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Tumor cell intrinsic RON signaling suppresses innate immune responses in breast cancer through inhibition of IRAK4 signaling.

Jennifer R. Bourn[1], **Sasha J. Ruiz-Torres**[1], **Brian G. Hunt**[1], **Nancy M. Benight**[1], **Susan E. Waltz**[1,2,*]

[1]Department of Cancer Biology, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0521, USA

[2]Research Service, Cincinnati Veterans Affairs Medical Center, Cincinnati, OH 45267, USA

Abstract

Increasing evidence suggests that cancer cells require both alterations in intrinsic cellular processes and the tumor microenvironment for tumor establishment, growth, and progression to metastatic disease. Despite this, knowledge of tumor-cell intrinsic molecular mechanisms controlling both tumor cell processes as well as the tumor microenvironment is limited. In this study, we provide evidence demonstrating the novel role of RON signaling in regulating breast cancer initiation, progression, and metastasis through modulation of tumor cell intrinsic processes and the tumor microenvironment. Using clinically relevant models of breast cancer, we show that RON signaling in the mammary epithelial tumor cells promotes tumor cell survival and proliferation as well as an immunopermissive microenvironment associated with decreased M1 macrophage, natural killer (NK) cell, and $CD8^+$ T cell recruitment. Moreover, we demonstrate that RON signaling supports these phenotypes through novel mechanisms involving suppression of IRAK4 signaling and inhibition of type I Interferons. Our studies indicate that activation of RON signaling within breast cancer cells promotes tumor cell intrinsic growth and immune evasion which support breast cancer progression and highlight the role of targeting RON signaling as a potential therapeutic strategy against breast cancer.

[*]**Address correspondence to:** Susan E. Waltz PhD, Department of Cancer Biology, Vontz Center for Molecular Studies, University of Cincinnati College of Medicine, 3125 Eden Ave, Cincinnati, OH 45267-0521, Tel: 513.558.8675, susan.waltz@uc.edu, **Twitter Handle:** @SusanWaltz_UC.

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The authors declare no conflicts of interest.

Ethics approval:

All mice were maintained under specific pathogen-free conditions and treated in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Cincinnati and the Cincinnati Children's Hospital Medical Center.

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Keywords

receptor tyrosine kinases; breast cancer; tumor microenvironment; type I IFN signaling; IRAK4 regulation

1. Introduction

Breast cancer is the most common cancer among women and accounts for greater than 15% of all new cancer cases in the United States, with an estimated 276,500 newly diagnosed patients this year alone [1, 2]. Despite significant advances in early detection and treatment strategies, an estimated 42,000 women will die from breast cancer in 2020 alone, with advanced stage disease responsible for most deaths [1, 2]. These sobering statistics drive the need for understanding the molecular mechanisms promoting breast cancer progression and the development of novel therapeutics for the prevention and treatment of advanced breast cancer. A strong topic in cancer therapeutic research is the evaluation of novel immunotherapies designed to stimulate a patient's own immune system to recognize and aid in the killing of tumor cells [3, 4]. Currently, immunotherapies in blood cancers and many solid tumors show promise; however, their use in treating breast cancers as single- or combinatorial therapies have not yet shown similar efficacy and requires further study [5–8].

RON, a member of the Met family of receptor tyrosine kinases, is expressed on macrophages and epithelial cells and is activated by its ligand, hepatocyte growth factor-like protein (HGFL) [9–12]. HGFL is secreted primarily by hepatocytes, functioning in an endocrine fashion, and by breast cancer cells, functioning in an autocrine fashion [10]. RON is overexpressed in several epithelial cancers including breast cancer [13, 14]. More than 50% of human breast cancers exhibit RON overexpression which is highly associated with metastasis and early death in patients [15–19]. We previously demonstrated that RON overexpression in the mouse mammary epithelium (MMTV-RON transgenic mouse model) is sufficient to cause spontaneous, metastatic mammary tumors in 100% of female mice [17, 20]. As a result of RON overexpression, several key downstream signaling pathways were shown to be aberrantly regulated including β-catenin, PI3K/Akt, and MAPK, which mediate cancer cell growth, migration, and invasion [10, 16, 17, 21–24]. In addition, we have also demonstrated that whole body loss of HGFL in MMTV-RON mice leads to decreased tumor growth and increased immune cell infiltration to the tumors [25]. More specifically, upon HGFL loss, reduced breast tumor growth was associated with increased infiltration and M1 (pro-inflammatory) activation status of macrophages and cytotoxic T-cells, as well as increased tumor cell apoptosis; however, the mechanisms regulating innate immune cell alterations by tumor cells are largely unknown [25]. Identification of these novel tumor cell intrinsic pathways within breast cancer cells is critical for the rational development of effective therapeutic strategies for breast cancer patients.

Over the past decade, studies have shown that crosstalk between tumor cells and the tumor microenvironment supports tumor development, growth, and progression to metastatic disease [26–31]. However, specific immune cell types within the tumor microenvironment, such as tumor infiltrating leukocytes, play a major role in regulating breast cancer

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progression and several studies have demonstrated that higher levels of leukocytes both within the tumor microenvironment and the bloodstream, is associated with better prognostic outcomes for breast cancer patients [32–34]. More specifically, macrophages and natural killer (NK) cells have been shown to suppress mammary tumorigenesis through activation of anti-tumor innate immune responses [35–37]. Innate immune signaling pathways are activated by signaling through pattern recognition receptors (PRRs), leading to immune responses designed to eliminate abnormal cells and pathogens [38]. Signaling through PRRs culminate in the expression of genes that encode key factors, including type I IFNs and proinflammatory cytokines, that regulate immune responses. Type I IFNs have been shown to regulate cell proliferation, death, and immune cell modulation [39–41]. Interferon response factors (IRFs), specifically IRF3 and IRF7, are considered master regulators of type I IFNs and can act in a feed forward mechanism that allows for self-propagation and signal amplification [42, 43]. IRFs are upregulated during an innate immune response downstream of toll-like receptors (TLRs) or interleukin receptors (ILs). TLRs recognize and bind specific pathogen antigens, leading to recruitment of MyD88 and subsequent binding and activation of IRAK4 resulting in the formation of the myddosome complex, which is crucial to induce signaling transduction through IRFs (for type I IFN production) and NF-κB signaling (proinflammatory cytokines) [44]. IRAK4 activation is critical for inducing type I IFNs innate and adaptive anti-tumor responses [45]. Previous studies have shown that type I IFNs stimulate macrophage cytotoxicity, phagocytosis, and M1 macrophage polarization leading to the production of pro-inflammatory cytokines [46, 47]. In addition, type I IFNs increase NK cell and $CD8^+$ T cell proliferation, survival, and cytotoxicity [7, 46, 47]. As these cells are considered “first-responders” in innate immunity, tumor cells with the capacity to escape initial innate mediated detection demonstrate measurable advantage in tumor establishment and progression [48, 49]. Therefore, it is imperative to understand the mechanisms underlying cross talk between tumor cells and immune cells and allow for tumor development and progression.

Based on this premise, we sought to investigate the role of RON signaling in breast cancer cells in suppression of anti-tumor immune responses to drive tumor progression. In this study, we show, using a transgenic mouse model of breast cancer, that RON signaling in the mammary epithelium promotes mammary tumor growth and metastasis. We also show that RON signaling in the mammary epithelium allows for tumor cell intrinsic survival and proliferation and supports an immunopermissive tumor microenvironment with decreased macrophage, NK cell, and $CD8^+$ T cell infiltration observed in RON expressing versus RON replete tumors. Additionally, we demonstrate that RON signaling in breast cancer cells supports tumor growth and an evasion of innate immune responses through a novel mechanism that involves suppression of IRAK4-mediated type I Interferon (IFN) production, which are key regulators of anti-tumor innate immune responses. Overall, our findings support that RON signaling in breast cancer cells promotes an immunosuppressive tumor microenvironment to aid in escape from immune cell attack and support breast cancer progression, highlighting the novel role of targeting the RON signaling as a potential therapeutic strategy against breast cancers to improve patient prognostic outcomes.

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2. Materials and Methods

2.1 Cell lines

The murine breast cancer cell line, R7, was derived from a mammary tumor obtained from a transgenic MMTV-RON mouse as previously described [17]. Generation of the R7 knockdown (KD), R7 shRON and 3F7G10, cell lines from the parental R7 cell line has been previously described [21, 23]. The human breast cancer cell lines, MCF-7 and T47D, and HEK-293T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Generation of the MCF-7 PCI-Neo empty vector (EV), MCF-7 PCI-Neo RON, T47D shNT, and T47D shRON cell lines has been previously described [23, 24]. R7 IRAK4 overexpressing (OE) and R7 empty vector (EV) cell lines were generated by transducing R7 cells with a pMSCV-pGK-IRAK4 (pMSCV-pGK control for EV cells) or pMSCV-IRES-mCherry-IRAK4 (pMSCV-IRES-mCherry control for EV cells) plasmid (gift from Dr. Daniel T. Starcynowski, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA) and sorted based on GFP or mCherry expression using the BD FACS ARIA-Illu Sorter (BD Biosciences, San Jose, CA, USA) [50].

2.2 Mice

The generation of homozygous floxed RON receptor tyrosine kinase ($TK^{FL/FL}$) mice has been previously described and were backcrossed into an FVB background (FVB $TK^{FL/FL}$) [20, 51, 52]. FVB MMTV-PyMT (polyoma virus middle T-antigen) mice were purchased from The Jackson Laboratory (The Jackson Laboratory, Bar Harbor, ME, USA) and FVB MMTV-Cre mice were a gift from Dr. William J. Muller (McGill University, Montreal, Canada). FVB $TK^{FL/FL}$ female mice were crossed to hemizygous $PyMT^{+/-}$ male mice and the resultant hemizygous PyMT $TK^{+/FL}$ male mice were bred to $TK^{FL/FL}$ female mice to generate hemizygous $PyMT^{+/-}$ $TK^{FL/FL}$ control mice (PyMT $TK^{FL/FL}$). Hemizygous MMTV-$Cre^{+/-}$ mice were crossed with $TK^{FL/FL}$ mice to generate the FVB $TK^{FL/FL}$ MMTV-$Cre^{+/-}$ mice and $PyMT^{+/-}$ $TK^{FL/FL}$ MMTV-$Cre^{+/-}$ study mice (PyMT $TK^{\Delta Epithelial}$) were generated by crossing $PyMT^{+/-}$ $TK^{FL/FL}$ male mice to $TK^{FL/FL}$ MMTV-$Cre^{+/-}$ female mice. Genotyping of transgenic mice was performed, and primer sets for the identification of the PyMT transgene, RON genomic locus status, and MMTV-Cre status are as follows: PyMT: 5'-GGAAGCAAGTACTTCACAAGGG-3' and 5'-GGAAAGTCACTAGGAGCAGGG-3'; RON: 5'-TCATTTGAATCAGTCCCCTCACTTTTCTCC-3', 5'-GGAACCAGTACACAGATGAGTAAACTGAGC-3', and 5'-TCGCTCAAGCCCAGGCAGGGCCTCACAGAG-3'; MMTV-Cre: 5'-AGGTGTAGAGAAGGCACTCAGC-3' and 5'-CTAATCGCCATCTTCCAGCAGG-3'.

For orthotopic transplantation studies, cells were injected into the inguinal fat pad of FVB wild-type (WT) and NOD scid gamma (NSG) mice [21, 23, 53]. Tumor development was monitored over time until end-point tumor size was reached in the R7 control group mice. Tumor volume was calculated using the formula: $V = (\pi/6) \times L \times W^2$ [53].

