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Supplementary Materials for

A Kinase-Independent Role for Unoccupied Insulin and IGF-1 Receptors in the Control of Apoptosis

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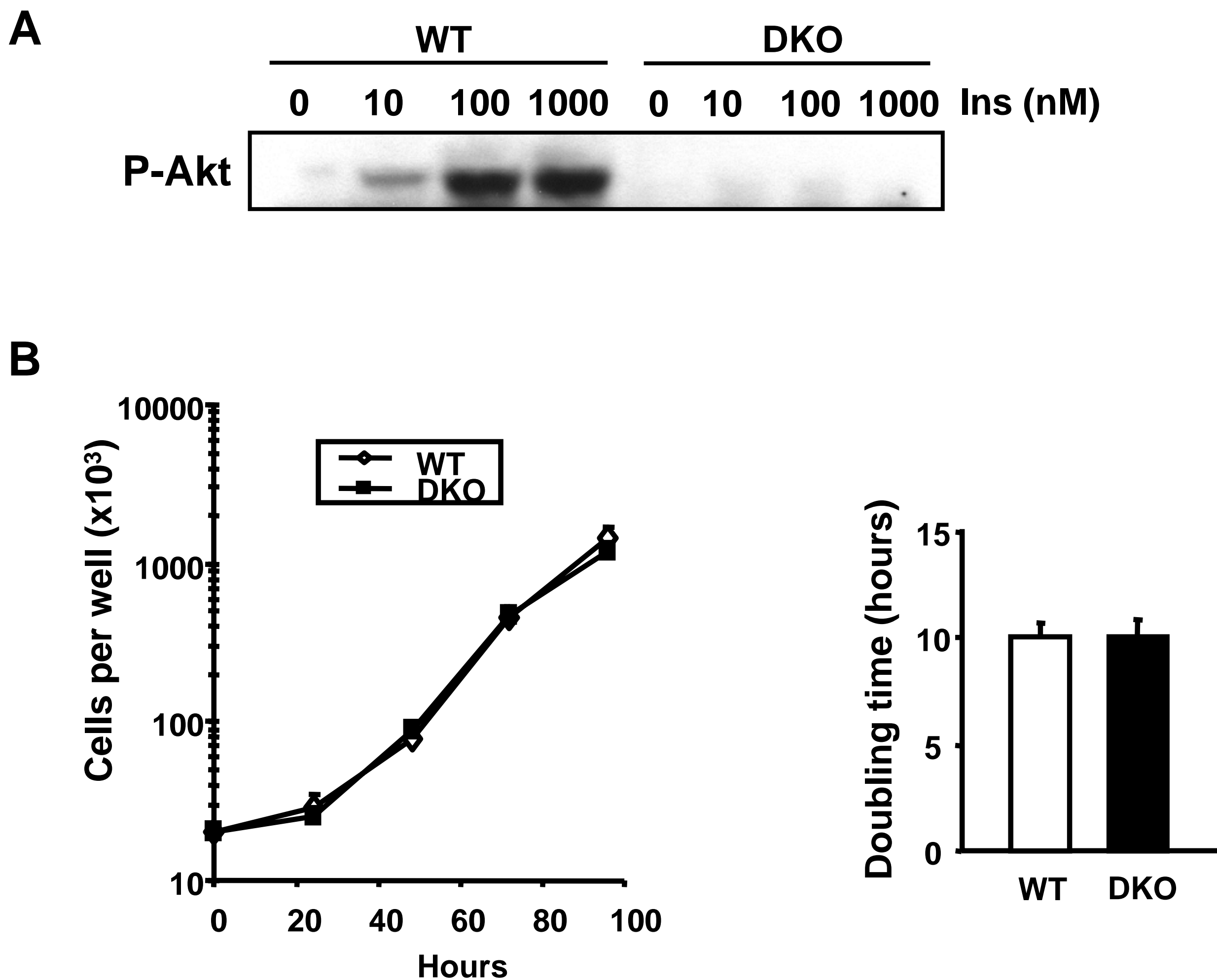

Fig. S1: Insulin signaling and growth rate in wild-type and DKO cells.
A) Confluent WT and DKO cells were serum deprived overnight and then incubated with various insulin concentrations of insulin for 5 minutes. Cells were harvested, and protein extracts were used to perform immunoblots to detect phosphorylation of Ser 473 on Akt. B) Cells were plated at a density of 20.000 cells per well and and their number determined at 24h intervals for 4 consecutive days. Doubling time was calculated during the exponential growth phase between day 3 and day 2 after plating.

