Figure S1: Generation and Analysis of *Bax/Bak*-deficient Embryonic Stem Cells

A

Wildtype ESC; $Bax^{f/f}$ $Bak^{-/-}$ ESC: Line A, Line B, Line C; Tail DNA: $Bak^{+/+}$, $Bak^{-/-}$

300 bp – ; ← f/f ; ← +/+

Bax Genotyping

Wildtype ESC; $Bax^{f/f}$ $Bak^{-/-}$ ESC: Line A, Line B, Line C; Tail DNA: $Bak^{+/+}$, $Bak^{-/-}$

500 bp – ; ← +/+ ; ← -/-

Bak Genotyping

B

Relative abundance of *Bax* mRNA: 0.0, 0.5, 1.0

Parental; Line #3, Line #17, Line #18 (DKO ESC)

C

ESC line: A ($Bax^{f/f}$); 3, 17, 18 ($Bax^{-/-}$)

28 kDa – ; ← Bax

49 kDa – ; ← ACTIN

Cre: -, +, +, +

D

DKO ESC: Line 3, Line 17, Line 18; Tail DNA: Wildtype, Cre^{tg}

500 bp – ; ← *Cre*

Cre Genotyping

E

Cell Count: 10^3, 10^4, 10^5, 10^6

d0, d4, d7

WT; DKO

F

← Nanog ; ← Oct4 ; ← Actin

WT; $Bax^{f/f}$ $Bak^{-/-}$; DKO; $Casp3^{-/-}$

G

DKO#17; DKO#18

H

Cre Genotyping

← *Cre*

24 h post-tfxn; #1, #2, #3, #4 (Independent WT ESC lines)

I

← Nanog ; ← Actin

WT #1 #2 (UT); WT #1 #2 (RA (d2))

J

← Oct4 ; ← Actin

WT #1 #2 (UT); WT #1 #2 (RA (d3))

K

← Nanog ; ← Oct4 ; ← Actin

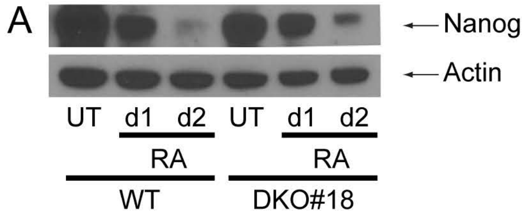
UT, RA (d4) (DKO); #1, #2 (DKO lines (post-recovery))

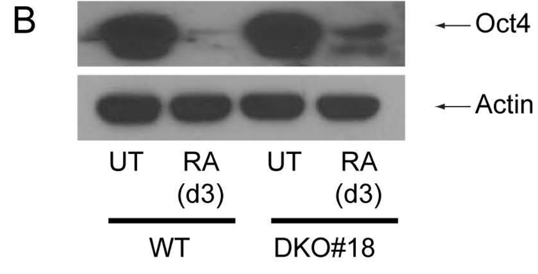
L

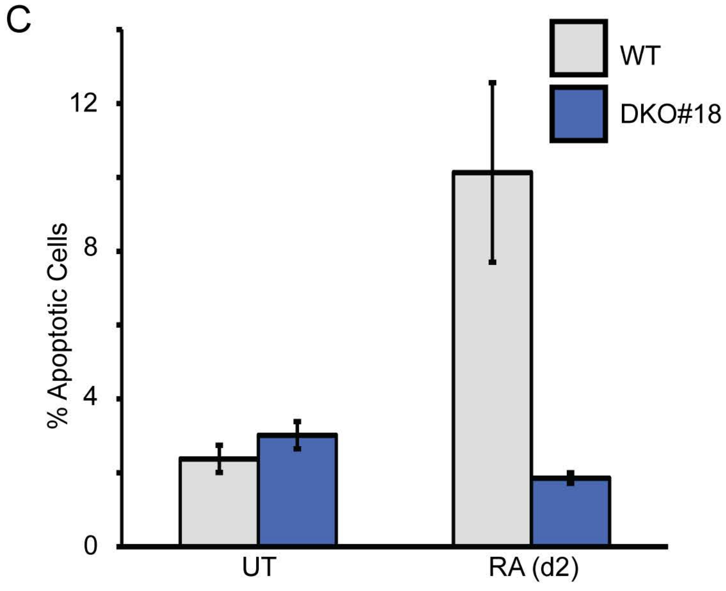
← Nanog ; ← Oct4 ; ← Actin

UT, RA (d2) (DKO recovered #1); UT, RA (d2) (DKO recovered #2)

