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# FUT3/6 DIFFERENTIALLY REGULATE DEATH RECEPTOR-SPECIFIC SENSITIVITY TO TRAIL IN COLON CANCER CELLS

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## ABSTRACT

The remarkable pro-apoptotic properties of tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) have raised considerable interest in this ligand as an anticancer therapeutic. However, TRAIL was found to be largely ineffective in inducing apoptosis in several cancer cells. In colon adenocarcinomas, post-translational modifications including *O*-glycosylation and fucosylation catalysed by the fucosyltransferases FUT3 and FUT6 regulate TRAIL-induced apoptosis. We have now analysed the specific contribution of FUT3/6-mediated fucosylation to the apoptosis induction of TRAIL via its two death receptors (DR4 and DR5), using agonistic receptor-specific TRAIL variants. Triggering of apoptosis by TRAIL revealed that the low FUT3/6 expressing DLD-1 and HCT116 cells are insensitive via DR5 but not to DR4-mediated apoptosis, contrasting to the efficient apoptosis mediated via both receptors in FUT3/6 high expressing Colo205 cells. Reconstitution of FUT3/6 expression in DR5-resistant cells completely restored TRAIL sensitivity via this receptor. Furthermore, we show that the addition of L-fucose restores DR5-mediated apoptosis in both DLD-1 and HCT116 cells. DR4-mediated apoptosis was also further enhanced in these cells upon overexpression of either FUT3 or FUT6, but as these cells were already highly responsive via DR4, we conclude that FUT3/6 may not be strictly required for efficient TRAIL-induced apoptosis via DR4. Taken together, these results provide evidence for a differential impact on the activity via DR4 and DR5 by fucosylation, and provide novel opportunities to enhance TRAIL sensitivity in colon adenocarcinoma cells that are highly resistant to DR5-mediated apoptosis.

## INTRODUCTION

Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females [1]. Although early detection methods, increased awareness and improved treatment modalities have led to reduction of both incidence and cancer death rates in several Western countries, colorectal neoplasias are still one of the most deadly cancers, causing around 300,000 deaths worldwide in 2008 alone [1]. The survival rate of colorectal cancer patients depends on the tumour-node-metastasis (TNM) classification with >90% 5-year survival rate for stage I tumours and 5-8% for stage IV tumours [2]. Thus novel therapies are required to improve the prognosis of colorectal patients.

The TNF superfamily member TRAIL has gathered considerable attention as a potential cancer therapeutic, as it is able to induce apoptosis selectively in tumour cells while leaving normal cells unharmed [3, 4]. TRAIL is capable of binding to two apoptosis-inducing transmembrane death receptors (DRs), namely DR4 (TRAIL-R1) and DR5 (TRAIL-R2). TRAIL also binds to three decoy receptors (DcRs); DcR1 (TRAIL-R3), DcR2 (TRAIL-R4) and the soluble decoy receptor osteoprotegerin (OPG) [5, 6]. Decoy receptors are unable to transduce death-inducing signals as they lack a functional intracellular death domain (DD) [6-9], and they can diminish apoptosis activation by competing with TRAIL-DR interactions [7, 10] or by forming non-signalling heterotrimeric complexes [11].



Binding of trimeric rhTRAIL to death receptors triggers the formation of the so-called death inducing signalling complex (DISC), consisting of Fas-associated death domain (FADD) [12-14], which further recruits and activates pro-caspase-8 and/or pro-caspase-10. Activation of initiator caspases leads to direct cleavage and activation of executioner caspases-3 and -7 and subsequent apoptosis induction through the extrinsic apoptotic pathway [15-17], or/and the cleavage of Bid (tBid), resulting in the release of mitochondrial factors, caspase-9 cleavage and activation of effector caspases, through the activation of the apoptosis intrinsic pathway [18]. Despite its tumour selective properties, several reports testing the effectiveness of TRAIL in human colorectal cancer cell lines have shown that approximately 50% of the cell lines tested are resistant to recombinant human (rh) TRAIL (rhTRAIL) [19, 20].

Fucosylation is an important type of post-translational modification in colon cancer [21], in which fucose residues are attached to *N*- or *O*-linked glycans or glycolipids [22, 23]. This modification of cell-surface and secreted glycoproteins is essential in numerous biological processes, such as ontogeny, cellular differentiation and a variety of signalling events [22, 24]. Several fucosyltransferases [21, 23] require the donor substrate GDP-fucose, which can be synthesized from GDP-mannose by the dominant *de novo* pathway or from free L-fucose by the salvage pathway [24]. Notably, fucosylation is often up-regulated in cancers, where it has been suggested to play a role in enhanced metastasis [21]. In the past years, several reports have described the importance of fucosylation in TRAIL-induced apoptosis in colon cancer. Wagner *et al*. firstly described the correlation

between TRAIL sensitivity and mRNA levels of *O*-glycosylation initiating enzyme GALNT3 and *O*-glycan processing fucosyltransferase enzymes FUT3 and FUT6 in a panel of colon adenocarcinoma cells [19]. Furthermore, TRAIL-induced clustering of *O*-glycosylated DR4 and DR5 was described as the mechanism that enhances TRAIL signalling in tumours [19]. Moriwaki *et al*. also demonstrated the importance of fucosylation on TRAIL sensitivity and natural killer (NK)-cell-mediated immune surveillance in colon cancer cell lines [25]. Mutation of the GDP-mannose-4,6-dehydratase (GMDS) gene leads to the inactivation of the *de novo* GDP-fucose pathway and decreased TRAIL sensitivity, resulting in accelerated tumour growth *in vivo,* due to a lack of NK cell-mediated tumour surveillance [25]. GMDS also plays an important role in the formation of secondary FADD-dependent complex II, which comprises FADD, caspase-8 and c-FLIP. Although only DR4 has been described to be fucosylated, GMDS deficiency inhibited both DR4- and DR5-mediated apoptosis by inhibiting the formation of secondary FADD-dependent complex II, while it did not affect formation of the DISC or recruitment to and activation of caspase-8 [26]. The same group showed that fucosylation can be regulated through DNA methylation. Treatment with the novel methyltransferase inhibitor zebularine was found to increase fucosylation levels, leading to enhanced TRAIL-induced apoptosis without increasing TRAIL receptor and caspase-8 levels [27].



Here we investigated the more precise role of fucosylation on DR4- and DR5-mediated apoptosis in colon adenocarcinomas, using TRAIL receptor-specific apoptosis-inducing variants that bind selectively and with high affinity to either DR4 or DR5 [28-32]. The demonstration that DR5- but not DR4-mediated apoptosis is highly dependent on expression of fucosyltransferases FUT3 and FUT6 in colon adenocarcinoma cell lines, and the restoration of DR5-mediated apoptosis by the enhanced activation of the salvage pathway through L-fucose addition, reveals a different regulation mechanism imposed by fucosylation on each of the two death receptors of TRAIL.

