Direct Inhibition of Elastase Activity by Indole-3-Carbinol Triggers a CD40-TRAF Regulatory Cascade That Disrupts NFκB Transcriptional Activity in Human Breast Cancer Cells

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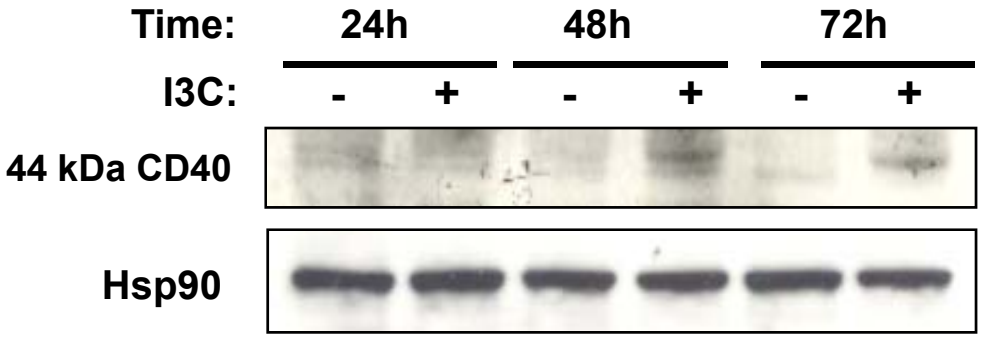
Running title: Phytochemical control of CD40 signaling

Supplemental Results and Supplemental Materials and Methods

MCF7 cells

A



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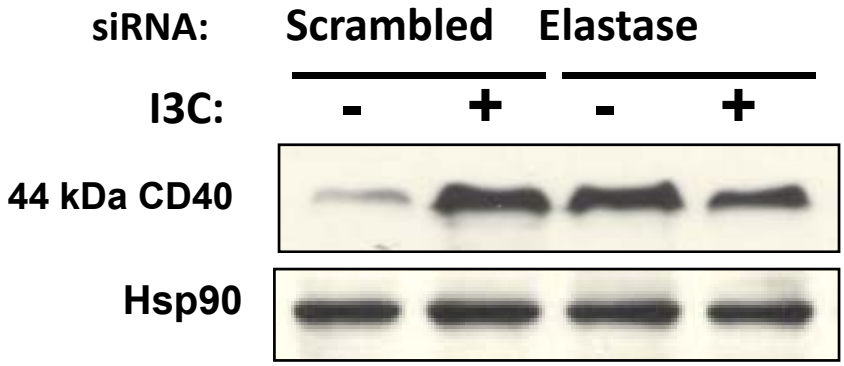

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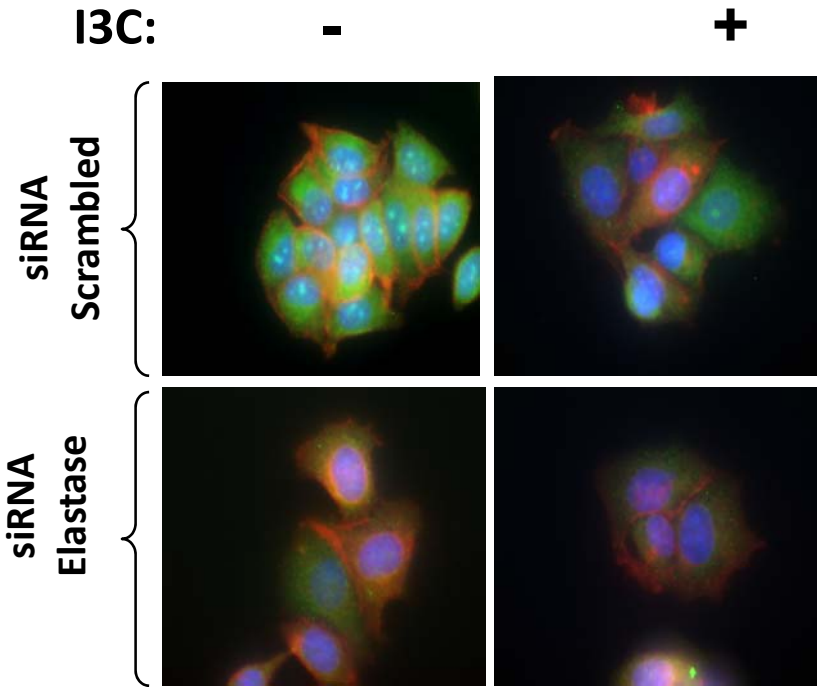

