The Role of GLI-1 in Endocrine Resistant Breast Cancer

Undergraduate Honors Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in Biomedical Science in the School of Health and Rehabilitation Sciences at The Ohio State University

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PROBLEM

Estrogen receptor positive (ER+) and estrogen receptor negative (ER-) are two major types of breast cancer. For women with ER+ positive breast cancer, patients are treated with the antiestrogenic compounds, tamoxifen or faslodex for five years, immediately after surgical resection of tumors. Unfortunately, 30-40% of these patients will develop resistance to endocrine therapy. Our recent study has shown that the Hedgehog (Hhg) signaling pathway plays a significant role in endocrine resistance and that the aberrantly activated transcription factor, GLI-1, is vital to the development of resistance. However, not much is known about the GLI-1 target genes that might contribute to endocrine resistance. Our goal is to determine novel target genes of GLI-1 and determine how these genes promote endocrine therapy resistance.

BACKGROUND

Breast Cancer

Breast cancer is the most prevalent and second most deadly cancer among women in the United States.[1] While breast cancer incidence has increased over the past 30 years partly because of increased screening programs and therefore earlier and efficient detection, breast cancer mortality has declined.[2] There are four subtypes of breast cancer: Luminal A, Luminal B, HER2 enriched (Human Epidermal Growth Factor-2), and triple negative. Each subtype is classified by the presence of four molecular markers: estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki-67, a proliferative marker (Fig.1).[3]

The involvement of the hormone estrogen in breast cancer progression has been known since 1896, when removal of the ovaries, the source of endogenous estrogen, led to the remission of metastatic breast cancer in some women.[4] The active form of estrogen, 17β-estradiol (E2), exerts its

Subtype	Characteristics
Luminal A	ER+, PR+, HER2- (low Ki-67)
Luminal B	ER+, PR+, HER2- (high Ki-67)
Triple Negative	ER-, PR-, HER2-
HER2 Enriched	ER-, PR-, HER2+

Figure 1. Clinical subtypes of breast cancer are classified by the presence of estrogen receptor (ER), progesterone receptor (PR), HER2, and the proliferative marker, Ki67.

biological effect upon binding to its receptor, estrogen receptor alpha (ERα). Following activation, the E2/ER complex acts as a transcription factor and activates several downstream target genes upon binding to a palindromic 13 base pair consensus sequence: 5'-GGTACnnnTGTTCT-3'.[5] Binding of E2 to ER also activates several signaling pathways leading to cell proliferation. It is well known that the activation of ERα results in cellular proliferation through genomic and non-genomic interactions.[6] Inhibiting the function of estrogen and ERα has been the cornerstone of therapy for women with estrogen receptor positive (ER+) breast cancer since the 1960s.

Estrogen Receptor Positive Breast Cancer Therapies

Tamoxifen (TAM) has been the first line of therapy for patients with ER+ breast cancer for over 40 years. TAM is a selective estrogen receptor modulator (SERM) where binding to ER, inhibits binding and activation of ER by 17β-estradiol. Women with ER+ breast cancer today undergo adjuvant therapy constituting surgical resection of the tumor followed by endocrine therapy. Therapy with TAM following surgery has been very effective. TAM treatment for 5 years following surgery resulted in a 51% reduction in recurrence and 28% reduction in cancer-related deaths. However, treatment with endocrine therapy for more than 5 years can result in endometrial cancer.[2] An alternative commonly used non-steroidal anti-estrogenic compound is faslodex (FAS). Faslodex is a selective estrogen receptor downregulator (SERD) that is also used to treat patients with ER+ breast

cancer. FAS binds to ER with high affinity and inhibits ER function through a mechanism that results in its nuclear export and degradation.[2] It is important to note that patients with Luminal A and Luminal B breast cancer are treated with endocrine therapies as they express ER (Fig. 1). As 30-40% of women with ER+ breast cancer develop resistance to endocrine therapy, it important to study what contributes to this resistance. Recent work in our laboratory demonstrated that aberrant activation of sonic hedgehog pathway promotes development of resistance to tamoxifen.[7]

The Hedgehog Pathway

The name hedgehog pathway was coined when scientists mutated the hedgehog (*Hh)* gene in *Drosophila melanogaster* embryos and observed that the embryos developed spiked-shaped projections, resembling the spikes of a hedgehog. In canonical hedgehog signaling, the pathway is regulated by one or more of three hedgehog ligands: Indian hedgehog (Ihh), desert hedgehog (Dhh) and sonic hedgehog (Shh). When the pathway is inactive, the hedgehog receptor, Patched (PTCH) inhibits the G-protein coupled receptor, Smoothend (SMO). However, upon binding of the ligand to PTCH, SMO is no longer inhibited and migrates to the cilium. This leads to activation of the Glioma-associated oncoproteins, the GLI family of transcription factors. Once activated, GLI proteins migrate to the nucleus and exert their regulatory effects on the genome. The family is made up of three zinc-fingered transcription factors: GLI-1, GLI-2, and GLI-3. GLI-1 is a known activator; GLI-3 is a known repressor; while GLI-2 can behave as both an activator and repressor of transcription.[8]

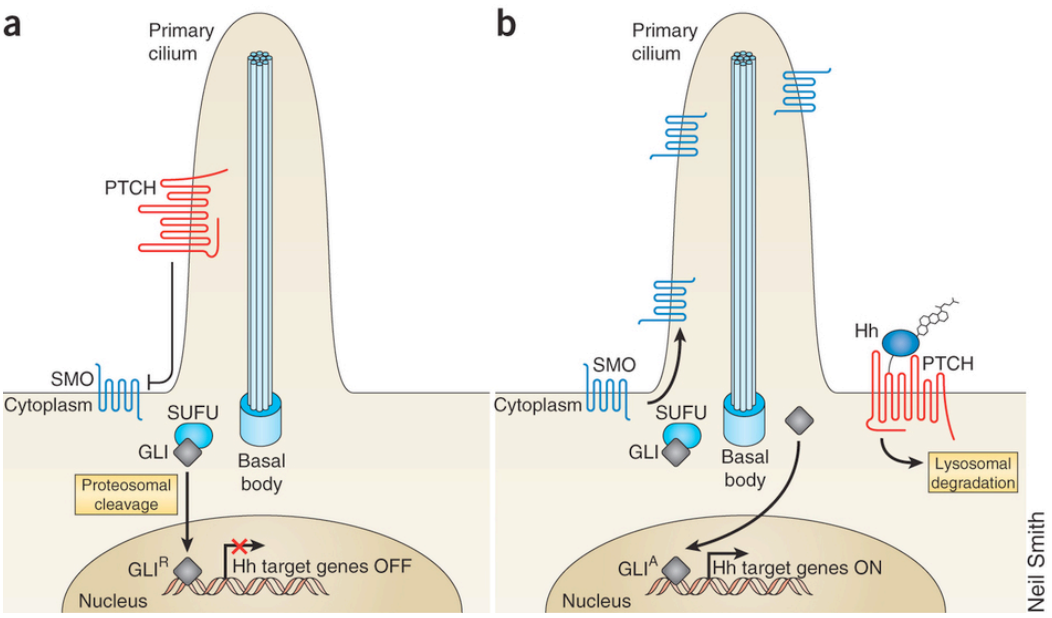


Figure 2. Canonical hedgehog signaling. **A)** Hedgehog signaling is inactive in the absence of the hedgehog ligand. PTCH inhibits SMO. **B)** Hedgehog signaling is active in the presence of the ligand. PTCH no longer inhibits SMO. SMO migrates to the cilium where it activates GLI transcription factors. GLI then migrates to the nucleus, activating or repressing target genes. Figure from: *Unraveling the therapeutic potential of the Hedgehog pathway in cancer*. *Nature Medicine*. 2013; 19; 1410-1422.

The hedgehog pathway is active during embryogenesis and is responsible for body plan development and segmentation. During adulthood, the pathway remains mostly silent but its aberrant activation has been observed in several types of cancer including: basal cell carcinoma, medulloblastoma, glioblastoma, chronic lymphoid leukemia, pancreatic cancer, and breast cancer.[9] The hedgehog pathway can be activated in ligand-independent manner. This is caused by inactivating mutations of PTCH or activating mutations of SMO, leading to overall activation of SMO and GLI proteins.[9] Ligand-independent activation of the hedgehog pathway can also occur through cross-talk with multiple signaling pathways and therefore dual inhibition of both hedgehog and EGFR, JAK/STAT, or PI3K pathways are currently underway in clinical trials.[10] Ligand-dependent activation of hedgehog signaling can occur in an autocrine manner where the tumors secreting the hedgehog ligand act upon themself, thereby stimulating the growth of tumor cells.[11] Studies also show that paracrine signaling can activate the hedgehog pathway in tumor cells where secretion of

hedgehog ligands from the surrounding tumor microenvironment acts upon the tumor cells and vice versa.[12]

Activation of the hedgehog pathway promotes tumor metastasis through upregulation of genes associated with epithelial to mesenchymal transition (EMT) such as SNAI1, SNAI2, TWIST2 and FOXC2.[13] EMT is a process where tumors begin to lose their cell-adhesion capability. Following EMT, tumor cells gain migratory and invasive properties, allowing them to metastasize to other parts of the body. One study showed that EMT could be inhibited in prostate cancer stem cells *in vivo* by treating mice with the SMO inhibitor, Erismodegib.[14] Targeting the hedgehog pathway could therefore be a valuable strategy to inhibit metastasis.

