EFFECTS OF TNFAIP8 KNOCKDOWN ON EGFR AND IGF-1R SIGNALING AND CYTOTOXICITIES OF TARGETED DRUGS IN NON-SMALL CELL LUNG CANCER CELLS

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EFFECTS OF TNFAIP8 KNOCKDOWN ON EGFR AND IGF-1R SIGNALING AND CYTOTOXICITIES OF TARGETED DRUGS IN NON-SMALL CELL LUNG CANCER CELLS

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ABSTRACT

Lung cancer is the most commonly diagnosed cancer and leading cause of cancer-related deaths worldwide. Non-small cell lung carcinoma (NSCLC) accounts for 85% of lung cancer cases. Molecular therapies targeting epidermal growth factor receptor (EGFR) have been developed for NSCLC. However, resistance of NSCLC to various therapies is a major clinical challenge. Previous studies from our laboratory and others have shown that tumor necrosis factor-α-inducible protein 8 (TNFAIP8), the first discovered member of a highly conserved TNFAIP8 family, is an oncogenic and metastatic molecule. TNFAIP8 (T8) expression is induced by NF-κB and is high in many cancers including NSCLC. This thesis project investigates the effects of T8 shRNA (shT8) on EGFR signaling in NSCLC cells. Since acquired resistance to EGFR-targeted tyrosine kinase inhibitors (TKIs) has been associated with activation of insulin-like growth factor-1 receptor (IGF-1R), the effects of shT8 on IGF-1R signaling were also studied. The shRNA silencing of T8 resulted in inhibition of growth factor (EGF, IGF-1, FGF-1, VEGF)-stimulated cell migration in A549 lung cancer cells. T8 knockdown cells showed increased expression of sorting nexin 1 (SNX1), a regulator of EGFR expression through endosomal trafficking, and siRNA silencing of SNX1 partially restored EGFR expression in shT8 cells. Decreased levels of EGF-inducible pEGFR and pERK were observed in shT8 cells as compared to control cells. T8 knockdown cells also showed increased expression of IGF-1
binding protein 3 (IGFBP3), a negative regulator of IGF-1R signaling. Consistently, IGF-1-inducible expression of pIGF-1R and pAKT was decreased, and siRNA silencing of IGFBP3 resulted in increased pIGF-1R and pAKT levels in shT8 cells. In preliminary studies, cytotoxic effects of EGFR and IGF-1R TKIs, gefitinib and AG-1024, were enhanced in shT8 cells. Aberrant regulations of EGFR and IGF-1R signaling are hallmarks of several other tumors including breast, melanoma and pancreatic cancers. Present studies suggest that TNFAIP8 plays an important role in EGFR and IGF-1R signaling in lung cancer cells, and offer a rationale for development of T8 knockdown models of a wide range of TKI-resistant tumor cells. Ultimately, these efforts may lead to a new strategy for preventing or delaying resistance to EGFR and IGF-1R-targeted therapeutics.
ACKNOWLEDGEMENTS

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Finally, I would like to thank my wife Ikuko. You have been my foundation throughout my doctoral studies. Together we have had so much joy and celebration, and, with our wonderful daughter Annabelle, I cannot think of a more perfect family. Thank you.

Many thanks,
Timothy Francis Day
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<th>Full Name/Phrase</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>amino acids</td>
</tr>
<tr>
<td>AKT/PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ALS</td>
<td>acid-labile subunit</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BRCA1</td>
<td>breast cancer type 1 susceptibility protein</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>c-MET</td>
<td>mesenchymal–epithelial transition factor</td>
</tr>
<tr>
<td>CRKL</td>
<td>CRK-like protein</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DED</td>
<td>death effector domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified essential medium</td>
</tr>
<tr>
<td>ECR</td>
<td>extracellular region</td>
</tr>
<tr>
<td>EE</td>
<td>early endosomes</td>
</tr>
<tr>
<td>EEA1</td>
<td>early endosome antigen 1</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>EMT</td>
<td>epithelial-to-mesenchymal transformation</td>
</tr>
<tr>
<td>EPS</td>
<td>epsin</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>ESCRT</td>
<td>endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>EV</td>
<td>empty vector control</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HER2/erbB-2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HER3/erbB-3</td>
<td>human epidermal growth factor receptor 3</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia-inducible factor</td>
</tr>
<tr>
<td>HRS</td>
<td>HGF-regulated tyrosine-kinase substrate</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<tr>
<td>IGF-1R</td>
<td>insulin-like growth factor receptor</td>
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<td>IGFBP</td>
<td>IGF binding protein</td>
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integral trafficking from the ER to the nuclear envelope transport

IP3  inositol triphosphate
IR  insulin receptor
IRS1/2  insulin receptor substrate 1/2
JAK  Janus kinase
JM  juxtamembrane domain
kb  kilobases
kDa  kilodaltons
MCBF  M-CAT binding factor
MEK  mitogen-activated protein kinase kinase
MES  2-(N-morpholino)ethanesulfonic acid
miRNA/miR  microRNA
MMP  matrix metalloproteinase
mTOR  mammalian target of rapamyocin
MVB  multivesicular body
nc  not changed
ng  nanogram
NSCLC  non-small cell lung cancer
P/S  100 μg/mL streptomycin, and 100 U/mL penicillin
PARP  poly ADP ribose polymerase
PBS  phosphate buffered saline
PDGF  platelet-derived growth factor
PDK1  3-phosphoinositide dependent protein kinase-1
PI3K  phosphatidylinositol-3-kinase
PIP2  phosphatidylinositol 4,5 bisphosphate
PIP3  phosphatidylinositol 3,4,5 triphosphate
PKC  protein kinase C
PLC  phospholipase C
PX  phox homology domain
qPCR  quantitative polymerase chain reaction
RIPA  radioimmunoprecipitation assay buffer
RISC  RNA-induced silencing complex
RPMI  Roswell Park Memorial Institute medium
RT  room temperature
RTK  receptor tyrosine kinase
SCLC  small cell lung cancer
scr  stably expressing scrambled shRNA
scr-cm  culture medium from cell line stably expressing scrambled shRNA
SDS  sodium dodecyl sulfate
SFM  serum free medium
SHC  SRC homologous and collagen protein
shRNA  small hairpin RNA
shT8  stably expressing TNFAIP8 shRNA
shT8-cm  culture medium from cell line stably expressing TNFAIP8 shRNA
siRNA  small interfering RNA
SMAD  C. elegans SMA/mothers against decapentaplegic
SNX1  sorting nexin 1
STAM  signal transducing adaptor molecule
STAT  Signal Transducer and Activator of Transcription
TBST  tris-buffered saline with tween
TEMED  N,N,N',N'-Tetramethylethlenediamine
TGF-α  transforming growth factor-α
TH domain  TIPE2 homology domain
TIPE1  tumor necrosis factor-α-induced protein 8-like 1
TIPE2  tumor necrosis factor-α-induced protein 8-like 2
TIPE3  tumor necrosis factor-α-induced protein 8-like 3
TKD  tyrosine kinase domain
TKI  tyrosine kinase inhibitor
TNFAIP8/T8  tumor necrosis factor-α-induced protein 8
TNF-α  tumor necrosis factor α
VEGF  vascular endothelial growth factor
VEGFR  vascular endothelial growth factor receptor
wt  wild type
2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

XTT  2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
Chapter 1. Introduction

1.1. Hypothesis, rationale, and specific aims

Molecular therapies targeting epidermal growth factor receptor (EGFR) have been developed for non-small cell lung carcinoma (NSCLC). However, primary and acquired resistance of NSCLC to various targeted therapeutics is a major clinical challenge. Previous studies from our laboratory and others have shown that tumor necrosis factor-α-induced protein 8 (TNFAIP8), the first discovered member of a highly conserved TNFAIP8 family of proteins, is an oncogenic and metastatic molecule. TNFAIP8 (T8) expression has been shown to be significantly higher in multiple tumor types including NSCLC as compared to benign tissues. Our primary hypothesis is that shRNA silencing of T8 will downregulate EGFR signaling by decreasing expression of EGFR in NSCLC cells. Since acquired resistance to EGFR-targeted tyrosine kinase inhibitors (TKIs) has been associated with activation of insulin-like growth factor-1 receptor (IGF-1R), we will also test the hypothesis that T8 shRNA (shT8) will downregulate IGF-1R signaling in NSCLC cells.

The rationale for our hypothesis is based on the following observations. First, our data suggested that EGF and IGF-1-induced cell migration is inhibited in shT8 transduced A549 lung cancer cells as compared to scrambled shRNA (scr) transduced A549 cells, suggesting that T8 may regulate signaling response to EGF and IGF-1 in these cells. Second, RNA array and antibody array profiling studies of T8 knockdown tumor cells (PC-3, MDA-MB-231, LM2-4175) showed that T8 knockdown was associated with high expression of sorting nexin 1 (SNX1), a known regulator of EGFR expression through the late endosomal trafficking. Consistently, antibody array profiling also revealed decreased expression of EGFR protein in T8 knockdown models of tumor cells tested (PC-3, MDA-MB-231, MDA-MB-435). These data
indicate that T8 may regulate endosomal processing of the internalized EGFR. Third, RNA array and antibody array profiling data indicated increased expression of IGF-1 binding protein 3 (IGFBP3), a negative regulator of IGF-1/IGF-1R signaling, in two T8 knockdown tumor cell models tested (PC-3 and C4-2B). Therefore, it is plausible that T8 may also impact IGF-1/IGF-1R signaling by regulating IGFBP3 expression.

This research has three specific aims.

**Aim 1.** To establish T8 shRNA knockdown model of A549 lung cancer cells, and determine the effects of silencing of T8 on EGF, IGF-1, FGF-1 and VEGF-induced cell migration in T8 knockdown A549 cells as compared to scr control transduced cells.

**Aim 2.** To investigate the effects of silencing of T8 on expression of SNX-1, EGFR, pEGFR and pERK in A549 cells. The effect of siRNA silencing of SNX1 on EGFR expression will be tested in T8 knockdown A549 cells.

**Aim 3.** To investigate the effects of shRNA depletion of T8 on expression of IGFBP3, and IGF-induced expression of pIGF-1R and pAKT. The effects of siRNA silencing of IGFBP3 on restoration of pIGF-1R and pAKT levels will be determined. We will also test the effects of T8 knockdown on cytotoxicities of EGFR TKI (gefitinib) and IGF-1R TKI (AG-1024) in A549 cells.

### 1.2. Introduction to non-small cell lung carcinoma (NSCLC)

Lung cancer is the most commonly diagnosed cancer and leading cause of cancer-related deaths throughout the world (Figure 1) (Jemal et al., 2011). In 2011, lung cancer was the leading cause of deaths in both men and women in the United States accounting for approximately 157,000 deaths and 27% of all cancer-related deaths (Siegel et al., 2015). Lung carcinoma has two major histopathologic types, small cell lung carcinoma (SCLC) and non-small cell
Figure 1. Estimated new cancer cases and deaths worldwide in 2008 for leading cancer types (modified from Jemal et al., 2011) (Used with permission from publisher).
carcinoma (NSCLC). SCLC is a rapidly progressing cancer. It accounts for 13% of newly diagnosed lung cancers, and only 5-10% patients diagnosed with SCLC have a life span of five years (Tognela et al., 2014). NSCLC accounts for 85% all lung cancer cases and is divided into three histological subtypes, adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (http://www.xalkori.com). Lung adenocarcinoma is the most common subtype of NSCLC lung cancers (Zer et al., 2014). It is localized to the periphery of the lung and is prevalent in current or past smokers. Squamous cell carcinoma is localized to epithelial cells lining the airways, and is also linked to smoking. Finally large cell carcinoma is an aggressive cancer which can appear anywhere in the lungs. Current treatments for stages I, II, and III NSCLC include a combination of surgery, chemotherapy, and radiotherapy. Lobectomy is the standard treatment for stage I NSCLC. For patients unable to undergo surgery, stereotactic ablative radiotherapy is an alternative treatment option. Stage II and stage III NSCLC patients receive post-surgical chemotherapy with a two-drug combination including cisplatin (Vansteenkiste et al., 2013).

1.2.1. **Representative targetable pathways in NSCLC.** Examination of lung adenocarcinoma biology has led to identification of receptor-activated signal transduction pathways and novel molecular targets, paving the way for personalized medicine treatments of NSCLC patients (Zer et al., 2014). In brief, three signaling pathways which affect NSCLC cell growth and proliferation are induced by binding of ligands hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) to their respective receptors mesenchymal–epithelial transition factor (c-MET), vascular endothelial growth factor receptor (VEGFR), and epidermal growth factor receptor (EGFR). EGFR is a well-known therapeutic target in lung cancer. Upon binding of EGF to EGFR, downstream intracellular signaling is activated through two major pathways. RAS activation leads to
signaling through BRAF, mitogen-activated protein kinase kinase (MEK), and extracellular-signal-regulated kinase (ERK). Fusion protein BCR-ABL may have bidirectional activity leading to activation of RAS and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways. The second pathway involves activation of phosphatidylinositol-3-kinase (PI3K)/3-phosphoinositide dependent protein kinase-1 (PDK1)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling and transcription changes. Other targets such as histone deacetylase (HDAC), poly ADP ribose polymerase (PARP), and heat shock proteins (HSP) in the nucleus also promote NSCLC cell survival and proliferation. Several molecular therapies targeting these pathways have been developed for treatment of NSCLC. As shown in Figure 2, HGF signaling can be blocked with antibodies ficlatuzmab and onartuzumab, and inhibitors of MET include TKIs crizotinib and tivantinib. Likewise, VEGFR signaling can be inhibited by anti-VEGF antibody bevacizumab and VEGFR TKI nintedanib. Many targeted therapeutics are also available for EGFR and its downstream targets. These include anti-EGFR antibody (cetuximab) and small molecule inhibitors (afatinib, dacomitinib, neratinib, erlotinib, and gefitinib). Small molecule inhibitors targeting downstream effectors are also in clinical trials for NSCLC patients. These include BRAF inhibitors vemurafenib and dabrafenib, MEK inhibitors selumetinib and trametinib, mTOR inhibitors everolimus and temsirolimus, HDAC inhibitor vorinostat, HSP inhibitor ganetespib, and PARP inhibitors veliparib and olaparib.

1.3. Introduction to T8

1.3.1. T8 protein family: an overview. The tumor necrosis factor-α-induced protein 8 (T8) family of proteins is a relatively newly discovered group of highly conserved proteins comprised of four major members, namely T8, TNFAIP8-like 1 (TIPE1),
Figure 2. Overview of representative targetable pathways in NSCLC cells (Zer et al., 2014) (Used with permission from publisher).
TNFAIP8-like 2 (TIPE2), and TNFAIP8-like 3 (TIPE3). All members of the family contain an atypical death effector domain (DED)-like sequence also referred to as the TIPE2 homology (TH) domain (Lou and Liu, 2011, Zhang et al., 2009a). Sequence alignment of human T8 family members along with mouse, xenopus, zebrafish and drosophila TIPE2 show highly conserved hydrophobic residues found to line the internal cavity of the protein fold (Figures 3-5) (Zhang et al., 2009a). Based on the crystal structure, TIPE2 consists of six antiparallel α-helices (Figure 4). The structure of TIPE2 is not consistent with that of a DED (Zhang et al., 2009a). Indeed, the ternary structure of the TH domain indicates a unique structure dissimilar from the DED fold in two ways; the TIPE2 sequence contributing to the structure is 60% larger than DED, and TH domain has an N-C topology which mirrors the C-N DED topology (Figure 4, panel B). The TIPE2 protein fold also contains a cavity cylindrically- shaped, 10 angstroms in diameter, and 20 angstroms in length (Figure 5, panel A). The residues lining the cavity are mostly hydrophobic (Figure 3, green residues, and Figure 5, panel B). The crystal structure analysis of TIPE3 protein also revealed a large centrally located hydrophobic cavity 10 angstrom in diameter and 20 angstrom in length in the TH domain (Fayngerts et al., 2014). This cavity is also lined with hydrophobic residues (Fayngerts et al., 2014). Currently structural analyses for T8 and TIPE1 have not been performed, but due to highly conserved sequence identity they are thought to contain similar protein folds as TIPE2 and TIPE3 (Figure 3) (Zhang et al., 2009a).

As described below, the members of T8 family have either proapoptotic (TIPE1 and TIPE 2) or prosurvival and oncogenic functions (TIPE3 and T8). Based on the crystal structure, the apparently conserved hydrophobic cavity (Figure 5) may bind to functionally distinct co-factors in various T8 members. Therefore, it appears that the
Figure 3. Sequence alignment of TIPE2 from five species and representative members of the TNFAIP8 family of proteins. Conserved sequences are highlighted in yellow. Residues located on the inner surface of the central cavity are colored green (Zhang et al., 2009a) (Used with permission from publisher).
Figure 4. A. The structure of TIPE2 is shown in two perpendicular views. The six-helices are rainbow colored. B. The topology of TIPE2 (above) is different from that of the DED (below) (Zhang et al., 2009a) (Used with permission from publisher).
Figure 5. A. The TIPE2 hydrophobic cavity is shown in three views. B. Residues within the central cavity are predominantly hydrophobic. The cavity represents a potential binding site for cofactors (modified from Zhang et al., 2009a) (Used with permission from publisher).
T8 family proteins may function as scaffold or adaptor proteins.

1.3.2. **TIPE1, an apoptosis inducer regulating the Rac1 pathway.** TIPE1 is the least studied member of the T8 family. It induces cell death by negatively regulating Rac1 activity resulting in inhibition of p65 and c-Jun (Zhang *et al*., 2014). It was found that TIPE1 is downregulated in hepatocellular carcinoma (HCC) compared to adjacent healthy tissue. In addition, overexpression of TIPE1 increased apoptosis and decreased colony formation in HCC. This indicates that loss of TIPE1 could be a prognostic marker in HCC patients (Zhang *et al*., 2014).

1.3.3. **TIPE2, a negative regulator of immune homeostasis.** TIPE2 was identified through a screen of highly expressed genes in inflamed nervous tissue but not in normal tissue (Sun *et al*., 2008). It is also expressed in thymus, spleen, lymph nodes, macrophages, and B and T lymphocytes. TIPE2 is a negative regulator of immune cell function and T-cell homeostasis. In *TIPE2* knockout mice, T-cell (CD4+ and CD8+) response to immune stimulation was increased compared to littermate controls. These mice were hypersensitive to septic shock and revealed multiorgan inflammation, enlarged spleens, and associated premature death. TIPE2 binds to caspase-8, inhibits AP-1 and NF-κB, and promotes Fas-induced apoptosis (Sun *et al*., 2008). Other studies have shown that porcine TIPE2 is expressed most highly in the spleen, followed by the lung and adipocytes, with small expression in the lymph nodes and brain (Li *et al*., 2010). TIPE2-GFP fusion protein was found to be localized in the cytoplasm, endoplasmic reticulum, and nucleus (Li *et al*., 2010). Promoter characterization of *TIPE2* gene showed functional binding sites for AP-1 and M-CAT binding factor (MCBF) (Li *et al*., 2010). Given the proliferative role of AP-1 in most cell types, TIPE2 expression may result in a feedback negative regulation of AP-1 activity. Consistently, TIPE2 was shown to have tumor suppressor
and pro-apoptotic properties. In brief, overexpression of TIPE2 in 293T cells led to downregulation of Ral-GTP by binding to the RAS activating domain of RalGEF protein RGL (Gus-Brautbar et al., 2012). Moreover, cells with overexpression of TIPE2 showed increased cell death and decreased cell motility. Conversely, cells from TIPE2 knockout cells showed increased constitutive pAKT expression and increased F-actin polymerization and motility (Gus-Brautbar et al., 2012).

