UNIVERSITY OF CALIFORNIA, SAN DIEGO

Regulation of Growth Factor Signaling, Golgi Orientation, and Cell Migration by GOLPH3

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Marshall Clarke Peterman

Committee in charge:

Professor Seth Field, Chair
Professor Marilyn Farquhar
Professor Kun-Liang Guan
Professor Alexandra Newton
Professor Reuben Shaw

2016

The dissertation of Marshall Clarke Peterman is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____

Chair

University of California, San Diego

2016

DEDICATION

To Kelsie,

my unwavering supporter and partner in life.
You helped me the whole way through.

To my parents,

for your unconditional love and support.

To the victims of cancer,

that you would find hope and your suffering would cease.

To God,
who said,

"Great are the works of the LORD, studied by all who delight in them."
Psalm 111:2

and

"Whatever you do, do all to the glory of God."
1 Corinthians 10:31

EPIGRAPH

It would be more of a gain for science if, abandoning the pretence of constructing edifices which all too often are reduced to castles in the air, we held ourselves, for the moment, to the modest task of studying the facts with patience!

Camillo Golgi

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SUPPLEMENTARY MOVIES

Movie 3.1: Effect of GOLPH3 overexpression on MDA-MB-231 cell migration following monolayer wounding. MDA-MB-231 cells overexpressing GOLPH3-IRES-GFP (top panel) or IRES-GFP (control, bottom panel) were cultured to a confluent monolayer before a scratch was created. Cells were incubated with calcein AM to stain the whole cell after wounding. Scratch healing cell migration following monolayer wounding is shown by time-lapse imaging, with the wound indicated by green lines. GOLPH3-overexpressing cells show more robust scratch wound healing.

Movie 3.2: Relationship between Golgi and mitochondrial movements following monolayer wounding. MDA-MB-231 cells were transfected with ManII-GFP to label the Golgi, and cultured to a confluent monolayer before incubating with MitoTracker to label the mitochondria. After a scratch was created, cells at wound edge were examined by time-lapse imaging. Movement of the Golgi (green) and mitochondria (magenta) in response to wounding was monitored. Open white arrowhead indicates position of the Golgi; filled black arrowheads indicate the ends of the mitochondrial ribbon. The Golgi moves faster than mitochondria toward the leading edge of the cell, resulting in movement relative to mitochondria and the rest of the cell.

Movie 3.3: Relationship between Golgi and nuclear movements following monolayer wounding. MDA-MB-231 cells were transfected with ManII-GFP to label the Golgi, and cultured to a confluent monolayer. After a scratch was created, time-lapse images were taken of cells at wound edge, where movement of the Golgi was examined by GFP and movement of the nucleus by DIC. The red line and the yellow line indicate the rotation of the Golgi and the nucleus, respectively. The Golgi rotates independently and at a faster speed than the nucleus following wounding.

Movie 3.4: Relationship between Golgi and lysosome movements following monolayer wounding. MDA-MB-231 cells were transfected with ManII-GFP to label the Golgi, and cultured to a confluent monolayer before incubating with LysoTracker to label lysosomes. After a scratch was created, cells at wound edge were examined by time-lapse imaging to monitor movement of the Golgi (green) and lysosomes (red) in response to wounding. The Golgi and lysosomes move in a concerted fashion toward the leading edge.

Movie 3.5: Effect of depolymerization of actin on the relationship between the Golgi and lysosomes. MDA-MB-231 cells were transfected with ManII-GFP to label the Golgi, and incubated with LysoTracker to label lysosomes. Cells were treated with DMSO (vehicle control, not shown), or Latrunculin B to depolymerize the actin cytoskeleton. Changes to morphologies of the Golgi and lysosomes were examined by time-lapse imaging following drug

treatment. The Golgi (green) and lysosomes (red) condense upon depolymerization of actin, while their association (merge) remains intact (also see Figure 3.10D).

Movie 3.6: Effect of depolymerization of microtubules on the relationship between the Golgi and lysosomes. MDA-MB-231 cells were transfected with ManII-GFP to label the Golgi, and incubated with LysoTracker to label lysosomes. Cells were treated with DMSO (vehicle control, not shown), or Nocodazole to depolymerize microtubules. Changes to morphologies of the Golgi and lysosomes were examined by time-lapse imaging following drug treatment. Nocodazole causes fragmentation of the Golgi (green) and lysosomes (red). The resulting puncta of the Golgi and lysosome become distinct after treatment with Nocodazole (merge, also see Figure 3.10D).

Movie 3.7: Effectiveness of NH_4Cl and Bafilomycin A1 at neutralizing lysosomes. MDA-MB-231 cells were incubated with LysoTracker to label lysosomes, and treated with DMSO (vehicle control), or each of two lysosomal inhibitors, NH_4Cl and Bafilomycin A1. Effectiveness of lysosome inhibition by both inhibitors is shown by loss of LysoTracker staining in time-lapse imaging following drug treatment.

ACKNOWLEDGEMENTS

Many special people have played instrumental roles along the way from nurturing my scientific beginnings to bringing this dissertation through to its completed form. It is a humbling task to attempt to compile a list of everyone who deserves to be thanked and it makes me realize how much of what I have been able to accomplish must be credited to them. Surely, my debt to each person would be impossible to repay but I will attempt to give at least some brief acknowledgment in this section.

I would like to thank Mark Hori, who opened the first door of opportunity for me to experience working in a research lab at the VA in North Hills, California, where he patiently taught me many skills that, unbeknownst to me at the time, I would continue to rely upon for the ensuing 17 years. The people at SGX Pharmaceuticals were exceedingly kind for giving me many opportunities to learn and grow as a scientist. I would like to thank Ingeborg Feil for taking a chance on me and training me during my internship as a member of her protein biochemistry team. After the internship, others at SGX continued to help me grow, teaching me valuable lessons, and giving me opportunities to learn. I cannot give a complete list of names here, but I thank everyone from that organization. Specifically, I thank Sean Buchanan for giving me the opportunity to join his group, Nathalie Lokker for giving me a chance to grow as a biologist, and Katti Jessen for giving me so many opportunities, so much of her time dedicated to teaching me, and being so fun

to work for. I thank Spencer Emtage for sharing his wisdom and telling me the importance of pursuing a Ph.D. if I intended to continue a career in science.

As I transitioned into the BMS Program at UCSD, I found myself extremely grateful to be among the cohort of students entering in 2008 who made for a friendly yet competitive environment. Starting out, I received a wonderful opportunity to begin looking for inhibitors of cancer cell invasion in Sara Courtneidge's lab and I am thankful to have benefited from very patient training from Bengoña Diaz. Alexandra Newton kindly took me into her lab for my second rotation project where I met many extraordinary people, including Lisa Gallegos who sacrificed so much of her time to help give me the tools I needed to study mutations in PKCβ. My thesis committee, Marilyn Farquhar, Kun-Liang Guan, Alexandra Newton, and Reuben Shaw all generously gave their time to provide feedback, insightful comments, and ideas to ensure my progress. Gina Butcher and Leanne Nordeman were always helpful advocates in the BMS office.

The members of the Field Lab must be thanked for warmly welcoming me when I was a new addition and for always being helpful during my years in the lab. Michelle Ng helped me to hit the ground running, taking time away from her own experiments to help me get started with my projects. Michelle, along with Holly Dippold, Suzette Farber-Katz, Patricia Wiharto, Chris Noakes, Matt Buschman, Yuli Rahajeng, Mengke Xing, Lacey Tran, and Stefanie Makowski never stopped sharing ideas, reagents, and doing favors. Dave Cowan deserves special mention for sharing the space closest to me in the

lab, finding himself the primary victim of my flaws, yet he proved himself to be a true friend at all times. Furthermore, Dave could always be relied upon to go above and beyond, making sure that everything in the lab ran smoothly and that I always had everything I needed. Dave provided assistance on numerous tasks and made my job infinitely easier.

I am sincerely grateful to my mentor, Seth Field, for everything he has invested in me. Seth has spent countless, painstakingly patient hours mentoring me, supplying massive doses of wisdom and knowledge of which I was only competent to ingest a very small portion. Having direct access to Seth's creative and brilliant mind has been one of the greatest privileges of graduate school. I thank him for all of his generosity toward me throughout my graduate school career, including his generous financial support.

The acknowledgments would be incomplete without mentioning my loving family to whom I owe so much. I thank them for their constant support and encouragement. My grandparents, Sam, Opal, Paul and Jean, left a great legacy for the rest of our family to follow and continue to encourage and support one another in higher education. My parents, Dave and Lynda, supported me in anything I ever did and gave me so much even at great cost to themselves. I am thankful that it was always clear that their support was an outpouring of their love rather than a burdensome duty. I thank my brother for always having my back and for the special camaraderie of a close brother. The blessing of growing up in such a loving home and the continued support of my self-sacrificing parents cannot be overstated.

Kelsie, my lovely bride, gave so much of herself to help me accomplish all I have done. I thank her for sticking with me through this whole endeavor from the very beginning, for doing everything she could to support me, and encouraging me every single day. I would not have been able to do it without her. And I am deeply grateful to Kelsie for being the very best mother to our precious daughters. It was a great comfort to know that our girls were in her care when I was away at work. And my sweet girls, Elise and Noelle, I thank them for giving me pictures that made me smile during breaks from the microscope and for filling me with joy when I came home.

Thank you to my fellow scientists for allowing me to use material they co-authored. The material contained in Chapter 2 is being prepared for publication: GOLPH3-dependent Golgi-to-plasma membrane trafficking controls growth factor signaling. Marshall C. Peterman, Mengke Xing, Matthew D. Buschman, Agnieszka T. Grezechnik, and Seth J. Field. The dissertation author was the primary investigator and author of this material.

The material contained in Chapter 3 has been submitted for publication, as it may appear: GOLPH3 drives cell migration by promoting Golgi reorientation and directional trafficking to the leading edge. Molecular Biology of the Cell, 2016. Mengke Xing, Marshall C. Peterman, and Seth J. Field.

VITA

2003 Bachelor of Science in General Biology,

University of California, San Diego

2003-2008 Senior Research Associate,

SGX Pharmaceuticals, San Diego, California

2016 Doctor of Philosophy in Biomedical Sciences,

University of California, San Diego

PUBLICATIONS

Peterman, M.C., Xing, M., Buschman, M.D., Grzechnik, A.T., and Field, S.J. GOLPH3-Dependent Golgi-to-Plasma Membrane Trafficking Controls Growth Factor Signaling. Manuscript in preparation.

Xing, M, **Peterman, M.C.**, and Field, S.J. GOLPH3 drives cell migration by promoting Golgi reorientation and directional trafficking to the leading edge. Submitted.

Farber-Katz, S.E., Dippold, H.C., Buschman, M.D., **Peterman, M.C.**, Xing, M., Noakes, C.J., Tat, J., Ng, M.M., Rahajeng, J., Cowan, D.M., Fuchs, G.J., Zhou, H., and Field, S.J. (2014). DNA damage triggers Golgi dispersal via DNA-PK and GOLPH3. Cell *156*, 413–427.

Buchanan, S.G., Hendle, J., Lee, P.S., Smith, C.R., Bounaud, P.-Y., Jessen, K.A., Tang, C.M., Huser, N.H., Felce, J.D., Froning, K.J., **Peterman, M.C.**, Aubol, B.E., Gessert, S.F., Sauder, J.M., Schwinn, K.D., Russell, M., Rooney, I.A., Adams, J., Leon, B.C., Do, T.H., Blaney, J.M., Sprengeler, P.A., Thompson, D.A., Smyth, L., Pelletier, L.A., Atwell, S., Holme, K., Wasserman, S.R., Emtage, S., Burley, S.K., and Reich, S.H. (2009). SGX523 is an exquisitely selective, ATP-competitive inhibitor of the MET receptor tyrosine kinase with antitumor activity in vivo. Mol. Cancer Ther. *8*, 3181–3190.

Dippold, H.C., Ng, M.M., Farber-Katz, S.E., Lee, S.-K., Kerr, M.L., **Peterman, M.C.**, Sim, R., Wiharto, P.A., Galbraith, K.A., Madhavarapu, S., Fuchs, G.J., Meerloo, T., Farquhar, M.G., Zhou, H., and Field, S.J. (2009). GOLPH3 bridges phosphatidylinositol-4- phosphate and actomyosin to stretch and shape the Golgi to promote budding. Cell *139*, 337–351.

ABSTRACT OF THE DISSERTATION

Regulation of Growth Factor Signaling, Golgi Orientation, and Cell Migration by GOLPH3

by

Marshall Clarke Peterman

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2016

Professor Seth Field, Chair

GOLPH3 is an oncogene that is amplified in many different tumor types and its overexpression often correlates with poor survival. Previously known functions of GOLPH3 include its participation in secretory trafficking from the Golgi and enhancing cell survival following exposure to DNA damaging

agents. In view of the fact that standard cancer chemotherapy commonly employs DNA damaging agents, the survival benefit conferred to cells by GOLPH3 overexpression provides a likely explanation for the correlation of high GOLPH3 expression with poor patient survival. However, this function of GOLPH3 in promoting cell survival seems insufficient to explain why GOLPH3 is an oncogene in the first place. It has been observed that GOLPH3 expression can modulate the strength of growth factor signaling, and inhibition of growth factor signaling ablates GOLPH3's transforming ability, but the mechanism for how GOLPH3 modulates growth factor signaling remains unclear. Chapter 2 of the dissertation documents a systematic investigation into the role of GOLPH3 in growth factor signaling and traces the requirement for GOLPH3 to the level of regulating growth factor receptors themselves. Both GOLPH3 and the unconventional myosin, MYO18A, which mediates the Golgi function of GOLPH3, are each required for growth factor signaling. Depletion of either GOLPH3 or MYO18A retains growth factor receptors at the Golgi, reducing their levels at the plasma membrane, thus explaining the impaired growth factor signaling upon GOLPH3 depletion. Conversely, overexpression of GOLPH3, as observed in many tumor types, is sufficient to increase plasma membrane levels of growth factor receptors, promoting ligand-stimulated receptor activation, and propagating enhanced downstream signaling. Chapter 3 of the dissertation describes the role for GOLPH3 in establishing cell polarity and migration, which are key features of aggressive cancers. Both of the functions of GOLPH3 described in Chapters 2 and 3

require the ability for GOLPH3 to bind phosphatidylinositol-4-phosphate (PtdIns(4)P), a lipid uniquely enriched at *trans*-Golgi membranes, suggesting that it is the PtdIns(4)P/GOLPH3/MYO18A pathway at the Golgi which is responsible. This pathway, therefore, potentially contains novel targets for therapeutic intervention. Chapter 4 of the dissertation provides a summary and implications for future research.

CHAPTER 1: Introduction

1.1 GOLPH3 is a Golgi protein

The structure of the mammalian Golgi was first described in 1898 by the man for whom the structure was named, Camillo Golgi (Golgi and Lipsky, 1989). At the time, many thought the internal reticular structure of neurons that Golgi described was merely an artifact. But in the years since, advances in electron microscopy have enabled clear resolution of discrete stacks of flattened cisternae which compose the characteristic morphology unique to the organelle we now refer to as the Golgi (Farquhar and Palade, 1981). And, although the Golgi's function as a central hub of the cell's secretory system has been universally accepted for quite some time, much less is known about how the Golgi is regulated and its physiological and pathophysiological roles.

One of the most striking features of the Golgi is the distinct, flattened shape of the Golgi cisternae. The maintenance of this unique morphology has perplexed past observers and been thought to be attributed to forces acting upon the Golgi by cytoskeletal components or due to intrinsic properties resulting from asymmetric lipid distribution, protein composition of the Golgi, or from curvature-inducing proteins (Burkhardt, 1998; Egea et al., 2006; Peter et al., 2004; Puthenveedu and Linstedt, 2005; Short et al., 2005). More recently, however, GOLPH3 was found to provide a linkage between the Golgi and the actin cytoskeleton, thereby applying a tensile force that stretches Golgi membranes (Dippold et al., 2009). Depletion of GOLPH3 disrupts vesicle

budding from the Golgi and impairs normal secretion (Bishé et al., 2012a; Dippold et al., 2009; Farber-Katz et al., 2014; Ng et al., 2013). Depletion of GOLPH3 also results in a visible collapse of the flattened Golgi cisternae, underscoring the inherent relationship between Golgi form and function which is regulated by GOLPH3. Inevitably, deeper insights into this mechanism responsible for maintaining the unique morphology of the Golgi will further inform our understanding of Golgi function.

GOLPH3 was first identified through proteomic mass spectroscopy analysis of Golgi proteins (Wu et al., 2000; Bell et al., 2001). These studies provided initial characterization of GOLPH3 as a novel Golgi protein, confirming its localization, identifying the protein sequence and its differentially phosphorylated forms. Work in yeast models led to the observation that deletion of *VPS74* (the *Saccharomyces cerevisiae* homolog of GOLPH3) resulted in aberrant intracellular trafficking of vacuolar proteases, hinting that GOLPH3 plays a critical role in the secretory pathway (Bonangelino et al., 2002). Subsequently, others demonstrated that Vps74p, like its mammalian homolog, localizes to the Golgi and, in yeast, is necessary for proper localization of Golgi-resident glycosyltransferases, protein glycosylation and Golgi secretion, (Schmitz et al., 2008; Tu et al., 2008). These studies helped to highlight the importance of GOLPH3 for Golgi biology but provided little mechanistic insight.

Independently, unbiased screening of the *Drosophila melanogaster* proteome for lipid binding proteins discovered that GOLPH3 binds specifically

to phosphatidylinositol-4-phosphate (PtdIns(4)P) (Dippold et al., 2009). Phosphoinositides may be differentially phosphorylated, generating seven distinct phosphoinositide species, of which PtdIns(4)P is uniquely enriched at *trans*-Golgi membranes (Godi et al., 2004, 1999). The compartmentalization of phosphoinositide species within the cell helps to define a membrane's unique identity and is responsible for the assembly of effector molecules contributing to the membrane's unique properties and functions. Since PtdIns(4)P is enriched at the *trans*-Golgi, the PtdIns(4)P-binding ability of GOLPH3 explains its targeting to the Golgi. Indeed, mutation of the PtdIns(4)P binding pocket of GOLPH3 with the point mutation, Arg to Leu at codon 90 (R90L), abolishes the ability of GOLPH3 to localize to the Golgi and its ability to maintain Golgi secretory function (Dippold et al., 2009). Thus, GOLPH3 is a PtdIns(4)P-effector that is required for Golgi secretory function. This role for GOLPH3 as a PtdIns(4)P-effector provides a missing link, explaining previous observations that PtdIns(4)P is required for Golgi-to-plasma membrane trafficking (Audhya et al., 2000; Hama et al., 1999; Walch-Solimena and Novick, 1999; Wang et al., 2003).

In addition to binding to PtdIns(4)P, GOLPH3 also interacts with the unconventional myosin, MYO18A (Dippold et al., 2009). This dual linkage, with GOLPH3 binding to both the PtdIns(4)P-rich Golgi membranes and MYO18A, thus constitutes a bridge between Golgi membranes and the actin cytoskeleton (Figure 1.1A). Functionally, the PtdIns(4)P/GOLPH3/MYO18A/F-actin apparatus is required for generating the tensile force necessary for

pulling vesicles from the Golgi membrane for efficient Golgi-to-plasma membrane trafficking (Figure 1.1B). Perturbation of any component of this apparatus results in defective secretory trafficking (Dippold et al., 2009; Bishé et al., 2012a; Ng et al., 2013). The tensile force applied to the Golgi by PtdIns(4)P/GOLPH3/MYO18A/F-actin, which is required for secretory trafficking, is also the mechanism behind the unique morphology of flat, stretched Golgi cisternae that had previously confounded decades of cell biologists. Therefore, the study of GOLPH3 highlights the interrelated nature of the structure, composition, and function of the Golgi and further investigation into the roles of GOLPH3 and its regulation are sure to advance our understanding of the roles for the Golgi in biology.

1.2 GOLPH3 is an oncogene

Genome-wide analysis of human cancers revealed that GOLPH3 is frequently amplified in solid tumors (Scott et al., 2009). Focal amplification of the GOLPH3 locus is observed in more than 56% of carcinomas of the lung, 37% of prostate, 38% of ovary, 32% of breast, and 32% of melanomas (Table 1.1). Such a high frequency of GOLPH3 amplification in human tumors raises questions about the function of GOLPH3 in cancer and how the secretory pathway, in general, contributes to tumor growth.

The ability of GOLPH3 to cause oncogenic transformation *in vitro* has been demonstrated to cooperate with other known oncogenes, namely HRAS G12V and BRAF V600E, in order to escape contact inhibition and gain

anchorage independent growth capability (Scott et al., 2009). *In vivo*, overexpression of GOLPH3 contributes to enhanced tumor growth in mouse xenograft models (Scott et al., 2009). These findings demonstrate that overexpression of GOLPH3 confers to cells the classical hallmarks of cancer cells (Hanahan and Weinberg, 2000). Notably, the enhanced tumor growth due to GOLPH3 overexpression is abrogated by treatment with the mTOR complex 1 (mTORC1) inhibitor, rapamycin, suggesting that mTOR signaling is a key mechanism for GOLPH3-driven oncogenic transformation (Scott et al., 2009).

Scott and colleagues, as well as others, have shown that perturbation of GOLPH3 levels influences phosphorylation of signaling proteins including AKT (Ser473), p70S6K (Thr389), GSK3β, and FOXO1 (Scott et al., 2009; Zeng et al., 2012). Each one of these proteins lies downstream of the phosphatidylinositol 3-kinase (PI-3-kinase) –AKT pathway (Manning and Cantley, 2007), which is one of the most frequently over-activated signaling pathways across the spectrum of human malignancies (Courtney et al., 2010; Vivanco and Sawyers, 2002; Yuan and Cantley, 2008). Activation of the AKT signaling pathway is sufficient to induce cellular transformation and targeted inhibition of the AKT signaling pathway is a common strategy behind current drug discovery efforts (Cheng et al., 2005).

Although, several models for the role of GOLPH3 in modulating signal transduction have been postulated, all of the models propose novel functions for GOLPH3, distinct from GOLPH3's known role at the Golgi, and none of

them are able to synthesize the various perturbations to signaling observed upon GOLPH3 knockdown or overexpression. At present, the seemingly most prominent model is based upon a putative interaction of GOLPH3 with a retromer component, VPS35, that somehow modulates mTOR signaling (Abraham, 2009; Scott et al., 2009; Scott and Chin, 2010). As of this writing, confirmation of the endogenous GOLPH3-VPS35 interaction nor a mechanistic basis is not found in the literature. It should also be taken into consideration that the evidence supporting the supposed interaction of GOLPH3 with VPS35 was generated using two systems that are notorious for detecting false-positive results: (1) yeast-two hybrid screening (which is estimated to have a false-positive rate between 44-91% (Mrowka et al., 2001)) and (2) co-immunoprecipitation of overexpressed and tagged exogenous proteins (overexpression and tagging can lead to protein mislocalization and non-native interactions (Kobayashi et al., 2000, p. 1; Yang et al., 1996)). Thus, in the absence of additional supporting evidence, a model for GOLPH3-VPS35 regulation of mTOR remains tentative.

Other models involving additional, novel roles for GOLPH3 have been proposed in order to explain the ability of GOLPH3 to regulate signaling and drive cancer. One group has suggested that GOLPH3 drives glioblastoma migration and invasion through the mTOR-YB1 pathway (X. Zhang et al., 2014). The authors argue that, in cancer, GOLPH3 overexpression correlates with elevated YB1 expression and, for their *in vitro* models, the ability of GOLPH3 to drive migration and invasion are abrogated by either mTOR

inhibition or YB1 silencing. Since YB1 is a mTOR target gene (Hsieh et al., 2012), this evidence points to GOLPH3 regulation of signaling at mTOR or higher.

Others have argued that GOLPH3 regulates FOXO1 by activation of AKT signaling (Zeng et al., 2012), which could be consistent with regulation of mTOR signaling, but may also point further upstream since AKT is both upstream and downstream from mTOR signaling (Huang and Manning, 2009). Adding to the mix, other groups have reported observing that overexpression or knockdown of GOLPH3 correlates with perturbations to PKD protein levels (Zhou et al., 2014), RhoA levels (Zhou et al., 2013), NF-kB signaling (Dai et al., 2015), and sialylation of N-glycans (Isaji et al., 2014). Clearly, a cohesive model accounting for the role of GOLPH3 in signaling has yet to be put forth.

Meanwhile, the evidence for GOLPH3 overexpression as a prognostic indicator for cancer has been mounting. Since Scott and colleagues first identified GOLPH3 as an oncogene, many other groups have now reported that GOLPH3 overexpression is a common feature of many different tumor types and its overexpression correlates with poor patient prognosis (Dai et al., 2015; Hua et al., 2012; Hu et al., 2013, 2014; JianXin et al., 2014; Kunigou et al., 2011; Li et al., 2014, 2012, 2011; Lu et al., 2014; Ma et al., 2014a, 2014b; Peng et al., 2014; Tokuda et al., 2014; Wang et al., 2012, 2014; Xue et al., 2014; Zeng et al., 2012; L.-J. Zhang et al., 2014; X. Zhang et al., 2014; Y. Zhang et al., 2014; Zhou et al., 2012, 2014, 2013). Despite the increasing recognition of a role for GOLPH3 in cancer biology, it still remains to be

answered how GOLPH3, a Golgi protein, is capable of modulating signal transduction and causing oncogenic transformation. Therefore, Chapter 2 will detail the results from a systematic investigation into the role of GOLPH3 in modulating the strength of intracellular signal transduction.

1.3 GOLPH3 and growth factor signaling

Cancer researchers have long been focused on gaining a better understanding of growth factor signaling (Gschwind et al., 2004). The implications of growth factor signaling for cancer were made abundantly clear upon the discovery that viral oncogenes often encode proteins similar to growth factors or constitutively active variants of growth factor receptors. One classic example is the cell transforming v-ErbB gene, which was isolated from Avian erythroblastosis virus (AEV), and shown to be highly similar to the human epidermal growth factor receptor (EGFR), but lacking the EGF-binding domain (Downward et al., 1984). This finding provided proof of concept that dysregulation of growth factor signaling, induced in this case by a viral oncogene that mimics the human EGFR, is sufficient to cause malignant cellular transformation.

The EGFR belongs to a class of growth factor receptors known as receptor tyrosine kinases (RTKs), which have an intracellular tyrosine kinase domain, transmembrane domain, and extracellular ligand-binding domain (Lemmon and Schlessinger, 2010). In the absence of ligand, EGFR normally remains *cis*-autoinhibited, but conformational changes occur upon ligand

binding that relieve autoinhibition, promote oligomerization, *trans*-phosphorylation of the intracellular tyrosine kinase domain and result in receptor activation (Burgess et al., 2003; Hubbard, 2004). Even though EGFR may pre-exist as a dimer in the unbound state, ligand binding is still required for full tyrosine kinase activity of the receptor (Gadella and Jovin, 1995; Moriki et al., 2001; Sako et al., 2000; Yu et al., 2002). The requirement for ligand binding imposes the requirement for EGFR to be localized to the plasma membrane in order to receive extracellular ligands. Furthermore, EGFR propagates its signaling primarily from the plasma membrane (Sousa et al., 2012). Thus, plasma membrane localization of the EGFR is an important aspect for its full activation.

Upon extracellular ligand binding, activation of growth factor receptors initiates assembly of intracellular signaling complexes, propagating downstream signaling cascades. The phosphorylated intracellular tyrosine residues of growth factor receptors recruit additional signaling molecules, either directly or indirectly via adaptor molecules and their Src homology-2 (SH2) domains and phosphotryosine-binding (PTB) domains (Pawson, 2004; Schlessinger and Lemmon, 2003). The assortment of growth factor receptors, coupled with the diversity of SH2-domain and PTB-domain containing proteins, along with the variety of docking proteins and effectors, all serve to generate a vast multiplicity of signaling outputs for growth factor signaling. Nevertheless, a few key pathways of particular importance will be described

here because of their pervasive dysregulation in cancer. A simplified diagram of the key signaling pathways discussed is presented in Figure 1.3.

The phosphatidylinositol 3-kinase (PI-3-kinase) signaling pathway is one of the most commonly overractivated pathways in cancer (Courtney et al., 2010; Vivanco and Sawyers, 2002; Yuan and Cantley, 2008). Many growth factor receptors can activate PI-3-kinase signaling by recruiting the regulatory subunit of class I_A PI-3-kinase, known as p85, which contains SH2-domains that specifically bind to phosphorylated tyrosines of growth factor receptors, or via adaptor molecules bound to the receptors (Carpenter et al., 1993; Kim et al., 1994; Ram and Ethier, 1996). For example, phosphorylation of EGFR on Tyr920 may directly recruit p85 (Stover et al., 1995). Furthermore, the possible mechanisms for p85 recruitment by activated EGFR are greatly multiplied by heterodimerization of other EGFR family members which also interact with p85 directly or via adaptors (Normanno et al., 2006). The recruitment of p85 by a growth factor receptor or its adaptor relieves the inhibition of the catalytic subunit p110, while simultaneously holding PI-3-kinase in close proximity to its substrate at the plasma membrane, so that PtdIns(4,5)P2 becomes phosphorylated, generating PtdIns(3,4,5)P3 at the plasma membrane (Cantley, 2002; Carpenter et al., 1993; Soltoff and Cantley, 1996; Zhao and Vogt, 2008). Thus, plasma membrane localization of growth factor receptors is important not only for ligand binding but also for proximity to PI-3-kinase substrate.

The generation of PtdIns(3,4,5)P3 prompts the recruitment of the PH-domains of two key signaling molecules, PDK1 and AKT, the former which directly phosphorylates AKT on Thr308 (Alessi et al., 1997, 1996; Cantley, 2002; Currie et al., 1999). Additional phosphorylation of AKT on Ser473 is required for its full activation and, though regulated by mTORC2 downstream of growth factor signaling, the precise mechanism is less clear than for PDK1 mediated phosphorylation of AKT on Thr308 (Hresko and Mueckler, 2005; Sarbassov et al., 2006, 2005; Xie and Guan, 2011; Zinzalla et al., 2011).

Activated AKT propagates further signaling by directly phosphorylating a number of different substrates. In response to growth factor signaling, Tuberin (TSC2) is phosphorylated on Thr1462 by AKT (Inoki et al., 2002; Manning et al., 2002). Tuberin, as a heterodimer with its binding partner Hamartin (TSC1), functions as a GTPase-activating protein (GAP) for the small GTPase, Rheb, which, in its GTP-bound state, is a potent activator of mTORC1 (Tee et al., 2003). Thus, the Tuberin-Hamartin heterodimer functions as a negative regulator of mTORC1 signaling by promoting the hydrolysis of Rheb-GTP to Rheb-GDP (Manning and Cantley, 2003). Growth factor stimulated phosphorylation of Tuberin on Thr1462 by AKT inhibits the GAP function of Tuberin-Hamartin, thereby promoting the accumulation of active Rheb-GTP and promoting mTORC1 signaling (Tee et al., 2003).

Like Tuberin, the proline-rich AKT substrate of 40 kDa (PRAS40) also functions as a negative regulator of mTORC1 (Manning and Cantley, 2007). Binding of PRAS40 to Raptor, a core component of mTORC1, competes for

substrates and inhibits mTORC1 kinase activity (Sancak et al., 2007; Wang et al., 2007). This negative regulation of mTORC1 is relieved by growth factor stimulated phosphorylation of PRAS40 on Thr246 by AKT (Kovacina et al., 2003). In summary, AKT phosphorylates Tuberin (Thr1462) and PRAS40 (Thr246), thereby promoting mTORC1 signaling.

The mTOR signaling axis coordinates a vast array of cellular processes required for cell growth, metabolism, proliferation, and survival (Laplante and Sabatini, 2009). One of the many inputs activating mTOR signaling is growth factor stimulation, as enumerated above through PI-3-kinase-AKT-PRAS40/Tuberin signaling. The Raptor containing mTOR complex, known as mTORC1, controls phosphorylation of p70S6K on Thr389, and functions as a regulator of protein translation (Burnett et al., 1998; Isotani et al., 1999). Therefore, PI-3-kinase-AKT-PRAS40/Tuberin signaling leads to mTORC1-dependent phosphorylation of p70S6K (Thr389) in response to growth factor stimulation.

Another key pathway stimulated by growth factor signaling is the Ras signaling pathway. Signaling through Ras is of particular interest because it is estimated that approximately 20% of all human tumors have activating mutations in Ras (Downward, 2003). Unlike PI-3-kinase, Ras is constitutively associated with the plasma membrane by its posttranslational modifications (Eisenberg and Henis, 2008). But similar to p85, growth factor signaling recruits the key regulator for Ras activity to the plasma membrane. Ras is a GTPase and GTP-bound Ras is active until it hydrolyzes GTP, converting into

Ras-GDP (Wennerberg et al., 2005). The GTPase activity of Ras can be accelerated by GAPs, speeding its inactivation, and then returned to its active state upon exchanging GDP for GTP, a process which is promoted by Guanine-nucleotide-Exchange-Factors (GEFs) (Wennerberg et al., 2005). Therefore, the activation of Ras is regulated by its association with a GEF.

One of the primary GEFs for Ras is Sos, which is constitutively associated with the adaptor molecule, Grb2 (Buday and Downward, 1993). Recruitment of Grb2 occurs via Grb2 SH2-domains which bind to phosphorylated EGFR Tyr1068 and Tyr1086, which brings Sos along with Grb2 in proximity to Ras at the plasma membrane (Batzer et al., 1994). Indirect recruitment of Grb2 to EGFR can also occur via EGF-stimulated tyrosine phosphorylation of Shc, bringing Grb2-Sos in proximity to Ras to promote Ras activation (Sasaoka et al., 1994). Once activated, Ras initiates further downstream signaling to Raf-1, leading to phosphorylation and nuclear translocation of ERK (Hallberg et al., 1994; Johnson and Vaillancourt, 1994). The key phosphorylation sites required for enzymatic activation of ERK are Thr202 and Tyr204, which are stimulated by growth factor signaling (Payne et al., 1991; Roskoski, 2012). Growth factor stimulated signaling through the Ras/Raf/ERK pathway results in cell proliferation and, in certain contexts, is sufficient to drive oncogenic transformation (Lefloch et al., 2009; McCubrey et al., 2007; Mercer et al., 2002; Roskoski, 2012).