There is also some evidence that the hedgehog pathway is involved in the maintenance of cancer stems cells (CSCs). Cancer stem cells are a small subpopulation of cancer cells that are implicated in tumor recurrence. CSCs are functionally defined by their self-renewal capability, their ability to differentiate, and their tumorgenicity (their ability to form tumors from a relatively few number of cells when implanted in mice). Importantly, the hedgehog proteins PTCH, GLI-1 and GLI-2 are present in breast cancer stem/progenitor cells. These genes are downregulated when these cells were induced to differentiate. In addition, inhibition of hedgehog signaling components reduced mammosphere formation, indicating that the hedgehog pathway may be involved in the self-renewal capability of breast cancer stem cells.[15]

The Hedgehog Pathway & Drug Resistance

A major problem in cancer therapy is the development of resistance to drug therapies. Multi-drug resistance (MDR) occurs when cancer cells become resistant to several drugs with different molecular structures. Several studies implicate role of hedgehog pathway activation to the

development of drug resistance in cancer. For example, GLI-1, directly activates several ATP Binding Cassette (ABC) transporters including ABCG2 and ABCB1 which mediate the efflux of several chemotherapeutic agents such as miloxantrone, daunorubicin, and doxorubicin in breast cancer.[16] Hedgehog pathway mediated activation of ABCG2 has also been associated with radiation resistance in pediatric medulloblastoma.[17] Hedgehog signaling also mediated resistance to anti-EGFR therapies in head and neck cancer.[18] Furthermore, CSCs are generally resistant to therapies and often express these ABC transporters[19]. It is important to note that these ABC transporters have not been implicated in TAM or FAS transport in cancer cells.

Non-canonical Hedgehog Signaling and Endocrine Resistance

Recent studies in our laboratory demonstrated that the PI3K/AKT pathway plays a key role in upregulation of the hedgehog pathway by stabilizing SMO and GLI-1 proteins, and conferring resistance to tamoxifen. Glycogen synthase kinase 3-beta (GSK3β) remains in its active form and phosphorylates SMO and GLI-1 when the PI3K pathway is inactive. This promotes proteasomal degradation of the two key Hedgehog signaling proteins. However, in tamoxifen resistant MCF7 cells, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) activates the serine-threonine protein kinase, AKT. Following activation, AKT inhibits GSK3β, and prevents phosphorylation and subsequent degradation of SMO and GLI-1. This promotes the non-canonical activation of the Hhg signaling pathway (Fig. 3).

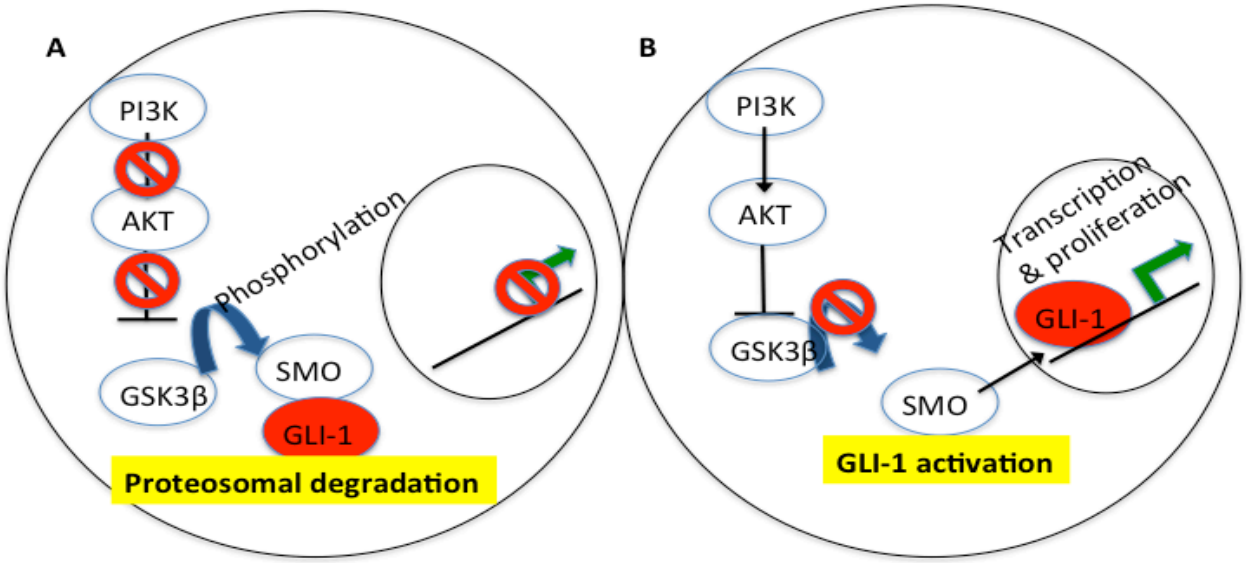


Figure 3. PI3K activation stabilizes SMO and GLI-1 leading to hedgehog pathway activation. **A)** When PI3K is inactive, GSK3β phosphorylates SMO and GLI-1, designating these proteins for protesomal degradation. **B)** When PI3K is active, GSK3β is inhibited. SMO and GLI-1 remain active allowing GLI-1 to transcriptionally activate genes.

Si-RNA knockdown of hedgehog genes, SMO and GLI-1, in tamoxifen resistant cells markedly reduced their sensitivity to tamoxifen *in vitro.* Treatment of mice harboring tamoxifen-resistance xenografts with Vismodegib (GDC-0449, Genentech) an inhibitor of SMO, inhibited the growth of these xenografts, suggesting that the hedgehog pathway is vital for growth of the endocrine resistant xenografts. In addition, high GLI-1 protein level inversely correlated with overall survival and disease free survival in primary human breast tumors.[7] Our previous work elucidated the importance of GLI-1 in development of resistance to endocrine therapy. It is therefore important to identify the GLI-1 target genes and study their functional significance in the development of anti-estrogen resistance.

Hedgehog Targeted Therapies

Vismodegib is the only clinically approved SMO inhibitor that is used currently to treat patients with basal cell carcinoma. Other SMO inhibitors such as Sonidegib and BMS-833923 are in

clinical trials.[10] In addition to SMO inhibitors, a screen of several small molecule inhibitory compounds targeting GLI-1 led to identification of GANT-61 and GANT58.[20] However the high toxicity of these compounds prohibits them from being used in clinical trials. Because the hedgehog pathway is often activated with other signaling pathways, several studies are now focusing on treating cancer patients with multiple inhibitors for better outcome.[10]

Solute-like Carrier Family 39 Member 6 (SLC-39A6)

Our gene expression analysis identified SLC-39A6 as a putative target of GLI-1 that could be a mediator of TAM or FAS sensitivity in breast cancer cells. SLC-39A6 is a zinc-influx transporter protein located on the cell membrane.[21] Knockdown of SLC-39A6 in T47D breast cancer cells reduced E-cadherin expression while increasing vimentin expression, indicating that SLC-39A6 may play a tumor suppressor role by inhibiting EMT. In addition, SLC-39A6 depletion was shown to cause a reduction in cytochrome C mediated apoptosis.[22] Previous studies have demonstrated that SLC-39A6 expression is induced by estrogen.[23] SLC-39A6 gene expression was also found to be significantly higher in breast tumors compared to normal mammary epithelium. Importantly, SLC-39A6 protein expression was correlated with lower grade tumors of smaller size. Patients with ER positive breast cancer expressing high levels of SLC-39A6 had longer relapse free survival (RFS) when compared to other patients in the cohort. This correlation was also observed at the protein level.[24] We are interested in seeing if SLC-39A6 plays a tumor suppressor role in the context of endocrine therapy. A reduction in SLC-39A6 may thus promote cell survival in TAM or FAS resistant cells. However, the tumor suppressor role of SLC-39A6 is not consistent among other cancers. In liver cancer cells, downregulation of SLC-39A6 repressed cell growth.[25] In esophageal squamous cell carcinoma (ESCC), SLC-39A6 protein expression was correlated with shorter length

of survival in patients with advanced ESCC.[26] Interestingly, one member of the SLC family of transporters, SLC22 is a known transporter of cisplatin and oxaliplatin.[27] It is possible that SLC-39A6 could be a transporter of TAM or FAS, and its repression may therefore reduce the efficacy of TAM or FAS. While it seems that patients with increased SLC-39A6 expression have better disease outcome in breast cancer, the role of SLC-39A6 in the development of breast cancer remains elusive and warrants further study.

SIGNIFICANCE

Endocrine therapy is the most effective therapy for estrogen receptor positive (ER+) breast cancer patients and thus resistance to endocrine therapy is an unfortunate reality for many of these patients. There are very few options and no additional approved targeted therapy for the patients who develop resistance to endocrine therapy. Our study has identified several novel genes that are either up or downregulated when the GLI-1 transcription factor was ectopically expressed in MCF7 cells. It is expected that detailed analysis of these genes will uncover pathways that promote resistance and provide additional targets for future therapy. The impact of this study is three fold:

1) This study will identify novel Hhg target genes that are regulated by the transcription factor, GLI-1.

2) It is important to note that GLI-1 is a known activator of transcription and not an inhibitor. This study could also elucidate a novel repressive role of GLI-1.

3) This study could delineate the role of novel Hhg target genes in endocrine resistance that in the future could be therapeutically targeted to improve patient outcome.

HYPOTHESIS

As our previous study demonstrated the importance of GLI-1 in tamoxifen resistance, we hypothesize that GLI-1 reduces sensitivity to endocrine therapy by upregulating or downregulating target genes that inhibit apoptosis, drug influx, or other cell death mechanisms. We also hypothesize that GLI-1 can act as a transcriptional repressor by binding alone or with other proteins to the GLI-1 consensus sequences on the promoter of putative target genes. Specifically, we will investigate the role of GLI-1 in regulation of SLC-3A96 and its potential tumor suppressor role.

SPECIFIC AIMS

Specific Aim 1: *Identify putative downstream targets of the transcription factor, GLI-1*.
Using MCF7 cells overexpressing GLI-1 (MCF7 GLI-1), we will perform gene expression analysis for 96 breast cancer-related genes. This will lead to the identification of novel GLI-1 target genes and we expect to validate already identified GLI-1 targets.

