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Original Articles

TRAIL receptor activation overcomes resistance to trastuzumab in HER2 positive breast cancer cells

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ABSTRACT

The appearance of resistance to the anti-HER2 targeted drug trastuzumab constitutes, nowadays, an important challenge in the oncology clinic. To fight such resistance, we searched for potential vulnerabilities in cells resistant to that drug. To that end, we used cell lines primary resistant to trastuzumab, as well as cells made secondarily resistant to the drug upon continuous exposure. Using genomic and proteomic approaches, a deregulation in cell death pathways was identified in trastuzumab-resistant cells. More precisely, an increased response to the death factor TRAIL, caused by an increase in the cellular receptors for this factor, was observed. In parallel, a decrease in inhibitory components of the pathway was detected. This combination produces a more efficient assembly of the functional complex in the trastuzumab-resistant cells that translates in the observed increased response to TRAIL. Analysis of HER2 positive patient samples confirmed deregulation of this pathway in trastuzumab-resistant patients. Taken together our data identify a vulnerability of trastuzumab-resistant cells that could be used to design new targeted therapies in that context.

1. Introduction

Breast cancers require pathological analyses to define the presence/absence of markers that guide therapeutic interventions. One group of breast tumors, comprising 20% of total breast cancers, is characterized by the presence of elevated levels of HER2, a transmembrane tyrosine kinase with oncogenic properties in preclinical models [1]. Those facts led to the development of therapies aimed at targeting this tyrosine kinase receptor [2,3]. Two types of drugs targeting HER2 have reached the clinic [2,4]. One strategy is based on antibodies directed against the extracellular domain of the receptor, while the second is based on small membrane-permeant molecules that specifically inhibit the tyrosine kinase activity of HER2. The first group of molecules includes trastuzumab and biosimilars [5], pertuzumab [6], and the antibody-drug conjugate trastuzumab-emtansine (T-DM1) [7,8]. The second includes lapatinib [9] or neratinib [10].

Trastuzumab-based therapeutic regimens, which include combinations with a chemotherapeutic agent, often a taxane, are first line standard of care options for patients with HER2+ breast cancer tumors [11]. Despite the fact that trastuzumab has revolutionized the prognosis of HER2+ breast tumors, one problem frequently found in the metastatic setting is the appearance of resistance to the drug which can be present from the beginning of the treatment (primary or de novo resistance) or developed along the treatment (secondary or acquired/adaptative resistance). If resistance develops, second line treatment options such as T-DM1 [12], neratinib, or a combination of lapatinib with capecitabine [13] are available. However, in most of the cases, these therapies fail to produce long lasting remissions, and disease relapses after a few months. Therefore, alternative strategies should be investigated for the therapy of trastuzumab-resistant tumors.

Several groups have reported studies describing mechanisms of resistance to anti-HER2 therapies [14–16] and defined resistance mechanisms depending on molecular alterations of HER2, such as deletions of its extracellular domain [17,18], and HER2-independent mechanisms of resistance, including gain of function alterations of downstream signaling pathways [19,20]. In fact, those reports have

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established the bases for the clinical evaluation of agents that target some of these pathways, as PI3K route inhibitors [21].

Aiming at better understanding the molecular bases of trastuzumab resistance, we generated models of resistance by continuous exposure of HER2 positive breast cancer cells to that drug. Genomic analyses followed by functional validation allowed identification of the TRAIL pathway as a druggable vulnerability that could be explored as a therapeutic strategy to overcome trastuzumab resistance.

2. Materials and methods

2.1. Reagents and antibodies

Cell culture media and MTT were purchased from Sigma, fetal bovine serum (FBS) and antibiotics from Life Technologies, Immobilon-P membranes from Millipore Corp and JetPEI™ reagent from Polyplus-transfections SA. Trastuzumab was obtained from the pharmacy. Other generic chemicals were from Sigma Chemical Co., Roche Biochemicals or Merck.

The anti-GAPDH (sc-166574), anti-PARP (sc-8007), anti-MCL1 (sc-20679), anti-PY (sc-7020), anti-ERK1/2 (sc-154), anti-pERK1/2 (sc-7383), anti-BAX (sc-493) and anti-FLIP (sc-8347) antibodies were purchased from Santa Cruz Biotechnology; anti-Caspase 3 (#610323), anti-XIAP (#610716) and anti-cIAP1 (#556533) and from BD Biosciences; anti-Caspase 8 (#9746), anti-Caspase 9 (#9502), anti-cleaved Caspase 3 (#9664), anti-Caspase 7 (#9492), anti-Bid (#2002), anti-AKT (#9272), anti-pAKT (#560397) and anti-FLIP (#8837) from Cell Signaling technologies; anti-HER2 (#OP15) from Calbiochem; anti-Calnexin (SPA-860) from Stressgen Bioreagents corporation and anti-FADD (#06–711) from Millipore.

Anti-DR4 and anti-DR5 specific antibodies were generated using GST fusion proteins which included amino acids 261–339 of DR4 or 242–313 for DR5. Purified fusion proteins were injected into rabbits following a previously described protocol [22]. Animals were manipulated according to Institutional guidelines for the Use of Laboratory Animals (University of Salamanca), with permission from the Ethical Committee for Animal Experimentation of the University and according to current Spanish laws on animal experimentation.

2.2. Cell lines, cell culture and viral infections

Human cell lines were obtained from the ATCC and grown in DMEM (BT474, BT-RH, SKBR3 and 293T) or RPMI medium (HCC1419, HCC1569, HCC1954, HCC2218 and MM1S) supplemented with 10% FBS.

To overexpress DR4, DR5 and FLIP, retroviral infections were used. cDNAs for DR4, DR5S or DR5L were amplified from MM1S cells [23] and subcloned into the retroviral vector pLZR-IRES-GFP-puro. pBABE-FLIP was generously gifted by Dr. Lopez-Rivas. Knockdown experiments were performed by lentiviral transduction with commercial vectors containing shRNAs for DR4 and DR5 (Open Biosystems) or donated by Dr. Lopez-Rivas ($FLIP_L$). Virus generation and infections were done as described [24].

2.3. Protein extraction, immunoprecipitation (IP), Western blot (WB), TRAIL treatment, and DISC pull-down

Cells were washed with PBS, lysed and IP and WB performed with the indicated antibodies as described [25].

Recombinant TRAIL was produced and quantified as described [26]. For TRAIL treatment experiments, cells were maintained for two days in the presence of 2 μg/ml TRAIL, unless otherwise indicated.

For co-IP, 5×10^6 BT474 or BT-RH cells were treated for 1 h with TRAIL or vehicle, lysed, protein quantified and 2 mg of cell extracts immunoprecipitated with anti-DR4 or -DR5 antibodies. For the pull-down experiments, 2 μg/ml biotinylated TRAIL were added to the cells for 30 or 60 min. For control time 0, cells were kept on ice, and biotinylated TRAIL added for 10 min on ice. Cells were lysed, protein quantified and the same amount in all the conditions precipitated with 50 μl of streptavidin-agarose (Sigma-Aldrich) for 3 h at 4 °C.

2.4. Cell cycle, proliferation, and apoptosis assays

Cell cycle analysis and apoptotic cell death were assessed as previously described [25]. To analyze cell proliferation conventional MTT assays [27] or cell counting experiments using a Z1 Coulter Particle Counter were performed.