Experimental perturbations of GOLPH3 protein expression have been shown to modulate intracellular signaling. Knockdown of GOLPH3 reduces

phosphorylation of AKT on Ser473 and p70S6K on Thr389, while overexpression of GOLPH3 has the opposite effect and increases phosphorylation on those sites (Scott et al., 2009). Others have noted changes to additional signaling molecules upon perturbation of GOLPH3, namely, altered phosphorylation of GSK3β and FOXO1 (Zeng et al., 2012). Different models have been proposed for how GOLPH3 modulates signaling, with Scott and colleagues arguing for a role through regulation of mTOR, while Zeng and colleagues argue for a role through FOXO1 (Abraham, 2009; Scott et al., 2009; Scott and Chin, 2010; Zeng et al., 2012). Still, others have argued for additional roles for how GOLPH3 modulates signaling (Dai et al., 2015; Isaji et al., 2014; X. Zhang et al., 2014; Zhou et al., 2014, 2013). These novel mechanisms that have been proposed are independent from GOLPH3's known function at the Golgi and it is unclear from them how GOLPH3 might act to influence signal transduction.

Although the mechanism(s) for how GOLPH3 modulates signaling remains poorly defined, it is evident that GOLPH3's role in modulating signaling is a key component of its contribution to tumor growth. Administration of the mTORC1 inhibitor, rapamycin, to a cohort of mice with tumor xenografts inhibited the growth advantage conferred by GOLPH3 overexpression (Scott et al., 2009). This finding, coupled with the fact that mTOR is a key node for many signaling pathways that are frequently dysregulated in cancer (Zoncu et al., 2011), strongly suggests that whatever the mechanism, modulation of signaling through mTOR is the critical aspect of GOLPH3-induced tumor

growth. Interestingly, GOLPH3 can modulate signaling to p70S6K (Thr389) and AKT (Ser473) when stimulated specifically by EGF (Scott et al., 2009). Since p70S6K (Thr389) and AKT (Ser473) are both known targets downstream of growth factor stimulated mTORC1 and mTORC2 signaling, respectively (Manning and Cantley, 2007), this suggests a role for GOLPH3 in the regulation of growth factor signaling. The ability of GOLPH3 to modulate growth factor signaling has interesting implications for cancer since growth factor receptors and their downstream signaling pathways are well-validated targets for cancer therapeutics (Gschwind et al., 2004). Thus, the GOLPH3 pathway involved with modulating growth factor signaling demands our attention since it contains potential therapeutic targets.

1.4 GOLPH3 and cell migration

The molecular mechanisms underlying cancer cell migration are an area of clinical interest because cell migration confers the ability to spread within a tissue, a key feature contributing to metastatic disease (Friedl and Wolf, 2003). The first step of cell migration is cellular polarization, which involves the assembly of filamentous actin at the leading edge and reorientation of the Golgi, along with the microtubule-organizing center, forward of the nucleus and facing toward the leading edge (Kupfer et al., 1982; Lauffenburger and Horwitz, 1996). Though some of the requisite elements and signals for cell polarization have been reported, the molecular interactions are

unknown which form the basis for the coordinated repositioning of organelles preceding directional migration.

Interestingly, the Golgi reorients in response to extrinsic cues. Signals such as growth factor stimulation are able to prompt reorientation of the Golgi toward the leading edge, which can be blocked by pharmacological inhibition of MEK/ERK signaling (Bisel et al., 2008). Others have documented groups of kinases and phosphatases required for maintaining Golgi structure and these are likewise required for directional migration (Farhan et al., 2010).

In addition to extrinsic cues, certain intrinsic Golgi properties are required for Golgi reorientation and directional cell migration. Depletion of Golgi-membrane associated golgins disrupts Golgi ultrastrucutre, dispersing the Golgi into mini-stacks, and prevents Golgi reorientation to the leading edge (Yadav et al., 2009). This defect in Golgi structure and reorientation prevents polarized secretion (though bulk secretion is unaltered) and is accompanied by defective directional migration. These suggest that Golgi associated proteins are required for proper organization and positioning of the Golgi, which is a key component for migration. Furthermore, the Golgi itself via GM130 is able to control directed secretion which maintains the spatial distribution of active Cdc42 required to establish cell polarity (Baschieri et al., 2014), arguing for a central role for the Golgi in coordinating directional migration. Still, the linkages required to apply the force which reorients the Golgi are unknown. Since GOLPH3 mediates a linkage between Golgi-PtdIns(4)P and MYO18A, it

seems reasonable to hypothesize that GOLPH3 is involved with the process of Golgi reorientation.

Since GOLPH3 expression correlates with advanced metastatic tumors (Dai et al., 2015; Hua et al., 2012; Hu et al., 2013, 2014; JianXin et al., 2014; Kunigou et al., 2011; Li et al., 2014, 2012, 2011; Lu et al., 2014; Ma et al., 2014a, 2014b; Peng et al., 2014; Tokuda et al., 2014; Wang et al., 2012, 2014; Xue et al., 2014; Zeng et al., 2012; L.-J. Zhang et al., 2014; X. Zhang et al., 2014; Y. Zhang et al., 2014; Zhou et al., 2012, 2014, 2013), it is reasonable to predict that GOLPH3 may contribute to a cancer cell's migratory ability. Following this line of reasoning, several groups have reported that GOLPH3 is indeed required for cancer cell migration (Isaji et al., 2014; X. Zhang et al., 2014; Zhou et al., 2013). Moreover, one group has shown that GOLPH3 must retain its ability to bind PtdIns(4)P in order to enhance cancer cell migration (Tokuda et al., 2014). This finding supports the idea that GOLPH3 acts at the Golgi, via PtdIns(4)P interaction, in order to enhance cell migration. Providing new insight for this function, Chapter 3 of the dissertation documents the investigation into the role for GOLPH3 in coordinating Golgi reorientation for cell migration.

1.5 GOLPH3 and the DNA damage response

In order to gain insight into the regulation of GOLPH3, which was originally identified as a phosphoprotein (Wu et al., 2000), further investigation into the specific phosphorylation sites of GOLPH3 was initiated. It was noted

that GOLPH3 peptides were phosphorylated on Thr143, followed immediately by a glutamine residue, comprising the consensus “TQ” motif preferred by the DNA damage activated protein kinase, DNA-PK (Farber-Katz et al., 2014). It was demonstrated that in response to DNA damaging agents, DNA-PK directly phosphorylates GOLPH3 on Thr143, enhancing the GOLPH3/MYO18A interaction, thereby increasing the tensile force applied to the Golgi and initiating a dramatic dispersal of the Golgi (Figure 1.2) (Farber-Katz et al., 2014). The exact function of Golgi dispersal is unclear, however, secretory trafficking is altered and cell survival is enhanced (Farber-Katz et al., 2014). Thus, phosphorylation of GOLPH3 in response to DNA damage confers resistance to killing by DNA damaging agents. Furthermore, the overexpression of GOLPH3, as observed in many tumor types, promotes enhanced cell survival (Farber-Katz et al., 2014). In this regard, GOLPH3 mediates a surprising cytoplasmic response to DNA damage, uncovering a previously unknown role for the Golgi in promoting cell survival following DNA damage.

Many cancer chemotherapy regimens rely upon the administration of DNA damaging agents (Azzoli et al., 2011; Swain, 2011). These agents function by intercalating into DNA and eliciting a cytotoxic DNA damage response (Ciccia and Elledge, 2010). The observation that GOLPH3 overexpression confers resistance to killing by DNA damaging agents (Farber-Katz et al., 2014) suggests that tumors with high GOLPH3 expression are likely refractory to conventional chemotherapy regimens involving the

administration of DNA damaging agents. This seems likely to be responsible, at least in part, for the correlation of high GOLPH3 expression with poor patient outcomes. While this function of GOLPH3 in mediating a survival response to DNA damaging agents seems to provide a plausible explanation for the correlation of GOLPH3 expression with poor prognosis, this aspect of GOLPH3 function seems unable to answer why GOLPH3 overexpression causes oncogenic transformation in the first place.

1.6 Summary

GOLPH3 performs a vital role at the Golgi by bridging PtdIns(4)P-rich Golgi membranes with MYO18A, serving to convey a tensile force necessary for Golgi secretion. The surprising identification of GOLPH3 as an oncogene provides an unprecedented link between the Golgi and cancer. The sheer frequency of GOLPH3 amplification and its correlation with patient mortality provide an urgent rationale for investigation into how GOLPH3 overexpression functions to cause oncogenic transformation. To date, there is no other known Golgi functioning oncoprotein, raising interesting questions about the intersection between the pathophysiology of cancer and regulation of the Golgi. More specifically, while it has been observed that GOLPH3 can modulate phosphorylation of signaling molecules, the mechanism has remained unclear for how a Golgi protein might be able to exert such influence over the strength of growth factor signaling. The role of GOLPH3 in enhancing cell survival following DNA damage lends some insight into why GOLPH3

overexpression in tumors correlates with poor patient prognosis, but it doesn't seem to account for the effects on signaling and oncogenic transformation that have been observed. Therefore, Chapter 2 provides a systematic experimental investigation into how GOLPH3 regulates signal transduction. Chapter 3 documents additional studies uncovering the role for GOLPH3 in cancer cell migration. Chapter 4 summarizes the current models for GOLPH3 in cancer and considers directions for further study.

1.7 Figures

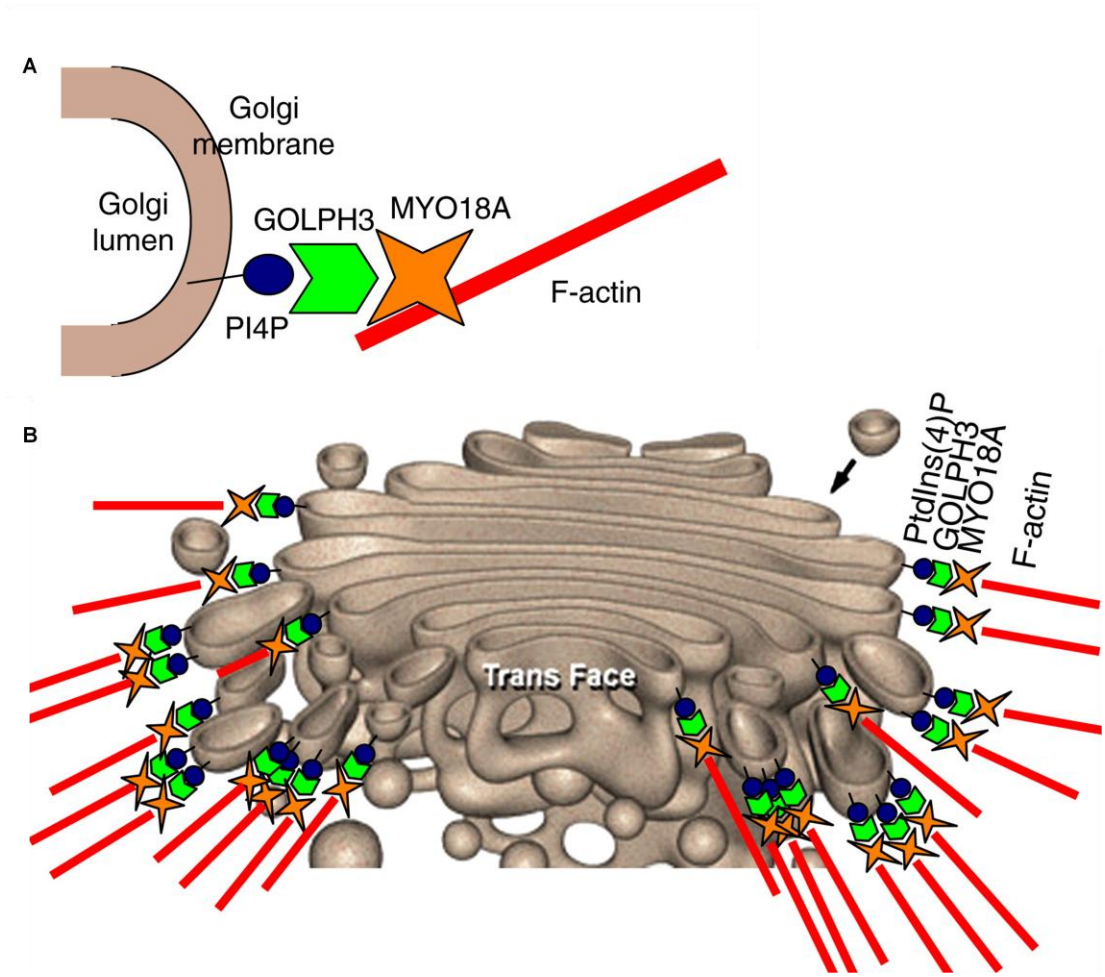

Figure 1.1: The PtdIns(4)/GOLPH3/MYO18A/F-actin pathway. (A) GOLPH3 binds to PtdIns(4)P-rich Golgi membranes and MYO18A creating a linkage between the Golgi and F-actin. (B) The PtdIns(4)P/GOLPH3/MYO18A/F-actin linkage applies a tensile force that pulls vesicles from the Golgi membrane. Each component of the linkage is required for efficient vesiculation and Golgi-to-plasma membrane trafficking. This figure is from Dippold et al., 2009.

Table 1.1: GOLPH3 is frequently amplified in various tumor types. The table summarizes results tumor microarray (TMA) samples subjected to fluorescence in situ hybridization (FISH) for the 5p13 locus. GOLPH3 is the functional target of 5p13 amplification. This table is from Scott, et al., 2009.

Cancer Type	Number Informative Cores	5p13 Status	
		Gain*	Amplification**
Lung Carcinoma	48	27 (56.3%)	16 (33.3%)
Ovarian Carcinoma	48	18 (37.5%)	12 (25.0%)
Pancreatic Carcinoma	12	4 (33.3%)	3 (25.0%)
Liver Carcinoma	17	5 (29.4%)	4 (23.5%)
Breast Carcinoma	31	10 (32.3%)	6 (19.4%)
Prostate Carcinoma	43	16 (37.2%)	8 (18.6%)
Melanoma	38	12 (31.6%)	7 (18.4%)
Colon Carcinoma	33	8 (24.2%)	4 (12.1%)
Multiple Myeloma	37	3 (8.1%)	0

*Gain = signal to reference ratio > 1.5

**Amplification = signal to reference ratio > 2.5

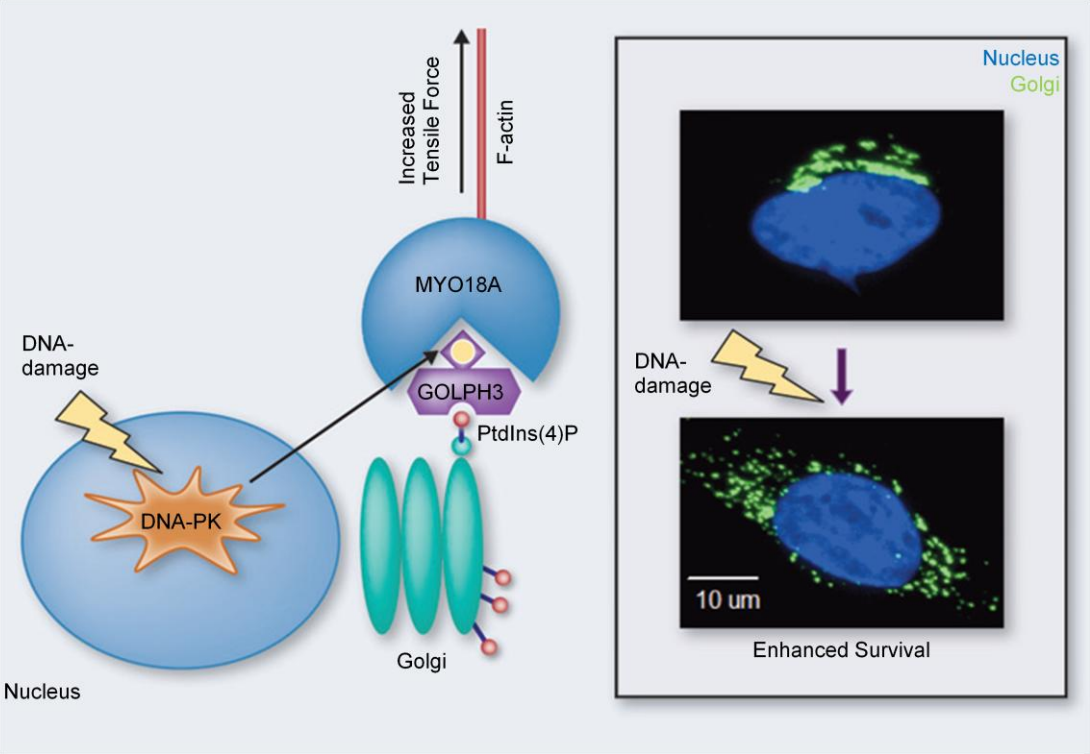

Figure 1.2: DNA damage-induces Golgi dispersal through the GOLPH3 pathway to enhance cell survival. Following DNA damage, DNA-PK phosphorylates GOLPH3 on Thr143. Phosphorylation of GOLPH3 increases the GOLPH3-MYO18A interaction, resulting in increased tensile force applied to the Golgi, and dispersal of Golgi vesicles throughout the cytoplasm. Overexpression of GOLPH3 enhances cell survival following DNA damage, conferring resistance to DNA damaging agents. This figure is from Buschman et al., 2015a.

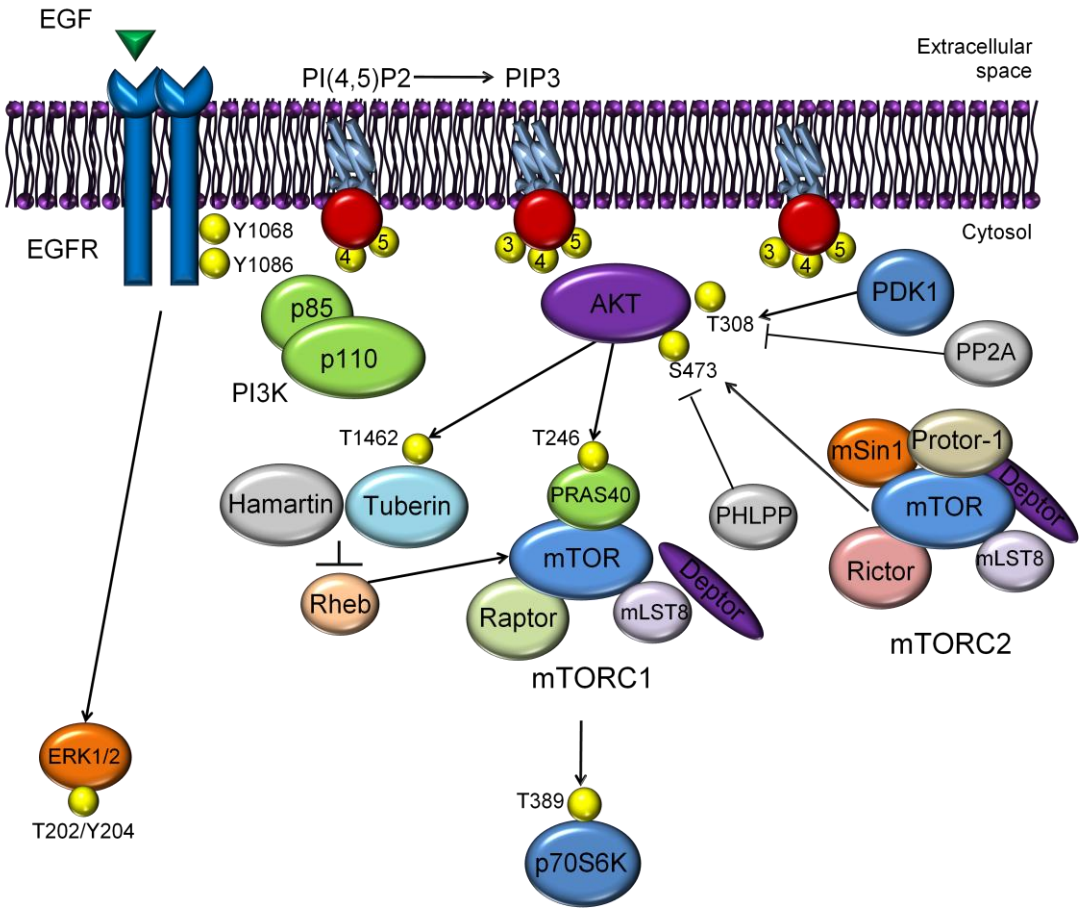

Figure 1.3: Simplified diagram of EGF-stimulated signaling. EGFR signaling is activated when extracellular EGF binds to the EGFR at the plasma membrane, facilitating receptor oligomerization and phosphorylation of cytoplasmic tyrosine residues. The phosphorylated tyrosines of EGFR nucleate the assembly of signaling complexes directly or via adaptor molecules, propagating multiple branches of signaling, including ERK1/2 phosphorylation and recruitment of PI-3-kinase to the plasma membrane where it catalyzes PIP3 production. Both AKT and PDK1 are recruited by PIP3 at the plasma membrane, where PDK1 phosphorylates AKT on Thr308. Then, AKT phosphorylates Tuberin on Thr1462 and PRAS40 on Thr246 to promote mTORC1 signaling, resulting in phosphorylation of p70S6K on Thr389. Phosphorylation of AKT on Ser473 is mediated by mTORC2, also in response to growth factor signaling.

CHAPTER 2: Regulation of growth factor signaling by GOLPH3

2.1 Summary

GOLPH3 is unique in being an oncogene that functions at the Golgi in secretory trafficking. Perturbation of GOLPH3 has been shown to affect phosphorylation events downstream of growth factor receptors. A number of models have been proposed to explain the mechanism, involving novel roles for GOLPH3. Here, using systematic analysis of signaling, we find that depletion of GOLPH3 or its partner MYO18A impairs signaling at the level of the growth factor receptors, and that this is a consequence of impaired trafficking of the receptors from the Golgi to the plasma membrane. Furthermore, we find that overexpression of GOLPH3 enhances growth factor signaling via its function at the Golgi by driving receptor trafficking to the plasma membrane. Thus, we conclude that GOLPH3-dependent Golgi-to-plasma membrane trafficking of growth factor receptors is both necessary for, and rate-limiting for growth factor signaling. The GOLPH3 pathway provides new insight into the role of the Golgi in establishing the strength of growth factor signaling, and provides new targets for therapeutics to interfere with growth factor signaling.

2.2 Introduction

We previously identified GOLPH3 as a novel PtdIns(4)P effector that links the *trans*-Golgi to the unconventional myosin, MYO18A, to convey a

tensile force that participates in vesicle exit for Golgi-to-plasma membrane trafficking (Dippold et al., 2009; Bishé et al., 2012a; Farber-Katz et al., 2014; Ng et al., 2013). Surprisingly, GOLPH3 is also an oncogene that is frequently amplified in human cancers (Buschman et al., 2015a; Scott et al., 2009). Overexpression of GOLPH3 is common in many cancers, and high expression correlates with poor patient prognosis (Scott et al., 2009; Kunigou et al., 2011; Li et al., 2011; Hua et al., 2012; Li et al., 2012; Wang et al., 2012; Zeng et al., 2012; Zhou et al., 2012; Hu et al., 2013; Zhou et al., 2013; Hu et al., 2014; JianXin et al., 2014; Li et al., 2014; Lu et al., 2014; Ma et al., 2014a, 2014b; Peng et al., 2014; Tokuda et al., 2014; Wang et al., 2014; Xue et al., 2014; Y. Zhang et al., 2014; L.-J. Zhang et al., 2014; X. Zhang et al., 2014; Zhou et al., 2014; Dai et al., 2015). Thus, GOLPH3 is the first example of an oncogene that functions in Golgi trafficking, raising questions about the mechanism of transformation. We previously demonstrated that GOLPH3 plays a key role in the Golgi response to DNA damage, which promotes cell survival following DNA damage (Farber-Katz et al., 2014). Since common cancer therapeutics function via DNA damage, GOLPH3's role in the DNA damage response provides a plausible explanation for the correlation between high expression and poor prognosis in cancer. However, this seems unable to explain the ability of GOLPH3 to cause oncogenic transformation.

Scott, et al. observed a role for GOLPH3 in modulating the strength of growth factor signaling (Scott et al., 2009). They proposed a novel function for GOLPH3 mediated by mTOR, independent of GOLPH3's function at the Golgi.

Here we systematically examine the ability of GOLPH3 to modulate the strength of growth factor signaling and trace it to regulation of the growth factor receptors. We find that both GOLPH3 and MYO18A are required for Golgi-to-plasma membrane trafficking of growth factor receptors. Therefore, interference with the GOLPH3 pathway impairs signaling by growth factor receptors, and everything downstream. We demonstrate that the requirement for the GOLPH3 pathway for growth factor signaling, rather than involving a novel signaling pathway, is a consequence of its function in Golgi-to-plasma membrane trafficking. Furthermore, overexpression of GOLPH3 is capable of driving enhanced growth factor signaling by driving increased receptor levels at the plasma membrane. Thus, surprisingly, we find that GOLPH3-dependent Golgi-to-plasma membrane trafficking is rate-limiting for growth factor signaling.

2.3 Results

2.3.1 GOLPH3 and MYO18A are required for growth factor signaling

It was previously shown that knockdown of GOLPH3 interferes with growth factor stimulated phosphorylation of AKT on Ser473 and p70S6K on Thr389 (Abraham, 2009; Scott et al., 2009; Scott and Chin, 2010; Zeng et al., 2012). Since these are dependent on the mTOR complexes mTORC2 and mTORC1, respectively, it was proposed that GOLPH3 may function to regulate mTOR, independent of GOLPH3's role at the Golgi (Scott et al.,

2009). To better understand how GOLPH3 influences growth factor signaling, we systematically assessed changes in signaling caused by perturbation of GOLPH3. First, we sought to reproduce the published result (Scott et al., 2009) that knockdown of GOLPH3 interferes with EGF-stimulated phosphorylation of AKT on Ser473 and p70S6K on Thr389 in A549 cells. We performed Western blots on whole cell SDS lysates of control or GOLPH3 knockdown A549 cells that were growth factor-starved or starved and stimulated with 10 ng/ml EGF for 5 minutes. As reported, we observed that although EGF robustly stimulated the phosphorylation of AKT on Ser473 and p70S6K on Thr389 in control A549 cells, knockdown of GOLPH3 significantly impaired this response to EGF (Figure 2.1).

p70S6K phosphorylation by mTORC1 is downstream of AKT (Manning and Cantley, 2007), and a defect in this phosphorylation might be due to impaired activation of AKT. To determine whether signaling downstream of AKT is generally impaired by knockdown of GOLPH3, we examined phosphorylation of two direct AKT substrates, PRAS40 on Thr246 (Kovacina et al., 2003) and Tuberin on Thr1462 (Inoki et al., 2002; Manning et al., 2002). We observed that these EGF-stimulated phosphorylation events were similarly impaired by knockdown of GOLPH3 (Figure 2.1).

Since phosphorylation of AKT on Ser473, as well as two AKT substrates, was reduced by GOLPH3 depletion, this suggested that the reduction in signaling might not be due to an effect on mTOR, but that GOLPH3 might influence signaling further upstream. To assess EGF-

stimulated signaling independent of the AKT pathway, we examined the role of GOLPH3 in the ERK branch of growth factor signaling, as measured by ERK1/2 phosphorylation on Thr202/Tyr204 (Figure 2.1). While EGF stimulated robust phosphorylation of ERK1/2 on Thr202/Tyr204 in control cells, it was significantly impaired by knockdown of GOLPH3. As expected, pre-treatment with the PI-3-kinase inhibitor wortmannin (100 nM for 10 minutes) abolished phosphorylation of AKT, its substrates PRAS40 and Tuberin, and the downstream target p70S6K, but did not impair phosphorylation of ERK1/2. However, wortmannin did not alter the requirement for GOLPH3 for phosphorylation of ERK1/2, indicating that GOLPH3's role is independent of the PI-3-kinase/AKT/mTORC1 pathway.

Since both the AKT and ERK branches of signaling are impaired by depletion of GOLPH3, we suspected that GOLPH3 acts at the level of the EGF receptor (EGFR) itself. We assessed phosphorylation of the EGFR on Tyr1068 and Tyr1086, observing that EGF stimulated phosphorylation in control cells, but this phosphorylation was significantly impaired upon knockdown of GOLPH3. Notably, this was also true in cells pre-treated with wortmannin, again indicating that GOLPH3's role is independent of events downstream of PI-3-kinase. The defect in signaling by the EGFR itself upon knockdown of GOLPH3 provides a parsimonious explanation for all of the downstream effects on signaling.

To determine if the role of GOLPH3 in signaling by the EGFR is restricted to A549 or is more general, we next examined signaling in HeLa

cells. Again, we observed that knockdown of GOLPH3 impairs signaling by the EGFR itself (Tyr1068), with consequently impaired downstream signaling, including defects in AKT phosphorylation (Ser473 and Thr308), ERK1/2 (Thr202/Tyr204), PRAS40 (Thr246), and Tuberin (Thr1462) (Figure 2.2A). Quantification of multiple independent experiments indicates a highly significant, >50% reduction in signaling upon knockdown of GOLPH3 (Figure 2.2B). Furthermore, we found that three independent siRNAs targeted against GOLPH3 similarly impair EGF-stimulated signaling (Figure 2.3), indicating that the effect on signaling is due to knockdown of GOLPH3, rather than an off-target effect of an siRNA. We also examined signaling in normal primary human umbilical vein endothelial cells (HUVECs) and human glioblastoma-derived U87 cells. In both, we again observed impaired EGF signaling upon depletion of GOLPH3 (Figures 2.4A and 2.5). We conclude that the requirement for GOLPH3 for growth factor signaling is common to many types of cells.

To determine whether GOLPH3's role in growth factor signaling occurs via its function in Golgi trafficking in the PtdIns(4)P/GOLPH3/MYO18A pathway, we examined whether MYO18A is also required for EGF signaling. In HeLa cells we observed that knockdown of MYO18A completely recapitulated the effect of knockdown of GOLPH3, resulting in a defect in EGF stimulated phosphorylation of the EGFR, and a defect in all downstream signaling as well (Figure 2.2A). Quantification revealed a significant, >45% reduction in signaling upon knockdown of MYO18A, similar to the effect of knockdown of

GOLPH3 (Figure 2.2B). Comparable results are observed with knockdown of MYO18A with each of three distinct siRNA oligonucleotides (Figure 2.6). MYO18A is likewise required for EGF signaling in HUVECs and U87 glioblastoma cells (Figures 2.4B and 2.5). Since GOLPH3 and MYO18A are both required for EGF signaling, we infer that the GOLPH3/MYO18A pathway that functions at the Golgi is required for EGF signaling.

Because we observed that GOLPH3 and MYO18A were required for efficient EGF-stimulated activation of EGFR, we wondered whether signaling by other growth factors also depends upon GOLPH3 and MYO18A. HeLa cells express detectable levels of insulin and IGF1-receptors (Giudice et al., 2013; McKeon et al., 1990), allowing detection of insulin-stimulated signaling (Figure 2.7A). While stimulation by insulin increased the phosphorylation of both AKT (Thr308 and Ser473) and ERK1/2 (Thr202/Tyr204) in control cells, knockdown of either GOLPH3 or MYO18A impaired both AKT and ERK signaling. Quantification of multiple independent experiments demonstrated a significant reduction in insulin-stimulated phosphorylation of AKT and ERK upon depletion of GOLPH3 or MYO18A (Figure 2.7B). As with the response to EGF, ablation of PI-3-kinase signaling by wortmannin abolished AKT phosphorylation, but did not abolish the effect of GOLPH3 or MYO18A knockdown on insulin-stimulated phosphorylation of ERK. These data demonstrate that the role of GOLPH3/MYO18A in modulating insulin signaling is independent of the PI-3-kinase/AKT/mTORC1 pathway.

Next, to test signaling stimulated by another growth factor, we examined signaling by the PDGF receptor. HeLa cells do not express PDGF receptors (Pietras et al., 2008), but PDGF responsiveness can be reconstituted by expression of exogenous PDGFRβ-GFP (Figure 2.8A) (Haj et al., 2002; Lim et al., 2010). In HeLa cells transfected with increasing amounts of a PDGFRβ-GFP expression vector we observed increasing PDGF-BB-stimulated phosphorylation of the receptor on Tyr751 and of AKT on Thr308 and Ser473 but not in mock transfected cells. The response to PDGF was impaired by knockdown of GOLPH3, where we observed reduced phosphorylation of the PDGF receptor, as well as reduced phosphorylation of AKT on Thr308 and Ser473. Quantification of multiple independent experiments demonstrated a significant reduction in PDGFRβ signaling upon depletion of GOLPH3 (Figure 2.8B). Collectively, our results lead to the conclusion that the GOLPH3/MYO18A pathway is required for efficient signal transduction by multiple growth factor receptors, due to impaired signaling by the receptors themselves.

Cell cycle entry is a pivotal consequence of growth factor stimulated signaling (Sherr, 1996). Thus, we asked whether EGF-stimulated cell cycle entry was affected by interference with the GOLPH3/MYO18A pathway. We synchronized HeLa cells in G_0 by removal of serum from the growth medium for 24 hours, stimulated re-entry into the cell cycle with EGF treatment, and at various timepoints measured the distribution of DNA content in each population of cells (Figure 2.9A). In control cells, following 20 hours of EGF

stimulation there is an increase in the fraction of cells with 4N DNA content, indicating progression to the G2/M phase of the cell cycle. By contrast, the ability of EGF treatment to stimulate re-entry into the cell cycle is significantly impaired by siRNA-mediated depletion of either GOLPH3 or MYO18A (Figure 2.9B). Our results demonstrate that growth factor stimulated signal transduction, from the activation of the receptor to the mitogenic response, is dependent upon the GOLPH3/MYO18A pathway.

2.3.2 GOLPH3 and MYO18A are required for Golgi-to-plasma membrane trafficking of the EGFR

The observations that GOLPH3 and MYO18A function in Golgi-to-plasma membrane trafficking (Bishé et al., 2012a; Dippold et al., 2009; Farber-Katz et al., 2014; Ng et al., 2013), and that both are required for efficient growth factor signaling, suggested a model whereby the requirement for GOLPH3 and MYO18A for growth factor signaling is due to the requirement for the receptor to traffic to the plasma membrane in order to encounter extracellular growth factors. We wondered whether knockdown of GOLPH3 or MYO18A would modify trafficking of growth factor receptors, thus altering the subcellular localization of the receptors. We examined the subcellular localization of the EGFR in control versus GOLPH3 or MYO18A knockdown HeLa cells (Figure 2.10A). Cells were serum-starved, reducing EGFR internalization, leading to its accumulation at the plasma membrane in control cells (Wiley et al., 1991). By contrast, knockdown of either GOLPH3 or

MYO18A resulted in significant intracellular localization of the EGFR, with a consequent decrease in the amount of EGFR at the plasma membrane. Quantification of the fraction of EGFR at the plasma membrane demonstrated a highly significant reduction caused by knockdown of GOLPH3 or MYO18A (Figure 2.10B).