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2.3 Tissue histology and immunohistochemistry

Tissues were processed for histological analyses as previously described [20]. Immunohistochemical staining and quantifications were performed as previously described [20, 25, 54–56].

2.4 Flow cytometry analyses

For immunophenotyping, mammary tumors were mechanically dissociated, enzymatically digested, and filtered to obtain single-cell suspensions as previously described [25, 54]. Isolated cells were stained with CD45 to detect the total immune cell population ($CD45^+$) and then stained for neutrophil or macrophage markers ($F4/80^+$ $CD11b^+$ $Ly6G^-$ M1 macrophages, $F4/80^+$ $CD11b^+$ $Ly6G^+$ granulocytes, $F4/80^+$ $CD11b^+$ $Ly6G^-$ $Ly6C^+$ M2 macrophages), B cells ($CD45R^+$), natural killer cells ($NK1.1^+$), $CD4^+$ T cells, and $CD8^+$ T cells with the following antibodies: CD45-APC (#103111; BioLegend, San Diego, CA, USA), F4/80-PE-Cy7 (#25-4801-82; eBioscience, San Diego, CA, USA), CD11b-FITC (#557396; BD Biosciences, San Jose, CA, USA), Ly6G-APC (#127613; BioLegend), Ly6C-Pacific Blue (#128014; BioLegend), CD45R/B220-Pacific Blue (#558108; BD Biosciences), NK1.1-APC (#108709; BioLegend), CD107A-FITC (#121606; Biolegend), CD4-PE-Cy7 (#25-0041-82; eBioscience), and CD8a-APC (#17-0081-82; eBioscience). Flow cytometry was performed and analyzed using the BD FACS ARIA-Illu or LSRFortessa and FACS Diva software (BD Biosciences).

For cell proliferation studies, cells were cultured in serum free media (SFM) for 24 hours, labeled with EdU for 6 hours and processed per manufacturer's instructions (Click-It™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 647 dye, #C10634; Invitrogen, Carlsbad, CA, USA). For apoptotic studies, cells were cultured in SFM for 24 hours and stained with Annexin V-APC (#550474) and Propidium Iodide (PI; #556463) per manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Flow cytometry was performed and analyzed using the LSRFortessa and FACS Diva software (BD Biosciences, San Jose, CA, USA). Cells in early apoptosis, late apoptosis, or dead were designated as apoptotic. Each *in vitro* experiment was performed in triplicate and repeated at least three times.

2.5 alamarBlue and MTT Cell Viability Assay

For growth curve analysis, cells were seeded at equal density in 96 well plates and cultured for 0 to 7 days under normal culture conditions. At each respective timepoint, 10 μL of resazurin reagent (#R7017; Sigma, St. Louis, MO, USA) was added to each well 4 hours before the plate was imaged using the CLARIOstar Plus plate reader system (BMG Labtech, Cary, NC, USA). The plate was imaged with the resazurin filter set (ex. 545±20 nm, em. 600±40 nm) to measure fluorescence intensity and average fluorescence intensity of the blank was subtracted from each of the wells. The average fluorescence intensity was plotted as mean ± SEM; representative experiments are shown.

R7 and R7 KD cells were seeded at equal density in 96-well plates and incubated under normal culture conditions for 24 hours. The next day, cells were treated with the IRAK4 inhibitor (IRAK4i), CA-4948, (5μM; #S8562, Selleckchem, Houston, TX, USA) for 48

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hours. The alamarBlue assay was used to quantify cell viability, percent viability was normalized to the control cells and plotted as mean ± SEM.

R7, R7 KD, and R7 IRAK4 OE cells were seeded at equal density in 96-well plates and incubated under normal culture conditions for 24 hours. The next day, cells were treated with FasL (10ng/mL; #310-03H; PeproTech, Inc., Rocky Hill, NJ, USA) and TRAIL (100ng/mL; #315-19; PeproTech, Inc., Rocky Hill, NJ, USA) for four hours. MTT (3-(4,5-Dimethythiaol-2-yl)-2, 5-diphenyltetrazolium bromide; #M5655; Sigma, St. Louis, MO, USA) was used to quantify cell viability as previously described [25]. The percent cell viability was normalized to the control, R7 cells, and plotted as mean ± SEM.

2.6 Enzyme-linked immunosorbent assay (ELISA)

Murine breast cancer cells were plated in 6-well tissue culture dishes at a density of 1×10^5 cells/well in complete media for 24 hours followed by an additional 24 hours in serum-free media. After 48 hours, the culture media were collected, and dead cells and debris were removed by centrifugation. Media was concentrated by using the Amicon® Ultra-4 3K Centrifugal Filter Devices (MilliporeSigma, St. Louis, MO, USA) and was used for analysis following the manufacturers protocol for the murine IFNα and IFNβ VeriKine ELISA kits (#42120-1 and #42400-1; R&D Systems, Inc., Minneapolis, MN, USA). IFNα and IFNβ concentration (pg/ml) was measured at an optical density of 450nm using a Synergy MX plate reader and a standard curve was generated by using the Gen5™ 4 parameter logistic curve fitting software (Bio-Tek Instruments, Winooski, VT, USA). The data are shown as mean ± SEM of three independent experiments.

2.7 CD11b and Natural Killer (NK) cell cytotoxic assays

Splenocytes were isolated from wild-type (WT) mice as previously described [25]. Isolation of $CD11b^+$ and Natural Killer (NK^+) cells from splenocytes was performed per manufacturer's instructions using the mouse CD11b (#130-049-601) or NK cell isolation Kits (#130-115-818) (Miltenyi Biotec, Auburn, CA, USA). Target cells, R7, R7 KD, and R7 IRAK4 OE, were plated at 1×10^3 cells/well and incubated for 24 hours under normal culture conditions. The next day, splenocyte-derived $CD11b^+$ or NK^+ cells were directly co-cultured with target cells at target to effector (T:E) ratios of 1:0, 1:3, 1:10, and 1:30 for two hours. Then, the number of viable target cells was determined by alamarBlue cell viability assay and analyzed using the CLARIOstar Plus plate reader system (BMG Labtech, Cary, NC, USA). For each group, the number of viable target cells at each ratio was normalized to the number of viable target cells at 1:0 ratio and then plotted.

2.8 RNA-Sequencing (RNA-Seq) and Quantitative Real-Time PCR (qRT-PCR) analyses

For RNA-Seq, RNA was isolated from T47D shNT and T47D shRON cells using the TRIzol method (#15596026; Invitrogen, Carlsbad, CA, USA) and libraries were prepared using the Illumina TruSeq RNA preparation kit and sequenced on the Illumina Hi-Seq 2000 (Illumina, San Diego, CA, USA). Data was analyzed using GeneSpring NGS software (Agilent Technologies, Santa Clara, CA, USA) as previously described [21]. For differential expression between T47D shNT and T47D shRON cells, a cutoff of ≥ 2-fold difference was applied with genes provided in Supplementary Table S1. Pathway enrichment analysis for

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differential gene lists was performed using ToppGene as previously described with adjusted P-values displayed using the Benjamini and Hochberg procedure for multiple hypothesis testing [21, 57]. qRT-PCR was performed as previously described [52] and the following primer sets were used for measurement of transcript levels: human *IFNA1* 5'-GACTCCATC TTGGCTGTGA-3' and 5'-TGATTTCTGCTCTGACAACCT-3'; human *IFNB1* 5'-AAACTCATGAGCAGTCTGCA-3' and 5'-AGGAGATCTTCAGTTTCGGAGG-3'; mouse *Ifna4* 5'-CCTGTGTGATGCAGGAACC-3' and 5'-TCACCTCCCAGGCACAGA-3'; mouse *Ifnb1* 5'-TGTCCTCAACTGCTCTCCAC-3' and 5'-CCTGCAACCACCACTCATTC-3'; mouse *Irf7* 5'- CTGGAAGACCAACTTCCGCT-3' and 5'-AGCATTGCTGAGGCTCACTT-3'; *18S* 5'-AGTCCCTGCCCTTTGTACACA-3' and 5'-GATCCGAGGGCCTCACTAAAC-3'. Data was normalized to *18S* reference gene and analyzed by $\Delta\Delta C_T$. Relative gene expression normalized to the reference gene is reported. Data can be accessed at NCBI's Gene Expression Omnibus (GEO) database (#GSE162532, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE162532).

2.9 Immunoprecipitation and Western blot analyses

For immunoprecipitation (IP) analyses, pcDNA plasmid constructs including Wild-type (WT) RON, kinase dead (KD) RON, IRAK4 OE were transiently transfected into HEK-293T cells; IP and western blot analyses were performed as previously described [50, 58, 59]. After protein collection, the solubilized proteins were immunoprecipitated with either RON-β (C-20) (#sc-322; Santa Cruz Biotechnology, Dallas, TX, USA), p-IRAK4 (Thr345/Ser346) (#11927; Cell Signaling Technology, Danvers, MA, USA), IRAK4 (#4363; Cell Signaling Technology, Danvers, MA, USA), TRAF6 (#ab33915; Abcam, Cambridge, MA, USA), or Rabbit IgG control (#1-1000-5; Vector Laboratories, Burlingame, CA, USA) antibody and 20 μl of a 0.25% slurry of Protein A/G PLUS-Agarose beads (#sc-2003; Santa Cruz Biotechnology, Dallas, TX, USA), followed by western blot analyses.

For western blot analyses, whole cell lysates were collected in RIPA buffer [21]. Western blot analyses were performed with antibodies: RON-β (E-9; #sc-374626), MyD88 (E-11; #sc-74532), and Actin (C-4; #sc-47778) (Santa Cruz Biotechnology, Dallas, TX, USA); phospho-p44/42 MAPK (Thr202/Tyr204; #4730), p44/42 MAPK (Erk1/2; #9107), phospho-IRAK4 (Thr345/Ser346; #11927), phospho-p38 (Thr180/Tyr182; #9216), p38 MAPK (#9212) (Cell Signaling Technology, Danvers, MA, USA); IRAK4 (#32511) and TRAF6 (#33915) (Abcam, Cambridge, MA, USA); and IRF7 (#PA520281; Invitrogen, Carlsbad, CA, USA). Peroxidase-conjugated secondary antibodies were applied (#711-035-152 anti-Rabbit and #715-035-150 anti-Mouse; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), and membranes were developed using Pierce ECL2 Western Blotting substrate (#P180196; Thermo Fisher Scientific, Waltham, MA, USA). Images were obtained using the UVP ChemStudio Imaging system and densitometry analysis performed with VisionWorks software (AnalytikJena, Upland, CA, USA).