2.5. Functional genomic and transcriptomic profiling

Isolated RNA was hybridized to HG-U133 plus 2.0 GeneChip oligonucleotide arrays (Affymetrix) and scanned as described [28]. Unprocessed files were normalized using the RMA algorithm implemented in the Affymetrix Expression Console 4. Cut-off for differentially expressed genes (DEGs) was ≥ 2 fold change and a P value ≤ 0.05.

Gene set enrichment analysis (GSEA) was performed to identify genesets with expression alterations [29]. The network of genesets interactions was constructed using Cytoscape software (version 3.6.0). Only genes with maximum 0.05 p-value differential expression were selected. Microarray data are now available through the GEO repository database (reference GSE 119397).

2.6. qPCR, PCR arrays and apoptosis antibody array

For quantitative RT-PCR (qRT-PCR), RNA was primed with oligo (dT) and cDNA synthesized with MMLV reverse transcriptase (Promega). qRT-PCR reactions were performed using iQTM SYBR Green Supermix (Bio-Rad Laboratories) and recorded with the Bio-Rad iQ5 software. Relative quantitation of gene expression was performed as described [30] and results normalized with GAPDH.

To analyze apoptosis pathway proteins the Human Apoptosis RT^2 Profiler PCR Array (Qiagen) was used following manufacturer instructions. Relative gene expression was normalized with GAPDH and compared to the parental cells using the 2ˆ(-ΔΔCt) method. For the graphical representation values smaller than one were transformed in their derived reciprocal and opposite (negative) numbers.

To determine the levels of different apoptotic proteins, the Proteome Profiler Human Apoptosis Array Kit (R&D Systems) was used following manufacturer's instructions. Quantitation of pixel densities was performed using the Image Studio V5.2 program (LI-COR).

2.7. Protein and gene expression correlation studies

DISC expression was defined by WB quantitation of the five DISC components (DR4, DR5, FADD, caspase 8 and FLIP) with the ImageJ software and its normalization to BT474 levels. Then, the amount of the four positive components was added, and that of the inhibitor Flip, subtracted to obtain the measure of DISC expression.

For patient analysis, mRNA data were extracted from the Gamez-Pozo study (GSE44272 [31]) and normalized against the most sensitive patient (the longest relapse free survivor) levels. DISC expression value was calculated as before.

2.8. Statistical analyses

In proliferation experiments each condition was analyzed in triplicate and data presented as mean ± SD of at least 2 independent experiments. Comparisons of continuous variables between two groups were performed using a two-sided Student's t-test. $P < 0.05$ was considered a statistically significant difference.

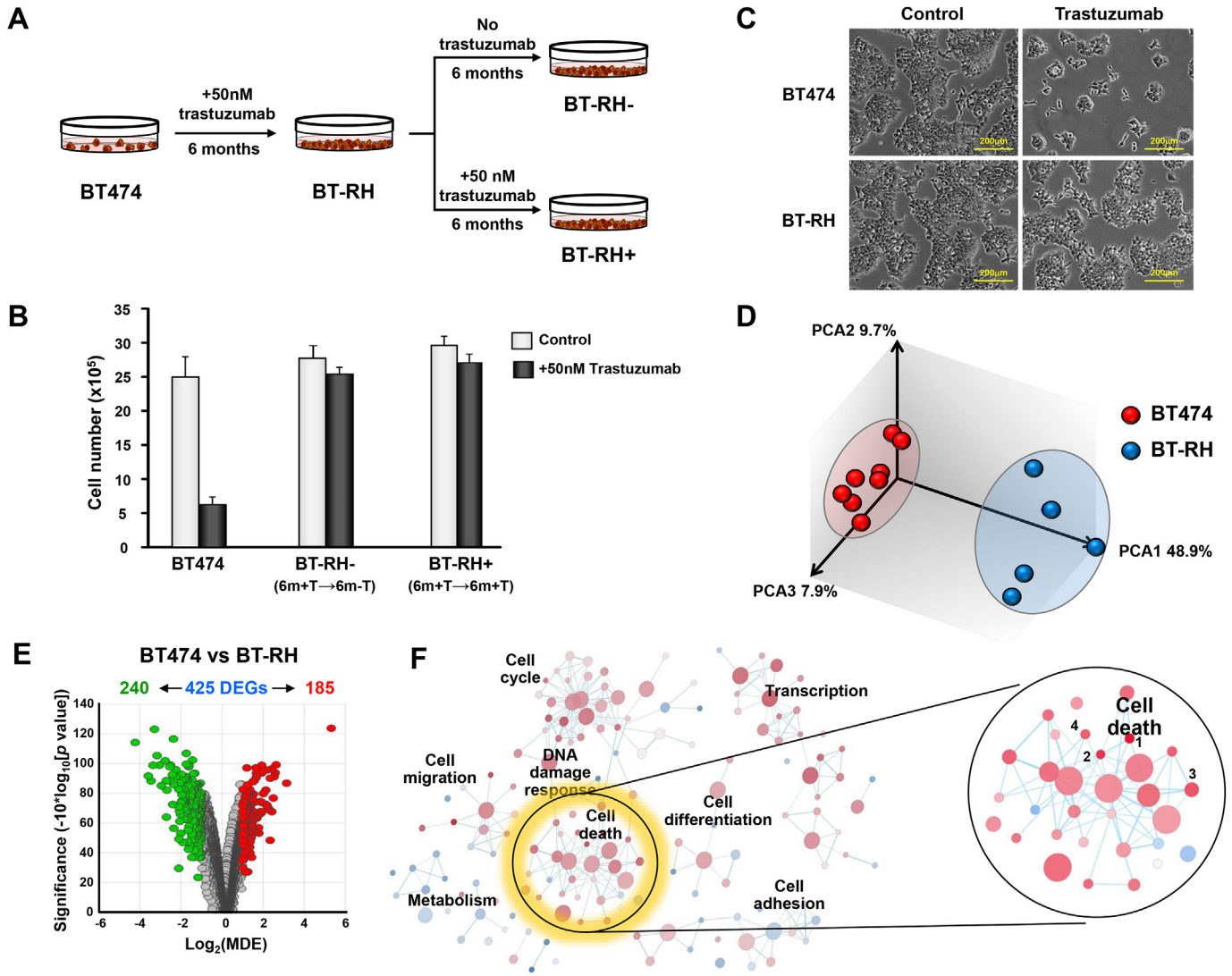

Fig. 1. Generation and characterization of trastuzumab-resistant HER2+ cells. (A). Generation of trastuzumab resistant cells. BT474 cells were treated for 6 months with 50 nM trastuzumab. (B) Pools of trastuzumab resistant cells (BT-RH- or BT-RH+) were collected and their response to an acute treatment with trastuzumab (10 days, 50 nM) was verified in cell counting experiments and compared to control previously untreated cells. (C) The general aspect and response of both populations was observed at the microscope. (D) Genomic characterization of trastuzumab resistant cells. RNA of BT474 and BT-RH cells was isolated and hybridized to Affymetrix oligonucleotide arrays. Principal component analysis (PCA) of variance between signal data is represented and replicates for each sample are shown in the same color. (E) Differentially expressed genes (DEGs) in BT-RH versus BT474 cells. Cut-off for DEGs was ≥2 fold change and a *P* value ≤ 0.05. Genes meeting both criteria are colored green, if downregulated, or red, if upregulated. The y-axis represents the significance as $-10*\log_{10}$ (*P*value) and x-axis the $\log_2$ of the mean differential expression (MDE). (F) Gene set enrichment expression network designed using Cytoscape to compare BT474 and BT-RH cells. Cell death genesets are clearly deregulated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Identification of vulnerabilities in trastuzumab-resistant cells

