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Supplementary data table 1

Primers	
chrebp-HA_fwd	CCA GTG TGG TGG AAT TCT GCA TGG CGC GCG CGC TGG CGG ATC TAT CCG TGA AC
chrebp-HA_rev	GCT GGA TAT CTT TAA GCG TAA TCT GGA ACA TCG TAT GGG TAT AAT GGT CTC
gibson-MLX_fw	CCA CTA GTC CAG TGT GGT GGG CGA TCG CCA TGA CGG AG
gibson-MLX_rv	ACT GTG CTG GAT ATC TGC AGT TAA ACC TTA TCG TCG TCA TCC TTG TAA TC
mm_MLX-alpha/beta_Rv	CTT GAC CCA AGG GTC CTC
mm_MLX-alpha/beta_Fw	GTC GAG TAT GCC TAC AGT GAC
mm_MLX-alpha_Rv	AGG ATC CAG GCT GTT GTC
mm_MLX-alpha_Fw	GAT GAT GAG GAC AGT GAT TAT CAG
mmMLX_S91AS94	AAG GGG GCT GTA GTG GCC AGA GCT AAT AGC ATC G
mmMLX_S86A	GTG GGC GGC TTC TAC AAA AAG CCC AGG ATC CAG GCT G
mmMLX_S101-106A-f	GCC GCC GCT GCC GCT GCT GTC CCC AAC ACA GAT GAT G
mmMLX_S98A-rv	GCC GAT GGC ATT AGC TCT GGA CAC TAC A
mmMLX_S115A-fw	GAG GAC GCT GAT TAT CAG CAG GAG TCC TAC AAG
mmMLX_T110A-rv	ATC ATC TGC GTT GGG GAC AGA AGA GGC ACT G
mmMLX_S86DT87D-rv	GTG GTC GTC TTC TAC AAA AAG CCC AGG ATC CAG GCT G
mmMLX_S91DS94D-fw	AAG GGG GAT GTA GTG GAC AGA GCT AAT AGC ATC G
mmMLX_S98D_rv	GCC GAT GTC ATT AGC TCT GGA CAC TAC A
mmMLX_S101106D-fw	GAC GAC GAT GCC GAT GAT GTC CCC AAC ACA GAT GAT G
mmMLX-T110D-rv	ATC ATC GTC GTT GGG GAC AGA AGA GGC ACT G
mmMLX_S115D-fw	GAG GAC GAT GAT TAT CAG CAG GAG TCC TAC AAG
mmMLX_105-115A_fw	GCT GCT GTC CCC AAC GCA GAT GAT GAG GAC GAT GAT TAT CAG CAG GAG TCC TAC AAG
mmMLX_94-103A_rv	GGC AGC GGC GGC GCC GAT GGC ATT AGC TCT GGC CAC TAC ACT CCC CTT GTG GG
mmMLX_105-115D_fw	GAT GAT GTC CCC AAC GAC GAT GAT GAG GAC GAT GAT TAT CAG CAG GAG TCC TAC AAG
mmMLX_94-103D_rv	GGC ATC GTC GTC GCC GAT GTC ATT AGC TCT GTC CAC TAC ACT CCC CTT GTG GG
mmMLX_105-110A_rv2	ATC ATC TGC GTT GGG GAC AGC AGC G
rnHK2-SA155 fw	TTC ACC TTC GCG TTC CCC TGC CAC CA
rnHK2-SA155 rv	CAG GGG AAC GCG AAG GTG AAA CCC AGA GGG
rnHK2-SA603 fw	ACA TTC GCC TTC CCT TGC CAG C
rnHK2-SA603 rv	AGG GAA GGC GAA TGT GAA ACC CAA AGG C
pUAST-mmMLX-dmKozak_EcoRI_fw	GGG AAT TCC AAA ATG ACG GAG CCG GGC GCC TC
MLX_cloning_XhoI_rv	GGC TCG AGT CAG TAG AGT TGG TTT TTC AAC TGA TGA AGG AC
gRNA1-GSK3A FW	CAC CGG GCC ACC CGG TAC ACT GTC T
gRNA1-GSK3A-RV	AAA CAG ACA GTG TAC CGG GTG GCC C
gRNA2-GSK3A-FW	CAC CGG GAA CTA GTC GCC ATC AAG A
gRNA2-GSK3A-RV	AAA CTC TTG ATG GCG ACT AGT TCC C
GSK3A-gRNA1-seq-FW	ATG CGT AAG CTG GAC CAC TG
GSK3A-gRNA1-seq-RV	ATT CAG TCA GGC CTT GCC TG
GSK3A-gRNA2-seq-FW	GTC CCC AAC GAG CTT CCT G
GSK3A-gRNA2-seq-RV	CAA GAA TTT GCT TAT TAA GCA CTT ACA TTG
mm_Fasn-fw	GCT GCG GAA ACT TCA GGA AAT
mm_Fasn-rv	AGA GAC GTG TCA CTC CTG GAC TT
mm_Acly-fw	GCC AGC GGG AGC ACA TC
mm_Acly-rv	CTT TGC AGG TGC CAC TTC ATC
pGEX4T2_fwd	CTA CAC GTA AGC GGC CGC ATC GTG ACT G
pGEX4T2_rev	TCC GTC ATA ACC TGG GGA TCC ACG CGG A
mlx_fwd	GAT CCC CAG GTT ATG ACG GAG CCG GGC G

