Cbl downregulation increases RBP4 expression in adipocytes of female mice

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Short title: Cbl regulation of RBP4 in adipocytes

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Abbreviations: RBP4: Retinol binding protein 4; Cbl: Casitas b-lymphoma; ER:estrogen receptor; ERE: estrogen responsive element, ERK: Extracellular signal-regulated kinase; PI3K:phosphatidylinositol 3-kinase; AMP: AMP regulated Kinase. ACRP30: Adipose complement related protein of 30 kDa.

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Abstract

Obesity leads to adipose tissue dysfunction, insulin resistance and diabetes. Adipose tissue produces adipokines,that contribute to regulate insulin sensitivity. In turn insulin stimulates the production and release of some adipokines. Casitas-b-lymphoma proteins (c-Cbl, Cbl-b and Cbl3) are intracellular adaptor signalling proteins that are rapidly phosphorylated by activation of tyrosine kinase receptors. c-Cbl is rapidly phosphorylated by insulin in adipocytes. Here we tested the hypothesis that Cbl signalling regulates adipokine expression in adipose tissue. We determined the adipokine profile of WAT of Cbl-/- and Cbl+/+ mice in the C57BL6 background. Female Cbl-/- mice exhibited altered expression of adiponectin, leptin and RBP4 in visceral adipose tissue while no significant changes were seen in male mice. TNFα and IL6 levels were unaffected by Cbl depletion. RBP4 expression was unchanged in liver. Adipose tissue of Cbl-/- animals showed increased basal activation of extracellular regulated kinases (ERK1/2) compared to Cbl+/+. c-Cbl knockdown in 3T3L1 adipocytes also increased basal ERK phosphorylation and RBP4 expression. Inhibition of ERK1/2 phosphorylation in Cbl-depleted 3T3L1 adipocytes or in adipose tissue explants of Cbl-/- mice reduced RBP4 mRNA. 17β-estradiol increased RBP4 mRNA in adipocytes. Cbl depletion did not change ER expression but increased phosphorylation of ERα at S118, a target site for ERK1/2. ERK1/2 inhibition reduced phosphoER and RBP4 levels. These findings suggest that Cbl contributes to regulate RBP4 expression in adipose of female mice through ERK1/2-mediated activation of ERα. Since Cbl signalling is compromised in diabetes, these data highlights a novel mechanism that upregulates RBP4 locally.

Introduction

Casitas b-lymphoma (Cbl) is a proximal insulin receptor adaptor protein identified as necessary for insulin-mediated activation of glucose transport in adipocytes (Baumann, et al. 2000; Chang, et al. 2002; Chiang, et al. 2001; Liu, et al. 2003; Liu, et al. 2002). Three Cbl isoforms are present in mammalian cells (c-Cbl, Cbl-b and Cbl3). Cbl3 is a truncated isoform present only in the skin, while c-Cbl and Cbl-b are quite ubiquitous and become rapidly phosphorylated on tyrosine residues in response to insulin and other growth factors c-Cbl is the predominant isoform expressed in mature adipocytes (Liu et al. 2003). We reported that C-Cbl (hereafter Cbl) is also quickly phosphorylated by insulin *in vivo* in heart and skeletal muscle albeit in these tissues it appears to regulate other intracellular proteins (Gupte and Mora 2006). Cbl phosphorylation and expression is compromised in diabetic animal models (Gupte and Mora 2006). In addition, Cbl proteins also contain a RING finger domain that allows them to function as E3-Ubiquitin ligase enzymes and thus facilitate protein degradation. Whole body disruption of c-Cbl gene in mice in the Jvs129 background resulted in reduced fat peripheral stores and increased fatty acid oxidation in skeletal muscle and whole body insulin sensitivity (Molero, et al. 2004).These mice were also exhibited resistance to the deleterious effects of a high fat diet (Molero, et al. 2006).

White adipose tissue (WAT) has an important role in maintaining glucose and lipid homeostasis and thus in maintaining health. WAT is a dynamic tissue that stores surplus energy in the form of triglycerides, but it also produces a plethora of bioactive molecules collectively called adipokines that help regulate biological functions in other cells and organs. Some of these proteins like adiponectin increase insulin sensitivity in peripheral organs, some arepro-inflammatory such as IL6 or TNFa and inhibit insulin action and others like leptin can function both ways..

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Adiponectin (also called ACRP30) is an adipokine with insulin sensitizing functions in liver and skeletal muscle (Berg, et al. 2002; Kadowaki, et al. 2006; Yamanuchi, et al. 2002), as well as anti-inflammatory (Mandal, et al. 2011; Ohashi, et al. 2010; Takemura, et al. 2007) and anti-atherogenic effects (Lovren, et al. 2010; Okamoto, et al. 2008; Okamoto, et al. 2002; Ouchi, et al. 2001). Adiponectin circulating levels are inversely correlated with BMI, visceral adiposity and insulin resistance (Arita, et al. 1999; Bouatia-Naji, et al. 2006; Pajvani and Scherer 2003) (For a recent reviews see (Kwon and Pessin 2013) (Ohashi, et al. 2014; Ohashi, et al. 2015; Ouchi, et al. 2011))

Leptin is a small WAT adipokine that regulates feeding behaviour through the hypothalamic regulation in central nervous system (Zhang, et al. 1994). Leptin plasma levels are elevated in obese rodents and humans due to increased adipose tissue content and leptin resistance (Friedman and Halaas 1998). Leptin stimulates monocytes and macrophages to produce pro-inflammatory cytokines including: IL6, TNFa, and IL12(Gainsford, et al. 1996) and stimulates CCL2 production in human hepatic stellate cells (Aleffi, et al. 2005).

