

SUPPLEMENTARY DATA

CRISPR/Cas9 Genome Editing to ablate REDD1 in R28 cells. pCas9-REDD1 guide plasmid and REDD1 donor plasmid were obtained from Blue Heron Biotech LLC (Bothell, WA). Rat REDD1/DDIT4 (NP_543182) guide and donor fragments were synthesized *de novo* according to the sequences found below. A functional GFP-Loxp-Puro-Loxp cassette was cloned into the linker between the homologous arms of the REDD1/DDIT4 donor sequence using *SpeI* and *Ascl* and inserted into pUC-MinusMCS (Blue Heron). The guide fragment was cloned into pCas-Guide (Origene). R28 cells were co-transfected with a 1:1 ratio of guide and donor plasmid using FugeneHD™ transfection reagent (Promega, Madison, WI) according to the manufacturer's instructions. After 48 hours of transfection, cells expressing GFP were selected using a BDFACsaria™ cell sorter, and single cells were isolated for culture. Cells were amplified for eight passages then treated with 4 µg/µL puromycin. After two weeks of selection, colonies were maintained in medium containing 1.3 µg/mL puromycin. Colonies were screened for stable genomic REDD1 gene deletion by fluorescence microscopy to confirm the presence of GFP protein (Fig S1A), PCR to confirm GFP insertion into the REDD1 gene (Fig S1B), and Western blotting for REDD1 and GFP protein expression (Fig S1C). A STR marker profile of wild-type and REDD1 CRISPR R28 cells was produced by IDEXX BioResearch (Columbia, MO) and confirmed the Sprague-Dawley rat genotype.

Rat REDD1/DDIT4 donor sequence:

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GGACCTTGGCAGCTGCTGAAAGCCGCCTCGGATCCCTTCCAGAAAGGGGGTGTGGCCGAGG
CTAAGGCGAGGGGAGAGGGGTTTCGGCCGGGAGCGTTCTGGGGCTTCGTGGGGGCGGGGCC
AGGCCGCTGTCACCAGGCAGGAGAGAACGTTGCGAACGTGCGCCCGGTGCCTATTGGATG
AAGTGGGCCCACTCCCGGGTCTCGATTGGGCTGAGACCTGGAGGGGGCGTGGTCTCGTTCG
GGAGAGCCCTTATAGGCTGCTGCTCGCTGGTGTAGGGATCACAGCAGGCAGGGGGGAGG
TGCAGAGGGGCTGGAAAGGACAGGTCCGGGCAGCGATCGGGGGTTGGCATCAGTTCGCTC
ACCCTTCGAGAGGCAGATCGCTCTTGTCCGCAATCTTCGCTGACCGCGCTAGCTGGTGAGT
GTCCCTCCCGGGTGTGCACATCTTGGCGGGGAGCGACCTCGGCTGTTGGGGCCCCAGAGCC
CTCAATCCTGTGCAACGCCAACACTGACGCCAAATCCTTGTCTCTCTTGTCTTTCAGCGGCT
TCTGTGCTCCTTCGCCGAACCTCATCAACCAGCGTCTCGGCGTCTGACCTCGCCACTAGTTT
CGCGTTGCAGCATCATTGCGGCGCGCCTGGGGGTCTGCGGCCAGAGAAGAGGGCCTTGACC
GCTGCGCGAGCCTGGAGAGCTCGGACTGCGAGTCCCTGGACAGCAGCAACAGTGGCTTTGG
GCCGGAGGAAGGTGAGTACTGAGCAAGTGCTGAATTGGAGGTGGGGACGAAGCGAGCTGA
ACCGGATTAAGCCACCTTAGTTTCCCAATCCACAGCACGAAAAAACAGATTATCCCAGGG
TTAGAAGGCACAAGAGTGGGATGGAACTGAGGCTCGGAATGGGAAGGAACGCCGTTTTTC
TTAGTAAACTGCACCTCCCCCTCCCGTACAGCTCCTTTGGAAGGCGCTCTAATACCCTTCC
CTGTGTTCTCTCTTCCAGACTCCTCATACTGGATGGGGTGTCTCTGCCTGACTTTGAGCTGC
TCAGTGACCCCGAGGATGAGCACCTGTGTGCCAACCTGATGCAGCTGCTGCAGGAGAGCCT
GTCCAGGCGCGATTGGGCTCGCGGCGCCCTGCGCGCCTGCTGATGCCGAGCCAGCTG
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pCas9-REDD1/Rat DDIT4 Guide Sequence:

GGAGCGCGGCGGCCGATCAG

PCR primers used to confirm insertion of GFP into the REDD1 gene:

REDD1 Rat 5' UTR Forward, 5'-GGAGGTGCGAGAGGGCTGGA-3'

GFP Reverse, 5'-TGCGGCACTCGATCTCCATG-3'

Flow Cytometry. Cell Apoptosis was characterized using the BD Pharmingen™ PE Annexin V Apoptosis Detection Kit I (Cat #559763 Lot # 6179925 BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. The percentage of live, early apoptotic (Annexin V positive/7-Aminoactinomycin D [7-AAD] negative), late-dual apoptotic (Annexin V positive/7-AAD positive) and

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Dead/Necrotic (Annexin V negative/7-AAD positive) were determined using a BDFACSCanto flow cytometer (San Jose, CA). The data was analyzed using BD FACSDiva (San Jose, CA).

Buffer Compositions.

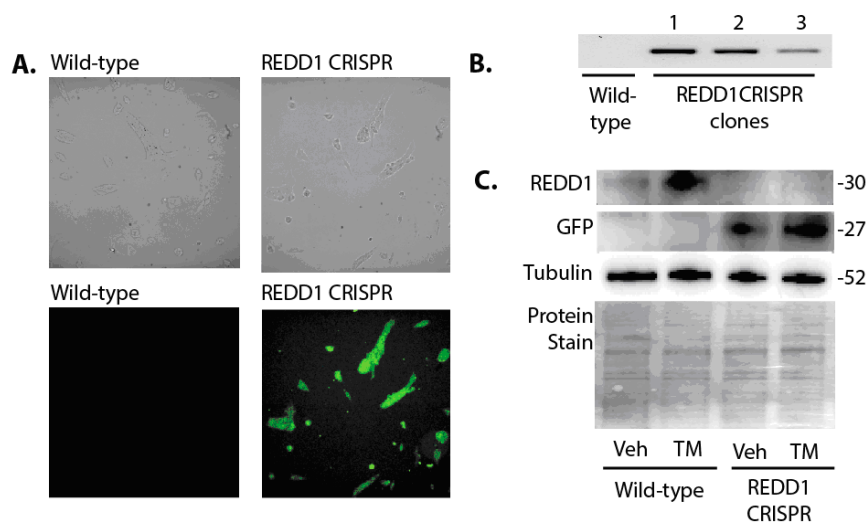
Buffer A: 20 mM HEPES, 2 mM EGTA, 100 mM KCl, 0.2 mM EDTA, 50 mM β -glycerophosphate, 0.5% sodium deoxycholate, 2.5% Triton-X 100, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM benzamidine, 10 μ M microcystin, and 10 μ L/mL protease inhibitor cocktail (PIC; P8340, Sigma-Aldrich).

Buffer B: 100 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 2 mM EDTA, 10 mM HEPES, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM benzamidine, 10 μ M microcystin, and PIC.

Buffer C: 1.0% Triton X-100, 50 mM β -glycerophosphate, 2 mM EDTA, 5 mM EGTA, 10 mM HEPES, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM benzamidine, 10 μ M microcystin, and PIC pH 7.2.

