ROLE OF *DE NOVO* FATTY ACID SYNTHESIS IN INTRINSIC ACTIVATION OF EGFR IN CANCER

A Dissertation Presented to

the Faculty of the Department of Biology & Biochemistry

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

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August 2014

ROLE OF *DE NOVO* FATTY ACID SYNTHESIS IN INTRINSIC ACTIVATION OF EGFR IN CANCER

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DEDICATION

It is the upmost honor and privilege to dedicate my PhD dissertation work to my nephew, Manas Reddy Bollu, who died with acute lymphoblastic leukemia (ALL) in 2011 at the age of 15 months.

I will always remember the 18th of June in 2010. It was a beautiful summer morning; I was outside my lab, ready to start my day, when I received a call from my older brother, Srikanth. His voice was filled with joy as he shared with me that his wife, Aruna, had blessed our family with a baby boy. Being the first child born to our family, this boy child brought new life and light into our lives.

He was so precious to us; his smile that could brighten a room never seemed to disappear. I will forever cherish the moments I had with him via video chat on the weekends. We were ecstatic as he grew and learned new things each day, not knowing that a deadly disease was also growing within his tiny body. He had already started walking with support and calling me Babai, a Telugu word for Uncle (dad's brother), when our lives were flooded with darkness. Sudden hospitalization of Manas with ALL threw my entire family into state of shock. Manas battled ALL for six months, fighting a fight no child should ever have to fight. He left us physically in September, 2011.

We love you and miss you, Manas. You and your charming smile are always with us; we wish you could play with your new sister, Akshaya. You are the driving force and inspiration for my career in cancer research.

ACKNOWLEGEMENTS

I would like to express my sincere gratitude to my PhD advisor, Dr. Zhang Weihua for giving me the opportunity to work with his research team. He provided guidance, patience, wonderful and innovative ideas for my dissertation projects throughout my tenure and research assistantship. I would also like to acknowledge my committee members, Dr. Huang Peng, Dr. Seema Khurana, Dr. William Widger and Dr. Cecilia Williams for their help and suggestions to my projects. I also like to take this opportunity to acknowledge Department of Biology and Biochemistry for admitting me into the graduate school, teaching assistantship and providing imaging core facility and common research equipments (CNRCS). I also sincerely express my gratitude to our funding agencies, Department of Defense and American Cancer Society.

Very special thanks to Alicia Blessing from Dr. Daniel Frigo's group at University of Houston for her help with my experiments, preparation of manuscripts, editing my dissertation writing and encouragement. I would like to acknowledge Dr. Selvaraj Muthusamy and late Dr. Stefan Andersson for their help with thin layer chromatography experiment. I am also very thankful to my former lab mate, Dr. Jinrong Wang for teaching me several techniques in the lab and for his preliminary work that started my projects. I also sincerely thankful to my former and current lab members, Jiangong Ren, Jinyu Chen, Xu Lei, Xuefeng Wu, Rajasekhara Katreddy, Andy Pham, Nilu Fateh, Mai Huynh and Nora Gao. I would also acknowledge our collaborators, at MD Anderson Cancer Institute, Dr. Yuenfei Zhou and Dr. Lee Ellis.

I am also thankful to my friends, Prasenjith, Fotis, Ana Dey, Efi, Kim Anthony, Sarah Nelson, Geetha, Sarah Rapp, Gayani, Nick, Srijitha, Vrutant, Igor, Mohan,

Jayantha, Ashwin Juvva, Karthik Karra, Karthik Mannepalli, Pranathi Abburi, Prakash and Swetha Kodipaka.

My sincere gratitude to my parents Vanna Reddy Bollu and Bharathi Bollu and my family members, Srikanth, Madhav, Aruna and Akshaya for their support and constant encouragement.

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ABSTRACT

De novo fatty acid synthesis is one of the major upregulated metabolic pathways in cancer. *De novo* fatty acid synthesis pathway is crucially involved in cell proliferation, survival and drug resistance of cancer cells. This study focused on the role of fatty acid synthesis in intrinsic activation of EGFR and drug resistance in cancer. EGFR is a receptor tyrosine kinase expressed in most human cancers of epithelial origin and advanced cancers. Besides the plasma membranous (pmEGFR) and nuclear localization, EGFR can also exist in mitochondria (mtEGFR). Our studies revealed that EGFR exists in the inner mitochondrial membrane of prostate and breast cancer cells and promotes mitochondrial fusion through increasing the protein levels of PHB2 and OPA1. Interestingly, in this study, we found that activated pmEGFR activates mtEGFR through *de novo* synthesized palmitate, through palmitoylation, and promotes mitochondrial fusion. Along with mtEGFR, we also found that pmEGFR also undergo *de novo* fatty acid synthesis dependent palmitoylation which is important for EGFR localization, stability and signaling. We also observed intrinsic activation of EGFR in response to anticancer drugs such as SN38 and oxaliplatin dependent on *de novo* fatty acid synthesis. Finally, targeting *de novo* fatty acid synthesis or palmitoylation significantly increased the sensitivity of cancer cells to EGFR TKIs or anticancer drugs. In conclusion, through this study we've uncovered the importance of *de novo* fatty acid synthesis in intrinsic activation and non-classical functions of EGFR in cancer.

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Introduction

1.1 Cancer and altered metabolism

Cancer is a disease of abnormal cell growth. Eight hallmarks of cancer have been identified are uncontrolled cell proliferation, evading contact inhibition and tumor suppressors, invasion and metastasis, unlimited replicative potential, angiogenesis, resisting cell death, altered metabolism and evading immune destruction (Hanahan and Weinberg, 2000, 2011). Rapid cell growth and proliferation in cancer requires abundant building blocks such as nucleic acids, proteins and lipids. In cancer, the carbon flux is diverted from energy production to biosynthesis of building blocks of cell and signaling molecules. Several metabolic pathways are altered in cancer as compared to its normal counterpart to support the higher demand of nutrients by cancer cells (Ortega et al., 2009; Romero-Garcia et al., 2011). These pathways include up-regulations of aerobic glycolysis, known as Warburg effect, of *de novo* fatty acid synthesis, of glutamine metabolism, and the pentose phosphate pathway (Ameer et al., 2014; Flavin et al., 2011; Menendez and Lupu, 2007; Mounier et al., 2014; Warburg, 1928; WARBURG, 1956; Warburg et al., 1927). Over expression or activation of oncogenes transform normal cells to cancer by manipulating several metabolic pathways. HER2 induces *de novo* fatty acid synthesis in breast cancer and promotes cell proliferation and survival (Jin et al., 2010). Over expression of FASN activates EGFR and HER2 signaling (Vazquez-Martin et al., 2008). EGFR is overexpressed in most cancers of epithelial origin. EGFR can promote glucose consumption and the Warburg effect through upregulation of glycolytic enzymes

(Yang et al., 2012), however, EGFR tyrosine kinase inhibitors have not produced satisfactory clinical outcomes. A deeper understanding of the involvement of altered metabolic pathways in EGFR signaling is needed to improve EGFR targeted cancer therapy.

1.2 ErbB family of receptor tyrosine kinases

EGFR is a receptor tyrosine kinase (RTK) and belongs to the family of ErbB. Along with EGFR (ErbB1), ErbB family contains three more RTKs, ErbB2 (neu, HER2), ErbB3 (HER3) and ErbB4 (HER4). EGFR was the first RTK to be discovered (Todaro et al., 1976). Each member of ErbB member has three functional domains; extra cellular domain, trans membrane (a hydrophobic) domain and an intracellular domain (Roskoski, 2004; Yarden and Pines, 2012). Extra cellular domain contains ligand binding domain and the intra cellular domain possesses the tyrosine kinase domain. Intra cellular domains of ErbB members are highly conserved compared to their extra cellular domains. The less conservation in extracellular region provides the opportunity to have more ligands for the receptors. Hetero dimerization, more ligands and highly conserved kinase domains increases the versatile nature and activation of ErbB members. Extra cellular domains of ErbB members have four sub domains I-IV. Sub domains I (L1) and III (L2) have the affinity to the ligands. Binding of EGF to the sub domains I and III protrudes the sub domain II which promotes the dimerization of receptor. Crystal structure of HER2 revealed that even in the absence of ligand, HER2 has activated conformation with sub domain II protruded out as in ligand activated EGFR structure. Because of this reason

HER2 doesn't have a ligand and readily available for hetero dimerization with other ErbB members.

Among the four receptors, EGFR, HER2 and HER4 are kinase active ErbB members where as HER3 is a kinase inactive. ErbB members are activated by extra cellular ligands either synthesized by the same cell (autocrine) or by neighbor cells (paracrine). So far EGFR has ten known ligands that can activate EGFR and the classical ligand is epidermal growth factor, EGF. TGF alpha, amphiregulin, betacellulin, heparin binding growth factor, epiregulin, neregulin are some of the ligands for ErbB members (Harris et al., 2003). Even though HER2 is kinase active member and has functional kinase domain, the ligand binding domain of HER2 is not functional and no ligand for HER2 has been described. The activation of HER2 depends on other ErbB members through hetero dimerization. HER3 is a kinase inactive RTK and its ligand (Heregulin) cannot activate HER3 kinase activity in the absence of other ErbB members. Hetero dimerization with EGFR or HER2 is the known mechanism of HER3 activation.

1.2.1 Epidermal Growth Factor Receptor (EGFR)

EGFR, an oncogenic receptor tyrosine kinase, is over-expressed/overactive in a majority of tumors of epithelial origin including prostate cancer. Our understandings on EGFR have been primarily focused on its tyrosine kinase dependent functions in the plasma membrane, based on which therapeutic agents have been developed and used clinically. However, interfering with the tyrosine kinase activity of EGFR cancer treatment has not produced satisfactory outcomes. Besides the canonical mechanisms that mediate the tyrosine kinase dependent plasma membrane EGFR, studies have also revealed some

non-canonical mechanisms that mediate the functions of non-plasma membrane localized EGFR and the tyrosine kinase independent functions of EGFR. EGFR mediates the proliferative effects of its primary ligand EGF. Identification of its tyrosine kinase domain by DNA and protein sequencing (Ullrich et al., 1984) immediately drew the attention of researchers to study tyrosine kinase dependent functions of EGFR localized in the plasma membrane. The current comprehensive understanding on EGFR tyrosine functions lead to the development of small molecules that inhibit the tyrosine kinase activity for the treatment of cancer. In recent years, challenged by the appearance of resistance (Wheeler et al., 2010) to EGFR tyrosine kinase inhibitors (TKI) in cancer treatment, researchers have started exploring the non-canonical mechanisms that contribute to the oncogenic functions of EGFR, which include the functions mediated by non-plasma membrane localized EGFR and the tyrosine kinase independent functions of EGFR. In revealing the non-canonical mechanisms of EGFR function, novel targetable mechanisms are emerging, and overcoming the resistance of EGFR targeted cancer therapy becomes more promising.

1.2.2 The canonical EGFR signaling

Upon ligand binding, EGFR undergoes dimerization. Dimerization can be homo dimerization with another EGFR or hetero dimerization with HER2 or HER3 (Roskoski, 2004; Yarden and Pines, 2012). Even though EGF induces EGFR dimerization, EGFR dimerization has been described in the absence of ligand also (Gan et al., 2007). Dimerization of EGF receptors leads to the activation of tyrosine kinase domain. Studies focusing on the tyrosine kinase activity have provided great insights into the underlying

molecular mechanisms of EGFR function. Ligand-binding-induced dimerization or ligand-independent dimerization of EGFR activates the intrinsic tyrosine kinase activity that causes the mutual phosphorylation of the tyrosine residues of the dimerized partners. ErbB members are involved in cell proliferation, motility, survival and development. Dysregulation of receptor activity results in neoplastic transformation which is commonly observed in epithelial cancers (Arteaga, 2011; Arteaga and Engelman, 2014). The activity of receptor is regulated through dephosphorylation of tyrosine residues by Protein Tyrosine Phosphatases (PTPs) (Tiganis, 2002). The consequences of the phosphorylation of the tyrosine residues are the recruitment of adaptor/effector proteins to EGFR and subsequently phosphorylation of some of the adaptor/effector proteins by EGFR. The phosphorylated tyrosine residues serves as docking sites for adaptor/effector proteins that often contain *Src* homology-2 domains (SH2) or phosphotyrosine binding (PTB) domains, such as enzyme phospholipase C gamma (PLCγ), Grb2, Gab1 (Mattoon et al., 2004; Normanno et al., 2006; Roskoski, 2004; Yarden and Pines, 2012). Because many of the adaptor/effector proteins can interact with phospholipids, nucleic acids or possess intrinsic enzymatic activity, cascades of downstream signaling pathways can be triggered by the activation of the tyrosine kinase activity of EGFR. The downstream signaling pathways activated by EGFR-dependent tyrosine kinase activity include activations of RAS/RAF/MAP/ERK, PI3K/AKT, Src tyrosine kinase, PLCγ, PKC, STAT, hedgehog signaling, and NFκB(Klein and Levitzki, 2009; Sebastian et al., 2006). The overall impact of EGFR tyrosine kinase activation is enhancement of cell growth and survival, which explains why EGFR is often over-expressed/over-activated in tumors of epithelial origin.

EGFR was primarily found on the plasma membrane, where it is activated by its extracellular ligands such as EGF. Besides the plasma membrane, EGFR can also exist in the nucleus and in the mitochondrion.

1.2.3 EGFR in the Nucleus

Nuclear localized EGFR was first found in regenerating hepatocytes (Biswas and Iglehart, 2006). Thereafter, nuclear EGFR signal has been detected in many other types of cells, which include hepatocyte, placenta, keratinocytes, thyroid, and a variety of tumor cells, such as skin, breast, bladder, glioma, and oral cavity (Brand et al., 2011; Taguchi, 2014).

The mechanism by which EGFR translocates to the nucleus is not clear and is one of the main research focuses on nuclear EGFR. Accumulating evidence indicates that plasma membrane EGFR can be internalized and transported to the nucleus, which is supported by studies showing that disruption of EGFR's endocytosis blocks nuclear import of EGFR (De Angelis Campos et al., 2011; Lo et al., 2006). The nuclear translocation of EGFR involves a phosphoinositide kinase, PIKfyve (Kim et al., 2007), the nuclear localization sequences within EGFR, and retrograde translocation of EGFR from the endoplasmic reticulum to the Golgi apparatus (Wang et al., 2010). In the nucleus, together with other transcriptional factors, EGFR can regulate the transcription of several genes, which include cyclin D1(Lin et al., 2001), aurora kinase A (Hung et al., 2008), B-Myb (Hanada et al., 2006), iNOS (Lo et al., 2005), COX2 (Lo et al., 2010), Myc (Jaganathan et al., 2011) and BCRP (Huang et al., 2011). EGFR can also phosphorylate and activate PCNA and DNA-PK in the nucleus.

While much work is needed to fully understand the functions of nuclear EGFR, the existing data support that nuclear EGFR is pro-survival and proliferative in cancer cells. Identifying targetable mechanisms of nuclear EGFR functions bears a great potential to improve EGFR targeted cancer therapy.

1.2.3 EGFR in the Mitochondria

EGFR signaling is linked with the mitochondria by observations that EGFR activation regulates a mitochondrial encoded gene, cytochrome C oxidase subunit II (Cox II), induces reactive oxygen species, and interacts with COX II (Sun et al., 2002). EGFR has been found to be able to translocate to the mitochondria in a murine cell line of fibroblasts transfected with exogenous EGFR, and this translocation process is regulated by EGFR's ligand induced endocytosis and Src activity (Boerner et al., 2004). Data from independent studies support a role of Src in regulating EGFR's mitochondrial translocation (Demory et al., 2009), however, it is still being debated about the role of tyrosine kinase activity and endocytosis of EGFR in its mitochondrial translocation (Cvrljevic et al., 2011). Interestingly, inhibition of the tyrosine kinase activity of EGFR increases the mitochondrial EGFR content in cancer cells, supporting that the tyrosine kinase activity of EGFR is not required for its mitochondrial localization (Cao et al., 2011). More studies are needed to define the exact mechanisms by which EGFR translocates to the mitochondria. Involvement of mitochondrial EGFR in metabolism is indicated by a recent study showing that glucose deprivation induced EGFR's mitochondrial translocation and mitochondrial EGFR enhances oxidative phosphorylation (Cvrljevic et al., 2011). Given the critical role of metabolic alteration in

tumor pathogenesis and the role of mitochondrial EGFR in regulating cancer metabolism demands more research attentions.

1.3 EGFR expression in normal and disease prostate

1.3.1 In Normal Prostate

EGFR is exclusively expressed in the basal cells of fetal prostate and remains in the basal layer of neonatal prostate (Leav et al., 1998). In the normal adult prostate, EGFR is strictly localized in basal cells and in the lateral plasma membranes of secretary epithelial cells (Cohen et al., 1994; Leav et al., 1998). EGFR expression in basal cells echoes with the proliferative potential and stem cell features of this special population of cells in the prostate. Recently, it was found that basal cells from human prostate tissue could initiate prostate cancer with similar morphologies as cancer initiated by cancerous epithelial cells in immunodeficient mice (Goldstein et al., 2010; Lawson et al., 2010). The role of EGFR in the basal cells of prostate in the pathogenesis of prostate cancer warrants further investigation.

1.3.2. In Benign Prostatic Hyperplasia (BPH)

A number of studies on the expression of EGFR in BPH tissues have presented consistent observations showing that EGFR is up-regulated in the basal and stromal cells of BPH (Cohen et al., 1994; Ibrahim et al., 1993; Turkeri et al., 1994). However, interfering EGFR signaling in the management of BPH is yet to be tested.

1.3.3. In Prostate Cancer

Consistently, EGFR has been found to be up-regulated in prostate cancers (Cohen et al., 1994; Dorkin et al., 1997; Ibrahim et al., 1993; Leav et al., 1998; Ow et al., 1995; Scher et al., 1995; Sherwood and Lee, 1995; Sherwood et al., 1998; Visakorpi et al., 1992) and negatively correlates with prognosis. Up-regulation of EGFR is associated with the development of androgen-independence of prostate cancers and is highly correlated with metastases. Gene amplification (Cho et al., 2008) and loss of androgen signaling (Traish and Morgentaler, 2009) are involved in EGFR's up-regulation. A reciprocal negative regulatory relationship between androgen receptor signaling and EGFR signaling may exist. Expression of EGFR facilitates prostate cancer cells to develop androgen independence by inhibiting the transcription of androgen receptor (Cai et al., 2009) , while expression of androgen receptor down regulates EGFR via a protein degradation pathway (Pignon et al., 2009).

1.4 The Tyrosine Kinase Independent Functions of EGFR

EGFR is known to enhance both cell proliferation and cell survival. However, evidence suggests that the kinase activity of EGFR has a more pronounced effect on cell proliferation than on cell survival. Studies clearly demonstrate that EGFR contributes to cell proliferation predominately through its tyrosine kinase activity. For example, expression of a constitutively active mutant of EGFR (Tang et al., 2000) or activation of EGFR by its ligands leads to accelerated cell growth (Chen et al., 1994). At the same time, expression of a mutated, kinase-dead EGFR negates the ability of EGF to induce proliferation (Shushan et al., 2007) and treatment of cells with EGFR tyrosine kinase

inhibitors decreases cell growth (Coker et al., 1994). Expression of a kinase-dead EGFR results in EGF-induced DNA amplification (Goel et al., 2007; Rodeck et al., 1997) suggesting a possible EGFR kinase-independent role in cell proliferation.

Although EGFR contributes to cell survival, data supporting a role for EGFR tyrosine kinase activity in this cellular function remains inconclusive. Two types of cell survival exist: basal-survival and survival-under-stress. Basal-survival refers to cells maintaining a viable status under normal physiological conditions while survival-under-stress refers to the ability of cells to remain viable under harmful conditions, such as exposure to cytotoxic compounds, irradiation, hypoxia, etc. The molecular mechanisms underlying basic-survival and survival-under-stress may not necessarily coincide. Treating cells with EGFR tyrosine kinase inhibitors alone normally leads to cell growth arrest, not cell death (Harari and Huang, 2004; Jost et al., 2001; Pu et al., 2006), but co-treatment of cells with EGFR kinase inhibitors and cytotoxic stimuli, such as chemotherapeutic drugs, apoptotic inducers, and irradiation, results in enhanced cell death (Caraglia et al., 2003). Overexpression of wild type EGFR promotes cell survival under conditions of cytotoxic stress (Leu et al., 2000)(Burdak-Rothkamm et al., 2005; Feng et al., 2007; Morelli et al., 2005). Conversely, pre-treatment with EGFR tyrosine kinase inhibitors protects cells from chemotherapeutic drugs (Abal et al., 2003; Liu et al., 2003). These conflicting data could be due to the timing of EGFR kinase inhibition. The synergistic cytotoxic effect produced by co-treatment of cells with EGFR tyrosine kinase inhibitors and cytotoxic drugs might be explained by the instant suppression of cell survival pathways, such as PI3/Akt and MAPK pathways, by tyrosine kinase inhibition. On the other hand, pre-

treatment of cells with tyrosine kinase inhibitors results in cell growth arrest at the G1 phase of cell cycle, and arrest at G1 promotes resistance to certain chemotherapeutic drugs (Ewald et al., 2003). In another study, both the wild-type and kinase-dead EGFR showed a pro-survial role in EGFR negative 32D hematopoietic cells, suggesting a kinase independent pro-survival role of EGFR (Threadgill et al., 1995). The existence of kinase independent pro-survival functions of EGFR is also supported by mouse models of EGFR mutant, EGFR knockout animals die soon after birth (Luetteke et al., 1994), but animals with severely compromised EGFR kinase activity are completely viable and display only some epithelial defects (Weihua et al., 2008).

The molecular mechanisms mediating EGFR's kinase independent pro-survival function remain unclear. One mechanism is that EGFR interacts with and stabilizes the active sodium glucose co-transporter 1 (SGLT1), by which EGFR helps to maintain high rate of glucose uptake in cancer cells to prevent cells from autophagic cell death (Zhu et al., 2010). Another mechanism uncovered is that EGFR kinase-independently interacts/sequesters a proapoptotic protein, PUMA, to prevent cells from undergoing apoptosis (Blackledge et al., 2000; Dhupkar et al., 2010; Karashima et al., 2002; Kondapaka and Reddy, 1996; Prewett et al., 1996; Sirotnak, 2003; Sirotnak et al., 2002). While the kinase independent pro-survival function of EGFR is being unfolded, its implication in EGFR targeted therapy is tremendous, i.e. targeting the tyrosine kinase independent pro-survival functions of EGFR might overcome the resistance to EGFR tyrosine kinase inhibitors universally seen in the clinic.

1.5 EGFR targeted therapy

1.5.1 Small Molecules of RTK and Monoclonal Antibodies Against EGFR

There are two classes of EGFR inhibitors, one is small molecules of ATP analog, and another is monoclonal antibodies. The FDA-approved ATP analogs (also called receptor tyrosine kinase inhibitors, RTKi) include Erlotinib, Gefitinib, Vandetanib, and Lapatinib, which block the access of ATP to its binding pocket in EGFR; the FDA-approved monloclonal antibodies include Cetuximab and Panitumumab, which compete with EGFR's ligands for binding to EGFR. Data from *in vitro* experiments and in vivo pre-clinical experiments have provided supportive evidence that inhibition of the tyrosine kinase activity can reduce the growth and invasive ability of prostate cancer cells (Dhupkar et al., 2010), although prostate cancer cells exhibit resistance to the monoclonal antibody Cetuximab *in vitro* (Blackledge et al., 2000; Gross et al., 2007; Pezaro et al., 2009; Salzberg et al., 2007). Clinical application of these EGFR inhibitors has produced therapeutic responses between 10-20% in cancers such as lung and colon; however RTKi has failed to show therapeutic effects for prostate cancer patients. There are a few reports of clinical trials of Gefitinib showing negative therapeutic outcomes (Sridhar et al., 2010), and one clinical trial of Lapatinib also ended with negative patient responses (Scher et al., 1995). Prostate cancers seem to be unresponsive to EGFR inhibitors in the clinical trials.

1.5.2 EGFR Down-Regulators

Considering that EGFR is over-expressed in more that 85% of prostate cancers of advanced stages (Scher et al., 1995b; Hernes et al., 2004; Di Lorenzo et al., 2002;

Sherwood et al., 1998b) (Rubenstein et al., 2003, 2007), it is intriguing that blocking EGFR's activity fails to generate positive clinical responses. While the molecular mechanisms by which prostate cancers resist EGFR inhibitors need to be investigated, down-regulating EGFR protein levels in *in vitro* and pre-clinical models of prostate cancer has produced impressive anti-survival effects. Reduction of EGFR expression by antisense oligonucleotides (Tsui et al., 2004, 2005) (Tsui et al., 2005) or by siRNA are equally efficient in inducing cell death of prostate cancer cells (Wedel et al., 2011). Histone deacetylase (HDAC) inhibitors also can down-regulate EGFR expression and sensitize prostate cancer cells to other anti-cancer agents (Xu and Weihua, 2011). Down-regulating EGFR expression by siRNA but not inhibiting the tyrosine kinase activity of EGFR sensitizes prostate cancer cells to chemotherapeutic compounds (Gabrielson et al., 2001; Kuhajda, 2000; Pizer et al., 1998; Wang et al., 2001). Molecules that can decrease the expression levels of EGFR, EGFR down-regulators, might improve the EGFR targeted therapy for prostate cancer.

1.6 *De novo* Fatty Acid Synthesis

De novo fatty acid synthesis is a very complex and highly regulated pathway and it is one of the altered metabolic pathways commonly observed in human cancers (Ameer et al., 2014; Eissing et al., 2013; Koo, 2013; Lambert et al., 2014; Pal et al., 1988; Zadra et al., 2013). In normal tissues, the cells utilize *de novo* pathway to convert excess carbohydrates into triacylglycerols (TG) for storage. The TGs will be later used in beta oxidation to produce ATP in mitochondria. Even though *de novo* fatty acid synthesis pathway is highly regulated, deregulation of this pathway is observed in metabolic disordered diseases such as obesity, non-alcoholic fatty liver disease, retro viral infections and cancer (Dufort et al., 2014; Eissing et al., 2013; Flavin et al., 2011). In cancer cells, *de novo* fatty acid synthesis is considered nearly as a universal phenomenon. Fatty acids can be used as substrates for making phospholipids and triglycerides for membranes, acts as substrates for beta oxidation, as secondary messengers or anchorage for membranous proteins. There are two sources of fatty acids for cells, one is dietary fatty acids and the other one is *de novo* synthesized fatty acids. Normal cells and tissues utilize exogenous circulating fatty acids for making membranous lipids where as cancer cells excessively depend on *de novo* pathway even in the presence of excess exogenous fatty acids. In well nourished and healthy individuals, *de novo* fatty acid synthesis has minor importance because of the availability of fatty acids in the circulation. Normal tissues such as adipose tissues, liver and breast epithelial cells are actively involved in *de novo* pathway to convert excess blood glucose into fatty acids and store them as triacylglycerides. *De novo* fatty acid synthesis is functionally linked to glucose

metabolism as glucose feeds into the acetyl Co-A and citrate pools which will be used for fatty acid synthesis (Basaranoglu et al., 2013; Ha et al., 2013). The plasma glucose levels and glucose uptake directly associated with increased *de novo* fatty acid synthesis. Simple sugars, Fructose in particular, are more potent in stimulating *de novo* fatty acid synthesis compared to complex sugars (Moon et al., 2002). In normal cells, serum fatty acids regulate *de novo* fatty acid synthesis especially polyunsaturated fatty acids (PUFs) decrease fatty acid synthesis by reducing the expression of lipogenesis enzymes (Menendez and Lupu, 2007). *De novo* fatty acid synthesis is also very active during embryogenesis and associated with endometrial cell proliferation during menstrual cycle. In cancer, the cells shift towards *de novo* pathway and It was determined using 14C glucose labeling that >90% of total lipids in cancer cells derived from *de novo* pathway.

1.6.1 Enzymes in *de novo* fatty acid synthesis

Three major enzymes are involved in *de novo* fatty acid synthesis namely; ATP citrate lyase (ACLy), Acetyl Co-A carboxylase (ACC) and Fatty acid synthase (FASN) (Flavin et al., 2010; Zhou et al., 2013). ACLy is the first enzyme that converts the 6-carbon citrate molecule derived from mitochondria through glucose metabolism into Acetyl CoA (Roughan, 1997; Sumper, 1974). ACly can be activated by protein kinase B (Akt) through serine 454 phosphorylation in response to growth factors and DNA damaging chemo therapeutic drugs (Zhou et al., 2013). ACC, catalyzes the committed and rate limiting step in fatty acid biosynthesis, carboxylates acetyl CoA and synthesizes malonyl CoA. ACC is positively regulated by citrate and negatively regulated by fatty acyl-CoAs (Ha et al., 1994). Under nutrient deprivation conditions or under increased AMP/ATP levels, ACC is negatively regulated by AMP activated protein kinase (AMPK) through

phosphorylation (Menendez and Lupu, 2007). FASN, the most studied fatty acid synthesis enzyme relevant to cancer (Shi et al., 2013; Gregory et al., 2011; Hartmann et al., 2013; Karahashi et al., 2013), carries out the terminal step in the synthesis of 16-carbon saturated fatty acid, palmitate, by condensing acetyl CoA and malonyl CoA using NADPH as reducing equivalent and ATP as energy source. The 16 carbon palmitate can be further modified by elongases to increase the carbon chain length or by desaturases to incorporate double bonds in the carbon chain (Furuta et al., 2008; Sano et al., 2014). These fatty acids can be used to make phospholipids, triglycerides, sterols or they can be stored.

1.6.2 *De novo* fatty acid synthesis in cancer

Many human cancers and their precursor lesions exhibit increased *de novo* fatty acid synthesis independent of circulating fatty acids. Increased *de novo* lipogenesis is significantly associated with increased activity or expression of all three lipogenic enzymes in tumour cells (Flavin et al., 2011; Menendez and Lupu, 2007). Increased *de novo* fatty acid synthesis is associated with increased levels of FASN in cancer. Up regulation and hyperactivity of FASN in precursor lesions might be due to the exposure to the hypoxic, low nutrient and low pH conditions in the micro environment of pre invasive cancers (Chirala and Wakil, 2004; Weiss et al., 1986). Kuhajda et al (1994) initially identified the oncogenic antigen-509 (OA-519) as FASN associated with poor prognosis in breast cancer patients.

1.6.3 Fatty Acid Synthase (FASN)

FASN is a multifunctional cytoplasmic enzyme with a molecular weight of 270 kDa (Chirala and Wakil, 2004; Weiss et al., 1986). FASN is involved in the terminal step of 16-carbon palmitate synthesis. Up regulation of FASN, the key metabolic enzyme in fatty acid synthesis represents a nearly-universal phenotypic alteration in most human malignancies. Many human cancers have been reported for up regulated FASN expression such as prostate, breast, colon, lung, bladder, ovarian, stomach, endometrium, kidney, skin, pancreas, head and neck and tongue (Flavin et al., 2011; Menendez and Lupu, 2007). In addition, FASN expression is also up regulated in pre cancerous tissues (Kuhajda et al., 2000, 2006; Menendez and Lupu, 2006, 2007). FASN has been considered as a metabolic oncogene because of its role in altered lipid metabolism linked to glucose metabolism, cancer cell proliferation and survival.

FASN contains seven catalytic domains and each domain carries out a specific reaction in the biosynthesis of palmitate. The catalytic domains are β-ketoacyl synthase (KS), malonyl/acetyltransferase (MAT), dehydrogenase (DH), enoylreductase (ER), β-ketoacyl reductase (KR), acyl carrier protein (ACP) and thioesterase (TE) (Jin et al., 2010a) . Human fatty acid synthase is a complex of homodimeric (~550-kDa) enzyme that catalyzes the *de novo* biosynthesis of long-chain fatty acids. This cytosolic enzyme catalyzes the formation of 16 carbon (C16) palmitate, from acetyl-coenzyme A (acetyl-CoA) and malonyl-coenzyme A (malonyl-CoA) in the presence of NADPH. This entire reaction is composed of seven sequential reactions catalyzed by specific enzyme activity in FASN. Even though up regulation of FASN expression is commonly associated with increased lipogenesis, post translational modifications such as tyrosine phosphorylation

by HER2 (Kusakabe et al., 2000; Wagle et al., 1999) and EGFR or increased dimerization by growth factors such as EGF increased *de novo* fatty acid synthesis.

