Quantitative Analysis of Signaling Networks in Proneural Glioblastoma

By

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ABSTRACT

Glioblastoma (GBM) is the most common malignant form of brain cancer. Even with treatment including surgery, radiation, and temozolomide chemotherapy, the 1 year survival rate is only 35%. To identify specific mediators of GBM progression in a genetically engineered murine model of proneural GBM, we quantified signaling networks using mass spectrometry. We identified oncogenic signaling associated with the GBM model, such as increased phosphorylation of ERK1/2, PI3K, and PDGFRA, relative to murine brain. Phosphorylation of CDK1 Y15, which causes G2/M cell cycle arrest, was measured to be the most differentially phosphorylated site, with a 14-fold increase in the tumors. We used syngeneic cell lines to investigate this checkpoint further and treated these cells with MK-1775, an inhibitor of Wee1, the kinase responsible for phosphorylation of CDK1 Y15. MK-1775 treatment resulted in mitotic catastrophe of these cells, as measured by increased DNA damage, abnormal percentages of cells in cell cycle phases, and death by apoptosis. This response was abrogated by inhibiting CDK1 with roscovitine, a CDK inhibitor, demonstrating the necessity of active CDK1 for MK-1775 induced mitotic catastrophe. To assess the extensibility of targeting Wee1 and the G2/M checkpoint in GBM, we treated patient-derived xenograft (PDX) cell lines with MK-1775. The response was more heterogeneous, but we measured decreased CDK1 phosphorylation, increased DNA damage, and death by apoptosis. These results were validated in a flank GBM PDX model where treatment with MK-1775 increased mouse survival by 1.74-fold. We also quantified the signaling differences in our murine GBM model after treatment with sunitinib, an inhibitor of its driver receptor tyrosine kinase, PDGFRA. Treatment increased survival but lead to a morphological change causing a more invasive phenotype. Pro-migratory signaling was characterized by mass spectrometry, such as increased phosphorylation of Enoi, ELMO2, and tubulins. Invasion was further characterized in a lung cancer model where we identified signaling specific to different ligands that result in similar levels of invasion. We have demonstrated that unbiased, quantitative phosphotyrosine proteomics has the ability to reveal therapeutic targets in tumor models and signaling differences between treatments.

Thesis Supervisor: Forest M. White
Title: Professor of Biological Engineering
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<tr>
<td>ANXA2</td>
<td>Annexin A2</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BIC</td>
<td>Bayesian information criterion</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescence imaging</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DPI</td>
<td>Days post injection</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GBM</td>
<td>Glioblastoma</td>
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<tr>
<td>GIST</td>
<td>Gastrointestinal stromal tumors</td>
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<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene Set Enrichment Analysis</td>
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<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IP</td>
<td>Immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>Isobaric Tag for Relative the Absolute Quantification</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography coupled to tandem mass spectrometry</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>M/Z</td>
<td>Mass to charge</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>OPC</td>
<td>Oligodendrocyte progenitor cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBS-TB</td>
<td>PBS supplemented with 1% BSA and 0.1% Tween20</td>
</tr>
<tr>
<td>PDGF/PGDF/PDGFRA</td>
<td>Platelet derived growth factor receptor alpha</td>
</tr>
<tr>
<td>PDX</td>
<td>Patient-derived xenograft</td>
</tr>
<tr>
<td>PIP_3</td>
<td>Phosphatidylinositol-3, 4, 5-trisphosphate</td>
</tr>
<tr>
<td>PIP_2</td>
<td>Phosphatidylinositol-4, 5-bisphosphate</td>
</tr>
<tr>
<td>pHH3</td>
<td>Phospho-Histone H3</td>
</tr>
<tr>
<td>PLSR</td>
<td>Partial least-squares regression</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine binding</td>
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<tr>
<td>PTM</td>
<td>Post-translational modification</td>
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<tr>
<td>Ros</td>
<td>Roscovitine</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate, polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
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<tr>
<td>SILAC</td>
<td>Stable Isotope Labeling by Amino acids in Cell culture</td>
</tr>
<tr>
<td>TBST</td>
<td>150 mM NaCl, 0.1% Tween20, 50 mM Tris, pH 8.0</td>
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<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
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<tr>
<td>TNS3</td>
<td>Tensin 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Thr/T</td>
<td>Threonine</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>Tyr/Y</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VIP</td>
<td>Variable Importance for the Projection</td>
</tr>
<tr>
<td>VSVG</td>
<td>Vesicular stomatitis virus G protein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
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Chapter 1: Introduction

Rebecca Lescarbeau

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Chapter 1: Introduction

Cancer is characterized as abnormal growth and spread of cells. External and internal or hereditary factors can lead to cancer. In 2015, it is estimated that 1.6 million new cases will be diagnosed in the United States and 600,000 deaths will occur due to cancer. This number makes cancer the second leading cause of death in the United States, after cardiovascular disease, accounting for 1 in 4 deaths (American Cancer Society, 2015). Brain and central nervous system (CNS) cancers are some of the most devastating cancers; while they will account for 23,000 new cases in 2015, they will cause 15,000 deaths.

Glioblastoma Characterization and Treatment

Glioblastoma (GBM) is the most common and malignant brain tumor. It accounts for 45.2% of malignant primary brain and CNS tumors (Ostrom et al., 2013). The one year survival rate is 35% and the five year survival rate is only 4.7%. GBM is a World Health Organization (WHO) grade IV glioma (scale: I-IV); a tumor of the neuroepithelial tissue that has a median diagnosis at age 64 (Fisher et al., 2007). GBM commonly presents as progressive cognitive deficits, headaches, and seizures. Diagnosis usually includes MRI, or contrast-enhanced CT, although CT provides inferior detail. The frontal lobes of the supratentorial compartment is the most common tumor location. Although the majority of the neoplastic cells are within the tumor bed or 2 cm of its boarders, migrating cells can be found many centimeters from the tumor due to the diffuse nature of GBM. These cells do not metastasize outside of the CNS, however (Adamson et al., 2009).
Standard of care currently includes surgery, radiation, and temozolomide. Additional therapies are often used to treat associated symptoms such as corticosteroids to reduce edema and anti-epileptics for patients presenting with seizure (Stupp et al., 2010). Surgery is often unable to remove all the tumor cells and recurrence usually occurs in the end stage of this disease. High proliferation and migration rates of the GBM cells make total surgical resection nearly impossible and limit surgeons’ ability to reach all the cancerous cells. However, increasing the percentage of the surgically removed mass increases the patient’s survival, with nearly full resection (>98% of T1-weighted enhancing volume on MRI) required for significant survival improvement (Lacroix et al., 2001). Significant effort has been invested into better resection measures such as fluorescence-guided surgery using 5-aminolevulinic acid (Valle et al., 2011) and fluorescein sodium-guided surgery (Koc et al., 2008; Shindoa et al., 2003). Enhancing contrast between tumor cells and brain matter is possible, particularly in the white matter, and this distinction significantly increases gross total resections.

Two seminal randomized, controlled trials in the 1960s-1980s demonstrated an increase in survival by about 6 months after the addition of radiation to surgery (Walker et al., 1979; Chin et al., 1981), which became the standard of care. This addition increased patient survival from about 4 months to 10 months. Currently 60 Gy of focal radiotherapy is administered in numerous fractions (e.g. 30 fractions of 2 Gy), but this can be adjusted based on patient age and health (Stupp et al., 2010).
Chemotherapy was added to the typical treatment regimen in the 1990s-early 2000s. The nitrosourea chemotherapeutic, carmustine (BCNU), became the standard of care, in addition to surgery and radiation, after multiple studies and a meta-analysis demonstrating marginal benefit (Glioma Meta-analysis Trialists Group, 2002). This chemotherapeutic is a mustard gas related compound that causes DNA crosslinks between strands. Since then, however, temozolomide has become the standard chemotherapeutic prescribed with surgery and radiation after a 2005 study demonstrated a 2.5 month increased survival (Stupp et al., 2005) relative to surgery and radiation alone and increased survival compared to other chemotherapies (Mineo et al., 2007). Current guidelines recommend low dose temozolomide (75 mg/m²) concomitant with radiotherapy. Following radiotherapy, the maintenance phase of treatment begins which consists of daily temozolomide at a higher dose (150-200 mg/m²) for five continuous days, every four weeks for six cycles (Stupp et al., 2010). Following patients for five years demonstrated a benefit of temozolomide even at 5 years (Stupp et al., 2009). However, even with these aggressive, yet untargeted approaches, patients inevitably succumb to the disease, with an average life expectancy of just over a year following diagnosis. In addition to these general therapies, molecular studies have been undertaken to stratify GBM tumors and to understand the changes occurring these cells in hopes of furthering the understanding of this disease and patient survival.

Temozolomide is a DNA damaging agent that induces O⁶-methylguanine lesions (Roos et al., 2007). Although these lesions form less frequently than N-alkyl adducts, they readily lead to base mismatch with thymine during DNA replication, thereby causing mutagenic and cytotoxic biological effects (Fu et al., 2012). One of the main effects of
these O⁶-methylguanine lesions is to trigger arrest in s-phase, as well as other parts of the cell cycle, in order to give the cells time for repair by mismatch repair pathways. If repair is not successful, the cell may undergo apoptosis, programmed cell death (Noonan et al., 2012). For full effectiveness, chemotherapies must damage DNA without inducing a DNA damage response (described below) or overwhelm the response and cause apoptosis (Sancar et al., 2004). While temozolomide is currently used to treat patients regardless of MGMT promoter methylation status, it is more beneficial to patients whose tumors have methylation of their MGMT promoter (Hegi et al., 2005). Methylation is an epigenetic silencing mechanism that causes loss of protein expression. MGMT is a DNA methyltransferase that removes alkyl groups from the O⁶ position of guanine, the typical site of functional lesions caused by temozolomide. Promoter methylation decreases the level of functional MGMT in the tumor and thereby reduces the cells’ ability to repair temozolomide-induced DNA damage, leading to increased patient survival. In the Hegi et al. study patients were stratified by molecular diagnosis, even though this has not yet led to a change in treatment overall.

Necrosis and vascular hyperproliferation set grade IV tumors apart from lower grade gliomas. Lower grade gliomas can progress into GBM and are termed secondary GBM, whereas grade IV GBM tumors arising de novo are termed primary GBM and account for 95% of GBMs. Although primary and secondary GBMs are histologically similar, they have distinct features. For instance, secondary GBM are diagnosed in younger patients (mean age is 45) and the survival outcomes are slightly increased. Primary GBMs often have EGFR overexpression or PTEN loss causing aberrant PI3K/AKT signaling, whereas secondary GBMs often present in younger patients and contain TP53 mutations and
amplification or increased expression platelet derived growth factor (PDGF) receptor (PDGFR) (Reardon et al., 2006; Ohgaki et al., 2004). Although these features differ between primary and secondary gliomas, additional molecular differences may occur in different subtypes of primary gliomas. To elucidate these molecular characteristics, The Cancer Genome Atlas (TCGA) underwent a large scale study to further understand the patient-to-patient differences in primary GBM.

**GBM Subtype Classification**

In a landmark study, 500 GBM tumors were analyzed by the TCGA for gene expression, DNA copy number, nucleotide sequence aberration, large scale chromosomal rearrangement, and DNA methylation status (TCGA Research Network, 2008; Brennan et al., 2013). TCGA was launched by the National Institutes of Health in 2005 with the goal of initiating a “comprehensive effort to accelerate our understanding of the molecular basis of cancer...” (NHGRI Communications, 2005) and due to the stagnated progress in treatment and availability of samples and other processing requirements, GBM was chosen as one of the first three cancers for study during the pilot phase of the program (NHGRI Communications, 2006). Common core pathways, such as receptor tyrosine kinases (RTKs), p53 signaling, and the RB pathway, were shown to be highly mutated in a large proportion of these tumors.

For example, 45% of GBMs analyzed had epidermal growth factor receptor (EGFR) mutations or significant copy number variation and 13% had PDGFR alpha (PDGFRA) amplifications. Both of these proteins are RTKs, cell surface receptors to many growth factors and cell-cell interacting ligands that transmit external signals into the cell
through tyrosine phosphorylation cascades that can affect cell form, function, growth, division, and survival. RTKs are typically composed of an extracellular domain for ligand binding, a transmembrane helix, a cytosolic kinase domain, and a cytosolic signaling domain. Similar to most RTKs, PDGFR is a monomeric protein whose ligand binding induces dimerization and autophosphorylation of tyrosine residues in the cytosolic domain. Phosphorylation of these sites leads to the recruitment and phosphorylation of additional proteins, thereby initiating signaling cascades that ultimately drive phenotypic change (cell biological response). These signaling cascades are linked through phosphorylation; for example, Src is activated through the binding of its SH2 domain to tyrosine phosphorylation sites in the C-terminal tail of PDGFR. Similar events occur in P85/P110 (PI3K) and PLCγ activation (Schlessinger et al., 2000). Tyrosine phosphorylation is one of a number of post-translational modifications (PTMs) that propagate signaling through intracellular cascades. These pathways have positive and negative feedback mechanisms in addition to redundancy, such as multiple RTKs, creating a tightly controlled cell phenotype until there is network dysregulation.

In addition to these mutations in the genes of proteins at the cell surface and the beginning of the signaling cascades, 36% of tumors had mutations or homozygous deletions in the tumor suppressor PTEN, further down the pathway (TCGA Research Network, 2008). PTEN is a bi-specific lipid and protein phosphatase whose primary function appear to be regulation of PI3K signaling. Upon ligand binding to RTKs and p85/p110 recruitment to the activated RTK and phosphorylation, phosphatidylinositol-4, 5-bisphosphate (PIP2) is phosphorylated by PI3K to generate the lipid second messenger phosphatidylinositol-3, 4, 5-trisphosphate (PIP3). PIP3 recruits proteins such
as AKT to the cell membrane through pleckstrin homology domains. At the membrane, these proteins are then phosphorylated (AKT by its kinase PDK1), thus initiating pro-growth and survival signaling in the cell. PTEN is an antagonist of this mechanism by dephosphorylating PIP3 at the D3 position to convert it back to its bisphosphate state, PIP2 (Chalhoub and Baker, 2009). The TCGA study greatly expanded the molecular understanding of GBMs. For instance, TP53 (described in detail below) was measured to have mutations or homozygous deletions in 35% of tumors in this cohort of patients with predominantly primary, over secondary, GBMs, suggesting that TP53 mutations are not exclusive to secondary GBM, as previously thought because most of these tumors were not known to have grown out from lower grade gliomas and were classified as primary GBM.

In addition to analysis of the entire GBM population as a whole, TCGA was able to identify four statistically significantly distinct subtypes using gene expression analysis and unbiased consensus clustering, Figure 1.1A, each with its own characteristic alterations (Verhaak et al., 2010). Prior to this study, Philips et al. completed a subtyping analysis that consisted of 78 high grade glioma tumors (WHO Grade III and IV) and identified three subtypes based on transcript expression data. Patients with one of the three subtypes experienced increased survival relative to the other two (Philips et al., 2006). TCGA expanded upon this study in terms of the number of tumors and the stricter criteria that they be GBM. Because data was gathered using three different gene expression platforms, 1740 genes were used in this analysis that provided consistent and variable expression across the tumors and platforms. Two hundred tumors plus two normal brain samples were used in creating these classifications. The four subtypes are
named proneural, neural, classical, and mesenchymal based on the expression of signature genes, Figure 1.1B. From this analysis, a 210-gene signature was identified for each subtype as the smallest gene signature with the lowest cross-validation and prediction error while preserving distinction between subtypes.
Figure 1.1. TCGA GBM Subtypes. A. Identified copy number events (cn), gene expression (ge), and mutations (mut), in characteristic alterations present across hundreds of GBM samples grouped by subtype. B. Comparison of gene expression signatures of four cell types (oligodendrocytic, neuronal, astrocytic, and cultured astroglia) and the tumor samples' expression profile. Positive enrichment score indicates positive correlation. Figure adapted from Verhaak et al., 2010.
Proneural

The proneural subtype expresses a gene signature similar to that of oligodendrocyte development and is characterized, specifically, by two main features: *PDGFRA* alterations and *IDH1* mutations. The proneural subtype harbors joint focal amplification of the gene and increased gene expression. While all subtypes contain tumors with *PDGFRA* focal amplifications, the rate is significantly higher in the proneural tumors. *IDH1* mutations were almost exclusive to this subtype, but they did not typically present with *PDGFRA* abnormalities. These tumors harbored the majority of *TP53* mutations and loss of heterozygosity (LOH) events. Using Gene Set Enrichment Analysis (GSEA) (Shi and Walker, 2007), proneural tumors were highly enriched for the oligodendrocytic gene expression signature. These patients were diagnosed at a younger age, on average, and Verhaak *et al.* hypothesize that some of these tumors may have been secondary GBM or may have developed from clinically silent lower grade gliomas. Patients with proneural tumors trend towards an increased survival time. However, a more aggressive therapy, denoted by additional rounds of chemotherapy or concurrent radiation and chemotherapy, does not provide clinical benefit (as is seen in other subtypes, below, Verhaak *et al.*, 2010).

Neural

The neuronal subtype is characterized by gene ontology (GO) categories such as neuron projection and axon and synaptic transmission. Increased relative expression was identified in neuronal markers *NEFL, GABRA1, SYT1*, and *SLC12A5* (Verhaak *et al.*, 2010).
Classical

The classical subtype has very consistent mutations. For example, high-level $EGFR$ amplification and increased $EGFR$ expression was measured in 97% of classical subtype tumors. $CDKN2A$ deletion (encoding $p14^{INK2A}$ and $p14^{ARF}$) occurred with 94% of $EGFR$ amplification. $EGFR$ vIII mutations were also present in >50% of classical tumors and $TP53$ mutations were noticeably absent from these tumors. Chromosome 7 amplification accompanied by chromosome 10 loss was seen in 100% of classical tumors. Classical tumor expression was similar to the murine astrocytic signature and these patients benefited from increased survival after aggressive therapy (Verhaak et al., 2010).

Mesenchymal

The mesenchymal subtype is characterized by reduced expression of $NF1$ in a majority of tumors and hemizygous deletion of the NF1-containing region of chromosome 17, 17q11.2. Additionally, co-mutation of $NF1$ and $PTEN$ were almost exclusively measured in this subtype. Mesenchymal markers were also expressed such as $CHI3L1$ and $MET$ along with NF-KB pathway members. The enriched gene set of these tumors strongly relates to the cultured astroglial signature and microglia markers using GSEA. As with classical tumors, patients with mesenchymal tumors experienced increased survival with aggressive therapy (Verhaak et al., 2010).

Targeted Cancer Therapies

A deeper understanding of cancer biology and fundamental cell biology has allowed for a wave of targeted treatment options for a variety of cancers in the past decade. Two of
the earliest approved therapies were trastuzumab and imatinib. Trastuzumab is a recombinant (humanized) monoclonal antibody that was approved by the FDA in 1998 for the treatment of HER2 (a member of the EGFR family)-overexpressing breast cancer (Glennie and Johnson, 2000). While this targeted treatment is successful for many patients and changed the approach to treating cancer, it is not a cure-all as nearly 60% of HER2-overexpressing metastatic breast cancer patients do not respond to trastuzumab-based treatment (Dean-Colomb and Esteva, 2008). Imatinib received approval in 2001 for treatment of chronic myelogenous leukemia (Cohen et al., 2002). Imatinib is an orally available small molecule inhibitor of the BCR-Abl tyrosine kinase, a product of gene fusion that promotes uncontrolled cell growth. Tyrosine kinases transmit their signal through transfer of a phosphate group usually from ATP to the target tyrosine of its target protein. Because of this conserved mechanism, the ATP-binding site is often similar between kinases. The small molecule inhibitor is often a competitive inhibitor to ATP at the ATP-binding site. Thus one therapeutic may target multiple kinases; as one such example, imatinib, also inhibits PDGFR. Targeted therapeutics may be either biologics, such as trastuzumab, or small molecules, such as imatinib, that target specific proteins or families of proteins that are involved with cell phenotypes such as survival, proliferation, and invasion. These hallmarks have been known for years, and were summarized into six categories by Hanahan and Weinberg in 2000 and updated in 2011 (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). These pathways are often hijacked by cancer cells causing abnormal growth and tumor formation, such as increased expression of PDGFRA in proneural GBM.
The goal of a targeted therapeutic is to increase on-target effects while reducing off-target effects and toxicity. Surgery, when available, is able to remove the main bulk of a tumor, but manually removing all GBM cells is nearly impossible. As highlighted in Lefranc et al., even with removal of a brain lesion and a second surgery to remove a recurrent lesion, the tumor continues to regrow and the location within the brain of that regrowth is new, highlighting the migratory nature of GBM cells (Lefranc et al., 2005). For these reasons, additional treatment, such as radiation and chemotherapy, is helpful in prolonging life with GBM. However, substantial gains are still needed.

Although we have not yet realized a change in GBM standard of care, targeted therapies have changed the course of treatment in other cancers. In addition to trastuzumab and imatinib, which has now also been approved for treatment of gastrointestinal stromal tumors (GIST), gefitinib, a small molecule inhibitor of the EGFR tyrosine kinase, gained FDA approval in 2003 (Cohen et al., 2003) for non-small cell lung cancer treatment in patients that have failed chemotherapy. Sunitinib malate (sutent) is a small molecule multi-kinase inhibitor that inhibits PDGFR, and vascular endothelial growth factor receptors (VEGFRs), among others (Pfizer Labs, 2011). This inhibitor was originally approved in 2006 and is now approved for GIST, advanced renal cell carcinoma, and pancreatic neuroendocrine tumors. VEGFR inhibition affects tumor cells expressing VEGFR and also the microenvironment necessary to allow some tumor growth via nutrients provided by the blood supply (Sawyers, 2004).
Cell Cycle Regulation

Cell duplication must be a tightly regulated process of ordered events in order to maintain genomic fidelity through cell generations. The cell cycle consists of several stages: $G_1 \rightarrow S \rightarrow G_2 \rightarrow M$, culminating in division, and a resting state $G_0$. This process is tightly controlled through a series of checkpoints that allow the cell to transition from one phase to another. These checkpoints are in turn tightly controlled by a number of positive and negative regulatory circuits. By monitoring the state of the cell and its DNA, these cell cycle regulators act as a surveillance mechanism, ensuring the cell state is appropriate for the subsequent transition to occur. These checkpoints are of extreme importance in the cell because their loss can lead to reduced cellular fidelity and chromosomal abnormalities.