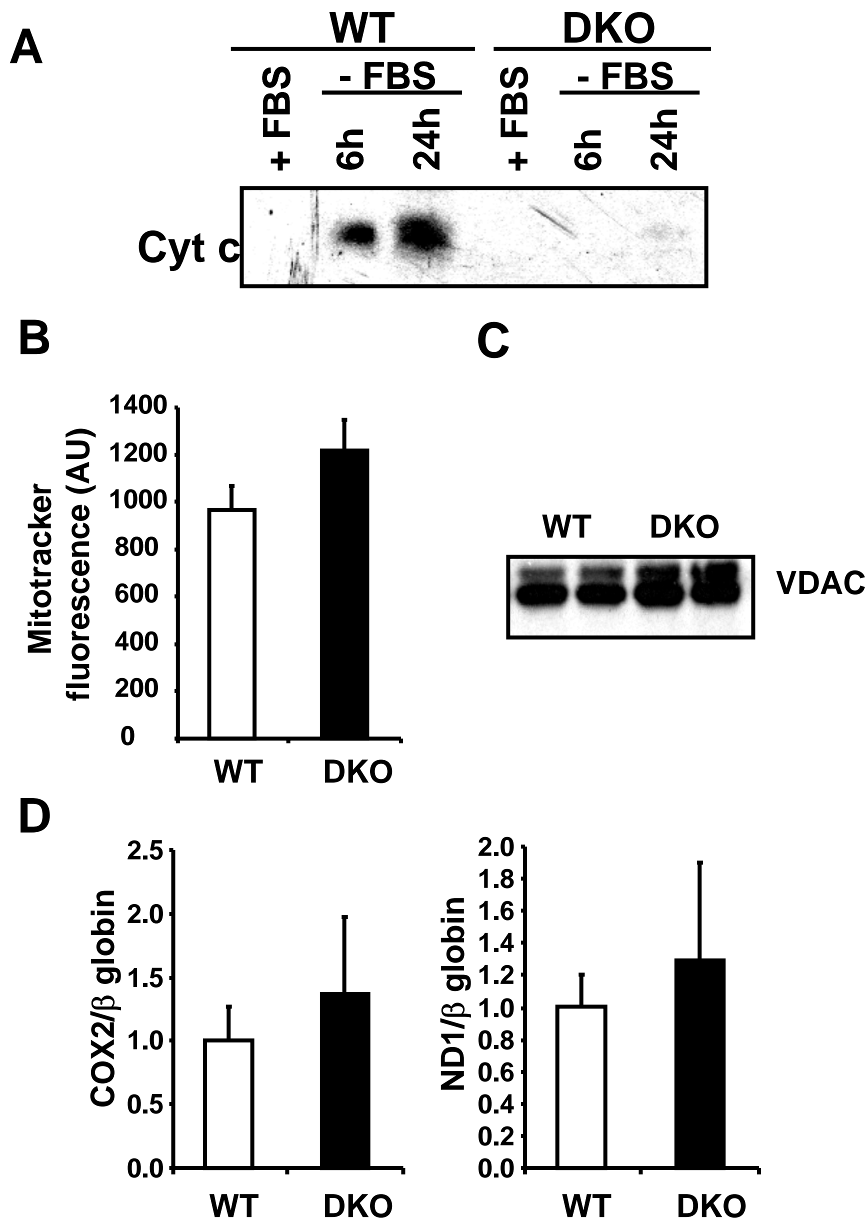

Fig. S2: DKO cells show reduced cytochrome c release from mitochondria but no difference in mitochondrial mass.

A) Confluent WT and DKO cells were serum deprived for 6 or 24 hours. Floating cells were collected and pooled with attached cells recovered after trypsin treatment. Protein extracts from the cytosolic fraction extracted using kit QIA88 from Calbiochem were used to perform immunoblots to detect cytochrome c accumulation. One representative blot from 2 independent experiments is shown. B) Confluent WT and DKO cells were incubated with MitoTracker green FM (Invitrogen) at the final concentration of 25 nM for 20 min at 37C. Cells were then trypsinized, washed with PBS and fluorescence was analyzed by FACS. Results represent average fluorescence ± S.E.M. from 5 independent experiments. C) Abundance of voltage-dependent anion channel (VDAC) was measured in confluent serum fed WT and DKO cells by western blot. D) DNA from confluent WT and DKO cells was extracted and relative levels of mitochondrial versus nuclear DNA was measured by real-time PCR using specific primers for mitochondrially encoded Cytochrome c oxydase subunit 2 (*Cox 2*), NADH dehydrogenase 1 (*ND1*) genes and the nuclear encoded beta globin gene. Results are mean ± S.E.M. from 6 independent experiments.

