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► **To cite this version:**

Xiaobo Wang, Karine Belguise, Christine F. O'Neill, Nuria Sánchez-Morgan, Mathilde Romagnoli, et al.. RelB NF-kappaB represses estrogen receptor alpha expression via induction of the zinc finger protein Blimp1.. *Molecular and Cellular Biology*, 2009, 29 (14), pp.3832-44. 10.1128/MCB.00032-09 . inserm-00396241

HAL Id: inserm-00396241

<https://inserm.hal.science/inserm-00396241v1>

Submitted on 17 Jun 2009

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1 **RelB NF- κ B Represses Estrogen Receptor α Expression via Induction of the Zinc Finger**
2 **Protein Blimp1**

3

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23

1 Short title: RelB represses ER α transcription by inducing Blimp1

2

3 Word counts:

4 Materials and Methods: 1,592

5 Introduction, Results, Discussion: 3,878

6

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

17

18

19 NF- κ B is a structurally and evolutionary conserved family of dimeric transcription factors with
20 subunits having an N-terminal region of approximately 300 amino acids that shares homology
21 with the v-Rel oncoprotein (17, 44). The conserved Rel homology domain (RHD) is responsible
22 for DNA binding, dimerization, nuclear translocation, and interaction with inhibitory proteins of
23 NF- κ B (I κ Bs). Mammals express five NF- κ B members, including c-Rel, RelB, RelA (p65), p50

1 and p52, which can form either homo-dimers or hetero-dimers. RelB differs from the other
2 members in that it only binds DNA as a hetero-dimer with either p52 or p50, and interacts only
3 poorly with the inhibitory protein I κ B α . In most untransformed cells, other than B lymphocytes,
4 NF- κ B complexes are sequestered in the cytoplasm bound to specific I κ B proteins.

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

20 While studying the mechanism of the inverse correlation between RelB and ER α levels
21 in breast cancer, more recently we observed that RelB complexes robustly inhibited *ER α* (*ESR1*)
22 gene expression. Since RelB is not by itself inhibitory, we postulated that it controls the
23 expression of an intermediate negative regulatory factor that represses *ER α* gene transcription.

1 TransFac analysis identified putative binding sites for the B lymphocyte-induced maturation
2 protein (Blimp1), which is expressed from the *PRDMI* gene (24) and has been reported to be
3 regulated by NF- κ B, although it is not known whether this control is exerted directly or
4 indirectly (22). The Zn finger protein Blimp1 is a repressor of transcription and has been shown
5 to function as a master regulator of development of antibody secreting B lymphocytes and more
6 recently of T cell homeostasis and function (6, 28), and specification of primordial germ cells
7 (33, 54). Blimp1 was originally identified as a silencer of *IFN- β* gene transcription (24).
8 Although the exact mechanism by which repression occurs is not fully understood, Blimp1
9 possesses DNA-binding activity and can recruit histone deacetylases, histone methyltransferases
10 and the co-repressor Groucho (19, 39, 63). Here, we demonstrate for the first time that Blimp1
11 functions as a potent repressor of ER α synthesis in breast cancer cells and is induced by
12 activation of a Bcl-2/Ras pathway by RelB.

13

14 MATERIALS AND METHODS

15 **Cell culture and treatment conditions.** ER α positive MCF-7, T47D, ZR-75 and BT474
16 cells, and ER α negative Hs578T and MDA-MB-231 cells were purchased from the American
17 Type Culture Collection (ATCC, Manassas, VA) and maintained in standard culturing medium
18 as recommended by ATCC. The NF639 cell line, kindly provided by Philip Leder (Harvard
19 Medical School, Boston MA) was derived from a mammary gland tumor in a mammary tumor
20 virus (MMTV)-*ERBB2* transgenic mouse and cultured as described (18). Hs578T cell lines
21 containing human *pRELB*-siRNA or control *pRELB* sense (si-Control) were established as
22 described (56) and grown in the presence of 1 μ g/ml puromycin (Sigma, St. Louis, MO). MCF-7
23 cell lines containing pBABE and pBABE-Bcl-2 were established by retroviral infection, as

1 described (56), and grown in the presence of 1 $\mu\text{g/ml}$ puromycin. Hs578T or NF639 cell lines
2 carrying pSM2c-sh-*BCL2* (V2HS_111806, Open Biosystems) or scrambled control were
3 established by retroviral infection, as above. MCF-7 cells containing inducible C4_{bsr}R(TO) or
4 C4_{bsr}R(TO-RelB) were established as described (56). Expression of RelB was induced with 1
5 $\mu\text{g/ml}$ doxycycline treatment for 48 h.

6

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

11 *KpnI*-proB -3489: 5'-CGCTCGGTACCTCCCATGCTCACCAAGCC-3'

12 *HindIII*-proB -1814: 5'-CGCTCAAGCTTTCAAGCCTACCCTGCTGG-3'

13 Following amplification, the product was digested with *KpnI* and *HindIII* and subcloned into
14 pGL3Basic; sequencing confirmed that no mutations were introduced during amplification. The
15 pC4_{bsr}R(TO-RelB) construct was generated by subcloning the *RelB* cDNA into the Eco RI
16 restriction site of the doxycycline inducible pC4_{bsr}R(TO) retroviral vector. The Ras C186S
17 mutant and the *ER α* expression vector were kindly provided by Mark R. Philips (NYU School of
18 Medicine, NY) and Pierre Chambon (Strasbourg, France), respectively. The Ras-EGFP-C1
19 vector was generously provided by Wen-Luan Wendy Hsiao (Hong Kong Baptist University)
20 The Ras CA (RasV12) vector was as previously described (20). The human full-length *PRDM1*
21 construct in pcDNA3 was as reported (39). The TBlimp vector expressing a dominant negative
22 (dn)Blimp1 protein, containing the DNA binding domain of Blimp1 in the Vxy-puro vector, and
23 the 7 kB human *PRDM1* promoter construct in pGL3 were kindly provided by Kathryn Calame

1 (Columbia University, NY) (1, 49). *pRELB* sense and *pRELB*-siRNA containing control or si-
2 *RELB* oligonucleotides in pSIREN-RetroQ vector were described previously (41). The human
3 full-length *BCL2* constructs in pcDNA3 or pBabe were as reported (42). The BCL-2 mutant
4 L14G, with the indicated point mutation, was kindly provided by Tristram G. Parslow (Emory
5 University School of Medicine, Atlanta GA). The pRc/CMV-Bcl-2, pRc/CMV-Bcl-acta and
6 pRc/CMV-Bcl-nt constructs, which were kindly provided by David W. Andrews (McMaster
7 University, Hamilton, Canada), were as previously described (64). Briefly, the Bcl-acta
8 (mitochondria-localized Bcl-2 mutant) and the Bcl-nt (cytoplasm-localized Bcl-2 mutant) were
9 established by exchanging the Bcl-2 carboxy-terminal insertion sequence for an equivalent
10 sequence from *Listeria ActA* or by deletion of the Bcl-2 carboxy-terminal insertion sequence,
11 respectively. The SV40 β -galactosidase (SV40- β -gal) reporter vector was as reported (57). The
12 estrogen response element (ERE)-TK luciferase construct was as reported (18). For transfection
13 into six well or P100 plates, 3 or 10 μ g total DNA was used, respectively. For transient
14 transfection, cultures were incubated for 48 h in the presence of DNA and Fugene6 (Roche
15 Diagnostics Co., Indianapolis, IN) or Geneporter2 (Gene Therapy System Inc., San Diego, CA)
16 Transfection Reagent. All transient transfection reporter assays were performed, in triplicate, a
17 minimum of three times. Luciferase assays were performed as described (40, 45). Co-
18 transfection of the SV40- β -gal expression vector was used to normalize for transfection
19 efficiency, as described (40). *PRDMI* siRNA duplex sequences have been previously described
20 (61). Duplexes (0.8 nM final) were introduced in cells using Lipofectamine 2000 (Invitrogen)
21 according to the manufacturer's protocol.

22

1 **Isolation of membrane and cytosolic fractions.** Membrane and cytosolic proteins were
2 extracted using Mem-PER Eukaryotic Membrane Protein Extraction Kit (PIERCE, 89826)
3 according to the manufacturer's protocol.

4
5 **Immunoblot analysis, antibodies, and immunoprecipitation.** Whole cells extracts (WCEs)
6 were prepared in RIPA buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 10 mM EDTA, 1% NP-40,
7 0.1% SDS, 1% sodium sarcosyl, 0.2 mM phenylmethylsulfonylfluoride, 10 µg/ml leupeptin, 1
8 mM dithiothreitol). Nuclear extracts were prepared as previously described (18). Immunoblotting
9 was performed as we have described (18). Antibodies against RelB (sc-226), Bcl-2 (sc-492), Ras
10 (sc-35), or Lamin B (sc-6217), were purchased from Santa Cruz Biotechnology (Santa Cruz,
11 CA). Antibody against ER α was purchased from NeoMarker (Fremont, CA). Antibodies against
12 E-cadherin, γ -catenin, and Ras (610001) used in immunoprecipitation analyses were purchased
13 from BD Transduction Lab (Franklin Lakes, NJ), and for β -actin (AC-15) from Sigma.
14 Antibodies against mouse Blimp1 (ab13700) and human Blimp1 (NB600-235) were purchased
15 from Novus Biologicals and Abcam, respectively. For immunoprecipitation, WCEs were
16 prepared in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EGTA, 1% NP-40, 0.5%
17 deoxycholate, 0.1% SDS, 10mM NaF, 4mM Na₃VO₄, 0.5 mM phenylmethylsulfonylfluoride, 10
18 µg/ml leupeptin, 10mM β -glycerophosphate) and precleared WCEs (500 µg) were incubated
19 overnight at 4°C with antibodies against Bcl-2 or Ras or the corresponding normal IgG in NETN
20 buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 0.5% NP-40, 1 mM EDTA, plus protease and
21 phosphatase inhibitors). Immunoprecipitates were collected using protein A-Sepharose beads,
22 washed four times in NETN buffer and then subjected to immunoblotting.

