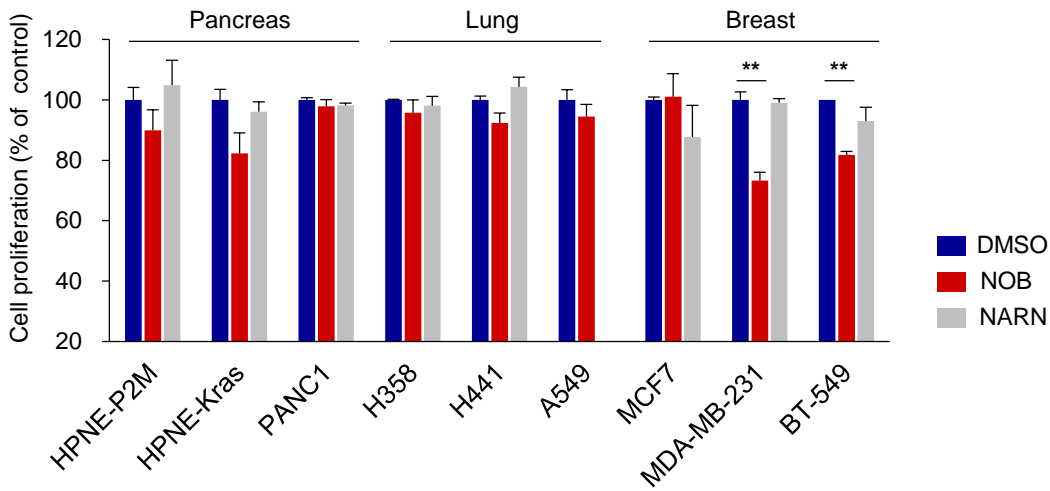
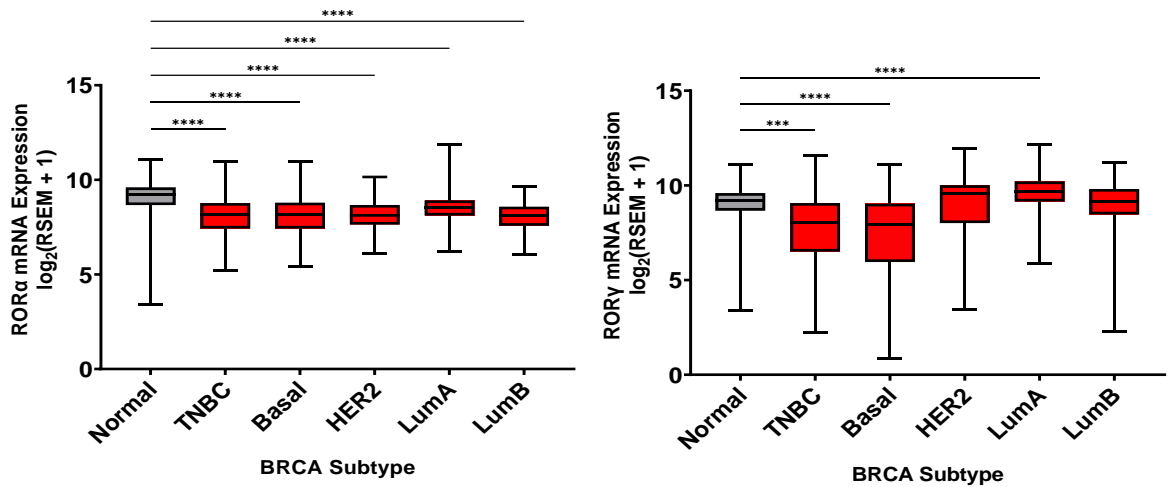
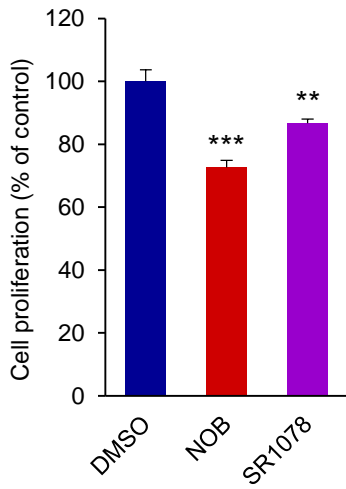