1.3.4. TIPE3, a novel phosphoinositides transfer protein that promotes cancer. Recent studies have shown that TIPE3 is a novel protein that captures two major lipid second messengers, phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P2, PIP2) and phosphatidylinositol 3,4,5 triphosphate (PtdIns(3,4,5)P3, PIP3) (Fayngerts et al., 2014). Indeed, TIPE3 is the first transfer protein shown to bind and transfer PIP2 (Moniz and Vanhaesebroeck, 2014). The large hydrophobic cavity in TIPE3 was found to bind either PIP2 or PIP3 (Figure 6). Whereas in normal cells the relative plasma membrane levels of PIP2 and PIP3 are regulated by PI3K and PTEN, increased TIPE3 expression in cancer cells may promote shuttling and elevated expression of plasma membrane phosphoinositides (PIP2 and PIP3). Consequently, increased membrane-bound PIP3 was found to lead to PI3K-mediated upregulation of pAKT. In addition, TIPE3 overexpression correlated with increased PLC-mediated cleavage of PIP2, resulting in high levels of DAG and IP3 and activation of PKC and its downstream effectors pERK and cyclin D1. In cells with overexpression of TIPE3, PDGF treatment also resulted in increased levels of pAKT and pERK. When TIPE3 was overexpressed in human cancer cell line 293T, there was an increase in soft agar colony formation and increased cell proliferation. Conversely, fewer soft agar colonies were observed with shRNA downregulation of TIPE3 (Fayngerts et al., 2014).
Figure 6. TIPE3 captures phosphoinositide second messengers and promotes pAKT and pERK signaling pathways (Fayngerts et al., 2014) (Used with permission from publisher).
Overexpression of TIPE3 also correlated with tumorigenesis. Overexpression of TIPE3 in mouse xenografts of NIH3T3-HRASV12 cell line promoted tumorigenesis. TIPE3 knockout mice had decreased ability to form carcinogen 3-methylcholanthrene–induced tumors. In addition, TIPE3 was found to be upregulated in human lung cancer, and cervical, esophageal, and colon adenocarcinomas compared to normal adjacent tissues. These results suggest TIPE3 is a cancer therapeutic target (Fayngerts et al., 2014).

1.3.5. T8 is a novel prosurvival, oncogenic and metastatic molecule: implications in cancer prognosis and therapy response. T8 is the first discovered member of the T8 family (Patel et al., 1997; Kumar et al., 2000). It was identified as a novel transcript overexpressed in metastatic and radioresistant head and neck squamous cell carcinoma cells as compared to their matched primary tumor-derived cells (Patel et al., 1997). Expression analysis of normal human tissues showed wide-spread mRNA expression significantly in endocrine compartments such as the thyroid, lymph nodes, and bone marrow, but absent in other normal tissues such as the lung and liver (Kumar et al., 2000). T8 protein is a predominantly cytosolic molecule (Kumar et al., 2004). There are four major isoforms of T8 (23 kDa, 22 kDa, 21.8 kDa, and 24 kDa) (www.uniprot.org). The T8 isoforms differ in short N-terminus sequences, while the majority of the isoform sequences share 100% identity within the C-terminus (Figure 7). T8 isoform 1 (23 kDa) and isoform 3 (SCC-S2/GG2-1/NDED/MDC3.13, 21.8 kDa) are highly expressed in most cancers cell lines (Kumar et al., 2004). T8 mRNA and protein expression was found to be induced by tumor necrosis factor α (TNF-α) in human tumor cells and endothelial cells (Horrevoets et al., 1999, Kumar et al., 2000). Subsequent studies showed that T8 expression is induced by NF-κB, and overexpression of T8 promotes cell survival in NF-κB null cells, suggesting that it is an
Figure 7. Alignment of the amino acid sequences of various human TNFAIP8 isoforms (www.uniprot.org).
important prosurvival molecule downstream of NF-κB (You et al., 2001). T8 overexpression was also found to inhibit caspase-8 activity, further supporting the prosurvival function of this molecule (You et al., 2001).

Promoter analysis of approximately 2.0 kb genomic sequence upstream of the translation start site indicated potential binding sites for multiple transcription factors including NF-κB, hypoxia-inducible factor (HIF), COUP-TFI, and androgen receptor (Zhang et al., 2013). COUP-TFI is a regulatory repressor which binds to the promoter of T8 and prevents transcription. A complex including the proapoptotic protein DBC1 and COUP-TFI was found to occupy the T8 promoter. This complex was found to be crucial for repression of the T8 promoter by COUP-TFI (Zhang et al., 2009b). T8 is also an androgen-inducible molecule in prostate cancer cells (Zhang et al., 2013).

T8 is a highly oncogenic and metastatic molecule. Overexpression of T8 resulted in changed in cell morphology and increased cell proliferation and migration and invasion in MDA-MB-435 melanoma cells (Kumar et al., 2004, Zhang et al., 2006). Furthermore, injection of T8 overexpressing cells into mammary fat pads in athymic female mice led to accelerated tumor formation as compared to control cells (Kumar et al., 2004). In other studies, tail-vein injection of MDA-MB-435 cells stably expressing T8 led to significant pulmonary colonization of tumor cells as compared to control cells (Zhang et al., 2006). In addition, systemic delivery of liposome-entrapped T8 targeted antisense oligos in MDA-MB-435 tumor bearing mice resulted in significant decrease in tumor growth and pulmonary colonization (Zhang et al., 2006).

T8 appears to be an important cancer biomarker. The publicly available datasets (Oncomine) show that T8 mRNA expression is increased in several tumor types.
including prostate, renal, liver, breast, head-neck cancers, brain, lung, melanoma, pancreas, and colorectal tumors (Zhang et al., 2013). Consistently, immunohistological studies have reported significantly higher T8 protein expression in multiple tumor types (Lou and Liu, 2011). These include NSCLC (Dong et al., 2010), esophageal carcinoma (Hadisaputri et al., 2012), pancreatic carcinoma (Liu et al., 2012), prostate carcinoma (Zhang et al., 2013), gastric adenocarcinoma (Yang et al., 2014), cervical cancer (Shi et al., 2013), endometrial cancer (Liu et al., 2014), thyroid cancer (Duan et al., 2014), and NSCLC (Wang et al., 2014).

Expression of T8 has been associated with increased resistance to chemotherapeutic drugs cytosine arabinoside and anthracyclines in acute myeloid leukemia (Eisele et al., 2007). Along these lines, antisense downregulation of T8 has been associated with enhanced sensitivities to paclitaxel and docetaxel in prostate tumors grown in athymic mice. Recently nuclear T8 expression has been correlated with worse prognosis in patients with prostatic adenocarcinomas (Zhang et al., 2013). Together, these observations suggest that T8 is a viable therapeutic target in cancer.

Mechanism of T8 action remains unclear. In melanoma and prostate cancer cells, antisense knockdown of T8 correlated concomitantly with decreased expression of angiogenic and metastatic molecules such as VEGFR2, matrix metalloproteinase 1 (MMP1), and MMP9 (Zhang et al., 2006). Depletion of T8 also correlated with decreased expression of VEGFR2 in endothelial cells. These data suggest that T8 may regulate VEGF-signaling and associated biological response. In yeast two hybrid studies, T8 was found to interact with Galphalpha(i) and inhibit cell death in caspase-independent manner in Balb-D2S cells (Laliberte et al., 2010). Other proteins interacting with T8 include GDNF family receptor α 1, PARP1, Serine/arginine-rich splicing factor 2, Karyopherin α 2 (RAG cohort 1, importin α 1), DEAD (Asp-Glu-Ala-Asp)
In pancreatic adenocarcinoma, T8 expression was found to strongly correlate with increased EGFR expression (r=0.671135, P<0.05) (Figure 8) (Liu et al., 2012). Further studies are necessary to understand the mechanism of T8 and how it may impact the biology and response to targeted therapies in diverse cancer types.

1.4. Introduction to shRNA and siRNA silencing of gene expression

The ability to specifically knockdown genes in human cancer cells has greatly aided functional genomics. Small interfering RNA (siRNA) and siRNA precursors, such as small hairpin RNA (shRNA), function to silence gene expression at the mRNA level (Paul et al., 2014). ShRNA is introduced into the cell through viral vectors and becomes stably integrated into the host genome through reverse transcription (Paul et al., 2014). Subsequently, the shRNA sequence is processed by Drosha and Dicer (Figure 9) into double-stranded RNA before export from the nucleus via RAN/GTP-dependent pathway. In the cytoplasm, it is recognized by RNA-induced silencing complex (RISC) to generate separated two single-stranded RNAs, with one strand, the guide strand, involved in forming the active RISC complex. Subsequently the guide strand recruits the RISC complex to mRNAs complementary to the guide strand (Mohr et al., 2014; Deng et al., 2014). The RNase H domain of argonaute (part of the RISC complex) then degrades the mRNA resulting in abrogation of translation. In contrast, siRNA is a synthetic double-stranded RNA. It is directly incorporated into the RISC complex followed by targeting mRNA for degradation.
Figure 8. Correlation of TNFAIP8 expression with EGFR in pancreatic adenocarcinoma (Laliberte et al., 2010) (Used with permission from publisher).
Figure 9. Schematic representation of gene silencing via siRNA and small hairpin RNA (Paul et al., 2014) (Used with permission from publisher).
1.5. Introduction to EGFR-induced signal transduction and endocytic mechanisms of EGF-induced internalization and degradation of EGFR

1.5.1. EGFR structure, ligand-induced activation, and signal transduction. EGFR is a receptor tyrosine kinase with an extracellular region (ECR), single transmembrane domain, and an intracellular region consisting of a juxtamembrane domain (JM) and a tyrosine kinase domain (TKD) (Figure 10) (Bessman et al., 2014). The ECR consists of four domains: I-IV. The TKD contains three important structures: an N-lobe including a helix α-C, an activation loop, and a C-lobe. In the absence of ligand, autoinhibitory interactions occur between ECR domains II and IV as well as intracellular helix α-C and activation loop as shown in Figure 10. The transmembrane domain of EGFR is 23 residues long (AA 620-643) and is followed by the intracellular domains, including the JM (AA 643-685), TKD (AA 685-953) and the autophosphorylation domain (AA 953 – 1186) (Flynn et al., 2009, Sharma et al., 2007). The transmembrane domain may be glycosylated and localized within caveolae or lipid rafts (Flynn et al., 2009), which could help regulate dimerization and rapid internalization. The JM domain has been shown to stabilize the asymmetric dimer of the kinase domains by forming an anti-parallel helix upon ligand binding and receptor dimerization (Figure 10) (Endres et al., 2011). The TKD contains various tyrosine residues which may become phosphorylated to regulate downstream signaling events.

Ligands with potential to bind to EGFR extracellular domains include EGF, transforming growth factor-α (TGF-α), heparin-binding EGF, β-cellulin, amphiregulin, epiregulin, and epigen (Roepstorff et al., 2008). Ligand binding to extracellular domains induces a conformation shift allowing dimerization and autophosphorylation of tyrosine residues in intracellular domains (Citri and Yarden, 2006, Gomez et al., 2013). ECR domains I and III are required for ligand
Figure 10. EGF-induced activation of EGFR as described in the text (Bessman et al., 2014) (Used with permission from publisher).
binding, II and IV regulate heterogeneous or homogenous dimerization with ErbB family receptors. Binding of a ligand to domains I and III promotes a close proximity of the two domains, exposing the dimerization loop in domain II and allowing stable interaction with a second receptor. Ligand binding stabilizes the interaction and induces a rotation in the transmembrane domain, resulting in subsequent rearrangement of the intracellular region (Bessman et al., 2014). After ligand-dependent dimerization and rearrangement, the N-lobe of TKD is arranged to the C-lobe of the partner receptor tyrosine kinase (RTK) TKD, and results in tyrosine kinase activation (Figure 10) (Endres et al., 2011, Flynn et al., 2009, Kumar et al., 2008). The activator TKD allosterically activates the receiver N-lobe. After a secondary ligand binds, the EGFR dimer presents a ‘staggered’ interface, resulting in increased extracellular and intracellular symmetry (Bessman et al., 2014). Finally the C-terminal autophosphorylation domain contains regulatory tyrosines which recruit adaptor molecules within the cell, and is relatively mobile compared to the TKD (Flynn et al., 2009). Phosphorylation of key tyrosines in the C-terminal domain, Y1045, 1068 and 1086, enable ubiquitination by E3 ligase CBL, promoting internalization and future sorting through multivesicular bodies (MVBs) (Roepstorff et al., 2008). Mutations either by deletion or point mutations in various domains have been shown to alter ligand-dependence and EGFR activity, regulating cell growth and proliferation.

Downstream signaling of EGFR involves two major pathways. Interaction with GRB2 and SOS leads to RAS activation, and a signal cascade through activation of RAF, MEK1 and ERK1/2 (Figure 11) (Siegelin and Borczuk, 2014). This, in turn, promotes cell survival, proliferation, migration, and metastasis. Also PI3K may be activated (through heterodimerization with another RTK, such as HER3), followed by increase in PIP3 and phosphorylation of AKT (Siegelin and
Borczuk, 2014). This pathway predominately results in inhibition of apoptosis, and may also promote cell survival, proliferation, migration, and

Figure 11. Two major EGFR signal transduction pathways and a well-known site of various mutations within the tyrosine kinase domain observed in lung adenocarcinoma (Siegelin and Borczuk, 2014) (Used with permission from publisher).
metastasis. Several types of mutations of EGFR can result in aberrant regulation of EGFR signaling. V-ErbB was found to be a truncated isoform of EGFR in breast cancer. Truncation mutations can induce constitutive activation of EGFR. EGFRv1 (∆1-51), EGFRvII (∆521-603) EGFRvIII (∆6-273) lack portions or all of ECR, and are constitutively active and slowly recycled (Gomez et al., 2013).

1.5.2. Regulation of EGFR expression via endocytic trafficking and other mechanisms.

After ligand binding and receptor dimerization on the membrane, EGFR dimer interacts with the adaptor protein GRB2 which recruits E3 ubiquitin ligase CBL (Figure 12) (Madshus and Stang, 2009). This results in ubiquitination of lysine residues in the kinase domain. Ubiquitinated EGFR, upon interaction with epsin1 (EPS1), and EPS15, and clathrin adaptor protein AP2 in the clathrin coat is then recruited to clathrin coated pits (Tomas et al., 2014). Inhibition of EGFR ubiquitination prevents its internalization and degradation, resulting in sustained EGFR signaling (Madshus and Stang, 2009). Uptake of EGFR into the clathrin coated pits is regulated by both lamellipodin and endophilin which also regulate vesicle scission (Vehlow et al., 2013). After internalization, ligand-bound EGFR becomes partially deubiquitinated and then reubiquitinated in early endosomes (Madshus and Stang, 2009). Early endosomes (EE) are an early destination of EGFR in the endocytic compartments. Proper sorting of ligand-bound EGFR into late endocytic compartments requires the endosomal sorting complex required for transport (ESCRT) machinery (Tomas et al., 2014). The initial ESCRT machinery (ESCRT-0) is comprised of two core proteins, HGF-regulated tyrosine-kinase substrate (HRS) and signal transducing adaptor molecule (SH3 domain and ITAM motif) 1 (STAM 1) which bind to ubiquitin on EGFR (Clague et al., 2012). ESCRT machinery mediated trafficking of the ligand-bound EGFR involves targeting of the receptor to the MVB and late endosomes/lysosomes for degradation (Figure 12)
Figure 12. A model illustrating endosomal trafficking of EGFR upon ligand stimulation (Madshus and Stang, 2009) (Used with permission from publisher).
(Tomas et al., 2014). Under low pH, partially ubiquitinated EGFR dissociates from the ligand and is targeted to recycling endosomes where it is deubiquitinated and recycled to the plasma membrane (Figure 12).

Recently it has been shown that mono- versus polyubiquitination can determine the final fate of RTKs. Lysine 63-linked polyubiquitination is required for EGFR targeting to and degradation by the lysosome (Huang et al., 2013). EGFR with only monoubiquitination shows decreased sorting to the late endosome and degradation, and increased sorting to the recycling endosomes (Huang et al., 2013). Furthermore, a complex consisting of RAB31, a member of the RAB5 family of small GTPases, and early endosome antigen 1 (EEA1) promotes EGFR trafficking to the late endosomes (Chua and Tang, 2014). In addition, loss of EEA1 reduces the interaction between EGFR and RAB31, indicating that EEA1-mediated interaction between EGFR and RAB31 occurs in the early endosome (Chua and Tang, 2014). Consistently, loss of RAB31 inhibits EGFR targeting to the late endosome, whereas overexpression of RAB31 promotes its degradation. Regulation of EGFR trafficking to the recycling endosome may also be regulated by other factors including RAB35, RAB11, and EPS15S (Tomas et al., 2014).

Similar to the ligand-activated EGFR, stress-activated EGFR and oncogenic mutants of EGFR also undergo internalization and endocytic compartmentalization. While recycling of the ligand-activated EGFR targets it to membrane, oncogenic mutants of EGFR and stress-activated EGFR (i.e. exposed to UV light, oxidative stress, chemotherapy or gamma radiation) may be transported to the nucleus. In addition, exposure to stress or EGF may result in mitochondrial localization of EGFR (Figure 13) (Tomas et al., 2014). Trafficking of EGFR to the plasma membrane through the recycling endosomes promotes cell survival and proliferation, while trafficking of EGFR to nucleus or mitochondria promotes cellular homeostasis. In contrast,
Figure 13. Biological outcomes of EGFR trafficking pathways (Tomas et al., 2014) (Used with permission from publisher).
enhanced degradation of EGFR via late endosomes seems to increase cell death (Tomas et al., 2014). Other protein complexes, such as the integral trafficking from the ER to the nuclear envelope transport (INTERNET) pathway, direct EGFR to the nucleus and promote cell survival, but their effects are not well understood (Wang et al., 2012, Tomas et al., 2014).

Another key protein for regulation of endocytic sorting and expression of EGFR is sorting nexin 1 (SNX1). SNX1 is a 66 kDa protein localized in large macromolecular complexes with various proteins involved in vesicular trafficking (Worby and Dixon, 2002). SNX1 contains a MVP1P homology domain and an EGFR binding domain (Figure 14) (Kurten et al., 1996). MVP1P, a yeast sorting nexin protein, plays a role in the vacuolar membrane protein sorting and binds to specific phosphoinositides through phox homology domain (PX) for subsequent endosomal localization (http://repository.upenn.edu/dissertations/AAI3363566/). Currently there are 28 known mammalian sorting nexins. All members of the sorting nexin family have a conserved PX domain required for binding to specific phosphoinositides (Bonaficino and Hurley, 2008). SNX1 protein is localized to the early endosomes. It was discovered as a binding partner of EGFR by a yeast two hybrid screen with EGFR as bait (Kurten et al., 1996). Overexpression of SNX1 leads to increased degradation of total EGFR (Kurten et al., 1996).

Recently it has been shown that HRS, a key component of the ESCRT-0 machinery, binds directly to SNX1 in endocytic vesicles (Chin et al., 2001). HRS and SNX1 seem to compete for binding to EGFR, and HRS may inhibit lysosomal trafficking of EGFR by sequestering SNX1 (Worby and Dixon, 2002). Indeed, overexpression of HRS inhibits ligand-induced degradation of EGFR indicating that interactions of HRS with SNX1 prevent EGFR trafficking to the late endosome. (Chin et al., 2001).
Figure 14. Predicted amino acid sequence of the SNX1 showing MVP1P homology domain (underlined once) and EGFR-binding domain (underlined twice) (Kurten et al., 1996) (Used with permission from publisher).
It has been shown that SNX1 also regulates EGFR expression transcriptionally. Down-regulation of SNX1 by transient transfection of SNX1 siRNA in NSCLC cell lines A549 and QG56 resulted in increased EGFR mRNA expression (Nishimura et al., 2012). In addition, microRNA (miRNA) miR-7 has been shown to target EGFR and multiple microRNAs seem to regulates EGFR signaling networks (Table 1) (Gomez et al., 2013).

1.6. EGFR targeted therapies and mechanisms of drug resistance

The EGFR gene is comprised of 28 exons spanning two ligand binding sites within the ECR, a single transmembrane region, and an intracellular region consisting of the TKD and a C-terminal autophosphorylation region (Figure 15) (Sharma et al., 2007, Flynn et al., 2009). Activating mutations within the TKD and aberrant EGFR signal transduction pathways and networks have been identified in many cancers including NSCLC. Consequently, a majority of the efforts for drug development have focused on the catalytic site of EGFR and multiple RTKs.