1.6.4 Regulation of FASN expression

FASN is primarily expressed in hormone-sensitive tissues such as liver and adipose where it is regulated by nutritional signals and hormones (Song et al., 2012). In a well-nourished individual, normal proliferating cells preferentially depend on circulating fatty acids when abundant in blood and reduce *de novo* fatty acid synthesis which requires lot of NADPH and ATP. Increased blood glucose levels after a meal and insulin induces *de novo* fatty acid synthesis in adipose tissue (Kusakabe et al., 2000) to remove excess blood glucose and convert them into fatty acids and store them as triglycerides. Serum response element binding proteins (SREBP) are the major transcriptional factors in regulating the transcription of FASN (Griffiths et al., 2013). SREBPs normally exists as membranous proteins as inactive precursor proteins. In response to reduced levels of fatty acids and sterols, the precursor SREBPs undergoes proteolytic cleavage and gets activated (Kim et al., 2002; Xiaoping and Fajun, 2012) (Chen et al., 2012; Shah et al., 2006; Yang et al., 2008). The activated SREBPs translocated to nucleus and induce FASN gene transcription (Ettinger et al., 2004). The expression of FASN in normal tissues is also regulated by hormones (insulin, thyroid hormone, estrogen, progesterone) (Kuhajda, 2000).

In cancer, in contrast to normal tissues, FASN expression is no longer under the regular control of nutritional signals or hormones (Baron et al., 2004) and is regulated at multiple steps including gene copy number (Shah et al., 2006), transcription and post translational level. Increased FASN levels were due to the increased gene copy number was reported

in prostate cancer cell lines PC-3 and LNCaP and metastatic cancer cells (Shah et al., 2006). Transcriptional regulation of FASN has been well studied in variety of cancers and is considered as the major contributor for increased FASN expression. The two major oncogenic pathways are involved in up regulation of FASN expression in cancer. They are the mitogen-activated protein kinase (MAPK) and PI3K/AKT pathways (Wang et al., 2005). Growth factors (EGF, neuregulin), steroid hormones (Androgens, estrogens) and their receptors increase the expression of FASN transcription through utilizing these pathways (MAPK or PI3K/Akt) in cancer cells (Li et al., 2014) (Gao et al., 2006; Menendez et al., 2004c) (Swinnen et al., 2002, 2004). FASN protein levels also regulated at post translational level. Increased ubiquitin protease (USP2a) in prostate cancer increases FASN stability by decreasing ubiquitination and proteosomal degradation (Kim et al., 2012; Tao et al., 2013) .

1.6.5 Significance of *de novo* fatty acid synthesis in cancer

Cancer is a disease of uncontrolled cell proliferation. Rapidly dividing cancer cells require a lot of fatty acids for membrane production. Increased *de novo* synthesis supplies the immediate demand of fatty acids for membrane production and lipid rafts creation (Flavin et al., 2010; 2000; Kuhajda, 2006; MEDES et al., 1953; Menendez and Lupu, 2006; Vazquez-Martin et al., 2008). Inhibition of fatty acid synthesis induces cell cycle arrest, decreased cell proliferation and apoptosis (Chen et al., 2012b; Shah et al., 2006; Yang et al., 2008). The cell cycle arrest could be due to the decreased synthesis of phospholipids which is needed for G1 and S phases of cell cycle. Decreased cell proliferation could be due to the unavailability of fatty acids for membrane production. Inhibition of FASN also results in accumulation of malonyl CoA. Accumulated malonyl

Co-A inhibits carnitine palmitoyl transferase and promotes apoptosis through ceramide mediated apoptotic pathway (Bandyopadhyay et al., 2006).

Lipid rafts are specialized membrane microdomains in plasma membranes. These microdomains are detergent resistant lipid aggregations. *De novo* fatty acid synthesis provides phospholipids for lipid rafts creation and inhibition of *de novo* pathway affects the assembly of lipid rafts and associated proteins such as caveolin1 (Coleman et al., 2009; Ravacci et al., 2013). FASN physically interact with caveolin1 at lipid rafts and provides lipids for lipid raft creation (Di Vizio et al., 2007, 2008; Witkiewicz et al., 2008). Lipid rafts are critical for the survival, proliferation and sensing extra cellular signals. The lipid microdomains harbor growth factor receptors such as EGFR and HER2 (Lambert et al., 2006; Pike et al., 2005;). These growth factor receptors interact with their growth factors and promote cell proliferation and survival. Lipid rafts also harbour sodoium dependent glucose transporters (SGLT1 and 2) (Lee et al., 2012) which promote glucose uptake into the cells. Weihua et al (2008) showed that EGFR interacts with SGLT1. Since both EGFR and SGLT1 localized in lipid rafts, it is possible that lipid rafts acts as platform for oncogenic activities for these membrane proteins in cancer. Insulin receptor (IR) also localized to the lipid rafts and disruption of lipid rafts affects the activation of IR (Sánchez-Wandelmer et al., 2009) and results in degradation of IR (Elena Gonzalez-Munoz, 2009).

It is common that cancer cells express elevated levels of fatty acid synthase (FASN) and epidermal growth factor receptor (EGFR). Accumulating evidence indicates that FASN activity is closely associated with the survival functions of receptor tyrosine kinases of

the EGFR family, which include EGFR/HER1, HER2, HER3, and HER4. Over expression of FASN activates EGFR and HER2 signaling in breast epithelial cells. Inhibition of FASN activity concomitantly suppresses EGFR and HER2.

If the increased *de novo* fatty acid synthesis is just to provide only membrane lipids, the pathway should be inhibited when excess amount of extracellular fatty acids are available like in well nourished individuals. In cancer irrespective of the levels of extra cellular lipids, the cells undergo *de novo* fatty acid synthesis suggesting that *de novo* fatty acid synthesis has roles beyond just providing lipids for membrane formation. Long chain fatty acids including palmitate act as ligands for transcription factors such as the peroxisome proliferator-activated receptors (PPAR) (Poulsen et al., 2012). A wide range of proteins undergo a reversible posttranslational modification by fatty acids called fatty acylation including palmitoylation and myristoylation. In humans, 23 palmitoyltransferases (PATs) have been reported for palmitoylation of proteins (Huang et al., 2011a; Ladygina et al., 2011; Resh, 2006). Palmitoylation increases the stability of proteins, regulates the cellular distribution of proteins and activity of proteins (Aicart-Ramos et al., 2011; Fukata and Fukata, 2010). Palmitoylation increases the activity of glucose transporter1 (Pouliot and Béliveau, 1995) and glutamate transporter (Alaluf et al., 1995; Thomas and Huganir, 2013). Palmitoylation activates Ras-GTPase by promoting plasma membranous localization (Aicart-Ramos et al., 2011; Eisenberg et al., 2013) . Elevated *de novo* palmitate synthesis is associated with activation of Wnt1 through palmitoylation resulting in stabilization and activation of beta-catenin and its downstream signaling pathway (Fiorentino et al., 2008). The fact that cancer cells rely on *de novo* synthesized fatty acids for survival suggests that the activity of FASN is critically

integrated into the basic survival mechanisms of cancer and blockade of this pathway through pharmacological inhibition or knocking down of FASN by siRNA induces apoptosis selectively in human cancer cells with minimal effect on non cancer cells.

In addition to the roles of FASN in cell proliferation and survival functions in cancer, FASN also plays very important role in drug resistance to anticancer drugs. Through proteomic approach, Liu et al in 2008 found that FASN was over expressed in Adriamycin resistant breast cancer cells and knocking down of FASN significantly increased the sensitivity of resistant cells to Adriamycin. In contrast to the drug mechanism described by Liu et al, Yang et al in 2011 found that FASN activity was upregulated (without increasing FASN levels) in gemcitabine resistant pancreatic cell line (Yang et al., 2011). The upregulated activity could be due to the post translational modifications. HER2 activates FASN through phosphorylating tyrosine residues in breast cancer cells (Jin et al., 2010b). In addition to the role of FASN in Adriamycin and gemicitabine resistance, FASN has been reported in involvement of drug resistance to anti-cancerous drugs, 5-FU (Vazquez-Martin et al., 2007), Paclitaxel (Meena et al., 2013), gamma irradiation therapy (Kao et al., 2013), receptor targeted therapies such as transtuzumab (for HER2) (Vazquez-Martin et al., 2007a) and TRAIL (Subbiah et al., 2012).

1.6.6 FASN Inhibitors

Considering the role and importance of FASN in cancer in cell proliferation, oncogene signaling and drug resistance, several inhibitors for FASN were developed as novel therapy for cancer treatment (Flavin et al., 2010; Olsen et al., 2010). FASN inhibitors that

have been in use at laboratory and clinical level are Cerulenin, C75, Orlistat, C93, Epigallocatechine-3-gallate (EGCG) and triclosan. All these inhibitors potently induce apoptosis in cancer cells with minimal effect on non-cancer cells. Cerulenin is the first known FASN inhibitor isolated from culture filtrates of fungus *Cephalosporum caerulens*. Cerulenin is a non-competitive irreversible inhibitor covalently binds to KS domain of FASN (Johansson et al., 2008). C75, an analog of cerulenin, was developed to increase the effects of cerulenin. C75 is a competitive irreversible inhibitor of FASN on KS domain, ER and TE domains. Orlistat is derivative (reduced form) of lipstatin and approved by FDA for the treatment of obesity. Orlistat acts as irreversible inhibitor of the TE domain of FASN. EGCG competitively inhibits KR domain of FASN whereas triclosan inhibits ER domain of FASN.

CHAPTER 2

Materials and Methods

2.1 Cell culture and Materials

Prostate cancer cell lines PC3, DU145, breast cancer cell lines MDA-MB-231, MCF-7, colon cancer cell lines HCT116 and HT29 and lung cancer cell lines A549 and CRL5908 cells were from American Type of Cell Culture. Cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5.5mM glucose, 1% penicillin/streptomycin mixture and 10% fetal bovine serum at 37 °C in a cell culture incubator with 5% CO2. Non-cancerous cell lines MCF10A, 3T3 and Human embryonic kidney cells (HEK 293T) were cultured in DMEM supplemented with 25 mM glucose. EGF, fatty acids and FASN inhibitor (Cerulenin, cat # C2389) were obtained from Sigma Aldrich (St. Louis, MO, USA). AEE788 (Cat# S1486) was purchased from Selleck Bio (Houston, TX, USA). For activity assay of fatty acid synthase, ^{14}C labeled 2-acetate and ^{14}C-palmitate were purchased from MP Biomedicals LLC (Solon, OH, USA). Antibodies for FASN (cat # sc-55580), EGFR (Cat # sc-03) beta actin (Cat # sc-1616), Na^+/K^+ ATPase (Cat # sc-21712), Monoamine oxidase (MAO) (Cat # sc-50333), calreticulin (Cat# sc-7431), Tubulin (Cat # sc-5286) and Glut1 (Cat # sc-7903) were purchased from Santa Cruz Biotechnology. EGFR monoclonal antibody, C225, was from EMD Millipore. Antibody against EGFR for immunostaining (Cat #4267), was obtained from Cell Signaling (Danvers, MA, USA). OPA 1 (Cat # NBP1-71656) antibody was from Novus Biologicals (CO, USA). PHB2 (Cat # AB10198) antibody was from Millipore. MTCO2 (Cat # Ab3298) was from Abcam (MA, USA). Anti-flag (M2) antibody was from Sigma

Aldrich. Anti-pEGFR (Y1137) (Cat # 44794G) antibody was from Invitrogen (CA, USA).

2.2 Plasmid constructions

Plasmids expressing wild type EGFR and kinase dead EGFR (R817M) were cloned by PCR and point mutagenesis and inserted into the pDNA3.1 vector. EGFR and PHB2 were amplified by PCR from cDNA isolated from HEK293T cell. The PCR products with a Flag tag sequence (DYKDDDDK) fused at their 3' were introduced into mammalian expression vector pRK5 (Clontech). Truncated mutations on EGFR were performed by two-rounds of overlapping PCR. EGFR 5' forward primer is TATCTCGAGATGCGACCCTCCGGGACGGC, common EGFR 3' reverse primer is ACTATCTAGATGCTCCAATAAATTCACTGC; the primer for L1 domain deletion (57-68Aa) is TGTTGCTGAGAAAGTCACTGCTATTGAACATCCTCTGGAG; the primer for deletion of fragment between the L domain and the furin-like cyctein rich (69-184AA) is TGGATCACACTTTTGGCAGCTGCCGACTATGTCCCGCCACTGGAT

GCT; the primer for deletion of the furin-like cyctein rich domain (185-337Aa) is TCACCAATACCTATTCCGTTCAGGTGGTTCTGGAAGTCCATC; the primer for deletion of the fragment between furin-like cyctein rich and the L2 domain (338-360 AA) is TCGCCAC-TGATGGAGGTGCAGTTACACACTTTGCGGCAAGGCCCT; the primer for deletion of the L2 domain (361-481AA) is GTTTTCTGACCGGAGGTCCCT

TTGAAGTGTTTAATATTCG; the primer for deletion of the fragment between the L2 domain and the furin–like cysteine rich region (482-496AA) is TGGCAGACCTGGCCT

GTGGCCTTGCAGCTGTTAAACAGTTTTTTCCAGTTTATTGTA; the primer for deletion of the furin-like cyctein rich region 2 (497-598 AA) is ACCAGGGTGTTGTTTTCTCCCATGACTTCACCTCTGTTGCTTATAAT; the primer for deletion of the fragment between the furin–like cysteine rich region 2 and the transmembrane domain (599-644 AA) is TGCGTCAAGACCTGCCCGGCAGGAATCC

CGTCCATCGCCACTGGGA; the primer for deletion of the transmembrane domain is AAGGCTGTCCAACGAATGGGCCTAAGCACATCGTTCGGAAGCGCAC; the primer for deletion of juxtamembrane domain is TGGCCCTGGGGATCGGCCTCTTCA

TGGAGAGGGAGCTTGTGGAGCCTCTT; the extracellular deletion (1-642 Aa) was directly amplified with a forward primer TATCTCGAGATCCCGTCCATCGCCACTGG

GA and reverse primer ACTATCTAGATGCTCCAATAAATTCACTGC; the intracellular domain deletion (684-1210 AA) was directly achieved using a forward primer TATCTCGA GATGCGACCCTCCGGGACGGC and a reverse primer ACTATCACTGCAGCAGCCTCCGCAGCGTGCGCTT. In order to specifically introduce EGFR into mitochondria, the DNA sequence of EGFR membrane signal peptide (1-24 AA) was replaced with the DNA sequence of mitochondria signal peptide, ACGCGTCGACATGTCCGTCCTGACGCCGCTGCTGCTGCGGGGCTTGACAGGT

CGGCCCGGCGGCTCCCAGTGCCGCGCGCCAAGATCCATTCGTTGCTGGAGGA

AA AGAAAGTT TGC. Specific cysteine mutations on mito EGFR constructs were created using stratagene Quickchange site directed mutagenesis kit purchased from Agilent technologies (Santa clara, CA).

2.3 Transfections

Plasmid transfections were performed on 60-70% confluent cells using lipofectmine 2000 reagent (Invitrogen) following manufacturer instructions. Briefly, plasmid DNA and lipofectamine reagent were mixed with Opti-MEM separately and incubated for 15 minutes room temperature. After 15 minutes of incubation, both DNA and lipofectamine reagents were mixed and incubated for 30 minutes. After 30 minutes, the mixed contents were added on to the cells and incubated for 4 hours. After 4 hours, the medium was removed and fresh full medium was added.

2.4 Protein sample preparation and western blotting

Protein samples were prepared by lysing cells in RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors on ice for 30 minutes. Lysed cell lysates were collected and centrifuged at 14000 rpm for 15 minutes at 4^{o}C. Concentration of protein samples were measured using Quibit (Invitrogen) according to the instructions given by the manufacturer. For SDS PAGE electrophoresis, the samples were prepared by adding equal amount of 2Xsample lamellae buffer with β-Mercaptoethanol. Before loading the samples on to the gel, the samples were boiled for 5 minutes at 100 ^{o}C. Equal amount of proteins were loaded on to the SDS PAGE and separated proteins by running 100V. The separated protein samples were transferred onto PVDF membrane (Biorad) at 90V for 90 minutes. Non-specific sites on transferred membrane were blocked by incubating with 5% milk in TBS buffer containing 0.5% Tween-20 (TBST) for 30 minutes to 1 hour. After blocking, primary antibodies were added at optimized concentrations and incubated for overnight at 4°C on a shaker. After primary antibody incubation, membrane was washed with TBST for 10 minutes for 3 times followed by incubation with secondary

antibodies conjugated with HRP diluted at 1:3000 in 5% milk for 1 hour at room temperature. Membrane was washed in 1XTBST 3 times followed by exposure to ECL solution for 1-2 minutes. Antibody complexes were visualized by exposing the membrane to X-ray film.

2.5 Immunoprecipitation

Equal amount (200ug) of protein samples were added to the eppendorf tube containing 25ul of protein A/G beads and 1ug of antibody and incubated at $4^{o}C$ for overnight on a shaker. After overnight incubation, the beads were washed with 600ul of RIPA buffer for three times. Immunoprecipitated proteins were eluted directly into lamellae buffer and boiled for 5 minutes and subjected to western blotting.

2.6 Detection of FASN dimers

Detection of FASN homodimer was performed according to a protocol published by others. Briefly, we treated PC3 cells with vehicle alone or EGF (10ng/ml) in the presence or absence of AEE788 (5uM) for 15 minutes and cells were lysed on ice in RIPA buffer followed by centrifugation to remove the cell debris. The cell lysates were mixed with 2x non-reducing sample buffer (lamellae sample buffer (Biorad) without dithiothreitol) at 1:1 ratio and loaded (without boiling) on to the 6% SDS PAGE. For control (Monomeric FASN), protein sample was prepared in reducing sample buffer (lamellae sample buffer with dithiothreitol) and boiled for five minutes. Western blotting was performed to identify the FASN dimers using FASN antibody.

2.7 Measurement of FASN phosphorylation

Serum-starved PC3 cells were treated with vehicle alone, EGF (20ng/ml) for different time intervals of 30 minutes in the presence or absence of AEE788 (5µM). FASN was immunoprecipitated with anti-FASN antibody. Tyrosine phosphorylation of FASN was determined using anti-phosphotyrosine antibody (Cat # 05-1050) (Millipore, MA) by Western blot analysis.

2.8 Mitochondrial extraction and *In vitro* phosphorylation assay

Mitochondria were isolated using ultra-centrifugation according the protocol used by others. To obtain pure mitochondrial pellets and separate from mitochondrial associated membranes, the mitochondrial pellets were centrifuged on 30% percoll medium at 94,000g for 30 minutes. Lower band was collected and further centrifuged at 3000 rpm to pellet pure mitochondria. Isolated mitochondria was then suspended in 20ul of kinase buffer (25mM HEPES at PH 7.5, 100mM NaCl, 5 mM $MnCl_2$, 0.5 mM Na_3VO_4, 5mM beta glycerophosphate and 20uM ATP) and treated with ethanol or 200uM palmitate for 15 minutes at 37°C. The reaction was stopped by adding 20 ul of lamellae sample buffer. The samples were subjected to Western blot analysis for total and phosphorylated EGFR. The inner membranes and outer membranes of mitochondria were separated according a protocol used by others.

2.9 Protease protection assay

Purified mitochondria were suspended in 60 ul of buffer A (50 mM HEPES-KOH pH 7.2, 0.75% bovine serum albumin, 0.5 M sorbitol, 80 mM KCl, 2.5 mM magnesium acetate, 1 mM potassium phosphate, 0.5 mM MnCl2). To prepare mitoplasts,

mitochondria were suspended in 60 ul of hypotonic (swelling) solution (20mM HEPES pH7.2, 0.3% BSA, 50mM sorbitol, 8mM KCl, 1mM magnesium acetate, 0.8mM potassium phosphate and 0.2 mM $MnCl_2$) and incubated on ice 10 minutes. 0.5 units of protease, Thrombin, was added to 25 ul of mitochondria either suspended in buffer A or swelling solution and incubated at 37 C for 15 minutes. Protease activity was stopped by adding 25 ul of reducing protein sample buffer and boiling for 5 minutes.

2.10 Acyl-Biotin exchange method for palmitoylation analysis of EGFR

The Acyl-Biotin exchange palmitoylation assay was carried out according the protocol used by others. Mitochondrial EGFR was immuno precipitated from purified mitochondrial protein samples using EGFR monoclonal antibody, C225. Acyl-Biotin exchange method was adopted to study the palmitoylation of EGFR as described elsewhere. Immuno precipitated EGFR was treated with 50mM NEM in RIPA buffer at 4^oC for 2 hours on a shaker. Excess NEM was removed by washing EGFR with RIPA buffer three times. EGFR was treated with hydroxylamine buffer (1M Hydroxylamine, 50mM tris, 150 mM Nacl, 5mM EDTA, 0.2% TX100, pH 7.4) at room temperature for 2 hrs on a shaker. For mock, EGFR was treated with hydroxylamine buffer without hydroxyl amine. EGFR was then treated with 4uM HPDP-biotin in 50mM tris, 150 mM Nacl, 5mM EDTA, 0.2% TX100, pH 6.2 for 2 hours followed by three washes to remove excess biotin. 60 ul of non reducing protein sample buffer was added and samples were boiled for two minutes to elute EGFR from beads. 20% of sample was loaded on to SDS PAGE gel. Membrane was blocked with 5% BSA for overnight followed by incubation with streptavidin conjugated with HRP at 1:30000 for 60 minutes at room temperature.

Biotin-streptavidin HRP complex was visualized by exposing the membrane to ECL and then to X-ray film.

2.11 Immunocytochemistry and Immunohistochemistry

For immunocytochemistry (ICC), cells , grown on cover slips, were fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature and permeabilized with 0.2% tritonX100 in PBST followed by 1 hour blocking with 2.5% normal donkey serum. The cells were incubated with optimized concentrations of primary antibodies in 2.5% NDS for 16hrs at 4°C. Cells were washed with PBST three times and incubated in dark with alexa fluor secondary antibodies (Invitrogen, CA) at RT for 1 hr followed by three washes with PBST. The cover slips were mounted on microscopic slides using ultracruz mounting medium (Santa Cruz Biotechnology, CA, USA) with DAPI. For immunofluorescent staining, formalin fixed, paraffin embedded human cancer tissue samples were used. Briefly, after the tissue sections were treated with xylene and ethanol to remove paraffin, tissues were blocked with 2.5% normal donkey serum for 1 hr followed by incubation with primary antibodies at optimized dilution for 16 hrs at 4°C. Tissues sections were then washed with PBS three times and incubated with AlexaFluor conjugated secondary antibodies (Invitrogen, CA) at 1:300 dilution for one hour at room temperature. The samples were again washed with PBS three times and mounted on to microscopic slides using mounting medium. The images were taken using a confocal microscope.

2.12 Mitochondrial imaging

For live-cell mitochondrial imaging, cells were grown in 10mm dishes with glass bottom. After treating the cells with respective agents, cells were incubated with Mitotracker red (Invitrogen, CA) at 1:6000 dilution for 5 minutes at 37°C. The cells were then washed with fresh medium and live mitochondrial images were taken by a confocal microscope for further analyses. Mitochondrial classification and quantification was performed according to the method used by the others. Cells with long tubular interconnecting mitochondria were counted as type I, cells with elongated mitochondria are counted as type II, cells with rod shaped mitochondria are counted as type III and cells with punctuated and round shaped mitochondria were counted as cells with fragmented mitochondria. We counted number of cells for each type of mitochondria in each group and calculated as percentage of cells of each type. For each group, we counted a total of 30-75 cells.

2.13 FASN activity assay

De novo synthesized palmitate was measured according a published protocol. Briefly, cells were cultured in serum free medium containing 1uCi/ml ^{14}C labeled acetate with/without addition of EGF (20ng/ml), AEE788 (5uM) or AEE788/EGF for 3 hours. Cells were washed and collected into PBS followed by total lipid extraction using Folsch reagent (chloroform and methanol mixture at 2:1). Samples were centrifuged and the lower phase of the samples was collected into new tubes and air dried. The dried lipid pellet was resuspended in 50ul of Folsch reagent. The radioactive lipid samples were then separated on thin layer chromatography. Hexane, diethyl ether, acetic acid and methanol

mixture (90:20:2:3) was used to develop the TLC chromatogram. The radiochromatogram was then read by an AR2000 TLC plate reader (BioScan technologies, Washington, DC, USA) to measure the *de novo* synthesized fatty acids. ^{14}C labeled palmitate was used as standard run in parallel to identify the peak of palmitate on TLC plate.

2.14 Adipo red assay

We performed adipored assay in 96-well plate following the instructions given by the manufacturer (Lonza Walkersville, Inc) with minor modifications. Briefly, after the treatments were performed the cell culture plate was removed from the incubator and allowed to cool to room temperature. The cell culture medium was removed and washed the cells with PBS. Working solution of adipored reagent was prepared by mixing 25 ul of adipored in 1ml of PBS. Then 200 ul of adipored working solution was added to each well of 96-well plate and incubated for 10-15 minutes at room temperature and absorbance was measured using fluorimeter (excitation/emission at 485/535).

2.15 MTS assay

Cell viability assay was performed using a MTS assay kit (Cat # G3582, Promega) following manufacturer instructions in 96 well plates.

2.16 Drug resistant cell lines

The human colorectal cancer cell lineHT29was obtained from the American Type Culture Collection (ATCC). Oxaliplatin-and 5-FU–resistant cell lines were developed in our laboratory as previously described (20, 21). SN38-resistant cell lines were developed by using a similar protocol. Briefly, parental HT29 cells were exposed to an initial SN38

dose of 1 nmol/L and cultured to a confluency of 80% for 3 passages (6 weeks). The cells that survived the initial SN38 treatment were then exposed to 5 nmol/L SN38 for 3 passages (8 weeks) and to 10 nmol/L for 3 more passages (8 weeks). Finally, the SN38 concentration was increased to the clinically relevant plasma drug concentration of 15 nmol/L for 3 passages (10 weeks). The surviving resistant cells were named HT29-SNR.

2.17 Flow cytometric analysis for cell death

Apoptotic cells and total dead cells (sub-G1 population) were quantified by Annexin V/propidium iodide (PI) staining and flow cytometry. Apoptosis was analyzed by an Annexin V assay kit (BD Biosciences Pharmingen) according to the manufacturer's instructions. For sub-G1 phase quantification, cells grown in 6-cm-diameter petri dishes were harvested with trypsin and washed in PBS. Cells were then resuspended in 10% ethanol for fixation. Next, 100 mL of a cell suspension (105 cells) was stained with PI at room temperature for at least 15 minutes in the dark. Cells were then analyzed in a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed by FlowJo software (Tree Star).

2.18 Lentivirus transduction

To stably overexpress ACLy in HT29 cells, lentivirus transduction particles were generated using 293 packaging cells transfected by plasmid constructs containing fulllength ACLy cDNA. ACLy cDNA was subcloned into the mammalian expression vector pCDNA3.1 (Invitrogen) from bacterial cloning vector pOTB6 (MG1813; ATCC) using EcoR1 and HindIII sites and subsequently cloned into a GFP-expressing vector for virus production. Virus particles and polybrene (10 uL/mL) were added to cells at 70% confluency in 6-well plates for 24 hours. After cell expansion for one passage, GFP-

positive cells were subjected to fluorescence-activated cell sorting (FACS) to obtain cells stably expressing ACLy.

2.19 RNA interference for ACLy knockdown

SmartPool siRNA oligonucleotides for ACLy and a negative control were purchased from Dharmacon. Four independently validated siRNAs were transfected into HT29-SNR cells together at a final concentration of 40 nmol/L by Lipofectamine (Invitrogen) according to the manufacturer's protocol. First, 120 pmol of siRNA and 16 mL of Lipofectamine were mixed in 500 mL of Opti-MEM medium (10 nmol/L final concentration for each individual siRNA in the SmartPool). After 20 minutes of incubation, the mixture was added to cells at 70% confluency plated in a 6-well plate (2mLfinal volume). Fresh medium was added to cells after 6 hours. Twenty-four hours after transfection, cells were either collected for protein harvest or continued for SN38 treatment for additional 48 hours before analysis for apoptosis markers by Western blotting.

2.20 Spectrophotometric assay for ACLy activity

ACLy activity was determined as described previously (24) in an assay mixture containing 100 mmol/L Tris-HCl (pH7.4), 10mmol/LMgCl2, 20mmol/L potassiumcitrate, 1mmol/L dithiothreitol, 10U of malic dehydrogenase, 300 mmol/L coenzyme A (CoA), 280 mmol/L NADH, and various amounts of purified ACLy (BPS Bioscience), and optimal conditions for measuring enzyme activities were identified. The reaction was initiated by adding 10 mmol/L ATP at 37^0C, andNADHoxidation, as evidenced by a decrease in absorbance at 340 nm, was monitored continuously for 10 minutes using a microplate reader. To measure the activity of GSK165, a small-molecule

inhibitor of ACLy (provided by GlaxoSmithKline under a material transfer agreement; 25), 400 mg of purified ACLy was used in a final volume of 100 ml. Controls not containing ACLy were used to account for nonspecific NADH oxidation.

2.21 Statistics

Student two-tailed *t*-test was used to compare the values (mean±SD) of triplicate control and experimental groups of three independent experiments. $P < 0.05$ is considered as significant difference.

CHAPTER 3

Project #1: Involvement of de novo synthesized palmitate and mitochondrial EGFR in EGF induced mitochondrial fusion of cancer cells.

3.1 Introduction to the problem

It is common that cancer cells express elevated levels of FASN and EGFR (Asturias et al., 2005). Homodimeric FASN catalyzes the *de novo* synthesis of fatty acids with palmitate as the predominant product (Menendez and Lupu, 2007). Tumor-associated *de novo* synthesized fatty acids provide tumor cells with growth and survival advantage independent of their roles as energy substrates (Westermann, 2010). EGFR is an oncogenic receptor tyrosine kinase involved in promoting tumorigenesis of a majority of tumors of epithelial origin. Besides the plasma membrane and nucleus, EGFR can also exists in the mitochondrion (mtEGFR) however signals that can activate the mtEGFR and the role of mtEGFR activation in survival of cancer cells remain to be investigated.

Besides performing metabolic reactions, mitochondria also undergo fission/fusion changes, namely the mitochondrial dynamics, which plays critical roles in regulating cell metabolism, survival, and proliferation (Lee et al., 2004), Fusion serves to unify the mitochondrial compartment, whereas fission generates morphologically and functionally distinct mitochondria. Mitochondrial fission often occurs early in the apoptotic event (Twig et al., 2008) and the autophagic process (Gomes and Scorrano, 2011). Fusion of mitochondria is associated with increased cell survival (Cerveny et al., 2007). The molecular mechanisms that govern the fission/fusion dynamics have been partially

illustrated. A number of dynamin-related GTPases, such as Drp1, Mfn1, Mfn2 and OPA1, play key roles in regulating mitochondrial dynamics. Translocation of Drp1 from cytosol to mitochondria promotes mitochondria fission, Mfn1 and Mfn2 are involved in the fusion of outer mitochondrial membrane, and OPA1 is needed for the fusion of inner mitochondrial membrane (Palorini et al., 2013). Signaling factors found to regulate the mitochondria dynamics include PKA (Hung et al., 2008), CaMKIα (Zunino et al., 2007), and activities of ubiquitin and SUMO ligases (Molina et al., 2009). Mitochondrial fusion could be increased by peroxisome proliferator-activated receptor-γ coactivator 1β (Park et al., 2011), which could be activated by intracellular fatty acids (Karbowski et al., 2004), suggesting signals initiated by fatty acids are involved in regulating the mitochondria dynamics.

The first part of my study focused on studying the role of EGFR in mitochondria and significance of *de novo* fatty acid synthesis in EGFR function in mitochondria. We found that EGFR exists in the mitochondria of cancer cells of prostate and breast. Activation of pmEGFR promotes mitochondrial fusion by increasing the mtEGFR's tyrosine kinase activity and increasing protein levels of PHB2 and OPA1. pmEGFR activation by EGF induces *de novo* palmitate synthesis; *de novo* synthesized palmitate activates mtEGFR via inducing palmitoylation of mtEGFR and the activated mtEGFR promotes mitochondrial fusion and cell survival via increasing the levels of mitochondrial prohibitin 2 (PHB2) and OPA1.

3.2 Specific Aims of Project #1

Our proteomics data from proteomics experiment aimed to identify proteins that interact with EGFR in kinase independently manner showed that EGFR interacted with FASN, a cytosolic protein, and Prohibitin 2, a mitochondrial inner membranous protein. Interaction with mitochondrial protein led us to focus on EGFR functions in mitochondria and its function in mitochondria of cancer cells. We also observed that FASN inhibitor blocked pmEGFR induced mitoEGFR activity which led us to study the role of *de novo* fatty acid synthesis in mitoEGFR activation. The present study characterizes mtEGFR in the mitochondria of cancer cells (prostate and breast) and reveals that mtEGFR can promote mitochondrial fusion through increasing the protein levels of fusion proteins, PHB2 and OPA1. The following three specific aims were addressed in the first project of my dissertation work.

