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Submitted on 17 Jun 2009

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RelB NF-κB Represses Estrogen Receptor α Expression via Induction of the Zinc Finger Protein Blimp1

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Short title: RelB represses ERα transcription by inducing Blimp1

Word counts:

Materials and Methods: 1,592

Introduction, Results, Discussion: 3,878
Aberrant constitutive expression of NF-κB subunits, reported in over 90% of breast cancers and multiple other malignancies, plays pivotal roles in tumorigenesis. Higher RelB subunit expression was demonstrated in estrogen receptor alpha (ERα) negative breast cancers vs ERα positive ones, due in part to repression of RelB synthesis by ERα signaling. Notably, RelB promoted a more invasive phenotype in ERα negative cancers via induction of the BCL2 gene. Here, we report that RelB reciprocally inhibits ERα synthesis in breast cancer cells, which contributes to a more migratory phenotype. Specifically, RelB is shown for the first time to induce expression of the zinc finger repressor protein Blimp1 (B lymphocyte-induced maturation protein), the critical mediator of B and T cell development, which is transcribed from the PRDM1 gene. Blimp1 protein repressed ERα (ESR1) gene transcription. Commensurately, higher Blimp1/PRDM1 expression was detected in ERα negative breast cancer cells and primary breast tumors. Induction of PRDM1 gene expression was mediated by interaction of Bcl-2, localized in the mitochondria, with Ras. Thus, induction of Blimp1 represents a novel mechanism whereby the RelB NF-κB subunit mediates repression, specifically of ERα, thereby promoting a more migratory phenotype.

NF-κB is a structurally and evolutionary conserved family of dimeric transcription factors with subunits having an N-terminal region of approximately 300 amino acids that shares homology with the v-Rel oncoprotein (17, 44). The conserved Rel homology domain (RHD) is responsible for DNA binding, dimerization, nuclear translocation, and interaction with inhibitory proteins of NF-κB (IκBs). Mammals express five NF-κB members, including c-Rel, RelB, RelA (p65), p50
and p52, which can form either homo-dimers or hetero-dimers. RelB differs from the other members in that it only binds DNA as a hetero-dimer with either p52 or p50, and interacts only poorly with the inhibitory protein IκBα. In most untransformed cells, other than B lymphocytes, NF-κB complexes are sequestered in the cytoplasm bound to specific IκB proteins.

Aberrant activation of NF-κB has been implicated in the pathogenesis of many carcinomas (37). Constitutive activation of c-Rel, RelA, p50, and p52 was first detected in breast cancer (9, 30, 45). RelB appeared to have more limited involvement and functioned predominantly in lymphoid organs and their malignancies (25, 58). For example, Stoffel and coworkers found p50/RelB complexes in mucosa-associated lymphoid tissue (MALT) lymphoma (46) and RelB complexes were implicated in Notch1-induced T-cell leukemia (53). More recently, RelB has been implicated in carcinomas of the breast and prostate. RelB was the most frequently detected NF-κB subunit in nuclear preparations from advanced prostate cancer tissue and correlated directly with Gleason score (26), suggesting an association with prostate cancer progression. We observed elevated nuclear RelB levels in mouse mammary tumors driven by ectopic c-Rel expression in transgenic mice or following carcinogen treatment (14, 40). More recently, we demonstrated that RelB synthesis, which is mediated via synergistic transactivation of the RELB promoter by p50/RelA NF-κB and c-Jun/Fra-2 AP-1 complexes, was selectively active in estrogen receptor (ER) α negative vs positive breast cancers and led to the induction of transcription of the BCL2 gene (56).

While studying the mechanism of the inverse correlation between RelB and ERα levels in breast cancer, more recently we observed that RelB complexes robustly inhibited ERα (ESR1) gene expression. Since RelB is not by itself inhibitory, we postulated that it controls the expression of an intermediate negative regulatory factor that represses ERα gene transcription.
TransFac analysis identified putative binding sites for the B lymphocyte-induced maturation protein (Blimp1), which is expressed from the PRDM1 gene (24) and has been reported to be regulated by NF-κB, although it is not known whether this control is exerted directly or indirectly (22). The Zn finger protein Blimp1 is a repressor of transcription and has been shown to function as a master regulator of development of antibody secreting B lymphocytes and more recently of T cell homeostasis and function (6, 28), and specification of primordial germ cells (33, 54). Blimp1 was originally identified as a silencer of IFN-β gene transcription (24).

Although the exact mechanism by which repression occurs is not fully understood, Blimp1 possesses DNA-binding activity and can recruit histone deacetylases, histone methyltransferases and the co-repressor Groucho (19, 39, 63). Here, we demonstrate for the first time that Blimp1 functions as a potent repressor of ERα synthesis in breast cancer cells and is induced by activation of a Bcl-2/Ras pathway by RelB.

MATERIALS AND METHODS

Cell culture and treatment conditions. ERα positive MCF-7, T47D, ZR-75 and BT474 cells, and ERα negative Hs578T and MDA-MB-231 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in standard culturing medium as recommended by ATCC. The NF639 cell line, kindly provided by Philip Leder (Harvard Medical School, Boston MA) was derived from a mammary gland tumor in a mammary tumor virus (MMTV)-ERBB2 transgenic mouse and cultured as described (18). Hs578T cell lines containing human pRELB-siRNA or control pRELB sense (si-Control) were established as described (56) and grown in the presence of 1 µg/ml puromycin (Sigma, St. Louis, MO). MCF-7 cell lines containing pBABE and pBABE-Bcl-2 were established by retroviral infection, as
described (56), and grown in the presence of 1 µg/ml puromycin. Hs578T or NF639 cell lines carrying pSM2c-sh-BCL2 (V2HS_111806, Open Biosystems) or scrambled control were established by retroviral infection, as above. MCF-7 cells containing inducible C4₄₃R(TO) or C4₄₃R(TO-RelB) were established as described (56). Expression of RelB was induced with 1 µg/ml doxycycline treatment for 48 h.

Plasmids and transfection analysis. A long promoter B ERα construct (ERα-proB) was cloned via PCR from a full-length ERα promoter (kindly provided by Ronald Weigel, University of Iowa College of Medicine, Iowa City, Iowa) using Pfu Hotstart Ultra with the following PCR primers (restriction sites are italicized and ERα sequence is underlined):

- **KpnI**-proB -3489: 5’-CGCTCCTTGTACCTCCCATGCTCACCACAAAGCC-3’
- **HindIII**-proB -1814: 5’-CGCTCAAGCTTTCAAGCCTACCCTGCTGG-3’