To determine if the reduction of EGFR at the plasma membrane was due to a defect in Golgi-to-plasma membrane trafficking, we examined whether there was a corresponding increase in the amount of EGFR at the Golgi. To allow for unambiguous identification of the Golgi we used a HeLa cell line that we generated that stably expresses the transmembrane Golgi localization domain of α-mannosidase II fused to Green Fluorescent Protein (ManII-GFP) (Llopis et al., 1998). Immunofluorescence to examine EGFR subcellular localization revealed an increase at the Golgi upon knockdown of GOLPH3 or MYO18A (Figure 2.11A). Quantification demonstrated the effect to approximately mirror the decrease in EGFR at the plasma membrane, and again was highly significant (Figure 2.11B).

Our data demonstrate a role for GOLPH3 and MYO18A in growth factor signaling due to their role in Golgi-to-plasma membrane trafficking to deliver receptors to the plasma membrane. Thus, the GOLPH3/MYO18A pathway is required for signaling at the level of the receptor, and therefore for all downstream signaling as well. We conclude that the requirement for GOLPH3 and MYO18A for growth factor signaling, rather than requiring a novel, unknown growth factor signaling pathway, is a predictable consequence of

their role in Golgi-to-plasma membrane trafficking, although unanticipated in the literature (Abraham, 2009; Scott et al., 2009; Scott and Chin, 2010; Zeng et al., 2012).

2.3.3 GOLPH3 overexpression at the Golgi enhances growth factor signaling

It is surprising that GOLPH3, a protein that functions in Golgi-to-plasma membrane trafficking, is an oncogene (Abraham, 2009; Buschman et al., 2015a; Dai et al., 2015; Hua et al., 2012; Hu et al., 2013, 2014; JianXin et al., 2014; Kunigou et al., 2011; Li et al., 2014, 2012, 2011; Lu et al., 2014; Ma et al., 2014a, 2014b; Peng et al., 2014; Scott et al., 2009; Scott and Chin, 2010; Tokuda et al., 2014; Wang et al., 2012, 2014; Xue et al., 2014; Zeng et al., 2012; L.-J. Zhang et al., 2014; X. Zhang et al., 2014; Y. Zhang et al., 2014; Zhou et al., 2012, 2014, 2013). We wondered whether GOLPH3 could be rate-limiting for growth factor signaling, at least in some circumstances. Previously, overexpression of GOLPH3 was reported to promote phosphorylation of AKT, p70S6K, FOXO1, and GSK3β (Scott et al., 2009; Zeng et al., 2012), all of which are known to occur downstream of growth factor receptor signaling (Alessi et al., 1996; Brunet et al., 1999; Cross et al., 1995; Dennis et al., 1996). Previous studies hypothesized that overexpression of GOLPH3 might enhance signaling via a direct effect on mTOR, independent of GOLPH3's role at the Golgi (Abraham, 2009; Scott et al., 2009; Scott and Chin, 2010). To examine the effect of overexpression of GOLPH3 on growth factor signaling

we generated a bicistronic GOLPH3-IRES-GFP expression vector to allow overexpression of untagged GOLPH3 along with GFP by transient transfection. We examined the effect of overexpression of GOLPH3 on EGFR signaling in the breast cancer-derived MDA-MB-231 cell line, to first recapitulate the published observation of increased phosphorylation of AKT on Ser473 (Zeng et al., 2012). By Western blot we observed that serum-starved, control IRES-GFP transfected MDA-MB-231 cells respond to EGF with increased phosphorylation of the EGFR at Tyr1068 and of AKT on Ser473 (Figure 2.12). Overexpression of GOLPH3 enhanced the response to EGF, with increased phosphorylation of AKT, as reported, but we also observed increased phosphorylation of the EGFR, as well. To test whether the ability of GOLPH3 to drive enhanced signaling is due to its function at the Golgi we compared wild-type (WT) GOLPH3 to the R90L point mutant that is unable to bind to PtdIns(4)P and thus unable to localize to the Golgi (Dippold et al., 2009). Unlike WT GOLPH3, GOLPH3(R90L) was unable to enhance EGF signaling, indicating that the ability of GOLPH3 to drive growth factor signaling depends on its function at the Golgi.

The effect on signaling we observed by Western blot was limited by the efficiency of transient transfection, and thus we next examined signaling using a single cell assay. We used GFP fluorescence to identify cells expressing either GOLPH3-IRES-GFP or the IRES-GFP control, and could detect in each cell phosphorylation of AKT on Ser473 using immunofluorescence microscopy (Figure 2.13A). Increased phosphorylation of AKT was detected in response to

stimulation with EGF, and this was blocked by pre-treatment with wortmannin, providing confidence in the specificity of the assay for phosphorylated AKT (Ser473) (Figure 2.13A). Furthermore, we observed increased phosphorylation of AKT on Ser473 in cells overexpressing GOLPH3. We quantified phospho-AKT (Ser473), pooling data from multiple experiments, observing a significant increase in EGF-stimulated phosphorylation of AKT caused by overexpression of GOLPH3 (Figure 2.13B).

To determine whether signaling stimulated by other growth factors was enhanced by GOLPH3 overexpression, we measured the response to insulin. We found that GOLPH3 overexpression significantly enhanced insulin-stimulated phosphorylation of AKT (Ser473) (Figure 2.14). To determine if the ability of GOLPH3 to drive increased signaling is dependent on its function at the Golgi, we examined the effect of the GOLPH3(R90L) point mutant. Since the R90L mutation ablates the ability of GOLPH3 to drive enhanced signaling, we conclude that overexpression of GOLPH3 influences signaling through its function at the Golgi.

Since Western blotting indicated that overexpression of GOLPH3 resulted in enhanced signaling at the level of the receptor itself, we further examined phosphorylation of the EGFR using single-cell immunofluorescence. We examined EGFR (Tyr1068) phosphorylation in response to EGF, using immunofluorescence to measure EGFR phosphorylation specifically in cells that were overexpressing GOLPH3-IRES-GFP or the IRES-GFP control (Figure 2.15A). We found that overexpression of GOLPH3 led to a significant

increase in EGF-stimulated EGFR (Tyr1068) phosphorylation (Figure 2.15B). Again, the GOLPH3(R90L) mutant had no effect. Thus, we conclude that GOLPH3's function at the Golgi is rate-limiting for growth factor receptor signaling.

2.3.4 GOLPH3 overexpression promotes trafficking of the EGFR to the plasma membrane

Given that GOLPH3 is required to maintain normal plasma membrane levels of EGFR (Figure 2.10, A and B), we wondered whether the ability of GOLPH3 to drive enhanced signaling is due to increased delivery of the growth factor receptor to the plasma membrane. Therefore, we examined EGFR localization to the plasma membrane upon overexpression of GOLPH3. We transiently transfected MDA-MB-231 cells with expression vectors for GOLPH3-IRES-GFP or IRES-GFP. We rendered the cells quiescent by serum-starvation, thus resulting in accumulation of the EGFR at the plasma membrane. We then used immunofluorescence to examine the subcellular localization of the EGFR in GFP-positive cells (Figure 2.16A). As shown, overexpression of GOLPH3 results in a significant increase in EGFR localization to the plasma membrane. We quantified EGFR localization at the plasma membrane relative to total expression, demonstrating a highly significant increase in EGFR localization to the plasma membrane caused by overexpression of GOLPH3 (Figure 2.16B). By comparison, the GOLPH3(R90L) point mutant has no effect, indicating that GOLPH3's ability to

drive the EGFR to the plasma membrane is dependent on GOLPH3's ability to bind to PtdIns(4)P, and thus to localize to the Golgi. The ability of GOLPH3 to control the delivery of the EGFR to the plasma membrane provides a satisfying explanation for the mechanism by which a Golgi protein can modulate the strength of growth factor signaling.

2.4 Discussion

2.4.1 Regulation of growth factor receptor trafficking and signaling

The discovery that GOLPH3 is an oncogene, together with the discovery that GOLPH3 functions at the Golgi, made an unprecedented link between cancer and secretory trafficking, although the mechanism remained unclear. Indeed, attempts to explain how GOLPH3 can modulate growth factor signaling involved the proposal of novel functions for GOLPH3 (Dai et al., 2015; Isaji et al., 2014; Scott et al., 2009; Scott and Chin, 2010; Zeng et al., 2012; X. Zhang et al., 2014; Zhou et al., 2014, 2013), unrelated to its well-documented role at the Golgi in trafficking to the plasma membrane (Bishé et al., 2012a; Dippold et al., 2009; Farber-Katz et al., 2014; Ng et al., 2013). Here, by using unbiased, systematic mapping of the effect of GOLPH3 on growth factor signaling, we found that the GOLPH3 pathway is required for growth factor signaling because it is required for trafficking of growth factor receptors from the Golgi to the plasma membrane. Trafficking to the plasma membrane is required for growth factor receptors to have access to

extracellular growth factor ligands. This provides a straightforward explanation for the role of a Golgi protein in growth factor signaling. Surprisingly, we find that GOLPH3-dependent Golgi-to-plasma membrane trafficking is rate-limiting for growth factor signaling. It will be interesting to determine whether regulation of GOLPH3-dependent trafficking might be used for physiological cell autonomous regulation of cellular reception of growth factor signals.

2.4.2 Quantitative changes in signaling

Throughout this study we attempt careful measurements of quantitative effects. Our measurements are sufficient to allow assessment of statistical significance of differences across treatments. Interpretation of the magnitude of observed effects is less certain since we cannot be confident of the linearity of all of the assays. Nevertheless, if taken at face value, we can use these data to calculate a Hill coefficient for the input/output relationship between EGFR concentration at the plasma membrane and EGFR phosphorylation. Using data from our knockdown and overexpression experiments we calculate a Hill coefficient of 3.8 ± 0.2 (mean ± SEM). While it is well known that the EGFR forms at least a dimer (Yarden and Schlessinger, 1987; Schlessinger, 2002), our results are consistent with several studies that conclude that the EGFR functions as a tetramer (van Belzen et al., 1988; Whitson et al., 2004; Clayton et al., 2005, 2008).

2.4.3 Regulatory feedback

Recent studies have indicated that growth factor signaling regulates PtdIns(4)P levels at the Golgi. In particular, Blagoveshchenskaya et al. observe that p38/MAPK activation downstream of growth factor receptor signaling leads to phosphorylation of SAC1, the PtdIns(4)P-4-phosphatase (Blagoveshchenskaya et al., 2008). This phosphorylation causes SAC1 to relocalize from the Golgi to the ER, resulting in elevated levels of PtdIns(4)P at the Golgi and consequently increased secretory trafficking following growth factor stimulation. GOLPH3 is a major effector of PtdIns(4)P, and thus taken together with the data presented here, this suggests a role for the GOLPH3 pathway in a positive feedback loop in growth factor signaling. It will be interesting to investigate the significance of this possibility.

2.4.4 Consequences for cancer biology

The present study demonstrates that the GOLPH3 pathway is required, and rate-limiting for delivery of growth factor receptors to the plasma membrane. More generally, we have observed that the GOLPH3 pathway is required for delivery to the plasma membrane of a wide variety of proteins (Bishé et al., 2012a; Dippold et al., 2009; Ng et al., 2013). Thus, we expect that perturbations of the GOLPH3 pathway likely will have pleiotropic effects on cell function. We suspect that, like many other oncogenes, there may exist multiple mechanisms for GOLPH3 to promote cancer initiation and/or progression via modulation of Golgi-to-plasma membrane trafficking. Notably, overexpression of GOLPH3, through its function at the Golgi, can confer

resistance to killing by DNA damaging agents (Farber-Katz et al., 2014). There also is evidence that overexpression of GOLPH3 can increase cell migration and invasion, again through its function at the Golgi (Isaji et al., 2014; Tokuda et al., 2014). It is possible that all of these effects are the result of enhanced growth factor signaling due to overexpression of GOLPH3. For example, EGFR signaling is known to modify the cellular response to DNA damage (Lee et al., 2012). However, we suspect that the consequence of overexpression of GOLPH3 likely results from perturbed trafficking of multiple cargoes, producing pleiotropic effects.

Nevertheless, GOLPH3's ability to modulate growth factor signaling is likely an important part of its role in cancer. Indeed, previous publications suggest that the ability of GOLPH3 to cause oncogenic transformation in xenograft mouse models is dependent on signaling downstream of mTORC1 (Scott et al., 2009). GOLPH3's role in growth factor signaling also has important therapeutic implications. Interference with growth factor signaling is now a well-validated strategy for cancer therapeutics. This therapeutic approach has been limited by the appearance of mutations in the drug binding site, or by activation of alternative growth factor receptors (Niederst and Engelman, 2013). Notably, we have demonstrated that interfering with the GOLPH3 pathway interferes with signaling by multiple growth factor receptors. Moreover, the mechanism, a defect in trafficking to the plasma membrane, suggests that interference with the GOLPH3 pathway likely impairs signaling by all growth factor receptors. We expect that even mutated, constitutively

active receptors will depend on trafficking to the plasma membrane, since PI-3-kinase signaling requires its substrate, PtdIns(4,5)P2, which is uniquely enriched at the plasma membrane. Thus, the GOLPH3 pathway presents an interesting target for novel cancer therapeutics. Regardless, the GOLPH3 pathway reveals an important, previously unappreciated role for the Golgi in regulating the strength of growth factor signaling.

2.5 Materials and methods

2.5.1 Cell culture

Mammalian cell lines including HeLa S3, A549, U87, and MDA-MB-231 cells were grown according to American Type Culture Collection (ATCC) guidelines. HUVECs were maintained in EGM-2 medium (Lonza). HeLa cells stably expressing α-mannosidaseII-GFP (Llopis et al., 1998) were generated by transfection using linear polyethyleneimine MW 25K and G418 resistant clones were selected. For serum-starvation, cells were washed once with PBS and placed in media lacking serum and growth factors for 18 hours (unless otherwise noted) prior to growth factor stimulation and lysis or fixation. Cells were stimulated with the indicated amount of recombinant human EGF (Life Technologies), recombinant human insulin (Sigma-Aldrich), or recombinant human PDGF-BB (Austral Biologicals) for the indicated time prior to lysis or fixation.

2.5.2 siRNA and plasmid transfections

siRNA sequences and transfections were carried out as previously described (Dippold et al., 2009) in HeLa, A549, and U87 cells. siRNA transfection into HUVECs was performed using DharmaFECT (Thermo Scientific). Unless indicated otherwise, GOLPH3 siRNA #3 and MYO18A siRNA #1 were used. GOLPH3-IRES-GFP and related overexpression plasmids were described previously (Farber-Katz et al., 2014). Plasmids were transiently transfected using *Trans*IT LT-1 transfection reagent (Mirus Bio).

2.5.3 Antibodies and reagents

AKT, pAKT (Ser473), pAKT (Thr308), pp70S6K (Thr389), Tuberin, pTuberin (Thr1462), PRAS40, pPRAS40 (Thr246), pERK 1/2 (Thr202/Tyr204), EGFR, pEGFR (Tyr1068), pEGFR (Tyr1086), PDGFRβ, PDGFRβ (Tyr751) and GAPDH antibodies were from Cell Signaling Technologies. GOLPH3 and MYO18A antibodies were previously described (Dippold et al., 2009). GFP and EGFR (528) antibodies were from Santa Cruz Biotechnology. TGN46 antibody was from Serotec. Horseradish peroxidase (HRP) conjugated mouse anti-rabbit immunoglobulin secondary for Western blot was from Jackson ImmunoResearch. AlexaFluor (488, 594, and 647)-conjugated secondary antibodies for immunofluorescence and Cy5-phalloidin were from Life Technologies.

2.5.4 Cell lysis and Western blotting

Cells were lysed by the addition of boiling SDS sample buffer, collected into microfuge tubes, and boiled at 100°C for 5 minutes. Lysates were resolved by SDS-PAGE, transferred to PVDF for Western blotting and detection by enhanced chemiluminescence. Western blot band intensities were measured with ImageJ software, subtracting the adjacent background, and normalized to loading assessed by GAPDH, and then normalized to the parallel control.

2.5.5 Fluorescence microscopy and image analysis

Cells were fixed in 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100 prior to immunostaining. Fluorescence microscopy was performed with an Olympus IX81-ZDC spinning-disk confocal microscope and Slidebook software. Image analysis was performed using CellProfiler software (Carpenter et al., 2006). Measurement of plasma membrane localization of the EGFR was determined for each cell by examining a single confocal z-plane in the middle of the cell, segmenting the cell into the plasma membrane as a 2-3 pixel thick rim located at the outer boundary of phalloidin staining or GFP fluorescence, and calculating the fraction of signal at the rim compared to total EGFR across the slice. For overexpression experiments, transfected cells were identified by expression of GFP fluorescence produced by the bicistronic IRES constructs. pAKT (Ser473) or pEGFR (Tyr1068) signal were measured from maximum projection images, with background determined from control images in the absence of primary antibody staining, and signal intensities were

normalized to the growth factor stimulated controls (transfected with IRES-GFP).

2.6 Acknowledgments

I thank Seth Field for his creative ideas, helpful data analysis, guidance and contributions throughout the entire project. Thank you to Mengke Xing for generating and characterizing the GOLPH3 expression vectors. Thank you to Matt Buschman for generating and characterizing the ManII-GFP HeLa cell line. Thank you to Agnes Grezechnik for help with experiments. Thank you to each person mentioned above for allowing me to use material that they co-authored. Thank you to Marilyn Farquhar, Alexandra Newton, Kun-Liang Guan, and Reuben Shaw for their insightful advice; Ciro Zanca, Frank Furnari, Perla Arcaira, and Roger Tsien for generous gifts of reagents.

The material contained in Chapter 2 is being prepared for publication: GOLPH3-Dependent Golgi-to-plasma membrane trafficking controls growth factor signaling, Marshall C. Peterman, Mengke Xing, Matthew D. Buschman, Agnieszka T. Grezechnik, and Seth J. Field. The dissertation author is the primary investigator and author of this material.

2.7 Figures

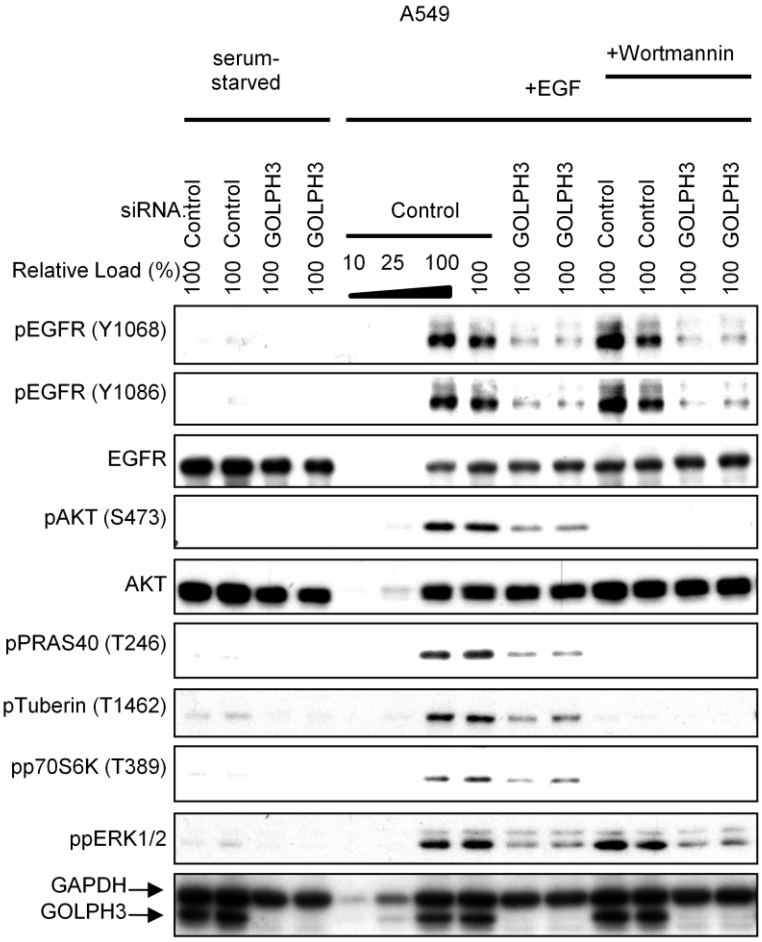

Figure 2.1: EGFR signaling in A549 cells requires GOLPH3. A549 cells were transfected with the indicated siRNAs, serum-starved, pre-treated (as indicated) with wortmannin (100 nM for 10 minutes), stimulated (or not) with EGF (10 ng/ml for 5 minutes), and then whole cell lysates analyzed by Western blotting using the indicated antibodies.

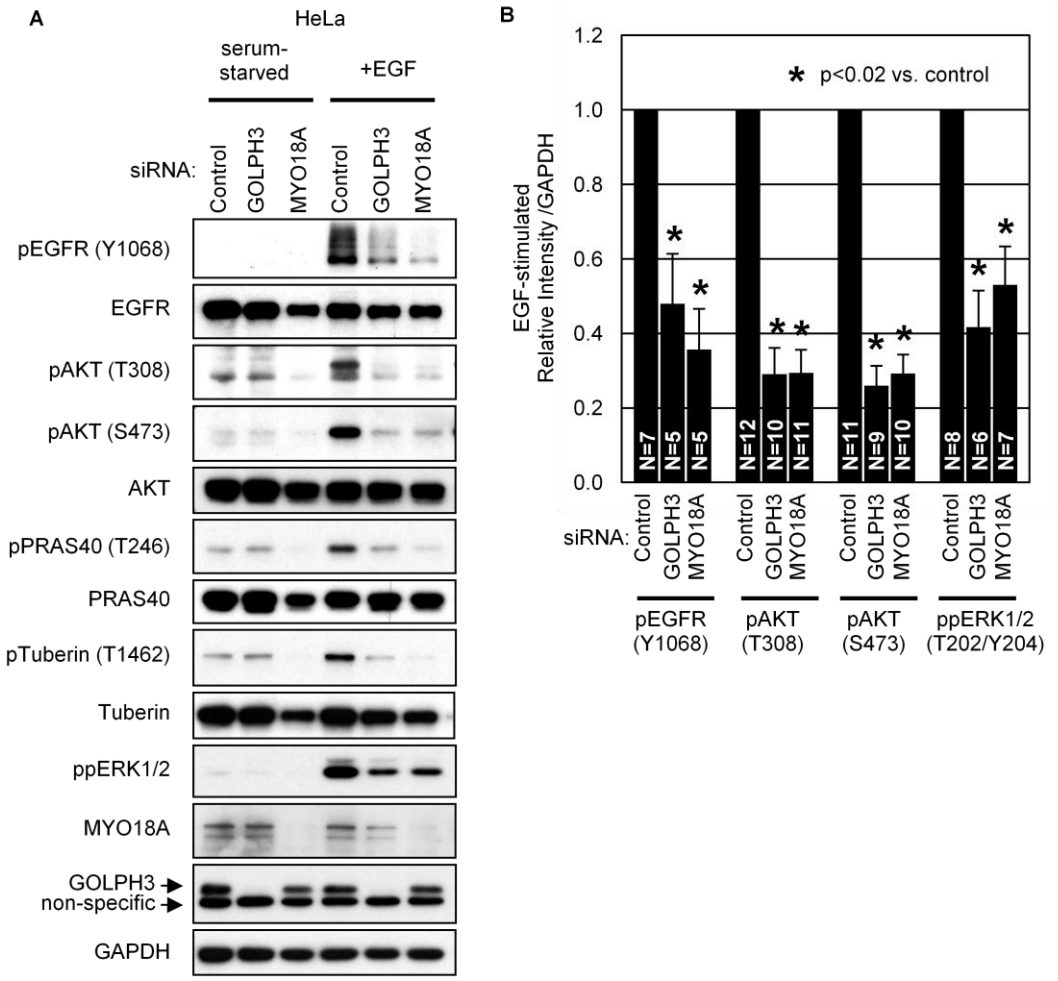

Figure 2.2: EGFR signaling in HeLa cells requires GOLPH3 and MYO18A. (A) HeLa cells were transfected with the indicated siRNAs, serum-starved, and then stimulated (or not) with EGF (10 ng/ml for 5 minutes), and then whole cell lysates analyzed by Western blotting using the indicated antibodies. (B) Quantification of phosphorylation of indicated proteins from (A) normalized to protein loading assessed by GAPDH. Mean and SEM are indicated for pooled data from N independent experiments, as indicated. p-values are calculated by t-test.

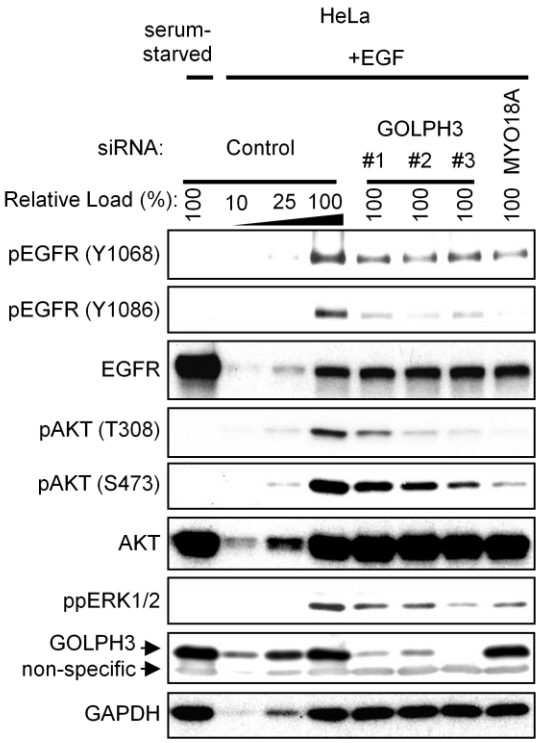

Figure 2.3: Knockdown of GOLPH3 with different siRNA oligonucleotides inhibits EGFR signaling. HeLa cells were transfected with the indicated siRNAs, serum-starved, stimulated (or not) with EGF (10 ng/ml for 5 minutes), and then whole cell lysates analyzed by Western blotting using the indicated antibodies.

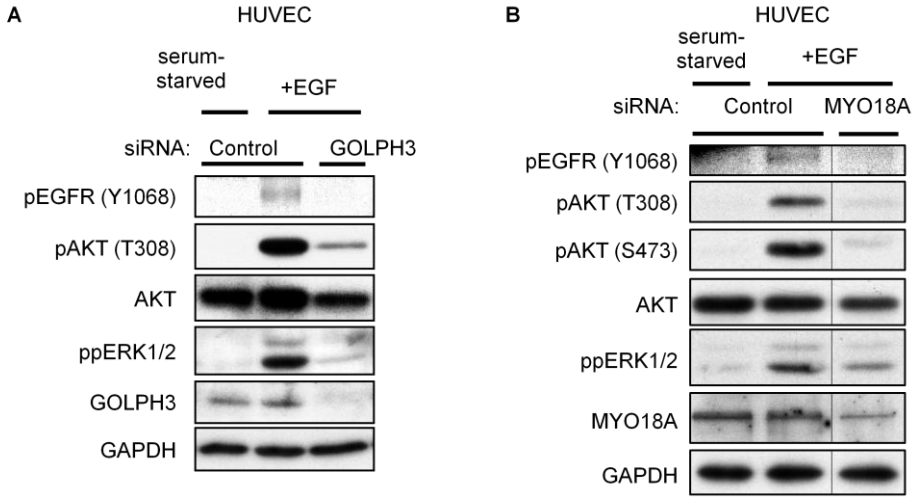

Figure 2.4: EGFR signaling in HUVECs requires GOLPH3 and MYO18A. (A) HUVECs were transfected with the indicated siRNAs, growth factor and serum-starved, and then stimulated (or not) with EGF (10 ng/ml for 5 minutes), and then whole cell lysates analyzed by Western blotting using the indicated antibodies. (B) HUVECs were transfected with the indicated siRNAs, treated and processed as in (A). Vertical line indicates omission of irrelevant intervening lane.

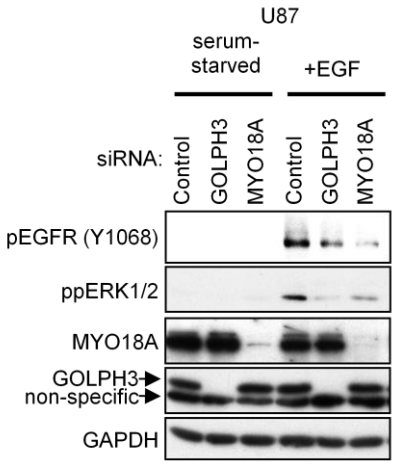

Figure 2.5: EGFR signaling in U87 cells requires GOLPH3 and MYO18A. U87 cells were transfected with the indicated siRNAs, serum-starved, stimulated (or not) with EGF (10 ng/ml for 5 minutes), and then whole cell lysates analyzed by Western blotting using the indicated antibodies.

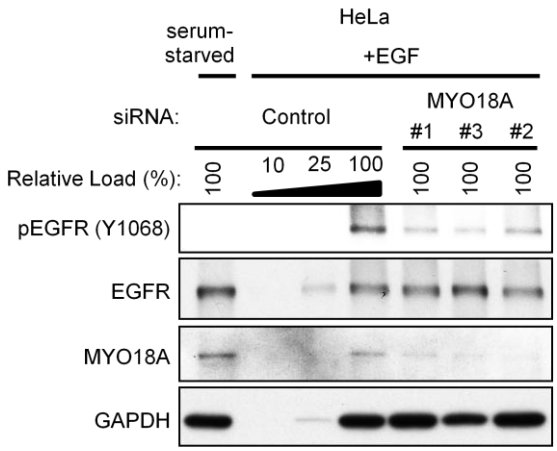

Figure 2.6: Knockdown of MYO18A with different siRNA oligonucleotides inhibits EGFR signaling. HeLa cells were transfected with the indicated siRNAs, serum-starved, stimulated (or not) with EGF (10 ng/ml for 5 minutes), and then whole cell lysates analyzed by Western blotting using the indicated antibodies.

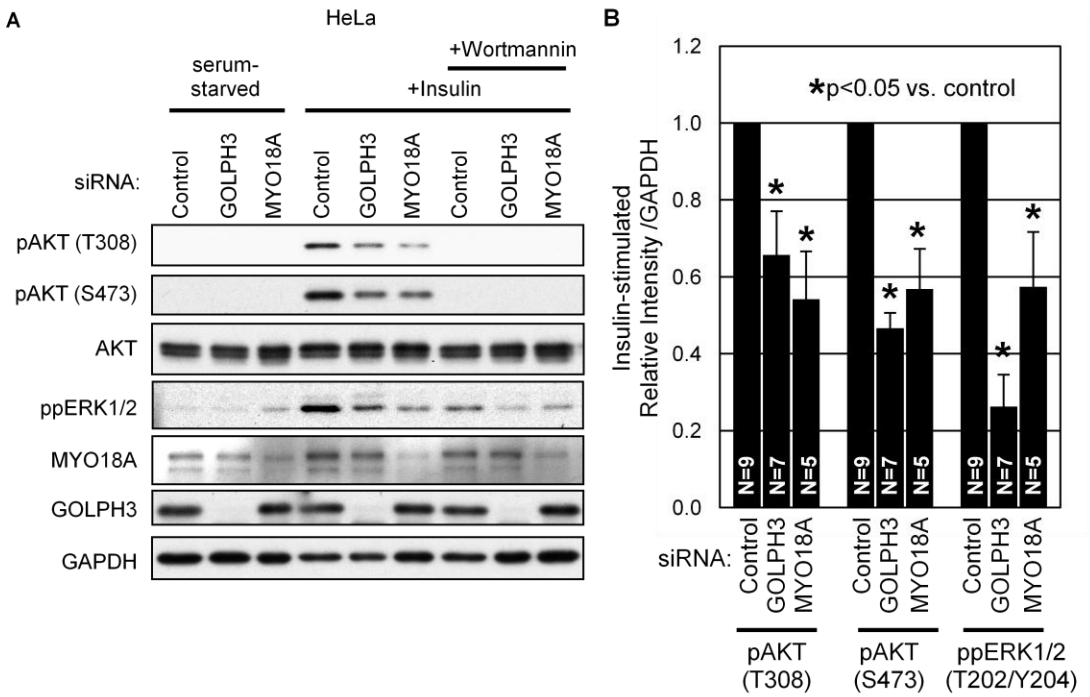

Figure 2.7: Insulin signaling requires GOLPH3 and MYO18A. (A) HeLa cells were transfected with the indicated siRNAs, serum-starved, pre-treated (as indicated) with wortmannin (100 nM for 10 minutes), stimulated (or not) with insulin (10 μg/ml for 5 minutes), and then whole cell lysates were Western blotted using the indicated antibodies. (B) Quantification of phosphorylation of indicated proteins from (A) normalized to protein loading assessed by GAPDH. Mean and SEM are indicated for pooled data from N independent experiments, as indicated. p-values are calculated by t-test.

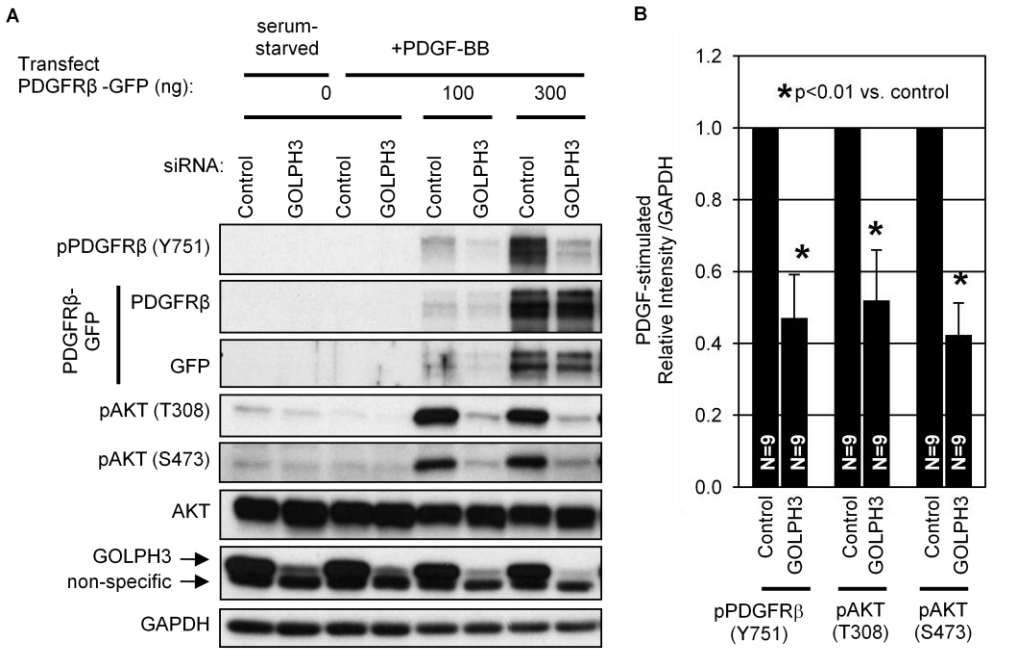

Figure 2.8: PDGF signaling requires GOLPH3. (A) HeLa cells were transfected with the indicated siRNAs and plasmids, serum-starved, stimulated (or not) with PDGF-BB (50 ng/ml for 15 minutes), and then whole cell lysates were Western blotted using the indicated antibodies. (B) Quantification of phosphorylation of indicated proteins from (A) normalized to protein loading assessed by GAPDH. Mean and SEM are indicated for pooled data from N independent experiments, as indicated. p-values are calculated by t-test.