Retinol Binding Protein 4 (RBP4) is both an adipokine and liver-derived protein that transports retinol (vitamin A) in the blood (Tamori, et al. 2006). It has been implicated in the metabolic syndrome and adipose tissue inflammation (Boyraz, et al. 2013; Kovacs, et al. 2007). RBP4 expression in adipose tissue correlates positively with inflammation (Yao-Borengasser, et al. 2007), expression of pro-inflammatory cytokines and adiposity (Klöting, et al. 2007) and inversely with GLUT4 protein levels (Yang, et al. 2005). Recombinant RBP4 administration induced insulin resistance in mice (Yang et al. 2005). RBP4 levels are increased in obese and insulin-resistant humans (Graham, et al. 2006) and mouse models. Genetic (Moraes-Vieira, et al.2014) or pharmacologic elevation of serum RBP4 causes insulin resistance and hepatic steatosis in mice (Lee, et al.2016). Recent studies in transgenic mice models overexpressing *Rbp4*gene in adipose tissue have revealed that RBP4 causes inflammation in adipose tissues by activating macrophages independently of

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its retinol binding status and via activation of the Toll-like 4 receptor (Norseen, et al. 2012), as well as activating innate immunity (Moraes-Vieira, et al. ; Moraes-Vieira et al.).

Here, we tested the hypothesis that Cbl signalling in adipose tissue may regulate adipokine expression. We examined the expression and circulating levels of several adipokines involved in insulin sensitivity in the c-Cbl null animal model and their wild type littermates and their expression in c-Cbl knocked-down 3T3L1 adipose cells. We report that Cbl depletion in adipocytes increases basal activation of extracellular regulated kinases (ERK1/2) and results in enhanced expression of RBP4 in adipocytes of female mice via activation of the estrogen receptor.

Materials and methods

Reagents plasmids and antibodies: Tissue culture reagents (FBS, trypsin, penicillin/streptomycin, insulin, IBMX, dexamethasone, puromycin, 17-β estradiol) were from Sigma-Aldrich. MEK1/2 inhibitors PD98059 and U0126 were obtained from Sigma-Aldrich. Adipokine ELISA kits for RBP4 (cat. Number DY3476, assay range: 93.8-6000 pg/ml), Adiponectin (cat. Number DY1119, assay sensitivity 31-2000 pg/ml) and leptin (cat. Number DY498, assay range 125-8000 pg/ml) were from R&D Systems (Minneapolis, Minnesota USA) , IL6 (cat. Numbers capture: detection: from and TNFa (cat number from BD Biosciences) ELISA antibodies were from Thermofisher Scientific and used at 2ug/ml). The PKLO.1 puro empty plasmid (cat number SHC001), the PKLO.1 plasmid carrying a non-targeting shRNA (NT shRNA) sequence (cat SHC002) and PKLO.1 puro vectors carrying shRNAs for cCBL proteins were from Sigma (MISSION shRNA). The PMDG2 and PMCV-dR8.74 vectors to generate the lentiviral vectors were a gift from Dr. Antonio Zorzano (IRB, Barcelona, Spain). Antibodies: anti IRS1 (cat number 2382, 1:1000), phospho AKT(Ser473)(cat.number 9271, 1:1000), total AKT (cat.number 2920, 1:1000), phosphoERK1/2 (T202/T204) (cat.number 910,1:1000) , total ERK1/2 (p44/p42) (cat.number 9107, 1:1000) and phosphoER (S118) (cat number 2511,1:1000) were from Cell Signalling.,

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Total ERa antibody was from Abcam (cat number ab108398,1:2000) , anti bTUBULIN (catalogue number T4026, 1:2000) antiACTIN(catalogue number A2066, 1:2000) antibodies were from Sigma-Aldrich, anti GLUT4 antibody was a gift from Dr. Jeffrey Pessin (Albert Einstein NY, USA)

Animals, genotyping and diet intervention. C-Cbl null mice on C57BL6/J background were obtained from the National Institute of Health (USA). C57BL6/J wild type controls (WT) were purchased from the Jackson Laboratories (B Harbour ME, USA). Cbl null and WT animals were bred to obtain heterozygous animals (Cbl $^{+/-}$) and the colony expanded through hetxhet crossings to obtain experimental cohort of animals. In all experiments age and gender wild type littermate control animals from our colony were used. For some experiments in addition to hetxhet crossings Cbl$^{-/-}$x Cbl$^{-/-}$ and Cbl$^{+/+}$x Cbl$^{+/+}$ pairs from our colony were established to assure enough animals per each genotype were available. Genotypes were determined by PCR of genomic DNA using the following primer sets: LOXP: 5' TGG CTG GAC GTA AAC TCC TCT TCA GAC CTA ATA AC 3'; CBL-10: 5' GAC GAT AGT CCC GTG GAA GAG CTT TCG ACA 3'; CBL-11: 5' CCT AAG TGG TAG GAT TAT AAT TGC AAG CCA CCA C 3' and CBL-13: 5' TCC CCT CCC CTT CCC ATG TTT TTA ATA GAC TC 3' which amplify the targeted and non-targeted genes respectively. Animals were housed at 12hr light/dark cycle and fed *at libitum* with standard chow diet. Weight of animals was monitored weekly. All procedures were carried out in accordance with the U.K. Animal (Scientific Procedures) Act 1986 and Home Office licenses

Glucose and insulin tolerance tests were carried out as we previously reported (Yang, et al. 2001) at week 12.