Supplementary Information

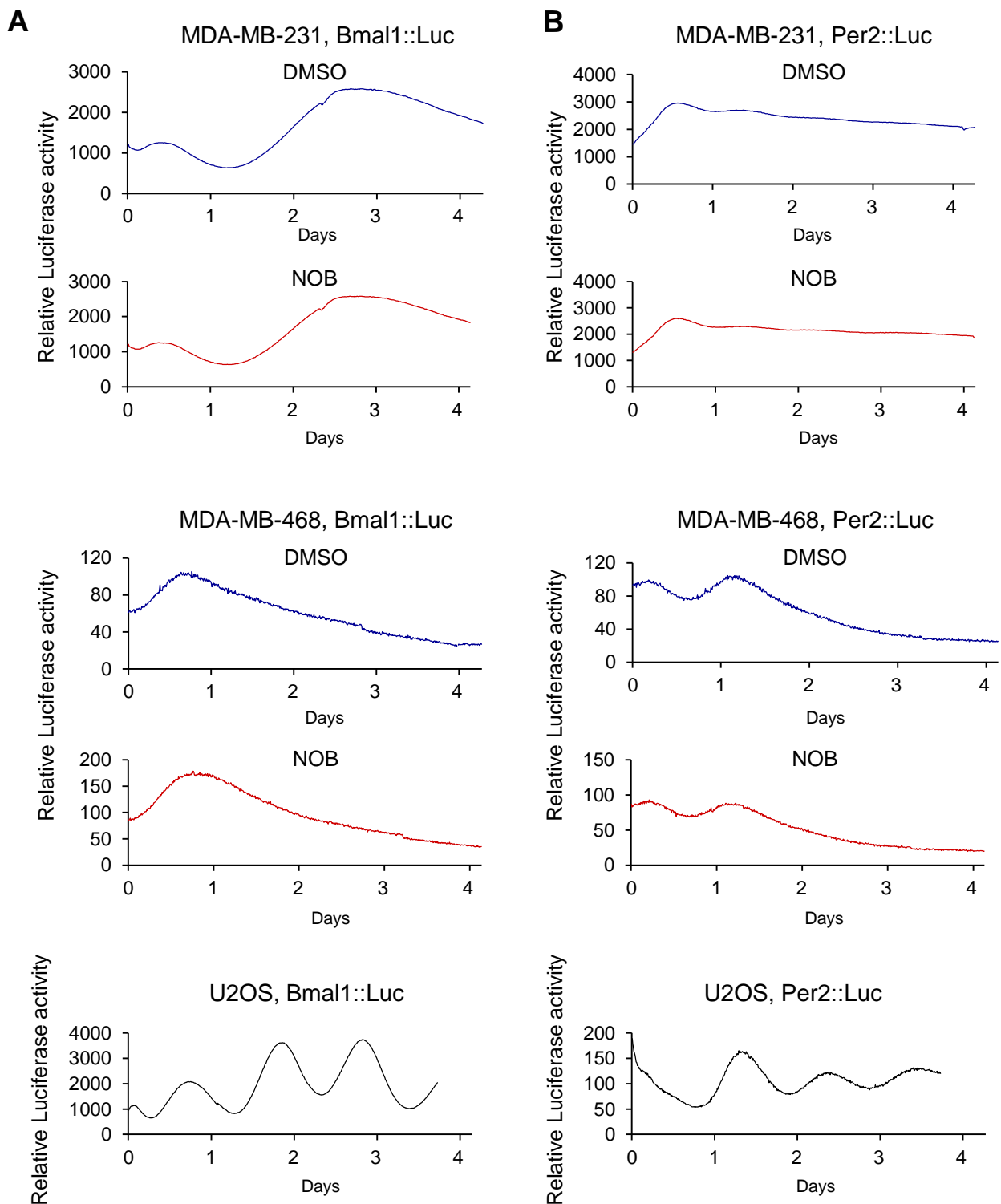
ROR activation by Nobiletin enhances anti-tumor efficacy via suppression of I κ B/NF- κ B signaling in triple-negative breast cancer

Eunju Kim, Yoon-Jin Kim, Zhiwei Ji, Jin Muk Kang, Marvin Wirianto, Keshav Raj Paudel, Joshua A. Smith, Kaori Ono, Jin-Ah Kim, Kristin Eckel-Mahan, Xiaobo Zhou, Hyun Kyoung Lee, Ji Young Yoo, Seung-Hee Yoo, Zheng Chen