Checkpoints are active throughout the cell cycle and can be activated to inhibit cell progression at multiple points. The response can include removal of damaged DNA and restoration of the original sequence, activation of DNA damage checkpoints to arrest the cell cycle and allow repair, transcription profile changes to increase repair enzyme content, and apoptosis to eliminate highly damaged cells (Sancar et al., 2004). For example, DNA-damage checkpoints are present throughout the cell cycle and if activated can generate a signal that causes cell cycle arrest and, through inducing transcription of DNA repair genes, lead to DNA repair. If DNA damage is detected in $G_1$, DNA synthesis (S)-phase can be slowed; arrests in $G_2$, post S-phase, causes activation of repair genes before the cell divides with an inaccurate genome (Elledge, 1996; Zhou and Elledge, 2000). In attempting to repair the DNA, if this is not possible, the cell may undergo apoptosis. While apoptosis kills the specific cell, this process is beneficial for
the organism as a whole; damaged DNA may harbor mutations leading to abnormal daughter cells.

The p53 tumor suppressor is primarily involved in the G\textsubscript{1} DNA damage checkpoint, but is also active in G\textsubscript{2} (Agarwal et al., 1995; Bunz et al., 1998; Goi et al., 1997). P53 is a transcription factor that is activated in response to DNA damage and causes increased transcription of repair proteins. Its gene is most frequently mutated in cancer and defective p53 reduces cell arrest and apoptosis. ATM, which is activated by DNA double strand breaks and linear DNA, and ATR, which is activated by base damages, phosphorylate p53 switching it into its activated state (Sancar et al., 2004). Activated p53 causes increased p21 expression which, in turn holds the G\textsubscript{1}/S and G\textsubscript{2}/M transitions in an arrested state to allow time for repair (Bunz et al., 1998). Mutations in the TP53 gene, as is often demonstrated in cancer, provide a growth advantage since the cells do not spend time assessing and repairing DNA damage before division. However, this deficiency allows these cells to continue growing and dividing even with substantial or significant DNA abnormalities such as those promoting tumor growth in cancer.

Cell cycle transitions are dependent upon checkpoint complexes that are composed of cyclin dependent kinases (CDKs) and cyclins that are tightly controlled by positive and negative effectors. There are many of these complexes in mammalian cells and they are active at different points of the cell cycle. Phosphorylation acts as a point of regulation, activating or inhibiting the CDK. Kinases and phosphatases are present throughout the cell to readily phosphorylate and dephosphorylate amino acids, respectively, on many proteins. CDK\textsubscript{1} (CDC2) is active during the G\textsubscript{2}/M transition with its partner cyclin,
CyclinB. Multiple kinases (Wee1, MIK1, and MYT1) and multiple phosphatases (CDC25 and PYP3) tightly control the phosphorylation of two amino acids on CDK1, threonine 14 (T14) and tyrosine 15 (Y15) (Elledge, 1996). Phosphorylation of these amino acids activates the cell cycle checkpoint, causing cell cycle arrest events and inhibiting entry into mitosis (Sancar et al., 2004; Donzelli and Draetta, 2003), Figure 1.2. Activation of Wee1 and inactivation of CDC25 by ATR-chk1 and ATM-chk2 initiate this cell cycle stalling.
**Figure 1.2. CDK Regulation.** A. G₁→S transition of the CDK2/CyclinE checkpoint pair with kinases (Wee1, MIK1, and MYT1) that inhibit cell cycle progression through phosphorylation and phosphatase (CDC25A) that promotes progression after dephosphorylation. B. G₂→M transition CDK1/CyclinB checkpoint pair with kinases (Wee1, MIK1, and MYT1) that inhibit cell cycle progression through phosphorylation and phosphatases (CDC25A, B, and C) that promote progression after dephosphorylation.
Mammalian cells have evolved to express multiple checkpoints throughout the cell cycle and multiple regulatory mechanisms at each checkpoint. These are often deregulated in cancer, such as p53, promoting inappropriate growth, but also causing cells to survive with damage that causes them to be closer to initiating apoptosis than other, healthy cells.

**Mitotic Catastrophe**

Cancer treatment strategies attempt to remove the abnormal cells in bulk (surgery), induce a level of DNA damage that is incompatible with cell life (DNA damaging agents such as chemotherapies, which induce non-specific damage), or fundamentally alter a critical pathway necessary for cell survival of the tumor specifically (targeted therapeutics). As discussed above, all cells rely on a tightly controlled cell cycle, including cancer cells. Synergizing with the abnormalities of the cancer cells' cell cycle regulation is one strategy to induce apoptosis by mitotic catastrophe, defined as cell death, during late stages of the cell cycle, caused by abrogation of the G2/M arrest point and cellular damage in order to avoid genomic instability (Vitale et al., 2011). Failing to induce apoptosis with substantial DNA damage will lead to aneuploidy in the daughter cells due to asymmetric division (Castedo et al, 2004). In addition to aneuploidy, aberrant mitosis can lead to large cells containing many micronuclei of uncondensed chromosomes (Castedo et al, 2002). Thus mitotic catastrophe may be a protective mechanism for the fitness of the organism at large.

Tumor cells rely on the advantageous mutations throughout their genome that have caused some amount of growth advantage for tumor maintenance and survival.
However, these cells cannot incur too much damage otherwise they will initiate apoptosis. Inducing apoptosis as a therapeutic strategy is built off the theory that cancer cells may have increased sensitivity to abrogation of cell cycle checkpoint regulators, especially in tumors already harboring the loss of a regulator, such as p53. Similarly, unscheduled activation of the “mitosis-promoting factor” CDK1, by dephosphorylation of T14/Y15, can result in mitotic catastrophe through the promotion of premature chromatin condensation (Castedo et al., 2004).

**Murine Proneural GBM Model System**

Model systems are used to learn about a disease in a controlled setting that allows for exploratory treatments and close control over handling and treatment design. The model system used in many of our studies is a murine proneural GBM that was developed in the Canoll Laboratory at Columbia University (Lei et al., 2011). To induce tumor formation, transgenic mice with floxed PTEN and TP53 and stop floxed luciferase or yellow fluorescent protein (YFP) were injected with a retrovirus into the subcortical white matter. The retrovirus was vesicular stomatitis virus G protein (VSVG)-pseudotyped PDGF-IRES-Cre (PIC) which expresses PDGF and Cre in a single transcript. This injection causes tumor formation with 100% penetrance and histological features similar to human GBM. These tumors are P53 and PTEN null and express reporter luciferase or YFP. Importantly only infected cells and their progeny express the reporter which allows for tumor monitoring. These tumors develop quickly; mouse survival is only about one month. Control studies confirmed the necessity of both a tumor driver (PDGF overexpression) and the loss of tumor suppressor(s) (PTEN and/or P53) for tumor growth and development.
Oligodendrocyte progenitor cells (OPCs) are a well characterized population of glial progenitor cells that are still cycling in the adult mouse brain, allowing for targeted transformation by retroviral injection (Dawson et al., 2003; Roy et al., 1999). Through site-controlled injection and fate mapping, Lei et al. showed selective infection of progenitor cells that develop into tumors cells of the oligodendrocyte lineage. Markers for these cells include PDGFRA, NG2, and Olig2 (Dawson et al., 2003; Lindberg et al., 2009), Figure 1.3A.
Figure 1.3. Proneural GBM Model. A. Immunostaining of end-stage proneural GBM murine model tumors in the right hemisphere with low (left) and high (right) power micrografts. Immunostaining (red) and Hoechst nuclear staining (blue) demonstrate decreased PTEN and astrocytic marker GFAP and increased Olig2 and PDGFRA. B. Enrichment of the OPC gene set is demonstrated in both mouse murine tumors and human proneural GBMs. Figure adapted from Lei et al., 2011.
These GBM-like tumors closely resemble the proneural subtype, Figure 1.3B. The combination of their increased expression of PDGF and genetic deletion of TP53 and PTEN, induced by the retroviral PIC injection, and the high expression of genes expressed by oligodendrocytes, due to the cells that are infected by the retrovirus, creates a model system that is valuable for studying GBM, and proneural GBM specifically.

Measuring Signaling Networks Using Mass Spectrometry

Cancer progression is characterized by alterations in pathways and molecular characterization has identified amplifications, over-expression, and mutations in proteins such as PDGFRA and EGFR (described above). Uncontrolled cell growth can be a direct result of these changes and the increased signaling caused by aberrant activation of their pathways.

PTMs are cellular signaling mechanisms that aid in propagating external cues to cause a cellular phenotypic response. These include phosphorylation, acetylation, methylation, glycosylation, and ubiquitylation. Proteins can contain multiple modification sites that affect their function. Phosphate groups effect the protein’s state, cellular location, enzymatic activity, and interactions with or affinities to other proteins. Activation through ligand stimulation or inhibition of a protein’s function with the use of small molecules or antibodies will change its downstream signaling and these perturbations provide a lens through which signaling dynamics can be studied, such as phosphotyrosine signaling cascades (Zhang et al., 2005; Wolf-Yadlin et al., 2006; Johnson et al., 2013).
Mass spectrometry-based approaches, specifically liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) through electrospray ionization, have the ability to probe signaling changes at a network scale. Recent advances in biochemistry, MS technology, and computational techniques have allowed for a great increase in the possible scale and scope of projects in terms of both the number of conditions or samples analyzed and the number of site-modifications quantified (Del Rosario and White, 2010). Site-specific resolution of phosphorylation events is possible with MS techniques, creating an ‘omics’-based approach to understanding a protein’s and PTM’s state relative to many other proteins and across multiple conditions.

Profiling the tyrosine phosphoproteome requires enrichment to extract these low abundance events from the complex cellular milieu. Immunoprecipitation (IP) and immobilized metal affinity chromatography (IMAC) are commonly used methods. IMAC separates phosphorylated from non-phosphorylated peptides due to the charged nature of phosphate group (Andersson and Porath, 1986) and can readily be used in preparation of MS samples (Ficarro et al., 2002). Protein IP with antibodies raised against short peptides containing phosphotyrosine is a necessary additional step for phosphotyrosine analysis because phosphoserine and phosphothreonine are about 3000 times more abundant, cumulatively (Hunter and Sefton, 1980), and phosphotyrosine represented <1% of measured phosphorylation events by Ficarro et al. (Ficarro et al., 2002). Combining these techniques led to an increase in the number of sites measured to tens of specifically tyrosine phosphorylation events (Salomon et al., 2003; Brill et al., 2004). IP can also be strengthened by digesting proteins into peptides prior to IP and
using multiple antibodies. Each antibody is raised against a target of interest, such as phosphotyrosine, but the exact sequence antigen or epitope to which the antibodies react can vary. The resulting phosphorylation sites measured will vary (Zhang and Neubert, 2006; personal communications with A. Del Rosario and V. Agrawal). Therefore, incubating samples with multiple antibodies will increase the phosphotyrosine sequences that are retained during an IP (Johnson et al., 2012). Through multiple method optimizations and instrument improvements, it is now possible to measure hundreds of unique tyrosine phosphorylation sites during one MS analysis (Gajadhar et al., 2015).

Labeling techniques such as Isobaric Tag for Relative the Absolute Quantification (iTRAQ), Tandem Mass Tags (TMT), and Stable Isotope Labeling by Amino acids in Cell culture (SILAC) allow for multiplexing, the simultaneous analysis of multiple samples by MS. These labeling methods allow for direct and quantitative comparisons of identified phosphorylation sites across samples. iTRAQ/TMT peptide labeling occurs by a chemical modification of amine-reactive tags to the peptides’ N-termini and free amines on lysines. Quantification is possible via peptide fragmentation during MS2 where the iTRAQ/TMT reporter ions are fragmented from the N-termini and iTRAQ/TMT balancing groups. Characterization of hundreds of sites of PTMs allows for network based analyses (Zhang et al., 2005; Wolf-Yadlin et al., 2006; Johnson et al., 2012; Johnson et al. 2013). These techniques can be applied to numerous types of studies such as those analyzing cell lines with different treatments or multiple tissue or tumor samples relative to each other. Intra-MS analysis allows for direct comparisons without the need for an exogenous standard.
Using an untargeted approach, where spectral peaks are selected for fragmentation based on intensity rather than predetermined mass to charge (M/Z) ratios, one can study a PTM generally without \textit{a priori} decisions on which proteins are of interest. Peptide sequence information, including PTM location, and relative quantification data, by iTRAQ/TMT, is obtained from these fragmentation ions. The untargeted nature, however, doesn’t guarantee that specific phosphosites will be measured. By using an untargeted approach, abundant proteins may overshadow less abundant ones and their peptides can be measured many times. Balancing the desire to measure a network of hundreds of phosphosites and specific phosphosites of interest is necessary.

\textbf{Overview of Current Study}

Tyrosine phosphorylation is commonly deregulated in cancer and connected to oncogenic phenotypes such as migration, invasion, and division. Despite GBM being heavily studied in the past decade, there have not been major gains in its treatment or the life expectancy of patients suffering with GBM. In the work presented here, I describe our comparison of the phosphotyrosine signaling differences between brain and GBM tumor and demonstrate the global differences of these tissues in Chapter 2. In Chapter 3, I demonstrate highly increased phosphorylation of CDK1 Y15 in murine GBM tumor samples. Abrogation of that signaling node, by inhibiting its kinase Wee1, in murine GBM cell lines causes mitotic catastrophe. Using human patient-derived xenografts (PDXs), we demonstrate increased mouse survival after treatment of PDX tumors with the Wee1 inhibitor, suggesting that this targeted strategy may useful in treating GBM. These results were born out of analyses of MS-based data, and the entire
dataset has yet to be explored; there may be additional interesting leads to follow up on.

In Chapter 4, we describe work comparing murine GBM tumors that have been treated with an RTK inhibitor, sunitinib malate, and its effects on tumor signaling. We also describe work to understand the signaling involved with invasion, in the context of ligand-stimulated RTKs in Chapter 5. Finally, we conclude in Chapter 6 with some discussion of the future directions of this work and the implications of our findings.

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Chapter 2: Characterization of Murine GBM Model Signaling

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Introduction

There is a need for a greater understanding of the molecular characteristics of GBM in order to identify new treatment strategies. Its importance was highlighted when GBM was named by the National Institutes of Health (NIH) as one of the first three cancers to be studied by TCGA (NHGRI Communications, 2006). With treatment, including surgery, radiation, and chemotherapy, the 5 year survival rate is only 4.7% (Ostrom et al., 2013). In addition to the genomic and expression-based measurements compiled by TCGA, additional analyses are necessary to understand the functional consequences of these mutations. Measuring changes in PTMs, such as phosphotyrosine, allows one to quantify the alterations in signaling networks that are affected by targeted therapeutics such as small molecule inhibitors of active sites which disrupt an enzymatic process.

Lei et al. have created a murine model system that forms with 100% penetrance and expresses signatures similar to GBM, specifically proneural GBM (Lei et al., 2011; Gill et al., 2014). The Canoll Laboratory has bred mice that have floxed TP53 and PTEN, and stop floxed luciferase or YFP. After a retroviral injection of PDGF-IRES-Cre into the subcortical white matter, proneural GBM-like tumors form. These tumors express PDGF, and lack PTEN and P53 due to their retroviral initiation. These characteristics are similar to those of the proneural subtype of GBM, which accounts for 28% of tumors analyzed by TCGA and is one of the four identified subtypes (Verhaak et al., 2010).
2010). This model provides a well characterized system to understand the effects of characteristic mutations, such as those that drive the proneural subtype.

Understanding the signaling differences between the brain and tumor may help to create a targeted therapeutic approach to treating GBM cells specifically. Historically, targeted therapies for GBM have been focused on local targeting, rather than molecular targeting. For instance, surgery and Gliadel, BCNU (carmustine) laden wafers, physically target the tumor, as Gliadel is implanted at the resection site after surgery to treat remaining tumor cells with the chemotherapy. This delivery system is preferable to systemic treatment because, while carmustine is able to cross the blood brain barrier, it has high systemic toxicities and a short \textit{in vivo} half-life of about 20 minutes, causing low effective concentrations at the tumor site (Westphal et al., 2003). A two month increase in survival time was demonstrated with the addition of Gliadel to surgery and radiation; however temozolomide provides similar benefits (Stupp et al., 2005) as an orally available therapeutic, making it the standard of care even for patient that previously received Gliadel treatment (McGirt et al., 2009). Gliadel makes use of physically targeted approaches (surgery and implantation), but molecular characteristics of the tumors, especially relative to the surrounding brain material, have yet to be fully utilized as a tool to treat this disease. Surgery and chemotherapy can remove and attack both tumor and brain cells. A caveat is that since the proliferation rate of the tumor cells is faster, adducts on their DNA are more prone to cause errors in replication and cell death.
In this study we characterize the phosphorylation signaling in two cohorts of seven murine GBM tumors each to understand the molecular changes that occur in our proneural GBM tumor model. Using an untargeted mass spectrometry-based approach, we quantify the global phosphotyrosine signaling in these tumors. These tumors are growing rapidly and causing mouse morbidity at the time of tumor extraction, allowing one to measure the drivers in these late stage, fast growing tumors. In this way, they are similar to the late stage of GBM tumors which is often when GBM is diagnosed in patients. We have also collected survival data on these mice and compare the signaling to this metric. Although we are studying one model system, there is biological variation across these tumors. This biological variability is present at the phospho-signaling level and macroscopic, mouse survival level. In addition to the GBM tumors, we have measured the phosphorylation signaling in 8 murine brain samples. These tissue samples were gathered from mice of the same genetic background as the mice harboring tumors. Using one mouse brain sample as a control and iTRAQ 8plex reagent, we simultaneously compared the signaling dynamics of these tumors relative to each other and the brain and are able to compare across the multiple analyses and study the signaling across the cohorts.

Results

Characterization of Murine Proneural GBM

With the goal of characterizing signaling network alterations associated with the proneural subtype of GBM, we have utilized a genetically engineered mouse model of proneural GBM. These model tumors lack P53 and PTEN, and overexpress PDGF, creating a molecular signature similar to that of human proneural GBM, Figure 1.3 (Lei
et al., 2011). To quantify cellular signaling network alterations in these tumors, global tyrosine phosphorylation profiles in tumors were analyzed by quantitative MS. After tumor initiation by a stereotactical retroviral injection, tumors develop with 100% penetrance and mice succumb to the tumors within about 1 month. Once the mice experienced tumor burden, such as peri-orbital hemorrhages, seizure, decreased alertness, or impaired motor function, among other symptoms, the mice are anaesthetized and the tumors are extracted and immediately flash frozen in liquid nitrogen. Frozen tissues were homogenized and their proteins were reduced, alkylated, and digested with trypsin to peptides. Peptides from each sample were differentially labeled with iTRAQ 8plex and phosphotyrosine-containing peptides were enriched for using IP and IMAC prior to LC-MS/MS analysis for identification and quantification, Figure 2.1. Using iTRAQ 8plex reagent to tag each specimen, we are able to simultaneously compare the signaling dynamics of these tumors relative to each other and the brain control.
Figure 2.1. Mass Spectrometry Experimental Workflow. Tumors were initiated by a retroviral injection and tumors grew until mice demonstrated morbidity. The mice were anaesthetized and tumors or normal brain tissues were excised and immediately flash frozen. Tissue samples were homogenized and proteins were reduced, alkylated, and digested with trypsin. Peptides were differentially labeled with iTRAQ, and phosphotyrosine peptides were enriched for using IP and IMAC. Phosphotyrosine peptides were then analyzed by LC-MS/MS. Peptide fragmentation generated sequence specific ions, enabling identification of the phosphosite (left) and iTRAQ marker ions (right), providing quantification of the phosphopeptide levels across various biological samples.
The initial analysis, Cohort 1, consisted of seven tumors for which the average mouse survival time was 39.3 days post-injection (DPI). One normal brain tissue specimen was used throughout the analyses as a control to compare across multiple iTRAQ analyses and filled the 8th iTRAQ channel. In this cohort from multiple replicate analyses, 249 phosphotyrosine (pTyr/pY) containing peptides from 185 proteins were identified and quantified. A separate cohort of seven additional tumors (average survival time: 25.3 dpi) was also analyzed relative to the same normal brain sample. In total from replicate analyses of this cohort, 212 pTyr sites on 176 proteins were identified and quantified. These cohorts were analyzed using Orbitrap Elite and Orbitrap Q Exactive mass spectrometers, respectively.

To assess the inherent variability in signaling across mouse normal brain tissue specimens, a separate analysis was performed to quantitatively compare tyrosine phosphorylation across 8 normal brain specimens in which we identified and quantified 276 pTyr sites on 201 proteins. To enable cross-analysis quantitative comparisons, the same normal brain tissue was included in all analyses as a normalization control.

After normalizing the iTRAQ quantification of the phosphorylation sites to the brain control and log2 transforming the data, hierarchical clustering was performed on the 125 phosphosites present in both the tumor Cohort 1 and the brain analyses, Figure 2.2A. As can be seen from the clustering results, tumor samples cluster together and separately from the brain samples, indicating that the overall signaling in the tumor is distinct from normal biological signaling in the brain. When comparing the second tumor cohort to the normal brain specimens using the 78 phosphorylation sites
identified in both analyses, the tumors again cluster together and separately from the brain samples, Figure 2.2B. The phosphosites in both panels of Figure 2.2 were clustered using Spearman correlation as the distance metric.
Figure 2.2. Global Differences between Tumors and Brain Samples. A. Heatmap of 125 phosphopeptides quantified in Tumor Cohort 1 and Brain Samples. B. Heatmap of 78 phosphopeptides quantified in Tumor Cohort 2 and Brain Samples. iTRAQ quantification is reported as the fold change normalized to one brain sample present in both analyses and log2 transformed. Phosphorylation sites were hierarchically clustered.
In addition to general separation of tumors from normal brain using their phosphorylation levels, phosphorylation signals characteristic of the PDGF-driven proneural mouse model were identified. We compared the phosphorylation levels of Cohort 1 to the brain samples. Phosphorylation of an activating site on PDGFRA, pY742 (Heldin and Ronnstrand, 1998), was increased by two-fold, on average, in the tumors compared to brain, Figure 2.3A. Downstream of the PDGF receptor tyrosine kinase, PI3K subunit P85 (Carracedo and Pandolfi, 2008) and the activation loops of MAPK cascade ERK1/2 proteins (Boulton and Cobb, 1991; Wolf et al., 2001) were also increased in the tumors relative to the normal brain control, Figure 2.3A. Phosphorylation of tyrosines of the cell-cell contact proteins, Eph receptors (Himanen et al., 2007) were decreased in the tumors, consistent with the diffuse invasive characteristic of this tumor type.
Figure 2.3. Phosphorylation Differences between Murine Tumors and Brain Tissue. A. Quantification of phosphotyrosine sites on PDGFRA, PI3K, ERK1 and ERK2, and Ephrin receptors in tumors of Cohort 1 and brain tissues relative to brain control. B. Quantification of phosphotyrosine sites on glutamate receptors and synapse-associated proteins in tumors of Cohort 1 and brain tissues relative to the brain control sample.
Our data also highlight that the brain is a highly active organ with many dynamic phospho-signaling cascades occurring during normal function. Most of the phosphorylation sites identified in both the tumors and the normal brain have higher amounts of phosphorylation in the brain tissues than the tumors, Figure 2.2. Neuronal proteins, including glutamate receptors and Dlg proteins, have decreased phosphorylation in the tumors, Figure 2.3B. These findings are consistent with a change in cell type from active neurons in the normal brain to oligodendrocytic precursor tumor cells in the proneural tumors, and likely reflect differences in cellular composition with neurons representing a smaller fraction of total cells in the tumors.