Cells.3T3L1 cells were obtained from the ATCC. Culture and differentiation of cells was carried out as we described previously (Xie, et al. 2006) in cell passages <p10.Generation of 3T3L1 cell lines stably expressing shRNAs for c-Cbl or empty PKLO puro vector or NT-shRNA were carried out as we described previously (Carson, et al. 2013).

Culturing of adipose tissue explants was carried out as described (Fried and Moustaid-Moussa 2001). *Briefly,* white adipose tissue was dissected and fragmented into small (1-2 mm) pieces under sterile conditions, and subsequently incubated for 2 hours in Dulbeco Modified Eagle's media (DMEM). Following the first 2 hrs of incubation, the media was changed to fresh DMEM and cultured further for up to 24hr. At that time insulin was added for 30 minutes at final concentrations of 0nM, 1nM, 10nM and 100nM insulin (or as indicated in figure legends), tissues were then snap frozen in liquid nitrogen and stored at -80°C until analysis.

Immunohistochemistry of Adipose tissue. Dissected perigonadal WAT adipose tissue was fixed in 4% paraformaldehyde/PBS overnight at 4°C, rinsed in PBS, dehydrated through a series of descending graded alcohols and embedded in paraffin. Tissue was sectioned in a microtome at 5-7 μm thick sections, dewaxed in histoclear (5 minutes, twice), rehydrated stained with Mayer's haematoxylin (8 min), rinsed in water and acidified water (10 seconds). Sections were counterstained with Eosin (2 min) washed in tap water, dehydrated in ascending graded alcohols, cleared in xylene and mounted using DPX mounting medium. Sections were visualized in a Leica inverted microscope at 40x magnification and pictures taken with color camera. Image analysis of histological tissue preparations (cell size and diameter) were carried out using Image J software (NIH, USA).

Tissue/cell lysate preparation and Immunoblotting. Tissue homogenates and whole cell lysates were obtained as previously described (Mora, et al. 2001). Briefly, tissue samples were homogenised in a DOUNCE glass homogenizer, in ice-cold lysis buffer (NaCl 100 mM; EDTA 1mM; 1% Triton x-100; NaF 50 mM; 2 mM sodium pyrophosphate; 1 mM sodium vanadate; 1 mM phenylmethylsulfonyl fluoride; 2 μg/ml aprotinin; 2 μg/ml pepstatin A and 2 μg/ml leupeptin) .Samples were incubated at 4°C for 30 minutes, and centrifuged 15,300 xg for 15 min at 4°C). Protein concentration of the supernatant was determined using the Bio-Rad Protein Assay Kit. Samples were separated on a SDS-PAGE, transferred to

nitrocellulose membranes, blotted in 5% non-fat milk in Tris-buffered saline (pH 7.6) and subsequently immunoblotted with primary antibodies and fluorescent labelled secondary antibodies IRDye 800 cw (cat number 92632210 at 1:15,000) and IRDye 680RD (cat number 926-68071 at 1:20,000) (LICOR). Membranes were washed in Tris Buffered Saline containing0.1% Tween and visualized in a LI-COR Odyssey system. Quantification of blots relative to reference protein as indicated in the figure legends was carried out using image J (NIH).

ELISA determination of adipokinecontent. Adipose tissue or 3T3L1 cells were obtained in lysis buffer by homogenization as described above and as we reported previously (Mora et al. 2001). Expression of adipokines (RBP4, adiponectin, leptin,TNFa and IL6) were determined by ELISA using commercially available kits and antibodies (indicated above) as we have previously reported (Carson et al. 2013; Xie, et al. 2008). Adipokine content was determined in a 10 µl aliquot of lysate using standards supplied by the manufacturer and the results were normalized to the total protein content in the lysate as determined by Bradford. Plasma from tail bleeds was collected in EDTA coated tubes. The sample was centrifuged 10 minutes at 2,000 rpm at room temperature and the supernatant containing the plasma was transferred to a new tube and frozen at -80ºC until used. For ELISA a 10 µl aliquot was used. A standard curve with recombinant protein provided by the kit was used in each assay, and when necessary the plasma was diluted in PBS so that the adipokine values were within the standard curve.

Total RNA extraction and qPCR. Total RNA was isolated using Tri reagent (Sigma-Aldrich) following the manufacturer's instructions. RNA was quantitated by spectrophotometry and visualized in an agarose gel. Total RNA was reverse transcribed to cDNA using an iScript cDNA synthesis kit (BIO-RAD) following the manufacturer's instructions. Validated Taqman probes for Rbp4 and 18S (assay IDs: Mn00803264-31 and Hs 99999901 respectively) were obtained from Life Technologies. The resulting cDNA was amplified using iTAQ probes and

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iSCript qPCR kit (Life Technologies). Estrogen receptor isoforms α and β (accession numbers NM007956.5 and BC145329.1 respectively) were amplified using Kapa Sybr green Fast mix from Roche and the following primers: ERα: F: 5'TGATTGGTCTCGTCTGGCG3'; R: 5'CATGCCCTCTACACATTTACC3'; ERβ: F: 5'CTGGCTAACCTCCTGATGCT3'; R: 5'CCACATTTTTGCACTTCATGTTG3' .The primers produce amplicons of 100 bp and 91bp respectively. The conditions of the reaction were: denaturation 95ºC 30 seconds, annealing 60ºC 20 seconds, extension 72ºC 30 seconds, for 40 cycles. A melting curve was run at the end of each run. Relative quantification was carried out using the ΔΔCt method using 18S gene expression (primer sequences: forward: 5'TCAAGAACGAAAGTCGGAGG 3' and reverse: 5'GGACATCTAAGGGCATCACA3': for normalization, as we have previously reported (Yang, et al. 2012).

