	0.213	-2.053	-0.927	0.999
Q9H3N1	TMX1	270	IRQRSLGPS	-1.303	-1.127		0.121	0.121		-1.336	0.049
Q9ULJ3	ZBTB21	411	LRSFSASQS		0.025	-3.360		0.169	-0.312	-1.596	0.999
Q6VY07	PACS1	355	LEHVSREQI	1.005	1.123		0.622	-0.314		0.909	0.999

Unique phosphosite changes in response to Vertex-11e

Uniprot ID	Gene	Position	9 aa window	SCH772984/DMSO[a]			Vertex-11e/DMSO[a]			SCH/VTX[b]	
				Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Combined	adj p-value
Q9UJX6	ANAPC2	314	ARPASPEAG	-0.133	0.131		-2.314	-0.074		1.192	0.999
P21359	NF1	2543	GTRKSFDHL	-0.808	-0.412		0.479	1.262		-1.480	0.999
P61247	RPS3A	236	HGEGSSSGK	-0.076	0.166		1.782	0.561		-1.126	0.999

Information from phosphoproteomics comparisons of the two MKK1/2 inhibitors and two ERK1/2 inhibitors can be combined to provide a deeper understanding of pathway specificity. The overlap between significant phosphosite changes with each inhibitor is summarized in Fig 2.12. I highlighted four comparisons of interest. First, I noted 150 phosphosites that were significantly responsive to all four inhibitors. These are candidates for bona fide targets downstream of the BRAF/MKK/ERK signaling pathway. Second, I noted 4 phosphosites that were significantly responsive to both MKK1/2 inhibitors but neither ERK1/2 inhibitor. These are candidates for pathway bifurcation at the level of MKK1/2, upstream of ERK1/2. Third, zero phosphosites were significantly responsive to both ERK1/2 inhibitors but neither MKK1/2 inhibitor. Finally, I noted 48 phosphosites that were responsive to only one inhibitor, and none of the other three. These are candidates for off-target effects of each compound. Vertex-11e was excluded from this analysis because of the limited data collection in the DMSO/Vertex-11e/SCH772984 experiment.
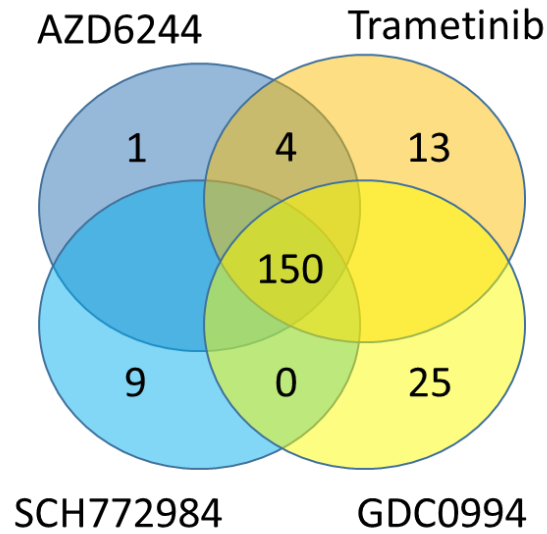
Figure 2.12: Counts of phosphosites that change significantly in response to each MKK1/2 and ERK1/2 inhibitor. Venn diagram summarizing phosphosites identified in each of three SILAC experiments, comparing DMSO-trametinib-SCH772984, DMSO-SCH772984-GDC0994, and DMSO-selumetinib (AZD6244)-vemurafenib, where the | $\log_2$ (combined ratio)| ≥ 0.84 in at least 2 replicates. In total, 3,382 phosphosites were quantified and comparable across all SILAC experiments. Phosphosites showing significant changes in both SCH772984 treatment experiments were combined. 3,021 phosphosites showed no significant change in response to any inhibitor. Additional overlap remains to be validated.

Phosphosites affected by all four treatments are those most likely to be bona fide targets of the BRAF-MKK1/2-ERK1/2 signaling pathway. I examined these for known and potentially novel targets of signaling. Of the 150 phosphosites that were significantly altered, 141 phosphosites decreased, while 9 increased in response to all inhibitors (Table 2.7). Two-thirds of these phosphosites corresponded to the canonical ERK1/2 substrate recognition motif (Ser/Thr-Pro). Of these, 40 phosphosites were located on proteins known to be ERK1/2 substrates, and 16 of these were known sites for regulation by phosphorylation (Carlson et al., 2011; Hornbeck et al., 2015; Yoon & Seger, 2006). Thus, the majority of phosphosites regulated by all four inhibitors either corresponded to novel MAPK pathway targets, or were uncharacterized with respect to function. This means that MAPK signaling potentially regulates many new processes, and that comparing phosphorylation responses to different small molecules enables the lengthy lists of phosphosites typically generated by these experiments to be filtered in a manner that prioritizes their importance. The subset of genes regulated by all four inhibitors at Ser/Thr-Pro sites (100 phosphosites on 88 genes) was analyzed using STRING software (http://string-db.org/) (Szklarczyk et al., 2015), which reports likely gene networks based on different types of interactions reported by literature curation and public datasets. Thirty-one of these genes fell into four subnetworks, including (i) growth factor signaling and transcriptional regulation, (ii) small GTPase signaling, (iii) nuclear pore complex, and (iv) cellular structure, localization and trafficking. Many proteins in these networks were previously identified as ERK1/2 pathway targets in our previous screen (Stuart et al., 2015) including the nucleoporins (Courcelles et al., 2013; Kosako et al., 2009), c-Jun (Pulverer, Kyriakis, Avruch, Nikolakaki, & Woodgett, 1991), and ERF (von Kriegsheim et al., 2009).

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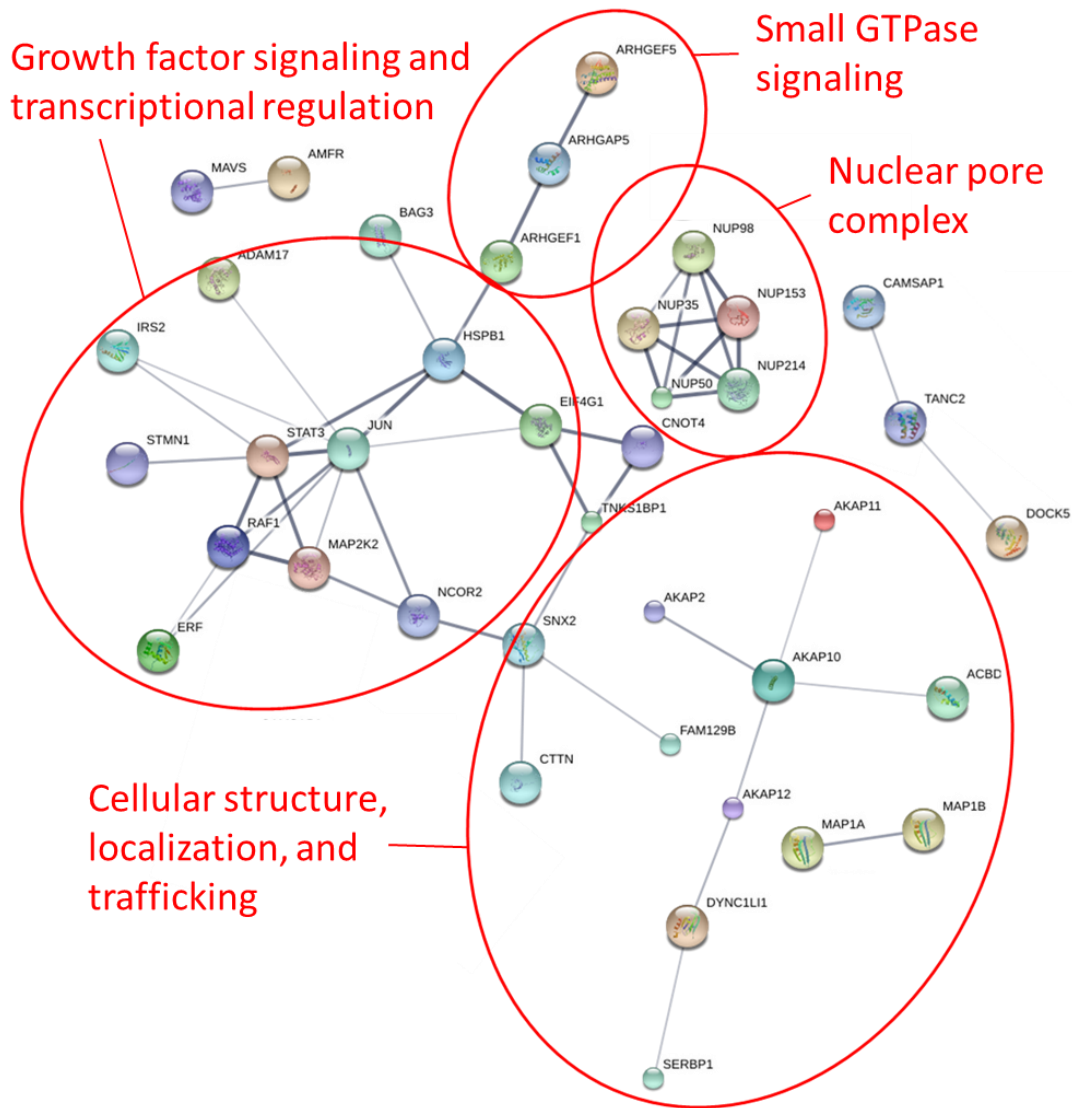
Figure 2.13: Subnetworks of proteins containing phosphosites responsive to all four MKK1/2 and ERK1/2 inhibitors. STRING software was used to examine protein networks among 88 genes containing 100 Ser/Thr-Pro phosphosites significantly regulated by selumetinib (AZD6244), trametinib, SCH772984, and GDC0994. Edges (interactions) between genes nodes (genes) were identified with medium confidence score of 0.7 (Szklarczyk et al., 2015). Color and size of nodes are arbitrary. Disconnected nodes that could not be linked to others are not shown. Edge thickness indicates confidence level of interaction.

Table 2.7. Phosphosites significantly regulated by all inhibitors (trametinib, selumetinib (AZD6244), SCH772984, GDC0994) in SILAC experiments. Log2 values of quantified ratios in individual SILAC replicates. Empirical bayes generated log2 combined ratio and adjusted p-value. Phosphosites below double line are significantly increased in response to inhibitor log2 combined ratio and adjusted p-value.