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mlx_rev	ATG CGG CCG CTT ACG TGT AGA GTT GGT TTT TCA ACT GAT G
RV_MLX_TID	CAC AGT ATT GTC TTT GCT AGC GTA GAG TTG GTT TTT CAA CTG ATG AAG GAC
Fwd_MLX_mini_and_TID	GGA CAG CAC CGC TAG CAT GAC GGA GCC GGG CG
pcdna3.1_MLX_fwd	CTG CAG ATA TCC AGC ACA GTG GCG G
pcdna3.1_MLX_rev	GAG CGG CCG CGT ACG CGT
eGFP_fwd	ACA CGC GTA CGC GGC CGC TCG TGA GCA AGG GCG AGG AG
eGFP_rev	ACT GTG CTG GAT ATC TGC AGT TAC TTG TAC AGC TCG TCC ATG
mmChREBP-S140A-fw	CGG AGG AAG GCC CCA GTG TGT GGT TTC G
mmChREBP-S140A-rv	CAC TGG GGC CTT CCT CCG TTG CAC ATA CTG
mmChREBP-S196A-fw	CGT AAG TCC GCC AGG GAA GGG GAT TTC CTG
mmChREBP-S196A-rv	TTC CCT GGC GGA CTT ACG GAG CCG CTT TTT G
mmChREBP-S626A-fw	CGG CGA CTA GCC GGG GAT CTC AAC TCC ATA C
mmChREBP-S626A-rv	GAG ATC CCC GGC TAG TCG CCG CTC ACT GCC
mmChREBP-T665A-fw	CGA CGT ATC GCC CAC ATC TCC GCG GAG CAG
mmChREBP-T665A-rv	GGA GAT GTG GGC GAT ACG TCG GTT CTC CAT CTT G