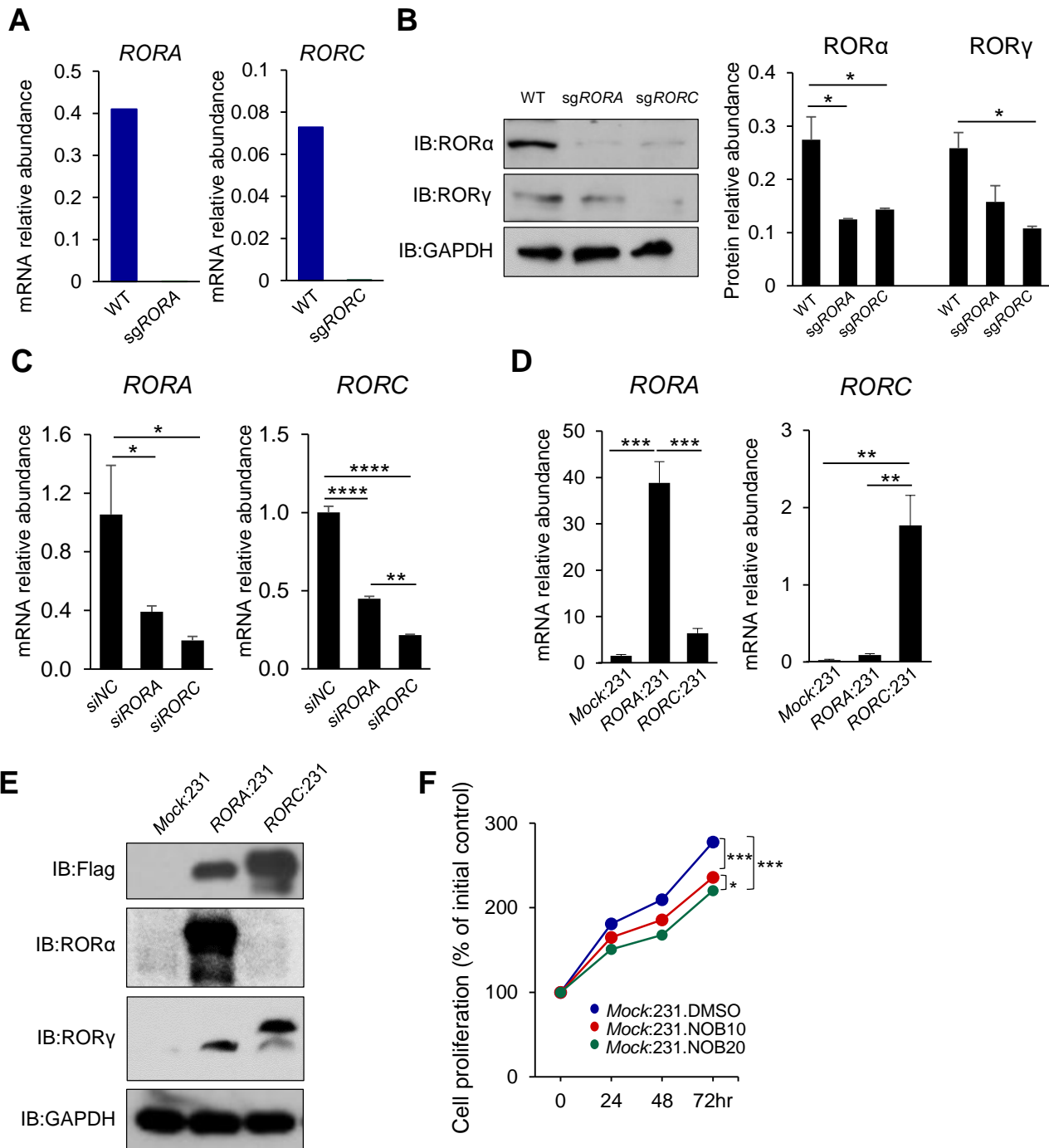
This SI file contains 7 supplementary figures/legends and 2 supplementary tables.