#	Uniprot ID	Gene	Position n	9aa Window	Exp1 SCH772984/DMSO Rep1	Rep2	Rep3	Combined	adj p-value	Exp1 Trametinib/DMSO Rep1	Rep2	Rep3	Combined	adj p-value	Exp2 AZD6244/DMSO Rep1	Rep2	Rep3	Combined	adj p-value	Exp2 SCH772984/DMSO Rep1	Rep2	Rep3	Combined	adj p-value	Exp3 GDC0994/DMSO Rep1	Rep2	Rep3	Combined	adj p-value
1	Q2M2I8	AAK1	846	QRLPSQTES		-1.935	-1.260	-1.644	<0.001		-2.735	-2.862	-2.752	<0.001	-1.645	-1.066		-1.328	<0.001	-1.677	-2.080		-1.880	<0.001	-1.714	-1.570		-1.640	<0.001
2	Q9H3P7	ACBD3	43	LPPPSPPGS	-1.273	-1.515	-0.990	-1.266	<0.001	-1.097	-1.511	-1.321	-1.303	<0.001	-1.433	-1.562	-1.548	-1.503	<0.001	-1.045	-1.421	-0.985	-1.156	<0.001	-1.274	-1.796	-1.642	-1.565	<0.001
3	Q9UKV3	ACIN1	710	ARRLSQPES	-0.985	-1.322	-1.352	-1.210	<0.001	-1.360	-1.302	-1.234	-1.309	<0.001	-1.366	-1.288	-1.641	-1.416	<0.001	-1.320	-1.474		-1.386	<0.001	-1.253	-1.359		-1.317	<0.001
4	P78536	ADAM17	735	PAPQTPGRL	-1.69	-1.72	-1.05	-1.49	<0.001	-1.78	-2.29	-1.88	-1.99	<0.001	-1.71	-1.57	-1.48	-1.57	<0.001	-1.58			-1.89	0.001	-1.31	-1.92		-1.60	0.002
5	Q8WVP5	AHCTF1	1232	ETRISFVEE	-1.195	-1.288	-1.206	-1.157	<0.001	-0.814	-1.275	-1.509	-1.272	<0.001	-0.839	-1.033	-1.105	-0.966	<0.001	-1.393	-0.874	-1.156	-1.134	<0.001	-1.620	-1.285	-1.281	-1.402	<0.001
6	Q09666	AHNAK	135	PRLKSEDGV	-6.353		-6.692	-6.525	<0.001	-5.923		-7.501	-6.709	<0.001	-6.230	-4.955	-3.147	-4.908	<0.001	-6.277	-6.680		-6.458	<0.001	-6.027	-5.653		-5.861	<0.001
7	Q09666	AHNAK	5110	APLPSPKLE	-3.983	-4.275	-3.296	-3.854	<0.001	-3.979	-4.560	-4.143	-4.224	<0.001	-3.773	-4.104	-3.925	-3.921	<0.001	-4.374	-3.687	-4.018	-4.026	<0.001	-4.234	-3.508	-3.339	-3.694	<0.001
8	Q09666	AHNAK	3426	LNLKSPKVK	-2.875		-1.878	-2.407	<0.001	-1.792		-3.336	-2.533	0.021	-2.733	-2.566	-2.089	-2.351	<0.001	-3.032		-2.558	-2.803	<0.001	-3.342	-2.715		-3.021	<0.001
9	Q09666	AHNAK	2397	LHLKSPKAK	-2.893	-2.888	-2.593	-2.844	<0.001	-2.367	-2.601	-2.437	-2.415	<0.001	-2.745	-2.708	-2.220	-2.511	<0.001	-2.453	-2.149	-3.341	-2.637	<0.001	-3.019	-2.637	-2.912	-2.867	<0.001
10	Q09666	AHNAK	511	LSLGSPKLK	-1.592	-1.553	-1.058	-1.389	<0.001	-0.895	-1.176	-1.305	-1.137	<0.001	-1.302	-1.290	-1.087	-1.216	<0.001	-1.793	-0.573	-1.144	-1.171	0.008	-1.920	-0.592	-0.519	-1.009	0.012
11	O43572	AKAP10	187	AEPVSPSKK		-1.13	-1.11	-1.16	<0.001		-1.07	-0.97	-0.98	<0.001		-0.92	-1.04	-0.97	<0.001	-0.67	-0.95		-0.84	0.002	-0.83	-1.20		-0.99	<0.001
12	Q9UKA4	AKAP11	448	SGLFSPIRS	-1.289	-1.279	-1.511	-1.334	<0.001	-1.130	-1.208	-1.283	-1.232	<0.001	-1.167	-1.305	-1.315	-1.267	<0.001	-1.448	-1.632	-1.275	-1.445	<0.001	-1.874	-1.767	-1.586	-1.749	<0.001
13	Q02952	AKAP12	286	ESPTSPVTS	-1.55	-1.98	-1.00	-1.50	<0.001	-1.23	-2.10	-1.79	-1.71	<0.001	-1.50	-1.58	-1.18	-1.42	<0.001	-0.80	-1.27	-0.85	-0.96	0.001	-1.07	-1.49	-1.69	-1.43	<0.001
14	Q9Y2D5	AKAP2	748	QSTQSPRTK	-2.975	-3.390		-3.225	<0.001	-3.090	-3.162		-3.083	<0.001	-3.035	-2.796	-3.146	-2.991	<0.001	-2.952	-2.741	-3.179	-2.967	<0.001	-2.978	-2.587	-2.708	-2.748	<0.001
15	Q9Y2D5	AKAP2	152	QVPVSPSST	-1.163	-1.057	-1.316	-1.172	<0.001	-1.373	-1.074	-1.202	-1.224	<0.001	-1.099	-0.822	-1.506	-1.147	<0.001	-1.232	-1.023	-1.137	-1.117	<0.001	-1.313	-1.227	-1.135	-1.239	<0.001
16	Q9UKV5	AMFR	542	PLDLSPRLE		-1.503	-2.591	-2.600	<0.001		-2.743	-2.580	-2.682	<0.001	-2.829	-2.692	-2.819	-2.774	<0.001	-2.548	-2.318	-2.329	-2.402	<0.001	-2.980	-3.199	-2.746	-2.972	<0.001
17	Q9HCE9	ANO8	641	LEEGSPTMV		-1.503	-1.268	-1.401	<0.001		-1.098	-1.015	-1.040	<0.001	-1.359	-1.177	-1.442	-1.318	<0.001	-0.854	-1.057	-1.189	-1.086	<0.001	-1.110	-1.252	-1.194	-1.133	<0.001
18	O14617	AP3D1	658	KHRPSEADE	-1.97	-2.14	-1.83	-1.98	<0.001	-1.88	-1.98	-1.96	-1.94	<0.001	-2.03	-2.15	-1.84	-2.02	<0.001	-2.18		-1.63	-1.91	<0.001	-2.22		-2.11	-2.15	<0.001
19	Q13017	ARHGAP5	968	VFLPSPRDC	-1.708	-1.881	-1.579	-1.722	<0.001	-1.606	-1.919	-1.953	-1.826	<0.001	-1.794	-1.883	-1.688	-1.775	<0.001	-1.770	-1.811	-1.491	-1.689	<0.001	-1.882	-2.197	-1.789	-1.957	<0.001
20	Q92888	ARHGEF1	863	GGPLSPART	-1.532	-1.204	-1.249	-1.346	<0.001	-1.767	-1.297	-1.383	-1.464	<0.001	-1.061	-1.078	-1.212	-1.122	<0.001	-1.031	-0.867	-0.854	-0.905	<0.001	-0.968	-1.177	-0.827	-1.003	<0.001
21	Q8TER5	ARHGEF40	1474	SGDVSPGPR	-1.763		-1.355	-1.540	<0.001	-1.136		-1.680	-1.427	<0.001	-1.110	-1.396	-1.357	-1.310	<0.001	-0.726	-1.093		-0.943	0.001	-1.668	-1.567		-1.584	<0.001
22	Q12774	ARHGEF5	450	PAALSPSLE	-1.983	-0.731	-1.516	-1.388	0.002	-1.678	-1.186	-2.207	-1.713	<0.001	-1.901	-2.487	-2.063	-2.165	<0.001	-2.251	-2.212	-1.349	-1.908	<0.001	-1.440	-2.479	-1.556	-1.855	<0.001
23	Q12774	ARHGEF5	445	AEELSPAAL	-1.349	-1.940	-1.183	-1.505	<0.001	-1.141	-1.873	-1.592	-1.521	<0.001	-1.139	-1.630	-1.320	-1.334	<0.001	-1.427	-1.352	-0.431	-1.084	0.006	-0.807	-1.787	-0.934	-1.161	<0.001
24	Q14155	ARHGEF7	694	ERKPSDEEF	-1.121	-1.312	-1.399	-1.283	<0.001	-1.209	-1.097	-1.220	-1.169	<0.001	-1.406	-1.702	-1.635	-1.584	<0.001	-1.826	-2.369	-1.687	-1.626	<0.001		-1.282	-1.366	-1.316	<0.001
25	P0C7T5	ATXN1L	284	VRRESEALD	-3.86		-3.09	-3.44	<0.001	-3.70		-3.72	-3.75	<0.001	-4.02	-4.02	-4.08	-4.06	<0.001	-3.91	-3.74		-3.82	<0.001	-3.34	-3.14		-3.24	<0.001
26	O95817	BAG3	377	CPPPSPGPS	-1.45	-1.53	-0.83	-1.29	<0.001	-1.26	-1.31	-0.97	-1.16	<0.001	-1.12	-0.95	-1.03	-1.06	<0.001	-1.41	-1.06	-0.79	-1.08	<0.001	-1.09	-1.10	-0.93	-1.05	<0.001
27	Q8NFC6	BOD1L1	2973	KRQKSVSDP	-1.191	-1.314	-0.746	-1.069	<0.001	-1.044	-1.455	-1.241	-1.261	<0.001	-1.637	-1.500	-1.409	-1.475	<0.001	-1.270	-0.637	-1.226	-1.321	0.012	-0.992	-1.043		-0.992	0.008
28	Q8NFC6	BOD1L1	2954	KPKRSLTVS	-1.317	-1.291	-1.330	-1.289	<0.001	-1.533	-0.893	-1.443	-1.313	<0.001	-1.416	-1.209		-1.343	<0.001	-1.462		-1.226	-1.321	<0.001	-1.091	-0.834		-0.986	<0.001
29	Q9H6R7	C2orf44	690	SFSHSPGAV	-1.242		-0.946	-1.129	<0.001	-1.648		-1.478	-1.528	<0.001	-1.205	-1.242	-1.062	-1.216	<0.001	-1.003	-1.213		-1.100	0.001	-1.493	-1.163		-1.336	<0.001
30	C9orf72	C9orf72	9	CPPPSPAVA	-2.559	-2.398	-2.393	-2.458	<0.001	-2.619	-2.622	-2.734	-2.651	<0.001	-2.707	-2.464	-2.905	-2.698	<0.001	-2.347	-2.304	-2.057	-2.202	<0.001	-2.502	-2.129	-2.362	-2.365	<0.001
31	Q5TSY3	CAMSAP1	1144	SHPRTPTDP	-1.811		-1.768	-1.787	<0.001	-1.843		-2.420	-2.134	<0.001	-2.130	-2.208	-1.796	-2.041	<0.001	-2.429		-1.426	-1.963	0.002	-2.110		-2.066	-2.053	<0.001
32	Q5TSY3	CAMSAP1	1080	VEPLSPTGV	-2.056		-2.074	-2.061	<0.001	-2.060		-2.498	-2.283	<0.001	-2.143	-1.961	-1.941	-1.985	<0.001	-1.826	-2.369	-1.687	-2.051	<0.001	-1.600	-2.049	-2.088	-1.822	<0.001
33	P48960	CD97	831	ALRASESGI	-2.63		-2.59	-2.61	<0.001	-2.73		-2.98	-2.86	<0.001	-2.90	-2.78	-2.77	-2.80	<0.001	-3.20	-3.09		-3.12	<0.001	-2.80	-2.81		-2.83	<0.001
34	Q9UKI2	CDC42EP3	144	LPRLSCEPV	-1.960	-1.755	-2.244	-1.976	<0.001	-2.137	-1.535	-2.020	-1.907	<0.001	-1.709	-1.658	-1.796	-1.710	<0.001	-2.515		-2.261	-2.464	<0.001	-2.953	-1.996		-2.398	<0.001
35	Q9UKI2	CDC42EP3	100	TETPSPVLK	-1.711	-1.729	-1.649	-1.692	<0.001	-1.692	-1.516	-1.871	-1.691	<0.001	-1.357	-1.765	-1.275	-1.466	<0.001	-1.619	-1.179	-1.473	-1.428	<0.001	-1.711	-1.360	-1.318	-1.458	<0.001
36	Q9Y4F5	CEP170B	1548	QSPPSPASA	-1.242		-1.689	-1.513	<0.001	-1.648		-1.743	-1.696	<0.001	-1.202	-1.387	-1.333	-1.326	<0.001	-0.887	-1.442		-1.177	0.002	-1.215	-1.168		-1.180	0.001
37	O14757	CHEK1	280	PRVTSGGVS	-1.99	-2.09	-2.06	-2.03	<0.001	-1.75	-0.92	-1.38	-1.35	0.001	-0.78	-1.05		-0.91	0.002	-1.44		-1.81	-1.62	<0.001	-1.95		-1.90	-1.93	<0.001
38	Q96S66	CLCC1	438	TGNKSPEVL	-1.490	-1.438	-1.061	-1.286	<0.001	-1.083	-1.748	-1.349	-1.437	<0.001	-1.324		-1.343	-1.362	<0.001	-1.279	-1.157		-0.893	0.115	-0.580	-1.777		-1.503	0.013
39	Q95628	CNOT4	432	QPSLSPTSL	-1.30		-0.84	-1.06	<0.001	-0.99		-0.84	-0.92	<0.001	-1.20	-1.32	-1.18	-1.23	<0.001	-1.05	-0.94		-1.01	<0.001	-1.27	-1.09		-1.17	<0.001
40	Q53SF7	COBLL1	993	EQTLSPLSK	-1.711	-1.540	-1.731	-1.666	<0.001	-1.633	-1.547	-1.838	-1.667	<0.001	-1.684	-1.768	-1.796	-1.744	<0.001	-1.520	-1.508	-1.429	-1.477	<0.001	-2.139	-1.960	-2.052	-2.058	<0.001
41	Q53ET0	CRTC2	433	RVPLSPLSL	-1.350		-1.689	-1.513	<0.001	-1.636		-1.743	-1.696	<0.001	-1.466	-1.623	-1.309	-1.454	<0.001	-1.401	-1.429	-1.824	-1.562	<0.001	-1.910	-1.694	-1.828	-1.800	<0.001
42	O43310	CTIF	299	EAPRSPDTL	-1.99	-2.09	-2.06	-2.03	<0.001	-2.07	-2.20	-2.36	-2.22	<0.001	-2.24	-2.27	-2.43	-2.31	<0.001	-2.18	-2.09	-1.87	-2.02	<0.001	-1.92	-2.10	-1.47	-1.85	<0.001
43	Q14247	CTTN	401	AKTQTPPVS	-1.891	-2.041	-2.257	-2.062	<0.001	-2.161	-1.774	-2.252	-2.064	<0.001	-1.759	-2.015	-1.800	-1.859	<0.001	-1.850		-2.107	-1.985	<0.001	-2.186	-2.392		-2.282	<0.001
44	Q14247	CTTN	405	TPPVSPAPQ	-1.891	-2.051	-2.257	-2.070	<0.001	-2.161	-1.781	-2.252	-2.061	<0.001	-1.759	-1.986	-1.806	-1.851	<0.001	-1.850	-1.815	-2.107	-1.937	<0.001	-2.186	-1.776	-2.392	-2.105	<0.001
45	Q14247	CTTN	418	RUPSSPVVE	-1.093	-1.358	-1.525	-1.321	<0.001	-1.408	-0.890	-1.828	-1.380	<0.001	-0.949	-1.228	-1.314	-1.169	<0.001	-0.981	-1.129	-0.992	-1.032	<0.001	-1.209	-1.059	-1.180	-1.151	<0.001
46	Q5VWQ8	DAB2IP	702	TRLPSPTPE		-1.474	-1.493	-1.466	<0.001		-1.432	-1.408	-1.437	<0.001	-1.359	-1.375	-1.290	-1.325	<0.001	-1.575		-1.459	-1.525	<0.001	-1.474	-1.589		-1.523	<0.001
47	Q9H7D0	DOCK5	1756	PDLMSPTRK	-2.628	-3.042		-2.850	<0.001	-2.530	-3.000		-2.750	<0.001	-2.681	-2.439	-2.549	-2.523	<0.001	-2.754	-2.543	-2.454	-2.581	<0.001	-2.976	-3.016	-2.763	-2.921	<0.001
48	Q9Y6G9	DYNC1LI1	516	TTPTSPTEG	-1.279	-1.160	-1.157	-1.198	<0.001	-1.831	-1.859	-1.726	-1.806	<0.001	-1.318	-1.495	-1.367	-1.389	<0.001	-1.485	-1.221	-1.016	-1.241	<0.001	-1.266	-1.415	-1.163	-1.281	<0.001
49	Q6P2E9	EDC4	780	LHLLSPRPR	-1.802	-2.063	-2.084	-1.973	<0.001	-1.832	-2.114	-1.619	-1.864	<0.001	-1.939	-2.031	-1.773	-1.888	<0.001	-1.910	-1.798	-1.754	-1.804	<0.001	-1.996	-1.798	-1.953	-1.932	<0.001
50	Q8NDI1	EHBP1	1058	QRKPSEDEV	-2.715	-2.782	-2.572	-2.677	<0.001	-2.256	-2.708	-2.819	-2.607	<0.001	-2.560	-2.625	-2.571	-2.589	<0.001	-2.475	-2.253	-2.170	-2.275	<0.001	-1.949	-2.407	-2.032	-2.154	<0.001

Table 2.7. Phosphosites significantly regulated by all inhibitors (trametinib, AZD6244, SCH772984, GDC0994) in SILAC experiments. Log2 values of quantified ratios in individual SILAC replicates. Empirical bayes generated log2 combined ratio and adjusted p-value. Phosphosites below double line are significantly increased in response to inhibitor.