Correlation Analysis and Survival

In addition to the overall phosphorylation differences in both cohorts relative to the brain, we also analyzed one cohort alone, Cohort 2, in order to increase the number of phosphorylation sites examined. These tumors were all initiated, handled, and processed together at one time. Survival data was also available for all 7 of these tumors.

We performed correlation analysis of the quantification of each of the 212 phosphopeptides relative to every other phosphopeptide. This analysis creates N x N matrix with values ranging from -1 to 1. The diagonal is a comparison of every phosphosite to itself and, therefore, has a correlation of 1. The matrix is a mirror image across the diagonal. While the correlation of one phosphosite to another does not change based on the ordering scheme, if one first clusters the data, it can help to visualize the data. The phosphosites were hierarchically clustered using Spearman correlation as the distance metric to order the peptides followed by a correlation
analysis of all the phosphosites to one another, Figure 2.4. Five main clusters formed. In addition to the clusters that form around the diagonal with correlation values very close to 1 (dark red), there are also phosphosites that are highly anti-correlated with each other (dark blue). For example, clusters 2 and 4 are anti-correlated with each other. Cluster 1 has been boxed and is discussed below.
Figure 2.4 Correlation Matrix of Phosphosites Quantified in Tumor Cohort 2. Correlation matrix 212 phosphosites that have been quantified by iTRAQ in 7 tumors. The phosphosites are clustered hierarchically then correlated to one another. Each phosphorylation quantification has been normalized to the brain control and log2 transformed.
In addition to correlating the phosphosites to each other, we have also correlated them to mouse survival. Of note, the 14 sites that comprise Cluster 1 in Figure 2.4 are all among the top 20 phosphorylation sites most highly correlated with survival, Figure 2.5A, and most are known to interact with each other, Figure 2.5B. Two of these proteins, GluR2 (Gria2, described above) and Ctnnd2 are neuronal-associated proteins. Ctnnd2 is expressed more highly in normal brain than GBM and its loss was found to be associated with a more aggressive phenotype in GBM (Frattini et al., 2013). Since proneural GBM is derived from the oligodendrocyte lineage, high phosphorylation in these neuronal proteins may be associated with a smaller percentage of tumor cells in these tumors than was typically resected or less aggressive tumors, thus longer mouse survival. This higher activation of neural processes was correlated with longer survival possibly due to a slower growing tumors or ones that have delayed tumor burden onset.
Figure 2.5. Phosphosites Highly Correlated to Each Other and Mouse Survival. A. Top 20 phosphorylation sites measured in Tumor Cohort 2 correlated to survival. Black bars indicates their presence in Cluster 1 of Figure 2.4. B. Protein interactions of 14 proteins whose phosphorylation is correlated to each other and survival (Cluster 1 of Figure 2.4) using String DB.
The second group of phosphorylation sites identified by this analysis includes adaptor and signal processing proteins. Nck1, for example, provides negative feedback downstream of RTKs, specifically VEGFRs. After phosphorylation at Y105 by c-ABL, through reduced signaling from Nck1 to PAK1/2, there is down regulation of p38 activity and p38 induced migration (Anselmi et al., 2012). When phosphorylated, this inhibitory site is thought to interfere with N-WASP and/or PAK1/2 binding and further signaling. It is less clear the mechanism by which PTK2 (FAK) phosphorylation is correlated to survival. Y925 is phosphorylated after stimulation by fibronectin and integrin engagement induces signaling through the oncogenic Ras pathway leading to cell-matrix adhesion, and pro-migration signaling (Schlaepfer et al., 1994).

Phosphorylation sites most anti-correlated with survival have not been studied specifically, but the proteins to which they belong have been. MEGF10 pY1061 was most anti-correlated to survival (-0.76), and MEG10 is more highly expressed in diffuse and invasive GBM xenograft cell lines than those that form more nodular tumors (Monticone et al., 2012). This phosphorylation site is also anti-correlated with Cluster 1 sites, -0.61 on average. The tensin family of proteins is downregulated in kidney cancer and inhibit cell migration, but the specific phosphorylation site we measured, Tensin2 pY483, has not been implicated in this role (Martuszewska et al., 2009) and is anti-correlated with survival (-0.73). We have focused on the first cluster and a few phosphorylation sites anti-correlated with Cluster 1, but there is much more data in this analysis that may also provide valuable insight into GBM signaling.
Discussion

Current GBM therapies include surgery, radiation, and temozolomide, but the median survival has remained relatively constant at approximately 12 months for the past decade (Furnari et al., 2007). To identify novel intervention points in tumor-specific signaling networks, here we compared the phosphotyrosine signaling of 14 proneural GBM murine tumors to 8 murine brain controls and found vastly different signaling between the two tissue types.

Hierarchical clustering of the phosphorylation data demonstrated that all tumors in both cohorts were more similar to each other than to the brain tissues, Figure 2.2. Of the sites identified in the tumor and brain samples, most had decreased phosphorylation in the tumors, demonstrating the high activity of signaling networks in the normal brain. Among the sites with decreased phosphorylation in the tumors, reduced glutamate receptor phosphorylation has been previously observed in GBM (van Vuurden et al., 2009; Markert et al., 2001); we measured decreased phosphorylation on multiple family members, Figure 2.3B. Dlg proteins 2 (PSD-93), 3, and 4 (PSD-95) are a family of neuronal pre- and post-synaptic proteins that organize signaling between cells (Brenman et al., 1996). Decreased phosphorylation of this protein family in our tumors highlights the oligodendrocytic, rather than neuronal or astrocytic, gene signature of proneural tumors (Verhaak et al., 2010), Figure 2.3B. By comparison, phosphorylation of proteins involved in canonical oncogenic signaling networks were increased in the tumor samples, Figure 2.3A. For example, phosphorylation of the ERK1/2 MAP kinases (T183/Y185 and T203/Y205 in the activation loop) (Boulton and Cobb, 1991; Wolf et al., 2001) and PI3K regulatory subunit p85a were increased, as were phosphorylation of
PDGFRA and the RTK scaffolds GAB1 and SHC. Given that the engineered tumor model is driven by PDGF expression, in the context of P53 and PTEN deletion, increased phosphorylation of the PDGFR network was anticipated.

Clustering analysis of phosphorylation sites of tumors from one cohort allowed us to analyze the entire dataset and also to understand the variation across tumors. While tumors were all initiated using the same retrovirus and mice all had the same genetic background, there is still meaningful variation that can be analyzed among the tumors. Phosphosite data is lost when comparing multiple mass spectrometry analyses because, when using an untargeted MS approach, not all of the same phosphosites are quantified in both (all) datasets. Additionally, the variation of a phosphorylation site between tumors can also effectively be lost even if it is present in both datasets if the differences in quantification between the samples of datasets is significantly larger than those within the individual datasets; effectively dampening the inter-tumor heterogeneity.

In analyzing the tumors separately, we identified multiple clusters of phosphorylation sites that are correlated together across all tumors. Two clusters were large, containing many tens of phosphorylation sites that were tightly correlated, and interestingly these clusters, on the whole, were anti-correlated to each other, Figure 2.4 Clusters 2 and 4. Further analysis of these clusters may provide understanding to the mechanisms which are functioning inversely to each other. For example, they could be compensatory mechanisms or pro-growth vs. anti-inhibitory phosphorylation sites. Correlation was used to understand the changes in signaling dynamics not to make quantitative
conclusions about their correlation values; they have not been corrected by a false discovery metric, but statistical significance has not been utilized either.

In addition to understanding the phosphorylation sites that signal similarly to one another, we analyzed the signaling that is correlated to mouse survival. While many proteins have been studied in a cancer or GBM background, many of these phosphorylation sites have not. Even so, the 14 phosphosites of Cluster 1 in our correlation heat map, Figure 2.4, are within the top 20 sites most correlated to survival, Figure 2.5. Similar to the comparison between tumors and normal brain, phosphosites on two neuronal proteins are associated with increased survival (Frattini et al., 2013; van Vuurden et al., 2009; Markert et al., 2001). Increased survival is indicative of slower tumor growth, although tumor burden, not size, determined survival. Nck1 pY105 is correlated with survival and has been shown to be a negative regulator of migration (Anselmi et al., 2012). MEGF10 expression has been associated with a more diffuse and invading GBM phenotype and is anti-correlated with survival (Monticone et al., 2012). These examples demonstrate the usefulness of such analyses and confirm our understanding of the model.

The role of the phosphorylation sites on the other proteins that were highlighted is less understood. FAK phosphorylation was correlated with survival, but the protein function is known to induce the Ras pathway (Schlaepfer et al., 1994). Similarly, tensin family proteins have been shown to be downregulated in kidney cancer but increased phosphorylation of Tensin2 Y483 is anti-correlated with mouse survival. We have highlighted phosphorylation sites that both positively (ERK1/2 activation loop double...
phosphorylation) and negatively (Nck1 pY150) effect protein function. Understanding not just the protein expression, but PTM state and role within the context of tumor growth will enhance our ability to identify therapeutic targets.

Materials and Methods

Tumor Initiation and Brain and Tumor Extraction

As described in detail in Lei et al., the tumors and brain samples were initiated and/or harvested in the Canoll Laboratory at Columbia University as approved by the Institutional Animal Care and Use Committee (IACUC) of Columbia University (Lei et al., 2011). PDGF-IRES-Cre was generated using the pQXIX vector (Clontech) and VSV-G pseudotyped retrovirus was generated in 293GP cells. Mice are of mixed genetic backgrounds from NOD/SCID mice purchased from Jackson Labs. They were generated by breeding floxed PTEN mice (Groszer et al., 2001), floxed P53 mice (Chen et al., 2005), and stop-floxed YEF mice (Srinivas et al., 2001) or stop-floxed luciferase mice.

Brain surgery to introduce the virus was performed using a stereotaxis platform, injecting retrovirus using a Hamilton syringe into anesthetized mice targeting the dorsal lateral corner of the subventricular zone. Following the injection, weight, appearance, and behavior was and observed and measured daily. Animals lived until humane endpoints were reached, as they experience neurological deficits due to the growing tumor. At terminal tumor burden, mice were anesthetized and a gross total resection of the tumor was performed. Terminal tumor burden was determined by the following indications: peri-orbital hemorrhages, papilledema, epistaxis (nose bleeds), seizures, decreased level of alertness, impaired motor function, and/or impaired ability to feed.
secondary to decreased motor function, paresis, or coma. Prior to the onset of symptoms, the animals usually developed weight loss of at least 20 percent.

Brain tissue controls were obtained from mice of the same genetic background that did not undergo injection. Adult mice were anesthetized and their cerebri were removed. Once removed, tumor and brain tissues were immediately flash frozen in liquid nitrogen to preserve their protein and PTM state.

**Tissue Homogenization**

Once excised, flash frozen tumors and brain tissues to be analyzed by MS and western blotting were homogenized (Polytron) in ice-cold lysis buffer consisting of 8M urea supplemented with 1 mM sodium orthovanadate, 0.1% Nonident P-40 (NP-40), and protease inhibitor and phosSTOP tablets (Roche). Samples were homogenized on ice using 5x10 sec pulses, with 10 sec intervening periods to prevent tissue heating. The lysates were cleared through centrifugation at 4,000 g for 15 min at 4°C. Protein concentrations were quantified by a bicinchoninic acid (BCA) assay (Pierce).

**Mass Spectrometry Preparation**

Homogenized tumor and brain samples were reduced in 10 mM DTT at 56°C for 45 min, and alkylated with 50 mM iodoacetamide at room temperature for 1 hour in the dark. Urea was diluted with 100 mM ammonium acetate, pH 8.9 to 0.8M. Proteins were digested to peptides with sequencing grade trypsin (Promega) at 1:100 enzyme/substrate ratio at room temperature overnight in 100 mM ammonium acetate, pH 8.9. Trypsin activity was then quenched with acetic acid at a final concentration of
10%. Urea was removed by reverse-phase desalting using C18 SepPak cartridges (Waters). Samples were then lyophilized and stored at -80°C.

iTRAQ Labeling
Peptides were labeled with iTRAQ 8plex isobaric mass tags (iTRAQ, AB Sciex) as previously described (Zhang et al., 2005). Each sample was labeled with a different iTRAQ tag. Two vials of iTRAQ dissolved in 140 μL isopropanol were reacted with 800 μg of protein (measured by BCA assay, above) dissolved in 60 μL 0.5 M triethylammonium bicarbonate at pH 8.5, vortexed, and incubated at room temperature for 2 hours, and concentrated to 30 μL. Samples labeled with the 8 different isobaric iTRAQ reagents were combined, concentrated, and resuspended in 400 μL IP buffer (100 mM Tris, 100 mM NaCl, and 1% NP-40 at pH 7.4). Three sets of analyses, with technical replicates for each analysis, were performed to quantify signaling across two cohorts of tumors, comprising 14 total tumors, and 8 total normal brain samples. In the first two sets of analyses, seven tumor specimens from Cohort 1 or 2, were compared to a single normal brain specimen, using the same normal brain as a constant normalization channel. To assess the variance across normal brain specimens, a third set of analyses was performed in which 7 additional normal brain specimens were compared to the normalization control brain tissue.

Phosphotyrosine Enrichment and Mass Spectrometry Analysis
Phosphotyrosine peptides were enriched by IP with a cocktail of three anti-phosphotyrosine antibodies followed by IMAC, as previously described (Arneja et al., 2014). Briefly, 60 μL of Protein G-agarose (EMD Millipore) beads were rinsed with IP
buffer and centrifuged. The beads were then incubated with 12 μg pY100 (Cell Signaling Technologies, CST), 12 μg PT66 (Sigma), and 12 μg 4G10 (EMD Millipore) antibodies in 200 μL IP buffer. After 8 hours of incubation at 4°C on the rotor, the beads were spun down and rinsed with 400 uL IP buffer. iTRAQ-labeled peptides were resuspended in 400 uL IP buffer and incubated with mixing with the antibody-conjugated protein G-agarose beads overnight at 4°C.

Beads were rinsed once with 400 uL IP buffer and four times with rinse buffer (100 mM Tris, pH 7.4). Peptides were then eluted from the antibody conjugation into 70 uL 100 mM glycine, pH2 and further enriched using an IMAC column. The retained peptides were loaded onto a C18 reverse-phase pre-column (100 μm ID, 10 cm packed bed length, YMC ODS-A, 10 μm), rinsed with 0.1% acetic acid (Sigma) to remove excess phosphate buffer, and attached to a C18 reverse-phase analytical column (50 μm ID, 10 cm packed bed length, YMC ODS-AQ, 5 μm) with integrated electrospray emitter tip. All columns were made in house. The peptides were then separated by reverse-phase HPLC (Agilent) over a 130 minute gradient, and injected into either an Orbitrap Elite or Q Exactive mass spectrometer (Thermo Scientific) using a custom ultra-low-flow nano-electrospray interface. The HPLC gradient was as follows: buffer A = 200 mM acetic acid, buffer B = 70% acetonitrile in 200 mM acetic acid; the percent buffer B was varied over the following steps: 0-10 min, 0-13% B; 10-105 min, 13-42% B; 105-115 min, 42-60% B; 115-120 min, 60-100% B; 120-128 min constant 100% B, and 128-130 min step down from 100% to 0% B. The mass spectrometers were operated in a data-dependent mode with full scan mass spectrum followed by tandem mass spectra. The Orbitrap Elite CID was set at 35% energy for sequence information and HCD was set at 75% energy for
iTRAQ quantification. Ion trap injection time was set to 100 ms and Fourier transform-MS (FTMS) was set to 1000 ms with a resolution of 60,000 across m/z 400-2,000. Fragmentation was carried out for the top 10 precursor ions of each MS spectrum above 500 counts with an FTMS resolution of 7,500 for IT and FT-MS/MS scans. Orbitrap Q Exactive ion trap injection time was set to 100 ms with a resolution of 70,000 across m/z 350-2000 for the full scan mass spectrum. Fragmentation was carried out with CID set at 32% energy for sequence information and iTRAQ quantification for the top 20 precursor ions of each MS1 spectrum with an AGC target of 1E5, a maximum injection time of 300 ms, and a resolution of 35,000.

Phosphotyrosine Data Analysis

Using DTAsupercharge 1.31 (http://msquant.sourceforge.net/) or Discoverer 1.4.0.288 (Thermo Scientific), raw mass spectra data files were converted to the .mgf file format. The MS/MS peak lists were searched against the National Center for Biotechnology Information Uniprot 2009 database containing Mus musculus protein sequences using Mascot 2.1.03 (Matrix Science) or SwissProt 2015_1 using Mascot 2.4.1. Trypsin enzyme specificity was applied with a maximum of 1 missed cleavage. Mass tolerance for precursor ions was set to 10 ppm. Fragment ion mass tolerance was 0.8 Da for samples analyzed by the Orbitrap Elite and 15 mmu for samples analyzed by the Orbitrap Q Exactive. MS/MS spectra searches incorporated fixed modifications of iTRAQ 8plex for peptide N-termini and lysines and carbamidomethylation of cysteines. Variable modifications included phosphorylation of tyrosine, serine, and threonine residues and oxidized methionine. Peptides were initially filtered by a Mascot score of 20, corresponding to a peptide FDR of 3%. Precursor ion and MS/MS spectra information
containing sequence and iTRAQ quantification data were extracted using CAMV (Curran et al., 2013). MS1 and MS/MS spectra were manually validated to remove spectra containing contamination within the precursor isolation window and to ensure peptide sequence and phosphorylation site localization, respectively, with the aid of CAMV (Curran et al., 2013). Contamination led us to discard peptides if ions were present with an intensity of >25% of the precursor base peak within 1 m/z on either side of the selected precursor ion for measurements made with the Orbitrap Elite and 0.5 m/z on either side for measurements made with the Orbitrap Q Exactive. These peptides were eliminated from our analysis because these contaminating ions may incorrectly contribute to the relative iTRAQ intensities. iTRAQ quantification data were corrected for isotopic overlap, as determined by AB Sciex. To correct for slight variations in protein loading, phosphotyrosine peptide iTRAQ ratios were normalized to mean relative protein quantification ratios obtained from the total protein of each iTRAQ channel.

**Clustering Analyses**

Quantitative phosphorylation sites across the different tissue cohorts were analyzed using Matlab R2013b, 8.2.0.701 (Mathworks). iTRAQ quantification values were normalized to the brain tissue control in each separate MS analysis then then Log2 transformed. Hierarchical clustering was performed using the Spearman distance metric (Best and Roberts, 1975). Correlation between phosphorylation sites of the untreated tumor Cohort 2 was performed in Matlab. Correlation of phosphosites to survival was performed in Excel 2010 (Microsoft). String-DB.org was used to analyze associations between the proteins of differentially express proteins (Jensen et al., 2009).
**Conclusions and Future Directions**

Using quantitative mass spectrometry, we have demonstrated that the signaling of murine GBM tumors and brain specimens vary greatly. Our data confirms the increased signaling through specific sites on proteins in tumor tissues that are consistent with our model system, such as increased phosphorylation of PDGFRA Y742 and MAPK cascade activation. We have also demonstrated that the signaling of these tumors vary, even though they are derived from the same retroviral system. We have highlighted phosphorylation sites that are correlated with each other and survival and also ones that are anti-correlated with survival.

Going forward there is still much to be explored in this dataset. The hundreds of quantified sites and the clustering that occurs can be studied further. As is similar to many PTM studies, the functional consequence of increased or decreased phosphorylation of many sites in these tissues remains to be determined and may provide further insight into GBM signaling and identification of additional therapeutic intervention points.

**References**


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Chapter 3: Wee1 as a Therapeutic Target

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Mass spectrometry analysis, cell line work, and tumor western blotting was performed by R Lescarbeau and F White; tumors were initiated, grown, treated, measured while in vivo, and extracted by K. Bakken and J Sarkaria.
Chapter 3: Wee1 as a Therapeutic Target

Introduction

In addition to the characterization of our proneural GBM model relative to normal brain that highlighted the differences in signaling in prototypical oncogenic signaling cascades, Chapter 2, the site measured with the highest increase in phosphorylation was Y15 on the cell cycle regulatory protein CDK1.

Cells are constantly under attack, especially their DNA which must be maintained at very high fidelity to ensure their and their progeny’s proper function. Normal organismal processes can cause DNA damage, such as the production of free oxygen radicals, as well as external DNA damaging agents, such as environmental ionizing radiation (Kastan and Bartek, 2004). Breaking of phosphodiester bonds of the DNA backbone in both strands can lead to double-strand breaks. Modification of bases with chemical adducts can cause mispairing of bases. Inter- and intra-strand crosslinking can occur. If these errors occur, are not repaired, and cause a functional advantage for that cell, they can be the initiating, oncogenic mutation of a cancer. If there is too much damage, however, cells are coded to undergo programmed cell death: apoptosis. Interestingly, as described by Kastan and Bartek, DNA damage can be a cause of cancer, the treatment for the disease (radiation and chemotherapies), and responsible for the toxicity that can kill the disease (Kastan and Bartek, 2004).

Mammalian cells are constantly checking the state of their DNA and many checkpoints have evolved throughout the cell cycle. $G_1$/S transition checkpoints are in place to
ensure cells only undergo DNA replication with intact DNA. G2/M transition
checkpoints inhibit cells from dividing with DNA damage (Hartwell and Weinert, 1989).
These checkpoints comprise a biochemical monitoring system of signal transduction
cascades that result in halting the cell cycle if damage is sensed. CDKs and cyclins create
checkpoint complexes and their regulation is just as important as their function
(Murray, 1994). For example, increased levels of P53 primarily inhibit G1/S transition
through P21 interactions to inhibit and regulate CDK2 and its interaction with Cyclins A,
E, and D1 and D2 (Harper et al., 1993). Wee1 is responsible for inhibiting the G2/M
transition by maintaining CDK1 (CDC2) in an inhibited state (phosphorylation of Y15)
until mitosis should occur. This role is especially important in its active organelle, the
nucleus (Heald et al., 1993). In addition to these transitions, intra-M-phase checkpoints
are also important and abnormalities in spindle assembly can initiate a delay in mitosis
(Murray, 1994). Having these checkpoints throughout the cell helps to ensure correct
DNA propagation, but also creates points of intervention. Wee1 has been shown to be
overexpressed in GBM (Mir et al., 2010); creating a potential point of intervention.
These intervention points can be deleterious to the organism leading to cancerous cells,
such as the loss of tumor suppressor p53, or beneficial in treating cancer, such as
activating mitotic catastrophe and apoptosis specifically in cancer cells.

