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Regulation of Cell Signaling by Mig6 and Sprouty2 in Cancers With Egfr Mutations

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Regulation of Cell Signaling by Mig6 and Sprouty2 in Cancers With Egfr Mutations

Abstract
Epidermal growth factor receptor (EGFR) mutation and overexpression promote tumorigenesis in multiple cancers. Understanding the complex EGFR regulatory network is critical for developing effective therapeutic interventions. To this end, this work investigated the functions of two incompletely characterized regulators of EGFR trafficking and signaling, mitogen-inducible gene 6 (MIG6) and Sprouty2 (SPRY2), in two cancer settings where EGFR mutation is common, non-small cell lung cancer (NSCLC) and glioblastoma multiforme (GBM). In NSCLC cells, results indicate that MIG6, an endogenous inhibitor of EGFR activity and endocytic adaptor, is surprisingly responsible for at least half of EGFR endocytosis, suggesting that a substantial fraction of internalized EGFR may not be competent to drive signaling. Computational modeling further suggested that in cells expressing kinase-activated, endocytosis-impaired EGFR mutants, the importance of MIG6 relative to other endocytic pathways is increased, but that MIG6 internalization capacity is reduced compared to cells expressing wild-type EGFR. Additional data indicate that SPRY2 expression reduces EGFR endocytosis rate primarily by promoting EGFR expression, which overwhelms the saturable EGFR endocytic pathway, but that SPRY2 also promotes ERK phosphorylation and resistance to EGFR inhibition independent of EGFR expression level. In GBM cell lines, our data demonstrate that SPRY2 expression promotes proliferation, anchorage-independent growth, resistance to EGFR and c-MET co-inhibition, and growth as mouse tumor xenografts. Additional studies identified SPRY2-mediated regulation of the strength and effects of JNK and p38 MAP kinase pathways as important for controlling GBM cell behaviors. Through analysis of public datasets and a collaborative analysis of human and rat tumors, we further found that elevated SPRY2 expression is associated with reduced patient survival and expression of EGFR variant III, an EGFR mutant linked to aggressive GBM. Thus, while SPRY2 is a candidate tumor suppressor in other contexts, our results support a tumor promoter role for SPRY2 in GBM and identify SPRY2 and the pathways it regulates as potential therapeutic targets or biomarkers for therapeutic response. Overall, these findings add new qualitative and quantitative understanding of the complexities of EGFR trafficking and signaling regulation and the functions of SPRY2 and MIG6 that may be leveraged to develop improved cancer therapies.

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REGULATION OF CELL SIGNALING BY MIG6 AND SPROUTY2 IN CANCERS WITH EGFR MUTATIONS

Alice Macdonald Walsh

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ABSTRACT

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Alice Macdonald Walsh
Matthew Lazzara, PhD

Epidermal growth factor receptor (EGFR) mutation and overexpression promote tumorigenesis in multiple cancers. Understanding the complex EGFR regulatory network is critical for developing effective therapeutic interventions. To this end, this work investigated the functions of two incompletely characterized regulators of EGFR trafficking and signaling, mitogen-inducible gene 6 (MIG6) and Sprouty2 (SPRY2), in two cancer settings where EGFR mutation is common, non-small cell lung cancer (NSCLC) and glioblastoma multiforme (GBM). In NSCLC cells, results indicate that MIG6, an endogenous inhibitor of EGFR activity and endocytic adaptor, is surprisingly responsible for at least half of EGFR endocytosis, suggesting that a substantial fraction of internalized EGFR may not be competent to drive signaling. Computational modeling further suggested that in cells expressing kinase-activated, endocytosis-impaired EGFR mutants, the importance of MIG6 relative to other endocytic pathways is increased, but that MIG6 internalization capacity is reduced compared to cells expressing wild-type EGFR. Additional data indicate that SPRY2 expression reduces EGFR endocytosis rate primarily by promoting EGFR expression, which overwhelms the saturable EGFR endocytic pathway, but that SPRY2 also promotes ERK phosphorylation and resistance to EGFR inhibition independent of EGFR expression level. In GBM cell lines, our data demonstrate that SPRY2 expression promotes proliferation, anchorage-independent growth, resistance to EGFR and c-MET co-inhibition, and growth as mouse tumor xenografts. Additional studies identified SPRY2-mediated regulation of the strength and effects of JNK and p38 MAP kinase pathways as important for controlling GBM cell behaviors. Through analysis of public datasets and a collaborative analysis of human and rat tumors, we further found that elevated SPRY2 expression is associated with reduced patient survival and expression of EGFR variant III, an EGFR mutant linked to aggressive GBM. Thus, while SPRY2 is a candidate tumor suppressor in other contexts, our results support a tumor promoter role for SPRY2 in GBM and identify SPRY2 and the pathways it regulates as potential therapeutic targets or biomarkers for therapeutic response. Overall, these findings add new qualitative and quantitative understanding of the complexities of EGFR trafficking and signaling regulation and the functions of SPRY2 and MIG6 that may be leveraged to develop improved cancer therapies.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS .......................................................................................................................... iii

ABSTRACT ...................................................................................................................................................... iv

TABLE OF CONTENTS ................................................................................................................................. v

LIST OF TABLES ................................................................................................................................................ vii

LIST OF FIGURES ............................................................................................................................................. viii

Chapter 1: Introduction .................................................................................................................................. 1
1-1. Challenges in cancer treatment and targeted therapeutics ................................................................. 1
1-2. EGFR signaling and EGFR targeted inhibitors ................................................................................. 2
1-3. EGFR trafficking .................................................................................................................................. 4
1-4. Feedback regulators of EGFR signaling ........................................................................................... 7
1-5. MIG6 ..................................................................................................................................................... 8
1-6. SPRY2 .................................................................................................................................................. 10
1-7. Overview of thesis work .................................................................................................................... 13

Chapter 2: Regulation of EGFR trafficking and cell signaling by Sprouty2 and MIG6 in lung cancer cells ........................................................................................................................................ 16
2-1. Abstract................................................................................................................................................ 16
2-2. Introduction ......................................................................................................................................... 17
2-3. Materials and Methods .................................................................................................................... 19
2-4. Results ................................................................................................................................................ 22
2-5. Discussion .......................................................................................................................................... 35
2-6. Acknowledgements .......................................................................................................................... 39
2-7. Supplemental Materials .................................................................................................................... 40

Chapter 3: Differential parsing of EGFR endocytic flux among parallel internalization pathways in lung cancer cells with EGFR-activating mutations .................................................................................................................. 51
3-1. Abstract................................................................................................................................................ 51
3-2. Introduction ......................................................................................................................................... 52
3-3. Materials and Methods .................................................................................................................... 54
3-4. Results ................................................................................................................................................ 58
3-5. Discussion .......................................................................................................................................... 72
3-6. Conclusion ......................................................................................................................................... 74
3-7. Acknowledgements .......................................................................................................................... 74
3-8. Supplemental Materials .................................................................................................................... 74

Chapter 4: Sprouty2 drives drug resistance and proliferation in glioblastoma ........................................ 90
4-1. Abstract................................................................................................................................................ 90
4-2. Introduction ......................................................................................................................................... 90
4-3. Materials and Methods .................................................................................................................... 92
4-4. Results ................................................................................................................................................ 95
4-5. Discussion .......................................................................................................................................... 107
4-6. Acknowledgements .......................................................................................................................... 110
4-7. Supplemental Data .......................................................................................................................... 111
4-8. Supplemental Materials and Methods ............................................................................................. 120

Chapter 5: Conclusions and future directions ......................................................................................... 125
5-1. Introduction ......................................................................................................................................... 125
5-2. Future work and interpretation related to SPRY2 and MIG6 function in NSCLC ......................... 125
5-3. Future work and interpretation related to predictions of the EGFR trafficking model ............ 127
5-4. Future work and interpretation related to study of SPRY2 in GBM ............................................ 130
5-5. Main conclusions ............................................................................................................................. 133

Appendix 1: Changes in cell adhesion in U87MG cells with SPRY2 depletion .................................... 135

v
A1-1. Results ..................................................................................................................... 135
A1-2. Materials and Methods .......................................................................................... 137
A1-3. Figures .................................................................................................................... 139

Appendix 2: SPRY1 and SPRY4 expression and patient survival in GBM .................. 143

BIBLIOGRAPHY .............................................................................................................. 144
LIST OF TABLES

Table 3-S1. Model equations for individual species. ................................................................. 83
Table 3-S2. Model reactions included in the ODEs in Table 3-S1. ........................................ 84
Table 3-S3. Parameters for model equations. ............................................................................. 85
Table 3-S4. Parameter values based on literature. .................................................................... 86
Table 3-S5. Estimated parameter values and initial model species concentrations.............. 87
Table 3-S6. Normalized experimental $k_e$ data. ................................................................. 88
Table 3-S7. Full results of local parameter sensitivity analysis. ............................................ 89
Table 4-S1. Upregulated genes shared by human GBMs expressing EGFRvIII and 9L.EGFRvIII rat tumors compared to wild-type EGFR human GBMs or 9L.EV rat tumors .................... 111
LIST OF FIGURES

Figure 1-1. EGFR trafficking. ................................................................. 6
Figure 1-2. MIG6 regulation of EGFR activity and internalization. ................ 9
Figure 1-3. Domain map of MIG6 and SPRY2. ....................................... 10
Figure 1-4. ERK regulation by SPRY2. .................................................. 12
Figure 2-1. SPRY2 and/or MIG6 knockdown perturb EGF-mediated EGFR endocytosis. 23
Figure 2-2. SPRY2 and/or MIG6 knockdown perturb ERK phosphorylation, and SPRY2 knockdown reduced EGFR expression. ........................................ 25
Figure 2-3. EGF-mediated EGFR ubiquitylation and CBL association occur in H1666 cells in a SPRY2-dependent manner, but ubiquitylation and CBL association do not occur in PC9 cells. ... 26
Figure 2-4. SPRY2 knockdown decreases EGFR mRNA and protein levels in an ERK-dependent manner in PC9 cells, but not in H1666 cells. ............................................... 28
Figure 2-5. EGFR reconstitution reverses the effect of SPRY2 knockdown on EGFR endocytosis, but not ERK phosphorylation. ........................................ 30
Figure 2-6. Monensin inhibits ERK phosphorylation, and SPRY2/EGFR levels control EGFR recycling. ................................................................. 32
Figure 2-7. SPRY2 knockdown alters cellular sensitivity to gefitinib in an ERK-dependent and EGFR level-independent manner. ................................. 34
Figure 2-8. SPRY2 and MIG6 regulate EGFR signaling with a complex network of feedback interactions. .......................................................... 35
Figure 2-S1. SPRY2 and MIG6 expression are efficiently reduced by shRNA-mediated knockdown. ............................................................................. 40
Figure 2-S2. SPRY2 and/or MIG6 knockdown perturb EGF-mediated EGFR endocytosis. 41
Figure 2-S3. Differences in EGF-mediated EGFR endocytosis measured with 1.5 ng/mL 125I-EGF are consistent with measurements using 10 ng/mL 125I-EGF (Figure 2-1), and treatment with the dynamin inhibitor Dynasore reduces $k_e$. ........................................ 42
Figure 2-S4. Phosphorylation of ERK and AKT in response to gefitinib is perturbed in NSCLC cells with SPRY2 and/or MIG6 knockdown relative to controls. ........................................ 43
Figure 2-S5. EGFR protein levels are reduced in PC9 and H1666 cells by a second independent shRNA, and SPRY2 knockdown reduces EGFR expression in a broader panel of NSCLC cell lines. .................................................. 44
Figure 2-S6. SPRY2 knockdown results in increased EGF-mediated EGFR ubiquitylation and CBL association in H1666 cells, but not in PC9 cells. ............................................................. 45
Figure 2-S7. EGFR mRNA levels are reduced in PC9 cells by a second independent shRNA. ... 46
Figure 2-S8. Reconstitution of EGFR in SPRY2 knockdown cells does not rescue diminished ERK phosphorylation. .................................................. 46
Figure 2-S9. Flow cytometry measurements demonstrate differences in surface EGFR expression in PC9 and H1666 cells with SPRY2 knockdown and EGFR reconstitution. ...................... 47
Figure 2-S10. SPRY2 knockdown alters cellular sensitivity to gefitinib in additional cell lines. ... 48
Figure 2-S11. Titration of the MEK inhibitor U0126 identifies a concentration of U0126 that reduces ERK phosphorylation to a similar level as SPRY2 knockdown. ...................... 49
Figure 2-S12. PTEN expression is not affected by SPRY2 knockdown. ............... 50
Figure 3-1. Key features of model topology. .......................................... 56
Figure 3-2. Model agreement with experimental data in wild-type EGFR-expressing H1666 cells and relative importance of MIG6 and CBL levels for EGFR internalization. .................. 59
Figure 3-3. Model agreement with experimental data in mutant EGFR-expressing PC9 cells and differences from results for wild-type EGFR-expressing H1666 cells. .................. 63
Figure 3-4. Effect of changing EGFR, SPRY2, CBL, and MIG6 levels on simulated EGFR $k_e$. 65
Figure 3-5. Local parameter sensitivity analysis for PC9 and H1666 models. ................................. 67
Figure 3-6. Robustness of fitted $k_{MIG6}$ and $k_{CBL}$ and model agreement with PC9 data. .......... 68
Figure 3-7. EGF-mediated EGFR degradation in wild-type and mutant EGFR-expressing NSCLC cells and the role of EGFR recycling differences in explaining the data. .................. 71
Figure 3-S1. Measurement of number of EGFR per cell in PC9 cells ................. 75
Figure 3-S2. Simulation of SPRY2 phosphorylation. .................................. 76
Figure 3-S3. Predicted and experimental measurements of internal and surface-bound EGF for H1666 cells. ................................................................. 77
Figure 3-S4. Values of fitted rate constants for a range of MIG6 and CBL concentrations ........ 78
Figure 3-S5. EGFR internalization flux over a range of MIG6 and CBL concentrations. .......... 79
Figure 3-S6. Effect of allowing basal MIG6/EGFR association on model fit to PC9 data. ....... 80
Figure 3-S7. Predicted effect of changing dimerization rate on relationship between EGFR expression and predicted EGFR k∞ ............................................. 80
Figure 3-S8. Model agreement with H1666 data. ......................................................... 81
Figure 3-S9. Fitted parameters when changes in EGFR expression due to SPRY2 knockdown are not considered .......................................................... 81
Figure 3-S10. Values of fitted rate constants when fi was set to experimentally determined values. ........................................................................ 82

Figure 4-1. SPRY2 knockdown reduces cellular proliferation and anchorage-independent growth and enhances cellular sensitivity to EGFR and c-MET co-inhibition. ............................................. 97
Figure 4-2. SPRY2 depletion suppresses xenograft growth ............................................ 98
Figure 4-3. p38 and JNK phosphorylation are increased in U87MG cells with SPRY2 knockdown and in cells co-treated with EGFR and c-MET inhibitors, and control cellular proliferation, anchorage-independent growth, and response to EGFR and c-MET co-inhibition. .................. 100
Figure 4-4. In U87MG cells, SPRY2 knockdown reduces MKP-1 and MKP-5 mRNA expression, and MKP-1 or MKP-5 knockdown enhances cellular response to EGFR and c-MET co-inhibition. ........................................................................... 103
Figure 4-5. SPRY2 protein expression in GBMs is confirmed by immunohistochemical analysis. ........................................................................................................ 104
Figure 4-6. TCGA GBM data reveals that elevated SPRY2 expression correlates with EGFRvIII expression, the classical GBM subtype, and reduced patient survival. .................. 106
Figure 4-7. SPRY2 promotes GBM cell proliferation, anchorage-independent growth, and resistance to tyrosine kinase inhibition. ............................................. 109
Figure 4-S1. SPRY2 is efficiently knocked down by shRNA in GBM cell lines. ................. 112
Figure 4-S2. SPRY2 depletion by a second non-overlapping shRNA reduces cellular proliferation, and SPRY2 depletion by siRNA transfection reduces colony formation in soft agar in EGFRvIII-expressing cells ............................................................................. 113
Figure 4-S3. SPRY2 depletion increases cellular sensitivity to EGFR and c-MET co-inhibition. 114
Figure 4-S4. SPRY2 knockdown promotes response to EGFR/c-MET co-inhibition in GSC cells. ....................................................................................................... 115
Figure 4-S5. p38 and JNK control anchorage-independent growth and response to EGFR and c-MET co-inhibition ........................................................................ 116
Figure 4-S6. shRNA-mediated knockdown of MKP-1 or MKP-5 reduces MKP-1 or MKP-5 mRNA level .................................................................................. 117
Figure 4-S7. SPRY2 protein expression in kidney and cerebellum sections by immunohistochemical analysis ........................................................................... 117
Figure 4-S8. SPRY2 protein expression is confirmed in patient-derived cells by immunoblot .... 118
Figure 4-S9. SPRY2 correlates well with ERK phosphorylation in a panel of GBM cell lines. .... 119
Figure 4-S10. TCGA GBM dataset analysis reveals that SPRY2 expression is associated with reduced patient survival. ........................................................................ 120
Figure A1-1. Cellular morphological changes resulting from SPRY2 knockdown. ............... 139
Figure A1-2. Effect of p38 and JNK inhibition on cellular morphology and cell spreading ....... 140
Figure A1-3. Effect of p38 and JNK inhibition on non-adherent cell culture. ...................... 141
Figure A1-4. Differential phosphorylation of signaling proteins in adherent and non-adherent cell populations ............................................................................. 142
Figure A2-1. SPRY1 and SPRY4 expression in EGFRvIII-positive samples and effect on patient survival. ........................................................................ 143
Chapter 1: Introduction

1-1. Challenges in cancer treatment and targeted therapeutics

Over the last 30 years, the molecular understanding of cancer has expanded dramatically. Disappointingly, over the same period, cancer death rates have remained relatively stagnant (Siegel et al., 2013), indicating a gap in progress translating advanced knowledge of tumor biology into effective medicines. Furthermore, some reductions in cancer deaths can be attributed to lower smoking rates and early detection rather than treatment advances (Siegel et al., 2013). For many cancers, treatment remains limited to surgical resection, radiation, and/or chemotherapy. However, with growing mechanistic understanding of the specific proteins that promote cancer pathogenesis, there is an opportunity to rationally design new treatments targeting specific proteins. Many such targeted therapies have been developed and approved for clinical use, and more are in development. Ultimately, these therapeutics could lead to the improved patient outcomes that have been elusive for the last 30 years. This work is focused on one such class of targeted therapeutics that inhibit the epidermal growth factor receptor (EGFR).

Before examining the details of EGFR inhibition, however, it is worth examining some general aspects of targeted therapeutics.

There are several challenges facing the development of successful targeted cancer therapeutics. As for pharmaceuticals in general, consideration of adverse side effects and drug delivery mechanisms is necessary. More specific to targeted cancer therapies, however, one must also consider that many cancers are heterogeneous diseases driven by several diverse molecular mechanisms such that a drug targeting one mechanism will fail to be successful for the entire patient population. Another challenge is acquired resistance to targeted therapeutics that develops in tumors that may initially respond to treatment (Arora and Scholar, 2005; Branford et al., 2003; Sosman et al., 2012). Imatinib (Gleevec) exemplifies a generally successful targeted therapeutic, but also serves as an example to illustrate the challenges facing the development of effective targeted therapeutics. Imatinib targets the oncogenic fusion protein, BCR-ABL, a
tyrosine kinase found in chronic myeloid leukemia. Imatinib has a response rate of up to 90% and minimal side effects (Arora and Scholar, 2005). However, imatinib is only effective for patients with tumors driven by the BCR-ABL fusion protein and patients may acquire resistance to imatinib. Primarily, this resistance occurs through the development of point mutations in the BCR-ABL kinase domain that interfere with imatinib binding (Shah et al., 2004). Additional examples of targeted cancer therapeutics include angiogenesis inhibitors that target vascular endothelial growth factor (VEGF) signaling, inhibitors of the kinase BRAF, inhibitors of aerobic glycolysis, cyclin-dependent kinase inhibitors, and the focus of this thesis, EGFR inhibitors (Ferrara and Kerbel, 2005; Flaherty et al., 2010; Hanahan and Weinberg, 2011).

EGFR is a representative case where targeted inhibitors show promise, but have been disappointing clinically to date (Dutta and Maity, 2007; Wheeler et al., 2010). While EGFR is one of the best-studied receptor tyrosine kinases (RTKs), the factors contributing to resistance to EGFR inhibition are not well understood. Understanding the determinants of sensitivity and acquired resistance to EGFR inhibitors in order to develop more effective treatments provides the motivation for this thesis. In this chapter, we begin by briefly describing EGFR signaling, EGFR targeted inhibitors, and EGFR regulation through trafficking and feedback regulation. Next, we define the current understanding of mitogen-inducible gene 6 (MIG6) and Sprouty2 (SPRY2), the two signaling regulators that are the central focus of this work. Finally, we outline the motivation for the study of MIG6 and SPRY2 in lung and brain cancer and summarize the work presented in the following chapters.

1-2. EGFR signaling and EGFR targeted inhibitors

There are many excellent reviews of EGFR signaling (Lemmon and Schlessinger, 2010; Shilo, 2005; Wieduwilt and Moasser, 2008; Yarden and Sliwkowski, 2001) that describe in greater detail the current knowledge of EGFR signaling than space allows here. The following brief review describes some of the key features of EGFR regulation and the challenges remaining in understanding EGFR-mediated signaling.
The ErbB family of RTKs consists of four family members: EGFR (ErbB1, HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). The ErbB receptors are single-chain transmembrane proteins with an extracellular domain (that mediates ligand binding), transmembrane domain, intracellular tyrosine kinase domain, and a C-terminal tail that contains multiple tyrosine residues. Receptors are activated through binding of the extracellular domain to one of several known peptide ligands (with the exception of ErbB2 that has no known ligand) that stabilize receptor homo- or hetero- dimers. For EGFR, the dimerization event creates an asymmetric dimer interface between the intracellular kinase domains, resulting in kinase activation and subsequent phosphorylation of C-terminal tyrosines (Zhang et al., 2006). These phosphotyrosine residues recruit cytosolic adaptor proteins that typically bind ErbB receptor phosphotyrosines through SH2 domains and lead to subsequent activation of several signaling pathways including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), protein kinase B (AKT), and signal transduction and activator of transcription (STAT) pathways.

Due to the prevalence of EGFR overexpression, gene amplification, mutation, and aberrant autocrine signaling in cancers, EGFR represents an attractive drug target (Dutta and Maity, 2007; Normanno et al., 2006). In multiple cancers, EGFR expression also correlates with poor patient prognosis (Nicholson et al., 2001). Two classes of drugs targeting EGFR have been approved for use in cancers: antibodies that bind the extracellular domain (e.g., cetuximab/Erbitux, panitumumab/Vectibix) and tyrosine kinase inhibitors (TKIs) (e.g., gefitinib/Iressa, erlotinib/Tarceva) that inhibit intracellular EGFR enzymatic activity. Other inhibitors have been developed or are in trials that inhibit multiple ErbB receptors or irreversibly inhibit the EGFR tyrosine kinase domain (Bianco et al., 2007; Wieduwilt and Moasser, 2008).

Despite the high incidence of EGFR activation observed in multiple cancers, EGFR inhibitors have not been overwhelmingly successful clinically. In non-small cell lung cancer (NSCLC), of the patients that respond to EGFR TKIs such as gefitinib or erlotinib, a large fraction express activating mutations of the EGFR tyrosine kinase domain (Lynch et al., 2004; Pao et al., 2004). These mutations are primarily point mutations (e.g., substitution of arginine for leucine at
residue 858, L858R) and small in-frame deletions (e.g., deletion of residues 746 to 750, del746-750), and alter the kinetics of ATP and drug binding, resulting in EGFR inhibition at lower drug concentrations (Carey et al., 2006; Yun et al., 2008). Unfortunately, the majority of tumors initially sensitive to EGFR inhibitors eventually develop resistance. Resistance mechanisms include upregulation of other receptors (Engelman et al., 2007; Sergina et al., 2007), secondary mutations in EGFR (e.g., substitution of methionine for threonine, T790M) (Kobayashi et al., 2005; Yun et al., 2008), and activation of downstream signaling pathways (Ercan et al., 2012; Sos et al., 2009; Yao et al., 2010).

Because tumors typically arise through multiple genetic alterations (Hanahan and Weinberg, 2011), it is likely that many tumors require interventions for more than a single target. There is promise in approaches that combine EGFR inhibitors with other inhibitors or traditional treatments such as radiation or chemotherapy or altering the scheduling of drugs (Dutta and Maity, 2007; Lee et al., 2012). Improving understanding of the complex network of EGFR regulation can help predict sensitivity to EGFR inhibitors as well as discover new drug targets that could be used alone or in combination with EGFR inhibitors in tumors that exhibit de novo or acquired EGFR inhibitor resistance. To this end, the work described here focuses on improving the understanding of EGFR trafficking and feedback regulation. These aspects of EGFR regulation are likely important for understanding drug response and are introduced in the following sections.

1-3. EGFR trafficking

Attenuation of EGFR-mediated signaling is achieved through dephosphorylation of EGFR cytoplasmic phosphotyrosines by protein tyrosine phosphatases including DEP1 and protein-tyrosine phosphatase 1B (PTP1B) (Berset et al., 2005; Haj et al., 2002), as well as endocytosis and subsequent degradation in lysosomes (Sorkin and Goh, 2009). The major features of EGFR trafficking are illustrated in Figure 1-1. Clathrin-mediated endocytosis is the best-studied pathway of EGFR endocytosis. This process involves the recruitment of EGFR to clathrin-coated pits and
subsequent pinching off of endosomes by the GTPase dynamin (Damke et al., 1994; Ferguson and De Camilli, 2012). The predominant view is that the E3 ubiquitin ligase CBL is recruited to tyrosine phosphorylated EGFR by the intracellular adaptor protein growth factor receptor-bound protein 2 (GRB2). CBL binding results in EGFR ubiquitylation and entrance into clathrin-coated pits (Schmidt and Dikic, 2005; Soubeyran et al., 2002). While clathrin-mediated endocytosis is generally accepted as the principal EGFR endocytosis pathway under normal conditions, several less well-understood pathways also exist (Sigismund et al., 2005; Sorkin and Goh, 2009). These pathways include caveolae-mediated endocytosis and basal membrane turnover (Kirkham and Parton, 2005). Because clathrin-mediated endocytosis is faster than these alternate mechanisms, if any components of clathrin-mediated endocytosis are limiting, the overall rate of endocytosis can be reduced (Lund et al., 1990; Sigismund et al., 2005). Once internalized, endosomes undergo acidification, altering the binding of ligands such as transforming growth factor alpha (TGFα), which dissociates from EGFR in endosomes, whereas EGF remains bound (Ebner and Derynck, 1991). Endosomal cargoes are recycled back to the plasma membrane or routed for degradation in lysosomes. This process may be regulated by EGFR ubiquitylation and is controlled by cytosolic protein complexes known as endosomal sorting complexes required for transport (ESCRT) (Sorkin and Goh, 2009).

Importantly, the processes of EGFR internalization and signaling are interconnected, and internalization can serve roles other than signal attenuation (Sorkin and von Zastrow, 2009). Localization of the receptor determines proximity to substrates such as phosphatidylinositol 4,5-bisphosphate (PIP₂) at the plasma membrane and adaptor proteins within the cell (Haugh, 2002). In some contexts, EGFR internalization is necessary for maximal signaling. For example, cells that cannot undergo clathrin-mediated endocytosis due to expression of a mutant form of dynamin undergo reduced EGF-stimulated ERK phosphorylation compared to wild-type dynamin expressing controls (Lazzara et al., 2010; Vieira et al., 1996).
Figure 1-1. EGFR trafficking.

Upon activation, EGFR is rapidly recruited to clathrin-coated pits and subsequently internalized to endosomes. The canonical view is that CBL binding initiates clathrin-mediated endocytosis. Non-clathrin dependent pathways can also internalize EGFR. Vesicle formation requires the GTPase dynamin. Once internalized, EGFR can continue to signal from endosomes and is routed for recycling or degradation in lysosomes.

EGFR trafficking has also been linked to response to EGFR inhibitors. When EGFR endocytosis was blocked by expression of a dynamin mutant, cells that were previously insensitive to EGFR inhibition died in response to the EGFR TKI gefitinib (Lazzara et al., 2010). In NSCLC, cells with EGFR-activating mutations exhibit impaired EGFR endocytosis (Hendriks et al., 2006; Lazzara et al., 2010). This defect in endocytosis is potentially linked to drug response, but the basis for impaired endocytosis and EGF-induced EGFR degradation is poorly understood.

In agreement with a relationship between EGFR internalization and sensitivity to EGFR inhibition, increased EGFR internalization was measured in NSCLC cells with acquired resistance to EGFR inhibition caused by ERK amplification (Ercan et al., 2012). Due to the complexity of EGFR trafficking processes, computational biology techniques that model EGFR trafficking have been very useful for understanding the system’s behavior and will be essential moving forward to improve the understanding of internalization regulation (Birtwistle and Kholodenko, 2009;
1-4. Feedback regulators of EGFR signaling

Similar to EGFR trafficking, feedback regulation of downstream signaling controls net EGFR signaling and therefore drug response. A traditional view of cell signaling frames signaling pathways as linear paths. In this view, ligand-binding initiates a signal at the cell surface, adaptors transport (and possibly amplify) this signal through the cell, and the ultimate result is changes in gene transcription in the nucleus or alternative non-transcriptional mechanisms that result in phenotypic changes. However, this interpretation disregards several essential aspects of signaling – mainly the importance of feedback loops, which are known to be an important feature of cell signaling systems. A feedback loop is generated when a downstream component affects an upstream component. For example, a protein might promote its own transcription, yielding a positive feedback loop. Feedback loops are essential for generating some of the basic behaviors observed in biology such as adaptation, oscillations, ultrasensitivity, and hysteresis (Kholodenko et al., 2010; Ma et al., 2009).

A drug designed to bind and inhibit a target upstream in a signaling pathway with high potency may fail due to unanticipated upregulation of feedback pathways that result in net increases in the signaling output the drug was designed to repress. Feedback in the ERK MAP kinase pathway is a useful example. Active RAF leads to ERK activation, which causes upregulation of a host of negative feedback mechanisms that limit the duration and strength of ERK signaling. ERK can directly inhibit upstream components through phosphorylation of proteins such as son-of-sevenless (SOS) and RAF, resulting in inhibition of their function (Dougherty et al., 2005; Douville and Downward, 1997). ERK can also initiate feedback regulation through inducing expression of proteins that negatively regulate the pathway such as dual-specificity phosphatases (DUSPs) that dephosphorylate ERK and other MAP kinases (Eblaghie et al., 2003). One example of the unforeseen consequence of feedback regulation is the case of melanomas with the BRAFV600E mutation. In these melanomas, high levels of negative
feedback driven by ERK results in suppressed RAS activity and insensitivity to growth factors (Lito et al., 2012). In these cells, RAF inhibition actually increases ERK activity. Another example was demonstrated in several cancer cell lines where MEK (the kinase that activates ERK) inhibition leads to activation of AKT, providing compensatory survival signaling and limiting the effectiveness of MEK inhibition (Turke et al., 2012). The work presented in this thesis focuses on two feedback regulators of EGFR signaling, mitogen-inducible gene 6 (MIG6; also known as RALT and gene 33) and Sprouty2 (SPRY2). The current understanding of MIG6 and SPRY2 function is described in the following sections.