23

1 **Electrophoretic Mobility Shift Assay (EMSA).** Nuclear extracts were prepared and samples
2 (5 µg) subjected to binding and EMSA as described (45). The sequences of the oligonucleotide
3 used are as follows:

4 *c-MYC* Blimp1 site, 5'-ACAGAAAGGGAAAGGACTAGCGGATC-3'

5 Puta-1 Blimp1 site, 5'-GATCCGGAAAGGAAAGGGGATCTG-3'

6 Puta-2 Blimp1 site, 5'-GATCCAGTTGAGAAAGTGGTCAAG-3'

7 Puta-3 Blimp1 site, 5'-GATCCGCCAGAGAAAGCTGTACTG-3'

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

12

13 **Chromatin Immunoprecipitation Assay (ChIP).** Cells were crosslinked with 1%
14 formaldehyde at room temperature for 10 min and the reaction stopped by addition of glycine to
15 a final concentration of 125 mM. After three washes in cold PBS, cell pellets were resuspended
16 in ChIP lysis buffer (50 mM Hepes pH 7.5, 140 mM NaCl, 1% Triton X-100 plus protease
17 inhibitor cocktail) and incubated 30 min on ice. Following sonication and centrifugation, the
18 supernatants were incubated with 2 µg salmon sperm DNA, 150 µg BSA and 50 µl protein G-
19 Sepharose for 2h at 4°C and centrifuged to pre-clear. The resulting supernatants were
20 immunoprecipitated overnight at 4°C with 2 µg Blimp1 antibody (ab13700) plus 50 µl protein
21 G-Sepharose and 2 µg salmon sperm DNA. Immunoprecipitates were washed sequentially for 5
22 min each time at 4°C with ChIP lysis buffer, ChIP lysis high salt buffer (50 mM Hepes pH 7.5,
23 500 mM NaCl, 1% Triton X-100), ChIP wash buffer (10 mM Tris pH 8, 250 mM LiCl, 0.5%

1 NP40, 1 mM EDTA) and twice with TE buffer. Immunoprecipitates were then eluted twice with
2 elution buffer (50 mM Tris pH 8, 1% SDS, 10 mM EDTA) at 65°C for 10 min and both input
3 and pooled eluates were incubated overnight at 65°C to reverse the crosslinking. DNA was
4 purified with QIAquick PCR purification kit and 1 µl from 50 µl DNA extraction was used for
5 PCR. (Information on PCR primers and conditions are presented in Supplementary Information).

6

7 **Reverse Transcriptase (RT)-PCR analysis.** RNA, isolated using Trizol reagent
8 (Invitrogen), was quantified by measuring A_{260} . RNA samples, with $A_{260}:A_{280}$ ratios between 1.8
9 and 2.0, were treated with RQ1 RNase-free DNase (Promega Corporation, Madison WI). For
10 RT-PCR, 1 µg RNA was reverse transcribed with Superscript RNase H-RT in the presence of
11 100 ng of random primers (Invitrogen). PCR for different targets was performed in a Thermal
12 Cycler. (Information on PCR primers and conditions are presented in Supplementary
13 Information).

14

15 **Migration assays.** Suspensions of 1 to 2.5×10^5 cells were layered, in triplicate, in the upper
16 compartments of a Transwell (Costar, Cambridge, MA) on an 8-mm diameter polycarbonate
17 filter (8 µm pore size), and incubated at 37°C for the indicated times. All migration assays were
18 performed three independent times, each in triplicate. Migration of the cells to the lower side of
19 the filter was evaluated with the acid phosphatase enzymatic assay using p-nitrophenyl
20 phosphate and OD_{410nm} determination. Assays were performed three independent times, each in
21 triplicate, and the mean \pm SD are presented.

22

1 **Confocal Laser Scanning Microscopy.** ZR 75 cells were transfected with 1 μ g Ras-GFP
2 expression plasmid or pEGFP-C1 EV DNA using Fugene. At 24 h, cultures were incubated for
3 15 min in 100 nM Mitotracker (Molecular Probes), washed with PBS, and incubated in fixative
4 solution [2% Electron Microscopy (EM)-grade paraformaldehyde (EMS), PBS, 5 mM $MgCl_2$].
5 Cells were blocked in PBS/2% bovine serum albumin for 30 min, and incubated in blocking
6 buffer containing Bcl-2 antibody (Santa Cruz; sc-492) for 1 h at 37°C. Cells were subsequently
7 washed in PBS and incubated in PBS/BSA containing an Alexa 647 secondary antibody
8 (Invitrogen; A2143) for 1 h, then washed in PBS and mounted in anti-fade medium (Invitrogen)
9 containing DAPI to stain nuclei. Fluorescent signals were visualized using a Zeiss LSM 510
10 200M Confocal Microscope (Thornwood, NY) using a x63 objective. Z-sections were taken at
11 an optical slice of 2 μ m to determine the localization of mitotracker (red), Bcl-2 (purple) and
12 Ras-GFP (green). Confocal settings were optimized to control for signal cross-over. Detector
13 gain and amplitude offset were set to maximize the linear range without saturation and were kept
14 consistent throughout experiments. Images were stacked and composites generated and an
15 orthogonal slice analysis was performed on the composite using LSM 510 Image Software.

16

17 **RESULTS**

18 **RelB represses synthesis of ER α .** The inverse relationship between NF- κ B and ER α that
19 typifies many breast cancer cells and tissues has previously been related to the inhibition of NF-
20 κ B synthesis or activity by ER α signaling (23, 51, 56). Unexpectedly, we observed the
21 reciprocal repression of ER α activity by RelB in cultures of stable clones of ER α positive MCF-
22 7 cells that ectopically express elevated RelB levels, as described previously (56). Specifically,
23 MCF-7 clones RELB(1) and RELB(2) showed marked reduction of estrogen response element

1 (ERE) driven reporter activity compared to control MCF-7 EV(1) and EV(2) clones, with
2 parental pcDNA3 empty vector (EV) DNA (Fig. 1A). To verify that the observed effects of RelB
3 on ERE activity were not due to clonal selection, transient transfection analysis was performed
4 using ER α positive ZR-75 cells with vectors expressing either RelB or binding partners p50 or
5 p52 alone, or in combination. RelB expression resulted in a substantial decrease in ERE driven
6 reporter activity, which was further decreased upon co-expression of p50 or p52 (Fig. 1B).
7 (Similar data were obtained using transient transfection of MCF-7 cells, not shown). To verify
8 the effects of RelB on endogenous expression of ER α target genes, RNA was isolated from
9 MCF-7/RELB(1) and MCF-7/EV(1) cells. RT-PCR analysis confirmed decreased RNA levels of
10 target genes *CATHEPSIN D*, *Retinoic Acid Receptor (RAR) α* , *pS2*, and *MTA3* in MCF-
11 7/RELB(1) compared to MCF-7/EV(1) cells (Fig. 1C). Similarly, endogenous mRNA levels of
12 *CATHEPSIN D* and *RAR α* were decreased in ZR-75 and MCF-7 cells upon transient transfection
13 of RelB/p50 or RelB/p52 (Fig. 1D, upper and lower panels). To determine whether RelB
14 decreases ER α levels, transient transfection analysis of vectors expressing RelB in the presence
15 of either p50 or p52 was performed in ZR-75 and MCF-7 cells. Decreased endogenous levels of
16 ER α protein (Fig. 1E) and *ER α* mRNA (Fig. 1F) were seen with both RelB complexes. (All
17 protein and RT-PCR samples in Fig. 1E and Fig. 1F, respectively were from the same gels.) ER α
18 has two major promoters, A and B, of which the latter is predominantly used in breast cancer
19 cells. Co-transfection of RelB and p52 expression vectors with the *ER α* promoter B reporter
20 decreases its activity approximately 5-fold in MCF-7 and ZR-75 cells (Fig. 1G). Lastly, we
21 monitored the effects of RelB on endogenous ER α levels and ER α promoter B reporter activity
22 in the stable MCF-7/RELB(1) and RELB(2) clones and the control MCF-7/EV(1) and EV(2)

1 clones. RelB expression reduced levels of ER α protein (Fig. 1H) and RNA (Fig. 1I) as well as
2 promoter activity (Fig. 1J). Thus, RelB complexes robustly inhibit ER α expression and activity.

3 **RelB induces expression of the zinc finger repressor protein Blimp1.** Since RelB has a
4 transactivation domain and has not been shown to function itself directly as an inhibitor of
5 transcription, we tested the hypothesis that it controls the expression of an intermediate negative
6 regulatory factor that represses ER α . TransFac analysis (at 80% certainty) of promoter B
7 sequences identified 3 putative binding sites for Blimp1, a master regulator of B and T cell
8 development. The Zn finger protein Blimp1, which functions as a repressor of key regulatory
9 genes in these cells, is expressed from the gene termed *PRDMI* (human) or *Blimp1* (mouse) (24).
10 Since expression of Blimp1 has not been reported in epithelial cells, we first tested for
11 endogenous Blimp1 protein in nuclear extracts of human and mouse breast cancer cells.
12 Substantial Blimp1 levels were detectable in ER α negative Hs578T human breast cancer cells,
13 and more moderate levels in ER α negative MDA-MB-231 cells (Fig. 2A). Very low, but
14 detectable Blimp1 levels were seen with ER α positive ZR-75, MCF-7, T47D, and BT474 human
15 breast cancer cell lines (better seen on darker exposures and see Fig. 2F below). The murine
16 NF639 Her-2/neu-driven ER α low breast cancer cell line displayed a moderate level of Blimp1
17 protein. RT-PCR confirmed the presence of *PRDMI* mRNA in human breast cancer cells and
18 demonstrated that the levels of expression were much higher in ER α negative vs positive lines
19 (Fig. 2B). To test whether the inverse correlation between Blimp1 and ER α predicted by these
20 findings exists in primary human breast cancers, microarray gene expression datasets publicly
21 available at Oncomine.org were analyzed. The levels of *PRDMI* mRNA were significantly
22 higher in ER α negative vs positive breast cancers (Fig. 2C, left panel) in the Van de
23 Vijver_Breast carcinoma microarray dataset (reporter number NM_001198) (50) ($P = 1.3e^{-6}$ by

1 Student's *t*-test), consistent with the analysis of the cell lines. Data from an additional microarray
2 study (by Bittner: <https://expo.intgen.org/expo/public/2005/01/15>) publicly available at:
3 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2109>) confirmed these findings ($P =$
4 $7.3e^{-7}$ by Student's *t*-test) (Fig. 2C, right panel). Thus, *PRDM1* gene expression occurs in patient
5 samples and breast cancer cell lines, with higher levels in ER α negative cells, consistent with the
6 pattern of RelB expression (56).

