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Original Article

miR-3132 upregulates surface TRAIL to induce apoptotic cell death in cancer cells

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Abstract: TRAIL-based therapies are of significant clinical interest because of its unique ability to induce apoptosis in cancer cells while sparing normal and untransformed cells. This selective antitumor potential of the TRAIL pathway has been harnessed by development of therapeutics including recombinant (rh)TRAIL and TRAIL-receptor agonist antibodies such as mapatumumab and lexatumumab. While these TRAIL-based therapies have proven successful in preclinical studies and safe in early phase clinical trials, the limited serum half-life has been a hurdle for further clinical development. Here we characterize miR-3132, a novel and first-in class TRAIL-inducing miRNA with potent anti-proliferative and pro-apoptotic effects in cancer cell lines. Initial mechanistic studies indicate that miR-3132 engages the interferon signaling pathway to induce TRAIL and subsequent TRAIL-dependent apoptosis in cancer cell lines. Our data further suggests that the binding of miR-3132 to toll-like receptors could be the upstream pathway for the interferon response. The current study the first report to demonstrate miR-3132's *in vitro* efficacy and preliminary mechanism of action in cancer cell lines.

Keywords: miRNA, TRAIL, cell death, colon cancer, TRAIL, miR-3132

Introduction

TNF superfamily member tumor necrosis factor related apoptosis-inducing ligand (TRAIL/Apo2L), an important pro-apoptotic effector, is a type II transmembrane protein that can be released from the cell surface in soluble form via proteolysis [1]. On release, soluble TRAIL induces rapid apoptosis through binding to death receptors DR4 or DR5, which are expressed on the surface of cancer cells [2]. Additionally, TRAIL plays an important role in innate immune surveillance, killing both tumor cells and cells infected with bacteria and viruses [3, 4]. Its quick and potent apoptotic properties keep the TRAIL gene under the tight regulation by transcriptional factors such as FOXO3a, p53, AP-1, CEBP, NFAT, GATA Stat-1, IRF-1, GSP1, GSP2 and GSP4. Following activation of an immune response, interferon signaling elements, particularly IFNα, IFNβ, IFNγ, mediate Stat-1 signaling and toll-like receptors (TLRs) (TLR3, TLR7 and TL8) control TRAIL upregulation. These pathways mediate TRAIL upregulation in immune cells including natural killer cells, macrophages, dendritic cells and cytotoxic T cells. Thus, TRAIL upregulation is a highly regulated event [3, 4].

There is significant interest in the regulation of the TRAIL gene because of its unique ability to induce apoptosis in cancer cells while sparing normal and untransformed cells [2, 5]. This selective antitumor potential of the TRAIL path-

way has been harnessed by development of different therapeutic approaches including recombinant (rh)TRAIL and TRAIL-receptor agonist antibodies such as mapatumumab and lexatumumab [6]. Many TRAIL-based therapies have proven successful in preclinical studies and safe in early phase clinical trials. Clinical trial results revealed several limitations of rhTRAIL and TRAIL receptor agonists with serum half-life, hepatotoxicity, stability, tumor-specific cytotoxicity and biodistribution being the most evident [7]. Hence, there is still an unmet clinical need for TRAIL-based anti-tumor agents with increased efficacy and safety.

miRNAs are 18-22 nucleotide small non-coding RNAs that can inhibit translation and/or affect mRNA stability by binding to the 3' untranslated region (UTR) of target genes [8, 9]. Studies in different tumor models have shown that miRNAs can either be oncogenic, as in the case of miR-155, miR-21 and the miR17~92 cluster, or tumor suppressive (miR-34a, let-7 family and miR-143) [10, 11]. Tumor suppressive miRNAs are dysregulated in cancer cells through multiple mechanisms including methylation of promoter sites, deletion, or loss of expression [12, 13]. Re-introduction of tumor suppressive miRNAs known as "miRNA mimics" has become an attractive therapeutic strategy. Many such miRNA mimics are currently in clinical or pre-clinical development for cancer [10]. Here we provide a mechanistic pre-clinical rationale for novel miRNA therapeutic-miR-3132 that has anti-proliferative and pro-apoptotic effects in a broad panel of cancer cell lines. Notably, miR-3132 induced surface TRAIL and subsequent TRAIL-dependent apoptosis in a broad range of cancer lines. Treating cells with TRAIL sequestering antibody RIK2 completely blocked miR-3132 induced apoptosis in cancer cells. Our study is the first evidence of a miRNA upregulating surface TRAIL and inducing TRAIL-dependent cell death. To gain insight into the putative mechanisms of miR-3132's pro-apoptotic effects, we have performed gene expression profiling following treatment with miR-3132. Microarray data analysis indicates that miR-3132 putatively binds to toll-like receptors (TLRs) such as TLR6 and up-regulates the interferon type I signaling, in cancer cells. Interferon signaling subsequently activates the JAK-STAT-IRF9 signaling, the direct upstream transcriptional complex for TRAIL induction. Knockdown studies indicate STAT1/STAT2 and interferons IFNB1 and IFNG are required for TRAIL indication, in agreement with the microarray data.

Materials and methods

Cell culture and reagents

All colorectal, breast, and lung cancer cell lines were obtained from American Type Culture Collection (ATCC) and maintained in the recommended media. Cells were routinely verified as free of mycoplasma contamination. miRNA mimic for hsa-miR-3132 (HMI1058) was purchased from Sigma-Aldrich. TRAIL neutralizing antibody RIK2 was purchased from Santa Cruz Biotechnology (sc-56246) and used at a concentration of 1:200 in all experiments. Cleaved Caspase 8 inhibitor Z-IETD-FMK was purchased from BD Biosciences (Cat. No. 550380) and used at final concentration of 10 μM in all experiments.

Transfection of miRNA mimics

miRNA mimic transfections were performed by reverse transfection using Lipofectamine RNAiMAX (Life technologies, Grand Island, NY). miRNA mimics were transfected at concentrations of either 25 nM or 50 or 100 nM, as indicated in respective assays.

Cell proliferation assays

A total of 5K-10K cells were transfected with either scrambled duplex or miRNA to a net concentration of 50 nM and plated in a 96-well plate. Cell viability was measured 72 hrs post-transfection using CellTiter-Glo® Luminescent Cell Viability Assay (Promega). The percent-cell viability was calculated by normalizing the luminescence signal to scramble duplex wells. All transfections were performed in triplicates and reported as % Viability + SEM, compared to scramble.

Colony formation assays

A total of 0.1×10^6 cells of each cell line were transfected with either scramble duplex or miR-3132 mimics to net concentration of 50 nM for 72 hrs. At 72 hrs, transfected cells were harvested and 500 cells per treatment group were plated in triplicate in 6-well plates for col-

ony formation. Colonies were stained with 0.25% crystal violet on Day 14, imaged, counted and reported as # of colonies ± SEM.

Cell cycle analysis

All cell lines were transfected with either scrambled duplex or miRNA mimic. At 72 hrs post-transfection, both floating and adherent cells were collected and fixed in 70% ethanol, followed by RNase A treatment and PI staining. Cell death (sub-G1) was quantified by propidium iodide (PI) staining and flow cytometry. Flow-Jo analysis was performed to quantify the distribution of cells in G1, S and G2-M phases of the cell cycle under different transfection conditions.

Cell surface staining for TRAIL

Cells transfected with SCR or miR-3132 were harvested using enzyme-free cell dissociation buffer (Life Technologies) at indicated time points. Cells were washed with FACS buffer (PBS with 1% FBS and 0.1% sodium azide) and stained with conjugated antibodies against TRAIL (Biolegend, 308205). Flow cytometry data was collected using LSR II flow cytometer (BD Biosciences). Flow-Jo software was used to exclude doublets and analyze data.

