Université de Montréal

Implicating the mechanisms of ADP-ribosylation factor activation in the resistance of invasive breast cancer cells to EGFR tyrosine kinase inhibitors

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Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de PhD
en Pharmacologie

March 2015

Université de Montréal

Faculté des études supérieures

Cette thèse intitulée :

p66Shc-mediated ARF1 activation in invasive breast cancer cells: implications in EGFR tyrosine kinase inhibitor resistance

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Résumé

ADP-ribosylation factor-1 (ARF1) est une petite GTPase principalement connue pour son rôle dans la formation de vésicules au niveau de l'appareil de Golgi. Récemment, dans des cellules de cancer du sein, nous avons démontré qu'ARF1 est aussi un médiateur important de la signalisation du récepteur du facteur de croissance épidermique (EGFR) contrôlant la prolifération, la migration et l'invasion cellulaire. Cependant, le mécanisme par lequel l'EGFR active la GTPase ainsi que le rôle de cette dernière dans la régulation de la fonction du récepteur demeure inconnue. Dans cette thèse, nous avions comme objectifs de définir le mécanisme d'activation de ARF1 dans les cellules de cancer du sein hautement invasif et démontrer que l'activation de cette isoforme de ARF joue un rôle essentiel dans la résistance de ces cellules aux inhibiteurs de l'EGFR. Nos études démontrent que les protéines d'adaptatrices Grb2 et p66Shc jouent un rôle important dans l'activation de ARF1. Alors que Grb2 favorise le recrutement d'ARF1 à l'EGFR ainsi que l'activation de cette petite GTPase, p66Shc inhibe le recrutement du complexe Grb2-ARF1 au récepteur et donc contribue à limiter l'activation d'ARF1.

De plus, nous démontrons que ARF1 favorise la résistance aux inhibiteurs des tyrosines kinases dans les cellules de cancer du sein hautement invasif. En effet, une diminution de l'expression de ARF1 a augmenté la sensibilité des cellules aux inhibiteurs de l'EGFR. Nous montrons également que de hauts niveaux de ARF1 contribuent à la résistance des cellules à ces médicaments en améliorant la survie et les signaux prolifératifs à travers ERK1/2, Src et AKT, tout en bloquant les voies apoptotiques (p38MAPK et JNK). Enfin, nous mettons en évidence le rôle de la protéine ARF1 dans l'apoptose en réponse aux traitements des inhibiteurs de l'EGFR. Nos résultats indiquent que la dépletion d'ARF1 promeut la mort cellulaire induite par gefitinib, en augmentant l'expression de facteurs pro-apoptotiques (p66shc, Bax), en altérant le potentiel de la membrane mitochondriale et la libération du cytochrome C.

Ensemble, nos résultats délimitent un nouveau mécanisme d'activation de ARF1 dans les cellules du cancer du sein hautement invasif et impliquent l'activité d'ARF1 comme un médiateur important de la résistance aux inhibiteurs EGFR.

Mots-clés : ARF1, EGFR, p66Shc, Grb2, Inhibiteurs des tyrosine kinases, résistance

Abstract

The small GTPase ADP-ribosylation factor-1 (ARF1) has been well described for its role in regulating transport within the Golgi. Recently, in breast cancer cells, we have characterized ARF1 as important mediator of epidermal growth factor receptor (EGFR) signals leading to cell proliferation, migration and invasion. However, the mechanisms regulating ARF1 activity downstream of the EGFR had yet to be defined. Here, we aim to characterize these mechanisms of ARF1 activation in invasive breast cancer cells and demonstrate that activated ARF1 plays an essential role in mediating the resistance of breast cancer cells to EGFR tyrosine kinase inhibitors. We show that the adaptor proteins Grb2 and p66Shc regulate EGF-dependent ARF1 activation. While Grb2 was shown to be essential in the recruitment of ARF1 to the EGFR as well as the activation of this small GTPase, p66Shc blocked the recruitment of this Grb2-ARF1 complex to the receptor and thus suppressed EGF-induced ARF1 activation.

Additionally, we demonstrate that ARF1 promotes EGFR tyrosine kinase inhibitor resistance in invasive breast cancer cells. Indeed, the depletion of ARF1 was associated with an increased sensitivity to EGFR inhibition. We show that ARF1 promotes resistance by enhancing survival and proliferative signals through Erk1/2, Src and AKT, while blocking the apoptotic p38MAPK and JNK pathways. Furthermore, ARF1 was shown to stabilize EGFR dynamics (Expression, activation, dimerization and down-regulation) in response to treatment with EGFR inhibitors

Finally, we highlight the role of ARF1 in mediating mitochondrial-dependent apoptosis in response to EGFR tyrosine kinase inhibitor treatment. The depletion of ARF1 was shown to promote gefitinib-induced cell death as measured by increase expression of pro-apoptotic factors (p66Shc, Bax), altered mitochondrial membrane potential and cytochrome C release.

Together, our results delineate a novel mechanism of ARF1 activation in breast cancer cells and implicate ARF1 activity as an important mediator of EGFR inhibitor resistance further supporting the importance of targeting this GTPase in breast cancer patients.

Keywords: ARF1, EGFR, p66Shc, Grb2, EGFR tyrosine kinase inhibitors, resistance

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List of Abbreviations

AKT: Protein kinase B

APAF-1: Apoptotic protease activating factor-1

ARF: ADP-ribosylation-factor

ARFGAP: ARF GTPase-activating protein

ARFGEF: ARF guanine exchange factor

ATP: Adenosine triphosphate

AXL: AXL receptor tyrosine kinase

Bax: Bcl2-associated X protein

Bcl2: B-cell lymphoma 2

BFA: Brefeldin A

BH3: Bcl2-homology domain 2

Bim: Bcl2-like protein 11 (Bcl2L11)

CB: Cytochrome C binding domain

Cbl: Casitas B-lineage Lymphoma

CH: Collagen homology domain

cMET: Hepatocyte growth factor receptor (HGFR)

CytC: Cytochrome C

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EGFRTKi: EGFR tyrosine kinase inhibitor

EMT: Epithelial-mesenchymal transformation

ER: Estrogen receptor

ERK1/2: Extracellular signal-regulated kinase 1/2

ERMES: Endoplasmic reticulum-mitochondria encounter structure complex

FGFR: Fibroblast growth factor receptor

Gab1: Grb2-associated binding protein 1

GAP: GTPase activating protein

GDP: guanosine diphosphate

GEF: Guanine exchange factor

GGA3: Golgi-localized, gamma adaptin ear-containing, ARF-binding protein 3

Grb2: Growth factor receptor-binding protein 2

GTP: Guanosine triphosphate

HER2: Epidermal growth factor receptor-2 (ErbB2)

HER3: Epidermal growth factor receptor-3 (ErbB3)

HER4: Epidermal growth factor receptor-4 (ErbB4)

HRG: Heregulin

HSP70: Heat shock protein 70

IC_{50}: Half maximal inhibitory concentration

IGF1R: Insulin growth factor-1 receptor

JNK: c-Jun N-terminal kinase

Mab: Monoclonal antibody

MAPK: Mitogen-activated protein kinase

MEK: Mitogen-activated protein kinase kinase

mTor: mammalian target of rapamycin

MTT: 3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide

OMM: Outer mitochondrial membrane

PIP2: Phosphatidylinositol 4, 5-bisphosphate

PIP3: Phosphatidylinositol (3, 4, 5)-trisphosphate

PI3K: Phosphatidylinositol-4, 5-bisphosphate 3-kinase

PKC: Protein kinase C

PLD: Phospholipase D

PR: Progesterone receptor

PTEN: Phosphatase and tensin homolog

PTB: Protein tyrosine binding domain

PTPC: Permeability transition pore complex

PUMA: p53 upregulated modulator of apoptosis

p38MAPK: P38 mitogen-activated protein kinase

pY: phosphotyrosine residue

Raf: Rapidly accelerated fibrosarcoma kinase

Ras: Rat sarcoma protein

ROS: Reactive oxygen species

RTK: Receptor tyrosine kinase

Shc: SH2-containing protein

SH2: Src homology-2 domain

SH3: Src homology-3 domain

siRNA: Small interference RNA

SOS: Son of sevenless

Src: Src tyrosine kinase

STAT: Signal transducer and activator of transcription

VEGFR: Vascular endothelial growth factor receptor

I would like to dedicate this thesis to my wife Sevine
and our children Spencer and Logan

Acknowledgements

It has been a long journey filled with many ups and downs. But looking back, I regret none of it. I have had the opportunity to meet a lot of great people over the years. Some have come and gone but others I will consider good friends for the rest of my life. I would like to thank you all for your support, help, laughs and countless pints of beer. Cheers!

Firstly, I would like to thank Audrey for always being there for me to bounce ideas off of and for giving me the creative liberties to go off on tangents and seek answer that might have been a little outside of the realm of the laboratory. I thank you for your supervision and guidance and I'll do you proud in Boston. I would also like to thank my fellow lab members for being there to hear and discuss my sometime crazy scientific ideas, putting up with my not always enjoyable music selections and for making each day a little more entertaining.

Next, I would like to briefly thank some of the important people that have helped me get through this long journey: Dr. Giovanni Hernandez for taking me under his wing and introducing me to the pleasures of scientific research, the Ali Lab, especially Naila, Jenny and Noor for keeping me semi-sane, H5 and H7 for the many good times had, GLG communications – you may of worked me crazy but you significantly improved my writing skills and the Piekny lab – Paul, Madhav, Husni and Neetha you have become my best of friends. I raise my pint to you all. I would also like to thank Ye Olde Orchards and Mr Man for the great service and cold beers.

I would like to now thank my parents. No matter my decision you have always supported me. Even though you have no clue what I do, I know that you are proud me and that is all that matters. I only hope to be as good as a parent to my boys as you are to me

Finally, I would like to thank my wife, Sevine. When we first met, I was lost. You gave me the direction needed to start this journey. More, importantly, you were there to support me through every step of the way. You put up with me bouncing around from lab to lab and if it wasn't for you I wouldn't have made it through it. I thank you and I love you.

CHAPTER I. Introduction

I.1 Cancer

Alterations in a cell's physiology are often associated with the development of disease, including cancer. Cancer is a complex pathology that effects multiple organ systems and tissues within the human body. While it is a dynamic disease, all cancers have similar properties and are characterized by an abnormal cell growth and increased capacity to invade both local and distant tissues. Many factors have been shown to contribute to the development of this disease. These include: tobacco/alcohol use, obesity, diet, physical inactivity as well as environmental and occupational risk factors. On a molecular level, cancer is caused by DNA damage. While DNA damage occurs in normal cells, cells have mechanisms to repair this damage. Furthermore, when these repair mechanisms fail, the cell undergoes programmed cell death, apoptosis, to ensure that cells with damaged DNA do not propagate. However, in cancer cells, DNA damage repair and cell death mechanisms are inhibited or impaired. This gives rise to a cancerous population of cells with mutated DNA.

Throughout our body, genes play an important role in mediating the functions of normal cells such as cell growth, maturity and death. However, alterations in these genes as simple as a single nucleotide point mutation or as broad as a gain or loss of entire chromosomes are associated with the development of disease. Genetic alterations have been linked to the development of cancer. Three genetic alterations leading to cancer have been proposed: 1- Genomic amplification where a cell gains many copies of a small chromosomal locus. Examples include: amplification of the *myc* oncogene in a variety of tumors and *HER2* in breast and ovarian cancer. 2- DNA mutations characterized by single nucleotide mutations and nucleotide deletions or insertions that alter the functionality or expression of their coded proteins. For instance, *BRCA1* and *BRCA2* mutations in breast and ovarian cancers or *p53* mutations and 3- Translocation where two separate chromosomal regions become abnormally fused. The best known example is the BCR-ABL fusion protein in chronic myelogenous leukemia.

There are two broad categories of genes that are affected by these alterations: 1- Oncogenes or genes known to cause cancer. While many oncogenes are expressed in normal/non-cancerous cells, cancer patients have been shown to have an abnormal increase in the expression or activity of these oncogenes. Furthermore, mutations in normal genes can alter the functionality of these genes and

promote oncogenesis. 2- Tumor suppressor genes or genes that generally inhibit the cell division and survival of cells that have damaged DNA. In cancer patients, these genes are disabled either through gene deletion or mutation. This allows for the growth and survival of cancer cells (Weinberg 1995).

The majority of rapidly dividing cancer cells will form solid tumors in their target tissues. However, blood cancers, such as leukemia, generally do not form solid tumors. Tumors are considered to be malignant because they can invade into nearby tissues. Additionally, as the tumor grows, some cancerous cells can travel through either the circulatory or lymphatic systems and form tumors in distant organs and/or tissues. This is known as a metastasis. The development and progression of cancer is depicted in the Figure 1 below.

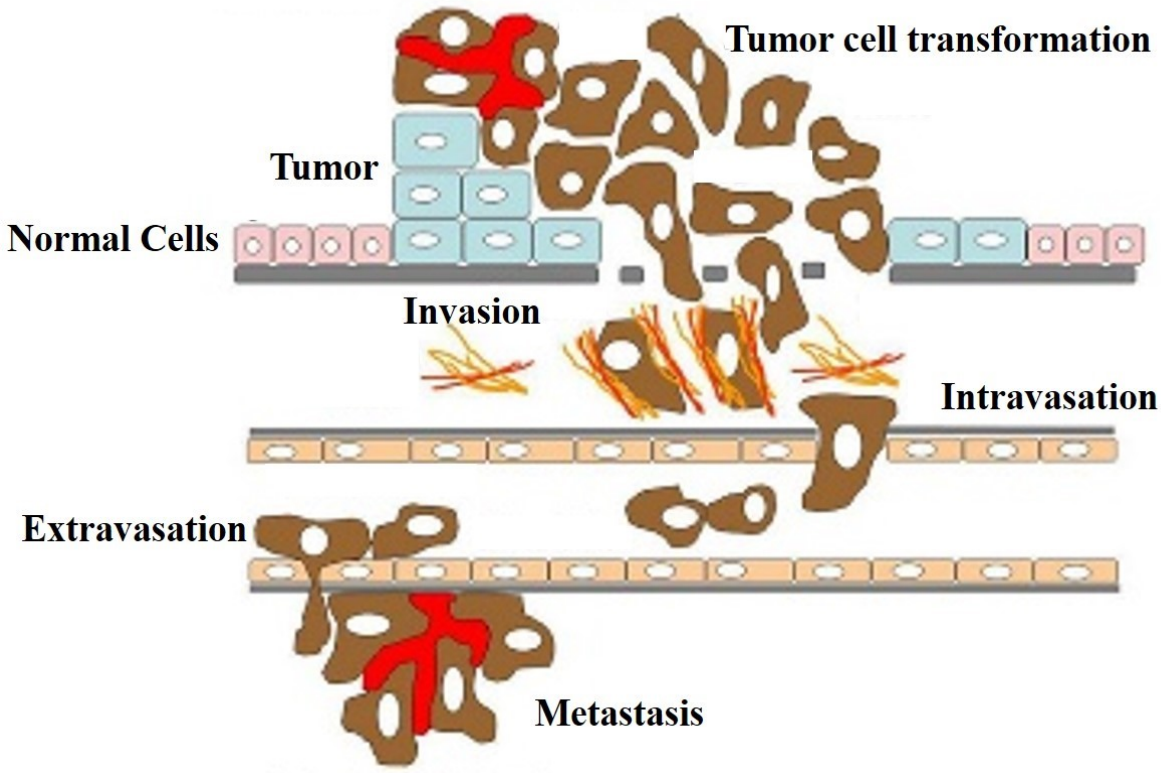

Figure 1. Cancer development and progression

Genetic alterations in normal cells results in the rapid division of cells and the formation of a primary tumor. As the tumor grows some cells transform into an invasive phenotype through a process known as epithelial-mesenchymal transformation. These cells can invade the basal membrane and enter the blood vessels, a process known as intravasation. Once in the circulatory system the tumor cell to transported to a distant organ or tissue. The cell exits the circulatory and invades this secondary site though a process known as extravasation. The tumor cell can now proliferation to form a secondary tumor or metastasis. Adapted from: (Freire-de-Lima 2014)

Within the last few decades, there has been an increased interest in cancer research and the development of therapeutics to counter this disease. Over these years, there has been significant progress which has led to the thorough characterization of the disease and the development of effective therapies. However, a large proportion of cancer patients are unresponsive to current available therapies and the majority of cancers remain incurable. Thus, it is important to further characterize the mechanisms that regulate the development and progression of cancer and to develop improved therapeutic agents.

I.2 Breast cancer

The genetic and cellular alterations described in the section above can result in the development of cancer in multiple organ systems. The most commonly affected tissues include: the lung, colon, breast and prostate (Canadian Cancer Society) In fact, breast cancer is the most commonly diagnosed cancer in Canadian women. It is estimated that in 2014, close to 24000 new cases of breast cancer were diagnosed. In fact, 1 in 9 women will be diagnosed with breast cancer within their lifetime and 1 in 30 women will succumb to the disease. However, improved diagnostics, screening techniques and therapeutics have decreased the overall mortality rate by almost 50% since 1986. Furthermore, approximately 90% of breast cancer patients survive for at least 5 years (Canadian Breast Cancer Foundation).

Breast cancer is a heterogeneous disease that is generally classified into four subtypes; Luminal A, Luminal B, HER2-positive and Triple negative (Basal-like) breast cancers. However, a 5th subtype, Normal-like breast cancer, has emerged. This subtype is characterized by a genetic profile similar to that of normal breast tissue, small tumor size and good prognosis (Carey, Perou et al. 2006). However, it is still unclear whether normal-like breast cancer is a distinct molecular subtype or just a collection of tumors that are difficult to classify in another subtype (Prat, Carey et al. 2014). The classification of cancers into these subtypes is based on the expression of therapeutically important genetic markers such as the estrogen receptor (ER), progesterone receptor (PR) and the epidermal growth factor-2 receptor (HER2) (Yersal and Barutca 2014).

I.2.1 Luminal A/B breast cancer

Luminal A breast cancer is the most common form of breast cancer, representing 50-60% of all diagnosed cases. Whereas. Luminal B breast cancer represents 15-20% of diagnosed cancers. These subtypes are characterized by a high ER and/or PR expression (Carey, Perou et al. 2006). In general, patients with luminal A/B breast cancer have a good prognosis, lower relapse rate and low incidence of metastatic disease (Kennecke, Yerushalmi et al. 2010). The primary difference between Luminal A and Luminal B breast cancer stems from the increased expression of proliferative genes in the Luminal B subtype (Reis-Filho, Weigelt et al. 2010). This increased proliferative index is associated with a worse prognosis, increased grade and aggressivity, higher recurrence and lower survival rate compared to Luminal A breast cancer (Ellis, Tao et al. 2008; Creighton 2012). Hormonal therapy is commonly used in the treatment of these subtypes (Ignatiadis and Sotiriou 2013).

I.2.2 HER2-positive breast cancer

The third subtype is known as HER2-positive breast cancer and is characterized by a high expression of the *HER2* gene. It represents 15-20% of diagnosed breast cancers and is associated with a poor prognosis, high proliferative index and aggressivity and low survival rate when left untreated (Tsutsui, Ohno et al. 2003; Staaf, Ringner et al. 2010). However, these patients are generally responsive to HER2-directed therapies (Ross, Fletcher et al. 2003). In fact, 75-80% of women diagnosed with HER2-poistive metastatic breast cancer have been shown to be responsive HER2-inhibition when treated in combination with chemotherapy (Slamon, Leyland-Jones et al. 2001). However, the overall survival of patients treated with HER2-targeted therapies after adjuvant chemotherapy was not different to that of patients left untreated (Piccart-Gebhart, Procter et al. 2005). Furthermore, there are many limitations to targeting the HER2 receptor. These limitations include: 1- Effective only in tumors expressing high levels of HER2 (low response rate in moderate and low HER2 expressing tumors) (Albanell, Codony et al. 2003), 2- Large proportion of patients develop resistance to HER2-targeted therapies (Romond, Perez et al. 2005) and 3- Generally an expensive therapy, but still considered cost-effective (Dedes, Szucs et al. 2007). Therefore, substantial work is required in this subtype to improve therapeutic outcomes.

I.2.3 Triple negative breast cancer

The final subtype is known as triple negative breast cancer (TNBC) or basal-like breast cancer and is characterized by a low expression of ER, PR and HER2 receptor and a high expression of the epidermal growth factor receptor (EGFR). It represents 8-37% of diagnosed breast cancers. The discrepancies in TNBC diagnoses stem from inconsistencies amongst clinicians and diagnostic definitions. For example, some institutes combine TNBC and basal-like breast cancer in the same subgroup, whereas others separate the two. In this case, TNBC is defined as ER, PR and HER2 negative and basal-like breast cancer as ER, PR, HER2 negative and either Cytokeratin5/6 or EGFR positive (Rakha, Elsheikh et al. 2009). The TNBC subtype is considered to have a poor prognosis, high incidence of metastasis, high proliferative index and aggressivity and poor survival rates (Heitz, Harter et al. 2009; Rakha, Elsheikh et al. 2009; Criscitiello, Azim et al. 2012). While targeted therapies are currently available for luminal and HER2-positive tumors, no targeted therapies are presently approved for the treatment of TNBC patients (Engebraaten, Vollan et al. 2013).

The TNBC subtype is known to be the most chemosensitive breast cancer subtype. Indeed, TNBC patients have increased sensitivity to chemotherapy compared to ER-positive breast cancers (Crown, O'Shaughnessy et al. 2012). This increased sensitivity has been shown to be the result of increased incidence of BRCA mutations in this subtype (Bhattacharyya, Ear et al. 2000; Moynahan, Cui et al. 2001). However, little is known on which chemotherapeutic agents elicit the best response in these patients (Cleator, Heller et al. 2007). In fact, there are no systematic therapeutic regiments recommended for the treatment of TNBC patients (Cleator, Heller et al. 2007). Furthermore, the use of standard chemotherapeutics leaves these patients at an increased risk of both local and systemic relapse (Cleator, Heller et al. 2007). Additionally, while approximately half of patients have been reported to respond to chemotherapeutic treatment with either paclitaxel (a mitotic inhibitor of the taxane family of chemotherapies) or doxorubicin (an anthracycline involved in DNA intercalation), TNBC patients have been reported to develop resistance to these treatments (Bhattacharyya, Ear et al. 2000; Quinn, Kennedy et al. 2003). Therefore, further investigation is required to better define the therapeutic benefits of chemotherapies in TNBC patients. Emerging therapies are focused on targeting oncogenic

pathways such as the PI3K/AKT and Ras/MAPK pathways as well as receptor tyrosine kinases (RTKs) such as the EGFR, fibroblast growth factor receptor-2 (FGFR2) and vascular endothelial growth factor (VEGFR) (Cunningham, Humblet et al. 2004; Mendelsohn and Baselga 2006; Cleator, Heller et al. 2007; O'Shaughnessy 2010; Turner, Lambros et al. 2010; Zhao and Adjei 2014). However, little clinical success has been demonstrated when targeting these factors. As our research is focused on the signals downstream of the EGFR, this family of RTKs and the therapeutic agents targeting it will be described in detail below.

I.3 EGFR inhibitors

The EGFR is a receptor tyrosine kinase (RTK) known to be expressed and activated in a variety of cancers. Upon ligand binding to the receptor, there is receptor dimerization and auto-phosphorylation. This allows for the recruitment of adaptors and other signaling mediators to receptor leading to the activation of important signaling cascades involved in cancer cell proliferation, survival, migration and invasion. This receptor will be discussed in more detail in section I.5.1. With the majority of TNBC patients expressing higher levels of the EGFR, the EGFR is a potential therapeutic target in this breast cancer subtype. Research within the field of EGFR signal transduction has led to the development of targeted EGFR cancer therapeutics such as the monoclonal antibodies that target the extracellular domain of the receptor preventing ligand binding and receptor dimerization (Fan, Lu et al. 1994; Mendelsohn 1997). Secondly, small molecule tyrosine kinase inhibitors have also been synthesized that target the intracellular kinase domain of the EGFR and prevent ATP-binding (Ward, Cook et al. 1994) (Figure 2). Several of these EGFR inhibitors have been approved for the treatment of EGFR-overexpressing cancers, whereas many more are currently being tested in clinical trials. The two families of EGFR inhibitors will be discussed further below.

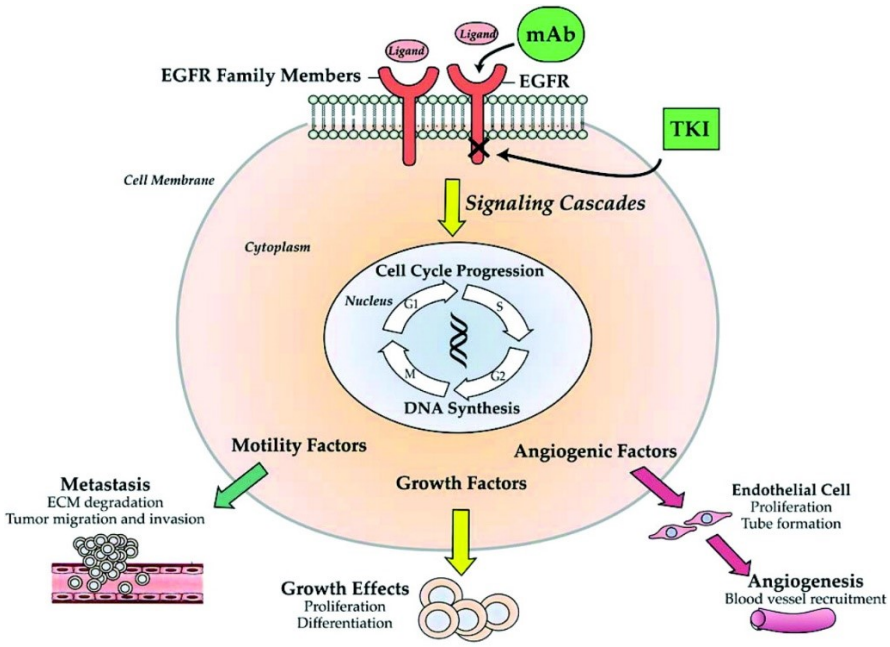

Figure 2. Targeting the EGFR

EGFR activity plays an important role in cancer cells to promote the activation of signaling cascades involved in the induction of cell cycle progression, proliferation, differentiation, cell motility and angiogenesis. There are currently two therapeutic means of targeting the EGFR in cancer: 1- Monoclonal antibodies (denoted Mab) which block the activation of the EGFR by preventing the ligand from binding to its receptor and 2- Tyrosine kinase inhibitors (denoted TKI) which block the activation of the kinase domain of the EGFR by competing with ATP for the ATP-binding domain. Taken from: (Harari 2004).

I.3.1 Monoclonal antibodies

Monoclonal antibodies (Mab) block EGFR family signaling by interacting with the extracellular domain of the receptor and blocking the binding of its ligand. This, in turn, prevents receptor dimerization and the induction of EGFR-dependent signal transduction (Fan, Lu et al. 1994; Mendelsohn 1997). The most common Mab, trastuzumad, targets the HER2 receptor and is currently the only EGFR family Mab therapy approved for the treatment of breast cancer patients (Huston and George 2001). Treatment of patients with this Mab has been shown to have positive effects on patient outcome and decreased tumor cell survival, proliferation and angiogenesis (Hudziak, Lewis et al. 1989; Karamouzis, Konstantinopoulos et al. 2007). Unfortunately, Mabs against the EGFR have shown disappointing results. While treatment with the EGFR Mab, cetuximab, has been shown to have growth inhibitory effects in both breast cancer cell lines and

tumor xenographs (Masui, Kawamoto et al. 1984; Mendelsohn 1997), it was shown to have little to no effect in the treatment of breast cancer patients (Modi, D'Andrea et al. 2006).

I.3.2 Tyrosine kinase inhibitors

The second EGFR-targeted therapies, the tyrosine kinase inhibitors (TKi), were first identified in the late 1980s as a negative regulator of EGFR auto-phosphorylation and EGF-dependent cell proliferation (Yaish, Gazit et al. 1988). Today, multiple tyrosine kinase inhibitors have been synthesized and approved for the treatment of several cancers and target multiple RTKs, including the EGFR. Additionally, many other TKis are currently being developed and tested within the clinic. The EGFR TKis are members of class of compounds known as the 4-anilinoquinazolines (See Figure 4) and primarily act by competing with ATP for binding sites within the EGFR kinase domain (Ward, Cook et al. 1994). As shown in Figure 3, a hydrogen bond is formed between the Met793 residue within the hinge region of the ATP-binding site of the EGFR and gefitinib. This blocks the binding of ATP and the activation of the receptor (Eck and Yun 2010).

TKis are more therapeutically advantageous than monoclonal antibodies because they are generally well tolerated and can be orally administered. Second, they have been shown to be active against the monoclonal antibody-resistant truncated form of HER2 (Xia, Liu et al. 2004). Finally, since the kinase domain of all members of the EGFR family are highly homologous, TKis can be designed to target multiple or all EGFR family members (Ekstrand, Longo et al. 1994) (Figure 3). The TKis currently approved for the treatment of cancer include:

1- Gefitinib: a reversible, EGFR-specific inhibitor currently approved for the treatment of non-small cell lung cancer
2- Erlotinib: a reversible, EGFR-specific inhibitor currently approved for the treatment of non-small cell lung cancer and pancreatic cancer
3- Lapatinib: a dual inhibitor of EGFR and HER2 currently approved for the treatment of hormone-positive and HER2-positive breast cancer

While significant therapeutic responses have been demonstrated in patients treated with these inhibitors, many of the patients develop TKi resistance upon continuous use of these inhibitors (Jackman, Pao et al. 2010). Furthermore, patients with mutations in either the EGFR and/or Ras/Raf have also been shown to be resistant to TKi treatment (Misale, Yaeger et al. 2012; Ohashi, Sequist et al. 2012; Yu, Arcila et al. 2013).

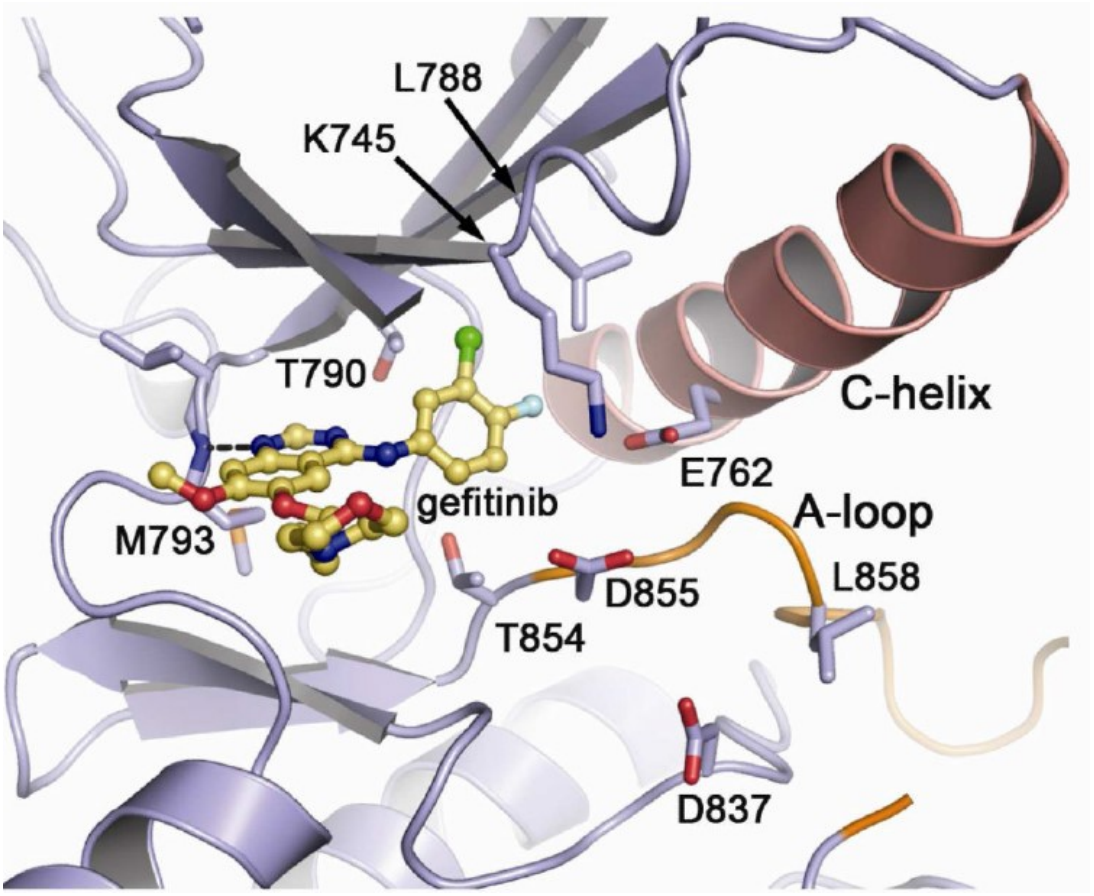

Figure 3. Crystalized structure of gefitinib binding the ATP-binding domain of the EGFR

The kinase domain of the EGFR is composed of a C-terminal-lobe and an N-terminal-lobe connected by a hinge region. This hinge region comprises part of the ATP-binding site. A hydrogen bond is formed between the Met793 residue of the EGFR and the quinazoline moiety of gefitinib, thus blocking ATP binding. Taken from: (Eck and Yun 2010).

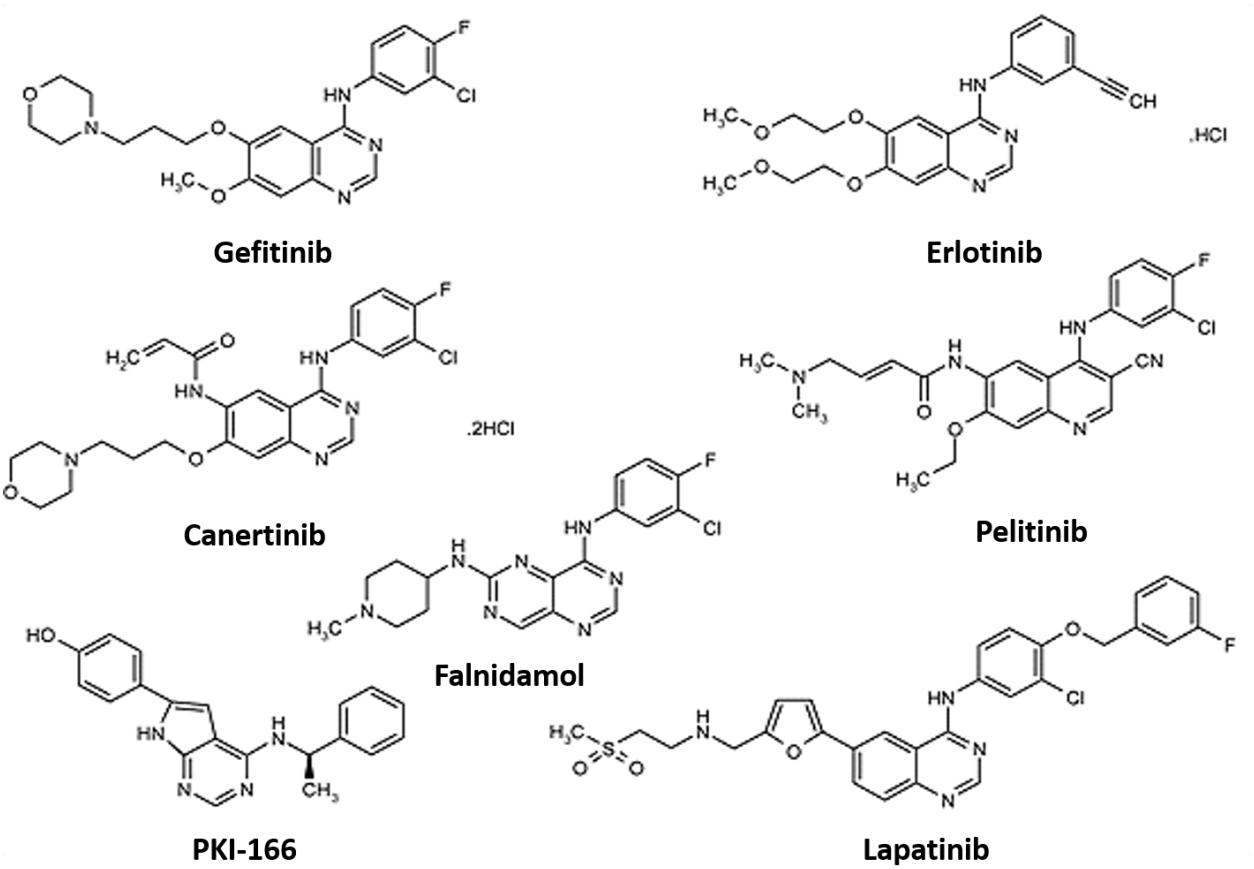

Figure 4. Chemical structures of EGFR tyrosine kinase inhibitors

EGFR tyrosine kinase inhibitors are members of the class of compounds known as the 4-anilinoquinazolines that target the ATP-binding pocket of the kinase domain of the EGFR. The quinazaline core is essential for its inhibitory effects on the EGFR. Furthermore, modifications to this core have been essential in the development of both second generation reversible and irreversible inhibitors. Adapted from: (van Montfort and Workman 2009; Hamed, Abou El Ella et al. 2013)

I.4 EGFR inhibitor resistance

Even though the majority of TNBCs overexpress the EGFR, attempts at targeting this receptor in patients has shown limited success. One reason these patients lack a response to EGFRTKis stems from the development of drug resistance. This resistance can be innate to the patient's cancer (i.e. mutations in the EGFR or downstream signaling mediators) known as intrinsic resistance or can be developed by the patient throughout the treatment regiment, known as acquired resistance. Since EGFRTKis are clinically approved for the treatment of lung cancer, it is important to note that the majority of studies in the literature examine EGFRTKi resistance in this cancer. However, key studies evaluating TKi resistance in breast cancer will be highlighted throughout this thesis.

I.4.1 Intrinsic resistance

Cancer cells have innate characteristics that make them resistant to the currently used therapeutics. In fact, approximately 50% of all cancer patients are resistant to chemotherapy before treatment (Lippert, Ruoff et al. 2008). This is known as intrinsic or primary resistance. The most common mechanisms of intrinsic EGFRTKi resistance include: EGFR mutations characterized by a loss in sensitivity to EGFRTKi treatment such as exon 20 insertions or duplications (Greulich, Chen et al. 2005; Yasuda, Kobayashi et al. 2012), the amplification of another RTK, cMET (Engelman, Zejnullahu et al. 2007) and altered survival (PIK3CA mutations) (Cizkova, Susini et al. 2012) and apoptotic (Bim expression) (Faber, Corcoran et al. 2011) pathways. As our research focuses primarily on the role of ARF1 in the development of acquired resistance to EGFRTKis, the remainder of this thesis will be dedicated to the description of important mechanisms of acquired resistance.

I.4.2 Acquired resistance

Acquired resistance or secondary resistance (Figure 5), unlike intrinsic resistance, occurs in patients that were previously responsive to therapy and is clinically defined as: a systematic progression of the disease after a complete or partial response or following a period of 6 months of stable disease in patients treated with a targeted therapy (Jackman, Pao et al. 2010). It is generally divided into two subgroups: 1- Genetic alterations in the primary oncogene that lead to increased downstream signaling. This normally occurs through either a secondary mutation in the target kinase (the EGFR) or amplification of the target kinase. Briefly, point mutations have been

identified in the EGFR that alter either the affinity of EGFRTKis for the receptor or enhance the activity of the receptor itself (Engelman and Janne 2008; Engelman and Settleman 2008; Sierra, Cepero et al. 2010). 2- Development of resistance that is independent of genetic changes in the EGFR, such as the activation of downstream signaling pathways or other receptors, changes in tumor histology, evasion of apoptosis and alterations in drug metabolism. Amplification of other members of the EGFR family as well as other RTKs such as cMET and AXL have all been implicated in the resistance to EGFRTKis. Additionally, up-regulation of signals through the Ras/MAPK and PI3K/AKT pathway also mediate resistance. Finally, epithelial-mesenchymal transformation (EMT), a process in which epithelial cancer cells transform into more invasive mesenchymal phenotype to evade the therapeutic effects of EGFRTKis (Engelman and Janne 2008; Engelman and Settleman 2008; Ellis and Hicklin 2009; Sierra, Cepero et al. 2010). The importance of altered RTK expression and signaling, as well as the activation of downstream signaling cascades in the regulation of acquired resistance will be further discussed throughout this thesis.

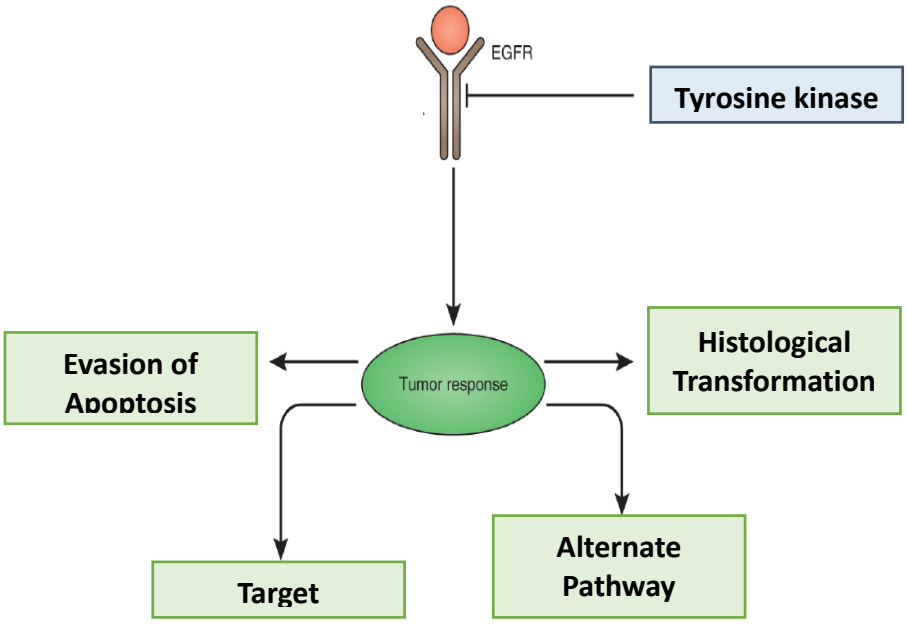

Figure 5. Mechanisms of EGFRTKi resistance

Acquired resistance plays an important role in the tumor response to EGFR inhibition. Four mechanisms of acquired resistance have been proposed: 1- Target modifications: Mutations in the EGFR itself enhance EGFR activation or block the binding of the inhibitor to the receptor, 2- Alternate pathway activation: The activation of other receptors (HER2, HER3, cMET, AXL) or signaling pathways (Ras/MAPK, PI3K/AKT) compensate for the loss of EGFR signals, 3- Evasion of apoptosis: modification in the tumor cell apoptotic machinery that prevents inhibitor-induced

cell death and 4- Histological transformation: processes such as epithelial-mesenchymal transformation that allow tumors cells to acquire new properties and decreased inhibitor sensitivity. Adapted from: (Chong and Janne 2013)

I.5. Membrane receptors

Membrane receptors act to relay signals between a cell and its environment. These receptors bind external stimuli such as peptides, hormones, growth factors and cytokines and relay their message to the nucleus through the activation of signaling pathways. Membrane receptors are generally divided into four families: 1- G-Protein-Coupled Receptors (GPCRs), 2- Catalytic Receptors 3- Channel-Linked Receptors, 4- Non-Catalytic Single Transmembrane Receptors. Briefly, GPCRs are a large superfamily of seven transmembrane receptors that relay their signals by binding guanine nucleotide-binding proteins (G-proteins). These G-proteins link the receptor to downstream effectors that regulate biological activities and cellular functions (Hamm 1998). Altered signaling through this receptor superfamily is associated with multiple diseases, including cancer. Secondly, the catalytic receptors are single transmembrane receptor that once bound to their ligand act directly as phosphorylating enzymes. In other words, these receptors possess enzymatic activity (Yarden and Ullrich 1988). This family is also implicated in the development and progression of cancer. As the EGFR is a member of this group of receptors, this family will be described in detail below. Next, the channel-linked receptors are generally hormone receptors that regulate the influx and efflux of ions through the cell membrane (Levitan 1988). Lastly, the non-catalytic receptors are best known to propagating signals downstream of the interleukins, peptides, hormones and neuronal cues. Like catalytic receptors, this family also consists of single transmembrane receptors. However, they do not possess catalytic activity and rely on interacting proteins to propagate its signals (Cooper and Qian 2008). In summary, membrane receptors play important roles in the communication between a cell and its environment and the de-regulation of these receptors has been linked to disease development.

I.5.1 Receptor tyrosine kinases

The RTK family of receptors are catalytic receptors that possess intrinsic kinase activity. Protein kinases are enzymes that are involved in the phosphorylation of tyrosine, serine or threonine residues (Tsai and Nussinov 2013). Families of protein phosphatases act to dephosphorylate proteins making phosphorylation a reversible process (Alonso, Sasin et al. 2004). Phosphorylation and dephosphorylation are very important in the regulation of cellular activities (Shah, Shah et al.

2013). Kinases are classified as protein-serine/threonine kinases (385 members), protein-tyrosine kinases (90 members) and tyrosine-kinase-like proteins (44 members). Of the 90 protein-tyrosine kinases, 58 are receptor-tyrosine kinases (RTKs) (Manning, Whyte et al. 2002). Alterations in RTK activity, receptor overexpression, chromosomal translocation, gene amplification, mutations and impaired receptor downregulation have all been associated with the development of cancer (Abella and Park 2009; Tsai and Nussinov 2013). In fact, 30 of the identified RTKs have been implicated in cancer (Weinstein 2000).

I.5.2 EGFR family

The EGFR family of RTKs, the most characterized members of the catalytic receptors, is composed of four members: EGFR, HER2, HER3 and HER4, also depicted ErbB1, ErbB2, ErbB3 and ErbB4 (Figure 6). These RTKs are ubiquitously expressed and play roles in the regulation of normal cell cycle progression, apoptosis, cell differentiation, development and gene transcription (Lemmon and Schlessinger 2010). The EGFRs are activated upon ligand binding. Presently, ten polypeptide growth factor ligands have been identified which include: EGF, amphiregulin (AR), transforming growth factor-α (TGF), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EPR) and neuregulins 1-4 (NRG1-4), which include the heregulins (HRG) (Roskoski 2014). These ligands have been shown to activate specific EGFR family members and favor distinct receptor dimerization patterns. The EGFR members have been shown to mediate oncogenesis via several mechanisms that include receptor overexpression, mutations and ligand-independent signaling (Burtness 2007). In addition, the expression of these receptors has been associated with a poor prognosis in most cancers. Indeed, the activity of EGFRs has been shown to promote cancer cell proliferation, survival, migration and invasion (Herbst 2004; Roskoski 2014).

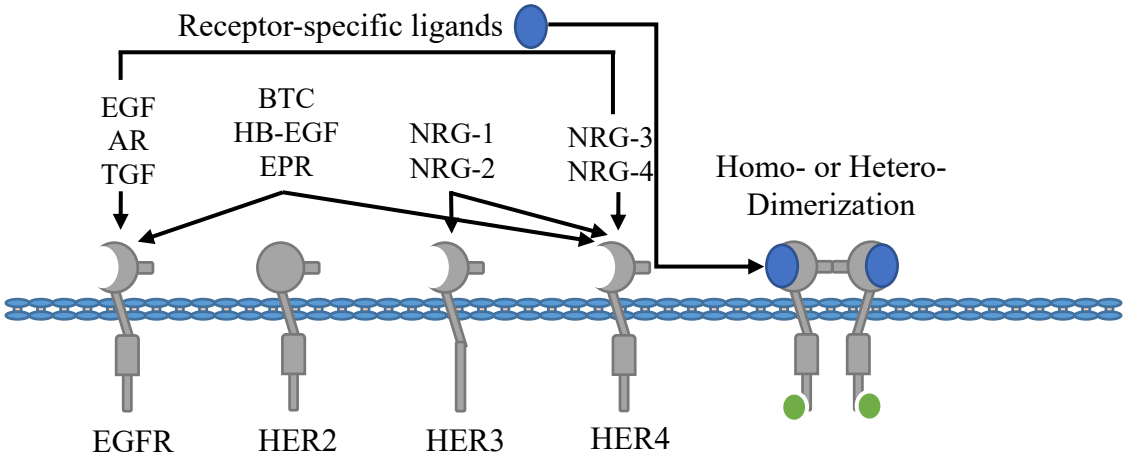

Figure 6. The EGFR family of receptor tyrosine kinases

The EGFR family of receptors is composed of four members: EGFR, HER2, HER3 and HER4. The activation of this family of receptors is governed by ligand-dependent homo- or heterodimerization. There are currently 11 identified ligands with specificity for certain EGFR family members, as illustrated above. HER2 differs from other members in that it has no ligand-binding domain and its activation is dependent on the formation of heterodimers. All EGFRs, except HER3, have intracellular kinase activity required for the initiation of downstream signaling cascades. Therefore, HER3 is also dependent on heterodimerization to potentiate its signals. Adapted from: (Itamochi 2010)

I.5.3 EGFR

The EGFR was the first receptor to be demonstrated to possess kinase activity and is the RTK that has been the best characterized (Carpenter and Cohen 1990). Its role in the regulation of proliferation, apoptosis and metastasis has been well defined in several cancer models, as well as in cancer patients (Herbst 2004; Roskoski 2014). Over 80% of TNBC patients have elevated EGFR expression levels and the activity of this receptor has been shown to play an important role in the oncogenic properties of this breast cancer subtype (Siziopikou, Ariga et al. 2006; Engebraaten, Vollan et al. 2013; Roskoski 2014). This makes the EGFR and its downstream effectors potential therapeutic targets in this patient subpopulation. However, attempts at targeting the EGFR have had limited success.

I.5.3.1 EGFR structure

The EGFR consists of an extracellular domain, a single transmembrane domain and an intracellular domain with protein kinase activity (Ullrich, Coussens et al. 1984) (Figure 7). The extracellular domain is glycosylated and consists of four domains: domain I through IV. Domains I and III mediate ligand binding, whereas, domains II and IV regulate receptor dimerization (Roskoski 2014). The intracellular domain consists of a juxtamembrane domain, a protein kinase domain and a carboxyl-terminal tail. The juxtamembrane domain has been shown to play an essential role in the tyrosine phosphorylation of the EGFR without regulating receptor dimerization and ligand binding (He and Hristova 2012). However, this domain plays an important role in the stabilization of receptor dimers and promotes receptor activation (Jura, Endres et al. 2009).The protein kinase domain is required for the activity of the receptor and promotes the activation of downstream signaling effectors (Roskoski 2014). Finally, the C-terminal tail contains essential phosphorylation sites that recruit signaling adaptors and effectors, thus promoting the activation of downstream signals (Yarden and Sliwkowski 2001). Furthermore, this tail has also been shown to function in a negative-feedback loop required for receptor inactivation (Gajiwala 2013).

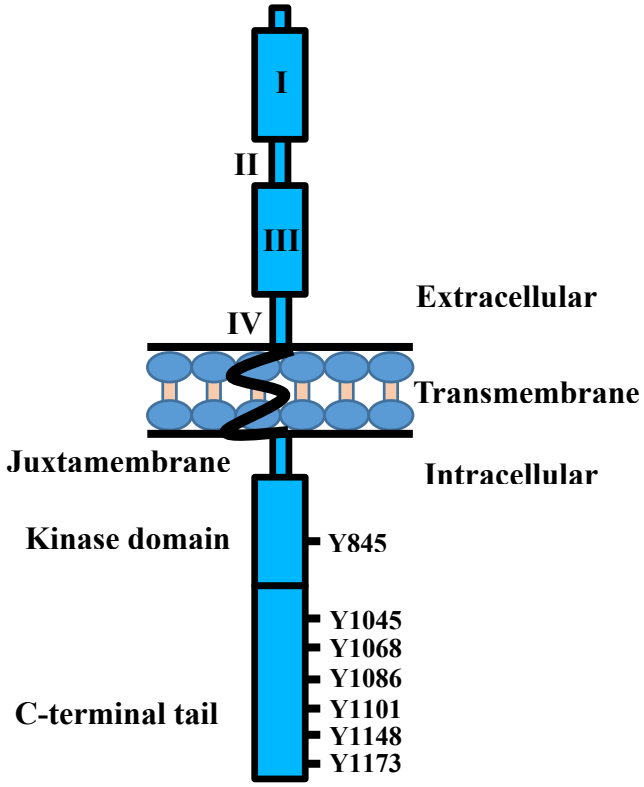

Figure 7. EGFR structure

The EGFR is a single transmembrane receptor with an extracellular, transmembrane and intracellular domains. The extracellular domain mediates ligand binding through it's I and III domains and receptor dimerization through its II and IV domains. The intracellular domain consists of a juxtamembrane domain that mediates receptor activation, dimerization and internalization, a kinase domain and c-terminal autophosphorylation domain that contains tyrosine residues that serve as recruitment sites for signaling mediators. Adapted from: (Bazley and Gullick 2005).

I.5.3.2 EGFR activation mechanism

The EGFR receptor exists as a monomer at the plasma membrane. Upon ligand binding, a large conformational change occurs in the extracellular domain. This removes the dimerization autoinhibition in domain IV and promotes homo and heterodimerization of the EGFR. The juxtamembrane domain then promotes the phosphorylation of the activation segment of the protein kinase domain leading to kinase activation. The activated kinase domain tyrosine phosphorylation residues on the EGFR creating docking sites for the recruitment of signaling adaptors and effectors which initiate the activation of signaling cascades. The kinase domain can also directly phosphorylate and activate other siganling mediators to initiate downstream signals (Burgess, Cho et al. 2003; Nolen, Taylor et al. 2004; Lemmon and Schlessinger 2010).