1-5. MIG6

MIG6 is a 50 kDa scaffolding protein without catalytic activity that binds to all ErbB family members and inhibits their tyrosine kinase activity (Anastasi et al., 2003). This regulation involves a unique mechanism where MIG6 binds EGFR at the asymmetric dimer interface between EGFR kinase domains and prevents kinase activation (Zhang et al., 2007a). Because MIG6 expression is induced by ERK activity, MIG6 functions as a negative feedback regulator of EGFR signaling (Fiorini et al., 2002). In addition to regulating tyrosine kinase activation, MIG6 also promotes EGFR internalization by acting as a scaffold for AP-2, Intersectin1 (ITSN1), and Intersectin2 (ITSN2), adaptors of clathrin-mediated endocytosis, and human syntaxin 8 (STX8), a protein required for late endosome trafficking (Frosi et al., 2010; Ying et al., 2010). These two main activities of MIG6 are illustrated in Figure 1-2.

In addition to regulating EGFR, MIG6 contributes to several other signaling pathways through known interacting proteins, although these aspects of MIG6 function are not as well understood (Figure 1-3). MIG6 binds to the small GTPase Cdc42, reducing cell migration (Makkinje et al., 2000; Pante et al., 2005). MIG6 also binds to the tyrosine kinase c-ABL, promoting apoptosis in mammary epithelial cells (Hopkins et al., 2012). Little is known about the regulation of MIG6 activity or the role of interactions with several other proteins such as 14-3-3, SRC kinase, GRB2, and phospholipase C gamma (PLC-γ) (Fiorentino et al., 2000; Makkinje et
Chk1 was shown to phosphorylate MIG6 at serine 251 and negatively regulate the inhibitory activity of MIG6 on EGFR activation (Liu et al., 2012). It is unknown how phosphorylation of serine 251 or other sites controls other aspects of MIG6 function.

**Figure 1-2. MIG6 regulation of EGFR activity and internalization.**

MIG6 is thought to function with two mechanisms to control EGFR signaling: (1) by blocking the key interactions at the asymmetric EGFR dimer interface thereby preventing EGFR phosphorylation and subsequent downstream signaling, and (2) by promoting interactions with other proteins, such as AP-2, that lead to internalization of EGFR and potential degradation.

Previous work has suggested that MIG6 may play an important role in some types of cancer. In mice, loss of *Mig6* results in lung, gallbladder, bile duct, and skin cancer (Ferby et al., 2006; Zhang et al., 2007b). In human cancers, such as breast, brain, skin, pancreatic, and ovarian, MIG6 expression is frequently reduced (Anastasi et al., 2005; Ferby et al., 2006; Ying et al., 2010). In addition, mutations in *MIG6* have been found in lung cancer cell lines (Zhang et al., 2007b). These studies point to a tumor suppressor role for MIG6 in cancer. However, it remains unknown how MIG6 functions in the context of cancer cells with EGFR-activating mutations. Because MIG6 expression is induced by ERK activity, MIG6 expression may be elevated in the context of tumors with elevated ERK signaling (Fiorini et al., 2002). Indeed, MIG6 expression was found to be elevated in NSCLC cells with EGFR-activating mutations (Nagashima et al., 2009).
Figure 1-3. Domain map of MIG6 and SPRY2.

(A) MIG6 and (B) SPRY2 interacting partners are shown at the regions where they bind. Proteins with unknown interaction sites are shown not contacting the protein. CRIB = Cdc42/Rac interactive binding; ED = endocytic domain; EBR = ErbB binding region; SH3 = SRC homology 3 domain.

1-6. SPRY2

SPRY2 is one of four mammalian sprouty proteins and a regulator of several RTKs including EGFR (Edwin et al., 2009; Kim and Bar-Sagi, 2004; Mason et al., 2006). Sprouty proteins were identified in Drosophila melanogaster, where a single isoform, Spry, regulates RTK-mediated processes including branching morphogenesis (Edwin et al., 2009; Hacohen et al., 1998). SPRY2 has no catalytic activity, but regulates cellular signaling through several known protein interactions, although some of these are incompletely characterized (Figure 1-3). SRC kinase phosphorylates SPRY2 at tyrosine 55, forming a binding site for CBL and the phosphatase PP2A. Upon CBL binding, SPRY2 is poly-ubiquitylated, targeting it for destruction in the proteasome. PP2A binding, on the other hand, leads to dephosphorylation of serines 112 and 121, which is thought to allow ERK inhibition via GRB2 interactions (Edwin et al., 2009).
addition, SPRY2 can bind itself and other sprouty family members, although little is known about the role of sprouty oligomerization (Ozaki et al., 2005; Wu et al., 2005). SPRY2 is also known to interact with several other proteins such as the kinase TESK1 (Chandramouli et al., 2008) and the phosphatase SHP2 (Hanafusa et al., 2004). These proteins are involved in other mechanisms of SPRY2 signaling regulation that are not well understood.

SPRY2 expression is promoted by ERK activity, but at the same time, SPRY2 functions to either inhibit or potentiate ERK activation, creating a feedback loop. SPRY2-mediated ERK regulation appears to be highly cell and context-specific. SPRY2 may inhibit ERK at the level of RAS by preventing GRB2/SOS recruitment (Hanafusa et al., 2004), preventing RAS activation downstream of GRB2/SOS (Gross et al., 2001), or at the level of RAF (Yusoff et al., 2002), depending on the cellular context. Although SPRY2 is generally thought to inhibit ERK signaling, SPRY2 can also potentiate ERK signaling downstream of EGFR. This occurs in a proposed mechanism where SPRY2 binds the ubiquitin ligase CBL, interfering with CBL-mediated EGFR internalization (Haglund et al., 2005; Rubin et al., 2003; Wong et al., 2002). It was also shown that SPRY2 can interact with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a protein that promotes EGFR progression from early to late endosomes (Kim et al., 2007). In this manner, SPRY2 reduces EGFR trafficking to late endosomes. Due to the large number of studies that demonstrate a variety of functions of SPRY2 depending on the cell system and growth factor stimulation, no agreement on the SPRY2 mechanism of action has been established. Therefore, the function of SPRY2 in any given setting is extremely difficult to predict. The proposed mechanisms of SPRY2-mediated ERK regulation are shown in Figure 1-4.
SPRY2 is proposed to function either (1) as an inhibitor of ERK signaling downstream of several RTKs that initiate ERK signaling or (2) as a promoter of ERK signaling downstream of EGFR. SPRY2 expression is promoted by ERK and can interfere with the ERK pathway by binding GRB2 or RAF. Conversely, SPRY2 can bind CBL and prevent EGFR/CBL association, preventing EGFR degradation and also the attenuation of EGFR signaling.

The role of SPRY2 has been studied in several cancers (Edwin et al., 2009; Normanno et al., 2006). In some cancers, such as hepatocellular carcinoma, breast cancer, and prostate cancer, SPRY2 expression is decreased and SPRY2 appears to act as a tumor suppressor (Fong et al., 2006a; Lo et al., 2006; Sutterluty et al., 2007). For example, in mice, Spry2 overexpression in osteosarcoma cells suppressed tumor growth and metastasis (Miyoshi et al., 2004). However, there is evidence that in colon cancer, SPRY2 expression may promote tumorigenesis (Barbachano et al., 2010; Holgren et al., 2010; Ordonez-Moran et al., 2013). There are several questions remaining about the role of SPRY2 in cancer. Because of the various proposed regulatory functions of SPRY2, it is difficult to predict the effect of SPRY2 expression in different settings or in the context of drug treatment. It is also unknown how SPRY2 regulates signaling in
cells with oncogenic receptors or receptor overexpression that could be driving high SPRY2 expression and/or phosphorylation.

1-7. Overview of thesis work

Significant progress has been made in recent years towards understanding response to EGFR inhibitors in cancer. However, several questions remain unanswered. One such poorly understood aspect of drug response is how feedback regulators such as MIG6 and SPRY2 regulate EGFR-mediated signaling and ultimately response to EGFR inhibitors in cancer. The work described in the following chapters attempts to address this question in the settings of NSCLC and glioblastoma multiforme (GBM). EGFR mutations and EGFR overexpression are common in NSCLC and GBM, potentially leading to unforeseen perturbations in signaling networks due to feedback mechanisms. Due to the complex and interconnected nature of EGFR signaling and regulation, our approach combines experimental methods with computational methods to enable interpretation of experimental results.

In Chapters 2 and 3, we sought to determine the role MIG6 and SPRY2 play in regulating the impaired EGFR endocytosis, altered cellular signaling, and enhanced cellular sensitivity to EGFR tyrosine kinase inhibitors observed in NSCLC cells with EGFR mutations. We find that perturbations to MIG6 and SPRY2 expression control EGFR endocytosis and subsequent signaling in cell lines expressing wild-type EGFR as well as those expressing EGFR mutants. Data demonstrate that the already low EGFR endocytosis rate constant measured in cells expressing an EGFR mutant can be driven even lower by reducing MIG6 expression. Although MIG6 was only recently shown to regulate EGFR endocytosis, results indicate that MIG6 internalizes a similar amount of wild-type EGFR as CBL. As suggested by previous studies, our analysis indicates that CBL-mediated internalization of mutant EGFR is impaired, but that MIG6-mediated internalization of mutant EGFR is also diminished. This work indicates that MIG6 has a previously underappreciated role in EGFR internalization in NSCLC cells. This finding has important implications because internalized EGFR bound to MIG6 may not be competent for
driving downstream signaling. In addition, this work uncovers and quantifies the importance of differences in EGFR recycling between wild-type and mutant EGFR-expressing cells. We measured significantly increased recycling of mutant EGFR relative to wild-type EGFR and find that this difference explains observed reductions in EGF-stimulated EGFR degradation. Finally, we find that, due to the complex functions of MIG6 on both EGFR activity and trafficking, MIG6 depletion does not significantly affect EGFR downstream signaling or response to EGFR inhibition. Conversely, we find that SPRY2 expression promotes ERK phosphorylation and resistance to EGFR inhibition. Data and modeling analysis demonstrated that SPRY2 depletion reduces EGFR expression, which results in increased endocytosis rate because of alleviated saturation of rapid endocytic pathways. Further detailed EGFR reconstitution studies indicated that, unlike endocytosis rate, the effects of SPRY2 on ERK phosphorylation and response to EGFR inhibition are independent of EGFR expression.

In Chapter 4, the role of SPRY2 is examined in GBM, the most common form of brain cancer in adults. Similarly to NSCLC, EGFR is frequently overexpressed and mutated in GBM, but a structurally distinct EGFR mutation is common. Our findings uncover an important role for SPRY2 in GBM tumorigenesis and response to EGFR and c-MET co-inhibition. We determined the expression of SPRY2 as a function of the most common EGFR mutation in GBM patients, the deletion mutation, EGFR variant III (EGFRvIII), which is associated with an aggressive tumor phenotype. We find that SPRY2 expression is elevated in EGFRvIII-positive human tumors and orthotopic rat tumors compared to EGFRvIII-negative tumors. Furthermore, SPRY2 expression is associated with the classical GBM subtype and reduced patient survival. In GBM cell lines, we find that SPRY2 promotes proliferation, colony formation in soft agar, and cellular resistance to EGFR and c-MET co-inhibition. In mouse xenografts, SPRY2 depletion significantly reduced proliferation. Interestingly, SPRY2 appears to control these phenotypes through regulation of p38 and JNK MAP kinases or other signaling pathways, but not through ERK regulation. In addition, our results point to a novel mechanism of SPRY2-mediated regulation involving p38 and JNK MAP kinase regulation by MAP kinase phosphatases. Overall, our data suggest that SPRY2 or
the pathways it regulates could be promising therapeutic targets or prognostic biomarkers in GBM.
Chapter 2: Regulation of EGFR trafficking and cell signaling by Sprouty2 and MIG6 in lung cancer cells

2-1. Abstract

The duration and specificity of epidermal growth factor receptor (EGFR) activation and signaling are determinants of cellular decision processes and are tightly regulated by receptor dephosphorylation, internalization, and degradation. In addition, regulatory proteins that are upregulated or activated post-transcriptionally upon receptor activation may initiate feedback loops that play crucial roles in spatiotemporal regulation of signaling. We examined the roles of Sprouty2 (SPRY2) and mitogen-inducible gene 6 (MIG6), two feedback regulators of EGFR trafficking and signaling, in lung cancer cells with or without EGFR-activating mutations. These mutations are of interest because they confer unusual cellular sensitivity to EGFR inhibition through a mechanism involving an impairment of EGFR endocytosis. We found that the endocytosis of wild-type and mutant EGFR was promoted by SPRY2 knockdown and antagonized by MIG6 knockdown. SPRY2 knockdown also significantly reduced extracellular signal-regulated kinase (ERK) phosphorylation, EGFR expression, and EGFR recycling. In a cell line expressing mutant EGFR, this effect on ERK led to a marked increase in cell death response to EGFR inhibition. The effects of SPRY2 knockdown on EGFR endocytosis and recycling were primarily the result of the concomitant change in EGFR expression, but this was not true for the observed changes in ERK phosphorylation. Thus, our study demonstrates that SPRY2 and MIG6 are important regulators of wild-type and mutant EGFR trafficking and points to an EGFR expression-independent function of SPRY2 in the regulation of ERK activity that may impact cellular sensitivity to EGFR inhibitors, especially in the context of EGFR mutation.

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**2-2. Introduction**

Epidermal growth factor receptor (EGFR)-mediated signaling must be tightly controlled in order to ensure appropriate cellular outcomes. Such control is achieved through a variety of mechanisms, including feedback. Studies of biological networks have shown that feedback regulation is necessary to generate biologically observed signaling patterns such as adaptation, oscillations, and switch-like responses (Kholodenko et al., 2010; Ma et al., 2009). In addition, multiple positive and negative feedback loops can create systems that are tunable and insensitive to noise (Brandman et al., 2005). These and other observations have made it increasingly clear that a complete understanding of signaling initiated by EGFR will require a deeper understanding of the feedback regulators that control EGFR-mediated signaling (Avraham and Yarden, 2011). Two such feedback regulators of EGFR-mediated signaling are Sprouty2 (SPRY2) and mitogen-inducible gene 6 (MIG6).

SPRY2 belongs to a family of four mammalian Sprouty proteins and regulates signaling downstream of multiple growth factor receptors. SPRY2 expression is induced by extracellular signal-regulated kinase (ERK) activity (Ozaki et al., 2001). When phosphorylated at tyrosine 55 in response to growth factors such as EGF, SPRY2 binds and sequesters the E3 ubiquitin ligase CBL, impeding EGFR ubiquitylation and degradation (Egan et al., 2002; Haglund et al., 2005; Rubin et al., 2003; Wong et al., 2002). SPRY2 also inhibits EGFR passage from early to late endosomes in a proposed mechanism involving SPRY2 binding to hepatocyte growth factor-regulated tyrosine kinase substrate (Kim et al., 2007). Downstream of receptors, SPRY2 may antagonize ERK activity by inhibiting RAS through binding to GRB2 or by inhibiting RAF, depending on the cellular context (Lao et al., 2006; Yusoff et al., 2002).

MIG6 (also known as RALT) is transcriptionally regulated by ERK downstream of EGFR (Fiorini et al., 2002; Hackel et al., 2001) and inhibits EGFR activation by binding at the asymmetric interface between dimerized EGFR kinases (Zhang et al., 2006; Zhang et al., 2007a). MIG6 also promotes EGFR endocytosis by coupling the receptor to AP-2 and Intersectins (Frosi et al., 2010; Ying et al., 2010).
Although elevated expression and activating mutations of EGFR occur frequently in multiple cancers (Hirsch et al., 2003; Itakura et al., 1994; Mellinghoff et al., 2005; Sheng and Liu, 2011), the functional role of EGFR feedback regulation by proteins such as SPRY2 and MIG6 has not been thoroughly studied. In cases where there is an important role for EGFR feedback regulation in oncogenesis or tumor progression, it could potentially be leveraged to overcome de novo or acquired resistance to EGFR inhibitors (Kobayashi et al., 2005; Kosaka et al., 2006; Mellinghoff et al., 2005; Sheng and Liu, 2011).

We studied the roles of SPRY2 and MIG6 in non-small cell lung cancer (NSCLC) cells, where EGFR is frequently expressed at elevated levels. In a small fraction of NSCLCs, the expression of kinase-activated EGFR mutants confers unusual cellular sensitivity to EGFR inhibitors (Lynch et al., 2004; Mitsudomi and Yatabe, 2007; Paez et al., 2004) and leads to increased expression and phosphorylation of SPRY2 and MIG6 (Guo et al., 2008; Nagashima et al., 2009; Rubin et al., 2003). These EGFR mutations also lead to dramatic impairment of EGFR endocytosis, which has been linked to differential cellular sensitivity to EGFR inhibitors (Hendriks et al., 2006; Lazzara et al., 2010). We hypothesized that SPRY2 and MIG6 could participate in this perturbation to EGFR mutant endocytosis and in turn serve as important determinants of cellular response to EGFR inhibitors.

In two different NSCLC cell lines, one with an EGFR-activating mutation and demonstrating the previously documented impairment in receptor internalization, EGFR endocytosis was augmented by SPRY2 knockdown and reduced by MIG6 knockdown. EGFR recycling, which we quantitatively determined to be roughly two-fold more efficient in the cell line with EGFR mutation, was also reduced by SPRY2 knockdown in both cell lines. Thus, SPRY2 may play two roles that promote EGFR expression in NSCLC cells with or without EGFR mutation. Interestingly, the effects of SPRY2 knockdown on receptor endocytosis and recycling were explained by a concomitant decrease in EGFR expression, as revealed by EGFR reconstitution experiments. Downstream of the receptor, SPRY2 knockdown significantly reduced ERK phosphorylation. However, MIG6 knockdown had a relatively modest effect on ERK
Moreover, as a result of reduced ERK phosphorylation, SPRY2 knockdown promoted apoptotic response to the EGFR inhibitor gefitinib. This increase in apoptosis was especially pronounced in PC9 cells, which express a deletion mutant of EGFR. Despite the rescue effects of EGFR reconstitution on top of SPRY2 knockdown on EGFR trafficking, EGFR reconstitution did not rescue the effects of SPRY2 knockdown on ERK phosphorylation or cellular response to gefitinib. Thus, our study identifies SPRY2 and MIG6 as important regulators of EGFR endocytosis and recycling in EGFR mutant-expressing cells, as well as cells expressing wild-type EGFR. Our results also point to an EGFR expression-independent function of SPRY2 in the regulation of ERK that impacts cellular sensitivity to EGFR inhibition. These findings provide new insights into the coupling between EGFR trafficking, signaling, and feedback regulation and suggest that interference with SPRY2 expression or function could be a useful therapeutic approach in lung cancer cells with acquired resistance to EGFR-targeted therapy.

2-3. Materials and Methods

Cell lines and cell culture. H1666 cells (EGFR wild-type) were obtained from the American Type Tissue Collection (Manassas, VA, USA) and maintained in ACL4 (Lazzara et al., 2010). PC9 cells (EGFR delE746-A750) were a generous gift of Dr. Douglas Lauffenburger (MIT, Cambridge, MA, USA) and were maintained in RPMI supplemented with 10% fetal bovine serum (FBS), 1 mM L-Glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Grand Island, NY, USA). For EGFR or MEK inhibition experiments, gefitinib or U0126 (both from LC Laboratories, Woburn, MA, USA) were added to the cells in complete media. PC9 cells are extremely sensitive to gefitinib, with an IC$_{50}$ for cellular proliferation << 1 μM (Guo et al., 2008; Noro et al., 2006; Tracy et al., 2004). H1666 cells are moderately sensitive to gefitinib with an IC$_{50}$ of ~2 μM (Tracy et al., 2004). Consistent with previously reported trends (Guo et al., 2008; Nagashima et al., 2009), PC9 cells expressed more MIG6 than H1666 cells (Figure 2-S1A). SPRY2 levels were similar for both cell lines. These general trends held in a broader panel of NSCLC cell lines (Figure 2-S1A).
SPRY2 and MIG6 knockdown. The pSuper retroviral shRNA vector with neomycin resistance was purchased from Oligoengine (Seattle, WA, USA), and the pSicoR lentiviral shRNA vector with puromycin resistance was a generous gift from Dr. Tyler Jacks (MIT Koch Institute for Integrative Cancer Research, Cambridge, MA, USA; (Ventura et al., 2004)). An oligonucleotide encoding a hairpin targeting nucleotides 1649-1667 of human MIG6 was cloned into the pSuper plasmid. Oligonucleotides encoding hairpins targeting nucleotides 2061-2079 (main sequence used) or 1195-1213 of human SPRY2 were cloned into pSicoR. Controls were created for each vector using hairpins that do not target a known human mRNA, and control cells for simultaneous knockdown of SPRY2 and MIG6 expressed both control shRNAs. All oligonucleotides were purchased from IDT (Coralville, IA, USA). Retrovirus was produced by calcium-phosphate-mediated transfection of amphotropic Phoenix cells (Dr. Gary Nolan, Stanford University, Stanford, CA, USA) with pSuper plasmids. Lentivirus was produced by transfection of 293FT cells (Life Technologies) with pSicoR, pCMV-VSVg, pMDL-gp-RRE, and pRSV-Rev plasmids (Dr. Marilyn Farquhar, UCSD, La Jolla, CA, USA) using calcium phosphate. Virus-containing supernatant was passed through 0.45 μm syringe filters prior to addition to target cells. Cells were selected in 1-2 μg/mL puromycin (Sigma, St. Louis, MO, USA) and/or 100-500 μg/mL geneticin (Life Technologies). Efficient stable knockdown of SPRY2 and MIG6 in PC9 and H1666 cells was confirmed by western blot (Figure 2-S1B).

EGFR expression. The pBabe.hygro wild-type human EGFR retroviral expression plasmid was constructed by sub-cloning from a pCDNA4/TO/Myc-HisB vector with a wild-type human EGFR insert (gift from Dr. Yi-Rong Chen, National Health Research Institutes, Taiwan). Retrovirus was prepared and cells were infected as described above. Cells were selected in 100 μg/mL hygromycin B (Sigma). To express the human EGFR delE746-A750 mutant, a lentiviral expression plasmid (pLenti6/V5-DEST) with the appropriate insert was used (gift of Dr. Daniel Haber, Harvard Medical School, Boston, MA, USA). Lentivirus was prepared and cells were infected as described above. Target cells were selected in 2 μg/mL blasticidin (Life Technologies).
Western blotting. Whole cell lysates were prepared in a standard cell extraction buffer (Life Technologies) supplemented with protease and phosphatase inhibitors (Sigma). Lysates were cleared by centrifugation at 13,200 rpm for 10 min, and total protein concentrations were determined by micro-bicinchoninic assay (Thermo Scientific, Rockford, IL, USA). Approximately 20 μg of total protein was loaded per lane on 4-12% gradient polyacrylamide gels (Life Technologies) under denaturing and reducing conditions and transferred to 0.2 μm nitrocellulose membranes (Life Technologies). After probing with antibodies, membranes were imaged on a LI-COR Odyssey scanner (LI-COR, Lincoln, NE, USA). Membranes were stripped with 0.2 M NaOH as needed.

EGFR immunoprecipitation. Whole cell lysates were prepared using a lysis buffer optimized for immunoprecipitation (Cell Signaling Technology, Danvers, MA, USA) supplemented with protease and phosphatase inhibitors. 600 μg of total protein was incubated overnight with protein G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) that were pre-conjugated to 400 ng of EGFR antibody. Immunoprecipitates were analyzed by western blot, as described above.

Antibodies. Antibodies against EGFR (#2232), AKT (#9272), p-AKT S473 (#9271), ERK (#4695), ubiquitin (#3933), and p-ERK T202/Y204 (#4377) were purchased from Cell Signaling Technology. The CBL antibody was from Epitomics (#1486; Burlingame, CA, USA). The EGFR immunoprecipitation antibody was from Thermo Scientific (Ab-12). The SPRY2 antibody was purchased from Sigma (#S1444). The MIG6 antibody was purchased from Santa Cruz Biotechnology (#sc-137155), and the actin antibody was purchased from Millipore (#MAB1501; Billerica, MA, USA). Infrared dye-conjugated secondary antibodies were purchased from Rockland Immunochemicals (Gilbertsville, PA, USA). All antibodies were used according to manufacturer recommendations.

Flow cytometry. Floating and adherent cells were pooled and stained with FITC-conjugated Annexin V (Southern Biotech, Birmingham, AL, USA). Cells were analyzed within 1 hr of staining using a Becton Dickinson FACS-Calibur cytometer, and data were analyzed using
EGFR endocytosis rate constant and recycling fraction measurements. Rate constants of EGF-mediated EGFR endocytosis ($k_e$) were measured using $^{125}$I-EGF and corrected for the effects of non-specific binding and surface spillover, as described previously (Lund et al., 1990; Wiley and Cunningham, 1982). For experiments using the dynamin inhibitor Dynasore (Millipore), cells were pretreated with 80 μM Dynasore or DMSO control for 2 hrs, and Dynasore was added to the $^{125}$I-EGF-containing media used to make $k_e$ measurements. Steady-state EGFR recycling fractions ($f_r$), defined as the fraction of intact internalized ligand that is returned to and released from the plasma membrane, were measured as described previously (French et al., 1995), with intact and degraded $^{125}$I-EGF separated with 5 kDa molecular weight cutoff centrifugal filters (Millipore).

qRT-PCR. Relative amounts of EGFR mRNA were determined using the comparative C_T method. RNA samples were prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) with on-column DNase I digestion. Equal amounts of RNA from each sample were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). qPCR was performed using SYBR Green PCR Master Mix (Life Technologies) on an Applied Biosystems 7300 Real-Time PCR System.

2-4. Results

EGF-mediated EGFR endocytosis is promoted by SPRY2 knockdown and impaired by MIG6 knockdown. We reduced SPRY2 and/or MIG6 expression in PC9 (delE746-A750 EGFR) and H1666 (wild-type EGFR) NSCLC cells through stable shRNA expression. As expected based on previous studies of NSCLC cells (Hendriks et al., 2006; Lazzara et al., 2010), the rate constant for EGF-mediated EGFR endocytosis ($k_e$) was larger in H1666 cells transduced with either of the control shRNA vectors than in corresponding PC9 cells (Figure 2-1). SPRY2 knockdown significantly increased $k_e$ in both cell lines (from 0.05 to 0.08 min$^{-1}$ in PC9 cells, and from 0.16 to 0.21 min$^{-1}$ in H1666 cells). MIG6 knockdown reduced $k_e$ in PC9 and H1666 cells (from 0.06 to
0.03 min\(^{-1}\) in PC9 cells, and from 0.18 to 0.12 min\(^{-1}\) in H1666 cells). When SPRY2 and MIG6 were simultaneously depleted, the net result was a decrease in \(k_e\) in both cell lines (from 0.07 to 0.045 min\(^{-1}\) in PC9 cells, and from 0.17 to 0.15 min\(^{-1}\) in H1666 cells). The effect of combined knockdown suggests a hierarchy between SPRY2 and MIG6 in controlling \(k_e\) for both wild-type and mutant EGFR. Data used to generate the \(k_e\) values are shown in Figure 2-S2.

**Figure 2-1. SPRY2 and/or MIG6 knockdown perturb EGF-mediated EGFR endocytosis.**

EGFR endocytosis rate constants (\(k_e\)) were measured using 10 ng/mL \(^{125}\)I-EGF for PC9 and H1666 cells with knockdown of SPRY2, MIG6, or SPRY2 and MIG6, as described in Materials and Methods. Data were corrected for the effects of non-specific binding and spillover and represent an average of three experiments ± s.e.m. Asterisks indicate \(p < 0.05\) for comparisons against controls.

At EGF concentrations above or near 10 ng/mL (the condition used in Figure 2-1), EGFR endocytosis may occur through clathrin dependent and independent processes (Sigismund et al., 2008; Sigismund et al., 2005). To determine whether the differences measured in Figure 2-1 might reflect effects other than those attributable to clathrin-mediated endocytosis, we repeated \(k_e\) measurements in H1666 cells at 1.5 ng/mL EGF. The differences observed at 10 ng/mL were
preserved at 1.5 ng/mL (Figure 2-S3A). We also found that inhibition of dynamin, a GTPase required for clathrin-mediated endocytosis of many receptor tyrosine kinases (Kirchhausen et al., 2008), reduced $k_e$ in PC9 and H1666 cells, indicating that clathrin mediated endocytosis was relevant in both cell lines (Figure 2-S3B). Both CBL- and MIG6-mediated endocytosis pathways are clathrin-mediated (Frosi et al., 2010; Swaminathan and Tsygankov, 2006).

**SPRY2 and MIG6 knockdown alter ERK phosphorylation in qualitatively different ways, and SPRY2 knockdown reduces EGFR expression.** Because normal EGFR trafficking is important for complete ERK activation in at least some cellular contexts (Vieira et al., 1996), and because ERK activity is a key determinant of NSCLC cell response to EGFR inhibition (Furccht et al., 2012; Lazzara et al., 2010), we tested whether the changes in endocytosis we measured due to SPRY2 and MIG6 knockdown correlated with any changes in ERK phosphorylation. In PC9 and H1666 cells, SPRY2 knockdown resulted in an approximately two-fold reduction in ERK phosphorylation with or without gefitinib present (Figure 2-2A and Figure 2-S4A, B). ERK phosphorylation was increased by MIG6 knockdown, but the magnitude of this effect was more modest. With combined SPRY2 and MIG6 knockdown, there was a small (but statistically insignificant) reduction in ERK phosphorylation in PC9 cells and no change in ERK phosphorylation in H1666 cells. Qualitatively similar, but generally smaller, changes in AKT phosphorylation were observed with SPRY2 and MIG6 knockdown (Figure 2-S4C).

In validating the stable knockdown of SPRY2 and MIG6, we also noticed a reduction in EGFR expression concomitant with SPRY2 knockdown. Western blot analysis revealed that, in PC9 and H1666 cells, SPRY2 knockdown resulted in decreases in EGFR protein levels of 54% and 40%, respectively (Figure 2-2B). Differences in absolute counts of $^{125}$I-EGF binding in PC9 and H1666 cell lines from $k_e$ measurements also confirmed these decreases in EGFR expression. This effect repeated with an independent SPRY2 shRNA (Figure 2-S5A) and generally held when we probed the effects of SPRY2 knockdown in a larger panel of NSCLC cells (Figure 2-S5B). As will be shown later, we also verified these changes by flow cytometry. Because SPRY2 knockdown led to the most dramatic changes in the activity of the ERK pathway and also
appeared to alter EGFR expression, effects that may alter cellular response to EGFR inhibition, we focused most of the remainder of our studies on the effects of SPRY2 knockdown.

Figure 2-2. SPRY2 and/or MIG6 knockdown perturb ERK phosphorylation, and SPRY2 knockdown reduced EGFR expression.