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

16 Next we sought to test whether the *ER* α promoter is indeed repressed by ectopic Blimp1
17 expression and to localize the functional Blimp1 binding element(s). An ~4-fold reduction in
18 activity of an *ER* α proB construct (Fig. 2J), which contains all three putative Blimp1 binding
19 sites, was seen with ectopic Blimp1 expression in co-transfection analysis in MCF-7 cells
20 compared to EV DNA. The three Blimp1 sites all contain the core GAAA sequence, but differ in
21 surrounding sequencing (see Materials and Methods). To identify the functional sites,
22 competition and supershift EMSA were performed using nuclear extracts of ZR-75 cells
23 ectopically expressing Blimp1 protein or control EV DNA and an oligonucleotide containing the

1 Blimp1 binding site of the *c-MYC* gene as probe. Putative site 2, but not sites 1 or 3, competed
2 well for binding to the *c-MYC* Blimp1 site (Fig. 3A). When used as a probe, the putative site 2
3 oligonucleotide effectively bound Blimp1 protein (Fig. 3B), as judged by supershift EMSA (Fig.
4 3C), confirming this site is a *bona fide* Blimp1 element. The position of this confirmed Blimp1
5 site is indicated on the map in Fig. 3D. ChIP analysis confirmed intracellular binding of Blimp1
6 following ectopic expression to the *ERα* promoter B region containing the element in MCF-7
7 cells (Fig. 3E, upper panels). Furthermore, ChIP analysis in Hs578T cells, which expressed the
8 highest levels of Blimp1, confirmed binding of endogenous Blimp1 to this *ERα* promoter B
9 region (Fig. 3E, lower panels).

10 To assess the functional role of the low endogenous levels of Blimp1 in the *ERα* positive
11 lines, a dominant negative form of Blimp1, termed TBlimp or dnBlimp (1), which contains only
12 the DNA binding domain, was used. Ectopic expression of the dnBlimp caused an increase in
13 *ERα* protein (Fig. 3F), indicating that endogenous Blimp1 represses *ERα* in these lines.
14 Consistently, as seen in Figure 4A below, knockdown of Blimp1 levels using an siRNA strategy
15 in NF639 cells induced *ERα* protein levels. Conversely, ectopic Blimp1 expression substantially
16 reduced *ERα* expression in ZR-75 and MCF-7 cells (Fig. 3G), indicating it can repress the
17 endogenous *ERα* gene in both lines. Blimp1 expression also led to reduced *ERα* RNA levels in
18 ZR-75 cells, while inhibition of Blimp1 activity with the dnBlimp led to their induction (Fig.
19 3H). Of note, expression of the dnBlimp ablated the decrease in *ERα* expression induced by
20 ectopic RelB in MCF7 cells (Fig. 3I), further implicating Blimp1 in RelB-induced
21 downregulation of *ERα* gene expression. Thus, Blimp1 is expressed in breast cancer cells and
22 mediates repression of *ERα*.

1 **Blimp1 promotes a more migratory phenotype in breast cancer cells via inhibition of**
2 **ER α .** In primordial germ cells, the absence of Blimp1 led to defects in cell migration (33). Given
3 our previous findings showing that ER α negative breast cancer cells have a more migratory
4 phenotype than ER α positive ones, we next tested whether Blimp1 regulated migration and
5 reduced expression of epithelial markers, which are required for maintenance of a non-migratory
6 phenotype. First, an siRNA was used to reduce Blimp1 levels in NF639 cells, which are highly
7 invasive and display robust Blimp1 expression. Knockdown of Blimp1 in NF639 cells decreased
8 migration (Fig. 4A, left panel) and increased expression of E-cadherin and ER α (Fig. 4A, right
9 panel). Conversely, ectopic Blimp1 expression in ER α positive ZR-75 cells, which repressed
10 ER α levels (see Fig. 3G above), substantially increased migration (Fig. 4B, left panel), and
11 commensurately decreased levels of E-cadherin and γ -catenin (Fig. 4B, right panel). We next
12 assessed the role of ER α in the ability of Blimp1 to promote a more migratory phenotype.
13 Expression of ER α prevented the increased ability of ZR-75 cells to migrate resulting from
14 Blimp1 expression (3.1-fold vs 1.1-fold) (Fig. 4C, left panel) as well as the decrease in E-
15 cadherin and γ -catenin induced by Blimp1 (Fig. 4C, right panel). Lastly, dnBlimp expression
16 induced the levels of E-cadherin expression in ZR-75 and MCF-7 cells, and these increases were
17 prevented upon knockdown of ER α using *siER α* RNA (Fig. 4D). Together, these results
18 demonstrate Blimp1 induces a more migratory phenotype and implicate the inhibition of ER α in
19 this control.

20 **Bcl-2 induces Blimp1 via functional interaction with Ras.** Previously, we demonstrated
21 *BCL2* is a critical RelB target that mediates the migratory phenotype of breast cancer cells (56).
22 For example, knockdown of Bcl-2 in Hs578T cells impaired the ability of the cells to migrate,
23 whereas stable ectopic expression of Bcl-2 in MCF-7 cells (MCF-7/Bcl-2 vs MCF-7/pBABE

1 stable cells) was sufficient to promote a more migratory phenotype via decreasing E-cadherin
2 levels (56). Thus, we tested the hypothesis that Bcl-2 mediates the induction of Blimp1 by RelB.
3 Knockdown of Bcl-2 with the *Bcl2* shRNA in NF639 cells led to increased ER α levels (Fig 5A,
4 left panel), whereas Bcl-2 overexpression decreased ER α level in ZR-75 and MCF-7 cells (Fig
5 5A, right panel), indicating Bcl-2 can negatively regulate ER α expression. Consistently,
6 knockdown of Bcl-2 levels led to downregulation of Blimp1 protein and *Blimp1* RNA levels in
7 NF639 cells (Fig. 5B, upper panels), whereas ectopic Bcl-2 expression robustly induced their
8 levels in these cells (Fig. 5B, lower panels). Similarly Bcl-2 led to substantial increases in the
9 levels of *PRDM1* RNA (Fig. 5C, upper panels) and Blimp1 protein expression (Fig. 5C, lower
10 panels) in both ZR-75 and MCF-7 cells, while ectopic *BCL2* shRNA, which inhibited RelB/p52-
11 mediated induction of Bcl-2 (56), prevented the induction of Blimp1 by RelB/p52 in ZR-75 cells
12 as well as the reduction of ER α levels (data not shown). Lastly, the dnBlimp1 robustly impaired
13 the previously observed ability of Bcl-2 to enhance migration of MCF-7 cells in MCF-7/Bcl-2
14 cells (Fig 5D).

15 WT Bcl-2, which has been shown to interact with and activate Ras in the mitochondria of
16 cells, leads to the induction of NF- κ B and AP-1, whereas a Bcl-2 14G mutant unable to associate
17 with Ras is functionally inactive (7, 15, 35). Notably, Ohkubo and coworkers identified an AP-1
18 element upstream of the *Blimp1* promoter (34) leading us to hypothesize a role for Bcl-2/Ras
19 interaction in the activation of Blimp1. As a first test, we compared the effects of the wild type
20 and Bcl-2 14G mutant on the *PRDM1* promoter reporter in ZR-75 cells. Transfection of a vector
21 expressing WT Bcl-2 resulted in a substantial induction of the *PRDM1* promoter activity in ZR-
22 75 cells (mean of 3.5 ± 1.7 -fold, $P = 0.05$), whereas the Bcl-2 14G mutant led to a reduction in
23 activity of approximately 5-fold [mean of 0.2 ± 0.06 -fold, $P = 0.002$]. To assess for association

1 of Bcl-2 and Ras in breast cancer cell lines, WCEs were prepared from ZR-75 cells transfected
2 with vectors expressing H-Ras and Bcl-2. Immunoprecipitation of Bcl-2 led to co-precipitation
3 of Ras, whereas, control rabbit IgG protein did not (Fig. 6A, left panel). Furthermore, co-
4 immunoprecipitation analysis of endogenous proteins in NF639 cell extracts similarly detected
5 association between endogenous Ras and Bcl-2 (Fig. 6A, right panels). Confocal microscopy
6 confirmed the partial association of transfected Ras-GFP with Bcl-2 in the mitochondria of ZR-
7 75 cells (Fig. 6B, and see projection images in Supplementary Information, Fig. S1).

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

21 To test whether Ras signaling is sufficient to induce Blimp1 expression, constitutively
22 active Ras was ectopically expressed in ZR-75 and MCF-7 cells; potent upregulation of Blimp1
23 was observed (Fig. 7A). We next assessed data publicly available on microarray gene expression

1 profiling of primary human mammary epithelial cells following transformation with various
2 genes. Consistent with the data presented above, a significant induction of *PRDMI* mRNA
3 expression was detected upon expression of activated H-Ras vs GFP ($P = 1.0e^{-7}$), but not with
4 E2F3, activated β -catenin, c-Myc or c-Src (2) (Fig. 7B). A significant difference was also
5 observed in *PRDMI* mRNA levels between the means of the H-Ras group vs all others combined
6 ($P = 1.7e^{-10}$). Lastly, we sought to elucidate the role of Ras on cell migration induced by Bcl-2 in
7 MCF-7 cells using the dominant negative function of the Ras C186S mutant. Ras C186S mutant,
8 but not Ras WT, inhibited the Bcl-2-mediated decrease in E-cadherin and ER α levels (Fig. 7C)
9 and the ability of MCF-7 cells to migrate (Fig. 7D), consistent with the effects seen above in ZR-
10 75 cells (Figs. 6D-F). Together these data strongly argue that Bcl-2 association with Ras, likely
11 in the mitochondrial membrane, leads to the induction of the levels of Blimp1 and to a more
12 migratory phenotype (see scheme in Fig. 7E).