Following amplification, the product was digested with KpnI and HindIII and subcloned into pGL3Basic; sequencing confirmed that no mutations were introduced during amplification. The pC4₄₃R(TO-RelB) construct was generated by subcloning the RelB cDNA into the Eco RI restriction site of the doxycycline inducible pC4₄₃R(TO) retroviral vector. The Ras C186S mutant and the ERα expression vector were kindly provided by Mark R. Philips (NYU School of Medicine, NY) and Pierre Chambon (Strasbourg, France), respectively. The Ras-EGFP-C1 vector was generously provided by Wen-Luan Wendy Hsiao (Hong Kong Baptist University). The Ras CA (RasV12) vector was as previously described (20). The human full-length PRDM1 construct in pcDNA3 was as reported (39). The TBlimp vector expressing a dominant negative (dn)Blimp1 protein, containing the DNA binding domain of Blimp1 in the Vxy-puro vector, and the 7 kB human PRDM1 promoter construct in pGL3 were kindly provided by Kathryn Calame.
(Columbia University, NY) (1, 49). pRELB sense and pRELB-siRNA containing control or si-
RELB oligonucleotides in pSIREN-RetroQ vector were described previously (41). The human
full-length BCL2 constructs in pcDNA3 or pBabe were as reported (42). The BCL-2 mutant
L14G, with the indicated point mutation, was kindly provided by Tristram G. Parslow (Emory
University School of Medicine, Atlanta GA). The pRc/CMV-Bcl-2, pRc/CMV-Bcl-acta and
pRc/CMV-Bcl-nt constructs, which were kindly provided by David W. Andrews (McMaster
University, Hamilton, Canada), were as previously described (64). Briefly, the Bcl-acta
(mitochondria-localized Bcl-2 mutant) and the Bcl-nt (cytoplasm-localized Bcl-2 mutant) were
established by exchanging the Bcl-2 carboxy-terminal insertion sequence for an equivalent
sequence from Listeria ActA or by deletion of the Bcl-2 carboxy-terminal insertion sequence,
respectively. The SV40 β-galactosidase (SV40-β-gal) reporter vector was as reported (57). The
estrogen response element (ERE)-TK luciferase construct was as reported (18). For transfection
into six well or P100 plates, 3 or 10 µg total DNA was used, respectively. For transient
transfection, cultures were incubated for 48 h in the presence of DNA and Fugene6 (Roche
Diagnostics Co., Indianapolis, IN) or Geneporter2 (Gene Therapy System Inc., San Diego, CA)
Transfection Reagent. All transient transfection reporter assays were performed, in triplicate, a
minimum of three times. Luciferase assays were performed as described (40, 45). Co-
transfection of the SV40-β-gal expression vector was used to normalize for transfection
efficiency, as described (40). PRDM1 siRNA duplex sequences have been previously described
(61). Duplexes (0.8 nM final) were introduced in cells using Lipofectamine 2000 (Invitrogen)
according to the manufacturer’s protocol.
Isolation of membrane and cytosolic fractions. Membrane and cytosolic proteins were extracted using Mem-PER Eukaryotic Membrane Protein Extraction Kit (PIERCE, 89826) according to the manufacturer’s protocol.

Immunoblot analysis, antibodies, and immunoprecipitation. Whole cells extracts (WCEs) were prepared in RIPA buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 10 mM EDTA, 1% NP-40, 0.1% SDS, 1% sodium sarcosyl, 0.2 mM phenylmethylsulfonylfluoride, 10 µg/ml leupeptin, 1 mM dithiothreitol). Nuclear extracts were prepared as previously described (18). Immunoblotting was performed as we have described (18). Antibodies against RelB (sc-226), Bcl-2 (sc-492), Ras (sc-35), or Lamin B (sc-6217), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against ERα was purchased from NeoMarker (Fremont, CA). Antibodies against E-cadherin, γ-catenin, and Ras (610001) used in immunoprecipitation analyses were purchased from BD Transduction Lab (Franklin Lakes, NJ), and for β-actin (AC-15) from Sigma. Antibodies against mouse Blimp1 (ab13700) and human Blimp1 (NB600-235) were purchased from Novus Biologicals and Abcam, respectively. For immunoprecipitation, WCEs were prepared in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EGTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 10mM NaF, 4mM Na3VO4, 0.5 mM phenylmethylsulfonylfluoride, 10 µg/ml leupeptin, 10mM β-glycerophosphate) and precleared WCEs (500 µg) were incubated overnight at 4°C with antibodies against Bcl-2 or Ras or the corresponding normal IgG in NETN buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 0.5% NP-40, 1 mM EDTA, plus protease and phosphatase inhibitors). Immunoprecipitates were collected using protein A-Sepharose beads, washed four times in NETN buffer and then subjected to immunoblotting.
**Electrophoretic Mobility Shift Assay (EMSA).** Nuclear extracts were prepared and samples (5 µg) subjected to binding and EMSA as described (45). The sequences of the oligonucleotide used are as follows:

4 c-MYC Blimp1 site, 5’-ACAGAAAGGAAAGGACTAGCGGATC-3’
5 Puta-1 Blimp1 site, 5’-GATCCGGAAGGAAAGGGATCTG-3’
6 Puta-2 Blimp1 site, 5’-GATCCAGTTGAGAAAGTGGTCAAG-3’
7 Puta-3 Blimp1 site, 5’-GATCCGCCAGAAAGCTGTACT-3’

The Oct-1 sequence is as reported previously (45). For supershift analysis, 200 ng goat anti-Blimp1 antibody (Abcam) was incubated overnight with 5 µg nuclear extract at 4°C and then labeled probe was added and the mixture incubated at room temperature for 30 min, as described previously (57).

**Chromatin Immunoprecipitation Assay (ChIP).** Cells were crosslinked with 1% formaldehyde at room temperature for 10 min and the reaction stopped by addition of glycine to a final concentration of 125 mM. After three washes in cold PBS, cell pellets were resuspended in ChIP lysis buffer (50 mM Hepes pH 7.5, 140 mM NaCl, 1% Triton X-100 plus protease inhibitor cocktail) and incubated 30 min on ice. Following sonication and centrifugation, the supernatants were incubated with 2 µg salmon sperm DNA, 150 µg BSA and 50 µl protein G-Sepharose for 2h at 4°C and centrifuged to pre-clear. The resulting supernatants were immunoprecipitated overnight at 4°C with 2 µg Blimp1 antibody (ab13700) plus 50 µl protein G-Sepharose and 2 µg salmon sperm DNA. Immunoprecipitates were washed sequentially for 5 min each time at 4°C with ChIP lysis buffer, ChIP lysis high salt buffer (50 mM Hepes pH 7.5, 500 mM NaCl, 1% Triton X-100), ChIP wash buffer (10 mM Tris pH 8, 250 mM LiCl, 0.5%
NP40, 1 mM EDTA) and twice with TE buffer. Immunoprecipitates were then eluted twice with elution buffer (50 mM Tris pH 8, 1% SDS, 10 mM EDTA) at 65°C for 10 min and both input and pooled eluates were incubated overnight at 65°C to reverse the crosslinking. DNA was purified with QIAquick PCR purification kit and 1 µl from 50 µl DNA extraction was used for PCR. (Information on PCR primers and conditions are presented in Supplementary Information).

Reverse Transcriptase (RT)-PCR analysis. RNA, isolated using Trizol reagent (Invitrogen), was quantified by measuring A_{260}. RNA samples, with A_{260}:A_{280} ratios between 1.8 and 2.0, were treated with RQ1 RNase-free DNase (Promega Corporation, Madison WI). For RT-PCR, 1 µg RNA was reverse transcribed with Superscript RNase H-RT in the presence of 100 ng of random primers (Invitrogen). PCR for different targets was performed in a Thermal Cycler. (Information on PCR primers and conditions are presented in Supplementary Information).