I.5.3.3 EGFR in resistance

With expression levels of the EGFR being elevated in TNBC patients, it would be considered a good therapeutic target. However, the majority of these patients are unresponsive to EGFRTKis. The development of drug resistance is one explanation why TNBC patients do not respond to these therapies. While less common in breast cancer patients, mutations in the EGFR have been identified as key mediators of EGFRTKi treatment response in lung cancer patients. Indeed, a high proportion of lung cancer patients develop resistance through EGFR mutations such as EGFRT790M that increases the affinity of the kinase domain of the EGFR for ATP and in turn reduces the sensitivity of ATP-competitive reversible EGFRTKis (Yun, Mengwasser et al. 2008; Chong and Janne 2013). These mutations can be either acquired throughout the treatment or innate (Lee, Shin et al. 2013). However, in breast cancer patients, these mutations in the EGFR are very rare (Bhargava, Gerald et al. 2005) and downstream signaling mediators play a more important role in acquired resistance (Ferrer-Soler, Vazquez-Martin et al. 2007). Additionally, increase expression and the nuclear translocation of the EGFR have also been implicated in resistance (Brand, Iida et al. 2014; Lee, Seo et al. 2014). Indeed, the inhibition of the nuclear translocation of the EGFR significantly improved the response to EGFR inhibitors. Briefly, the endocytosis of the EGFR has been implicated in its translocation from the membrane into the nucleus. However, it remains unknown how the EGFR is transported from the vesicle into the nucleus. Once in the nucleus, EGFR acts as a transcriptional co-activator for various oncogenes implicated in resistance (Brand, Iida et al. 2014). Additionally, AKT, an importance mediator of resistance, has been shown

to Serine phosphorylation EGFR leading to its nuclear translocation (Huang, Chen et al. 2011). While alterations in EGFR-mediated signals influence patient response to EGFR inhibitors, other EGFR family members as well as other RTKs have also been implicated in the development of resistance. A description of these receptors can be found below.

I.5.4 HER2

This EGFR family member, like the EGFR, is also well characterized for its role in mediating breast oncogenesis. In fact, 20-30% of breast cancers have HER2 receptor overexpression or gene amplification. Additionally, HER2 expression is associated with a poor prognosis and increased cancer cell proliferation (Roskoski 2014). Unlike other EGFR family members, HER2 has no ligand and exerts its oncogenic properties through the heterodimerization with other EGFRs. While HER2 has been reported to dimerization with both EGFR and HER3, studies have shown that it favors HER3 heterodimer formation (Citri, Skaria et al. 2003). This would suggest that targeting HER2 could significantly reduce signals downstream of both the EGFR and HER3, making HER2 a favorable therapeutic target. However, HER2 monoclonal antibodies have been shown to be most effective at inhibiting signals downstream of HER2 homodimers and to have no effect on the ability of HER2 to heterodimerize with the EGFR and HER3 (Ghosh, Narasanna et al. 2011). Additionally, the effectiveness of HER2-targeted therapies is hindered by the development of resistance (Rexer and Arteaga 2012). Therefore, there is significant room for improvement in the treatment regiments of HER2-positive breast cancer patients.

Like EGFR, this receptor has also been implicated in the development of resistance to EGFRTKis. In fact, the amplification of HER2 is commonly found in EGFRTKi resistance lung cancer patients that do not develop mutations in the EGFR. Interestingly, 12% of EGFRTKi resistance lung tumors had amplified HER2 expression compared to only 1% in non-resistant tumors (Takezawa, Pirazzoli et al. 2012). Conversely, HER2 expression is linked to EGFRTKi sensitivity in breast cancer. However, over time, mutations in the ATP-binding pocket of the kinase domain of HER2 have been observed and this leads to the development of acquired resistance in breast cancer (Piechocki, Yoo et al. 2007). Additionally, prolonged inhibitor treatment has been shown to enhance and stabilize HER2 heterodimerization with both EGFR and HER3 and potentiate downstream signaling (Jain, Penuel et al. 2010; DeFazio-Eli, Strommen et al. 2011). Together,

these findings demonstrate that HER2 mediates breast oncogenesis and that targeting this receptor could be important in the sensitivity of breast cancer patients to EGFR inhibitors.

I.5.5 HER3

The influence of the third member of this receptor family, HER3, in breast cancer has been less documented. However, 20-30% of invasive breast cancers have shown to overexpress the HER3 receptor (Karamouzis, Badra et al. 2007). Also, HER3 has been linked to HER2 positivity and decreased incidence of metastasis. However, no true correlations between HER3 and patient survival have been made within the literature (Lemoine, Barnes et al. 1992; Gasparini, Gullick et al. 1994; Bieche, Onody et al. 2003). Like HER2, the oncogenic properties of HER3 is highly dependent on its heterodimerization with other EGFRs (Koutras, Fountzilas et al. 2010). In fact, HER2-HER3 heterodimerization has been associated with a high mitogenic potential (Citri, Skaria et al. 2003).

Additionally, HER3 has been implicated in EGFRTKi resistance. Indeed, increased HER3 expression is associated with decreased sensitivity to EGFRTKis (Stegeman, Kaanders et al. 2013; Nakata, Tanaka et al. 2014). Furthermore, increased HER3 activation, heterodimerization as well as decreased in activity of phosphatases targeting the HER3 have all been linked to acquired resistance to EGFR inhibitors (Koizumi, Shimoyama et al. 2005; Sergina, Rausch et al. 2007; Xia, Petricoin et al. 2013). Additionally, the pharmacological targeting of HER3 has been shown to overcome EGFRTKi resistance (Huang, Li et al. 2013). The increased activation and dimerization is explained by an altered localization of HER3 in lung cancer cells. Indeed, prolonged EGFR inhibition promoted the membrane localization of HER3 (Sergina, Rausch et al. 2007). Additionally, HER3 has been shown to promote resistance through the activation of the PI3K/AKT pathway. In fact, heterodimerization of HER3 with either mutant EGFR (T790M) or another RTK cMET have been shown to promote PI3K activation in lung cancer cells (Engelman, Mukohara et al. 2006; Engelman, Zejnullahu et al. 2007). While the functions of HER3 in breast cancer has yet to be fully elucidated, it has been demonstrated in the literature that HER3 exerts its oncogenic properties through it dimerization with HER2 and is an important mediator of EGFRTKi sensitivity.

I.5.6 HER4

Of the four EGFR family members, the functions of HER4 in breast cancer are the least discussed. Its expression is detectable in approximately 50% of diagnosed breast cancers. However, unlike other EGFR family members, HER4 is associated with the inhibition of cellular proliferation and the induction of apoptosis (Sartor, Zhou et al. 2001; Naresh, Long et al. 2006). Interestingly, HER4 has been demonstrate to localize to the mitochondria and enhance the release of cytochrome C to promote apoptosis (Naresh, Long et al. 2006). Additionally, HER4 expression has been correlated with a good prognosis, increased patient survival, ER positivity, decreased HER2 signaling and increased response to hormonal therapy (Knowlden, Gee et al. 1998; Tang, Concepcion et al. 1999; Bieche, Onody et al. 2003; Witton, Reeves et al. 2003; Barnes, Khavari et al. 2005; Naresh, Thor et al. 2008). Meanwhile, the role of HER4 in EGFRTKi resistance has yet to be evaluated within the literature.

Together, the EGFR family of receptors are important mediators of oncogenesis and their activity and downstream signals have been implicated in the development of drug resistance.

I.5.7 Other RTK family members in resistance

While members of the EGFR family play an important role in mediating EGFRTKi resistance, other RTK family members have also been shown to compensate for the loss of EGFR signaling. First, the amplification cMET receptor, an oncogene overexpressed in breast cancer and associated with a poor prognosis, has been shown to promote EGFRTKi resistance in lung, brain and breast cancer cells (Engelman, Zejnullahu et al. 2007; Stommel, Kimmelman et al. 2007; Gastaldi, Comoglio et al. 2010; Raghav, Wang et al. 2012; Sohn, Liu et al. 2014). This receptor was shown to activate the PI3K pathway through the transactivation of HER3. Moreover the inhibition of this HER3 transactivation re-sensitized EGFRTKi resistance cells to EGFR inhibition (Engelman, Zejnullahu et al. 2007). Additionally, another RTK overexpressed in breast cancer and associated with a poor prognosis, AXL, has also been shown to mediate EGFRTKi resistance in both lung and breast cancer (Vuoriluoto, Haugen et al. 2011; Zhang, Lee et al. 2012; Byers, Diao et al. 2013; Meyer, Miller et al. 2013). Recently, AXL was shown to promote resistance by enhancing signals downstream of the EGFR. Indeed, the pharmacological inhibition or depletion of AXL significantly hindered the activation of the EGFR and its downstream signals. Furthermore, other RTKs such as the insulin growth factor-1 receptor (IGF1R) and fibroblast growth factor receptor

1 (FGFR1) have also been shown to be activated in EGFRTKi resistant cancer (Cortot, Repellin et al. 2013; Azuma, Kawahara et al. 2014). Inhibition of IGF1R was shown to prevent the development of EGFRTKi resistant lung cancer cells. (Cortot, Repellin et al. 2013) Whereas, the inhibition or depletion of FGFR1 was shown to decrease the activation of both AKT and ERK1/2, two important mediators of EGFRTKi resistance (Azuma, Kawahara et al. 2014).

Altogether, mutations in the EGFR itself or increased signaling through other RTKs (HER2, HER3, cMET, AXL) promote resistance to EGFRTKis. Therefore, targeting signaling mediators downstream of these receptor could improve the response of patients to EGFR inhibition and delay the onset of resistance to these inhibitors.

I.6 Signaling adaptors

Activated RTKs are linked to their downstream signaling pathways through the recruitment of adaptor and effector proteins. Briefly, the autophosphorylation of the EGFR or the transphosphorylation of tyrosine residues by other kinases such as Src within the c-terminal tail of the EGFR create docking sites for adaptor proteins including Grb2 and Shc (Rozakis-Adcock, McGlade et al. 1992; Biscardi, Maa et al. 1999; Roskoski 2014). The recruitment of these adaptors has been shown to greatly increase the ability of the EGFR to phosphorylate and activate its downstream signaling mediators (Rojas, Yao et al. 1996; Migliaccio, Mele et al. 1997). Furthermore, adaptors assist in the assembly of spatially organized signaling cascades leading to the induction of important physiological responses such as cell proliferation, survival, migration and invasion (Hsieh, Yang et al. 2010). In this next section, we will discuss the importance of adaptor proteins as mediators of signals downstream of the EGFR and their influence in the development of breast cancer.

I.6.1 Grb2

The best characterized adaptor recruited to the EGFR is Grb2. It is classically known to link activated RTKs to the Ras/MAPK pathway. Grb2 is constitutively bound to the Ras guanine exchange factor (GEF) son of sevenless (SOS). Upon RTK activation, the Grb2/SOS complex is recruited to the receptor bringing SOS into close proximity with the GTPase Ras. This leads to Ras activation and the initiation of the Ras/MAPK pathway (van der Geer, Hunter et al. 1994; Kairouz and Daly 2000). As Grb2 has been previously reported to recruit a GEF to the EGFR

leading to the activation of the small GTPase Ras, we highlight the importance of Grb2 in the activation of other small GTPases, ARF1 and ARF6, in Chapter II. Grb2 has also been shown to interact with other important signaling mediators such as Grb2-associated binding protein-1 (Gab1) which recruits phosphatidylinositol-4, 5-biphosphate 3 kinases (PI3K) to RTKs leading to its activation (Ong, Hadari et al. 2001).

The role of Grb2 in breast cancer remains controversial. An increased protein and mRNA expression of Grb2, as well as an amplification of the *GRB2* gene locus have been observed in breast cancer cells and primary breast tumors (Daly, Binder et al. 1994; Verbeek, Adriaansen-Slot et al. 1997; Yip, Crew et al. 2000). Additionally, depletion of Grb2 is associated with a decreased ERK1/2 activation in breast cancer cells and delayed onset of mammary tumors induced by the polyomavirus middle T antigen suggesting that Grb2 may play an important role in mammary oncogenesis (Gale, Kaplan et al. 1993; Suen, Bustelo et al. 1993). However, Grb2 has also been shown to negatively regulate signals downstream of the EGFR (Li, Couvillon et al. 2001; Haines, Minoo et al. 2009; Belov and Mohammadi 2012). Indeed, the tyrosine phosphorylation of Grb2 by the EGFR itself and the prolactin receptor, an important mediator of breast development and oncogenesis, has been shown to attenuate its interaction with SOS and thus block the activation Ras downstream of the EGFR (Li, Couvillon et al. 2001; Haines, Minoo et al. 2009). Additionally, Grb2 has been shown to recruit the ubiquitin ligase, Casitias B-lineage Lymphoma protein (Cbl), to the EGFR leading to receptor ubiquination and its down-regulation (Belov and Mohammadi 2012). On the same note, recent attempts at targeting Grb2 in cancer have had little to no therapeutic effects (Dharmawardana, Peruzzi et al. 2006).

Together, Grb2 has been shown in the literature to be recruited to activated RTKs and promote the activation of signaling cascades. However, its role in breast cancer is still controversial.

I.6.1.1 Grb2 structure

The adaptor Grb2 consists of a Src Homology 2 (SH2) domain flanked by two Src Homology 3 (SH3) domains (van der Geer, Hunter et al. 1994) (See Figure 8). Grb2 interacts with tyrosine phosphorylated residues such as those on the EGFR through its SH2 domain and proline-rich motifs such as those present on SOS through its SH3 domains (Lowenstein, Daly et al. 1992; van der Geer, Hunter et al. 1994; Kairouz and Daly 2000). Other proteins known to interact with the SH2 domain of Grb2 include: other RTKs and the adaptor Shc (Lowenstein, Daly et al. 1992;

Rozakis-Adcock, McGlade et al. 1992). Whereas, Grb2 binds proteins such as dynamin, a GTPase involved in endocytosis and Cbl, an E3 ubiquitin protein ligase involved in EGFR down-regulation through its SH3 domains (Lowenstein, Daly et al. 1992; Sparks, Rider et al. 1996; Yamazaki, Zaal et al. 2002).

Figure 8. Grb2 structural domains

The adaptor protein Grb2 contains a SH2 domain flanked by two SH3 domains. Classically, Grb2 is recruited to the activated EGFR through its SH2 domain. This recruits the exchange factor SOS, bound to its SH3 domain, leading to the activation of Ras/MAPK pathway. Other important signaling mediators such as the Gab1 and Shc family of adaptors as well as the ligase Cbl have all been shown to interact with Grb2. Adapted from: (Skolnik, Lee et al. 1993).

I.6.2 Shc family of adaptors

Another important family of adaptors characterize for their role downstream of RTKs is the Shc family of adaptors. It was first identified in a screen for novel SH2-containing proteins. Interestingly, Shc adaptors were discovered due to their high homology (~60%) with the tyrosine kinase, Src (Pelicci, Lanfrancone et al. 1992). They are best known for their role in mediating the activation of the Ras/MAPK and the PI3K/AKT pathways (Ravichandran 2001). Presently, four members of this family of adaptors have been identified and are designated: ShcA, ShcB, ShcC and ShcD (Wills and Jones 2012). While ShcB and ShcC are generally expressed within the central nervous system (CNS), ShcA is more ubiquitously expressed and highly expressed in epithelial cells (Pelicci, Lanfrancone et al. 1992; Nakamura, Sanokawa et al. 1996; O'Bryan, Songyang et al. 1996; Pelicci, Dente et al. 1996). Within the CNS, ShcB and ShcC have been shown to promote the activation of the Ras/MAPK pathway downstream of both the EGFR and Trk receptors, a family of neurotrophins important for the survival of neurons (Sakai, Henderson et al. 2000). This suggests that ShcB/C exert similar effects in CNS as ShcA does in both the CNS and epithelium (van der Geer, Hunter et al. 1994; Sakai, Henderson et al. 2000). As for ShcD, it has been shown

to be expressed in the CNS, muscle, epithelia and bone precursors. However, its role in these tissues has yet to be thoroughly characterized (Hawley, Wills et al. 2011; Wills and Jones 2012). Collectively, the Shc family of adaptors are important regulators of signals downstream of RTKs in a variety of tissues within the human body. As our research focusses on the role of Shc adaptors in mediating ARF1 activity in breast cancer cells, the structure of the ShcA isoforms as well as their functions and role in breast cancer will be detailed below.

I.6.2.1 ShcA isoform structures

The ShcA family is composed of three isoforms: p66Shc, p52Shc and p46Shc. While the three isoforms originate from the same gene, their expression are governed by different transcriptional initiation sites as well as translational start sites (Luzi, Confalonieri et al. 2000). Structurally, the three isoforms high homology, with differences only present within their N-terminus, and are composed of a protein tyrosine binding domain (PTB), a collagen homology domain (CH1) and a SH2 domain (Migliaccio, Mele et al. 1997) (Figure 9). Both the PTB and SH2 domains of ShcA interact with phosphotyrosine residues and have been implicated in its receptor recruitment (Pelicci, Lanfrancone et al. 1992; van der Geer, Wiley et al. 1996; Ravichandran 2001). The PTB and SH2 domains are connected via a CH1 domain which is rich in proline motifs and known to interact with SH3-containing proteins such as Src (Migliaccio, Mele et al. 1997; Luzi, Confalonieri et al. 2000; Ravichandran 2001). Additionally, three tyrosine residues (Y239, Y240 and Y317 in p52Shc) are phosphorylated upon the engagement of ShcA to an activated RTK. Two of these residues (Y239 and Y240) are known to be phosphorylated by Src, whereas the kinase involved in Y317 phosphorylation has yet to identified (Ravichandran 2001). The phosphorylation of these residues has been implicated in the interaction between Grb2 and Shc (van der Geer, Wiley et al. 1996). Indeed, mutation of these residues to alanine blocked the interaction between these two adaptors.

The expression and structure of the p66Shc isoform differs from the other two ShcA isoforms. While p52/46Shc has been reported to be ubiquitously expressed, p66Shc is specifically expressed within the epithelium (Pelicci, Lanfrancone et al. 1992). This isoform also differs from the other two isoforms in that it consists of an additional CH domain (CH2). The CH2 domain is best characterized for its functions in mediating oxidative stress upon the phosphorylation of serine 36 (Migliaccio, Giorgio et al. 1999). Oxidative stress, hydrogen peroxide treatment or irradiation,

promotes the serine phosphorylation of the CH2 domain leading to cellular apoptosis. Indeed, inhibition of this phosphorylation or the depletion of p66Shc attenuate oxidative stress-induced cell death. Interestingly, the knockout of p66shc in mice was shown to enhance the resistance of mice to the treatment with paraquat, an herbicide known to be highly toxic to both humans and animals. Moreover, the lifespan of these knockout mice was prolonged by 30% (Migliaccio, Giorgio et al. 1999). This suggested that p66Shc plays an important role in the aging process.

On another note, p66Shc also has a functional cytochrome C-binding domain (CB) involved in the production of reactive oxygen species (ROS). While the CB domain is also present in the p52Shc isoform, it has been shown to be functionally inactive (Giorgio, Migliaccio et al. 2005). Briefly, ROS-dependent apoptosis is attenuated in p66Shc knockout mice. Interestingly, p66Shc was shown to have redox activity and reduces oxygen to hydrogen peroxide. This leads to the production of ROS and the induction of apoptosis. The redox activity was shown to be dependent on its CB domain and its interaction between cytochrome C (CytC) and p66Shc.

Altogether, the three ShcA isoforms have been shown to be recruited to the EGFR and Grb2 to mediate signals leading to Ras/MAPK activation (Migliaccio, Mele et al. 1997). However, the CH2 and CB domains present in p66Shc regulate the isoform-specific properties of this adaptor, oxidative stress-induced apoptosis through its CH2 domain (Migliaccio, Giorgio et al. 1999) and ROS production and apoptosis through its CB domain (Giorgio, Migliaccio et al. 2005). Finally, p66Shc has been shown to mediate aging and age-related diseases (Migliaccio, Giorgio et al. 1999).

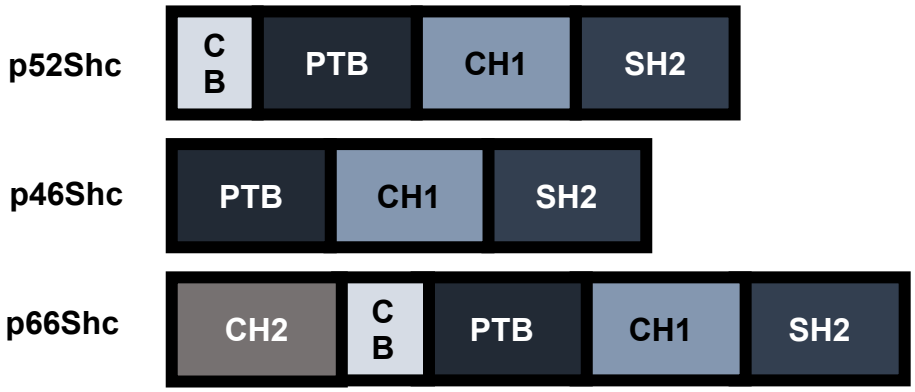

Figure 9. Shc isoforms

All three Shc isoforms (p52Shc, p46Shc and p66Shc) have been shown to be recruited to the EGFR via their protein tyrosine binding domain (PTB) and associate with the adaptor protein Grb2 via three tyrosine residues in their collagen homology 1 domain (CH1). Furthermore, the three isoforms can interact with tyrosine phosphorylated substrates through their SH2 domain. p66Shc differs from the other two isoforms in that it has an additional CH domain (denoted CH2). The phosphorylation of serine 36 in this domain is associated with the mitochondrial translocation and apoptotic functions of this isoform. It also has a cytochrome c-binding domain (CB) that is present but inactive in p52Shc. This domain allows p66Shc to associate with cytochrome c in the mitochondria and mediate the production of reactive oxygen species (ROS). Adapted from: (Alam, Rajendran et al. 2009)

I.6.2.2 p52Shc

Of all the ShcA isoforms, p52Shc is the best characterized for its role in mammary oncogenesis. Clinical studies have demonstrated that increased phosphorylation of Y317 in the CH1 domain of p52Shc is associated with advanced disease and increased risk of relapse in breast cancer patients. Additionally, blocking the recruitment of p52Shc to RTKs significantly delayed mammary tumor onset and progression. Furthermore, preventing the interactions between Grb2 and p52Shc by mutating the tyrosine residues within the CH1 domain of p52Shc was also associated with reduced tumorgenesis. Finally, p52Shc has been shown to promote cell proliferation, migration and invasion, as well as tumor angiogenesis (Dankort, Maslikowski et al. 2001; Saucier, Khoury et al. 2004; Lucs, Muller et al. 2010). Therefore, this Shc isoform has been well established for its role in the promotion of cellular malignancy.

I.6.2.3 p46Shc

While p46Shc and p52Shc are considered to have redundant cellular functions, the literature has described some minor differences between the two isoforms. First, p46Shc has a mitochondrial-targeting domain that isn't present in p52Shc and p66Shc. Indeed, p46Shc has been shown to localize to the mitochondrial matrix (Ventura, Maccarana et al. 2004). However, its role within the mitochondria has yet to be defined Secondly, the affinity of the PTB domain of p46Shc for phosphotyrosine residues has been shown to be 10 times lower than that of p52Shc (Ravichandran 2001). Interestingly, while the PTB-dependent tyrosine phosphorylation of p46Shc downstream of the EGFR has shown to be similar to that of p52Shc, p46Shc is unable to be phosphorylated by both the insulin receptor and the polyoma middle T antigen (Okada, Yamauchi et al. 1995). However, the differences in PTB-dependent phosphorylation amongst Shc isoforms need further investigation. Finally, the region of the CH1 domain of p52Shc and p66Shc known to interact with Src is not present in p46Shc (Sato, Nagao et al. 2002). Indeed, both the *in vitro* and *in vivo* activation of Src was regulated by the expression of p52Shc and p66Shc, but not p46Shc. While the literature may suggest that p52Shc and p46Shc play redundant roles within the cell, the differences between the two isoforms discussed in this section would suggest that p46Shc may have distinct functions. Therefore, future studies are required to better characterize the importance of p46Shc in mediating RTK signaling as well as cancer development and progression.

I.6.2.4 p66Shc

The p66Shc isoform contains the same PTB, SH2 and CH1 domains present in p52Shc and p46Shc. However, p66Shc differs from the other two isoforms in that it has an additional CH domain (CH2) and a functional CB domain (Giorgio, Migliaccio et al. 2005). Unlike p52Shc and p46Shc, the expression of p66Shc is cellular specific (Pelicci, Lanfrancone et al. 1992). This isoform is absent in the brain, most hematopoietic cell lines and peripheral blood lymphocytes. Additionally, its expression varies in different cancer cell lines as observed by an increased expression in invasive prostate and breast cancer cells and a reduced expression in HER2-postive breast cancer cells (Xie and Hung 1996; Stevenson and Frackelton 1998; Jackson, Yoneda et al. 2000; Veeramani, Igawa et al. 2005). All three isoforms have been shown to be recruited to activated RTKs and associate with the adaptor Grb2 (Migliaccio, Mele et al. 1997; Migliaccio, Giorgio et al. 1999). However, while p52Shc and p46Shc have been shown to promote the

activation of the MAPK pathway, p66Shc exerts a negative regulation on RTK-induced MAPK activation (Migliaccio, Mele et al. 1997). In fact, it has been demonstrated downstream of both the EGFR and the insulin growth factor-1 receptor (IGF1R) that p66Shc sequesters the Grb2/SOS complex away from Ras, thus blocking the activation of the Ras/MAPK pathway (Okada, Kao et al. 1997; Xi, Shen et al. 2010). This would suggest that p66Shc is an important mediator of the signals activated by the EGFR and may also play an important function in mediating signals downstream of this receptor. Since we and others have highlighted the importance of ADP-ribosylation factors (ARF) in mediating signals downstream of the EGFR, we will describe the importance of p66Shc in mediating the activity the small GTPases, ARF1 and ARF6, in breast cancer cells in Chapter II. Furthermore, p66Shc associates with Src kinase, another important mediator of RTK signaling, and impairs the activity of this kinase (Xi, Shen et al. 2010). Together, these findings highlight the role of p66Shc as a negative regulator of RTK-dependent signals.

The role of p66Shc in cancer is not well understood. While one would assume that p66Shc would have tumor suppressor properties since it negatively regulates RTK signaling, p66Shc has been associated with a poor prognosis in gastric, colorectal and prostate cancer (Lee, Igawa et al. 2004; Liu, Xie et al. 2014). Additionally, p66Shc has been shown to promote the invasion of both prostate cancer cells as well as hormone-sensitive breast cancer cells (Lee, Igawa et al. 2004; Rajendran, Thomes et al. 2010). Conversely, the expression of p66Shc in breast cancer patients has been associated with a good prognosis and reduced risk a disease relapse in addition to an increased response to hormonal therapies (Davol, Bagdasaryan et al. 2003; Frackelton, Lu et al. 2006). This would suggest that the functions of p66Shc in oncogenesis may be cancer specific. However, further investigation of the role of p66Shc in cancer is needed. Here, in this thesis, we will highlight the importance of p66Shc in mediating TNBC cell proliferation and migration, implicating the small GTPases, ARF1 and ARF6.

I.6.2.5 Mitochondrial p66Shc

The tumor suppressive properties of p66Shc may also stem from its role in apoptosis. While the majority of p66Shc has a cytosolic localization, a subpopulation of p66Shc has been shown to localize within the inter-membrane space of the mitochondria. It has been reported that MEK, JNK and protein kinase C beta (PKCβ) dependent phosphorylation on Serine 36 in its CH2 domain induces the mitochondrial localization of p66Shc (Okada, Kao et al. 1997; Orsini, Migliaccio et al. 2004). Since we found that ARF1 mediates gefitinib-dependent JNK and ERK activity (Chapter III), we depict an interplay between in ARF1 and p66Shc in the regulation of mitochondrial apoptosis. (See Chapter IV). In the mitochondria, p66Shc is maintained in an inactive state through its association with heat shock protein 70 (Hsp70) (Orsini, Migliaccio et al. 2004). In response to apoptotic signals (growth factor deprivation, chemotherapeutic treatment, oxidative stress), p66Shc is released from Hsp70 and promotes the production of reactive oxygen species (ROS) via the reduction of oxygen through its association with CytC (Orsini, Migliaccio et al. 2004; Giorgio, Migliaccio et al. 2005). This results in the opening of mitochondrial pores, the release of cytochrome c and the activation of caspase-dependent apoptosis (Orsini, Migliaccio et al. 2004) (See Figure 10).

Together, the literature suggests that p66Shc has two roles within the cells: 1- A cytoplasmic population that mediates RTK signaling and 2- A mitochondrial population that regulates cellular apoptosis. On the same note, the migratory properties of p66Shc may be mediated by the cytoplasmic pool. Whereas, it regulates cell death through its mitochondrial localization.

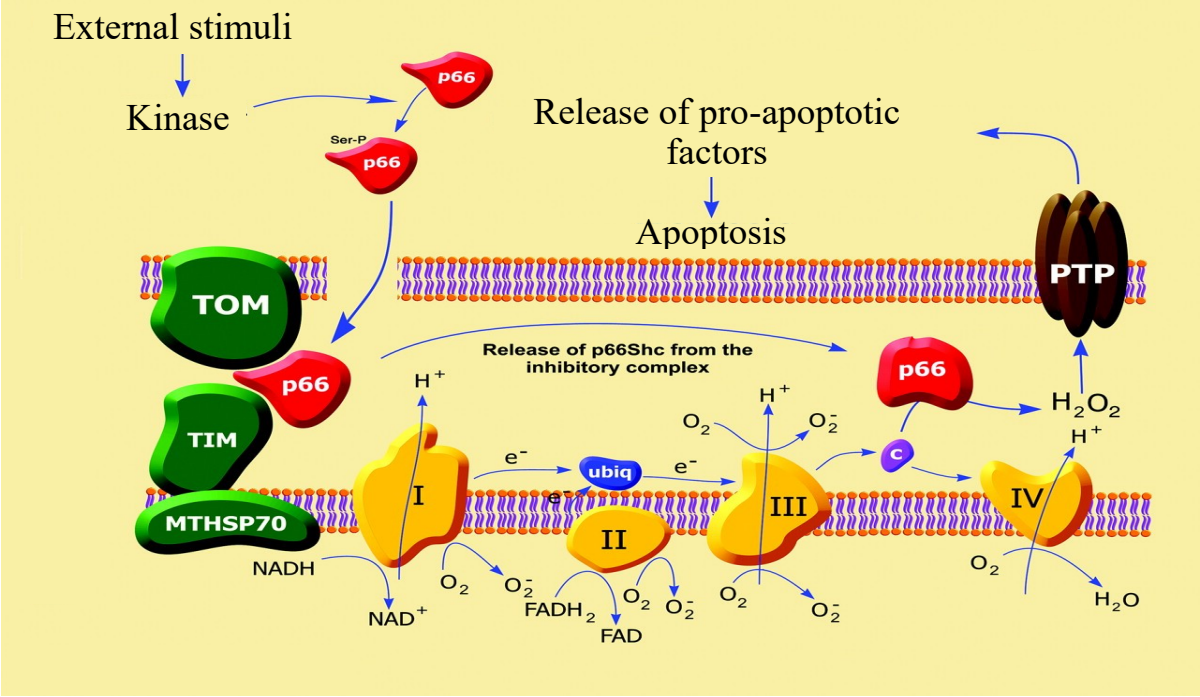

Figure 10. The role of p66Shc in the mitochondria

In response to external stimuli, p66Shc is serine phosphorylated. This allows for its translocation into the mitochondria where it forms an inactive complex with mitochondrial heat shock protein 70 (MTHSP70), transporter outer membrane complex (TOM) and transporter inner membrane complex (TIM). In response to an apoptotic signal, p66Shc is released from this complex, binds and oxidizes cytochrome c and catalyzes the reduction of O_2 to H_2O_2. H_2O_2 opens the mitochondrial permeability transition pore (PTP) leading to the release of pro-apoptotic factors and the induction of apoptosis. Adapted from: (Cosentino, Francia et al. 2008).

I.6.2.6 p66Shc in resistance

Since p66Shc is an important mediator signals downstream of the EGFR, it would be of interest to determine its role in mediating EGFRTKi resistance. However, few studies have evaluated the role of p66Shc in mediating drug resistance. It has been shown that taxol, a taxane chemotherapeutic agent known to disrupt the microtubules, promotes the MEK-dependent serine phosphorylation of the CH2-domain of p66Shc (Yang and Horwitz 2000). This serine phosphorylation is required for p66Shc dependent apoptosis (Rajendran, Thomes et al. 2010). Thus, taxol sensitivity may be regulated by the expression of p66Shc. Additionally, the expression of p66Shc has been shown to

be required for the induction of apoptosis in response to several apoptosis-inducing agents suggesting the p66Shc may sensitize cells to apoptotic inducers and signals (Clark, Faisal et al. 2010; Xiao and Singh 2010; Borkowska, Sielicka-Dudzin et al. 2012; Sakao and Singh 2012). While Shc proteins have been linked to the activation of the Ras/MAPK pathway, a key regulator of EGFRTKi resistance, the role of each Shc isoform in resistance has yet to be evaluated. However, one could hypothesize that apoptotic signals downstream of EGFR inhibition may be moderated by p66Shc expression and activity and we will highlight a possible role in for p66Shc in breast cancer cell sensitivity to EGFR inhibitors in Chapter IV.

I.7 Ras GTPase superfamily

Small GTP-binding proteins (GTPase) are important mediators of cellular function such as cell proliferation, cytoskeletal regulation, membrane trafficking and nucleo-cytoplasmic trafficking. They have been shown to play important roles in multiple physiological events including: embryogenesis, as well as cellular growth, polarity, adhesion, migration and differentiation. Disruption and/or alternation of the normal functions of these GTPases are linked to the development of multiple pathologies, which include several cancers (Williams and Rottner 2010). Therefore, GTPases are of great research interest.

Of the small GTPases, the Ras GTPase superfamily is best characterized for their roles in both normal cellular physiology and cancer. This superfamily is composed of low molecular weight (~20 KDa) monomeric GTP-binding/hydrolyzing proteins. They are considered active when bound to GTP and inactive when bound to GDP. The transition from inactive to active states acts as a switch that transduces extracellular signals to intracellular responses such as cell proliferation, survival, migration and invasion. The deregulation of several members of these GTPases have been associated with oncogenesis as well as the development of other pathological diseases (Goitre, Trapani et al. 2014). The superfamily has been divided into five major families:

A) Ras GTPases: Mediators of organism development, proliferation, differentiation and survival (Haigis, Kendall et al. 2008; Karnoub and Weinberg 2008).
B) Rho GTPases: Regulators of cytoskeletal organization, cell polarity, cell cycle progression and gene expression (Heasman and Ridley 2008).
C) Rab GTPases: Regulators of intracellular vesicular transport and the trafficking of proteins (Zerial and McBride 2001; Grosshans, Ortiz et al. 2006).

D) ARF GTPases: Mediators of vesicular transport and cytoskeletal organization (Donaldson and Jackson 2011).
E) Ran GTPases: Regulators of nucleo-cytoplasmic transport of RNA and proteins (Moore 1998).

Since our research is focused on the ARF GTPases subfamily, the characteristics and functionalities of these members of the Ras GTPase superfamily will be thoroughly discussed below.

I.7.1 ADP Ribosylation Factors (ARFs)

The ARF family of GTPases, and the interest of our laboratory, are low molecular weight proteins that were originally identified for their role as a cofactor for cholera-toxin-catalyzed ADP-ribosylation of the α subunit of heterotrimetric G proteins (Kahn and Gilman 1986). In this pivotal study, cholera toxin-mediated ADP ribosylation of G_s, G protein subunit involved in signaling through the cyclic AMP (cAMP), was shown to be dependent on the presence of a lipid membrane, nicotinamide adenine dinucleotide (NAD), GTP and a membrane-bound cofactor (Moss and Vaughan 1977; Enomoto and Gill 1979; Nakaya, Moss et al. 1980). This cofactor was coined ADP-ribosylation factor (ARF). This cofactor was latter purified from both bovine brain and rabbit liver and was shown to bind radiolabeled GDP and GTP suggesting that it had GTPase activity (Kahn and Gilman 1986). More recently, ARF proteins have been implicated in membrane trafficking and the maintenance of organelle structure (Donaldson and Jackson 2000). While, these GTPases are ubiquitously expressed, the unique cellular distribution (Golgi vs. plasma membrane) and interactions with effector molecules play an important role in defining the function of each ARF protein within the cell (D'Souza-Schorey and Chavrier 2006). There are 6 mammalian ARF proteins that have been identified and divided into three classes based on sequence homology: Class I (ARF1-3), Class II (ARF4, 5) and Class III (ARF6) (Donaldson and Jackson 2000). Class I ARF proteins control the assembly of vesicles in the secretary pathway and activate lipid-modifying enzymes (Bonifacino and Glick 2004). However ARF2 is not present in humans. Meanwhile, the functions of Class II ARFs have yet to be fully elucidated. However, ARF5 may regulate early Golgi transport and recruitment of coat components to the *trans*-Golgi membrane (Claude, Zhao et al. 1999; Takatsu, Yoshino et al. 2002). As for ARF6 (Class III), it regulates

endosomal membrane trafficking and modifies to the actin cytoskeleton (D'Souza-Schorey, Li et al. 1995; Peters, Hsu et al. 1995).

I.7.2 ARF structure

The ARF proteins are characterized by central switch domains (Switch I and Switch II) and an N-terminal amphipathic domain (See Figure 11) (Donaldson and Jackson 2011). They are unique to other small GTPases in that these proteins undergo two conformational changes in response to its GTP loading. Like other GTPases, the Switch I and Switch II domains undergo a conformational change which allows for its association with effector molecules. However, a second conformational change has been described in the amphipathic N-terminal domain of ARF GTPases. This promotes the membrane association of ARF proteins (Randazzo, Terui et al. 1995; Antonny, Beraud-Dufour et al. 1997; Pasqualato, Renault et al. 2002). These studies demonstrated that upon GTP loading of ARF proteins, the N-terminal helix is release from the protein core which allows its hydrophobic residues to interact with the membrane phospholipids, both at the plasma membrane as well as within the Golgi (Antonny, Beraud-Dufour et al. 1997). Therefore, GTP-loading promotes both the recruitment of signaling effectors and the transition from a cytosolic localization to membrane structures (Antonny, Beraud-Dufour et al. 1997; Donaldson and Jackson 2011). Additionally, ARF GTPases are myristoylated at their N-terminus. This myristoylation, an irreversible addition of a lipid, myristoyl group to the N-terminal glycine residues, is important for both the membrane recruitment and biological activity of these GTPases (Randazzo, Terui et al. 1995; Chavrier and Menetrey 2010). Both the membrane localization and N-terminal myristoylation play an important role in mediating the loading of GTP to ARF proteins. Indeed, a higher affinity for GTP was observed for myristoylated ARFs in the presence of phospholipids compared to this myristoylated GTPase in the absence of phospholipids (Randazzo, Terui et al. 1995). Together, the GTP-loading of ARF GTPases is dependent on multiple processes that include a conformational change in the interswitch domains, membrane localization and n-terminal myristoylation.

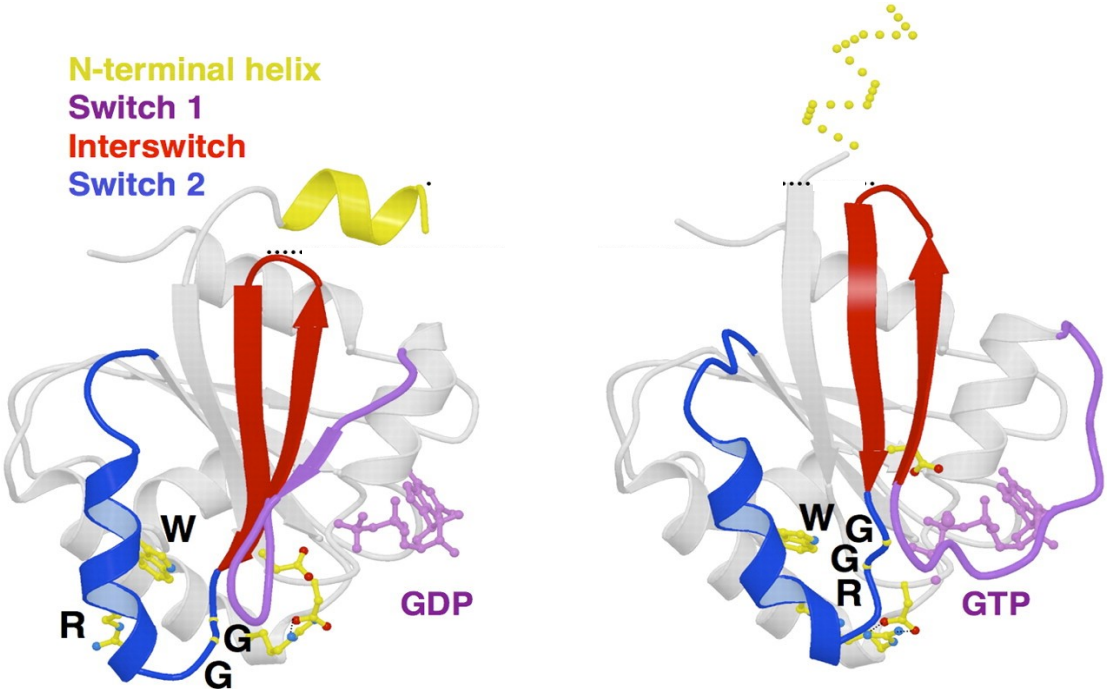

Figure 11. The structure of ARF GTPases

The above figure depicts the 3D structure of inactive, GDP-bound (Left side) and active, GTP-bound (Right side) ARF6. In its GDP-bound state, the interswitch domain (Red) of ARF proteins is retracted and fastened by its N-terminal helix (Yellow). When activated, a large conformational change involving a two residue β-strand shift results in an exposed conformation. The W/GG/R signature stabilizes this conformation and this allows the nucleotide-binding site to detect interactions with the N-terminal of the ARF GTPase. Adapted from: (Kahn, Cherfils et al. 2006).

I.7.3 ARF activation

Like all small GTPases, ARF proteins are considered in their active state when bound to GTP and their inactive state when GDP-bound. The transition from inactive to active state is moderated by guanine exchange factors (GEFs) and the inactivation of ARF proteins is regulated by GTPase activating proteins (GAPs) (Figure 12). Furthermore, the membrane association of ARF proteins is essential for their activation (Antonny, Beraud-Dufour et al. 1997). While several ARF-specific GEFs and GAPs have been shown to interact with one or more ARF protein, *in vitro*, *in vivo* analysis has demonstrated that distinct ARFGEFs and ARFGAPs mediate the GDP/GTP transition of individual ARF GTPases (Jackson and Casanova 2000; Randazzo and Hirsch 2004). Listed below is a brief description of the identified ARFGEFs and ARFGAPs.

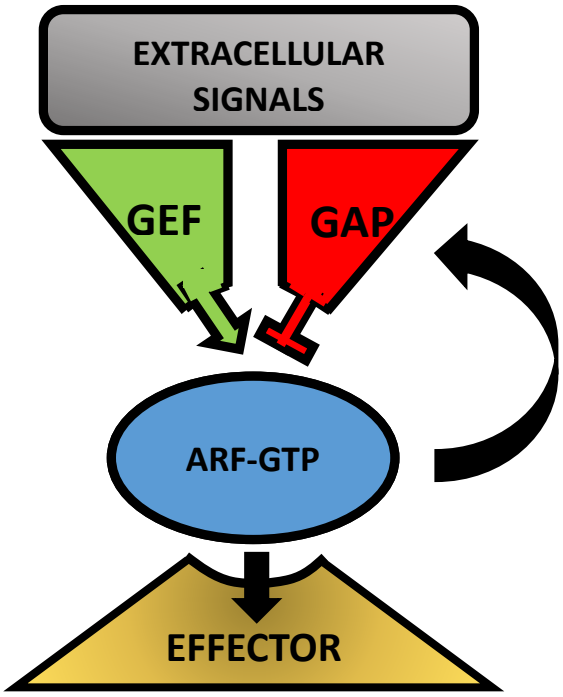

Figure 12. Regulation of ARF activity

ARF GTPases are considered inactive when bound to GDP and active when loaded with GTP. In response to extracellular signals, guanine exchange factors (GEF) mediate the enzymatic reaction that removes the GDP from small GTPase and adds GTP. Conversely, GTPase activating proteins (GAP) reduce GTP-bound ARF GTPases to the inactive GDP-bound form. Upon ARF activation (GTP-loading), the GTPase associates and mediates its effector molecules leading to the initiation of downstream signaling. Interestingly, GAPs can act like effectors. This is implicated in a negative feedback loop that regulates ARF activity. Adapted from: (Otey, Goicoechea et al. 2009)

I.7.3.1 ARFGEFs

All ARFGEFs contain a conserved Sec7 domain that catalyzes the GDP release and GTP binding on their target ARF protein. Briefly, the Sec7 domain is named for its homology with the yeast protein Sec7p, which was identified in a screen for protein secretion defects. There are presently 15 identified ARFGEFs in humans divided in 5 families based on overall structure and domain organization: 1- Golgi Brefeldin A (BFA)-resistance factor 1/BFA-inhibited GEF (GBF/BIG), 2- ARF nucleotide binding site opener (ARNO/Cytohesin), 3- Exchange factor for ARF6 (EFA6), 4- BFA-resistant ARFGEF (BRAG) and 5- F-box only protein 8 (FBX8) (Casanova 2007). The GBF/BIG GEFs are localized to the Golgi and regulate the activity of both class I and class II ARFs (Togawa, Morinaga et al. 1999). Whereas, the cytohesins are primarily localized to the cell

periphery and can be recruited to the plasma membrane through their PH domain. While this subtype of GEF favors the activation of Class I ARFs, it has been reported to also mediate ARF6 activity (Klarlund, Guilherme et al. 1997; Frank, Upender et al. 1998; Venkateswarlu, Gunn-Moore et al. 1998; Santy and Casanova 2001). The third and fourth GEF subgroups, EFA6 and BRAG, are localized to the plasma membrane and selectively activate ARF6 (Franco, Peters et al. 1999; Someya, Sata et al. 2001). Finally, the functions of FBX8 remain uncharacterized (Casanova 2007). Of interest, Cytohesin 2 (ARNO) has been demonstrated to mediate the dimerization of the EGFR. Indeed, the overexpression of Cytohesin 2 was shown to enhance EGFR signals in lung cancer cells (Bill, Schmitz et al. 2010). This would suggest that the activity of ARF GTPase may also mediate EGFR dynamics. While we have shown that ARF1 does not affect the activity, localization and internalization of the EGFR upon EGF stimulation (Boulay, Cotton et al. 2008), we highlight the importance of ARF1 in mediating EGFR internalization and degradation in breast cancer cells treatment with the EGFR inhibitor, gefitinib, in Chapter III of this theses. Moreover, we also show that ARF1 also mediates the activation and dimerization of EGFR family members in cells threated with this inhibitor (Chapter IV).

I.7.3.2 ARFGAPs

ARFGAPs catalyze the release of GTP from their target ARF isoform. Briefly, At least 24 ARFGAPs have been identified in humans and are divided into two subfamilies: 1- ARFGAP1 type and 2- AZAP type (Inoue and Randazzo 2007). The ARFGAP1s are divided into three subtypes: 1- ARFGAPs which mediate Golgi functions by acting on the ARF1 isoform (Tanigawa, Orci et al. 1993; Cukierman, Huber et al. 1995), 2- SMAPs which are both ARF1 and ARF6 GAPs and have been shown to mediate ARF6-dependent endocytosis (Tanabe, Torii et al. 2005; Natsume, Tanabe et al. 2006) and 3- GITs which regulate ARF6 activity, membrane trafficking and focal adhesions (Hoefen and Berk 2006). The AZAPs are divided into four subtypes: 1- ASAPs which mediate ARF1 activity, focal adhesions, invadopodia and podosomes (Randazzo, Andrade et al. 2000; Oda, Wada et al. 2003), 2- ACAPs which are ARF6 GAPs involved in cytokinesis, cell migration and actin cytoskeleton remodeling (Jackson, Brown et al. 2000), 3- ARAPs characterized for their role in mediating ARF1, 5 and 6 activity as well as membrane protrusions (Krugmann, Anderson et al. 2002; Krugmann, Andrews et al. 2006; Yoon, Miura et al. 2006) and

4- AGAPs that regulate ARF1 activity and may serve as a link between the endocytic pathway and the actin cytoskeleton (Nie, Stanley et al. 2002; Nie, Fei et al. 2005).

Together, the activation and inactivation of ARF GTPases are complex mechanisms involving the interplay of several GEFs and GAPs. However, understanding these mechanisms could help identify new therapeutic targets and develop novel cancer therapies.

I.7.3.3 Brefeldin A – ARFGEF inhibitor

There are currently developed ARF inhibitors available. Of these inhibitors, Brefeldin A (BFA), an ARF inhibitor of fungal origin is the best characterized within the literature. Structural studies have demonstrated that BFA binds the interface between ARF-GDP and the Sec7 domain of BFA-sensitive GEFs locking ARFs in an inactive state (Peyroche, Antonny et al. 1999; Renault, Guibert et al. 2003). Its actions are specific to Class I ARFs, specifically ARF1, as the activation of ARF6 is unaffected by BFA treatment (Menetrey, Macia et al. 2000). Physiologically, BFA has been shown to block the secretion of newly synthesized proteins from the endoplasm reticulum. This stems from morphological changes in the Golgi leading to its disassembly (Fujiwara, Oda et al. 1988; Lippincott-Schwartz, Yuan et al. 1989; Klausner, Donaldson et al. 1992). This process has been shown to have cytotoxic effects on several cancer cell lines (Ishii, Nagasawa et al. 1989). Indeed, BFA treatment induced the cellular death of lung, bladder, skin and gastric carcinoma cells, as well as in cellular models of leukemia. While this inhibitor has shown to be effective in the laboratory, multiple factors have hindered its progression into clinical trials. For instance, it has been associated with a poor solubility in biological fluids, an undesirable pharmacokinetic profile and neurotoxicity in animal models. This neurotoxicity was shown to originate from its ability to dissemble the Golgi (Dinter and Berger 1998; Kikuchi, Shinpo et al. 2003). However, its apoptotic properties have been shown to be independent of its actions within the Golgi (Lippincott-Schwartz, Glickman et al. 1991). While this inhibitor is plagued by its non-specificity and toxicity, it remains a useful tool used within a laboratory setting to evaluate the functions of ARF GTPases.

Overall, the activation of ARF GTPase is dynamic involving the interplay of ARFGEFs and GAPs. While inhibitors have been developed to regulate the activity of these GTPases, the pharmacological properties and toxicity have hindered their clinical applications. Therefore, extensive research is required to improve the understanding of the mechanisms of ARF activation

and to develop improved therapeutic tools. A novel mechanism of both ARF1 and ARF6 activation will be discussed in Chapter II and a thorough description of these two ARF isoforms is found below.

I.7.4 ARF1

Within the literature, the ARF isoforms, ARF1 and ARF6, are the best described. Here, I will provide a detailed description of ARF1. Whereas, ARF6 will be discussed in the next section (I.7.5). GDP-bound ARF1 is generally localized in the cytosol, whereas, activated ARF1 is primarily found in the Golgi. It is here where this ARF isoform regulates the secretion between this organelle and the endoplasmic reticulum. Briefly, the secretory pathway allows for the transport of proteins, carbohydrates and lipids within the cell. This pathway is made up of primarily the endoplasmic reticulum, the Golgi and the plasma membrane. The secretory cargo (proteins) are synthesized within the endoplasmic reticulum and are transported to the Golgi for further processing and maturation. The matured cargo is sorted and packaged within the trans-Golgi and then transported to their target location (i.e. plasma membrane) (Lippincott-Schwartz, Roberts et al. 2000). Within the Golgi, ARF1 mediates the recruitment of the vesicle coating protein complex, coat protein complex 1 (COPI), to the pre- and *cis*-Golgi structures. Briefly, there are two types of vesicles that mediate transport between the Golgi and the endoplasmic reticulum, COPII and COPI. While COPII vesicles are required for the concentration and export of secretory cargo from the endoplasmic reticulum (Kuehn, Herrmann et al. 1998; Matsuoka, Morimitsu et al. 1998), COPI vesicles mediate the retrograde transport of recycled cargo returning from the Golgi to the ER (Letourneur, Gaynor et al. 1994; Pelham 1994; Cosson and Letourneur 1997). Therefore, ARF1 is an important regulator of the transport from the Golgi back to the endoplasmic reticulum (Pepperkok, Whitney et al. 2000). Additionally, this GTPase recruits the adaptor proteins of clathrin-coated vesicles (AP1, AP3 and AP4) and the ubiquitous coat protein (GGA3) to the *trans*-Golgi and endosomal membrane (Bonifacino and Glick 2004). AP1 and AP4 are implicated in the bidirectional transport between the trans-Golgi and endosomes (Bonifacino and Traub 2003; Hirst, Irving et al. 2013), whereas, AP3 mediates the transport between the endosomes and the lysosome-related organelles (Dell'Angelica 2009). Meanwhile, GGA3 mediates clathrin assembly, intracellular transport and plasma membrane trafficking (Puertollano and Bonifacino 2004). Of interest, GGA3 has also been implicated in RTK internalization. Indeed, GGA3 was shown to

associate with the cMET receptor and promote the internalization and recycling of this receptor (Parachoniak, Luo et al. 2011). Therefore, ARF1 may mediate the internalization of RTKs through its regulation of GGA3.

In addition to its role in vesicle secretion, activated ARF1 has also been shown to stimulate the assembly of both spectrin and the actin cytoskeleton on the Golgi membrane (Godi, Santone et al. 1998; Fucini, Navarrete et al. 2000). Spectrin, involved in the maintenance of the Golgi organization and possibly vesicle secretion, is assembled through the ARF1-dependent activation of type I phosphatidylinositol-4-phosphate-5-kinase (PIP5K) (Godi, Santone et al. 1998; Jones, Morris et al. 2000). Whereas, actin cytoskeleton assembly was shown to be mediated by actin-related proteins 2- and 3- (Arp2/3) dependent actin polymerization involving the activity of CDC42 and its downstream effector Wiskott-Aldrich syndrome protein (WASP) (Chen, Lacomis et al. 2004; Matas, Martinez-Menarguez et al. 2004). Together, within normal cells, ARF1 plays an important role in Golgi function and organization.

