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Uncovering ubiquitylation pathways in liver metabolism by a proteomic approach

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Table of contents

Detailed table of contents

Table of Figures

Table of Tables

Abstract

Organisms depend on a constant supply of energy in order to survive. In vertebrates, the liver has developed to be a major metabolic organ able to control glucose and lipid homeostasis. It can sense the metabolic state of the organism and adapt to changes in nutrient availability and to food deprivation by inducing or repressing specific pathways. Importantly, deregulation of these specific responses contributes to the development of several metabolic diseases, for instance, non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes (T2D). Most of the regulation in metabolic pathways occurs through posttranslational modifications of key signaling molecules. Phosphorylation and acetylation are the most studied modifications in the context of metabolism. As compared to the latter modifications, our knowledge is relatively limited concerning ubiquitylation as another means to modify proteins. Emerging experimental evidence suggests that the ubiquitin conjugation system is engaged in response to different metabolic cues, regulating, for instance, hepatic glucose production during fasting. Based on the hypothesis that ubiquitin signaling could regulate hepatic metabolic pathways, as well as the emerging role of ubiquitylation in cellular processes, we have conducted a global proteomics analysis to identify ubiquitylated proteins in livers of mice subjected to fasting or refeeding with high sucrose diet. To this end we have taken two complementary approaches to purify ubiquitylated proteins from mice livers: pulldowns using tandem ubiquitin-binding entities (TUBEs) as well as UbiQapture. Using label-free quantification mass spectrometry we have identified 1641 putative ubiquitylated proteins, including 117 differentially ubiquitylated upon fasting or refeeding conditions. We selected 8 proteins for biochemical validation and, notably, we have confirmed the ubiquitylation of all them. In addition, we chose to study ubiquitylation of one of these proteins, complement component 3 (C3) in more details. We observed that C3 can be cleaved intracellularly in primary hepatocytes generating active C3a and C3b. Interestingly, intracellular C3a receptor was also detected. Moreover, we observed ubiquitylation of overexpressed C3a in primary hepatocytes. Thus, we suggest that the ubiquitylation of C3 plays a role in the regulation of inflammatory or metabolic functions of C3 in the liver. Future experiments are focusing on understanding the downstream pathways following C3 ubiquitylation. In conclusion, signaling through ubiquitylation provides another level of regulation of hepatic metabolism that might also be affected in livers in which adaption to fasting and feeding is strongly impaired, thereby contributing to pathogenic hallmarks of type 2 diabetes and non-alcoholic fatty liver disease.

Introduction

1. Energy homeostasis

Cells and organisms depend on a constant supply of energy in order to survive. For this reason, animals had to evolve a way to adapt to changes in nutrient availability and to food deprivation, depending on their environment. Energy homeostasis in multicellular organisms is reached with a combined action of multiple organs (Herman and Kahn, 2006; Tirone and Brunicardi, 2001). With the division of labor by all tissues, hormonal signals integrate and coordinate the metabolic activities and allocate specific fuels and precursors to each organ. The actions from the most important tissues involved in energy homeostasis will be discussed in this chapter.

1.1. Pancreas

The pancreas is a key organ in regulating energy homeostasis in mammals. More specifically, the endocrine pancreas secretes two hormones able to modulate the metabolic state of the organism: insulin and glucagon. These two hormones are synthesized by the β- and α-cells in the islets of Langerhans, respectively, which respond differently to changes in blood glucose concentrations. While high blood glucose levels will stimulate β-cells to secrete insulin, low levels of blood glucose will induce the secretion of glucagon by α-cells (Nadal et al, 1999; Quesada et al, 2006). These hormones will signal different states of energy levels in the body and will coordinate the responses of many tissues towards the energy availability. Importantly, an abnormal function of these cells and or the signaling cascades induced by them in different tissues will generate diverse problems in the control of blood glucose levels, which can lead to the development of diseases, such as diabetes.

1.1.1. Insulin

The pancreatic β-cells express GLUT2 as the main glucose transporter. GLUT transporters mediate a bidirectional and energy-independent process of glucose transport in most tissues and cells. GLUT2 is a low affinity glucose transporter, with a dissociation constant (K_m) of ~17

mM for glucose. Despite its low affinity, GLUT2 is a high capacity transporter, which makes the concentration inside the cells proportional to the extracellular glucose levels (Johnson et al, 1990; Schuit, 1997; Thorens, 1992; Thorens and Mueckler, 2010).

When blood glucose levels rise, glucose will stimulate the secretion of insulin. Following uptake from the blood, glucose is phosphorylated by glucokinase and subsequently metabolized, causing an increase in the ATP/ADP ratio, which, in turn, mediates the closure of ATP-sensitive potassium channels. This will depolarize the plasma membrane, causing voltage-gated calcium channels to open (Lenzen and Panten, 1988). The increase of intracellular calcium concentration results in the fusion of secretory granules with the plasma membrane and release insulin to the blood (Henquin, 2009; Baltrusch and Lenzen, 2007; Rorsman and Renstrom, 2003).

Insulin has an anabolic effect on different tissues: it promotes glucose uptake (especially on muscle and adipose tissue), while it inhibits the production of glucose by hepatocytes. In addition, insulin stimulates the storage of excess of glucose and fatty acids into glycogen (muscle and liver) and triacylglycerides (TAGs) (adipose tissue and liver), respectively.

1.1.2. Glucagon

Pancreatic α-cells express GLUT1 as the major glucose transporter. GLUT1 shows a high affinity for glucose, with the K_m of ~3 mM (Uldry et al, 2002). Glucagon secretion so far has been less studied than insulin secretion. The difference in stimulated secretion from α- to β-cells relies partially on the nature of the K_{ATP} channels present in these cells. At low glucose concentrations, the activity of K_{ATP} channels allows the opening of voltage-dependent Ca^{2+} and Na^{+} channels. The increase in Ca^{2+} influx causes the exocytosis of glucagon granules. However, high glucose concentrations block K_{ATP} channels, reducing glucagon secretion. One additional mechanism discussed in the context of α-cells is the paracrine inhibition of glucagon secretion by insulin signaling (Quesada et al, 2008).

Glucagon receptor is expressed in multiple tissues such as the liver, pancreas, heart, kidney, brain and muscle and its signaling has a rather catabolic effect. The main action of glucagon in regulating glucose homeostasis is due to its action on the liver, by increasing glucose production. The role of glucagon in the regulation of lipolysis is rather controversial. Although

glucagon was shown to stimulate lipolysis in isolated adipocytes and glucagon receptors were identified in the membrane of human adipose tissue, the role of glucagon signaling in stimulating lipolysis *in vivo* is still under investigation (Duncan et al, 2007).

1.2. Brain

The delivery of glucose to the brain is regulated by several different glucose transporters. GLUT3, for instance, is considered to be the main transporter expressed in neuronal populations, while GLUT1 is mostly expressed in the blood-brain barrier. Moreover, GLUT2 is expressed in the hypothalamus providing a glucose sensing function (Vannucci et al, 1997). GLUT1 and GLUT3 display a high affinity for glucose (K_m ~3 mM and ~1.4 mM, respectively) (Uldry et al, 2002). This high affinity ensures that even when blood glucose levels are low, neurons will have priority amongst other tissues to take up glucose.

The brain has a very active respiratory metabolism, using glucose as main energy source. As the brain contains very little glycogen, it is mostly dependent on glucose present in the blood. During fasting, the brain relies on glucose produced by the liver. Importantly, the brain cannot directly use free fatty acids or lipids from the blood as energy sources. Instead, it can use ketone bodies synthesized by the liver from fatty acids during prolonged fasting or starvation.

The hypothalamus in the brain is a coordinating center of the endocrine system; it contains neurons responsible for controlling food intake and energy expenditure. Various nutrients and hormones, such as insulin and leptin, can act on the hypothalamus and alter food intake (Morton et al, 2014).

1.3. White adipose tissue

Adipocytes express GLUT4 as a glucose transporter. GLUT4 has a high affinity for glucose, its K_m is approximately 5 mM, similar to GLUT1 (Zhao and Keating, 2007). GLUT4 is usually sequestered to intracellular vesicles in the absence of insulin. Upon insulin signaling, GLUT4 is rapidly translocated to the plasma membrane, leading to a dramatic increase in the glucose uptake (Gonzalez and McGraw, 2006; Herman and Kahn, 2006; Furtado et al, 2002; Bryant et

al, 2002; Dugani and Klip, 2005). When insulin stimulation ceases, GLUT4 proteins are endocytosed and recycled back to the intracellular storage vesicles.

In the postprandial period, adipocytes will take up glucose and fatty acids to generate ATP and store it in the form of TAG. TAGs are a great way to store energy due to the fact that they contain more energy per unit mass than carbohydrates, and they can be stored in the absence of water, in contrast to glycogen. During fasting, TAGs stored in adipocytes are hydrolyzed by lipases to release fatty acids and glycerol into the blood, a process called lipolysis. Fatty acids are used by the skeletal muscle, heart, and liver as energy source. In addition, glycerol can be used by the liver in the gluconeogenic pathway and fatty acids in the synthesis of ketone bodies. While epinephrine and glucagon will increase the activity of TAG lipases, insulin reduces its activity, thereby inhibiting lipolysis and reducing the amounts of non-esterified fatty acids (NEFAs) circulating in the blood (Gonzalez-Yanes and Sanchez-Margalet, 2006).

The white adipose tissue plays also a major role in energy homeostasis by secreting several hormones such as leptin, adiponectin, visfatin, omentin, resistin and others (Rosen and Spiegelman, 2006). Leptin, for instance, is one of the most well studied hormones secreted by adipocytes (Zhang et al, 1994). It circulates in the blood in proportion to body fat stores and acts on the hypothalamus to inhibit feeding behavior and to increase energy expenditure (Friedman, 1999; Friedman, 2002; Halaas et al, 1995; Pelleymounter, 1995; Gao and Horvath, 2007; Rosenbaum and Leibel, 2010; Morton et al, 2014).

1.4. Skeletal Muscle

Similarly to adipocytes, myocytes express the GLUT4 glucose transporter. Under insulin stimulation, GLUT4 is translocated to the plasma membrane, thus increasing dramatically the uptake of glucose and reducing blood glucose levels. In fact, a large portion of the glucose present in the blood in the post-prandial period is taken up by myocytes.

Active muscle cells will favor the use of glucose as energy source by breaking it down to lactate through fermentation. In addition, they can store glycogen as a resource to be used when glucose is needed. Differently from the liver, muscle cells do not contain glucose 6-phosphase (G6Pase) and cannot convert glucose 6-phosphate (G6P) into free glucose to be exported to the

blood. Thus, muscle glycogen is uniquely used to provide glucose for the muscle to be oxidized during glycolysis. The glycogen amount stored by the muscle is relatively small, which means that upon exercise, the glycogen stores run down and the amounts of lactate raise. The lactate can be converted back to glucose by the liver in the Cori cycle. In resting conditions, however, myocytes can use fatty acids or ketone bodies as a main source of energy.

1.5. Liver

The liver plays a central role in regulating glucose and lipid homeostasis in our organisms. It coordinates the breakdown, synthesis, storage and redistribution of nutrients. Under fasting/starvation conditions, hepatic glucose production is largely responsible for keeping blood glucose levels steady in the blood. It can also use fatty acids from TAG breakdown to produce ketone bodies that will be utilized by organs, such as the brain that cannot use fatty acids as energy source. On the other hand, in the postprandial period, the liver takes up glucose from the blood to re-build glycogen stores. During high carbohydrate intake, hepatocytes can convert glucose via pyruvate and acetyl-CoA into fatty acids in a pathway called *de novo* lipogenesis. These fatty acids can be used in the synthesis of TAG. Importantly, a deregulation of specific pathways in the liver are strongly associated with the development of diseases such as type 2 diabetes and non-alcoholic fatty liver disease.

1.5.1. Postprandial state

1.5.1.1. Main pathways

1.5.1.1.1. Glucose metabolism

During the postprandial state, about 10-25% of the ingested glucose is taken up by hepatocytes (Ferrannini et al, 1985; Woerle et al, 2003). The major glucose transporter in hepatocytes is GLUT2 (Mueckler and Thorens , 2013; Aschenbach et al, 2009), which is bi-directional and it is not induced by insulin. The rate of glucose transport into the liver is high, only saturating at glucose concentrations above 30 mM (Mueckler and Thorens, 2013), which allows the uptake of high amounts of glucose from the blood.

Once glucose enters hepatocytes it is phosphorylated by glucokinase into glucose-6-phosphate (G6P) (Wilson, 2003; Cardenas et al, 1998). Glucokinase is a glucose-sensing enzyme that can process a wide range of glucose concentrations and efficiently sequester glucose in hepatocytes in response to glycemic fluctuations. It has a lower affinity for glucose than hexokinase, and unlike hexokinase it is not inhibited by its product, G6P (Wilson, 2003; van Schaftingen et al, 1994). In addition, high glucose concentrations and insulin will increase the expression and activity of glucokinase (Heredia et al, 2006; Iynedjian, 2009). Finally, the combined action of GLUT2 and glucokinase delimit part of the fate of glucose in hepatocytes; blood glucose levels need to be high in order to be transported and sequestered intracellularly by phosphorylation. Also, it allows hepatocytes to keep taking up and phosphorylating glucose even when the blood glucose concentration is very high.

G6P can be used in different pathways depending on the metabolic needs of the organism. G6P is used in the synthesis of glycogen (glycogenesis) to replenish glycogen stores. As a substrate, G6P also allosterically activates glycogen synthase and inhibits glycogen phosphorylase, switching the balance towards glycogen synthesis (Aiston et al, 2004; von Wilamowitz-Moellendorff et al, 2013). Moreover, G6P is oxidized in glycolysis to generate pyruvate in the cytoplasm. Pyruvate is then transported into the mitochondria, where the pyruvate dehydrogenase complex will decarboxylate it until acetyl-CoA. In turn, acetyl-CoA can enter the tricarboxylic acid (TCA) cycle to produce ATP through oxidative phosphorylation. Alternatively, an intermediate of the TCA cycle, citrate, can be shuttled to the cytoplasm, where it is converted into oxaloacetate and acetyl-CoA. The cytoplasmic acetyl-CoA will serve as the precursor for the synthesis of fatty acids in the *de novo* lipogenenic pathway. Furthermore, G6P can also be used in the pentose phosphate pathway, to generate both reducing power with NADPH (needed for the biosynthesis of fatty acids and cholesterol) and D-ribose 5-phosphate, a precursor in the nucleotide biosynthesis.

1.5.1.1.2. Fatty-acid metabolism

In the postprandial state, fatty acids from the diet are packed by enterocytes with cholesterol and proteins into chylomicrons. These will transport the exogenous lipids through the blood to various tissues, including adipose tissue and muscles. In these tissues, the TAG components will be unloaded from the chylomicron and taken up by the cells. Chylomicron remnants are then taken up by the liver.

Another source of fatty acids in the liver after a meal is through the above mentioned *de novo* lipogenesis. In fact, the majority of the glucose in the liver that enters the glycolytic pathway will provide carbons to the formation of acetyl-CoA. Certain amino acids deriving from the diet can also be used to generate acetyl-CoA. In the mitochondria, acetyl-CoA and oxaloacetate are converted into citrate, which can be exported from the mitochondria and converted to acetyl-CoA by the ATP citrate lyase in the cytoplasm. The cytoplasmatic acetyl-CoA is then converted to malonyl-CoA by the acetyl-CoA carboxylase 1 (ACC1), which is subsequently converted to palmitate by the fatty-acid synthase (FAS). Fatty acids will then be elongated (fatty acyl-CoA elongase, ELOVL6) and desaturated (stearoyl-CoA desaturase 1, SCD1) before the process of esterification. These are key regulatory reactions in the *de novo* lipogenic pathway.

Liver fatty acids will go through esterification and formation of TAGs. The TAGs can then be stored in hepatocytes or exported to other tissues. Some of the acetyl-CoA derived from the oxidation of fatty acids and from glucose is also used for cholesterol biosynthesis. Cholesterol is essential in membrane biosynthesis, steroid hormones, and bile salts, which are essential for the digestion and absorption of lipids. Together, TAG, phospholipids, and cholesterol will be packed into very low-density lipoproteins (VLDLs) and exported to other tissues through the blood.

1.5.1.2. Main molecular players

1.5.1.2.1. Glucose and ChREBP

A major transcription factor in the liver that controls the expression of many glucose-sensing genes is the carbohydrate response element binding protein (ChREBP) (Jeong et al, 2011; Ma et al, 2006). Glucose activates ChREBP by regulating its entry from the cytosol to the nucleus (Dentin et al, 2005; Kawaguchi et al, 2001). The exact mechanisms of ChREBP activation are still under investigation, but it is known that its activation requires G6P (Li et al, 2010a; Dentin et al, 2012) and possibly fructose-2,6-biphosphate (Arden et al, 2012). In addition, it is believed that PTMs and allosteric modulation are involved in its activation (Li et al, 2006; Sakiyama et al, 2008; Davies et al, 2008). ChREBP activity is increased by acetylation and O-linked β-N-acetylglucosaminylation (Bricambert et al, 2010; Guinez C, 2011). Moreover, acetyl-CoA and liver x receptor (LXR) were also shown to modulate ChREBP transcriptional activity.

In the nucleus, ChREBP recognizes conserved carbohydrate response elements (ChoREs) present in promoters of glycolytic and lipogenic genes (Thompson and Towle, 1991; Shih et al, 1995; Towle, 2005; Filhoulaud et al, 2013). Targets of ChREBP include liver pyruvate kinase (L-PK), ACC, FAS and SCD1, the enzymes in the pentose-phosphate pathway that generates NADPH (e.g. G6P dehydrogenase), the enzyme of TAG synthesis (glycerol 3-phosphate dehydrogenase (GPDH)), the rate limiting enzyme of VLDL production (TAG transfer protein (MTP)), and ELOVL6 (Ma et al, 2006; Ma et al, 2007; Iizuka et al, 2004; Dentin et al, 2004; Wang et al, 2006).

1.5.1.2.2. Insulin

Insulin is the main hormone that orchestrates the metabolic pathways in the fed state. At the molecular level, insulin binds to the insulin receptor (IR) and causes receptor dimerization and auto-phosphorylation (Figure 1). The activation of IR tyrosine kinase recruits and phosphorylates several substrates, including IR substrates (IRS) 1-4, Src homology 2 domain containing transforming protein 1 (SHC), Grb-associated protein (GAB1). These substrates will provide docking sites for the recruitment of downstream targets which will ultimately activate Ras - MAPKs and PI(3)K – AKT signaling cascades (White, 2003). The activation of Ras-MAPKs will mediate the effect of insulin on cell growth, while the activation of AKT through PDK1 and mTORC1 will mediate the metabolic and pro-survival effects.

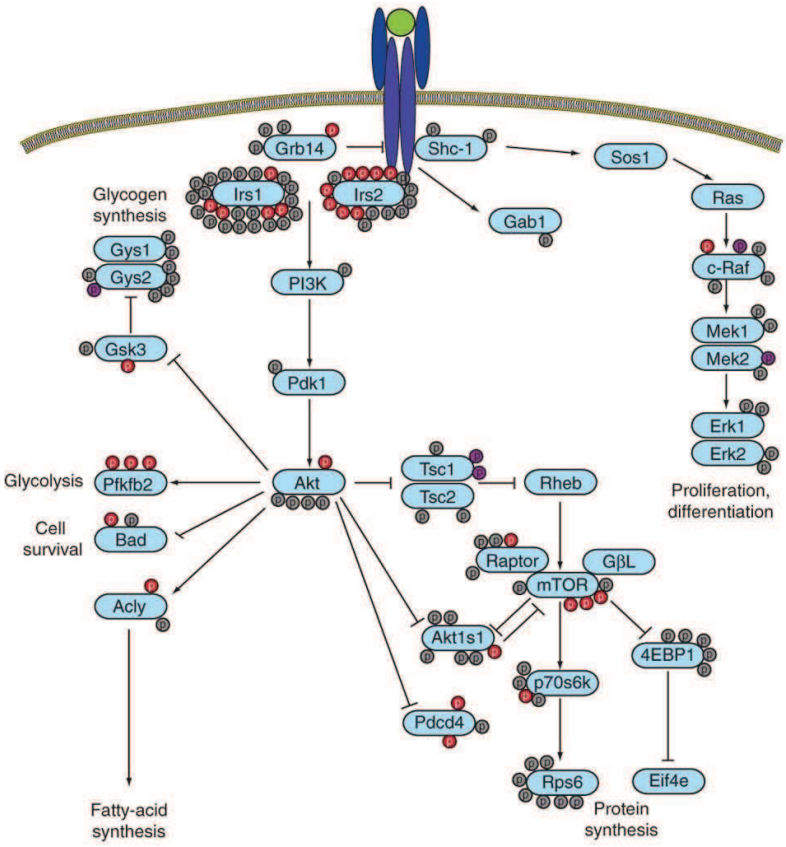


Figure 1: Components of insulin signaling in the liver shown to be activated upon insulin treatment

Insulin binding to its receptor leads to the recruitment of adaptors and activation of many downstream targets in the liver. This signaling will activate a whole plethora of cellular pathways regulating glycogen synthesis, glycolysis, cell survival, fatty acid synthesis, protein synthesis, proliferation, and differentiation. Remarkably, phosphorylation plays a crutial role in the regulation of insulin signaling as it is shown by all the phosphorylation sites identified by mass spectrometry. *Monetti et al, 2011.*

In livers, insulin stimulates glycogen accumulation through 2 coordinated ways: increasing glucose uptake by the hepatocytes and promoting the dephosphorylation and activation of glycogen synthase, a rate-limiting enzyme in the synthesis of glycogen. The effects of insulin on activating glycogen synthase are mediated by activation of protein phosphatase 1 (PP1) (Brady et al, 1997) and inhibition of protein kinase A (PKA) and glycogen synthase kinase-3 (GSK-3), which can phosphorylate and inhibit glycogen synthase (Cross et al, 1995; Lawrence and Roach, 1997).

Insulin directly controls metabolic enzymes by affecting both, transcriptional levels and post-translational modifications (e.g. phosphorylation) (Pilkis and Granner, 1992). It inhibits the transcription of key gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (F1,6Pase), and G6Pase (Sutherland et al, 1996), thus inhibiting gluconeogenesis. PEPCK is further regulated in the post- translational level by acetylation and ubiquitylation (Jiang et al, 2011). In addition, insulin increases the transcription of glycolytic enzymes (glucokinase, pyruvate kinase) and lipogenic enzymes (fatty acid synthase and acetyl-CoA carbolxylase), and promotes the synthesis of lipids and inhibits their degradation through action on the transcription factor SREBP-1c (sterol regulatory element-binding protein 1c) (Shimomura et al, 1999).

Disruption of insulin signaling in liver insulin receptor knockout (LIRKO) mice led to a lack of inhibition of hepatic glucose production, with high levels of PEPCK and G6Pase in the postprandial period. In addition, mice showed insulin resistance in other tissues, causing severe glucose intolerance (Michael et al, 2000).

1.5.1.2.3. AKT and mTOR

Insulin signaling promotes the phosphorylation and activation of AKT (Figure 1), which has several downstream targets that will mediate the functions to increase energy storage and oxidation of glucose. Many of the other targets of AKT will lead to GLUT4 translocation, apoptosis inhibition, and cAMP degradation. One AKT target is the glycogen synthase kinase 3β (GSK3β), which phosphorylates and inhibits glycogen synthase. Upon phospohorylation by AKT, GSK3β is inhibited and glycogen synthase becomes activated. Another target that is phosphorylated and inhibited is TSC2, thereby activating RhebGTPase and activating mTORC1 (Inoki et al, 2002). Yet, another target of AKT which gets phosphorylated and inhibited is the cAMP response element-binding protein (CREB)-regulated transcription coactivator 2 (CRTC2). CRTC2 is a CREB co-activator that increases hepatic gluconeogenesis (Wang et al, 2010a), but when phosphorylated, it is ubiquitylated and degraded in the cytosol (Dentin et al, 2007). Moreover, AKT increases the expression and activation of the sterol regulatory element-binding protein 1 (SREBP1) transcription factor (Yecies et al, 2011) and suppresses the transcription factor FOXO1, which promotes gluconeogenesis (Hagiwara et al, 2012).

mTOR is a highly conserved protein kinase able to control cell growth and metabolism in response to nutrients, growth factors and energy status of the cell. mTOR exists in 2 different complexes: mTORC1 and mTORC2 (Sengupta et al, 2010). mTORC2 phosphorylates and activates AKT, thus regulating cell survival and homeostasis (Sarbassov et al, 2006; Hagiwara et al, 2012). mTORC1, however, is activated via AKT downstream signaling, affecting protein synthesis by activating ribosomal protein S6 kinase (S6K) and eukaryotic initiation factos 4E-binding protein (eIF-4E) (Thomas and Hall, 1997; Miron et al, 2001). mTORC1 also promotes lipogenesis via the phosphatidic acid phosphatase lipin 1. Upon phosphorylation by mTORC1, lipin 1 translocates to the nucleus and it stimulates SREBP1c and lipogenesis (Li et al, 2010a; Peterson et al, 2011). mTORC1 is also activated by nutrients such as amino acids and it can suppress autophagy.

1.5.1.2.4. SREBP-1c

SREBP-1c is a major transcription factor that mediates the anabolic response in the liver (Figure 2). Insulin signaling through AKT-mediated phosphorylation stimulates SREBP1c to promote lipogenesis. AKT signaling suppresses INSIG2, an inhibitor of SREBP-1c, and mTORC1 signaling activates SREBP-1c (Yabe et al, 2002; Yecies et al, 2011). The combined action of both stimuli results in increased transcription of several enzymes involved in fatty acid synthesis (e.g. ACC, FAS, SCD1, ELOVL6) (Shimano et al, 1999; Horton et al, 2003; Moon et al, 2001) and TAG synthesis (e.g. GPAT) (Gonzalez-Baro et al, 2007).

1.5.1.2.5. FoxO1

Part of the inhibitory effect of insulin on gluconeogenesis is through AKT-mediated phosphorylation and inhibition of Forkhead transcription factor FoxO1 transcriptional activity, thereby suppressing glucose production in the liver. FoxO1 is a fasting response transcription factor that increases levels of PEPCK and G6Pase (Schmoll et al, 2000; Yeagley et al, 2001). Mice lacking hepatic FoxO1 shows lower hepatic glucose production and blood glucose levels (Estall, 2012). Phosphorylation of FoxO causes its cytoplasmic translocation from the nucleus and interaction with SKIP1 (TRIB1)/CUL1/-F-box protein complex which causes its ubiquitylation and subsequent proteasomal degradation, thereby reducing the expression of gluconeogenic genes (Huang et al, 2005).

1.5.1.2.6. LXR

Liver X receptors (LXR) are ligand-activated transcription factors that play a role in regulating expression of lipogenic genes (Figure 2), as well as genes involved in cholesterol and bile acid metabolism. These receptors directly regulate expression of FAS, ACC, and SCD1 (Schultz et al, 2000; Liang et al, 2002; Chu et al, 2006). In addition they regulate SREBP-1c (Chen et al, 2004; Chen et al, 2007; Schultz et al, 2000) and ChREBP (Cha and Repa, 2007; Denechaud et al, 2008), playing an important role in insulin-mediated anabolic effects.

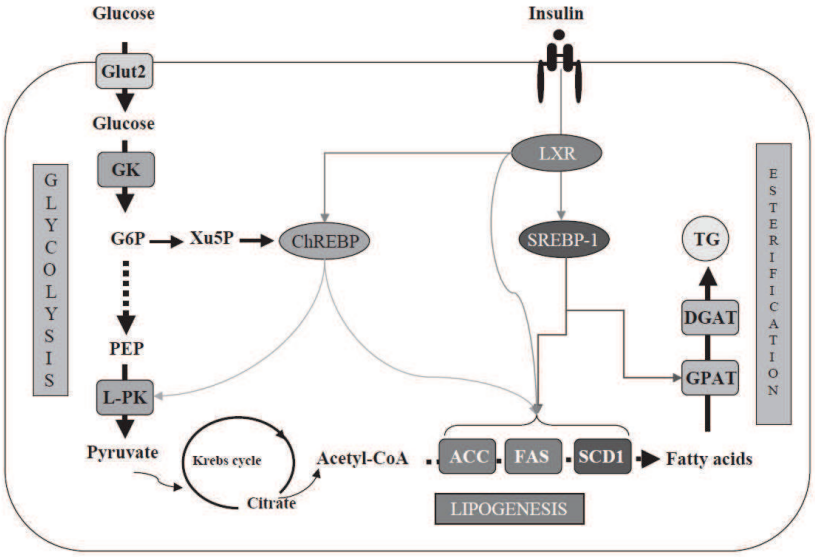


Figure 2: Glucose and insulin regulate the transcription of glycolytic and lipogenic genes

Glucose uptake and insulin signaling in hepatocytes regulate the activity and expression of proteins that will induce glycolysis, lipogenesis, and fatty acid esterification. *Postic and Girard, 2008b.*

1.5.2. Fasted state

1.5.2.1. Main pathways

1.5.2.1.1. Glucose metabolism

In a fasted state, the liver utilizes fatty acids as the main energy source and will make available glucose to other tissues that depend on glucose, such as the brain and erythrocytes. Hepatic glucose uptake and metabolism decreases as soon as glucose levels start dropping. Most tissues in our organism will rely on the production of glucose by the liver, or will switch to alternate energy sources.

When insulin signaling ceases and glucagon signaling starts, the liver will immediately start breaking down glycogen stores (glycogenolysis). These stores are able to keep blood glucose levels steady during the initial fasting hours. Another pathway that generates glucose to the blood is the gluconeogenic pathway. The liver is the major organ able to generate glucose from glucogenic amino acids, lactate, and glycerol as precursors. The final reaction of both glycogenolysis and gluconeogenesis is the dephosphorylation of G6P by G6Pase to yield free glucose, which will be transported to the blood via GLUT2.

The rate of gluconeogenesis is controlled by the activities of the three rate-limiting enzymes: PEPCK, F1,6Pase, and G6Pase (shown in Figure 3 and Figure 4). These enzymes are regulated at several levels; by transcription factors, allosteric regulators and post translational modifications. Importantly, animals are not able to convert fatty acids into glucose; fatty acids cannot be used as carbon source for the gluconeogenic pathway. From TAGs, the liver can only use the glycerol component to generate glucose *de novo*.

1.5.2.1.2. Fatty acids metabolism

During the fasted state, lipolysis in the adipose tissue will release non-esterified fatty acids (NEFAs) and glycerol into the plasma. While the NEFAs will be transported to several tissues in the form of albumin complexes, the glycerol will be transported to the liver to be used as a precursor of *de novo* glucose production.

NEFAs in the liver will go through the process of fatty acid β-oxidation which happens in the mitochondria to finally yield acetyl-CoA and NADH. Under normal conditions, acetyl-CoA will enter the TCA cycle and mitochondrial electron transport chain to be further oxidized and release ATP. However, under certain conditions, acetyl-CoA can accumulate in cells. For instance, it can accumulate when amounts of acetyl-CoA generated by β-oxidation are higher

than the processing capacity of the TCA cycle. Acetyl-CoA can also accumulate when the activity of the TCA cycle is low due to low amounts of intermediates (e.g. oxaloacetate), which happens due to active gluconeogenesis and it results in blocking the entry of acetyl-CoA in the TCA cycle. In such cases, acetyl-CoA is used instead in the biosynthesis of ketone bodies via acetoacyl-CoA and mitochondrial β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). Some amino acids that are ketogenic (e.g. leucine) also can be used for generation of ketones bodies.

Ketone bodies are comprised of three compounds: acetone, acetoacetic acid, and β-hydroxybutyric acid. The two latter ones can be used by tissues such as muscles and the brain as a source of acetyl-CoA to provide energy. Acetone, however, is usually excreted as a degradation product as it cannot be converted into acetyl-CoA. When ketogenesis exceeds the rate of ketone body utilization, their concentration in blood increases, in a state called ketosis. As ketone bodies are acidic, ketosis usually causes the pH of the blood to drop, resulting in ketoacidosis, a condition that occurs in some diabetic patients.

1.5.2.2. Main molecular players

1.5.2.2.1. Glucagon

Glucagon is a major hormone responsible for the fasting response in our organisms and its primary target is the liver. Glucagon stimulates glycogenolysis and gluconeogenesis, while blocking glycolysis and glycogenesis, thereby raising blood glucose levels. Binding of glucagon to its receptor, a seven-transmembrane G protein-coupled receptor, causes conformational changes of the latter, leading to subsequent activation of the G proteins $G_S\alpha$ and G_q (Figure 3). Activation of $G_S\alpha$ leads to the activation of adenylate cyclase, increase of cyclic AMP (cAMP) levels, and subsequent activation of protein kinase A (PKA). On the other hand, activation of G_q leads to the activation of phospholipase C, generation of inositol 1,4,5-triphosphate, and subsequent release of intracellular calcium (Figure 3) (Burcelin et al, 1996; Christophe, 1995; Jiang and Zhang, 2003).

PKA signaling will activate glycogen phosphorylase, which will phosphorylate glycogen and increase its breakdown to G6P. To the same end, glucagon phosphorylates and inhibits glycogen synthase, inhibiting glycogenesis (Akatsuka et al, 1985; Jiang and Zhang, 2003; Andersen et al, 1999).

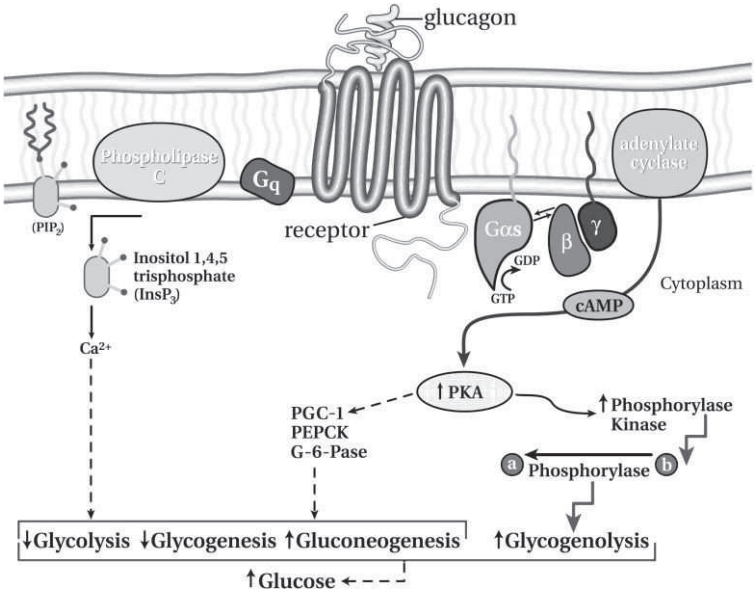


Figure 3: Glucagon signaling pathway leads to increased hepatic glucose production

Upon binding to its receptor, glucagon mediates the cAMP-dependent activation of PKA, which phosphorylates downstream targets responsible for the increased rates of gluconeogenesis, glycogenolysis and decresed rates of glycogenesis and glycolysis. *Jiang and Zhang, 2003.*

Glucagon signaling also inhibits the glycolytic enzyme pyruvate kinase, blocking the conversion of phosphoenolpyruvate (PEP) to pyruvate. This avoids the further oxidation of pyruvate by the TCA cycle and it favors the accumulation of PEP to be used in the gluconeogenic pathway (Slavin et al, 1994). PKA mediated-signaling also leads to the upregulation of key gluconeogenic enzymes: PEPCK and G6Pase. It also lowers the levels of fructose 2,6-biphosphate, which is an allosteric activator of the rate-limiting gluconeogenic enzyme fructose-1,6-biphosphatase and an activator of phosphofructokinase-1. Importantly, there are also other hormones able to elicit the fasting response, such as epinephrine and cortisol, which can also stimulate lipolysis and glycogenolysis in the liver.

1.5.2.2.2. Transcription factors

Cyclic AMP-responsive element (CRE)-binding protein (CREB) is a transcription factor responsible for a large part of the transcriptional regulation involving glucagon signaling (Figure 4). Activated PKA translocates into the nucleus and phosphorylates CREB. In fact,

phosphorylation activates CREB by stimulating its interaction with CREB-binding protein and p300, which are histone deacetylases (Chrivia et al, 1993; Kwok et al, 1994; Arias et al, 1994). Activated CREB, together with cAMP-regulated transcriptional co-activators (CRTCs), will bind the promoters of genes that contain CREs (cAMP-responsive elements). Interestingly, CRTCs significantly increase their activity and when phosphorylated, CRTCs translocate to the cytoplasm. In this way, two regulatory events have to exist for CREB to activate target genes: CREB phosphorylation and CRTCs dephosphorylation (Altarejos and Montminy, 2011).

During short-term fasting, CREB is responsible for the upregulation of gluconeogenic genes such as PEPCK and G6Pase by directly binding to their promoters (Figure 4) (Herzig et al, 2001; Quinn and Granner, 1990; Wynshaw-Boris et al, 1986). In parallel, a decrease in insulin signaling also stimulates transcription of these genes by dephosphorylation and nuclear translocation of the forkhead box O (FoxO) transcription factors (Haeusler et al, 2010; Eijkelenboom and Burgering, 2013).

CREB also upregulates the expression of peroxisome proliferator-activated receptor γ-coactivator-1α (PGC-1α) and of members of the nuclear receptor subfamily 4 group A (NR4A) family (Herzig et al, 2001; Pei et al, 2006). PGC-1α plays a role as a co-activator for glucocorticoid receptor (GR), hepatocytes nuclear factor 4 (HNF4) and FoxO transcription factors. Upregulation of PGC-1α and NR4A is a mechanism that strengthens the expression of gluconeogenic genes in response to prolonged fasting (Figure 4) (Altarejos and Montminy, 2011). During prolonged fasting or heavy exercise, the energy-sensing kinase AMPK (AMP-activated protein kinase) can inhibit hepatic gluconeogenesis by phosphorylating and inactivating CRTC2. In addition, AMPK also increases hepatic β-oxidation and the generation of ketone bodies (Koo et al, 2005; Inoki et al, 2012).

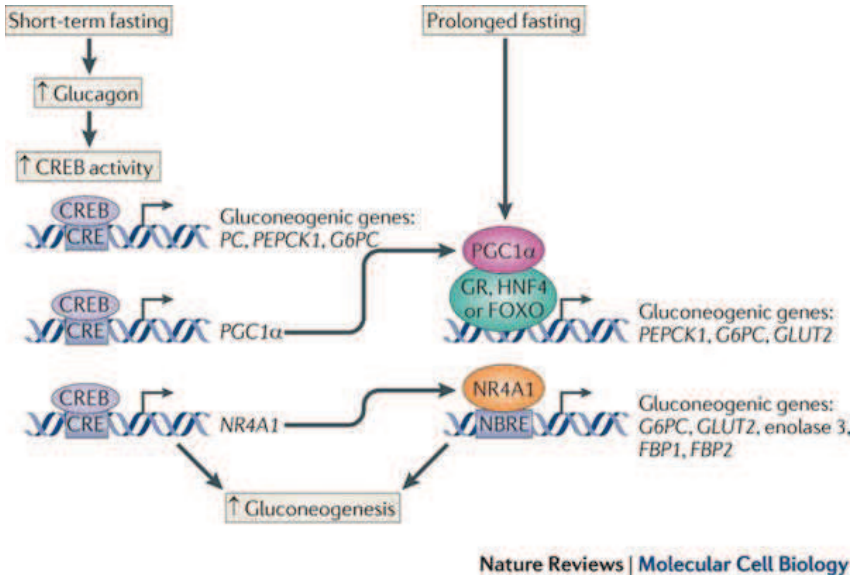


Figure 4: CREB activation increases the transcription of gluconeogenic genes

Glucagon signaling leads to the activation of CREB, increased expression of pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase 1 (PEPCK1), glucose 6-phosphatase (G6PC), peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α) and nuclear receptor subfamily 4 group A (NR4A). PCG1α and NR4A will further increase the transcription of gluconeogenic enzymes and the glucose transporter GLUT2. *Altarejos and Montminy, 2011.*

1.6. Liver glucose and lipid homeostasis - disorders

Due to the fact that the liver works as the central metabolic organ in our organisms, with specific pathways being switched on and off depending on the hormonal and metabolic stimulation, an alteration in the regulation of these specific pathways can lead to diseases. For example, anabolism and catabolism of lipids is in a tight balance in order to prevent excessive TAG storage in hepatocytes, but also to avoid release of additional glucose in prostprandial stage. If lipid metabolism is altered in the liver, leading to the abnormal accumulation of fatty acids and TAG, this will lead to the development of non-alcoholic fatty liver disease (NAFLD). On the other hand, if insulin is not able to suppress gluconeogenesis in the liver in the postprandial period, it will continue to raise blood glucose levels in the postprandial state, a characteristic feature of type-2 diabetes (T2D).

1.6.1. Non-alcoholic fatty liver disease (NAFLD)

NAFLD is a chronic liver disease characterized by the abnormal accumulation of TAG (steatosis) in the liver of people who consume little or no alcohol. The spectrum of the disease encompasses simple hepatic steatosis, non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma. Simple steatosis can be reversible, but it can also progress to NASH, when the liver shows signs of inflammation and fibrosis. NASH can continue to progress to cirrhosis, and hepatocellular carcinoma in a few cases (McCullough, 2006; Serfaty and Lemoine, 2008; Cohen et al, 2011; Gariani et al, 2013).

NAFLD is the most frequent form of chronic liver disease in developed countries. Its prevalence is constantly increasing with the growing epidemics of diabetes and obesity, especially in Western countries. In a recent study based on the population of the United States with 328 individuals, NAFLD had a prevalence of 46%. The prevalence for NASH was 12%, and, 2.7% of the analyzed individuals showed significant liver fibrosis (Williams et al, 2011).

There is a strong association between NAFLD and insulin resistance (Kumashiro et al, 2011; Jornayvaz and Schulman, 2012). More generally, any of the elements that are part of the metabolic syndrome, such as type 2 diabetes, hypertension and dyslepidaemia, are linked with NAFLD. It is estimated that around 85% of the patients with NAFLD also show at least one of the constituents of the metabolic syndrome (Gariani et al, 2013).

The diagnostics of NAFLD can be done based on imaging techniques, such as computed tomography or magnetic resonance imaging. In such cases, it is difficult to distinguish between the different stages of the disease. For this reason, the “gold standard” method to diagnose NAFLD is still based on liver biopsies, which allows determining the degree of steatosis, inflammation and fibrosis (Wong et al, 2010; Mazhar et al, 2009; Lee et al, 2010).

1.6.1.1. The “two-hit” theory of NAFLD development

The “two-hit” theory states that NAFLD is probably a result of two distinct steps of hepatic injury, similar to those observed in alcoholic fatty liver disease (James and Day, 1998; Lieber, 2004). The first “hit” is the development of hepatic steatosis as a result of an imbalance of normal hepatic lipid metabolism. At this point steatosis might be reversed and not necessarily lead to

hepatic injury (Burt et al, 1998). The second, less common, “hit” occurs in around 5% of the individuals with steatosis and is related to an inflammatory process, which leads to hepatocyte death, scarring and progression to NASH (Syn et al, 2009). It is estimated that about 25% of patients with NASH will develop cirrhosis in a process that can take only a few years (Brunt, 2009; Patton et al, 2006).

1.6.1.1.1. The first “hit”: hepatic steatosis

The sources of fatty acids that contribute to the development of liver steatosis include the (i) NEFAs released from the white adipose tissue, (ii) dietary fats through the uptake from chylomicron remnants, and (iii) the fatty acids derived from the *de novo* lipogenic pathway within the liver (Figure 5). In the liver, fatty acids can be oxidized to generate energy through β-oxidation, or they can go through the process of esterification and generation of TAG. TAG can be either stored within lipid droplets in hepatocytes or be exported to other organs via blood in the form of VLDL (Figure 5). Thus, an imbalance between the fatty acids input and output pathways can lead to excessive TAG accumulation in the liver (Bradbury and Berk, 2004; Koteish and Diehl, 2001; Lim et al, 2010; Postic and Girard, 2008a). This imbalance can, for instance, be generated by increased fat delivery by adipose tissue, increased uptake of fats from the diet (or reduced uptake by other organs), increased *de novo* synthesis of lipids, reduced β-oxidation or reduced VLDL export (Figure 5).

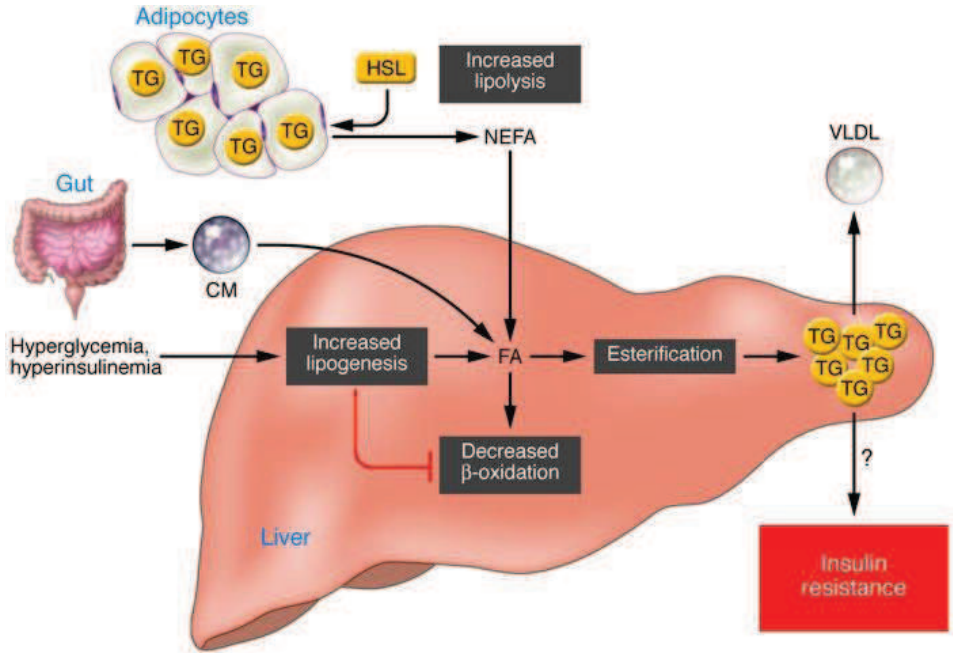


Figure 5: Pathways involving fatty acid metabolism in the liver

Fatty acids in the liver can come from the diet, from the *de novo* lipogenic pathway or from NEFA circulating in the blood. Fatty acids can then be oxidized or esterified into triacylglygerides (TG) to be finally exported or stored. An imbalance between these pathways can lead to the excessive accumulation of TG in the liver, a process that has been associated with insulin resistance. *Postic and Girard, 2008a.*

In NAFLD patients, insulin does not suppress lipolysis in the adipose tissue in the same ways as in healthy individuals, resulting in high levels of circulating NEFAs in the blood (Lewis et al, 2002). High release of NEFAs from visceral adipose tissue will circulate first through the liver and may contribute to steatosis (Anstee and Goldin, 2006; Roden M, 2006).

In the fasted state in healthy individuals, the *de novo* lipogenic pathway contributes to less than 3% of the fatty acids, TAG and VLDL synthesis in hepatocytes (Diraison and Beylot, 1998). However, conditions such as high carbohydrate intake, hyperglycemia and hyperinsulinemia are associated with increased rates of *de novo* lipogenesis, increasing the amounts of TAG produced from this source (Donnelly et al, 2005; Lim et al, 2010). Indeed, feeding mice, which were previously fasted, a low fat-high carbohydrate diet induces a strong *de novo* lipogenesis. It was shown that this protocol increases the expression of enzymes involved in glycolysis (glucokinase and liver-pyruvate kinase), lipogenesis (acetyl-CoA carboxylase and fatty acid

synthase), fatty acid elongation and desaturation (long-chain elongase, stearoyl-CoA desaturase 1) and mitochondrial enzymes for TAG synthesis (glycerol 3-phosphate acyltransferase and diacylglycerol acyltransferase) (Postic and Girard, 2008a).

β-oxidation is the main pathway for the metabolism of long-chain fatty acids under physiological conditions. If this process is disrupted in one of the key enzymatic stages, it may lead to a dramatic lipid accumulation in hepatocytes (Ferramosca and Zara, 2014). Excess of fatty acids can be esterified and incorporated into VLDLs to be exported to other tissues. It was observed that VLDL production in patients with NASH was decreased compared to healthy individuals (Charlton et al, 2002). If either the production or export of VLDLs is impaired, it might also lead to the accumulation of hepatic lipids.

1.6.1.1.2. Fructose metabolism

Each of these inputs and outputs, when disturbed, can lead to the TAG accumulation in hepatocytes. However, the fact that the prevalence and severity of NAFLD has been increasing, suggests that there might be lifestyle or diet changes that favors the TAG accumulation. The ingestion of fructose could be the answer to this dilemma. In the typical Western diet, the consumption of fructose in form of sucrose or as corn syrup has increased six times in the last 20 years (Bray, 2007).

The liver is the main organ responsible for metabolizing fructose in our organisms. It possesses the fructose-specific GLUT5 transporter (Douard and Ferraris, 2008). Due to the fact that fructose is chemically different from glucose, it is metabolized in a more similar way to ethanol than to glucose. In hepatocytes, fructose is phosphorylated by fructokinase yielding fructose 1-phosphate, which is subsequently metabolized into glyceraldehyde 3-phosphate (Gly-3P). Gly-3P will go through glycolysis to yield acetyl-CoA, which is either further metabolized in the TCA cycle, or converted into fatty acids via the *de novo* lipogenic pathway (Samuel, 2011; Lim et al, 2010). In this way, fructose metabolism activates the *de novo* lipogenic pathway by providing required substrates.

In addition, fructose metabolism also increases lipogenesis by inhibiting β-oxidation due to excess formation of malonyl-CoA. It will also lead to the activation of MAPK8 and PKCε, which will contribute to the phosphorylation of IRS-1 and development of hepatic insulin resistance.