#	Uniprot ID	Gene	Position n	9aa Window	Exp1 SCH772984/DMSO Rep 1	Rep 2	Rep 3	Combined	adj p-value	Exp1 Trametinib/DMSO Rep 1	Rep 2	Rep 3	Combined	adj p-value	Exp2 AZD6244/DMSO Rep 1	Rep 2	Rep 3	Combined	adj p-value	Exp2 SCH772984/DMSO Rep 1	Rep 2	Rep 3	Combined	adj p-value	Exp3 GDC0994/DMSO Rep 1	Rep 2	Rep 3	Combined	adj p-value
51	Q96KQ7	EHMT2	232	RKLNSGGGL	-1.373	-1.454	-1.449	-1.429	<0.001	-1.226	-1.183	-1.473	-1.290	<0.001	-1.575	-1.062	-1.132	-1.324	<0.001	-1.290	-2.058		-1.787	0.010		-1.578	-1.158	-1.255	0.029
52	Q04637	EIF4G1	1231	LPPVSPNLK	-2.01	-1.91	-1.79	-1.90	<0.001	-1.70	-1.86	-1.80	-1.79	<0.001	-1.74	-1.67	-1.89	-1.77	<0.001	-1.78	-1.60	-1.45	-1.60	<0.001	-1.63	-1.90	-1.38	-1.65	<0.001
53	Q32P44	EML3	198	DPLSSPGGP	-1.799	-1.750		-1.773	<0.001	-1.444	-1.652		-1.549	<0.001	-1.559	-1.421	-1.462	-1.494	<0.001	-1.669	-1.480	-1.172	-1.444	<0.001	-1.540	-1.433	-1.071	-1.344	<0.001
54	Q9Y6I3	EPN1	435	VPARSPGAF	-1.973	-1.976	-1.923	-1.965	<0.001	-2.158	-2.156	-2.237	-2.176	<0.001	-2.148	-2.020	-2.077	-2.074	<0.001	-1.850	-1.643	-1.776	-1.755	<0.001	-2.033	-1.991	-1.630	-1.886	<0.001
55	P50548	ERF	526	GGPLTPRRV	-2.89	-2.82	-3.38	-3.01	<0.001	-3.04	-2.67	-3.11	-2.96	<0.001	-2.63	-2.69	-2.99	-2.76	<0.001	-3.26	-2.81	-3.62	-3.21	<0.001	-3.59	-3.46	-3.14	-3.41	<0.001
56	Q96TA1	FAM129B	696	SPPASPLQH	-1.850	-2.022	-1.683	-1.861	<0.001	-1.785	-1.283	-1.898	-1.646	<0.001	-1.795	-2.070	-1.841	-1.900	<0.001	-1.867	-1.922	-1.484	-1.757	<0.001	-1.816	-1.896	-2.015	-1.910	<0.001
57	Q96TA1	FAM129B	692	PEASSPPAS	-1.831		-1.603	-1.725	<0.001	-1.792		-1.705	-1.740	<0.001	-1.841	-2.066	-1.763	-1.898	<0.001	-1.775		-1.484	-1.606	<0.001	-1.549		-2.015	-1.806	<0.001
58	Q86VR2	FAM134C	10	GVPTTPGPA	-2.212	-1.268	-2.069	-1.893	<0.001	-2.472	-1.808	-1.921	-2.023	<0.001	-1.848	-1.749	-1.728	-1.770	<0.001	-2.210	-1.849		-1.990	<0.001	-2.364	-2.221		-2.332	<0.001
59	Q4G0A6	FAM188B	392	EVILSPVPS	-2.071	-1.494		-1.759	<0.001	-1.786	-1.909		-1.871	<0.001	-1.751	-1.652	-1.336	-1.584	<0.001	-2.163	-2.056	-1.274	-1.832	<0.001	-2.086	-1.951	-1.559	-1.865	<0.001
60	P49327	FASN	2204	LACPTPKED	-0.81	-0.96	-0.95	-0.91	<0.001	-0.79	-1.06	-1.28	-1.04	<0.001	-1.19	-1.13	-1.14	-1.18	<0.001	-1.17	-0.81	-1.19	-1.07	<0.001	-1.56	-1.41	-1.27	-1.40	<0.001
61	Q9U1I4	GGT7	72	QRLPSSSSE	-2.608	-1.875	-2.096	-2.150	<0.001	-2.145	-2.046	-2.161	-2.161	<0.001	-2.873	-2.690	-2.306	-2.520	<0.001	-3.061	-3.430	-2.811	-3.084	<0.001	-3.575	-3.102	-3.606	-3.445	<0.001
62	Q6Y7W6	GIGYF2	593	RVPFSPGPA		-2.150	-1.749	-1.954	<0.001		-2.129	-1.814	-1.967	<0.001	-1.274	-2.204	-2.113	-1.863	<0.001	-1.694	-1.824	-1.749	-1.753	<0.001	-1.784	-2.032	-2.223	-2.016	<0.001
63	Q6Y7W6	GIGYF2	26	GSITSPPLS	-1.336	-1.409	-1.212	-1.314	<0.001	-1.082	-1.240	-1.333	-1.223	<0.001	-1.152	-1.231	-1.193	-1.183	<0.001	-0.896	-1.330	-1.211	-1.135	<0.001	-1.051	-1.658	-1.904	-1.548	<0.001
64	Q9UKJ3	GPATCH8	1107	ERKPSVSEE	-1.518	-1.152	-1.322	-1.322	<0.001	-1.539	-1.438	-1.501	-1.501	<0.001	-2.011	-1.802	-1.709	-1.824	<0.001	-1.748	-1.547		-1.662	<0.001	-1.851	-1.274		-1.548	<0.001
65	Q62VF9	GPRIN3	57	EPDLSPRAA	-0.823	-1.334	-1.224	-1.133	<0.001	-1.116	-0.916	-1.469	-1.161	<0.001	-1.204	-0.932	-1.086	-1.090	<0.001	-1.056	-1.063	-0.957	-0.982	<0.001	-1.127	-1.214	-1.214	-1.228	<0.001
66	P04792	HSPB1	65	AAIESPAVA	-1.70	-0.56		-1.17	0.016	-2.72	-2.16		-2.40	<0.001	-1.69	-1.51	-1.56	-1.58	<0.001	-1.60	-1.20	-0.30	-1.10	0.005	-1.44	-1.22	-1.19	-1.22	0.002
67	Q9Y4H2	IRS2	1203	EPPTSPRQL	-1.313	-1.673	-1.461	-1.461	<0.001	-1.514	-1.832	-1.706	-1.706	<0.001	-1.402	-1.508	-1.448	-1.438	<0.001	-1.028	-1.100	-1.266	-1.146	<0.001	-1.296	-1.172	-1.772	-1.398	<0.001
68	Q96ST2	IWS1	720	RRMNSTGGQ	-1.238	-1.282	-0.857	-1.117	0.002	-0.887	-1.440	-1.778	-1.377	<0.001	-1.136	-1.044	-1.017	-1.085	<0.001	-2.783	-0.231	-0.933	-1.153	0.110	-2.190	-0.144	-0.759	-1.194	0.069
69	P05412	JUN	63	DLLTSPDVG	-1.05		-1.42	-1.21	<0.001	-1.93		-2.17	-2.07	<0.001	-1.20	-0.65		-0.92	<0.001	-1.54	-0.04	-1.58	-1.05	0.045	-2.84	-1.43	-1.66	-1.98	0.001
70	Q2LD37	KIAA1109	3653	VDAASPGPR	-2.312	-0.141	-2.433	-1.646	0.021	-2.095	0.023	-2.544	-1.521	0.029	0.020	-2.505	-2.399	-1.708	0.013	-3.111	-2.893		-3.025	<0.001	-2.703	-2.564		-2.611	<0.001
71	Q62U35	KIAA1211	874	KRHSSTGDS	-1.080	-1.287	-1.569	-1.296	<0.001	-1.172	-0.788	-1.552	-1.187	<0.001	-1.449	-1.428	-1.369	-1.419	<0.001	-0.915	-1.645	-1.350	-1.304	0.002	-1.120	-0.873	-1.831	-1.273	0.001
72	O14686	KMT2D	2274	EPILSPPPF		-1.34	-1.34	-1.29	<0.001		-1.26	-1.66	-1.51	<0.001	-1.34	-1.39	-1.46	-1.38	<0.001	-1.37	-1.24	-1.78	-1.46	<0.001	-1.58	-1.34	-1.55	-1.49	<0.001
73	P78559	MAP1A	504	SEPQTPPAQ	-2.76	-3.39		-3.07	<0.001	-2.49	-3.08		-2.80	<0.001	-0.31	-2.33	-2.94	-1.92	<0.001	-2.23	-2.54		-2.57	<0.001	-2.02	-3.09		-2.37	<0.001
74	P46821	MAP1B	1797	SPLYSPTFS	-1.58	-1.58		-1.58	<0.001	-1.07	-1.68		-1.38	<0.001	-1.77	-1.56	-1.84	-1.74	<0.001	-2.13	-1.38		-1.74	0.002	-1.35	-1.52		-1.45	0.003
75	P11137	MAP2	821	SRLASVSAD	-1.28	-0.87	-1.43	-1.21	<0.001	-1.47	-1.17	-1.37	-1.32	<0.001	-0.89	-0.88	-0.87	-0.89	<0.001	-1.68	-0.66	-0.80	-1.03	0.005	-1.30	-0.93	-0.25	-0.84	0.011
76	P36507	MAP2K2	295	PHSISPRPR	-1.43	-1.64	-1.39	-1.48	<0.001	-1.25	-1.61	-1.61	-1.49	<0.001	-1.38	-1.44	-1.49	-1.44	<0.001	-1.38	-1.37	-1.37	-1.39	<0.001	-1.71	-1.81	-1.67	-1.71	<0.001
77	P36507	MAP2K2	293	GEPHSISPR	-1.65	-1.64	-0.09	-1.11	0.015	-1.77	-1.59	-1.23	-1.55	0.015	-0.98	-0.83	-1.60	-1.13	0.003	-1.47	-1.01	-1.15	-1.22	0.003	-1.62	-1.71	-1.37	-1.55	<0.001
78	P29966	MARCKS	101	EAGASPVEK	-1.55	-1.95	-1.97	-1.77	<0.001	-1.60	-1.64	-1.85	-1.75	<0.001	-1.30	-1.61	-1.81	-1.60	<0.001	-1.56	-1.57	-1.60	-1.57	<0.001	-1.66	-1.68	-1.78	-1.71	<0.001
79	P43243	MATR3	188	FRRDSFDDR	-1.27	-0.92	-1.42	-1.19	<0.001	-1.15	-0.91	-1.11	-1.07	<0.001	-0.89	-1.00	-0.96	-0.96	<0.001	-1.10	-1.04	-1.05	-1.04	<0.001	-0.92	-0.84	-0.81	-0.89	<0.001
80	Q7Z434	MAVS	222	RGPVSPSVS	-1.470	-1.635	-1.492	-1.535	<0.001	-1.622	-1.593	-1.812	-1.673	<0.001	-1.529	-1.794	-1.499	-1.609	<0.001	-1.349	-1.276		-1.292	<0.001	-1.667	-1.583		-1.646	<0.001
81	Q7RTP6	MICAL3	685	RRKTSQSEE	-2.148	-2.200	-2.041	-2.120	<0.001	-2.232	-2.085	-2.129	-2.158	<0.001	-2.320	-2.642	-2.295	-2.478	<0.001	-2.366	-2.369		-2.468	<0.001	-2.147	-2.880		-2.413	<0.001
82	Q8IY33	MICALL2	712	GRPLSPANV	-1.678	-2.297	-2.278	-2.117	<0.001	-2.126	-2.371	-2.545	-2.315	<0.001	-1.883	-1.913	-1.727	-1.846	<0.001	-1.700	-1.843		-1.770	<0.001	-2.297	-2.493		-2.397	<0.001
83	Q8IY33	MICALL2	726	ETVTSPVRL	-2.362		-2.213	-2.311	<0.001	-3.045		-3.058	-3.028	<0.001	-3.015	-2.560	-3.020	-2.863	<0.001	-2.291	-2.482		-2.413	<0.001	-2.370	-2.448		-2.383	<0.001
84	Q6WCQ1	MPRIP	619	EVDRSPGLP	-0.901	-1.112	-1.121	-1.059	<0.001	-0.926	-0.947	-1.000	-0.943	<0.001	-0.933	-0.909	-1.052	-0.975	<0.001	-1.349	-0.775	-1.157	-1.060	<0.001	-1.254	-0.876	-0.727	-0.986	<0.001
85	Q86UE4	MTDH	494	LKTISTSDP	-1.149	-1.724	-0.998	-1.266	<0.001	-0.694	-1.517	-1.662	-1.315	<0.001	-1.331	-0.624	-1.245	-1.069	<0.001	-1.865	-1.344	-1.101	-1.434	<0.001	-1.762	-1.006	-1.297	-1.357	<0.001
86	Q9Y217	MTMR6	561	VHPESPNLK	-1.327	-1.647	-1.025	-1.337	<0.001	-1.261	-1.494	-1.245	-1.330	<0.001	-1.283	-1.009	-1.085	-1.127	<0.001	-1.197	-0.684	-0.899	-0.877	0.010	-1.176	-0.495		-0.877	0.008
87	O75592	MYCBP2	3467	RRVNSGDTE	-1.78	-1.71	-2.19	-1.91	<0.001	-1.56	-1.25	-1.91	-1.56	<0.001	-1.16	-1.48	-1.49	-1.40	<0.001	-1.60	-1.81	-2.16	-1.85	<0.001	-1.64	-1.24	-1.93	-1.61	<0.001
88	Q09161	NCBP1	22	RRKTSDANE	-3.79	-4.02	-3.68	-3.86	<0.001	-3.71	-4.07	-4.20	-3.96	<0.001	-3.75	-3.89	-4.53	-4.04	<0.001	-3.27	-3.64	-3.47	-3.45	<0.001	-3.23	-3.90	-3.53	-3.56	<0.001
89	P16333	NCK1	85	KRKPSVPDS	-1.27	-1.16	-1.72	-1.38	<0.001	-1.34	-0.86	-1.13	-1.11	<0.001	-1.52	-1.43	-1.67	-1.55	<0.001	-1.77	-1.78	-1.51	-1.65	<0.001	-1.34	-1.19	-0.98	-1.20	<0.001
90	O60524	NEMF	831	KKLPSDSGD	-1.62		-1.82	-1.72	<0.001	-1.82		-1.86	-1.84	<0.001	-1.91	-1.98	-1.84	-1.89	<0.001	-1.95	-1.97	-2.16	-2.06	<0.001	-2.17	-2.11	-2.09	-2.09	<0.001
91	Q12968	NFATC3	344	TRKTSEDQA	-2.708	-2.726		-2.723	<0.001	-2.289	-2.187		-2.232	<0.001	-2.955	-2.819	-2.979	-2.943	<0.001	-2.777	-2.805		-2.773	<0.001	-2.016	-2.377		-2.214	<0.001
92	P46087	NOP2	58	RRLGSVEAP	-1.72	-2.02		-1.87	<0.001	-1.50	-1.85		-1.68	<0.001	-1.21	-1.26	-1.23	-1.24	<0.001	-1.71	-1.54	-1.71	-1.62	<0.001	-1.93	-1.42	-1.31	-1.59	<0.001
93	O00567	NOP56	563	KRKFSKEEP	-1.73	-1.87		-1.81	<0.001	-1.82	-1.76		-1.78	<0.001	-1.64	-1.69	-1.76	-1.73	<0.001	-1.94	-1.99		-1.91	<0.001	-1.80	-1.52		-1.72	<0.001
94	Q49790	NUP153	338	SPLNSPLDR	-1.33	-1.43	-1.37	-1.34	<0.001	-1.47	-1.57	-1.69	-1.61	<0.001	-1.80	-1.85	-1.87	-1.87	<0.001	-1.69	-1.70	-1.88	-1.75	<0.001	-1.52	-1.36	-1.55	-1.49	<0.001
95	Q8NFH5	NUP35	48	PAPVTPQPR	-2.040	-1.158		-1.505	0.002	-1.286	-1.153		-1.314	<0.001	-1.600	-1.426	-1.294	-1.450	<0.001	-1.921	-0.682		-1.465	0.011	-1.429	-0.847		-0.975	0.037
96	Q9UKX7	NUP50	221	AETQSPSLF		-0.989	-0.856	-0.930	<0.001		-1.234	-1.125	-1.172	<0.001	-1.011	-1.040	-0.931	-0.999	<0.001	-1.312	-0.920	-0.896	-1.039	<0.001	-1.579	-1.840	-1.544	-1.658	<0.001
97	P52948	NUP98	623	ENLASPSEY	-1.26	-1.47		-1.39	<0.001	-1.33	-1.57		-1.42	<0.001	-1.31	-1.42	-1.37	-1.37	<0.001	-1.06	-1.10	-1.54	-1.26	<0.001	-1.18	-0.97	-2.01	-1.36	<0.001
98	Q8WX93	PALLD	893	ARIASDEEI	-2.622	-3.176	-2.696	-2.812	<0.001	-2.785	-3.265	-3.113	-3.074	<0.001	-3.552	-3.499	-3.701	-3.550	<0.001	-3.601	-3.017		-3.274	<0.001	-3.821	-3.384		-3.637	<0.001
99	O94880	PHF14	835	GRKRSFVPE	-2.50	-2.66	-2.63	-2.59	<0.001	-2.75	-2.78	-3.06	-2.87	<0.001	-2.63	-2.60	-2.59	-2.61	<0.001	-2.68	-2.63		-2.65	<0.001	-2.68	-2.38		-2.53	<0.001
100	Q16513	PKN2	583	PRASSLGEI	-1.010	-0.817	-1.244	-1.012	<0.001	-1.270	-0.810	-1.290	-1.134	<0.001	-1.156	-1.237	-1.432	-1.283	<0.001	-1.108	-1.127	-0.777	-1.065	<0.001	-1.282	-1.132	-1.336	-1.189	<0.001