1: The first aim was to characterize mitochondrial EGFR using two pairs of cancer tissues and cell lines of prostate and breast.

2: The second aim was to study the function of mitochondrial EGFR.

3: The third aim was focused to study the involvement of *de novo* fatty acid synthesis in regulation of mitochondrial EGFR kinase activity.

3.3 Results of Project #1

3.3.1 Proteomic study reveals that EGFR interacts with mitochondrial protein, PHB2, and FASN.

Weihua et al (2008) identified a novel mechanism for EGFR, independent of its kinase activity, in promoting cell survival through a sodium dependent glucose transporter, SGLT1. To study kinase independent functions of EGFR, we aimed to identify proteins that interact with EGFR independent of kinase activity through co-immunoprecipitation assay and mass spectrometry. HEK293 cells were transfected with either WT EGFR or KD EGFR. 24 hours post transfection, the cells (WT EGFR) were treated with EGF in the presence or absence of EGFR kinase inhibitor. Protein samples were subjected for immunoprecipitation with flag antibody followed by separation of immunoprecipitated proteins on 10% SDS PAGE gel and silver staining to identify EGFR interacting proteins. As shown in the figure 1, through mass spectrometry analysis, we identified that a mitochondrial protein, prohibitin 2 (PHB2), and FASN as EGFR interacting proteins. The interaction of EGFR with a mitochondrial protein led us to study EGFR characterization and function in mitochondria.

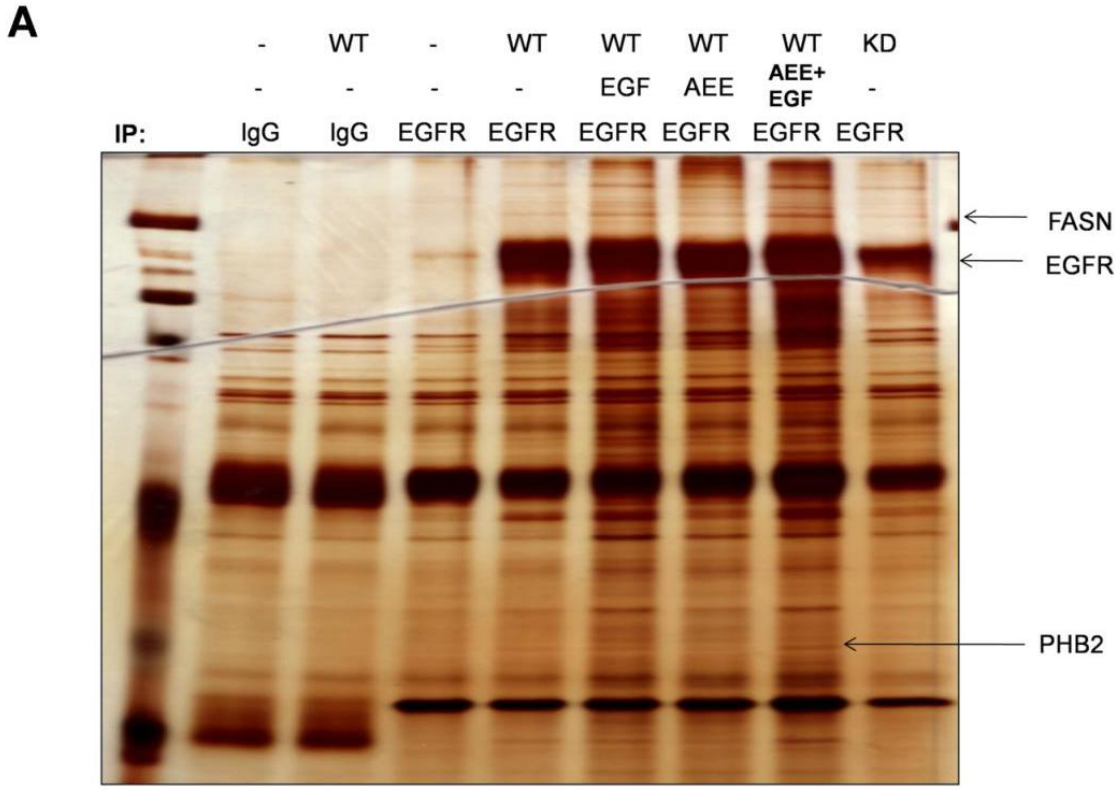


FIGURE. 1: PROTEOMIC ANALYSIS OF IMMUNOPRECIPITATED EGFR.

A: HEK293 cells were transfected with wild type (WT) or kinase dead (KD) EGFR and treated with EGF or AEE788 as shown in the figure. EGFR was immunoprecipitated with anti-flag antibody and immuno precipitated complexes were subjected to SDS PAGE and silver staining. Silver stained SDS-PAGE gel used for proteomic identification of EGFR interacting proteins. Bands of FASN, EGFR, and PHB2 are indicated by arrows.

3.3.2 Cancer Tissues and Cell Lines of Prostate, Breast, Contain Mitochondrial EGFR.

To determine whether cancer cells have mtEGFR, we performed immunofluorescent co-staining of EGFR with a mitochondrial specific protein, the MTCO2 (mitochondrial Cytochrome c oxidase subunit II) on tissue arrays of two types of cancer, prostate (n=63) and breast (n=41), and their corresponding cancer cell lines, PC3 (prostate) and MDA-MB-231 (breast). We found that all the EGFR positive cancer tissues (Figure 2A) and cell lines (Figure 2B) contain mtEGFR. The existence of mtEGFR in PC3 and MDA-MB-231 cells was also determined by Western blot analysis of mitochondria purified by ultra-centrifugation. To determine the purity of isolated mitochondrial fraction, we measured the levels of marker proteins for plasma membrane (glucose transporter 1), endoplasmic reticulum (ER) (calreticulin), cytosol (tubulin) and Golgi apparatus (syntaxin 6) in the mitochondrial fraction. As shown in Figure 3A, mtEGFR was found in the purified mitochondrial fraction of both PC3 and MDA-MB-231 cells. We also performed immunofluorescent co-staining of EGFR and MTCO2 on smears of the isolated mitochondria. The results show that EGFR exists in the purified mitochondria (Figure 3B). To further determine the localization of mtEGFR within the mitochondria, we performed protease (thrombin) protection assay with intact mitochondria and mitoplast (outer membrane deprived mitochondria). PHB2 was used as a positive control of inner membranous protein. As shown in Figure 3C, intact mitochondria prevented mtEGFR from Thrombin treatment but mitoplast failed to protect mtEGFR, suggesting that mtEGFR is localized in the inner membrane of mitochondrion. To further validate the localization of mtEGFR at the inner membrane of mitochondria, we prepared inner

and outer membrane protein fractions of mitochondria and performed Western blot analysis for mtEGFR, MTCO2 (an inner membrane protein), and MAO (monoamine oxidase, an outer membrane protein). Consistent with the results shown in Figure 3C and with the findings of other groups, mtEGFR in PC3 and MDA-MB-231 cells also exists at the mitochondrial inner membrane (Figure 3D). Together, these data suggest that cancer cells of prostate and breast harbor mtEGFR, and mtEGFR may play roles in regulating mitochondria based cellular events of cancer cells. To determine the functional domain that drives EGFR to mitochondria, we made deletions in EGFR as shown in the Figure 4A and transfected in HEK293 cells. As shown in the Figure 4B, deletion of transmembrane domain siginificantly reduced EGFR translocation to the mitochondria.

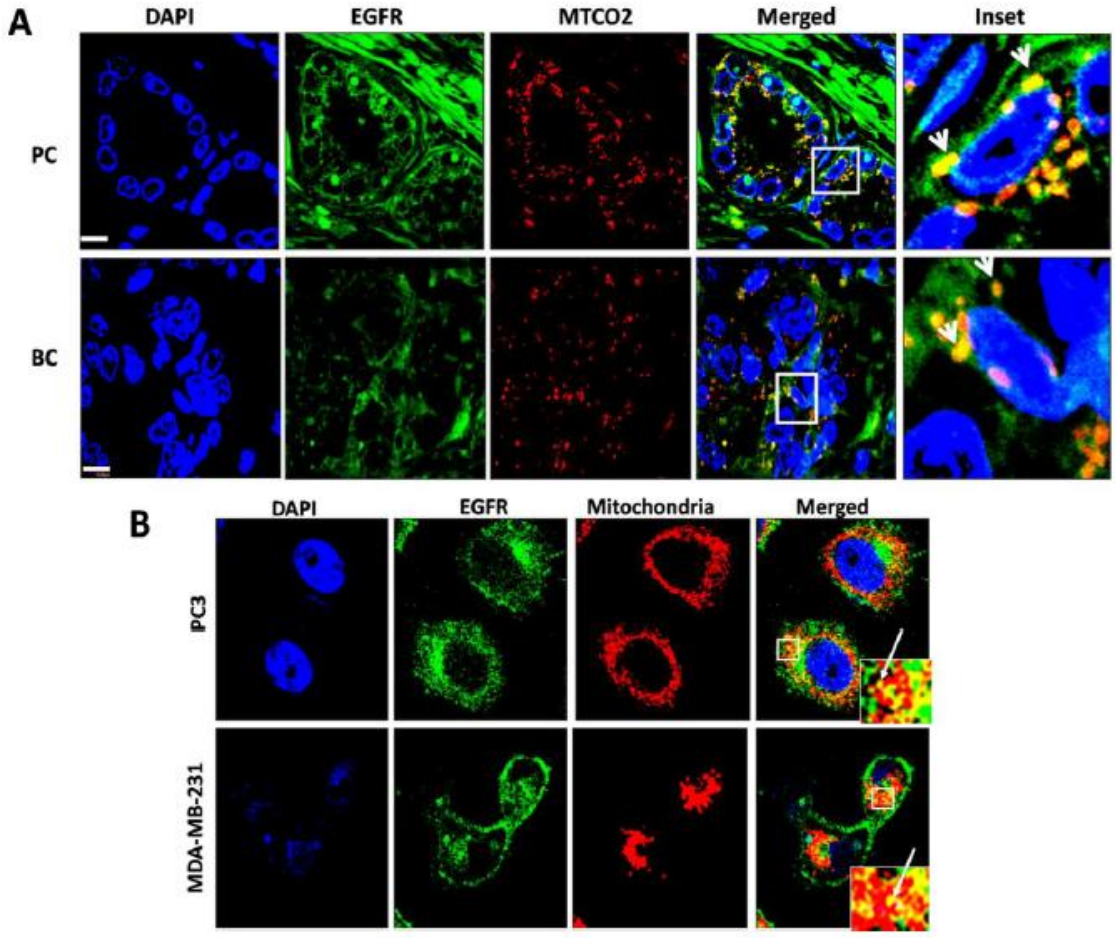


FIGURE 2. EGFR LOCALIZATION IN THE MITOCHONDRIA OF CANCER TISSUES AND CELL LINES.

A: Immuno fluorescent co-staining of EGFR (green) and MTCO2 for mitochondria (red) in prostate and breast cancer tissues. Scale bar is 10um.B: Immuno fluorescent co-staining of EGFR (green) and MTCO2 for mitochondria (red) in prostate and breast cancer cell lines. Scale bar is 30um.

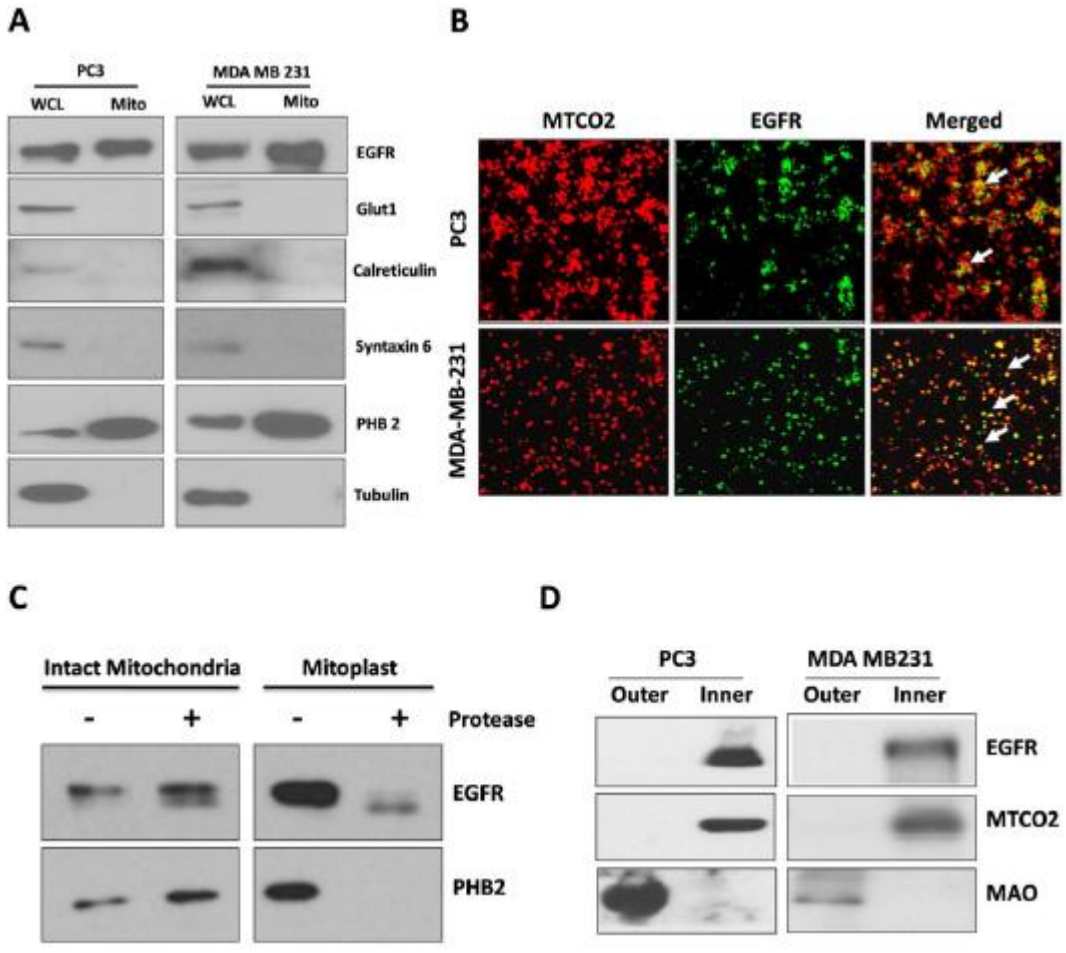


FIGURE 3. EGFR LOCALIZATION TO THE INNER MEMBRANE OF MITOCHONDRIA OF CANCER TISSUES AND CELL LINES.

A: Western blot analysis of mitochondrial protein samples prepared from PC3 and 231 cells for EGFR, Glut1, Calreticulin, Syntaxin 6, Tubulin and PHB2. B: Immunoflourescent co-staining of EGFR and MTCO2 on smears of isolated mitochondria from PC3 and MD-MB-231 cells. Colocalization of EGFR with mitochondria is in orange/yellow color in the merged confocal images (arrows). C: Western blot analysis of mitochondrial protein samples for EGFR and PHB2. Intact mitochondria or mitoplasts were treated with protease (Thrombin) at 37^0C for 15 minutes. D: Western blot analysis of mitochondrial (inner and outer membrane fractions) protein samples for EGFR, MTCO2 (inner membrane) and Mono Amino Oxidase (MAO,outer membrane).

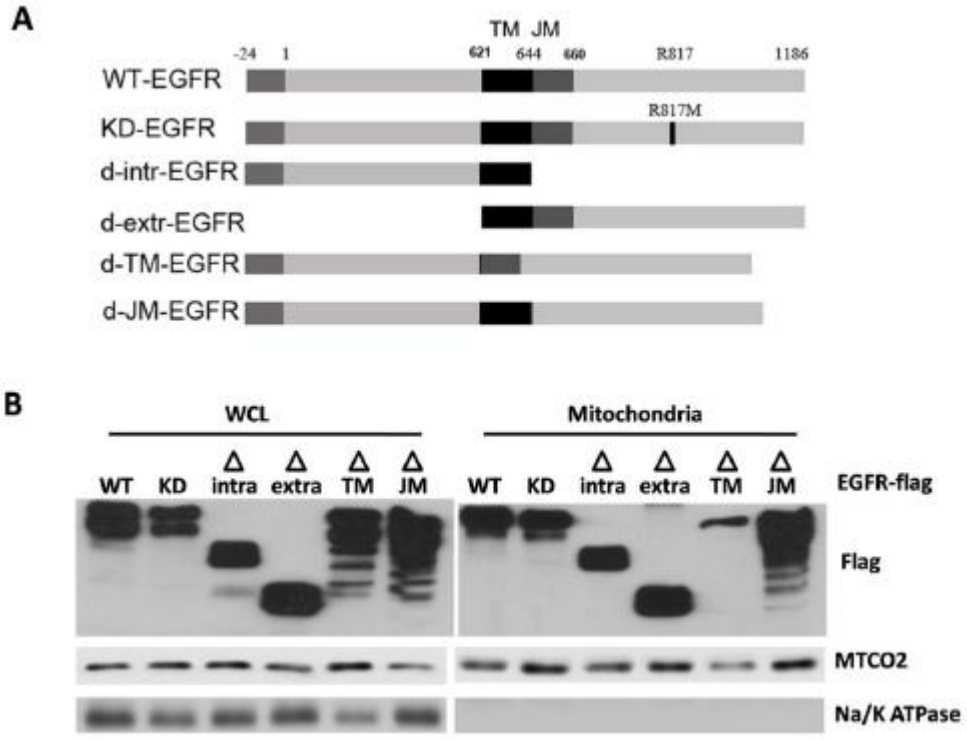


FIGURE 4: TRANS-MEMBRANE DOMAIN OF EGFR IS IMPORTANT FOR ITS LOCALIZATION TO MITOCHONDRIA.

A: Schematic diagram of EGFR truncations. B: Western blot analysis of whole cell lysates and mitochondrial samples for flag (EGFR), Na/K ATPase (plasma membrane) and MTCO2 (mitochondria). HEK 293T cells were transfected with indicated EGFR mutants as shown in the figure and processed for whole cell lysate and mitochondrial sample extraction.

3.3.3 Activation of mtEGFR promotes mitochondrial fusion .

Mitochondria are dynamic intracellular organelles that constantly undergo fusion and fission processes. Activation of fusion process leads to the formation of tubular, elongated and inter-connected mitochondria. We observed that mtEGFR in cancer cells was phosphorylated at Y1173 residue in the absence of external ligand. Treatment of serum-starved cells, PC3 with EGF showed an increased phosporylated levels of EGFR in the mitochondria and AEE788 blocked EGF induced EGFR phosphorylation in mitochondria (Figure 5A). It was reported that a tyrosine kinase inhibitor caused mitochondrial fission in Hela cells (Ishihara et al., 2006). Similarly, we found that inhibition of EGFR tyrosine kinase activity by AEE788 also resulted in mitochondrial fission and activation of pmEGFR by EGF promoted fusion of mitochondria in PC3 cells (Figure 5B and 5C). As shown in figure 5C, EGF significantly increased highly fused, interconnected and elongated mitochondria (Type I & II) and decreased rod-like mitochondria (Type III) and round shaped or fragmented mitochondria whereas EGFR kinase inhibitor (AEE788) decreased fused mitochondria (type I and II) and increased type III and fragmented mitochondria (Mitochondrial classification and quantification was performed according to the method used by others (Ehses et al., 2009). To explore the role of mtEGFR activation in regulating mitochondrial dynamics, we created plasmids expressing the kinase wild type (WT-EGFR) and the kinase dead (KD-EGFR) EGFR that can only localize to the inner membrane of mitochondria by replacing the membrane translocation signal sequence (the first 24 amino acids) of EGFR with the mitochondrial localization signal of cytochrome *c* oxidase subunit I. For detection purpose, we also fused a Flag tag to the C-terminus of the mitochondrial specific EGFRs.

We named these EGFRs as mito-WT–EGFR and mito-KD-EGFR (serves as a dominant negative mutant for the endogenous mtEGFR) respectively. We then transfected these mitochondrial specific EGFRs into PC3 cells and determined their effects on mitochondrial dynamics. At the 24 hr after transfection, we co-stained mitochondria with MTCO2 antibody and the mitochondrial specific EGFRs with Flag antibody for confocal imaging. We observed that, as compared to the control cells, the mito-WT-EGFR expressing cells exhibited less fragmented type of mitochondria and more fused mitochondria; the mitochondria of cells expressing the dominant negative mito-KD-EGFR were more fissed (Figure 6A and 6C). Figure 6 B shows that mito-KD-EGFR blocks mito-WT-EGFR phosphorylation. Together, these data suggest that the tyrosine kinase activation of mtEGFR can promote mitochondrial fusion.

FIGURE 5. EGFR kinase activity promotes mitochondrial fusion in PC3 cells.

A: Western blot analysis of whole cell lysates and mitochondrial samples for pEGFR, EGFR, Glut1 (plasma membrane) and MTCO2 (mitochondria). PC3 cells were treated with EGF +/- AEE788 as shown in the figure and processed for whole cell lysate and mitochondrial sample extraction. B: Representative images of mitochondria of PC3 cells treated with DMSO, EGF (20ng/ml), AEE788 (a small molecular EGFR inhibitor at 6uM) or AEE788+EGF for 24 hours. Mitochondria were stained with Mitotracker Red and images were taken with confocal microscope.C: The graph shows the quantification of mitochondrial dynamics in PC3 cells treated as shown in panel A. Data are means +/- SD of triplicates. Asterisk indicates the statistical significance between treated group and DMSO group. P is <0.05.

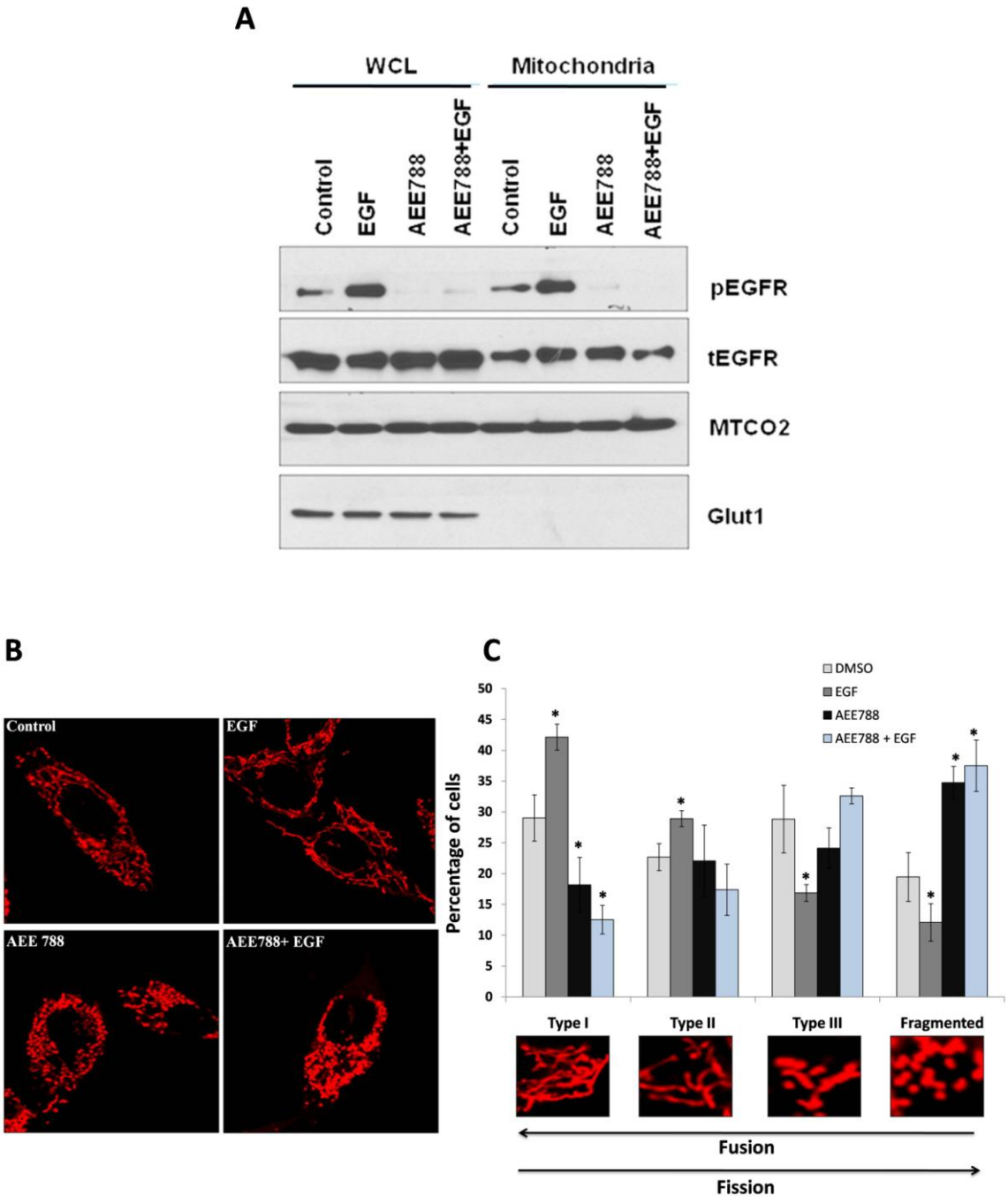


FIGURE 5. EGFR KINASE ACTIVITY PROMOTES MITOCHONDRIAL FUSION IN PC3 CELLS.

FIGURE 6. mtEGFR kinase activity promotes mitochondrial fusion in PC3 cells.

A: Representative confocal images of mitochondria of PC3 cells transfected with empty vector, mito-WT-EGFR or mito-KD-EGFR for 24 hours. Nuclei were stained with DAPI (blue), Mitochondria (red) were stained with MTCO2 and ectopic mitoEGFR (green) was stained with anti-Flag antibody. B: Western blot analysis of whole cell lysates and mitochondrial samples for pEGFR and myc tag. HEK 293T cells were transfected with indicated EGFR mutants as shown in the figure and whole cell lysates were prepared western blot after 24 hours of transfection. C: The graph shows the quantification of mitochondrial dynamics in PC3 cells transfected with mito EGFR as shown in panel C. Data are means +/- SD of triplicates. Asterisk indicates the statistical significance between treated group and DMSO. P is <0.05.

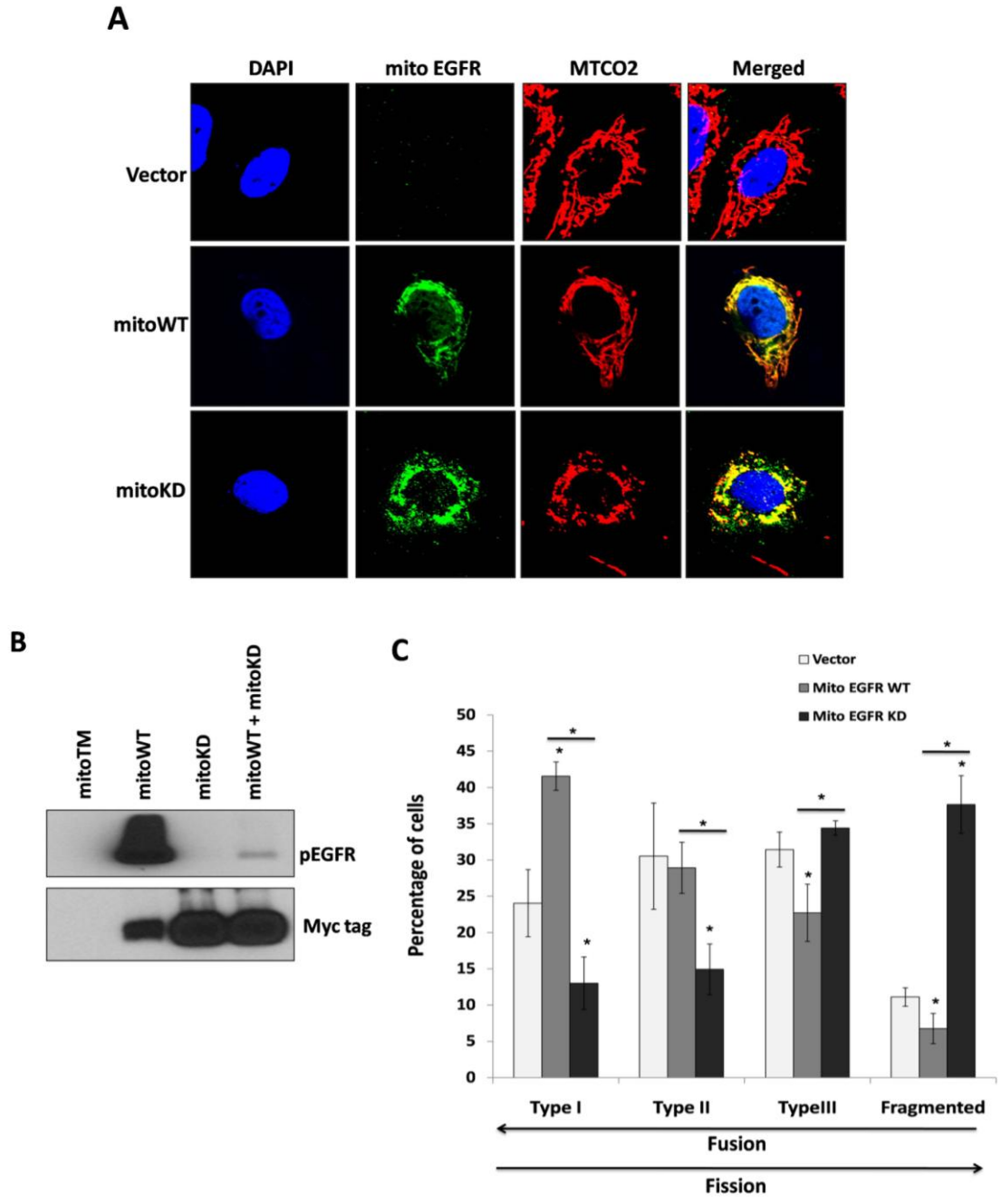


FIGURE 6. MT EGFR KINASE PROMOTES MITOCHONDRIAL FUSION IN PC3 CELLS.

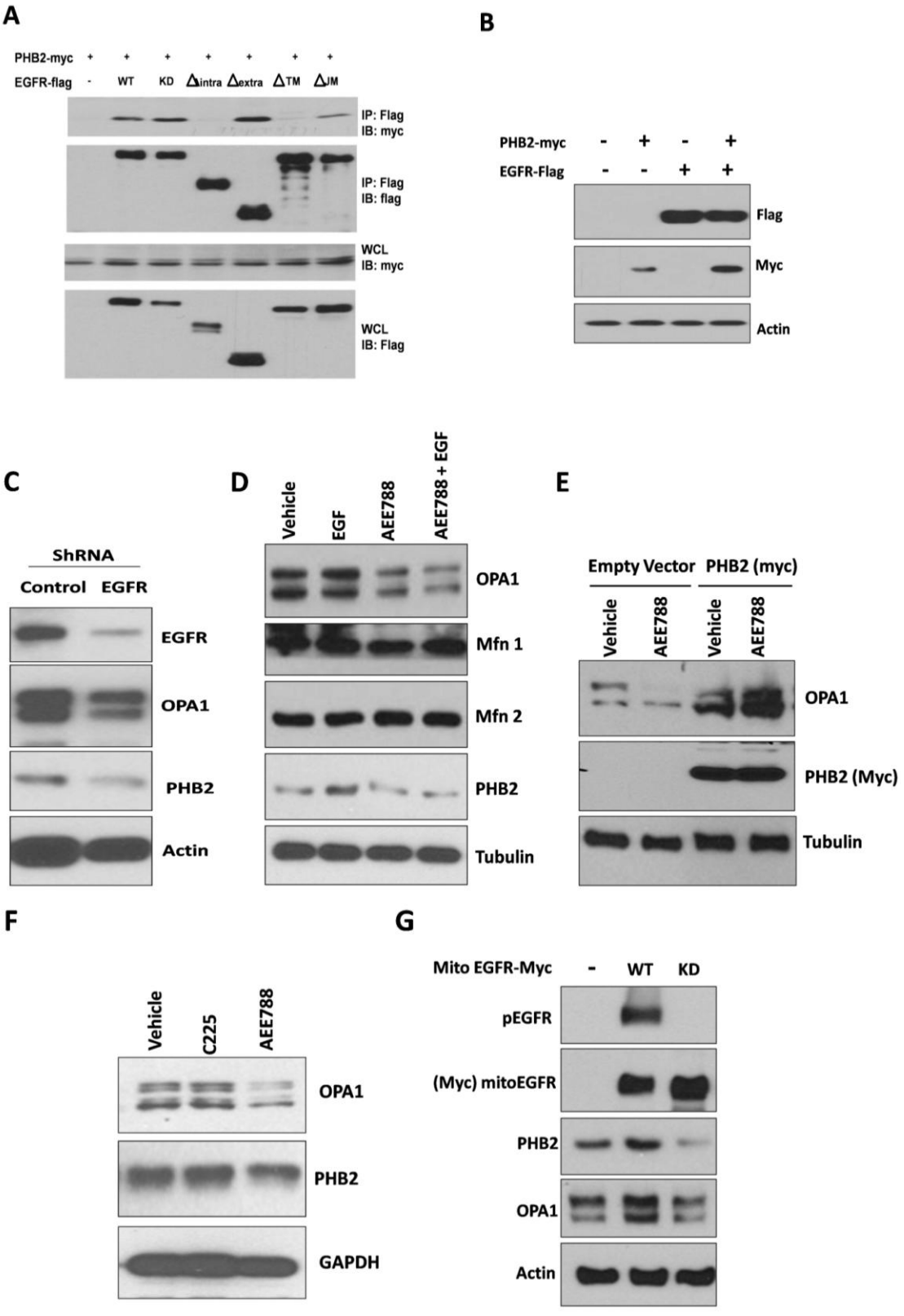
3.3.4 mtEGFR interacts with PHB2 and increases OPA1.