2.10 Statistical Analysis

Statistical analyses for tumor growth kinetics were performed using a 2-way ANOVA with Bonferroni post-test. Statistical analyses for the distribution of IFNα and IFNα mRNA expression in the breast cancer TCGA database were performed with Mann-Whitney rank-

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sum tests and probability of survival analysis was performed with Gehan-Breslow-Wilcoxon test or log-rank test; hazard ratio (HR) and P-value is provided. All other data are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by performing Student's t-test for pairwise comparisons or ANOVA for comparison of multiple groups using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Significance was set at *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

3. Results

3.1 RON signaling in the mammary epithelium promotes mammary tumor growth and progression.

We previously showed that whole-body loss of HGFL-RON signaling significantly reduces mammary tumor progression and metastasis [17, 20, 25]. However, the contributions of HGFL-RON signaling, specifically within mammary epithelium cells, to drive breast cancer progression remain unknown. To test this, we utilized the murine PyMT model of breast cancer with (PyMT $TK^{FL/FL}$) or without (PyMT $TK^{\Delta Epithelial}$) a conditional deletion of the RON tyrosine kinase (TK) domain selectively within the mammary epithelium, which was confirmed by western blot analyses on tumors isolated from both groups (Figure 1A). The role of epithelial RON expression on tumor burden was determined by measuring tumor kinetics. PyMT $TK^{\Delta Epithelial}$ mice showed a significant decrease in tumor growth compared to PyMT $TK^{FL/FL}$ controls (Figure 1B). To assess breast cancer metastasis, we examined PyMT $TK^{FL/FL}$ and PyMT $TK^{\Delta Epithelial}$ mice with similar primary tumor burden at time of harvest (Supplementary Figure S1A). In this group, there were no differences in the incidence of mice with metastases (Supplementary Figure S1B) with 100% of animals exhibiting metastases in both groups. However, a significant decrease in the number of lung metastases was observed in PyMT $TK^{\Delta Epithelial}$ mice compared to PyMT $TK^{FL/FL}$ control mice (Figure 1C).

Histological analyses of PyMT $TK^{FL/FL}$ and PyMT $TK^{\Delta Epithelial}$ tumors revealed a significant increase in necrosis in PyMT $TK^{\Delta Epithelial}$ tumors compared to PyMT $TK^{FL/FL}$ tumors (Figure 1D). Immunohistochemical analysis for Cleaved Caspase 3 indicated a significant increase in the number of apoptotic cells within PyMT $TK^{\Delta Epithelial}$ tumors compared to PyMT $TK^{FL/FL}$ control mice (Figure 1D). Immunohistochemical staining for cell proliferation (via assessment of BrdU incorporation) showed increased proliferation in tumors from PyMT $TK^{FL/FL}$ control mice compared to PyMT $TK^{\Delta Epithelial}$ tumors (Figure 1D). Taken together, these results suggest that RON signaling in the mammary epithelium promotes breast cancer survival, proliferation and metastasis leading to advanced disease.

3.2 RON signaling in the mammary epithelium is associated with an immunosuppressive microenvironment.

To assess the immune microenvironment in mice with a selective loss of RON in the mammary epithelium, tissue sections from PyMT $TK^{FL/FL}$ and PyMT $TK^{\Delta Epithelial}$ tumors were examined. Immunohistochemical analyses for the macrophage marker F4/80 showed a significant increase in macrophage infiltration in PyMT $TK^{\Delta Epithelial}$ tumors compared to control mice (Figure 2A). Subsequent analysis for the expression of the M1 polarization

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marker, iNOS, showed a significant increase in the number of iNOS positive cells in PyMT $TK^{\Delta Epithelial}$ tumors compared to controls (Figure 2A). Recruitment of cytotoxic $CD8^+$ T cells to the tumor was assessed and PyMT $TK^{\Delta Epithelial}$ tumors showed a significant increase in the number of CD8a positive cells compared to PyMT $TK^{FL/FL}$ tumors (Figure 2B). No significant difference was observed in the number of CD4 positive cells in tissue sections from PyMT $TK^{FL/FL}$ and PyMT $TK^{\Delta Epithelial}$ tumors (Figure 2B). The changes observed in the tumor microenvironment via immunohistochemistry analysis were also confirmed by immunophenotyping of isolated mammary tumors. PyMT $TK^{\Delta Epithelial}$ tumors showed a significant increase in the percent of macrophages, natural killer (NK) cells, and CD8a positive cells present compared to PyMT $TK^{FL/FL}$ tumors (Figure 2C and Supplemental Figure S2). Taken together, these data suggest that RON signaling within the mammary epithelium promotes breast cancer progression by stimulating an immunosuppressive tumor microenvironment associated with decreased M1 macrophage, NK cell, and cytotoxic $CD8^+$ T cell anti-tumor responses.

3.3 RON suppresses interferon and cytokine signaling in breast cancer cells.

To understand the mechanisms by which epithelial RON expression supports tumor growth and a pro-tumorigenic microenvironment, RNA-Sequencing (RNA-Seq) analyses was performed on human breast cancer cells with (T47D shNT) and without (T47D shRON) RON expression. Pathway enrichment analysis of up and down regulated genes ≥ 2-fold is shown in Figure 3A–B. Surprisingly, a dramatic upregulation of genes related to pro-inflammatory signaling pathways and anti-tumor responses was observed upon RON loss (Figure 3A and Supplemental Table S1). The most significant pathways negatively regulated by RON expression include type I Interferon (IFN) signaling (including IFNa and b), pro-inflammatory cytokine signaling pathways (including IFN-γ, TNF, NF-κB, HIF1α, and IL-17), and pattern-recognition receptor signaling pathways (such as NOD-like Receptor Signaling and RIG-I/MDA5 Signaling). In contrast, the most significant pathways that RON promotes includes activation of metabolic pathways (Figure 3B and Supplemental Table S1). These metabolic pathways included regulators of cholesterol biosynthesis, glucose metabolism, and alanine and aspartate metabolism, which have been linked to tumor cell growth and survival [60, 61]. To further interrogate the changes in type I IFN signaling upon loss of RON signaling, data was mined from The Cancer Genome Atlas (TCGA) breast cancer dataset for IFNα and IFNβ expression in human breast cancers. Interestingly, IFNα and IFNβ expression displayed two distinct subpopulations with high or low expression (Figure 3C). To determine whether the distinct subpopulations are associated with a breast cancer subtype, we further mined the TCGA breast cancer dataset utilizing the PAM50 gene signatures to stratify the samples included in Figure 3C based on molecular subtype (Figure 3D). Interestingly, we found that the two distinct subpopulations (IFNα/β high or low) are present within each molecular subtype. Based on these distributions, an overall and relapse-free survival analyses of the samples with high and low IFN expression were performed and found that patients with low IFNα expression show poorer survival compared to those with high IFNα expression with a hazard ratio (HR) = 1.190 (0.89 to 1.60) (Figure 3E and Supplemental Figure S3). These data suggest that suppression of in type I IFN signaling may play a strong role in breast cancer progression in a subset of breast cancer patients independent of molecular subtype.

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We next examined changes in type I IFN signaling in human and murine breast cancer cell lines with modulations in RON expression. qRT-PCR analyses showed an increase in the expression of IFNα (*IFNA1*) and IFNβ (*IFNB1*) in the RON replete human breast cancer cell lines (T47D shRON and MCF-7 PCI-Neo EV) compared to the RON expressing cells (Figure 3F–3G). Similarly, an increase in the expression of *Ifna4* and *Ifnb1* in the RON deficient murine breast cancer cells (R7 KD) compared with control R7 cells (Figure 3H) was observed, corroborating the results obtained from RNA-Seq analysis and suggesting that activation of RON signaling broadly inhibits the expression of IFNα and IFNβ in breast cancer cells. To further confirm that the changes in expression of IFNα and IFNβ production, we performed ELISAs for murine IFNα and IFNβ. Consistent with our expression data, we observed a significant increase in the production of IFNα and IFNβ in the RON deficient murine breast cancer cells (Figure 3I). Taken together, these results suggest that RON signaling in breast cancer cells alters the expression and production of type I IFNs.

Activation of pattern recognition receptors, such as toll-like receptors (TLRs), promote the secretion of type I IFNs to trigger innate immunity [38, 62]. More importantly, TLRs are known to play an important role in bridging innate and adaptive immune responses in cancer [42]. Given the changes in IFNα and IFNβ with RON loss, we examined the TLR signaling pathway, upstream of type I IFNs production, by Western blot analyses in breast cancer cells with and without RON expression. Figure 3J depicts increases in the activation (phosphorylation) of IRAK4 and p38 as well as a decrease in TRAF6 and phosphorylation of ERK1/2 expression with RON loss. These results are consistent with data from murine breast cancer cells, R7 and R7 KD, and further show an increase in IRF7 expression (a master transcriptional regulator of type I IFNs) with RON loss (Figure 3K). Taken together, these data suggest that RON signaling suppresses IRAK4 activation, a crucial upstream kinase in TLR activation, and subsequent production of type I IFNs.

3.4 RON signaling promotes tumor cell growth and inhibits apoptosis.

We next assessed the tumor cell intrinsic effects of RON signaling on tumor cell growth and apoptosis. RON proficient and deficient breast cancer cells were cultured under normal culture conditions and cell viability was measured temporally. A significant decrease in tumor cell growth was observed in RON deficient (T47D shRON, MCF-7 PCI-Neo EV) cells compared to RON proficient cells (T47D shNT, MCF-7 PCI-Neo RON) (Figure 4A). To further evaluate the changes observed in tumor cell growth, RON proficient and deficient cells were evaluated for changes cell proliferation and apoptosis. RON deficient cells exhibited reduced proliferation and increased apoptosis (Figure 4B, C, and Supplemental Figure S4). These results are comparable to breast cancer cell turnover observed in vivo with RON loss in the PyMT model (Figure 1) and suggest that RON signaling within breast cancer cells suppresses tumor cell intrinsic anti-neoplastic processes to support tumor growth.

To corroborate these cell intrinsic growth characteristics in vivo and to mechanistically investigate the role of IRAK4 suppression by RON, we generated an R7 IRAK4 overexpression (OE) cell line from the parental, R7, murine cell line. Activation of IRAK4

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signaling in these cells compared to the R7 empty vector (EV) control cells, was confirmed by western blot analyses and qRT-PCR for p-IRAK4 and IRF7 expression (Figure 5A). In addition, growth curves were examined for the murine breast cancer cell lines, R7, R7 KD, and R7 IRAK4 OE cells (Figure 5B). Interestingly, we found that overexpression of IRAK4 in R7 cells phenocopied the R7 KD cells suggesting that RON-mediated suppression of IRAK4 observed in R7 cells promotes tumor cell growth. To confirm that IRAK4 is a downstream target of RON, we performed rescue experiments using an IRAK4-specific inhibitor (IRAK4i), CA-4948, on the R7 KD cells [63].

Treatment with the IRAK4i was able to partially revert the phenotype of the R7 KD cells back to that of the parental, RON expressing, R7 cells which was measured by significant changes in cell growth (Figure 5C), IFNα/β mRNA expression (Figure 5D), and downstream signaling (Figure 5E). More importantly, treatment with IRAK4i was able to revert IRF7, p-ERK1/2, and p-p38 expression to that of the R7 cells which further support that IRAK4 is a downstream target of RON in breast cancer cells.

To examine in vivo growth characteristics, we orthotopically transplanted the murine cell lines into the mammary fat pad of NOD-Scid-Gamma (NSG) mice. All cell lines were able to efficiently form breast tumors in 100% of the mice (Table I). However, compared to control cells, breast cancer cell lines with either a knockdown of RON or IRAK4 overexpression had significant defects in tumor growth and metastasis (Figure 5F, Table I). Immunohistochemistry analyses of tissue sections stained for proliferation (Ki67) and apoptotic markers (cleaved caspase 3) showed increases in cell death with RON loss or IRAK4 overexpression while no significant differences were observed in proliferation at the same time point collected (Figure 5G). Taken together, these results show that tumor cell intrinsic RON expression promotes tumor cell growth and inhibits apoptosis and suggests this may occur through IRAK4-mediated suppression of type I IFNs.

