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CRHR2/Ucn2 signaling is a novel regulator of miR-7/YY1/Fas circuitry contributing to reversal of colorectal cancer cell resistance to Fas-mediated apoptosis

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Abstract

Colorectal cancer (CRC) responds poorly to immuno-mediated cytotoxicity. Underexpression of Corticotropin-releasing-hormone-receptor-2 (CRHR2) in CRC, promotes tumor survival, growth and Epithelial to Mesenchymal Transition (EMT), *in vitro* and *in vivo*. We explored the role of CRHR2 downregulation in CRC cell resistance to Fas/FasL-mediated apoptosis and the underlying molecular mechanism.

CRC cell sensitivity to CH11-induced apoptosis was compared between Urocortin-2 (Ucn2)-stimulated parental and CRHR2-overexpressing CRC cell lines and targets of CRHR2/Ucn2 signaling were identified through *in vitro* and *ex vivo* analyses. Induced CRHR2/Ucn2 signaling in SW620 and DLD1 cells increased specifically their sensitivity to CH11-mediated apoptosis, via Fas mRNA and protein upregulation. CRC compared to control tissues had reduced Fas expression that was associated with lost CRHR2 mRNA, poor tumor differentiation and high risk for distant metastasis. YY1 silencing increased Fas promoter activity in SW620 and re-sensitized them to CH11-apoptosis; suggesting YY1 as a putative transcriptional repressor of Fas in CRC. An inverse correlation between Fas and YY1 expression was confirmed in CRC tissue arrays, while elevated YY1 mRNA was clinically relevant with advanced CRC grade and higher risk for distant metastasis. CRHR2/Ucn2 signaling downregulated specifically YY1 expression through miR-7 elevation, while miR-7 modulation in miR-7^{high} SW620-CRHR2+ and miR-7^{low} HCT116 cells, had opposite effects on YY1 and Fas expressions and cell sensitivity to CH11-killing.

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CRHR2/Ucn2 signaling is a negative regulator of CRC cell resistance to Fas/FasL-apoptosis via targeting the miR-7/YY1/Fas circuitry. CRHR2 restoration might prove effective in managing CRC response to immune-mediated apoptotic stimuli.

Keywords

colorectal cancer; CRHR2; YY1; microRNA-7; Fas

INTRODUCTION

Colorectal carcinoma (CRC) is associated with increased patient mortality, attributed partially to acquisition of drug- and immuno-resistant tumor phenotypes. The most reported basis of CRC unresponsiveness to immuno-mediated cytotoxicity is through escaping Fas/FasL apoptosis.[1–3] Fas (Apo-1/CD95) is a cell surface receptor that mediates apoptotic cell death after cross-linking Fas ligand (FasL). Activation following Fas/FasL engagement represents a major cytotoxic effector mechanism of cytotoxic T lymphocytes (CTLs) resulting in elimination of tumor cells.[4] Fas is highly expressed in normal human colon epithelial cells but it is frequently diminished in primary CRC, and almost lost in metastatic forms, thus suggesting that downregulation or loss of Fas expression may represent an immune evasion and escape mechanism in CRC.[1–5] The diminished expression of functional Fas detected in primary and metastatic CRC tumors have not been attributed to inactivating mutations in CD95 gene, expression of antagonistic Fas receptor lacking a transmembrane domain, or to defective translocation of the receptor to the cell surface;[3, 6] however H3K9 trimethylation of the *Fas* promoter has been recently reported as a dominant mechanism underlying Fas silencing in CRC.[7] Fas counterattack, a molecular mechanism of tumor immune privilege, has also been described for CRC immunoescape, with CRC cells to overexpress FasL.[6, 8]

The Corticotropin-Releasing-Hormone (CRH) family of peptides (CRH, Ucn1, Ucn2 and Ucn3) and receptors (CRHR1 and CRHR2) beyond its role in stress regulation through the ACH- axis, it represents a critical regulator of gastrointestinal functions and has an established role in the modulation of immune responses related to intestinal inflammatory conditions, such as IBD.[9, 10] CRC development and progression is known to be favored in an inflamed colon with IBD history being a critical risk factor.[11, 12] We have recently reported that CRC tissues and cell lines have diminished or lost CRHR2 expression compared to their normal counterparts.[11] Diminished CRHR2 expression in CRC was further shown to favor tumor survival, proliferation and EMT, and to associate with poor prognosis and occurrence of distant metastasis.[11] However, our knowledge on the involvement of CRH family members in the regulation of CRC survival through modulation of the immune-mediated cytotoxicity against tumor cells is still unclear. The CRH system has been implicated in T-cell apoptosis via CRH-mediated FasL upregulation in the fetal-maternal interface, while tumor-produced CRH has been shown to modulate FasL expression in ovarian and cervical carcinomas, thus facilitating tumor immunoescape by activating T-cell apoptosis.[13–15] Another reported mechanism of CRH-induced cervical

cancer immunoescape under stress conditions is through downregulation of NKG2D expressed by NK cells.[16]

It is well established that tumor survival and progression partially depend on how efficient the host anti-tumor immune responses 'edit' the tumors and select for more aggressive variants, resulting in immune evasion and tumor escape. Up to date there are no available data in any type of cancer, including CRC, as to whether the differential expression of CRH family receptors may be directly or indirectly involved in tumor resistance to immuno-mediated apoptotic stimuli via regulation of death receptor signaling on tumor cells. The objective of the present study is to 1) define the biological role of CRHR2 elimination in CRC on tumor cell unresponsiveness to Fas-mediated apoptosis and 2) explore the underlying molecular mechanism *in vitro* and *ex vivo*. Our overall goal is to identify novel CRHR2 targets involved in modulation of CRC immune evasion through regulation of the commonly defected cytotoxic effector mechanisms in CRC.

MATERIALS AND METHODS

Lentiviral transduction of CRC cell lines

The human CRC cell lines HCT116, DLD1, HT29, SW480 and SW620 as well as the immortalized colonic epithelial cell line NCM460, were obtained from the ATCC (Manassas, VA, USA) and cultured as previously described.[11] CRC cells were transduced with CRHR2+ - or EV (empty vector-parental)-expressing MCS-IRES-Strawberry-hPGK-Puro lentivirus. The construction of the lentivirus and the stable cell transduction were performed as described previously.[11, 17] Transduced cells were cultured in presence of 10μg/ml puromycin (Sigma, Saint Louis, MI).

Cell treatments

Cells were treated with the CRHR2 specific agonist Urocortin 2 (Bachem, Torrance, CA), the CRHR2 specific antagonist Astressin 2B (Tocris Bioscience, Bristol, UK) and an agonist antibody for Fas, anti-Fas (clone CH11) (Millipore, Temecula, CA) used either as single agents or in combination at the indicated concentrations of each experimental condition.

Tissue samples for mRNA analysis

Fifty-two CRC samples were obtained from surgical resections from CRC patients diagnosed with established criteria (Supplementary Table 1). The control group included samples from disease-free donors (N=20). All samples were provided by the Department of Gastroenterology and Hepatology, Leiden University Medical Center, and confirmed by histopathological evaluation.[11] The samples were obtained according to the instructions and guidelines of the LUMC Medical Ethics Committee, in accordance with the Helsinki Declaration, as regulated and approved by the LUMC MDL biobanking protocol CuraRata. All experiments were approved by the Institutional Review Boards.

Immunohistochemistry (IHC)

Tissue microarray (TMA) blocks covering 21 randomly selected CRC and 6 controls from adjacent morphologically normal tissue were cut into 4-mm sections and arrayed according

to the Microarray technique with a semi-automatic ATA-100 Chemicon system (Chemicon's Advanced Tissue Array).[18] TMAs were processed for IHC as previously described,[11] using as primary antibodies anti-YY1 (1/500), anti-Fas (1/250) or normal IgG serum (negative control) (all from Santa Cruz Biotechnology, Santa Cruz, CA). Immunostaining was digitized and analyzed by an Aperio Scanscope CS (Aperio, Vista, CA), as previously described.[11] The sample collection and use was approved by the Ethics Committee of the Children Hospital of Mexico 'Federico Gomez' (Mexico City, Mexico). Both CRC and normal samples were collected by the Department of Pathology of Hospital General Regional No. 25, IMSS and Speciality Hospital CMN 'La Raza', IMSS (Mexico City, Mexico). All CRC cases were classified according to the Dukes or TNM staging.

Transient transfections

3×10^5 cells from CRC cell lines were reversely transfected with 50 nM of siYY1 or miRNA-7 precursor (pre-miR-7) or antagomiRNA-7 (anti-miR-7) or the corresponding controls (all from Ambion Inc., Austin, TX) using Lipofectamine RNAimax (Invitrogen, Carlsbad, CA) in 6-well culture plates, according to the manufacturer's instructions. NCM460 cells were similarly transfected with 50nM siCRHR2 or a negative sicontrol, at a concentration of $4x10^5$ cells/well.

Fas reporter assay

Fas promoter activity was assessed in SW620 cells using the commercial LightSwitch Luciferase Assay system (Active Motif, Carlsbad, CA), according to the manufacturer's instructions. Relative Luciferase Units (RLU) were assessed in a Luminometer and normalized using a positive (pLightSwitch-GAPDH-prom) and a negative (pLightSwitch-EMPTY-prom) control vector (both from Active Motif).

RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from cell pellets and tissues and reverse-transcribed (RT) as previously described.[11] For miRNA studies, total RNA was transcribed using a Universal cDNA synthesis kit II (Exiqon, Inc., Woburn, MA). Real-time PCR was performed using TaqMan universal PCR master mix and on-demand gene-specific primers for *CRHR2, YY1, Fas* and *18S* (all from Applied Biosystems).[11] Mature miR-7 was quantified using SYBR Green-based microRNA assays and specific primers (all from Exicon, Inc.). Reactions were run in an ABI 7500 Fast RT-PCR system (Applied Biosystems). Relative quantification was achieved by normalizing to gene expression of *18S* or *U6* for miR-7 expression.

Cell viability assay

Cell viability was assessed in 96-well plates using the CellTiter-Glo Luminescent cell viability assay (Promega, Madison, WI), according to the manufacturer's instructions.

Flow cytometry

For detection of apoptosis, 2×10^5 DLD1 and SW620 cells (EV and CRHR2+) were treated in 6-well plates with 0.1uM Ucn2 and 100ng/ml CH11. After 48h, floating cells were collected and pooled with the attached cells harvested by trypsinisation. Cells were fixed and

permiabilized with CytoFix/CytoPerm (Pharmingen, San Diego, CA, USA) and stained with PE-conjugated anti-active caspase-3 antibody (Pharmingen) or the corresponding isotype control, according to manufacturer's instructions. Samples were analyzed using a Becton Dickinson FACScan Flow cytometer (San Diego, CA). For determination of Fas surface expression, 2×10^5 cells from EV and CRHR2+ transduced CRC cell lines, treated with 0.1uM Ucn2 for 24 h, were detached using Accutase (Sigma) and stained with FITC-conjugated anti-CD95-FITC antibody (cloneDX2) (Miltenyl Biotech Inc, San Diego, CA), or the corresponding isotype control, according to manufacturer's instructions. Cells were analyzed by flow cytometry as described above.

Western blot

Western blot was performed by infrared Imaging technology (Odyssey, Li-Cor Biosciences) using 30ug of total protein lysates, as previously described.[19] The primary antibodies for YY1, cleaved PARP and GAPDH were all purchased from Cell Signaling Technology, Inc., (Danvers, MA) and used in 1/1000 dilution. The primary antibodies for Fas, cleaved caspase 3, β-tubulin and β-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA) were used in dilutions 1/500, 1/200, 1/1000 and 1/2000, respectively.

MicroRNA profiling

A high-throughput screen of the human microRNAome was performed in HT29, HCT116, SW620, SW480 and DLD1 cell lines transduced with EV or CRHR2 and treated with 0.1uM Ucn2 for 24h, using the Nanostring platform, as previously described.[20]

Processing of digital images

Photoshop software (CS6) was used as a digital tool to 1) adjust contrast, brightness, or color, in digital images after uniform application to the entire image and 2) crop fractions of no interest from gel images.

Statistical analysis

The results were expressed as mean±SEM. Statistical analysis was performed using the GraphPad Prism Software (v. 5, La Jolla, CA). Student's *t*-test or Mann–Whitney U test was performed to compare the variables of two groups. Differences between multiple groups were determined using analysis of variance (ANOVA). For correlation analyses Spearman and Pearson tests were used. Results were considered significant if $p \leq 0.05$.

RESULTS

CRHR2 restoration and activation by Ucn2 in CRC cell lines results in Fas upregulation

We have previously reported significantly diminished CRHR2 expression in CRC cell lines and tissues and presented evidence that this loss promotes tumor survival and proliferation.[11] (Supplementary Table 2). Since Fas downregulation in CRC is thought to be involved in tumor unresponsiveness to FasL-mediated killing, thus supporting tumor survival, we hypothesized that CRHR2 dysregulation in CRC might affect Fas expression levels. To test this hypothesis, we stably transduced 5 CRC cell lines with CRHR2 (CRHR2+ cells) or

empty vector (EV), and compared the Fas expression at the mRNA and protein levels. We have previously shown that the endogenous mRNA expression of the CRHR2 selective agonist Ucn2 is sharply reduced in CRHR2-transduced CRC cell lines and the baseline CRHR2 receptor activity (as it relates to cAMP production) is greatly enhanced in presence of exogenously administrated Ucn2[11]. As such, we initially performed a Ucn2-dose response analysis of Fas expression in representative CRC-CRHR2+ cell lines and compared the derived patterns. We showed that SW620-CRHR2+ cell exposure to exogenous ucn2 concentrations lower than 0.01uM resulted in significantly diminished Fas expression, indicating that the capability of CRHR2/Ucn2 signaling to induce Fas in CRC cells was Ucn2-dose dependent (Supplementary Figure 1A). Therefore, the comparison of Fas levels between CRHR2+ and EV CRC cells, as well as all the following experiments described herein, were performed under cell stimulation with 0.1uM of exogenous Ucn2.