Fructose metabolism was also shown to increase synthesis of FoxO1, which will promote gluconeogenesis and hyperglycemia. At last, fructose metabolism generates 100 times more reactive oxygen species than glucose metabolism which can lead to hepatocellular damage (Samuel, 2011; Lim et al, 2010). Thus, fructose is a monosaccharide with strong lipogenic potential that can lead to the development of liver steatosis and liver damage. In fact, many studies showed the implications of fructose in the pathogenesis of NAFLD, metabolic syndrome and type 2 diabetes (Thuy et al, 2008; Le and Tappy, 2006; Rutledge and Adeli, 2007; Havel, 2005; Gross et al, 2004; Elliott et al, 2002; Brown et al, 2008; Spruss and Bergheim, 2009; Le et al, 2009; Lim et al, 2010).

1.6.1.1.3. The second “hit”: inflammation

According to the “two-hit” theory, the increased inflammation deriving from fat accumulation leads to the progression of the disease into NASH (Day and James, 1998). A more recent theory proposes also a third “hit” as being important in the disease development; the third “hit” corresponds to hepatocytes death and lack of repair (Jou et al, 2008).

Oxidative stress has been associated with NAFLD (Sanyal et al, 2001; Bergheim et al, 2008) and it may be a result of the lipotoxicity of fat accumulation (Yamaguchi et al, 2007; McClain et al, 2007). Adipose tissue also contributes to the inflammatory process of NAFLD/NASH with the secretion of hormones and adipokines. TNF-α, for instance, is a pro-inflammatory cytokine that is known to be elevated in NAFLD (Kern et al, 1995) and its levels increase as the disease progresses (Jarrar et al, 2008). Likewise, increased levels of interleukin-6 (IL-6) are associated with increased inflammation and fibrosis in NAFLD (Van Der Poorten et al, 2008). On the contrary, adiponectin, an anti-inflammatory factor, is known to be decreased in NAFLD and to have a negative correlation with hepatic steatosis (Jarrar et al, 2008; Aller et al, 2008).

1.6.1.2. NAFLD linked to insulin resistance

Insulin resistance is defined as the lack of appropriate downstream effects of insulin signaling; and this is a feature that is almost always present in NAFLD patients (Farrell and Larter, 2006). A major unresolved question, however, is if NAFLD is a cause or a consequence of insulin resistance.

There are several evidences supporting the hypothesis that insulin resistance causes liver steatosis. For instance, it was shown that, when the liver becomes insulin resistant, the inhibitory effect of insulin on glucose production is diminished, while the stimulatory effect of insulin on lipogenesis is retained (Brown and Goldstein, 2008). In a similar manner, patients with AKT2 mutations show strong resistance to the glucoregulatory actions of insulin, but retain sensitivity to the lipogenic effects of the hormone (Semple et al, 2009). In addition, treatments that ameliorate the insulin resistance, lead to lower insulin levels and reduced liver fat accumulation (Cohen et al, 2011).

On the other hand, the coincident occurrence of hepatic steatosis and insulin resistance provokes the hypothesis that abnormal accumulation of TAG in the liver causes insulin resistance. Many groups have shown that decreasing hepatic TAG pools correlates with improved insulin sensitivity (Savage et al, 2006; Dentin et al, 2006; Neschen et al, 2005). Other groups have shown that accumulation of fatty acids in excess in TAG is not cytotoxic, but protective instead. This is because the inhibition of TAG synthesis in mice improved dramatically liver steatosis, but it increased liver damage. Showing that not TAG, but other lipid metabolites, including lysophosphatidic acid (LPA) and DAG, may contribute to the development of insulin resistance (Postic and Girard, 2008a).

1.6.2. Type 2 diabetes (T2D)

T2D is characterized by hyperglycemia in the context of insulin resistance and relative lack of insulin. The prevalence of T2D has been increasing dramatically since 1960 in parallel with obesity. In 2010 there were approximately 285 million people (around 6% of the world's adult population) diagnosed with the disease compared to around 30 million in 1985 (Smyth and Heron, 2006; Meetoo et al, 2007). Some long-term complications of T2D include cardiovascular diseases (including ischemic heart disease and stroke), retinopathy, and kidney failure.

T2D is characterized by peripheral insulin resistance that results in high blood glucose levels. The peripheral insulin resistance happens when tissues such as liver, muscle and adipocytes, do not respond to insulin signaling in a proper way and therefore need higher insulin concentrations to respond to the hormonal cue. The initial steps of insulin resistance can be compensated by the action of pancreatic β-cells. They can increase insulin secretion to sufficient levels in order to overcome the initial resistance and maintain normal glucose tolerance (Perley

and Kipnis, 1966; Polonsky et al, 1988; Kahn et al, 1993). The increased rates of insulin secretion can eventually cause beta cell dysfunction and gradual loss of insulin secretion. Thus, when β-cells cannot fully compensate for the decreased insulin sensitivity of peripheral organs, T2D may develop (Kahn et al, 1993; Kahn et al, 2006). More recently, this model has been challenged by a few groups that claim that insulin resistance is not the cause of T2D, but instead, it is an adaptation to increased insulin levels (Gray et al, 2010; Ning et al, 2011; Mehran et al, 2012).

Obesity is strongly associated with increased risk of developing T2D and insulin resistance. More specifically, the visceral fat is the one that has been linked with the development of type 2 diabetes (Rosen and Spiegelman, 2006). Adipose tissue modulates metabolism by releasing NEFAs, glycerol, hormones (e.g. leptin, adiponectin and resistin) and proinflammatory cytokines (Wellen and Hotamisligil, 2005; Scherer, 2006). Reduced levels of adiponectin release (insulin sensitizer) and increased levels of pro-inflammatory factors such as TNF-α and IL-6, might play a role in the development of insulin resistance (Wellen and Hotamisligil, 2005; Scherer, 2006; Fain et al, 2004). Increased NEFA levels are observed in obesity and in T2D patients and they were shown to be able to induce insulin resistance in muscle and liver (Roden et al, 1996; Reaven et al, 1988; Boden, 1997; Boden and Chen, 1995).

The liver plays a major role in the pathophysiology of T2D. Insulin normally suppresses glucose release by the liver. However, in the setting of insulin resistance, the liver inappropriately releases glucose into the blood. In addition, the aberrant glucose sensing by the liver leads to TAG accumulation and excessive glucose production (Dentin et al, 2008; Dentin et al, 2006). The excessive glucose production is a major contributor of fasting and postprandial hyperglycemia in T2D patients.

1.6.3. The regulation of metabolic pathways

Metabolic pathways can be regulated in three different manners: gene expression, allosteric modulation and posttranslational modifications (PTMs). PTMs are one of the fastest and reversible ways to control signaling events; so far many proteins in metabolism were described to be regulated by phosphorylation and acetylation. One emerging concept in signaling transduction is protein ubiquitylation, which will be introduced in Chapter 2. Ubiquitylation was shown to modulate signaling events and protein degradation in various fields, such as cell

signaling and DNA repair, but it is explored to a lesser extent in the field of metabolism. The regulation of metabolic pathways by ubiquitylation will be discussed in more details in Chapter 3.

2. Ubiquitylation

Ubiquitylation is a post-translational modification involved in most of the physiological processes in eukaryotes. In this very fascinating modification, ubiquitin is covalently attached to target proteins by an inducible cascade of 3 enzymes: E1 activating enzymes, E2 conjugating enzymes and E3 ligases. The ubiquitin conjugation is reversible; there are deubiquitinating enzymes able to remove ubiquitin from the target proteins. Altogether, the ubiquitin system comprises around 1000 proteins, which is equivalent to around 5% of the genome.

Ubiquitylation is an extremely versatile and diverse post-translational modification; ubiquitin can form chains with many different topologies. These different topologies can be recognized by different proteins and lead to different cellular outcomes. Once considered only a degradation mark due to its ability of targeting proteins to the proteasomal degradation, ubiquitin is now considered a signaling component able to trigger a whole plethora of molecular events in cells. Importantly, abnormalities in the ubiquitin system are strongly associated with the pathogenesis of various diseases, such as cancers, inflammatory disorders and neurogeneration. For this reason, the ubiquitin system has been used as a platform for the development of specific drugs.

2.1. Ubiquitin

Ubiquitin is a small regulatory protein with only 76 aminoacids (8.5 kDa) and highly conserved from yeast to humans. Ubiquitin can perform a variety of different functions when conjugated to larger, target proteins. In mammals, ubiquitin is encoded by four genes: *UBB*, *UBC*, *UBA52* and *RPS27A*. While the two first genes encode for a polyubiquitin precursor chain, the two latter ones encode for a single copy of ubiquitin fused to ribosomal proteins. Independently of the gene, the protein product has to be further processed by enzymes in order to generate the free ubiquitin pool in cells.

The structure of ubiquitin shows a compact β-grasp with a flexible C-terminal tail (Figure 6).The 7 lysines are distributed throughout the surfaces of ubiquitin pointing into different directions (Figure 6A) (Komander and Rape, 2012). Ubiquitin-binding proteins often recognize ubiquitin

through hydrophobic patches on the surface of ubiquitin, as depicted in Figure 6B. Most of the ubiquitin binding domains (UBDs), including the proteasome, interact with ubiquitin through the Ile44 patch (Dikic et al, 2009). The three other patches, centered on the Ile36, Phe4 and TEK-box were also shown to be essential to some cellular functions (e.g. cell cycle) and they can also be recognized by some UBDs, E3 ligases and deubiquitinases (Komander and Rape, 2012).

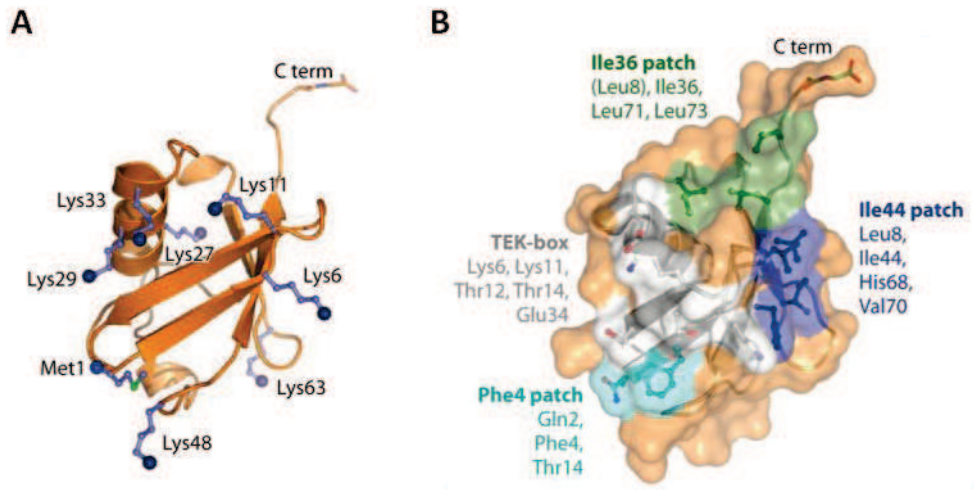


Figure 6: Ubiquitin structural properties

(A) Structure of ubiquitin indicating the C-terminal (C-term) tail and all the 8 residues used as attachment sites for chain formation: Met1, Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63 residues. (B) Surface of ubiquitin structure highlighting its 4 hydrophobic patches: Ile44, Ile36, Phe4, and TEK-box. *Adapted from Komander and Rape, 2012.*

Ubiquitin is a physically stable protein; it remains folded and active after exposure to extreme conditions (Ciechanover et al, 1980; Vijay-Kumar et al, 1987). Nevertheless, ubiquitin has a half-life of 10 hours and its degradation is proteasome-mediated following ubiquitylation (Shabek et al, 2007; Shabek et al, 2009; Carlson and Rechsteiner, 1987; Hiroi and Rechsteiner, 1992).

Many ubiquitin-like proteins have also been identified, including Nedd8, SUMO, FAT10 and ISG15. These proteins will be further discussed during this introduction. In addition, ubiquitin-like proteins were also found in prokaryotes (PUPs – prokaryotic ubiquitin-like proteins) (Darwin, 2009) and archaea (SAMPs – small archaeal modifier proteins) (Humbard et al, 2010).

2.2. Ubiquitin conjugation

Ubiquitylation requires three coordinated enzymatic reactions; ubiquitin activation (E1 activating enzymes), ubiquitin conjugation (E2 conjugating enzymes) and ubiquitin ligation to the substrate (E3 ligases) (Figure 7). In the human proteome there are two E1 activating enzymes, around 40 E2s and more than 600 E3 ligases.

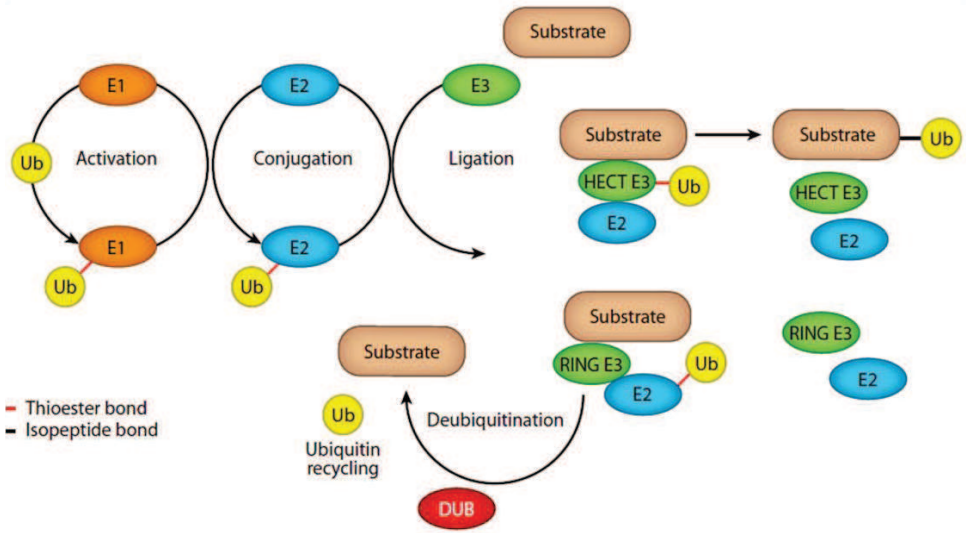


Figure 7: Ubiquitylation requires three coordinated reactions by E1, E2 and E3 enzymes

Three enzymes are necessary to covalently link an ubiquitin (Ub) molecule to a target substrate: E1, E2, E3. E3 ligases containing a HECT domain will be bound to ubiquitin via thioester bond, while in reactions with RING-domain containing E3 ligases, ubiquitin is directly transferred from the E2 to the target protein. Linkages indicated in red refer to thioester bonds, while the ones indicated in black refer to the covalent isopeptide bond. Deubiquitylating enzymes (DUBs) can remove ubiquitin from target proteins and from ubiquitin chains. HECT, Homologous to the E6-AP Carboxyl Terminus; RING, Really Interesting New Gene; Ub, ubiquitin. *Husnjak and Dikic, 2012.*

Ubiquitin conjugation starts by the activation of ubiquitin by the activating enzymes in the expense of one ATP (Figure 7). During this reaction, ubiquitin will form a thioester bond between the C-terminal carboxyl group of ubiquitin and the active site cysteine in an E1. In a second reaction, ubiquitin is then transferred to an E2 conjugating enzyme also via a thioester bond. The

third and final reaction will vary depending on the type of E3 ligase. For most of the E3 ligases (containing a RING or a U-box domains), the E3 will serve only as a scaffold bringing the substrate into close proximity to the E2 enzyme. In this case, the ubiquitin molecule is finally transferred from the E2 to form a covalent isopeptide bond with the substrate. On the other hand, E3 ligases containing HECT domains will be first bound to ubiquitin via a thioester bond before the final covalent attachment to the substrate.

In the conventional ubiquitylation, the α-carboxyl group of the C-terminal Gly76 residue of ubiquitin is covalently attached to the ε-amino group of an internal lysine by an isopeptide bond (Hershko and Ciechanover, 1998; Deshaies and Joazeiro, 2009). However, ubiquitin can also be conjugated to the α-amino group of the N-terminal Met residue of the substrate by a peptide bond (Ciechanover and Ben-Saadon, 2004). Interestingly, ubiquitin can even be attached to other aminoacids, such as Cys, Ser, Thr, and Tyr residues of target proteins by an thioester- or hydroxyester-based likage (Cadwell and Coscoy, 2005; Ravid and Hochstrasser, 2007; Wang et al, 2007; McDowell et al, 2010; Vosper et al, 2009; McDowell and Philpott, 2013).

An important and particular feature of ubiquitylation is that ubiquitin itself can be a target of ubiquitin conjugation. Ubiquitin has 7 lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) and they can all be used as attachment sites for the formation of ubiquitin chains. In addition, the N-terminal Met1 can also be as a site for the formation of linear 'head-to-tail' chains. These are inter-ubiquitin linkages in which the C-terminal α-carboxyl group of Gly76 of the incoming ubiquitin is conjugated to the α-amino group of Met1 of the preceding, substrate-attached ubiquitin (Ikeda and Dikic, 2008; Kirisako et al, 2006; Walczak et al, 2012). Importantly, all possible linkages have been detected in cells by mass spectrometry (Peng et al, 2003, Xu et al, 2009).

Finally, the action of the ubiquitylating enzymes can be counter-regulated by a family of isopeptidases known as the deubiquitylating enzymes (DUBs) (Figure 7). There are about 100 of them in the human proteome and they can cleave off ubiquitin molecules from the substrate, or from another ubiquitin to modify the ubiquitin chain (Clague et al, 2012). A coordinated activity of the E3 ligases and the DUBs ensures a tight regulation of the ubiquitylation.

2.3. Different topologies of ubiquitylation

The modification of proteins by ubiquitin can acquire different topologies; proteins can be monoubiquitylated, multiubiquitylated, and polyubiquitylated in homotypic, mixed linkage, or heterologous ubiquitin chains. All of these different types of ubiquitin linkages can recruit different receptors that lead to different cellular outcomes. Importantly, the different ubiquitin modifications are specifically made, recognized, and hydrolyzed in cells. For this reason, it is possible to state that differently linked ubiquitin chains are independent posttranslational modifications. The types of ubiquitylation and their respective commonly associated intracellular functions are shown in Figure 8.

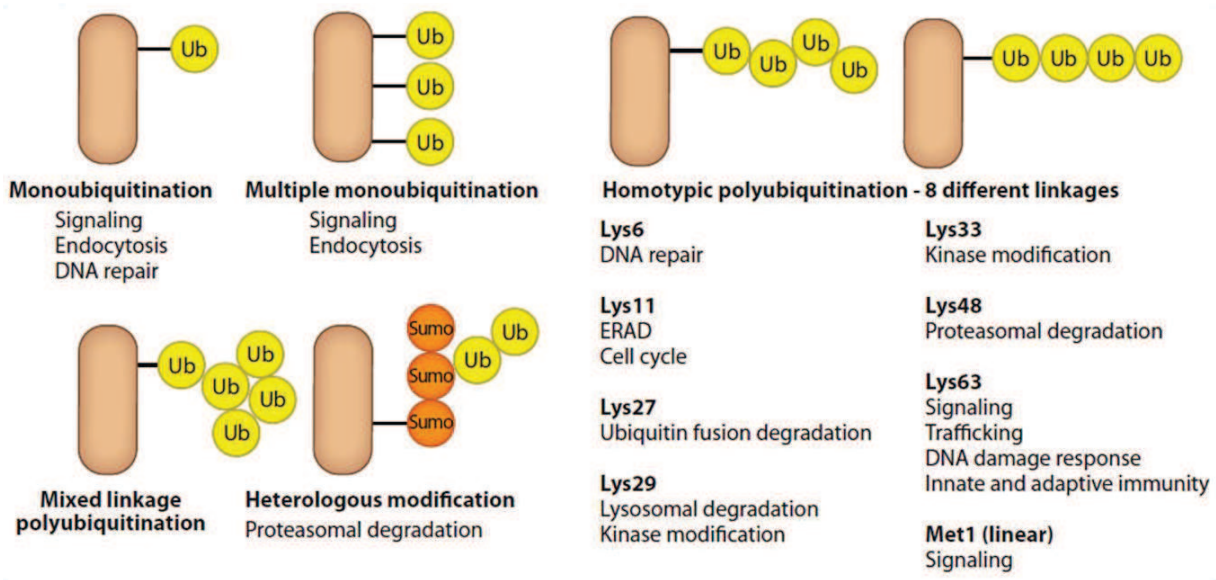


Figure 8: Ubiquitylation can lead to a large variety of modifications with different cellular outcomes

Proteins can be monoubiquitylated, multiubiquitylated and polyubiquitylated. Polyubiquitin chains can be formed through any of the 7 Lys residues or Met1, in a homotypic, mixed, or heterologous manner, when other ubiquitin-like modifiers are involved. Examples of cellular processes in which specific ubiquitylation types were shown to play a role. ERAD, endoplasmic reticulum-associated degradation; SUMO, small ubiquitin-like modifier. *Husnjak and Dikic, 2012.*

Ubiquitin chains can contain from 2 to more than 10 ubiquitin molecules per site. These chains are called homogeneous if the same residue is used for the chain formation by all the ubiquitin

molecules. Chains can also have a mixed topology, if different residues are used for chain formation, or be heterologous, if other ubiquitin-like modifiers are present in the same chain with ubiquitin (Komander and Rape, 2012). In addition, recent evidences support the idea that there are signals consisting of multiply branched, or forked, chains where more than one ubiquitin is linked to a single ubiquitin molecule. These forked chains were shown to be resistant to proteasomal degradation and serve non-proteolytic functions (Kim et al, 2007; Ben-Saadon et al, 2006; Kravtsova-Ivantsiv and Ciechanover, 2012).

While it is true that each type of ubiquitin modification can lead to a different cellular outcome, it is not true that each ubiquitylation type can be assigned to a specific cellular function. This will be explained into more details below.

2.3.1 Cellular functions of different types of ubiquitylation

The best ubiquitylation type studied so far is the polyubiquitylation through K48-linked ubiquitin chains. In the vast majority of cases, K48-linked chains will target proteins to proteasomal degradation and thereby regulates protein stability. In fact, Thrower et al (2000) defined a K48-linked tetraubiquitin chain to be the minimal proteasomal targeting signal. However, it is now known that not only K48-linked chains can lead to proteasomal degradation, but also, the 26S proteasome can recognize proteins that are modified by monoubiquitylation, multimonoubiquitylation and polyubiquitylation through other than K48-linked chains.

With the advancement of methodologies to analyse ubiquitylation, studies in both, yeast and mammalian cells, have shown that K6-, K11-, K27-, K29-, K33- and K48-linked chains are all able to target proteins for degradation (Bedford et al, 2011; Xu et al, 2009). Moreover, linear chains were also shown to be able to target proteins for degradation (Kirisako et al, 2006; Zhao and Ulrich, 2010). Although chains of all these linkages can target substrates to degradation, the abundance of all linkage types varies in cells, with the K48-linked chains being the most abundant modification identified (Dammer et al, 2011).

In addition, atypical linkages were also shown to be able to target proteins to the proteasome. For instance, the BH3 interacting-domain death agonist (BID), part of the B-cell lymphoma 2 (BCL2) family of anti-apoptotic proteins, can be ubiquitylated on serine, threonine and cysteine residues and degraded by the proteasome (Tait et al, 2007). In another example, it was shown

that the myoblast determination protein 1 (MyoD) can be a target for N-terminal ubiquitylation and subsequent targeting to proteasomal degradation (Breitschopf et al, 1998).

After K48-linked chains, K11-linked chains are the second most common linkage type to be known to target proteins for proteasomal degradation. These chains seem to be an important regulatory event during cell cycle, regulating the turnover of proteins (Wickliffe et al, 2011). In addition, the abundance of K11-linked chains increases when cells exit mitosis and the E3 ligase APC/C is activated (Matsumoto et al, 2010).

K63-linked chains have also been well-characterized, and they were shown to be mostly implicated in non-proteolytic intracellular signaling processes (Chen and Sun, 2009). Instead, they are important for the regulation of other processes, such as inflammation, protein translation, DNA-damage repair and vesicle trafficking (Miranda and Sorkin, 2007). More recently, K63-linked chains were shown to target membrane proteins for degradation in the lysosome (Lauwers et al, 2010).

A chain type that has been receiving a lot of attention lately is the linear chains. These are formed in a “head-to-tail” way, and not via isopeptide bonds. The E3 ligase responsible for the generation of these chains was called linear ubiquitin chain assembly complex (LUBAC) ubiquitin ligase. LUBAC was first thought to target proteins to proteasomal degradation (Kirisako et al, 2006). Later, however, it was discovered that LUBAC is involved in various signaling pathways, such as the ones triggered by TNF and interleukin (IL)-1β (Haas et al, 2009; Tokunaga et al, 2009).

Much less is known about the chains linked through K6, K27, K29, and K33, with only a few substrates described for each one of them. Chains linked through K27, K29 and K33 are the most challenging to be detected by mass spectrometry and they are also in low abundance in resting cells.

K6-linked chains are known to be assembled by the breast cancer type 1 susceptibility (BRCA1) – associated RING domain protein 1 (BARD1) complex. This type of ubiquitylation is involved in DNA repair pathways and acts on the substrate RNA Polymerase subunit RPB8 (Wu et al, 2007). These chains are recognized by receptor-associated protein 80 (RAP80), further linking it to the DNA damage response (Sobhian et al, 2007). Mass spectrometry studies have shown

that, similarly to K63-liked chains, K6-linked chains do not become more abundant following proteasomal inhibition, which would suggest that in most of the cases, this linkage has a non-proteolytic role.

K27-linked chains are known to be assembled by the E3 ligase parkin. Parkin can modify several mitochondrial proteins, following mitochondrial damage. One of these proteins is the voltage-dependent anion-selective channel protein 1 (VDAC1). Ubiquitylated VDAC1 is recognized by the autophagy adaptor p62, triggering clearance of damaged mitochondria by mitophagy (Glauser et al, 2011; Geisler et al, 2010).

Modification of proteins by monoubiquitylation is very frequently associated with non-proteolytic cellular signaling. The addition of one ubiquitin can have several functions in proteins. For instance, it can lead to intramolecular autoinhibition, if the target protein contains an ubiquitin-binding domain (Hicke et al, 2005). It can also affect intracellular localization, endocytosis, protein trafficking, and regulation of protein complex formation. For example, monoubiquitylation of H2B plays a role in transcription and in cell differentiation (Bonnet et al, 2014; Pavri et al, 2006).

2.3.2 Different topologies of ubiquitin conjugation and their 3-dimensional structures

The structural characterization of ubiquitin chains showed that different linkages result in different conformations. So far, five ubiquitin chain types have been structurally characterized by using X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and small angle X-ray scattering (SAXS). Together, these data suggested that the structure could be classified as either “compact” or “open” conformations. Ubiquitin chains can have a more compact conformation when two ubiquitin molecules interact with each other, or an open conformation, when the two ubiquitin molecules do not interact, apart from the linkage site.

The structure of 5 di-ubiquitin molecules is shown in Figure 9. From the conformation, K48-linked chains show a compact and symmetric structure. In addition, the two ubiquitin molecules interact via their Ile44 patches, which are buried in the structure. The Ile44 patch can also interact with the Ile36 patch, which might give the flexibility for binding partners to access the remaining Ile44 (Cook et al, 1992; Cook et al, 1994; Tenno et al, 2004; Varadan et al, 2002;

Eddins et al, 2007). Despite the compact structure, K11 and K6 chains have their Ile44 patches accessible for partners (Bremm et al, 2010; Matsumoto et al, 2010; Virdee et al, 2010). In contrast, K63 and Met1 linked chains show a more extended and flexible structure, with Ile44 patches accessible (Komander et al, 2009b; Weeks et al, 2009; Datta et al, 2009).

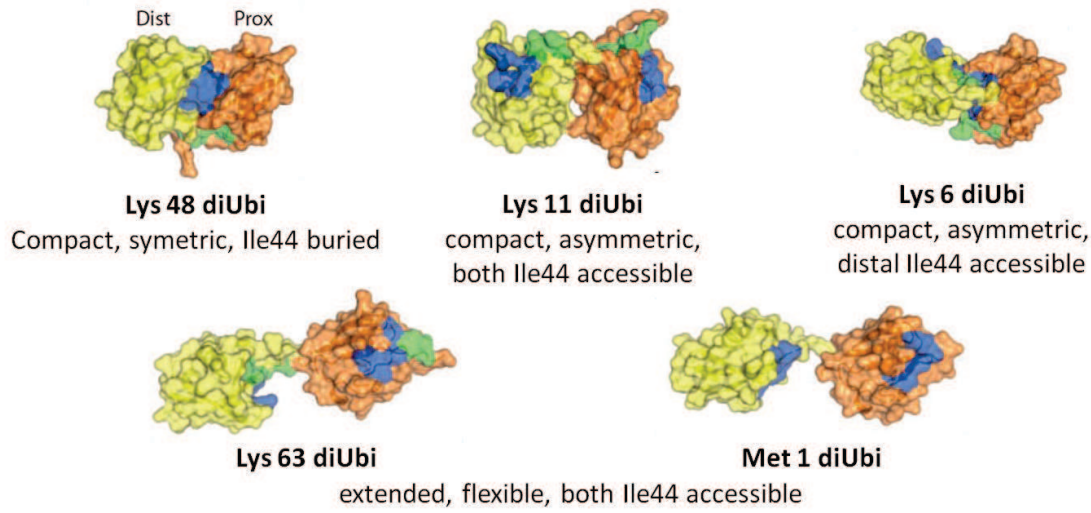


Figure 9: Ubiquitin chain structure for 5 different linkages

The structure of diubiquitin molecules are shown with the distal ubiquitin in yellow and the proximal ubiquitin in orange. The hydrophobic patch Ile44 is shown in blue and the Ile36 in green. While K48-, K11- and K6-linked chains show a more compact structure, K63- and Met1-linked chains show an extended structure. *Adapted from Komander and Rape 2012.*

The unique overall structure of ubiquitin chains given by the linkage type can be recognized by ubiquitin receptors. These receptors demonstrate different affinities towards different linkage types, thereby translating the structural information given by the ubiquitin code into specific regulated cellular functions.

2.4. The main effectors in the ubiquitin system

2.4.1. Ubiquitylating enzymes

Ubiquitin conjugation requires the activity of three enzymes; E1 activating, E2 conjugating, and E3 ligases. As ubiquitin attachment can result in different topologies, the specificity can be given

by the E2 or E3 individually, by the E2-E3 complex, or by the substrate-E3 complex. In this way, the same E3 ligase can catalyse monoubiquitylation or polyubiquitylation, depending on the E2, adaptor, or substrate. Moreover, E3 enzymes can block the ability of E2s to catalyse chain formation, leading to the monoubiquitylation of substrates. For example, the E2 Rad6 is blocked by the E3 Rad18 leading to the monoubiquitylation of PCNA (Hibbert et al, 2011).

There are three classes of E3 ligases, depending on the domains present in their sequence: RING domain, HECT domain, and RING-in-between-RING domains. The schematic representation of ubiquitin transfer to the substrate for each type of E3 ligase is shown in Figure 10.

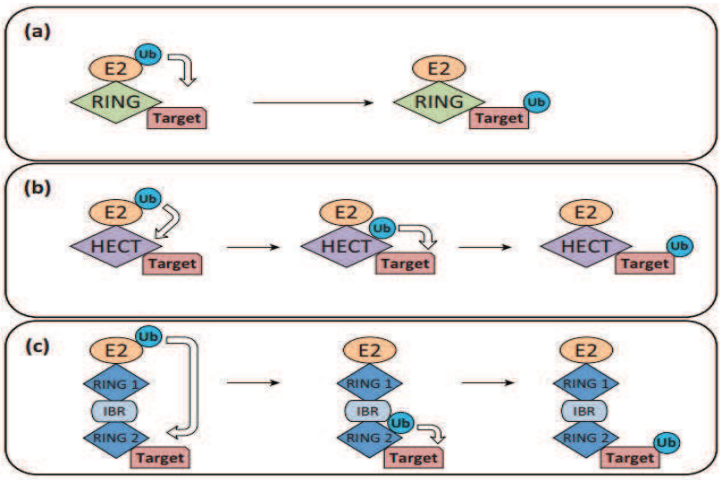


Figure 10: Model of ubiquitin conjugation catalyzed by different classes of E3 ligases
E3 ligases containing a (a)RING, (b)HECT, or (c)RBR catalyse ubiquitin chain formation by different mechanisms. RING, really interesting new gene; HECT, homology to E6AP C terminus; RBR, RING-between-RING. *Rieser et al, 2013.*

2.4.1.1. RING E3 ligases

E3 ligases containing a RING domain (Figure 10a) act as a scaffold protein, catalyzing the ubiquitin transfer from the E2 to the target protein. It is believed that the linkage specificity in these reactions is determined by the E2. This is supported by the observation of E3 ligases catalyzing the formation of different chains depending on the E2 enzyme (Ye and Rape, 2009). For example, the E3 Murf catalyses the formation of K63-linked chains with the E2 complex

Ube2N-Uev1A, and K48-linked chains with the E2 Ube2K (Christensen et al, 2007; Scaglione et al, 2011; Kim et al, 2007). Conversely, different RING E3 ligases that interact with the same E2 enzyme will very often show the specificity of that E2 (Ye and Rape, 2009).

In most cases, it is seen that initiating E2s cooperate with specific chain-elongating E2, which will determine the favored linkage. The E2s can select the correct lysine for chain elongation by recognizing a specific acceptor ubiquitin surface. For instance, the E2 Ube2N is a K63-linkage specific enzyme and it does so due to its interaction with an auxiliary subunit, Uev1A (Deng et al, 2000). Uev1A has a UBC-E2 domain that has lost its catalytic cysteine, but it can still recognize ubiquitin (Eddins et al, 2006). This interaction with ubiquitin allows the K63 to face the active site of Ube2N and thereby providing the specificity.

2.4.1.2. HECT E3 ligases

Ubiquitylation involving this class of E3 ligases is different from the previously mentioned RING-domain E3 ligases and this is due to the fact that HECT E3 ligases contain a catalytic cysteine (Rotin and Kumar, 2009). In the chain of reactions, the ubiquitin is first transferred from the E2 enzymes to the cysteine of E3 to form a thioester bond (Figure 10b).

HECT domain E3 ligases can synthesize chains of different topologies. For instance Nedd4 can make K63-linked chains, while E6AP can synthesize K48-linked chains (Kim and Huibregtse, 2009; Maspero et al, 2011). The linkage specificity is most likely given by the HECT E3 due to the fact that the acceptor lysine will attack the bond between the cysteine and ubiquitin. Many HECT E3s pair up with the nonspecific E2s or with E2s that do not promote chain formation (Kim and Huibregtse, 2009).

2.4.1.3. RING-in-between-RING E3 ligases

The last class of E3 ligases is quite special as it contains a RING domain, a RING-in-between-RING (RBR) and a RING-like domain (Figure 10c). These E3s are a combination of RING and HECT E3s. The reaction happens first by the recruitment of an E2 by the RING domain. The ubiquitin from the E2 is then transferred to the cysteine in the RING-like domain and finally linked to the substrate (Wenzel et al, 2011).

The linear ubiquitin chain assembly complex (LUBAC) is an example of RBR E3 that catalyses Met1-linked linear chains (Ikeda et al, 2011; Tokunaga et al, 2011; Rieser et al, 2013). Parkin is another example of RBR E3 and it can catalyze monoubiquitylation, K63-, K48- and K27-linked chains (Doss-Pepe et al, 2005; Geisler et al, 2010).

Many RBR E3 ligases pair with E2s without reactivity against lysines, such as Ube2L3, which suggests that the linkage specificity is given by the E3 ligase (Wenzel et al, 2011). In addition, it was observed that even if a RBR E3 pairs up with an E2 with a defined specificity, the linkage specificity will be given by the E3. For example, usually Ube2K synthesizes K48-linked chains, but when it is paired with LUBAC, it leads to the formation of linear chains (Tokunaga et al, 2009).

2.4.1.4. Mode of regulation of E3 ligase

A large number of known E3 ligases are regulated through ubiquitylation (or auto-ubiquitylation). The result of self-ubiquitylation is auto-regulation or targeting for self-destruction by the proteasome. The complexity of this regulation is increased by the fact that the self-ubiqutitylation may be regulated by the heterologous ubiquitylation by another E3 ligase (Weissman et al, 2011). There are also examples in which the activity of E3 ligases is regulated by UBLs modification. The most studied example of this case is the activating modification of cullin-RING ligases (CRL) by the UBL NEDD8 (Weissman et al, 2011).

2.4.2. Ubiquitin binding proteins

Ubiquitylated proteins with various chain topologies are recognized by effector proteins with ubiquitin-binding domains (UBDs). These ubiquitin-binding proteins (UBPs) will translate the ubiquitin signal into a specific cellular response (Dikic et al, 2009; Husnjak and Dikic, 2012; Komander and Rape, 2012; Rahighi and Dikic, 2012).

2.4.2.1. Ubiquitin binding domains

Ubiquitin binding domains (UBDs) are present in ubiquitin binding proteins and they recognize ubiquitin. So far, around 20 different families of UBDs have been described, and they are structurally and functionally different, being able to bind a variety of different ubiquitin signals. Based on the structural folds they contain, UBDs are categorized into 4 groups. In the first group, UBDs fold into a α-helix, and it includes UBA (ubiquitin-associated), UIM (ubiquitin-interacting motif), GAT (GGA and TOM), VHS (Vps27/Hrs/STAM), CUE (coupling ubiquitin conjugation to ER degradation), UBM (ubiquitin binding motif) and UBAN (ubiquitin-binding domain in ABINs and NEMO). In the second group, UBDs contain zinc fingers (ZnFs), such as NZF (Npl4 ZnF), A20 ZnF domains, ZnF UBP (ubiquitin-specific processing protease), and UBZ (ubiquitin-binding ZnF). The third group contains UBDs with a PH (Plekstrin homology) domain. And the fourth group includes UBDs with Ubc (ubiquitin-conjugating-like) domains (Hicke et al, 2005; Rahighi and Dikic, 2012).

2.4.2.2. Mechanisms of binding and specificity of UBDs to ubiquitin

An interesting question in the field of ubiquitin signaling is how the specificity of the various ubiquitin-binding modules for different ubiquitin species is determined. UBPs can make use of different features of ubiquitin modification topologies in order to increase the specificity. Several factors have been proposed so far and they are briefly discussed below.

2.4.2.2.1. Distance between ubiquitin molecules

Depending on the chain topology, the structure of the ubiquitin chain can have an open or compact conformation, with the distance between the ubiquitin molecules changing depending on the linkage type. Proteins with more than one UBD can have linkers in between the UBDs to adjust to the distance between the ubiquitin molecules. For example, some proteins contain several tandem repeats of ubiquitin-interacting motifs (UIMs). These motifs interact with ubiquitin through the Ile44 hydrophobic patch (Swanson et al, 2003). When the distance between these motifs is small, the UBP will most likely recognize compact structures, such as Ataxin-3 recognition of K48-linked chains. However, when the distance is longer, the UBP will favor more open structures, for instance the recognition of Rap80 of K63-linked chains (Sims and Cohen, 2009).

2.4.2.2.2. Chain flexibility

In cases when the structure of different linkage types is very similar, for instance for K63-linked and linear chains, it was observed that UBPs make use of the chain flexibility. For instance, the Npl4-like zinc fingers (NZF) domain of both TAB2 and TAB3 (proteins involved in the activation of the NF-κB pathway) bind preferentially K63-linked chains. It was shown that the NZF domain interacts with the Ile44 patches, which leads to a bending of the K63-chain structure. Such bending would not occur in linear chains, differentiating the two chains (Kulathu et al, 2009; Sato et al, 2009).

2.4.2.2.3. Linkage context

Some UBPs can also bind directly the isopeptide bond or the linkage context and thereby distinguish between different chain topologies. This is the case for the UBAN domain from the protein NEMO. NEMO recognizes linear diubiquitin chains by binding to the Ile44 patch, Phe4 patch and Gln2. Such interactions would not be possible in other linkage types, demonstrating how the specificity is achieved (Rahighi et al, 2009).

2.4.2.3. Mechanisms of affinity of UBDs to ubiquitin

The affinity of ubiquitin-binding molecules for ubiquitin is usually low (Husnjak and Dikic, 2012). For this reason, the system makes use of several strategies to increase the affinity and thus the specificity of the system. Some of these strategies include increased avidity, contribution of other domains in the target protein, post-translational modifications, and conformational changes.

The increased avidity is given by the recognition of more than one ubiquitin moiety in the structure. For instance, the dimerization of UBAN motif allows NEMO to recognize two linear chains, in either side of the coiled-coil structure (Rahighi et al, 2009). Likewise, the presence of post-translational modifications on the UBP can also change the affinity of UBDs for ubiquitin. For instance, the phosphorylation of the UBA domain of p62/SQSTM1 (protein that regulates selective autophagy) leads to an increase in its affinity towards polyubiquitin chains (Matsumoto et al, 2011).

2.4.3. Erasing the code

Deubiquitylating enzymes (DUBs) play an essential role in cells cleaving off ubiquitin molecules from target proteins and from ubiquitin chains. There are 5 classes of DUBs; 55 ubiquitin-specific proteases (USPs), 14 ovarian tumor DUBs (OTUs), 10 JAMM family DUBs, 4 ubiquitin C-terminal hydrolases (UCHs) and 4 Josephin and JAB1/MPN/MOV34 metalloenzymes (JAMMs) (Komander et al, 2009a; Clague et al, 2012).

2.4.3.1. Housekeeping enzymes

The ability of the ubiquitin system to modify several different targets and carry out different functions relies on the availability of free ubiquitin in cells. Housekeeping DUBs play a role guaranteeing there will be enough amounts of free ubiquitin to be conjugated. These DUBs are responsible for the processing of precursor ubiquitin chains originated from the 4 genes: *UBB*, *UBC*, *UBA52* and *RPS27A*. In addition, they are responsible for recycling the ubiquitin chains of proteins targeted to degradation. For instance, USP14 and UCH37/UCH-L5 are associated to the proteasome and therefore protect ubiquitin from degradation (Finley, 2009).

2.4.3.2. Substrate-specific enzymes

Some DUBs can dissociate chains independently of the linkage. Instead, the specificity is usually achieved by interaction domains between DUBs substrates, in the presence or absence of adaptor proteins (Komander et al, 2009a; Sowa et al, 2009). Most USP DUBs are active against all linkages and can also hydrolyze the isopeptide bond between the substrate and the first ubiquitin (Komander and Rape, 2012), but they can show specificity towards their substrates.

2.4.3.3. Linkage-specific enzymes

Some DUBs show specificity towards one, or a few linkage types. Most of these DUBs can be considered to be UBPs with catalytic activity. This is due to the fact that many of them contain UBDs able to recognize specific linkage types, providing the linkage-specificity to these enzymes. For instance, JAMM DUBs are often K63 specific (McCullough et al, 2004). Another

class of DUBs, the OTU enzymes, was also shown to display specific activities towards different linkage types. For instance, OTUB1 is specific for K48 chains (Edelmann et al, 2009), while Cezanne is specific for K11 chains (Bremm et al, 2010). Interestingly, linkage-specific DUBs were shown not to be able to cleave off the last ubiquitin in the chain (Bremm et al, 2010), thereby generating monoubiqutylated substrates, with possible distinct properties.

Independently of the mode of action, DUBs can also edit ubiquitin chains. This is a very fascinating process in which one chain type is replaced by one with different linkage and thus changing the fate of the target protein. The change of linkage type can be achieved by sequential action of a DUB/E3 ligase complex. In fact, some proteins have a combined DUB and E3 ligase activity as seen in the pathway that regulates NF-kB (Wertz et al, 2004). Alternatively, DUBs can cleave off ubiquitin chains, leaving the substrate monoubiquitylated, as already mentioned before.

2.5. Various functions of protein ubiquitylation

The first cellular function discovered for ubiquitylated proteins was its ability of targeting substrates to proteasomal degradation. Later this function was attributed to K48-linked ubiquitin chains (Chau et al, 1989), while K63-linked chains were discovered to have a nonproteolytic function. However, with the advancement of the study of ubiquitylated proteins, it is now understood that this classification is too simplified when it comes to the cellular functions of ubiquitylation. So far, multiple chains, including K63-linked chains, were also shown to be able to target proteins to degradation (Babst, 2014). On the other hand, K48-linked chains were also shown have a nonproteolytic function (Flick et al, 2006). Therefore, it has become clear that the functions of ubiquitylation depend not only on the chain topology, but also on the surroundings of the target proteins in the cells. Factors like the timing of ubiquitylation and deubiquitylation, the subcellular localization and protein-protein interactions will affect the fate of the target protein.

2.5.1. Degradative ubiquitylation

Intracellular proteolysis has several functions; elimination of misfolded or damaged proteins, source of amino acids in case of starvation, generation of specific protein fragments with other

functions in cells. Protein degradation is also important to control the half-life of a protein, or to control the degradation of a specific portion of a protein. In this way, protein degradation often surpasses the regulation through transcription and translation (Varshavsky, 2008). There are several systems in cells able to degrade proteins; ubiquitin proteasome system (UPS), ERAD, N-end rule, autophagy and lysosomal proteolysis.

2.5.1.1. Proteasomal degradation

Ubiquitin chains can target proteins to the 26S proteasome in all eukaryotic cells (Finley, 2009). Many E3 ligases studied so far, such as SCF, gp78, or E6AP can catalyze the formation of K48-linked ubiquitin chains and affect the turnover of intracellular proteins (Petroski and Deshaies, 2005; Li et al, 2007a; Kim and Huibregtse, 2009). According to quantitative proteomics studies, K48-linked chains are the most abundant lysine modification in cells and the levels increase dramatically when the proteasome is inhibited (Kim et al, 2011; Kaiser et al, 2011; Xu et al, 2009). As discussed previously, K11-linked chains were also shown to be a robust proteolytic signal important in cell division (Jin et al, 2008; Matsumoto et al, 2010). Interestingly, many K48- and K11-specific E3 ligases were shown to interact with the proteasome to efficiently couple ubiquitylation and degradation (Verma et al, 2000; Seeger et al, 2003).

Other chain types were shown to be recognized by the proteasome (Baboshina and Haas, 1996; Thrower et al, 2000) and the abundance of some linkage types increase upon proteasome inhibition (Xu et al, 2009). These observations show that other linkage types are also responsible for targeting proteins to the proteasome. Indeed, all the other linkages were shown to be able to target proteins to proteasomal degradation (Johnson et al, 1995; Koegl et al, 1999; Kirkpatrick et al, 2006; Saeki et al, 2009; Huang et al, 2013). However, these linkages target substrates to degradation much less frequently than K48- or K11-linked chains.

2.5.1.1.1. The 26S proteasome

The 26S proteasome is a 2MDa complex made of 2 subcomplexes, the 19S regulatory proteasome and the 20S catalytic proteasome. The catalytic subcomplex is composed of two β-rings that contain the catalytic sites, flanked by two α-rings. The regulatory subcomplex contains a base and a lid. Once the ubiquitylated substrate is recognized by the lid in the 19S proteasome, the ATPases in the base will unfold the substrate and open the α-rings of the 20S

proteasome, to allow the entry of the unfolded substrate into the catalytic chamber. Meanwhile, DUBs associate with the proteasome will remove ubiquitin molecules from the substrate in order to prevent its degradation (Weissman et al, 2011; Finley, 2009).

2.5.1.1.2. Degradation of monomeric ubiquitin

The degradation of monomeric ubiquitin is done by the proteasome. Ubiquitin itself needs to be ubiquitylated in order to be translocated into the proteasome and finally degraded. The ubiquitylation of monomeric ubiquitin is catalyzed by a specific E2 (UBE2K) able to add K48-linked chains in an E3-independent manner (Piotrowski et al, 1997) and by the thyroid receptor interacting protein (TRIP12), which is a HECT domain containing E3 ligase (Park et al, 2009). Alternatively, ubiquitin can also be degraded together with its target substrate, in a piggyback mode of degradation, or when conjugated to short peptides through its C-terminal end (Weissman et al, 2011).

2.5.1.2. Targeting to lysosomal degradation

Plasma membrane proteins are targeted to lysosomal degradation by monoubiquitylation or K63-linked polyubiquitin chains (Mukhopadhyay and Riezman, 2007). These proteins are recognized by different ESCRT (endosomal sorting complex required for transport) complexes, which contain ubiquitin-binding domains that interact with tagged cargo and initiate their targeting to lysosomes (Shields and Piper, 2011; Babst, 2014). Thus, K63-linked chains can trigger proteolysis by targeting proteins, or compartments, to lysosomal degradation.

2.5.2. Non-degradative ubiquitylation

The non-proteolytic function of ubiquitylation is more frequently a consequence of monoubiquitylation, linear or K63-linked chains. Ubiquitylation modifies the substrates' affinities to other proteins; it can affect activity of proteins; and even bring the substrate to a specific pathway or cellular compartment.

2.5.2.1. Modulate protein-protein interactions

Ubiquitylation can alter the interaction between two proteins. Often, ubiquitylation can increase the binding of two proteins by adding one extra site of recognition for the binding protein. For instance, the DNA damage response involves the monoubiquitylation of PCNA (Hoege et al, 2002) and its recognition by DNA polymerases (Bienko et al, 2005). These polymerases recognize both PCNA and ubiquitin through UBZ or UBM domains. In this way, in response to DNA damage, PCNA recruits a family of DNA polymerases that allows the repair of DNA. Once this is achieved, ubiquitin is removed by the DUB Usp1 and the replication machinery returns to continue their job (Huang et al, 2006).

In another example, the transcription factor Met4 was shown to be polyubiqutylated through K48-linked chains by SCF^{Met30}. This modification blocked the ability of Met4 to engage coactivators, demonstrating that K48-linked chains can also lead to other cellular outcomes than protein degradation (Flick et al, 2006).

2.5.2.2. Modulate protein activity

Ubiquitylation can modulate the activity of proteins by several different mechanisms. One of them is by targeting to degradation inhibitors of these proteins. This is the case for the transcription factor NF-κB, in which IκBα is polyubiquitylated with K48-chains by $SCF^{\beta TrCP}$ and degraded, thereby freeing NF-κB from inhibition (Winston et al, 1999).

2.5.2.3. Modulate subcellular localization

Subcellular localization is also affected by ubiquitylation. One mechanism is when UBPs bind concomitantly to ubiquitylated proteins and to proteins with determined subcellular localization. Alternatively, ubiquitin might facilitate the binding to a transport protein. For example, following its multimonoubiquitylation, p53 exits the nucleus binding to the nuclear export machinery (Li et al, 2003). The deubiquitylation by USP10 leads to the re-import of p53 into the nucleus (Yuan et al, 2010).