Fig S1

Figure S1. Effects of I3C and siRNA ablation of elastase expression on accumulation of the full-length CD40 protein and NFκB subcellular localization in MCF7 human breast cancer cells.

(*A*) MCF7 cells were treated with 200 μM I3C at the indicated times up to 72 hours and the level of the 44 kDa CD40 protein was monitored by western blot analysis using N-terminal specific CD40 antibodies. The level of Hsp90 was used as gel-loading control. (*B*) MCF7 cells were transfected with siRNA targeting elastase or a scrambled siRNA sequence for 72 hours, and treated with or without 200 μM I3C for the last 48 hours of the transfection. The effects of I3C and/or siRNA ablation of elastase on full-length CD40 protein levels were assessed by western blot of total cell lysates using antibodies targeting the N-terminus of CD40. (*C*) Localization of p65 subunit of NFκB was visualized by indirect immunofluorescence using p65 specific antibodies followed by secondary antibodies conjugated to AlexaFuor488. Rhodamine-phalloidin was used to visualize actin filaments and the outer boundary of the cells, and DAPI staining was used to visualize the nuclei.

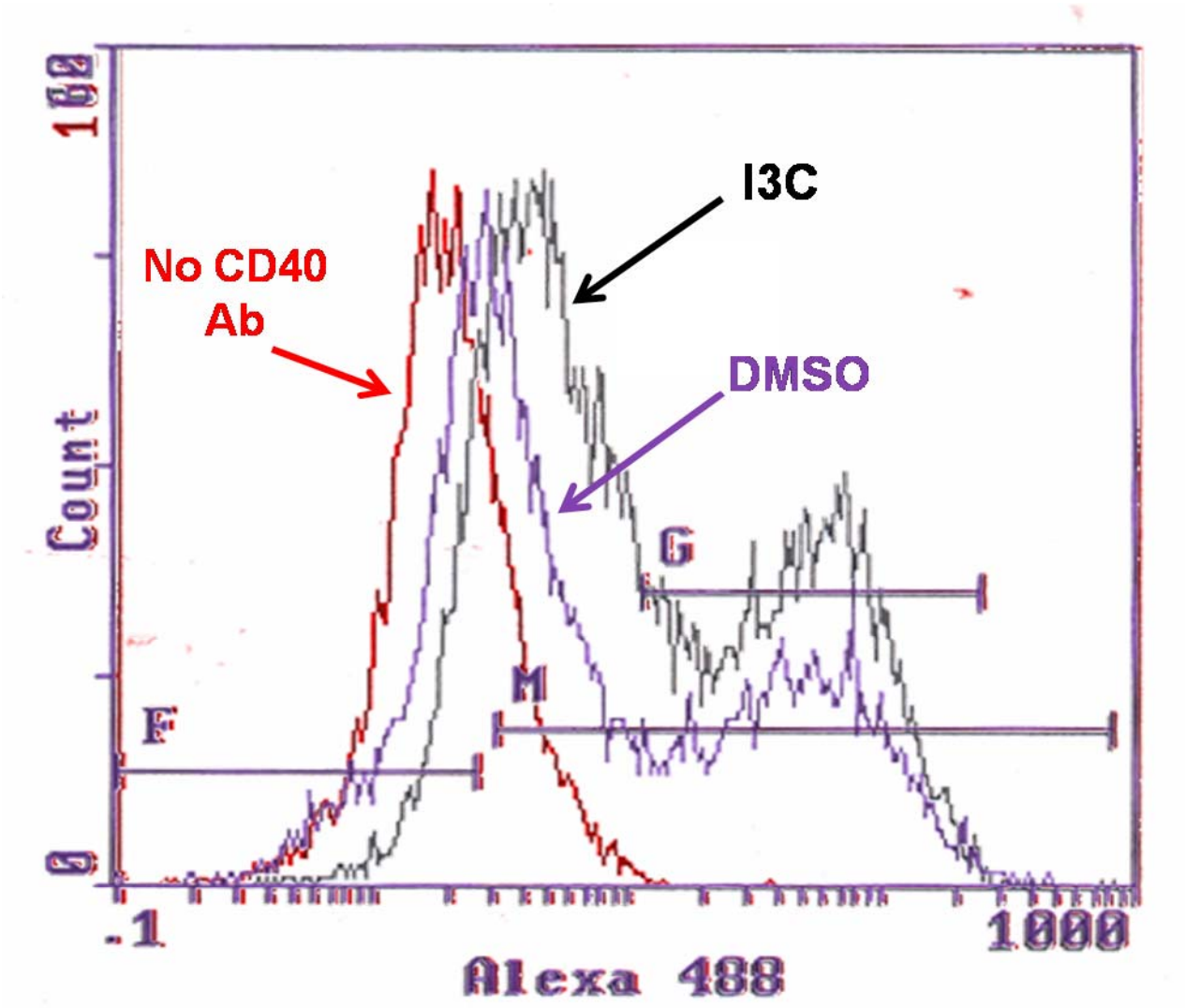

Fig S2

Figure S2. Effects of I3C on the level of cell-surface localized full-length CD40 protein.

MDA-MB-231 human breast cancer cells were treated with or without 200 μM I3C for 48 hours. The effect of I3C on the accumulation of cell-surface localized CD40 protein was assessed by fluorescent labeling of the molecule using specific primary directed against the N-terminal domain of CD40, followed by incubation with fluorescent-probe conjugated secondary antibodies. The fluorescence histograms of the FACS analysis shows the negative control (no primary CD40 antibody) in red, DMSO treated cells in purple and of I3C treated cells in black.