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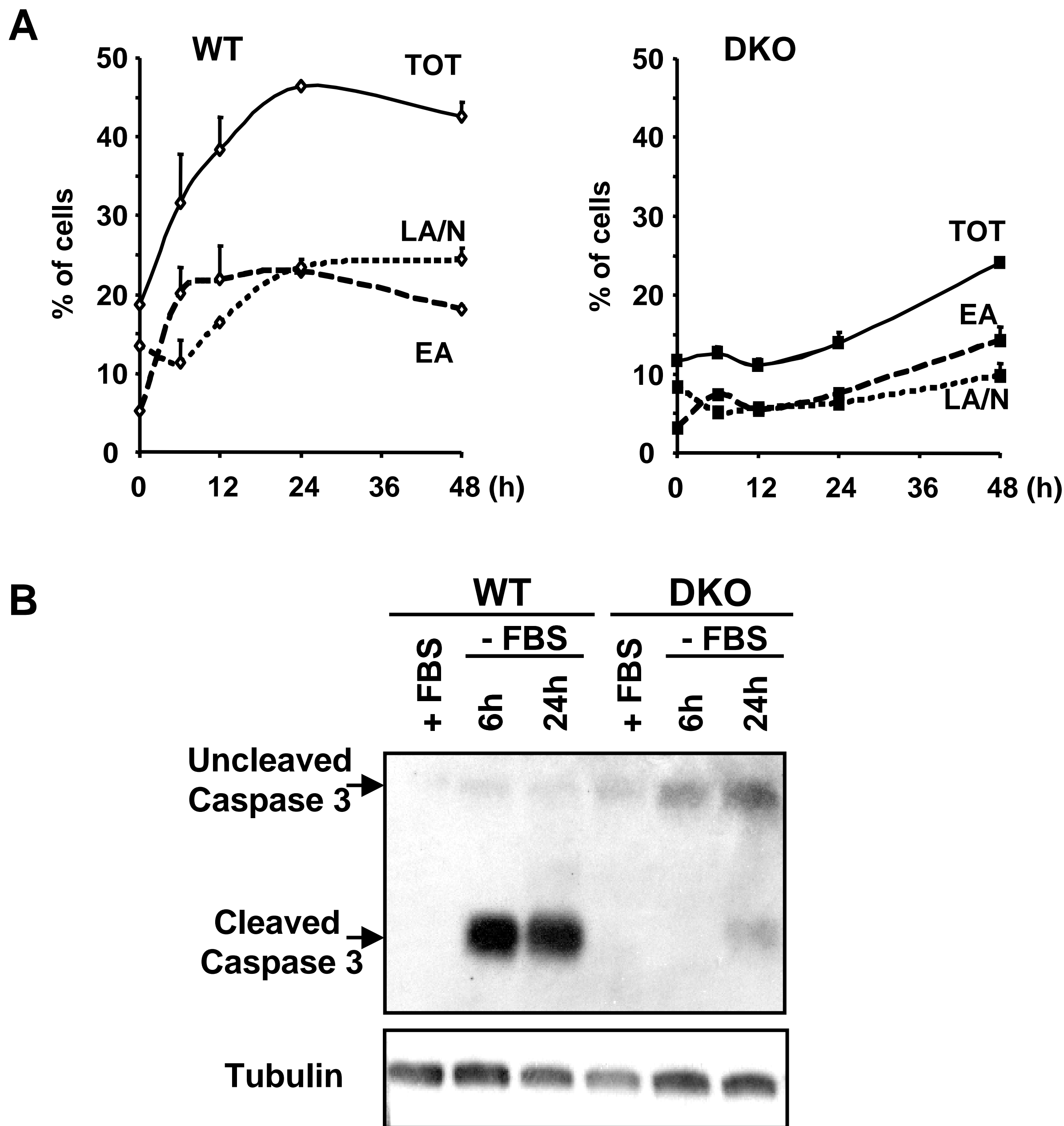