2.5.2.4. TNF signaling cascade as an example

The signaling cascade following tumor necrosis factor α (TNFα) binding is a good example of how interesting and diverse ubiquitylation can be. Once TNFα binds to its receptor, many different ubiquitylation events take place in the cytoplasm (Figure 11). Binding of TNF to TNFR1 leads to receptor trimerization, which induces a conformational change and subsequent recruitment of both TRADD (TNFR1-associated death domain) and receptor-interacting protein (RIP)1 (Hsu et al, 1995; Hsu et al, 1996a; Hsu et al, 1996b). TRADD then recruits TNFR-associated factor (TRAF)2, which, in turn, recruits cellular inhibitor-of-apoptosis protein (cIAP)1 and 2 (Shu et al, 1996; Vince et al, 2009).

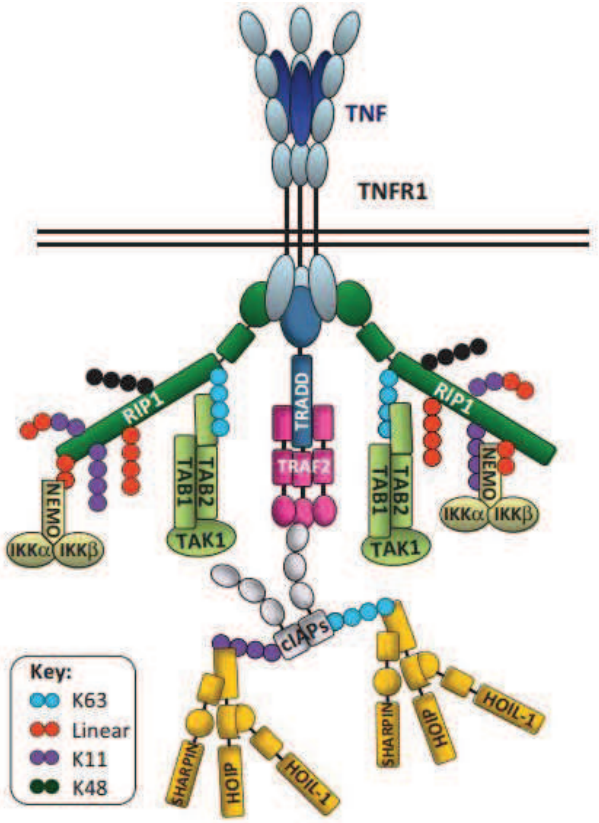


Figure 11: Model of the ubiquitylation events following TNFα binding to its receptor

Once activated, TNFR1 recruits TRADD/TRAF and RIP1. TRAF recruits cIAPs and LUBAC (yellow), E3 ligases that will polyubiquitylate different proteins in this pathway. The different ubiquitin linkages will activate and recruit two complexes: IKK and TAB/TAK. These complexes will mediate the activation of pathways leading to the transcriptional events related to proinflammatory and antiapoptotic functions. TNF, tumor necrosis factor; TNFR1, TNF receptor 1; TRADD, TNFR1-associated death domain; TRAF, TNFR-associated factor; cIAPs, cellular inhibitor-of-apoptosis protein; HOIL-1, heme-oxidized iron-regulatory protein 2 ubiquitin ligase-1; HOIP, HOIL1 interacting protein; SHARPIN, shank-associated RH (RBCK1

homology) domain-interacting protein; RIP, receptor-interacting protein; TAK, transforming growth factor-β activated kinase 1; TAB, TAK1-binding protein; NEMO, nuclear factor-κB-essential modulator; IKK IκBα kinase. *Rieser et al, 2013.*

cIAPs are RING-type E3 ligase that catalyses the formation of mixed K11/K63-linked ubiquitin chains (Dynek et al, 2010). Once recruited to the complex, cIAPs polyubiquitylate RIP1 with K11- or K63-linked chains. cIAP1/2 also generate ubiquitin linkages that are recognized by LUBAC.

LUBAC is a 600 kDa E3 complex composed of three subunits: shank-associated RH (RBCK1 homology) domain-interacting protein (SHARPIN), longer isoform of heme-oxidized iron-regulatory protein 2 ubiquitin ligase-1 (HOIL1) and HOIL1 interacting protein (HOIP). HOIL and HOIP are members of the RBR family of E3 ligases (Kirisako et al, 2006; Gerlach et al, 2011; Ikeda et al, 2011; Tokunaga et al, 2011). Once recruited to the complex, LUBAC catalyses the formation of linear chains on RIP1 and on nuclear factor-κB-essential modulator (NEMO) (Tokunaga et al, 2009). NEMO is a subunit of the IκBα kinase (IKK) complex. Interestingly, these Met1-linked chains in NEMO are recognized by the UBAN (ubiquitin binding in A20-binding inhibitor of NF-κB activation (ABIN) and NEMO) domain of NEMO itself. Upon recognition of liner chains, NEMO undergoes a conformational change, which leads to the allosteric activation of IKK (Rahighi et al, 2009). NEMO can also interact with mixed K11/K63-linked chains on the receptor interacting protein 1 (RIP1) through the C-terminal zinc finger domain (Lo et al, 2009; Wu et al, 2006; Dynek et al, 2010).

In this way, the ubiquitylated RIP1 is recognized by two complexes: IKK complex (inhibitor of κB kinase) through NEMO and TAK/TAB complex (transforming growth factor-β activated kinase 1; TAK1-binding protein) through TAB2/3 (Ea et al, 2006; Wu et al, 2006). Activated IKK will phosphorylate IκB, which will signal its polyubiquitylation of K48-linked chains and proteasomal degradation. As IkB masks the transcription factor NF-kB nuclear localization signal, the degradation of IkB leads to NF-kB nuclear translocation. Once in the nucleus, NF-kB is able to regulate the expression of many genes involved in the immune and proinflammatory responses (Magnani et al, 2000). Similarly, the recruitment of TAK/TAB complex will mediate the activation of mitogen-activated protein kinase (MAPK) cascades, leading to the transcriptional events related to antiapoptotic functions (Rieser et al, 2013).

In summary, many of the signaling events following TNFα binding to its receptor are regulated by ubiquitylation. In this example, we see that ubiquitin attachment can modulate proteasomal degradation, allosteric activation, or the recruitment of binding partners. The protein NEMO also provides an example of how the cooperation of multiple UBDs in the same protein might increase the affinity of UBPs for various ubiquitin chains rather than one specific chain type. Importantly, the exact length and position of all the chains and the mechanism behind all these different ubiquitin chains and recruitment of signaling components still needs to be studied.

2.6. Pharmacological targeting of UPS

Due to its involvement in a multitude of cellular processes, including protein degradation, abnormalities in the ubiquitin system often can lead to diseases. For this reason, drug discovery in the ubiquitin system is an emerging field of research. The system is being exploited for the identification of drug targets for potential novel treatments of neurogegenerative diseases, cancer, immunological disorders and microbial infection (Hol et al, 2006; Xolalpa et al, 2013; Edelmann et al, 2011; Nalepa et al, 2006).

Proteasome inhibitors were initially thought not to be potential drug targets, as they have a very general function in the cells. However, bortezomib, an inhibitor of 26S proteasome, was approved for the treatment of a few types of cancer. However, as expected, the use of proteasome inhibitors leads to several side effects. An alternative to overcome the general toxicity is to target specific proteasome subsets, which are expressed in only a subset of cells (Edelmann et al, 2011).

DUBs are also molecular targets, especially for the treatment of different types of cancers. For instance, USP7 is critical in cancer progression because of its role in destabilizing the tumor suppressor p53 (Nicholson et al, 2007). Also related to p53-role in cell proliferation and apoptosis, the E3 ligase HDM2 can polyubiquitylate p53, targeting it to proteasomal degradation. Thus, inhibition of HDM2 can lead to activation of the p53 pathway. The understanding of the exact role of many of the UPS components in disease processes is essential to finding possible drug targets (Xolalpa et al, 2013; Edelmann et al, 2011).

2.7. Ubiquitin like modifiers

Proteins related in sequence to ubiquitin and of a similar three-dimensional structure are referred as ubiquitin-like proteins (UBLs) (Hochstrasser, 2009; Komander, 2009; Schulman and Harper, 2009; van der Veen and Ploegh, 2012). Most UBLs are conjugated to proteins in a similar manner as ubiquitin, and so far, UBLs have much less known substrates when compared to ubiquitin.

The small-ubiquitin-related modifier (SUMO) is one of the best studied UBLs. SUMO was shown to be involved in the regulation of several cellular processes, such as nuclear transport and organization, transcription, chromatin remodeling, DNA repair and ribosomal biogenesis. Similarly to ubiquitin, SUMO can create or disrupt an interface for protein-protein interactions and it can induce a conformational change (Gareau and Lima, 2010; Wilkinson and Henley, 2010). The first substrate identified for SUMOylation was the protein RanGAP1 (Ran GTPase-activating protein). The modification increases affinity of RanGAP1 for the nuclear pore component RanBP2, causing a localization change from the cytosol to the nuclear pore (Matunis et al, 1996; Mahajan et al, 1997). Sumoylation is very often associated with a cellular stress response, as it was shown to increase in response to severe oxidative, hypoxic, osmotic, genotoxic, or heat stress (Saitoh and Hinchey, 2000; Golebiowski et al, 2009).

Other UBLs include: NEDD8, a known activator of Cullin RING E3 ligases; ISG15 (interferon-stimulated gene 15), involved in host defense; FAT10, involved in apoptosis and able to target proteins to proteasomal degradation; Ufm1, with unknown function so far; Atg8 and Atg12, regulators of autophagy; Urm1, involved in tRNA thiolation; Hub1, involved in RNA splicing (van der Veen and Ploegh, 2012).

2.8. Current tools to study ubiquitylation

Mass spectrometry-applied techniques have been very helpful in contributing to the understanding of the ubiquitin system. The mass spec-based methodologies used to define the ubiquitylated proteome of cells have improved since the first published article on the ubiquitylated proteome by Peng et al (2003).

The approach usually employed is the “shot-gun proteomics” in which samples are digested enzymatically and the peptide mixture is subsequently separated using liquid chromatography. The peptides are then subjected to electrospray ionization and measured by the mass spectrometer based on the mass-to-charge ratio of the eluting peptides. The identity of the analyzed peptide is obtained by matching the data acquired with the mass spectrometer and a database consisting of known protein sequences digested in silico (Choudhary and Mann, 2010). When analyzing PTMs, it is essential to use mass spectrometers with high speed, sensitivity and mass accuracy, as they are often present in low abundance in a complex peptide mixture (Cox and Mann, 2008; Wu et al, 2011).

Following trypsin digestion, the ubiquitylated peptides and possibly the sites are identified by the mass spectrometer by two specific features: one tryptic misscleavage on the lysine due to the ubiquitin attachment and a signature di-Glycine peptide covalently linked to the modified lysine leading to a mass shift of 114 Da to the peptide (Peng et al, 2003). The modified lysine can be identified with the information obtained in the MS/MS spectra for peaks with a mass difference.

The first study combining ubiquitylation with mass spectrometry made use of the mass shift and managed to identify more than 100 sites in yeast (Peng et al, 2003). Following this work, several studies tried to define the ubiquitylated proteome of cells (Table 1). Since ubiquitylation, like other PTMs, is usually present at substoichiometric levels, to increase the number of indentified ubiquitylated proteins requires improvement in both, the mass spectrometer and biochemical enrichment of ubiquitylated proteins. The biochemical improvement came from enrichment techniques performed in both, the protein- and peptide-level (Figure 12).

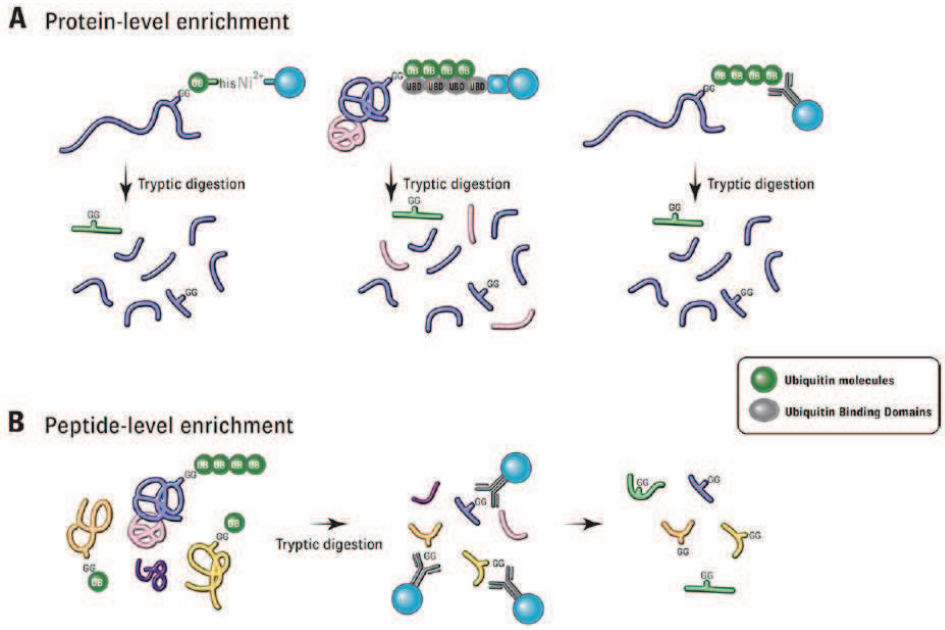


Figure 12: Schematic representation of biochemical enrichment techniques in both, protein and peptide levels

(A) Ubiquitylated proteins can be purified with the use of tagged ubiquitin (left), tandem ubiquitin-binding domains linked to beads (middle) or with ubiquitin antibodies (right). (B) Following the tryptic digestion of ubiquitylated proteins, peptides containing the di-Gly remant motif can be enriched with the use of antibodies. *Carrano and Bennett, 2013.*

2.8.1. Protein-based ubiquitin proteomics

In the protein level enrichment methodologies (Figure 12A), the ubiquitylated proteins are first purified and subsequently digested prior to analysis by the mass spec. The pioneer work used His-tagged ubiquitin in cells allowing the use of Ni-chelate chromatography under denaturing conditions to purify ubiquitylated proteins from cells (Figure 12A left) (Peng et al, 2003; Kirkpatrick et al, 2005). Other tags were used for purifying ubiquitylated proteins after that such as: His/biotin, Myc, HA and FLAG-tags (Tagwerker et al, 2006; Danielsen et al, 2011). A drawback of this approach is that the exogenous expression of tagged ubiquitin might lead to the modification of non-physiological substrates.

Another method to purify ubiquitylated proteins from a complex mixture is to use tandem repeats of ubiquitin binding domains as an affinity matrix (Figure 12A middle) (Hjerpe et al, 2009; Shi et al, 2011; Lopitz-Otsoa et al, 2012). When in tandem, UBDs can show a very high affinity towards

ubiquitin, with the dissociation constant in the nanomolar range (Hjerpe et al, 2009). Examples of these tools are TUBEs and UbiQapture. As these enrichments are not performed under denaturing conditions, the co-purification of binding partners and protein complexes cannot be excluded.

Antibodies recognizing ubiquitin have also been used to purify ubiquitylated proteins (Figure 12A right) (Matsumoto et al, 2005; Vasilescu et al, 2005; Schwertman et al, 2013). However, this was not the methodology of choice to many proteomic studies due to the lack of antibodies that recognize the various forms of ubiquitin with high affinity.

The protein-level enrichment of the ubiquitin-modified proteome greatly reduces the initial complexity of the analyzed sample. However, the percentage of peptides containing the di-Gly motif is still low, as with the digestion, most of the peptides in the mixture will not contain the modified lysine. As a result, these techniques based on the enrichment of ubiquitylated proteins provided very good insights into putative ubiquitylated proteins in cells. Table 1 summarizes many of the proteomic studies performed to identify ubiquitylated proteins. So far with these techniques, it was already possible to increase the number of identified sites to 1100 lysine residues and many putative ubiquitylated proteins (Danielsen et al, 2011; Meierhofer et al, 2008; Shi et al, 2011).

2.8.2. Peptide-based ubiquitin proteomics

The development of antibodies that recognize the di-Gly motif in peptides were a breAKThrough in the MS-based identification of ubiquitylated proteins. These antibodies are used after enzymatic digestion of the total cell lysate allowing the enrichment of di-Gly containing peptides (Figure 12B). In this way, this purification technique dramatically increases the amount of peptides containing the modified lysine, consequently indentifying a greater number of ubiquitylated proteins. By using this approach, several groups have demonstrated the power of this technique in identifying a comprehensive and extensive number of ubiquitylated proteins (Table 1) (Xu et al, 2010; Kim et al, 2011; Wagner et al, 2011; Emanuele et al, 2011; Lee et al, 2011; Na et al, 2012; Buckley et al, 2012; Povlsen et al, 2012; Udeshi et al, 2012; Wagner et al, 2012; Sarraf et al, 2013).

Despite the fact that this peptide-enrichment technique has allowed us to identify many ubiquitylated proteins in the human proteome, which can be used as a resource to study the ubiquitylation of proteins, the technique has some limitations. The first limitation is the bias introduced by the antibody used. This was demonstrated by the two large-scale studies by Kim et al (2011) and Wagner et al (2011). These two groups have used two different antibodies to enrich for peptides-containing di-Gly motif and together, they identified more than 23000 ubiquitylation sites. However, only 4300 of them were common to both studies. Taking a closer look at the peptides identified by these two studies, it is observed that there was a preference for certain amino acids adjacent to the ubiquitylated sites. This observation was further confirmed by Wagner et al (2012) by using both antibodies to enrich for ubiquitylated proteins from murine tissues. As distinct antibodies enrich a distinct subset of di-Gly sequences, an approach that uses a mixture of these antibodies might be an even more effective approach to identify di-Gly-containing peptides by reducing the bias.

Another issue with this technique is the fact that two other UBLs, NEDD8 and ISG15, also produce di-Gly remnant on target substrates which cannot be distinguished from those generated by ubiquitin digestion. It is estimated that up to 6% of the sites identified could be derived from NEDDylated and ISGylated proteins (Kim et al, 2011).

Table 1: Summary of studies identifying the ubiquitylated proteome by mass spectrometry

Reference	Purification method	Cell/tissue	Protein no	Sites no
Peng et al, Nat Biotechnol, 2003	His-Ubiquitin	Yeast	1,075	110
Meierhofer et al, J Proteome Res, 2008	His-Bio-Ubiquitin	HeLa	669	44
Danielsen et al, Mol Cell Prot, 2011	Strep-HA-Ubiquitin	U2OS and HEK293T	471	753
Shi et al, Mol Cell Prot, 2011	TUBEs1	HEK293T	223	294
Lopitz-Otsoa et al, J Proteomics, 2012	TUBEs2	MCF7	643	
Starita et al, Proteomics, 2012	His-Ubiquitin	Yeast	438	870
Zhou et al, Mol Cell Biochem, 2012	Poly-Ubi affinity matrix	MDA-MB-231	260	
Kim et al, The Plant Cell, 2013	TUBEs + His-Ubiquitin	Arabidopsis thaliana seedlings	941	

Xu et al, Nat Biotechnol, 2010	Anti-di-Gly	HEK293T	236	374
Kim et al, Mol Cell, 2011	Anti-di-Gly	HCT116 and 293T	~5000	~19000
Wagner et al, Mol Cell Prot, 2011	Anti-di-Gly	HEK293T	4273	11054
Emanuelle et al, Cell, 2011	Anti-di-Gly	HeLa	2814	
Udeshi et al, Mol Cell Prot, 2012	Anti-DiGly	Human Jurkat cell	2039	5533
Wagner et al, Mol Cell Prot, 2012	Anti-DiGly	Murine tissues		~20000

2.8.3. Identification of ubiquitin chain topologies

MS-based ubiquitin studies can also be used to determine which lysine in the ubiquitin peptide contained the di-Gly motif and thereby identifying the chain topology (Xu et al, 2009). More recently, it was shown to be possible to detect also branched ubiquitin chains by using a mild trypsinolysis (Valkevich et al, 2014). However, in a complex mixture of proteins, it is not possible to identify the chain topology for one specific protein. Instead, linkage-specific antibodies were developed to allow the identification of certain homotypic ubiquitin chains. So far, only K11-, K48- or K63-specific antibodies exist and they can be used to detect proteins by imaging or biochemical techniques. To date, they have not been used to purify a specific subset of ubiquitylated proteins, but in principle, they could be used to immunoprecipitate a subset of modified proteins prior to analysis by mass spec (Matsumoto et al, 2012; Matsumoto et al, 2010; Newton et al, 2008; Wang et al, 2008).

In addition, ubiquitin chain-specific sensors were developed based on ubiquitin binding domains of known proteins that bind specifically to one type of ubiquitin chain (Akimov et al, 2011). These sensors are based on the properties of UBDs and can be used to selectively detect, visualize and inhibit ubiquitin-dependent processes in cells. In combination with mass spectrometry, they can help to identify new ubiquitylation targets (van Wijk SJ et al, 2013).

Another technique used to determine the chain linkage in a protein of interest makes use of ubiquitin mutants. It is possible to use mutants in which the Lys residues are mutated to Arg residues, thereby preventing the formation of chains through this residues. However, it is

important to remember that the overexpression of ubiquitin constructs can lead to the ubiquitylation of proteins that are not physiologically ubiquitylated.

One very recent technique based on DUBs was developed to also detect the ubiquitin linkage type. In this technique, called ubiquitin chain restriction analysis, the ubiquitylated protein of interest is incubated with DUBs known to specifically cleave one chain type. In this way, if the reaction cleaved off the ubiquitin molecules from the protein of interest, it is possible to deduce the linkage type (Mevissen et al, 2013).

3. Ubiquitylation in metabolism

Mass spectrometry studies have shown that more than 4 thousand proteins in the human proteome are modified by ubiquitin (Wagner et al, 2011; Kim et al, 2011). Moreover, the ubiquitylation machinery contains more than 700 proteins, while the phosphorylation machinery contains around 650 proteins, and phosphorylation affects nearly all processes in cells. These observations suggest that ubiquitylation is a widespread PMT in the proteome.

PTMs are one of the ways of regulating metabolic pathways; they provide one of the fastest and regulated ways of cells to respond to environmental cues. Indeed, there are some proteins related to metabolism which were shown to be regulated by ubiquitylation. Table 2 summarizes some examples of such proteins. Except for two proteins, all of the others shown to be ubiquitylated in metabolism were targeted to proteasomal degradation.

Table 2: Summary of metabolic proteins shown to be modified by ubiquitylation

Protein name	Function of the protein	E3 ligase/ DUB responsible	Function of ubiquitylation	References
HMG-CoA reductase	Rate-limiting enzyme in cholesterol biosynthesis; converts HMG-CoA to mevalonate.	gp78 (E3)	Rapid sterol-promoted degradation of HMG-CoA leads to block in cholesterol biosynthesis.	Song et al, 2005a; Song et al, 2005b; Liu et al, 2012
FoxO	Transcription factor that regulates fasting response	SKIP1	Insulin signaling leads to its phosphorylation, cytoplasmic translocation and proteasomal degradation.	Huang et al, 2005
Adipophilin	Lipid droplet-associated protein	Not identified	Degradation of adipophilin leads to regression of lipid droplets	Masuda et al, 2006
Delta9 stearoyl-CoA desaturase 1 (SCD1)	Key enzyme in the biosynthesis of mono-unsaturated fatty acids	Not identified	Degradation of Scd1 is constitutive irrespectively of the cellular levels of unsaturated fatty acids	Kato et al, 2006
Insig-1	Key negative regulator of sterol-regulated proteolysis of SREBP.	gp78 (E3)	Degradation of Insig-1 stimulates lipogenesis.	Lee et al, 2006a; Lee et al, 2006b;

				Gong et al, 2006
CRTC2	Transcriptional co-activator of CREB (fasting response).	COP1 (E3)	Insulin signaling leads to degradation of CRTC2 and switches-off gluconeogenesis.	Dentin et al, 2007; Liu et al, 2008
AKT	Plays a central role in cell proliferation, survival, metabolism, and tumorigenesis.	TRAF6 (E3) Skp2 SCF (E3) CYLD (DUB)	Growth factor-mediated membrane recruitment and activation of AKT (K63 chains).	Yang et al, 2009; Yang et al, 2010; Chan et al, 2012; Yang et al, 2013
PEPCK1	Key gluconeogenic enzyme; converts oxaloacetate to phosphoenolpyruvate	UBR5 (E3)	Glucose stimulation leads to PEPCK degradation and switches-off gluconeogenesis	Jiang et al, 2011
PGC-1α	Transcriptional coactivator that regulates the fasting response	RNF34	Its ubiquitylation in the lack of cold leads to proteasomal degradation and reduced thermogenesis	Wei et al, 2012
fructose-1,6-bisphosphatase	Key gluconeogenic enzyme; converts of fructose-1,6-bisphosphate to fructose 6-phosphate.	Gid complex (E3)	Rapidly degraded upon glucose stimulation.	Menssen et al, 2012
Insulin receptor (IR) and IR substrate 1	Effectors of insulin signaling	MG53 (E3)	Degraded; negatively regulates skeletal myogenesis and insulin signalling	Song et al, 2013; Yi et al, 2013
APPL1	regulates both activity and substrate specificity of AKT	TRAF6 (E3)	Insulin induces Lys63-linked ubiquitination; membrane recruitment and activation of AKT	Cheng et al, 2013
ATP-citrate lyase (ACLY)	Cleavage of citrate to Acetyl-CoA (building block for *de novo* lipogenesis)	UBR4 (E3)	Degradation of ACLY; blocks *de novo* lipid synthesis, cell proliferation, and tumor growth	Lin et al, 2013
SREBP1c	key transcription factor for *de novo* lipogenesis during the postprandial state	RNF20 (E3)	Activation of PKA signaling (fasting) promotes its degradation	Lee et al, 2014a
p35	CDK5 activator (cyclin dependent kinase 5), which inhibits insulin secretion	PJA2 (E3)	Upon glucose stimulation, p35 is ubiquitylated, degraded; promote insulin secretion.	Sakamaki et al, 2014

The rate-limiting enzyme in the gluconeogenic pathway, phosphoenolpyruvate carboxykinase (PEPCK), catalyses the conversion of oxaloacetate to phosphoenolpyruvate. It is well established that this enzyme is regulated by transcription factors in response to hormones and diet (Granner and O'Brien, 1992; Hanson and Reshef, 1997). However, in order to assure the switching off of the gluconeogenic pathway after a meal, Jiang et al (2011) showed that high glucose stimulation leads to the acetylation of PEPCK by the P300 acetylatransferase. This modification increases the interaction between PEPCK and the HECT domain containing E3 ligase UBR5. UBR5 polyubiquitylates PEPCK, targeting it to proteasomal degradation. They also observed that the deacetylase SIRT2 is able to increase the stability of PEPCK, having an important role during the fasting response.

Another example in which ubiquitylation is important to switch off gluconeogenesis in the liver is the ubiquitylation of CRTC2 (Figure 13). CRTC2 plays an important role during fasting in which it stimulates the gluconeogenic program by binding to CREB upon glucagon stimulation. Dentin et al (2007) showed that insulin can lead to the activation of the Ser/Thr kinase SIK2, which phosphorylates CRTC2. The modified CRTC2 exits the nucleus and in the cytoplasm it associates with the E3 ligase COP1, which polyubiquitylates it, targeting it to degradation. This is another step to ensure that during nutrient rich conditions, gluconeogenesis is not is inactivated.

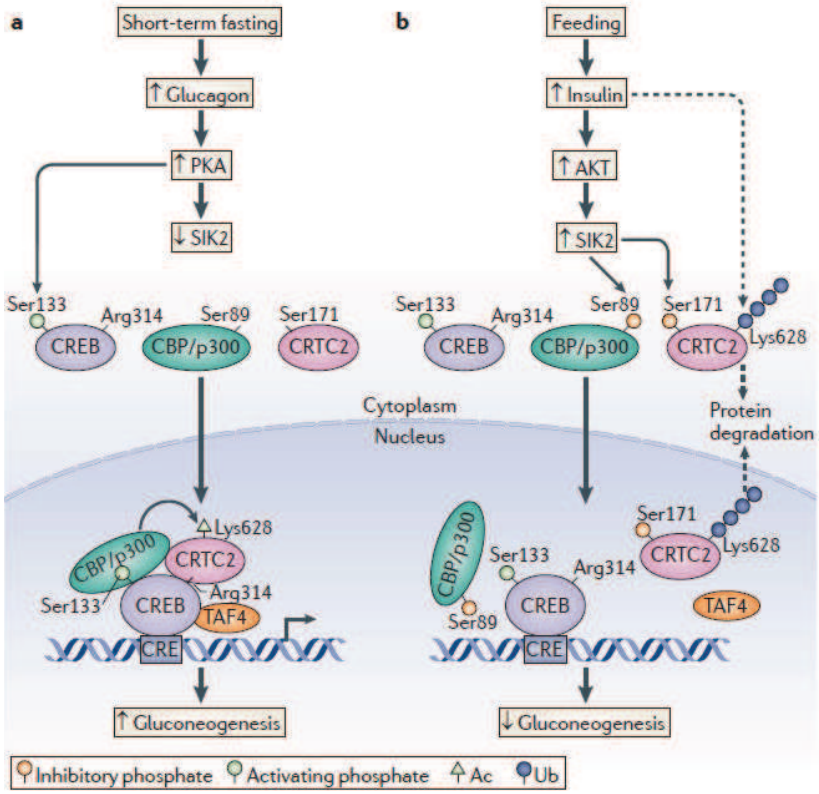


Figure 13: CRTC2 ubiquitylation regulates gluconeogenesis

During fasting, PKA-mediated signaling activates the activity of CREB to increase expression of gluconeogenic genes. However, insulin signaling provides an alternative way to ensure that this program is switched off by first phosphorylating CRTC2 and targeting it to porteasomal degradation through ubiquitylation. *Altarejos and Montminy, 2011.*

A protein shown to be ubiquitylated in which this modification did not lead to degradation is AKT. AKT is a key component of many cellular signaling pathways that regulate cell survival, metabolism, proliferation and apoptosis. Two groups (Yang et al, 2009; Yang et al, 2010; Chan et al, 2012) have shown that distinct ubiquitin E3 ligases can mediate the formation of K63-linked polyubiquitin chains in AKT, depending on the growth factors used for stimulation. Particularly, TRAF6 can ubiquitylate AKT in response to IGF-1 stimulation, while Skp2 SCF can polyubiquitylate AKT in response to EGF treatment. Importantly, the ubiquitylation of AKT leads to its translocation to the membrane and subsequent phosphorylation and activation. These groups also showed that, in starvation conditions, AKT is kept hypoubiquitylated (Yang et al, 2009; Yang et al, 2010; Chan et al, 2012). More recently, Yang et al (2013) have identified the cylindromatosis (CYLD) deubiquitinating enzyme able to cleave off ubiquitin chains from AKT.

They showed that, under serum-starved conditions, CYLD removed ubiquitin moieties from AKT, leading to its inactivation. Finally, they showed that the cycles of ubiquitin chain formation and cleavage are determining the plasma membrane localization and activation of AKT.

These are only three examples of proteins in metabolic pathways modulated by ubiquitylation (Table 2). Nevertheless, in the literature there are a really limited number of identified metabolic pathways modified by ubiquitylation. Especially when compared to the knowledge of metabolic pathways modulated by other PTMs, such as phosphrylation. Low- and high-throughput analysis of liver has shown many metabolic proteins to be phosphorylated and/or acetylated, and this modification would modulate their activities or target them to degradation (Zhao et al, 2010; Yang et al, 2011; Xiong et al, 2011; Kim et al, 2006). For instance, the activity of several transcriptional regulators of hepatic metabolism are modulated by acetylation; for instance, ChREBP, LXR, PGC-1α, and SREBP-1c (Li et al, 2007b; Liu et al, 2008; Rodgers et al, 2005; Mihaylova et al, 2011; von Meyenn et al, 2013; Kemper et al, 2009; Ponugoti et al, 2010; Walker et al, 2010).

As ubiquitylation is thought to be as widespread as phosphorylation, the number of modified substrates belonging to metabolic pathways modified by ubiquitylation is very limited when compared to the ones phosphorylated. With the advancement of the techniques available to study ubiquitylation, the number of ubiquitylated proteins in metabolism is very likely to increase seeing that ubiquitylation can provide a fine tuning of pathways as already demonstrated for other fields, such as cell cycle and inflammation. This is true especially because ubiquitylation can modulate proteins and pathways in several ways, and not only by targeting proteins to degradation, which include most of the examples present in the Table 2.

With the hypothesis that ubiquitylation might be regulating metabolic processes, we have designed a proteomic screen to identify new metabolic pathways and proteins regulated by ubiquitylation. To this end we decided to look at pathways in liver, as the liver constitutes a major metabolic hub in our organisms, able to switch on and off several pathways depending on the hormonal and diet input.

Aims of the project

Metabolic pathways are very tightly regulated to ensure energy homeostasis in our organism. This regulation is achieved by changes in gene expression, allosteric modulation of enzymes and posttranslational modifications (PTMs) of enzymes and components of signaling cascades. PTMs provide a fast, reversible and controlled way of regulating proteins and signaling pathways. While most attention has been focused on studying phosphorylation and acetylation, the role of ubiquitylation in regulating metabolic pathways is still poorly studied.

For this reason, the primary aim of this work was to establish a reproducible and reliable protocol to purify and identify ubiquitylated proteins from livers of mice. Moreover, I aimed at identifying proteins that are ubiquitylated in response to metabolic challenges, i.e. fasting and feeding. At last, I aimed at understanding the role of ubiquitylation in the function of one particular identified protein in the context of liver physiology. Collectively, our aim was to identify new ubiquitylation pathways that regulate fasting and feeding responses in liver.

Materials and Methods

1. Antibodies and Reagents

The following antibodies and reagents were commercially available: anti-ubiquitin (P4D1; Santa Cruz Biotechnology; WB1:1000), anti-IκB (Cell Signalling Technology; WB 1:1000), anti-GAPDH (G9545; Sigma Aldrich; WB 1:50000), anti-acetylated-lysine (Cell Signalling Technology; WB 1:1000), streptavidin-HRP (Thermo Fisher Scientific; WB 1:10000; ELISA 1:2000), anti-C3a (Hycult Biotech: FACS 1:30), anti-C3aR (Hycult Biotech; FACS 1:50), anti-C3b (Hycult Biotech; FACS 1:50; ELISA 1:50), anti-C3 (Santa Cruz Biotechnology; WB 1:1000; FACS 1:200; ELISA 1:200), anti-mouse-HRP (Cell Signalling Technology; WB 1:10000), anti-rat-HRP (Cell Signalling Technology; WB 1:2000; ELISA 1:2000), anti-rabbit-HRP (Thermo Fisher Scientific; WB 1:10000), anti-rabbit-HRP (DAKO; ELISA 1:2000), MG132 (Tocris Bioscience), PR619 (Enzo Life Sensors), TNFα (Immunotools). The following antibodies were provided by the antibody facility (IGBMC): anti-Tubulin (WB 1:5000), anti-Flag mouse (WB 1:1000), anti-SOD (WB 1:5000).

2. Cloning and cDNAs

The construct expressing His-Bio-Ubiquitin was kindly provided by Prof. Peter Kaiser, University of California, Irvine, and subcloned into the vector pAAV-MCS. The following cDNAs were subcloned into the vector pcDNA3.1 with a FLAG tag introduced by PCR cloning: Lpin1 (Addgene plasmid 32005), PDK4 (Addgene plasmid 23577), HSD11β1 (Addgene plasmid 24096), STBD1 (cDNA human bank), PDZK1 (cDNA human bank), C3 (cDNA human bank), PDHX (cDNA human bank), ENO1 (cDNA human bank).

3. Cell culture and transfection

HEK293T cells were obtained from ATCC and cultivated in a medium containing DMEM 1g/L glucose, 10% FCS and penicillin-streptomycin. These cells were transfected with JetPEI (Polyplus Transfection) following the manufacturer's protocol.

Unless indicated differently, whole-cell extracts were prepared as follows: cells were first washed with ice-cold PBS, and then scraped in the presence of lysis buffer (50 mM Tris pH 7.2, 50 mM NaCl, 1% Triton-X100, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 5 mM $Na_4P_2O_7$, 270 mM sucrose, 1 mM DTT, 0.2 mM PMSF, 10 mM NEM, 1x Complete protease inhibitor (Roche)), and finally incubated at 4°C for 20 minutes in the rotating wheel. After centrifugation at 13000 xg for 15 minutes the supernatant containing the total cell lysate was stored at -80°C until use.

For the experiments with IκB ubiquitylation, cells were treated with 10 ng/mL TNFα and 20 μM MG132 for 3 hours. For secondary screen experiments, cells were treated with 10 μM MG132 for 4 hours, 10 μM PR619 for 2 hours and, when indicated, the media was replaced with HBSS for 4 hours.

4. Primary hepatocytes isolation and culture

The protocol used to isolate murine primary hepatocytes was adapted from Li et al, 2010b. In brief, mice were anesthetized with ketamine and xylazine and while the heart was still beating, the portal vein was canulated. The liver was then perfused with 40 mL of Solution I (HBSS (Sigma H6648), 10mM HEPES) and subsequently perfused with 40 mL of Solution II (HBSS (Sigma H6648), 10mM HEPES, 4mM $CaCl_2$, 1 mg/mL Collagenase type I (Worthington Biochem)). After perfusion, the liver was placed in a petri dish with 10 mL of Solution B and the cells inside the capsule were released into the buffer. These cells were then centrifuged (50 g, 5 min, 4°C) and washed 3 times in hepatocyte medium (DMEM 1 g/L glucose, 10% FCS, 0.1 μM insulin, 1 mM glutamine, penicillin-streptomycin). The final solution of cells was passed through a 70um mesh and the viability was accessed by trypan blue exclusion.

The primary hepatocytes were plated with on collagen coated plates. To coat them, a solution of 0.05 mg/mL of rat tail collagen diluted in 0.2 N Acetic Acid was put on top of the plates and let

overnight. Plates and coverslips were washed with PBS and used immediately. The final cell density used was $1.5*10^6$ cells per 10 cm plate and $0.25*10^6$ cells per well in 24-well plates.

For the experiments using different media in the cells with the aim of mimicking the fasting-refeeding of mice, the following media were used: Growing Culture (GC), DMEM 1 g/L glucose, 10% FCS, 0.1 µM insulin, 1 mM glutamine, penicillin-streptomycin; Starvation Medium (SM), DMEM 1 g/L glucose, penicillin-streptomycin; Rich Medium (RM), DMEM 4.5 g/L glucose, 10% FCS, 0.1 µM insulin, 1 mM glutamine, penicillin-streptomycin.

5. Western blot

Protein samples were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked for 1 hour at room temperature with 5% skimmed milk in TBS-T (0.05% Tween) and incubated overnight at 4°C with the primary antibody also diluted in milk-TBS-T. Membranes were then washed in TBS-T prior to incubation with the secondary antibody HRP-conjugated for 1 hour at room temperature. Proteins were visualized using SuperSignal Chemiluminescent reagent (Thermo Scientific).

6. Mouse lines and genotyping

Wild-type mice from the strain C57BL/6J were obtained from the provider Charles River and were used between 8 – 10 weeks of age. The purified mitochondria from *Alb-Cre fxn*$^{f/f}$ were kindly provided by the group of Helene Puccio (IGBMC, France) (Martelli et al, 2012). All the experiments were performed using littermate mice as controls.

7. Fasting and Refeeding of mice

The fasting and refeeding of mice were performed as follows: mice were fasted for 16 hours and either sacrificed at 10 am or refed a high-sucrose diet (D00041102; Research Diets Inc) for 24 hours and sacrificed on the next day at 10 am. The mice were sacrificed by cervical dislocation and the liver was rapidly dissected, snap-frozen in liquid nitrogen and stored at -80°C.

To prepare liver lysates, livers were homogenized in ice-cold lysis buffer (as described for HEK293T cells) with an ultra-turrax and incubated at 4°C for 20 minutes in a rotating wheel. After centrifugation at 13000 g for 15 minutes the supernatant containing the total liver lysate was stored at -80°C until use.

8. Identification of differentially ubiquitylated proteins

The selection of the differentially identified proteins was done with the software Perseus (Cox and Mann, 2008). Firstly, all the proteins also identified in agarose beads were removed from the TUBEs 1 dataset. Also, TUBEs- and UbiQapture- identified proteins were analyzed separately. All the label-free quantification values (LFQ) were transformed to the $\log_2$ scale and zero values were transformed using the function “imputation” into the smallest detected value. A one-way ANOVA was performed with all the mice in fasted conditions (13 mice for TUBEs 1 and 10 mice for UbiQapture) and refed conditions (13 mice for TUBEs 1 and 10 mice for UbiQapture). With the p-values obtained from the ANOVA and the fold-change between refed and fasted-specific proteins, a volcano plot was built. The selection of the proteins was based on $p > 0.05$ (-log p-value > 1.3) and fold change > 4 ($\log_2$ fold change > 2).

9. Cellular compartments and pathways enrichment

All identified proteins or the fasted-refed-specific proteins were submitted to Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al, 2009a; Huang et al, 2009b) and to Reactome (Croft et al, 2014) online tools. The gene ontology for enriched cellular compartments was extracted from the DAVID analysis and the results with a significant p-value were used to generate graphs. With Reactome, only the pathways considered enriched according to the p-value were considered to build graphs.

10. TUBEs pulldown

TUBEs 1 and TUBEs 2 were purchased from LifeSensors. For experiments with cell lysates, 10 – 30 µL of TUBEs (50% slurry) were incubated with 1 mg of whole-cell lysates. For the screen 100 µL of TUBEs 1 were incubated with 50 mg of whole-liver lysates. After overnight incubation

at 4°C, the beads were left on ice to precipitate for 20 min and the flow-through was collected. The beads were then washed 2 times with lysis buffer + 400 mM NaCl, 2 times with lysis buffer and 2 times with 50 mM Tris pH 7.2. For the experiments with cell lysates, the bound proteins were eluted by addition of Laemmli buffer and incubation at 95°C for 5 min. For the proteomic screen, the bound proteins were eluted by 2 sequential elutions with 0.2 M Glycine pH 2.0 at 4°C for 30 min. Agarose beads were always incubated with protein lysates in parallel to control for unspecific binding.

11. UbiQapture pulldown

UbiQapture was purchased from Enzo Life Sciences. For experiments with cell lysates, 10 µL of UbiQapture (50% slurry) were incubated with 1 mg of whole-cell lysates. For the screen 100 µL of UbiQapture (50% slurry) were incubated with 50 mg of whole-liver lysates. After overnight incubation at 4°C, the beads were left on ice to precipitate for 20 min and the flow-through was collected. The beads were then washed 2 times with lysis buffer + 400 mM NaCl, 2 times with lysis buffer and 2 times with PBS. The bound proteins were eluted by addition of Laemmli buffer and incubation at 95°C for 5 min. For the proteomic screen, the proteins were ran on a SDS-PAGE only until the whole sample was inside the stacking gel and stained with Commassie Brilliant Blue. The stained samples were cut and kept in -20°C until further treatment.

12. FLAG and IκB immunoprecipitation

Total cell lysates of transfected HEK293T cells treated with MG132 or PR619 were incubated with FLAG M2 affinity gel (Sigma Aldrich) on a ratio of 3 mg of total protein to 40 µL FLAG beads (50% slurry). After overnight incubation at 4°C, beads were left on ice to precipitate for 20 min and the flow-through was collected. The beads were then washed 6 times PBS. The bound proteins were eluted in 2 sequential elutions of lysis buffer + 0.05 mg of FLAG peptide (provided by the Peptide Synthesis Group at the IGBMC) at 4°C for 6 hours (1st elution) and at room temperature for 30 min (2nd elution).

Anti-IκB conjugated to agarose beads (Cell Signalling Technology) were incubated with cell lysates of HEK293T cells treated with and without TNFα and MG132 on a ratio of 1 mg of total lysate to 10 uL of anti-IκB beads. After overnight incubation at 4°C, the beads were left on ice to

precipitate for 20 min and the flow-through was collected. The beads were then washed 2 times with lysis Buffer + 400 mM NaCl, 2 times with lysis buffer and 2 times with 50 mM Tris pH 7.2. The bound proteins were eluted by addition of Laemmli buffer and incubation at 95°C for 5 min.

13. Tandem affinity purification of ubiquitylated proteins

Tandem denaturing ubiquitin pulldowns were performed as described in Meierhofer et al (2008). Briefly, HEK293T cells transfected with the construct His-Bio-Ubiquitin and cultured in DMEM 1g/L glucose, 10% FCS and penicillin-streptomycin supplemented with 1 µM biotin. Cells were washed with ice-cold PBS and scraped on plates with Buffer A (8 M urea, 300 mM NaCl, 50 mM NaH_2PO_4, 0.5% NP-40), pH 8.0, and 1 mM PMSF. After centrifugation at 15000 g, 30 min, 20 °C, the clarified supernatants were stored at -80°C until further purification.

For the pulldown, for each 1 mg of protein from the whole-cell lysate, 35 µL of Ni^{2+} sepharose beads (GE Healthcare) were used. After overnight incubation at room temperature with supplemented 10 mM imidazole on a rotating wheel, beads were washed sequentially with Buffer A (pH 8.0), Buffer A (pH 6.3), and Buffer A (pH 6.3) with 10 mM imidazole. Proteins were then eluted twice for 1 h at room temperature with 5 bead volumes of Buffer B (8 M Urea, 200 mM NaCl, 50 mM Na_2HPO_4, 2% SDS, 10 mM EDTA, 100 mM Tris, 250 mM imidazole) pH 4.3. The pH of the elute was adjusted to pH 8.0 and 7 µL of streptavidin sepharose beads (Thermo Scientific, Rockford, IL) were added for each 1 mg of initial protein lysate. After overnight incubation at room temperature on a rotating wheel, beads were washed twice with 50 bead volumes of Buffer C (8 M Urea, 200 mM NaCl, 2% SDS, 100 mM Tris, pH 8.0), followed by 2 washes of 50 bead volumes of Buffer D (8 M Urea, 1.2 M NaCl, 0.2% SDS, 100 mM Tris, 10% EtOH, 10% Isopropanol, pH 8.0) and final 2 washes with 50 mM Tris pH 7.2. The bound proteins were eluted by addition of Laemmli buffer and incubation at 95°C for 5 min.

14. Flow cytometry analysis of murine primary hepatocytes

Freshly isolated primary mouse hepatocytes were stained with anti-C3a, anti-C3b, anti-C3aR and anti-C3 for both extracellular and intracellular proteins. After extracellular staining, cells were fixed and permeabilized using the kit BD Cytofix/Cytoperm™ (BD Biosciences) following the manufacturer's protocol. DAPI (4',6-Diamidino-2-phenylindole dihydrochloride; Sigma) was

added before permeabilization to differentiate between intact and dead cells. The samples were analysed using the BD LSR II Flow Cytometer and FlowJo software (Tree Star).

15. Mitochondrial purification

The purification of mitochondria from the livers of mice which underwent fasting – refeeding was done according to Pallotti and Lenaz (2007). The method used was based on the differential centrifugation of these organelles. Briefly, freshly isolated livers were washed in ice-cold Solution A (0.22 M Manitol, 0.07 M sucrose, 0.02 M HEPES, 1 mM EDTA, 2 mM Tris pH 7.2) with 0.4% BSA in order to remove the excess of blood and connective tissue. The liver is then homogenized in cold Solution A by using a Potter-Elvehjen glass homogenizer. After the first centrifugation at 3000 g for 1.5 min, the pellet was ressuspended in Solution A and it underwent another centrifugation. Both supernatants were combined and centrifuged at 17500 g for 2.5 min. The pellet was washed in Solution A and centrifuged at 17500 g for 4.5 min. The pellet was washed again in Solution B (0.22 M Manitol, 0.07 M sucrose, 1 mM EDTA, 0.01 M Tris pH 7.2) and centrifuged at 17500 g for 4.5 min. The pellet is finally ressuspended in Solution B and stored at -80°C until use.

16. Elisa for C3b detection

A monoclonal C3b specific antibody was immobilized in Maxisorp microtiter plates (Nunc) in bicarbonate buffer (28.5 mM Na_2CO_3, 71.4 mM $NaHCO_3$, pH 9.9) for 2 hours at room temperature. After blocking, liver lysates diluted 2 times in Dilution buffer (50 mM Tris pH 7.4, 150 mM NaCl, 20 mM Glycero-P, 1 mM Na_3VO_4, 100 mM NaF, 1X Complete protease inhibitor (Roche)) were incubated overnight at 4°C on a rocking platform. After washing, anti-C3 was added to detect captured C3b. To visualize the binding, 3,3',5,5'-tetramethylbenzidine (TMB) was added and the absorbance was measured at 450 nm by a plate reader.

17. RNA isolation, cDNA synthesis and quantitative real-time PCR

For liver RNA isolation, a small amount of liver was homogenized in 1 mL TRIzol reagent (Sigma) and lysed with a syringe. The RNA isolation was followed according to manufacturer's

instructions. cDNA was synthesized with Oligo dT primers using SuperScript® III First-Strand cDNA Synthesis kit (Invitrogen) according to the manufacturer's protocol. Quantitative real-time PCR was performed using SYBR Green (Roche Diagnostics) on the LightCycler 480 (Roche Diagnostics). The samples were individually normalized to the housekeeping gene HPRT. The gene-specific primers used were the following: C3 (NM_009778) fwd 5'-CCAGCTCCCCATTAGCTCTG-3' rev 5'- GCACTTGCCTCTTTAGGAAGTC-3'; CTSL (NM_009984) fwd 5'-ATCAAACCTTTAGTGCAGAGTGG-3' rev 5'-CTGTATTCCCCGTTGTGTAGC-3'; CPN1 (NM_030703) fwd 5'-TCCAAGTTTGTTACCCCGGTG-3' rev 5'-CTGCGCCCGATGTTGTAGAG-3'; CPB2 (NM_019775) fwd 5'- AGCATGGCTTCGCCTTTCA-3' rev 5'-GAGAACGACCTCATACGTTGTAG-3'; C3aR1 (NM_009779) fwd 5'-TCGATGCTGACACCAATTCAA-3' rev 5'-TCCCAATAGACAAGTGAGACCAA-3' ; C5L2 (NM_001146005) fwd 5'-TGGCCGACTTGCTTTGTTGT-3' rev 5'-CGCACCCCAAATGTTCGATG-3'; HPRT (NM_013556) fwd 5'-GTAATGATCAGTCAACGGGGGAC-3' rev 5'- CCAGCAAGCTTGCAACCTTAACCA-3'.