All tested CRHR2+ cell lines expressed significantly higher Fas mRNA (Figure 1A) and protein (Figure 1B) levels, compared to the corresponding EV clones. Further analysis of the Fas protein expression between the 2 cell groups, revealed that CRHR2+ cell populations had either significantly elevated Fas surface expression (SW480, DLD1) (Figure 1C) and/or higher percentages of Fas+ cells (Figure 1D). Fas mRNA induction in CRHR2+ cells was significantly reversed after cell treatment with the CRHR2 antagonist Ast2B, thus suggesting CRHR2 specificity (Figure 1E). Overall, the above findings identify Fas as one of the targets of CRHR2/Ucn2 signaling in CRC and suggest a potential role of CRHR2 dysregulation in CRC in tumor cell resistance to Fas/FasL-mediated apoptosis.

Low Fas mRNA expression in CRC tissues is positively associated with lost CRHR2 expression, poor tumor differentiation and high risk for distant metastasis

Significant Fas mRNA downregulation was detected in human CRC tissues compared to their normal counterparts (Figure 2A). Tumor samples with lost (undetectable) CRHR2 mRNA levels also had significantly lower Fas mRNA expression over those with low, but detectable CRHR2 mRNA. (Figure 2B). Immunihistochemical analysis of Fas expression in tissue arrays revealed increased Fas staining in CRC over control samples (Figure 2C), a result that might be explained by the high accumulation of Fas positive inflammatory cells in tumor microenviroment. Indeed when the observational analysis excluded the inflammatory immune cells, Fas expression was less dominant in CRC than in normal tissues. In addition, a marginally insignificant positive correlation was observed between Fas and CRHR2 expressions in CRC tissue arrays, which may be again attributed to the high prevalence of Fas-expressing immune cells and the low CRHR2 expression in CRC tissues (Figure 2D). The above findings corroborate our *in vitro* observations; however we cannot exclude the possibility that even CRHR2 can drive Fas expression, may not necessarily be required for it. Analysis of the available clinicopathological data showed that Fas mRNA expression was significantly associated with tumor differentiation status and recurrence, with the lowest Fas levels to be indicative of poorly differentiated tumors (Figure 2E, Supplementary Table 1) and tumors with the highest occurrence of distant metastases (Figure 2F, Supplementary Table 1). Our previous[11] and present findings corroborate a similar pattern of expression and clinical relevance for Fas and CRHR2 in CRC tissues.

CRHR2/Ucn2 signaling reverses specifically CRC resistance to CH11-mediated apoptosis

Given the elevated Fas expression observed in CRC-CRHR2+ cell lines and normal colonic tissues, we further examined the sensitivity of CRHR2+ CRC cells to FasL-mediated cytotoxicity. As such, we treated 2 representative CRHR2+ CRC cell lines, DLD1 (epithelial) and SW620 (stage III metastatic) with various concentrations of the Fas agonist antibody, CH11, in presence of Ucn2. Compared to EV cells, the viability of both CRHR2+ cell lines was significantly lower for all the CH11 concentrations used in a dose-dependant manner (Figure 3A). Control experiments performed in absence of exogenous Ucn2, revealed no response for the parental cells and a partial response to the highest CH11 concentration used for the CRHR2+ CRC cells (Supplementary Figure 1B). These control experiments signify the role of exogenously stimulated CRHR2/Ucn2 signaling to facilitate Fas-mediated cytotoxicity. Concomitantly, the increased CRHR2+ cell sensitivity to CH11-cytotoxicity was significantly reversed (up to 40%) after cell treatment with varying concentrations of the CRHR2 antagonist Ast2B (Figure 3B), therefore suggesting specificity to CRHR2/Ucn2 signaling.

To further test our present hypothesis for CRHR2/Ucn2-mediated Fas induction that results in increased CRC-CRHR2+ cell sensitization to CH11-cytotoxicity, we included in our study an immortalized colonic epithelial cell line, namely, NCM460 which is characterized by elevated baseline CRHR2 levels and low Ucn2 expression compared to most CRC cell lines[11]. Using NCM460 as a reference cell line, we monitored the cell response to CH11 in presence or absence of CRHR2 silencing. As shown in Figure 3C, wild type cells were resistant to CH11-mediated cytotoxicity in absence of exogenous CRHR2 stimulation by Ucn2. Cell treatment with Ucn2 increased cell viability, as expected[11, 19], while it sensitized them significantly to CH11. CRHR2 silencing resulted in significant loss of cell viability in absence or presence of exogenous Ucn2, while it made Ucn2-treated cells unresponsive to CH11, possibly through Fas inhibition (Figure 3C). The findings from the NCM460 cell model corroborate the findings obtained by the CRC-CRHR2+ cell lines and signify further the role of CRHR2/Ucn2 signaling in promoting Fas-mediated CRC killing through Fas induction.

The CH11-mediated effects on CRHR2+ cell viability, were associated with increased apoptosis, as evidenced by caspase 3 (Figure 3D) and PARP (Figure 3E) cleavage. The above findings were further corroborated by elevated percentage of cleaved-caspase 3 expressing cells in CRHR2+ clones, as shown by flow cytometry (Figure 3F). Notably, Bcl2 levels remained unchanged suggesting that CRHR2/Ucn2 signaling is less likely involved in activating an intrinsic apoptotic pathway in CRHR2+ CRC cells (Figure 3E).

YY1 is a putative transcriptional repressor of Fas in CRC; it is downregulated specifically by CRHR2/Ucn2 signaling and its inhibition re-sensitizes CRC cells to CH11-apoptosis through Fas induction

In an effort to understand the underlying molecular mechanism of CRHR2/Ucn2-mediated Fas upregulation in CRC and tumor resensitization to Fas/FasL-apoptosis, we hypothesized that CRHR2/Ucn2 signaling might interfere with the transcriptional regulation of Fas. The Fas promoter silence region contains 3 putative cis-acting clusters for the transcription factor

Yin Yang 1 (YY1) which has been previously reported to suppress Fas transcription in ovarian cancer.[21] YY1 is a zing finger transcription factor known to be overexpressed in several malignant tumors, including colorectal cancer.[22–24] YY1 silencing in SW620 cells transfected with a Fas-Luc reporter system, resulted in elevated Fas promoter activity (Figure 4A), suggesting YY1 as one putative transcriptional repressor of Fas in CRC. This notion is further corroborated by findings showing significant dominance of YY1 mRNA expression over Fas in parental CRC cell lines (Figure 4B). In contrast, YY1 expression was significantly inhibited in representative CRHR2+ DLD1 and SW620 cell clones, at both mRNA (Figure 4C) and protein (Figure 4D) levels. Figure 4E signifies the specificity of this YY1 inhibition to CRHR2/Ucn2 signaling, since Ast2B was able to significantly reverse it in representative SW620-CRHR2+ cells. The functional role of CRHR2-mediated YY1 downregulation in CRC sensitivity to CH11-mediated cytotoxicity was tested after YY1 silencing in DLD1 and SW620 parental cells. YY1 inhibition was able to reverse significantly tumor resistance to CH11-killing (Figure 4F) through Fas induction (Figure 4G). Although, YY1 inhibition by itself could also reduce considerably parental cell viability, inhibition of cell survival was significantly more pronounced in presence of CH11 (data not shown), indicating that YY1 knockdown alone is not sufficient to kill the cells drastically. CRHR2 mRNA levels were also found elevated after YY1 inhibition (data not shown). Our overall findings provide evidence that YY1 is negatively targeted by CRHR2/Ucn2 signaling in CRC resulting in CRHR2-specific tumor resensitization to CH11-apoptosis, through transcriptional de-repression of Fas.

High YY1 expression in CRC tissues is associated with diminished Fas and CRHR2 levels, advanced tumor grade and high risk for distant metastases

Analysis of YY1 mRNA expression in CRC tissues and normal controls revealed significantly higher YY1 levels in CRC over normal samples (Figure 5A, Supplementary Table 2), which were inversely associated to Fas mRNA expression assessed in the same tissues (Figure 5B). IHC performed in CRC tissue arrays, further confirmed significant dominance of YY1 nuclear expression in CRC tissues over normal (Figure 5C), that was also inversely correlated to Fas protein expression (Figure 5D). Although, a trend of negative correlation was detected between CRHR2 and YY1 expression in CRC tissue arrays ($r=-0.2757$), no statistical significance was found ($p=0.13$) (Figure 5E). This lack of significance might be explained by the low detectable CRHR2 levels in CRC samples. Based on the available clinicopathological data (Supplementary Table 1), elevated YY1 mRNA expression in CRC was evidenced in highly aggressive tumor stages (3&4), as per TNM classification, (Figure 5F, Supplementary Table 1) and in tumors with increased risk for distant metastases (Figure 5G, Supplementary Table 1). These findings, extent the clinical significance of YY1 upregulation in CRC on the repression of the host immunesurveillance mechanisms through Fas downregulation and underline the inhibiting role of CRHR2/Ucn2 signaling in YY1 expression.

Restoration of CRHR2/Ucn2 signaling in CRC inhibits YY1 expression via miR-7 upregulation resulting in tumor resensitization to Fas/FasL-apoptosis

Given the lack of any statistically significant differences in YY1 promoter activities between EV and CRHR2+ CRC cell lines transfected with a YY1-Luc reporter plasmid (data not

shown), we examined whether CRHR2/Ucn2 signaling regulates YY1 expression at a post-transcriptional level. To test this, we performed a high-throughput microRNA profiling in 5 pairs of parental and CRHR2-expressing CRC cell lines (HT29, HCT116, SW620, SW480 and DLD1) treated with Ucn2 using the Nanostring molecular platform. Within the panel of the differentially expressed miRNAs between EV and CRHR2+ clones (Figure 6A), we observed that miRNA-7-5p (miR-7) was upregulated in all CRHR2+ cell lines tested. MiR-7 has been previously reported to directly repress YY1 mRNA translation in CRC cell lines, and therefore its expression is diminished in most CRC cell lines and primary CRC tumors.[25] After validation of miR-7 induction in CRHR2+ CRC clones by qPCR (Figure 6B), we examined the potential regulatory effect of miR-7 on CRC sensitivity to CH11-cytotoxity through YY1 suppression. Our results showed that ectopic induction of miR-7 in parental HCT116 cells (low basal miR-7 expression)[25] by a miR-7 precursor, mimics CRHR2/Ucn2 signaling in resensitizing tumor cells to CH11-killing (Figure 6C) by YY1 inhibition and Fas induction (Figure 6D). In contrast, inhibition of the high basal levels of miR-7 in SW620-CRHR2+ cells by an antagomiR-7 resulted in significant reduction of tumor sensitivity to CH11 killing (Figure 6E), which might be associated with anti-miR-7-mediated derepression of YY1 and downregulation of Fas (Figure 6F).The finding that in the absence of exogenously administrated Ucn2 the desensitizing effect of antagomiR-7 was marginally significant ($p=0.049$), while in presence of Ucn2 the effect was significantly more pronounced ($p=0.021$), suggests that miR-7 is a critical component of the underlying molecular mechanism of CRHR2/Ucn2-mediated CRC resensitization to Fas/FasL-apoptosis. Our overall findings indicate that CRHR2/Ucn2 signaling confers to restoration of host immunosurveillance against CRC at least via regulation of the miR-7/YY1/Fas circuitry in tumor cells.

DISCUSSION

Although, recent studies have proposed the implication of CRH family members in different steps of tumorigenesis, including initiation, promotion, progression and metastasis,[26] their role in tumor apoptosis via regulation of host immuno-surveillance is still unclear. Jin et al, first bridged CRH receptor signaling with modulation of intrinsic apoptotic pathways in cancer cells by reporting a mechanism of CRHR1-mediated apoptosis of a mouse prostate cancer cell line (RM-1) to be a change of Bcl-2:Bax ratio via cytosolic calcium-dependent phospholipase A2 (cPLA2) upregulation.[27] To our knowledge, the current study is the first to link the colorectal cancer resistance to extrinsic apoptotic pathways, such as the Fas/FasL-death cascade, with the deregulated expression of CRH receptors in CRC cells. We show that diminished or lost CRHR2 expression, previously described in CRC[11], is associated with tumor resistance to Fas/FasL-cytotoxicity and CRHR2 restoration and stimulation with Ucn2, specifically resensitizes CRC cells to FasL-mediated apoptosis through Fas upregulation. As revealed here, one molecular mechanism by which CRHR2/Ucn2 signaling promotes Fas expression in CRC cells, involves induction of miR-7, which in turn results in downregulation of the transcriptional repressor of Fas, YY1. Taking into account previous studies that establish a connection between Fas/FasL expression and tumor immunoescape,[2] we present here CRHR2/Ucn2 signaling as a putative modulator of Fas expression in CRC

cells and propose that novel agents or other molecular approaches able to restore CRHR2 levels in CRC might positively affect host immunosurvaillance.

Emerging evidence suggests that the CRH receptors are novel angiogenic regulators in intestinal inflammation, such as IBD, with CRHR2 to inhibit inflammation-associated angiogenesis.[28, 29] Since angiogenesis is a critical component of CRC and chronic inflammation greatly increases the risk for developing CRC, one can speculate that CRHR2 signaling might oppose CRC development and progression. Consistent with this notion are our recently reported findings showing that CRHR2 underexpression in CRC favors tumor survival and EMT.[11] We have also identified that persistent IL-6/Stat3 signaling, observed under CRHR2 suppression, represents one of the underlying molecular mechanisms that could trigger CRC growth and EMT and provides further evidence for interdependence among CRHR2 levels, inflammation and CRC. However, it is likely that CRHR2 signaling might act at more than one level in controlling CRC survival and expansion.

The involvement of CRH system in cytototoxic mechanisms has been reported only in ovarian and cervical cancers, where CRH promoted Fas counterattack against host immune defense cells, by inducing FasL expression in tumor cells.[14, 15] In a non cancer-related context, uterine CRH has been shown to participate in local immune responses associated with early pregnancy tolerance and embryo implantation by increasing the apoptosis of activated T lymphocytes through FasL induction.[13] In addition, CRH seems to induce the expression of FasL in human macrophages and potentiates their ability to induce the apoptosis of Fas-expressing extravillous trophoblasts during pre-eclampsia.[30] Our findings, for the first time, identify Fas as a potent specific target of CRHR2/Ucn2 signaling in cancer cells resulting in its upregulation in CRC and thus increasing tumor susceptibility to FasL-mediated apoptosis. Low Fas mRNA expression in CRC tissues was further shown to be positively associated with lost CRHR2 expression and to be clinically relevant with poor tumor differentiation and high risk for distant metastases.