Fig. S3: Reduced apoptosis in DKO cells.
A) Quantification of annexin binding assays in WT and DKO cells after serum deprivation. The proportion of early apoptotic cells (annexin-PE positive cells) and late apoptotic or necrotic cells (annexin-PE positive cells and PI positive cells) were determined. Results are expressed as the percentage of total cell number. E.A = early apoptotic cells; LA/N = late apoptotic/necrotic cells; TOT = total number of apoptotic cells. Data are mean ± S.E.M from 3 independent experiments.
B) Proliferating (40% confluent) WT and DKO cells were serum deprived for 6 or 24 hours. Immunoblots to detect Caspase 3 cleavage were performed on protein lysates from floating and attached cells. One representative blot from 3 independent experiments is shown.

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A

WT DKO

+FBS Prolif +FBS Conf -FBS +FBS TNF +FBS Prolif +FBS Conf -FBS +FBS TNF

6 24 6 24 6 24 24 6 24 6 24 6 24 24 hours

Bik
Bak
Bim
Bid
T Bid
Bmf
Mcl-1
Puma
AIF
HSP90
HSP70
HSP60
HSP27

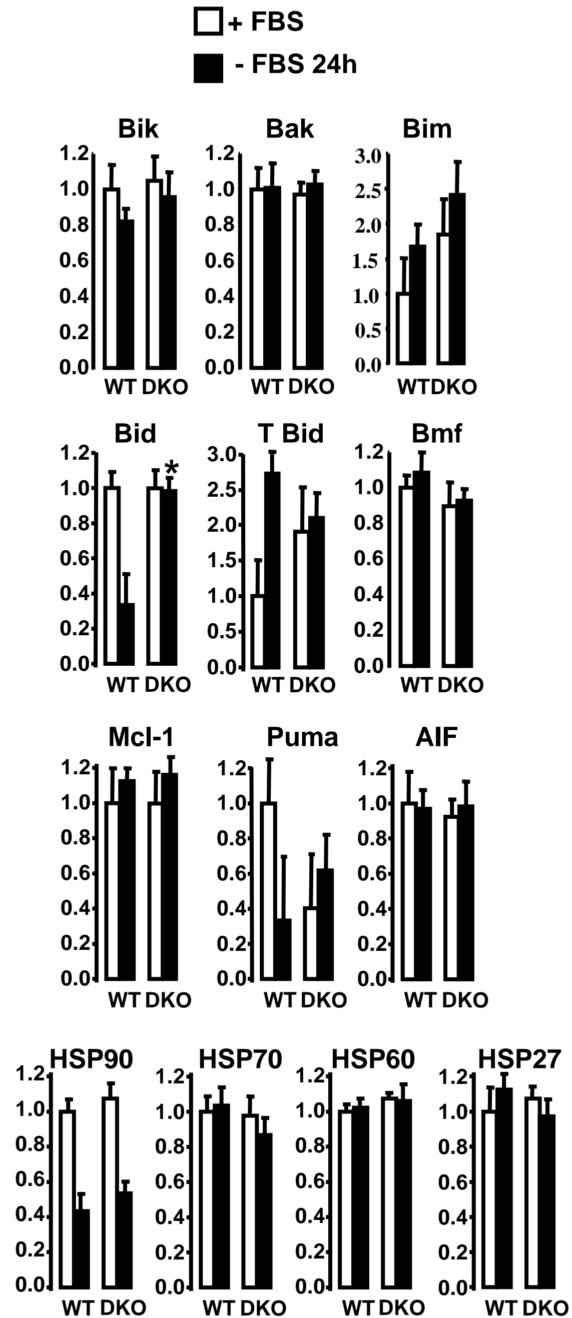

Fig. S4: Abundance of proteins involved in apoptosis in wild-type and DKO cells. Proliferating (50% confluent) and confluent WT and DKO cells were kept in the presence of serum for 6 or 24 hours. Confluent cells were also serum starved for 6 or 24 hours or treated with TNFα (50 ng/ml) for 24 hours. Immunoblots were performed on protein lysates from floating and attached cells. A. One representative Western blot from 3 independent experiments is shown. B. Immunoblots quantification for serum-fed or serum-deprived confluent cells. Results are normalized to β tubulin and are mean ± S.E.M. from 3 independent experiments. * indicates a significant difference compared to WT cells, p value <0.05 by Student's t test.