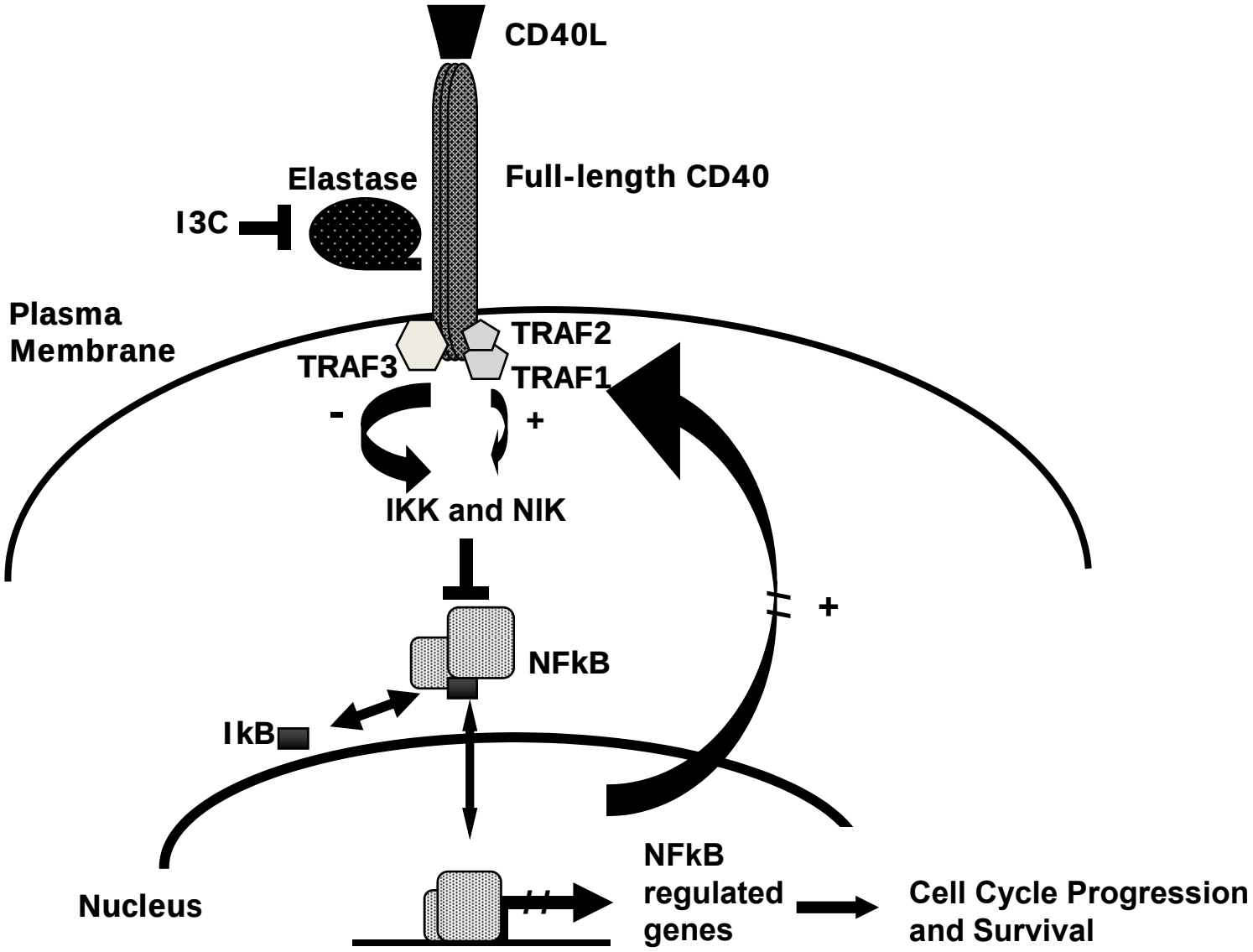
CD40L
Elastase
I3C
Full-length CD40
Plasma
Membrane
TRAF2
TRAF3
TRAF1
-
+
IKK and NIK
+
NFkB
IkB
Nucleus
NFkB
regulated
genes
Cell Cycle Progression
and Survival

Fig S3

Figure S3. Proposed mechanism by which the I3C inhibition of extracellular elastase activity disrupts NFκB nuclear localization and transcriptional activity through modulation of CD40 activated TRAF signaling.

We propose that the I3C inhibition of extracellular elastase prevents the proteolytic cleavage of membrane associated CD40 that results in the stable accumulation of its full-length form in the plasma membrane. The full-length CD40 is bound by its CD40L ligand, the cellular consequences are a shift in the signaling balance towards the negative regulation of NFκB activity and the down-regulated expression of NFκB activated genes such as TRAF1 and those associated with proliferation and survival of the breast cancer cells, such as cyclin D1. In the absence of I3C, the ligand independent signaling of the elastase-cleaved CD40 is mediated by the interactions of TRAF1 and TRAF2 with the intracellular domain of CD40 to activate the upstream activators of NFκB, IKK and NIK, resulting in the positive regulation of NFκB activity and nuclear localization. Because TRAF1 is an NFκB target gene, TRAF1 positive signaling to NFκB is further amplified by this positive feedback loop.