By investigating the underlying molecular mechanism of Fas upregulation and CH11-induced apoptosis in Ucn2-treated CRC-CRHR2+ cells, we identified CRHR2/Ucn2 signaling as a novel regulator of Fas transcriptional de-repression through miR-7-mediated YY1 inhibition. In normal intestine, YY1 has been shown to be indispensable for Lgr5+ intestinal stem cell renewal and de-activating mutations in YY1 result in stem cell loss from apoptosis and accelerated differentiation.[31] Evidence provided by us and others support a critical role of the multifunctional transcription factor YY1 in oncogenesis.[23, 32] YY1 overexpression detected in CRC and several other solid and hematological malignancies is linked to tumor growth, proliferation, migration and metastasis.[33–39] Consistent with previous reports indicating a significant decrease in overall survival of CRC patients with high YY1 protein levels[25], our present findings show a clinical association among high YY1 mRNA expression, advanced tumor grade and high risk for distant metastasis; suggesting that YY1 may serve as an independent prognostic biomarker for CRC patients. On the same context, we have previously identified a critical role of YY1 as mediator of tumor resistance to both chemo- and immuno-mediated apoptosis in various cancer models.[21, 37, 40–43] We and others have proposed that a molecular mechanism underlying YY1 effects on tumor immunoresistance is through suppression of death receptors DR5 and Fas transcriptional

activities in cancer cells.[21, 40, 44, 45] Transcriptional repression is mediated via direct binding of YY1 on YY1 responsive elements present into the DR5 and Fas promoter silencer regions as shown by reporter and mutational analyses in ovarian, prostate and other cancer cell lines.[21, 40, 46, 47] YY1 silencing in SW620 cells resulted in elevated Fas promoter activity, suggesting YY1 as one putative transcriptional repressor of Fas in CRC. We show that YY1 expression is significantly downregulated by restoration of CRHR2/Ucn2 signaling in CRC, resulting in Fas overexpression and increased tumor susceptibility to CH11-mediated apoptosis. These findings are consistent with the inverse association between YY1 and Fas expressions established in CRC tissues at both mRNA and protein levels. Inverse correlation between YY1 and Fas levels as well as oppose influence of the 2 gene products in the induction of apoptosis and the patient survival rates have also been described in other non-cancerous models.[48] Although, we failed to detect any statistically significant association between YY1 and CRHR2 expressions in CRC tissues, a trend of negative correlation was observed. Due to almost undetectable CRHR2 expression levels in CRC tissues, we believe that further studies with larger tissue sample number are needed to resolve the above issue.

YY1 overexpression in colon cancer has been reported in the absence of gene amplification and chromosomal translocation.[22] As such, the basis of aberrant YY1 expression in CRC should be investigated at the levels of gene transcriptional and/or post-transcriptional regulation. Restored CRHR2/Ucn2 signaling had no effect on YY1 promoter activity in CRC cell lines (data not shown), suggesting that CRHR2/Ucn2 most likely exhibits its inhibitory effects on YY1 expression at a post-transcriptional point. In CRC, YY1 has been reported to be targeted directly by miR-186[49], miR-34a[50] and miR-7[25] which act as tumor suppressors. High throughput microRNA profiling between CRC-CRHR2+ and EV cell lines treated with Ucn2, revealed a higher than 2-fold increase of miR-7 in all 5 CRC-CRHR2+ cell lines tested, suggesting that miR-7 could be a potential CRHR2/Ucn2 target. MiR-7 is significantly underexpressed in CRC, and its ectopic expression suppressed colon cancer cell proliferation, induced apoptosis and caused cell-cycle arrest *in vitro* and *in vivo*.[25] These tumor suppressing effects have been associated with the direct binding of miR-7 to YY1 3′UTR to negatively regulate YY1 translation in CRC cells.[25] The opposite functions of miR-7 and YY1 in CRC sensitivity to CH11-killing was shown by ectopic expression of miR-7 in miR-7^{low} HCT116 cells and miR-7 silencing in miR-7^{high} SW620-CRHR2+ cells. In both experimental settings modification of Fas expression was in linear association with miR-7 expression levels, suggesting that one molecular mechanism by which CRHR2/Ucn2 signaling increases CRC sensitivity to Fas/FasL-cytotoxicity is through dysregulation of miR-7/YY1/Fas circuitry.

As shown by us and others, YY1 knockdown in parental CRC cell lines consistently increased apoptosis, indicating the critical role of YY1 inhibition in cell suicidal programs.[25] Pathway analysis in shYY1-transduced CRC cell lines has revealed that the oncogenic and anti-apoptotic effect by YY1 in CRC was associated with p53 inhibition, modulation of its downstream effector caspase cascades and C-Jun, and Wnt signaling activation through induction of β-catenin and anti-apoptotic survivin.[25] These data together with our previously reported gene array-based findings on E2F1, p53 and caspase 3 induction and survivin downregulation in Ucn2-treated CRC-CRHR2+ cell lines[11], suggest that beyond Fas de-repression, CRHR2/Ucn2-mediated YY1 inhibition might eliminate CRC survival, through

regulation of additional apoptotic networks (Supplementary Figure 2). Our findings propose YY1 as a potential target of CRHR2/Ucn2 signaling and a putative mediator of CRHR2/Ucn2 effects on Fas expression in CRC. However, we recognize that further validation in *in vivo* experimental settings is necessary to corroborate the above findings and prove their physiological relevance.

Summarizing, we present for the first time a large body of *in vitro* and *ex vivo* data that support a critical involvement of the CRH system in regulation of host immunosurveillance during CRC progression. Specifically, we identified CRHR2/Ucn2 signaling as a potential mediator of CRC cell susceptibility to Fas/FasL-apoptosis through modulation of miR-7/YY1/Fas cascade. We further suggest that CRHR2 levels may have a prognostic significance in CRC response to immune-mediated cytotoxicity and CRHR2 restoration may prove effective in diminishing tumor immunoresistance through Fas upregulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Bcl-2	B-Cell CLL/Lymphoma 2
DR5	death receptor 5
CRC	colorectal cancer
CRH	corticotropin-releasing hormone
CRHR1	corticotropin-releasing hormone receptor 1
CRHR2	corticotropin-releasing hormone receptor 2
CTL	cytotoxic T-lymphocyte
EMT	epithelial-to-mesenchymal transition
Fas	FAS receptor
FasL	Fas ligand
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase

GI	gastrointestinal
IBD	Inflammatory Bowel Disease
IL	interleukin
miR	micro-RNA
PARP	Poly(ADP-ribose) Polymerase
Pre-miR-7	precursor molecules of miR-7
qRT-PCR	quantitative real-time polymerase chain reaction
siRNA	small interference RNA
TNFα	tumor necrosis factor alpha
Ucn	urocortin
3'-UTR	3' untranslated region
YY1	Yin Yang 1

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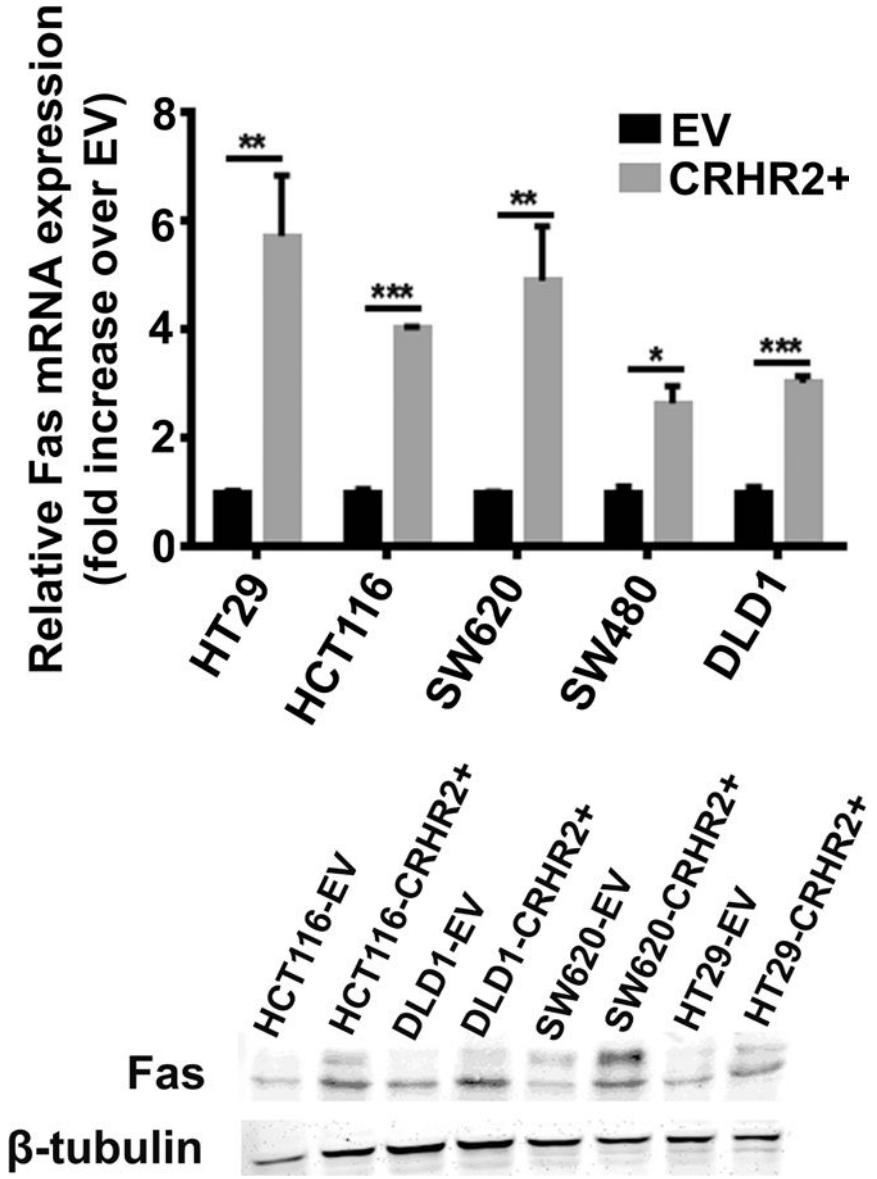
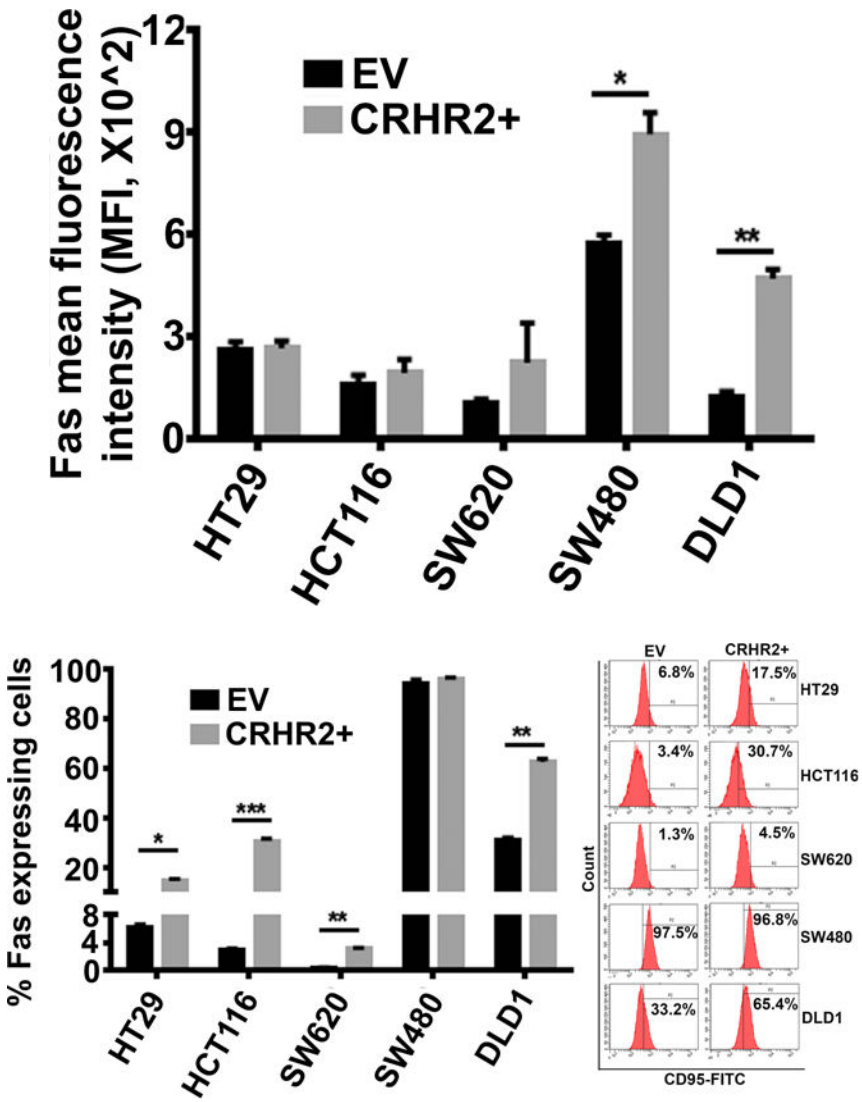
Novelty and impact statement

Colorectal cancer (CRC) is highly resistant to immuno-mediated cytotoxicity. This study uncovers a novel role of the stress regulatory CRHR2 receptor in reversing CRC resistance to extrinsic apoptotic stimuli. We show that CRHR2/Ucn2 signaling specifically resensitizes CRC cells to Fas/FasL-induced apoptosis through upregulation of Fas receptor and delineate the underlying molecular mechanism. Our findings suggest that CRHR2 expression and activation levels might have a prognostic and therapeutic impact in the management of CRC immunoresistance.