I.7.5 ARF6

Unlike ARF1, ARF6 does not play a regulatory role in the Golgi. Instead, this ARF isoform is localized to the plasma membrane and endosomal compartments and regulates membrane trafficking and actin cytoskeletal remodeling. Briefly, the endocytic pathway is involved in the uptake of macromolecules within the cell. Endocytosis is the process through which material from the cells environment or plasma membrane are internalized into the cell. Upon internalization, the cargo is transported within endosomes to the lysosome for degradation or recycled back to the plasma membrane or the *trans*-Golgi (Besterman and Low 1983; Soldati and Schliwa 2006; Mayor and Pagano 2007). In the endocytic pathway, ARF6 regulates phospholipid metabolism by activating both type I PIP5K and phospholipase D (PLD). This leads to the accumulation of PI (4, 5) P2 and the induction of clathrin-mediated endocytosis (Brown, Gutowski et al. 1993; Honda, Nogami et al. 1999; Wenk and De Camilli 2004). This process has been implicated in the internalization of GPCRs (Claing, Chen et al. 2001). ARF6 also mediates clathrin-independent endocytosis. Here, the inactivation of this GTPase promotes the trafficking throughout this pathway (Brown, Rozelle et al. 2001; Donaldson 2003). Additionally, ARF6 controls endosomal recycling (D'Souza-Schorey, van Donselaar et al. 1998).

ARF6 also modulates the actin cytoskeleton to form pseudopods, membrane ruffles, comet-tails on endosomes, structures involved in driving movement, as well as promote cell spreading, cell migration and phagocytosis (D'Souza-Schorey and Chavrier 2006). These processes are governed by the control of Rac1 activation by ARF6 (Radhakrishna, Al-Awar et al. 1999; Boshans, Szanto et al. 2000). Furthermore, ARF6-dependent Rac1 activity is correlated with a decreased Rho activity (Boshans, Szanto et al. 2000). This is important to promote cellular motility. This ARF isoform has also been shown to modulate the cytoskeleton through its regulation of the proteasome. However, the connection between the proteasome and actin remodeling has yet to be well defined (Rangone, Pardo et al. 2005; D'Souza-Schorey and Chavrier 2006). Finally, ARF6 remodels the actin cytoskeleton by regulating the lipid metabolism of $PI(4,5)P_2$ through the activation of both PLD and PIP5K (Hilpela, Vartiainen et al. 2004).

Altogether, in normal cells, ARF1 plays an important role in mediating the secretory pathway, whereas, ARF6 regulates the endocytic pathway. Moreover, ARF6 also mediates the organization of the actin cytoskeleton. This suggests that these two ARF isoforms have distinct functions within the cell.

I.7.6 ARF GTPases and breast cancer

While ARF1 and ARF6 exert distinct functions within non-cancerous cells, we and others have demonstrate that within invasive breast cancer cells, these two GTPases, both exert similar oncogenic properties. Recently, our laboratory has demonstrated that ARF1 is activated downstream of the EGFR in invasive breast cancer cells (MDA-MB-231, SKBR3, HCC70 cells) (Boulay, Cotton et al. 2008; Haines, Saucier et al. 2014). We have shown that ARF1 signaled through the PI3K/AKT pathway and mediated breast cancer cell proliferation, migration and invasion (Boulay, Cotton et al. 2008; Schlienger, Campbell et al. 2014). More specifically, ARF1 regulated cell proliferation by the induction cell-growth arrest and cellular senescence via a retinoblastoma protein (Rb)-dependent mechanism (Boulay, Schlienger et al. 2011). Whereas, the ARF1-dependent control of cell migration and invasion acted through the regulation of the small GTPases Rac1 and RhoA/C, respectively (Lewis-Saravalli, Campbell et al. 2013; Schlienger, Campbell et al. 2014). Furthermore, ARF1 has been demonstrated to mediate cancer cell invasion downstream of another RTK, ephrin-B1 (Tanaka, Sasaki et al. 2007). Additionally, pharmacologically targeting ARF1 with BFA has been shown to be cytotoxic in several cancer

cells. However, the poor bioavailability of this ARF1 inhibitor has hindered their progression into clinical trials (Ohashi, Iijima et al. 2012). We also have preliminary data that suggests that ARF1 expression is increased in patients with invasive TNBC and HER2-positive breast cancer (unpublished data). Additionally, ARF1 has also been shown to be overexpressed in both in gastric and ovarian carcinomas (Bani, Nicoletti et al. 2004; Tsai, Lin et al. 2012).

Similar to ARF1, ARF6 has also been implicated in breast cancer. It has been shown to signal downstream of the EGFR through the MAPK pathway (Tague, Muralidharan et al. 2004; Morishige, Hashimoto et al. 2008). It promotes breast cancer cell proliferation, migration, invasion, adhesion, angiogenesis and metastasis (Sabe 2003; Sabe, Hashimoto et al. 2009; Hashimoto, Hashimoto et al. 2011; Knizhnik, Kovaleva et al. 2012). Furthermore, the expression of ARF6, its GEF, GEP100 (BRAG2), and a downstream effector, AMAP1, have all been associated with a poor prognosis in breast, lung and head and neck cancer patients (Sabe, Hashimoto et al. 2009; Oka, Uramoto et al. 2014; Sato, Hatanaka et al. 2014). Additionally, they have also been linked to cancer reoccurrence following surgery to partially remove the affected region of the breast, known as breast conservative therapy (Kinoshita, Nam et al. 2013).

In summary, in normal cells, ARF1 regulates the secretory pathway within the Golgi and ARF6 mediates the endocytic pathway and actin cytoskeletal organization at the plasma membrane. However, in breast cancer cells both isoforms are localized to the plasma membrane and mediate oncogenic processes such as cell proliferation, migration and invasion.

I.7.7 Other ARF isoforms

While less characterized in the literature, interesting findings have been documented for other ARF isoforms (ARF3-5). Similar to ARF1, ARF3 has been shown to localize to the Golgi. However, it selectively localizes the trans-Golgi (Manolea, Chun et al. 2010). Whereas, ARF1 is not specifically localized to this region of the Golgi. Additionally, ARF3, along with ARF4, has been recently shown to mediate the integrity of recycling endosomes (Kondo, Hanai et al. 2012; Nakai, Kondo et al. 2013). Meanwhile, ARF4 and ARF5 have been shown to localize to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) and mediate COPI vesicle formation within the Golgi (Chun, Shapovalova et al. 2008; Popoff, Langer et al. 2011).

Of these three ARF isoforms, ARF4 is of most interest in terms of cancer. Para-methoxyamphetamine (PMA), a selective serotonin releasing agent, has been shown to enhance

the expression of ARF4 in glioblastoma cells. This increased ARF4 expression was associated with enhanced migration of these cells (Jang, Jang et al. 2012). Thus, ARF4 expression in glioblastoma cells exerts similar effects that ARF1 and ARF6 have been shown to exert in breast cancer cells. Additionally, ARF4 has been shown to promote the survival of glioblastoma cells. This was measured by a decrease in apoptotic signals through p38MAPK and JNK, decreased mitochondrial translocation of Bax, CytC release and caspase 3 activity. Also, a decrease in ROS production, a mediator of mitochondrial apoptosis, was observed in ARF4 overexpressing cells (Woo, Eun et al. 2009). Interestingly, we observed a similar role for ARF1 in mediating gefitinib-induced apoptosis in breast cancer cells (See Chapter IV).

Altogether, ARF1/6 and ARF4 mediate important oncogenic processes in breast cancer cells and glioblastoma cells, respectively (Boulay, Cotton et al. 2008; Sabe, Hashimoto et al. 2009; Woo, Eun et al. 2009; Jang, Jang et al. 2012; Haines, Saucier et al. 2014). This suggests that targeting the ARF family of GTPase may have significant therapeutic benefits in cancer patients. However, further characterization of these GTPases is needed. Since ARF GTPases have been implicated in the activation of classical signaling cascades, we will discuss the important signaling pathways involved in breast oncogenesis in detail below.

I.8 Signal transduction

Cells are highly responsive to their environment and transduce external stimuli into internal cellular responses. This process has been coined signal transduction. The majority of signal transduction cascade originate at the membrane through an interaction between an external stimulus such as a ligand, growth factor, hormone or inhibitor with a membrane-bound receptor, in our case the EGFR (Figure 13). The signal is then relayed through the cell via second messengers, adaptors and other signal mediators to the nucleus where it promotes the transcription of target genes. Phosphorylation is the major means through which signals are propagated from the receptor to nucleus. The disruption of normal signal transduction cascades has been well documented to play a role in oncogenesis. Described below are signaling pathways that mediate cellular responses in breast cancer. Furthermore, inhibitors of these signaling cascades as well as their role in EGFRTKi resistance are also discussed.

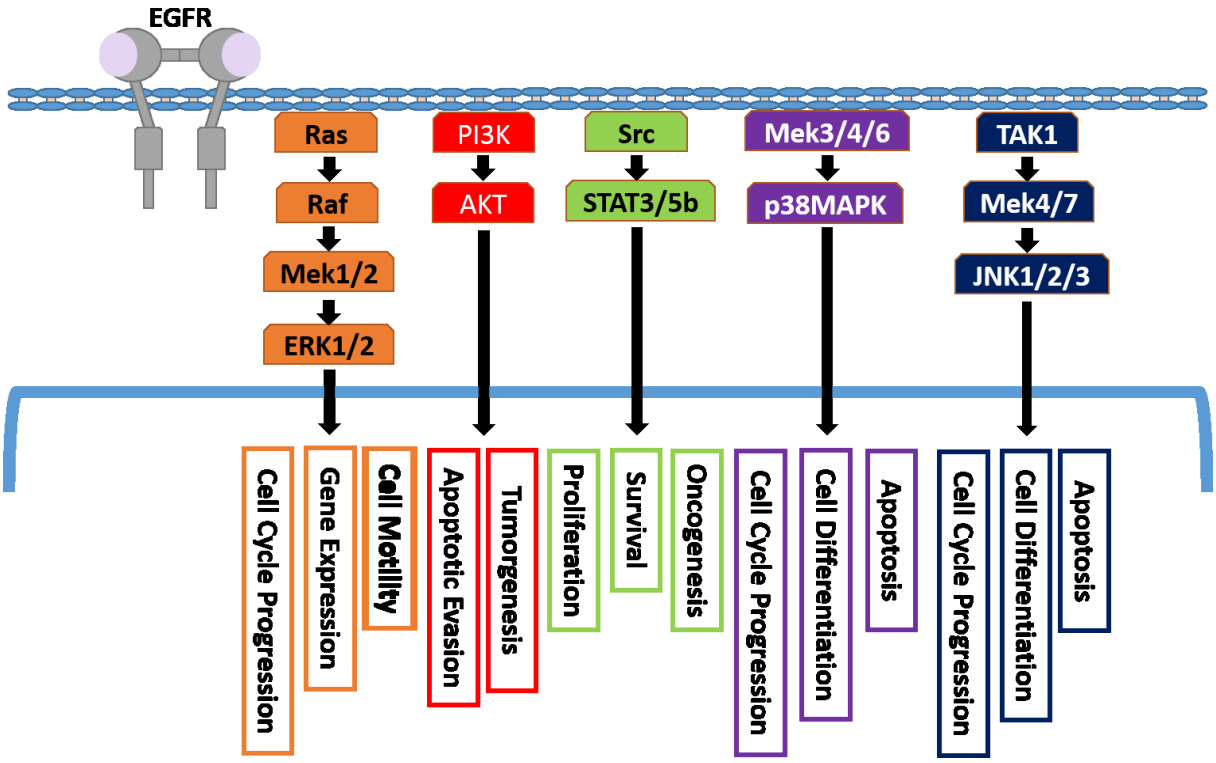

Figure 13. Signaling cascades activated downstream of the EGFR in cancer

Upon the activation of the EGFR, several signaling cascades can be initiated. 1- The Ras/MAPK (ERK1/2) pathway identified in a variety of oncogenic processes such as cell motility and cell cycle progression; 2- The PI3K/AKT pathway best characterized for its role in cancer cell survival; 3- Src kinase, a non-receptor tyrosine kinase, known to activate multiple signaling cascades such as the Signal transducer and activator of transcription (STAT) pathway to promote cell survival and proliferation. Additionally, Src kinase has been reported to tyrosine phosphorylate the EGFR to enhance receptor activation; 4- p38MAPK pathway and 5- JNK pathway are both known to regulate apoptosis, cell differentiation and cell cycle progression. Adapted from: (Nyati, Morgan et al. 2006; Roberts and Der 2007; Wagner and Nebreda 2009)

I.8.1 Ras/ERK1/2 pathway

Signal transduction is an essential in coordinating the normal functions within a cell. Over decades of research in cell signaling, the Ras/MAPK pathway has emerged as a central mediator of cell growth and survival. The extracellular-regulated kinases 1 and 2 (ERK1/2) or the mitogen-activated protein kinase (MAPK) pathway is activated upon RTK activation (Shaw and Cantley 2006). Classically, the adaptor Grb2 is recruited to an activated receptor bringing the GEF, SOS, into the proximity of the small GTPase Ras (Downward 2003; Shaw and Cantley 2006; Katz, Amit et al. 2007). Activated Ras interacts and activates RAF protein kinases, which in turn phosphorylate the MAPK kinases, MEK1 and MEK2 (Downward 2003; Katz, Amit et al. 2007). These MEK isoforms then activate ERK1 and ERK2 leading to their translocation into the nucleus where they promote the transcription of genes involved in cell proliferation, survival, migration and invasion (Downward 2003; Katz, Amit et al. 2007). In cancer, activation of the MAPK pathways occurs in response to constitutive activation of the EGFR as a result of somatic mutations, gene amplification and/or autocrine/paracrine signaling (Downward 2003; Katz, Amit et al. 2007). Additionally, mutations in other components of this signaling cascade such as the *RAS*, *BRAF* and *MEK* genes have also been implicated in cancer (Katz, Amit et al. 2007). However, mutations of these signaling mediators are rarely present in breast cancer patients (Normanno, Tejpar et al. 2009; Neuzillet, Tijeras-Raballand et al. 2014). The constitutive activation of this signaling cascade is commonly found in several cancers and has been shown to promote multiple oncogenic responses such as the transformation of mammalian cells, uncontrolled cell proliferation and resistance to apoptosis. Indeed, the inhibition of this pathway, either *in vitro* or *in vivo*, has been shown attenuate tumor growth, reduce cancer cell invasiveness and induce apoptosis (Wang, Boerner et al. 2007). Therefore, this pathway is an interesting therapeutic target in human malignancies.

I.8.1.1 MEK inhibitors

Since the Ras/MAPK pathway is often activated in cancer patients and plays an important role in cancer development and progression (McCubrey, Steelman et al. 2007), this pathway is a therapeutic target in cancer patients. Several MEK inhibitors are currently in development. In fact, some MEK inhibitors have been shown to be good potential therapies for some cancers, especially BRaf-mutated melanomas and BRaf/KRas-mutated colorectal cancers (Wang, Boerner et al. 2007). The benefit of these inhibitors is that unlike most kinase inhibitors, the majority of MEK inhibitors do not target the ATP-binding site, conferring high specificity (Wu and Park 2015). Instead, these inhibitors target the region around the ATP-binding site and lock MEK in a conformation that is unable to associate with ERK and activate it (Chappell, Steelman et al. 2011). Additionally, while EGFR inhibitors lose their activity in tumors with EGFR, Ras or Raf mutations, MEK inhibitors retain their activity (Wu and Park 2015). Indeed, co-treatment of EGFR-mutated lung cancer cells with an EGFR and MEK inhibitor re-sensitized cells to EGFR inhibition, induced apoptosis and attenuate tumor growth *in vivo* (Huang, Lee et al. 2013). Moreover, MEK inhibitors have also shown to reduce tumor growth of both KRas- and BRaf-mutant cancers (Hatzivassiliou, Haling et al. 2013). While MEK is a promising target in cancer, recent clinical trials have failed. The primary reasons for their poor success include a low bioavailability, rapid drug metabolism, increased toxicity and the development of drug resistance (Rinehart, Adjei et al. 2004; LoRusso, Krishnamurthi et al. 2010; Wagle, Emery et al. 2011). Therefore, targeting upstream mediators leading to ERK1/2 activation in cancer cells may have therapeutic benefits. Interestingly, the ARF proteins have recently been shown to mediate the activation of this pathway, see Chapter III, (Morishige, Hashimoto et al. 2008) and thus the pharmacological inhibition of this family of GTPases may show important biological responses in cancer patients. However, substantial research is required to better characterize the therapeutic benefits of targeting ARF GTPases.

I.8.1.2 ERK1/2 pathway and EGFRTKi resistance

The activation of the ERK1/2 pathway, an important mediator of oncogenesis, has been shown to be elevated in cancers that are resistant to EGFR inhibition (Chong and Janne 2013). Moreover, with the emergence of the importance of KRas mutations in the development of EGFRTKi resistance, there are presently clinical studies evaluating the efficacy and safety of targeting the MEK kinases in EGFRTKi-resistant cancers (Brand, Iida et al. 2011). However, KRas mutations are relatively rare in triple negative breast cancer patients. Yet, these patients are known to have an increased signaling through the MEK kinases that occurs through EGFR-independent mechanisms (Hoeflich, O'Brien et al. 2009; Mirzoeva, Das et al. 2009). Indeed, EGFRTKi-resistant cancers have increased and persistent MEK/ERK1/2 activity (McCubrey, Steelman et al. 2007). Furthermore, the treatment of gefitinib-resistant breast cancer cells with persistent ERK activation with a MEK inhibitor restored gefitinib sensitivity (Normanno, Campiglio et al. 2008). In this study, the overexpression of activated ERK1 was associated with the development of gefitinib resistance in breast cancer cells. In fact, the IC_{50} of gefitinib increased by approximately 5-fold in these cells. In addition, the combinational therapy of EGFR and MEK inhibitors has also been shown to be effective in the treatment of EGFRTKi-resistant colorectal and lung cancer cells (Ercan, Xu et al. 2012; Troiani, Napolitano et al. 2014).

Altogether, the Ras/MAPK pathway plays an important role in mediating cancer cell proliferation and survival. Inhibitors to this pathway are currently under development and being tested within clinical trials. However, there are many limitations to the uses of these inhibitors. Finally, increased activation of this pathway is associated with resistance to EGFR inhibitors and the treatment of EGFR inhibitor-resistant cancers with MEK inhibitors can re-sensitize cells to EGFR inhibition.

I.8.2 PI3K/AKT pathway

Another important pathway well characterized for its role in oncogenesis, is the PI3K/AKT pathway. It is best known for its role in the cancer cell survival, but has been implicated in other oncogenic processes. The phosphatidylinositol-4, 5-biphosphate 3 kinases (PI3K) are a family of kinases that phosphorylate the 3'-hydroxyl group of phosphoinositides (Yuan and Cantley 2008). They are activated upon RTK activation. Briefly, the SH2 domain of the p85 regulatory subunit of PI3K binds to the phosphorylated tyrosine residues of RTKs and/or adaptor proteins recruited to RTKs (Carpenter, Auger et al. 1993; Lam, Carpenter et al. 1994). This activates the p110 catalytic domain of PI3K which phosphorylates PIP_2 to form PIP_3. Protein phosphatase and known tumor suppressor (PTEN) negatively regulates the conversion of PIP_2 to PIP_3. Protein kinase B (AKT) is recruited to PIP_3 via its pleckstrin homology (PH) domain and is activated by 3-phosphoinositide-dependent kinase 1 (PDK1) and/or the second mammalian target of rapamycin (mTor) complex (mTORC2) (Massacesi, Di Tomaso et al. 2016). Activated AKT has been shown to primarily mediate cell survival (Datta, Dudek et al. 1997). In cancer, mutations or amplification of the PI3K catalytic gene *PIK3CA* and/or *AKT* and the loss of PTEN are commonly associated with mitogenesis. In fact, mutations in the *PIK3CA* gene have been identified in approximately a quarter of breast cancer patients (Liu, Cheng et al. 2009). Moreover, the activation of the PI3K/AKT pathway mediates breast cancer cell survival, migration, motility and the formation of metastasis in animal models of breast cancer (Arboleda, Lyons et al. 2003; Hutchinson, Jin et al. 2004). Indeed, the overexpression of AKT2 was shown to promote the invasiveness of both breast cancer and ovarian cancer cells. This invasiveness was hindered upon the treatment of PI3K inhibitors or by the overexpression of PTEN (Arboleda, Lyons et al. 2003). Moreover, the co-expression of HER2 and AKT1 produced mammary tumors in mice earlier than mice that only overexpressed HER2. However, these tumors were found to be less invasive compared to HER2-alone tumors (Hutchinson, Jin et al. 2004). This would suggest that while AKT2 promotes cancer cell invasion, the oncogenic properties of AKT1 are restricted to the induction of tumor cell proliferation. Furthermore, we have shown that ARF1, an important mediator of breast cancer cell proliferation, migration and invasion, signals through AKT activation (Boulay, Cotton et al. 2008; Lewis-Saravalli, Campbell et al. 2013; Schlienger, Campbell et al. 2014). This would suggest that ARF1 could activate both AKT1 (proliferation) and AKT2 (invasion). However, further examination is required.

I.8.2.1 PI3K inhibitors

With the PI3K/AKT pathway being the most frequently activated pathway in cancer, there are have been many attempts at targeting this pathway in patients. In fact, 30-50% of breast tumors have increased activation of this pathway. However, the importance of PI3K activation is more prevalent in ER-positive breast cancer compared to TNBC (Bachman, Argani et al. 2004; Cerami, Gao et al. 2012). With such an important role in a variety of cancers, this pathway is the focus of many clinical trials. Indeed, clinical trials for inhibitors of PI3K, AKT and mTORC2 are currently underway (Bauer, Patel et al. 2014). Furthermore, inhibitors of mTORC1 are approved for the treatment of several cancers (Bauer, Patel et al. 2014). While mTORC1 inhibitors have had clinical success, other than in hematologic malignancies, targeting upstream modulators such as PI3K and AKT has shown disappointing results (Bauer, Patel et al. 2014). The main reason for the lack of effectiveness of these inhibitors in the treatment of cancer is the doses required to significantly modulate this pathway in cancer patients, are associated with relatively severe adverse effects. Thus, drug tolerability is a major pillar for PI3K inhibitors (Bauer, Patel et al. 2014). However, the efficacy of these inhibitors in the treatment of either PI3KCA mutant or PTEN deficient breast cancer has been promising (DeGraffenried, Fulcher et al. 2004; Elkabets, Vora et al. 2013). While PTEN deficient breast cancer cells were shown to be highly dependent on the PI3K/AKT pathway for growth and survival, these cells were the most susceptible to PI3K inhibition (DeGraffenried, Fulcher et al. 2004). Additionally, PI3KCA mutant breast cancer cells with increased TORC1 activity were shown to have reduced activity both *in vitro* and *in vivo* cancer cell proliferation upon TORC1 inhibitor treatment (Elkabets, Vora et al. 2013). Therefore, while inhibition of the PI3K/AKT pathway is plagued by issues with drug toxicity, certain populations of breast cancer patients may benefit from the use of these inhibitors.

I.8.2.2 PI3K pathway and EGFRTKi resistance

The role of the PI3K/AKT pathway in EGFRTKi resistance is less clear compared the Ras/MAPK pathway. It is suggested that mutations in the catalytic domain of PI3K (PI3KCA) may promote intrinsic resistance to EGFRTKis (Engelman, Mukohara et al. 2006). In this study, signaling through the HER3/PI3K/AKT axis was implicated in resistance to the EGFR inhibitor, gefitinib, in lung cancer cells. Additionally, the overexpression of PI3KCA in these cells blocked gefitinib-induced apoptosis. Whereas, the inhibition of PI3K signals, sensitized cells to EGFR inhibition. However, the importance of PI3KCA mutations in EGFRTKi-resistant patients has yet to be well defined (Sequist, Waltman et al. 2011). Conversely, *in vitro* studies have mapped the important mutations involved in resistance to the exon 20 kinase domain of PI3KCA (Engelman, Mukohara et al. 2006). In addition to the PI3KCA mutation, loss of PTEN has also been associated with intrinsic resistance (Sos, Koker et al. 2009). Here, PTEN is shown to be essential for the inhibition of signals downstream of mutant/erlotinib-resistant-EGFR. Moreover, increased signaling from mutant-EGFR was observed in PTEN-depleted cells. Yet, again, the importance of activating mutations and PTEN expression in resistance needs to be validated in the clinic. Thus, the importance of the PI3K/AKT pathway in mediating intrinsic EGFRTKi resistance requires further examination.

An increased activation of this pathway has also been linked to acquired resistance. However, this activation has been shown to result from increased signaling through other RTKs such as HER3, cMET and IGF1R (Engelman, Zejnullahu et al. 2007; Frolov, Schuller et al. 2007; Zhang, Moerkens et al. 2011). In gefitinib-resistant lung cancer cells, amplification of cMET promoted the activation of HER3 and in turn downstream signals though PI3K/AKT (Engelman, Zejnullahu et al. 2007). Additionally, the expression, activation and dimerization of HER3 was shown to promote resistance to erlotinib in pancreatic cancer cells (Frolov, Schuller et al. 2007). Finally, IGF1R has been shown to promote resistance in breast cancer cells by enhancing signals through both the ERK1/2 and PI3K/AKT pathways (Zhang, Moerkens et al. 2011). In breast cancer, a decreased resistance to hormonal therapy was observed in patients co-treated with PI3K inhibitors (Provenzano, Kurian et al. 2013). Yet, its importance in EGFRTKIs resistance has yet to be examined. While trials examining the efficacy of the co-treatment of these inhibitors with EGFRTKis are currently underway, the combinational toxicity of these two families of inhibitors may hinder the success of these trials.

In summary, like the Ras/MAPK pathway, this pathway also plays an important role in mediating oncogenic signals and promoting resistance to EGFR inhibitors. However, the success of inhibiting this pathway in cancer patients has been limited.

I.8.3 p38MAPK pathway

Another signaling cascade known to regulate by EGFR signaling, as well as EGFR inhibitors, is the p38MAPK pathway. The p38MAPK family consists of four isoforms: p38MAPKα, p38MAPKβ, p38MAPKγ and p38MAPKδ. While both p38MAPKα and p38MAPKβ are ubiquitously expressed, the expression of the two other isoforms is tissue specific (Johnson and Lapadat 2002). In response to external signals, p38MAPKs are activated via phosphorylation by the MEK kinase, MKK3, MKK4 and MKK6 (Dong, Davis et al. 2002; Vander Griend, Kocherginsky et al. 2005; Cuadrado and Nebreda 2010). Once phosphorylated, p38MAPKs can translocate to the nucleus where they phosphorylate transcription factors involved in cell apoptosis, cell cycle arrest, survival, proliferation, migration and invasion (Bhowmick, Zent et al. 2001; Olson and Hallahan 2004; Wada and Penninger 2004). More specifically in breast cancer, activated p38MAPK is associated with increased invasion and metastasis, increased expression of EGFR and HER2 and resistance to hormonal therapy (Gutierrez, Detre et al. 2005; Han, Zeng et al. 2007). Indeed, a strong correlation between tamoxifen-resistance and the activation of p38MAPK was observed in tissues isolated from breast cancer patients. Moreover, cells collected from tamoxifen-resistant xenograft tumors also had elevated p38MAPK activity (Gutierrez, Detre et al. 2005).

I.8.3.1 p38MAPK inhibitors

Since p38MAPK has both the properties of a tumor suppressor (cell cycle arrest and apoptosis) as well as a role in promoting cancer progression (cellular migration), it still remains controversial whether this pathway should be targeted in cancer patients. However, p38MAPK inhibitors are currently available. Two families of p38MAPK inhibitors have been synthesized: 1- Inhibitors that directly target the ATP-binding site of the kinase domain, 2- Inhibitors that indirectly block the binding of ATP to the kinase domain (Yong, Koh et al. 2009). While both types of inhibitors have been shown to effectively inhibit the kinase activity of p38MAPK, only the inhibitors that indirectly block the binding of ATP to the kinase domain have been shown to have target specificity (Karaman, Herrgard et al. 2008). Preclinical studies have demonstrated the efficacy of

p38MAPK inhibitors in both cancer and inflammation. In inflammation, p38MAPK inhibitors have been shown to down-regulate an inflammatory response as measured by a decreased cytokine production and C-reactive protein release as well as a diminished leukocyte response (Branger, van den Blink et al. 2002). Alternatively, in squamous cell carcinoma cells, p38MAPK inhibition mediated cell survival, proliferation and invasion (Junttila, Ala-Aho et al. 2007). Additionally, its inhibition has been shown to sensitize colon cancer cells to growth inhibition and apoptosis, while sensitizing gastric cancer cells to chemotherapy (Lim, Lee et al. 2006; Guo, Ma et al. 2008). In fact, the treatment of resistant gastric cancer cells with a p38MAPK inhibitor enhanced their response to chemotherapeutic agents. While the inhibition of p38MAPK is associated with anti-tumorigenic properties, other studies have shown that treatment with these inhibitors can block the induction of apoptosis and thus promote oncogenesis (She, Bode et al. 2001; Bradham and McClay 2006). Interestingly, the depletion of p38MAPK phosphatases, thus increasing p38MAPK activity, was associated with a decrease in both *HRAS* and *HER2*-driven tumors. Moreover, the pharmacological inhibition of p38MAPK was shown to promote mammary-tumor formation (Bulavin, Phillips et al. 2004). Additionally, the inhibition of p38MAPK was shown to block the taxol-dependent apoptosis of HeLa cells. In fact, activating p38MAPK in these cells can enhance taxol-induced cell death by approximately 25-fold (Deacon, Mistry et al. 2003). Even though the role of p38MAPK in cancer remains controversial, clinical trials using p38MAPK inhibitors are currently underway. However, safety issues have plagued their success (Yong, Koh et al. 2009).

I.8.3.2 p38MAPK pathway and EGFRTKi resistance

As p38MAPK has been previously reported to play a pro-apoptotic function in cancer cells, it could be assumed that cellular apoptosis induced by EGFR inhibitors may be dependent on this pathway. However, the activation of p38MAPK has yet to be directly linked to EGFRTKi resistance. Meanwhile, it has been previously shown to mediate resistance to other chemotherapeutics such as cisplatin (Hernandez Losa, Parada Cobo et al. 2003). Indeed, the treatment of several human cancer cell lines with p38MAPK inhibitors reduced the sensitivity of these cells to cisplatin-induced apoptosis suggesting that p38MAPK is required for the chemosensitivity of these cells. Moreover, p38MAPK has been shown to mediate chemotherapy-induced EGFR internalization (Zwang and Yarden 2006). Briefly, p38MAPK has been shown to phosphorylate a threonine residue on the EGFR leading to its internalization. Thus, p38MAPK

may mediate resistance by controlling the availability of the EGFR. Furthermore, p38MAPK has also been linked to other important mediators of drug resistance such as p53 and c-ABL (Pandey, Raingeaud et al. 1996; Sanchez-Prieto, Rojas et al. 2000). In response to genotoxic stress, p38MAPK has been shown to directly associated with p53 leading to its serine phosphorylation. This promotes the apoptotic-inducing functions of p53 (Sanchez-Prieto, Rojas et al. 2000). While it seems that p38MAPK would be a prime candidate mediating EGFRTKi sensitivity, further investigation is required to better understand its role in mediating drug resistance.

Altogether, the role of p38MAPK and the use of p38MAPK inhibitors in cancer remains controversial. Some studies suggest that the inhibition of p38MAPK signals diminish the progression and spread of cancer. Whereas, others suggest that blocking this pathway could promote the survival of cancer cells. Furthermore, the role of this MAPK in EGFRTKi resistance has yet to be evaluate. However, in Chapter III, we will highlight an important role for this pathway in mediating gefitinib sensitivity in breast cancer cells depleted of the small GTPase ARF1.

I.8.4 JNK pathway

Another well characterized signaling pathway known to mediate cancer cell survival is the c-Jun NH2-terminal kinase (JNK) pathway. There have currently been three JNK genes: *JNK1*, *JNK2* and *JNK3*, which encode for 10 identified JNK isoforms (Zhou, Li et al. 2015). While JNK1 and JNK2 are ubiquitously expressed, JNK3 is primarily found in the brain, testis and heart. Like all MAPKs, the JNK isoforms are activated by MEK kinases, specifically MKK4 and MKK7 (Derijard, Raingeaud et al. 1995; Tournier, Whitmarsh et al. 1997). Activated JNK is translocated to the nucleus where it phosphorylates the proto-oncogene, c-Jun, which exerts its cellular response (Hibi, Lin et al. 1993; Derijard, Hibi et al. 1994). While transient JNK activation is associated cell survival, prolonged JNK activation has been shown to phosphorylate the anti-apoptotic factor Bcl2 and induce apoptosis (Bassik, Scorrano et al. 2004; Ventura, Hubner et al. 2006). Briefly, phosphorylation of Bcl-2 diminishes its ability to lower intracellular concentrations of calcium. This in turn leads to increased calcium-dependent death stimulation and the induction of apoptosis (Bassik, Scorrano et al. 2004). In breast cancer, JNK has been associated with a tumor suppressor function. Indeed, depletion of JNK1 and JNK2 in the mammary gland of mice was associated with an enhanced tumor development (Cellurale, Weston et al. 2010). Therefore, like the p38MAPK pathway, activation of JNK is associated with the induction of apoptosis.

I.8.4.1 JNK inhibitors

While JNK is best known for its role in promoting cell death, there have also been reports that JNK activation can enhance cancer cell survival (Ventura, Hubner et al. 2006). Therefore several JNK inhibitors have been developed and characterized for their effectiveness in cancer treatment. However, the majority of these inhibitors have low kinase specificity and do not affect the phosphorylation of downstream targets of JNK (Bennett, Sasaki et al. 2001). While others, require very high doses to mediate JNK activity (Gaillard, Jeanclaude-Etter et al. 2005). To date, no JNK inhibitors have been used in the clinic. However, their efficacy have been demonstrated in a variety of cell-based and animal models (Bubici and Papa 2014). In breast cancer, JNK2-specific kinase inhibitors have been shown to block the migration of polyoma middle T antigen mammary tumor cells (Kaoud, Mitra et al. 2011). Moreover, drugs targeting JNK1 blocked the formation of liver tumors (Hui, Zatloukal et al. 2008). Indeed, the inhibition or depletion of JNK1 was shown to mediate the proliferation of liver cancer cells by affecting the expression of p21, a cell cycle inhibitor. Therefore, while JNK is an important mediator of apoptosis, it can also promote pro-oncogenic processes such as cancer cell migration and proliferation. Thus, the development of JNK-targeting therapeutics may elicit interesting responses in cancer patients.

I.8.4.2 JNK pathway and EGFRTKi resistance

JNK activation has been shown to play an essential role in the induction of chemotherapeutic-induced cell death/apoptosis (Sunters, Madureira et al. 2006). It was shown in breast cancer cells that JNK was required for taxol-induced apoptosis by promoting FOXO3a nuclear translocation. FOXO3a is an important apoptotic trigger. Thus, JNK could promote apoptosis by mediating its nuclear transport. Additionally, JNK activation has been implicated in resistance by mediating EGFR expression (Kim, Park et al. 2009). More specifically, JNK activation was shown to down-regulate EGFR expression upon gefitinib treatment via the induction of cyclooxygenase-2 (COX-2). Thus, JNK activation plays an important role in mediating gefitinib sensitivity leading to the induction of apoptosis.

In summary, like for p38MAPK, the role for JNK in cancer and drug resistance remains controversial and requires further investigation. The inhibitors of this pathway currently available are inefficient. Therefore, the development of improved tools to characterize the importance of JNK in cancer and apoptosis are necessary.

I.8.5 Src kinase

Src kinase is one of the oldest oncogenes studied and was identified and sequenced in the mid-1970s (Stehelin, Guntaka et al. 1976). They are members of the non-receptor tyrosine kinase family and consists of nine members: Src, Lyn, Fyn, LCK, HCK, FGR, BLK, YRK and YES. These kinases have been shown to play an important role in mediating signal transduction. Of these, Src kinase is the best characterized and its role in oncogenesis has been well defined (Roskoski 2015). In fact, several cancers exhibit increased Src expression and activity (Liu, Kovacevic et al. 2015). Furthermore, this kinase has been linked to cancer cell proliferation, survival, migration, invasion and angiogenesis (Roskoski 2015). Src is activated upon the dephosphorylation of the auto-inhibitory tyrosine 530 residue which leads to the autophosphorylation of tyrosine 419 within its catalytic domain (Roskoski 2015). Its activation has been shown to be mediated by multiple factors including RTK activation (Parsons and Parsons 1997). Interestingly, Src has also been to shown to promote EGFR activation by tyrosine phosphorylating specific residues within its intercellular domain (Biscardi, Maa et al. 1999). Indeed, Src was shown to directly associated with the EGFR and phosphorylate tyrosine 845 on the receptor. This phosphorylation has been shown to enhance the activation of signals downstream of the EGFR. In fact, the overexpression of a mutant form the EGFR that cannot for phosphorylated on this residue by Src was shown to have decreased signaling. Thus, Src may promote its oncogenic properties through increased EGFR activation. In addition, Src activates the Ras/MAPK, Stat and PI3K/AKT pathways, three pathways shown to mediate oncogenic cellular responses (Martin 2001). Altogether, the literature demonstrates an important role for Src in mediating oncogenesis. This makes this kinase a potential therapeutic target in cancer patients.

I.8.5.1 Src kinase inhibitors

Several Src kinase inhibitors have been developed with dasatinib being the most commonly used. Dasatinib is a dual Src kinase/ BCR-ABL inhibitor effective in the treatment of chronic myeloid leukemia and Philadelphia chromosome positive acute lymphoblastic leukemia. However, its therapeutic benefits in these two diseases stems from its actions on BCR-ABL and not Src (Lindauer and Hochhaus 2014). With Src being overexpressed in approximately 80% of TNBCs (Tryfonopoulos, Walsh et al. 2011), there have been many attempts of targeting Src in these patients. However, clinical trials using dasatinib in the treatment of invasive breast cancer have demonstrated disappointing results (Herold, Chadaram et al. 2011). In fact, a phase II trial using dasatinib treatment as a single agent showed that treatment of this inhibitor had no effect in patients with metastatic breast cancer. Conversely, another Src kinase/BCL-ABL inhibitor, bosutinib, has shown effective activity in another trial (Campone, Bondarenko et al. 2012). However, it was only shown to be effective in hormone-positive breast cancer patients. Therefore, this compound needs to be thoroughly tested in all breast cancer populations.

I.8.5.2 Src kinase and EGFRTKi resistance

Since Src kinase is an important regulator of signals downstream of activated EGFR, it is easily speculated that this kinase may mediate the resistance of cancer cells to EGFRTKis. Indeed, an increased Src activity in EGFR-inhibitor resistant lung cancer and colon cancer cells has been documented (Lu, Li et al. 2007; Wheeler, Iida et al. 2009). Additionally, Src-dependent tyrosine phosphorylation of the EGFR, a process known to enhance EGFR signaling, was also enhanced in these cells (Wheeler, Iida et al. 2009). It has been previously shown that Src-dependent EGFR tyrosine phosphorylation can potentiate EGFR activation and signaling (Biscardi, Maa et al. 1999). Thus, Src can promote resistance by directly increasing the activation state of the receptor itself. More interestingly, treatment with a Src inhibitor can re-sensitize resistant cells to EGFR inhibition (Lu, Li et al. 2007; Wheeler, Iida et al. 2009). Indeed, increased cellular apoptosis was observed in gefitinib-resistant cells co-treated with an EGFR and Src inhibitor compared to just the EGFR inhibitor alone. Src has also been shown to promote the nuclear translocation of the EGFR which mediates the transcription of genes involved in resistance (Lin, Makino et al. 2001; Li, Iida et al. 2009). The nuclear accumulation of the EGFR has been associated with acquired resistance to the monoclonal antibody, cetuximab. Interestingly, treatment of non-small cell lung cancer cells with

the Src inhibitor, dasatinib, blocked the cetuximab-dependent nuclear sequestering of the EGFR (Li, Iida et al. 2009). Clinicians have recognized the importance of Src kinase in mediating the resistance to EGFRTKis. In fact, currently, the Src inhibitor, dasatinib, is being test in clinical trials of EGFR-inhibitor resistant colon, head and neck and lung cancers.

Src kinase is the first true oncogene and has been linked to many oncogenic processes as well as the development of EGFRTKi resistance. Pharmacological inhibition of the kinase activity of Src has been shown to have therapeutic benefits and re-sensitized EGFRTKi-resistant cancers to EGFR inhibition. However, larger and more specific clinical trials are required to further characterize these inhibitors.

Altogether, signal transduction is the process that transmits signals from the cell surface (receptor) to gene transcription (nucleus). De-regulation of these signaling networks are associated with the development of cancer as well as other pathologies. While we discuss each signaling cascade individually, it is important to understand that signal transduction is a complex notion involving the interplay and crosstalk between multiple signaling cascades. Therefore, it is best to discuss signal transduction in terms of a signaling network and not a single signaling pathway. This can explain why pharmacologically targeting a signal cascade has shown limited success in cancer patients and why future therapeutics are moving towards targeting multiple pathways in congruence. In this thesis, we will highlight the ARF GTPase family as key mediators of signaling networks leading to cell proliferation, migration and EGFRTKi resistance. Thus, targeting these small GTPases may lead to the inhibition of multiple oncogenic signaling cascades as well as blocking physiological events such as cancer cell proliferation, migration and invasion.

I.9 Cell death

The process of cell death plays an important role in disease. For example, in conditions such as Parkinson's disease, stroke and congestive heart disease, cell death is associated with loss of organ/tissue functionality and ultimately death. In this case therapeutics are used to prevent cell death. Conversely, in cancer, therapeutics are designed to promote the induction of cell death. Within the literature, three mechanisms of cell death have been described: 1- necrosis (death of a portion of tissue differentially affected by a local injury), 2- apoptosis (a genetically programmed mechanism that allows cells to commit suicide) and 3- autophagy (digestion of cellular constituents by enzyme of the same cell) (Henriquez, Armisen et al. 2008; He and Klionsky 2009; Hotchkiss,

Strasser et al. 2009). Since EGFR inhibitors have been reported to promote apoptosis in cancer cells, the remainder of this chapter of my thesis will describe this mode of cell death in more detail.

I.9.1 Apoptosis

Apoptosis is important process that ensure organism survival by eliminating damaged or infected cells. Apoptosis is generally accompanied by several distinct morphology events such as cytoplasmic shrinkage, chromosomal condensation and membrane blebbing as well as biochemical features such as the activation of the enzymatic caspases (Kerr, Wyllie et al. 1972; Galluzzi, Lopez-Soto et al. 2016). Altered apoptotic signaling has been linked to several pathologies such as cancer (Hotchkiss, Strasser et al. 2009). In fact, malignant cells are more resistant to apoptotic signals. This stems from the de-regulation of signals through the two apoptotic activating pathways (Brown and Attardi 2005; Pandey, Prasad et al. 2016). These pathways are known as: (See Figure 14) 1- The extrinsic pathway involving the activation of death receptors which recruit and activate caspases 3, 8 and 10 leading to DNA fragmentation, nuclear fragmentation and membrane blebbing and 2- The intrinsic pathway involving the Bcl-family-regulated release of CytC from the mitochondria leading the activation of caspases 3 and 9 (Duprez, Wirawan et al. 2009). Work depicted in this thesis will focus on intrinsic or mitochondrial-dependent apoptosis.

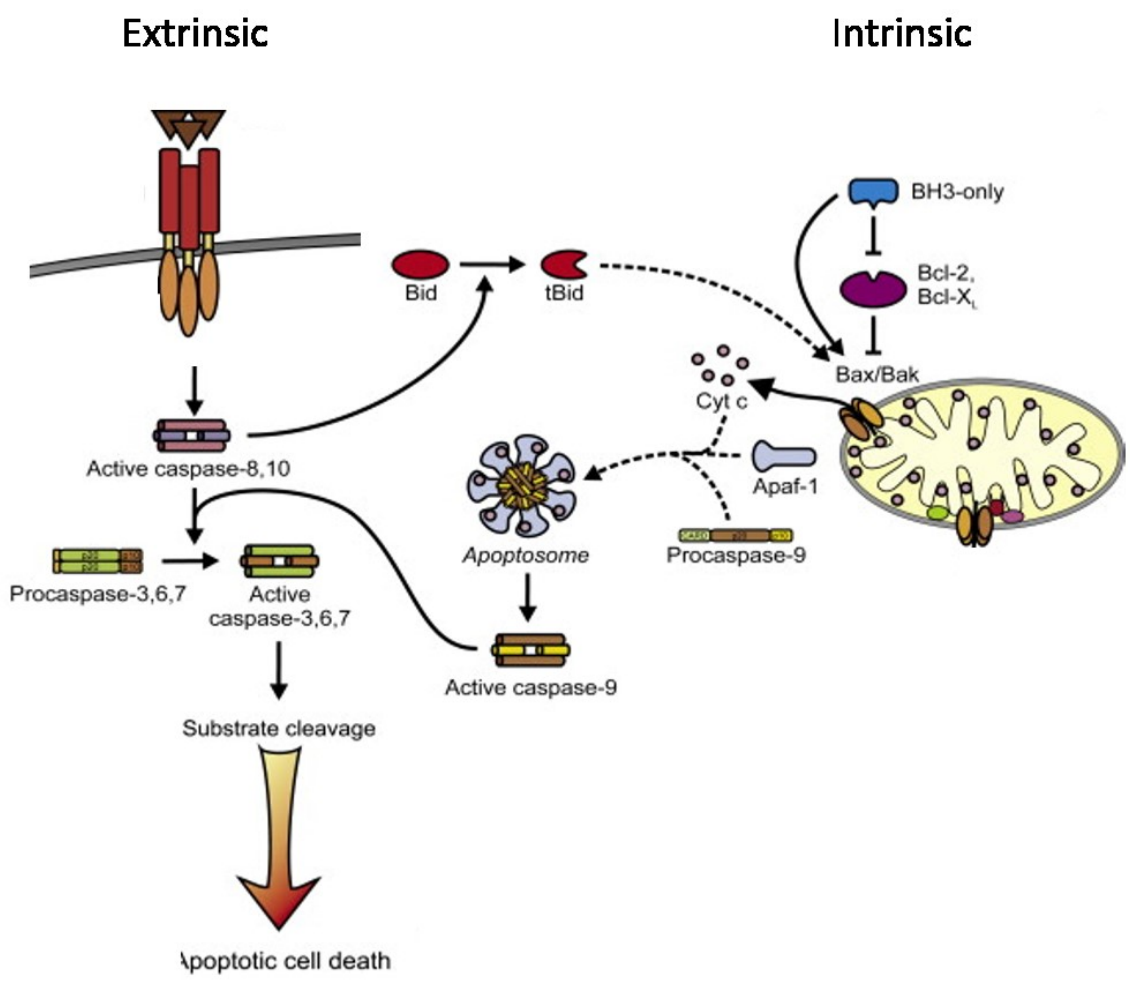

Figure 14. Intrinsic versus extrinsic apoptotic pathways

There are two major pathways that mediate apoptosis: 1- The extrinsic pathway that involves the induction of cell death in response to extracellular signals. Briefly, death ligands bind death receptors at the plasma membrane resulted in the recruitment of a death-inducing complex that plays a primary role in the activation of caspases 8 and 10. These initiator caspases activate effector caspases 3, 6 and 7 which cleave their substrates and induce apoptotic cell death; 2- The intrinsic pathway which incorporates death signals from within the cell, usually involving the mitochondria. In this pathway, the imbalance and activation of Bcl family members mediate the membrane potential of the mitochondria leading to the opening of pores within mitochondrial outer membrane. This allows for the efflux of pro-apoptotic regulators (Cytochrome c and APAF-1) leading to the formation of the apoptosome and the activation of caspase 9. Caspase 9 activates the effector caspases leading to substrate cleavage and the induction of apoptosis. Adapted from: (Duprez, Wirawan et al. 2009)

I.9.2 The mitochondria

Apoptosis is commonly mediated by the integrity of the mitochondria, small organelles that play an essential role in the control of cell life and death. They are responsible for the cells energy production through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. In addition to its role in energy production, the mitochondria mediate the synthesis of other important factors such as lipids, pyrimidines, heme moieties, some amino acids and other biomolecules. It is also the major intracellular source of reactive oxygen species (ROS). This organelle controls cell fate by; 1- mitochondrial outer membrane permeabilization leading to cell death by apoptosis, 2- mitochondrial permeability transition leading to cell death by necrosis, 3- controlling the cellular energy supply and 4- participating in the synthesis of essential molecules such as lipids and nucleotides (Green, Galluzzi et al. 2014). The mitochondria play a limiting role in the induction of apoptosis, as cells depleted of mitochondria are resistant to apoptotic signals (Tait, Oberst et al. 2013). The process of mitochondrial-dependent apoptosis will be further discussed below.

I.9.3 The mitochondria and Cancer

Since cancer cells need to evade apoptosis and alter their cellular respiration to survive, two cellular functions mediated by the mitochondria, it is highly likely that this organelle plays a role in oncogenesis. In fact, mitochondrial dysfunction is associated with the development and maintenance of several cancers including breast cancer (Wallace 2012). In response to hypoxia (low oxygen supply), cancer cells favor anaerobic glycolysis by shutting down mitochondrial functions (Zong, Rabinowitz et al. 2016). In fact, mutations in both mitochondrial DNA and enzymes have been identified in cancer patients. The majority of these mutations inhibit oxidative phosphorylation, the mitochondrial process involved in the production of ATP (Wallace 2012). In addition to altered mitochondrial respiration, cancer patients have been shown to have altered cellular metabolism and resistance to mitochondrial apoptosis (Zong, Rabinowitz et al. 2016). Furthermore, increase mitochondrial ROS production modulates important signaling cascades involved in mitogenesis (Wallace 2012). In summary, the mitochondria are organelles that play an important role in the induction of apoptosis and oxidative phosphorylation. To survive in low oxygen environments, cancer cells alter the functions of the mitochondria to evade apoptosis and favor anaerobic glycolysis as a mode of energy production.

I.9.4 Bcl Family

Multiple factors mediate the induction of apoptosis within the mitochondria. A group of these factors are known as the Bcl family of proteins, named after B-cell lymphoma 2 (Bcl2), are well characterized for their role in mediating cell death. This family consists of three subgroups: 1- BH3-only proteins (E.g. Bim) which consist of a single BH3 domain and act as initiators of apoptosis by either blocking the pro-survival Bcl members or directly activating the pro-apoptotic Bcl members (Kuwana, Bouchier-Hayes et al. 2005; Czabotar, Lessene et al. 2014), 2- Pro-survival Bcls (E.g. Bcl2) which are localized to the mitochondria and act by blocking the activation and oligomerization of pro-apoptotic Bcl members (Ku, Liang et al. 2011; Czabotar, Lessene et al. 2014) and 3- Pro-apoptotic effector proteins (E.g. Bax) that accumulate in the mitochondria in response to cytotoxic signals where they oligimerize and form pores in the mitochondrial membrane (Czabotar, Lessene et al. 2014). In the presence of an apoptotic signal, there is an accumulation of BH3-only proteins which either promote the activation and mitochondrial translocation of pro-apoptotic Bcls or inhibit the pro-survival Bcls. This allows for the oligomerization of apoptotic Bcls and the formation of pores within the outer mitochondrial membrane (OMM) and the initiation of caspase-dependent apoptosis (Suzuki, Youle et al. 2000; Czabotar, Lessene et al. 2014). Of interest, in Chapter IV, we present results demonstrating that ARF1 favors the actions of the anti-apoptotic Bcl factors to prevent cell death induced by the EGFRTKi, gefitinib. Therefore, controlling the interplay of Bcl family members could be beneficial in the treatment of cancers by favoring the actions of both the initiators of apoptosis and the pro-apoptotic effectors or by blocking the actions of the anti-apoptotic members. In fact, multiple Bcl inhibitors are currently being developed and characterized.

I.9.5 Mitochondrial membrane potential

The Bcl family described in the section above, exert the actions on apoptosis by mediating pore formation within the mitochondrial membrane. These pores alter the membrane potential of the mitochondria. The alterations in mitochondrial membrane potential have been proposed as an apoptotic mechanism (Figure 15). In normal physiological conditions, polarization of the outer mitochondrial membrane (OMM) opens the permeability transition pore complex (PTPC) and allows for the transport of metabolites, ions and other factors between the cytosol and the mitochondrial matrix. This process is important in the maintenance of mitochondrial functions (Kroemer, Galluzzi et al. 2007). However, in the presence of apoptotic signals, this controlled pore opening found in normal conditions is altered. It was originally proposed that the depolarization of the OMM results in a prolonged opening of the PTPC. This allows for the release of pro-apoptotic factors such as CytC (Shimizu, Narita et al. 1999; Kroemer, Galluzzi et al. 2007). However, more recently another mechanism of mitochondrial apoptosis has been proposed. In this case, hyperpolarization of the OMM leads to the closure of the PTPC. This results in the accumulation of factors within the mitochondria leading to mitochondrial swelling. Consequentially, the OMM bursts under pressure and pro-apoptotic factors are released (Vander Heiden, Chandel et al. 2000; Kroemer, Galluzzi et al. 2007). This would suggest that both the depolarization and hyperpolarization of the OMM could promote the induction of apoptosis and that the actions of Bcl members could mediate the polarization state of the OMM.