18. Statistical analysis

For the proteomic screen, one-way ANOVA was used to find proteins differentially ubiquitylated. Student's *t* test was used to compare two groups for the other experiments, unless stated otherwise. To identify if overlaps between 2 or more groups of proteins were random, we used the hypergeometric distribution, with assumption of the mouse proteome of 40000 proteins. For all the tests, statistical significance was considered when $p < 0.05$.

Results

Based on the hypothesis that ubiquitin signaling could regulate liver metabolic pathways, as well as the emerging role of ubiquitylation in cellular processes, we have conducted a global proteomics analysis to identify ubiquitylated proteins in livers of mice subjected to a fasting – refeeding protocol. Briefly, I fasted mice for 16 hours and, either sacrificed them, or refed the with a high-sucrose diet. The high-sucrose diet is known for inducing a synchronized and massive *de novo* lipogenesis in the livers (Cohen et al, 1972). Using this model, we aimed at synchronizing the mice in two opposite states, fasting and refeeding, in which gluconeogenesis and *de novo* lipogenesis, respectively, are activated.

1. TUBEs and UbiQapture can efficiently bind and purify ubiquitylated proteins

In order to purify ubiquitylated proteins from total liver lysates, I used two complementary approaches – Tandem Ubiquitin-Binding Entities (TUBEs) and UbiQapture pulldowns (Figure 14 and Figure 15). These two techniques are based on ubiquitin binding domains linked to beads, allowing a one-step pulldown of ubiquitylated proteins. We have decided to use both tools because the ubiquitin binding domains they contain are derived from different ubiquitin binding proteins. For this reason, they have different affinities towards different ubiquitylated proteins and different types of ubiquitylation. For instance, TUBEs 1 was shown to have higher affinity for K63-linked chains, TUBEs 2 has a higher affinity toward K48-linked chains and UbiQapture seem to have a broader spectrum, binding also monoubiquitylated proteins.

I first assessed TUBEs 1 and TUBEs 2 for efficient enrichment and affinity for ubiquitylated proteins in total extracts of HEK293T cells treated with the proteasome inhibitor MG132 (Figure 14A, Figure 14B). Proteasome inhibition was efficient, as there were more ubiquitylated proteins in the input of cells treated with MG132 as compared to DMSO control treatment. Importantly, both TUBEs were able to enrich for ubiquitylated proteins in the eluted fractions. Agarose beads, however, did not bind unspecifically to ubiquitylated proteins.

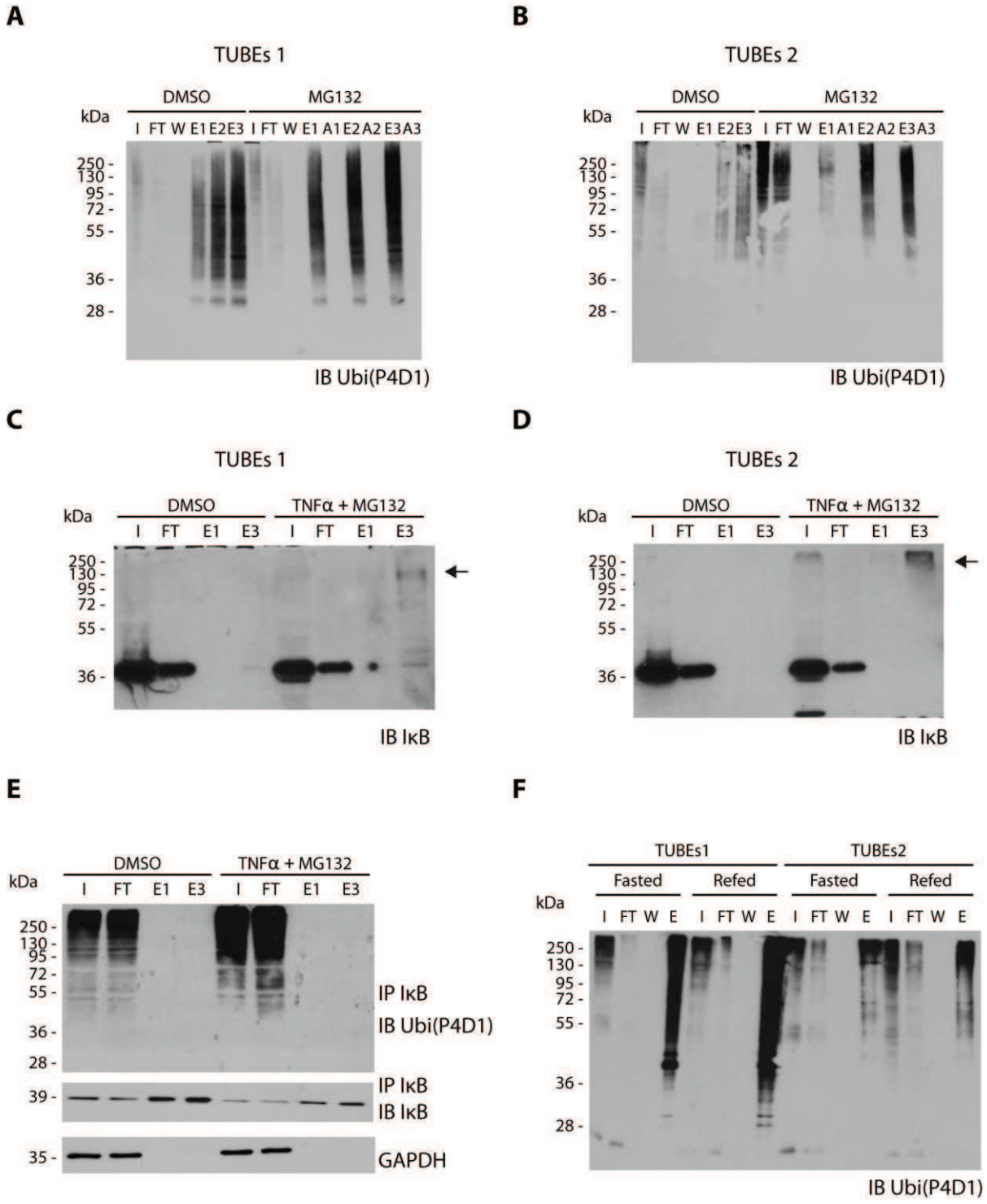


Figure 14: TUBEs can efficiently enrich for ubiquitylated proteins

(A,B) Western blot after TUBEs1 (A) or TUBEs2 (B) pulldowns from HEK293T whole-cell lysates treated with DMSO or 20 µM MG132 (proteasome inhibitor) for 3 hours. Antibody against ubiquitin (P4D1 clone) was used to detect ubiquitylated proteins.

(C,D) Western blot after TUBEs 1 (C) or TUBEs 2 (D) pulldowns from HEK293T whole-cell lysates treated with DMSO or 10 ng/mL TNFα and 20 μM MG132 (proteasome inhibitor) for 3 hours. Antibody against IκB was used. Arrows indicate polyubiquitylated IkB in the eluted fractions.
(E) Western blot after IkB immunoprecipitation from HEK293T whole-cell lysates treated with DMSO or 10 ng/mL TNFα and 20 μM MG132 (proteasome inhibitor) for 3 hours. Antibodies against ubiquitin (P4D1), IκB and GAPDH were used.
(F) Western blot after TUBEs1 and TUBEs2 pulldowns from liver lysates from mice fasted 16 hours or fasted and refed with a high-sucrose diet for 24 hours. Antibody against ubiquitin (P4D1) was used to detect ubiquitylated proteins.
I, input; FT, flow through; W, wash; E1, elution with 10uL TUBEs; E2, elution with 20uL TUBEs; E3, elution with 30uL TUBEs; A1, elution with 10uL agarose; A2, elution with 20uL agarose; A3, elution with 30uL agarose.

I next investigated whether a pulldown by TUBEs was able to enrich a known ubiquitylated protein (Figure 14C, Figure 14D). To this end, HEK293T cells were treated with MG132 and tumor necrosis factor α (TNFα), which leads to ubiquitylation and accumulation of IκB (Lang and Rodriguez, 2008; Hjerpe and Rodriguez, 2008). I observed that both TUBEs 1 and TUBEs 2 were able to capture ubiquitylated IκB from total cell lysates, demonstrated by the upshifted bands corresponding to the polyubiqutylated IκB (Figure 14C, Figure 14D). We have also tried to immunoprecipitate IκB from the same extracts (Figure 14E), but we were not successful to identify the ubiquitylated part of IκB, as we failed to see an ubiquitin signal in the eluted fractions. This might be due to the lack of sensitivity of the tools used under the conditions tested. Overall, these experiments strongly corroborate the suitability of TUBEs for purification and identification of ubiquitylated proteins.

We next tested whether TUBEs 1 or TUBEs 2 is better suited for a proteomic screen by performing pulldowns from liver lysates of fasted or refed mice (Figure 14F). I observed that TUBEs 1 purified a higher amount of ubiquitylated proteins, when compared to TUBEs 2. Notably, TUBEs 1 could enrich for low molecular weight proteins, which suggests that potentially monoubiquitylated proteins were also enriched. In fact, TUBEs 1 was previously shown to have a higher affinity towards K63 linked chains (Hjerpe et al, 2009), being associated with a functional ubiquitylation related to cellular signaling (Wang et al, 2008). As we were interested in identifying not only ubiquitylated proteins targeted for degradation, but also modified proteins

with other intracellular functions, we decided to use TUBEs 1 to purify ubiquitylated proteins from liver lysates for the analysis with mass spectrometry.

The second tool I used to purify ubiqutylated proteins prior to mass spectrometry analysis was UbiQapture. Less is known about this reagent apart from the fact that it is also based on ubiquitin binding domains and that it can bind many types of ubiquitylated proteins, including monoubiquitylation. I went on to investigate whether UbiQapture could also purify a known ubiquitylated protein. As for tests on TUBEs, I treated HEK293T cells with TNFα and MG132 in order to identify accumulated polyubiquitylated IκB. We observed that UbiQapture was also successfully enriching the polyubiqutylated fraction of IκB, demonstrated by the upshifted bands (Figure 15A). We subsequently tested the efficiency of UbiQapture to pulldown ubiquitylated proteins from liver lysates (Figure 15B). As expected, the eluted fractions after pulldown with UbiQapture were highly enriched with ubiquitylated proteins.

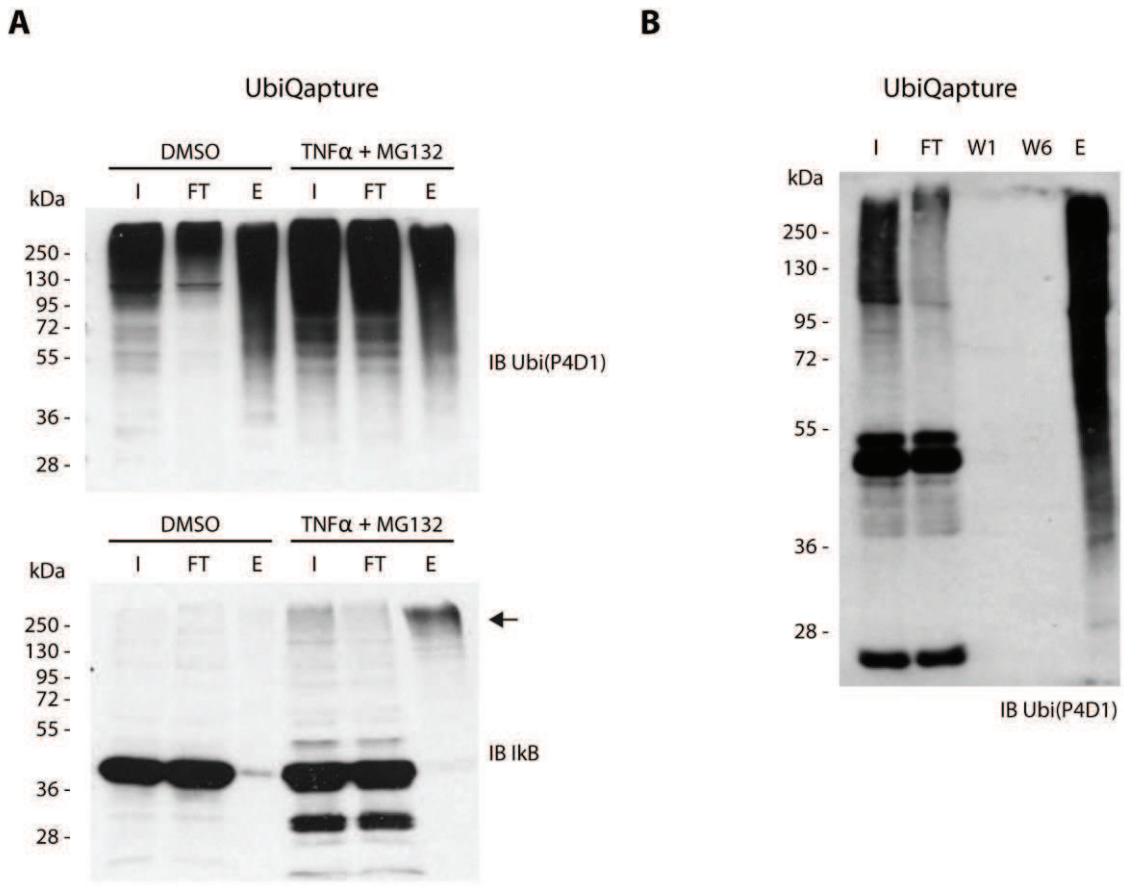


Figure 15: UbiQapture can efficiently enrich for ubiquitylated proteins

(A) Western blot after UbiQapture pulldowns from HEK293T whole-cell lysates treated with DMSO or 10 ng/mL TNFα and 20 μM MG132 (proteasome inhibitor) for 3 hours. Antibody against ubiquitin (P4D1 clone) was used to detect ubiquitylated proteins. Antibody against IκB was used to detect polyubiquitylated IkB in the eluted fractions (indicated by arrows).
(B) Western blot after UbiQapture pulldown from liver lysates from ad libitum mice. Antibody against ubiquitin (P4D1) was used to detect ubiquitylated proteins.
I, input; FT, flow through; W1, first wash after incubation with lysate; W6, last wash before elution of the protein; E, elution.

Altogether, these results show that both TUBEs 1 and UbiQapture could efficiently enrich for ubiquitylated proteins from lysates of liver as well as cells.

2. Proteomics leads to identification of ubiquitylated proteins in livers of fasted and refed mice

After assessing the efficiency of TUBEs and UbiQapture, I have used these tools to purify ubiqutylated proteins from liver lysates of mice subjected to a fasting – refeeding protocol. The detailed scheme of the performed screen can be seen in Figure 16A and is described in the Figure legend. In brief, I fasted the mice for 16 hours and then refed them with a high sucrose diet for 24 hours. This is leading to a massive and synchronized *de novo* lipogenesis in the liver (Cohen et al, 1972).

I subsequently purified ubiquitylated proteins from livers with TUBEs 1 and UbiQapture. An example of the purification efficiency for samples before the mass spectrometry analysis is shown in Figure 16B. The pattern of ubiquitylation that was purified with both tools seemed to be different, as UbiQapture was more specific to the higher molecular weight proteins. The eluted proteins were digested with trypsin, and indentified with an Orbitrap XL. The raw mass spectrometric data was analysed with Maxquant (Cox and Mann, 2008) with the Andromeda searching engine (Cox et al, 2011). The label-free quantification (LFQ) was performed in MaxQuant (Luber et al, 2010) and the statistics was done using Perseus.

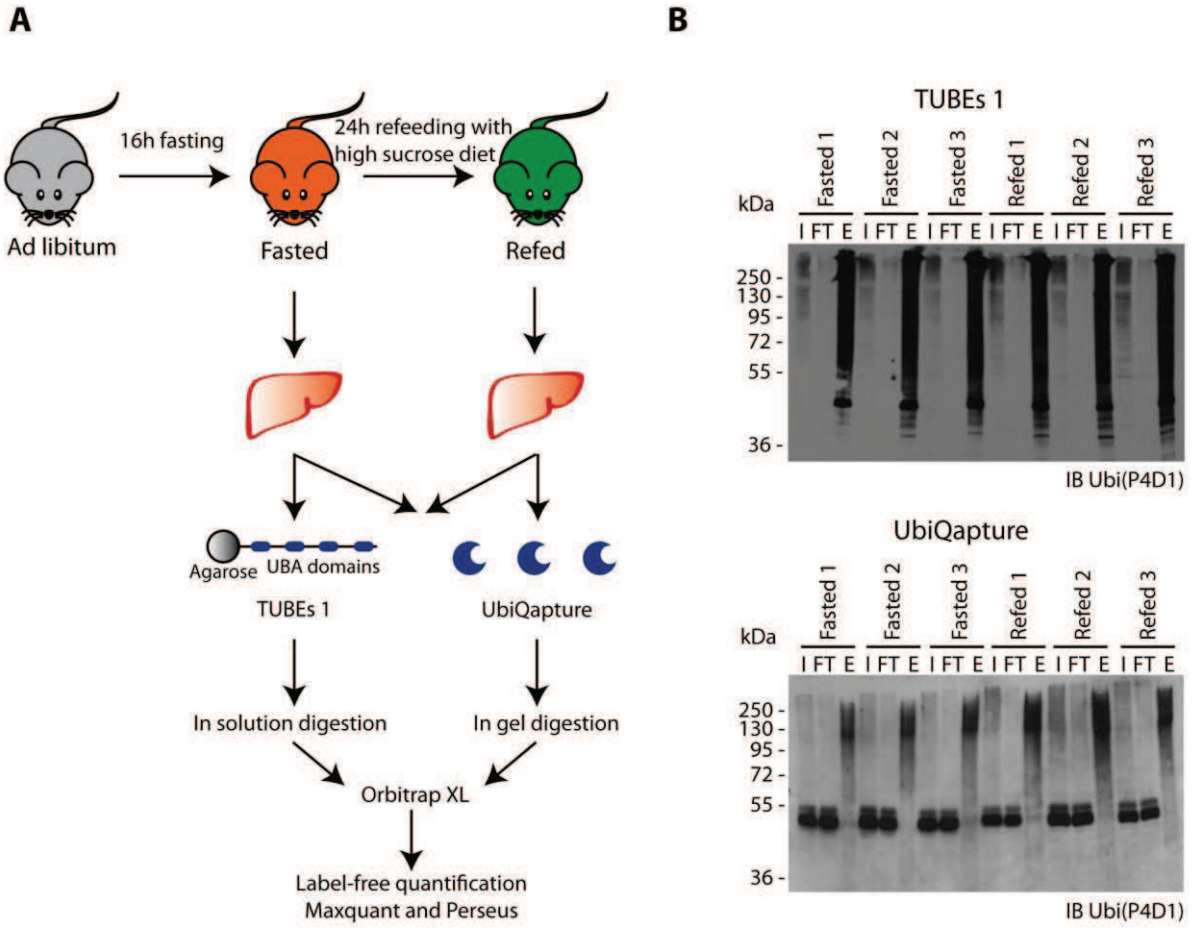


Figure 16: TUBEs and UbiQapture are used to purify ubiquitylated proteins prior to mass spectrometry analysis

(A) Schematic representation of the steps involved the proteomic screen. After submitting mice to the fasting-refeeding protocol, their livers were collected, ubiquitylated proteins were purified and analysed by an Orbitrap XL.

(B) Western blot after TUBEs1 and UbiQapture pulldowns from liver lysates from mice fasted 16 hours or fasted and refed with a high-sucrose diet for 24 hours. Antibody against ubiquitin (P4D1) was used to detect ubiquitylated proteins.

I, input; FT, flow through; E, elution.

We performed 3 experimental replicates, with 10 or 8 mice in each one, for the TUBEs purification and 2 experimental replicates, with 10 mice each, for UbiQapture purification. In total we used livers from 46 mice (23 fasted and 23 refed). Finally, we have identified 1641 unique

putative ubiquitylated proteins in the livers of fasted and refed mice. For 25 proteins, we identified potential ubiquitylation sites (Figure 17A).

There was a great overlap between experimental replicates – 50% of proteins identified with TUBEs were identified in at least 2 experiments and 73% of the proteins identified with UbiQapture were identified in both experiments (Figure 17B, Figure 17C). Using a hypergeometric distribution with the assumption for the mouse proteome consisting of 40000 proteins, these overlaps were statistically significant ($p<0.01$) and not random. Importantly, these overlaps confirm that these experiments were very reproducible. In addition, 22% of the identified proteins were identified with both techniques (Figure 17D, Figure 17E). These overlaps were also statistically significant ($p<0.01$). This observation indicates that some of the proteins are recognized by both UBA domains, but most of the proteins have different affinities towards these different domains. In consequence, these different tools (TUBEs 1 and UbiQapture) enrich a different fraction of the proteins.

It is important to remember that the pulldowns were performed under native conditions. While the used tools enriched for ubiquitylated proteins, other proteins could also be co-purified. For instance, it is possible that proteins belonging to the same protein complex would be purified together. Moreover, ubiquitin binding proteins could bind the ubiquitylated proteins and also be present in the eluted fractions, even though they were not initially ubiquitylated. For this reason, we believe that amongst the identified proteins, there is probably a mixture of ubiquitylated proteins and proteins that were co-purified together with the first ones. However, we suppose that these are not so many since we used very stringent washing conditions (0.4 M NaCl) during the pulldown.

The detection of ubiquitylated sites in the mass spectrometer would be the only direct way of confirming the ubiquitylation of proteins in the screen. Ubiquitylation sites are identified in mass spectrometry by to 2 features: a signature di-Gly motif left in the lysine that was ubiquitylated and 1 tryptic miss-cleavage. In our approach, we enriched for ubiquitylated proteins, but after trypsin digestion, one protein was cleaved into many peptides, which made it less likely to identify the modified lysine.

A

Purification method	Experiment number	No of proteins identified	Ubi-sites identified
TUBEs	Exp 1	173	2
	Exp 2	413	9
	Exp 3	577	21
	Total	732	25
UbiQapture	Exp 1	1102	3
	Exp 2	1108	2
	Total	1275	5
TOTAL		1641	25

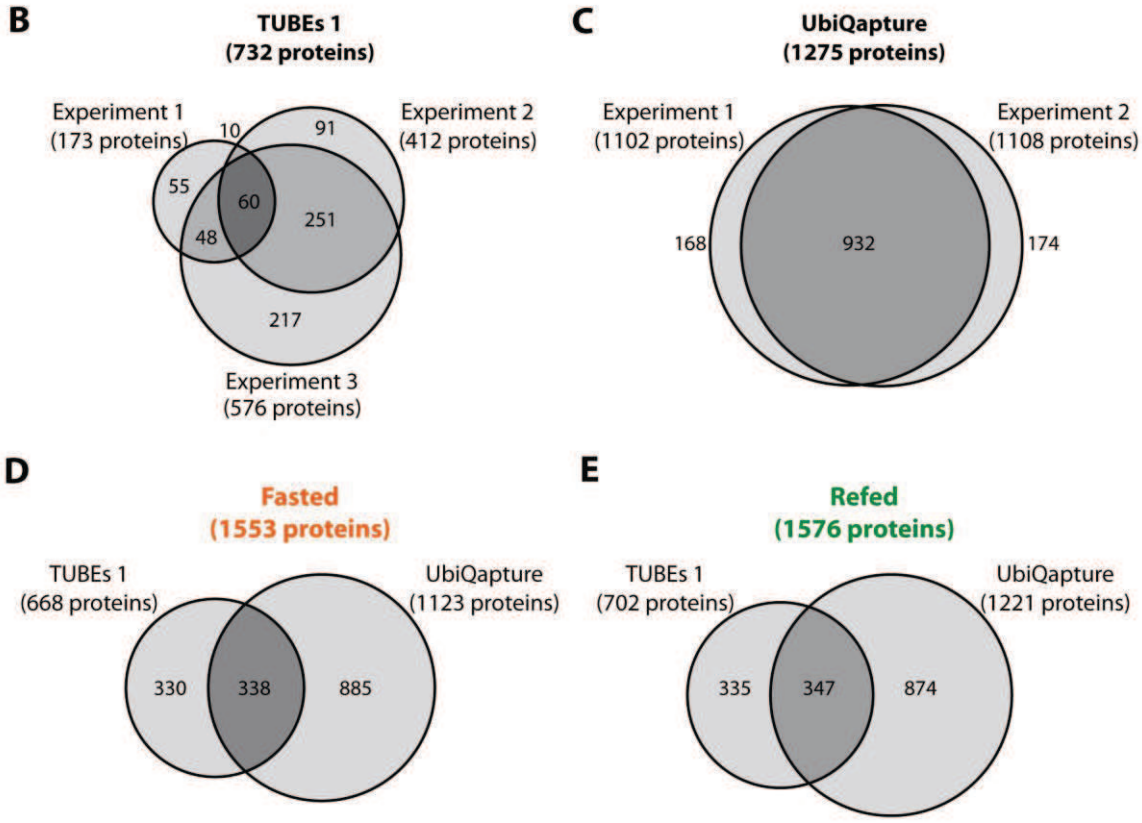


Figure 17: Mass spectrometry identifies 1641 proteins in the liver of mice with large reproducibility between experiments

(A) Table with the number of indentified proteins and proteins with ubiquitylation sites identified in each experiment. Three experiments were performed with TUBEs pulldown from 26 mice in total. Two experiments were performed with UbiQapture pulldown from 20 mice in total. In total, 1641 unique proteins were identified.

(B,C) Venn diagrams show the number of indentified proteins that overlap between different experiments. (B) More than 50%** of the TUBEs-identified proteins were identified in more than 2 independent experiments. (C) More than 73%** of the UbiQapture-identified proteins were identified in more than 2 independent experiments.
(D,E) Venn diagrams show the number of indentified proteins that overlap between different purification techniques. 22%** of the total number of identified proteins overlapped between (D) TUBEs1 and (E) UbiQapture purified proteins.
**Hypergeometric distribution with the hypothesis of overlap happening by chance (assumption mouse proteome of 40000 proteins), $p < 0.01$.

Indeed, the number of identified ubiquitylation sites in the screen was quite low - only 25 out of 1641 proteins (Figure 17A). In order to estimate the portion of proteins that we identified that were in fact ubiquitylated, I have compared our identified proteins with 2 other global mass spectrometry studies. In Wagner et al 2012 they identified 3946 ubiquitylated proteins in the livers of ad libitum fed mice. The purification technique used was the antibody that recognizes directly the di-Gly remnant motif. Because the purification is enriching directly at the peptide level, it is possible to identify the ubiquitylation site for many of the identified peptides. We observed that 38% of our identified proteins were also present in their list (Figure 18A), which suggests that many of our putative ubiquitylated proteins are indeed ubiquitylated. The ones that are not present in this published list could be specifically ubiquitiylated upon fasting-refeeding or they could be partners of some ubiquitylated proteins. Using the same technology, Kim and colleagues published an extensive catalogue of ubiquitylated proteins obtained in human cells (HCT116 cells and 293T cells) upon treatment with different proteasome inhibitors (Kim et al, 2011). We saw 46% of our proteins also present in their list. Together, 65% of our identified proteins were shown to be ubiquitylated by either one of these two studies. This is statistically significant ($p<0.01$) according to a hypergeometric distribution, which means that these overlaps were not random.

Collectively, these results show that we were able to purify and identify an extensive amount of proteins and our methods were very reproducible between replicates. Moreover, due to the fact that many of these proteins were shown to be ubiquitylated in other studies, we strongly believe that a large portion of the proteins we identified are indeed regulated by ubiquitylation.

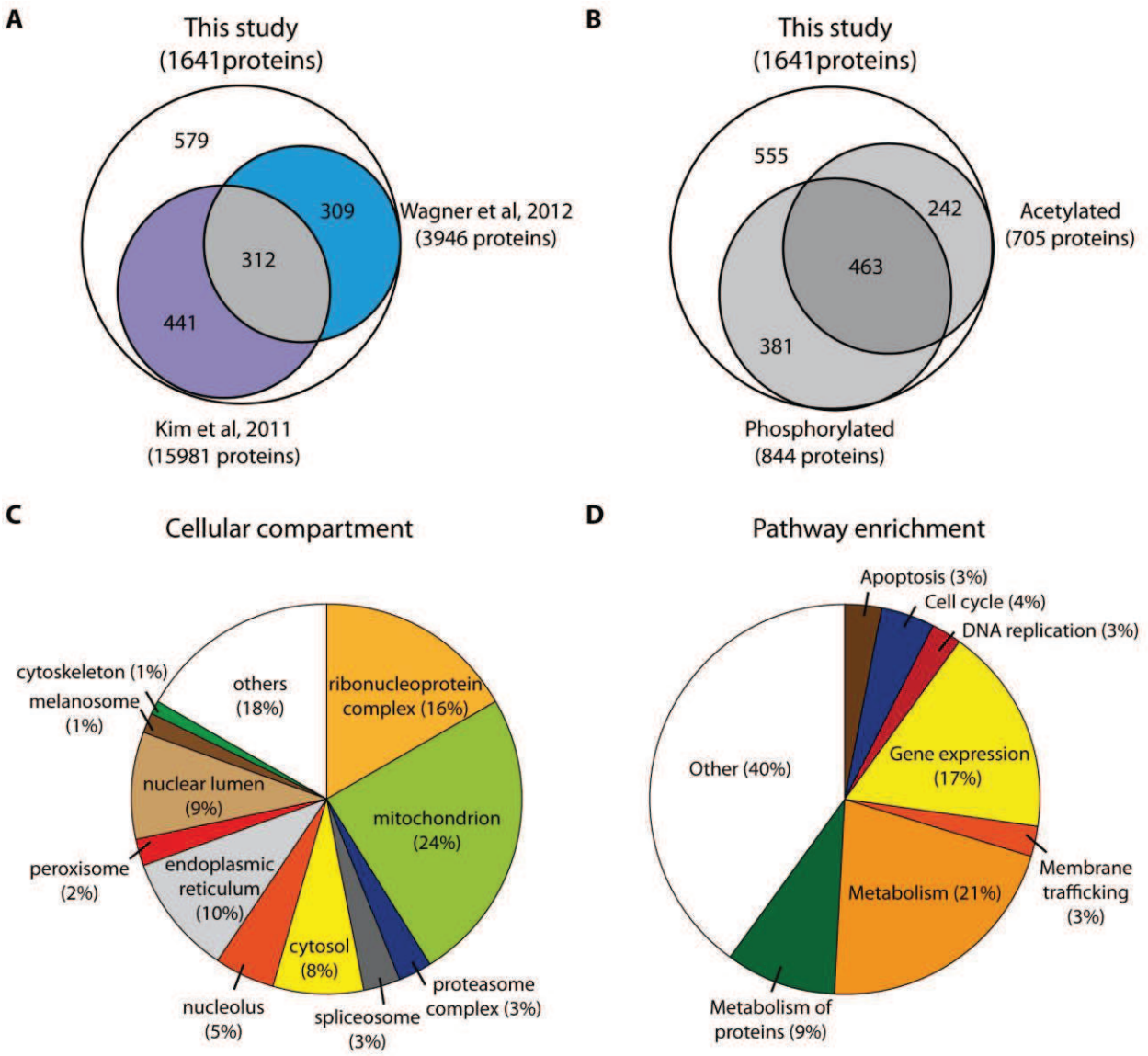


Figure 18: Proteins belonging to metabolic pathways are enriched amongst all the identified proteins

(A) Venn diagrams depict the amount of the identified proteins that were already shown to be ubiquitylated by two other studies, Kim et al (2011) and Wagner et al (2012). 65%** of the identified proteins in this screen have reported ubiquitylation sites by large-scale ubiquitylation studies.

(B) Venn diagrams represent the amount of the identified proteins that were already shown to be modified by other posttranslational modifications, phosphorylation and acetylation.

(C) A pie chart representing the amount of the identified proteins belonging to each Cellular Compartment Gene Ontology (GO) term. The percent contribution of each cellular compartment is indicated.

(D) A pie chart of the most representative pathways groups enriched within all the identified proteins. The percent contribution of each large group of pathways is indicated.
**Hypergeometric distribution with the hypothesis of overlap happening by chance (assumption mouse proteome of 40000 proteins), $p < 0.01$.

3. Functional analysis of identified proteins reveals enrichment of proteins belonging to metabolic pathways

It is known that there is a crosstalk between post-translational modifications in some proteins. For example, PEPCK needs to be acetylated first and this is the priming event for the ubiquitylation which marks it for degradation (Jiang et al, 2011). We observed that a large part of the identified proteins were already shown to be acetylated and/or phosphorylated (Figure 18B). 51% of the identified proteins were already demonstrated to be phosphorylated and 43 % of the identified proteins were already shown to be acetylated. Acetylation is particularly interesting as it is also a lysine modification.

Some of the proteins that we identified are related to the Ubiquitin Proteasome System (UPS). Out of 1641 proteins we have identified, 120 proteins are components of UPS (E1, E2, E3, DUBs, UBDs, adaptors, proteasome). It is well known that many of these proteins belonging to the UPS are themselves ubiquitylated, so this could be the reason why they were pulled down. Alternatively, these proteins could have strongly interacted with other ubiquitylated proteins during fasting or refeeding.

I proceeded to perform a gene ontology analysis to understand in which cellular compartments the proteins identified were most commonly found (Figure 18C). The enriched compartments with more proteins are the mitochondria, ribonucleoprotein complex, nucleus, and endoplasmatic reticulum. The fact that many of the identified proteins were mitochondrial was very interesting to us since the mitochondria are the main organelles responsible for the switch between fasting and refeeding and defects in mitochondria are a hallmark of fatty liver disease. This finding was also somehow surprising since not so much is known about ubiquitylation of mitochondrial proteins. It might be that these proteins are normally belonging to mitochondria, but they get ubiquitylated in the cytosol. It could also be that they get ubiquitylated before being shuttled into

the mitochondria. In addition, there are known E3 ligases that were shown to be associated to the mitochondria, so they could ubiquitylate proteins upon fusion and fission of mitochondria.

In a similar manner, I performed an analysis in Reactome with the aim of identifying the pathways in which the proteins identified were most commonly enriched (Figure 18D). The proteins identified in the screen showed a great enrichment in metabolic pathways, gene expression, membrane trafficking, cell cycle, DNA replication and apoptosis. It is well studied that ubiquitylation plays an essential role in the regulation of cell cycle and membrane trafficking, but so far it is not clear that it also plays an essential role in metabolism. The observation that metabolic pathways were also enriched amongst the identified proteins strongly suggests that ubiquitylation is playing an important role in metabolism.

4. Label-free quantification allows identification of 117 differentially detected proteins in livers of fasted and fed mice

The primary aim of our proteomic screen was to identify proteins that were ubiquitylated exclusively in the livers of fasted or refed mice. In order to identify these groups of proteins, we used label-free quantification and performed a one-way analysis of variance (ANOVA) to compare all fasted mice to all refed mice. With the *p*-value obtained from the ANOVA and the fold difference given by the quantification, we built volcano plots (Figure 19A, Figure 19B). I delimited areas by selecting proteins with a fold difference higher than 4 and *p*-values smaller than 0.05. With these parameters, we selected 26 proteins identified in fasted mice and 91 proteins identified in refed mice. There was little or no overlap between proteins identified with TUBEs and UbiQapture, which is consistent with the small overlap (22%) among all identified proteins (Figure 19C). The proteins identified only in the liver of fasted mice are listed in Table 3 and Table 4 contains the proteins identified only in the livers of refed mice. Both tables display the following information: uniprot accession number, gene name, protein name, method by which the protein was purified, fold difference between the 2 groups, *p*-value obtained with ANOVA, information whether the protein was already shown to be ubiquitylated by two other studies, and the biological function or the molecular process in which the protein is involved.

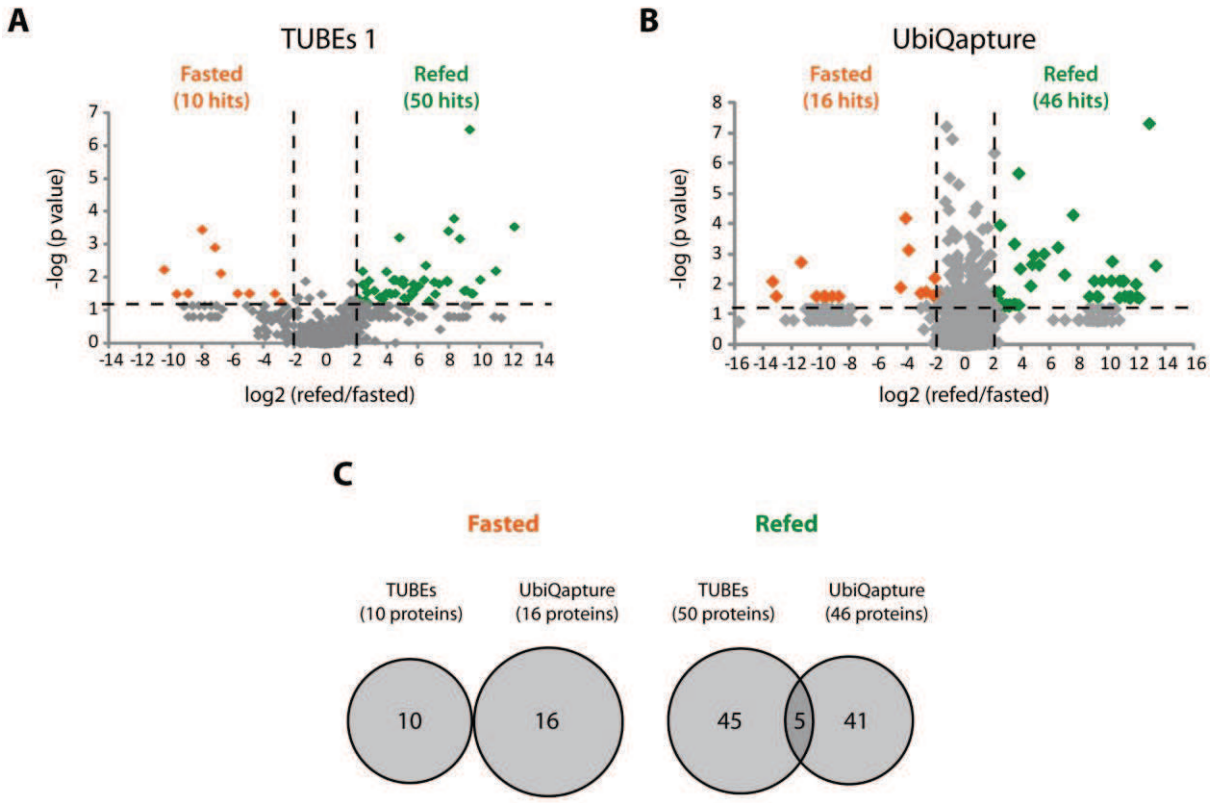


Figure 19: 117 proteins are differentially ubiquitylated in the livers of fasted and refed mice

(A,B) Volcano plots represent all of the identified proteins classified according to log2 of the difference between refed and fasted and the negative log of pvalue obtained by ANOVA. By selecting threshold of 4 times difference between fasted and refed and *p*-value < 0.05, areas with differentially identified proteins were selected for (A) TUBEs1 and (B) UbiQapture purified proteins.

(C) Venn diagrams depict the overlap between differentially identified proteins by TUBEs and UbiQapture purification.

Table 3: Proteins differentially identified in the livers of FASTED mice

Uniprot	Gene Name	Protein Name	Method	Fasted / Refed	pvalue	Wagner et al 2012	Kim et al 2011	
Q99PI5	*Lpin2*	Lipin-2	UbiQ	17,0	0,00006430	FALSE	FALSE	fa
Q9Z1Z0	*Uso1*	General vesicular transport factor p115	TUBE	249,1	0,00034507	TRUE	TRUE	v
Q91ZP3	*Lpin1*	Lipin-1	UbiQ	14,7	0,00072770	FALSE	FALSE	fa
Q8C7E7	*STBD1*	Starch-binding domain-containing protein 1	TUBE	140,4	0,00120168	TRUE	FALSE	
Q99KG3	*Rbm10*	RNA-binding motif protein 10	UbiQ	2646,7	0,00183999	FALSE	FALSE	
Q9JIL4	*PDZK1*	PDZ domain-containing protein 1	TUBE	1368,2	0,00566699	TRUE	FALSE	
Q80TR8	*Vprbp*	DDB1- and CUL4-associated factor 1	UbiQ	4,2	0,00610171	TRUE	TRUE	
Q6NXM2	*Rcbtb1*	RCC1 and BTB domain-containing protein 1	TUBE	107,5	0,00736712	TRUE	TRUE	
O70571	*PDK4*	Pyruvate dehydrogenase kinase isoform 4	UbiQ	10421,6	0,00805387	FALSE	TRUE	
P24788	*Cdc2l1*	Cell division cycle 2-like protein kinase 1	UbiQ	21,7	0,01264454	FALSE	TRUE	
Q3TC46	*Patl1*	PAT1-like protein 1	UbiQ	6,3	0,01766991	FALSE	FALSE	
Q8VC19	*Alas1*	5-aminolevulinate synthase, nonspecific, mitochondrial	UbiQ	8,2	0,01958358	FALSE	TRUE	h
Q62018	*Ctr9*	RNA polymerase-associated protein CTR9 homolog	UbiQ	4,5	0,02328407	TRUE	TRUE	
B8JJ92	*Acin1*	Apoptotic chromatin condensation inducer 1	UbiQ	595,1	0,02478380	FALSE	FALSE	
B7ZNE4	*Slc2a9*	Solute carrier family 2 (Facilitated glucose transporter), member 9	UbiQ	8787,0	0,02479080	FALSE	FALSE	
P26040	*Vil2*	Villin-2	UbiQ	1265,4	0,02483749	TRUE	FALSE	a

A2CF97	*Kif13b*	Kinesin family member 1C	UbiQ	828,6	0,02488284	FALSE	FALSE	re
Q99MB1	*Tlr3*	Toll-like receptor 3	UbiQ	426,6	0,02496248	FALSE	FALSE	
Q3UQ44	*Iqgap2*	Ras GTPase-activating-like protein IQGAP2	UbiQ	894,0	0,02497080	TRUE	TRUE	
P27786	*Cyp17*	Cytochrome P450 17A1	UbiQ	810,8	0,02599739	TRUE	FALSE	s
Q99MR8	*Mcca*	3-methylcrotonyl-CoA carboxylase 1	TUBE	468,7	0,02992495	FALSE	FALSE	
A2A8S9	*Cyp4a31*	Cytochrome P450, family 4, subfamily a, polypeptide 31	TUBE	29,5	0,03012892	FALSE	FALSE	m
Q6IS41	*Hdmcp*	Hepatocellular carcinoma down-regulated mitochondrial carrier homolog	TUBE	51,0	0,03018738	TRUE	FALSE	
P18581	*Slc7a2*	Low affinity cationic amino acid transporter 2	TUBE	30,2	0,03020211	FALSE	TRUE	t
Q8CC88	*1300010F03Rik*	Uncharacterized protein KIAA0564 homolog	TUBE	9,5	0,03045168	TRUE	FALSE	p
P21107	*Tpm3*	Tropomyosin alpha-3 chain	TUBE	778,4	0,03096125	TRUE	TRUE	c

Table 4: Proteins differentially identified in the livers of REFED mice

Uniprot	*Gene Name*	Protein Name	Method	Refed / Fasted	pvalue	Wagner et al 2012	Kim et al 2011	
Q8C163	*Endogl1*	Endonuclease G-like 1	UbiQ	7598,2	0,00000005	FALSE	FALSE	
P01027	*C3*	C3a anaphylatoxin	TUBE & UbiQ	652,8	0,00000031	TRUE	FALSE	
O70349	*SKI*	Putative uncharacterized protein SKI	UbiQ	4,3	0,00000045	FALSE	TRUE	
Q8BSE0	*Fam82a*	Regulator of microtubule dynamics protein 2	UbiQ	13,8	0,00000210	TRUE	FALSE	
Q8VCH6	*Dhcr24*	24-dehydrocholesterol reductase	UbiQ	5,7	0,00010912	FALSE	TRUE	
Q58ET4	*Osgin1*	Oxidative stress induced growth inhibitor 1	TUBE	322,6	0,00015865	FALSE	TRUE	
Q8C845	*Efhd2*	EF hand domain containing 2	TUBE	4818,5	0,00028049	FALSE	FALSE	
B1AWD9	*Clta*	Clathrin light polypeptide (Lca)	TUBE	251,9	0,00038126	TRUE	FALSE	in
P21981	*Tgm2*	Protein-glutamine gamma-glutamyltransferase 2	UbiQ	11,2	0,00045475	TRUE	FALSE	
Q920L1	*Fads1*	Fatty acid desaturase 1	TUBE	27,7	0,00060026	TRUE	TRUE	
P50172	*Hsd11b1*	11-beta-hydroxysteroid dehydrogenase 1	TUBE	420,9	0,00064841	TRUE	FALSE	
Q5SXR6	*Cltc*	Clathrin, heavy polypeptide (Hc)	UbiQ	46,8	0,00098125	FALSE	TRUE	in
B9EHT6	*Fn1*	Fibronectin	UbiQ	28,7	0,00108880	FALSE	FALSE	
Q9DBT9	*Dmgdh*	Dimethylglycine dehydrogenase, mitochondrial	UbiQ	1261,4	0,00174762	FALSE	FALSE	
P13516	*Scd1*	Acyl-CoA desaturase 1	TUBE & UbiQ	26,6	0,00219717	TRUE	FALSE	
Q9DCC8	*Tomm20*	Mitochondrial 20 kDa outer membrane protein	UbiQ	37,4	0,00228203	FALSE	TRUE	

Q3UW06	*C4bp*	C4b-binding protein alpha-chain	UbiQ	15,0	0,00303572	FALSE	FALSE	
Q5EBG8	*AU022252*	Uncharacterized protein C1orf50 homolog	TUBE	91,1	0,00423502	FALSE	FALSE	
Q8VC97	*Upb1*	Beta-alanine synthase	TUBE	2101,0	0,00619410	FALSE	FALSE	
P84078	*Arf1*	ADP-ribosylation factor 1	TUBE	5,4	0,00636091	FALSE	TRUE	
Q9D7M1	*Gid8*	Glucose-induced degradation protein 8 homolog	TUBE	15,6	0,00653903	FALSE	FALSE	
Q9QYJ3	*Dnajb1*	DnaJ homolog subfamily B member 1	UbiQ	772,5	0,00768675	FALSE	TRUE	
Q6Q899	*Ddx58*	DEAD box protein 58	UbiQ	1810,0	0,00768995	TRUE	TRUE	
Q8BKZ9	*PDHX*	Pyruvate dehydrogenase complex, component X	UbiQ	1210,5	0,00769173	FALSE	FALSE	
Q3UYZ5	*Ttc37*	Putative uncharacterized protein	UbiQ	2227,4	0,00770846	FALSE	TRUE	
P62334	*Psmc6*	26S protease regulatory subunit 10B	UbiQ	2090,9	0,00771224	TRUE	TRUE	
Q811J3	*Irp2*	Iron regulatory protein 2	UbiQ	529,9	0,00774854	TRUE	FALSE	
Q3TGQ4	*Secisbp2*	SECIS binding protein 2	UbiQ	24,8	0,01130512	FALSE	FALSE	
A3KGU5	*Sptan1*	Spectrin alpha chain, non-erythrocytic 1	TUBE & UbiQ	1047,6	0,01152780	FALSE	FALSE	
P60766	*Cdc42*	Cell division control protein 42 homolog	TUBE	22,3	0,01156413	TRUE	TRUE	
Q5M8Q6	*Cyp2d40*	Cytochrome P450, family 2, subfamily d, polypeptide 40	TUBE	84,0	0,01160932	TRUE	FALSE	
Q9ESP1	*Sdf2l1*	Stromal cell-derived factor 2-like protein 1	TUBE	21,2	0,01185180	FALSE	FALSE	
P70280	*Sybl1*	Synaptobrevin-like protein 1	TUBE	23,9	0,01187692	TRUE	FALSE	
P01898	*H2-Q10*	H-2 class I histocompatibility antigen, Q10 alpha chain	TUBE	30,5	0,01194287	FALSE	FALSE	
B2RT54	*Apol7a*	Apolipoprotein L 7a	TUBE	33,4	0,01197958	TRUE	FALSE	
Q9WV85	*Nme3*	Nucleoside diphosphate kinase 3	TUBE	31,8	0,01219858	TRUE	FALSE	

Q01237	*Hmgcr*	3-hydroxy-3-methylglutaryl-coenzyme A reductase	TUBE	236,3	0,01220377	FALSE	TRUE	
Q68FL6	*Mars*	Methionyl-tRNA synthetase	TUBE	7,1	0,01227007	FALSE	TRUE	
Q8R2L5	*Mrps18c*	28S ribosomal protein S18-1, mitochondrial	TUBE	31,8	0,01250919	FALSE	FALSE	
P08032	*Spna1*	Spectrin alpha chain, erythrocyte	TUBE	241,2	0,01333829	TRUE	FALSE	
P15508	*Spnb1*	Spectrin beta chain, erythrocyte	TUBE	132,9	0,01360930	FALSE	FALSE	
Q62264	*Thrsp*	Thyroid hormone-inducible hepatic protein	TUBE	30,5	0,01460107	FALSE	FALSE	
Q9WTI7	*Myo1c*	Myosin I beta	TUBE	165,0	0,01538782	TRUE	TRUE	
Q99JY9	*Actr3*	Actin-related protein 3	TUBE	52,3	0,01557441	FALSE	TRUE	
Q63880	*Es31*	Liver carboxylesterase 31	TUBE	5,3	0,01598127	TRUE	FALSE	
Q9CVB6	*Arpc2*	Actin-related protein 2/3 complex subunit 2	TUBE	33,5	0,01622488	FALSE	TRUE	
P58468	*1810008A 18Rik*	Uncharacterized protein C21orf70 homolog	UbiQ	4,2	0,01695773	FALSE	FALSE	
Q3UH74	*Apob*	Apolipoprotein B	TUBE	5,8	0,01788304	FALSE	TRUE	
P25688	*Uox*	Urate oxidase	TUBE & UbiQ	66,9	0,01792611	TRUE	FALSE	
Q5CZY4	*Nfix*	Nuclear factor 1 X-type	UbiQ	4,8	0,01917201	FALSE	FALSE	
P47754	*Capza2*	F-actin-capping protein subunit alpha-2	TUBE & UbiQ	60,4	0,02028582	TRUE	TRUE	
Q9ERG0	*Lima1*	LIM domain and actin-binding protein 1	TUBE	561,5	0,02427075	FALSE	TRUE	
Q91W67	*Ubl7*	Ubiquitin-like protein 7	TUBE	9,0	0,02457057	FALSE	TRUE	
P24369	*Ppib*	Peptidyl-prolyl cis-trans isomerase B	UbiQ	3101,7	0,02478865	TRUE	TRUE	
O70451	*Slc16a7*	Solute carrier family 16 member 7	UbiQ	2329,7	0,02492764	FALSE	TRUE	o

P16546	*Spna2*	Spectrin alpha 2	TUBE	511,1	0,02501875	FALSE	FALSE	
Q9Z321	*Top3b*	DNA topoisomerase 3-beta-1	UbiQ	603,0	0,02502901	FALSE	TRUE	
P23475	*Ku70*	ATP-dependent DNA helicase 2 subunit 1	UbiQ	421,1	0,02543661	FALSE	FALSE	
Q9JHJ0	*Tmod3*	Tropomodulin-3	TUBE	48,4	0,02546667	FALSE	TRUE	c
Q8BGY7	*Fam210a*	Uncharacterized protein C18orf19 homolog	UbiQ	650,1	0,02622491	FALSE	FALSE	
Q3TFC7	*Aldh7a1*	Aldehyde dehydrogenase family 7 member A1	TUBE	6,6	0,02794977	FALSE	FALSE	
Q6ZWQ9	*2900073 G15Rik*	Myosin regulatory light chain 12B	UbiQ	4618,5	0,02836994	TRUE	FALSE	
P20152	*Vim*	Vimentin	UbiQ	3003,6	0,02845936	TRUE	TRUE	
P11679	*Krt8*	Cytokeratin-8	UbiQ	4,9	0,02913849	TRUE	TRUE	
Q8R404	*Qil1*	Protein QIL1	TUBE	17,7	0,02948566	TRUE	FALSE	
Q9D1G1	*Rab1b*	Ras-related protein Rab-1B	TUBE	701,8	0,02984796	FALSE	TRUE	
Q99PG0	*Aadac*	Arylacetamide deacetylase	TUBE	15,3	0,02985934	TRUE	FALSE	
P17182	*ENO1*	Enolase 1	TUBE	23,2	0,02990757	TRUE	TRUE	g
Q9EQ32	*Bcap*	B-cell adapter for phosphoinositide 3-kinase	TUBE	750,8	0,03045409	TRUE	FALSE	
P01592	*Igj*	Immunoglobulin J chain	TUBE	51,4	0,03064026	FALSE	TRUE	h
Q99JY0	*Hadhb*	3-ketoacyl-CoA thiolase	TUBE	19,3	0,03144139	TRUE	FALSE	
Q91YP3	*Dera*	Phosphodeoxyriboaldolase	TUBE	11,5	0,03935364	TRUE	TRUE	P
Q91WT7	*Akr1c14*	Aldo-keto reductase family 1, member C14	TUBE	4,2	0,03990403	TRUE	FALSE	m
P07901	*Hsp86*	Heat shock protein HSP 90-alpha	TUBE	34,7	0,04101212	TRUE	FALSE	re
Q9Z1D1	*Eif3g*	Eukaryotic translation initiation factor 3 RNA-binding subunit	TUBE	41,3	0,04288126	FALSE	TRUE	
Q3UNY8	*Ghr*	Growth hormone receptor	TUBE	13,3	0,04348793	FALSE	FALSE	c
A2AKG8	*Kiaa1797*	Uncharacterized protein KIAA1797	UbiQ	11,0	0,04469444	TRUE	TRUE	

Q9CQ69	*Uqcrq*	Complex III subunit 8	UbiQ	12,1	0,04715072	TRUE	FALSE	
Q91VH2	*Snx9*	Sorting nexin-9	UbiQ	9,0	0,04738346	FALSE	TRUE	in
Q9D938	*Tmem160*	Transmembrane protein 160	TUBE	6,0	0,04805200	FALSE	FALSE	
P59999	*Arc20*	Actin-related protein 2/3 complex subunit 4	UbiQ	13,9	0,04846255	TRUE	FALSE	
Q7TMY8	*Huwe1*	E3 ubiquitin-protein ligase HUWE1	UbiQ	7,5	0,04860644	FALSE	TRUE	
P39054	*Dnm2*	Dynamin-2	UbiQ	7,4	0,04867494	FALSE	TRUE	
Q8BHN1	*Rbbp7*	Gamma-taxilin	UbiQ	7,4	0,04890478	FALSE	TRUE	
Q9CYV5	*Tmem135*	Transmembrane protein 135	UbiQ	8,0	0,04925651	FALSE	TRUE	
Q91Y97	*Aldob*	Fructose-bisphosphate aldolase B	UbiQ	7,1	0,05006854	FALSE	FALSE	f

Amongst the differentially ubiquitylated proteins, there were many mitochondrial proteins in both, lysates of fasted and refed mice (Figure 20A). An analysis in Reactome showed that metabolic pathways were also enriched in the differentially identified proteins (Figure 20B). For instance, in the livers of fasted mice we identified lipin 1 and lipin 2 as putative targets of ubiquitylation (Table 3). Comparatively, we identified enolase 1, fatty acid desaturase 1 and HMG-CoA reductase as putative targets of protein ubiquitylation upon refeeding. We believe that fasting-refeeding leads to a change in the ubiquitylation levels of some proteins which are involved in different metabolic pathways. Proteins belonging to the metabolism of lipids and lipoproteins were enriched in fasted conditions. On the other hand, proteins involved in cholesterol and carbohydrate metabolism were enriched in refed conditions. In addition, membrane trafficking proteins, such as ARF-1 and cytoskeleton-related proteins were enriched amongst the proteins identified upon refeeding. This is a very interesting observation since hepatocytes use the protein trafficking machinery to secrete VLDL, which is increased in high nutrient conditions. A more detailed view of all the pathways enriched amongst all proteins detected, fasted and refed hits is depicted in Figure 21.