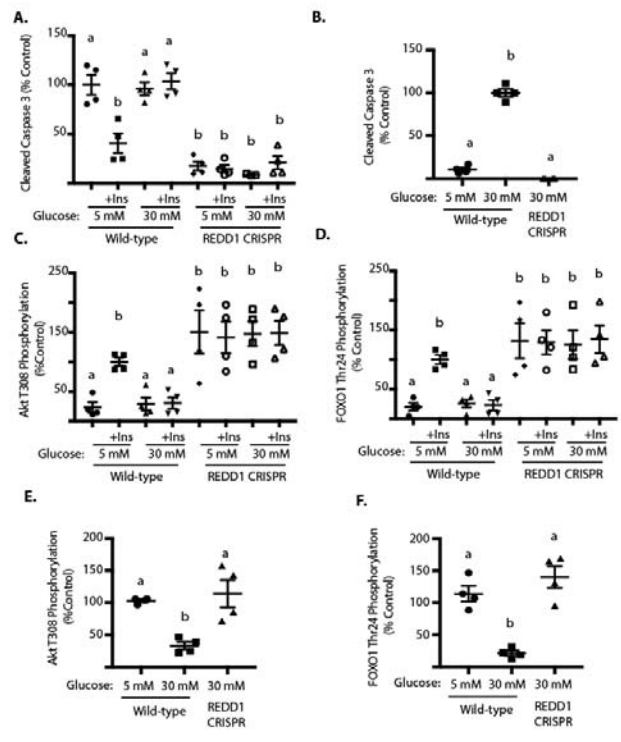
Buffer D: 10 mM Tris HCl, 50 mM β -glycerophosphate, 2 mM EDTA, 5 mM EGTA, 10 mM HEPES, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM benzamidine, 10 μ M microcystin, and PIC pH 7.2.

Supplementary Figure S1. Genomic deletion of REDD1 in R28 retinal cells by CRISPR. R28 cells were maintained in DMEM containing 5 mM glucose and supplemented with 10% FBS. REDD1 deficient R28 cells (REDD1 CRISPR) were generated using CRISPR. To facilitate selection, the donor cassette encoded for GFP expression in addition to puromycin resistance. Following transfection, R28 cells expressing GFP were selected using a cell sorter, GFP expressing cells were grown into colonies, and then selected for puromycin resistance. **A.** Stable GFP expression in REDD1 CRISPR cells was confirmed by fluorescence microscopy. **B.** Genomic DNA from wild-type R28 cells or REDD1 CRISPR colonies was screened by PCR to confirm insertion of GFP into the REDD1 gene. **C.** Western blotting for REDD1, GFP, and tubulin protein expression in wild-type and REDD1 CRISPR R28 cells treated with either vehicle (Veh) or 10 μ g/mL tunicamycin (TM) for 4 h. Protein molecular mass in kDa is indicated at *right* of blots. Gel loading was assessed by protein stain.



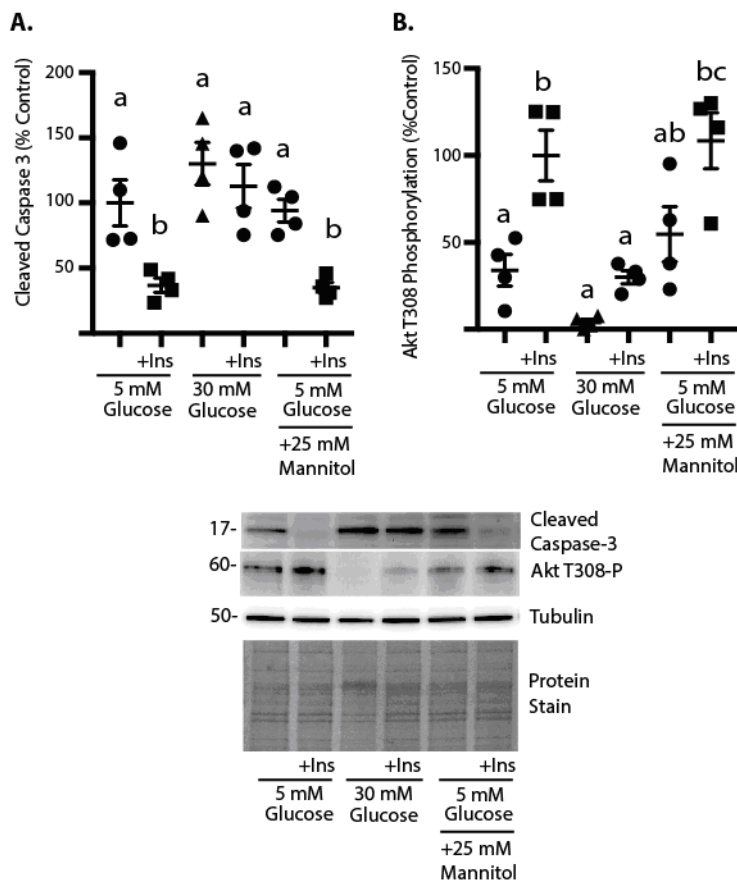
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Supplementary Figure S2. Quantification of Western Blots found in Figure 1. R28 cells were maintained in DMEM containing 5 mM glucose and supplemented with 10% FBS. *A, C, D.* Wild-type and REDD1 CRISPR R28 cells were serum deprived for 24 h in medium containing 5 or 30 mM glucose plus the presence or absence of insulin (+Ins). *B, E, F.* Wild-type and REDD1 CRISPR R28 cells were exposed to medium containing 10% FBS and either 5 or 30 mM glucose for 24 h. Caspase 3 cleavage, Akt, and FOXO1 expression, as well as phosphorylation of Akt and FOXO1 were assessed by Western blotting. Gel loading was assessed by protein stain. Values are means + S.E. for two independent experiments ($n=4$). Statistical significance is denoted by the presence of different letters above each scatter plot on the graphs. Scatter plots with different letters are statistically different, $p<0.05$.