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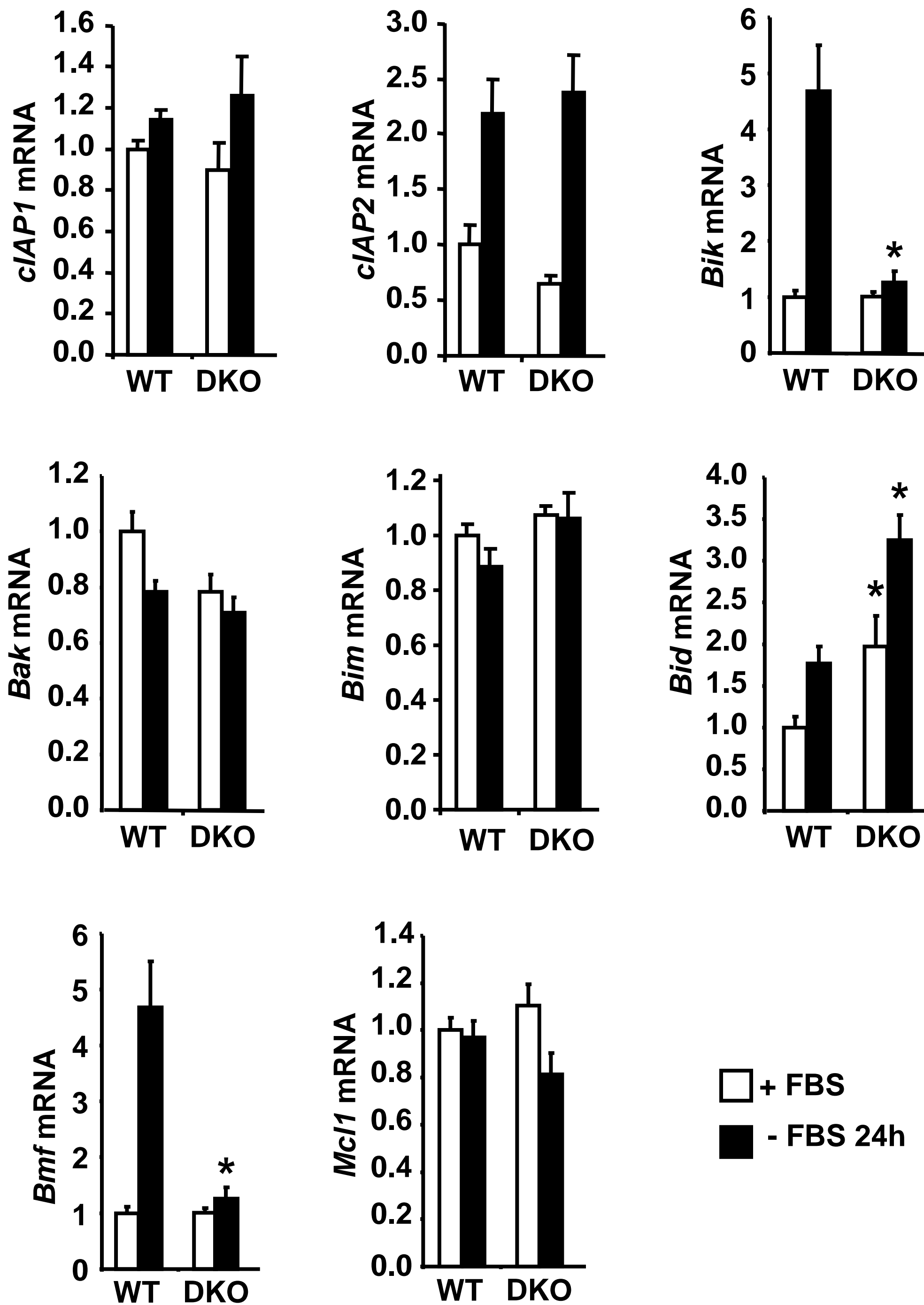

Fig. S5: mRNA abundance of proteins involved in apoptosis in wild-type and DKO cells. mRNA abundance was quantified by real-time PCR in confluent WT and DKO cells in the presence or in the absence of serum for 24 hours. Data were normalized to *TBP* mRNA levels. Results are mean ± S.E.M. from 5 independent experiments. * indicates a significant difference compared to WT cells, p value <0.05 by Student's t test.

