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A genome-wide loss-of-function screen identifies SLC26A2 as a novel mediator of TRAIL resistance

Lina Y. Dimberg[1,*], **Christina G. Towers**[1,*], **Kian Behbakht**[2], **Taylor J. Hotz**[1], **Jihye Kim**[3], **Susan Fosmire**[4], **Christopher C. Porter**[4], **Aik-Choon Tan**[3], **Andrew Thorburn**[1], and **Heide L. Ford**[1,2,#]

[1]Department of Pharmacology, University of Colorado Anschutz Medical Campus

[2]Department of Obstetrics and Gynecology, University of Colorado Anschutz Medical Campus

[3]Department of Medicine, Division of Medical Oncology, University of Colorado Anschutz Medical Campus

[4]Department of Pediatrics, Division of Pediatric Hematology and Oncology, University of Colorado Anschutz Medical Campus

Abstract

TNF-related apoptosis inducing ligand (TRAIL) is a potent death-inducing ligand that mediates apoptosis through the extrinsic pathway and serves as an important endogenous tumor suppressor mechanism. Because tumor cells are often killed by TRAIL and normal cells are not, drugs that activate the TRAIL pathway have been thought to have potential clinical value. However, to date, most TRAIL-related clinical trials have largely failed due to the tumor cells having intrinsic or acquired resistance to TRAIL-induced apoptosis. Previous studies to identify resistance mechanisms have focused on targeted analysis of the canonical apoptosis pathway and other known regulators of TRAIL receptor signaling. To identify novel mechanisms of TRAIL resistance in an unbiased way, we performed a genome wide shRNA screen for genes that regulate TRAIL sensitivity in sub-lines that had been selected for acquired TRAIL resistance. This screen identified previously unknown mediators of TRAIL resistance including Angiotensin II Receptor 2, Crk-like protein, T-Box Transcription Factor 2 and solute carrier family 26 member 2 (SLC26A2). SLC26A2 downregulates the TRAIL receptors, DR4 and DR5, and this downregulation is associated with resistance to TRAIL. Its expression is high in numerous tumor types compared to normal cells, and in breast cancer, SLC26A2 is associated with a significant decrease in relapse free survival.

Keywords

genome-wide shRNA screen; TRAIL sensitivity; SLC26A2

[#]Corresponding Author: Heide L. Ford, University of Colorado School of Medicine, 12800 E 19th Ave, P18-6115, Mail Stop 8303, Aurora, CO 80045, 303-724-3509, 303-724-3663 (Fax), heide.ford@ucdenver.edu.

[*]These authors contributed equally to this manuscript,

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Introduction

The TNF-related apoptosis inducing ligand (TRAIL) was discovered by virtue of its high sequence homology to Fas ligand (1) (2). In contrast to tumor necrosis factor (TNF) and FasL, TRAIL has a remarkable specificity for killing tumor cells while inducing little toxicity in normal cells. TRAIL induced apoptosis is one of the body's physiological mechanisms of tumor suppression, making it an ideal pathway to reactivate with therapeutics (3, 4). However, resistance to TRAIL-induced apoptosis is common in cancer and, the overall efficacies of both monotherapy and combined treatment have been disappointing(5). Nevertheless, in numerous clinical trials with TRAIL receptor agonists, positive responses have been achieved in small subsets of patients(6).

TRAIL binds to five receptors: the functional death receptors (DR) 4 and DR5, the non-functional decoy receptors (DcR) 1 and DcR2, and the soluble TNFR member osteoprotegerin (OPG). Upon ligand binding, the trimerized functional TRAIL receptors form larger aggregates enabling the recruitment of the Fas Associated Death Domain (FADD), and procaspase 8 to the receptor. Together, these proteins form the core of the Death Inducing Signaling Complex (DISC). Formation of the DISC enables dimerization of procaspase 8 into its active form, which is then usually cleaved to produce a soluble active protease. The activated caspase initiates activation of mitochondrial membrane permeabilization and downstream effector caspases, which ultimately cleave downstream proteins leading to the hallmarks of apoptosis including cleavage of structural proteins, DNA fragmentation and membrane blebbing.

Theoretically, modulation of any of the pro- or anti apoptotic players that are involved in TRAIL signaling could influence the response to TRAIL. Numerous studies have been performed to understand the physiological mechanisms that tumor cells employ to evolve resistance to TRAIL induced apoptosis(7). Many cellular pathways have been linked to TRAIL resistance including ER-stress, proteasome mediated degradation, protein folding, metabolism and autophagy(7) (8). They each converge on the death receptor mediated apoptosis pathway via multiple mechanisms, including regulation of the death receptors via protein translation(9, 10) and degradation(7, 11), as well as membrane localization via glycosylation (12). Other mechanisms include upregulation of competing pro-survival BH3 proteins(5), and regulation of DISC and apoptosome inhibitory molecules such as cellular FLICE-like inhibitory protein (FLIP) and the inhibitor of apoptosis protein, XIAP, respectively(13–15). All of these well-established resistance mechanisms involve interference with the core components of the TRAIL signaling pathway and many of these mechanisms affect any apoptosis signal. Insight into TRAIL mediated resistance may reveal which combination therapies will be effective alongside TRAIL activation. In some cases, such as with proteasome inhibitors, it may be possible to overcome a broad range of resistance mechanisms(16).

Because tumor cells can utilize numerous and often unanticipated means to evade cell death, unbiased approaches can be useful to identify novel pathways that, when inhibited, may synergize with TRAIL activation. In this study, we employed a genome-wide loss-of-function approach to discover novel mediators of TRAIL resistance. Using a lentiviral

shRNA library designed to target the entire human genome, we discovered novel genes that when lost in TRAIL resistant cells, can re-sensitize these cells to TRAIL agonists. These genes include, amongst others, the sulfate transporter SLC26A2. Here, we demonstrate that this novel mediator of TRAIL resistance evades apoptosis through its ability to downregulate the TRAIL receptors DR4 and DR5. Furthermore, we show that the expression of SLC26A2 is correlated with worsened prognosis in breast cancer.

Materials and Methods

Genome-wide Loss-of-Function shRNA Screen

For the genome wide shRNA screen(17), the HIV based GeneNet lentiviral Human 50K library (pSIH1-H1-Puro, System Biosciences (SBI), Mountain View, CA), containing 200,000 shRNAs (3–5 shRNAs for each of about 47,000 genes) with sequencing tags, was used with minor modifications to the manufacturer's protocol. Briefly, 2.5 x 10^6 BJAB LexR cells, a sub-line of BJABs that have been made resisitant to TRAIL and lexatumumab (an agonistic TRAIL-DR5 antibody)(18) through long-term culture in increasing doses of lexatumumab (were transduced with the library virus particles together with polybrene (final concentration 8 μg/mL). Parental, TRAIL sensitive BJAB cells were transduced or mock-transduced in parallel and exposed to puromycin selection to confirm transduction efficiency. One week post-transduction, 90 x 10^6 cells were set up at a concentration of 0.5 million cells/mL and 24 hours later exposed to selection with recombinant TRAIL (Genentech) at a concentration of 100 ng/mL. Untreated library-transduced cells and untreated non-transduced cells were cultured in parallel. After TRAIL selection for 24 hours, the cells were washed and cultured for an additional 3 days before the cells were harvested and total RNA was isolated from transduced TRAIL-treated and untreated cells as well as non-transduced cells (negative control for next step vector DNA preparation) using TRIZOL (Thermo Fisher Scientific). The RNA was reverse transcribed using the vector–specific cDNA synthesis GNF/GNH primer from SBI and M-MLV reverse transcriptase (Epicentre) and then amplified by nested PCR, using a first-step amplification with Fwd GNF/GNH primer (5′-TGC ATG TCG CTA TGT GTT CTG GGA-3′) and Rev GNF/GNH primer (5′-CTC CCA GGC TCA GAT CTG GTC TAA-3′)(SBI) and then a second-step amplification step with NRev Illumina (5′-CAA GCA GAA GAC GGC ATA CGA AGA AGC AAA AAG CAG AAT CGA AGA A-3′)and NFw Illumina specific primers (5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT TCC TGT CAG A-3′) containing Illumina-specific adapter sequences. The cDNA was quantified using a Bioanalyzer (Agilent) and sequenced on an Illumina Genome Analyzer (Illumina) using a specific sequencing primer (5′-ACA CTC TTT CCC TAC ACG ACG CTTCCT GCT AGA-3′), and the number of clusters for each shRNA sequence was identified.

Genome-wide Loss-of-Function shRNA Screening Analysis

The genome-wide loss-of-function shRNA screening data was analyzed using BiNGS! (Bioinformatics for Next Generation Sequencing)(19) as previously described and validated(17, 20–22). In brief, sequencing data were mapped against the shRNA reference library using Bowtie(23). We then employed Negative Binomial to model the count distribution in the sequencing data using edgeR(24). We computed the q-value of False

Discovery Rate for multiple comparisons for these shRNAs, and performed meta-analysis by combining adjusted p-values for all shRNAs representing the same gene using weighted Z-transformation(19). We used the associated p-value [P(wZ)] to sort lists of genes with differentially represented shRNAs.