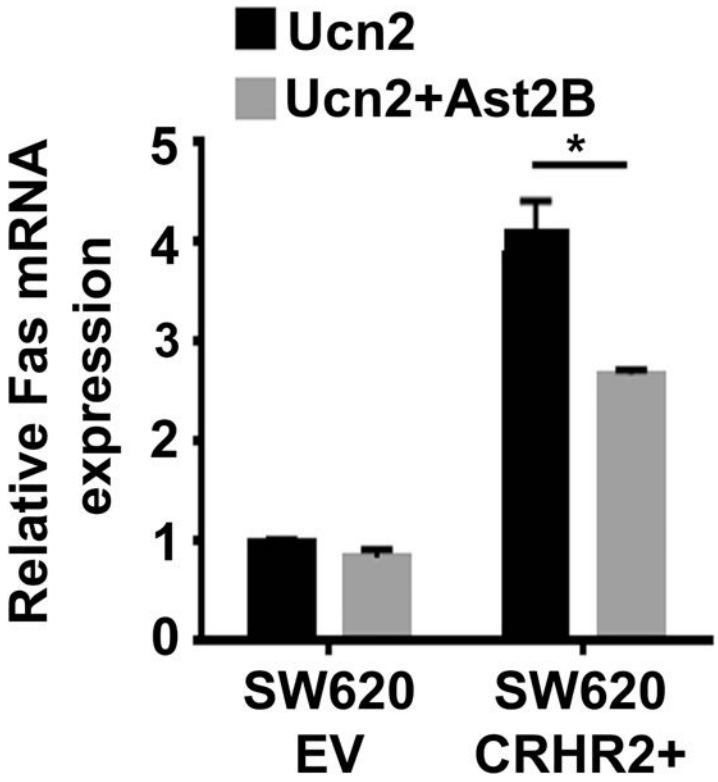
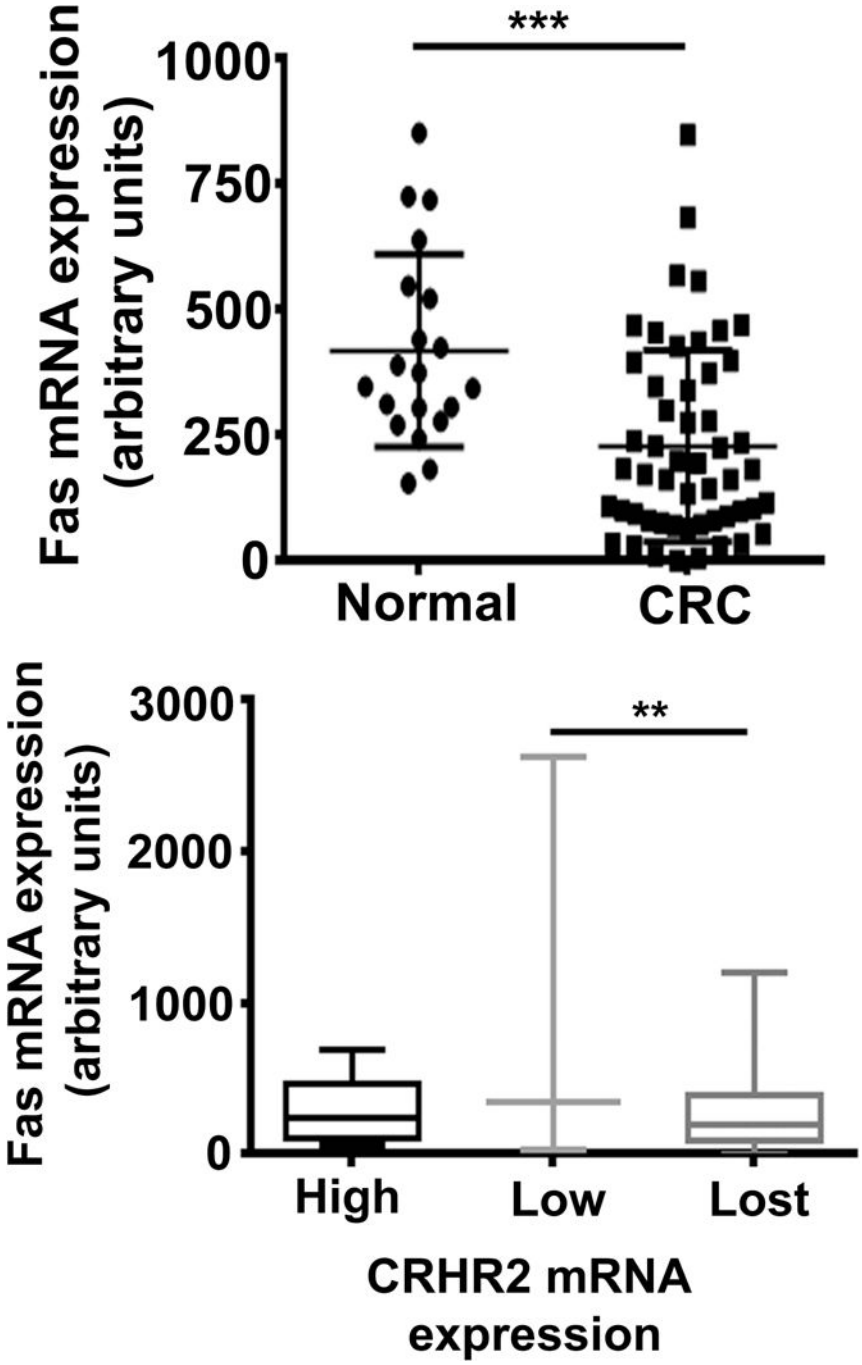
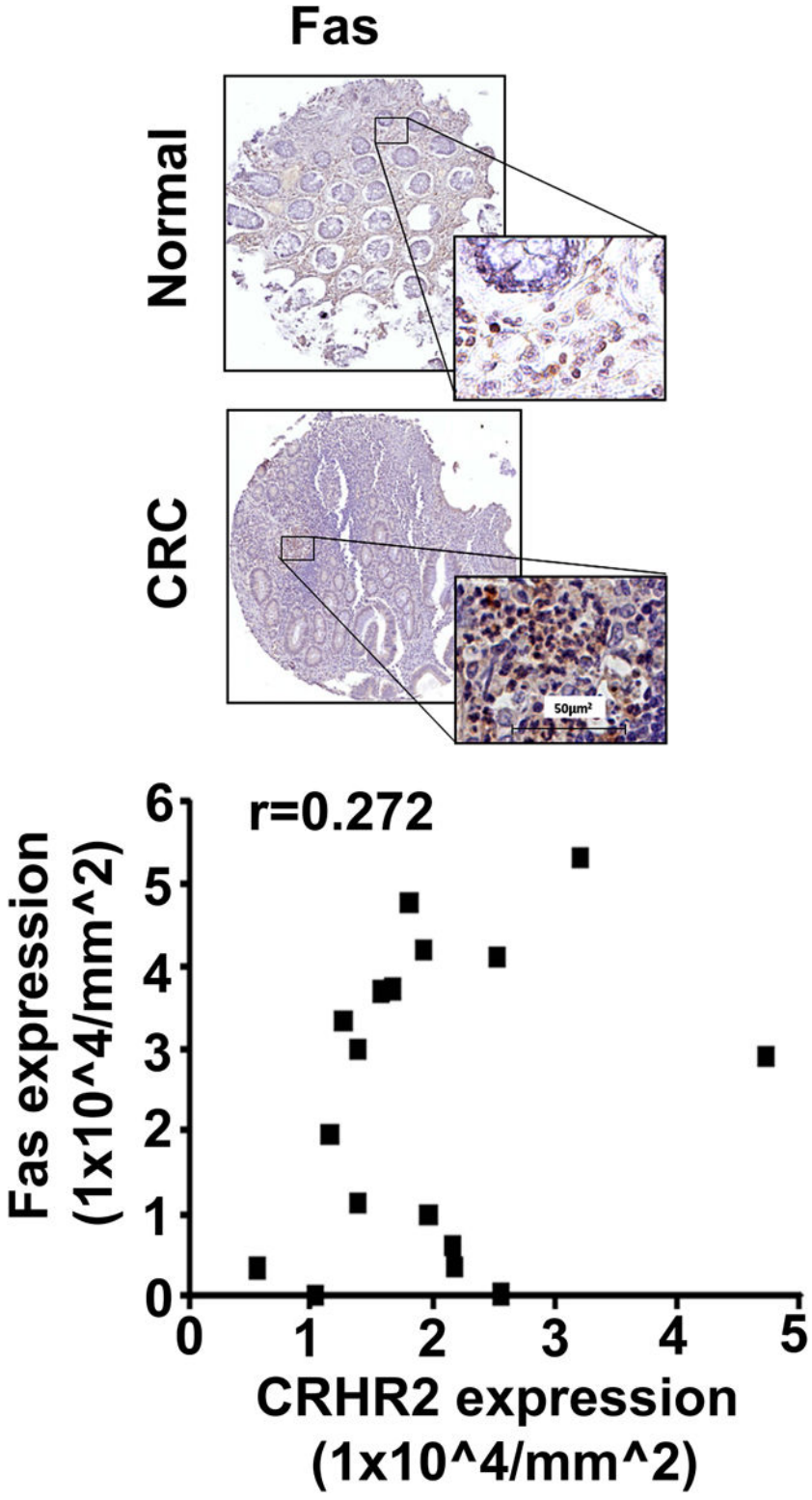


Figure 1. CRHR2/Ucn2 signaling specifically promotes Fas upregulation in CRC

CRHR2+ and EV CRC cell lines were treated for 24h with 0.1mM Ucn2 before evaluation of Fas expression at the mRNA and protein level. Significant elevation of Fas expression in A) mRNA or B) protein levels in CRHR2+ CRC cells compared to the corresponding EV counterparts, as assessed by qPCR or western blot, respectively. Flow cytometric analysis of extracellular Fas expression revealed that CRHR2+ CRC cells have either, C) increased surface expression of Fas (expressed as mean fluorescent intensity, MFI), or D) higher percentages of Fas expressing cells, or both, compared to EV cells. Representative flow cytometry histograms with the percentages of Fas positive cells in all EV and CRHR2+ cell lines tested, are also shown in Figure 1C. P2 regions were set based on isotype control background staining (not shown). E) Ast2B (0.5uM) partially reverses CRHR2/Ucn2-mediated Fas mRNA upregulation. CRHR2+ DLD1 and SW620 cells were treated for 24h with Ucn2 alone or in combination with Ast2B. *$p<0.05$, **$p<0.01$. ***$p<0.0001$.