## MATERIALS AND METHODS

### *Cell lines and reagents*

Human colorectal cancer cell lines Colo205, DLD-1 and HCT116 were cultured in RPMI1640 medium (GIBCO) supplemented with 10% foetal calf serum (FCS), 100 units/mL penicillin and 100 µg/mL streptomycin in a humidified incubator at 37 °C containing 5% $CO_2$. Human foreskin fibroblasts (a kind gift of prof. H.H. Kampinga) were cultured in Ham's F-10 medium (GIBCO) supplemented with 10% foetal calf serum (FCS), 100 units/mL penicillin and 100 µg/mL streptomycin. RhTRAIL wild type (WT), the DR4-specific TRAIL variant 4C7 and the DR5-specific TRAIL variant D269H/E195R (amino acids 114-281) were constructed and produced as previously described [28, 29]. L-(-)-Fucose (L-fucose, F2252) and benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (bGalNAc) (B4894) were purchased from Sigma.

**Cell viability and apoptosis assay**

Cell viability and proliferation assays were conducted using a MTS assay. Cells were seeded in triplicate in 96-well plates at a cell density of 10,000 cells/well in 0.1 mL medium. After 24 h, cells were treated with several rhTRAIL concentrations, ranging from 0 to 500 ng/mL of rhTRAIL WT, 4C7 or D269H/E195R for 16 h, to a final volume of 0.15 mL. In the case of L-fucose treatment, cells were incubated with different concentrations of L-fucose (0-100 mM) for 48 h to a final volume of 0.2 mL. For the combination of L-fucose and rhTRAIL, cells were initially incubated with 0 or 50 mM of L-fucose in a final volume of 0.15 mL. After 24 h, cells were treated with concentrations ranging from 0 to 500 ng/mL of rhTRAIL WT, 4C7 or D269H/E195R for 16 h to a final volume of 0.2 mL. Stock solutions of L-fucose were serially diluted in serum-free RPMI medium and TRAIL ligands in complete medium. Cells were incubated with MTS reagent according to manufacturer's instructions (G3581, Promega). Cell viability was determined by measuring the absorption at 492 nm using a microplate reader (Thermo Labsystems). For the inhibition of O-glycosylation, cells were pre-treated 24 h after seeding with 2 mM bGalNAc in a final volume of 0.15 mL. Afterwards, cells were treated with several rhTRAIL concentrations, ranging from 0 to 1000 ng/mL of rhTRAIL 4C7 or D269H/E195R for 16 h, to a final volume of 0.2 mL.

Apoptosis induction was measured using Annexin V-APC (IQP-120A) or Annexin V-FITC (IQP-120F, IC products) staining and quantified by flow cytometry. Cells were seeded in 6-well plates 24 h prior to treatment. The next day, cells were incubated with 0-500 ng/mL rhTRAIL WT, 4C7 or D269H/E195R for 16 h. After treatment, cells were harvested and washed with calcium buffer (10.9 µM HEPES, 140 µM NaCl, 2.5 µM $CaCl_2$). Cell pellets were resuspended in 60 µL calcium buffer complemented with 5 µL Annexin V-APC and incubated for 20 minutes on ice. Cells were washed and analysed using a FACSCalibur flow cytometer (BD).

**Western blotting**

Cells were harvested and lysed by using the Mammalian Protein Extraction Reagent (M-PER, PIERCE, Thermo Scientific) with additional Protease Inhibitor Cocktail, EDTA-Free (100x; Thermo Scientific). Protein concentrations were determined using a Bradford assay (Bio-Rad Laboratories). Equal amounts of protein (20 µg) for each sample were loaded per lane on pre-cast 4-12% SDS-PAGE gels (Invitrogen) and transferred onto Immobilon-FL PVDF 0.45 µm membranes (Millipore). Subsequently, the membranes were blocked for 1h at room temperature in blocking buffer (Rockland). Western Blot membranes were probed overnight at 4 °C. The following primary antibodies were used: caspase-8 (9746, Cell Signaling), FUT3 (ab110082, Abcam) and PARP (9542, Cell Signaling). Goat-α-mouse-IRDye 680 (926-32220, LI-COR Biosciences) or goat-α-rabbit-IRDye 800CW (926-32211, LI-COR Biosciences) secondary antibodies were used for detection using a LI-COR Odyssey Infrared Imaging System (Westburg). Membranes were probed with anti-γ-tubulin (T6557, Sigma) to confirm equal loading.

**TRAIL receptor membrane expression analysis**

Cells were harvested and washed with standard buffer (PBS/1% BSA). TRAIL receptor cell surface expression was determined using 10 µg/mL TRAIL-R1 (ALX-804-297), TRAIL-R2 (ALX-804-298), TRAIL-R3 (ALX-804-344), TRAIL-R4 (ALX-804-299) (Alexis Biochemicals, Enzo Life Sciences.), DR5-01-1 (EXB-11-461, Exbio) or negative control mouse IgG1 (X0931, DAKO). Cells were incubated with primary antibodies for 1h. Subsequently, the cells were washed and incubated for 1h with R-phycoerythrin (PE) conjugated goat anti-mouse antibody (1010-09, Southern Biotech) or Alexa Fluor 488 conjugated goat anti-mouse antibody (A-11001, Invitrogen). Receptor cell surface expression was analysed using a FACSCalibur flow cytometer (BD).

**Transient overexpression of FUT6-GFP and lentiviral overexpression of FUT3 and FUT6**

Cells were seeded in 6-wells plates at a density of 150,000 cells/well. The next day, the subconfluent cultures were transfected with plasmid containing GFP-conjugated α-1,3-fucosyltransferase 6, fuc-T6-GFP (FUT6-GFP) (a kind gift from prof. Jack Rohrer) using the FuGENE HD Transfection Reagent (E3211, Promega) according to manufacturer's instructions. 48 h after transfection, cells were seeded and treated with 50 ng/mL rhTRAIL WT, 4C7 or D269H/E195R for 16 h. Apoptosis induction was measured using Annexin V-APC staining.

