Role of the tumour suppressor pathway p53-p21 in the regulation of metabolism

Giuseppe Pulice

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UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

Departament de Bioquímica i Biologia Molecular

Programa de Doctorat en Biomedicina

Role of the tumour suppressor pathway p53-p21

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Memòria presentada per Giuseppe Pulice per optar al títol de doctor per la Universitat de Barcelona

Dra. Carme Caelles Franch

Giuseppe Pulice

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RESUMEN

INTRODUCCIÓN

La obesidad es una de las plagas de la sociedad moderna y se define como una excesiva acumulación de grasa que puede afectar la salud. Entre las varias causas, las más comunes son cambios en los hábitos alimentarios y una disminución de la actividad física, que juntos conducen a un desequilibrio entre ingesta y gasto energético. En 2008 el 35% de los adultos de 20 o más años estaban sobrepeso y el 11% era obeso.. En los últimos treinta años se ha observado un alarmante incremento de la incidencia de la obesidad en los países occidentales. La obesidad es el mayor factor de riesgo para el desarrollo de la diabetes de tipo 2 (T2D) así como, para otras enfermedades asociadas al síndrome metabólico, y al cáncer, patologías que representan un importante desafío social y sanitario. Por este motivo, es urgente el desarrollo de nuevas terapias y estrategias farmacológicas para la prevención y el tratamiento de la obesidad y sus enfermedades asociadas.

En la obesidad se produce, en primer lugar, una expansión anormal y excesiva del tejido adiposo (AT) debida a procesos de hiperplasia e hipertrofia de los adipocitos. Como consecuencia de esta situación se activan vías de señalización inducidas por estrés – como la respuesta al estrés del retículo endoplásmico (ER), y la activación de la vía de la c-Jun N-terminal quinasa (JNK), entre otras – y se induce la sobreproducción de especies reactivas de oxígeno (ROS) así como, el reclutamiento en el AT de células del sistema inmunitario para construir una respuesta inflamatoria crónica de baja intensidad que a la vez estimula aún más la mayoría de estas vías de señalización inducidas por estrés .

El AT es un tejido conectivo que clásicamente corresponde a la grasa corporal de un animal pero en los últimos años su definición se ha expandido a "órgano adiposo" porqué involucrado en procesos metabólicos y como órgano endocrino, no solo como mera reserva de energía. Se compone principalmente de adipocitos y también de pre-adipocitos, fibroblastos y macrófagos. Según criterios morfológicos y funcionales se clasifica en 3 tipos diferentes tipos: blanco (WAT), marrón (BAT) y beige. El WAT está formado por adipocitos que contienen

una única gota lipídica circundada por el citoplasma. Su función es almacenar energía en forma de triglicéridos (TG), pero también tienen función endocrina y sintetizan varias moléculas de señalización. Estas moléculas son las adipokinas y regulan varios procesos fisiológicos como apetito/saciedad, gasto energético, secreción y sensibilidad a insulina, inflamación, etc. La leptina es una adipokina fundamental y fue identificada como el producto del gen *ob* en ratones. Los niveles de leptina circulante son directamente proporcionales a la cantidad de grasa corporal y fluctúan con los cambios en la ingesta calórica: sus niveles suben después de comer, mientras que el ayuno resulta en un decremento. La leptina regula la ingesta de comida y la distribución de energía, así que controla el peso corporal. Es crucial para la homeostasis energética en respuesta a la falta de energía: esto sucede durante un ayuno agudo, el ejercicio físico, etc. En estos casos, los niveles de leptina caen independientemente de la cantidad de grasa corporal, provocando la activación de mecanismos para incrementar la ingesta energética y preservar el gasto energético. Independientemente de sus efectos sobre los hábitos alimenticios, la leptina tiene un fuerte efecto sobre la insulina y el metabolismo de carbohidratos y lípidos. La leptina promueve la oxidación lipídica y la síntesis proteica que resulta en una pérdida de adiposidad sin afectar la masa corporal magra. La leptina sistémica actúa independientemente del peso y las calorías reduciendo el contenido de TG en el WAT, sin incrementar los niveles de FFAs en círculación, y contrasrestando la acción de la insulina que estimula la lipogenesis.

El receptor de leptina (LepR), tras la unión de la adipokina, desencadena la activación de la vía de señalización de leptina. El LepR se expresa en el CNS así como en tejidos periféricos como el AT. Fue identificado como producto del gen *db* y pertenece a la familia de receptores de citokina de clase 3. Al menos 6 distintas isoformas (generadas por procesamiento alternativo) existen in *M. musculus.* Una de estas variantes, la isoforma b, se expresa en particular en el núcleo arcuato del hipotálamo, donde regula la homeostasis energética y la función neuroendocrina. Es además la variante con procesamiento anormal en ratones C57BL/Ks *db/db* y que resulta en una proteína mutante y defectuosa en la transducción de la señal. Eso sugirió que los efectos de reducción de peso por

leptina podían ser mediados por esa vía. Sin embargo, también se demostró que la sobrexpresión específica en el AT de un transgen LepR-b previene totalmente la hipertrofia/hiperplasia de los adipocitos y el incremento en grasa corporal inducido por dieta grasa en ratones. La leptina activa varias rutas de señalización como *JAK2/STAT3, SHP2/MAPK, PI3K/v-Akt, Akt/mTOR.*

La resistencia a leptina se origina a partir de la alteración de la señalización por leptina y se define como la incapacidad de promover efectos saludables como la supresión del apetito/aumento de peso y la estimulación de gasto energético. La resistencia a leptina se relaciona con estados energéticos excesivos y puede ocurrir a partir de la interferencia con la fosforilación de JAK2 de parte de moléculas como SOCS3, SHP2, and PTP1B. Otros mecanismos incluyen el estrés del ER relacionado a obesidad y la inflamación crónica de bajo nivel. El enfoque es ahora sobre el papel de la leptina en el mantenimiento de la pérdida de peso más que como hormona anti-obesidad. Es eficaz en pacientes con deficiencia de leptina congénita o adquirida, que se caracteriza por hiperfagia y obesidad. La FDA aprobó la leptina también para tratar la lipodistrofia generalizada. La deficiencia de leptina y la lipodistrofia se asocian a la resistencia a insulina y al metabolismo de la glucosa anómalo, así que el tratamiento con leptina puede mejorar el metabolismo de la glucosa. La leptina provoca un decremento del peso corporal y de la masa grasa, mejorando la resistencia a insulina y puede activar también tejidos sensibles a insulina.

En relación al estudio de la activación de JNK en obesidad, los ratones transgénicos MKK7D constituyen un modelo útil para el estudio de la activación de JNK en células β del páncreas y en la homeóstasis de la glucosa. Datos obtenidos por el Dr. Jordi Lanuza-Masdeu en nuestro laboratorio mostraron que la activación de JNK por MKK7D en células-β pancreáticas afectaba a la homeóstasis de la glucosa y comprometía la señalización de insulina pero, sorprendentemente, eso no resultaba en resistencia sistémica insulina inducida por obesidad. En condiciones de HFD, los ratones MKK7D desarrollan obesidad casi igual que los animales controlsin embargo, están totalmente protegidos del desarrollo de resistencia a la insulina e hiperinsulinemia.

OBJETIVOS, RESULTADOS Y DISCUSIÓN

Cambios en dieta y estilos de vida han causado un incremento alarmante en la incidencia de la obesidad, uno de los principales factores de riesgo para el síndrome metabólico. Uno de los eventos iniciales en la obesidad es la hipertrofia de los adipocitos, una condición de estrés que desencadena respuestas celulares como la inflamación que pueden conducir al desarrollo de resistencia a insulina e híperinsulinemia, que constituyen la principal conexión de la obesidad a otras patologías asociadas a obesidad como la T2D, enfermedades cardiovasculares, aterosclerosis y cáncer. El principal objetivo de esta tesis fue desvelar nuevos mecanismos moleculares a la base del desarrollo de obesidad y de la resistencia a la insulina e híperinsulinemia asociadas. Perseguimos este objetivo a través de dos distintos estudios:

1. Análisis del papel de la ruta supresora tumoral p53/p21 en obesidad.
2. Análisis del efecto de la activación de JNK en células-β pancreáticas en la resistencia a la insulina sistémica e híperinsulinemia asociadas a obesidad.

Al principio de nuestra investigación encontramos que la expresión del gen p21 estaba aumentada en el tejido adiposo – pero no en músculo esquelético o hígado – en distintos ratones modelos murinos de obesidad como en los ratones deficientes para el gen de la leptina (*ob/ob*) y en los ratones obesos debido a la ingesta de una dieta rica en grasas y carbohidratos (HFD). Además, observamos que la deficiencia de p21 protegía los ratones del desarrollo de DIO (de *diet induced obesity*) y de resistencia a insulina inducida por esta HFD. A continuación, un análisis de genómica funcional comparando los perfiles de expresión génica en el tejido adiposo blanco epididimal (e-WAT) de ratones de tipo silvestre (WT de *wild type*) vs heterocigotos y "knock out" (KO) para p21 en condiciones de dieta estándar (SD) demostró una correlación inversa entre la expresión génica de p21 y el receptor de leptina (LepR). Esta observación fue posteriormente validada por PCR cuantitativa en tiempo real (qRT-PCR) e inmunoblotting. Además, estos datos fueron validados por resultados genéticos demostrando que la deficiencia de p21

no ejercía ninguna acción protectora contra el desarrollo de la obesidad en el contexto de déficit genético del gen de leptina dado que ratones dobles KO para los genes de p21 y de leptina ($p21^{-/-}$ *ob/ob*) desarrollaban el mismo grado de obesidad que los ratones *ob/ob*. En resumen, estos datos moleculares y genéticos demuestran que el mecanismo por el que la ausencia del gen p21 protege de la obesidad depende de la señalización de la leptina. Además, mientras este trabajo estaba en marcha, fue demostrado que la ausencia del supresor tumoral – y regulador transcripcional del gen p21 – p53 específicamente en AT también protege contra DIO coincidiendo con el fenotipo mostrado por los ratones p21 KO. También en estos últimos años se han ido acumulando fuertes evidencias acerca de la importancia para la progresión tumoral de alteraciones metabólicas y de la implicación de la vía de p53 en el control metabólico. De manera consistente comprobamos que los niveles proteicos del receptor de leptina estaban también aumentados en AT epididimal de ratones KO para p53. Finalmente el análisis transcriptómico también mostró otros perfiles de expresión génica interesantes. Por ejemplo es notable como en el background del p21 KO la expresión de genes adipogénicos no estaba alterada, indicando que el déficit del gen p21 no modifica la capacidad de diferenciación de los adipocitos *in vivo*. Estos resultados son consistentes con la incapacidad de detectar diferencias en el número, tamaño o morfología de las células en WAT-I de ratones en dieta estándar WT vs p21 KO.

Basándonos en estas observaciones, nuestra hipótesis para explicar a nivel molecular el fenotipo de protección contra la obesidad mostrado por los s p21 KO, fue que estos animales podrían tener una mayor sensibilidad a leptina debida a mayores niveles de LepR en el AT.

La leptina es una adipoquina que actúa a nivel del sistema nervioso central como molécula anorexigénica que inhibe la ingesta de comida, mientras en tejido adiposo la leptina ejerce una acción paracrina incrementando el gasto energético gracias a la promoción de biogénesis mitocondrial o inhibiendo el tono canabinóide. De manera interesante no encontramos ninguna diferencias significativa en la ingesta de comida en ratones WT vs p21 KO sugiriendo que la acción relevante de la leptina en relación con el fenotipo ligado a obesidad no se ejercía en el sistema nervioso central sino en tejidos periféricos como el AT. De

hecho datos de literatura suportan esta posibilidad dado que la sobre expresión del LepR especificadamente en AT protege de la DIO. Los experimentos i*n vivo* tenían como objetivo el estudio de la sensibilidad a leptina en ratones p21 KO vs WT. En este sentido, ratones de ambos genotipos en SD fueron tratados diariamente con dosis subóptimas de leptina recombinante murina y sus pesos fueron seguidos durante dos semanas. Hay que hacer constar que los ratones p21 KO en SD no son significativamente más delgados de los WT, sugiriendo la regulación del peso corporal está controlada a un nivel más alto y más complejo, entonces no restringido solo a la actividad central y periférica de la leptina. También hay que considerar que los niveles de leptina circulante no son diferentes entre estos ratones. Sin embargo, cuando forzamos el sistema administrando leptina exógena, los animales p21 KO mostraron un decremento significativo en el peso corporal al contrario de los animales WT. No observamos diferencias significativas en la ingesta de comida durante el tratamiento.

Una segunda estrategia *in vivo* fue el análisis de la fosforilación de STAT3 en WAT después del tratamiento con leptina. STAT3 está involucrado en la señalización de leptina y está fosforilado tras la unión de la leptina a su receptor y concomitante activación del mismo. En relación a esto, los ratones fueron inyectados a nivel intraperitoneal y luego sacrificados al cabo de treinta minutos.

A continuación nos planteamos analizar la conexión a nivel molecular entre la expresión del LepR y la vía p53/p21. Mediante análisis *in silico* observamos que el gen del LepR contiene un posible sitio de unión para el factor de transcripción E2F. Esto podría ser relevante porqué p21 es uno de los principales reguladores del ciclo celular y puede modular la transcripción génica a través de la regulación de la fosforilación de pRB que a la vez regula negativamente la actividad del regulador transcripcional E2F. En favor de esta idea datos de la bibliografía demuestran que ratones KO para pRB especificadamente en el AT están también protegidos de DIO. Además, ha sido sugerido un papel de E2F en el control metabólico independientemente de su función en la regulación del ciclo celular. En relación a esto, E2F tendría un papel negativo previniendo la regulación de ciertos genes y proponemos que el gen LepR podría ser uno de ellos.

Para averiguar el efecto de la leptina sobre la adiposidad y el metabolismo de la glucosa analizamos el comportamiento de ratones deficientes en leptina y,como consecuencia, en señalización de leptina, los ratones ob/ob, animales modelos para la obesidad. Eso permitió estudiar los efectos generados por la ausencia de p21 en un contexto de ausencia de señalización de leptina, y analizar las conexiones moleculares entre p53-p21 and Lep/LepR. Los ratones doble KO ob/ob $p21^{-/-}$ son obesos, mientras los ob/+ $p21^{-/-}$ son delgados, debido a la presencia de una copia del gen de la leptina. Una vez más observamos que la ausencia de p21 provocaba un aumento en la expresión del LepR pero, sin señalización de leptina, el déficit de p21 no era suficiente para mejorar el fenotipo obeso. En relación a la sensibilidad a leptina, observamos que el tratamiento de adipocitos aislados del e-WAT de ratones ob/ob y ob/ob $p21^{-/-}$ con leptina produjo un pequeño incremento en la fosforilación de STAT3 en doble KO comparado con el ob/ob. Resumiendo, el fenotipo obeso en ratones ob/ob no se puede simplemente reducir por deleción de p21. Rsta observación nos indica que la señalización vía leptina/LepR tiene un papel fundamental en la protección frente a obesidad en un background de ausencia de p21.

En relación a la segunda parte de este trabajo, investigamos la activación de JNK y su papel en la obesidad. En el tejido adiposo blanco inguinal (e-WAT) observamos una situación mezclada: JNK estaba fosforilada y activada y los niveles de TNFα eran altos en ambos grupos de ratones control y MKK7D en dieta grasa (HFD) comparados con aquellos en dieta estándar (SD), indicando dos características de la inflamación asociada a obesidad, junto con unos mayores niveles de fosforilación de eIF2α (relacionados con el estrés del retículo endoplásmico). Sin embargo, estos eventos no son suficientes para el desarrollo de resistencia insulina sistémica. En hígado, la activación de JNK no resultó clara así como la presencia de un estado inflamatorio, mientras los niveles de IRS se mantuvieron en condiciones de HFD también en este tejido y eran similares entre los distintos grupos de ratones. Finalmente, basado en la observación que el músculo esquelético de ratones MKK7D en HFD mantuvo inalterados los niveles de expresión de IRS, en contraposición con los animales control, podemos especular

que el músculo esquelético posiblemente retiene la sensibilidad a insulina en los ratones MKK7D obesos.

CONCLUSIONES

Primera parte

1.- La expresión de p21 está aumentada específicamente en el AT de ratones obesos

2.- La deficiencia en p21 tiene efectos beneficiosos en ratones sujetos de una dieta rica en grasas dado que impide el desarrollo de obesidad y preserva la homeostasis glucídica.

3.- La ausencia de p21 o p53 provoca un incremento en la expresión de gen del LepR que conlleva a un incremento de la sensibilidad a leptina.

4.- la señalización Leptin/LepR media la protección frente al desarrollo de obesidad inducida por la inhibición de la ruta de p53/p21.

Segunda parte

1.- La inflamación y la respuesta de estrés en el retículo endoplasmático inducidos por una HFD en el AT no es suficiente para el desarrollo de Resistencia a la insulina sistémica.

2.- La secreción de insulina en respuesta a la glucemia es necesaria para el desarrollo de Resistencia a la insulina sistémica inducida por la obesidad.

3.- Nuestros datos apoyan que la exposición continuada a niveles plasmáticos elevados de insulina secretada por las células b del páncreas en respuesta a la ingesta continuada es necesaria para la Resistencia a la insulina sistémica inducida en la obesidad.

INTRODUCTION

Obesity: an epidemic of the 21st century

Obesity is one of the plagues of modern society and is defined as an excessive fat accumulation that may impair health. Among the causes of the increased-obesity incidence, changes in eating habits and decrease in physical activity, which together lead to an imbalance between energy intake and expenditure are the most common. Body mass index (BMI) is used to classify overweight and obesity in adults, and is defined as a person's weight in kilograms divided by the square of its height in meters (kg/m^2). According to the World Health Organization (WHO), a BMI≥25 is considered overweight, and ≥30 obesity [WHO Obesity and overweight, 2013]. BMI represents the most useful population-level measure of overweight and obesity, as it is the same for both sexes and for all ages of adults. However, it may not correspond to the same degree of fatness in different individuals or across different populations because it does not consider the differences in body proportions and variation of obesity among populations or single individuals [WHO Obesity and overweight, 2013; Kopelman, 2000].

The incidence of obesity has dramatically increased worldwide over the last decades, and has nearly doubled since 1980; in 2008 35% of adults aged 20 and over were overweight, and 11% were obese [WHO Obesity and overweight, 2013; The World Health Report, 2002; Finucane *et al.*, 2011]. (Fig.1). Dramatically, more than 40 million under the age of five were overweight in 2011 [WHO Obesity and overweight, 2013], a condition that is associated with a higher chance of obesity, premature death and disability in adulthood. Moreover, some low- and middle-income countries face a paradoxical situation, that is, while they are still dealing with infectious diseases and under-nutrition, they are also experiencing an increase of obesity/overweight, particularly in urban areas, where under-nutrition and obesity coexist [WHO Obesity and overweight, 2013; Popkin *et al.*, 2012]. Currently overweight/obesity is the fifth leading risk factor for global deaths. At least 2.8 million adults die each year as a result of being overweight/obese.

A high BMI is a major risk factor for some so called noncommunicable diseases (NCDs) such as type 2 diabetes (T2D), stroke, osteoarthritis and some cancers. In

this regard, 44% of the T2D burden, 23% of the ischemic heart disease burden and between 7% and 41% of certain cancer burdens are attributable to overweight and obesity [WHO Obesity and overweight, 2013]. Overweight/obesity, as well as their related NCDs, are preventable. On one hand people should receive support from education and other institutions to develop knowledge on the topic: this is fundamental to shape their personal choices in function of a healthy life. Simple advices for prevention would be for example limiting energy intake from total fats and sugars; increasing consumption of fruit and vegetables, as well as legumes, whole grains and nuts, and engagement in regular physical activity. Also the food industry should contribute by reducing the fat, sugar and salt content of processed foods; ensuring that healthy and nutritious choices are available and affordable to all consumers; practicing responsible marketing especially those aimed at children and teenagers; and supporting regular physical activity practice in the workplace. For this purpose the WHO Assembly adopted various strategies and policies aimed to publicize the actions needed to support healthy diets and regular physical activity and for the surveillance, prevention and management of NCDs [WHO Obesity and overweight, 2013].

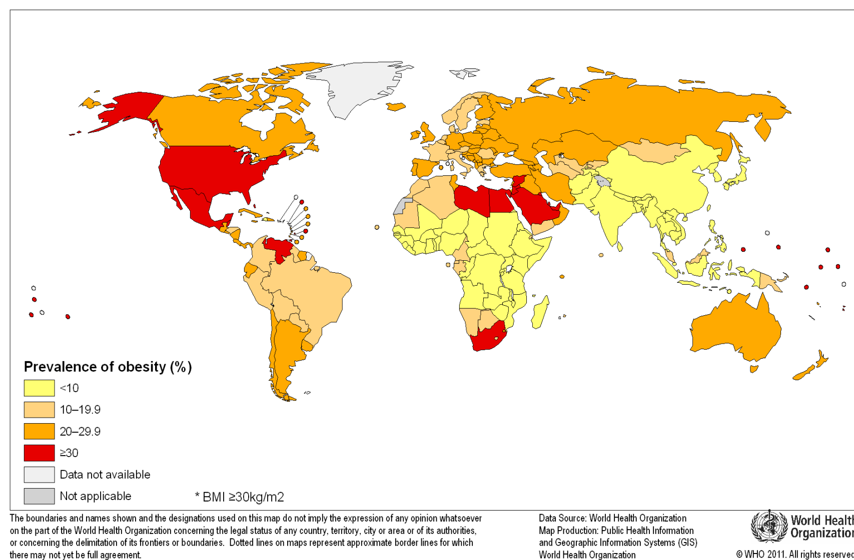


Figure 1. Prevalence of obesity*, ages 20+, age standardized, both sexes 2008 (source: WHO) The incidence of obesity has dramatically increased worldwide and nearly doubled since 1980; in 2008 35% of adults aged 20 and over were overweight, and 11% were obese.

As mentioned above, obesity is associated with various disorders like T2D, insulin resistance, fatty liver disease, atherosclerosis, cardiovascular diseases, degenerative disorders, airway disease and some cancers [Hotamisligil, 2006; Semenkovich, 2006]. These disorders generally are grouped under the metabolic syndrome, a cluster of chronic and complex diseases/conditions that feature metabolic deterioration are in close relationship among them. In particular, the strong association between obesity and insulin resistance constitutes an increased risk factor for the development not only of T2D but also for the rest of pathological processes.

Adipose tissue and adipose organ

Adipose tissue (AT) is a connective tissue that corresponds to the body fat of an animal. In the last years this definition has expanded, since AT has been involved in the regulation of metabolic processes as an endocrine organ and not just as an energy storage reservoir.

AT is mainly composed of adipocytes, stromal vascular fraction cells (SVF), including pre-adipocytes (APs), fibroblasts, vascular endothelial cells, and immune cells like macrophages. According to morphological and functional criteria AT have been classified in 3 different types; white AT (WAT), brown AT (BAT) and beige AT.

White adipose tissue (WAT)

The parenchymal element is constituted by white adipocytes. These are spherical cells containing a single large lipid droplet surrounded by the cytoplasm and the nucleus [Fantuzzi & Mazzone, 2014; Cinti, 1999]. They are specialized to store energy in the form of triglycerides (TG), available in the intervals between meals or during longer periods of starvation. Nonetheless, WAT is not only specialized in storing energy but it produces a vast array of signalling molecules with important paracrine and endocrine functions.

Brown adipose tissue (BAT)

Unlike white, brown adipocytes accumulate lipids in the form of multi-locular droplets. Their shape is polygonal or ellipsoid, and are characterised by a high concentration of mitochondria and high levels of uncoupling protein (UCP)1 expression to uncouple the electron chain from ATP synthesis and dissipate chemical energy as heat, so they are able to produce heat and counteract hypothermia [Wu, 2012; Fedorenko *et al.*, 2012]. BAT is abundant in rodents, but in larger mammals it almost disappears after infancy. BAT, for example from interscapular depots in rodents, derives from a myf-5 muscle-like cell lineage [Seale *et al.*, 2008].

Beige adipose tissue

Some WAT contain cells that, upon prolonged exposure to cold or stimulation by signals that increase cAMP level, can express high levels of UCP1, have high respiration rates and take on a multi-locular lipid droplet appearance [Wu, 2012; Cousin *et al.*, 1992[a]; Young *et al.,* 1984]. The "brown-like" cells have been called "beige" or "brite". They do not derive from the myf-5 lineage [Seale *et al.*, 2008; Ishibashi *et al.*, 2010; Petrovic *et al.*, 2010] and have a gene expression pattern distinct from either white or brown fat [Wu, 2012].

Anatomy of the adipose organ

Due to AT anatomy and especially for its role in metabolism it can also be considered as a complex multidepot organ (Fig. 2) [Fantuzzi & Mazzone, 2014; Cinti, 1999, 2001, 2002, 2005] that goes by the name of adipose organ.

In humans major adipose depots are located beneath the skin (subcutaneous fat), providing insulation from heat and cold; around organs providing protective padding (visceral fat), in bone marrow and breast. In mice there are different adipose depots in the abdominal cavity which constitute the visceral fat, and that is different from subcutaneous and intramuscular fat. Out of the abdominal cavity

there are other fat depots such as the inguinal depots, anterior to the upper segment of the hind limbs, underneath the skin and the subscapular depots of BAT adjacent to regions of WAT, under the skin between the dorsal crests of the scapulae. Minor depots include the pericardial (surrounding the heart) and the popliteal depots (behind the knees). The adipose organ receives a vascular network and nerve supply. The brown areas have a higher density of the capillaries [Fantuzzi & Mazzone, 2014; Cinti, 1999] and innervation, with noradrenergic fibres directly in contact with adipocytes [Yasuda *et al.*, 2005; De Matteis *et al.*, 1998]. The density of the parenchymal fibres increases in the brown part during cold exposure [De Matteis *et al.*, 1998] and in the white part during fasting [Giordano *et al.*, 2005].

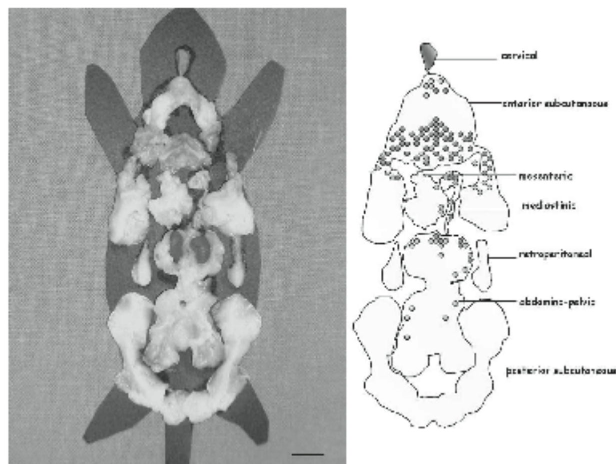


Figure 2. Gross anatomy of the adipose organ [Fantuzzi & Mazzone, 2014]. The abdominal cavity of mice hosts various adipose depots which constitute the visceral fat, different from subcutaneous and intramuscular fat. Fat depots out of the abdominal cavity: include the inguinal WAT depots and the subscapular depots of BAT adjacent to regions of WAT. Minor depots include the pericardial and the popliteal depots.

Adipogenesis

The expansion of WAT during normal development or obesity is due to an increase in the size and number of the adipocytes. Mature adipocytes do not divide *in vivo*, so the regeneration and expansion of the tissue during adult life depend on the pool of APs [Hauner *et al.,* 1989; Spalding *et al.*, 2008]. Adipogenesis consists in the generation of the APs followed by the terminal differentiation of these

precursor cells. In mouse, the source of APs include: the SVF of AT, mostly [Tang *et al.*, 2008]; the mural cell compartment of AT vasculature, with cells expressing APs markers and capable of *in vitro/in vivo* adipogenesis [Tang *et al.*, 2008]; bone marrow [Crossno *et al.*, 2006]; visceral fat depots, with hematopoietic–derived adipocytes accumulating with age and with high expression of inflammatory genes [Crossno *et al.*, 2006; Majka *et al.*, 2010; Cousin *et al.*, 1999[b]]. APs from different depots show differences in differentiation/proliferation, gene expression [Tchkonia *et al.*, 2007; Gesta *et al.*, 2006], adipocyte functionality and contribute differently to metabolic diseases.