To identify vulnerabilities that could help in overcoming trastuzumab resistance, an in vitro cellular model of trastuzumab resistance was established. The prototypical BT474 HER2+ breast cancer cell line was plated at low density and grown in 50 nM trastuzumab (7.5 μg/ml) for 6 months (Fig. 1A). Trastuzumab-resistant cells were pooled, generating a population named BT-RH ("Resistant to Herceptin™"). BT-RH cells were maintained in the presence (BT-RH+) or absence (BT-RH-) of trastuzumab for 6 additional months. The proliferation of BT474 cells was inhibited by trastuzumab while BT-RH- and BT-RH+ cells grew similarly in the presence or absence of trastuzumab (Fig. 1B). For the last five years, the resistance to trastuzumab of BT-RH- cells has been stable, even though these cells are regularly cultured in the absence of the drug (data not shown). Morphological inspection of cultures of BT-RH- cells (hereon called BT-RH) showed a similar aspect to parental BT474 cells and confirmed refractoriness of BT-RH cells to the anti-proliferative effects of trastuzumab (Fig. 1C). Such refractoriness could not be due to down-regulation of HER2, since BT474 and BT-RH cells expressed similar levels of that protein (Supplementary Fig. 1). Moreover, no differences were observed between both cell lines in the amounts of pHER2, pAKT, or pERK1/2.

Transcriptomic studies were performed to explore differences between BT474 and BT-RH cells. Principal component analysis (PCA) of microarray data showed differences between them (Fig. 1D). These studies defined the presence of 425 deregulated genes (DEGs) between both cell lines (Fig. 1E and Supplementary Table 1). Of them, 185 genes were up-regulated in BT-RH cells while 240 were down-regulated. To dissect the functional consequences of the genomic alterations present in trastuzumab-resistant cells, GSEA analyses were performed and

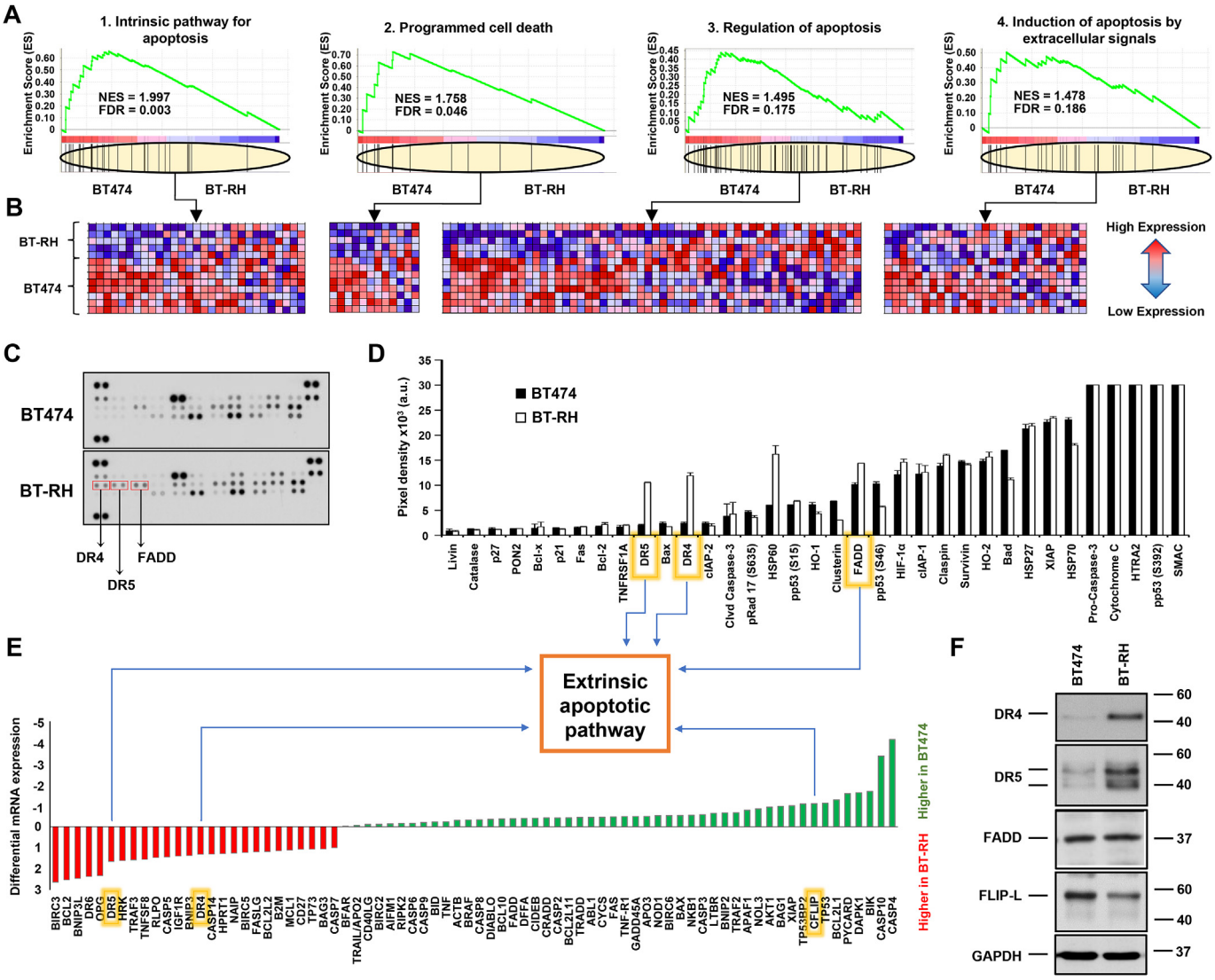

Fig. 2. Cell death is deregulated in BT-RH cells. (A) Enrichment score (ES) profile and location of GeneSet members on the rank ordered list of the cell death related pathways with the highest scores. (B) Blue-Pink diagram of genes included in cell death related pathways with the highest ES. Overexpressed genes are displayed in shades of pink-red and downregulated ones in shades of blue. (C) Image of the antibody array used to evaluate several apoptotic protein levels. (D) Quantification of protein levels from the array shown in C. (E) qPCR comparative expression level of 84 genes involved in apoptosis. Yellow highlighted genes are deregulated between BT474 and BT-RH in both analyses and are constitutive members of extrinsic apoptotic pathways, specifically of TRAIL pathway. (F) Protein levels of several proteins involved in TRAIL signaling such as DR4 and DR5 receptors, FLIP or FADD in BT474 or BT-RH cells by WB analysis. GAPDH was used as a loading control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

resulted in a functional transcriptomic map that indicated deregulation of cellular functions related to cell death, metabolism, migration, DNA damage response, cell cycle, transcription, differentiation or adhesion (Fig. 1F).