Table 2.7. Phosphosites significantly regulated by all inhibitors (trametinib, AZD6244, SCH772984, GDC0994) in SILAC experiments. Log2 values of quantified ratios in individual SILAC replicates. Empirical bayes generated log2 combined ratio and adjusted p-value. Phosphosites below double line are significantly increased in response to inhibitor

#	Uniprot ID	Gene	Position n	9aa window	Exp1 Tram Rep1	Rep2	Rep3	Comb	adj p	Exp1 SCH Rep1	Rep2	Rep3	Comb	adj p	Exp2 AZD Rep1	Rep2	Rep3	Comb	adj p	Exp2 SCH Rep1	Rep2	Rep3	Comb	adj p	Exp3 GDC Rep1	Rep2	Rep3	Comb	adj p
101	P47712	PLA2G4A	727	PSRCSVSLS		-0.93	-1.44	-1.18	0.004	-0.92	-1.77		-1.35	0.002	-0.99	-0.74	-0.83	-0.84	<0.001	-1.71	-1.03		-1.26	0.002	-1.17	-0.97		-1.18	0.002
102	O14974	PPP1R12A	507	RRLASTSDI	-4.71	-5.29	-5.47	-5.10	<0.001	-4.81	-5.07	-4.63	-4.89	<0.001	-5.45	-4.96	-5.27	-5.23	<0.001	-6.17	-5.23	-4.72	-5.36	<0.001	-5.42	-5.89	-5.30	-5.55	<0.001
103	Q86WR7	PROSER2	215	LSPTSPFRE		-2.028	-1.994	-1.999	<0.001	-2.077	-1.665	-1.24	-1.883	<0.001	-1.523	-1.557	-1.130	-1.413	<0.001	-1.688	-1.451	-1.498	-1.555	<0.001	-1.971	-1.441	-0.775	-1.387	<0.001
104	P48634	PRRC2A	1219	PGPLSPVAR	-1.00	-0.98	-1.09	-1.03	<0.001	-1.17	-1.15	-1.24	-1.18	<0.001	-1.05	-1.05	-1.07	-1.04	<0.001	-0.99	-0.85	-0.80	-0.90	<0.001	-0.98	-0.94	-0.83	-0.89	<0.001
105	P04049	RAF1	642	TLTTSPRLP	-2.91	-3.31		-3.19	<0.001	-2.40	-3.63		-2.94	<0.001		-3.45	-3.47	-3.43	<0.001		-3.15	-2.94	-3.04	<0.001	-0.98	-3.46	-3.87	-3.66	<0.001
106	Q72SI4	RAI1	1068	SEPRTPGPP	-2.213	-2.162	-2.706	-2.342	<0.001	-2.298	-2.260	-2.288	-2.300	<0.001	-2.530	-2.316	-2.348	-2.347	<0.001	-2.915	-2.411	-2.479	-2.597	<0.001	-2.796	-2.381	-2.579	-2.590	<0.001
107	Q70E73	RAPH1	1154	QVPTSPKSS	-1.429		-0.806	-1.061	0.006	-1.053		-0.581	-0.873	0.006	-0.922	-1.152	-0.606	-0.897	<0.001	-1.283		-0.866	-1.091	0.001	-1.080	-1.168		-1.108	<0.001
108	Q8IY67	RAVER1	463	AAQLTPPPA	-1.135	-1.223	-1.392	-1.221	<0.001	-0.893	-1.182	-1.167	-1.110	<0.001	-1.172	-1.493	-1.238	-1.295	<0.001	-1.130	-1.181	-1.107	-1.127	<0.001	-1.399	-1.456	-1.604	-1.498	<0.001
109	Q96D71	REPS1	709	RRLKSEDEL	-1.972	-2.514	-2.468	-2.307	<0.001	-2.290	-2.135	-1.972	-2.143	<0.001	-1.891	-1.911	-1.700	-1.818	<0.001	-1.598	-1.717	-1.502	-1.616	<0.001	-1.630	-1.243	-1.332	-1.391	<0.001
110	Q9NZJ4	SACS	1779	ADLQSPLFR		-2.573	-3.096	-2.887	<0.001		-1.905	-2.266	-2.083	<0.001	-2.281	-2.559	-2.326	-2.402	<0.001		-2.114	-1.948	-2.097	<0.001		-2.734	-2.244	-2.424	<0.001
111	Q9NZJ4	SACS	4264	STPTSPTEF	-2.245		-2.264	-2.264	<0.001	-1.714		-1.917	-1.807	<0.001	-1.677	-2.093	-1.781	-1.829	<0.001	-1.766	-1.753	-2.029	-1.833	<0.001	-1.607	-1.618	-2.062	-1.778	<0.001
112	O43290	SART1	448	RRRVSEVEE	-1.17	-1.78	-1.58	-1.52	<0.001	-1.34	-1.55	-0.95	-1.27	<0.001	-2.05	-1.77	-1.80	-1.83	<0.001	-1.83	-0.76	-1.18	-1.29	<0.001	-1.78	-0.82	-1.32	-1.28	<0.001
113	O15027	SEC16A	1786	GPLASPARV	-1.40	-1.54	-1.50	-1.47	<0.001	-1.64	-1.81	-1.73	-1.74	<0.001	-1.46	-1.55	-1.46	-1.47	<0.001	-1.56	-1.68	-1.64	-1.62	<0.001	-2.34	-2.04	-2.36	-2.25	<0.001
114	Q8NCS1	SERBP1	234	ELTESPVYI	-1.856	-2.149	-1.644	-1.891	<0.001	-2.278	-2.332	-1.658	-2.082	<0.001	-1.833	-1.683	-1.845	-1.787	<0.001	-2.085	-1.697	-1.745	-1.850	<0.001	-1.833	-1.541	-1.468	-1.606	<0.001
115	Q7Z333	SETX	1366	RRRLSDCES	-2.409	-2.147	-2.303	-2.283	<0.001	-2.298	-1.621	-2.270	-2.066	<0.001	-2.382	-2.517	-2.319	-2.363	<0.001	-2.418		-2.386	-2.421	<0.001	-2.059		-2.483	-2.252	<0.001
116	Q86VV5	SGK223	745	AESLSPSFR		-1.542	-1.865	-1.705	<0.001		-1.710	-1.898	-1.803	<0.001	-1.755	-1.952	-2.044	-1.930	<0.001	-1.703	-1.816	-2.011	-1.838	<0.001	-1.734	-1.734	-1.877	-1.785	<0.001
117	Q9P2F8	SIPA1L2	1461	MLPDSPLVE	-1.878		-2.364	-2.211	<0.001	-2.310		-2.493	-2.312	<0.001	-2.046	-2.448	-2.206	-2.194	<0.001	-2.462	-2.192	-2.315	-2.304	<0.001	-2.394	-2.079	-2.098	-2.209	<0.001
118	Q08357	SLC20A2	268	QEAESPVFK	-1.32	-1.24	-1.21	-1.26	<0.001	-1.56	-1.63	-1.42	-1.53	<0.001	-1.40	-1.28	-1.44	-1.36	<0.001	-1.45	-1.33	-1.05	-1.29	<0.001	-1.30	-1.32	-0.78	-1.12	<0.001
119	P19634	SLC9A1	703	ARIGSDPLA		-2.08	-2.44	-2.18	<0.001		-2.08	-2.30	-2.18	<0.001	-2.18	-1.81	-2.35	-2.15	<0.001	-2.35	-2.35	-1.93	-2.22	<0.001	-2.05	-2.40	-1.90	-2.10	<0.001
120	O60749	SNX2	104	VTPVTPTTL	-2.77		-3.10	-2.93	<0.001	-2.38		-2.70	-2.55	<0.001	-2.76	-3.04	-1.57	-2.42	<0.001	-2.61	-2.35	-2.45	-2.55	<0.001	-2.54	-2.47	-2.41	-2.48	<0.001
121	P40763	STAT3	727	DLPMSPRTL		-1.23	-1.12	-1.18	<0.001		-1.31	-1.67	-1.49	<0.001	-1.37	-1.48	-1.33	-1.39	<0.001	-1.26	-1.51	-1.63	-1.49	<0.001	-1.58	-1.35	-1.49	-1.45	<0.001
122	O94804	STK10	952	PNPSTPSKA	-1.61	-2.17	-2.75	-2.20	<0.001	-2.20	-2.85	-1.82	-2.27	<0.001	-2.49	-2.36	-1.96	-2.29	<0.001	-2.43	-2.44	-1.85	-2.26	<0.001	-2.35	-2.53	-2.64	-2.49	<0.001
123	P16949	STMN1	25	ELILSPRSK	-1.72	-1.58	-1.53	-1.63	<0.001	-1.65	-1.35	-1.49	-1.48	<0.001	-1.73	-1.72	-1.84	-1.76	<0.001	-1.64	-1.60	-1.46	-1.58	<0.001	-2.13	-2.15	-1.52	-1.92	<0.001
124	Q9Y2Z0	SUGT1	265	GDVPTPKQF	-1.650	-1.674	-1.507	-1.608	<0.001	-1.538	-1.523	-1.458	-1.509	<0.001	-1.642	-1.299	-1.502	-1.476	<0.001	-1.773	-1.315	-1.424	-1.300	<0.001	-2.269	-2.270	-1.782	-2.311	<0.001
125	O95425	SVIL	547	VRTRSLSDF		-0.46	-1.60	-1.06	0.017		-0.89	-1.51	-1.18	0.024	-1.02	-0.86	-0.89	-0.92	0.007	-1.28		-1.74	-1.50	<0.001	-1.68		-1.71	-1.71	<0.001
126	Q9ULH1	TACC2	2317	NTPASPPRS	-2.10	-2.10		-2.08	<0.001	-2.35	-1.99		-2.19	<0.001	-1.66	-1.67	-2.34	-1.92	<0.001	-2.62	-2.22		-2.40	<0.001	-2.37		-1.48	-1.95	<0.001
127	Q9ULH1	TACC2	2321	SPPRSPAEP	-2.10	-2.10		-2.19	<0.001	-2.35	-1.99		-2.19	<0.001	-1.66	-1.67	-2.34	-1.92	<0.001	-2.62	-2.22		-2.40	<0.001	-2.37		-1.48	-1.95	<0.001
128	Q13769	THOC5	328	RRRPTLGVQ	-3.568	-3.597	-4.127	-3.736	<0.001	-2.653	-2.764	-2.268	-2.589	<0.001	-2.937	-2.882	-2.132	-2.659	<0.001	-2.761	-2.874		-2.809	<0.001	-2.027	-2.179		-2.112	<0.001
129	Q12893	TMEM115	320	AKVDSPUPS	-2.357	-2.388	-2.624	-2.456	<0.001	-2.712	-2.607	-2.534	-2.618	<0.001	-2.513	-2.345	-2.296	-2.417	<0.001	-2.638	-2.533	-2.198	-2.475	<0.001	-2.598	-2.848	-2.241	-2.544	<0.001
130	Q6PI48	TMEM55B	162	PGPLSPEPQ	-1.558	-1.049	-1.501	-1.375	<0.001	-1.198	-1.207	-1.794	-1.394	<0.001	-1.401	-1.381	-1.398	-1.395	<0.001	-1.079	-1.057	-1.984	-1.352	<0.001	-1.332	-0.959	-2.014	-1.457	<0.001
131	Q9UQ2	TNKS1BP1	131	PPPLTPPAR	-2.637	-3.230	-3.205	-2.994	<0.001	-2.822	-3.418	-2.800	-3.043	<0.001	-3.063	-3.035	-2.903	-2.997	<0.001	-2.866		-2.766	-2.746	<0.001	-2.955		-2.882	-2.988	<0.001
132	Q9UQ2	TNKS1BP1	1666	PRNRSAEEG	-0.738	-0.981	-0.856	-0.872	<0.001	-0.996	-1.024	-0.644	-0.874	<0.001	-1.003	-1.090	-1.081	-1.038	<0.001	-1.279		-0.290	-0.915	0.026	-1.232		-1.220	-1.096	0.009
133	Q13625	TP53BP2	556	RVILSPSIP	-0.807	-1.084	-1.244	-1.045	<0.001	-0.955	-1.010	-1.109	-1.025	<0.001	-1.010	-1.138	-0.921	-1.029	<0.001	-1.147	-1.133	-1.354	-1.212	<0.001	-1.400	-1.326	-1.370	-1.365	<0.001
134	Q8WVT3	TRAPPC12	182	QMVKSPSFG		-2.470	-2.612	-2.540	<0.001		-2.494	-2.406	-2.451	<0.001	-2.462	-2.511	-2.357	-2.434	<0.001	-2.265	-2.347	-2.465	-2.356	<0.001	-2.639	-2.671	-2.451	-2.590	<0.001
135	Q96LD4	TRIM47	588	GIPASPIDP		-3.005	-3.952	-3.478	<0.001		-3.189	-2.569	-2.880	<0.001	-2.803	-3.159	-3.087	-3.016	<0.001	-2.771	-2.632	-2.966	-2.774	<0.001	-2.632	-2.635	-2.628	-2.647	<0.001
136	Q9Y3D8	TSC22D4	279	LVHKSPRDF	-2.589	-3.117	-3.512	-3.095	<0.001	-2.344	-3.160	-2.773	-2.737	<0.001	-2.801	-2.247		-2.646	<0.001	-2.607	-2.497	-2.511	-2.549	<0.001	-2.445	-2.720	-2.462	-2.551	<0.001
137	Q3B726	TWISTNB	316	KRKHSEEAE	-1.329	-1.598	-1.592	-1.506	<0.001	-1.449	-1.387	-1.223	-1.353	<0.001	-1.598	-1.668	-1.689	-1.610	<0.001	-1.524	-1.587	-3.233	-1.946	<0.001	-1.500	-1.486	-1.529	-1.674	<0.001
138	P40222	TXLNA	515	QAPSSPRVT	-1.06	-1.30	-1.18	-1.17	<0.001	-1.22	-1.31	-1.08	-1.21	<0.001	-1.33	-1.12	-1.42	-1.27	<0.001	-1.27	-1.22	-1.12	-1.22	<0.001	-0.95	-1.22	-0.89	-1.00	<0.001
139	Q9C0C9	UBE2O	839	GSPTSPTVE	-1.743	-1.729	-1.781	-1.769	<0.001	-1.529	-1.635	-1.520	-1.543	<0.001	-1.637	-1.860	-1.899	-1.791	<0.001	-1.574	-1.807		-1.702	<0.001	-1.557	-1.984		-1.760	<0.001
140	O75152	ZC3H11A	758	TRRLSSAST	-1.65	-1.33	-2.25	-1.70	<0.001	-1.41	-1.86	-1.51	-1.64	<0.001	-1.54	-1.53	-1.24	-1.48	<0.001	-1.38	-1.72	-1.31	-1.44	<0.001	-1.32	-0.88	-1.64	-1.31	<0.001
141	O43149	ZZEF1	1518	EEPLSPSTP	-1.77	-1.87		-1.80	<0.001	-1.74	-1.43		-1.61	<0.001	-1.55	-1.82	-1.62	-1.67	<0.001	-1.55	-1.59	-1.55	-1.53	<0.001	-1.11	-1.25	-0.90	-1.11	<0.001
142	Q09666	AHNAK	5739	SISGSKGDL	1.220	1.011	1.329	1.183	<0.001	0.712	0.779	1.124	0.875	<0.001	1.129	1.025	1.083	1.090	<0.001	0.950	1.501	0.187	0.902	0.070	0.223	1.383	1.296	0.945	0.040
143	P0CT75	ATXN1L	330	AHRGTPDTD	1.105	1.190	1.216	1.179	<0.001	1.061	1.211	1.085	1.110	<0.001	0.605	1.329	1.202	1.052	<0.001	0.946	0.716	1.203	0.931	<0.001	0.800	0.802	1.066	0.913	<0.001
144	P04626	ERBB2	1054	SSTRSGGGD	1.134	0.834	1.015	0.966	<0.001	0.961	0.894	1.050	0.997	<0.001	1.409	1.479	1.514	1.475	<0.001	2.428	1.812	1.691	1.979	<0.001	2.235	1.933	2.005	2.049	<0.001
145	Q9UJ14	GGT7	83	SQDGSPLRE	1.174	1.243	1.011	1.140	<0.001	1.256	0.830	0.980	1.024	<0.001	1.539	1.701	1.451	1.583	<0.001	1.651		1.162	1.479	0.002	1.354		1.384	1.296	0.002
146	Q9Y618	NCOR2	121	LLRPSPLLA	1.337	1.370		1.371	<0.001	1.399	1.574		1.469	<0.001	2.030	1.434		1.714	<0.001	1.813	1.385		1.630	0.010	1.111	2.088		1.568	0.009
147	P35658	NUP214	430	RQPKSPGST	2.379	2.270	2.606	2.397	<0.001	2.205	2.257	2.353	2.293	<0.001	2.210	2.353		2.315	<0.001	2.544	3.254		2.810	<0.001	2.748	2.511		2.718	<0.001
148	Q9HCD6	TANC2	1530	KEYPSPPPS		0.747	1.063	0.908	<0.001		0.910	1.151	1.028	<0.001	1.075	1.378	1.796	1.407	<0.001	1.798		2.063	1.923	<0.001	1.937		1.953	1.952	<0.001
149	Q9UQ2	TNKS1BP1	429	QRRFSEGVL	1.257	1.763	1.561	1.515	<0.001	1.577	1.204	0.782	1.200	<0.001	1.151	0.991	0.775	0.966	<0.001	2.145	0.394	1.487	1.342	0.015	2.423	1.003	0.890	1.438	0.007
150	Q3B726	TWISTNB	328	PLKCSPKRK	1.233	0.490	1.189	1.076	<0.001	1.352	1.447	1.230	1.238	<0.001	1.682	1.788	1.956	1.828	<0.001	1.566	1.780	1.698	1.635	<0.001	1.039	2.136	1.741	1.686	<0.001

My comparison of SILAC datasets allowed me to identify phosphosites that were significantly responsive to the MKK1/2 inhibitors (trametinib and selumetinib) but not ERK1/2 inhibitors (SCH772984 and GDC0994), or vice versa. Only four phosphosites were differentially regulated by one set of inhibitors and not the other (Table 2.8), and all were regulated by MKK1/2 inhibitor, with no sites uniquely regulated by ERK1/2 inhibitors across all three experiments. These sites are intriguing candidates for targets of MKK1/2 which bifurcate upstream of ERK1/2.

These phosphosites included Ser612 in nuclear pore complex protein 98 (NUP98), Thr5824 in giant neuroblast differentiation-associated protein (AHNAK), Ser455 in mitogen activated protein kinase kinase kinase 7 (MAP3K7/TAK1), and Ser893 in DNA double strand break repair G family endonuclease 1 (GEN1). Three of these phosphosites showed significant differences between $\log_2$(combined ratio for MKK1/2 inhibitor response) vs $\log_2$(combined ratio for ERK1/2 inhibitor response), using a Students t-test with p value < 0.05. Thr5824 in AHNAK failed this test (Table 2.8). But the other three phosphosites support the existence of potential branchpoints upstream of ERK1/2, breaking the standard paradigm of linear signaling in the MAPK cascade.

Ser612 in NUP98 has been identified as a target of ERK1/2 inhibition in previous phosphoproteomics experiments (Courcelles et al., 2013; Kosako et al., 2009; Stuart et al., 2015) monitoring responses to MKK1/2 and BRAF V600E/K inhibitors. Six other NUP98 phosphorylation sites identified previously showed similar responses to all four MKK1/2 and ERK1/2 inhibitors, highlighting Ser612 as a particularly interesting site for regulation through a branching pathway. Thr5824 in AHNAK was also identified in previous phosphoproteomic analyses as a site responsive to a MKK1/2 inhibitor but not an ERK1/2 inhibitor (Pan et al., 2009). TAK1 is a MEKK, which is known to phosphorylate and activate MKK3/6 in response to

cytokine signaling, and is also subject to negative feedback inhibition by p38α through its'
binding partner TAB1 (Cheung, Campbell, Nebreda, & Cohen, 2003; Cuadrado & Nebreda, 2010).

Table 2.8. Phosphosites significantly regulated with MKK1/2 inhibitors but not ERK1/2 inhibitors. Log$_2$ values of quantified ratios in individual SILAC replicates. Empirical bayes generated log2 combined ratio. Phosphosites significantly inhibited <0.84 are highlighted in green. Phospohosites significantly increased by drug are highlighted in red.