AT can undergo both hyperplasia (increase in adipocyte number) and hypertrophy (increase in adipocyte volume). Hypertrophy in general is a typical condition found in overweight and obese people and it is also an independent risk factor for developing T2D [Wren *et al.*, 2008; Lonn *et al.*, 2010]. Hyperplasia correlates more with severe obesity [Hirsch *et al.*, 1976]. Despite initial observations, it is now known that adipocytes are a dynamic and regulated population of cells: new adipocytes constantly form to replace lost cells, such that almost 50% of human subcutaneous adipocytes are replaced every eight years [Spalding *et al.*, 2008].

Adipogenesis is a multi-step process that involves the activation of cascades of transcription factors, cell-cycle regulators and regulation of gene expression [Lefterova *et al.*, 2009]. The first step of the process consists of a determination phase when the stem cell, converted to AP, loses its potential to differentiate into other cell types. This is followed by a differentiation phase, which is accompanied by major morphological changes. The AP shifts from a fibroblast-like to a spherical shape due to changes in the cytoskeleton [Gregoire *et al.*, 1998] as well as in the extracellular matrix [Selvarajan *et al.*, 2001]. The last stage is a terminal differentiation phase, when the cell gains the typical features of a mature adipocyte (lipid transport and synthesis, adipokine secretion, etc.). This involves increased levels of enzymes for TG synthesis/degradation, glucose transporters, insulin receptor, and adipokines such as leptin, adipsin, resistin, among others.

The main cellular models used to study the differentiation are AP cell lines, already committed to the adipocyte lineage (for example the 3T3-F442A and 3T3-L1 cells, derived from disaggregated 17-19 day old Swiss 3T3 mouse embryos [Green *et al.*, 1974; Green *et al.*, 1976]); and multipotent stem cell lines. For instance, differentiation of confluent cell-cycle arrested 3T3-L1 APs can be induced by a cocktail of insulin, 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone (DXM). After 48 hours insulin alone is required to continue the program [Cornelius *et al.*, 1994]. The first stage of adipogenesis involves the transcriptional induction of C/EBP-β and δ within 24 hours [Ramji *et al.*, 2002]. Later, the cell enters again the cell cycle and undergoes a phase of multiclonal expansion (MCE) (Fig.3) [Cornelius *et al.*, 1994; Ntambi *et al.*, 2000; Tang *et al.*, 2003]. These events depend on a cascade of proteins, such as E2Fs and retinoblastoma protein (pRB) [Fajas *et al.*, 2002[a,b]]. At a molecular level, C/EBP-β is hyperphosphorylated and activated by glycogen synthase kinase-3β and mitogen-activated protein kinase (MAPK) during the G1 to S transition. Then C/EBP-β and δ induce the expression of the master adipogenic regulators, PPAR-γ and C/EBP–α (Fig.3), which start a positive feedback mechanism enhancing their own expression. By day 2 of differentiation, C/EBP–α starts to accumulate and is phosphorylated by cyclin D3-cdk4/cdk6 complexes, inhibiting the proliferation and thus allowing the start of the final differentiation phase and the expression of adipose genes [Wang *et al.*, 2006]. By day 8 more than 90% of adipocytes are mature. PPAR-γ, a member of the nuclear receptor family of ligand-activated transcription factors, is necessary and sufficient for AT formation [Farmer *et al., 2006*], and fundamental for both white and brown adipocytes differentiation [Kajimura *et al.,* 2008]. C/EBP–α directly induces the transcription of many AT genes but when ectopically expressed can promote adipogenesis only in presence of PPAR-γ [Freytag *et al.*, 1994; Rosen *et al.*, 2002].

Other transcription factors involved in adipogenesis include: SREBPc1, which mediates the induction of lipid biosynthesis by insulin and induces PPAR-γ expression [Kim *et al.*, 1996; Kim *et al.*, 1998[a,b]]; the Krupple-like factors (KLFs) [Gray *et al.,* 2002; Mori *et al.,* 2005]; cAMP response element-binding protein

(CREBP) [Zhang *et al.*, 2004]. While others, belonging to the GATA-binding and forkhead protein families, are involved in the repression of adipogenesis.

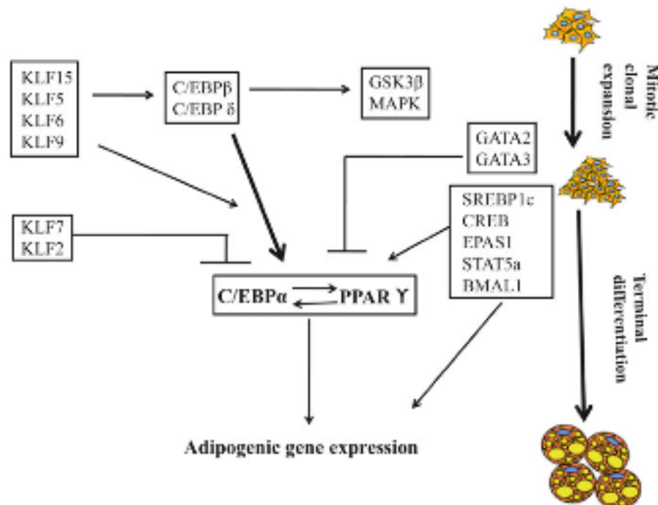


Figure 3. Transcriptional regulation during 3T3-L1-clonal expansion and terminal adipocyte differentiation [Symonds, 2011]. Adipogenesis starts with the induction of C/EBP-β and δ. Then the cells undergo a phase of multiclonal expansion. C/EBP-β and δ induce the expression of the master adipogenic regulators, PPAR-γ and C/EBP–α. By day 2 of differentiation, C/EBP–α starts to accumulate and is phosphorylated by cyclin D3-cdk4/cdk6 complexes, inhibiting the proliferation and thus allowing the start of the final differentiation phase and the expression of adipose genes

Signals for the induction of adipogenesis include hormones, cytokines, growth factors and probably also epigenetic factors, miRNAs and circadian clock genes. Positive regulators are: insulin and components of the insulin/insulin-like growth factor (IGF)-1 pathway [Kim & Chen, 2004; Klemm *et al.*, 2001]; the thyroid hormone (T3) and glucocorticoids [Joiner *et al.*, 2000]. Among negative regulators: Wnt and the Wnt/β-catenin pathway [Ross *et al.*, 2000; Kanazawa *et al.*, 2005[a,b]; Singh *et al.* 2006]; the transforming growth factor (TGF) β; tumour necrosis factor (TNF)–α and interleukine (IL)-1β, [Suzawa *et al.*, 2003].

Metabolic function of adipose tissue

Most of the body's energy reserves are stored in adipocytes in the form of TGs, which may originate from two major routes: *de novo* lipogenesis from non-lipid precursors or uptake of free fatty acids (FFAs) from the plasma. On the other

hand, energy release from AT is achieved by the breakdown of TGs to FFAs and glycerol by lipolysis.

Figure 4. Overview of TG metabolism in adipocytes. Regulation of lipid metabolism in adipocytes is controlled by 3 basic cellular functions: FA uptake, lipogenesis (FA and TG synthesis), and lipolysis (TG hydrolysis) [Avram *et al.*, 2005]

De novo lipogenesis is the synthesis of fatty acids from non-lipid substrates. It takes place in AT of small animals on a “rich in carbohydrate-low in fat” diet. In mammals the proper sites are liver, WAT, and the mammary glands during lactation. In humans, the contribution of WAT to whole-body lipogenesis is very low and less than that of liver. The capacity of the lipogenic pathway of AT is higher in rats compared to humans and it is related with the abundance of mRNA of the two transcription factors controlling the lipogenic pathways: SREBP-1c and CREBP.

Regarding the uptake of FFAs, circulating TGs exist in chylomicrons or in very-low density lipoprotein (VLDL) particles, originated in the intestine and the liver, respectively. The uptake of fatty acids from these lipoproteins constitutes the major pathway for TG accumulation in human WAT (Fig. 5). VLDL and chylomicron particles are processed by lipoprotein lipases (LPL) in the luminal space because they are too large to penetrate the endothelial lining of AT capillaries [Frayn *et al.*, 2002]. Thereafter, long-chain fatty acids (LCFA) can diffuse passively across the

plasma membrane, but transport can be accelerated by membrane proteins or moving lipid rafts [Schaffer *et al.*, 2002]. Fatty acid transport proteins (FATPs) have been shown to enhance LCFA uptake [Stahl *et al.,* 2002]. FATPs expression is negatively regulated by insulin, whereas starvation and PPARγ and PPARα agonists increase FATP mRNA levels [Martin *et al.*, 1997; Martin *et al.,* 2000]. On the other hand, fatty acid translocase CD36 is involved in regulating the raft-dependent uptake of LCFA into muscle cells and also in AT [Hajri *et al.,* 2002; Pohl *et al.,* 2005]. Alterations in fatty acid metabolism and FFA plasma level are involved in the onset of insulin resistance, dyslipidemia and hypertension, and could interfere with TG synthesis in AT and other FFA-mediated intracellular actions.

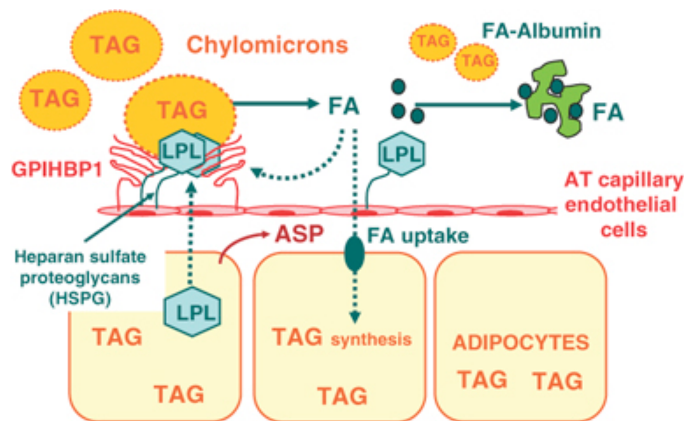


Figure 5. Binding of lipoprotein lipase (LPL) and of a chylomicron particle on the surface of a capillary microvascular endothelial cell [Lafontan *et al.*, 2008]. LPL-dependent lipolysis occupies key position in lipid and lipoprotein metabolism and fatty acid trapping is a fundamental buffering process of normal AT activity. The hydrolysis of lipoproteins TAGs by LPL provides non-esterified fatty acids (NEFA) for storage as TAG in adipocytes. Untrapped NEFAs bind to albumin and flow outside the adipose tissue.

During fasting or exercise, when the organism needs of energy reserves, TGs from AT are hydrolysed, and the liberated fatty acids are destined to the plasma [Langin *et al.,* 2000; Langin *et al.*, 2006]. The whole process takes the name of lipolysis, and involves several lipases and lipid-droplet associated proteins such as perilipins that coat the lipid droplets blocking access to the lipases [Brasaemle *et al.*, 2007].
In rodents and humans, the fatty acids released into the cytosol can be removed by mitochondrial β-oxidation, though this is a minor pathway in white adipocytes.

In normal fed rats just 0.2% of endogenous fatty acids are oxidized. Fasting pushes towards oxidation but without a significant impact [Wang *et al.*, 2003]. Despite human AT is rich in mitochondria, O_2 consumption per kg of wet weight of AT is very low compared to skeletal muscle and is not affected by obesity or insulin resistance [Frayn *et al.*, 1995] In the postprandial state, fatty acid oxidation represents only a small percent of the rate of fatty acid release from adipocytes [Coppack *et al.*, 1990]. Elevated fatty acid concentrations in the cytosol of adipocytes may induce mitochondrial uncoupling and thereby allow mitochondria to remove more fatty acids. The ectopic expression of PGC1-α induces the expression of the *UCP1* and glycerol kinase and other genes related to fatty acid oxidation and mitochondrial respiratory chain [Mazzucotelli *et al.*, 2007]. In brown fat cells UCP1 is located in the inner membrane of mitochondria and uncouples oxygen consumption from ATP synthesis [Tiraby *et al.*, 2003[a]]. Increased expression of PGC-1α in adipocytes may promote the utilization of fatty acids within the cells and limit their release into the blood [Tiraby *et al.*, 2003[b]]. Leptin stimulates fatty acid oxidation by activating AMP-activated protein kinase [Minokoshi *et al.*, 2002] and also stimulates peroxisome proliferator-activated receptor alpha [Suzuki *et al.*, 2007].

Endocrine function of adipose tissue

AT is not just a passive reservoir for energy storage, in 1987 it was already identified as a site for sex steroid metabolism [Siiteri, 1987] and for the production of adipsin [Flier *et al.*, 1987]. Later, in 1994, leptin was identified and characterised [Zhang *et al.*, 1994]. In the following years many other signalling molecules were discovered, including efferent molecules and receptors (Table 1) [Kershaw *et al.*, 2004]. Adipokines (signalling proteins products of AT) regulate various physiological processes like appetite/satiety, energy expenditure, insulin secretion and sensitivity, inflammation, etc., in an endocrine manner but they also exert autocrine and paracrine functions regulating adipogenesis and adipocyte metabolism (Table 1).

Adipokine	Primary source(s)	Binding partner or receptor	Function
Leptin	Adipocytes	Leptin receptor	Appetite control through the central nervous system
Resistin	Peripheral blood mononuclear cells (human), adipocytes (rodent)	Unknown	Promotes insulin resistance and inflammation through IL-6 and TNF secretion from macrophages
RBP4	Liver, adipocytes, macrophages	Retinol (vitamin A), transthyretin	Implicated in systemic insulin resistance
Lipocalin 2	Adipocytes, macrophages	Unknown	Promotes insulin resistance and inflammation through TNF secretion from adipocytes
ANGPTL2	Adipocytes, other cells	Unknown	Local and vascular inflammation
TNF	Stromal vascular fraction cells, adipocytes	TNF receptor	Inflammation, antagonism of insulin signalling
IL-6	Adipocytes, stromal vascular fraction cells, liver, muscle	IL-6 receptor	Changes with source and target tissue
IL-18	Stromal vascular fraction cells	IL-18 receptor, IL-18 binding protein	Broad-spectrum inflammation
CCL2	Adipocytes, stromal vascular fraction cells	CCR2	Monocyte recruitment
CXCL5	Stromal vascular fraction cells (macrophages)	CXCR2	Antagonism of insulin signalling through the JAK–STAT pathway
NAMPT	Adipocytes, macrophages, other cells	Unknown	Monocyte chemotactic activity
Adiponectin	Adipocytes	Adiponectin receptors 1 and 2, T-cadherin, calreticulin–CD91	Insulin sensitizer, anti-inflammatory
SFRP5	Adipocytes	WNT5a	Suppression of pro-inflammatory WNT signalling

ANGPTL2, angiopoietin-like protein 2; CCL2, CC-chemokine ligand 2; CXCL5, CXC-chemokine ligand 5; IL, interleukin; JAK, Janus kinase; NAMPT, nicotinamide phosphoribosyltransferase; RBP4, retinol-binding protein 4; SFRP5, secreted frizzled-related protein 5; STAT, signal transducer and activator of transcription; TNF, tumour necrosis factor.

Table 1. Overview of adipokines' primary sources, binding partners and functions [Ouchi, 2001]. Adipokines are signalling molecules synthesized mostly by of AT) that regulate physiological processes like appetite/satiety, energy expenditure, insulin secretion and sensitivity, inflammation acting as endocrine factors. They also exert autocrine and paracrine functions in the regulation of adipogenesis and adipocyte metabolism.

LEPTIN

Leptin is a 16 KDa protein that is primarily produced in AT [Dalamaga *et al.*, 2013]. It was identified as the product of the *ob* gene in mice which, in humans, is located on chromosome 7 [Zhang *et al.*, 1994]. Circulating leptin levels are directly proportional to the amount of body fat and fluctuate with changes in caloric intake: its levels raise after feeding, whereas fasting results in a decrease of leptin levels [Chan *et al.*, 2003]. Leptin regulates food intake and energy distribution, thus, eventually controls body weight. Leptin is crucial for energy homeostasis in response to energy deprivation: this happens during acute fasting, exercise, etc. In these circumstances leptin levels fall independently of the amount of body fat [Boden *et al.*, 1996], causing the initiation of mechanisms to increase energy intake and preserve energy expenditure.

Independently of its effects on feeding behaviour, leptin has strong effect on insulin action and carbohydrate and lipid metabolism [Barzilai *et al.*, 1997; Halaas *et al.*, 1997]. Leptin promotes lipid oxidation and protein synthesis and curtails lipogenesis, which results in loss of adiposity without affecting lean body mass [Shimabukuro *et al.*, 1997, Wang *et al.*, 1999]. Systemic leptin acts independently of weight and calories by, in contrast to insulin that stimulates lipogenesis, depleting TG in the WAT, but without raising the levels of circulating FFAs, thanks to their augmented AMPK-dependent mitochondrial oxidation, the induction of futile metabolic pathways and the suppression of lipogenesis [Shimabukuro *et al.*, 1997, Wang *et al.*, 1999]. Additionally, leptin prevents the accumulation of lipids in ectopic tissues alleviating lipotoxicity [Minokoshi *et al.*, 2002]. Leptin also regulates the endocannabinoid tone. Endocannabinoids are lipid mediators synthetized in the CNS and peripheral tissues that regulate food intake and lipogenesis through their receptor, CB1. In obesity the increased endocannabinoid tone is related to a malfunctioning central leptin signalling in controlling peripheral endocannabinoids [Buettner *et al.*, 2008]. Finally, leptin actions also affect neuroendocrine function, bone metabolism and the immune system [Dalamaga *et al.*, 2013; Carbone *et al.*, 2012].

Leptin receptor (LepR)

The Leptin Receptor (LepR) is the receptor binding the hormone/cytokine leptin and triggers, upon hormone binding, the activation of leptin signalling. The LepR belongs to the class 3-cytokine receptor family and is expressed in the CNS as well as in several peripheral tissues including AT. LepR was identified as the product of the *db* gene that maps within the 6-cM interval on the mouse chromosome 4 [Tartaglia *et al.*, 2005] and from which originate at least six alternatively spliced isoforms (a-f) (shown as Ob-R in the figure). All share identical extracellular ligand-binding domains but they differ at the C-terminus. Five of the six have transmembrane domains but only LepR-b encodes all protein motifs capable of activating the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signal transduction pathway. JAKs associate constitutively with a

conserved box 1 motif (intracellular aa 6–17), which is critical for JAK2 activation. A putative Box 2 motif (intracellular aa 49–60), apparently required for maximal activation of JAK2, has also been identified. Additionally, LepR-b has three conserved tyrosines in its cytoplasmic domain, corresponding to positions Y985, Y1077, and Y1138. The latter functions as a docking site for STAT3. LepR-e is truncated before the membrane-spanning domain and is secreted (Fig. 6) [Ceddia, 2005].

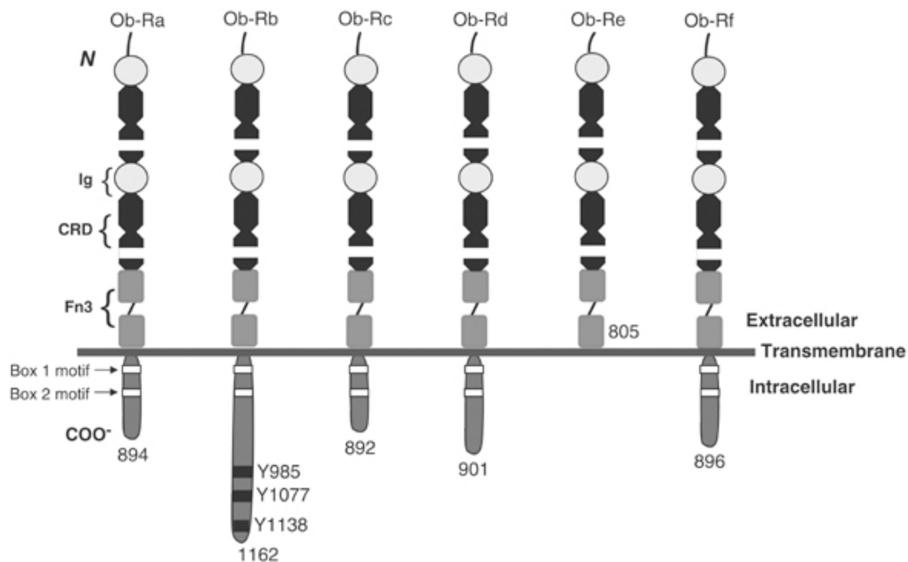


Figure 6. LepR isoforms. In mouse there are at least six different isoforms of the LepR (shown as Ob-R in the figure). All share the same extracellular ligand-binding domains but they differ at the C-terminus. Only LepR-b encodes all the motifs for the activation of JAK/STAT signal transduction pathway [Ceddia, 2005].

One of these spliced variants, the isoform b, is expressed throughout the CNS, particularly in the arcuate nucleus (ARC) of hypothalamus: here it regulates energy homeostasis and neuroendocrine function [Kelesidis *et al.*, 2010]. It is also abnormally spliced in C57BL/Ks *db/db* mice: the resulting mutant protein lacks the cytoplasmic region, and is likely to be defective in signal transduction [Lee *et al.*, 1996]. This suggests that the weight-reducing effects of leptin may be mediated by signal transduction through a leptin receptor in the hypothalamus [Lee *et al.*, 1996]. However, it was also shown that the adipocyte-specific LepR-b overexpression of a LepR-b transgene completely prevented adipocyte

hypertrophy, hyperplasia and the increase in body fat induced in wild-type mice by a high fat diet (HFD) [Wang *et al.*, 2005].

Upon binding to its receptor leptin activates several signalling pathways (Fig 7).

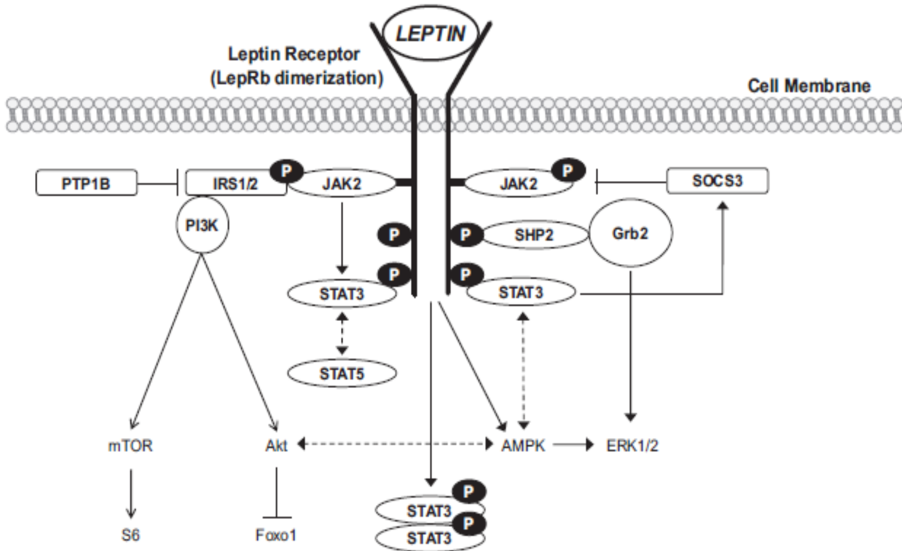


Figure 7. Intracellular Leptin Signalling Pathways [Dalamaga *et al.*, 2013]. Leptin binding to its receptor prompts activation of the JAK2/STAT3 and other downstream related pathways.

Binding of leptin results in the dimerization of its receptor, prompts JAK2 phosphorylation and consequent activation of the JAK2/STAT3 pathway. Phosphorylated STAT3 dissociates from the LepR and (as active dimer) regulates gene expression after translocation to the nucleus. STAT3 signalling may activate STAT5, which in turn can regulate STAT3-dependent gene expression. JAK2 phosphorylation also activates src homology-2-containing protein tyrosine phosphatase 2 (SHP2), which recruits the adaptor protein Growth factor Receptor-Bound protein-2 (Grb2) for the activation of Extracellular signal-Regulated Kinase 1/2 (ERK1/2). Upon LepR dimerization, STAT3 induces Suppressor Of Cytokine Signaling 3 (SOCS3) expression, which acts as a negative-feedback signaling inhibiting leptin-stimulated phosphorylation of JAK2. Leptin-mediated Phosphatidyl-Inositol 3 Kinase (PI3K activation) via Insulin Receptor Substrate 1/2 (IRS1/2) causes Akt/mTOR (v-Akt murine Thymoma viral oncogene homolog/mammalian Target Of Rapamycin) phosphorylation and subsequent

regulation of ribosomal protein 6 (S6) and FOrkhead boX O1 (FOXO1) activity. Protein Tyrosine Phosphatase 1B **(**PTP1B) may interfere with PI3K activation by inhibiting leptin-stimulated IRS-1/2 phosphorylation. Table 2 illustrates the Leptin-activated signalling pathways and the effects of their activation.