Statistical analysis. Statistical analyses were carried out using GraphPad Prism 6 software (GraphPad software). Student t-tests or analysis of variance (ANOVA) analyses with Sidak's post hoc test were carried out as indicated in the figure legends, with a confidence interval of 95% and statistical significance was considered if $p < 0.05$.

Results

c-Cbl depletion increases whole body glucose sensitivity in male mice and alters adipokine expression in WAT of female mice.

c-Cbl null mice on a Jvs129 background were shown to have reduced adiposity and to be more insulin sensitive than wild type mice, even on a high fat diet (Molero et al. 2004; Molero et al. 2006). Because the c-Cbl^{-/-} used in our studies was generated in a C57BL6 background, we first sought to determine growth, adiposity and *in vivo* whole body glucose tolerance and insulin sensitivity of ourmice. As shown in Fig.1A, female mice were slightly smaller than male mice but there were no differences in the weight of c-Cbl^{-/-} mice compared to c-Cbl^{+/+}. We did not detect any significant differences in adipose tissue morphology, as revealed by eosin and hematoxylin staining of visceral adipose depots and

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adipocyte cell size quantification (Fig.1 B and C).Similarly to what was reported previously

(Molero et al. 2004), the null mice showed a tendency towards an increase in glucose

tolerance although but it did not reach statistical significance for neither gender (Fig1.D).

Male mice however, showed improved insulin sensitivity (Fig.1E) whereas this was not

observed in female mice.

We next determined the adipokine profile expression in visceral adipose tissue and

plasma by ELISA. In the steady state, there was a marked gender dimorphism in the

content of some adipokines in white adipose tissue. In adipose tissue of female mice,

adiponectin levels were decreased whereas leptin and RBP4 levels were increased. No

significant changes were observed in male Cbl$^{-/-}$ mice compared to Cbl$^{+/+}$ mice. TNFα or IL6

in WAT were not significantly different to those obtained in the wild type mice for either

gender (Fig 2A). Circulating plasma levels for adiponectin, leptin or RBP4 were

unremarkable in the Cbl null animals compared to wild type mice (Fig.2B).

Despite the changes in RBP4 protein levels in white adipose tissue no significant

changes were detected in the liver of Cbl$^{-/-}$ mice compared to Cbl$^{+/+}$ animals (Fig.3).

Depletion of c-Cbl increases ERK1/2 activation in WAT of mice and in cultured 3T3L1

adipose cells.

In order to determine the molecular mechanisms that may be involved in the

upregulation of RBP4 expression in adipose tissue we examined the insulin signalling

pathways in the adipose tissue of c-Cbl null mice and wild type animals. Expression of the

insulin responsive substrate 1 (IRS1) or the glucose transporter GLUT4 were not altered in

visceral WAT of null mice compared to controls (Fig4.A and B). Perigonadal visceral adipose

tissue explants were obtained from Cbl$^{-/-}$ or Cbl$^{+/+}$ animals and were incubated in the absence

or presence insulin at concentrations ranging from 0 to 100nM. We then determined the

activation of the phosphatidylinositol 3-kinase and ERK1/2 signalling pathways by

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monitoring the phosphorylation levels of the downstream serine threonine kinase AKT at Ser473 and the phosphorylation levels of p44/p42 ERK1/2 at Thr202/204. Insulin effectively activated phosphatidylinositol 3 kinase and AKT to a similar extent in both c-Cbl null and wild type mice (Fig.4,male animals panels B, C; female animals panels D,E). However, while activation of ERK1/2 proteins was achieved to a similar extent with submaximal insulin concentrations in the two genotypes, the c-Cbl null mice displayed a greater ERK1/2 phosphorylation in the basal (untreated) state, with approximately 40% more phosphorylation of p44/p42 compared to the wild type (Fig.4 panels C,E). Interestingly, this increase in ERK1/2 phosphorylation was not seen in the liver (Fig.4.F).

To confirm that the *Cbl* gene depletion was directly responsible for the increased basal ERK1/2 phosphorylation levels we turned to the well characterized 3T3L1 adipose cell line model. We postulated that if the changes in ERK1/2 signalling were solely due to the depletion of c-CBL in adipose cells and not an indirect or compensatory effect caused by the depletion of c-CBLl expression elsewhere, reduction of c-CBLexpression in the 3T3L1 cell line using a shRNA-mediated lentiviral approach would replicate the findings seen in the adipose tissue of the mice. To this end, undifferentiated 3T3L1 cells were either left untreated or infected with lentiviral particles expressing either validated shRNAs for the *Cbl* gene or with lentiviral particles expressing a non-targeting shRNA (NT-shRNA) or with lentiviral particles containing an empty vector as an additional control. Stable cell lines were selected in the presence of puromycin and following amplification, the selected cells were differentiated to obtain fully differentiated adipocytes as we reported previously (Carson et al. 2013). Of note inhibition of *Cbl* by shRNA did not result in changes in the differentiation of 3T3L1 cells into adipocytes (Data not shown).