Lentiviral plasmids encoding FUT3 (LV164063) or FUT6 (LV164081) and the control plasmid (LV590) were purchased from Applied Biological Materials Inc. For the packaging of the lentiviral particles, $2*10^6$ cells HEK293 cells were plated in 94.0 mm cell culture dishes. The following day, cells were transfected with either pLenti-CMV-GFP vector expressing FUT3, FUT6 or the control vector, using $CaCl_2$. After 24 h, the medium containing virus particles was harvested, filtered, mixed with polybrene and added to DLD-1 and HCT116 cells, which were plated the day before at a density of $0.25*10^6$ cells in wells of a 6-wells plate; the final concentration of polybrene was 10 µg/mL. The following day, the previous steps were repeated. The cells were exposed to the viral particles for 48 h in total after which the virus was removed and fresh medium was added. Mixed populations of control-, FUT3- or FUT6-overexpressing cells were cultured in RPMI supplemented with 10% foetal calf serum, 100 units/mL penicillin, 100 µg/mL streptomycin and 2 µg/mL puromycin in a humidified incubator at 37 °C containing 5% $CO_2$. Single clones that expressed GFP were subcloned.

**qRT-PCR**

RNA was isolated from DLD-1 and HCT116 cells transduced with control, FUT3 or FUT6 lentiviral plasmids using the RNeasy Mini Kit (QIAGEN) according to manufacturer's instructions. cDNA was synthesized from 4 µg total RNA using oligo dT primers and M-MLV Reverse Transcriptase (28025-013, Invitrogen) in a total volume of 80 µL.

Quantitative real-time (qRT)-PCR was performed to determine the mRNA expression levels of FUT3 (5'- GGACATGGCCTTTCCACATC-3' and 5'-TCCAGGTGCTGGCAGTTAGG-3'), FUT6 (5'-CGCTTCCCAGACAGCACAGG-3' and 5'-TCCGTCCATGGCTTTCAGCTGCCA-3') and the housekeeping gene *RPL27* (5'-TCCGGACGCAAAGCTGTCATCG-3' and 5'-TCTTGCC CATGGCAGCTGTCAC-3') using SsoAdvanced Universal SYBR Green Supermix (172-5274, BioRad) on the CFX Connect Real-Time PCR Detection System (Bio-Rad). The protocol was as follows: initial denaturation at 98 °C for 3 min, followed by 45 cycles of amplification (5 sec at 98 °C and 20 sec at 65 °C). Finally, a melting curve was performed to ensure that only a single PCR amplicon was produced.

**Immunostaining of surface DR4 and DR5**

DLD-1:control, DLD-1:FUT3 and DLD-1:FUT6 cells were seeded at a density of 150,000 cells on poly-L-lysine (P8920, Sigma) coated coverslips. Cells were fixed using 4% Formaldehyde solution (F1635, Sigma-Aldrich) for 15 minutes at room temperature. The cells were then stained for 1h with 1:50 in PBS diluted TRAIL-R1 (ALX-804-297, Alexis Biochemicals, Enzo Life Sciences), DR5-01-1 (EXB-11-461, Exbio) or IgG1 negative control (X0931, DAKO). After washing with PBS three times, cells were incubated with secondary antibody donkey anti-mouse IgG (H+L) Alexa Fluor 647 (715-605-150, Jackson ImmunoResearch) at a concentration of 1:100 for 1h. Nuclei were counterstained with 0.2 µg/mL DAPI (D1306, Molecular Probes) for 10 minutes. The coverslips were mounted with CitiFluor (AF1, Agar Scientific). Slides were photographed using a Leica DMI 6000 Inverted microscope.



## RESULTS

**Different sensitivities of colon adenocarcinomas to DR4- and DR5-mediated apoptosis**

The colon adenocarcinoma cells Colo205, DLD-1 and HCT116 showed dissimilar sensitivities to rhTRAIL WT or the death receptor-specific variants. In concert with previous observations, Colo205 (figure 1A), as well as CL-34 [29] cells were highly sensitive to TRAIL-mediated cell death via both DR4 and DR5, with the DR-specific variants 4C7 (DR4-specific) and D269H/E195R (DR5-specific) exhibiting higher potency compared to rhTRAIL WT already at low concentrations. Interestingly, apoptosis induction in DLD-1 and HCT116 cells was primarily mediated by DR4 but not DR5 (figure 1B,C), as shown by the high Annexin V levels seen upon 4C7 incubation but not D269H/E195R in both cell lines. Even at relatively high concentrations of the ligand (500 ng/mL treatment with rhTRAIL D269H/E195R), Annexin V positivity in DLD-1 and HCT116 cells was nearly absent, (figure 1B,C). We next determined if differences in surface expression of TRAIL receptors could explain the differential activities observed when directly inducing apoptosis via DR4 or DR5 in these cells. Analysis of receptor expression by flow cytometry revealed that all the three cell lines express both DR4 and DR5 on their surface (figure 1D). These results reinforce the notion that death receptor expression alone

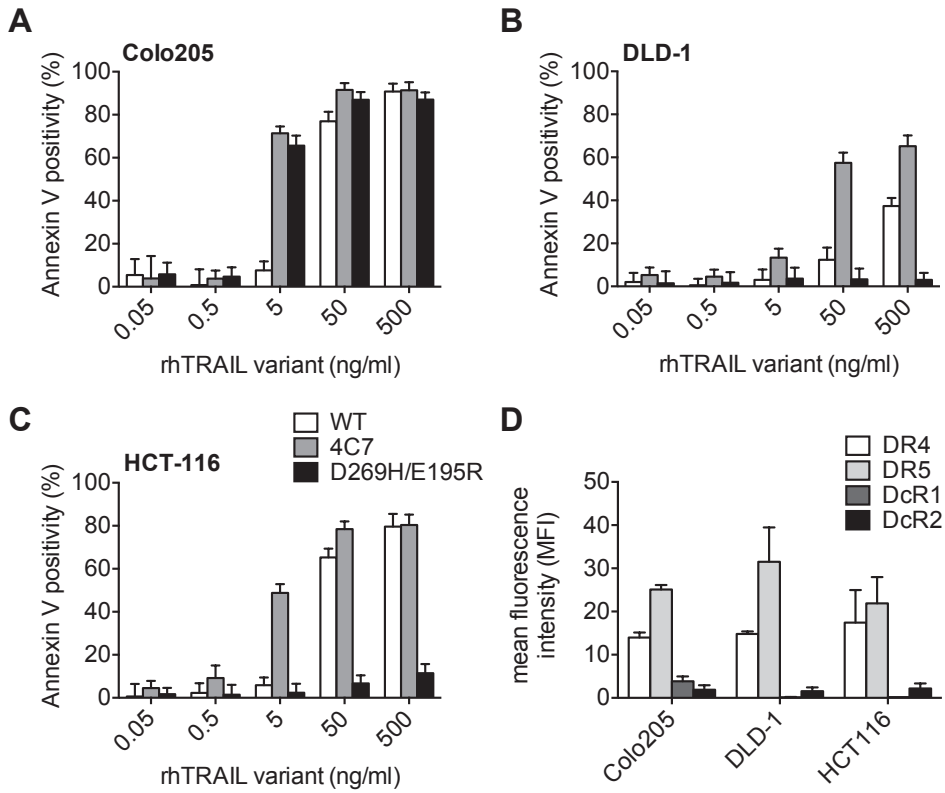


**Figure 1. Different colon adenocarcinoma cell lines exhibit differential sensitivities via DR4 and DR5.** Apoptosis inducing potential of rhTRAIL WT, 4C7 and D269H/E195R (0.5-500 ng/mL) in Colo205 (**A**), DLD-1 (**B**) and HCT116 (**C**) was determined after 16 h treatment using Annexin V-APC by flow cytometry. (**D**) Cell surface expression of TRAIL receptors was determined in Colo205, DLD-1 and HCT116 cells using flow cytometry analysis and expressed as the Mean Fluorescence Intensity (MFI) ratio compared to binding of isotype antibody. The data are presented as mean values ± S.D. (*n=3*).

