Increased IGFBP-1 phosphorylation in response to leucine deprivation is mediated by CK2 and PKC

Supplementary Legends

Supplementary Figure 1. Schematic diagram outlining the methods used in this study

Supplementary Figure 2. Dose-dependent changes in IGFBP-1 phosphorylation with Bisindolylmaleimide (BIS).

A significant decrease in IGFBP-1 phosphorylation was seen at 10 μM BIS which remained consistent at twice the inhibitor concentration (20 μM). A mid concentration between 5μM and 10μM BIS (7.5 μM) was used in subsequent experiments. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3.

Supplementary Figure 3A. HepG2 cell vitality after treatment with BIS (7.5 μM) for 24 hours

A graphical representation of cell vitality between leucine plus or minus treatments with or without BIS. A Trypan Blue exclusion assay was conducted to assess cell viability, illustrated as the ratio of live to total cells. Values are normalized to viability in control samples (leucine plus, no inhibitor). BIS inhibition did not compromise cell survival. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. C:450 Control, 450 μM leucine. C:0: Leucine deprivation, 0 μM leucine. BIS:450: Bisindolylmaleimide (7.5 μM), 450 μM leucine. BIS:0: Bisindolylmaleimide (7.5 μM), 0 μM leucine.

Supplementary Figure 3B. HepG2 cell vitality after treatment with TBB (1 μM) for 24 hours

A graphical representation of cell vitality between leucine plus or minus treatments with or without TBB. A Trypan Blue exclusion assay was conducted to assess cell viability, illustrated as the ratio of live to total cells. Values are normalized to viability in control samples (leucine plus, no inhibitor). TBB did not compromise cell survival. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. C:450 Control, 450 μM leucine. C:0: Leucine deprivation, 0 μM leucine. TBB:450: TBB (1 μM), 450 μM leucine. TBB:0: TBB (1 μM), 0 μM leucine.

Supplementary Figure 4. **MRM spectra of non phosphorylated synthetic IGFBP-1 peptides used for CK2 *in vitro* kinase assay**

MRM/MS successfully detected synthetic peptides corresponding to the digested fragments of IGFBP-1 containing Ser101, Ser119 or Ser169 sites.

Supplementary Figure 5. Efficiency of CK2*α*+α'+β silencing

A representative immunoblot of HepG2 cell lysates (50 μg per lane) demonstrates knockdown efficiencies of each CK2 subunit. CK2α, CK2α'and CK2β expression were decreased (-45-55%) regardless of leucine status in HepG2 cells treated with siRNA against all three CK2 subunits. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. Sc: Scrambled, 450 μM

leucine. Sc:0: Scrambled, 0 μM leucine. CK2:450: CK2α+α'+β siRNA, 450 μM leucine. CK2:0: CK2α+α'+β siRNA, 0 μM leucine.

Supplementary Figure 6. Efficiency of pan-PKC silencing

A representative immunoblot of HepG2 cell lysates (50 μg per lane) assayed for PKCδ and PKCε expression following siRNA silencing of pan-PKC. The expression of A. PKCδ and B. PKCε were both reduced (-50%) regardless of leucine status by pan-PKC siRNA in HepG2 cells. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.0001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. Sc: Scrambled, 450 μM leucine. Sc:0: Scrambled, 0 μM leucine. PKC:450: pan-PKC siRNA, 450 μM leucine.PKC:0: pan-PKC siRNA, 0 μM leucine.

Supplementary Figure 7. Effects of conditioned HepG2 cell media immuno-depleted for IGFBP-1 on IGF-1R autophosphorylation

An IGF-1R autophosphorylation assay in P6 cells treated with conditioned HepG2 cell media depleted of IGFBP-1 verifies that the reduction of IGF-I bioactivity (IGF-1Rβ (Tyr1135)) is due to IGFBP-1 and independent of other cell media components. Values are displayed as mean + SEM. *p< 0.05, **p= 0.001-0.05, ***p < 0.001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. – IGF-I: Negative control, no IGF-I, no IGFBP-1. +IGF-I: Positive control, 25 ng/mL IGF-I, no IGFBP-1. Control: Intact HepG2 cell media. Control (Dep): IGFBP-1-depleted HepG2 cell media.

Supplementary Figure 8. Effects of TBB and BIS treatments on CK2 activity

A. A CK2 activity assay demonstrates that treatment with TBB, but not BIS, reduces CK2 activity. **B.** BIS, but not TBB, reduces PKC activity. TBB and BIS do not have off-target effects on PKC and CK2 activity, respectively, under control (leucine plus) conditions. Values are displayed as mean + SEM. **p*< 0.05, ***p*= 0.001-0.05, ****p* < 0.0001 versus control; One-way analysis of variance; Dunnet's Multiple Comparison Test; n=3. Control: 450 μM leucine. BIS: Bisindolylmaleimide (7.5 μM), 450 μM leucine. TBB: TBB (1 μM), 450 μM leucine.