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Supplemental Information

BAK/BAX-Mediated Apoptosis Is a *Myc*-Induced Roadblock to Reprogramming

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Supplemental Information comprises:

Figure S1

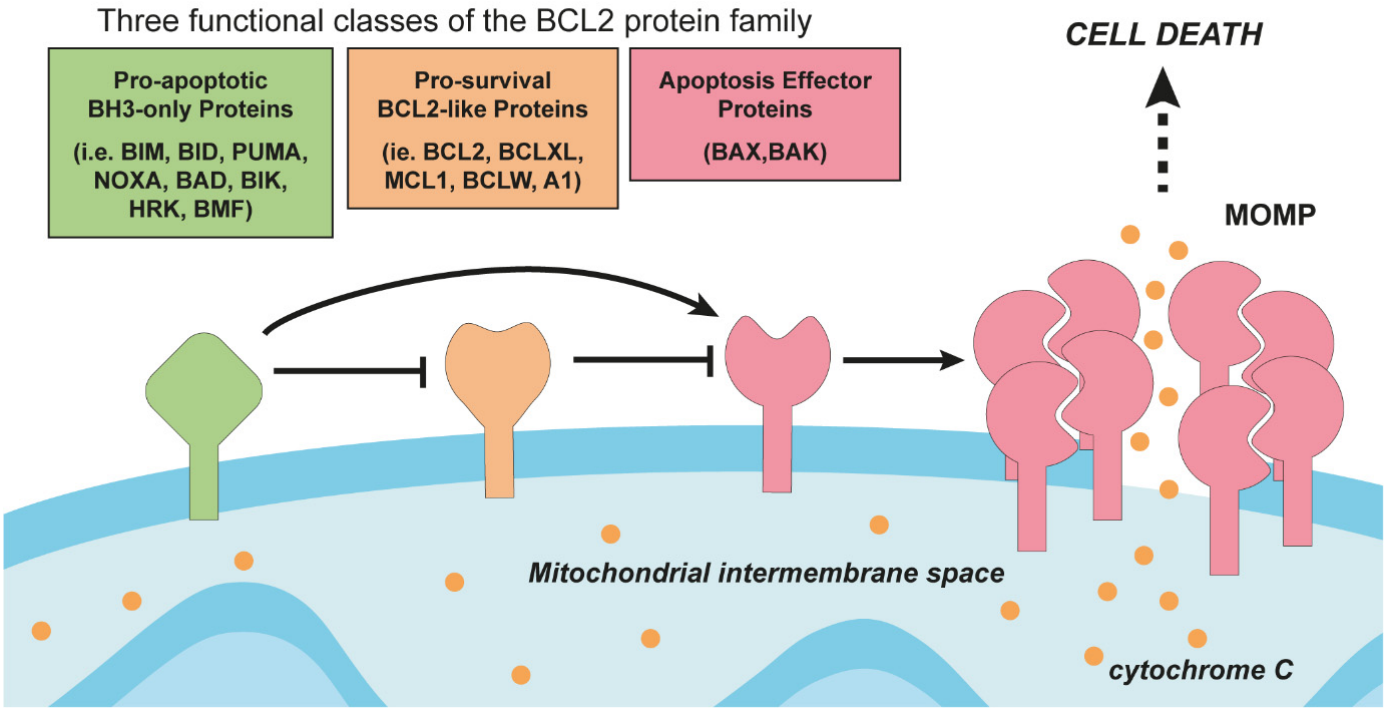

Three functional classes of the BCL2 protein family
Pro-apoptotic BH3-only Proteins
(i.e. BIM, BID, PUMA, NOXA, BAD, BIK, HRK, BMF)
Pro-survival BCL2-like Proteins
(ie. BCL2, BCLXL, MCL1, BCLW, A1)
Apoptosis Effector Proteins
(BAX,BAK)
CELL DEATH
MOMP
Mitochondrial intermembrane space
cytochrome C