is not predictive of TRAIL susceptibility to either DR4 or DR5, as seen in the DR4/5 sensitive Colo205 and DR5-insensitive DLD-1 and HCT116 cell lines.

### O-glycosylation and FUT6 expression influence TRAIL sensitivity

A role for fucosylation in TRAIL-induced apoptosis of colon adenocarcinomas has been previously established [19, 25-27]. Wagner *et al*. tested a panel of 36 colorectal adenocarcinoma cell lines and found that sensitivity to TRAIL correlated with increased mRNA levels of the *O*-glycosylation initiating enzyme GALNT3, as well as the *O*-glycan processing fucosyltransferase enzymes FUT3 and FUT6 [19]. Interestingly, when we compared the reported mRNA levels of FUT3 and FUT6 with our own TRAIL sensitivity data, we noted that FUT3 and FUT6 mRNA levels were low in the DR5-insensitive DLD-1 and HCT116 cells. We confirmed that *O*-glycosylation is important for TRAIL-induced

apoptosis using our DR-specific ligands. The Colo205 and HCT116 cell lines were pre-incubated with 2 mM of the pan *O*-glycosylation enzyme inhibitor benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (bGalNAc) for 24 h, followed by the addition of rhTRAIL 4C7 or rhTRAIL D269H/E195R for another 16 h. As expected, cell death induction via both DR4 and DR5 was hampered in Colo205 upon bGalNAc pre-treatment (figure 2A). DR4-

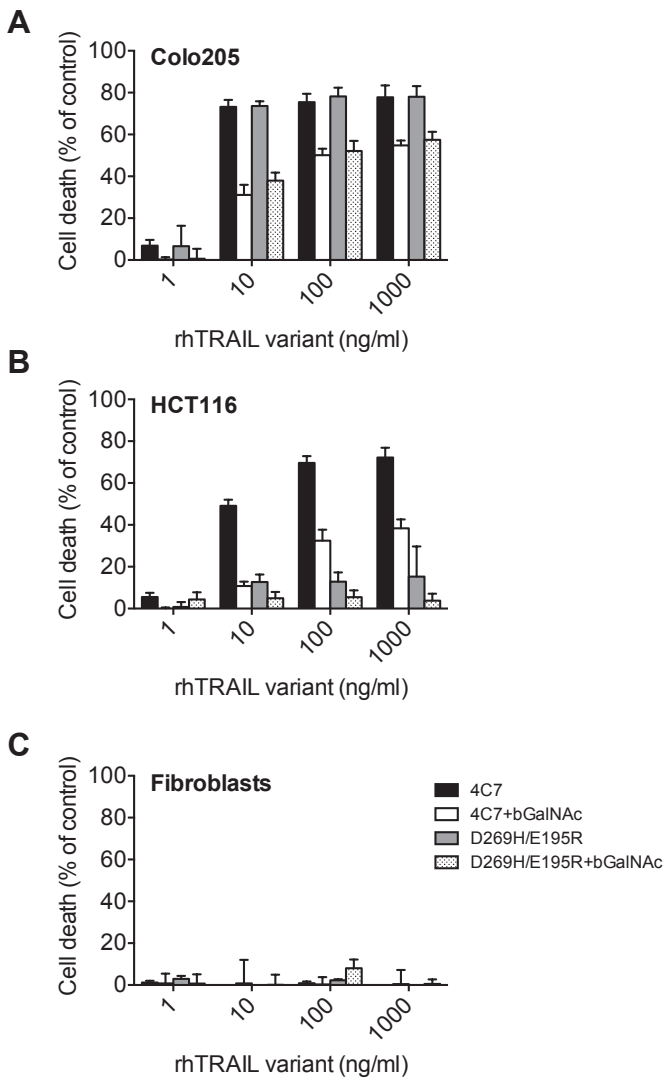


**Figure 2. Inhibition of O-glycosylation decreases TRAIL sensitivity.** Colo205 (**A**), HCT116 (**B**) and fibroblasts (**C**) were pre-treated with 2 mM bGalNAc for 24 h, after which cells were stimulated with 1-1000 ng/mL rhTRAIL 4C7 or D269H/E195R for 16 h. Cell death levels were determined by MTS assay. The data are presented as mean values ± S.D. (*n=3*).

mediated apoptosis was also significantly decreased in HCT116 (figure 2B). We have not detected any changes in the amount of cell death in the DR4/5-resistant human fibroblasts or in HCT116 cells treated with DR5-specific rhTRAIL D269H/E195R (figure 2B,C). These results substantiate the importance of *O*-glycosylation for TRAIL-induced cell death via both death receptors.

We then tested if the enhancement of fucosylation, by transient overexpression of FUT6, could sensitize DLD-1 and HCT116 cells to DR5-mediated apoptosis. Cells were transiently transfected with control (GFP) and FUT6-GFP, treated with 50 ng/mL of rhTRAIL, and apoptosis induction of GFP-positive cells was further measured by Annexin V-APC staining. Apoptosis induction with rhTRAIL WT and the DR5-specific D269H/E195R variant was clearly enhanced in FUT6-GFP transfected cells, while apoptosis levels remained the same when cells were treated with the DR4-specific 4C7 variant (supplementary figure 1).

**Stable ectopic overexpression of FUT3 and FUT6 enhances TRAIL sensitivity via both death receptors**



We generated stable cell lines expressing either FUT3 or FUT6 in both DR5-insensitive DLD-1 and HCT116. Cells were transduced either with an empty lentiviral vector (control) or a vector expressing FUT3 or FUT6. Transduced cells further expressed GFP, allowing for the isolation of cells. Western blotting and qRT-PCR were used to assess the FUT3 and FUT6 expression levels in these cells. Transduced cells showed a clear increase in relative FUT3 or FUT6 mRNA levels compared to control, with transduced DLD-1 cells expressing higher levels of FUT3 and FUT6 compared to HCT116 (figure 3A,B). Notably, stable overexpression of FUT3 in HCT116, but not in DLD-1, also slightly increased the mRNA levels of FUT6 (figure 3B). Further western blot analysis of FUT3 expression, showed a significant increase in FUT3 protein levels in both DLD-1-FUT3 and HCT116-FUT3 cells, when compared to control and FUT6 overexpressing cells (figure 3C). Flow cytometry analysis showed that overexpression of FUT3 and FUT6 had no effect on DR4 and DR5 expression levels in transduced DLD-1 and HCT116 cells (data not shown).