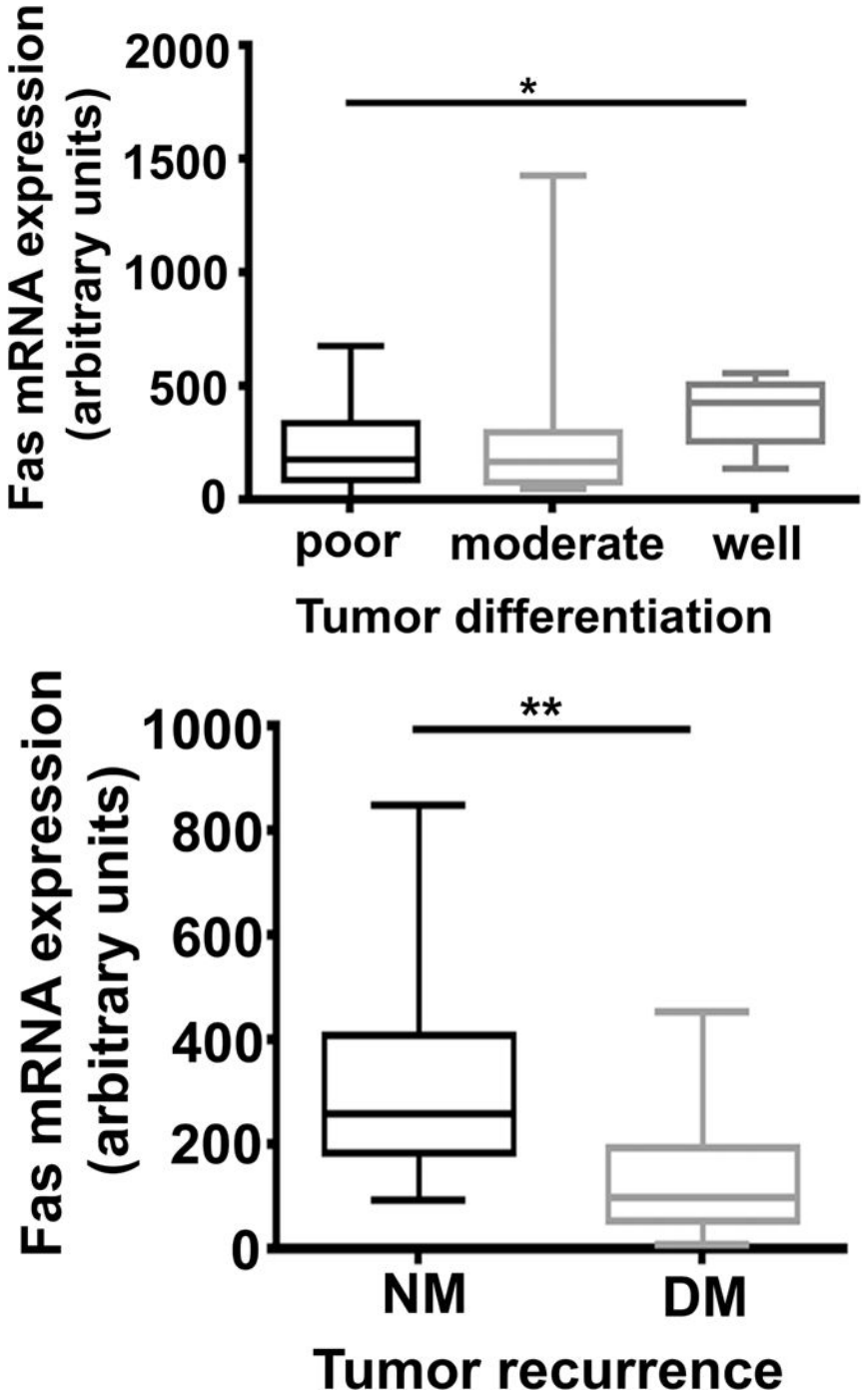
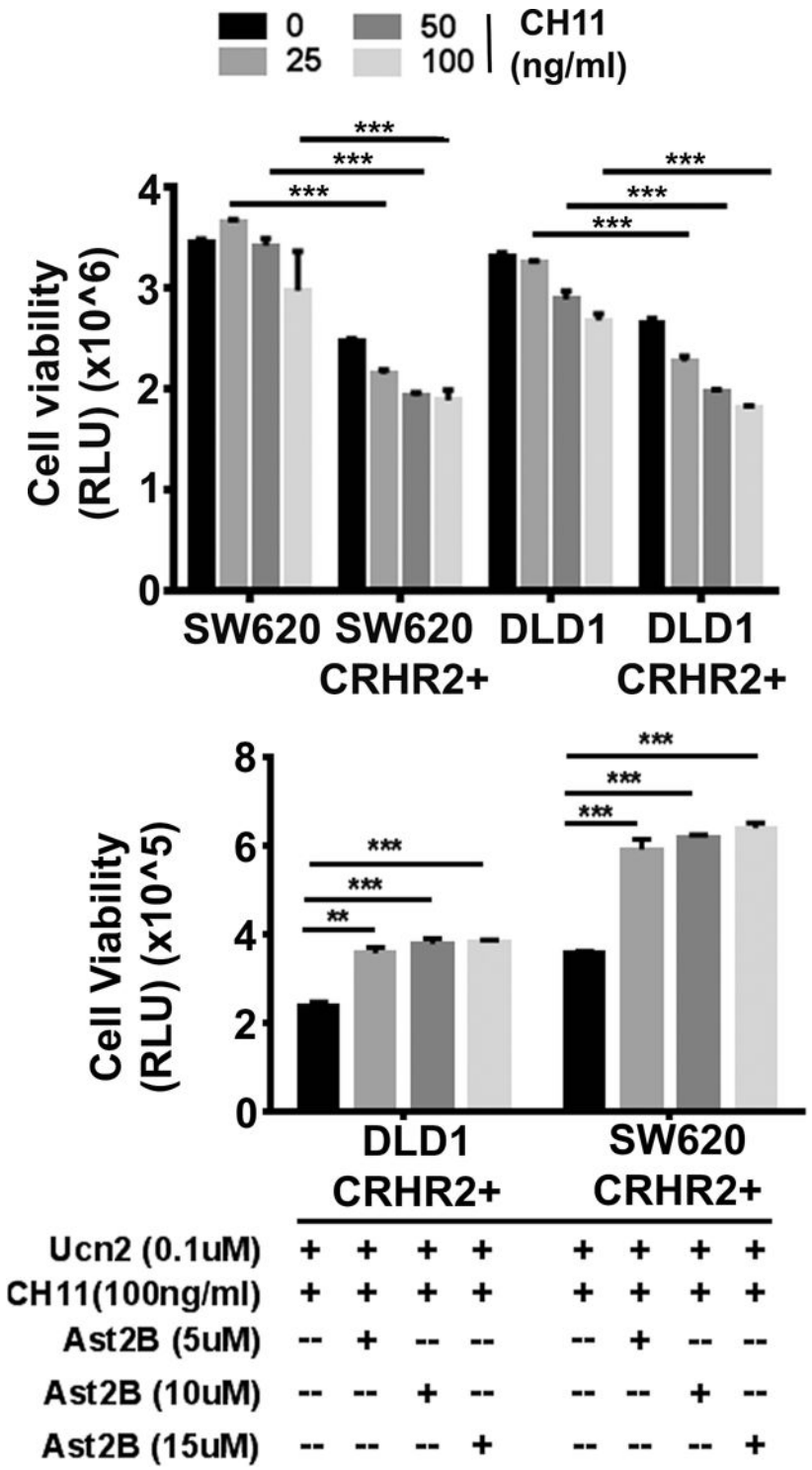
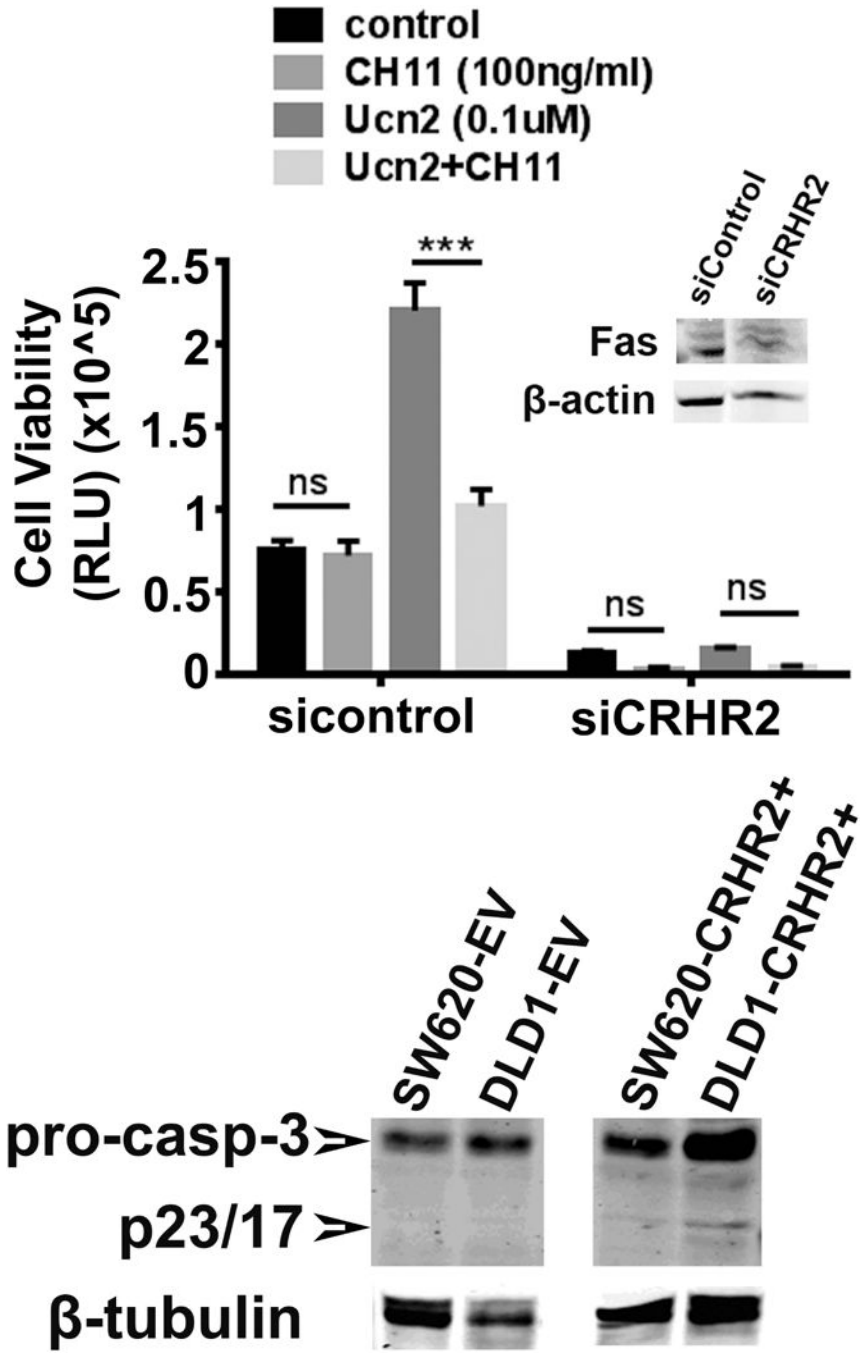


Figure 2. Low Fas mRNA expression in CRC tissues is positively associated with lost CRHR2 expression, poor tumor differentiation and high risk for distant metastasis

A) Fas mRNA is significantly underexpressed in CRC vs normal colorectal human tissues. RNA was extracted by 52 CRC and 20 normal human colorectal tissues and subjected to qPCR for Fas mRNA detection. B) CRC tissues with lost CRHR2 mRNA expression have significantly lower Fas mRNA compared to those with low CRHR2 mRNA. C) Representative Fas protein expression in CRC and normal tissue arrays. Aberrant Fas

staining in CRC mainly concerns inflammatory immune cells. Original magnifications x20 and x40, bar scale: 50μm. D) Marginally insignificant positive correlation between CRHR2 and Fas expressions in CRC tissue arrays (N=17, r=0.272, 90% CI). Positive association between low Fas mRNA levels in CRC tissues and E) poor tumor differentiation (poor: N=26, moderate: N=16, well: N=5) and F) occurrence of distant metastases. NM: no metastasis (N=26); DM: distant metastasis (N=15). CRHR2 mRNA expression was set as lost when Ct value was above 40 cycles (N=38), low when expression <mean+SEM (N=3) and high for values >mean+SEM (N=11), see Supplementary Table 2. *p<0.05, **p<0.01.