To investigate the mechanism by which mtEGFR regulates mitochondrial dynamics, we probed whether EGFR interacts with any mitochondrial proteins that are involved in the regulation of mitochondrial dynamics. Through proteomic approach, we found that a mitochondrial specific protein, prohibitin 2 (PHB2), was co-precipitated with EGFR independent of EGFR's tyrosine kinase activity (Figure 1). We co-transfected myc-tagged PHB2 and flag-tagged wild type and functional domain mutated EGFRs into HE293 cells. The EGFR-PHB2 interaction was then validated and characterized by immnoprecipitation coupled Western blot analysis. We found that the EGFR-PHB2 interaction was indeed independent of the tyrosine kinase activity of EGFR and the transmembrane and the intracellular domain of EGFR are critical for the interaction (Figure 7A). Overexpression of WT EGFR increased the protein levels of PHB2 when expressed in HEK 293 cells (Figure 7B). OPA1 is a GTPase involved in the fusion process of mitochondria. Stabilization of OPA1 is required for promoting mitochondrial fusion, and PHB2 facilitates mitochondrial fusion by preventing OPA1 from cleavage by mitochondrial proteases such as the mAAA proteases (Merkwirth et al., 2008) or by the ATP-independent metalloprotease OMA1 (Zaidi et al., 2012). As shown in the Figure 7C, knockdown of EGFR using shRNA reduced PHB2 levels and also OPA1 protein. We found that the EGFR tyrosine kinase inhibitor AEE788 also significantly reduced the levels of EGF induced OPA1 and PHB2 in PC3 cells without changing the levels of other mitochondrial fusion related proteins such as Mfn1 and Mfn2 (Figure 7D), suggesting that PHB2 and OPA1 are also involved in EGFR regulated mitochondrial dynamics. Interestingly, the EGFR monoclonal antibody inhibitor, C225 that inhibits

pmEGFR's kinase activity, did not reduce OPA1 level (Figure 7F), suggesting that the tyrosine kinase activity of pmEGFR is not directly involved in AEE788 caused down-regulation of OPA1. Because PHB2 is a mitochondrial protein, EGFR exists in mitochondria, EGFR interacts with PHB2, and AEE788 down-regulates OPA1 and induces fission, we tested the tyrosine kinase activity of mtEGFR in regulating the levels of PHB2 and OPA1. We transfected the mito-WT-EGFR and the mito-KD-EGFR into PC3 cells, and determine their effects on PHB2 and OPA1. As shown in Figure 7G, the mito-WT-EGFR increased the levels of PHB2 and OPA1, the mito-KD-EGFR decreased them, suggesting that EGFR within the mitochondria upregulates PHB2 and OPA1 protein levels dependent of its tyrosine kinase activity. It is known that PHB2 protects OPA1 from proteolysis (Fiorentino et al., 2008). mtEGFR may protect OPA1 via PHB2, which is supported by the data that the AEE788 induced down-regulation of OPA1 was prevented by over-expression of PHB2-myc in PC3 cells (Figure 7E). Together, these data suggest that mtEGFR can increase OPA1 by interacting with (independent of mtEGFR's tyrosine kinase activity) and increasing PHB2 (dependent of mtEGFR's tyrosine kinase activity).

FIGURE 7. MITOEGFR REGULATES PROTEIN LEVELS OF FUSION PROTEINS, PHB2 AND OPA 1.

A: Western blot analysis of immunoprecipitated samples for flag and myc antibodies. HEK 293T cells were transfected with PHB2-myc alone or with flag tagged EGFR mutants including wild type (WT) and EGFR was immunoprecipitated with Flag antibody. B: HEK293 cells were transfected with PHB2 +/- EGFR for 24 hours and protein samples were subjected to western blot analysis for Flag (EGFR), Myc (PHB2) and Actin. C: PC3 cells were transfected with scrabled or EGFR ShRNA for 48 hours and protein samples were subjected for western blot analysis of EGFR, OPA1, PHB2 and Actin. D: Western blot analysis of protein samples for PHB2, OPA 1, Mfn1, Mfn2 and Tubulin. PC3 cells were treated with EGF (20ng/ml) in the presence or absence of AEE788 (5uM) for 30 hours. E: PC3 cells were transfected with empty vector or PHB2-myc for 24 hours followed by AEE788 for another 24 hours. Protein samples were analyzed on western blot for OPA 1, myc (PHB2) and Tubulin. F: PC3 cells were treated with vehicle, EGFR monoclonal antibody (inhibits plasma membranous EGFR) or AEE 788 for 24 hours and isolated proteins were analyzed for OPA1 levels. GAPDH serves as loading control. G: HEK 293T cells were transfected with MitoEGFR WT or KD for 24 hours and protein samples were analyzed on western blot for proteins as shown in the figure.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3.3.5. EGFR kinase inhibitor, AEE788, reduces mitochondrial content and aerobic glycolysis.

Mitochondrial fusion is important for biogenesis of mitochondria. In mitochondrial dynamics experiment, we observed that EGFR TKI, AEE788, reduced the volume of mitochondria per cell. We did not see any significant increase in mitochondrial volume by EGF but AEE788 significantly decreased mitochondrial volume (Figure 8A). Cancer cells show altered glucose metabolism known as the Warburg effect. Increased mitochondrial fusion is associated with aerobic glycolysis. Prostate cancer cell line, PC3, exhibit highly fused mitochondria and aerobic glycolysis. We tested whether induction of mitochondrial fission by EGFR TKI reduce aerobic glycolysis. As shown in the Figure 8, AEE788 significantly decreased glucose consumption (figure 8C) and lactate production (Figure 8D). Like AEE788, other EGFR TKIs showed same effect on aerobic glycolysis in PC3 cells (Figure 9A and 9B). HIF1 alpha is a master regulator of aerobic glycolysis. We tested whether the TKIs (Erlotinib and Iressa) have any effect on HIF1 alpha. As shown in the figure 9C, the TKIs reduced HIF1 alpha levels. These results suggest that, EGFR tyrosine kinase activity is important for mitochondrial fusion, mitochondrial biogenesis and aerobic glycolysis.

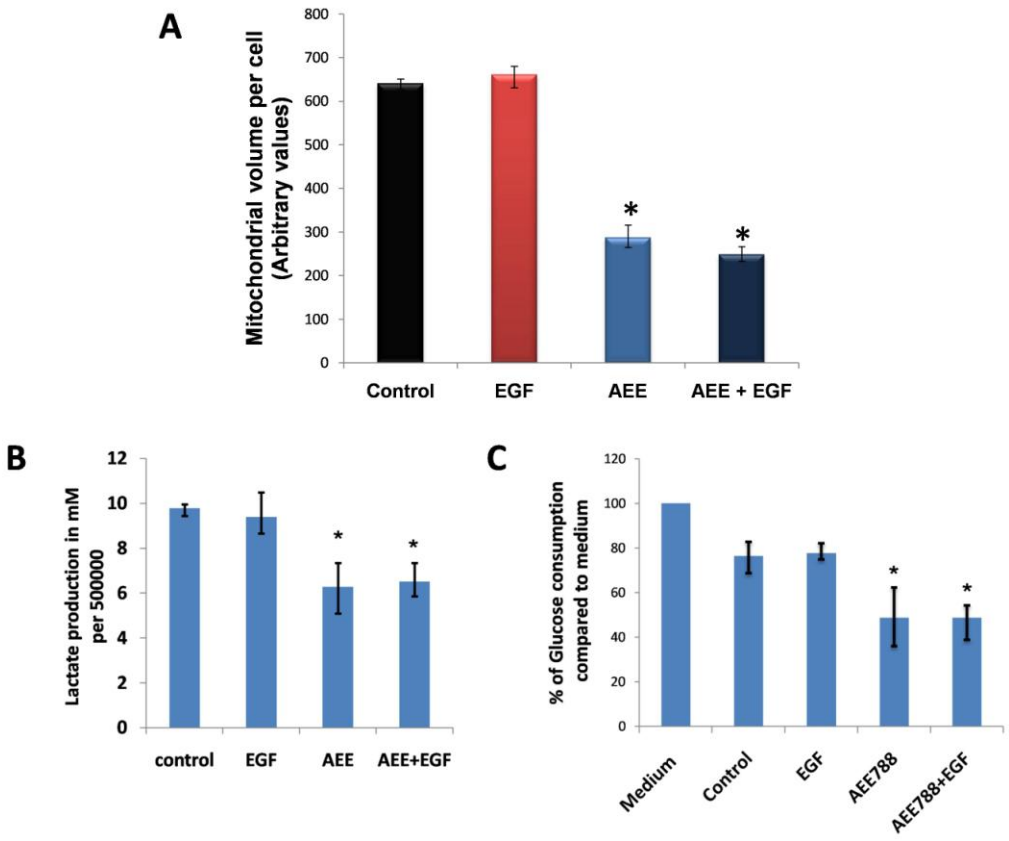

FIGURE 8. EGFR KINASE ACTIVITY REGULATES MITOCHONDRIAL CONTENT AND AEROBIC GLYCOLYSIS.

A: Quantification of mitochondrial volume in PC3 cells treated as shown in the figure. Data are means +/- SD of triplicates. Asterisk indicates the statistical significance between treated group and DMSO. B: Secreted lactate was measured in the medium collected from PC3 cells treated with EGF +/- AEE788 for 24 hours. Data are means +/- SD of triplicates. Asterisk indicates the statistical significance between treated group and DMSO. C: Glucose was measured in the medium collected from PC3 cells treated with EGF +/- AEE788 for 24 hours and presented percentage glucose consumed. Data are means +/- SD of triplicates. Asterisk indicates the statistical significance between treated group and DMSO. P is <0.05.

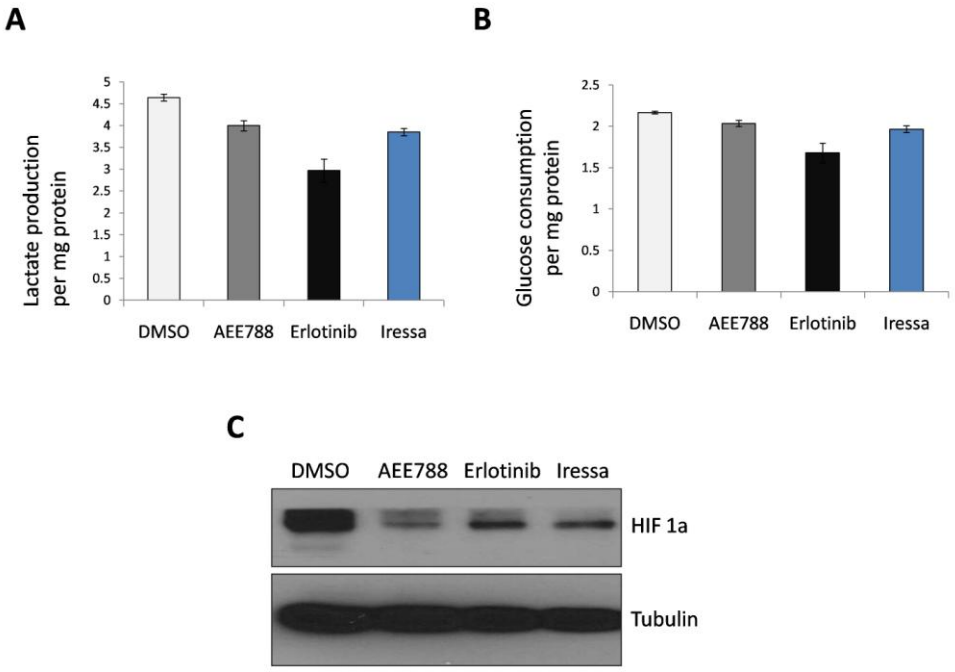

FIGURE 9. EGFR KINASE INHIBITORS DOWN REGULATE AEROBIC GLYCOLYSIS BY REDUCING HIF1 ALPHA PROTEIN LEVELS.

A: Secreted lactate was measured in the medium collected from PC3 cells treated with EGFR kinase inhibitors as shown in the figure for 24 hours. Data are means +/- SD of triplicates. Asterisk indicates the statistical significance between treated group and DMSO. P is <0.05. B: Glucose was measured in the medium collected from PC3 cells treated with EGFR small molecule kinase inhibitors for 24 hours and presented percentage glucose consumed. Data are means +/- SD of triplicates. Asterisk indicates the statistical significance between treated group and DMSO. P is <0.05. C: Western blot analysis of HIF 1a and Tubulin on protein samples isolated from PC3 cells treated with EGFR kinase inhibitors in panel B.

3.3.6. EGFR interacts with FASN independent of the kinase activity of EGFR.

In looking for upstream signals that may regulate the activity of mtEGFR, we focused on *de novo* synthesized palmitate because our proteomic analysis had identified that FASN interacted with EGFR independent of EGFR's tyrosine kinase activity (Figure 1). Because both EGFR and FASN are frequently over-expressed in cancer cells, we determined their location relationship in cancer cells of prostate and breast using immunofluorescent co-staining based confocal imaging. It was found that FASN co-localized with pmEGFR at the plasma membrane of cells of cancer tissues and cell lines (Figure 10A and 10B). Immunoprecipitations of EGFR from PC3 cells treated with EGF with/without AEE788 show that FASN interacts with EGFR regardless of AEE788 (Figure 11A). To further characterize the EGFR-FASN interaction, we created a panel of mutated/truncated forms of EGFR with a Flag tag at their C-termini (Figure 11B). We determined the interaction between these mutated EGFRs and FASN in HEK293T cells using co-immunoprecipitation coupled Western blot analysis. We confirmed that EGFR interacts with FASN independent of EGFR's tyrosine kinase activity, and found that deletion of the transmembrane domain (645-671AA) or the intracellular domain (684-1210AA) of EGFR demolished the interaction (Figure 11C), suggesting that membranous localization of EGFR and the intracellular domain of EGFR, but not the tyrosine kinase activity of EGFR, are critical for its interaction with FASN. These data suggest that pmEGFR may regulate the function of FASN of cancer cells.

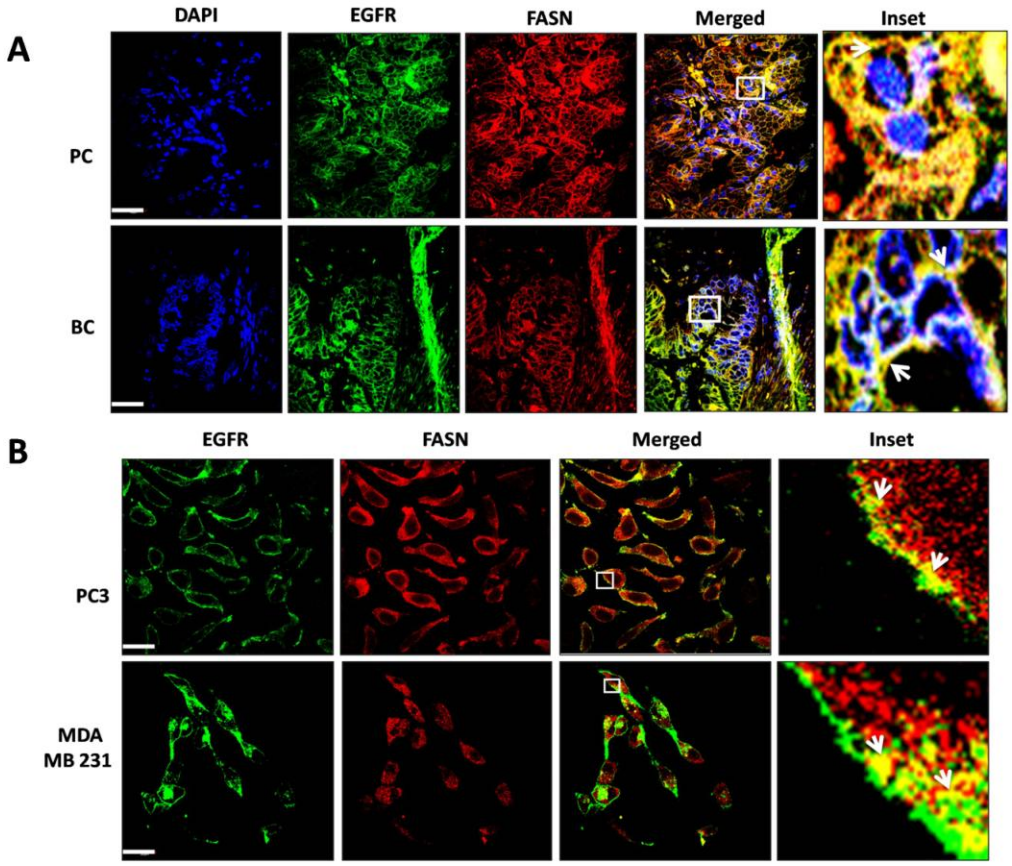

FIGURE 10. EGFR INTERACTS WITH FASN IN CANCER CELLS OF PROSTATE AND BREAST.

A: Immuno fluorescent co-staining of EGFR (green) and FASN (red) in prostate and breast cancer tissues. Scale bar is 40 um. B: Immuno fluorescent co-staining of EGFR (green) and FASN (red) in prostate and breast cancer cell lines. Scale bar is 30 um.

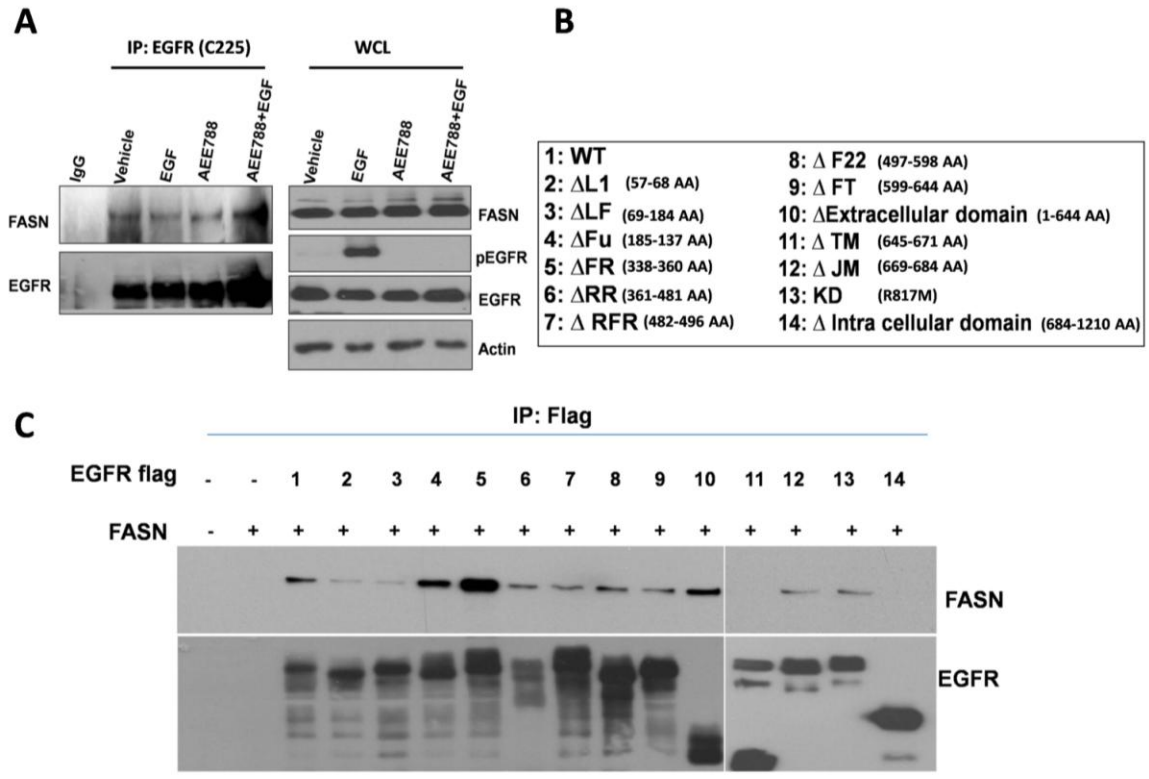

FIGURE 11. EGFR INTERACTS WITH FASN INDEPENDENT OF EGFR KINASE ACTIVITY.

A: Co-immunoprecipitation of endogenous EGFR (using C225) and endogenous FASN from PC3 cells treated with EGF+/-AEE788 for 30 minutes. Left panel shows western blot analysis of immunoprecipitated samples and right panel shows the analysis of whole cell lysates. B: Schematic diagram of EGFR truncations (mutants) used in pane C. C: Co-immunoprecipitation of mutated flagged EGFRs (using anti-flag antibodies) with exogenous FASN transfected into HEK293 cells.

3.3.7. pmEGFR activation by EGF increased *de novo* palmitate synthesis.

To determine the functional significance of the pmEGFR-FASN interaction, we measured the activity of *de novo* palmitate synthesis in EGFR positive PC3 cells treated with EGF in presence/absence of AEE788. ^{14}C-acetate was used as a substrate to trace the *de novo* synthesized palmitate. Palmitate levels were measured by thin layer chromatography (TLC) using ^{14}C-palmitate as a standard (Figure 12). We found that EGF treatment for 3 hours significantly increased the levels of *de novo* synthesized lipids (Figue 13A) and palmitate (Figure 13B), which was inhibited by AEE788 without changing FASN protein levels (Figure 13C). Similarly, EGF treatment also promoted the *de novo* palmitate synthesis of MCF-7 cells transfected with wild type EGFR but not with the kinase dead EGFR (Fig. 13D and 13E). Given the facts that the function of FASN depends on the formation of FASN homodimer and EGFR physically interacts with FASN, we speculated that EGF induced dimerization of pmEGFR might promote dimerization of FASN. To test this possibility, we treated PC3 cells with EGF in the presence/absence of AEE788 and conducted Western blot analysis on proteins isolated under reducing and non-reducing conditions. As shown in Figure 14A, EGF caused a molecular weight shift of FASN from 270kDa to about 540kDa regardless of AEE788, suggesting that EGF induced dimerization of FASN is independent of EGFR's tyrosine kinase activity. ATP citrate lyase (ACLy) is another critical enzyme regulating *de novo* palmitate synthesis. Phosphorylation at S454 activates ACLy, which results in the breakdown of citrate into oxaloacetate and acetyl CoA, and the later is a substrate for palmitate synthesis (Vazquez-Martin et al., 2008). We found that activation of pmEGFR by EGF lead to activation of ACLy, which was blocked by AEE788 (Figure 14B).

Because EGF increased palmitate synthesis without increasing the protein levels of FASN (Figure 13B and 13C) and AEE788 decreased palmitate synthesis without affecting EGF induced FASN dimerization, we tested the possibility that pmEGFR activates *de novo* fatty acid synthesis through phosphorylation of tyrosine in FASN. To do this, we immunoprecipitated FASN and performed Western blot analysis for phosphorylated tyrosine (pTyr) residues in FASN using anti-pTyr antibody. It was found that EGF treatment increased the levels of phosphorylated tyrosine of FASN, which was blocked by EGFR kinase inhibitor (AEE788) (Figure 14C and 14D). We also performed *in vitro* kinase assay using immunoprecipitated FASN and EGFR. As shown in the Figure 14 E, EGFR induces tyrosine phosphorylation on FASN. These data suggest that EGF increased *de novo* palmitate synthesis requires the tyrosine kinase activity of EGFR, although the tyrosine kinase activity of EGFR is neither needed for EGFR-FASN interaction nor for EGF induced dimerization of FASN.

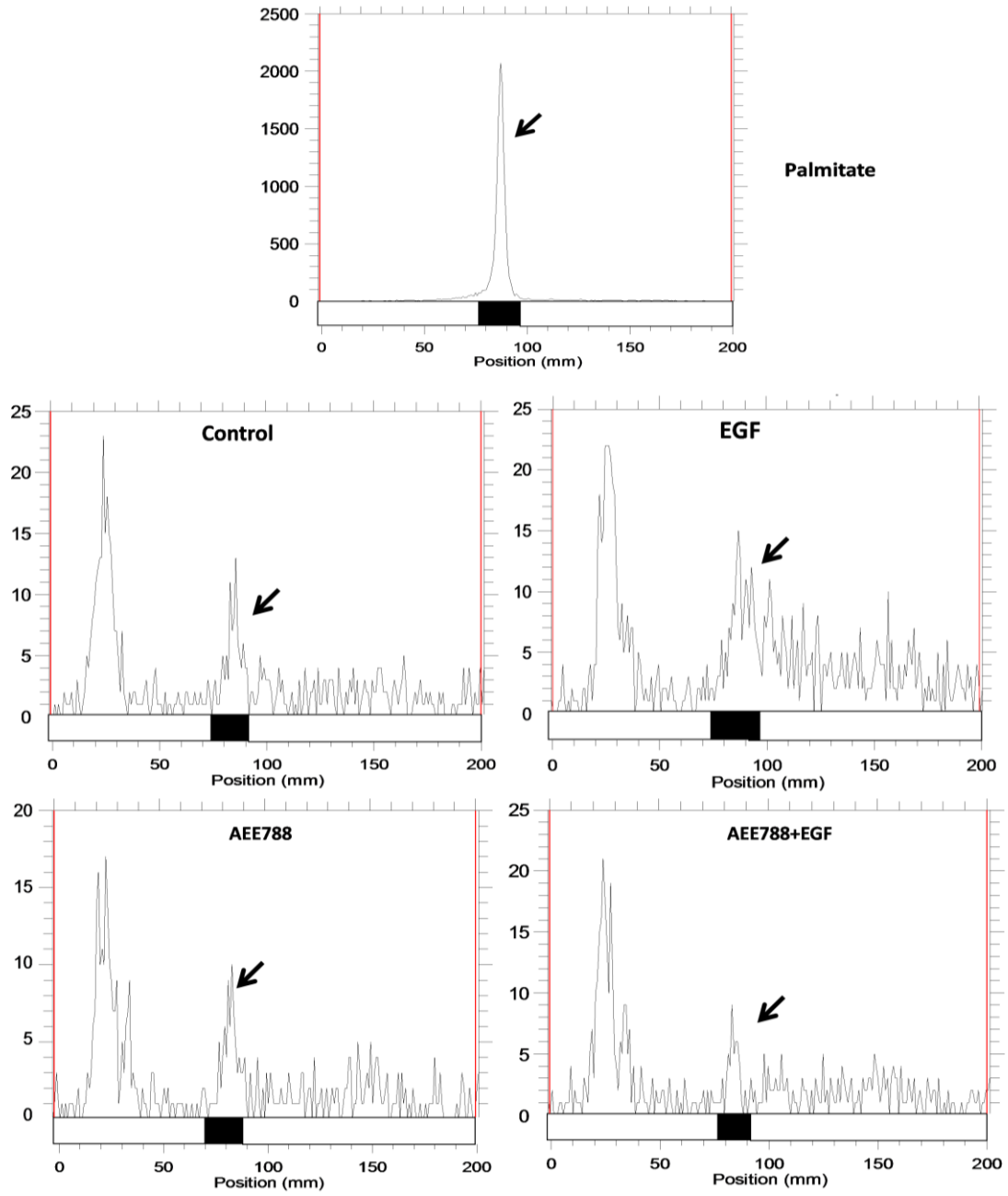

FIGURE 12. THIN LAYER CHROMATOGRAPHY (TLC) ANALYSIS OF RADIO LABELED FATTY ACIDS.

TLC radiochromatograms of lipid samples. PC3 cells were treated with EGF+/- AEE788 for 3 hours in the presence of ^{14}C Acetate and total lipids were extracted as described in the methods and separated on TLC. Arrows indicate the palmitate peak in the radiochromatogram. ^{14}C-Palmitate was used as the positive control.

FIGURE 13. EGF induces *de novo* synthesis of palmitate.

A: Quantification of *de novo* synthesized ^{14}C total fatty acids in PC3 cells treated with EGF (20nM) in the presence or absence of AEE788 (5uM) for 3 hours. Error bars indicate the mean +/- SD of triplicate samples. B: Quantification of *de novo* synthesized ^{14}C palmitate in PC3 cells treated with EGF (20nM) in the presence or absence of AEE788 (5uM) for 3 hours. Error bars indicate the mean +/- SD of triplicate samples. C: Western blot analysis of protein samples for FASN and Tubulin. Protein samples were prepared from PC3 cells treated with EGF +/- AEE788 for 3 hours. D: EGF promotes *de novo* palmitate synthesis of MCF-7 cells transfected with wild type EGFR (WT EGFR), but not the kinase dead EGFR (KD- EGFR). Quantification of *de novo* synthesized ^{14}C palmitate in MCF-7 cells. Cells were treated with EGF for 3 hr. Error bars indicate the mean +/- SD of triplicate samples. Asterisk marks indicate statistical significance between the indicated groups ($P<0.05$). E. Western blot analysis of the level of endogenous and exogenous EGFR and pEGFR.

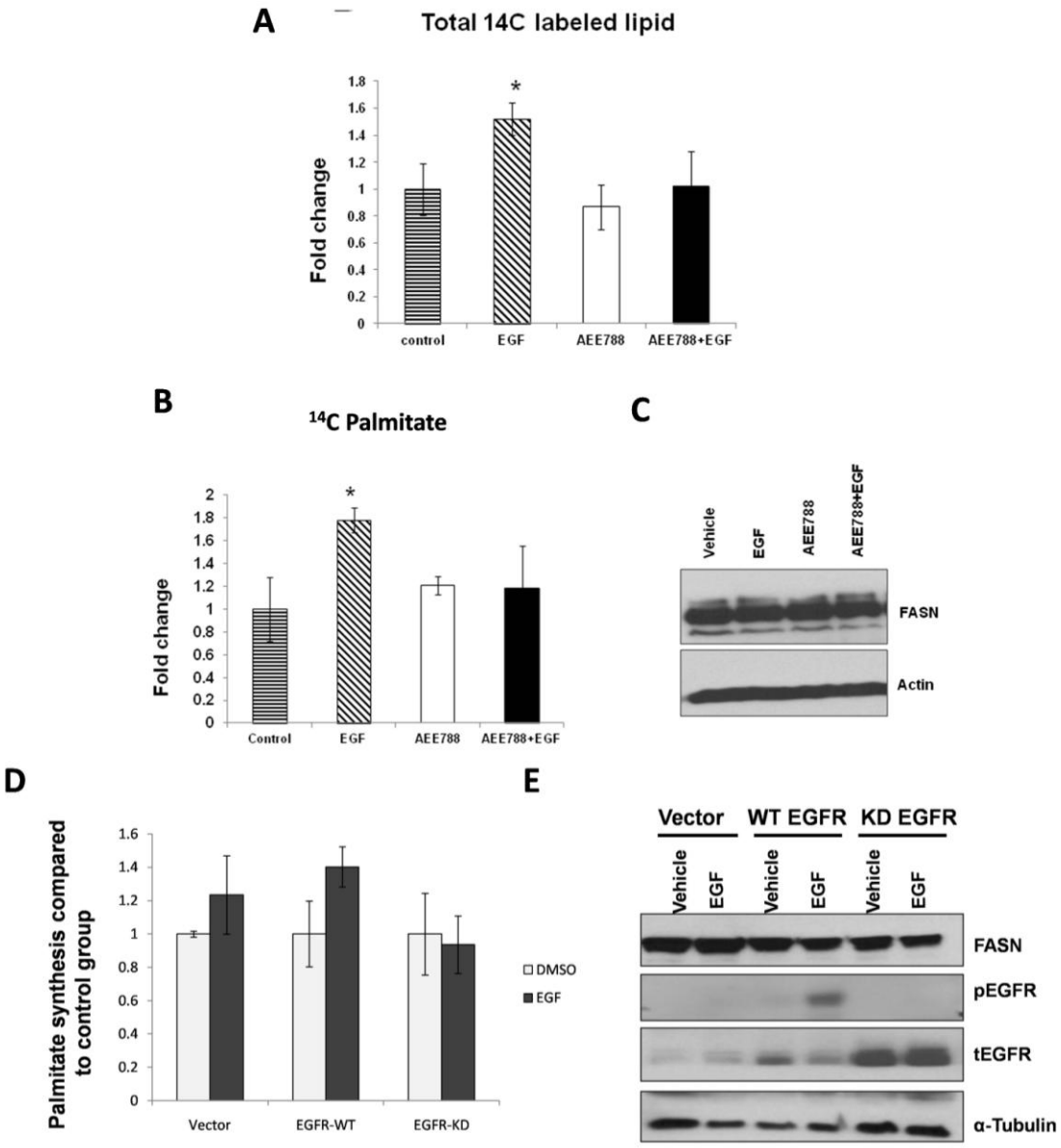

FIGURE 13. EGF INDUCES *DE NOVO* SYNTHESIS OF PALMITATE.