1.6.1. EGFR targeted therapies. EGFR targeted therapies include monoclonal antibodies and small molecule TKIs. Cetuximab and other monoclonal antibody therapies bind to the ECR of EGFR and prevent receptor dimerization and subsequent activation of the catalytic domain (Ciardiello et al., 2008, Rolfo et al., 2014). The most widely used TKIs of EGFR are gefitinib and erlotinib (Figure 16) (Ciardiello et al., 2009, Antonicelli et al., 2013, Rolfo et al., 2014). These inhibitors bind directly within the TKD (amino acids (AA) 685-953, coded by exons 18-24), specifically competing with adenosine triphosphate (ATP) for inclusion in the catalytic domain of the TKD (Ciardiello et al., 2009). With the catalytic domain occupied by a TKI, ATP is unable to bind to the receptor, resulting in inhibition of TKD phosphorylation and downstream signaling. EGFR TKIs bind to EGFR with a moderate degree of specificity, but can also inhibit other EGFR family members (Ciardiello et al., 2009).
Table 1. Regulation of EGFR signaling networks via miRNAs.*

<table>
<thead>
<tr>
<th>miRNA name</th>
<th>miRNA regulator</th>
<th>miRNA Targets</th>
</tr>
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<tbody>
<tr>
<td>miR-7</td>
<td>EGFR</td>
<td>EGFR, IRS1/2, RAF-1</td>
</tr>
<tr>
<td>miR-21</td>
<td>EGFR, ErbB2, c-MET, AP-1</td>
<td>PTEN, SPRY, PDCD4</td>
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<td>BIM</td>
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<td>PTEN, Apaf-1</td>
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<tr>
<td>let-7</td>
<td>EGFR, C-Myc, LIN28</td>
<td>Ras, C-Myc</td>
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</tbody>
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*Gomez et al., 2013.

(Used with permission from publisher)
Figure 15. Mutations in EGFR associated with sensitivity or resistance to gefitinib and/or erlotinib in NSCLC. Relevant mutations are localized within exons 18-21 representing tyrosine kinase domain of the receptor (Sharma et al., 2007) (Used with permission from publisher).
Figure 16. A. Structures of two EGFR TKIs used in the treatment of NSCLC. B. Ribbon diagram of wild-type human EGFR illustrating binding of gefitinib to the active site of the kinase (Antonicelli et al., 2013) (Used with permission from publisher).
Clinical trials in NSCLC patients have shown mixed results of the efficacies of gefitinib and erlotinib (Rolfo et al., 2014). Mutations within the TKD of EGFR can result in its constitutive activation and are found in 15% of Caucasians and 40% of Asians with lung cancer (Zer et al., 2014). Interestingly, most prevalent mutations seen within exons 18-21 enhance sensitivity to TKIs. Indeed 45% of drug-sensitizing EGFR mutations in NSCLC are located in exon 19 near the conserved LREA (4AA) motif at AA residues 747-750 (Figure 15). Multiple small in-frame deletions that include Δ746-750 and Δ746-751 have been associated with hyperactivation of EGFR and increased sensitivity to TKIs gefitinib and erlotinib (Sharma et al., 2007). Other hyperactivating mutations include point mutation within exon 21 (L858R) near the beginning of the activation loop accounting for 45% of EGFR mutations, and nucleotide substitutions within exon 18, including G719, seen in 5% of NSCLC cases (Sharma et al., 2007). All of the above mentioned mutations are associated with NSCLC cellular “addiction” to the EGFR pathway and increased sensitivity to TKIs (Figure 15) (Sharma et al., 2007).

1.6.2. Mechanisms of resistance to EGFR targeted therapies. Multiple factors are associated with the primary or acquired resistance of NSCLC to EGFR targeted therapies. Primary resistance to TKIs has been attributed to EGFR mutations T790M in exon 20 and D761Y near the end of exon 19 (Figure 15) (Sharma et al., 2007). The T790M mutation may confer insensitivity to TKIs by a conformational change resulting in greater affinity of the TKD for ATP (Yun et al., 2008). Other mechanisms of primary resistance include increased expression of breast cancer type 1 susceptibility protein (BRCA1) (Rolfo et al., 2014), NF-κB hyperactivation (Rolfo et al., 2014), and low mRNA expression of pro-apoptotic protein BIM (Rolfo et al., 2014). An upfront combined modality approach, including TKI may offer improved outcomes of an aggressive primary disease.
Tumors may acquire resistance to treatment with gefitinib or erlotinib through several mechanisms. Fifty percent of NSCLC tumors with acquired resistance to EGFR TKIs show EGFR T790M mutation. Amplifications of MET and HGF, and upregulation of AXL occur in 20% of resistant NSCLC (Rolfo et al., 2014). MET amplification and heterodimerization with EGFR family member ErbB2 leads to activation of PI3K/pAKT survival signaling and promotion of cell survival. HGF signaling is amplified by clonal selection after treatment with EGFR TKI, and this also activates the PI3K/pAKT signaling (Figure 17) (Lin et al., 2014). Other processes such as epithelial to mesenchymal transformation, morphological conversion of NSCLC to SCLC, and CRK-like protein (CRKL) signaling amplification may also promote acquired resistance of cancer cells to EGFR TKI therapy (Rolfo et al., 2014).

Activation of other RTK pathways can also confer resistance of NSCLC to EGFR targeted TKIs gefitinib and erlotinib. These receptors include HER2, HER3, MET, and IGF-1R (Figure 17) (Niederst and Engelmann, 2013, Lin et al., 2014). These RTKs may heterodimerize with TKI targeted EGFR or bypass EGFR signaling by homodimerization, activating multiple downstream pathways including the PI3K/pAKT, RAS-RAF-ERK and JAK/STAT pathways and leading to enhanced cell proliferation and inhibition of apoptosis (Figure 17) (Niederst and Engelmann, 2013, Lin et al., 2014).

Regulation of EGFR endocytosis potentially impacts resistance to EGFR targeted therapies. Cell lines insensitive to EGFR TKI gefitinib were found to be more sensitive if EGFR endocytosis was suppressed (Jo et al., 2014). Additionally, downregulation of RAB25, differentially expressed between insensitive and sensitive cells lines and a known regulator of
Figure 17. Multiple RTK-dependent mechanisms of EGFR-TKI resistance in NSCLC (Lin et al., 2014) (Used with permission from publisher).
endocytic vesicle processing, led to changes in EGFR endocytosis and increased sensitivity to gefitinib (Jo et al., 2014).

MiRNA expression has also been associated with regulation of resistance to EGFR targeting drugs. By targeting tumor suppressor and apoptotic molecules such as PTEN, APAF-1, BIM, and TIMP3 expression, miRNAs miR-221 and miR-222 may confer resistance to gefitinib treatment (Table 2) (Lin et al., 2014). MiRNAs may also function as repressors of oncogenes. For example miR-103 and miR-203 which target SRC and PKC-ε, and miR-128b which targets EGFR are downregulated in TKI-resistant NSCLC cells (Lin et al., 2014). Targeting specific miRNAs in NSCLCs may improve response to EGFR-TKIs.

Understanding the mechanisms of acquired resistance of NSCLC to EGFR TKIs is essential for developing a strategy to overcome resistance (Figure 18) (Chong and Janne, 2013). For example, relatively infrequent EGFR ectodomain mutation S492R prevents binding of EGFR monoclonal antibody cetuximab but not panitumumab to EGFR. Therefore, panitumumab may be substituted for cetuximab in these patients. Likewise, NSCLC tumors carrying EGFR T790M mutation may respond effectively to a combination of EGFR specific TKI afatinib and cetuximab. Additional T790M mutation specific small molecules AZD9291, CO-1686, and dacomitinib are also in clinical trials (Chong and Janne, 2013, Rolfo et al., 2014). Histological transformation cases such as epithelial-to-mesenchymal transformation (EMT) and SCLC may be treated with combination chemotherapies such as cisplatin and etoposide. In addition, a multi-targeted approach involving a combination of agents targeting EGFR and amplified MET or HER2, activated IGF-1R, or mutated KRAS may be used to treat drug-resistant NSCLC (Chong and Janne, 2013). Finally, broad spectrum small molecules (e.g. XL647) targeting multiple RTKs may be used in the management of resistant disease (Rolfo et al., 2014).
Table 2. Role of miRNAs in EGFR-TKI resistance in NSCLC.*

<table>
<thead>
<tr>
<th>miRNA name</th>
<th>Expression in resistant NSCLC</th>
<th>Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>Up</td>
<td>PDCD4, MDR1, PTEN and bel-2</td>
</tr>
<tr>
<td>miR-103 and -203</td>
<td>Down</td>
<td>PKC-ε, SRC, Dicer.</td>
</tr>
<tr>
<td>miR-126</td>
<td>Down</td>
<td>PIK3R2, CRK, VEGF</td>
</tr>
<tr>
<td>miR-128b</td>
<td>Down</td>
<td>EGFR</td>
</tr>
<tr>
<td>miR-214</td>
<td>Up</td>
<td>PTEN, p38, MAPK</td>
</tr>
<tr>
<td>miR-221, miR-222</td>
<td>Up</td>
<td>PTEN, APAF-1, BIM, TIMP3</td>
</tr>
<tr>
<td>MiR-145</td>
<td>Down</td>
<td>c-MYC, AKT, ERK, OCT4</td>
</tr>
</tbody>
</table>

*Lin et al., 2014
(Used with permission from publisher)
Figure 18. New strategies for targeting EGFR TKI-resistant NSCLC (Chong and Janne., 2013) (Used with permission from publisher).
1.7. Introduction to IGF-1R structure and signal transduction

IGF-1R is closely related to the insulin receptor (IR) and a member of the superfamily of RTKs which comprises 59 members in humans. Unlike many other RTKs, IGF-1R and IR form disulfide-linked covalent homodimers. IGF-1R is processed from a monomeric pro-receptor by cleavage into extracellular ligand binding α-subunit, and a β-subunit that contains the transmembrane and the intracellular domains (Figure 19, left panel) (Gray et al., 2003, De Meyts, 2008). The α-subunits are linked by multiple disulfide bonds, whereas a single disulfide bond links the α- and β-subunits. The ligand-binding extracellular α-subunits preferentially bind only one molecule of ligand (Siddle, 2012). The intracellular domain of IGF-1R contains a short juxtamembrane domain, a tyrosine kinase domain, followed by a C-terminal domain (Figures 19 and 20) (Siddle, 2012). The juxtamembrane domain contains binding sites for insulin receptor substrate (IRS1/2) and SHC proteins (Figure 20) (Siddle, 2012).

Activation of the IGF-1R signaling influences cancer progression in several ways (Figure 19, right panel) (Seccareccia and Brodt, 2012). IGF-1 signaling can promote VEGF expression, initiating angiogenesis. Epithelial to mesenchymal transition (EMT) is also promoted by activation of IGF-1R and downstream activation of transcription factor Snail. In addition, IGF-1R signaling activates MMPs involved in cell invasion through the extracellular matrix. Activation of the IGF-1R signaling pathway can confer resistance of cells to immune response. Finally, it has been shown that IGF signaling helps maintain cancer stem cell population (Seccareccia and Brodt, 2012). IGF-1R signaling is implicated in increased risk of breast, prostate, and lung cancers (Butt et al., 2000). Aberrant IGF-1R signaling contributes to development of NSCLC tumors (Scagliotti and Novello, 2012).
Figure 19. Left panel, Schematic representation of the IGF-1R structure (modified from Gray et al., 2003). Right panel, Role of IGF-1 in angiogenesis, invasion, immune escape and maintenance of cancer stem cell population (Seccareccia and Brodt, 2012) (Used with permission from publisher).
Figure 20. IGF/IGF-1R-initiated canonical signaling pathway (Siddle, 2012). (JM, juxtamembrane; TK, tyrosine kinase; CT, carboxyl-terminal; PTB, phosphotyrosine-binding; PH, pleckstrin homology, SH2, Src homology-2; KRLB, kinase regulatory loop binding) (Used with permission from publisher).
IGF-1R signaling pathway is initiated by binding of insulin-like growth factor (IGF-1/IGF-2) to the receptor. Upon ligand binding, autophosphorylation of the IGF-1R TKD (Y1131, Y1135, and Y1136) results in conformational changes allowing intracellular substrates bind to the phosphorylated residues in the receptor (Siddle, 2012). In brief, phosphorylated IGF-1R recruits insulin receptor substrate (IRS 1/2), activating the two major survival and oncogenic signaling pathways, PI3K/PIP3/PDK/AKT and RAS/RAF/MEK/ERK pathways (Figure 20) (Siddle, 2012, Desbuquois et al., 2013). Activated IGF-1R may also bind to SRC homologous and collagen protein (SHC) via GRB2 and SOS, resulting in the activation of RAS-RAF-MAP kinase cascade and promoting cell proliferation (Figure 20) (Fürstenberger and Senn, 2002, Siddle 2012).

Limited information is available on the efficacies of IGF-1R-targeted drugs in advanced cancers. Several IGF-IR inhibitors have been developed as potential therapies of NSCLCs (Scagliotti and Novello, 2011, Haisa, 2013). These include monoclonal antibodies cixutumumab and figitumumab targeting the extracellular domains of IGF-1R and orally available small molecule tyrosine kinase inhibitors (INSM-18, OSI-906 (linsitinib)), functioning as ATP antagonists (Table 3) (Scagliotti and Novello, 2012, Haisa, 2013). INSM-18 is of particular interest as it can directly inhibit EGFR, HER2, and IGF-1R tyrosine kinase (Haisa, 2013). Small molecule TKIs may have more metabolic side effects, as they target both insulin receptor and IGF-1R (Haisa, 2013), but have a greater range of inhibition if IR is contributing to tumor progression (Haisa, 2013). Other IGF-1R targeted small molecules at preclinical stages include both non-ATP competitive (AG-1024) and ATP competitive molecules (BMS-536924) (Table 3).
Table 3. Small molecule inhibitors targeting IGF-1R in clinical trials.*

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Company</th>
<th>Class</th>
<th>Trial status</th>
</tr>
</thead>
<tbody>
<tr>
<td>INSM-18</td>
<td>Insmed</td>
<td>Reversible, ATP-competitive</td>
<td>Phase I/II</td>
</tr>
<tr>
<td></td>
<td>UCSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSI-906</td>
<td>OSI Pharmaceuticals</td>
<td>Reversible, Oral small molecule, ATP-competitive</td>
<td>Phase I</td>
</tr>
<tr>
<td>XL-228</td>
<td>Exelixis</td>
<td>Oral small molecule, Reversible, ATP-competitive</td>
<td>Phase I</td>
</tr>
<tr>
<td>NVP-ADW742</td>
<td>Novartis</td>
<td>Reversible, ATP-competitive</td>
<td>Preclinical</td>
</tr>
<tr>
<td>NVP-AEW541</td>
<td>Novartis</td>
<td>Reversible, ATP-competitive</td>
<td>Preclinical</td>
</tr>
<tr>
<td>AG-1024</td>
<td>Merck</td>
<td>Non ATP-competitive</td>
<td>Preclinical</td>
</tr>
<tr>
<td>BMS-536924</td>
<td>Bristol-Myers Squibb</td>
<td>ATP-competitive</td>
<td>Preclinical</td>
</tr>
<tr>
<td>BMS-554417</td>
<td>Bristol-Myers Squibb</td>
<td>Oral small molecule, Reversible ATP-competitive</td>
<td>Preclinical</td>
</tr>
<tr>
<td>BVP-51004 (Cyclolignan PPP)</td>
<td>Karolinska Cancer Institute, Biovitrum</td>
<td>Oral small molecule, Non ATP-competitive</td>
<td>Preclinical</td>
</tr>
</tbody>
</table>

*Haisa, 2013.

(Used with permission from publisher)
1.8. IGFBP3-mediated regulation of IGF/IGF-1R signal transduction

IGF-1R signaling is regulated by IGF binding proteins (IGFBPs), a family of secreted factors comprised of six members (IGFBP1-IGFBP6), through their high affinity binding to IGF-1 and IGF-2. IGF secreted into circulation by the liver, kidney and other tissues forms a binary or ternary complex with circulating IGFBP and ALS (acid-labile subunit) (Figure 21, panel A). While free IGF peptides have a circulating half-life of minutes, bound IGF seems to have a prolonged half-life of up to 24 hours (Baxter, 2014).

Binding of IGFBP to IGF blocks the ability of IGF to activate IGF-1R (Figure 21) (Baxter, 2014). Crystal structural analysis for the basis of the binding of IGFBP to IGF-1 and how this may inhibit interaction of IGF-1 with IGF-1R indicates that IGF binds with high affinity to the N- and C-terminal regions of IGFBP (Sitar et al., 2006). Upon reaching the target tissue such as tumor cells expressing IGF-1R, limited proteolysis of IGFBP in extracellular matrix seems to release IGF and allow its binding to IGF-1R. Alternatively, IGFBPs may preferentially bind to cell surface IGFBP receptor (IGFBPR) and facilitate release of IGF (Figure 21) (Baxter, 2014). Intracellularly, IGFBPs may be translocated to the nucleus via importin and influence gene transcription by binding to other nuclear receptors. Cytosolic IGFBP3 has been shown to enhance caspase-dependent cell death (Baxter, 2014). IGFBP-3 may also promote tumor suppression by binding to TGFβ receptor II and activating growth inhibitory TGFβI/II/SMAD2-SMAD3/SMAD4 pathway (Figure 21).

Mechanism of regulation of IGFBP3 expression and function is unclear. In this context, FOXA1 promotes tumor progression in prostate cancer cells, in part, by downregulating IGFBP3 (Imamura et al., 2012). Consistently, depletion of FOXA1 in human prostate cancer cells by
Figure 21. A. IGFBP shows high affinity binding to circulating IGF-1/IGF-2. B. Role of IGFBP in regulating IGF-1R, TGFβ, and caspase-dependent cell death signaling pathways (Baxter, 2014) (Used with permission from publisher).
siRNA led to an increase in IGFBP3 protein expression, and increased expression of IGFBP3 has been associated with inhibition of pAKT expression and enhanced expression of cell cycle arrest proteins p21 and p27 (Imamura et al., 2012).

IGFBP3 expression is increased in certain head and neck squamous cell carcinoma, pancreatic ductal adenocarcinoma, renal clear cell carcinoma, melanoma, oesophageal cancer and breast cancer (Baxter, 2014). Consistent with an apparent role of IGFBP3 in cell growth suppression, overexpression of IGFBP3 mRNA and protein has been associated with improved prognosis in breast cancer patients (Mu et al., 2009).

1.9. Acquired resistance to EGFR TKIs is associated with activation of IGF-1R pathway: implications of combined targeting of EGFR and IGF-1R in treatment of resistant NSCLC cells.

EGFR TKIs have shown significant benefits in the treatment of NSCLC patients. However, these responses are not durable. Several reports indicate that prolonged treatment with EGFR TKIs gefitinib and erlotinib results in the activation of IGF-1R via heterodimerization with EGFR (Morgillo et al., 2006, Morgillo et al., 2007). Activation of IGF-1R in gefitinib or erlotinib treated cells has been associated with activation of PI3K/AKT and RAS/RAF/MEK/ERK pathways and overexpression of EGFR and anti-apoptotic protein survivin (Figure 22) (Morgillo et al., 2006, Morgillo et al., 2007). In addition, NSCLC cell line H1650, which has high resistance to gefitinib, was found to have sustained IGF-1R signaling in the presence of EGFR TKIs (Choi et al., 2010). Therefore, dual targeting of EGFR and IGF-1R may prevent or delay development of acquired resistance to EGFR TKIs in NSCLC tumors. Indeed, synergistic effect of a combination of EGFR TKI gefitinib or erlotinib and IGF-1R TKI AG-1024 has been observed in EGFR TKI resistant NSCLC cells (H460) (Table 4) (Morgillo et al., 2006, Morgillo
Figure 22. Acquired resistance to EGFR-targeted small molecule involves activation of IGF-1R pathway leading to overexpression of EGFR and Survivin (Morgillo et al., 2006) (Used with permission from publisher).
Table 4. Synergistic effect of targeting both EGFR and IGF-1R

<table>
<thead>
<tr>
<th>Treatment A</th>
<th>Treatment B</th>
<th>Combination treatment</th>
<th>Index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug</td>
<td>Concentration (μmol/L)</td>
<td>MGI</td>
<td>P value</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>1</td>
<td>0.85</td>
<td>5.3E-05</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.65</td>
<td>1.9E-09</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.59</td>
<td>4.3E-10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Ha60 cell proliferation treated with indicated concentrations of gefitinib, AG1024, or their combinations was calculated by the MTT assay. Abbreviation: MGI, mean growth inhibition rate = growth rate of treated group/growth rate of untreated group.