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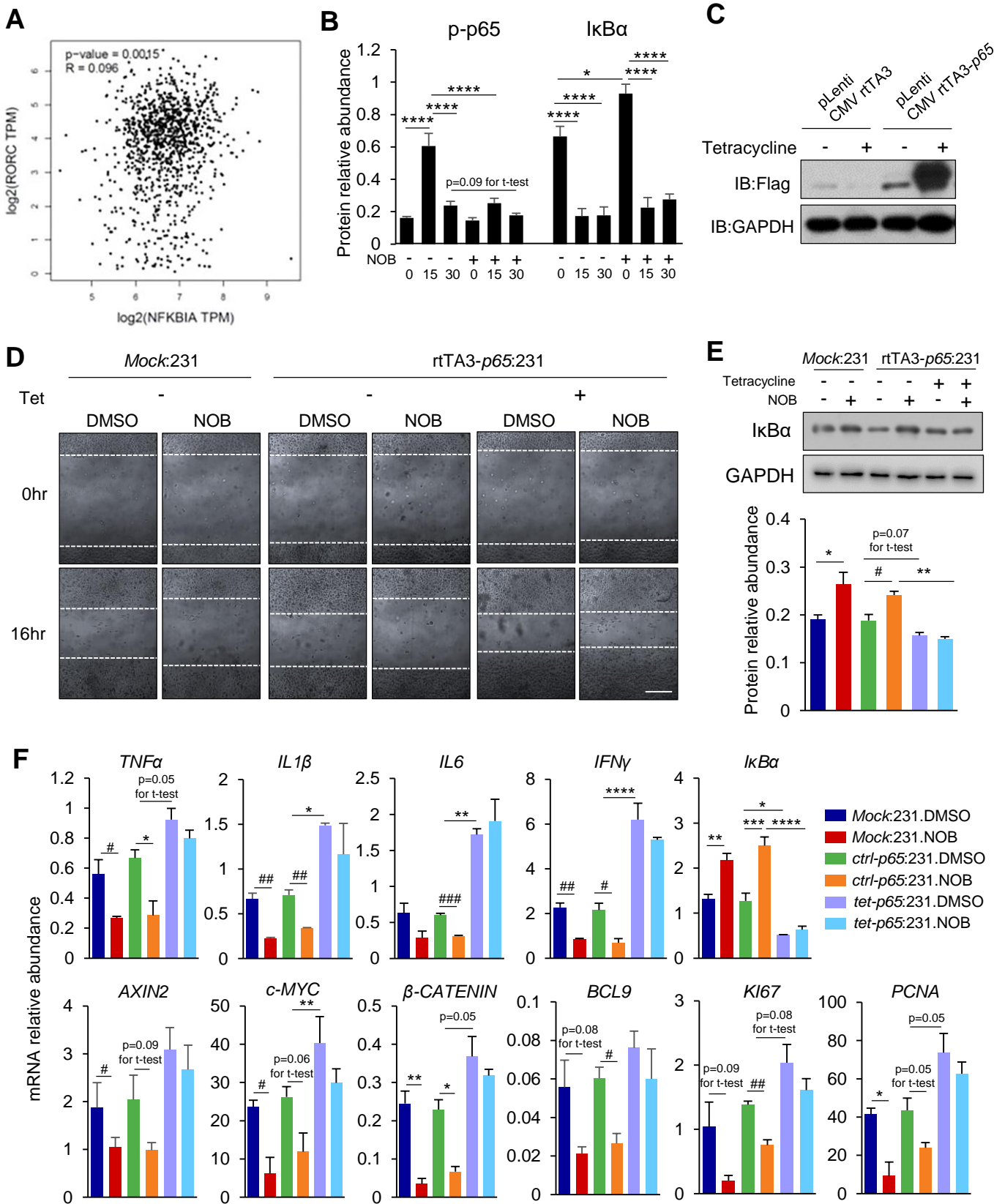
Supplementary Fig. 1. Anti-TNBC effects of NOB-ROR. A. Screening of NOB inhibitory effects in cancer cell lines. Pancreas, lung and breast cancer cell lines were seeded in 96-well plate overnight and treated with 10 μ M NOB. Cell proliferation was measured by WST-1 assays 72 hrs after NOB treatment. Data represent mean \pm SEM. Two-tailed Student's t-test, ** $p < 0.01$ vs. control. **B.** TCGA database search revealed that *RORA* and *RORC* expression was significantly reduced in TNBC and some other subtypes. **C.** *ROR* α/γ agonists decreased cell proliferation. MDA-MB-231 cells were treated with NOB 10 μ M and SR1078 10 μ M for 72 hrs. Cell proliferation was determined by WST-1 assays. DMSO (0.1%) was used as a solvent control. Data represent mean \pm SEM. One-way ANOVA with Tukey's post hoc test. Significantly different compared with DMSO ** $p < 0.01$ and *** $p < 0.001$.



Supplementary Fig. 2. Absence of robust circadian rhythms in MDA-MB-231 and MDA-MB-468. A-B. MDA-MB-231 or MDA-MB-468 cells were transfected with Bmal1::Luc (A) and Per2::Luc (B) reporter constructs and stable cell lines were established. Cells were synchronized and treated with NOB 10 μ M. Real-time bioluminescence recordings were analyzed by Lumicycle analysis program (Actimetrics). Lower panels: U2OS cells transfected with these reporter constructs show circadian rhythms.