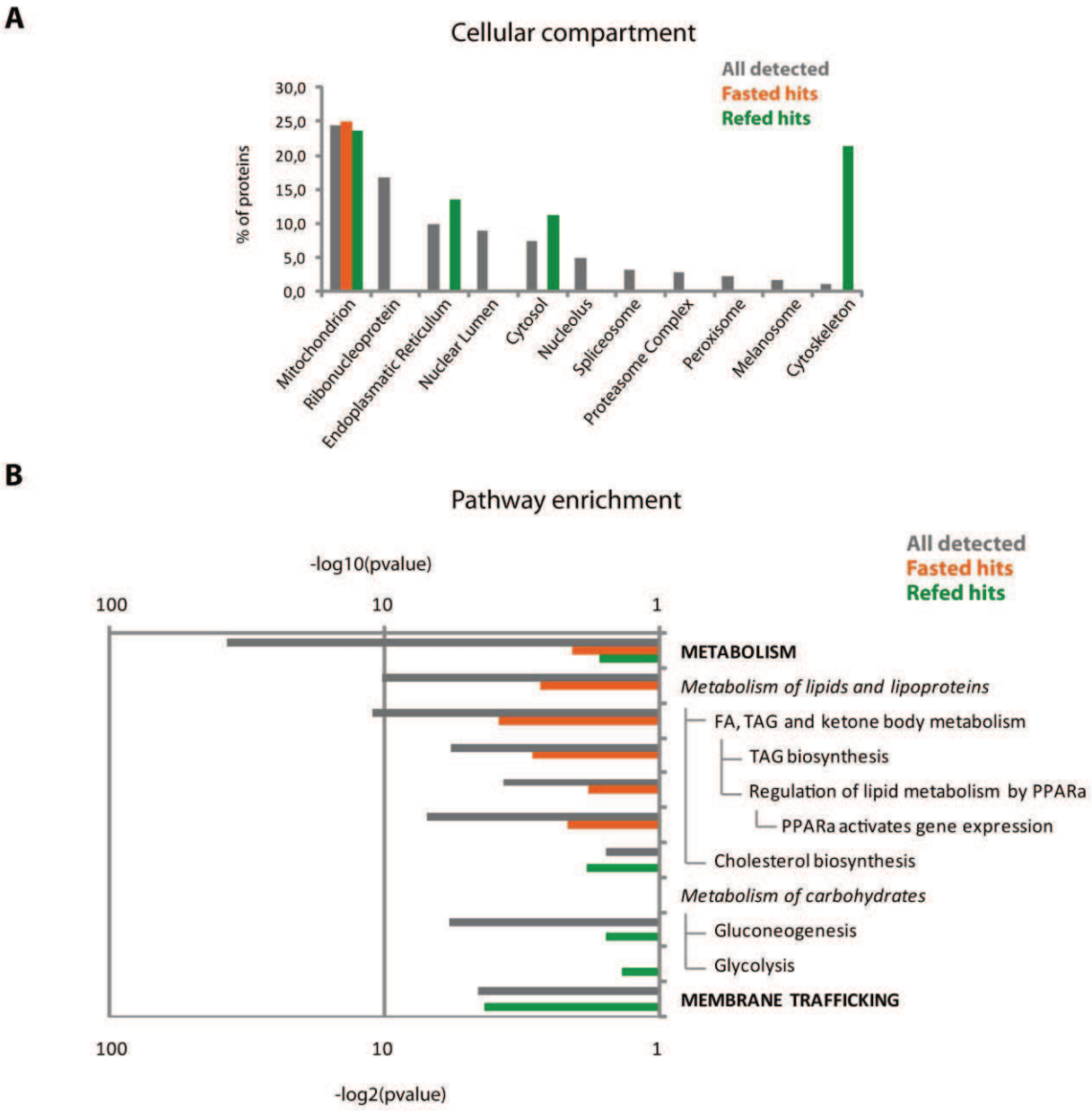


Figure 20: Metabolic pathways are enriched amongst differentially identified proteins

(A) Bar chart representing the percentage of proteins in the group belong to each Cellular Compartment Gene Ontology (GO). Depicted are all identified proteins, differentially identified proteins in the livers of fasted (orange) and refed (green) mice.

(B) Bar chart depicting the significance (pvalue) for each pathway enriched in all the proteins, compared to the differentially identified proteins in the livers of fasted (orange) and refed (green) mice. Only a selected group of pathways is shown. –log10 of *p*-value is used to all detected proteins and –log2 of *p*-value is used to fasted and refed hits.

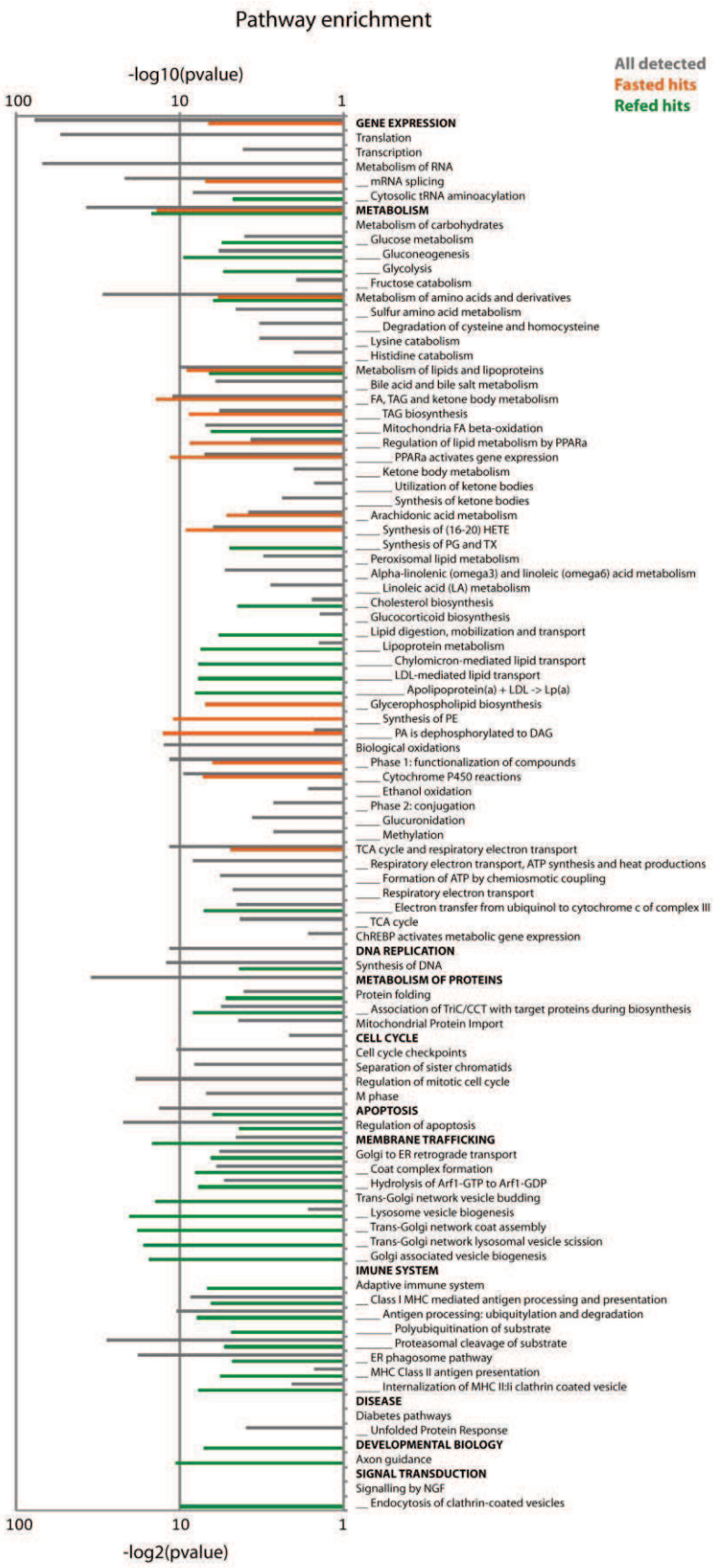

Pathway enrichment
All detected
Fasted hits
Refed hits
-log10(pvalue)
100
10
1
GENE EXPRESSION
Translation
Transcription
Metabolism of RNA
__ mRNA splicing
__ Cytosolic tRNA aminoacylation
METABOLISM
Metabolism of carbohydrates
__ Glucose metabolism
____ Gluconeogenesis
____ Glycolysis
__ Fructose catabolism
Metabolism of amino acids and derivatives
__ Sulfur amino acid metabolism
____ Degradation of cysteine and homocysteine
__ Lysine catabolism
__ Histidine catabolism
Metabolism of lipids and lipoproteins
__ Bile acid and bile salt metabolism
__ FA, TAG and ketone body metabolism
____ TAG biosynthesis
____ Mitochondria FA beta-oxidation
____ Regulation of lipid metabolism by PPARa
_____ PPARa activates gene expression
____ Ketone body metabolism
_____ Utilization of ketone bodies
_____ Synthesis of ketone bodies
__ Arachidonic acid metabolism
____ Synthesis of (16-20) HETE
____ Synthesis of PG and TX
__ Peroxisomal lipid metabolism
__ Alpha-linolenic (omega3) and linoleic (omega6) acid metabolism
____ Linoleic acid (LA) metabolism
__ Cholesterol biosynthesis
__ Glucocorticoid biosynthesis
__ Lipid digestion, mobilization and transport
____ Lipoprotein metabolism
_____ Chylomicron-mediated lipid transport
_____ LDL-mediated lipid transport
_____ Apolipoprotein(a) + LDL -> Lp(a)
__ Glycerophospholipid biosynthesis
____ Synthesis of PE
_____ PA is dephosphorylated to DAG
Biological oxidations
__ Phase 1: functionalization of compounds
____ Cytochrome P450 reactions
____ Ethanol oxidation
__ Phase 2: conjugation
____ Glucuronidation
____ Methylation
TCA cycle and respiratory electron transport
__ Respiratory electron transport, ATP synthesis and heat productions
____ Formation of ATP by chemiosmotic coupling
____ Respiratory electron transport
_____ Electron transfer from ubiquinol to cytochrome c of complex III
__ TCA cycle
ChREBP activates metabolic gene expression
DNA REPLICATION
Synthesis of DNA
METABOLISM OF PROTEINS
Protein folding
__ Association of TriC/CCT with target proteins during biosynthesis
Mitochondrial Protein Import
CELL CYCLE
Cell cycle checkpoints
Separation of sister chromatids
Regulation of mitotic cell cycle
M phase
APOPTOSIS
Regulation of apoptosis
MEMBRANE TRAFFICKING
Golgi to ER retrograde transport
__ Coat complex formation
__ Hydrolysis of Arf1-GTP to Arf1-GDP
Trans-Golgi network vesicle budding
__ Lysosome vesicle biogenesis
__ Trans-Golgi network coat assembly
__ Trans-Golgi network lysosomal vesicle scission
__ Golgi associated vesicle biogenesis
IMUNE SYSTEM
Adaptive immune system
__ Class I MHC mediated antigen processing and presentation
____ Antigen processing: ubiquitylation and degradation
_____ Polyubiquitination of substrate
_____ Proteasomal cleavage of substrate
__ ER phagosome pathway
__ MHC Class II antigen presentation
____ Internalization of MHC II:Ii clathrin coated vesicle
DISEASE
Diabetes pathways
__ Unfolded Protein Response
DEVELOPMENTAL BIOLOGY
Axon guidance
SIGNAL TRANSDUCTION
Signalling by NGF
__ Endocytosis of clathrin-coated vesicles
100
10
1
-log2(pvalue)

Figure 21: Pathways related to metabolism, membrane trafficking, cell cycle and immune system are enriched amongst all identified proteins

Bar chart with a detailed view of indicated pathways enriched amongst detected proteins, with the respective significance levels. –log10 of *p*-value is given to all detected proteins and –log2 of *p*-value is given to differentially identified proteins in fasted (orange) and refed (green) mice.

5. Post-translational modifications in the mitochondria are associated with mitochondrial function

The observed high number of mitochondrial proteins identified in the screen prompted us to investigate the levels of ubiquitylation in the mitochondria purified from livers of ad libitum, fasted and refed mice (Figure 22A). To this end, mitochondria were isolated from freshly dissected livers by using a differential centrifugation method (Pallotti and Lenaz, 2007). In this method, purified mitochondria are intact and functional, while most of the cytosol and organelles are excluded. However, depending on the preparation, the mitochondrial fractions can be contaminated with low levels of cytoplasmic components (e.g. tubulin).

We observed that the total levels of ubiquitylation in isolated mitochondrial fractions do not change upon fasting-refeeding. This observation is consistent with the mass spectrometry data, where mitochondria are the cellular compartment enriched in both, fasted- and refed-specific identified proteins. However, the acetylation levels of a few proteins were slightly increased in the mitochondria from fasted mice, in contrary to what was shown by Hirschey et al (2010) (Figure 22B).

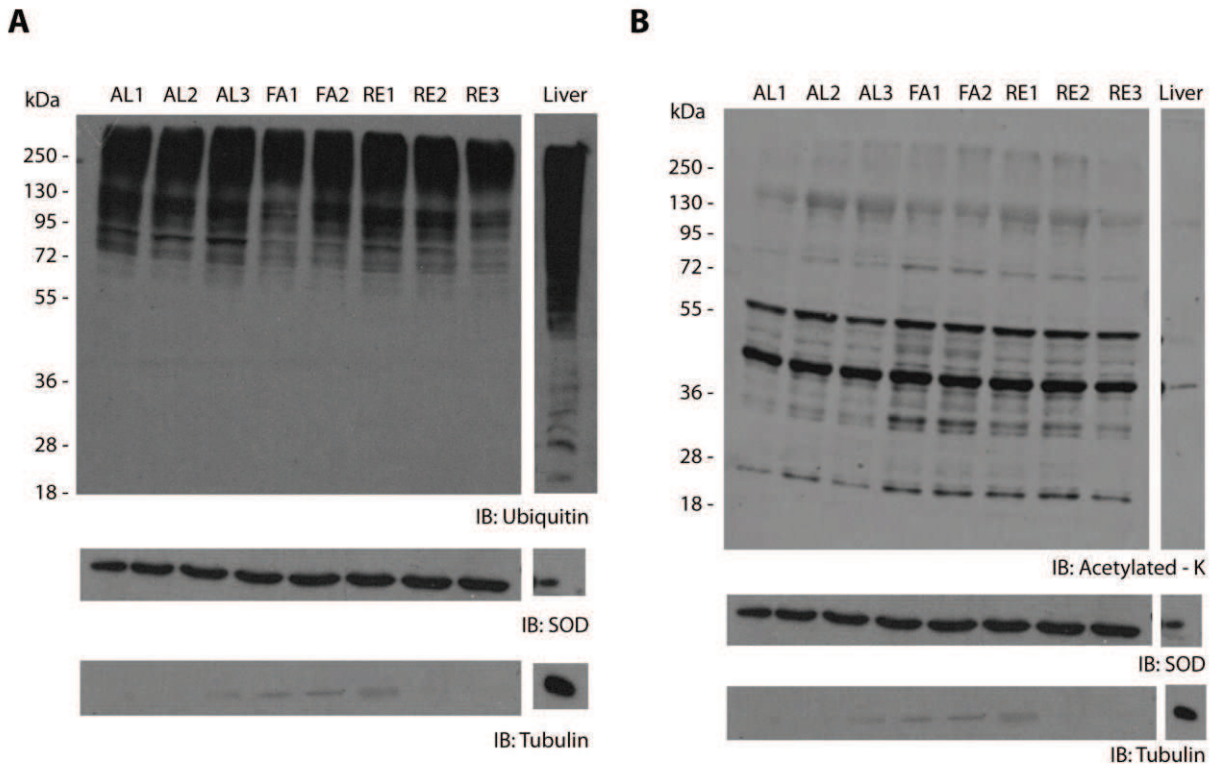


Figure 22: Ubiquitylation levels in the mitochondria do not vary upon fasting/refeeding
(A,B) Western blots of mitochondrial fractions isolated from the livers of 3 ad libitum mice, 2 16-hours fasted mice and 3 24-hours refed. Antibodies used were against: ubiquitin (A), to detect ubiquitylated proteins; acetylated-K (B), to detect acetylated proteins; superoxide dismutase (SOD), as loading control for mitochondria; tubulin, as a purity control for mitochondrial fractions.
AL, ad-libitum; FA, fasted; RE, refed.

Hypothesizing that ubiquitylation is regulating mitochondrial pathways upon fasting and refeeding, and thus affecting mitochondrial function, we decided to make use of a model in which mitochondria are dysfunctional. Frataxin (FXN) is a mitochondrial protein involved in the assembly of iron-sulfur clusters (Pastore and Puccio, 2013). The disruption of frataxin gene was shown to cause severe mitochondrial dysfunction. At 2 weeks of age liver-specific FXN knock-out (KO) mice do not show impaired iron-metabolism or mitochondria function, while at 4 weeks of age, mice exhibit impaired iron-metabolism, and damaged and non-functional mitochondria (Martelli et al, 2012).

As these mice exhibit different levels of mitochondrial dysfunction, we decided to look at ubiquitylation and acetylation in purified mitochondria from livers of 2 and 4 weeks old mice (Figure 23A, Figure 23B). When compared to wild-type (WT) controls, mitochondria from FXN KO mice showed reduced ubiquitylation at both ages, with a more dramatic reduction in the ubiquitylation levels at 4 weeks of age. Acetylation was also affected in mitochondria isolated from 4 weeks old mice; it was increased when compared to the WT control from the same age.

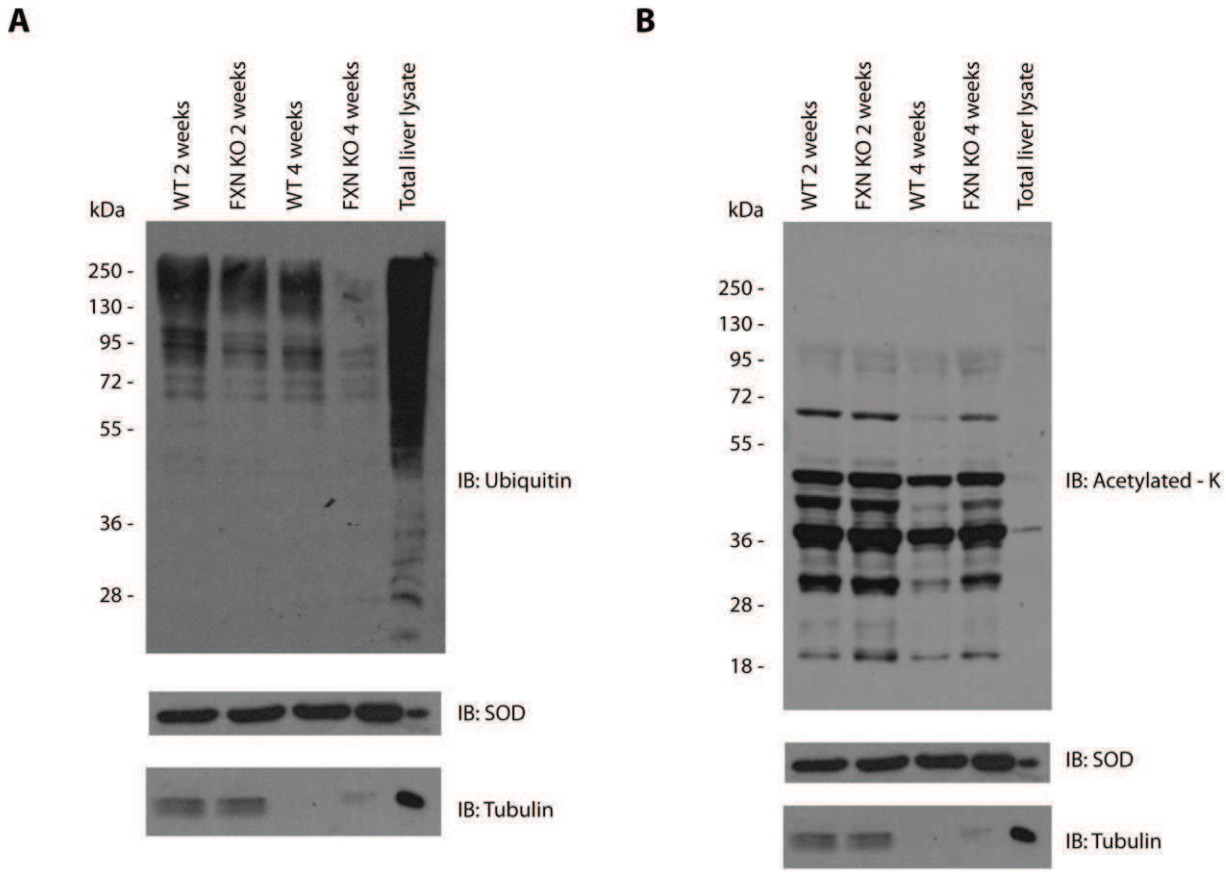


Figure 23: Ubiquitylation levels in the mitochondria of frataxin KO mice are dramatically reduced

(A,B) Western blots of mitochondrial fractions isolated from the livers of wild-type (WT) or frataxin knockout (FXN KO) mice at 2 and 4 weeks of age. Antibodies used were against: ubiquitin (A), to detect ubiquitylated proteins; acetylated-K (B), to detect acetylated proteins; superoxide dismutase (SOD), as loading control for mitochondria; tubulin, as a purity control for mitochondrial fractions.

These data suggests that essential functional pathways in mitochondria might be regulated by ubiquitylation. With the present data is not possible to decipher whether alteration in the ubiquitylation levels are a cause or a consequence of loss of function and more experiments need to be designed to address this question.

6. A biochemical validation in HEK293T cells and primary hepatocytes confirms ubiquitylation of proteins identified in proteomic screen

With the aim of validating the proteomic screen, we selected 8 proteins, out of the 117 differentially identified proteins, to undergo a biochemical confirmation of their ubiquitylation. These 8 proteins (Figure 24) – 4 from fasted and 4 from refed mice – were selected based on their described cellular function and on the significance of the difference between livers of fasted and refed mice.

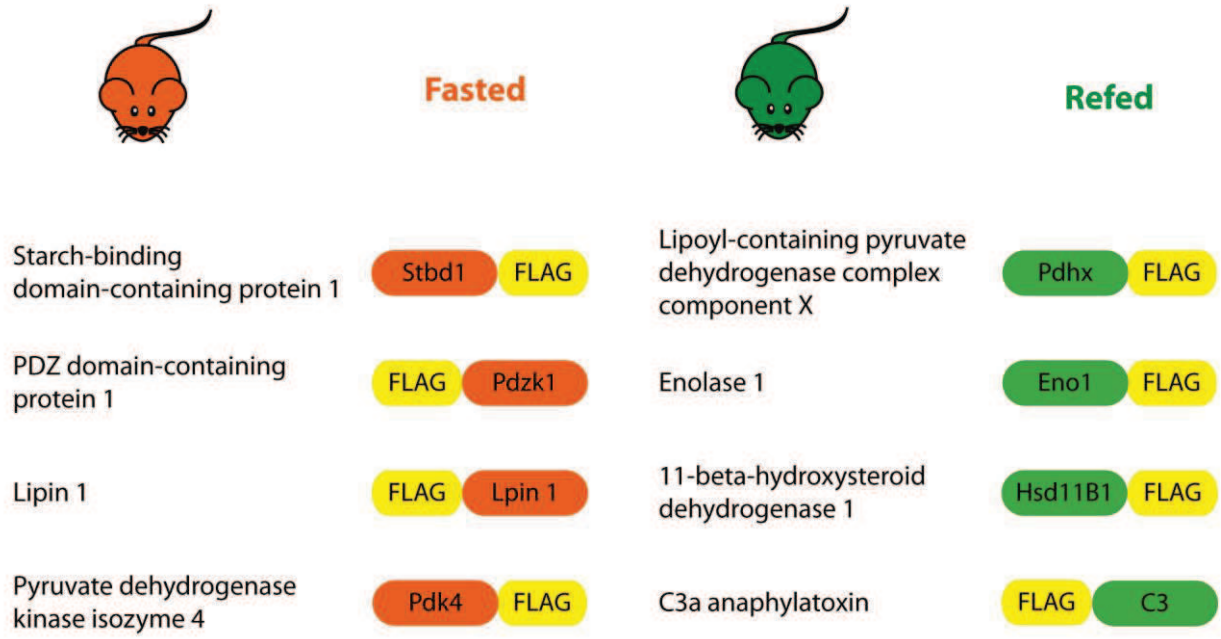


Figure 24: Selected proteins are tagged with FLAG for biochemical validation of proteomic screen

Illustration of the 8 proteins from the group of differentially identified proteins, selected for biochemical validation. The 4 proteins selected from fasted mice are depicted in orange, and

the 4 from refed mice, in green. FLAG tag was added to either N- or C-terminus depending on the domains present in the protein.

The biochemical validation was based on immunoprecipitation (IP) and pulldown assays. I subcloned cDNAs encoding for the human isoform of the 8 selected proteins with a FLAG tag, which allows for efficient IPs and simple detection (Figure 24). Each of these 8 proteins was expressed in HEK293T cells and in primary hepatocytes together with a tagged ubiquitin. The biochemical validation was then comprised of FLAG IPs and ubiquitin pulldowns. FLAG IPs enriched the protein of interest and I analyzed their co-immunoprecipitation with ubiquitin. It is important to note that, since FLAG IPs were performed under native conditions, protein complexes could also be immunoprecipitated, which could complicate the interpretation of these assays.

The complementary ubiquitin pulldowns allowed a two-step purification of ubiquitylated proteins under fully denaturing conditions. To this end, I used an ubiquitin construct tagged with 6xHis and a signal for biotinylation *in vivo* (Figure 25A). These 2 tags allowed tandem purification with Nickel and streptavidin beads (Figure 25B), with the eluate fraction from nickel purification being directly used for streptavidin purification. Using this approach, I observed a very remarkable enrichment of ubiquitylated proteins. The control pulldowns were performed using only the tag (6xHis-Bio), and I did not observe any unspecific binding of ubiquitylated proteins (Figure 25C). Differently from FLAG IPs, ubiquitin pulldowns are performed under fully denaturing conditions, implying that only proteins that are covalently attached to ubiquitin will remain bound to ubiquitin and be eluted.

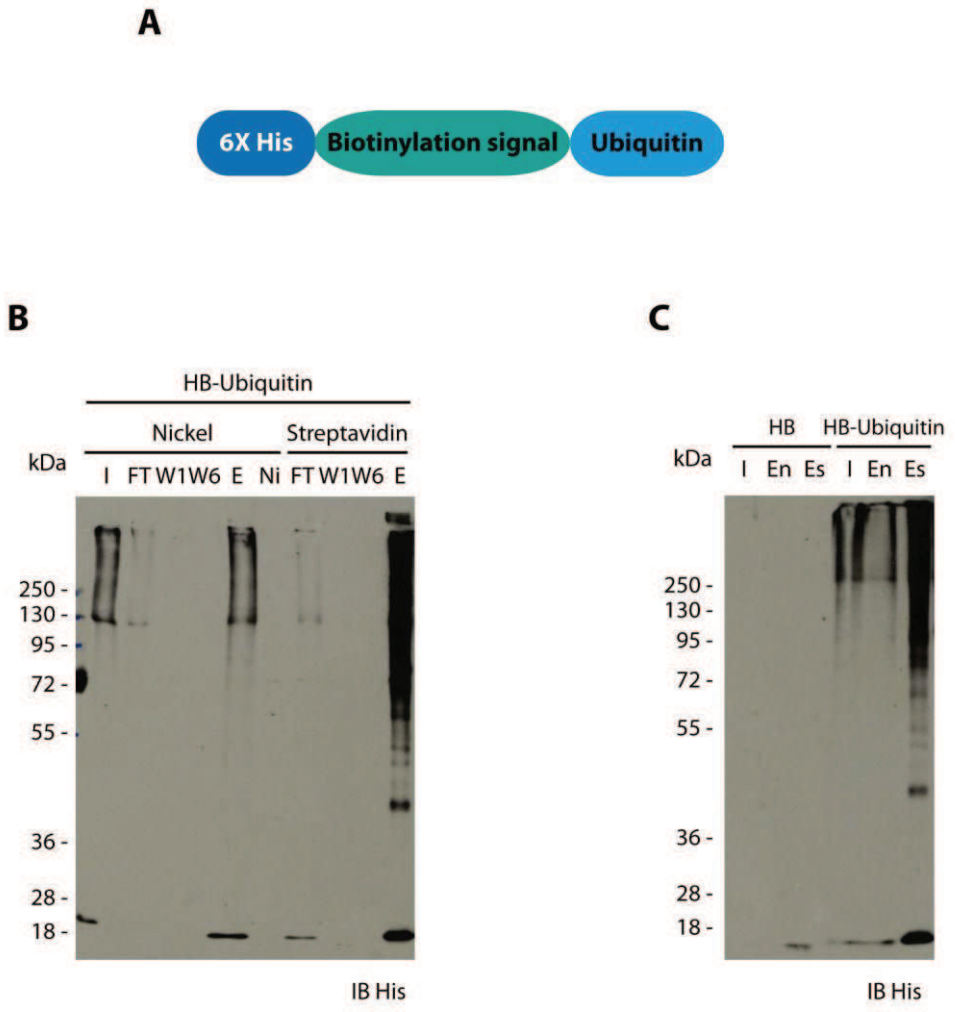


Figure 25: Denaturing ubiquitin pulldowns can efficiently enrich ubiquitylated proteins

(A) Illustration of the construct used to overexpress ubiquitin in cells with two tags: His tag and a biotinylation signal *in vivo*.

(B) Western blot after nickel and streptavidin pulldowns from HEK293T whole-cell lysates. Antibody against the His tag was used to detect ubiquitylated proteins.

(C) Western blot after nickel and streptavidin pulldowns from HEK293T whole-cell lysates. The first pulldown shown is is from cells expressing only the tag His-Biotin, while the second one used His-Biotin-Ubiquitin expressing cells. Antibody against the His tag was used to detect ubiquitylated proteins.

I, input; FT, flow through; W1, first wash after beads incubation; W6 last wash before elution; E, elution; Ni; Nickel beads after elution with imidazole.

Initially we chose to use HEK293T cells as they represent a cellular model easy to manipulate. In addition, we decided to include pulldowns performed in primary hepatocytes that better represent the liver physiology. In fact, I co-transfected these cells to express tagged ubiquitin and one of the 8 proteins of interest. I subsequently treated cells with DMSO, MG132 and PR619 (DUB inhibitor). When proteins are targeted for degradation, treatment with MG132 will increase the ubiquitylated form of this protein. Treatment with PR619 increases ubiquitylation of proteins in general, but usually if proteins were targeted to degradation, treatment with PR619 will also affect its degradation. Finally, the size of the bands can indicate whether the protein is monoubiquitylated or there is more than one ubiquitin linked to the protein.

6.1. Starch-binding domain-containing protein 1 (STBD1)

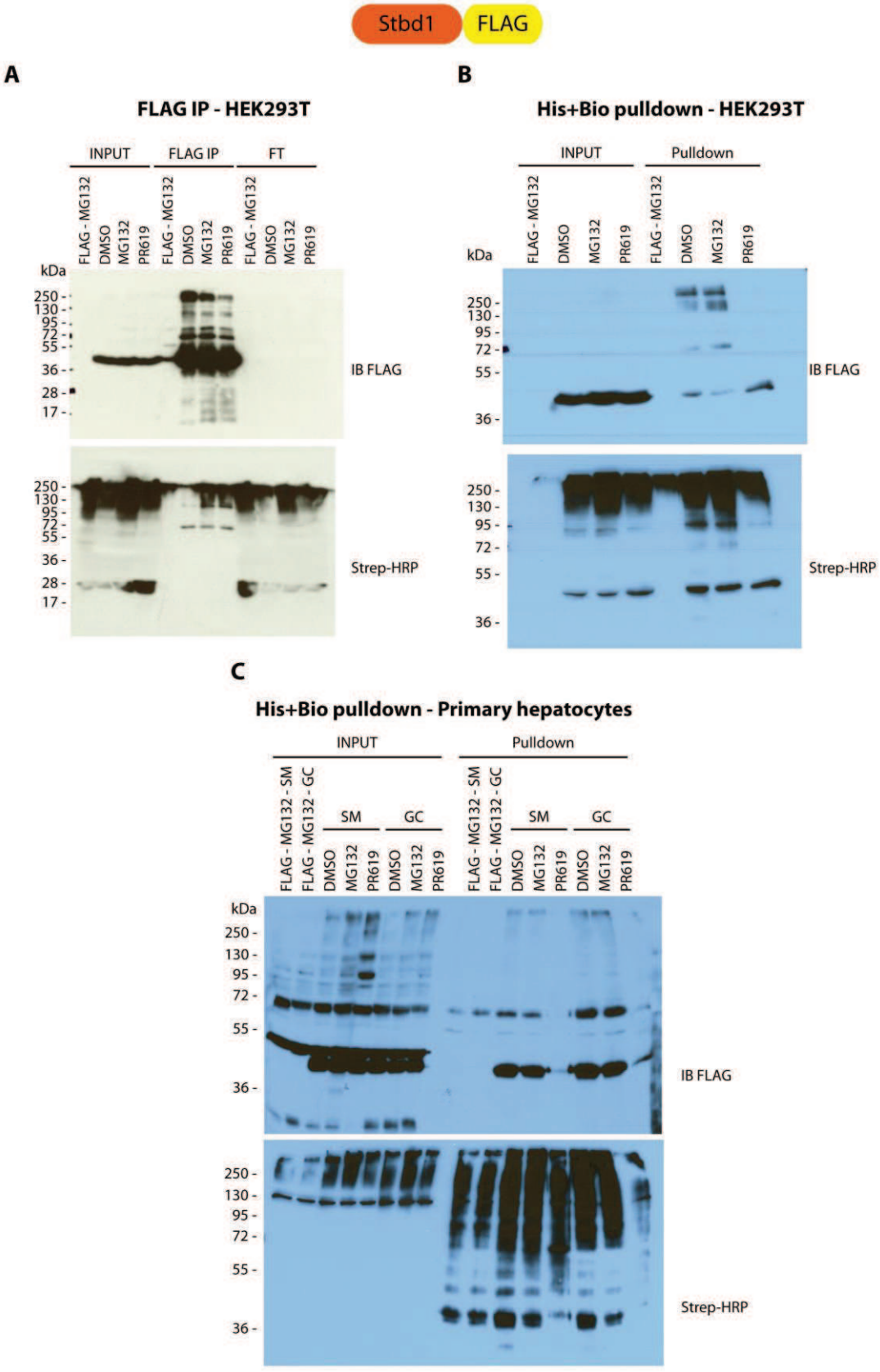
Starch-binding domain-containing protein 1 (STBD1 – 39kDa) was found as a putative target of ubiquitylation in fasted conditions, using TUBEs as purification method (Table 3). In addition, Wagner et al (2012) reported 7 ubiquitylation sites in STBD1 when analyzed murine liver proteins. STBD1 is involved in glycogen metabolism; it can bind glycogen via its carbohydrate binding domain. It has been reported that when STBD1 is bound to glycogen it can anchor it to membranes, thereby affecting its cellular localization (Jiang et al, 2010). Moreover, STBD1 contains an AIM (Atg8 interacting motif), which can interact with the autophagy machinery and target glycogen to lysosomal degradation (Jiang et al, 2011). Since STBD1 was identified in fasted mice, we hypothesized that ubiquitylation could affect its binding to glycogen, or it could increase targeting of glycogen to lysosomes.

With the aim of confirming STBD1 ubiquitylation I performed FLAG IPs and ubiquitin pulldowns. FLAG IPs from HEK293T cell lysates showed that the protein was ubiquitylated in all the conditions tested (Figure 26A). Interestingly, the ubiquitylation was increased when MG132 or PR619 was used, indicating that STBD1 might be ubiquitylated in different ways, leading to different cellular outcomes. For instance, while one fraction of the protein in the cell might be targeted to degradation, another fraction might target a specific subcellular localization. With denaturing pulldowns, both DMSO and MG132 confirmed the ubiquitylation of STBD1 (Figure 26B). Similarly, there was a strong indication that part of the protein is targeted to proteasomal degradation, but part of it is not increased upon MG132 treatment, hinting rather at functional

polyubiquitylation. STBD1 was also ubiquitylated in primary hepatocytes, and in this case the addition of MG132 did not seem to alter the amount of the ubiquitylated fraction (Figure 26C).

Primary mouse hepatocytes are a great cellular model to study liver metabolism since they can respond for a few days to hormonal and nutrients stimulation. For this reason we have decided to simulate a nutrient poor condition with a starvation media (SM) (containing no serum) and compare it with the media used in growing culture (GC) (containing serum, insulin and glutamine). We observed that these different media in primary hepatocytes did not modify the ubiquitylation levels of STBD1 (Figure 26C). Despite the responsiveness of primary hepatocytes to stimulation, the fasting and feeding response in mice is a complex mixture of hormones, nutrients and metabolites, which are difficult to reproduce in a cellular system.

In conclusion, we confirmed the ubiquitylation of STBD1 in HEK293T cells and in primary mouse hepatocytes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A
FLAG IP - HEK293T
INPUT
FLAG IP
FT
FLAG - MG132
DMSO
MG132
PR619
kDa
250 -
130 -
95 -
72 -
55 -
36 -
28 -
17 -
IB FLAG
Strep-HRP
B
His+Bio pulldown - HEK293T
INPUT
Pulldown
C
His+Bio pulldown - Primary hepatocytes
FLAG - MG132 - SM
FLAG - MG132 - GC
SM
GC

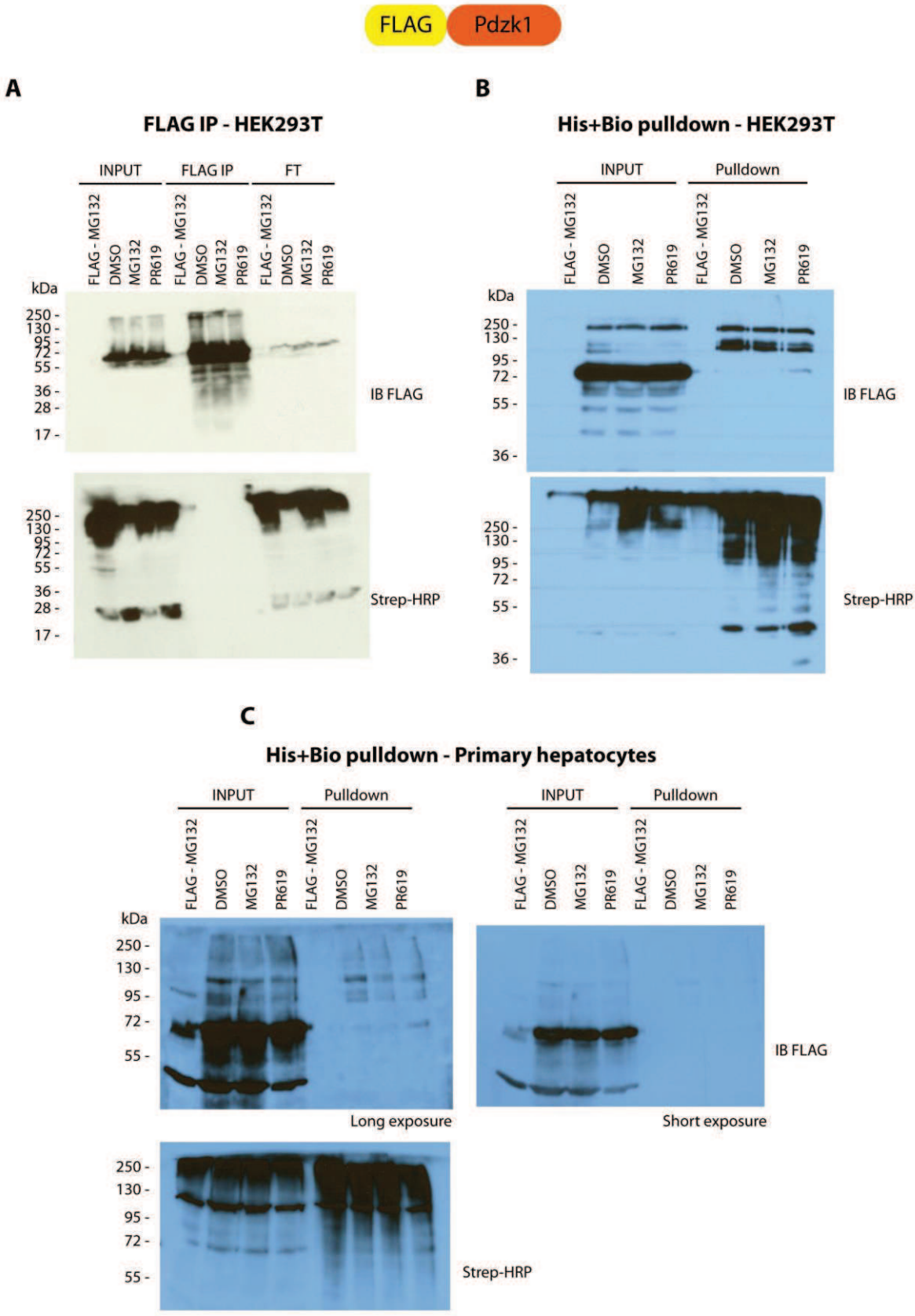
Figure 26: Starch-binding protein 1 is ubiquitylated in HEK293T cells and in primary hepatocytes

(A) Western blot after Anti-FLAG immunoprecipitation from HEK293T whole-cell lysates. FLAG immunoprecipitation, input and flow-through (FT) are shown as indicated.
(B,C) Western blot after His-Biotin pulldown from (B) HEK239T and (C) primary hepatocytes whole-cell lysates. His-Bio pulldown and input are shown as indicated.
Both, HEK293T cells and primary hepatocytes expressed His-Bio-Ubiquitin and either FLAG or FLAG-STBD1. Cells were treated with DMSO, 10 µM MG132 (proteasome inhibitor) for 4 hours, or 10 µM PR619 (DUB inhibitor) for 2 hours. Primary hepatocytes (C) were additionally treated with starvation media (SM) or growing culture media (GC). Antibody against FLAG was used to detect exogenous STBD1 and Streptavidin-HRP was used to detect ubiquitylated proteins with exogenous ubiquitin.

6.2. PDZ domain-containing protein 1 (PDZK1)

PDZ domain-containing protein 1 (PDZK1 – 57kDa) was found as a putative target of ubiquitylation in livers of fasted mice, using TUBEs as purification method (Table 3). Also, there were 25 lysines reported as being ubiquitylation sites (Wagner et al, 2012). PDZK1 is involved in a variety of cellular functions, acting as a scaffold that connects plasma membrane proteins to complexes in the cytosol. One of these proteins in hepatocytes is the HDL receptor scavenger receptor class B type I (SR-BI). PDZK1 showed to be necessary to promote normal hepatic expression, intracellular localization, and function of SR-BI, thereby influencing lipoprotein metabolism (Kocher and Krieger, 2009). Due to its function in hepatocytes, we hypothesized that ubiquitylation could affect either its subcellular localization, or its ability to recruit the HDL receptor to the membrane.

The FLAG IP of PDZK1 from HEK293T cell lysates did not detect ubiquitylated PDZK1 in western blots (Figure 27A). On the other hand, the denaturing ubiquitin pulldown showed very clear ubiquitylated PDZK1, in all the conditions tested (Figure 27B). We also observed PDZK1 to be a target of ubiquitylation in primary hepatocytes (Figure 27C). The treatment with MG132 and PR619 did not alter the ubiquitylation levels of PDZK1, which led us to suggest that PDZK1 is most likely a target of polyubiquitylation with non-degradative functions in the cell.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Pdzk1
A
FLAG IP - HEK293T
INPUT
FLAG IP
FT
FLAG - MG132
DMSO
MG132
PR619
kDa
250 -
130 -
95 -
72 -
55 -
36 -
28 -
17 -
IB FLAG
Strep-HRP
B
His+Bio pulldown - HEK293T
INPUT
Pulldown
C
His+Bio pulldown - Primary hepatocytes
Long exposure
Short exposure

Figure 27: Pdz-domain containing protein 1 is ubiquitylated in HEK293T cells and in primary hepatocytes

(A) Western blot after Anti-FLAG immunoprecipitation from HEK293T whole-cell lysates. FLAG immunoprecipitation, input and flow-through (FT) are shown as indicated.