To evaluate the impact of FUT3 or FUT6 stable overexpression in TRAIL-mediated apoptosis via either DR4 or DR5, transduced cells were further treated with 50 ng/mL of the DR4-specific rhTRAIL 4C7 and the DR5-specific rhTRAIL D269H/E195R, respectively. Indeed, DLD-1 and HCT116 overexpressing either FUT3 or FUT6 were significantly sensitized to TRAIL-induced apoptosis via DR5, whereas the maximum amount of cell death at the concentration used (50 ng/mL), remained largely unaltered in control versus FUT3 or FUT6 overexpressing cells, when treating them with rhTRAIL 4C7 (figure 3D,E). Although DLD-1 and HCT116 cells overexpressing either FUT3 or FUT6 are sensitized primarily to DR5 at high concentrations, we have also detected an increase in apoptosis induction via DR4, especially when using low concentrations of rhTRAIL 4C7 (5 ng/mL),

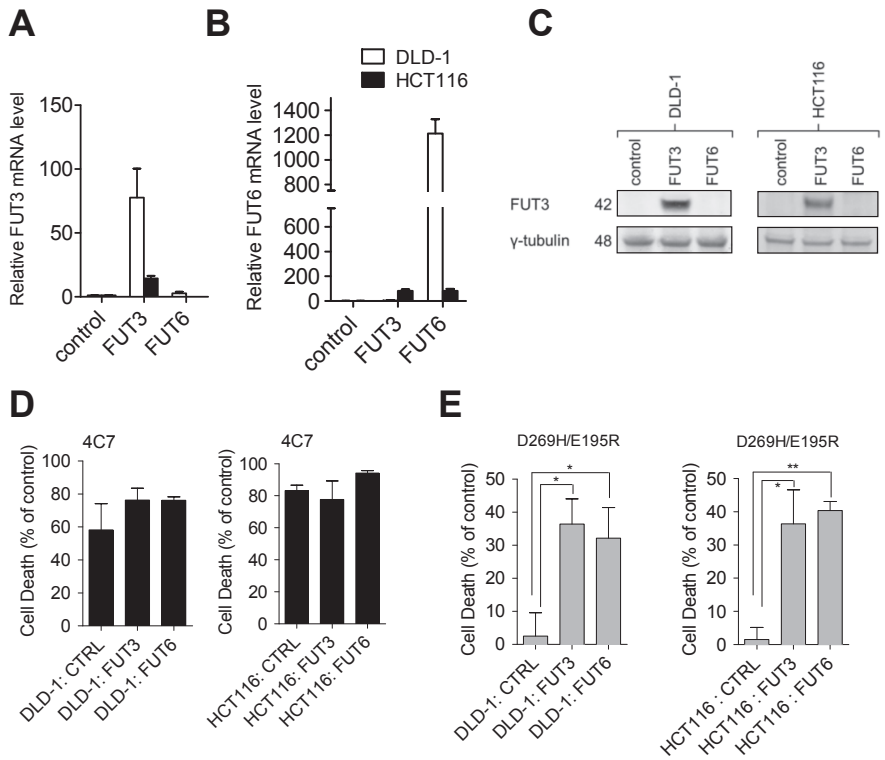


**Figure 3. FUT3 and FUT6 overexpression enhances TRAIL sensitivity of DLD-1 and HCT116 cells via both death receptors.** Overexpression of FUT3 and FUT6 was analysed by qRT-PCR of DLD-1 and HCT116 cells transduced with control, FUT3 or FUT6 overexpressing plasmid. The amount of FUT3 (**A**) or FUT6 (**B**) amplicon was relative to the endogenous reference RPL27 and normalized to the control cells. (**C**) Western blot analysis of control or FUT3 transduced DLD-1 and HCT116 cells. Lysates were examined for FUT3 expression levels; γ-tubulin served as a loading control. Cell death of transduced DLD-1 and HCT116 cells overexpressing FUT3 or FUT6 was assessed after treatment with 50 ng/mL rhTRAIL 4C7 (**D**) or D269H/E195R (**E**) for 16 h as measured by MTS assay. The data are presented as mean values ± S.D. (*n=3*).

indicating that DR4-mediated apoptosis is also improved by the overexpression of these fucosyltransferases (supplementary figure 2).

### Activation of the salvage pathway by L-fucose treatment augments TRAIL sensitivity primarily via DR5

Although total fucosyltransferase protein expression levels are important for triggering TRAIL-mediated apoptosis, targeting the *de novo* pathway or inducing the salvage pathway by increasing the level of donor substrate GDP-fucose may also enhance fucosylation. Recently, Moriwaki *et al*. showed that HCT116 cells are less able to synthesize GDP-fucose, due to mutations in the *GMDS* gene that plays a critical role in the *de novo* GDP-fucose pathway [25]. We therefore investigated whether the salvage pathway could

be activated by the addition of L-fucose, and thereby potentially sensitize these cells to TRAIL-induced apoptosis. To do this, DLD-1 and HCT116 cells were firstly incubated with several concentrations of L-fucose (0-100 mM). Significant levels of apoptosis (relative to control) were not detected after incubating HCT116 with several concentrations of L-fucose for 48 h (supplementary figure 3B-D). However, we did detect a decrease in the proliferation rate of both DLD-1 and HCT116 cells, especially at high concentrations of L-fucose (>50 mM) (supplementary figure 3A).

Next, we investigated the effects of L-fucose incubation on death receptor surface expression, using flow cytometry. Indeed, DLD-1 and HCT116 cells showed no increase in DR4 and DR5 levels after treatment with 50 mM L-fucose, when compared to cells left untreated (figure 4A). DLD-1 and HCT116 cells were then pre-incubated with L-fucose, and after 24 h further incubated with rhTRAIL WT, 4C7 or D269H/E195R for 16 h (0.05-500 ng/mL). This combination treatment revealed that the induction of DR5-mediated cell death is clearly enhanced upon pre-treatment with L-fucose in both cell lines (figure 4B,C), with rhTRAIL WT and rhTRAIL D269H/E195R activities increasing in the presence of L-fucose across different concentrations of rhTRAIL. In contrast with the increase in apoptosis seen in FUT3/6 overexpressing cells, we could not detect any enhancement of TRAIL-induced apoptosis in rhTRAIL 4C7 treated cells after L-fucose treatment (supplementary figure 4). Taken together, these results indicate that L-fucose primarily sensitizes DLD-1 and HCT116 to apoptosis, mainly through the activation of DR5.