Acting on cell death mechanisms appeared attractive because operating on them could result in tumor cell death. Moreover, drugs targeting proteins involved in cell death are available [32,33]. Further genomic and proteomic analyses aimed at identifying actionable targets within cell death routes were carried out and showed deregulation of programmed cell death genes, including some that participate in the intrinsic and extrinsic apoptotic routes (Fig. 2A). Deregulated cell death-related genes included in those gene sets are represented by columns in Fig. 2B and detailed in Supplementary Table 2. In parallel, a commercially available antibody array allowed exploration of the levels of apoptotic proteins in BT474 and BT-RH cells (Fig. 2C and D). These studies showed that the most deregulated proteins corresponded to TRAIL receptors Death Receptor 4 (DR4) and Death Receptor 5 (DR5), as well as Hsp60. Besides, increased levels of the adaptor protein FADD in BT-RH cells were shown. qPCR expression studies of 84 apoptotic genes confirmed higher levels of DR4 and DR5 in BT-RH cells (Fig. 2E). Besides the TRAIL pathway inhibitor FLIP was down-regulated in BT-RH cells. Higher levels of DR4 and DR5 as well as lower levels of FLIP in BT-RH cells were also confirmed by WB (Fig. 2F). DR4 migrated as a single band, while DR5 appeared as a doublet, due to the presence of two alternatively spliced forms [34]. GSEA analysis of TRAIL pathway demonstrated that multiple genes of this route were deregulated in BT-RH cells (Supplementary Fig. 2).

3.2. Augmented sensitivity of BT-RH cells to TRAIL

These data indicated that several genes participating in the extrinsic apoptotic pathway triggered by death ligands such as TRAIL were deregulated in BT-RH cells. We postulated that since resistant cells expressed higher levels of the receptors and lower levels of the inhibitor FLIP, such altered levels could represent a vulnerability susceptible of exploitation to overcome trastuzumab resistance (Fig. 3A). To explore

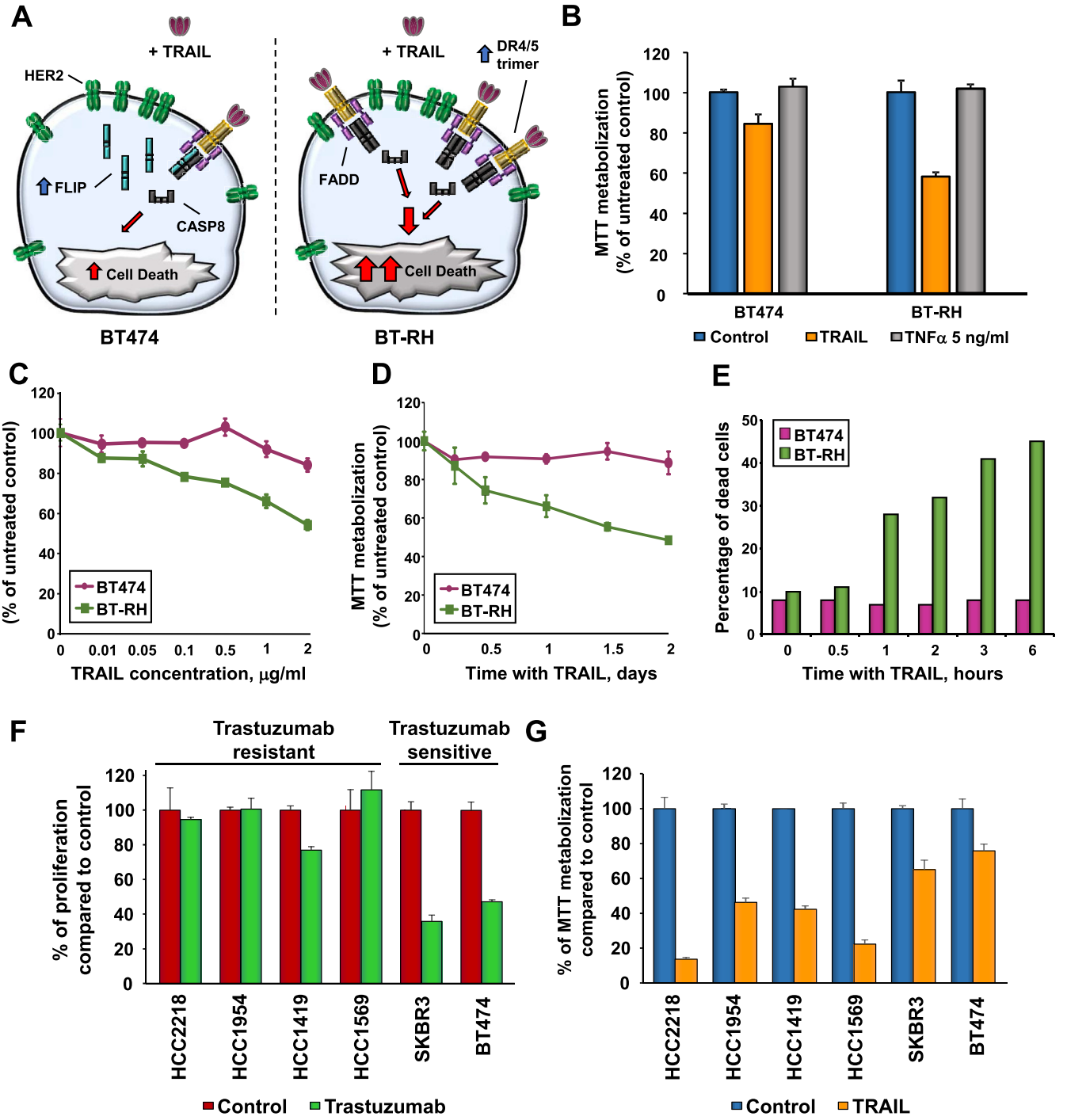

Fig. 3. BT-RH cells are more sensitive to the trophic factor TRAIL. (A) Schematic representation of BT474 and BT-RH cells in terms of their content in TRAIL apoptotic pathway components and cell surface receptors in both cells. (B) TRAIL (2 μg/ml) causes a decrease in cell proliferation in BT-RH cells larger than in BT474 when analyzed in MTT metabolization experiments. Treatment with TNFα had no effect on any of these cell lines. (C, D) The differential effect of TRAIL on BT-RH proliferation was both time (C) and dose (D) dependent, as demonstrated in MTT experiments. (E) Such effect was due to an increase in cell death, as demonstrated in flow cytometry experiments. Data were acquired, the percentage of death cells calculated and represented in the graph. An increase of double stained Annexin V/PI population was detected only in BT-RH TRAIL treated cells. Data from a representative experiment repeated at least twice are shown. (F) Susceptibility of HER2+ cell lines to trastuzumab. Cells were plated in 6 well plates and treated with 50 nM trastuzumab for 10 days. Cells were then detached and counted in a Z1 Coulter particle counter and cell numbers normalized to untreated controls. (G) Analysis of cell sensibility to TRAIL in those cell lines by MTT assay. Data are represented as mean ± SD of triplicates of an experiment that was repeated at least twice.

that possibility BT474 and BT-RH cells were treated with TRAIL and their proliferation assessed in MTT metabolization assays. Incubation of BT474 cells with TRAIL slightly reduced their MTT metabolization (Fig. 3B). Similar treatment of BT-RH cells had a much more pronounced effect. Treatment with TNFα, another death activating ligand expressed in these cell lines, did not affect MTT metabolization of BT474 or BT-RH cells (Fig. 3B), as expected by their equally low levels of the TNF receptor 1 (TNFRSF1A in Fig. 2D). The increased sensitivity of BT-RH cells to TRAIL was confirmed in dose-dependent and time-course experiments (Fig. 3C and D).

