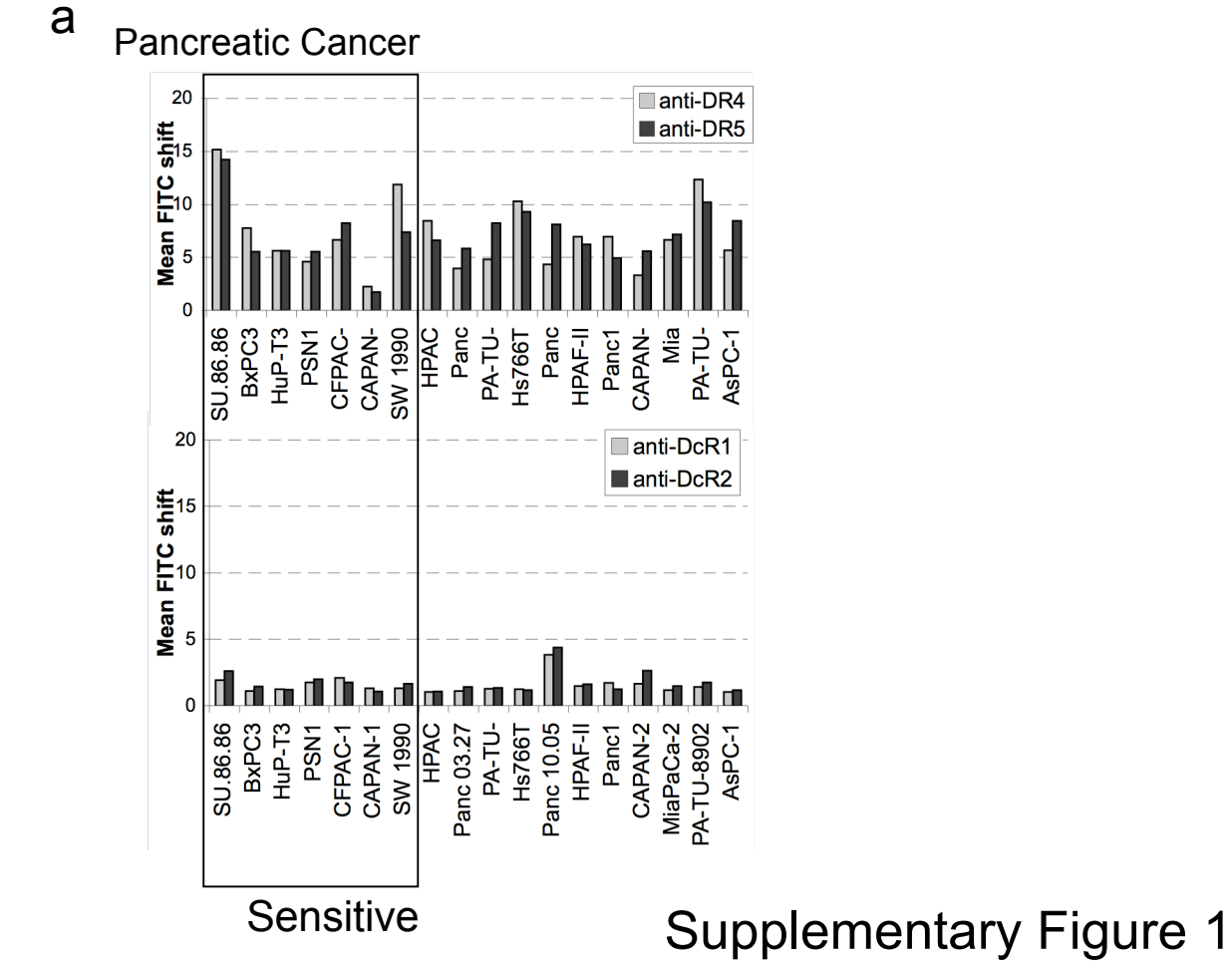
Death receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL.

Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K, Lee D, Von Goetz M, Yee SF, Totpal K, Huw L, Katta V, Cavet G, Hymowitz SG, Amler L and Ashkenazi A.

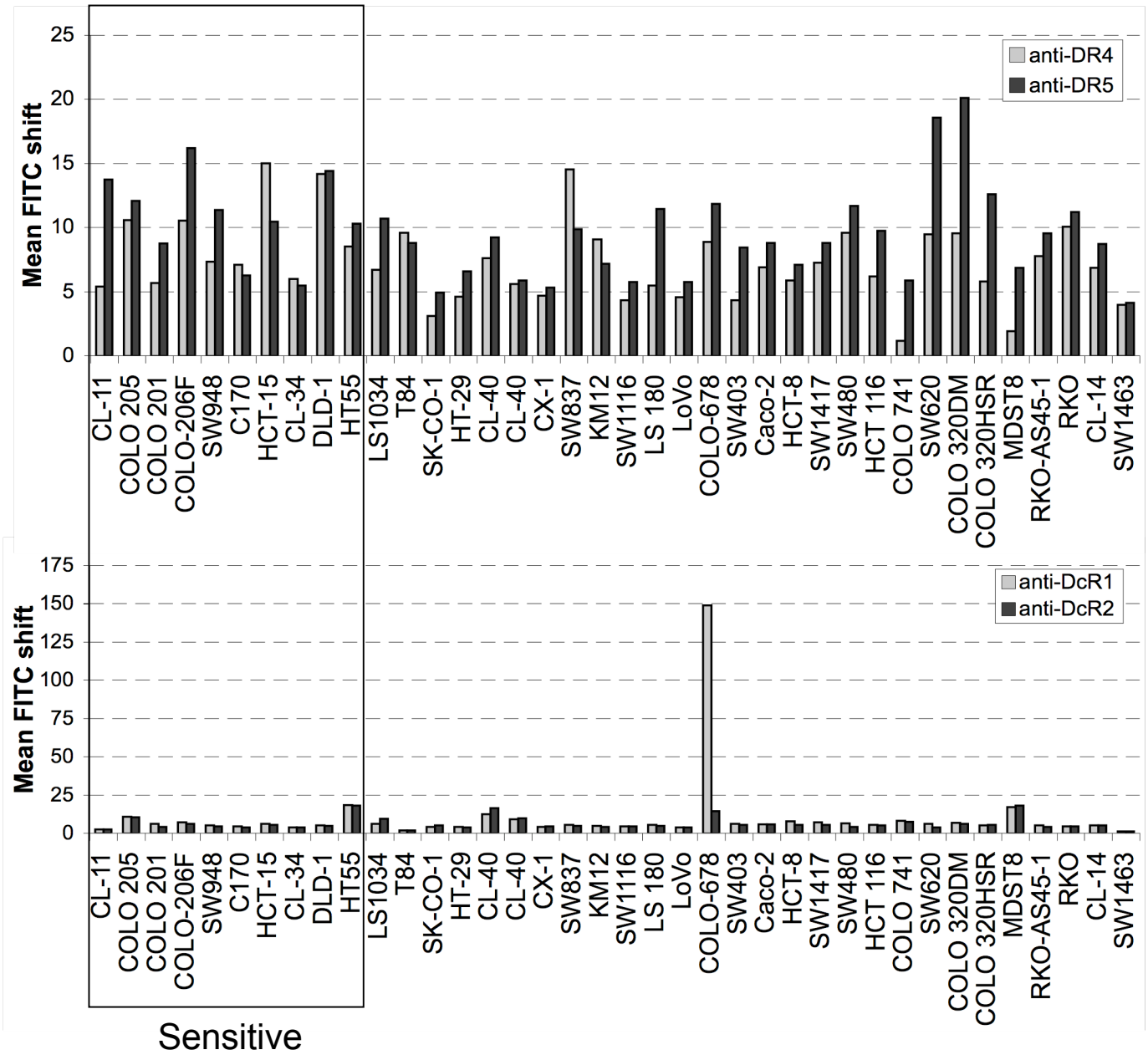
Supplementary Figure 1: Sensitivity to Apo2L/TRAIL in cancer cell lines is not widely correlated to expression of recptors and intracellular anti-apoptotic factors.
(**a**) Cell surface expression of DR4 and DR5 (top panel) and DcR1 and DcR2 (bottom panel) was measured by FACS analysis in the pancreatic cell line panel. Black box denotes Apo2L/TRAIL sensitive cell lines. (**b**) Receptor expression was measured in the colorectal cancer cell line panel as described in **a**. (**c**) Western blot analysis comparing expression of anti-apoptotic molecules (c-FLIP, Bcl-2, Mcl-1, Bcl-XL, XIAP) and GALNT14 in lysates from Apo2L/TRAIL sensitive and resistant pancreatic cancer cell lines. (**d**) Comparison of anti-apoptotic molecules and FUT6 expression in colorectal cancer cell lines, as described in **c**. Comparison of proliferation rates between Apo2L/TRAIL sensitive and resistant cell lines by counting cells *in vitro* (**e**) or measuring tumor doubling time *in vivo* (**f**). P-values indicate no significant difference ($p>0.05$) between sensitive and resistant cells as evaluated by a student t-test.