Uniprot ID	Gene	Position	9aa window	p-value	MKK1/2 inhibitors												ERK1/2 inhibitors							
					Trametinib/DMSO Experiment 1				AZD6244/DMSO Experiment 2				SCH772984/DMSO Experiment 1				SCH772984/DMSO Experiment 3				GDC0994/DMSO Experiment 3			
					Rep 1	Rep 2	Rep 3	Combined	Rep 1	Rep 2	Rep 3	Combined	Rep 1	Rep 2	Rep 3	Combined	Rep 1	Rep 2	Rep 3	Combined	Rep 1	Rep 2	Rep 3	Combined
P52948	NUP98	612	SNLFSPVNR	0.027	-1.36	-0.65	-1.01	-1.00		-0.79	-0.87	-0.85	-0.07	-0.48	-0.91	-0.49	-0.69			-0.04	0.31	0.36		0.32
Q09666	AHNAK	5824	LKFGTFGGL	0.136	0.66	0.67	1.22	0.85	0.91	1.15	1.12	1.09	0.32	0.59	0.85	0.58	0.17	1.37	0.75	0.80	-0.55	0.54	1.05	0.32
O43318	MAP3K7	455	GQVSSRSSS	0.004	1.04	0.76	0.82	0.86	0.55	0.70	1.30	0.86	0.16	0.34	0.56	0.37		0.41	0.67	0.38		0.37	-0.02	0.08
Q17RS7	GEN1	893	TCLDSPLPL	0.019	1.24		0.89	1.08	0.96	1.25	2.02	1.40	0.45		0.25	0.33	-0.20	0.49	-0.17	0.31	-0.28	0.76	1.65	0.67

Comparison of these datasets can also yield insight about off-target effects of individual compounds, which will most likely be reflected by the phosphosites responding uniquely to one inhibitor, but none of the other three. This was exemplified by the case described above of p38α MAPK phosphorylation, which appears to be an off-target of only trametinib and none of the other MKK1/2 or ERK1/2 inhibitors.

Table 2.9 describes the phosphosites that can be quantified across all four treatments among three experiments, but change in response to only one drug. In total, 48 phosphosites were identified as significantly altered by only one compound. Eight of these are in proteins previously validated as known components of the RAF-MKK-ERK pathway or downstream targets of pathway signaling (Carlson et al., 2011; Yoon & Seger, 2006). Among these 48 phosphosites, 21 contain the canonical Ser/Thr-Pro motif associated with direct substrates of phosphorylation by ERK1/2. However, none of these were validated by more than one inhibitor, therefore I believe that all 48 phosphosites most likely reflect off-target effects of single compounds. A striking observation was how many phosphosites were targeted only by GDC0994, an ERK1/2 inhibitor which is currently being tested in early stage clinical trials. Twenty-five phosphosites were uniquely responsive to GDC0994, which therefore shows more potential off-targets than the other three drugs combined. The difference was not explained by greater inhibition of ERK1/2 signaling at the 10 µM concentration used, because GDC0994 shows a similar IC_{50} for ERK1/2 inhibition as the other inhibitors (Fig. 1.4), and among the 150 phosphosites significantly affected by all four inhibitors, only 33% were most strongly responsive to GDC0994 (Table 2.7). I found no specificity motif among the 25 phosphosites uniquely responsive to GDC0994 that would indicate regulation of a specific set of kinases. In

fact, the range of sequence motifs suggested substrate specificities comparable to the other

MKK1/2 and ERK1/2 inhibitors analyzed.

Among the unique GDC0994 off-target candidates were Ser196 on the transcription

factor, ELK1. ERK1/2 is known to phosphorylate and activate ELK1, but at a different residue

than Ser196, which has no known function (Hornbeck et al., 2015; Stuart et al., 2015). The off-

target candidates for GDC0994 were also examined by STRING, which reported a network of

genes known to regulate mRNA binding and translational initiation, including the highest

ranking target uniquely responsive to GDC0994, eukaryotic translation initiation factor 4 gamma

1 (EIF4G1) (Fig. 2.14). Three additional genes containing phosphosites unique to GDC0994, la

ribonucleoprotein domain family member 1 (LARP1), la ribonucleoprotein domain family 1B

(LARP1B), and heterogeneous nuclear ribonucleoprotein M (HNRNPM), are among those in the

STRING network (Fig. 2.14). These genes are RNA binding proteins, known to function in pre-

mRNA processing and specificity of mRNA translation. Further studies are now needed to verify

if GDC0994 indeed affects RNA processing or translation in a manner that is independent of

MKK1/2-ERK1/2 signaling. Understanding off-targets of drug candidates may provide useful

insight about relative frequencies of off-target effects, at early stages of inhibitor screening.

Table 2.9. Phosphosites significantly regulated in only one MKK1/2 or ERK1/2 inhibitor. $\log_2$ values of quantified ratios in individual SILAC replicates. Empirical bayes generated $\log_2$ combined ratio and adjusted p-value. Phosphosites significantly inhibited <0.84 are highlighted in green. Phosphosites significantly increased by inhibitor are highlighted in red.