**FUT3 and FUT6 overexpression leads to pre-clustering of DR4 and DR5 and enhances caspase-8 activation**

We investigated whether fucosylation enhanced TRAIL sensitivity by pre-clustering of DR4 and DR5 on the cell surface using immunofluorescent staining. Indeed, DLD-1:FUT3 and DLD-1:FUT6 cells showed more pre-clustering of DR4 and DR5 compared to control cells (figure 5A). In addition, we tested the activation of caspase-8 and PARP by Western blot to assess whether fucosylation modified DLD-1 cells up-stream or down-stream in the apoptotic pathway. Transduced DLD-1 cells were treated with 500 ng/mL rhTRAIL WT, 4C7 or D269H/E195R for 1h. Afterwards, Western blot analysis of caspase-8 and PARP activation profiles demonstrated that cleavage of these proteins was more pronounced in FUT3 and FUT6 overexpressing cells compared to control (figure 5B). These results confirm that fucosylation by FUT3 and FUT6 enhances TRAIL-induced apoptosis by modifications up-stream of caspase-8.

## DISCUSSION

TRAIL is an interesting anticancer therapeutic agent, as it selectively induces apoptosis in tumour cells without affecting normal cells and tissues [3, 4]. However, several reports have shown that approximately 50% of human colorectal cancer cell lines are highly resistant to TRAIL-induced apoptosis [19, 20]. Recently, fucosylation has been associated

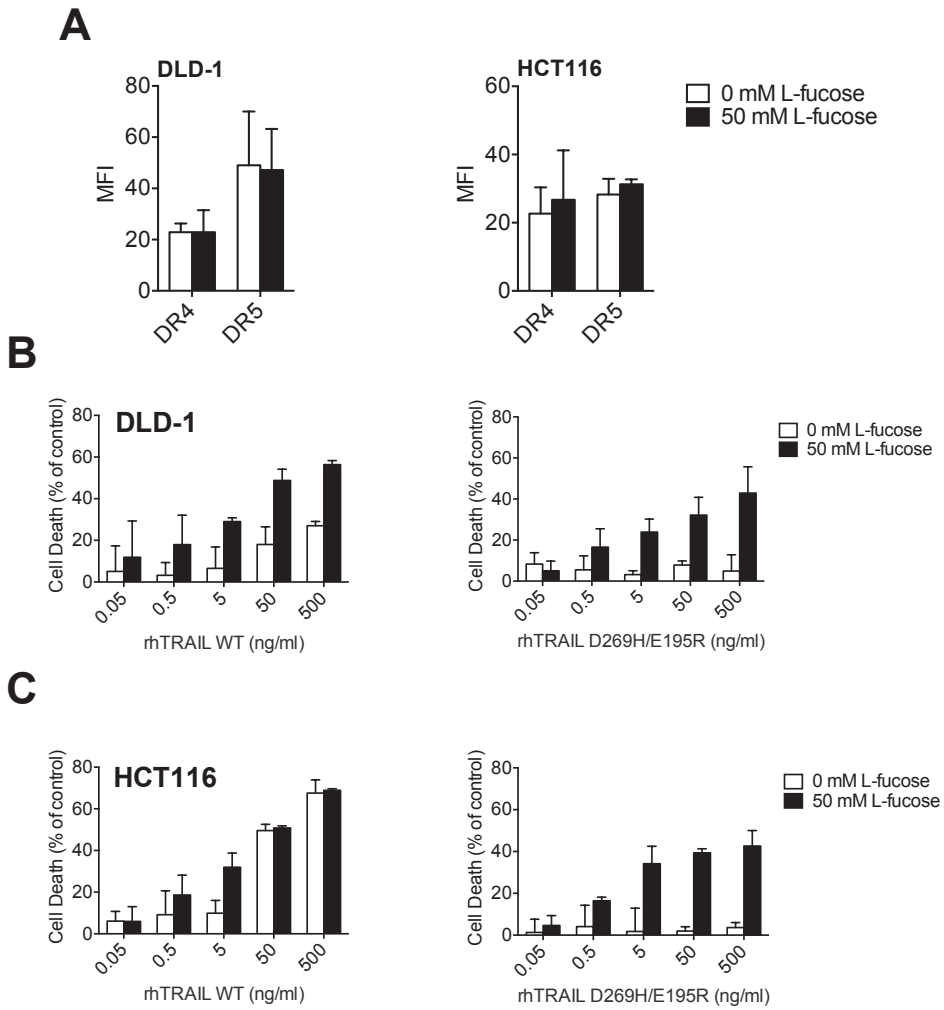


**Figure 4. L-fucose treatment augments TRAIL-induced apoptosis predominantly via activation of DR5.** (**A**) Cell surface expression of TRAIL receptors was determined in DLD-1 and HCT116 cells treated with 0 or 50 mM L-fucose using flow cytometry analysis and expressed as the Mean Fluorescence Intensity (MFI) ratio compared to binding of isotype antibody. DLD-1 (**B**) and HCT116 (**C**) were pre-treated with 0 or 50 mM L-fucose for 24 h and subsequently incubated with 0.05-500 ng/mL rhTRAIL WT or D269H/E195R for another 16 h. Cell death was assessed using MTS assay. The data are presented as mean values ± S.D. (*n=3*).

with TRAIL signalling in colon cancer. Specifically, FUT3 and FUT6 expression was shown to correlate with TRAIL sensitivity in a large panel of colorectal adenocarcinoma cell lines [19]. Moreover, fucosylation was recently found to decelerate tumour growth and metastasis *in vivo* [25], due to formation of FADD-dependent complex II at both death receptors [26]. Here, we investigated the role of fucosylation in the specific TRAIL apoptosis induction via either DR4 or DR5, using designed receptor-specific TRAIL variants.