*Calculated by dividing the expected growth inhibition rate by the observed growth inhibition rate. An index more than 1 indicates synergistic effect and <1 indicates less than additive effect.

1 P value (two-sided) was calculated by t-test compared with no treatment.

1 Growth inhibition rate of treatment A = growth inhibition rate of treatment B.

1 Growth inhibition rate of combined treatment on treatments A and B.

*Modified from Morgillo et al., 2007

(Used with permission from publisher)
et al., 2007). In other studies, combination of gefitinib and monoclonal anti-IGF-1R antibody (MK0646) or IGF-1R TKI (AEW541) resulted in enhanced suppression of cell growth and prevented recurrence of tumors in a mouse model of gefitinib-resistant A431 squamous carcinoma cells (Guix et al., 2008). Likewise, synergistic cytotoxic effects of a combination of EGFR TKI gefitinib and anti-IGF-1R antibody (α-IR3) or IGF-1R TKI (AG-1024) was observed in EGFR TKI resistant H1650 NSCLC cells (Figure 23) (Choi et al., 2010). Combined inhibition of EGFR and IGF-1R in gefitinib-resistant NSCLC cell line PC9/G has been associated with decreased pAKT expression, increased apoptosis, and inhibited cell proliferation (Qi et al., 2011).

As mentioned earlier, IGFBP3 is a negative regulator of IGF-1R signaling. Loss of IGFBP3 and IGFBP4 expression has been attributed to in vitro acquired resistance of A431 squamous cancer cells to EGFR TKI gefitinib (Guix et al., 2008). In these studies, IGFBP-3 and IGFBP-4 expression was significantly reduced in gefitinib-resistant A431 cells as compared to parental A431 cells. Furthermore, treatment of gefitinib-resistant A431 cells with a combination of gefitinib and recombinant IGFBP3 protein resulted in enhanced cell growth suppression as compared to single agents (Figure 24) (Guix et al. 2008), implying that acquired resistance to EGFR TKI is, in part, mediated loss of IGFBP3 protein expression in EGFR TKI-resistant cells.
Figure 23. Synergistic cytotoxic effect of a combination of gefitinib and AG-1024 in EGFR TKI resistant H1650 NSCLC cells (Choi et al., 2010) (Used with permission from publisher).
Figure 24. Synergistic cytotoxic effect of a combination of gefitinib and recombinant IGFBP3 protein in gefitinib resistant A431 cells (Guix et al., 2008) (Used with permission from publisher).
Chapter 2. Materials and Methods

2.1. Cell lines and culture conditions

Prostate cancer cell lines PC-3, C4-2B and DU-145, melanoma cell line MDA-MB-435, and pancreatic cancer cell lines PANC-1 and MIA PaCa-2 (MIAPaC) (Lombardi Comprehensive Cancer Center Tissue Culture Shared Resource (TCSR)), prostate cancer cell line PC-3M (Dr. Isaiah J. Fidler, M.D. Anderson Cancer Center), breast cancer cell line LM2-4175 (Dr. Joan Massague, Memorial Sloan-Kettering Cancer Center), and HEK293T cells (ACS-4500) (ATCC, Manassas, VA) were maintained in DMEM culture medium (Dulbecco’s modified essential medium with Glutamax and high glucose, Life Technologies, Grand Island, NY, cat #10566) supplemented with heat inactivated 10% fetal bovine serum (FBS) (Life Technologies), and 100 μg/mL streptomycin and 100 U/mL penicillin (P/S) (Life Technologies). Non-small cell lung cancer cell lines A549 (TCSR) and H1299 (ATCC) were maintained in RPMI complete medium (RPMI 1640, Life Technologies, cat #11875) supplemented with 10% FBS and P/S. All cultures were maintained in humidified incubators at 37°C with 5% CO₂.

2.2. Antibodies, growth factors, reagents and chemicals

A majority of the antibodies were commercially obtained as detailed below. Rabbit polyclonal anti-EGFR (D38B1), rabbit polyclonal anti-pEGFR Y1068 (D7A5), rabbit polyclonal anti-AKT (pan) (11E7), rabbit polyclonal anti-pAKT S473 (D9E), rabbit polyclonal anti-IGF-1 Receptorβ (IGF-1R), rabbit polyclonal anti-pIGF-1R Y1135 (DA7A8), rabbit polyclonal anti-ERK1/2 (p44/42 MAPK) (13F5), and mouse monoclonal anti-pERK1/2 (p-p44/42 T202/Y204) (E10) were obtained from Cell Signaling (Beverely, MA). Mouse monoclonal anti-SNX1 (611482) and mouse monoclonal anti-EEA1 (610456) antibodies were obtained from BD Biosciences (Franklin Lakes, NJ). Rabbit polyclonal anti-IGFBP3 (H-98) and mouse
monoclonal anti-β-ACTIN (AC-15) were obtained from Santa Cruz Biotechnology (Dallas, TX). Rabbit polyclonal anti-peptide T8 antibody was custom made from Covance Antibody Products (BioLegend) (Dedham, MA) as described earlier (Kumar et al., 2004). Conjugated secondary goat polyclonal anti-rabbit immunoglobulins-horseradish peroxidase (HRP) and goat polyclonal anti-mouse immunoglobulins-HRP were obtained from Dako (Carpinteria, CA). Alexa Fluor 488 goat anti-rabbit (A11034) and Alexa Fluor 546 goat anti-mouse IgG1 (y1) (A21123) were obtained from Life Technologies. Additional reagents and chemicals used were as follows. Recombinant proteins EGF (236-EG), IGF-1 (291-G1), VEGF (293-VE), and FGF-1 (231-BC) (all from R&D Biosystems, Minneapolis, MN); FuGENE6 (Roche, Nutley, NJ), Lipofectamine2000 and Optimem medium (Life Technologies); pLKO.1 plasmid Mission shRNA scrambled control (Addgene, catalogue # 1864), pLKO.1 vector (Moffat et al., 2006) and T8 plasmid mission shRNAs 1-5 (pLKO.1) (Sigma-Aldrich, St. Louis, MO); lentiviral packaging vectors pMD2.G and psPax2 (Addgene, Cambridge, MA); Fluorogel (Electron Microscopy Sciences, Hatfield, PA); gefitinib (Tocris, Minneapolis, MN); AG1024 (Santa Cruz Biotechnology); 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) cell viability assay kit (Biotium, Hayward, CA, cat 30007); DNA miniprep kit, Endofree plasmid maxi kit, and RNeasy RNA miniprep kit (Qiagen, Germantown, MD); custom made SNX1 siRNA (GE Dharmaco, Lafayette, CO) (Nishimura et al., 2012); IGFBP3 siRNA (On-Targetplus SMARTpool) and On-Targetplus SMARTpool control siRNA (GE Dharmaco); DH5α chemically competent cells (Cat #18258), ampicillin, S.O.C. medium, Platinum PCR Supermix kit, and SuperScriptIII First-Strand Synthesis SuperMix for qRT-PCR kit (Life Technologies); and Fast SYBR Green Master Mix (Life Technologies); restriction enzyme Pvu II (New England Biolabs, Ipswitch, MA); IGEPAL CA-630, protease inhibitor cocktail (P8340),
and phosphatase inhibitor cocktail #2 (P5726) (all from Sigma-Aldrich); Pierce BCA Protein Assay kit (Thermo Scientific, Waltham, MA); and phosphate buffered saline pH 7.6 (PBS), Trypsin-EDTA, ethidium bromide, LDS NuPAGE sample buffer, NuPAGE sample reducing buffer, NuPAGE antioxidant, precast NuPAGE 4-12% polyacrylamide gels, MES running buffer, Tris-glycine transfer buffer, Seeblueplus2 protein standard, and 0.45 μm PVDF membrane (all from Life Technologies). HyGLO chemiluminescent detection reagent and HyBlot autoradiography films were obtained from Denville Scientific (Metuchen, NJ). Glycerol (G5516-1L), bovine serum albumin fraction V (BSA) (cat # A9647), sodium chloride (S-3014), deoxycholic acid (D6750), Tween-20 (P1379), puromycin dihydrochloride (P7255), HEPES (H6147), dimethyl sulphoxide (DMSO)(D2438), and puromycin dihydrochloride (puromycin) were obtained from Sigma-Aldrich. TRIzol reagent, MES SDS running buffer, NuPAGE transfer buffer (20x), and Microamp fast optical 96-well reaction plates were all obtained from Life Technologies. Bis N,N’-methylene-bisAcrylamide, acrylamide, glycine, sodium dodecyl sulfate (SDS), blotting-grade blocker non-fat dry milk, and TEMED were all obtained from Bio-Rad (Hercules, CA). Tris-HCl 1M pH 8.0 and Tris-HCl 1M pH 7.6 were obtained from Quality Biological (Gaithersburg, MD). Parafilm was obtained from American National Can (Menasha, WI). Agar, tryptone, and yeast extract were obtained from Fisher Scientific (Hanover Park, IL).

2.3. Plasmid DNA preparations

Plasmid constructs (shRNA and lentiviral packing plasmids) were amplified using protocols provided by the manufacturer (Life Technologies, Addgene, and Sigma-Aldrich). For plasmid DNA transformation, 0.1 μg of plasmid DNA in 5 μL of DNase free/RNase free water was added to 50 μL of DH5α chemically competent cells (Life Technologies) for 30 minutes on ice. Mixture was heat shocked at 42°C for 45 seconds, incubated on ice for two minutes, and
added to 450 μL of S.O.C. medium, followed by bacterial growth in a shaking incubator for 1 hour at 37°C. Bacteria were streaked on an LB-agar plate prepared using LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl), 1.5% agar and 100 μg/mL ampicillin, and incubated at 37°C overnight (O/N). The LB-agar plates were stored at 4°C. For mini plasmid DNA preparation, independent bacterial colonies were picked using a pipette tip and resuspended into 10 mL of LB broth supplemented with 100 μg/mL ampicillin, and incubated O/N at 37°C in a shaking incubator. The bacterial culture (3 mL) was microcentrifuged for one minute at 4°C and the bacterial pellet was processed for plasmid DNA isolation using DNA mini-prep kit. The pLKO.1 vector has a length of 7,052 bp. With T8 shRNA insert, the length of the shRNA plasmid is 7,086 bp (Sigma-Aldrich). Purified plasmid DNA preparation was cleaved with PvuII and the band sizes of the shRNA containing vector were found to be approximately 3800 bp, 2500 bp, and 780 bp, verifying T8 shRNA containing pLKO.1 plasmid (Sigma-Aldrich). For maxi plasmid DNA preparations, DNA was isolated using maxi-prep kit and the manufacturer’s protocol (Qiagen).

2.4. T8 shRNA transient transfections

Transient T8 shRNA transfections were performed following the manufacturer’s protocol (Addgene) ([https://www.addgene.org/tools/protocols/plko/#H](https://www.addgene.org/tools/protocols/plko/#H)). The following T8 shRNAs in pLKO.1 plasmid (Figure 25) were used for transient transfections.
Figure 25. pLKO.1 – puro vector map (Addgene).
T8 shRNA #1: 5’-
CCGGTTGGATGAAGAGAACAATATCTCGAGATATGTTCCTCTTCATCCAAACATTTTTG-3’,
T8 shRNA #2: 5’-
CCGGAGATGCTGCACCAAATCATTCTCAGGAATGATTTGTCAGCATCTTTT
TG-3’,
T8 shRNA #3: 5’-
CCGGCATCAAGCTGGCCATTCTTTACTCGAGTAAGAATGGCCAGCTTGATGTTTTTG
G-3’,
T8 shRNA #4: 5’-
CCGGATGAATAATGTCCAGTATAAGCTCGAGCTTATACTGGACATTATTTCTTT
TG-3’,
T8 shRNA #5: 5’-
CCGGCCAAGAGAATTTCTCTTGCTATCTCGAGATAGCAAGAGAAAGTTCTTGGTTTT
G-3’.

The T8 mRNA target sequences corresponding to various T8 shRNAs were ascertained by using BLAT software for sequence homology (UCSC Genome Browser) (Figure 26). T8 shRNA containing plasmid DNA was transfected into the PC-3 cells using FuGENE6. Cells were seeded in a 6-well plate at 0.3 x 10^6 cells per well and grown in DMEM with 10% FBS and P/S for 24 hours. Next day, medium was removed and replaced with 900 μL of Optimem medium per well. Six μL of FuGENE6 was mixed with 74 μL of Optimem,
Figure 26. Human TNFAIP8 mRNA target sequences corresponding to various T8 shRNAs used were first ascertained by using BLAT software for sequence homology (UCSC Genome Browser)
and added to 20 μL of Optimem containing 1 μg of T8 shRNA or empty vector (pLKO.1). The mixture was incubated for 30 minutes at room temperature (RT), and added to the PC-3 cells, followed by incubation for 6 hours. One mL of DMEM supplemented with 10% FBS and P/S was added to the cells containing Optimem medium with shRNA or control vector. After 24 hours, medium was replaced with DMEM culture medium. Cells were grown in DMEM culture medium for additional 48 hours and subsequently lysed for immunoblotting.

2.5. T8 shRNA lentivirus and stable transductions

Cancer cell lines stably expressing T8 shRNA, scrambled shRNA (5'-CCTAAGGTATAGCCCTCGGAGCGAGGGCGACCTAACCCTTAGG-3'), or empty vector (pLKO.1) were established using the pLKO.1 lentiviral transduction procedure (Addgene) as outlined in Figure 27. For preparation of lentiviral stocks, HEK293T cells were seeded at 0.3 x 10^6 cells per well in 6-well dishes and grown to 70% confluency in DMEM culture medium. The next day medium was replaced with 900 μL of Optimem medium. One μg of plasmid DNA (T8 shRNA, empty vector, or scrambled shRNA) was resuspended in 20 μL of Optimem medium containing 0.75 μg of psPAX2 packaging vector and 0.25 μg pMD2.G envelope vector. The transfection mixture was combined with 80 μL of Optimem medium containing 6 μL of FuGENE6, and incubated at RT for 30 minutes and then added to cells for 6 hours at 37°C. One mL of DMEM culture medium was added to the cells for O/N and the medium was replaced with fresh 5 mL DMEM culture medium. Twenty-four hours later medium containing packaged lentiviruses was collected and stored at 4°C. Additional 5 mL of fresh DMEM culture medium was added to the cells and medium containing viruses was collected at 24 hours. Pooled lentiviral stocks were centrifuged to remove any residual HEK293T cells. The pooled supernatant containing the lentiviral particles was aliquoted and stored at -20°C.
Figure 27. Stable knockdown of TNFAIP8 using lentiviral pLKO.1 shRNA expression vector.
Cancer cells were grown to 70% confluency in 6-well dishes containing complete medium (RPMI or DMEM). On the day of the infection, medium was replaced with 1 mL serum free medium with P/S (SFM), and 50-100 µL of lentiviral T8 shRNA (1-5) (shT8), empty vector (EV), or scrambled shRNA (scr), or left untransfected (three wells per treatment group). After 24-48 hours the lentiviral medium was replaced with complete medium. After 72 hours, medium was replaced with complete medium with 1 µg/mL of puromycin. After 5-10 days of growth in selection medium, 100% cell death was verified in the untransfected group. Surviving colonies in the lentiviral transfection groups were pooled after trypsinization and cultures were maintained in complete medium containing puromycin. T8 knockdown efficiency in various shT8 treatment groups as compared to scr and EV control groups was verified by immunoblotting as described below.

2.6. SNX1 and IGFBP3 siRNA transfections

A549 cells stably expressing shT8 shRNA (shT8) were seeded in 12-well tissue culture plates with 0.1 x 10^6 cells per well in complete medium. When cells were 70% confluent, medium was replaced with 400 µL of Optimem medium. Mixture of siRNA (0.25 µL of 100 µM stock) and LipofectAmine 2000 (4 µL) was resuspended in 100 µL Optimem medium and incubated at RT for 20 minutes. The mixture was added to the cells for a final concentration of 50 nM siRNA (siSNX1 (#1, On-Targetplus SMARTpool Cat#L-017518-00-0), siSNX1 (#2, 5’-AAGAACAAGACCAAGAGCCACUU-3’), siIGFBP3 (On-Targetplus SMARTpool Cat#L-00477700), or siRNA On-Targetplus SMARTpool control). After 6 hours, 500 µL of complete medium was added per well. After 48 hours, culture medium was changed to SFM and with or without 50 nM of siRNA for 12 hours followed by cell lysis and immunoblotting. For growth
factor treatment, siRNA containing medium was replaced with fresh SFM and growth factor as detailed below.

2.7. Growth factor treatments

To study the effects of EGF and IGF-1 on EGFR and IGF-1R signaling, including expression of EGFR, pEGFR, pERK, pIGF-1R, and pAKT, growth factor treatment conditions were followed as described before (Morgillo et al., 2006, Morgillo et al., 2007, Guix et al., 2008). Stock solutions of EGF and IGF-1 were prepared at 500 µg/mL in PBS and aliquots were stored at -80°C. Cells were grown in complete medium for 24-48 hours, medium was replaced with SFM, and incubations continued for 12 hours. The next day, SFM was replaced with fresh SFM containing desired final concentration of EGF or IGF-1 for various times. Cells were scraped in lysis buffer and the lysates were immediately processed for immunoblotting as detailed below.

2.8. A549 scr and A549 shT8 culture medium treatments

To obtain medium from stable A549 scr and A549 shT8 cell cultures, cells were seeded in duplicate 6-well plates at 0.3 x 10^6 cells per well and grown to 90% confluency in complete medium. Culture medium was changed to SFM (2 mL/well) for 12 hours. Culture medium was pooled from 3 wells seeded with A549 scr cells (scr-cm) and 3 wells seeded with A549 shT8 cells (shT8-cm) from six well plate 1 as shown in Figure 28. For treatment of cells seeded in six well plate 2 with scr-cm or shT8-cm, medium was removed and replaced with 2 mL of scr-cm or shT8-cm or left unchanged (nc) as shown in Figure 28. The recipient cells were then incubated for 30 minutes, followed by cell lysis and immunoblotting as described below.
Figure 28. Strategy for collecting culture medium from A549 scr cells and A549 shT8 cells and subsequent treatment of T8 knockdown and scrambled control cells. nc = no change.
2.9. Wound healing assay for cell migration

A549 scr and A549 shT8 cells were seeded in 6-well plates at 0.3x10^6 cells/well and grown to 90% confluency in complete medium. Medium was replaced with SFM for 12 hours. The strategy for scratching the cells and monitoring wound closure is shown in Figure 29. A 200 µL pipette tip was used to scrape the cells through the center of the well longitudinally, and a line was drawn with a felt tip pen on the outside of the well perpendicular to the scratch as described earlier (Kim et al., 2010). EGF and IGF-1 were reconstituted in PBS at 500 µg/mL as in section 2.7. VEGF and FGF-1 were reconstituted in PBS containing 0.1% BSA at 25 µg/mL and 100 µg/mL, respectively. Immediately after marking the scratch, cells were rinsed in SFM, and medium was replaced with fresh SFM containing growth factor (EGF 50 ng/mL, IGF-1 50 ng/mL, FGF-1 20 ng/mL, or VEGF 50 ng/mL) or 10% FBS. Images of the wound were captured immediately after wound generation, and 6, 12, 18, and 24 hours post-scratch. Two images were captured per scratch per time point, above and below the felt marked line (green, Figure 29) at 10x magnification using a phase contrast microscope (Nikon). At various times post-scratch, the wound area was quantified using ImageJ software (National Institutes of Health, USA) and data (mean +/- s.d.) were plotted relative to initial wound area (0 hour).

2.10. RNA isolation, RT-PCR and qRT-PCR

Total cell RNA was extracted using RNeasy RNA miniprep kit according to manufacturer’s instructions (Qiagen). RNA concentration was measured using a Nanodrop spectrophotometer (Thermoscientific, Waltham, MA), and 1 µg of RNA was reverse-transcribed to cDNA using SuperScriptIII First-Strand Synthesis SuperMix for qRT-PCR kit according to manufacturer’s protocol (Life Technologies). Reverse transcription reaction conditions were
Figure 29. Schematic diagram showing strategy for scoring wound healing. For each treatment group, cells were seeded in 9-12 wells in six well tissue culture dishes. Each well received one scratch (shown as solid black line) and two fields per scratch (white squares) were photographed at various times post-scratch, followed by scoring. Field area was calibrated by a horizontal marker shown in green line.
25°C for 10 minutes, 50°C for 30 minutes, and 85°C for 5 minutes, followed by a 20 minute digestion with RnaseH.