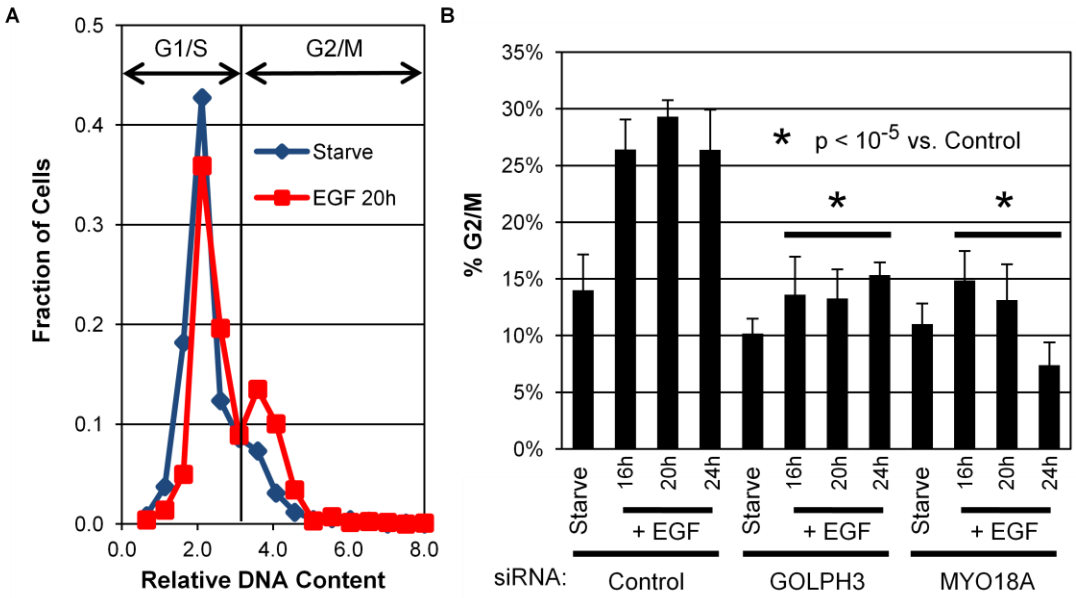

Figure 2.9: Cell-cycle re-entry requires GOLPH3 and MYO18A. (A) HeLa cell cycle was analyzed by measuring DNA content following 24 hour serum-starvation (blue diamond) and EGF stimulation (50 ng/ml) for 20 hours (red square). (B) HeLa cells were transfected with the indicated siRNAs, serum-starved as in (A) followed by stimulation (or not) with EGF (50 ng/ml) for the indicated times and percent of cells in G2/M was measured as in (A). Graphed are mean and SEM of data pooled from three independent experiments. p-values are calculated by two-way ANOVA.

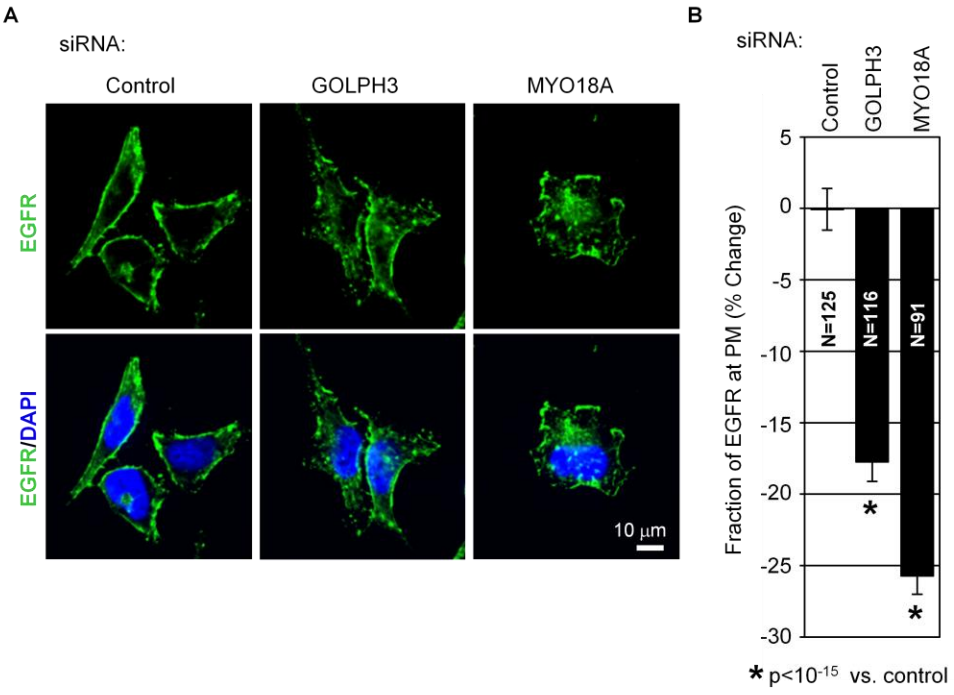

Figure 2.10: Plasma membrane EGFR is reduced by knockdown of GOLPH3 or MYO18A. (A) HeLa cells were transfected with the indicated siRNAs, serum-starved prior to fixation, permeabilization, and staining with DAPI and EGFR antibody. (B) Quantification of the fraction of EGFR at the plasma membrane, relative to control cells. Data are pooled from four independent experiments, with N indicating the number of cells analyzed. Graphed are mean and SEM. p-values are calculated by t-test.

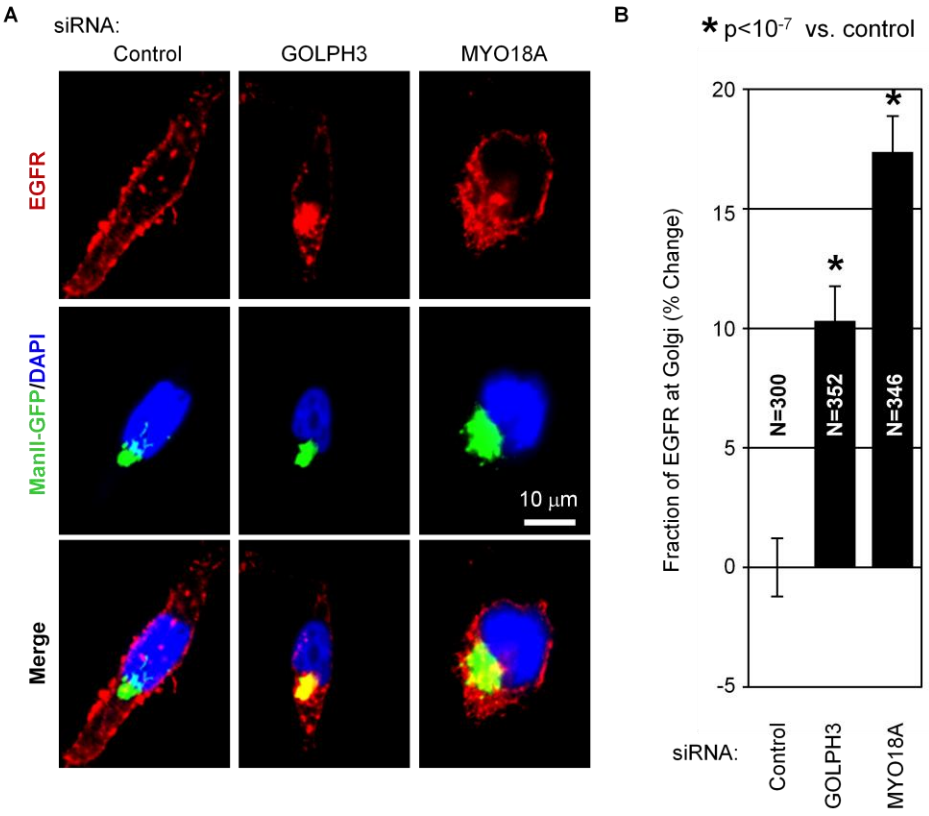

Figure 2.11: GOLPH3 and MYO18A are required for Golgi-to-plasma membrane trafficking of the EGFR. (A) HeLa cells stably expressing the Golgi marker ManII-GFP were transfected with the indicated siRNAs, serum-starved prior to fixation, permeabilization, and staining with DAPI and EGFR antibody. (B) Quantification of the fraction of EGFR at the Golgi, relative to control cells. Data are pooled from two independent experiments, with N indicating the number of cells analyzed. Graphed are mean and SEM. p-values are calculated by t-test.

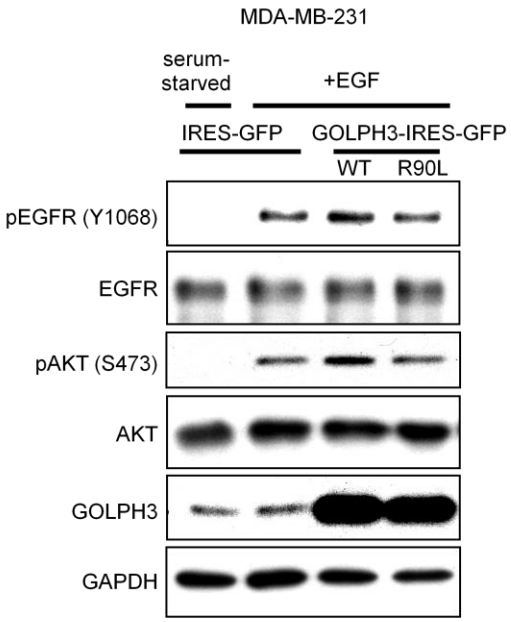

Figure 2.12: GOLPH3 overexpression enhances EGF signaling. MDA-MB-231 cells were transfected with IRES-GFP, GOLPH3(WT)-IRES-GFP, or GOLPH3(R90L)-IRES-GFP expression plasmids, serum-starved, and stimulated (or not) with EGF (100 ng/ml for 5 minutes). Western blots were performed on whole cell lysates using the indicated antibodies.

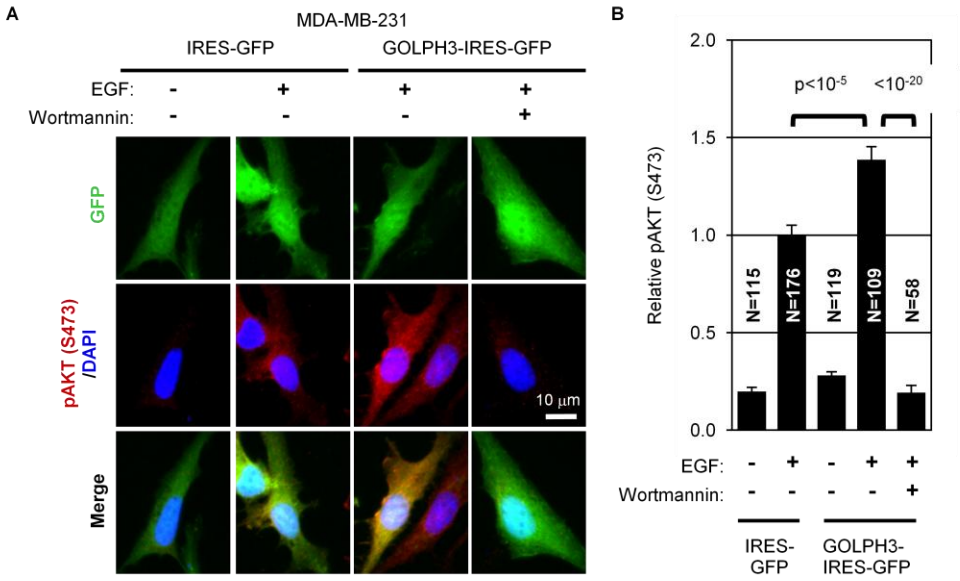

Figure 2.13: GOLPH3 overexpression enhances EGF-stimulated AKT phosphorylation. (A) MDA-MB-231 cells were transfected with IRES-GFP or GOLPH3-IRES-GFP plasmid, serum-starved, pre-treated with wortmannin (100 nM for 10 minutes) where indicated, then stimulated (or not) with EGF (100 ng/ml for 5 minutes) before fixation. Immunostaining for pAKT (Ser473) is shown in red and transfected cells were identified by GFP green fluorescence. DAPI (blue) identifies the nucleus. (B) Quantification of relative pAKT (Ser473) levels from (A), adjusted for background (assessed by omission of primary antibody) and normalized to EGF-stimulated, IRES-GFP expressing cells. Data are pooled from two independent experiments. Graphed are mean and SEM, with the number of cells analyzed (N) indicated. p-values calculated by t-test.

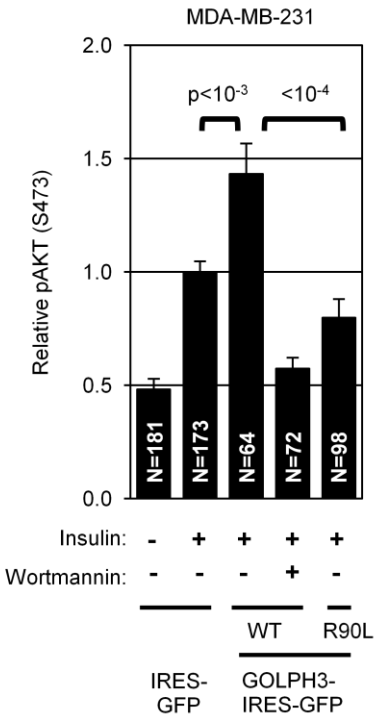

Figure 2.14: GOLPH3 overexpression enhances insulin-stimulated AKT phosphorylation. MDA-MD-231 cells were transfected with IRES-GFP, GOLPH3(WT)-IRES-GFP, or GOLPH3(R90L)-IRES-GFP plasmid, serum-starved, pre-treated with wortmannin (100 nM for 10 minutes) where indicated, then stimulated (or not) with insulin (10 μg/ml for 5 minutes) before fixation and immunostaining for pAKT (Ser473). Relative pAKT (Ser473) levels from transfected cells (identified by GFP green fluorescence) are indicated, adjusted for background (assessed by omission of primary antibody) and normalized to insulin-stimulated, IRES-GFP expressing cells. Data are pooled from two independent experiments. Graphed are mean and SEM, with the number of cells analyzed (N) indicated. p-values calculated by t-test.

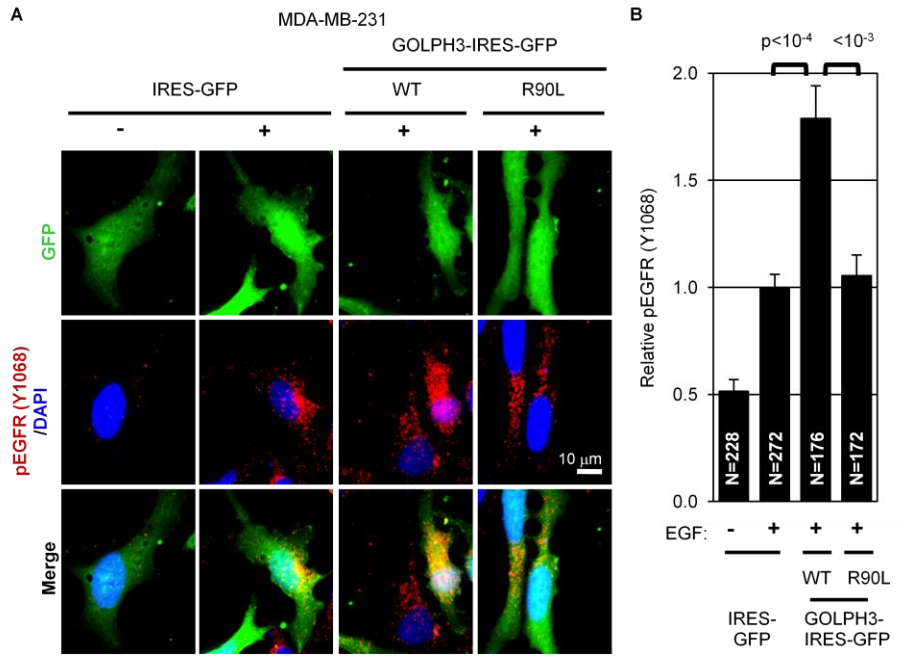

Figure 2.15: GOLPH3 overexpression enhances EGF-stimulated EGFR phosphorylation. (A) MDA-MB-231 cells were transfected with IRES-GFP, GOLPH3(WT)-IRES-GFP, or GOLPH3(R90L)-IRES-GFP plasmid, serum-starved, then stimulated with EGF (100 ng/ml for 5 minutes) before fixation and permeabilization. Immunostaining for pEGFR (Tyr1068) is shown in red and transfected cells were identified by GFP green fluorescence. DAPI (blue) identifies the nucleus. (B) Quantification of relative pEGFR (Tyr1068) levels are indicated, adjusted for background (assessed by omission of primary antibody) and normalized to EGF-stimulated, IRES-GFP expressing cells. Data are pooled from three independent experiments. Graphed are mean and SEM, with the number of cells analyzed (N) indicated. p-values calculated by t-test.

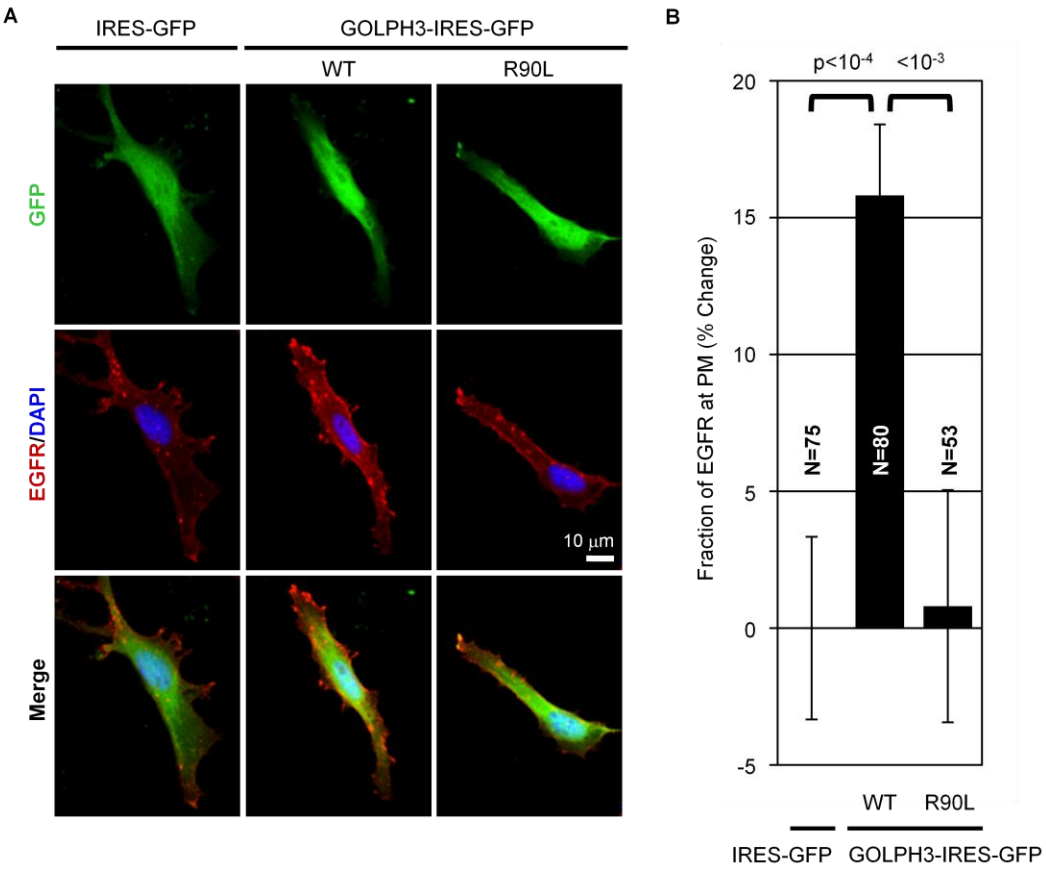

Figure 2.16: GOLPH3 overexpression increases EGFR levels at the plasma membrane. (A) MDA-MB-231 cells were transfected with IRES-GFP, GOLPH3(WT)-IRES-GFP, or GOLPH3(R90L)-IRES-GFP plasmid and serum-starved prior to fixation and permeabilization. Transfected cells were identified by GFP green fluorescence for inclusion in analysis. Immunostaining of EGFR (red) is shown overlayed with DAPI (blue). (B) Quantification of the fraction of EGFR at the plasma membrane, relative to control (IRES-GFP expressing) cells. Data are pooled from four independent experiments, with N indicating the number of cells analyzed. Graphed are mean and SEM. p-values are calculated by t-test.

CHAPTER 3: GOLPH3 drives cell migration by promoting Golgi reorientation and directional trafficking to the leading edge

3.1 Summary

The mechanism of directional cell migration remains an important problem with relevance to cancer invasion and metastasis. GOLPH3 is a common oncogenic driver of human cancers, and is the first oncogene that functions at the Golgi in trafficking to the plasma membrane. Overexpression of GOLPH3 has been reported to drive enhanced cell migration. Here we show that the PtdIns(4)P/GOLPH3/MYO18A/F-actin pathway that is critical for Golgi-to-plasma membrane trafficking is necessary and limiting for directional cell migration. By linking the Golgi to the actin cytoskeleton, GOLPH3 promotes reorientation of the Golgi toward the leading edge. GOLPH3 also promotes reorientation of lysosomes (but not other organelles) toward the leading edge, although lysosome function is dispensable for migration and the GOLPH3-dependence of their movement is indirect, via GOLPH3's effect on the Golgi. By driving reorientation of the Golgi to the leading edge, and driving forward trafficking, particularly to the leading edge, overexpression of GOLPH3 drives trafficking to the leading edge of the cell, which is functionally important for directional cell migration. Our identification of a novel pathway for Golgi reorientation controlled by GOLPH3 provides new insight into the mechanism of directional cell migration with important implications for understanding GOLPH3's role in cancer.

3.2 Introduction

Cell migration is critical to a range of normal biological processes during development and for adaptive and regenerative changes in adult organisms. Importantly, cell migration is also at the heart of the pathophysiology of cell invasion and metastasis that render cancers lethal. Understanding the cellular mechanism of cell migration, and the components that are limiting, and thus susceptible to pathophysiologic enhancement and therapeutic intervention, remains an important biological problem. Directional cell migration involves reorganization of the actin cytoskeleton, for example at lamellipodia at the leading edge of the cell (Insall and Machesky, 2009; Krause and Gautreau, 2014; Ridley, 2011). Interestingly, directional cell migration also involves reorientation of the Golgi toward the leading edge (Kupfer et al., 1982; Millarte and Farhan, 2012). However, the link between actin-mediated migration and Golgi reorientation remains poorly understood.

We previously identified GOLPH3 as an effector of PtdIns(4)P that bridges the Golgi to the actin cytoskeleton and is required for efficient Golgi-to-plasma membrane trafficking (Bishé et al., 2012a; Buschman et al., 2015b; Dippold et al., 2009; Farber-Katz et al., 2014; Ng et al., 2013). We showed that GOLPH3 binds to PtdIns(4)P and thus localizes to the PtdIns(4)P-rich *trans*-Golgi. GOLPH3 also binds to the unconventional myosin, MYO18A, thus linking the Golgi to F-actin (Bishé et al., 2012a; Dippold et al., 2009; Farber-Katz et al., 2014; Ng et al., 2013; Taft et al., 2013). The

PtdIns(4)P/GOLPH3/MYO18A/F-actin complex applies a tensile force to Golgi membranes that is essential for efficient vesicle exit for transport to the plasma membrane. Surprisingly, GOLPH3 has been discovered to be an oncogene, commonly overexpressed in human cancers (Buschman et al., 2015a; Scott et al., 2009). Thus, GOLPH3 is the first example of an oncogene that functions in the secretory pathway at the Golgi, raising hope that it will provide fresh insights into mechanisms of oncogenesis.

Overexpression of GOLPH3 has been reported to enhance cell migration, with obvious implications for understanding GOLPH3's role in driving cancer mortality that results from cancer invasion and metastasis (Buschman et al., 2015a; Isaji et al., 2014; Tokuda et al., 2014; X. Zhang et al., 2014; Zhou et al., 2013). Here we examine the consequence of overexpression of GOLPH3, and investigate the mechanism of enhanced cell migration. We demonstrate that the PtdIns(4)P/GOLPH3/MYO18A/F-actin pathway is required for cell migration by enabling reorientation of the Golgi toward the wound edge for directional secretion, and that overexpression of GOLPH3, as observed in many human cancers, is sufficient to drive this pathway to produce increased cell migration. Our study of the GOLPH3 pathway sheds light on the cellular basis of cell migration, and provides new insight into cancer biology and potential targets for therapeutic intervention.

3.3 Results

3.3.1 GOLPH3 overexpression promotes scratch assay wound healing

To test the consequences of overexpression of GOLPH3, we generated a bicistronic retroviral expression vector to overexpress untagged GOLPH3 together with GFP, or a control vector to express GFP alone. We used these vectors to produce pools of MDA-MB-231 human breast cancer cells that stably overexpress GOLPH3 plus GFP or GFP alone. Overexpression of GOLPH3 was validated by Western blot and immunofluorescence (Figure 3.1, A and B).

GOLPH3 overexpression has been reported to drive increased wound healing as observed in cell culture scratch assays (Isaji et al., 2014; Tokuda et al., 2014). We sought to recapitulate the published result. We examined wound healing by creating a scratch in a confluent monolayer of MDA-MB-231 cells. First, we used live imaging of cells stained with calcein AM to examine cell migration. This revealed enhanced cell migration into the wound in cells that overexpress GOLPH3 (Movie 3.1). Next, we performed endpoint assays to measure the number of cells that migrated into the monolayer wound after 15 hours. As shown in the images (Figure 3.1C) and quantification (Figure 3.1D), enhanced migration into the wound was observed in cells that overexpress GOLPH3, recapitulating the published reports.

To determine whether the ability of GOLPH3 to drive increased wound healing depends on its function at the Golgi, we made use of a previously described mutant. The R90L mutation in the PtdIns(4)P binding pocket largely abolishes the ability of GOLPH3 to bind to PtdIns(4)P, and thus GOLPH3-

R90L is unable to localize to the Golgi (Dippold et al., 2009). We generated a bicistronic retroviral vector to express GOLPH3-R90L together with GFP and produced pools of MDA-MB-231 cells that stably overexpress GOLPH3-R90L (Figure 3.1, A and B). When compared in cell culture scratch assays, these cells behave similarly to GFP-expressing control cells, unlike GOLPH3-overexpressing cells (Figure 3.1, C and D). Therefore, we conclude that the ability of GOLPH3 to drive cell culture wound healing is dependent on its ability to localize to the Golgi.

3.3.2 GOLPH3 and MYO18A are required for scratch assay wound healing

Since GOLPH3 is capable of driving enhanced wound healing in scratch assays, we wondered whether it is generally required for scratch wound healing. Specific siRNA to knock-down GOLPH3 was compared to a control siRNA. As shown in Figure 3.2A, knockdown of GOLPH3 significantly impaired scratch assay wound healing by MDA-MB-231 cells. To test whether the requirement for GOLPH3 is due to its function in the PtdIns(4)P/GOLPH3/MYO18A/F-actin pathway, we examined the effect of siRNA knockdown of MYO18A. We observed that MYO18A knockdown also significantly impaired wound healing by MDA-MB-231 cells. To determine whether the requirement for GOLPH3 and MYO18A is unique to MDA-MB-231 cells or is more generally true, we also examined wound healing in another, unrelated cell type, NRK (normal rat kidney) cells. Again, GOLPH3 and

MYO18A were each required for scratch assay wound healing (Figure 3.2B). Thus, we conclude that the PtdIns(4)P/GOLPH3/MYO18A/F-actin pathway is generally required for scratch assay wound healing.

3.3.3 GOLPH3 does not affect cell proliferation or sensing of loss-of-contact, but drives cell migration speed

To determine the mechanism by which the GOLPH3 pathway contributes to enhanced cell migration, we considered a range of possibilities. We first examined whether overexpression of GOLPH3 led to increased cell proliferation. We compared the rate of proliferation of GOLPH3 overexpressing MDA-MB-231 cells to the parental, GFP only and GOLPH3-R90L controls. We found that all four proliferated at essentially identical rates (Figure 3.3, A and B).

Next, we investigated whether GOLPH3 affects cell migration by modulating cells' sensing of loss-of-contact upon monolayer wounding. Cell confluence is known to regulate the subcellular localization of YAP between the nucleus and cytoplasm via the Hippo pathway (Zhao et al., 2007). Therefore, we could use YAP subcellular localization as a readout of Hippo pathway activity, and thus a readout of cellular sensing of confluence. We measured the relative levels of YAP in the nucleus versus cytoplasm using immunofluorescence in confluent cells compared to cells at the wound edge. We compared control cells to cells overexpressing GOLPH3, and also examined cells following siRNA knockdown of MYO18A versus siRNA control.

As expected, we observed significant translocation of YAP from the cytoplasm into the nucleus in cells at the wound edge compared to confluent cells (Figure 3.3C). However, overexpression of GOLPH3 or knockdown of MYO18A did not significantly alter the subcellular localization of YAP. We conclude that perturbation of the GOLPH3 pathway does not significantly affect the ability of MDA-MB-231 cells to sense a change in cell-cell contact upon wounding.

Next, we considered the possibility that overexpression of GOLPH3 might enhance wound healing by increasing the speed of cell migration. Using time-lapse imaging, we measured the traveling speed of actively migrating cells following wounding. We observed that overexpression of GOLPH3 resulted in a significant increase in the speed of cell migration (Figure 3.3D).

3.3.4 GOLPH3 overexpression promotes Golgi reorientation in wound healing

Reorientation of the Golgi toward the leading edge is known to be an early, limiting step in cell migration (Kupfer et al., 1982; Millarte and Farhan, 2012; Nobes and Hall, 1999). Since GOLPH3 serves to bridge the Golgi and the actin cytoskeleton, we wondered whether GOLPH3 plays a role in cell migration by facilitating Golgi reorientation in wound healing. Thus, we examined Golgi repositioning in MDA-MB-231 cells that overexpress GOLPH3 compared to control cells after monolayer wounding. In control cells, we observed that the Golgi had a random orientation prior to wounding. Following wounding, in cells at the edge of the wound the Golgi began to polarize toward

the wound edge, and by 5 hours was generally pointing toward the wound (Figure 3.4A). By comparison, cells overexpressing GOLPH3 exhibited a more rapid and robust reorientation of the Golgi toward the wound edge. To quantify Golgi reorientation, for each cell we measured the angle between a line from the nucleus to the wound edge and from the nucleus to the center of the Golgi (Figure 3.4B). Quantification showed Golgi reorientation toward the leading edge upon wounding is significantly enhanced by overexpression of GOLPH3 (Figure 3.4C).

In order to determine whether the observed effect of GOLPH3 overexpression on Golgi reorientation involves a novel signaling pathway or occurs through the PtdIns(4)P/GOLPH3/MYO18A/F-actin pathway, we first examined Golgi reorientation in cells overexpressing GOLPH3-R90L. We noted that they behaved similar to the control cells (Figure 3.4, A and C), indicating that GOLPH3-driven enhancement of Golgi repositioning depends on GOLPH3's interaction with PtdIns(4)P. We also examined the requirement for MYO18A in Golgi reorientation by siRNA knockdown of MYO18A in the MDA-MB-231 cell lines prior to wounding. As shown in Figure 3.4, D and E, we recapitulated the enhancement of Golgi reorientation due to overexpression of GOLPH3 in cells transfected with control siRNA. However, siRNA depletion of MYO18A significantly impaired Golgi polarization toward the wound and eliminated any effect due to overexpression of GOLPH3. Since the ability of overexpressed GOLPH3 to promote Golgi reorientation in wound healing depends on its ability to bind to PtdIns(4)P and on MYO18A, we

conclude that it acts through the PtdIns(4)P/GOLPH3/MYO18A/F-actin pathway.

3.3.5 GOLPH3 and MYO18A are required for Golgi reorientation in wound healing

Having established that overexpression of GOLPH3 is able to drive Golgi reorientation upon wounding, we wondered whether the PtdIns(4)P/GOLPH3/MYO18A/F-actin pathway is generally required for Golgi reorientation. We depleted GOLPH3 and MYO18A in MDA-MB-231 cells using three different siRNA oligos for each (Figure 3.5A). In control siRNA transfected cells, the Golgi was oriented randomly prior to wounding, and polarized toward the wound edge within 5 hours after monolayer wounding, as expected (Figure 3.6, A and B). However, Golgi reorientation was significantly impaired upon knockdown of either GOLPH3 or MYO18A. To determine if the requirement for GOLPH3 and MYO18A for Golgi reorientation in wound healing is also true in other cell types, we performed a similar experiment in NRK cells. Here, again, we observed impaired Golgi reorientation after knockdown of either GOLPH3 or MYO18A (Figure 3.5B and Figure 3.6, C and D). Thus, we conclude that the PtdIns(4)P/GOLPH3/MYO18A/F-actin pathway is generally required for efficient Golgi polarization toward the wound edge in response to monolayer wounding.

3.3.6 Lysosomes reorient with the Golgi in wound healing

In order to know whether GOLPH3's effect is restricted to the Golgi or also affects other organelles, we examined the movement of the Golgi and other cellular organelles upon wounding using time-lapse imaging of live cells. We first compared movement of mitochondria to movement of the Golgi (Movie 3.2). Upon wounding, we observed general rotation of the whole cell, and the movement of mitochondria reflected that rotation. In comparison, the Golgi actively moved around the nucleus toward the wound edge at a faster speed, resulting in faster movement of the Golgi relative to mitochondria and the rest of the cell. To assess the effect of overexpression of GOLPH3 on mitochondrial (versus Golgi) reorientation, we measured the orientation of mitochondria and the Golgi relative to the direction of the monolayer wound 5 hours after wounding (Figure 3.7A). We found that while Golgi reorientation was significantly enhanced by overexpression of GOLPH3, reorientation of mitochondria was insensitive to overexpression of GOLPH3 (Figure 3.7B). Thus, the movement of mitochondria and the Golgi do not share the same mechanism.