Migration assays. Suspensions of 1 to 2.5×10^5 cells were layered, in triplicate, in the upper compartments of a Transwell (Costar, Cambridge, MA) on an 8-mm diameter polycarbonate filter (8 µm pore size), and incubated at 37°C for the indicated times. All migration assays were performed three independent times, each in triplicate. Migration of the cells to the lower side of the filter was evaluated with the acid phosphatase enzymatic assay using p-nitrophenyl phosphate and OD_{410nm} determination. Assays were performed three independent times, each in triplicate, and the mean ± SD are presented.
Confocal Laser Scanning Microscopy. ZR 75 cells were transfected with 1 µg Ras-GFP expression plasmid or pEGFP-C1 EV DNA using Fugene. At 24 h, cultures were incubated for 15 min in 100 nM Mitotracker (Molecular Probes), washed with PBS, and incubated in fixative solution [2% Electron Microscopy (EM)-grade paraformaldehyde (EMS), PBS, 5 mM MgCl₂]. Cells were blocked in PBS/2% bovine serum albumin for 30 min, and incubated in blocking buffer containing Bcl-2 antibody (Santa Cruz; sc-492) for 1 h at 37°C. Cells were subsequently washed in PBS and incubated in PBS/BSA containing an Alexa 647 secondary antibody (Invitrogen; A2143) for 1 h, then washed in PBS and mounted in anti-fade medium (Invitrogen) containing DAPI to stain nuclei. Fluorescent signals were visualized using a Zeiss LSM 510 200M Confocal Microscope (Thornwood, NY) using a x63 objective. Z-sections were taken at an optical slice of 2 µm to determine the localization of mitotracker (red), Bcl-2 (purple) and Ras-GFP (green). Confocal settings were optimized to control for signal cross-over. Detector gain and amplitude offset were set to maximize the linear range without saturation and were kept consistent throughout experiments. Images were stacked and composites generated and an orthogonal slice analysis was performed on the composite using LSM 510 Image Software.

RESULTS

RelB represses synthesis of ERα. The inverse relationship between NF-κB and ERα that typifies many breast cancer cells and tissues has previously been related to the inhibition of NF-κB synthesis or activity by ERα signaling (23, 51, 56). Unexpectedly, we observed the reciprocal repression of ERα activity by RelB in cultures of stable clones of ERα positive MCF-7 cells that ectopically express elevated RelB levels, as described previously (56). Specifically, MCF-7 clones RELB(1) and RELB(2) showed marked reduction of estrogen response element
(ERE) driven reporter activity compared to control MCF-7 EV(1) and EV(2) clones, with parental pcDNA3 empty vector (EV) DNA (Fig. 1A). To verify that the observed effects of RelB on ERE activity were not due to clonal selection, transient transfection analysis was performed using ERα positive ZR-75 cells with vectors expressing either RelB or binding partners p50 or p52 alone, or in combination. RelB expression resulted in a substantial decrease in ERE driven reporter activity, which was further decreased upon co-expression of p50 or p52 (Fig. 1B). (Similar data were obtained using transient transfection of MCF-7 cells, not shown). To verify the effects of RelB on endogenous expression of ERα target genes, RNA was isolated from MCF-7/RELB(1) and MCF-7/EV(1) cells. RT-PCR analysis confirmed decreased RNA levels of target genes CATHEPSIN D, Retinoic Acid Receptor (RAR)α, pS2, and MTA3 in MCF-7/RELB(1) compared to MCF-7/EV(1) cells (Fig. 1C). Similarly, endogenous mRNA levels of CATHEPSIN D and RARα were decreased in ZR-75 and MCF-7 cells upon transient transfection of RelB/p50 or RelB/p52 (Fig. 1D, upper and lower panels). To determine whether RelB decreases ERα levels, transient transfection analysis of vectors expressing RelB in the presence of either p50 or p52 was performed in ZR-75 and MCF-7 cells. Decreased endogenous levels of ERα protein (Fig. 1E) and ERα mRNA (Fig. 1F) were seen with both RelB complexes. (All protein and RT-PCR samples in Fig. 1E and Fig. 1F, respectively were from the same gels.) ERα has two major promoters, A and B, of which the latter is predominantly used in breast cancer cells. Co-transfection of RelB and p52 expression vectors with the ERα promoter B reporter decreases its activity approximately 5-fold in MCF-7 and ZR-75 cells (Fig. 1G). Lastly, we monitored the effects of RelB on endogenous ERα levels and ERα promoter B reporter activity in the stable MCF-7/RELB(1) and RELB(2) clones and the control MCF-7/EV(1) and EV(2)
clones. RelB expression reduced levels of ERα protein (Fig. 1H) and RNA (Fig. 1I) as well as promoter activity (Fig. 1J). Thus, RelB complexes robustly inhibit ERα expression and activity.

**RelB induces expression of the zinc finger repressor protein Blimp1.** Since RelB has a transactivation domain and has not been shown to function itself directly as an inhibitor of transcription, we tested the hypothesis that it controls the expression of an intermediate negative regulatory factor that represses ERα. TransFac analysis (at 80% certainty) of promoter B sequences identified 3 putative binding sites for Blimp1, a master regulator of B and T cell development. The Zn finger protein Blimp1, which functions as a repressor of key regulatory genes in these cells, is expressed from the gene termed *PRDM1* (human) or *Blimp1* (mouse) (24). Since expression of Blimp1 has not been reported in epithelial cells, we first tested for endogenous Blimp1 protein in nuclear extracts of human and mouse breast cancer cells. Substantial Blimp1 levels were detectable in ERα negative Hs578T human breast cancer cells, and more moderate levels in ERα negative MDA-MB-231 cells (Fig. 2A). Very low, but detectable Blimp1 levels were seen with ERα positive ZR-75, MCF-7, T47D, and BT474 human breast cancer cell lines (better seen on darker exposures and see Fig. 2F below). The murine NF639 Her-2/neu-driven ERα low breast cancer cell line displayed a moderate level of Blimp1 protein. RT-PCR confirmed the presence of *PRDM1* mRNA in human breast cancer cells and demonstrated that the levels of expression were much higher in ERα negative vs positive lines (Fig. 2B). To test whether the inverse correlation between Blimp1 and ERα predicted by these findings exists in primary human breast cancers, microarray gene expression datasets publicly available at Oncomine.org were analyzed. The levels of *PRDM1* mRNA were significantly higher in ERα negative vs positive breast cancers (Fig. 2C, left panel) in the Van de Vijver_Breast carcinoma microarray dataset (reporter number NM_001198) (50) (P = 1.3e-6 by
Student’s $t$-test), consistent with the analysis of the cell lines. Data from an additional microarray study (by Bittner: https://expo.intgen.org/expo/public/2005/01/15) publicly available at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2109 confirmed these findings ($P = 7.3 \times 10^{-7}$ by Student’s $t$-test) (Fig. 2C, right panel). Thus, PRDM1 gene expression occurs in patient samples and breast cancer cell lines, with higher levels in ERα negative cells, consistent with the pattern of RelB expression (56).