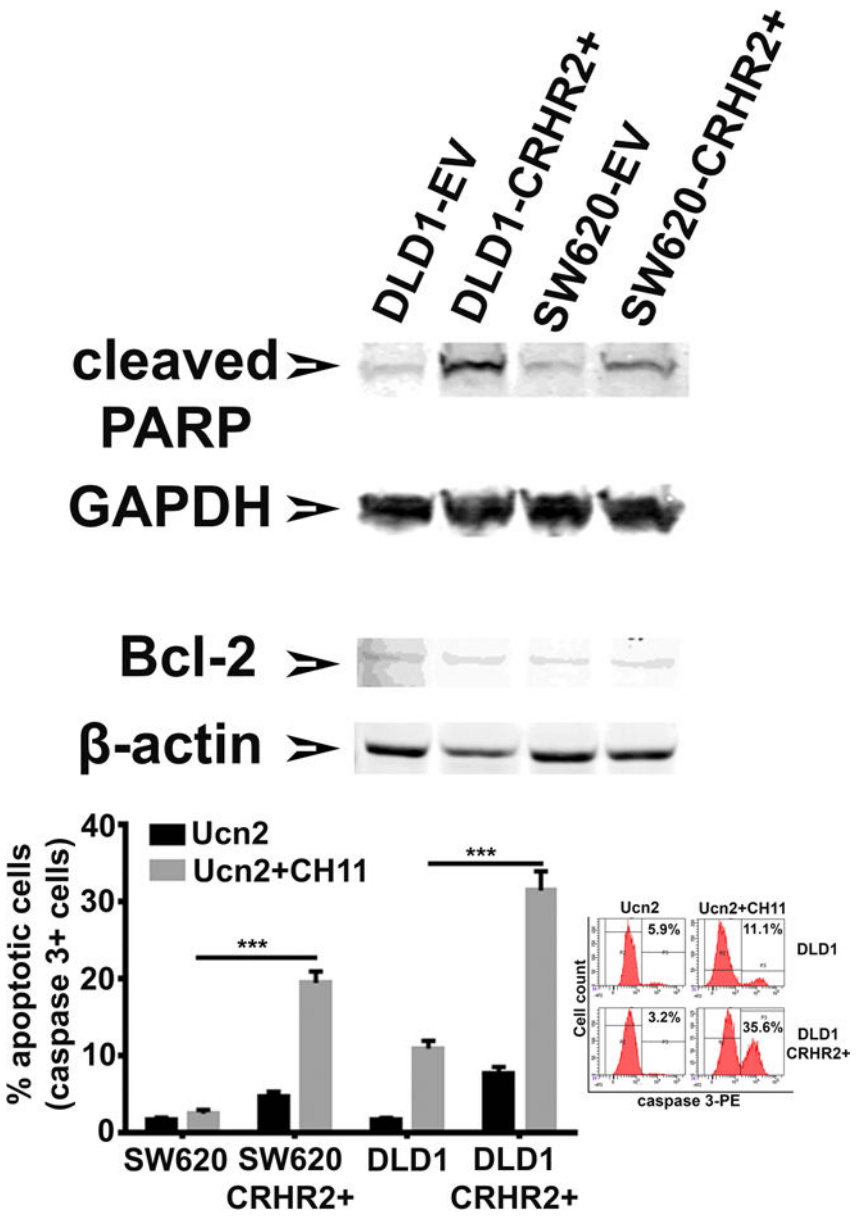
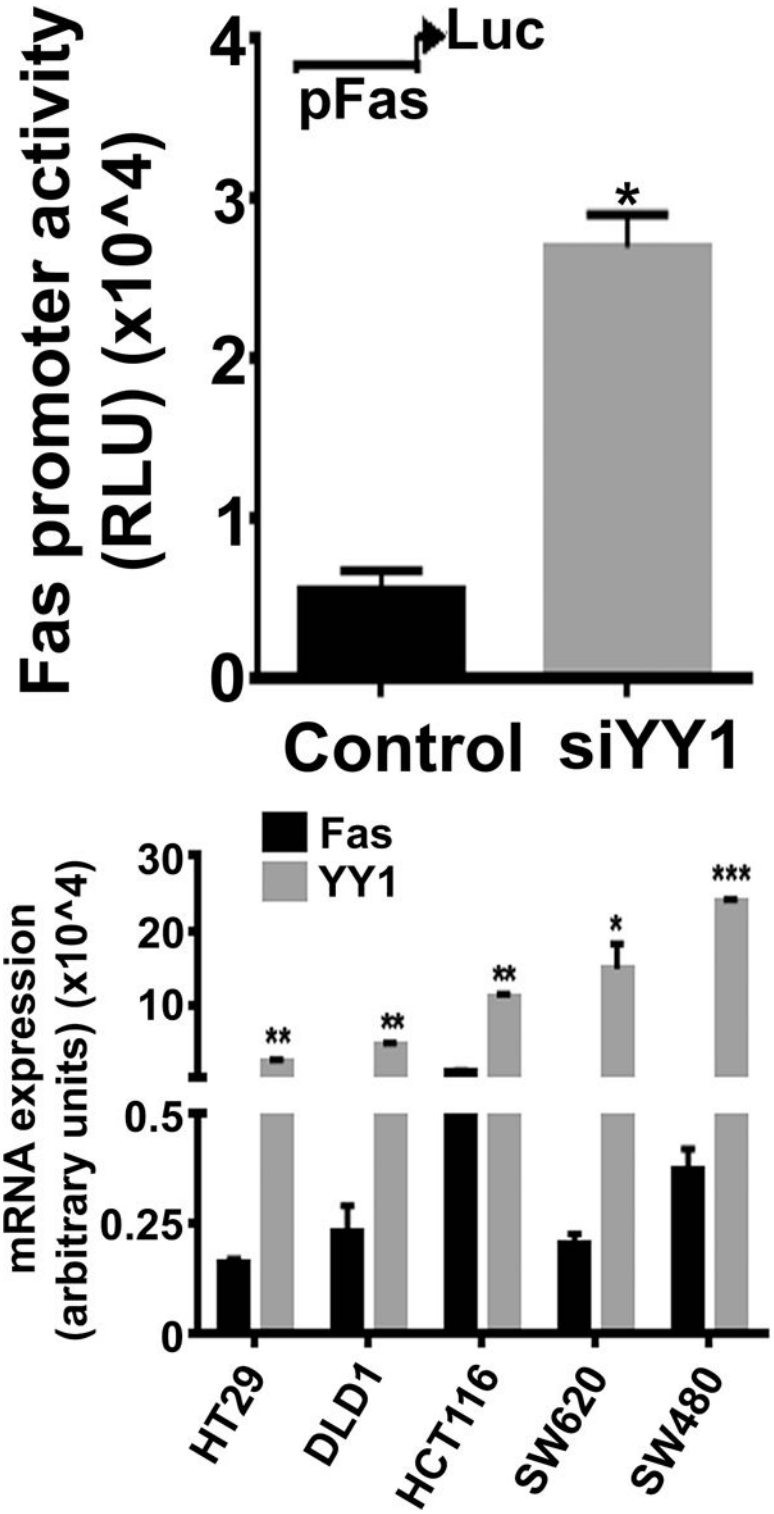
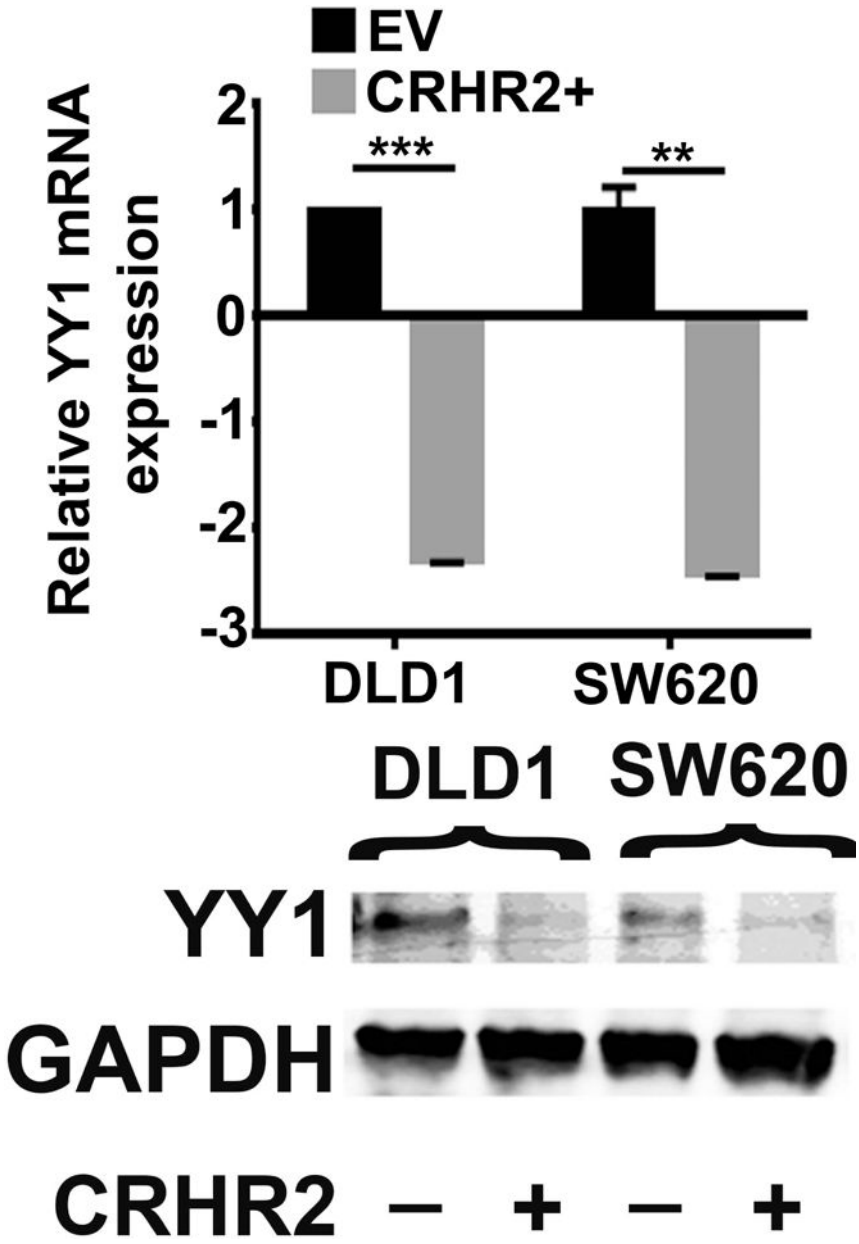


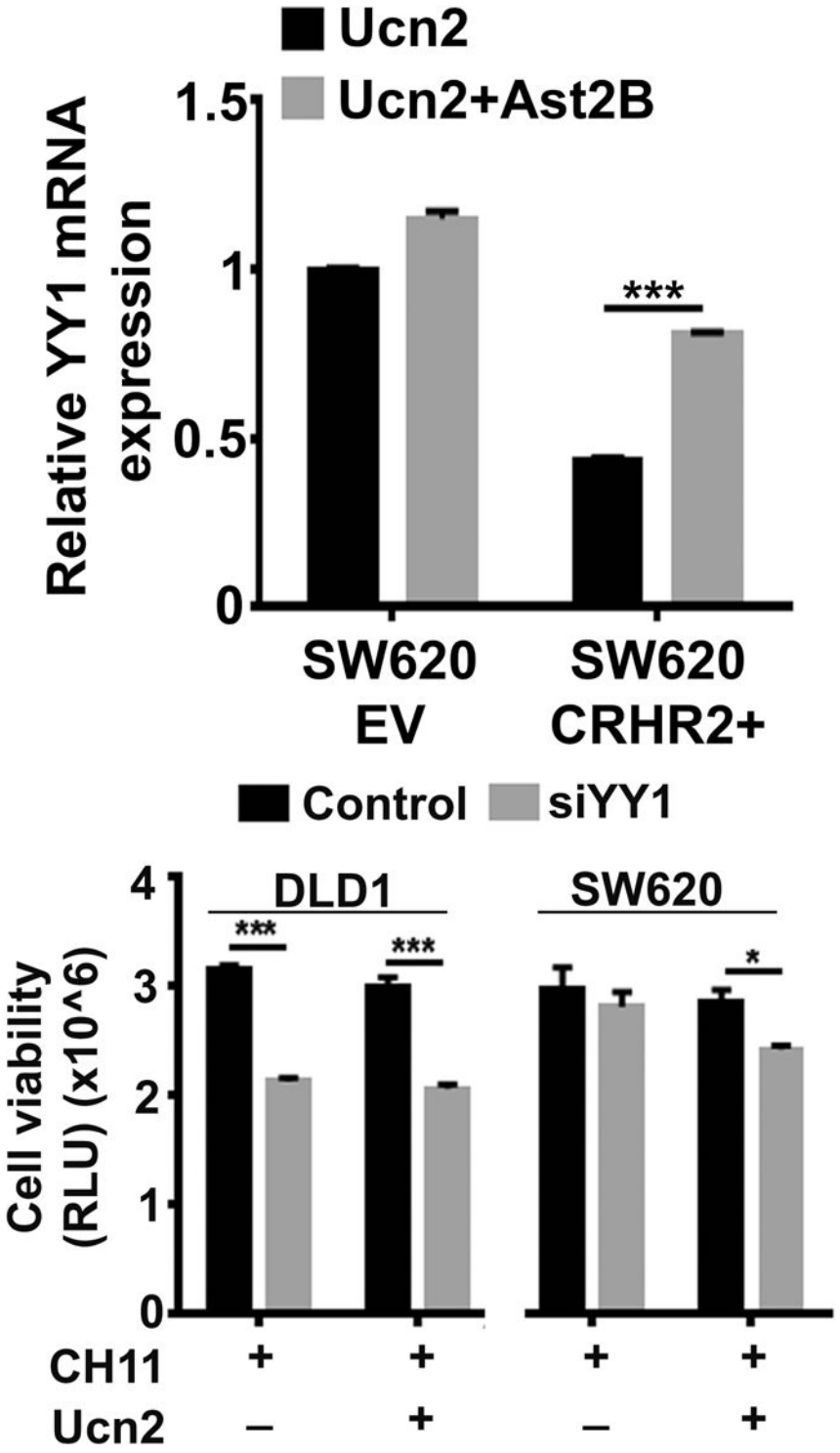
Figure 3. CRHR2/Ucn2 signaling reverses specifically CRC resistance to CH11-mediated apoptosis

A) Increased cytotoxicity of the Fas agonist antibody, CH11, in CRC-CRHR2+ cell lines. EV and CRHR2+ DLD1 and SW620 cells were treated with 0.1uM Ucn2 and different concentrations of CH11 for 48h. Cell viability was assessed by CellTiter-Glo Luminescent cell viability assay. B) Ast2B significantly reverses DLD1- and SW620-CRHR2+ cell sensitization to CH11-mediated cytotoxicity. DLD1 and SW620 EV and CRHR2+ cells were treated with 100ng/ml CH11 alone or in combination with varying concentrations of Ast2B (ranging from 5 to 15uM), in presence of 0.1uM Ucn2 for 48h before assessment of cell viability. C) Reversal of NCM460 sensitivity to CH11-mediated cytotoxicity after CRHR2 silencing via Fas inhibition. NCM460 cells were transiently transfected with siCRHR2 or negative control, followed by 48h exposure to 0.1M Ucn2, or 100ng/ml CH11,

or the combination. Cell viability was assessed by the CellTiter-Glo Luminescent cell viability assay. Fas expression was assessed by Western blot in NCM460 cells transfected with either siCRHR2 or negative control and treated with 0.1uM Ucn2 for 48h. Increased cleavage of the pro-apoptotic markers D) caspase 3 (p21/17 fragments) and E) PARP in CRHR2+ CRC cell clones, as assessed by Western Blot. The mitochondria associated pro-apoptotic marker Bcl2, remained unchanged. Total cell lysates were collected from EV and CRHR2+ DLD1 and SW620 cells treated with 0.1uM Ucn2 and 100ng/ml CH11 for 48h. GAPDH, b-actin or b-tubulin were used as loading controls. F) Compared to EV, CRHR2+ CRC cells treated with 100ng/ml CH11 and 0.1uM Ucn2 have higher percentages of apoptotic cells [expressed as % of cleaved-caspase 3 positive (+) cells]. Representative flow cytometry histograms with the percentages of cleaved-caspase 3 positive cells (+) in EV and CRHR2+ DLD1 cell clones treated with 0.1uM Ucn2 alone or in combination with 100ng/ml CH11, are also shown (right panel). *$p<0.05$, **$p<0.01$, ***$p<0.0001$.