Next, we compared movement of the nucleus to movement of the Golgi after cell monolayer wounding. As shown in Movie 3.3, structural features of the nucleus visible by differential interference contrast (DIC) imaging allowed us to detect rotation of the nucleus. We observed general rotation of the whole cell that was accompanied by rotation of the nucleus. The Golgi, on the other hand, moved at a faster speed around the nucleus toward the wound edge. By measuring the rotation speed of the Golgi and the nucleus we noted that the

Golgi exhibited a significantly higher rotation speed compared to the nucleus (Figure 3.7C). Thus, we demonstrate that the nucleus, too, moves differently from the Golgi in response to wounding.

Finally, we examined lysosome movement in response to wounding. Unexpectedly, as shown in Movie 3.4, the Golgi and lysosomes localized in close proximity to each other, and reoriented in a concerted fashion upon wounding. By 5 hours, both the Golgi and lysosomes were pointed toward the leading edge and remained co-localized (Figure 3.7D). Quantification showed that lysosome reorientation was significantly enhanced by overexpression of WT GOLPH3, but not GOLPH3-R90L, similar to the effect on reorientation of the Golgi (Figure 3.7E). Moreover, GOLPH3's ability to enhance both Golgi and lysosome reorientation is through the PtdIns(4)P/GOLPH3/MYO18A/F-actin pathway, because, in addition to GOLPH3-R90L being ineffective, either siRNA depletion of MYO18A or depolymerization of F-actin by latrunculin B significantly impaired reorientation and abolished the ability of GOLPH3 to promote reorientation of both lysosomes and the Golgi (Figure 3.8, A-D).

We also tested whether GOLPH3 and MYO18A are required for lysosome reorientation in response to a monolayer scratch. In NRK cells, knock-down of either GOLPH3 or MYO18A not only impaired Golgi reorientation, as observed previously, but also significantly impaired lysosome reorientation (Figure 3.8, E and F). In HeLa cells we observed a similar requirement for both GOLPH3 and MYO18A for reorientation of both the Golgi and lysosomes following cell culture wounding (Figure 3.9, A and B). Taken

together, we come to the surprising conclusion that lysosomes move with the Golgi to polarize toward the leading edge in response to wounding, which is dependent on, and also driven by, the PtdIns(4)P/GOLPH3/MYO18A/F-actin pathway.

3.3.7 Lysosome reorientation is indirect, through microtubule tethering to the Golgi

The question follows how GOLPH3, a protein found at the Golgi, regulates lysosome reorientation in response to wounding. As published previously (Bell et al., 2001; Dippold et al., 2009; Farber-Katz et al., 2014; Ng et al., 2013; Wu et al., 2000), we confirmed that GOLPH3 localizes to the Golgi, not lysosomes (Figure 3.10A). Thus, we hypothesized that GOLPH3's effect on lysosomes may be indirect, via the Golgi. To test this possibility, we first determined whether lysosome reorientation depends on the Golgi. We treated MDA-MB-231 cells with Brefeldin A (BFA) to disrupt the Golgi or methanol (vehicle control) at the time of monolayer wounding, and measured lysosome reorientation after 5 hours (Figure 3.10, B and C). In control cells, we recapitulated the enhanced reorientation of both the Golgi and lysosomes in response to overexpression of GOLPH3. In BFA treated cells, the Golgi was effectively disrupted within 15 minutes of drug treatment (Figure 3.10B and data not shown). Consequently, lysosome reorientation was significantly impaired. Moreover, the ability of overexpression of GOLPH3 to augment

lysosome reorientation was abolished by BFA disruption of the Golgi. We conclude that GOLPH3 acts on lysosomes indirectly through the Golgi.

Given the close association between the Golgi and lysosomes (Movie 3.4), we reasoned that a possible explanation for the GOLPH3-driven, Golgi-dependent reorientation of lysosomes could be due to structural tethering of lysosomes to the Golgi. Top candidates that could mediate this tethering would be actin or microtubule cytoskeletal elements. Thus, we first investigated whether F-actin is responsible for the link between the Golgi and lysosomes. We depolymerized actin with Latrunculin B, and examined the effect on the relationship between the Golgi and lysosomes (Movie 3.5). Treatment with Latrunculin B led to condensation of the Golgi, consistent with previous observations (Dippold et al., 2009). Lysosomes condensed simultaneously with the Golgi and the two organelles remained similarly co-localized after drug treatment (Figure 3.10D), suggesting that F-actin is not required to link lysosomes to the Golgi.

Next, we investigated the role of microtubules in tethering lysosomes to the Golgi. Published data indicate that lysosomes are associated with, and depend on, microtubules to maintain normal morphology (Collot et al., 1984; Harada et al., 1998; Matteoni and Kreis, 1987; Scheel et al., 1990). The Golgi also requires microtubules to maintain normal ribbon morphology (Rogalski and Singer, 1984; Sandoval et al., 1984; Thyberg and Moskalewski, 1999). However, little is known about the structural relationship between the two organelles. To examine a possible physical association between the Golgi and

lysosomes mediated by microtubules, we observed the consequences of depolymerization of microtubules. Treatment with nocodazole to depolymerize microtubules caused fragmentation and dispersal of both the Golgi and lysosomes, as expected (Movie 3.6). More importantly, we noted that upon nocodazole treatment that the Golgi and lysosome puncta moved away from each other, becoming spatially distinct. Measurement of the overlap between the Golgi and lysosome compartments showed significant dissociation upon treatment with nocodazole (Figure 3.10D), in contrast to the effect of Latrunculin B treatment. These data suggest that microtubules serve to link the Golgi and lysosomes. Overall, we conclude that GOLPH3's effect on lysosome reorientation is secondary to its effect on the Golgi, mediated by microtubule tethering of lysosomes to the Golgi.

3.3.8 GOLPH3 overexpression promotes directional trafficking toward the leading edge

Having discovered that GOLPH3 drives both the Golgi and lysosomes to reorient toward the leading edge upon wounding, we examined the functional requirement for each organelle for cell migration. We used inhibitors of Golgi or lysosome function to ask whether they interfere with monolayer scratch healing by NRK and MDA-MB-231 cells. Consistent with previous findings (Tseng et al., 2014), inhibition of Golgi function with BFA significantly reduced monolayer wound healing in both NRK and MDA-MB-231 cells (Figure 3.11, A and B). We note that the reduction in wound healing caused by

treatment with BFA is comparable to that due to siRNA depletion of either GOLPH3 or MYO18A (Figure 3.2, A and B). By contrast, inhibition of lysosomal function by NH_4Cl or Bafilomycin A1 had a minimal, non-significant effect on scratch wound healing (Figure 3.11, A and B). The effectiveness of lysosome inhibition was verified by loss of LysoTracker staining upon treatment with NH_4Cl or Bafilomycin A1 (see Movie 3.7). Therefore, we conclude that while the lysosome is dispensable, the Golgi plays an important role in cell migration that is enhanced by overexpression of GOLPH3.

Finally, we sought to elucidate the functional significance of GOLPH3-driven Golgi polarization in directional cell migration. Given that directed trafficking toward the leading edge occurs in migrating cells (Bergmann et al., 1983; Yadav et al., 2009), and that the GOLPH3 pathway is critical for Golgi-to-plasma membrane trafficking (Bishé et al., 2012a; Dippold et al., 2009; Farber-Katz et al., 2014; Ng et al., 2013), we examined the effect of overexpression of GOLPH3 on trafficking from the Golgi to the leading edge in wound healing. We transfected MDA-MB-231 cells overexpressing GOLPH3 or control cells with the trafficking reporter ts045-VSVG-GFP, which is released from the ER and traffics through the Golgi to the plasma membrane upon shift from the restrictive temperature (40^oC) to the permissive temperature (32^oC) (Presley et al., 1997). We created a monolayer scratch while cells were at the restrictive temperature and then allowed the Golgi to reorient for 4 hours. We then shifted to the permissive temperature for one hour to allow trafficking of VSVG. By using an exofacial antibody to VSVG on

unpermeablized cells, we measured surface VSVG at the front and back of cells at the edge of the monolayer wound (Figure 3.11C). We defined the front of the cell by a vector drawn from the centroid of the nucleus to the centroid of the Golgi. In GOLPH3 overexpressing cells, we observed significantly higher surface VSVG compared to control cells, especially at the front of the cell, with a smaller increase at the back of the cell (Figure 3.11, C and D). We calculated the ratio of surface VSVG at the front versus at the back of the cell as a measure of directional trafficking, and found a significant increase in directional trafficking in response to overexpression of GOLPH3 (Figure 3.11E). The ability of GOLPH3 to drive increased trafficking of VSVG to the plasma membrane, and increased directional trafficking to the front of the cell is dependent on GOLPH3's function at the Golgi, since the R90L mutant is inactive. Taken together, we conclude that overexpression of GOLPH3 promotes directional trafficking from the Golgi to the leading edge in wound healing.

3.4 Discussion

3.4.1 GOLPH3 drives Golgi-to-plasma membrane trafficking toward the wound edge

A large body of literature has demonstrated that extensive remodeling of the actin cytoskeleton occurs at the plasma membrane facing a monolayer wound, resulting in dramatic cell asymmetry with particular increases in the

steady-state and dynamic actin cytoskeleton toward the wound edge (Cau and Hall, 2005; Li and Gundersen, 2008; Nobes and Hall, 1999). Our data indicate that by linking the Golgi to the actin cytoskeleton, the GOLPH3 pathway transmits these changes in the actin cytoskeleton to the Golgi (Figure 3.12). Since the same GOLPH3 linkage of the Golgi to actin is important for trafficking, overexpression of GOLPH3 enhances both Golgi repositioning and forward trafficking.

We note that the Golgi is also known to be linked to microtubules, and, in fact, can nucleate microtubule growth (Chabin-Brion et al., 2001). Since the Golgi is linked to both microtubules and to actin microfilaments, it is perhaps not surprising that depolymerization of microtubules (Yadav et al., 2009) or F-actin (this study) each impair Golgi repositioning toward the wound. Our data indicate that the Golgi, and indirectly lysosomes via a linkage to the Golgi, are dependent on the PtdIns(4)P/GOLPH3/MYO18A/F-actin pathway for efficient reorientation toward the wound.

Our data demonstrate three consequences of overexpression of GOLPH3 that drive increased trafficking from the Golgi to the plasma membrane at the wound edge. First, overexpression of GOLPH3 increases overall trafficking to the plasma membrane. This is consistent with previous data demonstrating that the PtdIns(4)P/GOLPH3/MYO18A/F-actin pathway is important for the majority of trafficking from the Golgi to the plasma membrane (Bishé et al., 2012a; Dippold et al., 2009; Farber-Katz et al., 2014; Ng et al., 2013). Second, we also observe that overexpression of GOLPH3 promotes

trafficking preferentially toward the front of the cell, as defined by the position of the Golgi relative to the nucleus. Third, overexpression of GOLPH3 promotes reorientation of the Golgi toward the wound edge, thus causing the front of the cell to point toward the wound. All three of these effects contribute toward driving trafficking to the wound edge.

3.4.2 Golgi cargoes important for directed cell migration

Our VSVG reporter experiments demonstrate that GOLPH3 drives increased trafficking from the Golgi to the plasma membrane at the monolayer wound edge. VSVG serves as a general reporter for trafficking, suggesting that many cargoes undergo enhanced delivery to the plasma membrane at the wound edge. A few cargoes have been reported to be important for cell migration. General delivery of membrane lipids to build the plasma membrane at the leading edge has been suggested to be important to allow the generation of plasma membrane protrusions (lamellipodia and filopodia) that contribute to cell migration (Bergmann et al., 1983). In addition, the delivery of integrins to the leading edge is also known to be important for directional cell migration (Danen et al., 2005; Etienne-Manneville and Hall, 2001). We suspect that there is not a single cargo, but rather an ensemble of cargoes that are important for cell migration that are delivered to the leading edge via the Golgi GOLPH3 pathway. Future work will be required to identify critical cargoes.

3.4.3 Role of the GOLPH3 pathway in cancer

A growing body of evidence indicates that GOLPH3 is an oncogene, frequently amplified and overexpressed in human cancers (Buschman et al., 2015a; Scott et al., 2009). Furthermore, high levels of GOLPH3 predict poor prognosis in a wide variety of cancers, independent of other prognostic indicators. Cancer morbidity and mortality are largely a consequence of invasion and metastasis, processes driven by cell migration. Thus, the ability of GOLPH3 to drive cell migration may provide mechanistic insight into GOLPH3's contribution to the pathophysiology of cancer. GOLPH3 is also known to play a central role in the Golgi response to DNA damage that promotes cell survival following DNA damage (Farber-Katz et al., 2014). This provides another mechanism by which GOLPH3 can contribute to cancer progression. GOLPH3, like many clinically important oncogenes (Bieging et al., 2014; Gabay et al., 2014; Stephen et al., 2014), appears to have multiple mechanisms by which it can contribute to the pathophysiology of cancer. In addition to providing insights into the mechanism of important physiology and pathophysiology, the GOLPH3 pathway may provide new targets for novel cancer therapeutic agents.

3.5 Materials and methods

3.5.1 Cell culture and DNA construction

MDA-MB-231, NRK, and HeLa S3 cell lines were grown according to ATCC guidelines. The stable MDA-MB-231 cell lines that overexpress

GOLPH3-IRES-GFP, GOLPH3-R90L-IRES-GFP, or IRES-GFP were constructed with the pBABE-Puro retroviral system (Morgenstern and Land, 1990). Empty retroviral expression vector pBABE-Puro and packaging vectors pUMVC and pVSV-G were a kind gift from Dr. Jing Yang (University of California, San Diego). The expression vector of wild-type GOLPH3 was generated by serial cloning to combine GOLPH3 (Dippold et al., 2009) and IRES-hrGFP (Stratagene) into the BamHI and EcoRI sites of pBABE-Puro vector. Expression and packaging vectors were transfected into HEK293T packaging cells using TransIT-LT1 (Mirus). Viral supernatants harvested at 48 and 72 hrs after transfection were filtered through a 0.45 μm filter (Thermo Fisher Scientific) and incubated with target MDA-MB-231 cells in the presence of 6 μg/ml protamine (Sigma). 24 hrs after the second viral infection, 0.5 μg/ml puromycin (InvivoGen) was used to select and maintain stable cell pools. The cell lines overexpressing IRES-GFP and R90L-IRES-GFP were generated in similar fashion.

3.5.2 Cell proliferation assay

On day 0, cells were seeded in replicate wells at 20,000 cells/well in 6-well plates, and grown in normal medium. On day 1, 3, and 5 each, triplicate wells for each cell line were trypsinized and counted using a hemocytometer. Growth rate was measured by taking the binary logarithm of cell counts, normalized to day 1.

3.5.3 Scratch wound healing assay

Cells were seeded in 35 mm gridded glass-bottom culture dishes (MatTek), and maintained in normal growth medium until reaching a confluent monolayer. A 200 µl pipette tip was used to scratch the cell monolayer, followed by two rinses with PBS. For end-point analysis, random fields at the scratch were selected and captured by 10x phase contrast imaging with an Olympus IX81-ZDC microscope. Cells were then incubated in normal growth medium for 15 hrs before fixation, followed by permeablization and DAPI staining. DAPI images were taken at the same selected fields. For each field, the number of cells that migrated into the pre-defined scratch area were counted, and normalized to confluent density. For time-lapse imaging, cells were incubated in normal growth medium containing calcein AM (Life Technologies) after wounding. Time-lapse images were taken at 15 min intervals for 15 hrs.

For scratch assays with siRNA knockdowns, siRNA oligos were transfected into cells 48 hrs prior to monolayer wounding. For scratch assays with Golgi or lysosome inhibitors, after making the scratch cells were incubated in normal growth media containing 0.1% DMSO (Sigma), 0.1% MeOH, 5 ng/ml Brefeldin A (Sigma), 10 mM NH_4Cl (Sigma), or 100 nM Bafilomycin A1 (Sigma) for 8 hrs before fixation. DMSO was used as vehicle control for Bafilomycin A1. MeOH was used as vehicle control for Brefeldin A. Since DMSO and MeOH gave identical results, their data are pooled in Figure 3.11, A and B.

3.5.4 Fluorescence microscopy

Fluorescent microscopy was performed on an Olympus IX81-ZDC spinning disk confocal microscope, and images were analyzed with Slidebook and ImageJ software. All images shown represent maximum projections in z of confocal stacks with spacing of 0.5 µM. Movies show a single confocal plane.

3.5.5 Measurement of cell migration speed

Time-lapse images of scratch wound healing assay were analyzed in ImageJ of actively migrating cells, defining the center of the nucleus between consecutive frames, and measuring the traveling distance of the nucleus divided by the time interval.

3.5.6 Measurement of Golgi/lysosome/mitochondria orientation angle

Immunofluorescent images of cells at wound edge stained with a Golgi marker and DAPI were analyzed in ImageJ, by drawing a first line from the centroid of the nucleus to the wound edge and a second line from the centroid of the nucleus to the centroid of the Golgi. The angle formed in between the two lines was measured. The orientation angles of mitochondria and lysosome were measured in similar fashion. For cells prior to wounding, a fixed direction was chosen arbitrarily and the angle between said direction and the Golgi was measured for each cell.

3.5.7 Time-lapse imaging of cellular organelles after wounding

Cells were transfected with the expression vector for ManII-GFP to mark the Golgi and grown to confluence. Prior to making the scratch wound, cells were incubated with MitoTracker Deep Red or LysoTracker Red (Life Technologies) following the manufacturer's instructions to label mitochondria and lysosomes, respectively. A wound was made in the same way as described for the scratch wound healing assay. After wounding, time-lapse images were taken at 3 min intervals for 5 hrs.

3.5.8 Measurement of Golgi/nucleus rotation speed

Time-lapse fluorescent and DIC images of cells at the wound edge were analyzed in ImageJ, in each frame identifying the direction of the nucleus with the aid of structural features visible by DIC imaging, and identifying the direction of the Golgi by drawing a line through the center of the nucleus and the center of the Golgi (marked by ManII-GFP). Rotation speed was determined by measuring the rotation angle between consecutive frames divided by the time interval.

3.5.9 Measurement of surface ts045-VSVG-GFP in wound healing

Cells were transiently transfected with an expression vector for temperature-sensitive, GFP-tagged ts045-VSVG-GFP and grown to a confluent monolayer. 36 hrs after transfection, cells were maintained at the restrictive temperature (40^{o}C) for 12 hrs before a razor blade was used to

create a wound. Cells were returned to 40^{o}C for 4 hrs to allow Golgi reorientation, and then shifted to the permissive temperature (32^{o}C) for 1 hr to allow VSVG trafficking before fixation. An exofacial antibody specific to VSVG was used on unpermeabilized cells to detect surface VSVG, followed by permeabilization and staining with GOLPH3 antibody to label the Golgi. IF images were taken of transfected cells at the edge of the wound. Surface VSVG was analyzed in ImageJ by drawing a line through the nucleus and the Golgi with a width of 10 pixels and measuring the average intensity at the plasma membrane at the front and back of the cell (seen as two peaks in histogram), with background subtracted.

3.5.10 siRNA knockdown

siRNA oligonucleotides containing Stealth modifications were synthesized by LifeTechnologies and transfected using RNAiMAX according to the manufacturer's instructions. Negative control, GOLPH3, and MYO18A sequences were the same as those described previously (Dippold et al., 2009). If not otherwise specified in the figures, GOLPH3 siRNA #3 and MYO18A siRNA #1 were used. Knockdowns were verified by Western blots performed in parallel for each experiment.

3.5.11 Antibodies

The GOLPH3 rabbit polyclonal antibody was described previously (Dippold et al., 2009). The MYO18A antibody was a generous gift from Dr.

Zissis Chroneos (University of Texas, Tyler, TX). The GM130 antibody was from BD Biosciences. The YAP antibody H-125 was from Santa Cruz Biotechnologies. The ATP synthase subunit IF1 antibody 5E2D7 used to mark mitochondria was from Invitrogen. The LAMP1 and GAPDH antibodies were from Cell Signaling Technology. The VSVG exofacial monoclonal antibody 8G5F11 was from KeraFAST. The horseradish peroxidase-conjugated mouse anti-rabbit secondary antibody for Western blot was from Jackson ImmunoReasearch. AlexaFluor (488, 594, and 647)-conjugated secondary antibodies for immunofluorescence were from Invitrogen.

3.5.12 Statistics

Two-tailed Student's unpaired t-test was calculated using Microsoft Excel. Analysis of variance (ANOVA) and least squares linear regression were performed using PastProject statistical software (Hammer et al., 2001).

3.6 Acknowledgments

We would like to thank Ana Zubiaga, Alexandra Newton, Joan Heller Brown, Jing Yang, and Kun-Liang Guan for insightful comments and discussions. We thank Spencer Wei, Jing Yang, Corina Antal, and Mike Whitney for helpful reagents. Special thanks to Nuo Wang for drafting the model diagram.

The material contained in Chapter 3 has been submitted for publication, as it may appear: GOLPH3 drives cell migration by promoting Golgi

reorientation and directional trafficking to the leading edge. Molecular Biology of the Cell, 2016. Mengke Xing, Marshall C. Peterman, and Seth J. Field.

3.7 Figures

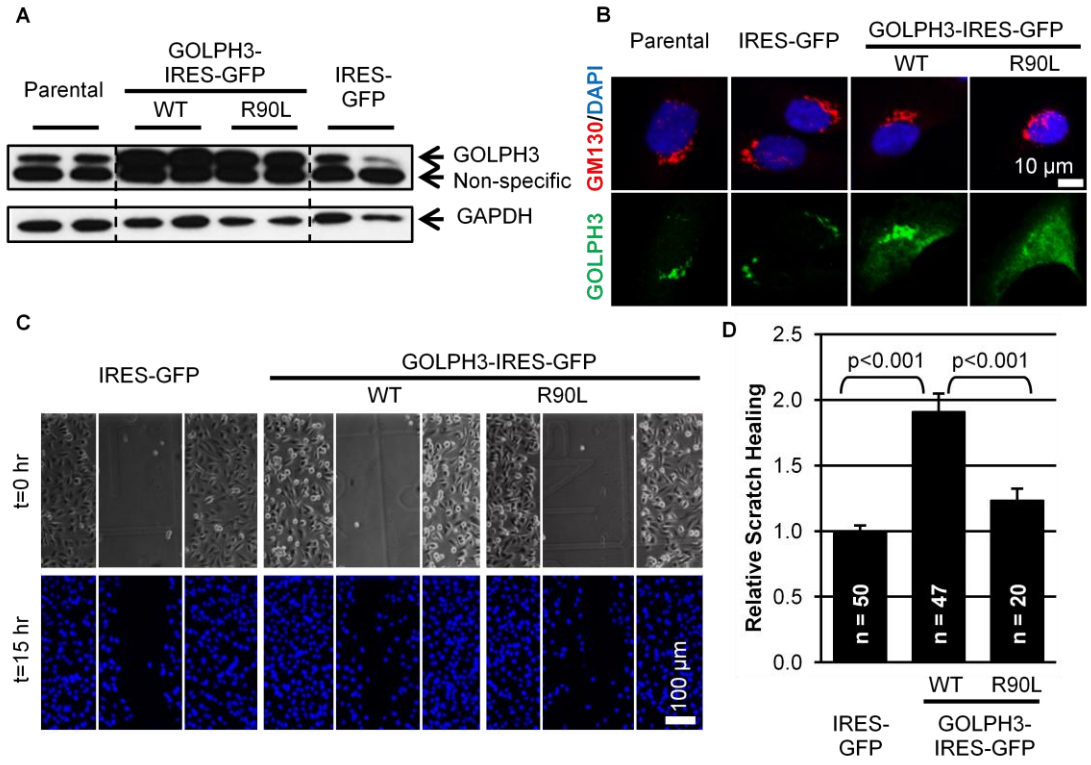

Figure 3.1: GOLPH3 overexpression promotes scratch wound healing. (A) Western blot of duplicate SDS lysates demonstrates increased expression of GOLPH3 (WT or R90L mutant) compared to parental cells or IRES-GFP control cells. GAPDH provides a control to demonstrate similar loading. Dashed lines indicate omission of irrelevant intervening lanes. (B) IF demonstrates endogenous GOLPH3 (green) in parental and IRES-GFP control cells, increased GOLPH3 at the Golgi in cells overexpressing WT GOLPH3, and increased GOLPH3 throughout the cytosol in cells overexpressing GOLPH3-R90L, a mutant unable to bind to PtdIns(4)P. GM130 marks the Golgi (red) and DAPI marks the nucleus (blue). (C) Representative images of scratch wound healing by MDA-MB-231 cells overexpressing IRES-GFP (control), GOLPH3-IRES-GFP, or GOLPH3-R90L-IRES-GFP. Top row, images of random fields at the scratch were taken at time of wounding (t=0 hr), with the scratch area indicated by the white box. Bottom row, the same fields after 15 hr, fixed and stained with DAPI for cell counting (t=15 hr). (D) Quantification of wound healing from (C) relative to control. Overexpression of GOLPH3 results in a significant, ~2-fold increase in cell migration into the scratch compared to control or GOLPH3-R90L expressing cells. Graphed are mean and standard error of the mean (SEM). The number of fields measured (n, pooled from four independent experiments) and p-values (t-test) are indicated.

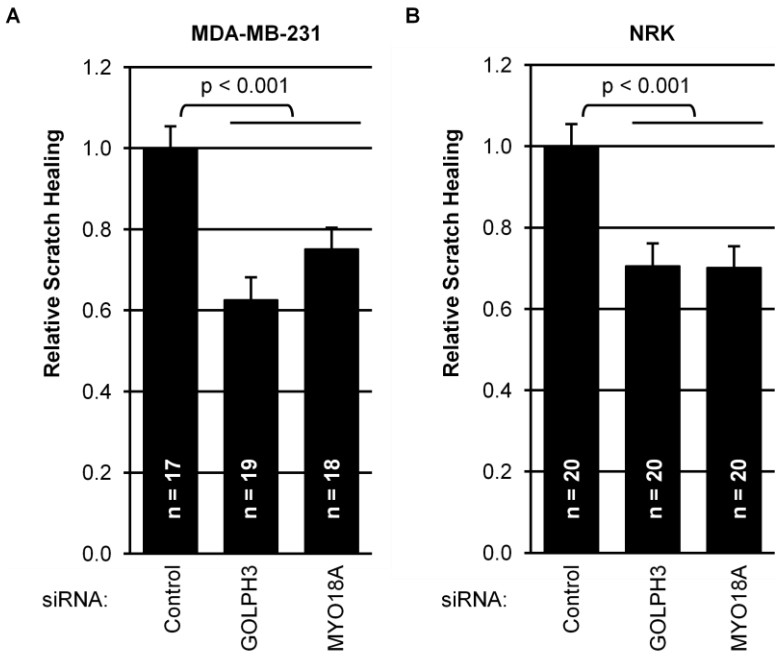

Figure 3.2: GOLPH3 and MYO18A are required for scratch wound healing. (A) (B) Quantification of scratch assay wound healing by MDA-MB-231 cells and NRK cells, respectively. Cells were transfected with control siRNA or siRNA targeting GOLPH3 or MYO18A prior to monolayer wounding. Effectiveness of knockdown was confirmed by parallel Western blots (not shown, see Figure 3.5 for representative examples). Scratch wound healing is expressed relative to control. Interference with the GOLPH3/MYO18A pathway significantly impairs wound healing in both MDA-MB-231 and NRK cells. Graphed are mean ± SEM pooled from two independent experiments. Number of fields measured (n) and p-values (t-test) are indicated.

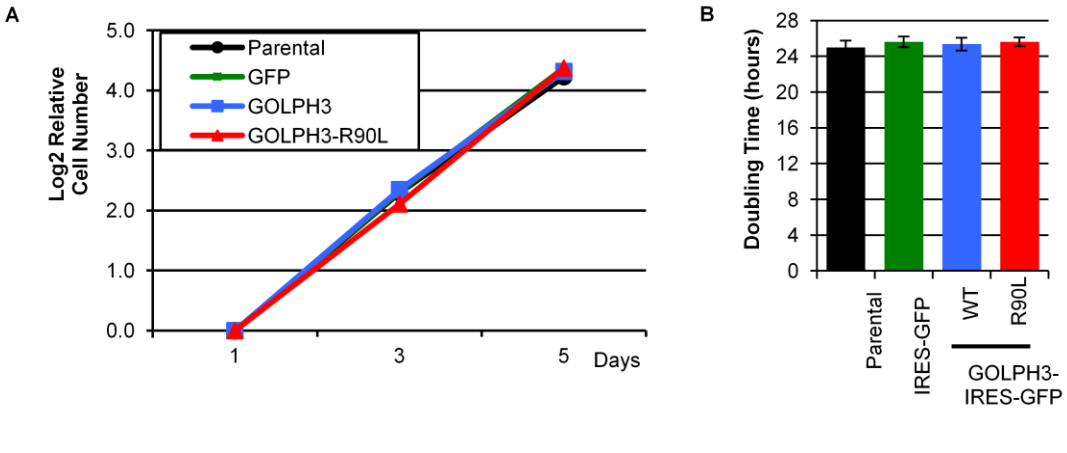

Figure 3.3: GOLPH3 does not affect MDA-MB-231 proliferation or sensing of loss-of-contact, but drives cell migration speed. (A) Rate of proliferation of cell lines was measured by counting on days 1, 3, and 5 following initial seeding of equal numbers of cells, and expressed as the binary logarithm of cell count normalized to day 1. (B) Doubling time for each cell line was calculated from (A) and shown in corresponding color. GOLPH3 overexpression has no significant effect on cell proliferation rate. (A) and (B), graphs indicate mean ± SEM. For each data point, n=3. (C) MDA-MB-231 cells overexpressing GFP (control), GOLPH3, or GOLPH3-R90L were grown to confluence and transfected with control siRNA or MYO18A siRNA prior to wounding. Subcellular YAP distribution of confluent cells and cells at wound edge was measured as the ratio of nuclear YAP versus cytoplasmic YAP. As expected, monolayer wounding caused loss of confluence and YAP translocation to the nucleus. However, neither GOLPH3 overexpression nor MYO18A knockdown had any effect on YAP distribution. Graphed are mean ± SEM pooled from two independent experiments. Number of cells measured (n) and p-values (t-test) are indicated. MYO18A knockdown was confirmed by parallel Western blots (data not shown, comparable to Figure 3.5) (D) Speed of actively migrating MDA-MB-231 cells overexpressing GFP (control) or GOLPH3 were measured from time-lapse images following wounding (Movie 3.1). Overexpression of GOLPH3 enhances cell migration. Graphed are mean ± SEM, with number of cells (n) and p-value (t-test) indicated.

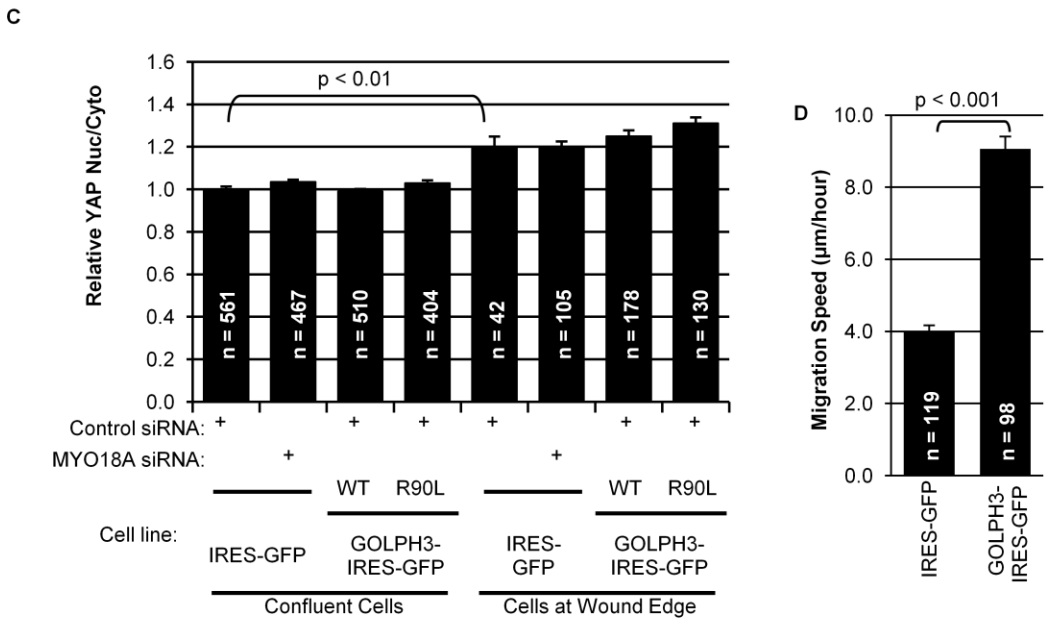

Figure 3.3: GOLPH3 does not affect MDA-MB-231 proliferation or sensing of loss-of-contact, but drives cell migration speed, continued. (C) MDA-MB-231 cells overexpressing GFP (control), GOLPH3, or GOLPH3-R90L were grown to confluence and transfected with control siRNA or MYO18A siRNA prior to wounding. Subcellular YAP distribution of confluent cells and cells at wound edge was measured as the ratio of nuclear YAP versus cytoplasmic YAP. As expected, monolayer wounding caused loss of confluence and YAP translocation to the nucleus. However, neither GOLPH3 overexpression nor MYO18A knockdown had any effect on YAP distribution. Graphed are mean ± SEM pooled from two independent experiments. Number of cells measured (n) and p-values (t-test) are indicated. MYO18A knockdown was confirmed by parallel Western blots (data not shown, comparable to Figure 3.5) (D) Speed of actively migrating MDA-MB-231 cells overexpressing GFP (control) or GOLPH3 were measured from time-lapse images following wounding (Movie 3.1). Overexpression of GOLPH3 enhances cell migration. Graphed are mean ± SEM, with number of cells (n) and p-value (t-test) indicated.