Quantitative RT-PCR (qRT-PCR)

Total RNA, which includes miRNA, was isolated using the Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, CA). 1 µg of total RNA from each sample was subjected to cDNA synthesis using SuperScript® III Reverse Transcriptase kit (Life technologies, Grand Island, NY), for detection of target genes and housekeeping genes. For detection of miRNAs, 0.5 µg of total RNA was reverse transcribed using TaqMan® MicroRNA Reverse Transcription Kit (Life technologies, Grand Island, NY). The relative expression of the reported genes and miRNAs was determined using real-time PCR performed on an Applied Biosystems 7900HT Fast Real-Time PCR system. For copy number analysis, a standard curve of miR-3132 copies ranging from 1.56×10^{7} to 1.56×10^{12} was used to quantify the copy number of miR-3132 at baseline for each cell lines. GAPDH and RNU6B were used as the endogenous controls for mRNA and miRNA samples, respectively. Each cDNA sample was amplified using Power SYBR Green (Applied Biosystems, CA) and miRNA components were quantified using TaqMan® Universal Master Mix II, no UNG (Applied Biosystems, CA). TaqMan miRNA assays were purchased from Applied Biosystems and used as per the manufacturer's instructions. ΔΔCt analysis was performed to calculate the fold-change for each gene.

Western blot

Western blotting was performed as described previously by Lulla et al. (https://etda.libraries.psu.edu/catalog/15216arl189). The following antibodies were used: BCL_{XL} (CST, 2764S), PARP (CST, 9542), p53-DO1 (Santa Cruz Biotechnology, sc-126), p21 (Calbiochem, OP64), β-actin (Sigma, A5441), XIAP (CST, 242S), FOXM1 (CST, D12D5), p-FOXM1 (CST, 14170S), Cyclin B1 (Santa Cruz Biotechnology, sc-245), CC3 (CST Asp175, cat. no. 9661), CC8 (CST Asp391, cat. no. 9496) and CC9 (CST Asp330, cat. no. 7237). Secondary antibodies acquired from Jackson Laboratories were horseradish-peroxidase conjugated.

Microarray analysis

A total of 0.5×10^{6} HT-29 were transfected in duplicate wells with either SCR RNA or 50 nM miR-3132 mimics for 48 hours. Cells were harvested and RNA was isolated using Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, CA) according to manufacturer's instructions and submitted to the FCCC Genomics Facility for gene expression analyses by microarray. The quality of RNA specimens was determined by Agilent Bioanalyzer RNA kits and RNA was amplified and labeled using the low RNA input linear amplification kit (Agilent, Santa Clara, CA, USA). Labeled cDNA targets were hybridized onto Affymetrix Human Gene 2.0-ST array. Raw data were quantile-normalized using RMA method. Ratios of gene expression level in miR-3132 transfected versus SCR were calculated and those with at least log 2-fold difference (up or down) were considered genes of interest.

Bioinformatics analysis

Gene ontology (GO) analysis: It was performed to identify overrepresentation of gene ontologies or families in the log 2-fold gene list data.

Pathway and network analysis by IPA (ingenuity pathway analysis): It was performed to identify key biological processes, canonical pathways,

upstream transcriptional regulators and gene networks.

GSEA (Gene set enrichment analysis): It was performed by ranking genes first by highest to lowest log 2-fold change. The ranked gene list was then queried using GSEA software to known Molecular Signature Database (MsigDB). Known pathways from curated databases and published studies that matched our gene signature were then reported in the analysis.

Statistical analysis

Data are presented as the mean ± standard error of the mean from at least three replicates. The Student's two-tailed t-test in GraphPad Prism was used for pairwise analysis. Statistically significant changes ($^{*}P\leq0.05$, $^{**}P\leq0.01$ and $^{***}P\leq0.001$) are indicated.

Results

Expression of miR-3132 has anti-proliferative and pro-apoptotic effects in cancer cells

miR-3132 was discovered as part of a cell-based high-throughput screen (HTS) to identify miRNAs that would selectively reduce proliferation in colorectal cancer (CRC) cell lines. Having screened a library of 2,754 miRNA mimics, miR-3132 was among the top 5 candidates that reduced viability in CRC cell lines [14] (https://etda.libraries.psu.edu/catalog/15216arl189). miR-3132 is an intronic miRNA transcribed as part of the TMEM198 gene, the function of which remains uncharacterized. In order to understand the role of miR-3132 in cancer, we first checked the expression of miR-3132 in tumor vs. non-transformed cell lines by qRT-PCR based copy number analysis. As seen in Supplementary Figure 1A, we observed that miR-3132 expression is much lower in cancer cells when compared to non-transformed cell lines in different tissue types. HCT-116 cancer line was the only exception that appeared to have higher expression than its normal counterpart. Next, we determined the functional effects of restoring the expression of miR-3132 in cancer cell lines. We first tested the short-term and long-term anti-proliferative effects of miR-3132. Overexpression of miR-3132 showed a broad therapeutic index in all cancer cell lines tested, evident by the 50-80% inhibition in cell viability compared to 5-20% inhibition seen in normal cell lines (**Figure 1A**). Similar results were noted in long-term colony formation assay. As seen in **Figure 1B-D**, over 60-90% inhibition of the long-term proliferation of cancer cells was observed. RKO and H460 cancer cell lines were the only exception that did not show the growth inhibition, despite optimal transfection efficiency. Thus, miR-3132 has potent anti-proliferative effects in most cancer cell lines tested. We then tested if miR-3132 could induce cell death in cancer cell lines. With the exception of MDA-MB-231, at 72 hours post-transfection we observed a 15-45% increase in the sub-G1 DNA content in all cancer cell lines tested (**Figure 2A**). Western blot analysis under similar experimental conditions confirmed these results. We observed cleavage of PARP and caspase-3 in all cell lines except SW-480 and MD-MBA-231 (**Figure 2B** and Supplementary Figure 1B and 1C). Thus, miR-3132 is a potent anti-proliferative and pro-apoptotic agent with a broad therapeutic index.

miR-3132 activates the extrinsic apoptotic pathway in cancer cell line

To further characterize the apoptosis induced by miR-3132, we performed a detailed analysis of the different markers of intrinsic and extrinsic apoptosis. As seen in **Figure 2B**, miR-3132 induced apoptosis through the extrinsic pathway in all CRC cell lines evident by increased levels of cleaved caspase-8. In Type II cell lines such as HCT-116, this further led to increase in cleaved caspase-9 levels and subsequent apoptosis. Of note, apoptosis was p53-independent as seen by unchanged levels of WT p53 in HCT-116 p53 +/+ cells treated with miR-3132 (**Figure 2B**, lanes 1 and 2). We also observed decreases in anti-apoptotic markers such as BCL_{xL} and XIAP. These results replicated in additional breast cancer cell lines (Supplementary Figure 1B) and lung cancer cell lines (Supplementary Figure 1C). WT p53 levels were unaltered in both MCF7 (Supplementary Figure 1B, lanes 1 and 2) and H460 (Supplementary Figure 1C, lanes 1 and 2) cells post miR-3132 treatment, further confirming p53-independent cell death. Overall 10/13 (exceptions were RKO, H460 and MD-MBA-231) cell lines were highly sensitive and underwent apoptosis following restoration of miR-3132 expression.