Supplementary Figure 1

b

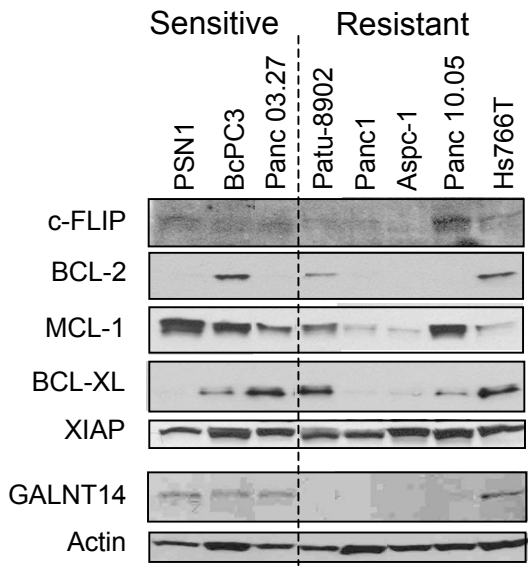
Colorectal Cancer



Supplementary Figure 1

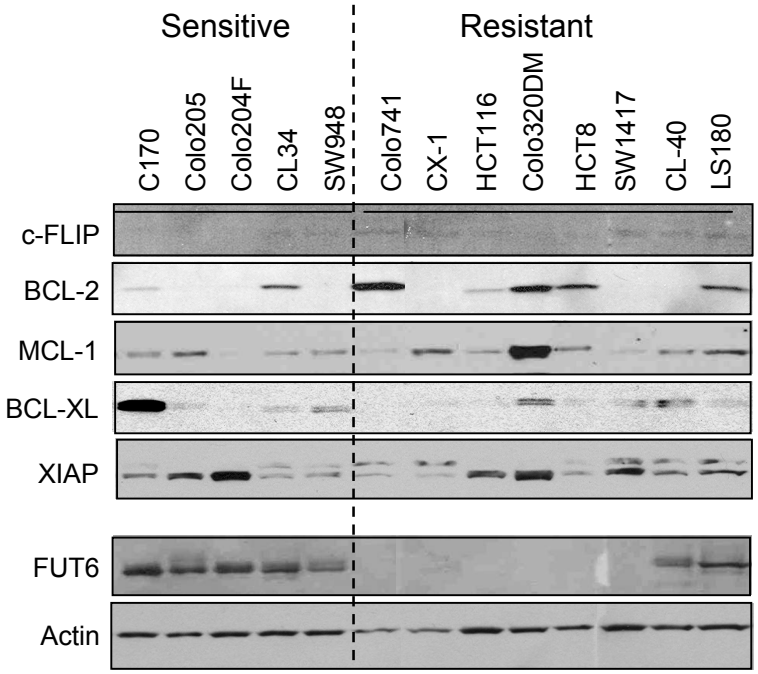
c

Pancreatic Cancer



d

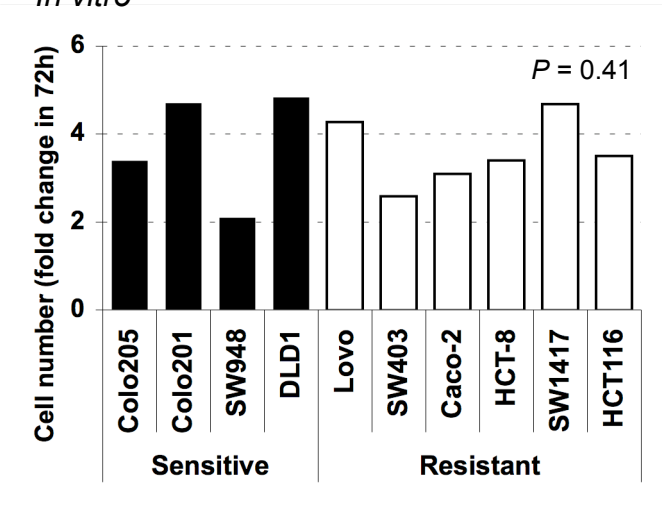
Colorectal Cancer



Supplementary Figure 1

e

In vitro



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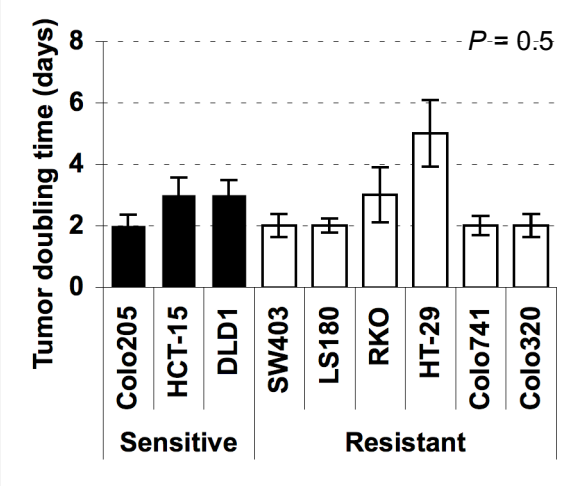
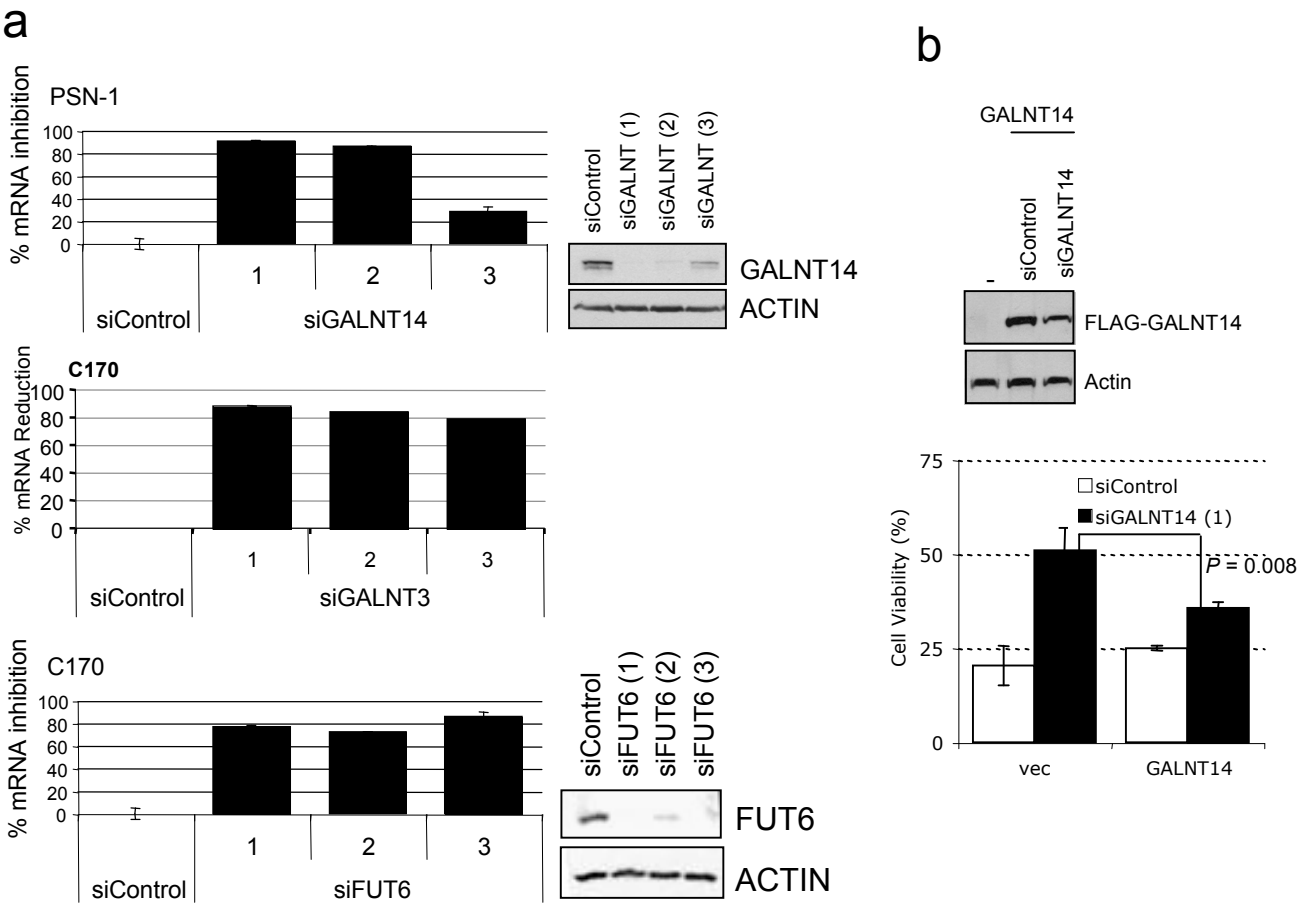
In vivo