Previously, we prepared stable Hs578T transfectants expressing either RELB siRNA (Hs578T RELB siRNA) or sense RELB control siRNA (Hs578T Control siRNA) (56). Analysis of these cells demonstrated that knockdown of RelB leads to decreased levels of PRDM1 mRNA (Fig. 2D) and Blimp1 protein (Fig. 2E). Conversely, RelB/p52 expression in human ZR-75 and MCF-7 cells, and in mouse NF639 cells effectively induced PRDM1 or Blimp1 RNA, respectively (Fig. 2F) and Blimp1 protein (Fig. 2G). Furthermore, RelB increased levels of Blimp1 protein (Fig. 2H) and PRDM1 RNA (Fig. 2I) in the stable MCF-7/RELB(1) and RELB(2) clones, indicating functional RelB complexes can induce Blimp1 expression in ERα positive or low breast cancer cells.

Next we sought to test whether the ERα promoter is indeed repressed by ectopic Blimp1 expression and to localize the functional Blimp1 binding element(s). An ~4-fold reduction in activity of an ERα proB construct (Fig. 2J), which contains all three putative Blimp1 binding sites, was seen with ectopic Blimp1 expression in co-transfection analysis in MCF-7 cells compared to EV DNA. The three Blimp1 sites all contain the core GAAA sequence, but differ in surrounding sequencing (see Materials and Methods). To identify the functional sites, competition and supershift EMSA were performed using nuclear extracts of ZR-75 cells ectopically expressing Blimp1 protein or control EV DNA and an oligonucleotide containing the
Blimp1 binding site of the c-\textit{MYC} gene as probe. Putative site 2, but not sites 1 or 3, competed well for binding to the c-\textit{MYC} Blimp1 site (Fig. 3A). When used as a probe, the putative site 2 oligonucleotide effectively bound Blimp1 protein (Fig. 3B), as judged by supershift EMSA (Fig. 3C), confirming this site is a \textit{bona fide} Blimp1 element. The position of this confirmed Blimp1 site is indicated on the map in Fig. 3D. ChIP analysis confirmed intracellular binding of Blimp1 following ectopic expression to the \textit{ER}\textsubscript{α} promoter B region containing the element in MCF-7 cells (Fig. 3E, upper panels). Furthermore, ChIP analysis in Hs578T cells, which expressed the highest levels of Blimp1, confirmed binding of endogenous Blimp1 to this \textit{ER}\textsubscript{α} promoter B region (Fig. 3E, lower panels).

To assess the functional role of the low endogenous levels of Blimp1 in the \textit{ER}\textsubscript{α} positive lines, a dominant negative form of Blimp1, termed TBlimp or dnBlimp (1), which contains only the DNA binding domain, was used. Ectopic expression of the dnBlimp caused an increase in \textit{ER}\textsubscript{α} protein (Fig. 3F), indicating that endogenous Blimp1 represses \textit{ER}\textsubscript{α} in these lines. Consistently, as seen in Figure 4A below, knockdown of Blimp1 levels using an siRNA strategy in NF639 cells induced \textit{ER}\textsubscript{α} protein levels. Conversely, ectopic Blimp1 expression substantially reduced \textit{ER}\textsubscript{α} expression in ZR-75 and MCF-7 cells (Fig. 3G), indicating it can repress the endogenous \textit{ER}\textsubscript{α} gene in both lines. Blimp1 expression also led to reduced \textit{ER}\textsubscript{α} RNA levels in ZR-75 cells, while inhibition of Blimp1 activity with the dnBlimp led to their induction (Fig. 3H). Of note, expression of the dnBlimp ablated the decrease in \textit{ER}\textsubscript{α} expression induced by ectopic RelB in MCF7 cells (Fig. 3I), further implicating Blimp1 in RelB-induced downregulation of \textit{ER}\textsubscript{α} gene expression. Thus, Blimp1 is expressed in breast cancer cells and mediates repression of \textit{ER}\textsubscript{α}. 
Blimp1 promotes a more migratory phenotype in breast cancer cells via inhibition of ERα. In primordial germ cells, the absence of Blimp1 led to defects in cell migration (33). Given our previous findings showing that ERα negative breast cancer cells have a more migratory phenotype than ERα positive ones, we next tested whether Blimp1 regulated migration and reduced expression of epithelial markers, which are required for maintenance of a non-migratory phenotype. First, an siRNA was used to reduce Blimp1 levels in NF639 cells, which are highly invasive and display robust Blimp1 expression. Knockdown of Blimp1 in NF639 cells decreased migration (Fig. 4A, left panel) and increased expression of E-cadherin and ERα (Fig. 4A, right panel). Conversely, ectopic Blimp1 expression in ERα positive ZR-75 cells, which repressed ERα levels (see Fig. 3G above), substantially increased migration (Fig. 4B, left panel), and commensurately decreased levels of E-cadherin and γ-catenin (Fig. 4B, right panel). We next assessed the role of ERα in the ability of Blimp1 to promote a more migratory phenotype. Expression of ERα prevented the increased ability of ZR-75 cells to migrate resulting from Blimp1 expression (3.1-fold vs 1.1-fold) (Fig. 4C, left panel) as well as the decrease in E-cadherin and γ-catenin induced by Blimp1 (Fig. 4C, right panel). Lastly, dnBlimp expression induced the levels of E-cadherin expression in ZR-75 and MCF-7 cells, and these increases were prevented upon knockdown of ERα using siERα RNA (Fig. 4D). Together, these results demonstrate Blimp1 induces a more migratory phenotype and implicate the inhibition of ERα in this control.

Bcl-2 induces Blimp1 via functional interaction with Ras. Previously, we demonstrated BCL2 is a critical RelB target that mediates the migratory phenotype of breast cancer cells (56). For example, knockdown of Bcl-2 in Hs578T cells impaired the ability of the cells to migrate, whereas stable ectopic expression of Bcl-2 in MCF-7 cells (MCF-7/Bcl-2 vs MCF-7/pBABE
stable cells) was sufficient to promote a more migratory phenotype via decreasing E-cadherin levels (56). Thus, we tested the hypothesis that Bcl-2 mediates the induction of Blimp1 by RelB. Knockdown of Bcl-2 with the Bcl2 shRNA in NF639 cells led to increased ERα levels (Fig 5A, left panel), whereas Bcl-2 overexpression decreased ERα level in ZR-75 and MCF-7 cells (Fig 5A, right panel), indicating Bcl-2 can negatively regulate ERα expression. Consistently, knockdown of Bcl-2 levels led to downregulation of Blimp1 protein and Blimp1 RNA levels in NF639 cells (Fig. 5B, upper panels), whereas ectopic Bcl-2 expression robustly induced their levels in these cells (Fig. 5B, lower panels). Similarly Bcl-2 led to substantial increases in the levels of PRDM1 RNA (Fig. 5C, upper panels) and Blimp1 protein expression (Fig. 5C, lower panels) in both ZR-75 and MCF-7 cells, while ectopic BCL2 shRNA, which inhibited RelB/p52-mediated induction of Bcl-2 (56), prevented the induction of Blimp1 by RelB/p52 in ZR-75 cells as well as the reduction of ERα levels (data not shown). Lastly, the dnBlimp1 robustly impaired the previously observed ability of Bcl-2 to enhance migration of MCF-7 cells in MCF-7/Bcl-2 cells (Fig 5D).