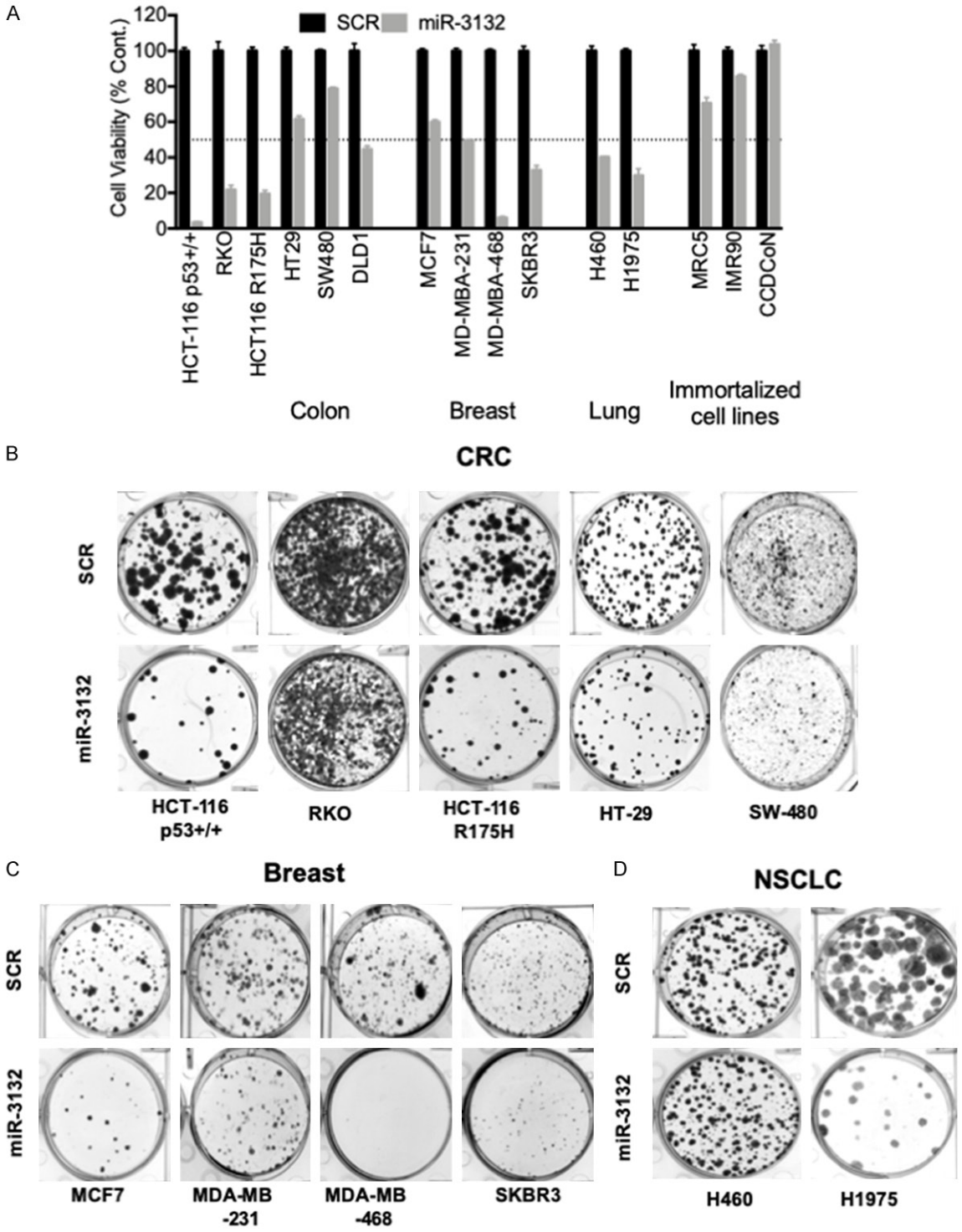

Figure 1. miR-3132 has anti-proliferative and pro-apoptotic effects in a broad panel of cancer cell lines. (A) A panel of cancer or normal cell lines was transfected with either 50 nM SCR or 50 nM of miR-3132 mimic. Effects on cell viability were measured at 72 hrs post-transfection using the CellTiter-Glo assay. (B-D) The effects of the miRNA mimics on long-term cell proliferation of CRC (B), breast (C) and NSCLC (D) cancer cell lines was assessed by colony formation assays done in 6-well plates. Cells were transfected with 50 nM of SCR or miR-3132 mimic. After 72 hrs, 500 cells were seeded per well in triplicate for each condition and stained with crystal violet on Day 14. Representative images of cells stained with crystal violet are shown.

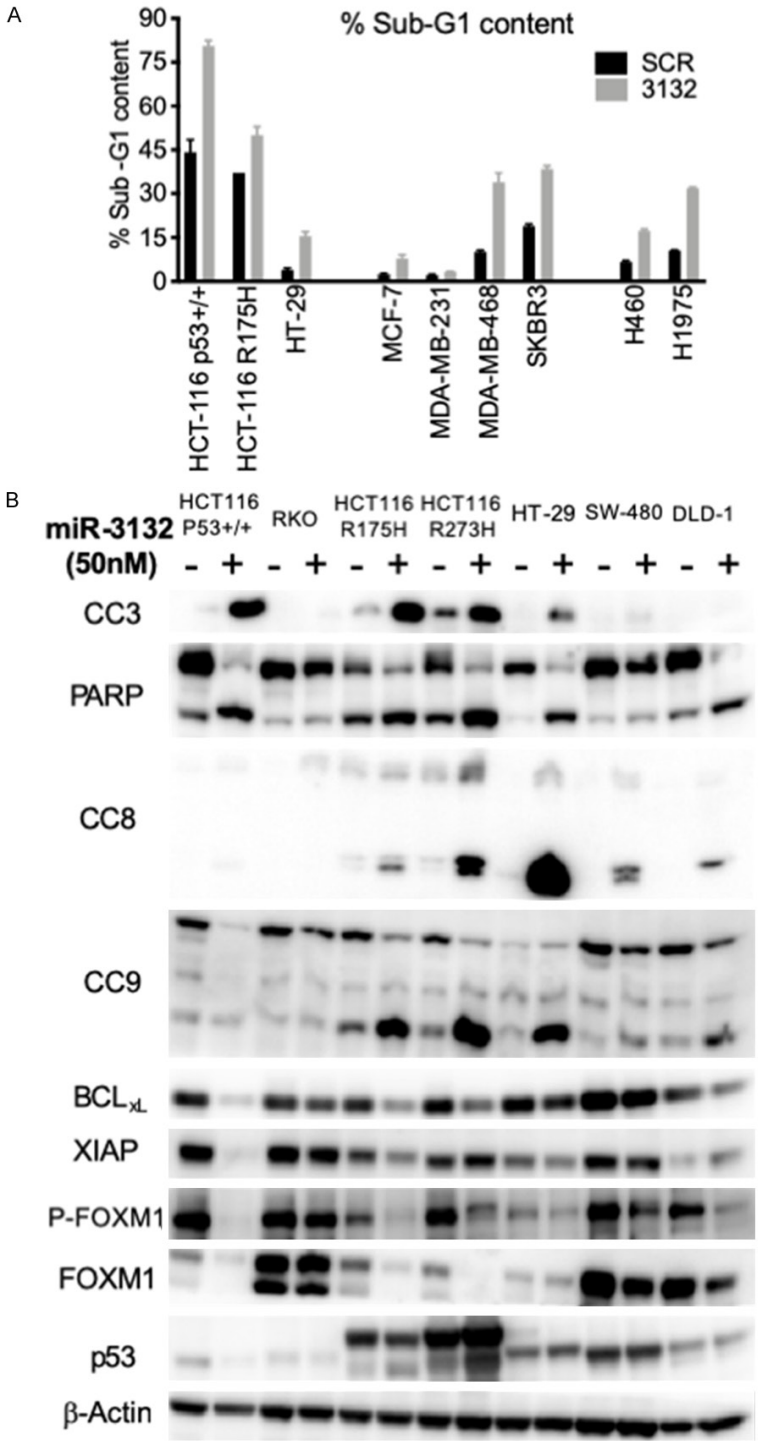

Figure 2. miR-3132 activates the extrinsic apoptosis pathway in cancer cell lines. A. Cell cycle profiles and apoptotic cells were assessed in the indicated cell lines by transfecting with either SCR or 50 nM miR-3132 mimic. At 72 hrs post-transfection cells were fixed, stained with PI and analyzed by FACS. Representative results of changes in sub-G1 phase of cell cycle are graphically represented (n=3). B. Cells were transfected with SCR or 50 nM miR-3132 mimic. 72 hrs post-transfection cells were harvested and western blot analysis for different markers of extrinsic and intrinsic were assessed in a panel CRC cell lines.