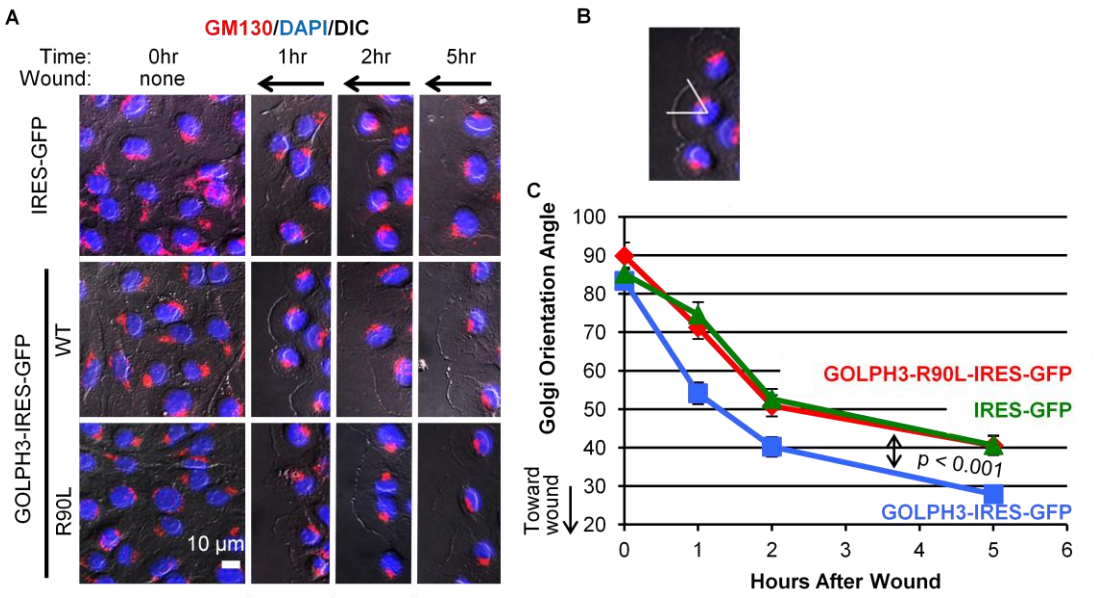

Figure 3.4: GOLPH3 overexpression promotes Golgi reorientation toward the wound edge. (A) Representative IF images of confluent monolayer of MDA-MB-231 cells overexpressing GFP (control), GOLPH3 or GOLPH3-R90L before and 1, 2, and 5 hrs after wounding are shown, with the Golgi marked by GM130 (red) and the nucleus by DAPI (blue). (B) Illustration of measurement of Golgi orientation angle. For each cell, a first line was drawn from the center of the nucleus to the wound edge, a second line was drawn from the center of the nucleus to the center of the Golgi. The angle formed between was measured. (C) Quantification of Golgi orientation angles from (A). The Golgi polarizes toward the wound edge upon monolayer wounding. GOLPH3 overexpression significantly enhances Golgi reorientation. Graphed are mean ± SEM pooled from two independent experiments, with p-values (ANOVA) indicated. (D) Representative immunofluorescence (IF) images of MDA-MB-231 cells overexpressing GFP, GOLPH3 or GOLPH3-R90L 5 hrs after wounding are shown. Each cell line was transfected with control siRNA or MYO18A siRNA prior to induction of wound. (E) Quantification of Golgi orientation angles from (D). GOLPH3-driven enhancement on Golgi reorientation toward the leading edge is abrogated by MYO18A depletion. Graphed are mean ± SEM pooled from two independent experiments. Number of cells measured (n) and p-values (t-test) are indicated.

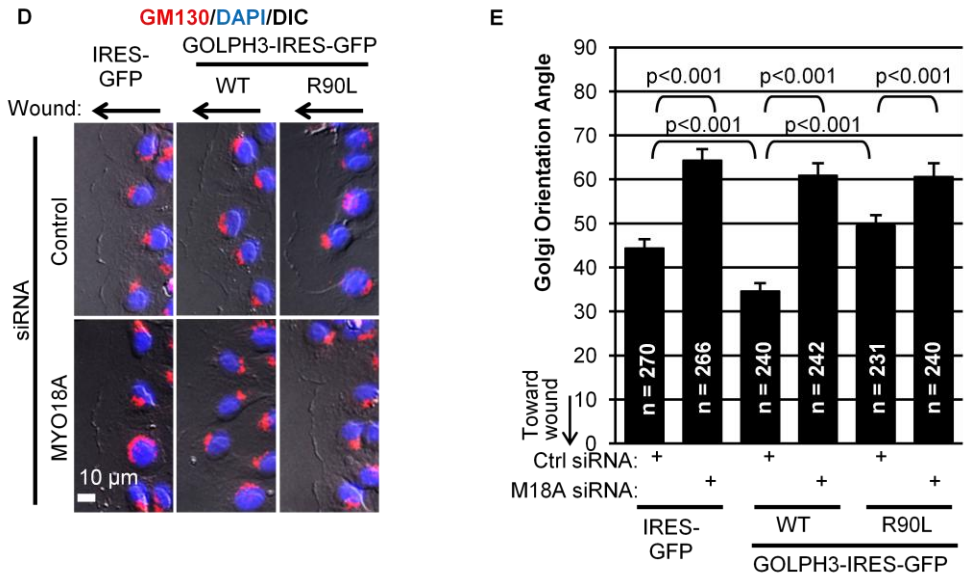

Figure 3.4: GOLPH3 overexpression promotes Golgi reorientation toward the wound edge, continued. (D) Representative immunofluorescence (IF) images of MDA-MB-231 cells overexpressing GFP, GOLPH3 or GOLPH3-R90L 5 hrs after wounding are shown. Each cell line was transfected with control siRNA or MYO18A siRNA prior to induction of wound. (E) Quantification of Golgi orientation angles from (D). GOLPH3-driven enhancement on Golgi reorientation toward the leading edge is abrogated by MYO18A depletion. Graphed are mean ± SEM pooled from two independent experiments. Number of cells measured (n) and p-values (t-test) are indicated.

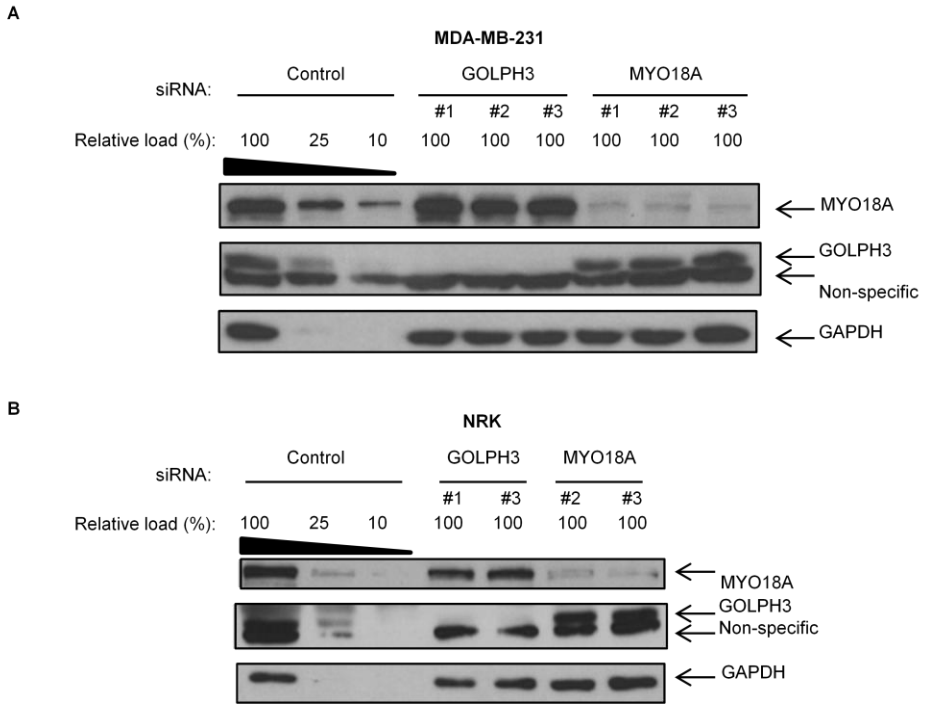

Figure 3.5: Efficient knockdown of GOLPH3 and MYO18A. (A) MDA-MB-231 and (B) NRK cells were transfected with control siRNA, siRNAs specific to GOLPH3, and siRNAs specific to MYO18A as indicated. Lysates were Western blotted for MYO18A and GOLPH3 with GAPDH as a loading control. Lysates from control siRNA-treated cells were loaded at different amounts to allow semiquantitative assessment of knockdown efficiency. Transfection with each of the GOLPH3 or MYO18A siRNA oligos results in at least 90% knockdown.

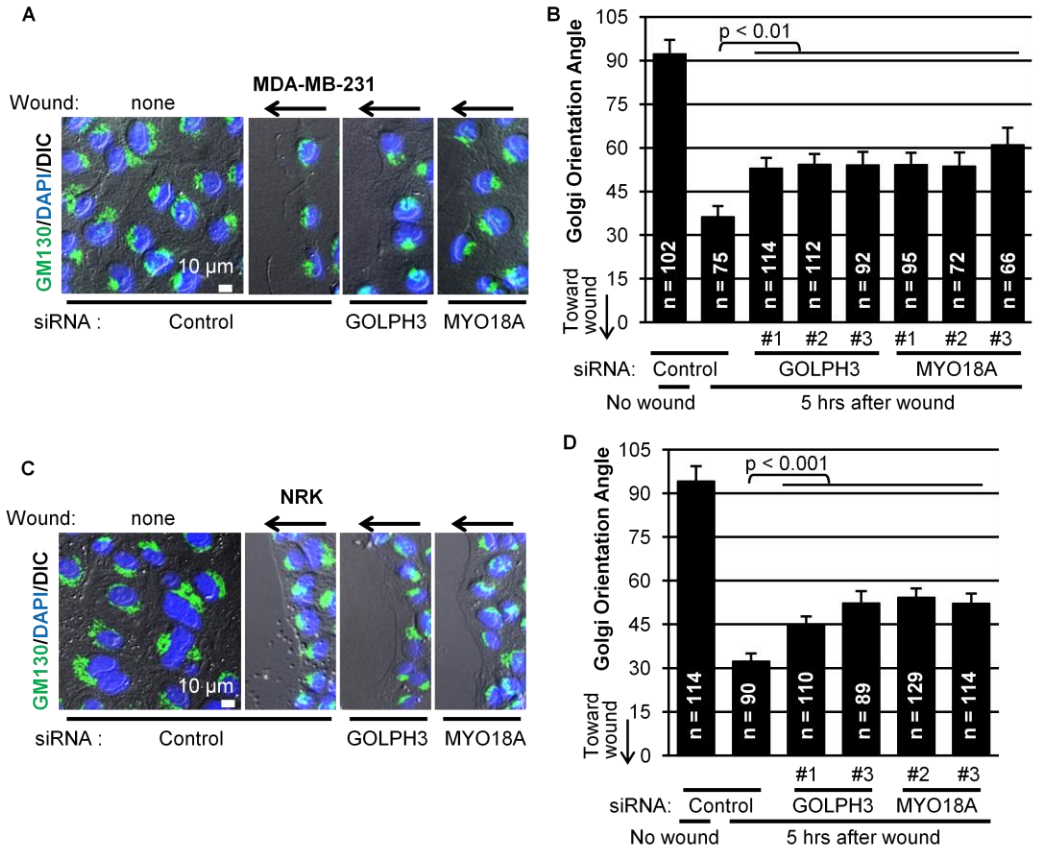

Figure 3.6: GOLPH3 and MYO18A are required for normal Golgi reorientation toward the wound edge. (A) MDA-MB-231 cells and (C) NRK cells were transfected with control siRNA, or siRNA oligos targeting GOLPH3 or MYO18A prior to wounding. Representative IF images of cells before and 5 hrs after wounding are shown, with the Golgi marked by GM130 (green) and the nucleus marked by DAPI (blue). (B) (D) Quantification of Golgi orientation angles from (A) and (C), respectively. The Golgi had a random orientation prior to wounding and polarized toward the leading edge after wounding. Golgi reorientation is significantly impaired if either GOLPH3 or MYO18A is depleted with different siRNA oligos. Graphed are mean ± SEM. The number of cells measured (n) and p-values (t-test) are indicated.

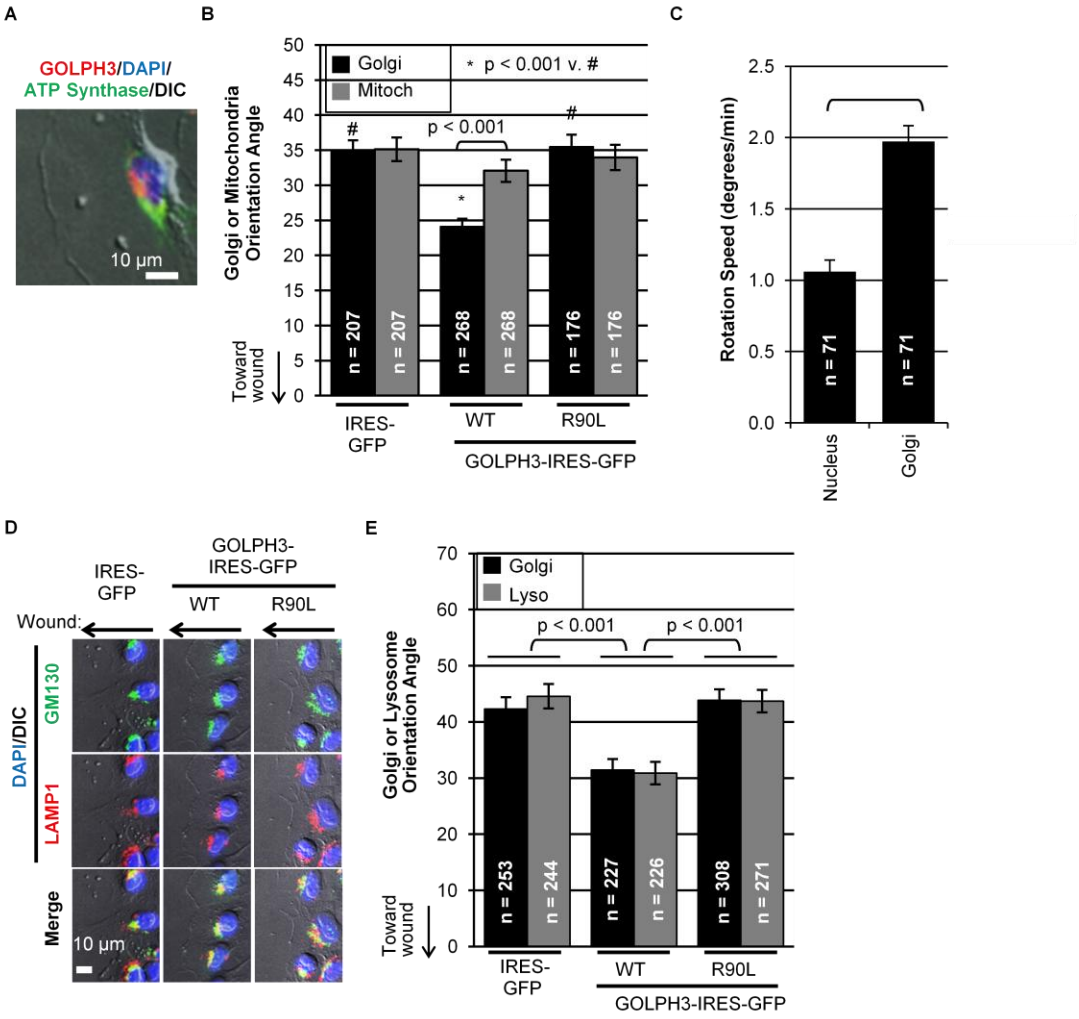

Figure 3.7: Comparison of Golgi to mitochondria, nucleus, and lysosomes in response to monolayer wounding. (A) IF example of cell at wound edge with mitochondria marked by ATP synthase (green), Golgi marked by GOLPH3 (red), and the nucleus marked by DAPI (blue). (B) Quantification of Golgi and mitochondria orientation angles from MDA-MB-231 cells overexpressing GFP (control), GOLPH3 or GOLPH3-R90L. Mitochondria orientation is insensitive to GOLPH3 overexpression, whereas Golgi reorientation is driven by GOLPH3 overexpression. (C) Measurement of Golgi and nuclear rotation speed upon wounding. The Golgi rotates significantly faster than the nucleus. Data are pooled from ten movies (e.g., Movie 3.3) with p-value indicated (t-test). (D) Representative IF images of cells at wound edge with Golgi marked by GM130 (green), lysosomes marked by LAMP1 (red), and the nucleus marked by DAPI (blue). (E) Quantification of Golgi and lysosome orientation angles from (D), showing GOLPH3-driven reorientation upon wounding for both organelles. (B) and (E) represent mean ± SEM pooled from two independent experiments with number of cells (n) and p-values (t-test) indicated.

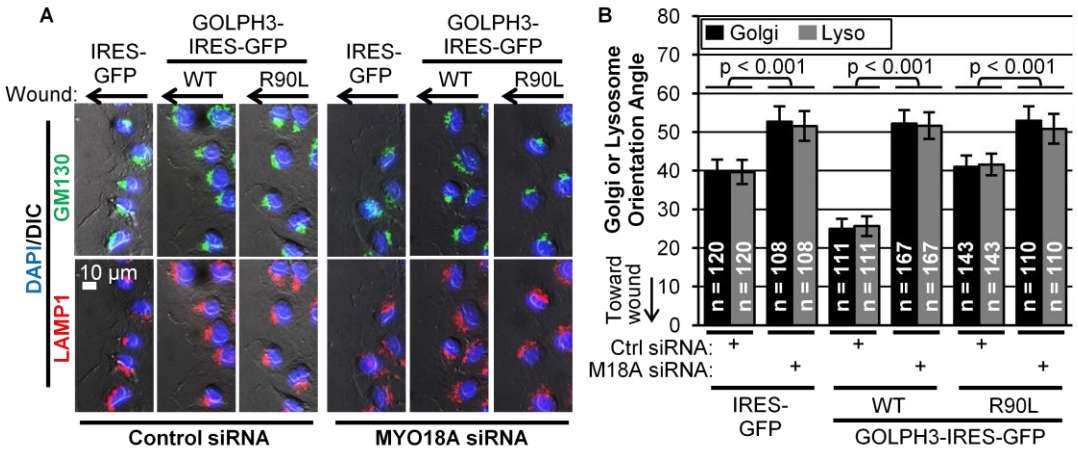

Figure 3.8: GOLPH3 drives Golgi and lysosome reorientation via the GOLPH3/MYO18A/F-actin pathway. (A) Representative IF images of MDA-MB-231 cells overexpressing GFP (control), GOLPH3, or GOLPH3-R90L at wound edge with Golgi labeled in green, lysosome in red, and the nucleus in blue. Cells were transfected with control siRNA or MYO18A siRNA for 48 hrs prior to monolayer wounding, and fixed 5 hrs after wounding. (B) Quantification of Golgi and lysosome orientation angles from (A). Depletion of MYO18A significantly impairs Golgi and lysosome reorientation, rendering them insensitive to overexpression of GOLPH3. (C) Representative IF images of MDA-MB-231 cells at wound edge treated with DMSO (vehicle control) or 250 nM Latrunculin B at time of monolayer wounding and fixed 5 hrs after wounding. (D) Quantification of Golgi and lysosome orientation angles from (C). Depolymerizing F-actin significantly impairs Golgi and lysosome reorientation. (E) Representative IF images of NRK cells at wound edge, transfected with control siRNA or siRNA targeting GOLPH3 or MYO18A 48 hrs prior to wounding and fixed 5 hrs after wounding. (F) Quantification of Golgi and lysosome orientation angles from (E). Golgi and lysosome reorientation toward the leading edge upon wounding is significantly impaired by interference with GOLPH3 or MYO18A. (B), (D), and (F), graphed are mean ± SEM pooled from two independent experiments, with number of cells measured (n) and p-values (t-test) indicated.

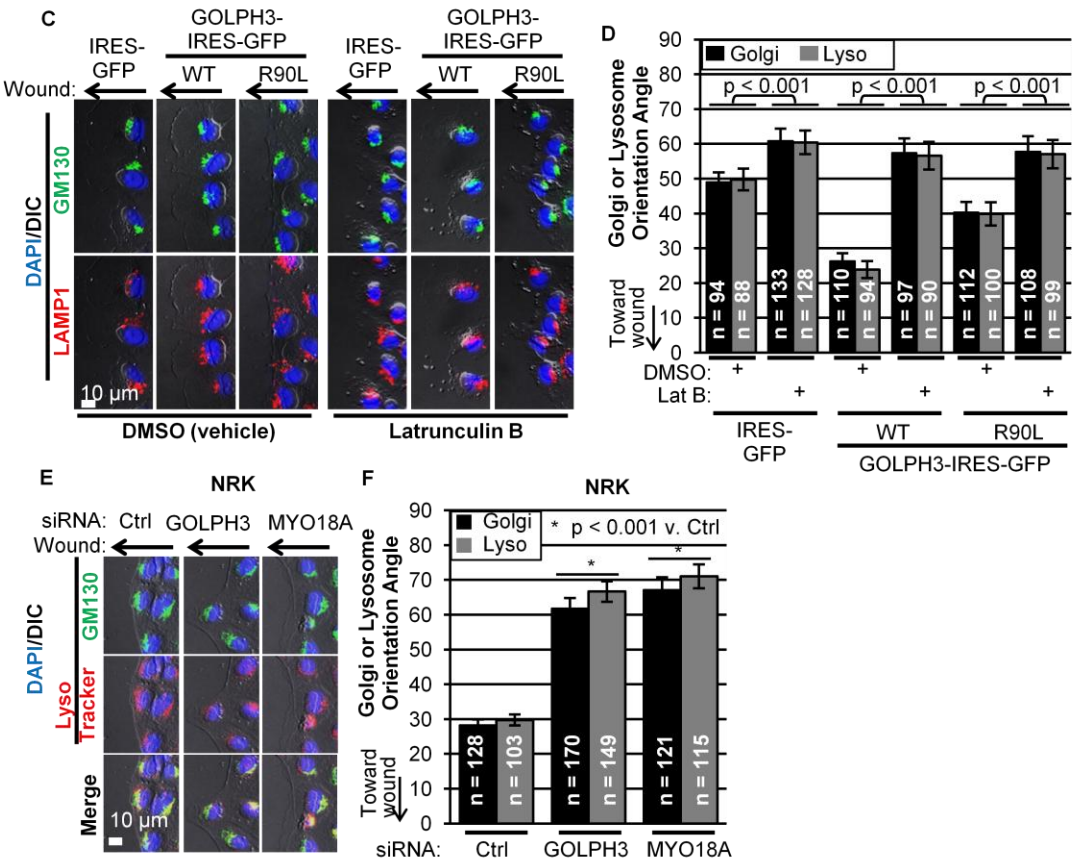

Figure 3.8: GOLPH3 drives Golgi and lysosome reorientation via the GOLPH3/MYO18A/F-actin pathway, continued. (C) Representative IF images of MDA-MB-231 cells at wound edge treated with DMSO (vehicle control) or 250 nM Latrunculin B at time of monolayer wounding and fixed 5 hrs after wounding. (D) Quantification of Golgi and lysosome orientation angles from (C). Depolymerizing F-actin significantly impairs Golgi and lysosome reorientation. (E) Representative IF images of NRK cells at wound edge, transfected with control siRNA or siRNA targeting GOLPH3 or MYO18A 48 hrs prior to wounding and fixed 5 hrs after wounding. (F) Quantification of Golgi and lysosome orientation angles from (E). Golgi and lysosome reorientation toward the leading edge upon wounding is significantly impaired by interference with GOLPH3 or MYO18A. (B), (D), and (F), graphed are mean ± SEM pooled from two independent experiments, with number of cells measured (n) and p-values (t-test) indicated.

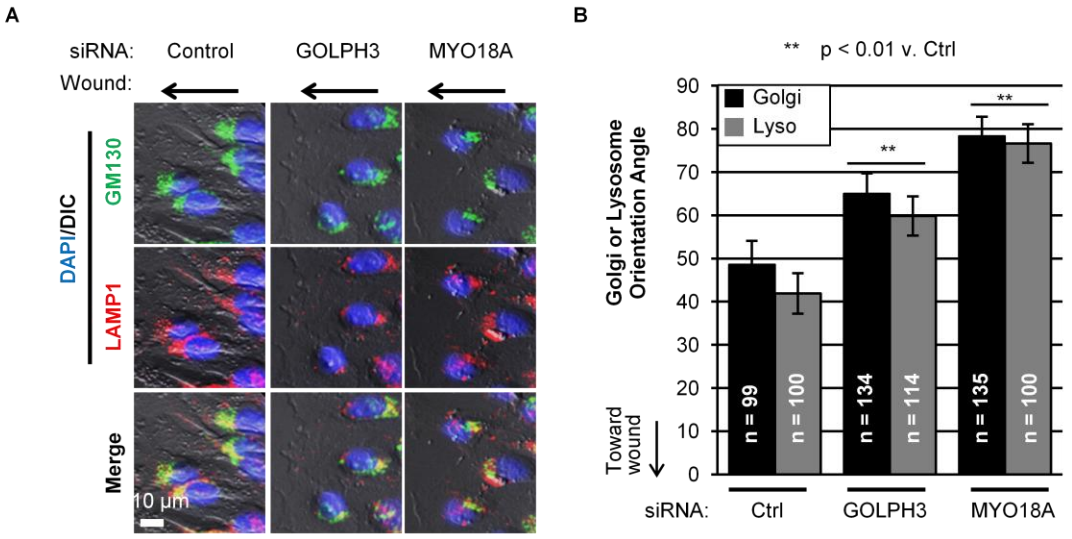

Figure 3.9: GOLPH3 and MYO18A are required for Golgi and lysosome reorientation at cell culture wound edge in Hela cells. (A) Representative IF images of Hela cells at wound edge. Cells were transfected with control siRNA or siRNA targeting GOLPH3 or MYO18A 48 hrs prior to wounding and fixed 5 hrs after wounding. The Golgi is labeled in green (GM130), lysosome in red (LAMP1), and the nucleus in blue (DAPI). (B) Quantification of Golgi and lysosome orientation angles from (A). Reorientation of the Golgi and lysosome toward the leading edge upon wounding is significantly impaired by depletion of GOLPH3 or MYO18A. Graphed are mean ± SEM pooled from two independent experiments, with number of cells (n) measured and p-values (t-test) indicated.

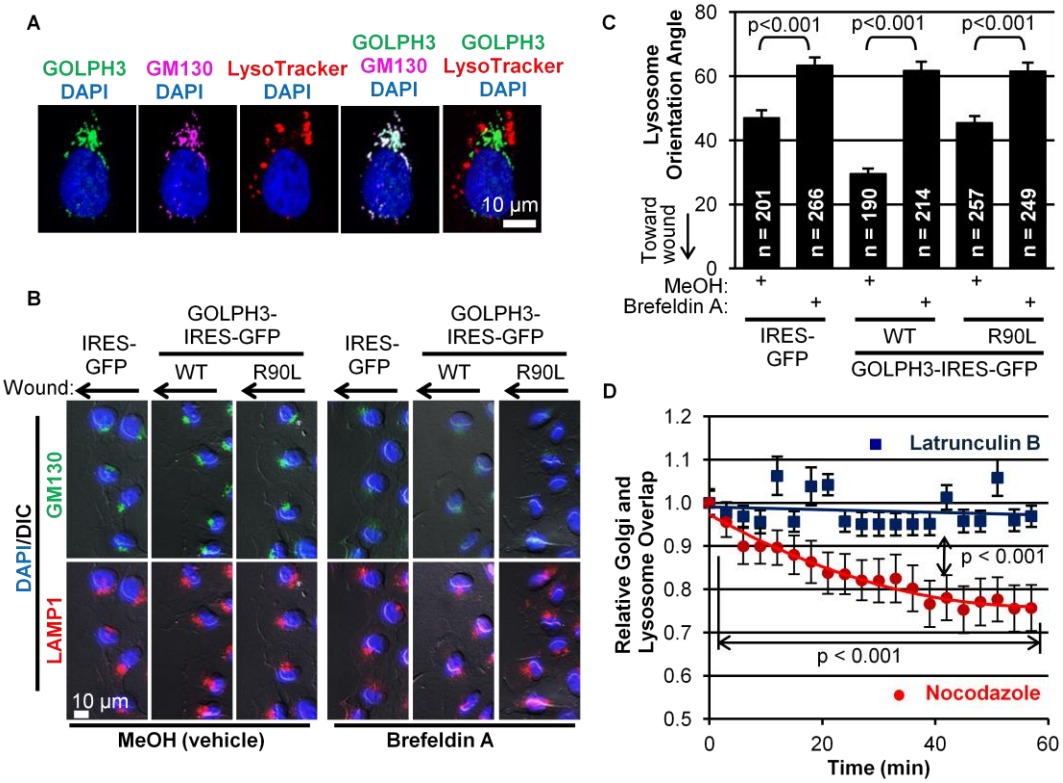

Figure 3.10: GOLPH3 acts indirectly on lysosome reorientation via the Golgi. (A) Representative IF image of cell stained for GOLPH3 (green), with Golgi marked by GM130 (magenta), lysosomes labeled with LysoTracker (red), and the nucleus marked by DAPI (blue). The staining pattern of GOLPH3 closely mimics GM130, but is distinct from LysoTracker. (B) Representative IF images of cells at wound edge treated with MeOH (vehicle) or Brefeldin A (BFA) at time of wounding, with Golgi labeled in green, lysosome in red, and the nucleus in blue. (C) Quantification of lysosome orientation angles from (B). Lysosome reorientation toward the wound is significantly impaired by disruption of the Golgi. Likewise, the ability of overexpressed GOLPH3 to accelerate lysosome reorientation is abolished by BFA treatment. Graphed are mean ± SEM pooled from two independent experiments. Number of cells measured (n) and p-values (t-test) are indicated. (D) Measurement of overlap of the Golgi labeled by ManII-GFP and lysosome labeled by LysoTracker from time-lapse imaging upon treatment with Latrunculin B to depolymerize F-actin or Nocodazole to depolymerize microtubules. The overlap between the Golgi and lysosome compartments remains intact with Latrunculin B treatment, but significantly decreases with Nocodazole treatment. Graphed are mean and SEM, and p-values (two-way ANOVA) are indicated.

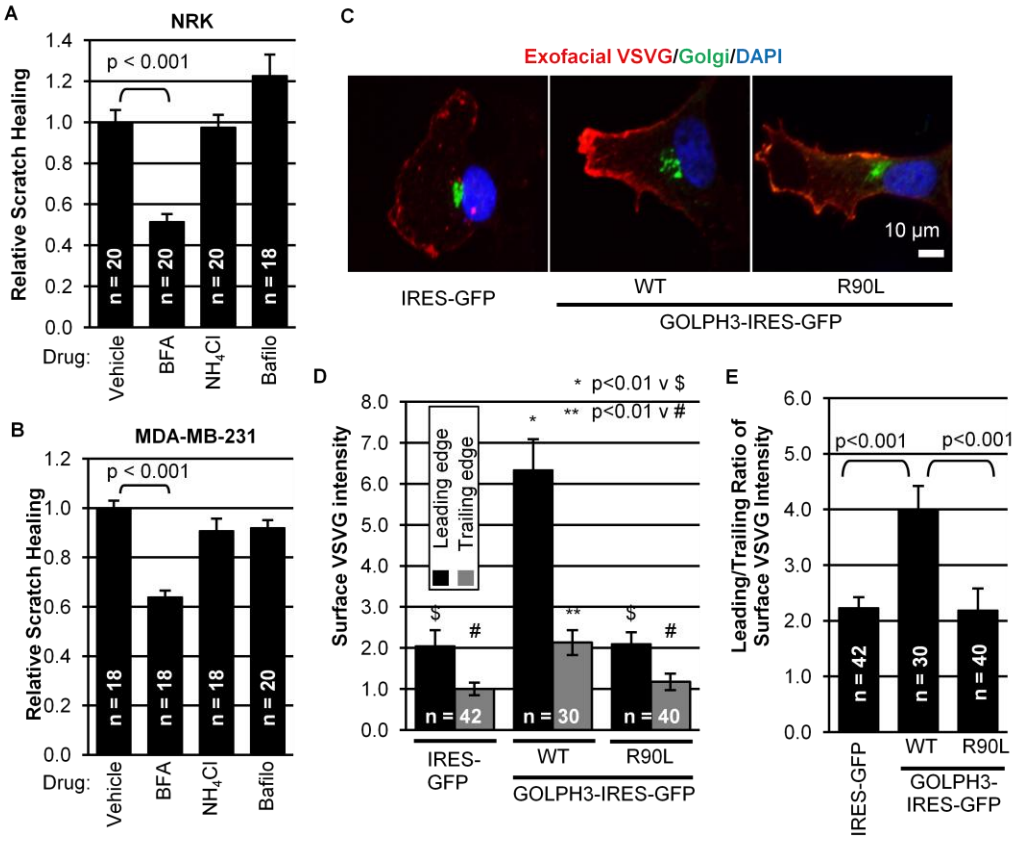

Figure 3.11: GOLPH3 promotes directional trafficking to the leading edge in wound healing. (A) (B) Quantification of scratch wound healing in NRK and MDA-MB-231 cells, treated with inhibitors of Golgi or lysosome function at time of wounding. Graphs indicate cell migration into the wound relative to vehicle control. Treatment with Golgi inhibitor BFA results in a significant reduction in cell migration, while treatment with lysosome inhibitors NH_4Cl or Bafilomycin A1 has minimal effect. (C) Representative IF images of migrating cells expressing ts045-VSVG-GFP at wound. Surface VSVG (red) was detected by using an exofacial VSVG antibody on unpermeablized cells. The Golgi is shown in green and nucleus in blue. (D) Quantification of surface VSVG at the leading and trailing edges of cells from (C). GOLPH3 overexpression results in a significant increase in surface VSVG in both leading and trailing edges compared to GFP (control) or GOLPH3-R90L. (E) Quantification of directional VSVG trafficking expressed as the ratio of leading over trailing surface VSVG intensity. (A), (B), (D) and (E), graphed are mean ± SEM pooled from two independent experiments. Number of fields (A, B) or number of cells (D, E) measured (n) and p-values (t-test) are indicated.