Supplementary data table 2

Antibodies		
Akt (pan)	Cell Signaling Technology, Inc	Cat#2920, (40D4)
Phospho-Akt (Ser473)	Cell Signaling Technology, Inc	Cat#4060, (D9E)
CK2α	Cell Signaling Technology, Inc	Cat#2656
DYKDDDDK Tag	Cell Signaling Technology, Inc	Cat#8146, (9A3)
GSK-3α/β	Cell Signaling Technology, Inc	Cat#5676, (D75D3)
HA-Tag	Cell Signaling Technology, Inc	Cat#3724,(C29F4)
HA-Tag	Cell Signaling Technology, Inc	Cat#2367,(6E2)
Hexokinase II	Cell Signaling Technology, Inc	Cat#2867,(C64G5)
MLX	Cell Signaling Technology, Inc	Cat#85570,(D8G6W)
S6 Ribosomal Protein	Cell Signaling Technology, Inc	Cat#2217,(5G10)
Phospho-S6 Ribosomal Protein (Ser235/236)	Cell Signaling Technology, Inc	Cat#4856,(2F9)
Calnexin	Invitrogene	Cat#PA5-34754
Anti-Thiophosphate ester antibody	Abcam	Cat#ab92570
IRDye® 800CW Goat anti-Rabbit IgG	LI-COR Biotech	Cat#926-32211
IRDye® 680RD Goat anti-Mouse IgG Secondary Antibody	LI-COR Biotech	Cat#926-68070
Anti-FLAG® M2 Affinity Gel	Sigma-Aldrich	A2220

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Supplementary data table 3

Plasmids		
pMSCV-ChREBPalpha	Michael Schupp Lab (PMID: 26181104)	N/A
pCMV-MLX-myc-FLAG	Origene	Cat#MR204137
pCDNA 3.1 -ChREBP-HA	This paper	N/A
pCDNA 3.1 -MLX	This paper	N/A
pCDNA 3.1 -MLX-A (94-115)	This paper	N/A
pCDNA 3.1 -MLX-D (94-115)	This paper	N/A
pCDNA 3.1 -MLX-S110-115A	This paper	N/A
pGL3-ChoRE-luc	Michael Schupp Lab (PMID: 33023907)	N/A
pNL1.1[Nluc/TK]	Promega	Cat#N150A
pCDNA 3.1 -rnHK2	This paper	N/A
Lenti-CRISPR-V2-GSK3A-1	This paper	N/A
Lenti-CRISPR-V2-GSK3A-2	This paper	N/A
pCDNA 3.1 -MLX-alpha	This paper	N/A
pCDNA 3.1 -MLX-beta	This paper	N/A
3xHA-TurboID-NLS_pCDNA3	Alice Ting Lab (Addgene)	Addgene plasmid Cat#107171
pCDNA 3.1 -MLX-TurboID	This paper	N/A
lentiCRISPR v2	Feng Zhang Lab (Addgene)	Addgene plasmid Cat#52961
pGEX-MLX-WT	This paper	N/A
pGEX-MLX-WT	This paper	N/A
pUAST-mMLX-WT	This paper	N/A
pUAST-mMLX-WT	This paper	N/A

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Figure supplementary 1. MLX phosphorylation promotes ChREBP-MLX activity, related to Fig. 1.

a. Phosphorylation sites on MLX alpha, beta, or gamma isoforms according to PhosphoSitePlus® (https://phosphosite.org).

b. MLX phosphorylation in 293T cells expressing MLX-WT or -A. Protein lysates were treated with calf intestine phosphatase (CIP) or CIP and phosphatase (PP) inhibitors. N=3.

c. Luciferase reporter assay for ChREBP-MLX activity.

d. ChREBP-MLX luciferase reporter activity in 293T expressing ChREBP, MLX-WT, and either HK2 or GCK. Cells were starved for glucose and treated with 25 mM glucose for 3 hours. One-way ANOVA, ***p<0.001, ****p<0.0001. N=3.

e. ChREBP-MLX luciferase reporter activity in 293T cells expressing ChREBP, MLX-WT, and either HK2 or kinase dead HK2 (HK2-KD). Cells were starved for glucose and treated with 25 mM glucose for 3 hours. One-way ANOVA, ***p<0.001, ****p<0.0001. N=3.

f. ChREBP-MLX luciferase reporter activity in 293T expressing HK2, MLX-WT or -A and either ChREBP-WT or -4A. Cells were starved for glucose and treated with 25 mM glucose for 3 hours. One-way ANOVA, **p<0.01, ****p<0.0001, ns=not significant. N=3.