FIGURE 14. EGF induces *de novo* fatty acid synthesis by activating FASN and ACLy through phosphorylation.

A: Western blot analysis of protein samples for FASN and Tubulin. Protein samples were prepared under reducing or non-reducing conditions from PC3 cells treated with EGF +/- AEE788 for 15 minutes. B: Western blot analysis of protein samples for pACLY, ACLY and Tubulin. Protein samples were prepared from PC3 cells treated with agents for one hour as shown in the figure. C: FASN was immunoprecipitated from PC3 cells treated with EGF+/-AEE788 for 30 minutes and subjected western blot analysis for phosphorylated tyrosine (pTyr) and FASN. D: Western blot analysis of whole cell lysates for FASN, pEGFR, EGFR and Tubulin as indicated in the figure. E: In vitro kinase assay shows that EGFR phosphorylates FASN on tyrosine residues. HEK 293T cells were transfected with EGFR WT or FASN flag. Immuno precipitated FASN was mixed with EGFR in kinase buffer and incubated for 20 minutes. The samples were subjected for detecting FASN (flag), pTyrosune on FASN and EGFR through western blotting.

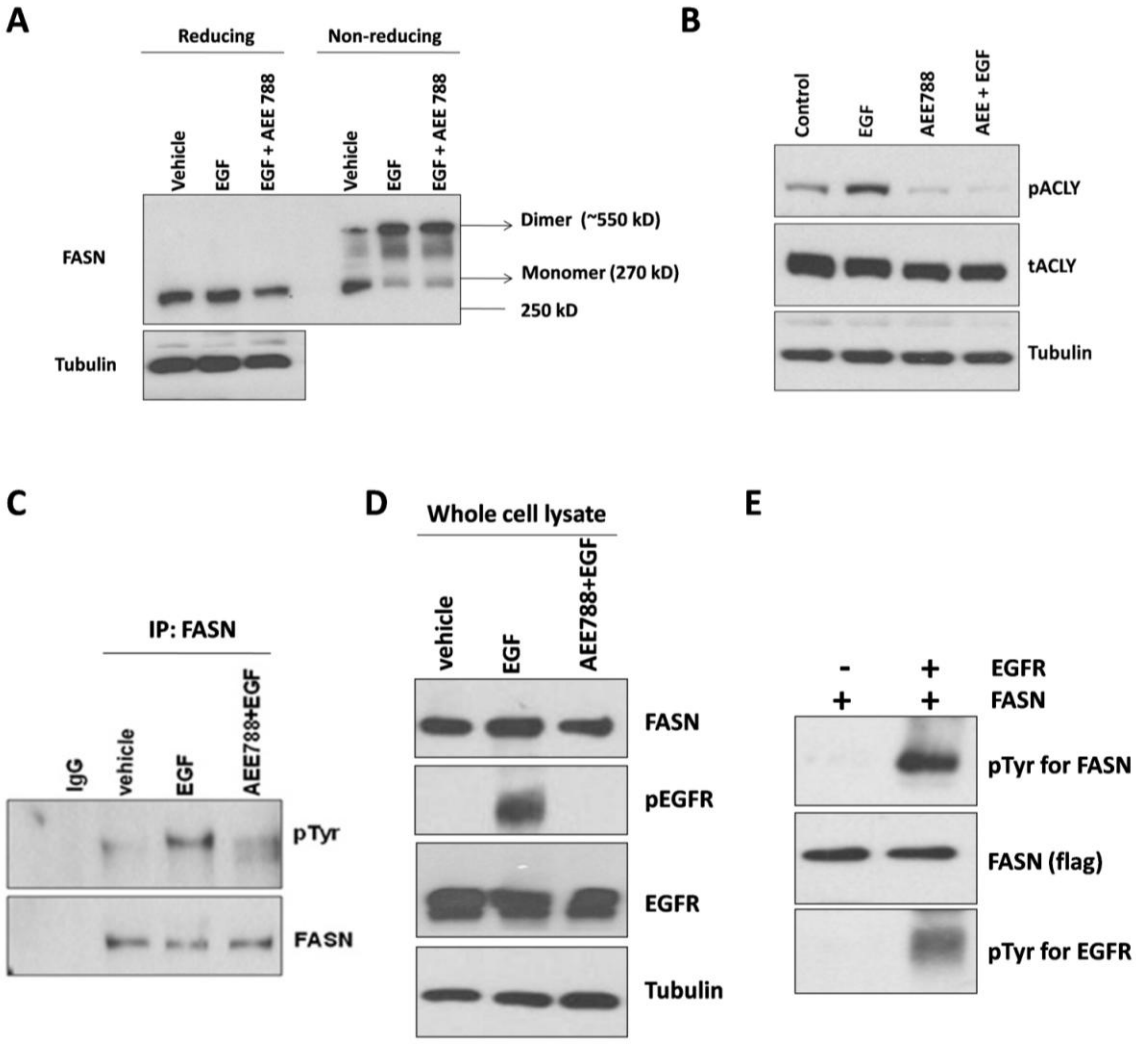

FIGURE 14. EGF INDUCES *DE NOVO* FATTY ACID SYNTHESIS BY ACTIVATING FASN AND ACLY THROUGH PHOSPHORYLATION.

3.3.8. *De novo* synthesized palmitate activated mtEGFR via palmitoylation.

It has been reported that *de novo* synthesized fatty acids act as ligands for PPAR, actives Wnt1 signaling pathway through palmitoylation (Hanahan and Weinberg, 2011). Over expression of FASN increases the oncogenic activity of ErbB members (Cantor and Sabatini, 2012). We hypothesized that EGF induced *de novo* synthesized palimitate may activate mtEGFR. To test this hypothesis, we treated PC3 cells with EGF in the presence or absence of FASN inhibitor, cerulenin, and measured the phosphorylation levels of mtEGFR in isolated mitochondria using Western blot analysis. We also treated cells with AEE788 as a positive control for the inhibition of mtEGFR activity. We observed that EGF treatment increased phosphorylation of mtEGFR, which could be inhibited by both AEE788 and cerulenin (Figure 15A), suggesting that *de novo* synthesized palmitate mediates the EGF induced mtEGFR activation. This possibility was further supported by the immunocytochemistry data showing that the phosphorylation of mtEGFR by EGF was inhibited by cerulenin (Figure 15C). To further probe the mechanism by which *de novo* synthesized palmitate activates mtEGFR, we performed the following experiments. First, we tested whether palmitate can activate mtEGFR *in vitro* by treating isolated mitochondria with palmitate in a kinase reaction buffer and measured the levels of phosphorylated mtEGFR by Western blot analysis. Indeed, palmitate potently increased the mitochondrial pEGFR levels in a reaction of 15 minutes (Figure 16A and 16B). These results suggest that *de novo* synthesized palmitate can serve as a signal molecule from pmEGFR to activate mtEGFR. Furthermore, we determined whether mtEGFR can be palmityolated (Figure 17A) and in response to pmEGFR activation (Figure 17B). We treated PC3 cells with EGF without/with AEE788 or cerulenin and immunoprecipitated

mtEGFR from isolated mitochondria, and measured palmityolation level of the mtEGFR using the Acyl-Biotin exchange assay. It was found that in deed EGF increased the level of palmitoylated mtEGFR, which was inhibited by both AEE788 (Figure 17B). Knowing that mtEGFR can be palmitoylated, we analyzed the amino acid sequence of EGFR for possible palmitoylation sites using the CSS-Palm 3.0 program (http://csspalm.biocuckoo.org/). The program predicted cysteine residues of 781, 797, 1058 and 1146 as highly possible sites for palmitoylation. We then mutated each of these cysteines to glycine in the mito-WT-EGFR and determined their effects on mito-WT-EGFR activation using HEK293T cells. We found that C781G and C797G significantly reduced the phosphorylation of mtEGFR, and mutations on cysteine 1058 and 1146 had no effect on the phosphorylation status of mito-WT-EGFR (Figure 17C). Therefore, we further determined the role of C797 on the palmitoylation of mito-WT-EGFR. As shown in Figure 17D, C797G mutation in the mito-WT-EGFR significantly reduced the palmitoylated level of mito-WT-EGFR transfected into HEK293T cells. These data suggest that induction of palmitoylation of mtEGFR might be a mechanism by which *de novo* synthesized palmitate activates mtEGFR.

FIGURE 15. Inhibition of *De novo* palmitate synthesis blocks EGF induced mitochondrial EGFR phosphorylation.

A: Western blot analysis of mitochondrial protein samples for EGFR and pEGFR prepared from PC3 cells treated with EGF (20 nM) +/- AEE788 (5uM) or Cerulenin (pretreated at 5ug/ml for 3 hours) for 30 minutes. MTCO2 was used as a loading control. Na^+/K^+ ATPase serves as plasma membrane marker. B: Western blot analysis of whole cell lysates for EGFR, pEGFR and Tubulin prepared from PC3 cells treated with EGF (20 nM) +/- AEE788 (5uM) or Cerulenin (pretreated at 5ug/ml for 3 hours) for 30 minutes. C: Immunofluorescent co-staining of mitochondria (using antibody against MTCO2, red) and pEGFR (pY1173, green) in PC3 cells treated with EGF+/-cerulenin. Co-localized signals of pEGFR and mitochondria are in yellow color. Nucleus was stained by DAPI (blue).

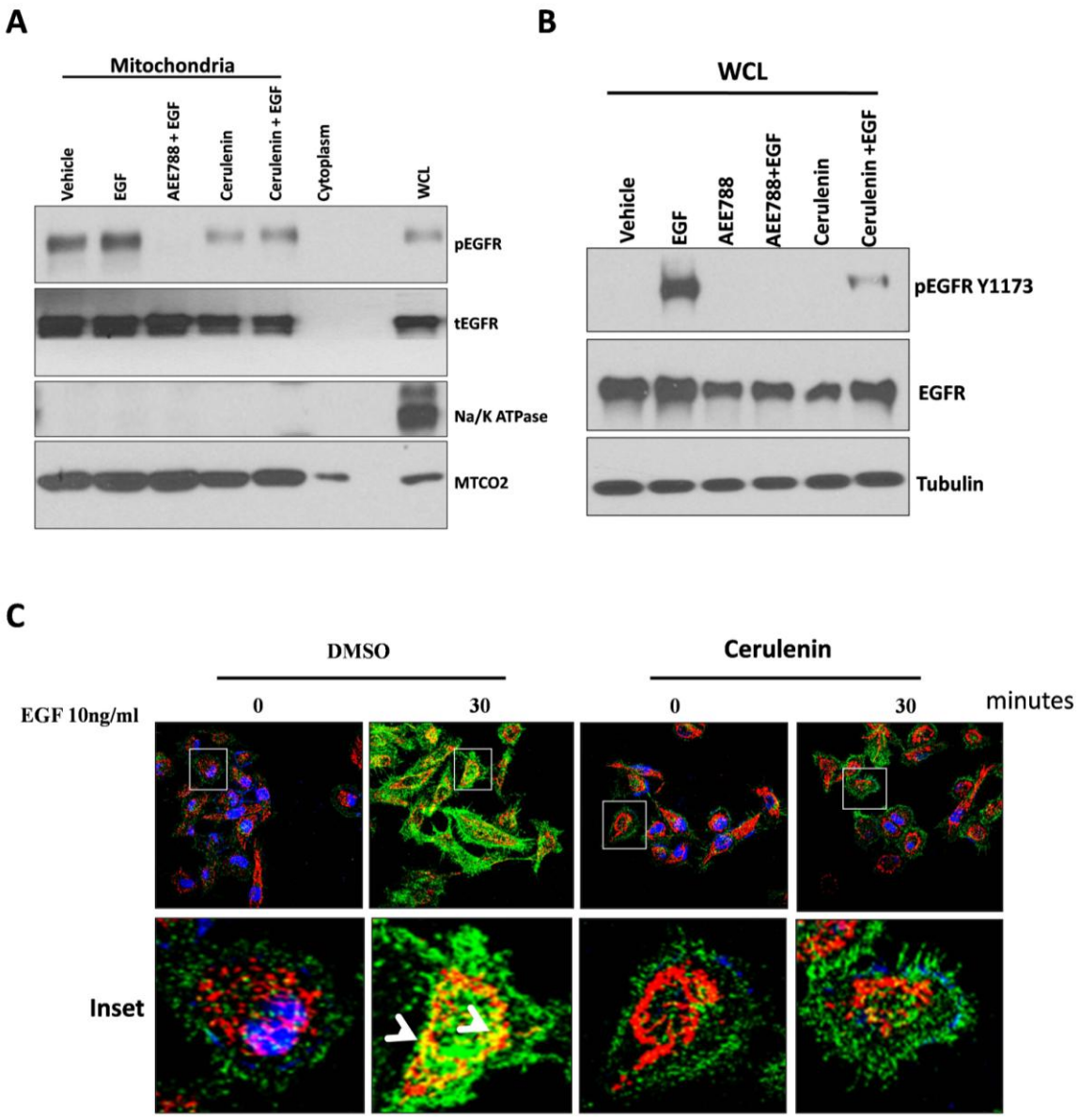

FIGURE 15. INHIBITION OF *DE NOVO* PALMITATE SYNTHESIS BLOCKS EGF INDUCED MITOCHONDRIAL EGFR PHOSPHORYLATION.

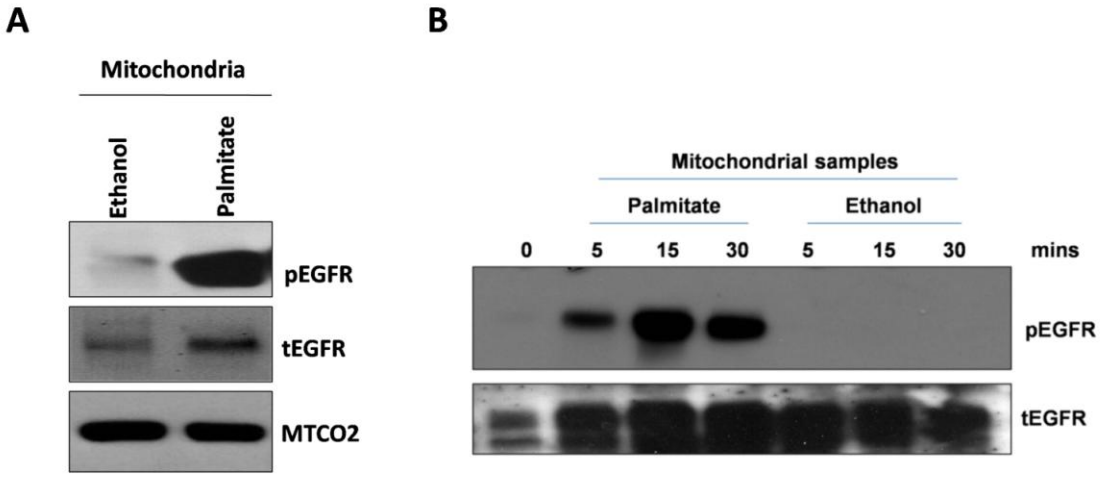

FIGURE 16. PALMITATE ACTIVATES MITOCHONDRIAL EGFR.

A: Western blot analysis of mitochondrial samples for pEGFR and EGFR. Purified mitochondria were treated with ethanol or palmitate at 200 uM for 15 minutes in in vitro kinase buffer at 37 C. assay- palmitate activates mitoEGFR. B: Western blot analysis of mitochondrial samples for pEGFR and EGFR. Purified mitochondria were treated with ethanol or palmitate at 200 uM for different time points as shown in the figure in in vitro kinase buffer at 37 C.

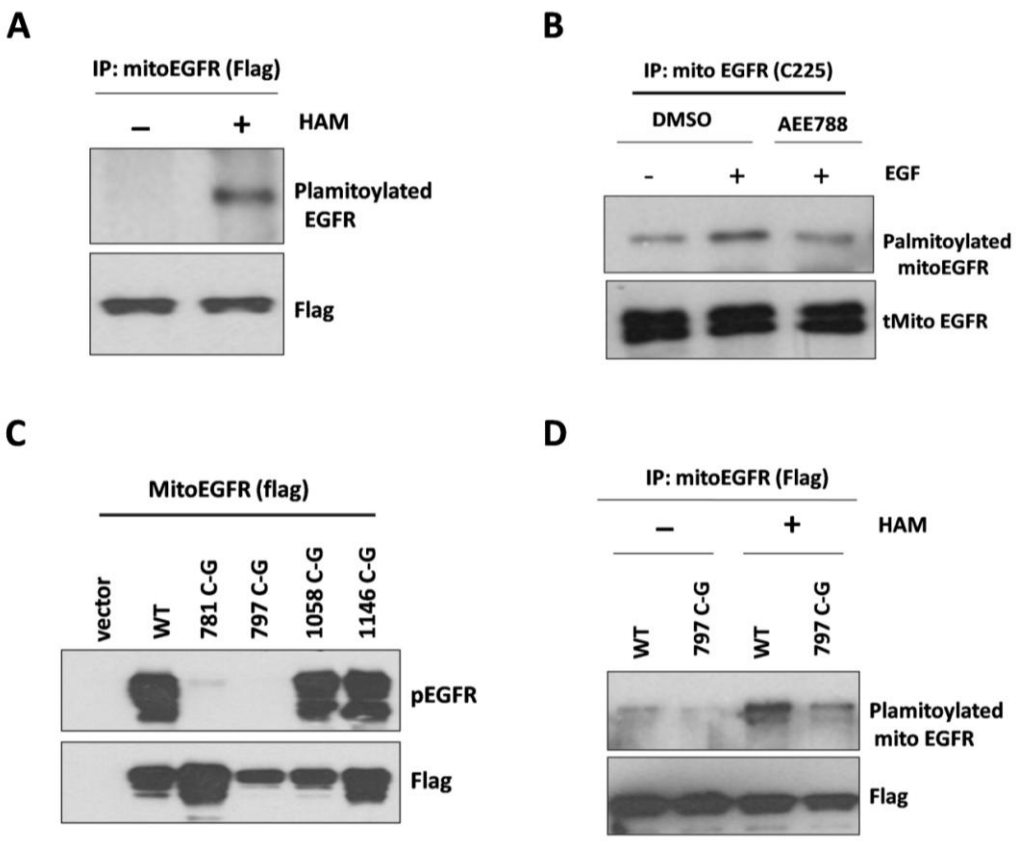

FIGURE 17. *DE NOVO* SYNTHESIZED PALMITATE ACTIVATES MITO-EGFR VIA PALMITOYLATION.

A: Palmitoylation of mitoEGFR using Acyl-Biotin exchange assay as described in the method section. MitoEGFR (flag) was immunoprecipitated from HEK 293 cells that were transfected with MitoEGFR WT. B: Palmitoylation of mitoEGFR using Acyl-Biotin exchange assay as described in the method section. MitoEGFR was immunoprecipitated from purified mitochondria isolated from PC3 cells that were treated with 20 nM of EGF +/- AEE788 (5 uM) for 30 minutes. C: Western blot analysis of mitoEGFR Cysteine mutants for their kinase activity (pEGFR). D: Palmitoylation assay of mitoEGFR WT and C797G mutant HEK 293T cells.

3.3.9. pmEGFR activates mtEGFR via *de novo* synthesized fatty acids to promote mitochondrial fusion and cell survival.

Supported by the data that activation of pmEGFR enhances FASN activity (Figure 13), *de novo* synthesized palmitate can activate mtEGFR (Figure 16), and activation of mtEGFR promotes mitochondrial fusion (Figure 5 and 6), we hypothesized that activation of pmEGFR may promote mitochondrial fusion via *de novo* synthesized palmitate induced activation of mtEGFR. First we stested whether EGF induced mtEGFR palmitoylation is dependent on *de novo* fatty acid synthesis. As shown in the figure 18A, inhibition of FASN significantly reduced mtEGFR palmitoylation suggesting that EGF induced mtEGFR palmitoylation is dependent on *de novo* fatty acid synthesis. To test the role *de novo*, we treated EGFR positive PC3 cells with EGF in the presence/absence of cerulenin, and determined the mitochondrial changes using confocal fluorescent image analysis. EGF treatment promoted mitochondrial fusion, which was inhibited by cerulenin and induced mitochondrial fission (Figure 18B and 18C). Supportively, cerulenin treatment decreased the levels of OPA1 and PHB2 without changing the levels of other mitochondrial fusion promoting proteins such as Mfn1 and Mfn2 (Figure 18D) and caused recruitment of DRP1 to the mitochondria (Figure 19A) without reducing mitochondrial membrane potential (Figure 19B). Considering *de novo* fatty acid synthesis participates in EGFR's prosurvival functions, we thought to test the effect of inhibition of FASN on the sensitivity of prostate cancer cells to EGFR tyrosine kinase inhibitor. Inhibition of FASN by cerulenin increased the sensitivity of PC3 cells to AEE788 (Figure 19C). These data suggest that FASN is involved in mtEGFR activation by pmEGFR to

promote mitochondrial fusion through increasing PHB2 and OPA1, and co-targeting EGFR and FASN may enhance the effects of EGFR tyrosine kinase inhibitors.

FIGURE 18. Inhibition of FASN inhibits EGF induced mitochondrial fusion.

A: Palmitoylation assay of endogenous mitoEGFR isolated from PC3 cells treated with 20 nM EGF +/- Cerulenin at 5ug/ml (pretreated for 12 hours) for 30 minutes. B: Representative confocal images of PC3 cells treated with 20nM of EGF +/- cerulenin (5ug/ml) for 24 hours. Mitochondria were stained with MTCO2 (red). C: The graph shows the quantification of mitochondrial dynamics in PC3 cells treated as shown in panel 7A. Y-axis represents the percentage of cells in each group containing different types of mitochondria from experiments. Data are means +/- SD of triplicates. Asterisk indicates the statistical significance between treated group and DMSO group. P is <0.05. D: Western blot analysis of OPA1, Mfn1, Mfn2 and PHB2 in PC3 cells treated with cerulenin +/- EGF. Actin was used as a loading control.

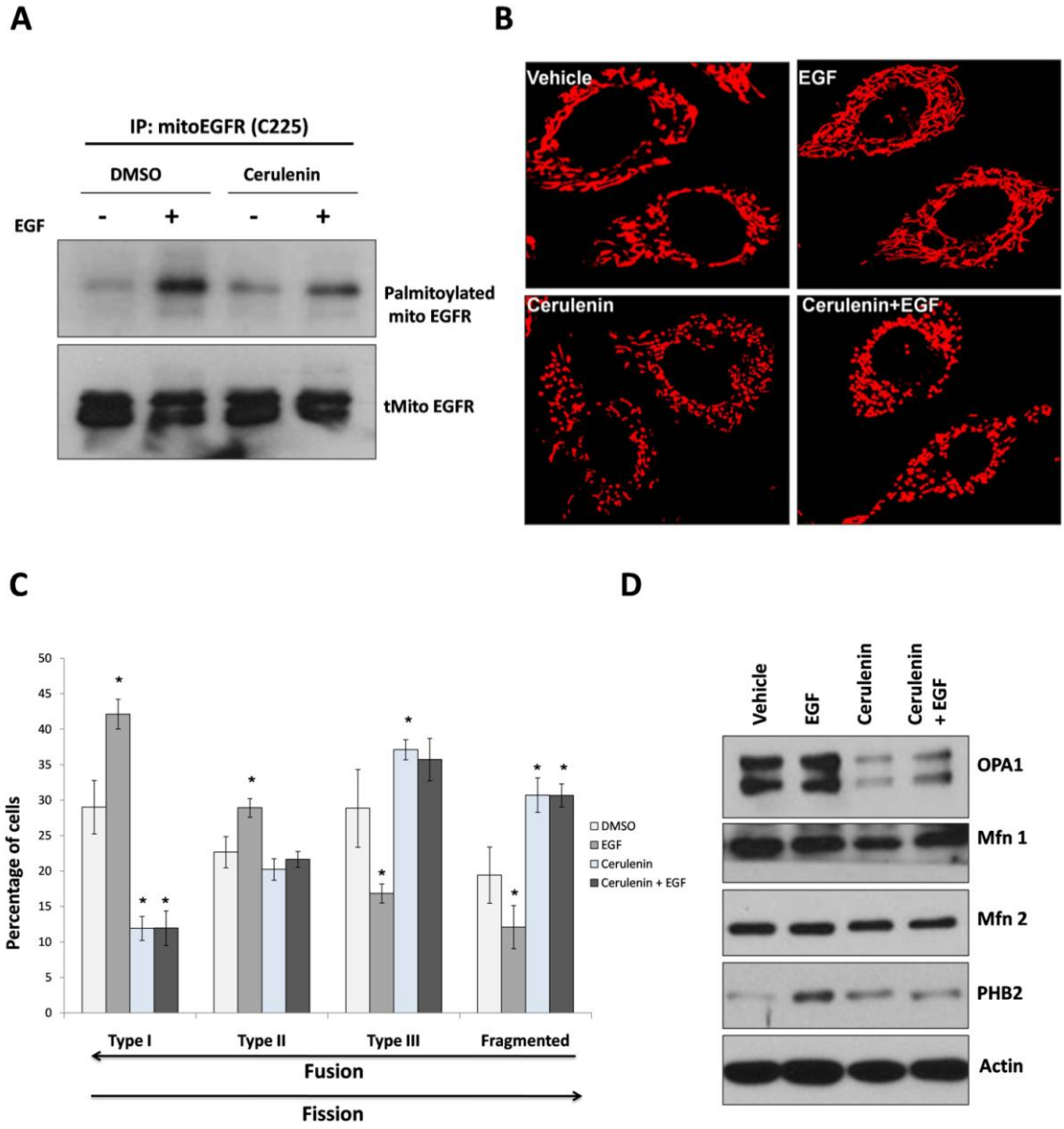

FIGURE 18. INHIBITION OF FASN INHIBITS EGF INDUCED MITOCHONDRIAL FUSION.

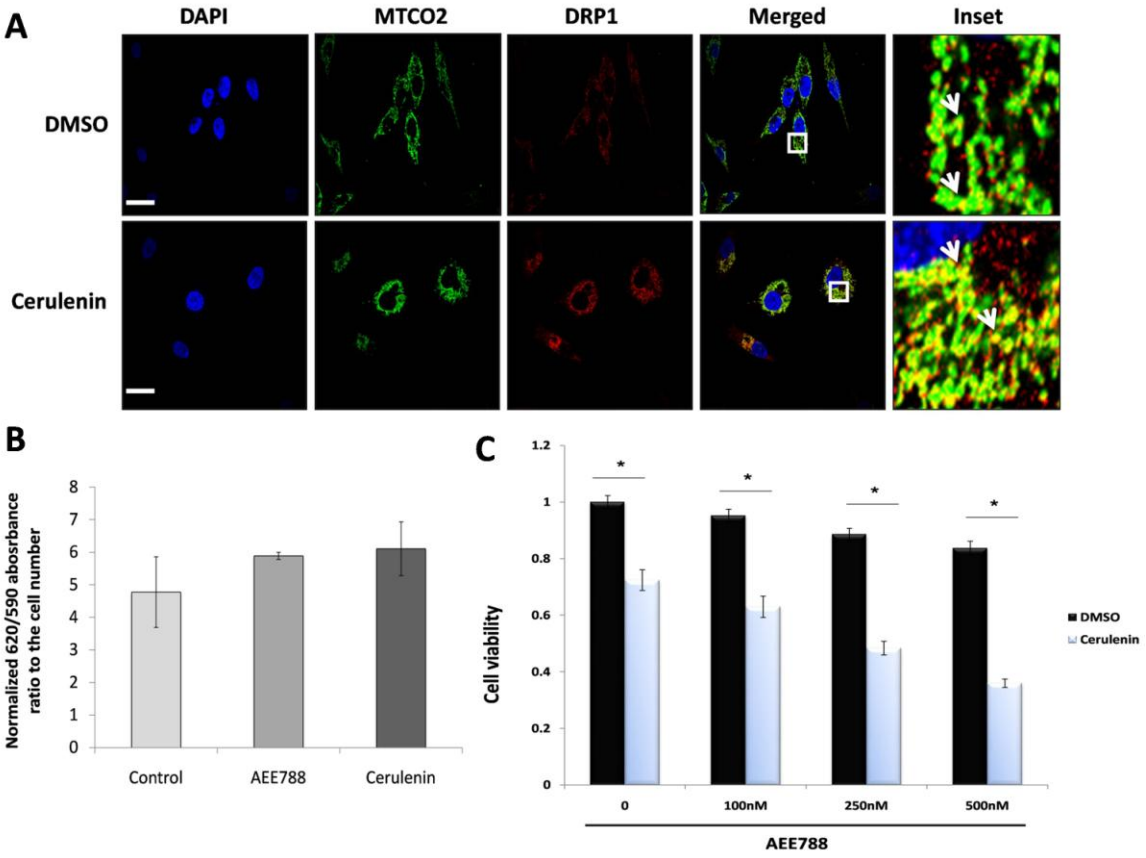

FIGURE 19. INHIBITION OF FASN INCREASES THE SENSITIVITY OF PC3 CELLS TO THE GROWTH INHIBITORY EFFECT OF AEE788.

A: PC3 cells were treated with DMSO or Cerulenin (5ug/ml) for 24 hours and immuno stained for MTCO2 and DRP1. Inset shows the enlarged portion of the cell and yellow staining (white arrow heads) indicates DRP1 recruited to the mitochondria. Nuclei were stained with DAPI. Scale bar is 30um. B: JC 1 analysis of PC3 cells treated with DMSO (control) or Ceruelnin (5ug/ml) for 24 hours. Absorbance of JC1 dye was measured using plate reader at 620nm and 590 nm and normalized to cell number and calculated ratio of 620/590 for each group. C: MTS assay of PC3 cells treated with increasing dose of AEE788 with/without a constant dose of cerulenin (5µg/ml) for 24hr. Asterisk marks indicate statistical significance between the groups as shown in the figure.

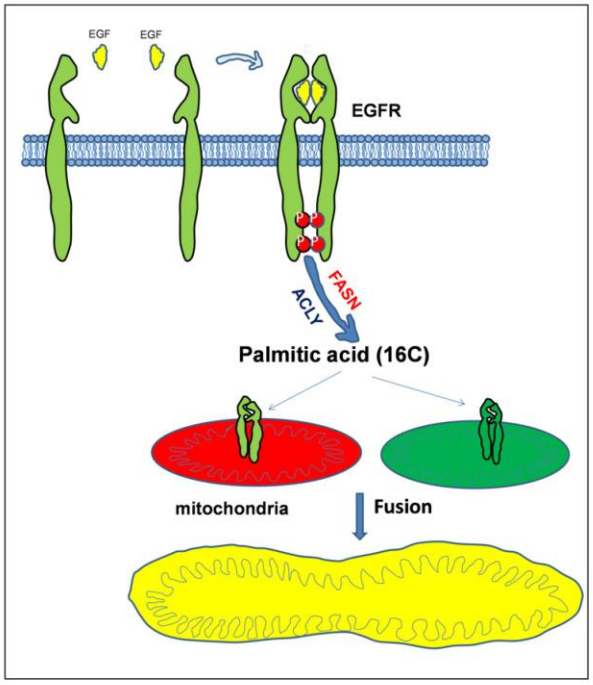

FIGURE 20. SUMMARY DIAGRAM OF THE FIRST PART OF MY WORK.