3.5 RON expression is required for tumor establishment and immune escape.

Since type I IFNs mediate anti-tumor immune responses and our data show that RON signaling suppresses IRAK4 signaling upstream of IRF7 and type I IFN production, we next sought to assess whether RON-mediated IRAK4 suppression would affect tumorigenesis in immunocompetent mice. R7 breast cancer cells with modulations in RON or IRAK4 expression were orthotopically injected into the mammary gland of immunocompetent syngeneic FVB mice. Tumor formation and growth was monitored over time (Table II and Figure 6). As noted in Table II, RON knockdown (KD) in the control R7 cells significantly inhibited tumor establishment in FVB hosts with only one mouse in the RON KD group that developed a mammary tumor. Similarly, overexpression of IRAK4 in the control R7 cells reduced tumor cell growth to an equivalent extent as RON loss (Table II). Tumor volume measurements were recorded for the tumors that formed in each group, with RON deficient cells or IRAK4 overexpressing showing a major defect in growth over time compared to R7 control cells (Figure 6A). To evaluate immune cell infiltration during tumor establishment, we performed immunophenotyping on mammary glands isolated from mice one week following orthoptopic implantation of the tumor cells. Interestingly, no significant differences in the recruitment of immune cells were found between the groups suggesting

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that RON signaling within the tumor cell plays an important role in regulating and responding to the immune cells within the tumor microenvironment. While further studies are required to evaluate the role of RON signaling in altering immune cell activation to promote a pro-tumor microenvironment, our combined studies show that RON-mediated IRAK4 suppression is required for mammary tumor growth establishment and growth.

Since type I IFNs are established mediators of innate and adaptive immune responses, we next sought to examine whether the activation of RON signaling within breast cancer cells protects the tumor cells from immune cell clearance. R7, R7 KD, and R7 IRAK4 OE cells were co-cultured with different target to effector (T:E) ratios of innate immune cells ($CD11b^+$ and NK^+ cells) isolated from the spleens of FVB WT mice and breast cancer cell viability was examined after 2 hours of co-culture. Consistent with in vivo studies, R7 cells exhibited greater cell viability and were less susceptible to innate immune cell killing compared to the same cells with either a RON KD or IRAK4 overexpression (Figure 6B). Furthermore, we show that RON KD and IRAK4 overexpressing cells are more susceptible to FasL or TRAIL induced death compared to control R7 cells (Figure 6C) supporting resistance of the R7 cells to several immunomodulators and providing further support that activation of RON signaling protects the tumor cells from immune cell clearance and allows for efficient tumor establishment in immunocompetent mice.

3.6 RON interacts with IRAK4 and prevents activation of the myddosome complex.

As several of our studies demonstrate that overexpression of IRAK4 in RON expressing cells phenocopies loss of RON signaling in breast cancer cells, we next sought to investigate whether RON may interact with IRAK4 to regulate IRAK4 signaling. We co-transfected 293T cells with either wild-type RON (WT RON) or a kinase dead RON (KD RON) expression plasmid in combination with IRAK4 and performed co-immunoprecipitation experiments. Based on western blot analyses, we found that WT RON interacts with IRAK4 and that this interaction occurs independently of RON kinase activity (Figure 7A, 7B). To further support this physical interaction, we used the human breast cancer cell lines (T47D shNT, T47D shRON, MCF-7 PCI-Neo RON and MCF-7 PCI-Neo EV) and show an endogenous interaction between RON and IRAK4 in RON expressing cells which was not observed in the RON deficient cell lines (Figure 7C). We also show that in RON proficient breast cancer cells, an interaction between TRAF6 and Myd88, two key regulators of the myddosome complex, which suggests that in RON proficient cells, the interaction between RON and IRAK4 blocks the activation of the myddosome complex and further downstream signaling (Figure 7C). Overall, these results support a working model wherein suppression of IRAK4-mediated type I IFN production and subsequent innate immune responses is driven by an interaction between RON and IRAK4 (Figure 8).

4. Discussion

Despite significant advances in early detection and treatment strategies, approximately 42,000 patients will die from breast cancer every year, highlighting the need for the development of novel therapeutic strategies [1, 2]. Evidence suggests that tumor cell intrinsic signaling can exhibit crosstalk with the tumor microenvironment to support tumor

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establishment, growth, and progression to metastatic disease [26–31]. However, current understanding of the underlying tumor cell intrinsic molecular mechanisms regulating the cross talk between tumor cells and immune cells is limited. In this study, we provide evidence demonstrating a novel role of tumor cell intrinsic RON signaling in regulating breast cancer initiation, growth, and metastasis through modulations of tumor cell intrinsic processes and the tumor microenvironment.

Previous studies have demonstrated that HGFL-RON signaling is a major regulator of breast cancer development, progression, and metastasis [16, 17, 20, 25, 64, 65]. However, such studies were limited in the capacity to evaluate cell-type specific (epithelial or macrophage) contributions of RON signaling in breast cancer. Herein, we present the first evidence that loss of RON signaling specifically in the mammary epithelium significantly delays tumor growth and metastatic burden as well as increases tumor cell apoptosis and implicates RON signaling as a key driver of mammary tumorigenesis. Furthermore, RON expression is a predictor of poor survival independent of molecular subtype, tumor stage, or nodal status which highlights the need to understand the underlying molecular mechanisms of how RON signaling promotes breast cancer progression [19]. In addition to a significant delay in tumor progression, we observed alterations in the tumor microenvironment upon loss of RON signaling in the mammary epithelium, including suppression of M1 macrophages, natural killer (NK) cells, and $CD8^+$ T cells in the tumor microenvironment. These results are consistent with a previous study where whole-body loss of HGFL signaling is associated with increased M1 macrophage and $CD8^+$ T cell anti-tumor responses [25].

Based on the changes observed in mammary tumor growth/progression and the tumor microenvironment upon loss of mammary epithelial RON signaling *in vivo*, we sought to investigate the underlying molecular mechanisms of how RON signaling drives tumor growth and progression *in vitro*. We show here that loss of RON signaling in tumor epithelial cells results in upregulated gene expression associated with increased innate immune cell signaling, specifically type I interferon (IFN) signaling suggesting that tumor cell intrinsic RON signaling negatively regulates type I IFN production in breast cancer cells. Interestingly, TCGA breast cancer patient data showed a distinct bimodal distribution of type I IFN expression independent of molecular subtype, and that low levels of IFNα/β in human breast cancer patients is associated with poor overall and relapse-free survival. Deeper investigation into the prognostic/predictive significance of RON expression and type I IFN expression is warranted, however our published work demonstrates RON protein expression has greater prognostic capacity, thus limiting the extent by which existing datasets of RNA sequencing data could be mined. These results are consistent with previous studies demonstrating that repression of type I IFN signaling promotes tumor progression and metastasis in breast and pancreatic cancer [66, 67].

Interestingly, we have shown that upon loss of RON signaling in human and murine breast cancer cell lines there is a significant increase in type I IFN expression and production. To further evaluate these findings, we investigated the upstream signaling pathways of type I IFNs production and we found decreased levels of the adaptor protein TRAF6 and phosphorylated ERK1/2, and increased levels of MyD88, and phosphorylated p38 in the RON-deficient breast cancer cell lines. Previous studies demonstrated that ERK1/2 plays a

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role in inhibiting the activation of type I IFN signaling and p38 plays a regulatory role for promoting cell death through type I IFN activation [68, 69]. More importantly, we found significant suppression of IRAK4 activation when RON was present in breast cancer cells. Taken together, our data supports that RON signaling elicits suppressive signals to dampen type I IFN expression, with a particularly striking effect on the downstream targets of myddosome complex formation through IRAK4. Functionally, exogenous expression of IRAK4 in the control R7 cells (R7 IRAK4 OE) was able to revert the growth of the R7 cells to resemble that of the R7 KD cells. Conversely, the inhibition of IRAK4 in the R7 KD cells was able to revert the R7 KD phenotype to that of the R7 RON expressing cells. Taken together, these results indicate that IRAK4 is a downstream target of RON. When R7, R7 KD, and R7 IRAK4 OE cells were orthotopically injected into the mammary glands of NSG mice to evaluate tumor cell intrinsic phenotypes, tumors uniformly established across all groups, but the tumor growth kinetics and metastatic burden significantly differed. The RON overexpressing R7 cells grew significantly faster and the mice had increased metastatic burden compared to the tumors formed from RON-deficient cells, R7 KD, or R7 cells overexpressing IRAK4. These data support that robust tumor cell intrinsic growth and metastasis is seen in RON-overexpressing breast cancer cells compared with isogenic counterparts deficient in RON, and further suggests that IRAK4 overexpression can compensate to override this tumor cell intrinsic phenotype. Thus, we posit that suppression of IRAK4 and its downstream targets by RON in the absence of microenvironmental immune effectors leads to enhanced tumor growth and reduced cell death. To examine the impact of the immune system on tumor establishment and growth, we orthotopically injected the same cell lines into FVB WT mice (from which R7 cells were derived) and we found that loss of RON signaling or IRAK4 overexpression in these cells reduced the ability of to establish tumors suggesting a mounted anti-tumor immune response given that tumor establishment was uniform in NSG mice. These data support that IRAK4 overexpression can also compensate to normalize the anti-tumor immune response; we posit that suppression of IRAK4 and its downstream targets by RON in the presence of microenvironmental immune effectors leads to dampening of the anti-tumor immune response. The few tumors that did establish grew at a significantly reduced rate compared to the R7 control cells. *In vitro*, RON overexpressing cells are less susceptible to innate immune cell attack (CD11b and NK cell) and treatment by FasL and TRAIL, soluble factors excreted by adaptive immune cells, compared with that of RON deficient, or IRAK4 overexpressing isogenic counterparts supporting that RON signaling confers tolerance to immune cell attack. Taken together, these data highlight the novel role of tumor cell intrinsic RON expression in regulation of tumor growth as well as suggest that this expression affects tumor establishment by protecting the cancer cells from an early innate immune response and ultimate tumor cell death through IRAK4-mediated suppression of type I IFNs production.

In this study, we have shown that tumor cell intrinsic RON signaling suppresses the activation of IRAK4 to promote tumor cell growth and suppress type I IFN expression. However, exogenous expression of IRAK4 in the R7 control cells phenocopies that of the RON-deficient, R7 KD cells. We also demonstrate that RON directly interacts with IRAK4 independent of RON kinase activity to impact IRAK4 kinase activity which implicates this novel interaction to suppress IRAK4 activation and subsequent type I IFN production.

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Structurally, IRAK4 contains three domains: a N-terminal death domain, a proST domain, and a kinase domain [70]. The structure of IRAK4 provides several potential mechanisms for the RON-mediated suppression of IRAK4, including sequestration of IRAK4 from the myddosome complex or binding to the kinase domain to IRAK4 to prevent autophosphorylation. Future studies are required to investigate the mechanisms through which RON binds to IRAK4 to suppress type I IFNs production.

The role of type I IFNs in autoimmunity and cancer has been a focus of research for more than 50 years since early studies demonstrated the anti-tumor effects of IFNα/β, which fast-tracked IFNs to the clinical setting as a novel therapeutic option for cancer patients [71, 72]. Unfortunately, their early use as primary cancer treatments resulted in severe side effects and unforeseen complications which led to a discontinuation of IFNs as a primary treatment option for patients [7, 73]. Retrospective studies have shown that these adverse effects were associated with high doses of IFNs treatment over a continuous period of time which was a result of lack of knowledge about the mechanisms of action for these drugs before they were approved for clinical use but despite this, interest in utilizing IFNs as a therapeutic strategy is still limited [74, 75]. However, their reemergence as treatment adjuvants rather than a primary therapeutic option has begun to show promise after careful mechanistic studies have illustrated their potential as an adjuvant therapy [6, 76–78]. Recently, several clinical trials have been implemented utilizing type I IFNs alone or in combination with other immunotherapies or chemotherapies to improve anti-tumor immune responses and breast cancer patient outcomes. In this study, we have demonstrated that RON signaling in breast cancer cells suppresses IRAK4-mediated type I IFN production which is associated with tumor cell growth and pro-tumor immune responses. Targeting RON signaling alone or in combination with immunotherapies may prevent the RON-mediated IRAK4 suppression of type I IFNs, which will result in anti-tumor immune responses and improved prognostic outcomes for patients with advanced breast cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- RON expression promotes mammary tumor development, growth, and metastasis in breast cancer mouse models.
- RON expression supports a pro-tumor microenvironment and protection from immune cell attack.
- Tumor cell intrinsic RON signaling promotes cell proliferation, inhibits apoptosis, and suppresses type I IFNs in breast cancer cells.
- RON-mediated suppression of type I IFNs production is dependent on the direct interaction of RON and IRAK4.
- Inhibition of RON signaling in combination with immunotherapy may represent a novel therapeutic strategy for patients diagnosed with advanced stage or recurrent breast cancer.