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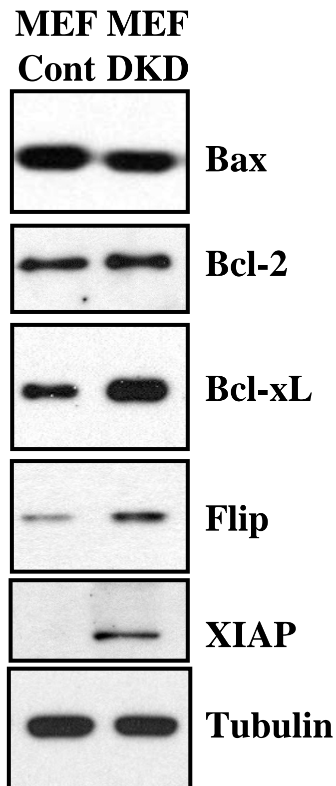

Fig. S6: Abundance of apoptotic proteins in control and DKD MEFs.
Bax, Bcl-2, Bcl-xL, Flip, XIAP and beta tubulin protein levels were measured in confluent serum fed MEF Cont and MEF DKD cells. One representative blot from 3 independent experiments is shown.

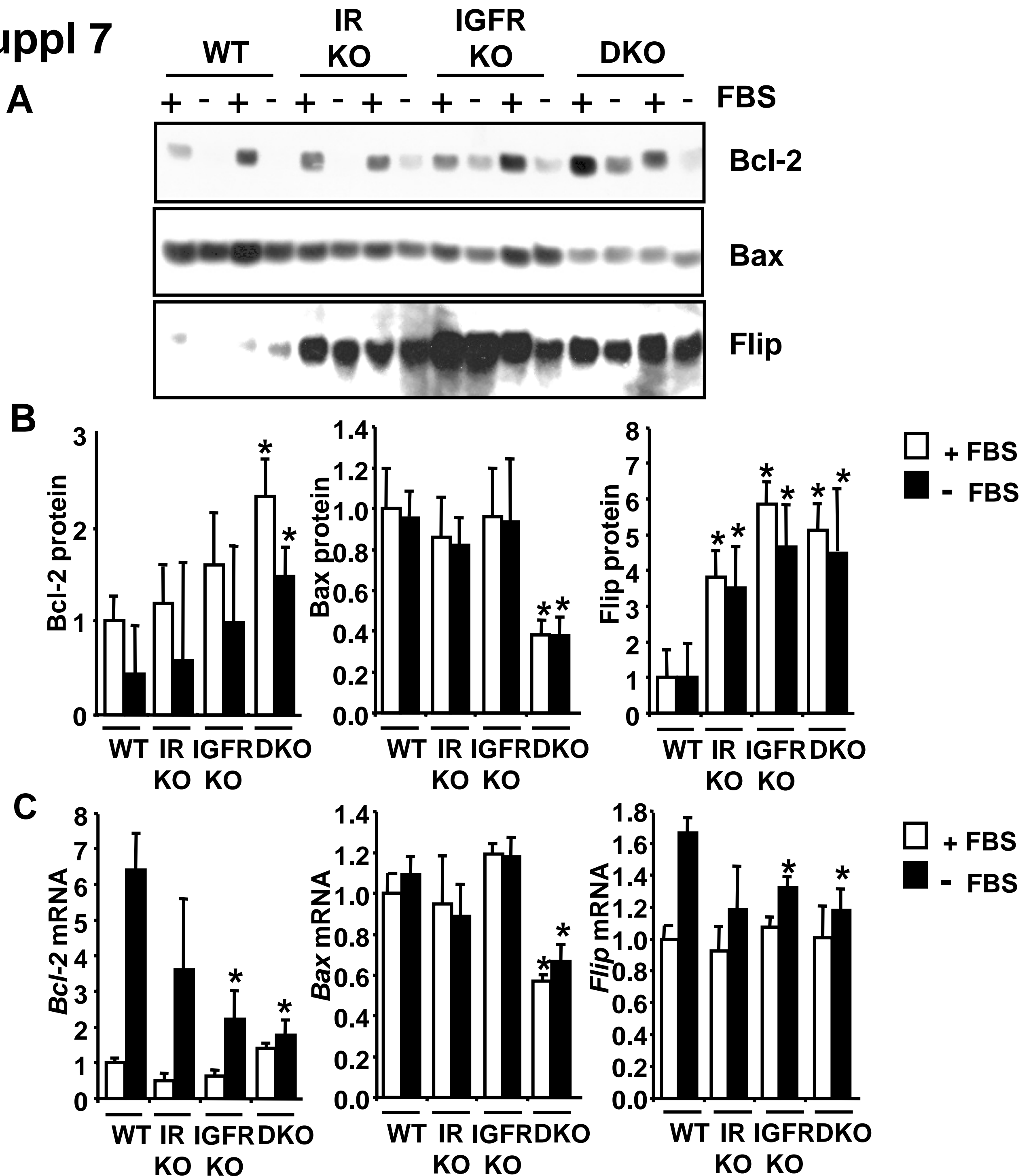

Fig. S7: Bcl-2, Bax, and Flip abundance in IRKO and IGFRKO cells.
A) Confluent WT, IRKO, IGFRKO and DKO cells were serum starved for 6 hours. Immunoblots were performed on protein lysates from floating and attached cells. One representative western blot from 4 independent experiments is shown. B) Quantification of Bcl-2, Bax and Flip in WT, IRKO, IGFRKO and DKO confluent cells kept in the presence or in the absence of serum for 6 hours. Results are normalized to beta tubulin levels and are mean ± S.E.M. from 