Abrogation of these checkpoint inhibitors will cause the cell to progress through the cell
cycle without correct cellular surveillance and can cause too much damage and lead to
mitotic catastrophe. Vakifahmetoglu et al. described mitotic catastrophe as an aberrant
form of mitosis that is correlated with incomplete DNA synthesis (Vakifahmetoglu et al.,
2008). This premature DNA condensation (Castedo et al., 2004), which regularly
promotes division, can be thought of as a cellular “admission” of insurmountable damage. This damage can induce both death and cell senescence (Roninson et al., 2001), creating populations of cells that no longer divide. Inducing mitotic catastrophe, therefore, may be a viable cancer treatment strategy.

Previous studies have caused cancerous cells to undergo mitotic catastrophe. Inhibitors of Wee1 have been developed as cancer therapeutics in hopes of abrogating the G2/M checkpoint and pushing cells that have not fully completed DNA replication or DNA damage repair into mitosis. However, in these studies, additional DNA damage is usually induced. Wee1 inhibition, using the potent ATP-competitive inhibitor MK-1775, synergizes with a chemotherapeutic such as gemcitabine to force s-phase arrested breast cancer cells into mitosis, which is particularly strong in P53-independent cell lines (Aarts et al., 2012). This unscheduled entry into mitosis leads to apoptosis. Similarly, PDX pancreatic tumors demonstrated enhanced tumor regression after a combination treatment of gemcitabine and MK-1775 that was dependent on the combination treatment and P53-deficient tumors (Rajeshkumar et al., 2011). Using data published from Lee et al., Mir et al. demonstrate that there is a significant increase in lifespan for GBM patients with low Wee1 expression (Lee et al., 2008, Mir et al., 2010). Knockdown of Wee1 by siRNA or inhibition by PDO166285 in GBM cell lines demonstrated abrogation of the DNA damage-checkpoint induced by radiation or temozolomide (Mir et al., 2010). The use of MK-1775 as a means to independently abrogate the G2/M transition was demonstrated within sarcoma cells (Kreahling et al., 2012). Based on these studies, in conjunction with the results from our untargeted mass spectrometry analyses highlighting CDK1 Y15 phosphorylation as being highly distinct between tumor
and brain samples, we decided to explore the use of MK-1775 to independently treat our proneural GBM model.

**Results**

**Quantification of CDK pY15**

Comparing our proneural GBM model tumors to the brain samples identified one phosphorylation site that had consistently higher levels of phosphorylation in the tumors, CDK1 pY15. The singly phosphorylated version of this peptide, pY15, was identified in both tumor cohorts of 7 tumors each, Chapter 2, with an average of 14-fold increase in phosphorylation relative to the control brain tissue. The doubly phosphorylated version, phosphothreonine (pT14)/pY15 was detected in one cohort and had an average of 6-fold increased phosphorylation in the tumors, Figure 3.1A. Phosphorylation of these sites was low enough in the normal brain that they were not detected in the untargeted mass spectrometry analysis of the 8 normal brain samples, which was dominated by signaling due to normal brain function. Thus, mass spectrometry quantification for these peptides was based on the comparison between the brain normalization channel and the tumors in Cohort 1 or 2; only one brain sample is present in the plots. Additionally, due to the inherent variability in identifications during non-targeted mass spectrometry analysis, the doubly phosphorylated peptide was only detected in Cohort 1. The sequences of CDK1 and CDK2 are identical in the region surrounding this phosphorylation site, and thus phosphorylation at T14 and Y15 on CDK1 or CDK2 are indistinguishable by either mass spectrometry or western blot. For simplicity, we will refer to these site as CDK1 T14/Y15, although it is possible that there is some contribution from both CDK1 and CDK2.
Figure 3.1. CDK1 Phosphorylation in our Murine Proneural GBM Model. A. iTRAQ quantification of singly (pY15) and doubly phosphorylated (pT14/pY15) CDK1 in two and one cohort(s), respectively, relative to the brain control. B. Immunoblotting of CDK1 pY15, CDK1, and β-tubulin used as a loading control for three tumors and three brain samples. Each sample is a unique biological tissue sample.
To confirm this increase in tumors relative to multiple brain samples, western blots were performed with antibodies recognizing CDK1 and phospho-CDK1/2 (Y15), Figure 3.1B. These blots demonstrate a significant \( p=3.38\times10^{-4} \) increase in protein expression and confirm the significant \( p=9.33\times10^{-4} \) increase in phosphorylation in the tumors relative to the brain tissue samples.

**CDK1 Phosphorylation Reduced after Wee1 Kinase Inhibition**

Increased phosphorylation on CDK1 Y15 in the tumor samples was initially unexpected because phosphorylation of this site causes cell cycle inhibition and delays cell cycle progression in the context of DNA damage (Perry and Kornbluth, 2007), and yet the tumor cells rapidly proliferate in this mouse model (Lei et al., 2011). Given the role of this phosphorylation site as a cell cycle checkpoint, we reasoned that inhibition of Wee1, the kinase responsible for phosphorylation of this site, might lead to progression with DNA damage and potentially to mitotic catastrophe in these cells. To test this hypothesis, we treated syngeneic primary cell lines MGPP6 and MGPP7 (P53- and PTEN-deficient), Figure 3.2A, developed from the same murine GBM tumor model, with the Wee1 inhibitor MK-1775 and observed a significant dose dependent decrease \( p<0.05 \) at 0.1\( \mu \)M for MGPP6 and 0.3\( \mu \)M for MGPP7 in cell number after 48 hours of treatment, Figure 3.2B. At 1 \( \mu \)M MK-1775 treatment, the cell counts were 17.8\% \( p=1.32\times10^{-6} \) and 15.7\% \( p=3.58\times10^{-6} \) of the DMSO control treatment for MGPP6 and MGPP7, respectively. This dose was used throughout this study, similar to a study treating sarcoma cells with MK-1775 as a single agent at 500 nM (Kreahling et al., 2012).
Figure 3.2. Primary Murine Cell Lines and Their Response to MK-1775. A. Immunoblotting of P53, PTEN, and GAPDH used as a loading control for the two primary murine cell lines, MGPP6 and MGPP7, and the control cell line mouse embryonic fibroblasts (MEF). B. Cell counts at 48 hours of MGPP6 and MPGG7 primary murine cell lines in response to MK-1775 treatment at the indicated concentrations, normalized to the DMSO control. Each point is the mean of three biological replicates and error bars indicate the standard deviation.
Treatment with MK-1775 effectively decreased levels of phosphorylation at CDK1 Y15, indicating on-target inhibition of Wee1, Figure 3.3A-B. The levels were quantified using ImageJ (NIH) and demonstrate a trend of reduced overall phosphorylation in MGPP6 cells (not significant) and significant reduction in MGPP7 cells ($p=7.5\times10^{-5}$, $p=0.049$, $p=0.11$, and $p=5.3\times10^{-3}$ at 8, 24, 36, and 48 hours of treatment, respectively), Figure 3.3C-D. The level of CDK1 pY15 was normalized to the β-tubulin expression. Additionally, the decreased level of phosphorylation relative to total CDK1 expression was significant at all four measured time points for MGPP6 ($p=5.8\times10^{-3}$, $p=2.0\times10^{-3}$, $p=7.3\times10^{-5}$, and $p=0.041$ for 8, 24, 36, and 48 hours of treatment, respectively) and three of four for MGPP7 ($p=1.5\times10^{-5}$, $p=9.7\times10^{-3}$, and $4.7\times10^{-3}$ for 8, 24, and 48 hours of treatment, respectively), Figure 3.3E-F. This measurement accounts for changes in the total CDK1 levels that may occur throughout a 48 hour treatment to its kinase.
Figure 3.3. Primary GBM Cell Lines' CDK1 Response to MK-1775. A and B. Immunoblotting of CDK1 pY15, CDK1, and the loading control β-tubulin of MGPP6 and MGPP7 cell lines, respectively, after treatment with 1μM MK-1775 or DMSO for the indicated lengths of time. Immunoblots are representative of three biological replicates. C and D. Quantification of CDK1 pY15 for each condition at each time point for MGPP6.
and MGPP7 cells, respectively, as measured by ImageJ, normalized to β-tubulin. Each point is the mean of three biological replicates and error bars indicate the standard deviation. E and F. Quantification of CDK1 pY15 relative to CDK1 expression for each condition at each time point for MGPP6 and MGPP7 cells, respectively, as measured by ImageJ. Each point is the mean of three biological replicates and error bars indicate the standard deviation. * Indicates p<0.05 between the treated and control at that time point.
Mitotic Catastrophe and Apoptosis Induced after MK-1775 Treatment

To define the mechanism by which Wee1 kinase inhibition led to decreased cell counts, we used western blotting and flow cytometry to quantify DNA damage and DNA content following MK-1775 treatment. Intriguingly, as illustrated in Figure 3.4A, γH2AX, a marker of DNA damage (Rogakou et al., 1998), was strongly increased within 8 hours after cells were exposed to the drug. Even without P53, these cells are not activating the DNA damage pathway to a large extent in the control, demonstrating one reason why these cells are dividing rapidly and continuing to grow in both the microenvironment of the mouse brain and on coated tissue culture plastic.

Using matched time points, we demonstrate that DNA damage response activation (γH2AX) occurs prior to any substantial changes in gross DNA content, Figure 3.4B-C, as measured using Hoechst staining. At 8 hours the histograms between populations appear similar with only slight variations in the S-phase population (cells with DNA content between 2N and 4N), and the major peak is still the G1 (2N) population of cells. Increased DNA damage following MK-1775 treatment does occur, however. Changes in total DNA content occur starting at the 24 hour time point and continue throughout the rest of the time course. A significant increase in the percentage of cells with DNA content greater than 2N after treatment is measured at 24 hours (p=1.59E-4 and p=1.45E-3 at 24 hours for MGPP6 and MGPP7, respectively), Figure 3.4D. As treatment length continues, the distinction between 2N and 4N is lost because the percentage of cells in each portion of the cell cycle is increasingly becoming abnormal, with cells with DNA content between 2N and 4N steadily increasing between 24 and 48 hours.
A. DMSO MK-1775

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<td>y-H2AX</td>
<td>36</td>
<td>36 48</td>
</tr>
<tr>
<td>MGPP7</td>
<td>48</td>
<td>8 24 36</td>
</tr>
<tr>
<td>y-H2AX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-tubulin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. MGPP6

C. MGPP7

D. MGPP6

MGPP7

>2N DNA Content

% of Population

0 10 20 30 40 50

Treatment Length (H)
Figure 3.4. MK-1775 Induces DNA Damage in Primary Murine GBM Cells. A. Immunoblotting of γH2AX and the loading control β-tubulin of MGPP6 and MGPP7 cell lines after treatment with 1μM MK-1775 or DMSO for the indicated lengths of time. Immunoblots are representative of three biological replicates. B and C. DNA content histogram of MGPP6 and MGPP7, respectively, after treatment with 1μM MK-1775 or DMSO for the indicated lengths of time with 2N and 4N chromosomal content indicated. Flow cytometry histograms are representative of three biological replicates. D. Percentage of cells with >2N DNA content with and without 1μM MK-1775 treatment. Each point is the mean of three biological replicates and error bars indicate the standard deviation, * indicates p<0.05.
Having documented a decrease in cell count, DNA damage pathway activation, and a disruption in the DNA content occurring after MK-1775 treatment, we investigated cell death. We quantified two apoptotic pathway markers, cleaved PARP (Kaufmann et al., 1993) and cleaved Caspase-3 (Jänicke et al., 1998) by flow cytometry in both of our cell lines, Figure 3.5A and C. After MK-1775 treatment, there was a statistically significant increase in the percentage of cells expressing the cleaved forms of these proteins, Figures 3.5B and D, as early as 24 hours (p=0.039 and p=0.042 for MGPP6 cleaved PARP and cleaved Caspase-3, respectively; p=9.25E-3 and p=0.017 for MGPP7 cleaved PARP and cleaved Caspase-3, respectively). Further increases in exposure time led to a greater effect. The quadrants for quantification were set based on the DMSO, 8 hour condition in each biological replicate.
A. MGPP6

Cleaved PARP

DMSO

MK-1775

Cleaved Caspase-3

B. MGPP6

Cleaved Caspase-3 Positive Cells

Cleaved PARP Positive Cells

% of Population

DMSO

MK-1775

Treatment Length (H)

C. MGPP7

Cleaved PARP

DMSO

MK-1775

Cleaved Caspase-3

D. MGPP7

Cleaved Caspase-3 Positive Cells

Cleaved PARP Positive Cells

% of Population

DMSO

MK-1775

Treatment Length (H)
Figure 3.5. MK-1775 Induces Apoptosis in Primary Murine GBM Cells. A and C. Representative bivariate dot plots of MGPP6 and MGPP7 cells, respectively, treated with 1μM MK-1775 or DMSO for the indicated lengths of time. The vertical axis shows anti-cleaved PARP staining and the horizontal axis shows anti-cleaved Caspase-3 staining. The quadrants are set from the DMSO 8 hour control. Flow cytometry dot plots are representative of three biological replicates. B and D. Percentage of cleaved PARP positive and cleaved Caspase-3 positive MGPP6 and MGPP7 cells, respectively, after treatment with 1μM MK-1775 or DMSO. Each point is the mean of three biological replicates and error bars indicate the standard deviation, * indicates p<0.05.
In addition to the statistical significance between DMSO and MK-1775 treatment populations, the control cells only ever reached 2% of their populations staining positive for either apoptotic marker. None of the control MGPP7 cells had significant differences in cleaved PARP or cleaved Caspase-3 positive cells over the length of the experiment (24, 36, and 48 hour time point measurements relative to the 8 hour time point). Only the 48 hour time point had significantly increased cleaved PARP and cleaved Caspase-3 positive cells relative to the 8 hour time point for the MGPP6 cells. Therefore, the increase in cell death and apoptotic markers was due to the effect of MK-1775, not the experimental conditions.

**Rescuing Cells from MK-1775 Treatment**

To understand the consequence of MK-1775, we hypothesized that treatment with roscovitine (rosc, seliciclib), a general CDK inhibitor, would reduce the effects of MK-1775 and rescue the cellular response. We have demonstrated that treatment with MK-1775 causes DNA damage and abnormal cellular content. MK-1775, however, acts by inhibiting Wee1, upstream of the cell cycle modifiers. We investigated whether these results were dependent on CDK activity by inhibiting activity with rosc. We measured response after 24 hours of combined treatment of MK-1775 (1μM) and rosc (10 μM) in both murine GBM cell lines, MGPP6 and MGPP7. This rosc dosage was similar to other studies (Qi et al., 2014; Wang et al., 2014). The combined treatment normalized DNA content similarly to DMSO, Figure 3.6A. γH2AX was significantly reduced compared to treatment with MK-1775 alone in both murine cell lines, MGPP6 and MGPP7, Figure 3.6B and C. There was a significant increase γH2AX positive cells after MK-1775 treatment compared to DMSO (p=3.67E-4 for MGPP6, p=7.64E-6 for MGPP7) and a
significant decrease with combined treatment relative to MK-1775 alone (p=2.04E-4 for MGPP6, p=3.55E-6 for MGPP7).
Figure 3.6. Altered DNA and DNA Damage Response in Murine GBM Cells.
A. Representative DNA content histograms of MGPP6 and MGPP7 cells after DMSO, 10μM rosc, 1μM MK-1775, or 1μM MK-1775 and 10μM rosc treatment for 24 hours. B and C. Quantification of γH2AX positive MGPP6 and MGPP7 cells, respectively, after 24 hours of treatment. Means and standard deviations of three biological replicates are plotted. * indicates p<0.05.
We also quantified phospho-Histone H3 (pHH3) positive MGPP6 and MGPP7 cells after 24 hours of treatment, Figure 3.7, a measure of mitotic cells (Noonan et al., 2012). Cells should only divide when their DNA content is 4N. During mitotic catastrophe, cells divide without proper DNA content and/or with extensive DNA damage. In the control condition, 90% and 93% of pHH3 positive cells, MGPP6 and MGPP7, respectively, had 4N DNA content. With MK-1775 treatment, 85% and 84% of pHH3 MGPP6 and MGPP7 cells, respectively, are 4N. This difference is significant for MGPP7 (p=0.034), but not MGPP7 (p=0.10). Treatment with both MK-1775 and rosc increases pHH3 positive, 4N cells to 87% and 93% in MGPP6 and MGPP7 cells, respectively, which is significantly increased in MGPP7 (p=0.009) but not MGPP6 (p=0.23), Figure 3.7B and C.
Figure 3.7. Mitosis Marker in Murine GBM Cell Lines. A. Representative bivariate dot plots of MGPP6 and MGPP7 cells after DMSO, 10µM rosc, 1µM MK-1775, and 1µM MK-1775 and 10µM rosc treatment for 24 hours. The vertical axis shows anti-pHH3 staining and the horizontal axis shows DNA content. pHH3 positive (set by DMSO), 2N and 4N cells are quantified. B and C. Quantification of 4N, pHH3 positive cells relative to all pHH3 positive cells for MGPP6 and MGPP7 cell lines, respectively. Means and standard deviations of three biological replicates are plotted. * indicates p<0.05.
Confirmation of Results in Patient-Derived Xenograft (PDX) Cell Lines

Induction of apoptosis with MK-1775 in primary cells derived from the engineered mouse model of proneural GBM led us to test our theory in a different GBM system, human GBM PDX tumors and cell lines. As previously described (Sarkaria et al., 2006), serially passaged xenografts were derived from tumor tissue of different patients undergoing surgical treatment that were diagnosed as GBM. GBM PDX models have been shown to harbor many of the same characteristics as the parental tumors from which they were derived, such as MGMT methylation status (Carlson et al., 2009). We chose three PDX lines with similar genomic markers to the mouse model: GBM6, GBM36, and GBM38 are all P53 deficient and GBM36 is also PTEN deficient and has been subtyped as proneural. Using these cell lines we have studied the effect of MK-1775 on human GBM cell lines as a single agent.

Treatment of the PDX cell lines for 48 hours with 1μM MK-1775 led to a decrease in cell counts for all three lines, although the extent of the response varied. On average, there was a 40% reduction in the number of cells. GBM6 and GBM36 displayed increased sensitivity to MK-1775 compared to GBM38, Figure 3.8A. At 1μM MK-1775, the on-target inhibition of CDK1 phosphorylation, analyzed by western blot, reflected the phenotypic cell count response, with decreased phospho-CDK1 as early as 8 hours in GBM6 and GBM36, and by 48 hours in GBM38, Figure 3.8B.
A. GBM PDX Cells

![GBM PDX Cells Graph]

B. CDK1 pY15, CDK1, and β-tubulin Immunoblots for GBM 6, GBM 36, and GBM 38.

**Figure 3.8. PDX GBM Cell Line Responses to MK-1775.** A. Cell counts of three PDX GBM models, GBM6, GBM36, and GBM38, after treatment with MK-1775 at the indicated concentrations for 48 hours. All measurements are relative to the DMSO control. The mean of three biological replicates is plotted and error bars indicate the standard deviation. * indicates p<0.05. B. Immunoblotting of CDK1 pY15, CDK1, and the loading control β-tubulin for the three PDX models after 8 and 48 hours of treatment with 1μM MK-1775 or DMSO. Immunoblots are representative of three biological replicates.
Similarly to the primary murine cell lines, we investigated the effect of MK-1775 on the DNA of the GBM PDX cell lines. The DNA damage response pathway (measured by γH2AX expression) was activated in all three cell lines in response to MK-1775 treatment for 8 hours, Figure 3.9A. This robust response, relative to the control treatment, was seen in all cell lines, independent of the reduction in phosphorylation level of CDK Y15 at 8 hours. To further characterize the PDX models’ cellular response to MK-1775, we used flow cytometry to quantify DNA content, Figure 3.9B, and apoptosis, Figure 3.10. Although minimal change in the cellular DNA content was detected at 8 hours, there was a substantial increase in the population of cells that had a >2N DNA content after 48 hours of treatment, Figure 3.9B. This result was particularly strong for GBM6 and GBM36 cells, consistent with the greater impact on these PDX lines relative to GBM38. The delay between DNA damage response pathway activation (γH2AX expression) and abnormal DNA content ratio is consistent with the murine model.
Figure 3.9 DNA Damage Response and Altered DNA in GBM PDX Cell Lines.

A. Immunoblotting of γH2AX and the loading control β-tubulin for three GBM PDX models after 8 and 48 hours of treatment of 1µM MK-1775 or DMSO. Immunoblots are representative of three biological replicates. B. DNA content histogram of GBM6, GBM36, and GBM38 after 1µM MK-1775 or DMSO treatment for 8 or 48 hours with 2N and 4N chromosomal content indicated. Flow cytometry histograms are representative of three biological replicates.
Figure 3.10. Apoptotic Markers in MK-1775 treated GBM PDX Models. A. Representative bivariate dot plots of GBM6, GBM36, and GBM38 cells after 1μM MK-1775 or DMSO treatment for 8 or 48 hours. The vertical axis shows anti-cleaved PARP staining and the horizontal axis shows anti-cleaved Caspase-3 staining. The quadrants are set from the DMSO 8 hour control. Flow cytometry dot plots are representative of three biological replicates. B. Quantification of cleaved PARP positive and cleaved Caspase-3 positive cells after treatment of MK-1775 1μM for 8 or 48 hours relative to DMSO control.
Significant increases in cleaved PARP and cleaved Caspase-3 were also observed after 48 hours of treatment for all three cell lines, again with the greatest increase occurring in GBM6, Figure 3.10. Comparing the fold change values in Figure 3.10B, there is an increase in these apoptotic markers in as little as 8 hours of treatment, but the response is more robust after 48 hours.

PDX Flank Tumors Respond to MK-1775

After successfully inducing an apoptotic response and cell cycle stalling in cell culture, we tested MK-1775 treatment in vivo using a flank tumor GBM6 PDX model. A short-term, 5 day treatment study was used to assess molecular changes, while a long-term treatment was used to investigate if MK-1775 treatment changes the time for tumor outgrowth. Once flank tumors were large enough mice were treated orally with vehicle or MK-1775 until the pre-established endpoint (5 days for short-term study; tumor volume $>1500$ mm$^3$ for two measurements or $>2000$ mm$^3$ for a single measurement for the long-term study).