(A) PC9 and H1666 cells expressing shRNAs targeting SPRY2, MIG6, or SPRY2 and MIG6 (KD, knockdown) or non-targeting control shRNAs were treated with the indicated concentrations of gefitinib for 24 (PC9) or 48 hrs (H1666), and western blots of whole cell lysates were probed with antibodies against phosphorylated or total ERK. Normalized densitometry data represent an average of three independent experiments ± s.e.m. Asterisks indicate $p < 0.05$ for comparisons against controls. (B) PC9 and H1666 cells expressing a SPRY2-targeting shRNA or a control shRNA (indicated by “-”) were cultured in complete media, and western blots of whole cell lysates were probed with antibodies against indicated proteins. Normalized densitometry data represent an average of three independent experiments ± s.e.m. Asterisks indicate $p < 0.05$ for comparison against control.
EGFR ubiquitylation and CBL association are enhanced by SPRY2 knockdown in cells expressing wild-type, but not mutant, EGFR. To further probe the mechanism of SPRY2-mediated regulation of EGFR internalization and expression, we measured EGFR ubiquitylation and association with CBL. A previous report found that EGFR mutants are poorly ubiquitylated and do not associate with CBL in response to EGF (Padron et al., 2007), but the effects of SPRY2 on EGFR ubiquitylation and CBL association has not previously been explored in NSCLC cells with EGFR mutation. We did not detect significant EGFR ubiquitylation or CBL association in EGFR immunoprecipitates of PC9 cells treated with EGF (Figure 2-3A). In contrast, and consistent with previously hypothesized mechanisms of wild-type EGFR regulation by SPRY2 (Egan et al., 2002; Haglund et al., 2005; Rubin et al., 2003; Wong et al., 2002), EGFR was ubiquitylated and CBL-associated in response to 10 ng/mL EGF in H1666 cells, and these effects were augmented by SPRY2 knockdown (Figure 2-3B). Even with EGF treatments for longer times and at higher concentrations than used in Figure 2-3, these differences between PC9 and H1666 cells persisted (Figure 2-S6A-D). The absence of non-specific immunoprecipitation of EGFR, ubiquitin, and CBL was confirmed in a separate experiment (Figure 2-S6E).

**Figure 2-3.** EGF-mediated EGFR ubiquitylation and CBL association occur in H1666 cells in a SPRY2-dependent manner, but ubiquitylation and CBL association do not occur in PC9 cells.

PC9 and H1666 cells expressing SPRY2-targeting shRNA or a non-targeting control shRNA were serum starved overnight and treated with or without 10 ng/mL EGF for 2 min. EGFR was immunoprecipitated (IP) from whole cells lysates of (A) PC9 cells and (B) H1666 cells, and
immunoprecipitates were analyzed by western blot (WB) using antibodies against the indicated proteins. Densitometry data for H1666 cells represent averages from three independent experiments ± s.e.m. Asterisks indicate $p < 0.05$ for comparison against control.

**SPRY2 regulates EGFR transcription and ERK regulates EGFR expression in cells expressing an EGFR mutant.** Because no differences in EGFR ubiquitylation or CBL association were detected in PC9 cells with SPRY2 knockdown (where EGFR expression was substantially reduced), we probed the effect of SPRY2 knockdown on EGFR mRNA levels. In PC9 cells, SPRY2 knockdown reduced EGFR mRNA levels by ~75% (Figure 2-4A). A reduction in EGFR mRNA was also measured for a second non-overlapping SPRY2 shRNA (Figure 2-S7). In contrast, no change in EGFR mRNA level was detected in H1666 cells with SPRY2 knockdown (Figure 2-4A and Figure 2-S7).

Because EGFR expression can be regulated by ERK activity (Grassian et al., 2011), and since a large effect on ERK phosphorylation was found in cells with SPRY2 knockdown, we tested the effect of MEK inhibition on EGFR expression. As determined by western blot analysis, MEK inhibition decreased EGFR expression in PC9 cells, but had no effect in H1666 cells (Figure 2-4B). Thus, changes in EGFR expression with SPRY2 knockdown are likely to occur via transcriptional effects in PC9 cells (due to decreased ERK phosphorylation) and through changes in trafficking (increased endocytosis and degradation) in H1666 cells.
Figure 2-4. SPRY2 knockdown decreases EGFR mRNA and protein levels in an ERK-dependent manner in PC9 cells, but not in H1666 cells.

(A) EGFR mRNA levels were determined by qRT-PCR in PC9 and H1666 cells expressing control or SPRY2 shRNA. Data represent an average of three independent experiments performed in triplicate wells ± s.e.m. Asterisks indicate $p < 0.05$ for comparison against control. (B) Parental PC9 and H1666 cells were treated for 24 hrs with the indicated concentrations of U0126, and whole cell lysates were analyzed by western blot using antibodies against indicated proteins. Images are representative of multiple independent experiments. Changes in EGFR/actin levels were quantified by densitometry and reported as values normalized to the untreated condition. Data reflect an average of three independent experiments ± s.e.m. Asterisks indicate $p < 0.05$ for comparison against the 0 μM U0126 condition.

EGFR reconstitution in SPRY2 knockdown cells rescues changes in EGFR $k_e$, but not changes in ERK phosphorylation. Because we found that SPRY2 knockdown increased $k_e$ in PC9 cells without increasing EGFR ubiquitylation or CBL association, we examined whether SPRY2
knockdown could affect $k_e$ through changes in EGFR expression. This hypothesis was motivated by knowledge that clathrin-mediated endocytosis is a saturable process wherein relatively large numbers of receptors may decrease $k_e$ as clathrin-mediated machinery becomes limiting and receptors are forced to internalize through slower, non-clathrin-mediated pathways (Lund et al., 1990; Sigismund et al., 2005). In PC9 and H1666 cells, reconstitution of mutant and wild-type EGFR, respectively, rescued the effect on $k_e$ observed with SPRY2 knockdown (Figure 2-5A, B). In the H1666 EGFR-reconstituted cells, $k_e$ was slightly lower than in the appropriate control cells (transduced with SPRY2 shRNA and empty expression vector). This difference may have occurred because EGFR reconstitution increased EGFR levels beyond those seen in the control cells. Data used to calculate these $k_e$ values are shown in Figure 2-S2. Despite the ability of EGFR reconstitution to rescue the effects of SPRY2 knockdown on EGFR $k_e$, EGFR reconstitution did not augment ERK phosphorylation basally, in the presence of gefitinib, or with EGF stimulation compared to SPRY2 knockdown without EGFR reconstitution (Figure 2-5C, Figure 2-S8). We verified that effects due to EGFR reconstitution were not due to small sub-populations of cells or mis-localized EGFR expression by measuring EGFR surface expression with flow cytometry (Figure 2-S9).
Figure 2-5. EGFR reconstitution reverses the effect of SPRY2 knockdown on EGFR endocytosis, but not ERK phosphorylation.

PC9 and H1666 cells expressing SPRY2-targeting shRNA or non-targeting control shRNA were transduced with a retroviral vector encoding EGFR or an empty expression vector (EV). (A) Lysates were prepared from cells maintained in complete media and analyzed by western blot using antibodies against the indicated proteins. Images are representative of multiple independent experiments. (B) Endocytosis rate constants ($k_e$) were measured with 10 ng/mL $^{125}$I-EGF, as described in Materials and Methods and represent an average of three experiments ± s.e.m. Asterisks indicate $p < 0.05$ for comparisons indicated by horizontal bars. (C) Cells were treated with indicated concentrations of gefitinib for 24 (PC9) or 48 (H1666) hrs, and western blots of whole cell lysates were probed with antibodies against phosphorylated or total ERK. Densitometry data represent an average of three independent experiments ± s.e.m. Asterisks indicate $p < 0.05$ for comparisons with cells transduced with control shRNA and EV.
SPRY2 controls EGFR sorting in an EGFR expression-dependent manner. We further hypothesized that the reduction in ERK phosphorylation observed with SPRY2 depletion could be the result of changes in recycling of endocytosed EGFR that, unlike the effects on $k_e$, might not be rescued by EGFR reconstitution. The general notion of a connection between receptor endocytic recycling and ERK activation has been previously discussed (Parachoniak et al., 2011; Robertson et al., 2006). In support of this hypothesis, treatment of PC9 or H1666 control cells with the trafficking inhibitor monensin reduced ERK phosphorylation to a similar degree as SPRY2 knockdown in cells with or without EGFR reconstitution (Figure 2-6A). We verified an effect of monensin on trafficking by showing that PC9 and H1666 cells pre-treated with 10 μM monensin released ~60% and 80% less internalized $^{125}$I-EGF compared to untreated controls (data not shown). We also directly measured the recycling fraction of internalized EGF ($f_r$) in PC9 and H1666 cells with SPRY2 knockdown and EGFR reconstitution (Figure 2-6B). It has been suggested previously based on receptor localization that mutant EGFR is preferentially recycled (Chung et al., 2009). However, enhanced recycling of mutant EGFR has never been quantified. The $f_r$ for mutant EGFR in PC9 cells was significantly higher than for wild-type EGFR in H1666 cells (over 0.9 for control PC9 cells and 0.5 for H1666 cells). Interestingly, $f_r$ was reduced by SPRY2 knockdown in both cell lines, but this change was at least partially rescued by EGFR reconstitution. Thus, it seems unlikely that the change in EGFR recycling is responsible for the observed reductions in ERK phosphorylation with SPRY2 knockdown.
Figure 2-6. Monensin inhibits ERK phosphorylation, and SPRY2/EGFR levels control EGFR recycling.

PC9 and H1666 cells expressing SPRY2-targeting shRNA or non-targeting control shRNA were transduced with a retroviral vector encoding EGFR or an empty expression vector (EV). (A) Western blots of whole cell lysates from PC9 and H1666 cells treated with or without 10 μM monensin in complete media for 4 hrs were probed with antibodies against indicated proteins. Images are representative of three independent experiments. Densitometry data reflect an average of three independent experiments ± s.e.m. Asterisks indicate $p < 0.05$ for comparisons with untreated cells transduced with the control shRNA and EV. (B) EGFR recycling fraction ($f_r$) was measured using 10 ng/mL $^{125}$I-EGF, as described in Materials and Methods. Data reflect an average of three independent experiments ± s.e.m, with each experiment performed in triplicate ($n = 3$). Asterisks indicate $p < 0.05$ for comparisons indicated by horizontal bars.
*SPRY2* knockdown increases cellular sensitivity to gefitinib, especially in a cell line expressing an EGFR mutant, in an ERK activity-dependent manner. To test whether the changes in EGFR trafficking, EGFR expression, and ERK phosphorylation due to SPRY2 and MIG6 knockdown altered cellular response to EGFR inhibitors, we first treated PC9 and H1666 cells with gefitinib at appropriate doses to induce cell death. For PC9 cells treated with 0.1 μM gefitinib for 24 hrs, Annexin V staining increased from low basal levels for shRNA controls to > 25% with SPRY2 knockdown (Figure 2-7A). In contrast, Annexin V staining did not change significantly in response to gefitinib in PC9 cells as a result of MIG6 knockdown. There were significant increases in Annexin V staining in PC9 cells, however, when SPRY2 and MIG6 were simultaneously depleted. Qualitatively similar changes were observed in H1666 cells, but they were small by comparison to those observed in PC9 cells. This difference is likely a result of wild-type EGFR expression in H1666 cells, which generally confers a much greater degree of cellular resistance to interference with survival signaling. Increased cell death in response to gefitinib was also observed in H1975 and H358 cell lines with SPRY2 knockdown compared to controls (Figure 2-S10).

To determine whether the reduction in ERK phosphorylation observed with SPRY2 knockdown could cause increased cell death response to gefitinib, we used U0126 to reduce ERK phosphorylation to a similar degree in control PC9 and H1666 cells as observed with SPRY2 knockdown. Appropriate U0126 concentrations were found by U0126 titration in the presence of gefitinib to recapitulate the conditions in Figure 2-3 (Figure 2-S11). PC9 and H1666 cells expressing control shRNA treated with the concentrations of U0126 we identified showed the anticipated augmentation in cell death response to gefitinib (Figure 2-7B). Consistent with the fact that EGFR reconstitution did not rescue the decrease in ERK phosphorylation due to SPRY2 knockdown (Figure 2-5C), there was no effect of EGFR reconstitution on cellular response to gefitinib in H1666 or PC9 cells (Figure 2-7C).
Figure 2-7. SPRY2 knockdown alters cellular sensitivity to gefitinib in an ERK-dependent and EGFR level-independent manner.

(A) For PC9 and H1666 cells with knockdown of SPRY2, MIG6, or SPRY2 and MIG6, Annexin V staining was measured by flow cytometry for cells treated with gefitinib (PC9: 0.1 μM for 24 hrs; H1666: 0.1 μM for 48 hrs) or DMSO control. Data represent an average of three replicates ± s.e.m. Asterisks indicate $p < 0.05$ for comparisons indicated by horizontal bars. (B) PC9 and H1666 cells with or without SPRY2 knockdown were analyzed for Annexin V staining by flow cytometry after exposure to U0126 and/or gefitinib (PC9: 2 μM U0126 and 0.1 μM gefitinib for 24 hrs; H1666: 0.5 μM U0126 and 0.1 μM gefitinib for 48 hrs) or DMSO control. Data represent an average of three replicates ± s.e.m. Asterisks indicate $p < 0.05$ for comparisons indicated by horizontal bars. (C) PC9 and H1666 cells expressing SPRY2-targeting shRNA or non-targeting control shRNA were transduced with a retroviral vector encoding EGFR or an empty expression vector (EV). Cells were analyzed for Annexin V staining by flow cytometry after exposure to gefitinib (PC9: 0.1 μM for 24 hrs; H1666: 1 μM for 72 hrs) or DMSO control. Data represent an average of three replicates ± s.e.m. Asterisks indicate $p < 0.05$ for comparisons with gefitinib-treated cells transduced with control shRNA and EV.
2-5. Discussion

To our knowledge, the data presented here constitute the first study of the functional roles of SPRY2 and MIG6 in EGFR endocytosis and recycling, EGFR-mediated signaling, and cellular response to EGFR kinase inhibitors in cells expressing the constitutively active EGFR mutants that arise in NSCLC. Our data demonstrate that feedback regulation can indeed play important roles in determining receptor trafficking and signaling in cells characterized by EGFR overexpression and activating mutations. Since the effects we measured were generally largest in a cell line with *EGFR* mutation, our results suggest that the role of feedback regulation through SPRY2 and MIG6 may be especially important in the context of receptor mutation. The main trends we found are illustrated schematically in Figure 2-8 and discussed in further detail below.

![Diagram of key regulatory mechanisms among EGFR, ERK, SPRY2, and MIG6.](image)

**Figure 2-8.** SPRY2 and MIG6 regulate EGFR signaling with a complex network of feedback interactions.

(A) Diagram of key regulatory mechanisms among EGFR, ERK, SPRY2, and MIG6. SPRY2 and MIG6 expression are promoted by ERK activity. ERK activity is driven by EGFR and promoted by SPRY2 independent of EGFR expression. SPRY2 can promote EGFR stability (and reduce EGFR endocytosis) by reducing EGFR/CBL association (as in H1666 cells) and through regulation of ERK (as in PC9 cells). MIG6 inhibits EGFR activity and promotes EGFR internalization. Ultimately, ERK activity promotes survival in gefitinib. (B) When SPRY2 is depleted, EGFR expression is reduced, resulting in faster EGFR endocytosis and reduced EGFR recycling. SPRY2 depletion also results in decreased ERK phosphorylation, which can reduce EGFR expression via transcriptional regulation (as in PC9 cells). Decreased ERK activity potentiates cellular death response to EGFR inhibition by gefitinib. (C) When MIG6 is depleted, EGFR endocytosis rate is reduced, and a modest increase in ERK phosphorylation is observed. Cellular response to EGFR inhibition is not altered.
We first hypothesized that SPRY2 and MIG6 might regulate cell signaling and cell fate determination through their effects on EGFR trafficking. Several studies have reported impaired ligand-mediated EGFR endocytosis in NSCLC cells expressing EGFR mutants versus those expressing wild-type EGFR (Hendriks et al., 2006; Lazzara et al., 2010). These studies point to EGFR mutation itself as playing a direct role in defective endocytosis in a receptor expression-dependent manner, but the molecular basis for this defect is not fully understood. Consistent with the reported roles of MIG6 and SPRY2 in the endocytosis of wild-type EGFR in other cell types, we found that the endocytosis of mutant EGFR is promoted by MIG6 and antagonized by SPRY2. Since MIG6 expression tends to be elevated in NSCLC cells with EGFR mutation (Guo et al., 2008; Nagashima et al., 2009), the finding that EGFR $k_e$ is greatly reduced in mutant-expressing cells relative to wild-type could indicate that MIG6-mediated endocytosis does not occur as efficiently for EGFR mutants as for wild-type EGFR. We do not have direct evidence for this hypothesis, but it should be noted that even with SPRY2 knockdown the measured $k_e$ value in PC9 cells was still lower than values typically measured in cell lines expressing wild-type EGFR. It is also worth noting that MIG6 is functionally impaired in other cancers through mechanisms including downregulation, deletion, or loss-of-function mutation (Anastasi et al., 2005; Li et al., 2012; Ying et al., 2010; Zhang et al., 2007b). Thus, disruption of MIG6 function may be a general feedback perturbation across several cancer types.

Focusing on SPRY2, we also found that SPRY2 exerts control over EGFR endocytosis rates in PC9 and H1666 cells by influencing EGFR expression. Specifically, the increased $k_e$ values we measured in PC9 and H1666 cells with SPRY2 knockdown were accompanied by reduced EGFR expression, and rescuing EGFR expression on top of SPRY2 knockdown returned $k_e$ values to their levels prior to SPRY2 knockdown. These data indicate that some component of the EGFR endocytosis rate process is at or near saturation in the context of the elevated EGFR expression characteristic of PC9 and H1666 cells. These results suggest that future studies of SPRY2-mediated EGFR regulation should control for EGFR levels to determine
whether the effects of SPRY2 are an indirect consequence of changes in EGFR expression.

We also explored the basis of reduced EGFR expression with SPRY2 knockdown. In H1666 cells, SPRY2 knockdown promoted EGFR-CBL association and EGFR ubiquitylation, effects that are consistent with reduced EGFR expression. In PC9 cells, where SPRY2 knockdown also reduced EGFR expression, we did not detect any EGFR-CBL association or EGFR ubiquitylation with or without SPRY2 knockdown. We did, however, find that impaired ERK activity reduced EGFR expression in PC9 cells, but not in H1666 cells. Consistent with this finding, PC9 cells with SPRY2 knockdown also displayed significant reductions in EGFR mRNA levels. Thus, reduced EGFR expression with SPRY2 knockdown resulted from the effect on ERK phosphorylation in PC9 cells uniquely. The fact that EGFR reconstitution did not restore ERK phosphorylation levels in PC9 cells identifies the ERK/EGFR connection as a one-way coupling and suggests that SPRY2 controls ERK phosphorylation in an EGFR expression-independent manner.

In addition to the finding of receptor expression level-dependent endocytosis, we also found that the recycling of endocytosed EGFR was SPRY2 and EGFR expression level-dependent. In both cell lines, SPRY2 knockdown reduced $f_r$, and this change was at least partially reversed by EGFR reconstitution in cells with SPRY2 knockdown. These constitute the first results to examine the relationship between SPRY2 and EGFR expression on EGFR recycling in NSCLC. Although it was previously reported that mutant EGFR co-localized with transferrin (Chung et al., 2009), suggesting that mutant EGFR is preferentially recycled, our measurements are the first quantitative comparison of recycling between wild-type and mutant EGFR. We found that mutant EGFR was almost entirely sorted for recycling ($f_r > 0.9$), whereas wild-type EGFR was split between degradation and recycling ($f_r \sim 0.5$). Overall, these recycling results demonstrate an additional mode of regulation utilized by SPRY2 in setting EGFR expression levels.

Part of our initial hypothesis was that SPRY2 and MIG6 could play a role in the previously documented impairment of ERK activation in the context of mutant EGFR expression (Lazzara et
This hypothesis was based in part upon the previously reported requirement of normal EGFR endocytosis for complete activation of ERK downstream of EGFR (Lazzara et al., 2010; Vieira et al., 1996). The trends we identified in this study did not align in a straightforward way with this previously established relationship between $k_e$ and ERK activity, due to the complex, coupled, and multi-faceted processes governed by SPRY2 and MIG6. For example, MIG6 knockdown decreased $k_e$ but promoted ERK activity. This net effect of MIG6 knockdown may have been observed because, in addition to participating in EGFR endocytosis, MIG6 plays an important role as an inhibitor of the EGFR kinase. Although SPRY2 knockdown increased $k_e$ in PC9 and H1666 cells, ERK phosphorylation was reduced rather than enhanced. As already mentioned, this effect on ERK was not the result of reduced EGFR expression, but instead could have resulted from perturbations to signaling downstream of other RTKs such as c-MET, FGFR, and PDGFR, that are also regulated by CBL (Petrelli et al., 2002; Swaminathan and Tsygankov, 2006). Our EGFR recycling results could also be partially explanatory of the reduction in ERK phosphorylation observed in PC9 cells since $f_r$ did not fully return to its control value with EGFR reconstitution.

We briefly investigated the effects of SPRY2 and MIG6 knockdown on AKT phosphorylation as well, and found qualitatively similar but smaller effects as with ERK. Interestingly, previous studies in HeLa cells showed that SPRY2 expression promoted PTEN expression and reduced AKT phosphorylation (Edwin et al., 2006). However, our data indicate that SPRY2 promotes AKT phosphorylation in NSCLC cells, which would be inconsistent with decreased PTEN expression. Indeed, we found no change in PTEN expression with SPRY2 knockdown in any of the cell lines we studied (Figure 2-S12).

Our findings regarding SPRY2 expression and NSCLC cellular sensitivity to gefitinib are distinct from the findings of Feng et al. (Feng et al., 2010) in colon cancer cells where SPRY2 expression correlated with cellular sensitivity to gefitinib. While the authors observed a relationship between SPRY2 and EGFR expression similar to what we observed, they did not investigate any possible perturbations to downstream signaling. Thus, the net effects of SPRY2
expression may have been qualitatively different from those we observed in NSCLC cells.

While our study utilized NSCLC cells as a model cell background, feedback regulation has recently emerged as an important determinant of response to clinically relevant inhibitors in many different cancers. ERK signaling drives oncogenic processes (e.g., proliferation and migration) and is often dysregulated in cancer, but MEK inhibitors have been largely unsuccessful in clinical trials (Adjei et al., 2008; Haura et al., 2010; Rinehart et al., 2004). It has been proposed that inhibition of the ERK pathway relieves negative feedback loops generated by ERK activity and that inhibition therefore has a net neutral effect or may even promote other signaling pathways such as AKT (Mirzoeva et al., 2009; Pratilas et al., 2009). Similarly to MEK inhibition, AKT inhibitors can induce the expression and phosphorylation of several receptor tyrosine kinases due to relief of feedback inhibition (Chandarlapaty et al., 2011). Along with the data presented in our study, these findings additionally suggest that much more must be known about the mechanisms of feedback regulation of cell signaling in order to design successful therapies for disease.

2-6. Acknowledgements

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Figure 2-S1. SPRY2 and MIG6 expression are efficiently reduced by shRNA-mediated knockdown.

(A) Western blots of whole cell lysates from six NSCLC cell lines maintained in complete media were probed with antibodies against indicated proteins. HCC827 (delE746-A750 EGFR; Douglas Lauffenburger, MIT, Cambridge, MA, USA), H358 (wild-type EGFR; Russ Carstens, University of Pennsylvania, Philadelphia, PA, USA), and H1975 (L858R/T790M EGFR; Eric Haura, Moffitt Cancer Center, Tampa, FL, USA) cells were grown in RPMI (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Life Technologies), 1 mM L-Glutamine (Life Technologies), 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). H3255 (L858R EGFR; Pasi Jänne, Dana-Farber Cancer Institute, Boston, MA, USA) were maintained in ACL4. H1666 (wild-type EGFR) and PC9 (delE746-A750 EGFR) were maintained as described in Materials and Methods. (B) Stable knockdown of SPRY2 and/or MIG6 was generated using pSicoR and pSuper vectors in PC9 and H1666 cells. Western blots of whole cell lysates from the indicated cells maintained in complete media were probed with antibodies against indicated proteins. Images are representative of at least three independent experiments. Percent knockdown (“KD”) calculations were made for each shRNA, and results are indicated in the figure.
**Figure 2-S2. SPRY2 and/or MIG6 knockdown perturb EGF-mediated EGFR endocytosis.**

EGFR endocytosis rate constants ($k_e$) were measured using 10 ng/mL $^{125}$I-EGF as described in *Materials and Methods*. Data represent an average of three experiments ± s.e.m. and were corrected for the effects of non-specific binding and spillover and then normalized to the maximum internal $^{125}$I-EGF or integral with respect to time of surface $^{125}$I-EGF value. The slopes of the linear fits shown were used to calculate the $k_e$ values in (A) Figure 2-1 for PC9 and H1666 cells with knockdown ("KD") of SPRY2, MIG6, or SPRY2 and MIG6; and in (B) Figure 2-5B PC9 and H1666 cells expressing SPRY2-targeting shRNA or non-targeting control shRNA were transduced with a retroviral vector encoding EGFR or an empty expression vector (EV).
Figure 2-S3. Differences in EGF-mediated EGFR endocytosis measured with 1.5 ng/mL $^{125}$I-EGF are consistent with measurements using 10 ng/mL $^{125}$I-EGF (Figure 2-1), and treatment with the dynamin inhibitor Dynasore reduces $k_e$.

(A) $k_e$ values were measured using 1.5 ng/mL $^{125}$I-EGF for H1666 cells with knockdown of SPRY2, MIG6, or SPRY2 and MIG6, as described in Materials and Methods. Data were corrected for the effects of non-specific binding and spillover and represent an average of three experiments ± s.e.m. Asterisks indicate $p < 0.05$ for comparisons against controls. (B) $k_e$ values were measured using 10 ng/mL $^{125}$I-EGF for PC9 and H1666 cells that were pre-treated with 80 μM Dynasore (Millipore, Billerica, MA, USA). Cells were pretreated with 80 μM Dynasore or DMSO control for 2 hrs, and Dynasore was added to the $^{125}$I-EGF-containing media used to make $k_e$ measurements. Data were corrected for the effects of non-specific binding and spillover and represent an average of three experiments ± s.e.m. Asterisks indicate $p < 0.05$ for comparisons indicated by horizontal bars.
Figure 2-S4. Phosphorylation of ERK and AKT in response to gefitinib is perturbed in NSCLC cells with SPRY2 and/or MIG6 knockdown relative to controls.

Whole cell lysates from PC9 (A) or H1666 (B) cells treated as described for Figure 2-2 were probed with antibodies against the indicated proteins. Images are representative of at least three independent experiments. (C) Band intensities were quantified and phosphorylated levels were normalized to total protein levels for AKT. Densitometry data represent an average of three independent experiments ± s.e.m. Asterisks indicate $p < 0.05$ for comparisons against controls.
Figure 2-S5. EGFR protein levels are reduced in PC9 and H1666 cells by a second independent shRNA, and SPRY2 knockdown reduces EGFR expression in a broader panel of NSCLC cell lines.

(A) Western blots of whole cell lysates from PC9 and H1666 cells expressing control non-targeting or two independent SPRY2-targeting shRNAs were probed with antibodies against indicated proteins. Cell lines were maintained in complete media prior to lysis. SPRY2 sh1 was used for results shown in the main figures. Images are representative of three independent experiments. Band intensities were quantified and data represent an average of three independent experiments ± s.e.m. Asterisks indicate p < 0.05 for comparisons against controls.

(B) Five of six NSCLC cell lines had significantly reduced EGFR levels upon SPRY2 knockdown. Western blots of whole cell lysates from the indicated cell lines grown in complete media were probed with antibodies against the indicated proteins. Non-targeting control shRNA is indicated by "-". Images are representative of three independent experiments. Band intensities were quantified and data represent an average of three independent experiments ± s.e.m. Asterisks indicate p < 0.05 for comparisons against controls.
Figure 2-S6. SPRY2 knockdown results in increased EGF-mediated EGFR ubiquitylation and CBL association in H1666 cells, but not in PC9 cells.

PC9 and H1666 cells expressing SPRY2-targeting or control shRNA were serum starved and treated with or without 10 ng/mL EGF for 10 min (A and C) or 100 ng/mL EGF for 10 min (B and D). EGFR was immunoprecipitated (IP) from whole cells lysates of PC9 and H1666 cells, and immunoprecipitates were analyzed by western blot (WB) using antibodies against the indicated proteins. Images are representative of three independent experiments. (E) A control IgG did not immunoprecipitate ubiquitin, CBL, or EGFR from PC9 and H1666 lysates. PC9 and H1666 cells were serum starved prior to treatment with 10 ng/mL EGF for 2 min, as in Figure 2-3. Whole cells lysates were incubated with agarose beads conjugated to a control mouse IgG, following the immunoprecipitation protocol described in Materials and Methods. Immunoprecipitates (capture) and supernatants were analyzed by western blot (WB) using antibodies against the indicated proteins.
Figure 2-S7. *EGFR* mRNA levels are reduced in PC9 cells by a second independent shRNA. *EGFR* mRNA levels were measured by qRT-PCR in PC9 and H1666 cells expressing control non-targeting or two independent *SPRY2*-targeting shRNAs. SPRY2 sh1 was used for results shown in the main figures. Data represent an average of triplicate wells and are representative of multiple independent experiments. Error bars represent the range of possible relative mRNA values calculated from the standard error of the ΔC_Ts.

Figure 2-S8. Reconstitution of *EGFR* in *SPRY2* knockdown cells does not rescue diminished ERK phosphorylation. PC9 and H1666 cells expressing *SPRY2*-targeting or control shRNA were transduced with a retroviral vector encoding *EGFR* or an empty expression vector (EV). Cells were treated with the indicated concentrations of gefitinib for 24 (PC9) or 48 (H1666) hrs, and western blots of whole cell lysates were probed with antibodies against indicated proteins. Images are representative of three independent experiments.
Figure 2-S9. Flow cytometry measurements demonstrate differences in surface EGFR expression in PC9 and H1666 cells with SPRY2 knockdown and EGFR reconstitution.

PC9 and H1666 cells expressing SPRY2-targeting or control shRNA were transduced with a retroviral vector encoding EGFR or an empty expression vector (EV). Cells were collected by trypsinization and stained with an anti-EGFR antibody (Ab225; K. Dane Wittrup, MIT, Cambridge, MA, USA) and a secondary antibody conjugated to Alexa-Fluor 488 (Cell Signaling Technology, Danvers, MA, USA). Cells were not permeabilized. Samples were analyzed as described in Materials and Methods. Data represent an average of duplicate samples from each of two independent experiments.
Stable knockdown of SPRY2 was generated using pSicoR vectors in H1975 and H358 cells. Western blots of whole cell lysates from the indicated cells maintained in complete media were probed with antibodies against indicated proteins. Expression of non-targeting control shRNA is indicated by “-”. Images are representative of three independent experiments. Annexin V staining was measured by flow cytometry for cells treated with gefitinib (H1975: 10 μM for 72 hrs; H358: 5 μM for 72 hrs) or DMSO control. Data represent an average of three replicates ± s.e.m. Asterisks indicate $p < 0.05$ for comparisons indicated by horizontal bars.
Figure 2-S11. Titration of the MEK inhibitor U0126 identifies a concentration of U0126 that reduces ERK phosphorylation to a similar level as SPRY2 knockdown.