Signaling Pathway	Effects of Pathway Activation	Evidence
JAK2/STAT3: tyrosine kinase signaling pathway responsible for leptin signal transduction	CNS regulation of adipose tissue maintenance	Neural disruption of the STAT3 pathway results in severe obesity in mice
	Stimulation of anorectic pathways in ARC of hypothalamus	$\uparrow$POMC transcription; $\downarrow$AgRP/NPY[a] transcription
SHP2/MAPK: upstream activator of the MAPK pathway	CNS regulation of energy homeostasis	ERK1/2 activation by JAK2; ERK1/2 is the primary MAPK implicated in leptin's central regulation of energy homeostasis
PI3K/Akt/mTOR: kinase pathway that mediates leptin's effects on insulin metabolism and cellular proliferation	Activation of growth pathways/cellular proliferation pathways	Akt activation corresponds with cellular proliferation in several murine peripheral tissue cell lines
	Anorectic central effects	Initiation of the PI3K pathway activates POMC-expressing neurons
	Mediation of leptin's impacts on insulin metabolism	The PI3K/Akt/mTOR pathway may play a role in pancreatic islet function
FoxO1: orexigenic downstream mediator of the PI3K pathway	Orexigenic central effects	FoxO1 activation the expression of POMC and promotes the expression of AgRP and NPY in the CNS; leptin administration inhibits FoxO1 activity and expression in a PI3K-dependent manner
	Point of intersection for leptin and insulin signaling	Deletion of IRS2 in LepRb CNS neurons deregulates PI3K signaling and results in increased FoxO1 signaling
AMPK: serine/threonine protein kinase that modulates energy regulation	Peripheral adipose tissue maintenance	Peripherally promotes increased fatty-acid oxidation and various insulin-sensitizing effects
	Orexigenic central effects	In the CNS promotes increased food intake and weight gain; leptin inhibits AMPK centrally and activates it peripherally
SOCS3: key negative feedback molecule in leptin signaling	Possibly mediates leptin resistance in obesity	Interferes with JAK2/STAT3 activation SOCS3 mRNA is increased in leptin-resistant mouse model of obesity
SHP2: Src homology-2-containing tyrosine phosphatase implicated in growth-factor and cytokine signaling	May be a therapeutic target for treatment of leptin resistance; possibly mediates leptin resistance in obesity	SHP2's interference in JAK2/STAT3 activation is counteracted by the SHP2-mediated upregulation of the leptin-ERK1/2 pathway
PTP1B: negative regulator of leptin-stimulated pathways	Possibly mediates leptin resistance in obesity	PTP1B binds to and dephosphorylates JAK2

Table 2. Leptin-activated signalling pathways (modified from Dalamaga *et al.*, 2013)
The signalling pathways include the JAK2/STAT3, SHP2/MAPK, PI3K/Akt/mTOR, FoxO1 and AMPK pathways [Kelesidis *et al.*, 2010]

Leptin resistance and leptin deficiency

Leptin resistance originates from disrupted leptin signalling and is generically defined as the failure to promote salutary metabolic outcomes, such as suppression of appetite/weight gain and stimulation of energy expenditure. Leptin resistance is linked to excessive energy states and may result from interference with JAK2 phosphorylation by signalling molecules such as suppressor of cytokine signalling 3 (SOCS3), SHP2, and protein tyrosine phosphatase 1B (PTP1B) [Myers *et al.*, 2010]. Other mechanisms include: impaired blood brain barrier leptin transport, defective LepR-b trafficking from the Golgi to the cellular membrane;

obesity-associated endoplasmic reticulum (ER) stress and chronic low-level inflammation [Moon *et al.*, 2011]. These mechanisms differ from other models of genetic obesity in which mutations in the LepRb gene or downstream in the pathway block proper leptin response [Myers *et al.*, 2010]. Table 3 summarizes disease states associated with leptin deficiency and leptin resistance**.**

Disease States	Estimated Prevalence	Clinical Features
Fat Loss Associated with Leptin Deficiency		
Congenital generalized lipoatrophy	Rare	Generalized fat wasting, insulin resistance, impaired glucose tolerance, type 2 diabetes mellitus, dyslipidemia, hepatic steatosis, acanthosis nigricans
HAART-induced lipoatrophy	15%–36% of HIV-infected patients	Fat wasting of the face, arms, legs and buttocks, insulin resistance, impaired glucose tolerance, type 2 diabetes mellitus, hypertriglyceridemia, hepatic steatosis
Hypothalamic amenorrhea (functional)	3%–7.6% in women aged 13–44 years; up to 69% of trained female athletes	Strenuous exercise, psychogenic stress, energy deficit, low bone mass and increased bone loss, neuroendocrine dysfunction with decreased GnRH pulsatility and estradiol levels, decreased thyroid and IGF-1 levels, increased growth hormone levels
Anorexia nervosa	1.2%–2.2% lifetime prevalence for women	Weight loss to body weight <85% of expected, refusal to maintain normal body weight, fear of gaining weight, disturbed body image, severe restriction of food intake, amenorrhea and other neuroendocrine dysfunction
Obesity as a Manifestation of Leptin Deficiency		
Complete congenital leptin deficiency	Rare	Hyperphagia, early-onset morbid obesity, hypogonadotrophic hypogonadism, hyperinsulinemia and type 2 diabetes mellitus, dyslipidemia, immune dysfunction
Heterozygous leptin deficiency	≤6% in obese subjects	Garden-variety obesity with hypoleptinemia relative to fat mass, normal neuroendocrine function
Obesity Associated with Leptin Resistance (Involving Leptin and Molecular Signaling Pathways Downstream of the Leptin Receptor)		
Leptin receptor gene mutations	Rare	Phenotype similar to congenital leptin deficiency, hyperphagia, less-remarkable hyperinsulinemia, hypogonadotrophic hypogonadism, abnormal growth hormone secretion with mild growth delay, hypothalamic hypothyroidism, immune dysfunction
POMC mutations	Rare	Hyperphagia, early-onset obesity, ACTH deficiency with adrenal insufficiency/crisis, lack of MSH function at MC1Rs resulting in pale skin and red hair
Prohormone convertase 1 deficiency	Rare	Hyperphagia, early-onset obesity, hypogonadotrophic hypogonadism, abnormal glucose homeostasis, hypoinsulinemia, hypocortisolemia
Melanocortin 4 receptor mutations	5%–8% of childhood obesity	Hyperphagia, early-onset obesity, increased fat and lean body mass, increased linear growth and bone density, severe hyperinsulinemia
Melanin-concentrating hormone receptor-1 mutations (loss of function)	Rare	Markedly underweight individuals
Neurotropin receptor tropomyosin-related kinase B mutations	Rare	BDNF deficiency resulting in hyperphagia, severe obesity, developmental delay, cognitive dysfunction
Mutations of other molecules downstream of leptin receptor	Rare	Obesity with onset in childhood
Mechanism to be discovered	>90% of obese individuals	Garden-variety obesity

Table 3 Disease states associated with leptin deficiency and leptin resistance (modified from Dalamaga *et al.*, 2013). The table summarises prevalence and clinical features of disorders and pathological states related to leptin and leptin signalling. These conditions may concern: fat loss associated with leptin deficiency; obesity as manifestation of leptin deficiency; obesity associated to leptin resistance involving molecules and pathways downstream of leptin receptor.

Nowadays the focus on leptin is on its role in weight loss maintenance more than as an anti-obesity hormone. It is efficacious in patients with congenital and acquired leptin deficiency, characterized by hyperphagia and obesity [Cahn *et al.*, 2006; Farooqi *et al.*, 2002; Licinio *et al.*, 2004] but does not work in obese humans who show leptin resistance [Heymsfield *et al.*, 1999]. US Food and Drug Administration (FDA) recently approved metreleptin also to treat generalized lipodystrophy [Sinha *et al.*, 2014; Kelesidis *et al.*, 2010]. Both leptin deficiency and lipodystrophy are associated with insulin resistance and impaired glucose metabolism, so leptin replacement can improve glucose metabolism. Leptin provokes a decrease in body weight and fat mass (especially ectopic or intra-abdominal fat) ameliorating insulin resistance [Morandi *et al.*, 2004], and can also activate insulin-sensitive tissues.

A useful model to study the relation between leptin and diabetes is the uncontrolled, insulin-deficient diabetes (uDM), a pathology that causes insulin resistance [DeFronzo *et al.*, 1982; DeFronzo *et al.*, 1986; Yki-Järvinen *et al.*, 1986; Perseghin *et al.*, 2003; German *et al.*, 2010], impaired glucose metabolism [German *et al.*, 2010; Schwartz *et al.*, 1996; Chinookoswong *et al.*, 1999; Morton *et al.*, 2005; Coppari *et al.*, 2005] and neuroendocrine derangements. Administration of leptin at a dose that maintains physiological leptinemia prevents the development of severe, progressive insulin resistance in rats with uDM; it also reduces body fat while sparing lean mass, and attenuates the increased energy expenditure [Nair *et al.*, 1984]. Insulin resistance occurs in patients with T2D [DeFronzo *et al.*, 1982; DeFronzo *et al.*, 1986; Yki-Järvinen *et al.*, 1986; Perseghin *et al.*, 2003] and is also a risk factor for vascular complications [Chaturvedi *et al.*, 2001; Soedamah-Muthu *et al.*, 2004]. Insulin therapy combined with lifestyle modifications [Rosenfalck, 2006; Yki-Jarvinen *et al.*, 1984] and drugs like thiazolidinediones (TZD) [Strowig *et al.*, 2005] improves insulin sensitivity and preserves pancreatic β-cell function but these benefits are offset by severe hypoglycemia and weight gain that prevent a total glycemic regulation [The DCCT Research Group, 1988, 1993, 1997]. A therapy including also leptin may reduce the

amount of insulin required to achieve glycemic control and also limit weight gain [Miyanaga *et al.*, 2003].

Large-scale clinical and epidemiological trials are still necessary to investigate potential negative effects of leptin therapy and its real efficacy, to determine whether other leptin sensitizers would be useful in the treatment of obesity or in the promotion of weight maintenance; to determine the appropriate doses of leptin to optimize metabolic response and avoid leptin resistance, but also the diagnostic criteria for hypo- and hyperleptinemia.

Obesity as pathological condition and a risk factor for many other diseases

Obesity is a heterogeneous group of disorders and conditions originated from many different causes. The body weight itself depends on the interaction of genetic, environmental and psychosocial factors. Genetic differences are important but as stated before the huge rise in the prevalence of obesity in nowadays may be better explained by behavioural, environmental and technological changes that affect the balance between energy intake and expenditure [Kopelman, 2000]. From an evolutionary point of view the ability to withstand starvation is critical for species survival and is based on the storage of excess calories when food is accessible. However, in the presence continuous of nutritional surplus, this mechanism could lead to an excess of adiposity and to metabolic dysfunctions [Hotamisligil, 2006].

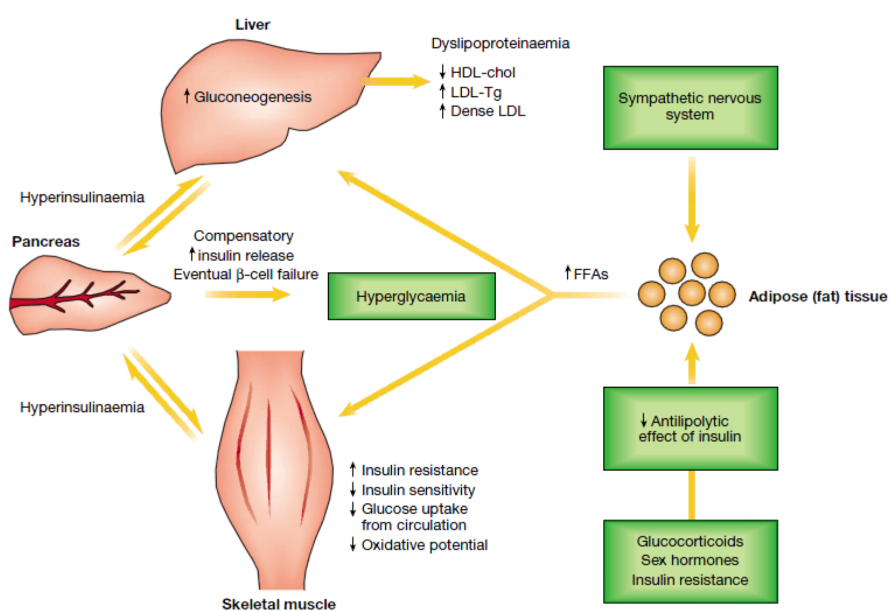
In the contest of gene-susceptible obesity, more than 300 locus in the human genome have been involved, nonetheless, the influence of the genotype may be affected by non-genetic factors and, apart from rare obesity-associated syndromes, the genetic component of obesity seems to operate through susceptibility genes. These genes increase the risk for developing some features even if they are not essential o sufficient by themselves for the onset of the pathology [Kopelman, 2000]. According to studies conducted in pairs of twins,

variations in genetic susceptibility determine who will probably develop obesity in certain environmental conditions [Kopelman, 2000]. Rodent models have been used in the search for obesity candidate genes and their effect on fat composition and distribution, food intake and energy expenditure. These monogenic rodent models of obesity are characterised by early onset of obesity concomitant with hyperinsulinemia and insulin resistance. One model is represented by the *ob*/*ob* mouse that carries a mutation for the leptin gene, thus lacking leptin signalling [Zhang *et al.*, 1994]. Intraperitoneal injections of leptin in these mice lead to a decrease in body weight, fat, food intake and insulinemia while injections in the lateral or third ventricle of the brain reduce weight [Campfield *et al.*, 1995]. A second model is the *db*/*db* mouse, characterised by high leptin levels and for which leptin injections have no effect: in fact the mutation affects the LepR and, therefore, leptin signalling. In humans, obesity does not generally depend in a relative or absolute absence of leptin, in fact most obese subjects show high levels of circulating leptin in proportion to their fat depots [Maffei *et al.*, 1995] and only few individuals with severe obesity have been reported to be deficient or harbour mutations in the LepR gene. Moreover, many others genes, including factors involved in thermogenesis and appetite regulation are more often associated with obesity.

In addition, obesity is also a feature associated to many single-gene (like the Prader-Willi syndrome, PWS) and more heterogeneous (like the Bardet-Biedl syndrome) disorders in humans. Overall, obesity represents of course a huge medical problem as body fatness is accompanied by changes in the physiology of the organism, which at least in part depend on the distribution of AT. Obesity results in variation of total blood volume and alterations of the cardiac function, whereas the presence of fat in the thorax and abdomen compromises the respiratory function and mechanics that may result in hypoxia, cardiac arrhythmias and stress and disturbed sleep.

In particular, the accumulation of intra-abdominal visceral AT is one of the factors that lead to hypertension an excessive release of FFAs into the portal system [Frayn *et al.,* 1996]. Elevated FFAs concentration in plasma leads to the impairment of insulin action in liver causing the impropriate maintenance of

hepatic glucose production and the impairment of hepatic glucose utilization, which result in glucose intolerance. For instance in non-obese conditions, insulin would exert its post-prandial action, avoiding this malfunctioning. So obesity-induced insulin insensitivity is not restricted to just AT, but it is systemic, affecting other insulin-target tissues such as liver, skeletal muscle and pancreas. In addition, hepatic insulin clearance is reduced, leading to increased systemic insulin levels and further down regulation of insulin receptors. At the beginning of this process the pancreas can compensate by increasing insulin release, which leads to hyperinsulinemia, but with continuously increasing concentrations of FFAs, hyperglycemia prevails. This interaction of conditions is related to a dyslipoproteinemic state, with alterations of plasma lipid profile typical of obesity: high fasting triglyceridemia, and LDL-cholesterol levels, and reduced level of HDL-cholesterol [Sniderman *et al.*,1995].



Box 1. Obesity and T2D (modified from Kopelman, 2000). The scheme illustrates the deleterious effect of adipose tissue accumulation on insulin sensitivity and glucose tolerance. FFAs have a detrimental action on the uptake of insulin by the liver, which results in increased liver gluconeogenesis and systemic dyslipidemia. This contributes to the systemic hyperinsulinemia and decreased skeletal insulin sensitivity with reduced glucose uptake. β-cells of the pancreas initially compensate with insulin hyper production but fail in time, with consequent increment of hyperglycemia, and finally onset of type 2 diabetes.

As stated before, obesity is one of the pathological processes included in the so called metabolic syndrome and which generally implies a cluster of chronic and complex diseases/disorders, all of which feature metabolic deterioration and are in close relationship/occurrence among them. Nonetheless, obesity is also a risk factor for other pathological processes out of the metabolic syndrome such as degenerative disorders, airway disease and some cancers [Semenkovich, 2006] [Fig. 8]. Components found in all these obesity-associated disorders are either insulin resistance/hyperinsulinemia or chronic low-grade inflammatory response, or both.

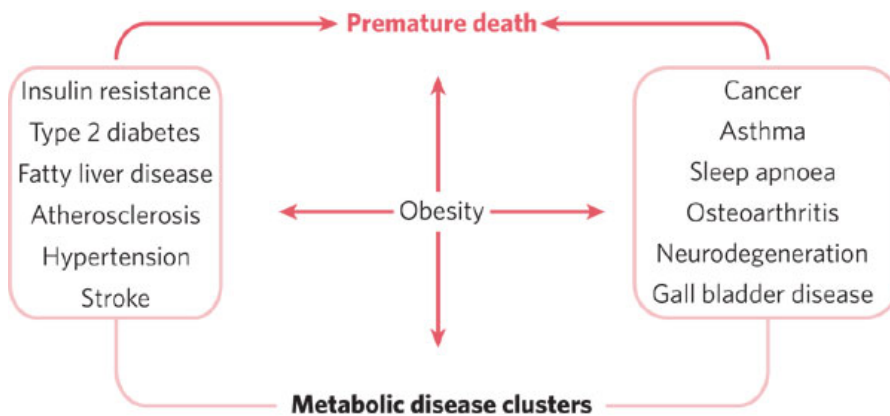


Figure 8. Obesity is a central feature that increases the risk for many pathological conditions, (modified from Hotamisligil, 2006). Obesity is one of the pathological conditions included in the metabolic syndrome, which represents a cluster of related diseases/disorders featuring metabolic deterioration. Obesity is also a risk factor for other pathological processes such as degenerative disorders, airway diseases and some cancers. Components found in all these obesity-associated disorders are either insulin resistance/hyperinsulinemia or chronic low-grade inflammatory response, or both.

Insulin signalling, insulin resistance and T2D

Insulin circulating in the blood stream binds its Insulin Receptor (IR) on the cells membrane. The main endocrine insulin target tissues are skeletal muscle, liver and AT. As an autocrine factor, in pancreatic β-cell it stimulates the release of insulin and induces its own gene expression [Muller *et al.*, 2006], together with other glucose homeostasis related genes such as Glut2. The binding to IR causes

structural changes within the receptor leading to autophosphorylation of various intracellular tyrosine residues, followed by the recruitment of specific adapter proteins such as the IR substrate proteins (IRS). Multiple tyrosine residues of IRS are then phosphorylated by insulin-bound IR. IRS are thus active and can trigger various insulin signal transduction pathways. Among them, the PI3K pathway which leads to the activation of the Akt/PKB cascade [Cross *et al.*, 1995; Alessi *et al.* 1996]. Depending on the insulin-target tissue, activated Akt elicits glycogen synthesis through inhibition of GSK-3 [Weeren *et al.,* 1998]; protein synthesis via mTOR and downstream effectors [Pullen *et al.,* 1998]; cell survival, through inhibition of apoptosis [Datta *et al.,* 1997] and glucose uptake through Glut4 translocation from vesicles to the cytoplasmic membrane [Tanti *et al.,* 1997]. In general in all cell types analysed up to now, an insulin-induced JNK-mediated negative feedback results in serine phosphorylation and inactivation of IRS signalling [Lee *et al.*, 2003] (Fig. 9). Of particular interest is mTOR, a conserved Ser/Thr kinase target of rapamycin that (integrating various stimuli coming from growth factors, nutrients availability, etc.) regulates metabolism and cell growth through protein/lipid/nucleotide synthesis and inhibition of autophagy. mTORC1 controls protein synthesis by inducing ribosome biogenesis and mRNA translation [Shimobayashi & Hall, 2014]. TOR exists in two functionally and structurally distinct complexes, TOR complex 1 (TORC1) and 2 [Wullschleger *et al.,* 2006; Laplante & Sabatini, 2012]. mTORC1 activity is stimulated by growth factors, nutrients and the cellular energy status [Dibble & Manning, 2013]. Insulin for example activates mTORC1 through the PI3K–phosphoinositide-dependent kinase 1 (PDK1)–AKT pathway. Activated mTORC1 phosphorylates various substrates such as S6K (ribosomal S6 kinase), which has itself several targets, including IRS1, upstream of mTORC1 [Ma & Blenis, 2009; Mamane *et al.,* 2006; Jewell *et al.*, 2013; Mieulet *et al.*, 2010]. Phosphorylation of IRS1 by S6K inhibits IRS1 and PI3K activation, thus forming a negative feedback loop in the regulation of mTOR [Mieulet *et al.*, 2010]. Furthermore mTORC1 promotes lipogenesis by activating SREBPs [Duvel e*t al.*, 2010; Porstmann e*t al.*, 2008; Peterson e*t al.*, 2011]. I*n vivo,* mTORC1, mTORC2 and AKT are required for activation of SREBPs and lipogenesis

[Hagiwara et *al.*, 2012; Sengupta et *al.*, 2010; Kenerson et *al.,* 2011; Yecies et *al.*, 2011; Yuan et *al.*, 2012].

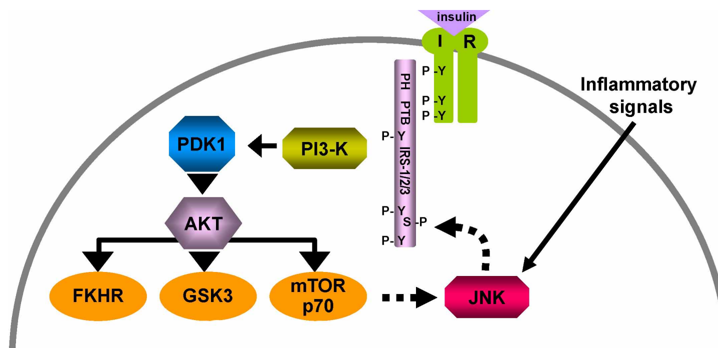


Figure 9. JNK pathway in the regulation of insulin signalling [Yang & Trevillyan, 2008]. Insulin acts on its receptor to increase phosphorylation of the IR and IRS. Phosphorylated IRS lead to activation of the PI3 kinase pathway to increase AKT phosphorylation, which in turn regulates the activation of mTOR/p70(S6K). mTOR signaling triggers IRS-1degradation, a feedback inhibition for insulin signalling, through kinases that increase serine phosphorylation of IRS-1, such as JNK. Inflammatory signals lead to insulin resistance through several inflammatory kinases, including JNK [Yang & Trevillyan, 2008].

Animals are heterotrophic and have evolved in an environment where nutrient sources are often scarce and the caloric demands change. So they had to develop mechanisms to promote anabolism or catabolism depending on the balance between energy demand and supply**.** Insulin secretion and action promote carbohydrate uptake, prompting the conversion of carbohydrates and protein to storage lipids, constituting for this purpose a well-functioning mechanism (Fig. 10). But the environment in which we live today dramatically changed towards an abundance of caloric supply with very little caloric demands. This alteration lead to an increase in metabolic disorders like obesity and related anomalies, with insulin resistance playing an important role [Samuel *et al.*, 2012]. Insulin resistance is always associated with hyperinsulinemia. Currently, there is a debate about which of them is the cause and or the effect. The most accepted idea is that obesity-induced insulin resistance increases insulin demand and leads to hyperinsulinemia. However, an alternative point of view is that the continuous exposure to caloric surplus causes chronic increased levels of plasma insulin to maintain normoglycemia and which finally leads to insulin signalling down-regulation and, hence, insulin resistance.

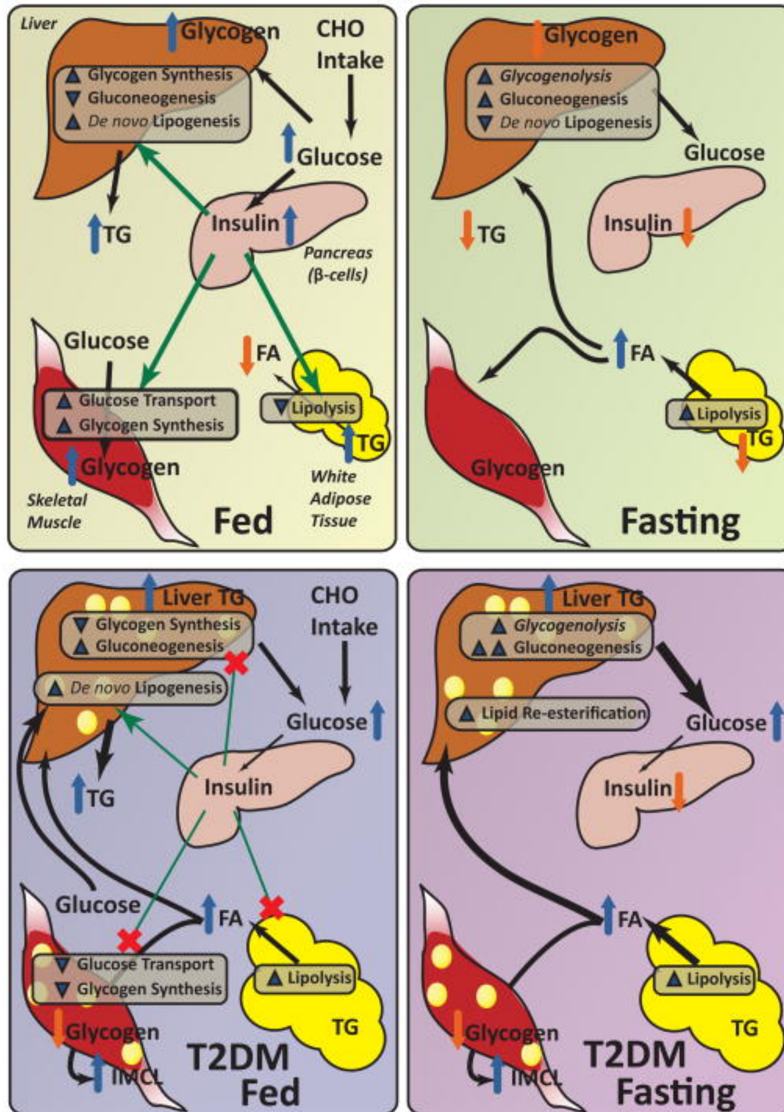


Figure 10. Insulin provides an integrated set of signals that balance nutrient availability and demands (modified from Samuel, 2012). In the fed state, dietary carbohydrates (CHO) increase glycemia and promote insulin secretion from the pancreatic β-cells. In the skeletal muscle, insulin increases glucose entry and glycogen synthesis. In the liver, insulin promotes glycogen synthesis and *de novo* lipogenesis, and inhibits gluconeogenesis. In the adipose tissue, insulin suppresses lipolysis and promotes lipogenesis. In the fasted state, insulin secretion is decreased. Hepatic gluconeogenesis increases and glycogenolysis is promoted. Hepatic lipogenesis diminishes whereas lipolysis increases. In type 2 diabetes, ectopic intramyocellular lipid (IMCL) accumulation impairs the insulin-mediated skeletal muscle glucose uptake. Glucose is diverted to the liver, where lipid increase impairs gluconeogenesis and glycogen synthesis, while lipogenesis remains unaffected. This may result in non-alcoholic fatty liver disease. In adipose tissue, compromised insulin function allows for increased lipolysis, which promotes ectopic re-esterification of lipids in liver and exacerbates insulin resistance. Coupled with a decline in pancreatic β-cells, hyperglycemia develops.

Insulin resistance is a complex metabolic disorder, impossible to relate to a single pathway. The main mechanisms proposed for the pathogenesis of insulin resistance are ectopic lipid accumulation, ER stress (and UPR) and finally chronic inflammation. Insulin resistance is a key feature for the development of T2D, which is characterised by impaired insulin-mediated glucose uptake due to diminished insulin-sensitivity in peripheral tissues [Pettersson *et al.*, 2013]. Progression from obesity to T2D in rodents and humans is dependent on genetic background [Guilherme *et al.*, 2008]. Inbred mouse strains respond in different manners to HFD diets and to the impact of obesity on insulin sensitivity [Clee, 2007]. Despite an increased risk, many obese human patients do not progress to diabetes, suggesting the importance of genetic and environmental factors [Guilherme *et al.*, 2008]. Two features seem to be critical for obesity to elicit T2D: a) impaired responsiveness to insulin in skeletal muscle [Sims *et al.*, 1973, Freidenberg *et al.*, 1988]; b) failure of β-cells to secrete the required levels of insulin to maintain normal fasting blood glucose levels (or to compensate insulin resistance in non diabetic individuals) [Kahn, 2003; Butler *et al.*, 2003; Rhodes, 2005].Various factors contribute to the development of peripheral insulin resistance: increased levels of pro-inflammatory cytokines as TNF-α [Gregor & Hotamisligil, 2011], ER stress caused by adipocyte hypertrophy and the increased plasma level of FFAs [Ozcan *et al.*, 2004; Solinas *et al.*, 2006]. Ser/Thr protein kinases like JNK are activated by these stimuli. As a result, IRS-1 recruitment to the IR is inhibited, thereby preventing activation of the insulin-signalling cascade [Solinas & Karin, 2010]. In normal conditions insulin-bound IR triggers IRS-1 phosphorylation, with the recruitment and activation of downstream effectors, such as the PI3K-Akt cascade [Taniguchi Solinas G & Karin, 2006].

In mouse models of obesity, JNK activity is increased in peripheral insulin-target tissues, (skeletal muscle, AT and liver) with consequent promotion of insulin resistance [Hirosumi *et al.*, 2002]. The administration of the JNK inhibitory peptide JNKi-1 to these mice strongly improves insulin signalling by reducing IRS-1 serine phosphorylation and increasing Akt phosphorylation [Kaneto *et al.*, 2004].