13

14

DISCUSSION

15 Our findings identify a novel mechanism whereby the RelB NF- κ B family member acts to
16 repress ER α gene expression via induction of the zinc finger protein Blimp1. Previously, we
17 demonstrated RelB induces transcription of the *BCL2* gene via interaction with C/EBP binding to
18 an upstream CREB site (56) (Figure 7E). Here, we show that the association of Bcl-2 with Ras,
19 activates this proto-oncogene and leads to expression of the Zn finger repressor Blimp1. In turn,
20 Blimp1 binds to an element upstream of *ER α* promoter B, reducing promoter activity and ER α
21 levels, and thereby causing a reduction in the levels of E-cadherin and γ -catenin and a
22 commensurate increase in migratory phenotype in breast cancer cells (see scheme in Figure 7E).
23 To our knowledge, this is the first study identifying a crucial role for Blimp1 in repression of

1 *ERα* transcription and also in increased migration of cancer cells. Indeed, Blimp1 is a well-
2 known zinc finger transcriptional repressor that is critical for terminal differentiation of B cells
3 into immunoglobulin-secreting plasma cells and functions by attenuating proliferation while
4 promoting the differentiated B-cell gene expression program (10). More recently, Blimp1 has
5 been implicated in T cell homeostasis (16), where it similarly represses expression of genes
6 promoting proliferation, e.g., IL-2 and Bcl-6 while enhancing IL-10 production. Interestingly,
7 studies have also indicated that Blimp1 is a critical determinant of primordial germ cells
8 differentiation (33). The absence of Blimp1 led to the formation of primordial germ cells
9 clusters, and to defects in primordial germ cells cell migration, as well as to apoptosis (33). Our
10 findings identify one mechanism for the effects of Blimp1 on migration of breast cancer cells
11 related, in part, to the downregulation of *ERα* gene expression. It remains to be determined
12 whether similar regulation of *ERα* by Blimp1 occurs in B cells and plays a role in autoimmune
13 disease. Overall, our data identify Blimp1 as a potent negative regulator of *ERα* expression and
14 thereby as an activator of migration of breast cancer cells.

15 RelB has been reported to activate gene transcription via several different mechanisms. RelB has
16 been shown to induce transcription of the *c-Myb* oncogene via releasing pausing that occurs
17 during elongation (47). The activation of the *Bcl*, *Elc*, *Sdf-1*, and *Slc* promoters by RelB required
18 IKKα (4). Interestingly activation of the *dapk1*, *dapk3*, *c-flip* and *birc3* promoters by RelB can
19 be prevented by Daxx, which interacts selectively with RelB but not with RelA or c-Rel (36). In
20 addition to the *BCL2* gene (56), RelB induces transcription of several other pro-survival genes,
21 including *MNSOD* in prostate cancer (60) and *MNSOD* and *SURVIVIN* in breast cancer (29).
22 However, RelB was unable to bind to the NF-κB elements upstream of the *Bcl-xl* promoter and
23 led to decreased *Bcl-xl* gene expression, in contrast to the potent activation seen upon co-

1 expression of either c-Rel or RelA (21). In fibroblasts, RelB has also been reported to suppress
2 expression of several genes, including IL-1 α , IL-1 β and TNF α , indirectly via modulating I κ B α
3 stability (59), and to inactivate p65 via the formation of dimeric complex (27). Here, we report
4 that RelB can repress expression of the *ER α* promoter via activation of the zinc finger repressor
5 protein Blimp1. Consistent with this finding, *ER α* and RelB levels display an inverse pattern of
6 expression in many breast cancer cells and tissues, including inflammatory breast cancers (23, 30,
7 52, 56).

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

18 Activation of Ras via interaction with Bcl-2 has been previously reported in the context
19 of Ras-mediated apoptosis of T cells (15, 38) and of adipocytes (31), although, the findings were
20 somewhat contradictory. Ras-Bcl-2 interaction in T cells blocked Ras-induced apoptosis,
21 whereas in adipocytes it led to the induction of apoptosis via inactivation of Bcl-2 pro-survival
22 function. Bcl-2 has also been shown to interact with Raf, although the resulting effects differed
23 depending upon the cellular conditions. In murine myeloid progenitor cells, Bcl-2 targeted Raf to

1 mitochondrial membranes allowing this kinase to protect against apoptosis by phosphorylating
2 BAD (55), whereas Taxol-induced activation of Raf was accompanied by the loss of Bcl-2 anti-
3 apoptotic function in MCF-7 cells (3). Of note, we observed that a constitutively active Raf
4 could similarly induce Blimp1 expression, implicating this Ras-induced pathway in signaling
5 events (not shown). Interestingly, El-Ashry and coworkers showed that inhibition of Ras or Raf
6 activities led to re-expression of ER α in MCF-7 cells (32). Our findings that Blimp1 mediates
7 repression of ER α can explain these observations. Overall, our study indicates that RelB-
8 mediated induction of Bcl-2 leads to the repression of *ER α* transcription and thus to a more
9 migratory phenotype of breast cancer cells by activating Ras.

10

11

ACKNOWLEDGEMENTS

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

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

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Figure Legends

1
2 FIG. 1 RelB represses ER α synthesis in breast cancer cells. (A) Stable MCF-7 clones
3 expressing either RELB or EV DNA, isolated as described previously (56) and termed RELB(1),
4 RELB(2), EV(1), and EV(2), were transfected with 0.5 μ g ERE-TK luciferase reporter, 0.5 μ g
5 SV40- β -gal. Normalized ERE activity, set to 100% in the stable EV cells, was decreased in both
6 RelB-expressing lines (mean \pm S.D. from three separate experiments). (B) ZR-75 cells were
7 transiently transfected with 0.5 μ g ERE-TK Luciferase DNA plus 0.5 μ g of the indicated NF- κ B
8 subunit expression vectors, 0.5 μ g SV40- β -gal and EV pcDNA3 DNA to a 3.0 μ g DNA total.
9 Normalized ERE activity, set to 100% in the EV-transfected cells, was reduced by RelB alone or
10 in combination with p50 or p52 (mean \pm S.D. from three separate experiments). (C) RNA,
11 isolated from EV(1) and RELB(1) stable MCF-7 clones, was subjected to RT-PCR analysis for
12 expression of ER α target genes *CATHEPSIN D (CATHD)*, *RAR α* , *pS2*, *MTA3*, and *GAPDH*, as
13 loading control. (D) ZR-75 or MCF-7 cells were transiently transfected with 3 μ g each of vectors
14 expressing RelB and either p50 or p52 or 6.0 μ g of EV DNA. Isolated RNA was subjected to
15 RT-PCR analysis for *RAR α* , *CATHEPSIN D (CATHD)*, and *GAPDH*. (E-F) ZR-75 or MCF-7
16 cells were transiently transfected with 3.0 μ g of vectors expressing RelB, and p50 or p52 or EV
17 DNA, and WCEs and RNA isolated, which were subjected to immunoblot analysis for ER α and
18 β -actin (E), and RT-PCR for *ER α* and *GAPDH* (F), respectively. (Samples in E and F were run
19 on the same gels and the lanes brought into contiguous positions.) (G) ZR-75 and MCF-7 cells
20 were transiently transfected with 0.5 μ g of ER α proB luciferase reporter plus 0.25 μ g of vectors
21 expressing RelB and p52 or 0.5 μ g EV pcDNA3 DNA, 0.5 μ g SV40- β -gal and EV DNA to a 3.0
22 μ g DNA total. Normalized proB activity values are presented as the mean \pm S.D. from three

1 experiments (control EV DNA set to 1). (H-I). Protein and RNA, isolated from RELB(1),
2 RELB(2), EV(1), and EV(2) stable MCF-7 clones, were subjected to immunoblotting for
3 expression of ER α , RelB and β -actin (H), and to RT-PCR analysis for expression of ER α and
4 *GAPDH* (I), respectively. (J) RELB(1), RELB(2), EV(1), and EV(2) MCF7 clones were
5 transiently transfected with 0.5 μ g of ER α proB luciferase reporter, 0.5 μ g SV40- β -gal and EV
6 DNA to a 3.0 μ g DNA total. Normalized proB activity values are presented as the mean \pm S.D.
7 from three experiments (control EV DNA set to 1).

8
9 FIG. 2 RelB induces Blimp1 in breast cancer cells. (A) Nuclear extracts were isolated from
10 the indicated human and mouse breast cancer cell lines and subjected to immunoblotting for
11 Blimp1 and β -actin, which confirmed equal loading. (B) RNA, isolated from the indicated
12 human breast cancer lines, was subjected to RT-PCR for *PRDMI* for either 28 or 30 cycles (as
13 indicated) and for *GAPDH* levels. (C) (left panel) Box plot of data from the Van de
14 Vijver_Breast carcinoma microarray dataset (reporter number NM_001198) (50) was accessed
15 using the ONCOMINETM Cancer Profiling Database (www.oncomine.org) and is plotted on a
16 log scale. The dataset includes 69 ER α negative and 226 ER α positive human primary breast
17 carcinoma samples. A Student's *t*-test, performed directly through the Oncomine 3.0 software,
18 showed the difference in *PRDMI* expression between the two groups was significant ($P = 1.3e^{-6}$).
19 (right panel) Box plot of data from the Bittner study: [https://expo.intgen.org/expo/public/
20 2005/01/15](https://expo.intgen.org/expo/public/2005/01/15)) publicly available at:
21 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2109>) showed the difference in
22 *PRDMI* expression between the two groups was significant ($P = 7.3e^{-7}$). (D) RNA was isolated
23 from stable Hs578T transfectants expressing either *RELB* siRNA (Hs578T *RELB* siRNA) or

1 sense *RELB* (Hs578T Control siRNA) (Con) and analyzed for *PRDMI* levels. (E) Nuclear
2 proteins were isolated from Hs578T *RELB* siRNA and Hs578T Control siRNA (Con) cells and
3 analyzed for Blimp1, Lamin B and RelB levels by immunoblotting. (F) ZR-75, MCF-7 and
4 NF639 cells were transiently transfected with 3 μ g each of RelB and p52 expression vectors or 6
5 μ g of EV DNA and RNA subjected to RT-PCR for human *PRDMI* or mouse *Blimp1* RNA
6 levels. (G) ZR-75, MCF-7 and NF639 cells were transiently transfected with 3 μ g each of RelB
7 and p52 expression vectors or 6 μ g of EV DNA. Nuclear extracts (ZR-75, MCF-7; left panels)
8 and WCEs (NF639, right panels) were analyzed by immunoblotting for Blimp1 and either Lamin
9 B or β -actin, respectively. (H-I). Nuclear extracts and RNA, isolated from RELB(1), RELB(2),
10 EV(1), and EV(2) stable MCF-7 clones, were subjected to immunoblotting for expression of
11 Blimp1 and Lamin B (H), and to RT-PCR analysis for expression of *PRDMI* and *GAPDH* (I),
12 respectively. (J) MCF-7 cells were transiently transfected with 0.5 μ g of ER α proB luciferase
13 reporter, 0.5 μ g SV40- β -gal and EV DNA to a 3.0 μ g DNA total. Normalized proB activity
14 values are presented as the mean \pm S.D. from three experiments (control EV DNA set to 1).