In the short-term study, 5 GBM6 PDX tumors (3 vehicle control and 2 MK-1775 treated) were analyzed. GBM6 PDX tumors have been shown to express high levels of EGFR (Johnson and White, 2014). One of the tumors we analyzed had significantly less EGFR than the others, Figure 3.11A; normalized to GAPDH, signal was 5.8 standard deviations from the other four, Figure 3.11B. There will be some biological variation, but tumor was unexpectedly low and was not further analyzed.
Figure 3.11. In vivo Short-term Response to MK-1775 in Flank GBM6 PDX Tumors. A. Immunoblotting of EGFR and loading control GAPDH of tumors from MK-1775 and vehicle control treated mice. B. Relative EGFR expression normalized to GAPDH in A quantified by ImageJ. C. Immunoblotting of CDK1 pY15, CDK1, γ-H2AX, and the loading control β-tubulin of tumors from MK-1775 and vehicle control treated mice. These are the same four right-most tumors presented in A and B. D-F. Relative CDK1, phospho-CDK/CDK1, and γH2AX normalized to β-tubulin in C quantified by ImageJ.
We measured phospho-CDK1, total CDK1, and γ-H2AX in the remaining 4 tumors, Figure 3.11C. Although the two MK-1775 treated tumors had similar total CDK1 expression compared to the control, vehicle treated tumors, Figure 3.11D, their phospho-CDK1 levels were substantially decreased by treatment with the inhibitor, Figure 3.11E. Tumors treated with MK-1775 demonstrated increased γ-H2AX, Figure 3.11F, consistent with the results generated in our cell line model systems. With oral administration of MK-1775, the GBM6 PDX flank tumors had an on-target response of decreased CDK1 pY15 and DNA damage was induced.

The long-term in vivo study measured the effects of MK-1775 on the tumor size and therefore mouse survival, as mice were sacrificed when tumors reached a size of >1500 mm³. Once tumors were established (as measured by tumor volume), oral treatment of MK-1775 or vehicle began. There was a significant increase in time until endpoint tumor volume (log-rank p=0.02) with MK-1775 treatment, Figure 3.12A. The average survival time after treatment was 22 days with MK-1775 treatment, but only 13 days with the control. The tumor volume data was binned into three day increments, Figure 3.12B, which averages the day-to-day variation and allows one to better visualize the stratification of treatment types. Treatment has an effect on the growth rate of the tumors within the first week of treatment and changes the course of the mice’s survival. These results demonstrate the applicability of this treatment to in vivo tumors and suggest that Wee1 inhibition might be an effective treatment strategy for P53-deficient GBM tumors expressing high CDK1 phosphorylation.
Figure 3.12 Survival of Mice with GBM6 Flank Tumors Treated with MK-1775. Mice with flank tumors have been treated with either MK-1775 or vehicle once tumors were established. They were treated 12 times per week until they succumbed to their tumors (tumors > 1500 mm³). A. Kaplan-Meier plot demonstrating significant increase in survival time with MK-1775 treatment. B. The average tumor volume of tumors measured within each three day window after treatment begins.
Discussion

The phosphorylation site with the largest increase in phosphorylation in the murine tumor tissues was on the cell cycle regulator, CDK1 pY15. CDK1 Y15 phosphorylation by the Wee1 kinase holds the protein in an inactive state until the DNA is fully replicated and any necessary DNA repair occurs, causing a cell cycle arrest (Perry and Kornbluth, 2007). Although genetic aberrations and mutations occur, cancer cells still must maintain a functional genome capable of sustaining growth and division. Similarly, if too much DNA damage is incurred, the cells need to be able to initiate repair pathways to survive. CDKs and their partner, cyclins, allow cell cycle transitions in a unidirectional and tightly controlled manner, providing opportunities for DNA repair, if necessary. The P53 tumor suppressor protein is an upstream inhibitor of cyclins after DNA damage, causing cell cycle arrest primarily in G1, but also in G2 (Agarwal et al., 2005; Bunz et al., 1998; Goi et al., 1997). In the presence of TP53 mutations or deletions, such as in our genetically engineered proneural murine tumors, cells may be able to progress through the cell cycle with DNA damage, depending on the status of other cell cycle checkpoints. Therefore, in these cells, Wee1 phosphorylation of CDK1 at tyrosine 15 may be critical to inhibit cell entry into mitosis after DNA damage (McGowan and Russell, 1995).

Previous studies have demonstrated the efficacy of inhibiting Wee1 in combination with DNA damaging agents, such as gemcitabine (Rajeshkumar et al., 2011; Aarts et al., 2012; Hirai et al., 2010; Sarcar et al., 2011) in the context of non-functional P53. MK-1775 monotherapy has been previously documented by Kreahling et al. where tumor explants treated with 500 nM MK-1775 for 24 hours demonstrated decreased CDK1
phosphorylation and features of cell death (Kreahling et al., 2012). Building on these results, here we have used multiple human and murine tumor derived cell lines and a flank PDX model to assess the effectiveness of monotherapy with MK-1775 in \textit{in vitro} and \textit{in vivo} models of GBM. Following exposure to MK-1775, tumor derived human and murine cell lines experience DNA damage, Figures 3.4A and 3.9A, have abnormal DNA content, Figures 3.4B-D and 3.9B, and die by apoptosis, Figures 3.5 and 3.10, suggesting they undergo mitotic catastrophe. These results can be abrogated by introducing rosc, demonstrating the importance of active CDKs, Figure 3.6. Intriguingly, even without the addition of an exogenous DNA-damaging agent, which has been previously typically used in conjunction with MK-1775, we observed rapid and strong increases in $\gamma$H2AX and decreased cell number after treatment compared with the DMSO-treated control.

These models were all P53 independent, removing a cell cycle checkpoint, possibly paving the way for this therapy to be effective in these cells. Without treatment, the cells are able to thrive under our typical growth conditions; there was minimal amounts of apoptotic cells or $\gamma$H2AX expression in our control conditions. These cells, however, are strongly and rapidly affected by MK-1775. The $\gamma$H2AX expression is activated to induce cellular arrest. However, the cells, after treatment with MK-1775, can no longer stall the cell cycle at the G$_2$\textrightarrow M transition and therefore they continue through the cell cycle without arresting in G$_2$ and undergo mitosis, Figure 3.7. A significantly larger percentage of cells are undergoing mitosis (pHH3$^+$ cells) after treatment with MK-1775 or MK-1775 and rosc compared to DMSO or rosc alone. Rosc is a general CDK inhibitor, and its addition to MK-1775 treatment does not significantly reduce the percentage of pHH3$^+$ cells. The addition of rosc does, however, significantly increase the percentage
of cells undergoing mitosis that have 4N DNA content, compared to MK-1775 alone, in MGPP7 cells, Figure 3.7C. These results suggest that our cells are under constant mutational stress that they are, under typical conditions, able to address, and that Wee1 activation and CDK1 Y15 phosphorylation may be primarily responsible for inhibiting cell cycle progression to repair damage in this system, especially in the context of a P53 deficiency.

Even a slight reduction in CDK1 pY15, as is exhibited in GBM38 after 8 hours of treatment, Figure 3.8B, leads to a strong increase in γH2AX, Figure 3.9A. A 6.7-fold increase in cleaved PARP positive cells and 5.1-fold increase in cleaved Caspase-3 positive cells, Figure 3.10, is also measured after the same 8 hour treatment period. These data suggest that even small changes in phosphorylation may have a profound impact on cell survival. This result is similar to that discussed by Heald et al. which emphasized the importance of nuclear phospho-CDK1, compared to total cellular phospho-CDK1, the distinction of which is measured neither by mass spectrometry nor western blotting (Heald et al., 1993). Thus even a slight change in total CDK1 pY15, as is seen in our GBM38 cells, may have a large functional effect if the change is primarily in the nuclear portion.

Perhaps not surprisingly, there was greater variability across the PDX models derived from three different patients compared to the syngeneic murine models. Relative to the other PDX models, GBM38 demonstrated increased cell count at 48 hour of 1μM MK-1775 treatment and we observe a corresponding delay in decreased phosphorylation of CDK1, Figure 3.8B. Although the percentage of GBM38 cells in S/G2/M increased after
treatment, G₁ (2N) was still the dominant peak in the histogram, unlike the other two cell lines that have more similar percentages of cells in G₁ and G₂/M, Figure 3.9B. These endpoints complement each other and highlight the “patient” specific effect of treatment.

Finally, we tested the effect of monotherapy MK-1775 in vivo on flank GBM6 PDX tumors. Flank PDX tumors exposed to MK-1775 demonstrated decreased phosphorylation of CDK1 Y15 and increased DNA damage. Treatment with the inhibitor slowed tumor growth and thereby increased survival time, Figure 3.12. MK-1775 treatment led to a 1.74-fold improvement in survival, while treatment with temozolomide in a previous study using this same GBM6 PDX model demonstrated only a 1.39-fold improvement (Carlson et al., 2009). Treating with a specific inhibitor as a single agent not only increases the survival benefit in this model, but would decrease off target effects of a general DNA damaging, O⁶-methylguanine lesion inducing agent (Roos et al., 2007). We focused on inhibiting the kinase of CDK1 pY15, Wee1, and successfully induced mitotic catastrophe and measured increased apoptosis after inhibition by MK-1775.

Materials and Methods

Cell Culture

Primary cell lines (MGPP6 and MGPP7) derived from PTEN- and P53-deficient proneural murine tumors (described above) were cultured in at 37°C and 5% CO₂ in Dulbecco’s Modified of Eagle’s Medium with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (DMEM, Corning), supplemented with 0.5% fetal bovine serum (FBS), 1X
Antibiotic-Antimycotic (Anti-Anti, Gibco), 1X N2 Supplement (Gibco), 20 μg/L 3,3',5'-Triiodo-L-thyronine (Sigma), 10 ng/mL PDGF-AA (Peprotech), and 10 ng/mL FGF-Basic (Peprotech) for general growth and maintenance. MGPP6 and MGPP7 cells were plated on poly-L-lysine coated, tissue culture treated polystyrene plates. PDX cell lines (GBM6, GBM36, and GBM38) were cultured at 37°C and 5% CO₂ in DMEM media supplemented with 20% FBS and 1X Penicillin-Streptomycin (Pen-Strep, Gibco) on tissue cultured treated polystyrene plates for general growth and maintenance, as described previously (Pokorny et al., 2015).

**MK-1775 and Roscovitine Treatment for Cell Lines**

*Cell Counts*

Primary and PDX cells were plated and allowed to adhere to plates overnight. The media was then replaced with media containing the indicated MK-1775 (Selleckchem) concentrations (0, 0.1, 0.3, 1, 3, and 10 μM) and the cells were allowed to grow for an additional 48 hours. Cells were washed with phosphate-buffered saline (PBS) and trypsinized from the plates, centrifuged to concentrate them, and resuspended in 1 mL media. Cells were then counted using a Cellometer (Nexcelom) and the cell counts were normalized to the number of cells grown in media without MK-1775 for three biological replicates in each of the five cell lines.

*Western Blots and Flow Cytometry*

Prior to treatment with DMSO (Sigma) or 1 μM MK-1775, primary and PDX cells were grown in their respective media for 16 hours, then their media was replaced with media containing 1 μM MK-1775 or DMSO for the indicated time period (8, 24, 36, and 48
hours for primary cells; 8 and 48 hours for PDX cells). For the rescue experiments, primary cells were grown in their media for 16 hours, then the media was replaced with media containing DMSO, 10 μM rosc (Selleckchem), 1 μM MK-1775, or 1 μM MK-1775 and 10 μM rosc for 24 hours. For western blotting, cells were then rinsed with PBS and were lysed on ice in 1X RIPA buffer (Boston Bioproducts) supplemented with 1X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) and 0.1 mM phenylmethanesulfonyl fluoride (Sigma). The lysates were cleared through centrifugation at 10,000 g for 15 min at 4°C. Protein concentrations were quantified by BCA assay. For flow cytometry, all media was collected and cells were washed with PBS and trypsinized from the plates and added to the collected media. This solution was then centrifuged to concentrate the cells, and they were then washed with PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, washed again with PBS, and permeabilized and stored in methanol at -20°C.

**Western Blotting**

Sample lysates were boiled with laemmli sample buffer at 95°C for 5 min. Proteins from cell line samples were separated using 10 or 12% sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) and proteins from tissue samples were separated using 4M urea, 10 or 12% SDS-PAGE at 120V for 2 hours. The proteins were then transferred onto nitrocellulose membranes (Biorad) at 100V and 0.4A for 2 hours. Nitrocellulose was blocked with blocking buffer (5% bovine serum albumin (BSA), 150 mM NaCl, 0.1% Tween20, 50 mM Tris (TBST), pH 8.0). Membranes were probed with antibodies diluted in blocking buffer overnight with gentle rocking at 4°C and washed three times with TBST. They were then probed with horseradish peroxidase-conjugated goat anti-
mouse or goat anti-rabbit secondary antibodies diluted in TBST for 1 hour with gentle rocking at room temperature and washed three times with TBST. The blots were developed using SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Scientific) and imaged using film. Protein expression was quantified using scanned images of the film with ImageJ 1.48V (NIH). Anti-P53, and anti-CDK1 were purchased from Calbiochem and BD Biosciences, respectively. Anti-PTEN and anti-EGFR were purchased from Millipore. Anti-pCDK1 (pY15), anti-β-tubulin, anti-γH2AX (pS139), and anti-GAPDH were purchased from CST.

**Flow Cytometry**

Fixed cells were blocked with 1% BSA in PBS. They were incubated with anti-cleaved Caspase-3 (BD Biosciences) and anti-cleaved PARP (BD Biosciences) or anti-pHH3 (Millipore) and anti-γH2AX (Millipore) primary antibodies diluted in PBS supplemented with 1% BSA and 0.1% Tween20 (PBS-TB) for 1 hour at room temperature then PE-conjugated goat anti-rabbit (Molecular Probes, Invitrogen) and Alexa Fluor 647-conjugated goat anti-mouse (Molecular Probes, Invitrogen) secondary antibodies diluted in PBS-TB for 1 hour at room temperature. Cells were stained with 1.2 μg/mL Hoechst (Molecular Probes, Invitrogen) and resuspended in PBS-TB. Samples were analyzed using a BD LSR II HTS Flow Cytometer (BD Bioscience) and the data were analyzed using FlowJo (V10, Tree Star). Flow cytometry events were acquired ungated, and live cell gates were created based on their forward and side light scatter profiles using FlowJo.
Flank Tumors

Previously established xenografts were used in this study as approved by the IACUC of the Mayo Clinic (Sarkaria et al., 2006). These models were established from resected tumor tissue of patients with GBM. Briefly, after the xenografts were harvested, they were mechanically disaggregated, and grown in culture to create cell lines, one of which is GBM6. Athymic mice (Harlan) were injected, as previously described (Carlson et al., 2011), with 2E6 GBM6 cells. Once tumors were of the appropriate size for their respective study (500 mm³ for short-term and 140 mm³ for long-term), mice were treated with MK-1775 or vehicle control (0.5% methoxycellulose) via oral gavage.

For the short-term study, mice were treated with 100 mg/kg MK-1775 or vehicle twice daily for four days then once on the fifth day before tissue harvest that day. These tumors were immediately flash frozen and homogenized (Polytron) in ice-cold lysis buffer consisting of 8M urea supplemented with 1 mM sodium orthovanadate, 0.1% Nonident P-40 (NP-40), and protease inhibitor and phosSTOP tablets (Roche). Using 5x10 sec pulses on ice, with 10 sec intervening periods to prevent tissue heating, samples were disaggregated. The lysates were cleared through centrifugation at 4,000 g for 15 min at 4°C. Protein concentrations were quantified by a BCA assay (Pierce) and stored at -80°C until western blotting analysis (described above).

The mice in the long-term study were treated with 75 mg/kg MK-1775 or vehicle using the following scheme: twice daily for five days followed by once daily for two days until mice sacrifice. When the tumors reached 1500 mm³ (two measurements of 1500 mm³ or
a single measurement of 2000 mm$^3$) the mice were euthanized, marking the end of humane survival while harboring a tumor.

**Statistical Analysis**

Data are expressed as means and standard deviations of at least three biological replicates. For experiments in which two groups are compared, statistical analysis was performed using Student $t$ test in Excel 2010 (Microsoft) or log-rank in Prism 5.03 (GraphPad). Statistical significance was defined as $p \leq 0.05$.

**Conclusions and Future Directions**

In this chapter, we have focused on the targeted inhibition of CDK1 pY15 to induce mitotic catastrophe. This protein, and this phosphosite specifically, became a target of study due to our quantitative phosphoproteomic analysis described in Chapter 2, demonstrating the utility of quantitative mass spectrometry to highlight signaling differences between groups. The enhanced phosphorylation of CDK1 Y15 in tumor tissues relative to their normal tissue counterparts reveal a potential therapeutic target and through further analysis we demonstrated that reducing phosphorylation of this site was accompanied by decreased cell counts, DNA damage, abnormal DNA content, and increased apoptosis. We demonstrated that MK-1775 induced mitotic catastrophe in both murine and human PDX models. There is an increase in cells undergoing mitosis without 4N DNA content after treatment with MK-1775. These results suggest that abrogating this checkpoint may be a viable treatment option for GBM patients. Additionally, Mir *et al.* demonstrate that GBM patients with low *Wee1* expression live for a significantly longer period of time than patients with high *Wee1* expression,
demonstrating the importance of this cell cycle checkpoint in GBM patients (Mir et al., 2010).

Going forward, it may be interesting to characterize the compartmentalization of CDK1 and measure the percentage of nuclear phospho-CDK1 relative to total nuclear CDK1 and also the percentage of nuclear CDK1 to cytosolic CDK1. These characterizations would help to further understand what we have demonstrated in this chapter. As MK-1775 is poorly brain penetrant (Pokorny et al., 2015), our in vivo study was conducted in the flank. This work provides a proof of principle that Wee1 inhibition slows tumor growth; we have demonstrated that our murine and human GBM cell line models undergo mitotic catastrophe after treatment with MK-1775 that causes Wee1 inhibition and that Wee1 inhibition may be a strategy in vivo as well.

References


Carlson BL, Pokorny JL, Schroeder MA, Sarkaria JN. Establishment, Maintenance, and In Vitro and In Vivo Applications of Primary Human Glioblastoma Multiforme (GBM)


Chapter 4: Comparison of Murine GBM Tumors Treated with Sunitinib, a Multi-kinase Inhibitor

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Tumors were initiated, grown, treated, measured while in vivo, and extracted by L Lei and P Canoll; mass spectrometry data and quantitative analysis was performed by R Lescarbeau and F White.
Chapter 4: Comparison of Murine GBM Tumors Treated with Sunitinib, a Multi-kinase Inhibitor

Introduction

Our murine GBM tumors are a model system that is molecularly similar to the proneural subtype of GBM. As described in Chapter 1, the gene expression patterns are similar to those used by the TCGA to define the proneural subtype in human GBM (Verhaak et al., 2010). We have studied the signaling that occurs after tumor growth when mice experience tumor burden, Chapters 2 and 3. Treatment currently lacks long-term clinical benefit, however, as recurrence is almost guaranteed (Stupp et al., 2005; Stupp et al., 2009). Therefore, in addition to understanding the molecular characteristics of the late stage, untreated tumors, studying tumors after treatment is important to understanding the signaling in resistant tumors. Resistance after targeted therapy is well documented in many cancer types (Rini and Atkins, 2009; Nahta et al., 2006; Heinrich et al., 2006), and understanding the changes in signaling can help guide the process of continued treatment.

Sunitinib is an ATP-competitive, multi-kinase inhibitor that is FDA approved for use in GIST, advanced renal cell carcinoma, and pancreatic neuroendocrine tumors. It is an inhibitor of PDGFR, VEGFRs, and other RTKs (Pfizer Labs, 2011). This inhibitor has been shown to have cytotoxic effects in GBM and glioma cell lines (Costa et al., 2013). Studying the changes, both molecularly and phenotypically, that occur after treatment with this drug will help to provide an understanding of phosphorylation signaling remodeling that occurs and the compensatory pathways that are used to overcome
inhibition of these RTKs, specifically an oncogenic driver such as PDGFRA in our model system and its inhibition.

Bioluminescence imaging (BLI) of firefly luciferase is used as an in vivo reporter system. Luciferase catalyzes the oxidation of D-luciferin in the presence of ATP-Mg$^{2+}$ and O$_2$ to generate oxyluciferin and light. This reaction is possible at physiological temperature and pH (Gould and Subramani, 1988), but requires the presence D-luciferin, which is possible by intraperitoneal delivery in mice and allowing the circulatory system to carry it to the GBM tumor, that expresses luciferase, as described in Chapter 1. BLI has been used to assess genetically modified tumors that express luciferase. Tumor growth has been assessed by BLI in GBM-like tumors that formed from PDGF+, PTEN-/-, P53-/-, luciferase+ glioma cells, syngeneic to our primary cell lines discussed in Chapter 3. Cell number correlated to BLI signal and serial BLI measurements of tumor growth correlated with size and location measurements by ex vivo imaging (Sonabend et al., 2013). BLI allows measurement collection during tumor growth in a minimally-invasive fashion that only temporarily effects the mice and is therefore a valuable way to measure tumor size throughout growth.

In this chapter we have analyzed the phenotypic and molecular differences between tumors that have been treated with sunitinib and those that have not. We have analyzed tumors with multiple treatment schedules by mass spectrometry. Eleven tumors were randomly assigned to be treated with either vehicle (n=6) or sunitinib (n=5, Cohort 1) starting from 14 DPI. These 11 tumors were initiated at the same time. Another 4 tumors were also treated with sunitinib starting at 14 DPI but were initiated at an earlier time.
(Cohort 2). An additional 4 tumors were treated starting at 21 DPI. Treatment followed a 5 days on/2 days off schedule of a 60 mg/kg dosage. These tumors all become resistant to sunitinib and were extracted when tumor burden was indicated. Using luciferase signaling, we compared tumor size during growth in the vehicle treated and sunitinib Cohort 1 tumors. We have also analyzed differential gene expression using RNA-seq analysis. Finally, we have collected survival data on these 11 mice and Cohort 2 mice.

We have also analyzed 3 tumors by mass spectrometry that were treated once with sunitinib (60 mg/kg) as an acute treatment. These mice were treated when they began to exhibit tumor burden and were then extracted 6 hours after treatment. The signaling differences between these tumors and untreated ones allows for the study of initial signaling cascade changes prior to a resistance mechanism arising. These tumors are compared to untreated tumors that were initiated at the same time.