JNK with IR signalling is also related to insulin resistance in pancreatic β-cells. Treatment with FFAs results in sustained JNK activation together with the inhibition of the autocrine insulin action due to JNK-dependent IRS-1 and 2 phosphorylation at serine residues that interfere with the IR binding [Solinas *et al.*, 2006]. Treatment with JNKi-1 or *jnk1* deficiency relieves the inhibition of glucose-induced insulin transcription by FFAs and enhances obesity-inhibited and glucose-induced insulin secretion in isolated pancreatic islets [Kaneto *et al.*, 2004; Solinas *et al.*, 2006; Bennett, 2003; Varona-Santos *et al.*, 2008]. Insulin signalling is also crucial for maintaining the essential function of the β-cells [Wang *et al.*, 2010]. JNK is also involved in the loss of β-cells induced caused by pro-inflammatory stimuli such as IL-1β [Ammendrup *et al.*, 2000; Kim *et al.*, 2005].

The interaction between metabolic and inflammatory pathways

The connection between obesity and T2D highlights the links between nutrient- and pathogen-sensing pathways, and the interface of metabolic and inflammatory responses as the mechanistic core of common chronic metabolic diseases [Hotamisligil, 2006]. A feature key of these two related pathologies seems to be inflammation [Wellen & Hotamisligil, 2005]. Inflammation is the principal response of the organism against injuries, involves systemic signalling and is fundamental for tissue repair [Larsen & Henson, 1983]. However, the consequences of prolonged inflammation may not be beneficial. An example is metabolic diseases, which show a low-grade chronic inflammatory response or 'metaflammation', a condition triggered by nutrients and metabolic surplus, which involves molecules and signalling pathways similar to those of classical inflammation [Hotamisligil, 2006]. Dynamic interactions between immune and metabolic responses could also establish communications with other sites such as pancreatic islets and skeletal muscle, in relation to the development of obesity and T2D [Wellen & Hotamisligil, 2005; Shoelson *et al.*, 2006]. Common or overlapping pathways may regulate both metabolic and immune functions through common key regulatory molecules and signalling systems [Beutler, 2004; Sondergaard, 1993; Song *et al.*, 2006; Shi *et al.*, 2006].

However, in perturbed long-term conditions, it would be hard for the organism to maintain this balance with short-term and adaptive responses. In a similar fashion, chronic disturbance of metabolic homeostasis due to malnutrition or over nutrition, could lead to aberrant immune responses [Wellen & Hotamisligil, 2005]. Unbalanced nutritional habits and lifestyles favour metabolic overload with diminishing physical activity. Under such conditions, these evolutionary advantageous features and the connection between nutrient and pathogen responses have established the groundwork for the explosion of chronic metabolic diseases [Hotamisligil, 2006]. From a clinical point of view**,** obesity, insulin resistance and T2D are associated with chronic inflammation that is characterized by abnormal cytokine production, increased acute-phase mediators, and activation of inflammatory signalling pathways [Wellen & Hotamisligil, 2005]. Experimental evidences connect chronic inflammation to the development of these metabolic diseases and their complications, particularly in the context of obesity and T2D [Wellen & Hotamisligil, 2005; Shoelson *et al.*, 2006; Dandona *et al.*, 2004].

AT appears to be the central metabolic site from where the inflammatory response related to obesity arises as hypertrophic adipocytes generates inflammatory mediators and interact with other effectors of the immune system [Wellen & Hotamisligil, 2005; Shoelson *et al.*, 2006]. In fact, macrophages infiltrate the AT in obese mice and humans [Weisberg *et al.*, 2003; Xu *et al.*, 2003] probably recruited by chemotactic signals produced by AT [Wellen & Hotamisligil, 2005]. The convergence of macrophages could represent an attempt to limit the expansion of AT [Wellen & Hotamisligil, 2005] or it could be aimed for clearance of dead adipocytes [Cinti *et al.*, 2005]. However, the contribution to the emergence and maintenance of obesity-induced inflammatory responses and the functional involvement of macrophages in systemic metabolism are still unclear. Among the different pro-inflammatory mediators, TNF-α has a prominent role in this obesity-associated chronic inflammatory response. TNF-α is a pro-inflammatory cytokine that has a crucial role in septic shock, induction of cytokines, proliferation, differentiation and apoptosis. It acts through its TNF receptors, TNFR1 and TNFR2

[Loetscher *et al.,* 1990; Smith *et al.,* 1990] and IKK/NF-κB and MAP kinase pathways, including JNK pathway, are activated in most types of cells and apoptosis or necrosis could also be induced [Smith *et al.*, 2004]. It is mainly produced by the activated macrophages and is overexpressed in the AT of obese individuals [Hotamisligil *et al.*, 1993]. TNF–α can also activate pathways, such as the JNK pathway, that are critical inhibitors of insulin action, in fact, when administered exogenously, leads to insulin resistance [Hotamisligil *et al.*, 1995; Krogh-Madsen *et al.*, 2006]. Moreover, obese mouse models that lack of TNF–α function show improved insulin sensitivity and glucose homeostasis [Uysal *et al.*, 1997; Ventre *et al.*, 1997]. However, other inflammatory mediators and cytokines are also overexpressed in adipose and other tissues in mice models of obesity and in humans and are also involved in the promotion of insulin resistance [Wellen & Hotamisligil, 2005].

The inflammatory network activated in obesity is able to interact with and disrupt insulin action and signalling. Insulin and IGF receptors belong to a receptor tyrosine kinase family but they use docking proteins to mediate their signalling. Among their intracellular substrates are found the IRS proteins [Taniguchi *et al.*, 2006; White, 2002]. Insulin stimulates tyrosine phosphorylation of IRS proteins: this crucial step is defective in most cases of systemic insulin resistance [Wellen & Hotamisligil, 2005]. TNF–α also targets these elements through inhibitory serine phosphorylation of IRS-1 [Hotamisligil *et al.,* 1996]: this post-translational modification has also been detected in insulin-resistant cells and tissues. IRS-1 is phosphorylated at serine residues by various kinases, interfering with the signalling and altering insulin action [Taniguchi *et al.*, 2006; Aguirre *et al.*, 2000; Paz *et al.*, 1997]. Of particular importance, involved in this mechanism are JNK, IKK [Arkan *et al.* 2005; Yuan *et al.,* 2001; Hundal *et al.*, 2002; Cai *et al.*, 2005] and PKC [Yu *et al,.* 2002; Kim *et al.*, 2004; Boden *et al.*, 2005] which mediate serine phosphorylation of IRS-1 in response to stress and pro-inflammatory signals [Aguirre *et al.*, 2000; Gao *et al.*, 2002; Griffin *et al.*, 1999]. The disruption of IR signalling through IRS-1 serine phosphorylation blocks insulin action [Aguirre *et al.*, 2000; Baud & Karin, 2001], but the effect of these kinases affects also gene expression, for example promoting further inflammatory gene transcription

through activation of activator protein-1 [AP-1] complexes and NF-κB [Baud & Karin, 2001]. In obesity there is a strong increase of JNK activity in AT, liver [Hirosumi *et al.*, 2002] and also hypothalamus [Prada *et al.,* 2005]. JNK may be activated upon exposure to cytokines as TNF–α or by FFA or ER stress, and all of them can relate to an obese condition [Wellen & Hotamisligil, 2005; Aguirre *et al.*, 2000; Ozcan *et al.,* 2004]. Mice deficient for *jnk-1* are protected from obesity-induced JNK activation, IRS-1 serine phosphorylation, and, hence, insulin resistance, fatty liver and diabetes [Hirosumi *et al.*, 2002; Tuncman *et al.,* 2006]. Blocking JNK activity in models of obesity and diabetes improves systemic glucose homeostasis and insulin sensitivity, as well as protects from the development of atherosclerosis [Ricci *et al.*, 2004; Kaneto *et al.*, 2004; Liu. & Rondinone 2005].
Finally, other evidences of the intersection between metabolic and inflammatory pathways come from lipids and the activation of transcription factors of the peroxisome-proliferator activated receptor (PPAR) and liver X receptor (LXR) families that can inhibit the expression of inflammatory genes in macrophages and adipocytes [Hotamisligil, 2006; Chawla *et al.*, 2001; Glass. & Ogawa, 2006; Joseph *et al.,* 2003].

ER stress

A critical stress situation that connects inflammation and insulin signalling in obesity and T2D is represented by the ER stress. The ER is a network of membranes where folding, maturation, storage and transport of secreted and membrane proteins take place. Unfolded or misfolded proteins are removed from the ER and degraded by the 26S proteasome [Marciniak & Ron, 2006; Schroder & Kaufman, 2005]. The stress condition originates because of the increased accumulation of unfolded proteins, hypoxia, toxins, nutrient fluctuations etc. The response to this perturbed state is known as unfolded protein response (UPR) and is aimed to restore the functional integrity of the organelle [Marciniak & Ron, 2006; Schroder & Kaufman, 2005]. The principal molecular mediators of this complex response are: IRE-1, PERK and ATF6 [Harding *et al.*, 1999; Harding *et al.*, 2000]. The two principal inflammatory pathways that disrupt insulin action, JNK–AP-1 and IKK–NF-kB, are respectively linked to IRE-1 and PERK activity during ER

stress [Urano *et al.*, 2000; Deng *et al.*, 2004; Hu *et al.*, 2006]. Inflammatory mediators can trigger ER stress and lead to the propagation of general stress responses [Zhang *et al.*, 2006]. In the hypertrophic adipocyte the ER might be in the first place responsible for sensing the metabolic stress, which in turn is translated into inflammatory signalling [Hotamisligil, 2006]. In obesity ER functionality may be compromised due to increased demand on the synthetic capacity of organs such as the liver and the AT. Furthermore, increased adiposity could affect the ER structure and architecture, challenging the function and capacity of the cells. This affects energy and nutrient availability and could create a stressful condition. In dietary and genetic obesity, ER stress is increased in adipose and liver tissues [Ozcan *et al.*, 2004]. In cellular systems, the induction of ER stress leads to insulin resistance, in part through IRE-1-dependent activation of JNK. Modulation of ER-folding capacity showed a link between ER function and insulin action and sensitivity [Ozawa *et al.*, 2005; Nakatani *et al.*, 2005]. In mice chemical chaperones are effective in alleviating obesity-induced ER stress and preventing JNK activation, and preventing insulin resistance and T2D [Ozcan *et al.*, 2006].

The ER is also a great source of reactive oxygen species (ROS) and, therefore, of the consequent oxidative stress in the cell [Cullinan *et al.*, 2006; Xue *et al.*, 2005], which is another condition related to obesity and to the development of insulin resistance, as well as mitochondrial dysfunction in T2D [Lowell & Shulman, 2005]. Oxidative stress is coupled to the activation of inflammatory pathways (NF-kB and JNK pathways) and, therefore promotes insulin resistance in adipocytes and muscle cells, and impairs insulin secretion in pancreatic β-cells [Furukawa *et al.*, 2004; Lin *et al.,* 2005; Houstis *et al.*, 2006]. Moreover, all the pathways that act at the interface of metabolic and inflammatory responses have crucial direct/indirect effects on β-cells, particularly in the case of ER stress and its related signalling pathways [Marciniak & Ron, 2006; Schroder & Kaufman, 2005]. This broad range of action could explain the pathological T2D phenotype that is characterized by both insulin resistance and defective insulin secretion. In conclusion, inflammatory mediators alone are able to trigger insulin resistance in cells in certain conditions (infections, trauma, etc.) and they have a similar role in certain metabolic

disorders. Furthermore, metabolic dysfunction can be triggered by chronic excess of nutrients but, however, this happens simultaneously to inflammatory responses. So it is more likely that, in a context of metabolic excess, metabolic signals trigger the inflammatory responses, which then further disrupt metabolic functions, leading to more stress and inflammation, creating a vicious cycle that leads to metabolic deterioration.

JNK and obesity

JNK has a central role in obesity and insulin resistance, especially in the peripheral tissues.

In fact, JNK activity is increased (with consequent promotion of insulin resistance) in insulin-target tissues like skeletal muscle, AT and liver of mouse models for obesity [Hirosumi *et al.*, 2002]. JNK1 KO mice are protected from diet-induced obesity, glucose intolerance, and insulin resistance [Hirosumi *et al.*, 2002]. JNK1 has been conditionally inactivated in insulin sensitive tissues like adipose tissue, muscle and liver [Sabio *et al.,* 2008; Sabio *et al.;* 2009;

Sabio *et al.*, 2010] and also in the hypothalamus and the pituitary gland [Velloso *et al.*, 2008; De Souza *et al.*, 2005; Bruning *et al.*, 2000]. Moreover, JNK inhibition prevents a β-cell dysfunction [Kaneto *et al.*, 2004; Bennett, 2003]. Other studies link the lack of insulin secretion and the protection against high-fat diet-induced obesity, for examples are the cholecystokinin gut-deficient mice [Lo *et al.*, 2010; Lo *et al.*, 2011], the β-pancreatic cells specific fatty acid receptor GPR40 KO mice [Kebede *et al.*, 2008; Steneberg *et al.*, 2005] and the Cav1.2 calcium channel β-cell depletion [Schulla *et al.*, 2003; Uebele *et al.*, 2009]: these animals are significantly leaner and they do not develop HFD induced-insulin resistance. Obesity is also associated with increased risk of Acute Pancreatitis. Rosiglitazone prevents the onset of pancreatitis in HFD-fed mice through a mechanism including PPARγ [Pini *et al.*, 2012]. JNK1 signalling is important in macrophage induced β-cell apoptosis and in the development of hyperglycemia in streptozotocin-induced pancreatic injury [Fukuda *et al.*, 2008].

Cell cycle regulation and metabolism

It is now clear the connection between cell cycle and metabolic regulation and the case of adipogenesis is a good example: the process requires different stages and the intervention of several factors to proceed correctly, APs have to stop proliferating and arrest their growth before undergoing a stage of clonal expansion and finally stop again for final differentiation and lipid accumulation. In fact, cell cycle regulators participate in the control of lipid synthesis, insulin secretion, etc. Alteration of these pathways can provoke the onset of metabolic perturbations and pathologies like obesity and T2D.

p53/p21 pathway

p21$^{WAF1/CIP1}$ (or CDKN1A cyclin-dependent kinase inhibitor 1A) is the major CDK inhibitor, causes cell cycle arrest mainly in G1 [Inoue *et al.*, 2008] and it also protects cells from apoptosis [Polyak *et al.*, 1996]. It was the first identified transcriptional target of the tumour suppressor p53 [el-Deiry *et al.*, 1993]. In addition to cell cycle regulation, p21 is also involved in metabolism, in fact it is also a transcriptional target of SREBPS, which are fundamental transcription factors for lipogenesis and adipogenesis [Inoue *et al.*, 2005; Shimomura *et al.*, 1998] as well as of others transcription factors including some nuclear receptors such as the glucocorticoid receptor. Furthermore, p21 is induced by FOXO1 during the clonal expansion step of adipogenesis [Morrison *et al.*, 1999; Nakae *et al.*, 2003; Sakai *et al.*, 2007]. At a tissue level, p21 is highly expressed in hypertrophic AT and in the fat liver of obese animal [Yahagi *et al.*, 2003; Yahagi *et al.*, 2004]. According to its role in adipocyte differentiation and apoptosis, studies carried in the 3T3-L1 cell line showed that p21 is crucial in later stages of adipocyte differentiation and hypertrophy. Consistently, p21 expression is induced during adipogenesis and then sustained during terminal differentiation, even if it is not strictly required.

In parallel with the development of this PhD thesis, several research papers have been published related to the role of p21 in obesity and which partially overlap with the results of this thesis. These studies suggested that p21 is more important

in adipocyte hypertrophy rather than in the early stages of differentiation [Inoue et al., 2008]. *In vivo,* p21 expression is upregulated in the AT of diet-induced obese (DIO) mice, while p21 deficiency ameliorates the obese phenotype with improved insulin sensitivity and a reduction of TNFα levels and macrophage infiltration in the AT [Inoue *et al.*, 2008]. In contrast to p27-deficient mice this phenotype was not related to β-cell function [Uchida *et al.*, 2005; Auld *et al.*, 2006; Auld *et al.*, 2006; Rane *et al.*, 1999]. Furthermore, p21 deficient macrophages could contribute to the inhibition of AT inflammation as in the case of atherosclerosis [Merched& Chan, 2004]. The positive effects on metabolism seem to be restricted to the AT, in fact in liver and skeletal muscle not relevant changes were observed in genes related to metabolism [Inoue et al., 2008]. In contrast to knock-down experiments performed in 3T3-L1, AT from HFD p21-deficient mice did not show apoptosis, and the same happened for p21-deficient differentiating MEFs, probably because cells did not reach an hypertrophic state due to p21-deficiency in first place [Inoue et al., 2008]. Under physiological conditions the accumulation of intracellular lipids into large adipocytes may constitute a stress for the cell, hence provoking a response and the activation of p53/p21 pathway, with p21 exerting its anti-apoptotic pathway rather than arresting the growth [Inoue et al., 2008].

During the first phase of adipocytes differentiation, several transcription factors are involved (FOXO1, SREBP-1c/ADD1, C/EBP family) and show transcriptional activity on the p21 promoter [Inoue *et al.*, 2005; Nakae *et al.*, 2003; Cram *et al.*, 1998; Gartel *et al., 1999*]. In the 3T3-L1 cell line the expression of p53 is stable during the differentiation process but at later stages Ser 15 and 20 are phosphorylated enhancing p53 transactivation capacity [Inoue *et al.*, 2008, Shieh *et al.*, 2000; Ashcroft *et al.,* 1999]. Accordingly with its potential role in this later stage of differentiation, the p53/p21 pathway is activated in fatty livers and in hypertrophic adipocytes of *ob*/*ob* and DIO mice [Inoue et al., 2008, Yahagi *et al.*, 2003; Yahagi *et al.*, 2004]. p53 is the crucial sensor for cellular stress and determines whether the cell stops proliferation or enters into apoptosis. As speculated in the previous paragraph, if excessive lipid accumulation represents a

stress for the cell (adipocyte) that activates p53, p21 could function as a protector against the induction of apoptosis.

As commented before, changes in diet and life style have caused a worldwide increase in metabolic diseases. Along with these new habits came the improvement in hygiene, medical technology, and quality of life in general (at least in economically healthy countries). This led to the extension of life span and consequent increase of age-related pathologies like cardiovascular and neurodegenerative diseases and cancer. But with aging also grows the chance to develop the metabolic syndrome [Ahima, 2009]. Which like in a vicious cycle, in turn accelerates the process of aging. The deterioration of the structure and function of organs during aging is associated with oxidative stress, genetic instability and disruption of homeostatic pathways [Russell & Kahn]. At a cellular level, ROS can increase genomic damage overtime and obesity and diabetes are associated with the overproduction and the inability to detoxify ROS, respectively [Valko *et al.*, 2007]. The shortening of telomeres is a feature of normal somatic cells: this process leads to the activation of tumour suppressors like p53, which induces cell cycle arrest and senescence [Deng *et al.*, 2008]. In this regard, telomere shortening has been already linked to obesity and diabetes [Fuster & Andrés, 2006]. The existence of these similarities suggested that metabolic disorders and aging might share similar pathways. In the last few years the link between obesity (and altered metabolism) and aging has become clear: in obese conditions, AT is subjected to oxidative stress, resulting in aging, production of pro-inflammatory cytokines, accumulation of macrophages, induction of insulin resistance and suppression of adiponectin expression. It is suggested that aging of AT may induce insulin resistance also in liver and skeletal muscle, and mediate the progression to T2D [Minamino *et al.*, 2009; Ahima, 2009]. p53 protein, activated by the obesity-related aging process, may be the crucial factor mediating the process: in fact p53 deficiency specifically in the AT lowers the inflammation and improves insulin sensitivity in obese mice. Furthermore, when bone marrow cells are transplanted from normal mice to mice p53-deficient mice in the AT, an improvement of glucose homeostasis is observed, underlining the importance of

macrophages in the process. Finally, transgenic overexpression of p53 and cdkn1a genes in AT induces inflammation and insulin resistance [Minamino *et al.*, 2009].

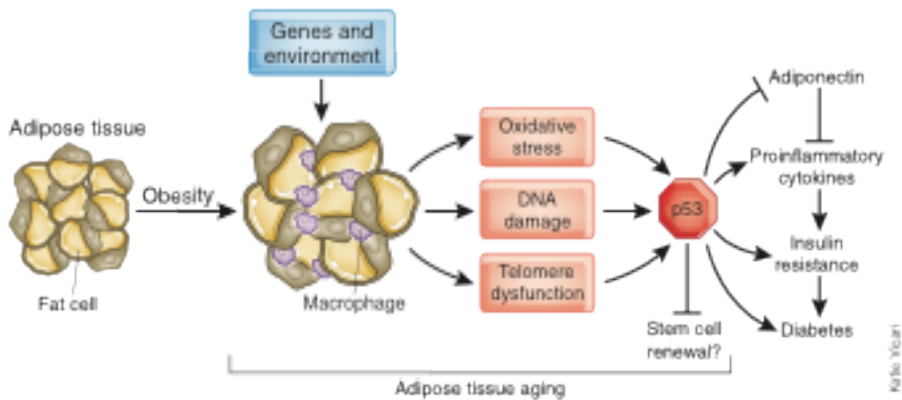


Figure 11. The connection between obesity-mediated aging of adipose tissue and diabetes [Ahima, 2009]. In obesity, adipose tissue is subjected to oxidative stress, resulting in aging, accumulation of macrophages, production of proinflammatory cytokines and suppression of adiponectin. Activation of p53 is crucial for aging, stimulates inflammation and may attenuate stem cell renewal capacity. The aging of adipose tissue induces insulin resistance in adipose tissue, liver and muscle and mediates the progression to diabetes.

On the other hand p53 is a key tumour suppressor protein with a diverse range of functions (promotion of apoptosis, cellular senescence and DNA repair) that help to prevent cancer development [Vousden & Ryan, 2009]. It is now well-established role in regulating metabolic pathways suggests another mechanism by which p53 helps to stall malignant progression. Metabolic alterations are common features of cancer cells and have recently been shown to have an important role in the maintenance of malignancies. Several functions of p53 promote oxidative phosphorylation and reduce glycolysis in cells; disruption of this balance is associated with mutations in p53 and oncogenic transformation [Vousden & Ryan, 2009]. p53 also has a key role in regulating cell growth and autophagy, thereby helping to coordinate cellular response to nutrient starvation. Altered metabolism can contribute to malignant transformation, and cancer cells become dependent on these changes. Understanding the role of p53 in the regulation of metabolism may provide some interesting potential targets for the development of new cancer therapies [Vousden & Ryan, 2009].

The CDK4- pRB-E2F1 pathway in the regulation of metabolism

E2F transcription factors are implicated in the G1/S cell-cycle transition, DNA repair, apoptosis, development and differentiation (including adipocyte differentiation) [Dimova & Dyson, 2005; Sardet *et al.*, 1997; Fajas *et al.*, 2002] and can regulate expression of both proliferative and metabolic genes [Dimova & Dyson, 2005; Sardet *et al.*, 1997]. So they are able to coordinate and modulate the response to a stressful condition. In a starvation state, for instance, cells need to reduce proliferative (glycolytic) and activate energy productive (oxidative) pathways [Warburg, 1930].

E2F transcription factors can be found associated in large complexes including members of the retinoblastoma (pRB) and of the cyclin/cdk families. The association of E2F with pRB facilitates transcriptional repression thanks to the recruitment of histone deacetylase [Brehm *et al.*, 1998] and Lys/Arg metyl-transferase [Fabbrizio *et al*., 2002]. G1 cyclin/cdk complexes are able to phosphorylate pRB, causing the release of E2Fs and, concomitantly, activation of E2F-target gene transcription for the G1-S phase transition [Helin, 1998].

Regarding whole body metabolism, CDK4, pRB and E2F1 are expressed in pancreatic β-cells independently of their proliferation, suggesting a role in β-cell function apart from their action as cell cycle regulators (i.e. control of β-cell number). In this regard, CDK4-deficient mice show reduced islet mass with consequent development of insulin-dependent diabetes [Uchida *et al.*, 2005; Rane *et al.*, 1999]. In addition, E2F1-deficient mice exhibit reduction in pancreatic size caused by affected pancreatic growth after birth and are glucose intolerant due to impaired insulin secretion in response to glucose [Fajas *et al*., 2004]. Therefore, E2F1 function is important for the maintenance of normal blood glucose levels through the regulation of insulin secretion, whereas it does not affect insulin synthesis: in fact there are no differences in insulin content between wild type (WT) vs E2F1-deficient islets [Annicotte *et al*., 2009]. Furthermore E2F1-deficient mice are insensitive to the antidiabetic drug glibenclamide that stimulates insulin secretion and blocks ATP-sensitive potassium channels in β cells, lowering blood

glucose levels, so the mechanism of action of E2F1 is different. E2F1 directly regulates the expression of Kir6.2 which is a crucial element of the K_{ATP} channel involved in the regulation of glucose-induced insulin secretion. The CDK4-pRB-E2F1 pathway regulates the expression of Kir6.2 at a promoter level and in mice inhibition of CDK4 or genetic inactivation of E2F1 results in decreased expression of the Kir6.2 gene, impaired insulin secretion and glucose intolerance [Annicotte *et al*., 2009]. In normal pancreas glucose increases CDK4 activity, alleviates repression by pRB (through its phosphorylation) and activates E2F1 for Kir6.2 transcription [Annicotte *et al*., 2009]. These effects are mediated by an autocrine action of insulin, after the primary insulin secretion stimulated by glucose [Annicotte *et al*., 2009]. Notably kir- and E2F1-deficient mice show impaired insulin secretion but they are not diabetic because insulin sensitivity at a peripheral level is increased [Fajas *et al*., 2004; Annicotte *et al*., 2009; Fajas *et al.*, 2002; Remedi *et al.,* 2004, Miki *et al.*, 2002]. Recently, it was found out that Cyclin D1-CDK4 controls hepatic glucose metabolism also independently of cell cycle progression: in mice insulin activates Cyclin D1-CDK4 which increases the activity of the histone acetyltransferase general control non-repressed protein (GCN) 5 and suppresses glucose production in the liver. Insulin/GSK-3β signalling provokes the sequestration of cyclin D1 in the nucleus, in parallel with the action of dietary aminoacids that increase cyclin D1 mRNA transcripts in this organ. Activated Cyclin D1-CDK4 phosphorylates and activates GCN5, which inhibits PGC-1α activity on gluconeogenic genes [Lee *et al*., 2014]. Consistently with its role in metabolism, E2F1 deficient mice are resistant to diet-induced obesity [Fajas *et al.*, 2002]. In basal conditions, E2F/pRB complex represses genes that regulate energy homeostasis and mitochondrial functions in skeletal muscle and BAT [Lee *et al*., 2014]. This repression is alleviated in a constitutively active CDK4 mouse model or when adaptation to energy demand is needed [Blanchet *et al*., 2011].

pRb

The pRb is the product of the first tumor suppressor gene to be characterized and belongs to the family of pocket proteins composed of pRb, p107, and p130 proteins. They induce growth arrest through the interaction with other transcription factors such as E2Fs [Nguyen & McCance, 2005; Lipinski *et al*., 1999], playing an important role in cell cycle regulation. They are also involved in the control of apoptosis [Chau & Wang, 2003] and cell differentiation [Lipinski *et al*., 1999]. pRb germ-line knock out animals die prematurely because of developmental defects comprising lack of differentiation of brain, skeletal muscle, and cells of the erythropoietic lineage [Jacks *et al*., 1992; Lee *et al*., 1992; Clarke *et al*., 1992].

pRb facilitates the differentiation of APs and mouse embryonic fibroblasts (MEFs) into adipocytes [Scime *et al*., 2005; Chen *et al*., 1996; Hansem *et al*., 2004]. pRb–deficient 3T3 cells fail to undergo terminal adipocyte differentiation and overexpression of pRb promotes adipocyte differentiation in WT cells [Classon *et al*., 2000]. Moreover, the SV40 large antigen blocks adipocyte differentiation of 3T3-L1 cells by inhibiting pRb's positive effect on this process [Higgins *et al*., 1996]. So, during the clonal expansion and the early stages of adipogenesis, pRb inhibits differentiation [Fajas *et al.*, 2002[a]]. Thereafter, pRb rather activates adipogenesis by allowing the cells to exit the cell cycle for terminal differentiation [Richon *et al*., 1997].