PC9 and H1666 cells expressing SPRY2-targeting shRNA or a non-targeting control shRNA (indicated by “-”) were treated with the indicated concentrations of U0126 for 24 hrs in the presence of 0.001 μM gefitinib (PC9) or 48 hrs in the presence of 0.01 μM gefitinib (H1666). (A) Western blots of whole cell lysates were probed with antibodies against phosphorylated or total ERK. Images are representative of at least three independent experiments. (B) Normalized densitometry data represent an average of three independent experiments ± s.e.m. Asterisks indicate $p < 0.05$ for comparisons indicated by horizontal bars.
Figure 2-S12. PTEN expression is not affected by SPRY2 knockdown.
NSCLC cells maintained in complete media expressing non-targeting control or SPRY2-targeting shRNA were probed by western blot for PTEN. Bands were quantified from a single experiment.
Chapter 3: Differential parsing of EGFR endocytic flux among parallel internalization pathways in lung cancer cells with EGFR-activating mutations

3-1. Abstract

Due to the existence of parallel pathways for receptor endocytosis and the complexity of their regulation, a quantitative understanding of receptor endocytosis in normal and pathological settings requires computational analysis. Here, we develop a mechanistic model of epidermal growth factor receptor (EGFR) endocytosis to determine the relative contributions of three parallel internalization pathways: clathrin-dependent internalization mediated by mitogen-induced gene 6 (MIG6), clathrin-dependent internalization mediated by CBL, or alternative pathways that may be non-clathrin mediated. We applied the model to interpret our previously reported measurements of EGFR endocytosis in non-small cell lung cancer (NSCLC) cells where expression of MIG6 and Sprouty2, two regulators of EGFR endocytosis, as well as the expression of EGFR itself, were perturbed. Interestingly, our results suggest that MIG6, an endogenous inhibitor of EGFR kinase activity recently discovered to regulate EGFR endocytosis, is responsible for approximately half of wild-type EGFR internalization. This MIG6 function appears to be impaired for EGFR kinase-activated mutants that arise in NSCLC and display reduced endocytosis. Our results also suggest that Sprouty2 controls EGFR endocytosis primarily by regulating EGFR expression, rather than by sequestering CBL, and support the notion that CBL-mediated internalization is impaired for EGFR mutants. We further demonstrate that differences in internalization between wild-type and mutant EGFR cannot explain observed differences in EGF-mediated EGFR degradation without concomitant changes in EGFR recycling, which we previously quantified. This work provides new insight into the trafficking of wild-type and mutant EGFR in NSCLC and provides a framework for studying parallel endocytosis pathways for other receptors.

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Endocytosis and degradation are important mechanisms in EGFR signaling regulation that involve the concerted action of many proteins connected through a complicated regulatory network (Sorkin and von Zastrow, 2009). Impaired receptor trafficking is observed in several cancers, promoting inappropriate growth and survival signaling (Abella and Park, 2009; Mosesson et al., 2008; Sorkin and Goh, 2009; Sorkin and von Zastrow, 2009). The complexity of the internalization process is due in part to the existence of multiple intracellular adaptor proteins that can facilitate clathrin-mediated internalization through parallel pathways, as well as the existence of non-clathrin-mediated internalization pathways. Few attempts have been made to quantitatively determine the relative importance of different adaptor proteins involved in mediating EGFR endocytosis through parallel pathways or to determine how these roles may be perturbed in various diseases. Computational modeling is a natural and useful approach for interpreting experimental data generated to probe this issue.

The CBL ubiquitin ligase is one the most well-known mediators of ligand-mediated EGFR endocytosis. In the prevailing view, CBL is recruited to tyrosine phosphorylated EGFR by the intracellular adaptor GRB2, resulting in EGFR ubiquitylation and entrance into clathrin-coated pits (Schmidt and Dikic, 2005; Soubeyran et al., 2002). In contrast to CBL, mitogen inducible gene 6 (MIG6) was only recently discovered to regulate EGFR endocytosis. MIG6 binds EGFR, simultaneously inhibiting kinase activity and promoting internalization by linking EGFR to AP-2 and Intersectins (Anastasi et al., 2003; Frosi et al., 2010; Ying et al., 2010; Zhang et al., 2007a). The importance of MIG6-mediated endocytosis relative to CBL-mediated endocytosis has never been directly assessed (Frosi et al., 2010; Schmidt and Dikic, 2005; Soubeyran et al., 2002; Ying et al., 2010). Adding to this complexity, CBL can be regulated by proteins such as Sprouty2 (SPRY2), a candidate tumor suppressor whose most well-studied function is to inhibit extracellular signal-regulated kinase (ERK) activity downstream of several receptor tyrosine kinases but may also antagonize EGFR internalization by binding and sequestering CBL (Haglund et al., 2005; Rubin et al., 2003; Wong et al., 2002). While CBL and MIG6 regulate
clathrin-mediated endocytosis, non-clathrin mediated pathways also exist. These are generally less well understood, but are thought to account for a relatively small fraction of EGFR endocytosis under normal conditions (Sigismund et al., 2005; Sorkin and Goh, 2009).

Due to these and other complexities, computational models of EGFR-mediated signaling that include EGFR trafficking typically approximate receptor endocytosis with a coarse-grained approach. In such models, EGFR at the plasma membrane is converted to internalized EGFR in a reaction described by a single kinetic rate constant (Hendriks et al., 2006; Schoeberl et al., 2009; Wiley and Cunningham, 1981). Some models have introduced additional steps by requiring binding of an intermediate species, such as a coated-pit protein, followed by EGFR endocytosis (Gex-Fabry and DeLisi, 1984; Starbuck and Lauffenburger, 1992), or by considering the potential influence of EGFR dephosphorylation at the plasma membrane (Monast et al., 2012). These models have been informative, but they do not provide insight into the parsing of EGFR endocytic flux among the various parallel pathways. In fact, to our knowledge, no EGFR model has explicitly included multiple internalization pathways, aside from consideration of ligand-induced and constitutive/basal internalization.

We previously studied the effects of shRNA-mediated depletion of MIG6 and SPRY2 on EGFR endocytosis in non-small cell lung cancer (NSCLC) cells expressing wild-type EGFR or kinase-activated EGFR mutants that display inefficient endocytosis and degradation in response to EGF (Amann et al., 2005; Hendriks et al., 2006; Lazzara et al., 2010; Lynch et al., 2004; Padron et al., 2007; Paez et al., 2004; Walsh and Lazzara, 2013; Yang et al., 2006). Our data showed significant and complex changes in EGFR endocytosis rates due to perturbations in MIG6, SPRY2, and EGFR expression for both wild-type and mutant EGFR. Here, we use that data to develop and apply a mechanistic model of EGFR endocytosis to determine the relative importance of parallel internalization pathways and to identify differences in such parsing between cells expressing wild-type EGFR or kinase-activated EGFR mutants that display impaired EGF-mediated EGFR endocytosis (Hendriks et al., 2006; Lazzara et al., 2010). Our model accounts for EGFR internalization regulated by MIG6 or CBL, as well as a third pathway
intended to account for other modes of EGFR internalization, and accounts for the ability of SPRY2 to regulate the pool of CBL available for EGFR binding. Our results indicate that MIG6-mediated EGFR internalization accounts for at least half of wild-type EGFR endocytic flux and almost all of mutant EGFR endocytic flux, despite the fact that MIG6-mediated internalization appears to be less efficient for EGFR mutants than for the wild-type receptor. This surprising finding has important implications for EGFR signaling because MIG6 inhibits EGFR activity, thus potentially resulting in internalized receptors unable to activate downstream signaling pathways. Our model results are also consistent with previous experimental studies showing that CBL function is impaired for EGFR mutants relative to wild-type. We further show that these differences in internalization alone cannot explain observed differences in EGF-mediated EGFR degradation between mutant EGFR and wild-type EGFR-expressing cells without simultaneously considering our previous measurements of differences in recycling of wild-type versus mutant EGFR. The basic methodology we develop here could be applied to future efforts to model EGFR in different settings and to better understand the trafficking of other receptors.

3-3. Materials and Methods

*Model topology.* A diagram showing the major features of the model topology and parameters is shown in Figure 3-1. The model includes EGF binding to unoccupied receptor monomers and unoccupied and singly-occupied receptor dimers at the plasma membrane. EGFR dimerization is allowed for all receptor monomers, occupied or unoccupied. To reduce model complexity, EGFR tyrosine auto-phosphorylation is not explicitly modeled. Rather, ligand-bound EGFR dimers are considered phosphorylated unless bound to MIG6. MIG6 and CBL bind ligand-bound EGFR dimers, in agreement with findings that these proteins are recruited to EGFR upon receptor activation and phosphorylation (Anastasi et al., 2003; Soubeyran et al., 2002; Ying et al., 2010). However, binding of MIG6 and CBL to the same receptor complex is not allowed because MIG6-bound receptors are considered kinase-inactive and un-phosphorylated, which would prevent CBL binding (Schmidt and Dikic, 2005; Soubeyran et al., 2002; Zhang et al., 2007a).
MIG6- or CBL-bound receptors are internalized via reactions characterized by the rate constants \( k_{i,MIG6} \) and \( k_{i,CBL} \), respectively. To account for other mechanisms of EGFR endocytosis (e.g., non-clathrin mediated internalization or clathrin-mediated internalization facilitated by other adaptor proteins (Sigismund et al., 2008; Sorkin and Goh, 2009)), internalization of ligand-bound dimers not bound to MIG6 or CBL is also allowed and characterized by rate constant \( k_{i,\text{other}} \). The model includes basal internalization of all receptor species with a rate constant, \( k_{i,\text{basal}} \), which is fitted to yield a simulated EGFR endocytosis rate constant \( k_e = 0.03 \text{ min}^{-1} \) in the absence of EGF, a typical value reported in the literature for basal EGFR internalization (Lund et al., 1990; Sigismund et al., 2008). EGFR recycling to the plasma membrane and degradation is modeled as described previously (Hendriks et al., 2006; Schoeberl et al., 2009). Briefly, a fraction of internalized ligand-bound receptors are recycled \((f_i)\) with a rate constant \( k_{\text{rec}} \). The remaining fraction of internalized receptors \((1-f_i)\) is degraded with a rate constant \( k_{\text{deg}} \) (Hendriks et al., 2006; Schoeberl et al., 2009). All unoccupied receptors are assumed to recycle. Because SPRY2 phosphorylation occurs in response to EGFR activation and is important for SPRY2 regulatory function (Mason et al., 2004), we assumed that SPRY2 binds its kinase (referred to as “SPRY2 kinase”) with a rate proportional to the fraction of ligand-bound EGFR dimers. SPRY2 phosphorylation is required for CBL binding (Rubin et al., 2003), and phosphorylated SPRY2 is assumed to become dephosphorylated in a first-order reaction.

**Model implementation.** Model rate equations were formulated assuming mass action kinetics with proteins associating reversibly with a forward rate constant \( k_{\text{on}} \) and a reverse rate constant \( k_{\text{off}} \). SPRY2 phosphorylation was modeled as the reversible formation of a substrate/enzyme intermediate followed by catalysis characterized by a rate constant \( k_{\text{cat}} \). The resulting system of ordinary differential equations (ODEs) describing the change in concentration with respect to time for all model species was numerically integrated in MATLAB using the function ode15s. The basic model topology requires 25 model species and 24 unique kinetic parameters. The model equations are provided in Tables 3-S1, 3-S2, and 3-S3.
Figure 3-1. Key features of model topology.

The model accounts for three parallel internalization pathways for ligand-bound EGFR and a basal internalization pathway for all receptor species. Although the kinetics of EGFR phosphorylation are not explicitly considered in the model, receptor phosphorylation status is shown in the figure to highlight that CBL binds phosphorylated EGFR and that MIG6-bound EGFR is kinase-inhibited and therefore assumed to be not phosphorylated. This assumption allows us to consider CBL- and MIG6-mediated EGFR internalization as independent processes. The model also includes the effects of EGFR recycling, degradation, dimerization, and ligand binding.

To simulate an EGFR $k_e$ measurement, the model is initiated in the absence of EGF until a steady-state is reached (i.e., no model species are changing with respect to time). 10 ng/mL EGF is then added, and the model is numerically integrated for up to $t = 7.5$ min, the length of our experimental $k_e$ measurements. The simulated EGFR $k_e$ is calculated in the same way as the experimental $k_e$, that is, by determining the slope of a plot of internalized ligand against the integral with respect to time of surface associated ligand for a series of time points up to and including 7.5 min (Lund et al., 1990). For simulations of EGF-induced EGFR degradation, we include EGFR synthesis, with a synthesis rate fit to maintain EGFR levels in the absence of EGF.

Parameters. Where possible, kinetic parameters and protein concentrations were chosen based on published experimentally determined values. Parameters available from the literature are summarized in Table 3-S4. The model parameters and species concentrations we estimated are summarized in Table 3-S5. We set unknown cytosolic model species concentrations to be
near that of other cytosolic proteins that have been measured (Schoeberl et al., 2009). The relative values of total MIG6 and SPRY2 per cell line were set to match the relative differences measured by western blot for H1666 and PC9 cells. The cellular concentration of EGFR was based on measurements of $^{125}$I-EGF binding to PC9 cells (Figure 3-S1) and western blotting comparisons of PC9 cells to H1666 cells (Walsh and Lazzara, 2013). In our previous experiments, we found that EGFR expression was decreased by SPRY2 knockdown and combined MIG6/SPRY2 knockdown (Walsh and Lazzara, 2013). In a subset of experiments, we reconstituted EGFR expression in the context of SPRY2 knockdown. For model calculations, EGFR concentrations were scaled accordingly. The parameters for SPRY2 binding to its kinase were set to $10^5$ M$^{-1}$s$^{-1}$ for $k_{on}$ and 0.1 s$^{-1}$ for $k_{off}$, similar to values used in previous models based on assumptions of diffusion-limited protein interactions and measurements of other protein interactions (Kholodenko et al., 1999; Northrup and Erickson, 1992). The parameters for SPRY2 phosphorylation and dephosphorylation ($k_{cat}$ and $k_{dephos}$, respectively) were estimated to agree with SPRY2 phosphotyrosine immunoprecipitation data from Mason et al. (Mason et al., 2004) demonstrating that peak SPRY2 tyrosine phosphorylation was reached 3 min after EGF addition (Figure 3-S2).

The model parameters $k_{i,MIG6}$, $k_{i,CBL}$, and $k_{i,other}$ were fit to allow for model recapitulation of our previously measured EGFR $k_e$ values in H1666 and PC9 cells using a nonlinear optimization algorithm to minimize the sum of the squares error between the simulated and experimental data. Presumably because different vector backbones and control non-targeting shRNA sequences were used for each knockdown (SPRY2 or MIG6) and because cells were selected in different antibiotics, the measured $k_e$ values for the individual controls were slightly different. To address this difference, the $k_e$ values were scaled so that differences between each knockdown and its respective control were maintained compared to a single control value set to the average of the individual control values. MIG6, SPRY2, and EGFR concentrations for control or knockdown conditions were set to agree with differences observed by western blot (Walsh and Lazzara, 2013).
Local sensitivity analysis and model robustness to perturbations in parameters. Normalized local sensitivity of predicted EGFR $k_e$ to perturbations in model parameters was calculated as described previously, with 10% perturbations to parameter values (Hendriks et al., 2006; Schoeberl et al., 2009). The robustness of the fitted $k_i$ values to changes in other model parameters was calculated by randomly sampling selected parameters in a range an order of magnitude above or below their base values. The $k_i$ values were then fit to experimental data with this random parameter set. This process was repeated for 300 random parameter sets to calculate the range of fitted $k_i$ values.

EGF-mediated EGFR degradation experimental data. H1666 (American Type Culture Collection) and PC9 cells (Douglas Lauffenburger, MIT) were maintained as described previously (Walsh and Lazzara, 2013). Cells were starved overnight in media containing 0.1% FBS and then treated with 10 ng/mL EGF (Peprotech) for up to 180 min. Cells were lysed in a standard buffer, and lysates were probed by western blot with antibodies against EGFR (no. MS-400, Thermo Scientific) or actin (no. MAB1501, Millipore). See Supplemental Materials and Methods for complete details on cell lysis and western blotting procedures.

3-4. Results

Model fit to experimental data from wild-type EGFR-expressing NSCLC cells. We previously measured the effects of shRNA-mediated depletion of MIG6 and SPRY2 (alone or together) on EGFR $k_e$ in the wild-type EGFR-expressing NSCLC cell line H1666 (Walsh and Lazzara, 2013). Consistent with previous studies on the effects of MIG6 and SPRY2 on wild-type EGFR (Frosi et al., 2010; Wong et al., 2002), we found that MIG6 depletion reduced $k_e$ and that SPRY2 depletion increased EGFR $k_e$. Combined knockdown of MIG6 and SPRY2 reduced $k_e$ to a similar degree as MIG6 knockdown alone. With SPRY2 knockdown, we also noticed significantly decreased EGFR expression, and we found that the increase in EGFR $k_e$ due to SPRY2 depletion was reversed by EGFR reconstitution in cells with SPRY2 depletion. Together, these findings suggest that the major effect of SPRY2 on $k_e$ in H1666 cells was related to relieving a
partial saturation of endocytosis at basal EGFR expression levels, rather than to antagonism of CBL sequestration. The same qualitative trends were also observed in PC9 cells that express one of the kinase-activated EGFR mutants observed in NSCLC (delE746-A750), for which the effects of SPRY2 and MIG6 on $k_e$ had not previously been investigated.

Figure 3-2. Model agreement with experimental data in wild-type EGFR-expressing H1666 cells and relative importance of MIG6 and CBL levels for EGFR internalization. (A) For base MIG6 and CBL concentrations ([MIG6] = 5×10⁴ cell⁻¹ and [CBL] = 1×10⁵ cell⁻¹), experimentally measured $k_e$ values (± s.e.m.) in H1666 cells are compared to $k_e$ values simulated using $k_i$ parameters fit to data for control and knockdown of MIG6 or SPRY2 alone. “KD” indicates knockdown, and “predicted” identifies data not used to fit model parameters. (B) Values of the fitted $k_i$ parameters are plotted. (C) The integral of EGFR internalized by MIG6- or CBL-mediated mechanisms over the time course of a $k_e$ measurement is plotted for a range of MIG6 and CBL concentrations. To make these calculations, $k_i$ parameters were fit to $k_e$ data for control and knockdown of MIG6 or SPRY2 alone for all combinations of MIG6 and CBL concentrations. The star indicates MIG6 and CBL concentrations used in A, B, and C. (D) The log of the sum of squares error (SSE) of the fit for the same range of MIG6 and CBL concentrations is plotted. The star indicates the base MIG6 and CBL concentrations used in A, B, and C.

To determine whether the model could recapitulate those experimental findings, the model was first fit to our experimentally determined $k_e$ values for H1666 cells with MIG6 knockdown, SPRY2 knockdown, or controls (Table 3-S6), with $k_{i,MIG6}$, $k_{i,CBL}$, and $k_{i,other}$ as the fitted parameters. As described in Materials and Methods, the condition of SPRY2 knockdown was simulated with reduced EGFR expression, consistent with our experimental findings that EGFR
expression was reduced by 40% with SPRY2 knockdown in H1666 cells. The resulting model fit of $k_e$ values for the individual knockdowns was good (Figure 3-2A). Based on these fit results, the model accurately predicted $k_e$ values for conditions of combined MIG6/SPRY2 knockdown or SPRY2 knockdown with EGFR reconstitution, neither of which were included in the fit. The model fit to experimental data suggests that the model topology allows for appropriate recapitulation of the behavior of the endocytic system and its dependence on MIG6, SPRY2, and EGFR expression. The internal and surface-bound EGF levels from model simulations are compared to experimental measurements used to calculate $k_e$ values in Figure 3-S3.

For these model conditions, the fitted $k_i$ values are shown in Figure 3-2B. The fitted $k_{i,MIG6}$ and $k_{i,CBL}$ values are similar to each other and higher than the fitted value for $k_{i,other}$, indicating that CBL- and MIG6-mediated pathways (both of which depend upon clathrin) are responsible for the vast majority of EGF-mediated EGFR internalization in H1666 cells. While the fitted rate constants may seem to suggest that MIG6- and CBL-mediated internalization occur at similar rates, the MIG6 and CBL concentrations and binding parameters are not equivalent (Table 3-S5), which may skew the extent to which relative $k_i$ values reflect relative endocytic fluxes. Moreover, uncertainty in the expression of MIG6 and CBL may also affect the fit results in Figures 3-2A and 3-2B and their interpretation. To determine the relative contributions of MIG6 and CBL for EGFR internalization and to simultaneously determine the sensitivity of our results to changes in MIG6 and CBL expression, we fit the model to the experimental data for a range of MIG6 and CBL concentrations ($1 \times 10^3$ to $1 \times 10^7$ cell$^{-1}$), including the base values for our model reflected in the results of Figures 3-2A and 3-2B. The parameters were fit to data for single knockdowns of MIG6 or SPRY2 and simultaneous MIG6/SPRY2 knockdown, and the best-fit results are shown in Figure 3-S4. We used these results to calculate the integral of EGFR internalized by each pathway (MIG6- or CBL-mediated) over the time course of the simulated $k_e$ experiment (Figure 3-2C). Interestingly, except where CBL levels were approximately an order of magnitude higher than MIG6 levels, MIG6 was predicted to drive more EGFR internalization than CBL. The fluxes of internalized EGFR at two time points after EGF addition demonstrate a similar trend (Figure 3-
It is important to note, however, that not all combinations of MIG6 and CBL concentrations fit the data equally well, as shown by calculation of the sum of squares errors (SSE) for each concentration combination (Figure 3-2D). Moderate MIG6 and CBL concentrations (~1×10^4 to 1×10^5) fit the data best, suggesting that the estimated MIG6 and CBL concentrations used initially were reasonable. The quality of the fit to data for these concentrations is equivalent to the best fit generated in Figure 3-2A. For most of the region where CBL-mediated internalization dominated (high CBL and low MIG6), the model did not fit the data well. Therefore, in order to fit experimental data, MIG6-mediated internalization must account for a relatively large fraction of overall wild-type EGFR internalization in H1666 cells.

**Model fit to experimental data from mutant EGFR-expressing NSCLC cells.** We also previously measured EGFR \( k_e \) with the same perturbations to MIG6, SPRY2, and EGFR expression using PC9 cells that express one of the most common NSCLC-associated EGFR mutants (delE746-A750). These mutants fail to undergo efficient ligand-mediated endocytosis, which has been linked to the increased cellular sensitivity to EGFR inhibition that accompanies expression of the mutants in NSCLC cells (Furcht et al., 2012; Lazzara et al., 2010). A possible explanation for the observed defect in mutant EGFR internalization is that CBL fails to associate with mutant EGFR, but that other internalization pathways such as MIG6-mediated internalization remain fully functional. This hypothesis is based on previous findings that the EGFR mutants are poorly ubiquitylated and fail to associate with CBL in response to EGF (Padron et al., 2007; Walsh and Lazzara, 2013; Yang et al., 2006). To simulate this possibility, the CBL binding parameters were set to zero, and the \( k_{i,MIG6} \) and \( k_{i,other} \) parameters were set to the values fit from consideration of the H1666 data. Concentrations of model species, such as EGFR, MIG6, and SPRY2, were set to values estimated for PC9 cells (Table 3-S5). As shown in Figure 3-3A, these assumptions yielded predicted \( k_e \) values that greatly exceed PC9 experimental results, indicating that loss-of-function of CBL-mediated internalization alone is not sufficient to explain reduced mutant EGFR internalization. This poor prediction is also qualitatively consistent with our findings that MIG6 drives more than half of wild-type EGFR endocytosis in H1666 cells and that the EGFR
$k_e$ for PC9 cells is less than half that of H1666 cells. Fitting the model to PC9 data while allowing CBL binding and including results with MIG6 knockdown, SPRY2 knockdown, or control, yielded much more reasonable agreement between the model and experimental data (Figure 3-3B).

When compared to parameters fit to H1666 data, the fitted value of $k_{i,MIG6}$ was approximately three-fold lower for PC9 cells than H1666, and the fitted value of $k_{i,CBL}$ was over ten-fold lower (Figure 3-3C). Note that the slight differences in $k_i$ values for H1666 cells between Figures 3-2B and 3-3C arise because the fitted $k_i$ values in Figure 3-3C come from consideration of all $k_e$ data, including that for simultaneous knockdown of SPRY2 and MIG6 and for EGFR reconstitution on top of SPRY2 knockdown. The very low fitted value of $k_{i,CBL}$ for PC9 cells could suggest very slow CBL-mediated internalization or poor CBL/EGFR association. If the parameters are re-fit assuming no CBL binding, the quality of the fit is not greatly reduced (data not shown).

We calculated the integral of EGFR internalized over 7.5 min (for standard model conditions) and normalized to the total number of EGFR for H1666 or PC9 conditions. This normalized integral of EGFR internalized by MIG6 was ~25% lower for PC9 than H1666, and the integral of EGFR internalized by CBL was ~90% lower for PC9 than H1666 (Figure 3-3D).

As described previously for H1666 cells, we went on to fit the internalization parameters for a range of possible MIG6 and CBL concentrations and calculated the integral of EGFR internalized by MIG6 or CBL (Figure 3-3E). The fitted $k_i$ parameter values are shown in Figure 3-S4. For most MIG6 and CBL concentrations, more EGFR was internalized by MIG6 than CBL. The fluxes of internalized EGFR at two time points after EGF addition demonstrate a similar trend (Figure 3-S5). As observed with H1666 cells, the model could not fit experimental data well in the region of parameter space where CBL-mediated internalization dominated (Figure 3-S3F).
Figure 3-3. Model agreement with experimental data in mutant EGFR-expressing PC9 cells and differences from results for wild-type EGFR-expressing H1666 cells.

(A) Experimentally measured $k_e$ values in PC9 cells are compared to $k_e$ values (± s.e.m.) simulated using $k_i$ parameters from the fit to H1666 data but with CBL binding parameters set to zero. “KD” indicates knockdown. (B) For base MIG6 and CBL concentrations ([MIG6] = $1.2 \times 10^5$ cell$^{-1}$ and [CBL] = $1 \times 10^5$ cell$^{-1}$), experimentally measured $k_e$ values (± s.e.m.) in PC9 cells are compared to $k_e$ values simulated using $k_i$ parameters fit to PC9 cell data for control and knockdown of MIG6 or SPRY2 alone. “Predicted” identifies data not used to fit model parameters.

(C) Values of the $k_i$ parameters found by fitting to all data points (including conditions for simultaneous knockdown of SPRY2 and MIG6 and for EGFR reconstitution on top of SPRY2 knockdown) are plotted for H1666 and PC9 cells using base MIG6 and CBL concentrations. (D) The integral of EGFR internalized by MIG6 or CBL is plotted for H1666 or PC9 parameters normalized to the total EGFR cell$^{-1}$ with the base model conditions. (E) The integral of EGFR internalized by MIG6 or CBL is plotted for a range of MIG6 and CBL concentrations. To make these calculations, $k_i$ parameters were fit to $k_e$ data for control and knockdown of MIG6 or SPRY2 alone for all combinations of MIG6 and CBL concentrations. The star indicates the base MIG6 and CBL concentrations used in A and B. (F) The log of the sum of squares error (SSE) of the fit for the same range of MIG6 and CBL concentrations is plotted. The star indicates the base MIG6 and CBL concentrations used in A and B.
We also considered the possible effects of basal EGFR/MIG6 association due to basal phosphorylation of EGFR mutants (Greulich et al., 2005; Guo et al., 2008). In these calculations, MIG6 was allowed to bind non-ligand bound EGFR dimers (assumed here to be phosphorylated in the absence of ligand) and drive their internalization. Including these effects slightly lowered the fitted values of $k_{i,CBL}$ and $k_{i,MIG6}$, as expected due to the slight augmentation of ligand-mediated internalization arising from the existence of dimers bound by MIG6 prior to ligand binding. This modification did not change the result that the best fit to PC9 data required higher MIG6 internalization than CBL-mediated internalization (Figure 3-S6). As another way to assess the potential impact of such basal MIG6/EGFR binding, we simulated a measurement of non-ligand mediated EGFR internalization made using the EGFR antibody cetuximab, with binding constants for cetuximab taken from published studies (Patel et al., 2007). Such experimental measurements are made as a means to determine fluid-phase rates of basal EGFR internalization. Allowing basal EGFR/MIG6 association increased the model-predicted basal non-ligand mediated EGFR $k_e$ from 0.027 to 0.036 min$^{-1}$. Overall, these modest effects of basal MIG6/EGFR mutant binding suggest that our basic model conclusions are not strongly dependent upon the capacity of EGFR mutants to demonstrate basal binding to some MIG6.

Predicted effects of changes in EGFR, SPRY2, CBL, or MIG6 expression on EGFR $k_e$. As discussed previously, rapid EGFR internalization is a saturable process, and sufficiently high receptor EGF occupancy can lead to reductions in EGFR $k_e$ (Lund et al., 1990). For our base model parameters fit to H1666 or PC9 data, we examined the predicted effects of changing EGFR expression (base EGFR cell$^{-1}$ = 6×10$^5$ for H1666 and 8×10$^5$ for PC9) (Figure 3-4A). For standard model conditions, the predicted EGFR $k_e$ increases with increasing EGFR expression for < ~1×10$^5$ EGFR cell$^{-1}$ due to the increasing driving force for EGFR dimerization (Figure 3-S7). However, the predicted EGFR $k_e$ sharply decreased for > ~1×10$^5$ EGFR cell$^{-1}$, reflecting saturation effects. Increasing MIG6 or CBL expression ten-fold caused the downturn in $k_e$ to shift to a higher EGFR expression.
Figure 3-4. Effect of changing EGFR, SPRY2, CBL, and MIG6 levels on simulated EGFR $k_e$.

(A) EGFR concentration was varied from $10^4$ to $10^7$ cell$^{-1}$, and the predicted EGFR $k_e$ was calculated for base model conditions or higher levels of MIG6, CBL, or both MIG6 and CBL for H1666 or PC9 cells. (B) SPRY2 concentration was varied from $10^2$ to $10^6$ cell$^{-1}$, and the predicted EGFR $k_e$ was calculated for base model conditions for H1666 or PC9 cells. (C) CBL or MIG6 concentration was varied from $10^2$ to $10^6$ cell$^{-1}$, and the predicted EGFR $k_e$ was simulated for base model conditions for H1666 or PC9 cells. All panels use $k_i$ parameters fit to all data for H1666 or PC9 cells. The vertical dashed lines indicate the base EGFR, SPRY2, CBL, and MIG6 concentrations used in Figure 3-2 and Figure 3-3.
We also plotted the predicted effect of changing the concentration of SPRY2 (keeping EGFR and all other model species concentrations constant) on $k_e$ (Figure 3-S4B). For SPRY2 expression $> \sim 1 \times 10^4$ cell$^{-1}$, the predicted $k_e$ decreased as SPRY2 levels increased, but the magnitude of change in $k_e$ was relatively small. In order for SPRY2 to have a larger influence on EGFR $k_e$, SPRY2 would need to bind all CBL and CBL-mediated internalization would need to dominate other internalization pathways. Thus, for the best fit to our data, the difference in EGFR $k_e$ due to SPRY2 knockdown is attributable mainly to differences in EGFR levels and, to a lesser degree, to changes in available CBL levels. Similar plots are shown for changing expression levels of MIG6 and CBL with expected trends (Figure 3-S4C). For PC9 parameters, increasing CBL expression can actually decrease the predicted $k_e$ because higher CBL levels compete with MIG6 for EGFR binding. This occurs for PC9, but not for H1666, because CBL-mediated internalization is much slower than MIG-mediated internalization for PC9 parameters.