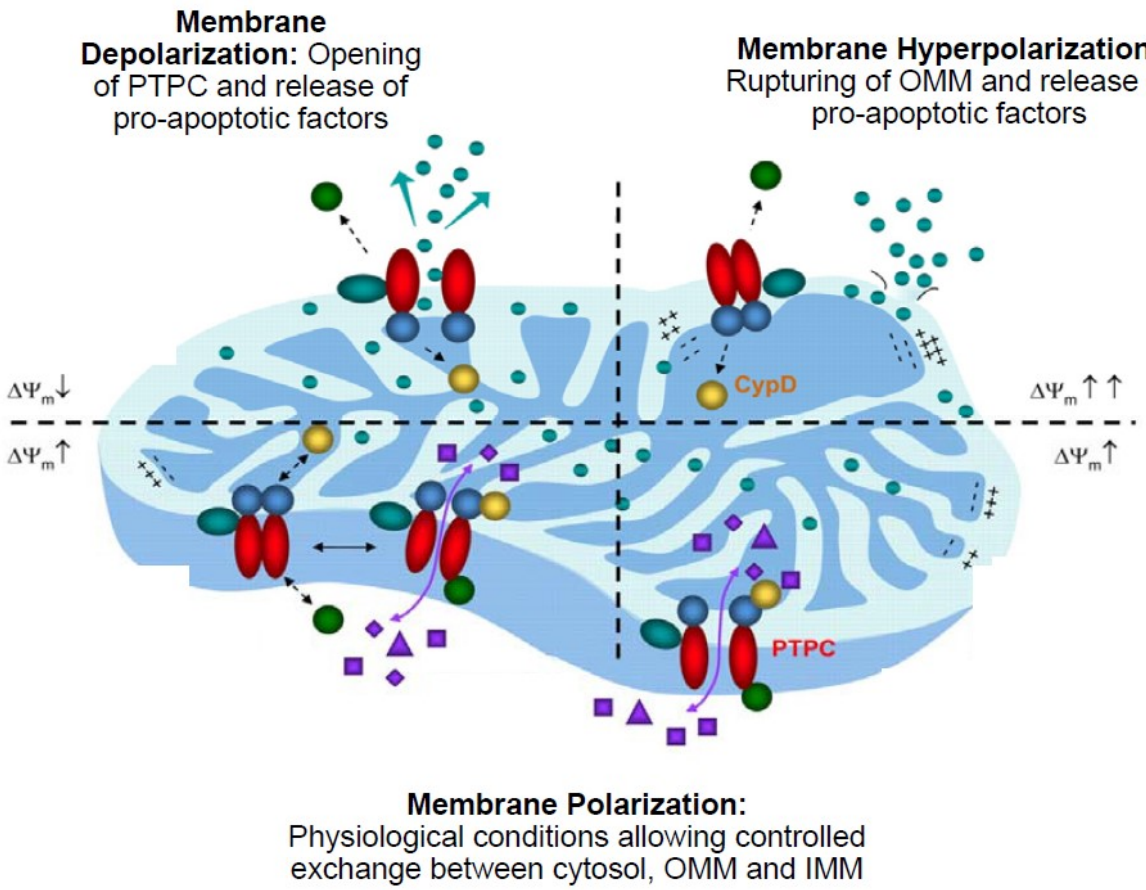

Figure 15. Mediation of apoptosis by mitochondrial membrane potential

In physiological conditions, interactions between the permeability transition pore complex (PTPC), hexokinase (green circle) and cyclophilin D (yellow circle) result in the opening of the PTPC, the polarization of the outer mitochondrial membrane (OMM) and the influx and/or efflux of metabolites (purple). The mitochondrial membrane potential can influence the induction of apoptosis 2-fold: 1- Depolarization of the OMM prolongs the opening of the PTPC allowing for the release of pro-apoptotic factors (cyan circles) into the cytosol. 2- Hyperpolarization of the OMM closes the PTPC leading to the accumulation of factors in the mitochondrial. This results in the swelling of the mitochondria, bursting of the OMM and the release of pro-apoptotic factors. Adapted from: (Kroemer, Galluzzi et al. 2007)

I.9.6 Cytochrome C

The opening of pores within the mitochondrial membrane results in the release of pro-apoptotic factors that lead to the activation of proteolytic enzymes within the cytosol of the cell. One of these pro-apoptotic factors released from the mitochondria is cytochrome C (Cytc). It is a heme-protein localized between the outer and inner membrane of the mitochondria that mediates both cellular respiration and apoptosis (Ow, Green et al. 2008). In respiration, it functions to transfer electrons from complex III to complex IV of the respiratory chain (Lenaz and Genova 2010). In apoptosis, CytC is released into the cytosol through pores within the mitochondrial membrane. Interestingly, we found that CytC release in response to gefitinib treatment of breast cancer cells is mediated by the expression of the small GTPase ARF1 (See Chapter III). Once in the cytosol and in the presence of ATP, it promotes the activation and oligomerization of the adaptor-molecule-apoptosis-protease-activating-factor-1 (APAF-1) which forms the complex known as the apoptosome. Each apoptosome can recruit and activate the proteinase activity of seven caspase 9 molecules. This promotes apoptosis (Zhou, Chou et al. 1999; Twiddy, Brown et al. 2004; Garrido, Galluzzi et al. 2006).

I.9.7 Caspases

The formation of the apoptosome upon CytC release from the mitochondrial leads to the activation of caspases, a family of endoproteases that hydrolyze peptide bonds that are involved in both apoptosis (caspases 3, 6, 7, 8 and 9) and inflammation (caspases 1, 4, 5, 12). These enzymes exist as inactivate monomers and generally require both dimerization and prodomain cleavage for activation. Briefly, in inflammation, caspases play an essential role in the cleavage and activation of pro-inflammatory cytokines such as, IL-1β and IL-18 (Martinon and Tschopp 2004). In apoptosis, initiator caspases are activated by either the extrinsic (caspase 8) or intrinsic (caspase 9) pathways. These initiator caspases activate executioner caspases (caspases 3, 6 and 7) which demolish important structural proteins and activate other enzymes involved in apoptosis. While caspases mediate key oncogenic processes, they are rarely mutated in cancer. In fact, in cancer, the deregulation of these enzymes is commonly the result of mutations in their upstream regulators (McIlwain, Berger et al. 2013). Furthermore, the inactivation of a single caspase is not sufficient to inhibit apoptosis. Thus, malignant cells survive by inhibiting upstream mediators of multiple caspases.

Together, the mitochondria is an important mediator of apoptosis in both normal and cancer cells. Cancer cells evade apoptosis by countering the mitochondrial functions by acting on the expression and activity of Bcl family members, controlling the mitochondria membrane potential and CytC release and by mediating the activity of the caspase enzymes. Interestingly, therapeutics and mechanisms that counter these properties in cancer cells, re-sensitize malignant cells to apoptotic signals.

I.9.8 Apoptosis and resistance

The evasion of apoptosis is considered an important mechanism through which cancer cells develop resistance to EGFRTKis (Chong and Janne 2013). It is well documented that EGFR inhibitors activate mitochondrial apoptosis. Indeed, the treatment with EGFRTKis activates the pro-apoptotic factors Bax and Bak and decreases the expression of the anti-apoptotic Bcl2 (Hopfner, Sutter et al. 2004; Ling, Lin et al. 2008). While the literature suggests that decreased expression or polymorphisms in the pro-apoptotic *BIM* gene are associated with acquired resistance (Faber, Corcoran et al. 2011; Ng, Hillmer et al. 2012), little is known on the mechanisms through which resistant cells evade apoptosis. It has been suggested that the EGFR may have effects directly on the mitochondria. In fact, inhibitor treatment has been shown to translocate EGFR into this organelle (Cao, Zhu et al. 2011). Furthermore, EGFR was shown to sequester the pro-apoptotic p53 up-regulated modulator of apoptosis (PUMA) away from the mitochondria and block its function (Zhu, Cao et al. 2010). Additionally, gefitinib-mediated alterations in mitochondrial membrane potential were shown to be essential for the induction of apoptosis (Hopfner, Sutter et al. 2004). It has been also suggested that cells may escape apoptosis through increased upstream survival signals such as the AKT/mTor pathway or through mutations in or loss of PTEN (Bianco, Shin et al. 2003; Chong and Janne 2013).

Altogether, apoptosis is a complex process involving the mitochondria. Cancer cells can evade the induction of apoptosis by altering multiple mitochondrial functions. Cancer cells can also develop resistance to cancer therapeutics by moderating the apoptotic pathways. Therefore, blocking survival mechanisms utilized by cancer cells can re-sensitize these cells to apoptotic signals and cancer therapeutics such as EGFRTKis. Chapter IV of this thesis to dedicated to characterizing the importance of ARF1 in mediating the anti-apoptotic signals present in breast cancer cells treated

with gefitinib. Findings from this chapter suggest that targeting this GTPase could re-sensitize resistant cancer cells to EGFR inhibition.

I.10 Study objectives and hypothesis

A high proportion of TNBC patients have an increased expression of the EGFR. However, attempts at targeting the EGFR in these patients have shown limited success. Therefore, it is important to identify and characterize novel therapeutic targets in this breast cancer subtype. To date, we have demonstrated that the small GTPase ARF1, a possible TNBC therapeutic target, signals downstream of the EGFR in invasive breast cancer cells to mediate proliferation, migration and invasion (Boulay, Cotton et al. 2008; Boulay, Schlienger et al. 2011; Schlienger, Campbell et al. 2014). However, we have yet to identify important mediators of ARF1 activation in breast cancer cells and characterize the mechanism through which ARF1 is recruited to the EGFR and activated. Here, in our first article (Chapter II), we hypothesized that adaptor proteins known to be recruited to the activated EGFR such as Grb2 and Shc may play an important role in the regulation of ARF1 activity. It has been previously shown that ARF6 was recruited to the EGFR through a direct interaction with the EGFR and an ARF6GEF (Morishige, Hashimoto et al. 2008). Interestingly, the sites on the EGFR shown to interact with this GEF were known interaction sites for the adaptors Grb2 and Shc. Indeed, we show that Grb2 was essential for both ARF1 and ARF6 activity downstream of the EGFR. Whereas, the Shc isoform, p66Shc, attenuated ARF1 activation by blocking the recruitment of the ARF1/Grb2 complex to the receptor, while potential signals through the ARF6-Ras/MAPK signaling axis leading to cell proliferation and migration.

Next, our second article (Chapter III), evaluated the importance of ARF1 in modulating EGFRTKi sensitivity in EGFR-positive breast cancer cells. Since ARF1 acts as a signaling switch downstream of the EGFR, known to be involved in EGFRTKi resistance, we proposed that the depletion or pharmacological inhibition of ARF1 could block these signals and re-sensitize cells to EGFR inhibition. We hypothesized that breast cancer cells evade the cytotoxic properties of EGFRTKis by increasing survival signals through ARF1 activation. Actually, ARF1 was shown to promote resistance by enhancing survival signals while blocking pro-apoptotic events. Additionally, we showed that ARF1 was essential in stabilizing EGFR expression in response to gefitinib treatment by blocking p38MAPK-dependent receptor internalization and degradation.

Finally, in Chapter IV, we further highlight the importance of ARF1 in mediate gefitinib sensitivity and demonstrate that ARF1 may play a role within the mitochondria and mediate p66Shc activity in this organelle. Since the adaptor p66Shc has been shown to be an important regulator of mitochondria-dependent apoptosis and we showed that this adaptor mediates ARF1 activity, an

important regulator of EGFRTKi resistance, we proposed to determine the interplay between ARF1 and p66Shc in mediating EGFRTKi-induced cell death. Indeed, p66Shc was shown to be required for gefitinib sensitivity. Furthermore, we highlight a novel role for ARF1 as a mediator of mitochondrial integrity. The depletion of ARF1 was associated with an up-regulation of gefitinib-mediated mitochondrial-dependent apoptosis as measured by p66Shc-mitochondrial translocation, mitochondrial membrane hyperpolarization and CytC release. We also demonstrate that ARF1 plays a key role mediating the dimerization and activation of EGFR family members. Subsequently, we demonstrated a role for this small GTPase in the induction of EGFRTKi resistance.

Currently, there is a lack of effective treatment options available for TNBC patients. In fact, there are no targeting therapies accepted for the treatment of this subtype of breast cancer. Therefore, it is essential to characterize and identify novel therapeutic targets in this cancer population. We have characterized the small GTPase ARF1 as an important mediator of signals leading to cell proliferation, migration and invasion in a cellular model of TNBC. Furthermore, ARF1 was shown to be activated downstream of the EGFR, a receptor overexpressed in TNBC patients. Here, we delineate the mechanism leading to the EGFR-dependent ARF1 activation, implicating the adaptor proteins Grb2 and p66Shc. Furthermore, we demonstrate that the activation of ARF1 mediates the resistance to EGFR inhibitors, commonly found in TNBC patients. Therefore, pharmacologically inhibited this GTPase in TNBC patients could block tumor cell proliferation, migration and invasion, while sensitizing these tumors to EGFR inhibition.

CHAPTER II: The adaptor proteins p66Shc and Grb2 regulate the activation of the GTPases ARF1 and ARF6 in invasive breast cancer cells

Published in the journal: Journal of Biological Chemistry. 2014; 289, 5687-5703

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Short title: p66Shc mediates ARF activation in breast cancer cells

Author contributions:
EH: experimental conception, experimental execution, data analysis and writing
CS: experimental conception.
AC: experimental conception and writing

II.1 Abstract

Signals downstream of growth factor receptors play an important role in mammary carcinogenesis. Recently, we demonstrated that the small GTPases ARF1 and ARF6 were shown to be activated downstream of the epidermal growth factor receptor (EGFR) and act as a key regulator of growth, migration, and invasion of breast cancer cells. However, the mechanism via which the EGFR recruits and activates ARF1 and ARF6 to transmit signals has yet to be fully elucidated. Here, we identify adaptor proteins Grb2 and p66Shc as important regulators mediating ARF activation. We demonstrate that ARF1 can be found in complex with Grb2 and p66Shc upon EGF stimulation of the basal-like breast cancer MDA-MB-231 cell line. However, we report that these two adaptors regulate ARF1 activation differently, with Grb2 promoting ARF1 activation and p66Shc blocking this response. Furthermore, we show that Grb2 is essential for the recruitment of ARF1 to the EGFR, whereas p66Shc hindered ARF1 receptor recruitment. We demonstrate that the negative regulatory role of p66Shc stemmed from its ability to block the recruitment of Grb2/ARF1 to the EGFR. Conversely, p66Shc potentiates ARF6 activation as well as the recruitment of this ARF isoform to the EGFR. Interestingly, we demonstrate that Grb2 is also required for the activation and receptor recruitment of ARF6. Additionally, we show an important role for p66Shc in modulating ARF activation, cell growth, and migration in HER2-positive breast cancer cells. Together, our results highlight a central role for adaptor proteins p66Shc and Grb2 in the regulation of ARF1 and ARF6 activation in invasive breast cancer cells.

II.2 Introduction

The epidermal growth factor receptor (EGFR), one of the best characterized tyrosine kinase receptors, has been shown to be highly expressed in certain breast cancer patients (1). Activation of this receptor by the binding of a variety of ligands, including the epidermal growth factor (EGF), has been implicated in breast cancer cell proliferation, survival, migration, and invasion (2). Upon binding of EGF, the EGFR can homodimerize or heterodimerize with other EGFR family members, ErbB2 and/or ErbB3 (3-5). This leads to the autophosphorylation of several tyrosine residues on the intracellular domains of the receptor. These residues serve as docking sites for a variety of adaptor proteins that are essential for the initiation of downstream signaling (6-8), such as the phosphoinositide 3-kinase (PI3K) and mitogen-activating protein kinase (MAPK) pathways (2, 9, 10).

One family of adaptors that are recruited to the EGFR are the Src homology 2 domain-containing proteins (Shc), which consists of four members, ShcA, -B, -C, and –D (11-15). Although ShcB and -C have been shown to be primarily present within the central nervous system and ShcD has only been identified in mice, ShcA is ubiquitously expressed and has been implicated in breast cancer (11-13, 15). ShcA consists of three isoforms: p46Shc, p52Shc, and p66Shc, which result either from alternative translational initiation sites (p46Shc and p52Shc) or mRNA splicing (p66Shc) (16, 17). Although ShcA is generally considered as an adaptor protein mediating EGFR-dependent activation of the MAPK pathway (16), the function of each isoform, especially p66Shc, in different physiological and pathological settings, remains controversial. Like p52Shc, p66Shc has also been reported to be recruited to the EGFR and associate with Grb2 upon stimulation (16). However, unlike the other Shc isoforms, p66Shc blocked the recruitment of Grb2 to the EGFR and insulin-like growth factor receptor. It was proposed that p66Shc may complete for similar binding sites on the EGFR. Furthermore, a non-receptor pool of p66Shc may sequester Grb2 away from the EGFR. This was shown to lead to an inhibition of the Ras/MAPK pathway (18, 19). Furthermore, the expression of p46Shc and p52Shc was shown to be elevated in tumors isolated from transgenic breast cancer mouse models, whereas p66Shc levels were undetectable (20, 21). In fact, recent studies have reported that p66Shc expression in breast cancer patients may be predictive of node negativity, reduced disease stage, and decreased incidence of patient relapse (22, 23). However, the same group demonstrated that p66Shc expression was associated with a poor prognosis in colorectal cancer patients (24). Interestingly, p66Shc expression has been

demonstrated to be up-regulated by steroid hormones in differentiated hormone-sensitive cancer cells and elevated in the highly invasive breast cancer MDA-MB-231 cell line, as well as in invasive prostate cancer cells (25-27) suggesting a role for this isoform in cancer progression. Moreover, LNCaP cell proliferation and motility was shown to be significantly hindered upon the depletion of p66Shc (27). In summary, the role of p66Shc in cancer remains highly controversial and the exact role for p66Shc in invasive breast cancer has yet to be examined. Recently, members of the ADP-ribosylation factor (ARF) family of small GTPases have been shown to be activated downstream of the EGFR in highly invasive breast cancer cells such as MDA-MB-231, MDA-MB-435, and SKBR3. Moreover, these ARF proteins play an essential role in the proliferation, migration, and invasion of these cells (28, 29). Briefly, ARFs are members of the Ras superfamily of small monomeric G proteins and consist of six isoforms divided into three distinct classes: Class I, consisting of ARF1–3, which is known to regulate the secretory pathway; Class II, ARF4 and ARF5, in which their role has yet to be fully elucidated; and Class III, ARF6, known to modulate intracellular trafficking between the plasma membrane and the endosomes and play an essential role in the organization of the actin cytoskeleton (30). In breast cancer, isoforms ARF1 and ARF6 have been the best characterized. Both GTPases play critical roles in the proliferation and migration of invasive breast cancer cells (28, 29). Although ARF6 has been shown to exert its oncogenic properties via the ERK1/2 pathway, we demonstrated that ARF1 signals primarily via the PI 3-kinase/AKT signaling axis (28, 29, 31). Little is known on the molecular mechanism downstream of the EGFR that leads to activation of ARFs. It was suggested that for ARF6, the guanine nucleotide exchange factor GEP100 (BRAG2) directly bound to the EGFR to mediate the activation of this small GTPase (32). However, it is important to define whether classical adaptor proteins contribute to regulate ARF activation.

Here, for the first time, we show that the adaptor proteins p66Shc and Grb2 are key proteins controlling EGF-dependent ARF1 and ARF6 activation in invasive breast cancer cells. We demonstrate that whereas p66Shc attenuates ARF1 activation, it potentiates ARF6 activation. Furthermore, we demonstrate that another adaptor, Grb2, is essential for the activation of both ARF1 and ARF6. More specifically, we show that p66Shc mediates ARF1 activation by blocking recruitment of the Grb2-ARF1 complex to the EGFR. Conversely, we demonstrate that p66Shc potentiates ARF6 activation by favoring its Grb2-dependent recruitment to the EGFR.

II.3 Materials and Methods

Reagents and Antibodies

Lipofectamine 2000^{TM} was purchased from Invitrogen. EGF was purchased from Fitzgerald Industries International, Inc. (Concord, MA). Monoclonal antibodies used in the study were ARF1 (Sigma), p66Shc (Invitrogen), ARF6 (Santa Cruz Biotechnology, Dallas, TX), and phosphotyrosine (Millipore, Billerica, MA). Polyclonal antibodies used were EGFR, HER2, Pan-actin, pERK1/2, pAKT, AKT (Cell Signaling, Danvers, MA), ARF1 (Proteintech Group, Chicago, IL), Grb2, HA tag, H-Ras, ERK1/2 (Santa Cruz Biotechnology), and Shc (BD Transduction Laboratories, Mississauga, Ontario, Canada). Other reagents used were goat anti-mouse antibody-horseradish peroxidase and goat anti-rabbit antibody-horseradish peroxidase (R & D Systems, Minneapolis, MN) and protein G-agarose plus beads (Santa Cruz Biotechnology).

DNA Plasmids and siRNAs

HA-p66Shc cloned into a pcDNA3 vector was a gift from Dr. Nagamine (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland) (33). Double-stranded scrambled with 19-nucleotide duplex RNA and 2-nucleotide 3′-dTdT overhangs were previously described (34). The 19-nucleotide sequence for the human Grb2 siRNA target was 5′-GAA AGG AGC TTG CCA CGG G-3′. The 21-nucleotide sequence for the human p66Shc siRNA target was 5′-GAA UGA GUC UCU GUC AUC GUC-3′ as previously described (33). All siRNA include 2-nucleotide 3′-dTdT overhangs and were purchased from Dharmacon Inc. (Lafayette, CO).

Cell Culture and Transfection

MDA-MB-231, SkBr3, and MCF7 cells were maintained at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HCC70 cells were maintained at 37 °C, 5% CO_2 in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS. Cells were transfected with plasmid cDNA and/or siRNA using Lipofectamine 2000 according to the manufacturer's instructions. Briefly, for overexpression experiments, cells were mock transfected or transfected with HA-p66Shc cDNA for 6 h prior to being serum starved overnight and then stimulated with EGF for the indicated time points. In siRNA experiments, MDA-MB-231 cells were transfected with 50

nm siRNA for 72 h, serum-starved overnight, and then stimulated with EGF for the indicated time points.

Co-immunoprecipitation and Western Blot Analysis

Serum-starved cells from confluent 10-cm dishes were harvested in 500 µl of lysis buffer (20 mm Tris-HCl, pH 8, 1% Triton X-100, 10% glycerol, 140 mm NaCl, 5 mm EDTA, 1 nm sodium orthovanadate (Na_3VO_4)) complemented with protease inhibitors aprotinin (5 µg/ml), benzamidine (150 µg/ml), leupeptin (5 µg/ml), pepstatin (4 µg/ml), and phenylmethylsulfonyl fluoride (20 mg/ml). Cell lysates were solubilized at 4 °C for 30 min and total soluble proteins were run on polyacrylamide gels and transferred onto nitrocellulose membranes. The transferred proteins were detected using specific primary antibodies (see each experiment for details). Secondary antibodies were all horseradish peroxidase-conjugated, and chemiluminescence was used to detect protein expression. The quantification of the digital images obtained was performed using ImageQuant TL (GE Healthcare Life Sciences). For immunoprecipitation experiments, lysates from the serum-starved cells described above were incubated with agitation with the indicated antibodies and protein G-agarose plus beads at 4 °C for 3 h. Proteins were eluted in SDS-sample buffer by heating to 65 °C for 15 min. Protein interactions and receptor phosphorylation were assessed by Western blot analysis.

ARF Activation Assay

Cells were plated into 10-cm dishes, transfected for the indicated times, and serum-starved overnight. Cells were then stimulated with EGF (10 ng/ml) at 37 °C for the indicated times, and the activation of ARF1 was measured as previously described (34). Briefly, cells were lysed in 400 µl of ice-cold lysis buffer E(pH 7.4, 50 mm Tris-HCl, 1% Nonidet P-40, 137 mm NaCl, 10% glycerol, 5 mm $MgCl_2$, 20 mm NaF, 1 mm $NaPP_i$, 1 mm Na_3VO_4, and the protease inhibitors: aprotinin (5 µg/ml), benzamidine (150 µg/ml), leupeptin (5 µg/ml), pepstatin (4 µg/ml), and phenylmethylsulfonyl fluoride (20 mg/ml)). Samples were spun for 5 min at 10,000 rpm. GST-GGA3-(1–316) (35) coupled to glutathione-Sepharose 4B was added to each tube, and the samples were rotated at 4 °C for 45 min. Proteins were eluted in 20 µl of SDS-sample buffer by heating to 65 °C for 15 min. The detection of ARF1-GTP or ARF6-GTP was performed by immunoblot analysis using specific antibodies to ARF1 and ARF6, respectively.

Ras Activation Assay

Cells were plated into 6-well plates, transfected for the indicated times, and serum-starved overnight. Cells were then stimulated with EGF (10 ng/ml) at 37 °C for the indicated times, and the activation of Ras was measured. Briefly, cells were lysed in 200 μl of ice-cold lysis buffer E (pH 7.4, 50 mm Tris-HCl, 1% Nonidet P-40, 137 mm NaCl, 10% glycerol, 5 mm $MgCl_2$, 20 mm NaF, 1 mm $NaPP_i$, 1 mm Na_3VO_4, and the protease inhibitors: aprotinin (5 μg/ml), benzamidine (150 μg/ml), leupeptin (5 μg/ml), pepstatin (4 μg/ml) and phenylmethylsulfonyl fluoride (20 mg/ml)). Samples were spun for 5 min at 10,000 rpm. GST-Raf-binding domain coupled to glutathione-Sepharose 4B was added to each tube, and the samples were rotated at 4 °C for 45 min. Proteins were eluted in 20 μl of SDS-sample buffer by heating to 65 °C for 15 min. The detection of Ras-GTP was performed by immunoblot analysis using a specific antibody to H-Ras.

Cell Counting Assay

Cells were transiently transfected with 50 nm scrambled siRNA or p66Shc siRNA for 48 h for knockdown experiments or an empty vector or HA-p66Shc cDNA for 24 h for overexpression experiments, trypsinized, and an equal cell number (1×10^4 cells) were reseeded in a 6-cm dish for 24, 48, and 72 h. For each indicated time point, cells were trypsinized, stained with trypan blue, and live cells were manually counted.

Cell Viability Assay

Cells were transiently transfected with 50 nm scrambled siRNA or p66Shc siRNA for 48 h for knockdown experiments or an empty vector or HA-p66Shc cDNA for 24 h for overexpression experiments, trypsinized, and equal cell numbers (1000 cells) were reseeded in a 96-well plate for 72 h. Cells were then stained with thiazolyl blue tetrazolium bromide (Sigma) for 2 h before being solubilized in 20% SDS, 50% dimethylformamide solution overnight. Absorbance was measured at 570 nm with a reference wavelength at 450 nm using a plate reader.

Cell Migration Assay

Cells were transiently transfected with 50 nm scrambled siRNA or p66Shc siRNA for 72 h or an empty vector or HA-p66Shc cDNA for 24 h. Cells were then seeded onto Boyden Chambers (8 μm pores) (Corning, New York) and incubated with or without EGF (10 ng/ml) for 6 h at 37 °C. Cells were fixed in 4% paraformaldehyde and stained with crystal violet for 16 h. Cells present in the upper chamber were removed with a cotton swab and the migrated cells, present in the lower chamber, were quantified by manual counting. Images were acquired using an epifluorescent inverted microscope (Carl Zeiss Axio Observer A1) with ZEN Pro 2011 software Blue edition.

***Statistical* Analysis**

Statistical analysis was performed using either a one-way or two-way analysis of variance followed by a Bonferroni's multiple comparison test using GraphPad Prism (version 5, San Diego, CA).

II.4 Results

p66Shc Modulates ARF1 Activation in Invasive Breast Cancer Cells

Knowing that ARF1 is activated downstream of the EGFR, we sought to determine whether key adaptor proteins, such as Grb2 and Shc, may play a role in the recruitment of this GTPase to the activated receptor. Therefore, we first evaluated the expression levels of Grb2 and the three isoforms of ShcA (p46Shc, p52Shc, and p66Shc) in the non-invasive MCF7 cells (low EGFR, ARF1/ARF6-expressing) and the invasive MDA-MB-231 cell line (high EGFR, ARF1/ARF6-expressing) (Fig. 1*A*). Although we observed no significant difference in expression of Grb2, p46Shc, and p52Shc between the two cell types, p66Shc was found to be present only in the MDA-MB-231 cells. This is in accordance with previously published data highlighting an increased expression of p66Shc in this invasive breast cancer cell line (26). We next evaluated whether ARF1 could form a complex with p66Shc. As shown in Fig. 1*B*, ARF1 co-immunoprecipitated p66Shc and this association was enhanced upon EGF stimulation. Additionally, we detected an association between ARF1 and Grb2 and p52Shc, but not p46Shc. These associations were also enhanced by EGF treatment. Because the function of p66Shc still remains ill-defined in breast cancer and this isoform is specifically expressed in MDA-MB-231 breast cancer cells, we further examined the role of this Shc isoform in the activation process of ARF proteins. Therefore, we next assessed the importance of p66Shc in the regulation of EGF-induced ARF1 activation. To do this, we measured the levels of GTP-bound ARF1 in EGF-stimulated MDA-MB-231 cells that were either transfected with scrambled or p66Shc siRNA. As shown in Fig. 1*C*, EGF induced the activation of ARF1 in cells transfected with a control siRNA. However, a significant increase in ARF1 activation was observed in cells where endogenous expression of p66Shc was reduced suggesting that this Shc isoform, in MDA-MB-231 cells, might act to limit ARF1 activation. For all experiments described in this study, we observed an average inhibition of p66Shc expression by 67%, when cells were transfected with a specific siRNA. To further evaluate the role of p66Shc, we next overexpressed an HA-tagged p66Shc in MDA-MB-231 cells. In these conditions, activation of ARF1 was significantly decreased (Fig. 1*D*), further supporting the role of p66Shc in controlling ARF1 activation downstream of the EGFR. Next, we confirmed our finding in another basal-like breast cancer cell model, the HCC70 cell line (Fig. 1*E*). Indeed, similar to what was observed in MDA-MB-231 cells, ARF1 activation was significantly reduced when p66Shc was overexpressed

compared with control cells. In summary, high expression of p66Shc in invasive breast cancer cells acts to negatively regulate ARF1 activation, upon EGF stimulation.

p66Shc Regulates the Activation of the Ras/MAPK and AKT Pathways

To further characterize the regulation of signaling cascades downstream of the EGFR, we next aimed to define the role of p66Shc on the regulation of ARF-dependent signals such as the PI 3-kinase and MAPK pathways (29). First, we determined whether p66Shc mediated the activation of another small GTPase known to be activated by the EGFR, Ras (36). To do this, we measured the levels of GTP-bound Ras in EGF-stimulated MDA-MB-231 cells that were either transfected with scrambled or p66Shc siRNA (Fig. 2*A*). Interestingly, knockdown of p66Shc significantly decreased Ras activation compared with control cells. Next, we compared the Ras activation profile to that of ERK1/2 activation. As shown in Fig. 2*A*, whereas the initial activation of ERK1/2 by EGF stimulation was diminished by depletion of p66Shc, no significant alterations in ERK activation were observed during later time points. These findings differ from previous observations in other cell types in which p66Shc has been shown to negatively regulate the activation of both Ras and ERK1/2 (16, 19, 37, 38). No significant effects on AKT phosphorylation were observed in p66Shc knockdown cells. To further evaluate the role of p66Shc in regulation Ras/MAPK activation in MDA-MB-231 cells, we next overexpressed a HA-tagged p66Shc in these cells. Surprisingly, overexpression of p66Shc resulted in a significant increase in the basal activation of both Ras and ERK1/2 (Fig. 2*B*). However, a significant decrease in Ras and MAPK activation that is consistent with previous reports was observed at later time points (16, 19, 37, 38). Additionally, we observed a delay in the phosphorylation of AKT in cells overexpressing p66Shc compared with control cells. Together, our results demonstrate that p66Shc mediates important signaling cascades known to be regulated by ARF proteins.

p66Shc Mediates Breast Cancer Cell Growth and Migration

Because ARF1 and p66Shc have been previously reported to mediate cell growth and migration (29, 37, 39, 40), we next evaluated the physiological role of p66Shc in MDA-MB-231 cells. First, we evaluated the importance of p66Shc expression in cell proliferation using a cell counting assay. As shown in Fig. 3*A*, knockdown of p66Shc, by siRNA significantly reduced both the basal and EGF-mediated growth rate of MDA-MD-231 cells at both 48 and 72 h compared with the control scrambled siRNA-transfected cells. Next, we overexpressed p66Shc in MDA-MB-231 cells and evaluated cell growth at 24, 48, and 72 h (Fig. 3*B*). A significant decrease in basal and EGF-dependent cell growth was observed in cells overexpressing HA-p66Shc for all tested time points. More interestingly, the EGF-independent cell number of HA-p66Shc overexpressing cells was found to be lower than the cell number originally seeded. This would suggest that overexpression of p66Shc may induce MDA-MB-231 cell death. We further confirmed the above regulation of cell growth using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide cell viability assay. Although no significant difference in basal cell growth was observed in p66Shc siRNA-transfected cells compared with control, EGF-induced cell growth was significantly reduced upon p66Shc knockdown (Fig. 3*C*). Additionally, we also observed a decrease in both basal and EGF-dependent proliferation in HA-p66Shc overexpressing conditions (Fig. 3*D*). Together, our results show that whereas p66Shc plays an important role in breast cancer cell growth, elevated expression of p66Shc may promote cell death.

Next, we examined the role of p66Shc in cellular migration using Boyden chambers. As illustrated in Fig. 3*E*, EGF induced the migration of control MDA-MB-231 cells. However, EGF-mediated migration was significantly reduced upon the depletion of p66Shc. Furthermore, we were able to enhance the basal, but not EGF-dependent, migration of MDA-MB-231 cells by overexpressing p66Shc (Fig. 3*E*). Together, our results demonstrate that p66Shc is an important mediator of invasive breast cancer cell growth and migration.

p66Shc Regulates ARF1 Activity, Cell Growth, and Migration of HER2-positive Breast Cancer Cells

Thus far, we have demonstrated that p66Shc is an important regulator of the growth and migration of triple negative breast cancer cells. Moreover, we show that p66Shc is an important mediator of signaling events downstream of the EGFR such as ARF1 and Ras/MAPK activation and AKT phosphorylation. However, triple negative breast cancer represents only ∼15% of all breast cancer cases (41). Therefore, we next evaluated the role of p66Shc in a more prominent cellular model of HER2 positive breast cancer, the SKBR3 cell line. In fact, approximately, 20–40% of breast cancer patients have an amplified HER2 receptor expression (42). First, we assessed the expression of p66Shc in SKBR3 cells (HER2 and EGFR positive) compared with MDA-MB-231 cells (HER2 negative and EGFR positive). As shown in Fig. 4*A*, MDA-MB-231 cells express high levels of p66Shc compared with SKBR3 cells. These findings are in accordance with previously published observations showing that p66Shc expression is negatively correlated with expression of the HER2 receptor (43). Furthermore, we previously reported that SKBR3 cells were shown to have a higher expression level of ARF1 compared with MDA-MB-231 cells (29). Comparable expression of EGFR, Grb2, and ARF6 were observed for both cell lines. Next, we examined the effect of overexpressing HA-tagged p66Shc on ARF1 activation in this cell type (Fig. 4*B*). In control cells, EGF stimulation induced the activation of ARF1. Interestingly, overexpression of p66Shc was shown to also reduce the activation of ARF1 further emphasizing the role of p66Shc as a negative regulator of ARF1 activation. Knowing that p66Shc influenced ARF1 activation in these cells and that we previously found that the proliferation and migration of SKBR3 cells was dependent on ARF1 expression (29), we next assessed the physiological role of p66Shc in this HER2 positive background. First, we evaluated its regulation of SKBR3 cell growth. As shown in Fig. 4*D*, EGF stimulation of control cells significantly promoted proliferation. Whereas, cells overexpressing p66Shc were shown to have an increased growth rate after 24 h. However, a significant decrease in both basal and EGF-dependent cellular growth was observed by 72 h. Second, we assessed the migration of mock-transfected or p66Shc overexpressing SKBR3 cells in the absence and presence of EGF stimulation (Fig. 4*E*). EGF was shown to enhance the migration of control SKBR3 cells. Interestingly, both EGF-independent and -dependent migration was enhanced in cells overexpressing p66Shc. Together, our results demonstrated that whereas p66Shc negatively regulated EGF-mediated signaling cascades such as ARF1 activation and cellular growth in HER2

positive breast cancer cells, it significantly enhanced cellular migration. These findings would suggest that p66Shc would be positively acting on other key proteins mediating this important cellular response.

p66Shc Attenuates the Recruitment of ARF1 to the EGFR

Having characterized the role of p66Shc in mediating ARF1 activation, cell growth, and cell migration in both a cellular model of triple negative and HER2 positive breast cancer, we next sought to delineate the mechanism via which p66Shc regulated ARF1 activation. Because p66Shc is endogenously expressed and ARF1 activation has been previously described in MDA-MB-231 cells, we used this cellular model to characterize the mechanism of negative regulation of ARF1 activity by p66Shc. First, we asked whether this ARF isoform was recruited to the EGFR in p66Shc-depleted cells (Fig. 5*A*). To do this, we immunoprecipitated EGFR from lysates of cells either transfected with control scrambled or p66Shc siRNA and immunodetected associated ARF1 using specific antibodies. EGF induced the recruitment of ARF1 to the EGFR in control siRNA cells. However, knockdown of p66Shc resulted in an increased EGFR recruitment of ARF1 suggesting that p66Shc blocks ARF1 receptor recruitment. To further confirm these results, we either mock transfected MDA-MB-231 cells or overexpressed HA-tagged p66Shc. As shown in Fig. 5*B*, the overexpression of p66Shc significantly attenuated the EGFR recruitment of ARF1 compared with the mock transfected condition. Together, our findings suggest that p66Shc attenuates ARF1 activation by blocking the recruitment of this GTPase to the EGFR.

Recruitment of the Grb2-ARF1 Complex to the EGFR is attenuated by p66Shc

It has been previously demonstrated that p66Shc can block the recruitment of Grb2 to the EGFR and insulin-like growth factor receptor leading to decreased receptor signaling (18, 19). Therefore, we next evaluated whether in our cellular model p66Shc could block the recruitment of Grb2 to the EGFR thereby impacting the recruitment and activation of ARF1. In MDA-MB-231 cells transfected with a control siRNA or p66Shc-specific siRNA, we immunoprecipitated the EGFR and examined Grb2 (Fig. 6*A*). As expected Grb2 was recruited to the EGFR upon its activation in control cells. However, the recruitment of Grb2 to the EGFR was enhanced upon knockdown of p66Shc. Furthermore, reduced expression of p66Shc was associated with a delay in the activation of the EGFR compared with control conditions (Fig. 6*A*). Here, EGF was shown to transiently induce tyrosine phosphorylation of the EGFR at 1 and 2 min before returning to basal levels by 5

min. A significant increase in EGFR tyrosine phosphorylation was observed at 1 min compared with p66Shc overexpressing cells, and this increase was maintained above basal levels for up to 15 min. We also observed an enhanced recruitment of endogenous p66Shc to the EGFR that was maintained throughout the 60 min of stimulation in cells overexpressing HA-p66Shc (Fig. 6*B*). Together, these results suggest that p66Shc may block activation and receptor recruitment of ARF1 by blocking Grb2 recruitment to the EGFR. Furthermore, p66Shc and Grb2 may compete for similar binding sites on the EGFR and thus the Grb2-ARF1 complex would not be recruited to the receptor because Grb2 binding sites, on the receptor, are occupied by p66Shc.

Grb2 Is Essential for the Recruitment of ARF1 to the EGFR and Activation of GTPase

With evidence demonstrating that p66Shc blocks the recruitment of Grb2 to the EGFR, we next examined the influence of p66Shc on association between ARF1 and Grb2. Here, we immunoprecipitated ARF1 from lysates obtained from either scrambled or p66Shc siRNA-transfected cells and measured the level of associated Grb2 (Fig. 7*A*). Interestingly, knockdown of p66Shc resulted in an increased association between Grb2 and ARF1 compared with control cells. Alternatively, we next determined the influence of Grb2 on interaction between p66Shc and ARF1. To do this, we immunoprecipitated ARF1 from lysates obtained from cells depleted of Grb2. As shown in Fig. 7*B*, the association between p66Shc and ARF1 was decreased in cells depleted of this adaptor. For this study, the average percent inhibition of Grb2 expression of siRNA was 95%. Together, our results suggest that regulation of ARF1 activation by p66Shc may stem from its negative effect on the Grb2/ARF1 interaction. Our results further suggest that the formation of a complex between p66Shc and ARF1 may occur indirectly, via the adaptor Grb2.

Subsequently, we sought to determine whether Grb2 was essential for ARF1 activation and the recruitment of this GTPase to the EGFR. As shown in Fig. 7*C*, depletion of Grb2 significantly suppressed EGF-induced ARF1 activation compared with control conditions further suggesting that p66Shc may inhibit ARF1 activation by blocking the actions of Grb2. Next, we determined whether Grb2 was required for the recruitment of ARF1 to the EGFR. To do this, we immunoprecipitated the EGFR from cells either transfected with scrambled or Grb2 siRNA and immunodetected the presence of ARF1. As shown in Fig. 7*D*, EGF- induced recruitment of ARF1 to the EGFR in control conditions. However, the recruitment of this ARF isoform to the receptor

was significantly blocked in cells depleted of Grb2. Together, these findings highlight the importance of Grb2 in receptor recruitment and activation of ARF1.

ARF6 Activation and Its Recruitment to the EGFR Is Potentiated by p66Shc

Because ARF6 has also been shown to be activated downstream of the EGFR (29, 31), we next examined whether p66Shc could regulate ARF6 activation in highly invasive breast cancer cells. First, EGF stimulation promoted GTP loading on this other ARF isoform in control MDA-MB-231 cells (Fig. 8*A*). Although, knockdown of p66Shc expression attenuated ARF6 activation. The contribution of p66Shc in ARF6 activation was confirmed by overexpressing HA-tagged p66Shc. As illustrated in Fig. 8*B*, overexpression of p66Shc increased ARF6 activation. In fact, the potentiated activation of ARF6 was shown to be independent of EGF stimulation as observed by a significant increase in ARF6 activation at the basal level that was not altered upon EGF stimulation. Next, we examined the role of p66Shc in mediating ARF6 activation in the HER2 positive breast cancer SKBR3 cell line (Fig. 8*C*). Similar to what was observed in MDA-MB-231 cells, a significant increase in basal ARF6 activation was observed in SKBR3 cells overexpressing HA-p66Shc compared with control conditions. Together, our results demonstrate that activation of both ARF1 and ARF6 are regulated by p66Shc. However, whereas p66Shc blocked both the basal and EGF-dependent activation of ARF1, it significantly increased EGF-independent ARF6 activation.

We next examined the role of p66Shc in the recruitment of ARF6 to the EGFR. As seen in Fig. 8*D*, EGFR stimulation of control cells induced the recruitment of this ARF isoform to the EGFR. However, knockdown of p66Shc expression reduced the ability of this ARF isoform to associate with the receptor. In contrast, overexpression of p66Shc resulted in an increased recruitment of ARF6 to the EGFR (Fig. 8*E*). This recruitment to the EGFR, in cells overexpressing p66Shc, was shown to be independent of EGF stimulation as an increased association with the EGFR was equally observed in untreated and EGF-treated cells. Collectively, our results illustrate that whereas p66Shc attenuates ARF1 activation by blocking its recruitment to the EGFR, ARF6 activation and recruitment to the activated receptor is dependent on p66Shc.

Grb2 Is Required for Activation of ARF6 and Its Recruitment to the EGFR

We next examined whether Grb2 was also required for ARF6 activation. Interestingly, similar to what was observed for ARF1, the depletion of Grb2 was associated with a decreased activation of ARF6 (Fig. 9*A*), suggesting that the activation process of both ARF isoforms requires this adaptor. Last, we evaluated the role of Grb2 in recruitment of ARF6 to the EGFR. Once again, as observed for ARF1, depletion of Grb2 was associated with a diminished association between the receptor and ARF6 (Fig. 9*B*). These data show that Grb2 is essential for activation and EGFR recruitment of both ARF isoforms. To further define the role of p66Shc in the activation process of ARF6, we examined its ability to regulate the Grb2/ARF6 interaction. As illustrated in Fig. 9*C*, depletion of p66Shc blocked the ability of ARF6 to associate with Grb2 suggesting that Grb2-dependent recruitment of ARF6 to the EGFR may be acting through p66Shc. Together, our results suggest that Grb2 is required for activation and receptor recruitment of ARF6 and that this small GTP-binding protein is in complex with Grb2 via its association with p66Shc.

Altogether, these results demonstrate that the adaptor proteins p66Shc and Grb2 regulate both ARF1 and ARF6 activation. We show that ARF1 is recruited to the EGFR and activated through its association with Grb2. Furthermore, we demonstrate that p66Shc decreases the activation of ARF1 by blocking recruitment of the Grb2-ARF1 complex to the EGFR. Additionally, we demonstrate that ARF6 activation and EGFR recruitment are dependent on both p66Shc and Grb2, where in which ARF6 is recruited to the EGFR via Grb2 by means of its association with p66Shc.

II.5 Discussion

Because ARF1 and ARF6 are small GTPases activated downstream of the EGFR in invasive breast cancer cells, we aimed at defining the molecular mechanisms by which EGF stimulation leads to their activation. Classically, stimulation of the EGFR by its ligands results in the engagement of adaptor proteins assuring downstream signaling. Here, we show that p66Shc and Grb2 play an important role in the recruitment of ARF1 and ARF6 to the EGFR as well as in the activation process of these small GTP-binding proteins. Specifically, we demonstrate that Grb2 is essential for GTP loading of the two ARF isoforms, whereas p66Shc has opposite effects on ARF1 and ARF6 activation, attenuating ARF1 and potentiating ARF6, respectively.

The role of p66Shc in breast cancer remains highly controversial and not fully understood. In the highly invasive MDA-MB-231 cell line, expression of p66Shc is elevated similar to what we have observed for ARF1 and ARF6, suggesting a possible role for this adaptor in breast cancer progression. Using knockdown and overexpression approaches, we have shown that p66Shc limits ARF1 activation and recruitment of this GTPase to the EGFR. Alternatively, we have demonstrated that p66Shc enhances ARF6 activation. We propose that this may represent another mechanism through which p66Shc acts as a key mediator of breast cancer progression.

Although p66Shc has been reported to influence cellular growth and migration on a variety of cell types (37, 39, 40, 44, 45), we demonstrate for the first time its physiological role in invasive breast cancer cells. We show that knockdown of p66Shc significantly attenuates the growth of MDA-MB-231 cells. It has been previously reported that depletion of p66Shc in normal bronchial cells lead to cell growth arrest by increasing the expression of the cyclin-dependent kinase 4 inhibitor, p16, and by decreasing phosphorylation of the retinoblastoma protein, a key cell cycle regulator (37). Interestingly, we observed a similar profile (increased p16 expression, decreased retinoblastoma protein phosphorylation) when we transfected p66Shc siRNA in MDA-MB-231 cells (data not shown) suggesting that this adaptor may play an important role in mediating cell cycle progression. Surprisingly, we also observed reduced proliferation in cells overexpressing p66Shc. It has been previously reported that increased expression of p66Shc is associated with the induction of apoptosis (46–49). In our experiments, we observed a decreased expression of pro-apoptotic factors in p66Shc knockdown cells (data not shown).

We also demonstrated that cellular migration of MDA-MB-231 and SKBR3 cells was regulated by p66Shc. Although depletion of p66Shc significantly blocked cell migration, an enhanced effect

was observed in cells overexpressing p66Shc. To this day, the role of p66Shc, in cancer cell migration, remains controversial. Although this Shc isoform has been shown to induce the migration of prostate and colorectal cancer cells, it was reported to suppress the migration of lung cancer cells (37, 40). We have previously demonstrated that ARF1 is essential for the migration of both MDA-MB-231 and SKBR3 cells (29). However, we report here that a negative regulator of ARF1 enhances cellular migration. We hypothesize that p66Shc may promote migration through an ARF1-independent pathway such as via activation of the ARF6/Ras/MAPK pathway. A signaling cascade also implicated in the induction of breast cancer cell migration (32). Additionally, p66Shc has been shown to induce migration via the activation of another small GTPase, Rac1 (39). Recently, we have demonstrated that Rac1 was a downstream effector of ARF1 signals in MDA-MB-231 cells and the overexpression of a constitutively active form of Rac1 could reverse the inhibitory effect of ARF1 depletion on cell migration (50). Therefore, p66Shc may promote cell migration via the activation of Rac1. Together, our findings demonstrate that p66Shc is a key regulator of breast cancer cell growth and migration and may play an important role in breast cancer progression.

The majority of mechanistic studies have examined the signaling role of p52Shc. Upon EGFR stimulation, this Shc isoform is recruited to the receptor and assembled into a complex with Grb2 (51–53). However, p66Shc has also been reported to be recruited to both EGFR and Grb2 (16). In smooth muscle cells, p66Shc sequesters Grb2 away from the insulin-like growth factor receptor and EGFR blocking the activation of downstream signals (18, 54). Here, we show that p66Shc can also block the recruitment of Grb2 to the EGFR in invasive breast cancer cells. Additionally, unlike p46Shc and p52Shc, which activate ERK1/2 when overexpressed in HeLa cells, the overexpression of p66Shc has been shown to have little effect on ERK1/2 activation (16). Meanwhile, p66Shc was shown to block ERK1/2 activation in a variety of other cell lines such as mouse renal proximal tube cells, mouse splenic T cells, and porcine smooth muscle cells (18, 47, 54). In MDA-MB-231 cells, overexpression of p66Shc potentiated the basal ERK1/2 phosphorylation, a process we and others have shown to be mediated by ARF6 (29, 31). Furthermore, this increase in basal ERK activation correlated with an increase in Ras activation. We therefore propose that this increase in Ras/ERK activation observed in p66Shc overexpressing cells stems from an increased ARF6 activation. Interestingly, overexpression of p66Shc was shown to block the activation of both Ras and ERK1/2 following prolonged stimulation with EGF

(15–60 min). Our findings also show that p66Shc can negatively regulate AKT phosphorylation, an effect previously described in a variety of cell types (55, 56). This attenuation of AKT phosphorylation has been associated with the apoptotic properties of p66Shc (56), thus suggesting a possible role for p66Shc in mediating MDA-MB-231 cell survival. Together, our data reveals a mechanism through which p66Shc attenuates ARF1/AKT activation by blocking the recruitment of Grb2 to the EGFR and potentiates basal ARF6/Ras/ERK activation by promoting the receptor recruitment of ARF6.

Similar to p66Shc, the role of the adaptor Grb2 in breast cancer is ill-defined. The expression of Grb2 has been shown to be elevated in primary breast tumors (57) as well as in estrogen receptor positive breast cancer cells (58). In our experiments, we observed a slightly higher expression of Grb2 in MCF7 cells (prototypical estrogen receptor positive cell line) compared with the MDA-MB-231 cells (prototypical triple negative cell line). Although it has previously been reported that overexpression of this adaptor alone is insufficient to transform cells, Grb2 is well known to promote the activation of the Ras/MAPK pathway (59–61). Furthermore, delayed Poly Middle T Antigen-induced mammary tumor formation was observed in Grb2 knock-out mice suggesting a role for this adaptor in mammary tumorgenesis (62). Here, we show that Grb2 is essential for the activation of both ARF1 and ARF6 and thus may contribute in mediating the cellular responses associated with activation of this GTPase: proliferation, migration, and invasion.

It was proposed that GEP100 (BRAG2), an ARF GEF, could directly interact with the phosphorylated Tyr-1068 and Tyr-1086 residues on the EGFR through its PH domain and therefore act as an intermediate mediating ARF activation following EGF stimulation (32). Interestingly, we show that overexpression of p66Shc enhances total phosphorylation of the EGFR, including residues Tyr-1068 and Tyr-1086 (data not shown). This suggests that p66Shc may enhance ARF6 activation by potentiating the tyrosine phosphorylation of the EGFR. The conclusions demonstrated by Morishige and colleagues (32) were obtained from *in vitro* evidences, where phosphopeptides that mimicked residues Tyr-1068 and Tyr-1086 of EGFR could directly interact with the PH domain of GEP100 (63). *In vivo*, these two phosphorylated residues have been characterized as Grb2 binding sites (64, 65). Here, we show that classical EGFR adaptors do play an important role in the activation process of ARF1 and ARF6 similar to what has been reported for other GEFs. Namely, Grb2 is well known to interact with the GEF Sos to promote its recruitment to the EGFR (66, 67). Through its PH domain, SOS interacts with the plasma

membrane, where it activates the small GTPase Ras (67, 68). Interestingly, we show here that p66Shc also plays a role in mediating Ras activation, possibly by regulating the recruitment of a GEF. Additionally, Grb2 has also been reported to recruit another PH domain containing GEF, Vav2, to HER2 to promote activation of both Ras and Rac1 (69). Together, this suggests that Grb2 may play a conserved role in the recruitment of GEFs to the EGFR. Furthermore, p66Shc may also modulate ARF1 activation selectively by either blocking the recruitment of ARF1/GEFs or promoting the recruitment of ARF6/GEFs. Additionally, p66Shc may function to regulate the association of ARF GTPases with their GEFs. This adaptor may promote ARF6/GEF interactions and dissolve ARF1/GEF interactions. Altogether, the recruitment of GEFs and ARF activation may be mediated by both EGFR tyrosine phosphorylation and adaptor recruitment.