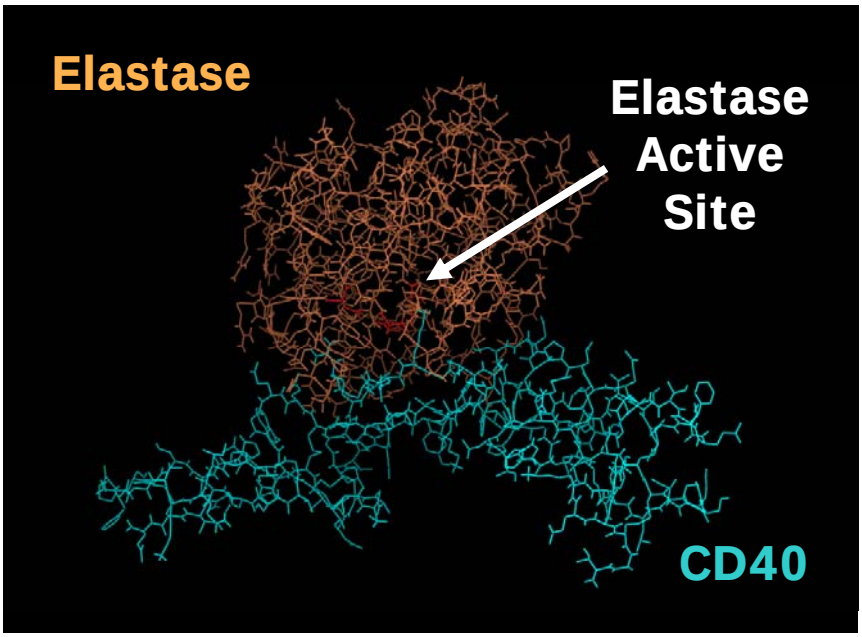

Fig 1A: color version: Computer-aided model of the molecular interactions between elastase and CD40 From figure 1 of the manuscript.

SUPPLEMENTAL Materials and Methods

Materials. MDA-MB-231 cells were obtained from ATCC (Manasas, VA). IMDM cell media, fetal bovine serum, PBS and trypsin were supplied by Lonza and Cambrex/Bohittaker (Walkersville, MD). I3C was purchased from LKT laboratories (St. Paul, MN). Human Neutrophil Elastase was purchased from Calbiochem/EMD (Los Angeles, CA). GenomeWide siRNA for elastase, CD40, TRAF3, IκB as well as validated siRNA for PTEN and scrambled siRNA negative control were purchased from Qiagen (Valencia, CA). P65-GFP-Luciferase plasmid was a kind gift from Dr. William Sha at UC Berkeley. HiPerFect and SuperFect transfection reagents were also supplied by Qiagen (Valencia, CA) and used for RNA and DNA transfections respectively. CD40 Chimera was obtained from R&D Systems (Minneapolis, MN). Antibody to Hsp90 was purchased from BD Biosciences (Franklin Lakes, NJ) antibodies to CD40 N-terminus and CD40L were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IκB, p65 NFκB and TRAF3 targeting antibodies were acquired from Cell Signaling Technology (Beverly MA). The secondary anti-mouse and anti-rabbit antibodies conjugated with HRP were obtained from Bio-Rad Laboratories (Hercules, CA). Secondary Antibodies conjugated to fluorescent probes as well as rhodamine-phalloidin for Immunofluorescence were purchased from Molecular Probes/ Invitrogen (Eugene, OR). All primers used in RT-PCR reactions were synthesized by IDT technologies (San Diego, CA). MG-132 was purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture and treatments with I3C and MG-132. MDA-MB-231 cells were cultured in IMDM media, supplemented by 2 mM L-glutamine, 10% Fetal Bovine Serum and 1.25mL 20,000 U/mL Penicillin/Streptomycin mixture. MCF7 cells were cultured in DMEM media, supplemented by 2 mM L-glutamine, 100 μg/ml bovine insulin, 10% Fetal Bovine Serum and 1.25mL 20,000 U/mL Penicillin/Streptomycin mixture. The cells were incubated at 37 ^{o}C with controlled humidity and 5% CO2 content of the air. I3C was dissolved in DMSO 99.9% HPLC grade (Sigma Aldrich Milwaukee, WI) and the final dilution was performed in the media aliquots used for treatment. Negative control samples were treated with volume of the DMSO vehicle control equivalent to that used for indole treatment. In the experiment using the MG-132 specific 26S proteasome inhibitor, MDA-MB-231 cells were treated with I3C for 48 hours, and on the day of the collection the cell media was aspirated four hours prior to the end of the experimental term and replaced with media containing either10 μM MG-132 or equivalent amount of the DMSO vehicle control. The concentration and duration of MG-132 treatment have been previously established to induce extensive proteasome inhibition while inducing minimum additional cytotoxicity