Figure supplementary 2. MLX phosphorylation on an evolutionarily conserved motif promotes sugar response in *Drosophila*, related to Fig. 2.

a. Evolutionary relationships of the MLX sequence among animal species. Branching order shows the relationships between species and branch length displays the amount of evolutionary change between the nodes.

b. Quantification of lipid staining in Fig. 2g, each point represents lipid signals per cell. Two-way ANOVA, ****p<0.0001. n=5.

c. Quantification of lipid staining in Fig. 2g, each point represents lipid signals normalized by cell volume. Two-way ANOVA, *p<0.05, **p<0.01, ****p<0.0001, ns=not significant. n=5.

d. Hemolymph glucose levels in 3rd instar control, *mlx*^{1}, *mMlx-WT* or *mMlx-A* grown in LSD or MSD. Two-way ANOVA, *p<0.05, ***p<0.001, ns=not significant. N=3.

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Figure supplementary 3. MLX phosphorylation is required for the binding of ChREBP-MLX heterotetrametric complex on the ChoRE, related to Fig. 3.

a. Model of ChREBP-phosphorylated MLX (pMLX) structure by AlphaFold 3 with confidence scores.

b. Immunoprecipitated MLX and ChREBP proteins from in 293T cells expressing MLX-WT or -A with or without ChREBP for Electrophoretic mobility shift assay (EMSA) experiments in Fig. 3d.

c. EMSA with antibodies against ChREBP or MLX validating band identity from Fig. 3d and 5j.

d-g. Quantification in Fig. 3D. t test, *p<0.05, ****p<0.0001, ns=not significant. N=3-4.

Figure supplementary 4. Identification of CK2 and GSK3 as MLX kinases, related to Fig. 4.

a. MLX phosphorylation in 293T cells treated with the mTORC1 inhibitor rapamycin or mTORC1/mTORC2 dual inhibitor torin2. Cells were starved for glucose overnight, pretreated with 100 nM rapamycin or 250 nM torin2 for 30 min, and treated with 25 mM glucose for 60 min. S6-pS235/236 and AKT-pS473 serve as positive controls for mTORC1 and mTORC2 inhibition, respectively. CALX serves as a loading control. N=3.

b. MLX phosphorylation in 293T cells expressing TurboID-tagged MLX- WT, -A and -D. N=3.

c. Biotinylated proteins in 293T cells expressing TurboID-tagged MLX-WT, HK2 and ChREBP. Streptavidin blot serves as a control for biotinylation. N=3.

d-e. MLX phosphorylation in WT, GSK3-a KO, GSK3-b KD or GSK3-a/b KO/KD 293T cells. GSK3-a/b serves as a control for KO/KD. One-way ANOVA, *p<0.05. N=3.

f. ChREBP-MLX luciferase reporter activity in 293T cells expressing ChREBP and HK2 with MLX-WT, 94-115A, or 110-115A. Cells were starved for glucose overnight and treated with 25 mM glucose for 3 hours. One-way ANOVA, *p<0.05, ****p<0.0001, ns=not significant. N=3.

Figure supplementary 5. G6P accumulation inhibits CK2-mediated MLX phosphorylation and the binding of ChREBP-MLX tetramer on the ChoRE, related to Fig. 5.

a. MLX phosphorylation in 293T cells transfected with HK2 or empty plasmid. Cells were starved for glucose overnight and refeed with 2DG for 60 min. CALX serves as a loading control. N=3.

b. *Acly* and *Fasn* mRNA levels in 3T3 L1 adipocytes treated with 25 μM glucose or 2DG for 30 min. one-way ANOVA, ****p<0.0001, ns=not significant. N=6.