Local parameter sensitivity analysis. To identify the key model reactions and species that influence the calculated value of $k_e$, we computed the normalized sensitivities of simulated $k_e$ to 10% perturbations in each parameter. The ten parameters with the largest normalized sensitivities for H1666 are plotted and compared to the same sensitivities for PC9 (Figure 3-S5A). Complete results for all parameters are shown in Table 3-S7. We also determined the sensitivity to changes in model species concentrations (Figure 3-S5B). Notably, the sensitivities to changes in $k_{i,CBL}$ or CBL concentration were relatively large for H1666, but very low for PC9. Similarly, the sensitivities associated with parameters for CBL binding to EGFR were also lower for PC9. The sensitivities to changes in $k_{i,MIG6}$ or MIG6 concentration, however, were higher for PC9 than H1666. The large sensitivity to changes in $k_{i,MIG6}$ for PC9 conditions reflects that MIG6-mediated internalization is substantially more important than CBL-mediated internalization in that cell line. Both H1666 and PC9 models were very sensitive to changes in EGFR levels, consistent with the results of Figure 3-4. As SPRY2 can modulate internalization via CBL, which is more important for H1666 conditions, a larger sensitivity to changes in SPRY2 concentration was found for H1666 conditions versus PC9 conditions. Of note, the sensitivities for perturbations in parameters
related to recycling \( (k_{\text{rec}} \text{ and } f_r) \) were relatively high, indicating that recycling processes could be contributing to the EGFR \( k_e \) calculation even for experiments lasting only 7.5 min after EGF addition. Finally, we note that the parameters in the top ten for PC9 that were not in the top ten for H1666 were the reverse rate constant for MIG6 binding \( (k_{\text{off,M}}) \), the EGFR dimerization rate constant \( (k_{\text{dimer}}) \), and the rate constant for EGFR degradation \( (k_{\text{deg}}) \), reflecting the reduced relative importance of CBL-associated parameters and increased relative importance of other model parameters.

**Figure 3-5. Local parameter sensitivity analysis for PC9 and H1666 models.**

(A) The ten model parameters with the highest normalized sensitivities for predicted \( k_e \) for H1666 parameters are plotted with the corresponding sensitivities for PC9 parameters. (B) The normalized sensitivities of predicted \( k_e \) to changes in model species concentrations in PC9 or H1666 cells are plotted.

*Robustness of and basis for differences in internalization parameters for MIG6 and CBL.*

The best-fit values of the internalization rate constants depend on MIG6 and CBL expression (as described above), their binding parameters, and, of course, all other model parameters to at least some extent. We calculated the robustness of the fitted \( k_i \) values to changes in other model parameters by randomly sampling the ten non-internalization parameters with the highest sensitivity for PC9 or H1666 conditions in a range an order of magnitude above or below their normal values. We fit the model with these random parameter sets and found that for H1666 conditions, the fitted \( k_{i,MIG6} \) value was higher than the fitted \( k_{i,CBL} \) value for 61% of the parameter.
sets (183/300 fits) (Figure 3-6A). For PC9 conditions, the fitted $k_{i,MIG6}$ value was higher than the fitted $k_{i,CBL}$ value for 97% of the parameter sets (290/300 fits). This result suggests that even if the model parameters are somewhat poorly estimated, MIG6 is still likely to be responsible for a significant fraction of EGFR internalization. Indeed, MIG6 is responsible for at least 25% of EGFR endocytosis in 88% of fits for H1666 and 98% of fits for PC9.

Figure 3-6. Robustness of fitted $k_{i,MIG6}$ and $k_{i,CBL}$ and model agreement with PC9 data. (A) The model was fit to H1666 or PC9 data for controls, MIG6 knockdown, SPRY2 knockdown, and MIG6/SPRY2 knockdown for 100 random parameter sets where the ten parameters with the largest normalized sensitivities for predicted $k_e$ were randomly sampled in a range an order of magnitude above or below their normal values. Model error was calculated for a range of $k_{i,MIG6}$ and $k_{i,CBL}$ values considering: (B) all data points with standard model conditions, (C) data excluding MIG6 knockdown data with $[MIG6] = [CBL] = 1 \times 10^5$ cell$^{-1}$, $k_{on,M} = k_{on,C}$, and $k_{on,S} = 0$, and (D) the same conditions for C including all data. Error is reported as the log of the sum of the squares error (SSE). Error minima are indicated by red circles, and the dashed lines represent $k_{i,MIG6} = k_{i,CBL}$.
To identify what specific experimental data led to particular model conclusions and to assess the ability of the data to constrain the fitted parameters, the model error was calculated as a function of $k_{i,MIG6}$ and $k_{i,CBL}$ for several conditions, focusing first on PC9 cells. Unlike the fits considered in Figures 3-2A and 3-3B, the model error here is calculated considering all experimental data, including effects of simultaneous MIG6 and SPRY2 knockdown and EGFR reconstitution on top of SPRY2 knockdown. Under normal model conditions for PC9 cells, the data constrained the parameters, and the error was minimized where $k_{i,MIG6} > k_{i,CBL}$ (Figure 3-6B), as expected based upon results from Figure 3-3C. As a control setting to demonstrate that there is no inherent bias in the model topology that causes MIG6-mediated internalization to be faster, the MIG6 concentration and binding parameters were set equal to those for CBL. In addition, SPRY2 binding to CBL was not permitted because such binding would decrease the amount of CBL available to bind EGFR. With these changes, CBL and MIG6 become equally capable of driving EGFR internalization. As expected, when the MIG6 knockdown and MIG6/SPRY2 knockdown data were excluded, combinations of $k_{i,MIG6}$ and $k_{i,CBL}$ with the same total sum fit the data equally well, indicating that MIG6 and CBL contribute equally in describing saturable EGFR endocytosis for this control case (Figure 3-6C). Thus, for this scenario, there is no single error minimum, but a family of minima that minimize the error equally. A similar result would be obtained if the SPRY2 knockdown data were excluded instead of MIG6 knockdown data. When the entire data set including the MIG6 knockdown data was considered (keeping MIG6 and CBL parameters and concentrations equal and without SPRY2 binding), the model again fit a $k_{i,MIG6} > k_{i,CBL}$ (Figure 3-6D). Similar error plots for H1666 data are shown in Figure 3-S8. Thus, the magnitude of change in EGFR $k_e$ due to MIG6 knockdown requires that $k_{i,MIG6}$ be larger than $k_{i,CBL}$, even when CBL and MIG6 levels and binding parameters are considered equal. If EGFR expression did not decrease with SPRY2 knockdown, however, the best fit value for $k_{i,CBL}$ would increase by ~2-fold for both H1666 and PC9 data (Figure 3-S9). Without changes in EGFR expression, however, the model error is increased.

To summarize, optimally fitting the PC9 data requires a higher value of $k_{i,MIG6}$ than $k_{i,CBL}$.
for several reasons. Internalization by CBL and MIG6 are both saturated at high EGFR expression. If all factors such as binding rates and expression levels were equivalent, MIG6 and CBL would be equally capable of driving EGFR internalization and any combination of MIG6 and CBL internalization rates that yield the correct rate to match changes in $k_e$ due to changes in EGFR expression would fit the data. However, the magnitude of the decrease in EGFR $k_e$ due to MIG6 knockdown, along with the fact that EGFR expression is reduced by SPRY2 knockdown, require MIG6 internalization to contribute more than CBL mediated internalization. Furthermore, perturbations to specific parameters and species concentrations used can increase the difference between the fitted $k_{i,MIG6}$ and $k_{i,CBL}$ values, but our robustness calculations find that $k_{i,MIG6}$ is still likely to be greater than $k_{i,CBL}$ when parameters are altered.

**Model fit to EGFR degradation data and differences in EGFR recycling.** Cells expressing mutant EGFR do not display normal rapid EGFR degradation upon treatment with EGF (Yang et al.). EGFR degradation was quantified in H1666 and PC9 cells upon treatment with 10 ng/mL EGF by examining total EGFR levels by western blot. After 2 hours of EGF treatment, ~80% of EGFR in wild-type expressing H1666 cells had disappeared (Figure 3-7A). However, there was no measurable degradation of mutant EGFR in PC9 cells. The model parameter that determines sorting of internalized EGFR to recycling or degradation is the sorting fraction ($f_r$). An $f_r = 0$ or 1 indicates that all receptors are degraded or recycled, respectively. All model simulations described above for PC9 and H1666 cells were performed with $f_r = 0.5$, a value chosen based on measurements typical of other cell lines (French et al., 1994; Hendriks et al., 2003). With these conditions and the previously fit $k_i$ values, the model predicted far too much degradation for PC9 cells, but did a reasonable job for H1666 cells (Figure 3-7B). Therefore, differences in internalization rates alone cannot explain the differences in EGFR stability between wild-type and mutant EGFR-expressing cells. We previously measured $f_r$ in H1666 and PC9 cells and found that $f_r$ was 0.57 ± 0.024 for H1666 cells and 0.90 ± 0.013 for PC9 cells (Walsh and Lazzara, 2013). When the model $f_r$ was set to match experimental measurements, the simulated EGFR degradation fit experimental data much better (Figure 3-7C). Note that if the $k_i$ parameters are
refit using the experimentally measured $f_r$ values for H1666 and PC9 cells that the resulting values are not substantially different from those shown in Figures 3-2 and 3-3 (Figure 3-S10).

The higher sorting fraction in PC9 cells could have an effect on the measured EGFR $k_e$. Indeed, the parameter sensitivity analysis shown in Figure 3-5A suggests that the simulated $k_e$ is moderately sensitive to changes in parameters associated with recycling. Keeping other parameters constant for H1666 and PC9 parameters, $f_r$ was varied and the simulated $k_e$ calculated (Figure 3-7D). The simulated $k_e$ decreased as $f_r$ increased, but the simulated $k_e$ for PC9 cells never reached the value for H1666 cells. This result suggests that differences in recycling are not explanatory of the low EGFR $k_e$ in mutant EGFR-expressing cells.

**Figure 3-7.** EGF-mediated EGFR degradation in wild-type and mutant EGFR-expressing NSCLC cells and the role of EGFR recycling differences in explaining the data. 
(A) Whole cell lysates from PC9 and H1666 cells treated with EGF were probed by western blotting with antibodies against EGFR and actin. (B) Experimental data are compared to simulations of EGFR degradation in response to 10 ng/mL EGF with the sorting fraction ($f_r$) set to 0.5 for both H1666 and PC9 parameters. Western blot band intensities from three independent experiments were quantified and normalized to the $t = 0$ point. Markers represent mean values ± s.e.m. (C) Experimental data are compared to simulations of EGFR degradation in response to 10 ng/mL EGF with $f_r$ set to experimentally measured values for the two cell lines. (D) EGFR $k_e$ was simulated over the full range of $f_r$ for H1666 and PC9 parameters.
3.5. Discussion

By considering the specific roles of SPRY2 and MIG6 in the regulation of EGFR endocytosis, the model developed here allows for a new level of quantitative understanding of how ligand-mediated EGFR endocytosis occurs in the wild-type setting and how endocytic flux may be perturbed for specific cancer-relevant EGFR mutations. The realistic development of these models depends on the availability of quantitative EGFR trafficking data, such as the data we previously gathered in NSCLC cell lines where alterations in SPRY2, MIG6, and EGFR expression had been engineered. In addition to endocytosis, we examined differences in EGFR recycling between the cell lines examined that were critical for explaining apparent differences in EGF-mediated EGFR degradation.

We found that MIG6-mediated internalization was comparable to or more important than CBL-mediated internalization in both cell lines examined, and this result was robust to fluctuations in model parameters. Setting CBL expression to greatly exceed MIG6 expression can force a situation where CBL-mediated internalization dominates, but only at the cost of model agreement with experimental data. These results suggest that, at least in the context of the NSCLC cells examined, MIG6-mediated internalization is a major component of overall EGFR endocytosis and should be further studied to determine the extent to which the internalization function of MIG6 may play a role in tumor suppression. Because MIG6 functions to inhibit EGFR tyrosine kinase activity, another important implication of our findings is that a significant fraction of internalized EGFR could be unable to signal within the cell interior.

The results presented here also indicate that MIG6-driven EGFR internalization is impaired in mutant EGFR-expressing cells relative to wild-type EGFR-expressing cells. There are several possible explanations for this finding. A previous in vitro study found that MIG6 does not bind mutant EGFR as well as wild-type EGFR (Wang et al.). Because MIG6 binds EGFR at the asymmetric dimer interface, (Zhang et al., 2006; Zhang et al., 2007a) it is possible that this site is not as accessible in mutant EGFR. A second possibility is that MIG6 phosphorylation, which is elevated in NSCLC cells expressing mutant EGFR (Guo et al., 2008), antagonizes MIG6’s ability
to facilitate EGFR internalization. A recent study found that depletion of the Chk1, a kinase that phosphorylates MIG6 on Ser251, reduced MIG6 phosphorylation and decreased phosphorylation of EGFR (Liu et al.). This study suggests that MIG6 phosphorylation impairs MIG6’s ability to inhibit EGFR kinase activity, but it is unknown whether phosphorylation also reduces MIG6-mediated EGFR internalization. Another explanation for our modeling results could be that some component not explicitly modeled, but whose function is lumped into the MIG6 internalization parameter, is impaired. This missing component could be a different part of internalization machinery that affects multiple internalization pathways.

Another important result is that model fits are consistent with experimental studies showing that CBL/EGFR associations are impaired for EGFR mutants (Padron et al., 2007; Walsh and Lazzara, 2013). Yet, our experimental and model results suggest that SPRY2, which can regulate EGFR endocytosis by sequestering CBL, exerts control over mutant EGFR internalization. This apparent paradox is resolved by our demonstration that SPRY2 can also regulate EGFR endocytosis by regulating EGFR expression levels. This same mechanism of SPRY2-mediated control of \( k_e \) was also identified in H1666 cells expressing wild-type EGFR.

While it is tempting to propose that the reduced overall rates of EGF-induced EGFR mutant internalization explain the significant impairment of EGF-mediated EGFR mutant degradation that we and others have noted, our quantitative model demonstrates that EGFR mutants must also recycle more efficiently than wild-type receptors to explain experimental data. Indeed, when the sorting fraction was set equal for PC9 and H1666 parameters (using a value typical for wild-type EGFR), the model predicted far too much degradation in PC9 cells over a 2 hr period, even with the reduced mutant endocytosis rate. Molecular mechanisms that could explain differential sorting of EGFR mutants have not been extensively researched, but our results highlight the need for further studies on the mechanisms of EGFR sorting and their potential role in aberrant signaling in disease.
3.6. Conclusion

We created a mechanistic model of EGFR trafficking that captures EGFR internalization and degradation dynamics in NSCLC cells expressing wild-type or mutant EGFR through consideration of EGFR’s ability to internalize through parallel pathways. We find that MIG6 is a key mediator of EGFR internalization for wild-type and mutant EGFR, but that MIG6’s apparently impaired ability to drive mutant EGFR internalization underlies the overall impaired ability of mutant EGFR, which also fails to bind CBL efficiently, to undergo ligand-mediated endocytosis. Our analysis further demonstrates that large differences in wild-type and mutant EGFR recycling are also required to explain reduced EGF-mediated degradation of EGFR mutants, but that these differences in recycling do not account for reduced EGFR internalization rate constants. Our method for modeling distinct EGFR internalization pathways could be included in future models of EGFR dynamics and used to gain insight into the trafficking dynamics of other receptors.

3.7. Acknowledgements

Dr. Douglas Lauffenburger (MIT) generously provided reagents for this work.

3.8. Supplemental Materials

*Cell lysis and western blotting.* Whole cell lysates were prepared in a standard cell extraction buffer (Life Technologies) supplemented with protease and phosphatase inhibitors (Sigma). Lysates were cleared by centrifugation at 13,200 rpm for 10 min, and total protein concentrations were determined by micro-bicinchoninic assay (Thermo Scientific). Approximately 20 μg of total protein was loaded per lane on 4-12% gradient polyacrylamide gels (Life Technologies) under denaturing and reducing conditions and transferred to 0.2 μm nitrocellulose membranes (Life Technologies). After probing with antibodies, membranes were imaged on a LI-COR Odyssey scanner (LI-COR). Membranes were stripped with 0.2 M NaOH as needed.

*Estimation of number of EGFR per cell.* Recombinant human EGF (Peprotech) was
labeled with $^{125}\text{I}$ as described previously (Walsh and Lazzara, 2013). Cells were starved overnight in media containing 0.1% FBS (Life Technologies) and then treated with 10 ng/mL $^{125}\text{I}$-EGF on ice for 30 min. After washing with buffer to remove un-bound $^{125}\text{I}$-EGF, the amount of cell surface-associated radioactivity was quantified by stripping surface-bound ligand from receptors using a mild acid strip. These samples were used to calculate the number of EGFR per cell based on the known EGF/EGFR dissociation constant and the specific activity of the labeled EGF. Three plates were reserved to determine the number of cells per plate by counting with a hemocytometer.

Figure 3-S1. Measurement of number of EGFR per cell in PC9 cells.

The number of EGFR per cell in PC9 cells was calculated using $^{125}\text{I}$-EGF binding as described in *Supplemental Materials and Methods*. Data represents the mean of three replicates ± s.d.
Figure 3-S2. Simulation of SPRY2 phosphorylation.

SPRY2 phosphorylation with 10 ng/mL EGF treatment was simulated over the time course of an EGFR $k_e$ measurement. Base model conditions with standard parameters for H1666 cells were used. SPRY2 phosphorylation parameters were estimated to agree with data from Mason et al. (Mason et al., 2004) such that peak SPRY2 phosphorylation occurred by 3 min after EGF addition.
Figure 3-S3. Predicted and experimental measurements of internal and surface-bound EGF for H1666 cells.

The $k_i$ parameters were fit to $k_e$ data from H1666 cells as described in Figure 3-2A. Shown here are the primary experimental data from the study by Walsh and Lazzara (Walsh and Lazzara, 2013) used to experimentally determine $k_e$ values and the ability of the model to recapitulate the dynamics of surface and internal $^{125}$I-EGF. Markers represent the mean of three experimental replicates ± s.d.
Figure 3-S4. Values of fitted rate constants for a range of MIG6 and CBL concentrations. The $k_i$ parameters were fit to data from (A) H1666 cells or (B) PC9 cells as described in Figures 3-2C and 3-3C. Stars indicate the base MIG6 and CBL concentrations ([MIG6] = 5×10^4 cell$^{-1}$ (H1666) or 1.2×10^5 cell$^{-1}$ (PC9) and [CBL] = 1×10^5 cell$^{-1}$).
Figure 3-S5. EGFR internalization flux over a range of MIG6 and CBL concentrations.
EGFR flux is plotted for receptors internalized by MIG6 or CBL pathways at $t = 3.5$ min or 7 min for (A) H1666 cells and (B) PC9 cells. Stars indicate the base MIG6 and CBL concentrations ($[\text{MIG6}] = 5 \times 10^4$ cell$^{-1}$ (H1666) or $1.2 \times 10^5$ cell$^{-1}$ (PC9) and $[\text{CBL}] = 1 \times 10^5$ cell$^{-1}$).
Figure 3-S6. Effect of allowing basal MIG6/EGFR association on model fit to PC9 data.
The $k_i$ parameters were fit to data from PC9 cells as in Figure 3-3 and then by allowing basal MIG6/EGFR association for non-ligand-bound EGFR dimers using standard MIG6 and CBL concentrations.

Figure 3-S7. Predicted effect of changing dimerization rate on relationship between EGFR expression and predicted EGFR $k_e$.
For parameters fit to data from H1666 cells, $k_{dim}$ was set to values between $10^{-3}$ and $10^{-7}$ cell $\text{#}^{-1}$ s$^{-1}$, and the predicted $k_e$ was calculated for a range of EGFR concentrations as in Figure 2-4A. This demonstrates that the increase in $k_e$ with increasing EGFR expression arises due to an increased driving force for EGFR dimerization.
Figure 3-S8. Model agreement with H1666 data.

Model error was calculated considering: (A) all data points with normal model conditions, (B) data excluding MIG6 knockdown data with $[\text{MIG6}] = [\text{CBL}] = 1 \times 10^5 \text{ cell}^{-1}$, $k_{\text{on, MIG6}} = k_{\text{on, CBL}}$, and $k_{\text{on, S}} = 0$, and (C) the same conditions for C including all data. The log of the sum of the squares error (SSE) is plotted for a range of $k_{i, \text{MIG6}}$ and $k_{i, \text{CBL}}$. Red circles indicate error minima. The dashed lines represent $k_{i, \text{MIG6}} = k_{i, \text{CBL}}$.

Figure 3-S9. Fitted parameters when changes in EGFR expression due to SPRY2 knockdown are not considered.

The $k_i$ parameters were fit to experimental EGFR $k_s$ data for controls, MIG6 knockdown, and SPRY2 knockdown from H1666 or PC9 cells using standard MIG6 and CBL concentrations as described for Figs. 2 and 3. The $k_i$ parameters were also fit without changing EGFR concentration for SPRY2 knockdown conditions.
Figure 3-S10. Values of fitted rate constants when $f_r$ was set to experimentally determined values.

The $k_i$ parameters were fit to all data points from H1666 or PC9 cells using standard MIG6 and CBL concentrations ([MIG6] = 5×10^4 cell⁻¹ (H1666) or 1.2×10^5 cell⁻¹ (PC9) and [CBL] = 1×10^5 cell⁻¹) and setting $f_r$ to experimentally determined values.
### Table 3-S1. Model equations for individual species.

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<td>k29<em>RL - kr</em>Rli*fr</td>
<td>Internalization/recycling</td>
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<tr>
<td>R30</td>
<td>k30<em>D- kr</em>D<em>i</em>fr</td>
<td>Internalization/recycling</td>
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</tr>
<tr>
<td>R31</td>
<td>k31<em>DL - kr</em>Dli*fr</td>
<td>Internalization/recycling</td>
<td></td>
</tr>
<tr>
<td>R32</td>
<td>k32<em>DLL - kr</em>Dli*fr</td>
<td>Internalization/recycling</td>
<td></td>
</tr>
<tr>
<td>R37</td>
<td>k37<em>DLLM - kr</em>Dli*fr</td>
<td>Internalization/recycling</td>
<td></td>
</tr>
<tr>
<td>R39</td>
<td>k39<em>DLLM - kr</em>Dli*fr</td>
<td>Internalization/recycling</td>
<td></td>
</tr>
<tr>
<td>R41</td>
<td>kd<em>Ri</em>(fd)</td>
<td>Degradation</td>
<td></td>
</tr>
<tr>
<td>R43</td>
<td>kd<em>Rli</em>(fd)</td>
<td>Degradation</td>
<td></td>
</tr>
<tr>
<td>R44</td>
<td>kd<em>Dli</em>(fd)</td>
<td>Degradation</td>
<td></td>
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<td>R45</td>
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</tr>
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<td>R46</td>
<td>kd<em>Dli</em>(fd)</td>
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<td></td>
</tr>
<tr>
<td>R51</td>
<td>kd<em>DLLMi</em>(fd)</td>
<td>Degradation</td>
<td></td>
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<tr>
<td>R53</td>
<td>kd<em>DLLMi</em>(fd)</td>
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</tr>
<tr>
<td>R56</td>
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<td>2<em>f57</em>DL<em>C - r57</em>DLC</td>
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<tr>
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<td>CBL binding</td>
<td></td>
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<tr>
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<td>k59<em>DL</em>C - kr<em>DLCi</em>fr</td>
<td>Internalization/recycling</td>
<td></td>
</tr>
<tr>
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<td>k60<em>DLLC - kr</em>DLCi*fr</td>
<td>Internalization/recycling</td>
<td></td>
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<tr>
<td>R61</td>
<td>kd<em>DLCi</em>(fd)</td>
<td>Degradation</td>
<td></td>
</tr>
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<td>R62</td>
<td>kd<em>DLCi</em>(fd)</td>
<td>Degradation</td>
<td></td>
</tr>
<tr>
<td>R63</td>
<td>f63<em>C</em>Sp - r63<em>C</em>Sp</td>
<td>CBL binding</td>
<td></td>
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<tr>
<td>R68</td>
<td>f68<em>S</em>kin*(Ligand-bound EGFR dimers/total EGFR) - r68*kinS;</td>
<td>SPRY2/kinase binding</td>
<td></td>
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<tr>
<td>R69</td>
<td>f69*kinS</td>
<td>SPRY2 phosphorylation</td>
<td></td>
</tr>
<tr>
<td>R70</td>
<td>r70*Sp</td>
<td>SPRY2 dephosphorylation</td>
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Table 3-S3. Parameters for model equations.

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<td>EGFR synthesis</td>
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<td>f2, f4, f3, f5</td>
<td>$k_{on,L}$</td>
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</tr>
<tr>
<td>f6, f22, f56</td>
<td>$k_{on,L2}$</td>
<td>$1\times10^5$ M$^{-1}$s$^{-1}$</td>
</tr>
<tr>
<td>f7</td>
<td>$k_{on,L2}$</td>
<td>$2.6\times10^5$ cell #/s$^{-1}$</td>
</tr>
<tr>
<td>f18, f23</td>
<td>$k_{on,L}$</td>
<td>$2\times10^7$ cell #/s$^{-1}$</td>
</tr>
<tr>
<td>r2, r4, r6, r22, r56</td>
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<td>$2.7\times10^{-3}$ s$^{-1}$</td>
</tr>
<tr>
<td>r3, r5, r7</td>
<td>$k_{off,M}$</td>
<td>$1\times10^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>r18, r23</td>
<td>$k_{off,M}$</td>
<td>$1\times10^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>k28</td>
<td>R internalization</td>
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<td>k29</td>
<td>RL internalization</td>
<td>0</td>
</tr>
<tr>
<td>k30</td>
<td>D internalization</td>
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</tr>
<tr>
<td>k31, k32</td>
<td>Other internalization</td>
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</tr>
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<td>k37, k39</td>
<td>MIG6 internalization</td>
<td>$k_{i,MIG6}$ (fitted)</td>
</tr>
<tr>
<td>k59, k60</td>
<td>CBL internalization</td>
<td>$k_{i,CBL}$ (fitted)</td>
</tr>
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<td>$k_{deg}$</td>
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<td>kr</td>
<td>$k_{rec}$</td>
<td>$3.4\times10^{-1}$ s$^{-1}$</td>
</tr>
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<td>fr</td>
<td>$f_r$</td>
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</tr>
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<td>$f_{r, unbound}$</td>
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</tr>
<tr>
<td>f57, f58</td>
<td>$k_{on,C}$</td>
<td>$4\times10^{-5}$ cell #/s$^{-1}$</td>
</tr>
<tr>
<td>r57, r58</td>
<td>$k_{off,C}$</td>
<td>1 s</td>
</tr>
<tr>
<td>f63</td>
<td>$k_{on,S}$</td>
<td>$1\times10^{-5}$ cell #/s$^{-1}$</td>
</tr>
<tr>
<td>r63</td>
<td>$k_{off,S}$</td>
<td>$1\times10^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>f68</td>
<td>SPRY2/kinase $k_{on}$</td>
<td>$1\times10^{-5}$ cell #/s$^{-1}$</td>
</tr>
<tr>
<td>r68</td>
<td>SPRY2/kinase $k_{off}$</td>
<td>$1\times10^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>f69</td>
<td>SPRY2 phosphorylation</td>
<td>$1\times10^{-1}$ s$^{-1}$</td>
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<tr>
<td>r70</td>
<td>SPRY2 dephosphorylation</td>
<td>$1\times10^{-3}$ s$^{-1}$</td>
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Table 3-S4. Parameter values based on literature.

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<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference</th>
</tr>
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<tr>
<td>$k_{on,L}$ [M$^{-1}$s$^{-1}$]</td>
<td>1×10$^6$</td>
<td>(Berkers et al., 1991; Felder et al., 1992; French et al., 1995)</td>
</tr>
<tr>
<td>$k_{off,L}$ [s$^{-1}$]</td>
<td>2.7×10$^{-3}$</td>
<td>(Berkers et al., 1991; Felder et al., 1992; French et al., 1995)</td>
</tr>
<tr>
<td>$k_{on,L2}$ [M$^{-1}$s$^{-1}$]</td>
<td>1×10$^5$</td>
<td>(Berkers et al., 1991; Felder et al., 1992; Macdonald-Obermann and Pike, 2009)</td>
</tr>
<tr>
<td>$k_{+dim}$ [cell #$^{-1}$s$^{-1}$]</td>
<td>2.6×10$^{-8}$</td>
<td>(Macdonald-Obermann and Pike, 2009)</td>
</tr>
<tr>
<td>$k_{+dim2}$ [cell #$^{-1}$s$^{-1}$]</td>
<td>2.6×10$^{-5}$</td>
<td>(Kholodenko et al., 1999; Monast et al., 2012; Schoeberl et al., 2009)</td>
</tr>
<tr>
<td>$k_{-dim}$ [s$^{-1}$]</td>
<td>1×10$^{-1}$</td>
<td>(Kholodenko et al., 1999; Schoeberl et al., 2009)</td>
</tr>
<tr>
<td>$k_{deg}$ [s$^{-1}$]</td>
<td>6×10$^{-4}$</td>
<td>(Hendriks et al., 2006; Hendriks et al., 2003; Schoeberl et al., 2009)</td>
</tr>
<tr>
<td>$k_{rec}$ [s$^{-1}$]</td>
<td>3.4×10$^{-3}$</td>
<td>(Hendriks et al., 2006; Hendriks et al., 2003; Schoeberl et al., 2009)</td>
</tr>
<tr>
<td>Cell volume [L]</td>
<td>5.2×10$^{-13}$</td>
<td>Calculated</td>
</tr>
<tr>
<td>H1666 $f_r$</td>
<td>0.574</td>
<td>(Walsh and Lazzara, 2013)</td>
</tr>
<tr>
<td>PC9 $f_r$</td>
<td>0.899</td>
<td>(Walsh and Lazzara, 2013)</td>
</tr>
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</table>
Table 3-S5. Estimated parameter values and initial model species concentrations.