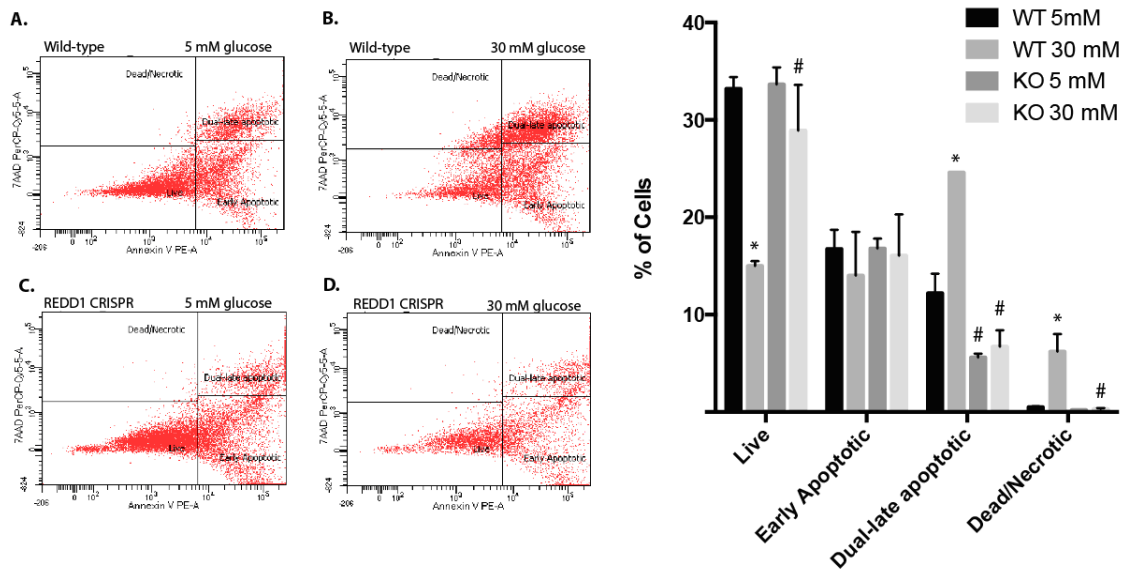
SUPPLEMENTARY DATA

Supplementary Figure S3. Hyperglycemic conditions promote caspase 3 cleavage and attenuate insulin stimulated Akt Thr308 phosphorylation. R28 cells were maintained in DMEM containing 5 mM glucose and supplemented with 10% FBS. Cells were serum deprived for 24 h in medium containing 5 or 30 mM glucose plus the presence or absence of insulin (+Ins). As an osmotic control, cells were serum deprived in 5 mM glucose plus 25 mM mannitol plus the presence or absence of Ins. Caspase 3 cleavage, Akt phosphorylation, and tubulin expression were assessed by Western blotting. Gel loading was assessed by protein stain. Protein molecular mass in kDa is indicated at the *left* of blots. Values are means + S.E. for two independent experiments ($n=4$). Statistical significance is denoted by the presence of different letters above each scatter plot on the graphs. Scatter plots with different letters are statistically different, $p<0.05$.