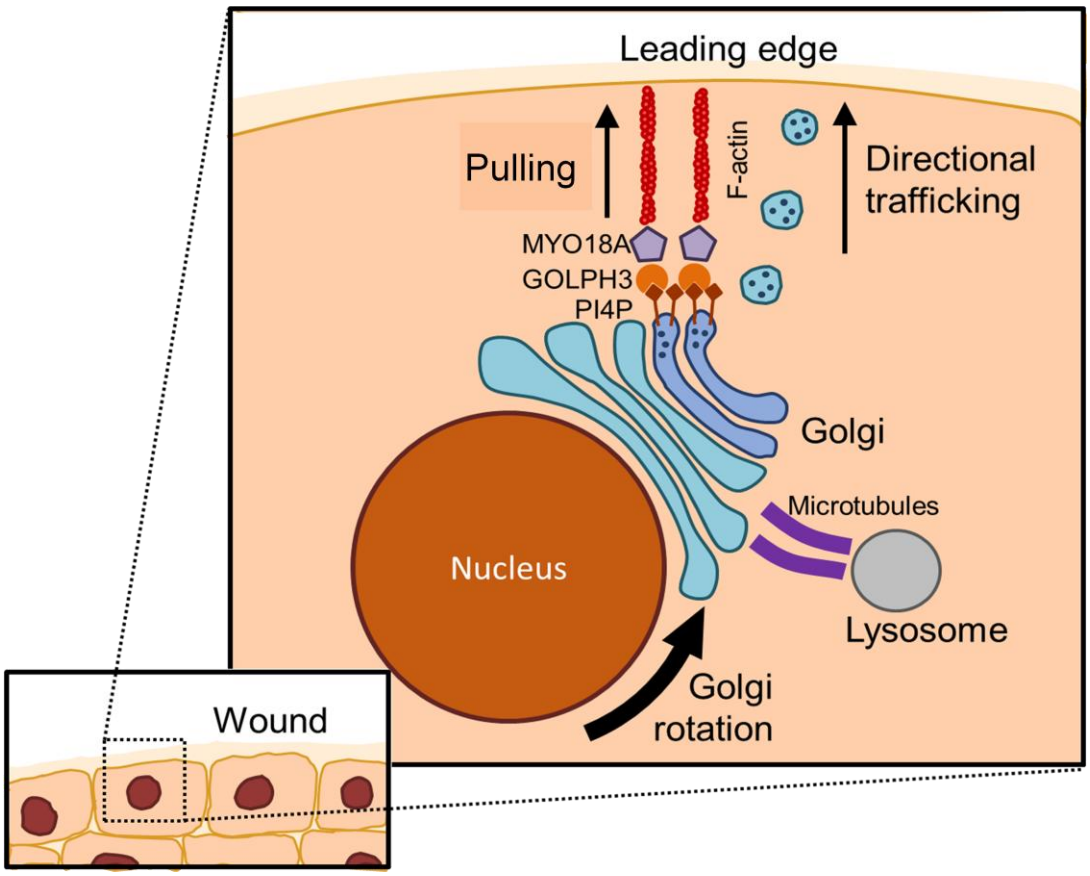

Figure 3.12: Model for GOLPH3-dependent Golgi reorientation and directional trafficking in wound healing. Upon wounding, the GOLPH3/MYO18A/F-actin machinery pulls the PtdIns(4)P-rich Golgi to actively reorient toward the leading edge. Lysosomes move in concert with the Golgi due to linkage to the Golgi mediated by microtubules. As a functional consequence of Golgi polarization, directional trafficking occurs toward the leading edge, which is required for cell migration. Overexpression of GOLPH3 drives enhanced reorientation of the Golgi toward the leading edge, increased overall Golgi-to-plasma membrane trafficking, and increased trafficking preferentially toward the front of the cell. All three contribute to increased directional trafficking to the wound edge, driving enhanced cell migration.

CHAPTER 4: Conclusions and future directions

4.1 The Roles of GOLPH3

GOLPH3 functions at the Golgi to promote secretory trafficking (Bishé et al., 2012a; Dippold et al., 2009; Ng et al., 2013). Deeper investigations into the implications for this function of GOLPH3 promise to yield insights into not only basic biology of the Golgi, but also insights that translate into clinical benefits. The high frequency of GOLPH3 amplification, in 32% to 56% of the most common human tumors, and its correlation with patient mortality are striking signposts pointing to the clinical relevance of GOLPH3 (Buschman et al., 2015a; Dai et al., 2015; Hua et al., 2012; Hu et al., 2013, 2014; JianXin et al., 2014; Kunigou et al., 2011; Li et al., 2014, 2012, 2011; Lu et al., 2014; Ma et al., 2014a, 2014b; Peng et al., 2014; Scott et al., 2009; Tokuda et al., 2014; Wang et al., 2012, 2014; Xue et al., 2014; Zeng et al., 2012; L.-J. Zhang et al., 2014; X. Zhang et al., 2014; Y. Zhang et al., 2014; Zhou et al., 2012, 2014, 2013).

The known functions of GOLPH3 occur at the Golgi, and enhancement of the GOLPH3 pathway at the Golgi, by overexpressing GOLPH3, confers resistance to killing by DNA damaging agents (Farber-Katz et al., 2014). The role for GOLPH3 in the DNA damage response provides a plausible explanation for why it is so frequently amplified in refractory tumors. However, this role does not adequately account for how GOLPH3 functions as an oncogene that is capable of causing cellular transformation.

Overexpression of GOLPH3 drives increased cell migration, a phenotype likely related to the observed correlation of GOLPH3 overexpression with advanced metastatic disease. The contribution of GOLPH3 to promote cell migration is also through its Golgi function. However, like its role in the DNA damage response, the role for GOLPH3 in promoting cell migration seems able only to explain its frequent overexpression observed in tumors, but unable to explain why GOLPH3 is an oncogene in the first place.

Others had previously shown that GOLPH3 modulates intracellular signaling (Scott et al., 2009; Zeng et al., 2012). We have now shown that GOLPH3's role in modulating signaling is a consequence of its function at the Golgi, rather than functioning through a novel signaling pathway. We show that GOLPH3 is required for growth factor signaling because it is required for trafficking of growth factor receptors to the plasma membrane. Since the trafficking and signaling of growth factor receptors also require MYO18A, we infer that it is indeed the Golgi function of GOLPH3, which is mediated by MYO18A, that is required for its ability to regulate growth factor receptor trafficking and, therefore, growth factor receptor signaling as a consequence.

It is surprising that GOLPH3 overexpression is sufficient to increase the delivery of growth factor receptors to the plasma membrane, pointing to the fact that GOLPH3 is a limiting factor for their trafficking. Since GOLPH3 must retain the ability to bind PtdIns(4)P in order to enhance receptor trafficking to the plasma membrane, we infer that it is the Golgi function of GOLPH3 which

is responsible for our observations. The consequence of GOLPH3 overexpression-enhanced Golgi-to-plasma membrane trafficking is enhanced growth factor signaling. Therefore, GOLPH3-dependent Golgi-to-plasma membrane trafficking is limiting for growth factor signaling. A model summarizing the role of GOLPH3 in controlling growth factor receptor trafficking and signaling is depicted in Figure 4.1. The function of GOLPH3 in regulating Golgi-to-plasma membrane trafficking, thereby controlling growth factor signaling, provides a parsimonious explanation for the role of GOLPH3 in causing oncogenic transformation, since enhanced growth factor signaling is known to cause cellular transformation and mTORC1 signaling is a ciritcal requirement for GOLPH3-overexpression-induced cellular transformation (Scott et al., 2009; Velu et al., 1987). This function of GOLPH3 at the Golgi highlights a novel aspect of growth factor signaling and its regulation by the cell's secretory system. Further experiments will need to be done to address whether other components of the secretory system are capable of driving cellular transformation and what are the critical cargoes.

4.2 Dependence of growth factor receptors on GOLPH3

We have shown that GOLPH3 is required for growth factor signaling because of its role in Golgi-to-plasma membrane trafficking of growth factor receptors. This raises questions about the types of cargo dependent on the GOLPH3 pathway and whether the dependence of growth factor receptors is in some way unique. Our model would predict that many different secretory

cargoes rely on the GOLPH3-dependent trafficking pathway and we have shown this is indeed the case (Bishé et al., 2012a; Dippold et al., 2009; Farber-Katz et al., 2014; Ng et al., 2013). However, we would also predict that the types of cargo most acutely dependent on the GOLPH3 pathway are those that are most rapidly turned-over at the plasma membrane, thus relying on efficient trafficking from the Golgi to the plasma membrane in order to be replenished with nascent protein.

Since growth factor receptors have relatively short half-lives, they may have greater dependence than other cargoes for efficient GOLPH3-dependent Golgi-to-plasma membrane trafficking. For example, the half-life of PDGFR is only 45 minutes in the presence of ligand, while many other cell surface proteins have half lives >75 hours (Hare and Taylor, 1991; Keating and Williams, 1987). Because of this relatively rapid turnover, PDGFR levels at the plasma membrane may be more tightly regulated by GOLPH3 than the average, longer-lived plasma membrane protein. The half-life of a growth factor receptor depends on its expression level, growth conditions, and many other factors, and is therefore variable from one context to another. Perhaps certain contexts and conditions lend themselves to greater dependence and regulation by the GOLPH3 pathway.

The effect of increased growth factor receptor delivery to the plasma membrane may also be compounded by effects on the receptor internalization machinery. If the abundance of active EGFR at the plasma membrane becomes too high, the clathrin-mediated endocytosis machinery becomes

saturated (Goh and Sorkin, 2013; Wiley, 1988). Thus, not only does GOLPH3 overexpression drive signaling by driving increasing levels of growth factor receptors at the plasma membrane, it has the potential to overload the endocytic machinery necessary for terminating signaling. Elegant studies will need to be designed for studying the effects of GOLPH3 perturbations on trafficking kinetics. It will also be interesting to investigate the effects on the entire cell surface proteome in order to identify whether sets of GOLPH3-dependent and independent cargoes may be classified.

4.3 Implications for constitutively active growth factor receptors

Growth factor receptors normally become activated upon ligand binding at the plasma membrane (Lemmon and Schlessinger, 2010). However, many tumors are driven by mutated growth factor receptors that are constitutively active, having gained ligand-independence. For example, a constitutively active EGFR variant is harbored by 25% to 64% of patients with of glioblastoma multiforme (GBM), the most common and lethal brain tumor in adults (Gan et al., 2013). This variant, termed EGFRvIII, is a truncated form of EGFR which lacks the regulation of the extracellular ligand-binding domain, yielding a receptor that is not internalized and has constitutively activated and prolonged signaling (Huang et al., 1997). In another example, a mutation of FLT3 (FLT3/ITD) resulting in constitutively active RTK activity occurs in approximately 23% of adult acute myeloid leukemia (AML) patients (Levis and Small, 2003, p. 3). In both cases, the mutant receptors are active without the

input signal of ligand binging at the plasma membrane. Even so, the spatial regulation of growth factor receptors is an important determinant of their signaling output as observed with FLT3/ITD, which normally signals through the PI-3-kinase, ERK, and STAT5 pathways, but mislocalization of the constitutively active receptor alters the signaling output to these pathways (Choudhary et al., 2009). Reasoning that PI-3-kinase signaling occurs at the locale of its substrate PtdIns(4,5)P2, which is primarily at the plasma membrane, we would predict that even constitutively active growth factor receptors would require trafficking to the plasma membrane for class I_A PI-3-kinase signaling. Therefore, we anticipate that inhibition of the GOLPH3 pathway will likely interfere with signaling, even by constitutively active growth factor receptors. It will be interesting to experimentally investigate this possibility.

4.4 Therapeutic implications of the GOLPH3 pathway

Many, if not most, of the current efforts for developing targeted cancer therapeutics are focused on inhibiting growth factor receptors and other components of their downstream signaling pathways. Inhibition of growth factor receptors is a clinically validated therapeutic strategy for several cancers (Gschwind et al., 2004). Despite their successes, the emergence of resistance to growth factor receptor-targeted inhibitors is a perennial problem resulting from mutations of the target or activation of alternate pathways (Chen and Fu, 2011; Niederst and Engelman, 2013). To combat this problem it will

be important to continue innovating new therapeutic strategies that can be used to overcome drug resistance.

Toward this end, we anticipate that the GOLPH3 pathway we have identified contains new targets for therapeutics. Because of the high frequency of GOLPH3 overexpression in cancer and the common theme of dysregulated growth factor signaling found in cancer, it seems likely that inhibition of the GOLPH3 pathway will be a broadly applicable strategy. Moreover, because GOLPH3 is required for signaling by multiple growth factor receptors, we expect that inhibition of GOLPH3 will be helpful for overcoming common resistance mechanisms. This is because amplification of alternate receptor signaling pathways in response to individual receptor inhibition is a known mechanism of resistance for growth factor receptor-targeted therapeutics (Niederst and Engelman, 2013; Shattuck et al., 2008). Indeed, *in vitro* evidence indicates that inhibition of more than one growth factor receptor is required for killing GBM tumor cells, while targeted inhibition of a single receptor is largely ineffective (Stommel et al., 2007). For this reason, the GOLPH3 pathway will perhaps be a particularly efficacious therapeutic target because of the capacity for regulating signaling by multiple receptors.

Beyond cancer, the GOLPH3 pathway may have therapeutic implications for other human diseases. Thus far, virion secretion of hepatitis C virus (HCV) and replication of human immunodeficiency virus (HIV) have been shown to depend on GOLPH3 (Bishé et al., 2012a, 2012b; Brass et al., 2008). In light of these studies, it seems likely there are additional viruses that require

GOLPH3 for secretion. It will be important to further validate the efficacy and safety of GOLPH3 as a therapeutic target, using cell-based and animal models of disease. Currently, our lab is testing a mouse model of GOLPH3 overexpression and screening compound libraries for inhibitors of the GOLPH3 pathway.

4.5 Regulation of GOLPH3

Further insights into the regulation of GOLPH3 will enhance our understanding of the functions of the GOLPH3 pathway in biology. Originally, GOLPH3 was first identified as a phosphoprotein (Bell et al., 2001; Wu et al., 2000). Characterization of the regulation of several of the phosphorylation sites on GOLPH3 helped reveal its role in the cytoplasmic response to DNA damage and its role in promoting cell survival (Farber-Katz et al., 2014). The phosphorylation of GOLPH3 in response to DNA damage enhances its interaction with MYO18A and secretory trafficking is altered. Therefore, because of the increased interaction with MYO18A, GOLPH3 phosphorylation leads to activation of the pathway which we would predict has consequences for growth factor receptor trafficking and thus growth factor signaling. It remains to be tested whether DNA damage or regulation of specific phosphorylation sites on GOLPH3 by additional kinases or phosphatases has consequences for regulating its function in modulating growth factor receptor trafficking and signaling.

Additional roles for GOLPH3 will likely be revealed by further investigation into regulation of its other phosphorylation sites and posttranslational modifications (PTMs), especially since PTMs can regulate the activity, interactions, and stability of a protein. To date, there are ten phosphorylation sites and two ubiqutinated lysines observed for GOLPH3 whose regulation and function remain largely uncharacterized (Figure 4.2).

The regulation of GOLPH3 in cancer appears to be primarily related to its overexpression by gene amplification (Buschman et al., 2015a). Moreover, we have shown that GOLPH3 is rate-limiting for growth factor signaling and its overexpression enhances signaling. Therefore, it seems likely that mechanisms regulating GOLPH3 expression will have consequences for oncogenic signaling. Thus far, it has been shown that GOLPH3 expression levels are under the control of androgen signaling (Romanuik et al., 2009). Since androgens and many other regulatory factors depend on the secretory pathway it will be interesting to investigate the potential for feedback mechanisms regulating GOLPH3 expression and their effects on growth factor signaling.

Enhancement of growth factor signaling by GOLPH3 overexpression requires that GOLPH3 retains the ability to bind PtdIns(4)P, as evidenced by the lack of effects observed upon GOLPH3(R90L) compared with WT GOLPH3 overexpression. Thus, GOLPH3 functions as a PtdIns(4)P-effector in order to enhance growth factor signaling, suggesting the requirement for adequate PtdIns(4)P/GOLPH3 stoichiometry to mediate the effects of

GOLPH3 overexpression. For this reason, we predict that regulation of PtdIns(4)P levels, in addition to GOLPH3 levels will have consequences for growth factor signaling. Indeed, growth factor signaling regulates the PtdIns(4)P-4-phosphatase SAC1 to increase PtdIns(4)P levels at the Golgi (Blagoveshchenskaya et al., 2008). Further investigation into the regulation of PtdIns(4)P and GOLPH3's other binding partner, MYO18A, will likely reveal additional mechanisms for regulating growth factor receptor trafficking and signaling.

In conclusion, the study of the Golgi protein, GOLPH3, has generated significant new insights into the regulation of the Golgi, with consequences for cell survival and, as we now understand, growth factor signaling and cell migration. We have discovered that the PtdIns(4)P/GOLPH3/MYO18A pathway is required for growth factor signaling because it is required for trafficking growth factor receptors to the plasma membrane. Surprisingly, GOLPH3 is rate-limiting and overexpression of GOLPH3 is sufficient to drive increased levels of growth factor receptors to the plasma membrane, consequently driving increased growth factor signaling. This mechanism presents a new paradigm for regulation of growth factor signaling from the Golgi. We find that the PtdIns(4)P/GOLPH3/MYO18A pathway is also required for Golgi reorientation and that overexpression of GOLPH3 is able to drive the reorientation process, thereby driving cell migration. These roles for GOLPH3 highlight a previously underappreciated role for the Golgi in key biological

processes with consequences for human disease, and the GOLPH3 pathway described here contains novel targets for therapeutics.

4.6 Figures

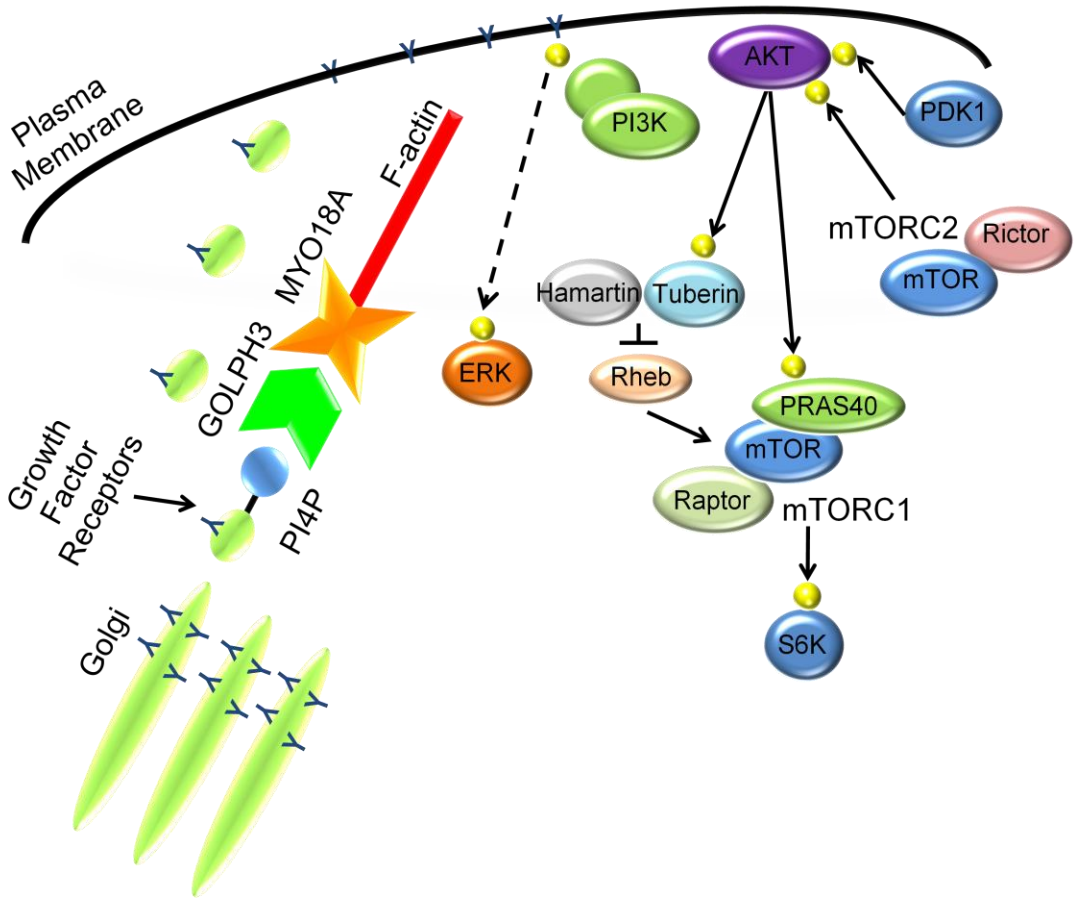

Figure 4.1: Model for GOLPH3-dependent regulation of growth factor signaling. A tensile force is applied to the Golgi membrane by the PtdIns(4)P/GOLPH3/MYO18A/F-actin pathway that pulls vesicles from the Golgi and assists in Golgi-to-plasma membrane trafficking of vesicle cargo, including growth factor receptors. GOLPH3 is required and, in some cases, rate-limiting for trafficking of growth factor receptors, having consequences for ligand-stimulated growth factor signaling through the receptor/PI-3-kinase/AKT/mTOR and receptor/Ras/Raf/Mek/Erk pathways.

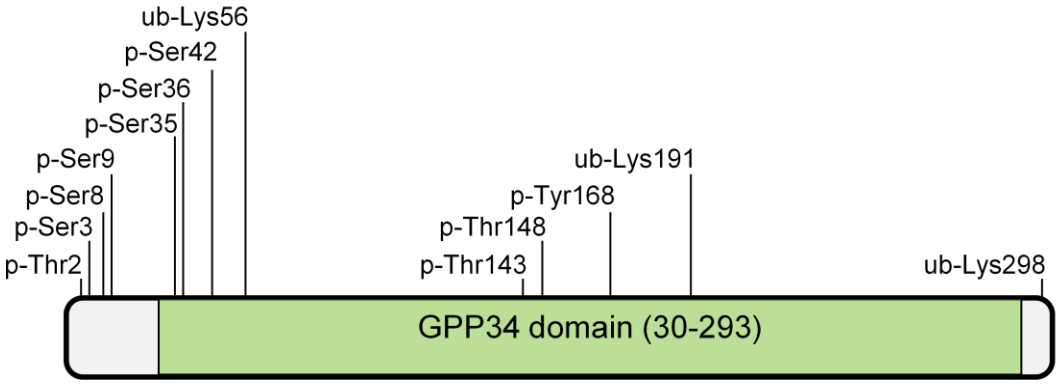

Figure 4.2: Post-translational modifications and domains of GOLPH3. The majority of GOLPH3 is comprised of the GPP34 domain which is required for PtdIns(4)P binding. Ten different phosphorylation (p) sites have been mapped for human GOLPH3. Of these, only phosphorylations of Thr143 and Thr148 have known functions. Phosphorylation of Thr143 and Thr148 are phosphorylated by DNA-PK to mediate the Golgi response to DNA damage. The regulation and function of the other eight phosphorylation sites and two ubiquitinated (ub) lysines remain uncharacterized. (Dippold, et al., 2009, Farber-Katz, et al., 2014, www.phosphosite.org)

REFERENCES

Abraham, R.T., 2009. GOLPH3 links the Golgi network to mTOR signaling and human cancer. Pigment Cell Melanoma Res 22, 378–379. doi:10.1111/j.1755-148X.2009.00596.x

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., Hemmings, B.A., 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J 15, 6541–6551.

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B., Cohen, P., 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr. Biol. 7, 261–269.

Audhya, A., Foti, M., Emr, S.D., 2000. Distinct roles for the yeast phosphatidylinositol 4-kinases, Stt4p and Pik1p, in secretion, cell growth, and organelle membrane dynamics. Mol. Biol. Cell 11, 2673–2689.

Azzoli, C.G., Temin, S., Aliff, T., Baker, S., Brahmer, J., Johnson, D.H., Laskin, J.L., Masters, G., Milton, D., Nordquist, L., Pao, W., Pfister, D.G., Piantadosi, S., Schiller, J.H., Smith, R., Smith, T.J., Strawn, J.R., Trent, D., Giaccone, G., 2011. 2011 Focused Update of 2009 American Society of Clinical Oncology Clinical Practice Guideline Update on Chemotherapy for Stage IV Non–Small-Cell Lung Cancer. JCO 29, 3825–3831. doi:10.1200/JCO.2010.34.2774

Baschieri, F., Confalonieri, S., Bertalot, G., Di Fiore, P.P., Dietmaier, W., Leist, M., Crespo, P., Macara, I.G., Farhan, H., 2014. Spatial control of Cdc42 signalling by a GM130–RasGRF complex regulates polarity and tumorigenesis. Nat Commun 5, 4839. doi:10.1038/ncomms5839

Batzer, A.G., Rotin, D., Ureña, J.M., Skolnik, E.Y., Schlessinger, J., 1994. Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. Mol. Cell. Biol. 14, 5192–5201.

Bell, A.W., Ward, M.A., Blackstock, W.P., Freeman, H.N., Choudhary, J.S., Lewis, A.P., Chotai, D., Fazel, A., Gushue, J.N., Paiement, J., Palcy, S., Chevet, E., Lafrenière-Roula, M., Solari, R., Thomas, D.Y., Rowley, A., Bergeron, J.J., 2001. Proteomics characterization of abundant Golgi membrane proteins. J. Biol. Chem. 276, 5152–5165. doi:10.1074/jbc.M006143200

Bergmann, J.E., Kupfer, A., Singer, S.J., 1983. Membrane insertion at the leading edge of motile fibroblasts. Proc Natl Acad Sci U S A 80, 1367–1371.

Bieging, K.T., Mello, S.S., Attardi, L.D., 2014. Unravelling mechanisms of p53-mediated tumour suppression. Nat Rev Cancer 14, 359–370. doi:10.1038/nrc3711

Bisel, B., Wang, Y., Wei, J.-H., Xiang, Y., Tang, D., Miron-Mendoza, M., Yoshimura, S., Nakamura, N., Seemann, J., 2008. ERK regulates Golgi and centrosome orientation towards the leading edge through GRASP65. J Cell Biol 182, 837–843. doi:10.1083/jcb.200805045

Bishé, B., Syed, G.H., Field, S.J., Siddiqui, A., 2012a. Role of phosphatidylinositol 4-phosphate (PI4P) and its binding protein GOLPH3 in hepatitis C virus secretion. J. Biol. Chem. 287, 27637–27647. doi:10.1074/jbc.M112.346569

Bishé, B., Syed, G., Siddiqui, A., 2012b. Phosphoinositides in the hepatitis C virus life cycle. Viruses 4, 2340–2358. doi:10.3390/v4102340

Blagoveshchenskaya, A., Cheong, F.Y., Rohde, H.M., Glover, G., Knodler, A., Nicolson, T., Boehmelt, G., Mayinger, P., 2008. Integration of Golgi trafficking and growth factor signaling by the lipid phosphatase SAC1. J Cell Biol 180, 803–812. doi:10.1083/jcb.200708109

Bonangelino, C.J., Chavez, E.M., Bonifacino, J.S., 2002. Genomic screen for vacuolar protein sorting genes in Saccharomyces cerevisiae. Mol. Biol. Cell 13, 2486–2501. doi:10.1091/mbc.02-01-0005

Brass, A.L., Dykxhoorn, D.M., Benita, Y., Yan, N., Engelman, A., Xavier, R.J., Lieberman, J., Elledge, S.J., 2008. Identification of host proteins required for HIV infection through a functional genomic screen. Science 319, 921–926. doi:10.1126/science.1152725

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., Greenberg, M.E., 1999. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. Cell 96, 857–868.

Buday, L., Downward, J., 1993. Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell 73, 611–620.

Burgess, A.W., Cho, H.-S., Eigenbrot, C., Ferguson, K.M., Garrett, T.P.J., Leahy, D.J., Lemmon, M.A., Sliwkowski, M.X., Ward, C.W., Yokoyama, S., 2003. An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. Mol. Cell 12, 541–552.

Burkhardt, J.K., 1998. The role of microtubule-based motor proteins in maintaining the structure and function of the Golgi complex. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1404, 113–126. doi:10.1016/S0167-4889(98)00052-4

Burnett, P.E., Barrow, R.K., Cohen, N.A., Snyder, S.H., Sabatini, D.M., 1998. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proc. Natl. Acad. Sci. U.S.A. 95, 1432–1437.

Buschman, M.D., Rahajeng, J., Field, S.J., 2015a. GOLPH3 Links the Golgi, DNA Damage, and Cancer. Cancer Res. 75, 624–627. doi:10.1158/0008-5472.CAN-14-3081

Buschman, M.D., Xing, M., Field, S.J., 2015b. The GOLPH3 pathway regulates Golgi shape and function and is activated by DNA damage. Front. Neurosci 362. doi:10.3389/fnins.2015.00362

Cantley, L.C., 2002. The phosphoinositide 3-kinase pathway. Science 296, 1655–1657. doi:10.1126/science.296.5573.1655

Carpenter, A.E., Jones, T.R., Lamprecht, M.R., Clarke, C., Kang, I.H., Friman, O., Guertin, D.A., Chang, J.H., Lindquist, R.A., Moffat, J., Golland, P., Sabatini, D.M., 2006. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biology 7, R100. doi:10.1186/gb-2006-7-10-r100

Carpenter, C.L., Auger, K.R., Chanudhuri, M., Yoakim, M., Schaffhausen, B., Shoelson, S., Cantley, L.C., 1993. Phosphoinositide 3-kinase is activated by phosphopeptides that bind to the SH2 domains of the 85-kDa subunit. J. Biol. Chem. 268, 9478–9483.

Cau, J., Hall, A., 2005. Cdc42 controls the polarity of the actin and microtubule cytoskeletons through two distinct signal transduction pathways. Journal of Cell Science 118, 2579–2587. doi:10.1242/jcs.02385

Chabin-Brion, K., Marceiller, J., Perez, F., Settegrana, C., Drechou, A., Durand, G., Poüs, C., 2001. The Golgi Complex Is a Microtubule-organizing Organelle. Mol Biol Cell 12, 2047–2060.

Cheng, J.Q., Lindsley, C.W., Cheng, G.Z., Yang, H., Nicosia, S.V., 2005. The Akt/PKB pathway: molecular target for cancer drug discovery. Oncogene 24, 7482–7492. doi:10.1038/sj.onc.1209088

Chen, Y., Fu, L., 2011. Mechanisms of acquired resistance to tyrosine kinase inhibitors. Acta Pharmaceutica Sinica B 1, 197–207. doi:10.1016/j.apsb.2011.10.007

Choudhary, C., Olsen, J.V., Brandts, C., Cox, J., Reddy, P.N.G., Böhmer, F.D., Gerke, V., Schmidt-Arras, D.-E., Berdel, W.E., Müller-Tidow, C., Mann, M., Serve, H., 2009. Mislocalized Activation of Oncogenic RTKs Switches Downstream Signaling Outcomes. Molecular Cell 36, 326–339. doi:10.1016/j.molcel.2009.09.019

Ciccia, A., Elledge, S.J., 2010. The DNA damage response: making it safe to play with knives. Mol. Cell 40, 179–204. doi:10.1016/j.molcel.2010.09.019
Clayton, A.H.A., Orchard, S.G., Nice, E.C., Posner, R.G., Burgess, A.W., 2008. Predominance of activated EGFR higher-order oligomers on the cell surface. Growth Factors 26, 316–324. doi:10.1080/08977190802442187

Clayton, A.H.A., Walker, F., Orchard, S.G., Henderson, C., Fuchs, D., Rothacker, J., Nice, E.C., Burgess, A.W., 2005. Ligand-induced dimer-tetramer transition during the activation of the cell surface epidermal growth factor receptor-A multidimensional microscopy analysis. J. Biol. Chem. 280, 30392–30399. doi:10.1074/jbc.M504770200

Collot, M., Louvard, D., Singer, S.J., 1984. Lysosomes are associated with microtubules and not with intermediate filaments in cultured fibroblasts. Proc Natl Acad Sci U S A 81, 788–792.

Courtney, K.D., Corcoran, R.B., Engelman, J.A., 2010. The PI3K pathway as drug target in human cancer. J. Clin. Oncol. 28, 1075–1083. doi:10.1200/JCO.2009.25.3641

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., Hemmings, B.A., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785–789. doi:10.1038/378785a0

Currie, R.A., Walker, K.S., Gray, A., Deak, M., Casamayor, A., Downes, C.P., Cohen, P., Alessi, D.R., Lucocq, J., 1999. Role of phosphatidylinositol 3,4,5-trisphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1. Biochem. J. 337 (Pt 3), 575–583.

Dai, T., Zhang, D., Cai, M., Wang, C., Wu, Z., Ying, Z., Wu, J., Li, M., Xie, D., Li, J., Song, L., 2015. Golgi phosphoprotein 3 (GOLPH3) promotes hepatocellular carcinoma cell aggressiveness by activating the NF-κB pathway. J. Pathol. 235, 490–501. doi:10.1002/path.4479

Danen, E.H.J., Rheenen, J. van, Franken, W., Huveneers, S., Sonneveld, P., Jalink, K., Sonnenberg, A., 2005. Integrins control motile strategy through a Rho–cofilin pathway. J Cell Biol 169, 515–526. doi:10.1083/jcb.200412081

Dennis, P.B., Pullen, N., Kozma, S.C., Thomas, G., 1996. The principal rapamycin-sensitive p70(s6k) phosphorylation sites, T-229 and T-389, are differentially regulated by rapamycin-insensitive kinase kinases. Mol. Cell. Biol. 16, 6242–6251.

Dippold, H.C., Ng, M.M., Farber-Katz, S.E., Lee, S.-K., Kerr, M.L., Peterman, M.C., Sim, R., Wiharto, P.A., Galbraith, K.A., Madhavarapu, S., Fuchs, G.J., Meerloo, T., Farquhar, M.G., Zhou, H., Field, S.J., 2009. GOLPH3 bridges phosphatidylinositol-4- phosphate and actomyosin to stretch and shape the Golgi to promote budding. Cell 139, 337–351. doi:10.1016/j.cell.2009.07.052

Downward, J., 2003. Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer 3, 11–22. doi:10.1038/nrc969

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., Waterfield, M.D., 1984. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature 307, 521–527. doi:10.1038/307521a0

Egea, G., Lázaro-Diéguez, F., Vilella, M., 2006. Actin dynamics at the Golgi complex in mammalian cells. Curr. Opin. Cell Biol. 18, 168–178. doi:10.1016/j.ceb.2006.02.007

Eisenberg, S., Henis, Y.I., 2008. Interactions of Ras proteins with the plasma membrane and their roles in signaling. Cell. Signal. 20, 31–39. doi:10.1016/j.cellsig.2007.07.012

Etienne-Manneville, S., Hall, A., 2001. Integrin-Mediated Activation of Cdc42 Controls Cell Polarity in Migrating Astrocytes through PKCζ. Cell 106, 489–498. doi:10.1016/S0092-8674(01)00471-8

Farber-Katz, S.E., Dippold, H.C., Buschman, M.D., Peterman, M.C., Xing, M., Noakes, C.J., Tat, J., Ng, M.M., Rahajeng, J., Cowan, D.M., Fuchs, G.J., Zhou, H., Field, S.J., 2014. DNA damage triggers Golgi dispersal via DNA-PK and GOLPH3. Cell 156, 413–427. doi:10.1016/j.cell.2013.12.023

Farhan, H., Wendeler, M.W., Mitrovic, S., Fava, E., Silberberg, Y., Sharan, R., Zerial, M., Hauri, H.-P., 2010. MAPK signaling to the early secretory pathway revealed by kinase/phosphatase functional screening. J Cell Biol 189, 997–1011. doi:10.1083/jcb.200912082

Farquhar, M.G., Palade, G.E., 1981. The Golgi apparatus (complex)-(1954-1981)-from artifact to center stage. J Cell Biol 91, 77s–103s.