15
16 FIG. 3 Blimp1 represses *ER α* gene expression in breast cancer cells. (A) Nuclear extracts,
17 prepared from ZR-75 cells transfected with Blimp1 expression vector, were used in competition
18 EMSA with the Blimp1 element from the *c-MYC* gene as probe and 50x molar excess of
19 oligonucleotides containing the *c-MYC* or putative *ER α* promoter Blimp1 sites 1,2 or 3 (P1 –
20 P3). (B) Nuclear extracts, prepared from ZR-75 cells transfected with EV (-) or Blimp1
21 expression vector (+) were used in EMSA with the putative *ER α* gene P1, P2 and P3 Blimp1
22 sites, as probe. (C) Nuclear extracts, prepared from ZR-75 cells transfected with Blimp1
23 expression vector were used in supershift EMSA in the absence (-) or presence (+) of 200 ng of

1 Blimp1 antibody and the putative *ERα* gene Blimp1 P2 site, as probe. (D) *ERα* promoter A and
2 B regions with confirmed Blimp1 site indicated with the element sequence given below, where
3 core is underlined. (E) ChIP analyses were performed on MCF-7 cells transiently transfected
4 with Blimp1 (top panels) or Hs578T cells (bottom panels) using anti-Blimp1 antibody or the
5 corresponding normal IgG as indicated. The DNA, extracted from the immunoprecipitates or the
6 input, were amplified by PCR using pairs of primers specific to the confirmed Blimp1 site of
7 *ERα* promoter (Blimp1 site) (left panels) or an upstream region as negative control (right
8 panels). (F) ZR-75 and MCF-7 cells were transiently transfected with 5.0 μg Vxy-puro TBlimp
9 vector expressing the 35 kDa dnBlimp protein or EV DNA, and WCEs subjected to
10 immunoblotting for *ERα*, dnBlimp and β-actin. (G) ZR-75 and MCF-7 cells were transiently
11 transfected with 5.0 μg of Blimp1 expression vector or EV DNA, and WCEs subjected to
12 immunoblotting for *ERα*, Blimp1 and β-actin. Densitometry indicated the *ERα* levels in the ZR-
13 75 and MCF-7 cells were reduced to 28.0 ± 3.4 and 73.9 ± 5.0 , respectively upon ectopic Blimp1
14 expression compared to EV DNA set at 100] (H) ZR-75 cells were transiently transfected with
15 5.0 μg of vector expressing either Blimp1 or dnBlimp protein (left or right panels, respectively)
16 or the corresponding EV DNA, and RNA subjected to RT-PCR for *ERα* and *GAPDH*. (I) MCF-7
17 cells carrying an inducible C4_{bsr}R(TO-RelB) RelB construct, plated in the absence or presence of
18 1 μg/ml doxycycline, were transiently transfected with 6.0 μg dnBlimp expression vector. After
19 48 h, WCEs were analyzed for levels of *ERα*, RelB, dnBlimp and β-actin.

20

21 FIG. 4 Blimp1 induces a migratory phenotype in breast cancer cells via repression of *ERα*.
22 (A) NF639 cells were transiently transfected with control *siRNA* (si-con) or *siBlimp1* RNA. (left
23 panel) Cells were subjected, in triplicate, to a migration assay for 4 h. Cells that migrated to the

1 lower side of the filter were quantified by spectrometric determination at OD_{410nm}. Average
2 migration from three independent experiments ± S.D. is presented relative to si-cont (set at
3 100%) (**P* = 7.0e⁻⁵). (right panel) WCEs were subjected to immunoblotting for E-cadherin (E-
4 Cadh), ERα, Blimp1, and β-actin. (B) ZR-75 cells were transiently transfected with 6 μg of EV
5 or Blimp1 expression vector. (left panel) Transfect ZR-75 cells were subjected, in triplicate, to a
6 migration assay for 24 h, as in part (A). **P* = 0.0007. (right panel) WCEs were analyzed by
7 immunoblotting for Blimp1, E-cadherin, γ-catenin (γ-Caten) and β-actin. (C) ZR-75 cells were
8 transiently transfected with 6 μg of Blimp1 expression vector or EV DNA in the absence or
9 presence of ERα expression vector (1.5 μg) or EV DNA, as indicated. (left panel) After 24 h,
10 ZR-75 cells were subjected to migration assays for 24 h, as in part (A). The enhanced migration
11 induced by Blimp1 is significantly decreased by ERα. **P* = 0.0001. (right panel) After 48 h,
12 WCEs were prepared and analyzed by immunoblotting for E-cadherin, γ-catenin, ERα, Blimp1
13 and β-actin. (D) ZR75 and MCF7 cells were transiently transfected with dnBlimp (+) or EV
14 DNA (-). Twenty four h later, ERα validated StealthTM RNAi (+) or Stealth RNAi negative
15 control (-) were introduced in cells using Lipofectamine RNAiMAX reagent by reverse
16 transfection as previously described (56). After 24 h, WCEs were prepared and analyzed by
17 immunoblotting for E-cadherin (E-Cadh), ERα, and β-actin.

18
19 FIG. 5 Induction of Blimp1 is mediated via Bcl-2. (A) (left panels) NF639 cells were
20 transiently transfected with expression vectors for either control shRNA (contl shRNA) or *Bcl2*
21 shRNA and WCEs analyzed for ERα, Bcl-2 (which confirmed effective knockdown) and β-actin
22 protein levels. (right panels) ZR-75 and MCF-7 cells were transiently transfected with EV or full
23 length Bcl-2 expression vector. WCEs were analyzed for ERα and β-actin protein levels, and for

1 Bcl-2 (which confirmed the expected changes in levels, not shown). (B) NF639 cells were
2 transiently transfected with expression vectors for either control shRNA (contl shRNA) or *Bcl2*
3 shRNA (upper panels) or EV or full length Bcl-2 (bottom panels). (left panels) WCEs were
4 analyzed for Blimp1 and β -actin. (right panels) RNA was subjected to RT-PCR analysis for
5 levels of *Blimp1* and *Gapdh*. (C) RNA (top panels) and nuclear extracts (bottom panels) were
6 prepared from ZR-75 and MCF-7 cells transfected with control EV DNA or vector expressing
7 Bcl-2 and subjected to RT-PCR for *PRDM1* and *GAPDH* expression (upper panels) and Blimp1
8 and Lamin B levels (lower panels), respectively. (D) Stable mixed populations of MCF-7 cells
9 expressing Bcl-2 or pBabe (pB) DNA were transiently transfected with EV DNA (-) or vector
10 expressing dnBlimp (+). Cells were subjected, in triplicate, to a migration assay for 24 h, as in
11 Fig. 4A. The increase in migration induced by Bcl-2 is significantly reduced by dnBlimp, $P =$
12 $3.8e^{-7}$. Western blot analysis confirmed the expression of the dnBlimp (not shown).

13

14 FIG. 6 Bcl-2 interaction with Ras induces Blimp1. (A) WCEs (500 μ g), prepared from ZR-
15 75 cells transiently transfected with Bcl-2 and Ras expression vectors (left panels) or from
16 untransfected NF639 cells (right panels), were immunoprecipitated with rabbit anti-Bcl-2 or
17 mouse anti-Ras antibodies or the corresponding normal IgG as indicated. Immunoprecipitated
18 proteins were analysed by immunoblotting for Bcl-2 and Ras. WCEs (5 μ g) were used as input.
19 (B) ZR75 cells, grown on coverslips in 6-well dishes, were transfected with 1 μ g Ras-GFP
20 expression plasmid. Following staining with Mitotracker, cells were fixed, and subjected to
21 immunohistochemistry for Bcl-2 using an Alexa 647 labeled secondary antibody (purple 694).
22 Mitochondria are labeled in red with Mitotracker, Ras-GFP is green and nuclei are labeled with
23 DAPI stain (blue). A composite image was generated from the z-sections and an Orthoganol