Expression of c-Cbl in differentiated 3T3L1 cells was significantly reduced by the expression of *c-Cbl* shRNAs compared to control cells expressing an empty vector or a non-targeting shRNA (NT-shRNA) (Fig.5A). Insulin treatment resulted in the activation of

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phosphatydilinositol 3-kinase cascade and phosphorylation of AKT in Ser473 to a similar

extent in CBL knock down cells compared to control cells (Fig 5 A and B). However, as

detected with the primary adipose tissue of the Cbl$^{-/-}$ we found enhanced basal ERK1/2

phosphorylation levels in the c-Cbl Knock down cells compared to control cells (Fig.5A and

5B left panel). Interestingly, concomitantly with these data we found that RBP4 levels were

significantly increased in the c-CBL knockdown cells compared to control cells. Leptin levels

were slightly elevated in the CBL depleted cells albeit this did not reach statistical

significance. No changes were observed in adiponectin levels compared to control cells

(Fig.5C).

Chemical inhibition of ERK1/2 decreases RBP4 expression in adipose tissue explants

of CBL null mice and in CBL knockdown 3T3L1 adipocytes

Based on the above data, we postulated that the increase in ERK1/2 activation seen

in adipocytes depleted of CBLl may be responsible for the elevated expression of RPB4. To

test this hypothesis, we examined the effects of chemical inhibition of ERK1/2 in the adipose

tissue explants of the *Cbl* null mice and in 3T3L1 *Cbl* KD adipocytes.   First, visceral adipose

tissue explants were obtained and cultured *in vitro* in DMEM media in the absence or

presence of two chemical inhibitors (10 µM of PD98059 and 20 µM U0126) of MEK1/2, the

upstream kinases that regulate ERK1/2. Subsequently, adipose tissue lysates were obtained

and immunoblotted with antibodies for phospho-ERK1/2 to determine the level of inhibition.

Both inhibitors significantly decreased ERK1/2 phosphorylation after 24hr treatment although

U0126 seemed to achieve greater inhibition (Fig.6A). We then determined the effect of this

inhibition on RBP4 expression. Concomitantly with the reduction in ERK1/2 activation the

content of RBP4 protein in the tissue was reduced with U0126 treatment (Fig.6B). At the

mRNA level, treatment with both inhibitors reduced *Rbp4* mRNA levels maximally after 3 hrs

of treatment (Fig. 6C). *Rbp4* mRNA levels recovered completely after 24hr following the

treatment with PD98059 whereas a 50% decrease was still noted for U0126 at this time

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point (Fig. 6C). As expected, the inhibitor PD98059 did not reduce the expression of *Rbp4* in adipose tissue of wild type mice (Fig.6C).

We confirmed this data in the 3T3L1 adipocytes cell line and found that chemical inhibition of ERK1/2 activation in these cells (Fig.6D) also reduced the levels of mRNA for *Rbp4* (Fig. 6E).

Estrogen receptor regulates RBP4 expression in adipocytes

Since our findings on RBP4 expression in adipose tissue were confined to female mice and white adipose tissue expresses abundant ER receptors we postulated that 17β-estradiol may be involved in regulating RBP4. To assess this we first used UCSC Genome Browser (http://genome.UCSC.edu) to examine the human and mouse gene sequences. We found Estrogen Receptor alpha responsive elements (ERE) in the human promoter, in an area that is highly conserved to the mouse gene (data not shown).This suggested that estrogens may contribute to RBP4 gene regulation . To determine whether 17β-estradiol regulates *Rbp4* expression in adipocytes we treated differentiated 3T3L1 adipocytes with increasing concentrations (0, 1, 10 and 100 nM) of 17β-estradiol for 15hrs. Total RNA was subsequently harvested and mRNA levels of *Rbp4* were determined by qPCR. We found that 17β-estradiol increased *Rbp4* mRNA levels in adipocytes (Fig7A).

Cbl depletion increases the phosphorylation of Estrogen receptor alpha

Next, we questioned whether *Cbl* depletion in adipocytes would increase the expression or activation of ER receptor. We determined the expression of *Era* and *Erb* by qPCR in control cells and in cells stably expressing shRNAs *Cbl* KD. We found that the mRNA levels for ER receptors in *Cbl* depleted cells was similar to that observed in control cells (Fig.7B).

Existing literature demonstrates that ERa can be activated by phosphorylation at Ser118, a consensus phosphorylation site for ERK1/2. We next tested whether *Cbl* depletion could result in increased phosphorylation of ERa. To this end we immunoblotted white

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adipose tissue extracts obtained from c-Cbl$^{-/-}$ and c-Cbl $^{+/+}$ mice and 3T3L1 lysates obtained

from control or CBL knock down cells, with a phosphoERa S118 specific antibody and a

total ERa antibody as loading control. We found that the phospho ER (S118) levels

normalized to total ER receptor were slightly elevated in the CBLnull mice, albeit it did not

reach statistical significance (Fig.8A). In 3T3L1 adipocytes, CBLdepleted cells showed

increased ER S118 phosphorylation, and as expected, this decreased dramatically upon

exposure to MEK1 and MEK2inhibitors PD98059 and U0126 (Fig.8 B).