Figure S2: Analysis of DKO#18 ESC Line







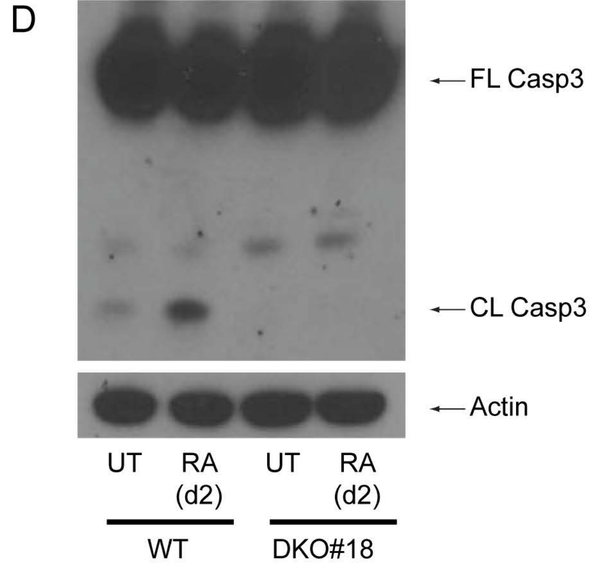
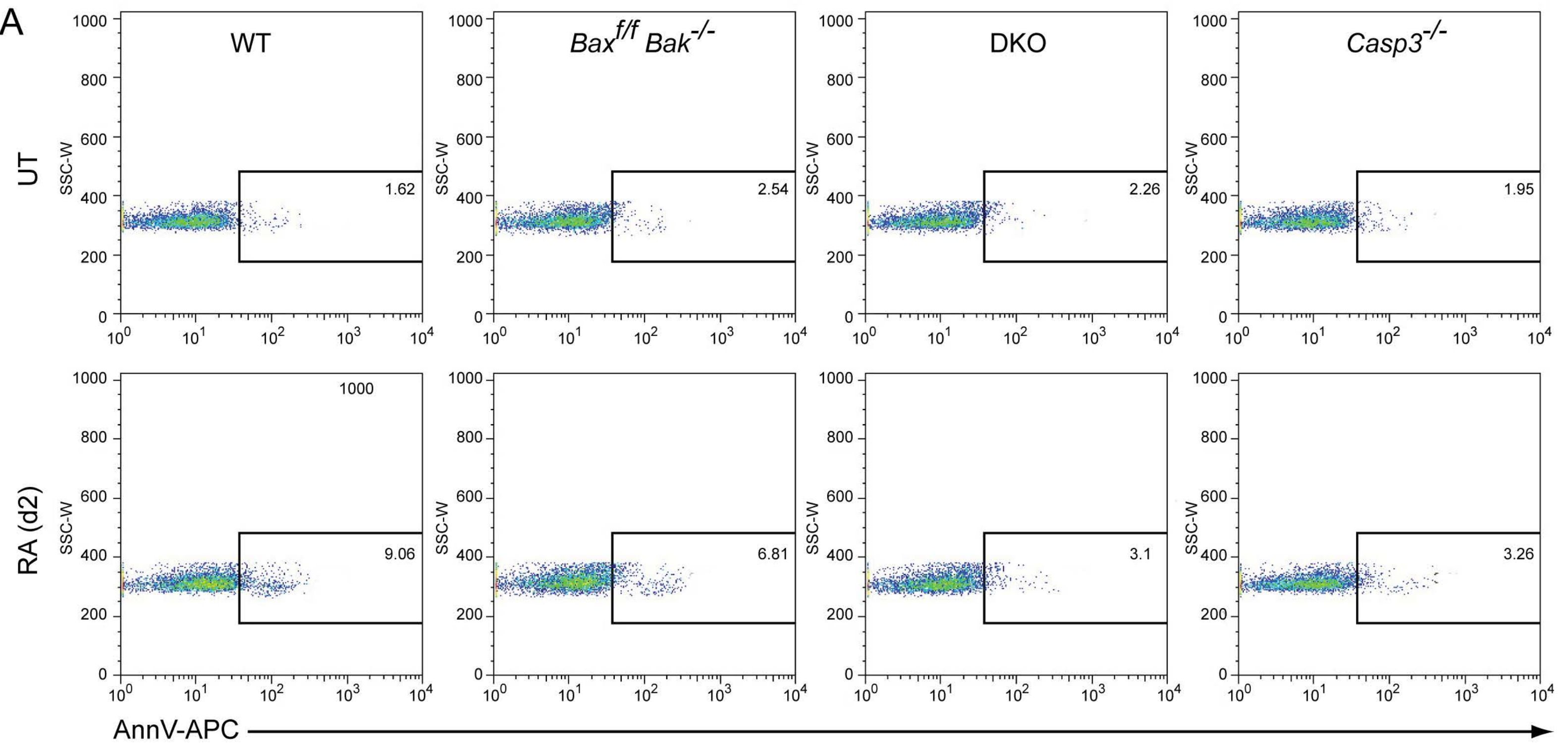