Friedl, P., Wolf, K., 2003. Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer 3, 362–374. doi:10.1038/nrc1075

Gabay, M., Li, Y., Felsher, D.W., 2014. MYC Activation Is a Hallmark of Cancer Initiation and Maintenance. Cold Spring Harb Perspect Med 4, a014241. doi:10.1101/cshperspect.a014241

Gadella, T.W., Jovin, T.M., 1995. Oligomerization of epidermal growth factor receptors on A431 cells studied by time-resolved fluorescence imaging microscopy. A stereochemical model for tyrosine kinase receptor activation. J. Cell Biol. 129, 1543–1558.

Gan, H.K., Cvrljevic, A.N., Johns, T.G., 2013. The epidermal growth factor receptor variant III (EGFRvIII): where wild things are altered. FEBS J 280, 5350–5370. doi:10.1111/febs.12393

Giudice, J., Barcos, L.S., Guaimas, F.F., Penas-Steinhardt, A., Giordano, L., Jares-Erijman, E.A., Leskow, F.C., 2013. Insulin and insulin like growth factor II endocytosis and signaling via insulin receptor B. Cell Communication and Signaling 11, 18. doi:10.1186/1478-811X-11-18

Godi, A., Di Campli, A., Konstantakopoulos, A., Di Tullio, G., Alessi, D.R., Kular, G.S., Daniele, T., Marra, P., Lucocq, J.M., De Matteis, M.A., 2004. FAPPs control Golgi-to-cell-surface membrane traffic by binding to ARF and PtdIns(4)P. Nat. Cell Biol. 6, 393–404. doi:10.1038/ncb1119

Godi, A., Pertile, P., Meyers, R., Marra, P., Di Tullio, G., Iurisci, C., Luini, A., Corda, D., De Matteis, M.A., 1999. ARF mediates recruitment of PtdIns-4-OH kinase-beta and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex. Nat. Cell Biol. 1, 280–287. doi:10.1038/12993

Goh, L.K., Sorkin, A., 2013. Endocytosis of Receptor Tyrosine Kinases. Cold Spring Harb Perspect Biol 5, a017459. doi:10.1101/cshperspect.a017459

Golgi, C., Lipsky, N.G., 1989. On the structure of nerve cells1. Journal of Microscopy 155, 3–7. doi:10.1111/j.1365-2818.1989.tb04294.x

Gschwind, A., Fischer, O.M., Ullrich, A., 2004. The discovery of receptor tyrosine kinases: targets for cancer therapy. Nat Rev Cancer 4, 361–370. doi:10.1038/nrc1360

Haj, F.G., Verveer, P.J., Squire, A., Neel, B.G., Bastiaens, P.I.H., 2002. Imaging Sites of Receptor Dephosphorylation by PTP1B on the Surface of the Endoplasmic Reticulum. Science 295, 1708–1711. doi:10.1126/science.1067566

Hallberg, B., Rayter, S.I., Downward, J., 1994. Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. J. Biol. Chem. 269, 3913–3916.

Hama, H., Schnieders, E.A., Thorner, J., Takemoto, J.Y., DeWald, D.B., 1999. Direct involvement of phosphatidylinositol 4-phosphate in secretion in the yeast Saccharomyces cerevisiae. J. Biol. Chem. 274, 34294–34300.

Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4.

Hanahan, D., Weinberg, R.A., 2000. The Hallmarks of Cancer. Cell 100, 57–70. doi:10.1016/S0092-8674(00)81683-9

Harada, A., Takei, Y., Kanai, Y., Tanaka, Y., Nonaka, S., Hirokawa, N., 1998. Golgi Vesiculation and Lysosome Dispersion in Cells Lacking Cytoplasmic Dynein. J Cell Biol 141, 51–59.

Hare, J.F., Taylor, K., 1991. Mechanisms of plasma membrane protein degradation: recycling proteins are degraded more rapidly than those confined to the cell surface. Proc. Natl. Acad. Sci. U.S.A. 88, 5902–5906.

Hresko, R.C., Mueckler, M., 2005. mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. J. Biol. Chem. 280, 40406–40416. doi:10.1074/jbc.M508361200

Hsieh, A.C., Liu, Y., Edlind, M.P., Ingolia, N.T., Janes, M.R., Sher, A., Shi, E.Y., Stumpf, C.R., Christensen, C., Bonham, M.J., Wang, S., Ren, P., Martin, M., Jessen, K., Feldman, M.E., Weissman, J.S., Shokat, K.M., Rommel, C., Ruggero, D., 2012. The translational landscape of mTOR signalling steers cancer initiation and metastasis. Nature 485, 55–61. doi:10.1038/nature10912

Huang, H.S., Nagane, M., Klingbeil, C.K., Lin, H., Nishikawa, R., Ji, X.D., Huang, C.M., Gill, G.N., Wiley, H.S., Cavenee, W.K., 1997. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. J. Biol. Chem. 272, 2927–2935.

Huang, J., Manning, B.D., 2009. A complex interplay between Akt, TSC2, and the two mTOR complexes. Biochem Soc Trans 37, 217–222. doi:10.1042/BST0370217

Hua, X., Yu, L., Pan, W., Huang, X., Liao, Z., Xian, Q., Fang, L., Shen, H., 2012. Increased expression of Golgi phosphoprotein-3 is associated with tumor aggressiveness and poor prognosis of prostate cancer. Diagn Pathol 7, 127. doi:10.1186/1746-1596-7-127

Hubbard, S.R., 2004. Juxtamembrane autoinhibition in receptor tyrosine kinases. Nat. Rev. Mol. Cell Biol. 5, 464–471. doi:10.1038/nrm1399

Hu, B.-S., Hu, H., Zhu, C.-Y., Gu, Y.-L., Li, J.-P., 2013. Overexpression of GOLPH3 is associated with poor clinical outcome in gastric cancer. Tumour Biol. 34, 515–520. doi:10.1007/s13277-012-0576-z

Hu, G.-S., Li, Y.-Q., Yang, Y.-M., Shi, W., Liao, A.-J., Yao, Y.-H., Zeng, B., Yuan, J., 2014. High expression of Golgi phosphoprotein-3 is associated with poor survival in patients with hepatocellular carcinoma. Tumour Biol. 35, 8625–8632. doi:10.1007/s13277-014-2105-8

Inoki, K., Li, Y., Zhu, T., Wu, J., Guan, K.-L., 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat. Cell Biol. 4, 648–657. doi:10.1038/ncb839

Insall, R.H., Machesky, L.M., 2009. Actin dynamics at the leading edge: from simple machinery to complex networks. Dev. Cell 17, 310–322. doi:10.1016/j.devcel.2009.08.012

Isaji, T., Im, S., Gu, W., Wang, Y., Hang, Q., Lu, J., Fukuda, T., Hashii, N., Takakura, D., Kawasaki, N., Miyoshi, H., Gu, J., 2014. An oncogenic protein Golgi phosphoprotein 3 up-regulates cell migration via sialylation. J. Biol. Chem. 289, 20694–20705. doi:10.1074/jbc.M113.542688

Isotani, S., Hara, K., Tokunaga, C., Inoue, H., Avruch, J., Yonezawa, K., 1999. Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase alpha in vitro. J. Biol. Chem. 274, 34493–34498.

JianXin, J., Cha, Y., ZhiPeng, L., Jie, X., Hao, Z., Meiyuan, C., ChengYi, S., 2014. GOLP3 is a predictor of survival in patients with hepatocellular carcinoma. Clin Invest Med 37, E233.

Johnson, G.L., Vaillancourt, R.R., 1994. Sequential protein kinase reactions controlling cell growth and differentiation. Curr. Opin. Cell Biol. 6, 230–238.

Keating, M.T., Williams, L.T., 1987. Processing of the platelet-derived growth factor receptor. Biosynthetic and degradation studies using anti-receptor antibodies. J. Biol. Chem. 262, 7932–7937.

Kim, H.H., Sierke, S.L., Koland, J.G., 1994. Epidermal growth factor-dependent association of phosphatidylinositol 3-kinase with the erbB3 gene product. J. Biol. Chem. 269, 24747–24755.

Kobayashi, S., Morimoto, K., Shimizu, T., Takahashi, M., Kurosawa, H., Shirasawa, T., 2000. Association of EXT1 and EXT2, Hereditary Multiple Exostoses Gene Products, in Golgi Apparatus. Biochemical and Biophysical Research Communications 268, 860–867. doi:10.1006/bbrc.2000.2219

Kovacina, K.S., Park, G.Y., Bae, S.S., Guzzetta, A.W., Schaefer, E., Birnbaum, M.J., Roth, R.A., 2003. Identification of a proline-rich Akt substrate as a 14-3-3 binding partner. J. Biol. Chem. 278, 10189–10194. doi:10.1074/jbc.M210837200

Krause, M., Gautreau, A., 2014. Steering cell migration: lamellipodium dynamics and the regulation of directional persistence. Nat Rev Mol Cell Biol 15, 577–590. doi:10.1038/nrm3861

Kunigou, O., Nagao, H., Kawabata, N., Ishidou, Y., Nagano, S., Maeda, S., Komiya, S., Setoguchi, T., 2011. Role of GOLPH3 and GOLPH3L in the proliferation of human rhabdomyosarcoma. Oncol. Rep. 26, 1337–1342. doi:10.3892/or.2011.1413

Kupfer, A., Louvard, D., Singer, S.J., 1982. Polarization of the Golgi apparatus and the microtubule-organizing center in cultured fibroblasts at the edge of an experimental wound. PNAS 79, 2603–2607.

Laplante, M., Sabatini, D.M., 2009. mTOR signaling at a glance. J Cell Sci 122, 3589–3594. doi:10.1242/jcs.051011

Lauffenburger, D.A., Horwitz, A.F., 1996. Cell Migration: A Physically Integrated Molecular Process. Cell 84, 359–369. doi:10.1016/S0092-8674(00)81280-5

Lee, M.J., Ye, A.S., Gardino, A.K., Heijink, A.M., Sorger, P.K., MacBeath, G., Yaffe, M.B., 2012. Sequential application of anticancer drugs enhances cell death by rewiring apoptotic signaling networks. Cell 149, 780–794. doi:10.1016/j.cell.2012.03.031

Lefloch, R., Pouysségur, J., Lenormand, P., 2009. Total ERK1/2 activity regulates cell proliferation. Cell Cycle 8, 705–711. doi:10.4161/cc.8.5.7734

Lemmon, M.A., Schlessinger, J., 2010. Cell signaling by receptor tyrosine kinases. Cell 141, 1117–1134. doi:10.1016/j.cell.2010.06.011

Levis, M., Small, D., 2003. FLT3: ITDoes matter in. Leukemia 17, 1738–1752. doi:10.1038/sj.leu.2403099

Li, H., Guo, L., Chen, S.-W., Zhao, X.-H., Zhuang, S.-M., Wang, L.-P., Song, L.-B., Song, M., 2012. GOLPH3 overexpression correlates with tumor progression and poor prognosis in patients with clinically N0 oral tongue cancer. J Transl Med 10, 168. doi:10.1186/1479-5876-10-168

Li, H., Meng, F., Ma, J., Yu, Y., Hua, X., Qin, J., Li, Y., 2014. Insulin receptor substrate-1 and Golgi phosphoprotein 3 are downstream targets of miR‐126 in esophageal squamous cell carcinoma. Oncol. Rep. 32, 1225–1233. doi:10.3892/or.2014.3327

Lim, J., Zhou, M., Veenstra, T.D., Morrison, D.K., 2010. The CNK1 scaffold binds cytohesins and promotes insulin pathway signaling. Genes Dev. 24, 1496–1506. doi:10.1101/gad.1904610

Li, R., Gundersen, G.G., 2008. Beyond polymer polarity: how the cytoskeleton builds a polarized cell. Nat. Rev. Mol. Cell Biol. 9, 860–873. doi:10.1038/nrm2522

Li, X.-Y., Liu, W., Chen, S.-F., Zhang, L.-Q., Li, X.-G., Wang, L.-X., 2011. Expression of the Golgi phosphoprotein-3 gene in human gliomas: a pilot study. J. Neurooncol. 105, 159–163. doi:10.1007/s11060-011-0573-x

Llopis, J., McCaffery, J.M., Miyawaki, A., Farquhar, M.G., Tsien, R.Y., 1998. Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. PNAS 95, 6803–6808.

Lu, M., Tian, Y., Yue, W.-M., Li, L., Li, S.-H., Qi, L., Hu, W.-S., Gao, C., Si, L.-B., Tian, H., 2014. GOLPH3, a Good Prognostic Indicator in Early-stage NSCLC Related to Tumor Angiogenesis. Asian Pac. J. Cancer Prev. 15, 5793–5798.

Manning, B.D., Cantley, L.C., 2007. AKT/PKB Signaling: Navigating Downstream. Cell 129, 1261–1274. doi:10.1016/j.cell.2007.06.009

Manning, B.D., Cantley, L.C., 2003. Rheb fills a GAP between TSC and TOR. Trends in Biochemical Sciences 28, 573–576. doi:10.1016/j.tibs.2003.09.003

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., Cantley, L.C., 2002. Identification of the tuberous sclerosis complex-2 tumor suppressor gene

product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol. Cell 10, 151–162.

Matteoni, R., Kreis, T.E., 1987. Translocation and clustering of endosomes and lysosomes depends on microtubules. J. Cell Biol. 105, 1253–1265.

Ma, Y., Ren, Y., Zhang, X., Lin, L., Liu, Y., Rong, F., Wen, W., Li, F., 2014a. High GOLPH3 expression is associated with a more aggressive behavior of epithelial ovarian carcinoma. Virchows Arch. 464, 443–452. doi:10.1007/s00428-014-1536-3

Ma, Y., Wang, X., Wu, Y., Sun, B., Lv, H., Rong, F., Zheng, X., 2014b. Overexpression of GOLPH3 protein is associated with worse prognosis in patients with epithelial ovarian cancer. Tumor Biol. 35, 11845–11849. doi:10.1007/s13277-014-2411-1

McCubrey, J.A., Steelman, L.S., Chappell, W.H., Abrams, S.L., Wong, E.W., Chang, F., Lehmann, B., Terrian, D.M., Milella, M., Tafuri, A., Stivala, F., Libra, M., Basecke, J., Evangelisti, C., Martelli, A.M., Franklin, R.A., 2007. ROLES OF THE RAF/MEK/ERK PATHWAY IN CELL GROWTH, MALIGNANT TRANSFORMATION AND DRUG RESISTANCE. Biochim Biophys Acta 1773, 1263–1284. doi:10.1016/j.bbamcr.2006.10.001

McKeon, C., Moncada, V., Pham, T., Salvatore, P., Kadowaki, T., Accili, D., Taylor, S.I., 1990. Structural and functional analysis of the insulin receptor promoter. Mol. Endocrinol. 4, 647–656. doi:10.1210/mend-4-4-647

Mercer, K., Chiloeches, A., Hüser, M., Kiernan, M., Marais, R., Pritchard, C., 2002. ERK signalling and oncogene transformation are not impaired in cells lacking A-Raf. Oncogene 21, 347–355. doi:10.1038/sj.onc.1205101

Millarte, V., Farhan, H., 2012. The Golgi in cell migration: regulation by signal transduction and its implications for cancer cell metastasis. ScientificWorldJournal 2012, 498278. doi:10.1100/2012/498278

Morgenstern, J.P., Land, H., 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. 18, 3587–3596.

Moriki, T., Maruyama, H., Maruyama, I.N., 2001. Activation of preformed EGF receptor dimers by ligand-induced rotation of the transmembrane domain. J. Mol. Biol. 311, 1011–1026. doi:10.1006/jmbi.2001.4923

Mrowka, R., Patzak, A., Herzel, H., 2001. Is There a Bias in Proteome Research? Genome Res. 11, 1971–1973. doi:10.1101/gr.206701

Ng, M.M., Dippold, H.C., Buschman, M.D., Noakes, C.J., Field, S.J., 2013. GOLPH3L antagonizes GOLPH3 to determine Golgi morphology. Mol. Biol. Cell 24, 796–808. doi:10.1091/mbc.E12-07-0525

Niederst, M.J., Engelman, J.A., 2013. Bypass Mechanisms of Resistance to Receptor Tyrosine Kinase Inhibition in Lung Cancer. Sci. Signal. 6, re6. doi:10.1126/scisignal.2004652

Nobes, C.D., Hall, A., 1999. Rho GTPases control polarity, protrusion, and adhesion during cell movement. J. Cell Biol. 144, 1235–1244.

Normanno, N., De Luca, A., Bianco, C., Strizzi, L., Mancino, M., Maiello, M.R., Carotenuto, A., De Feo, G., Caponigro, F., Salomon, D.S., 2006. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene 366, 2–16. doi:10.1016/j.gene.2005.10.018

Pawson, T., 2004. Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. Cell 116, 191–203.

Payne, D.M., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J.H., Shabanowitz, J., Hunt, D.F., Weber, M.J., Sturgill, T.W., 1991. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J 10, 885–892.

Peng, J., Fang, Y., Tao, Y., Li, K., Su, T., Nong, Y., Xie, F., Lai, M., 2014. Mechanisms of GOLPH3 Associated with the Progression of Gastric Cancer: A Preliminary Study. PLoS ONE 9, e107362. doi:10.1371/journal.pone.0107362

Peter, B.J., Kent, H.M., Mills, I.G., Vallis, Y., Butler, P.J.G., Evans, P.R., McMahon, H.T., 2004. BAR Domains as Sensors of Membrane Curvature: The Amphiphysin BAR Structure. Science 303, 495–499. doi:10.1126/science.1092586

Pietras, K., Pahler, J., Bergers, G., Hanahan, D., 2008. Functions of Paracrine PDGF Signaling in the Proangiogenic Tumor Stroma Revealed by Pharmacological Targeting. PLoS Med 5, e19. doi:10.1371/journal.pmed.0050019

Presley, J.F., Cole, N.B., Schroer, T.A., Hirschberg, K., Zaal, K.J., Lippincott-Schwartz, J., 1997. ER-to-Golgi transport visualized in living cells. Nature 389, 81–85. doi:10.1038/38001

Puthenveedu, M.A., Linstedt, A.D., 2005. Subcompartmentalizing the Golgi apparatus. Curr. Opin. Cell Biol. 17, 369–375. doi:10.1016/j.ceb.2005.06.006

Ram, T.G., Ethier, S.P., 1996. Phosphatidylinositol 3-kinase recruitment by p185erbB-2 and erbB-3 is potently induced by neu differentiation factor/heregulin during mitogenesis and is constitutively elevated in growth factor-independent breast carcinoma cells with c-erbB-2 gene amplification. Cell Growth Differ. 7, 551–561.

Ridley, A.J., 2011. Life at the leading edge. Cell 145, 1012–1022. doi:10.1016/j.cell.2011.06.010

Rogalski, A.A., Singer, S.J., 1984. Associations of elements of the Golgi apparatus with microtubules. J. Cell Biol. 99, 1092–1100.

Romanuik, T.L., Wang, G., Holt, R.A., Jones, S.J., Marra, M.A., Sadar, M.D., 2009. Identification of novel androgen-responsive genes by sequencing of LongSAGE libraries. BMC Genomics 10, 476. doi:10.1186/1471-2164-10-476

Roskoski, R., 2012. ERK1/2 MAP kinases: structure, function, and regulation. Pharmacol. Res. 66, 105–143. doi:10.1016/j.phrs.2012.04.005

Sako, Y., Minoghchi, S., Yanagida, T., 2000. Single-molecule imaging of EGFR signalling on the surface of living cells. Nat. Cell Biol. 2, 168–172. doi:10.1038/35004044

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., Sabatini, D.M., 2007. PRAS40 Is an Insulin-Regulated Inhibitor of the mTORC1 Protein Kinase. Molecular Cell 25, 903–915. doi:10.1016/j.molcel.2007.03.003

Sandoval, I.V., Bonifacino, J.S., Klausner, R.D., Henkart, M., Wehland, J., 1984. Role of microtubules in the organization and localization of the Golgi apparatus. J Cell Biol 99, 113s–118s.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.-H., Hsu, P.P., Bagley, A.F., Markhard, A.L., Sabatini, D.M., 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol. Cell 22, 159–168. doi:10.1016/j.molcel.2006.03.029

Sarbassov, D.D., Guertin, D.A., Ali, S.M., Sabatini, D.M., 2005. Phosphorylation and Regulation of Akt/PKB by the Rictor-mTOR Complex. Science 307, 1098–1101. doi:10.1126/science.1106148

Sasaoka, T., Langlois, W.J., Leitner, J.W., Draznin, B., Olefsky, J.M., 1994. The signaling pathway coupling epidermal growth factor receptors to activation of p21ras. J. Biol. Chem. 269, 32621–32625.

Scheel, J., Matteoni, R., Ludwig, T., Hoflack, B., Kreis, T.E., 1990. Microtubule depolymerization inhibits transport of cathepsin D from the Golgi apparatus to lysosomes. J. Cell. Sci. 96 (Pt 4), 711–720.

Schlessinger, J., 2002. Ligand-Induced, Receptor-Mediated Dimerization and Activation of EGF Receptor. Cell 110, 669–672. doi:10.1016/S0092-8674(02)00966-2

Schlessinger, J., Lemmon, M.A., 2003. SH2 and PTB domains in tyrosine kinase signaling. Sci. STKE 2003, RE12. doi:10.1126/stke.2003.191.re12

Schmitz, K.R., Liu, J., Li, S., Setty, T.G., Wood, C.S., Burd, C.G., Ferguson, K.M., 2008. Golgi Localization of Glycosyltransferases Requires a Vps74p Oligomer. Developmental Cell 14, 523–534. doi:10.1016/j.devcel.2008.02.016

Scott, K.L., Chin, L., 2010. Signaling from the Golgi: mechanisms and models for Golgi phosphoprotein 3-mediated oncogenesis. Clin. Cancer Res. 16, 2229–2234. doi:10.1158/1078-0432.CCR-09-1695

Scott, K.L., Kabbarah, O., Liang, M.-C., Ivanova, E., Anagnostou, V., Wu, J., Dhakal, S., Wu, M., Chen, S., Feinberg, T., Huang, J., Saci, A., Widlund, H.R., Fisher, D.E., Xiao, Y., Rimm, D.L., Protopopov, A., Wong, K.-K., Chin, L., 2009. GOLPH3 modulates mTOR signalling and rapamycin sensitivity in cancer. Nature 459, 1085–1090. doi:10.1038/nature08109

Shattuck, D.L., Miller, J.K., Carraway, K.L., Sweeney, C., 2008. Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. Cancer Res. 68, 1471–1477. doi:10.1158/0008-5472.CAN-07-5962
Sherr, C.J., 1996. Cancer cell cycles. Science 274, 1672–1677.

Short, B., Haas, A., Barr, F.A., 2005. Golgins and GTPases, giving identity and structure to the Golgi apparatus. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, The Golgi Complex 1744, 383–395. doi:10.1016/j.bbamcr.2005.02.001

Soltoff, S.P., Cantley, L.C., 1996. p120cbl is a cytosolic adapter protein that associates with phosphoinositide 3-kinase in response to epidermal growth factor in PC12 and other cells. J. Biol. Chem. 271, 563–567.

Sousa, L.P., Lax, I., Shen, H., Ferguson, S.M., De Camilli, P., Schlessinger, J., 2012. Suppression of EGFR endocytosis by dynamin depletion reveals that

EGFR signaling occurs primarily at the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. 109, 4419–4424. doi:10.1073/pnas.1200164109

Stephen, A.G., Esposito, D., Bagni, R.K., McCormick, F., 2014. Dragging Ras Back in the Ring. Cancer Cell 25, 272–281. doi:10.1016/j.ccr.2014.02.017

Stommel, J.M., Kimmelman, A.C., Ying, H., Nabioullin, R., Ponugoti, A.H., Wiedemeyer, R., Stegh, A.H., Bradner, J.E., Ligon, K.L., Brennan, C., Chin, L., DePinho, R.A., 2007. Coactivation of Receptor Tyrosine Kinases Affects the Response of Tumor Cells to Targeted Therapies. Science 318, 287–290. doi:10.1126/science.1142946

Stover, D.R., Becker, M., Liebetanz, J., Lydon, N.B., 1995. Src phosphorylation of the epidermal growth factor receptor at novel sites mediates receptor interaction with Src and P85 alpha. J. Biol. Chem. 270, 15591–15597.

Swain, S.M., 2011. Chemotherapy: updates and new perspectives. Oncologist 16 Suppl 1, 30–39. doi:10.1634/theoncologist.2011-S1-30

Taft, M.H., Behrmann, E., Munske-Weidemann, L.-C., Thiel, C., Raunser, S., Manstein, D.J., 2013. Functional characterization of human myosin-18A and its interaction with F-actin and GOLPH3. J. Biol. Chem. 288, 30029–30041. doi:10.1074/jbc.M113.497180

Tee, A.R., Manning, B.D., Roux, P.P., Cantley, L.C., Blenis, J., 2003. Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr. Biol. 13, 1259–1268.

Thyberg, J., Moskalewski, S., 1999. Role of Microtubules in the Organization of the Golgi Complex. Experimental Cell Research 246, 263–279. doi:10.1006/excr.1998.4326

Tokuda, E., Itoh, T., Hasegawa, J., Ijuin, T., Takeuchi, Y., Irino, Y., Fukumoto, M., Takenawa, T., 2014. Phosphatidylinositol 4-phosphate in the Golgi apparatus regulates cell-cell adhesion and invasive cell migration in human breast cancer. Cancer Res. 74, 3054–3066. doi:10.1158/0008-5472.CAN-13-2441

Tseng, C.-N., Hong, Y.-R., Chang, H.-W., Yu, T.-J., Hung, T.-W., Hou, M.-F., Yuan, S.-S.F., Cho, C.-L., Liu, C.-T., Chiu, C.-C., Huang, C.-J., 2014. Brefeldin A reduces anchorage-independent survival, cancer stem cell potential and migration of MDA-MB-231 human breast cancer cells. Molecules 19, 17464–17477. doi:10.3390/molecules191117464

Tu, L., Tai, W.C.S., Chen, L., Banfield, D.K., 2008. Signal-Mediated Dynamic Retention of Glycosyltransferases in the Golgi. Science 321, 404–407. doi:10.1126/science.1159411

Van Belzen, N., Rijken, P.J., Hage, W.J., de Laat, S.W., Verkleij, A.J., Boonstra, J., 1988. Direct visualization and quantitative analysis of epidermal growth factor-induced receptor clustering. J. Cell. Physiol. 134, 413–420. doi:10.1002/jcp.1041340312

Velu, T.J., Beguinot, L., Vass, W.C., Willingham, M.C., Merlino, G.T., Pastan, I., Lowy, D.R., 1987. Epidermal-growth-factor-dependent transformation by a human EGF receptor proto-oncogene. Science 238, 1408–1410.

Vivanco, I., Sawyers, C.L., 2002. The phosphatidylinositol 3-Kinase–AKT pathway in human cancer. Nat Rev Cancer 2, 489–501. doi:10.1038/nrc839

Walch-Solimena, C., Novick, P., 1999. The yeast phosphatidylinositol-4-OH kinase pik1 regulates secretion at the Golgi. Nat. Cell Biol. 1, 523–525. doi:10.1038/70319

Wang, J.-H., Chen, X.-T., Wen, Z.-S., Zheng, M., Deng, J.-M., Wang, M.-Z., Lin, H.-X., Chen, K., Li, J., Yun, J.-P., Luo, R.-Z., Song, L.-B., 2012. High expression of GOLPH3 in esophageal squamous cell carcinoma correlates with poor prognosis. PLoS ONE 7, e45622. doi:10.1371/journal.pone.0045622

Wang, L., Harris, T.E., Roth, R.A., Lawrence, J.C., 2007. PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. J. Biol. Chem. 282, 20036–20044. doi:10.1074/jbc.M702376200

Wang, Y.J., Wang, J., Sun, H.Q., Martinez, M., Sun, Y.X., Macia, E., Kirchhausen, T., Albanesi, J.P., Roth, M.G., Yin, H.L., 2003. Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. Cell 114, 299–310.

Wang, Z., Jiang, B., Chen, L., Di, J., Cui, M., Liu, M., Ma, Y., Yang, H., Xing, J., Zhang, C., Yao, Z., Zhang, N., Dong, B., Ji, J., Su, X., 2014. GOLPH3 predicts survival of colorectal cancer patients treated with 5-fluorouracil-based adjuvant chemotherapy. J Transl Med 12, 15. doi:10.1186/1479-5876-12-15

Wennerberg, K., Rossman, K.L., Der, C.J., 2005. The Ras superfamily at a glance. J Cell Sci 118, 843–846. doi:10.1242/jcs.01660

Whitson, K.B., Beechem, J.M., Beth, A.H., Staros, J.V., 2004. Preparation and characterization of Alexa Fluor 594-labeled epidermal growth factor for

fluorescence resonance energy transfer studies: application to the epidermal growth factor receptor. Analytical Biochemistry 324, 227–236. doi:10.1016/j.ab.2003.09.023

Wiley, H.S., 1988. Anomalous binding of epidermal growth factor to A431 cells is due to the effect of high receptor densities and a saturable endocytic system. J. Cell Biol. 107, 801–810.

Wiley, H.S., Herbst, J.J., Walsh, B.J., Lauffenburger, D.A., Rosenfeld, M.G., Gill, G.N., 1991. The role of tyrosine kinase activity in endocytosis, compartmentation, and down-regulation of the epidermal growth factor receptor. J. Biol. Chem. 266, 11083–11094.

Wu, C.C., Taylor, R.S., Lane, D.R., Ladinsky, M.S., Weisz, J.A., Howell, K.E., 2000. GMx33: A novel family of trans-golgi proteins identified by proteomics. Traffic 1, 963–975.

Xie, X., Guan, K.-L., 2011. The ribosome and TORC2: collaborators for cell growth. Cell 144, 640–642. doi:10.1016/j.cell.2011.02.029

Xue, Y., Wu, G., Liao, Y., Xiao, G., Ma, X., Zou, X., Zhang, G., Xiao, R., Wang, X., Liu, Q., Long, D., Yang, J., Xu, H., Liu, F., Liu, M., Xie, K., Huang, R., 2014. GOLPH3 is a novel marker of poor prognosis and a potential therapeutic target in human renal cell carcinoma. Br. J. Cancer 110, 2250–2260. doi:10.1038/bjc.2014.124

Yadav, S., Puri, S., Linstedt, A.D., 2009. A Primary Role for Golgi Positioning in Directed Secretion, Cell Polarity, and Wound Healing. Mol. Biol. Cell 20, 1728–1736. doi:10.1091/mbc.E08-10-1077

Yang, W., Pepperkok, R., Bender, P., Kreis, T.E., Storrie, B., 1996. Modification of the cytoplasmic domain affects the subcellular localization of Golgi glycosyl-transferases. Eur. J. Cell Biol. 71, 53–61.

Yarden, Y., Schlessinger, J., 1987. Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. Biochemistry 26, 1443–1451.

Yuan, T.L., Cantley, L.C., 2008. PI3K pathway alterations in cancer: variations on a theme. Oncogene 27, 5497–5510. doi:10.1038/onc.2008.245

Yu, X., Sharma, K.D., Takahashi, T., Iwamoto, R., Mekada, E., 2002. Ligand-independent dimer formation of epidermal growth factor receptor (EGFR) is a step separable from ligand-induced EGFR signaling. Mol. Biol. Cell 13, 2547–2557. doi:10.1091/mbc.01-08-0411

Zeng, Z., Lin, H., Zhao, X., Liu, G., Wang, X., Xu, R., Chen, K., Li, J., Song, L., 2012. Overexpression of GOLPH3 promotes proliferation and tumorigenicity in breast cancer via suppression of the FOXO1 transcription factor. Clin. Cancer Res. 18, 4059–4069. doi:10.1158/1078-0432.CCR-11-3156

Zhang, L.-J., Wang, K.-B., Liu, L.-S., Chen, L.-Z., Peng, B.-G., Liang, L.-J., Li, Z., Xue, L., Li, W., Xia, J.-T., 2014. Overexpression of GOLPH3 is associated with poor prognosis and clinical progression in pancreatic ductal adenocarcinoma. BMC Cancer 14, 571. doi:10.1186/1471-2407-14-571

Zhang, X., Ding, Z., Mo, J., Sang, B., Shi, Q., Hu, J., Xie, S., Zhan, W., Lu, D., Yang, M., Bian, W., Zhou, X., Yu, R., 2014. GOLPH3 promotes glioblastoma cell migration and invasion via the mTOR-YB1 pathway in vitro. Mol. Carcinog. doi:10.1002/mc.22197

Zhang, Y., Ma, M., Han, B., 2014. GOLPH3 high expression predicts poor prognosis in patients with resected non-small cell lung cancer: an immunohistochemical analysis. Tumour Biol. 35, 10833–10839. doi:10.1007/s13277-014-2357-3

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., Zheng, P., Ye, K., Chinnaiyan, A., Halder, G., Lai, Z.-C., Guan, K.-L., 2007. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev. 21, 2747–2761. doi:10.1101/gad.1602907

Zhao, L., Vogt, P.K., 2008. Class I PI3K in oncogenic cellular transformation. Oncogene 27, 5486–5496. doi:10.1038/onc.2008.244

Zhou, J., Xu, T., Qin, R., Yan, Y., Chen, C., Chen, Y., Yu, H., Xia, C., Lu, Y., Ding, X., Wang, Y., Cai, X., Chen, J., 2012. Overexpression of Golgi phosphoprotein-3 (GOLPH3) in glioblastoma multiforme is associated with worse prognosis. J. Neurooncol. 110, 195–203. doi:10.1007/s11060-012-0970-9

Zhou, X., Xue, P., Yang, M., Shi, H., Lu, D., Wang, Z., Shi, Q., Hu, J., Xie, S., Zhan, W., Yu, R., 2014. Protein kinase D2 promotes the proliferation of glioma cells by regulating Golgi phosphoprotein 3. Cancer Letters 355, 121–129. doi:10.1016/j.canlet.2014.09.008

Zhou, X., Zhan, W., Bian, W., Hua, L., Shi, Q., Xie, S., Yang, D., Li, Y., Zhang, X., Liu, G., Yu, R., 2013. GOLPH3 regulates the migration and invasion of glioma cells though RhoA. Biochem. Biophys. Res. Commun. 433, 338–344. doi:10.1016/j.bbrc.2013.03.003

Zinzalla, V., Stracka, D., Oppliger, W., Hall, M.N., 2011. Activation of mTORC2 by association with the ribosome. Cell 144, 757–768. doi:10.1016/j.cell.2011.02.014

Zoncu, R., Efeyan, A., Sabatini, D.M., 2011. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat. Rev. Mol. Cell Biol. 12, 21–35. doi:10.1038/nrm3025