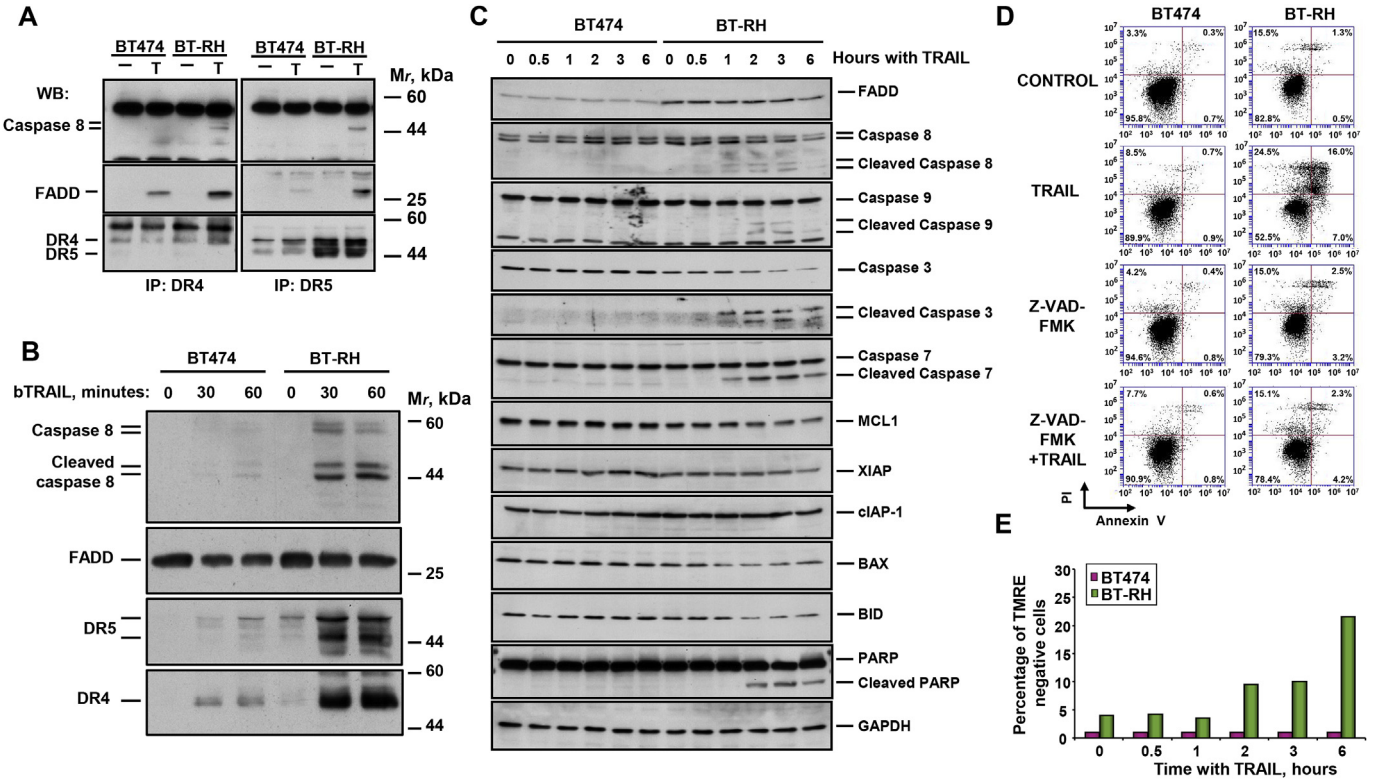

Fig. 4. An increased engagement of the functional DISC leads to augmented apoptosis in BT-RH cells. (A) The formation of the DISC is more efficient in BT-RH cells. BT474 and BT-RH cells were treated for 1 h with TRAIL, and regular co-IP experiments after TRAIL treatment (T) carried out using anti-DR4 (upper panel) or anti-DR5 (lower panel) antibodies. The WB were probed with anti-caspase 8 or -FADD antibodies. (B) DISC pull down experiments. BT474 and BT-RH cells were treated for the indicated times with biotinylated TRAIL (bTRAIL) and the functional complex precipitated by pull-down with streptavidin-sepharose. WB were probed with antibodies to Caspase 8, DR4, DR5, or FADD. (C) TRAIL treatment induces the activation of downstream caspases in BT-RH cells, but not BT474. Cells were treated with TRAIL for the indicated times and cell extracts prepared. The status and amount of different downstream proteins was analyzed by direct WB. GAPDH was used as a loading control. (D) TRAIL induced cell death is caspase dependent. BT474 or BT-RH cells were treated with TRAIL, the caspase inhibitor Z-VAD-MFK (50 μM) or both, and cell death determined by double staining with annexin V and PI, and flow cytometry analysis. (F) TRAIL induces mitochondrial membrane potential loss in BT-RH cells. Cells were treated with TRAIL and, at the indicated times, stained with 100 nM TRME. Loss of mitochondrial membrane potential was determined both on BT474 and BT-RH by flow cytometry. The percentage of cells that had loss such potential in shown in the graph. A representative experiment that was repeated twice is shown.

To analyze if TRAIL effect was due to stimulation of apoptotic cell death, BT474 and BT-RH cells were treated for different times (up to 6 h) with 2 μg/ml TRAIL and apoptotic cell death was measured by flow cytometry after Annexin V staining. The percentage of Annexin V-stained cells progressively increased along time in TRAIL-treated BT-RH cells (Fig. 3E). In contrast, TRAIL failed to provoke changes in Annexin V staining in BT474 cells.

3.3. Primary trastuzumab-resistant cells are sensitive to TRAIL

We next explored TRAIL sensitivity of other HER2+ cell lines. Trastuzumab inhibited the proliferation of SKBR3 cells similarly to BT474 (Fig. 3F). The proliferation of HCC1419 was slightly inhibited, while HCC1569, HCC1954 and HCC2218 were insensitive to this agent, indicating its primary or de novo resistance to trastuzumab. TRAIL treatment reduced MTT metabolization of all tested cell lines. In the trastuzumab-sensitive cell lines BT474 and SKBR3 the effect was less marked than in the primary trastuzumab-resistant ones (Fig. 3G).

3.4. DISC complex formation and apoptotic signaling in BT-RH cells

Stimulation of cell death by TRAIL involves engagement of DR4/DR5 within a multiprotein complex termed death-inducing signaling complex (DISC), which includes the receptors, procaspase 8 and the adaptor protein FADD [35,36]. DISC assembly is required for procaspase 8 cleavage that leads to generation of active caspase 8, needed for the activation of the extrinsic apoptotic cascade [37]. Assembly of the DISC was explored in BT474 and BT-RH cells by stimulation with 2 μg/ml TRAIL, and precipitation with anti-DR4 or anti-DR5 antibodies, followed by analyses of co-precipitating FADD and caspase 8 by WB. TRAIL stimulation attracted FADD to DR4 and DR5 in both cell types (Fig. 4A), although the degree of coprecipitating FADD was higher in BT-RH cells, and was only observed after TRAIL treatment. DR4 and DR5-associated caspase 8 was only detected in TRAIL-treated BT-RH cells. This result was confirmed by precipitation of biotinylated TRAIL with streptavidin sepharose, and detecting caspase 8, DR4, DR5 and FADD by Western (Fig. 4B).