Discussion

CBL null mice made in the C57BL6 background did not display any significant growth

differences compared with the wild type mice. The male mice replicated the insulin sensitivity

phenotype described previously on the Jvs129 background (Molero et al. 2004; Molero et al.

2006) whereas females were not significantly different than wild type. It was reported that

CBL depletion increases muscle metabolism (Molero et al. 2004) enhanced energy

expenditure and reduced adiposity. The c-Cbl null mice used in our studies did not show any

adipose tissue morphology abnormalities or differences adipocyte cell size. These findings

suggest small differences in the phenotype that may be attributable to distinct genomic

background.

However, we detected some differences in the adipokine content of the white

adipose tissue of female c-CBL null mice. Content of adiponectin was decreased while leptin

and RBP4 were increased in the female Cbl$^{-/-}$ compared to Cbl$^{+/+}$ mice. Reduced adiponectin

levels and increased RBP4 plasma levels have been shown to correlate with states of insulin

resistance such as obesity in both animal and humans (Tan, et al. 2007; Yang et al. 2005).

The increase in RBP4 content was relatively small and only detected in WAT with no

changes seen in the liver or in the circulating plasma levels. Since the liver is the main

producer of RBP4 protein contributing to 80% of the plasma levels, that explains the limited

impact on circulating RBP4 and in overall insulin sensitivity. However, local changes of

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RBP4 levels in WAT are important in determining a local inflammatory response (Moraes-Vieira et al. 2016). Norseen et al,(Norseen et al. 2012) have recently reported that transgenic overexpression of RBP4 in adipose tissue increases adipose tissue inflammation through the activation of macrophages via JNK, TLR4 and NFkB signalling pathways. We did not detect any local increase in the abundance of proinflammatory cytokines IL6 or TNFa in WAT, which suggests RBP4 levels in the CBL null mice did not reach the threshold necessary to increase local macrophage inflammation response.

We found increased basal activation of ERK1/2 in the adipose tissue of c-Cbl$^{-/-}$ mice and in 3T3L1 adipocyte cells depleted of c-CBL. This correlated with the increase in RBP4 and leptin expression in adipose tissue. We did not detect any increase in the expression of RBP4 or raised basal ERK1/2 phosphorylation in the liver of c-Cbl$^{-/-}$ mice (data not shown). Furthermore, chemical inhibition of ERK pathway in c-CBL-depleted adipocytes decreased RBP4 levels. These findings suggest that c-CBL may regulate RBP4 expression through ERK1/2 activation. Indeed c-CBLproteins have dual functionalities in cells. They act as adaptor proteins for tyrosine kinase receptors, including the insulin receptor, and recruit signalling proteins in the caveolae of adipose cells (Watson and Pessin 2001). In addition, c-CBL proteins can function as an E3-ubiquitin ligase enzymes, thus facilitating the degradation of proteins. Molero et al.(Molero et al. 2004), had found increased insulin receptor expression in the skeletal muscle of Cbl$^{-/-}$ mice. However, we did not detect any changes in the expression levels of the glucose transporter GLUT4 or IRS1 . Furthermore signalling through PI 3-Kinase in response to insulin was unremarkable compared to the wild type mice indicating equal insulin receptor substrate1 and PI 3-Kinase activation. It remains possible however, that the effects seen on Increased ERK1/2 activation may be dependent on CBL action as an E3-Ubiquitin ligase on ERK1/2 regulatory proteins, for example targeting an upstream ERK kinase or through an inhibitory effect of an ERK phosphatase. Further research is necessary to identify the molecular mechanisms for increased basal ERK1/2 phosphorylation in c-CBL depleted adipose cells.

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There was a clear gender dimorphism effect observed with RBP4 seen elevated only in female mice. Similar findings were found previously in humans (Kos, et al. 2011). Both ERa and ERb Estrogen eceptors are present in white adipose tissue with ERa being the most abundant. These receptors play an important role during adipose tissue differentiation. Binding of estradiol to the ERa facilitates ligand-dependent activation and transactivation of estrogen response elements (ERE) in target genes which activate or repress gene expression. The human *Rbp4* promoter has ERE sequences in conserved regions, which suggest a role for ER in the expression of RBP4, in support of this, we found that incubation of mouse adipose cells with 17b-estradiol increased *Rbp4* mRNA levels. Our data is consistent with previous findings by Jung et al. (Jung, et al. 2013) who found that in 3T3L1 adipocytes, ERa but not ERb activation increased *Rbp4* mRNA levels.

We did not determine the oestrous cycle in our female mice or indeed measured the circulating estradiol levels to correlate them with *Rbp4* expression levels. However, the existing literature in human studies, have shown a correlation of RBP4 serum levels with estradiol and estradiol/testosterone levels (Mohasseb and Khalil 2014) which supports the role of ER in the in vivo regulation of *Rbp4* expression. The effects of other female hormones released during the oestrous cycle on the expression of *Rbp4* however, have not been extensively studied. A recent study in heifers showed Rbp4 mRNA and protein levels raised in the endometrium during the dioestrus phase with elevated progesterone levels (Mullen, et al. 2012). This may be related to the role of retinoic acid signalling in the expression of proteins important for embryo implantation (Ma, et al. 2012). Unfortunately, the authors did not measure *Rbp4* levels in WAT of these animals.