Figure S2

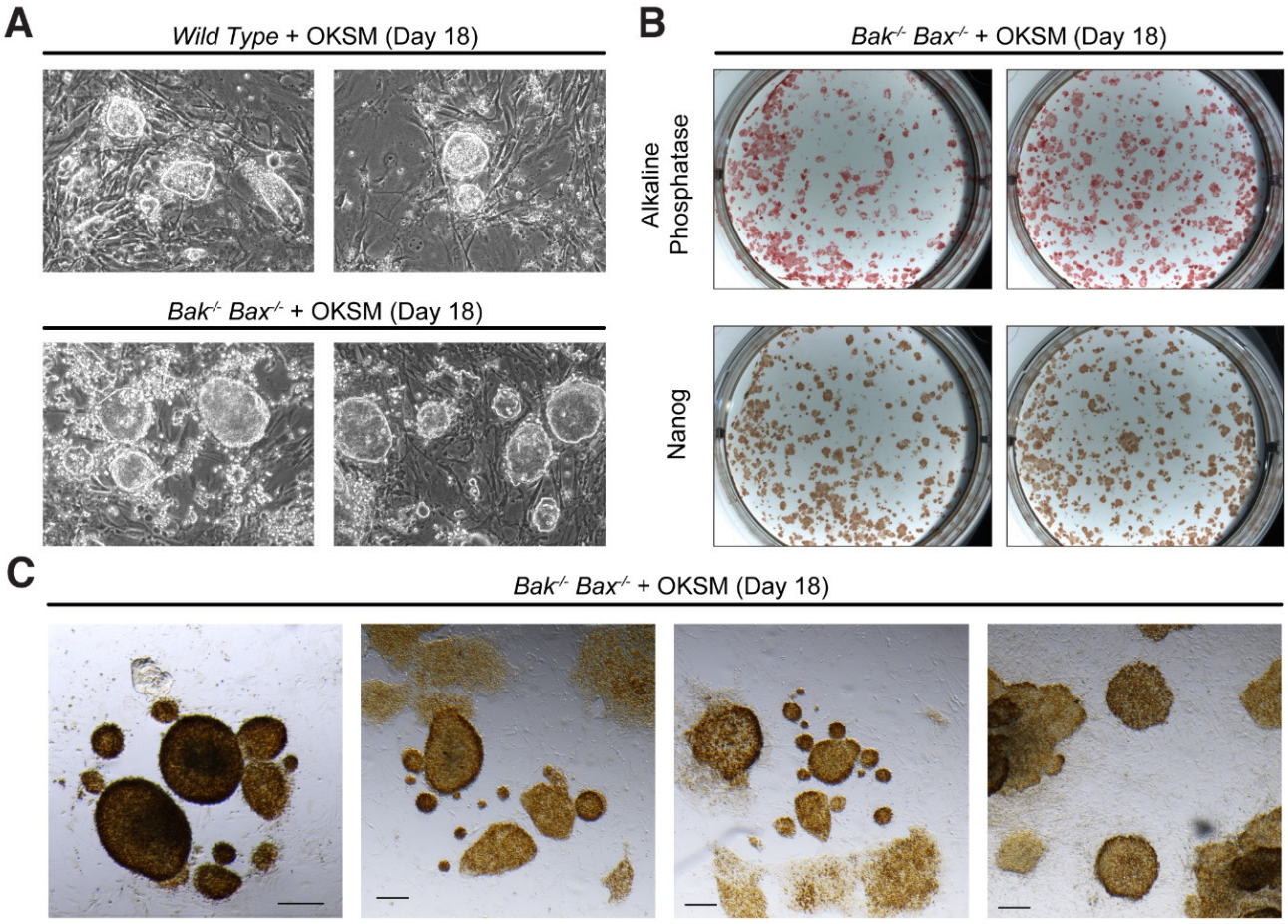

A
Wild Type + OKSM (Day 18)
Bak-/- Bax-/- + OKSM (Day 18)
B
Bak-/- Bax-/- + OKSM (Day 18)
Alkaline Phosphatase
Nanog
C
Bak-/- Bax-/- + OKSM (Day 18)