In addition to caspase 8, WB analyses of other proteins involved in downstream apoptotic cell death showed induction of cleavage of caspases 9, 3, and 7 in TRAIL-treated BT-RH cells, but not in BT474 (Fig. 4C). Cleavage was detected 1 h after TRAIL treatment as evidenced by the appearance of faster migrating bands of those caspases. A decrease in BID or BAX was also observed in BT-RH cells treated with TRAIL. Biochemically, TRAIL treatment provoked proteolytic processing of PARP, a readout of caspase-mediated apoptotic signaling. Similar data were obtained at longer incubation times with TRAIL (Supplementary Fig. 3). The fact that the apoptotic death observed in the BT-RH cells was caspase dependent was supported by the elimination of TRAIL-induced cell death in BT-RH cells by preincubation with the pan-caspase inhibitor Z-VAD-FMK (Fig. 4D).

Mitochondrial membrane potential, indicative of mitochondrial integrity, was measured by flow cytometry by using the fluorescent dye

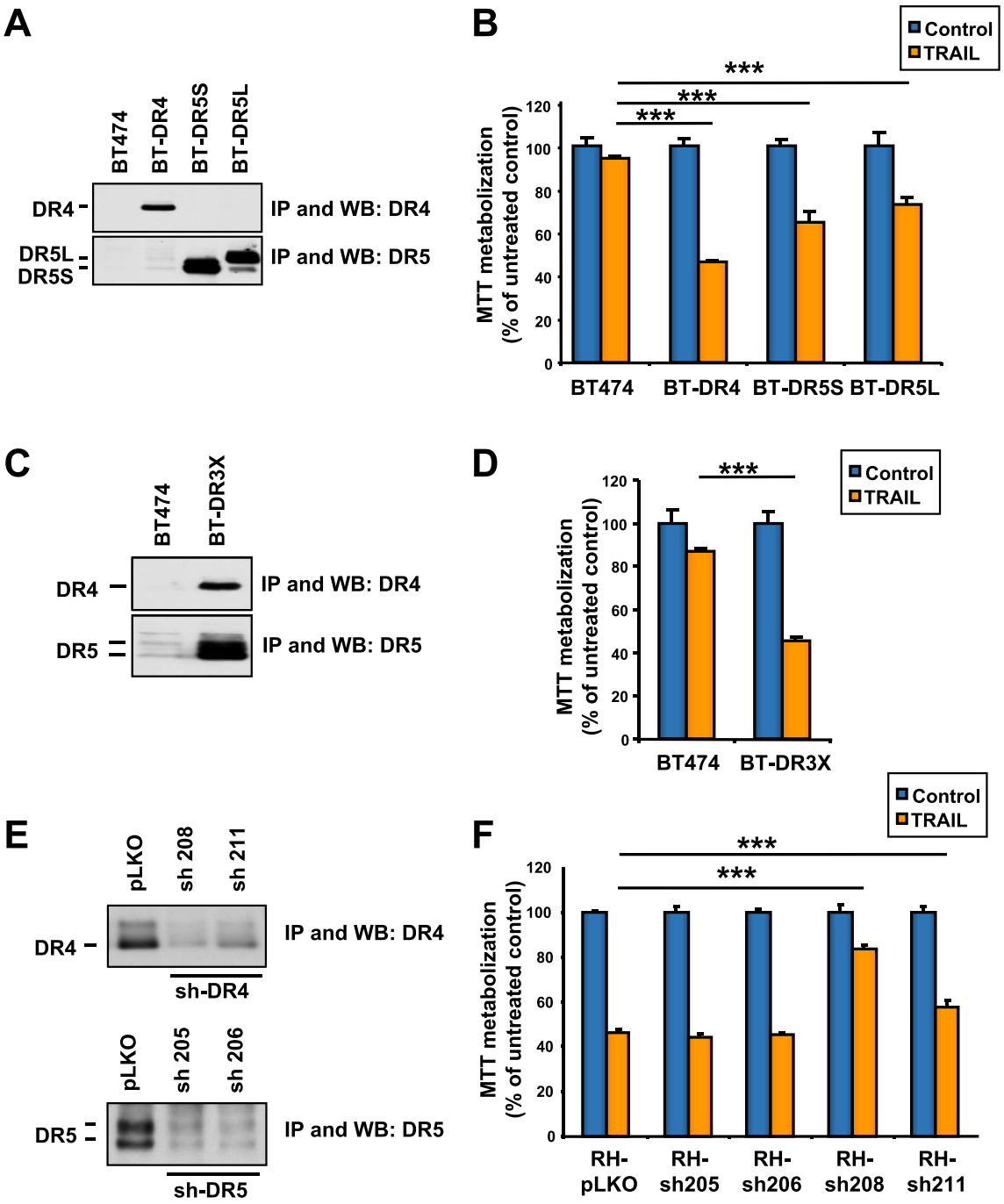

Fig. 5. Importance of DR4 and DR5 receptors in TRAIL induced cell death. (A) The increase of death receptors in BT474 is enough to increase BT474 susceptibility to TRAIL. The cDNAS coding for DR4 and the small or large isoforms of DR5 (DR5S or DR5L, respectively) were subcloned into appropriate expression vectors based on retroviral infection. BT474 cells were transduced with retroviral-containing supernatants and positive cells purified. The levels of the receptors were verified by IP and WB, as indicated. (B) TRAIL response of those cells with increased receptors was determined in conventional MTT assays. (C and D) Similarly, a population enriched in the three receptors was generated and isolated. The levels of the receptors as well as the response to TRAIL was determined as in A and B. (E, F) Importance of DR receptors validated in loss-of-function experiments. DR4 and DR5 levels were diminished in BT-RH cells by lentiviral infection using commercial plasmids carrying shRNAs for them. The performance of the transduction was validated in IP and WB experiments (E). (F) Besides, the sensitivity to TRAIL of these populations was determined in MTT assays. In all the MTT assays, the mean absorbance values of the untreated samples were considered as 100%. Data are represented as mean ± SD of triplicates of an experiment that was repeated at least twice. *** indicate statistically significant differences.

TMRE. After 6 h of TRAIL treatment a slight increase in TMRE negative cells could be observed in the BT-RH cells (Fig. 4E).

3.5. Modulation of TRAIL-induced cell death by DISC components

Transcriptomic and biochemical analyses suggested that changes in DISC-components expression could be responsible for the different sensitivity of BT474 and BT-RH cells to TRAIL. To explore that possibility, DR4 and DR5 levels were manipulated and its effect on TRAIL-induced responses analyzed.

cDNAs coding for DR4 and DR5 were amplified from MM1S cells by PCR, sequenced and subcloned into appropriate retroviral vectors. BT474 cells were transduced with the corresponding viruses. Amplification of DR5 gave rise to two different isoforms whose