**Results**

**Mouse and Tumor Characteristics**

The matched vehicle and sunitinib treated tumors were analyzed by BLI at 14 and 21 DPI. Tumor size measurements are possible because the cells infected with the retrovirus, and their progeny, express luciferase, as described in Chapter 2. These 11 tumors were randomly assigned at 14 DPI to be treated with the vehicle or sunitinib and there is no statistical difference between the luciferase signal in these groups at day 14 (measurement was taken prior to starting the treatment, p=0.45), Figure 4.1A.
Figure 4.1. **Mouse and Tumor Characteristics with and without Sunitinib Treatment.** Sunitinib treated tumors in Panel A are Cohort 1 in Panel B. A. Luciferase signal at 14 DPI, prior to treatment, a proxy for tumor size. Mean and standard deviation are plotted; N.S. not significant. B. Survival, in days, of vehicle treated (n=6) and sunitinib treated tumors (Cohort 1, n=5; Cohort 2, n=4) when treatment started at 14 DPI (indicated by →). Cohort 1 and vehicle treated tumors initiated at the same time and randomly assigned to those groups. Log-rank p value between vehicle treated and all sunitinib treated mice. C. Hematoxylin and Eosin staining of representative untreated (left) or sunitinib treated (right) tumors with low (top) and high (bottom) power micrografts. Micrografts taken by L Lei.
Treatment with sunitinib had a significant impact on mouse survival. Mice treated with sunitinib lived significantly longer than those that were vehicle treated: 37.4 (Cohort 1) versus 26.1 DPI for matched vehicle treated samples and 36.3 DPI for the 4 tumors in Cohort 2. The log-rank p value is 1E-3 for the matched cohorts, and the log-rank p value 2E-4 for the vehicle compared to all sunitinib treated tumors starting at 14 DPI, Figure 4.1B. Macroscopically, they appear different as well, Figure 4.1C. After treatment with sunitinib, tumors become more diffusely infiltrative.

As discussed above, the vehicle treated and Cohort 1 sunitinib treated tumors were imaged using BLI prior to treatment. They were also imaged after 1 week of treatment began. The fold changes in their luminescence signal between 21 DPI and 14 DPI were, on average, 35.9 for the vehicle treated and 5.2 for the sunitinib treated, Figure 4.2A. While the standard deviations are large, the difference is significant, p=0.03.
Figure 4.2. Luciferase Signal. A. Luciferase signal fold change between 21 DPI and 14 DPI for vehicle (n=6) and sunitinib (n=5) treated mice. Treatment started at 14 DPI. Means and standard deviations are plotted. * Indicates p<0.05. B. Fold change of luciferase signal (Panel A) versus survival of sunitinib treated tumors and linear regression.
Sunitinib is a multi-kinase inhibitor that is known to inhibit PDGFR, but also VEGFRs and KIT (Pfizer Labs, 2011). It is, therefore, potentially inappropriate to compare BLI values between vehicle treated and sunitinib treated tumors because inhibiting RTKs responsible for angiogenesis and regulation of vascular permeability may have an effect on luciferase signaling. Luciferase signal is the product of a luciferase reacting with D-luciferin, which was administered intraperitoneally and carried by the blood stream to the luciferase (tumors); therefore, shutting down angiogenesis RTKs may have an effect on luciferase signaling. To understand this further, we analyzed only the sunitinib treated tumors. Comparing the fold change in luciferase signal between 21 and 14 DPI and overall survival, using simple linear regression, the correlation is -0.72 ($R^2=0.52$), Figure 4.2B. The more luciferase signal changes in the first week of treatment, the shorter the mouse survives. There may be an effect of luciferin reaching the tumors after sunitinib treatment that is not measured in this analysis, but 52% of the variance in survival of the sunitinib treated mice is explained by the difference in signal before and after one week of treatment alone.

**Mass Spectrometry Analysis of Vehicle and Sunitinib Treated Tumors**

We have compared the signaling of 6 vehicle treated tumors starting at 14 DPI, 9 sunitinib treated tumors starting at 14 DPI, and 4 sunitinib treated tumors starting at 21 DPI. These tumors were extracted and immediately flash frozen. They were then homogenized in 8M urea and their proteins were reduced, alkylated, and digested with trypsin prior to differential labeling with iTRAQ 8plex. Using IP and IMAC, phosphotyrosine peptides were enriched for and analyzed by LC–MS/MS as described in Chapter 2, Figure 2.1. This large number of samples requires multiple mass
spectrometry analyses to be completed, even using multiplexing chemical tags such as iTRAQ. These analyses were also completed on different mass spectrometers due to laboratory logistics and the timeframe over which these samples were analyzed, i.e. years. In order to quantify as many phosphorylation sites as possible, we run our mass spectrometers in a data dependent mode. This method inherently quantifies different peptides in each analysis, dependent on the peptides' relative intensity compared to other peptides being analyzed by the mass spectrometer at that time. We have analyzed our samples in technical duplicate to increase the number of phosphorylation sites we quantified and our confidence in the quantification.

In one analysis, we compared 2 vehicle treated tumors and 4 of the matched sunitinib treated tumors (Cohort 1) where treatment for every tumor started at 14 DPI. We identified and quantified 210 phosphosites on 166 proteins in this group, light blue line of MS Analysis in Figure 4.3. We identified and quantified 304 phosphosites on 232 proteins on 4 tumors treated at 21 DPI and 3 vehicle treated at 14 DPI, red line of MS Analysis in Figure 4.3. We measured 412 phosphosites on 288 proteins in the last vehicle and sunitinib treated tumor at 14 DPI, purple line of MS Analysis in Figure 4.3. In the second cohort of sunitinib treated tumors, Cohort 2 of Figure 4.1B, 254 phosphosites on 191 proteins were identified and quantified, green line of MS Analysis in Figure 4.3. Phosphopeptides quantified in each dataset vary; while some are measured in all, others are measured in only one or two, etc., datasets. When comparing data from multiple mass spectrometry analyses, reduced set of phosphorylation sites that have been quantified in all tumors are compared. Additionally, although we used a normalization control in all analyses, a brain tissue sample control described in Chapter
2, we have seen batch effects when comparing across the analyses, Figure 4.3. Here we have hierarchically clustered the 87 phosphorylation sites on 75 proteins measured in all four cohorts, listed above, using Spearman correlation as the distance metric. Similarly to the tumors analyzed in Chapter 2, the phosphorylation sites have been normalized to the brain control present in each analysis, and log2 transformed.
Figure 4.3. Hierarchical Clustering of Vehicle and Sunitinib Treated Tumors. Clustering of 19 vehicle or sunitinib treated tumors. Columns represent tumors and rows represent the 87 phosphopeptides measured in all four analyses. Phosphorylation site quantification was normalized to a control present in each iTRAQ 8plex mass spectrometry analysis then log2 transformed. The tumors quantified in the same mass spectrometry analysis are colored the same in MS Analysis row below the heat map. Tumors treated with the same treatment schedule are colored the same in the Tumor Type row below the heat map where blue indicates tumors were treated with sunitinib starting at 14 DPI (dark blue Cohort 1, light blue Cohort 2), green indicates tumors were vehicle treated at 14 DPI, and orange indicates tumors were sunitinib treated starting at 21 DPI.
Macro-scale descriptors such as survival time, tumor morphology, and luciferase signal, Figures 4.1 and 4.2, demonstrate a separation between the tumors when they have and have not been treated with sunitinib. While we don’t expect all our tumors of each treatment type to cluster together perfectly, due to the natural biological variability, we did expect to have more similarity in the phospho-signatures of tumors treated in the same manner than occurred, as shown in the heat map. Vehicle treated tumors are indicated by green, sunitinib treated at 14 DPI are indicated by dark (Cohort 1) and light (Cohort 2) blue, and sunitinib treated at 21 DPI are indicated by orange in the Tumor Type row below the heat map in Figure 4.3. The tumors clustered more often by analysis (MS Analysis row below heat map) than by tumor type.

In order to take advantage of the hundreds of phosphorylation sites quantified, rather than just the tens of sites present in all analyses, and to reduce confounding the batch effects, we have further analyzed the differences in within each mass spectrometry analysis using the vehicle control and sunitinib treated tumors (Cohort 1), both starting at 14 DPI. For example, we hypothesized that the treatment of sunitinib would decrease activity of PDGFRA, its target. A phosphosite responsible for its activity, pY742 (Heldin and Ronnstrand, 1998), was measured in the vehicle and treated tumors. When we compare all 6 vehicle treated tumors to all 5 sunitinib treated, Cohort 1 tumors, there is not a statistically significant difference between the phosphorylation in groups, Figure 4.4A, and the average fold change between them (treated/vehicle) is 0.90. These 11 tumors have been quantified in three mass spectrometry analyses and they have all been normalized to the signaling from the same brain control that was present in each of the mass spectrometry analyses. However, when we measure the fold change of tumors
from the same analyses, the average fold changes are 0.61 (for analysis labeled MS1) and 0.38 (for analysis labeled MS2), Figure 4.4B. Note that the third mass spectrometry analysis did not contain tumors treated with sunitinib starting at 14 DPI; therefore the vehicle treated tumors from that analysis are not represented in Figure 4.4B.
Figure 4.4. Examples of Phosphorylation Levels Affected by Sunitinib Treatment and Batch Effects. A. Relative phosphorylation of PDGFRA pY742 at sacrifice in vehicle (n=6) and sunitinib treated tumors (n=5) starting at 14 DPI. Mean and standard deviation are plotted; N.S. not significant. B and C. Relative fold change of sunitinib treated and control treated tumors separated by individual mass spectrometry analyses. In MS1, 2 vehicle treated and 4 sunitinib treated tumors are compared. One of each is compared in MS2. Fold change of MS1 is 0.61 and the fold change of MS2 is 0.38 in B. Selected phosphorylation sites in C that have differential relative phosphorylation in MS1 and their relative phosphorylation measured in MS2.
We further analyzed the phosphorylation data within the same mass spectrometry analysis (MS1 in Figure 4.4) and have found differences in phosphorylation between treatment groups; the proteins of the top 25 most differentially phosphorylation sites are presented in Figure 4.5. Adaptor and signaling proteins have increased phosphorylation in the treated tumors, such as Doki and ERK1, while CDK1 has decreased phosphorylation. ERK1 pY205 has differentially increased phosphorylation, but the doubly phosphorylated (pT203/pY205) is considered the fully active state of the protein (Boulton and Cobb, 1991). These tumors are no longer inhibiting their cell cycling by CDK1 pT14/pY15, discussed in Chapter 3, to such an extent. Phosphorylation of motility-associated and cytoskeletal rearrangement proteins are also increased in the treated tumors, such as Eno1, Elmo2, and Tubulins, consistent with the phenotype demonstrated in these tumors. Of the proteins described above, all phosphosites quantified in MS2 (Doki pY450 was not measured in MS2) follow the same trend, Figure 4.4C. With sunitinib treatment the mice live significantly longer than without, and their signaling has changed to reflect their phenotype.
Figure 4.5. Top Differentially Phosphorylated Proteins after Sunitinib Treatment. The proteins of the top 25 differentially phosphorylated peptides between vehicle and sunitinib treated tumors are shown using String DB (V10) with their associations (identified by co-occurrence, experimentally determined, published in databases, through text mining, or homology) shown as lines.
RNA-seq Analysis of Vehicle and Sunitinib Treated Tumors

In addition to the phosphorylation data collected on these vehicle and sunitinib treated tumors, RNA-seq analysis and ArrayCGH was also performed (vehicle and sunitinib Cohort 1). We have begun our analysis of the RNA-seq data. Using raw count data we identified differentially expressed genes using the DEseq2 algorithm (Love et al., 2015) followed by SPIA, a pathway based analysis method that uses the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Tarca et al., 2009). The top general descriptors of the differences between vehicle and treated tumors include infections and inflammatory responses. These pathways are increased in tumors treated with sunitinib. Connecting this result to the known changes in the tumors as they become resistant to treatment and continue to grow may provide valuable information on understanding the differences between succumbing to an untreated/vehicle treated tumor and a tumor in which the major RTK has been shut down or hindered from functioning fully.

Acutely Treated Tumors

We analyzed 3 acutely treated tumors that were treated right before terminal tumor burden was reached. These mice were treated with one dose of sunitinib and then the tumors were extracted for analysis 6 hours later. These tumors were initiated at the same time as 9 untreated tumors, 7 of which formed Cohort 2 from Chapter 2, and were quantified in the same mass spectrometry analysis as the remaining 2 of these untreated tumors.

We analyzed the signaling differences in the untreated and acutely treated tumors. We quantified 4 phosphorylation sites on PDGFRA in the tumors measured during the same
mass spectrometry analysis and the average fold change in the phosphorylation after the acute treatment was 0.48, Figure 4.6A. We also quantified 4 phosphorylation sites on Afap1L2, which has been associated with proliferation and invasion (Shi et al., 2014), but the specific phosphorylation sites have not been implicated in the phenotypes, Figure 4.6B, and the differences in phosphorylation varied. Two of the most differentially phosphorylated proteins are mitochondrial proteins SLC25A and NIPSNAP1, which has strictly neuronal expression in rats (Nautiyal et al., 2010). These sites have decreased phosphorylation in the acutely treated tumors relative to the brain control and further reduced phosphorylation in the untreated tumors, Figure 4.6C. Five phosphorylation sites were measured on SLC25 family member proteins (one of which is also plotted in Figure 4.6C) and all had an increased level of phosphorylation (average increase of 2.06) in the acutely treated tumors, Figure 4.6D. These proteins are expressed in the central nervous system (Haitina et al., 2006), however their cell type specific expression was not documented. After 6 hours of treatment, we hypothesized that inhibition, rather than resistance, would be the primary driver in tumor signaling. However, even at 6 hours of treatment, we measure increases in the phosphorylation of neuronal and mitochondrial proteins that are expressed in the central nervous system. As these are proneural tumors and, we hypothesized, not yet invading, an increase in neuronal protein phosphorylation was unexpected.
Figure 4.6. Phosphorylation of Specific Proteins in Acutely Treated and Untreated Tumors. Quantification of phosphorylation levels all measured in the same mass spectrometry analysis. Compared are 3 sunitinib acutely treated (6 hours) tumors, 2 untreated tumors, and 1 brain control (plotted in C). A. Fold change of phosphorylation of 4 measured PDGFRA tyrosine sites. B. Fold change of phosphorylation of 4 AFAP1L2 tyrosine sites measured. C. Relative phosphorylation of mitochondrial proteins with the most differential signaling between acutely treated and untreated tumors, plotted relative to the brain control. D. Relative phosphorylation SLC25 family proteins of acutely treated and untreated tumors. E. Relative phosphorylation in untreated and acutely treated tumors of sites previously quantified in Figure 4.4C.
Of the 25 most differentially phosphorylation peptides shown in Figure 4.5, three of the top four phosphorylation sites are present on proteins involved in RNA and protein synthesis. Phosphorylation of Prpf4b Y849, CNP Y110, and eEF1A1 Y141 are all increased in the sunitinib treated tumors relative to the control, Figure 4.7A. This effect is not demonstrated in the acutely treated tumors, relative to the untreated tumors from the same MS analysis, Figure 4.7B. Variation between these treatment types highlights the signaling differences the sunitinib causes after long-term, sustained treatment that does not occur immediately.
Figure 4.7. Differential Phosphorylation in Long-term and Acutely Sunitinib Treated Tumors. Phosphorylation sites Prpf4b pY849, CNP pY110, and eEF1A1 pY141 are differentially phosphorylated in long-term sunitinib treated tumors relative to vehicle control tumors (A), but not in acutely treated relative to untreated control tumors (B).
Overall Survival Analysis

We analyzed the 34 tumors for which we had survival data (not including acutely treated tumors) and regressed the 43 phosphosites on 41 proteins to that survival data. Using regularized regression, we were able to explain 58% of the variance in survival. Even though the data has batch effects, treatment types varied, there were multiple cohorts of mice, and the overlap between all the analyses left us with quantitative data on only 43 phosphorylation sites, we were able to explain 58% of the variation with 10-fold cross validated results. We analyzed 15 untreated tumors (2 cohorts), 6 vehicle treated tumors (starting at 14 DPI), 9 sunitinib treated tumors starting at 14 DPI (2 cohorts), and 4 sunitinib treated tumors starting at 21 DPI. The acutely treated tumors were not analyzed because their survival date was determined independent of treatment (and the phosphorylation due to that treatment).

Regularized regression minimizes the number of variables used in a model and the reduced model is comprised of 27 phosphosites, Figure 4.8. Although this analysis has not been completely explored, there are a few noteworthy sites. For instance, Vimentin pY117, Elmo2 pY48, and Afap1L2 pY413 are all negatively regressed to survival (negative Variable Importance for the Projection (VIP) score). These proteins have all been associated with migration, invasion, and tumor growth (Gilles et al., 1996; Hiramoto-Yamaki et al., 2010; Shi et al., 2014); they, understandably, are not pro-(mouse) survival proteins. CDK1 pY15 was also negatively associated with survival, and we have demonstrated that decreasing phosphorylation on this site increases mouse survival with flank tumors, Figures 3.11 and 3.12. However, Elmo2 pY48 is increased in sunitinib
treated tumors compared to vehicle control (both starting at 14 DPI, Figure 4.4C), and these sunitinib treated mice live longer.
Figure 4.8. Regularized Regression Variables. Phosphorylation sites with non-zero VIP values contribute to the model predicting survival. This model explains 58% of the variation in survival of 34 tumors treated in distinctly different ways: 15 tumors were untreated (2 cohorts), 6 tumors were vehicle treated (starting at 14 dpi), 9 tumors were sunitinib treated starting at 14 DPI (2 cohorts), and 4 tumors were sunitinib treated starting at 21 DPI.
ERK proteins and their phosphorylation is associated with pro-tumor growth and cell survival which should, in theory, be anti-correlated with mouse survival. However, ERK1 pY205 and ERK2 pY185 are associated with survival (positive VIP score). These proteins are part of RTK signaling cascades, and these peptides contain two phosphorylation sites that comprise the activation loop of these proteins (Roux and Blenis, 2004). The peptides measured here were singly, not doubly, phosphorylated. The singling phosphorylated versions are, by definition, not doubly phosphorylated as both phosphorylation sites appear on the same peptide when digested by trypsin as they were in our analysis; therefore they may represent a protein that is not active, or fully active.

Discussion

Sunitinib malate is a multi-kinase inhibitor that targets the receptor of the PDGF, the ligand that is overexpressed in our tumors due to a retroviral injection. Studying tumors that are treated against their main driver allows one to gather data about tumors’ abilities to become resistant to therapies, as is often seen in cancer treatment (Rini and Atkins, 2009; Nahta et al., 2006; Heinrich et al., 2006). In addition to mouse and tumor macro-scale phenotypes, we have also measured their phospho-signaling with and without treatment.

Treatment of our murine proneural GBM model with sunitinib increases mouse survival, but the mice still ultimately succumb to the tumor. On average, the overall mouse survive 41% longer from injection (tumor initiation) with treatment. After treatment begins, at 14 DPI, the mice survive 81% longer (23 days of sunitinib treatment
verses 12 days of vehicle treatment, both with a 5 day on/2 day off schedule), Figure 4.1. After 1 week of treatment, the tumors have grown significantly less than those without treatment, as measured by the fold change of BLI measurements, Figure 4.2. In addition to the increased survival, there is a phenotypic change in these tumors where they become more diffusively infiltrative. We quantified the signaling differences between sunitinib and vehicle treated tumors. These tumors were extracted at tumor burden; therefore, the tumors have become resistant to treatment by this point. We also quantified the signaling differences in acutely treated (6 hours) and untreated tumors to measure the initial effects of sunitinib on signaling.

BLI is inherently ‘noisy’ and the standard deviations are large in our data, ranging from 5.4 to 85.9-fold increases in the vehicle treated tumors over 1 week and from 2.2 to 10.8-fold increases in the sunitinib treated tumors. However, the measurement is negatively correlated with survival after sunitinib treatment, Figure 4.2B. Even with concerns of reduced signaling due to reduced D-luciferin reaching the tumor luciferase after treatment with a VEGFR inhibitor such as sunitinib, the vasculature of sunitinib treated mice are all equally challenged and only sunitinib treated tumors were compared in Figure 4.2B.

We quantified phosphorylation levels of tumors treated in different fashions and because of the number of tumors, they were measured across multiple mass spectrometry analyses. Because of the batch effects, Figures 4.3 and 4.4A and B, we restricted some of our comparisons to within one mass spectrometry analysis. This restriction reduced the number of tumors analyzed in each comparison, so we do not
always have standard deviations to report with sample size of at least 3, but we have commented on the trends that have been identified. Whether we treat for 6 hours or 23 days, there is a reduction in phosphorylation in PDGFRA, Figures 4.4B and 4.6A, demonstrating the on-target effect of sunitinib. Not all differential phosphorylation is similar between these two treatments, however. The phosphorylation sites identified to highlight the consistency with the pro-migratory phenotype in the sunitinib treated tumors versus the vehicle control treated tumors starting at 14 DPI, Figure 4.4C, do not have consistently increased phosphorylation in the tumors treated for 6 hours relative to untreated tumors, Figure 4.6E. These tumors were analyzed in the same mass spectrometry analysis as MS2 in Figure 4.4C, thus this is not analysis by analysis variability but differences in short-term and long-term treatment effects. This short treatment was able to reduce the phosphorylation occurring at its target, but not able to effect these pro-migratory sites to the extent that was seen in the longer term treatment.

Regressing phosphorylation to survival highlights trends that are consistent through various treatment schemes and multiple mass spectrometry analyses. The phosphorylation sites compared in Figure 4.8 were quantified in all our untargeted mass spectrometry analyses, suggesting they are common and abundant phosphorylated peptides in our samples. Consistent with the analysis of vehicle and sunitinib treated tumors at 14 DPI (which live longer than vehicle treated ones), Figure 4.4C, overall, ERK1 pY205 was associated with survival. Similarly, CDK1 pY15 negatively regresses to survival overall and there is decreased relative phosphorylation in the sunitinib treated compared vehicle treated tumors (MS1 Cohort). This trend is not seen in the doubly phosphorylated version, CDK1 pT14/pY15, Figure 4.4C, however, where
phosphorylation is greatly reduced in the sunitinib treated tumors. Conversely, Elmo2 pY48 has increased phosphorylation in the sunitinib treated tumors in Figure 4.4C, but negatively regresses to survival in the large analysis of 37 tumors, Figure 4.8. The regression analysis did not consider tumor type, just survival time, which could account for some of the different findings.

**Materials and Methods**

**Tumor Initiation and Brain and Tumor Extraction**

Tumor initiation and extraction were prepared for mass spectrometry as described in Chapter 2.