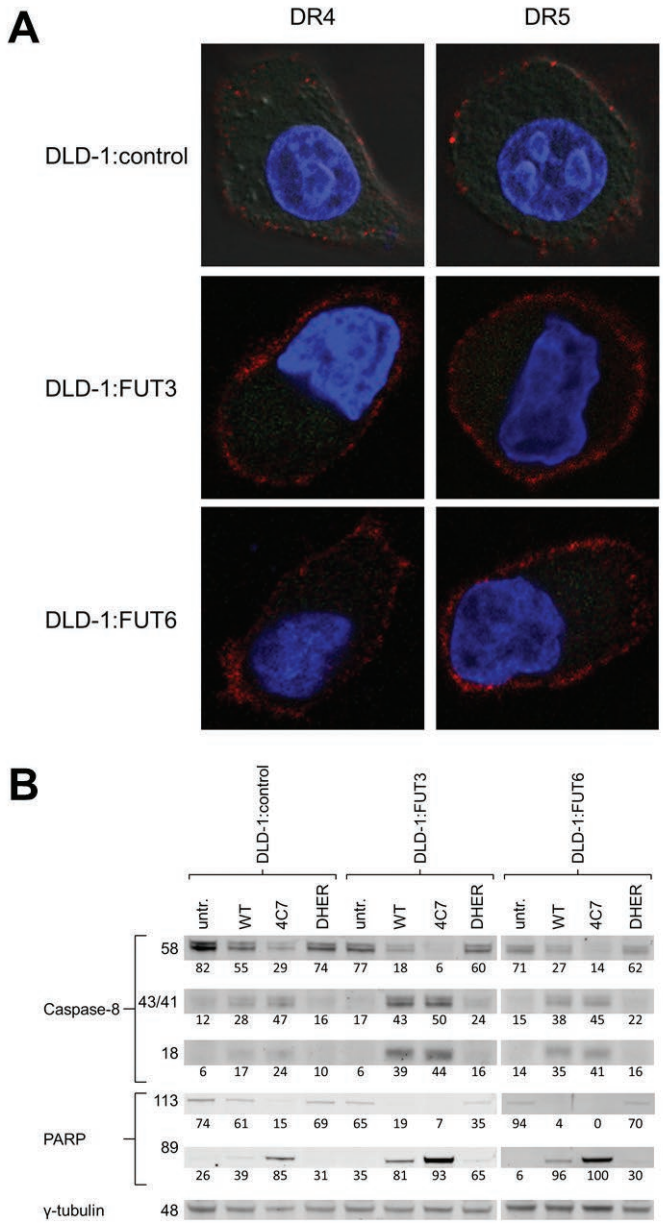


**Figure 5. FUT3 and FUT6 overexpression leads to clustering of DR4 and DR5.** (**A**) Immunostaining of DLD-1 cells transduced with control, FUT3 or FUT6 plasmids, containing the *GFP* gene. Cells were seeded on poly-L-lysine coated coverslips and stained with primary antibodies against DR4 or DR5, and secondary antibody conjugated with Alexa Fluor 647. Nuclei were counterstained with DAPI. (**B**) Western blot analysis of FUT3 or FUT6 transduced DLD-1 cells treated with 500 ng/mL rhTRAIL WT, 4C7 or D269H/E195R for 1h. Caspase-8 and PARP activation was examined and analysed using densitometry (values depicted as % of total protein); γ-tubulin served as a loading control.

Contrasting to the high sensitivity to both DR4- and DR5-mediated apoptosis in the colon adenocarcinoma Colo205 cell line (figure 1A), DLD-1 and HCT116 cells were shown to be highly insensitive to DR5-mediated apoptosis (figure 1B,C). The preference for death receptor-signalling via DR4 in these cell lines was not related to differential surface levels of both death receptors of TRAIL (figure 1D).

Given the differences in the mRNA levels of FUT3 and FUT6 reported by Wagner *et al*. using several colon adenocarcinoma cell lines [19], we hypothesized that the low expression of *O*-glycan processing FUT3 and FUT6 in both DLD-1 and HCT116 could be responsible for the insensitivity of these cells to DR5-mediated apoptosis. General inhibition of *O*-glycosylation by pre-treatment with the inhibitor bGalNAc led to decreased TRAIL sensitivity via DR5, as determined using TRAIL-death receptor specific variants (figure 2). Reciprocally, the enhanced fucosylation upon transient overexpression of FUT6-GFP re-sensitized DLD-1 and HCT116 cells to DR5-mediated apoptosis (supplementary figure 1). DLD-1 and HCT116 stably overexpressing FUT3 and FUT6 cells showed mainly enhanced TRAIL-mediated cell death via activation of DR5 (figure 3). These results clearly indicate that low FUT3 and FUT6 expression levels render colon adenocarcinoma cells completely resistant to DR5- but not DR4-mediated apoptosis, and that overexpression of either fucosyltransferases enhances TRAIL sensitivity via both death receptors. Interestingly, while FUT3 and FUT6 overexpression proved to be very important for DR5-induced apoptosis in DLD-1 and HCT116 cells, it does not seem to be as important to the overall sensitivity via DR4. Flow cytometry analysis demonstrated that the enhanced TRAIL sensitivity could not be assigned to an increase in death receptor surface expression levels (data not shown).



We also demonstrate here that incubation with L-fucose efficiently reduced cell proliferation and initiates the salvage pathway, which restores DR5-mediated apoptosis in DLD-1 and HCT116 cells (figure 4B-C and supplementary figure 4). L-Fucose treatment proved to enhance TRAIL-induced cell death independent of death receptor surface expression levels (figure 4A). These results are of interest since dietary fucose therapy is a simple, non-invasive therapy that was found to successfully increase fucosylation in a patient with leukocyte adhesion deficiency type II (LAD II), a rare inherited disorder of fucose metabolism [33]. Therefore, targeting the salvage pathway may provide a simple treatment for cancers in which the *de novo* GDP-fucose pathway is inactivated.

Finally, we demonstrate that pre-clustering of DR4 and DR5 occurs more in DLD-1:FUT3 and DLD-1:FUT6 cells compared to control cells (figure 5A). Western blot analysis of caspase-8 and PARP activation profiles shows that both proteins are more rapidly cleaved in FUT3 and FUT6 overexpressing cells compared to control (figure 5B), indicating modifications up-stream of caspase-8. However, more experiments are needed in order to determine the specific molecular mechanisms that explain the differential activation of DR4 and DR5.

In conclusion, enhancement of fucosylation by FUT3 and FUT6 overexpression or L-fucose treatment restored DR5-mediated apoptosis, which is, at least in part, due to pre-clustering of DR4 and DR5 on the membrane. Our findings indicate that fucosylation represents a promising novel approach for restoring TRAIL sensitivity in resistant cancer cells.