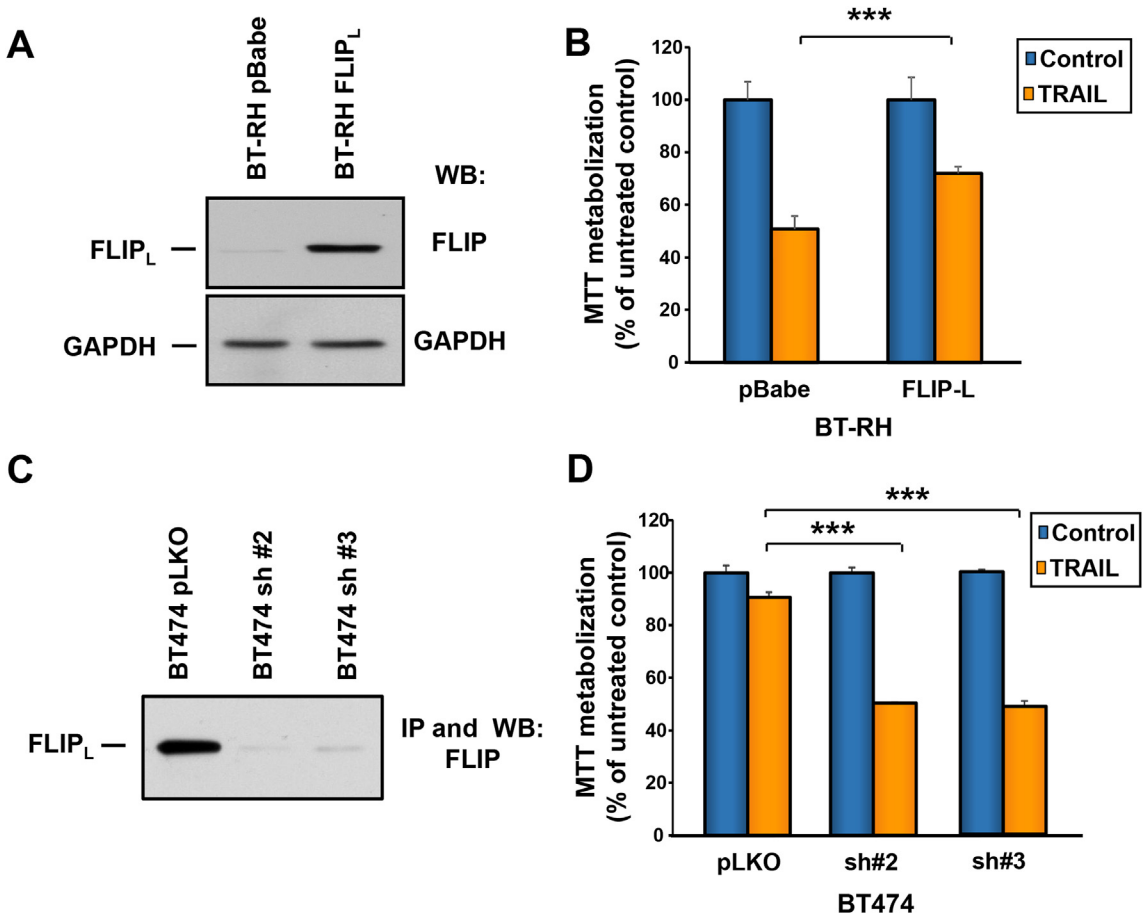

Fig. 6. Importance of cFLIP levels in TRAIL response. (A) Analysis of the importance of cFLIP on TRAIL response in gain of function experiments. cFLIP levels were increased in BT-RH cells by retroviral transduction and verified in WB experiments. (B) Such increase made BT-RH cells more resistant to TRAIL treatment as demonstrated in MTT assays. (C) In parallel, cFLIP levels were diminished in BT474 cells by lentiviral transduction using commercial plasmids carrying shRNAs for this protein. The effectiveness of the reduction was demonstrated in IP and WB with anti-FLIP antibodies. (D) FLIP downregulation made BT474 cells more sensitive to TRAIL treatment, as demonstrated in MTT experiments. Mean absorbance values of untreated samples were considered as 100%. Data are represented as mean ± SD of triplicates of an experiment that was repeated at least twice. *** indicate statistically significant differences.

introduction in BT474 cells resulted in expression of two closely-migrating bands (Fig. 5A) corresponding to the two isoforms of the molecule [34]. These two isoforms were named DR5S (short) or DR5L (large). When the response to TRAIL of these cells was tested, an inhibition of MTT metabolization was observed (Fig. 5B). We then explored the impact of the combined expression of the three receptors on TRAIL-induced responses. Infection of BT474 cells with the three different viruses resulted in expression of high levels (higher than in BT-RH cells) of DR4, DR5S and DR5L (BT-DR3X, Fig. 5C). MTT assays demonstrated that expression of the three receptors conferred sensitivity to TRAIL (Fig. 5D).

Reduction in receptor levels in BT-RH cells should decrease the proapoptotic action of TRAIL. We used RNA interference to reduce DR4 or DR5 levels in BT-RH cells. Two shRNA lentiviral vectors carrying different target sequences for each of the receptors were chosen. Those selected induced a substantial reduction of the correspondent receptor levels (Fig. 5E), without affecting the other (data not shown). In the case of DR5, selected sequences reduced expression of both isoforms of the receptor. Downregulation of DR4 decreased the sensitivity to TRAIL in BT-RH cells. In the case of DR5, analogous decreases in receptor level did not substantially affect the response to TRAIL (Fig. 5F).

The initial analyses of deregulated apoptotic proteins showed decreased levels of the inhibitory protein FLIP/cFLAR in the resistant cells (Fig. 2E and F). Gain- and loss-of-function experiments were carried out to investigate the regulatory action of FLIP on death receptor signaling. Since FLIP levels were decreased in BT-RH cells, the cDNA coding for FLIP was retrovirally transduced in BT-RH cells (Fig. 6A). Such increased expression significantly reduced their response to TRAIL (Fig. 6B). Besides, when FLIP levels were downregulated in BT474 by lentiviral short-hairpin infections (Fig. 6C), a higher sensitivity of this population to TRAIL was demonstrated by MTT metabolization assays (Fig. 6D).

3.6. Correlation between DISC expression and TRAIL sensitivity in HER2+ cell lines and patients

We next evaluated if the increased sensitivity to TRAIL observed in HER2+ cell lines correlated with increased levels of DISC components. With this purpose the amount of the different components was determined and quantified as described in the material and methods section. Statistical studies indicated a correlation between DISC expression and TRAIL sensitivity of 0.624 (Fig. 7A).

To evaluate the importance of this pathway in patient samples, we used transcriptomic data from GSE44272 study [31]. DISC mRNA expression levels appeared to be significantly (p-value = 0.007) higher in those patients who relapsed sooner.

4. Discussion

Resistance to antitumoral therapies represents a major clinical problem in oncology, which demands solution. In this paper, we explored transcriptomic profiles that could uncover potential