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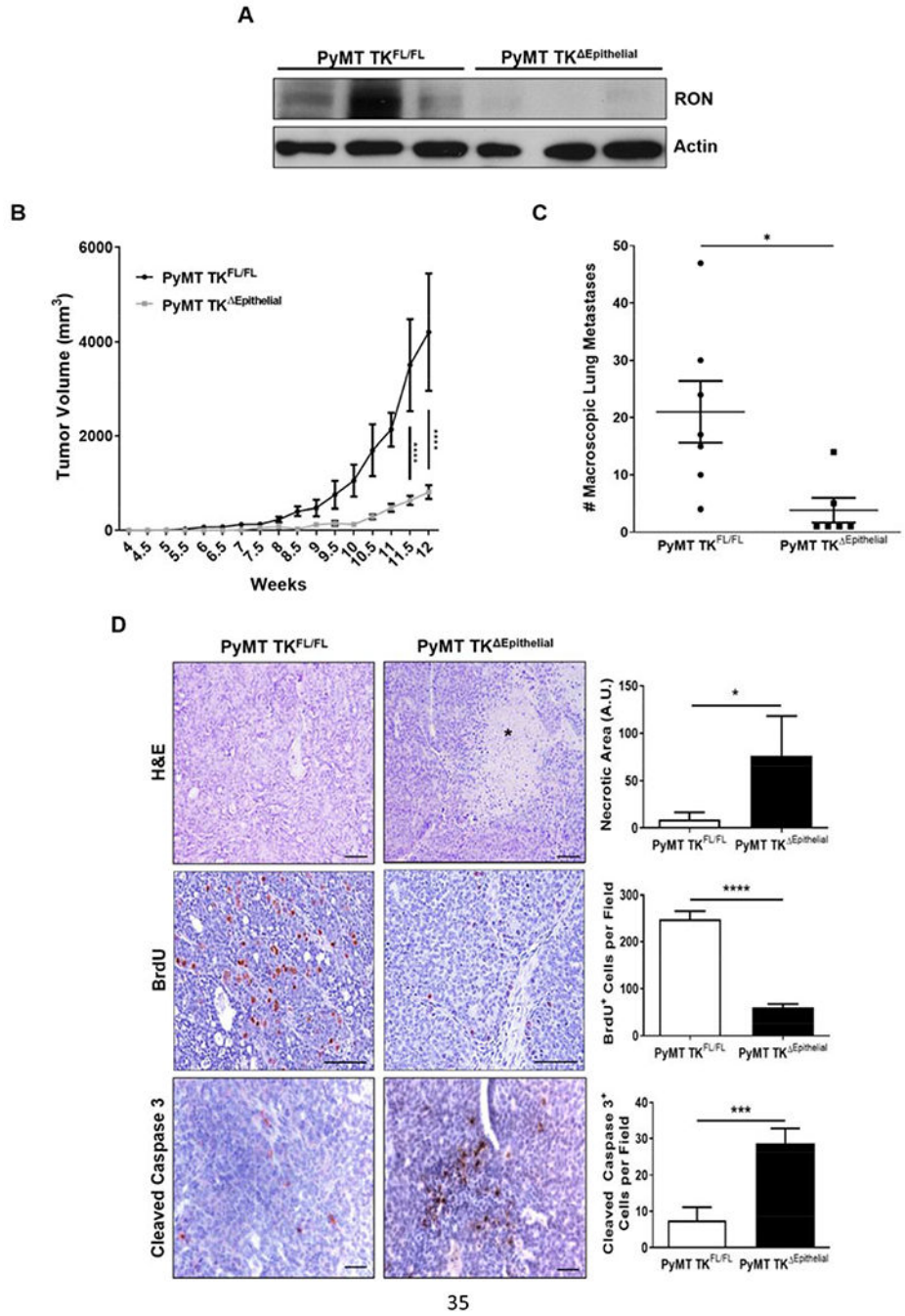

Figure 1. RON expression in the mammary epithelium is required for mammary tumor growth and metastasis in the PyMT model.

A) Western blot image showing RON expression in PyMT TK$^{FL/FL}$ and PyMT TK$^{\Delta Epithelial}$ tumors. Actin was used as a loading control. **B)** Tumor volume over time in PyMT TK$^{FL/FL}$ and PyMT TK$^{\Delta Epithelial}$ mice (n>22). **C)** Number of macroscopic lung metastatic metastases in size matched mammary tumors from PyMT TK$^{FL/FL}$ and PyMT TK$^{\Delta Epithelial}$ (n=6-7 lungs/group) mice. **D)** Representative images and quantifications of PyMT TK$^{FL/FL}$ and

PyMT TK$^{\Delta Epithelial}$ tumors stained for H&E (*, necrotic regions), Cleaved Caspase 3, and BrdU. Data represent means ± SEM from 3-5 mice/tumors/genotype; scale bar=100μm.

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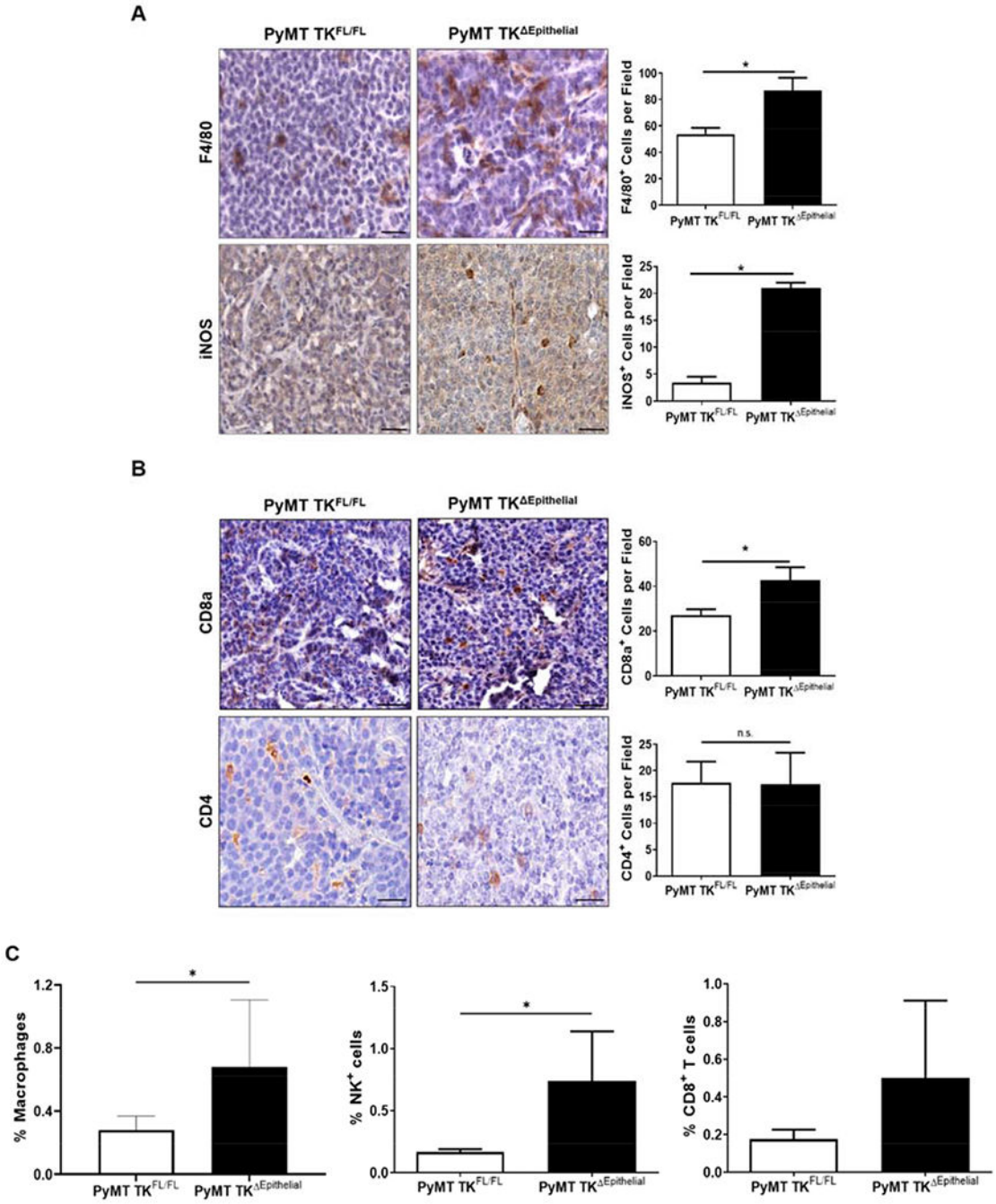

Figure 2. RON signaling in the mammary epithelium is associated with an immunosuppressive microenvironment.

A-B) Representative images and quantifications of PyMT TK$^{FL/FL}$ and PyMT TK$^{\Delta Epithelial}$ tissue sections stained for F4/80 and iNOS (**A**, n=3-8; scale bar=50μm) and for CD8a and CD4 (**B**, n=3-4; scale bar=100μm; n.s.=not significant). **C)** Immunophenotyping analyses of immune cells isolated from PyMT TK$^{FL/FL}$ and PyMT TK$^{\Delta Epithelial}$ tumors showing the percent of F4/80^{+} CD11b^{+} Ly6G^{-} macrophages, CD8^{+} T cells, and NK+ cells within the bulk tumor population (n=2-6).