(B,C) Western blot after His-Biotin pulldown from (B) HEK239T and (C) primary hepatocytes whole-cell lysates. His-Bio pulldown and input are shown as indicated.

Both, HEK293T cells and primary hepatocytes expressed His-Bio-Ubiquitin and either FLAG or FLAG-PDZK1. Cells were treated with DMSO, 10 µM MG132 (proteasome inhibitor) for 4 hours, or 10 µM PR619 (DUB inhibitor) for 2 hours. Antibody against FLAG was used to detect exogenous PDZK1 and Streptavidin-HRP was used to detect ubiquitylated proteins with exogenous ubiquitin.

6.3. Lipin 1

Lipin 1 (99kDa) was found to be a putative target of ubiquitylation in the livers of fasted mice, using UbiQapture as purification method (Table 3). Lipin 1 is known for its essential role in the control of fatty acid metabolism at different levels. In high nutrient conditions, lipin 1 catalyzes the conversion of phosphatidic acid to diacylglycerol during triglyceride biosynthesis in the reticulum endoplasmic membrane (Reue and Zhang, 2008). However, in low nutrient conditions, lipin 1 acts inhibiting SREBP1 and reducing expression of SREBP target genes, thereby blocking lipogenesis (Peterson et al, 2011). This dual function in lipid metabolism led us to hypothesize that ubiquitylation during fasting modulates its ability to bind other partners and to change its subcellular localization.

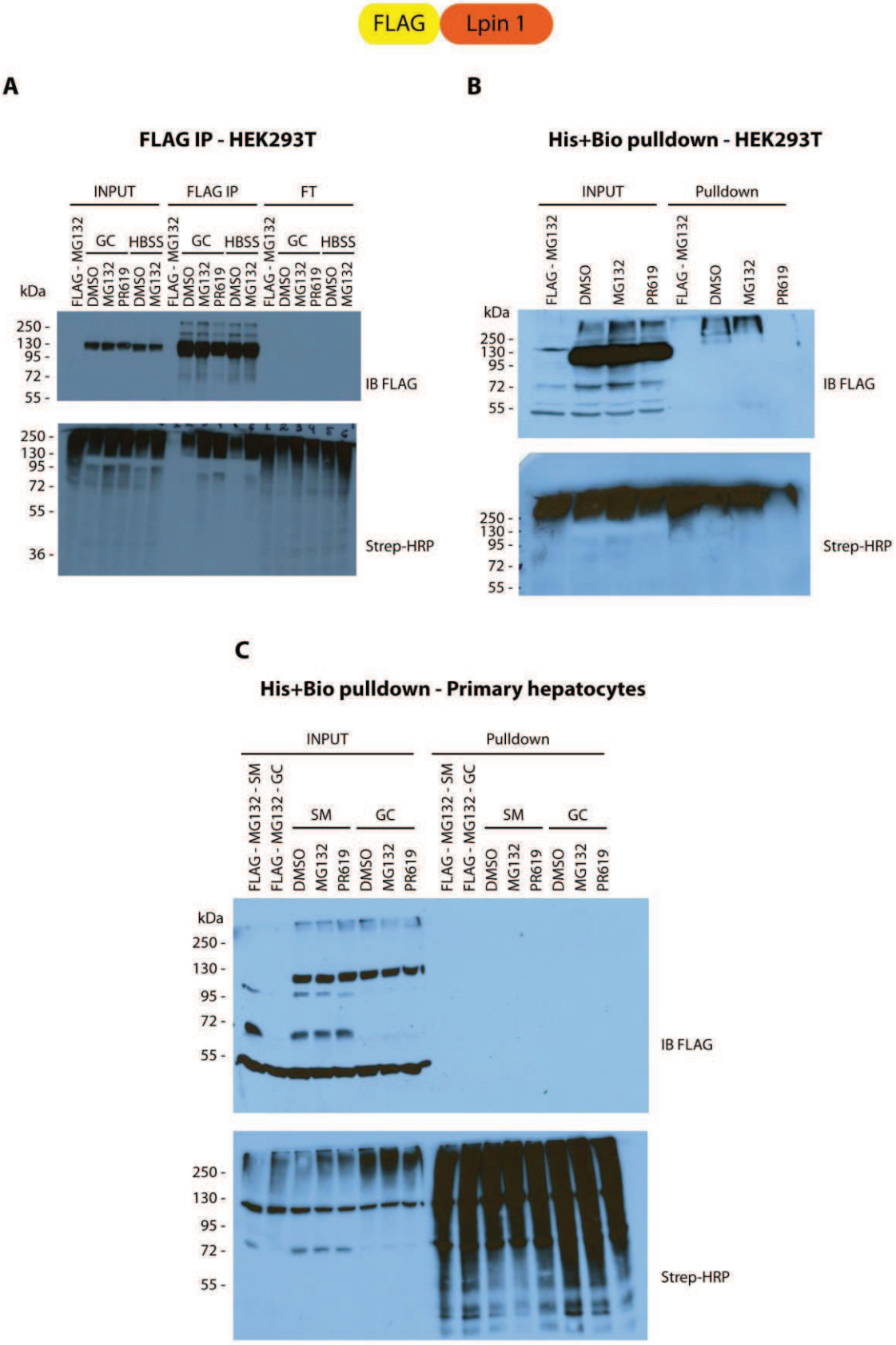

FLAG
Lpin 1
A
FLAG IP - HEK293T
INPUT
FLAG IP
FT
GC
HBSS
FLAG - MG132
DMSO
MG132
PR619
kDa
250 -
130 -
95 -
72 -
55 -
36 -
IB FLAG
Strep-HRP
B
His+Bio pulldown - HEK293T
INPUT
Pulldown
C
His+Bio pulldown - Primary hepatocytes
FLAG - MG132 - SM
FLAG - MG132 - GC
SM
GC

Figure 28: Lipin1 is ubiquitylated in HEK293T cells

(A) Western blot after Anti-FLAG immunoprecipitation from HEK293T whole-cell lysates. FLAG immunoprecipitation, input and flow-through (FT) are shown as indicated.

(B,C) Western blot after His-Biotin pulldown from (B) HEK239T and (C) primary hepatocytes whole-cell lysates. His-Bio pulldown and input are shown as indicated.

Both, HEK293T cells and primary hepatocytes expressed His-Bio-Ubiquitin and either FLAG or FLAG-lipin1. Cells were treated with DMSO, 10 µM MG132 (proteasome inhibitor) for 4 hours, or 10 µM PR619 (DUB inhibitor) for 2 hours. HEK293T cells (A) were additionally treated either with growing culture media (GC) or with Hank's balanced salt solution (HBSS). Primary hepatocytes (C) were additionally treated with starvation media (SM) or growing culture media (GC). Antibody against FLAG was used to detect exogenous lipin1 and Streptavidin-HRP was used to detect ubiquitylated proteins with exogenous ubiquitin.

The FLAG IP from HEK293T cell lysates showed very clear ubiquitylated lipin 1 in all the conditions tested (Figure 28A). As HEK293T cells can respond to the lack of nutrients, I also tested the ubiquitylation once the cells were starved (HBSS), which did not alter the levels of ubiquitylation. Also in HEK293T cells, the denaturing pulldown showed that lipin 1 is ubiquitylated in both, DMSO and MG132 conditions (Figure 28B). In the conditions tested, however, I was not able to see ubiquitylated lipin1 in primary hepatocytes (Figure 28C). I have also performed the experiment under starvation media (SM) (containing no serum) and compared it with the media used in growing culture (GC) (containing serum, insulin and glutamine), but in either case lipin 1 was not ubiquitylated in primary hepatocytes. These data suggests that lipin 1 can be indeed ubiquitylated, but we either did not find the conditions that lead to the ubiquitylation in primary hepatocytes, or the assay was not sensitive enough for detection. Another possibility is that lipin 1 was not ubiquitylated in livers during the screen, but it was rather part of a protein complex that was pulled down together with an ubiquitylated protein. This is a possibility due to the fact that the screen was performed under native conditions and therefore identified proteins can actually be binding partners of ubiquitylated proteins.

6.4. Pyruvate dehydrogenase kinase isozyme 4 (PDK4)

Pyruvate dehydrogenase kinase isozyme 4 (PDK4 – 46kDa) was also identified in the livers of fasted mice by using UbiQapture as purification method (Table 3). Two ubiquitylation sites were

reported by Kim et al (2011). PDK4 is a mitochondrial kinase that belongs to the pyruvate dehydrogensase complex (PDC) and is able to inhibit it. The PDC catalyzes the conversion of pyruvate to acetyl-CoA, a rate-limiting reaction that determines the fate of pyruvate in the cell. Once converted to acetyl-CoA, this metabolite can no longer be used in the gluconeogenic pathway. As PDK4 can inhibit this PDC, it actually blocks the reaction, making pyruvate available in the cytoplasm to undergo the gluconeogenic pathway (Sugden and Holness, 2006). Based on the function of PDK4 in fasted conditions, we hypothesized that ubiquitylation could affect its targeting to the mitochondria or its activity.

FLAG IPs from HEK293T cell lysates showed a robust ubiquitylation of PDK4 (Figure 29A). I also tested the ubiquitylation once the cells were starved (HBSS), which did not alter the levels of ubiquitylation. Similarly, in denaturing conditions, I could also confirm the ubiquitylation of PDK4 in HEK293T cells (Figure 29B). One technical complication of the assay was the low level of detection of PDK4. This could arise from the low expression of the construct or the inability of solubilizing PDK4 during cell lysis. In the input fractions, there is a faint band in the western blot corresponding to the tagged PDK4. Since the transfection efficiency in primary hepatocytes is usually low when compared to the efficiency in HEK293T cells, it was not possible to perform the same experiment in primary hepatocytes, as they did not show detectable expression of exogenous PDK4. Nevertheless we could confirm the ubiquitylation of PDK4 in HEK293T cells.

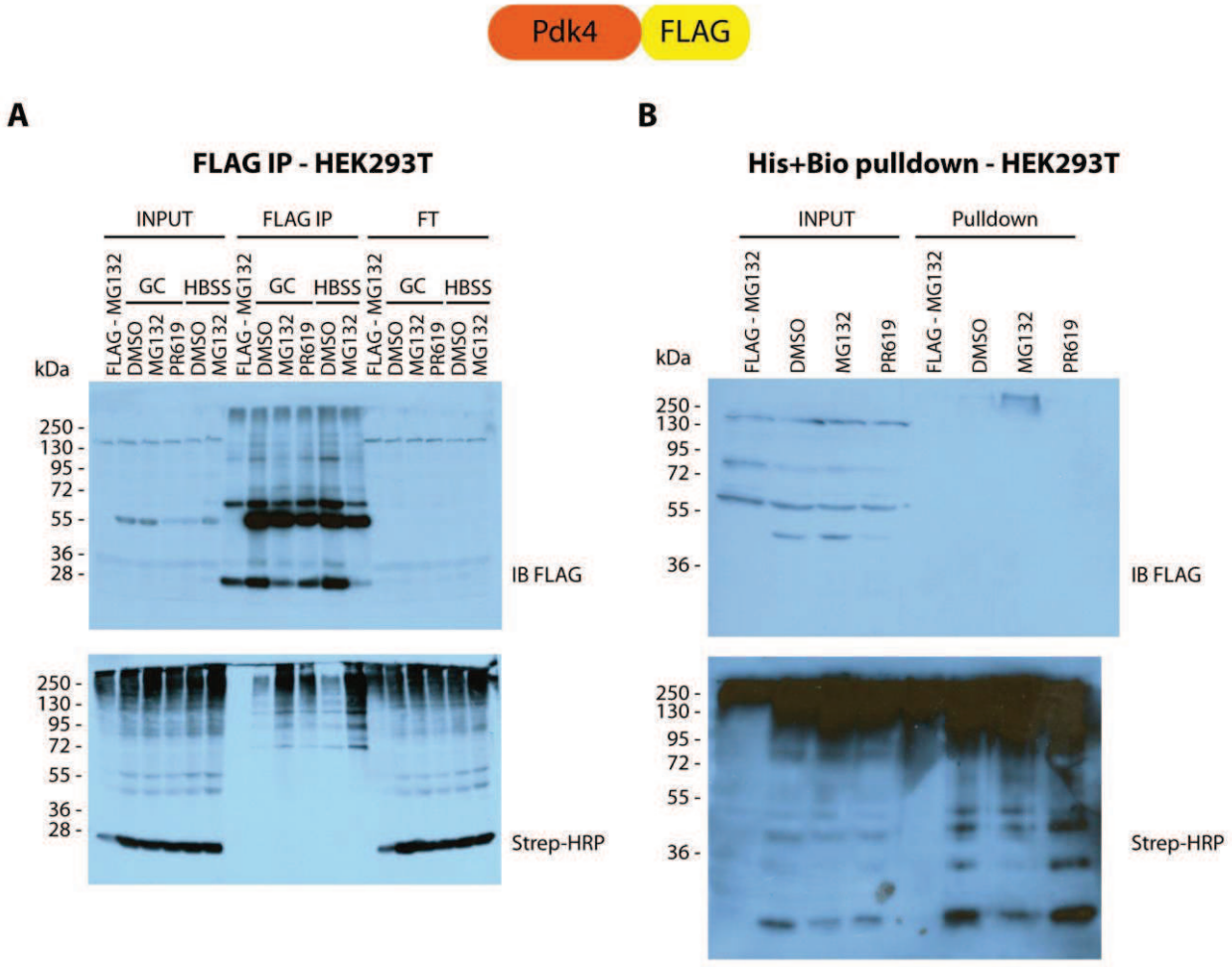


Figure 29: Pyruvate dehydrogenase kinase isosyme 4 is ubiquitylated in HEK293T cells

(A) Western blot after Anti-FLAG immunoprecipitation from HEK293T whole-cell lysates. FLAG immunoprecipitation, input and flow-through (FT) are shown as indicated.

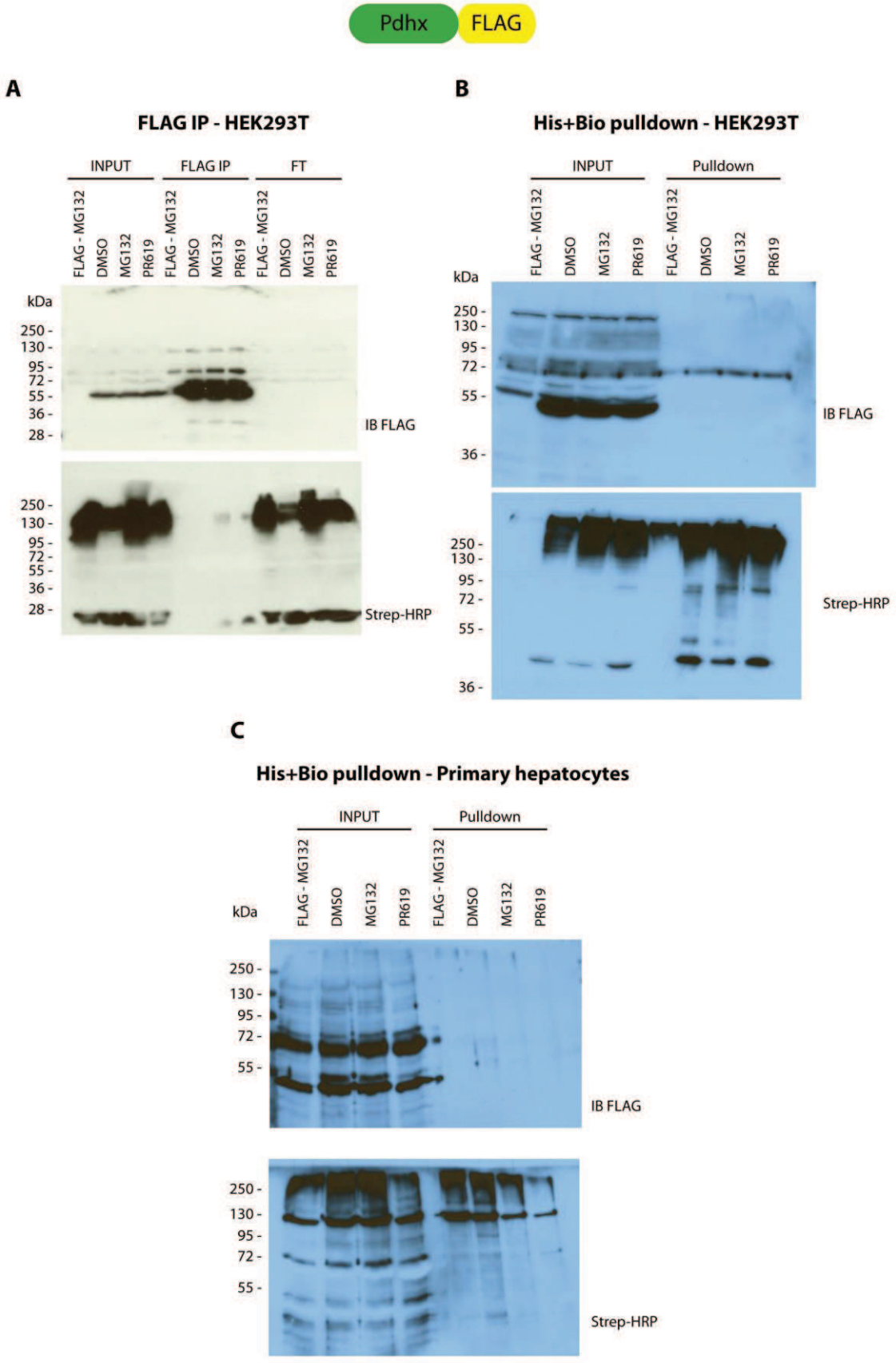
(B) Western blot after His-Biotin pulldown from HEK239T whole-cell lysates. His-Bio pulldown and input are shown as indicated.

HEK293T cells expressed His-Bio-Ubiquitin and either FLAG or FLAG-PDK4. Cells were treated with DMSO, 10 µM MG132 (proteasome inhibitor) for 4 hours, or 10 µM PR619 (DUB inhibitor) for 2 hours. HEK293T cells (A) were additionally treated either with growing culture media (GC) or with Hank's balanced salt solution (HBSS). Antibody against FLAG was used to detect exogenous PDK4 and streptavidin-HRP was used to detect ubiquitylated proteins with exogenous ubiquitin.

6.5. Pyruvate dehydrogenase complex component X (PDHX)

Another component of the PDC was found ubiquitylated specifically upon refeeding of mice - pyruvate dehydrogenase complex component X (PDHX – 54kDa). PDHX was identified by using UbiQapture as purification method (Table 4). PDHX plays a structural role in PDC, keeping other components bound to the catalytic core, which is essential for PDC activity. Taking into account the role of PDHX during the fed state, we hypothesized that ubiquitylation of PDHX might affect its binding to the protein complex.

The performed FLAG IPs from HEK293T cell lysates identified ubiquitylated PDHX upon MG132 treatment (Figure 30A). However, upon denaturing conditions, PDHX was not identified as being ubiquitylated in the conditions tested, both in HEK293T cells as well as in primary hepatocytes (Figure 30B, Figure 30C). These results led us to conclude that PDHX is ubiquitylated in HEK293T cells, but it might have been identified in the proteomic screen due to its interaction with another ubiquitylated protein.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FT
FLAG - MG132
DMSO
MG132
PR619
kDa
250 -
130 -
95 -
72 -
55 -
36 -
28 -
IB FLAG
Strep-HRP
B
His+Bio pulldown - HEK293T
INPUT
Pulldown
C
His+Bio pulldown - Primary hepatocytes

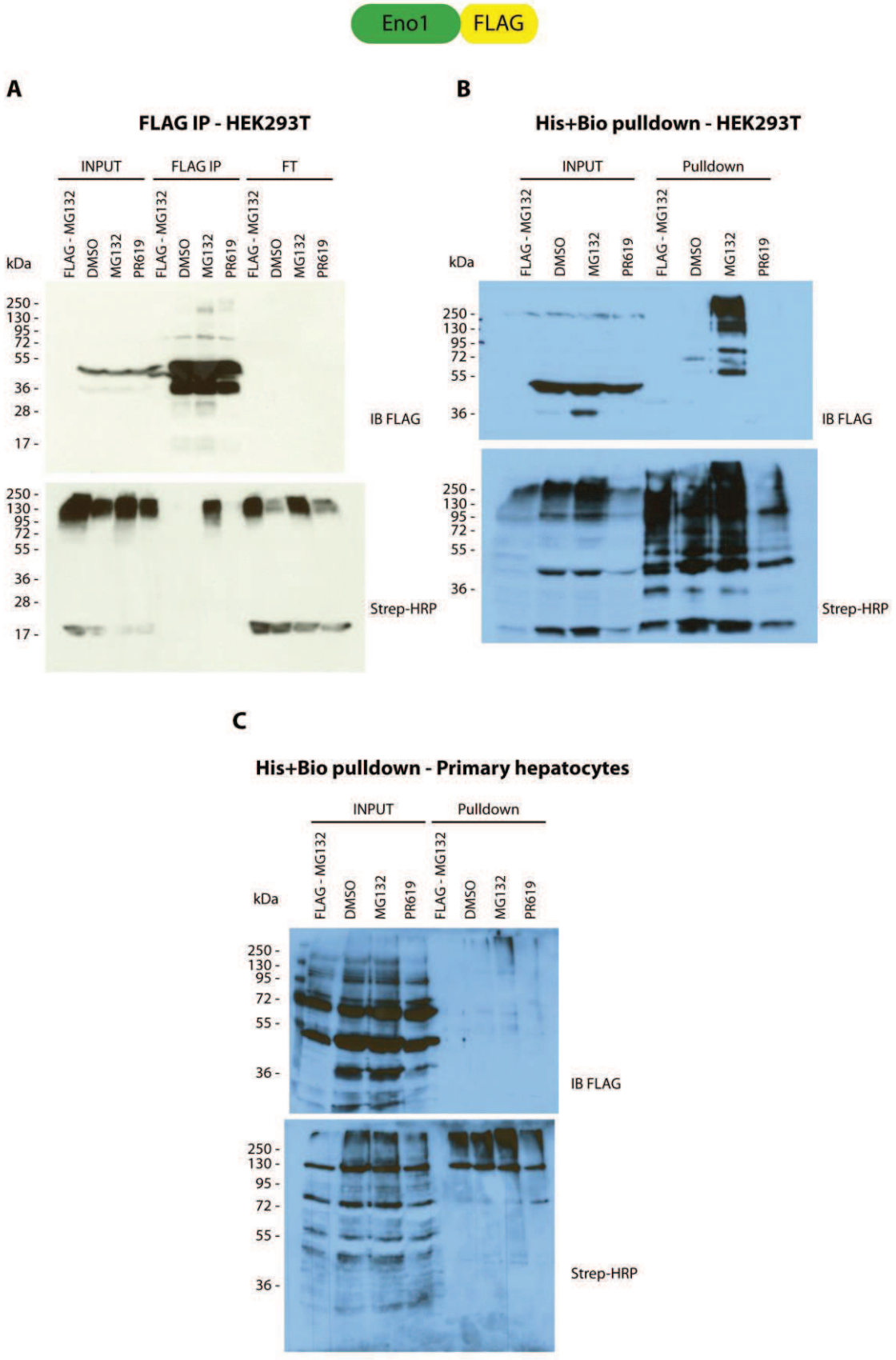
Figure 30: Pyruvate dehydrogenase complex component X is ubiquitylated in HEK293T cells

(A) Western blot after Anti-FLAG immunoprecipitation from HEK293T whole-cell lysates. FLAG immunoprecipitation, input and flow-through (FT) are shown as indicated.
(B,C) Western blot after His-Biotin pulldown from (B) HEK239T and (C) primary hepatocytes whole-cell lysates. His-Bio pulldown and input are shown as indicated.
Both, HEK293T cells and primary hepatocytes expressed His-Bio-Ubiquitin and either FLAG or FLAG-PDHX. Cells were treated with DMSO, 10 µM MG132 (proteasome inhibitor) for 4 hours, or 10 µM PR619 (DUB inhibitor) for 2 hours. HEK293T cells (A) were additionally treated either with growing culture media (GC) or with Hank's balanced salt solution (HBSS). Antibody against FLAG was used to detect exogenous PDHX and Streptavidin-HRP was used to detect ubiquitylated proteins with exogenous ubiquitin.

6.6. Enolase 1 (ENO1)

Enolase 1 (ENO1 – 47kDa) was identified as a putative ubiquitylation target in livers of refed mice by using TUBE as purification method (Table 4). Large-scale ubiquitylation studies identified 27 lysines in ENO1 as ubiquitylation sites (Kim et al 2011; Wagner et al 2012). ENO1 plays a role in glycolysis catalyzing the conversion of 2-phosphoglycerate to phosphoenolpyruvate. In fact, ENO1 is a multifunctional enzyme that plays also a role in other processes, such as growth control, hypoxia tolerance and allergic responses (Díaz-Ramos et al, 2012). Due to its various roles in cells, including a major role in glycolysis, we hypothesized that ubiquitylation could affect its catalytic role in glycolysis. Alternatively, ubiquitylation could affect ENO1 subcellular localization and target it to a different function.

The FLAG IP from HEK293T cell lysates identified ENO1 to be ubiquitylated upon MG132 treatment (Figure 31A). This observation strongly indicates that ENO1 is ubiquitylated and targeted for degradation. I confirmed this observation with the denaturing ubiquitin pulldown, in which HEK293T cells treated with MG132 showed polyubiquitylation of ENO1 (Figure 31B). At last, these results were also confirmed in primary hepatocytes (Figure 31C). Together, these data suggests that ENO1 is polyubiquitylated and targeted to degradation in livers.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A
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INPUT
FLAG IP
FT
FLAG - MG132
DMSO
MG132
PR619
kDa
250 -
130 -
95 -
72 -
55 -
36 -
28 -
17 -
IB FLAG
Strep-HRP
B
His+Bio pulldown - HEK293T
INPUT
Pulldown
C
His+Bio pulldown - Primary hepatocytes

Figure 31: Enolase 1 is ubiquitylated in HEK293T cells and in primary hepatocytes
(A) Western blot after Anti-FLAG immunoprecipitation from HEK293T whole-cell lysates. FLAG immunoprecipitation, input and flow-through (FT) are shown as indicated.
(B,C) Western blot after His-Biotin pulldown from (B) HEK239T and (C) primary hepatocytes whole-cell lysates. His-Bio pulldown and input are shown as indicated.
Both, HEK293T cells and primary hepatocytes expressed His-Bio-Ubiquitin and either FLAG or FLAG-ENO1. Cells were treated with DMSO, 10 μM MG132 (proteasome inhibitor) for 4 hours, or 10 μM PR619 (DUB inhibitor) for 2 hours. HEK293T cells (A) were additionally treated either with growing culture media (GC) or with Hank's balanced salt solution (HBSS). Antibody against FLAG was used to detect exogenous ENO1 and streptavidin-HRP was used to detect ubiquitylated proteins with exogenous ubiquitin.

6.7. Corticosteroid 11-beta-dehydrogenase isozyme 1 (HSD11β1)

Corticosteroid 11-beta-dehydrogenase isozyme 1 (HSD11β1 – 32 kDa) was indentified in the livers of refed mice and it was purified with TUBEs technique (Table 4). Wagner et al (2012) reported 2 lysines as acceptor sites for ubiquitylation in livers. HSD11β1 is a microsomal enzyme that activates cortisone to cortisol, and thereby amplifies glucocorticoid concentrations in key metabolic tissues including liver and adipose tissue. Glucocorticoids, such as cortisol, participate together with glucagon and epinephrine in the fasting/starvation response. For this reason, chronic glucocorticoid excess is strongly associated with components of the metabolic syndrome and inhibitors of HSD11β1 are being tested as drugs with benefits in metabolic syndrome (Hughes et al, 2008; Pereira et al, 2012). As glucocorticoid pathway should not be activated upon refeeding, we hypothesized that HSD11β1 ubiquitylation could lead to its inhibition or degradation.

FLAG IP from HEK293T cell lysates identified ubiquitylated HSD11β1 in all the conditions tested (Figure 32). Starved HEK293T cells (HBSS condition) did not alter the ubiquitylation levels. Even though the FLAG IP was very efficient and I could enrich HSD11β1 in the eluted fraction, it was difficult to detect the protein in the whole-cell lysates. This technical difficulty might be due to the low expression of HSD11β1 or the lack of complete solubilization in the lysis buffer. For this reason, when we performed ubiquitin pulldowns from both HEK293T and primary hepatocytes

we did not observe any ubiquitylated HSD11β1, which could be explained by the low protein levels.

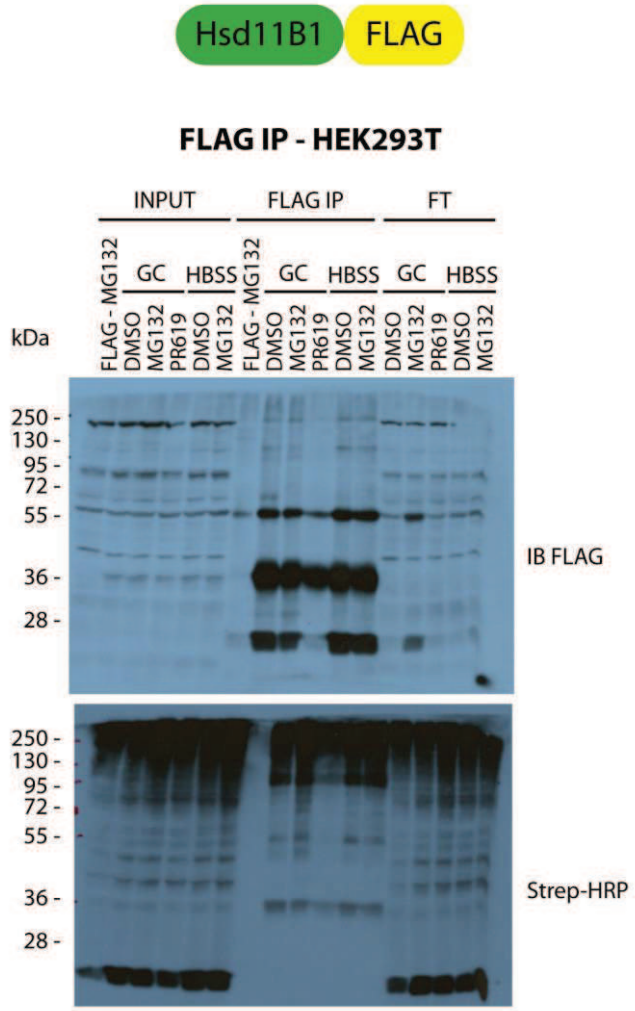


Figure 32: Hormone-sensitive dehydrogenase11 beta 1 is ubiquitylated in HEK293T cells

Western blot after Anti-FLAG immunoprecipitation from HEK293T whole-cell lysates. Cells expressed His-Bio-Ubiquitin and either FLAG or FLAG-HSD11b1. Cells were treated with DMSO, 10 µM MG132 (proteasome inhibitor) for 4 hours, or 10 µM PR619 (DUB inhibitor) for 2 hours. Cells were additionally treated either with growing culture media (GC) or with Hank's balanced salt solution (HBSS). Antibody against FLAG was used to detect exogenous HSD11b1 and streptavidin-HRP was used to detect ubiquitylated proteins with exogenous ubiquitin. FLAG immunoprecipitation, input and flow-through (FT) are shown as indicated.

6.8. Complement 3 (C3)

Complement 3 (C3 – 187kDa) was identified upon refeeding of mice. Interestingly, C3 was one of the few proteins identified with both purification techniques, TUBEs and UbiQapture (Table 4). C3 contains 15 ubiquitylation sites reported in murine livers (Wagner et al, 2012).

C3 plays a central role in the activation of the complement system, which is a humoral component of the innate immune response and is composed out of more than 60 proteins (Walport MJ, 2001a; Walport MJ, 2001b). Most of the soluble complement proteins are expressed by the liver and secreted to the blood in an inactive form. In the presence of a trigger such as damaged cells or pathogens, the complement system works as a protein activation cascade. All activation cascades of complement will lead to the formation of the C3 activating protein complex, the C3 convertase. Once C3 convertase is formed, it cleaves C3 into C3a and C3b. C3a is an anaphylatoxin and it binds C3a receptor (C3aR) on cell membranes, thereby stimulating lymphocytes chemotaxis and cytokine production. C3b, on the other hand, has several roles, including formation of membrane attack complex, opsonization and amplification of complement activity (Sahu and Lambris, 2001).

In the blood, C3a is rapidly inactivated from its immunological functions by proteolytic cleavage. The enzymes carboxypeptidase N1 (CPN1) and carboxypeptidase B2 (CPB2) cleave the C-terminal arginine of C3a, generating C3adesArg (also known as acylation stimulating protein - ASP). C3adesArg has its own receptor, C5L2, and signaling through this receptor in adipocytes leads to a strong lipogenic activity, increasing glucose uptake, TAG storage and reducing lipolysis (Cianflone et al, 2003). Thus, C3 also plays a role in postprandial lipid metabolism (Hertle et al, 2014). In addition, C3 has been associated with liver steatosis: 74% of the NAFLD patients showed hepatic deposition of activated C3 around hepatocytes with macrovesicular steatosis (Rensen et al, 2009). Due to its known functions in inflammation and lipid metabolism, the identified ubiquitylation of C3 might be a regulator for C3 activities.

FLAG IP from HEK293T cell lysates showed that C3 was ubiquitylated in independently of treatment with MG132 or PR619 (Figure 33A). In the same way, denaturing ubiquitin pulldowns in HEK293T cell lysates confirmed that C3 molecule is ubiquitylated and that part of the ubiquitylated C3 is degraded, as the treatment with MG132 increased a fraction of ubiquitylated C3 (Figure 33B). Denaturing ubiquitin pulldowns from primary hepatocytes were quite

encouraging, as C3 was also ubiquitylated in these cells under treatment of MG132, which indicates that overexpressed C3 is ubiquitylated and degraded in primary hepatocytes (Figure 33C). Interestingly, different media seem to alter the ubiquitylation levels of C3; there is a higher amount of ubiquitylated C3 in the growing culture (GC) conditions, as compared to the starvation media (SM), in agreement to what we observed in the proteomic screen *in vivo*.

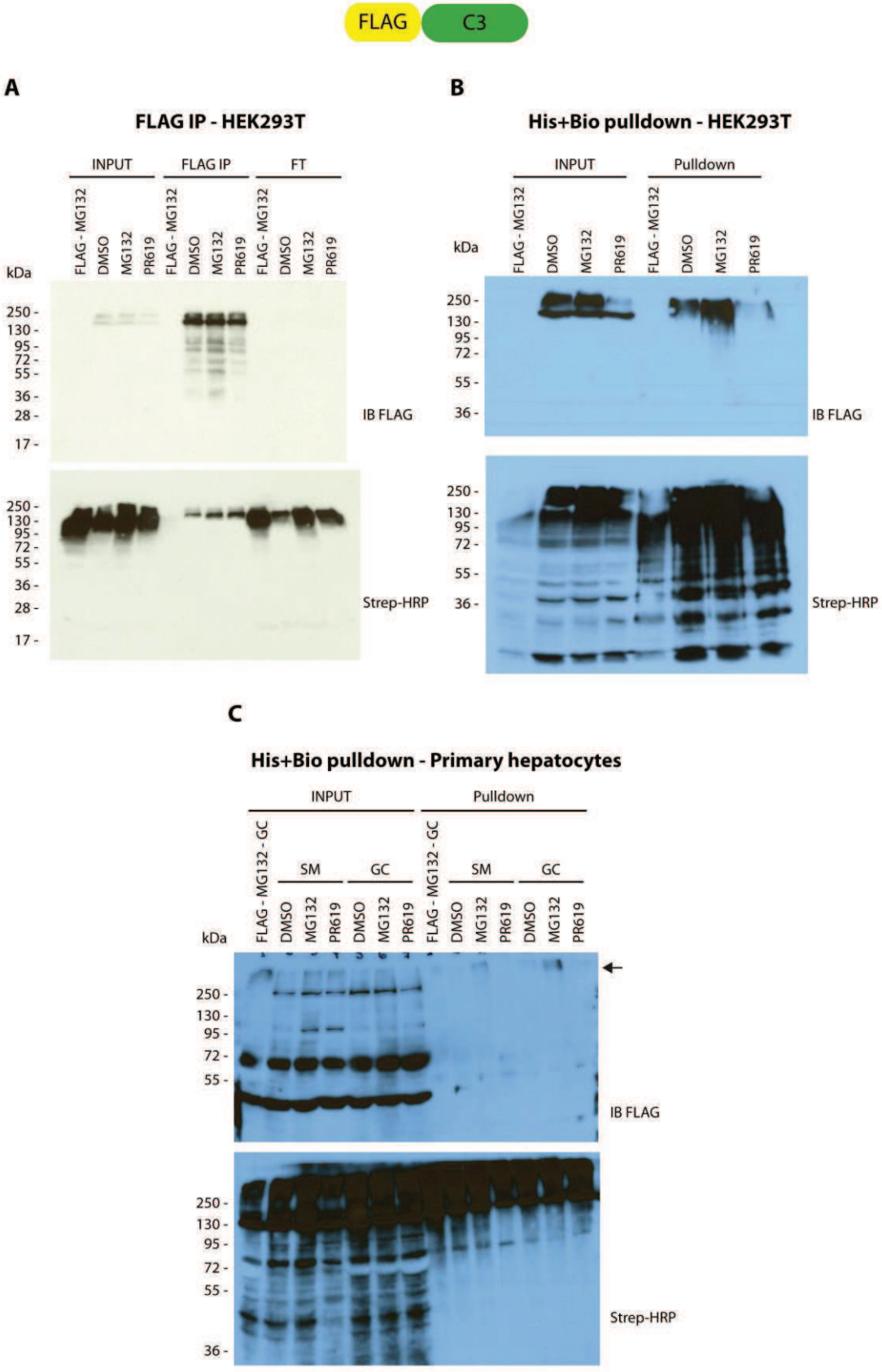

FLAG
C3
A
FLAG IP - HEK293T
INPUT
FLAG IP
FT
FLAG - MG132
DMSO
MG132
PR619
kDa
250 -
130 -
95 -
72 -
55 -
36 -
28 -
17 -
IB FLAG
Strep-HRP
B
His+Bio pulldown - HEK293T
INPUT
Pulldown
C
His+Bio pulldown - Primary hepatocytes
FLAG - MG132 - GC
SM
GC

Figure 33: Complement 3 is ubiquitylated in HEK293T cells and in primary hepatocytes
(A) Western blot after Anti-FLAG immunoprecipitation from HEK293T whole-cell lysates. FLAG immunoprecipitation, input and flow-through (FT) are shown as indicated.
(B,C) Western blot after His-Biotin pulldown from (B) HEK239T and (C) primary hepatocytes whole-cell lysates. His-Bio pulldown and input are shown as indicated. Arrow indicates ubiquitylated C3.
Both, HEK293T cells and primary hepatocytes expressed His-Bio-Ubiquitin and either FLAG or FLAG-C3. Cells were treated with DMSO, 10 µM MG132 (proteasome inhibitor) for 4 hours, or 10 µM PR619 (DUB inhibitor) for 2 hours. Primary hepatocytes (C) were additionally treated with starvation media (SM) or growing culture media (GC). Antibody against FLAG was used to detect exogenous C3 and Streptavidin-HRP was used to detect ubiquitylated proteins with exogenous ubiquitin.

6.9. Overview of the biochemical validation

Collectively, the IPs and pulldowns performed confirmed that all the 8 proteins can indeed be ubiquitylated. We showed the ubiquitylation of all of them in HEK293T cells and of 4 in primary hepatocytes (Figure 34).

	FLAG IP (native conditions)	His+Bio Pulldown (denatured conditions)	
	HEK293T	HEK293T	Primary hepatocytes
Stbd1 FLAG	Yes	Yes	Yes
FLAG Pdzk1	No	Yes	Yes
FLAG Lpin 1	Yes	Yes	No
Pdk4 FLAG	Yes	Yes	LD
Pdhx FLAG	Yes	No	No
Eno1 FLAG	Yes	Yes	Yes
Hsd11B1 FLAG	Yes	LD	LD
FLAG C3	Yes	Yes	Yes

LD: Low detection

Figure 34: All of the 8 proteins tested are ubiquitylated in cells

Summary of all the IPs and pulldowns performed to confirm the ubiquitylation of 8 proteins selected for biochemical validation. “Yes” refers to ubiquitylation identified in at least one of the conditions tested; “No” refers to ubiquitylation not identified in the conditions tested.
LD, low detection, protein was not detected in the input.

The data from the proteomic screen, together with the biochemical validation provided strong evidences that C3 is differentially ubiquitylated in the livers of mice upon fasting and refeeding. C3 was the only protein amongst the 8 validated proteins to be identified in the mass spectrometry screen with both techniques, TUBEs and UbiQapture. When TUBEs was used to purify proteins, C3 was identified in 11 out of 13 analyzed refed mice and in none of the 13 analyzed fasted mice divided into three independent experiments. In a similar manner, when UbiQapture was used to purify proteins, C3 was identified in 9 out of 10 analyzed refed mice and in 5 of the 10 analyzed fasted mice divided into two independent experiments. By assuming that whenever proteins were not detected they were under the detection limit, we used a function of imputation by Perseus. With this function we could perform the label-free quantification and statistical analysis. For the experiments with TUBEs, C3 levels were around 650 times higher in refed over fasted mice, ($p \leq 0.001$). With the same tendency, for the experiments with

UbiQapture, the fold difference of refed over fasted was around 5 times, ($p \leq 0.018$). In summary, there was a dramatic difference between the amount of ubiquitylated protein identified in refed versus fasted mice. In addition, C3 was also confirmed to be ubiquitylated with by IPs and pulldowns from HEK293T cells and primary hepatocytes.

Due to the ability of C3 to mediate inflammatory and metabolic functions, together with the strong biochemical data, we studied the ubiquitylation of C3 into more details to understand the behavior of this protein upon fasting and refeeding of mice. Moreover, we wanted to address conditions at which C3 gets ubiquitylated and whether this ubiquitylation regulate C3 functionality.

7. C3 is cleaved and ubiquitylated intracellularly

I first addressed whether the different ubiquitylation levels of C3 during fasting – refeeding of mice was due to variations of the total C3 levels. Therefore I examined the mRNA and protein levels of C3. Although *C3* mRNA levels were slightly increased upon refeeding, the total protein level did not seem to be affected (Figure 35A, Figure 35B). As the protein lysates used for western blotting were reduced with DTT, C3 was detected by several bands reflecting the structure C3. These results indicate that the identification of C3 in the proteomic screen is a result of increased ubiquitylation upon refeeding and not due to changed abundance of C3.

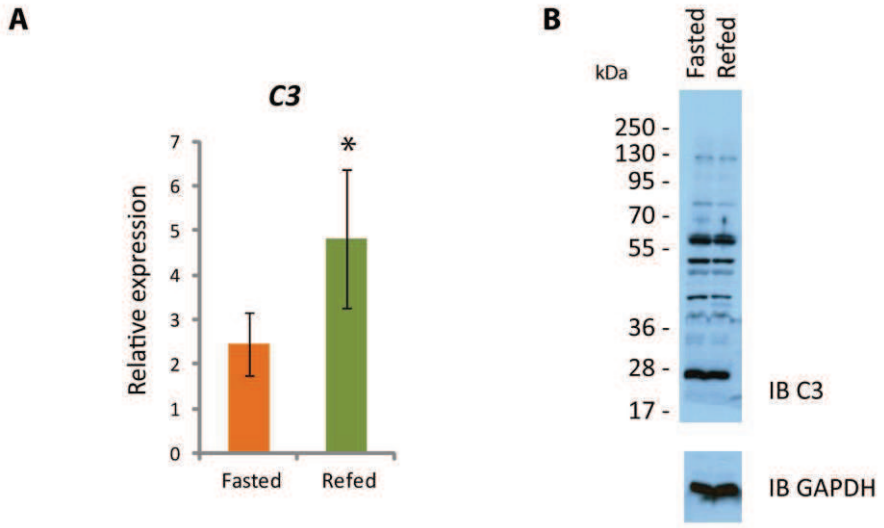
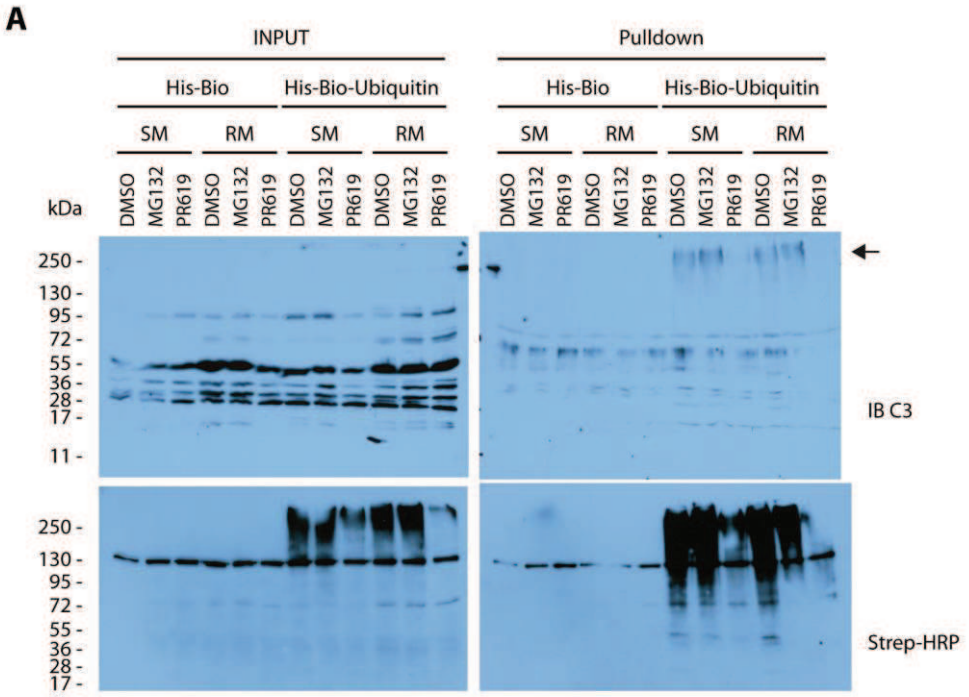


Figure 35: C3 levels are slightly increased in the livers of refed mice

(A) qPCR analysis depicts the expression levels of *C3* in the livers of fasted and refed mice. Data are presented as means ± SDV of 2 individual experiments with mRNAs from 3 different livers each. Statistical significance calculated using t-test, $p<0.05$.

(B) Western blot of whole-liver lysates of fasted and refed mice. Liver lysates from 9 different livers (3 independent experiments) were pooled together and used for this experiment. Antibodies against C3 were used to detect endogenous C3 and GAPDH is used as loading control.

We next addressed if the endogenous C3 would also be ubiquitylated in primary hepatocytes, since the validation of the ubiquitylation was performed with overexpressed protein C3. To this end, primary hepatocytes were transfected with His-Bio-Ubiquitin, and polyubiquitylated C3 was successfully pulled down in cells treated with DMSO and MG132 (Figure 36A). These results demonstrate the ubiquitylation of endogenous C3 molecule and that ubiquitylation of endogenous C3 does not lead to protein degradation. By using different cell culture media conditions I wanted to investigate the ubiquitylation during fasting and refeeding *in vitro*. I kept hepatocytes overnight in starvation media (SM) (low glucose, no serum) and exchanged to either the same SM or to a rich media (RM) (high glucose, insulin, serum, and glutamine). However, I did not observe a difference in the ubiquitylation levels with this *ex vivo* protocol.


A
INPUT
Pulldown
His-Bio
His-Bio-Ubiquitin
SM
RM
DMSO
MG132
PR619
kDa
250 -
130 -
95 -
72 -
55 -
36 -
28 -
17 -
11 -
IB C3
Strep-HRP

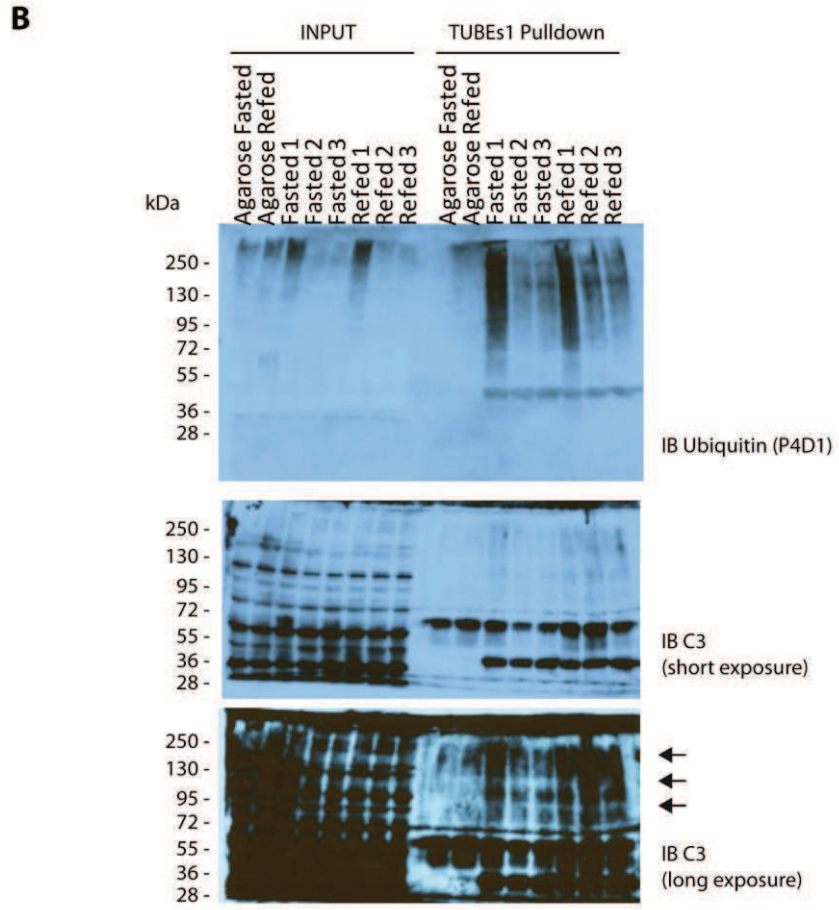

B
INPUT
TUBEs1 Pulldown
Agarose Fasted
Agarose Refed
Fasted 1
Fasted 2
Fasted 3
Refed 1
Refed 2
Refed 3
kDa
250 -
130 -
95 -
72 -
55 -
36 -
28 -
IB Ubiquitin (P4D1)
IB C3
(short exposure)
IB C3
(long exposure)

Figure 36: C3 is endogenously ubiquitylated in livers and in primary hepatocytes

(A) Western blot after His-Biotin pulldown from primary hepatocytes whole-cell lysates. Primary hepatocytes expressed either His-Bio or His-Bio-Ubiquitin. Cells were treated with DMSO, 10 µM MG132 (proteasome inhibitor) for 4 hours, or 10 µM PR619 (DUB inhibitor) for 2 hours in the presence of starvation media (SM) or rich media (RM). Antibody against C3 was used to detect endogenous C3 levels and Streptavidin-HRP was used to detect ubiquitylated proteins with exogenous ubiquitin. His-Bio pulldown and input are shown as indicated. Arrows indicate ubiquitylated C3.