Secondary Screen

For the secondary screen, a custom-made pooled “mini-library” of lentiviral HIV based shRNA plasmids (pLKO.1) were ordered from the Functional Genomics Facilities, University of Colorado at Boulder. For each of 183 genes, 2 to 5 shRNAs were ordered. On Day 1, HEK293FT cells were plated in antibiotic-free DMEM media on 15 cm plates to achieve 60–80% confluency on the day of transfection, Day 2. The mini shRNA library was transfected into the cells along with packaging vectors pVSV-G, pPACKH1-GAG and pPACKH1-REV in the presence of Turbofect (Fermenta). In parallel, transfections were performed with a negative control vector and with a GFP-vector for confirming transduction efficiency. At Day 3, the media was replaced and at Day 4 and 5, virus particles were harvested. BJAB-LexR cells and BJAB cells were infected with the virus particles and cells were left untreated or were treated with recombinant TRAIL as described above. Genomic DNA was isolated using Qiagen DNeasy blood and tissue (Qiagen, cat #69504). Amplification was performed by PCR with specific pLKO primers (Fwd: 5′-CTT GTG GAA AGG ACG AAA CAC CG-3′, Rev: 5′-CCA AAG ATC TCT GCT GCT C-S3′_) which were then digested with Xho I and ligated to previously annealed barcode linkers. A second round PCR was performed with Illumina adapter primers left (5′-CAA GCA GAA GAC GGC ATA CGA TGG AAA GGA CGA AAC ACC GG-3′) and right (5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T-3′). The DNA was quantified on a Bioanalyzer (Agilent) and sequenced on the Illumina Sequencer using custom sequencing primer FGS pLKO primer (5′-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT-3′)

Gene-by-Gene Validations

For gene-by gene validations, 2–3 shRNAs per gene in lentiviral HIV based TRC shRNA plasmids (pLKO.1) were ordered from the Functional Genomics Facilities, University of Colorado at Boulder. The shRNAs are ordered by TCR number and the sequences are identical to those of Sigma-Aldrich Mission® pLKO.1-puro vectors. Lentiviral particles were generated as described above and transduced into BJAB-LexR cells or MDA-MB231-TRAILR cells as described. As negative controls, non-targeting Mission® pLKO.1-puro Non Target viral particles were generated and transduced to cells in parallel.

Cell culture

All cells were cultured at 37°C in humidified air supplemented with 5% CO_2. 293FT cells were cultured in DMEM cells supplemented with 10% FBS and penicillin/streptomycin. MDA MB231 cells were cultured in DMEM supplemented with 5% FBS, insulin, Hepes, non-essential amino acids (Sigma). BJAB lymphoma cells were cultured in RPMI 1640 with 10% FBS. The SLC26A2 expression plasmid was a kind gift from Dr. Antonio Rossi(25).

Cell line authentication

The BJAB-wt and LexR cell lines and the MDA-MB-231 SEN and TRAIL R cells were also recently profiled (07/2016) to confirm their identity.

qRT-PCR

cDNA synthesis was performed using the iScript kit (Biorad) from total RNA that was isolated using the RNAeasy extraction kit (Qiagen). qRT-PCR reactions were run using ssoFast Evagreen supermix (Biorad) and primers for either SLC26A2, (AGCTCCAAGGGATCATGGGAAAGTTGC, CATACTCAGCTTTCTGGTGTGGTAACAGC), DR4 (TGTACAATCACCGACCTTGACCA, AGCTAAGTCCCTGCACCACGA), DR5 (TCCTGGACTTCCATTTCCTG, TGCAGCCGTAGTCTTGATTG), CRKL, AGTR2, TBX2 (TTCCACAAACTGAAGCTGAC, GCTGTGTAATCTTGTCATTCTG), GAPDH (ACCCAGAAGACTGTGGATGG, TCTAGACGGCAGGTCAGGTC) or 18s (ACCCGTTGAACCCCATTCGTGA, GCCTCACTAAACCATCCAATCGG) with the Biorad CFX96.

Western blot analysis

Whole cell extracts were harvested from equal numbers of cells for all conditions tested. After lysates were sonicated, they were loaded onto acrylamide gels and electophoresed, after which they were transferred to PVDF membranes. After blocking in 5% milk in Tris-buffered saline with 0.1% tween (TBST) for 1 hour, membranes were incubated in primary antibodies overnight with constant rocking at 4°C. After washing in TBST, membranes were incubated in the appropriate HRP-conjugated secondary antibodies for 1 hour at room temperature with constant rocking. The primary antibodies used were SLC26A2 ((1:500, Novus Biologicals 3F6), DR4 (1:500, AbCam #ab8414)), DR5 (1:500, AbCam #ab8416), β-Actin (1:10,000 Sigma-Aldrich).

Cell Viability Assays

The number of viable cells in transduced and treated cells was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega), a luminescent assay that quantifies ATP which is directly proportional to the number of viable cells, per manufacturer's instructions. Cells were set up at 0.25 x 10^6 cells/ml in white 96 well plates and 24 hours later serial dilutions of reagents (recombinant TRAIL (Genentech), doxorubicin (Sigma) or etoposide(Sigma)) were added in triplicates. Cells were incubated for 24 hours, luminescence was assayed within 5 hours of adding the CellTiter-Glo® using a using a Turner Biosystems Modulus Microplate reader.

PI staining

To assess PI positive BJAB cells via flow cytometry we utilized the FITC Annexin-V apoptosis detection kit I (BD Biosciences #556547) according to manufacturer's instructions. Specifically, 0.25 cells/mL were washed with phosphate buffer saline solution (PBS) and then resuspended in 1X binding buffer to 1X10^6 cells/mL. 100μL of the cell solution was transferred to a culture tube and incubated with 5μL of FITC-Annexin V and

5μL of PI (although FITC-Annxin V was not used for the current analysis) for 15 minutes at room temperature in the dark. 400μL of 1X binding buffer was added to stained cells prior to analysis with a Beckman Coulter FC500. Cells that underwent heat shock at 42° for 1hour were used as a positive control for dead cells for gating purposes.

Caspase 3/7 apoptosis assay

1,000 MDA-MB-231 cells were plated in replicates of 6 in 96 well plates. 24 hours later cells were treated with serial dilutions of TRAIL and 5μM CellEvent caspase 3/7 (Invitrogen C10423). Cells were monitored every 2 hours with live cell, in-vitro microscopy imaging via Incucyte Zoom (Essen BioScience).

Flow cytometry

Equal numbers of cells for all conditions tested were harvested and spun down via centrifugation and then washed 3X in (PBS) supplemented with 0.5% BSA. Cells were resuspended in 100μl of Flow Cytometry Staining Buffer (PBS + 1mM EDTA + 25mM HEPES pH7.0 + 1.0% FBS) per $1X10^6$ cells and were blocked with $1μg/1x10^6$ cells of anti-mouse IgG (Fc specific) antibody (M4280, Sigma Aldrich) for 15 minutes at room temperature. $10μl/1X10^6$ of PE conjugated antibodies against DR4 (FAB347P, R&D Systems) and DR5 (FAB6311P, R&D Systems) were then added and allowed to incubate for 30 minutes at room temperature in the dark followed by 3 washes with Flow Cytometry Staining Buffer. The X-median or the geometric mean for percent positive cells was then analyzed with a Beckman Coulter FC500 where populations were gated based on unstained samples for each condition.

Datamining

Publicly available data sets were examined using either Oncomine or kmplot.com. For data analyzed though Oncomine, studies with a p value of 0.05 or greater and a fold change of 2 or greater were reported. For data analyzed through kmplot (2014 version), probe set 205097_at was used to analyze SLC26A2 expression in systemically untreated breast cancer patients, redundant samples were removed, unbiased data was excluded and proportional hazards assumptions were checked. Results were split by the “auto select best cut off” and graphed such that the approximate bottom quartile are considered “low” expression and the remaining top 3 quartiles are considered “high” expression. Results are censored at threshold(26).

Micorarray Gene Expression Data Analysis

Parental and TRAIL-resistant MDA-MB 231 cells and WT BJAB and LEXR cells were treated with recombinant TRAIL at 100 ng/mL for 2 hours. RNA was isolated using Trizol (Thermo Fisher Scientific) and was further purified (including a gDNA removal step) using the RNeasy PLUS micro kit (Qiagen). Isolated RNA from these samples were hybridized on Affymetrix HuGene 1.0 ST microarrays according to manufacturer’s manual (Affymetrix, Santa Clara, CA). Gene expressions were normalized by using Robust Multiarray Average (RMA)(27) method as previously described using Affymetrix Power Tool. Gene Set Enrichment Analysis (GSEA)(28) was performed on the normalized data using KEGG gene

sets. Raw microarray data has been deposited into NCBI Gene Expression Omnibus with GSE82047.

Pathway analysis

Genome-scale integrated analysis of gene networks (GIANT(29)) was used with a confidence cut off of 0.5 and a maximum gene cut off of 20 across all tissue types.

Results

A genome wide loss-of-function screen in a cell model of acquired TRAIL resistance

In order to perform a screen to identify novel mechanisms of TRAIL resistance, we selected the Burkitt's Lymphoma cell line, BJAB, which is inherently sensitive to TRAIL, and its TRAIL resistant sub-line, LexR. The BJAB LexR sub-line was previously made resistant to TRAIL-induced apoptosis via increasing exposure to Lexatumumab, an agonistic antibody to the functional TRAIL receptor, TNF, and has been previously characterized to show a marked increase in cell growth in the presence of Lexatumumab as compared to parental counterparts, BJAB-wild type (wt) cells (16). A cell viability assay confirmed that BJAB-LexR cells are significantly more resistant to TRAIL than are BJAB-wt cells (Figure 1A). Importantly, this induced resistance is specific to the TRAIL pathway, as the BJAB-LexR cells do not show increased resistance to other apoptosis inducing chemotherapeutics including doxorubicin or etoposide (Supplemental Figure 1A,B).

Using the BJAB system, we performed a genome-wide loss of function screen. To perform the screen, we introduced a library of lentiviral particles expressing shRNAs targeting the entire human genome to BJAB cells with forced resistance to LexR (BJAB-LexR) and then subjected the cells to either no treatment or TRAIL treatment (Figure 1B). In this screen, over-representation of a particular shRNA after TRAIL treatment implies that the target gene promotes TRAIL-induced apoptosis and, conversely, an under-representation of a certain shRNA after TRAIL treatment implies that the target promotes resistance against TRAIL-induced apoptosis. As shown in Figure 1C, there were clear differences in the representation of different shRNAs before and after TRAIL treatment, indicating that selection of certain shRNAs has, in fact, taken place. After analysis of the altered shRNAs in the BJAB-LexR TRAIL treated and untreated cells, we identified 580 candidate resistance genes that were within the statistical cut-off, $E<2$. We selected 182 candidates from this gene list, and constructed a secondary library consisting of pooled shRNAs (2–5 shRNAs per gene) specifically targeting these genes. The top candidates confirmed in this secondary screen were then validated using specific shRNAs. We confirmed that knock down of Angiotensin II Receptor 2 (AGTR2), Crk-like protein (CRKL), T-Box Transcription Factor 2 (TBX2) and solute carrier family 26 (anion exchanger), member 2 (SLC26A2) in LexR cells (Figure 2A–D) significantly reduced TRAIL resistance (Figure 2E–H) with the reduction of resistance correlating to the level of knockdown seen with the two shRNAs.