Figure S3A

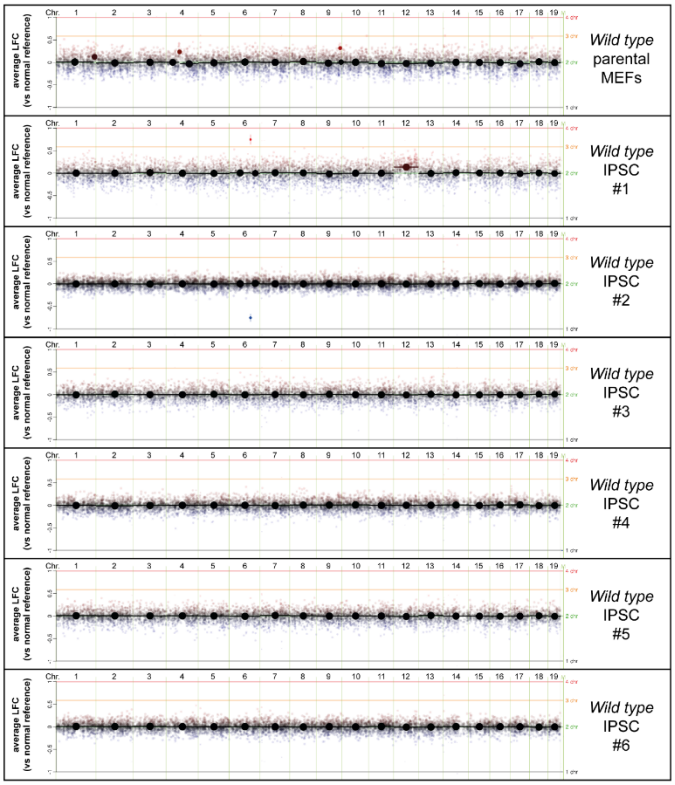


Figure S3B

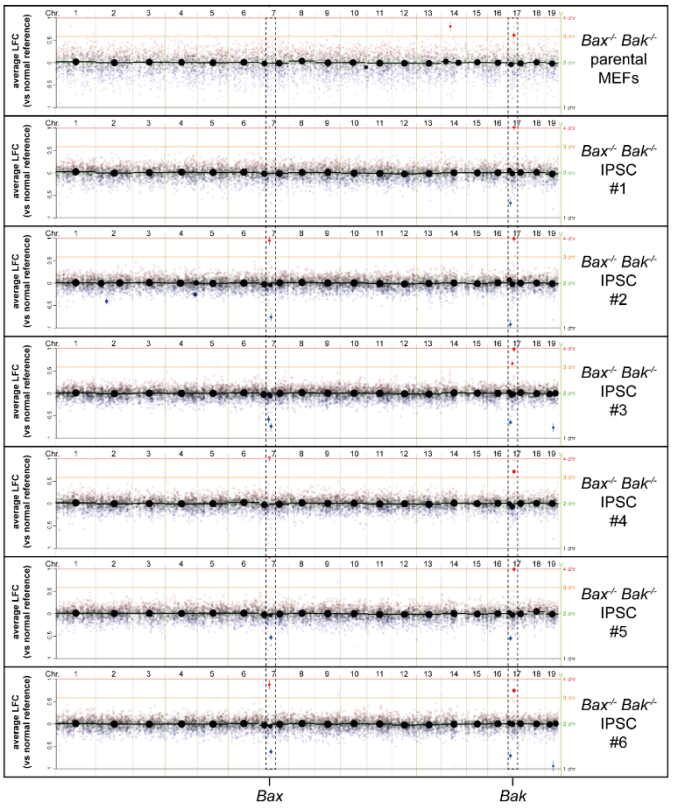
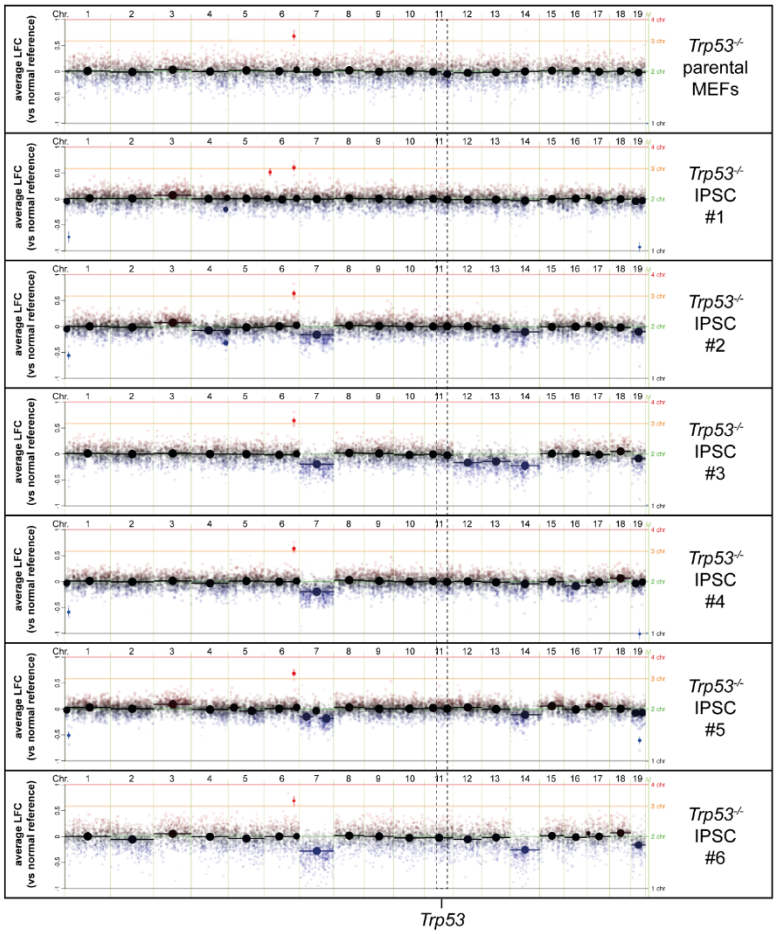