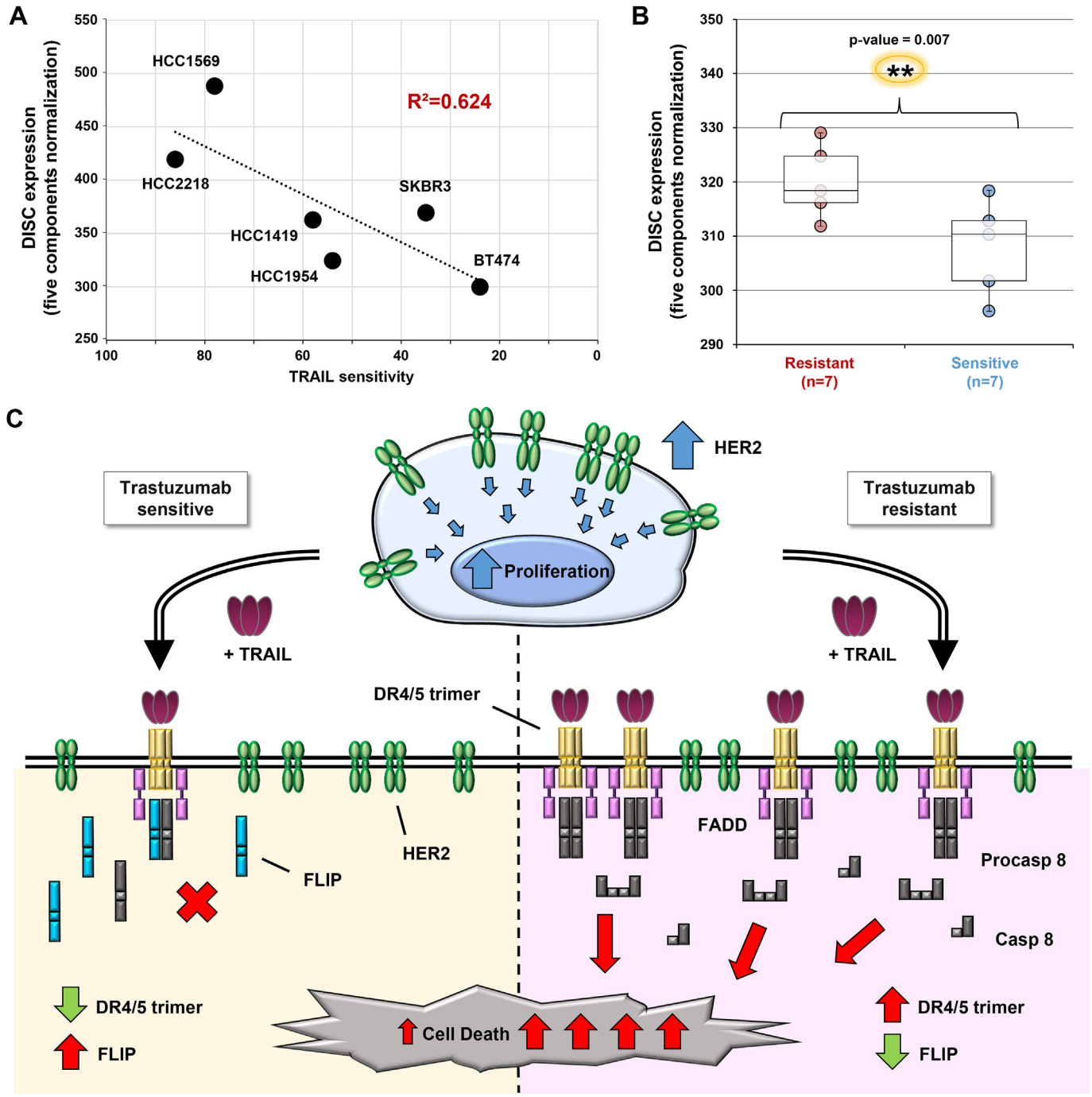

Fig. 7. DISC components expression correlates with TRAIL sensitivity in HER2 positive cell lines and patient samples. (A) Analysis of the correlation between TRAIL sensitivity and the protein levels of DISC components. DISC component levels (DR4, DR5, FADD, caspase 8 and cFlip) were determined by WB and normalized to BT474 levels. The amount of the positive components was added, and that of the inhibitor Flip, substracted to obtain a measure of DISC expression. Pearson's coefficient correlation value between DISC expression and TRAIL sensitivity was calculated and is shown in red. (B) Analysis of DISC expression (the five components) in HER2+ patients. Patients with a longer RFS were considered trastuzumab sensitive while those with a shorter RFS, resistant. Statistically significant differences are shown. (C) Schematic representation of a proposed trastuzumab resistant model in which an increased response to the trophic factor TRAIL was observed in trastuzumab resistant cells due to the upregulation of pro-apoptotic factors (TRAIL receptors), and the downregulation of anti-apoptotic components (FLIP). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

vulnerabilities to overcome resistance to trastuzumab, a gold standard therapy used in the treatment of HER2+ breast cancer [21]. While patients respond to the use of this anti-HER2 antibody, in advanced metastatic stages resistance to the drug is frequent [4]. Therefore, uncovering strategies to overcome such resistance must represent the first step towards successful therapy of trastuzumab-resistant-patients.

We developed a model to explore potential vulnerabilities that could be exploited to fight trastuzumab resistance. The model was created by continuous treatment with trastuzumab of the HER2 overexpressing cell line BT474, and resulted in the generation of a population resistant to the drug. Gene profiling combined with functional transcriptomics identified functions differentially affected in the resistant cells, as compared to the parental trastuzumab-sensitive ones. Among them, we identified differences in cell death mechanisms that

could be used to attack trastuzumab-resistant cells, such as the TRAIL signaling route, a pathway known to specifically act on cancer cells [38–41]. Components of that pathway such as TRAIL receptors DR4 and DR5 were up-regulated in the resistant cells, opening the possibility of acting on them to induce death of those cells. Functional studies confirmed that TRAIL killed BT-RH cells more efficiently than BT474. Biochemical studies indicated that TRAIL-induced DISC formation was more efficient in BT-RH cells. In these cells, TRAIL facilitated the integration of active caspase 8 into the DISC complex, an effect less marked in BT474 cells. Such increased efficiency of DISC formation translated into a more robust proapoptotic response in BT-RH cells, as demonstrated by the presence of cleaved forms of other caspases or PARP.

TRAIL receptor levels appeared to be a critical component dictating TRAIL sensitivity. This concept was explored using gain- and loss-of-function experiments. In BT-RH cells, reduction of DR4 by shRNA resulted in TRAIL resistance. That effect was more evident in DR4 than in DR5 knock down, suggesting that in this system DR4 is more active in transducing death signaling responses than DR5. This concept was supported by increasing DR4 and DR5 levels in BT474 cells, which resulted in an augmented sensitivity to TRAIL. However, such increase was higher with DR4 than with the long or short forms of DR5. Such finding falls in line with data which indicated that apoptosis induced by TRAIL preferentially occurs through DR4 [42]. The overexpression experiments confirmed the relevance of TRAIL receptor levels in dictating sensitivity to this peptide, and added evidence supporting the concept that death signaling through DR4 is more efficient than signaling through DR5 in BT474 and BT-RH cells, although in other systems DR5 seems to be the highest affinity receptor [43,44].

In addition to the receptors, other components of the DISC could regulate responses to TRAIL. In fact, a decrease in FLIP could facilitate its proapoptotic action [45,46]. FLIP levels were down-regulated in BT-RH cells, as indicated by gene expression and WB analyses. Manipulation of FLIP levels supported the concept that it could be involved in regulating death responses to TRAIL. Thus, transfection of FLIP in BT-RH cells caused resistance to TRAIL. Furthermore, FLIP down-regulation in BT474 cells rendered these cells highly sensitive to the proapoptotic action of TRAIL. These data are summarized in Fig. 7D.

The levels of different components of the DISC complex regulate the sensitivity of BT474 and BT-RH cells to TRAIL. These data corroborate a recent report describing the deregulation of TRAIL pathway in two different cellular models of resistance to the anti-HER2 therapeutic agent lapatinib [47]. Moreover, when transcriptomic analyses were carried out in trastuzumab-resistant HER2+ patients, a clear increase in DISC components was found, opening the possibility of acting on this pathway to treat resistance to anti-HER2 therapies and taking advantage of the vulnerability we have described. Strategies targeting TRAIL cell death pathway are now under development [48], some of them are being tested in clinical trials [33].

Conflict of interest

The authors declare no competing interests.

Authors' contributions

Experimental design, EDR and AP; Experimental execution EDR and CRL; Computational analysis EDR and JPP; Manuscript writing, review and discussion EDR, JPP, CRL, JA, AO and AP.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.042.

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