WT Bcl-2, which has been shown to interact with and activate Ras in the mitochondria of cells, leads to the induction of NF-κB and AP-1, whereas a Bcl-2 14G mutant unable to associate with Ras is functionally inactive (7, 15, 35). Notably, Ohkubo and coworkers identified an AP-1 element upstream of the Blimp1 promoter (34) leading us to hypothesize a role for Bcl-2/Ras interaction in the activation of Blimp1. As a first test, we compared the effects of the wild type and Bcl-2 14G mutant on the PRDM1 promoter reporter in ZR-75 cells. Transfection of a vector expressing WT Bcl-2 resulted in a substantial induction of the PRDM1 promoter activity in ZR-75 cells (mean of 3.5 ± 1.7-fold, \( P = 0.05 \)), whereas the Bcl-2 14G mutant led to a reduction in activity of approximately 5-fold [mean of 0.2 ± 0.06-fold, \( P = 0.002 \)]. To assess for association
of Bcl-2 and Ras in breast cancer cell lines, WCEs were prepared from ZR-75 cells transfected
with vectors expressing H-Ras and Bcl-2. Immunoprecipitation of Bcl-2 led to co-precipitation
of Ras, whereas, control rabbit IgG protein did not (Fig. 6A, left panel). Furthermore, co-
immunoprecipitation analysis of endogenous proteins in NF639 cell extracts similarly detected
association between endogenous Ras and Bcl-2 (Fig. 6A, right panels). Confocal microscopy
confirmed the partial association of transfected Ras-GFP with Bcl-2 in the mitochondria of ZR-
75 cells (Fig. 6B, and see projection images in Supplementary Information, Fig. S1).

Interestingly, ectopic Bcl-2 expression led to enhanced membrane and reduced cytosolic
localization of endogenous Ras protein in ZR-75 and MCF-7 cells (Fig. 6C). To evaluate the
requirement for Ras membrane localization, we compared the effects on ERα levels of
expression of Ras WT vs a Ras C186S mutant, which is unable to localize to the membrane (5,
8). The Ras C186S mutant, which interacts with Bcl-2 as well as Ras WT (data not shown), was
unable to reduce ERα levels or to induce Blimp1 expression in contrast to findings with Ras WT
(Fig. 6D). Furthermore, Ras C186S prevented the reduction in ERα levels mediated by Bcl-2
(Fig. 6E) and activation of the PRDM1 promoter (Fig. 6F), suggesting it functions as a dominant
negative variant. Lastly, we tested the role of mitochondrial localization using two Bcl-2
mutants: Bcl-acta, which localizes to the mitochondria, and Bcl-nt, which predominantly
localizes to the cytoplasm (64). Whereas Bcl-acta and Bcl-2 WT cooperated with Ras to induce
the PRDM1 promoter, the Bcl-nt was unable to induce its activity (Fig. 6G), consistent with an
important role for co-localization in the mitochondria.

To test whether Ras signaling is sufficient to induce Blimp1 expression, constitutively
active Ras was ectopically expressed in ZR-75 and MCF-7 cells; potent upregulation of Blimp1
was observed (Fig. 7A). We next assessed data publicly available on microarray gene expression
profiling of primary human mammary epithelial cells following transformation with various genes. Consistent with the data presented above, a significant induction of PRDM1 mRNA expression was detected upon expression of activated H-Ras vs GFP ($P = 1.0 \times 10^{-7}$), but not with E2F3, activated β-catenin, c-Myc or c-Src (2) (Fig. 7B). A significant difference was also observed in PRDM1 mRNA levels between the means of the H-Ras group vs all others combined ($P = 1.7 \times 10^{-10}$). Lastly, we sought to elucidate the role of Ras on cell migration induced by Bcl-2 in MCF-7 cells using the dominant negative function of the Ras C186S mutant. Ras C186S mutant, but not Ras WT, inhibited the Bcl-2-mediated decrease in E-cadherin and ERα levels (Fig. 7C) and the ability of MCF-7 cells to migrate (Fig. 7D), consistent with the effects seen above in ZR-75 cells (Figs. 6D-F). Together these data strongly argue that Bcl-2 association with Ras, likely in the mitochondrial membrane, leads to the induction of the levels of Blimp1 and to a more migratory phenotype (see scheme in Fig. 7E).

**DISCUSSION**

Our findings identify a novel mechanism whereby the RelB NF-κB family member acts to repress ERα gene expression via induction of the zinc finger protein Blimp1. Previously, we demonstrated RelB induces transcription of the BCL2 gene via interaction with C/EBP binding to an upstream CREB site (56) (Figure 7E). Here, we show that the association of Bcl-2 with Ras, activates this proto-oncogene and leads to expression of the Zn finger repressor Blimp1. In turn, Blimp1 binds to an element upstream of ERα promoter B, reducing promoter activity and ERα levels, and thereby causing a reduction in the levels of E-cadherin and γ-catenin and a commensurate increase in migratory phenotype in breast cancer cells (see scheme in Figure 7E). To our knowledge, this is the first study identifying a crucial role for Blimp1 in repression of
ERα transcription and also in increased migration of cancer cells. Indeed, Blimp1 is a well-known zinc finger transcriptional repressor that is critical for terminal differentiation of B cells into immunoglobulin-secreting plasma cells and functions by attenuating proliferation while promoting the differentiated B-cell gene expression program (10). More recently, Blimp1 has been implicated in T cell homeostasis (16), where it similarly represses expression of genes promoting proliferation, e.g., IL-2 and Bcl-6 while enhancing IL-10 production. Interestingly, studies have also indicated that Blimp1 is a critical determinant of primordial germ cells differentiation (33). The absence of Blimp1 led to the formation of primordial germ cells clusters, and to defects in primordial germ cells cell migration, as well as to apoptosis (33). Our findings identify one mechanism for the effects of Blimp1 on migration of breast cancer cells related, in part, to the downregulation of ERα gene expression. It remains to be determined whether similar regulation of ERα by Blimp1 occurs in B cells and plays a role in autoimmune disease. Overall, our data identify Blimp1 as a potent negative regulator of ERα expression and thereby as an activator of migration of breast cancer cells.