The ER receptors are members of the nuclear hormone receptor superfamily of transcription factors that bind ERE sequences as homo or heterodimers. ERs contain two transcription activation functions: AF1 located in the N terminal A/B domain and the AF2 located in the C terminal domain. AF2 is activated through ligand (hormone) binding,

whereas AF1 can modulate gene transcription in the absence of ligand (Murphy, et al. 2011), but this is weak.

ER activity can be modulated by intracellular signalling pathways (Barone, et al. 2010) that phosphorylate ERa. AKT and ERK1/2 phosphorylation sites on ERa map at S167 for AKT and S118 for ERK1/2 both within the AF1 domain. While phosphorylation at these sites leads to ligand-independent activation of ERa (Murphy et al. 2011), AF1 works to synergize with AF2 in the promotion of ligand-dependent transcription activation by the receptor (*reviewed byTsai and O'Malley 1994).* Thus, we postulate that this mechanism may operate in the female CBLnull mice to enhance expression of *Rbp4*. Based on our findings, we propose that along with the increased number of ER receptors and estrogen circulating levels present in females, c-CBL depletion may potentiate ER activity through ERK1/2-mediated phosphorylation of ER at S118 which results in higher RBP4 expression.

In all our data suggest that inhibition of *Cbl* in adipose tissue will increase RBP4 expression locally. We previously reported that CBL signalling is impaired in animal models of insulin deficiency and in obesity (Gupte and Mora 2006). While our findings need to be explored in the context of human adipose tissue, this study reveals a potential new molecular mechanism that may contribute locally to the dysregulation of RBP4 that occurs in obesity and insulin resistance.

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Figure legends

Fig. 1.A) Growth curve of wild type and CBL null mice. Data shows mean± SEM of weekly weight. Cbl $^{+/+}$: male n=18, female n=12; Cbl $^{-/-}$: male n=14,female n=17. Statiscal analysis multiple t-test, not significant. B) Haematoxylin-eosin staining of perigonadal white adipose tissue of Cbl$^{+/+}$ and Cbl $^{-/-}$ mice. Representative images shown of pictures obtained at 40X magnification of Cbl $^{+/+}$ n=9 and Cbl $^{-/-}$ n=12 mice. Panels a and b are male and panels c and d are female mice. C) Quantification: Image J was used to quantify cell diameter of n=100 cells and cell area n=50 cells per genotype and gender. Top graphs correspond to male and bottom to female mice Graphs show mean± SEM. Statistical Analysis: paired t-test, Not significant. D) Glucose tolerance test (GTT) and E) Insulin tolerance test (ITT) Tests were carried out at 14 weeks. Mice were injected with glucose 2 g/Kg of body weight (GTT) or insulin 0.75 U/Kg of body weight (ITT) and at the times indicated blood tail samples were obtained and glucose measured using a glucose meter. Graph show mean+ SEM of values. Male mice n=4, female mice n=3. Statistical analysis: paired t-test. Area under the curve (AUC) p values: female: GTT: p=0.079, ITT: p=0.442; male: GTT: p=0.428, ITT: p=0.0035

Fig.2. Cbl $^{-/-}$ mice shows altered expression of adipokines in WAT of female mice.A)Tissue lysates were obtained from perigonadal WAT and adipokines quantified by ELISA as specified in the methods section. Adipokine levels were normalized by the total protein content in the lysate. Graphs show mean±SEM. For ADIPONECTIN,LEPTIN and RBP4 data from Cbl$^{+/+}$: n=15 males and n=13 female mice; Cbl$^{-/-}$:n=6 males and n=12 females. For IL6 and TNFa Cbl$^{-/-}$ n=4 and cbl$^{+/+}$ n=10 mice. Statistical analysis: paired t-Test. * indicates p<0.05; ** indicates p<0.01.B) Plasma levels of adipokines. Mice were fasted for 6 hours and plasma drawn from the tail. Plasma adipokines were quantitated by ELISA. Graphs Show mean± SEM of n=10 Cbl$^{+/+}$ and n=5 Cbl$^{-/-}$. Statistical analysis paired t-test. Non-significant.

Fig.3. RBP4 expression in liver. Liver tissue lysates were obtained and RBP4 quantified by ELISA. Graphs show mean±SEM of RBP4 normalized to total protein. Cbl $^{+/+}$:male n=4, female n=5; Cbl $^{-/-}$: male n=4,female n=8. paired t-test, * indicates p<0.05.

Fig.4. Increased activation of ERK in adipose tissue of c-Cbl$^{-/-}$ .A) White adipose tissue extracts of c-Cbl$^{+/+}$ and c-Cbl $^{-/-}$ mice were immunobloted for IRS1andGLUT4, quantification vs actin is shown on the right panel)B-D) White adipose tissue explants from c-Cbl$^{+/+}$ and c-Cbl $^{-/-}$ mice were obtained and either left untreated or treated with insulin at the indicated concentrations (0-100nM). Tissue extracts were obtained, loaded onto SDS PAGE and immunoblotted with antibodies as indicated. Panel B shows data from male mice and D) female mice. Panels C) and E)show quantifications of AKT and ERK1/2 phosphorylation relative to the total AKT or ERK1/2 protein respectively from 5 experiments. Graph shows Mean±SEM of phosphorylation over basal as % of Ser473(AKT) or ERK T202/204 and Thr185/Tyr187, respectively. Statistical analysisTwo way ANOVA with Sidak's test, , * indicates p<0.05; ** indicates p<0.01. F) Liver extracts were obtained from c-Cbl$^{+/+}$ and c-Cbl $^{-/-}$ mice (n=4 per group) and immunoblotted for phosphoERK1/2 and tubulin. Graph shows mean±SEM of the quantification over tubulin (arbitrary units).Statical analysis: t-test, not significant.