In addition, pRb acts as a molecular switch between WAT and BAT differentiation. pRb deficient MEFs display features of BAT, with activation of BAT-specific genes, such as UCP1 and PGC-1, and an increase in mitochondria number [Hansem *et al*., 2004]. In transgenic mice overexpressing the SV40 antigen specifically in WAT, the impaired pRb activity leads to the conversion of WAT into BAT [Ross *et al*., 1992]. In p107 deficient mice, WAT is poorly differentiated with increased expression of UCP1 and PGC-1 and in p107 deficient AOs, pRb levels were also reduced [Scime *et al*., 2005]. Remarkably, specific ablation of pRb in adult AT leads to mitochondrial activation in WAT and BAT and protects from diet-induced obesity [Dali-Youcef *et al*., 2007].

OBJECTIVES

One of the initial events in obesity is adipocyte hypertrophy, a stressful condition that triggers other cellular responses, such as the inflammatory response, which eventually will implement a vicious circle leading to the development of insulin resistance and hyperinsulinemia, a pair of pathological conditions to which is the cause and the consequence remains under debate in nowadays. Moreover, insulin resistance and hyperinsulinemia are suggested to be the main link of obesity to the other obesity-associated disorders such as T2D, cardiovascular diseases, atherosclerosis and cancer. Therefore, the major goal of this thesis was to unravel novel molecular mechanisms underlying the development of obesity and the associated insulin resistance and hyperinsulinemia. We pursued this major goal performing two different studies:

1. Analysis of the role of the p53/p21 tumour suppressor pathway in obesity

2. Analysis of the effect of JNK activation in pancreatic β cells in obesity-associated systemic insulin resistance and hyperinsulinemia.

MATERIAL AND METHODS

Animals and *in vivo* studies

$p21^{-/-}$ mice were obtained from Jackson laboratories (Bar Harbor, Maine USA). In this knock out strain a neo cassette replaces exon 2 of the of the cyclin-dependent kinase inhibitor 1A (*Cdkn1a* or $p21^{CIP1/WAF1}$) gene. Homozygotes are viable, fertile, and normal in size. These mice belonged to the strain $\mathrm{B6.129S6(Cg)}\text{-}Cdkn1a^{tm1Led}/\mathrm{J}$. and were backcrossed with C57BL/6 mice strain in the animal facility of University of Barcelona.

$p53^{-/-}$ mice were kindly provided by Dr. José Yelamos López of the Cancer Research Program, IMIM (Hospital del Mar Research Institute), Barcelona

$E2F1^{-/-}$ mice were kindly provided by Dr. Ana María Zubiaga of the Department of Genetics, Physical Anthropology and Animal Physiology, University of the Basque Country (Bilbao, Spain)

ob/ob mice were obtained from Jackson laboratories sand back crossed with p21 $^{+/-}$ mice in in the C57BL/6 background to obtain ob/ob p21 $^{+/-}$ and ob/ob p21 $^{-/-}$ mice.

Transgenic mice C57BL/6J-Tg(Gfp^{loxP}-MKK7D)Ccf were generated by microinjection of oocytes with the transgene GFP^{loxP}-MKK7D, and two independent lines were selected for this study. All the experiments described in this study were performed in both transgenic mouse lines, and comparable results were obtained. The RIP-Cre (C57BL/6-Tg(Ins2-Cre)25Mgn) mice strain was obtained from the Jackson Laboratory. The mice used in this study came from crosses of heterozygous Cre recombinase male mice with homozygous GFP^{loxP}-MKK7D female mice.

All transgenic mice were in the C57BL/6 background.
All animal procedures were approved by the Animal Care Research Committee of the University of Barcelona.

Tissues isolation from mice

The animals were sacrificed by cervical dislocation. Tissues were collected and immediately stored at -80°C.

Adipocytes isolation and incubation with leptin

Mice were sacrificed and e-WAT was isolated and digested at 37ºC in oxygenated/carbonated "Digestion Buffer" (NaCl 120 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1 mM, KCl 6mM, NaH_2PO_4 1 mM, Na_2HPO_4 1 mM, Hepes pH 7.5 12.5 mM, Sodium Pyruvate 2mM, BSA 3.0-3.5%, type I Collagenase 0.66 mg/mL) for 20-30 minutes, then filtered and transferred to a new tube. Adipocytes were washed in "Washing Buffer" (NaCl 120 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1 mM, KCl 6mM, NaH_2PO_4 1 mM, Na_2HPO_4 1 mM, Hepes pH 7.5 12.5 mM) and then resuspended in oxygenated/carbonated "Incubation Buffer" at 37 ºC (NaCl 120 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1 mM, KCl 6mM, NaH_2PO_4 1 mM, Na_2HPO_4 1 mM, Hepes pH 7.5 12.5 mM, Sodium Pyruvate 2mM, BSA 3.0-3.5%). Cells were incubated with different dosis of leptin 100X at 37ºC. At the end of the incubation the adipocytes were washed in washing buffer and then lysed in Lysis buffer plus inhibitors.

Preparation of whole cell extracts

Cells were scraped from the tissue culture plates in 1 mL of phosphate buffered saline (PBS) and collected by centrifugation at 6000xg for 2 min at RT. After centrifugation cells were lysed in lysis buffer (Hepes.Na pH7.5 20 mM, EGTA 10 mM, β-glycerophosphate 40 mM, $MgCl_2$ 2.5 mM, NP-40 1% sodium orthovanadate 2mM**,** DTT 1mM, PMSF 0.5 mM, aprotinin 1mg/mL and leupeptin 1mg/mL) and incubated at 4°C for 10 min. Extracts were clarified by centrifugation and supernatants were stored at -80°C.

Tissue samples were homogenized in lysis buffer using a Polytron/ Ultratorrax. After ultracentrifugation supernatants were collected and stored at -80°C.

Protein concentration

Protein concentration was determined by Bio-Rad Protein Assay Dye Reagent following manufacturer's recommendations.

Immunoblot analysis

Immunoblots were performed after separation of proteins by SDS-PAGE and transference to PVDF (Immobilon-P Transfer Membrane, Millipore) or nitrocellulose membranes. Membranes were probed with the following primary and secondary antibodies (Table 4 and 5) and were detected by ECL Chemiluminiscense (Thermo Scientific).

Antigen	Company / Reference
ERK-1	Santa Cruz / sc-93
JNK/SAPK1	BD Biosciences – Pharmingen #610627
eIF2a	Cell Signalling #9722
Phospho-eIF2α (Ser51)	Cell Signalling #9721
Nek-9	Roig, Alexei, Christopher, & Jul, 2002
p70 S6 kinase alpha (H-160)	Santa Cruz sc-9027
p70 S6 kinase alpha (C-18)	Santa Cruz sc-230
Phospho-p70 S6 Kinase	Cell Signalling # 9205
IRS-1 (C-20)	Santa Cruz sc-559
IRS-2 (H-205)	Santa Cruz sc-8299
LepR (B-3) (mouse)	Santa Cruz sc-8391
LepR (C-20) (goat)	Santa Cruz sc-1832
STAT3	Cell Signalling # 9132 (now # 9139)
p-STAT3 Tyr 705	Cell Signalling # 9131
pRb (IF8)	Santa Cruz sc-102
p53 (1c12)	Cell Signalling #2524
p21	BD Pharmingen #556430
p-ERK (E-4)	Santa Cruz sc-7383
gamma-Tubulin (GTU-88)	Sigma #T5326

Table 4: Primary antibodies list.

Antigen	Conjugated	Company / Reference
Rabbit IgG	Peroxidase	Jackson / 115-095-003
Mouse IgG	Peroxidase	Jackson / 115-035-003
Goat IgG	Peroxidase	Jackson / 705-035-003
Mouse IgG	Peroxidase	Jackson / 115-035-174

RNA extraction and purification

RNA was extracted from cells or tissue samples using the TRIZOL® reagent following the manufacturer's recommendations. In brief, tissues were homogenized in TRIZOL® using a Polytron/Ultratorrax. After centrifugation the supernatant was transferred to a new tube, mixed with chloroform and centrifuged again. The aqueous phase was transferred and mixed with isopropanol to precipitate the RNA. After centrifugation, the pellet was washed in 75% ethanol, dissolved in RNase-free water and incubated for 10 min at 55 °C. Afterwards, the RNA preparation was further purified using the RNeasy kit (QIAGEN).

Quantitative real-time PCR (qRT-PCR)

RNA was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) and quantified by qRT-PCR using SYBR Green (Applied Biosystems). The pairs of primers that were used are shown in Table 6.

Gene	Forward primer	Reverse primer
β-actin	CTAGGCACCAGGGTGTGAT	CCATGTTCAATGGGGTACTT
CB1	AAGTCGATCTTAGACGGCCTT	TCCTAATTTGGATGCCATGTCT
GAPDH	AAGGGCTCATGACCACAGT	GATGCAGGGATGATGTTCTG
Insig1	CCTTGAATCAAAATGAATTACC	CACTATAGGTCCTACTGGGAGA
L14	TCCCAGGCTGTTAACGCGGT	GCGCTGGCTGAATGCTCTG
LepR	ACTCAACTACGCTCTTCTGATG	CTAAGGGTGGATCGGGTTTC
LepRb	TATCGACAAGCAGCAGAATG	AAATGTTTCAGGCTTTTGGAA
NRF1	AGCACGGAGTGACCCAAAC	TGTACGTGGCTACATGGACCT
PGC1α	AGACAGCTATGGTTTCATCACC	GTCAAAATCGTCTGAGTTGGTA
SOCS3	CTAGTCATCTCTCGGTCAGTAG	CCATACCTGACTTCAGAAAACTT
TNFα	CCAGTGTGGGAAGCTGTCTT	AAGCAAAAGAGGAGGCAACA
UCP1	ATGCTTACAGAGTTATAGCCAC	TCATCTGCCAGTATTTTGTTGTTT
UCP2	CAGTTTACTACCAACAGCTGAC	TAAGGTCTCTGCTATGCTGTTTT
UCP3	AGGAGAAAGAACACATTGATA	CCTCAGGCTTACATTTGTAGATG

Table 6: List of genes and primers analysed by quantitative real-time PCR.

Cell culture

HEK293T, MCF-7, 3T3-L1 and mouse embryonic fibroblasts (MEFs) cells were cultured in a 5% CO_2 atmosphere at 37°C in Dulbecco's modified medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (or 10% new born calf serum (NCS) in the case of 3T3-L1 APs), $NaHCO_3$ 1.5 g/L, L-glutamine 2 mM, penicillin 100 I.U./mL, streptomycin 100 µg/mL and Fungizone.

Cell Transfection

HEK293T and HeLa cells were transfected with different expression plasmids using PEI (Polyscience, Inc) [Boussif *et al.*, 1995]. The plasmid DNA and the PEI were mixed in the proportion 1 ug DNA 1ug/ul : 5 ul PEI 0.1 ug/ul in 150 mM NaCl and after 30 minutes of incubation at room temperature (RT), the transfection mixture was added to the cells. After at least 5 hours and up to 24 hours of incubation, the medium was changed and cells were kept for at least 24 hours to enable gene expression.

MEFs cells were transfected using Lipofectamine® 2000 reagent (Invitrogen - Life Technologies) following the manufacturer's recommendations.

3T3L1 differentiation

3T3-L1 cells were cultured in 10% FCS – DMEM 1X at. When cells reached confluence, they were incubated for 48 hours in Induction medium (10% FBS, 5 ug/ul Insulin, 0.25 um DXM, 0.5 mM IBMX in DMEM 1X). After 48h, the medium was removed to add the Insulin medium (10% FBS, 5 ug/ul Insulin, in DMEM 1X). After 48 hours more, the medium was removed to add the differentiation medium 3 (10% FBS-DMEM 1X), renewed every 48 hours. Full differentiation was achieved by day 8.

Lentivirus production and infection protocol

On day 1 293T cells were seeded in complete DMEM + antibiotics ($2.7x10^6$ cells for a p90 culture dish). Cells were transfected upon attachment (4-6 hours later) with a mix of plasmid DNA (VSVG, envelope vector; RTR2, REV expressing vector, KGP1R packaging vector; vector of interest) and PEI. After an O.N. incubation at 37°C, on day 2 fresh complete DMEM was added and cells were incubated at 33°C until 48h post-transfection. On day 3, 48 hours post-transfection the medium from the 293T cells was collected to infect the chosen cell line (i.e. 3T3L1) seeded at low confluence. The infected cells were incubated for 24 hours at 33°C. On day 4 a second round of infection was performed. On day 5 the infection medium was removed from the infected cells and replaced by fresh complete DMEM**.** Cells were incubated at 37°C O.N. On day 6, puromycin was added to the infected cells to select positive clones, and cells were incubated 48h at 37°C.

Transcriptomic analysis

The transcriptomic analysis was performed at IRB's transcriptomic facility using an Affymetrix 3'-IVT Expression Array. The array type was the Affymetrix Mouse 430 2.0

Statistical analysis

Data were analysed with a two-tailed unpaired Student's test. Values are presented as means plus SE (Standard Error).

RESULTS

1.- ANALYSIS OF THE ROLE OF THE P53/P21 TUMOUR SUPPRESSOR PATHWAY IN OBESITY

1.1 Analysis of p21 gene expression in obesity.

The expansion of AT that takes place in the development of obesity relays in processes of hyperplasia and hypertrophy. While the former requires *de novo* adipogenesis from APs, a multistep process that includes cell cycle arrest and clonal expansion phases, the later represents a stressful condition that may trigger pathways involved in protection from cell death. Taking this in account, we decided to analyse the potential role of p21, a cell cycle regulator and apoptosis inhibitor, in obesity. First, we confirmed that p21 expression was regulated during the *in vitro* differentiation of 3T3-L1 APs to adipocytes by a time course experiment (Fig. 12) (ref).

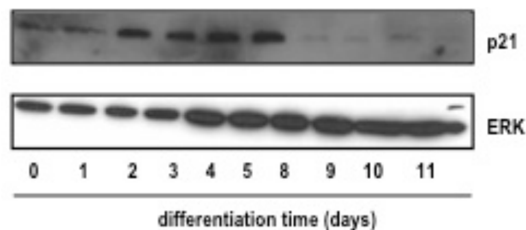


Figure 12. Analysis of p21 protein expression during 3T3-L1 AP differentiation to adipocytes. 3T3-L1 APs were subjected to the adipogenic differentiation procedure (see Materials and Methods). At the indicated time points WCE were prepared and analysed (10 μg of WCE/lane) by immunoblotting using an antibody against p21 (upper panel) and ERK (lower panel).

Afterwards**,** p21 gene expression was analysed in two different models of obesity in mice: the *ob/ob* mice, a genetically-induced obesity model, and the DIO mice. Semiquantitative RT-PCR and immunoblot analysis showed that p21 expression was significantly increased in epididymal WAT from the *ob/ob* mice when compared to lean mice. (Fig. 13 A and B). Immunoblot analysis of p21 of extracts prepared from subcutaneous WAT showed a similar result (Fig. 13C). In contrast,

no increase in p21 expression was detected in extracts from liver or skeletal muscle of these animals (Fig. 13 D and E).

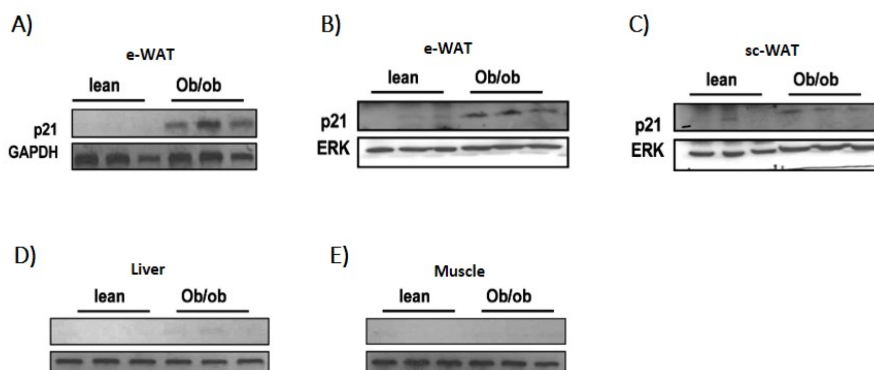


Figure 13. p21Waf1/Cip1 expression is upregulated in WAT from *ob/ob* mice. Semiquantitative RT-PCR analysis of p21 (upper panel) and GAPDH (lower panel) expression in epididymal WAT (e-WAT) (A) and immunoblot analysis (10 μg/lane) of p21 (upper panel) and ERK (lower panel) in extracts from epididymal WAT (B), subcutaneous WAT (sc-WAT) (C), liver (D) and skeletal muscle (E) of 8-week old lean and *ob/ob* mice, as indicated.

Regarding the DIO mice, upregulation of p21 was also observed in these animals, when compared to mice fed with standard chow (SD), in epididymal WAT but, again, not in liver or skeletal muscle (Fig. 14).

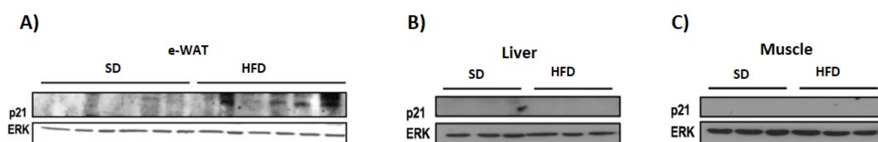


Figure 14. p21Waf1/Cip1 expression is upregulated in WAT from DIO mice. Immunoblot analysis of p21 (upper panel) and ERK (lower panel) in extracts from epididymal WAT (e-WAT)(A), liver (B) and skeletal muscle (C) of 16-week old mice fed with standard diet (SD) or, for the last 12 weeks, with HFD, as indicated.

A role of p21Waf1/Cip1 in HFD-induced obesity

To confirm the potential role of p21 in the development of obesity we followed the weight curve of mice heterozygous and deficient for p21 (p21+/- and p21-/-, respectively) in comparison to WT mice fed with SD or HFD. On SD, growth curves were nearly identical among the three genotypes (Fig. 15A). In contrast, on HFD, p21 KO mice showed almost complete resistance to the development of obesity

and heterozygous mice showed an intermediated phenotype when compared with their WT littermates (Fig. 15 A and B)). This phenotype was observed as well in male as in female mice. Furthermore, no changes were observed among these different mice in feeding behaviour or body temperature

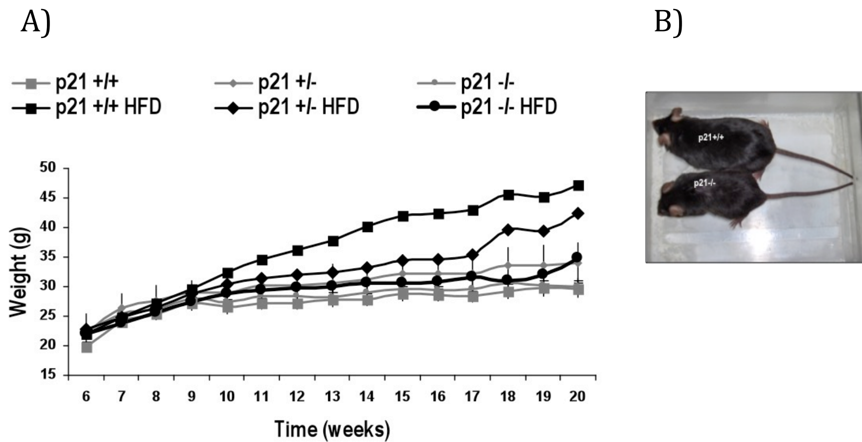


Figure 15. p21Waf1/Cip1 deficiency protected against the development of HFD-induced obesity. A: Weight curves of WT, $p21^{+/-}$ and $p21^{-/-}$ mice fed with SD or HFD for 14 weeks (n=6) B: Photograph showing a 20-week old WT and $p21^{-/-}$ mice fed with HFD.

Regarding lipid metabolism, in response to a HFD, plasma triglycerides level was increased in WT and p21+/- mice while in p21 deficient mice it remained similar to the animals on standard diet (Fig. 16).

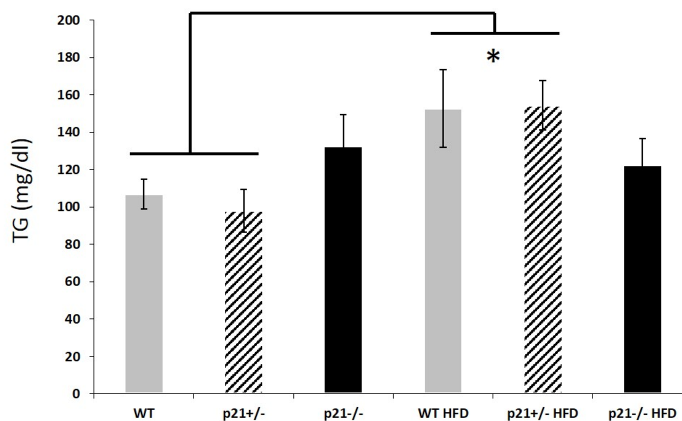


Figure 16. p21Waf1/Cip1 deficiency protected against the development of HFD-induced obesity. Analysis of the triglyceridemia of 20 week old WT, $p21^{+/-}$ and $p21^{-/-}$ mice fed with SD or HFD for 14 weeks, as indicated. (n=6)

Consistently, the weight of epididymal fat pads of p21$^{-/-}$ mice on HFD was significantly lower than those from the WT littermates (Fig. 17 A, B). Moreover, histological analysis of WAT and BAT from p21$^{-/-}$ and WT mice on SD showed no marked differences as in size as in number of adipocytes (Fig. 17 C).

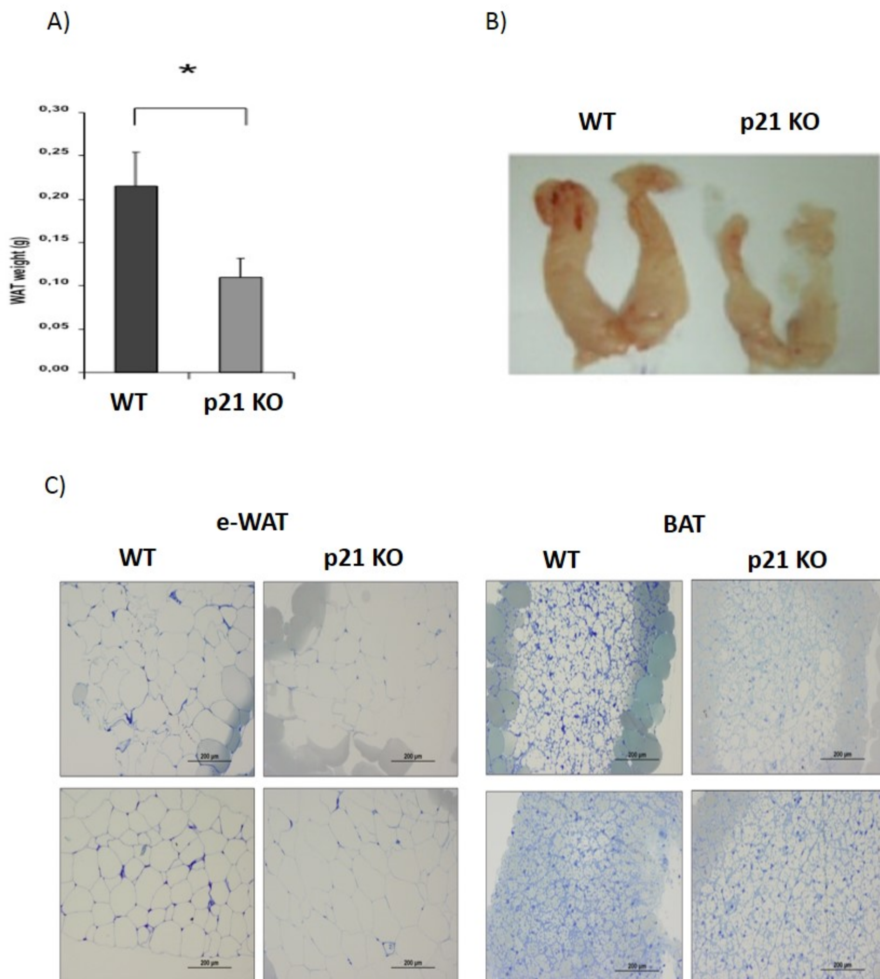


Figure 17. Comparative analysis of fad pads and adipocyte morphology in p21 deficient and WT mice. Analysis of weight (A) and size (B) of the epididymal fat pads of 20-week old p21$^{-/-}$ and WT mice fed with HFD for 14 weeks. (n=3) C: histological analysis of epididymal WAT (e-WAT) and BAT in p21$^{-/-}$ and WT mice in SD (n=3).

p21Waf1/Cip1 and DIO-associated insulin resistance.

Next, WT and p21-/- mice fed with SD and HFD were subjected to glucose-tolerance test (GTT). Regardless the phenotype, all animals fed with SD performed similar in the GTT (Fig. 18). As expected, HFD induced glucose intolerance in the WT mice, however, this diet had no effect on glucose tolerance in the p21-deficient mice. p21 heterozygous mice showed and intermediated phenotype (Fig. 18).

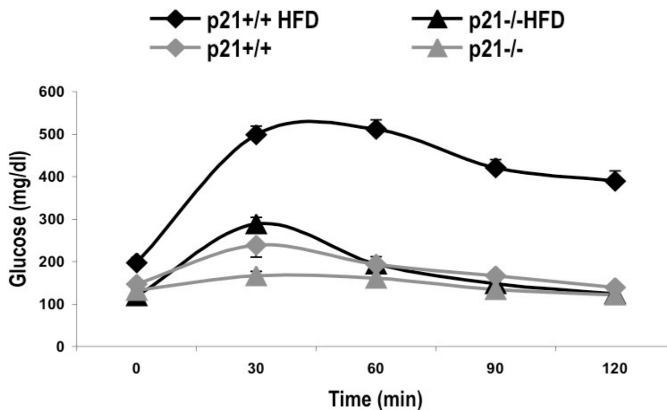


Figure 18. p21Waf1/Cip1 deficiency protects against the development of HFD-induced glucose intolerance. GTT were performed in 20-week old WT and p21$^{-/-}$ mice fed with SD or HFD as indicated (n=6).

In addition, mice were also analysed for their sensitivity to insulin subjecting them to insulin-tolerance test (ITT). Results of this test were consistent with the previous GTT results as p21-deficient mice subjected to a HFD showed a sensitivity to insulin similar to those animals fed with SD while WT in the same HFD conditions developed insulin resistance (Fig 19).

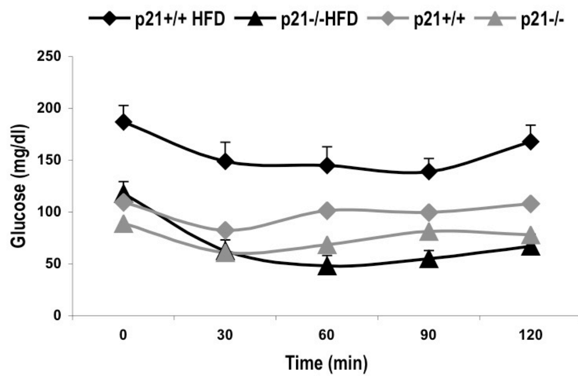


Figure 19. p21Waf1/Cip1 deficiency protects against the development of HFD-induced insulin resistance. ITT were performed in 20-week old WT and $p21^{-/-}$ mice fed with SD or HFD as indicated (n=6).