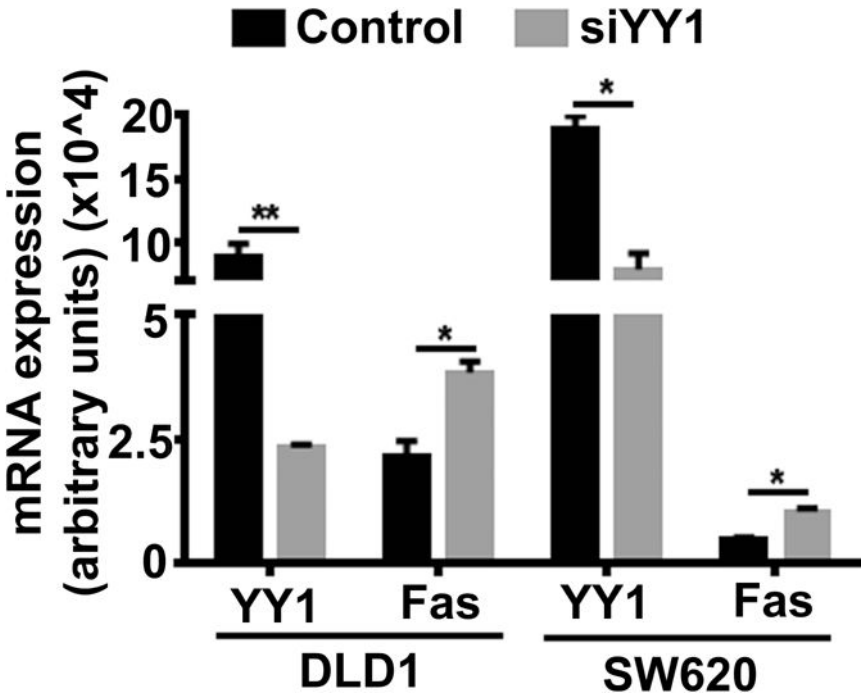
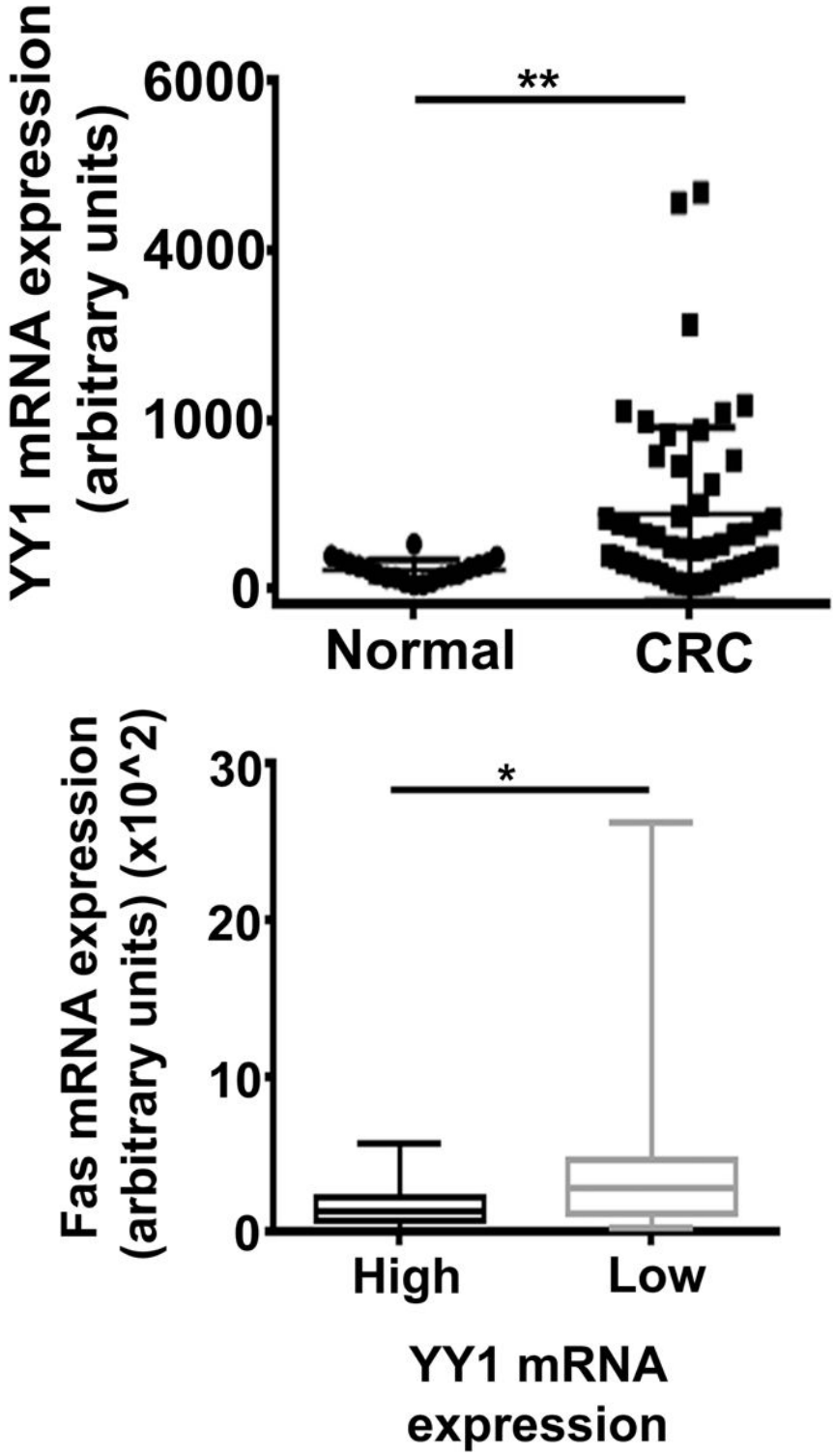
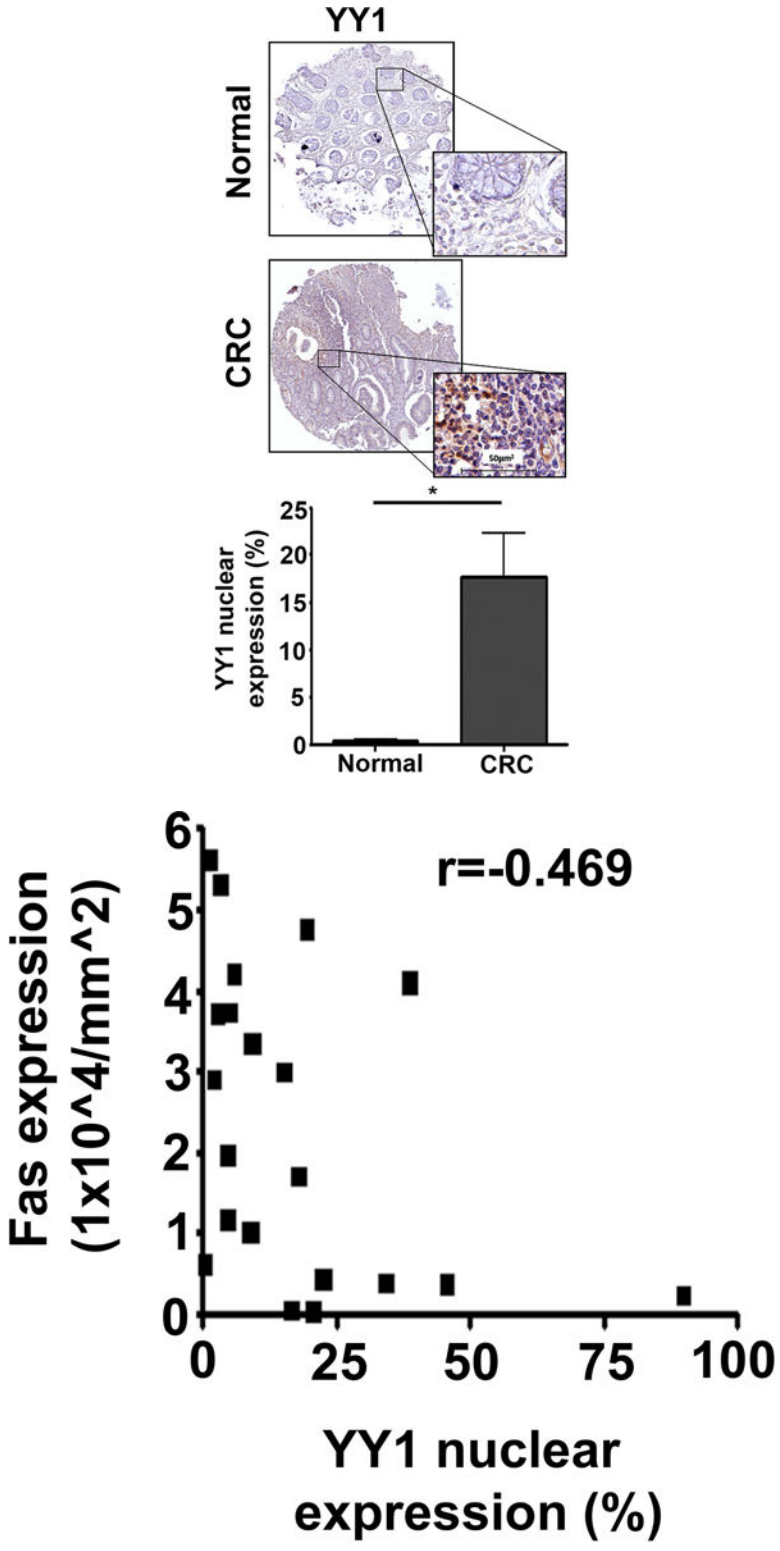
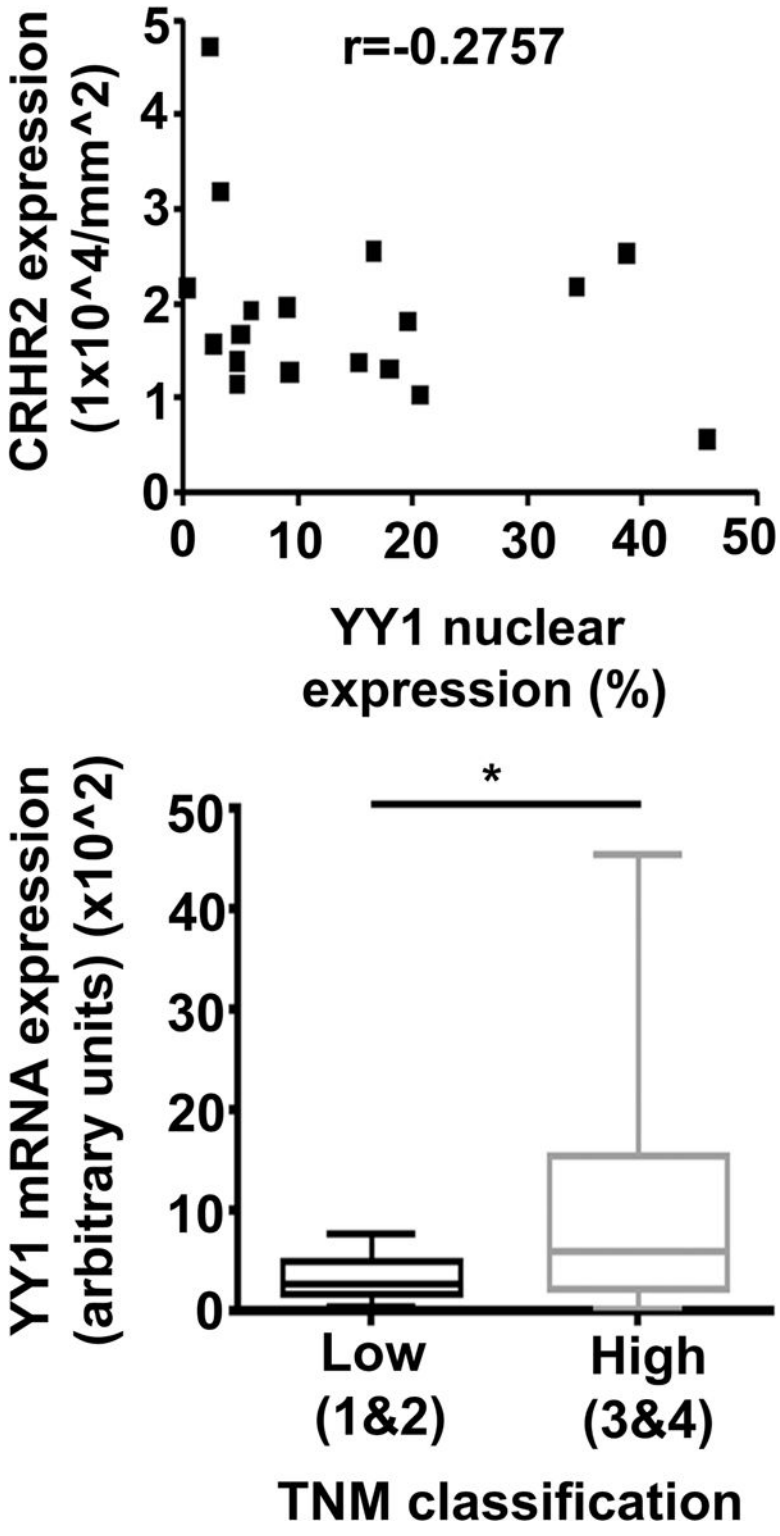


Figure 4. YY1 is a transcriptional repressor of Fas in CRC; it is specifically downregulated by CRHR2/Ucn2 signaling and its inhibition re-sensitizes CRC cells to CH11-killing through Fas induction

A) Fas promoter activity is significantly increased after YY1 silencing in SW620 cell line. Cells were transiently transfected with a pSwitchLight-Fas-promoter reporter construct or the corresponding GAPDH-promoter positive or negative control vectors for 24h, followed by transfection with siYY1 or a scrabbled control siRNA for 48 more hours. RLU: Relative luciferase units. B) Inverse association between YY1 and Fas mRNA baseline expression in parental CRC cells, as assessed by qPCR. Restored CRHR2/Ucn2 signaling in CRC cell lines decreases significantly YY1 expression at C) mRNA and D) protein level. EV and CRHR2+ SW620 and DLD1 cells were treated with 0.1M Ucn2 for 24h. Total RNA and protein were harvested for qPCR and western analysis, respectively. E) Ast2B inhibits specifically CRHR2/Ucn2-mediated YY1 mRNA downregulation in CRHR2+ CRC cell clones. EV and CRHR2+ SW620 cells were treated for 24h with 0.1uM Ucn2 alone or in combination with 0.5uM Ast2B before RNA extraction. F) Inhibition of YY1 re-sensitizes CRC cells to CH11-cytotoxicity. SW620 and DLD1 parental cells were transfected with siYY1 or control siRNA for 48 h followed by exposure to 100ng/ml CH11 for 48 more hours in presence or absence of 0.1M Ucn2. Cell viability was assessed by CellTiter-Glo Luminescent cell viability assay. G) Fas mRNA induction in siYY1- and Ucn2 -treated SW620 and DLD1 cells. *$p<0.05$, **$p<0.01$, ***$p<0.0001$.