Surface TRAIL upregulation mediates and is necessary for miR-3132's pro-apoptotic functions in cancer cell lines

Having characterized the apoptotic phenotype post miR-3132 treatment, we wanted to identify the pathways altered by miR-3132 that could explain p53-independent apoptosis. To this end we performed gene expression profiling using microarray in HT-29 cells and identified a total of 296 genes that were differentially expressed between miR-3132 and control scramble miR transfected cells. Of these 296, 98 genes were down-regulated by log 2-fold or lower and 198 genes were up-regulated by log 2-fold or higher. Examination of the differentially expressed genes showed that a key gene that could explain miR-3132 mediated apoptosis is TRAIL, a pro-apoptotic ligand that selectively induces apoptosis in tumor cells (**Figure 3A**). Additionally, a number of genes in the death receptor signaling were altered (**Figure 3A**), validating the phenotypic observations. No changes in p53 target genes such as p21, PUMA, NOXA, DR5 was observed (Supplementary Figure 2A), confirming that apoptosis was indeed p53-independent. The transcription of the TRAIL gene is a tightly regulated process, that could be induced by transcription factors such as STAT1, NFAT, FOXO proteins etc. [3]. Further, cytokine signaling could also upregulate TRAIL directly through interferons such as interferon γ or through interferon induced STAT signaling [15]. In order to understand miR-3132's me-

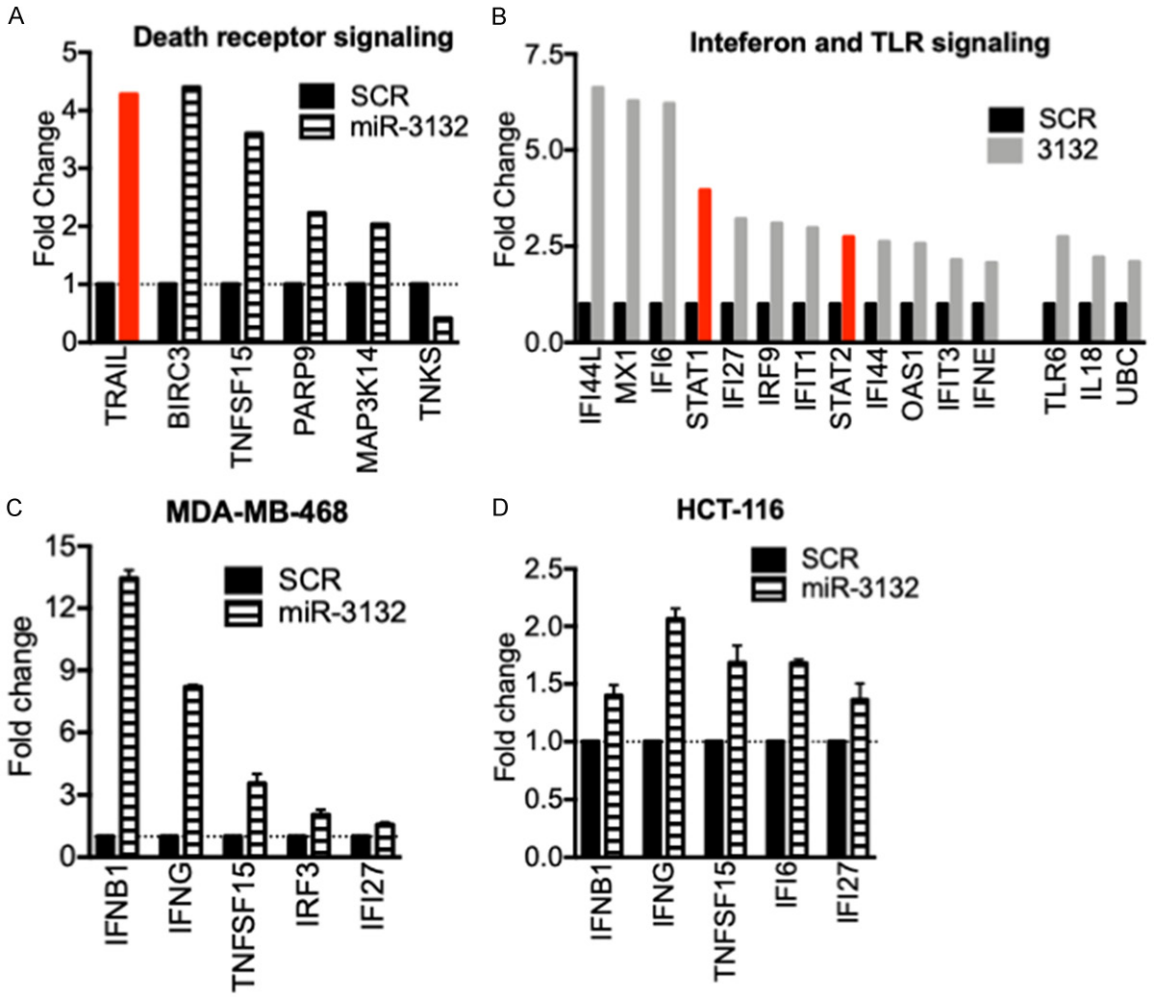

Figure 3. TRAIL induction is a downstream event of Interferon signaling (IFN) and Toll-Like Receptor signaling in cancer cells. A, B. HT-29 cells were transfected with either SCR or 50 nM miR-3132 for 48 hours, subsequent gene expression profiling by microarray and Ingenuity pathway analysis (IPA). Death Receptor signaling, Interferon signaling and Toll-like receptor signaling, the primary pathways modulated by miR-3132 and key genes altered are shown. C, D. MDA-MB-468 and HCT-116 cells were transfected with either 50 nM SCR or miR-3132. Genes involved in interferon signaling were assessed by qRT-PCR, 18 hours post-transfection.

chanism of TRAIL upregulation, we therefore performed both GSEA and Ingenuity pathway analysis (IPA) and identified Interferon Signaling the top activated pathway in response to miR-3132 (Supplementary Figure 2B and 2C). Examination of the upstream regulators (transcription factors mainly) further showed that STAT1 and IRFs were activated upon miR-3132 treatment (**Figure 3B**). No activation of NFκB or FOXO proteins were noted. These results were validated by performing qRT-PCR analysis in cancer cell lines (MDA-MB-468 and HCT-116), at 48 hrs post miR-3132 transfection (**Figure 3C** and **3D**). Thus, gene expression analysis reveals that miR-3132 could upregulate TRAIL via interferon signaling in cancer cells, which leads to extrinsic apoptosis.