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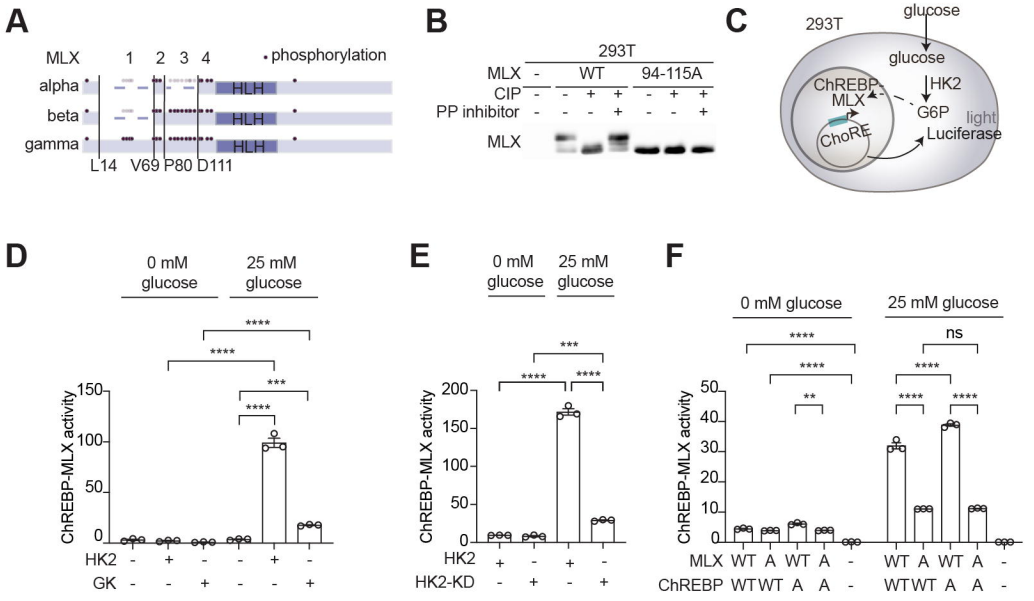
c. Immunoprecipitated MLX and ChREBP proteins used for EMSA experiments in Fig. 5J. 293T cells expressing MLX-WT with or without ChREBP were treated with glucose or 2DG for 60 min.

d-i. Quantification in Fig. 5J. t-test, *p<0.05, ***p<0.001, ****p<0.0001, ns=not significant. N=3-4.

Figure S1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gamma
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MLX
C
293T
glucose
glucose
ChREBP
MLX
HK2
G6P
ChoRE
light
Luciferase
D
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ChREBP-MLX activity
HK2
GK
E
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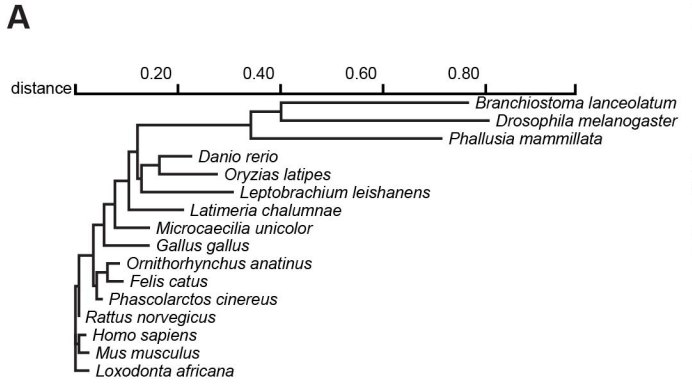
ChREBP-MLX activity
MLX
ChREBP