Altogether, we demonstrate a role for adaptor proteins p66Shc and Grb2 in mediating EGF-induced ARF1 and ARF6 activation, as well as their recruitment to the EGFR. More specifically, whereas we demonstrate that p66Shc negatively regulates ARF1 signals, ARF6 activation was potentiated by this adaptor. Furthermore, we show that the adaptor Grb2 plays an essential role in the activation of both ARF1 and ARF6. We propose that p66Shc blocks recruitment of the ARF1-Grb2 complex to the EGFR by either competing with Grb2 for common recruitment sites on the receptor or by sequestering Grb2 away from it. This could allow for an increased recruitment of p66Shc/ARF6 to the EGFR and increased ARF6 activation. Based on our findings, we propose the following model of ARF activation in MDA-MB-231 cells (Fig. 10). When the expression levels of Grb2 and p66Shc are in equilibrium, ligand binding induces the activation of the EGFR leading to its autophosphorylation. This first event allows recruitment of the adaptor Grb2. Grb2 acts to recruit ARF1 to the EGFR where it becomes activated by a guanine nucleotide exchange factor leading to activation of the PI3K/AKT pathway. Grb2 also recruits ARF6 to the EGFR, via p66Shc, resulting in its activation and engagement of the Ras/ERK1/2 pathway. Additionally, p66Shc acts as a negative regulator of ARF1 activation. Together these two pathways promote breast cancer cell growth and migration. In conditions where levels of p66Shc are reduced, ARF6 is no longer recruited to Grb2 or the EGFR, thus blocking the activation of both ARF6 and Ras/MAPK. In turn, an enhanced recruitment of the Grb2-ARF1 complex to the EGFR is observed promoting ARF1 activation. Together, this leads to a reduction in cell growth and migration. Upon overexpression of p66Shc, Grb2 and ARF1 are no longer recruited to the EGFR leading to a diminished ARF1 activation and a delayed phosphorylation of AKT. Conversely, receptor

recruitment and activation of ARF6 are potentiated resulting in a basal increase of Ras/MAPK activation, migration, and the induction of cell death. When Grb2 levels are depleted, ARF1 and the p66Shc-ARF6 complex cannot be recruited to the EGFR and thus neither GTPase are activated. In conclusion, we demonstrate for the first time the importance of adaptor proteins in the regulation of ARF activity in invasive breast cancer cells. More importantly, we demonstrate that certain adaptors (Grb2) can have similar effects on the activation of different ARF isoforms and others (p66Shc) can have opposing effects. Thus, characterization of the signaling mechanisms leading to breast cancer cell proliferation, migration, and invasion can help discover more specific and effective therapeutic targets.

II.6 Figure Legends

FIGURE 1. p66Shc negatively regulates ARF1 activation. *A,* the endogenous expression of Shc isoforms (p46Shc, p52Shc, and p66Shc), Grb2, EGFR, ARF1, ARF6, and actin were measured by Western blot (*IB*, immunoblot) analysis of lysates obtained from either confluent MCF7 or MDA-MB-231 cells. *B*, endogenous ARF1 was immunoprecipitated from lysates obtained from serum-starved MDA-MDA-231 cells that were stimulated with EGF (10 ng/ml) for the given time points and associated Shc isoforms and Grb2 were detected by Western blot analysis. The *upper arrow* indicates the p66Shc isoform, the *middle arrow* the p52Shc, and the *lower arrow* the p46Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal and are normalized to total protein content. *C*, MDA-MB-231 cells transfected with a scrambled (*CTL*) or p66Shc siRNA were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed, and a GST pulldown assay using GST-GGA3 coupled to glutathione-Sepharose 4B beads was used to capture activated ARF1. Endogenous levels of activated ARF1 and the total protein levels of ARF1 in cell lysates were assessed by Western blot analysis. Additionally, Western blot analysis was used to confirm the depletion of p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content and are the mean ± S.E. with (*) $p < 0.05$ and (***) $p < 0.001$, compared with the control condition. *D,* MDA-MB-231 cells transfected with an empty vector (*CTL*) or HA-p66Shc were stimulated with EGF (10 ng/ml) for the indicated times. Endogenous levels of activated ARF1 and total protein levels of ARF1 were captured and detected as in *C*. Western blot analysis was used to confirm the overexpression of HA-p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content and are the mean ± S.E. with (*) $p < 0.05$ and (***) $p < 0.001$. *E,* HCC70 cells were transfected and stimulated as in *D*. Endogenous levels of activated ARF1 and total protein levels of ARF1 were captured and detected as in *C*. Western blot analysis was used to confirm the overexpression of HA-p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the

total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (***) $p < 0.001$.

FIGURE 2. p66Shc regulates activation of the Ras/MAPK and AKT pathways. *A,* MDA-MB-231 cells transfected with a scrambled (*CTL*) or p66Shc siRNA were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed, and a GST pulldown assay using the GST-Raf binding domain coupled to glutathione-Sepharose 4B beads was used to capture activated Ras. Endogenous levels of activated Ras and the total protein levels of Ras in cell lysates were assessed by Western blot analysis. Activated ERK1/2 and AKT were assessed by measuring phosphorylation levels of these proteins using phosphospecific antibodies. Equal total protein expression of ERK1/2 and AKT was confirmed by Western blot (*IB*, immunoblot) analysis. The depletion of p66Shc was confirmed using a monoclonal antibody against p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (**) $p < 0.01$ and (***) $p < 0.001$. *B,* MDA-MB-231 cells transfected with an empty vector (*CTL*) or HA-p66Shc were stimulated with EGF (10 ng/ml) for the indicated times. Ras, ERK1/2, and AKT activation were assessed as in *A*. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

FIGURE 3. p66Shc mediates MDA-MB-231 cell growth and migration. *A,* MDA-MB-231 cells transfected with a scrambled (*CTL*) or p66Shc siRNA were left untreated or stimulated with EGF (10 ng/ml) for the indicated times. Cell numbers were determined by a trypan blue exclusion assay via manual counting. Results presented are representative of three independent experiments. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (*) $p < 0.05$ and (***) $p < 0.001$. *B,* MDA-MB-231 cells transfected with an empty vector (*CTL*) or HA-p66Shc were left untreated or stimulated with EGF (10 ng/ml) for the indicated times. Cell numbers were determined as in *A*. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (**) $p < 0.01$ and (***) $p < 0.001$. *C*, MDA-MB-231 cells were transfected and stimulated as in *A*. Cell growth at 72 h was determined via a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described under "Experimental Procedures." The quantifications of each experiment are presented as fold-increase in absorbance over basal absorbance and are the mean ± S.E. with (***) $p < 0.001$. *D,* MDA-MB-231 cells were transfected and stimulated as in *B*. Cell growth was determined as in *C*. The quantifications of each experiment are presented as fold-increase in absorbance over basal absorbance and are the mean ± S.E. with (**) $p < 0.01$ and (***) $p < 0.001$. *E,* MDA-MB-231 cells were transfected as in *A*. Cells were then seeded onto Boyden chambers and stimulated or not with EGF (10 ng/ml). Migration was assessed after 6 h. Results presented are representative of three independent experiments. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (***) $p < 0.001$. *F,* MDA-MB-231 cells were transfected as in *B* and migration was assessed as described in *E*. Results presented are representative of three independent experiments. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (***) $p < 0.001$. The depletion of p66Shc and the overexpression of HA-tagged p66Shc was confirmed by Western blot analysis for all physiological assays. *IB*, immunoblot.

FIGURE 4. Signals downstream of the HER2 receptor are mediated by p66Shc. *A,* the endogenous expression of p66Shc, Grb2, EGFR HER2, ARF1, ARF6, and actin were measured by Western blot (*IB*, immunoblot) analysis of lysates obtained from either confluent SKBR3 or MDA-MB-231 cells. *B,* SKBR3 cells transfected with an empty vector (*CTL*) or HA-p66Shc were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed, and a GST pulldown assay using GST-GGA3 coupled to glutathione-Sepharose 4B beads was used to capture activated ARF1. Endogenous levels of activated ARF1 and the total protein levels of ARF1 in cell lysates were assessed by Western blot analysis. Additionally, Western blot analysis was used to confirm the overexpression of HA-p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (**) $p < 0.01$. *C,* SKBR3 cells transfected as in *A* were left untreated or stimulated with EGF (10 ng/ml) for the indicated times. Cell numbers were determined by a trypan blue exclusion assays via manual counting. Results presented are representative of three independent experiments. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (***) $p < 0.001$. *D,* SKBR3 cells were transfected as in *A*. Cells were then seeded onto Boyden chambers and stimulated or not with EGF (10 ng/ml). Migration was assessed after 6 h. Results presented are representative of three independent experiments. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (*) $p < 0.05$ and (**) $p < 0.01$.

FIGURE 5. The recruitment of ARF1 to the EGFR is attenuated by p66Shc. *A*, MDA-MB-231 cells transfected with a scrambled (*CTL*) or p66Shc siRNA were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed and endogenous EGFR was immunoprecipitated (*IP*). Associated ARF1 was detected by Western blot (*IB*, immunoblot) analysis. Western blot analysis was also used to confirm the total protein expression of ARF1 and EGFR as well as the depletion of p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (*) $p < 0.05$. *B,* MDA-MB-231 cells transfected with an empty vector (*CTL*) or HA-p66Shc were stimulated with EGF (10 ng/ml) for the indicated times. The immunoprecipitation of endogenous EGFR was performed as described in *A*. The overexpression of HA-p66Shc was confirmed by Western blot analysis. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (**) $p < 0.01$ and (***) $p < 0.001$.

FIGURE 6. p66Shc blocks the recruitment of Grb2 to the activated EGFR. *A*, MDA-MB-231 cells transfected with a scrambled (*CTL*) or p66Shc siRNA were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed and endogenous EGFR was immunoprecipitated (*IP*). Associated Grb2 was detected by Western blot (*IB*, immunoblot) analysis. The tyrosine phosphorylation of the EGFR was detected using a phospho-specific monoclonal antibody against phosphorylated tyrosine residues. Western blot analysis was used to confirm the protein expression of Grb2 and EGFR and the depletion of p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (*) $p < 0.05$ and (**) $p < 0.01$. *B,* MDA-MB-231 cells transfected with an empty vector (*CTL*) or HA-p66Shc were stimulated with EGF (10 ng/ml) for the indicated times. The immunoprecipitation of endogenous EGFR was done using a polyclonal antibody to the EGFR and associated p66Shc and Grb2 were detected using a monoclonal antibody to p66Shc and a polyclonal antibody to Grb2. Western blot analysis was used to confirm the protein expression of p66Shc, Grb2, and EGFR. The tyrosine phosphorylation of

the EGFR was detected as described in *A*. The overexpression of HA-p66Shc was confirmed by Western blot analysis. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (**) $p < 0.01$ and (***) $p < 0.001$.

FIGURE 7. Grb2 is essential for activation and EGFR recruitment of ARF1. *A,* MDA-MB-231 cells transfected with a scrambled (*CTL*) or p66Shc siRNA were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed and endogenous ARF1 was immunoprecipitated (*IP*). Associated Grb2 was detected by Western blot analysis. Western blot (*IB*, immunoblot) analysis was used to confirm total expression levels of Grb2 and ARF1 and the depletion of p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (***) $p < 0.001$. *B,* MDA-MB-231 cells transfected with a scrambled (*CTL*) or Grb2 siRNA were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed and endogenous ARF1 was immunoprecipitated. Associated p66Shc was detected by Western blot analysis. Western blot analysis was also used to confirm the protein expression of p66Shc and ARF1 as well as the depletion of Grb2. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (*) $p < 0.05$ and (***) $p < 0.001$. *C,* MDA-MB-231 cells transfected with a scrambled (*CTL*) or Grb2 siRNA were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed, and a GST pulldown assay using GST-GGA3 coupled to glutathione-Sepharose 4B beads was used to capture activated ARF1. Endogenous levels of activated ARF1 and the levels of ARF1 protein in total cell lysates were assessed by Western blot analysis. Additionally, Western blot analysis was used to confirm the depletion of Grb2. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (*) $p < 0.05$ and (***) $p < 0.001$. *D*, MDA-MB-231 cells were transfected and stimulated as in *A*. Cells

were lysed and endogenous EGFR was immunoprecipitated. Associated ARF1 was detected by Western blot analysis. Western blot analysis was also used to confirm expression levels of ARF1 and EGFR as well as the depletion of Grb2. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (***) $p < 0.001$.

FIGURE 8. p66Shc enhances ARF6 activation and the recruitment of this GTPase to the EGFR. *A,* MDA-MB-231 cells transfected with a scrambled (*CTL*) or p66Shc siRNA were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed, and a GST pulldown assay using GST-GGA3 coupled to glutathione-Sepharose 4B beads was used to capture activated ARF6. Endogenous levels of activated ARF6 and the levels of ARF6 protein in total cell lysates were assessed by Western blot (*IB*, immunoblot) analysis. Additionally, the Western blot analysis was used to confirm the depletion of p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (**) $p < 0.01$ and (***) $p < 0.001$. *B,* MDA-MB-231 cells transfected with an empty vector (*CTL*) or HA-p66Shc were stimulated with EGF (10 ng/ml) for the indicated times. Endogenous levels of activated ARF6 were captured and detected as in *A*. Western blot analysis was used to confirm the overexpression of HA-p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (***) $p < 0.001$. *C,* SKBR3 cells transfected with an empty vector (*CTL*) or HA-p66Shc were stimulated with EGF (10 ng/ml) for the indicated times. Endogenous levels of activated ARF6 were captured and detected as in *A*. Western blot analysis was used to confirm the overexpression of HA-p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (**) $p < 0.01$. *D,* MDA-MB-231 cells were transfected and stimulated as in *A*. Cells were lysed and endogenous EGFR was immunoprecipitated (*IP*). Associated ARF6 was

detected by Western blot analysis. Western blot analysis was also used to confirm the protein expression of ARF6 and EGFR as well as the depletion of p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (***) $p < 0.001$. *E*, MDA-MB-231 cells were transfected and stimulated as in *B*. The immunoprecipitation of endogenous EGFR was completed as described in *D*. The overexpression of HA-p66Shc was confirmed by Western blot analysis. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, normalized to total protein content, and are the mean ± S.E. with (***) $p < 0.001$.

FIGURE 9. Grb2 is also essential for ARF1 activation and its recruitment to the EGFR. *A,* MDA-MB-231 cells transfected with a scrambled (*CTL*) or Grb2 siRNA were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed, and a GST pulldown assay using GST-GGA3 coupled to glutathione-Sepharose 4B beads was used to capture activated ARF6. Endogenous levels of activated ARF6 and the levels of ARF6 protein in total cell lysates were assessed by Western blot (*IB*, immunoblot) analysis. Additionally, Western blot analysis was used to confirm the depletion of Grb2. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (*) $p < 0.05$ and (***) $p < 0.001$. *B,* MDA-MB-231 cells were transfected and stimulated as in *A*. Cells were lysed and endogenous EGFR was immunoprecipitated (*IP*). Associated ARF6 was detected by Western blot analysis. Western blot analysis was also used to confirm the protein expression of ARF6 and EGFR as well as the depletion of Grb2. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (**) $p < 0.01$ and (***) $p < 0.001$. *C,* MDA-MB-231 cells transfected with a scrambled (*CTL*) or p66Shc siRNA were stimulated with EGF (10 ng/ml) for the indicated times. Cells were lysed and endogenous ARF6 was immunoprecipitated. Associated p66Shc was detected by Western blot

analysis. Western blot analysis was also used to confirm the protein expression of p66Shc and ARF6 as well as the depletion of p66Shc. Results presented are representative of three independent experiments and the inputs represent 5% of the total protein present in each sample. The quantifications of each experiment are presented as fold-increase over basal, are normalized to total protein content, and are the mean ± S.E. with (***) $p < 0.001$.

FIGURE 10. Model of ARF1 and ARF6 activation downstream of the EGFR in MDA-MB-231 cells. *A,* in normal conditions, the expression of the adaptors p66Shc and Grb2 is at equilibrium. Upon activation of the EGFR, Grb2 is recruited to the EGFR. This leads to the recruitment of ARF1 and the activation of this GTPase. Furthermore, Grb2 recruits the p66Shc-ARF6 complex to the EGFR leading to the activation of ARF6. *B,* the depletion of p66Shc is associated with an increased recruitment of Grb2 and ARF1 to the EGFR and an increase in ARF1 activation. Alternatively, a decrease in ARF6 activation stemming from a decreased EGFR recruitment of this GTPase was observed upon the depletion of p66Shc. *C,* when p66Shc levels are elevated, the recruitment of Grb2 and ARF1 to the EGFR is blocked and the activation of ARF1 is significantly decreased. Additionally, elevated p66Shc levels increased the recruitment of ARF6 to the EGFR resulting in an increase in ARF6 activation. *D,* the depletion of Grb2 blocks the recruitment of ARF1 to the EGFR and blocks ARF1 activation. In these conditions, p66Shc is no longer recruited to the EGFR. This leads to the attenuation of ARF6 activation and recruitment to the EGFR.

Figure 1
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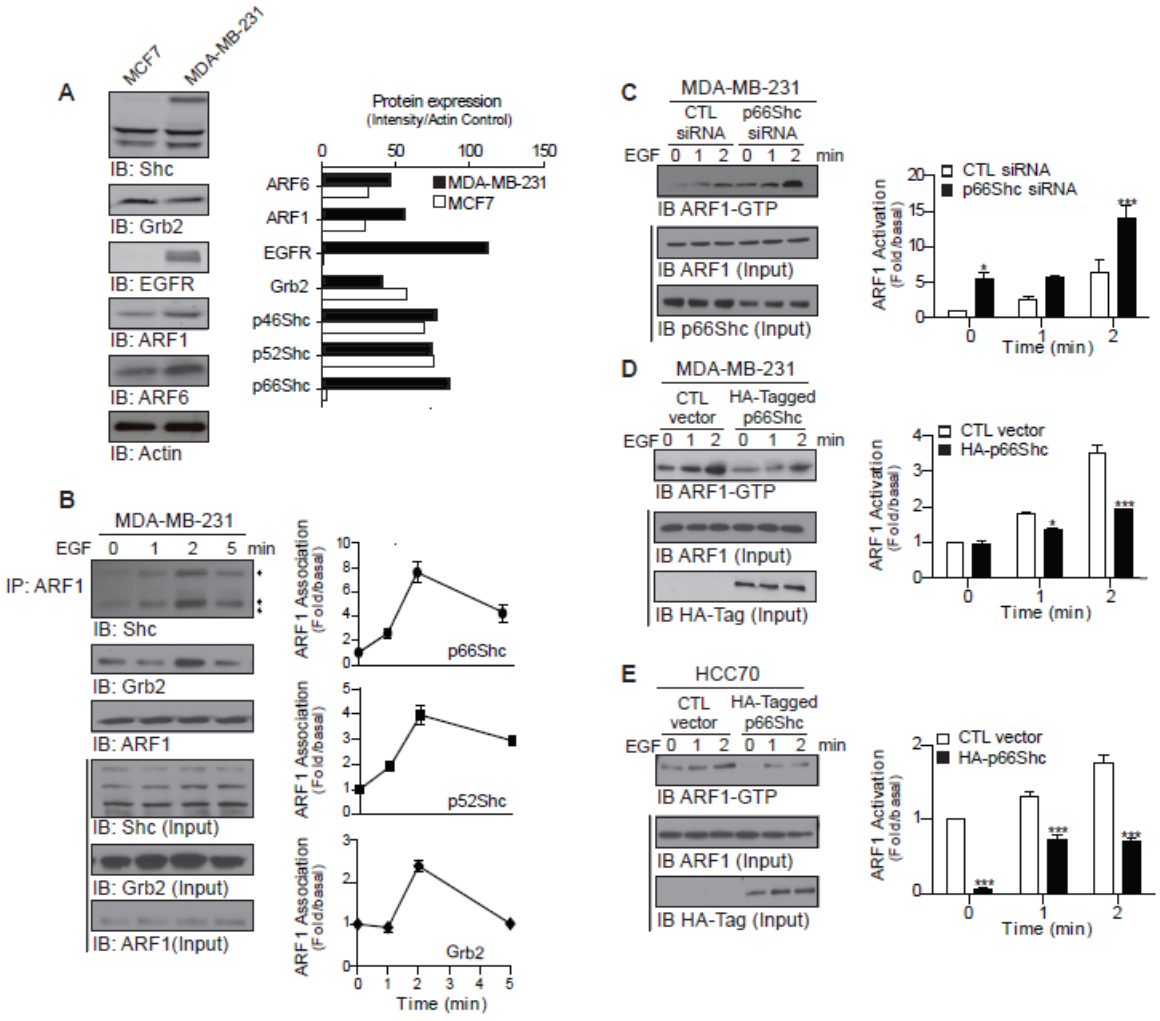

Figure 2
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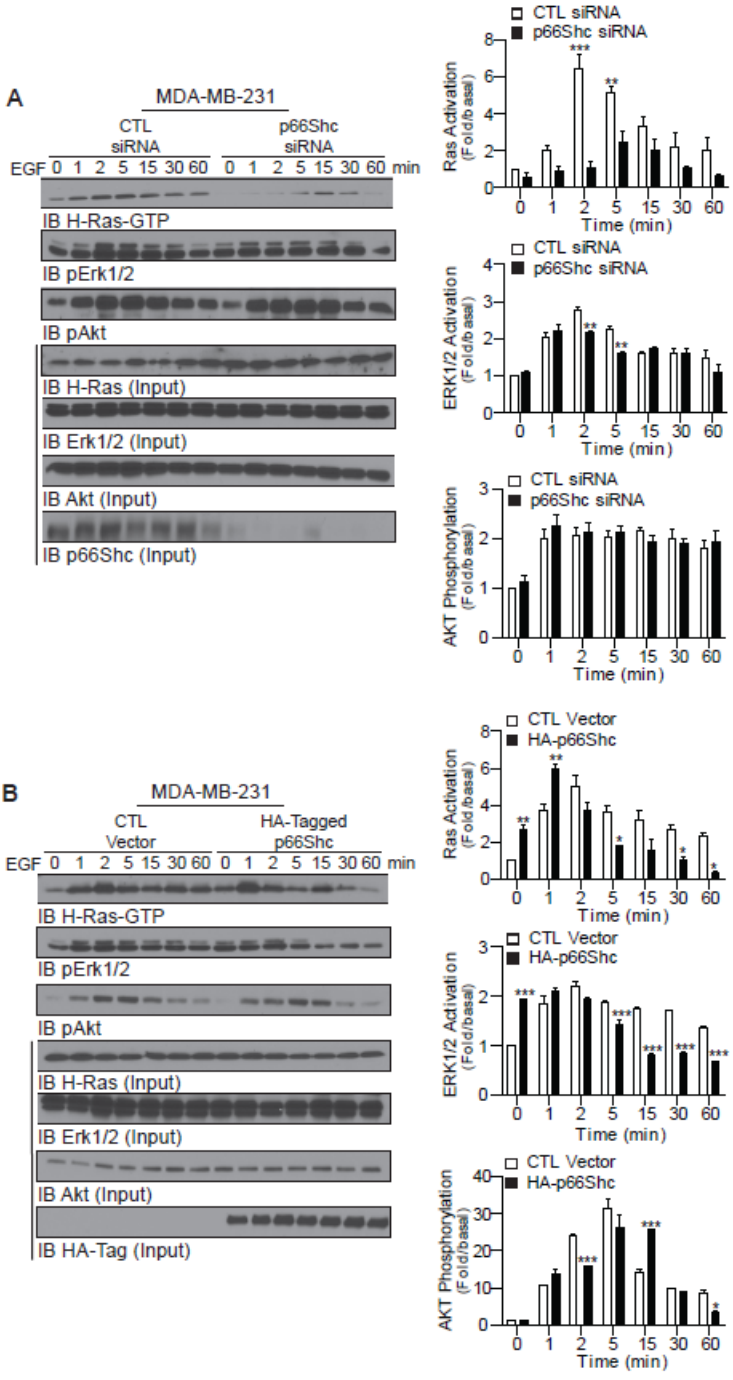

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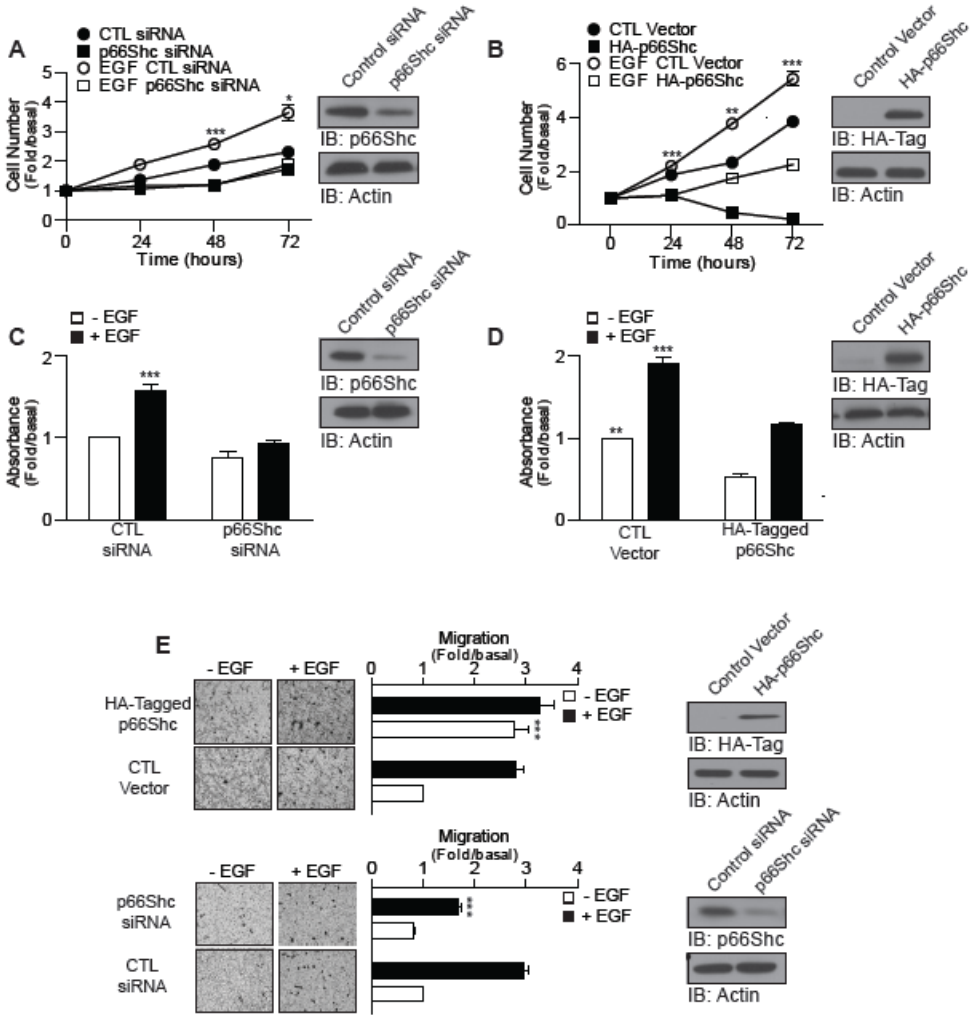

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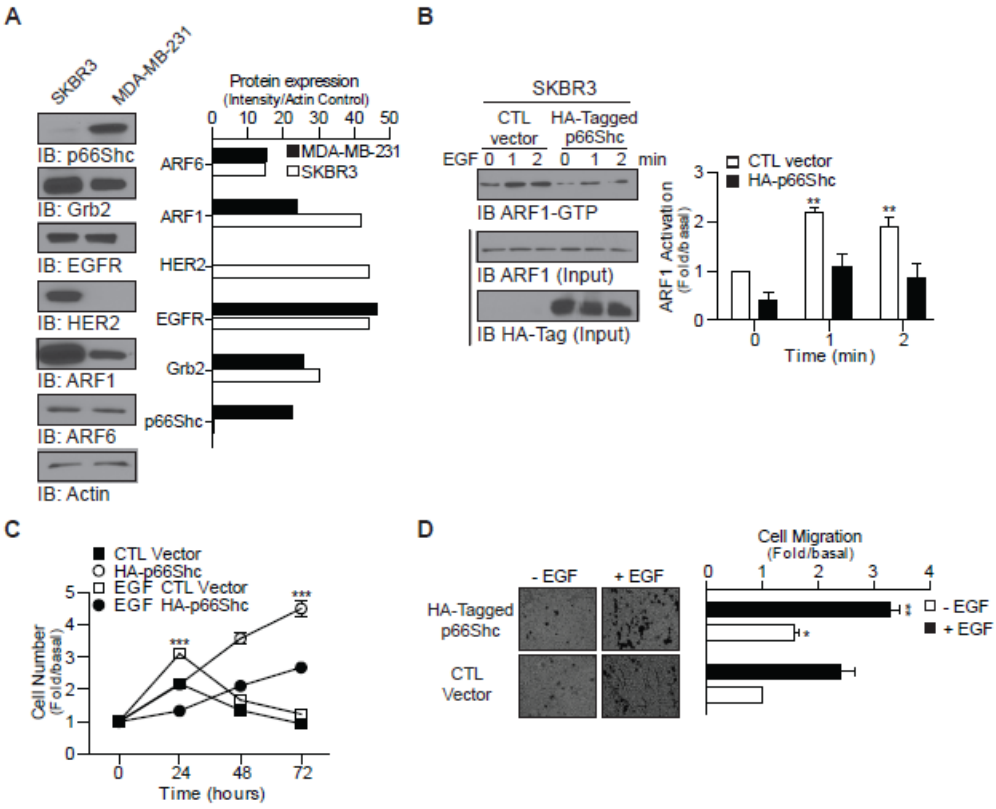

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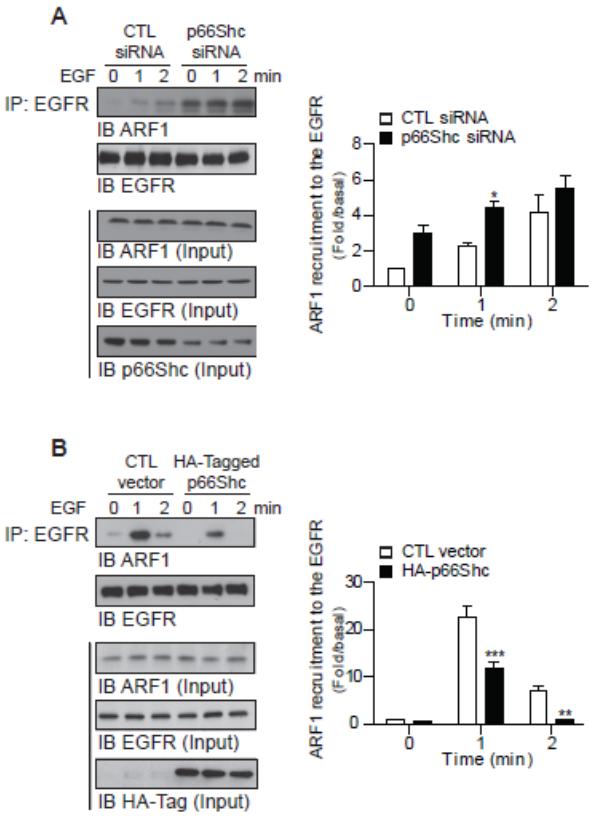

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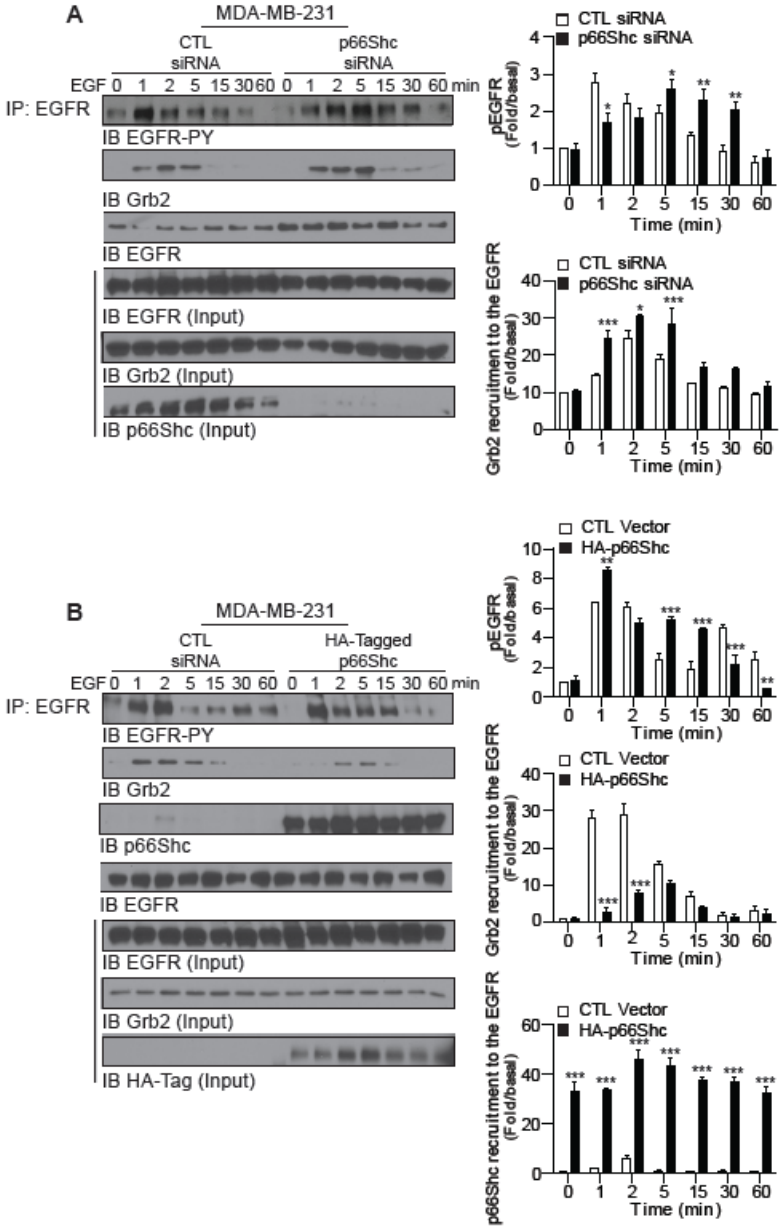

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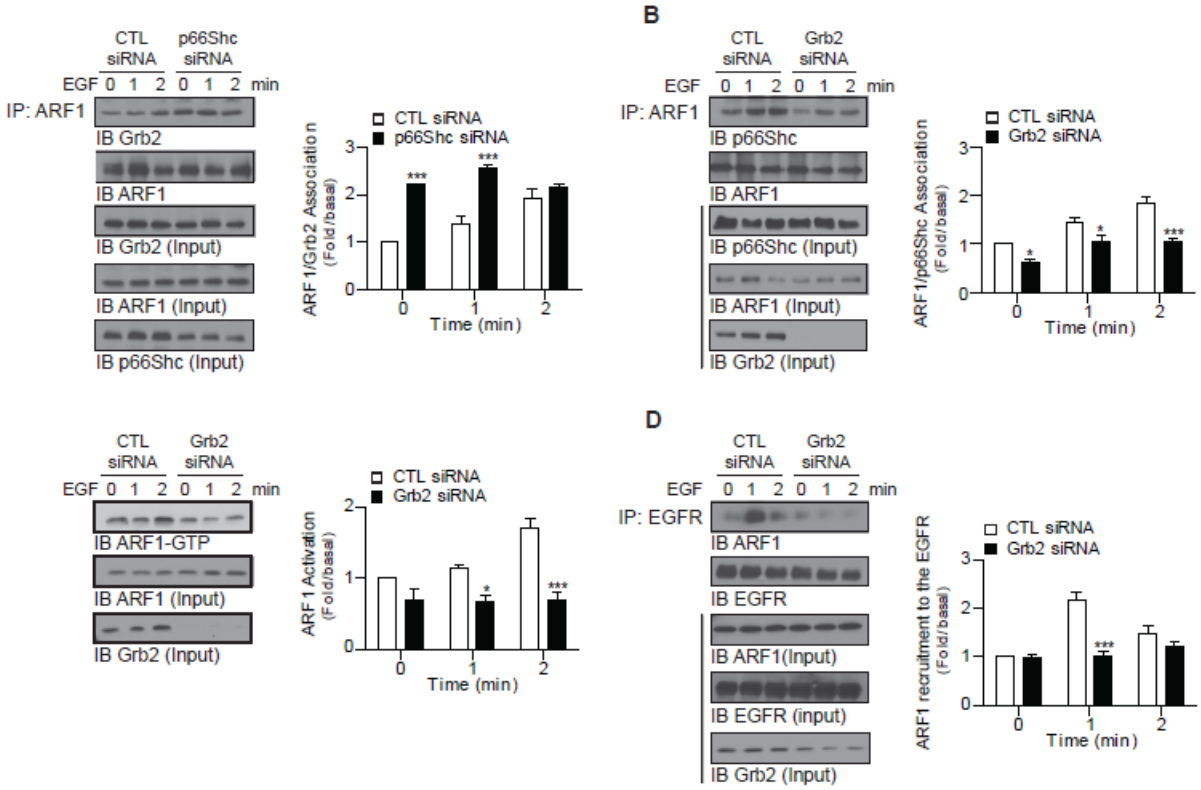

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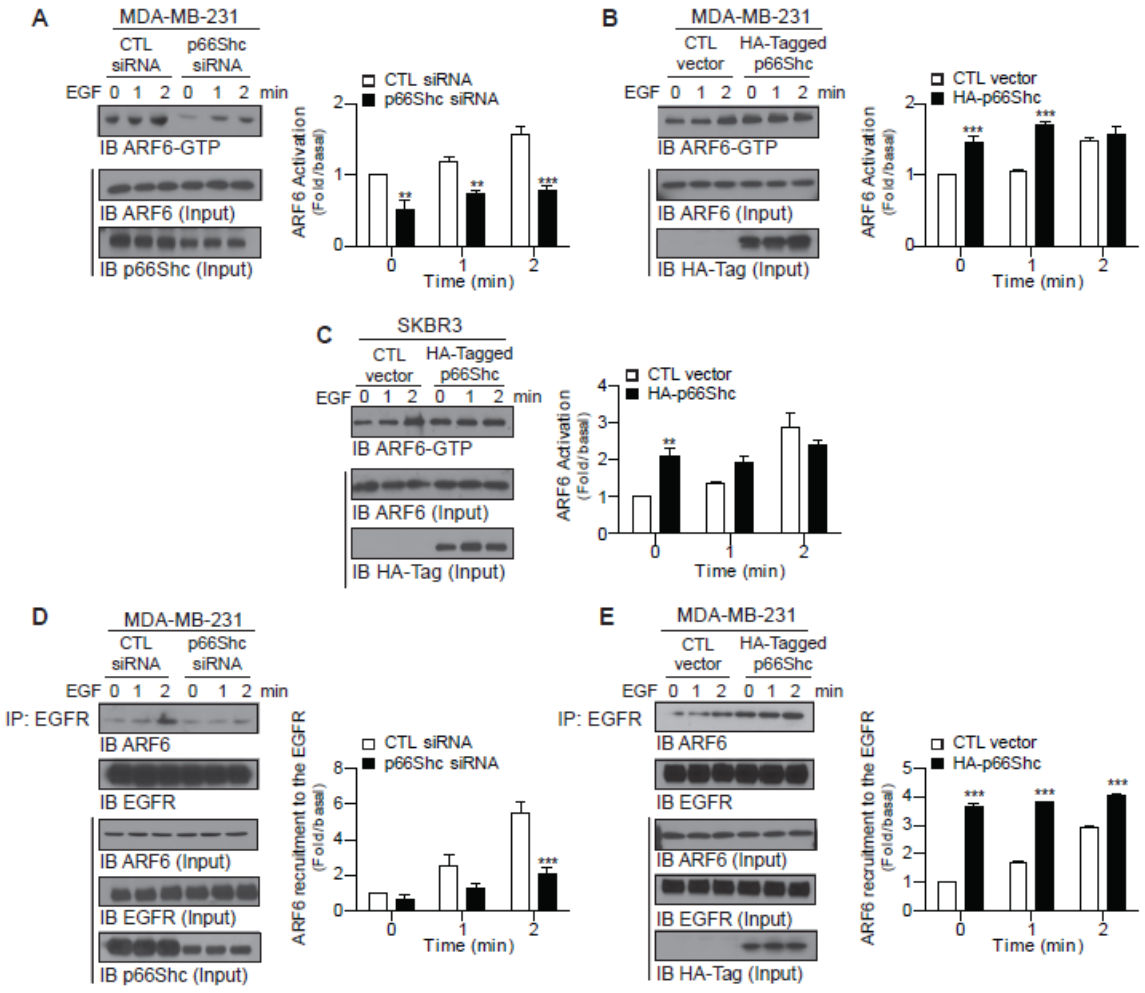

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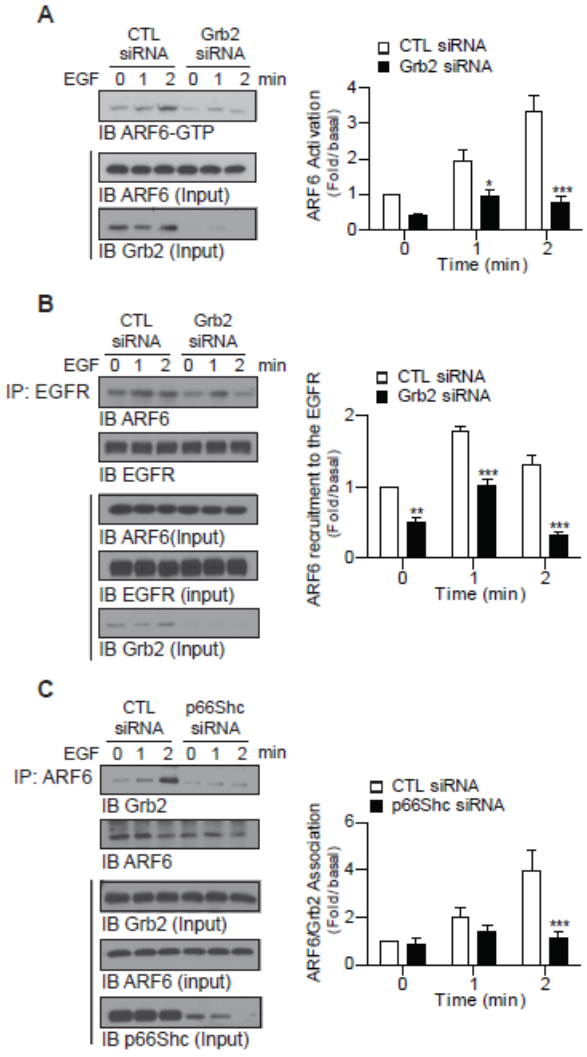

Figure 10
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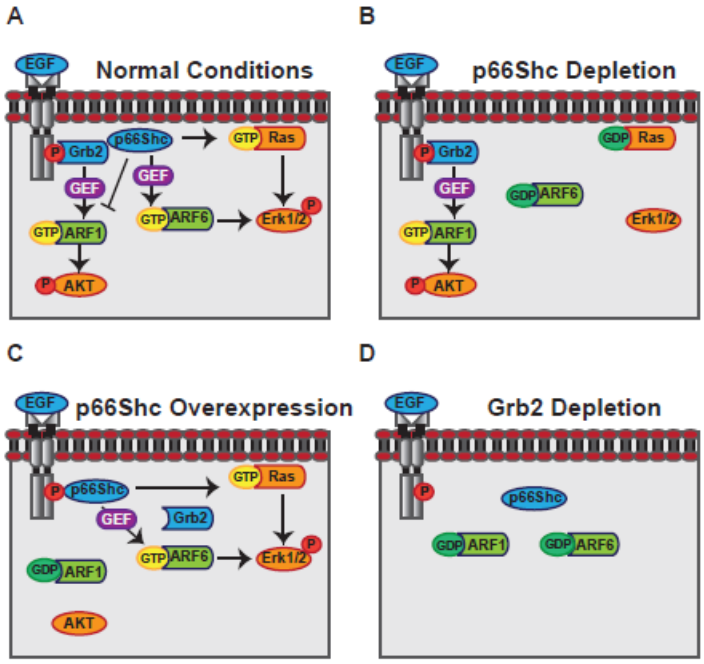

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CHAPTER III: The small GTPase ADP-Ribosylation Factor 1 mediates sensitivity to EGFR tyrosine kinase inhibitors in triple negative breast cancer cells

Accepted: *Cancer Biol Ther* 2015 15;16(10):1535-47.

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III.1 Abstract

The clinical use of EGFR-targeted therapy, in triple negative breast cancer patients, has been limited by the development of resistance to these drugs. Although activated signaling molecules contribute to this process, the molecular mechanisms remain relatively unknown. We have previously reported that the small GTPase ADP-Ribosylation Factor 1 (ARF1) is highly expressed in invasive breast cancer cells and acts as a molecular switch to activate EGF-mediated responses. In this study, we aimed at defining whether the high expression of ARF1 limits sensitivity of these tumor cells to EGFR inhibitors, such as gefitinib. Here, we show that the knock down of ARF1 expression or activity decreased the dose and latency time required by tyrosine kinase inhibitors to induce cell death. This may be explained by the observation that the depletion of ARF1 suppressed gefitinib-mediated activation of key mediators of survival such as ERK1/2, AKT and Src, while enhancing cascades leading to apoptosis such as the p38MAPK and JNK pathways, modifying the Bax/Bcl2 ratio and cytochrome c release. In addition, inhibiting ARF1 expression and activation also results in an increase in gefitinib-mediated EGFR internalization and degradation further limiting the ability of this receptor to promote its effects. Interestingly, we observed that gefitinib treatment resulted in the enhanced activation of ARF1 by promoting its recruitment to the receptor AXL, an important mediator of EGFR inhibition suggesting that ARF1 may promote its pro-survival effects by coupling to alternative mitogenic receptors in conditions where the EGFR is inhibited. Together our results uncover a new role for ARF1 in mediating the sensitivity to EGFR inhibition and thus suggest that limiting the activation of this GTPase could improve the therapeutic efficacy of EGFR inhibitors.

III.2 Introduction

The triple negative breast cancer (TNBC) subtype is characterized by the lack of expression of the estrogen, progesterone and HER2 receptors. Approximately 15-20% of global breast cancers are diagnosed as TNBC (1). This breast cancer subtype is considered to have an aggressive phenotype with high histological grade and metastatic potential (2,3). Moreover, disease recurrence has been shown to occur earlier in TNBC patients (4). This results in an overall poor patient prognosis (5). Since, current cytotoxic chemotherapeutics have shown to be effective only in a small proportion of patients (6), there are present attempts to identify and characterize agents that therapeutically target specific oncogenic factors.

The epidermal growth factor receptor (EGFR) is highly expressed in the majority of TNBC patients (7) and is associated with a poor prognosis making this receptor tyrosine kinase (RTK) a potential therapeutic target for the treatment of this aggressive form of breast cancer (7,8). Since the EGFR and HER2 are the two EGFR family members best characterized for their role in cancer, the majority of drugs targeting the EGFR family blocks these two members. There are two predominant types of EGFR-targeted therapies: monoclonal antibodies targeting the extracellular domain of the receptor and tyrosine kinase inhibitors targeting the kinase activity of the receptor (9,10). However, little to no therapeutic benefits have been observed in recent attempts at targeting the EGFR in TNBC patients (11,12). The development of EGFR inhibitor resistance (either innate or acquired) has been shown to play a major impact on the lack of response observed in these patients (13). Multiple mechanisms of resistance such as mutations in the EGFR itself and its downstream signaling effectors, increased expression of receptor tyrosine kinases (RTKs) (EGFR, HER2-3, AXL, cMET), and activation of other signaling regulators (Src, ERK1/2, AKT) have been described in the literature (12,14-19). Inhibiting these mechanisms of acquired resistance is an effective strategy to improve the sensitivity of these patients to EGFRTKis (20-22). In fact, the inhibition of the ERK1/2 pathway, as well as the AXL and cMET receptors have been shown to decrease cell growth and tumor formation of gefitinib-resistant cancer cells (20-22). However, the underlying mechanisms of acquired EGFRTKi resistance in highly invasive breast cancer cells have yet to be fully characterized.

Recently, we demonstrated that ADP-Ribosylation Factors 1 (ARF1), one of the six identified ARF isoform (ARF1 to 6) members of the superfamily of Ras GTPases, is activated downstream

of the EGFR in MDA-MB-231 cells, a cellular model of TNBC (23). ARF proteins are broadly known for their role in vesicular trafficking, membrane lipid remodeling and reorganization of the actin cytoskeleton. Of the ARF isoforms, ARF1 and ARF6 are the best characterized. Like all GTPases, these proteins are inactive when bound to GDP, but become active when GTP is loaded by specific guanine nucleotide exchange factors (GEF). While ARF1 was first identified as a key regulator of Golgi trafficking, in the most invasive breast cancer cell lines, we reported that this ARF isoform was overexpressed and localized to the plasma membrane where it could be activated by the EGFR to control signaling to the PI3K survival pathway (23). We further demonstrated that activation of this protein following EGF stimulation is dependent upon the recruitment of the classical EGFR adaptor proteins, Grb2 and p66Shc (24). Depletion of ARF1 markedly impairs migration, invasion and proliferation of highly invasive breast cancer cells (23,25,26).

In this study, we aimed at defining whether this small GTP-binding protein could also play a role in mediating the sensitivity of TNBC cells to EGFR tyrosine kinase inhibitors (EGFRTKis) since it is activated by the EGFR and acts to regulate numerous downstream signaling events coordinating key physiological responses characteristic of tumor cells and associated with invasiveness. Here, we report that ARF1 plays a key role in mediating EGFRTKi sensitivity. The knockdown or inhibition of this GTPase activity significantly improved the sensitivity of breast cancer cells to gefitinib. Our results suggest that targeting this key protein in combination with EGFR inhibitors may enhance their effectiveness and efficiency.

III.3 Materials and Methods

Reagents and Antibodies

Lipofectamine 2000™ was purchased from Invitrogen (Burlington, Ontario, Canada). Epidermal growth factor was purchased from Fitzgerald Industries International, Inc. (Concord, MA). Inhibitors used were gefitinib (Biovision Inc. Milpitas, CA), tivantinib (Selleckchem, Houston, TX), R428 (Abmole Bioscience, Houston, TX), lapatinib, MG132, PD0325901, PP2, LY294002 and SB220025 *(*Sigma-Aldrich, Oakville, Ontario, Canada). Polyclonal antibodies used were EGFR, HER2, HER3, HER4, AXL, cMET, pAXL, pcMET, pErk1/2, pAKT, AKT, pSrc, pp38MAPK, p38MAPK, pJNK, JNK, pan-actin, Bax, Bcl2, Cytochrome C, CoxIV (Cell Signaling, Danvers, MA), ARF1 (Proteintech Group, Chicago, IL), HA-Tag, Erk1/2 (Santa Cruz Biotechnology, Dallas, TX). Monoclonal antibodies used were pan-PY (Santa Cruz Biotechnology), Src (Millipore, Etobicoke, Ontario, Canada). Other reagents used were goat anti-mouse antibody-horseradish peroxidase and goat anti-rabbit antibody-horseradish peroxidase (RD Systems, Minneapolis, MN) and Protein G-Agarose Plus beads (Santa Cruz Biotechnology).

DNA Plasmids and siRNAs

HA-tagged ARF1WT and ARF1WTMut cDNAs were cloned into a pcDNA3 vector, the double-stranded scrambled with 19-nucleotide duplex RNA and 2-nucleotide 3′ dTdT overhangs, ARF1 siRNA was previously described (Cotton, Boulay et al. 2007; Boulay, Cotton et al. 2008; Schlienger, Campbell et al. 2014)(Boulay, et al. 2008; Cotton, et al. 2007; Schlienger, et al. 2014)(23,25,54). All siRNAs include 2-nucleotide 3’ dTdT overhangs and were purchased from Dharmacon Inc. (Lafayette, CO).

Cell Culture and Transfection

MDA-MB-231, MCF7, SKBR3, MDA-MB-157 cells were maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HCC70 cells were maintained at 37°C, 5% CO_2 in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS. Cells were transfected with siRNA or plasmid DNA using *Lipofectamine 2000*™ according to the manufacturer’s instructions. Briefly, cells were transfected

with 25 nM siRNA for 72 hours prior to treatment with inhibitors at indicated concentrations and for indicated time points.

Co-immunoprecipitation and Western Blot Analysis

Cells from confluent 10 cm dishes were harvested in 700 µl of Lysis buffer (20 mM Tris-HCl pH 8, 1% Triton X-100, 10% glycerol, 140 mM NaCl, 5 mM EDTA, 1 nM sodium orthovanadate (Na_3VO_4) complemented with the protease inhibitors aprotinin (5 µg/ml), benzamidine (150 µg/ml), leupeptin (5 µg/ml), pepstatin (4 µg/ml) and phenylmethylsulfonyl fluoride (20 mg/ml). Lysates were solubilized at 4°C for 30 minutes and total soluble proteins were run on polyacrylamide gels and transferred onto nitrocellulose membranes. Proteins were than detected using indicated specific primary antibodies. Secondary antibodies were all horseradish peroxidase-conjugated, and chemiluminescence was used to visualize protein expression. The quantification of the digital images obtained was performed using ImageJ 1.46o software (National Institutes of Health, USA). For immunoprecipitation experiments, cell lysates described above were agitated with indicated antibodies and protein G-Agarose plus beads at 4°C for 3 hours. Proteins were eluted in SDS-sample buffer by heating to 65°C for 15 minutes. Protein interaction and tyrosine phosphorylation were measured by western blot analysis.

ARF Activation Assay

Cells were left untreated or treated with indicated concentrations of gefitinib for indicated time points. Activated ARF1 was measured as previously described (54). Briefly, cells were lysed in 400 µl of Lysis buffer E (pH 7.4, 50 mM Tris HCl, 1% NP-40, 137 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 20 mM NaF, 1 mM NaPPi, 1 mM Na_3VO_4 and the protease inhibitors: aprotinin (5 µg/ml), benzamidine (150 µg/ml), leupeptin (5 µg/ml), pepstatin (4 µg/ml) and phenylmethylsulfonyl fluoride (20 mg/ml)). GST-GGA3-(1–316) (55) coupled to glutathione-Sepharose 4B was added to each sample. The samples were then rotated at 4°C for 45 minutes. Proteins were eluted in 20 µl of SDS-sample buffer by heating to 65°C for 15 minutes. The detection of ARF1-GTP or ARF6-GTP was performed by western blot analysis using specific antibodies to ARF1 and ARF6, respectively.