1 slice analysis was performed on the composite where XZ is depicted on the horizontal and YZ on
2 the vertical axis outside the dimension of the composite. Co-localization of Ras and Bcl-2 to the
3 mitochondria is demonstrated by the overlap of signals yielding white spots. 3-D projection
4 images were made from the composites. The Inset shows a forty-five degree rotation of the
5 image of the cell, with arrows indicating areas of co-localization of Ras and Bcl-2 in the
6 mitochondria. (C) ZR-75 and MCF-7 cells were transiently transfected with EV or full length
7 Bcl-2 DNA. After 48 h cytosol and membrane fractions were prepared as described in Materials
8 and Methods and subjected to immunoblotting for Ras and β -actin. Immunoblotting for VDAC,
9 a mitochondrial ion channel membrane protein, confirmed effective separation of the membrane
10 from the cytoplasmic compartment (not shown). (D) ZR-75 cells were transiently transfected
11 with EV (-) or vectors expressing Ras WT or Ras C186S mutant protein. After 48 h, WCEs and
12 nuclear extracts were analyzed for levels of ER α and β -actin (upper panels) and Blimp1 and
13 Lamin B (lower panels). (E) ZR-75 cells were transiently transfected with EV or vector
14 expressing full length Bcl-2 in the absence (-) or presence (+) of a vector expressing Ras C186S
15 mutant protein. After 48 h, WCEs were analyzed for ER α protein levels. (F) ZR-75 cells were
16 transiently transfected in triplicate, with 0.5 μ g of the 7 kB human *PRDMI* promoter-Luc vector,
17 0.5 μ g of Bcl-2 with 0.5 μ g Ras WT (Ras) or 0.5 μ g Ras C186S (C186S) expression vector, 0.5
18 μ g SV40- β -gal and pcDNA3 (EV) to make a total of 3.0 μ g DNA. Luciferase and β -gal activities
19 were determined and normalized *PRDMI* promoter activity values are presented as the mean \pm
20 S.D. (control EV DNA set to 1). (G) ZR-75 cells were transiently transfected in triplicate, with
21 0.5 μ g of the 7 kB human *PRDMI* promoter-Luc vector, 0.5 μ g of Ras expression vector with
22 0.5 μ g of either EV DNA, WT Bcl-2, Bcl-acta (mitochondria-localized Bcl-2 mutant) or Bcl-nt
23 (cytoplasm-localized Bcl-2 mutant) expression vectors (64), 0.5 μ g SV40- β -gal and pRc/CMV

1 (EV) to make a total of 3.0 mg DNA. Forty-eight h after transfection, luciferase and β -gal
2 activities were determined, and normalized *PRDMI* promoter activity values are presented as the
3 mean \pm S.D. from three separate experiments (control EV DNA set to 1). Using a Mann Whitney
4 test, a $P < 0.05$ was seen between each condition, except for Ras + WT Bcl-2 and Ras + Bcl-acta.

5
6 FIG. 7 Induction of Blimp1 is mediated via Bcl-2 and Ras activation. (A) ZR-75 and MCF-7
7 cells were transiently transfected with EV or vector expressing constitutively active Ras (Ras
8 CA), and nuclear extracts analyzed for Blimp1 and Lamin B. (B) Box plot of data from the
9 Bild_CellLine microarray dataset of primary human mammary epithelial cells transfected with
10 the vectors expressing GFP, E2F3, β -catenin (β -cat), c-Myc, c-Src or H-Ras (reporter number
11 228964_at) was accessed for *PRDMI* mRNA levels using the ONCOMINETM Cancer Profiling
12 Database (www.oncomine.org) and is plotted on a log scale (2). A Student's *t*-test, performed
13 directly through the Oncomine 3.0 software, showed the difference in *PRDMI* mRNA levels
14 between the means of the H-Ras vs all others groups combined was significant ($*P = 1.7e^{-10}$). (C-
15 D) Stable mixed populations of MCF-7 cells expressing Bcl-2 or pBabe DNA were transiently
16 transfected with 6 μ g of vectors expressing Ras WT or Ras C186S, as indicated. (C) After 48 h,
17 WCEs were prepared and analyzed by immunoblotting for E-cadherin (E-Cadh), ER α , Bcl-2 and
18 β -actin. (D) Alternatively, cells were subjected to migration assays. ANOVA shows the
19 difference between MCF-7/pBABE + EV vs MCF-7/Bcl-2 + Ras C186S was not statistically
20 significant [$P = 0.84$], whereas the differences between MCF-7/pBABE + EV vs MCF-7/Bcl-2
21 +EV or MCF-7/Bcl-2 + Ras WT were significant, as indicated. (E) Summary scheme. Bcl-2,
22 induced by RelB/p52 as shown previously (56), interacts with and activates Ras, apparently in
23 the mitochondrial membrane. In turn, Ras induces expression of Blimp1, which represses ER α .

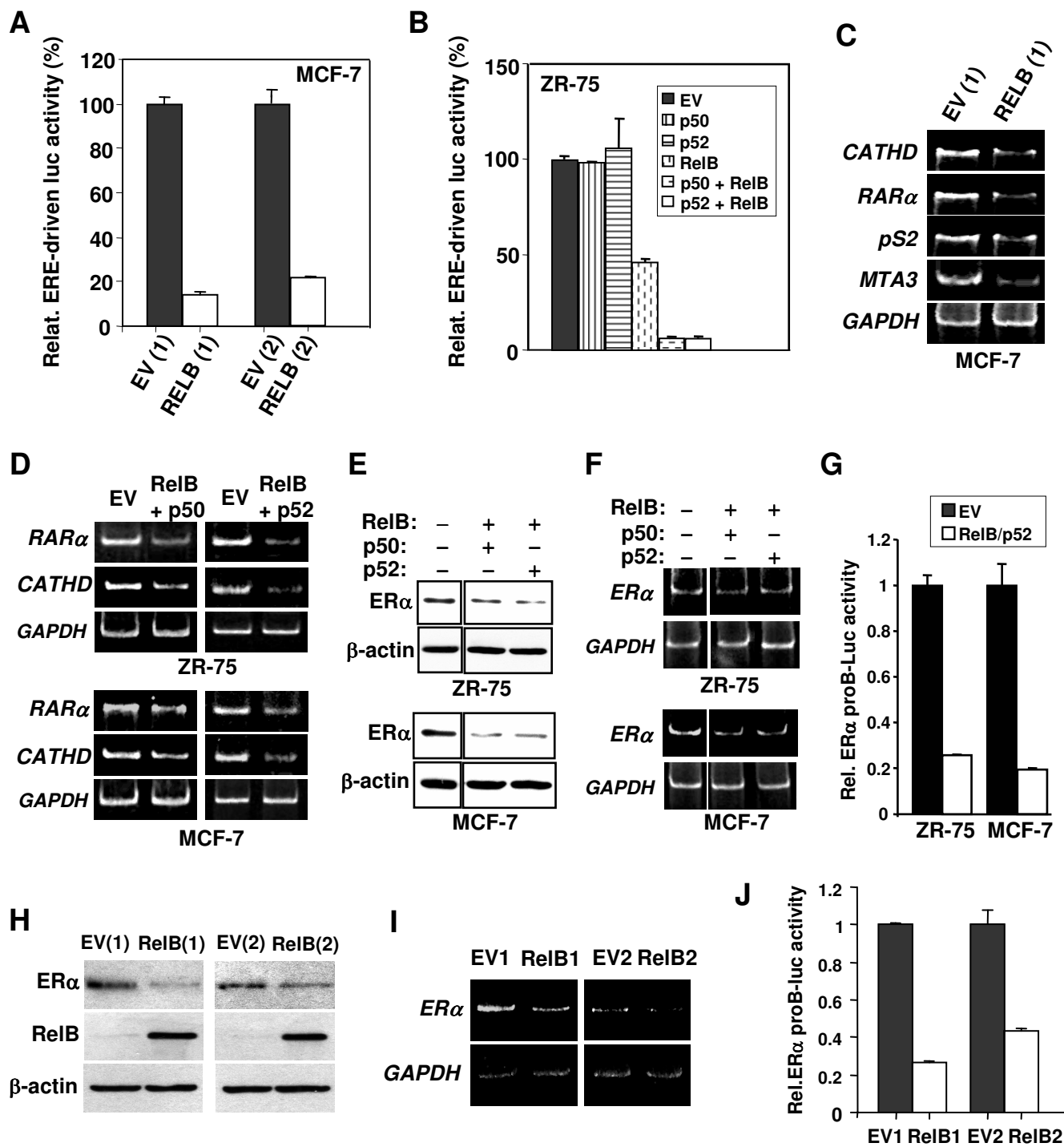


Fig. 1
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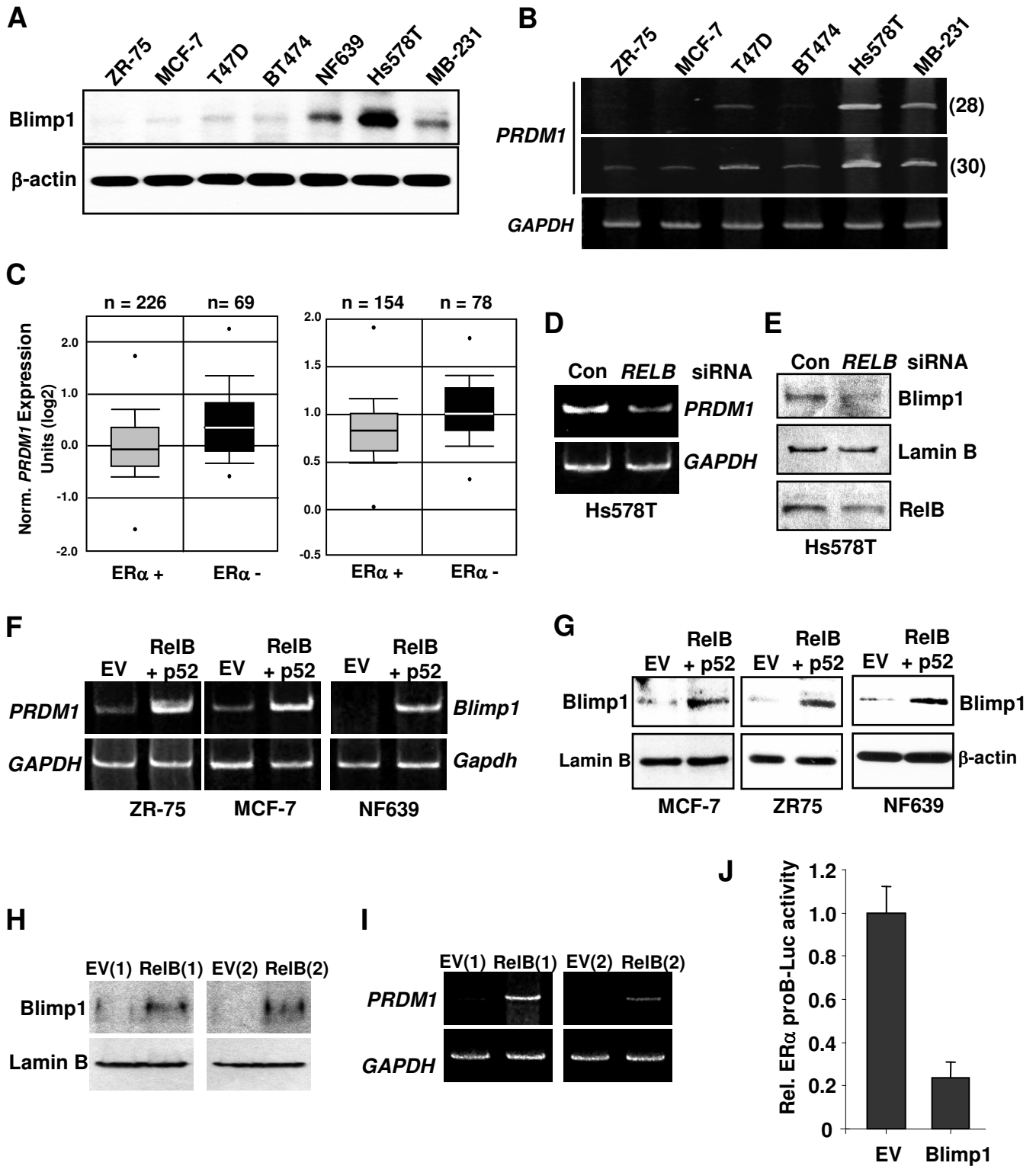


Fig. 2 Wang et al.