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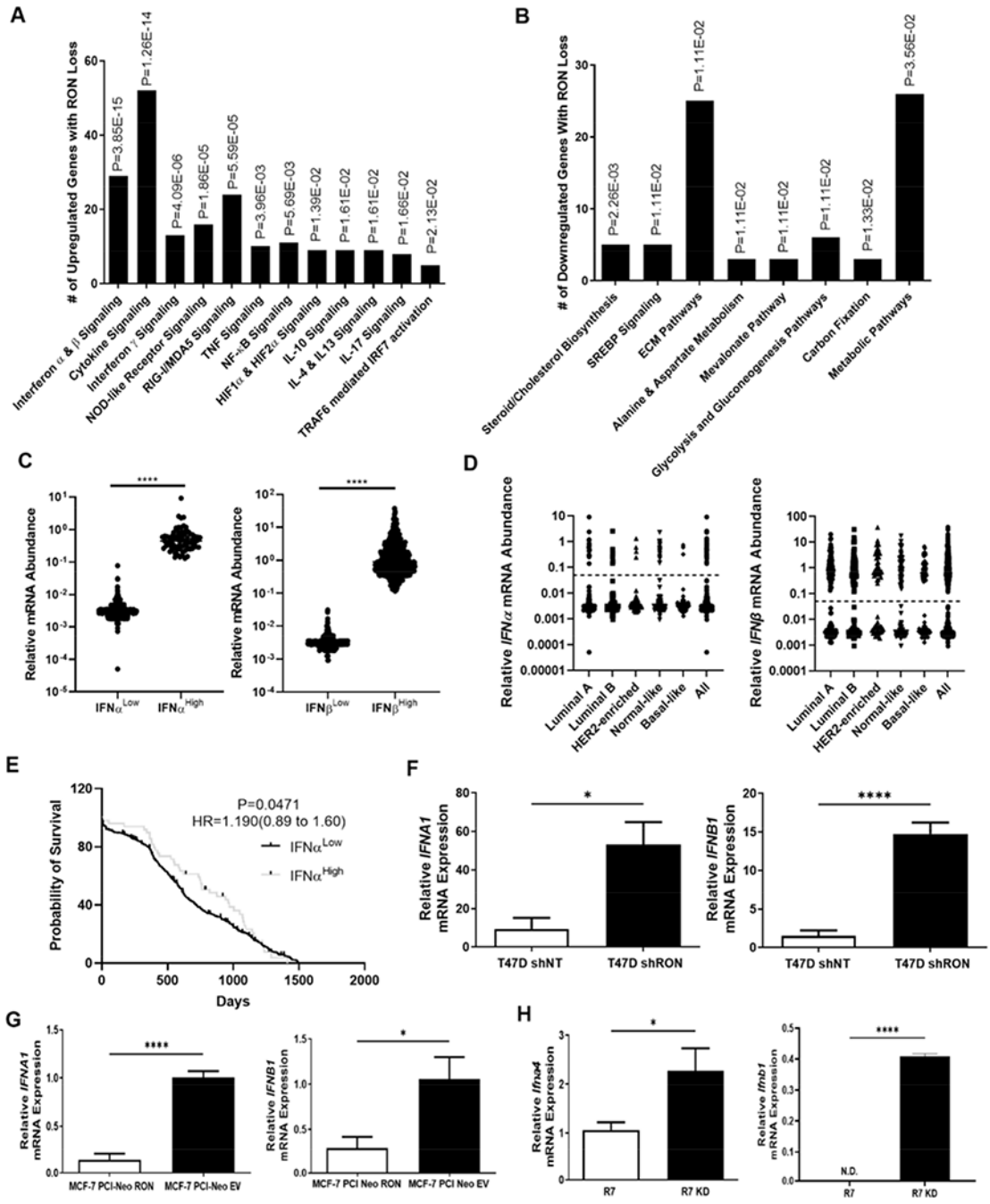

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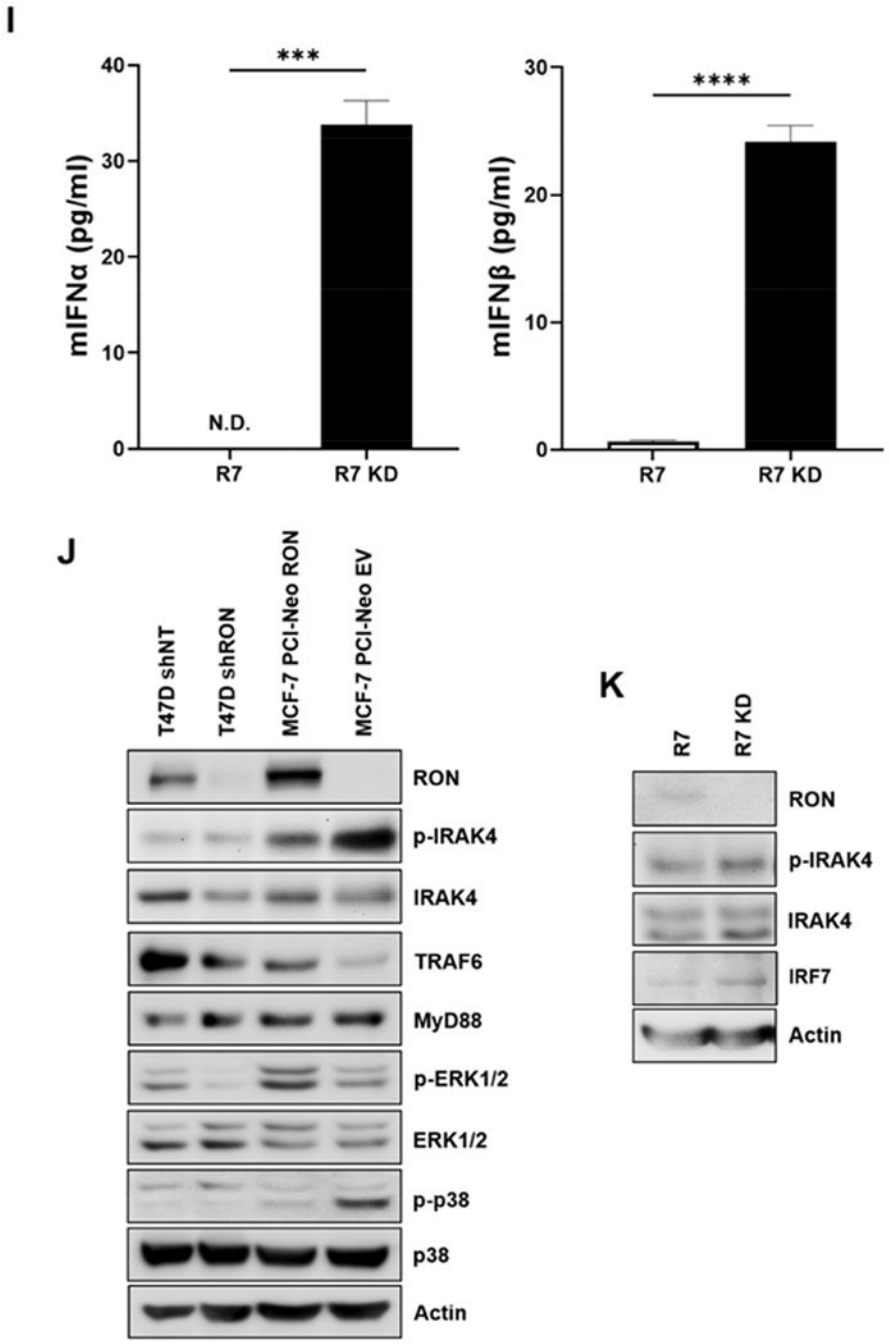

Figure 3. RON signaling suppresses type I Interferon (IFN) production and promotes metabolic pathways in breast cancer.

A-B) Bar graphs depicting the top biological pathways that are upregulated (**A**) and downregulated (**B**) from RNA-Seq analysis of human breast cancer cells with a RON knockdown (T47D shRON) compared to RON expressing (T47D shNT) control cells. **C)** Distribution of IFNα and IFNβ mRNA expression in the breast cancer TCGA database. **D)** Distribution of IFNα and IFNβ mRNA expression in the breast cancer TCGA database stratified by PAM50 molecular subtype classification. **E)** Probability of survival between

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patients with high IFNα and low IFNα mRNA expression. **F-G)** qRT-PCR analyses for *IFNA1* and *IFNB1* from T47D shNT and T47D shRON (**F**) or MCF-7 PCI-Neo EV and MCF-7 PCI-Neo RON human breast cancer cells (**G**). **H)** qRT-PCR analyses for *Ifna4* and *Ifnb1* from R7 and R7 KD murine breast cancer cells. All qRT-PCR analyses were performed from at least 2 independent isolations in triplicate and expression levels were normalized to *18S.* **I)** ELISA analyses for IFNα and IFNβ levels (pg/ml) produced by murine breast cancer cells (R7 and R7 KD). All ELISA analyses were performed in duplicate from three independent experiments. **J)** Representative western blot images showing the expression of RON, phosphorylated (p)-IRAK4, IRAK4, TRAF6, MyD88, p-ERK1/2, ERK1/2, p-p38, and p38 in human breast cancer cells. **K)** Representative western blot images showing the expression of RON, p-IRAK4, IRAK4, and IRF7 in murine breast cancer cells. Actin was used as a loading control.

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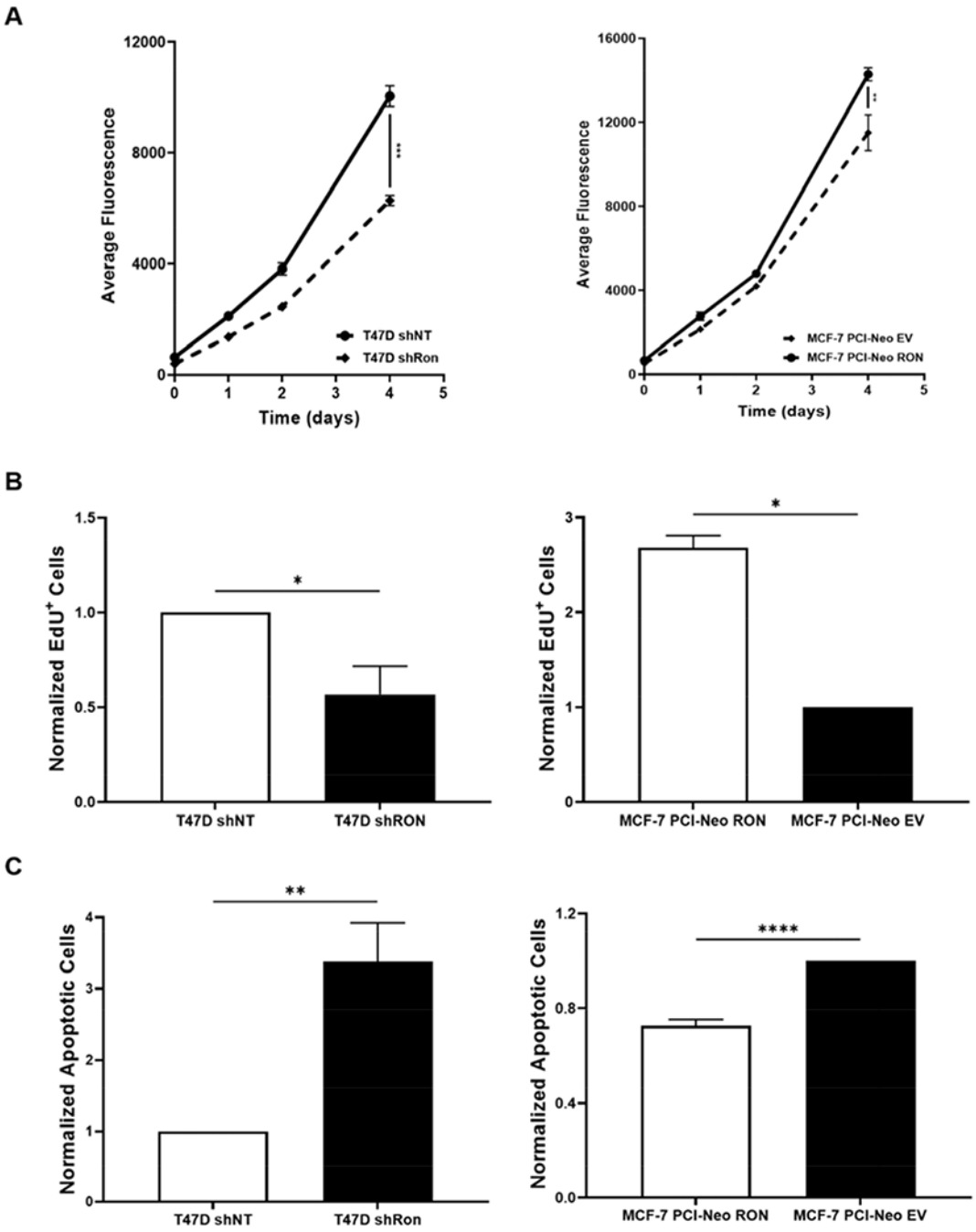

Figure 4. RON signaling promotes tumor cell growth and inhibits apoptosis in human breast cancer cells *in vitro.*

A) Representative cell growth curves of human breast cancer cells (T47D shNT, T47D shRON, MCF-7 PCI-Neo RON, and MCF-7 PCI-Neo EV) over time (0, 1, 2, and 4 days). **B-C)** Cell proliferation (**B**) and apoptosis (**C**) as determined by flow cytometry analysis of EDU incorporation (**B**) or Annexin V/Propidium Iodide staining (**C**) for T47D shNT, T47D shRON, MCF-7 PCI-Neo RON, and MCF-7 PCI-Neo EV cells. All *in vitro* analyses are

from at least three independent experiments in triplicate; data normalized to parental control cell line.