T8 mRNA variants were detected by PCR using Platinum PCR Supermix kit according to manufacturer’s instructions (Life Technologies) and agarose gel electrophoresis. In brief, 10 ng of cDNA was mixed with 45 µL of Platinum PCR Supermix, 0.1 nM forward and reverse isoform specific primers, and RNase/DNase free water to a final volume of 50 µL. PCR reaction conditions were 94°C for 5 minutes, and 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, followed by 72°C for 15 minutes. Twenty µL of cDNA was electrophoresed on a 2% agarose gel containing 0.0005% ethidium bromide in Tris-Acetate-EDTA buffer, and photographs were taken by digital camera Fluorchem 8900 (Alpha Innotech, San Leandro, CA). DNA ladder was GeneRuler 100 bp T8 mRNA variant specific primers were designed based on the T8 sequence at UCSC Genome Browser (http://genome.ucsc.edu/).

T8 variant 1 F1, 5'-GTCGTGTGCGGATTTCGCTGCAG -3’;

T8 variant (X) F(X), 5'-GAACGTGCAGTTCTTAAGCCTTAG-3’;

T8 variant 2 F2, 5'-GGAACCGGTAGCCACCGAGAGAGCA-3' ;

T8 variant 4 F4, 5'-GTACACGTCAACGGGTGGTTGCATG-3’;

T8 reverse primer (R1), 5'-CTGGTCACTCTGTAGCTCATC-3',

GAPDH F 5’ – AGAAGGCTGGGTTGAGGAGGAGG – 3’;

GAPDH R 5’ – AGGGGCAATCCACAGCTTTC – 3’.
For qPCR assays, Fast SYBR Green Master Mix kit was used according to manufacturer’s instructions (Life Technologies). Ten ng of cDNA was mixed with 10 µL of Fast SYBR green master mix, 0.1 nM forward and reverse gene specific primers, and RNase/DNase free water to a total volume of 20 µL. At least 12 reactions per experiment were performed for each set of gene specific primers. The qPCR reactions were performed using 7900HT Fast Real-Time PCR system (Applied Biosystems). The qPCR reaction conditions were 95°C 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. Various CT values obtained were normalized against mean CT value of ribosomal protein, large P0 (RPLP0) as internal control by subtraction (i.e. ΔCT) and fold change in mRNA expression in shT8 cells versus scr cells was calculated by the 2^ΔΔCT method (Livak and Schmittgen, 2001). T8 isoform 3 and IGFBP3 specific primers were designed based on gene sequence information at UCSC Genome Browser (http://genome.ucsc.edu/). SNX1 and EGFR specific primers were designed as reported earlier (Nishimura et al., 2012).

Various gene specific primers used for qPCR are as follows:

IGFBP3 F, 5’- GAGGACGTGCACGTGCTACAGCATGC -3’

IGFBP3 R, 5’ – GGTCATGTCCTTGGCAGTCTTTTGT -3’

SNX1 F, 5’-AGCCCCAGCCAACCTATGA-3’

SNX1 R, 5’-TCAGGATCAGTTATAACCGACTGT-3’

EGFR F, 5’-GCATTCCGCACGGTGTATAA-3’

EGFR R, 5’-GGCTTTCCGGAGATGTGCTTC-3’
RPLP0 F, 5’ – ATCCATCTGCCTTTGCGTGC -3’

RPLP0 R, 5’ – CTCCGACTCTTCCCTGCTTCA – 3’

T8 variant 2 F2, 5’-GGAACCGGTGAGCCACCGAGAGCA-3’

T8 reverse primer, 5’-CTGGTCACCTGAGCTAGCCTC-3’

2.11. Immunoblotting

Immunoblotting was performed following the AbCAM Western Blotting guide (http://www.abcam.com/index.html?pageconfig=resource&rid=11375). Culture medium was removed and cells were washed once with ice cold PBS, and scraped in RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail P8340, phosphatase inhibitor cocktail #2 P5726, and 1mM PMSF) or a modified lysis buffer (100 mM HEPES, 150 mM NaCl, 1% IGEPAL CA-630, 1mM EDTA, 10% glycerol, 1 mM PMSF, protease inhibitor cocktail P8340, phosphatase inhibitor cocktail #2 P5726). The lysate (200 µL per well in 6-well plates) was collected in an eppendorf tube and incubated on ice for 30 minutes, followed by sonication for 20 seconds 3 times in a Sonicator (Misonix). The supernatant was collected by microcentrifugation at 13,000 rpm (15890 xg), for 30 minutes at 4°C. Protein concentration was measured using Pierce BCA Protein Assay and absorbance at 562 nm using a spectrophotometer (DU 640, Beckman Coulter, Brea, CA). Lysate was mixed with LDS NuPAGE sample buffer containing sample reducing buffer (1:1) and boiled for 5 minutes and then run on a pre-cast 4-12% polyacrylamide gel in MES running buffer containing antioxidant at 90V for 2 hours at RT. Ten µL of the molecular weight standard (Seeblueplus2 protein standard, size range 3 kDa to 188 kDa) was included in each gel. Proteins were transferred to a 0.45 µm PVDF membrane in transfer buffer (25 mM
Bicine, 25 mM Bis-Tris, 1 mM EDTA, pH 7.2) using the X-Cell II blot module (LifeTechnologies) at 30V for 1 hour at RT. Membrane was washed twice in TBST buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Tween-20) and blocked in either 3% non-fat milk or 3% bovine serum albumin (BSA) in TBST at RT on a shaking platform. Blots were incubated with primary antibody in fresh blocking buffer O/N at 4°C on a shaking platform. For immunoblotting with anti-EGFR (1/2000 dilution), anti-pEGFR Y1068 (1/1000 dilution), anti-AKT (pan) (1/2000 dilution), anti-pAKT S473 (1/1000 dilution), anti-IGF-1R (1/2000 dilution), anti-pIGF-1R Y1135 (1/1000 dilution), anti-ERK1/2 (p44/42 MAPK) (1/2000 dilution), and anti-pERK1/2 (1/1000 dilution) antibodies, membranes were blocked in 3% BSA in TBST, and then incubated with indicated dilution of the primary antibody in 3% BSA in TBST O/N at 4°C, and then washed three times in TBST buffer at RT. The membranes were then incubated with either anti-mouse or anti-rabbit secondary antibody HRP conjugate (1/2000 dilution) in 3% BSA in TBST for two hours at RT. For immunoblotting with anti-SNX1 (1/1000 dilution), anti-β-ACTIN (1/5000 dilution) and anti-T8 (1/5000 dilution) antibodies, membranes were blocked in 3% non-fat milk in TBST, incubated with primary antibody in 3% non-fat milk in TBST O/N at 4°C, and then washed three times in TBST buffer at RT. The membranes were incubated with either anti-mouse or anti-rabbit secondary antibody HRP conjugate (1/2000 dilution) in 3% non-fat milk in TBST for two hours at RT. For immunoblotting with anti-IGFBP3 antibody (1/1000 dilution), membranes were blocked in 3% non-fat milk in TBST, and incubated with primary antibody in 3% non-fat milk in TBST for 8 hours at RT. Membranes were washed three times in TBST buffer at RT, followed by incubation with anti-rabbit secondary antibody HRP conjugate (1/2000 dilution) in 3% non-fat milk in TBST for two hours at RT. Following treatment with the secondary antibody, membranes were washed three times in
TBST, incubated with HyGLO chemiluminescent detection reagent for one minute, and exposed to HyBlot autoradiography film. For reprobing, blot was sequentially washed once in PBS and once in deionized water, and then incubated in stripping buffer (15 g/L glycine, 1 g/L SDS, 0.1% Tween-20, pH 2.2) for 20 minutes prior to reuse. The autoradiography films were scanned and band density was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). For evaluation of the T8 isoforms, 15% SDS-PAGE was performed using 50 mL of separating gel (15% acrylamide/N,N’-methylene-bis acrylamide (15% T, 2.67%C) in 0.375 M Tris, pH 8.8, 0.1% SDS, 0.1% ammonium persulfate and 20 μL TEMED) and 10 mL of stacking gel (4% acrylamide/N,N’-methylene-bis acrylamide (4% T, 2.67%C) in 0.125 M Tris, pH 6.8, 0.1% SDS, 0.1% ammonium persulfate and 10 μL TEMED). The lysate in RIPA lysis buffer was mixed with NuPAGE LDS sample buffer containing sample reducing buffer (1:1) and boiled prior to electrophoresis using running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) at 24 mA for 6 hours at RT. Immunoblotting using anti-T8 antibody was performed as described above.

2.12. Immunofluorescence

To examine the colocalization of EFGR and SNX1 or EEA1 in A549 shT8 cells and A549 scr control cells in response to EGF, logarithmically growing cells were trypsinized and seeded at 1 x 10^5 cells on a sterile glass coverslip placed in a 12-well plate in complete medium. Three coverslips were used per treatment group. After 48 hours, cells were switched to SFM for 12 hours, and treated for 10 minutes or 30 minutes with 50 ng/mL EGF or left untreated (-EGF). Cells were fixed in 4% paraformaldehyde in PBS for 10 minutes, washed in PBS three times for 5 minutes each, and permeabilized in 0.1% Triton X-100 in PBS for 20 minutes. After washing with PBS, blocking was done in 3% normal goat serum for 20 minutes. Subsequently the cover
slips were removed and cells were immunostained using an inverted staining method as recommended (Microscopy and Imaging Shared Resource, Lombardi Comprehensive Cancer Center). For immunodetection, combinations of two primary antibodies were mixed in 3% goat serum in PBS for a final dilution of 1:200 per antibody: rabbit anti-EGFR antibody plus mouse SNX1 antibody, and rabbit anti-EGFR antibody plus mouse anti-EEA1 antibody. One hundred μL of the antibody mixture was dropped on a parafilm, and the coverslip was placed on the drop with the cells in contact with the antibody. The cells were incubated with a mixture of the two primary antibodies for 1 hour at RT, washed with PBS three times for 5 minutes each, followed by treatment with a mixture of two secondary antibodies Alexa Fluor 488 goat anti-rabbit A11034 (1/200 dilution), Alexa Fluor 546 goat anti-mouse IgG1 (γ1) A21123 (1/200 dilution), and 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI) in 3% goat serum in PBS. After 1 hour, coverslips were washed three times in PBS and mounted on a prewashed glass slide in Fluorogel, and air-dried for 24 hours at RT. The colocalization of EGFR and SNX1 (green and red) or EGFR and EEA1 (green and red) was detected by confocal immunofluorescence microscopy using a Zeiss LSM510 Confocal and Deep tissue imaging microscope (Zeiss, Oberkochen, Germany). Photomicrographs were visually scored for EGFR+ve puncta (green), EEA1+ve puncta (red), and EGFR+ve and EEA1+ve puncta (merge, yellow) in various treatment groups as reported earlier (Nishimura et al., 2012). For the 10 minutes EGF group, three fields were scored with total approximately 15-20 cells, and for the 30 minutes EGF group, five fields were scored with a total of 50-60 cells.

2.13. XTT assay for cell viability
The effects of T8 knockdown on cytotoxicities of gefitinib and AG1024 were tested using the XTT cell viability assay kit. The stock solutions of gefitinib (100 mM) and AG1024 (10 mM) were prepared in DMSO (100%), and aliquots were stored at -20°C. One thousand cells were seeded per well (six wells per treatment group) in a 96-well tissue culture dish in complete medium. The next day medium was replaced with SFM containing gefitinib (0.25 µM, 0.5 µM, or 1 µM), AG1024 (0.5 µM, or 1 µM), or vehicle control (DMSO) for 72 hours. Twenty-five µL of activated XTT reagent was added to each well and incubated for 3 hours. XTT in the culture medium is reduced from a colorless tetrazolium salt to a brightly orange XTT formazan by breaking apart a positively-charged quaternary tetrazole ring. Reduction of the tetrazolium salt is accomplished by the succinate dehydrogenase system of the mitochondrial respiratory chain in viable cells. Hence the intensity of the orange color is proportional to the number of metabolically active cells. Absorbance in each well was measured at 450 nm on a Victor III spectrophotometer plate reader (Perkin Elmer, Waltham, MA). Mean +/- s.d, values were obtained after correction for the absorbance values from the drug alone (no cells) group.

2.14. Statistical analysis

Statistical significance was analyzed using the Student’s T-test. All means and confidence intervals were calculated using Microsoft Excel (Microsoft Corporation, Seattle, WA). In all statistical analyses, two-sided P-values were calculated and P <0.05 was considered statistically significant.
Chapter 3. Results

3.1. Study of the effects of T8 knockdown on EGF-induced signal transduction in non-small cell lung cancer cells

3.1.1. Analysis of T8 transcripts and protein isoforms in various tumor cells. Genomic organization of human T8 and T8 mRNA transcripts and protein isoforms are shown in Figures 30 and 31. Human T8 gene is located on chromosome 5q23.1. Genomic T8 is 125,912 bp long and is comprised of 7 introns and 8 exons. Analysis of the alternative splicing graph from the Swiss Institute of Bioinformatics (35392) indicates a total of 10 exons, where the last exon 8 seems to be alternatively spliced into two additional exons (Figure Supplement S1) (http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg38&position=chr5%3A119237213-119426083&hgsid=399825221_VfDB9aASTHw0cOPHNrkmoa6WjnhP). Four major transcript variants of T8 mRNA and corresponding open reading frames are shown in Figure 31. Transcript variants 1 and 3 code for T8 protein isoform 1. Variants (X), 2 and 4 code for protein isoforms 2, 3, and 4, respectively (Figures 30 and 31). To analyze the expression of various T8 mRNA transcripts (variants 1, (X), 2 and 4) in cancer cell lines, forward and reverse primers were designed from within adjacent exons to ensure that the PCR product originated exclusively from the spliced mRNA (Figure 32, panel A). In addition, exon locations of the forward primers were unique to each transcript variant. RNA was isolated from five cancer cell lines derived from metastatic tumors of the prostate (C4-2B, DU-145, PC-3, and PC-3M) and breast (LM2-4175). The anticipated sizes of the PCR products are shown in the legend to Figure 32. Differential expression of variants 1 and 2 was observed in the five cell lines tested, whereas variants (X) and 4 were found to be the least abundant transcripts in these
Figure 30. Human TNFAIP8: schematic map of genomic organization, alternative transcripts, and protein isoforms. Genomic localization, mRNA transcripts, and protein isoforms of human TNFAIP8 shown are based on data obtained from the NCBI [http://www.ncbi.nlm.nih.gov/gene/25816], UCSC genome browser [http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg38&position=chr5%3A119237213-119426083&hgsid=399825221_VfDB9aASTHw0cOPHNrkmOa6Wjnhp] and Uniprot websites [http://www.uniprot.org/uniprot/O95379]. Map was created by blasting TNFAIP8 transcript sequences against genomic TNFAIP8 sequence and alignment of each exon on the genomic sequence. Accession numbers: Variant 1, NM_014350.3; Variant 3, NM_001286813.1; Variant (X), AF099936; Variant 2, NM_001077654.2; and Variant 4, NM_001286814.1.
Figure 31. Human TNFAIP8: predicted alternative transcripts including coding and untranslated sequences and corresponding protein isoforms are shown. Protein isoform sequences were obtained from Uniprot website (http://www.uniprot.org/uniprot/O95379).
Figure 32. Validation of TNFAIP8 transcript variants in representative cancer cell lines. A. Locations of the variant specific forward primers (F1, F(X), F2, and F4), and the reverse primer (R1) designed from within the highly conserved exon 10 are shown by arrows. B. Agarose gel electrophoresis showing PCR products representative of transcript variants V1-V4. L, DNA ladder; V1, variant 1 (268 bp), V(X), variant (X) (188 bp), V2, variant 2 (241 bp), and V4, variant 4 (255 bp); G, Gapdh (285 bp)
cell lines (Figure 32, panel B). Of particular note, LNCaP derived cell line C4-2B showed high expression of variant 2, while PC-3 and its metastatic derivative cell line PC-3M showed high expression of variant 1. Comparable expression of variants 1 and 2 were seen in DU-145 cells and LM2-4175 cells. Since T8 protein isoform 3 was found to be highly expressed in A549 NSCLC cells (data shown later), subsequent mRNA expression studies of T8 were performed using primers specific for transcript variant 2.

Expression of T8 protein in prostate and breast cancer cell lines was analyzed by immunoblotting using a custom-made anti-peptide T8 specific antibody (Figure 33). The epitope for the T8 peptide antibody corresponds to exon 8 as reported earlier (Kumar et al., 2004). Differential expression of two major isoforms, 21 kDa and 23 kDa, corresponding to transcript variants 2 and 1, respectively, was observed in various cancer cell lines (Figure 33, panel A and panel B). These data indicate that transcripts variants 1 and 2, and corresponding protein isoforms 1 (23 kDa) and 3 (21 kDa), are highly abundant in cancer cells tested.

3.1.2. Establishment of the T8 knockdown models in cancer cell line. Pilot transfection experiments were performed to test the T8 knockdown efficiencies of five T8 shRNAs in cancer cells. As shown in Figure 34, panel A, T8 shRNAs 1, 2, and 4 were found to be most efficient in silencing T8 protein expression in transient transfection experiments as compared to pLKO.1 empty vector transfected cells (EV). Interestingly, in stably transfected prostate, breast and melanoma cancer cell lines T8 shRNA 3 (shT8) was found to be the most potent construct in silencing T8 expression (Figure 34, panel B- panel E). Subsequently, knockdown efficiency of shT8 lentiviral vector was validated in comparison with the scrambled shRNA containing vector (scr). As shown in Figure 34, approximately 60-90% inhibition of T8 expression was found in shT8.
Figure 3. Expression analysis of TNFAIP8 protein isoforms in representative prostate (PC-3, PC-3M, DU-145, C4-2B) and breast cancer cell lines (LM2-4175). Immunoblotting was performed using anti-TNFAIP8 antibody as described in Materials and Methods.
Figure 34. TNFAIP8 silencing by transient and stable transductions of lentiviral TNFAIP8 shRNA expression vectors. A. Immunoblot analysis of TNFAIP8 knockdown by transient transfection of TNFAIP8 shRNA plasmid DNA (1-5) into PC-3 cells. B-D. Immunoblot analysis of TNFAIP8 knockdown by stable transfection of TNFAIP8 shRNA plasmid DNA into various cancer cell lines. E. PC-3 cells were stably transfected with TNFAIP8 shRNA 3 (shT8) or empty vector (EV). The blot was reprobed with anti-β-ACTIN antibody and fold change in TNFAIP8 expression relative to EV was obtained after normalizing against β-ACTIN expression in the corresponding lanes.
transfected lung cancer cell lines A549 and H1299 (Figure 35, panel A), prostate cancer cell lines PC-3 and C4-2B (Figure 35, panel B), and pancreatic cancer cell line PANC-1 (Figure 35, panel C). These data established the effectiveness of shT8 (T8 shRNA 3) in silencing T8 in various tumor cell models used in this project.