Specific Aim 2: *Determine if **A.**GLI-1 directly represses SLC-39A6 by binding to its promoter and **B.** SLC-39A6 is involved in mediating the response to endocrine therapy.*

SLC-39A6 is one novel gene significantly downregulated in GLI-1 overexpressing cells. We plan to elucidate how GLI-1 represses SLC-39A6. If GLI-1 represses SLC-39A6 by directly binding to its promoter, this could shed light on a new regulatory mechanism of GLI-1.

SLC-39A6 expression is directly correlated with longer RFS in breast cancer patients and inversely correlated with tumor size and grade. Downregulation of SLC-39A6 in the GLI-1 overexpressing and endocrine resistant cells raises the probability that SLC-39A6 is involved in sensitizing the cells to endocrine therapy.

Specific Aim 3: *Determine if GLI-1 correlates with the expression of putative target genes in breast cancer patients and if these targets are predictive of disease outcome.*

We will look at the correlation between expression of GLI-1 and the putative target genes identified in specific aim 1 in ER+ breast cancer. We will also determine if combined expression of GLI-1 and its targets are better prognostic markers and correlate with disease recurrence. This data could also help design new combinatorial therapy targeting GLI-1 and its downstream effector genes.

STUDY DESIGN

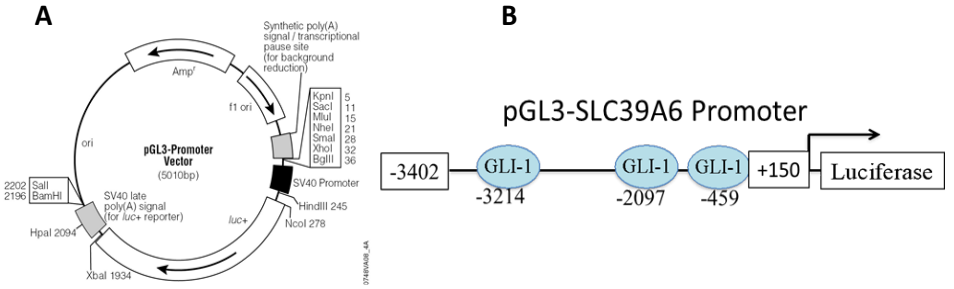
Specific Aim 1: *Identify putative downstream targets of the transcription factor, GLI-1*.

Using MCF7-GLI-1 and MCF7-vector cells we will compare expression of 96 breast cancer-related genes by PCR-based microarray analysis. Expression of newly identified genes will be validated by real-time PCR in these two cell lines. In addition, expression of these genes will be determined in MCF7 parental cells and MCF7 cells resistant to tamoxifen (TAM-R) and faslodex (FAS-R).

Specific Aim 2 A. *Determine if GLI-1 represses SLC-39A6 by directly binding to the promoter.*

The SLC-39A6 promoter will be analyzed using the rVista software (http://rvista.dcode.org) for putative GLI-1 binding sites. Human SLC-39A6 promoter spanning three putative GLI-1 binding sites will be cloned into the pGL3 luciferase reporter vector. MCF7-GLI-1 and MCF7-vector cells will be transfected with this plasmid along with Renila-Luciferase vector (Promega) as an internal control. Luciferase activity will be measured using dual luciferase assays (Promega) and SLC-39A6 promoter activity will be measured by firefly luciferase expression and normalized to

renilla luciferase expression. To assess the functional significance of the GLI-1 binding sites, each site will be individually mutated using Quik Change Lightening Site Directed Mutagenesis Kit (Agilent).



C

Site	Wild Type	Mutant
-459	5'-g C A T C A C C C A g-3'	5'-g G A T A A C A C g-3'
-2097	5'-g G T C C T C C C A g-3'	5'-g G T A C T C A C A g-3'
-3214	5'-a G A C C T C C A A g -3'	5'-a G A C A T C A A A g -3'

Figure 4. Promoter construct for SLC-39A6 **A)** pGL3 Basic reporter vector. **B)**SLC-39A6 and ER α promoter map showing putative GLI-1 binding sites. Numbers indicate the distance in base pairs these putative sites are from the transcriptional start site. **C)**Wild-type and mutated sequences for the three putative GLI-1 binding sites.

Specific Aim 2B) *Determine if SLC-39A6 is involved in mediating the response to endocrine therapy*

To determine the effect of SLC-39A6 on tamoxifen and faslodex sensitivity, we will generate stable cell lines of MCF7 cells transduced with sh-SLC-39A6 shRNA. We will monitor the cytotoxic effects of tamoxifen and faslodex with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] cell viability assay.

Specific Aim 3: *Determine if GLI-1 correlates with the expression of putative target genes in breast cancer patients and if these targets are predictive of disease outcome*

Patient data will be taken from the Hatzis data set where 298 ER+ patients were treated with tamoxifen for 5 years. Bioinformatic analysis will be handled by Steven Sizemore (Ohio State Comprehensive Cancer Center).

MATERIALS & METHODS

qPCR Array: The mRNA expression of 96 breast cancer related genes will be performed using PCR array from SABiosciences (Qiagen) and total RNA isolated from MCF7-GLI-1 and MCF7-vector cells.

Cell culture: Established MCF7 cell lines will be grown in phenol red containing containing DMEM (Sigma Aldrich) with 10% fetal bovine serum (FBS, Sigma Aldrich), non-essential amino acids (Cellgro), antibiotic/antimycotic, and 6ng/mL insulin (Complete DMEM). Tamoxifen (TAM-R) and faslodex resistant (FAS-R) cells were obtained from Dr. Kenneth P. Nephew (Indiana university) and will be cultured in their respective antiestrogen at different concentrations (100, 250, 500, and 1000 nanomolar).[28] Unless otherwise noted, the FAS-R and TAM-R cells used in this study will be cultured in 500 nM of the respective antiestrogen. Media without phenol red (phenol red free DMEM) will be supplemented with 10% charcoal-stripped FBS, antibiotic/antimycotic, 6ng/mL insulin, and 2mmol/L L-glutamine.

RNA: Cells will be grown to 70-80% confluency, washed twice with PBS, and RNA will be isolated using Trizol (Life Technologies). cDNA will be made using High Capacity cDNA kit (Applied

Biosystems). Real-Time PCR will be preformed using SYBR Green PCR Master Mix (Applied Biosystems).

Protein: Whole cell extracts will be prepared in SDS lysis buffer (1% SDS, 50mM Tris HCL, 10mM EDTA) and briefly sonicated. Proteins will be separated on SDS-PAGE gels and transferred to PVDF membrane. Blots will be blocked for 30 minutes in 0.1% TBST and 5% milk and incubated overnight with primary antibody. Blots will be reprobed with GAPDH antibody and incubated for one hour. This will be followed by incubation with anti-rabbit or anti-mouse secondary antibody for one hour (GE Life Sciences) and developed using ECL reagent (Pierce).

Antibodies: SLC-39A6 rabbit polyclonal antibody (#042377, Sigma-Aldrich); Estrogen Receptor alpha mouse monoclonal (#066-100, Diagenode); GAPDH polyclonal antibody (#25778 Santa Cruz); EGFR (#1005, Santa Cruz); p-EGFR Y1068 (#2234s, Cell Signaling); ERK1-2 (#4695s, Cell Signaling); p-ERK1-2 (#4376s, Cell Signaling).

Luciferase Assay: Cells will be seeded in 24-well plates in phenol red containing complete DMEM. The following day, cells will be transfected with100 ng of their respective pGL3 promoter-reporter vector (Promega) and the 1.0 ng pRLTK (thymidylate kinase promoter driven Renila Luciferase) as an internal control in DMEM with 5% FBS using Lipofectamine 2000 (Life Technologies). The following day, cells will be washed with 1x PBS and incubated in complete DMEM. Cells will be harvested 48 hours following transfection and promoter activity will be determined using the Dual Luciferase Reporter Assay kit (Promega)

Bioinformatics: Promoter sequence of SLC-39A6 will be obtained from the University of California at Santa Cruz (UCSC) genome browser. GLI-1 consensus binding sites will be located using rVista 2.0 online transcription factor consensus sequence database (http://rvista.dcode.org).

Cloning & Mutations: The SLC-39A6 promoter will be amplified from MCF7 genomic DNA and ligated into the pGL3-Basic reporter vector. Mutations of the putative GLI-1 binding sites will be performed using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and verified by sequencing at The Ohio State University Nucleic Acid Core Facility.

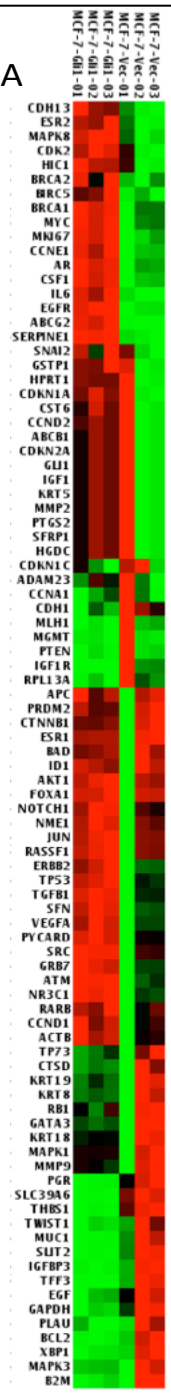
MTT Assay: Cells will be seeded in 96 well plates in phenol red free media. About 24 hours after seeding the cells will be starved overnight in 0.5% charcoal-stripped FBS containing PR-free DMEM. The next day, cells will be treated with 5μM tamoxifen or 5μM faslodex in the presence of 10nmol E2 for 72 hours. The drugs will be replenished after 48 hours.