To validate the gene expression data at the protein level, we started with investigating if TRAIL was induced at the cell surface in response to miR-3132. As early as 48 hrs post miR-3132 transfection, we observed a robust increase in surface TRAIL in three cell lines tested-HCT-116, HT-29 and SKBR3 (**Figure 4A** and Supplementary Figure 3A). Then to check if TRAIL was dispensable for miR-3132 mediated apoptosis through the extrinsic pathway in cancer cells, we co-treated HCT-116 and HT-29 cell lines with miR-3132 mimics and

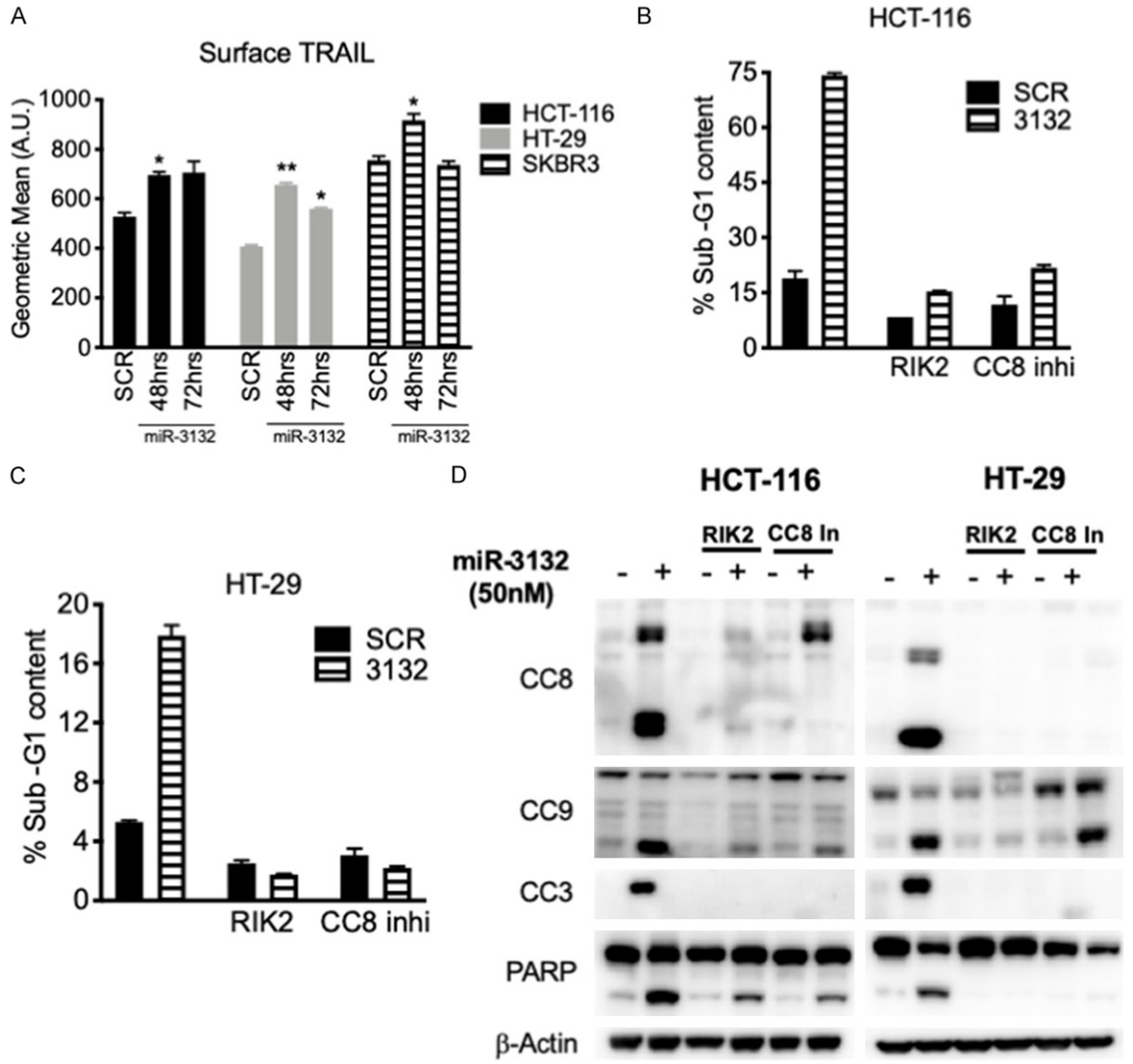

Figure 4. TRAIL is required for miR-3132 dependent apoptosis in cancer cells. (A) Expression of TRAIL at the cell surface post-transfection with 50 nM miR-3132 was tested at the indicated times by flow cytometry. Graphs indicate geometric mean (arbitrary units) of TRAIL expression from duplicate samples. (B, C) Sub-G1 analysis in HCT-116 and HT29 cells, to measure cell death post-treatment with miR-3132, in the presence/absence of RIK2 or CC8 inhibitor. (D) Using same experimental conditions as (B, C), markers of apoptosis were assessed by western blot. 48 hrs co-treatment samples for HCT-116 and 72 hrs co-treatment samples for HT-29 for results in (B-D).

either RIK2 (TRAIL neutralizing antibody) or cleaved caspase-8 inhibitor Z-IETD-FMK. miR-3132 induced cell death, measured by sub-G1 DNA content analysis, was abrogated in presence of RIK2 and Z-IETD-FMK (**Figure 4B** and **4C**). As early as 48 hrs post-treatment, both RIK2 and Z-IETD-FMK blocked the miR-3132 induced apoptosis markers cleaved caspase-8 and downstream cleaved caspase-9, cleaved caspase-3, and PARP in HCT-116 cells (**Figure 4D**, left). Similar results were observed in HT-29 cells, 72 hours post treatment (**Figure 4D**, right). This indicates the TRAIL is required and indispensable for miR-3132 induced apoptosis. It also validates that cell death by miR-3132 is primarily due to engagement of the extrinsic apoptosis pathway.

miR-3132-induces type I interferon signaling to mediate TRAIL upregulation in cancer cells

Having confirmed the role of TRAIL in miR-3132 induced apoptosis, we next investigated the upstream mechanism of TRAIL induction. IPA