RelB has been reported to activate gene transcription via several different mechanisms. RelB has been shown to induce transcription of the c-Myb oncogene via releasing pausing that occurs during elongation (47). The activation of the Blc, Elc, Sdf-1, and Slc promoters by RelB required IKKα (4). Interestingly activation of the dapk1, dapk3, c-flip and birc3 promoters by RelB can be prevented by Daxx, which interacts selectively with RelB but not with RelA or c-Rel (36). In addition to the BCL2 gene (56), RelB induces transcription of several other pro-survival genes, including MNSOD in prostate cancer (60) and MNSOD and SURVIVIN in breast cancer (29). However, RelB was unable to bind to the NF-κB elements upstream of the Bcl-xl promoter and led to decreased Bcl-xl gene expression, in contrast to the potent activation seen upon co-
expression of either c-Rel or RelA (21). In fibroblasts, RelB has also been reported to suppress expression of several genes, including IL-1α, IL-1β and TNFα, indirectly via modulating IκBα stability (59), and to inactivate p65 via the formation of dimeric complex (27). Here, we report that RelB can repress expression of the ERα promoter via activation of the zinc finger repressor protein Blimp1. Consistent with this finding, ERα and RelB levels display an inverse pattern of expression in many breast cancer cells and tissues, including inflammatory breast cancers (23, 30, 52, 56).

To our knowledge, this is the first report of a transcription factor that acts as a repressor of ERα promoter activity. Previous work showing repression of ERα promoter transcription in breast cancer cells implicated DNA methyltransferases and histone deacetylases in the regulation (43, 62). To date, the identified transcription factors controlling ERα promoter activity have all been shown to act as positive regulators. Fuqua, Clark and coworkers have identified SP1, USF-1 and ERα itself as essential factors required for full ERα gene transcription (12, 13). The estrogen receptor factor (ERF-1) and the estrogen receptor promoter B associated factor 1 (ERBF-1) have been implicated in positive control of ERα promoter A and B activity, respectively (11, 48). Moreover, our previous study also indicated that the Forkhead box O protein 3a (FOXO3a) activates ERα promoter B transcription in breast cancer cells (18).

Activation of Ras via interaction with Bcl-2 has been previously reported in the context of Ras-mediated apoptosis of T cells (15, 38) and of adipocytes (31), although, the findings were somewhat contradictory. Ras-Bcl-2 interaction in T cells blocked Ras-induced apoptosis, whereas in adipocytes it led to the induction of apoptosis via inactivation of Bcl-2 pro-survival function. Bcl-2 has also been shown to interact with Raf, although the resulting effects differed depending upon the cellular conditions. In murine myeloid progenitor cells, Bcl-2 targeted Raf to
mitochondrial membranes allowing this kinase to protect against apoptosis by phosphorylating BAD (55), whereas Taxol-induced activation of Raf was accompanied by the loss of Bcl-2 anti-apoptotic function in MCF-7 cells (3). Of note, we observed that a constitutively active Raf could similarly induce Blimp1 expression, implicating this Ras-induced pathway in signaling events (not shown). Interestingly, El-Ashry and coworkers showed that inhibition of Ras or Raf activities led to re-expression of ERα in MCF-7 cells (32). Our findings that Blimp1 mediates repression of ERα can explain these observations. Overall, our study indicates that RelB-mediated induction of Bcl-2 leads to the repression of ERα transcription and thus to a more migratory phenotype of breast cancer cells by activating Ras.

ACKNOWLEDGEMENTS

We thank Mark R. Philips, Pierre Chambon, Kathryn Calame, Wen-Luan Wendy Hsiao, David W. Andrews and Tristram G. Parslow and Philip Leder for generously providing cloned DNAs and cell lines, respectively.

These studies were supported by NIH grants P01 ES11624, R01 CA129129, R01 CA36355, R01 EY06000 and training grant T32 HL007501 and a fellowship from the American Institute for Cancer Research with funding from the Derx Foundation.

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FIG. 1 RelB represses ERα synthesis in breast cancer cells. (A) Stable MCF-7 clones expressing either RELB or EV DNA, isolated as described previously (56) and termed RELB(1), RELB(2), EV(1), and EV(2), were transfected with 0.5 µg ERE-TK luciferase reporter, 0.5 µg SV40-β-gal. Normalized ERE activity, set to 100% in the stable EV cells, was decreased in both RelB-expressing lines (mean ± S.D. from three separate experiments). (B) ZR-75 cells were transiently transfected with 0.5 µg ERE-TK Luciferase DNA plus 0.5 µg of the indicated NF-κB subunit expression vectors, 0.5 µg SV40-β-gal and EV pcDNA3 DNA to a 3.0 µg DNA total. Normalized ERE activity, set to 100% in the EV-transfected cells, was reduced by RelB alone or in combination with p50 or p52 (mean ± S.D. from three separate experiments). (C) RNA, isolated from EV(1) and RELB(1) stable MCF-7 clones, was subjected to RT-PCR analysis for expression of ERα target genes CATHEPSIN D (CATHD), RARα, pS2, MTA3, and GAPDH, as loading control. (D) ZR-75 or MCF-7 cells were transiently transfected with 3 µg each of vectors expressing RelB and either p50 or p52 or 6.0 µg of EV DNA. Isolated RNA was subjected to RT-PCR analysis for RARα, CATHEPSIN D (CATHD), and GAPDH. (E-F) ZR-75 or MCF-7 cells were transiently transfected with 3.0 µg of vectors expressing RelB, and p50 or p52 or EV DNA, and WCEs and RNA isolated, which were subjected to immunoblot analysis for ERα and β-actin (E), and RT-PCR for ERα and GAPDH (F), respectively. (Samples in E and F were run on the same gels and the lanes brought into contiguous positions.) (G) ZR-75 and MCF-7 cells were transiently transfected with 0.5 µg of ERα proB luciferase reporter plus 0.25 µg of vectors expressing RelB and p52 or 0.5 µg EV pcDNA3 DNA, 0.5 µg SV40-β-gal and EV DNA to a 3.0 µg DNA total. Normalized proB activity values are presented as the mean ± S.D. from three
experiments (control EV DNA set to 1). (H-I). Protein and RNA, isolated from RELB(1), RELB(2), EV(1), and EV(2) stable MCF-7 clones, were subjected to immunoblotting for expression of ERα, RelB and β-actin (H), and to RT-PCR analysis for expression of ERα and GAPDH (I), respectively. (J) RELB(1), RELB(2), EV(1), and EV(2) MCF7 clones were transiently transfected with 0.5 µg of ERα proB luciferase reporter, 0.5 µg SV40-β-gal and EV DNA to a 3.0 µg DNA total. Normalized proB activity values are presented as the mean ± S.D. from three experiments (control EV DNA set to 1).