Activation of pmEGFR induces *de novo* synthesis of palmitate through activation of ACLY and FASN. *De novo* synthesized palmitate activates mitoEGFR and promotes mitochondrial fusion and cell survival.

3.4 Discussion of Project #1

One of the hallmarks of cancer is that cancer cells depend on altered metabolism for survival and growth (Jin et al., 2010b; Vazquez-Martin et al., 2008). Enhanced aerobic glycolysis and elevated *de novo* fatty acid synthesis are common in cancer cells (Huang et al., 2011). Cellular events regulated by oncogenes are a major component of the survival machinery of cancer cells. Emerging evidence indicates that *de novo* fatty acid synthesis is connected with oncogenic pathways. The relationship between *de novo* fatty acid synthesis and members of the EGFR family is especially close and bi-directional (Boerner et al., 2004; Demory et al., 2009; Yao et al., 2010). Our finding, pmEGFR signaling promotes mitochondrial fusion by interacting with/activating FASN to elevate the levels of *de novo* synthesized palmitate that in turn activates mtEGFR to promote mitochondrial fusion, further supports that FASN or *de novo* fatty acid synthesis is a part of EGFR's oncogenic machinery.

Our understanding of the canonical EGFR functions is primarily from studies of EGFR in the plasma membrane, where it receives extracellular signals by the binding of its extracellular ligands. Recent years have witnessed expansions of understanding of EGFR and HER2 functions from the plasma membrane to the nucleus (Mollner et al., 1998; Resh, 2006) and to the mitochondria (Westermann, 2010). Our data reveal that cancer tissues contain mtEGFR and that mtEGFR play important roles in cancer cells. One of the questions that need to be addressed regarding the non-plasma membranous intracellular EGFRs is that what intracellular signal(s) is (are) involved in regulating their tyrosine kinase activity. Activation of mtEGFR by *de novo* synthesized palmitate

suggests that *de novo* synthesized palmitate is a signal molecule that can activate non-plasma membranous EGFRs. Protein palmitoylation is one of the powerful posttranslational protein modifications that regulate protein functions (Di Vizio et al., 2008). Our data indicate that induction of palmitoylation of mtEGFR might be a major mechanism by which *de novo* synthesized palmitate activates mtEGFR, however, deeper molecular mechanisms behind this event warrant further investigations. It is also possible that activation of FASN may also affect the activity of the p lasma membranous EGFR.

The fission and fusion processes of mitochondria are tightly controlled by yet to be better understood mechanisms involving both cytoplasmic and mitochondrial signals (Ren et al., 2013; Weihua et al., 2008). Our data that activation of mtEGFR enhances mitochondrial fusion suggest that promoting mitochondrial fusion is a part of the prosurvival funciton of EGFR. Importantly, this function of mtEGFR involves both of its kinase dependent and kinase independent functions, i.e. activation of the kinase activity of mtEGFR inhibits the cleavage of OPA1 and mtEGFR interacts with PHB2 independent of its kinase activity. Because the endogenous pre-existing PHB2 was oppositely regulated by our mitochondrial specific mito-WT-EGFR and mito-KD-EGFR (Figure 7G), it is most likely that mtEGFR stabilizes PHB2 protein within the mitochondria. Further studies are needed to identify the mechanism whereby mtEGFR stabilizes PHB2.

As a cytoplasmic protein, FASN was found to participate in molecular events associated with the plasma membrane. FASN can accumulate at membrane lipid rafts where it interacts with caveolin-1 to promote the survival of prostate cancer cells (Cohen et al.,

2003; Dancey and Freidlin, 2003; Weiss and Stinchcombe, 2013). The increase in FASN activity by pmEGFR activation was not associated with up-regulation of the protein levels of FASN (Figure 13B and 13C), which indicates that pmEGFR dimerization induced FASN's dimerization (Figure 14A) and consequential phosphorylation on FASN (Figure 14C and 14E) are likely the mechanisms by which pmEGFR activates FASN. In this mechanism, it is worth of notice that the EGFR-FASN interaction and the EGF induced dimerization of FASN are independent of EGFR's tyrosine kinase activity. Previously, we have reported that EGFR interacts and stabilizes the sodium/glucose co-transporter 1 (SGLT1) independent of EGFR's tyrosine kinase activity (Arteaga, 2002; Arteaga and Engelman, 2014). It is intriguing that the proteins found to interact with EGFR independent of EGFR's tyrosine kinase activity all own prosurvival functions, SGLT1 for glucose uptake, PHB2 for mitochondrial integrity, FASN for *de novo* fatty acids synthesis. Considering the facts that only about 10-20% of patients of solid cancers respond to treatment of EGFR tyrosine kinase inhibitors (Davies and Goldberg, 2008) and EGFR is overexpressed in the majority of cancers of epithelial origin (Tol et al., 2009), i.e, there is a majority of patients with EGFR positive cancers do not respond well to the inhibition of EGFR's tyrosine kinase, we argue that a possibility exists, which is that EGFR can promote cancer progression via mechanisms that are independent of its tyrosine kinase activity.

This study not only unveiled a mechanism by which *de novo* synthesized fatty acids participate in EGFR's signaling, but also identified a role of mtEGFR in regulating mitochondrial dynamics. The activation of intracellular EGFR by *de novo* synthesized

palmitate may contribute to the development of resistance of cancer cells to EGFR tyrosine kinase inhibitors due to a possibility that the constant high levels of *de novo* synthesized palmitate in cancer cells may offset the effects of tyrosine kinase inhibitors during the intervals of drug administration at the clinic. Co-targeting EGFR and FASN may have a therapeutic application in treating cancers that are resistant to EGFR tyrosine kinase inhibitors.

CHAPTER 4

Project #2: *De novo* fatty acid synthesis dependent EGFR palmitoylation and activation.

4.1. Introduction to the problem

EGFR is over expressed in many cancers of epithelial origin and also in advanced cancers. Over expression of EGFR is associated with constitutive activation of EGFR signaling in cancer. Constitutive or ligand independent activation of EGFR was observed in the absence of its ligand, EGF. Ligand independent activation of EGFR is well described by EGFR mutant, EGFRVII, expressed in glioblastoma. EGFRVIII lacks ligand binding domain but still constitutively active in these cancers suggesting that traditional extracellular ligand, EGF, is not required. We observed that inhibition of FASN blocked constitutive activation of EGFR. The objective of the present study was to understand the role of FASN in EGFR intrinsic activation and its significance in EGFR targeted therapy.

EGFR has an extra cellular, trans membrane and intra cellular domains. Extracellular domain has ligand binding domain. Upon ligand binding, EGFR undergoes dimerization. Dimerization induces auto phosphorylation of EGFR and activation of downstream signaling pathways. It has already been shown that extra cellular ligand is not absolutely required for egfr dimerization and activation which was supported by constitutively active EGFRvIII in glioblastoma. In EGFRvIII, the ligand domain, exons 2-7, was deleted making it unable to bind to the extra cellular ligand. Activation of EGFRvIII supports the hypothesis of intra cellular ligands/activating molecules for EGFR

activation. Intra cellular activation of mitoEGFR by *de novo* synthesized fatty acids supported our hypothesis and led us to study intrinsic activation of EGFR in cancer and drug resistance.

4.2. Specific Aims of Study

1: The first aim was to investigate the mechanism of intrinsic activation EGFR in cancer cell.

2: The second aim was to study the role of EGFR palmitoylation in EGFR signaling.

3: The third aim focused on targeting palmitoylation to increase the sensitivity of cancer cells to EGFR tyrosine kinase inhibitors.

4.3. Results of Project #2

4.3.1. *De novo* fatty acid synthesis dependent and EGF independent activation of EGFR.

Constitutive activation or phosphorylation of EGFR in the absence of extracellular ligands is very common in many human cancers that over express EGFR. As shown in the Figure 21A, PC3 cells exhibit constitutive activation of EGFR under serum free conditions and small molecule EGFR tyrosine kinase inhibitor, AEE788, inhibits the constitutive and EGF induced EGFR phosphorylation. AEE788 is an ATP analogue and competes with ATP for binding to the EGFR kinase domain. AEE788 inhibited EGFR constitutive activation within 10 minutes, suggesting that EGFR can be activated in the absence of extra cellular ligands. EGFR monoclonal antibody, cetuximab (C225), has been approved for colon cancer treatment. C225 inhibits EGFR activation by competing with extra cellular ligands. As shown in the Figure 21B, C225 completely inhibits EGF induced EGFR phosphorylation but failed to inhibit the constitutive EGFR phosphorylation (Figure 21B) suggesting that extra cellular ligand independent activation for EGFR. Extra cellular ligand independent constitutive activation of EGFR is well represented by EGFR vIII in glioblastoma. EGFR vIII lacks ligand binding domain but it is highly active in glioblastoma suggesting non-classical ligand independent activation of EGFR or intra cellular signal mediated EGFR activation. As shown in the figure 21C, complete deletion of the extra cellular domain of EGFR (delExtra) did not affect EGFR activation; in contrast it is highly active like WT EGFR when ectopically expressed in HEK 293T cells. Inhibition of *de novo* fatty acid synthesis by FASN inhibitor, Cerulenin,

inhibited constitutive activation of EGFR (Figure 21D) in PC3 and DU145 cells suggesting that *de novo* synthesized fatty acids activates EGFR in the absence of extra cellular ligands. To test this hypothesis, we over expressed FASN, to increase *de novo* synthesized fatty acids, and found that phosphorylation of EGFR was increased with increasing FASN levels (Figure 21E).

4.3.2. *De novo* fatty acid synthesis dependent activation of EGFR through palmitoylation.

Several reports have shown that *de novo* synthesized palmitate activates and stabilizes proteins through palmitoylation. We hypothesized that palmitoylation constitutively activates EGFR in cancer cells. Using an in vitro Acyl-Biotin exchange method we found that WT EGFR is palmitoylated (Figure 22A). Treatment of prostate cancer cells with palmitoylation inhibitor, 2-Bromopalmitate, reduced EGFR palmitoylation (Figure 22C and 22D). We tested whether EGF induces EGFR palmitoylation, as we expected and shown in the Figure 22C, EGF induced EGFR palmitoylation within 15 minutes of treatment with EGF. Inhibition of palmitoyl transferases with 2-Bromopalmitate completely blocked EGF induced EGFR palmitoylation (Figure 22C). To show that EGF induced EGFR palmlitoylation is dependent on *de novo* palmitate synthesis, we blocked FASN by cerulenin. As shown in Figure 22D, inhibition of *de novo* fatty acid synthesis by cerulenin blocked EGF induced EGFR palmitoylation in PC3 cells suggesting that EGFR utilizes *de novo* synthesized palmitate for its palmitoylation. To test the effect of *de novo* fatty acid synthesis and palmitoyion on EGF induced cell proliferation. For this

FIGURE 21. *De novo* fatty acid synthesis dependent and EGF independent activation of EGFR.

A: Western blot analysis of protein samples for pEGFR and EGFR isolated from PC3 cells treated with EGF +/- AEE788 for 15 minutes. Actin was used as a loading control. B: Western blot analysis of protein samples for pEGFR and EGFR isolated from PC3 cells treated with EGF +/- C225 for 15 minutes. C: Western blot analysis of protein samples for pEGFR and EGFR (flag) isolated from PC3 cells transfected with WT EGFR or Del Extra EGFR for 24 hours. D: Western blot analysis of protein samples for pEGFR and EGFR isolated from PC3 and DU145 cells treated with FASN inhibitor, cerulenin at 5ug/ml for 16 hours. E: Inhibition of FASN reduces phosphorylation of Del Extra EGFR in HEK 293T cells. F: Western blot analysis of protein samples for pEGFR, EGFR and actin isolated from MCF10A cells transfected with FASN flag with increasing FASN concentration for 24 hours followed by serum starvation for 12 hours.

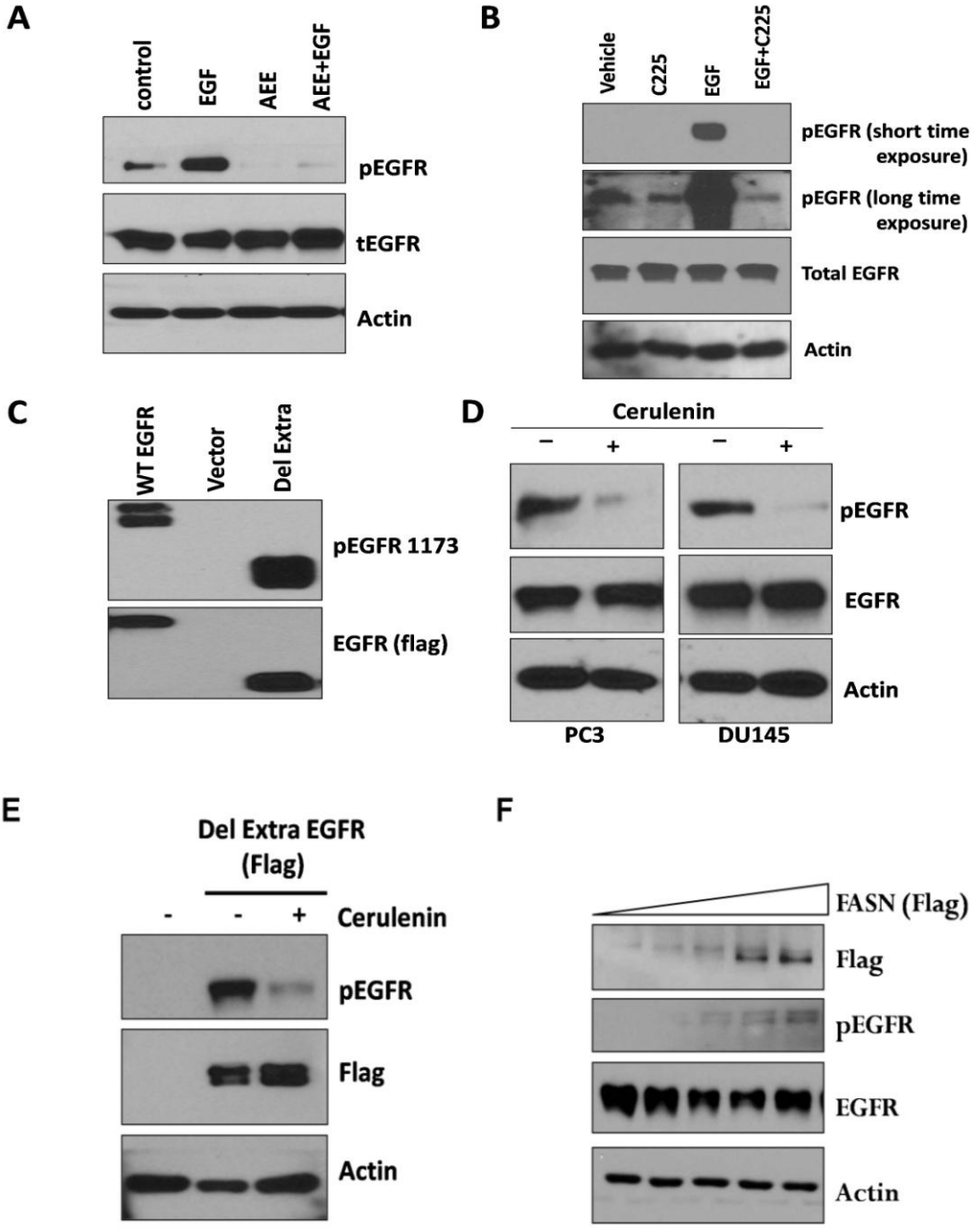

FIGURE 21. ***DE NOVO*** **FATTY ACID SYNTHESIS DEPENDENT AND EGF INDEPENDENT ACTIVATION OF EGFR.**

FIGURE 22. *De novo* fatty acid synthesis dependent activation of EGFR through palmitoylation.

A: Palmitoylation of EGFR using Acyl-Biotin exchange assay (ABE). WT EGFR (flag) was transfected into HEK 293 cells and immuno precipitated using flag antibody for ABE assay. B: Inhibition of palmitoyltransferases, reduces EGFR palmitoylation in PC3 cells. PC3 cells were treated with 2-bromopalmitate (2-BP), palmitoyl transferases, for 18 hours and EGFR palmitoylation was measured using ABE assay coupled with western blotting. C: EGF treatment increases EGFR palmitoylation in PC3 cells and 2-BP blocks EGF induced EGFR palmitoylation. D: EGF treatment increases EGFR palmitoylation in PC3 cells and inhibition of *de novo* fatty acid synthesis blocked EGF induced EGFR palmitoylation. E. Inhibition of *de novo* fatty acid synthesis blocked EGF induced cell proliferation in LNCap cells. F. Inhibition of palmitoyl transferases significantly reduced EGF induced cell proliferation in LNCap cells.

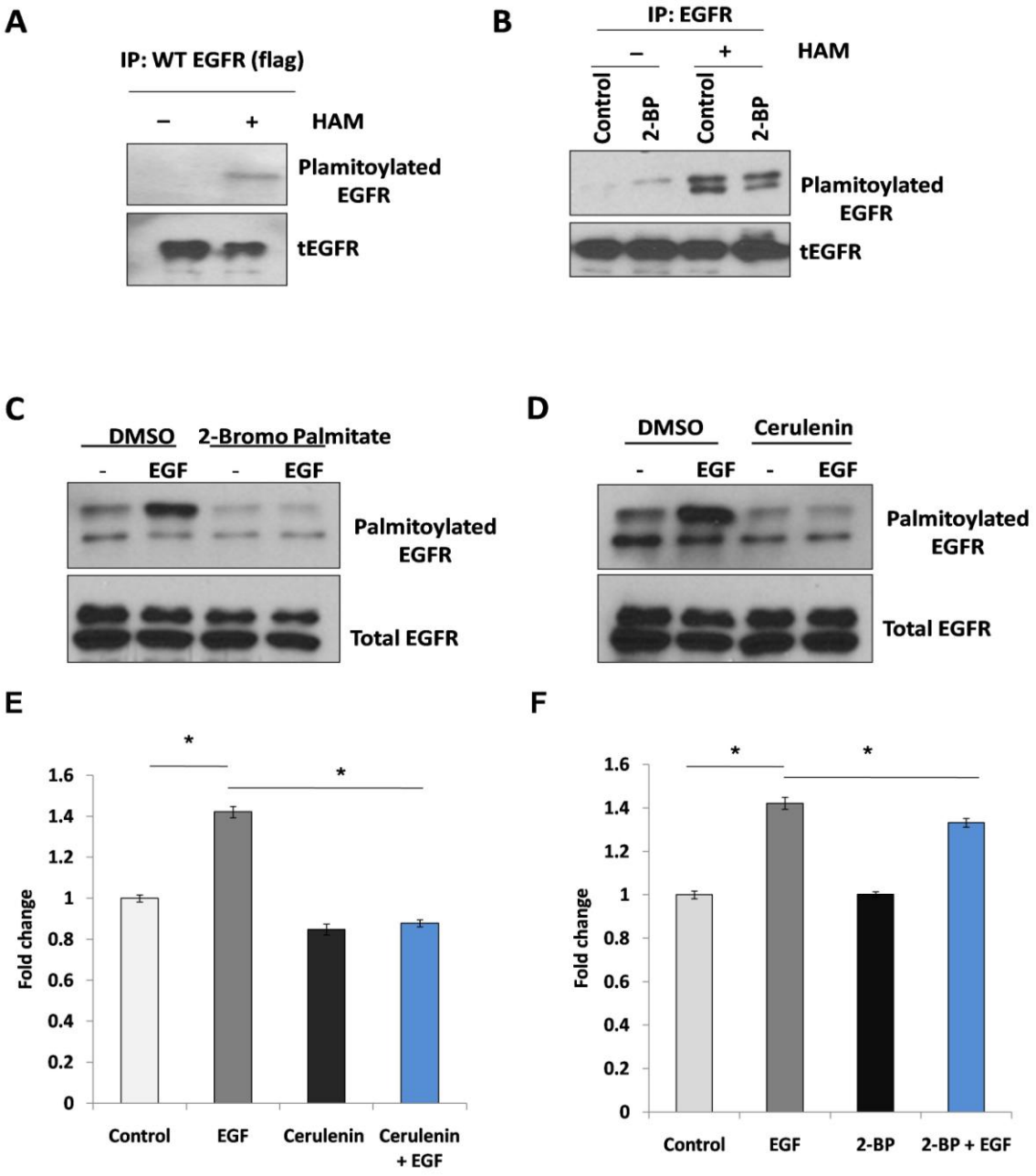

FIGURE 22. *DE NOVO* FATTY ACID SYNTHESIS DEPENDENT ACTIVATION OF EGFR THROUGH PALMITOYLATION.

study, we used LNCaP cells which express EGFR and FASN and respond to EGF treatment. As shown in the Figure 22E, EGF increased the cell proliferation in LNCap cells and inhibition of *de novo* fatty acid synthesis blocked the EGF induced cell proliferation. Inhibition of palmitoyltransferases (2-BP) significantly reduced EGF induced cell proliferation (Figure 22F).

4.3.3. EGFR palmitoylation on cysteine 797 (C797) residue is important for EGFR constitutive activation and palmitoylation.

We also found that inhibition of *de novo* fatty acid synthesis by FASN inhibitor also inhibited EGF induced EGFR activation and its downstream signaling pathway (Figure 23A). To probe palmitoylation sites on EGFR, using a bioinformatic program (CSS-Palm 3.0), we predicted four cysteine residues in the intra cellular domain of EGFR. Out of four residues, mutating cys797 to glycine significantly reduced EGFR palmitoylation (Figure 23B). We also tested whether the mutation C797G has any effect on the constitutive activation of delExtra EGFR. As shown in the Figure 23C, the C797G mutation blocked the delExtra EGFR activation. To study the role of C797 in EGFR signaling, we expressed WT EGFR and the C797G mutant and compared the MAPK activity in HEK 293T cells. As expected WT EGFR expression induced activation of MAPK in 293T cells where as mutation of C797G in EGFR failed to induce MAPK activation (Figure 23D). These results suggest the importance of C797 residue in EGFR activation and signaling.

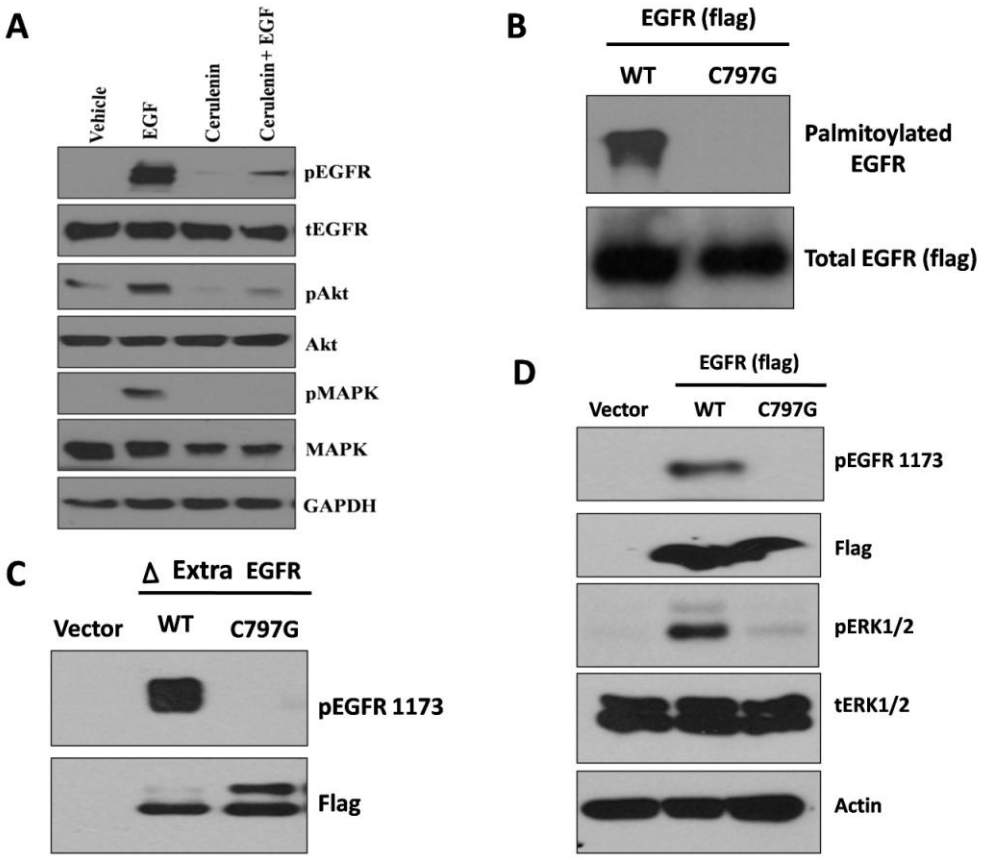

FIGURE 23. EGFR PALMITOYLATION ON CYSTEINE 797 RESIDUE IS IMPORTANT FOR EGFR CONSTITUTIVE ACTIVATION AND PALMITOYLATION.

A: Western blot analysis of protein samples for pEGFR, EGFR, pAkt, Akt, pMAPK, MAPK and GAPDH isolated from PC3 cells treated with 10ng of EGF +/- Cerulenin (5ug/ml) for 15 minutes. Cells were pretreated with cerulenin for 4 hours for maximum inhibition of FASN. B: Mutation of 797 cysteine residue to glycine blocks EGFR palmitoylation. C: C797G mutation blocks del extra EGFR phosphorylation when expressed in HEK 293 cells. D: C797G mutation blocks EGFR phosphorylation and its signaling pathway when expressed in HEK 293 cells.

4.3.4. C797 palmitoylation site is conserved in kinase active ErbB members.

We also tested whether the other members of EGFR family also undergo palmitoylation. Like EGFR, all of the members of ErbB, HER2, 3 and 4, also can be palmitoylated when expressed in HEK 293T cells, however palmitoylation level of Her3 is lower compared to the other members (Figure 24A and 24B). We also compared the other ErbB members whether C797 site (FG**C**LLDYV**)** of EGFR is conserved among ErbB members. Protein sequence alignment analysis showed that the palmitoylation site C797 is conserved in kinase active ErbB members, EGFR (C797), Her2 (C805) and Her4 (C803) but not in the kinase inactive member of ErbB member, HER 3 (Figure 24C). We mutated serine residue to cysteine in HER3 to determine whether creating a functional palmitoylation site would increase palmitoylation of Her3. As shown in the figure 24D, S-C increased Her3 palmitoylation. We also mutated the Cysteine residue in HER2 and 4 to glycine to test the significance of palmitoylation site on MAPK activation when transfected into HEK 293T cells. Substitution of Cysteine residue to glycine in Her2 and Her4 failed to activate MAPK as WT HER2 and 4, suggesting the importance of conserved cysteine (palmitoylation site) in constitutive activation of ErbB members (Figure 24E). Even though S-C mutation increased Her3 palmitoylation, we failed to observe any increase in MAPK signaling by Her3 (Figure 24E). Dimerization of EGFR is an important step for its activation. We tested whether C797 mutation is important for EGFR dimerization. As shown in the figure 25A, WT EGFR can form dimers in the absence of ligands and upon EGF addition EGFR dimers increased even more, importantly the C797G EGFR mutant failed to form dimers regardless of EGF. Supportively, the S794C mutation in HER3

increased the ability of its dimerization. As shown in the Figure 25B and 25C S794C Her3 formed more dimers compared to the WT Her3.

4.3.5. Palmitoylation is important for EGFR localization to plasma membrane and its stability.

Even though EGFR can exist in the mitochondria and nucleus, predominant levels of EGFR exists in the plasma membrane. Palmitoylation or myristoylation of proteins tethers them to the plasma membrane and provide flat form for their interaction with other proteins and other ErbB members and activation by ligands. To test the importance of palmitoylation for EGFR membranous localization, we inhibited EGFR palmitoylation using the FASN inhibitor, cerulenin. As shown in the Figure 26, the control cells have EGFR predominantly localized at the plasma membrane where as when FASN was inhibited EGFR was appeared to localized more in the cytoplasm compared to the control cells. We also observed that cerulenin reduced EGFR levels and induced ubiquitination (Figure 27A and 27B). Co-staining of EGFR showed that cerulenin treatment resulted in EGFR translocation to the lysosomes (Figure 27C), suggesting that *de novo* synthesis fatty acids is important for EGFR plasma membranous localization and inhibition of FASN results in reduced EGFR plasma membranous localization and increased lysosomal degradation.

FIGURE 24. C797 palmitoylation site is conserved in kinase active ErbB members.

A: Palmitoylation assay of ErbB members using ABE assay in HEK 293 cells. B: Quantification of palmitoylation of ErbB members and y-axis represents the normalized palmitoylated ErbB. C: Comparison of C797 palmitoylation sequence of EGFR with HER2, HER3 and HER4. D: S794 in HER3 increases HER3 palmitoylation. E: Western blot analysis of protein samples for flag, pMAPK, MAPK and GAPDH. Protein samples were prepared from HEK293 cells transfected with WT or cysteine mutants of ErbB members.

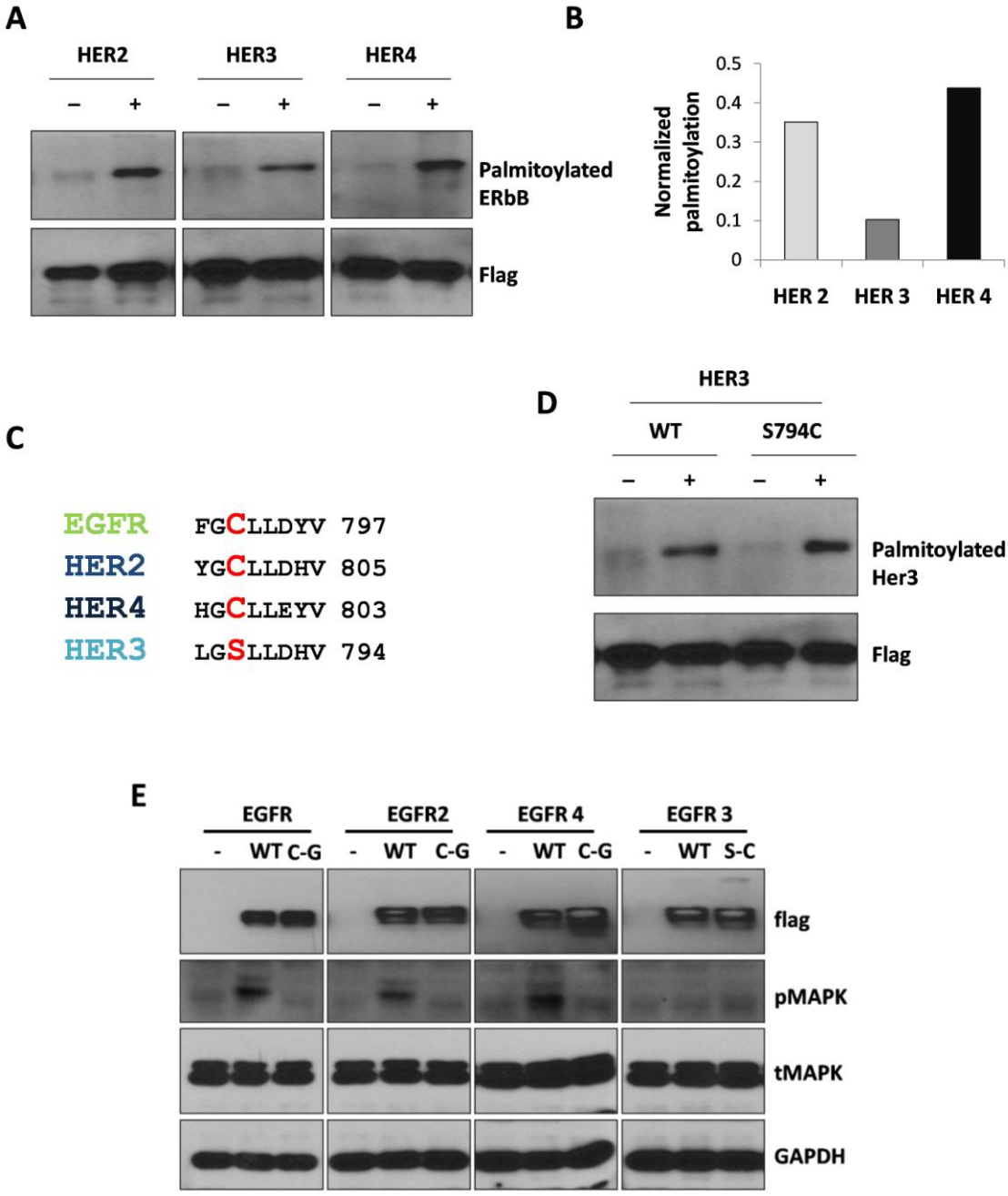

FIGURE 24. C797 PALMITOYLATION SITE IS CONSERVED IN KINASE ACTIVE ERBB MEMBERS.