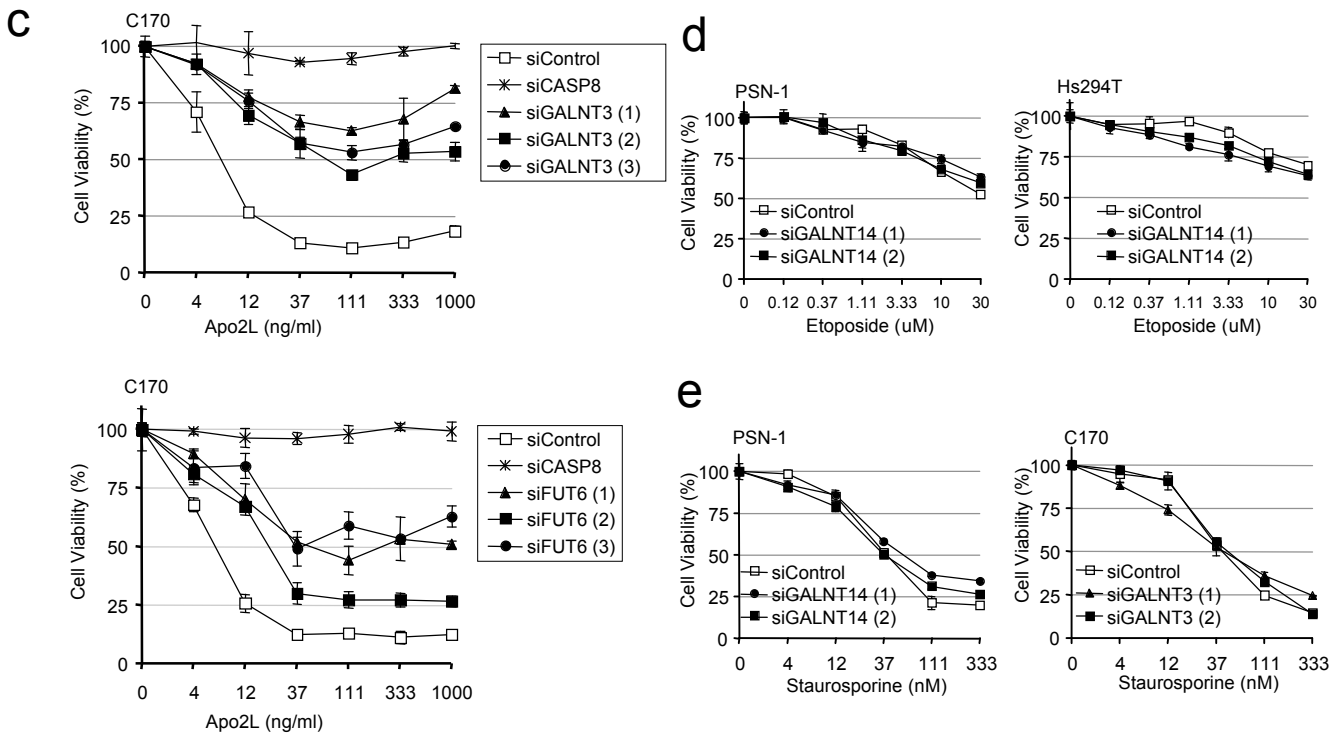


Supplementary Figure 1

Supplemetary Figure 2: RNA interference of specific O-glycosylation enzymes selectively alters sensitivity to Apo2L/TRAIL.
(**a**) The mRNA expression of *GALNT14*, *GALNT3* or *FUT6* was determined in PSN-1 or DLD-1 cells after 48 h siRNA knockdown by qPCR analysis. Western blots (WB) are shown for GALNT14 and FUT6 protein using antibodies against these proteins. (**b**) PSN-1 cells that are stably overexpressing GALNT14 or an empty vector were transfected with siGALNT14 (1) or siControl for 48 h. They were then treated with or without Apo2L/TRAIL (0.1 μg/mL) for another 48 h and cell viability measured. *P*-value was calculated by a student t-test comparing percent cell viability upon GALNT14 knockdown in vector and GALNT14 overexpressing cells. A western blot with an Flag antibody is used to detect overexpressed GALNT14. (**c**) Cell viability assay in C170 cells transfected with the indicated siRNA (48 h), followed by stimulation with Apo2L/TRAIL (48 h). (**d, e**) Cell viability assay was measured upon knockdown of the indicated genes (as in **c**) followed by a 24 h stimulation with etoposide (**d**) or staurosporine (STS) (**e**). Graphs are representative data from one of three experiments done. Each experiment was performed in triplicate and data points correspond to mean±S.D.