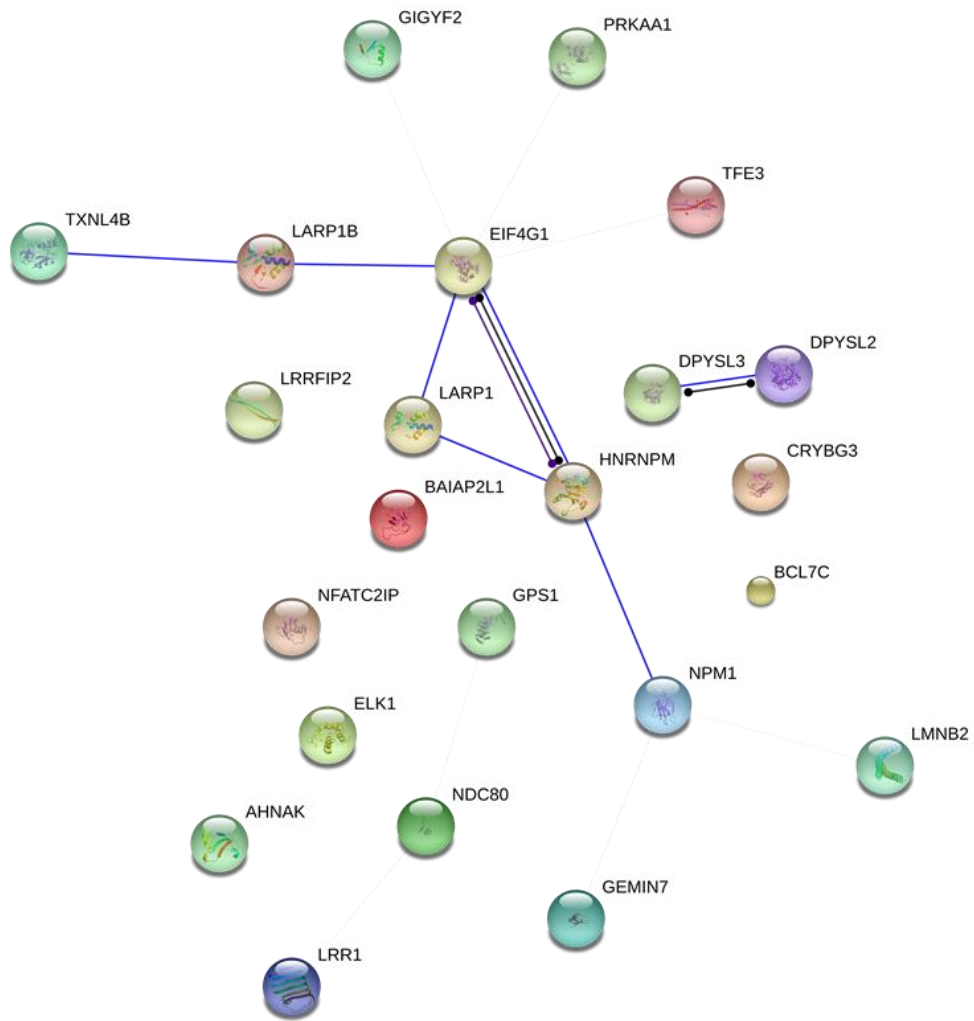| #	Unique to	Uniprot ID	Gene	Position	9aa window	E1 Trametinib/DMSO Rep1	Rep2	Rep3	Combined	adj pvalue	E1 SCH772984/DMSO Rep1	Rep2	Rep3	Combined	adj pvalue	E2 AZD6244/DMSO Rep1	Rep2	Rep3	Combined	adj pvalue	E3 SCH772984/DMSO Rep1	Rep2	Rep3	Combined	adj pvalue	E3 GDC0994/DMSO Rep1	Rep2	Rep3	Combined	adj pvalue
1	AZD6244	Q9BR22	TRIM56	471	LKSISREPS	0.342	0.325	0.221	0.236	0.036	-0.165	0.206	0.160	0.127	0.263	0.267	-0.709	-0.954	-0.857	<0.001	-	-	-	0.343	-	-	-	-	0.348	-
2	Trametinib	Q99704	DOK1	269	LRADSHEGE	-0.849	-1.540	-1.176	-1.191	<0.001	0.057	-0.223	0.146	-0.004	0.990	-0.432	-0.170	-0.424	-0.128	0.455	-0.642	-0.293	-0.473	-0.449	0.010	-0.230	0.136	0.281	0.042	0.811
3	Trametinib	P42566	EPS15	796	NKLDSPDPF	-1.322	-1.230	-1.495	-1.378	<0.001	-0.703	-0.483	-0.670	-0.589	<0.001		-0.441	-0.325	-0.412	<0.001										
4	Trametinib	Q68DA7	FMN1	243	DIPKTPDTD	-1.285	-1.522	-1.933	-1.595	<0.001	-0.198	-0.730	-0.606	-0.496	0.011		-	-	-	-										
5	Trametinib	Q16539	MAPK14	180	DDEMTGYVA	-0.958	-1.135	-1.413	-1.124	<0.001	-0.028	0.080	0.377	0.099	0.593		0.006	0.304	0.145	0.492										
6	Trametinib	Q16539	MAPK14	182	EMTGYVATR	-0.850	-1.183	-0.728	-0.914	<0.001	0.004	0.103	0.202	0.097	0.542	-0.091	0.043	-0.063	-0.038	0.723			-1.341					0.393		
7	Trametinib	Q86UE4	MTDH	298	SSQISAGEE	-0.826	-1.093	-1.042	-1.004	<0.001	-0.095	-0.130	0.117	-0.019	0.920		-0.048	-0.239	-0.134	0.397		-0.086	0.748	0.258	0.404		0.045	0.290	0.240	0.375
8	Trametinib	Q15154	PCM1	93	MSQMSVPEQ	-0.533	-1.136	-0.958	-0.879	0.010	0.000	-0.176	0.750	0.195	0.593	-0.275	-0.101	0.253	-0.046	0.870	-0.121	0.290	0.798	0.319	0.270	0.620	0.280	0.807	0.572	0.029
9	Trametinib	Q5I525	PRRC2B	480	FRQQSIEDK		-0.775	-1.196	-0.991	<0.001	0.000	-0.020	-0.080	-0.044	0.830		-	-	-	-										
10	Trametinib	P04920	SLC4A2	144	LTQPSPVST	-1.251	-0.861	-0.793	-0.973	<0.001	-0.942	-0.420	-0.803	-0.717	0.001	1.583		0.663	1.128	<0.001	1.424	-0.608	0.258	0.833	0.288	1.303	-1.176	-0.767	0.276	0.713
11	Trametinib	Q9BWH2	FUNDC2	151	KIRKSNQIP	1.313	0.975	0.791	1.016	<0.001	0.349	-0.036	0.269	0.204	0.223	0.099	-0.051	0.043	-0.008	0.974										
12	Trametinib	Q8TD22	MICAL1	1057	ERRLSELAL		0.967	1.349	1.088	0.002	-0.234	0.036	-0.177	-0.001	0.999	-0.234	0.428		0.079	0.818										
13	Trametinib	Q9BXB5	OSBPL10	30	SAGSSPSCS	1.494	1.238	1.191	1.304	<0.001	0.416	0.018	0.348	0.264	0.057		0.569													
14	Trametinib	O14828	SCAMP3	32	QHRPSRQYA		1.010	0.853	0.931	0.001	-0.268	0.333	-0.099	0.117	0.571	-0.268	-0.038	-0.805	-0.373	0.046	0.649	-0.539		0.043	0.962	1.479	0.171		0.837	0.100
15	SCH772984	Q9BXS5	AP1M1	223	LFDNTGRGK	0.376	0.214	-0.002	0.088	0.528	-1.256	-1.598	-1.815	-1.689	<0.001		-	-	-	-										
16	SCH772984	Q03252	LMNB2	419	SRATSSSSG		0.107	0.014	0.166	0.257	-1.256	-0.981	-1.235	-1.158	<0.001		-	-	-	-										
17	SCH772984	Q8IXT5	RBM12B	562	FHHSSEDFR	0.346	0.008	-0.194	0.042	0.842	-0.886	-0.957	-0.794	-0.868	<0.001	-0.190	-0.120	0.001	-0.108	0.339										
18	SCH772984	P38159	RBMX	88	ATKPSFESG	0.044	-0.329	0.114	-0.061	0.681	-1.045	-0.973	-0.850	-0.952	<0.001	-0.102	0.024	-0.012	-0.020	0.856	-1.385	-0.920		-1.169	0.006	0.139	-0.336		-0.082	0.807
19	SCH772984	Q9NWH9	SLTM	550	KKRISSKSP		-0.242	0.212	0.013	0.968		-1.094	-0.799	-0.974	0.001	-0.152		-0.200	-0.165	0.181										
20	SCH772984	Q9NWH9	SLTM	553	ISSKSPGHM		-0.242	0.103	-0.019	0.947		-1.094	-0.733	-0.964	0.001	-0.217	-0.011	-0.178	-0.130	0.161										
21	SCH772984	Q8N1F8	STK11IP	481	ARPPSPPQE	0.579	-0.081		0.196	0.556	-1.297	-0.963		-1.077	0.006	-0.911	0.048	0.885	0.016	0.972										
22	SCH772984	Q8NI27	THOC2	1285	KKEKTPATT	0.192	0.219	0.086	0.141	0.509	-2.041	-1.231	-1.442	-1.547	<0.001		-	-	-	-										
23	SCH772984	Q9H3N1	TMX1	270	IRQRSLGPS	0.634	0.424	0.308	0.442	0.039	-1.447	-1.396	-0.855	-1.219	<0.001	-0.003	0.110	0.269	0.098	0.553	-1.030	-1.254	-0.512	-0.928	0.001	-0.511	-0.456	-0.787	-0.589	0.014
24	GDC0994	Q09666	AHNAK	210	IRUPSGSGA	-0.298	-0.219	-0.209	-0.226	0.033	-0.311	-0.579	-0.624	-0.589	<0.001	-0.096	-0.134	-0.096	-0.105	0.037	-0.799	0.048	-0.927	-0.548	0.065	-2.054	-1.150	-1.415	-1.551	<0.001
25	GDC0994	Q09666	AHNAK	216	SGAASPTGS	-0.298	-0.219	-0.209	-0.242	0.023	-0.311	-0.579	-0.624	-0.505	<0.001	-0.019	-0.205	-0.017	-0.053	0.508	-0.750	0.048	-0.840	-0.508	0.087	-2.014	-1.150	-1.259	-1.480	<0.001
26	GDC0994	Q68DQ2	CRYBG3	2902	GGRDTPGAK		0.137	-0.290	-0.055	0.789		0.248	0.012	0.108	0.551	0.165	0.189	0.115	0.155	0.129	0.063		0.015	0.065	0.607	-1.219		-1.271	-1.272	<0.001
27	GDC0994	Q16555	DPYSL2	509	EVSVTPKTV	0.297	-0.023	0.408	0.234	0.055	0.045	-0.010	0.124	0.046	0.768	0.192	0.185	0.214	0.210	0.010	-0.070	0.566	0.298	0.248	0.218	-1.729	-1.751	-1.235	-1.555	<0.001
28	GDC0994	Q14195	DPYSL3	509	DLTTTPKGG	0.336	0.536	0.162	0.333	0.017	0.279	0.142	-0.165	0.096	0.516	0.298	0.163	0.335	0.246	0.009	0.287	0.068	-0.026	0.142	0.522	-1.089	-1.138	-1.721	-1.348	<0.001
29	GDC0994	Q04637	EIF4G1	1209	RKAASLTED	0.452	0.201	0.332	0.335	0.103	-0.563	0.030	0.113	-0.146	0.524	0.143	0.099	0.321	0.184	0.176	-0.373	-0.427	0.418	-0.206	0.517	-2.905	-3.068	-2.325	-2.688	<0.001
30	GDC0994	P19419	ELK1	196	PPSGSRSTS	-0.384	0.048	0.115	-0.086	0.608	-0.433	-0.142	-0.246	-0.261	0.081	0.104	0.121	-0.099	0.042	0.719	-0.257	-0.271	-0.763	-0.371	0.019	-1.317	-1.170	-1.212	-1.293	<0.001
31	GDC0994	Q9H840	GEMIN7	3	MQTPVNII	0.039	0.337	0.508	0.295	0.041	0.233	0.407	0.180	0.272	0.061	0.123	0.315	0.279	0.238	0.018	-0.098	0.305	0.222	0.140	0.302	-1.164	-1.103	-1.010	-1.090	<0.001
32	GDC0994	Q6Y7W6	GIGYF2	189	GGPTSVGRK	0.479	-0.333	-0.234	-0.051	0.916	-0.325	-0.274	0.213	-0.107	0.796	-0.656	0.135	-0.193	-0.258	0.269		0.414	0.585	0.377	0.184		-1.225	-0.880	-0.930	0.005
33	GDC0994	Q13098	GPS1	479	QGELTPANS	0.010	0.040	-0.135	-0.026	0.802	0.119	0.029	0.051	0.064	0.464	-0.082	0.125	0.259	0.127	0.082	-0.070	0.265	-0.019	0.072	0.936	-0.795	-0.493	-2.517	-1.281	0.018
34	GDC0994	P52272	HNRNPM	452	ERMGSGIER		-	-	-	-		-	-	-	-		-	-	-	-	-0.070	0.076	0.116	0.090	0.653	-0.070	-2.153	-2.448	-2.294	<0.001
35	GDC0994	Q659C4	LARP1B	900	VPINSPRRN		0.050	0.195	0.098	0.683		-0.067	0.389	0.186	0.391	0.078	0.078	0.305	0.179	0.086	0.342	0.292	0.323	0.323	0.018	-1.084	-1.063		-1.080	<0.001
36	GDC0994	Q03252	LMNB2	17	ATPLSPTRL		-0.227	-0.330	-0.261	0.063		-0.129	-0.402	-0.283	0.051	0.032	0.018		0.023	0.822	-0.079	-0.063	-0.059	-0.059	0.944	-1.988	-0.806		-1.409	0.015
37	GDC0994	Q96L50	LRR1	124	VSTLTPVKT			0.203					-0.082			0.267	0.304	0.114	0.228		0.076	0.080	0.168	0.168	0.679	-0.965	-0.668		-0.907	0.014
38	GDC0994	Q9Y608	LRRFIP2	320	TTPLSGNSS		-0.001					0.620				0.122	0.588	0.031	0.247		0.385	0.310	0.105	0.118	0.866	-1.113	-1.175	-1.234	-1.025	0.023
39	GDC0994	O14777	NDC80	69	GSRNSQLGI		-0.131	-0.123	-0.134	0.469	-0.001	-0.042	0.257	0.115	0.547	0.336	0.035	0.239	0.182	0.113	-0.199	0.117	0.012	0.012	0.972	-0.680	-0.929		-0.858	0.003