Flow Cytometry. MDA-MB-231 cells were plated on 6-well plates at 30-40% confluency, cultured and treated with I3C or DMSO as described above. On the day of the collection the cells were washed with PBS twice, harvested using rubber cell lifter and hypotonically lysed in propidium iodide containing buffer. 10,000 nuclei from each sample were analyzed for PI emitted fluorescence proportional to DNA binding of the dye using Coulter Elite flow cytometer (Beckman-Coulter) as previously described . The initial analysis was performed by EPICS software, with further quantification and visualization using Multicycle for Windows software and Excel (Microsoft Office) for graphical representation of the results.

Immunofluorescence. MDA-MB-231 cells or MCF7, were plated on Lab-Tek Permanox slides (Nunc International, Naperville, IL), and treated with the indicated combinations of siRNA and/or 200 μM I3C. On the day of the collection, the cells were fixed with 3.7% formaldehyde in PBS for 15 minutes and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. After blocking with 3% BSA for 1 hour, the cells were incubated with antibodies targeting p65, CD40L or CD40 N-terminus, which is the extracellular domain of CD40 contained in the full length molecule (Santa Cruz Biotechnologies) for 1 hour, followed by 3 washes with PBS and then incubation for 1 hour with anti-rabbit Alexa Fluor 488 or goat anti-rabbit Texas-Red IgG and rhodamine-phalloidin (Molecular Probes, Eugene, OR). Coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing nuclear stain DAPI and then visualized using a Zeiss Axiophot 381 epifluorescence microscope, with a 3.3 MPix Qimaging, MicroPublisher CCD color digital camera and Zeiss AxioImager M1 microscope with a Hamamatsu Orca camera.

siRNA Transfections. Transfections were carried out according to Qiagen HiPerFect transfection protocol. Briefly, MDA-MB-231 or MCF7 cells were plated at 50% confluency on 6-well plates in full growth media on the day of the transfection. For each treatment 150 ng of corresponding siRNA was mixed with 100 μL of growth media without serum or antibiotics. HiPerFect transfection reagent was added to the siRNA suspensions and after vortexing the samples were allowed to incubate for 10 minutes at room temperature. The resulting transfection mixtures were added to the cells in dropwise manner and the cells were gently agitated to assure even distribution of the treatment. The cells were incubated at normal conditions for 24 hours, then the media was aspirated and replaced by full growth media containing 200 μM I3C or DMSO as vehicle control.

NFκB Reporter Plasmid Transfections, GFP Cell Sorting and Luciferase Assays. MDA-MB-231 were seeded on 60 mm plates and following siRNA and/or I3C treatment, were transfected according to SuperFect transfection protocol guidelines (Qiagen, Valencia, CA). Briefly, 2.5 μg of DNA were mixed in with 300 μL of media not containing growth factors or antibiotics. 2 μL of Superfect reagent were added to each DNA-media mixture and after vortexing the samples were allowed to incubate at room temperature for 15 minutes. While the incubation took place growth media was aspirated from the cells and, after washing with PBS, the media was replaced with 2 mL of fresh full media. DNA was added to the plates in drop-wise manner and the plates were agitated to assure even transfection treatment distribution. After 3 hours the media was replaced with 4 mL containing 200 μM I3C or equivalent volume of DMSO. The cells were collected 24 hours following plasmid transfection in 1 mL of ice-cold PBS and analyzed immediately or stored at –20 ^{o}C. 250 μL of each sample was analyzed by FACS analysis for percentage of cells expressing GFP, and therefore containing the response plasmid. 10,000 cells were sorted using Coulter Elite flow cytometer (Beckman-Coulter) and percentage of GFP expressing cells was evaluated using EPICS software, with graphical interpretation using Excel (Microsoft Office). For luciferase assay the cells were pelleted by 15,000 rpm centrifugation for 5 minutes at 4 ^{o}C, then combined with 1x passive lysis buffer (Promega, San Luis Obispo, CA). The pellets were gently resuspended and incubated for 15 minutes at room temperature, followed by 15 minute incubation on ice. Following incubation 15 μL of each sample were combined with 100 μL of luciferase reagent (Promega) and light emission was immediately measured using luminometer, Lumat LB 9507 (EG&G Berthold). The relative light units were recorded and data interpretation was carried using Excel (Microsoft Office).