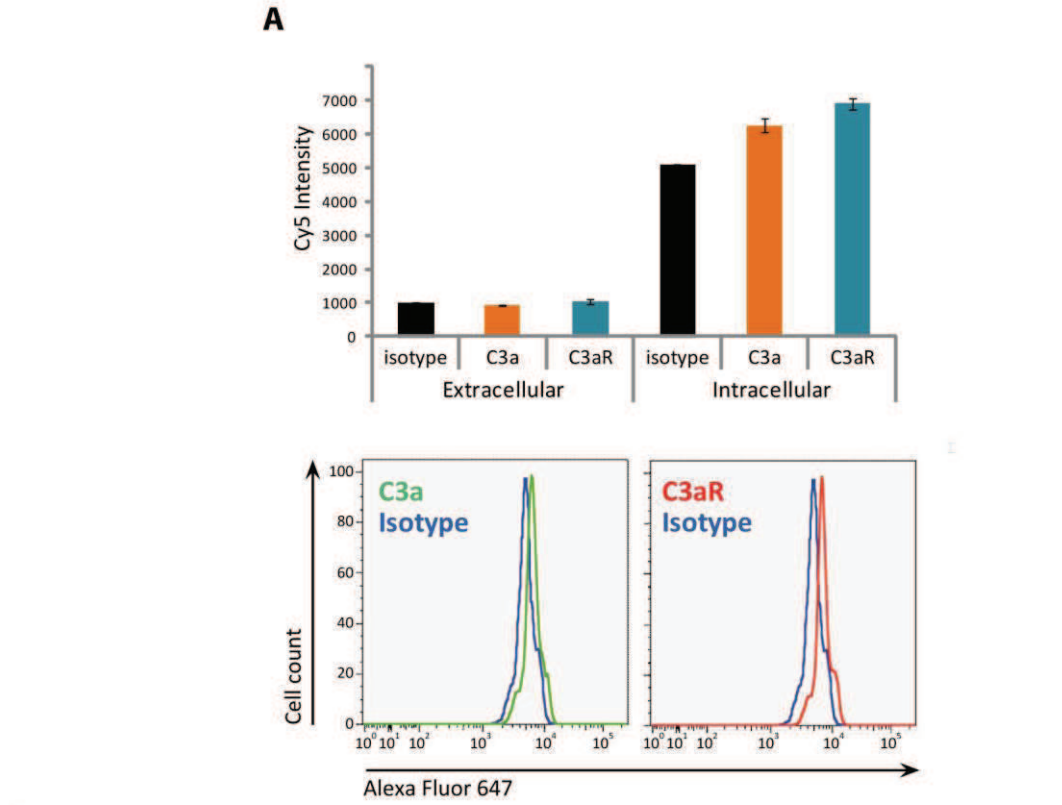
(B) Western blot after TUBEs1 pulldown from liver lysates of fasted and refed mice. Liver lysates from 3 different livers were pooled together and used for this experiment as 1 sample; numbers 1, 2, and 3 refer to pooled liver lysates obtained from independent fasting-refeeding experiments. Antibodies were used against: C3, to detect endogenous C3 and ubiquitin, to detect endogenous ubiquitylated proteins. Arrows indicates ubiquitylated C3.

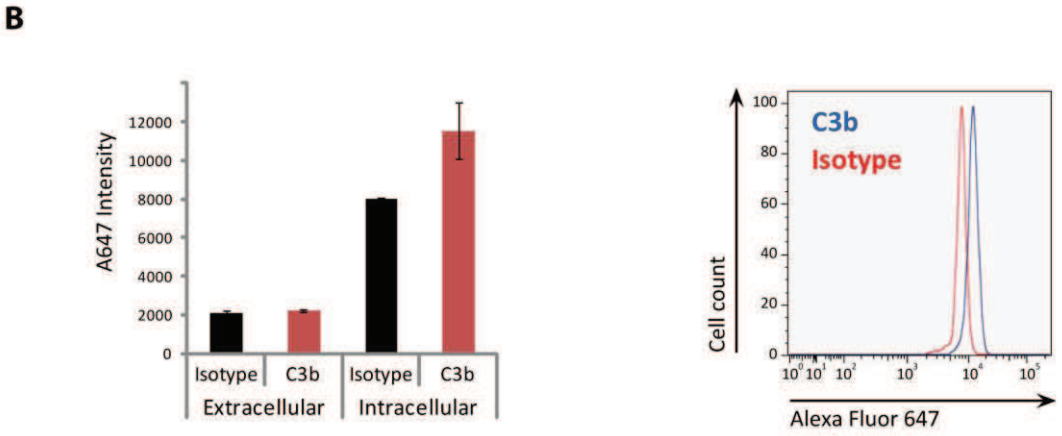
In order to analyze C3 ubiquitylation *in vivo* in mouse livers, I performed a pulldown using TUBEs 1 under native conditions from liver lysates (Figure 36B). I pooled liver lysates from 3 mice for each sample and numbers 1, 2, and 3 correspond to independent fasting-refeeding experiments. In this preliminary experiment, we observed ubiquitylated C3 in liver of fasted and refed mice, when compared to agarose controls. The amount of ubiquitylated C3 in the livers of refed mice was slightly higher, when compared to fasted conditions (Figure 36B), which goes in agreement with the proteomic screen.

As ubiquitylation regulate intracellular protein activity, we wondered if ubiquitylation could play a functional role for intracellular C3. In fact, an intracellular function of C3 has been discovered recently. Liszewski et al (2013) showed that the protease cathepsin L (CTSL) can cleave C3 into biologically active C3a and C3b in lysosomes and endosomes in resting T-cells. Moreover, they showed that C3a binds to intracellular C3aR, and this binding is essential for T-cell survival in a mechanism involving activation of mTOR signaling pathway. Once the T-cells were stimulated, the C3 activation system was shuttled to the cell surface and served as an autocrine system to induce a pro-inflammatory cytokine production. Importantly, how the intracellular cleavage of C3 is regulated still needs to be investigated.

Based on the described intracellular function of C3 and the hypothesis that ubiquitylation could regulate it, we wondered if C3 was also cleaved in hepatocytes. To this end, I performed flow

cytometry in primary hepatocytes in suspension right after their isolation. I stained extracellular C3, C3a and C3b on the surface of living intact hepatocytes. After fixation and permeabilization, the intracellular fraction of the C3 forms was stained. C3 and its cleavage products C3a and C3b were detected intracellularly, but not on the cell surface (Figure 37A, Figure 37B, Figure 37C).


A
Cy5 Intensity
7000
6000
5000
4000
3000
2000
1000
0
isotype
C3a
C3aR
isotype
C3a
C3aR
Extracellular
Intracellular
C3a
Isotype
C3aR
Isotype
Cell count
100
80
60
40
20
0
Alexa Fluor 647


B
A647 Intensity
12000
10000
8000
6000
4000
2000
0
Isotype
C3b
Isotype
C3b
Extracellular
Intracellular
C3b
Isotype
Cell count
Alexa Fluor 647

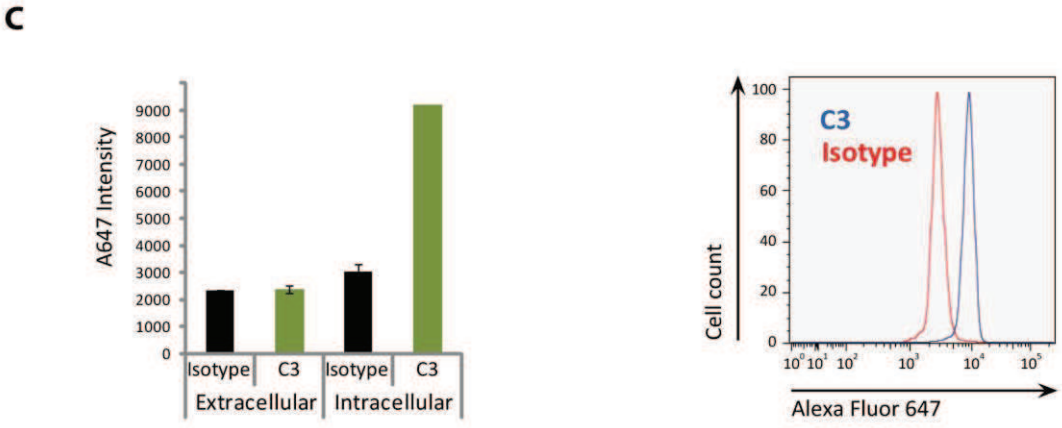

C
A647 Intensity
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Isotype
C3
Isotype
C3
Extracellular
Intracellular
C3
Isotype
Cell count
Alexa Fluor 647

Figure 37: C3 is activated and cleaved intracellularly in primary hepatocytes

(A,B,C) Bar chart indicating the fluorescence intensity in flow cytometry analysis for both, extracellular surface staining and intracellular staining. Spectra depict cell count over fluorescence intensity (Alexa-647) for intracellular stainings. Antibodies were used against C3a, C3aR (A), C3b (B), and C3 (C). Isotype control is indicated in all the charts and spectra.

We next questioned if the receptor for C3a (C3aR) is expressed intracellular of hepatocytes. Also using flow cytometry, I observed that C3aR was present intracellularly, but not on the cell surface (Figure 37A). These results suggest that C3 can be cleaved intracellularly in hepatocytes and that C3aR might be the receptor for intracellular C3a.

With a possible role of the ubiquitylation in the intracellular functions of C3, we sought to understand more about the metabolism of C3a and C3b in mouse hepatocytes. In human T-cells, it was shown that the enzyme responsible for intracellular cleavage of C3 into C3a and C3b was cathepsin L (CTSL) (Liszewski et al, 2013). We then assessed the expression of *Ctsl* in livers of fasted and refed mice. We observed that *Ctsl* mRNA was abundant in livers; with an increased amount in livers of fasted mice (Figure 38A).

C3a is known to be cleaved into C3adesArg, which has lipogenic functions in adipocytes. Although these functions have not yet been studied in hepatocytes, we wondered if this could be a possible mechanism of action for intracellular C3a. Assessment of the expression levels revealed that both enzymes responsible for C3a processing, *Cpn1* and *Cpb2*, were expressed in livers and their expression was increased upon refeeding of mice (Figure 38B, Figure 38C). I also checked the expression of the receptors *C3aR* and *C5l2*, which were expressed in livers, and their levels were not modified upon fasting and refeeding of mice (Figure 38D, Figure 38E).

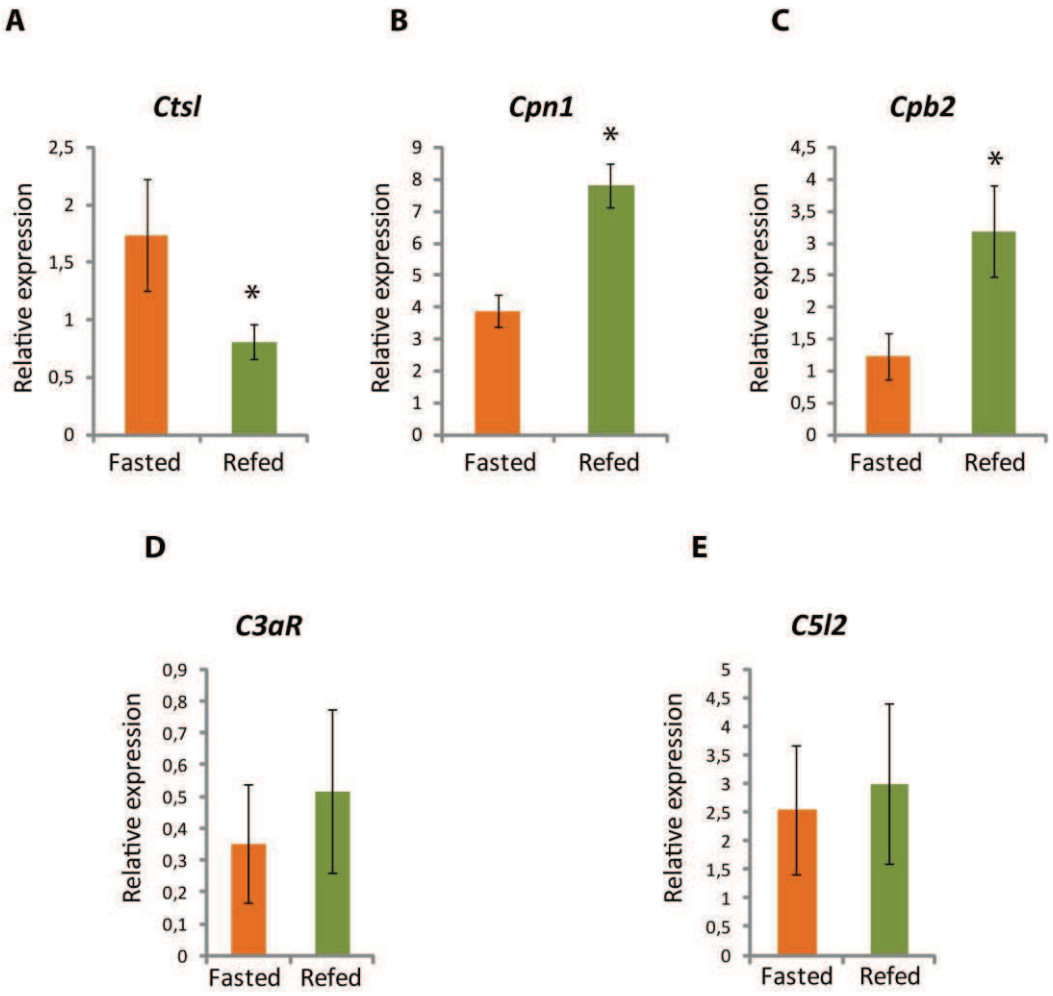


Figure 38: Catepsin L expression levels are higher during fasting, while carboxypeptidase N1 and B2 levels are increased upon refeding

qPCR analysis depicts the expression levels of *Ctsl* (A), *Cpn1* (B), *Cpb2* (C), *C3aR* (D), and *C5l2* (E) in the livers of fasted and refed mice. Data are presented as means ± SDV of 2 individual experiments with mRNAs from 3 different livers each. Statistical significance calculated using t-test, $p<0.05$.

With the goal of understanding if cleavage of C3 to C3a and C3b is affected upon fasting – refeeding of mice, we subsequently looked at the levels of C3b in the livers of fasted and refed mice by ELISA. Interestingly, we observed that C3b levels were increased upon refeeding of mice (Figure 39: C3b levels are increased in the livers of mice refed with high-sucrose diet). These data showed a tendency in the direction of enhanced intracellular cleavage of C3 into C3a and C3b in livers of refed mice as compared to fasted mice.

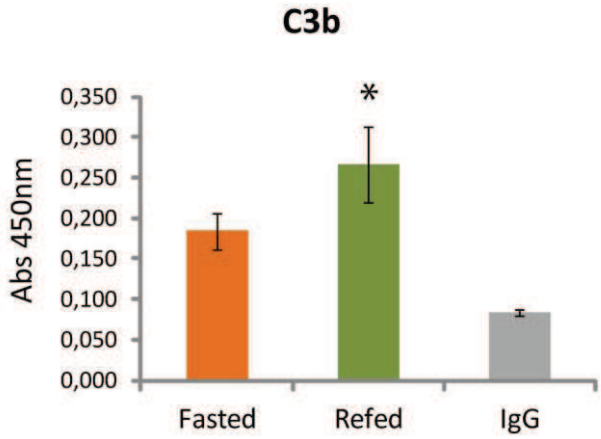


Figure 39: C3b levels are increased in the livers of mice refed with high-sucrose diet
Sandwich ELISA with C3b antibody used to immobilize proteins and C3 antibody used for detection. Liver lysates from 9 different livers (3 independent experiments) were pooled together and used for this experiment. IgG isotype control is shown as indicated. Statistical significance calculated using t-test, $p<0.05$.

In the proteomic screen that we performed, the peptides identified from C3 molecule were belonging to C3a, which could indicate that this is the modified part of the protein. We next investigated if C3a could be ubiquitylated in primary hepatocytes. We subcloned the C3a fraction with a FLAG tag and performed denaturing ubiquitin pulldowns in primary hepatocytes using the same treatment with SM and RM as described above. We saw that C3a was ubiquitylated, as observed by the upshifted bands (Figure 40A). According to the molecular weight, these bands could correspond to mono-, di- and polyubiquitylation of C3a. In addition, overexpressed and ubiquitylated C3a was partially targeted for degradation, as MG132 treatment increased the amount of precipitated C3a.

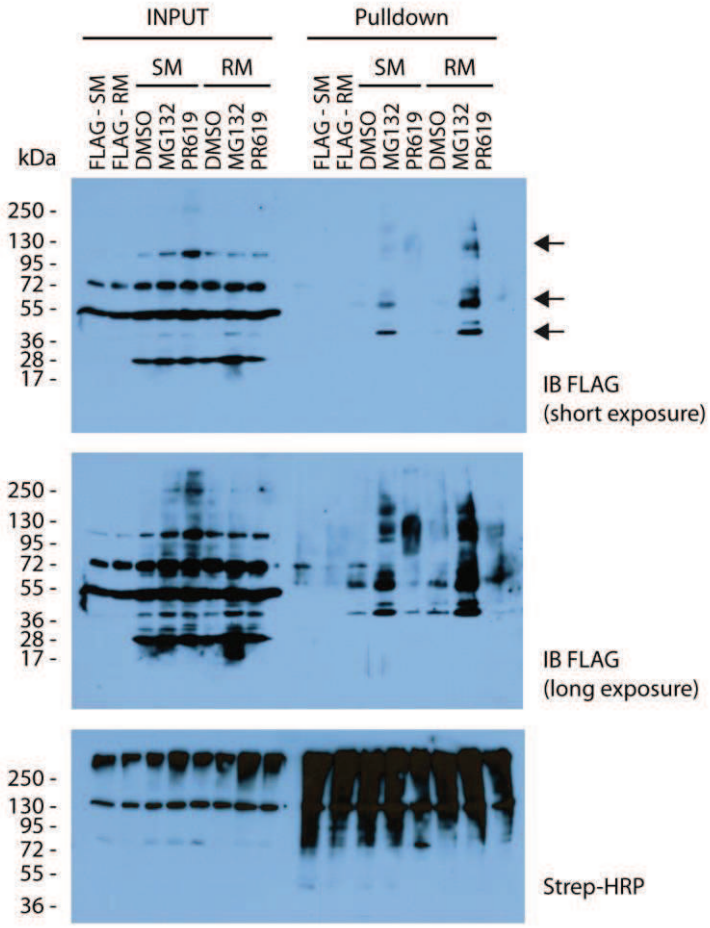


Figure 40: C3a is differentially ubiquitylated in primary hepatocytes

Western blot after His-Biotin pulldown from primary hepatocytes whole-cell lysates. Primary hepatocytes expressed His-Bio-Ubiquitin and either FLAG or FLAG-C3a. Cells were treated with DMSO, 10 µM MG132 (proteasome inhibitor) for 4 hours, or 10 µM PR619 (DUB inhibitor) for 2 hours. Primary hepatocytes (C) were additionally treated with starvation media (SM) or rich media (RM). Antibody against FLAG was used to detect exogenous C3a and Streptavidin-HRP was used to detect ubiquitylated proteins with exogenous ubiquitin. His-Bio pulldown and input are shown as indicated. Arrows indicate upshifted ubiquitylated C3a.

Collectively, these results demonstrate for the first time, that C3 can be processed to C3a and C3b in hepatocytes and that C3 is differentially ubiquitylated. I observed ubiquitylated C3 in HEK293T cells as well as in primary hepatocytes and preliminary results with TUBEs 1 pulldowns from livers showed higher levels of ubiquitylated C3 in the livers of refed mice.

Together, this confirms the proteomic screen. *In vitro*, the C3a fragment of C3 can indeed be ubiquitylated in primary hepatocytes, which might affect its function in metabolism and/or inflammation.

We have also observed that *Ctsl* is expressed and it is potentially the enzyme responsible for this cleavage which will need additional investigations. In addition, C3aR is not only expressed by hepatocytes, but also present intracellularly. Whether intracellular C3a can activate its intracellular receptor also needs to be verified. In addition, we have also observed that C3b is present in higher levels in refed livers, suggesting that the cleavage of C3 into C3a and C3b might be regulated by ubiquitylation of C3.

Moreover, due to the expression of *Cpn1* and *Cpb2*, C3a can potentially be further cleaved into C3adesArg and activate its receptor C5L2. Whether these enzymes and receptor are present intracellular in hepatocytes and have a physiological relevance still need to be tested.

Discussion

In vertebrates, the liver has developed to be a major metabolic organ able to control glucose and lipid homeostasis. It can store polysaccharides in the form of glycogen, and catabolize them when blood sugar levels are low. When glycogen stores run out, the liver can also produce glucose *de novo* (gluconeogenesis) from some amino acids, glycerol and pyruvate. On the other hand, in the post-prandial period, the liver switches off gluconeogenesis and it starts taking up glucose from the blood to restore the glycogen levels. And when the carbohydrate levels remain elevated, the liver can also produce fat *de novo* from carbohydrate precursors via the lipogenic pathway (*de novo* lipogenesis). Importantly, due to its central role in metabolism, a deregulation of these specific responses contributes to the development of several metabolic diseases, for instance, non-alcoholic fatty liver disease (NAFLD) and type two diabetes (T2D).

Metabolic pathways can be regulated by gene expression, allosteric modulation and post-translation modifications (PTMs). PTMs provide one of the fastest ways for cells to adapt to different stimuli and environmental cues. Phosphorylation and acetylation have been well studied thus far related to regulation of metabolic patyhways. In spite of the fact that ubiquitylation is an emerging concept in the field of signal transduction and was already well studied in cell cycle and inflammation fields, it has not received much attention in the field of metabolism. Ubiquitylation can modulate proteins in different ways; it can affect protein activity, subcellular localization, protein-protein interactions, and target proteins to proteolysis. For this reason, we sought to look at proteins in the liver of mice that are modified by ubiquitylation. Importantly, we screened for proteins which were differentially ubiquitylated in the livers of mice upon a metabolic challenge; fasting and refeeding with high sucrose diet.

1. Purification of ubiquitylated proteins with UBD-based technologies

The purification and identification of ubiquitylated proteins, especially from murine tissues, represented the first challenge of this study. Thus far, there is no “gold standard” technique considered to be the best method to purify ubiquitylated proteins to be further analyzed by mass spectrometry. Also, the analysis of ubiquitylated peptides by mass spectrometry is rather challenging due to the need of high sensitivity and accuracy.

We have decided to use tools based on ubiquitin-binding domains (UBDs) to enrich for ubiquitylated proteins, which were subsequently digested and analyzed by mass spectrometry. As TUBEs and UbiQapture are based on different UBDs, each of them enriched for a different subset of ubiquitylated proteins, showing the advantage of using this complementary approach. TUBEs 1 are based on the UBA domains of the protein ubiquilin and it shows a K_D of 9 nM for K48-linked chains and a K_D of 0.7 nM for K63-linked chains (Hjerpe et al, 2009). On the other hand, less is known about UbiQapture, but it is considered to be able to detect many different ubiquitylation forms, including monoubiquitylation. We observed that both of these tools were very efficient to enrich for ubiquitylated proteins in the eluted fractions, which can be seen by the ubiquitin western blots. Altogether, we identified 1641 proteins with high confidence levels, and from these proteins, 25 had their ubiquitylation sites identified. The low number of identified sites is expected due to the technique used that enriches for modified proteins. Once these proteins are digested, the peptide mixture will be comprised of a complex mixture of peptides with very few peptides containing the di-Gly motif characteristic of an ubiquitylation site.

More recently, the use of antibodies that enrich for ubiquitylation at the peptide level have been proven to be useful to increase the number of identified proteins and ubiquitylation sites (Kim et al, 2011; Wagner et al, 2011; Wagner et al, 2012; Emanuelle et al, 2012). With this technique, many groups have identified more than 20000 ubiquitylation sites. It has to be noted that all of these studies were performed in cultured cell lines with exception of Wagner et al (2012). When performed in cell lines, they made use of drugs to inhibit proteasome, such as MG132 and bortezomid. One criticism that this method has been receiving is the fact that due to its high sensitivity and the combination with drugs that affect ubiquitylation levels and the cell machinery, it might be that they identify many false-positive proteins. This is a criticism that arises from the fact that they identified several extracellular domains of proteins to be ubiquitylated, which nobody has ever shown that it exists. Another criticism is based on the two distinct antibodies developed so far; they show a bias towards a different sequence of amino acids. This issue was addressed by Wagner et al (2012) and the group confirmed that there was a small overlap between the proteins identified with each antibody.

Yet another possible way to purify ubiquitylated proteins would be to express a tagged ubiquitin in the livers of mice, either by generating a transgenic mouse line or by overexpressing it with adeno-associated viruses, for instance. This technique would allow introducing different tags to

ubiquitin, including tags that permit denaturing pulldowns, which would remove all of the proteins interacting with ubiquitylated proteins. A criticism to this technology is the fact that the exogenous expression of ubiquitin might lead to non-physiological ubiquitylation of certain proteins. This difficulty could be overcome by the generation of an endogenously tagged ubiquitin, for instance using the CRISPR system. Due to the fact that ubiquitin is expressed from 4 different genes and they encode ubiquitin in tandem with either a long chain of ubiquitin molecules or in tandem with ribosomal proteins, it could be difficult to modify the endogenous loci. We have also tried to generate a knock-in mouse expressing His-Bio-Ubiquitin in the endogenous locus of Rosa26 promoter. We failed to see expression of ubiquitin, due to the fact that ubiquitin might be a very well controlled transcript, and if not in tandem with ribosomal proteins or with other ubiquitin molecules, the transcript is not translated.

Our study could eventually be complemented with the use of these other methods, such as the anti-di-Gly antibody and tagged ubiquitin pulldowns from livers. In addition, a combination of both di-Gly antibodies developed so far can reduce the bias and increase the specificity for ubiquitylated proteins. Also, a combination of techniques that enrich ubiquitylated proteins first, such as TUBEs or UbiQapture, followed by peptide-level enrichment might be a very successful way to identify ubiquitylated proteins in the physiological context and increase the number of ubiquitylated sites, reducing the drawbacks of both techniques.

2. Putative ubiquitylated proteins are identified with mass spectrometry in the liver of mice

Most of the mass spectrometry studies published so far with ubiquitylation aimed at providing a catalogue of ubiquitylated proteins in different cell lines and in the presence and absence of different proteasomal inhibitor drugs (Kim et al, 2011; Wagner et al, 2011). These both studies looked at ubiquitylated proteins in HCT116 and 293T cells. Another study used mass spectrometry to look at targets of the E3 ligase parkin in response to mitochondrial depolarization in HCT116 or neuronal SH-SY5Y cells (Sarraf et al, 2013). It is important to note that all of the studies published so far were performed using cell lines, with one exception. Wagner et al (2012) identified ubiquitylated proteins in the following murine tissues: kidney, liver, muscle, brain and heart. Interestingly, the tissue in which most ubiquitylated proteins were identified was the liver.

Based on this background, we are first at presenting data on ubiquitylation in tissues in a physiological context; i.e. fasting and refeeding of mice. We chose to look at the fasting-refeeding response for several reasons. Firstly, we wanted to synchronize mice at a specific stage. Secondly, refeeding with a high sucrose diet leads to a strong induction of *de novo* lipogenesis. And thirdly, it is known that a deregulation of pathways, such as gluconeogenesis and lipogenesis, leads to the development of diseases. To this end, we fasted all the mice for 16 hours and half of them we sacrificed after fasting, while the other half was refed with a high sucrose diet containing 70% of sucrose (50% glucose and 50% fructose) and no fat. The high carbohydrate content of this diet induces the liver to activate the *de novo* lipogenic pathway, one of the pathways that contribute ultimately to the development of liver steatosis and NAFLD. The presence of fructose also acts as a strong lipogenic compound due to its particular metabolism in the liver.

In this screen we performed 5 independent fasting-refeeding experiments with 8 to 10 mice in each experiment. TUBEs were used in 3 of these experiments, with 26 mice in total – 13 in each condition, fasted and refed. UbiQapture was used in 2 experiments, with 20 mice in total – 10 in each condition, fasted and refed. We observed a high number of proteins identified in common between the experimental replicates, which showed the great reproducibility between the experimental replicates. Around 50% of the TUBEs-identified proteins were identified in at least 2 independent experiments, while around 70% of the UbiQapture-identified proteins were identified in both experiments. As expected, there was a small overlap between identified proteins from TUBEs and UbiQapture pulldowns, which demonstrated the advantage of using both techniques in a complementary manner.

As already mentioned before, one limitation of the protein-based enrichment of ubiquitylated proteins for mass spectrometry is the low number of identified ubiquitylated sites (also discussed in Carrano and Bennett, 2013). For this reason, we searched in 2 large-scale ubiquitylation studies performed in HCT116 and HEK293T cells (Kim et al, 2011) and in murine tissues (Wagner et al, 2012) to understand which of the proteins identified by us were already shown to be ubiquitylated. In fact, around 65% of the proteins we identified were shown to be ubiquitylated by either one of these studies. This overlap may indicate that probably a large amount of the proteins we identified were indeed ubiquitylated in the livers. We also believe that part of the proteins we identified will be ubiquitylated only in the liver upon a certain stimulus, which would

not be seen in HCT116 and HEK293T cells or in ad libitum livers. Nonetheless, probably part of the proteins identified in this study were in a complex with an ubiquitylated protein. In this case, these binders will be pulled down together and be equally identified by mass spectrometry. This happens due to the fact that these pulldowns are performed under native conditions, and allow protein complexes to form and proteins to interact. However, we tried to reduce this binding by increasing the stringency during the pulldown. In fact, we washed the beads after the pulldowns with 400 mM of NaCl, to reduce non-specific binding to the UBDs.

We also identified proteins related to metabolism that were already studied to be ubiquitylated. For instance, acyl-CoA desaturase 1 (SCD1) is a protein belonging to the lipogenic pathway. It participates in the desaturation of fatty acids, essential steps in lipid biogenesis. SCD1 was already shown to be ubiquitylated and degraded when lipogenesis has to be switched off (Kato et al, 2006). Another example of a protein shown to be ubiquitylated is 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR). This enzyme participates in the rate limiting step of the cholesterol biosynthesis and it was shown to be ubiquitylated and degraded when there is no cholesterol biosynthesis (Song et al, 2005a; Song et al, 2005b).

3. Differentially identified proteins are selected based on label-free quantification

The initial aim of our study was to detect proteins in liver metabolism that were regulated by ubiquitylation in different conditions of nutrient availability; i.e. fasting and refeeding. For this reason we made use of a label-free quantification to enable the comparison of identified proteins between these two separate conditions. Label-free quantification (MS/MS) provides a good estimation of the levels of the peptides identified (Zhang et al, 2010; Wong and Cagney, 2010). However, it is known that SILAC (stable isotope labeling amino acids in cell culture) provides a more precise quantification of peptides identified (Zhang et al, 2010). SILAC is very widely used in mass spectrometry to compare 2 or 3 different conditions in cell culture. However, very few groups so far could combine the SILAC technique with the use of mouse or human tissues. One group managed to label a mouse line by feeding the mice with labeled food over several generations (Zanivan et al, 2012). Another group used a technique called spike-in SILAC to quantify protein phosphorylation in murine livers (Monetti et al, 2011). In this technique, the protein extracted from SILAC labeled cells is added (or spiked-in) to the samples to be

measured before trypsin degradation. In this way, it is possible to compare relative amounts of the identified proteins to the labeled proteins that were spiked-in, and ultimately compare the relative amounts in 2 conditions. However, the spike-in technique has not been used yet in combination with the biochemical enrichment of proteins, which is required in the identification of ubiquitylated proteins. Thus, the quantification of ubiquitylated proteins with SILAC in tissues still needs to be further developed.

For these reasons, in order to select the differentially identified proteins we used the quantification levels provided by the label-free quantification in Maxquant and analysed them with Perseus. Finally, we looked at the ratio between refed- and fasted- identified proteins and the statistical significance provided by the ANOVA statistical analysis. In this way, we selected 117 proteins that were potentially ubiquitylated or bound to ubiquitylated proteins specifically during refeeding or fasting, respectively.

Due to the fact that ubiquitylation of proteins changes their functions in the cell in many ways, including their targeting to proteasomal degradation, we believe that individual assessment of each protein is needed to be able to conclude the function of ubiquitylation events. However, a few facts have to be pointed out. Identification of proteins under refeeding and fasting, respectively, can arise from two possible scenarios. 1) Ubiquitylation of proteins is specifically induced under a specific condition, while the abundance of these proteins does not change. 2) The abundance of proteins changed upon a particular metabolic challenge allowing us to detect ubiquitylated forms only in one condition. Importantly, in both cases, ubiquitylation events may entail specific functions upon one particular metabolic challenge. Moreover, as we did not use proteasome inhibitors, we believe that most of the proteins belonging to the list will not be targeted for degradation, but that ubiquitylation is involved in signaling. However, we cannot exclude, depending on the kinetics of degradation and levels of the proteins, that some of the identified proteins are indeed targeted for degradation.

4. Pathway enrichment analysis reveals that many metabolic proteins are enriched amongst all identified proteins

With pathway enrichment analysis we observed that many of the identified proteins were belonging to metabolic pathways. This enrichment remained the same when we looked at all 117 differentially identified proteins.

4.1. Pathways enriched in fasting conditions

During fasting conditions, we observed proteins involved in lipid metabolism, such as lipin 1 and lipin 2. Both of these proteins belong to the lipin protein family, which consists of three members with highly conserved N- and C-terminal regions. Lipin 1 is the most-well studied protein in the family and it was shown to play an essential role in the glycerolipid biosynthesis as well as in gene regulation. In high-nutrient state, lipin 1 localizes to the endoplasmic reticulum membrane and acts as a phosphatidate phosphatase catalysing the convertion of phosphatidic acid to diacylglycerol in (Reue and Dwyer 2009; Dwyer et al, 2012). In low-nutrient conditions, however, lipin 1 translocates to the nucleus and may function as a transcriptional coactivator of fatty acid oxidation genes as it can interact with PPARα (peroxisome proliferator-activated receptor α) and PGC-1α (PPARγ coactivator 1α) (Finck et al, 2006). It was also shown that lipin 1 can increase nuclear SREBP abundance and promoter activity, thereby enhancing the expression of SREBP target genes (Peterson et al, 2011). The subcellular localization is regulated by phosphorylation by mTORC1; when lipin 1 is phosphorylated it remains in the cytoplasm, while its dephosphorylated state is nuclear (Peterson et al, 2011). Interestingly, lipin 1 is also called fatty liver dystrophy, due to its deficiency in mice leading to hypertriglyceridemia, lipodystrophy, insulin resistance and increased risk for atherosclerosis (Reue et al, 2000). Both lipin 1 and lipin 2 are potential targets of differential ubiquitylation, according to our screen. Additionally, we also confirmed lipin 1 to be ubiquitylated in HEK293T cells. In general, bifunctional enzymes (or moonlighting enzymes) (Moore 2004), such as lipin 1, have several ways of regulation and we speculate that ubiquitylation, together with phosphorylation, can play this role during fasting conditions.

Another metabolic protein we identified upon fasting of mice was starch-binding protein domain-containing 1 (STBD1). STBD1 contains two functional domains: a carbohydrate binding domain that can bind glycogen, and, an AIM (Atg8 interacting motif) that interacts with the autophagy machinery and target glycogen to lysosomal degradation (Jiang et al, 2010; Jiang et al, 2011). Very recently it was shown that the carbohydrate domain can also mediate interactions with

glycogen synthase, glycogen debranching enzyme, and the polysaccharide amylase and mutations of W293 in the carbohydrate domain abolished these interactions (Zhu et al, 2014). This group also showed that exogenously expressed STBD1 in COS cells is polyubiquitylated. Our screen has identified STBD1 as a putative target of ubiquitylation in fasted conditions. In addition, we confirmed the ubiquitylation in HEK293T cells and in primary murine hepatocytes. STBD1 ubiquitylation in fasting conditions could mediate its affinity to glycogen, and thereby increase its lysosomal degradation to generate glucose monomers. Ubiquitylation could also modulate its interaction with other proteins, such as glycogen synthase and glycogen debranching enzyme, which play roles in the glycogenesis and glycogenolysis, respectively. At last, ubiquitylation of STBD1 can also modulate its half-life, targeting it to proteasomal degradation, as it has been demonstrated by Zhu et al (2014). In this case, it is possible that STBD1 play a role in glycogen metabolism during fed conditions.

An interesting group of proteins identified during fasting were related to transmembrane transport of molecules, for instance the protein solute carrier family 2, facilitated glucose transporter member 9 (Slc2a9 or GLUT9). Despite its name, this is not a glucose transporter, but a urate uniporter instead (Preitner et al, 2009; Bibert et al, 2009). Another transporter identified specifically in fasted conditions was the protein solute carrier family 7, cationic amino acid transporter, Y+ system (Slc7a2). This is known to be responsible for the cellular uptake of arginine, lysine and ornithine (Kavanaugh et al, 1994). The uptake of amino acids is an important function of liver during fasting since they can be incorporated into certain pathways. Arginine, for instance, is a glucogenic amino acid, while lysine is ketogenic; participating in the generation of glucose and ketone bodies, respectively. Yet another protein related to transmembrane transport, is the protein PDZ domain-containing protein 1 (PDZK1). PDZK1 contains 4 PDZ domains that mediate a variety of cellular functions by interacting with proteins. It can act as a scaffold protein connecting plasma membrane proteins to regulatory intracellular components. For instance, it is involved in the regulation of Na^+/H^+ exchange playing an important role in tubule function (Cunningham et al, 2010). In the liver, it has been reported to regulate the expression of the HDL receptor scavenger receptor class B type I (SR-BI), which mediates some of the intracellular responses of HDL metabolism. PDZK1 was shown to be necessary to maintain SR-BI levels, thereby regulating lipoprotein metabolism. In addition, animal models have shown that PDZK1 expression is protective against the development of atherosclerosis (Kocher and Krieger, 2009; Clapéron et al, 2011). Our screen has identified STBD1 as a putative target of ubiquitylation in fasted conditions and we also confirmed its

ubiquitylation in HEK293T cells and in primary murine hepatocytes. Due to its role in lipoprotein metabolism, PDZK1 ubiquitylation during fasting might reduce its ability to bind SR-BI and recruit it to the membrane.

4.2. Pathways enriched in refeeding conditions

Proteins identified upon refeeding of mice also were enriched in metabolic pathways. For instance, enolase 1 (ENO1) and fructose-bisphosphate aldolase B (ALDOB) play a role in glycolysis and gluconeogenesis. ENO1 catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate, and ALDOB catalyses the reaction of either fructose 1,6-bisphosphate or fructose 1-phosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Importantly, both enzymes can catalyse the reverse reaction, depending on environmental concentrations of substrates. Moreover, they both participate in the metabolism of glucose and fructose, with ALDOB playing a crucial role in the initial steps after fructose uptake. ALDOB was shown by one group to be ubiquitylated and degraded within autolysosomes in response to amino acid and serum starvation (Lenk et al, 1999).

Despite the identification of 27 ubiquitin acceptor sites in enolase 1 discovered by large-scale mass spectrometry studies (Kim et al 2011; Wagner et al 2012), the regulation of enolase 1 by ubiquitylation was still not further investigated. We identified enolase 1 as a putative target of ubiquitylation during refeeding of mice. Also, we showed that it can be ubiquitylated in HEK293T cells and in primary hepatocytes. In addition, ubiquitylation was clearly increased when we treated cells with proteasome inhibitor, which suggests a rather degradative role to this modification.

Other proteins involved in metabolic pathways identified in mice upon refeeding were related to fatty acid desaturation (Fatty acid desaturase 1 (FADS1) and stearoyl-CoA desaturase-1 (SCD1)), cholesterol biosynthesis (24-dehydrocholesterol reductase (DHCR24) and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR)), lipid and cholesterol transport (Apolipoprotein L 7a (APOL7a), apolipoprotein B (APOB)) and steroid metabolism (11β-hydroxysteroid dehydrogenase type 1 (HSD11b1)). The role of ubiquitylation in the regulation of ApoB will be further discussed below.

In the liver, HSD11b1 is an enzyme able to reduce cortisone to the hormone cortisol. Active cortisol can bind and activate the nuclear glucocorticoid receptors, which will regulate gene transcription of many metabolic enzymes. In the fasted state, cortisol can stimulate the expression of gluconeogenic genes, and it has been reported that too much cortisol can lead to central obesity (Lee et al, 2014b). In the proteomic screen we identified HSD11b1 to be a putative target of ubiquitylation upon refeeding of mice. Also, we observed ubiquitylated HSD11b1 in HEK293T cells.

Also during refeeding, there were many proteins involved in vesicle transport, for instance, regulators of microtubule dynamics (regulator of microtubule dynamics protein 2), regulators of actin dynamics (spectrin proteins, actin-related protein 3, actin-related protein 2/3 complex subunit 2, F-actin-capping protein subunit alpha-2). Indeed, cytoskeleton remodelers play a key role in the transport of vesicles that will undergo exocytosis. The liver is an organ responsible for the secretion of several proteins. The most prominent function of liver during fed state related is the secretion of very low-density lipoprotein (VLDL) particles. VLDLs are synthesized in the lumen of the endoplasmic reticulum, they are transported to the Golgi and finally the mature VLDL particles are transported to the plasma membrane and secreted to the bloodstream (Tiwari and Siddiqi, 2012). It is very possible that the modulation of one of these proteins mentioned above by ubiquitylation will affect the secretion of proteins by the liver, including VLDL particles.

Interestingly, it has been shown that an unsuccessful VLDL assembly can result in the targeting of APOB for degradation. APOB is the main lipoprotein present in VLDLs and it is known to be needed for its assembly and secretion. In fact, APOB was detected to undergo endoplasmic reticulum associated degradation (ERAD) associated with proteasomal degradation or autophagy (Fisher et al, 2011; Fisher et al, 2014). The degradation of APOB directly affects VLDL secretion. This mechanism of degradation can happen in response to a problem in the folding during VLDL assembly as well as due to an active regulated process such as insulin signaling (Pan et al, 2004). Indeed, APOB is one of the proteins we discovered in the proteomic screen to be differentially ubiquitylated only upon refeeding of mice, constituting one of the positive controls.

5. Mitochondrial proteins were enriched amongst identified proteins

With cellular compartment gene ontology analysis we identified mitochondria as one of the common compartments amongst all identified proteins. The pattern was the same when we looked at proteins differentially ubiquitylated in the livers of fasted and refed mice.

Some of the mitochondrial proteins identified specifically during fasting conditions were involved in the conversion of pyruvate to acetyl-CoA (PDK4 - pyruvate dehydrogenase lipoamide kinase isozyme 4), in the mammalian heme biosynthetic pathway (Delta-aminolevulinate synthase 1), in the metabolism of branched-chain amino acids to eventually yield acetyl CoA and acetoacetate (methylcrotonoyl-Coenzyme A carboxylase 1), and in the mitochondrial oxidative phosphorylation as an uncoupling protein (hepatocelluar downregulated mitochondrial carrier protein).

On the other hand, some of the mitochondrial proteins identified during refeeding of mice were involved in conversion of pyruvate to acetyl-CoA (PDHX - pyruvate dehydrogenase complex, component X), in the catabolism of choline (dimethylglycine dehydrogenase), in the mitochondrial import (TOMM20), and in the β-oxidation of long chain fatty acids (3-ketoacyl-CoA thiolase).

One interesting observation is that PDK4, identified in fasted mice, and PDHX, identified in refed mice, belong to the same complex (pyruvate dehydrogenase complex – PDC) responsible for the conversion of pyruvate to acetyl-CoA. This is a reaction that delimits the fate of pyruvate in the cell. If pyruvate is converted to acetyl-CoA, this molecule will be either further oxidized in the TCA cycle or be used in the biosynthesis of lipids. On the other hand, if pyruvate is not converted to acetyl-CoA, it remains available as a substrate for gluconeogenesis. PDK4 can phosphorylate and inhibit the activity of PDC, which happens during fasting conditions in order to have pyruvate for the gluconeogenic pathway. On the other hand, PDHX provides a scaffold necessary for the complex formation. During postprandial conditions, PDK4 has to be inhibited and PDC active. According to our data, PDK4 and PDHX were identified as putative targets of ubiquitylation in fasted and refed conditions in the proteomic screen. We also identified both of them as being targets of ubiquitylation in HEK293T cells. For this reason, it would be interesting

to investigate whether ubiquitylation is regulating the activity of the PDC by modifying its subunits.

Many mitochondrial proteins from all mitochondrial compartments were already shown to be modified by ubiquitylation such as mitofusin 1 and 2, TOM20, TOM70 (Chan et al, 2011), uncoupling proteins 2 and 3 (Azzu and Brand, 2010) and oligomycin sensitivity conferral protein (Margineantu et al, 2007). In addition, other mass spectrometry-based studies in purified mitochondria have identified components of ubiquitin system (Livnat-Levanon and Glickman, 2011). For instance, MITOL/MARCH5 (Nagashima et al, 2014) and MULAN (Li et al, 2008) are E3 ligases located in the outer mitochondrial membrane. In addition, USP30 is a DUB located also in the outer mitochondrial membrane (Nakamura and Hirose, 2008).

In all identified cases so far, ubiquitylation of these proteins was related to degradation. In fact, the ubiquitin proteasome system is known to be involved in mitochondrial protein quality control, which is a mechanism that protects mitochondrial integrity. However, it is still not clear how the cytosolic ubiquitin system modifies and recognizes the mitochondrial proteins, in particular the ones that are not exposed to the cytosol. One possibility would be that these proteins are recognized in the mitochondria by other proteins, translocated to the cytosol, modified and targeted to the proteasome. Recent studies have provided evidences that the protein CDC48/p97 might be playing a role as an adaptor protein linking mitochondrial proteins to the cytosolic degradation components (Heo and Rutter, 2011). So far it was not shown that proteins inside the mitochondria can be ubiquitylated, there are no reported E3 ligases or DUBs in the mitochondrial matrix. It is speculated that one of the mechanisms by which proteins inside mitochondria could be ubiquitylated is during the process of fission and fusion. Also, proteins could already be imported to the mitochondria modified with ubiquitylation.

Ubiquitylation also plays a role in regulating mitochondrial dynamics (fission and fusion) by targeting proteins such as mitofusin 1 and 2, FIS1 and DRP1 to proteasomal degradation. These are major regulators of mitochondrial dynamics (Tanaka et al 2010; Chan et al, 2011; Wang et al, 2011). Mitophagy, a mechanism by which mitochondria are selectively targeted to autophagy, also requires the ubiquitin system. A cytoplasmic E3 ligase, PARKIN, is known to play a role in the initiation of mitophagy (Tanaka et al, 2010; Chan et al, 2011).

Due to the essential role of mitochondria in cells in generating ATP and reactive oxygen species, the accumulation of damaged mitochondria or of unfolded and non-functional proteins whitin mitochondria can affect the cellular integrity. The presence of dysfunctional mitochondria leads to many diseases, such as neurodegenerative diseases. Friedreich's ataxia is genetic disease that leads to a progressive neuronal degeneration and cardiomyopathy, and is caused by reduced levels of frataxin (FXN) protein. This is a mitochondrial protein involved in the formation of iron-sulfur clusters. Animal models have shown that prior to the degenerative phenotype, FXNKO mice show dysfunctional mitochondria (Puccio, 2007). By looking at the mitochondria purified from liver specific FXNKO mice we observed that ubiquitylation levels are dramatically reduced, evidence that could link ubiquitylation to mitochondrial function. However, in this model we could not decipher whether reduced ubiquitylation was a cause or a consequence of dysfunctional mitochondria.

The metabolic function of mitochondria has been linked to the postrranslational modification acetylation. According to mass spectrometry studies, approximately 35% of the mitochondrial proteome are modified by acetylation. Moreover, the acetylation levels increase in the liver during fasting (Kim et al, 2006; Wang et al, 2010b; Zhao et al, 2010; Anderson and Hirschey, 2012). The deacetylating protein sirtuin 3 was identified as a mitochondrial specific deacetylase that is able to remove the acetyl group from many proteins (Schwer et al, 2002; Schwer et al, 2006). Interestingly, a mitochondrial specific acetylase wasn’t identified. In sirtuin 3 KO mice, proteins in the mitochondria were hyperacetylated, had an altered function and mitochondria became dysfunctional (Anderson and Hirschey, 2012; He et al, 2012). In one study sirtuin 3 was shown to deacetylate the enzyme long-chain acyl-CoA dehydrogenase (LCAD) and thus stimulate fatty acid oxidation (Hirschey et al, 2010). Another described target of sirtuin 3 is the enzyme involved in the ketone body synthesis 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), which is activated when deacetylated (Shimazu et al, 2010).