Figure S3: Representative FACS Plots



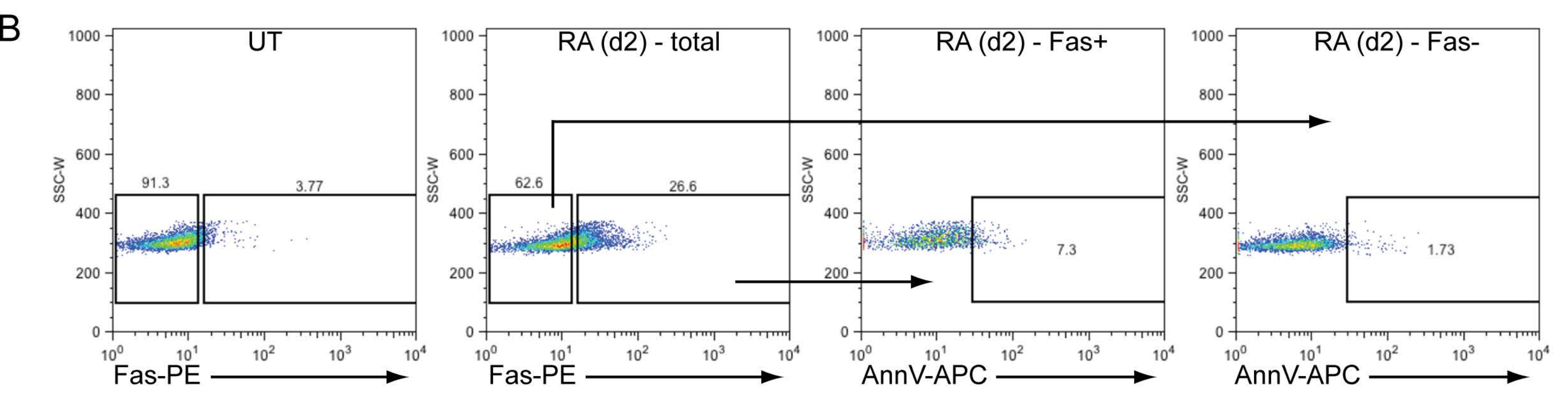


Figure S4: Core Pluripotency Factors Do Not Transcriptionally Regulate Expression of Aurora Kinase A

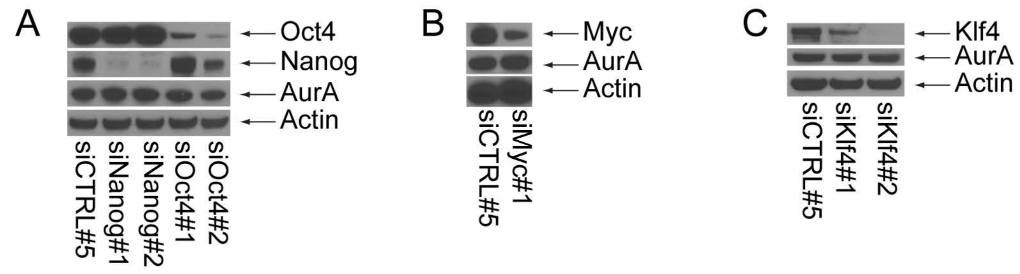


Figure S1, related to Figure 1. Generation and analysis of *Bax/Bak*-deficient Embryonic Stem Cells.