3.1.3. Effect of stable knockdown of T8 on growth factor-induced cell migration in A549 cell. As a step toward understanding the role of T8 in growth factor-induced mechanisms of signal transduction and biological response in non-small cell lung cancer, the effect of T8 knockdown on growth factor-induced cell migration was investigated in A549 cells. The wound healing assay was performed to study the effects of stable knockdown of T8 on cell migration in A549 cells treated with 10% FBS and five growth factors EGF, IGF-1, FGF-1, and VEGF. In a time course experiment, a significant decrease in wound closure (i.e. cell migration) was observed in A549 shT8 cells as compared to A549 scr control cells in response to 50 ng/mL EGF (% wound healed post-scratch (mean +/- s.d., n = 3 wells): 6 hours, scr, 20.06 +/- 4.47, shT8, 13.19 +/- 5.02, P-value < 0.05; 12 hours, scr, 51.13 +/- 6.70, shT8, 25.06 +/- 9.46, P-value < 0.001; 18 hours, scr, 63.62 +/- 7.04, shT8, 32.03 +/- 11.66, P-value < 0.001; 24 hours, scr, 71.33 +/- 8.87, shT8, 35.80 +/- 12.15, P-value < 0.001) (Figure 36, panel A). Under control serum free medium conditions or 10% FBS, no significant difference was noted in cell migration in A549 shT8 and A549 scr cells in this experiment (SFM: % wound healed post-scratch (mean +/- s.d., n = 3 wells, all P-values > 0.05): 6 hours, scr, 11.43 +/- 4.41, shT8, 10.06 +/- 1.31; 12 hours, scr, 22.74 +/- 8.32, shT8, 15.28 +/- 2.51; 18 hours, scr, 22.96 +/- 7.78, shT8, 19.41 +/- 4.00; 24 hours, scr, 25.51 +/- 7.09, shT8, 21.21 +/- 4.18; 10%FBS: % wound healed post-scratch (mean +/- s.d., n = 3 wells, all P-values > 0.05): 6 hours, scr, 36.80 +/- 2.47, shT8, 29.53 +/- 12.62; 12 hours, scr, 64.64 +/- 3.08, shT8, 50.23 +/- 21.51; 18 hours, scr,
Figure 35. Efficient silencing of TNFAIP8 by stable transduction of lentiviral TNFAIP8 shRNA expression vector. Cancer cell lines were stably transfected with TNFAIP8 shRNA 3 (shT8) or scrambled shRNA (scr) as described in Materials and Methods. The blots were reprobed with anti-β-ACTIN antibody and fold change in TNFAIP8 expression relative to scr was obtained after normalizing against β-ACTIN expression in the corresponding lanes. A. Lung cancer cell lines. B. Prostate cancer cell lines. C. Pancreatic cancer cell line.
Figure 36. Time-course experiment showing stable T8 knockdown inhibits cell migration in response to EGF in A549 lung carcinoma cells. The wound healing assay was performed as described in Materials and Methods. Top panels. Images of wound closures at various time points post-scratch in the presence of EGF 50 ng/mL (A), SFM (B), or 10% FBS (C) as observed by phase contrast microscopy. Bottom panels. Quantification data (mean +/- s.d.) are shown as % of wound healed relative to 0 hour wound in A549 scr cells (hatched bars) and A549 shT8 cells (solid bar). Each data point represents two fields per scratch per well (n=3). P values, A549 scr versus A549 shT8 at various time points: A. 50 ng/mL EGF, *, P-value < 0.05, **, P-value < 0.001. B. SFM, all P-values > 0.05; C. 10% FBS, all P-values > 0.05.
76.94+/- 5.23, shT8, 64.47 +/- 19.90; 24 hours, scr, 86.75 +/- 4.76, shT8, 70.75 +/- 21.54) (Figure 36, panel B and panel C).

The effect of T8 knockdown on cell migration in A549 cells was further investigated in an independent set of three experiments (n = 3 wells per time point per experiment). As shown in Figure 37 (panel A and panel B), T8 knockdown was found to significantly delay wound closure at 24 hours post-scratch in all three growth conditions tested (% wound healed, mean +/- s.d., P-value < 0.001, n=18: 50 ng/mL EGF, A549 scr, 63.7 +/- 14.3, A549 shT8, 29.7 +/- 11.4 (Figure 37, panel B, left); SFM, A549 scr, 25.3 +/- 5.25, A549 shT8, 18.3 +/- 3.73 (Figure 37, panel B, middle); 10% FBS, A549 scr, 79.0 +/- 13.3, A549 shT8, 60.3 +/- 18.9 (Figure 37, panel B, right).

Similar results were obtained in the presence of three additional growth factors tested, IGF-1, FGF-1, and VEGF (Figure 38, panel A and panel B). In A549 scr control cells treated with IGF-1 (50 ng/mL) for 24 hours, the wound had closed 33.2 +/- 8.9% relative to the 0 hour, while in A549 shT8 cells, the wound had closed 21.7 +/- 5.5% (n=24 wells, P-value < 0.001) (Figure 38, panel B, left). Likewise, FGF-1 treatment (20 ng/mL, 24 hours) induced 26.9 +/- 5.4% cell migration in A549 scr cells relative to 0 hour and 16.1 +/- 5.9% in A549 shT8 cells (n= 18 wells, P-value < 0.001) (Figure 38, panel B, middle). T8 knockdown also impacted cell migration in response VEGF in A549 cells (50 ng/mL, 24 hours) (% wound closure, A549 scr, 31.7 +/- 6.81, A549 shT8, 15.1 +/- 5.1, n= 18 wells, P-value < 0.001) (Figure 38, panel B, right). As shown in Table 5, all growth factors except VEGF were found to enhance cell migration in both A549 scr control cells as well as A549 shT8 cells. In addition, the above data demonstrate that silencing of T8 expression significantly impairs cell migration in A549 cells in response to all four growth factors tested.
Figure 37. Stable knockdown of T8 inhibits EGF-induced cell migration in A549 lung carcinoma cells. The wound healing assay was performed as described in Materials and Methods. A. Images of wound closures at various time points post-scratch in the presence of EGF 50 ng/mL, SFM or 10% FBS as observed by phase contrast microscopy. B. Quantification data (mean +/- s.d.) are shown as % of wound healed at 24 hours relative to 0 hour wound in scr A549 and shT8 A549 cells. Each data point represents three independent experiments (n=3 wells per experiment, P-values were as shown above).
Figure 38. Stable knockdown of T8 inhibits IGF-1, FGF-1 and VEGF-induced cell migration in A549 lung carcinoma cells. The wound healing assay was performed as described in Materials and Methods. A. Images of wound closures at various time points post-scratch in the presence of IGF-1 50 ng/mL, FGF-1 (20 ng/mL), or VEGF as observed by phase contrast microscopy. B. Quantification data (mean +/- s.d.) are shown as % of wound healed at 24 hours relative to 0 hour wound in scr A549 and shT8 A549 cells. Each data point represents three to four independent experiments (n=3 wells per experiment, P-values were as shown above).
Table 5. Effects of growth factors on cell migration in A549 scr and A549 shT8 cells.

<table>
<thead>
<tr>
<th>Growth Factor (GF)</th>
<th>A549 scr cell migration SFM (24hr) vs GF (24hr) P-value (n)</th>
<th>A549 shT8 cell migration SFM (24hr) vs GF (24hr) P-value (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% FBS</td>
<td>0.000 (18)</td>
<td>0.000 (18)</td>
</tr>
<tr>
<td>EGF (50 ng/mL)</td>
<td>0.000 (18)</td>
<td>0.000 (18)</td>
</tr>
<tr>
<td>IGF-1 (50 ng/mL)</td>
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<td>0.047 (24)</td>
</tr>
<tr>
<td>FGF-1 (20 ng/mL)</td>
<td>0.003 (18)</td>
<td>0.032 (18)</td>
</tr>
<tr>
<td>VEGF (50 ng/mL)</td>
<td>0.303 (18)</td>
<td>0.179 (18)</td>
</tr>
</tbody>
</table>
3.1.4. Effect of T8 knockdown on EGFR expression in A549 cells. In the antibody microarray analysis, decreased constitutive expression of EGFR was seen in T8 knockdown models of PC-3, MDA-MB-231 and MDA-MB-435 cell lines (shT8 vs scr, PC-3, -1.7, MDA-231, -1.3, MDA-435, -1.3) (Supplemental Table S1). To address whether T8 knockdown modulates EGFR expression in NSCLC cells, immunoblotting was performed using A549 shT8 and A549 scr cells. As shown in Figure 39, panel A, A549 shT8 cells showed decreased constitutive expression of EGFR protein as compared to A549 scr cells. A549 shT8 cells had 0.78 fold EGFR protein expression compared to scr control (Figure 39, panel A). However, using qRT-PCR assay, EGFR mRNA expression was not decreased in A549 shT8 cells relative to scr control (fold decrease in A549 shT8 cells, 0.91 +/- 0.09 (mean +/- s.d., n=24) (Figure 39, panel B). In addition, EGFR protein expression was found to be reduced in T8 knockdown model of prostate cancer (PC-3). PC3 shT8 showed a 0.71 fold decrease in EGFR expression relative to PC3 scr control cells (Figure 39, panel C).

3.1.5. Effect of T8 knockdown on endosomal sorting protein sorting nexin 1 (SNX1) expression involved in EGF-induced late endosomal/lysosomal targeting of EGFR. To determine whether T8 knockdown is associated with modulation of known regulator(s) of EGFR expression in cancer cells, the RNA microarray expression profiling of T8 knockdown cells was performed using the Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA) at the Genomics and Epigenomics Shared Resource of the Georgetown-Lombardi Comprehensive Cancer Center. The total RNA microarray analysis revealed that expression of sorting nexin 1 (SNX1) mRNA was increased 1.4 fold in PC-3 shT8 cells as compared to PC-3 scr cells (Supplemental Table S1). In addition, antibody array expression profiling of T8 knockdown MDA-MB-231 and LM2-4175 cells was performed using the BD Clontech Antibody Microarray
Figure 39. A. Immunoblot analysis showing constitutive downregulation of EGFR protein expression in T8 knockdown A549 cells as compared to scr control cells. A549 cells stably transfected with T8 shRNA or scr shRNA lentivirus were cultured in complete medium containing 10%FBS, followed by cell lysis and immunoblotting. B. qRT-PCR analysis of T8 knockdown A549 cells as compared to scr control cells. EGFR scores were normalized to ribosomal protein large, P0 (RPLP0) control. Quantification data (mean +/- s.d.) shown are from 24 wells per treatment group from one experiment performed. C. Immunoblot analysis showing constitutive downregulation of EGFR protein expression in T8 knockdown PC-3 cells as compared to PC-3 scr control cells.
500 at the Proteomic Center at the Uniformed Services University of Health Sciences, Bethesda, MD. In these experiments, SNX1 protein expression was detected to be increased 1.5 fold in T8 knockdown models of MDA-231 and LM2-4175 relative to control cells, (Supplemental Table S1). Furthermore, immunoblot analysis revealed increased SNX1 expression in representative T8 knockdown models of prostate (C4-2B) and pancreatic cell lines (PANC-1) (Figure 40).

To investigate the effects of T8 knockdown on mRNA and protein expression of SNX1 in non-small cell lung cancers, we performed immunoblot analysis and qRT-PCR analysis of T8 and SNX1 in A549 shT8 and A549 scr cells. A549 cells with stable expression of shT8 resulted in silencing of T8 protein and mRNA expression relative to A549 scr control (Figure 41, panel A - panel D). Immunoblotting revealed that approximately 70% inhibition of T8 protein expression in stably transfected A549 shT8 cells as compared to A549 scr cells (0.28 +/- 0.14 (mean +/- s.d., n=3)) (Figure 41, panel A). Expression of T8 mRNA was also inhibited approximately 60% in A549 shT8 cells relative to A549 scr cells (0.42 +/- 0.20 (mean +/- s.d., n=12)) (Figure 41, panel B). Consistent with the RNA microarray and antibody microarray analysis, both mRNA and protein expression of SNX1 was increased by approximately 3 fold in A549 shT8 cells relative to A549 scr cells (SNX1 mRNA fold increase, 3.37 +/- 0.87 (mean +/- s.d., n=24); SNX1 protein fold increase, 2.92 +/- 0.99 (mean +/- s.d., n=8)) (Figure 41, panel C and panel D). A five-fold increase in expression of SNX1 was noted in another non-small cell lung cancer cell line H1299 stably transfected with shT8 (Figure 41, panel E). These data demonstrate that knockdown of T8 expression may drive increase in both SNX1 mRNA and SNX1 protein expression in the non-small cell lung carcinoma cell lines.

Earlier reports have shown that overexpression of SNX1 in lung adenocarcinoma cells leads to direct binding of SNX1 with EGFR, and accelerated EGF-induced degradation of EGFR
Figure 40. Preliminary immunoblot analysis suggests increased expression of SNX1 protein in T8 knockdowns in two additional cancer lines tested.
Figure 41. T8 knockdown correlates with increased steady state expression of SNX1 in nonsmall cell lung cancer cell lines.  

A. T8 knockdown correlates with increased steady state expression of SNX1 mRNA and protein in A549 cells.  

Left panel, Immunoblot showing stable knock down of TNFAIP8 protein in A549 cells transfected with lentiviral TNFAIP8 shRNA or scrambled control shRNA (scr).  

Right panel, bar graph showing quantification data (mean +/- s.d.) from 3 independent experiments.  

B. TNFAIP8 mRNA expression was assessed by qRT-PCR in A549 cells as described in Materials and Methods. TNFAIP8 scores were normalized to ribosomal protein large P0 (RPLP0) mRNA.  

Quantification data (mean +/- s.d.) shown are from three independent experiments using 12 wells per treatment group in each experiment.  

C. Left panel, Immunoblot analysis showing increased expression of SNX1 protein in A549 cells transfected as in panel A.  

Right panel, bar graph showing quantification data (mean +/- s.d.) from 8 independent experiments.  

D. SNX1 mRNA expression was assessed by qRT-PCR in A549 cells as described in Materials and Methods. SNX1 scores were normalized to ribosomal protein large, P0 (RPLP0) control.  

Quantification data (mean +/- s.d.) shown are from 24 wells per treatment group from one experiment.  

E. T8 knockdown correlates with increased steady state expression of SNX1 protein in H1299 cells. The blot was sequentially probed with anti-snx1 and anti-β-ACTIN antibodies.
through the endocytic-lysosomal pathways (Kurten et al., 1996). In the context of present studies, it is plausible that T8 knockdown may offer a novel regulatory mechanism by enhancing steady state expression of SNX1 leading to downregulation of EGFR via lysosomal degradation and subsequent decrease in downstream effectors of EGF/EGFR signaling including pERK (Figure 42).

3.1.6. Effects of the EGF on expression levels of total EGFR and phosphorylated EGFR in T8 knockdown A549 cells. To investigate the effect of T8 knockdown on EGF-induced downregulation of EGFR, logarithmically growing A549 shT8 and A549 scr cells were grown in serum free medium (SFM) for 12 hours, and then treated with various doses of EGF in SFM for 30 minutes, followed by sequential immunoblotting with anti-EGFR, anti-T8, and β-ACTIN antibodies (Figure 43, panel A). A dose-dependent downregulation of EGFR expression EGFR expression was observed in A549 shT8 cells relative to A549 scr cells (EGFR expression in A549 shT8 cells relative to scr cells: 25 ng/mL, 0.85; 50 ng/mL, 0.31 +/- 0.04; 75 ng/mL, 0.57; 100 ng/mL, 0.22 +/- 0.14 (mean +/- s.d.)) (Figure 43, panel B). Likewise, robust EGF-responsive downregulation of EGFR expression (> 80%) was observed in independently generated stable T8 knockdown A549 cells as compared to A549 scr cells (Figure 43, panel C). Consistent with these observations, EGF treatment led to significant decrease in expression of phosphorylated EGFR (pEGFR) at all EGF doses tested in A549 shT8 cells as compared to A549 scr cells (Figure 43, panel D). Our data suggest that loss of T8 promotes inhibition of EGF-induced EGFR/pEGFR signaling in NSCLC cells.

3.1.7. Effect of SNX1 knockdown on total EGFR expression in A549 shT8 cells in the presence of EGF. To verify that increased expression of SNX1 in T8 knockdown A549 cells is directly associated with enhanced downregulation of EGFR expression in the presence of EGF,
Figure 42. A working model of role of TNFAIP8 in EGF-induced regulation of EGFR and pERK expression in non-small cell lung cancer cells (NSCLC). In response to EGF, EGFR undergoes dimerization and internalization and is processed through the endosomes for either recycling to the plasma membrane or SNX-1-mediated lysosomal degradation. Increased levels of SNX-1 in T8 knockdown cells may enhance EGF-induced targeting of EGFR to late endosomes in NSCLC and downregulate EGF-induced pERK expression in NSCLC.
Figure 43. Decreased expression of EGFR and pEGFR in EGF-treated T8 knockdown A549 cells relative to scrambled control cells. A. Dose response experiment showing downregulation of total EGFR expression in EGF-treated A549 shT8 cells. Logarithmically growing cells were seeded in 6-well tissue culture dishes (3x10^5 cells per well) in complete medium containing 10% FBS. At approximately 90% confluency, medium was replaced with serum free RPMI 1640 with penicillin/streptomycin (SFM) for 12 hours. The medium was then changed to SFM containing indicated doses of EGF for 30 minutes, followed by cell lysis and immunoblotting. The blot was sequentially probed with anti-EGFR, anti-TNFAIP8 and anti-β-ACTIN antibodies. B. Bar graph of total EGFR expression levels in A549 shT8 cells relative to A549 scr cells treated with indicated doses of EGF. Values shown for 50 ng/mL and 100 ng/mL represent mean +/- s.d., n=2. C. An independent experiment showing significant decrease in total EGFR expression in EGF-treated A549 shT8 cells relative to A549 scr control cells. D. Pilot experiment showing pEGFR levels are also reduced in response to EGF in A549 shT8 cells as compared A549 scr control cells. nt, non-transfected.
SNX1 expression in A549 shT8 cells was silenced using SNX1 siRNA #1, siRNA #2 or a combination thereof (Figure 44, panel A). As shown in Figure 44, panel A, siSNX1 #1 treatment resulted in approximately 70% decrease of SNX1 expression relative to siControl, whereas treatment with siSNX1#2 resulted in an 80% decrease in SNX1. Apparently, the combination of these two siRNA seemed to less potent in inhibition of SNX1 expression in this experiment (47% relative to siControl). We therefore used siSNX1 #2 to test the effect of SNX1 knockdown on EGF-induced downregulation of EGFR in A549shT8 cells. EGFR expression level in A548 shT8 cells treated with siControl was inhibited approximately 62% at 30 minutes in the presence of EGF relative to untreated cells, whereas in cells treated with siSNX1#2 EGFR expression level was inhibited only 27% at 30 minutes in the presence of EGF relative to untreated cells (Figure 44, panel B). Similar results were obtained in SNX1 knockdown A549 shT8 cells treated with EGF for 15 minutes (Figure 44, panel B). These data show that knockdown of SNX1 partially restores EGFR expression in A549 shT8 cells in the presence of EGF, implicating that T8 plays an important role in SNX1-mediated ligand-induced degradation of EGFR in NSCLC cells.

3.1.8. Analysis of co-localization of EGFR and SNX1 and time course study of EGFR localization within early endosomes in response to EGF in A549 shT8 and A549 scr cells. We first verified EGF-responsive internalization and colocalization of EGFR and SNX1 in A549 cells. In the absence of EGF, endosomal puncta were observed to be SNX1 positive but EGFR negative in both A549 scr and A549 shT8 groups (Figure 45, panel A and panel B, top rows). In contrast, after 10 minutes of EGF treatment of A549 scr and A549 shT8 cells, puncta were both EGFR positive and SNX1 positive, indicative of EGF induced EGFR internalization and
colocalization of EGFR and SNX1 (Figure 45, panel A and panel B, bottom rows). This indicates that in both A549 scr and A549 shT8 cells, treatment of EGF induces EGFR

Figure 44. Pilot experiment showing siRNA knockdown of SNX1 in T8 knockdown A549 cells restores total EGFR expression in the presence of EGF. A. A549 cells stably expressing TNFAIP8 shRNA (A549 shT8) were transfected with indicated SNX1 siRNAs or siControl as described in Materials and Methods. After 6 hours, 500 μL of complete medium was added per well. After 48 hours, culture medium was changed to SFM and 50 nM of siRNA for 12 hours followed by cell lysis and immunoblotting. B. For EGF treatment, siRNA containing medium was replaced with fresh SFM and EGF for indicated times, followed by cell lysis and immunoblotting. ut, untreated.
Figure 45. Pilot immunofluorescence data showing co-localization of EGFR and SNX1 within 10 min post EGF-treatment of A549 cells. A. A549 scr cells. B. A549 shT8 cells.
trafficking to the same subcellular compartment as SNX1, where they may interact. To investigate the effects of downregulation of T8 on subcellular compartmentalization of EGFR, immunofluorescence staining was performed using anti-EEA1 antibody to identify early endosome antigen 1 (EEA1), a marker of early endosomes, and anti-EGFR antibody. Without stimulation with EGF, EGFR was not found to be colocalized with EEA1 in A549 scr or A549 shT8 groups (Figure 46 and Figure 47, top rows). However after exposure with EGF for 10 minutes, EGFR and EEA1 show strong colocalization in both groups, demonstrating ligand-induced localization of EGFR in the early endosome (Figure 46 and Figure 47, middle rows). After 30 minutes exposure to EGF, the colocalization of EGFR with EEA1 was reduced in both A549 scr and A549 shT8 groups. However, the reduction of EGFR localization to early endosomes was significantly higher in A549 shT8 cells as compared A549 scr cells (Figure 46 and Figure 47, bottom rows). Quantification of EGFR and EEA1 colocalization is shown in Figure 18. These and other data indicate an early existing of EGFR from early endosomes in A549 shT8 cells as compared to a549 scr cells (ratio of EGFR+/EEA1+ puncta to total EGFR+ puncta ( mean +/- s.d): EGF 10 minutes, A549 scr, 0.52 +/- 0.06, A549 shT8, 0.50 +/- 0.04, P-value > 0.05; EGF 30 minutes, A549 scr, 0.44 +/- 0.05, A549 shT8, 0.19 +/- 0.05, P-value < 0.001; ratio of EGFR+/EEA1+ puncta to total EEA1+ puncta, EGF 10 minutes, A549 scr 0.53 +/- 0.06, A549 shT8 0.47 +/- 0.05, P-value > 0.05; EGF 30 minutes, A549 scr, 0.37 +/- 0.06, A549 shT8, 0.27 +/- 0.04, P-value < 0.05) (Figure 48).