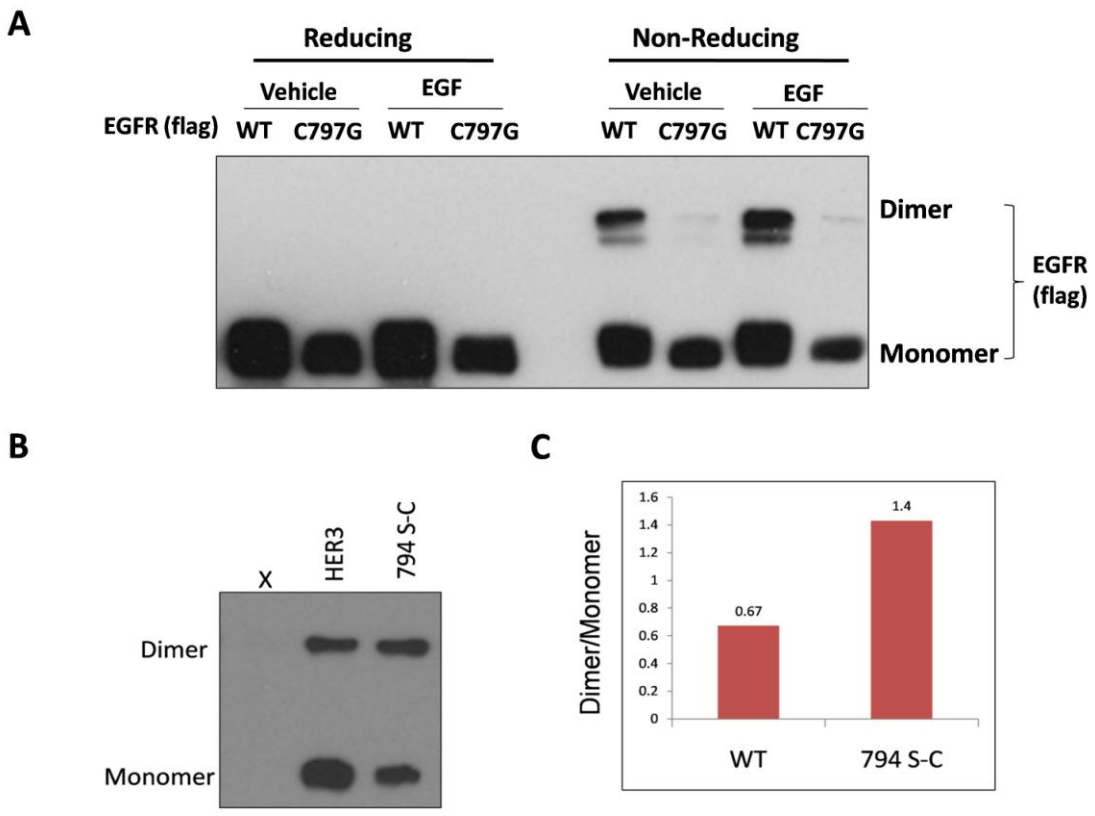

FIGURE 25. C797 IS IMPORTANT FOR EGFR DIMERIZATION.

A: HEK 293 cells were transfected with WT or C797G EGFR for 24 hours and treated with DMSO or EGF at 20ng/ml for 20 minutes and protein samples were analyzed for EGFR dimers through western blotting under reducing or non-reducing conditions. B: Dimerization of WT or S794C HER3 in HEK 293 cells. C: Quantification of HER3 dimers presented as a ratio of dimer to monomer.

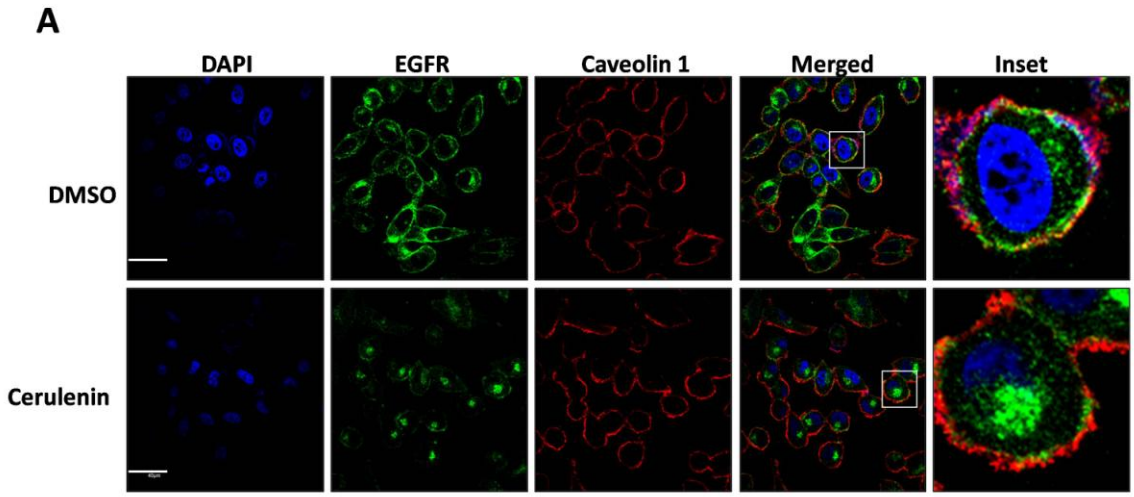

FIGURE 26. PALMITOYLATION IS IMPORTANT FOR EGFR LOCALIZATION TO PLASMA MEMBRANE.

A: Immuno fluorescent images of PC3 cells stained for nucleus (DAPI), EGFR (green), and Caveolin1 (red, plasma membrane). PC3 cells were treated with DMSO or cerulenin (5ug/ml) for 24 hours and stained for EGFR and Caveolin1.

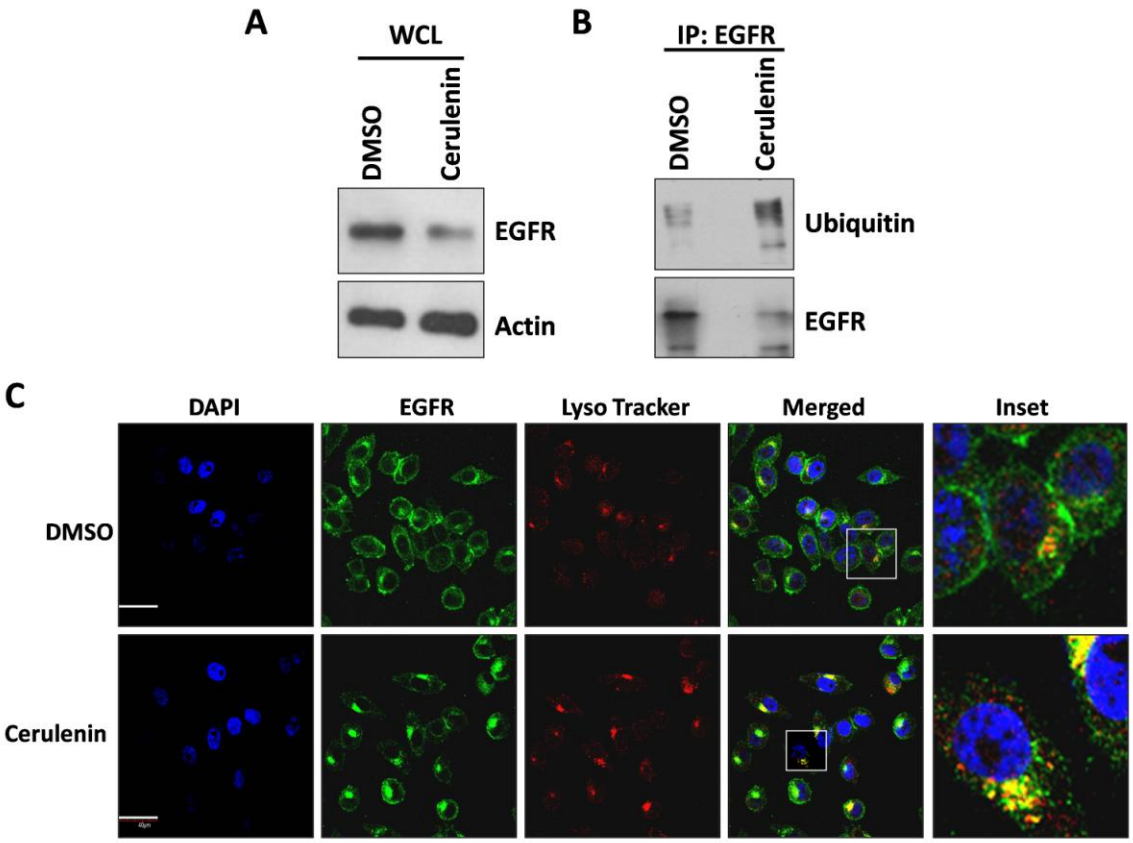

FIGURE 27. INHIBTION OF *DE NOVO* FATTY ACID SYNTHESIS EGFR UBIQUITINATION AND LYSOSOMAL DEGRADATION.

A: Western blot analysis of protein samples for EGFR and Actin isolated from PC3 cells treated with FASN inhibitor, cerulenin at 5ug/ml for 16 hours. B: Ubiquitination of EGFR in PC3 cells. EGFR was immuno precipitated from PC3 cells treated with DMSO and Cerulenin for 24 hours and analyzed for ubiquitin and EGFR. C: Immuno fluorescent images of PC3 cells. PC3 cells were treated with DMSO or cerulenin at 5ug/ml for 24 hours and stained for EGFR (green), lysosomes (red) and nucleus (DAPI, blue).

4.3.6. EGFR tyrosine kinase inhibitors promote *de novo* lipogenesis in prostate cancer cells.

As shown in the Figure 13 and 14 that EGF increases *de novo* fatty acid synthesis through EGFR-FASN interaction and FASN dimerization. We found that EGFR interacts with FASN independent of EGFR's kinase activity. As shown in the Figure 28 A, both WT and KD EGFR interacts with FASN. Treatment of cells EGFR TKIs did not inhibit EGFR-FASN interaction in PC3 cells (Figure 11A). Additionally we also observed that EGFR TKI, AEE788, induced dimerization of EGFR (Figure 28B). As EGFR interacts with FASN, we hypothesized that dimerization of EGFR could facilitates the dimerization of FASN. As shown in the Figure 28C, AEE788 indeed induced FASN dimerization. To study the importance of AEE788 can induce FASN dimerization, we measured lipid content in EGFR TKI treated cells (residual cells after 48 hours of drug treatment). As shown in the Figure 28E, EGFR kinase inhibitors significantly increased the lipid content. To test whether the increased lipid content is due to the *de novo* synthesis we inhibited FASN. Inhibition of FASN blocked the AEE788 induced lipid content (Figure 28F) suggesting the involvement of *de novo* fatty acid synthesis in TKI induced lipid content. Knowing the role of FASN in EGFR intrinsic activation, palmitoylation and increased *de novo* lipogenesis by TKI, we determined the effects of inhibitions of FASN or palmitoyl transferases on cell growth. Cerulenin and 2-BP significantly sensitized prostate cancer cells to the growth inhibitory effects of EGFR kinase inhibitors (Figure 29A, 29B and 29D).

FIGURE 28. EGFR tyrosine kinase inhibitors increase lipid content in drug residual cells dependent on FASN.

A: EGFR tyrosine kinase independent interaction with FASN. HEK 293 cells were transfected with FASN +/- WT or KD EGFR for 24 hours and EGFR was pulled down using flag antibody. Immunoprecipitated complexes were analyzed through western blot for FASN and EGFR (flag). B: PC3 cells were treated with DMSO or AEE788 at 5uM for 30 minutes and protein samples were analyzed for EGFR dimers through western blotting under reducing or non-reducing conditions. C: PC3 cells were treated with DMSO, EGF or AEE788 at 5uM for 30 minutes and protein samples were analyzed for FASN dimers through western blotting under reducing or non-reducing conditions. D: DU145 cells were treated with DMSO or AEE788 at 5uM for 30 minutes and protein samples were analyzed for FASN dimers through western blotting under reducing or non-reducing conditions. E: Adipored assay on PC3 cells treated with EGFR TKIs, AEE788 (10uM), Erlotinib (20uM), Iressa (20uM) and AG1478 (12uM) for 48 hours. F: Adipored assay on PC3 cells treated with AEE788 (10uM) +/- cerulenin for 48 hours. Total lipid content is normalized to cell number and expressed as fold change.

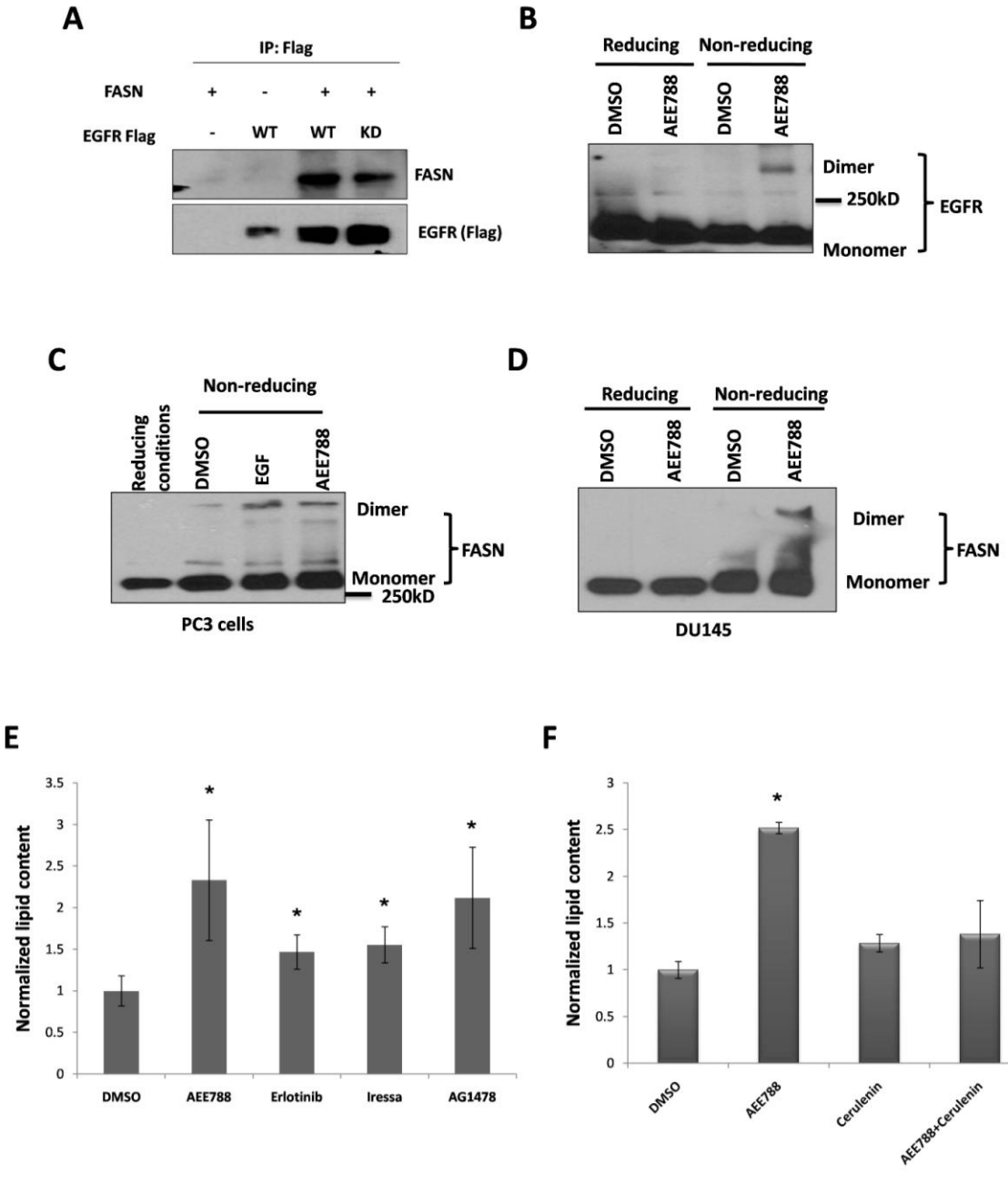

FIGURE 28. EGFR TYROSINE KINASE INHIBITORS INCREASES LIPID CONTENT IN DRUG RESIDUAL CELLS DEPENDENT ON FASN.

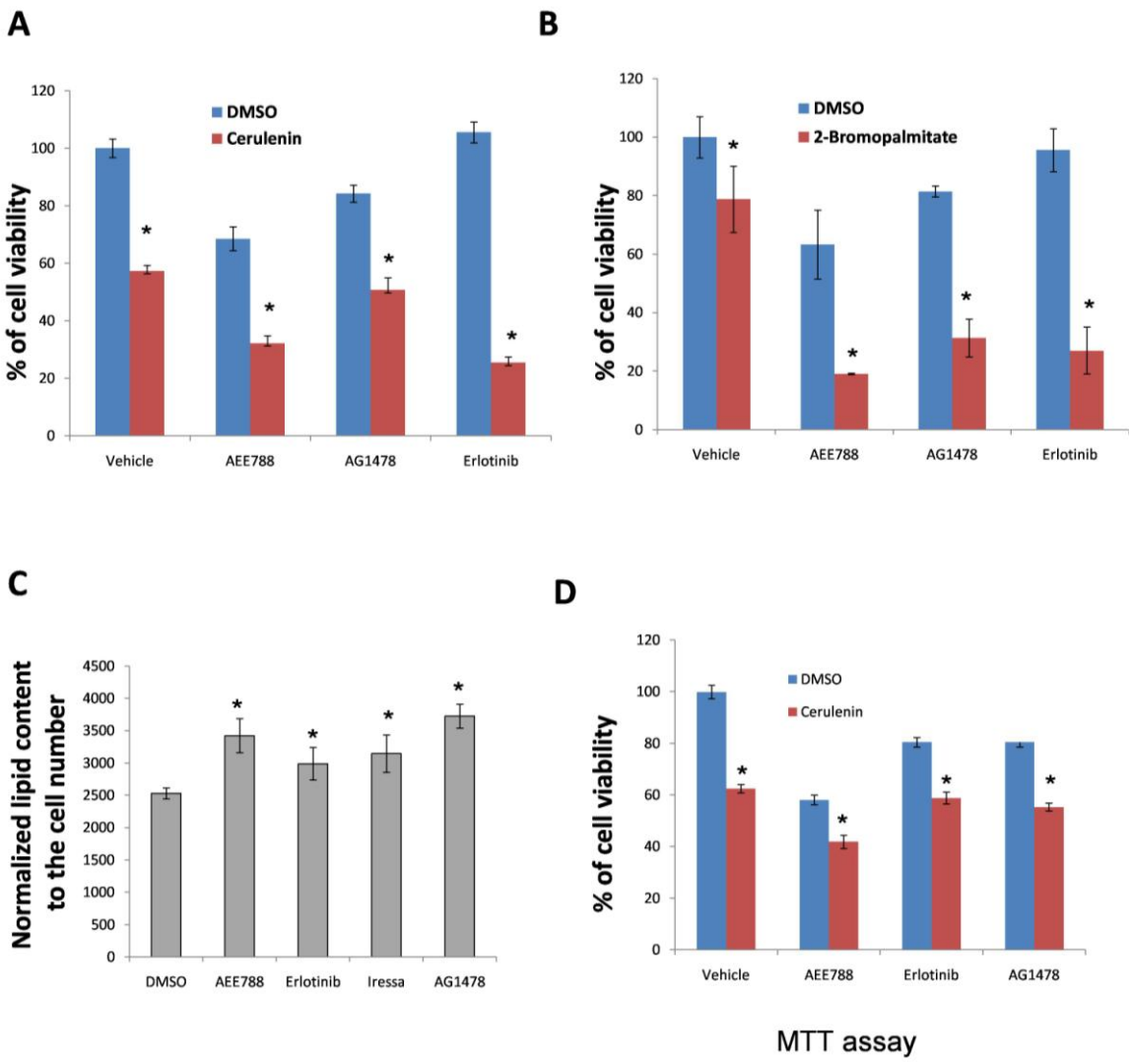

FIGURE 29. CERULENIN AND 2-BP SENSITIZES PROSTATE CANCER CELLS TO THE GROWTH INHIBITORY EFFECTS OF EGFR KINASE INHIBITORS.

A: Cell viability assay in PC3 cells treated with EGFR TKI alone or in combination with Cerulenin for 48 hours. B: Cell viability assay in PC3 cells treated with EGFR TKI alone or in combination with 2-Bromopalmitate for 48 hours. C: Adipored assay on DU145 cells treated with EGFR TKIs, AEE788 (10uM), Erlotinib (20uM), Iressa (20uM) and AG1478 (12uM) for 48 hours. D: Cell viability assay in DU145 cells treated with EGFR TKI alone or in combination with Cerulenin for 48 hours.

4.4. Discussion of Project #2

EGFR is often overexpressed/overactivated in many human cancers of epithelial origin (Di Lorenzo et al., 2002; Normanno et al., 2006; Ow et al., 1995; Scher et al., 1995a; Turkeri et al., 1994). It has been observed by several studies including our study that EGFR constitutively active in the absence of external ligands. In glioblastoma, over expression of EGFR vIII, in which subdomains I-II are deleted, is commonly observed (Gan et al., 2013). The deletion of sub-domains I-II results in impaired binding of EGFR to its ligand but this form of EGFR is highly active. Similar to EGFR vIII, HER2 has ligand binding domain but it cannot bind to any ligands and when it is over expressed in HEK 293T cells, which doesn't express any ErbB members, it was found that HER2 is constitutive phosphorylated and is able to activate MAPK signaling (Figue 24E). All these studies suggest that the classical ligand or ligand binding domain is not required for the constitutive activation of EGFR. We observed that inhibition of *de novo* fatty acid synthesis repressed EGFR phosphorylation and over expression of FASN increased EGFR activation in serum free conditions suggesting the importance of *de novo* fatty acid synthesis in intrinsic or constitutive activation of EGFR in cancer. We observed that EGFR can undergo palmitoylation which could be inhibited by cerulenin or 2-BP (figure 22C and D). Based on these observations we hypothesized that *de novo* synthesized palmitate intrinsically activates EGFR through palmitoylation. Palmitoylation is a post translational modification of proteins catalyzed by palmitatoyl acyl transferases (PATs). *De novo* fatty acid synthesis associated is with increased palmitoylation and activation of EGFR as inhibition of FASN blocked EGF induced palmitoylation and activation.

Palmitoyaltion of proteins is associated with activation of proteins. Increased *de novo* fatty acid synthesis is associated with palmitoylation of Wnt1 (Fiorentino et al., 2008). Wnt1 palmitoylation stabilizes and activates beta catenin signaling pathway. Inhibition of *de novo* fatty acid synthesis reduced beta-catenin signaling. In consistent with this data, inhibition of *de novo* fatty acid synthesis dependent EGFR by cerulenin blocked EGF induced EGFR phosphorylation, MAPK and Akt activation. Our findings are further supported by the observation of Jin et al (2010) in which inhibition of FASN blocked HER2 and HER3 mediated signaling in breast cancer cells. Palmitoylation/depalmitoylation is a dynamic and reversible process occurs on Cysteine residues. Mutational studies showed that C797 is important for EGFR activation and palmitoylation. Bioinformatic comparison studies showed that C797 of EGFR is conserved among kinase active ErbB members (Her2 and 4) but not in Her3 which is a kinase inactive member suggesting the importance of C797 residue in ErbB family proteins activation and mutating C797 blocked EGFR palmitoylation and its signaling. Palmitoylation increases the hydrophobicity of proteins and their association with the plasma membrane. In consistent with others findings (Aicart-Ramos et al., 2011; Eisenberg et al., 2013; Resh, 2006), palmitoylation promotes membrane localization, stability and activation of EGFR. The mechanism of intrinsic activation of EGFR through palmitoylation could serve as a potential targetable mechanism for increasing EGFR targeted therapies efficacy as supported by our data (Figure 29).

CHAPTER 5

Project #3: Role of *de novo* fatty acid synthesis in Intrinsic Activation of EGFR in chemoresistant cancer cells

5.1. Introduction to the problem

Current standard combination chemotherapy regimens for patients with metastatic colorectal cancer contain 5-fluorouracil (5-FU) in combination with oxaliplatin or irinotecan. Althoughthe response rate to systemic therapies is about 50% drug resistance develops in nearly all patients with metastatic colorectal cancer, leading to about 50,000 deaths each year in the United States (Chen et al., 1999; Schellens et al., 2000). Advances in targeted therapy have not dramatically improved the outcomes of patients with metastatic colorectal cancer, and the majority of those with unresectable metastatic disease still die within 2 years of the diagnosis of metastasis (Giovanella et al., 1989; Kanzawa et al., 1990). A better understanding of the mechanisms by which cancer cells develop resistance to individual chemotherapeutic agents is urgently needed to identify novel therapeutic targets and methods that would improve survival. Cancer cells may resist SN38-mediated cell death by various mechanisms: upregulation of drug efflux (Desai et al., 2001; Saleem et al., 2000), reduction of topoisomerase I levels (Huang et al., 2000), development of mutations in topoisomerase I (Djiogue et al., 2013; Hiller and Metallo, 2013; Tamada et al., 2012), enhancement of DNA repair (Desai et al., 2001), and activation of NF-kB–regulated pathways (Cairns et al., 2011; Hanahan and Weinberg, 2011; Mashima et al., 2009). Emerging evidence suggests that altered metabolism in cancer cells is fundamentally involved in the development of drug

resistance (Hiller and Metallo, 2013; Zhang and Yang, 2013). Many of the above mechanisms may result from a reprogramming of cellular metabolism, one of the hallmarks of cancer, as in enhanced aerobic glycolysis, glutaminolysis, and *de novo* lipogenesis (Gatti and Zunino, 2005; Huang et al., 2000; Kanzawa et al., 1990; Perego et al., 2006; Saleem et al., 2000). The targeting of key metabolic enzymes sustaining these cancerous metabolic adaptations and associated growth factor receptor activation bears great promise for improving treatment efficacy in patients with metastatic diseases (Xu and Villalona-Calero, 2002).

5.2. Specific Aims

1: To study the role of *de novo* fatty acid synthesis in SN38 resistant cancer cells

2: To study the involvement of *de novo* fatty acid synthesis in activation of EGFR in SN38 resistant cells

5.3. Results of Project #3

5.3.1. SN38 increases *de novo* lipogenesis in colorectal cancer cells.

SN38 is an active metabolite of Irinotican, an FDA approved anti-cancer drug for colorectal cancer. We found that the chemoresistant cells for SN38 and Oxaliplatin showed increased lipogenesis. We measured lipid content in parental cells and resistant cells to SN38, 5-fluoro uracil and oxaliplatin (SNR, FUR and OXR) by counting lipid droplet number per cell or through adipored assay. Transmission electron microscopic images were utilized for counting lipid droplets. Figure 30A represents the TEM images of parental and resistant cells. Figure 30B represents the quantification of lipid droplets which shows that lipid content was increased in drug resistant cells compared to parental cells but not in FUR cells. We also measured total neutral lipid content using adipored assay. As shown in the figure 30C the lipid content was increased in SNR compared to parental cells. We tested lipogenic enzymes ACLy and FASN whether they are upregulated or activated in SNR cells. As shown in the Figure 31A, the increased lipogenesis was due to the increased phoshorylation of ACLy (S454) and increased protein levels of FASN in SN38 resistant cells (Figure 31B). Activation of ACLy is due to the activation of Akt. SN38 treatment acutely increased lipid content in HT29 parental cells and inhibition of FASN blocks SN38 induced lipogensis (Figure 31C). We also treated HT29 SNR cells with cerulenin which showed that the inhibition of FASN decreased lipid content in HT29 SNR cells (Figure 31D) suggesting the importance of *de novo* pathway in increased lipogensis in HT29SNR cells.

FIGURE 30. Increased lipid dropls in HT29 cells that are resistant to SN38 (HT29-SNR) or Oxaliplatin (HT29-OXR).

A. Images of HT29, HT29-SNR, HT29-and HT29-OxR cells under transmission electron microscope (TEM). B: Clusters of round shaped homogeneous lipid droplets were identified in both parental and drug resistant cells. Increased amount of lipid droplet in HT29-SNR and HT29-OxR cells, but not in HT29-FUR cells compared with the parental HT29 cells by quantification of individual cells under TEM (n=20), * $p < 0.05$ for both comparisons. C: Intracellular lipid content is increased in HT29-SNR cells compared p with parental HT29 cells as measured by AdipoRed assay.

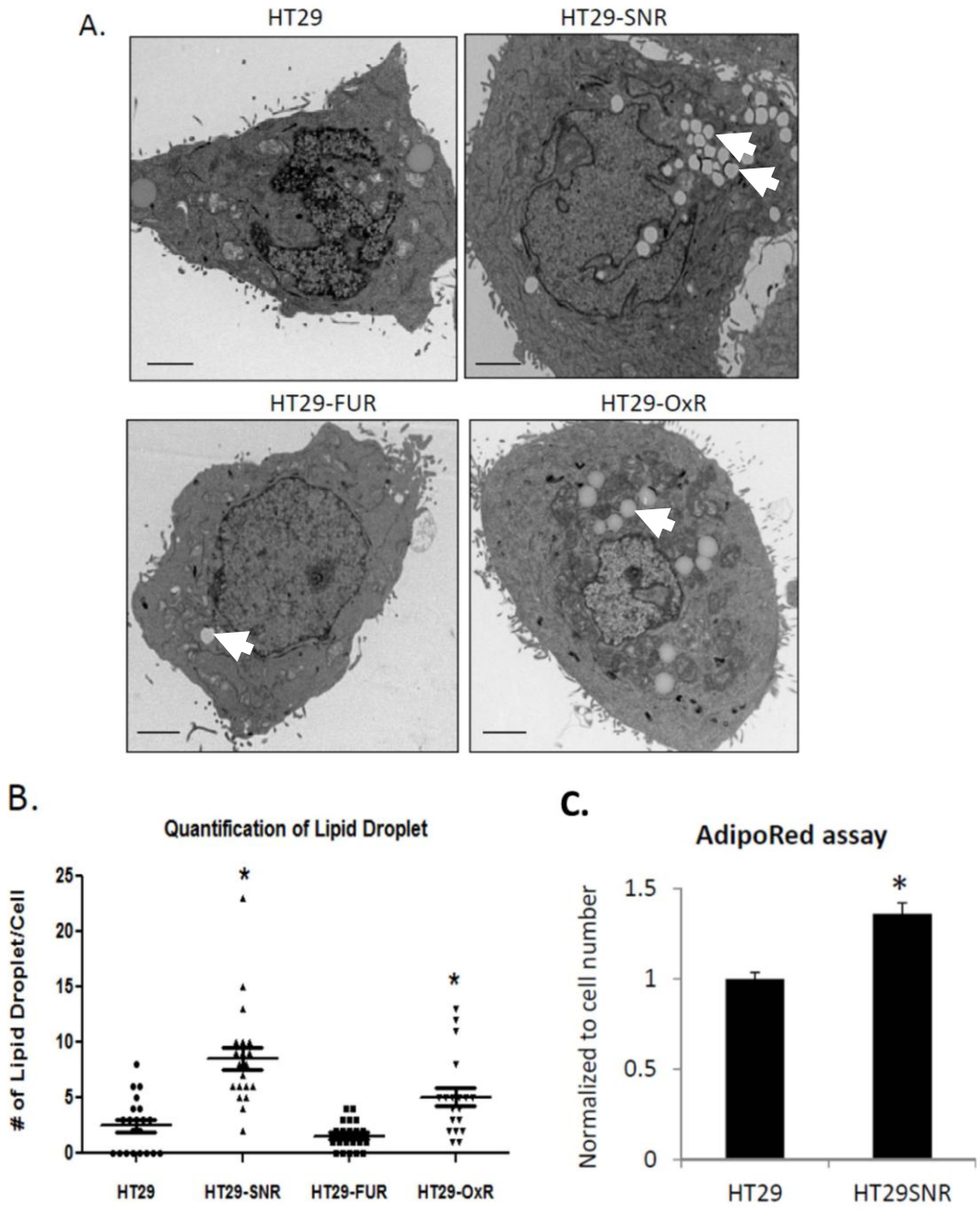

FIGURE 30. INCREASED LIPID DROPLETS IN HT29 CELLS THAT ARE RESISTANT TO SN38 (HT29-SNR) OR OXALIPLATIN (HT29-OXR).