Figure S2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Leptobrachium leishanens
Latimeria chalumnae
Microcaecilia unicolor
Gallus gallus
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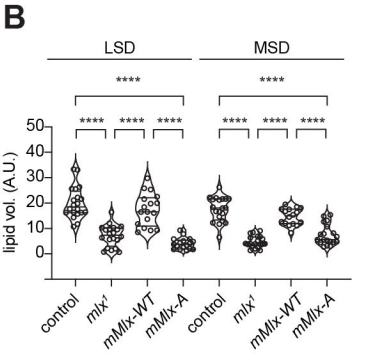
Loxodonta africana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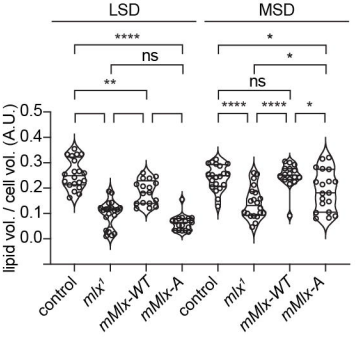
mMlx-A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MSD
lipid vol. / cell vol. (A.U.)
control
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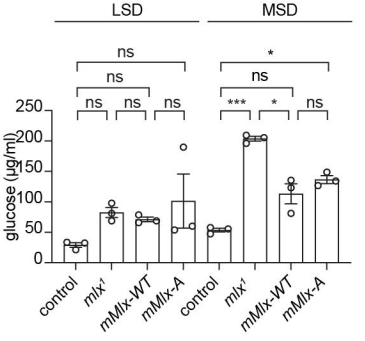
mMlx-A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MSD
glucose (μg/ml)
control
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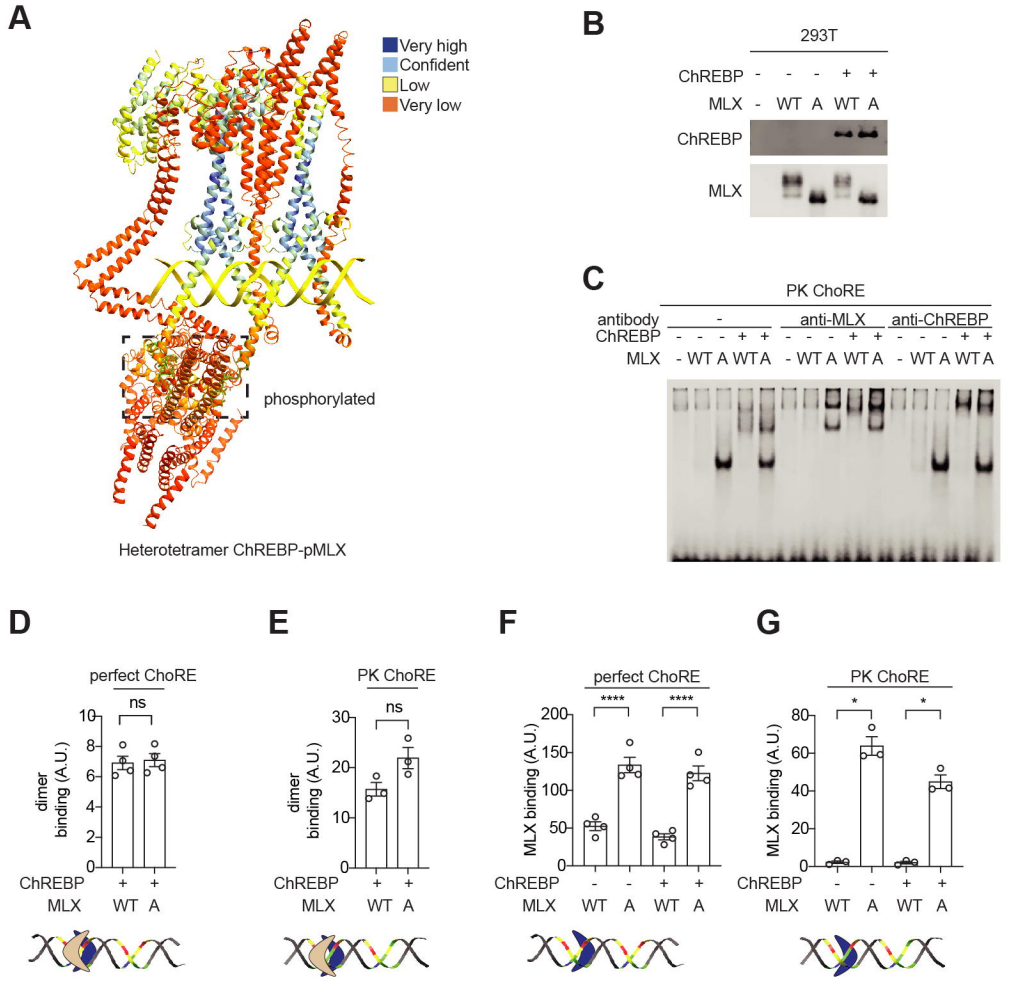
mMlx-A