Novel TRAIL resistance genes identified in a lymphoma model also mediate resistance in breast cancer

Next, we asked whether the effect of AGTR2, CRKL-1, TBX-2 and SLC26A2 on TRAIL resistance were unique to BJAB cells or if similar results could be observed in other tumor types. To this end, we developed a similar model of TRAIL resistance in MDA-MB-231 breast cancer cells through long term exposure to increasing concentrations of a recombinant TRAIL ligand, generating a TRAIL resistant sub-line, 231-TRAILR. 231-TRAILR cells are resistant to TRAIL specifically, but are not generally resistant to apoptosis because they retain sensitivity to doxorubicin and etoposide, similar to that of the parental cells (Figure 3A, Supplemental Figure 2A,B). In concordance with what we found in the BJAB-LexR system, knocking down AGTR2, CRKL, TBX2 and SLC26A2 (Figure 3B–E) all rendered the MDA-MB-231-TRAILR breast cancer cells significantly more sensitive to TRAIL-induced apoptosis (Figure 3F–I).

Of the genes identified, the sulfate transporter, SLC26A2, had the most robust effect when assessed across both systems. This gene is understudied in the context of cancer or drug resistance, and thus we decided to pursue it further. To better explore the mechanism by which SLC26A2 mediates resistance in breast cancer cells, we established clonal isolate sub-lines of the 231-TRAILR line with stable knock down of SLC26A2 expression using two different shRNA constructs. shRNA1 (sh1) targets SLC26A2 in the 3′UTR of the gene, while shRNA2 (sh2) targets the coding region. Knock down (KD) of SLC26A2 with either of these constructs selectively enhanced TRAIL sensitivity in 231-TRAILR cells, but did not affect sensitivity to other general chemotherapeutics including doxorubicin and etoposide (Figure 4A–B, Supplemental Figure 3A–B). As a test of the specificity of the knockdown, we rescued SLC26A2 expression with a construct containing only the coding sequence. This construct restored TRAIL resistance in the cells expressing the shRNA1 knockdown since it lacks the 3′UTR and thus is resistant to shRNA1 knockdown, but did not restore resistance in cells expressing the shRNA2 construct that targets the coding region (Figure 4A,B), thus demonstrating that the knock-down of SLC26A2 is sequence-specific. Taken together, these data demonstrate that SLC26A2 is a novel mediator of TRAIL resistance.

Knock down of SLC26A2 leads to enhanced expression of DR4 and DR5

To determine if the increase in viability observed with SLC26A2 KD in LEXR cells was due to cell death, we stained BJAB-LEXR cells with propidium iodine, a nuclear stain that is excluded by live cells and therefore indicative of late-apoptotic or necrotic cells. Uptake of PI, corresponding to cell death was quantified by flow cytometry. Cell death was dramatically increased in both BJAB-SLC26A2 KD lines as compared to the non-targeting control line (Figure 5A). In the adherent MDA-MB-231 cell lines, we performed an activated-caspase 3/7 assay by measuring green fluorescence with Incucyte live cell imaging after the addition of CellEvent caspase-3/7 and TRAIL. Indeed, we observed a significant increase in activated caspase 3/7 in the MDA-MB-231 TRAILR SLC26A2 KD lines as compared to the non-targeting control (Figure 5B). Taken together, these results indicate that the decrease of viable cells observed with loss of SLC26A2 is due to increased apoptosis in TRAIL resistant cell lines.

To understand mechanistically how SLC26A2 could affect cell death, we performed gene expression analysis in the MDA-MB-231 SENS and TRAILR cell lines and cross referenced this data with that of our shRNA screen. This analysis showed increased SLC26A2 expression in the MDA-MB-231-TRAILR cell line as compared to its sensitive counterpart (Figure 5C). Interestingly, we also noted that expression of the TRAIL receptors, DR4 and DR5, were decreased in the array analyses (Figure 5C). The increase of SLC26A2 and concomitant decrease of DR4 and DR5 expression in MDA-MB-231-TRAILR cells when compared to their sensitive counterparts was confirmed using quantitative real time PCR (qRT-PCR) (Figure 5D–F) as well as Western blot analysis (Figure 5G). Importantly, there was also reduced surface protein expression of DR4 and DR5 as assessed by flow cytometry in the MDA-MB-231-TRAILR cells as compared the SEN cells (Figure 5H–I). These results were confirmed in the BJAB cell system, where we again observed an increase in SLC26A2 mRNA expression and a corresponding decrease in surface protein expression of DR4 and DR5 in the BJAB-LexR cells as compared to the BJAB-wt cells (Supplemental Figure 4A–C).

Because knockdown of SLC26A2 reversed resistance to TRAIL and because downregulation of DR4 and DR5, crucial mediators of TRAIL apoptosis, is associated with acquired TRAIL resistance, we next assessed whether TRAIL sensitivity, SLC26A2 expression and expression of DR4 and DR5 were linked. To this end, we analyzed the expression of these receptors in MDA-MB-231-TRAILR sub-lines with stable SLC26A2 knockdown. Notably, TRAIL resistant MDA-MB-231 cells transduced with non-targeting shRNA had approximately the same DR4 and DR5 expression as non-transduced TRAIL resistant cells. However, in MDA-MB-231-TRAILR cells transduced with shRNAs targeting SLC26A2, the mRNA, protein in whole cell lysates, and surface protein levels of DR4 and DR5 were restored (Figure 5H–I). Together, these data demonstrate that SLC26A2 is regulating the overall levels of DR4 and DR5, and more importantly, surface levels of DR4 and DR5.

SLC26A2 expression correlates with worsened disease in human tumors

Because many tumors are known to be resistant to TRAIL, we asked whether the levels of SLC26A2 may be elevated in human tumors, as a means to induce TRAIL resistance. Indeed, numerous public gene expression datasets, spanning multiple tumor types, show a significant increase in SLC26A2 expression in tumor as compared to normal tissue (Table 1) (30–34) (35–40). In addition, elevated SLC26A2 expression correlates with metastasis or worsened prognosis in numerous tumor types (41–47). Importantly, in a cohort of 1000 untreated breast cancer patients (taken from multiple studies using kmplot)(26) high SLC26A2 expression correlated with a significant decrease in relapse free survival and almost significant decrease in distant metastasis free survival (Figure 6, Supplemental Figure 5). Together, these data demonstrate that SLC26A2 is a marker for cancer versus normal as well as an indicator for poor prognosis in breast cancer and other cancers, and suggest that it may regulate tumor progression at least in part via its ability to mediate TRAIL resistance.

Discussion

Much enthusiasm initially surrounded the promise of TRAIL agonist therapy as a specific means to target cancers. However, the lack of anticancer activity in a significant number of patients displayed during phase II clinical trials has been disappointing(5). The last decade has revealed insight into multiple missteps that occurred with the initial TRAIL agonist therapies, most specifically that because there are many routes of resistance that tumors can acquire to escape death induced by TRAIL, it is critical to both develop strategies to circumvent resistance and identify up front the most likely responders for successful use of TRAIL receptor-targeted therapies. Despite this new understanding, neither a successful combination therapy nor the use of biomarkers for TRAIL sensitivity, to identify the patients who are likely to benefit, have moved into clinical trials.

In order to find novel resistance markers and obtain a more general view of TRAIL resistance mechanisms beyond the known mechanisms that generally involve direct participants in the core TRAIL pathway, we performed a genome wide shRNA loss of function screen in TRAIL resistant cell lines (Figure 1). This unbiased approach allowed for the discovery of unique genes that were not previously linked to TRAIL signaling or drug resistance in general. Using gene specific shRNAs in two different cell line systems (encompassing different tumor types), we confirmed that downregulation of four specific genes re-sensitize resistant cell lines to TRAIL agonist therapy (AGTR2, CRKL, TBX2, and SLC26A2, Figure 2–3). Importantly none of these genes are involved in the core TRAIL signaling pathway, nor are they general regulators of apoptosis, emphasizing the ability of the genome wide unbiased screen to find new unanticipated regulators of TRAIL resistance/sensitivity.

Interestingly, we discovered a connection between these seemingly unrelated TRAIL resistance genes discovered in our screen. This connection was discovered by performing a genome-scale integrated analysis of gene networks (GIANT(29)) across all tissues. Restricting the analysis to highly significant relationships (relationship confidence >0.5) with a maximum number of 20 genes to form the network, we found that all four genes are connected (Figure 7a) through a functional network that is significantly associated with a number of pathways, processes, and diseases, the majority of which are relevant to cancer (Supplemental Table 1). Interestingly, microarray analysis of BJAB WT and LEXR TRAIL treated samples as well as MDA-231 SENS and TRAILR TRAIL treated samples, followed by gene set enrichment analysis (GSEA) of significantly changed genes reveals that a number of similar pathways are enriched (Supplemental Table 2 and 3). In particular, the ErbB/EGFR signaling pathway (or KEGG pathways that contain large portions of this pathway) is significantly associated with the functional network created between the four confirmed genes, as well as in the microarray analyses from both the BJAB and MDA-MB-231 cell lines (Figure 7B–C). These results strongly suggest that ErbB signaling may be relevant for TRAIL resistance. Importantly, downregulation of ErbB/EGFR has been previously associated with increasing TRAIL sensitivity. Specifically, decreased expression of ErbB with either Trastuzumab or antisense oligodeoxynucleotides sensitizes ErbB overexpressing breast and ovarian cells to TRAIL mediated apoptosis (48). Additional studies have mechanistically linked EGFR to TRAIL resistance via the Bcl-2 family member

myeloid cell leukemia 1 (Mcl-1)(49) as well as cytochrome C release and caspase 3-like activation(50).