RESULTS

Overexpression of GLI-1 in MCF7 cells leads to differential expression of several genes

As our previous study showed an increase in GLI-1 expression in tamoxifen resistant cells, we ectopically expressed GLI-1 in MCF7 cells and performed microarray analysis in order to determine putative GLI-1 targets. Overexpression of GLI-1 led to differential expression of several genes, shown in the heat map (Fig.5A) An increase in expression of several genes namely: IL-6, serpine-1, AR, EGFR, CCNE1, CSF1, ESR2, and ERBB2 was observed (Fig 5B). Some of these genes, such as ABCG2, MYC, VEGF, SNAI2 were previously shown to be GLI1 target genes. These genes could be directly or indirectly regulated by GLI-1. Interestingly, several genes were markedly downregulated upon ectopic expression of GLI-1, namely: IGF1R, SLC-39A6, SLIT2, TFF3, THBS1, and PGR. (Fig. 5C). This led us to hypothesize that GLI-1 could have a direct repressive role on gene expression.

A



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Gene	Fold Change
ABCG2	16.04
IL-6	14.01
SERPINE1	12.39
AR	7.84
EGFR	7.36
MYC	6.04
CCNE1	5
CSF1	4.81
ESR2	4.64
BRCA1	3.86
VEGFA	3.78
ATM	3.56
ERBB2	3.52
HIC1	3.27
SFN	3.02
GRB7	2.9
TP53	2.78
CDH13	2.75
SRC	2.61
TGFB1	2.58
NOTCH1	2.3
NR3C1	2.3
FOXA1	2.28
JUN	2.26
AKT1	2.17
GSTP1	2.16
SNAI2	2.09

C

Gene	Fold Change
IGF1R	-17.36
SLC-39A6	-14.44
SLIT2	-13.82
TFF3	-8.03
THBS1	-4.72
PGR	-4.02
MUC1	-3.76
EGF	-2.86
PLAU	-2.34
TWIST1	-2.21
XBP1	-2.12

Key
Green: Validated genes
Yellow: Known GLI-1 targets

Figure 5. Ectopic expression of GLI-1 in MCF7-GLI cells alters expression of several genes **A)** Heat map of MCF7 GLI-1 cells showing differential expression of 96 genes. Red signifies an upregulation in gene expression; green signifies a downregulation in gene expression. **B)** Chart showing fold change in gene expression in upregulated genes and in **C)** downregulated genes.

Validation of Putative GLI-1 Targets

In order to validate the putative target genes of GLI-1, we analyzed expression of several upregulated and downregulated genes in MCF7 GLI-1 #1, MCF7 GLI-1 #2 clones (Fig. 6A & 7A, respectively). In addition, we also analyzed expression of these genes in MCF7 parental, FAS-R, and TAM-R cells (Fig 6B & 7B respectively). Several of the upregulated genes identified by the microarray demonstrated higher expression in both the GLI-1 overexpressing clones, TAM-R and FAS-R cells compared to MCF7-vector cells and parental MCF7 cells respectively. Similarly, we confirmed that several downregulated genes had reduced expression in the GLI-1 overexpressing cells and the endocrine resistant cells.

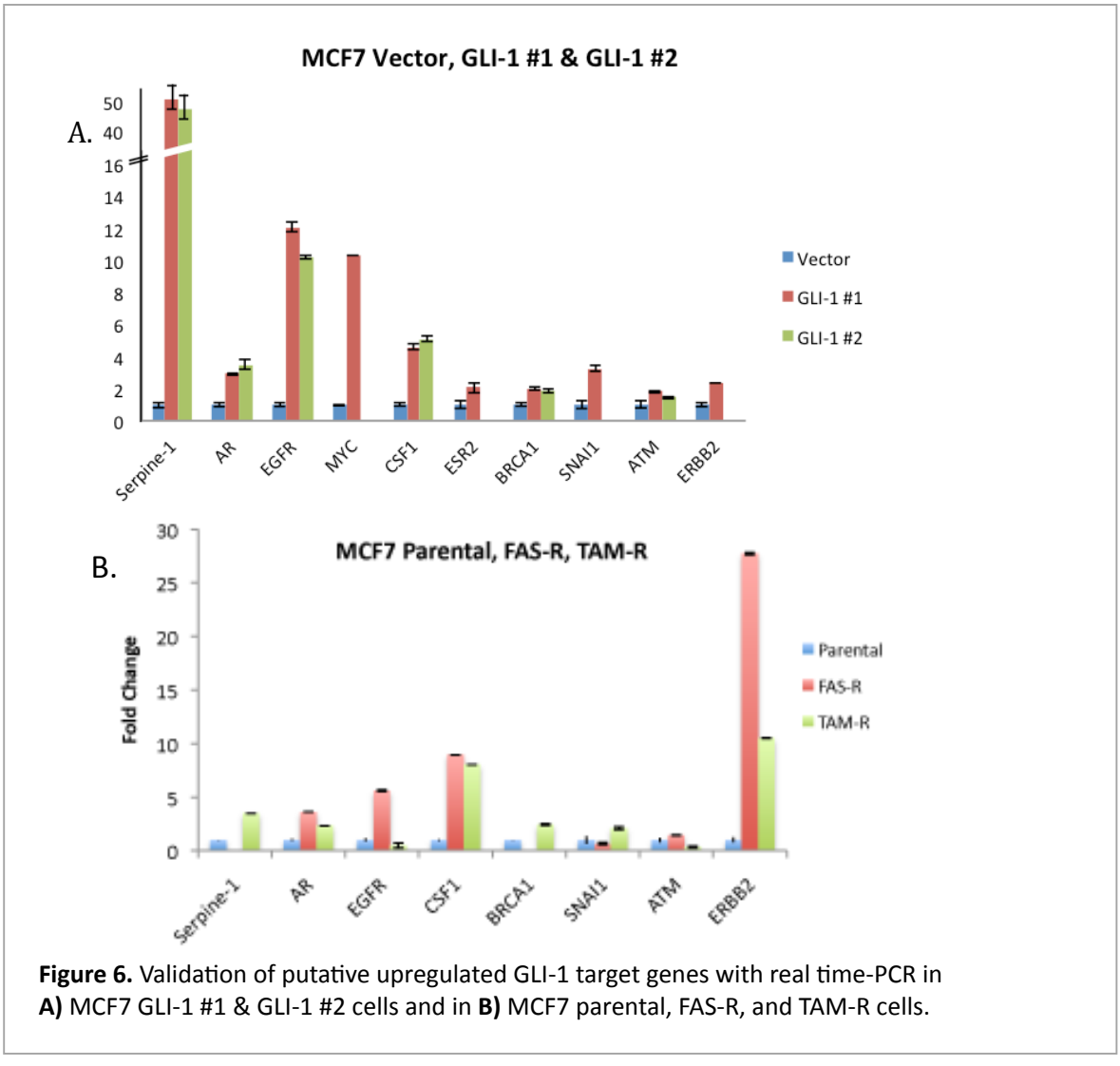


Figure 6. Validation of putative upregulated GLI-1 target genes with real time-PCR in **A)** MCF7 GLI-1 #1 & GLI-1 #2 cells and in **B)** MCF7 parental, FAS-R, and TAM-R cells.

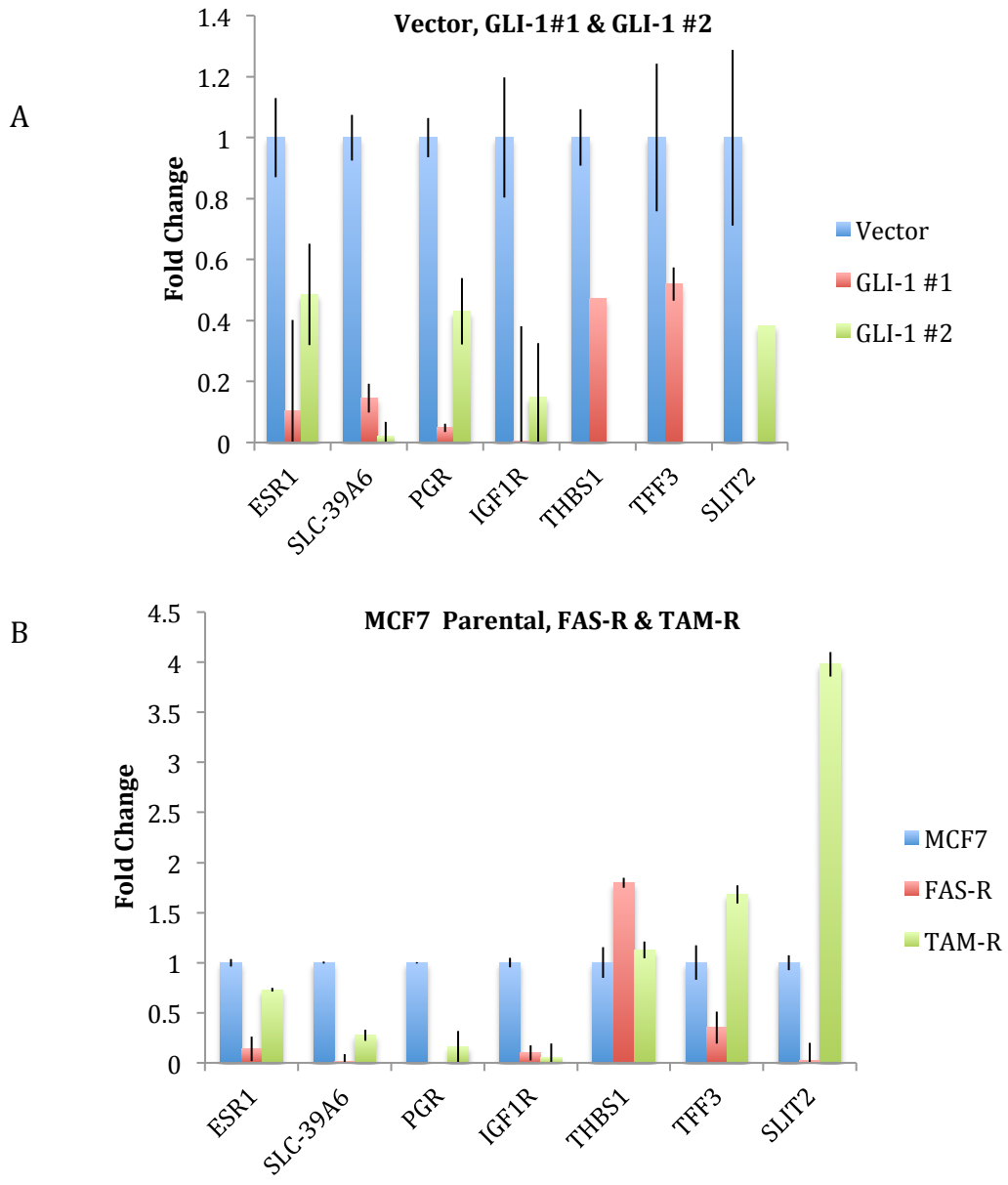


Figure 7. Validation of putative downregulated GLI-1 target genes with real time-PCR in **A)** MCF7 GLI-1 #1 & GLI-1 #2 cells and in **B)** MCF7 parental, FAS-R, and TAM-R cells.