5.3.2. Over expression of ACLy or FASN protects HT29 cells from growth inhibitory effects of SN38.

To determine the role of ACLy in drug resistance, we stably overexpressed ACLy protein by lentivirus transduction in HT29 cells (Figure 32A), which have relatively low levels of basal ACLy and p-ACLy expression. Overexpression of ACLy decreased the sensitivity of HT29 cells to SN38, as evidenced by decreased levels of cleaved PARP, an apoptosis marker, on Western blotting (Figure 32B). Furthermore, PI staining and flow cytometry showed that ACLy over expression inhibited cell killing by SN38. Figure 32C shows that the sub-G1 (apoptotic and necrotic) cell population decreased from 46.2% +/- 2.5% to 5.9% +/- 0.5% ($P < 0.05$) after ACLy overexpression following SN38 treatment but to a less extent from 7.5% +/- 0.5% to 2.8% +/- 0.5% ($P < 0.05$) following oxaliplatin treatment. Like ACLy, over expression of FASN significantly protected HT29 cells from cytotoxic effects of SN38 as measured by MTS assay.

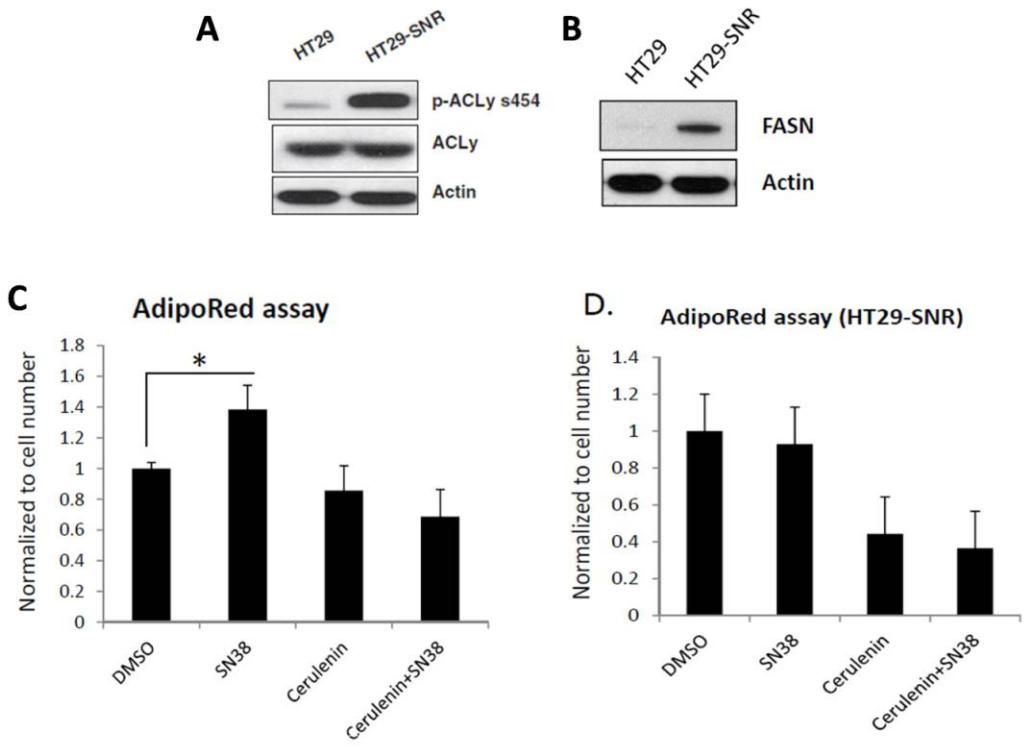

FIGURE 31. INCREASED LIPID CONTENT IN HT29SNR CELLS IS ASSOCIATED WITH INCREASED ACLY PHOSPHORYLATION AND FASN PROTEIN LEVELS.

A: Western blot analysis of protein samples for pACLy, ACLy and actin in HT29 and HT29 SNR cells. B: Western blot analysis of protein samples for FASN and actin in HT29 and HT29 SNR cells. C: Adipored assay on HT29 cells treated with SN38 (20nM) +/- cerulenin for 48 hours. Total lipid content is normalized to cell number and expressed as fold change. D: Adipored assay on HT29 SNR cells treated with SN38 +/- cerulenin for 48 hours. Total lipid content is normalized to cell number and expressed as fold change.

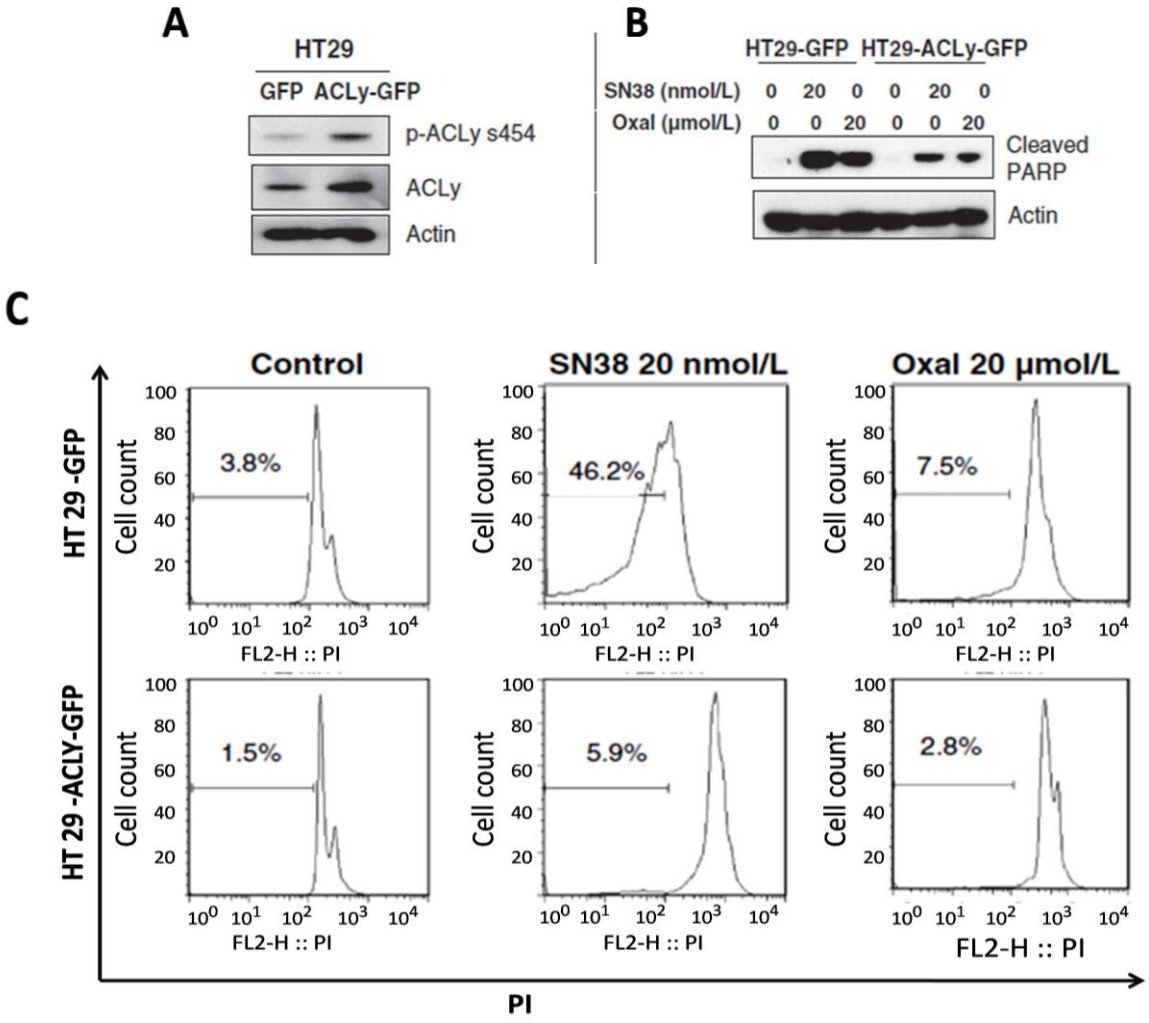

FIGURE 32. OVEREXPRESSION OF ACLY INDUCED CHEMORESISTANCE IN CHEMO-NAÏVE HT29 CELLS.

A, Stable overexpression of ACly was achieved by lentivirus transduction. B, Overexpression of ACLy partially blocked induction of cleaved PARP by SN38 and oxaliplatin (Oxal) treatment (48hours) in HT29 cells. C, ACLy overexpression blocked cell killing by SN38 (but to less extent by oxaliplatin), as indicated by the decreased sub-G1 cell count.

5.3.3. Knocking down or Inhibition of ACLy increases the sensitivity of HT29 cells to SN38 and reduces cell survival.

We next explored the relationship between SN38 and ACLy activity, and the effect of targeting ACLy in combination with SN38 treatment on chemo-naive HT29 cells. Surprisingly, SN38 activated both AKT and ACLy. A transient transfection of ACLy siRNA efficiently knocked down ACLy protein for 24 to 72 hours. After a 24-hour ACLy knockdown, we exposed the cells to 20 nmol/L of SN38 for an additional 48 hours. Knockdown of ACLy sensitized HT29 cells to SN38 without affecting AKT activity, as evidenced by increased PARP and caspase-3 cleavage (Figure 33A) and enhanced cell growth-inhibitory effect of SN38 by MTT assay (Figure 33B).We next investigated the effect of GSK165, which is a novel ACLy inhibitor modified from 2-hydroxy-N-phenylbenzenesulfonamide pharmacophore, structurally different from the known ACLy inhibitor SB-20499 and its prodrug SB-201076. As shown in Figure 33C and D, GSK165 inhibited ACLy activity in a concentration dependent manner and had a growth-inhibitory effect on cancer cells as a single agent with an IC50 of about 30 mmol/L. When 40 mmol/L GSK165 was combined with SN38 treatment, GSK165 sensitized the cells to SN38, as shown by Western blot analysis of PARP and caspase-3 cleavage (Figure 33E).

FIGURE 33. Inhibition of ACLy sensitized chemo-naïve HT29 cells to SN38.

A, SN38 activated AKT and ACLy, and knockdown of ACLy by siRNA (si) enhanced apoptosis induction by SN38, as indicated by increased levels of cleaved PARP and cleaved caspase (Casp)-3 in chemo-naïve HT29 cells. Con, control. B, Combination treatment of ACLy siRNA (24 hours) and SN38 (48 hours) has significantly enhanced growth inhibitory effect compared with SN38 single agent in HT29 cells by MTT assay. C, Concentration dependent inhibition of ACLy activity by GSK165 in a cell-free biochemical assay. D, Concentrationdependent inhibition of cell proliferation by GSK165 in HT29 cells. E, GSK165 in combination with SN38 treatment (48 hours) induced enhanced apoptosis, as indicated by increased cleavage of PARP and caspase-3 in a Western blot analysis. $P < 0.05$.

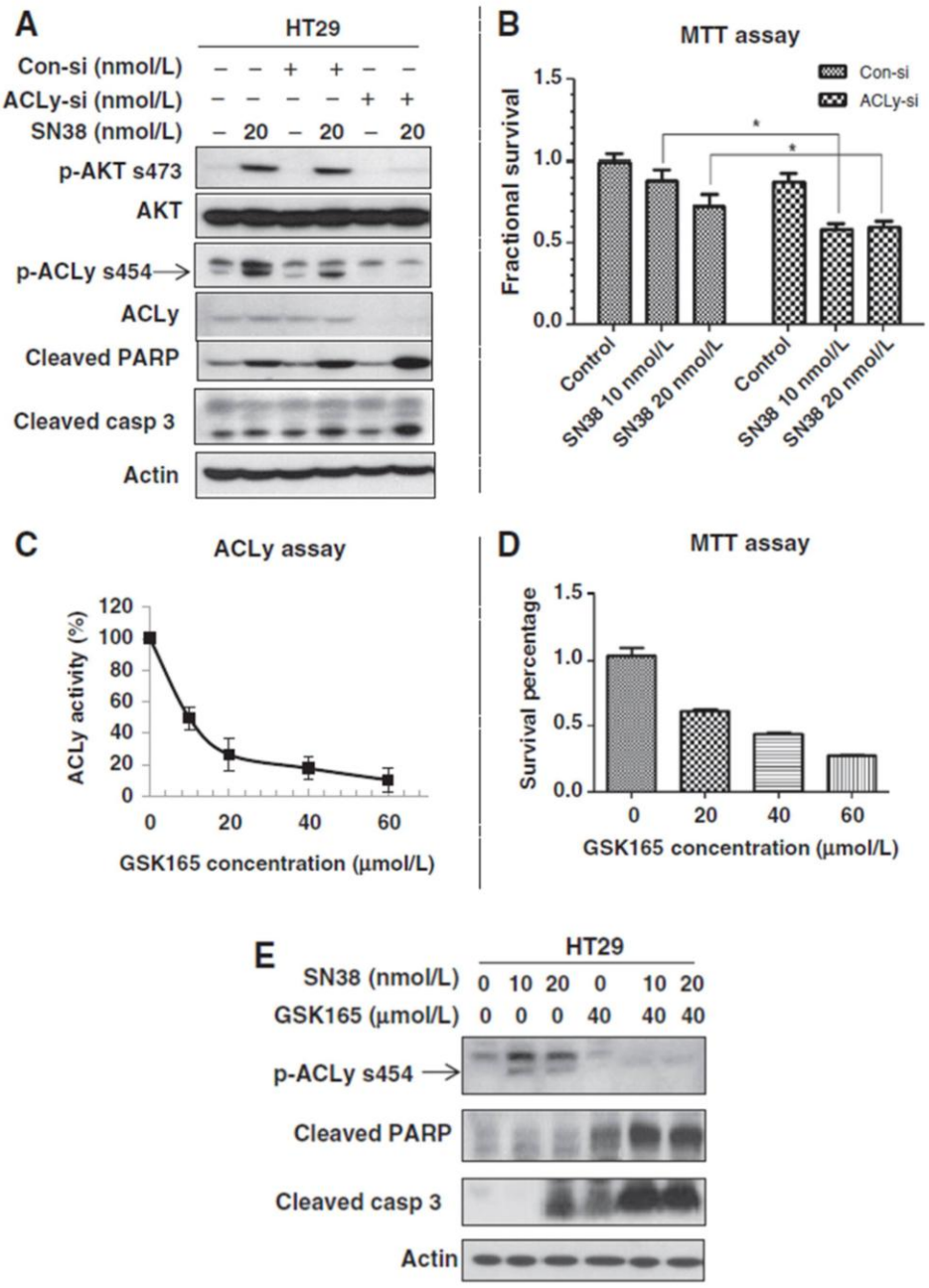

FIGURE 33. INHIBITION OF ACLY SENSITIZED CHEMO-NAIVE HT29 CELLS TO SN38.

5.3.4. SN38 activates EGFR through increased *de novo* fatty acid synthesis.

SN38 induces lipogenesis through upregulation of FASN and Akt mediated activation of ACLy. Inhibtion of FASN by cerulenin reduced SN38 induced lipid content. We observed that SN38 activates Akt and ACLy in HT29 cells. As shown in the Figure 34A, SN38 increased the activation of ACLy and Akt in a time dependent manner. SN38 increased the activity of Akt and ACLy as quickly as 15 minutes. Activation of Akt could be due to the activation of growth factor receptors. We wanted to test the whether SN38 activates EGFR. As shown in the Figure 34B, SN38 increased the phosphorylation of EGFR in a time dependent manner. To test whether *de novo* fatty acid synthesis is involved in the activation of EGFR, we inhibited FASN by cerulenin. As shown in the Figure 34C, SN38 activates EGFR in colon cancer cell line HT29 and inhibition of *de novo* fatty acid synthesis (FASN) by cerulenin blocked the SN38 induced activation of EGFR, suggesting the role of *de novo* fatty acid synthesis in SN38 induced activation of EGFR in cancer. In consistent with these data, Oxaliplatin also induced EGFR phosphorylation and blocking FASN activity inhibited EGFR phosphorylation (Figure 34D). Finally, treatment of HT29 cells with EGFR TKI increased the sensitivity of HT29 and HCT116 cells to SN38 and Oxaliplatin (Figure 34 E and 34F).

FIGURE 34. *De novo* fatty acid synthesis dependent activation of EGFR by SN38 and oxaliplatin.

A: Western blot analysis of protein samples for pACLy, ACLy, pAkt and actin in HT29 cells treated with SN38 at 30nM for different time intervals as shown in the figure. B: SN38 activates EGFR through phosphorylation. Western blot analysis of protein samples for pEGFR, EGFR and actin in HT29 cells treated with SN38 at 30nM for different time intervals as shown in the figure. C: Cerulenin blocks SN38 induced EGFR phosphorylation. D: Cerulenin blocks Oxaliplatin induced EGFR phosphorylation.

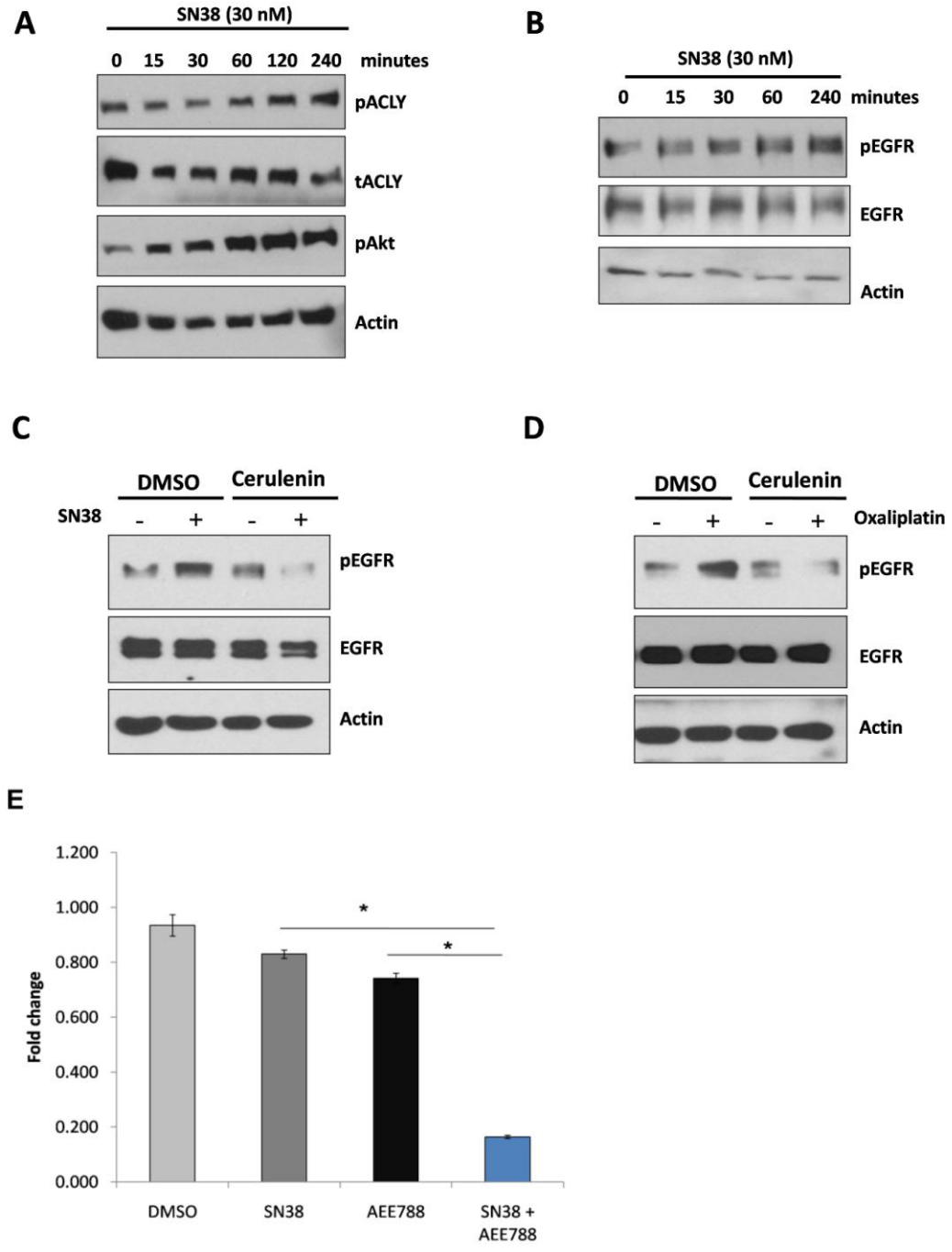

FIGURE 34. *DE NOVO* FATTY ACID SYNTHESIS DEPENDENT ACTIVATION OF EGFR BY SN38 AND OXALIPLATIN.

5.4. Discussion of Project #3

An understanding of the mechanisms of resistance to individual drugs is critical to develop more effective treatment strategies. Irinotecan is commonly used as part of the first-line chemotherapy backbone for patients with metastatic colorectal cancer. Chemoresistance in cancer cells is known to be mediated by one or more of the following mechanisms: increased drug efflux, enhanced drug inactivation, enhanced DNA damage repair, mutated survival-related genes, deregulated growth factor signaling pathways, increased expression of anti-apoptotic genes, and/or activated intracellular survival signaling following chemotherapeutic stress (Zhou et al., 2012; Pearce et al., 1998; Wellen et al., 2009). However, little is known about the role of metabolic changes in drug-resistant cancer cells. Previous findings indicated that metabolic changes are critically involved in the development of cross-chemoresistance in colorectal cancer cells(Beckner et al., 2010; Kwiatkowski et al., 1974; Migita et al., 2008; Szutowicz et al., 1979; Varis et al., 2002; Wang et al., 2012). This study revealed a novel mechanism specific to SN38 resistance that involves activation of ACLy. ACLy is the major enzyme for producing non mitochondrial acetyl-CoA that is needed for *de novo* lipogenesis and substrate acetylation (Menendez and Lupu, 2007). Upregulation of ACLy has been found in several types of cancer (Menendez and Lupu, 2007). The mechanism by which ACLy is involved in the survival machinery of cancer cells might be complex. Together with the other 2 critical lipogenic enzymes, acetyl-CoA carboxylase and fatty acid synthase, ACLy can promote cell growth and survival by increasing *de novo* synthesized fatty acids on which cancer cells depend (Zambell et al., 2003). ACLy can participate in epigenetic

events of cancer cells by regulating the levels of the key substrate of histone acetyltransferase, acetyl-CoA (Wellen et al., 2009). ACLy can regulate cell survival through coordination with cellular prosurvival metabolic network by controlling cytosolic levels of citrate and oxaloacetate, that is, decrease of cytosolic citrate levels can promote glycolsis by activating phosphofructose kinase (Migita et al., 2008;) and increase of oxaloacetate levels can facilitate gluconeogenesis and glycolysis. Enhanced *de novo* lipogenesis is one of the major metabolic alterations used by cancer cells to sustain survival and growth (Yoshii et al., 2009). Almost all kinds of carbohydrate can be sources of lipid synthesis, directly or indirectly. As one of the direct substrates (not carbon source) for cytoplasmic fatty acid synthase, acetyl-CoA is mainly derived from the citrate effluxes from the mitochondria, however studies have shown that colonic epithelial cells may have alternative pathways of synthesizing fatty acids independent of ACLy (Wagner and Vu, 1995), which is likely to be catalyzed by cytosolic acetyl-CoA synthase using acetate as its substrate (Potapova et al., 2000).

Our data suggest that ACLy-dependent *de novo* lipogenesis plays a critical role in SN38 resistance. Several protein kinases have been found to activate ACLy by phosphorylation, which include the nucleotide diphosphate kinase (Berwick et al., 2002), the cAMP-dependent protein kinase (Berwick et al., 2002), and the AKT (Berwick et al., 2002). The concurrent activations of AKT and ACLy by SN38 in the chemo-naive cells (Figure 33A) suggest that AKT plays a major role in SN38-induced ACLy activation which is supported by the data that inhibition of AKT almost completely abolished the phosphorylation of ACLy at its S454. Metabolic reprogram in adaptation to chemotherapeutic stresses may be a fundamental mechanism of drug resistance, although

cancer cells may achieve some common metabolic alterations, such as elevations of glycolysis and lipogenesis, via different mechanisms in response to different chemotherapeutic reagents. A better understanding of the mechanisms by which cancer cells gain resistance to commonly used chemotherapeutic reagents through metabolic adaptation bears a great potential of identifying novel targets for preventing and overcoming chemoresistance. One of the mechanisms involved in drug resistance are activation of growth factor receptors by *de novo* fatty acid synthesis. This study clearly shows that intrinsic activation of EGFR dependent on increased *de novo* fatty acid synthesis is associated with drug resistance to SN38 and Oxaliplatin. Blocking either EGFR activity or *de novo* fatty acid synthesis sensitizes colon cancer cells to anticancer drugs.

Summary

Cancer is a disease of abnormal cell growth. Altered metabolism in cancer is a recently appreciated hallmark of cancer even though the Warburg effect (aerobic glycolysis) was observed in early 1900s. Altered metabolism includes upregulation of aerobic glycolysis, known as Warburg effect, increase of *de novo* fatty acid synthesis, dysregulated glutamine metabolism and upregulation of the pentose phosphate pathway. Altered metabolism in cancer diverts the carbon flux from energy production to meet the abundant requirement of building blocks in rapidly proliferating cancer cells. *De novo* fatty acid synthesis, one of the altered metabolic pathways, is important for cell profileration, survival and drug resistance in cancer. EGFR is a receptor tyrosine kinase expressed in many human cancers of epithelial origin and metastasized cancers. Over expression or activation of EGFR family members can induce the transformation of normal cells to cancer by manipulating several metabolic pathways. EGFR and FASN are commonly co-over expressed in many human cancers. These oncogenic proteins regulate the functions of each other interdependently at transcriptional and post translational levels. The objective of my dissertation was to study the role of *de novo* fatty acid synthesis in intrinsic activation of EGFR and drug resistance in cancer.

Besides plasma membranous and nuclear localization, EGFR also exists in the mitochondria. In this study we extensively characterized mitochondrial EGFR and its role in the mitochondria. Utilizing immunostaining techniques and subcellular fractionation assays, we found that prostate and breast cancer cells contain EGFR in the inner membrane of mitochondria. Using EGFR kinase inhibitors and plasmids expressing

mitochondrial specific EGFR, we found that mitoEGFR activation promotes mitochondrial fusion by increasing the protein levels of PHB2 and OPA1.

Plasma membranous EGFR is activated by extracellular ligands such as EGF. MitoEGFR cannot be activated by EGF since EGF is a polypeptide protein and is not permeable through the plasma membrane to activate mitoEGFR. Activation of pmEGFR increased the activity of mitoEGFR and *de novo* fatty acid synthesis through activations of ACLy and FASN. When we inhibited FASN, it blocked pmEGFR induced activation of mitoEGFR and mitochondrial fusion. These observations led us to hypothesize that *de novo* synthesized fatty acids activate intra cellular EGFRs such as mitoEGFR. Our *in vitro* kinase assays with isolated mitochondria revealed that palmitate, the major product of *de novo* fatty acid synthesis, activated mitoEGFR. *In vitro* palmitoylation assay and mutational studies showed that *de novo* synthesized palmitate activates mitoEGFR through palmitoylation at Cysteine 797 residue. Along with mitoEGFR, we also found that pmEGFR can also undergo *de novo* fatty acid synthesis dependent palmitoylation which is important for EGFR activation, dimerization, plasma membranous localization, stability and signaling. Palmitoylation of EGFR is also important for EGFR mediated cell proliferation as FASN inhibitor or palmitoylation inhibitor blocked EGF induced proliferation. Knowing the significant role of palmitoylation in EGFR mediated functions, targeting *de novo* fatty acid synthesis or palmitoylation may significantly improve the sensitivity of cancer cells to EGFR TKIs.

EGFR can promote glucose consumption and the Warburg effect through upregulation of glycolytic enzymes, however, EGFR tyrosine kinase inhibitors have not produced

satisfactory clinical outcomes. A deeper understanding of the involvement of altered metabolic pathways in EGFR signaling is needed to improve EGFR targeted cancer therapy. In this study, we also found that EGFR (both kinase dependently and independently) promotes *de novo* fatty acid synthesis and promotes cell survival and drug resistance and inhibiting *de novo* fatty acid synthesis highly sensitized prostate cancer cells to low concentrations of EGFR TKIs. Resistance to anticancer drugs, SN38 and oxaliplatin, also mediated, in part, by upregulated *de novo* fatty acid synthesis. In revealing the survival functions of upregulated *de novo* fatty acid synthesis by these anticancer agents, we found that SN38 and oxaliplatin activated EGFR dependent on *de novo* fatty acid synthesis. Blocking of fatty acid synthesis or EGFR tyrosine kinase activity significantly reduced the cell survival. In conclusion, through this study we uncovered that *de novo* fatty acid synthesis plays critical role in intrinsic activation and non-classical functions of EGFR in cancer.

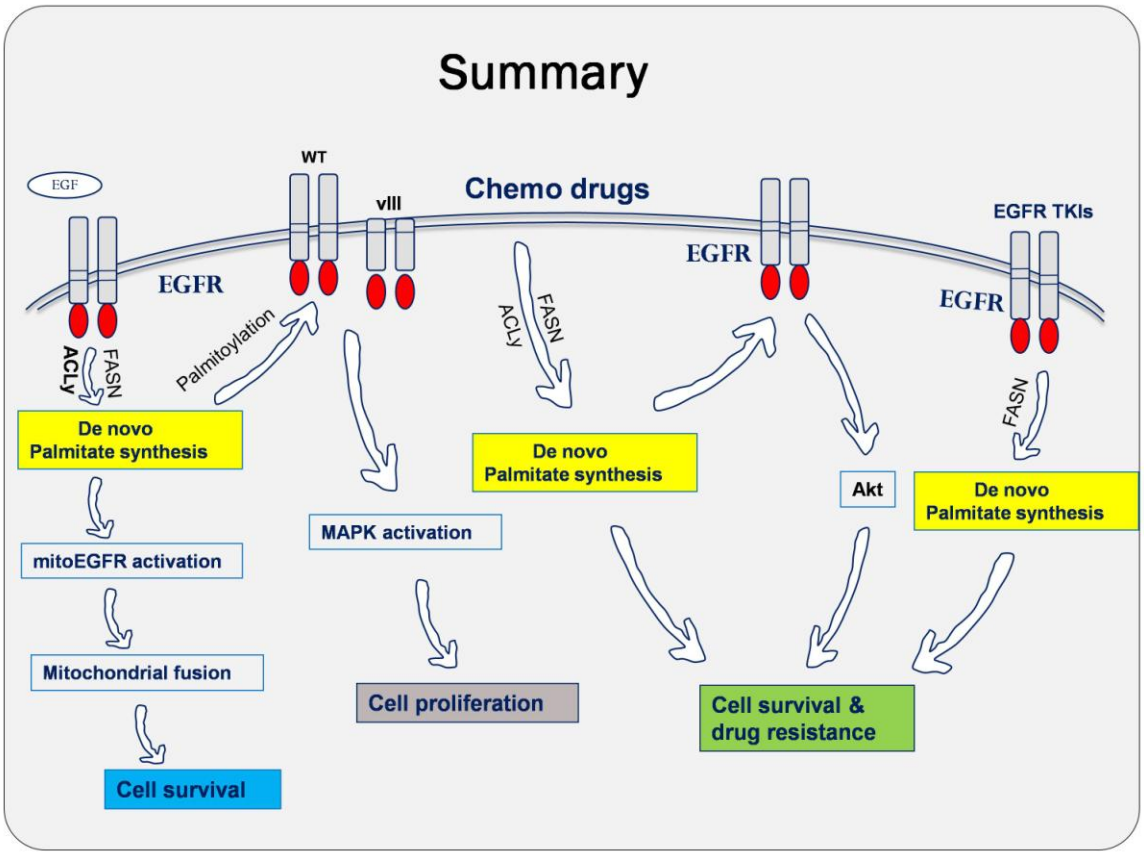

FIGURE 35. SUMMARY OF PROJECTS. INCREASED *DE NOVO* PALMITATE SYNTHESIS ASSOCIATED WITH INTRINSIC ACTIVATION OF EGFR AND PROMOTES CELL PROLIFERATION, SURVIVAL AND DRUG RESISTANCE.

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