Figure S3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ChREBP
MLX
C
PK ChoRE
antibody -
anti-MLX
anti-ChREBP
ChREBP - - - + + - - - + + - - - + +
MLX - WT A WT A - WT A WT A - WT A WT A
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ns
dimer binding (A.U.)
ChREBP + +
MLX WT A
E
PK ChoRE
ns
dimer binding (A.U.)
ChREBP + +
MLX WT A
F
perfect ChoRE
****
****
MLX binding (A.U.)
ChREBP - - + +
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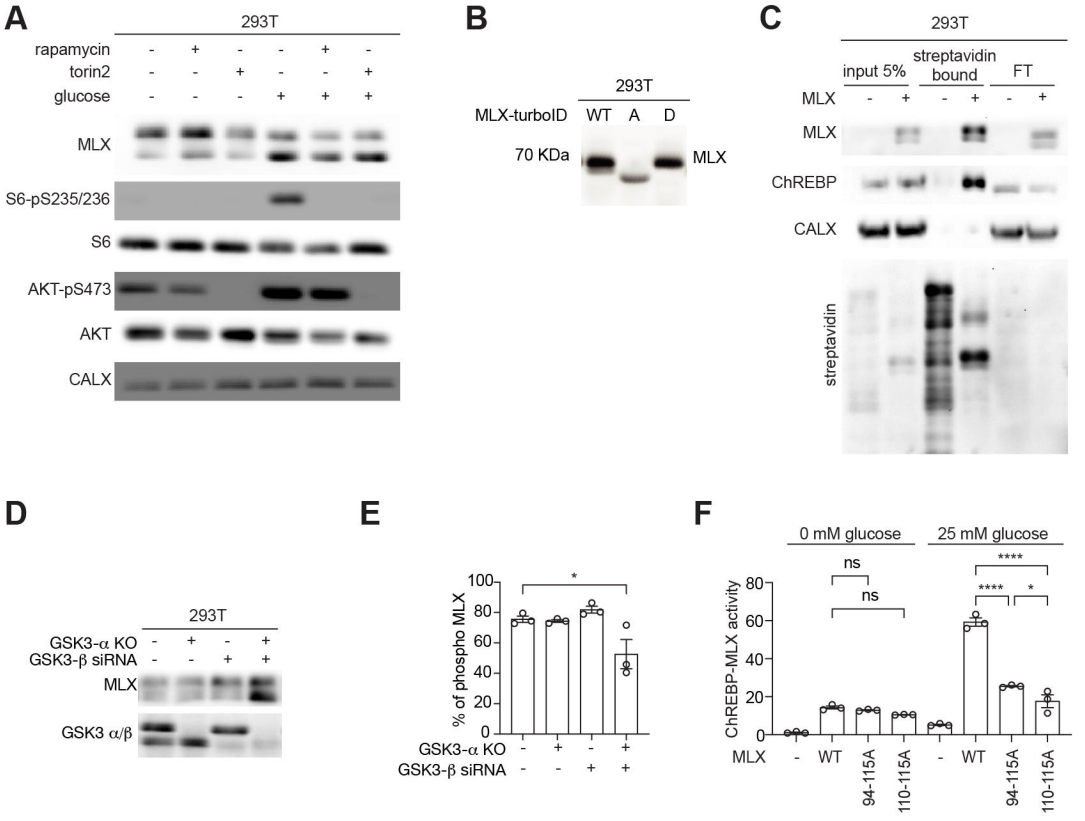
G
PK ChoRE
*
*
MLX binding (A.U.)
ChREBP - - + +
MLX WT A WT A