Mitochondrial Fractionation

MDA-MB-231 cells were treated with indicated concentrations of gefitinib for 72 hours. Cells were collected and sonicated in CHM buffer (10mM Tris-HCl pH 6.7, 10mM KCl, 150mM MgCl2). 0.25M sucrose was added and cells were spun at 1000g for 10 min and supernatant was collected as cytoplasmic fraction. Pellet was resuspended in SM buffer (10 mM Tris-HCl pH 6.7, 0.15M MgCl2, 0.25M sucrose) and spun 15 minutes at 5000g. Mitochondrial pellet was lysed in MLB buffer (50 mM Tris-HCl, pH 7.4, 150mM NaCl, 2mM EDTA, 2mM EGTA, 0.2% Triton X 100, 0.3% NP-40). Cytoplasmic and mitochondrial Cytochrome C expression was assessed by western blot analysis.

Membrane extraction

MDA-MB-231 cells were treated with indicated with gefitinib (10 μM) for indicated time points. Membrane extracts were isolated as previously described (56). Briefly, cells were harvested in an ice-cold hypotonic lysis buffer (10 mm Tris, pH 7.4, 1.5 mm $MgCl_2$, 5 mm KCl, 1 mm dithiothreitol, 0.2 mm sodium orthovanadate, leupeptin (10 μg/ml), 4-(2-aminoethyl)benzenesulfonyl fluoride (400 μm), NaF (1 mm), pepstatin 1 μg/ml, aprotinin 1 mg/ml). Cells homogenates were centrifuged at 700 × *g* for 10 min to pellet nuclei and intact cells. Supernatants were spun at 100,000 × *g* for 30 min at 4 °C to collect the membrane pellet. The pellet was lysed in hypotonic lysis buffer supplemented with 1% Nonidet P-40 before being spun at 100,000 × *g* for 30 min at 4 °C. The expression of the EGFR in the supernatant was assessed by western blot analysis.

Cell Viability Assay

MTT assay was used as a measure of cell viability/death. Cells were transfected with CTL siRNA, ARF1 siRNA or ARF6 siRNA for 24 hours. Cells were then trypsinized and plated at confluency on a 96-well plate in medium supplemented with 10% FBS overnight. The next day, cells were left untreated or treated in serum free medium with the specified concentrations of inhibitor for 12, 24, 48 or 72 hours, as indicated. Following the treatment, cells were stained with Thiazolyl Blue Tetrazolium Bromide (5 mg/ml) (Sigma-Aldrich) for 2 hours. The produced formazan product was than solubilized overnight in 20% SDS/50% Dimethyl-formamide solution (pH 4.7). Absorbance was measured at 570 nm with a reference wavelength at 450 nm using a plate reader.

Cell counting assay used an equal cell number (1×10^4 cells) seeded in a 6-cm dish for 24h. For each indicated treatment, cells were trypsinized, stained with trypan blue, and live cells were manually counted.

Statistical Analysis

Statistical analysis was performed using either a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or a two-way ANOVA followed by a Bonferroni's multiple comparison test using GraphPad Prism version 5 (San Diego, CA). The calculation of IC_{50} were also performed using GraphPad Prism version 5. CalcuSyn (Biosoft, Cambridge, Great Britain, UK) utilizing the Chou-Talalay combination index equation was used to calculate synergic relationships between tested inhibitors.

III.4 Results

ARF1 knockdown sensitizes breast cancer cells to gefitinib treatment.

We have recently identified ARF1 as a key downstream effector of EGFR signals (23,24) and asked whether this small GTP-binding protein could mediate the sensitivity of breast cancer cells to EGFRTKis. We first used gefitinib-resistant TNBC cells that highly express the EGFR and ARF1 (24) to examine the role of this small GTP-binding protein in mediating gefitinib sensitivity. In control conditions, MDA-MB-231 cell were found resistant to gefitinib when doses effective in other cell lines were used (0.1, 1, 10 μM) (27,28). A modest decrease in viability was however observed when these cells were treated with high doses of this inhibitor (25, 50 μM) as measured by MTT assay (24 hours) (Figure 1A). This is consistent with previously published findings that demonstrated that MDA-MB-231 cells are considered resistant to this EGFRTKi (29-31). When levels of ARF1 proteins were knocked down using two different siRNAs, gefitinib significantly reduced viability at a dose as low as 1 μM and this effect was maintained throughout all tested doses (1-50 μM). The IC_{50} for gefitinib treatment were 34.4 μM in control cells and 19.1 and 16.7 μM in ARF1 siRNA #1 and ARF1 siRNA #2 transfected cells, respectively (Table 1). A second approach was used to confirm these results. Counting of the cells also showed that depletion of ARF1 was an effective strategy to sensitize cells to gefitinib (Figure 1B). To demonstrate that the effects we observed were specific, we next performed a rescue experiment and overexpressed an ARF1 cDNA mutant (ARF1Mut) that contained the same sequence as the wild type ARF1, but was not targeted by the siRNA. As shown in Figure 1C and D, the enhanced sensitivity to gefitinib observed in ARF1-depleted cells was reversed upon the overexpression of ARF1Mut. Finally, the effect of gefitinib (10 μM) was further examined at varying times of exposure. As illustrated in Figure 1F, the knockdown of ARF1 potentiated the effect of this inhibitor in all examined time points (12 – 72 hours). Together these results suggest that ARF1 depletion not only improves the efficacy of gefitinib to kill tumor cells, but reduces the minimal required dose of this inhibitor to mediate its effects.

To further investigate the role of ARF1 in mediating the resistance of TNBC to EGFRTKi, we used two other cell lines, HCC70 and MDA-MB-157. Depletion of this ARF isoform significantly enhanced gefitinib sensitivity in these cells (Supplemental Figures 1A, B, and Table 1).

Furthermore, to demonstrate that the effects of ARF1 depletion was not specific to TNBC cells, we next examined gefitinib sensitivity in a cellular model of HER2-positive breast cancer that also expresses high levels of the EGFR and ARF1 (24), but are non-resistant to this RTKi (28). In control SKBR3 cells, increasing concentrations of gefitinib induced cell death (IC_{50}: 1.1 μM). However, this response was potentiated in cells depleted of ARF1 (IC_{50}: 0.5 μM) (Supplementary Figure 1C; Table 1). In contrast, our data indicate that while the ER-positive, low EGFR/ARF1 expressing MCF7 cells were sensitive to EGFR inhibitors, the knockdown of this ARF isoform had no effect on gefitinib sensitivity (24) (Supplementary Figure 1D; Table 1). All doses used in these experiments significantly inhibited EGF-dependent EGFR, ERK1/2 and AKT activation in all tested cell types (Supplementary Figure 2).

To further demonstrate the importance of ARF1 in mediating gefitinib sensitivity, we used the gefitinib-responsive MCF7 cell line that express low levels of ARF1 and transfected them with an HA-Tagged ARF1 cDNA. In these experiments, overexpression of this ARF had a reduced sensitivity to gefitinib treatment compared to control cells (Figure 1E). The IC_{50} for gefitinib treatment were 20.2 μM in control cells and 60.9 μM in ARF1 overexpressing cells, respectively (Table 1). These results were confirmed using a cell counting assay (Supplemental Figure 1E). This further supports the importance of ARF1 in mediating the sensitivity of breast cancer cells to EGFR inhibition.

Because compounds blocking ARF1 activity as well as mutants mimicking the inactive and active forms of ARF1 have been useful in demonstrating the function this small GTPase plays in cells, we further examined sensitivity to RTKis using these alternative approaches. First, treatment of the MDA-MB-231 cells with Brefeldin A (BFA, 10 nM), an ARFGEF inhibitor (32), induced 50% cell death due to the induction of apoptosis, a well-documented effect of this compound (33). However, BFA treatment markedly potentiated the effect of gefitinib as indicated in Supplemental Figure 3A. The IC_{50} of gefitinib was 26.6 μM in control conditions and 13.7 μM in BFA/gefitinib co-treated cells. The indicated dose of BFA blocked gefitinib-induced ARF1 activation as well as down-regulation of ERK1/2 and AKT activation (Data not Shown). Using the Chou-Talalay combination index equation, we determined a synergic relationship between gefitinib and BFA (Supplemental Figure 3B) (34,35). Additionally, the overexpression of a constitutively inactive ARF1 mutant (ARF1TN), but not an active form (ARF1QL) significantly enhanced gefitinib

sensitivity of MDA-MB-231 cells (Supplementary Figure 3C). Together, these findings demonstrate that the activity of ARF1 is essential in mediating gefitinib sensitivity of invasive breast cancer cells.

To better understand the role of ARF1 in mediating resistance to RTKi in general, we examined other EGFR and RTK inhibitors. Control and ARF1-depleted MDA-MB-231 cells were treated first with lapatinib, a dual EGFR/HER2 inhibitor, for 24 hours at doses ranging from 0.1 to 50 μM (Figure 1G). While this drug induced approximately 50% cell death in control cells at a concentration of 10 μM, a similar effect was observed in ARF1-depleted conditions, but at a dose of 1 μM. The IC_{50} for lapatinib was therefore 9.7 μM in control conditions and 2.5 μM in ARF1 knockdown cells (Table 1). Because lapatinib blocks the kinase activity of both EGFR and HER2, we evaluated the sensitivity of HER2-positive, low EGFR expressing SKBR3 breast cancer cells to this inhibitor (Figure 1H). In this cell line, ARF1-depletion was also associated with an increased sensitivity to lapatinib treatment (IC_{50}: 23.6 μM for controls versus 8.4 μM for ARF1 depleted cells; Table 1). Finally, we observed that ARF1-depletion had no effect on the sensitivity of MDA-MB-231 to both tivantinib and R428, cMet and AXL inhibitors, respectively (Supplemental Figure 1F, Table 1). Altogether, our results demonstrate that ARF1 plays a key role in mediating the sensitivity of TNBC and HER2-positive breast cancer cells to EGFRTKi, but not all RTK inhibitors.

ARF1 promotes gefitinib-mediated survival signals while blocking apoptosis.

Next, we sought to define the molecular mechanisms by which ARF1 mediated resistance. It is generally accepted that activation of certain signaling mediators such as ERK1/2 and Src contribute to EGFRTKi sensitivity, although the exact mechanism remains unclear (16,19). We therefore next examined the activation of these signaling pathways in gefitinib-treated, ARF1-depleted cells. Drug treatment for up to 72 hours induced the activation of both ERK1/2 and Src in control cells (Figure 2A). ARF1 depletion however delayed these signaling events. While gefitinib treatment of control cell was associated with a decreased AKT activation over time, this inhibition was more substantial and occurred earlier in cells depleted of ARF1 (24 hours vs. 48 hours in control cells) demonstrating that cell survival is mostly affected in gefitinib-treated and ARF1 knocked down conditions. Next, we evaluated whether the overexpression of ARF1 in the

non-invasive and low ARF expressing MCF7 cells could modulate the activation of ERK1/2 and AKT activation upon gefitinib treatment (Figure 2B). As shown in Figure 2B, gefitinib only slightly enhanced ERK1/2 activation while inhibiting AKT phosphorylation. Interestingly, overexpression of ARF1 markedly enhanced the effect of this EGFRTKi on the MAPK pathway while blocking the inactivation of the survival pathway further supporting a role for ARF1 as a key mediator regulating survival signals upon gefitinib treatment.

The co-administration of specific inhibitors of the MAPK and PI3K/AKT pathways, in combination with EGFRTKis, was reported to be an effective strategy to improved clinical outcomes (36-38). Here, we therefore examined whether the depletion of ARF1 could further enhance the synergy between gefitinib and a MEK (PD0325901), a PI3Kinase (LY294002) and a Src kinase inhibitor (PP2). While all the inhibitors, when used alone, significantly reduced the viability of MDA-MB-231 cells, their effects were not altered by the depletion of ARF1 (Figure 2C). Interestingly, co-treatment with gefitinib was an effective strategy to improve their efficacy. Namely, the depletion of ARF1 significantly enhanced the effects of the co-treatment of gefitinib and the MEK inhibitor as well as the Src inhibitor, but not the PI3Kinase (Figure 3C). The Chou-Talalay combination index equation (34,35) highlighted a synergic relationship between gefitinib and each of the inhibitors tested. However, the synergy between gefitinib and the inhibitors was not enhanced in ARF1-depleted cells (Data not shown) suggesting that the depletion of ARF1 has an additive effect, and not a synergic effect, on these co-treatments.

We next confirmed our findings using the ARF inhibitor. While treatment with the MEK inhibitor alone or in combination with gefitinib significantly enhanced cell death, no effect was observed when the cells were treated with PD0325901 in combination with BFA or in combination with both gefitinib and BFA (Supplementary Figure 3D). Treatment with the PI3K inhibitor, alone or in combination with gefitinib or BFA, significantly enhanced cell death (Supplemental Figure 3E). However, no significant increase was observed in cells treated with the combination of gefitinib, BFA and LY294002 compared to cells treated only with gefitinib in combination with BFA. Finally, we evaluated these inhibitor combinations with the Src kinase inhibitor (Supplementary Figure 3F). PP2 alone, and in combination with gefitinib or BFA, considerably promoted cellular death compared to control conditions. More importantly, the co-treatment of gefitinib, BFA and the Src kinase inhibitor robustly induced cell death compared to cells treated with only gefitinib

and BFA. Together, our results suggest that targeting ARF1 can enhance the sensitivity to gefitinib alone, but it can also enhance the effect of co-treatment of this EGFRTKi with other clinically relevant inhibitors such as the Src kinase inhibitors.

With ARF1 promoting the activation of survival cascades in gefitinib treated MDA-MB-231 cells, we next examined the importance of this GTPase in the induction of gefitinib-mediated apoptotic signals. Both p38MAPK and JNK have been shown to play an important role in promoting the activation of apoptotic pathways and blocking the survival pathway (39,40). Therefore, we next investigated the role of ARF1 in mediating the activation of these pathways, upon gefitinib treatment. As shown in Figure 3A, control cells treated with gefitinib-induced the activation of both p38MAPK and JNK. Interestingly, the activation of these kinases was augmented in ARF1-depleted cells compared to control conditions suggesting that ARF1 may prevent gefitinib-dependent activation of these apoptotic pathways. Alternatively, we examined activation of these pathways in control and ARF1 overexpressing MCF7 cells. As illustrated in 3B, ARF1 overexpression reduced gefitinib-induced activation of the apoptotic p38MAPK and JNK pathways compared to control conditions.

To confirm the role of ARF1 in regulating apoptotic pathways, we next examined the expression profile of specific markers. As shown in Figure 3C, we found that the Bax to Bcl2 protein expression ratio, an indicator of apoptosis, was significantly increased only in ARF1-depleted cells treated with gefitinib. Additionally, knockdown of ARF1 was associated with an increased release of Cytochrome C from the mitochondria into the cytoplasm (Figure 3D).

Altogether, these results suggest that high ARF1 expression, in highly invasive breast cancer cells, regulates anti-apoptotic pathways while promoting signals leading to cell survival.

ARF1 is essential for gefitinib-induced EGFR function.

In our attempt to further understand the role ARF1 plays in EGFRTKi resistance, we focused on the function of the receptor itself. An increased expression and activation of the EGFR and other members of the EGFR family has been reported to limit EGFRTKi sensitivity (10,17,41). Here, we examined the expression of these receptors upon gefitinib treatment (10 μM) for up to 72 hours. In control MDA-MB-231 cells, an increase in EGFR expression was observed following 12 and 24 hours of treatment with the inhibitor, followed by a return to basal EGFR expression by 48 hours (Figure 4A). Meanwhile, the expression levels of HER2 were reduced whereas HER3 levels remained unaffected by gefitinib treatment. However, we were unable to detect HER4 expression in MDA-MB-231 cells. In ARF1-depleted conditions, gefitinib treatment did not increase EGFR expression. Following a 48 and 72 hours exposure, EGFR expression was however found reduced compared to basal. Similarly, a decrease in HER2 expression at both 48 and 72 hours was also observed in cells depleted of ARF1 compared to control cells. No difference in HER3 expression was observed. These results suggest that the high expression of ARF1 may act to limit sensitivity to gefitinib by decreasing the expression levels of both EGFR and HER2.

Next, we examined the expression of EGFR family members in MCF7 cells. In control conditions, gefitinib treatment increased both EGFR and HER3 expression. However, a reduced HER2 expression was observed upon gefitinib treatment (Figure 4B). While a basal increase in EGFR and HER3 expression was detected in cells overexpressing ARF1, this ARF isoform was shown to only enhance gefitinib-mediated HER3 expression in this cell line. Moreover, the gefitinib-induced reduction in HER2 was reduced in ARF1-expressing MCF7 cells. No effect of ARF1 and gefitinib treatment on HER4 expression was observed. This further demonstrates the importance of ARF1 in mediating the expression of EGFR family members and that ARF1 may regulate gefitinib sensitivity in MCF7 cells by promoting signals downstream of HER2 and HER3.

We next confirmed our finding using the ARF inhibitor. As shown in Supplemental Figure 3G, the treatment of MDA-MB-231 cells with gefitinib was associated with an increased expression of EGFR and a decreased HER2 expression. More interestingly, when cells were co-treated with gefitinib and BFA, a significant reduction in EGFR expression levels was observed. Furthermore, the rate of reduction in HER2 expression was enhanced in BFA/gefitinib co-treated cells compared to cells treated with gefitinib alone. Together, these results demonstrate that ARF1 plays an

important role in mediating the expression of the EGFR and HER2 in gefitinib treated breast cancer cells.

Knowing that the depletion of ARF1 enhanced the gefitinib-dependent downregulation of the EGFR, we next examined whether treatment with this EGFRTKi enhanced receptor internalization. As shown in Figure 5A, gefitinib treatment promoted the internalization of the EGFR in control cells. Interestingly, this response occurred much faster (5 minutes compared to 30 minutes in control cells) in ARF1-depleted cells suggesting that ARF1 may mediate gefitinib sensitivity by controlling the membrane levels of the EGFR. Next, we investigated whether the internalized EGFR was targeted for degradation. To do this, we utilized the proteosomal inhibitor, MG132. As depicted in Figure 5B, the downregulation of EGFR expression in ARF1-depleted cells treated with gefitinib was partially recovered upon proteosomal inhibition. These results suggest that ARF1 may block the degradation of the EGFR in response to gefitinib treatment and thus reduce the sensitivity of these cells to EGFR inhibition.

Because ARF1-depletion enhanced gefitinib mediated p38MAPK activation (Figure 3A) and this specific MAPK has been previously reported to promote the internalization of the EGFR through the threonine phosphorylation of residue T669 on the receptor (42), we next examine this molecular event. While gefitinib treatment enhanced the threonine phosphorylation of the EGFR in control cells, an increased phosphorylation was observed in ARF1-depleted conditions (Figure 5C). These observations suggest that ARF1 may act to block the p38MAPK-dependent internalization of the EGFR and thus reduce the sensitivity of these cells to EGFR inhibition.

Finally, we determined whether the activation of p38MAPK was essential in mediating the cytotoxic properties of gefitinib in ARF1-depleted MDA-MB-231 cells. As shown in Figure 5D, we examined the induction of death in cells treated with the p38MAPK inhibitor, SB220025, alone or in combination with gefitinib. No difference in cell death was observed between ARF1-depleted and control cells treated with the p38MAPK inhibitor alone. As expected, treatment with gefitinib alone induced a 30% higher incidence of cell death in ARF1-depleted cells compared to control conditions. Remarkably, this gefitinib-dependent cell death in ARF1 knockdown MDA-MB-231 cells was decreased upon the co-treatment with the p38MAPK inhibitor further emphasizing the importance of this ARF isoform in mediating signals through the p38MAPK pathway leading to EGFR internalization and the induction of apoptosis.

Together our results suggest that ARF1 mediates gefitinib sensitivity by blocking the internalization and degradation of the EGFR through a p38MAPK-dependent mechanism.

Gefitinib promotes the activation of ARF1 by enhancing its recruitment to the RTK AXL.

Now that we have demonstrated that ARF1 plays an essential role in mediating gefitinib sensitivity, we asked whether gefitinib could in turn modulate the activity of the small GTPase. As shown in Figure 6A, treatment of MDA-MB-231 cells with this inhibitor resulted in an increased ARF1 activation. Similar effects were observed in HCC70 and MDA-MB-157 cells (Figure 6B, C).

We next asked how gefitinib promoted the activation of ARF1 in MDA-MB-231 cells. As the expression and activity of other RTKs such as HER2, cMet and AXL have all been implicated in gefitinib resistance (13,18,29), we determined whether gefitinib treatment could enhance the recruitment of ARF1 to these receptors. Indeed, gefitinib treatment was associated with an enhanced recruitment of ARF1 to HER2, cMet and AXL, but not to the EGFR (Figure 6D). This would suggest that upon gefitinib treatment, other mitogenic receptors may promote ARF1 activation. Therefore, we first attempted to examine the necessity of these RTK in mediating gefitinib-induced ARF1 activation using pharmacological inhibitors. As shown in Figure 6E, the dual inhibition of EGFR and HER2 by lapatinib resulted in a similar degree of ARF1 activation compared to gefitinib treatment alone suggesting that HER2 is not required for gefitinib-mediated ARF1 activity. Additionally, tivantinib treatment alone was unable to promote the activation of this ARF isoform. Furthermore, this cMet inhibitor was unable to block gefitinib-induced ARF1 activation suggesting that cMet is not required for gefitinib-dependent ARF1 activation. Interestingly, like in the EGFR inhibitor treatment, the AXL inhibitor, R428, was effective to enhance ARF1 activity. But, more importantly, this inhibitor blocked gefitinib-induced ARF1 activation. Together, these findings suggest that in gefitinib treated invasive breast cancer cells, ARF1 is activated via its recruitment to AXL.

Finally, we determined whether ARF1 depletion could enhance the efficacy of co-inhibiting other RTKs with the EGFR. As previously shown, ARF1 depletion enhanced the efficacy of both gefitinib- and lapatinib-, but not tivantinib- and R428-treated MDA-MB-231 cells (Figure 6F). Interestingly, a significant increase in cellular death was observed in ARF1-depleted cells co-

treated with gefitinib and tivantinib compared to control conditions. However, in cells co-treated with the AXL inhibitor, R428, and gefitinib, the depletion of ARF1 was shown to have no effect further suggesting that ARF1 is signaling downstream of AXL in gefitinib treated cells.

These data therefore provides a mechanism by which activation of ARF1 may contribute to potentiate survival and signaling of mitogenic receptors in conditions where cells are treated with EGFRTKi. By continuously activating intermediates regulating EGFR expression, internalization and signaling ARF contributes to EGFRTKi resistance of highly invasive breast cancer cells.

III.5 Discussion

Although inhibitors of mitogenic receptor activity remains a therapy of choice to treat cancer, the development of resistance to these drugs, by numerous tumor cells, has greatly limited their broad use in patients. The research of strategies to overcome resistance has identified key events contributing together to this cellular response. However, the identification of the most upstream events and master regulators as well as the mechanisms whereby they mediate resistance have yet to be elucidated.

We have recently demonstrated that ARF1 is highly expressed in the most invasive types of breast cancer cells and that stimulation of the EGFR leads to activation of this molecular switch and ultimately proliferation, migration and invasion, by a mechanisms involving the recruitment of classical adaptor proteins (23-25). Here, we show a novel role for ARF1 in mediating EGFRTKi sensitivity of these tumor cells. The depletion or inhibition of this ARF isoform significantly enhanced the sensitivity of resistant invasive breast cancer cells to the EGFRTKi, gefitinib. In these conditions, clinically relevant doses of this inhibitor now become effective in inducing signals leading to cell death. We show that upon gefitinib treatment ARF1 is activated upon its recruitment to the receptor AXL. This promotes the activation of survival signals through ERK1/2, Src and AKT, while, blocking apoptotic signals through the p38MAPK and JNK pathways. Additionally, we demonstrate that ARF1 plays an important role in the internalization and degradation of the EGFR observed upon gefitinib treatment. Indeed, enhanced signals through the p38MAPK pathway enhanced the internalization and in turn, the degradation of EGFR in gefitinib-treated, ARF1-depleted cells (Figure 7). From these data, we can conclude that ARF1 is an important regulator of EGFRTKi sensitivity in invasive breast cancer cells and its inhibition could improve therapeutic outcomes in patients treated with these drugs.

Acquired resistance is a major factor that markedly reduces the efficacy of EGFRTKis in the clinical setting (5,14,43). A variety of mechanisms have been proposed to mediate this response of tumor cells. Firstly, modified expression and activation of the EGFR as well as mutations within the receptor have all been implicated in this process (44). It was reported that a point mutation ($T^{790}M$), present within the kinase domain and targeted by EGFRTKis, significantly inhibited the functionality of these inhibitors. This mutation is commonly found in EGFRTKi treated patients

(44). In this study, we have primarily used the MDA-MB-231 cell model, which does not possess this mutation (45). Our data indicate that treatment with gefitinib increased EGFR expression without affecting its activation. Interestingly, the depletion of ARF1 blocked this augmentation in EGFR expression and promoted its internalization and degradation.

It has also been proposed that EGFR family members can compensate for the loss of EGFR signals (44). In our cell model, EGFR, HER2 and HER3, but not HER4 are expressed. While EGFR expression was increased by gefitinib treatment, gefitinib decreased HER2 expression while not affecting HER3. However, a significant decrease in HER2 expression was observed in cells depleted of ARF1, and treated with gefitinib. Thus, ARF1 may block EGFRTKi sensitivity and promote resistance by stabilizing the expression both EGFR and HER2. It is important to note that other tyrosine kinase receptors have also been shown to be implicated in acquired resistance. For instance, the amplification of both the cMET and AXL receptors as well as their ligands hepatocyte growth factor (HGF) and Gas6, respectively, has been reported in EGFRTKi resistant cancers (18,44). In our experiments, no observable increase in cMET or AXL expression was detected in gefitinib-resistant MDA-MB-231 cells (Data not shown). We did however observe an increased activity of these two receptors, but this activation was shown to be independent of ARF1 expression. Additionally, we demonstrated that ARF1 is activated downstream of AXL in gefitinib-treated cells. Thus, ARF1 may mediate EGFR inhibitor sensitivity by propagating signals downstream of activated AXL. Others have also reported that an increased activation of the insulin growth factor receptor (IGFR) and the fibroblast growth factor receptor (FGFR) are associated with acquired resistance (44,46). Therefore, it would be of interest to examine the role of ARF1 downstream of these RTKs.

In addition to altered RTK signals, the activation of downstream key pathways have also been implicated in EGFRTKi resistance. In fact, point mutations in either Ras or PTEN, resulting in the constitutive activation of both the Ras/ERK1/2 and the PI3Kinase/AKT pathways, have been linked to drug resistance (47,48). Independent of these mutations, it has been shown that ERK1/2 can be reactivated via either a HER2- or Src-dependent mechanism; whereas, PI3Kinase/AKT activation has been primarily shown to be dependent of signals from either HER3 or MET receptor (19,44). In our gefitinib-insensitive MDA-MB-231 cells, we observed an increase ERK1/2 and Src activation and this activation was significantly reduced by ARF1 depletion. Furthermore, while

gefitinib treatment was shown to reduce AKT phosphorylation in control cells, this decreased activation was found to be more pronounced in ARF1 depleted cells. This suggests that ARF1 may play an important role in drug sensitivity by activating survival pathways in TNBC cells.

Also, we observed an increase in p38MAPK and JNK activity in cells depleted of ARF1. The activation of these pathways have been linked to the gefitinib-induced cell death (49). Therefore, the increased gefitinib-sensitivity we observed in ARF1-depleted cells may stem from the enhance activation of these pathways. Furthermore, p38MAPK has been reported to promote the internalization of the EGFR, another mechanism known to promote EGFR inhibitor sensitivity of lung cancer cells (42,50). Indeed, for the first time in breast cancer cells, we observed an enhanced EGFR internalization in ARF1-depleted cells treated with gefitinib. Therefore, ARF1 may mediated gefitinib sensitivity by blocking p38MAPK signals to apoptosis and EGFR internalization

Although ARF1 plays key roles in physiology and diseases, successfully targeting small GTP-binding proteins as therapeutics targets remains a challenge. The design of molecular tools or drugs that specifically block the ability of a small G protein to become activated and interact with their effectors is of great interest (51,52). For ARF1, only a few inhibitors have been characterized in the literature (53). All of which have their limits regarding their potential use as therapeutics. Nevertheless, our demonstration that ARF1 is a key mediator of EGFRTKi sensitivity further support the relevance of studying the mechanisms by which ARF and other GTPases might control such phenomenon. Additionally, our demonstration that ARF1, namely, plays pleiotropic roles in tumorgenesis (23,25,26) further supports the benefits of targeting this ARF isoform as an anti-cancer treatment. Here, we demonstrate that the small GTPase ARF1, a downstream molecular switch activated by the EGFR, is a key player in mediating the sensitivity of invasive breast cancer cells to the EGFRTKi, gefitinib. Our results suggests that while inhibiting ARF1 alone may have some therapeutic benefits such as reduced cancer cell proliferation, migration and invasion (23,25), a strategy where ARF1 would be inhibited together with EGFRTKis could serve to improve efficacy of a compound such as gefitinib by increasing its cellular sensitivity as well as possibly decreasing the incidence of acquired resistance.

III.6 Figure Legends

Figure 1: ARF1 mediates gefitinib sensitivity in invasive breast cancer cells. *A,* Percent cell death was assessed by a MTT assay in MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then treated 24 hours with indicated concentrations of gefitinib. Western blot analysis confirmed the depletion of ARF1. *B,* Percent cell death was assessed by a cell counting assay in MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then treated 24 hours with indicated concentrations of gefitinib. *C,* Percent cell death was assessed by a MTT assay in MDA-MB-231 cells that were transfected with CTL siRNA, ARF1 siRNA alone or ARF1 siRNA and ARF1Mut cDNA and then treated 24 hours with indicated concentrations of gefitinib. Western blot analysis confirmed the depletion of ARF1 and the expression of HA-tagged ARF1Mut. *D,* Percent cell death was assessed by a cell counting assay in MDA-MB-231 cells that were transfected with CTL siRNA, ARF1 siRNA alone or ARF1 siRNA and ARF1Mut cDNA and then treated 24 hours with indicated concentrations of gefitinib. *E,* Percent cell death was assessed by a MTT assay in MCF7 cells that were transfected with CTL or HA-tagged ARF1 cDNA and then treated 24 hours with indicated concentrations of gefitinib. Western blot analysis confirmed the expression of HA-tagged ARF1. *F,* Percent cell death of MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then treated with 10 μM gefitinib for indicated time points as assessed by a MTT assay. *G,* Percent cell death was assessed by a MTT assay in MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then treated 24 hours with indicated concentrations of lapatinib. *H,* Percent cell death of SKBR3 cells as assessed as in (G). For all experiments, data shown are mean ± Standard error the mean (SEM). Significance was measured by a two-way ANOVA with n=3; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$.

Figure 2 : Gefitinib-induced survival signaling is altered in ARF1 depleted cells. *A,* Western blot analysis utilizing phospho-specific antibodies was used to measure the activation of ERK1/2, AKT and Src in cell lysates obtained from MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then treated with 10 μM gefitinib for the indicated time points. Data is presented as mean fold over basal activation ± SEM with n=3. Significance was measured by a two-way ANOVA; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$. *B,* Western blot analysis utilizing phospho-specific antibodies was used to measure the activation of ERK1/2 and AKT in cell lysates obtained from MCF7 cells that were transfected with CTL or HA-tagged ARF1 cDNA and then treated with 10 μM gefitinib for 24 hours. Data is presented as mean fold over basal activation ± SEM with n=3. Significance was measured by a two-way ANOVA; (*) $P < 0.05$. *C,* MDA-MB-231 percent cell death was assessed via a MTT assay in cells that were transfected with CTL or ARF1 siRNA and then treated with either PD0325901 (10 μM), LY294002 (15μM) or PP2 (1 μM) alone or in combination with gefitinib (10 μM) for 24 hours. Data shown are mean ± SEM. Significance was measured by a two-way ANOVA with n=3; (*) $P < 0.05$, (***) $P < 0.001$.

Figure 3 : Enhanced gefitinib-mediated apoptotic signals in ARF1 depleted cells. *A,* Western blot analysis utilizing phospho-specific antibodies was used to measure the activation of p38MAPK and pJNK in cell lysates obtained from MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then treated with 10 μM gefitinib for the indicated time points. Data is presented as mean fold over basal activation ± SEM with n=3. Significance was measured by a two-way ANOVA; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$. *B,* Western blot analysis utilizing phospho-specific antibodies was used to measure the activation of p38MAPK and pJNK in cell lysates obtained from MCF7 cells that were transfected with CTL or HA-tagged ARF1 cDNA and then treated with 10 μM gefitinib for 72 hours. Data is presented as mean fold over basal activation ± SEM with n=3. Significance was measured by a two-way ANOVA; (*) $P < 0.05$, (***) $P < 0.001$. *C,* The expression of Bcl-2 and Bax was measured by western blot analysis in cell lysates obtained from MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then left untreated or treated with 10 μM gefitinib for 72 hours. Data is presented as the mean ratio of Bax expression over Bcl-2 expression ± SEM with n=3. Significance was measured by a two-way ANOVA; (**) $P < 0.01$. *D,* The cytoplasmic and mitochondrial expression of Cytochrome C were

measured by western blot analysis in cell lysates obtained from MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then left untreated or treated with 10 μM gefitinib for 72 hours. Data is presented as mean fold Cytochrome C release ± SEM with n=3. Significance was measured by a two-way ANOVA; (**) $P < 0.01$.

Figure 4 : Gefitinib-induced EGFR family member expression is mediated by ARF1 expression. *A,* The protein expression of EGFR, HER2, HER3 and HER4 was assessed in lysates obtained from MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then treated with 10 μM gefitinib for the indicated time points using western blot analysis. Data is presented as mean fold over basal ± SEM with n=3. Significance was measured by a two-way ANOVA; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$. *B,* Western blot analysis was used to measure the expression of EGFR, HER2, HER3 and HER4 in cell lysates obtained from MCF7 cells that were transfected with CTL or HA-tagged ARF1 cDNA and then treated with 10 μM gefitinib for 72 hours. Data is presented as mean fold over basal expression ± SEM with n=3. Significance was measured by a two-way ANOVA; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$.

Figure 5 : Gefitinib-dependent EGFR internalization and degradation is enhanced by ARF1 depletion. *A,* The protein expression of EGFR was assessed in membrane extracts obtained from MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then treated with 10 μM gefitinib for the indicated time points using western blot analysis. Data is presented as mean fold over basal ± SEM with n=3. Significance was measured by a two-way ANOVA; *(****) $P < 0.001$. *B,* The protein expression of EGFR was assessed in lysates obtained from MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then treated with 10 μM gefitinib alone, 1 μM MG132 alone or the combination of gefitinib (10 μM) and MG132 (1 μM) for 24 hours using western blot analysis. Data is presented as mean fold over basal ± SEM with n=3. Significance was measured by a two-way ANOVA; (***) $P < 0.001$. *C,* The threonine phosphorylation of the EGFR was assessed in lysates obtained from MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then treated with 10 μM gefitinib for 72 hours using a phospho-specific antibody. Data is presented as mean fold over basal ± SEM with n=3. Significance was measured by a two-way ANOVA; (**) $P < 0.01$. *D,* MDA-MB-231 percent cell death was assessed via a

MTT assay in cells that were transfected with CTL or ARF1 siRNA and then treated with SD220025 (100 nM) alone or in combination with gefitinib (10 μM) for 24 hours. Data shown are mean ± SEM. Significance was measured by a one-way ANOVA with n=3; (**) $P < 0.01$.

Figure 6: Gefitinib promotes ARF1 activation through the recruitment of this GTPase to AXL. *A,* MDA-MB-231 cells were treated with indicated concentrations of gefitinib for 1 hour. A GST-GGA3 pulldown assay was used to capture activated ARF1 from cell lysates. Endogenous levels of activated ARF1 and the total protein levels of ARF1 were assessed by western blot analysis. Data shown are mean ± SEM. Significance was measured by a one-way ANOVA with n=3; (***) $P < 0.001$. *B,* HCC70 cells were treated with indicated concentrations of gefitinib for 1 hour. ARF1 activation was assessed as described in (A). Data shown are mean ± SEM. Significance was measured by a one-way ANOVA with n=3; (*) $P < 0.05$, (**) $P < 0.01$. *C,* MDA-MB-157 cells were treated with indicated concentrations of gefitinib for 1 hour. ARF1 activation was assessed as described in (A). Data shown are mean ± SEM. Significance was measured by a one-way ANOVA with n=3; (*)) $P < 0.05$, (***) $P < 0.001$. *D,* Co-immunoprecipitation experiments were used to assess the recruitment of ARF1 to the EGFR, HER2, cMet and AXL in MDA-MB-231 cells treated with gefitinib (10 μM) for 1 hour. Data is presented as mean receptor recruitment ± SEM with n=3. Significance was measured by a two-way ANOVA; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$. *E,* MDA-MB-231 cells were treated with gefitinib (10 μM), lapatinib (10 μM), tivantinib (10 μM) and R428 (1 μM) alone or tivantinib (10 μM) and R428 (1 μM) in combination with gefitinib (10 μM) for 1 hour. ARF1 activation was assessed as described in (A). Data shown are mean ± SEM. Significance was measured by a one-way ANOVA with n=3; (**) $P < 0.01$, (***) $P < 0.001$. *F,* Percent cell death was assessed by a MTT assay in MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then treated 24 hours with gefitinib (10 μM), lapatinib (10 μM), tivantinib (10 μM) and R428 (1 μM) alone or tivantinib (10 μM) and R428 (1 μM) in combination with gefitinib (10 μM).

Figure 7: The role of ARF1 in mediating gefitinib sensitivity in breast cancer cells. *A,* Upon treatment with the EGFRTKi, gefitinib, ARF1 is recruited to the RTK, AXL, leading to the activation of this GTPase. Activated ARF1 promotes signals through the proliferative/survival pathways, ERK1/2, Src and AKT. Furthermore, ARF1 attenuates gefitinib-induced apoptotic signals through p38MAPK and JNK activation. Additionally, the actions of ARF1 on the p38MAPK mediate the internalization and degradation of the EGFR. Altogether, ARF1 mediates gefitinib sensitivity in breast cancer cells by promoting cell survival and EGFR stability.

Fig. 1
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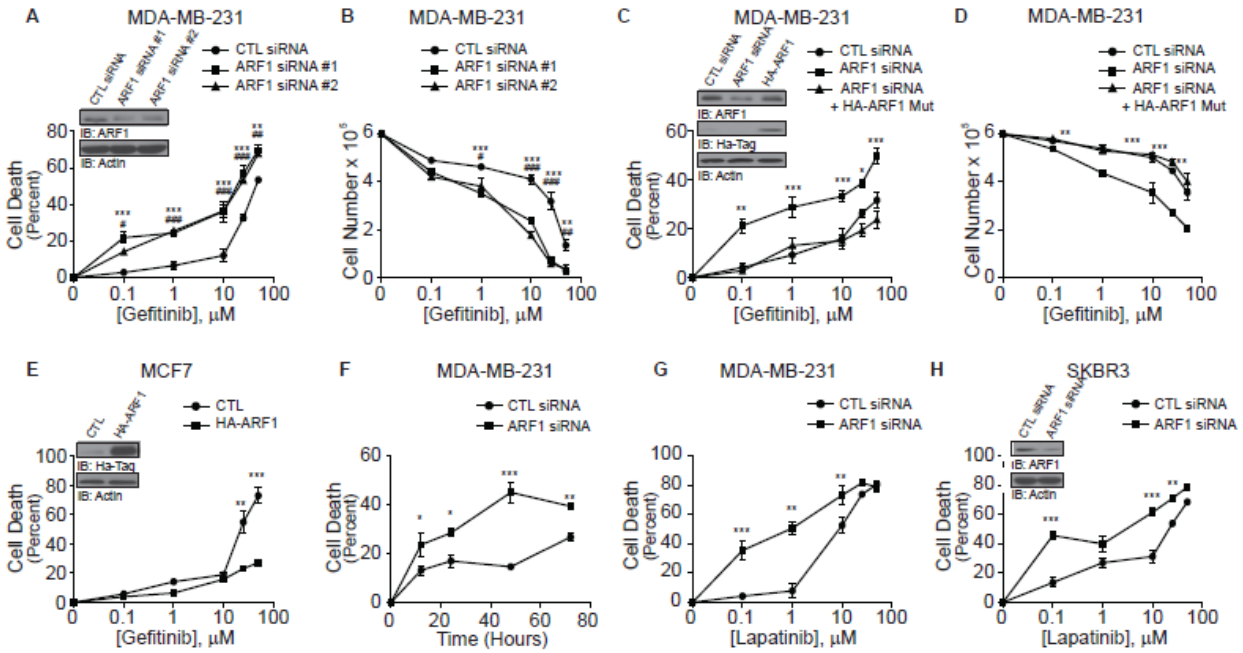

Fig. 2
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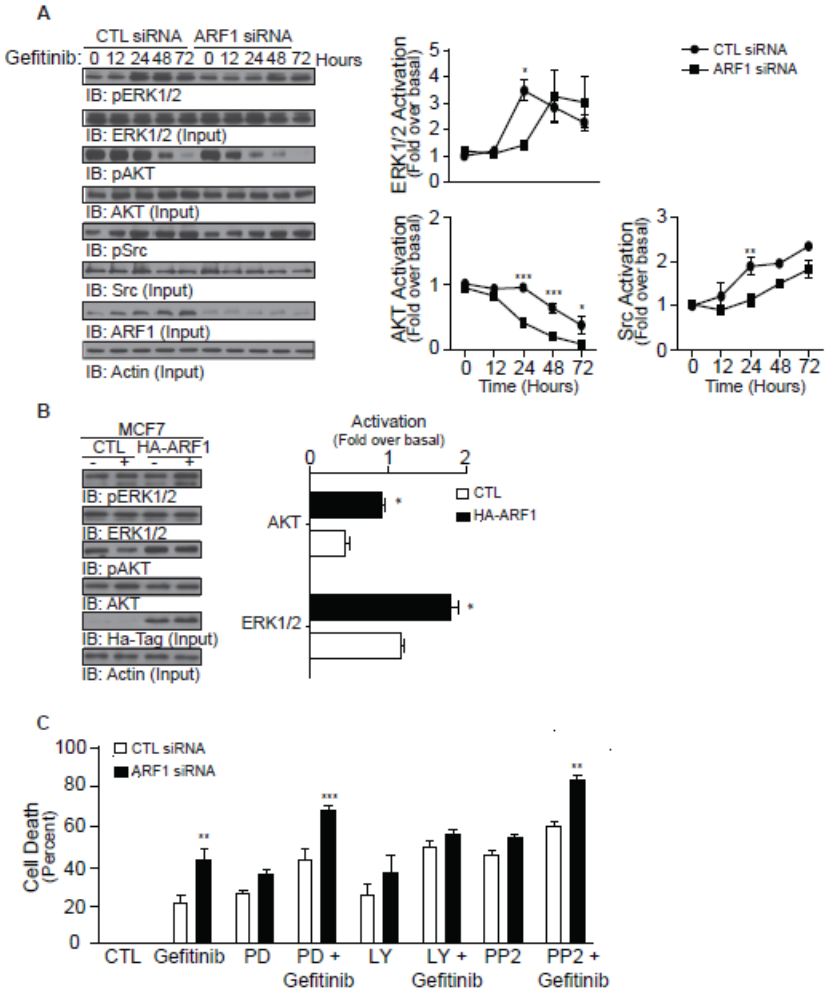

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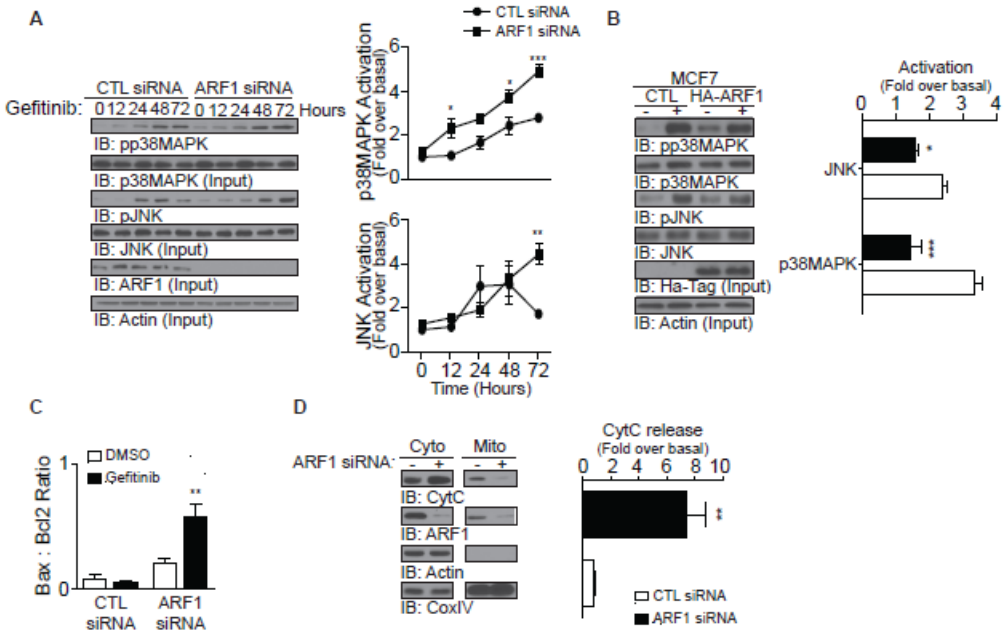

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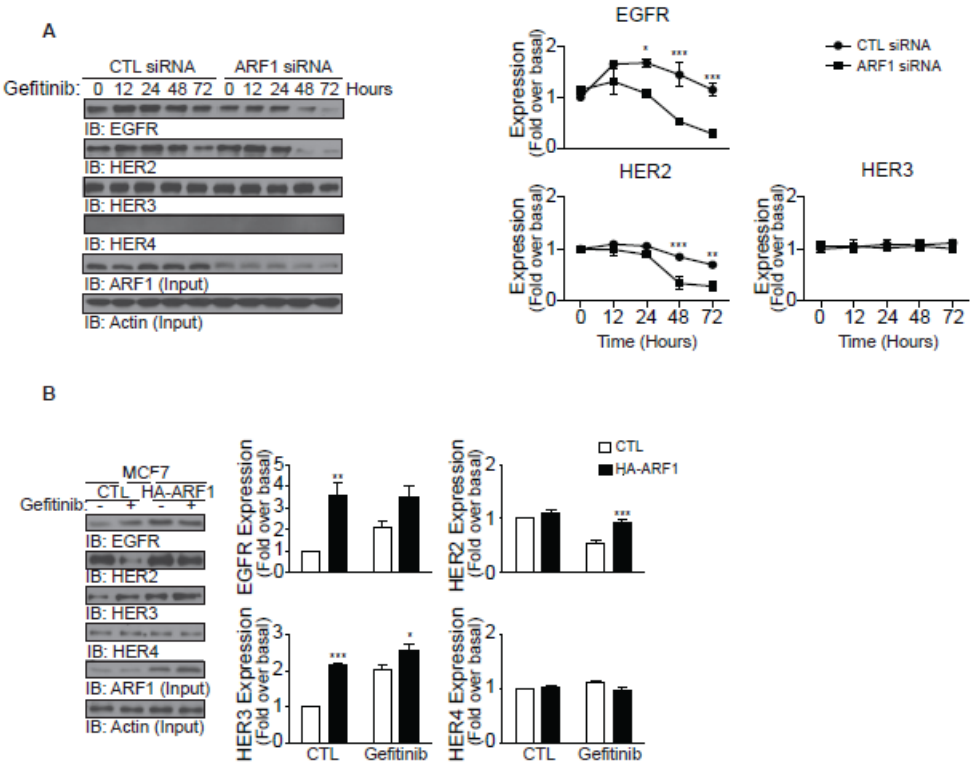

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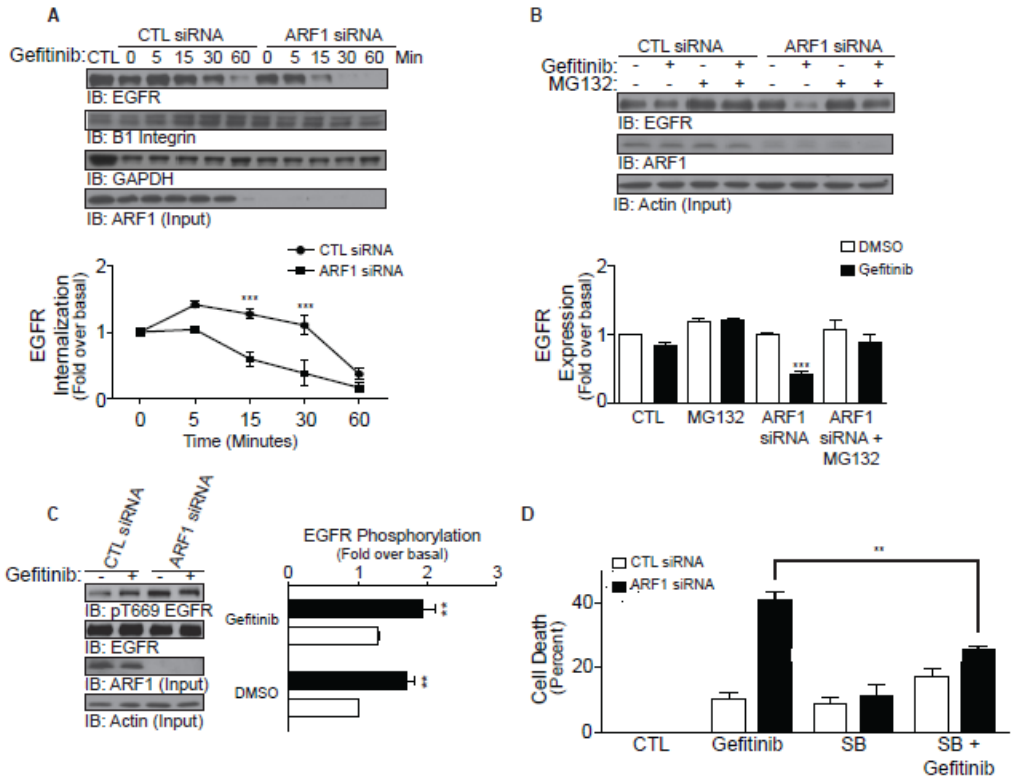

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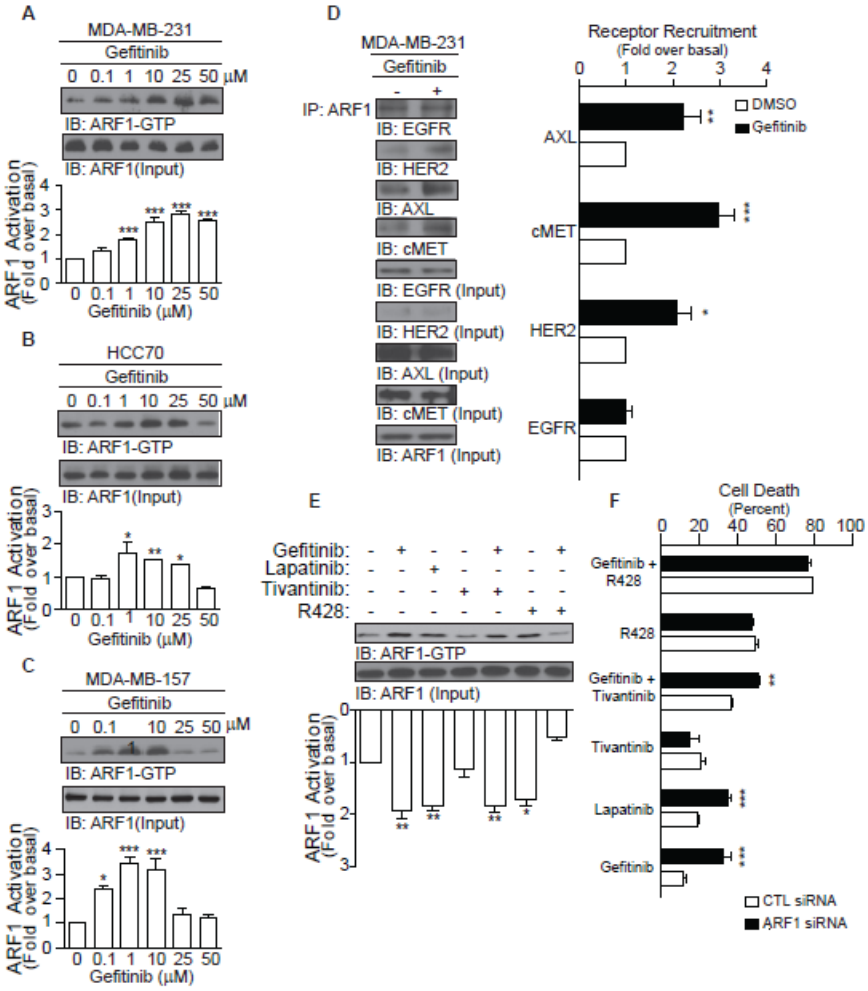

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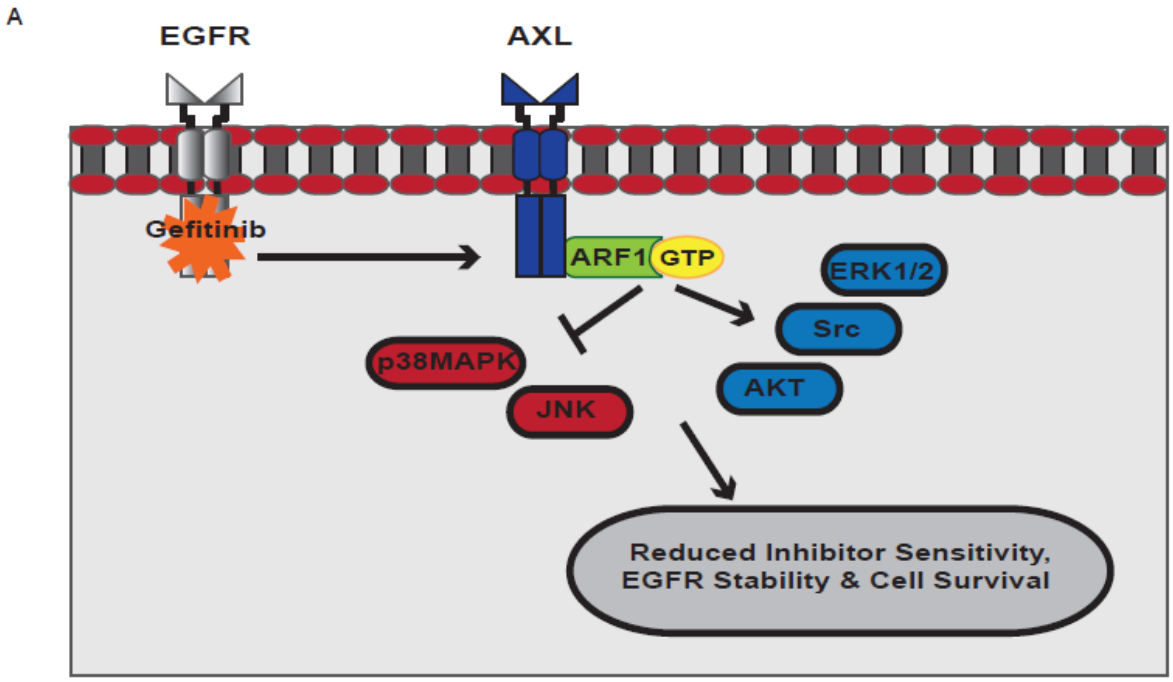

III.7 Tables

Table 1: Effect of ARF1 depletion on the IC50 of EGFRTKis in breast cancer cells. The IC50 for control cells or ARF1 knockdown cells treated with either gefitinib, tivantinib, R428 or lapatinib for 24 hours. Data shown are mean values. Significance was measured using an unpaired, two-tailed T-test with n=3; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$.