**Sunitinib Treatment**

Sunitinib treatment was given to mice bearing tumors starting at either 14 or 21 DPI. Either the carrier, DMSO, or sunitinib dissolved in DMSO was administered by oral gavage once daily in a 5 days on/2 days off schedule until morbidity, which was determined as described in Chapter 2.

**Bioluminescence Imaging**

BLI was performed as described in Sonabend et al., 2013. Briefly, mice were anaesthetized at 14 and 21 DPI with 2.5% isofluorane and intraperitoneally injected with 3 mg D-Luciferin. Imaging was performed 10 min after injection with the mouse’s head centered as the region of interest (2.25 cm).
Preparation for and Performance of Mass Spectrometry Analysis

Tissue homogenization, mass spectrometry preparation, iTRAQ labeling, phosphotyrosine enrichment and mass spectrometry analysis, and phosphotyrosine data analysis were performed as described in Chapter 2. All data was collected using an Orbitrap Elite with the exception of the vehicle and sunitinib treated tumors described as MS2 in Figure 4.4 and the untreated and acutely treated tumors described in detail in Figure 4.6 which were analyzed together using an Orbitrap Q Exactive. All quantification of phosphorylation sites were normalized to the brain control as described in Chapter 2.

Clustering and Regression Analyses

Hierarchical clustering was performed and String-DB.org (Jensen et al., 2009) was used as described in Chapter 2. Regression analysis on the log2 phosphorylation data was performed using R (R Core Team, 2013). The glmnet package (Friedman et al., 2010) was used to perform regularized regression while the caret package was used to perform cross validation and calculate performance numbers (Kuhn, 2008). The R-squared value was calculated from the average of 25 repeats of 10 fold cross validation. VIP values were calculated from caret by taking the regression coefficients of the regularized logistic regression model returned.

Conclusions and Future Directions

Treatment with sunitinib in our mouse model increases mouse lifespan, but the tumors spread and become diffusively infiltrative, and the mice do eventually die from their tumor burden. We have quantified phospho-signaling after a single dose of sunitinib
and after treatment for, on average, 23 days during which time resistance occurs and the mice succumb to the tumor. There are multiple phosphorylation sites that have differential signaling between treated and untreated or vehicle treated controls. We limited the scope of these specific phosphosite comparisons to tumors quantified within a single mass spectrometry analysis, but further analysis, incorporating both the larger number of tumors of each type and the batch effects of the mass spectrometry analyses would provide further insight and statistical significance between groups.

We have measured an on-target effect, and a difference in types of phosphorylation sites affected (relative to their respective controls) by a single or continuous treatment with sunitinib. Single dose treatment does not affect the phosphorylation status of pro-migratory or cytoskeletal rearrangement proteins that are affected in the long-term treatment. Long-term treatment also changes the gene expression in these tumors and inflammation and infections are the major pathways increased after treatment. Incorporating this data and that from the ArrayCGH that was performed would strengthen our understanding of resistance after treatment with an RTK inhibitor.

References


Chapter 5: Phosphorylation Profiling after Growth Factor Stimulation Reveals Mediators of Invasion in NSCLC

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Chapter 5: Phosphorylation Profiling after Growth Factor Stimulation Reveals Mediators of Invasion in NSCLC

Introduction

The role of HGF and EGF driven signaling in invasion and migration in cancer pathogenesis has long since been established (de Larco and Todaro, 1978; Sporn and Roberts, 1985). Binding of HGF to the RTK encoded by the c-Met proto-oncogene (1-Naldini et al., 1991; Bottaro et al., 1991; 2-Naldini et al., 1991) leads to dimerization and activation through phosphorylation on tyrosine residues in the intracellular kinase domain of the receptor (3-Naldini et al., 1991). Kinase activation leads to trans-autophosphorylation on tyrosine residues in the cytoplasmic tail. These phosphorylation events form docking sites allowing adaptor molecules such as Gab1, Grb2, PLC-γ, and Src to bind and perpetuate downstream signaling, eventually leading to cellular phenotypes such as migration, invasion and proliferation (Ponzetto et al., 1994; Weidner et al., 1996). HGF signaling through Met is known to play a significant role in promoting tumor cell invasion and metastasis in several different cell types such as lung and pancreatic carcinomas (Rong et al., 1994; Brinkmann et al., 1995). Deregulated Met signaling has been implicated in NSCLC and disruption of this pathway by Met-directed inhibitors decreases tumorigenic potential, yet these inhibitors have been prone to failure due to adaptation of the tumor microenvironment (Stabile et al., 2004).

EGFR is an RTK that binds EGF to cause dimerization, trans-autophosphorylation, activation, and downstream signaling (Normanno et al., 2006). Similar to Met, EGFR activation leads to phosphorylation of specific tyrosine residues within the cytoplasmic
tail that serve as docking sites for proteins containing Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains, the recruitment of which leads to activation of intracellular signaling pathways (Yarden, 2001; Marmor and Yarden, 2004; Yaffe, 2002). Signaling downstream of EGFR regulates proliferation, migration, and invasion (Hynes and Lane, 2005). Due to its increased activity in several cancers, including NSCLC, EGFR has emerged as a therapeutic target (Hynes and Lane, 2005). Despite the high efficacy of EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib, innate or adaptive resistance associated with compensatory signaling through additional RTKs has been detected in almost all cases (Hynes and Lane, 2005). Met is frequently coexpressed with EGFR family members in human tumors and cross-talk between these RTKs have been described in several contexts, including invasive growth (Fischer et al., 2004; Jo et al., 2000; Engelman et al., 2007; Huang et al., 2010). Aberrant EGFR activation increases protein expression and phosphorylation of Met in thyroid carcinoma and GBM cell lines (Huang et al., 2007; Bergstrom et al., 2000). Furthermore, characterization of phosphotyrosine signaling downstream of the EGFR receptor in NSCLC cell lines and Met in a gastric cancer cell line demonstrated increased phosphorylation of EGFR in a Met amplified cell line and increased Met phosphorylation in mutated and amplified EGFR cell lines (Guo et al., 2008). Additionally, inhibition of EGFR signaling with EGFR TKIs can lead to compensatory signaling through Met (Engelman et al., 2007). These studies demonstrate cross-talk between EGFR and Met signaling in driving invasive cancer progression, yet the specific and common nodes involved in these networks have not yet been characterized at a systems level.
Although the extent to which the EGF and HGF signaling pathways overlap is not fully understood, many of the canonical adaptor proteins and downstream pathways are known to be common to both receptors. To determine the overlap between these networks, a recent study utilized a quantitative mass spectrometry analysis of proteins following phosphotyrosine IP after stimulation with EGF or HGF (Hammond et al., 2010). While a significant number of common proteins were immunoprecipitated after EGF and HGF stimulation, phosphorylation site-specific information was lacking, and therefore it was unclear as to whether both stimulations phosphorylated the same sites on these proteins.

In this study, to determine EGF and HGF network specific mediators of invasion, we characterized temporal site-specific phosphotyrosine signaling in A549 cells by quantitative mass spectrometry. We quantified invasion and tyrosine phosphorylation signaling networks at four growth factor concentrations determined using a standard Boyden chamber invasion assay; EC₀ (no growth factor), EC₅₀ (the effective concentration that results in 50% of the maximum invasion), EC₁₀₀ (the effective concentration that results in 100%, i.e. maximum, invasion), and EC₅₀₅₀ (the effective concentration above EC₁₀₀ that results in 50% of the maximum invasion). These analyses led to the identification and quantification of 131 phosphotyrosine peptides following EGF stimulation and 139 phosphotyrosine peptides following HGF stimulation; of these, a total of 78 phosphorylation sites were quantified across all EGF and HGF stimulation conditions. Integration of the quantitative temporal phosphotyrosine data enabled us to calculate the total signal from 0 to 20 min and regress these measurements to invasion using partial least-squared regression (PLSR).
Through these efforts, we have uncovered phosphorylation sites that are associated with invasion and highlight both common and differential tyrosine phosphorylation sites regulating invasion downstream of EGFR and Met.

**Results**

**Characterization of A549 Cell Invasion in Response to EGF or HGF**

To verify protein expression levels of EGFR and Met in the A549 cells, immunoblotting was carried out for total EGFR and Met, Figure 5.1A. The protein expression of EGFR and Met was not affected by EGF or HGF stimulation at 30 min. While EGF stimulation increased EGFR Y1173 phosphorylation and HGF stimulation increased Met Y1234/5 phosphorylation, EGF stimulation did not increase Met pY1234/5, and HGF did not increase EGFR pY1173, Figure 5.1A. This result demonstrates that there is minimal crosstalk at the receptor level in terms of tyrosine phosphorylation in these cells under these conditions. To confirm that the signaling networks downstream of both receptors were functional under these stimulation conditions, we immunoblotted cell lysates for total phosphotyrosine after stimulation with EGF or HGF, Figure 5.1A. We observed a global increase in tyrosine phosphorylation in response to EGF and HGF, but did not identify any significant differences between the two growth factors other than the receptors at 180 kDa (EGFR) and 120 kDa (Met).
Figure 5.1. EGF and HGF Dose Dependent Stimulation and in vitro Invasion in A549 Cells. A. Immunoblotting of EGFR, EGFR pY1173, Met, Met pY1234/5, total phosphotyrosine (pY), and loading control β-tubulin in A549 cells stimulated with EGF or HGF at the specified times. Immunoblots are representative of 3 biological replicates. B and C. Boyden chamber invasion assay of A549 cells in response to EGF and HGF, respectively, at the indicated concentrations. Bar charts are representative of 2 biological replicates for all growth factor concentrations and EC\textsubscript{0} measurements and 4 biological replicates for 10% FBS. Error bars indicate standard error. The data were normalized to the 10% FBS condition and the EC\textsubscript{0}, EC\textsubscript{50}, EC\textsubscript{100}, and EC\textsubscript{am50} are indicated. Figure adapted from Johnson et al., 2013.
To quantify the cell biological response to stimulation of these cells with EGF or HGF, we carried out Boyden chamber invasion assays using Matrigel coated membranes. Invasion through Matrigel was quantified in response to 7 different EGF concentrations, Figure 5.1B, and 7 different HGF concentrations, Figure 5.1C, in comparison to 10% FBS. The invasion of A549 cells in response to EGF and HGF was quantitatively distinct, and the level of growth factor required to induce maximal invasion was much higher for HGF (300 ng/mL= 3.7 nM) than EGF (10 ng/mL= 1.6 nM). These data allowed us to identify the effective concentrations for invasion in A549 cells.

**Quantification of Signaling in A549 Cells in Response to EGF or HGF**

To determine the phosphorylation signaling networks driving invasion under EGF or HGF stimulation conditions, the EC₀, EC₅₀, EC₁₀₀, and EC₅₀₅₀ were selected for phosphotyrosine signaling analysis using quantitative mass spectrometry. Cells were stimulated for 5 or 20 min with the indicated effective concentrations or 10% FBS, which was used as the control. Cells were lysed with 8M urea and proteins were reduced, alkylated, and digested with trypsin prior to differential labeling with iTRAQ 8plex reagent and LC–MS/MS analysis similarly to the tumors described in Chapter 2, Figure 2.1. To calculate the total signal for each effective concentration, we calculated the area under the curve (AUC) using the EC₀ as the 0 min and the 5 and 20 min time points for EC₅₀, EC₁₀₀, and EC₅₀₅₀, Figure 5.2A. The basal level of phosphorylation was calculated by extrapolating the level of the EC₀ 0 min measurement out to 5 and 20 min and calculating the AUC. In total, 131 phosphotyrosine peptides in the EGF dataset and 139 phosphotyrosine peptides in the HGF dataset were identified and quantified across 3 different effective concentrations at 2 different time points.
Figure 5.2. Phosphorylation Response after EGF and HGF Stimulation. A. The AUC was calculated for each peptide at EC0, EC50, EC100, and ECam50 using the 5 and 20 min time points. B and C. Heat maps of the 68 EGF responsive and 61 HGF responsive phosphotyrosine peptides, respectively. The heat maps are a representation of iTRAQ 8plex fold changes normalized to the 10% FBS condition, normalized to the mean of all 8 channels, and then Log2 transformed. Phosphorylation sites were clustered using affinity propagation. Response sites are defined as a ≥1.5 fold change relative to EC0 in any of the growth factor iTRAQ channels. D. EGF and HGF responsive tyrosine phosphorylation sites (y) mapped onto a signaling network diagram. HGF responsive phosphorylation sites are indicated in green and EGF responsive phosphorylation sites are indicated in blue. Figure adapted from Johnson et al., 2013.
To identify phosphorylation sites that were either EGF or HGF responsive, we filtered the quantitative information to those peptides with a \( \geq 1.5 \) fold change relative to EC\(_0\) in any of the growth factor iTRAQ channels. This threshold was determined because the median coefficient of variation was found to be 15.7\% for EGF analyses and 14.4\% for HGF analyses, thus \( \geq 1.5 \) fold changes are statistically different. This filtering resulted in 68 EGF responsive phosphorylation sites and 61 HGF responsive phosphorylation sites.

To define sets of similarly regulated phosphorylation sites, responsive sites were clustered using affinity propagation, Figure 5.2B and 5.2C.

To visualize the effect of EGF and HGF stimulation on the signaling pathways downstream of the receptors, we mapped the responsive phosphorylation sites to a canonical network diagram, Figure 5.2D, where EGF responsive phosphotyrosine sites are represented in blue and HGF responsive phosphotyrosine sites are green. Phosphorylation sites on PLC-\( \gamma \) and Crk were detected in the EGF responsive dataset, but not in the HGF dataset; these are examples of the differences between these two signaling networks.

**Regression of EGF and HGF Phosphotyrosine Signaling to Invasion with a Combined PLSR Model**

To identify the common signaling pathways mediating invasion downstream of EGFR and Met, we selected the 78 overlapping phosphorylation sites that were quantified across both the EGF and HGF datasets. The calculated AUC measurements for the 3 EGF or HGF effective concentrations in addition to the EC\(_0\) are displayed in a heat map in Figure 5.3A. It is important to note that while the EC\(_0\) measurements should
theoretically be identical, some variation in selected phosphorylation sites can be seen across the different biological replicates. Despite this biological variation, distinct differences in signaling between EGF and HGF were detectable, Figure 5.3A. To identify the sites most strongly associated with invasion downstream of EGFR or Met activation, the 78 common phosphorylation sites were regressed against the quantitative invasion data at each of the effective concentrations using PLSR. The PLSR model contained 3 latent variables, or principal components, which explained 89% of the variation in the data. Figure 5.3B displays the scores plot of principal component 1 and 2 (PC1 and PC2) from the resulting model; EGF data are highlighted in blue and HGF data are highlighted in green. PC1 discriminated the data based on increasing invasion following growth factor stimulation, where A549 cells stimulated with EC100 separated from the EC50 and ECam50 cells. PC2 discriminated the data based on the differential quantitative invasion profiles resulting from the 2 different growth factors, where EGF and HGF data points separate.
Figure 5.3. Quantification and PLSR Modeling of the 78 Phosphorylation Sites Measured in both EGF and HGF Datasets. A. Heat map of the AUC of the 78 phosphotyrosine peptides quantified across HGF and EGF datasets and at EC₀, EC₅₀, EC₁₀₀, and EC₁₅₀. B. Scores plot of the EGF and HGF combined model showing the relationship between the signaling measured in A549 cells in response to EGF and HGF stimulation in terms of PC₁ (x-axis) versus PC₂ (y-axis) from a PLSR analysis of measured invasion data. The model uses 3 principal components. Growth factor conditions are color coded; EGF is blue and HGF is green. C. Plot of experimentally determined invasion (x-axis) versus predicted invasion (y-axis) as determined by the linear EGF and HGF combined invasion model. Invasion data was normalized to the mean of all 8 conditions and Log₂ transformed. D. Bar chart displays the VIPs for each phosphorylation site with a score above 1. Figure adapted from Johnson et al., 2013.
To identify the quality of the combined model, we calculated the linear regression of the measured invasion versus the predicted invasion and identified an $R^2$ value of 0.89, Figure 5.3C. To identify phosphotyrosine sites that were associated most highly with the prediction of invasion in response to either EGF or HGF in A549 cells, phosphorylation sites with VIP scores above 1 were selected and are listed in Figure 5.3D. Phosphotyrosine sites at the top of the canonical signaling networks (e.g., EGFR, Met, Gab1, Shc1, Ship2, ERK1, and ERK2) were most important in driving the phenotypic response in this combined model.

Identification of EGF and HGF Specific Mediators of Invasion

To parse out differences in EGF and HGF signaling in relation to invasion in A549 cells, we used the EGF and HGF datasets individually to specifically identify phosphorylation sites correlated with invasion after EGF or HGF stimulation. We built an EGF-only model with the quantitative data from the 78 overlapping phosphorylation sites shown in Figure 5.3A. The top 26 phosphorylation sites that contributed most significantly to the model are shown in Figure 5.4A along with their respective VIP scores. We generated a reduced “EGF-only” model, based on these 26 sites, which was then used to predict invasion. The predictive power of the reduced EGF model was the same as the full 78 phosphorylation site EGF model, with both models displaying an $R^2$ value of 0.99, Figure 5.4B. To identify how specific these 26 phosphorylation sites were for predicting the pattern of EGF driven invasion, we used the reduced EGF model to predict invasion following HGF stimulation. Intriguingly, the EGF-only reduced model could not predict the measured HGF invasion ($R^2 = 0.07$), Figure 5.4C. To ensure that this reduction in the predictive power of the model was not solely due to the receptor,
we removed EGFR from the model. A model comprised of the remaining 25 phosphorylation sites still failed to predict HGF invasion ($R^2 = 0.29$).
Figure 5.4. Individual EGF or HGF reduced linear models predict network specific mediators of invasion. A and D. Bar charts display the VIPs for the 26 most important variables from the EGF-only and HGF-only models, respectively. The dotted line indicates the score of 1 and asterisks indicate phosphorylation sites specific to each model. B and E. Plots of experimentally determined invasion (x-axis) versus predicted invasion (y-axis) as determined by the reduced linear EGF and HGF invasion models, respectively, which used 26 phosphorylation sites to predict invasion. C. Plot of experimentally determined HGF invasion (x-axis) versus predicted invasion (y-axis) as determined by the reduced linear EGF invasion model. F. Plot of experimentally determined EGF invasion (x-axis) versus predicted invasion (y-axis) as determined by the reduced linear HGF invasion model. Blue data points and bars indicate EGF data and green data points and bars indicate HGF data. Figure adapted from Johnson et al., 2013.
To identify HGF specific mediators of invasion, we built an HGF-only model with the quantitative data from the 78 overlapping phosphorylation sites shown in Figure 5.3A. The top 26 phosphorylation sites that contributed most significantly to the model were again selected, Figure 5.4D, and used to generate a reduced “HGF-only” model. The predictive power of the reduced HGF model was similar to the full 78 phosphorylation site HGF model, with the full model demonstrating an $R^2$ value of 0.97 and the reduced model providing an $R^2$ value of 0.96, Figure 5.4E. To confirm the specificity of these 26 phosphorylation sites for predicting HGF driven invasion, we used the reduced HGF model to predict EGF invasion. The HGF data also performed poorly at predicting the measured EGF invasion ($R^2 = 0.32$), Figure 5.4F. To assess the role of Met in affecting the model specificity, we removed Met from the model. A model comprised of the resulting 25 phosphorylation sites could still not predict EGF invasion ($R^2 = 0.27$). It is worth noting that the same 78 phosphorylation sites were used in both of the individual models. The difference between the models is therefore not due to qualitatively different sites, but is instead due to the quantitative differences in site-specific phosphorylation associated with the distinct growth factor stimulations. Since the same sites were used to construct both models, the two individual models can be directly compared to determine the contribution of specific phosphorylation sites to each model.

**Discussion**

Treatment with two growth factors, EGF and HGF, results in signaling dynamics that contain both similar and distinct cascades to each pathway. As expected, stimulation of A549 cells with EGF resulted in a robust phosphorylation increase on EGFR Y1173 and Y1148 along with known immediate downstream signaling proteins such as Gab1, Shc1,
and INPPL1 (Ship2) (Yarden, 2001). Overall the response to HGF stimulation was more muted than EGF, and the phosphorylation sites with the largest response were those at the receptor and those known to signal directly downstream of Met such as Gab1, GRB2, and Ship2 (Zhang and Woude, 2003). EGFR phosphorylation on Y1068 and Y1173 both increased by approximately 1.5 fold following HGF stimulation, despite no perceptible increase in phosphorylation of these sites in the immunoblot data in Figure 5.1A. This difference is likely due to the fold-change of 1.5 fold that was detected by mass spectrometry being below the limits of detection of immunoblotting in this instance. Several EGF and HGF responsive phosphorylation sites were identified on proteins known to be involved in cell adhesion, migration, and invasion. For instance, phosphorylation of Y30 and Y188 on Annexin A2 (ANXA2) and Y780, Y823, Y855, and Y333/Y354 on Tensin 3 (TNS3) were identified as EGF and HGF responsive. ANXA2 has been shown to play an important role in tumor progression and invasion in gliomas and ovarian cancers, and TNS3 is known to inhibit cell migration in breast and kidney cancer, yet the role of the phosphorylation sites identified here is not known (Lokman et al., 2011; Zhai et al., 2011; Katz et al., 2007; Martuszewska et al., 2009).

While it is known that Crk and PLC-γ play a role in signaling downstream of EGFR (Yarden, 2001; Birge et al., 1992) and Met (Zhang and Woude, 2003), they were only detected in the EGF dataset. This difference demonstrates the quantitative differences in the signaling pathways. Because we analyzed the phosphorylation levels by mass spectrometry in a data-dependent, untargeted mode, not all sites measured in one analysis will be present in another. If the peptide ions are not present at a high enough intensity, relative to other ions being measured at that time, they will not be selected for
MS/MS analysis and their iTRAQ quantitation will not be measured. This scenario may have occurred during the HGF analysis leading to the PLC-γ and Crk peptides not being quantified. HGF stimulation did result in responsive phosphorylation across multiple RTKs, however, with AXL, FGFR, and IGF1R phosphorylation sites increasing in response to this HGF stimulation, thus indicating crosstalk between these receptors. These data demonstrate distinct qualitative differences in the EGF and HGF signaling networks.

The PLSR modeling was used to understand the differences in signaling after stimulation with growth factor, Figure 5.3. PLSR predicted invasion via a linear regression of the datasets in a reduced-dimensionality principal component space with regression coefficients associated with principal components. The linear modeling is used to understand the invasion and phosphorylation data collected here; it was not meant to be used as a predictive model going forward as the data was not cross validated. With the small number of data points and the (relatively) large number of phosphorylation sites by comparison, it is not surprising that the prediction is very well correlated to the actual invasion data and likely reflects overfitting. However, we can still use this data to understand our model system presented here.