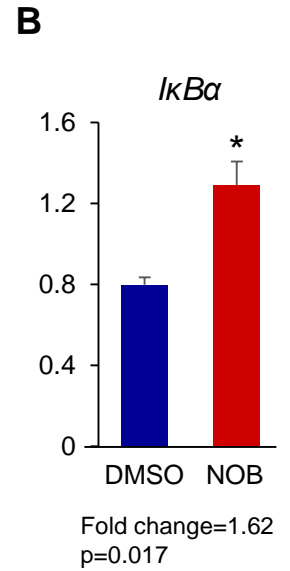
Supplementary Fig. 3. Loss- and gain-of-function studies of RORs. **A.** Validation of *RORA* and *RORC* expression in CRSPR knockdown cells by qPCR analysis. *GAPDH* was used as a reference gene. **B.** Validation of *RORA* and *RORC* expressions in knockdown cells by Western blotting using ROR α and ROR γ antibodies. One-way ANOVA with Tukey's multiple comparisons test, * $p < 0.05$ and *** $p < 0.001$. **C.** Validation of *RORA* and *RORC* expressions in si*RORA* and si*RORC* MDA-MB-231 cells by qPCR. One-way ANOVA with Tukey's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$. **D.** Validation of *RORA* and *RORC* expressions in *RORA* or *RORC* over-expression MDA-MB-231 cells by qPCR. One-way ANOVA with Tukey's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. **E.** ROR α or ROR γ protein expression in *RORA* or *RORC* over-expression MDA-MB-231 cell lines. Western blotting was performed using Flag, ROR α , and ROR γ antibodies. **F.** NOB inhibited cell proliferation of MDA-MB-231 cells transfected with the empty vector (P3Xflag-CMV10-Neo^r) dose-dependently. Mock indicates P3Xflag-CMV10-Neo^r (Empty vector) as the control. DMSO was used as a solvent control, NOB10, 10 μ M; NOB 20, 20 μ M. Data represent mean \pm SEM. One-way ANOVA with Tukey's multiple comparisons test, * $p < 0.05$ and *** $p < 0.001$.



Supplementary Fig. 4. Regulation of the NF- κ B pathway by NOB. **A.** TCGA database analysis revealed a significant correlation of *RORC* and *NFKBIA* (encoding I κ B α) expression in breast cancer. **B.** Quantification of p-p65 and I κ B α expressions in Fig. 4D. Data represent mean \pm SEM. Two-way ANOVA with Sidak's multiple comparisons test, * $p < 0.05$; **** $p < 0.0001$. **C.** Inducible p65 expression using pLenti CMV rtTA3-p65 in MDA-MB-231 cell line. **D.** Representative images of cell motility measured by wound healing assays (x 100 magnification, scale bar = 277.3 μ m). DMSO was used as a vehicle control; NOB, 20 μ M. **E.** I κ B α expression in MDA-MB-231 cells after p65 expression induction. **F.** mRNA expression of cancer related-genes for pro-inflammatory cytokines, Wnt/ β -catenin signaling, and cell proliferation. Data represent mean \pm SEM. Two-way ANOVA with Tukey's multiple comparisons test showed significant difference, * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. Student t-test. Significantly different, # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$.