Consistently with these results and in contrast to WT mice, HFD-induced expression of pro-inflammatory markers in AT was prevented in p21-deficient mice (Fig. 20)

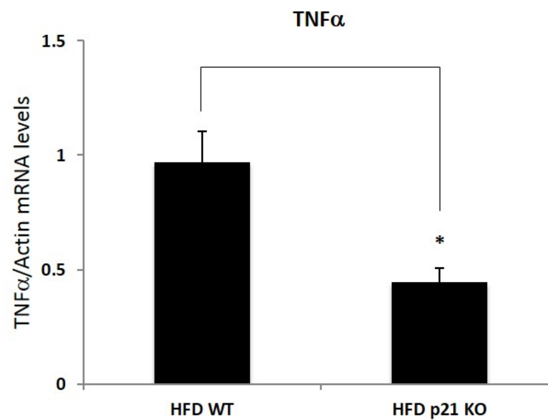


Figure 20. p21-deficiency prevents the expression of HFD-induced pro-inflammatory markers. q-RT PCR for TNFα in e-WAT of WT and p21 deficient mice on HFD for 14 weeks (n=4).

Transcriptomic analysis in AT of WT, p21+/- and $p21^{-/-}$ mice.

We performed a comparative study of gene expression using e-WAT RNA extracts from 8-week old WT, p21+/- and $p21^{-/-}$ mice subjected to SD. We decided to include heterozygous animals in this analysis because our previous results indicated that the obesity phenotype was dependent on p21 gene dosage. Table 7

shows the results of this transcriptomic analysis for some genes relevant to AT physiology and metabolism (the full list of genes is provided in Appendix I).

Microarray gene expression data

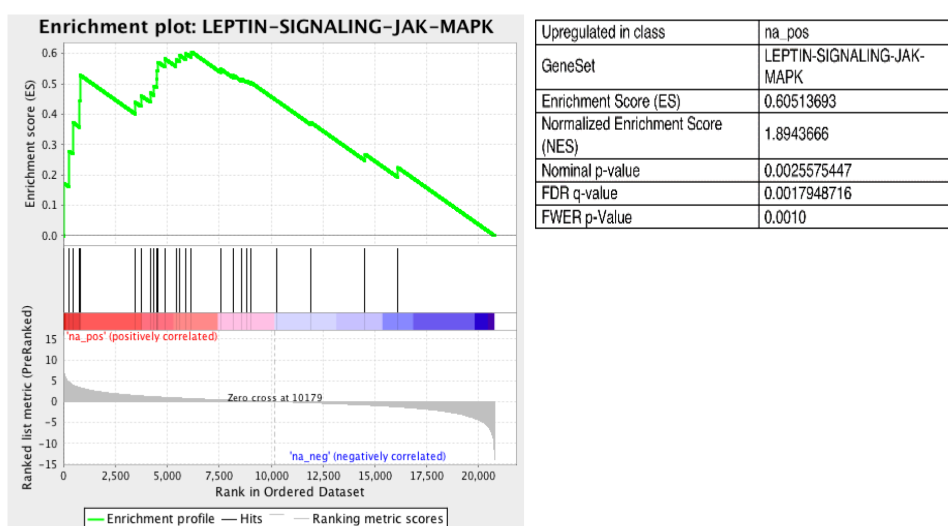
Probesets	Gene Title	Gene Symbol	KO vs WT (log2)	p-value	KO vs +/- (log2)	p-value
421679_a_at	Cyclin-dependent kinase inhibitor 1A (p21)	Cdkn1a	-5.299	0.000	- 4.146	0.001
1456156_at	Leptin receptor	LepR	0.925	0.011	0.466	0.008
1418901_at	CCAAT/enhancer binding protein (C/EBP), beta	Cebpb	0.106	0.496	-0.070	0.470
1418982_at	CCAAT/enhancer binding protein (C/EBP), alpha	Cebpa	0.034	0.724	-0.063	0.470
1420715_a_at	Peroxisome proliferator activated receptor, gamma	Pparg	0.055	0.744	0.096	0.643
1426381_at	Peroxisome proliferative activated receptor, gamma, coactivator-related 1	Pprc1	0.262	0.109	0.117	0.48
1418197_at	Uncoupling protein 1	Ucp1	0.065	0.805	-0.274	0.523
1448188_at	Uncoupling protein 2	Ucp2	0.266	0.130	0.294	0.096

*** Values represent n=3 mice per group**

Table 7. e-WAT transcriptomic analysis. Results of the indicated genes are shown as relative expression between p21 deficient (KO) and WT (Column 4) in e-WAT and KO and p21 heterozygous (+/-) mice.

The microarray gene expression analysis showed that there were no significant differences in adipogenic genes between WT, p21-heterozygous and -deficient mice, such as PPARγ or C/EBP. This observation lead us to speculate that p21 either might not be involved in adipocyte differentiation or other functionally redundant genes undertake this particular task. Remarkably, transcriptomic data showed a statistically significant increase in the expression of the LepR in the WAT

of p21-deficient mice when compared to the WT animals. Leptin receptor is almost two times up regulated in p21-knockout mice, when compared with their WT littermates. In agreement with the obesity phenotype, p21-heterozygous mice showed an intermediated phenotype, when compared with their WT and knockout littermates, suggesting a dose effect of p21 in the regulation of LepR expression. Accordingly, a gene set enrichment analysis (GSEA) performed *in silico* and screening among the genes analysed by transcriptomic evidenced that p21 deficiency causes an upregulation of genes related to leptin signalling (Fig. 21).



Upregulated in class	na_pos
GeneSet	LEPTIN-SIGNALING-JAK-MAPK
Enrichment Score (ES)	0.60513693
Normalized Enrichment Score (NES)	1.8943666
Nominal p-value	0.0025575447
FDR q-value	0.0017948716
FWER p-Value	0.0010

Figure 21. p21 deficiency causes an upregulation in genes related to leptin signalling. GSEA analysis screening data related to genes from the transcriptomic analysis

Data from the literature had previously shown that adipocyte-specific LepR over expression completely prevented HFD-induced increase in adiposity due to the increase in mitochondrial activity and consequent energy expenditure, results that support for a leptin paracrine action on the AT [Wang, *et al.*, 2005]. In addition, specific deletion of pRB in both WAT and BAT lead mice to be protected from the HFD-induced obesity [Dali-Youcef *et al.*, 2007]. Altogether, these data prompted us to continue the analysis of the up regulation of LepR as the protective mechanism for the development of HFD-induced adiposity in the p21 deficient mice.

Validation of the transcriptomic analysis data

The transcriptomic analysis showed that LepR mRNA levels were significantly ($p \leq 0.05$) higher in the e-WAT of p21-deficient mice compared to WT mice (Table 8).

symbol	genename	pval	fc	means.WT	means.KO
Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	1.28E-08	-39.35884127	8.894210734	3.595594891
Lepr	leptin receptor	0.002051121	1.898510414	5.161881883	6.086749795

Table 8. Analysis for the microarray gene expression data. Differential expression analysis of p21 and LepR mRNA expression in the e-WAT of p21 KO vs WT mice. Values represent n=3 mice per group.

The next step was to confirm the transcriptomic data that came from the microarray analysis. Figure 22 A shows the result of a qRT-PCR for LepR in the e-WAT of 8-week old mice in SD. LepR mRNA was significantly upregulated in p21 deficient mice compared to WT animals, thus confirming the data from the previous analysis. We observed the same evidence also in p21 KO animals fed with a HFD (Fig. 22 B), which interestingly have significantly higher isoform b mRNA levels compared to WT (Fig. 22 C).

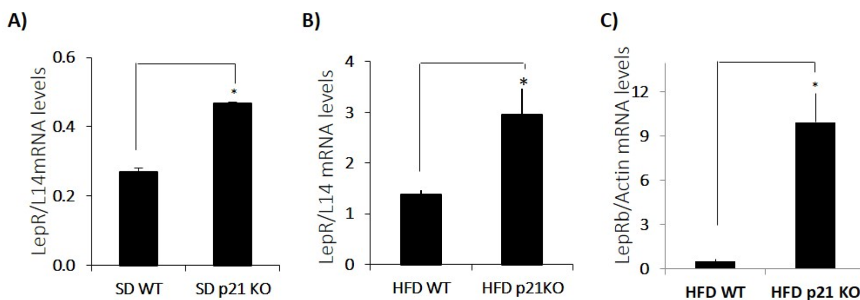


Figure 22. p21Waf1/Cip1 deficiency induces LepR gene upregulation in e-WAT. qRT-PCR for LepR mRNA levels in e-WAT of 8-week old WT and p21 deficient (p21 KO) mice in SD (n=3 mice per group) (A) and (B) 20-week old mice WT and p21 deficient (p21 KO) mice in HFD for 14 weeks (n=5 mice per group) (B) . C: qRT-PCR for LepR isoform b mRNA levels in e-WAT 20-week old mice WT and p21 deficient (p21 KO) mice in HFD for 14 weeks (n=4 mice per group).

To further validate the data from the transcriptomic analysis we checked for LepR protein expression levels in AT of mice. The predicted molecular weight for LepR is 92 KDa but according to literature, it can vary between 120-150 KDa or even 170 KDa due to N-glycosylation post-translational modifications [Haniu *et al.*, 1998; Liu *et al.*, 1997; Bjørbaek *et al.*, 1997]. Due to these discrepancies, together with the low LepR expression level in AT, we first compared the detection of LepR by a polyclonal goat and a monoclonal mouse antibodies raised against the LepR in brain and hypothalamic extracts, major sites for LepR expression. In brain extracts, both antibodies interact with two major bands around 95 KDa (corresponding to shorter isoform) and 170 KDa (corresponding to the isoform b) (Fig. 23A). Similar results were obtained analysing WCE from the hypothalamus (Fig. 23B) or LepR immunoprecipitates from hypothalamic WCE (Fig 23C). Although we were able to detect LepR in these extracts, we did not observe any differences in LepR protein levels between WT and p21-deficient mice.

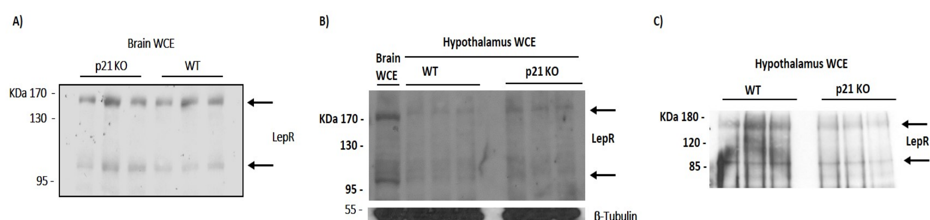


Figure 23. Immunoblot analysis of LepR in brain and hypothalamus. LepR immunoblot analysis (100 μg/lane) of (A) brain WCE; hypothalamic WCE and (C) LepR immunoprecipitates from hypothalamic extracts of 14-week old WT and p21 deficient (p21 KO) mice in SD (n=3 mice per group)

Next, we analysed LepR protein content in WCE from epididymal WAT by immunoblotting. In agreement with the transcriptomic data, at the age of 8 weeks, LepR levels were detectably higher for p21-deficient mice compared to WT mice (Fig 24A). This differential expression was maintained in mice subjected to a HFD (Fig. 24B).

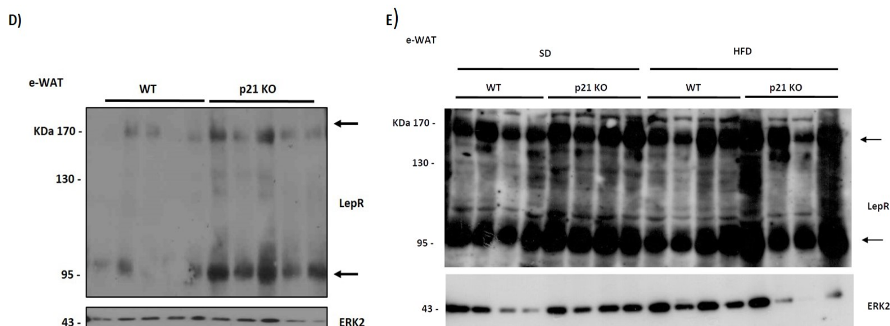


Figure 24. Immunoblot analysis (100 μg/lane) of LepR (upper panel) and ERK (lower panel) in epididymal WAT WCE of (A) 8-week old WT and p21 deficient (p21 KO) mice in SD and (B) 20-week old WT and p21 deficient (p21 KO) mice in SD and HFD, as indicated.

LepR expression in AT of p53-deficient mice

It has become clear in the last years how p53 is also involved in the regulation of metabolism, reinforcing its role as key regulator of most cellular processes. The first historically identified target gene of p53 was p21 (ref). For these reasons we wanted to check if p53 deficiency could recapitulate the phenotype observed in the p21-deficient mice. Firstly, we confirmed that p53 deficiency causes a down regulation of p21 gene expression in AT by RT-PCR analysis of RNA extracts from epididymal WAT of p53-deficient vs WT mice (not shown). Immunoblot analysis showed that LepR protein levels in epididymal WAT were similar in p53- and p21-deficient mice and incremented in comparison to WT mice in SD conditions (Fig. 25).

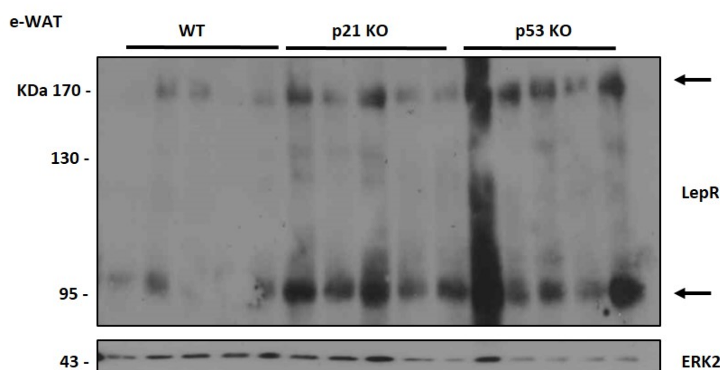


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Figure 25. LepR protein levels are increased in AT of p53 deficient mice. LepR immunoblot analysis in epididymal WAT WCE 8-week old WT and p21- and p53-deficient mice in SD.

p21 KO and mitochondrial metabolism genes

As stated before, mice protection to weight gain by HFD could be due to an increase of mitochondrial activity and, as a consequence, of energy expenditure in HFD conditions. However, the transcriptomic data revealed no significant up regulation of gene expression for NRF1, UCPs or either PGC-1α (which stimulates mitochondrial biogenesis) in the e-WAT of mice in SD (Table 9).

Probesets	Gene Title	Gene Symbol	KO vs WT (log2)	p-value
421679_a_at	cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	-5.299 *	0.000
1439545_at	nuclear respiratory factor 1	Nrf1	0.809	0.004
1456395_at	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	Ppargc1α (PGC1α)	0.360	0.365
1418197_at	Protein 1 (mitochondrial, proton carrier	Ucp1	0.065	0.805
1448188_at	protein 2 (mitochondrial, proton carrier	Ucp2	0.266	0.130
1420658_at	protein 3 (mitochondrial, proton carrier)	Ucp3	0.308	0.176

Table 9. p21Waf1/Cip1 deficiency revealed no increase in mitochondrial marker genes. Analysis of the microarray gene expression data regarding mitochondrial genes.

These results were kind to be expected since samples were collected from mice subjected to a SD. So we also analysed the expression of these genes in WT and p21-deficient mice subjected to HFD. Regardless the diet conditions, qRT-PCR analysis showed no changes in NRF1 mRNA expression levels (Fig. 26 A and B).

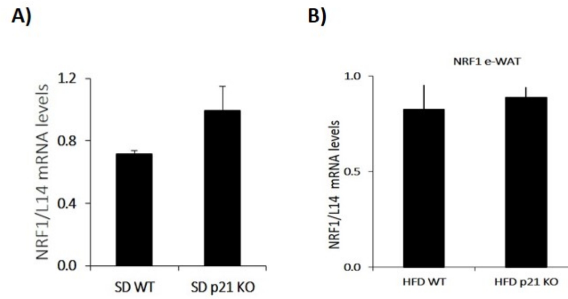


Figure 26. p21Waf1/Cip1 deficiency revealed no increase in NRF1 expression levels. qRT-PCR analysis of NRF1 mRNA levels in e-WAT of (A) 8 week old WT and p21-deficient mice on SD and (B) 20 -week old WT and p21-deficient mice on HFD (n=4 mice per group).

Similarly, no upregulation was observed either for UCP-1, 2, 3 mRNA expression levels (Fig. 27 A-C). Strikingly in the case of UCP2 we observed a significant decrease of expression in the p21 KO compared to WT samples.

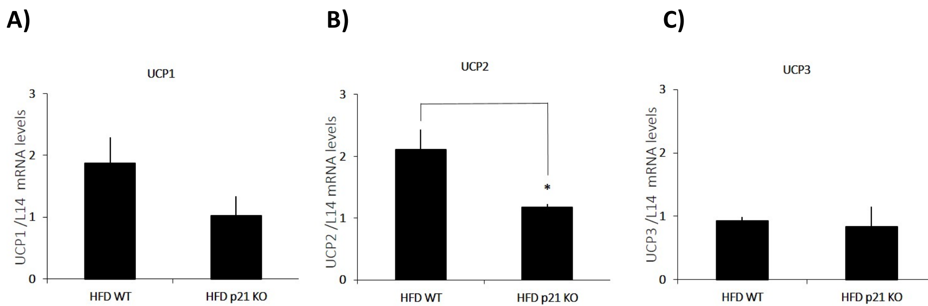


Figure 27. p21Waf1/Cip1 deficiency revealed no upregulation of UCPs expression levels. qRT-PCR analysis of mRNA levels for UCP1 (A), UCP2 (B), UCP3(C) in e-WAT) of 20-week old WT and p21-deficient mice on HFD (n=4 mice per group).

Interestingly, p21 deficiency caused an increase in the epididymal WAT expression level of PGC1α in conditions of HFD (Fig 28).

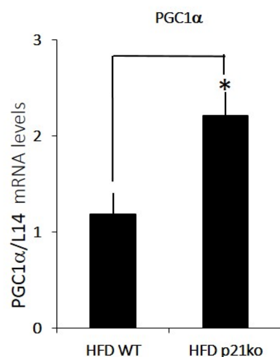


Figure 28. p21Waf1/Cip1 deficiency caused upregulation of PGC1α expression levels. qRT-PCR analysis of PGC1α mRNA levels in e-WAT-I 20-week old WT and p21-deficient mice on HfD (n=4 mice per group).

Leptin and the regulation of endocannabinoid tone

The endocannabinoid system (ECS) has emerged as an important modulator of energy balance. Indeed, ECS modulates several physiologic functions through central and peripheral mechanisms, and dysregulation of the ECS is linked to abdominal obesity and other risk factors for cardiovascular disease and T2D [Buettner *et al.*, 2008]. The CB1 is widely expressed in the brain, including brain areas associated with the regulation of energy homeostasis, but is also present in peripheral tissues, including liver, pancreas, muscle, and AT. The analysis of the formerly described microarray data showed a low but significant decrease of CB1 expression in the e-WAT p21 KO compared to WT mice in SD conditions (Table 10).

Probesets	Gene Title	Gene Symbol	KO vs WT (log2)	p-value
421679_a_at	cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	-5,299 *	0,000
1459425_at	cannabinoid receptor 1	Cnr1	-0.287*	0,010

Table 10. p21Waf1/Cip1 deficiency and CB1 expression. Analysis of the microarray gene expression data regarding CB1 gene.

Genetic or pharmacological reduction of CB1 activity decreases body weight and fat mass to a higher extent than that expected from the reduction of caloric intake alone. However, whether the metabolic amelioration associated with the

administration of a CB1 antagonist is dependent on its appetite suppressant effect is still a matter of debate. Regarding these results, we analysed the possible molecular mechanism between these two energy balance systems. It has been described that, in CB1 mice, low plasma leptin levels accompanied low adiposity and suppressive effects of exogenous leptin on feeding behaviour and body weight were enhanced. However, we were not able to validate the data from transcriptomic analysis at least in SD conditions (Fig. 29A), whereas we observed a significant downregulation of CB1 expression in p21 KO mice in conditions of HFD compared to WT (Fig. 29B).

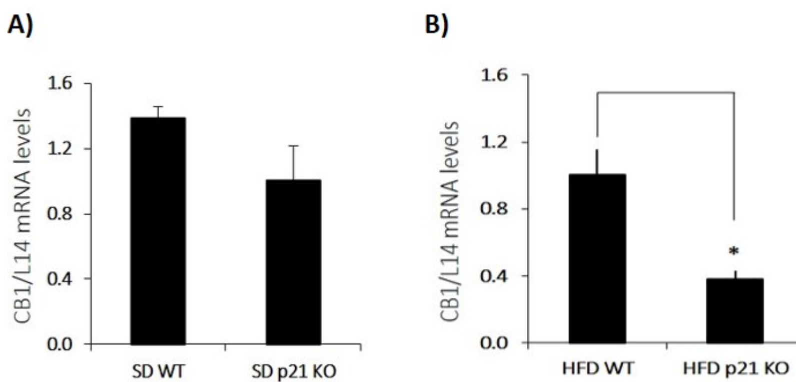


Figure 29. p21 deficiency induces down-regulation of CB1 gene expression in HFD condition. qRT-PCR for CB1 mRNA in e-WAT of (A) 8 week old WT and p21-deficient mice on SD and (B) 20-week old WT and p21-deficient mice on HFD (n=4 mice per group).

Potential involvement of E2F in the regulation of LepR gene expression by p21

As an inhibitor of the cyclin D/CDK 4/6 complexes, and hence of the G1 to S transition, of the cell cycle, p21 regulates the transcriptional regulatory activity of the pRB/E2F complexes. So we wanted to investigate a potential involvement of E2F in the regulation of LepR gene expression by p21. First of all we performed an *in silico* analysis of the LepR regulatory region using the TRANSFAC© (TRANScription FACtor database) and we identified two potential E2F binding sites located in the first intron of the LepR gene (Fig. 30A). Another tool, PROMO©, was also able to identify several potential target sequences in the promoter region (Fig. 30B).

A)

B)

Figure 30. Potential E2F binding sites in the LepR gene. *In silico* analysis for E2F binding sites in the LepR gene using both (A) TRANSFAC and (B) PROMO tools. E2F consensus: TTTCGCGC. Factors predicted within a dissimilarity margin less or equal than 20%.

We also analysed the LepR mRNA and protein expression level in epididymal WAT of E2F1-deficient mice relative to that in WT littermates in SD conditions.. Despite we performed qRT-PCR and immunoblot analysis no significant differences were detected (Fig. 31).

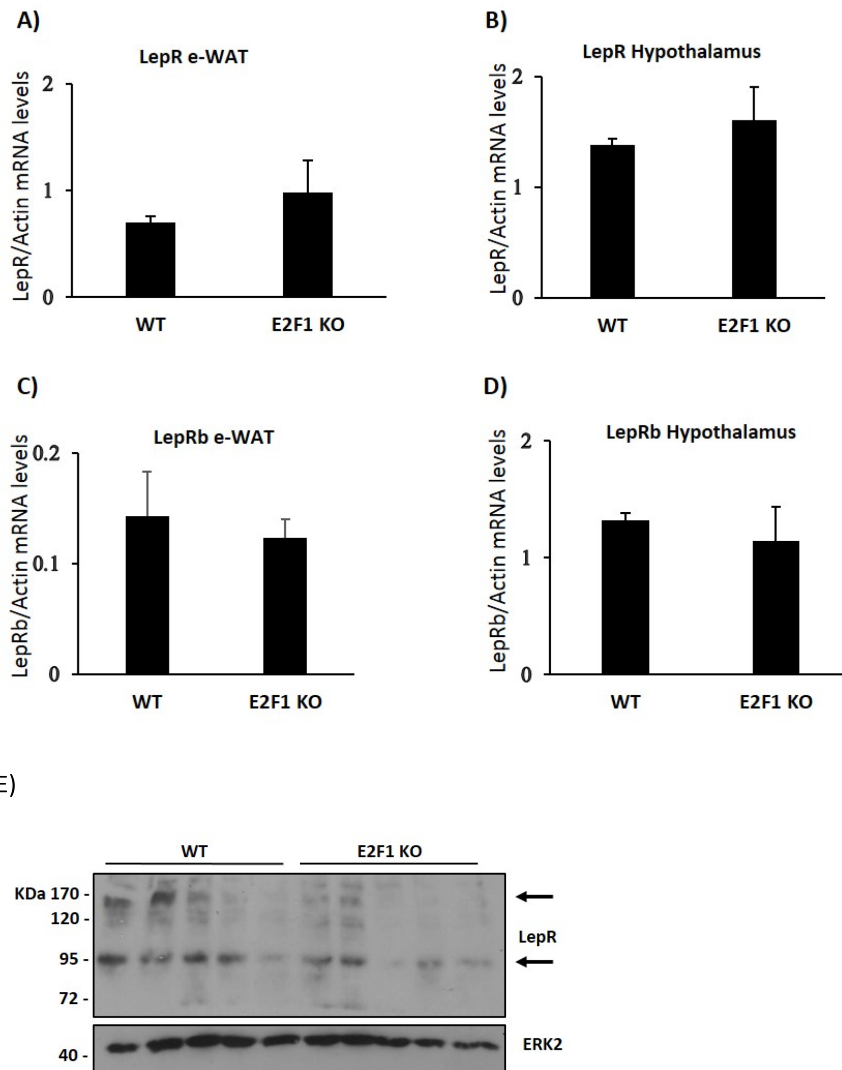


Figure 31. LepR gene expression in E2F1 deficient mice. qRT-PCR analysis for LepR in (A) e-WAT and (B) hypothalamus and LepRb in (C) e-WAT and (D) hypothalamus; and in immunoblot analysis for LepR in e-WAT (E) of E2F1 KO mice and WT mice in SD.

Once established that E2F1 did not regulate LepR gene expression by p21, we wanted to check if p21 or p53 directly regulated LepR protein levels at least in cell culture. Fig. 32 A shows that we were able to knockdown p21 protein expression (which is undetectable in undifferentiated cells). However, we failed to find a working vector to knockdown p53 expression (so this vector was used as further negative control). Fig. 32 B shows that, in in a pool of 3T3L1 cells infected with viral particles carrying a shRNA specific for p21, the levels of LepR were unvaried, though p21 expression was knocked down, not enough to cause changes in LepR levels. So neither p21 alone was responsible for a direct regulation of leptin receptor in this cellular system.

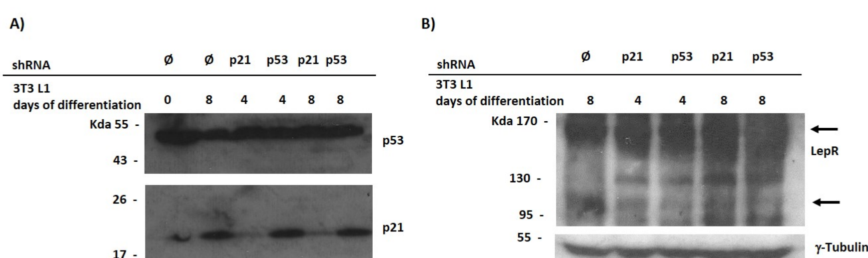


Figure 32. E2F does not regulate LepR gene expression by p21 in 3T3-L1 cell line. Immunoblot of (A) p53 and p21 and (B) LepR protein levels in 3T3L1 cell line after knockdown via shRNA for p21 and 53 knockdown carried out through viral infection.

Regulation of leptin sensitivity by 21- and p53-deficiency

In order to attribute the HFD-induced obesity protective action of p21 and p53 deficiency to the upregulation of LepR we performed experiments directed to assess leptin sensitivity of these mice. In this regard, we undertook two different experimental approaches: 1) a short-term treatment with leptin to study leptin signalling and 2) a diary administration of leptin to follow leptin action on body weight.

In the first case, leptin (1 mg/Kg) was administered intravenously after 6 hours of fasting to 10-week old female mice in SD condition; 10 minutes later the animals were sacrificed and immunoblot analysis to detect STAT3 phosphorylation were

performed. As shown in Fig. 33, the p21 KO mice showed a higher level of STAT3 phosphorylation in response to leptin than the WT mice.