40	GDC0994	Q8NCF5	NFATC2IP	84	GPVASRDNS	0.261	0.440	0.235	0.318	0.006	0.080	0.043	-0.062	0.014	0.926	0.376	0.145	0.156	0.239	0.006	0.270	0.137	-0.095	0.106	0.414	-0.693	-0.814	-1.053	-0.855	<0.001
41	GDC0994	P06748	NPM1	254	KMQASIEKG	0.070		0.178	0.158	0.186	0.250		0.086	0.134	0.265		0.503	0.368	0.436	0.001	0.461	0.491	0.262	0.462	0.014	-1.010	-0.535	-0.922	-0.880	<0.001
42	GDC0994	Q13131	PRKAA1	382	LVAETPRAR			0.048					-0.098			0.119	1.115	1.096	0.777	0.001	-0.133	0.135	-0.096	-0.096	0.654	-1.198	-0.956		-0.980	0.001
43	GDC0994	Q9NX01	TXNL4B	132	LIVQSPIDP		-0.179					-0.404					-0.128	-0.309	-0.146		-0.118	0.098	-0.054	-0.054	0.825	-1.414	-1.123		-1.225	<0.001
44	GDC0994	Q09666	AHNAK	4564	VGIDTPDID	0.279	-0.525	-0.415	-0.219	0.523	-0.391	-0.965	-0.049	-0.469	0.135	0.012	-0.134	1.002	0.311	0.264	0.004	0.229	-0.203	0.002	0.995	1.083	0.747	1.266	1.018	0.010
45	GDC0994	Q9UHR4	BAIAP2L1	261	TPQASPMIE		-0.298	0.132	-0.104	0.760		0.123	-0.292	-0.063	0.867	-0.116	-0.418	-0.790	-0.460	0.036		-0.110	-0.029	-0.029	0.917		0.733		0.884	0.001
46	GDC0994	Q8WUZ0	BCL7C	126	SRPVSPAGP	0.133	-0.449	-0.544	-0.267	0.282	-0.001	-0.225	0.168	-0.039	0.911	-0.625	-0.581	-0.546	-0.567	0.001	-0.240	-0.412	-0.371	-0.289	0.009	1.074	0.898	0.851	0.889	<0.001
47	GDC0994	Q6PKG0	LARP1	766	TIARSLPTT	-0.010	-0.047	-0.603	-0.221	0.185	-0.135	-0.017	-0.544	-0.231	0.168	-0.186	0.166	0.062	0.016	0.941	-0.346	0.393	-0.273	-0.064	0.852	1.641	2.357	2.029	1.998	<0.001
48	GDC0994	P19532	TFE3	548	LRAASDPLL		-0.456	0.084	-0.194	0.380		-0.102	0.295	0.104	0.677	0.105	0.012	-0.308	-0.055	0.674	-0.093	0.230	0.101	0.101	0.573	1.098	1.086		1.060	<0.001

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Figure 2.14: A STRING network of proteins with phosphosites significantly regulated only in response to GDC0994. Edges indicate evidence of molecular action such as binding or other reactions, with confidence at the lowest setting (>0.150). Color and size of nodes are arbitrary. Disconnected nodes are not shown.

2.6 Discussion

In summary, I have compared a series of phosphoproteomics experiments to interrogate molecular responses to drugs and drug candidates which inhibit different enzymes in the RAF-MKK-ERK MAPK pathway. From these, I can begin to classify responses to different tiers of the signaling pathway. These can be used to validate known and novel targets of signaling, based on stringent criteria requiring agreement between all four inhibitors. Importantly, the comparison can be used to identify candidates regulated by MKK1/2, which bifurcate upstream of ERK1/2, thus potentially reporting deviations from a linear pathway organization. Finally, compound-specific phosphorylation responses are likely to report off-targets, that can be useful for evaluating specificity of drug candidates.

Previously, our lab found that the BRAF V600E/K inhibitor, vemurafenib, and the MKK1/2 inhibitor, selumetinib, show a very high degree of overlap in their phosphosite responses (Stuart et al., 2015), reinforcing the idea that signaling from BRAF V600E/K to MKK1/2 is remarkably linear. The responses to submaximal concentrations of each inhibitor, singly and in combination, were additive, suggesting that the clinical effectiveness of cotreatment with BRAF V600E/K and MKK1/2 inhibitors in patients is likely due to an additive suppression of ERK1/2 signaling, rather than synergy between unique targets of each treatment individually. With the emergence of ERK1/2 inhibitors as tools to overcome resistance to BRAF V600E/K and MKK1/2 inhibitors, my first aim was to measure overlap in molecular responses to the ERK1/2 inhibitor, SCH772984, and the FDA-approved MKK1/2 inhibitor, trametinib. I found that among the 4.7% of phosphosites which changed significantly in response to either inhibitor, only 5.3% were exclusive to only one inhibitor. This further supports the previous findings of linearity and additivity among inhibitors of the BRAF V600E/K-MKK1/2-ERK1/2

pathway. Several ERK1/2 inhibitors are currently in early stage clinical trials, and may be successful in combination with BRAF V600E/K inhibitors This is supported by known mechanisms of resistance to the BRAF V600E/K+MKK1/2 inhibitor combination which in the majority of cases lead to the reactivation of ERK1/2.

My next aim was to identify specific targets of MKK1/2 and ERK1/2 inhibitors with known functions. I found that p38α MAPK phosphorylation sites are novel off-targets of trametinib, but not SCH772984 or GDC0994 in my study, and selumetinib and vemurafenib in our lab's previous study (Stuart et al., 2015). Inhibition of p38α MAPK phosphorylation occurs at ~10 μM, concentrations higher than those achieved in plasma during clinical studies. Nevertheless, this concentration range is commonly used in preclinical research studies and should be considered in studies of this drug. Further analysis revealed that MKK6 but not MKK3 can be inhibited *in vitro* by trametinib, and I initially assumed that this explains inhibition of p38α MAPK downstream. However, unexpectedly, the dose response for MKK6 inhibition was in a concentration range greater than 10 μM. The results suggests that MKK6 may in fact not be the direct target of trametinib relevant to its cellular inhibition of p38α MAPK. Further studies are needed to resolve this issue. Meanwhile, I observed that the p38 MAPK inhibitor, SB203580, decreases cell viability when used in combination with selumetinib, but not trametinib. Potentially, an off-target effect of trametinib on p38α MAPK may beneficially impact the efficacy of this drug in suppressing cell viability, by supplementing effects of RAF-MKK-ERK pathway inhibition.

My final aim was to compare molecular responses between the two different ERK1/2 inhibitors, SCH772984 and GDC0994. Some but not all ERK1/2 inhibitors show properties of conformational selection, based on NMR relaxation studies (Rudolph et al., 2015; Xiao et al.,

2014). While Vertex-11e preferentially binds phosphorylated, active ERK2 in a conformer unique to the active state, unpublished studies show that SCH772984 preferentially binds and locks phosphorylated, active ERK2 in the opposite conformer, characteristic of the inactive, unphosphorylated state. In contrast, GDC0994 binds the phosphorylated, active ERK2 in a manner which allows equilibrium interconversion between conformers, comparable to the apoenzyme, and favoring the new conformer characteristic of the active state (Y. Xiao, J. Rudolph and N. Ahn, unpublished). Thus, SCH772984 favors the inactive conformer, while GDC0994 interconverts but favors the active conformer. It is not yet clear whether conformational selection to shift the equilibrium towards one or the other conformers affects the specificity of inhibition of downstream substrates, or whether one conformer is more important than the other. Conceivably, the molecular responses to SCH772984 and GDC0994 might reflect these differences.