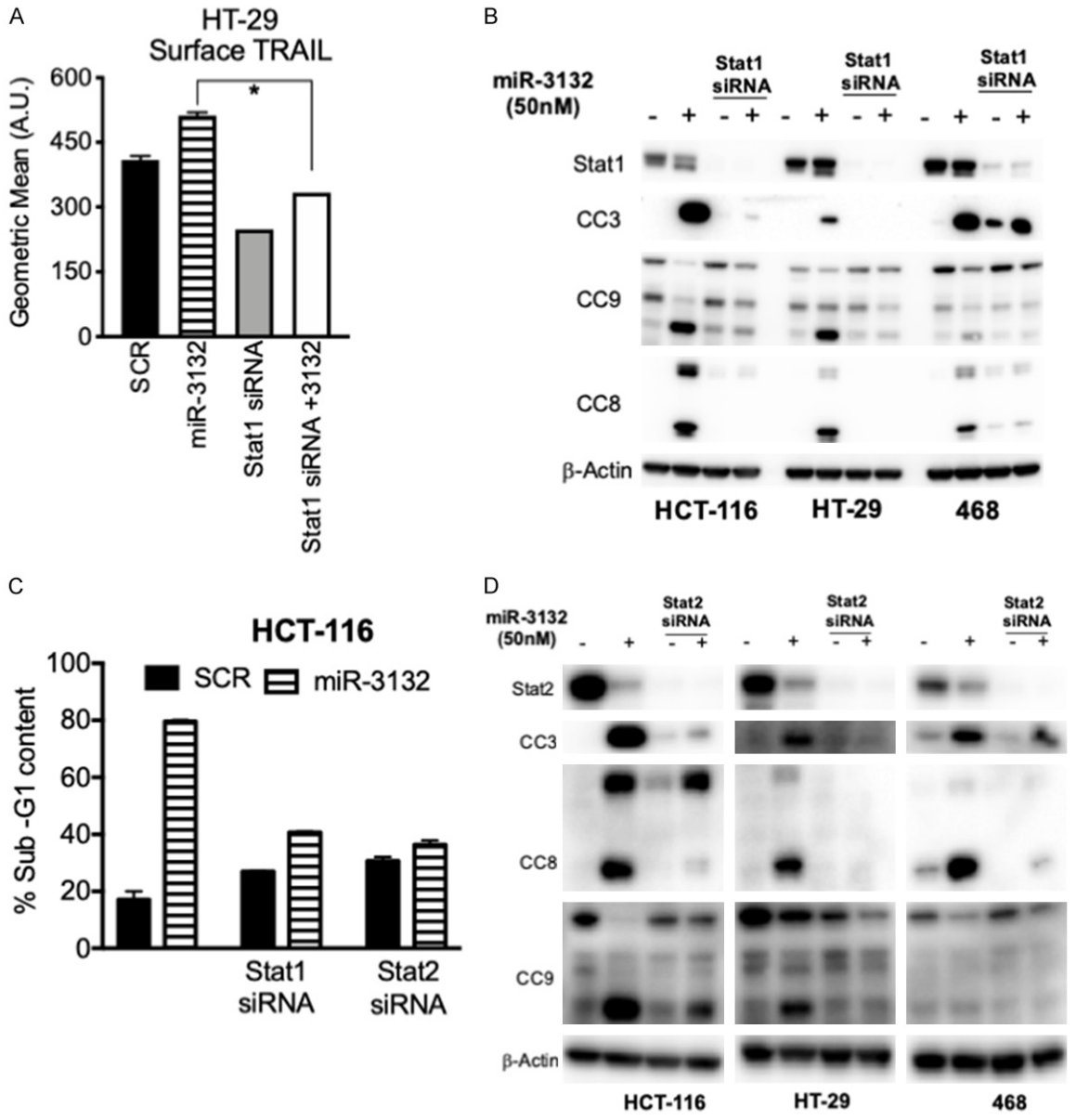

Figure 5. Stat-1 and Stat-2 are downstream effectors of IFN signaling. (A) HT-29 cells were transfected with either SCR, 50 nM miR-3132, 40 nM Stat-1 siRNA or co-transfection with Stat-1 siRNA. Expression of TRAIL at the cell surface, 48 hours post-transfection with was tested by flow cytometry. Graphs indicate geometric mean (arbitrary units) of TRAIL expression from duplicate samples. (B, D) Under similar experimental conditions as (A), indicated cell lines were assessed for expression of apoptotic markers by western blot analysis. For HT-29 cells alone, western blot analyses were done 72 hours post-transfection. (C) Cell cycle profiles and percentage of dead cells (sub-G_1) were assessed in HCT-116 using same conditions as (A). 72 hrs post-transfection cells were fixed, stained with PI and analyzed by FACS. Representative results of changes in sub-G_1 phase of cell cycle are graphically represented (n=3).

analysis was shown in Supplementary Figure 3B shows that the STAT1-STAT2-IRF9 complex could be an upstream transcription factor for TRAIL induction. To validate this, we looked at the upregulation of surface TRAIL post miR-3132 transfection, under conditions of -/+ STAT1 siRNA (**Figure 5A**). The data shows that STAT1 knockdown abrogated miR-3132 induced surface TRAIL in HT-29 cells. Further, knockdown of STAT1 and STAT2 also blocked the miR-3132 induced apoptosis in cancer cells (**Figure 5B-D**) in all cancer cells investigated. Taken together, these analyses indicate that miR-3132 can induce the production of

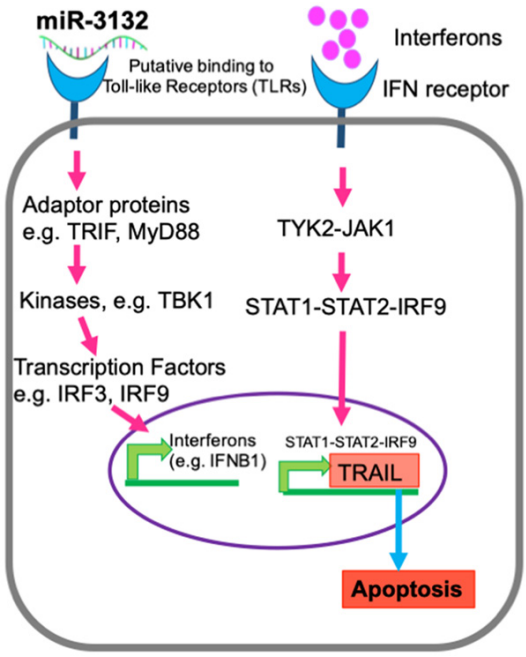

Figure 6. Model of miR-3132's proposed mechanism of action. miR-3132 induced TRAIL-dependent apoptosis could be attributed to its putative binding to TLR6. This binding results in upregulation of the type I interferon response in cancer cells, which in turn activate the JAK-STAT pathway. Subsequently, Stat1/Stat2 are responsible for the transcriptional upregulation of TRAIL and TRAIL-dependent apoptosis in cancer cells.

cytokines such as interferon β1 and interferon, which in turn can activate the JAK-STAT pathway and subsequent TRAIL induction. TLR6 could be the putative binding receptor that is needed for miR-3132 induced TRAIL and subsequent apoptosis (**Figure 6**).

Discussion

The upregulation of TRAIL as an effector molecule is highly regulated and context-dependent. In case of immune surveillance, IFN-γ induced expression of TRAIL on NK cells and other immune cells is well established [16-18]. TRAIL expressing immune cells are known to keep a check on tumor development and enhance efficacy of chemotherapeutics such as 5-fluorouracil [19]. TRAIL is also selectively cytotoxic to tumor cells [20]. Hence, activation of TRAIL-dependent death receptor signaling in cancer cells by rhTRAIL or TRAIL receptor agonistic antibodies is an ongoing therapeutic strategy. However, the limitations in bioavailability associated with rhTRAIL, or efficacy with DR4/DR5 monoclonal antibodies and atrimers has limited their clinical use [2]. Hence, there is a need for the development of novel non-toxic and highly efficacious TRAIL-based therapies.

We serendipitously discovered miR-3132 in a high-throughput screen that was designed to identify miRNAs that could selectively inhibit the proliferation/induce apoptosis of mutant p53-expressing cell lines. Our interest to investigate to miR-3132 stemmed from the phenotype that it was the only miRNA that was potent in inducing apoptosis in cancer cells, at par with chemotherapeutics or other drug based targeted therapies. Hence, regardless of p53 status, in the present study we have reported the preliminary mechanistic studies of miR-3132.

The detailed characterization of miR-3132 in a broad panel of cancer cell lines shows that it is selective for cancer cells and can effectively inhibit both the short-term and long-term proliferation of all cell lines tested. Further, miR-3132 engaged the extrinsic pathway of apoptosis in at least ten of the thirteen cell lines tested. This miR-3132 induced extrinsic apoptosis is a consequence of surface TRAIL upregulation, which was observed to be required for induction of apoptosis in all cell lines. Our study is the first to report a miRNA-mediated TRAIL upregulation in cancer cell lines. miRNAs normally inhibit protein synthesis by binding to the 3'UTR regions of mRNAs [21]. Therefore, we hypothesized that TRAIL induction might be a consequence of miR-3132 inhibiting synthesis of a TRAIL repressor proteins. However, to our surprise, microarray analysis indicated a robust induction of Type I interferon (IFN) signaling and toll-like receptor signaling (TLR). Both type I IFN and TLR signaling are well characterized and context dependent upregulators of TRAIL [3, 15]. Specifically, TLR are well characterized dsDNA/RNA and ssRNA sensors, which upon stimulation can activate IFN signaling [22]. Recent reports suggest that select miRNAs could be ligands for TLRs and induce downstream immune pathways and IFN signaling and that these binding properties are miRNA-specific [23]. In our current preliminary study on miR-3132 we have started delineating the downstream effectors of the putative miR-3132-TLR interaction. Specifically, transcriptions factors Stat1, Stat2, IRF9, IFNs (α, β, ε) and upstream TLR signaling via TLR6,

IRF3 were seen to be upregulated in miR-3132 microarray data. Starting with the transcription factors, we report that STAT1 and STAT2 are required for miR-3132 induced surface TRAIL expression and subsequent apoptosis. We further show that interferons β1 and γ are putative upstream activators of the JAK-STAT signaling. Future studies could focus on delineating the specific interferons and IRFs that are required for miR-3132 mediated TRAIL induction. Our preliminary literature search leans towards IFNβ1 as the putative upstream mediator of TRAIL induction [24]. Upcoming experimental studies will be performed to address these mechanistic questions. Future studies could also focus on addressing the miR-specific binding of miR-3132 to toll-like receptors (putatively TLR6) which induce production of Type I interferons. However, we cannot rule out that miR-3132 may also be negatively regulating factors that lead to Type I interferon production. In this regard, various knockdown studies or a pathway specific CRISPR screen could be performed, to understand the miR-3132 mediated TRAIL upregulation. In summary, we propose miR-3132 as a novel TRAIL-inducing therapeutic to be tested in different *in vivo* models of cancer, as an alternative to existing TRAIL based therapies.