## ACKNOWLEDGEMENTS

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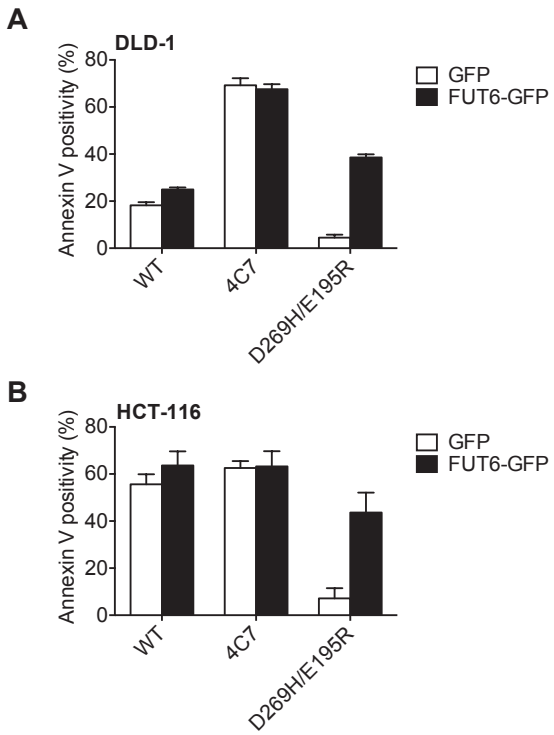
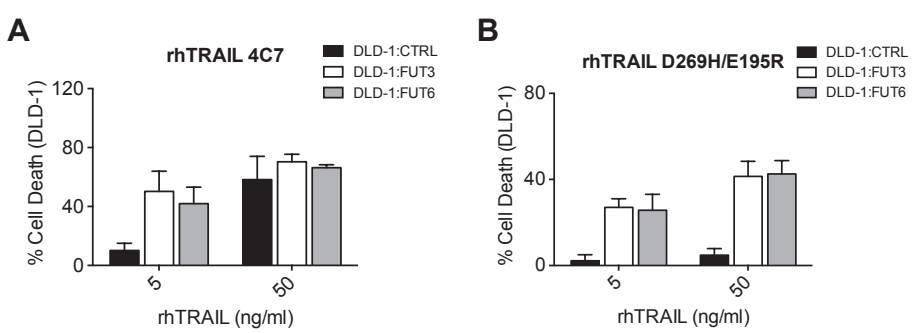
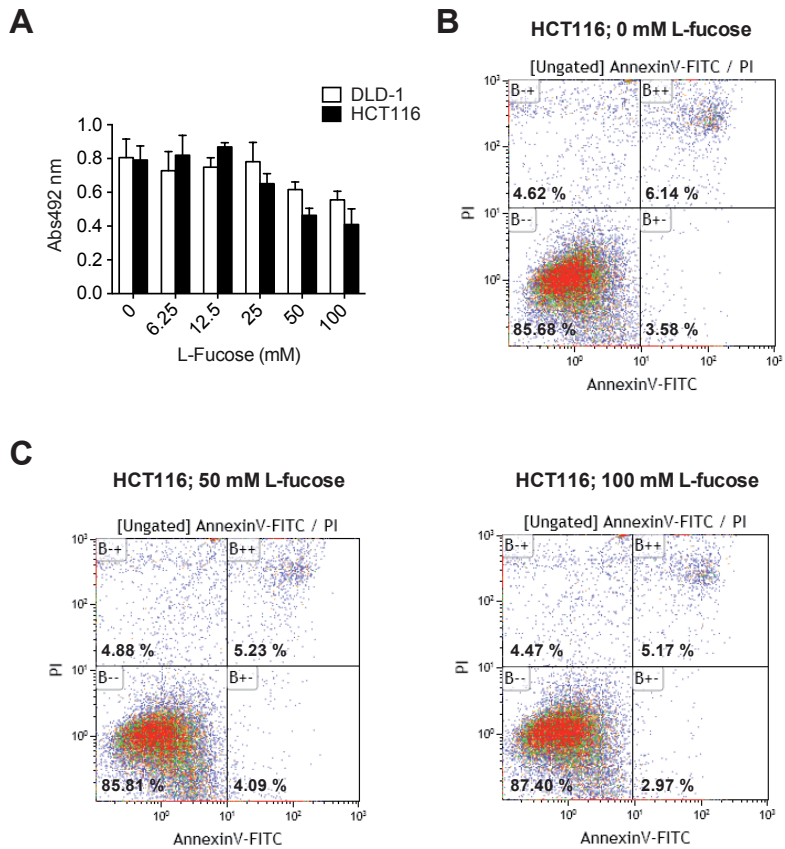
## SUPPLEMENTARY INFORMATION



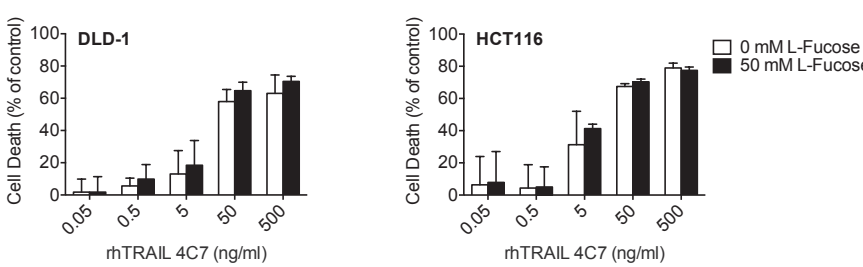

**Supplementary figure 1. Transient overexpression of FUT6-GFP increases TRAIL sensitivity via DR5.** DLD-1 (**A**) and HCT116 (**B**) cells transiently transfected with the FUT6-GFP construct were treated with 50 ng/mL rhTRAIL WT, 4C7 and D269H/E195R. Apoptosis induction was assessed after 16 h using Annexin V-APC staining measured by flow cytometry. The data are presented as mean values ± S.D. (*n=2*).




**Supplementary figure 2. FUT3 and FUT6 overexpression enhances TRAIL sensitivity of DLD-1 cells via both death receptors.** Cell death of transduced DLD-1 cells overexpressing FUT3 or FUT6 was assessed after treatment with 5 or 50 ng/mL rhTRAIL 4C7 (**A**) or D269H/E195R (**B**) for 16 h as measured by MTS assay. The data are presented as mean values ± S.D. (*n=3*).




**Supplementary figure 3. L-Fucose treatment reduces proliferation rates of DLD-1 and HCT116.** (**A**) DLD-1 and HCT116 cells were treated with 0-100 mM L-fucose. Proliferation was determined after 48 h treatment by MTS assay. Apoptosis inducing potential of 0 (**B**), 50 (**C**) or 100 (**D**) mM L-fucose in HCT116 was determined after 48 h treatment using Annexin V-FITC and PI using flow cytometry. The data are presented as mean values ± S.D. (*n=3*).




**Supplementary figure 4. Pre-treatment with L-fucose slightly enhanced cell death induction by DR4-specific rhTRAIL 4C7.** DLD-1 and HCT116 were pre-treated with 0 or 50 mM L-fucose for 24 h and subsequently incubated with 0.05-500 ng/mL rhTRAIL 4C7 for another 16 h. Cell death was assessed using MTS assay. The data are presented as mean values ± S.D. (*n=3*).