Figure S3C



SUPPLEMENTAL FIGURE TITLES AND LEGENDS

Figure S1. Regulation of mitochondrial apoptosis by the BCL2 family of proteins (related to Introduction). In healthy cells, BCL2-like proteins (BCL2, BCLXL, BCLW, MCL1, A1) restrain the apoptosis effector proteins BAK and BAX to permit cell survival. During apoptosis, BH3-only proteins (BIM, BID, BAD, PUMA, NOXA, BMF, BIK, HRK) become activated by stress signals and prevent BCL2-like proteins from inhibiting BAK and BAX. Some BH3-only proteins (e.g. BIM, BID) also activate BAK and BAX directly. Once active, BAK and BAX form dimers that assemble into higher order oligomers, which cause mitochondrial outer membrane permeabilization (MOMP). This leads to the efflux of cytochrome c to the cytosol where it activates caspase proteases, leading to the demolition of cellular components and cell death.

Figure S2. iPSC Morphology and Marker Expression (related to Figure 1). (**A**) MEFs transduced with OKSM yielded shiny, rounded colonies characteristic of pluripotent stem cells. The morphology of iPSC colonies is comparable between *WT* and *Bak*$^{-/-}$*;Bax*$^{-/-}$ cultures. (**B**) *Bak*$^{-/-}$;*Bax*$^{-/-}$ MEFs transduced with OKSM in duplicate wells were cultured for 18 d and then fixed and stained. One replicate well was stained with Vector Red Alkaline Phosphatase substrate. The other was stained with anti-NANOG primary antibody, which was detected with an HRP-conjugated secondary antibody and Vector DAB peroxidase substrate. Duplicate wells from two independent *Bak*$^{-/-}$;*Bax*$^{-/-}$ MEF clones are shown. **(C)** Higher magnification images of reprogrammed *Bak*$^{-/-}$;*Bax*$^{-/-}$ MEFs stained for NANOG protein (brown) reveal cells at different stages of reprogramming, as well as unstained cells that failed to be reprogrammed. Scale bar = 200μm.

Figure S3. Genomic Integrity of *Bak*$^{-/-}$;*Bax*$^{-/-}$ iPSCs is Comparable to *WT* iPSCs (related to Figure 4). Segmented copy number data are shown for each sample. The log fold change (LFC) is plotted for each 100kb interval, relative to the *WT* reference set. The chromosome number is listed at the top of each panel. Each segment is denoted by a point with a horizontal line extending over the length of the segment and a colour gradient that reflects the copy number status (blue for loss, red for gain and black for unchanged). Error bars represent 70% confidence intervals. Individual sample names are listed (at right). (**A, B**) Very few copy number alterations (CNAs) were detected in passage 2 *WT* and *Bak*$^{-/-}$;*Bax*$^{-/-}$ iPSCs, apart from several small variations (<100kb) found in close proximity to the targeted alleles in *Bak*$^{-/-}$;*Bax*$^{-/-}$ iPSCs (i.e. *Bak*, *Bax* on chromosomes 17 and 7, respectively). These were also detected in the parental MEFs and do not reflect CNAs arising during the reprogramming process. Instead, they reflect strain-specific variations from the reference C57BL/6 genome linked to the *Bax* and *Bak* loci, which have been maintained despite extensive backcrossing (N.B. the *Bax*$^{-/-}$ and *Bak*$^{-/-}$ alleles were originally established on a 129/Sv background). Detection of these events indicates that the low pass whole genome sequencing we conducted was sensitive and that most of the spontaneous CNAs observed in the iPSC lines were sub-clonal. (**C**) The genomes of iPSCs derived from p53-deficient MEFs harboured a significantly higher number of CNAs, consistent with many previous reports that iPSCs derived from p53-deficient MEFs exhibit elevated genome instability.