We also wondered if, similarly to acetylation, the levels of ubiquitylation were going to be modified upon fasting feeding, and we didn’t observe any global changes in western blots of mitochondrial isolated preparations. However, in the mass spectrometry data we observed that despite mitochondrial proteins being enriched in both, fasting and refeeding conditions, there were different proteins enriched in each case. This indicates that ubiquitylation is important to maintain proper metabolic mitochondrial function, since in the fasting-refeeding switch,

mitochondria play an essential role to switch from ketone bodies and fatty acid oxidation to pyruvate oxidation.

6. Eight selected proteins were shown to be ubiquitylated in a biochemical validation

We selected 8 proteins identified in the proteomic screen to undergo a biochemical validation in cellular models. These proteins were selected based on their cellular function in combination with the significance of the difference between fasted and refed conditions. We were able to confirm the ubiquitylation of all of these proteins in cellular models. It has to be noted that we considered the protein to be ubiquitylated during this validation, if it was identified with at least one of the techniques and cell lines. However, it is possible that, if proteins were only identified with FLAG IPs and not with denaturing ubiquitin pulldowns, they might not be ubiquitylated, but belong to a complex in which one of the proteins was ubiquitylated. On the other hand, this situation could also represent a scenario in which ubiquitin pulldowns were not sensitive enough to identify the protein of interest.

The protein C3 was one of the hits identified with higher confidence levels. In addition, both techniques, TUBEs and UbiQapture pulled down C3 in refeeding conditions. The biochemical validation confirmed its ubiquitylation in both, HEK293T cells and primary hepatocytes.

7. C3 is a key component of the innate immune system

The complement component 3 (C3) is a protein that belongs to the complement system and C3 plays a central role in this immune defense cascade. The complement system comprises more than 60 proteins and most of them are synthesized and secreted by the liver in an inactive state. The complement system is one component of innate immunity, participating in the clearance of pathogens and damaged cells and in the modulation of adaptive immune responses (Ricklin et al, 2010). In fact, it recognizes exogenous and endogenous danger motifs, such as pathogen-associated molecular patterns (Zipfel et al, 2007). The activation the complement system can go through 3 pathways: classical, lectin and alternative (Noris and Remuzzi, 2013). Independently on the pathway utilized, it results in the activation and cleavage of C3 into C3a anaphylatoxin and C3b. C3a can bind its G-coupled protein receptor C3aR on the surface of cells. C3b can

either bind its receptor (CD11b) on cell surfaces inducing phagocytosis and cell activity, or it can be incorporated into a complex (C3 convertase) that will further cleave C3 and C5, amplifying complement activation. C5 cleavage into C5a anaphylatoxin and C5b mediates the lysis of pathogens and unprotected host cells and activates cellular immune responses. Importantly, human cells express complement regulators that protect them from harmful complement attack (Ricklin and Lambris, 2013). The anaphylatoxins C3a and C5a are pro-inflammatory peptides that can mediate a large variety of cellular functions, such as enhancement of phagocytosis, recruitment and activation of inflammatory cells, (Ricklin et al, 2010). They can also modulate secretion of cytokines such as IL-6 and TNF-α from B-cells and monocytes (Fischer and Hugli, 1997; Fischer et al., 1999). In addition, they also mediate tissue regeneration and fibrosis (Klos et al, 2009).

Very recently, a new function and activation mechanism was identified. By investigating the mechanisms underlying T-cell activation and production of complement proteins, Liszewski et al. (2013) identified a new intracellular feature of the complement system. Based on the fact that T-cells express various endosomal and lysosomal proteases, they investigated the presence of intracellular cleavage of C3. In fact, they identified that cathepsin L (CTSL) could cleave C3 into C3a and C3b intracellularly, and this cleavage generated active C3a and C3b. In addition, they showed that these cells express intracellular C3aR, which upon C3a binding, led to the activation of mTOR signaling pathways that regulate survival of resting T-cells. Upon activation, cells switched off this mechanism and translocated the machinery to the cell surface. In this way, T-cells have a pool of C3a for rapid release during activation. However, the mechanism how the activation and cleavage of C3 is regulated is unclear and posttranslational modifications like ubiquitin might be involved.

8. C3 can also modulate postprandial lipid metabolism

Many studies so far have associated the activation of the complement system with components of the metabolic syndrome. The plasma levels of C3 and C3a were found to be higher in obese compared to non-obese subjects. Moreover, circulating concentrations of C3 were associated with central adiposity. In addition, C3, C3a and C5a levels were associated with systemic low-grade inflammation and systemic insulin resistance. Several studies have suggested that the

role of C3 in the metabolic syndrome is not only related to inflammation, but possibly also involving a role of C3 in postprandial lipid metabolism (Hertle et al, 2014; Cianflone et al, 2003).

C3 levels were also associated with hepatic steatosis, liver dysfunction, NAFLD and AFLD (alcoholic fatty liver disease) (Hertle et al, 2014). Patients with severe NAFLD showed increased C3 mRNA level in the liver (Sreekumar et al, 2003) and increased C3 plasma levels (Yesilova et al, 2005). Interestingly, their C3 levels were even higher than in patients with chronic viral hepatitis, which agrees with a potential role of C3 beyond inflammation. C3a levels were also associated with liver fat percentage and hepatocellular damage in patients independently on the alcohol consumption (Wlazlo et al, 2013). Moreover, patients with NAFLD showed increased activity of complement and deposition of activated complement proteins around hepatocytes with macrovesicular steatosis (Rensen et al, 2009). In the same study, patients that had activated C3 deposits had higher levels of hepatic neutrophil infiltration and IL-6 expression. Importantly, the progression of NAFLD to NASH was more prevalent in patients with activated complement (Rensen et al, 2009).

Experiments performed in C3 knockout (KO) mice showed that these mice developed more liver steatosis upon high-fat diet feeding than wild-type controls, indicating that probably basal levels of C3 are beneficial for liver function (Bykov et al, 2006; Bykov et al, 2007). In contrast, C3KO mice fed ethanol in a chronic or acute manner showed reduced levels of TAG accumulation in the liver when compared to wild-type controls, suggesting that C3 contributes to the development of ethanol-induced fatty liver in mice (Bykov et al, 2006). In addition, C3KO mice under ethanol treatment showed downregulation of lipogenic enzymes (Bykov et al, 2007).

The adipose tissue is both an effector and a target of the complement system. Patients with insulin resistance or type 2 diabetes have shown to have a higher expression of C3 in adipocytes, which was associated with higher plasma lipids and larger waist circumference (van Greevenbroek et al, 2012). In fact, the complement system seems to modulate both adipose tissue inflammation and metabolism, being associated with the progression of obesity. The modulation of adipose tissue metabolism by the complement system goes through the molecule C3adesArg or ASP (acylation-stimulating protein). C3adesArg is generated by the cleavage of the C-terminal arginine residue from C3a by the carboxypeptidases B2 or N1 (Bokisch and Müller-Eberhard, 1970; Matthews et al, 2004). C3adesArg was shown to be immunologically inactive (Sayah et al, 2003), but it possesses a metabolic activity in adipocytes (Cianflone et al,

2003). Actually, C3adesArg was shown to be a powerful stimulator of triacylglycerides (TAG) synthesis through stimulation of fatty acid incorporation into adipose triglyceride (FIAT) (Cianflone et al, 1999). C3adesArg can also increase glucose uptake in adipocytes by enhancing the translocation of glucose transporters (GLUT1, GLUT3 and GLUT4). In addition, C3adesArg can inhibit hormone-sensitive lipase (HSL)-mediated lipolysis (Van Harmelen et al, 1999). Moreover, these effects seem to be independent of insulin signaling (Germinario et al, 1993; Maslowska et al, 1997). Some studies have shown that C3a can also be a stimulator of TAG synthesis in adipocytes, meaning that the conversion from C3a to C3adesArg might not be necessary for its metabolic function (Cianflone et al, 1994; Murray et al, 1997). The downstream mechanisms that mediate the metabolic functions of C3adesArg, and possibly C3a, are still under investigation. It has been proposed that C3adesArg can bind to the C5aR-like receptor 2 (C5L2). In addition, in 3T3-L1 adipocytes, C3adesArg was shown to activate AKT, MAPK, ERK1, and NFκB signaling pathways (Maslowska et al, 2006; Poursharifi et al, 2013; Tom et al, 2013). More specifically, AKT and ERK pathways were shown to be involved to mediate the effects of C3adesArg in TAG metabolism in adipocytes (Maslowska et al, 2006).

9. Ubiquitylation might regulate the function of C3 in livers

In our proteomic screen we identified C3 as being a putative target of ubiquitylation in the livers of mice refed a high sucrose diet. More specifically, the peptides identified were belonging to the C3a part of C3, which could indicate that C3a was the modified molecule. In addition, we showed that overexpressed C3 is ubiquitylated in HEK293T cells and in primary hepatocytes and that endogenous C3 is also ubiquitylated in primary hepatocytes. Altough the overexpressed C3 seemed to be targeted to degradation due to increased ubiquitylation upon MG132 treatment, the endogenous C3 ubiquitylation was not affected by MG132 treatment, indicating a non-degradative role. Preliminary results using TUBEs and liver lysates confirmed the proteomic screen showing that more ubiquitylated C3 is pulled down from the livers of refed mice. At last, I observed that overexpressed C3a is ubiquitylated in primary hepatocytes.

Due to the fact that C3 is an extracellular protein, the identification of its ubiquitylation was surprising, since ubiquitylated extracellular proteins have never been reported, apart from one study in mammalian epididymis (Baska et al, 2008). However, it has to be noted that C3 and

many other components of the complement system are synthesized and secreted in large part by the liver.

Motivated by the recent work from Liszewski et al (2013) showing that C3 can be cleaved in T-cells into C3a and C3b, we checked whether this mechanism also happens intracellularly in hepatocytes. Indeed, we observed that C3 can be cleaved in hepatocytes into C3a and C3b. In addition, the enzyme responsible for the cleavage in T-cells, CTSL, was also expressed in livers. In fact, we observed increased expression of CTSL in the livers of fasted mice. This observation is somewhat expected due to the broad functions of CTSL in lysosomal degradation, which is activated upon fasting to degrade intracellular proteins. Conversely, we observed higher levels of C3b in the livers of refed mice along with enhanced ubiquitylation. This finding indicates increased cleavage of C3 into C3a and C3b during refeeding and that ubiquitylation might affect this process. Thus, intracellular hepatocyte C3 cleavage might depend on the energetic state of the organism. Further investigations will be necessary to show whether ubiquitylation of C3 directly affect this cleavage and if this mechanism also exists in T-cells.

According to previously published data, C3 plays not only an inflammatory role in our organisms, but also a metabolic role with the stimulation of lipogenesis. Importantly, many groups discuss the local generation of activated complement components within tissues and that this activation seems to play roles beyond inflammation (Klos et al, 2009). Based on my findings so far and on the published functions of C3 I have built three hypotheses that involve possible roles of the ubiquitylation of C3 in hepatocytes. These hypotheses will be discussed below and are summarized in Figure 41.

9.1. The role of C3 in immunology and inflammation

In the first hypothesis (Figure 41) ubiquitylation plays a role in C3-mediated local inflammatory processes. In this case, ubiquitylation in refeeding conditions could affect the secretion of C3, the cleavage of C3 into C3a and C3b, or even the secretion of C3a.

C3a in the liver activates Kupffer and stellate cells, since they express the C3a receptor (C3aR) (Strey et al, 2003; Qin and Gao, 2006; Xu et al, 2013). Kupffer cells are specialized macrophages that can secrete cytokines, including TNFα, which activate stellate cells resulting

in collagen synthesis and fibrosis. Kupffer cells also secrete cytokines that will recruit neutrophils. Remarkably, the inappropriate recruitment and activation of neutrophils results in liver injury and disease (Xu et al, 2014).

In this hypothesis, the ubiquitylation in refeeding conditions can increase the secretion of C3a, which would lead to the activation of Kupffer and stellate cells, contributing to the inflammatory process that is strongly associated with the development of hepatic steatosis and liver fibrosis. In addition, the increased secretion of C3a would go in agreement with the high levels of C3a deposition around hepatocytes with steatosis observed by Rensen et al (2009).

This hypothesis can be corroborated by looking at liver levels of C3a in different animal models that would complement the findings in the model used by this study, such as chronic high-sucrose diet feeding. Also, primary hepatocytes can be used to assess C3a secretion, which should be decreased under inhibition of CTSL or of C3 ubiquitylation. The function of the ubiquitylation in this hypothetic pathway can be assessed by the use of C3 mutants with the mutated ubiquitylated Lys residue.

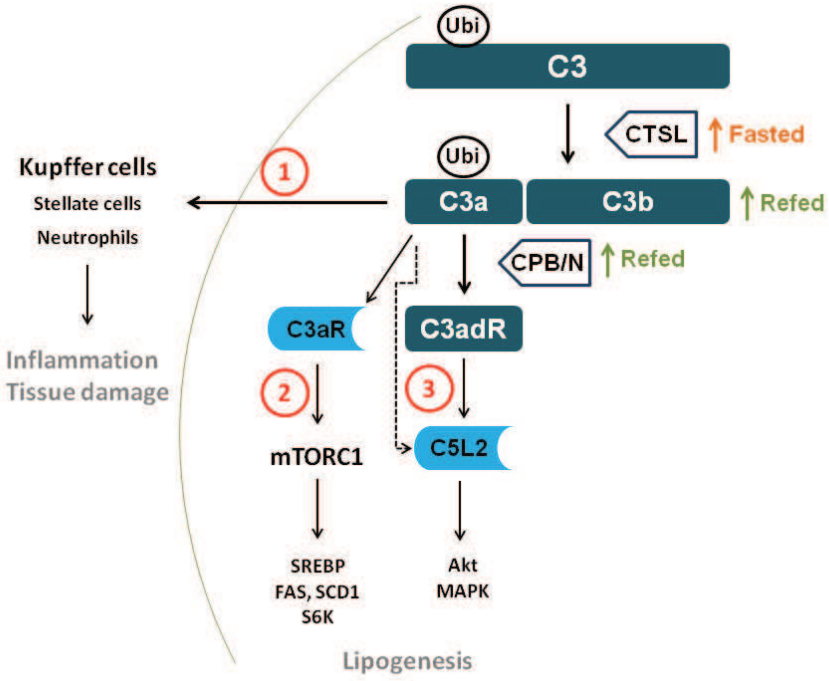


Figure 41: Model with 3 hypotheses for the role of ubiquitylation in C3-mediated liver metabolic and inflammatory functions

We suggest 3 possible roles for the regulation of C3 in the liver by fasting-refeeding and ubiquitylation. (1) Diet and ubiquitylation modulate the secretion of inflammatory C3a, which activates Kupffer cells, initiating the inflammatory response. (2) Diet and ubiquitylation modulate the activation of intracellular C3aR by C3a binding and activation of mTORC1 signaling. (3) Diet and ubiquitylation modulate the activation of intracellular C5L2 by C3adesArg binding and activation of AKT/MAPK signaling. Both hypothesis 2 and 3 will lead to an increase of lipogenesis in the liver.

9.2. The role of C3 as a nutrient sensor

In the second hypothesis (Figure 41) ubiquitylation regulates intracellular functions of C3a. In this case, ubiquitylation in refeeding conditions could affect the cleavage of C3 into C3a and C3b, C3a stability or C3a interaction with its receptor.

Similarly to T-cells, we have observed the presence of C3aR intracellularly in hepatocytes. C3aR is a G-protein coupled receptor that leads to the activation of mTORC1 signaling pathways in T-cells when activated (Liszewski et al. 2013). mTORC1 is an energy sensor in cells, and its activation leads to increased protein synthesis, increased lipogenesis, increased glycolysis and inhibition of autophagy.

If ubiquitylation in refeeding conditions could increase C3a intracellular signaling, this would lead to activation of C3aR and mTORC1. Thus, mTORC1 would contribute to the lipogenic effects of high-sucrose feeding through a C3a-mediated pathway. The observation that C3KO mice under ethanol treatment showed downregulation of lipogenic enzymes in comparison to wild-type controls goes in agreement with this hypothesis (Bykov et al, 2007), especially due to the similarities in between fructose and ethanol metabolism.

This hypothesis can be corroborated by experiments in primary hepatocytes in which CTSL is inhibited and intracellular levels of C3a are measured. Moreover, if mTORC1 is also activated by C3aR signaling, looking at the downstream signaling events following its activation can provide evidences of C3aR signaling. To this end, we can assess lipogenesis (SREBP, FAS, SCD), protein synthesis (S6K1 phosphorylation) and glucose metabolism (pyruvate tolerance test) in C3KO mice. In addition, we can use primary hepatocytes with CTSL inhibitor and siRNA against

C3aR. In a similar manner to the previous hypothesis, the use of C3 mutants with the ubiquitylated Lys mutated to Arg will help identifying the role of ubiquitylation in this pathway.

9.3. The role of C3 as a lipogenic molecule

The third hypothesis (Figure 41) suggests that ubiquitylation plays a role in the intracellular functions of C3adesArg to activate lipogenesis. In this case, ubiquitylation in refeeding conditions could affect the cleavage of C3 into C3a and C3b, C3a cleavage into C3adesArg, C3adesArg stability and C3adesArg affinity for its receptor.

The first condition for this hypothesis to be confirmed would be the actual processing of C3a into C3adesArg intracellularly in hepatocytes. So far, I have only confirmed that livers express the carboxypeptidases B2 and N1 (*Cpb2* and *Cpn1*), which can mediate the C-terminal arginine cleavage, and the receptor C5L2. Interestingly, the levels of Cpb2 and Cpn1 were increased upon refeeding conditions. Based on the role of C3adesArg in adipocytes, we could check for intracellular levels of C3adesArg and C5L2 as well as the activation of some of the downstream targets identified in adipocytes, such as AKT. These experiments could be performed in C3KO mice and in primary hepatocytes treated with siRNA against C5L2.

10. Conclusions

Taken together, we identified several putative ubiquitylated proteins in the livers of mice fasted and refed a high sucrose diet. The biochemical validation confirmed the ubiquitylation of all the proteins tested, reassuring the power of the approach used to identify ubiquitylated proteins. Due to the interesting function of C3 as a molecule able to mediate both, inflammatory and metabolic functions, and its identification with high confidence levels in the proteomic screen, we selected C3 to study into further detail. Based on our preliminary data, we believe that C3 might be playing a role in the development of liver steatosis and liver injury by a combination of its inflammatory and metabolic functions and that ubiquitylation can control C3 activity. Moreover, these functions might be regulated by ubiquitylation. By studying the regulation of main players involved in liver metabolism we will be able to better understand development of metabolic diseases.

Résume de thèse

Pour survivre, les organismes nécessitent un apport constant en énergie. Pour cette raison, les humains et les animaux ont évolués de manière á s'adapter aux périodes de déprivation de nourriture. Chez les vertébrés, le foie est ainsi l'organe majeur du métabolisme en étant le siège de la régulation des différentes voies du métabolisme qui vont contrôler l'homéostasie du glucose et des lipides. Il est essentiel que ces différentes voies métaboliques soient régulées le plus finement possible car une dérégulation de l'une d'elle peut entrainer une maladie métabolique sévère telle une stéatopathie hépatique non alcoolique NAFLD (Cohen *et al*, 2011) ou un diabète de type 2 (T2D) (Lin *et al*, 2009).

La phosphorylation et l'acétylation sont des modifications post translationnelles dont la capacité de réguler les voies métaboliques est connue (Oliveira *et al*, 2012 ; He *et al*, 2012), mais des travaux de recherche récents ont montré que l'ubiquitylation dans le foie est essentielle pour arrêter la production de glucose (gluconéogenèse) en réponse á une période de jeune (Liu *et al*, 2008; Jiang *et al*, 2011). Le rôle et le mécanisme d'action de l'ubiquitine sont l'objet de nombreuses recherches dans les domaines du cycle cellulaire et du cancer, mais peu ou pas dans la régulation du métabolisme.

Un exemple montrant l'importance de l'ubiquitination pour l'arrêt de la néoglucogenèse dans le foie est l'ubiquitination de CRTC2 (Figure 1). CRTC2 joue un rôle au cours du jeûne, durant lequel il stimule la gluconéogenèse en se liant à CREB après stimulation par le glucagon. Dentin *et al* (2007) ont montré que l'insuline peut conduire à l'activation de la kinase Ser/Thr SIK2 , qui phosphoryle CRTC2. La CRTC2 phosphorylée quitte le noyau et, dans le cytoplasme, elle s'associe à la ligase E3 COP1. COP1 peut polyubiquityler CRTC2 qui deviendra ainsi une cible à dégrader. C'est un des systèmes qui permet l'inactivation de la neoglucogenese dans des conditions riches en éléments nutritifs.

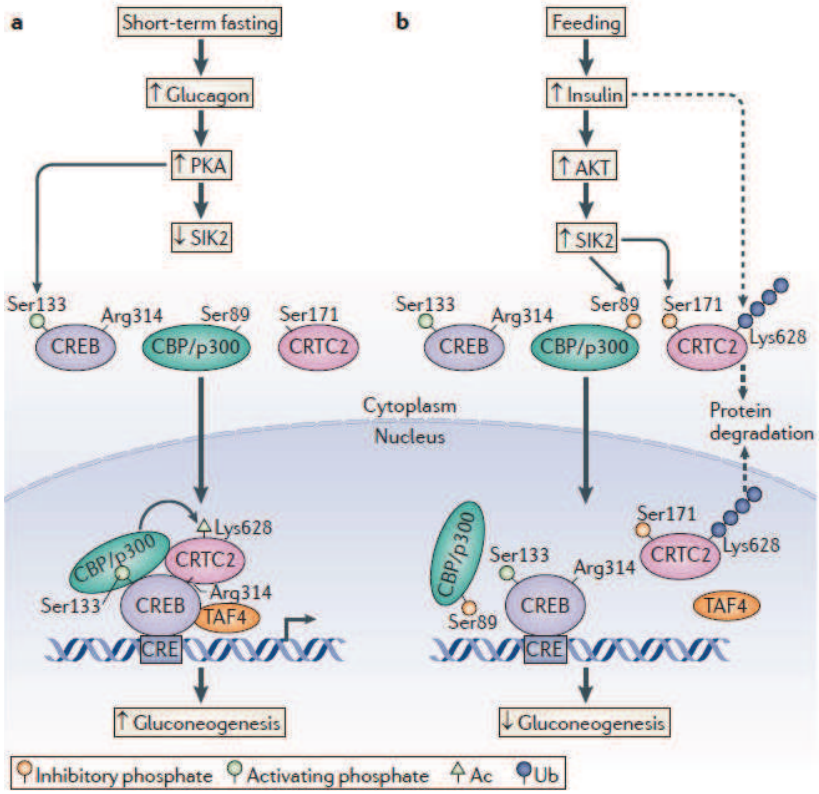


Figure 1: L'ubiquitylation de CRTC2 régule la néoglucogenèse

Au cours du jeûne, la voie de signalisation régulée par l'activité PKA active CREB pour augmenter l'expression des gènes gluconéogéniques. La signalisation par l'insuline permet d' arrêter ce processus en phosphorylant CRTC2 qui deviendra ainsi une cible pour la dégradation protéasomale via son ubiquitination. Altarejos et Montminy, 2011.

En se basant sur l'hypothèse que la signalisation par l'ubiquitine régule les voies métaboliques dans le foie, nous avons réalisé une analyse protéomique globale dans le but d'identifier des protéines ubiquitylées dans le foie de souris soumises á un protocole de jeûne – re-nourrissage (Figure 2). Il s'agit là de la première étude qui cherche á identifié des protéines ubiquitylées, dans le foie, dans des conditions physiologiques. En utilisant un protocole de jeûne – re-nourrissage (Cohen *et al*, 1972), nous avons cherché á synchroniser les souris dans deux états opposés qui sont, d'une part, le jeune, les animaux dans cet état présenteront une activation de la gluconéogenèse, et, d'autre part, le nourrissage, les animaux dans cet état présenteront une forte lipogenèse *de novo*.

Pour purifier les protéines ubiquitylées, nous avons, en collaboration avec le laboratoire du Pr Rudolf Aebersold (ETH Zurich, Suisse) (Figure 2A), utilisé une approche complémentaire en utilisant le système TUBEs1 (Hjerpe *et al*, 2009) et le kit UbiQaptureTM (Figure 2B). La purification a été suivie par une analyse très fine avec un spectromètre de masse de type Orbitrap. Cette analyse a permis d'identifier 1641 protéines possiblement ubiquitylées, avec un degré de confiance élevé grâce á l'utilisation de 40 souris au total (Figure 3A). L'homogénéité des replicats entre les différents échantillons et les différents essais, prouve que notre méthode est reproductible (Figure 3B).

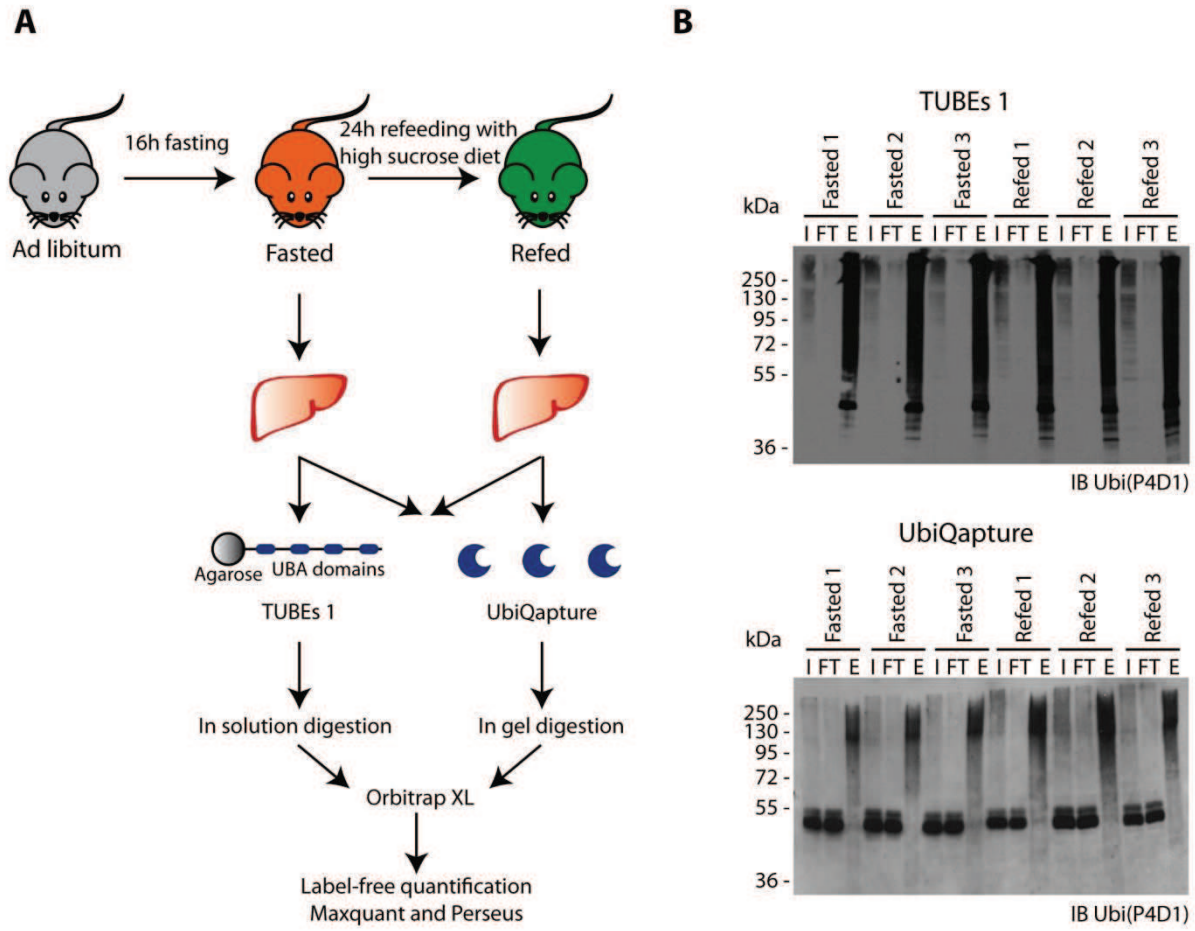


Figure 2: TUBEs et UbiQapture sont utilisés pour purifier les protéines ubiquitylées avant analyse par spectrométrie de masse

(A) Représentation schématique des étapes du criblage protéomique. Après avoir soumis les souris à un protocole de jeune et de re-nourrissage, les foies ont été prélevées, les protéines ubiquitylées ont été purifiées et analysées par un Orbitrap XL.

(B) Western blot, après pulldowns des protéines ubiquitinées par TUBEs1 ou UbiQapture, á partir de lysats de foie de souris à jeun pendant 16 heures ou à jeun et re-nourries avec un aliment riche en saccharose pendant 24 heures. Un anticorps contre l'ubiquitine (P4D1) a été utilisé pour détecter les protéines ubiquitylées.
I, input; FT, flow through; E, elution.

A

Purification method	Experiment number	No of proteins identified	Ubi-sites identified
TUBEs	Exp 1	173	2
	Exp 2	413	9
	Exp 3	577	21
	Total	732	25
UbiQapture	Exp 1	1102	3
	Exp 2	1108	2
	Total	1275	5
TOTAL		1641	25

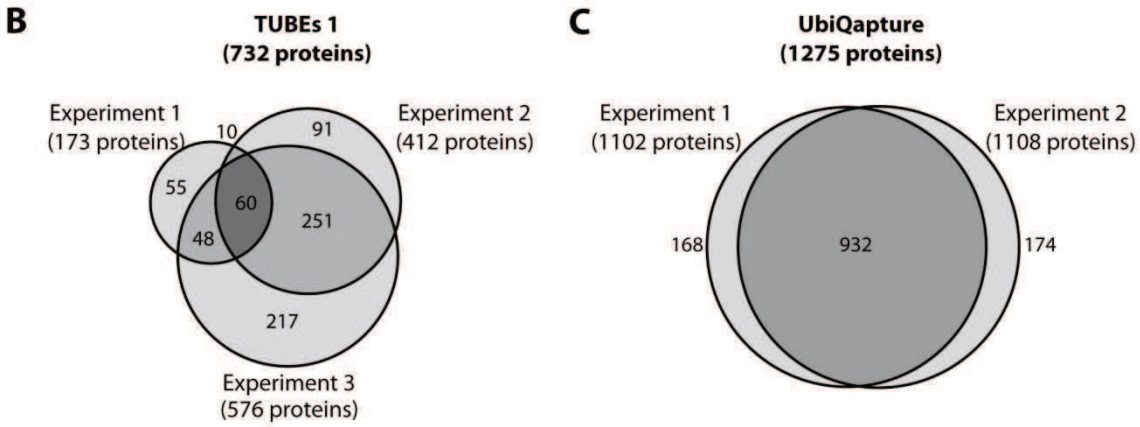


Figure 3: 1641 protéines ubiquitylées sont identifiées par spectrométrie de masse dans le foie de souris avec une grande reproductibilité entre les expériences

(A) Tableau indiquant le nombre de protéines identifiées et le nombre de protéines présentant un ou des sites d'ubiquitylation dans chaque expérience. Trois expériences ont été réalisées avec le systeme TUBEs sur 26 souris au total et deux expériences ont été réalisées avec le systeme UbiQapture sur 20 souris au total. Au total, 1641 protéines uniques ont été identifiées.

(B, C) Diagrammes de Venn montrant le nombre de protéines identifiées se chevauchant entre les différentes expériences. (B) Plus de 50% ** des protéines purifiées avec TUBEs ont

été identifiées dans plus de 2 expériences indépendantes. (C) Plus de 73% ** des protéines purifiées avec UbiQapture ont été identifiées dans plus de 2 expériences indépendantes.
** La distribution hypergéométrique avec l'hypothèse de chevauchement au hasard (en admettant un protéome de la souris de 40 000 protéines), p <0,01.

En procédant á une analyse ontologique des gènes, il s'est avéré que la mitochondrie est le compartiment cellulaire le plus enrichi (Figure 4A). La plupart des protéines identifiées font partie des voies de signalisation du métabolisme (Figure 4B). L'analyse permettant de déterminer les voies de signalisation les plus impliquées a aussi identifié les voies de signalisation métaboliques. Ces deux enrichissements sont très intéressants car ils suggèrent que beaucoup de protéines impliquées dans le métabolisme hépatique sont régulées par l'ubiquitylation.

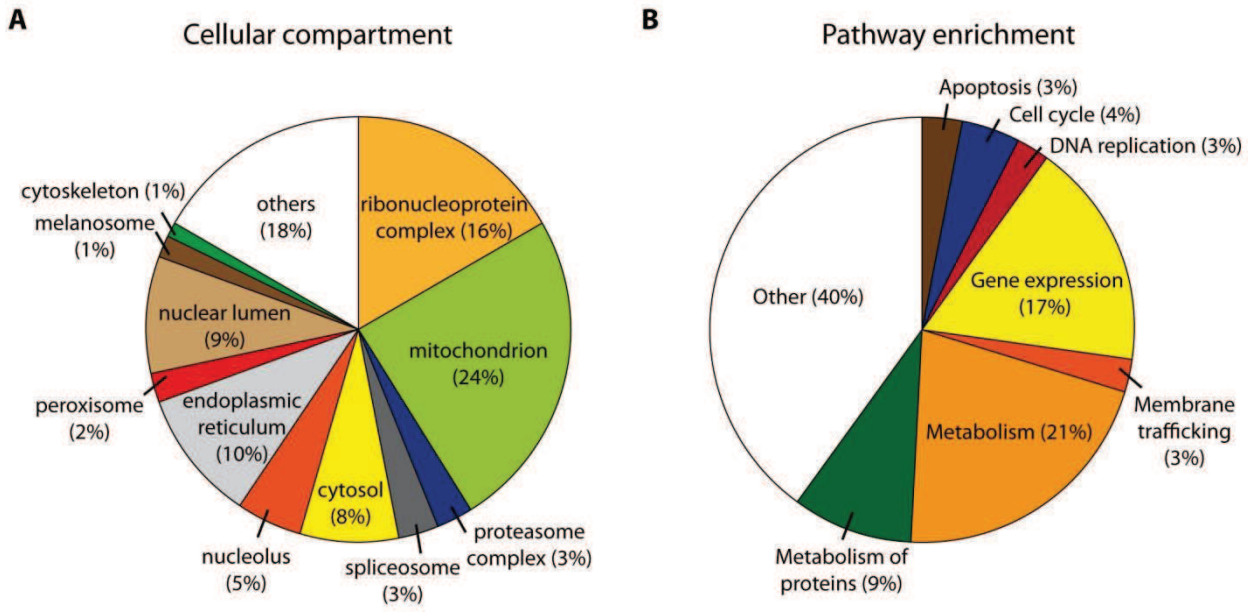


Figure 4: Les protéines appartenant à des voies métaboliques sont enrichies parmi toutes les protéines identifiées

(A) Diagramme circulaire représentant la quantité de protéines identifiées appartenant à chaque compartiment cellulaire en terrme Gene Ontology (GO). Le pourcentage de chaque compartiment cellulaire est indiqué.
(B) Diagramme circulaire représentant les principaux groupes de voies de signalisation enrichis par les protéines identifiées. Le pourcentage de contribution de chaque grand groupe est indiqué.

Pour identifier les protéines différemment ubiquitylées selon l'état de jeune ou de re-nourrissage, nous avons utilisé la méthode de Maxquant (Cox and Mann, 2008). Cette analyse statistique nous a permis d'identifier 117 protéines qui sont ubiquitylées de façon différente selon les deux conditions étudiées. Parmi ces 117 protéines, nous en avons sélectionnées 8 dont l'ubiquitylation sera validée biochimiquement. Pour la validation, nous avons surexprimé ces protéines, ainsi qu'une ubiquitine marquée, dans des hépatocytes primaires de souris, puis nous avons immunoprécipité (IP) les protéines natives et procédé au pulldown des protéines dénaturées. Sur les 8 protéines testées, nous avons confirmé l'ubiquitylation de toutes (Figure 5). Le fait que 8 des 8 protéines testées soient ubiquitylées valide le screen et nous permet de l'utiliser comme une source fiable pour l'étude des modifications protéiques qui ont lieu dans le foie après un challenge métabolique.

	FLAG IP (native conditions)	His+Bio Pulldown (denatured conditions)	
	HEK293T	HEK293T	Primary hepatocytes
Stbd1 FLAG	Yes	Yes	Yes
FLAG Pdzk1	No	Yes	Yes
FLAG Lpin 1	Yes	Yes	No
Pdk4 FLAG	Yes	Yes	LD
Pdhx FLAG	Yes	No	No
Eno1 FLAG	Yes	Yes	Yes
Hsd11B1 FLAG	Yes	LD	LD
FLAG C3	Yes	Yes	Yes

LD: Low detection

Figure 5: Toutes les 8 protéines testées sont ubiquitylé dans les cellules

Résumé de toutes les immunoprécipitations et pulldowns effectués pour confirmer l'ubiquitination des 8 protéines sélectionnées pour la validation biochimique dans les cellules HEK293T et les hépatocytes primaires de souris. "Yes" signifie une ubiquitylation identifiée

dans au moins l'une des conditions testées; "No" signifie pas d'ubiquitination identifiée dans les conditions testées.
LD signifie détection faible, la protéine n'a pas été détectée dans l'input.

Le complément C3 est une des protéines ubiquitylée dans les hépatocytes primaires. De plus, elle a été identifiée avec les 2 techniques (TUBEs et UbiQapture) lors de tous les essais. La protéine complément C3 fait partie du système du complément, essentiel dans le système immunitaire inné. Ce système est composé de différentes protéines plasmatiques qui s'activent en cascade lors d'une réaction immunitaire, afin d'opsoniser les pathogènes et de créer une réaction inflammatoire qui aidera á lutter contre l'infection (Sarma and Ward, 2011). Dans le plasma, après certains stimuli (pathogènes, tissus endommage), C3 est clivée en deux formes actives C3a et C3b. La plupart des protéines du complément sont produites et secrétées par les hépatocytes, ce qui a fait supposer que C3 est ubiquitylée avant sa sécrétion. Etant donné la fonction pro-inflammatoire de C3 dans le système immunitaire, l'ubiquitylation spécifique de cette protéine après nourrissage s'avère très intéressante car le T2D et le NAFLD sont caractérisés par une forte réponse inflammatoire (Emanuela, 2012). De plus, la forme immuno-inactive issue du clivage de C3, C3adesarg, est fortement associée á la lipogenèse dans les adipocytes (Maslowska *et al*, 2005). Cependant, sa fonction n'a pas encore été étudiée dans d'autres tissus mais elle pourrait avoir une fonction similaire dans les hépatocytes, étant donne que ces derniers expriment le récepteur spécifique á C3adesarg. Enfin, il a été montré récemment que dans les lymphocytes T, C3 est clivée en C3a et C3b, C3a étant requise pour la survie des lymphocytes T (Liszewski *et al*, 2013).

Nos résultats montrent que C3 est aussi clivé dans les hépatocytes primaires, indiquant que l'ubiquitylation pourrait aussi affecter des fonctions intracellulaires. En la sur exprimant dans des hépatocytes primaires et en réalisant des pulldown en conditions dénaturantes, nous avons montré que C3a est spécifiquement ubiquitylée et dégradée dans des conditions simulant un re-nourrissage de ces cellules (Figure 6). Ceci corrobore le criblage réalisé sur les foies de souris durant lequel nous avons identifié C3 comme une des protéines ubiquitylées après re-nourrissage des souris.

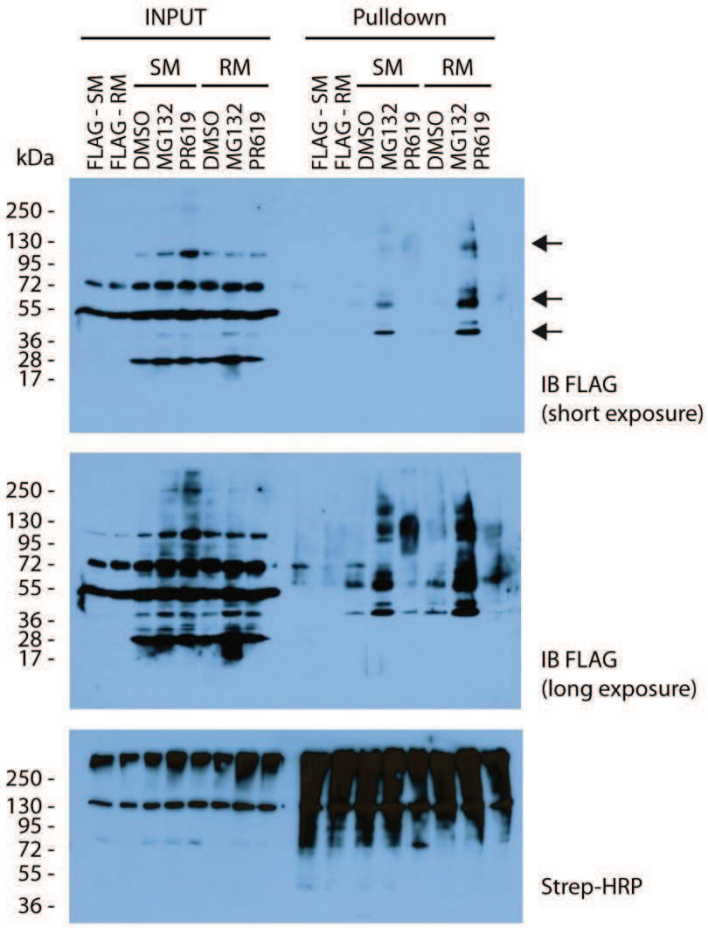


Figure 6: C3a est ubiquitylé différemment dans les hépatocytes primaires des souris

Western blot après pull down His-biotine de lysats d'hépatocytes primaires. Ces cellules expriment His-Bio-ubiquitine et FLAG ou FLAG-C3a. Les cellules ont été traitées ou au DMSO, ou au MG132 (inhibiteur du protéasome) 10 uM pendant 4 heures, ou au PR619 (inhibiteur DUB) 10 uM pendant 2 heures. Les hépatocytes primaires (C) ont en outre été cultivés dans un milieu pauvre (SM) ou riche (RM) en nutriments. Un anticorps dirigé contre FLAG a été utilisé pour détecter la C3a exogène et la streptavidine-HRP a été utilisée pour détecter les protéines ubiquitinées par l'ubiquitine exogène. Le pulldown par His Bio et l'input apparaissent comme indiqués.

En se basant sur nos résultats et sur les fonctions publiées de C3, nous avons émis trois hypothèses sur les rôles possibles de l'ubiquitination de C3 dans les hépatocytes (Figure 7). Dans la première hypothèse, nous suggérons que l'ubiquitination joue un rôle dans les

processus inflammatoires locaux induits par la sécrétion de C3a et l'activation subséquente des cellules de Kupffer. Dans la seconde hypothèse, nous suggérons que l'ubiquitination joue un rôle dans les fonctions intracellulaires de C3a, avec l'activation du récepteur de C3a (C3aR), et l'activation subséquente des voies de signalisation mTORC1. Enfin, la troisième hypothèse suggère que l'ubiquitination joue un rôle dans les fonctions intracellulaires de C3adesArg pour activer la lipogenèse dans les hépatocytes. De futures expériences testeront ces hypothèses afin de mieux comprendre le rôle de l'ubiquitination de C3 dans le foie.

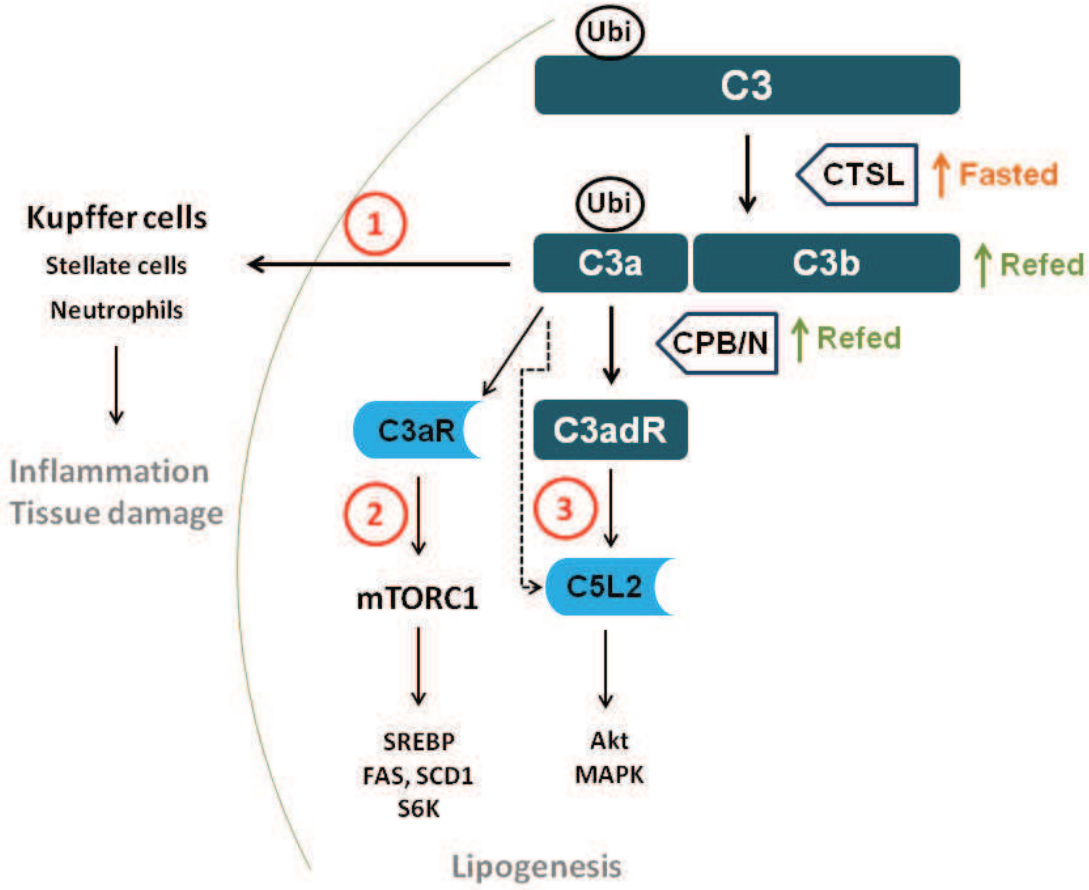


Figure 7: Modèle présentant 3 hypothèses sur le rôle de l'ubiquitination de C3 dans les fonctions métaboliques et inflammatoires du foie

Nous proposons 3 rôles possibles pour l'ubiquitination de C3 dans le foie lors du jeûne – re–nourrissage. (1) Le régime alimentaire et l'ubiquitination modulent la sécrétion de C3a inflammatoire, ce qui active les cellules de Kupffer, initialisant la réponse inflammatoire. (2) Le régime alimentaire et l'ubiquitination modulent l'activation du C3aR intracellulaire en se liant á C3a et en activant la signalisation par mTORC1. (3) Le régime alimentaire et l'ubiquitination modulent l'activation intracellulaire de C5L2 en se liant á C3adesArg

provoquant l'activation de la voie de signalisation Akt/MAPK. Les hypothèses 2 et 3 entraîneront une augmentation de la lipogenèse dans le foie.

Dans l'ensemble, nos résultats décrivent une analyse inédite au cours de laquelle nous avons observé de façon globale les protéines ubiquitylées dans le foie, dans un contexte physiologique, au cours d'un cycle jeûne - re-nourrissage. La validation biochimique a confirmé l'ubiquitination de toutes les protéines testées, confirmant ainsi la validité de l'approche utilisée pour identifier les protéines ubiquitylées. De part la fonction intéressante de C3, molécule impliquée dans les processus métaboliques et inflammatoires, et son identification avec un niveau de confiance élevé dans le criblage protéomique, nous avons décidé d'étudier C3 plus en détail. Sur la base de nos données préliminaires, nous pensons que C3 pourrait jouer un rôle dans le développement de la stéatose du foie et des lésions hépatiques par une combinaison de ses fonctions métaboliques et inflammatoires. De plus, ces fonctions pourraient être régulées par l'ubiquitination. Nous pensons que l'ubiquitination apporte un autre niveau de régulation dans le métabolisme hépatique, régulation qui pourrait être affectée lors de maladies métaboliques telles le T2D ou le NAFLD.

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Uncovering ubiquitylation pathways in liver metabolism by a proteomic approach

Résumé

Chez les vertébrés, le foie est un des organes majeurs du métabolisme en étant le siège de la régulation de différentes voies du métabolisme qui contrôlent l'homéostasie du glucose et des lipides. Cette régulation peut être réalisée par des modifications post-traductionnelles de molécules de signalisation. En se basant sur des travaux de recherche récents suggérant que le système de conjugaison de l'ubiquitine est engagé en réponse à différents signaux métaboliques, nous avons réalisé une analyse protéomique globale dans le but d'identifier des protéines ubiquitylées dans le foie de souris soumises á un protocole de jeûne – re-nourrissage. Parmi les 1641 protéines identifiées, 117 ont été différemment ubiquitylé sur le jeûne ou le re-nourrissage. En particulier, l'immunité innée composant complément 3 (C3) a été identifiée dans le foie de souris réalimentées. Nous avons observé qu'un produit d'activation de C3, C3a, est ubiquitylé dans les hépatocytes primaires traités avec les médias riches en nutriments. Ainsi, nous proposons que l'ubiquitination de C3 joue un rôle dans la régulation des fonctions inflammatoires ou métaboliques de C3 dans le foie.

Résumé en anglais

In vertebrates, the liver has developed to be a major metabolic organ able to control glucose and lipid homeostasis. It activates or inhibits specific pathways in a regulated manner, depending on the metabolic state of our organism. This regulation can be achieved by posttranslational modifications of key signaling molecules. Based on the emerging experimental evidence suggesting that the ubiquitin conjugation system is engaged in response to different metabolic cues, we conducted a global proteomic analysis to identify metabolic pathways modified by ubiquitylation. To this end, we used livers of mice subjected to a fasting – refeeding protocol. Amongst the 1641 identified proteins, 117 were differentially ubiquitylated upon fasting or refeeding conditions. In particular, the innate immunity component complement 3 (C3) was identified to be ubiquitinated in livers of refed mice. We observed that an activation product of C3, C3a, is ubiquitylated in primary hepatocytes treated with nutrient-rich media. Thus, we suggest that the ubiquitylation of C3 plays a role in the regulation of inflammatory or metabolic functions of C3 in the liver.