SLC-39A6 Protein is Reduced in MCF7 GLI-1, FAS-R, & TAM-R cells

As SLC-39A6 expression was correlated with better overall survival and disease free survival in ER+ patients and is downregulated in the resistant cells, we were interested in studying if loss of

this gene promotes endocrine resistance. As observed for mRNA expression, SLC-39A6 protein is also reduced in the MCF7 GLI-1 cells (Fig 8A), FAS-R, and TAM-R cells (Fig. 8B).

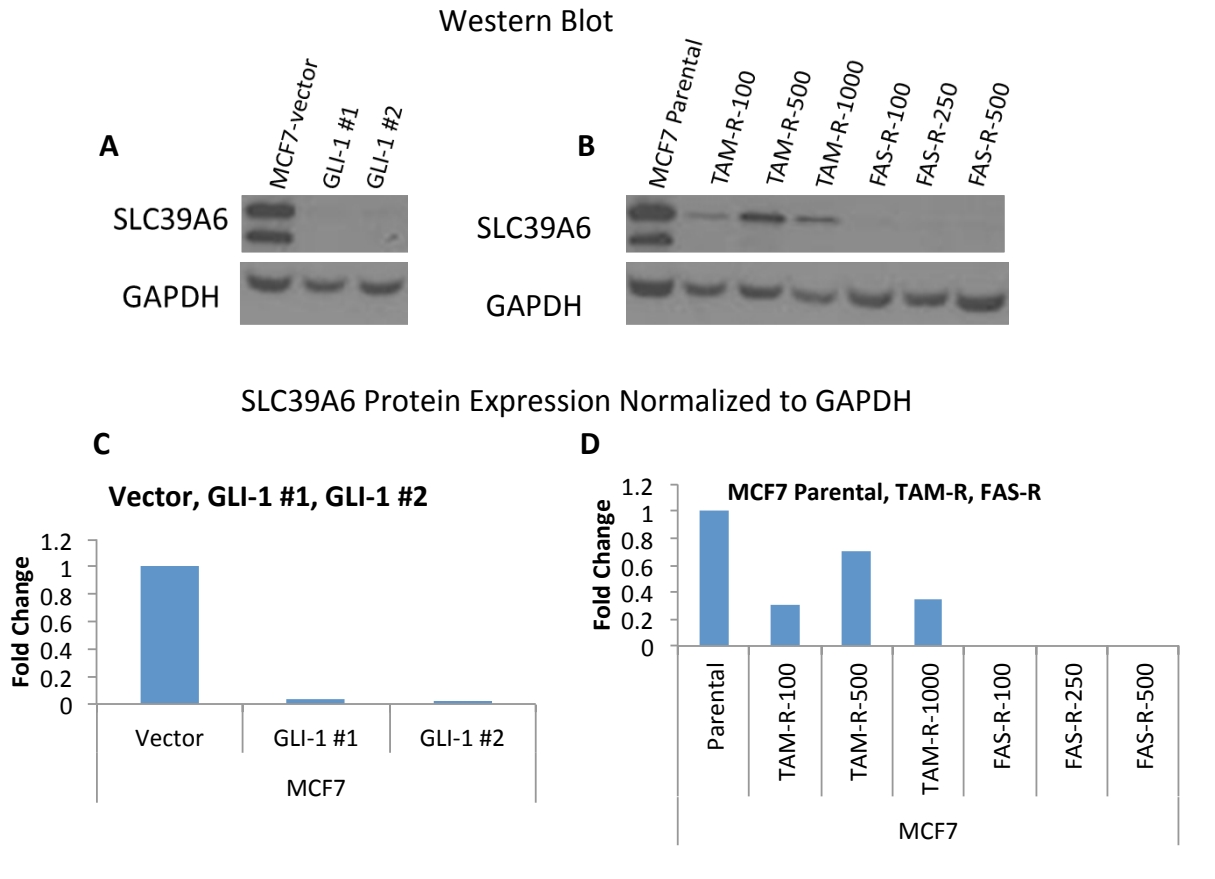


Figure 8. Western blot analysis and quantification of SLC-39A6 protein normalized to GAPDH in **A,C)**MCF7-vector, GLI-1 #1, and GLI-1 #2 and **B,D)**MCF7 parental and TAM-R and FAS-R cells. Numbers indicate the concentration (nM) of antiestrogen that the cells are maintained in.

SLC-39A6 Promoter Activity is Reduced in MCF7 GLI-1, FAS-R and TAM-R Cells

We hypothesized that SLC-39A6 was directly inhibited by GLI-1 binding to the promoter. Using rVista 2.0, we found three putative GLI-1 binding sites within 3.5kb upstream of the transcriptional start site. This region was cloned into the pGL3-Basic vector to determine SLC39A6

promoter activity. Luciferase assay determined that the SLC-39A6 promoter activity was reduced in MCF7 GLI-1 cells (Fig. 9A) in addition to FAS-R and TAM-R cells (Fig. 9B).

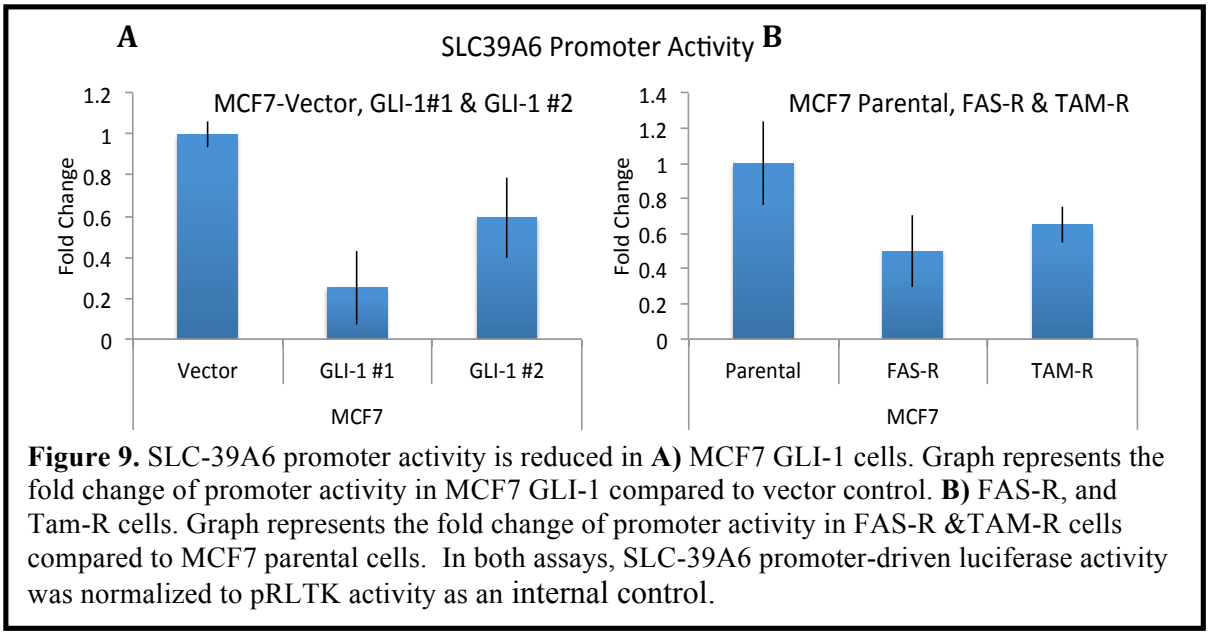


Figure 9. SLC-39A6 promoter activity is reduced in **A)** MCF7 GLI-1 cells. Graph represents the fold change of promoter activity in MCF7 GLI-1 compared to vector control. **B)** FAS-R, and Tam-R cells. Graph represents the fold change of promoter activity in FAS-R &TAM-R cells compared to MCF7 parental cells. In both assays, SLC-39A6 promoter-driven luciferase activity was normalized to pRLTK activity as an internal control.