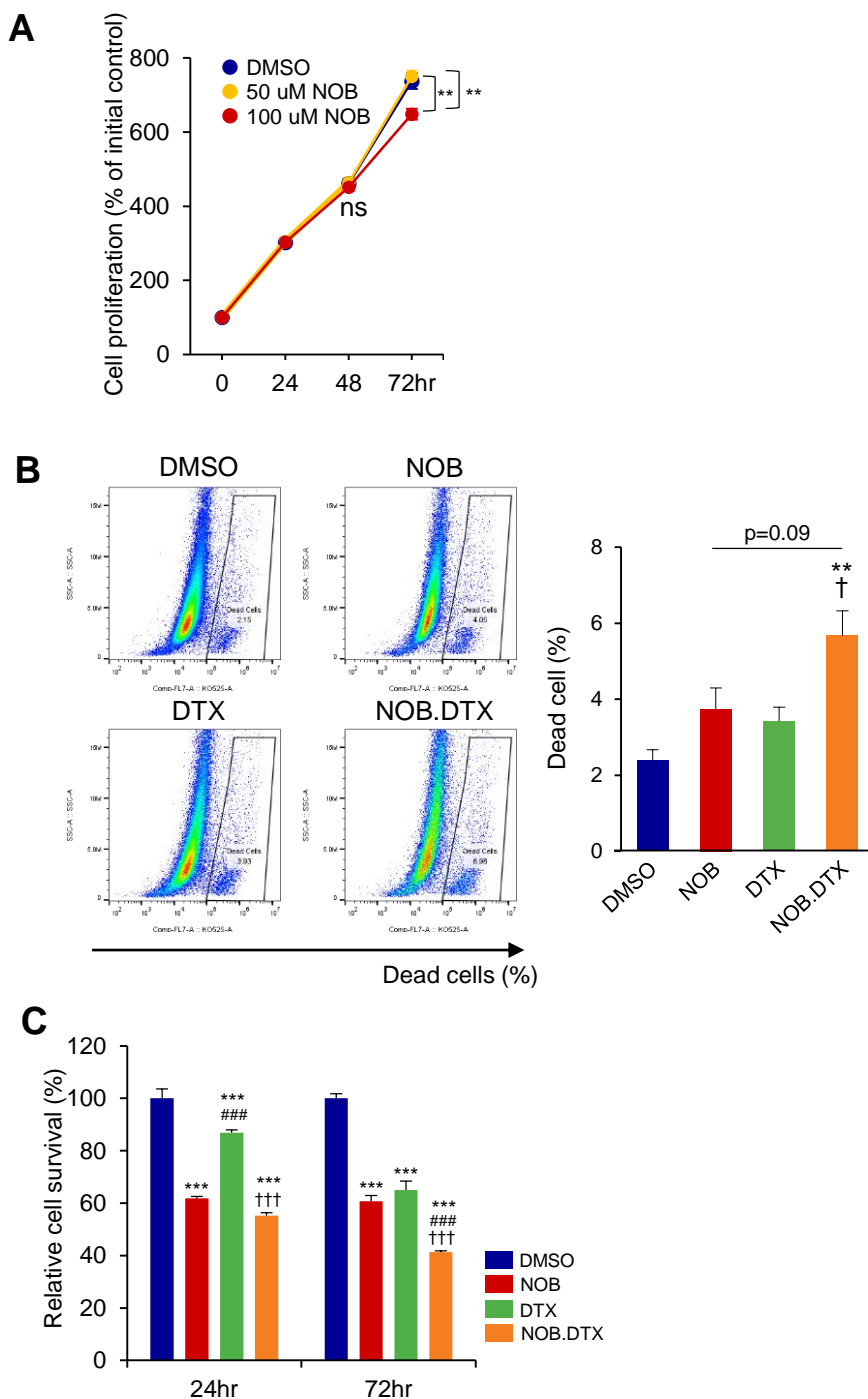
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	Gene name
Positive >2-fold change	TTC25, SLC25A41, TLE6, ICAM2, SLC25A21-AS1, LINC01173, ZNF132, PADI3, TNIP3, FPR1, SLC38A5, CCR7, C3AR1, HPD, CFAP58, MKRN3, LACTB2-AS1, QRFP, TUBAL3, CFAP69, BDKRB1, MIR5689HG, C9orf43, LLPH-DT, CPA5, GAL3ST4, ACSM4, CA3, MIR181A2HG, GRIP1, CCDC180, C6orf99, HHIPL2, CXCL3, FLNB-AS1, IL3RA, LINC02601, SH2D1B, MIR4292, USP50, USP27X-AS1, FAM166B, CCDC173, POPDC2, SERPIND1, C4orf47, STK4-AS1, ACRV1, LINC01812, SIRT4, S100A6, CLEC18B, LINC01179, FOXO6-AS1, ABI3BP
Negative >2-fold change	ABCA12, MYO16-AS1, ATP13A5, RGMB-AS1, NLRP14, ADAM21, LINC01714, MS4A4A, LRRC19, TTLL13P, STAG3, KLHL10, XKRX, BTBD18, DDIT4L, ABCA4, TSTD2, STRA6, C3orf49, PP2D1, REL, ERCC5, FARSA-AS1, LYG1, MLANA