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Disclosure of conflict of interest

None.

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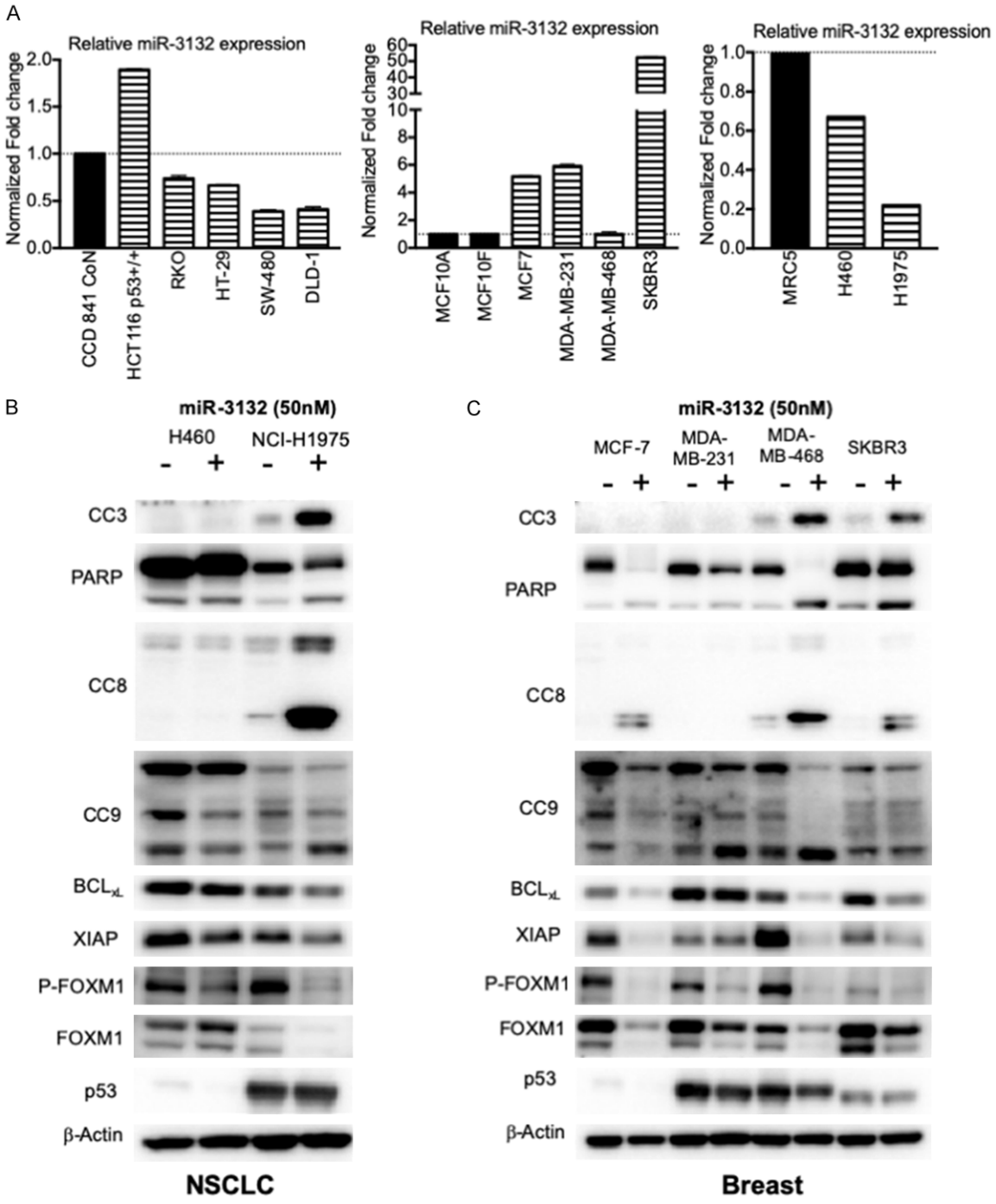
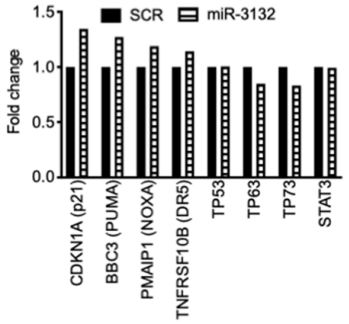


Supplementary Figure 1. (A) Baseline expression of mature miR-3132 was compared between immortalized and cancer cell lines from same tissues of origin. Fold change of miR-3132 in cancer cell lines is expression relative to immortalized cell line(s) from the same tissue. (B, C) Cells were transfected with SCR or 50 nM miR-3132 mimic. 72 hrs post-transfection cells were harvested and western blot analysis for different markers of extrinsic and intrinsic were assessed in (B), NSCLC (C) and breast cancer cell lines.

A



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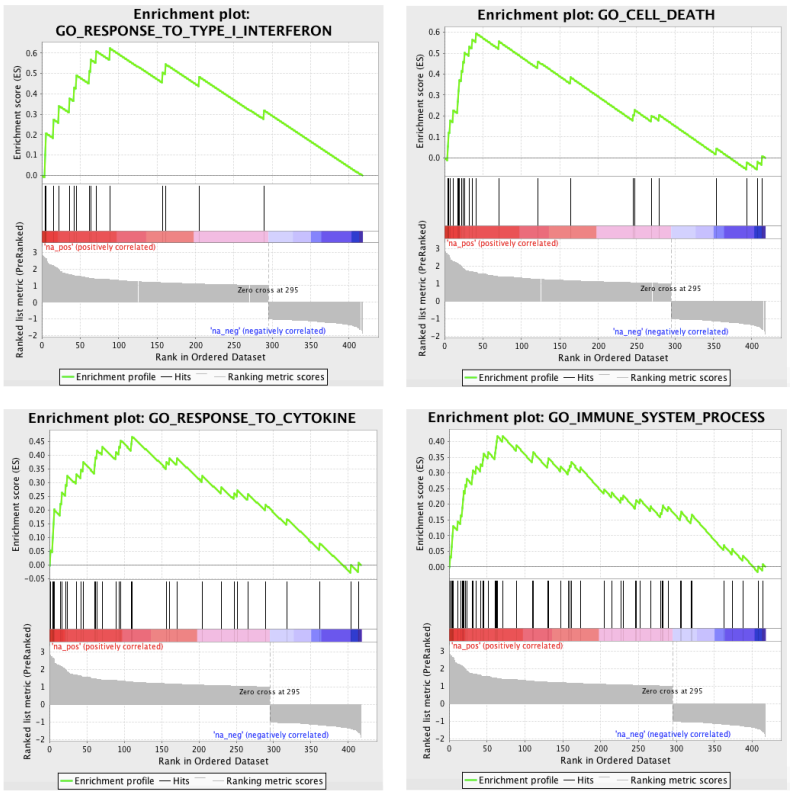