(A) *Bax* and *Bak* genotyping results from three independent $Bax^{f/f}Bak^{-/-}$ ESCs lines. Wildtype ESCs are shown as a control. (B) *Bax* mRNA levels in three independently transfected lines via quantitative RT-PCR. Clones were derived from transfection of *Cre* recombinase in the parental line, “ESC line B,” followed by selection in puromycin. (C) Bax protein levels after *Cre* mediated excision. (D) *Cre* genotyping results in three DKO lines. DKO, $Bax^{-/-}Bak^{-/-}$ double knockout. (E) Cell count of undifferentiated WT and DKO ESCs over time. (F) Immunoblot of Nanog and Oct4 from undifferentiated ESCs. (G) Representative metaphase spreads from DKO#17 (20/25 had 40XY karyotype) and DKO#18 (18/20 had 40XY karyotype) ESCs. (H) *Cre* genotyping from wildtype ESC 24 hours post-transfection and from individual ESC lines. (I) Immunoblot for Nanog from wildtype and two *Cre*-negative wildtype ESC lines after 2 days of RA treatment. (J) Immunoblot for Oct4 from wildtype and two *Cre*-negative wildtype ESC lines after 3 days of RA treatment. (K) Immunoblot for Nanog and Oct4 from DKO ESCs after 4 days of RA treatment and DKO ESCs that were allowed to recover in pluripotent conditions after RA-mediated differentiation. (L) Immunoblot for Nanog and Oct4 from DKO ESCs that were allowed to recover in pluripotent conditions after RA-mediated differentiation and then re-treated with RA for 2 days.

Figure S2, related to Figure 1. Analysis of DKO#18 ESC Line.

(A) Immunoblot for Nanog from wildtype or DKO#18 ESCs after 2 days of RA treatment. (B) Immunoblot for Oct4 from wildtype or DKO#18 ESCs after 3 days of RA treatment.

(C) Quantification of percent apoptotic cells (AnnexinV positive/PI negative) during RA-mediated differentiation of DKO#18 ESCs. (D) Immunoblot for full-length (FL) and cleaved (CL) Caspase-3 from wildtype and DKO#18 ESCs after 2 days of RA treatment.

Figure S3, related to Figure 2. Representative FACS Plots.

(A) Representative FACS plots of AnnexinV-stained ESCs after RA treatment. (B) Representative FACS plots of untreated and RA-treated WT ESCs stained for Fas and AnnexinV.

Figure S4, related to Figure 4. Core Pluripotency Factors do not Transcriptionally Regulate Expression of Aurora Kinase A

Immunoblot for AurA after siRNA knockdowns of (A) Oct4 and Nanog, (B) c-Myc, and (C) Klf4.

Table S1: Microarray Analysis for Apoptotic Proteins

SYMBOL	FOLD REGULATION
Fas	31.2691
Cdkn2a	20.7734
Egfr	16.374
Cdkn1a	14.2874
Jun	8.0371
B2m	7.7097
Mdm2	5.6178
Btg2	4.122
Wt1	3.793
Fasl	2.783
Rprm	2.7447
Apaf1	2.7321
Pmaip1	2.682
Tnfrsf10b	2.5609
Actb	2.4852
Prkca	2.468
Bcl2	2.2658
Rb1	2.0898
Rela	2.0046
Sfn	1.7901
Ercc1	1.5764
Bag1	1.5298
Nfkb1	1.4439
Fadd	1.3755
Cul9	1.3535
Serpinb5	1.2599
Traf1	1.2541
Esr1	1.217
Myc	1.1514
Trp53bp2	1.1514
Nf1	1.0994
Ppm1d	1.0943
Hif1a	1.0718
Gapdh	1
Zmat3	-1.0644
Foxo3	-1.0792
Gusb	-1.0943
Dapk1	-1.0994
Stat1	-1.1277
Lig4	-1.192
Hsp90ab1	-1.2311
Sesn2	-1.2687
Ep300	-1.2716
Gadd45a	-1.3013
Ccng1	-1.3226
Pten	-1.3256
Mcl1	-1.3692
Ccnh	-1.434