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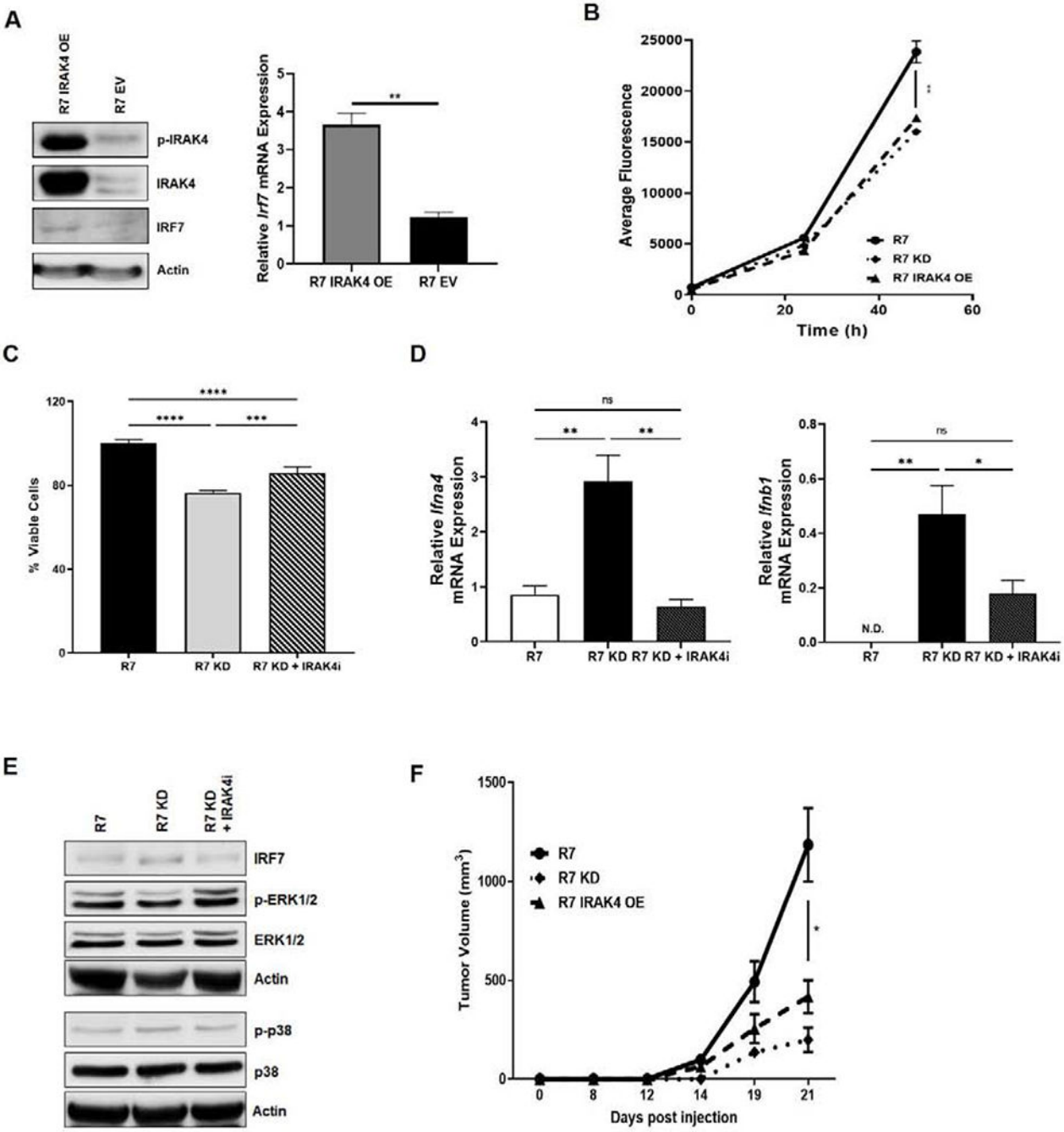

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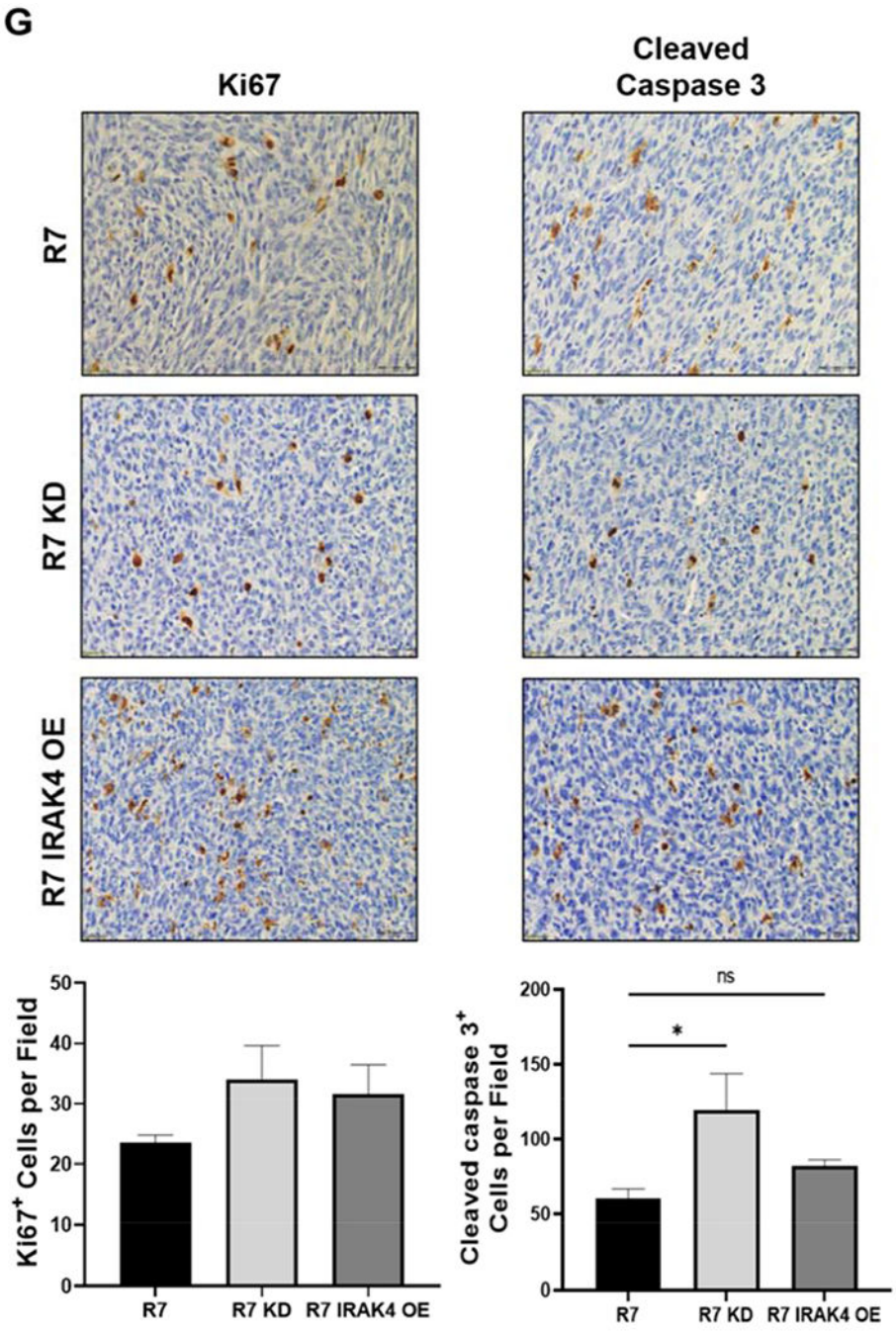

Figure 5. RON signaling promotes tumor cell growth and inhibits apoptosis in murine breast cancer cells.

A) Representative western blot images showing the expression of p-IRAK4, IRAK4, and IRF7 in R7 IRAK4 OE and R7 empty vector (EV) cells. Actin was used as a loading control. qRT-PCR analyses for *Irf7* from R7 IRAK4 OE and R7 empty vector (EV) cells. Expression levels were normalized to *18S.* **B)** Representative growth curve of murine breast cancer cells (R7, R7 KD, and R7 IRAK4 OE) over time (0, 24, and 48 hours). **C)** Change in cell viability for R7, R7 KD, and R7 KD + IRAK4 inhibitor (IRAK4i; 5μM) after 48h. **D)** qRT-PCR

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analyses for *IFNα4* and *Ifnb1* from R7, R7 KD, and R7 KD + IRAK4i cells. Expression levels were normalized to *18S.* **E)** Representative western blot images showing the expression of IRF7, p-ERK1/2, ERK1/2, p-p38, and p38 in R7, R7 KD, and R7 KD + IRAK4i cells. Actin was used as a loading control. **F)** Tumor kinetics of R7, R7 KD, and R7 IRAK4 OE murine breast cancer cells orthotopically injected into female NSG mice (n=5). **G)** Images and quantifications of tissue sections from R7, R7 KD, and R7 IRAK4 OE orthotopic tumors stained for Ki67 and Cleaved Caspase 3 (n=2 tissue sections/group; scale bar = 50μm).