Mutation of Putative GLI-1 Binding Sites had no effect on SLC-39A6 Promoter Activity

Hypothesizing that GLI-1 may have a novel repressive role in the regulation of SLC-39A6 we mutated the three putative GLI-1 binding sites on the promoter. If GLI-1 is a direct negative regulator of gene expression, we expect an increase in SLC-39A6 promoter activity upon mutation of the GLI-

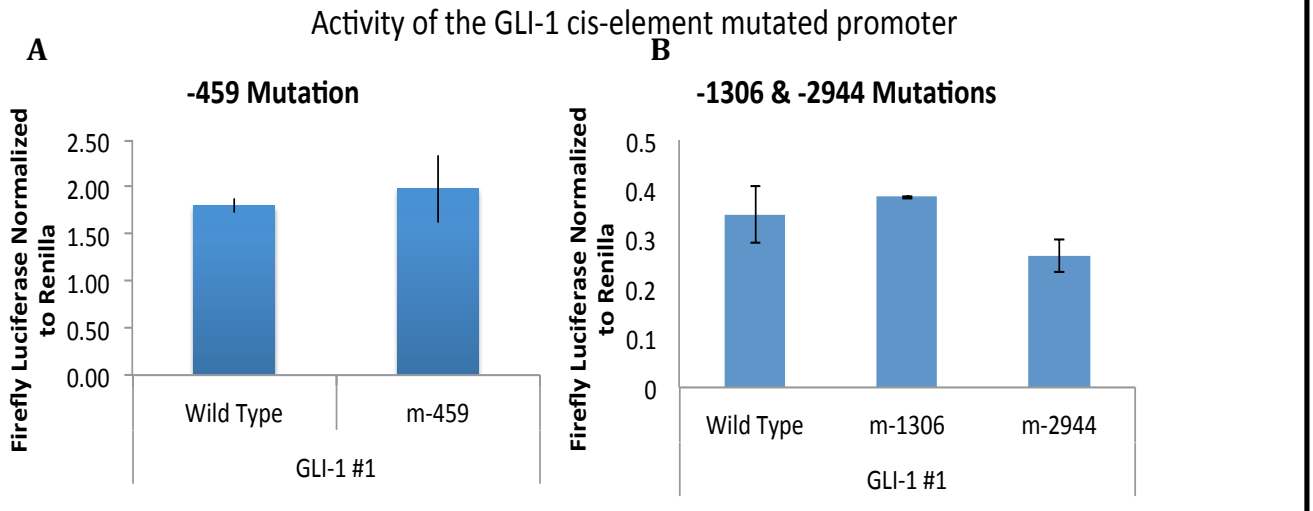


Figure 10. Activity of the SLC-39A6 promoter in MCF7 GLI-1 #1 cells, where putative GLI-1 binding site. **A)** at 459 base pairs and **B)** 1306 and 2944 base pairs upstream of transcription start site was mutated .

1 binding sites. Mutation of all three sites: 459, 1306, and 2944 base pairs upstream of the TSS had no effect on the promoter activity (Fig. 10A & 10B). This suggested that GLI-1 does not act as direct repressor of SLC-39A6 and it is likely regulated through a different mechanism.

Repression of SLC-39A6 does not increase FAS or TAM sensitivity

Hypothesizing that loss of SLC-39A6 promotes endocrine resistance in ER+ breast cancer, we knocked down SLC-39A6 protein in MCF7 cells and tested the resulting sensitivity to TAM or FAS. For this we expressed SLC-39A6 shRNA in MCF7 cells to mimic the SLC-39A6 loss observed in MCF7 GLI-1, FAS-R, and TAM-R cells. Western blot confirmed that SLC-39A6 expression was indeed reduced in this cell line (Fig. 11A). We then performed MTT assay with MCF7-vector and MCF7-sh-SLC-39A6 cells to compare the response of these cells to TAM and FAS (Fig. 11B). Cells were treated with DMSO (control), 5µM FAS, or 5µM TAM for 72 hours and cell viability was assessed. Although we expected the MCF7-sh-SLC-39A6 cells to be more sensitive to TAM and FAS, we did not observe any significant difference between MCF7-vector cells and MCF7-sh-SLC-39A6 cells in their responses to endocrine therapy (Fig 11B). This suggests that SLC-39A6 does not play a role in mediating anti-estrogen sensitivity.

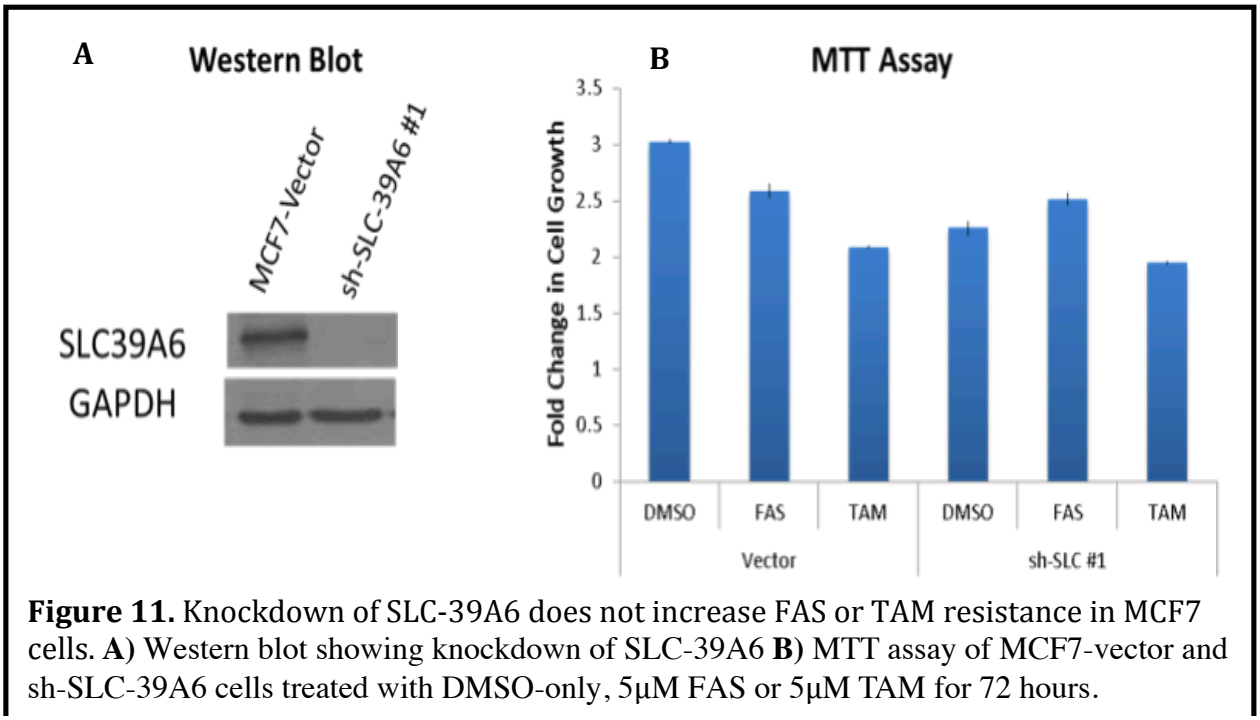


Figure 11. Knockdown of SLC-39A6 does not increase FAS or TAM resistance in MCF7 cells. **A)** Western blot showing knockdown of SLC-39A6 **B)** MTT assay of MCF7-vector and sh-SLC-39A6 cells treated with DMSO-only, 5µM FAS or 5µM TAM for 72 hours.

ERa expression is downregulated in GLI-1 overexpressing cells:

As mutation of the GLI-1 binding sites on the SLC-39A6 promoter failed to alleviate the repressive effect of GLI-1 on SLC-39A6 promoter activity, we entertained the possibility that GLI-1 is inhibiting ERα transcription and in turn down regulating ERα target genes. Analysis by real-time PCR showed significant decrease in ERα gene (ESR1) expression in MCF7-GLI-1 cells compared to the MCF7 –vector tor cells (Fig. 7A & 7B). Furthermore, previous studies have shown that ERα protein is reduced in GLI-1 overexpressing cells, supporting our observations.[29] Our computational analysis revealed presence of several putative GLI-1 binding sites in the promoter region of ERα. We are in the process of cloning this region in pGL3 vector for further analysis.

GLI-1 expression Correlates with expression of its Target Genes in Breast Cancer Patients

We next attempted to determine if expression of these novel GLI-1 target genes correlates with GLI-1 expression in primary human breast cancer. For this, we analyzed Hatzis data set that contains gene expression data of 298 ER+ breast cancer patients.[30] We found a statistically significant correlation of GLI-1 expression with several upregulated and downregulated genes (Fig

Expected Upregulated Genes		
A	**All Samples**	**Luminal A Samples**
ABCG2	1.15E-13	5.85E-07
ATM	0.006	0.001
BRCA1	6.30E-08	9.12E-05
EGFR	4.54E-40	6.90E-17
ESR2	3.17E-43	7.30E-17
SERPINE1	5.22E-10	2.57E-07
SNAI1	2.18E-15	1.68E-08
IL6	0.12	0.034
NOTCH	0.13	0.41

Expected Downregulated Genes		
B	**All Samples**	**Luminal A Samples**
IGFR1	(-)0.001	(-)0.001
MUC1	(-)0.06	(-)0.078
PGR	(-)0.002	(-)0.001
SLC39A6	(-)0.0001	(-)0.052
TFF3	(-)0.19	(-)0.047
THBS1	(-)9.4E-06	(-)0.02
XBP1	(-)0.008	(-)0.048

Negative sign (-) identifies genes that inversely correlate with *GLI1*

Figure 12. GLI-1 mRNA correlates with mRNA expression with several putative genes obtained from the MCF7 GLI-1 microarray. **A)** Genes that positively correlate with GLI-1 **B)** Genes that negatively correlate with GLI-1.

12A, B). Interestingly, when ER+ patients were sub-grouped in Luminal A and Luminal B (that are usually Her2 positive) patients, the correlation is significant only in patients with Luminal A subtype of breast cancer. This data validates our microarray analysis and leads us to identify an important difference between Luminal A and B patients.

Combined expression of GLI-1 and SNAI1 or EGFR is predictive of metastatic recurrence in the patients with Luminal A Breast Cancer

We next asked the question if combined expression of GLI-1 and its target genes have improved prognostic ability in ER+ patients compared to GLI-1 alone. Our analysis revealed that GLI-1 along with SNAI1 (Fig 13A, p value= 0.0019) or EGFR (Fig. 13B, p value= 0.0065) are predictive of metastatic recurrence in patients with Luminal A subtype of breast cancer. Expression of GLI-1 alone or SNAI1 or EGFR was not predictive of metastatic recurrence in this patient population.