Fig.5.A) Increased activation of ERK in *Cbl* knockdown 3T3L1 adipocytes. Fully differentiated 3T3L1 adipocyte cells expressing control plasmids (empty vector (EV) or NT-shRNA) or expressing shRNAs for c-*Cbl* (CblKD) were left untreated or stimulated with insulin for 20 minutes. Cellular lysates were obtained, loaded onto SDS-PAGE and immunoblotted with the indicated antibodies. Representative blot of 5 experiments is shown. B) shows the quantification in mean±SEM of intensities in arbitrary units of phospho-AKT (Ser473) and phospho- ERK (T202/204 and Thr185/Tyr187) respectively, obtained in 5 independent experiments. ** indicates p<0.05. C) Knock down of *Cbl* in 3T3L1 adipocytes

increases RBP4 expression. Adipokine expression (RBP4, ADIPONECTIN and LEPTIN) were determined by ELISA in whole cellular lysates obtained from control (expressing empty vector (EV) or Non-targeting shRNAs) or *Cbl* KD cells (expressing shRNAs for c-*Cbl*). Graphs show the mean± SEM of N=5 sample replicates. Statistical analysis: One way ANOVA with Tukey's test. ** indicates p<0.01.

Fig.6. Inhibition of ERK in adipose tissue explants of c-Cbl$^{-/-}$ mice and in Cbl depleted 3T3L1 adipocytes reduces RBP4 levels. A) Treatment of adipose tissue explants of CBL null mice reduces phosphorylation of ERK. Adipose explants were treated with PD98059 or U0126 for 24hrs. Tissue lysates were analysed by western blot. Left shows a representative blot, right graph shows quantification of data obtained from n=3 animals with each inhibitor. B) RBP4 quantification by ELISA in tissue lysates of white adipose tissue explants treated with ERK inhibitors for 24hr. Data is the mean± SEM from n=3 mice for each inhibitor. C) mRNA quantification of RBP4 in white adipose tissue explants from Cbl-/- mice left untreated or treated with ERK inhibitors for 3hrs or 24hrs. Graphs are mean mean± SEM of relative quantification to 18S as house keeping gene, data obtained from n=2 mice for each inhibitor, each sample quantified in triplicate. Right panel: mRNA quantification of RBP4 in white adipose tissue explants from Cbl+/+ mice incubated for 3hr with PD98059. D) *Cbl* KD 3T3L1 adipocytes were left untreated or treated with ERK inhibitors for 15hr. Cell lysates were obtained and analysed by western blot. Graph is quantitation of an experiment done in triplicate cell dishes. Representative of two independent experiments done in triplicate. E) mRNA levels of *Rbp4* were quantitated in *Cbl* Depleted 3T3L1 adipocytes left untreated or incubated with inhibitors for 15hrs. Graph shows mean and SEM. Representative experiment of n=2, with 3 biological replicates. Statistical analysis: One-Way ANOVA vs untreated with Sidak's posthoc test * indicates<0.05, ** p<0.01; ****p<0.0001.

Fig.7. A) 17 β-estradiol increases *Rbp4*mRNA in 3T3l1 cells**.** Differentiated adipocytes were treated with E2 at a range of concentrations from 0 to 10^{-7}M as indicated for 15hr. Total RNA was isolated and *Rbp4* mRNA levels analysed by real time PCR as described in the methods. Graphs shows mean and SEM of n=3 cell dishes. One-Way ANOVA *** indicates<0.01**.** B) Expression of ER receptors in Cbl KD 3T3L1 cells. Total RNA was isolated from control or *Cbl* depleted 3T3L1 adipocytes and the mRNA levels of ERα and ERβ were determined by qPCR as described in the methods. Graphs shows mean±SEM of n=3 cell dishes. Statistical analysis: One way ANOVA,non-significant.

Fig.8. Higher levels of ERa S118 phosphorylation in *Cbl* depleted adipocytes. A) White adipose tissue extracts obtained from c-Cbl$^{-/-}$ or c-Cbl$^{+/+}$ mice (n=3 male and n=3 female for each genotype) were immunoblotted with anti-phospho ERα (S118) antibody or ER antibody as loading control. The blot was quantified with Image J. Graph shows mean±SEM of intensities in arbitrary units. Statistical analysis:ANOVA with Sidak's multiple comparison . * indicates p<0.05. B) Whole cell lysates obtained from either control 3T3L1 adipocytes or *Cbl* depleted cells were immunoblotted with with anti-phospho ERa (S118) antibody or total ER as loading control. Graph shows a the quantification in Image J of n=3-5 replicate samples per group and represents the mean±SEM of phospho ERa/ER ratio in arbitrary units as % of control cells which do not express shRNAs for *Cbl.* Statistical analysis:ANOVA with Dunnett's test. * indicates p<0.05 vs control.

Fig.1

Fig.2

Fig.3

RBP4

RBP4

Fig.4

A)

B)

C)

AKT Phosphorylation

ERK1/2 phosphorylation

D)

E)

AKT phosphorylation

ERK1/2 phosphorylation

F)

Liver

Fig.5

A)

B)

C)

Fig.6

Fig.7

A)

B)

Fig. 8

A)

B)