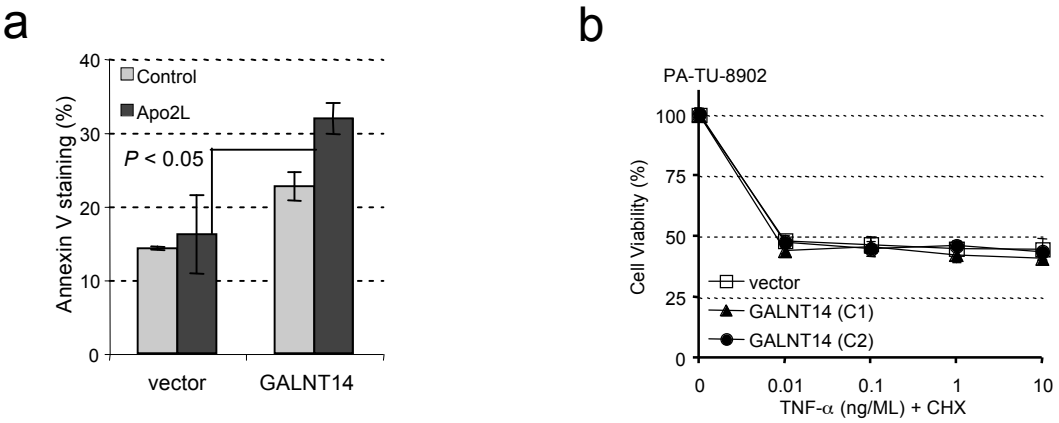


Supplementary Figure 2



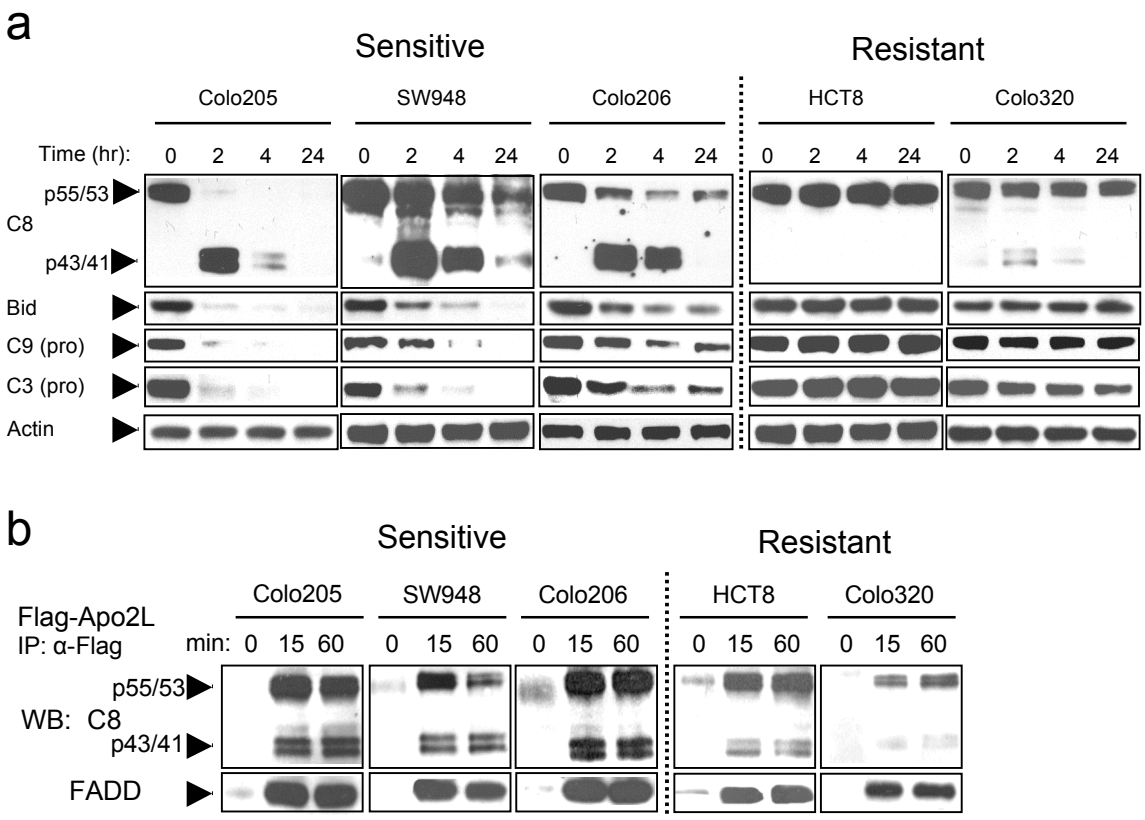
Supplementary Figure 2

Supplementary Figure 3: Overexpression of GALNT14 selectively sensitizes to Apo2L/TRAIL.
(**a**) HEK293 cells were co-transfected with trace amounts of DR5 and either GALNT14 or an empty vector for 48 h, followed by stimulation with Apo2L/TRAIL (1000 ng/ml) for an additional 24h and apoptosis measured by Annexin V staining. P-values were calculated from student t-tests for the indicated comparisons. (**b**) Cell viability assay of PA-TU-8902 stable lines after a 48 h stimulation with increasing doses of TNF-α with cycloheximide (10 μg/ml).



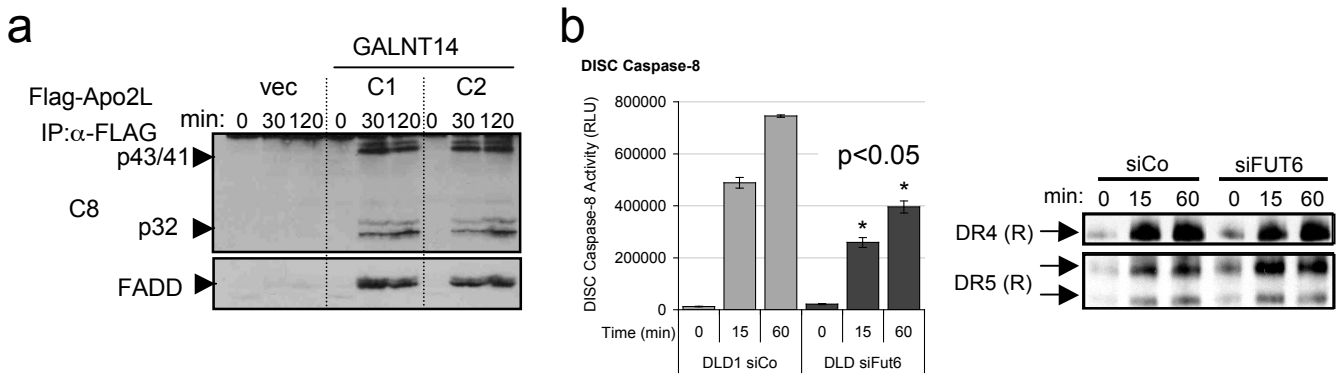
Supplementary Figure 3

Supplemental Figure 4: Apo2L/TRAIL-resistant cell lines are deficient in ligand induced DISC formation and caspase-8 activation.
(**a**)Apo2L/TRAIL sensitive and resistant colorectal cancer cell lines were treated with Apo2L/TRAIL (1 μg/mL) for the indicated amounts of time and cell lysates were analyzed by immunoblot with antibodies to caspase-8, Bid, caspase-9, or caspase-3. (**b**) Analysis of the Apo2L/TRAIL DISC. To immunoprecipitate the DISC, cells were incubated with FLAG-Apo2L/TRAIL (1 μg/ml) for either 15 or 60 min or after lysis for the 0 timepoint, and all samples subjected to immunoprecipitation with an FLAG antibody. DISC-associated FADD, caspase-8, DR4, and DR5 were detected by immunoblot.