<table>
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<tr>
<th>Parameter</th>
<th>H1666</th>
<th>PC9</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIG6 [# cell⁻¹]</td>
<td>1.2×10⁵</td>
<td>5×10⁴</td>
<td>Estimated and western blotting (Walsh and Lazzara, 2013)</td>
</tr>
<tr>
<td>SPRY2 [# cell⁻¹]</td>
<td>5×10⁴</td>
<td>5×10⁴</td>
<td>Estimated and western blotting (Walsh and Lazzara, 2013)</td>
</tr>
<tr>
<td>CBL [# cell⁻¹]</td>
<td>1×10⁸</td>
<td>1×10⁸</td>
<td>Estimated</td>
</tr>
<tr>
<td>EGFR [# cell⁻¹]</td>
<td>6×10⁵</td>
<td>8×10⁵</td>
<td>Based on ²²⁵I-EGF binding and western blotting (Walsh and Lazzara, 2013)</td>
</tr>
<tr>
<td>EGFR (SPRY2 KD) [# cell⁻¹]</td>
<td>3.6×10⁵</td>
<td>4×10⁵</td>
<td>(Walsh and Lazzara, 2013)</td>
</tr>
<tr>
<td>EGFR (SPRY2 KD+EGFR) [# cell⁻¹]</td>
<td>1.2×10⁶</td>
<td>8×10⁵</td>
<td>(Walsh and Lazzara, 2013)</td>
</tr>
<tr>
<td>SPRY2 kinase [# cell⁻¹]</td>
<td>1×10⁵</td>
<td>1×10⁵</td>
<td>Estimated</td>
</tr>
<tr>
<td>(k_{on,basal} [s⁻¹])</td>
<td>3.8×10⁻⁴</td>
<td>3.8×10⁻⁴</td>
<td>fitted</td>
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<tr>
<td>(k_{off,C} [cell #⁻¹ s⁻¹])</td>
<td>4×10⁻⁶</td>
<td>4×10⁻⁶</td>
<td>(Hsieh et al., 2010; Ng et al., 2008; Nguyen et al., 2000)</td>
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<tr>
<td>(k_{eff,C} [s⁻¹])</td>
<td>1</td>
<td>1</td>
<td>(Hsieh et al., 2010; Ng et al., 2008; Nguyen et al., 2000)</td>
</tr>
<tr>
<td>(k_{on,M} [cell #⁻¹ s⁻¹])</td>
<td>2×10⁻⁹</td>
<td>2×10⁻⁹</td>
<td>(Zhang et al., 2007a)</td>
</tr>
<tr>
<td>(k_{off,M} [s⁻¹])</td>
<td>1</td>
<td>1</td>
<td>(Zhang et al., 2007a)</td>
</tr>
<tr>
<td>(k_{on,S} [cell #⁻¹ s⁻¹])</td>
<td>1×10⁻⁹</td>
<td>1×10⁻⁹</td>
<td>(Ng et al., 2008)</td>
</tr>
<tr>
<td>(k_{off,S} [s⁻¹])</td>
<td>1×10⁻¹</td>
<td>1×10⁻¹</td>
<td>(Ng et al., 2008)</td>
</tr>
<tr>
<td>SPRY2/kinase binding</td>
<td>(k_{on} = 1×10⁻⁸) s⁻¹ cell⁻¹; (k_{off} = 1×10⁻¹) s⁻¹</td>
<td>(k_{on} = 1×10⁻⁸) s⁻¹ cell⁻¹; (k_{off} = 1×10⁻¹) s⁻¹</td>
<td>(Kholodenko et al., 1999; Northrup and Erickson, 1992)</td>
</tr>
<tr>
<td>SPRY2 phosphorylation (k_{cell} [s⁻¹])</td>
<td>1×10⁻¹</td>
<td>1×10⁻¹</td>
<td>Estimated based on Mason 2004 (Mason et al., 2004)</td>
</tr>
<tr>
<td>SPRY2 dephosphorylation [s⁻¹]</td>
<td>1×10⁻³</td>
<td>1×10⁻³</td>
<td>Estimated based on Mason 2004 (Mason et al., 2004)</td>
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Table 3-S6. Normalized experimental $k_e$ data.

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<tr>
<td>$k_e$ (min$^{-1}$) control</td>
<td>0.170</td>
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<tr>
<td>$k_e$ (min$^{-1}$) MIG6 KD</td>
<td>0.116</td>
<td>0.034</td>
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<tr>
<td>$k_e$ (min$^{-1}$) SPRY2 KD</td>
<td>0.219</td>
<td>0.091</td>
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<tr>
<td>$k_e$ (min$^{-1}$) MIG6/SPRY2 KD</td>
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<td>0.038</td>
</tr>
<tr>
<td>$k_e$ (min$^{-1}$) SPRY2 KD + EGFR</td>
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<td>0.058</td>
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Table 3-S7. Full results of local parameter sensitivity analysis.

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<td>$k_{off,kinS}$</td>
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<td>$k_{i,other}$</td>
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<td>$k_{dephos}$</td>
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<td>$k_{dephos}$</td>
<td>1.96×10⁻⁵</td>
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<td>$k_{on,kinS}$</td>
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<td>$k_{cat,S}$</td>
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<td>$k_{cat,S}$</td>
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<td>$k_{off,kinS}$</td>
<td>8.73×10⁻⁵</td>
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<td>$k_{on,L2}$</td>
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<td>$k_{on,kinS}$</td>
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</tr>
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<td>$f_{r,unbound}$</td>
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<td>$k_{on,S}$</td>
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<td>$k_{off,L}$</td>
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<td>$k_{+dim2}$</td>
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<tr>
<td>$k_{+dim}$</td>
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<td>$k_{off,C}$</td>
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<td>$k_{deg}$</td>
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<td>$k_{i,MIG6}$</td>
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<td>$k_{i,MIG6}$</td>
<td>0.629</td>
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Chapter 4: Sprouty2 drives drug resistance and proliferation in glioblastoma

4-1. Abstract

Due to the heterogeneity of cellular protein expression profiles in glioblastoma multiforme (GBM), the discovery of durable cures will require identification of broadly relevant regulators of tumorigenicity and survival. Here, we identify Sprouty2 (SPRY2) as one such regulator. SPRY2 knockdown reduced proliferation in GBM cell lines and in mouse xenografts. Additionally, SPRY2 knockdown reduced anchorage-independent growth and enhanced cell death response to co-inhibition of EGFR and c-MET in cell lines, an effect that appears to involve regulation of the ability of the p38 mitogen activated protein kinase to drive cell death. Furthermore, analysis of data from human and rat tumors demonstrated that SPRY2 expression is elevated in especially aggressive GBMs (i.e., those expressing epidermal growth factor receptor variant III) and that elevated SPRY2 expression portends reduced GBM patient survival. Our results identify SPRY2 and the pathways it regulates as biomarkers for response to therapy and potentially useful druggable targets in GBM.

4-2. Introduction

Glioblastoma multiforme (GBM) is the most common and deadliest form of brain cancer (Louis et al., 2007). Standard treatment includes surgical resection, radiotherapy, and chemotherapy, resulting in an average survival of 12-15 months (Wen and Kesari, 2008). Targeted approaches based on the molecular pathogenesis of GBM have shown some promise in clinical trials, but have failed to demonstrate significant benefit over traditional treatments (De Witt Hamer, 2010; Sathornsumetee et al., 2007). A significant challenge limiting the effectiveness

3 The work presented in this chapter has been submitted for peer-reviewed publication, with Janine M. Buonato, Lijoy K. Matthew, Gurpreet S. Kapoor, Yingtao Bi, Ramana V. Davuluri, M. Celeste Simon, Donald M. O’Rourke, and Matthew J. Lazzara as co-authors. The human and rat tumor gene expression studies we performed by GSK and DMO. The TCGA analysis was performed by AMW, YB, and RVD. The mouse xenograft studies were performed by JMB, LKM, and MCS. The GSC drug treatment was performed by JMB. All other studies were performed by AMW and the manuscript was written by AMW and MJL.
of available GBM treatments is diffuse spreading of GBM tumors and heterogeneity in the expression profiles of GBM cells (Bonavia et al., 2011; Claes et al., 2007). Therefore, identification of broadly relevant regulators of tumorigenicity and survival is necessary to improve GBM patient outcomes. Here, we examine the expression and function of one such signaling regulator, Sprouty2 (SPRY2), in GBM.

SPRY2 is a regulator of receptor tyrosine kinase signaling whose expression is promoted by extracellular signal-regulated kinase (ERK) activity (Egan et al., 2002; Reich et al., 1999), and whose most well-characterized role is regulation of ERK (Yusoff et al., 2002). However, studies in different systems have demonstrated varied, and sometimes conflicting, roles for SPRY2. SPRY2 inhibits ERK downstream of several receptor tyrosine kinases through regulation of RAS, by preventing growth factor receptor-bound protein 2 (GRB2)-son of sevenless (SOS) binding (Hanafusa et al., 2002) or by preventing RAS activation downstream of GRB2-SOS (Gross et al., 2001), or through regulation of RAF (Yusoff et al., 2002), depending on the cellular context. In contrast, SPRY2 can also potentiate epidermal growth factor (EGF)-induced ERK activation by interfering with CBL-mediated EGF receptor (EGFR) downregulation (Wong et al., 2002). Downstream of the fibroblast growth factor receptor, SPRY2 specifically inhibits ERK activation, without effect on p38 or JUN N-terminal kinase (JNK) activation, two other members of the mitogen-activated protein (MAP) kinase family (Yusoff et al., 2002). However, results showing that interferon-stimulated p38 phosphorylation is enhanced in SPRY1/SPRY2/SPRY4 knockout murine embryonic fibroblasts (Sharma et al., 2012) could indicate a broader role for SPRY2 in MAP kinase regulation.

Given that the signaling regulatory functions of SPRY2 appear to be highly context-dependent, it is difficult to predict SPRY2’s regulatory role in GBM, where the functional role of SPRY2 has not been explored in any detail. In several other human cancers, SPRY2 expression is reduced compared to normal tissue, and SPRY2 has been proposed to function as a tumor suppressor (Fong et al., 2006b; Lo et al., 2006; Sutterluty et al.). In addition, SPRY2 is downregulated by microRNA-21, which is elevated in some cancers, including GBM (Kwak et al.,
SPRY2 promotes cellular resistance to EGFR tyrosine kinase inhibitors in lung cancer cells (Walsh and Lazzara, 2013), but enhances cellular response to EGFR inhibition in colon cancer cells (Feng et al., 2010), highlighting the notion that SPRY2 function is highly context-dependent.

Here, we investigated the expression and function of SPRY2 in GBM cell lines, mouse xenografts, and human tumor samples. In a panel of GBM cell lines, SPRY2 knockdown reduced proliferation, antagonized colony formation in soft agar, and potentiated response to EGFR and c-MET co-inhibition. Interestingly, SPRY2’s control of cell death response to inhibitors appears to involve regulation of the ability of p38, which is phosphorylated in response to EGFR and c-MET co-inhibition, to promote cell death. Moreover, in some cells, SPRY2 controls p38 activity directly through regulation of the expression of the dual specificity phosphatases MKP-1 and MKP-5. In a mouse xenograft model, SPRY2 knockdown highly impaired tumor proliferation. Analysis of gene expression from primary human tumor samples and rat tumor allografts revealed that SPRY2 is upregulated in EGFR variant III (EGFRvIII)-positive tumors compared to EGFRvIII-negative tumors. This is of interest because EGFRvIII is present in 41-67% of GBMs with EGFR amplification (Aldape et al., 2004; Frederick et al., 2000; Mellinghoff et al., 2005), promotes an invasive and proliferative GBM cell phenotype (Koochekpour et al., 1997; Nishikawa et al., 1994), and is associated with poor prognosis for patients with EGFR amplification (Shinojima et al., 2003). SPRY2 protein expression was confirmed by immunohistochemical analysis of human tumor sections. Further demonstrating an important role for SPRY2 in GBM, we find that elevated SPRY2 expression portends reduced patient survival in The Cancer Genome Atlas (TCGA) GBM data set, regardless of EGFRvIII status. Overall, our study identifies SPRY2 and the pathways it regulates as useful prognostic biomarkers and candidate therapeutic targets in GBM.

4-3. Materials and Methods

Cell culture. Parental U87MG cells and U87MG cells and U373MG cells with EGFRvIII expression or expression of a kinase dead EGFRvIII were described previously (Huang et al.,
1997; Huang et al., 2007) and were provided by Dr. Frank Furnari (UCSD, La Jolla, CA, USA). U251 and SF188 cells were a gift from Dr. Celeste Simon (University of Pennsylvania, Philadelphia, PA, USA). LN18, T98G, 9L, and U118MG cells were obtained from the American Type Culture Collection. All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 1 mM L-Glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). Gefitinib, U0126, SB203580, SP600125 (all from LC Laboratories), and PHA665752 (Santa Cruz Biotechnology) were reconstituted in DMSO and added to cells in complete plates media. To measure cellular proliferation, cells were seeded at 50,000 cells per well in 6-well plates and counted with a hemocytometer 5 days later.

**Knockdown of SPRY2, MKP-1, and MKP-5.** Oligonucleotides encoding hairpins targeting nucleotides 2061-2079 (main sequence used; shRNA #1) or 1195-1213 (shRNA #2) of human SPRY2, nucleotides 2041-2059 of human MKP-1, or nucleotides 935-953 of human MKP-5 were purchased from IDT and inserted into pSicoR.puro (Dr. Tyler Jacks, MIT, Cambridge, MA, USA; Ventura et al., 2004). A control shRNA was created using a hairpin that does not target a known human mRNA. Lentivirus was produced by calcium-phosphate-mediated transfection of 293FT cells (Life Technologies) with pSicoR.puro, pCMV-VSVg, pMDL-gp-RRE, and pRSV-Rev plasmids (Dr. Marilyn Farquhar, UCSD, La Jolla, CA, USA). Virus-containing supernatant was filtered and target cells were selected in 1-2 μg/mL puromycin (Sigma).

**Retroviral protein expression.** MEK2DD cDNA in the pBabe.puro vector was provided by Dr. Sylvain Meloche (Université de Montréal, Montreal, Quebec, Canada). Y55F SPRY2 cDNA (Dr. Dafna Bar-Sagi, NYU, New York, NY, USA) and MKK3 cDNA (Dr. Margaret Chou, University of Pennsylvania, Philadelphia, PA, USA) were sub-cloned into pBabe.hygro For all constructs, retrovirus was produced by calcium-phosphate-mediated transfection of amphotropic Phoenix cells (Dr. Gary Nolan, Stanford University, Stanford, CA, USA). Virus-containing supernatant was filtered and target cells were selected in 150 μg/mL hygromycin B (Sigma) or 2 μg/mL puromycin (Sigma).

**Tumor xenografts.** 8 female NIH-III mice (Charles River) were subcutaneously injected in
each flank with either 2 million control (left side) or SPRY2-depleted (right side) U87MG-L cells. Tumors were measured with a caliper starting 7 days later when measurable tumors had formed and every 2-3 days afterwards. Tumor volume was calculated as $\pi/6 \times A \times B^2$, where $A$ and $B$ are the larger and smaller tumor diameters, respectively. After final caliper measurements, animals were sacrificed and dissected tumors from the 6 animals with both control and SPRY2 knockdown tumors were weighed. All experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and performed in accordance with NIH guidelines.

**Western blotting.** Whole cell lysates were prepared and western blotting performed as described previously (Walsh and Lazzara, 2013). Details, including information on antibodies used, are provided in Supplemental Materials and Methods.

**Flow cytometry.** Floating and adherent cells were pooled and stained with ToPro3 (Life Technologies). Cells were analyzed using a Becton Dickinson FACS-Calibur cytometer.

**Quantitative real-time PCR.** RNA was extracted from cells using the RNeasy kit (Qiagen) with on-column DNase I digestion. Equal amounts of RNA were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). PCR was performed using SYBR Green PCR Master Mix (Life Technologies) on an Applied Biosystems 7300 Real-Time PCR System. Relative amounts of mRNA were determined using the comparative $C_T$ method.

**Anchorage-independent growth assay.** 5,000 cells were seeded per 35 mm dish in 0.3% low melting temperature agarose (Lonza) on top of a bottom layer of 0.6% agarose. Media was replaced every three days with or without inhibitors. At three weeks, plates were stained with 0.1% crystal violet (Sigma) and colonies were counted.

**Immunohistochemistry.** Immunohistochemistry of formalin fixed paraffin embedded tissue was performed on a Leica Bond instrument using the Bond Polymer Refine Detection System and antibodies against SPRY2 (Sigma, #S1444; 1:200 dilution). Heat-induced epitope retrieval was required for SPRY2 and done for 20 min with ER1 solution (Leica Microsystems).

**Analysis of TCGA GBM data set.** Methods for preprocessing of TCGA exon-array data
A two-sided student’s t-test was applied to determine whether SPRY2 expression varied among GBM subtypes and normal brain samples using TCGA exon-array data. A student’s t-test was also used to determine whether SPRY2 was differentially expressed between EGFRvIII-positive and EGFRvIII-negative samples based on TCGA RNA-seq samples. The upper quartile normalized RSEM (Li and Dewey, 2011) count estimates were base-10 log transformed before the t-test. The R package “survival” was used to analyze the TCGA survival data (Therneau and Grambsch, 2000). The log-rank test was applied to test for differences between the survival curves.

Statistics. Data were analyzed by a student’s t-test, and differences with p-values < 0.05 were considered statistically significant.

Accession numbers. Microarray data presented in this study were deposited in the National Center for Biotechnology Information’s GEO database (GSE51062, GSE51147).

4-4. Results

SPRY2 knockdown slows cellular proliferation and reduces anchorage-independent growth. Using a set of commonly used GBM cell lines, including U87MG GBM cell lines with low (U87MG-L), medium (U87MG-M), or high (U87MG-H) engineered ectopic EGFRvIII expression or high expression of a control dead kinase EGFRvIII (U87MG-DK; K721M) (Huang et al., 2007), we sought to investigate the effects of SPRY2 in GBM cell lines. SPRY2 was depleted by stable expression of SPRY2-targeting shRNA (Figure 4-S1). SPRY2 knockdown reduced cellular proliferation compared to controls in U87MG-DK, U87MG-L, U251, and U118MG cells (Figure 4-1A). A second independent shRNA was expressed in a subset of the cell lines and produced a similar effect (Figure 4-S2A). Colony formation in soft agar was also reduced by > 50% in U87MG-L, U87MG-H, U251, and U118MG cells with SPRY2 knockdown compared to controls (Figure 4-1B). Note that data are not available for U87MG-DK cells due to lack of colony formation. Similar reductions in colony formation were observed in a panel of EGFRvIII-
expressing cell lines transfected with SPRY2 siRNA compared to control siRNA (Figure 4-S2B).

**SPRY2 knockdown enhances cellular sensitivity to EGFR and c-MET co-inhibition.**

Despite frequent EGFR overexpression and mutation in GBM, EGFR kinase inhibitors are not clinically effective in GBM (Dutta and Maity, 2007; Mellinghoff et al., 2005; Thiessen et al., 2010; Vivanco et al., 2012). GBM cell lines are generally resistant to EGFR kinase inhibitors, but co-inhibition of c-MET can augment response (Furcht et al., In press; Huang et al., 2007; Mellinghoff et al., 2005). We thus tested whether SPRY2 knockdown affects cellular response to EGFR and c-MET co-inhibition using the EGFR inhibitor gefitinib and the c-MET inhibitor PHA665752. This is a highly clinically relevant target combination as multiple therapeutics that simultaneously target EGFR and c-MET are currently in pre-clinical and clinical development (Castoldi et al., 2013). In multiple cell lines, the amount of cell death observed was significantly higher with SPRY2 knockdown than for controls (Figure 4-1C). In U87MG-DK cells, SPRY2 knockdown also promoted cleavage of poly ADP ribose polymerase (PARP) in response to EGFR and c-MET co-inhibition compared to controls (Figure 4-1D). The same general effect on response to inhibitors was also observed in parental U87MG cells with stable SPRY2 knockdown and U87MG-DK cells with transient SPRY2 knockdown (Figure 4-S3A, B). Because the expression of functional phosphatase and tensin homolog (PTEN) has been shown to regulate GBM cell response to EGFR inhibition (Mellinghoff et al., 2007; Mellinghoff et al., 2005), we also investigated whether PTEN status influenced the effects of SPRY2 knockdown. Ectopic expression of wild-type PTEN in U87MG cells, which do not express functional PTEN, did not reverse the increased cellular sensitivity to PHA665752 and gefitinib observed with SPRY2 knockdown (Figure 4-S3C). In two cell lines that express wild-type PTEN, LN18 and SF188, SPRY2 knockdown also increased death response to the inhibitors (Figure 4-S3D). Consistent with the effects of SPRY2 knockdown, expression of dominant negative Y55F SPRY2 in U87MG-DK cells also enhanced cellular sensitivity to EGFR and c-MET co-inhibition (Figure 4-1E). This effect also repeated in multiple cell lines expressing a second non-overlapping SPRY2-targeting shRNA (Figure 4-S3E). Enhanced response to EGFR and c-MET inhibitors due to SPRY2 knockdown was also observed
in a glioma stem cell (GSC) cell line maintained in non-adherent sphere culture conditions (Figure 4-S4).

Figure 4-1. SPRY2 knockdown reduces cellular proliferation and anchorage-independent growth and enhances cellular sensitivity to EGFR and c-MET co-inhibition.

(A) Cellular proliferation and (B) colony formation in soft agar were measured with expression of control or SPRY2-targeting shRNA in a panel of GBM cell lines including U87MG cells expressing dead kinase ("DK"), low ("L"), or high ("H") EGFRvIII. (C) The indicated cell lines were treated with DMSO or a combination of PHA665752 and gefitinib ("PHA+gef"; 5 μM PHA665752+10 μM gefitinib for U87MG-DK, U87MG-L, U118MG; 2 μM PHA665752+10 μM gefitinib for U251) for 48 hrs prior to flow cytometry analysis for ToPro3 permeability. (D) U87MG cells expressing DK EGFRvIII and control or SPRY2-targeting shRNA were treated with DMSO or PHA+gef (2 μM PHA665752+20 μM gefitinib) for 72 hrs. Whole cell lysates were probed by western blot using
antibodies against the indicated proteins. (E) Whole cell lysates of U87MG cells expressing DK EGFRvIII and transduced with an empty vector (“EV”) or Y55F SPRY2 were probed by western blot using antibodies against the indicated proteins. Cells were also treated with DMSO or PHA+gef (5 μM PHA665752+10 μM gefitinib) for 48 hrs prior to flow cytometry analysis for ToPro3 permeability. Throughout the panels, data are represented as the average of three independent experiments ± s.e.m., and asterisks indicate $p < 0.05$.

**SPRY2 knockdown inhibits tumor xenograft growth.** Female NIH-III mice were injected subcutaneously with U87MG-L cells with control or SPRY2-targeting shRNA. Tumors arising from SPRY2 knockdown cells were significantly smaller than those arising from control cells at all time points measured (Figure 4-2). Strikingly, SPRY2 knockdown cells failed to give rise to discernable tumors in 2 out of 8 mice. At 16 days post-injection, the average volume and weight of tumors from SPRY2 knockdown cells was approximately one-quarter of that for control tumors.

![Image](image.png)

**Figure 4-2. SPRY2 depletion suppresses xenograft growth.** Mice were subcutaneously injected with U87MG cells expressing low EGFRvIII and either control or SPRY2-targeting shRNA. (A) 16 days post-injection, animals were sacrificed and representative images of resected tumors were taken. (B) Tumor volume was measured every 2-3 days (n=8 mice; $p < 0.05$ at all time points). (C) Tumor weight was measured 16 days post-injection (n=6 mice with both control and SPRY2 knockdown tumors; $p < 0.05$). Data are represented as the mean ± s.e.m.

**SPRY2 controls p38’s ability to regulate anchorage-independent growth and response to inhibitors.** In multiple cellular settings, SPRY2 regulates signaling through the ERK pathway.
(Gross et al., 2001; Hanafusa et al., 2002; Yusoff et al., 2002). In the panel of U87MG cells, SPRY2 knockdown produced a small increase in basal ERK phosphorylation in U87MG-DK cells, but did not affect ERK phosphorylation in cells expressing active EGFRvIII (Figure 4-3A), where SPRY2 knockdown produces phenotypic effects. Although SPRY2 has been reported to specifically inhibit ERK among MAP kinase pathways (Yusoff et al., 2002), a recent study found enhanced p38 phosphorylation in SPRY1/SPRY2/SPRY4 knockout murine embryonic fibroblasts (Sharma et al., 2012). We thus hypothesized that JNK and/or p38 MAP kinases could be affected by SPRY2 knockdown. Indeed, basal JNK and p38 phosphorylation were increased in U87MG cells with SPRY2 knockdown compared to controls (Figure 4-3A). We did not observe similar basal increases in p38 or JNK phosphorylation in other GBM cell lines with SPRY2 knockdown, but we did observe increased p38 and JNK phosphorylation in U87MG-DK, U251, and U118MG cells in response to EGFR and c-MET co-inhibition (Figure 4-3B, Figure 4-S5A). We also observed increased p38 and JNK phosphorylation in U87MG cells expressing Y55F SPRY2 (Figure 4-S5B).

To determine whether JNK and/or p38 activity control the cellular phenotypes affected by SPRY2 knockdown, we treated cells with the p38 inhibitor SB203580 and/or the JNK inhibitor SP600125. p38 inhibition promoted colony formation, but JNK inhibition decreased colony formation (Figure 4-3C). When p38 and JNK inhibitors were combined, colony formation was reduced compared to DMSO-treated controls, but increased compared to JNK inhibition alone (Figure 4-S5C). While neither inhibitor rescued the decreased cellular proliferation observed in U87MG-DK cells with SPRY2 knockdown, JNK inhibition decreased cellular proliferation relative to DMSO-treated controls (Figure 4-3D), suggesting an important role for JNK in cellular proliferation.
Figure 4-3. p38 and JNK phosphorylation are increased in U87MG cells with SPRY2 knockdown and in cells co-treated with EGFR and c-MET inhibitors, and control cellular proliferation, anchorage-independent growth, and response to EGFR and c-MET co-inhibition.

(A) Whole cell lysates from U87MG cells expressing dead kinase ("DK"), low ("L"), medium ("M"), or high ("H") EGFRvIII and control ("-".) or SPRY2-targeting ("+".) shRNA were probed...
by western blot using antibodies against the indicated proteins, and blots were analyzed by densitometry. (B) The indicated cell lines expressing control (“-“) or SPRY2-targeting (“+“) shRNA were treated with DMSO or a combination of PHA665752 and gefitinib (“PHA+gef”) (5 μM PHA665752+10 μM gefitinib for U87MG and U118MG; 2 μM PHA665752+10 μM gefitinib for U251) for 48 hrs. Whole cell lysates were probed by western blot using antibodies against the indicated proteins. (C) Colony formation in soft agar was measured in the indicated cell lines treated with DMSO, 20 μM SB203580 (“p38i”), or 20 μM SP600125 (“JNKi”). (D) Cellular proliferation was measured in U87MG cells expressing DK EGFRvIII and control or SPRY2-targeting shRNA treated with DMSO, 20 μM p38i, 20 μM JNKi, or both 20 μM p38i and 20 μM JNKi (“p38i+JNKi”). (E) U87MG DK EGFRvIII and U251 cells expressing control or SPRY2-targeting shRNA were treated with DMSO or 20 μM p38i for 24 hrs and then treated with DMSO or PHA+gef (5 μM PHA665752+10 μM gefitinib for U87MG; 3 μM PHA665752+10 μM gefitinib for U251) for 48 hrs prior to flow cytometry analysis for ToPro3 permeability. (F) Whole cell lysates from U87MG DK EGFRvIII and U251 cells expressing control empty vector (“EV”), constitutively active MKK3 (“MKK3-Glu”), or inactive MKK3 (“MKK3-Ala”) were probed by western blot with a Flag antibody to detect Flag-MKK3 expression. (G) MKK3-expressing cells were treated with DMSO or PHA+gef (5 μM PHA665752+10 μM gefitinib for U87MG; 3 μM PHA665752+10 μM gefitinib for U251) for 48 hrs prior to flow cytometry analysis for ToPro3 permeability. (H) Whole cell lysates from the indicated EV control or MKK3-expressing cells were probed by western blot using antibodies against the indicated proteins. Throughout the panels, data are represented as the average of at least three independent experiments ± s.e.m. Asterisks indicate p < 0.05, and “NS” indicates lack of statistical significance.

We further found that pre-treatment of U87MG-DK and U251 cells with the p38 inhibitor eliminated the increase in cell death response to co-inhibition of EGFR and c-MET in cells with SPRY2 knockdown compared to controls (Figure 4-3E). Conversely, expression of constitutively active mitogen-activated protein kinase kinase 3 (MKK3; (Raingeaud et al., 1996)), the kinase for p38, enhanced cell death response to EGFR and c-MET co-inhibition in U87MG-DK and U251 cells (Figure 4-3F, G). Expression of an inactive MKK3 mutant did not affect drug response in either cell line. As expected, expression of constitutively active MKK3 increased p38 phosphorylation in cells where EGFR and c-MET were co-inhibited (Figure 4-3H, Figure 4-S5D). Note that U87MG-DK cells were used for these studies because they exhibited a larger increase in cell death due to SPRY2 knockdown than U87MG cells with active EGFRvIII expression.
SPRY2 knockdown reduces MKP-1 and MKP-5 mRNA levels in U87MG cells. Although we observed basal increases in p38 and JNK phosphorylation in U87MG cells with SPRY2 knockdown, there is no known mechanism for SPRY2-mediated inhibition of p38 or JNK. We postulated that SPRY2 could negatively regulate JNK and p38 phosphorylation by promoting expression of regulatory phosphatases, as had been shown with the protein tyrosine phosphatase PTP1B in the cytosolic fraction of HeLa cells (Yigzaw et al., 2003). We found that mRNA levels of the dual-specificity MAP kinase phosphatases MKP-1 and MKP-5, which act upon JNK and p38 (Owens and Keyse, 2007; Theodosiou and Ashworth, 2002), were indeed reduced in U87MG-DK cells with SPRY2 knockdown compared to controls (Figure 4-4A). We measured no change in MKP-3 mRNA levels, which primarily acts upon ERK (Owens and Keyse, 2007; Theodosiou and Ashworth, 2002). We reduced MKP-1 and MKP-5 expression in U87MG-DK and U87MG-H cells using shRNA (Figure 4-S6), which enhanced phosphorylation of p38, JNK, and ERK (Figure 4-4B) and significantly increased cell death response to EGFR and c-MET co-inhibition (Figure 4-4C). The effects were greatest with MKP-5 knockdown, perhaps due to relatively poor reduction of MKP-1 mRNA levels by MKP-1-targeting shRNA (Figure 4-S6).
Figure 4-4. In U87MG cells, SPRY2 knockdown reduces *MKP*-1 and *MKP*-5 mRNA expression, and MKP-1 or MKP-5 knockdown enhances cellular response to EGFR and c-MET co-inhibition.

(A) *MKP*-1, *MKP*-5, and *MKP*-3 mRNA levels were measured in U87MG dead kinase ("DK") EGFRvIII cells expressing control or SPRY2-targeting shRNA. (B) Whole cell lysates from U87MG DK or high ("H") EGFRvIII cells expressing a control, *MKP*-1-targeting, or *MKP*-5-targeting shRNA were probed by western blot using antibodies against the indicated proteins, and blots were analyzed by densitometry. (C) The indicated cell lines were treated with DMSO or 5 μM PHA665752+10 μM gefitinib ("PHA+gef") for 48 hrs prior to flow cytometry analysis for ToPro3 permeability. Throughout the panels, data are represented as the average of three independent experiments ± s.e.m. Asterisks indicate *p* < 0.05, and "NS" indicates lack of statistical significance.