RT-PCR assay. PCR conditions were as follows: GAPDH: 30s at 94 ^{o}C, 30s at 55 ^{o}C and 30s at 72 ^{o}C for 28 cycles, CD40: 1 min at 94 ^{o}C, 2 min at 60 ^{o}C, 1 min 72 ^{o}C for 33 cycles, cyclin D1 and TRAF1: 1 min at 95 ^{o}C, 1 min at 60 ^{o}C and 2 min at 72 ^{o}C for a total of 32 cycles. TRAF 2 and TRAF3: 1 min at 95 ^{o}C, 1 min at 58 ^{o}C and 2 min at 72 ^{o}C for a total of 32 cycles.

PCR products were assessed by electrophoresis on 1.5 % agarose gel containing 0.01% Gel Red (Biotium, Hayward, CA) for DNA staining along with 1 kb DNA Ladder Plus (Fermentas, Glen Burnie, MD) and further visualized by a UV transilluminator. The data was scanned and analyzed for densitometry using open source graphic software GNU Image Manipulation Program (http://www.gimp.org).

Western Blot Analysis. After the indicated treatments, cells were washed with ice-cold PBS, harvested using rubber cell lifters and lysed in the RIPA lysis buffer, supplemented with protease inhibitor cocktail (Roche Applied Sciences, Chicago, IL). Western blot analysis of samples electrophoretically fractionated on a 10% acrylamide gels was carried out as previously described . After incubation with the primary antibodies, ECL Lightening reagents (GE Healthcare, Piscataway, NJ) were used to visualize the immunoreactive proteins in nitrocellulose membranes and captured on ECL Autoradiography Film (GE Healthcare, Piscataway, NJ).

Analysis of cell surface CD40 by Fluorescence-Activate Cell Sorting (FACS). The procedure was based on Chemicon Direct Staining Protocol for Flow Cytometry. Briefly, MDA-MB-231 cells were grown on 10 cm plates and treated for the specified time periods. On the day of the experiment, the cells were washed twice with PBS and collected into 1.5 ml eppendorf test tubes and placed on ice. At 4 ^{o}C, the cells were centrifuged at 4000 rpm, the pellets were isolated and re-suspended in 100 μL of the primary anti-CD40 antibody targeting the N-terminus of the molecule diluted 1:100 in PBS. The tubes were then incubated on a nutator with gentle agitation for 1hour, after which the cells were centrifuged. The cell pellets were washed 3x with PBS, then re-suspended in 100 μL of the secondary antibody conjugated to Alexa Fluor 488 fluorescent probe diluted 1:200 in PBS. The cells were returned to the nutator for 1 hour. The pellets were washed 3 times in PBS and re-suspended in 350 μL of PBS and subjected to FACS analysis. Within each set of reactions, the negative control contained samples of cells not labeled with the primary antibody, or the secondary antibody or both. The graphed data represents an average of two experimental sets.

References

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2. Nguyen HH, Aronchik I, Brar GA, Nguyen DH, Bjeldanes LF, Firestone GL. The dietary phytochemical indole-3-carbinol is a natural elastase enzymatic inhibitor that disrupts cyclin E protein processing. Proc Natl Acad Sci USA 2008; 105:19750-19755.