While numerous potential TRAIL resistance genes were identified in our screen, here we focused on the anion exchange channel SLC26A2. We demonstrate that knocking down this gene, which has never been previously been implicated in cancer drug sensitivity, significantly re-sensitizes resistant cells to TRAIL agonist therapy in both lymphoma cells and breast cancer cells. The effect was not a general pro-apoptotic effect since apoptosis induced by other apoptosis-inducing agents were not affected. Moreover, when SLC26A2 was reintroduced into cells with knocked down SLC26A2, the cells became re-sensitized to TRAIL-induced apoptosis indicating that expression of this gene is both necessary and sufficient to confer selective TRAIL resistance. Expression of SLC26A2 led to downregulation of the death receptors DR4 and DR5, suggesting a plausible mechanism by which SLC26A2 counteracts TRAIL-induced apoptosis.

While SLC26A2 has not previously been associated with pro-tumorigenic phenotypes, examination of tumor microarray datasets reveals increased expression of SLC26A2 in tumor tissue as compared to normal tissue across multiple tumor types including Wilms tumor, glioblastoma, lymphoma, ovarian, breast, hepatocellular, skin and renal carcinoma (Table 1)(30–47). Furthermore, other datasets show a correlation between elevated SLC26A2 expression and worsened prognosis or metastasis, particularly in glioblastoma, melanoma, breast and lung cancer (Table 1, Figure 6). As SLC26A2 is understudied, there are no known specific inhibitors to block this channel, although other anion channel inhibitors have already moved into clinical trials for the treatment of cancer, suggesting that targeting SLC26A2 may be feasible(51).

TRAIL receptor-targeted therapy has so far been disappointing in the clinic, underscoring how inadequate understanding of potential resistance mechanisms prior to starting clinical studies can contribute to the failure of targeted therapies(52). Innovative, genome wide studies, such as that described here, provide a way to gain a comprehensive understanding of potential resistance mechanisms that cancer cells can employ to evade TRAIL induced apoptosis, or to evade apoptosis induced by other targeted agents. Additionally, these approaches can also uncover strategic combinational therapies that could synergize with TRAIL agonist therapies in clinic. While the majority of patients seem unresponsive to TRAIL monotherapies, survival curves indicate that a small percentage of patients do benefit. Development of a panel of strategic molecular biomarkers using the approaches described here may allow a way to better identify those patients who may benefit from TRAIL receptor-targeted therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J Biol Chem. 1996 May 31; 271(22):12687–90. [PubMed: 8663110]
2. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity. 1995 Dec; 3(6):673–82. [PubMed: 8777713]
3. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest. 1999 Jul; 104(2):155–62. [PubMed: 10411544]
4. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med. 1999 Feb; 5(2):157–63. [PubMed: 9930862]
5. Dimberg LY, Anderson CK, Camidge R, Behbakht K, Thorburn A, Ford HL. On the TRAIL to successful cancer therapy? Predicting and counteracting resistance against TRAIL-based therapeutics. Oncogene. 2013 Mar 14; 32(11):1341–50. [PubMed: 22580613]
6. von Pawel J, Harvey JH, Spigel DR, Dediu M, Reck M, Cebotaru CL, et al. Phase II trial of mapatumumab, a fully human agonist monoclonal antibody to tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1), in combination with paclitaxel and carboplatin in patients with advanced non-small-cell lung cancer. Clin Lung Cancer. 2014 May; 15(3):188–96. e2. [PubMed: 24560012]
7. Trivedi R, Mishra DP. Trailing TRAIL Resistance: Novel Targets for TRAIL Sensitization in Cancer Cells. Front Oncol. 2015; 5:69. [PubMed: 25883904]
8. Gump JM, Staskiewicz L, Morgan MJ, Bamberg A, Riches DW, Thorburn A. Autophagy variation within a cell population determines cell fate through selective degradation of Fap-1. Nat Cell Biol. 2014 Jan; 16(1):47–54. [PubMed: 24316673]
9. Graff JR, Konicek BW, Carter JH, Marcusson EG. Targeting the eukaryotic translation initiation factor 4E for cancer therapy. Cancer Res. 2008 Feb 1; 68(3):631–4. [PubMed: 18245460]
10. Fan S, Li Y, Yue P, Khuri FR, Sun SY. The eIF4E/eIF4G interaction inhibitor 4EGI-1 augments TRAIL-mediated apoptosis through c-FLIP Down-regulation and DR5 induction independent of inhibition of cap-dependent protein translation. Neoplasia. 2010 Apr; 12(4):346–56. [PubMed: 20360945]
11. Johnson TR, Stone K, Nikrad M, Yeh T, Zong WX, Thompson CB, et al. The proteasome inhibitor PS-341 overcomes TRAIL resistance in Bax and caspase 9-negative or Bcl-xL overexpressing cells. Oncogene. 2003 Aug 7; 22(32):4953–63. [PubMed: 12902978]
12. Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K, et al. Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. Nat Med. 2007 Sep; 13(9):1070–7. [PubMed: 17767167]
13. Yang JK. FLIP as an anti-cancer therapeutic target. Yonsei Med J. 2008 Feb 29; 49(1):19–27. [PubMed: 18306465]
14. Cummins JM, Kohli M, Rago C, Kinzler KW, Vogelstein B, Bunz F. X-linked inhibitor of apoptosis protein (XIAP) is a nonredundant modulator of tumor necrosis factor-related apoptosis-

inducing ligand (TRAIL)-mediated apoptosis in human cancer cells. Cancer Res. 2004 May 1; 64(9):3006–8. [PubMed: 15126334]

15. Ng CP, Bonavida B. X-linked inhibitor of apoptosis (XIAP) blocks Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis of prostate cancer cells in the presence of mitochondrial activation: sensitization by overexpression of second mitochondria-derived activator of caspase/direct IAP-binding protein with low pl (Smac/DIABLO). Mol Cancer Ther. 2002 Oct; 1(12):1051–8. [PubMed: 12481428]
16. Menke C, Bin L, Thorburn J, Behbakht K, Ford HL, Thorburn A. Distinct TRAIL resistance mechanisms can be overcome by proteasome inhibition but not generally by synergizing agents. Cancer Res. 2011 Mar 1; 71(5):1883–92. [PubMed: 21363923]
17. Porter CC, Kim J, Fosmire S, Gearheart CM, van Linden A, Baturin D, et al. Integrated genomic analyses identify WEE1 as a critical mediator of cell fate and a novel therapeutic target in acute myeloid leukemia. Leukemia. 2012 Jun; 26(6):1266–76. [PubMed: 22289989]
18. Menke C, Goncharov T, Qamar L, Korch C, Ford HL, Behbakht K, et al. TRAIL receptor signaling regulation of chemosensitivity in vivo but not in vitro. PLoS One. 2011; 6(1):e14527. [PubMed: 21264287]
19. Kim J, Tan AC. BiNGS!SL-seq: a bioinformatics pipeline for the analysis and interpretation of deep sequencing genome-wide synthetic lethal screen. Methods Mol Biol. 2012; 802:389–98. [PubMed: 22130895]
20. Spreafico A, Tentler JJ, Pitts TM, Tan AC, Gregory MA, Arcaroli JJ, et al. Rational combination of a MEK inhibitor, selumetinib, and the Wnt/calcium pathway modulator, cyclosporin A, in preclinical models of colorectal cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2013 Aug 1; 19(15):4149–62. [PubMed: 23757356]
21. Sullivan KD, Padilla-Just N, Henry RE, Porter CC, Kim J, Tentler JJ, et al. ATM and MET kinases are synthetic lethal with nongenotoxic activation of p53. Nat Chem Biol. 2012 Jul; 8(7):646–54. [PubMed: 22660439]
22. Casas-Selves M, Kim J, Zhang Z, Helfrich BA, Gao D, Porter CC, et al. Tankyrase and the canonical Wnt pathway protect lung cancer cells from EGFR inhibition. Cancer Res. 2012 Aug 15; 72(16):4154–64. [PubMed: 22738915]
23. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009; 10(3):R25. [PubMed: 19261174]
24. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010 Jan 1; 26(1):139–40. [PubMed: 19910308]
25. Bonafe L, Hastbacka J, de la Chapelle A, Campos-Xavier AB, Chiesa C, Forlino A, et al. A novel mutation in the sulfate transporter gene SLC26A2 (DTDST) specific to the Finnish population causes de la Chapelle dysplasia. Journal of medical genetics. 2008 Dec; 45(12):827–31. [PubMed: 18708426]
26. Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat. 2010 Oct; 123(3):725–31. [PubMed: 20020197]
27. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003 Apr; 4(2):249–64. [PubMed: 12925520]
28. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 2005 Oct 25; 102(43):15545–50. [PubMed: 16199517]
29. Greene CS, Krishnan A, Wong AK, Ricciotti E, Zelaya RA, Himmelstein DS, et al. Understanding multicellular function and disease with human tissue-specific networks. Nat Genet. 2015 Jun; 47(6):569–76. [PubMed: 25915600]
30. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature. 2007 Oct 4; 449(7162):557–63. [PubMed: 17914389]