4 independent experiments. * indicates p value <0.05 by Student's t test. C) Bcl-2, Bax and Flip mRNA levels were measured in WT, IRKO, IGFRKO and DKO confluent cells kept in the presence or in the absence of serum for 24 hours by real time PCR and were normalized to *TBP* mRNA levels. Results are mean ± S.E.M from 4 to 5 independent experiments. * indicates a significant difference compared to WT cells, p value <0.05 by Student's t test.

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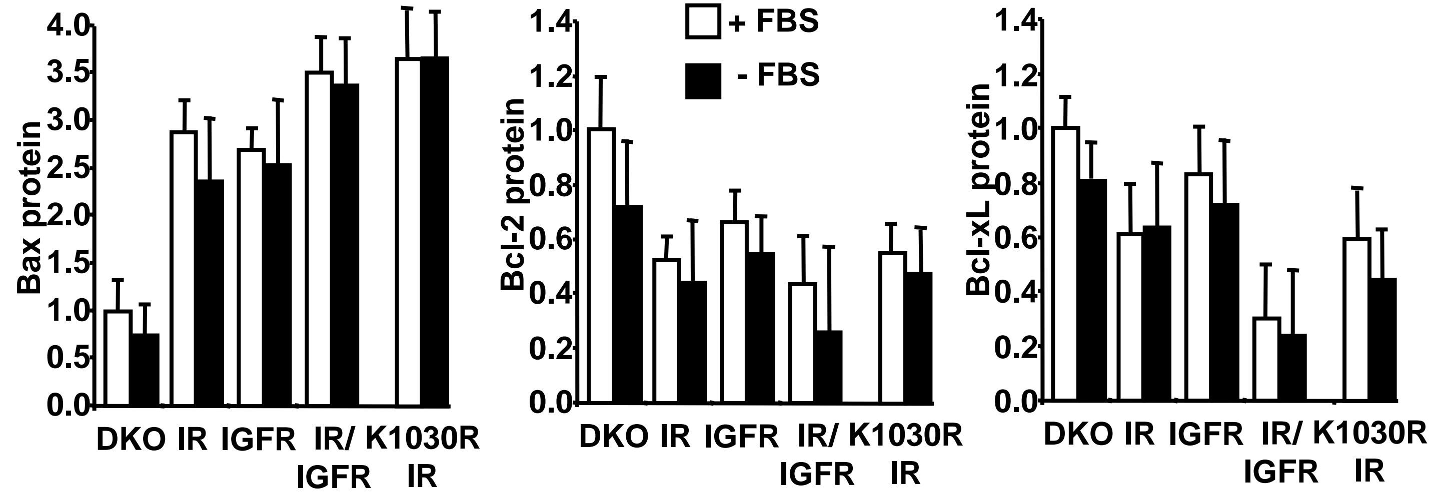

Fig. S8: Bax, Bcl-2, and Bcl-xL abundance in DKO cells expressing IR, IGF1R, or an inactive IR mutant.

Confluent DKO cells expressing hIR, hIGFR, or both or K1030R mutant IR were maintained in the presence or in the absence of serum for 6 hours and immunoblots were performed. Results are normalized to β tubulin and are mean ± S.E.M. from 3 independent experiments.