3.1.9. Examination of the effects of T8 knockdown on EGF-induced pERK expression in A549 cells. To investigate the effect of T8 knockdown on downstream effectors of ligand-activated EGFR, logarithmically growing A549 shT8 and A549 scr cells were grown in
Figure 46. Pilot data showing localization of EGFR to early endosomes (EEA1 marker) in response to EGF in A549 scr cells as shown under merge panels.
Figure 47. Pilot data showing localization of EGFR to early endosomes (EEA1) in response to EGF in A549 shT8 cells.
Figure 48. Pilot quantification data showing apparently decreased localization of EGFR in the early endosomal compartment in response to EGF in A549 shT8 cell as compared to A549 scr cells. For 10 minutes EGF treatment groups, three fields were scored with approximately 15-20 cells total, in both A549 scr and A549 shT8 groups. For 30 minutes EGF treatment groups, five fields were scored with a total of 50-60 cells in both A549 scr and A549 shT8 groups. Values shown mean +/- s.d., from one experiment performed using number of cells/per time point as indicated above.
serum free medium (SFM) for 12 hours, and then treated with 50 ng/mL EGF in SFM for various time lengths, followed by sequential immunoblotting with anti-pERK 1/2 and β-ACTIN antibodies. In two independent experiments, EGF-induced expression of pERK at various times post-EGF treatment was inhibited in A549 shT8 cells relative to A549 scr cells (pERK expression in A549 shT8 cells relative to scr cells: untreated (ut) 0.90; EGF, 15 minutes, 0.28, Figure 49, panel A, top, and panel B; EGF 30 minutes, 0.98, EGF 45 minutes, 0.76, EGF 60 minutes, 0.79, EGF 120 minutes, 0.71, Figure 49, panel C). In these experiments, total ERK expression does not seem change post EGF treatment in both A549 scr and A549 shT8 groups (Figure 49, panel A, bottom). Above data suggest that loss of T8 suppresses EGF-induced pERK signaling in NSCLC cells.

3.2. Study of the effects of T8 knockdown on IGF-1-induced signal transduction and response to EGFR and IGF-1R-targeted small molecules in non-small cell lung carcinoma cells.

3.2.1. Validation of the RNA array and antibody array data showing upregulation of insulin-like growth factor binding protein 3 (IGFBP3) in T8 knockdown NSCLC cells. The RNA array profiling of T8 knockdown C4-2B cells versus scr control cells revealed that expression of insulin-like growth factor binding protein 3 (IGFBP3) mRNA was increased approximately 5-fold in C4-2B shT8 cells as compared to C4-B scr cells (Supplemental Table S2). In addition, the antibody array expression profiling of T8 knockdown PC-3 cells showed that IGFBP3 protein expression was increased approximately 2-fold in T8 knockdown cells relative to control cells (Supplemental Table S2). We validated these observations in T8 knockdown model of A549 NSCLC cells. Immunoblot analysis and qRT-PCR analysis of
Figure 49. A. Pilot time course experiments showing decreased EGF-induced expression of pERK in A549 shT8 cells relative to A549 scr control cells. The blots were reprobed with β-ACTIN. The cell lysates from this experiment were also probed with total ERK1/2 antibody, followed by reprobing of the blot with β-Actin. B. Bar graph showing quantification of pERK levels in untreated cells and 15 minutes post EGF-treatment relative to A549 scr control in one experiment performed. C. Extended time course experiment showing decreased EGF-induced expression of pERK in A549 shT8 cells relative to A549 scr control cells. ut, untreated. Quantification was done using Image J software and data were normalized against β-ACTIN in corresponding lanes.
IGFBP3 in A549 shT8 and A549 scr cells were performed. Consistent with the array analyses, protein expression of IGFBP3 was increased by approximately 5-fold, whereas IGFBP3 mRNA was increased approximately 30% in A549 shT8 cells relative to A549 scr cells (IGFBP3 protein fold increase in A549 shT8 cells, 4.90 +/- 1.82 (mean +/- s.d., n=4); IGFBP3 mRNA fold increase in A549 shT8 cells, 1.27 +/- 0.10 (mean +/- s.d., n=24 wells)) (Figures 50, panel A – panel C). Similar observations were made in C4-2B prostate cancer cells with knockdown of T8 expression (T8 mRNA fold decrease in C4-2B shT8 cells vs C4-2B scr cells: 0.38 +/- 0.13 (n=4); IGFBP3 mRNA fold increase in C4-2B shT8 cells vs C4-2B scr cells: 1.59 +/- 0.19 (N=3)) (Figure 50, panel D). These data demonstrate that knockdown of T8 is associated with increased expression of both IGFBP3 mRNA and protein in A549 non-small cell lung carcinoma cells and other cancer cells.

3.2.2. Working model of regulation of IGF-1 signaling and biological response in T8 knockdown NSCLC. Given that A549 shT8 cells showed significant increase in expression of IGFBP3, a negative regulator of IGF-1 signaling, and IGF-1R-targeted small molecules have been tested as anti-cancer therapeutics in NSCLC patients (Scagliotti and Novello, 2011), it is plausible that T8 knockdown may offer a novel regulatory mechanism of downregulation of IGF-1R activation and subsequent decrease in downstream effectors of IGF-1R signaling including pAKT (Figure 51). In support of this rationale, our data shows constitutively decreased pAKT levels in A549 shT8 cells as compared to A549 scr control cells (pAKT protein fold change, 0.29 +/- 0.25, (mean +/- s.d., n=4) ) (Figure 52).

3.2.3. Effects of T8 knockdown on IGF-1-induced pIGF-1R and pAKT levels in NSCLC cells. To investigate the effect of T8 knockdown on downregulation of the IGF-1R pathway, A549 shT8 and A549 scr cells were treated with IGF-1 for 30 minutes as described in
Figure 50. TNFAIP8 knockdown is associated with upregulation of IGFBP3 mRNA and protein in cancer cells. A. Immunoblot analysis showing increased expression of IGFBP3 protein in T8 knockdown A549 cells as compared to scr A549 control cells. A549 scr and A549 shT8 cells were seeded in 10 cm tissue culture plates at 1 x 10^6 cells per dish and grown in complete medium with 10% FBS until 90% confluent, followed by lysis and immunoblotting. B. Bar graph showing quantification (mean +/- s.d.) of immunoblot data from 4 independent experiments. C. IGFBP3 mRNA expression was quantified by qRT-PCR as described in Materials and Methods. IGFBP3 scores were normalized to ribosomal protein large, P0 (RPLP0) control. Quantification data (mean +/- s.d.) shown are from 24 wells per treatment group from one experiment performed. D. C4-2B scr and C4-2B shT8 cells were seeded in 10 cm tissue culture plates at 1 x 10^6 cells per dish and grown in complete medium with 10% FBS until 90% confluent, followed by qRT-PCR analysis of TNFAIP8 and IGFBP3 mRNA as described in Materials and Methods. Expression scores were normalized to ribosomal protein large, P0 (RPLP0) control. Quantification data (mean +/- s.d.) shown are from 3-4 independent experiments with 3 wells per treatment group.
Figure 51. Working hypothesis of role TNFAIP8 in IGF-1-induced signaling and response to EGFR- and IGF-1R-targeted therapies in NSCLC. TNFAIP8 knockdown downregulates IGF-1 signaling in A549 cells by modulation of pIGF-1R, IGFBP3 and pAKT expression and enhances cytotoxic effects of gefitinib and AG1024. Silencing of IGFBP3 is expected to restore IGF-1 induced pIGF-1R and pAKT expression in TNFAIP8 knockdown A549 cells.
Figure 52. Right panel, Constitutive downregulation of pAKT expression in T8 knockdown A549 cells relative to scrambled control cells. Logarithmically growing cells were seeded in 6-well tissue culture dishes (3x10^5 cells per well) in complete medium containing 10% FBS. At approximately 90% confluency, medium was replaced with serum free RPMI 1640 with penicillin/streptomycin (SFM) for 12 hours, followed by cell lysis and immunoblotting. The blot was sequentially probed with pAKT and β-ACTIN antibodies. The cell lysates were also immunoblotted with total anti-AKT antibody followed by β-ACTIN antibody. Left panel, bar graph of pAKT expression levels normalized against β-ACTIN expression in corresponding lanes (mean +/- s.d., n=4).
Materials and Methods. In A549 scr control cells and A549 shT8 cells, no basal level of pIGF-1R expression was detectable. Following IGF-1 treatment, A549 scr cells showed pIGF-1R expression. In contrast, in A549 shT8 cells IGF-1-stimulation of pIGF-1R was suppressed at all doses tested (IGF-1R expression in A549 shT8 cells relative to scr cells: IGF-1, 10 ng/mL, 0.31, 50 ng/mL, 0.11, 75 ng/mL, 0.57, 100 ng/mL, 0.30) (Figure 53, panel A and panel B). In this experiment, total IGF-1R expression does not seem to change post IGF-1 treatment in A549 scr and A549 shT8 groups (Figure 53, panel A, bottom). In independent dose-response and time-course experiments performed, IGF-1-stimulated pAKT expression was also suppressed in A549 shT8 cells as compared to A549 scr cells (Figure 54, panel A-panel C) (pAKT expression in A549 shT8 cells relative to A549 scr, mean +/- s.d., n=2; IGF-1 (10 ng/mL): 15 minutes, 0.58 +/- 0.35, 30 minutes, 0.40 +/- 0.39, 45 minutes, 0.34 +/- 0.14, 60 minutes, 0.30 +/- 0.19 (Figure 54, panel B); pAKT expression in A549 shT8 cells relative to A549 scr, mean +/- s.d., n=1-5 experiments per data point; IGF-1 (50 ng/mL): 15 minutes, 0.77 +/- 0.33, 30 minutes, 0.62 +/- 0.33, 45 minutes, 0.68, 60 minutes 0.55 +/- 0.15, 120 minutes, 0.41 +/- 0.54) (Figure 54, panel C). These data demonstrate that T8 knockdown is associated with suppression of IGF-1-induced pIGF-1R and pAKT levels in A549 cells.

3.2.4. Effects of silencing of IGFBP3 expression on IGF-1-inducible expression of pIGF-1R and pAKT in T8 knockdown NSCLC cells. To verify that increased expression of IGFBP3 in T8 knockdown A549 cells is directly associated with enhanced downregulation of IGF-1-stimulated pIGF-1R and pAKT expression, IGFBP3 expression in A549 shT8 cells was transiently silenced using IGFBP3 siRNA as described in Materials and Methods. As shown in Figure 55, panel A, siIGFBP3 siRNA treatment resulted in approximately 80% inhibition of IGFBP3 expression relative to siControl. As expected, we have also observed reduced levels of
Figure 53. Decreased IGF-1-induced pIGF-1R expression in T8 knockdown A549 cells. A. Pilot experiment showing decreased IGF-1-induced expression of pIGF-1R in A549 shT8 cells relative to A549 scr control cells. The blot was reprobed with β-ACTIN. The cell lysates from this experiment were also probed with total IGF-1R antibody, followed by reprobing of the blot with β-ACTIN. B. Pilot dose response experiment showing decreased pIGF-1R expression in response to IGF-1 in A549 shT8 cells relative to scr control cells. Quantification was done using Image J software and data were normalized against β-ACTIN in the corresponding lanes in one experiment performed. ut, untreated.
Figure 54. Decreased IGF-1-induced pAKT expression in T8 knockdown A549 cells. A. Time course experiment showing decreased IGF-1-induced expression of pAKT in A549 shT8 cells relative to A549 scr control cells. The blot was reprobed with β-ACTIN. The cell lysates from this experiment were also probed with total AKT antibody, followed by reprobing of the blot with β-ACTIN. B. Quantification of low dose IGF-1-induced inhibition of pAKT expression in A549 shT8 cells relative to scr control cells. Quantification was done using Image J software and data were normalized against β-ACTIN in the corresponding lanes. Data shown are from two experiments (mean +/- s.d., n=2). C. Quantification of high dose IGF-1-induced inhibition of pAKT expression in A549 shT8 cells relative to scr control cells. Quantification was done using Image J software and data were normalized against β-ACTIN in the corresponding lanes. Data (mean +/- s.d.) shown are from one-five experiments per data point. ut, untreated.
Figure 55. SiRNA silencing of IGFBP3 protein expression in T8 knockdown A549 cells. A549 cells stably expressing shTNFAIP8 shRNA (shT8) were transfected with indicated siRNA (50 nM) as described in Materials and Methods. After 6 hours, 500 µL of complete medium was added per well. After 48 hours, culture medium was changed to serum free medium containing P/S (SFM) for 12 hours followed by cell lysis and sequential immunoblotting with anti-IGFBP3 and anti-β-ACTIN antibodies. B, Decreased expression of IGFBP3 in culture medium from siIGFBP3 treated A549 shT8 cells as compared to culture medium from siControl treated A549 shT8 cells. siControl, scrambled SiRNA, siIGFBP3, IGFBP3 siRNA.
IGFBP3 protein expression secreted in culture medium from siIGFBP3 treated A549 shT8 cells relative to siControl treated A549 shT8 cells (Figure 55, panel B). Next expression of pIGF-1R and pAKT was analyzed in A549 shT8 cells treated with siIGFBP3 or siControl siRNA. In the presence of siIGFBP3, IGF-1-induced expression of pIGF-1R and pAKT was increased at 30 minutes and 60 minutes of IGF-1 exposure (Figure 56, panel A) (pIGF-1R expression in the siIGFBP3 treatment group relative to siControl group, mean +/- s.d.: IGF-1 (50 ng/mL), 30 minutes, 1.91 +/- 0.68, 60 minutes, 3.69 +/- 0.06 (Figure 56, panel B); pAKT expression in siIGFBP3 treatment group relative to siControl group: IGF-1 (50 ng/mL), 30 minutes, 1.68 +/- 0.41, 60 minutes, 2.25 +/- 0.66 (Figure 56, panel C)). In this experiment, total levels of IGF-1R and AKT do not seem to change post IGF-1 treatment in A549 shT8 cells treated with siIGFBP3 or siControl (Figure 56, panel A). In other experiments, it is noteworthy that silencing of IGFBP3 resulted in detectable basal levels of pIGF-1R and significantly enhanced expression of basal pAKT in A549 shT8 cells (Figure 57). This indicates that IGFBP3 plays an important role in inactivation of constitutive IGF-1R signaling in NSCLC cells. In addition, low dose IGF-1 (10 ng/mL) was found to modestly increase expression of pIGF-1R at 15 minutes (approximately 60% as compared to untreated A549 shT8 cells treated with siIGFBP3) (Figure 57). In contrast, siControl treated A549 shT8 cells showed no detectable expression of pIGF-1R following low dose IGF-1 at all times tested. Low dose IGF-1 did not seem to induce pAKT expression over the high baseline level of pAKT seen in this experiment (Figure 57). These data show that silencing of IGFBP3 expression in A549 shT8 cells results in increased IGF-1/IGF-1R signaling. Thus T8 knockdown seems to attenuate ligand-induced activation of pIGF-1R and pAKT in NSCLC cells.
Figure 56. SiRNA silencing of IGFBP3 protein expression in T8 knockdown A549 cells results in increased IGF-1-induced expression of pIGF-1R and pAKT. A549 cells stably expressing shTNFAIP8 shRNA (shT8) were transfected with indicated siRNA (50 nM) as described in Materials and Methods. After 6 hours, 500 µL of complete medium was added per well. After 48 hours, culture medium was changed to SFM for 12 hours and siRNA containing medium was then replaced with fresh SFM and IGF-1 for indicated times, followed by cell lysis and immunoblotting. A. Time course experiment showing increased IGF-1-induced expression of pIGF-1R and pAKT in siIGFBP3 transfected T8 knockdown A549 cells compared to siRNA control cells. The blots were reprobed with β-ACTIN. The cell lysates from this experiment were also probed with total IGF-1R or total AKT antibody, followed by reprobing of the blots with β-ACTIN. B. Bar graph showing quantification of pIGF-1R expression in siIGFBP3 treated cells relative to siRNA control cells from 2 independent experiments (mean +/- s.d.). C. Bar graph showing quantification of pAKT expression in siIGFBP3 treated cells relative to siRNA control cells from 2 independent experiments (mean +/- s.d.). ut, untreated.
Figure 57. Time course experiments using low dose IGF-1 (10 ng/mL) treatment of A549 shT8 cells also show that siRNA silencing of IGFBP3 protein expression results in a modest increase in IGF-1-induced expression of pIGF-1R in these cells. A549 cells stably expressing shTNFAIP8 shRNA (shT8) were transfected with indicated siRNA as described in Materials and Methods. After 6 hours, 500 µL of complete medium was added per well. After 48 hours, culture medium was changed to SFM and 50 nM of siRNA for 12 hours. siRNA containing medium was replaced with fresh SFM and IGF-1 for indicated times, followed by cell lysis and sequential immunoblotting with anti-pIGF-1R and anti-β-ACTIN antibodies. The cell lysates from this experiment were also sequentially immunoblotted with anti-pAKT and anti-β-ACTIN antibodies.
3.2.5. Examination of the effect of culture medium from high IGFBP3 expressing T8 knockdown A549 cells on pAKT expression. We reasoned that culture medium from T8 knockdown cells with an increased level of IGFBP3 may suppress pAKT expression more effectively as compared to culture medium from scrambled control A549 cells. To assess the relative levels of secreted IGFBP3 in culture medium, logarithmically growing A549 shT8 and A549 scr cells were grown in serum free medium for 12 hours, and then culture medium was collected for immunoblotting with anti-IGFBP3 antibody as explained in legend to Figure 58, panel A. In three independent experiments performed, IGFBP3 levels seem to be higher in culture medium from A549 shT8 cells (shT8-cm) as compared to culture medium from A549 scr cells (scr-cm). Representative data are shown in Figure 58, panel A.