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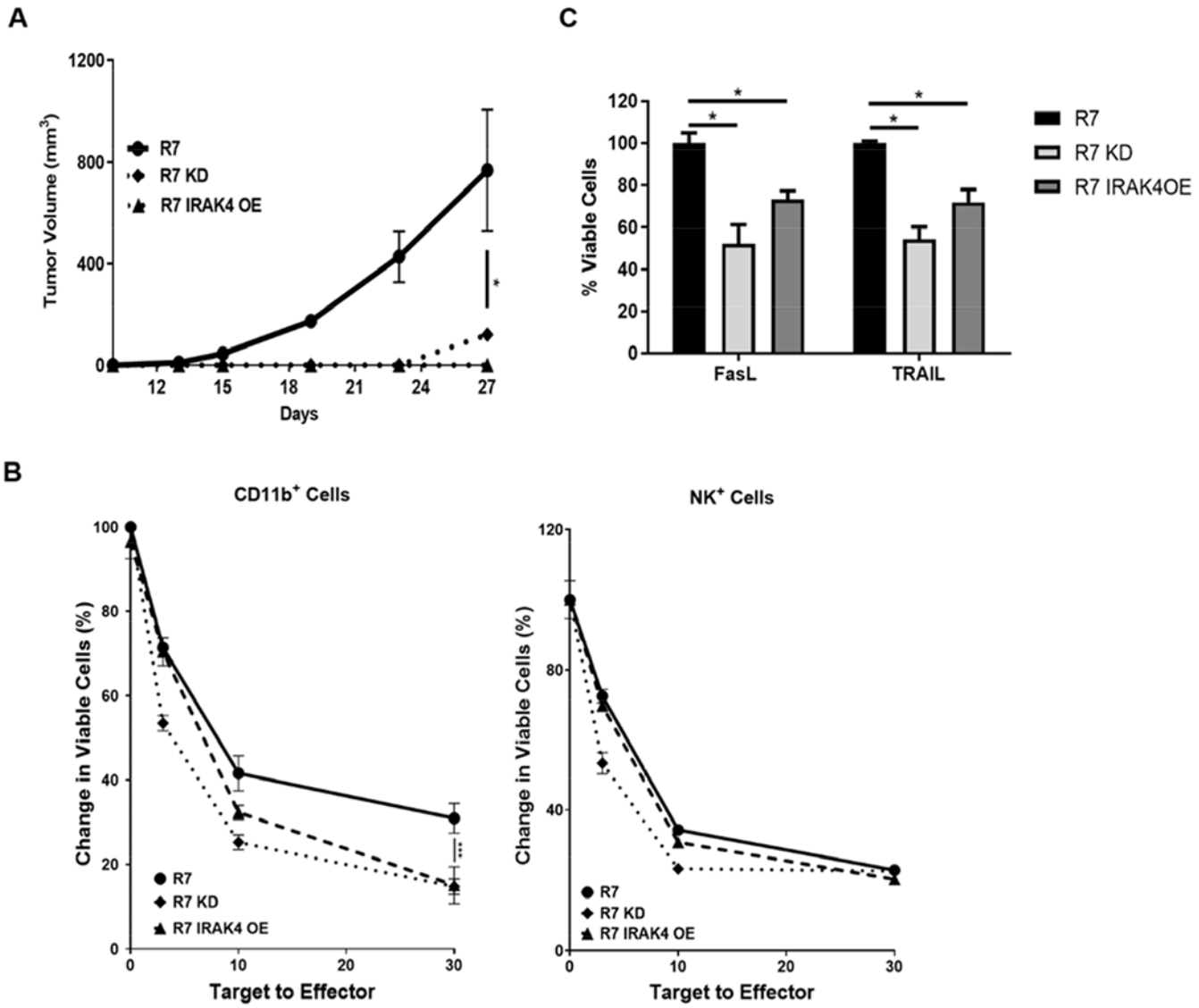

Figure 6. RON expression supports tumor establishment and resistance to innate immune cell attack.

A) Tumor kinetics of R7, R7 KD, and R7 IRAK4 OE murine breast cancer cells orthotopically injected into female FVB WT mice. **B)** Change in cell viability for R7, R7 KD, and R7 IRAK4 OE target cells (T) when cocultured with different ratios of $CD11b^+$ or NK^+ effector (E) cells. Results shown are representative of three independent experiments. **C)** Change in cell viability after 4-hour treatment with FasL (10 ng/mL) or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (100 ng/mL) (n = 3).

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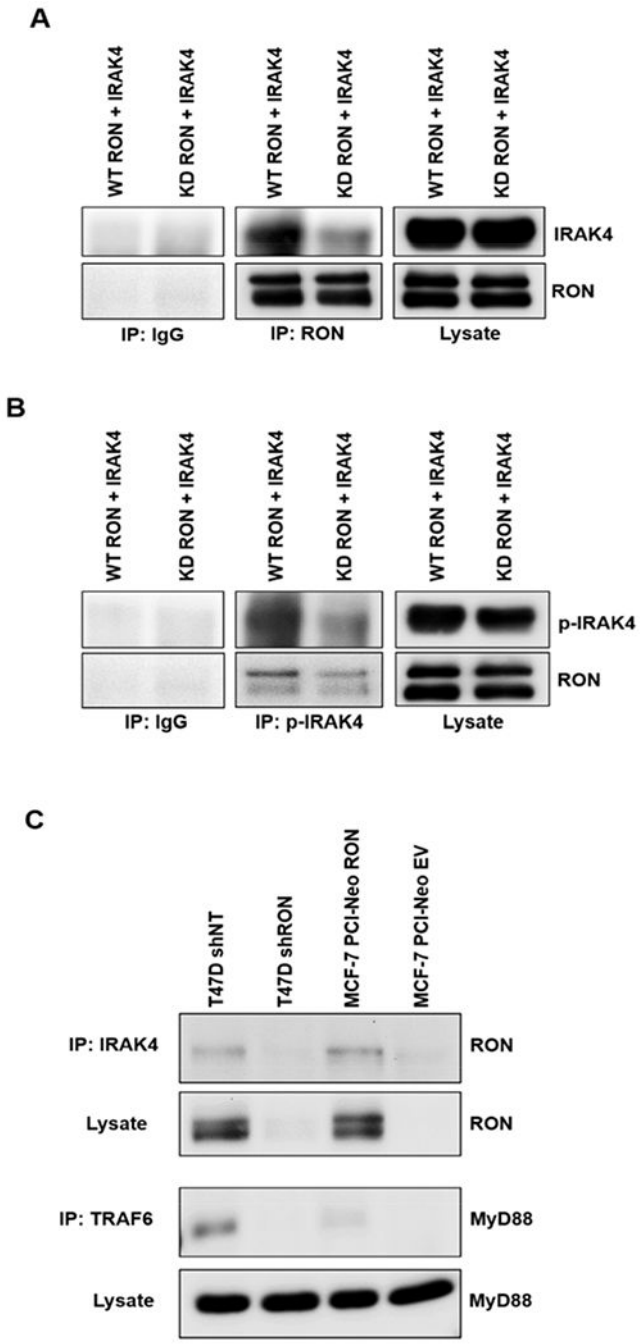

Figure 7. RON interacts with IRAK4 and disrupts myddosome complex activation.

A) Representative immunoblot analysis showing co-immunoprecipitation of RON and IRAK4 using a Ron antibody in HEK-293T cells. IgG was used as a negative control. **B)** Representative immunoblot analysis showing a reciprocal co-immunoprecipitation of IRAK4 and RON using an IRAK4 antibody in HEK-293T cells. IgG was used as a negative control. **C)** Representative immunoblot analysis showing an endogenous co-immunoprecipitation of RON and IRAK4 and co-immunoprecipitation of TRAF6 and

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MyD88 in T47D shNT, T47D shRON, MCF-7 PCI-Neo RON, and MCF-7 PCI-Neo EV human breast cancer cells.

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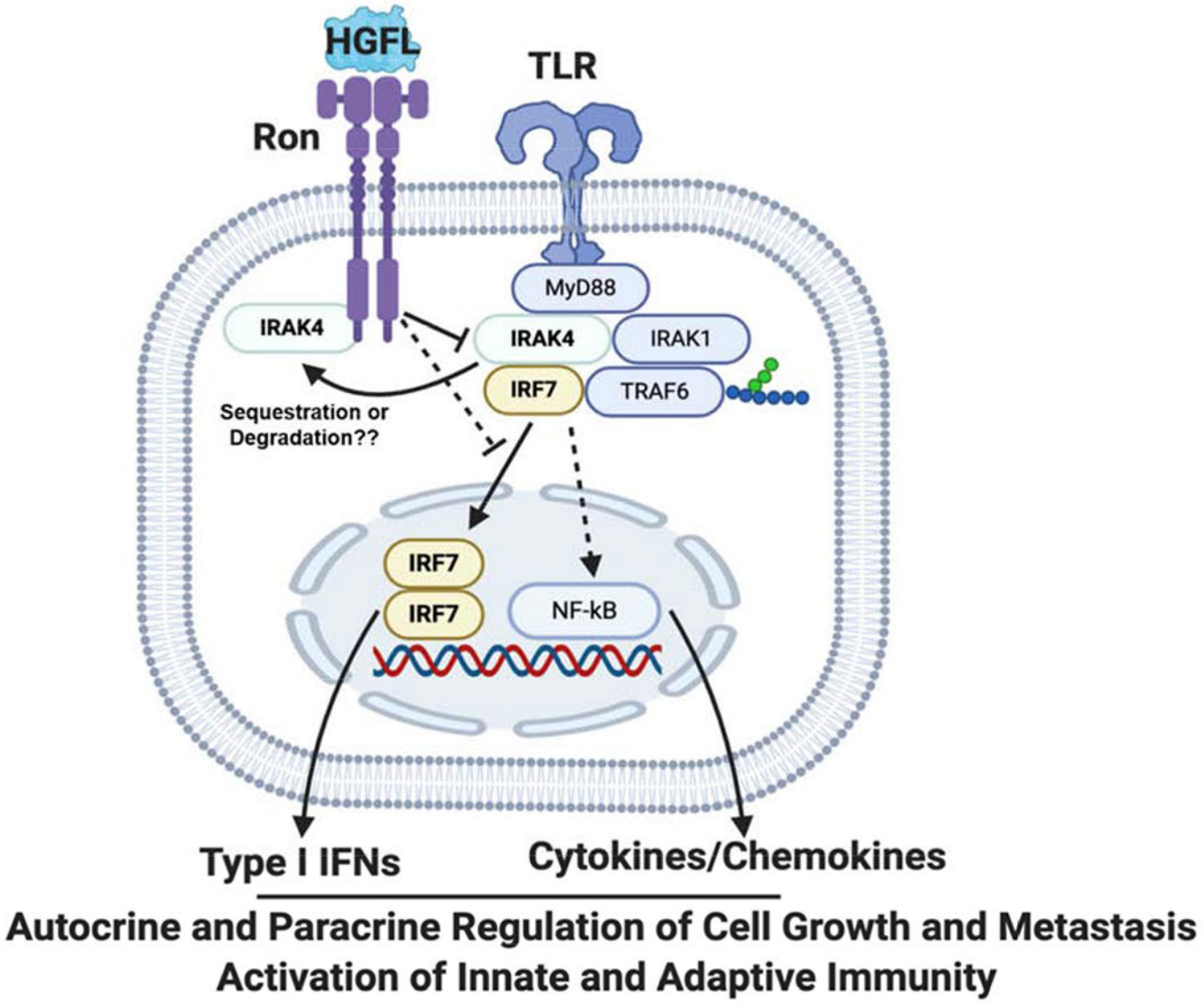

Figure 8. Schematic model of RON-mediated IRAK4 signaling inhibition and suppression of type I IFN production.

Schematic of the tumor cell intrinsic signaling network wherein RON directly interacts with IRAK4 to disrupt IRAK4 activation and further downstream TLR signaling. Consequently, through the inhibition of IRAK4 activation, RON signaling suppresses type I IFNs production to promote tumor cell growth and alter innate immune responses in breast cancer. Illustration generated courtesy of BioRender.

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Table I.

Tumor formation and metastatic progression of R7, R7 KD, and R7 IRAK4 OE breast cancer cells following orthotopic transplantation into NSG mice.

Recipient Mouse	Cells Injected	% Tumor Formation (n=5)	% Metastasis (at similar tumor size)	
			Intestine	Liver
NSG	R7	100.0	60.0	20.0
	R7 KD	100.0	0.0	0.0
	R7 IRAK4 OE	100.0	0.0	0.0

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Table II.

Tumor formation of R7, R7 KD, and R7 IRAK4 OE breast cancer cells following orthotopic transplantation into syngeneic FVB WT mice.

Recipient Mouse	Cells Injected	% Tumor Formation
FVB	R7	89.5 (n = 17/19)
	R7 KD	8.3 (n = 1/12)
	R7 IRAK4 OE	12.5 (n = 1/8)