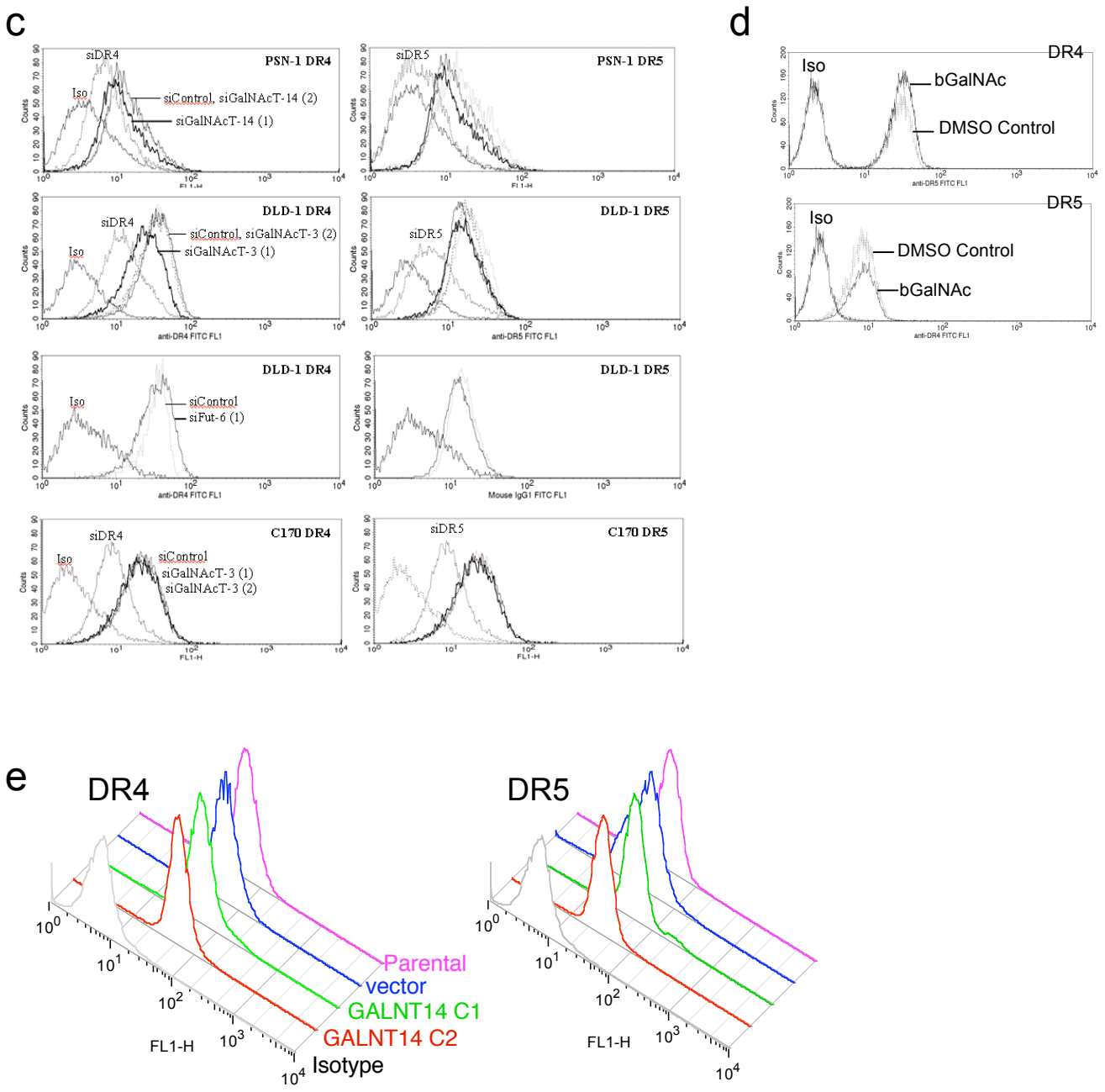


Supplementary Figure 4

Supplemental Figure 5: Modulating GALNT14 expression affects DISC formation and caspase-8 activation.
(**a**) PA-TU-8902 stable cell lines were stimulated with Flag-Apo2L/TRAIL for the indicated times and DISC components immunoprecipitated with an FLAG antibody and immunoblotted with antibodies against FADD and caspase-8. (**b**) DLD-1 cells were transfected with FUT6 siRNA, treated 48 h later with Flag-Apo2L/TRAIL and DISC-associated caspase-8 activity was measured in DISC immunoprecipitates. DISC associated receptor levels were analyzed by immunoblot (**right panel**). Asterisks indicate $p<0.05$ based on a t-test. (**c, d**) Cell surface expression of DR4 and DR5 was measured by FACS in cells that were subjected to (**c**) a 48 h siRNA knockdown with the indicated siRNA or (**d**) upon a 72h treatment with benzyl-GalNAc (bGalNAc) or vehicle (DMSO) or (**e**) upon stable overexpression of GALNT14 in PA-TU-8902 cells.