To determine the effect of culture medium from shT8 cells (shT8-cm) on pAKT expression, A549 scr and A549 shT8 cells were treated culture medium from scr cells (scr-cm) or shT8 cells (shT8-cm) as explained in legend to Figure 58, panel B. Immunoblotting analysis showed significant decrease in pAKT expression levels in both A549 scr cells and A549 shT8 cells treated with culture medium from shT8 cells (shT8-cm) as compared to scr cells (scr-cm) (pAKT expression relative to untreated A549 scr cells (nc group) (mean +/- s.d): scr, scr-cm 1.15 +/- 0.08, shT8-cm, 0.95 +/- 0.21; shT8, nc, 0.58 +/- 0.14, scr-cm, 1.30 +/- 0.25, shT8-cm, 0.48 +/- 0.17) (Figure 58, panel B and panel C). In this experiment, total levels of AKT do not seem to change post culture medium treatment in all groups tested (Figure 28, panel B, bottom). These experiments demonstrate distinct effects of culture media from A549 scr cells and A549 shT8 cells on pAKT expression. Together with data shown above (Figure 51), our data demonstrate that T8 knockdown suppresses pAKT expression, in part, by increasing steady state levels of secreted IGFBP3 protein.
Figure 58. Culture medium from T8 knockdown A549 cells (shT8-cm) showed increased expression of IGFBP3, and treatment with shT8-cm suppressed pAKT expression in both A549 scr and A549 shT8 cells. A. Logarithmically growing A549 scr and A549 shT8 cells were seeded in 6-well tissue culture dishes in complete medium containing 10% FBS. At approximately 90% confluency, medium was replaced with serum free RPMI 1640 with penicillin/streptomycin (SFM) for 12 hours. Culture media from A549 scr cells (scr-cm) and A549 shT8 cells (shT8-cm) were then collected in 15 mL tubes and stored on ice. Normalized volume of culture medium (equivalent to approximately equal number of viable cells) was loaded per lane, followed by immunoblotting with anti-IGFBP3 antibody. Expt. 1, scr-cm, 15 µL (2x10^5 cells), shT8-cm, 15 µL (1.3 x 10^4 cells); Expt. 2, scr-cm, Lane 1, 10 µL and Lane 2, 15.6 µL, shT8-cm, Lane 3, 11.5 µL and Lane 4, 12.9 µL. In lanes 1-4, culture medium volume represents approximately 1.3 x 10^4 cells. B. Logarithmically growing cells were seeded in 6-well tissue culture dishes in complete medium containing 10% FBS. At approximately 90% confluency, medium was replaced with serum free RPMI 1640 with penicillin/streptomycin (SFM) for 12 hours. Medium was removed and replaced with 2 mL culture medium from scr control cells (scr-cm), or shT8 cells (shT8-cm), or left untreated (nc) for 30 minutes, followed by cell lysis and immunoblotting. The cell lysates from this experiment were also probed with total AKT antibody, followed by reprobing of the blot with β-ACTIN antibody. C. Bar graph showing quantification of immunoblot data from 3 independent experiments (mean +/- s.d., P-values as shown above).
EGFR and IGF-1R are well known targets in NSCLC. EGFR-targeted small molecule gefitinib and a monoclonal antibody against IGF-1R figitumumab are in clinical trials in NSCLC patients (Scagliotti and Novello, 2011). T8 may suppress IGFBP3 mRNA and protein expression, and promote NSCLC progression and resistance to targeted therapies by enhancing IGF-1/pIGF-1R/pAKT axis in NSCLC cells. In preliminary studies, cytotoxic effects of these small molecules were found to be enhanced in A549 shT8 cells as compared to A549 scr cells (cell viability of T8 knockdown cells relative to scr controls: Gefitinib, 0.25 µM, 0.52 +/- 0.01, 0.5 µM, 0.45 +/- 0.02, 1 µM, 0.59 +/- 0.15; AG1024 0.5 µM, 1.01 +/- 0.38, 1 uM, 0.63 +/- 0.06, n=6) (Figure 59, panel A and panel B). Further investigations are necessary to establish combination of EGFR/IGF-1R-targeted small molecules and T8 knockdown as a viable therapeutic strategy in NSCLC cells.
Figure 59. Preliminary survival dose response data suggests that T8 knockdown in A549 cells enhances cytotoxicities of EGFR and IGF-1R targeted drugs, gefitinib and AG1024. Cells were seeded (10x3/well, n = 6) in complete medium containing 10% FBS for 24 hours. Medium was switched to SFM containing indicated doses of gefitinib (A) or AG1024 (B) in DMSO for 72 hours, followed by the XTT assay as described in Materials and Methods. Data shown (mean +/- s.d.) are from one experiment performed with each drug using number of wells per dose as shown above.
Chapter 4. Discussion

The EGFR and IGF-1R signaling pathways are prominent druggable targets in several malignancies (Scagliotti and Novello, 2012). Accordingly, numerous studies have focused on developing small molecule inhibitors and antibodies as anticancer agents targeting these RTKs (Lin et al., 2014, Haisa et al., 2013). However, primary or acquired resistance to the targeted therapies and disease progression remain an unmet clinical challenge, underscoring the need for evaluation of new targets and mechanisms regulating these pathways. Present research has demonstrated that T8, a novel NF-κB inducible oncogenic and metastatic molecule, is an important regulator of EGFR and IGF-1R signal transduction pathways in A549 lung cancer cells. Our data suggest that T8 knockdown A549 cells exhibit significant inhibition of cell motility in response to all four growth factors tested (Figures 36-38). In addition, it is likely that T8 may also function as a scaffold or adaptor protein facilitating/stabilizing multiple protein-protein interactions downstream of the ligand-activated RTKs.

As a step toward understanding the mechanism underlying T8 function in EGFR signaling response, depletion of T8 was found to be associated with a decrease in steady state expression of EGFR in A549 cells (Figure 39) and an increase in expression of SNX1 (Figure 41, panels C-E). SNX1 has been shown to promote targeting of EGFR to late endosomes for lysosomal degradation (Worby and Dixon, 2002). Presently, it is unclear how T8 might regulate SNX-1 mRNA and protein expression in these cells. Nonetheless, silencing of SNX1 was found to partially restore EGFR expression in T8 knockdown A549 cells (Figure 44). Furthermore, EGFR seemed to exit early endosomes relatively early on in T8 knockdown cells as compared to control cells (Figure 48). A549 cells express wild type (wt) EGFR (Van Schaeybroek et al., 2006). It would be important to test whether T8 knockdown enhances late endosomal targeting
of activated EGFR in relatively resistant NSCLC cells such as H1975 (EGFR, L858R/T790M) (Chung et al., 2009).

Other mechanisms of T8-mediated regulation of EGFR expression cannot be ruled out. For example, EGFR is a known target of miR-7 (Webster et al., 2009). In microRNA expression profiling studies, high expression of miR-7 was observed after silencing of T8 in C4-2B prostate cancer cells as compared to control cells (Supplemental Table S3). Consistent with enhanced downregulation of EGFR expression in T8 knockdown A549 cells, we have observed decreased levels of pEGFR in EGF-stimulated T8 knockdown cells as compared to control cells (Figure 43). In pilot time course experiments, decreased expression of pERK, a known effector of ligand-activated EGFR, was also observed in EGF-treated T8 knockdown cells as compared to control cells (Figure 49). These data demonstrate T8 knockdown impact signaling downstream of EGFR.

Significant increase in expression of IGFBP3 protein was seen in T8 knockdown A549 cells (Figure 50). In addition, IGF-1-treated T8 knockdown cells showed loss of pIGF-1R and its downstream effector pAKT (Figures 53 and 54). Furthermore, silencing of IGFBP3 resulted in restoration of pIGF-1R and pAKT expression in T8 knockdown cells, implying that T8 regulation of IGF-1R pathway is via modulation of IGFBP3 expression (Figures 56 and 57). Earlier, silencing of transcription factor FOXA1 has been associated with an increased expression of IGFBP3 in prostate cancer cells (Imamura et al., 2012). Interestingly, our RNA array and qRT-PCR validation studies suggest that FOXA1 expression is significantly decreased in T8 knockdown cancer cells (Supplemental Table S4, and Table 6 and Figure 60). Therefore, upregulation of IGFBP3 observed in T8 knockdown cells may be via downregulation of FOXA1.
Table 6. Primer sequences and anticipated RT-PCR product sizes of various genes tested in TNFAIP8 knockdown tumor cell models.*

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<th>Custom (UCSC gene ID) (<a href="http://genome.ucsc.edu/cgi-bin/hgGene">http://genome.ucsc.edu/cgi-bin/hgGene</a>)</th>
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*RNA isolation and RT-PCR was performed as described in Materials and Methods.
Figure 60. Changes in mRNA expression profiles detected by qRT-PCR assay in TNFAIP8 knockdown tumor cells (shT8) versus scrambled control cells (scr). RNA isolation and qRT-PCR was performed as described in Materials and Methods. The known functional categories of various genes are as follows. Fatty acid metabolism, ACADL; FGF signaling, FLRT2; Invasion and metastasis, S100P and OSTF1; and Tumor progression, FOXA1, KRAS, MAP2K6, NFAT5; and Alternative splicing, MALAT1. Data shown are from one representative experiment performed. (n), number of independent experiments performed using each cell line.
Tumor suppressor PTEN negatively regulates pAKT expression by downregulating the PIP3 level in the membrane. PTEN is targeted by miR-221 (Garofalo et al., 2009). In miRNA array studies, T8 knockdown cells showed decreased expression of miR-221 (Supplemental Table S3). Therefore, T8 may also regulate pAKT indirectly by regulating expression of miR-221 and PTEN. Recent studies have shown the T8 family member TIPE3 captures PIP2 and PIP3 in a large hydrophobic cavity and promotes tumorigenesis by increasing membrane levels of these phospholipid second messengers (Figure 6). TIPE3 shares significant homology of this hydrophobic region with other members of the T8 family including T8 (Figure 3). Further studies are necessary to address as yet another possibility that T8-mediated activation of pAKT involves high levels of PIP3 in the membrane and increased phosphorylation of AKT via activated membrane-bound PDK1/PDK2 serine threonine kinase.

T8 may also regulate oncogenic mechanisms in cancer cells via non-canonical pathways and gene networks. Indeed, our transcriptome analysis indicated a number of integrative pathways and networks signifying T8 function in cancer cells (Day et al., 2012). In brief, in addition to modulation of EGFR, SNX1 and IGFBP3 expression as discussed above, we have identified fourteen genes belonging to five distinct functional categories that are modulated in a T8 knockdown-specific manner in cancer cells (Supplemental Tables S4 and S5, Table 6, and Figures 60 and 61). These genes and functional categories are: ACADL, fatty acid metabolism; FLRT2, FGF signaling; S100P, OSTF1, invasion and metastasis; FOXA1, KRAS, MAP2K6, NFAT, MALAT1, MET, tumor progression; IL24 (mda-7), FAT3, LPHN2, and EPHA3, tumor suppression.

T8 is a prosurvival molecule. Earlier, we have demonstrated that antisense downregulation of T8 enhances cytotoxic effects chemotherapeutic drugs and radiation in
prostate tumor xenograft models (Zhang et al., 2013). Currently, the role of T8 in resistance of cancer cells to EGFR/IGF-1R TKIs is unknown. In pilot studies, T8 knockdown A549 cells with wt EGFR were found be more sensitive to EGFR TKI (gefitinib) and IGF-1R TKI (AG-1024) as compared to control cells (Figure 59).

In summary, aberrant regulations of EGFR and IGF-1R signaling and activation of pERK and pAKT are hallmarks of many tumor types including lung, breast, prostate, melanoma, and pancreatic cancers. This research demonstrates that T8 plays an important role in regulation of EGFR and IGF-1R signaling in A549 lung cancer cells. Further studies using T8 knockdown models of a wide range of TKI-resistant cells may ultimately lead to a new strategy for preventing or delaying resistance to targeted therapeutics in NSCLC and other cancers.

In present studies, A549 cells with wt EGFR were used to test the significance of T8 knockdown in EGFR and IGF-1R signaling. Other NSCLC cells with wt EGFR have shown differential survival response to EGFR TKIs. For example, H460 cells (wt EGFR) are relatively resistant to TKIs, in part, due to constitutive activation of pAKT via IGF-1R pathway. In contrast, H322 cells (wt EGFR) exhibit relatively higher sensitivity to TKIs perhaps associated with decreased expression of constitutive pAKT (Van Schaeybroek et al., 2006, Morgillo et al., 2006, Morgillo et al., 2007). Furthermore, resistance of NSCLC cells to TKIs has been associated with expression of certain mutations/deletions of EGFR (Sharma et al., 2007). Our data provide a rationale for future investigations using paired TKI-sensitive HCC827 (EGFR Δ746-750) and resistant cell lines (HCC8271R1 (EGFRT790M), HCC827R2 (MET amplification)) (Jia et al., 2013) to elucidate the significance of T8 knockdown in TKI-resistant NSCLC cells in vitro and in vivo. Ultimately, these efforts may lead to a new strategy for preventing or delaying resistance to EGFR and IGF-1R-targeted therapeutics.
Figure 61. Changes in mRNA expression profiles detected by qRT-PCR assay in TNFAIP8 knockdown tumor cells (shT8) versus scrambled control cells (scr). RNA isolation and qRT-PCR was performed as described in Materials and Methods. The known functional categories of various genes are as follows. Tumor progression, MET, TNFAIP8; Tumor suppression, IL24 (mda-7); FAT3, LPHN2, EPHA3, and IGFBP3. Data shown are from one representative experiment performed. (n), number of independent experiments performed using each cell line.
Supplemental Figure S1. Alternatively spliced variants of TNFAIP8 transcripts

Analysis of the alternative splicing graph from the Swiss Institute of Bioinformatics (35392) indicates a total of 10 exons, where the last exon 8 seems to be alternatively spliced into two additional exons (http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg38&position=chr5%3A119237213-119426083&hgsid=399825221_VfDB9aASTHw0cOPHNrkmo6WjnhP).
Supplemental Table S1. RNA and antibody array analyses showed increased expression of SNX1 and decreased expression of EGFR in T8 knockdown cancer cells*

### RNA Arrays**

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### Antibody Arrays***

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<td>Epidermal Growth Factor Receptor</td>
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<td>-1.7</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
<td>N/A</td>
<td>-1.3</td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
<td>N/A</td>
<td>-1.3</td>
</tr>
</tbody>
</table>

* Mewani, Day, Chakravarty et al (Review Article in prep)
** Total RNA was isolated from T8 knockdown and scr control PC-3 cells using Trizol reagent and samples, in triplicate, were processed for RNA profiling using RNA arrays (Human Genome U133 Plus 2.0 array (Affymetrix) at the Genomics and Epigenomics Shared Resource Facility of the Lombardi Comprehensive Cancer Center.). P-value was calculated by Student’s T test, two-tailed, equal variance.
*** Cell lysates were processed for antibody array (BD Clontech Antibody Microarray 500) analysis as described before (Zhang et al., 2013).
Supplemental Table S2. RNA and antibody array analyses showed increased expression of IGFBP3 in T8 knockdown cancer cells*

**RNA Arrays**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gene Symbol</th>
<th>Name</th>
<th>P-value</th>
<th>Fold change (shT8 vs. scr cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4-2B</td>
<td>IGFBP3</td>
<td>Insulin-like Growth Factor Binding Protein 3</td>
<td>0.0016681</td>
<td>+4.4</td>
</tr>
<tr>
<td>C4-2B</td>
<td>IGFBP3</td>
<td>Insulin-like Growth Factor Binding Protein 3</td>
<td>0.0001626</td>
<td>+5.2</td>
</tr>
<tr>
<td>C4-2B</td>
<td>TNFAIP8</td>
<td>TNFα induced protein 8</td>
<td>0.000117</td>
<td>-2.6</td>
</tr>
</tbody>
</table>

***Antibody Arrays***

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gene Symbol</th>
<th>Name</th>
<th>P-value</th>
<th>Fold change (shT8 vs. scr cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td>IGFBP3</td>
<td>Insulin-like Growth Factor Binding Protein 3</td>
<td>N/A</td>
<td>+1.7</td>
</tr>
</tbody>
</table>

* Mewani, Day, Chakravarty et al. (Review Article in prep)
** Total RNA was isolated from T8 knockdown and scr control C4-2B cells using Trizol reagent and samples, in triplicate, were processed for RNA profiling using RNA arrays (Human Genome U133 Plus 2.0 array (Affymetrix) at the Genomics and Epigenomics Shared Resource Facility of the Lombardi Comprehensive Cancer Center.
*** Cell lysates were processed for antibody array (BD Clontech Antibody Microarray 500) analysis as described before (Zhang et al., 2013).
Supplemental Table S3. Representative microRNA array data showing changes in miRNA expression in TNFAIP8 knockdown models of PC-3 and C4-2B prostate cancer cells*

<table>
<thead>
<tr>
<th>Human miRNA (TNFAIP8 knockdown tumor model)</th>
<th>MicroRNA Array data</th>
<th>P-value (vs. scrambled shRNA transduced cells)</th>
<th>Known target(s)</th>
<th>Known function in cancer progression</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-mir-429 (PC-3)</td>
<td>+2.4</td>
<td>0.036</td>
<td>FLK1 (VEGFR2) c-MYC (Sun et al., 2011)</td>
<td>Inhibits cell viability (Sun et al., 2011); Downregulation associated with tumor progression (Gregory et al., 2008)</td>
</tr>
<tr>
<td>hsa-mir-720 (PC-3)</td>
<td>+2.1</td>
<td>0.049</td>
<td>HECTD1</td>
<td></td>
</tr>
<tr>
<td>hsa-mir-134 (PC-3)</td>
<td>-2.2</td>
<td>0.031</td>
<td>Integrin β1, IGF2, SERPINE1 (Balakrishnan et al., 2014)</td>
<td>Regulates cell proliferation, apoptosis, and migration (Zhang et al., 2012)</td>
</tr>
<tr>
<td>hsa-mir-221 (PC-3)</td>
<td>-2.2</td>
<td>0.001</td>
<td>HECTD2 (Papadopoulos et al., 2008), PTEN (Garofalo et al., 2009)</td>
<td>Functions in tumor progression, EMT (Garofalo et al., 2012)</td>
</tr>
<tr>
<td>hsa-miR-29b-1 (C4-2B)</td>
<td>+2.2</td>
<td>0.021</td>
<td></td>
<td>Suppresses metastasis by regulating EMT signaling (Ru et al., 2012)</td>
</tr>
<tr>
<td>hsa-mir-7 (C4-2B)</td>
<td>+3.8</td>
<td>0.008</td>
<td>IGF-1R, IRS1/2, RAF-1, FAK, EGFR (Webster et al., 2009, Gomez et al., 2013)</td>
<td>Functions as an anti-metastatic microRNA by targeting IGF-1R (Zhao et al., 2013); Inhibits EMT by targeting FAK (Kong et al., 2012)</td>
</tr>
</tbody>
</table>

* Mewani, Day, Chakravarty et al., (Review article in prep)
Supplemental Tables S4 and S5. Representative RNA Array data showing changes in mRNA expression in TNFAIP8 knockdown models of PC-3 and C4-2B prostate cancer cells*

**Table S4**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>PC-3 RNA Array data Fold change in mRNA +, high in sh T8 vs scr, - low in sh T8 vs scr (FDR&lt;1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPHA3</td>
<td>+3.4**</td>
</tr>
<tr>
<td>FAT3</td>
<td>+6.3**</td>
</tr>
<tr>
<td>FOXA1</td>
<td>-3.1 (0.148)</td>
</tr>
<tr>
<td>IL24(mda-7)</td>
<td>+6.3 (0.573)</td>
</tr>
<tr>
<td>KRAS</td>
<td>-2.2**</td>
</tr>
<tr>
<td>LPHN2</td>
<td>+5.9 (0.712)</td>
</tr>
<tr>
<td>MALAT1</td>
<td>-5.9**</td>
</tr>
<tr>
<td>MAP2K6</td>
<td>-2.1**</td>
</tr>
<tr>
<td>MET</td>
<td>-3.4**</td>
</tr>
<tr>
<td>NFAT5</td>
<td>-9.1**</td>
</tr>
<tr>
<td>TNFAIP8</td>
<td>-2.9 (0.843)</td>
</tr>
</tbody>
</table>

**Table S5**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>C4-2B RNA Array data Fold change in mRNA +, high in sh T8 vs scr, - low in sh T8 vs scr (FDR&lt;1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACADL</td>
<td>+15.1 (0.00729)</td>
</tr>
<tr>
<td>FLRT2</td>
<td>-4.0 (0.0839)</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>+5.2 (0.0432)</td>
</tr>
<tr>
<td>OSTF1</td>
<td>-2.4 (&lt;1e-07)</td>
</tr>
<tr>
<td>S100P</td>
<td>-7.7 (0.0219)</td>
</tr>
<tr>
<td>TNFAIP8</td>
<td>-2.6 (0.0395)</td>
</tr>
</tbody>
</table>

* Mewani, Day. Chakravarty et al., (Review article in prep); **P-value, <0.05.
REFERENCES


Chua CE, Tang BL. Engagement of the small GTPase Rab31 protein and its effector, early endosome antigen 1, is important for trafficking of the ligand-bound epidermal growth factor receptor from the early to the late endosome. J Biol Chem. 2014;289:12375-12389.