SUPPLEMENTAL TABLES

Table S1. Primary antibodies used in this study

PRIMARY ANTIBODY	WORKING DILUTION	SUPPLIER	CATALOGUE NUMBER
Mouse monoclonal anti-OCT3/4	1:100	Santa Cruz	sc-5279
Rabbit polyclonal anti NANOG	1:150	Abcam	ab-80892
Mouse monoclonal anti-SSEA1	1:800	Cell Signaling Technology	4744
Rabbit polyclonal anti-α1-Fetoprotein	1:200	Dako	A0008
Mouse monoclonal anti-βIII Tubulin	1:1000	Promega	G7121
Mouse monoclonal anti-α Smooth Muscle Actin	1:400	Sigma	2547
Mouse monoclonal anti-p53	1:500	BD Pharmingen	554147
Mouse Monoclonal anti-phospho (γ) H2A-X	1:500	Merck Millipore	05-636

Table S2. Secondary antibodies used in this study

SECONDARY ANTIBODY	WORKING DILUTION	SUPPLIER	CATALOGUE NUMBER
Mouse anti-rabbit IgG FITC (H+L chain specific)	1:200	Southern Biotech	4050-02
Goat anti-mouse IgG PE (H+L chain specific)	1:200	Southern Biotech	1031-09

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of Mouse iPSCs

Primary MEFs at passage 3-4 were seeded onto 6-well plates (5 x 10^4 cells per well) pre-coated with 0.1% gelatin. These were transduced by centrifuging (45 min, 1100x*g*) with the lentiviral supernatant followed by overnight incubation. Transduced cells were cultured in iPSC medium (15% Knockout Serum Replacement, 2mM GlutaMAX-CTS, 1mM non-essential amino acids, 1000U/ml leukemia inhibitory factor (LIF), 100μM β-mercaptoethanol in Knockout DMEM) for 18 d. Media was changed every 48 h. On day 18 reprogramming efficiency was assessed using the Vector Red Alkaline Phosphatase Substrate Kit according to the manufacturer's instructions, or by NANOG immunocytochemistry (see Tables S1, S2). The high magnification images of NANOG staining were acquired with a SMZ25 stereomicroscope (Nikon) and DS-Ri2 high definition colour camera (Nikon).

Teratoma Formation

1 x 10^6 cells of each iPSC clone were subcutaneously injected into NOD/SCID mice. Teratomas were surgically removed 4-5 weeks post-injection. Tissues were fixed overnight at 4°C in 4% paraformaldehyde and embedded in paraffin. 2-3 μm sections were taken from samples and stained with hematoxylin and eosin for pathological examination.

Immunofluorescence

The primary and secondary antibodies used in this study are described in Tables S1 and S2. iPSC clones were fixed with 4% PFA for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 2% BSA in PBS for 1 h at room temperature (R.T). After incubation with antibodies against mouse SSEA1, NANOG, OCT4 or p53 overnight at 4°C, cells were washed with PBS with 0.1% Tween 20 and incubated either with secondary

antibodies conjugated with R-phycoerythrin or FITC for 1 h at R.T. For SSEA1 staining, the permeabilization step was not necessary. Washed coverslips were mounted on slides with Prolong Gold Antifade Reagent (Invitrogen) with DAPI. Images were acquired under non-saturating conditions on a Zeiss LSM 780 confocal microscope.

In Vitro Differentiation Assay

iPSCs were harvested and re-suspended at a concentration of 2.5 x 10^4 cells/ml in iPSC medium without LIF. Hanging droplets (20 µl) were made on the underside of a 9 cm cell culture plate lid, incubated for 2 d and then transferred to non-adhesive plates and cultured in suspension for 3 d to form embryoid bodies (EBs). EBs were then transferred to gelatin-coated tissue culture plates to differentiate for 9 - 14 d. Cells were stained for α-smooth muscle actin, βIII tubulin and α fetoprotein, along with DAPI.

Detection of DNA Double-Strand Breaks

DNA double-strand breaks were detected by immunofluorescence using a primary antibody against mouse H2AX (Table S1). Fluorescence intensity threshold was set by a positive control of *WT* iPSC colonies treated with 5 Gray of γ-irradiation. Foci in 100-150 nuclei per genotype per experiment were counted. DNA double-strand breaks were quantified automatically with Image J based on thresholds set by positive controls.

Image Analysis

All images were analysed with the Fiji image processing package.

Western Blot Analysis

MEFs were treated with doxorubicin (0.5µg/ml) for 18 h to increase p53 levels and protein extracts were prepared by lysis in RIPA buffer supplemented with protease inhibitors

(Roche). Proteins were quantified using the Bradford assay (Bio-Rad), separated by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with 5% (w/v) skimmed milk powder in PBS-Tween20 (0.1%), the membrane was incubated with primary antibody against p53 (Table S1).