31. Sun L, Hui AM, Su Q, Vortmeyer A, Kotliarov Y, Pastorino S, et al. Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain. Cancer cell. 2006 Apr; 9(4):287–300. [PubMed: 16616334]
32. Piccaluga PP, Agostinelli C, Califano A, Rossi M, Basso K, Zupo S, et al. Gene expression analysis of peripheral T cell lymphoma, unspecified, reveals distinct profiles and new potential therapeutic targets. The Journal of clinical investigation. 2007 Mar; 117(3):823–34. [PubMed: 17304354]
33. Lu KH, Patterson AP, Wang L, Marquez RT, Atkinson EN, Baggerly KA, et al. Selection of potential markers for epithelial ovarian cancer with gene expression arrays and recursive descent partition analysis. Clinical cancer research : an official journal of the American Association for Cancer Research. 2004 May 15; 10(10):3291–300. [PubMed: 15161682]
34. Tomlins SA, Mehra R, Rhodes DR, Cao X, Wang L, Dhanasekaran SM, et al. Integrative molecular concept modeling of prostate cancer progression. Nature genetics. 2007 Jan; 39(1):41–51. [PubMed: 17173048]
35. Wurmbach E, Chen YB, Khitrov G, Zhang W, Roayaie S, Schwartz M, et al. Genome-wide molecular profiles of HCV-induced dysplasia and hepatocellular carcinoma. Hepatology. 2007 Apr; 45(4):938–47. [PubMed: 17393520]
36. Beroukhim R, Brunet JP, Di Napoli A, Mertz KD, Seeley A, Pires MM, et al. Patterns of gene expression and copy-number alterations in von-hippel lindau disease-associated and sporadic clear cell carcinoma of the kidney. Cancer research. 2009 Jun 1; 69(11):4674–81. [PubMed: 19470766]
37. Riker AI, Enkemann SA, Fodstad O, Liu S, Ren S, Morris C, et al. The gene expression profiles of primary and metastatic melanoma yields a transition point of tumor progression and metastasis. BMC medical genomics. 2008; 1:13. [PubMed: 18442402]
38. Yusenko MV, Kuiper RP, Boethe T, Ljungberg B, van Kessel AG, Kovacs G. High-resolution DNA copy number and gene expression analyses distinguish chromophobe renal cell carcinomas and renal oncocytomas. BMC cancer. 2009; 9:152. [PubMed: 19445733]
39. Murat A, Migliavacca E, Gorlia T, Lambiv WL, Shay T, Hamou MF, et al. Stem cell-related "self-renewal" signature and high epidermal growth factor receptor expression associated with resistance to concomitant chemoradiotherapy in glioblastoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008 Jun 20; 26(18):3015–24. [PubMed: 18565887]
40. French PJ, Swagemakers SM, Nagel JH, Kouwenhoven MC, Brouwer E, van der Spek P, et al. Gene expression profiles associated with treatment response in oligodendrogliomas. Cancer research. 2005 Dec 15; 65(24):11335–44. [PubMed: 16357140]
41. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, et al. Genes that mediate breast cancer metastasis to lung. Nature. 2005 Jul 28; 436(7050):518–24. [PubMed: 16049480]
42. Weigelt B, Glas AM, Wessels LF, Witteveen AT, Peterse JL, van't Veer LJ. Gene expression profiles of primary breast tumors maintained in distant metastases. Proceedings of the National Academy of Sciences of the United States of America. 2003 Dec 23; 100(26):15901–5. [PubMed: 14665696]
43. Nutt CL, Mani DR, Betensky RA, Tamayo P, Cairncross JG, Ladd C, et al. Gene expression-based classification of malignant gliomas correlates better with survival than histological classification. Cancer research. 2003 Apr 1; 63(7):1602–7. [PubMed: 12670911]
44. Beer DG, Kardia SL, Huang CC, Giordano TJ, Levin AM, Misek DE, et al. Gene-expression profiles predict survival of patients with lung adenocarcinoma. Nature medicine. 2002 Aug; 8(8): 816–24.
45. Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, et al. X chromosomal abnormalities in basal-like human breast cancer. Cancer cell. 2006 Feb; 9(2):121–32. [PubMed: 16473279]
46. Network CGA. Comprehensie molecular characterization of human colon and rectal cancer. Nature. 2012; 487(7407):330–7. [PubMed: 22810696]
47. Xu L, Shen SS, Hoshida Y, Subramanian A, Ross K, Brunet JP, et al. Gene expression changes in an animal melanoma model correlate with aggressiveness of human melanoma metastases. Molecular cancer research : MCR. 2008 May; 6(5):760–9. [PubMed: 18505921]

48. Cuello M, Ettenberg SA, Clark AS, Keane MM, Posner RH, Nau MM, et al. Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2. Cancer Res. 2001 Jun 15; 61(12):4892–900. [PubMed: 11406568]

49. Henson ES, Gibson EM, Villanueva J, Bristow NA, Haney N, Gibson SB. Increased expression of Mcl-1 is responsible for the blockage of TRAIL-induced apoptosis mediated by EGF/ErbB1 signaling pathway. J Cell Biochem. 2003 Aug 15; 89(6):1177–92. [PubMed: 12898516]

50. Gibson EM, Henson ES, Haney N, Villanueva J, Gibson SB. Epidermal growth factor protects epithelial-derived cells from tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by inhibiting cytochrome c release. Cancer Res. 2002 Jan 15; 62(2):488–96. [PubMed: 11809700]

51. Lang F, Stournaras C. Ion channels in cancer: future perspectives and clinical potential. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2014 Mar 19.369(1638):20130108. [PubMed: 24493756]

52. Lemke J, von Karstedt S, Zinngrebe J, Walczak H. Getting TRAIL back on track for cancer therapy. Cell Death Differ. 2014 Sep; 21(9):1350–64. [PubMed: 24948009]

53. Gyorffy B, Surowiak P, Budczies J, Lanczky A. Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer. PloS one. 2013; 8(12):e82241. [PubMed: 24367507]

Implication

Our results shed light on novel resistance mechanisms that could affect the efficacy of TRAIL agonist therapies and highlight the possibility of using these proteins as biomarkers to identify TRAIL resistance tumors, or as potential therapeutic targets in combination with TRAIL.

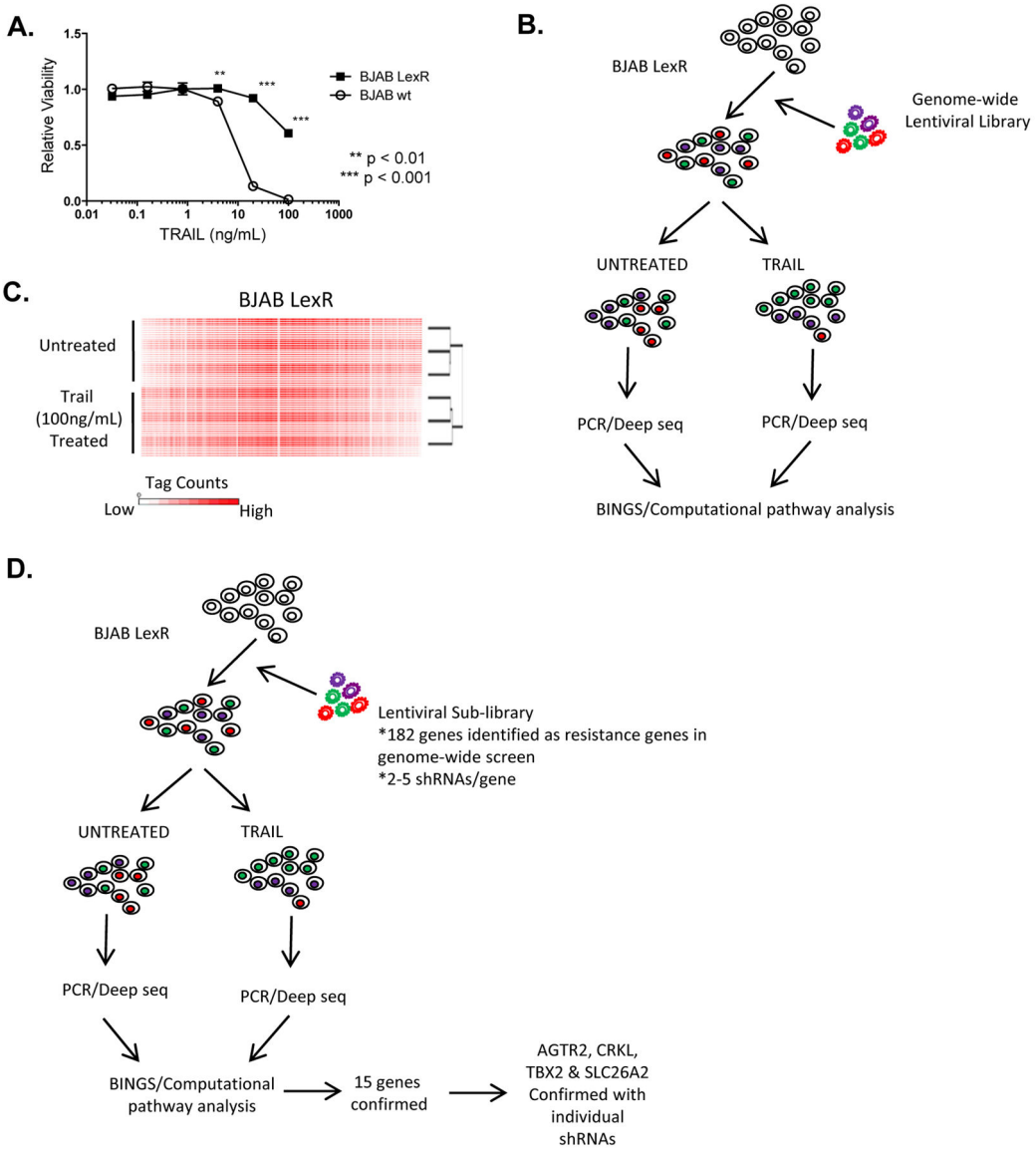

Figure 1. Genome wide loss-of-function shRNA screening of a TRAIL-resistant lymphoma cell line

Lymphoma cell lines BJAB-LexR (TRAIL resistant) were (A) treated with indicated concentrations of recombinant TRAIL for 24 hours. Relative viability compared to untreated cells was determined by CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay. Representative experiments are shown (of ≥ 3 experiments) with each experiment performed in triplicate. Statistical significance assessed by 2-way ANOVA with a Bonferroni post-test. (B) Schematic of shRNA screen: BJAB-LexR cells were transduced with a genome wide lentiviral shRNA library and treated with recombinant TRAIL (100 ng/ml) for 24 hours. (C) RNA was extracted after transduction with the shRNA library and pre and post-treatment with TRAIL and analyzed by deep sequencing. Results show clear differences in the shRNA representation between untreated and TRAIL treated BJAB-LexR cells. (D) Schematic of secondary shRNA screen: BJAB-LexR cells were treated with a lentiviral shRNA sub-library

and treated with recombinant TRAIL (100 ng/ml) for 24 hours. Hits confirmed from the primary and secondary screens were then individually tested with individual shRNAs.