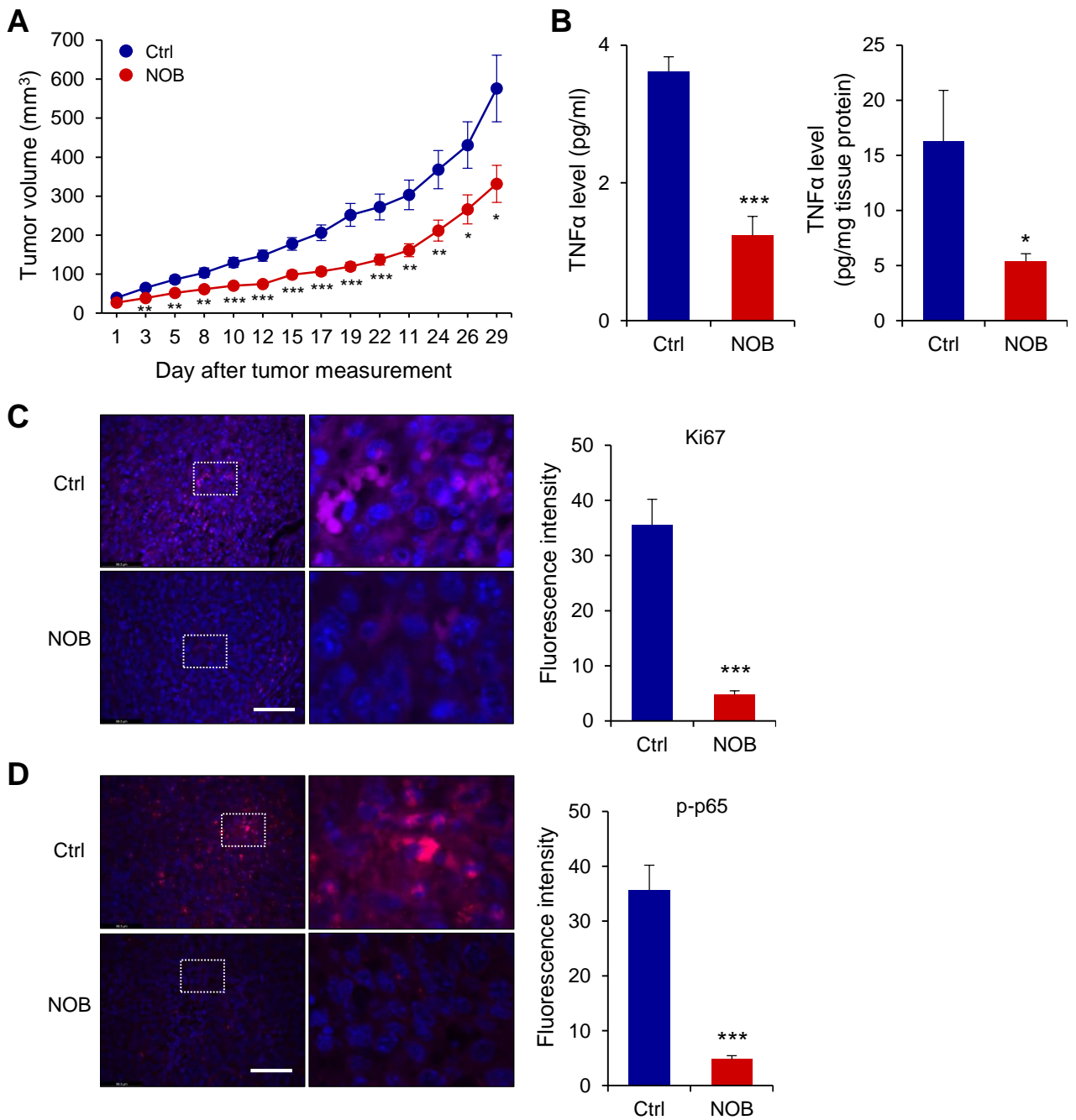


Supplementary Fig. 5. RNA-seq analysis using NOB-treated MDA-MB-231 cells.

A. Overlapping genes between differentially expressed genes (DEGs) and ROR γ target genes (GSE126380) in the GEO database in breast cancer. DEGs showing 2-fold or more changes in expression are shown. **B.** qPCR validation of *IkB α* mRNA induction by NOB in MDA-MB-231 cells.