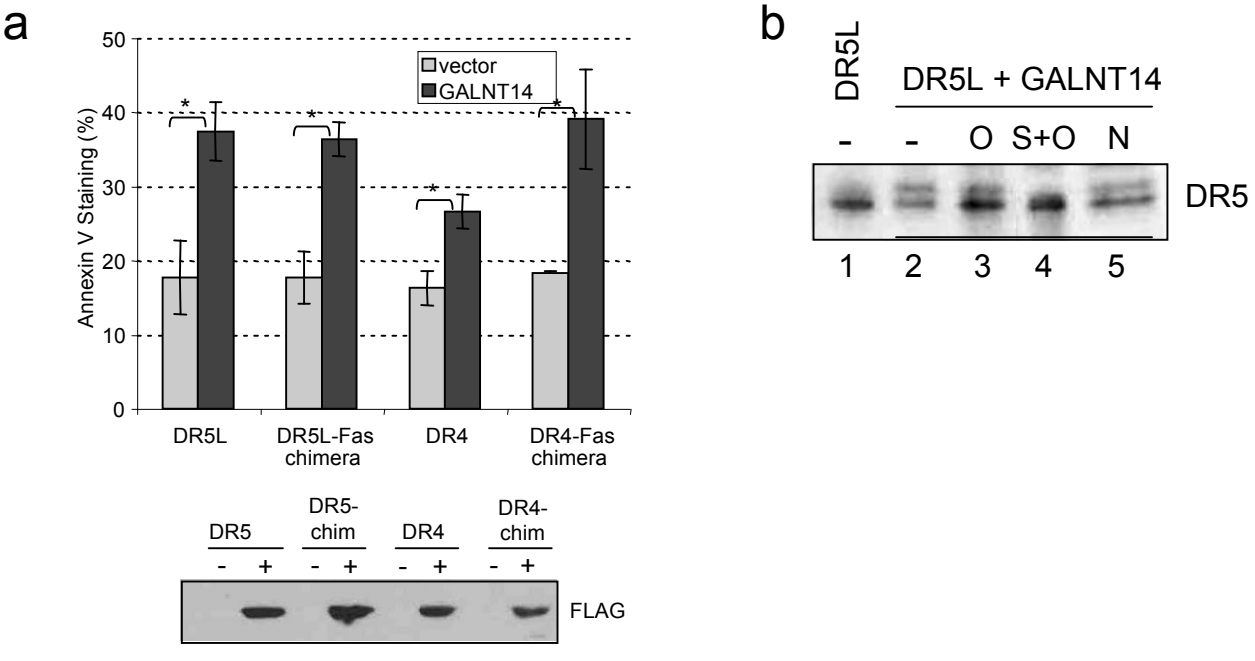


Supplementary Figure 5

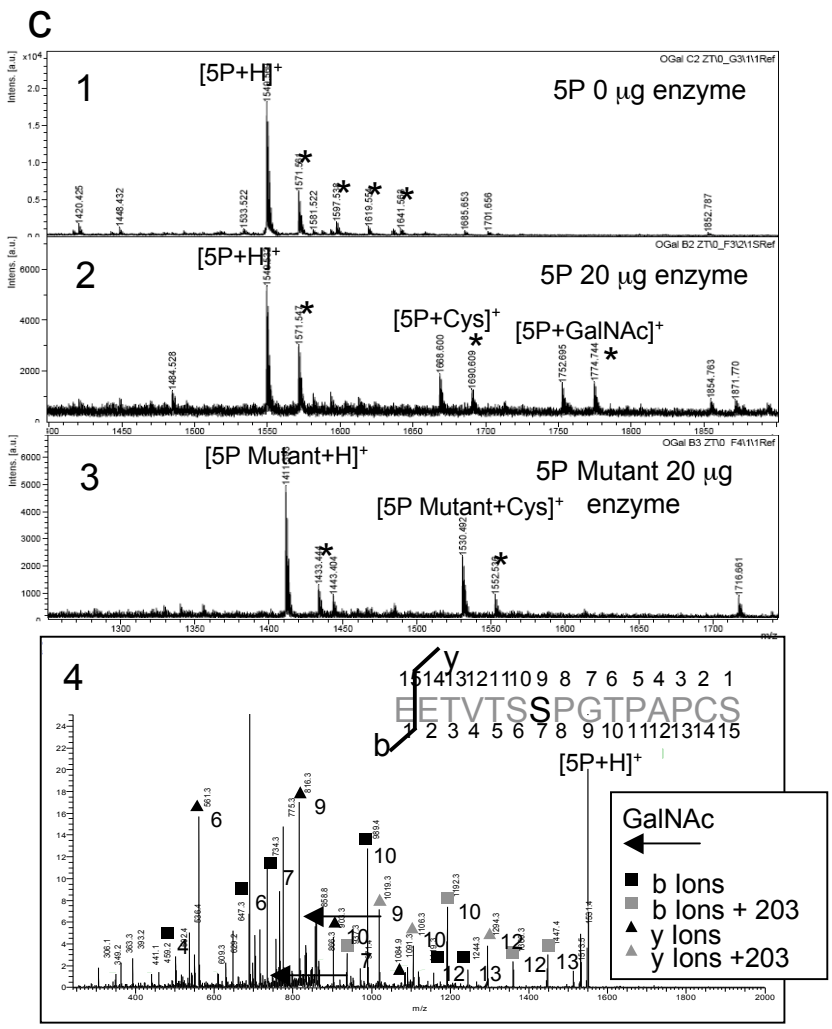


Supplementary Figure 5

Supplementary Figure 6: The ECD of DR4 and DR5 is the target of modification by GALNT14.
(**a**) HEK293 cells were cotransfected with the indicated death receptor chimeric constructs DR4 or DR5 extracellular domain (ECD) fused to Fas transmembrane and intracellular region) in combination with GALNT14 or an empty vector. Apoptosis was measured after 48 h by annexin V staining. A FLAG western blot indicates expression of Flag tagged GALNT14. Asterisks indicate $P < 0.05$ based on a t-test. (**b**) Immunoblots of DR5 from immunoprecipitates of DR5 from HEK293 expressing the receptor either alone (lane 1) or coexpressing GALNT14 (lanes 2-5) and subjected to 12 h incubation with deglycosylation enzymes: O-glycosidase (O), sialidase (S) or N-glycanase (N). (**c**) Mass spectra of a DR5 peptide (5P; spanning aa195-210) prior to GALNT14 exposure (**panel 1**), following GALNT14 exposure (**panel 2**) and 5P Mutant peptide (serine and threonines changed to alanine) following GALNT14 exposure (**panel 3**). The unmodified peptide is observed as a protonated ion $[5P+H]^+$. Following GALNT14 exposure, an additional ion corresponding to 5P with GalNAc modification [5P+GalNAc] is observed at m/z 1753 Da (**panel 2**) and is not observed in panel 1 or 3. Peptides with a free, unbound cysteine [5P+Cys]+ or with sodium adducts (asterisks) are indicated. Tandem mass spectrometry identifies the glycosylation site on 5P (**panel 4**) Mass spectrum obtained using tandem mass spectrometry of the 5P peptide following GALNT14 exposure. The b and y ions representing the directional ionization and fracture of the peptides are indicated (see key in figure) and the loss of the glycan moiety is indicated by arrow. The modified serine was identified (bold) due to the large mass difference between its two forms, corresponding to a mass difference of 203 Da or the mass of GalNAc.

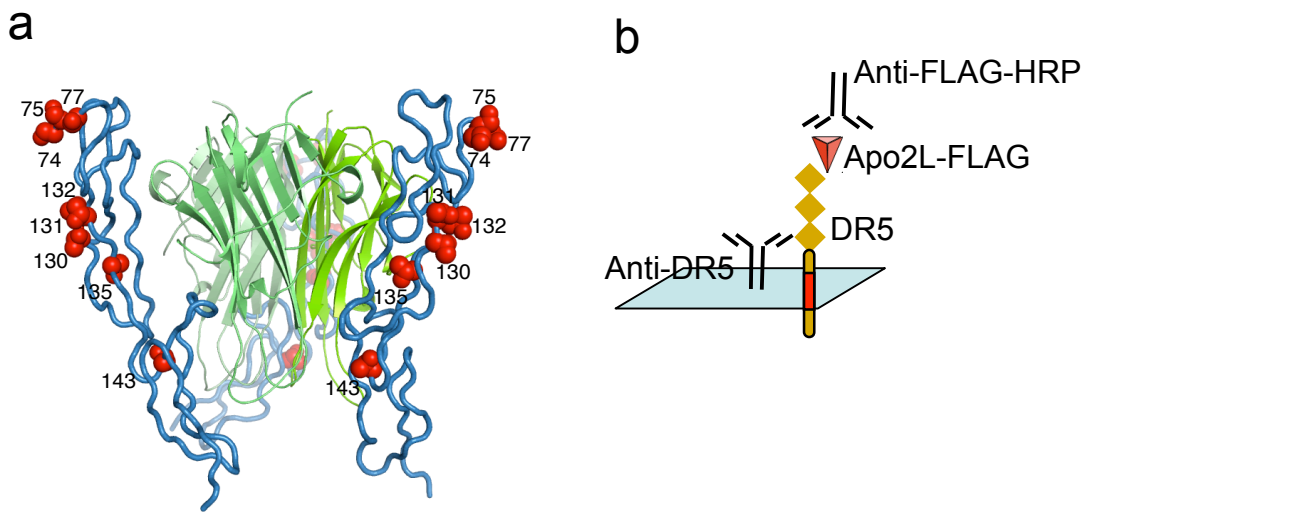


Supplementary Figure 6

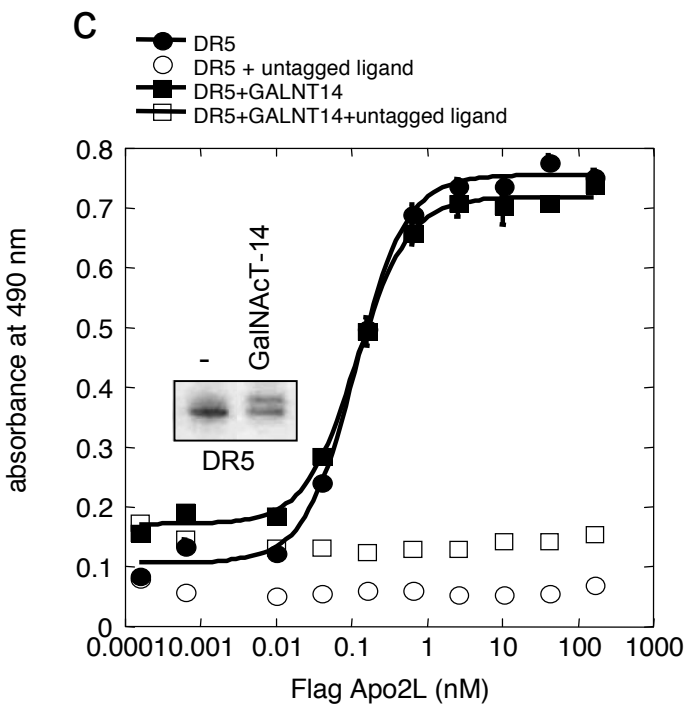


Supplementary Figure 6

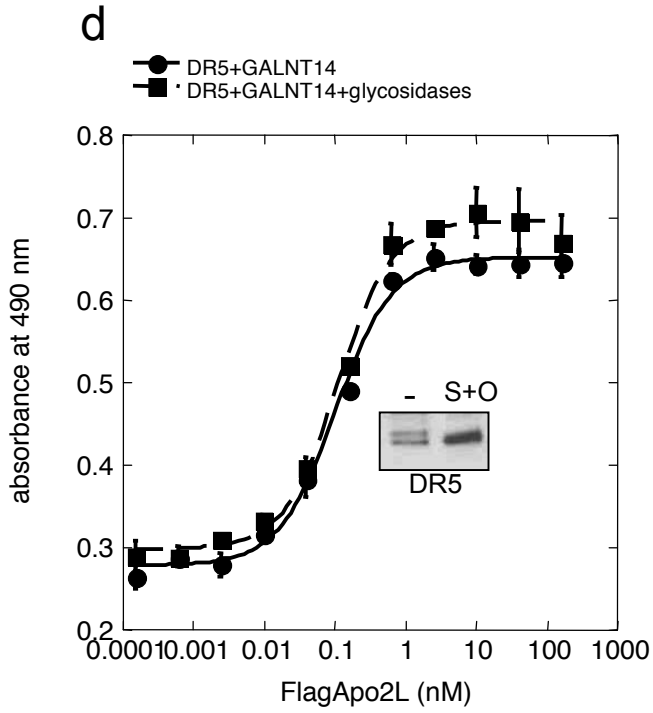
Supplementary Figure 7: O-glycosylation of DR5 does not affect affinity for Apo2L/TRAIL. (**a**) Mapping of the O-glycosylation sites on the crystal structure of the Apo2L/TRAIL DR5 complex. Residues of region 1 and 2 (aa74-77 and 130-144 respectively) are shown in red spheres (amino acid numbers are shown with the initial methionine as residue 1). The trimeric ligand (green) is shown in the center, with its 3 bound DR5 molecules (blue). (**b**) Schematic of the plate based Apo2L/TRAIL ligand binding assay. (**c, d**) Binding curves of Flag-Apo2L/TRAIL to DR5. (**c**) Plate-bound DR5 captured by immunoprecipitation from lysates of HEK293 cells transfected with DR5 alone (unglycosylated DR5) or in combination with GALNT14 (glycosylated DR5) were incubated with increasing concentration of Flag-Apo2L/TRAIL (closed symbols). Bound ligand was detected with an Flag-HRP antibody and apparent Kd calculated. Incubations with Flag-Apo2L/TRAIL were also done with saturating amounts of untagged Apo2L/TRAIL to determine non-specific binding (open symbols). (**d**) DR5 was captured from HEK293 lysates expressing DR5 and GALNT14 as described in **c** and incubated with the deglycosylation enzymes, sialidase (S) and O-glyconase (O) or left untreated. Ligand binding assay was done as in **c**.