SPRY2 is expressed in human GBM tumors and elevated in EGFRvIII-positive tumors in humans and rats. SPRY2 protein expression in human tumors was analyzed by immunohistochemistry. The majority of tumors analyzed showed positive SPRY2 staining. SPRY2 staining patterns included moderate focal, moderate granular, and strong diffuse staining (Figure 4-5). As a positive control, SPRY2 protein expression was also probed by immunohistochemistry in sections of kidney and cerebellum (Figure 4-S7). We additionally probed SPRY2 protein expression by western blot in patient-derived xenografts, GSC cell lines,
and a neurosphere cell line and observed SPRY2 protein levels comparable or greater than that observed in representative GBM cell lines (Figure 4-S8). In the analysis of GBM tumors, it was noted that there was a possible trend for lower intensity staining overall in tumors without expression of EGFRvIII compared to those positive for EGFRvIII expression.

**Figure 4-5. SPRY2 protein expression in GBMs is confirmed by immunohistochemical analysis.**

SPRY2 protein expression was probed in sections of EGFRvIII+ and EGFRvIII- GBMs. In both tumor types, the majority of tumors analyzed showed definitive SPRY2 staining with SPRY2 patterns including moderate focal, moderate granular, and strong diffuse staining. The neuropathologist who analyzed these images noted a possible tendency for less intense staining overall in EGFRvIII- tumor sections.

This trend for higher SPRY2 expression in EGFRvIII-positive GBMs was confirmed in an analysis of EGFRvIII-induced gene signatures in orthotopic rat tumor allografts and primary human GBM tumors. For the analysis in rats, 12 animals received intracranial injections of 9L rat gliosarcoma cells expressing EGFRvIII or an empty vector control. Three weeks after implantation and subsequent growth, tumors were analyzed for gene expression profiling using the RatRef12 Illumina chip array. This analysis revealed 1498 gene probes that were increased \((p < 0.05)\) by at least 1.5-fold in EGFRvIII-expressing tumors compared to empty vector control tumors. We also examined gene expression in 52 primary GBM tumors, which were stratified based on the presence or absence of EGFRvIII expression using EGFRvIII-specific RT-PCR (Tykocinski et al., 2012). The extracted RNA samples from these tumors were subjected to
microarray analysis using HG-U133 2.0 Plus Affymetrix gene chips. This analysis revealed 355 gene probes whose expression was significantly increased ($p < 0.05$) by at least 1.5-fold in EGFRvIII-positive tumors compared to EGFRvIII-negative tumors. These gene probes were aligned against the gene probes upregulated in EGFRvIII-positive rat tumors. SPRY2 was one of 9 upregulated genes that were shared by both rat 9L EGFRvIII-expressing tumors and human EGFRvIII-expressing GBMs (Table 4-S1).

We further examined SPRY2 expression in GBM cell lines, where we observed a positive correlation between SPRY2 expression and ERK phosphorylation that was supported by pharmacological and genetic perturbation (Figure 4-S9A, B, C), consistent with the documented ability of ERK to regulate SPRY2 expression (Egan et al., 2002; Reich et al., 1999). Among cell lines engineered to express EGFRvIII, we continued to observe good correlation between SPRY2 expression and ERK phosphorylation, but we did not observe a consistent trend between EGFRvIII and SPRY2 expression (Figure 4-S9E). The apparent lack of good SPRY2/EGFRvIII expression correlation may arise because of the limited number of isogenic cell backgrounds tested or because the method used to drive EGFRvIII expression in cell lines differs drastically from the way that EGFRvIII expression is regulated in GBM (Li et al., 2008a).

*Analysis of TCGA data reveals that elevated SPRY2 expression correlates with EGFRvIII expression, the classical GBM subtype, and reduced patient survival.* We next probed the TCGA GBM data set to ask if SPRY2 expression correlates with EGFRvIII expression in a larger set of patient samples and to determine whether SPRY2 expression correlates with previously defined GBM tumor subtypes and patient survival. TCGA samples were classified by EGFRvIII expression status based on RNA-seq data, which was available for 161 samples. 41/161 samples (25%) were EGFRvIII-positive. SPRY2 expression was increased in EGFRvIII-positive samples compared to EGFRvIII-negative samples ($p = 1.00 \times 10^{-8}$), with a fold difference of 1.49 (Figure 4-6A), consistent with the results in Table 4-S1. Based on the definitions of four clinically relevant GBM subtypes determined by gene signatures (Verhaak et al., 2010) and using TCGA GBM exon-array data (173 “core” representative samples), we further found that SPRY2 expression
was lower in the proneural subtype than the other three subtypes ($p = 8.06\times10^{-9}$ and fold difference of 0.60) (Figure 4-6B). SPRY2 expression was highest in the classical subtype ($p < 2.20\times10^{-16}$ and fold difference of 1.58 for comparison to other subtypes), which exhibits a high rate of EGFR amplification and mutation.

Figure 4-6. TCGA GBM data reveals that elevated SPRY2 expression correlates with EGFRvIII expression, the classical GBM subtype, and reduced patient survival. (A) SPRY2 expression (log base 10 transformed RPKM) is higher in EGFRvIII-positive RNA-seq samples ($n = 41$) than EGFRvIII-negative RNA-seq samples ($n = 120$) ($p = 1.00\times10^{-8}$, fold difference of 1.49). (B) SPRY2 expression (log base 2 transformed data) is shown for normal brain samples ($n = 10$) and the four GBM subtypes: proneural ($n = 54$, $p = 8.06\times10^{-9}$ and fold difference of 0.60 compared to other three subtypes), neural ($n = 27$), mesenchymal ($n = 55$), and classical ($n = 37$, $p < 2.20\times10^{-16}$ and fold difference of 1.58 compared to other three subtypes). The $p$-value for tumor versus normal brain samples is 0.154 with a fold difference of 1.13. (C) Survival probability is shown as a function of time after diagnosis classified by SPRY2 expression with the median SPRY2 expression used as a cutoff for all patients and patients parsed by age using the median age (59 yrs) as a cutoff.

We also determined the relationship between SPRY2 expression and patient survival (Figure 4-6C), using median SPRY2 expression as a cutoff between low and high expression. Across all patients, low SPRY2 expression was associated with reduced mortality compared to high SPRY2 expression ($p = 0.0123$), with median survival for low or high SPRY2 expression of 469 or 393 days, respectively. Because age is an established important prognostic factor in GBM (Lee et al., 2008; Siker et al., 2011), we further looked at the effect of SPRY2 expression in
patients stratified by age. Parsing patients into age groups using the median age (59 yrs) as a cutoff, we found that the effect of SPRY2 expression differences was more pronounced in the younger patient cohort ($p = 0.000416$), with median survival of 631 or 451 days for low or high SPRY2 expression, respectively, and that there was no effect of SPRY2 expression on survival in the older cohort. The difference in survival was even further increased using an age cutoff of 40 yrs ($p = 0.00316$), with median survival of 1024 or 538 days for low or high SPRY2 expression, respectively (Figure 4-S10).

4-5. Discussion

In GBM cell lines with or without EGFRvIII expression, we find that SPRY2 acts as a driver of GBM cell proliferation, anchorage-independent growth, and resistance to inhibition of receptor tyrosine kinases that promote GBM survival. Furthermore, SPRY2 knockdown had a dramatic effect on tumor xenograft growth. In at least some cases, SPRY2 may exert this control over GBM cell phenotypes by regulating the ability of p38 and JNK to influence cell outcomes, as summarized in Figure 4-7. We also find that SPRY2 gene expression is elevated in human GBMs and rat tumor grafts expressing EGFRvIII, which tend to be especially aggressive, and that high SPRY2 expression is associated with reduced patient survival and the classical GBM subtype. These central findings point to SPRY2 as a potential therapeutic target in GBM and may help to explain the established connection between EGFRvIII expression and GBM aggressiveness.

The finding that SPRY2 promotes growth and resistance to inhibition of receptor tyrosine kinases was initially surprising given SPRY2’s purported role as a tumor suppressor in hepatocellular carcinoma, lung cancer, breast cancer, and prostate cancer (Fong et al., 2006b; Lo et al., 2006; Sutterluty et al., 2007). While SPRY2 appears to negatively regulate tumor growth in these other settings, an oncogenic role for SPRY2 has recently been demonstrated in colon cancer where elevated SPRY2 expression is a marker of poor prognosis (Holgren et al., 2010; Ordonez-Moran et al., 2013). In colon cancer cells, SPRY2 may promote tumorigenesis through regulation of c-MET (Holgren et al., 2010) or E-cadherin (Barbachano et al., 2010). Because the
net outcome of SPRY2 expression and its mechanism of action appear highly dependent on cellular context, additional work is clearly needed to clarify the determinants of SPRY2’s function in different contexts and to develop strategies to interrupt SPRY2’s pro-tumorigenic functions in GBM and other cancers.

Another question raised by our study is how SPRY2 expression is promoted in EGFRvIII-positive tumors. Across many GBM cell lines and with multiple genetic and pharmacological perturbations, SPRY2 expression correlated very well with ERK phosphorylation. It has been suggested, however, that EGFRvIII-driven tumors do not activate canonical EGFR downstream signaling pathways such as ERK, STAT3, and AKT (Zhu et al., 2009). It is possible that tumor microenvironment features altered by EGFRvIII expression allow for increased ERK activation, or that an alternative pathway not explored here promotes SPRY2 expression in GBM. If it is true that ERK regulates SPRY2 expression in vivo, MEK inhibition could be a useful approach to downregulate SPRY2 expression in GBM. It should also be noted that SPRY2 is post-transcriptionally regulated by miR-21, which could potentially complicate the relationship between SPRY2 transcript and protein levels in some settings (Kwak et al., 2011; Sayed et al., 2008).

Although the best-studied role of SPRY2 is regulation of the ERK pathway, our results suggest that p38 and JNK activity are important for regulating anchorage-independent growth, proliferation, and response to tyrosine kinase inhibitors in GBM. Specifically, we interpret the results of Figure 4-3 to indicate that JNK activity is required for normal GBM cell proliferation and anchorage-independent growth, but that p38 activity antagonizes anchorage-independent growth and promotes cellular response to EGFR and c-MET co-inhibition. Since p38 inhibition mitigated the effects of SPRY2 knockdown on death response to EGFR and c-MET co-inhibition, even in a cell line where SPRY2 knockdown did not generate basal increases in p38 phosphorylation (U251), p38 activation may only be a necessary step along the path leading to apoptosis. SPRY2’s absence (or reduced expression) may potentiate this effect of p38 activity through specific mechanisms yet to be identified. The results of Figure 4-3 also suggest JNK as an interesting therapeutic target to consider in GBM moving forward.
Figure 4-7. SPRY2 promotes GBM cell proliferation, anchorage-independent growth, and resistance to tyrosine kinase inhibition.

The schematic highlights our study's key findings regarding regulation of SPRY2 expression and SPRY2-mediated regulation of signaling and cellular phenotypes. In tumors expressing EGFRvIII, SPRY2 expression is increased compared to tumors lacking EGFRvIII, an effect that may involve differential ERK activity in the tumor context of EGFRvIII expression. In GBM cells, SPRY2 promotes anchorage-independent growth and proliferation in adherent cultures, and drives resistance to targeted inhibitors of oncogenic kinases. SPRY2 regulates these phenotypes by permitting p38 activity to play a more prominent role in cell fate determination, and this regulation of p38 may involve regulation of the expression of the MKP-1 or MKP-5 dual specificity phosphatases in some cases. JNK activity and functional role may also be regulated by SPRY2, but JNK and p38 generally play opposing roles in the regulation of GBM cell phenotypes.

To our knowledge, our data constitutes the first report of SPRY2’s ability to regulate p38 and JNK MAP kinase signaling through regulation of the expression of dual specificity phosphatases (MKP-1 and MKP-5). This is also the first study relating MKP-1 and MKP-5 expression and response to clinically relevant inhibitors in GBM. Previous studies have not focused on MKP-1 or MKP-5 in GBM, but published gene expression data indicate that MKP-1 expression is higher in GBM samples than normal brain (Bredel et al., 2005; Lee et al., 2006). Together with our finding that MKP-1 or MKP-5 knockdown potentiated U87MG cell response to EGFR and c-MET co-inhibition, this suggests that MKPs merit further study in GBMs and could be useful therapeutic targets.
Overall, our findings highlight the surprising results that in GBM SPRY2 appears to play the role of promoting cancer phenotypes, including proliferation, anchorage-independent growth, and resistance to targeted inhibitors of oncogenic kinases, and that elevated SPRY2 expression is associated with expression of EGFRvIII (which tends to make GBM tumors especially aggressive) and reduced patient survival. These results stand in stark contrast to findings in several other cancer contexts where SPRY2 has been reported to function as a tumor suppressor. Looking forward, SPRY2 and the pathways it regulates should be assessed for their value as therapeutic targets or as prognostic markers for response to therapy in GBM.

4-6. Acknowledgements

Dr. Frank Furnari (UCSD, La Jolla, CA, USA), Dr. Celeste Simon (University of Pennsylvania, Philadelphia, PA, USA), Dr. Tyler Jacks (MIT, Cambridge, MA, USA), Dr. Marilyn Farquhar (UCSD, La Jolla, CA, USA), Dr. Margaret Chou (University of Pennsylvania, Philadelphia, PA, USA), Dr. Dafna Bar-Sagi (NYU, New York, NY, USA), and Dr. Sylvain Meloche (Université de Montréal, Montreal, Quebec, Canada) generously provided reagents for this work.
### Table 4-S1. Upregulated genes shared by human GBMs expressing EGFRvIII and 9L.EGFRvIII rat tumors compared to wild-type EGFR human GBMs or 9L.EV rat tumors.

<table>
<thead>
<tr>
<th>gene symbol</th>
<th>gene name</th>
<th>probe sets</th>
<th>Human GBM cohort (n=52)</th>
<th>Rat tumors (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>p-value</td>
<td>fold difference</td>
</tr>
<tr>
<td>CKAP4</td>
<td>cytoskeleton-associated protein 4</td>
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<td>0.0033</td>
<td>2.2</td>
</tr>
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<td>LRP5</td>
<td>low density lipoprotein receptor-related protein 5</td>
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<td>0.0086</td>
<td>1.6</td>
</tr>
<tr>
<td>FAT3</td>
<td>fat tumor suppressor homolog 3</td>
<td>2</td>
<td>0.0131</td>
<td>1.5</td>
</tr>
<tr>
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<td>sprouty homolog 2</td>
<td>1</td>
<td>0.0191</td>
<td>1.5</td>
</tr>
<tr>
<td>SLC7A1</td>
<td>solute carrier family 7 (cationic amino acid transporter, y+ system)</td>
<td>1</td>
<td>0.0279</td>
<td>1.6</td>
</tr>
<tr>
<td>AEBP1</td>
<td>ae binding protein 1</td>
<td>1</td>
<td>0.0287</td>
<td>1.5</td>
</tr>
<tr>
<td>CDK6</td>
<td>cyclin-dependent kinase 6</td>
<td>3</td>
<td>0.0299 range (0.012-0.041)</td>
<td>1.9 range (1.8-2.1)</td>
</tr>
<tr>
<td>SOCS2</td>
<td>suppressor of cytokine signaling 2</td>
<td>3</td>
<td>0.0299 range(0.012-0.041)</td>
<td>1.9 range (1.8-2.1)</td>
</tr>
<tr>
<td>AQP1</td>
<td>aquaporin 1 (colton blood group)</td>
<td>1</td>
<td>0.0405</td>
<td>1.7</td>
</tr>
</tbody>
</table>
Figure 4-S1. SPRY2 is efficiently knocked down by shRNA in GBM cell lines.

Whole cell lysates from U87MG cells with dead kinase (“DK”), low (“L”), medium (“M”), or high (“H”) EGFRvIII expression and U251, SF188, LN18, U118MG, and T98G cells with expression of control or one of two SPRY2-targeting shRNA were probed by western blot using antibodies against the indicated proteins. shRNA #1 is the hairpin used throughout the main figures, and shRNA #2 is an independent, non-overlapping shRNA made to validate the effects of shRNA #1. ERK was used as a loading control. Data are representative of three independent experiments.
Figure 4-S2. SPRY2 depletion by a second non-overlapping shRNA reduces cellular proliferation, and SPRY2 depletion by siRNA transfection reduces colony formation in soft agar in EGFRvIII-expressing cells.

(A) Cellular proliferation was measured by counting cells 5 days after plating for the indicated cell lines with expression of control or a second non-overlapping SPRY2-targeting shRNA (shRNA #2, described in Experimental Procedures). Data are represented as the average of three independent experiments ± s.e.m., and asterisks indicate $p < 0.05$. (B) Colony formation in soft agar was assessed in the indicated cell lines with EGFRvIII expression transfected with control or SPRY2-targeting siRNA. Images are representative of two experiments.
Figure 4-S3. SPRY2 depletion increases cellular sensitivity to EGFR and c-MET co-inhibition.

(A) Whole cell lysates of parental U87MG cells (without EGFRvIII expression) expressing control or SPRY2-targeting shRNA were probed by western blot using antibodies against the indicated proteins. Cells were treated with DMSO or PHA665752 and gefitinib ("PHA+gef") (5 μM PHA665752 and 10 μM gefitinib) for 48 hrs prior to cell death analysis. (B) Whole cell lysates of U87MG cell expressing dead kinase ("DK") EGFRvIII transfected with control or SPRY2-targeting siRNA smartpools were probed by western blot using antibodies against the indicated proteins. Cells were also treated with DMSO or PHA+gef (5 μM PHA665752 and 10 μM gefitinib) for 48 hrs prior to cell death analysis. (C) Parental U87MG cells expressing an empty vector control ("EV") or wild-type PTEN and either control or SPRY2-targeting shRNA were probed by western blot using antibodies against the indicated proteins. Cells were treated with DMSO or PHA+gef (5 μM PHA665752+10 μM gefitinib) for 48 hrs prior to cell death analysis. (D) SF188 and LN18...
cells with control or SPRY2-targeting shRNA were treated with DMSO or PHA+gef (5 μM PHA665752+10 μM gefitinib for SF188; 2 μM PHA665752+10 μM gefitinib for LN18) for 48 hrs prior to cell death analysis. (E) U87MG cells with DK, low ("L"), or medium ("M") EGFRvIII expression, U251, and SF188 cells expressing control or a second non-overlapping SPRY2-targeting shRNA (shRNA #2, described in Materials and Methods) were treated with DMSO or PHA+gef (5 μM PHA665752+10 μM gefitinib for U87MG and SF188; 2 μM PHA665752+10 μM gefitinib for U251) for 48 hrs prior to cell death analysis. Throughout the panels, cell death was measured by flow cytometry for ToPro3 permeability, and data are represented as the average of three independent experiments ± s.e.m. Asterisks indicate p < 0.05.

Figure 4-S4. SPRY2 knockdown promotes response to EGFR/c-MET co-inhibition in GSC cells.

The distribution of sphere size is shown for 3691 GSC cells transduced with control or SPRY2-targeting shRNA that were treated with DMSO or 3 μM PHA665752 ("PHA") and 20 μM gefitinib. Cells were seeded at a density of 10 cells per well in 96-well format (16 wells per treatment per cell line) and then treated with DMSO or PHA and gefitinib at 72 hrs post-plating and every 2-3 days subsequently. Cell spheres were analyzed after 8 days of treatment. Analysis included spheres with volume above 50,000 μm³, below which cells clusters were loosely associated and non-spherical. Representative spheres at equal magnification for different size bins are shown.
Figure 4-S5. p38 and JNK control anchorage-independent growth and response to EGFR and c-MET co-inhibition.

(A) Whole cell lysates from U87MG dead kinase ("DK") EGFRvIII cells, U251 cells, or U118MG cells expressing control or SPRY2-targeting shRNA were treated with DMSO or a combination of PHA665752 and gefitinib ("PHA+gef") (5 μM PHA665752+10 μM gefitinib for U87MG and U118MG; 2 μM PHA665752+10 μM gefitinib for U251) for 48 hrs. Whole cell lysates were probed by western blot and analyzed by densitometry. (B) Whole cell lysates from U87MG DK EGFRvIII cells transduced with empty vector control ("EV") or Y55F SPRY2 were probed by western blot using antibodies against the indicated proteins. (C) Colony formation in soft agar was measured in U87MG cells with low ("L") EGFRvIII expression that were treated with DMSO, 20 μM SB203580 ("p38i"), or a combination of 20 μM SB203580 and 20 μM SP600125 ("p38i+JNKi"). (D) The indicated cell lines expressing control EV, constitutively active MKK3 ("MKK3-Glu"), or inactive MKK3 ("MKK3-Ala") were treated with DMSO or PHA+gef (5 μM PHA665752+10 μM gefitinib for U87MG-DK; 3 μM PHA665752+10 μM gefitinib for U251). Whole cell lysates were probed by western blot using antibodies against phosphorylated p38 and ERK. Blots were analyzed by densitometry. Throughout the panels, data are represented as the average of at
least three independent experiments ± s.e.m., and asterisks indicate \( p < 0.05 \).

**Figure 4-S6.** shRNA-mediated knockdown of MKP-1 or MKP-5 reduces MKP-1 or MKP-5 mRNA level.

MKP-1 or MKP-5 mRNA levels were measured in U87MG cells expressing dead kinase ("DK") EGFRvIII and control, MKP-1-targeting, or MKP-5-targeting shRNA. mRNA expression is shown relative to cells expressing control shRNA. Data are represented as the average of three independent experiments ± s.e.m., and asterisks indicate \( p < 0.05 \).

**Figure 4-S7.** SPRY2 protein expression in kidney and cerebellum sections by immunohistochemical analysis.

SPRY2 protein expression was probed in sections of kidney and cerebellum as positive controls. In the kidney, SPRY2 was observed in the tubules ("T") but not in glomeruli ("G"). In cerebellum, diffuse SPRY2 expression was observed throughout the molecular layer ("ML") and in the dentate nucleus ("DN"), with moderate staining in Purkinje cells ("PC") and strong staining in Bergmann glia ("BG"), but was not observed in the granule cell layer ("GCL") or white matter ("WM").
Figure 4-S8. SPRY2 protein expression is confirmed in patient-derived cells by immunoblot.

SPRY2 protein expression in patient-derived xenografts ("PDX") exhibiting EGFRvIII expression was compared against that observed in two glioma stem cell ("GSC") lines, a neurosphere ("NS") line with EGFRvIII expression, and in a panel of GBM cell lines and one lung cancer cell line (H1666) cultured on tissue culture polystyrene ("TCPS"). For U251 cells, lysates are shown for cells transduced with control ("ctrl") or SPRY2-targeting shRNA ("sh").
Figure 4-S9. SPRY2 correlates well with ERK phosphorylation in a panel of GBM cell lines.

(A) Whole cell lysates from the indicated cell lines were probed by western blot using antibodies against the indicated proteins, and blots were analyzed by densitometry. (B) U87MG cells with dead kinase ("DK") or high ("H") EGFRvIII expression were treated with the indicated concentrations of U0126 in complete media for 24 hrs. Whole cell lysates were probed by western blot using antibodies against the indicated proteins. (C) SPRY2 mRNA levels were measured in U87MG cells with DK or H EGFRvIII expression after 24 hrs treatment with 25 μM U0126. (D) U87MG cells with H EGFRvIII expression and transduced with either control empty vector ("EV") or constitutively active MEK ("MEK2DD") were probed by western blot using antibodies against the indicated proteins. (E) Whole cell lysates from the indicated cell lines with EGFRvIII expression ("+") control EV expression, or control DK EGFRvIII expression were probed by western blot using antibodies against the indicated proteins, and blots were analyzed by densitometry. In U87MG cells, "+" indicates high EGFRvIII expression. Throughout the figure panels, data are represented as averages of three independent experiments ± s.e.m., and asterisks indicate $p < 0.05$. 
Figure 4-S10. TCGA GBM dataset analysis reveals that SPRY2 expression is associated with reduced patient survival.

Survival probability is shown as a function of time after diagnosis for patients classified by SPRY2 expression using the median SPRY2 expression used as a cutoff. This analysis is shown for patients of all ages (left panel) and for patients parsed by age using 40 yrs as a cutoff (center and right panels).

4-8. Supplemental Materials and Methods

*Western blotting.* Whole cell lysates were prepared in a standard cell extraction buffer (Life Technologies) supplemented with protease and phosphatase inhibitors (Sigma). Total protein concentrations were determined by micro-bicinchoninic assay (Thermo Scientific). Lysates were loaded on 4-12% gradient polyacrylamide gels (Life Technologies) under denaturing and reducing conditions and transferred to 0.2 μm nitrocellulose membranes (Bio-Rad). Membranes were imaged on a LI-COR Odyssey scanner (LI-COR) and stripped with 0.2 M NaOH as needed.

*Antibodies.* Antibodies against PARP (#9542), p-p38 (#4631), p-JNK (#4671), PTEN (#9552), ERK (#4695), and p-ERK T202/Y204 (#4377) were purchased from Cell Signaling Technology. The EGFR antibody (Ab-12) was purchased from Thermo Scientific. The SPRY2 antibody (#S1444) and the Flag antibody (#F3165) were purchased from Sigma. The p-EGFR antibody (#1727) was purchased from Epitomics. The GAPDH antibody (#sc-32233) was purchased from Santa Cruz Biotechnology. Infrared dye-conjugated secondary antibodies were
purchased from Rockland Immunochemicals. All antibodies were used according to manufacturer recommendations.

**Cell culture for supplemental data.** All cells (except GSC cells) were maintained in DMEM supplemented with 10% fetal bovine serum, 1 mM L-Glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). LN229 cells were obtained from the American Type Culture Collection. EGFRvIII or empty vector expressing 9L, U373MG, and LN229 cells for anchorage-independent growth assay were created by transfection with pcDNA3.1(+) (Life Technologies) using Lipofectamine 2000 (Life Technologies) according to manufacturer recommendations. Transfected 9L cells were supplemented with 600μg/ml zeocin (Life Technologies). Transfected U373MG and LN229 cells were supplemented with 2μg/ml of puromycin (Sigma). U87MG cells with PTEN expression were created by infection with retrovirus as described in Methods using PTEN cDNA (Dr. Frank Furnari, UCSD, La Jolla, CA, USA) subcloned into pBabe.hygro. 3691 GSC cells (Dr. Celeste Simon, University of Pennsylvania, Philadelphia, PA, USA) were maintained in neurobasal medium supplemented with B27 1:50 (Life Technologies), 20 ng/mL EGF (Sigma), and 20 ng/mL basic fibroblast growth factor. Sphere cross-sectional areas were analyzed using ImageJ and were used to calculate sphere volume. PDX, GSC, and NS cell lysates (Figure 4-S8) were provided by Dr. Frank Furnari (UCSD, La Jolla, CA, USA). H1666 lysates were prepared as described in Walsh et al. (Walsh and Lazzara, 2013).

**Transient knockdown of SPRY2.** For U87MG cells (Figure 4-S3B), cells were transfected with a smartpool of four SPRY2 siRNAs or a control siRNA (Thermo Scientific) using DharmaFECT1 transfection reagent (Thermo Scientific) according to the manufacturer’s recommendations. EGFRvIII-expressing U373MG, LN229, and 9L cells (Figure 4-S2B) were transfected with control or SPRY2-targeting siRNA using Lipofectamine RNAiMAX transfection reagent (Life Technologies) according to the manufacturer’s recommendations. The siRNA sequences used were: SPRY2 siRNA, 5’-CCUGUGGCUGAUGGCAUAA-3’ and control siRNA, 5’-CCUUCGUGUAGACGUGUA-3’. 

121
Rat tumor allografts and gene expression analysis. EGFRvIII-expressing 9L rat gliosarcoma cells (Sibenaller et al., 2005) were created by transfection with pcDNA3.1(+) (Life Technologies) with Lipofectamine 2000 (Life Technologies) according to the manufacturer’s recommendations and selected with 600 μg/ml zeocin (Life Technologies) for 2-3 weeks. Syngeneic female Fischer rats (4-6 weeks old, 120–150 g) bearing either 9L.empty vector (n = 6) or 9L.EGFRvIII (n = 6) tumors were used. General anesthesia was induced by intraperitoneal injection of a ketamine/acepromazine mixture at a dose of 91/9.1 mg/kg. A 10 μL suspension of $5 \times 10^4$ 9L.empty vector or 9L.EGFRvIII cells in phosphate buffered saline was injected into the cortex at a depth of 2 mm using a stereotactic apparatus (3 mm lateral and 3 mm posterior to the bregma). Three weeks after orthotopic implantation, tumors were removed and stored at -80°C in RNALater (Life Technologies). RNA was extracted using the TriZol method and purified using the RNeasy kit (Qiagen). 100 ng of RNA was amplified into biotinylated cRNAs using the Illumina TotalPrep RNA amplification kit (Ambion) according to manufacturer recommendations. The labeled cRNAs were subjected to hybridization on RatRef-12 Expression BeadChip array (Illumina Inc.). Gene identities and expression intensities were determined using the Partek Genomic suite (Partek Incorporated). The raw data were subjected to quantile normalization. The protocol was approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Human GBM samples. A retrospective analysis was conducted of 52 patients with primary GBMs (WHO grade IV), who had tissue harvested from standard image-guided surgical resections and banked between January 2002 and January 2009 at the Hospital of the University of Pennsylvania (HUP). There were no exclusion criteria. The protocol was approved by the Institutional Review Board at HUP, and informed consent from each participant or the participant’s guardian was obtained. An experienced neuropathologist performed the histopathologic evaluation, using the WHO classification criteria. Tumors were categorized on the basis of EGFRvIII expression using EGFRvIII-specific RT-PCR reactions (28). The 52 patients used in this study are a sub-set of the 132 patients studied by Tykocinski et al. (28). 13/52 (25%)
tumors were EGFRvIII-positive, and 39/52 (75%) tumors were EGFRvIII-negative. The median patient age was 63.1 years with EGFRvIII-positive tumors and 60 years with EGFRvIII-negative tumors. All personnel were blinded to all clinical data, including outcome.

**Human GBM gene expression analysis.** HG-U133 2.0 Plus Affymetrix Gene Chips (Affymetrix Inc.) were used to profile primary human GBMs according to the manufacturer’s protocol. Briefly, ~1 μg of total RNA was converted to first-strand cDNA using Superscript II reverse transcriptase (Life Technologies) primed by a poly(T) oligomer that incorporated the T7 promoter. Second-strand cDNA synthesis was followed by *in vitro* transcription to generate labeled cRNAs. The cRNAs were subjected to hybridization at 45°C for 16 hrs, followed by washing, staining, and scanning of the chip according to the manufacturer’s instructions. A confocal scanner was used to collect fluorescence signal at 3 μm resolution after excitation at 570 nm. The average signal from two sequential scans was calculated for each microarray feature. Affymetrix Microarray Suite 5.0 was used to quantify expression levels for targeted genes. The raw data were subjected to quantile normalization. The default values provided by Affymetrix were applied to all analysis parameters.

**Preprocessing of TCGA exon-array data.** The unprocessed Affymetrix exon-array datasets for 173 GBM core samples used by Verhaak et al. (Verhaak et al., 2010) and 10 normal brain control samples were downloaded from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga). The gene-level expression estimates were obtained by the Multi-Mapping Bayesian Gene eXpression (MMBGX) algorithm (Turro et al., 2010) for Affymetrix whole-transcript arrays, based on Ensemble database (version 56). The estimated expression values were then normalized across the samples, using the locally weighted scatter plot smoothing (loess) algorithm (Workman et al., 2002).