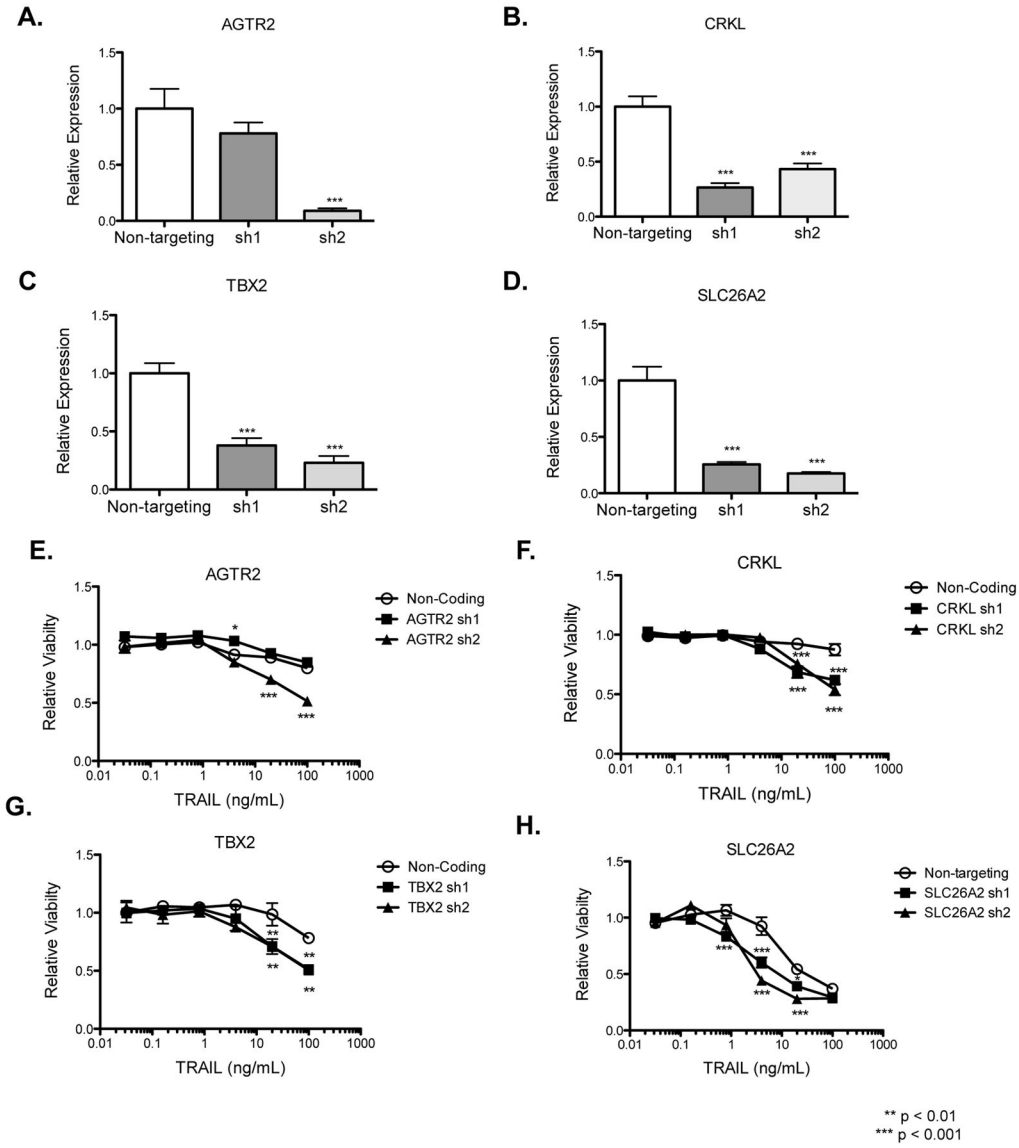

Figure 2. AGTR2, CRKL1, TBX2 and SLC26A2 mediate resistance to TRAIL in BJAB cells

TRAIL resistant BJAB-LexR cells were lentivirally transduced with either control, non-targeting shRNA vector (Non-Targeting) or with either of two different individual shRNAs (sh1, sh2) targeting (A) AGTR2, (B) CRKL1, (C) TBX2 and (D) SLC26A2. Knockdown was determined by real time quantitative PCR. The expression is relative to the expression of GAPDH and was normalized to non-targeting shRNA vector-expressing cells. Representative experiments are shown (of ≥ 3 experiments) with each experiment performed in triplicate. Statistical significance assessed by 2-way ANOVA with a Bonferroni post-test. BJAB-LexR cells transduced with either Non-Targeting shRNA vector or with either of two different individual shRNAs targeting (E) AGTR2, (F) CRKL1, (G) TBX2 and (H) SLC26A2 were treated with varying concentrations of recombinant TRAIL for 24 hours. Survival as a percentage of untreated cells was determined by CellTiter-Glo® Luminescent

Cell Viability Assay. Representative experiments are shown (of ≥ 3 experiments) with each experiment performed in triplicate. Statistical significance assessed by 2-way ANOVA.

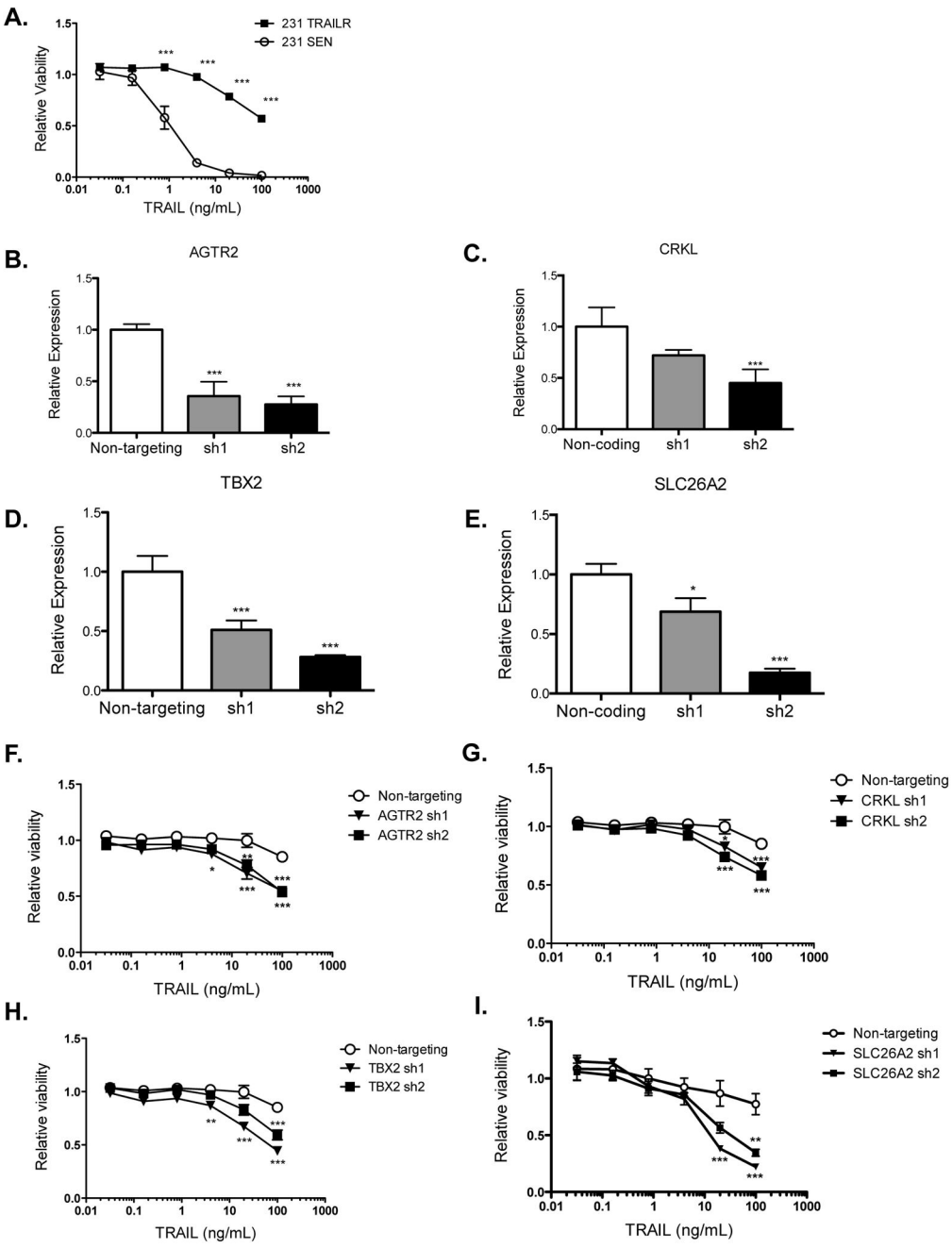

Figure 3. AGTR2, CRKL1, TBX2 and SLC26A2 mediate resistance to TRAIL in MDA-MB-231 breast cancer cells

(A) Parental, TRAIL sensitive MDA-MB-231 cells (231 SEN) and MDA-MB-231 cells made TRAIL resistant through long term culture in increasing concentrations of recombinant TRAIL (231 TRAILR) were treated at indicated concentrations of recombinant TRAIL for 24 hours. Relative viability compared to untreated cells was determined by CellTiter-Glo®Luminescent Cell Viability Assay. Representative experiments are shown (of ≥ 3 experiments) with each experiment performed in triplicate. Statistical significance assessed by 2-way ANOVA with a Bonferroni post-test. (B-E) 231 TRAILR cells were

lentivirally transduced with either control, non-targeting shRNA vector (Non-Targeting) or with either of two different individual shRNAs (sh1, sh2) targeting (B) AGTR2, (C) CRKL1, (D) TBX2 and (E) SLC26A2. Knockdown was determined by real time quantitative PCR. The expression is relative to the expression of GAPDH and was normalized to non-targeting shRNA vector-expressing cells. Representative experiments are shown (of ≥ 3 experiments) with each experiment performed in triplicate. Statistical significance assessed by 2-way ANOVA with a Bonferroni post-test. (F–I) 231 TRAILR cells transduced with either control, non targeting shRNA vector (Non-Targeting) or with either of two different individual shRNAs (sh1, sh2) targeting (F) AGTR2, (G) CRKL1, (H) TBX2 and (I) SLC26A2 were treated with varying concentrations of recombinant TRAIL for 24 hours. Survival as a percentage of untreated cells was determined by CellTiter-Glo® Luminescent Cell Viability Assay. Representative experiments are shown (of ≥ 3 experiments) with each experiment performed in triplicate. Statistical significance assessed by 2-way ANOVA.