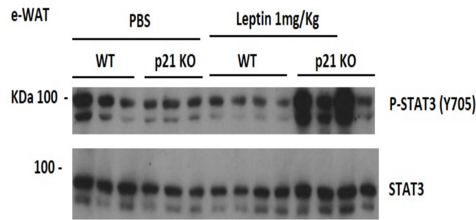


Figure 33. Leptin induced STAT3 phosphorylation in p21 KO vs WT mice. Immunoblot analysis of STAT3 phosphorylation (upper panel) and protein expression level (lower panel) in WCE from epididymal WAT of 10-week old female p21-deficient (p21 KO) and WT mice to which leptin (1mg/kg) or saline, as indicated, was administrated intravenously 10 min before to sacrifice the animals.

Regarding the second type of approach, 10-week old p21-deficient mice and WT littermates were daily injected intraperitoneally 2 h before the dark cycle with a suboptimal dose of leptin (0.1 mg/Kg/day) during 3 weeks. Food consumption was followed during the treatment period, and no differences were detected between the two groups of animals. The effects of the treatment were analysed in terms of weight loss and percentage of weight decrease (Fig. 34).

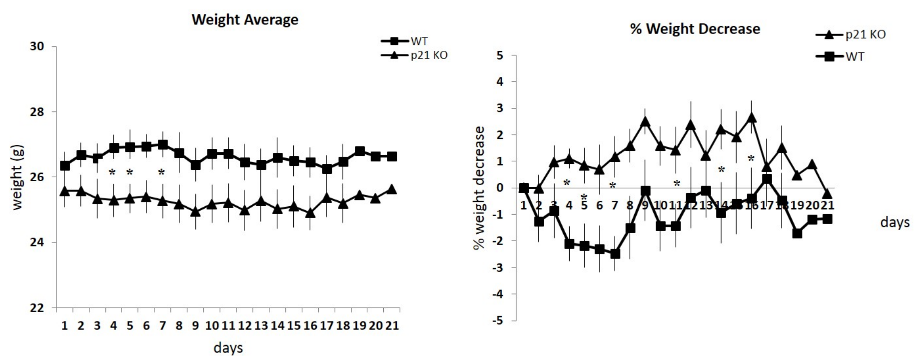


Figure 34. Effect of suboptimal doses of leptin in p21-deficient and WT mice. Graphic representation of net weight (A) and percentage of weight decrease (B) of p21-deficient (p21 KO) and WT mice daily injected intraperitoneally 2 h before the dark cycle with a suboptimal dose of leptin (0.1 mg/Kg/day) (n=4 animals per group).

As for p21 KO mice, the same leptin treatment was also performed in 12-16-week old p53 KO mice for 5 days and body weight was followed (Fig. 35).

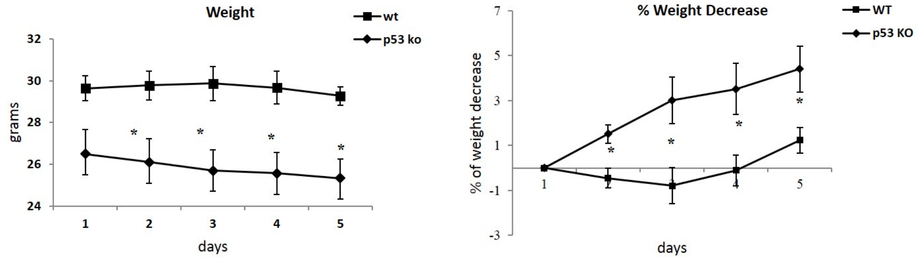


Figure 35. Effect of suboptimal doses of leptin in p53-deficient and WT mice. Graphic representation of net weight (A) and percentage of weight decrease (B) of p53-deficient (p53 KO) and WT mice daily injected intraperitoneally 2 h before the dark cycle with a suboptimal dose of leptin (0.1 mg/Kg/day) (n=4 animals per group).

Effects of the p21-deficiency in the *ob/ob* genetic background

In order to assess genetically the involvement of leptin and LepR signalling in the obesity protective action of the p21-deficiency, we generated double p21/*ob*-deficient mice. These mice preserved p21-deficiency-dependent LepR upregulation in the AT as assessed by qRT-PCR (Fig. 36 A) and immunoblot analysis (Fig 36 B)

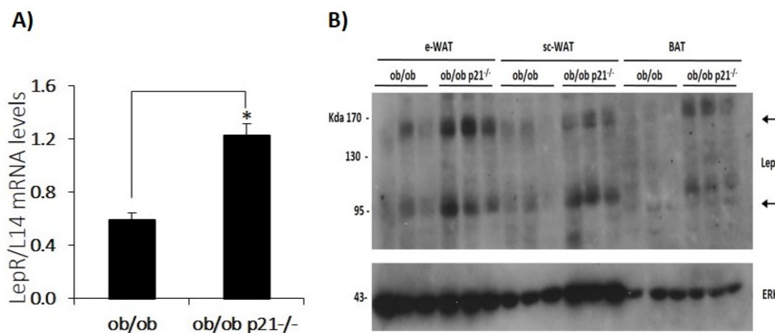


Figure 36. p21Waf1/Cip1 deficiency induces LepR gene upregulation in *ob/ob* genetic background. qRT-PCR analysis of LepR mRNA in e-WAT (A) and immunoblot analysis for LepR (upper panel) and ERK (lower panel) in e-WAT, sc-WAT and BAT (C) of 14-week old *ob/ob vs ob/ob* p21 $^{-/-}$ mice, as indicated.

Next, we followed the development of obesity of p21-/- *ob/ob versus ob/ob* mice and observed the failure of p21-deficiency to protect from obesity in the *ob/ob* genetic background (Fig. 37). These results provide a genetic proof for a critical role of leptin and LepR signalling in the obesity protective action of the p21-deficiency and strongly support our previous transcriptomic data.

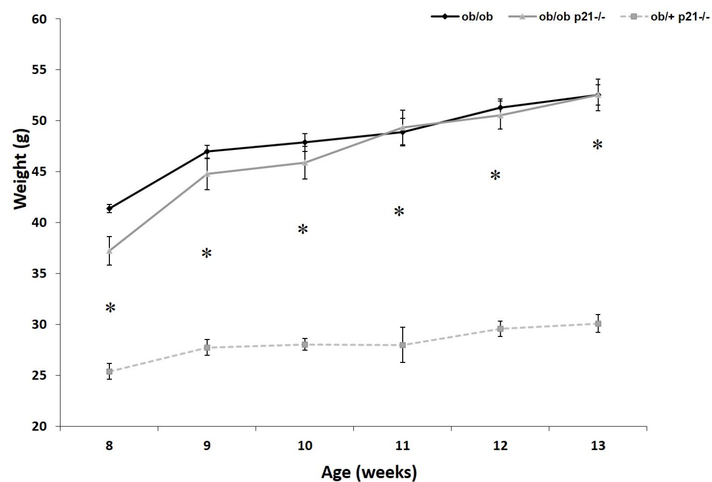


Figure 37. p21Waf1/Cip1 deficiency did not protect from adiposity in the *ob/ob* genetic background. Weight curves for *ob/ob*, *ob/ob* p21$^{-/-}$ and lean *ob/*$^{+}$ p21$^{-/-}$ mice.

Furthermore, the failure of the p21-deficiency to protect from adiposity in the absence of leptin correlated with the development of glucose intolerance to a similar degree as the *ob/ob* mice (Fig. 38 A) and a partial but statistically significant lost of insulin sensitivity (Fig. 38 B).

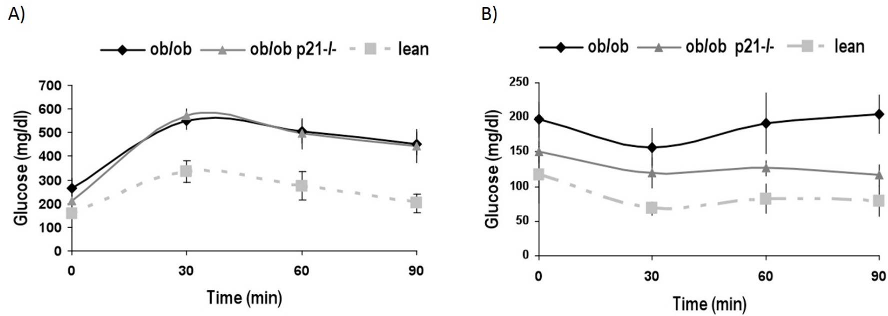


Figure 38. p21Waf1/Cip1 deficiency did not protect from glucose intolerance and only partially maintains insulin sensitivity in the *ob/ob* genetic background . GTT (A) and ITT (B) performed in 13-week old *ob/ob*, *ob/ob* p21$^{-/-}$ and *ob/+* p21$^{-/-}$ mice, as indicated.

Regarding the expression of genes related to mitochondria, again no differences were observed when analysing the expression levels of NRF1 gene in the e-WAT of *ob/ob* p21-/- *versus ob/ob* mice (Fig. 39 A). Moreover, in contrast to the results obtained in the HDF obesity model, in mice lacking leptin p21-deficinecy failed to cause an upregulation of *pgc1*α gene (Fig. 39 B), strongly suggesting that this action relays on leptin signalling.

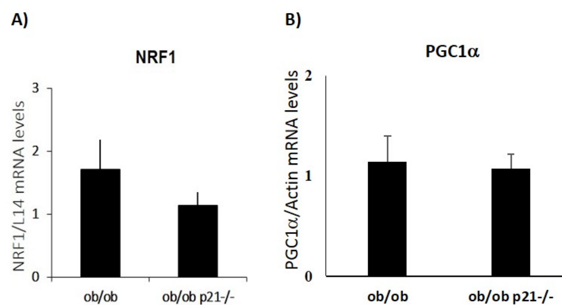


Figure 39. p21.deficiency does not induce upregulation of PGC1α **gene expression in the *ob/ob* genetic background.** qRT-PCR analysis of (A) NRF1 and (B) PGC1α in RNA extracts from the e-WAT of 14-week old *ob/ob* and *ob/ob* p21 $^{-/-}$ mice, as indicated. (n=)

In relation to the expression levels of the UCPs genes in the e-WAT, sc-WAT and BAT *ob/ob* and *ob/ob* p21 $^{-/-}$ mice. No major changes were detected for UCP2 and UCP3 gene expression. In the case of UCP1 expression a tendency to increase in WAT and a statistically significant decrease in the BAT was observed (Fig. 40).

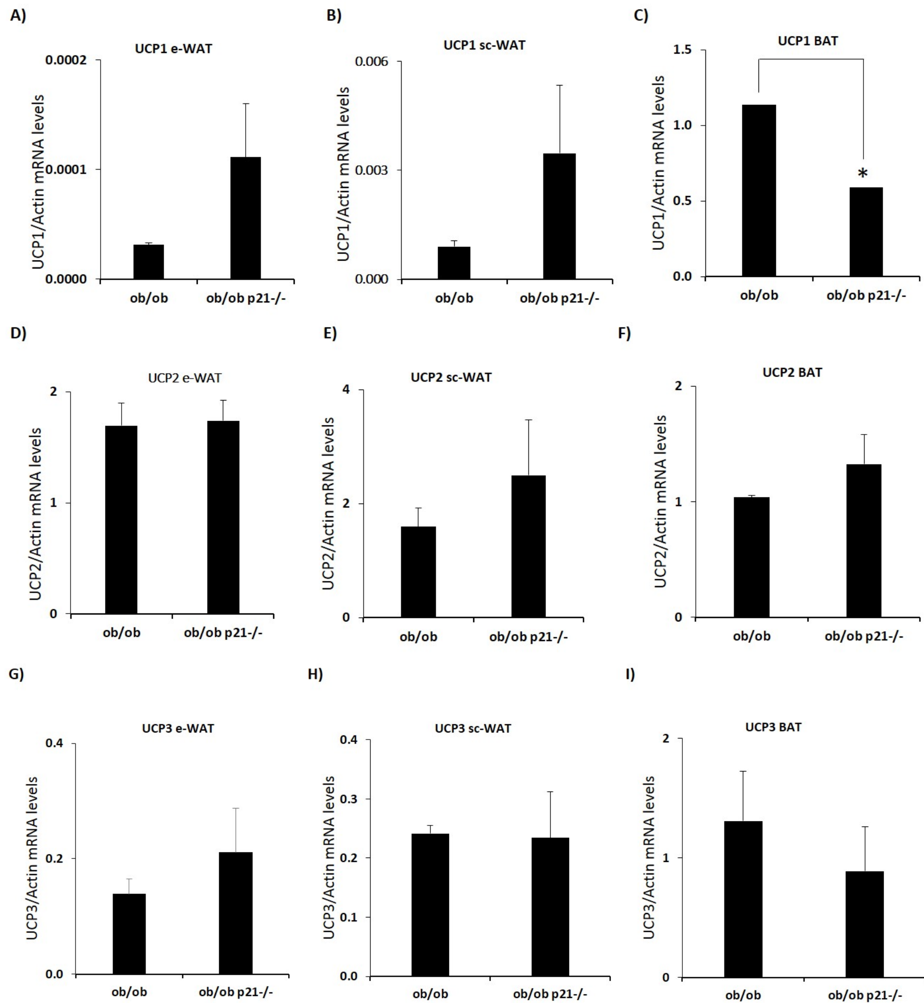


Figure 40. UCP1, UCP2 and UCP3 gene expression in *ob/ob* and *ob/ob* p21-/- mice. qRT-PCR analysis of UCP1 (A, B, and C), UCP2 (D, E and F) and UCP3 (G, H and I) in RNA extracts from e-WAT, sc-WAT, and BAT, respectively, of 14-week old *ob/ob* and *ob/ob* p21 $^{-/-}$ mice, as indicated.

The disruption of leptin signalling due to genetic leptin deficiency also affected the regulation of CB1 expression. In fact, the p21 deficiency by itself did not suffice for a CB1 downregulation (Fig. 41). So, the regulation of the endocannabinoid tone depends on a correct leptin signalling.

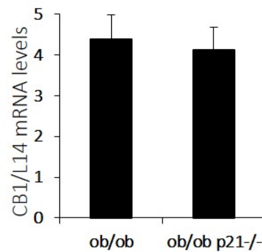


Figure 41. CB1 gene expression in *ob/ob* and *ob/ob* p21-/- mice. qRT-PCR analysis in RNA extracts from e-WAT of 14-week old *ob/ob* and *ob/ob* p21 $^{-/-}$ mice, as indicated.

We also checked the activation of leptin signalling in isolated adipocytes (whereas mixed unclear information resulted from in vivo experiments). So we proceeded investigating leptin sensitivity in adipocytes form ob/ob and ob/ob p21 KO. The ob/ob of course presents a useful study since it lacks leptin expression and leptin signalling. Adipocytes were isolated from e-WAT of 9-10 weeks ob and ob p21 mice and incubated with leptin. To detect the activation of leptin signalling we performed an immunoblot analysis for STAT3 phosphorylation. We observed a higher STAT3 phosphorylation level (Fig. 42). As expected, due to basal STAT3 phosphorylation levels, the difference is not big but still detectable.

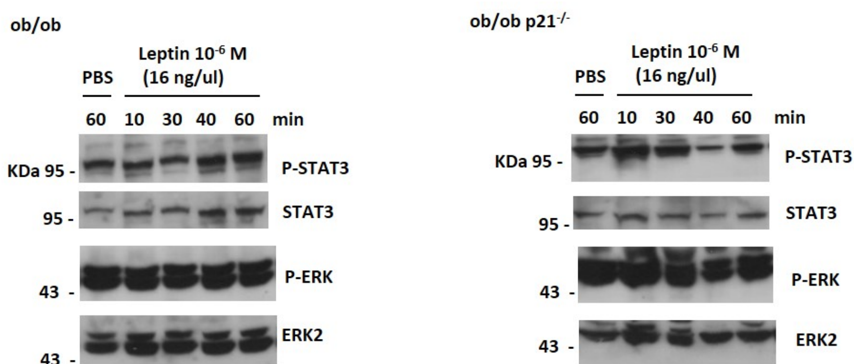


Figure 42. Leptin sensitivity in isolated adipocytes. Immunoblot analysis of STAT3 phosphorylation. The samples proceeded from adipocytes isolated from the e-WAT of 9-10 weeks mice and treated *in vitro* with leptin

2.- ANALYSIS OF THE EFFECT OF JNK ACTIVATION IN PANCREATIC β CELLS IN OBESITY-ASSOCIATED SYSTEMIC INSULIN RESISTANCE AND HYPERINSULINEMIA.

GFP-MKK7D mouse is a transgenic murine model generated in our laboratory which has shown to be useful to study the effects of JNK activation in pancreatic β-cells regarding glucose homeostasis and β-cell function. The transgene encodes for a conditional Cre-recombinase-dependent expression form of MKK7 (a MAP2K specific for JNK) which carries two mutations (MKK7D) mimicking the active form of the enzyme and upon expression can trigger JNK activity. MKK7D expression is under the control of a constitutive promoter but prevented by a stop cassette which encodes for the green fluorescent protein (GFP) and which is flanked by two LoxP sites (Lanuza-Masdeu et al., 2013) To activate JNK in pancreatic β-cells, homozygous GFP-MKK7D female mice were crossed with heterozygous RIP-Cre male mice to obtain animals expressing either GFP (Control mice) or, due to the Cre recombinase–dependent excision of the GFP expression cassette, MKK7D (MKK7D mice). Thesis' work by Dr. Jordi Lanuza-Masdeu showed how JNK activation in pancreatic β-cells induced glucose intolerance due to the impairment of the paracrine insulin signalling. Remarkably, MKK7D mice developed obesity in response to a HFD but, unlike Control animals, were protected from HFD-induced insulin resistance and hyperinsulinemia (Fig. 43).

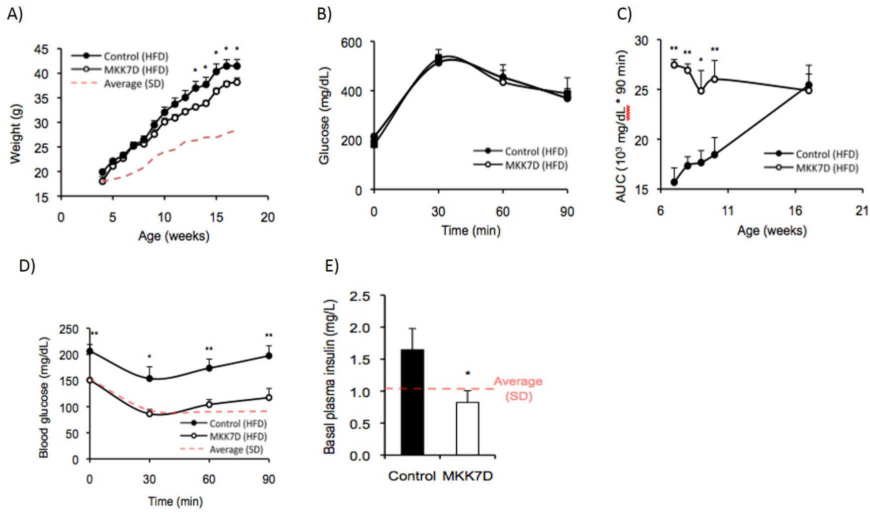


Figure 43. In HDF conditions, MKK7D mice dissociate obesity from insulin resistance and hyperinsulinemia. Weight curve (A), GTT (B), progression of AUC (C), ITT (D) and insulinemia (E) of MKK7D and Control mice fed for 12 weeks with HFD, as indicated. Red dotted lines represent values in SD.

Therefore, we decided to further characterize this phenotype at molecular level. Experiments that follow were performed in female mice. In this regard, we analysed the expression of inflammatory markers such as TNFα and MCP1 in AT. As shown in figure 44, there was a significant increase in TNFα levels due to HFD but there were no differences between Control and MKK7D mice indicating a similar level of inflammatory response at this tissue induced by HFD.

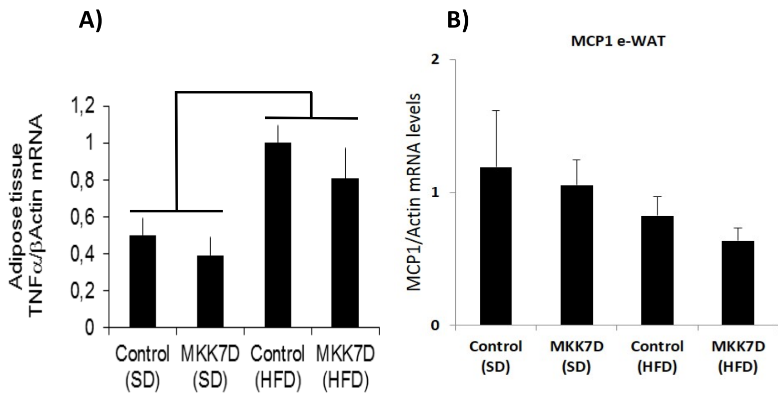


Figure 44. Inflammatory markers induced by obesity. qRT-PCR analysis for (A) TNFα and (B) MCP1 mRNA levels in the e-WAT of MKK7D and Control mice in SD and HFD conditions, as indicated.

This obesity-induced inflammation state in AT was also sustained by an increased JNK activity in both groups of mice in HFD (Fig. 45). In addition, we also observed higher phosphorylation levels for eIF2α, a marker for ER stress, in response to HFD (Fig. 43).

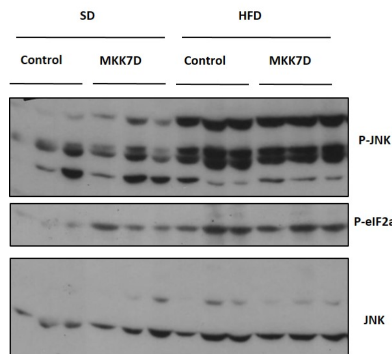


Figure 45. Obesity induced JNK activity and ER stress in AT. Immunoblot analysis for phospho-JNK (P-JNK)(upper panel), phospho-eIF2α (P-eIF2α) and JNK in extracts of e-WAT of MKK7D and Control mice, in SD and HFD, as indicated.

On the other hand, the expression of IRS-1 and IRS-2 (Fig. 46 A)(Fig. 46 B) was sustained and not compromised by HFD-induced obesity (Fig 45 A). Finally, GLUT4 expression was similarly decreased by HFD in both groups of mice (Fig. 46 B). In summary, obesity-induced inflammatory and ER stress responses in AT were similarly induced in Control and MKK7D mice however, in the later, these responses were not sufficient to induce systemic insulin resistance nor hyperinsulinemia.

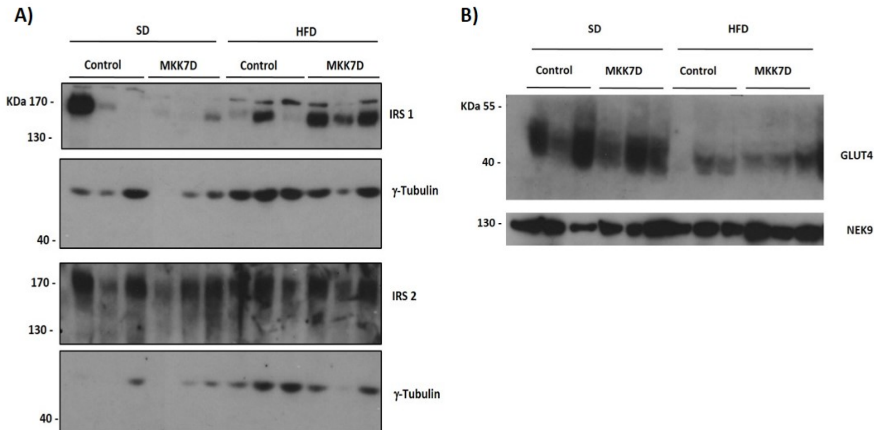


Figure 46. IRS-1, IRS-2 and GLUT4 protein levels in AT. Immunoblot analysis of (A) IRS-1 and IRS-2 and (B) GLUT4 in the e-WAT of Control and MKK7D mice in SD and HFD conditions, as indicated.

With respect to the liver, no significant changes were detected in the expression levels of the inflammatory markers TNFα and MCP1 (Fig. 47 A and B).

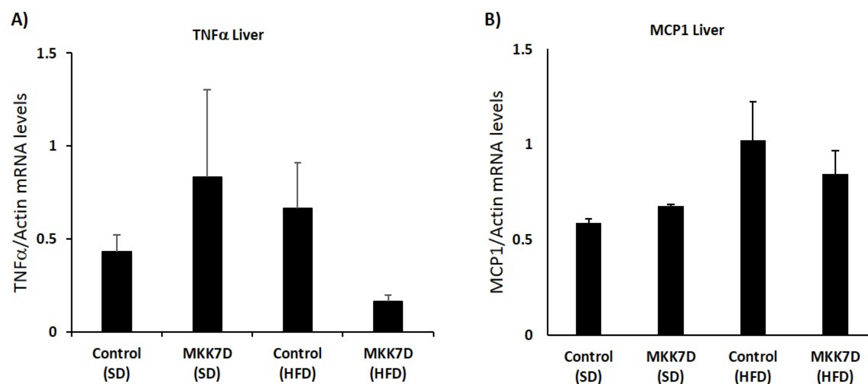


Figure 47. Analysis of inflammatory markers in the liver. qRT-PCR analysis for (A) TNFα and (B) MCP1 mRNA levels in the liver of MKK7D and Control mice in SD and HFD conditions, as indicated.

Yet we observed an increase in JNK activity in the liver of both Control and MKK7D mice in HFD. Furthermore there was no evidence for HFD-induced eIF2α phosphorylation in this tissue (Fig. 48).

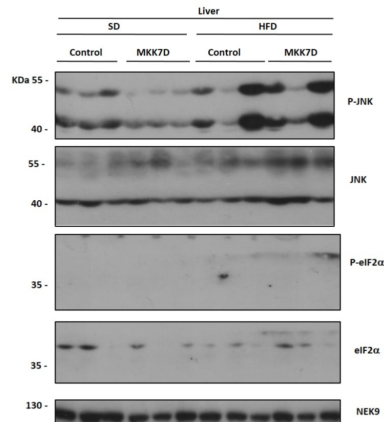


Fig 48. Obesity-induced JNK activity and ER stress in liver. Immunoblot analysis for phospho-JNK (P-JNK)(first panel), JNK (second panel), phospho-eIF2α (P-eIF2α) (third panel), eIF2α (forth panel) and NEK9 (fifth panel) in liver extracts of MKK7D and Control mice, in SD and HFD, as indicated.

In relation to liver, IRS-2, the dominant IRS isoform expressed in liver, was unchanged by HFD and similar in both groups of mice (Fig. 49).

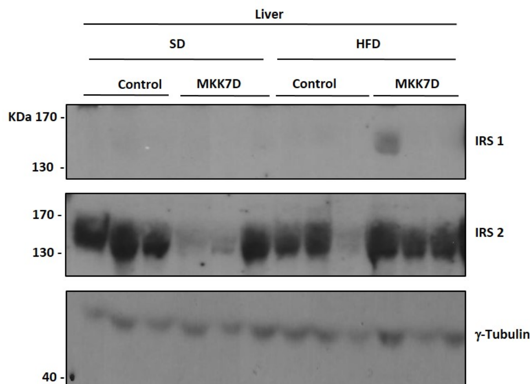


Figure 49. IRS-1 and IRS-2 protein levels in liver. Immunoblot analysis of IRS-1 (upper panel) and IRS-2 (central panel) and γ-tubulin (lower panel) in the liver of Control and MKK7D mice in SD and HFD conditions, as indicated.

In skeletal muscle, we were not able to detect any JNK phosphorylation (Fig. 50) in Control nor MKK7D mice. So JNK was not activated even in HFD conditions in this tissue.