C

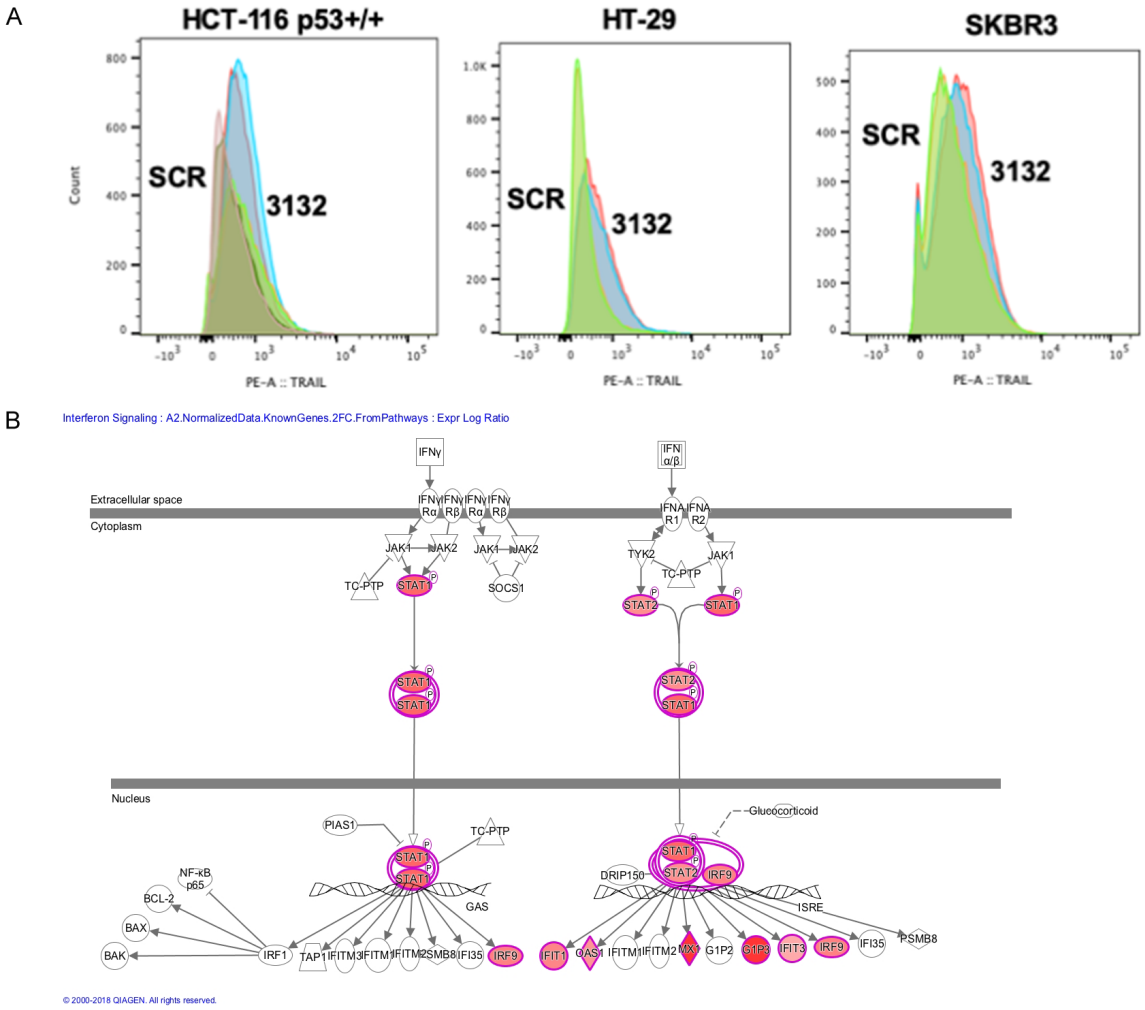
No	GSEA Set Name	Biological features	ES*	NES**	Nominal p-value	FDR Q value
1	BROWNE_INTERFERON RESPONSIVE GENES	Genes upregaulted in response to IFNα	0.587	2.395	0.00	8.52E-04
2	REACTOME_INTERFERON SIGNALING	Genes involved in interferon signaling	0.628	2.654	0.00	2.45E-04
3	GO_RESPONSE TO TYPE I INTERFERON	Response to Type 1 α, β, δ, κ,τ and gene family	0.622	2.473	0.00	9.17E-04
4	GO_CELL DEATH	Biological process that results in permanent cessation on vital functions of a cell	0.594	2.793	0.00	0.00
5	GO_REGULATION OF CELL DEATH	Any process that modulates the rate or frequecny of cell death	0.491	2.549	0.00	1.64E-04
6	REACTOME_CYTOKINE SIGNALING IN IMMUNE SYSTEM	Genes involved in Cytokine Signaling in Immune system	0.573	2.617	0.00	2.71E-04
7	GO_RESPONSE TO CYTOKINE	Process that results in change in state/activity of a cell as a result of cytokine stimulus	0.467	2.444	0.00	8.47E-04
8	REACTOME_IMMUNE SYSTEM	Genes involved in Immune system	0.486	2.404	0.00	7.23E-04
9	GO_IMMUNE SYSTEM PROCESS	Any process involved in the development and functioning of the immune system	0.418	2.607	0.00	0.00
10	GO_DEFENSE RESPONSE	Process triggered in response to presence of a foreign body or the occurrence of an injury	0.492	2.863	0.00	0.00
11	GO_REGUALTION OF RESPONSE TO STRESS	Any process that modulates the rate, extent or frequecny of stress	0.464	2.434	0.00	8.73E-04

*ES= ENRICHMENT SCORE, **NES= NORMALIZEDENRICHMENT SCORE

D

Upstream Regulator	Predicted State	Activation z-score	p-value of overlap
IRF7	Activated	4.554	6.45E-19
IRF3	Activated	3.801	5.29E-17
IRF1	Activated	3.752	8.59E-17
STAT1	Activated	4.448	1.11E-15
STAT2	Activated	2.57	1.22E-13
TP53	Activated	2.328	6.83E-12
IRF9	Activated	2.4	6.63E-11
NFKB1	Activated	3.066	1.48E-09
IRF5	Activated	3.094	2.18E-09
MYC		-1.487	2.47E-08
IFI16	Activated	2.583	0.0000218
YAP1	Activated	2.421	0.00185
FOXO1	Activated	2.376	0.0138

Supplementary Figure 2. A. Microarray data for the indicated p53 target genes is presented graphically. No change is p53 target genes were seen in miR-3132 transfected HT-29 cancer cells. B, C. GSEA analysis was performed using the microarray data described in **Figure 3**. B. Shows the GSEA plots for Response to Type I Interferon, Cell death, Response to cytokines and immune system process. These gene sets represent the top pathways upstream of TRAIL induction, that are enriched in miR-3132 transfected HT-29 cancer cells. C. Is the table listing the top 12 gene sets that were enriched in miR-3132 treated HT-29 cells. D. IPA analysis was done to identify transcription factors activated upstream of TRAIL signaling. The table lists only significant transcription factor activated, alongside their activation Z-score.



Supplementary Figure 3. A. Histograms showing expression of surface TRAIL transfected with either SCR or 50 nM miR-3132 for 48 hours. Note here, a shift on the X-axis to the right (blue, red) indicates increase in surface TRAIL expression compared to control (light and dark green). Y-axis indicates cell count. B. Detailed pathways built using IPA analysis and KEGG pathways are shown. Red coloring indicates upregulation of expression in microarray results. Here Type I Interferon signaling with Stat1, Stat2 and different downstream target genes altered are shown.