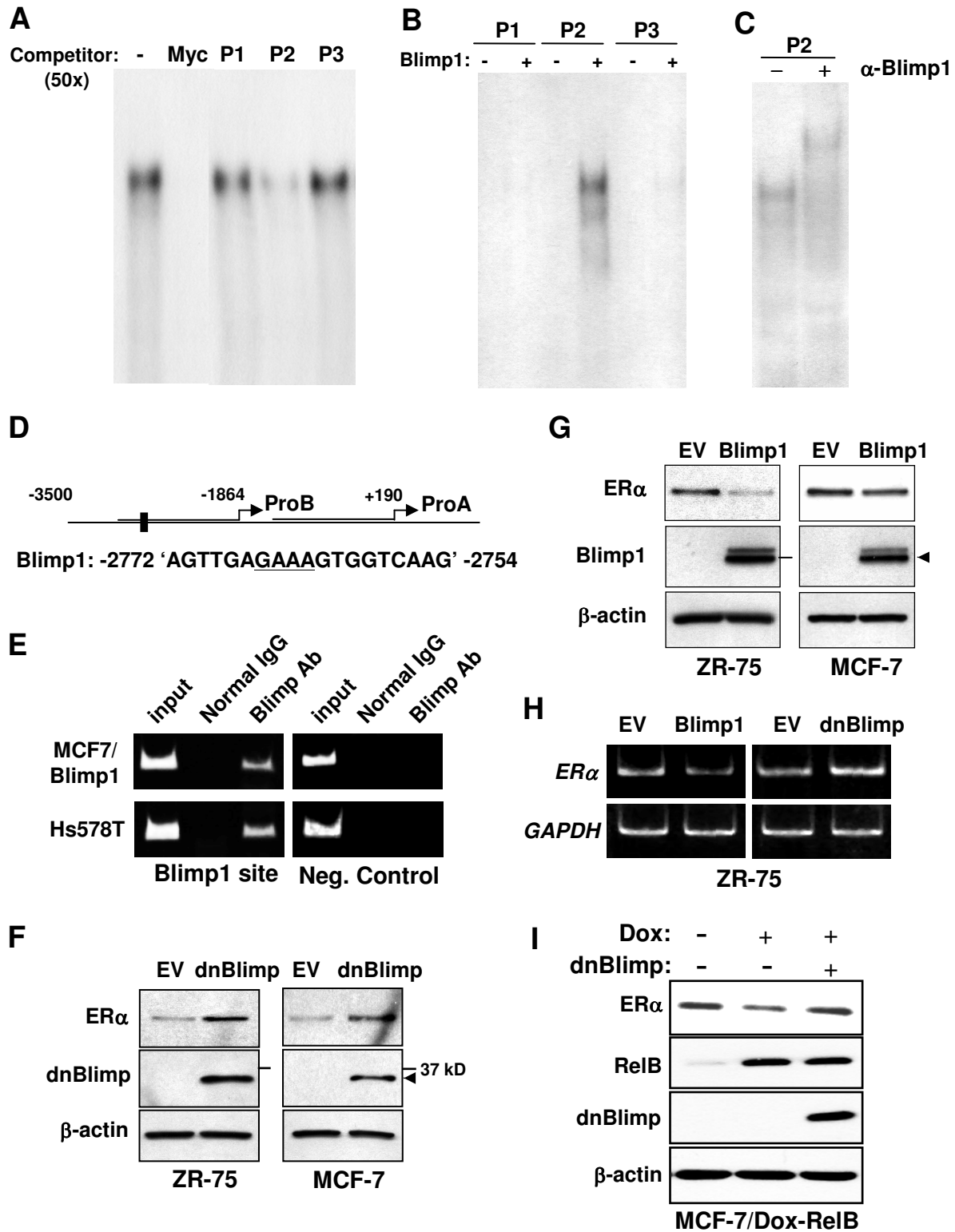


Fig. 3 Wang et al.