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Cell Line	Control Condition	Experimental Condition	Inhibitor	IC_{50} Control (mM)	IC_{50} Experimental (mM)
MDA-MB-231	CTL siRNA	ARF1 siRNA #1	Gefitinib	34.37	19.06 **
MDA-MB-231	CTL siRNA	ARF1 siRNA #2	Gefitinib	34.37	16.72 **
MDA-MB-231	CTL siRNA	ARF1 siRNA #1	Lapatinib	9.74	2.46 *
MDA-MB-231	CTL siRNA	ARF1 siRNA #1	Tivantinib	40.48	38.37
MDA-MB-231	CTL siRNA	ARF1 siRNA #1	R428	5.46	5.85
MDA-MB-231	DMSO	Brefeldin A	Gefitinib	45.96	18.53 *
HCC70	CTL siRNA	ARF1 siRNA #1	Gefitinib	53.14	12.95 *
MDA-MB-157	CTL siRNA	ARF1 siRNA #1	Gefitinib	46.46	14.04 **
SKBR3	CTL siRNA	ARF1 siRNA #1	Gefitinib	1.07	0.49 **
SKBR3	CTL siRNA	ARF1 siRNA #1	Lapatinib	23.63	8.39 ***
MCF7	CTL siRNA	ARF1 siRNA #1	Gefitinib	18.56	20.74
MCF7	Vector	HA-ARF1 cDNA	Gefitinib	20.15	60.86 ***

III.8 Supplementary data

Supplementary Figure 1 : ARF1 mediates the sensitivity of breast cancer cells to EGFR inhibitors. *A,* Percent cell death was assessed by a MTT assay in HCC70 cells that were transfected with CTL or ARF1 siRNA and then treated 24 hours with indicated concentrations of gefitinib. Western blot analysis confirmed the depletion of ARF1. *B,* Percent cell death of MDA-MB-157 cells as assessed as in (A). *C,* Percent cell death of SKBR3 cells as assessed as in (A). *D,* Percent cell death of MCF7 cells as assessed as in (A). *E,* Percent cell death was assessed by a cell counting assay in MCF7 cells that were transfected with CTL or HA-ARF1 cDNAs and then treated 24 hours with gefitinib (25 μM). *F,* Percent cell death was assessed by a MTT assay in MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then treated 24 hours with indicated concentrations of either tivantinib or R428. For all experiments, data shown are mean ± Standard error the mean (SEM). Significance was measured by a two-way ANOVA with n=3; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$.

Supplementary Figure 2 : Gefitinib blocks EGF-dependent activation of the EGFR, ERK1/2 and AKT. *A,* EGFR activation was assessed by immunoprecipitating the EGFR and immunoblotting with a phospho-specific antibody against total phospho-tyrosine sites (pan-PY) using lysates obtained from MDA-MB-231 cells that were left untreated or treated with indicated concentrations of gefitinib for 1 hour before being stimulated with EGF (10 ng/ml) for 1 minute. Activation of ERK1/2 and AKT were measured by western blot analysis using phospho-specific antibodies. *B,* The activation of EGFR, ERK1/2, and AKT in HCC70 cells was assessed as described in (A). *C,* The activation of EGFR, ERK1/2, and AKT in MDA-MB-157 cells was assessed as described in (A). *D,* The activation of EGFR, ERK1/2, and AKT in SKBR3 cells was assessed as described in (A). *E,* The activation of EGFR, ERK1/2, and AKT in MCF7 cells was assessed as described in (A)

Supplementary Figure 3 : The ARF inhibitor, brefeldin A, sensitizes MDA-MB-231 cells to EGFR inhibition. *A,* Percent cell death was assessed by a MTT assay in MDA-MB-231 cells that were left untreated or treated 24 hours with BFA (10 nM) alone, indicated concentrations of gefitinib alone or the combination of BFA (10 nM) and indicated concentrations of gefitinib. Data shown are mean ± SEM. Significance was measured by a two-way ANOVA with n=3; (*) $P < 0.05$, (**) $P < 0.01$. *B*, The combination indexes (CI) for the co-treatment of BFA and gefitinib were calculated using the Chou-Talalay combination index equation. CI values below 1 (dotted line) are indicative of a synergic relationship. Western blot analysis confirmed the efficacy of BFA in inhibiting ARF1 activity. *C*, Cell viability was assessed by a MTT assay in MDA-MB-231 cells that were transfected with CTL vector, ARF1WT, ARF1QL or ARF1TN cDNAs and then treated 24 hours with indicated concentrations of gefitinib. Western blot analysis confirmed the expression of the HA-Tagged ARF1 cDNAs. For all experiments, data shown are mean ± Standard error the mean (SEM). Significance was measured by a two-way ANOVA with n=3; (**) $P < 0.01$. *D*, MDA-MB-231 percent cell death was assessed via a MTT assay in cells that were left untreated (DMSO) or treated with either BFA (0.01 μM) alone, gefitinib (10 μM) alone or the combination of BFA and gefitinib in the presence or absence of PD0325901 (10 μM) for 24 hours. Data shown are mean ± SEM. Significance was measured by a two-way ANOVA with n=3; (**) $P < 0.01$, (***) $P < 0.001$. *E*, MDA-MB-231 percent cell death was assessed via a MTT assay in cells that were left untreated (DMSO) or treated with either BFA (0.01 μM) alone, gefitinib (10 μM) alone or the combination of BFA and gefitinib in the presence or absence of LY294002 (15μM) for 24 hours. Data shown are mean ± SEM. Significance was measured by a two-way ANOVA with n=3; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$. *F*, MDA-MB-231 percent cell death was assessed via a MTT assay in cells that were left untreated (DMSO) or treated with either BFA (0.01 μM) alone, gefitinib (10 μM) alone or the combination of BFA and gefitinib in the presence or absence of PP2 (1 μM) for 24 hours. Data shown are mean ± SEM. Significance was measured by a two-way ANOVA with n=3; (*) $P < 0.05$, (**) $P < 0.01$. *G,* The protein expression of EGFR and HER2 was assessed in lysates obtained from MDA-MB-231 cells that were left untreated, treated with gefitinib (10 μM) alone or the combination of BFA (0.01 μM) and gefitinib (10 μM) using western blot analysis. Data is presented as mean fold over basal ± SEM with n=3. Significance was measured by a two-way ANOVA; (*) $P < 0.05$, (***) $P < 0.001$.

Supplemental Fig.1
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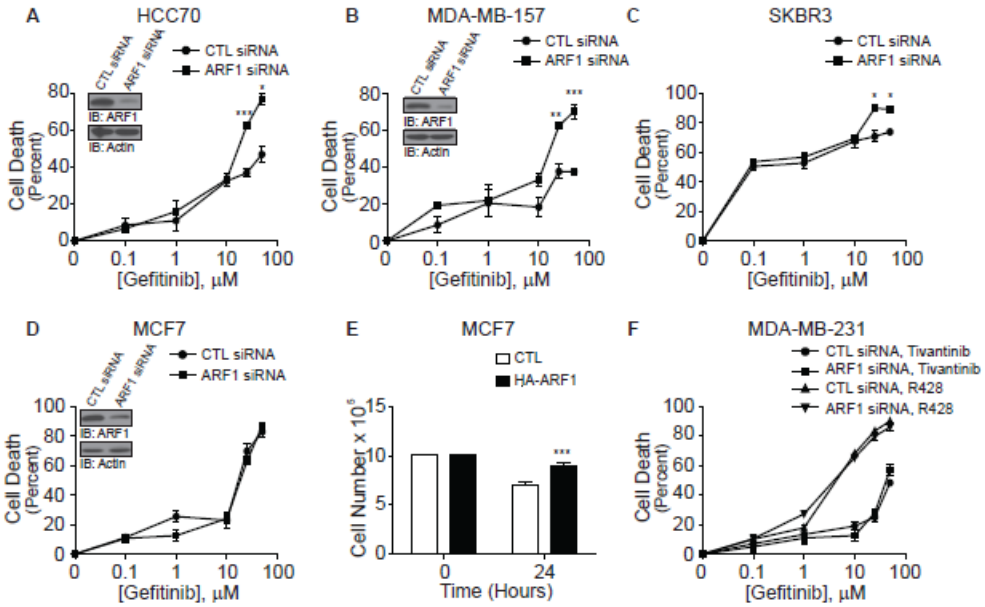

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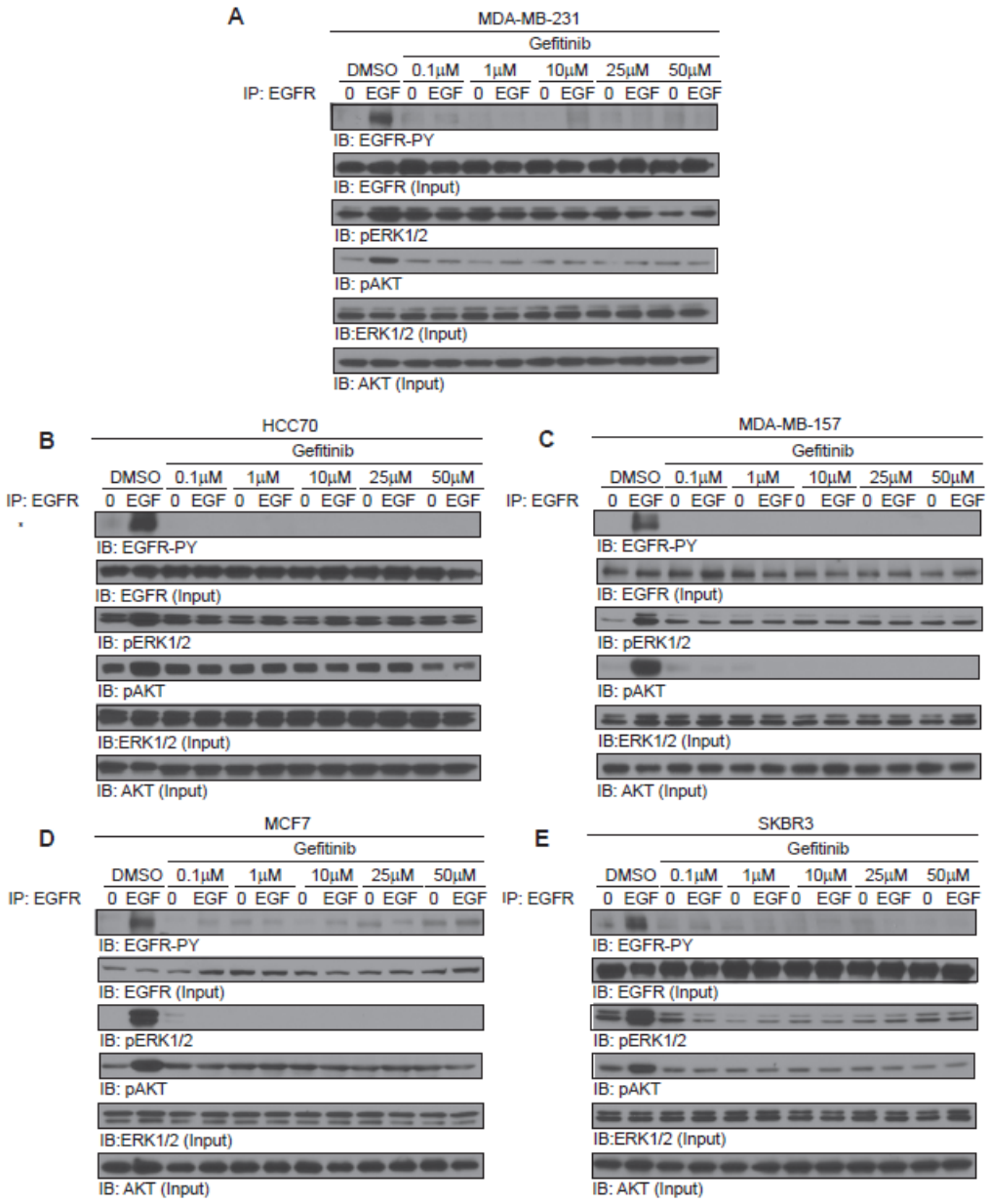

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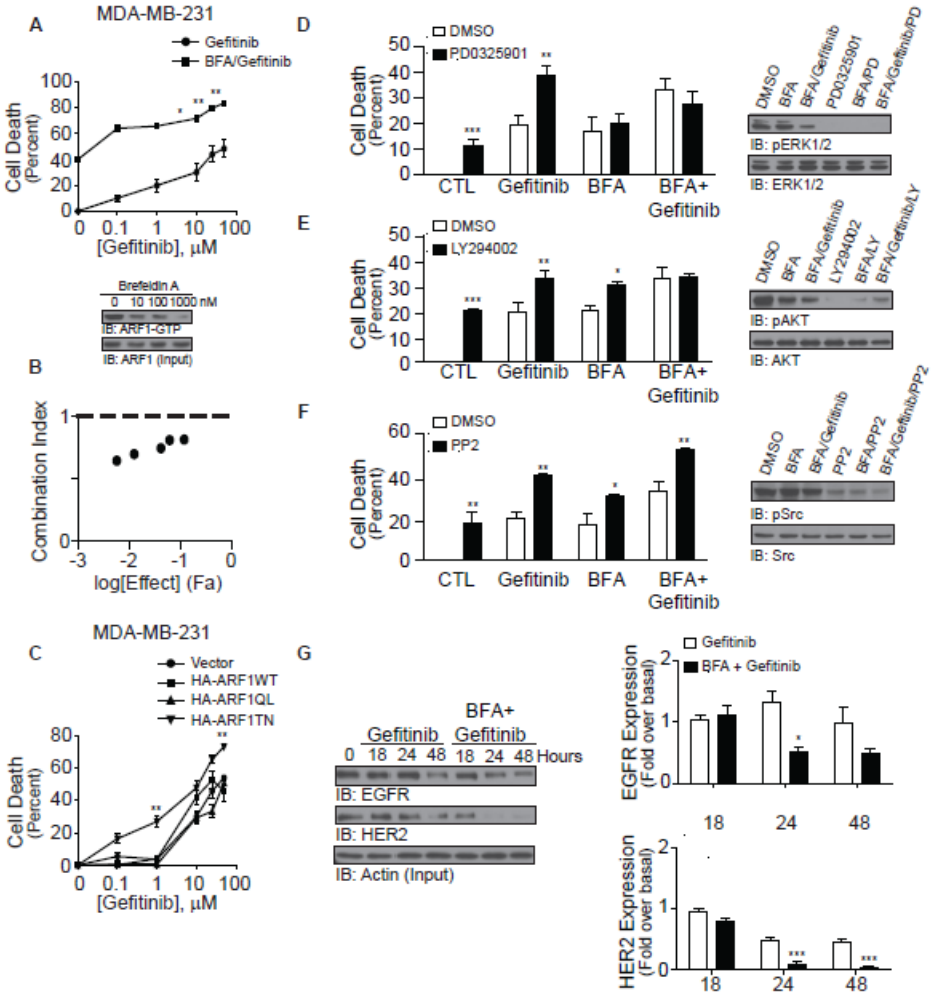

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CHAPTER IV: Further characterization of the role of ARF GTPases in mediating gefitinib sensitivity in breast cancer cells

Unpublished Data

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EH: experimental conception, experimental execution, data analysis and writing
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IV.1 Abstract

The majority of triple negative breast cancer (TNBC) patients have an increased epidermal growth factor receptor (EGFR) expression. However, pharmaceutically targeting this receptor, within this cancer population, has shown limited success. The patient response to EGFR tyrosine kinase inhibitors has been shown to be mediated by an innate insensitivity to these inhibitors as well as the development of resistance. Recently, we have demonstrated that the small GTPase ARF1 mediates signals downstream of the EGFR leading to cell proliferation, migration and invasion. Furthermore, this GTPase was shown to regulate the gefitinib sensitivity of TNBC cells. Here, we further characterize the importance of ARF1 in mediate gefitinib sensitivity and implicate this GTPase in the development of resistance. First, we demonstrate that p66Shc, a key mediator of ARF1 activity, and another ARF isoform, ARF6, also regulate gefitinib sensitivity. Next, we show that ARF1 regulates mitochondrial-dependent apoptosis in gefitinib treated cells. Indeed, reduced mitochondrial Bcl2 expression, increased mitochondrial p66Shc translocation, mitochondrial membrane hyperpolarization and caspase cleavage were observed in ARF1-depleted cells upon gefitinib treatment. Subsequently, we show that ARF1 mediates the gefitinib-dependent activation and dimerization of EGFR family members, important processes implicated in the sensitivity of cancer cells to EGFR inhibition. Finally, we show that a gefitinib-resistant cell population has elevated ARF1 activity and that the depletion of this GTPase can re-sensitize these cells to EGFR inhibition. Together, we further demonstrate the importance of ARF1 in mediating the response of TNBC cells to gefitinib treatment and highlight novel mechanisms, both through receptor dimerization and mitochondrial-dependent apoptosis, utilized by ARF1 to regulate gefitinib sensitivity. These findings further emphasize the importance of targeting ARF1 to improve the response of TNBC patients to EGFR inhibitors.

IV.2 Introduction

With 15-20% of global breast cancers being diagnosed as triple negative breast cancer (TNBC), an aggressive cancer with high metastatic potential, there are present attempts to identify novel therapeutic targets and improve currently available therapies (1-3). While current chemotherapeutics have shown some success in the treatment of TNBC, only a small portion of patients respond to these agents, whereas other develop drug resistance (4). Therefore, it is essential to better understand the mechanisms underlying the response to TNBC therapeutic agents. This could help improve the efficacy and response rate to these agents.

One oncogenic factor, the epidermal growth factor receptor (EGFR), has been shown to be elevated in these patients (5). Moreover, it is associated with a poor prognosis making it an interesting therapeutic target in this breast cancer subpopulation (5, 6). However, clinical trials targeting this receptor, either using tyrosine kinase inhibitors or monoclonal antibodies, have shown limited success (7, 8). This lack of response is associated, in part, by the development of drug resistance, a process well documented in lung cancer patients, but less understood in breast cancer (9). Briefly, multiple mechanisms of resistance of have been proposed which include: modifications to the EGFR that counter the effects of the drug, altered signaling through other receptors and signaling cascades (HER2-3, AXL, cMET, ERK1/2, Src and AKT), evasion of apoptosis and, finally, histological changes in the cancer cell phenotype (epithelial-mesenchymal transformation) (7, 10-16).

Recently, we have implicated the small GTPase, ADP-Ribosylation Factor 1(ARF1) as a key mediator of EGFR inhibitor sensitivity in breast cancer cells. We had previously shown that this GTPase, a member of the Ras superfamily of GTPases, mediates important signals downstream of the EGFR leading to cell proliferation, migration and invasion (17-19). Additionally, we showed that the depletion of ARF1 sensitized the gefitinib-resistant MDA-MB-231 cell line to EGFR inhibition. Moreover, ARF1 was shown to promote survival signals and EGFR stability while blocking apoptotic signals in gefitinib treated cells. However, further investigation into the role of this ARF isoform in EGFR inhibitor sensitivity and resistance is required.

In this study, we aimed to further characterize the mechanism through which ARF1 mediates gefitinib sensitivity and to define whether this small GTP-binding protein could also play a role in mediating the resistance of TNBC cells to EGFR tyrosine kinase inhibitors (EGFRTKis). Here,

we report that ARF1 plays a key role in mediating EGFRTKi sensitivity by regulating important mitochondrial dynamics that control cell survival as well as mediating EGFR family dimerization and activation. Furthermore, we show that a key regulator of ARF1 activity, p66Shc (20), and another ARF isoform, ARF6, also play a role in mediating gefitinib sensitivity. Finally, we demonstrate that ARF1 also regulates the development of EGFRTKi resistance. Our results suggest that targeting this key protein in combination with EGFR inhibitors may enhance their effectiveness and efficiency and in turn prevent the development of drug resistance in TNBC patients.

IV.3 Materials and Methods

Reagents and Antibodies

Lipofectamine 2000™ was purchased from Invitrogen (Burlington, Ontario, Canada). Epidermal growth factor was purchased from Fitzgerald Industries International, Inc. (Concord, MA) and heregulin was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Polyclonal antibodies used were EGFR, HER2, HER3, pErk1/2, pAKT, AKT, pp38MAPK, p38MAPK, pJNK, JNK, pan-actin, Bim, Bcl2, CoxIV, Caspase 3, Caspase 9 (Cell Signaling, Danvers, MA), ARF1 (Proteintech Group, Chicago, IL) HA-Tag, Erk1/2 (Santa Cruz Biotechnology, Dallas, TX). Monoclonal antibodies used were pan-PY (Santa Cruz Biotechnology). Other reagents used were goat anti-mouse antibody-horseradish peroxidase and goat anti-rabbit antibody-horseradish peroxidase (RD Systems, Minneapolis, MN) and Protein G-Agarose Plus beads (Santa Cruz Biotechnology).

DNA Plasmids and siRNAs

HA-tagged p66Shc, p66ShcS36A and p66ShcS36D cDNAs were cloned into a pcDNA3 vector and previously described (21, 22), the double-stranded scrambled with 19-nucleotide duplex RNA, ARF1 siRNA and ARF6 siRNA were previously described (Cotton, Boulay et al. 2007; Boulay, Cotton et al. 2008; Schlienger, Campbell et al. 2014). All siRNAs include 2-nucleotide 3' dTdT overhangs and were purchased from Dharmacon Inc. (Lafayette, CO).

Cell Culture and Transfection

MDA-MB-231, MCF7, SKBR3, MDA-MB-157 cells were maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HCC70 cells were maintained at 37°C, 5% CO_2 in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS. Cells were transfected with siRNA or plasmid DNA using *Lipofectamine 2000*™ according to the manufacturer's instructions. Briefly, cells were transfected with 25 nM siRNA for 72 hours or indicated cDNAs for 48 hours prior to treatment with inhibitors at indicated concentrations for indicated time points.

Co-immunoprecipitation and Western Blot Analysis

Cells from confluent 10 cm dishes were harvested in 700 µl of Lysis buffer (20 mM Tris-HCl pH 8, 1% Triton X-100, 10% glycerol, 140 mM NaCl, 5 mM EDTA, 1 nM sodium orthovanadate (Na_3VO_4) complemented with the protease inhibitors aprotinin (5 µg/ml), benzamidine (150 µg/ml), leupeptin (5 µg/ml), pepstatin (4 µg/ml) and phenylmethylsulfonyl fluoride (20 mg/ml). Lysates were solubilized at 4°C for 30 minutes and total soluble proteins were run on polyacrylamide gels and transferred onto nitrocellulose membranes. Proteins were than detected using indicated specific primary antibodies. Secondary antibodies were all horseradish peroxidase-conjugated, and chemiluminescence was used to visualize protein expression. The quantification of the digital images obtained was performed using ImageJ 1.46o software (National Institutes of Health, USA). For immunoprecipitation experiments, cell lysates described above were agitated with indicated antibodies and protein G-Agarose plus beads at 4°C for 3 hours. Proteins were eluted in SDS-sample buffer by heating to 65°C for 15 minutes. Protein interaction and tyrosine phosphorylation were measured by western blot analysis.

ARF Activation Assay

Cells were left untreated or treated with indicated concentrations of gefitinib for indicated time points. Activated ARF1 or ARF6 were measured as previously described (23). Briefly, cells were lysed in 400 µl of Lysis buffer E (pH 7.4, 50 mM Tris HCl, 1% NP-40, 137 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 20 mM NaF, 1 mM NaPPi, 1 mM Na_3VO_4 and the protease inhibitors: aprotinin (5 µg/ml), benzamidine (150 µg/ml), leupeptin (5 µg/ml), pepstatin (4 µg/ml) and phenylmethylsulfonyl fluoride (20 mg/ml)). GST-GGA3-(1–316) (24) coupled to glutathione-Sepharose 4B was added to each sample. The samples were then rotated at 4°C for 45 minutes. Proteins were eluted in 20 µl of SDS-sample buffer by heating to 65°C for 15 minutes. The detection of ARF1-GTP or ARF6-GTP was performed by western blot analysis using specific antibodies to ARF1 and ARF6, respectively.

Mitochondrial Fractionation

MDA-MB-231 cells were treated with indicated concentrations of gefitinib for 72 hours. Cells were collected and sonicated in CHM buffer (10mM Tris-HCl pH 6.7, 10mM KCl, 150mM MgCl2). 0.25M sucrose was added and cells were spun at 1000g for 10 min and supernatant was collected as the cytoplasmic fraction. Pellet was resuspended in SM buffer (10 mM Tris-HCl pH 6.7, 0.15M MgCl2, 0.25M sucrose) and spun 15 minutes at 5000g. Mitochondrial pellet was lysed in MLB buffer (50 mM Tris-HCl, pH 7.4, 150mM NaCl, 2mM EDTA, 2mM EGTA, 0.2% Triton X 100, 0.3% NP-40). Cytoplasmic and mitochondrial protein expression was assessed by western blot analysis.

Mitochondrial Membrane Potential

MDA-MB-231 cell membrane potential was assessed using JC-1 dye according to manufacturer's instructions. Briefly, 15,000 cells were plated on a 96-well plate and allowed to adhere overnight. Cells were treated with gefitinib (10 μM) for indicated time points. Cells were than washed with Dilution buffer, stained with 20 mM JC-1 for 10 minutes at 37°C, and washed twice in Dilution buffer. Fluorescence was measured using a plate reader (Excitation 475nm and Emission 590nm).

Cell Viability Assay

MTT assay was used as a measure of cell viability/death. Cells were transfected with CTL siRNA, ARF1 siRNA, ARF6 siRNA and p66Shc siRNA or p66Shc cDNAs for 24 hours. Cells were then trypsinized and plated at confluency on a 96-well plate in medium supplemented with 10% FBS overnight. The next day, cells were left untreated or treated in serum free medium with the specified concentrations of inhibitor for 12, 24, 48 or 72 hours, as indicated. Following the treatment, cells were stained with Thiazolyl Blue Tetrazolium Bromide (5 mg/ml) (Sigma-Aldrich) for 2 hours. The produced formazan product was than solubilized overnight in 20% SDS/50% Dimethyl-formamide solution (pH 4.7). Absorbance was measured at 570 nm with a reference wavelength at 450 nm using a plate reader.

Cell Migration Assay

Cells were transiently transfected with 50 nm scrambled siRNA or ARF1 siRNA for 72 h. Cells were then seeded onto Boyden Chambers (8 μm pores) (Corning, New York) and incubated with or without EGF (10 ng/ml) or HRG (100 ng/ml) for 6 h at 37 °C. Cells were fixed in 4% paraformaldehyde and stained with crystal violet for 16 h. Cells present in the upper chamber were removed with a cotton swab and the migrated cells, present in the lower chamber, were quantified by manual counting. Images were acquired using an epifluorescent inverted microscope (Carl Zeiss Axio Observer A1) with ZEN Pro 2011 software Blue edition.

Gefitinib-Resistant Clone Isolation

MDA-MB-231 cells were grown in media supplemented with 5 μM gefitinib (MDA-MB-231Gres) or DMSO (MDA-MB-231CTL) as control for 4 weeks. Cells that survived the 4 weeks of treatment and proliferated in the presence of gefitinib were considered to be gefitinib-resistant. Cells were maintained in medium supplemented with 10% FBS and 5 μM gefitinib or DMSO for the entirety of the experiments.

Statistical Analysis

Statistical analysis was performed using either a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or a two-way ANOVA followed by a Bonferroni's multiple comparison test using GraphPad Prism version 5 (San Diego, CA). The calculation of IC_{50} were also performed using GraphPad Prism version 5.

IV.4 Results

The adaptor p66Shc mediates gefitinib sensitivity in breast cancer cells.

The adaptor p66Shc has been shown to play an important role in mediating signals in response to oxidative stress and regulating mitochondrial functions leading to apoptosis. Additionally, this adaptor is a key mediator of signals downstream of the EGFR leading to Ras/MAPK activation (25, 26). Recently, we have shown that this adaptor attenuates EGF-induced ARF1 activation in invasive breast cancer cells by interfering with the recruitment of ARF1 to the EGFR (20). Since, we have shown that the activity of ARF1 mediates gefitinib sensitivity, we asked whether p66Shc, a key regulator of ARF1 activation, could also influence the sensitivity of these cells to EGFR inhibition. To do this, we depleted MDA-MB-231 cells of p66Shc using siRNA and assessed cell viability using a MTT assay following 72 hours of gefitinib (10 μM) treatment (Figure 1A). Interestingly, a decreased sensitivity to gefitinib was observed in p66Shc-depleted cells compared to control conditions. Since p66Shc-depleted cells have elevated ARF1 activity and the expression of ARF1 reduces the sensitivity of MDA-MB-231 cells to EGFR inhibitors, it is possible that p66Shc may mediate gefitinib sensitivity through its actions on ARF1 activity. However, it remains possible that p66Shc actions on gefitinib sensitivity are independent of ARF1; as p66Shc has been shown to mediate signals through oxidative stress and apoptosis. Thus, p66Shc may mediate gefitinib sensitive through its regulation of oxidative stress. Next, we overexpressed an HA-tagged p66Shc cDNA in these cells and measured cell viability (Figure 1B). The overexpression of p66Shc, associated with decreased ARF1 activity, was associated with an increased gefitinib-induced cell death further emphasizing the importance of ARF1 and mediators of its activity in the regulation of EGFR inhibitor sensitivity.

Additionally, we overexpressed two mutant forms of p66Shc that have altered serine phosphorylation on residue S36, HA-p66ShcS36A and HA-p66ShcS36D (Figure 1B). The phosphorylation of this serine residue has been implicated in the ability of p66Shc to translocate into the mitochondria and to mediate the induction of apoptosis (22}. Interestingly, unlike for wild-type p66Shc, the overexpression of both mutant forms of this adaptor were unable to promote gefitinib sensitivity suggesting that the mitochondrial targeting and/or apoptotic properties of p66Shc are required to induce gefitinib-dependent cell death.

Together, our results show that a key mediator of ARF1 activity can also regulate gefitinib sensitivity further highlight the importance of this GTPase in EGFR inhibition. Additionally, we show that the targeting of p66Shc as well as its apoptotic properties may be required for this adaptor to mediate EGFR inhibitor sensitivity.

ARF1 mediates mitochondrial-dependent apoptosis induced by gefitinib treatment.

Since p66Shc mediates mitochondrial dynamics leading to the induction of apoptosis and this adaptor mediates ARF1 activity (20, 22), we proposed that there may by an interplay between these two molecules that mediates mitochondrial functions in gefitinib-treated breast cancer cells. First, we examined the translocation of these two factors in MDA-MB-231 cells treated with gefitinib (10 μM) for 72 hours (Figure 2). While p66Shc was observed in isolated mitochondrial fractions, gefitinib treatment did not enhance the mitochondrial expression levels of this adaptor. Interestingly, we observed an increased ARF1 mitochondrial translocation upon gefitinib treatment and this was shown to be specific to this ARF isoform as gefitinib did not promote the mitochondria targeting of ARF6. Thus, suggesting that upon gefitinib treatment, ARF1 can translocate to the mitochondria and mediate the induction of apoptosis. However, we did observed some cytoplasmic contamination in our mitochondria extracts. Thus, ARF1 expression may stem from cytoplasmic contaminants. Interestingly, ARF1 has been previously shown to localize to mitochondrial-endoplasmic reticulum interface and mediate the transport between the two organelles and maintain mitochondrial functionality (27). However, here, we show for the first time that ARF1 can translocate to the mitochondria in cancer cells upon EGFR inhibition.

Next, we sought to characterize the functions of ARF1 in the mitochondria. To do this, we first depleted MDA-MB-231 cells of ARF1 and measured the expression of important mediators of mitochondrial integrity, p66Shc, Bcl2 and Bim (Figure 3). Interestingly, the depletion of ARF1 was associated with an enhanced p66Shc mitochondrial translocation suggesting that gefitinib could induce apoptosis in ARF1-depleted cells through a p66Shc-dependent mechanism. This increased mitochondrial influx of p66Shc correlated with an increased expression of the apoptotic initiator Bim and a decreased expression of the anti-apoptotic factor Bcl2 further highlighting the importance of ARF1 in blocking gefitinib-induced apoptosis of breast cancer cells. Together, these findings could suggest that ARF1 plays an important role in maintaining the cytosolic pool of

p66Shc and decreases the sensitivity to gefitinib by blocking the mitochondrial functions of p66Shc.

Since these apoptotic factors have been shown to promote cell death by mediate the opening of pores within the outer mitochondrial membrane which regulates the mitochondrial membrane potential (22, 28), we next assessed changes in mitochondrial membrane potential upon gefitinib treatment using the fluorescent marker, JC-1. Briefly, this marker has been shown to be an indicator of mitochondrial health and its accumulation within the mitochondrial is dependent on the organelle's membrane potential. While membrane depolarization is associated with increased green fluorescence, hyperpolarization is associated with red fluorescence (29). As shown in Figure 4A, gefitinib treatment of control cells exerted a slight hyperpolarization of the outer mitochondrial membrane, an indication of the induction of apoptosis. Whereas, in ARF1-depleted cells this hyperpolarization was significantly increased suggesting that ARF1 controls the induction of apoptosis upon gefitinib treatment.

Finally, we determined whether the activity of both initiator and effector caspases were enhanced by gefitinib treatment of ARF1-depleted cells (Figure 4B). Indeed, an increase in cleavage of both caspase 3 and 9 were observed in ARF1 knockdown cells compared to controls further emphasizing the importance of ARF1 in mediating gefitinib sensitivity and mitochondrial apoptosis.

Altogether, our findings demonstrate that gefitinib treatment of MDA-MB-231 cells promotes the mitochondrial translocation of ARF1. Furthermore, this GTPase is important in mediating the mitochondrial expression of p66Shc, Bcl2 and Bim. Moreover, we observed a hyperpolarization of the outer mitochondrial membrane as well as increased cleavage of both caspase 3 and 9 in ARF1-depleted cells compared to controls upon EGFR inhibition. This highlights a novel mechanism through which this ARF isoform mediates the sensitivity of breast cancer cells to EGFR inhibitors implicating the mitochondria.

Another ARF GTPase, ARF6, mediates gefitinib sensitivity.

Now that we have implicated ARF1 as a mediator of gefitinib sensitivity in breast cancer cells, we asked whether another ARF isoform, ARF6, implicated in signals downstream of the EGFR (30) could also regulate the inhibition of this receptor. Therefore, we depleted MDA-MB-231 cells of

ARF6 using siRNA and measured cell viability upon the treatment of indicated concentrations of gefitinib (Figure 5A). Similar to what we observed in ARF1 knockdown cells, the depletion of ARF6 was also associated with an increased sensitivity to EGFR inhibition compared to control cells. Indeed, compared to control cells, cells depleted of ARF6 showed increased sensitivity to gefitinib treatment at all doses above 1 μM. This further emphasizes the therapeutic benefit of targeting the ARF family of GTPases in breast cancer patients. It also suggests that not only member of the ARF GTPase, but at least two function to mediate the sensitivity to EGFR inhibitors.

As we demonstrated that ARF1 is activated in gefitinib treated cells and that this activation is important in mediating the sensitivity of cells to this inhibitor, we next determined if gefitinib treatment also promoted ARF6 activation (Figure 5B). Interestingly, lower doses of gefitinib shown to activate ARF1, blocked the activation of ARF6. However, increased doses of gefitinib were able to promote ARF6 activation. But, this increased ARF6 activity was shown not to be significant. Interestingly, this decreased ARF6 activity correlated with an increased gefitinib sensitivity. Thus suggesting that the blockade of ARF6 may mediate the response to this drug. However, it still remains plausible that the expression of ARF6 alone and not its activity may be required to mediate gefitinib sensitivity.

In summary, both ARF1 and ARF6 regulate the sensitivity of invasive breast cancer cells to EGFR inhibitors. However, while all tested concentrations of gefitinib enhanced ARF1 activity, only high doses of this inhibitor were sufficient to activate ARF6.

ARF1 is essential for gefitinib-induced EGFR activation and dimerization.

Since an increased activation of EGFR family members has been associated with reduced sensitivity to EGFR inhibitors (7, 31, 32) and we have demonstrated that gefitinib mediates the expression of both EGFR and HER2 in breast cancer cells, we next determined the activation state of EGFR family members (EGFR, HER2 and HER3). Control and ARF1-depleted MDA-MB-231 cells were treated with the inhibitor for 24 hours. Gefitinib (10 μM) had no effect on the ligand-independent activation of HER2 in control conditions (Figure 6A, C). However, a decrease in HER3 tyrosine phosphorylation was observed. In ARF1 knockdown cells, a decrease in tyrosine phosphorylation of all three EGFR family members was noted following gefitinib treatment. Thus,

ARF1 depletion is associated with both a decrease in EGFR family member expression and activation, which may explain the importance of ARF1 in mediating gefitinib sensitivity.

Since EGFR heterodimerization with other EGFR family members has also been associated with acquired resistance to EGFRTKis (33), we next examined EGFR dimerization patterns in gefitinib-treated cells. As shown in Figure 6B and D, treatment of control cells with gefitinib (10 μM) for 24 hours enhanced EGFR-HER2 heterodimer formation. In addition, this dimerization was attenuated by ARF1 depletion. Furthermore, gefitinib treatment alone, ARF1 depletion alone or gefitinib-treatment of ARF-depleted cells all decreased the heterodimerization between EGFR and HER3. ARF1 depletion also resulted in an increase of HER2-HER3 dimerization, which was attenuated in cells treated with gefitinib. Together, these results suggest that ARF1 depletion is associated with a decreased EGFR expression, activation and heterodimerization.

Knowing that ARF1 mediates the dimerization of EGFR family members and that EGF, which favors EGFR homodimers or HER2 heterodimers, promotes ARF1 activation, we next asked whether ligands that favor HER3 signaling could also promote ARF1 activity (Figure 7). To do this, we treated MDA-MB-231 with heregulin (HRG), a ligand known to promote HER3 heterodimerization with both EGFR and HER2 (34), and measured ARF1 activity using a classical GST-GGA3 pulldown activation assay. As shown in Figure 7A, Unlike EGF which exerted a prolonged activation of ARF1, HRG only induced a transient activation of ARF1 in MDA-MB-231 cells at 5 minutes. A similar activation pattern for EGF and HRG was observed in the HER2 positive SKBR3 breast cancer cell line (Figure 7B). Interestingly, the hormone responsive MCF7 cells which express higher HER3 levels compare to EGFR, showed a more rapid and longer activation of ARF1 upon HRG stimulation compared to EGF (Figure 7C). Together, we demonstrate that both EGF and HRG can activate the small GTPase ARF1 and that this ligand-dependent activation profile is mediated by EGFR family member expression.

As we have demonstrated that EGF mediates MDA-MB-231 cell proliferation and migration through the activation of ARF1 (17), we next asked if HRG could also promote these physiological cellular responses. As shown in Figure 8A, EGF, but not HRG, induced the proliferation of MDA-MB-231 cells and as previously reported EGF-mediated cell growth was hindered upon the depletion of ARF1 (17). Meanwhile, in MCF7 cells (Figure 8B), the proliferation of control cells was induced by HRG and not EGF. Interestingly, we were able to block HRG-dependent

proliferation of these cells by depleting ARF1 suggesting that ARF1 can mediate both EGF and HRG-induced cell proliferation. Next, we examined the migration of both MDA-MB-231 and MCF7 cells in response to both EGF and HRG stimulation, Figures 8C and 8D, respectively. EGF stimulation was shown to promote the migration of both cell lines, whereas, HRG only promoted that migration of MCF7 cells. Furthermore, the HRG-induced MCF7 cell migration was significantly higher than that of EGF stimulated cells. Interestingly, ARF1-depletion was shown to attenuate EGF-dependent MDA-MB-231 cell migration, but not in EGF stimulated MCF7 cells. Additionally, HRG-induced MCF7 migration was attenuated in cell depleted of ARF1 suggesting that both EGF and HRG-induced ARF1 activation play an important role in mediating cellular proliferation and migration.

Altogether, ARF1 can be activated downstream of multiple EGFR family members upon ligand stimulation and this GTPase plays an important role in mediate gefitinib sensitivity by governing the activation and dimerization of this receptor family.

ARF1 depletion re-sensitizes resistant cells to gefitinib treatment.

Thus far, our results have demonstrated that the depletion of ARF1 enhances the sensitivity of invasive breast cancer cells to gefitinib. We therefore sought to determine whether ARF1 may play a role in the acquired resistance to EGFRTKis in an isolated MDA-MB-231 gefitinib-resistant cell line (MDA-MB-231Gres). Clinically, acquired resistance is defined as a lack of therapeutic response to EGFRTKis in a patient that previously responded for a period greater than 6 months (35) . Therefore, we attempted to mimic this clinical situation by treating parental MDA-MB-231 cells with an effective dose of gefitinib (5 μM) for a month until cells became completely unresponsive to the cytotoxic properties of the treatment and began to survive and proliferate in the presence of this inhibitor. These cells were considered to have developed acquired resistance to gefitinib (MDA-MB-231Gres). Control parental MDA-MB-231 cells were treated with DMSO vehicle for a similar time period (MDA-MB-231CTL). These cells were non-resistant to high doses of gefitinib. First, we examined the expression and activation of ARF1 in our isolated clones. In control cells, EGF stimulation enhanced ARF1 activation, but this response was abolished in resistant clones (Figure 9A). Interestingly, the basal activity of ARF1 was significantly enhanced in MDA-MB-231Gres cells suggesting that mechanisms leading to enhanced ARF1 activation may

contribute to acquired gefitinib-resistance. Furthermore, in these cells, EGF stimulation was unable to promote phosphorylation of its receptor (Figure 9B). We next determined whether the depletion of ARF1 in MDA-MB-231Gres cells could sensitize these clones to gefitinib-induced cell death. As expected, ARF1 depletion in control DMSO-treated cells increased gefitinib sensitivity compared to non-depleted control cells (Figure 9C). This increased sensitivity was associated with a decreased IC_{50} in ARF1-depleted cells (IC_{50}: 5.5 μM) compared to control conditions (IC_{50}: 25.4 μM). By definition, the MDA-MB-231Gres cells were shown to have a decreased sensitivity to all examined gefitinib doses as measured by an increased IC_{50} of 72.1 μM (compared to 25.4 μM in non-resistant cells). More importantly, we observed that the depletion of ARF1, in these resistant clones, re-sensitized the cells to gefitinib treatment as measured by an increased cell death induced by the drug. This was marked by a significant decrease in the IC_{50} of resistant cells from 72.1 μM in non-depleted to 16.8 μM in ARF1-depleted conditions. Together, our results demonstrate that gefitinib resistant cells have an increased ARF1 activation and that this ARF isoform is required for gefitinib resistance.

The acquisition of EGFRTKi resistance is often associated with altered receptor function and signaling profile. In fact, resistance has been associated with an increased Src, AKT and ERK1/2 signaling in a variety of cancer cell types (7, 10-16). First, we examined expression of the EGFR family members in MDA-MB-231CTL and MDA-MB-231Gres cells transfected with either CTL or ARF1 siRNA (Figure 10A). While the expression of HER2 and HER3 was found to be higher in the resistant clones compared to controls, EGFR expression was unaffected. More interestingly, whereas the depletion of ARF1 had no effect on receptor expression in control clones, a significant reduction in EGFR (55%), HER2 (50%) and HER3 (73%) was observed in gefitinib-resistant cells suggesting that ARF1 depletion restores gefitinib sensitivity by regulating expression of EGFR family members present in this cell line. Furthermore, we examined the expression of two other RTKs previously reported to be increased in EGFRTKi-resistant cells, AXL and cMET (14, 36). In our gefitinib-resistant cell model, we were unable to detect any difference in the expression profile of those RTKs (Figure 10A). Additionally, the depletion of ARF1 in either control or gefitinib resistant clones had no effect on both AXL and cMET expression. However, a detectable increase in AXL and cMET activity was observed. This activity was independent of ARF1 expression (Figure 10A). Together, our results demonstrate that gefitinib resistance is associated

with increased ARF1 activation and expression of EGFRs and that the depletion of ARF1 is associated with a decreased expression of these receptors without affecting other RTKs.

Next, we analyzed the activation of signaling cascades in our gefitinib-resistant clones compared to control cells. Interestingly, an increase in AKT and p38MAPK signals, but not ERK1/2, Src and JNK was detected in resistant clones (Figure 10B). While the depletion of ARF1 in control clones had no effect on all examined signaling proteins, a significant reduction in both AKT and p38MAPK activation was observed in MDA-MB-231Gres cells depleted of ARF1. This result suggests that ARF1 may play a role in the maintenance of EGFRTKi resistance by promoting the activation of survival signals through the AKT and p38MAPK pathways. Together, our results demonstrate that in gefitinib resistant invasive breast cancer cells, ARF1 is over-activated and associated with an increased EGFRs expression and enhanced AKT and p38MAPK signaling. We propose that all these changes regulated by ARF1 expression contribute to promote gefitinib resistance.

Since the therapeutic benefits of targeting these signaling cascades in EGFRTKi resistant cancer has been previously shown (37-39), we finally aimed to determine whether the depletion of ARF1, in our gefitinib-resistant clones, could sensitize these cells to inhibition of other signaling mediators such as ERK1/2, AKT, Src and p38MAPK. We therefore measured the viability of control and gefitinib-resistant cells that were transfected with either a control or ARF1 siRNA upon 24 hours of inhibitor treatment. In control clones, ARF1 depletion did not enhance the efficacy of all tested inhibitors (Figure 10C). However, the depletion of ARF1, in gefitinib-resistant clones, significantly enhanced death of cells treated with the MEK, PI3Kinase and p38MAPK, but not the Src inhibitors. Together, our results demonstrate that ARF1 may promote gefitinib resistance by moderating important signaling effectors that are known to play an important role in acquired resistance. Furthermore, ARF1 depletion can enhance the effects of inhibitors of these pathways in gefitinib-resistant breast cancer cells.

I propose to further characterize the gefitinib-resistant clones. It would be of interest to examine the activity of other ARF isoforms such as ARF6. Additionally, the role p66Shc plays in these clones in mediating their resistant will also be important to evaluate.

IV.5 Discussion

Our laboratory has well characterized the small GTPase ARF1 as a key mediator of signals downstream of the EGFR leading to breast cancer cell proliferation, migration and invasion (17-20). More recently we implicated this ARF isoform in the sensitivity of these cells to EGFR inhibitors. Here, we further characterize ARF1 as an important regulator of gefitinib sensitivity in TNBC cells and show that it acts as a signaling switch that promotes resistance to EGFR inhibition. First, we demonstrate that a regulator of ARF1 activity, p66Shc, is also an essential in gefitinib sensitivity. Indeed, the overexpression of p66Shc, associated with decrease ARF1 activity, enhanced the sensitivity of MDA-MB-231 to gefitinib treatment. Moreover, decreased sensitivity was observed when cells were depleted of p66Shc, a condition shown to augment ARF1 activation. Additionally, we showed that another ARF isoform, ARF6, could also mediate EGFR inhibitor sensitivity. Next, we highlight a role for ARF1 in controlling mitochondrial functionality upon gefitinib treatment. This GTPase was shown to localize to the mitochondria in gefitinib-treated cells and the depletion of ARF1 enhanced gefitinib-induced apoptosis as measured by increase p66Shc and Bim mitochondrial translocation, decreased mitochondrial Bcl2, hyperpolarization of the mitochondrial membrane and increase caspase activity. Subsequently, we further demonstrate that ARF1 control the signaling dynamics of EGFR family members by both regulating their activity and dimerization. Finally, using an isolate cell line that showed resistance to gefitinib, we showed that depletion of ARF1 in these cells could re-sensitize them to EGFR inhibition implicating ARF1 in both gefitinib sensitivity and resistance in TNBC cells.

The adaptor p66Shc has been previously shown to negatively regulate RTK signaling. Indeed, its overexpression has been associated with decreased signaling through the Ras/MAPK pathway (25). Additionally, this adaptor has been implicated in the induction of apoptosis in response to cytotoxic agents (40-42). Recently, we demonstrated that p66Shc blocks the activation of ARF1 downstream of the EGFR by interfering with the recruitment of a Grb2-ARF1 complex to the receptor (20). Subsequently, we demonstrated that the activity of ARF1 is essential in mediating the sensitivity of breast cancer cells to EGFR inhibitors. Therefore, mediators of its GTPase activity should sensitize these cells to EGFR inhibition. Here, we show that p66Shc is required for gefitinib-induced breast cancer cell death. Indeed, the overexpression of this Shc isoform

sensitized cells to EGFR inhibitor treatment. However, further investigation into the mechanism through which p66Shc regulated gefitinib-induced cell death and whether this mechanism is dependent on the activity of ARF1 is required.

Interestingly, the mitochondrial translocation of p66Shc through serine phosphorylation of this adaptor has been implicated in the induction of apoptosis (22). We show that ARF1-depletion enhances the mitochondrial translocation of p66Shc. Furthermore, gefitinib treatment of ARF1 knockdown cells was associated with increased JNK activation, a serine kinase previously implicated in the phosphorylation of p66Shc (22, 26). Furthermore, when mutant forms of p66Shc that have altered serine phosphorylation on residue 36 were overexpressed in MDA-MB-231 cells, gefitinib sensitivity was no longer enhanced. Interestingly, blockade of the serine phosphorylation of p66Shc has been shown to block its mitochondrial transport (22) further emphasizing the importance of mitochondrial p66Shc in gefitinib sensitivity.

Knowing that ARF1 mediated the translocation of p66Shc into the mitochondria, we next asked whether this ARF isoform could also localize within this organelle upon gefitinib treatment. Interestingly, it has been recently shown that ARF1 can localize within an endoplasmic reticulum-mitochondria complex where it mediates the transport between the two organelles and maintains mitochondrial hemostasis and functionality (27). Here, we show that gefitinib treatment enhanced the translocation of ARF1 to the mitochondria. Moreover, this was shown to be specific to this ARF isoform as gefitinib did not mediate the mitochondrial localization of ARF6. While our data suggests that ARF1 plays a role in mediating cell death and that gefitinib promotes its mitochondrial translocation, we have yet to identify and characterize the role of this GTPase within the mitochondria of our cellular model. Further studies are required to better understand this novel functionality of ARF1 in gefitinib treated cells and whether this ARF1 population plays a role in mediated the sensitivity and/or resistance of these cells to EGFR inhibition.

To further characterize the mitochondrial functions of ARF1, we next examined mitochondrial dynamics in gefitinib-treated cells depleted of ARF1. Interestingly, the mitochondrial expression of Bim, an apoptotic initiator was enhanced. Whereas, the expression of Bcl2, an inhibitor of apoptosis, was reduced. Furthermore, we observed a hyperpolarization of the outer membrane of the mitochondria, a process involved in mitochondrial swelling and the induction of apoptosis (43). Finally, inhibitor treatment of ARF1-depleted cells was associated with both Cytochrome C release

from the mitochondria and the activation of caspase 3 and 9. Together, this data suggests that ARF1 may play a role in stabilizing the mitochondria and blocks the induction of mitochondrial-dependent apoptosis. However, whether ARF1 is acting upstream of the mitochondria or within the mitochondria to mediate these effects requires further investigation.

It has also been proposed that other EGFR family members can compensate for the loss of EGFR signals (36). Indeed, increased signaling through both HER2 and HER3 have been associated with EGFR inhibitor insensitivity and resistance. In our cell model, ARF1 depletion also significantly reduced HER2 activation upon gefitinib treatment. Additionally, the depletion of this small GTPase was associated with a reduction in the heterodimerization of HER2 with HER3. We have previously demonstrated that ARF1 stabilizes the expression of both EGFR and HER2 without effecting HER3 levels. This downregulation was associated with an increased degradation of these receptors in ARF1-depleted cells Thus, ARF1 may promote EGFRTKi resistance by stabilizing the expression and activation of both EGFR and HER2, as well as promoting HER2 heterodimerization.

The development of resistance to EGFR inhibitors can significantly hinder their therapeutic benefits. Multiple mechanisms have been implicated in the development of resistance. These include: mutations in the EGFR itself (5), increased signaling through other RTKs (HER2-3 AXL, cMET) (14, 44), altered downstream signaling through ERK1/2 and/or AKT, evasion of apoptosis and the induction of epithelial-mesenchymal transformation (44). Here, we show that gefitinib-resistant breast cancer cells have elevated ARF1 activity. Interestingly, the knockdown of this GTPase re-sensitized cells to EGFR inhibition. Furthermore, the depletion of ARF1 in these cells significantly reduced the expression of EGFR family members (EGFR, HER2 and HER3) resulting in decreased survival signals through AKT activation.