**Analysis of TCGA RNA-seq data to identify EGFRvIII-positive samples.** Level 3 GBM RNA-seq data that had been analyzed by the MapSpliceRSEM pipeline were downloaded from the TCGA data portal. The MapSplice genome alignment was used for overlap counting. The exon level quantifications were provided by normalized number of reads mapping to each exon.
(RPKM). 161 RNA-seq samples were available for further analysis. For each tumor sample, the difference between mean RPKM of exon 2-7 and the other 22 exons were computed. In EGFRvIII-positive samples, exon 2-7 should have lower signal than other exons. The null empirical distribution of the signal difference between any 5 exons and the others was computed by randomly sampling the 28 exons of the 161 samples 3000 times. Samples with a low p-value are considered likely EGFRvIII-positive candidates. Using a p-value threshold of 0.05, 41 out of 161 samples were considered EGFRvIII-positive.
Chapter 5: Conclusions and future directions

5-1. Introduction

With the introduction of targeted therapeutics for cancer treatment, it has become clear that in order to create successful drug interventions and identify the patients who could benefit from these drugs, the incredible complexity of cellular signaling needs to be taken into consideration. With this motivation in mind, the work presented here has attempted to uncover the function of two regulators of EGFR signaling and trafficking in the context of cancer cells where aberrant EGFR signaling frequently occurs. This work uncovers important roles for MIG6 and SPRY2 in regulating EGFR trafficking in NSCLC, develops a framework for studying multiple EGFR endocytosis pathways with computational models, and points to a previously unknown and surprising role for SPRY2 in promoting GBM tumorigenesis. Given the complexity of the processes studied, it is perhaps not surprising that there remain several important questions related to this work that could be addressed in the future.

5-2. Future work and interpretation related to SPRY2 and MIG6 function in NSCLC

This investigation began by questioning the role of known EGFR feedback regulators in NSCLC cells with or without EGFR-activating mutations. It was unknown how MIG6 or SPRY2 would control mutant EGFR trafficking and signaling. This question was of clinical importance because EGFR inhibitors have enhanced efficacy against EGFR mutants found in NSCLC, but little was known about the mechanisms underlying impaired internalization of mutant EGFR. We measured EGFR endocytosis rate constants in NSCLC cells expressing wild-type EGFR or a common EGFR mutant. We found that MIG6 and/or SPRY2 knockdown modulated the measured EGFR endocytosis rate constant in both cells lines, demonstrating that MIG6 and SPRY2 were able to regulate endocytosis of internalization impaired mutant EGFR. Indeed, reduction of MIG6 expression reduced the measured EGFR endocytosis rate constant in cells expressing mutant EGFR even lower than controls. As a result of the complexity of signaling involved, there are
several aspects of this experimental study that could be pursued further.

For one, we found that SPRY2 depletion significantly reduced EGFR protein levels in most NSCLC cell lines tested, independent of EGFR mutational status. Additional experiments could be performed to understand how SPRY2 functions to control EGFR expression. While other groups have reported that SPRY2 expression modulates EGFR expression (Edwin and Patel, 2008; Feng et al., 2010; Grassian et al., 2011), these studies did not examine SPRY2 function independent of EGFR expression changes or investigate the mechanism of EGFR expression regulation. In PC9 cells that express mutant EGFR, we observed that SPRY2 depletion reduced EGFR mRNA levels, an effect that is potentially linked to reduced ERK activity in those cells. It is known that ERK can directly phosphorylate EGFR and ERK-mediated phosphorylation of threonine 669 has been implicated in EGFR degradation. In a published study of CHO cells, expression of EGFR with threonine 669 mutated to alanine, resulted in rapid EGF-induced EGFR degradation compared to cells expressing wild-type EGFR (Li et al., 2008b). These results suggest that ERK could directly regulate EGFR expression in some settings, but do not propose any possible mechanisms of ERK-mediated EGFR transcriptional or post-transcriptional regulation. An investigation to determine what cell-context-dependent features dictate whether EGFR transcription is controlled by SPRY2 through ERK activity would be of interest to many researchers both in cancer biology and other fields where EGFR or SPRY2 have been shown to play important roles.

While we found that SPRY2 depletion promoted cellular sensitivity to EGFR inhibition in NSCLC cells, MIG6 depletion had a more modest effect on drug response. This result was initially surprising, given that MIG6 is known to negatively regulate both EGFR tyrosine kinase activity and surface expression through internalization. Interestingly, another group recently published a study on MIG6 expression and response to EGFR inhibitors. These researchers found that MIG6 expression correlated with resistance to EGFR inhibitors in several cancers that did not express the EGFR-activating mutations observed in lung cancer patients (Chang et al., 2013). While their study agrees with our work that MIG6 depletion does not affect cellular sensitivity to EGFR
inhibitors, they propose that MIG6 expression could be a useful marker for identifying patients most likely to benefit from anti-EGFR therapeutics. In a panel of cell lines from multiple cancer types, increased MIG6 expression was associated with resistance to EGFR inhibition. In our study, MIG6 expression had the opposite relationship to response to EGFR inhibition. Mutant EGFR-expressing cell lines had higher MIG6 expression and also demonstrated enhanced response to EGFR inhibitors compared to wild-type EGFR expressing cells. In fact, H1975 cells that express the secondary T790M "gatekeeper" mutation and are resistant to gefitinib treatment (Pao et al., 2005), had lower MIG6 expression than cells expressing L858R EGFR, suggesting that MIG6 expression correlates well with response. Overall, the use of MIG6 expression as a marker for response to EGFR inhibition is potentially complicated by the presence or absence of sensitizing EGFR mutations. To summarize, MIG6 expression may be associated with resistance to EGFR inhibition in cells expressing wild-type EGFR, but not in cells with mutant EGFR expression, where MIG6 expression is increased.

5-3. Future work and interpretation related to predictions of the EGFR trafficking model

Our study of EGFR endocytosis in NSCLC cells led naturally to computational analysis. Models of EGFR trafficking and signaling have been essential to the understanding of these processes. However, existing models of EGFR endocytosis were insufficient for our purposes and we required a new model to analyze our complex data set. One of the key results of the computational study presented in Chapter 3 was the finding that MIG6 is responsible for at least half of EGFR endocytosis in NSCLC cells, suggesting that a substantial fraction of internalized EGFR may not be competent to drive downstream signaling. Additionally, analysis suggested that MIG6-mediated internalization was impaired for mutant EGFR expressing cells compared to wild-type EGFR expressing cells. The model results also pointed to the importance of differences in EGFR recycling for maintaining high levels of EGFR expression in mutant EGFR expressing cells. Besides the results presented, this work also generated some testable hypotheses that could be pursued in the future.
We hypothesized that impaired MIG6 internalization capacity in cells with mutant EGFR expression could be the result of impaired MIG6 and EGFR association. MIG6 binding to EGFR could be measured with and without EGF addition by EGFR immunoprecipitation to compare the relative association between MIG6 and wild-type or mutant EGFR. This study would potentially be best undertaken in an isogenic background with exogenous wild-type and mutant EGFR are expressed to a similar level. Zhang and coworkers published in 2007 a crystal structure of a 25 residue fragment of MIG6 bound to the distal surface of the C-lobe of the EGFR kinase domain (Zhang et al., 2007a). This study identified methionine 346, phenylalanine 352, and tyrosine 358 as key residues of MIG6 required for this binding event as mutation of any of these residues decreased MIG6 and EGFR binding. This study suggests the possibility that phosphorylation of MIG6 at tyrosine 358 (or serine 337 and serine 361, which were also near the MIG6 and EGFR interface) could impair MIG6 binding to EGFR. Furthermore, Wang and coworkers demonstrated in 2011 that near full-length EGFR (residues 25-1022) was able to tyrosine phosphorylate a 77 residue fragment of MIG6 containing the EGFR binding site in vitro (Wang et al., 2011). Therefore, it is possible that direct MIG6 phosphorylation by EGFR, which might be expected to be enhanced in cells expressing EGFR-activating mutations, could impair MIG6’s affinity for EGFR. To test whether enhanced MIG6 phosphorylation downstream of EGFR mutation inhibits MIG6’s ability to bind EGFR, MIG6 mutants could be expressed. Endogenous MIG6 could be depleted using shRNA and then shRNA-resistant MIG6 with candidate important phosphorylation sites mutated to alanine could be introduced followed by measurements of MIG6 binding to EGFR.

As an alternative possibility to explain the reduction of MIG6 internalization capacity in cells expressing mutant EGFR, we hypothesized that MIG6 activity could be reduced due to phosphorylation in mutant EGFR expressing cells. If expression of MIG6 mutants as discussed above does not affect MIG6 binding to EGFR, but still results in increased mutant EGFR internalization, this would support an important role for that phosphorylation site in inhibiting MIG6-mediated EGFR endocytosis. Recent studies have suggested candidate MIG6 residues to
mutate in addition to those identified by Zhang et al. (Zhang et al., 2007a). Serine 302 and serine 251 of MIG6 can be phosphorylated by the kinase Chk1 and may be involved in MIG6’s ability to inhibit EGFR activity (Liu et al., 2012). In addition, tyrosine 394 of MIG6 was identified using mass spectrometry as differentially phosphorylated in human bronchial epithelial cells expressing mutant EGFR compared to wild-type EGFR (Guha et al., 2008).

In addition to the main findings described in Chapter 3, our modeling study also has implications for designing experiments to measure internalization rate constants. Local parameter sensitivity analysis revealed that the simulated EGFR $k_e$ was relatively sensitive to changes in parameters that determined recycling ($k_{rec}$ and $f_r$). This sensitivity to recycling parameters was greater for simulations with mutant EGFR than wild-type EGFR. Ideally, an experimental measurement of EGFR $k_e$ should be designed so that recycling and degradation do not affect the measurement. The measurement of EGFR $k_e$ using radiolabeled EGF was introduced in a 1981 study by Wiley and Cunningham (Wiley and Cunningham, 1981). Using human fibroblasts and 1 ng/mL $^{125}$I-EGF, they determined that EGFR $k_e$ could be measured with time points up to 15 min. Their study did not explicitly consider receptor recycling, but they noted that other cell lines tested had different "observational windows" for determining EGFR $k_e$, suggesting that different cell types require different measurement times. Our model does not include endosomal retention components that delay the processes of recycling or degradation (French and Lauffenburger, 1996), due to lack of data to constrain the unknown parameters related to these processes. Therefore, the simulated recycling and degradation processes affect the simulated EGFR $k_e$ at time points as early as 1-2 min after EGF addition, but the magnitude of the resulting effect on EGFR $k_e$ is more pronounced at late time points. Another consideration is that in cell lines with high EGFR expression, saturation of rapid internalization mechanisms also leads to time-dependent EGFR $k_e$ measurements. The initial rate of internalization could be fast, but then decrease as early as 1-2 min because rapid internalization becomes limited. These details should be considered when designing experiments to measure EGFR endocytosis rates in the future. For cell lines with high EGFR expression or high receptor recycling, lower EGF concentrations
and shorter measurements could improve accuracy.

More generally, our work on EGFR trafficking has implications beyond diseases involving EGFR signaling. Impaired receptor trafficking has emerged as a common theme in several cancers, including leukemia, breast cancer, lung cancer, and GBM (Abella and Park, 2009; Mosesson et al., 2008). Maintenance of polarity in epithelial cells and adhesion structures such as adherens junctions are regulated by endocytosis, recycling, and transcytosis. Loss of polarity and adhesion are thought to be crucial steps in metastasis, and therefore improper trafficking can contribute to metastasis (Ramsay et al., 2007). In addition, several regulators of endocytosis are downregulated or mutated in human cancers. For example, CBL missense mutations have been identified in leukemia (Sargin et al., 2007; Slape et al., 2008). Therefore, methods developed to study EGFR trafficking have implications beyond the EGFR system.

5.4. Future work and interpretation related to study of SPRY2 in GBM

Our findings on the role of SPRY2 in NSCLC cells led us to think about SPRY2 function in other cancers where EGFR mutations are common and SPRY2 function is poorly defined. Further motivation was provided to investigate GBM because there is a large unmet clinical need to develop novel treatments or advance existing treatments to improve patient outcomes. Our study is one of the first to investigate functional role of SPRY2 in GBM and significantly advances the knowledge of SPRY2 function in GBM. We demonstrated an important role for SPRY2 in promoting GBM proliferation, anchorage-independence, and resistance to RTK inhibitors. We also found that SPRY2 expression promotes proliferation of GBM cells in mouse tumor xenografts. We also presented evidence supporting the usefulness of SPRY2 as a therapeutic target or prognostic biomarker by examining SPRY2 mRNA expression and protein expression in human patient samples. All these studies provide a convincing argument that SPRY2 is important for GBM tumorigenesis and has potential therapeutic and prognostic significance. There are several directions that could be pursued to continue the work presented here.

In addition to the study presented in Chapter 4, we also examined the effect of SPRY2 on
cellular adhesion and the observation that SPRY2 knockdown promoted non-adherent cell growth (Appendix 1). We found that SPRY2 depletion and EGFRvIII expression promoted a non-adherent growth pattern in U87MG cells and that p38 and JNK signaling controlled this effect. This observation of non-adherent growth patterns is of interest because of its relationship to the study of so-called glioma stem cells (GSCs). It is known that primary GBM cells can be isolated and cultured in a non-adherent “neurosphere” system with serum-free media supplemented with EGF and fibroblast growth factor (FGF) (Tabatabai and Weller, 2011). These cells are referred to as GSCs or tumor-initiating cells because they express markers of neural stem cells, exhibit self-renewal, and form tumors that better resemble GBM in animal models compared to traditional GBM cell lines. These GSCs grown as neurospheres are visually reminiscent of the non-adherent cell growth we observed in U87MG cells with SPRY2 depletion or high EGFRvIII expression. Our preliminary results using established GSC cells presented in Chapter 4 indicate that SPRY2 may function similarly in GSCs to the other traditional cell lines examined. Therefore, further investigation of the role of SPRY2 in GSC survival and development is merited. Additional studies could be undertaken to evaluate whether SPRY2 expression controls cellular markers of neural stem cells that are typically measured in GSCs. To date, our studies have focused on a relatively limited panel of proteins assayed by western blot, but in future studies, a broader “omics” approach would be advantageous to examine a large range of proteins or genes for changes in phosphorylation and expression.

Another important consideration for future studies is the potential compensatory effect of other members of the sprouty family. SPRY1, SPRY2, and SPRY4 are expressed in all tissues (Minowada et al., 1999), while much less is known about the final sprouty family member, SPRY3. There appears to be some degree of redundancy in function of sprouty proteins, but they cannot fully compensate for the loss of a different sprouty family member. This is illustrated by the finding that while single Spry2 or Spry4 knockout mice are viable, Spry2/Spry4 double knockout mice are embryonic lethal (Taniguchi et al., 2007). Like SPRY2, SPRY1 and SPRY4 gene expression is increased in EGFRvIII-positive samples compared to EGFRvIII-negative samples in
TCGA GBM data set and high SPRY1 or SPRY4 expression are associated with reduced survival in patients (Appendix 2). Therefore, concurrent knockdown of SPRY1/SPRY2/SPRY4 could demonstrate a more potent effect than reduction of SPRY2 alone.

Another area of future work that is suggested by our GBM study concerns the relationship between SPRY2 and EGFRvIII expression and how SPRY2 expression is driven in GBM. As described in Chapter 4, in the tumor setting, EGFRvIII expression could promote ERK signaling and therefore promote SPRY2 expression. To test this possibility, ERK phosphorylation could be examined by immunohistochemistry or western blotting in tumor samples with or without EGFRvIII-expression. Another possible explanation is that an alternate pathway is elevated in tumors expressing EGFRvIII and this pathway promotes SPRY2 expression in vivo. A promising candidate pathway is the Wnt/β-catenin pathway that plays critical roles in development, proliferation, and migration. In colon cancer cells, SPRY2 was identified as a target gene of the Wnt/β-catenin pathway and SPRY2 expression was induced by β-catenin through the transcription factor FOXO3a (Ordonez-Moran et al., 2013). There are also studies that suggest that Wnt signaling is dysregulated in GBM (Zhang et al., 2012). In TCGA GBM RNA-seq data, four Wnt genes (WNT10A, WNT16, WNT3, and WNT7A) were significantly upregulated ($p < 0.05$) in EGFRvIII-positive samples compared to EGFRvIII-negative samples. However, four other Wnt genes (WNT4, WNT5A, WNT7B, and WNT2) were also significantly decreased ($p < 0.05$) by the same comparison. To test whether SPRY2 expression is induced by β-catenin and FOXO3a in GBM cells, FOXO3a and stabilized β-catenin could be expressed in cells and resulting changes in SPRY2 expression could be measured.

While we found that SPRY2 gene expression was elevated in tumors expressing EGFRvIII and this relationship was supported by further analysis of patient-derived xenograft samples and immunohistochemistry of human tumors, this relationship was not found in all cell lines tested. It should be noted that this result that data from ectopic EGFRvIII expression in GBM cells lines does not always agree with tumor findings implies (as has been suggested by others (Johnson et al., 2012; Tabatabai and Weller, 2011)) that traditional immortalized GBM cell lines
with ectopic EGFRvIII expression are poor models of GBM in patients. Therefore, other models, such as the GSC cultures discussed above may be more appropriate models for any ongoing studies.

A final consideration that arises from the identification of SPRY2 as a potential therapeutic target is how to develop strategies to interrupt SPRY2 function. Because SPRY2 has no catalytic activity, a molecule that binds and prevents the binding of key interacting partners would be necessary. There is precedent for this type of inhibitor. For example, researchers have designed small molecule inhibitors to block interactions essential for adaptor protein function such as inhibitors of the SH2 domain of GRB2 (Gay et al., 1999). To pursue SPRY2 inhibitors, it will first be important to determine what molecular interactions are of the greatest importance for the pro-oncogenic properties of SPRY2 and whether systemic inhibition of SPRY2 would lead to side effects outside the brain. As discussed previously, if SPRY1 and SPRY4 act similarly in GBM, this could provide clues as to what conserved domains of these sprouty proteins are essential for their pro-tumor properties. Of course, an alternative strategy would be a different therapeutic platform, such as RNAi-based treatment, or inhibition of a key SPRY2-interacting partner or a different protein in the key pathway controlled by SPRY2.

5-5. Main conclusions

Overall, the work presented in this thesis contributes significantly to the knowledge of EGFR function in the context of two deadly cancers where EGFR mutation is common. We focused on two protein regulators of EGFR trafficking and downstream signaling that we hypothesized could be differentially important in the context of cells expressing mutant EGFR. First, we quantified the effects of MIG6 and SPRY2 knockdown on EGFR trafficking and signaling in NSCLC cells to better understand differences between wild-type EGFR and clinically relevant EGFR mutants. Second, we developed a novel model of EGFR trafficking to develop testable hypotheses about mutant EGFR trafficking based on independent pathways of endocytosis. Third, we identified SPRY2 as an important regulator of GBM that is potentially upregulated in the
context of an *EGFR* mutation common in GBM. While SPRY2 may function as a tumor suppressor in other cancers, we found that SPRY2 promoted proliferation, anchorage-independent growth, resistance to EGFR and c-MET co-inhibition in GBM cells, and proliferation as mouse tumor xenografts. As a whole, this work supplements the understanding of the incredibly complex signaling regulatory network in cancer, underscoring the diversity of cellular signaling in different tumor settings and the need to develop novel targeted treatment strategies.
Appendix 1: Changes in cell adhesion in U87MG cells with SPRY2 depletion

A1-1. Results

SPRY2 depletion promotes non-adherent cell growth and decreases cell spreading. To determine the effect of decreased SPRY2 expression, SPRY2 was depleted by stable expression of SPRY2-targeting shRNA in U87MG cells (Figure 4-S1A). It was observed that SPRY2 depletion caused morphological changes to cells in culture (Figure A1-1). While U87MG cells with dead kinase EGFRvIII expression (U87MG-DK) typically formed adherent monolayers, SPRY2 depletion caused the cells to prefer cell-cell contacts and form spheres that could become detached from the surface and continue to proliferate in suspension. This effect was also observed with SPRY2 depletion in SF188 cells that express wild-type EGFR. The non-adherent proliferation of cells with SPRY2 depletion was similar to the growth observed in U87MG cells with high expression levels of EGFRvIII (U87MG-H). Cells with SPRY2 knockdown had a smaller spread area (Figure A1-1B) and spread less quickly (Figure A1-1C) than controls. Although U87-H cells had reduced SPRY2 expression compared to U87MG-DK, U87MG-H cells did not have reduced cell spreading or cell area similarly to U87MG-DK cells with SPRY2 knockdown. As determined by phalloidin staining, F-actin organization was also disrupted in cells expressing SPRY2-targeting shRNA compared to controls (Figure A1-1D). Phalloidin staining indicated that the F-actin organization could be impaired somewhat in U87MG-H cells compared to control U87MG-DK cells.

p38 and JNK inhibition control cell area. Treatment with the p38 inhibitor, SB203580, and the JNK inhibitor, SP600125, suggested that elevated JNK phosphorylation was responsible for the changes in cell area due to SPRY2 depletion in U87MG cells. JNK inhibition resulted in large actin stress fibers and strong phosphorylated paxillin staining at the cell periphery in immunofluorescence images of U87MG-DK cells (Figure A1-2A). p38 inhibition reduced actin organization and focal adhesion size. Consistent with this, JNK inhibition partially rescued the decreased cell area in SPRY2 knockdown cells compared to controls. (Figure A1-2B). Co-treatment with both inhibitors averaged their contrasting effects.
**p38 and JNK inhibition prevent formation of non-adherent cellular aggregates.** We tested whether p38 or JNK inhibition affected the formation of cellular aggregates that proliferated as floating, non-adherent spheres of cells. Cells were plated as single cells in the presence of DMSO control, the p38 inhibitor, SB203580, or the JNK inhibitor, SP600125. Non-adherent spheres of cells formed in DMSO-treated wells for both U87MG-DK cells with SPRY2 knockdown and U87MG-H cells (Figure A1-3A). No non-adherent cells were observed with JNK inhibition, but proliferation was greatly reduced (in agreement with the findings in Chapter 4), and therefore it is possible that there were not enough cells to form spheres. However, p38 inhibition, while having no effect on proliferation, prevented the formation of non-adherent cell spheres. Cells appeared to be growing very densely or even on top on one another, but remained attached to the substrate. When the large multi-cellular spheres formed by U87MG-DK cells with SPRY2 knockdown were re-plated in the presence of p38 or JNK inhibitors, the cells continued to proliferate and grow to be very large spheres of cells (Figure A1-3B). Interestingly, cell spheres cultured in the presence of the JNK inhibitor re-attached and began growing out onto the substrate. Taken together, these results suggest that p38 activity promotes signaling that allows cells to proliferate in suspension.

**Adherent and non-adherent cell populations have different patterns of signaling protein phosphorylation.** To test whether any important signaling pathways were altered between the cells growing attached to the substrate and those growing in non-adherent spheres, we lysed floating and adherent U87MG cells separately to analyze by western blotting (Figure A1-4). There were no floating cells for U87MG-DK cells and so only adherent cells were analyzed. There were decreases in focal adhesion kinase (FAK) phosphorylation in floating cells compared to adherent cells as would be expected for cells not forming focal adhesions. Interestingly, c-MET, ERK, and JNK phosphorylation were also decreased in floating cells compared to adherent cells. In agreement with generally opposing roles for p38 and JNK, p38 phosphorylation was significantly increased in floating cells compared to adherent cells.
Materials and Methods

Cell culture. U87MG cells with three different levels of EGFRvIII expression or expression of a kinase dead EGFRvIII were described previously (Huang et al., 2007) (Dr. Frank Furnari, UCSD, La Jolla, CA, USA). SF188 cells were a gift from Dr. Celeste Simon (University of Pennsylvania, Philadelphia, PA, USA). All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 1 mM L-Glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). SB203580, SP600125 (both from LC Laboratories) were reconstituted in DMSO and added to cells in complete media.

Knockdown of SPRY2. Oligonucleotides encoding hairpins targeting nucleotides 2061-2079 of human SPRY2 were purchased from IDT and cloned into pSicoR.puro (Dr. Tyler Jacks, MIT Koch Institute for Integrative Cancer Research, Cambridge, MA, USA; (Ventura et al., 2004)). A control shRNA was created using a hairpin that does not target a known human mRNA. Lentivirus was produced by calcium-phosphate-mediated transfection of 293FT cells (Life Technologies) with pSicoR.puro, pCMV-VSVg, pMDL-gp-RRE, and pRSV-Rev plasmids (Dr. Marilyn Farquhar, UCSD, La Jolla, CA, USA). Virus-containing supernatant was passed through 0.45 μm syringe filters prior to addition to target cells, and target cells were selected in 1-2 μg/mL puromycin (Sigma).

Western blotting. Whole cell lysates were prepared in a standard cell extraction buffer (Life Technologies) supplemented with protease and phosphatase inhibitors (Sigma). Total protein concentrations were determined by micro-bicinchoninic assay (Thermo Scientific). Lysates were loaded on 4-12% gradient polyacrylamide gels (Life Technologies) under denaturing and reducing conditions and transferred to 0.2 μm nitrocellulose membranes (Bio-Rad). Membranes were imaged on a LI-COR Odyssey scanner (LI-COR) and stripped with 0.2 M NaOH as needed.

Antibodies. Antibodies against p-paxillin (#2541), p-p38 (#4631), p-JNK (#4671), p-FAK (#3283), p-MET (#3126), ERK (#4695), and p-ERK T202/Y204 (#4377) were purchased from Cell Signaling Technology. Infrared dye-conjugated secondary antibodies were purchased from
Rockland Immunochemicals (Gilbertsville, PA, USA). The anti-rabbit Alexa-Fluor 488 secondary antibody for immunofluorescence was purchased from Life Technologies. All antibodies were used according to manufacturer recommendations.

**Cell spreading assay and cell area measurements.** Cells were imaged 45 min after plating on tissue culture plastic in complete media. For inhibitor treatments, cells were pre-treated with inhibitors for 24 hrs prior to plating and maintained in inhibitors. The fraction of cells that were spread versus rounded was quantified. Cells were imaged again 16 hrs after plating and individual cell areas were determined using ImageJ software (NIH). 100 or more cells were measured per experiment.

**Immunofluorescence.** Cells were plated on glass coverslips and cultured overnight in complete growth media with or without inhibitors. Immunofluorescence staining was performed as described previously (Furcht et al., 2012). Hoechst (Life Technologies) and phallolidin-Alexa488 (Life Technologies) were used according to manufacturer recommendations. Cells were washed with PBS and fixed for 20 min in 4% paraformaldehyde in PBS and then permeabilized for 5 min with 0.25% Triton X-100 (Sigma) in PBS. Coverslips were incubated with primary antibodies for 3 hrs at 37°C. After washing in 0.1% Tween-20 (Bio-Rad) in PBS, coverslips were incubated with secondary antibodies, Hoechst (Life Technologies), and phallolidin-Alexa488 (Life Technologies) for 1 hr at 37°C. After washing again, coverslips were mounted onto glass slides with Prolong Gold antifade (Life Technologies). Images were obtained with a Zeiss Axiovert 40 CFL microscope and 100x objective.
Figure A1-1. Cellular morphological changes resulting from SPRY2 knockdown.

(A) Phase contrast images of U87MG cells with expression of dead kinase ("DK") EGFRvIII or high expression of EGFRvIII and SF188 cells with control shRNA or SPRY2-targeting shRNA. (B) 16 hrs after plating, areas of individual cells were quantified for U87MG cells with DK or high EGFRvIII expression and control shRNA or SPRY2-targeting shRNA. (C) Cell spreading at 20 min was measured for U87MG cells with DK or high EGFRvIII expression and control shRNA or SPRY2-targeting shRNA. (D) Immunofluorescence images of U87MG cells with DK or high EGFRvIII expression and control shRNA or SPRY2-targeting shRNA. Cells were stained with Hoechst to visualize the nucleus (blue) and phalloidin-Alexa488 to visualize F-actin (green). Throughout the panels, data are represented as the average of three independent experiments ± s.e.m., and asterisks indicate $p < 0.05$. Images are representative of multiple cells and experiments.
Figure A1-2. Effect of p38 and JNK inhibition on cellular morphology and cell spreading.

(A) Immunofluorescence images of U87MG cells expressing dead kinase (“DK”) EGFRvIII treated with DMSO, 20 μM SB203580 (“p38i”), or 20 μM SP600125 (“JNKi”). Cells were stained with Hoechst to visualize the nucleus (blue), phalloidin-Alexa488 to visualize F-actin (green), and an antibody against phosphorylated paxillin (“p-paxillin”, red). Images are representative of multiple cells. (B) Cell area was quantified in U87MG cells expressing DK EGFRvIII and control shRNA or SPRY2-targeting shRNA were treated with DMSO, 20 μM p38i, 20 μM JNKi, or both 20 μM p38i and 20 μM JNKi. Data are represented as the average of three independent experiments ± s.e.m., and asterisks indicate $p < 0.05$. 
Figure A1-3. Effect of p38 and JNK inhibition on non-adherent cell culture.

(A) U87MG cells with expression of dead kinase (“DK”) EGFRvIII or high expression of EGFRvIII with control shRNA or SPRY2-targeting shRNA were plated as single cells in the presence of DMSO, 20 μM SB203580 (“p38i”), or 20 μM SP600125 (“JNKi”). (B) U87MG cells with expression of DK EGFRvIII and SPRY2-targeting shRNA were cultured to form spheres and then spheres were re-plated in the presence of DMSO, 20 μM p38i, or 20 μM JNKi. Images are representative of multiple experiments.
Figure A1-4. Differential phosphorylation of signaling proteins in adherent and non-adherent cell populations.

(A) Whole cell lysates of U87MG cells with dead kinase ("DK"), low ("L"), medium ("M"), or high ("H") EGFRvIII expression were probed by western blot using antibodies against the indicated proteins. Floating non-adherent cells were lysed separately from adherent cells. (B) Western blots were analyzed by densitometry. Data are represented as the average of three independent experiments ± s.e.m., and asterisks indicate $p < 0.05$. 
Appendix 2: SPRY1 and SPRY4 expression and patient survival in GBM

The TCGA GBM data set was analyzed as in Chapter 4 for changes in expression of other members of the sprouty family. As shown in Figure A2-1, both SPRY1 and SPRY4 expression were elevated in GBM samples with EGFRvIII expression, similarly to what was found for SPRY2. When differences in patient survival were examined, high expression of SPRY4 resulted in reduced patient survival ($p = 0.00955$) with a median survival time of 406 days for high SPRY4 expression and 434 days for low SPRY4 expression. High SPRY1 expression also resulted in reduced patient survival, but with a borderline significant $p$-value of 0.0529. The methods used for this study are described in section 4-3, Materials and Methods.

Figure A2-1. SPRY1 and SPRY4 expression in EGFRvIII-positive samples and effect on patient survival.

(A) SPRY1 and SPRY4 expression (log base 10 transformed RPKM) are higher for EGFRvIII-positive RNA-seq samples ($n = 41$) than EGFRvIII-negative RNA-seq samples ($n = 120$) ($p = 3.64 \times 10^{-7}$ with a fold change of 1.54 for SPRY1 and $p = 2.96 \times 10^{-8}$ and fold change of 1.69 for SPRY4). (B) Survival probability is shown as a function of time after diagnosis for patients classified by SPRY1 or SPRY4 expression, with the median SPRY1 or SPRY4 expression used as a cutoff ($p = 0.0529$ for SPRY1 and $p = 0.00955$ for SPRY4).


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