Supplementary Fig. 6. Combination studies show the efficacy of NOB with DTX in MDA-MB-468 and DB7 cells. **A.** Cell proliferation of the non-tumor MCF10A cells was measured by WST-1 assays for 72 hrs after NOB (50 or 100 μ M) treatment. One-way ANOVA with Tukey's post hoc test. ** $p < 0.01$. **B.** MDA-MB-468 cells were treated with the NOB (50 μ M) and DTX (2.5 nM) combination for 48 hrs and cell death was determined by FACS analysis. Left panel; Representative images; right panel; quantification. Data represent mean \pm SEM. Two-way ANOVA with Tukey's multiple comparisons test showed significant difference compared to DMSO, ** $p < 0.05$, DTX, † $p < 0.05$. **C.** DB7 cells were treated with the NOB (100 μ M) and DTX (2.5 nM) combination for 24 and 72 hrs and cell proliferation was determined by MTT assay. Data represent mean \pm SEM. Two-way ANOVA with Tukey's multiple comparisons test showed significant difference compared to DMSO, *** $p < 0.001$, compared to NOB, ### $p < 0.001$, and compared to DTX.NOB, ††† $p < 0.001$.



Supplementary Fig. 7. NOB suppresses tumorigenesis in DB7 xenograft mice. **A.** NOB induced anti-tumorigenesis effects in DB7 xenograft mice (n=17 for Ctrl and n=18 for NOB). Data represent mean \pm SEM. Student *t*-test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. **B.** TNF- α level of plasma (left panel) and tumor (right panel) of DB7 xenograft mice model. We randomly chose eight samples per group for ELISA measurement. Data represent mean \pm SEM. Student *t*-test, * $p < 0.05$ and *** $p < 0.001$. **C.** NOB reduced Ki67 expression from DB7 xenograft tumor. Representative images of the Ki67 (pink) and DAPI (blue) immunofluorescence in the lesion area (x 400 magnification, scale bar = 69.3 μ m). Quantitative analysis of the percentage of Ki67 immunoreactive area. Data represent mean \pm SEM. Student *t*-test. Significantly different compared with Ctrl, *** $p < 0.001$. **D.** NOB reduced p-p65 expression from DB7 xenograft tumor. Representative images of the p-p65 (red) and DAPI (blue) immunofluorescence in the lesion area. (x 400 magnification, scale bar = 69.3 μ m). Quantitative analysis of the percentage of p-p65 immunoreactive area. Data represent mean \pm SEM. Student *t*-test. Significantly different compared with Ctrl, *** $p < 0.001$.

Supplementary table 1. Primer sequences for qPCR analysis.

	Forward	Reverse
<i>RORA</i>	CTTCTTTCCCTACTGTTTCGTTTC	GCTCTTCTCTCAAGTATTGGC
<i>RORC</i>	GTGGGGACAAGTCGTCTGG	AGTGCTGGCATCGGTTTCG
<i>TNFα</i>	CTCTTCTGCCTGCTGCACTTTG	ATGGGCTACAGGCTTGCTACTC
<i>IL1β</i>	CACAGACCTTCCAGGAGAATG	GCAGTTCAGTGATCGTACAGG
<i>IL6</i>	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
<i>IFNγ</i>	GAGTGTGGAGACCATCAAGGA	GCTTTGCGTTGGACATTCAAGTC
<i>Axin2</i>	TACACTCCTTATTGGGCGATCA	AAGTTCGGAACAGGTAAGCAC
<i>c-MYC</i>	GGAACGAGCTAAAACGGAG	GGCCTTTTCATTGTTTTCCAAC
<i>β-CATENIN</i>	CACAAGCAGAGTGCTGAAGGTG	GATTCCTGAGAGTCCAAAGACAG
<i>BCL9</i>	TCCAGCTCGTTCTCCCAACTTG	GATTGGAGTGAGAAAGTGGCTGG
<i>KI67</i>	CAAGGAACAGCCTCAACCAT	ACCAAGCTTTGTGCCTTCAC
<i>PCNA</i>	CAAGTAATGTCGATAAAGAGGAGGA	TTCAGGTACCTCAGTGCAAAAG
<i>GAPDH</i>	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG

Supplementary Table. 2. Mean tumor growth inhibition rate in breast cancer mice on Day 45 since the 1st measurement.

	MGI ^a	Expected ^b	CI ^c
Ctrl			
NOB	0.69		
DTX.Ctrl	0.36		
DTX.NOB	0.21	0.26	1.22

^a Mean tumor growth inhibition rate (MGI) = tumor volume on day 43 - tumor volume on day 0 of the treated group/tumor volume on day 43 - tumor volume on day 0 of the control group

^b Expected growth inhibition rate = growth inhibition rate of NOB x growth inhibition rate of DTX.Ctrl.

^c Combination index (CI) = expected growth inhibition rate/observed growth inhibition rate (MGI).

^d An index >1.1 indicates a synergistic effect, between 0.9 and 1.1 indicates an additive effect, and <0.9 indicates a less than additive effect.