The majority of significantly affected phosphosites responded to both drugs, suggesting that inhibition of ERK1/2 is nearly complete at the 10 µM concentrations tested. Among the phosphosites uniquely responsive to one but not both ERK1/2 inhibitors, 25 phosphosites changed only in response to GDC0994 and 3 changed only in response to SCH772984. The 25 phosphosites that changed in response to GDC0994 alone included five proteins which are known downstream substrates of ERK1/2, LARP1, ELK1, AHNAK, GIGYF2, and LMNB2. STRING analysis of this gene subset (i.e., significantly inhibited by GDC0994 but not SCH772984) identified a network which included EIF4G1, LARP1, LARP1B, and HNRNPM, all of which are involved in regulating RNA processing and translation. Thus, they are interesting targets which may potentially be useful to ask whether the different conformers that predominate upon binding GDC0994 vs SCH772984 might account for these differences in

target specificity, and whether these phosphoproteins may be markers of the conformational

selective binding. Further studies are needed to address this intriguing question.

Chapter 3

Conclusions and future directions

3.1. Summary and conclusions

In this thesis I describe the overlap and specificity of MKK1/2 and ERK1/2 inhibitor targets in WM239a melanoma cells. Using phosphoproteomics, these changes were profiled across three experiments using the MKK1/2 inhibitor trametinib, and ERK1/2 inhibitors SCH772984, GDC0994, and Vertex-11e. These datasets, combined with a previously published study by our lap profiling the phosphoproteomic changes in response to the BRAF V600E/K inhibitor, vemurafenib, and the MKK1/2 inhibitor, selumetinib, constitute a unique resource for probing known and novel processes regulated by targeted MAPK inhibitor therapy in melanoma.

MKK1/2 and ERK1/2 inhibitor comparison by phosphoproteomics

Trametinib is a MKK1/2 inhibitor approved for clinical use as monotherapy or combination therapy with the BRAF V600E/K inhibitor, vemurafenib, in patients with metastatic melanoma. Previously, our lab demonstrated a high degree of overlap between inhibitors, suggesting that beneficial results observed in patients treated with combination therapies are most likely due to additive effects on the inhibition of downstream signaling targets. Preclinical studies established trametinib as a potent, selective inhibitor of MKK1/2 using *in vitro* kinase assays on 100 potential kinase targets (Yamaguchi et al., 2011). Although the prior studies showed few off-target effects of trametinib on other protein kinases, the regulation of downstream effectors such as transcription factors remained unknown. My SILAC-based phosphoproteomics study of responses to trametinib and SCH772984 identified 12,924 phosphosites affected by drug treatment, 8,577 of which were reproducibly quantified in multiple

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replicate experiments. Overall, I observed significant changes to approximately 5% of all phosphosites identified, and very few targets specific for only one inhibitor. This indicates a high degree of pathway linearity in the MAPK signaling cascade upstream of ERK1/2, and further supports the additive model of vertical targeting in combination therapies.

Inhibition of novel off-target p38α by trametinib

Among the small subset of targets uniquely affected by only one inhibitor, the canonical activation sites Thr180 and Tyr182 on p38α MAPK were significantly inhibited by trametinib but not SCH772984. Intriguingly, Ser796 on the epidermal growth factor receptor pathway substrate 15 (EPS15), which has previously been shown to respond to p38 MAPK inhibition (SB203580) but not MKK1/2 inhibition (U0126) or JNK inhibition (SP600125) in HeLa cells (Zhou et al., 2014), was also inhibited by trametinib, but not SCH772984 (Table 2.9). These findings, showing significant changes in the phosphorylation of p38α MAPK, as well as a known substrate specifically targeted by p38 MAPK, provide evidence that trametinib effects on p38α may regulate cellular processes not associated with the RAF-MKK-ERK pathway. To determine if p38α inhibition is shared among MKK1/2 inhibitors, I compared my datasets to previous SILAC datasets examining phosphoproteomics responses to the MKK1/2 inhibitor, selumetinib (Stuart et al., 2015) (Table 2.3), and again found that inhibition of Thr180 and Tyr182 is unique to trametinib.

Biochemical assays suggested that this unique inhibition of p38α MAPK phosphorylation by trametinib may be explained by its ability to inhibit MKK6, a kinase upstream of p38α MAPK. Supporting this hypothesis, MKK6 was identified as a direct target of trametinib but not selumetinib, leading to decreased p38α MAPK phosphorylation *in vitro*. Arguing against this

hypothesis, trametinib inhibited MKK6 with IC$_{50}$ 10-fold higher than its inhibition of p38α

MAPK in cells. Therefore, the jury is still out as to whether MKK6 is the direct target of

trametinib in cells explaining its effect on p38α MAPK. No crystal structures of trametinib-

bound MKK1/2 are available and the canonical activation motifs differ. MKK1 is activated by

dual phosphorylation at S218 and S222 while MKK6 is activated at S207 and T211. These

activation sites, as well as the HRD and DFG motifs important for catalytic activity, overlap

using NCBI BLAST protein sequence alignment, and there is a 38% sequence identity between

MKK1 and MKK6. Nevertheless, cells treated with the p38α inhibitor, SB203580 + trametinib

show small decreases in viability compared to trametinib alone. However, SB203580 +

selumetinib (which does not inhibit p38α) show larger decreases in cell viability compared to

selumetinib alone. The findings suggest that p38α inhibition functionally reduces cell viability of

melanoma cells. Taken together, this evidence supports p38α MAPK as a previously

uncharacterized off-target effect of trametinib, which may enhance the beneficial cellular

response to this drug. In any case, although this effect is only observed at drug concentrations

~300-fold higher than clinical dosing in patients, preclinical studies often use trametinib

concentrations of 10 µM or higher. Therefore, off-target effects on p38α MAPK must be

considered in studies of trametinib.

Comparing ERK1/2 inhibitors using phosphoproteomics

ERK1/2 inhibitors are emerging as a promising strategy to build on the success seen in

targeting multiple points upstream in the MAPK pathway, and to combat the development of

resistance to targeted BRAF V600E/K and MKK1/2 inhibitors that are commonly seen in

patients. Many of these resistance mechanisms involve re-activation of ERK1/2, and preclinical

studies have shown that inhibitors targeting ERK1/2 are able to overcome resistance to BRAF

V600E/K and MKK1/2 inhibitors (Morris et al., 2013). Our lab has previously published

evidence that ERK1/2 inhibitors show differences in conformational selectivity upon binding

ERK2 (Rudolph et al., 2015). Therefore, I performed phosphoproteomic SILAC experiments

comparing ERK1/2 inhibitors, in order to determine if these conformational selection preferences

were accompanied by differences in downstream phosphorylation events. In one experiment, the

ERK1/2 inhibitor SCH772984 was compared to Vertex-11e, which is structurally related to

Vertex-745 currently undergoing clinical trials. A triple labeled SILAC screen (DMSO-

SCH772984-Vertex-11e) identified 2,701 phosphosites, 1,163 of which were reproducibly

quantified in two or more replicate experiments. Like the previous comparisons, the phosphosites

regulated by SCH772984 and Vertex-11e showed almost complete overlap, indicating that

different ERK conformational binding characteristics had little to no effect on downstream drug

targets. The second screen compared SCH772984 to GDC0994, which is currently in clinical

trials (DMSO-SCH772984-GDC0994). I identified 6,893 phosphosites in total, and among the

4,436 phosphosites quantified in two or three replicates, 25 were significantly regulated by

GDC0994 and not SCH772984 while only 3 were unique to SCH772984 (Fig. 2.8G, Table 2.4).

These unique targets were also unaffected by treatment with other MKK1/2 and ERK1/2

inhibitors (Table 2.9). Potentially, the targets unique to GDC0994 may reflect the distinct

conformational selection by this compound, compared to SCH772984. This finding is relevant to

current and future clinical trials involving GDC0994, and requires further studies to correlate the

effects across many different compounds. Among the novel off-targets, a group of genes

regulating protein translation mRNA binding and initiation was identified using STRING protein

interaction mapping and included EIF4G1, LARP1, LARP1B, and HNRNPM (Fig. 2.14).

Coordinated regulation of this group of genes might reflect selective regulation of mRNA

processing and/or translation, and further studies are needed to investigate this possibility.

Combined phosphoproteomics MAPK inhibitor datasets provide an unparalleled resource for discovery of off target effects

With the completion of my thesis work, our lab has now generated SILAC-based

phosphoproteomics data in a single melanoma cell line, WM239a, treated with the BRAF

V600E/K inhibitor vemurafenib, the MKK1/2 inhibitors selumetinib and trametinib, and the

ERK1/2 inhibitors SCH772984, GDC0994, and Vertex-11e. Comparing the cellular response to

inhibitors targeting different levels of the MAPK pathway will lead to a better understanding of

the network of signaling responses to MAPK inhibition and interactions with other pathways.

Table 2.7 lists 150 phosphosite targets significantly regulated by all MAPK inhibitors

tested and reveals both known and novel targets. Nine of these sites significantly increase

phosphorylation in response to inhibitor, and may indicate changes in feedback inhibition or

reductions in phosphatase activity. In order to determine potential direct targets, I have compiled

information from several sources. First, ERK1/2 is known to preferentially phosphorylate proline

driven Ser and Thr residues with a sequence (S/T)P or PX(S/T)P motif. Phosphosites with these

motifs underwent further validating by identifying proteins with the ERK binding d-domain and

DEF domains. DEF domains were identified manually 6-20 residues C-terminal of the

phosphorylated residue (Cargnello & Roux, 2011) and d-domains were identified using a large-

scale d-domain profiling study (Zeke et al., 2015) and ScanSite3 high confidence identification

(Obenauer, Cantley, & Yaffe, 2003), and mapped to protein sequences to determine the distance

from the identified phosphorylated residue. Next, known ERK substrates and phosphorylated

residues were compiled from (Carlson et al., 2011; Yoon & Seger, 2006) and ERK interactors were identified from (von Kriegsheim et al., 2009). Finally, the phosphosite plus database (Hornbeck et al., 2015) downloads for phosphosites with known kinase regulation and protein regulatory function indicate sites that are known to be directly regulated by ERK. These different sources of information allow us to locate likely direct ERK target phosphosites and identify potential novel substrates of ERK.

My findings support previous work revealing linear pathway organization of RAF-MKK-ERK signaling, by identifying only three phosphosites uniquely regulated by two MKK1/2 inhibitors but not two ERK1/2 inhibitors tested (S893-GEN1, S612-NUP98, and S455-MAP3K7/TAK1, Table 2.8). TAK1 is a known to activate MKK3/6 and undergoes negative feedback regulation through p38α signaling to its binding partner TAB1. It is interesting that both trametinib and selumetinib increase the phosphorylation at this site, yet only trametinib inhibits phosphorylation of downstream p38α MAPK and the p38α substrate EPS15. Although p38 MAPK is the primary substrate of MKK3/6 (similar to ERK1/2 and MKK1/2), upstream activation of MKK3/6 by cellular and environmental stress signals is not yet fully understood.

My composite phosphoproteomic dataset is also useful for identifying unique targets that reflect potential off-target effects of individual compounds. I have identified 48 phosphosites that specifically respond to only one MKK1/2 or ERK1/2 inhibitor. These phosphosites are likely off-target effects specific to each compound and are good candidates for follow up analysis, using methods similar to those I used in my investigations of p38α MAPK inhibition by trametinib. I have now built a database of phosphoproteomics changes in response to BRAF V600E/K, MKK1/2, and ERK1/2 inhibitors across four separate SILAC experiments. This dataset will be a valuable resource for the development of new targeted therapies in melanoma. Future studies

may use this database to quickly profile the targets in common with these MAPK inhibitors, in order to identify shared or off-target effects during drug development and identify potential biomarkers for drug treatment.

3.2 Future experiments

The significance of inhibiting p38α MAPK activity in melanoma is largely unknown, considering that high concentrations of trametinib only decrease p38α phosphorylation ~50% in cells. The p38 pathway has been reported to have both oncogenic and tumor suppressive effects depending on both the cell type and the type of pathway stimulation. But there is evidence that p38 signaling protects UV-treated melanoma cells from apoptosis (Ivanov & Ronai, 2000), given that melanoma cells expressing constitutively active MKK6 show decreased basal and UV-induced cell death compared to controls, while a catalytically inactive mutant increased the percentage of cell death in response to UV irradiation. To determine if p38 acts as a pro-survival pathway vs inhibits cell viability under normal conditions, future studies are needed to treat cancer cell lines with p38α/β inhibitors (e.g. SB203580), or by siRNA knockdowns or gene editing of MKK3, MKK6, p38α, and p38β, measuring cell viability using the CellTiter Glo assay and apoptosis using annexin V-PI flow cytometry. These results can then be compared to cells where p38 MAPK signaling is activated by hyperosmotic stress or UV-irradiation. Any differences can be followed up by a phosphoproteomic screen of cells treated in parallel to look for p38-specific targets and manually validate candidate effectors that may contribute to apoptosis or survival.

Appendix I. Human Phospho-Kinase Antibody Array. Changes in kinase phosphorylation in response to 10 µM treatment for 4 h with MKK1/2 inhibitor selumetinib (AZD) or ERK1/2 inhibitors SCH772984 (SCH) and Vertex-11e (VTX)

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