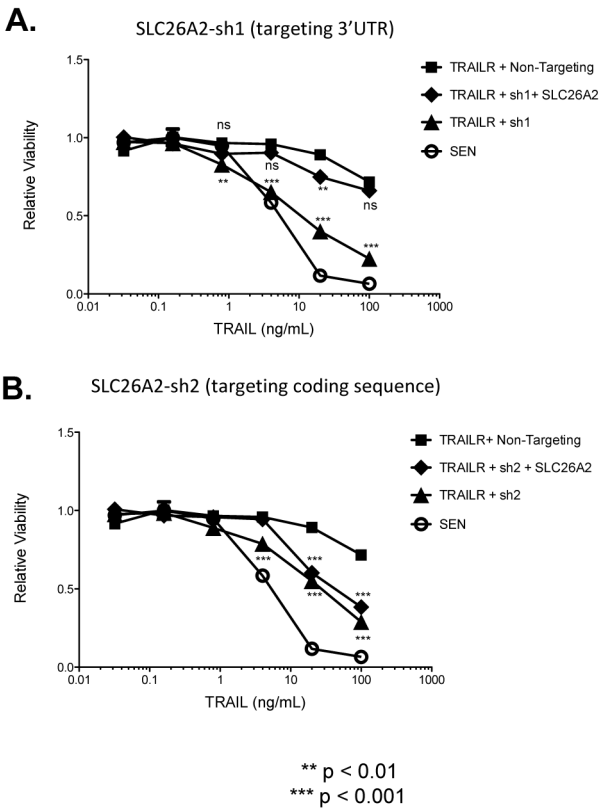

Figure 4. TRAIL resistant MDA-MB-231 are re-sensitized to TRAIL by knock-down of SLC26A2

231 SEN and 231 TRAILR cells transduced with non-targeting control shRNA or with (A) shRNA 1 that targets SLC26A2 in the 3′UTR of the gene (RES + sh1), such that the shRNA will not knock down exogenous SLC26A2 or with (B) shRNA 2 that targets SLC26A2 in the coding region and can target both exogenous and endogenous proteins were treated with indicated concentrations of recombinant TRAIL for 24 hours. Survival as a percentage of untreated cells was determined by CellTiter-Glo® Luminescent Cell Viability Assay. Representative experiments are shown (of ≥ 3 experiments) with each experiment performed in triplicate. Statistical significance assessed by 2-way ANOVA with a Bonferroni post-test.

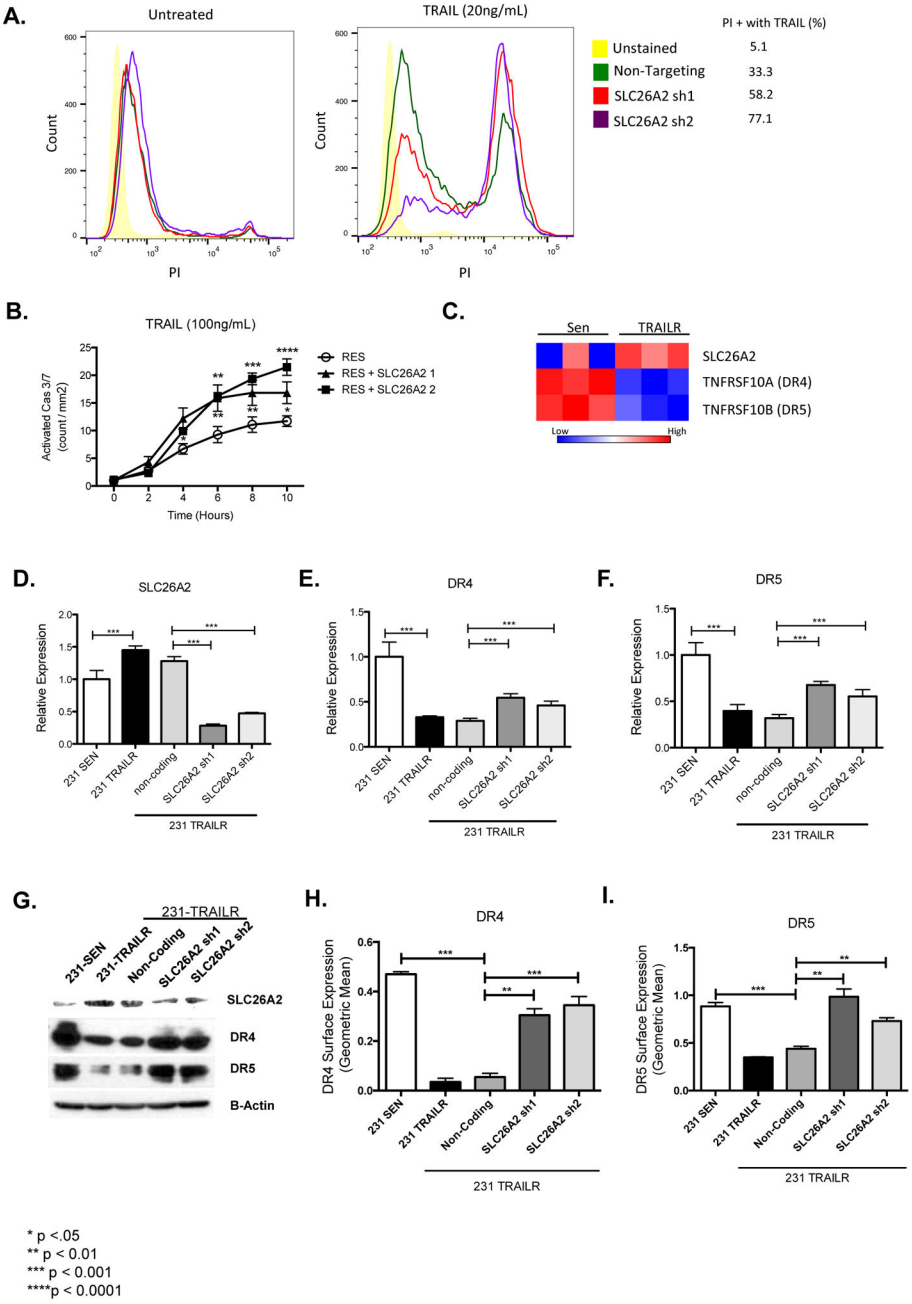

Figure 5. SLC26A2 regulates The TRAIL receptors, DR4 and DR5, in MDA-MB-231 cells

(A) Flow cytometry analysis in BJAB-LEXR cells with stable, lentiviral, shRNAs against SLC26A2 or a Non-targeting shRNA after 24hrs of treatment with TRAIL (20ng/mL) to assess propidium iodide (PI) positive cells as an indication of dead or late apoptotic cells. Representative experiment is shown (of 2 experiments). (B) MDA-231 SENS and TRAILR cells plated in replicates of 6 were treated with TRAIL (100ng/mL) and a green fluoregenic substrate for activated-caspase3/7. Green fluorescence was measured every 2 hours with 100ng/mL TRAIL by light microscopy using real time in-vitro imaging. Statistical significance assessed by 2-way ANOVA with a Tukey post-test. A representative figure is

shown (of 2 experiments). (C) Triplicate samples of 231 SEN and 231 TRAILR were analyzed for RNA expression of SLC26A2, DR4, and DR5, using microarray analysis. The expression is presented as a heat map relative to an average expression of a combination of constitutive housekeeping genes. RNA and protein were isolated from 231 SEN cells and 231 TRAILR cells as well as 231 TRAILR cells transduced with non-targeting control shRNA (non-coding) or with either of 2 shRNA targeting SLC26A2 (SLC26A2 sh1 and SLC26A2 sh2, respectively). mRNA expression of (D) SLC26A2, (E) DR4 and (F) DR5 was determined by real time quantitative PCR. The expression is relative to the expression of GAPDH and was normalized to non-targeting shRNA vector-expressing cells. Representative experiments are shown (of ≥ 3 experiments) with each experiment performed in triplicate. Statistical significance assessed by 1-way ANOVA. Protein expression of these genes as well as β-actin was analyzed by Western blot as shown in (G). Surface expression of DR4 and DR5 was analyzed by flow cytometry as depicted in (H) and (I), respectively. Representative experiments are shown (of ≥ 3 experiments). Statistical significance assessed by 1-way ANOVA.