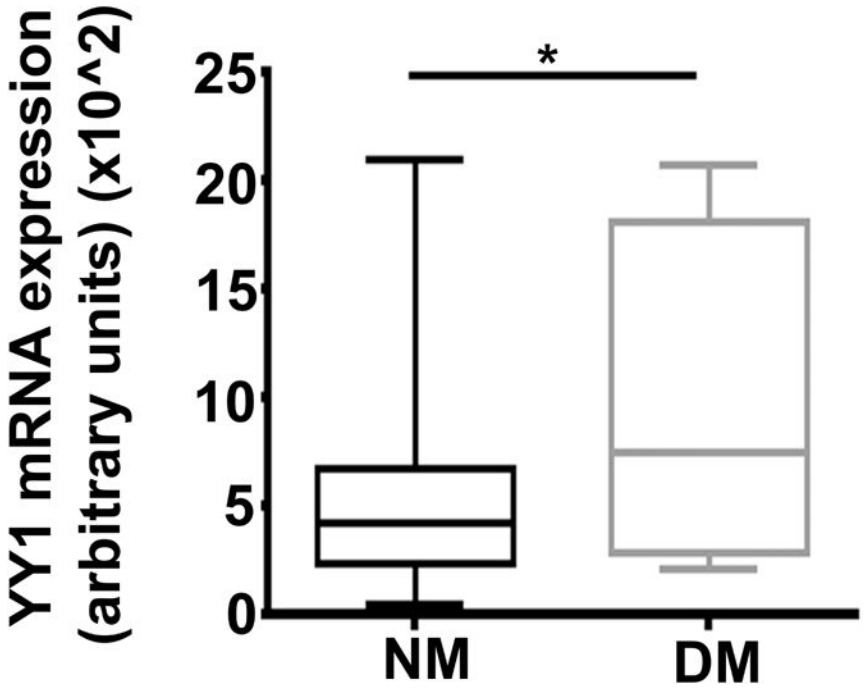
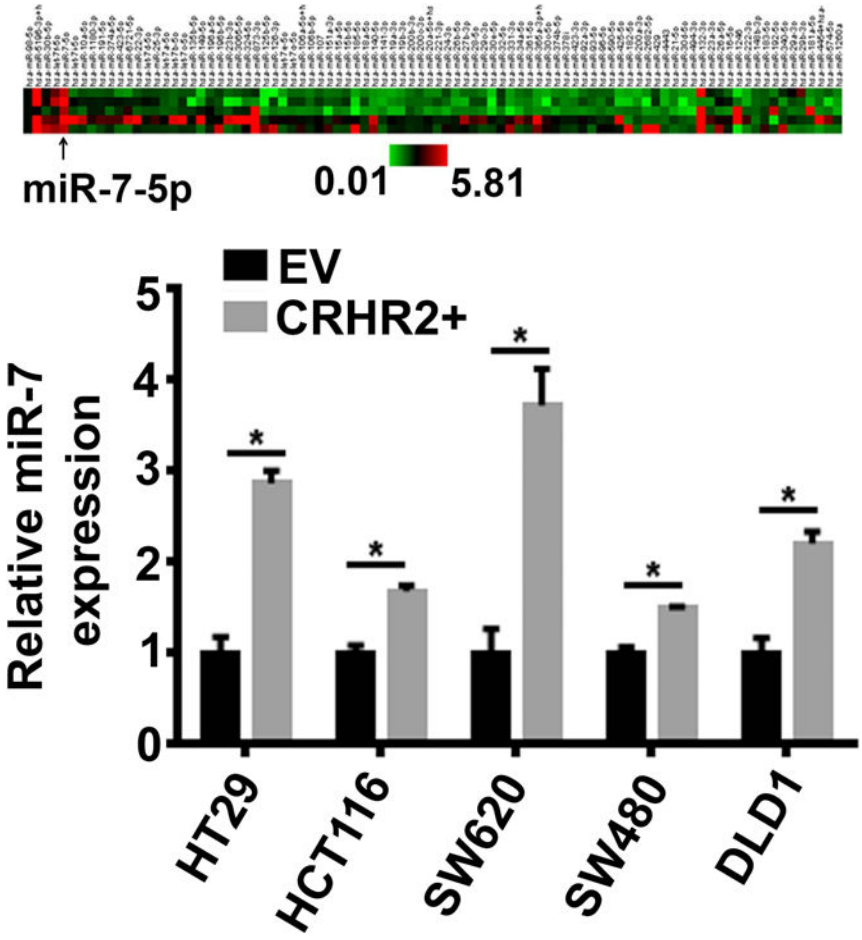
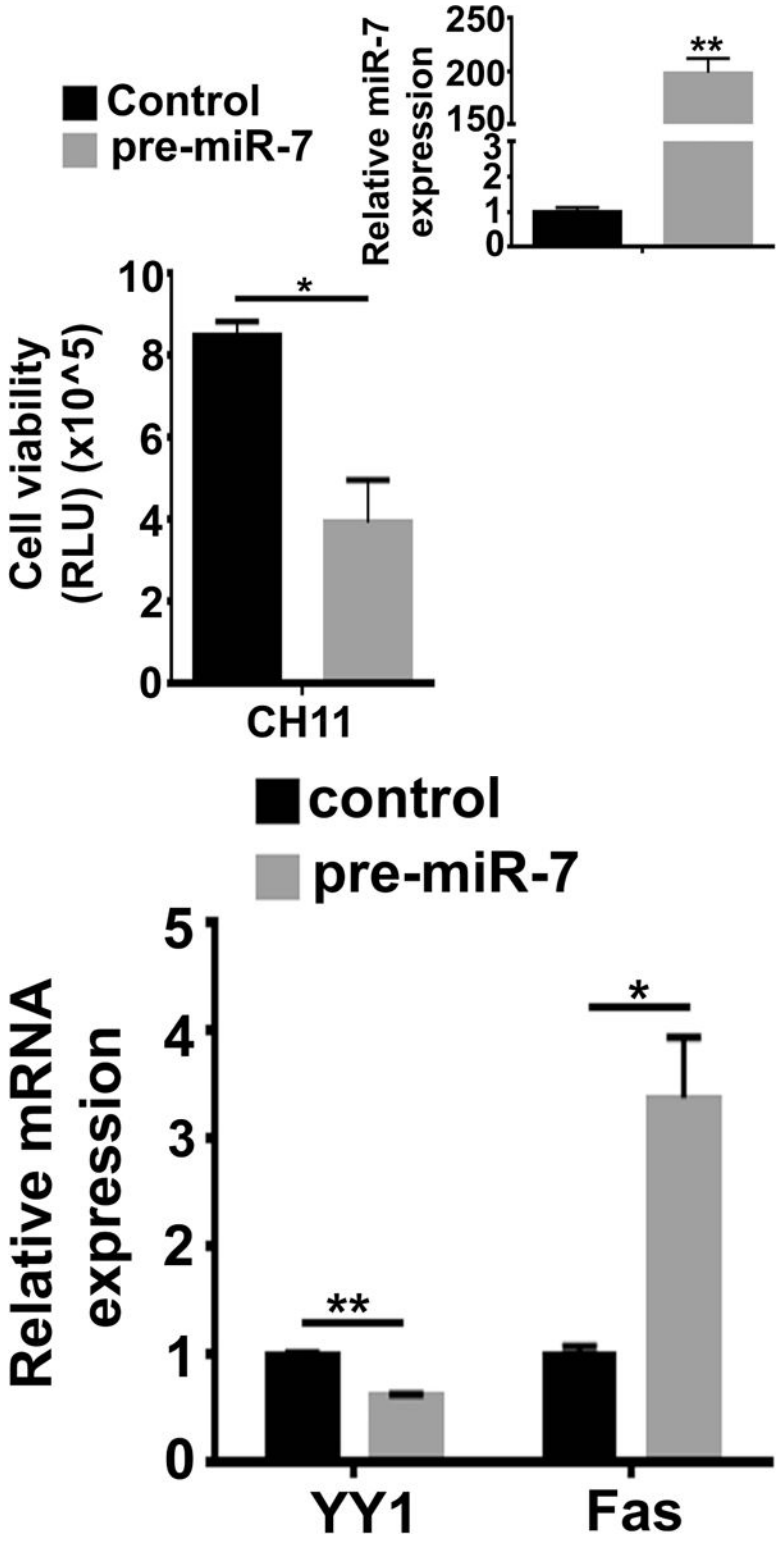


Figure 5. YY1 overexpression in CRC tissues associates significantly with reduced Fas and CRHR2 expression as well as with increased tumor aggressiveness

A) Significant overexpression of YY1 mRNA in CRC tissues (N=52) compared to normal controls (N=20), as assessed by qPCR. B) Inverse association between Fas and YY1 mRNA levels in CRC tissues (low and high YY1 expression set as < or >mean+SEM, respectively, see Supplementary Table 2; low N=31, high N=21). C) Increased nuclear YY1 protein expression (% positive cells) in CRC (N=21) over normal (N=6) tissue arrays, as assessed by IHC. Representative YY1 protein expression in CRC and normal tissue arrays also shown. Original magnifications x20 and x40, bar scale: 50μm. D) YY1 nuclear expression (% positive cells) in CRC tissue arrays is inversely correlated with Fas protein levels (N=21, Spearman r = −0.4690, 95% CI, p=0.032). E) A trend of negative correlation between YY1 and CRHR2 expressions in CRC tissue arrays, although without statistical significance (N=18, r=-0.2757, p=0.12). F) Increased YY1 mRNA expression in CRC samples associates with advanced tumor stage based on TNM classification [low (1&2), N=15; high (3&4), N=37) and G) occurrence of distant metastases NM: no metastasis (N=26); DM: distant metastasis (N=15). *p<0.05, **p<0.01, ***p<0.0001.





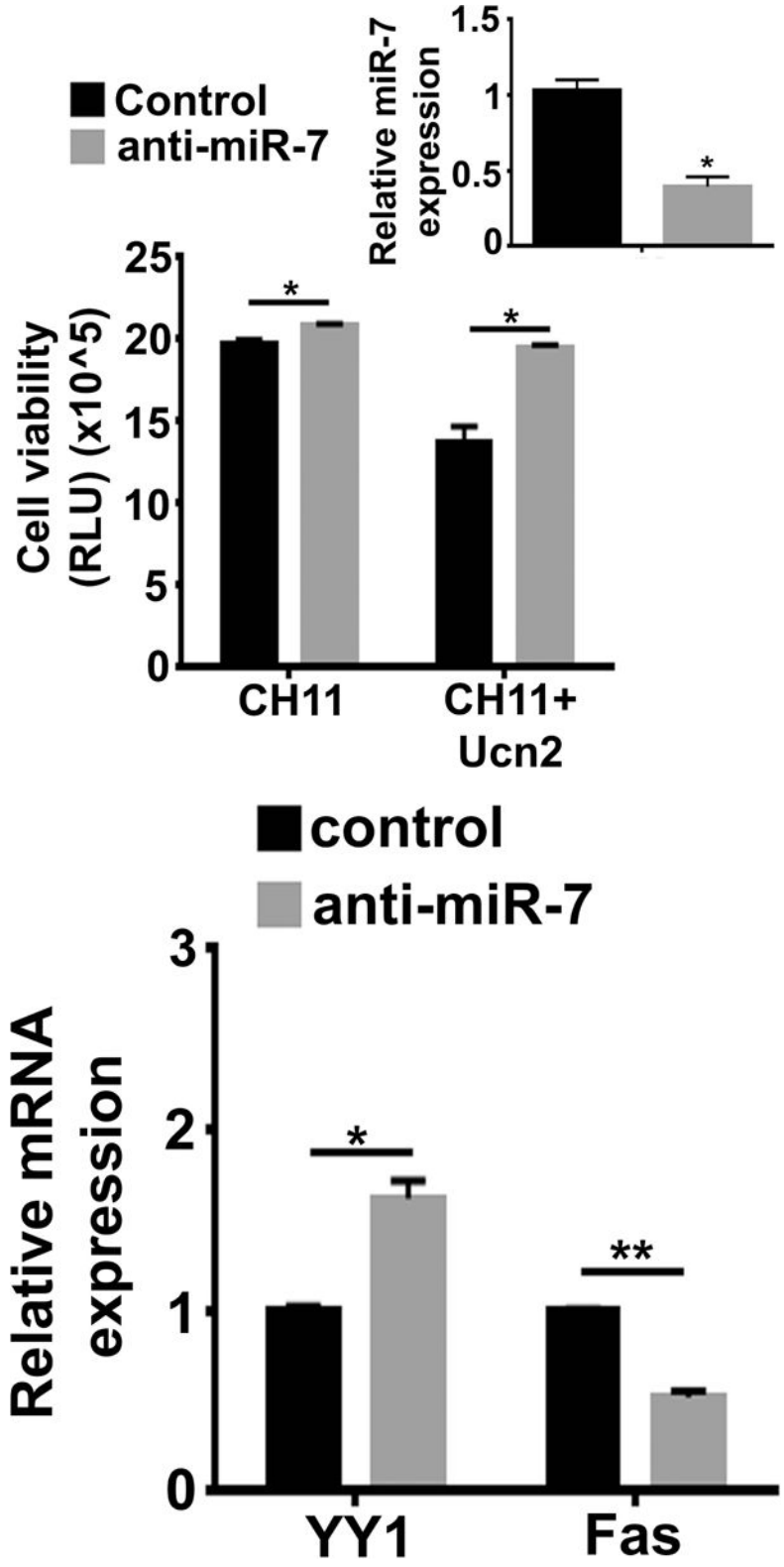


Figure 6. miR-7 is upregulated in CRHR2+ CRC cells and involved in CRHR2/Ucn2-mediated tumor sensitization to Fas/FasL-killing through YY1 downregulation

A) Heat map of differentially expressed miRNAs in CRHR2+ HT29, HCT116, SW620, SW480 and DLD1 cell lines (from up to down) after treatment with 0.1uM Ucn2 for 24h. The profiling of 850 miRNAs was performed using Nanostring technology. Gene expression changes >1.5 fold (compared to corresponding EV controls) were considered to be of biological and statistical significance. B) MicroRNA-7 is significantly overexpressed in CRHR2+ CRC cell lines. MiR-7 expression was quantified in 5 pairs (EV and CRHR2+) of CRC cell lines treated with Ucn2 and normalized to U6 expression. C) Ectopic expression of miR-7 in HCT116 increased cell response to CH11-cytotoxicity. HCT116 cells were transfected with either miR-7 precursor (pre-miR-7) or control for 48h, followed by treatment with 100ng/ml CH11 for 48 more hours and then analyzed for cell viability by CellTiter-Glo Luminescent cell viability assay. D) Pre-miR-7 suppressed YY1 and elevated Fas mRNA expressions in HCT116 cells, respectively, as assessed by qPCR. E) Inhibition of miR-7 in SW620-CRHR2+ cells reverses significantly cell sensitivity to CH11-mediated killing. Cell viability was analyzed in SW620-CRHR2+ cells transfected with either antagomiR-7 (anti-miR-7) or control and treated with CH11 (100ng/ml) in presence or absence of Ucn2 (0.1uM). F) Anti-miR-7 restored YY1 expression and dowregulated Fas levels in SW620-CRHR2+ cells. *$p<0.05$, **$p<0.01$.