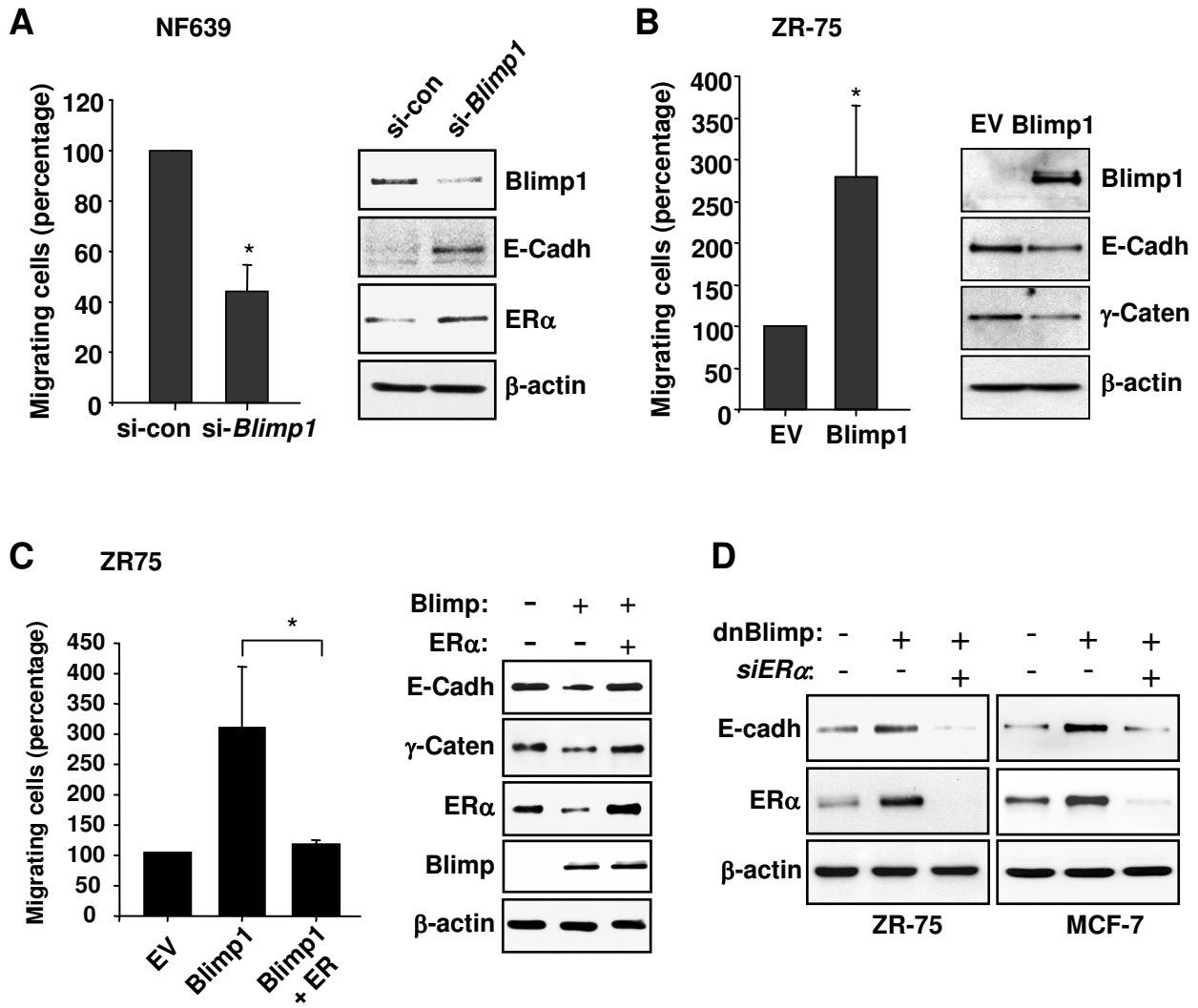


Fig. 4
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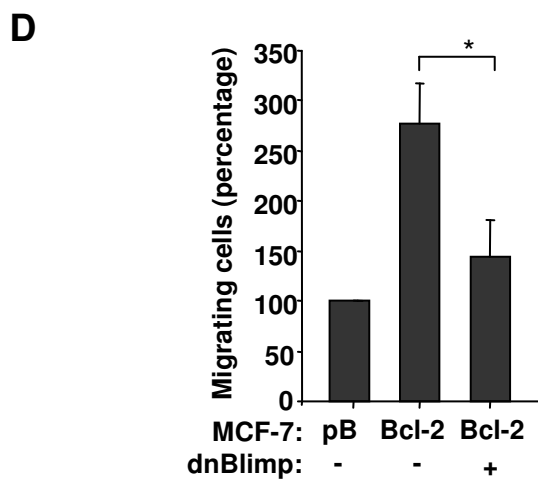
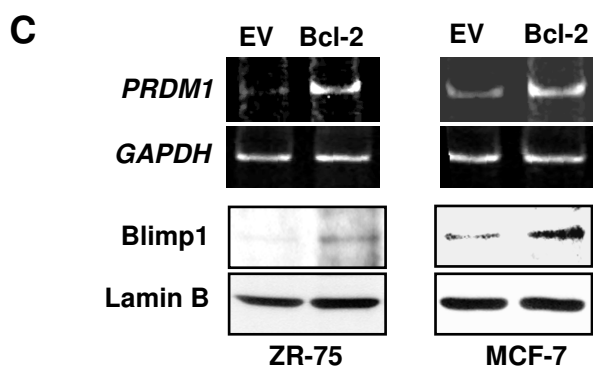
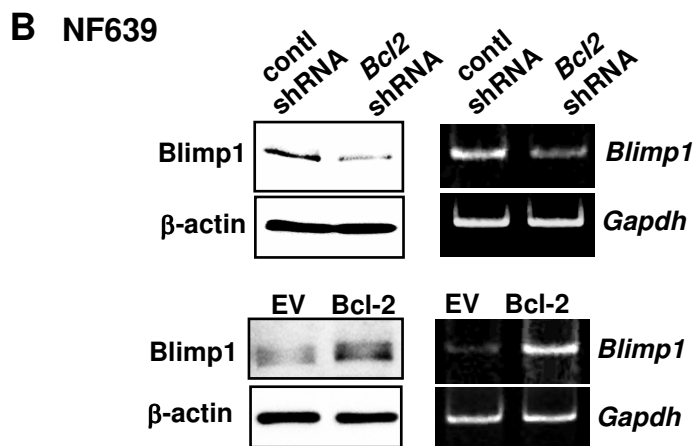
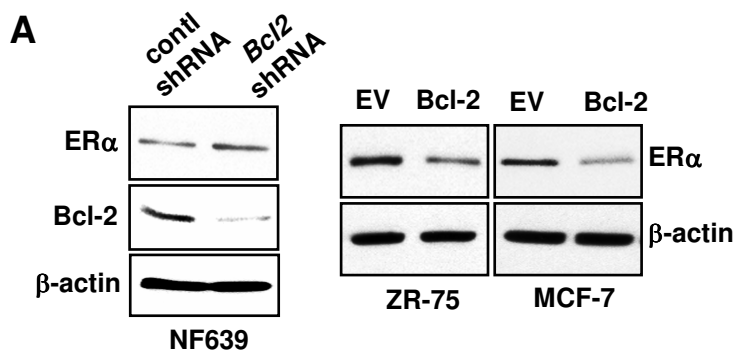


Fig. 5
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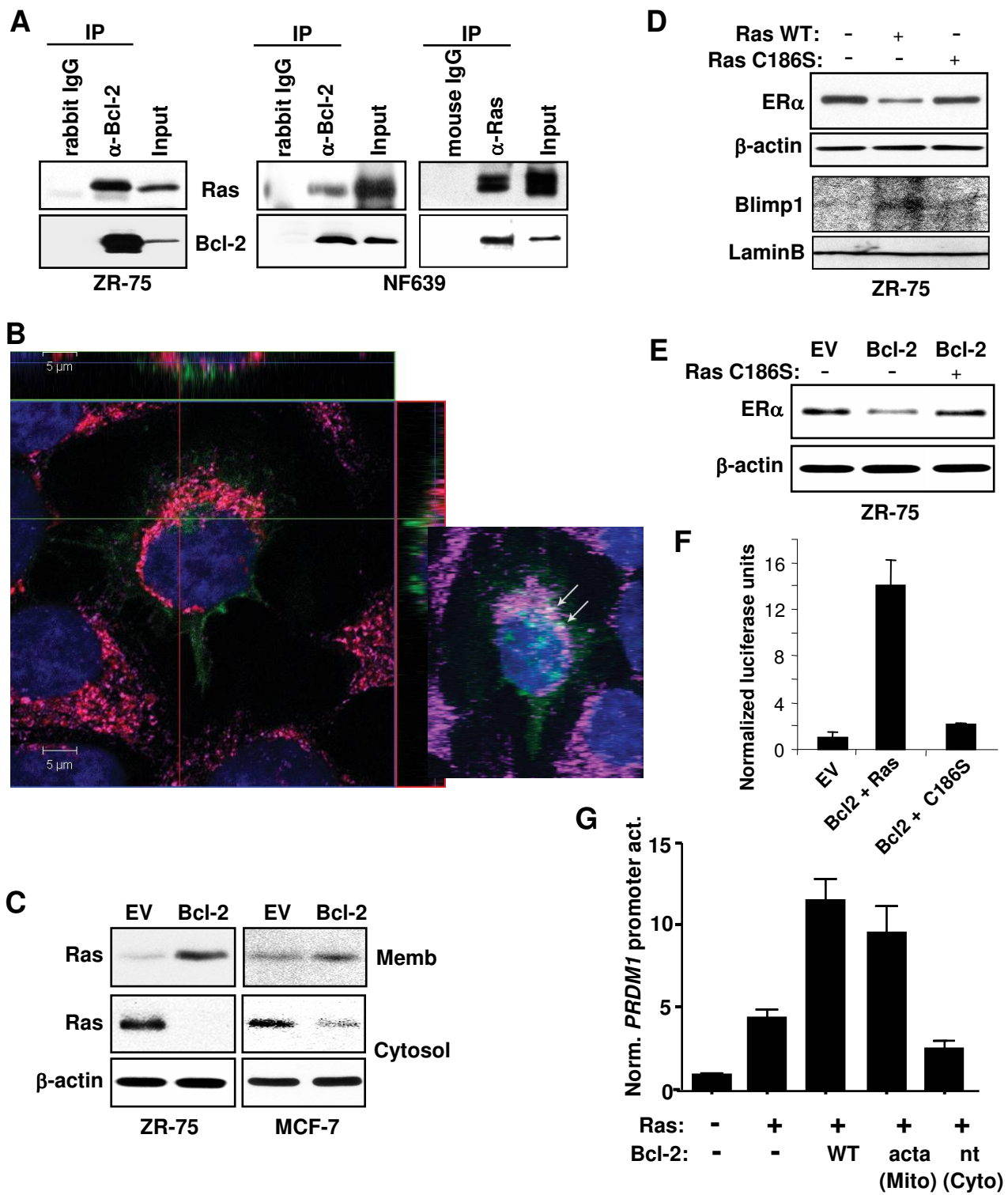


Fig. 6, Wang et al.

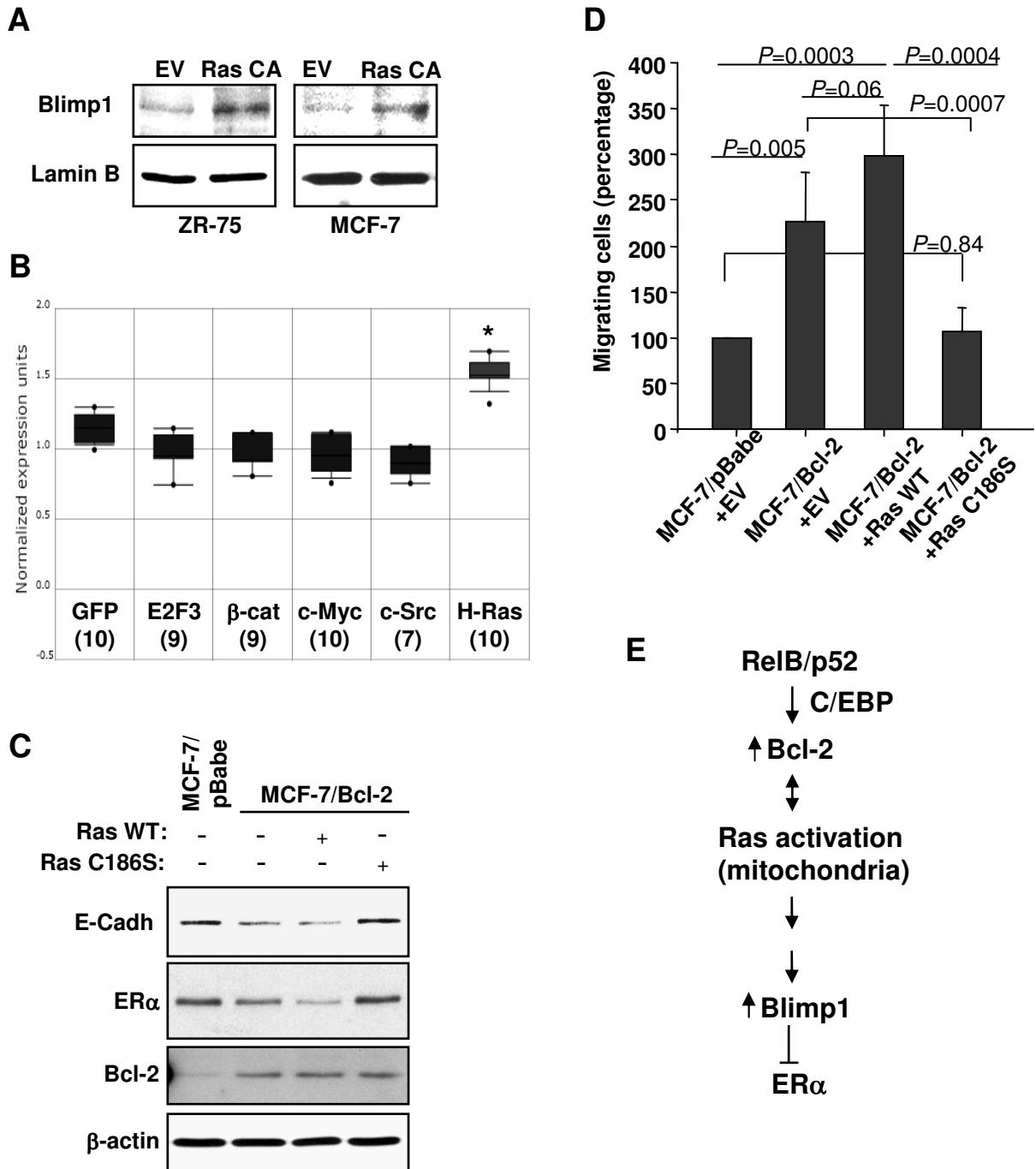


Fig. 7
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