FIG. 2 RelB induces Blimp1 in breast cancer cells. (A) Nuclear extracts were isolated from the indicated human and mouse breast cancer cell lines and subjected to immunoblotting for Blimp1 and β-actin, which confirmed equal loading. (B) RNA, isolated from the indicated human breast cancer lines, was subjected to RT-PCR for PRDM1 for either 28 or 30 cycles (as indicated) and for GAPDH levels. (C) (left panel) Box plot of data from the Van de Vijver_Breast carcinoma microarray dataset (reporter number NM_001198) (50) was accessed using the ONCOMINE™ Cancer Profiling Database (www.oncomine.org) and is plotted on a log scale. The dataset includes 69 ERα negative and 226 ERα positive human primary breast carcinoma samples. A Student’s t-test, performed directly through the Oncomine 3.0 software, showed the difference in PRDM1 expression between the two groups was significant ($P = 1.3e^{-6}$). (right panel) Box plot of data from the Bittner study: https://expo.intgen.org/expo/public/2005/01/15) publicly available at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2109) showed the difference in PRDM1 expression between the two groups was significant ($P = 7.3e^{-7}$). (D) RNA was isolated from stable Hs578T transfectants expressing either RELB siRNA (Hs578T RELB siRNA) or
sense RELB (Hs578T Control siRNA) (Con) and analyzed for PRDM1 levels. (E) Nuclear proteins were isolated from Hs578T RELB siRNA and Hs578T Control siRNA (Con) cells and analyzed for Blimp1, Lamin B and RelB levels by immunoblotting. (F) ZR-75, MCF-7 and NF639 cells were transiently transfected with 3 µg each of RelB and p52 expression vectors or 6 µg of EV DNA and RNA subjected to RT-PCR for human PRDM1 or mouse Blimp1 RNA levels. (G) ZR-75, MCF-7 and NF639 cells were transiently transfected with 3 µg each of RelB and p52 expression vectors or 6 µg of EV DNA. Nuclear extracts (ZR-75, MCF-7; left panels) and WCEs (NF639, right panels) were analyzed by immunoblotting for Blimp1 and either Lamin B or β-actin, respectively. (H-I). Nuclear extracts and RNA, isolated from RELB(1), RELB(2), EV(1), and EV(2) stable MCF-7 clones, were subjected to immunoblotting for expression of Blimp1 and Lamin B (H), and to RT-PCR analysis for expression of PRDM1 and GAPDH (I), respectively. (J) MCF-7 cells were transiently transfected with 0.5 µg of ERα proB luciferase reporter, 0.5 µg SV40-β-gal and EV DNA to a 3.0 µg DNA total. Normalized proB activity values are presented as the mean ± S.D. from three experiments (control EV DNA set to 1).

FIG. 3 Blimp1 represses ERα gene expression in breast cancer cells. (A) Nuclear extracts, prepared from ZR-75 cells transfected with Blimp1 expression vector, were used in competition EMSA with the Blimp1 element from the c-MYC gene as probe and 50x molar excess of oligonucleotides containing the c-MYC or putative ERα promoter Blimp1 sites 1, 2 or 3 (P1 – P3). (B) Nuclear extracts, prepared from ZR-75 cells transfected with EV (-) or Blimp1 expression vector (+) were used in EMSA with the putative ERα gene P1, P2 and P3 Blimp1 sites, as probe. (C) Nuclear extracts, prepared from ZR-75 cells transfected with Blimp1 expression vector were used in supershift EMSA in the absence (-) or presence (+) of 200 ng of
Blimp1 antibody and the putative \( \text{ER}\alpha \) gene Blimp1 P2 site, as probe. (D) \( \text{ER}\alpha \) promoter A and B regions with confirmed Blimp1 site indicated with the element sequence given below, where core is underlined. (E) ChIP analyses were performed on MCF-7 cells transiently transfected with Blimp1 (top panels) or Hs578T cells (bottom panels) using anti-Blimp1 antibody or the corresponding normal IgG as indicated. The DNA, extracted from the immunoprecipitates or the input, were amplified by PCR using pairs of primers specific to the confirmed Blimp1 site of \( \text{ER}\alpha \) promoter (Blimp1 site) (left panels) or an upstream region as negative control (right panels). (F) ZR-75 and MCF-7 cells were transiently transfected with 5.0 µg Vxy-puro TBlimp vector expressing the 35 kDa dnBlimp protein or EV DNA, and WCEs subjected to immunoblotting for ER\( \alpha \), dnBlimp and β-actin. (G) ZR-75 and MCF-7 cells were transiently transfected with 5.0 µg of Blimp1 expression vector or EV DNA, and WCEs subjected to immunoblotting for ER\( \alpha \), Blimp1 and β-actin. Densitometry indicated the ER\( \alpha \) levels in the ZR-75 and MCF-7 cells were reduced to 28.0 ± 3.4 and 73.9 ± 5.0, respectively upon ectopic Blimp1 expression compared to EV DNA set at 100]. (H) ZR-75 cells were transiently transfected with 5.0 µg of vector expressing either Blimp1 or dnBlimp protein (left or right panels, respectively) or the corresponding EV DNA, and RNA subjected to RT-PCR for \( \text{ER}\alpha \) and \( \text{GAPDH} \). (I) MCF-7 cells carrying an inducible C4bsR(TO-RelB) RelB construct, plated in the absence or presence of 1 µg/ml doxycycline, were transiently transfected with 6.0 µg dnBlimp expression vector. After 48 h, WCEs were analyzed for levels of ER\( \alpha \), RelB, dnBlimp and β-actin.

FIG. 4 Blimp1 induces a migratory phenotype in breast cancer cells via repression of ER\( \alpha \).

(A) NF639 cells were transiently transfected with control siRNA (si-con) or siBlimp1 RNA. (left panel) Cells were subjected, in triplicate, to a migration assay for 4 h. Cells that migrated to the
lower side of the filter were quantified by spectrometric determination at OD\textsubscript{410nm}. Average migration from three independent experiments ± S.D. is presented relative to si-cont (set at 100%) (*\(P = 7.0\times10^{-5}\)). (right panel) WCEs were subjected to immunoblotting for E-cadherin (E-Cadh), ER\(\alpha\), Blimp1, and β-actin. (B) ZR-75 cells were transiently transfected with 6 µg of EV or Blimp1 expression vector. (left panel) Transfect ZR-75 cells were subjected, in triplicate, to a migration assay for 24 h, as in part (A). *\(P = 0.0007\). (right panel) WCEs were analyzed by immunoblotting for Blimp1, E-cadherin, γ-catenin (γ-Caten) and β-actin. (C) ZR-75 cells were transiently transfected with 6 µg of Blimp1 expression vector or EV DNA in the absence or presence of ER\(\alpha\) expression vector (1.5 µg) or EV DNA, as indicated. (left panel) After 24 h, ZR-75 cells were subjected to migration assays for 24 h, as in part (A). The enhanced migration induced by Blimp1 is significantly decreased by ER\(\alpha\). *\(P = 0.0001\). (right panel) After 48 h, WCEs were prepared and analyzed by immunoblotting for E-cadherin, γ-catenin, ER\(\alpha\), Blimp1 and β-actin. (D) ZR75 and MCF7 cells were transiently transfected with dnBlimp (+) or EV DNA (-). Twenty four h later, ER\(\alpha\) validated Stealth™ RNAi (+) or Stealth RNAi negative control (-) were introduced in cells using Lipofectamine RNAiMAX reagent by reverse transfection as previously described (56). After 24 h, WCEs were prepared and analyzed by immunoblotting for E-cadherin (E-Cadh), ER\(\alpha\), and β-actin.