Supplementary Figure 7



	Kd (pM)
DR5	117
DR5+GALNT14	121



	Kd (pM)
DR5+GALNT14	114
DR5+GALNT14+glycosidases	106

Supplementary Figure 7

Supplemetary Table 1: Sensitivity to Apo2L/TRAIL in cancer cell lines is correlated with mRNA expression of specific O-glycosylation enzymes but not related family members. (**a**) mRNA expression of GalNAcT family members present on the Affymetrix U133P microarrays were compared against sensitivity to Apo2L/TRAIL across pancreatic cancer, non-small cell lung cancer and melanoma cell lines. Associations between mRNA expression of GalNAcT family members and Apo2L sensitivity was evaluated with t-tests using Bayesian variance estimates. (**b**) mRNA expression data from colorectal cancer cell lines were analyzed for associations between Fucosyltransferase family members and Apo2L/TRAIL sensitivity as described in **a** (**top panel**). A similar comparison for the GalNAc transferases in colorectal cancer cell lines identifies a significant correlation with GALNT3 and not GALNT14 expression (**bottom panel**).

a

GalNAc transferases

ProbeID	Symbol	p-value
219271_at	GALNT14	3.08E-06
228303_at	GALNT6	0.0125765
218885_s_at	GALNT12	0.0170342
229555_at	GALNT5	0.0521631
230906_at	GALNT10	0.0651716
219013_at	GALNT11	0.0707984
203397_s_at	GALNT3	0.113974
229451_at	GALNT9	0.2411783
230417_at	GALNTL1	0.2580999
239461_at	GALNT7	0.3185005
217788_s_at	GALNT2	0.3276176
236536_at	GALNT13	0.5226145
220929_at	GALNT8	0.6521618
233150_at	GALNT15	0.6771117
201723_s_at	GALNT1	0.733725

b

Fucosyltransferases

ProbeID	Symbol	p-value
214088_s_at	FUT3	9.21E-07
211885_x_at	FUT6	1.00E-06
208505_s_at	FUT2	0.03769637
226348_at	FUT11	0.03820284
209892_at	FUT4	0.09537817
211411_at	FUT1	0.13560032
203988_s_at	FUT8	0.13804822
210433_at	POFUT1	0.20354208
210506_at	FUT7	0.24325787
223976_at	FUT10	0.2837863
211225_at	FUT5	0.43394026
207696_at	FUT9	0.67825179

ProbeID	Symbol	p-value
203397_s_at	GALNT3	0.01499513
219271_at	GALNT14	0.35122026

Supplementary Table 2: Summary of siRNA knockdown phenotypes.
Cell lines, in which downregulation of GALNT14 or GALNT3 and FUT6 resulted in protection from Apo2L/TRAIL, are marked by pluses indicating less (+) or more (++) than 50% protection with at least one siRNA oligonucleotide tested. (0) indicates the absence of protection against Apo2L/TRAIL.

Tumor Type	Cell Line	Transient	Retroviral	Apo2L protection by knock-down		
		RNAi	shRNA	GALNT14	GALNT3	FUT6
Pancreatic Cancer	PSN-1	Yes		+ +		
	Panc 05.04	Yes		+ +		
	Panc 03.27	Yes		+		
	Su.86.86	No	Yes	+		
	Hup-T3	Yes		0		
				4/5		
Melanoma	A375	Yes		+ +		
	Hs294T	Yes		+ +		
				2/2		
Colorectal Cancer	C170	Yes			+ +	+ +
	DLD-1	Yes			+ +	+ +
	SW948	Yes			0	0
					2/3	2/3

SUPPLEMENTARY METHODS

Reagents and cell lines

Cell culture reagents were purchased from Gibco (Invitrogen), nontagged human recombinant soluble Apo2L/TRAIL (amino acids 114-281) was prepared as described[1]. The O-linked glycosylation inhibitor Benzyl-a-GalNAc was from Calbiochem; TNF-α (BD Pharmingen) Etoposide, Staurosporine, Cycloheximide (Sigma-Aldrich). All human cancer cell lines were obtained from ATCC (American Type Culture Collection) or DSMZ (Braunschweig, Germany) and cultured at 37°C and 5% CO_2 in RPMI1640 supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine and 10 mM HEPES without antibiotics. HEK293 human embryonic kidney cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS.

Cell viability assays and apoptosis assays

To determine sensitivity to Apo2L/TRAIL, cells were plated in triplicate in 96-well plates, allowed to adhere for 24 h and then treated with recombinant human Apo2L/TRAIL in various concentrations, up to 1000 ng/ml. After a 72-h incubation, the cells were subjected to a viability assay (MTT Assay, Pierce; or CellTiter-Glo Luminescent Cell Viability Assay, Promega) following manufacturer's protocols. Apoptosis was quantified by flow cytometric analysis of the average percentage of harvested cells (adherent + floating in the medium) stained with Annexin V (BD Pharmingen).

Caspase assays

Caspase-3/7 activity in cells was assayed using the Caspase-Glo 3/7 assay (Promega), that utilizes a luminogenic caspase 3/7 substrate to detect activity. Activity measurements were made using a luminescence reader (Envision, Perkin Elmer), according to Promega's recommended protocol. Caspase-8 activity in the DISC was measured by immunoprecipitating DISC components from stimulated cells using a ligand pulldown or receptor pull down, followed by a Caspase-Glo 8 assay (Promega), according to manufacturer's protocol.

Microarray hybridization and data analysis

Scanned image files from the oligonucleotide microarrays were analyzed with GENECHIP 3.1 (Affymetrix), Spotfire, GenePattern, Cluster/TreeView and R (http://www.r-project.org). Genes differentially expressed between sensitive and resistant cell lines were identified using a modified t-test with Bayesian variance estimate[2], and false discovery rates (FDR) were estimated by the q-value method of Storey and Tibshirani[3]. Probe sets with less than 2-fold or less than 250 intensity units difference between sensitive and resistant cell lines were filtered out. GALNT14 (probe set 219271_at) was the most significant overexpressed gene in sensitive vs. resistant pancreatic, NSCLC and melanoma cell lines ($p=3.1x10^{-6}$, FDR=0.08). FUT3 (214088_s_at) and FUT6 (211885_x_at) were, respectively, the most significant overexpressed genes in sensitive vs. resistant colorectal cell lines ($p=9.2x10^{-7}$ and $1.0x10^{-6}$ respectively, FDR=0.08). Microarray data can be found on GEO (http://www.ncbi.nlm.nih.gov/geo); accession number:

Mice and xenograft studies

Tumor measurements were collected by a digital caliper and tumor volumes calculated using the formula p/6 (A= length) (B=width)^2. If tumors ulcerated before completion of study, the study was stopped and last data point corresponds to measurement prior to ulceration. Statistical analysis for *Established Subcutaneous (s.c.) Xenograft Model:* The log-rank test was used to analyze the significance of the differences between the time to tumor progression (TTP) values of the treated and the control groups, using two-tailed statistical analysis to generate a *P* value. *Adjuvant Subcutaneous (s.c.) Xenograft Model.* Percent tumor growth was calculated using log transformed values of tumor volume measurements on termination of study (last day of treatment), taking the geometric mean (10 mice) and using the formula: anti-log(GALNT14 Apo2L- GALNT14 vehicle)-(Vector Apo2L – Vector vehicle). The standard error was used to compute the 95% confidence interval. All of the experimental procedures were approved by Genentech's Institutional Animal Care and Use Committee (IACAUC).