Figure S4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torin2
glucose
MLX
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AKT-pS473
AKT
CALX
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WT
A
D
70 KDa
MLX
C
293T
input 5%
streptavidin bound
FT
MLX
MLX
ChREBP
CALX
streptavidin
D
293T
GSK3-α KO
GSK3-β siRNA
MLX
GSK3 α/β
E
% of phospho MLX
GSK3-α KO
GSK3-β siRNA
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ChREBP-MLX activity
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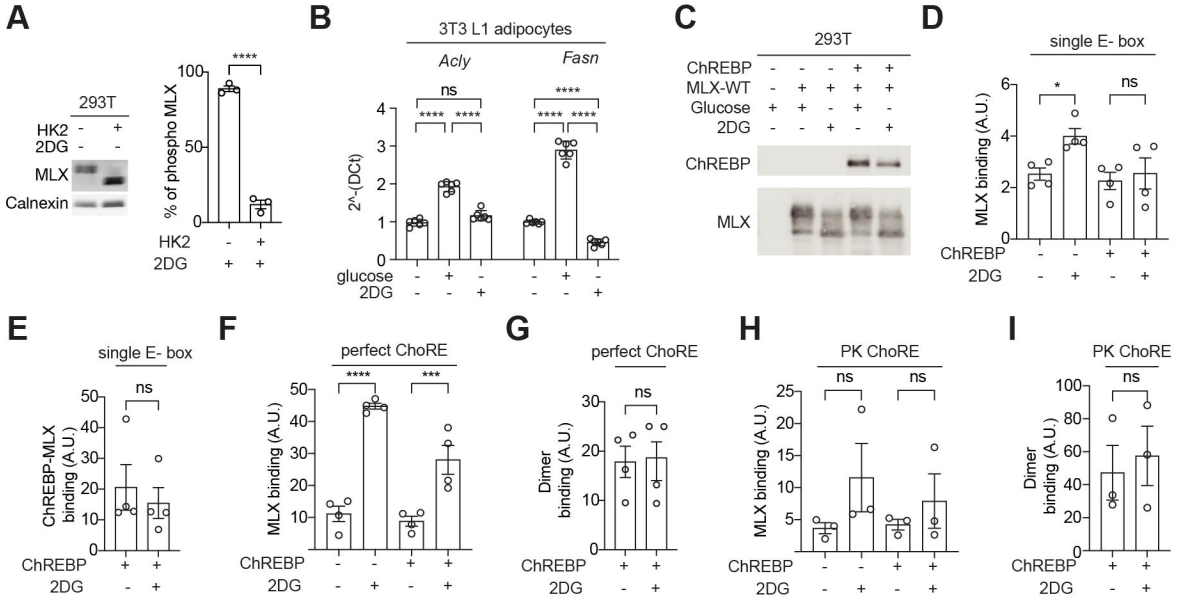
****
*
MLX
WT
94-115A
110-115A

Figure S5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293T
HK2 - +
2DG - -
MLX
Calnexin
% of phospho MLX
****
HK2 - +
2DG + +
B
3T3 L1 adipocytes
Acly
Fasn
2^-(DCt)
ns
****
glucose - + - - + -
2DG - - + - - +
C
293T
ChREBP - - - + +
MLX-WT - + + + +
Glucose + + - + -
2DG - - + - +
ChREBP
MLX
D
single E- box
MLX binding (A.U.)
*
ns
ChREBP - - + +
2DG - + - +
E
single E- box
ChREBP-MLX binding (A.U.)
ns
ChREBP + +
2DG - +
F
perfect ChoRE
MLX binding (A.U.)
****
***
ChREBP - - + +
2DG - + - +
G
perfect ChoRE
Dimer binding (A.U.)
ns
ChREBP + +
2DG - +
H
PK ChoRE
MLX binding (A.U.)
ns
ns
ChREBP - - + +
2DG - + - +
I
PK ChoRE
Dimer binding (A.U.)
ns
ChREBP + +
2DG - +