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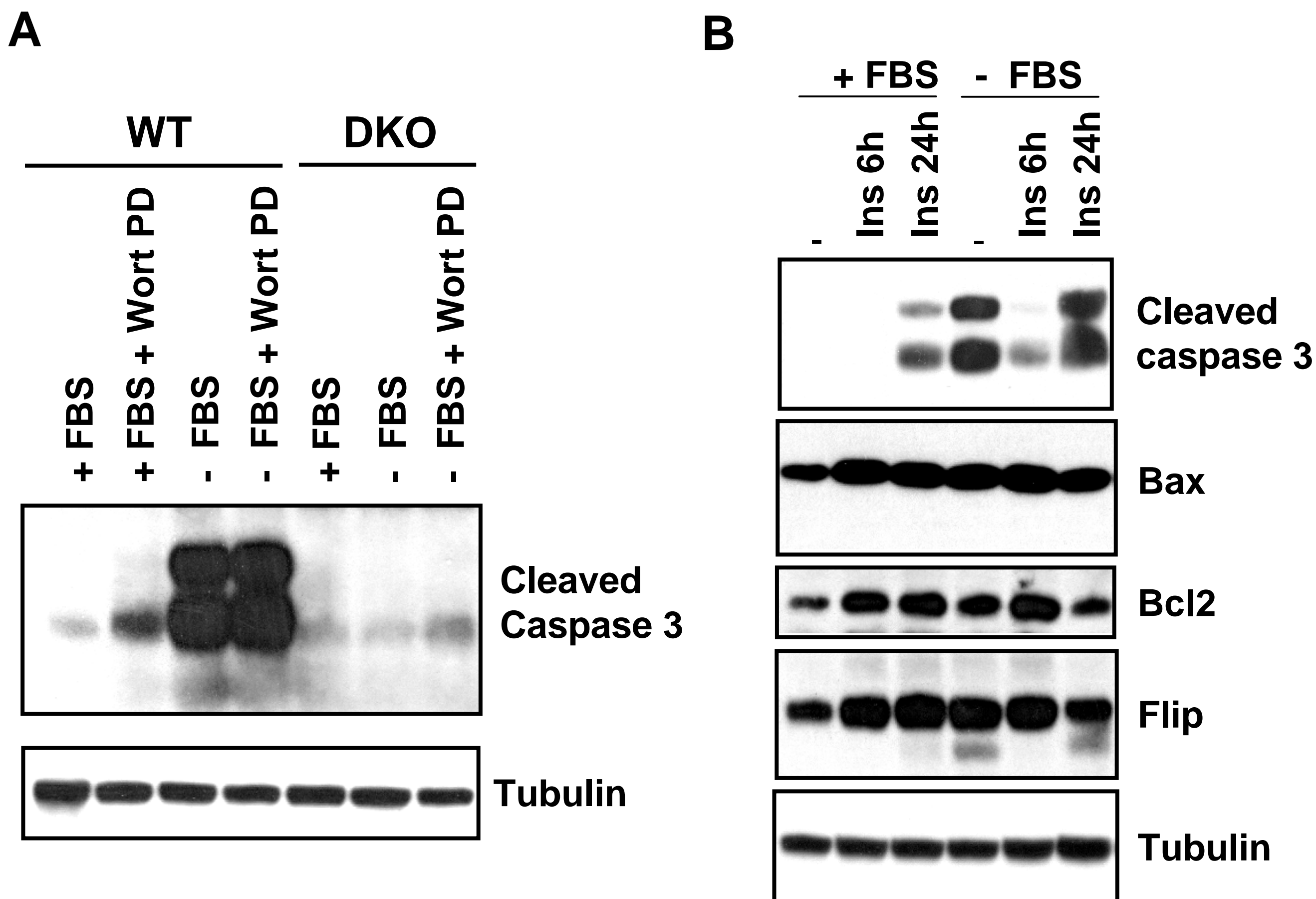

Fig. S9: Effect of inhibition or activation of insulin signaling on caspase 3 cleavage and Bax, Bcl-2, and Flip abundance in wild-type cells.
A) Confluent WT cells were pretreated or not with a combination of wortmannin (100 nM) and PD98059 (20 nM) for 1 hour and then incubated in medium with or without serum containing or not the inhibitors for 6 hours. Caspase 3 immunoblot was performed on protein lysates from floating and attached cells. One representative blot from 3 independent experiments is shown.
B) Confluent WT and DKO cells were treated with insulin (100 nM) for 6 or 24 hours in the presence or in the absence of serum. Immunoblots were performed on protein lysates from floating and attached cells. One representative blot from 3 independent experiments is shown.