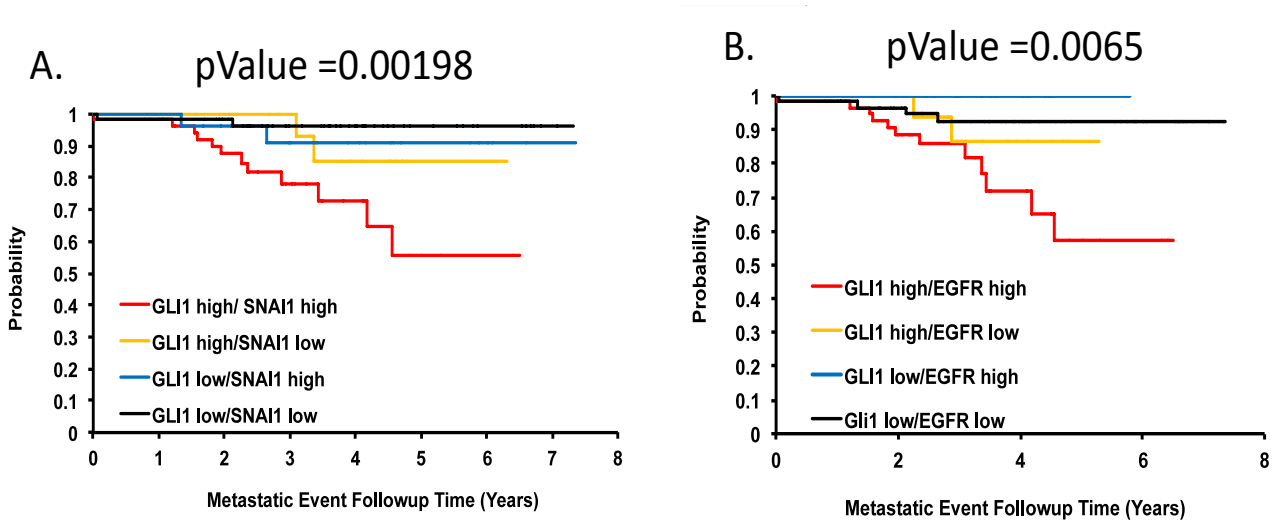


Figure 13. Kaplan-Meyer Curves predicting metastatic recurrence in patients with Luminal A breast cancer when GLI-1 expression is combined with **A)** SNAI1 and **B)** EGFR.

Expression And Phosphorylation Of EGFR Is Upregulated In MCF7-GLI-1 Cells

As expression of GLI-1 is significantly correlated with that of EGFR in Luminal A patients (Fig. 12A) and since patients with high GLI-1 and high EGFR had higher probability of metastatic recurrence (Fig. 13B) we were interested in the role of GLI-1 on EGFR expression and its activation. Western blot analysis showed upregulated EGFR protein in MCF7-GLI-1 cells compared to MCF7-vector control (Fig. 14A). In addition, MCF7-GLI-1 cells had increased phosphorylated EGFR at tyrosine 1068 (Y1068) compared to MCF7-vector (Fig. 14A). Phosphorylated EGFR at Y1068 demonstrates that EGFR is active and may be contributing to cell survival. The phosphorylation status of extracellular signal regulate kinases 1 and 2 (ERK-1-2), the downstream kinases commonly activated by EGFR was also assessed. Our western blot analysis demonstrates that ERK1/2 are also increasingly phosphorylated in GLI1 overexpressing cells (Fig. 14B).

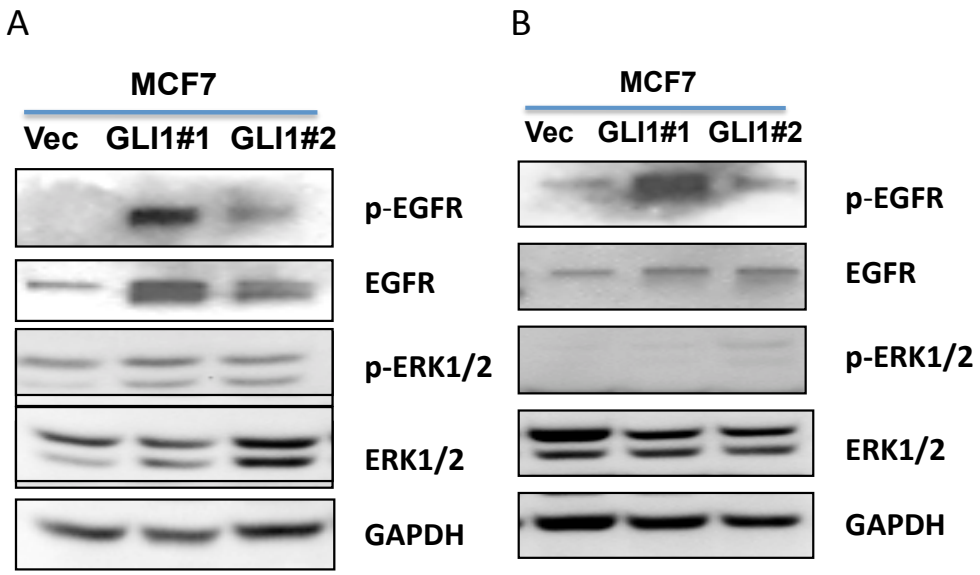
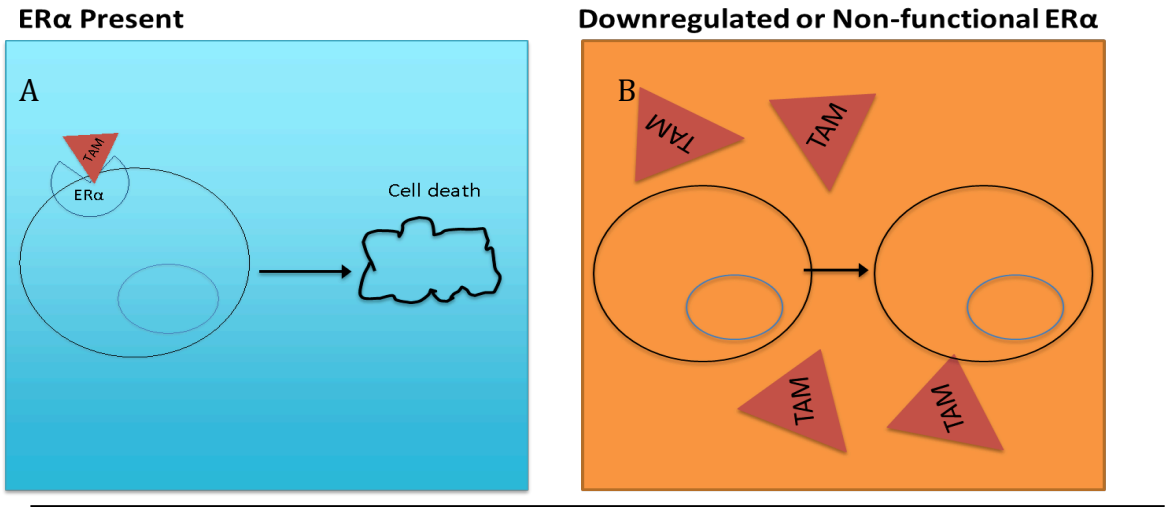


Figure 14. Total EGFR and phosphorylated EGFR are increased in MCF7-GLI-1 compared to MCF7-vector controls. A) Basal level protein expression B) Protein expression and phosphorylation status with 48-hour starvation.

DISCUSSION

This study is a work in progress and we will continue to evaluate the role of the hedgehog pathway in regulating novel targets obtained from the PCR microarray and their importance in endocrine resistance. We examined the potential role of GLI-1 as a transcriptional repressor of the zinc transporter protein, SLC-39A6. As mutation of the putative GLI-1 binding sites failed to increase SLC-39A6 promoter activity, we expect GLI-1 suppresses SLC-39A6 in an indirect manner. This is most likely due to an inhibitory effect of GLI-1 on ERα. We observed a significant decrease in ERα gene expression in MCF7 GLI-1 cells. Furthermore, of the top six genes (IGF1R, SLC-39A6, SLIT2, TFF3, THBS1, PGR) that are maximally reduced in MCF7-GLI-1 cells (Fig. 3C), all were previously reported to be induced by estrogen treatment. Treatment of breast cancer cell lines with estrogen led to the induction of IGF1R,[31] SLC-39A6[32], and THBS1.[33] Similarly, SLIT2 was shown to be induced by estrogen in the human endometrial Ishikawa cancer cell line[34] and TFF3 was shown to be induced by E2 in endometrial adenocarcinoma cell lines.[35] Although we did not validate downregulation of MUC1 and XBP1 in MCF7-GLI-1, TAM-R and FAS-R cells, it is important to note that MUC1[25] and XBP1[36], too, are regulated by ERα in Ishikawa endometrial and MCF7 breast epithelial cell lines, respectively. These data led us to believe that GLI-1 interferes with ERα expression. If GLI-1 activation is concurrent with loss in ERα expression or activity, this provides a plausible alternative mechanism for endocrine resistance. It is expected that repression of ERα and activation of an alternative survival mechanism (Hedgehog pathway), that is not a target of anti-estrogenic compounds will reduce the efficacy of TAM or FAS and increase resistance to endocrine therapy. (Fig 15)

Figure 15. Possible mechanism of resistance through downregulation of ERα. **A)** When ERα is present, TAM binds to ERα and causes cell death. **B)** In the absence of ER (or functional ERα) there is no substrate for TAM, and therefore therapy is not effective. The cells are no longer targetable by anti-estrogenic compound and evade cell death.



We also investigated the role of SLC-39A6 in increasing sensitivity to endocrine therapy. Our study did not suggest that SLC-39A6 plays any significant role in sensitizing the cells to TAM or FAS. Although previous studies demonstrated SLC-39A6 is associated with better disease free survival, it appears to be a marker of functional ERα in the cells. More studies are warranted to understand the role of this protein in breast carcinogenesis.