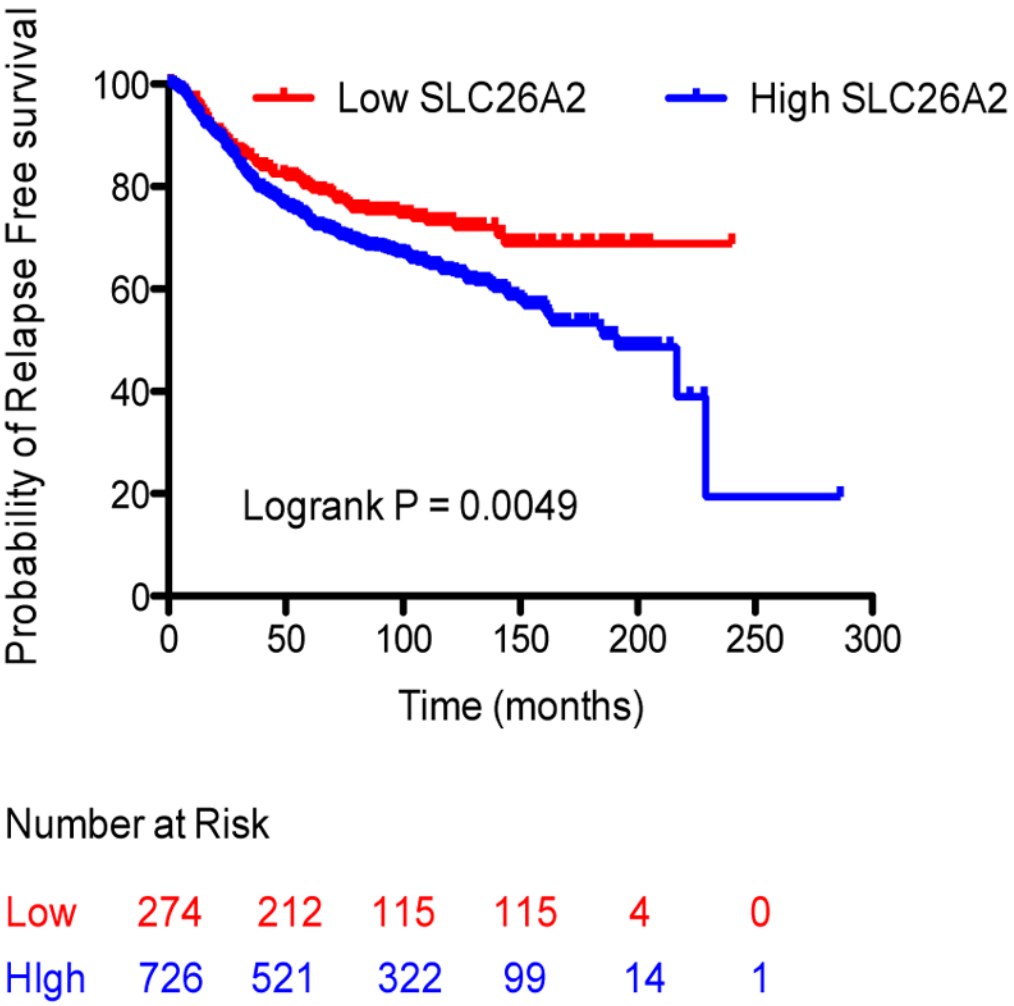

Figure 6. SLC26A2 expression correlates with worsened prognosis in breast cancer

With the use of kmplot(53), 1000 untreated breast cancer patients were divided into two groups with either SL26A2 expression above (high) or below (low) the median expression level. Patients with high SLC26A2 expression have a decreased probability of relapse free survival.

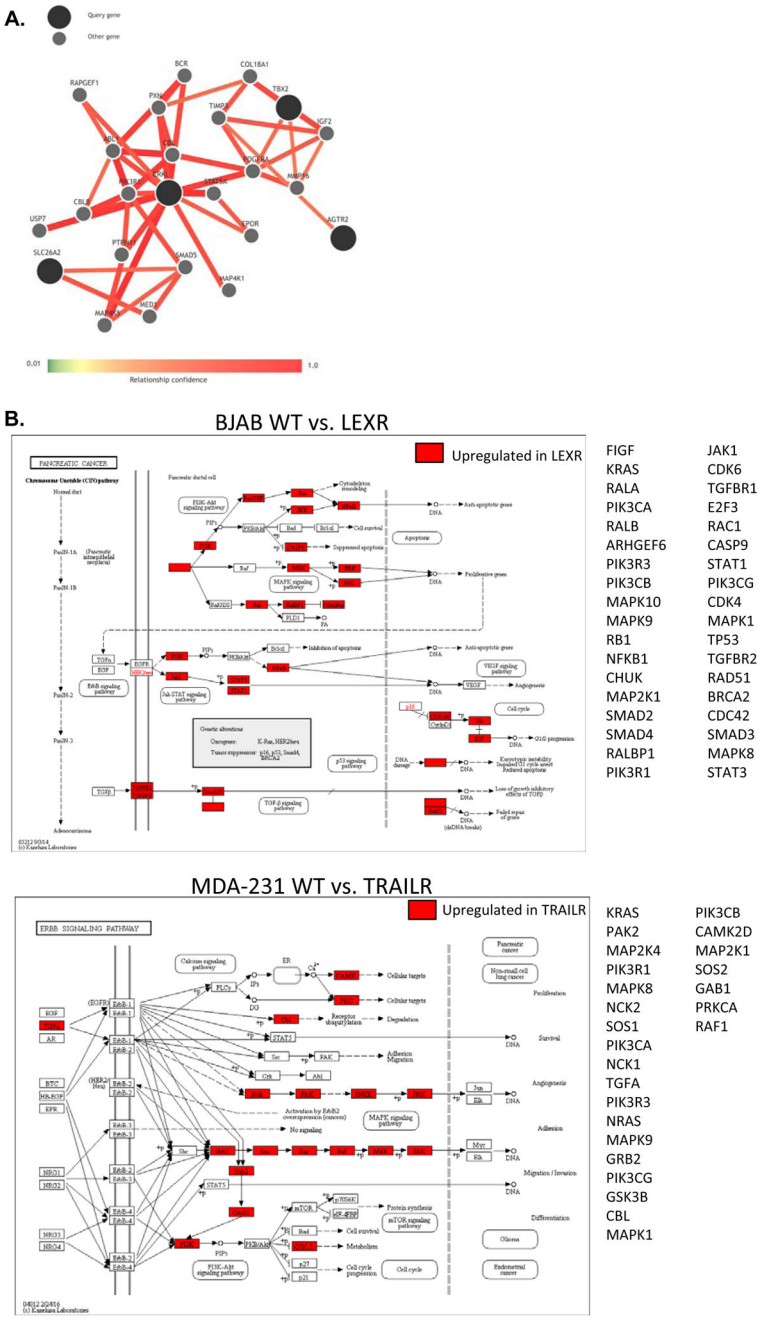

Figure 7. Pathway analysis identifies a functional network between AGTR2, CRKL, TBX2, and SLC26A2

(A) Use of a genome-scale integrated analysis of gene networks (GIANT(29)) with AGTR2, CRKL, TBX2, and SLC26A2 as inputs analyzed across all tissue types (relationship confidence > 0.5 and max number of genes set to 20). (B–C) Microarray analysis was performed on mRNA from (B) BJAB WT and LEXR samples and (C) MDA-231 SENS and TRAILR samples after treatment with TRAIL (100ng/mL), followed by GSEA analysis. (B) The KEGG Pancreatic signaling pathway (consisting mostly of ErbB/EGFR signaling pathways) is displayed with core genes upregulated (enriched) in the trail treated BJAB-

LEXR samples as compared to trail treated BJAB-WT samples in red. (C) The KEGG ErbB signaling pathway is displayed with core genes upregulated (enriched) in the trail treated MDA-231 TRAILR samples as compared to the trail treated MDA-231 SENS samples.

Table 1

SLC26A2 overexpression is correlated with worsened disease

Tumor Type	Differential Expression	Fold Change	P Value	Data Set
Breast	Metastatic Event at 1 Year vs. No event at 1 year	2.282	9.88E-10	Minn Breast 2
Breast	Metastatic Site vs. Primary Site	2.485	0.005	Weigelt Breast
Brain	Glioblastoma Dead at 5 years vs. Alive	2.531	6.02E-4	Nutt Brain
Lung	Lung Adenocarcioma-Advanced N stage	2.125	0.001	Beer Lung
Breast	Ductal Breast Carcinoma-ERBB2/ER/PR Negative vs. Positive	5.313	5.75E-6	Richardson Breast 2
Breast	Invasive Ductal Breast Carcinoma Stroma vs. Normal	2.214	8.23E-5	Karnoub Breast
Colon	Colon Adenocarcinoma Dead at 1 year vs. Alive	2.695	0.003	TCGA Colorectal
Brain	Glioblastoma vs. Normal	2.002	6.38E-18	Sun Brain
Lymphoma	Anaplastic Large Cell Lymphoma vs. Normal	5.641	2.79E-8	Piccaluga Lymphoma
Lymphoma	Angioimmunoblastiv T-cell Lymphoma vs. Normal	3.689	5.32E-8	Piccaluga Lymphoma
Lymphoma	Unspecified Peripheral T-cell Lymphoma vs. Normal	3.489	6.22E-13	Piccaluga Lymphoma
Ovarian	Ovarian Clear Cell Adenocarcinoma vs. Normal	2.353	8.41E-4	Lu Ovarain
Prostate	Prostatic Intraepithelial Neoplasia Epithelia vs. Normal	3.646	1.53E-4	Tomlins Prostate
Liver	Hepatocellular Carcinoma vs. Normal	3.239	5.64E-5	Wurmbach Liver
Renal	Non-Hereditary Clear Cell Renal Carcinoma vs. Normal	2.371	6.45E-7	Beroukhim Renal
Melanoma	Skin Basal Cell Carcinoma vs. Normal	2.337	2.15E-4	Riker Melanoma
Melanoma	Metastatic Site vs. Primary Site	2.079	5.01E-5	Xu Melanoma
Renal	Renal Wilms Tumor vs. Normal	2.210	0.004	Yusenko Renal
Brain	Glioblastoma vs. Normal	2.328	5.46E-5	Murat Brain
Brain	Anaplastic Oligodendroglioma vs. Normal	2.384	1.86E-5	French Brain