Expression constructs, transfections and retroviral transduction

A DNA fragment encoding GALNT14 was cloned from cDNA pooled from Apo2L/TRAIL sensitive cell lines and inserted into the expression plasmid pcDNA3.1 (Invitrogen) with an N-terminal Flag tag. The DNA sequences for DR5L and DR5S, DR4, murine TRAIL receptor (DR5), DR4, Fas (variant 1), TNFR1 and Bax (beta variant) were cloned from cDNA pools and inserted into the pRK expression vector (Genentech). O-glycosylation mutants of DR5L and DR5S were generated by site-specific mutation of three serine residues (S74, S75, S77) or five threonine residues (T130, T131, T132, T135, T143) to alanine. Chimeric receptors were generated by fusing the extracellular domain of DR4 and DR5 with the intracellular domain of Fas. In brief, PCR products were generated of the extracellular domain of DR4 or DR5 with 3'

overhangs (10 basepairs) complementary to the intracellular domain of Fas. Conversely PCR products of the intracellular domain of Fas was generated with 5'overhangs complementary to DR4 and DR5. The PCR products were combined, followed by primer extension and further PCR amplification to generate the chimeric receptor product which was then cloned into the pRK vector. Transient transfection into HEK293 cells with expression constructs of proapoptotic molecules were done in 6 well plates with 0.5 μg/well of the proapoptotic molecule and 2.0 μg of GALNT14 or a vector control. Cells were transfected using Lipofectamine 2000 according to the manufacturer's protocol. Following a 48-h incubation, cells were subjected to apoptosis analysis. To study the effect of GALNT14 on ligand induced apoptosis in HEK293 cells, the above protocol was modified such that 0.01 μg/well of DR5 was transfected with 2.0 μg of GALNT14 or a vector control. Following 48 h, cells were stimulated with 1μg/mlApo2L/TRAIL for an additional 24 h and subjected to apoptosis analysis.

To generate retroviral constructs GALNT14 were cloned into the pQCXIP retroviral vector (Clontech). High titer retroviral supernatants were generated using the ΦNX-Ampho helper cell line. Packaging cells were transfected using Calcium Phosphate (Invitrogen). Supernatants were isolated 48 h after transfection and added to target cells along with 10μg/ml polybrene (Sigma), followed by a 1h centrifugation step at 2700 rpm to enhance infection. Following transduction, cells were subjected to selection with 2 μg/ml puromycin (Sigma). Stable pools were plated thinly to allow growth of stable colonies which were picked and expanded to generate stable clonal lines.

siRNA studies

The siRNAs against GALNT14, GALNT3, Caspase-8 and DR5 were designed by Dharmacon (Lafayette, CO) using their proprietary selection criteria. The selected sequences were:

siGALNT14 (1): 5' GACCATCCGCAGTGTATTA-dTdT 3' (=14-4)

siGALNT14 (2): 5' ATACAGATATGTTCGGTGA-dTdT 3' (=14-6)

siGALNT3 (1): 5' CCATAGATCTGAACACGTT-dTdT 3' (=3-2)

siGALNT3 (2): 5' GCAAGGATATTATACAGCA-dTdT 3' (=3-7)

siFUT6 (1) 5' GUACCAGACACGCGGCAUA-dTdT 3' (=6-1)

siFUT6 (2) 5' ACCGAGAGGUCAUGUACAA-dTdT 3' (=6-2)

siCaspase-8: 5' GGACAAAGTTTACCAAATG-dTdT 3'

Cells were transfected using Lipofectamine2000 (Invitrogen) by reverse transfection where cells are added in suspension to the pre-plated lipid-siRNA complexes. Concentrations for Lipofectamine2000 (Invitrogen) were as per the manufacturer's protocol.

Quantitative RT-PCR

GALNT14 and *GALNT3* transcript expression levels were assessed by quantitative RT-PCR using standard Taqman techniques. Transcript levels were normalized to the housekeeping gene, *GAPDH* and results are expressed as normalized expression values ($=2^{-\Delta Ct}$). Primer/probe sets for the *GALNT14* (cat#: Hs00226180_m1_GT14), *GALNT3* (cat#:Hs00237084_m1_GT3) and *GAPDH* (cat#: 402869) were purchased from Applied Biosystems.

Immunoprecipitation, Western blot analysis and antibodies

Immunoprecipitation (IP): DR4 (3G1 and 4G7) and DR5 (3H3 and 5C7) monoclonal antibodies were generated at Genentech, Inc. using receptor-Fc fusion proteins as antigens. DR4 (4G7) and

DR5 (5C7) monoclonal antibodies, used for receptor immunoprecipitations or to immunoprecipitate the Apo2L/TRAIL DISC, were conjugated to agarose using the ImmunoPure Protein G IgG Plus orientation kit (Pierce, USA, catalog #44990). DISC immunoprecipitations were also done using a FLAG antibody M2 (Sigma) under conditions when cells were stimulated with Flag-Apo2L/TRAIL. These experiments were done as previously described for Apo2L/TRAIL-FLAG + anti-FLAG DISC analysis[4].

Immunoblot (IB): For Western blot analysis $5x10^5$ cells were lysed in 1% Triton X-100 containing hypotonic lysis buffer (20 mM HEPES pH 7.5, 10 mM KCL, 1.5 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT). Post-nuclear lysates were analyzed by SDS-PAGE. Antibodies used for Western blot: Caspase-3 (R&D), DR4, Caspase-8, Bid and BCL-XL (BD Pharmingen), Caspase-9 (MBL), DR5 (Cayman), FLIP NF6 (Alexis Biochemicals), MCL-1 and Actin (Santa Cruz Biotechnology), XIAP and FADD (BD Transduction Laboratories). Mouse monoclonal antibodies against GALNT14 and FUT6 were generated at Genentech by immunizing mice with purified full length protein.

Flow cytometry

Surface expression of DR4, DR5, DcR1 and DcR2 was determined by florescence-activated cell sorting (FACS). Cells stained with 10 μg/ml primary antibody, DR4 (4G7), DR5 (3H3), DcR1 (6G9), DcR2 (1G9.91) or a mouse IgG control antibody (Sigma) for 1 h at 4°C. All receptor antibodies were generated at Genentech. Cells were then washed with PBS and then incubated with a flouroscein (FITC) conjugated goat mouse secondary antibody (Jackson Laboratories) for 30 min at 4°C. Cells were then analyzed by flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry, CA).

Ligand affinity assay

Microtiter plates (Nunc) were coated with monoclonal antibody to DR5 (3H1) overnight at 4 degrees C in 0.05 M carbonate buffer, pH 9.6. Cleared lysates prepared with TBS/1.0% NP40 were loaded into the prepared mictrotiter plate and incubated for 1 hour at room temperature. The plates were blocked with 0.5 % BSA in PBS. Varying concentrations of Flag-Apo2L/TRAIL were added and incubated for 1 h. For competition experiments, Flag-Apo2L/TRAIL was added in the presence of saturating amounts of (unlabeled) Apo2L/TRAIL. The bound ligand was detected with Flag-HRP antibody (Sigma) and OPD (Sigma) substrate. Ligand binding data were analyzed by 4-parameter non-linear curve fit (Kaleidagraph), and apparent Kd values were determined from half-maximum absorbance values.

Cell proliferation Studies

To assess the proliferative capacity of different cell lines *in vitro*, 50,000 cells of each cell line were plated in 6 well plates in triplicate in complete medium, followed by a 72 h incubation. Cell numbers were then counted with a Vi-CELL cell counter (Beckman Coulter), and average fold change computed. Proliferative capacity of cell lines to grow as xenograft tumors was assessed by tumor Doubling Time (*DT*), which is determined by allowing tumors to grow to a size of 100 mm^3 (day 0) and then measuring the time when tumor volume has doubled from its initial tumor volume on day 0. It is calculated for each animal using the following equation: DT (days) = ($T - T_0$) x log2/log$V$ – logV_0, where $T - T_0$ indicates the length of time between two measurements and V_0 and V denote the tumor volume at two points of measurement. If the tumor volume is above 2x on the next day of measurement, Doubling Time is linearly interpolated to be between the previous day of measurement and the day of the measurement when the tumor is above 2x

volume.

In vitro GALNT14 glycosylation assay and mass spec analysis

Substrate glycosylation of the DR5 peptide (aa195-210) by GALNT14 was carried out by combing DR5 peptide (1 μM) with 20 ug of recombinant human GALNT14 (produced in CHO cells) with the buffer containing the following: 50mM Tris pH7.4, 5mMMnCl2, 250 μM UDP-GalNAc (Sigma). The enzymatic reaction was carried out for 16h reaction at 37 degree C. The peptide was then analyzed by mass spec analysis using Bruker UltraFlex. The matrix used was 2,5-dihydroxybenzioc acid in a one to one ratio following sample desalting with C18 ZipTip (Millipore). Tandem Mass spectrometry was performed on an ThermoScientific LTQ instrument with regular spray mode following sample desalting as previously described.

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