The primary separation was due to invasion, demonstrating that the overall signaling occurring when cells undergo a phenotypic change is stronger than the cause (EGF or HGF stimulation). PC2, however, separates the cells based treatment. The sites with a high VIP score, a weighted representation of the importance of each of the variables, phosphosites, in the model, are primarily driving invasion, the factor to which the
variables were regressed. These sites are the primary docking and activation sites that mediate downstream network activity following RTK activation, so while their association with invasion was expected, this result highlights the power of the model to identify crucial nodes in the network. Several less well characterized phosphorylation sites (e.g., Csk pY184, intersectin2 pY940, Cbl pY371, calmodulin (CALM1) pY100, ANXA2 pY188, and TNS3 pY333/pY354) were also found to be strongly correlated with invasion in this model. Further work is needed to determine the mechanistic contribution of these phosphorylation events to invasion downstream of HGF or EGF stimulation of these cells.

Studying the phosphosites with PC2 coefficients on either end of the spectrum can help us to understand EGF- versus HGF-driven invasion, Figure 5.3B. Phosphorylation sites with the PC2 lowest coefficients were HGF specific phosphorylation sites such as Gab1 (pY406 and pY689), Lyn (pY316), PTPRA (pY798), intersectin2 (ITSN2; pY940) and MPP5 (pY243). EGF specific phosphorylation sites defined by the highest PC2 coefficients were EGFR (pY1173), Lyn (pY397), Ship2 (pY986 and pY1135), MAPK1 (ERK2) (pY187/pT185), Shc1 (pY349/pY350) and BCAR1 (p130Cas; pY249). While many of these phosphorylation sites have not been previously attributed specifically to the EGF or HGF signaling networks, these data suggest specific mediation of these phosphorylation sites downstream of the respective receptors.

In addition to studying PC2 of the original PLSR model, we built the EGF-only and HGF-only models. Specific EGF mediators are highlighted in Figure 5.4A with asterisks. Several of these proteins have been previously associated with cell migration and/or
invasion, including ELMO2, involved in cytoskeletal rearrangement and cell motility (Hiramoto-Yamaki, 2010), Syntenin (SDBP), which promotes cell invasion in multiple cancer models (Meerschaert et al., 2007; Koo et al., 2002), p130Cas, which is involved in focal adhesion kinase (FAK) promoted invasion (Cary et al., 1998), and Cysteine rich protein 1 (CRIP1), which is regulated by the EGFR ligand transforming growth factor-β (TGF-β) and mediates cell contractility (Jarvinen et al., 2011). None of these proteins have previously been specifically linked to EGFR mediated invasion.

HGF specific mediators of invasion are indicated in Figure 5.4D. The association between these proteins and cell migration or invasion is less well characterized, but pragmin (DKFZp761P0423) has been shown to be involved in migration of colorectal cancer cells; mutation of Y391 significantly reduced migration, yet phosphorylation of Y132 has not been characterized (Leroy et al., 2009). ADAM9, a secreted protein, increases cell invasion in human liver adenocarcinoma and uveal melanoma (Mazzocca et al., 2005), but the role of phosphorylation of this protein is not known. Membrane protein, palmitoylated 5 (MPP5) is localized to the tight junctions of epithelial cells, although the functional role of this protein has not been elucidated (Stohr et al., 2005). Intriguingly, none of these proteins have been specifically related to Met mediated invasion. Thus, the proteins highlighted specifically for EGF- or HGF-mediated invasion warrant further studies to elucidate their role in growth factor mediated invasion.
Materials and Methods

Cell Invasion Assay

*In vitro* invasion of A549 cells was performed using Boyden chambers of 8.0 μm pore size, Transwell cell culture inserts coated with 5 μg Matrigel (BD Biosciences). Prior to the assay, cells were serum depleted for 24 hours in RPMI-1640 (Corning) supplemented with 0.1% BSA. Cell suspensions containing $5 \times 10^5$ cells were seeded into the upper region of a Boyden chamber. RPMI-1640 containing EGF (0, 0.01, 0.03, 0.1, 0.3, 1, 10, or 50 ng/mL), HGF (0, 3, 10, 30, 100, 300, 1000, or 3000 ng/mL), or 10% FBS was then placed in the lower chamber. Cells were allowed to invade through the Matrigel-coated membrane for 20 hours, after which the cells on the filter were stained with 0.1% crystal violet and scanned into ImageJ. Invasive cells on the bottom of the membrane were then quantified using ImageJ. Percent invasion was calculated relative to invasion induced by 10% FBS and EC₀, EC₅₀, EC₁₀₀ and EC₅₀ were determined for EGF and HGF.

Cell Lysis

Cells were lysed as described in Chapter 3.

Immunoblotting

Cell lysates were separated using 7.5% SDS-PAGE as described in Chapter 3. The membranes were probed with anti-EGFR (BD Biosciences), anti-EGFR pY1173 (Epitomics), anti-c-Met (Epitomics), anti-c-Met pY1234/5 (Epitomics), antiphosphotyrosine (4G10, Millipore), and anti-β-tubulin (CST).
Mass Spectrometry Sample Preparation

Proteins were prepared for mass spectrometry as described in Chapter 2.

iTRAQ Labeling

Peptide labeling was performed as described in Chapter 2. Growth factor stimulated A549 cells were labeled using iTRAQ 8plex. Three biological replicates were performed for each of the EGF and HGF stimulation conditions. EGF and HGF were analyzed in separate mass spectrometry analyses such that AUC measurements for EGF or HGF were all calculated from data measured the same mass spectrometry analyses.

Phosphotyrosine Enrichment and Mass Spectrometry Analysis

Phosphotyrosine analysis was performed as described in Chapter 2 with the following deviations. Following enrichment by IP and IMAC and separation chromatographically, peptides were nanoelectrosprayed directly into an Orbitrap XL mass spectrometer (Thermo Scientific). The mass spectrometer was operated in data-dependent mode with a full scan MS spectrum followed by MS/MS (CID was set at 35% energy for sequence information and HCD at 75% energy for iTRAQ quantification) for the top 10 precursor ions in each cycle. Ion trap injection time was set to 100 ms and FTMS injection time was set to 1,000 ms with a resolution of 60,000 across m/z 400–2,000. For IT and FT-MS/MS scans, fragmentation was carried out on ions above a threshold of 500 counts and an FTMS resolution of 7,500.
Phosphotyrosine Data Analysis

Mass spectra files were processed as described in Chapter 2 with the following differences. DTAsupercharge 1.31 was used to convert .RAW files. All resulting MS/MS peak lists were searched against a UniProt database containing Homo sapiens protein sequences using Mascot (Matrix Science, version 2.1.03). The MS/MS peak lists were searched with the same peptide modifications listed in Chapter 2, a precursor ion mass tolerance of 10 ppm, and a fragment ion mass tolerance of 0.8 Da.

Precursor ions were manually evaluated and peptides with contaminating peaks present within the isolation window (ions with intensity >25% of the base peak ±1.5 m/z around the selected precursor ion m/z) were discarded as they may contribute to the relative iTRAQ intensities. MS/MS spectra of the tyrosine phosphorylated peptides were manually validated to confirm peptide identification and phosphorylation site localization. EGFR phosphorylation site numbering excludes the 24 amino acid signal peptide sequence.

iTRAQ intensity values were extracted from HCD scans using an in house python script which converted iTRAQ intensities into .txt format. To determine the relative phosphorylation level of each phosphosite, the iTRAQ quantification data were corrected for isotopic overlap, as previously determined by AB Sciex, normalized to total protein signal of each sample, and normalized to the 10% FBS condition for both the EGF and HGF experiments. To quantify the overall phosphorylation signal for each peptide at EC₀, EC₅₀, EC₁₀₀ and EC₅₀₅₀, the AUC was calculated using relative intensities at 0, 5, and 20 min post growth factor stimulation. The normalized signal across time
was integrated from $EC_0$ (set as our initial measurement) to the 5 and 20 min phosphorylation level at each concentration prior to PLSR.

**Affinity Propagation Clustering Analysis**

Phosphorylation sites of the EGF and HGF, separate and combined, datasets were clustered using affinity propagation as previously described (Johnson et al., 2012) and proposed (Frey and Dueck, 2007). The similarity metric used was Euclidean distance. The clustering solution was chosen using a Gaussian mixture model based on exemplar clustering and the Bayesian information criterion (BIC)-scoring metric that penalized complexity. The highest scoring model was selected where scoring depended upon varying self-similarity values and calculating a BIC score for each clustering solution.

**Partial Least-Squares Regression**

The PLSR model was generated using SIMCA-P 11.5 (Umetrics) as previously described (Wolf-Yadlin et al., 2006). Briefly, an MxN matrix (X) was generated from the AUC (0 to 20 min) quantified for each of the phosphorylation sites. Each of the M rows corresponded to a different treatment. Each of the N columns corresponded to one phosphorylated peptide. A vector (Mx1, Y) was generated that described the invasion (cellular output). The invasion data and quantitative data for each phosphorylated peptide were mean centered across the cellular conditions and then Log2 transformed. Models were generated using three principal components (combined EGF and HGF model) or two principal components (individual EGF or HGF models) under the standard optimization criteria. The VIP score was used to identify explanatory variables.
that contribute the most to the prediction of invasion in response to EGF or HGF in A549 cells.

**Conclusions and Future Directions**

Here we have quantified phosphotyrosine signaling following HGF or EGF stimulation at multiple time points and concentrations. These temporal phosphorylation profiles have significantly increased the site specific phosphotyrosine information pertaining to EGFR and Met signaling networks, and have highlighted common and distinct points downstream of these receptors. The integration of this temporal tyrosine phosphorylation data with the quantitative invasion data through PLSR analysis has enabled identification of network specific mediators of invasion in A549 cells. By using either a combined or two individual models of EGF and HGF mediated invasion, we have been able to extract overlapping and distinct pathways by which EGFR or c-Met may mediate invasion. Nodes which are differentially important in the two models may also offer insight into the distinct signaling pathways activated by these two receptors. The common interaction points between the networks may provide insight into the compensatory mechanisms by which Met can mediate growth and invasion in the presence of EGFR inhibitors, as resistance after TKIs has been well documented. These same common nodes may represent potential therapeutic targets to inhibit invasion driven by either growth factor, offering a more general therapeutic mechanism that should prevent compensatory resistance. Overall, the quantitative data acquired in this study can be directly applied to the annotation of EGFR and Met signaling networks in NSCLC. Improved models of the signaling networks downstream of these receptors will
enable the identification of potential therapeutic targets directly involved in invasive
cancer growth.

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Chapter 6: Discussion

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Chapter 6: Discussion

Major Findings and Scientific Contributions

Targeting CDK1 pY15 as a Therapy in GBM Models

In this study, we used cancer models to investigate drivers of GBM. Using a murine proneural GBM model, we quantified signaling differences in tumors and compared them to normal brain controls. Basic research to further understand this disease and the identification of new therapeutic targets is gravely needed as the average life span of a patient with GBM is just over a year with aggressive therapy including surgery, radiation, and chemotherapy (Ostrom et al., 2013). These murine tumors form spontaneously after a retroviral injection of PDGF-IRES-Cre into mice with floxed PTEN, floxed P53, and stop-floxed Luciferase or YFP (Lei et al., 2011). Therefore, they are PTEN- and P53-deficient and are driven by excess PDGF, the ligand for PDGFRA. Similar to the late stages of disease that GBM is in when it is diagnosed in patients (Fisher et al., 2007), these tumors were harvested when mice experienced tumor burden. By enriching for phosphorylation sites using IP and IMAC and quantifying phosphorylation sites using iTRAQ and mass spectrometry techniques optimized in our laboratory (Johnson et al., 2013; Johnson et al., 2012; Wolf-Yadlin et al., 2006), we were able to identify and quantify hundreds of phosphorylation sites on 56 tumors and brain tissue specimens using untargeted, data-dependent MS/MS.

Our initial studies compared two cohorts of tumors to normal brain samples and overall our mass spectrometry data highlighted the vastly different signaling that occurs in these proneural, oligodendrocytic lineage tumors compared to the brain. Proteins
expressed in neurons had increased phosphorylation in the brain samples. Proteins that drive our tumor, such as PDGFRA, and its downstream signals through the MAPK cascade had increased phosphorylation in our tumors. The largest difference in phosphorylation, however, was measured on a cell cycle checkpoint protein, CDK1.

CDK1 pY15 had a 14-fold increase in phosphorylation in the tumors relative to the brain. The doubly phosphorylated version was measured to have a 6-fold increase relative to the brain. These phosphorylation sites are inhibitory sites that, when phosphorylated, induce cell cycle arrest (Heald et al., 1993). Therefore, although the tumor cells are rapidly dividing and the tumors are causing physiological defects within a month of initiation, there is still some attempt by the cells to reduce the speed at which division occurs and to allow DNA repair prior to entering mitosis. The peptide measured by mass spectrometry and the amino acid sequence used as an antigen for anti-phospho-CDK antibodies for use in western blotting is identical in CDK1 and CDK2. For simplicity, we have used CDK1 in this document, but CDK1 and CDK2 do serve different functions and our measurements are likely a summation of the two. CDK1 is a G₂/M arrest protein while CDK2 is a G₁/S arrest protein (Harper et al., 1993). Due to their identical sequence around these phosphorylation sites, while the target of phosphorylation by Wee1 is primarily CDK1, Wee1 is able to phosphorylate both.

In addition to the tumor analysis, we continued our study of this protein in murine cell lines that are syngeneic to these tumors, MGPP6 and MGPP7. The in vitro models provide tractability and homogeneity of cell type for further analysis. In vitro, we studied the effects of inhibiting the kinase of CDK1 pY15, Wee1, with a small molecule
inhibitor, MK-1775. We were able to induce mitotic catastrophe after treatment with MK-1775 which caused significant reduction in cell number. We characterized this cell death as mitotic catastrophe because the treated cells had DNA damage, there were abnormal percentages of cells in cell cycle phases, and they were dying by apoptosis.

We quantified DNA damage using a marker of activation of the DNA damage response pathway, γH2AX, by flow cytometry and western blotting. Using flow cytometry and Hoechst staining, we quantified the percentage of cells in each portion of the cell cycle. Using anti-pHH3 antibodies and Hoechst staining we measured whether cells were undergoing mitosis with the correct DNA content using flow cytometry. We characterized cell death by measuring cells expressing two markers of apoptosis, cleaved-PARP and cleaved-Caspase 3. These measurements confirm mitotic catastrophe whereby the cells die after abrogation of the G2/M arrest in late stages of the cell cycle with DNA damage (Vitale et al., 2011).

We confirmed these results in three GBM PDX cell lines. They all lack P53 expression, as our murine cell lines do, and one was also PTEN-deficient and subtyped as proneural. All three cell lines underwent mitotic catastrophe with DNA damage, abnormal percentages of cells in each phase of the cell cycle, and death by apoptosis. Significant increases in these metrics were reached after treatment with MK-1775 compared to the control, DMSO treatment. These cell lines are not syngeneic and their varied responses reflect this.
MK-1775 is described as potent and specific inhibitor for Wee1; we confirmed this and demonstrated the necessity of CDK1 activity for this therapeutic to have its intended effect. Phosphorylation of CDK1 Y15 was reduced after treatment with MK-1775, relative to total CDK1 expression levels. Adding roscovitine, a general CDK inhibitor, to treatment with MK-1775 suppresses the effects of MK-1775 and rescues the cells from mitotic catastrophe. The percentage of cells in each phase of the cell cycle is restored to control treatment percentages and cells with DNA damage (γH2AX positive cells) are reduced when the cells are treated with roscovitine in addition to MK-1775. These experiments demonstrate that not only is MK-1775 effective, but for it to be beneficial, cells must have active CDKs, potentially CDK1 specifically. This result is important in the context of our GBM and brain tissue analysis because the phosphorylation was significantly increased in the tumors, thus the brain tissue that is functioning normally has greatly reduced CDK1 inhibition activity. Since the cells of the brain are not often turning over and should not be incurring as much mutagenic stress, this cell cycle arrest may not be systemically necessary in normal brain cells and may make inhibiting Wee1 a more targeted therapy against the rapidly dividing GBM cells.

In addition to studying MK-1775 in murine and human cell lines, we treated mice harboring flank GBM PDX tumors orally with MK-1775. Again, we measured a reduction in phospho-CDK1 and increases in DNA damage, γH2AX, after 5 days of treatment. In addition to the molecular effect of MK-1775, we demonstrated a significant increase in survival for mice treated with MK-1775 compared to those that did not and the effect on the tumor size was significant within the first week.
All of the tumor models we investigated in this study were P53-deficient and therefore are already missing a major cell cycle checkpoint tumor suppressor. These tumors were able to compensate for this loss when they were not challenged with an inhibitor, as little DNA damage was measured in these models without MK-1775. However, a significant increase in DNA damage occurred after MK-1775 treatment. The response to MK-1775 was very strong in our model and did not require priming by a chemotherapeutic, as was needed in other studies (Mir et al., 2010; Rajeshkumar et al., 2011; Aarts et al., 2012), to induce mitotic catastrophe. This result points to the importance of the G2/M checkpoint, especially in the context of P53 deficiencies, a common mutation in cancer generally.

Beginning with two differentially phosphorylated amino acids (CDK1 pT14/pY15) measured by mass spectrometry between murine GBM tumors and brain samples, we were able to identify a potential therapeutic target (Wee1 kinase inhibition), study the pathways effected in cells by documenting an on-target effect of the inhibitor and measuring the response of mitotic catastrophe. We demonstrated that this response was due to CDK1 activity by inhibiting its activity with roscovitine. We measured mitotic catastrophe after treatment with MK-1775 in human GBM PDX cell lines. Finally, we quantified an in vivo molecular response and mouse survival benefit after treating mice harboring GBM flank tumors with MK-1775. This investigation proved valuable in itself, but also highlights the importance of discovery-based analyses such as untargeted mass spectrometry and our dataset is not fully explored.
Treatment of Tumors with a PDGFR Inhibitor, Sunitinib

In addition to the Wee1/CDK1 studies, we analyzed the effects of sunitinib. Sunitinib is a multi-kinase inhibitor that targets the driver receptor in our murine model, PDGFRA, among other RTKs. We analyzed tumors that were treated for weeks, as a long-term therapy, until they succumbed to the tumor burden and also tumors that were treated only once, to measure the initial effects of sunitinib.

Sunitinib treatment causes a significant increase in mouse life span, but does not cure the disease. The change in tumor size, as measured by BLI, after one week of treatment was predictive of and anti-correlated to mouse survival. These tumors demonstrate a diffusely infiltrative state where they invade the normal tissue to a greater extent than their untreated or vehicle treated counterparts. While sunitinib does increase lifespan, it also alters the tumor.

To understand the drivers of this altered tumor, we measured phosphorylation signaling in these tumors once tumor burden was reached and compared this to the signaling of vehicle treated tumors. We measured an on-target effect of decreased phosphorylation of PDGFRA Y742, an activating phosphorylation site of PDGFA (Heldin and Ronnstrand, 1998). We also measured increases in phosphorylation of pro-migratory proteins, such as Eno1 and Elmo2 (Hiramoto-Yamaki, 2010). The phosphorylation increase is relative to vehicle treated tumors; these increases are consistent with the phenotypic changes that occur due to treatment and begin to explain a possible resistance mechanism that occurs after tumors are challenged with sunitinib.
We also compared acutely treated tumors to untreated controls. These tumors were treated with sunitinib once at the end stage of the disease. These tumors do have decreased phosphorylation of PDGFRA, but there was not a difference in the phosphorylation of the pro-migratory proteins. While the number of tumors analyzed is small, we have begun to characterize the short- and long-term effects of sunitinib, as a model of an RTK, targeted inhibitor. By understanding the signaling through which the tumors become resistant to TKIs, better TKIs or multiple therapies may be proposed.

**Invasion of NSCLC Cells after Growth Factor Simulation**

NSCLC A549 cells maximally invade through a Boyden chamber after EGF and HGF stimulation to approximately the same amount (with different concentrations), relative to invasion driven by 10% FBS. We, however, measured differences in the phosphorylation signaling occurring in these cells resulting from the stimulations. While overall invasion was driven primarily by the phosphorylation of well characterized proteins just downstream of RTKs and the RTKs themselves (EGFR, Met, Gab1, SHC1, INPPL1 (Ship2), ERK1, and ERK2), we were able to separate signaling by growth factor as well. EGF-mediated invasion was driven by phosphorylation of Lyn, Ship2, and ERK2 while HGF-mediated invasion was driven by Gab1, Lyn, PTPRA, and Intersectin 2. Building on these differences will help our understanding of resistance to TKIs that is often observed in patients (Rini and Atkins, 2009; Nahta et al., 2006; Heinrich et al., 2006) and will help to propose additional therapies to treat this disease prior to or following tumor resistance.
Conclusions and Future Directions

The hundreds of phosphorylation sites identified and quantified in this work and those similar to it provide a wealth of data and a challenge to understand it. We have investigated the effect of inhibiting Wee1 on CDK1 phosphorylation and mitotic catastrophe. This study leaves many more signaling patterns and changes that could be analyzed further. Detailed studies of PTMs, such as phosphorylation status, are limited. Even if a protein’s function is known, the consequence of a measured phosphorylation site might not be. Computational techniques are necessary to understand this data and follow up studies can be limited by both scope and reagents. Phospho-specific antibodies are imperfect, inducing point mutations (such as Y to F mutations to remove a phosphorylation site while maintaining approximate residue size) or overexpression of a mutant protein is time and energy intensive; therefore, fully understanding and utilizing the phosphorylation data acquired prior to further analysis is necessary.

To this end, we have begun an analysis on the sunitinib treated tumors. Across multiple mass spectrometry analyses we have a reduced set of phosphorylation sites measured in all samples and we measured batch effects after data collection; therefore, we studied comparisons in Chapter 4 within single 8plex iTRAQ analyses. As our mass spectrometers continue to improve and our datasets continue to increase in size, computer-aided or fully automated spectra validation will only be more important. During the scope of this project, CAMV (Curran et al., 2013) reduced our manual validation time by at least a factor of ten. These types of advancements will continue to push this field forward. Coupling technical advancements with strong, well thought out experimental designs will greatly enhance our research.
Within the data collected here, we have characterized differences between sunitinib resistant tumors and unchallenged ones that are consistent with the known changes occurring in these tumors that allow them to adapt to and continue to grow in the presence of treatment. We have identified differences at both the gene expression and PTM levels and ArrayCGH data has also been collected for these matched samples. We are confident that the signaling data collected and the incorporation of data from different experimental platforms will lead to a better understanding of TKI resistance, much like the contribution we have made to understanding Wee1 inhibition in which we characterized the cellular response in murine and human models of GBM.

References