Importantly our bioinformatic analysis revealed a strong correlation between expression of GLI-1 and putative target genes in ER+ patients. Among the ER+ patients, Luminal A subtypes are expected to perform better on endocrine therapy compared to Luminal B patients. However there is a subpopulation of Luminal A patients that fail to respond to anti estrogenic compounds and the reason for such failure is largely unknown.[3] Our bioinformatic analysis correlating GLI-1 with SNAI1 and EGFR in the prediction of worse metastatic recurrence in breast cancer patients with Luminal A subtype provides a plausible cause for failure of endocrine therapy. It is conceivable that de novo or drug inducible activation of Hedgehog pathway in ER + breast cancer is one of the key mechanisms how these cancer cells fail to respond to anti-estrogenic compounds.

Along with increased expression of EGFR in GLI-1 overexpressing MCF7 cells, our studies also eluded to increased phosphorylation of EGFR and its down stream gene ERK1/2. Based on this observation we believe that therapeutic targeting of GLI-1 and EGFR could be a potential option for patients with Luminal A breast cancer who fails endocrine therapy. Importantly, several EGFR inhibitors like Gefitinib and Erlotinib are approved for human trials. Currently there is a phase one clinical trial using therapies that target both SMO and EGFR in patients with metastatic pancreatic cancer.[10]

All of the hedgehog pathway inhibitors approved for therapy or in clinical trials target and inhibit SMO. However, one study showed that GLI-1 is activated by mechanisms that are not reliant on SMO activation. For example, the cytokine osteopontin (OPN) was shown to increase GLI-1 transcription even in the presence of SMO inhibitors. This alternative pathway is likely to render SMO targeted therapy ineffective.[37] In a medulloblastoma patient, an acquired SMO mutation rendered the patient refractory to the SMO inhibitor, Vismodegib.[38] Therefore, as an alternative to SMO targeting it is expected that developing GLI-1 inhibitors will be beneficial for such patients. This will directly target the protein that is ultimately responsible for genomic regulation and drug resistance in many types of cancers. Developmental and preclinical studies along this line are in progress.

1. http://www.cdc.gov/cancer/dcpc/data/women.htm

2. Ali S, Buluwela L, Coombes RC. Antiestrogens and Their Therapeutic Application in Breast Cancer and Other Diseases. *Annual Review of Med.* 2011; 62:217-32

3. Zhang MH, Man HT, Zhao XD. Estrogen receptor-positive breast cancer molecular signatures and therapeutic potentials (Review). *Biomedical Reports*. 2013; 2;41-52.

4. Ali S Coombes RC. Endocrine-Responsive Breast Cancer and Strategies for Combating Resistance. *Nature Reviews Cancer.* 2002; 101-115

5. Klinge CM. Estrogen Receptor Interaction with Estrogen Response Elements. *Nucleic Acids Research.* 2001; 29:2905-2919

6. Bjornstrom L, Sjoberg M. Mechanisms of Estrogen Receptor Signaling: Convergence of Genomic and Nongenomic Actions on Target Genes. *Molecular Endocrinology* 19(4):833-842.

7. Ramaswamy B, Lu Y et al. Hedgehog signaling is a novel therapeutic target of tamoxifen-resistant breast cancer aberrantly activated by PI3K/AKT pathway. *Cancer Research* 2012 19;5048-5059.

8. Briscoe J Therond P. The Mechanisms of Hedgehog Signaling and its Roles in the Development of Disease. *Nature Reviews* 2013; 14; 416-429.

9. Scales SJ, de Sauvage FJ. Mechanisms of hedgehog pathway activation in cancer and implications for therapy. *Trends Pharmacol Sci* 2009; 30:303-312.

10. Brechbiel J et al. Crosstalk between hedgehog and other signaling pathways as a basis for combination therapies in cancer. Cancer Treat Rev (2014).

11. Amakye D, Jagani Z, Dorsch M. Unraveling the therapeutic potential of the Hedgehog pathway in cancer. *Nature Medicine*. 2013; 19;1410-1422.

12. Theunissen JW, de Sauvage FJ. Paracrine hedgehog signaling in cancer. *Cancer Research* 2009;69:6007-6010.

13. Katoh Y, M Katoh M. Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation. *Current Molecular Medicine*. 2009; 9(7)873-886.

14. Nanta R, Kumar D, Meeker D, et al. NVP-LDE-225 inhibits epithelial-mesenchymal transition and human prostate cancer stem cell growth in NOD/SCID IL2R null mice by regulating Bmi-1 and microRNA-128. *Oncogenesis* 2013; 2:e42.

15. S. Liu, G. Dontu, I.D. Mantle *et al.*Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells Cancer Res, 66 (2006), pp. 6063–6071

16.Natarajan K, Xie Y, Baer MR, Ross DD. Role of breast Cancer Resistance Protein (BCRP/ABCG2) in cancer drug resistance. *Biochem Pharmacol* 2012;83:1084–1103.

17. Ingram W, Crowther LM, Little EB, *et al.* ABC transporter activity linked to radiation resistance and molecular subtype in pediatric medulloblastoma. *Experimental Hematology & Oncology* 2013; 2:26.

18. Keysar SB, Le PN, A RT et al. Hedgehog Signaling Alters Reliance on EGF Receptor Signaling and mediates anti-EGFR Therapeutic Resistance in Head and Neck Cancer. 2013; 73; 3381-3392.

19. Holohan C, Schaeybroeck S, Longley D et al. Cancer drug resistance: an evolving paradigm. *Nature Reviews Cancer*. 2013 13;714-726.

20. Lauth M, Bergstrom A, Shimokawa T et al. Inhibition of GLI-mediated transcription and tumor cell growth by small-molecule antagonists. *PNAS*. 2007. 104;8455-8460.

[21] Grattan BJ, Freake HC. Zinc and Cancer: Implications for LIV-1 in Breast Cancer. *Nutrients*. 2012; 4:648-675.

22. Lopez V, Kelleher S. Zips6-attenuation promotes epithelial-to-mesenchymal transition in ductal breast tumor T47D cells

23. Manning DL, Daly, RJ, Lord PG, Kelly KF, Green CD. Effects of oestrogen on the expression of a 4.4 kb mRNA in the ZR-75-1 human breast cancer cell line. *Molecular and Cellular Endocrinology.* 1988; 59:205-212

24. Kasper G, Weiser A, Rump A, Sparbier K, Dahl E, Hartmann A, Wild P, Schwidetzky U, velez EC, Lehmann K. Expression levels of the putative zinc transporter LIV-1 are associated with a better outcome of breast cancer patients. *International Journal of Cancer*. 2005; 117:961-973.

[25] Shen R, Xie F, Shen H. Negative Correlation of LIV-1 and E-Cadherin Expression in Hepatocellular Carcinoma Cells. *Plos one*. 2013; 8

26. Wu C, Li D, Jia W et al. Genome-wide association study identifies common variants in SLC39A6 associated with length of survival in esophageal squamous cell carcinoma. *Nature Genetics*. 2013;45:632-638.

27 Yonezawa A, Masuda S, Toshiya S et al. Cisplatin and Oxaliplatin, but Not Carboplatin and Nedaplatin, Are Substrates for Human Organic Cation Transporters (SLC22A1–3 and Multidrug and Toxin Extrusion Family). *Pharamacology and Experimental Therapeutics* 2006

28. M Fan, PS Yan, Hartman-Frey C, Chen L, Paik H, Oyer SL, et al. Diverse Gene Expression and DNA methylation profiles correlate with differential adaptation of breast cancer cells to the antiestrogens tamoxifen and faslodex. *Cancer Res 2006*;66: 11954-11966.

29 Zhao J, Chen G, Cao D. et al. Expression of GLI-1 correlates with transition of breast cancer cells to estrogen independent growth. *Breast Cancer Research Treatment*. 2010; 119:39-51.

30. Symmans WF, Hatzis C, Sotiriou C et al. Genomic index of sensitivity to endocrine therapy for breast cancer. *Journal of Clinical Oncology*. 2010; 28:4111-9.

31. Lee AV, Jackson JG, Gooch JL et al. Enhancement of Insulin-Like Growth Factor Signaling in Human Breast Cancer: Estrogen Regulation of Insulin Receptor Substrate-1 Expression *in Vitro* and *in Vivo*. *Molecular Endocrinology* 1999; 10; 787-796/

32. Manning DL, Daly RJ, Lord PG, et al. Effects of oestrogen on the expression of a 4.4 kb mRNA in the ZR-75-1 human breast cancer cell line. *Molecular and Cellular Endocrinology*. 1988;59;205-212.

33. Hyder SM, Liang Y, Wu J. Estrogen regulation of thombospondin-1 in breast cancer cells. *Int. J. of Cancer* 2009; 125; 1045-1053.

34. Tamm-Rosenstein K, Simm J, Suhorutshenko M et al. Changes in the Transcriptome of the Human Endometrial Ishikawa Cancer Cell Line Induced by Estrogen, Progesterone, Tamoxifen, and Mifepristone (RU486) as Detected by RNA-Sequencing. *Plos One*. 2013; 8(7).

35. P Mhawech-Fauceglia, Wang D, D Samrao. Trefoil factor 3 (TFF3) expression and its interaction with estrogen receptor (ERα) in endometrial adenocarcinoma. *Gynecology Oncology.* 2013; 130; 174-180.

36. Sengupta S, CG Charma, Jordan VC. Estrogen regulation of X-box binding protein-1 and its role in estrogen induced growth of breast and endometrial cancer cells. *Hormone Molecular Biology and Clinical Investigation Journal*. 2010. 2; 235-243.

37. DAS S, Samant RS, Shevde LA. Nonclassical activation of Hedgehog Signaling enhances multidrug resistance and makes cancer cells refractory to Smoothened-targeting Hedgehog inhibition. *Journal of Biological Chemistry*. 2013;17;11824-33.

38. Yauch RL, Dijkgraaf GJ, Alicke B, Januario T et al. Smoothened mutation confers resistance to a Hedgehog pathway inhibitor in medulloblastoma. *Science* 2009;326:572-574.