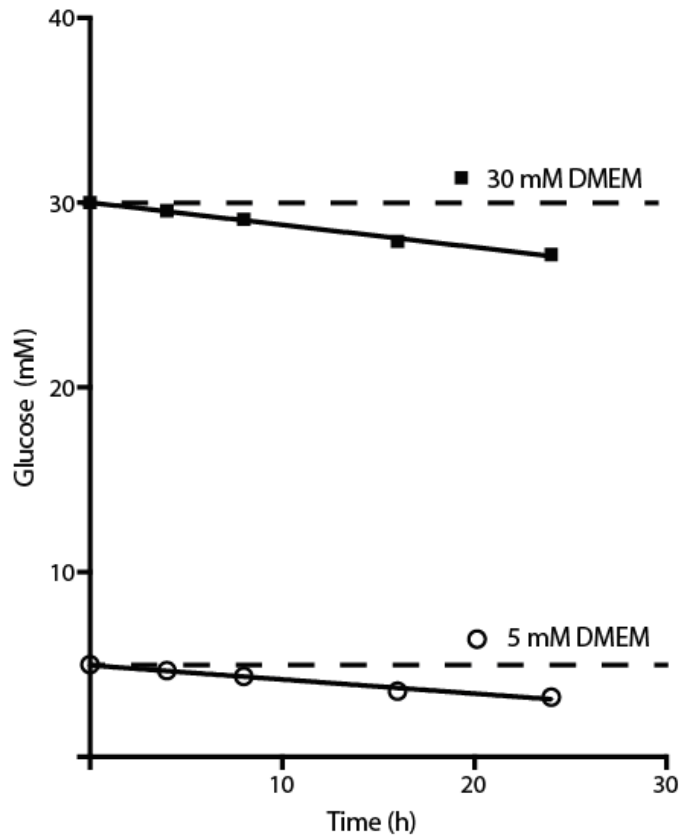
SUPPLEMENTARY DATA

Supplementary Figure S4. REDD1 deletion attenuates hyperglycemia-induced cell death in R28 cells. Wild-type (WT) and REDD1 CRISPR (KO) R28 cells were maintained in DMEM containing 5 mM glucose and supplemented with 10% FBS. Wild-type (A-B.) or REDD1 CRISPR cells (C-D.) were exposed to medium containing 10% FBS and either 5 (A. & C.) or 30 mM glucose (B. & D.) for 24 h. The effect of hyperglycemic conditions on cell death was assessed by treating cells with PE Annexin V and 7-amino-actinomycin D (7-AAD) followed by flow cytometry. Results are representative of two independent experiments; within each experiment, two independent replicates were analyzed. Statistically significant differences ($p < 0.05$) are denoted by * *versus* cells treated with medium containing 5 mM glucose and # *versus* wild-type.



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Supplementary Figure S5. Glucose consumption by R28 retinal cells in culture. R28 cells were maintained in DMEM containing 5 mM glucose and supplemented with 10% FBS. Cells were serum deprived for 24 h in medium containing 5 or 30 mM glucose as described in Fig 1A. Values are means + S.E. ($n=3$). Error bars are obscured by data points.



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Supplementary Table S1. Antibodies used for Western blotting. Primary and secondary antibodies used for Western blotting are listed by manufacturer and catalogue number. Antigen-antibody interaction was visualized with enhanced chemiluminescence (Clarity Reagent; Bio-Rad Laboratories, Inc; Hercules, CA) using a ProteinSimple Fluorochem E imaging system (Santa Clara, CA).

Cell Signaling Technology Antibodies	Cat #
AKT-p (T308)	9275L
AKT-T	9272S
FOXO1-p (T24)/ FOXO3a-p (T32)	9464S
FOXO1-T	2880S
Cleaved Caspase 3	9664S
Protein Tech Antibodies	Cat #
REDD1-T	10638-1-AP
Santa Cruz Antibodies	Cat #
GAPDH	6C5
Bethyl Laboratories, Inc	Cat #
Goat anti-Rabbit IgG-heavy and light	A120-101P
Goat anit-Mouse IgG-heavy and light	A90-116P