SYMBOL	FOLD REGULATION
Kras	-1.4506
Il6	-1.4845
RTC	-1.4983
E2f3	-1.5157
RTC	-1.5547
Cdk1	-1.6283
Bid	-1.7411
PPC	-1.7613
Cdc25a	-1.7654
Xrcc4	-1.7695
Bax	-1.7859
Ccne1	-1.8025
Myod1	-1.8361
Tnf	-1.8361
Trp63	-1.8361
MGDC	-1.8361
Bnip3	-1.9141
E2f1	-1.9274
RTC	-1.9498
PPC	-1.9543
Casp9	-1.9679
PPC	-2.0139
Cdk4	-2.0849
Trp53	-2.2243
Casp2	-2.2501
Dnmt1	-2.2658
Apex1	-2.555
Birc5	-2.6451
Prc1	-2.9828
Atm	-3.3636
Cdc25c	-3.6893
Msh2	-3.7064
Chek1	-3.7235
Mlh1	-3.9177
Pcna	-4.0465
Ccnb1	-4.1506
Trp73	-4.1506
Atr	-4.3169
Pttg1	-4.5002
Sirt1	-4.6482
Bbc3	-5.0397
Mdm4	-5.0747
Brca1	-5.4139
Xrcc5	-5.4139
Cradd	-5.5404
Egr1	-5.6308
Chek2	-6.4234
Brca2	-7.447

*normalized to GAPDH

Supplemental Experimental Procedures

Generation of $Bax^{-/-}Bak^{-/-}$ ESCs

ESCs were obtained from timed matings of $Bax^{f/f}Bak^{-/-}$ animals. Bax^{flox} [S1] and $Bak^{-/-}$ [S2] mice were backcrossed to C57BL/6J background for 6 generations before breeding together. Blastocysts were harvested at E3.5, plated on irradiated MEFs, trypsinized and allowed to grow until confluency. Three independent lines were obtained and transfected with pCre-pac. Cells were selected with puromycin at 2 µg/ml for 1 week until visible colonies formed. Four independent clones were screened for *Bax* mRNA and protein levels, and genotyped for *Cre* recombinase.

Western Blots and Antibodies

Cells were lysed in M-PER buffer (Thermo Scientific) containing protease/phosphatase inhibitor cocktail (Cell Signaling). Protein concentration was measured using a BCA assay (Pierce). Equivalent amounts of each sample were loaded on 10% Bis-Tris gels (Invitrogen), transferred to PVDF membranes, and immunoblotted with antibodies against Bax, Nanog, p53, γ-H2AX, FAS (human), Casp3, Casp8, and cleaved Casp8 (Cell Signaling Technology); Fas (M-20), Fas-L (N-20), and Oct4 (Santa Cruz Biotechnology); Bid (R&D Biosystems); AurA (BD Biosciences), and Actin (Sigma). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch. Membranes were developed with SuperSignal West Chemiluminescent Substrate (Thermo Scientific).

Karyotyping Analysis

DKO#18 ESCs were submitted to Cell Line Genetics (Madison, WI) for analysis, while DKO#17 ESCs were analyzed in-house. Briefly, embryonic stem cells were grown to confluency in a well of a 6-well plate. The cells were harvested and incubated in 8ml of 0.56% (w/v) KCl for 10 min at 37C. 2ml of fixative solution (3:1 Methanol:Glacial acetic acid) were then added. The cells were subsequently washed three times in fixative solution. After the washes, the cells were resuspended in 1ml fixative solution. Pre-cooled slides (Superfrost Plus slides incubated at -20°C in EtOH) were cleaned and moistened, and single drops of cells in fixative were dropped onto slides from a height of 0.5m. Slides were allowed to dry for 24-72 hours at room temperature. The slides were stained with Leishman's stain in Gurr's buffer for 8 min, rinsed in running water and cleared twice with Histoclear. Metaphase spreads were visualized and documented on an epi microscope at 40X magnification.

Flow Cytometry and Antibodies

Cells were harvested, washed in PBS twice, and incubated with AnnexinV-FITC (Biovision) or AnnexinV-APC (BD Pharmingen). Surface expression of FAS was measured using anti-mouse CD95-PE (clone 15A7, eBioscience) or a matched IgG1-PE isotype control (eBioscience); for analysis of Fas, cells were collected in citric saline (135mM potassium chloride, 15mM sodium citrate) with 1mM EDTA to avoid enzymatic digest of cell surface proteins. Propidium iodide (Biovision) or 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) was added to cells immediately before flow cytometry analysis.

Quantitative RT-PCR

Total RNA was extracted from ESCs using Trizol (Invitrogen) or RNeasy Mini Kit (Qiagen) (for *Bax* transcript levels). cDNA was generated using the SuperScript II Reverse Transcriptase Kit (Invitrogen). Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems). *Bax* transcript levels were normalized to hypoxanthine phosphoribosyltransferase (*HPRT*), while other targets were normalized to 60S ribosomal protein-7 (*RPL7*). Samples were run in triplicate. Primer sequences are listed below.