Together, our finding further emphasize the importance of ARF isoforms and mediators of their activity in the sensitivity to gefitinib treatment via both a mitochondrial dependent mechanism and the regulation of EGFR dimerization and activation. Furthermore, ARF1 activity governs the development of resistance to these inhibitors. Therefore, targeting this ARF isoform in TNBC could help improve the therapeutic outcome of patients treated with EGFR inhibitors.

IV.6 Figure Legends

Figure 1: p66Shc mediates gefitinib sensitivity of MDA-MB-231) cells. *A*, Percent cell death was assessed by a MTT assay in MDA-MB-231 cells that were transfected with CTL or p66Shc siRNA and then treated 72 hours with gefitinib (10 μM). Data shown are mean ± SEM. Significance was measured by a T-test with n=3; (***) $P < 0.001$. *B*, Percent cell death was assessed by a MTT assay in MDA-MB-231 cells that were transfected with CTL, p66Shc, p66ShcS36A or p66ShcS36D cDNAs and then treated 24 hours with gefitinib (10 μM). Data shown are mean ± SEM. Significance was measured by a one-way ANOVA with n=3; (**) $P < 0.01$.

Figure 2: Gefitinib induces the translocation of ARF1 into the mitochondria. *A*, Cytoplasmic and mitochondrial extracts were isolated from MDA-MB-231 cells that were treated with gefitinib (10 μM) for 72 hours. Expression levels of ARF1, ARF6, p66SHC, JNK and Bcl2 were assessed by western blot analysis. Actin was used as a marker of cytoplasmic fractions and CoxIV as a marker of mitochondrial extracts. Data shown are mean ± SEM. Significance was measured by a two-way ANOVA with n=3; (**) $P < 0.01$, (***) $P < 0.001$.

Figure 3: ARF1 is required for p66Shc translocation into the mitochondria. *A*, Mitochondrial extracts were isolated from MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA before being treated with gefitinib (10 μM) for 72 hours. Expression levels of ARF1, p66SHC, Bcl2 and Bim were assessed by western blot analysis. CoxIV was used as a marker of mitochondrial extracts. Data shown are mean ± SEM. Significance was measured by a two-way ANOVA with n=3; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$.

Figure 4: ARF1 depletion promotes gefitinib-induced mitochondrial apoptosis. *A*, Mitochondrial membrane potential was measured in MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA treated with gefitinib (10 μM) for indicated times. JC-1 oligomerization was used as a measure of membrane hyperpolarization. Data shown are mean ± SEM. Significance was measured by a two-way ANOVA with n=2; (**) $P < 0.01$, (***) $P < 0.001$.

Figure 5: ARF6 depletion enhances gefitinib sensitivity. *A*, Cell viability was assessed by a MTT assay in MDA-MB-231 cells that were transfected with CTL or ARF6 siRNA and then treated 24 hours with indicated concentrations of gefitinib. Data shown are mean ± SEM. Significance was measured by a two-way ANOVA with n=3; (*) $P < 0.05$, (**) $P < 0.01$. Western blot analysis confirmed the depletion of ARF6. *B*, MDA-MB-231 cells were treated with indicated concentrations of gefitinib for 1 hour. A GST-GGA3 pulldown assay was used to capture activated ARF6 from cell lysates. Endogenous levels of activated ARF6 and the total protein levels of ARF6 were assessed by western blot analysis. Data shown are mean ± SEM. Significance was measured by a one-way ANOVA with n=3; (**) $P < 0.01$, (***) $P < 0.001$.

Figure 6: ARF1 depletion mediates gefitinib-dependent EGFR activation and dimerization. *A,* EGFR, HER2 and HER3 activation was assessed by immunoprecipitating the indicated receptors and immunoblotting with a phospho-specific antibody against total phospho-tyrosine sites (pan-PY) using lysates obtained from MDA-MB-231 cells that were transfected with CTL or ARF1 siRNA and then treated 24 hours with 10 μM gefitinib. *B*, Co-immunoprecipitation experiments were used to assess EGFR family member heterodimerization using the cell lysates described in (A). *C*, Quantification of receptor tyrosine phosphorylation in (A). Data is presented as mean percent decrease in receptor tyrosine phosphorylation ± SEM with n=3. Significance was measured by a two-way ANOVA; (***) $P < 0.001$. *D*, Quantification of receptor dimerization in (B). Data is presented as mean percent decrease of dimerization for EGFR/HER2 and EGFR/HER3 and mean percent increase of dimerization for HER2/HER3 dimers ± SEM with n=3. Significance was measured by a two-way ANOVA; (*) $P < 0.05$, (**) $P < 0.01$.

Figure 7: Heregulin-induced ARF1 activation. *A-C*, MDA-MB-231, SKBR3 or MCF7 cells, respectively, were stimulated with either EGF (10 ng/ml) of HRG (100 ng/ml) for the indicated times. Cells were lysed, and a GST pulldown assay using GST-GGA3 coupled to glutathione-Sepharose 4B beads was used to capture activated ARF1. Endogenous levels of activated ARF1 and the total protein levels of ARF1 in cell lysates were assessed by western blot analysis.

Figure 8: ARF1 is required for heregulin-induced proliferation and migration. *A,* MDA-MB-231 cells were left untreated or stimulated with EGF (10 ng/ml) or HRG (100 ng/ml) for 24 hours. Cell growth was determined by MTT assay. Results presented are representative of three independent experiments. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (***) $p < 0.001$. *B,* MCF7 cells were left untreated or stimulated with EGF (10 ng/ml) or HRG (100 ng/ml) for 24 hours. Cell growth was determined as in (A). The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (*) $p < 0.05$. *C*, MDA-MB-231 cells were seeded onto Boyden chambers and stimulated or not with EGF (10 ng/ml) or HRG (100 ng/ml). Migration was assessed after 6 h. Results presented are representative of three independent experiments. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (***) $p < 0.001$. *D,* MCF7 cells were treated and migration was assessed as described in (C). Results presented are representative of three independent experiments. The quantifications of each experiment are presented as fold-increase over basal and are the mean ± S.E. with (***) $p < 0.001$.

Figure 9 : Gefitinib resistance is abolished upon ARF1 depletion. *A,* Control-DMSO (CTL) or gefitinib-resistant (GRes) clones were left untreated or treated with EGF (10 ng/ml) for 1 minute and a GST-GGA3 pulldown assay assessed ARF1 activation. Data shown are mean ± SEM. Significance was measured by a two-way ANOVA with n=3; (**) $P < 0.01$. *B,* EGFR activation was assessed by immunoprecipitating the EGFR and immunoblotting with a phospho-specific antibody against total phospho-tyrosine sites (pan-PY) using lysates obtained from CTL or GRes clones that were left untreated or treated with EGF (10 ng/ml) for 1 minute. Data shown are mean ± SEM. Significance was measured by a two-way ANOVA with n=3; (***) $P < 0.001$. *C,* Percent cell death of MDA-MB-231CTL and MDA-MB-231Gres clones that were either transfected with

CTL or ARF1 siRNA and then treated 24 hours with indicated concentrations of gefitinib was assessed by a MTT assay. Data shown are mean ± SEM. Significance was measured by a two-way ANOVA with n=3; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$. * Indicative of significance between CTL siRNA and ARF1 siRNA in non-resistant cells. # Indicative of significance between non-resistant and resistant cells transfected with CTL siRNA. ϕ Indicative of significance between CTL siRNA and ARF1 siRNA in resistant cells.

Figure 10: The receptor and signaling profile of gefitinib resistant cells is regulated by ARF1. *A,* The protein expression of EGFR, HER2, HER3, cMET and AXL as well as the activation of cMET and AXL was assessed in lysates obtained from in cell lysates obtained from Control-DMSO (CTL) or gefitinib-resistant (GRes) clones that were transfected with CTL or ARF1 siRNA by western blot analysis. Data is presented as mean percent decrease in receptor expression/activation ± SEM with n=3. Significance was measured by a two-way ANOVA; (***) $P < 0.001$. *B,* Western blot analysis was utilized to measure the activation of ERK1/2, AKT, Src, JNK and p38MAPK in cell lysates obtained from Control-DMSO (CTL) or gefitinib-resistant (GRes) clones that were transfected with CTL or ARF1 siRNA. Data is presented as mean percent decrease in pathway activation ± SEM with n=3. Significance was measured by a two-way ANOVA; (*) $P < 0.05$, (***) $P < 0.001$. *C,* Cell viability of CTL and GRes clones that were either transfected with CTL or ARF1 siRNA and then treated 24 hours with either PP2 (1 μM), PD0325901 (10 μM), LY294002 (15μM) or SB220025 (100 nM) was assessed by a MTT assay. Data shown are mean ± SEM. Significance was measured by a two-way ANOVA with n=3; (*) $P < 0.05$, (**) $P < 0.01$.

Fig. 1
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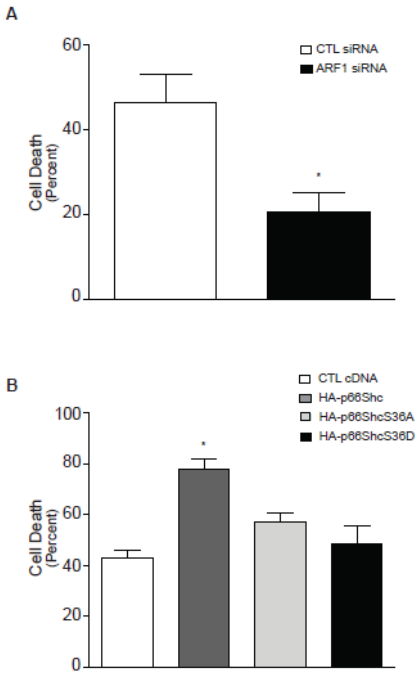

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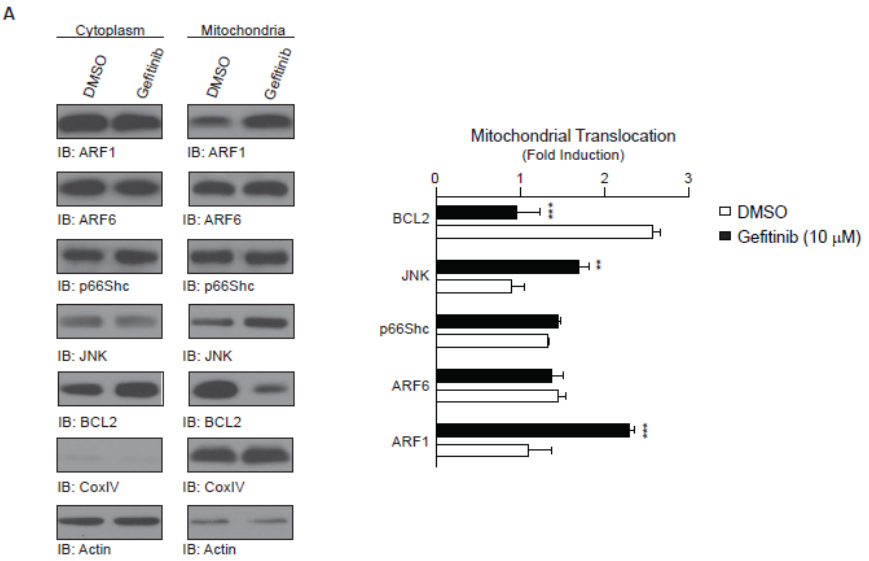

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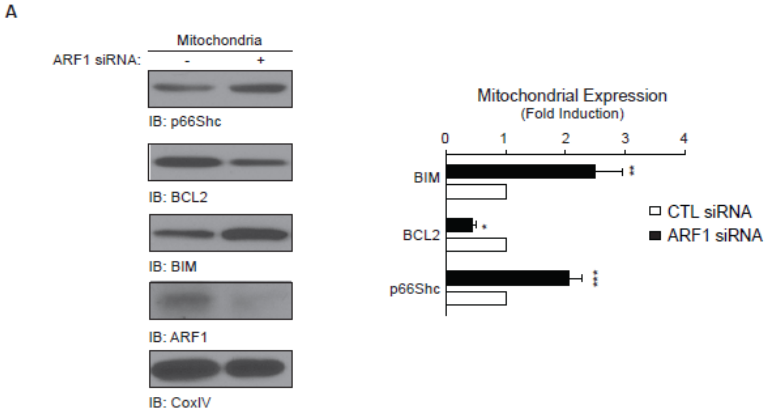

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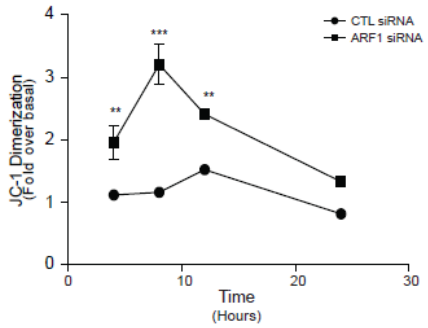

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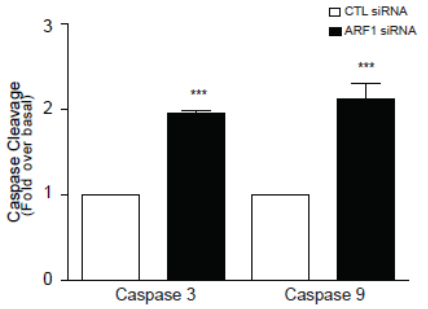

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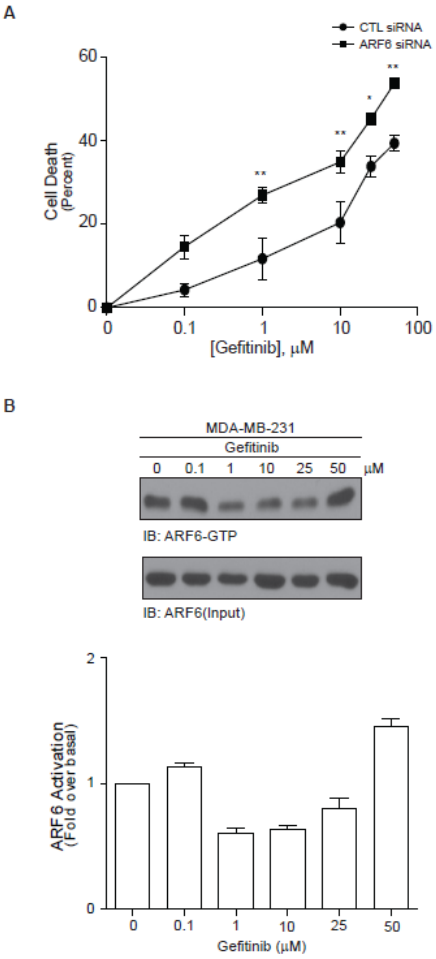

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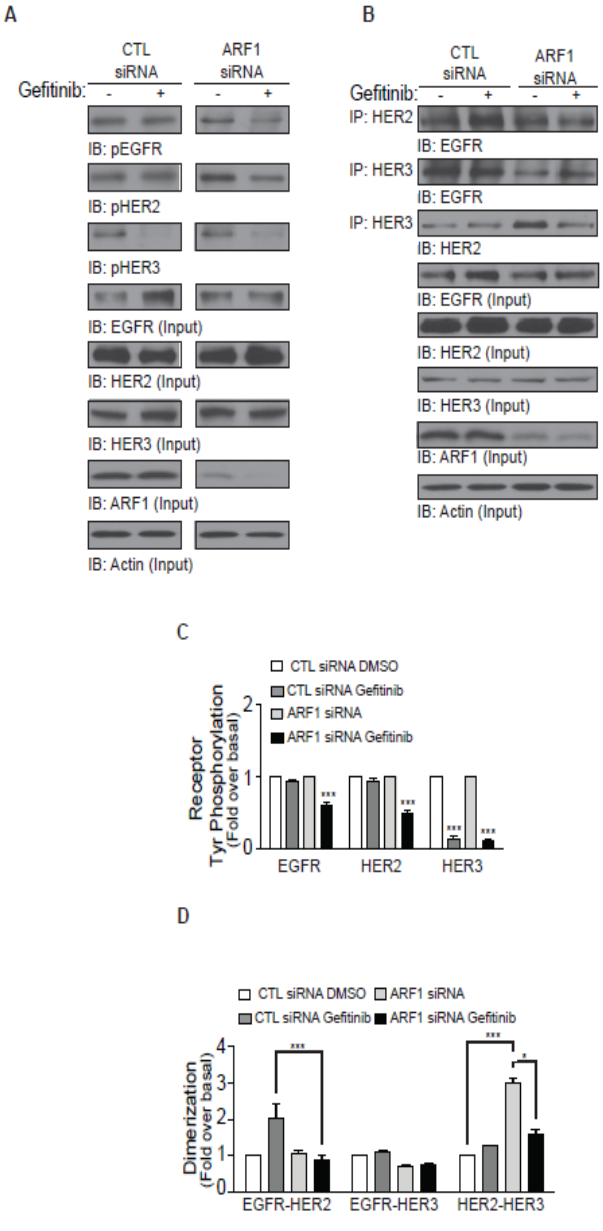

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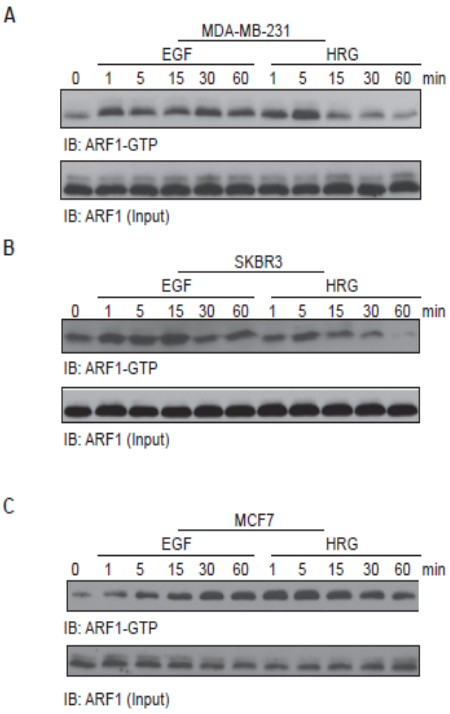

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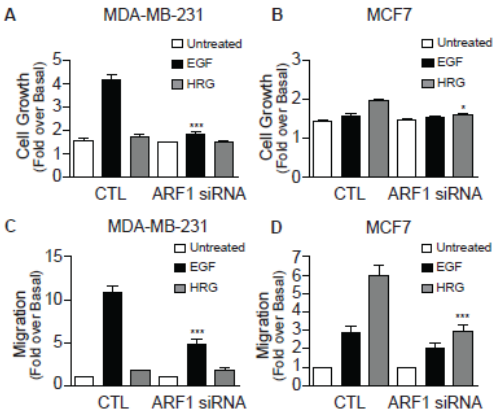

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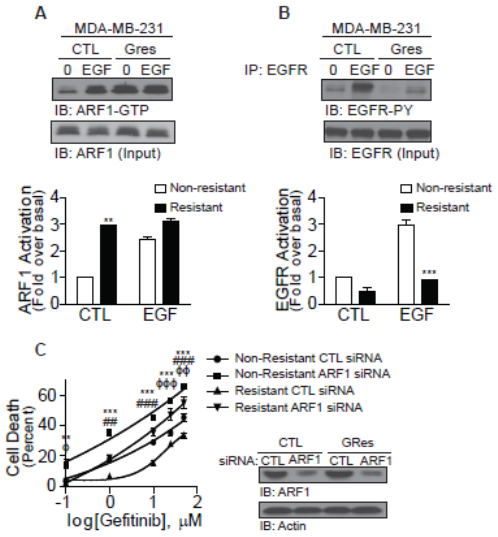

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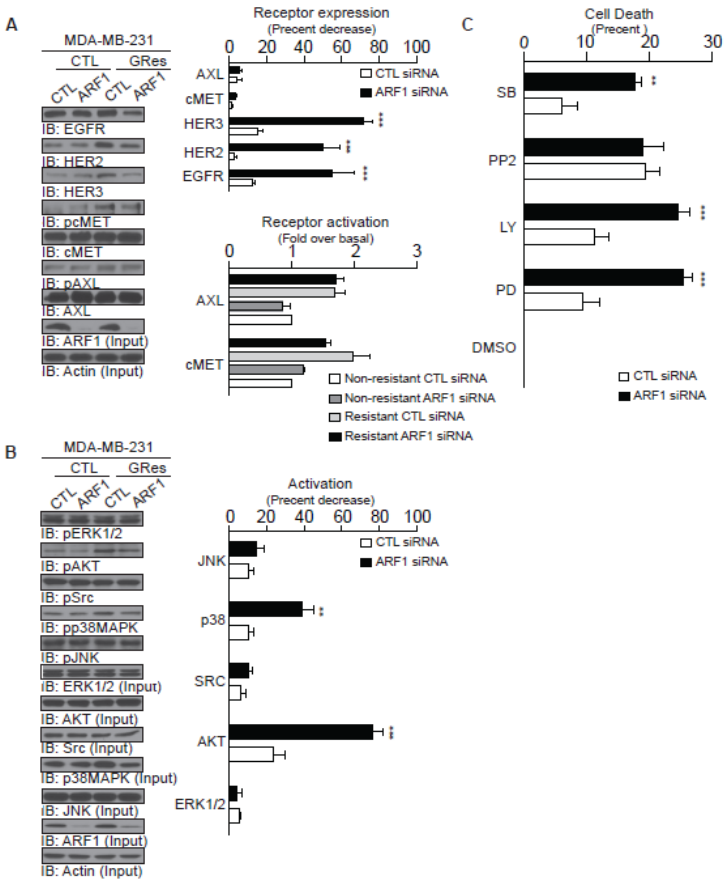

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CHAPTER V: Discussion

As no targeted therapies are currently available for the treatment of TNBC and many of these are unresponsive or develop resistance to treatments with classical chemotherapeutics, it is essential to identify and characterize novel malignancy-inducing targets within this breast cancer population. This study investigated the mechanisms leading to the activation of the small GTPases ARF1 and ARF6 downstream of the EGFR in cellular models of TNBC and their implications in gefitinib sensitivity and resistance. We show that the adaptor proteins, Grb2 and p66Shc, mediate the recruitment of ARF GTPases to the EGFR and their activation. Next, we highlight the importance of ARF1 in the regulation of EGFR inhibitor sensitivity and resistance in TNBC cells. ARF1 was shown to mediate these processes by promoting cell survival, implicating novel actions within the mitochondria, and by stabilizing the expression and activity of EGFR family members. Our findings and their therapeutic implications will be further discussed in this section.

V.1 Adaptor proteins modulate EGFR-dependent ARF activation in breast cancer cells

Adaptor proteins play essential roles in the propagation of signals originating from RTKs. They have been well characterized for their roles in the recruitment of signaling mediators such as Gab1, SOS and PI3K to RTKs (van der Geer, Hunter et al. 1994; Kairouz and Daly 2000; Ong, Hadari et al. 2001). This allows for the initiation of important signaling cascades such as the Ras/ERK1/2 and PI3K/AKT pathways (van der Geer, Hunter et al. 1994). Indeed, the depletion of adaptor proteins such as Grb2 and Shc is linked to decreased ligand-dependent signals downstream of RTK activation (Gale, Kaplan et al. 1993; Sweet and Tzima 2009). Here, we demonstrate that these adaptor proteins also mediate the activation of the ARF family of small GTPases. Interestingly, we found that Grb2 as well as the Shc isoform, p66Shc, associate with ARF1 upon EGF stimulation of MDA-MB-231 cells. Moreover, the depletion of these two adaptor proteins altered the activation state of ARF1. While Grb2 was shown to be essential for ARF1 activation, p66Shc attenuated the activation of this GTPase. Additionally, we showed that Grb2 also promoted the activation of ARF6. However, unlike ARF1, p66Shc was shown to also to be essential for ARF6 activation.

Since these adaptors are best characterized for their recruitment to the EGFR, we next determined whether they mediated the recruitment of ARF proteins to this receptor. Grb2 was shown to be

required for the recruitment of both ARF1 and ARF6 to the EGFR. Whereas, p66Shc blocked the ability of Grb2 to recruit ARF1 to the EGFR, but enhanced the association between EGFR and ARF6. These findings are consistent with previously published findings that demonstrated that p66Shc blocks the recruitment of Grb2 to both the EGFR and IGF1R by either competing for similar binding sites on the receptors or by sequestering Grb2 away from the receptors (Okada, Kao et al. 1997; Xi, Shen et al. 2010). Thus, we propose that upon EGFR activation, Grb2 recruits ARF1 and an unidentified guanine exchange factor (GEF) to the receptor to induce the GTPase activity of ARF1. Meanwhile, p66Shc acts to block ARF1 activation by blocking the recruitment of this Grb2-ARF1 complex to the receptor. Additionally, p66Shc can enhance the EGFR recruitment and activation of ARF6 (Chapter II, Fig. 10).

Recent findings from our lab and others have demonstrated that ARF1 and ARF6 play redundant roles in mediating signals downstream of the EGFR leading to breast cancer cell proliferation, migration and invasion (Boulay, Cotton et al. 2008; Sabe, Hashimoto et al. 2009; Boulay, Schlienger et al. 2011; Schlienger, Campbell et al. 2014). Our findings demonstrate that in conditions in which ARF1 activity is inhibited (i.e. high p66Shc expression), we observe an increased activation of ARF6. Furthermore, an increase cellular migration was observed in p66Shc overexpressing MDA-MB-231 cells. This could suggest that these two GTPases may act as compensatory mechanism utilized by cancer cells to proliferate and survive. Therefore, it may be essential to target both of these ARF isoforms in breast cancer patients. Furthermore, it would important to characterize the role of other ARF isoforms in breast cancer as ARF4 has been implicated in the migration of glioblastoma cells.

Here, we show that the activation and receptor recruitment of ARF6 is dependent on both the adaptors Grb2 and p66Shc. Interestingly, the tyrosine residues 1068 and 1086 on the EGFR, key recruitment sites for the adaptor Grb2, were previously shown to promote the EGF-dependent activation of ARF6 in invasive breast cancer cells. In this study, it was shown that the ARFGEF, GEP100 (BRAG2), could directly interact with these tyrosine residues through its PH domain. These conclusions were obtained in an *in vitro* binding assay that illustrated that phosphopeptides that mimicked tyrosine 1068 and 1086 could associate with GEP100 (Morishige, Hashimoto et al. 2008). However, in an *in vivo* setting, these residues are known to recruit Grb2 (Batzer, Rotin et al. 1994; Ono and Kuwano 2006). This would suggest that Grb2 promotes ARF6 activation by

recruiting this GTPase and possibly a GEF to the EGFR via its interactions with these tyrosine residues within the cytoplasmic tail of the receptor.

Grb2 is classically known to recruit GEFs to GTPases to promote their activity. Indeed, Grb2 brings the GEF, SOS, in the proximity of Ras GTPase leading to its activation (Chardin, Camonis et al. 1993; Li, Batzer et al. 1993). Furthermore, Grb2 has also been shown to recruit the GEF Vav2 to the HER2 receptor to promote both Ras and Rac1 activity (Bourguignon, Zhu et al. 2001). Thus, we propose that Grb2 may also utilize a similar mechanism in the recruitment of an ARFGEF to ARF proteins at the EGFR to promote their activation. However, this Grb2-dependent GEF remains to be identified.

V.2 p66Shc modulates breast cancer cell migration and apoptosis

Within the literature, the role p66Shc is cellular specific. While in prostate cancer and hormone-responsive breast cancer cells, p66Shc has been implicated in cell migration (Lee, Igawa et al. 2004; Rajendran, Thomes et al. 2010), p66Shc has shown play a role in the induction of apoptosis in other cellular systems (Orsini, Migliaccio et al. 2004; Giorgio, Migliaccio et al. 2005). Additionally, p66Shc has been implicated in tumor initiation but also as an important tumor suppressor when in complex with p53 (Beltrami, Valtorta et al. 2013). Here, we show that p66Shc via the activation of the ARF6-Ras-MAPK pathway promotes the migration of invasive breast cancer cells. However, its overexpression was observed to be associated with cellular death. Moreover, the depletion of p66Shc was associated with a reduced sensitivity of MDA-MB-231 cells to EGFR inhibition. Thus, we propose a dual role for p66Shc in breast cancer cells: 1- As an inducer of cell migration via the activation of ARF6 and 2- As a promoter of apoptosis via its inhibition of ARF1.

The migratory role of p66Shc has been linked to its ability to activate Rac1, promote ROS production and induce Ras/MAPK signaling (Bhat, Baba et al. 2014). Interestingly, we also demonstrate that p66Shc signals through the Ras/MAPK pathway to promote cell migration. Furthermore, we recently have implicated both ARF1 and ARF6 in the activation of Rac1 (Cotton, Boulay et al. 2007; Lewis-Saravalli, Campbell et al. 2013). Thus, p66Shc may mediate Rac1-dependent migration via the activation of ARF6. ARF6 has also been implicated in the production of ROS. Indeed, ARF6 is required for vascular endothelial growth factor (VEGF)-induced Rac1

activation and ROS production in endothelial cells (Ikeda, Ushio-Fukai et al. 2005). Moreover, the migration of MDA-MB-231 cells has been shown to be dependent on ROS production. In fact, the inhibition of ROS has been shown to block the migration of these cells (Liu, Cui et al. 2014). Thus, p66Shc may promote MDA-MB-231 cell migration by enhancing ROS production. Finally, p66Shc may induce cellular migration via the activation of the MAPK pathway either via Rac1 (Bhat, Baba et al. 2014), as previously demonstrated, or through ARF6 activation, shown in this thesis. The importance of p66Shc-induced Erk1/2 activity is highlighted by a decreased migration of p66Shc overexpressing cells when treated with a MEK inhibitor (Natalicchio, Laviola et al. 2004). Together, these observations implicate p66Shc as an important mediator of migration.

The second function of p66Shc described in the literature is its role in mediating oxidative stress and apoptosis. This adaptor, when serine phosphorylated within its CH2 domain, can translocate to the mitochondria. Here, in combination with an apoptotic signal, it interacts with cytochrome C which allows for the reduction of oxygen and the production of mitochondrial ROS. This ROS production opens pores within the outer mitochondrial membrane leading to the release of pro-apoptotic factors and the induction of apoptosis (Orsini, Migliaccio et al. 2004; Giorgio, Migliaccio et al. 2005). Interestingly, the depletion of ARF1 enhanced gefitinib-induced activation of JNK, a serine kinase previously demonstrated to phosphorylate p66Shc and promote its translocation into the mitochondria (Orsini, Migliaccio et al. 2004; Giorgio, Migliaccio et al. 2005; Smith, Norton et al. 2005). Furthermore, as shown in Chapter IV, the depletion of ARF1 enhanced the translocation of p66Shc into the mitochondrial where it regulated the mitochondrial membrane potential and the release of the pro-apoptotic factor, cytochrome C. This would suggest that p66Shc may mediate the sensitivity of MDA-MB-231 cells to gefitinib-treatment. Indeed, depletion of p66Shc significantly reduced cellular death in response to EGFR inhibition (See Chapter IV).

Together these findings would suggest that p66Shc plays an important role in the regulation of both cell migration and survival in invasive breast cancer cells. They also suggest that these roles are mediated by ARF6 and ARF1, respectively. With p66Shc-mediated ARF6 activation promoting cell migration and ARF1 blocking the apoptotic properties of p66Shc to promote cell survival.

V.3 ARF1 mediates sensitivity and resistance of breast cancer cells to EGFR inhibition

Mechanisms of resistance regulating EGFR inhibitors have been well documented in lung cancer. Amplification of other RTKs such as cMET, AXL, ERBB2 and ERBB3 have all been shown to decrease the sensitivity of lung cancer cells to EGFR inhibition (Chong and Janne 2013). In fact, gefitinib treatment promotes both cMET and AXL activation leading to increased AKT phosphorylation and decreased drug cytotoxicity (Rho, Choi et al. 2014). Furthermore, cMET has been shown to associate with both inactive EGFR and HER3 leading to the activation of the PI3K/AKT pathway (Engelman, Zejnullahu et al. 2007; Gusenbauer, Vlaicu et al. 2013; Meyer, Miller et al. 2013). However, many of these mechanisms aren't present in breast cancer patients.

While gefitinib has been shown to increase the activity of cMET in MDA-MB-231 cells, co-treatment of cells with gefitinib and a cMET inhibitor did not significantly enhance cellular death (Sohn, Liu et al. 2014). Additionally, EGFR inhibition was observed to enhance PI3K signals in TNBCs. Yet, inhibition of the PI3K/AKT pathway had no effect on the cytotoxic properties of gefitinib (Yi, Hong et al. 2013). These findings suggest that other mechanisms may be regulating the sensitivity of breast cancer cells to EGFR inhibition. Here, we show that the small GTPase ARF1 is recruited to AXL, cMET and HER2 upon gefitinib treatment. However, the inhibition of two of these receptors (cMET and HER2) did not mediate the gefitinib-dependent activation of ARF1. Conversely, inhibition of the other RTK, AXL, significantly impaired the activity of this GTPase suggesting that gefitinib promotes the activity of ARF1 through this receptor. Additionally, we show that either the depletion or the pharmacological inhibition of ARF1 significantly enhanced the sensitivity of invasive breast cancer cells to gefitinib treatment. Moreover, the overexpression of ARF1 in MCF7 cells reduced gefitinib sensitivity. ARF1 depletion was also shown to decrease signals downstream of these receptors involved in gefitinib sensitivity such as ERK1/2 and AKT. This would suggest that ARF1 activity may govern the sensitivity of TNBCs to EGFR inhibition and that targeting this GTPase could improve the efficacy of these inhibitors.

Recently, another RTK, AXL, was shown to play a central role in mediating sensitivity of TNBCs to EGFR inhibitors. It was demonstrated that AXL can bind and transactivate the EGFR as well as other RTKs such as cMET and PDGFR. Additionally, the depletion of AXL significantly reduced EGF-dependent signals to ERK1/2, Src and AKT activation (Meyer, Miller et al. 2013). Together,

these results suggest that AXL may regulate the sensitivity of cells to EGFR inhibition by regulating the activity of the EGFR and its downstream signaling effectors. Interestingly, we observed an enhanced AXL activation upon gefitinib treatment in our cellular system. Additionally, gefitinib promoted the recruitment of ARF1 to AXL and that the activity of AXL was essential in the gefitinib-dependent activation of this GTPase. Together, we demonstrate that AXL may play an important role in the regulation of gefitinib sensitivity by mediating the activity of ARF1 in MDA-MB-231 cells.

We also demonstrated that ARF1 depletion, as well as pharmacological inhibition, promoted the gefitinib-dependent down-regulation of the EGFR and HER2 as measured by a decreased protein expression, activation and heterodimerization. Interestingly, defects in EGFR internalization and degradation have been linked to EGFRTKi resistance. In effect, gefitinib has been shown to impair EGFR endocytosis and delay the trafficking of this receptor to the lysosomes, as well as trap RTKs in early endosomes (Nishimura, Bereczky et al. 2007; Nishimura, Yoshioka et al. 2008). Moreover, resistant cells have shown to have a steady-state EGFR expression when treated with gefitinib compared to the internalized and degraded receptor observed in non-resistant cells. This stable EGFR expression is associated with an increased heterodimerization with either HER2 or HER3 and drug resistance (Wheeler, Huang et al. 2008). We show that ARF1-depletion decreased the expression and dimerization of the EGFR family members. Thus, suggesting that ARF1 may promote resistance by stabilizing the EGFR. Indeed, inhibition of the proteasome partially restored EGFR expression suggesting that ARF1 mediates the degradation of EGFR. However, transcriptional regulation EGFR expression by ARF1 cannot be ruled out. The importance of ARF proteins in mediating receptor signaling is further highlighted by role of ARFGAPs in mediating the endocytosis, trafficking and degradation of RTKs, as well as ARF6-dependent endocytosis of GPCRs (Claing, Chen et al. 2001; Kon, Kobayashi et al. 2014). Additionally, the ARFGEFs, cytohesins, have been shown to promote EGFR dimerization (Bill, Schmitz et al. 2010). Therefore, we hypothesize that ARF1, like ARF6, ARFGAPs and ARFGEFs, may play an important role in the membrane dynamics of the EGFR and thus mediates gefitinib sensitivity and resistance by maintaining EGFR signals at the plasma membrane.

Finally, our work highlights a role for ARF1 in the propagation of survival signals and an inhibition of apoptotic signals in gefitinib-resistant breast cancer cells. Indeed, the knockdown of ARF1 was

associated with a decreased gefitinib-dependent activation of ERK1/2, AKT and Src and increased signaling via the JNK and p38MAPK pathways. Up regulation of ERK1/2, PI3K/AKT and Src signals have all been implicated in promoting resistance and sensitivity to EGFR inhibition (McCubrey, Steelman et al. 2007; Engelman and Settleman 2008; Wheeler, Iida et al. 2009). Moreover, the pharmacological inhibition of these three signaling cascades have been associated with improved therapeutic outcomes in EGFRTKi resistant cancers (Wheeler, Iida et al. 2009; Brand, Iida et al. 2011; Chong and Janne 2013). Meanwhile, the cytotoxic properties of gefitinib have been linked to increased signaling via both p38MAPK and JNK (Kim, Park et al. 2009; Ko, Chiu et al. 2013). Thus, increased signaling through these pathways in ARF1-depleted cells treated with gefitinib is suggestive of increased cellular death. Indeed, increased expression of mitochondrial apoptotic markers (Bim, p66Shc), cytoplasmic-released CytC, mitochondrial membrane hyperpolarization and caspase cleavage was observed in ARF1-depleted cells upon gefitinib treatment.

Together, our findings demonstrate that ARF1 has a dimensional regulation of EGFRTKi sensitivity by: 1- Propagation of signals downstream of alternate RTKs such as AXL, 2- EGFR stabilization, activation and dimerization and 3- Activation of survival signals while inhibiting apoptotic signals.

V.4 ARF1 and oncogene addiction and shock

Weinstein proposed that cancer cells can become “addicted” to an oncogene. In other words, a cancer cell becomes physiologically dependent on the continued activity and/or expression of an oncogene for the maintenance of their malignant phenotype (Weinstein 2002). A good example of oncogene addiction is described for the myc oncogene in hematopoietic cells or HRas in melanoma. Indeed, switching off these genes results in both growth arrest and apoptosis (Chin, Tam et al. 1999; Felsher and Bishop 1999). This would suggest that identifying and targeting a cancer’s addiction could have substantial therapeutic benefits (Weinstein 2002). This can be seen in cancer cells with mutations in the *EGFR* gene that cause increased expression or activity. These mutations enhance tumor growth but also sensitize cancer cells to EGFR inhibition (Gazdar, Shigematsu et al. 2004). As we show that ARF1 modulates both the response of breast cancer cells to EGFR inhibition as well as the growth and survival of these cells, we propose that triple negative

breast cancer cells may be “addicted” to the GTPase ARF1. Furthermore, the mediation EGFR dynamic by ARF1 could promote EGFR addiction in breast cancer cells. Therefore, making ARF1 an important therapeutic target in these cancer patients.

Another hypothesis, the oncogenic shock hypothesis, states that the same oncogene can promote proliferation and survival at the same time as paradoxically activating signals leading the induction of apoptosis. Furthermore, it is the length of activation in response to an oncogenic inhibitor that governs the cellular response. In other words, proliferative/survival signals upon oncogenic inhibition are rather short, whereas, the apoptotic signals in response to this inhibition are longer-lived. Therefore, the cellular response would be the induction of apoptosis. This can explain the development of resistance to EGFR inhibition. In this case the rapid inhibition of the EGFR receptor would only affect the proliferative/survival signals while allowing the apoptotic signals to prevail. However, the ability of EGFR signals to rapidly reset and re-promote survival signals, observed in resistant cells, would counter the apoptotic signals, allow the cells to survive and result in the development of drug resistance (Pagliarini, Shao et al. 2015). We observe this process in our cellular model. Indeed, gefitinib treatment of MDA-MB-231 cells was associated with a rapid inhibition of AKT. Meanwhile, apoptotic signals through p38MAPK and JNK were unaffected or moderately elevated. Interestingly, this “oncogenic shock” was shown to be enhanced in cells depleted of ARF1 compared to control conditions. This was observed by a shorter-lived AKT activation and an increased and prolonged p38MAPK and JNK activation. This is marked by the attenuation of survival signals (ERK1/2, AKT, Src) without the inhibition of apoptosis (p38MAPK, JNK) leading to cell death.

Together, our results would suggest that triple negative breast cancer cells are addicted to the expression and activity of ARF1 and that ARF1 may mediate the sensitivity of these cells to EGFR inhibition by favoring this receptor’s signals to proliferative and survival over its apoptotic signals. Moreover, therapeutically targeting this GTPase would block cell proliferation and survival as well as enhance apoptotic signaling.

V.5 A role for mitochondrial ARF1 in regulating gefitinib sensitivity

The small GTPase, ARF1, plays an essential role in membrane trafficking via the recruitment of coat proteins and the modulation of lipid-modifying enzymes (Godi, Santone et al. 1998; Jones, Morris et al. 2000; Bonifacino and Glick 2004). It is best characterized for its role within the Golgi (Bonifacino and Glick 2004). However, we have recently demonstrated that ARF1 also plays an important role at the plasma membrane (Boulay, Cotton et al. 2008). Thus, ARF1 is essential in the modulation signals originating from membrane structures. We hypothesized that ARF1 may also play a role at the mitochondrial membrane. Actually, the yeast (*saccharomyces cervisiae*) ARFGAP, Gcs1p, and ARFGEF, GBF1, have been shown to localize to the mitochondria and play a role in the maintenance of mitochondrial morphology, dynamics and homeostasis (Huang, Chen et al. 2002; Ackema, Hench et al. 2014). More recently, both yeast and *Caenorhaditis elegan* ARF1 were shown to be present in the mitochondria and to regulate mitochondrial homeostasis. Additionally, the knockdown of GBF1 in these model system was associated with mitochondrial abnormalities. The mitochondrial importance of ARF1 was confirmed in mammalian cell systems (Ackema, Hench et al. 2014). We show that ARF1 can localize to mitochondria, as measured by ARF1 expression within mitochondrial extracts isolated from MDA-MB-231 cells (See Chapter IV), upon gefitinib treatment. As the cytotoxic properties of gefitinib are mediated by mitochondrial-dependent apoptosis (Wu, Min et al. 2011) and ARF1 significantly reduced the sensitivity of breast cancer cells to gefitinib, ARF1 may promote resistance to EGFR inhibition by acting within the mitochondria or at the mitochondrial membrane. However, the role of mitochondrial ARF1 within our cellular model of EGFRTKi resistance needs to be further investigated.

ARF1 has also been shown to regulate mitochondrial functionality through its actions on the ER-mitochondria encounter structure complex (ERMES) which connects the endoplasmic reticulum to the mitochondria (Kornmann, Osman et al. 2011; Ackema, Hench et al. 2014). It consists of 4 components: 1- outer mitochondrial membrane proteins Mdm10 and 2- Mdm34, 3- endoplasmic reticulum membrane protein, Mmm1 and 4- the cytoplasmic protein Mdm12. This complex mediates multiple mitochondrial functions such as mitochondrial motility, genome replication, mitochondrial protein import, calcium transport and phospholipid homeostasis. Morphological defects are observed upon mutation or depletion of ERMES members (Kornmann and Walter

2010). Loss of ARF1 has been shown to disrupt the ERMES complex, impairing lipid transport between the mitochondria and endoplasmic reticulum leading to mitochondrial fragmentation and degradation (Ackema, Hench et al. 2014). Thus, ARF1 may counter the cytotoxic effects of gefitinib by stabilizing this ERMES complex and promoting mitochondrial homeostasis.

V.6 ARF1 promotes mitochondrial-dependent apoptosis

EGFR inhibition is associated with both mitochondrial-independent and –dependent apoptosis (Hofer and Frei 2007). Increased ROS production, p38MAPK/JNK activation, Bax activation and mitochondrial localization, increased caspase activity and decreased signals through ERK1/2 and AKT have all been linked to EGFRTKi-dependent cell death (Janmaat, Kruyt et al. 2003; Ariyama, Qin et al. 2006; Qian, Li et al. 2009; Palanivel, Kanimozhi et al. 2014). Interestingly, we show that the depletion of ARF1 enhanced that majority of these apoptotic characteristics associated with EGFRTKi mediated apoptosis. Indeed, we observed a decreased signaling via the ERK1/2 and AKT pathways, increased p38MAPK/JNK activation and elevated Bax to Bcl2 ratio, indicative of apoptosis. Furthermore, as discussed above in detail, ARF1 depletion may also enhance the pro-apoptotic activity of p66Shc (Chapter IV).

The pharmacological inhibition of ARF1 activity has been demonstrated to induce the apoptosis of both corneal and cancer cells (Dai, Liu et al. 2012; Ohashi, Iijima et al. 2012). Moreover, the overexpression of an inactive form of ARF1, ARF1TN, blocked cadmium-induced proximal tubule cell death (Wolff, Lee et al. 2011). While we have demonstrated that ARF1 promotes the activation of the cell survival, PI3K/AKT, pathway in breast cancer cells (Boulay, Cotton et al. 2008), the role of this GTPase in mediating apoptosis has yet to be characterized. Meanwhile, another ARF isoform, ARF4, has been shown to promote glioblastoma cell survival by blocking the activation of pro-apoptotic signals via the p38MAPK and JNK pathways. Additionally, ARF4 overexpression was shown to decrease the mitochondrial translocation of Bax1, the cytoplasmic release of CytC and the activation of caspase 3 (Woo, Eun et al. 2009). Interestingly, we found that ARF1 depletion was associated with an enhanced gefitinib-induced CytC release, mitochondrial membrane hyperpolarization and a decrease mitochondrial expression of Bcl2 (Chapter III & IV). Thus, suggesting that both ARF1 and ARF4 may play important roles in the regulation of mitochondrial functions leading to the induction of apoptosis.

V.7 Conclusion

In this study, we demonstrated that the adaptor proteins Grb2 and p66Shc are important mediators of ARF activation downstream of the EGFR in invasive breast cancer cells. Grb2 was shown to be essential for the activation and EGFR recruitment of both ARF1 and ARF6. Meanwhile, p66Shc promoted the activation of ARF6 while blocking EGF-dependent ARF1 activation. We showed that p66Shc mediated ARF1 activation by blocking the recruitment of the Grb2-ARF1 complex to the EGFR. Additionally, we highlight a role for ARF1 in mediating the EGFR inhibitor resistance and sensitivity in breast cancer cells. We show that ARF1 maintains EGFR expression, activation and dimerization in cells treated with gefitinib. Furthermore, ARF1 promoted gefitinib-induced proliferative/survival signals while inhibiting pro-apoptotic signals. Finally, we determined that ARF1 regulates mitochondrial-dependent apoptosis in response to EGFR inhibition. Briefly, depletion of ARF1 enhanced gefitinib-induced p66Shc mitochondrial translocation, mitochondrial membrane hyperpolarization and CytC release.

These results demonstrate the importance of adaptor proteins in mediating EGF-dependent ARF activity. The characterization of the signaling mechanisms leading to breast cancer cell proliferation, migration, and invasion, such as ARF activation, can allow for the discovery of novel breast cancer therapeutics and improve current therapies. Additionally, we illustrate that ARF1 is important mediator of EGFRTKi sensitivity and resistance in breast cancer cells. This would suggest that targeting ARF1 could improve the therapeutic response of breast cancer patients to EGFR inhibitors. Furthermore, ARF1 activity could be utilized as an indicator of patient response to EGFR inhibition. Together, our finding highlight ARF1 as an important regulator of breast cancer development, progression and therapeutic response.

CHAPTER VI: Future perspectives

VI.1 Characterize the inhibitory role of p66Shc on ARF1 activation

In Chapter II, we highlight the role of p66Shc in the negative regulation of ARF1 receptor recruitment and activation. We propose that p66Shc blocks ARF1 activation by interfering with the Grb2-dependent recruitment of ARF1 to the EGFR. However, it would be of interest to further characterize this mechanism. First, I propose to identify whether p66Shc interacts directly with ARF1 and where on p66Shc ARF1 associates. I would hypothesize that this interaction would be dependent on the CH2 domain of p66Shc since both p52Shc and p46Shc were shown not to associate with ARF1. Secondly, I propose to identify the regions of p66Shc that are essential in the regulation of ARF1 activity, If p66Shc blocks ARF1 activation through its regulation of Grb2, then mutating the tyrosine residues within the CH1 domain of p66Shc required for its association with Grb2 should reverse its negative regulation of ARF1 activity. Finally, we show that ARF1 mediates the mitochondrial translocation of p66Shc. Thus, it would be interesting to determine the mechanisms through which ARF1 mediates the functions of p66Shc. Moreover, we could determine whether mitochondrial p66Shc mediates ARF1 activity within this organelle. This thorough characterization of the negative regulation of ARF1 activity could lead to the development of therapeutics the effectively and specifically inhibit the oncogenic properties of ARF1 in breast cancer, while sparing the essential functions of ARF1 within the Golgi of non-cancerous cells.

VI.2 Identify the GEFs and GAPs involved in p66Shc-mediated ARF activation

ARF activation is primarily mediated by guanine exchange factors (GEFs) and GTPase activating proteins (GAPs). While GEP100 has been shown to be required for EGF-dependent activation of ARF6 (Sabe, Hashimoto et al. 2009), the GEF responsible for ARF1 activation downstream of the EGFR has yet to be identified. As Grb2 has been characterized for its role in recruitment of the GEF SOS to the EGFR leading to Ras activation (van der Geer, Hunter et al. 1994; Kairouz and Daly 2000), it would be interesting to determine whether Grb2 plays a similar role leading to the activation of ARF1. Moreover, p66Shc may block ARF1 activation by either blocking the recruitment of a GEF or promoting the recruitment of a GAP. Thus, understanding the important

GEFs and GAPs involved in p66Shc-mediated ARF activation could identify novel therapeutics in TNBC patients.

VI.3 Determine the role of ARF1 in gefitinib-induced EGFR down-regulation

In Chapter III, we argue that the depletion of ARF1 enhances the sensitivity of breast cancer cells to gefitinib treatment by down-regulating the expression, activation and dimerization of the EGFR. However, mechanism through which ARF1 mediates EGFR dynamics is not clear. The two major means that ARF1 may mediate EGFR expression include: 1- transcriptional regulation and 2- receptor degradation. We show that proteosomal inhibition blocks the ability of gefitinib to down-regulate EGFR expression in ARF1 depleted cells. Additionally, preliminary data not presented in this thesis suggest that the depletion of ARF1 favors the rapid activation and internalization of the EGFR. It would be of interest to monitor the trafficking of the EGFR upon gefitinib treatment of ARF1-depleted cells. This would allow us to determine whether the rapidly internalized receptor is sent to lysosomes and degraded or recycled back to the membrane. It has been previously demonstrated that the degradation of the EGFR can sensitize resistant-lung cancer cells to EGFR inhibition (Nishimura, Bereczky et al. 2007; Nishimura, Yoshioka et al. 2008). Thus, this would help better demonstrate the importance of ARF1 in mediating drug resistance. We show that the treatment of MDA-MB-231 cells with a proteosomal inhibitor can partially restore EGFR expression. This would suggest that ARF1 could transcriptionally mediate EGFR expression as well as regulating EGFR degradation. Additionally, an altered localization of the EGFR, either to the nucleus or mitochondria, has been proposed as a mechanism of EGFRTKi resistance (Cao, Zhu et al. 2011; Brand, Iida et al. 2014). Together, these experiments would demonstrate that targeting ARF1 could not only block the oncogenic properties of ARF1, but also effect another important mediator of oncogenesis in TNBC, the EGFR.

VI.4 Characterize the role of ARF1 within the mitochondria

Recently, ARF1 has been shown in yeasts and *c elegans* to localize to the mitochondrial and mediate the transport between this organelle and the endoplasmic reticulum (Ackema, Hench et al. 2014). Interestingly, we show that in breast cancer cells treated with gefitinib ARF1 translocates to the mitochondria. However, we have yet to characterize the mitochondrial functions of this small GTPase. It would be interesting to determine whether ARF1 mediates the ERMES complex in breast cancer cells and what are the oncogenic properties of this complex as well as the interplay between the mitochondria and the endoplasmic reticulum. Furthermore, the mechanism through which ARF1 is transported into the mitochondria and what is regulating its mitochondrial activity also needs to be elucidated. Preliminary sequence homology analyses suggest that ARF1 may possess a mitochondrial targeting sequence. However, these results need to be further accessed. As we have shown that p66Shc mediates ARF1 activity downstream of the EGFR, we hypothesize that mitochondrial p66Shc could also mediate ARF1 activity in the mitochondria. Thus, p66Shc may mediate gefitinib-induced apoptosis by regulating the activity of mitochondrial ARF1. Finally and most importantly, I propose to characterize the importance of mitochondrial ARF1 in mediating resistance to EGFR inhibition in TNBC cells. We will determine whether it is specifically this mitochondrial pool of ARF1 that mediates resistance and design means of specifically inhibiting this small pool of ARF1 by targeting its mitochondrial regulators. This would allow us to improve the therapeutics efficacy of EGFRTKis in these patients by targeting ARF1 without the negative effects associated with targeting ARF1 within the Golgi.

VI.5 Identify and characterize novel ARF1 inhibitors

Even though there is growing evidence demonstrating the importance of ARF GTPases in mediating cancer development and progression, there are currently no clinical trials underway testing ARF inhibitors. The lack of interest in targeting ARF proteins stems from results obtained testing the ARFGEF inhibitor, BFA. This inhibitor has been associated with a poor solubility in biological fluids, undesirable pharmacokinetic profile and neurotoxicity in animal models. Additionally, BFA toxicity was shown to originate from its ability to dissemble the Golgi (Dinter and Berger 1998; Kikuchi, Shinpo et al. 2003). However, its apoptotic properties have been shown to be independent on its actions within the Golgi (Lippincott-Schwartz, Glickman et al. 1991).

Using small inhibitor screens, we could identify novel ARF inhibitors with improved solubility and reduced toxicity. Our results, thus far, would suggest that these inhibitors would inhibit breast cancer cell proliferation, migration and invasion while decreasing the resistance of these cells to EGFR inhibition.

ANNEX I. References

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