FIG. 5 Induction of Blimp1 is mediated via Bcl-2. (A) (left panels) NF639 cells were transiently transfected with expression vectors for either control shRNA (contl shRNA) or Bcl2 shRNA and WCEs analyzed for ER\(\alpha\), Bcl-2 (which confirmed effective knockdown) and β-actin protein levels. (right panels) ZR-75 and MCF-7 cells were transiently transfected with EV or full length Bcl-2 expression vector. WCEs were analyzed for ER\(\alpha\) and β-actin protein levels, and for
Bcl-2 (which confirmed the expected changes in levels, not shown). (B) NF639 cells were transiently transfected with expression vectors for either control shRNA (contl shRNA) or Bcl2 shRNA (upper panels) or EV or full length Bcl-2 (bottom panels). (left panels) WCEs were analyzed for Blimp1 and β-actin. (right panels) RNA was subjected to RT-PCR analysis for levels of Blimp1 and Gapdh. (C) RNA (top panels) and nuclear extracts (bottom panels) were prepared from ZR-75 and MCF-7 cells transfected with control EV DNA or vector expressing Bcl-2 and subjected to RT-PCR for PRDM1 and GAPDH expression (upper panels) and Blimp1 and Lamin B levels (lower panels), respectively. (D) Stable mixed populations of MCF-7 cells expressing Bcl-2 or pBabe (pB) DNA were transiently transfected with EV DNA (-) or vector expressing dnBlimp (+). Cells were subjected, in triplicate, to a migration assay for 24 h, as in Fig. 4A. The increase in migration induced by Bcl-2 is significantly reduced by dnBlimp, $P = 3.8 \times 10^{-7}$. Western blot analysis confirmed the expression of the dnBlimp (not shown).

FIG. 6 Bcl-2 interaction with Ras induces Blimp1. (A) WCEs (500 µg), prepared from ZR-75 cells transiently transfected with Bcl-2 and Ras expression vectors (left panels) or from untransfected NF639 cells (right panels), were immunoprecipitated with rabbit anti-Bcl-2 or mouse anti-Ras antibodies or the corresponding normal IgG as indicated. Immunoprecipitated proteins were analysed by immunoblotting for Bcl-2 and Ras. WCEs (5 µg) were used as input. (B) ZR75 cells, grown on coverslips in 6-well dishes, were transfected with 1 µg Ras-GFP expression plasmid. Following staining with Mitotracker, cells were fixed, and subjected to immunohistochemistry for Bcl-2 using an Alexa 647 labeled secondary antibody (purple 694). Mitochondria are labeled in red with Mitotracker, Ras-GFP is green and nuclei are labeled with DAPI stain (blue). A composite image was generated from the z-sections and an Orthoganol
slice analysis was performed on the composite where XZ is depicted on the horizontal and YZ on
the vertical axis outside the dimension of the composite. Co-localization of Ras and Bcl-2 to the
mitochondria is demonstrated by the overlap of signals yielding white spots. 3-D projection
images were made from the composites. The Inset shows a forty-five degree rotation of the
image of the cell, with arrows indicating areas of co-localization of Ras and Bcl-2 in the
mitochondria. (C) ZR-75 and MCF-7 cells were transiently transfected with EV or full length
Bcl-2 DNA. After 48 h cytosol and membrane fractions were prepared as described in Materials
and Methods and subjected to immunoblotting for Ras and β-actin. Immunoblotting for VDAC,
a mitochondrial ion channel membrane protein, confirmed effective separation of the membrane
from the cytoplasmic compartment (not shown). (D) ZR-75 cells were transiently transfected
with EV (-) or vectors expressing Ras WT or Ras C186S mutant protein. After 48 h, WCEs and
nuclear extracts were analyzed for levels of ERα and β-actin (upper panels) and Blimp1 and
Lamin B (lower panels). (E) ZR-75 cells were transiently transfected with EV or vector
expressing full length Bcl-2 in the absence (-) or presence (+) of a vector expressing Ras C186S
mutant protein. After 48 h, WCEs were analyzed for ERα protein levels. (F) ZR-75 cells were
transiently transfected in triplicate, with 0.5 µg of the 7 kB human PRDM1 promoter-Luc vector,
0.5 µg of Bcl-2 with 0.5 µg Ras WT (Ras) or 0.5 µg Ras C186S (C186S) expression vector, 0.5
µg SV40-β-gal and pcDNA3 (EV) to make a total of 3.0 µg DNA. Luciferase and β-gal activities
were determined and normalized PRDM1 promoter activity values are presented as the mean ±
S.D. (control EV DNA set to 1). (G) ZR-75 cells were transiently transfected in triplicate, with
0.5 µg of the 7 kB human PRDM1 promoter-Luc vector, 0.5 µg of Ras expression vector with
0.5 µg of either EV DNA, WT Bcl-2, Bcl-acta (mitochondria-localized Bcl-2 mutant) or Bcl-nt
(cytoplasm-localized Bcl-2 mutant) expression vectors (64), 0.5 µg SV40-β-gal and pRc/CMV
(EV) to make a total of 3.0 mg DNA. Forty-eight h after transfection, luciferase and β-gal
activities were determined, and normalized PRDM1 promoter activity values are presented as the
mean ± S.D. from three separate experiments (control EV DNA set to 1). Using a Mann Whitney
test, a $P < 0.05$ was seen between each condition, except for Ras + WT Bcl-2 and Ras + Bcl-acta.

FIG. 7 Induction of Blimp1 is mediated via Bcl-2 and Ras activation. (A) ZR-75 and MCF-7
cells were transiently transfected with EV or vector expressing constitutively active Ras (Ras
CA), and nuclear extracts analyzed for Blimp1 and Lamin B. (B) Box plot of data from the
Bild_CellLine microarray dataset of primary human mammary epithelial cells transfected with
the vectors expressing GFP, E2F3, β-catenin (β-cat), c-Myc, c-Src or H-Ras (reporter number
228964_at) was accessed for PRDM1 mRNA levels using the ONCOMINE™ Cancer Profiling
Database (www.oncomine.org) and is plotted on a log scale (2). A Student’s $t$-test, performed
directly through the Oncomine 3.0 software, showed the difference in PRDM1 mRNA levels
between the means of the H-Ras vs all others groups combined was significant (*$P = 1.7e^{-10}$). (C-
D) Stable mixed populations of MCF-7 cells expressing Bcl-2 or pBabe DNA were transiently
transfected with 6 µg of vectors expressing Ras WT or Ras C186S, as indicated. (C) After 48 h,
WCEs were prepared and analyzed by immunoblotting for E-cadherin (E-Cadh), ERα, Bcl-2 and
β-actin. (D) Alternatively, cells were subjected to migration assays. ANOVA shows the
difference between MCF-7/pBABE + EV vs MCF-7/Bcl-2 + Ras C186S was not statistically
significant [$P = 0.84$], whereas the differences between MCF-7/pBABE + EV vs MCF-7/Bcl-2
+EV or MCF-7/Bcl-2 + Ras WT were significant, as indicated. (E) Summary scheme. Bcl-2,
induced by RelB/p52 as shown previously (56), interacts with and activates Ras, apparently in
the mitochondrial membrane. In turn, Ras induces expression of Blimp1, which represses ERα.
Fig. 1
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**Fig. 4**
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Fig. 5
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