Oligonucleotides Used for Quantitative RT-PCR

	Forward Primer (5' – 3')	**Reverse Primer (5' – 3')**
Cre	CTGCATTACCGGTCGATGCAA	TGCTAACCAGCGTTTTCGTTCTGCC
Bax	GCTGACATGTTTGCTGATGG	GATCAGCTCGGGCACTTTAG
Oct4	AAAGCCCTGCAGAAGGAGCTAGAA	AACACCTTTCCAAAGAGAACGCCC
Nanog	AAAGCCCTGCAGAAGGAGCTAGAA	TCCAGATGCGTTCACCAGATAG
RPL7	GATTGTGGAGCCATACATTGCA	TGCCGTAGCCTCGCTTGT
Actin	CACAGCTTCTTTGCAGCTCCT	CGTCATCCATGGCGAACTG
Fas#1	TGAATGGGGGTACACCAACC	TTCCATGTTCACACGAGGCG
Fas#2	AGATGCACACTCTGCGATGA	AGGCGATTTCTGGGACTTTGT
FasL#1	GAACTGGCAGAACTCCGTGA	TGAGTGGGGGTTCCCTGTTA
FasL#2	GGCTCTGGTTGGAATGGGAT	GGTGTACTGGGGTTGGCTATT
p53#1	CGAAGACTGGATGACTGCCA	CGTCCATGCAGTGAGGTGAT
p53#2	TGCTCACCCTGGCTAAAGTT	TCCGACTGTGACTCCTCCAT
AurA#1	TGGATGCTGCAAACGGATAG	TGAGACGGAATCTGCTCAGTC
AurA#2	ACTCTCCAAGTTTGACGAGCA	ACACATTGTGGTTCTCCTGGAAG

Microarray Analysis

Wildtype ESCs were left untreated or differentiated for 4 days with RA, and relative message levels of select targets were analyzed using a SABioscience microarray (pamm-027Z). Data are displayed as fold change relative to untreated after normalization to GAPDH.

Alkaline Phosphatase Staining

Untreated or RA treated ESCs were plated onto irradiated MEFs in a 6-well plate at 2000 cells/well and allowed to grow for a week or until visible colonies formed. Cells were washed with PBS, fixed, and stained for alkaline phosphatase using the Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories).

Teratoma Formation

One million wildtype (*Bax*$^{+/+}$*Bak*$^{+/+}$), parental (*Bax*$^{f/f}$ *Bak*$^{-/-}$), DKO (*Bax*$^{-/-}$*Bak*$^{-/-}$), or *Casp3*$^{-/-}$ ESCs were injected subcutaneously into nude mice. Tumors that formed were isolated, sectioned, and stained with hematoxylin and eosin or anti-Oct4 (H-134; Santa Cruz Biotechnology #9081).

siRNA Knockdown

ESCs were plated away from MEFs the day before transfection with Dharmafect1 (Dharmacon) and 50 nM siRNA. 24 hrs after transfection, the cells were treated with RA. siRNAs were purchased from Thermo Fisher Scientific against *Casp8* (D0-43044-

02 and -03), *Fas* (D-045283-19 and -20), and *AurA* (D-065109-01 and -03); a non-targeting siRNA (D-001210-05) was used as a control.

Immunofluorescence

ESCs were left in undifferentiated conditions or treated with RA. Subsequently, they were collected and spun onto frosted glass slides (Fisher) using a Cytospin 4 (Thermo Scientific). Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Trixon-X, and stained with anti-γ-H2AX (Cell Signaling) or by using an ApopTag (Millipore). γ-H2AX-positive or TUNEL-positive nuclei were quantified using Metamorph (Molecular Device Inc.). Results shown are mean +/- SD from at least three independent samples.

Supplemental References

S1. Takeuchi, O., Fisher, J., Suh, H., Harada, H., Malynn, B.A., and Korsmeyer, S.J. (2005). Essential role of BAX,BAK in B cell homeostasis and prevention of autoimmune disease. Proceedings of the National Academy of Sciences of the United States of America *102*, 11272-11277.

S2. Lindsten, T., Ross, A.J., King, A., Zong, W.X., Rathmell, J.C., Shiels, H.A., Ulrich, E., Waymire, K.G., Mahar, P., Frauwirth, K., et al. (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Molecular cell *6*, 1389-1399.