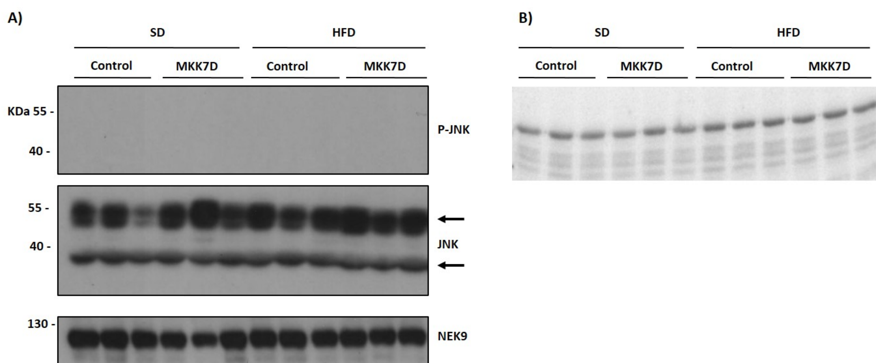


Figure 50. JNK is not activated in the skeletal muscle DIO female mice. A: Immunoblot analysis of phospho-JNK (P-JNK)(upper panel), JNK (central panel) and NEK9 (lower panel) in skeletal muscle of Control and MKK7D mice SD and HFD conditions, as indicated.
B: phosphoimager scan of WCE of skeletal muscle from control and MKK7 mice in both SD and HFD conditions, incubated with c-Jun (substrate of JNK [γ-^{32}P] ATP.

Remarkably, IRS1 and IRS2 protein levels remained higher in obese-insulin-sensitive MKK7D mice compared to obese-insulin-resistant Control mice (Fig. 51).

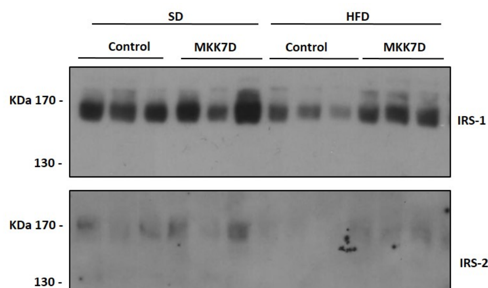


Figure 51. IRS-1 and IRS-2 protein levels in skeletal muscle. Immunoblot analysis of IRS-1 (upper panel) and IRS-2 (lower panel) in skeletal muscle of Control and MKK7D mice in SD and HFD conditions, as indicated.

Similarly, increased phosphorylation SK6 levels were observed in MKK7D mice in HFD conditions (Fig.52).

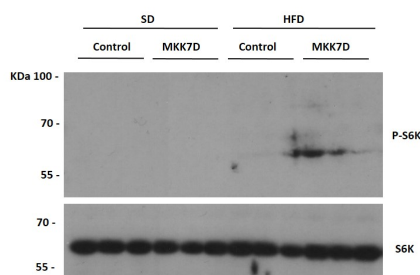


Figure 52. S6K phosphorylation and protein levels in skeletal muscle. Immunoblot analysis of phospho-S6K (P-S6K) (upper panel) and S6K (lower panel) in skeletal muscle of Control and MKK7D mice in SD and HFD conditions, as indicated.

DISCUSSION

PART I

Obesity and related metabolic diseases have spread worldwide and become a sanitary emergency in the last years, affecting both sexes, adults and children. The seed of this plague finds its origin in the huge changes in society, technology that came out in the last centuries, in particular starting from Industrial Revolution. While our organism has developed to survive in certain conditions of caloric restriction, food deprivation, and long periods of cold, these conditions have changed in a very short period of time that impaired the proper adaptation to this new environment. From an evolutionary point of view, our organism has evolved through the centuries facing each sort of environment. Ice age, starvation, viruses, wars constitute just some and quite different tasks that the humans had (and still have) to fight for survival and reproduction of the species. In the past years we have been experiencing a huge economic and population expansion, due to migrations, globalization and technology advance. In addition, changes get faster and faster and affect millions of people at a time. The same can be said for behaviours and feeding habits, lifestyles, in relation to new society's organization/structure and kind of life: city life vs country life, supermarket vs self-sustainment agriculture. Regarding physical activity and energy expenditure, it is not just a matter of lack of exercise: people do not have to hunt or provide for their own meals anymore, they can buy food in a single place that they reach by car; the possibility to regulate the temperature of the environment we live and work in; these and many other changes led to many transformations that our organisms in some cases cannot withstand. The genetic components is still the first determining factor when it comes to body responses and physiology with regards to the environment we live in. But of course still a life without a dietary attention can be deleterious for people genetically predisposed to certain metabolic disorders. Along with changes in diet and life style came the improvement in hygiene, medical technology, with consequent extension of life span, an increase of age-related pathologies (cardiovascular and neurodegenerative diseases, cancer, etc.) and of course, the chance to develop the metabolic syndrome [Ahima, 2009].

Obesity is in essence a metabolic disorder, though in recent years evidence has shown that other responses such as the inflammatory and the ER stress response are also relevant to the disorder. Therefore, to understand the changes driving the obesity-associated pathological process is necessary to have a broader look, expanding the investigation to other possible players that, until some years ago, were not considered as part of metabolic processes. Of course, this also opened the path to the development of new therapies and drugs to target different processes/factors for the treatment of obesity-associated metabolic anomalies. As already stated in the introductory part, in addition to cancer, factors that regulate cell cycle play also a role in metabolic regulation. At the beginning of our search we were interested in explore the role of p21 in the regulation of metabolism. p21 is the major CDK inhibitor and a very relevant p53 target gene [el-Deiry *et al.*, 1993], arresting cells in G1 phase [Inoue et al., 2008] and protecting from apoptosis [Polyak *et al.*, 1996]. Concerning adipogenesis, transcription factors like FOXO1, SREBP-1c/ADD1, C/EBP family show transcriptional activity on the p21 promoter [Inoue *et al.*, 2005; 228, 237, 238].

The accumulation of lipids into the adipocytes represent a stressful condition that could trigger the activation of the p53/p21 pathway, as in the case of hypertrophic adipocytes of obese mice [Inoue et al., 2008, Yahagi *et al.*, 2003; Yahagi *et al.*, 2004]. In these conditions, activation of p21 expression would have an anti-apoptotic effect rather than stopping proliferation the growth [Inoue et al., 2008].

In has been shown that p53 is activated by the obesity-related aging process, characterised by oxidative stress, production of pro-inflammatory cytokines, accumulation of macrophages in the AT, induction of insulin resistance and suppression of adiponectin expression. Accordingly, specific p53 KO at the AT lowers inflammation and improves insulin sensitivity in obese mice; on the other hand, transgenic overexpression of p53 and cdkn1a genes in AT induces inflammation and insulin resistance [Minamino *et al.*, 2009].

Despite p21 participates in the early stages of differentiation (ref), it seems that the importance of p21 is linked to adipocyte hypertrophy [Inoue et al., 2008]. This idea can be supported by the fact that AT in p21 deficient mice develops normally.

At a histological level, the analysis of WAT and BAT did not show differences in size, morphology or number of adipocytes between p21 KO and WT suggesting that p21 absence *per se* does not compromise or alter the anatomy-physiology of AT (Fig. 17). However, we observed that p21 gene expression is specifically enhanced in the epididymal WAT of ob/ob mice compared to lean mice (Fig. 13). An upregulation of p21 protein levels was also observed in e-WAT of HFD-induce obese mice when compared to mice fed with normal chow (standard diet) (Fig. 14). Moreover, this increased expression was restricted to the AT since p21 expression in liver (a slight increase in obese condition may be observed in liver but its origin it is likely to be the fat accumulated in this organ) and skeletal muscle remained undetectable in obese mice.

Once established that p21 is expressed and accumulates in the e-WAT of adult mice, we proposed to determine whether the lack of p21 may affect the onset of obesity. In terms of body weight p21-deficient mice fed with a HFD were significantly leaner than WT mice in the same conditions, and their average weight was comparable to mice in SD conditions (Fig. 15 A). It is worthwhile to notice that the protection from diet-induced obesity was dependent in the p21-gene dosage, as p21 heterozygous mice showed and intermediate phenotype. This phenotype was due to the lack of AT expansion in HFD conditions (Fig 17). Interestingly, we did not observe differences in food intake nor in body temperature between WT and p21 deficient mice. Furthermore p21 KO, in HFD, were not only protected against the development of HFD-induced adiposity but also to the associated hypertriglyceridemia (Fig. 16).

Finally, we also observed a sensible amelioration of the insulin resistance triggered by diet-induced obesity in the p21-deficient mice. In this regard, p21 deficient mice subjected to HFD preserved glucose tolerance and insulin sensitivity similar of that of mice in SD as shown by GTT and ITT, respectively (Fig 18).

So the following step was to perform a genome-wide comparative study of gene expression using e-WAT RNA extracts from WT and p21-heterozygous and -deficient mice in SD conditions. The idea was to find out differences in the expression levels of genes related to metabolism and adipogenesis in particular, trying to see a direct effect of p21 deficiency, independently of the diet-induced

obesity, on gene regulation. We focused on the e-WAT as our previous data showed that the upregulation of p21 in obesity was restricted to that rather than other tissues. Heterozygous mice were also introduced in this analysis because we expected to be helpful for relevant gene identification since its expression, as the phenotype, should be affected by p21 gene dosage.

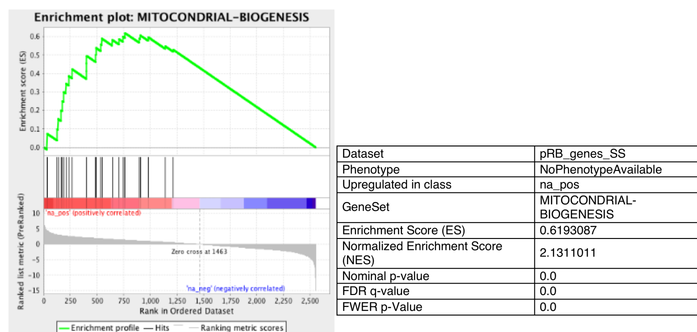
As first result, it was clear that no significant differences existed between WT, heterozygous and KO mice, in adipogenic/adipose genes. On the basis of these results and the former analysis of AT, we speculated that p21 might not be strictly necessary for adipocyte differentiation or maybe there are functional redundancies with other genes that are able to suffice for this task. Then we moved on the analysis of other possible pathways involved in the amelioration of the obese phenotype observed for the p21 KO mice in HFD. An enhanced mitochondrial activity and energy expenditure could give to mice the resistance to weight gain by HFD. However, the analysis of NRF1, UCPs or PGC-1a came back without any result. No changes were observed in genes related to energy expenditure and mitochondrial biogenesis. This was to be expected since mice were subjected to a SD and once again confirmed that the mere p21 deficiency was not enough to determine any changes in gene expression levels. To find out if any changes would appear in HFD conditions, we analysed samples by qRT-PCR in search for a possible increase of mitochondrial activity represented by an upregulation of mitochondrial genes expression due to p21 deficiency. As mentioned before we focused our attention on e-WAT samples from p21 KO mice in HFD. However, no changes were detected for NRF1 (Fig. 23 A and B) and for UCP-1 and 3 (Fig. 24 A-C). As a paradox, for UCP2 we registered an unexpected but significant decrease of expression in p21 KO compared to WT samples. But it was quite interesting to observe that p21 deficiency in the e-WAT of animals in HFD caused an increase in the levels of PGC1a levels compared to WT. This may represent a marker for a potential increase in mitochondrial biogenesis, even if the data regarding the uncoupling and respiration genes did not support the hypothesis that energy expenditure is higher in the e-WAT p21 KO vs WT mice in HFD. Shortly, it has to be mentioned also something about the endocannabinoid receptor CB1. Endocannabinoids have an important role both in CNS and

periphery in the control of energy homeostasis, and they expression is found to be enhanced in obese individuals. CB1 expression levels did not change between WT and p21 KO in SD conditions, in contrast with the transcriptomic data, whereas we observed a significant decrease in HFD p21 KO vs WT mice, confirming once again that p21 KO mice do not show many characteristics typical of obesity and are in some way protected.

In this scenario took an enormous significance the analysis of LepR. In fact, according to the transcriptomic data, LepR levels were upregulated (almost two times higher) in the e-WAT of p21 KO vs WT mice in SD. It was also interesting to notice that the p21-heterozygous mice showed an intermediated phenotype, between WT and p21 KO littermates, that could unravel a possible genetic dose effect of p21 in the regulation of LepR expression. In the context of obesity, in which leptin levels increase in proportion to the amount of fat, the importance of LepR uregulation may be crucial to preserve a functional leptin signalling and thus avoid the onset of leptin resistance, at least in the paracrine/autocrine system of e-WAT An important functional role of the LepR in mediating leptin paracrine functions came from the literature . At a systemic level, soluble LepR plasma levels are significantly decreased, whereas leptin levels are significantly increased in morbidly obese subjects compared to lean patients [van Dielen *et al.*, 2002]. Furthermore adipocyte-specific LepR over expression by transgenesis prevented HFD-induced increase in adiposity in WT mice and evidenced leptin paracrine actions [Wang *et al.*, 2005]. Finally according to the hypothesis that LepR is regulated by p21 through the regulation of pRb/E2F complex, specific deletion of pRb in AT lead mice to increased energy expenditure, weight loss and protection from diabetes [Dali-Youcef *et al.*, 2007]. These models supported the idea that resistance to obesity is due an increase of mitochondrial activity and consequent energy expenditure (on HFD conditions). In our case, we observed an upregulation just for PGC1a gene in e-WA. From all these data we generated the hypothesis of the upregulation of LepR gene expression as the mechanism for the protection to diet-induced of the p21-deficient mice. A further data supporting this hypothesis came again form the transcriptomic data. A gene set enrichment analysis (GSEA) evidenced that p21 deficiency causes an upregulation in the expression of genes

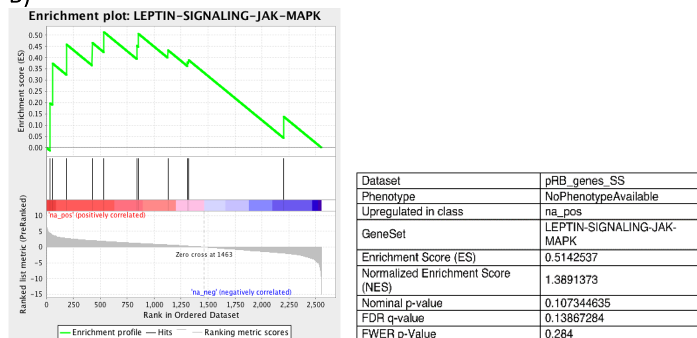
related to leptin signalling (Fig. 19). Another interesting observation emerged when we performed a GSEA (together with the statics facility in IRB) on a list of genes from a work analysing the transcriptional regulatory program in WT and pRB deficient MEFs during adipocyte differentiation [Hakim-Weber *et al.*, 2002]. As shown in Fig. 53, both mitochondrial biogenesis and leptin signalling pathways were upregulated in the AT-specific pRB KO background.

A)



Dataset	pRB_genes_SS
Phenotype	NoPhenotypeAvailable
Upregulated in class	na_pos
GeneSet	MITOCONDRIAL-BIOGENESIS
Enrichment Score (ES)	0.6193087
Normalized Enrichment Score (NES)	2.1311011
Nominal p-value	0.0
FDR q-value	0.0
FWER p-Value	0.0

B)



Dataset	pRB_genes_SS
Phenotype	NoPhenotypeAvailable
Upregulated in class	na_pos
GeneSet	LEPTIN-SIGNALING-JAK-MAPK
Enrichment Score (ES)	0.5142537
Normalized Enrichment Score (NES)	1.3891373
Nominal p-value	0.107344635
FDR q-value	0.13867284
FWER p-Value	0.284

Figure 53. pRB deficiency causes an upregulation in genes related to mitochondrial biogenesis and leptin signalling. GSEA analysis screening data related to genes from the transcriptomical analysis of WT and pRB deficient MEFs during adipocyte differentiation [Hakim-Weber *et al.*, 2002]. Profiles of (A) mitochondrial biogenesis and (B) leptin signalling pathways.

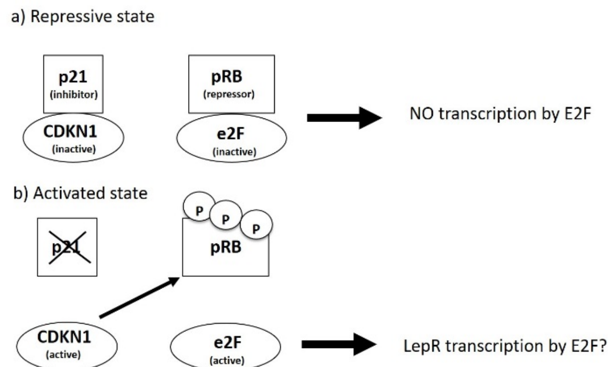
We confirmed the transcriptomic data related to LepR expression. Indeed the p21 deficiency (compared to WT littermates) provokes an upregulation of LepR gene expression and protein levels in the e-WAT of both SD and HFD-fed mice (Fig. 20 and 21). As already shown in the results chapter, it was interesting to observe the

expression of various LepR isoform, with a stronger expression of the isoform b (related to total LepR levels) in p21 KO vs WT mice in HFD. The importance of LepR isoforms is relevant in both humans and mice, for which long (LepRb) and short (LepR a, c, d) isoforms are generated by alternative splicing. Most of leptin's effects are believed to be mediated by the b isoform. However, the role of the other short isoforms and existence of heteromers are yet scarcely understood. A very interesting work found LEPRa/b and LEPRb/c heteromers located at the plasma membrane and stabilized by leptin [Bacart et *al.*, 2010]. Thus, considering the co-expression of a and b isoforms, in various tissues LEPRa/b heteromers may represent a major receptor species in the e-WAT. The function of the other LepR is yet to be unravelled but we can speculate that some isoform may help in the enhancement of Leptin-LepR bond; or they may exert their function far from the plasma membrane as soluble receptors in the blood stream; or work as scaffold proteins in the intracellular lumen to stabilize/amplify the signalling. Moreover, LepR protein levels were down-regulated in HDF only in WT mice. At the end of this other round of experiments we were able to conclude that p21 deficiency induced LepR gene upregulation in the AT independently of the diet that may enhance leptin paracrine action.

In addition to p21 we also checked if p53 deficiency could recapitulate the same phenotype as p21 deficit. It has become clear in the last years how p53 is also involved in the regulation of metabolism, reinforcing its role as key regulator of most cellular processes. The first historical identified target of p53 was p21. For these reasons we wanted to check if p53 deficiency could recapitulate the phenotype of p21 KO mice and indeed we detected an increase in LepR protein levels compared to WT mice in SD conditions (Fig.22). So these results support for the existence of a p53-p21-LepR pathway.

Once established the correlation between p21 and LepR we aimed to find the molecular link between LepR expression and the p53/p21 pathway. p21 can modulate gene transcription through the regulation of pRB, which in turn negatively regulates the activity of the E2F transcriptional regulator. For E2F it has already been proposed a role in metabolic control independent of its function in

cell cycle regulation (ref). In this regard, E2F would have a positive/negative role preventing up regulation of certain genes and we propose that the LepR gene would be one of them. The scheme below represents a simplified model for a possible LepR expression modulation by p21, pRB and E2F, linking cell cycle regulation and metabolism.



So we looked for the potential involvement of E2F in the regulation of LepR gene expression by p21, as summarized in the scheme. An *in silico* analysis of the LepR gene identified several putative E2F binding sites located in the first intron of the LepR gene, suggesting the existence of regulation sites for LepR by E2F. However, due to unsuccessful transcription reporter assays, we were not able to confirm or disprove a transcriptional regulation on LepR by E2F. We also analysed the phenotype of E2F1 KO mice, but at mRNA and protein level we did not observed any increase in LepR in p21 KO vs WT. Nonetheless, we cannot discard the involvement of E2F1 in p21-dependent regulation of the LepR gene transcription because of functional redundancy with other members of the E2F family. . In the attempt to reconstitute our model in a simpler system we initiated studies in the like 3T3-L1 APs. Unfortunately the knockdown of p21 expression in this system did not affect LepR protein levels in proliferating or differentiating cells. It is also probable that in order to see any changes cells have to be fully differentiated and we cannot exclude a residual p21 activity or the fact the cellular system is too simple compared to whole organism regulation, especially if LepR regulation is not directly controlled. Even though the mechanisms of LepR modulation by p21 remain unclear and yet to be defined, it is clear that the deficit of p21 provokes an

amelioration of the obese phenotype for mice under a diet rich in lipids and carbohydrates. Again, our hypothesis speculated that the upregulation of LepR in the e-WAT is responsible for an enhanced response to leptin and, as a consequence, for a better leptin signalling, and at least in part this may have beneficial effect at a systemic level. The adipokine leptin in the CNS as an anorexigenic molecule and thus inhibits food intake, whereas at a peripheral level it exerts a paracrine action by promoting mitochondrial biogenesis and increasing energy expenditure or by the inhibition of the cannabinoid tone. In the analysis of p21 KO mice's phenotype we found no significant differences in food intake in WT versus p21 KO mice suggesting that the relevant action of leptin (in relation to this phenotype) was not exerted in the CNS but in peripheral tissues such as the AT. We reasoned that increased expression of LepR will lead to an enhancement of sensitivity to leptin. To demonstrate this hypothesis we analysed activation of leptin signalling in AT and primary adipocytes from WT and p21-deficient mice and ob/ob mice versus ob/ob p21-/- mice, respectively, using as proxy the phosphorylation status of STAT3. Regarding the *in vivo* study, p21-deficient female mice showed an increased STAT3 phosphorylation in response to leptin treatment. Accordingly, primary adipocytes from double ob/ob p21-deficient mice showed a stronger response to leptin when compared to those isolated from ob/ob mice. Increased leptin sensitivity by genetic inhibition of the p53-p21 pathway was also evidenced by the decrease in body weight produced by a suboptimal dose of leptin in both p21- and p53-deficinet mice compared with WT animals Finally, an indirect evidence that p21-deficient mice were indeed more sensitive to leptin might come from the ITT in which ob/ob p21-deficient mice were more sensitive to insulin than the ob/ob counterpart despite having developed a similar grade of obesity and glucose intolerance. Insulin and leptin resistance are closely associated and though the mechanism responsible for this strong link remains to be elucidated, one explanation is that they share common intracellular steps in the signalling pathways.

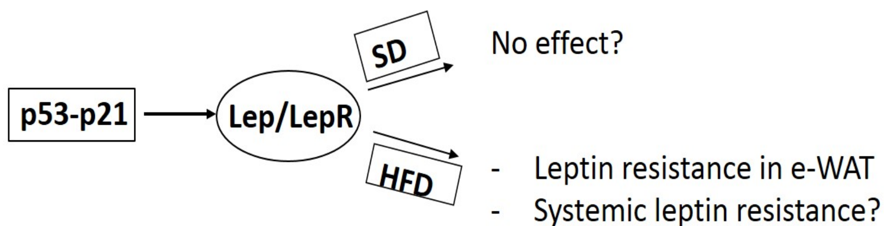
However, the stronger evidence supporting the increase in LepR gene expression as the mechanism mediating the obesity-protective action derived from the

inhibition of the p53/p21 pathway came from the study of p21 deficiency on the development of obesity in the ob/ob genetic background. Unlike the DIO model, ob/ob p21–deficient mice developed obesity to the same extend as the ob/ob counterpart. These double KO mice preserve the up regulation of the LepR gene expression as well as, LepR protein levels were higher than in the ob/ob mice (Fig. 36), nonetheless, in the absence of leptin, the mere p21 deficit did not suffice for the amelioration of the obese phenotype.. The ob/ob mice present a highly deteriorated and altered metabolism, for example we observed that mitochondrial function was compromised as well as in the double KO in ob/ob when we analysed the expression of genes related to energy expenditure. Even the regulation of endocannabinoid tone depends on a proper leptin signalling, in fact CB1 is not downregulated in the double KO mice compared to the ob/ob. In summary, regulation of expression of the other two genes we found altered in our analysis of the p21-deficient mice, PGC1a and CB1 lays downstream of the leptin/LepR signalling pathway.

The table below was realised according to data from the literature and our results about Lep and LepR plasma levels and expression in the e-WAT, highlighting the advantage that p21 deficiency may have in individuals subjected to a HFD-induced obesity.

		WT	p21 KO
SD	Lep	⬌	⬌
SD	LepR	⬌	⬆
HFD	Lep	⬆	⬆
HFD	LepR	⬇	⬆

From our data it is not yet clear if adipocytes are able to respond to leptin treatment in an autocrine fashion to signal or potentiate their response, or to improve leptin sensitivity in the peripheral tissue and systemically. In this context, p21 in first place may have a different role at a peripheral vs central level, and exert a different cytoplasmic vs nuclear functions. Apart for cell cycle regulation, it may be important for proper lipid storage in e-WAT against the accumulation of ectopic fat (typical of an obese phenotype), thus regulating lipid metabolism via Lep-LepR. To sustain the specificity of this peripheral model and better understand if its action is extended to the whole organism we would need to analyse an AT-specific p21 KO model, nonetheless data from the literature indicating that AT-specific p53 KO suffices to protect from adiposity strongly suggest that the p53/p21 pathway exerts its regulation of obesity by its actions in the AT. . In conclusion, we propose a model (schematized below) in which the p53-p21 axis interacts with leptin signalling in the e-WAT, leading probably to the onset of peripheral and systemic leptin resistance (among other anomalies) in condition of HFD.



Part II: MKK7

MKK7D transgenic mouse constitutes a useful model to study JNK activation in pancreatic β-cells and glucose homeostasis. Previous data from Dr. Jordi Lanuza-Masdeu in our laboratory showed that the activation of JNK (by MKK7D) in pancreatic β cells disrupts glucose homeostasis and impairs insulin signaling but, surprisingly, does not result in systemic insulin resistance induced by HFD. Furthermore MKK7D mice develop HFD-induced obesity to an almost similar degree that Control animals however, are fully preserved by the onset of fasted hyperglycemia and hyperinsulinemia (Fig. 44). This may be due to the existence of several protective or compensating mechanisms in other tissues. In the e-WAT we observed that JNK was phosphorylated/activated, and eIF2α phosphorylation TNFα levels were increased in response to HFD both in control and MKK7D mice, indicating that two distinctive features of the obesity supposed to be the link to insulin resistance, increased inflammatory and ER stress responses occur to the same extend in both groups of mice. This situation in the AT suggest the existence of a local insulin resistance. However, in contrast to Control animals, these features are not sufficient to develop systemic insulin resistance in MKK7D mice in which glucose-induced insulin secretion is impaired. While further studies are required, the observation that skeletal muscle preserve the expression level of IRS-1, and IRS-2, even in HFD conditions explains why MKK7D mice retain insulin sensitivity in obese conditions as this tissue is known to be the responsible for glucose uptake in response to insulin.. In conclusion, the outcome of these results is quite interesting because it permits to dissociate two strongly linked events like obesity/inflammation and insulin resistance, and even more specifically local vs systemic insulin resistance. Finally, these results also support the idea that hyperinsulinemia is required to develop insulin resistance, adding light to the causal and effect debate on this highly associated pair of conditions.

CONCLUSIONS

Part I

1.- p21 expression is upregulated specifically in the AT tissue of different mice model for obesity.

2.- p21 deficiency has beneficial effects in mice subjected to HFD in terms of resistance to diet-induced body weight gain and alteration of glucose homeostasis.

3.- The absence of p21 or p53 provokes a significant increase of LepR gene expression which leads to increased sensitivity to leptin.

4.- Leptin/LepR signalling mediates the obesity-protective action of the inhibition of the p53/p21 pathway..

Part II

1.- Diet-induced inflammatory and ER stress responses in AT trigger local insulin resistance but do not suffice to induce systemic insulin resistance

2.- Insulin secretion in response to glycemia is required for diet-induced systemic insulin resistance.

3.- Our data supports that chronic exposure to increased plasma insulin levels secreted by pancreatic β cells in response to frequent food intake is required for obesity-induced systemic insulin resistance.

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LIST OF ABBREVIATIONS

AP-1	Activator Protein-1
APs	Pre-adipocytes or adipocyte precursors
AT	Adipose tissue
BAT	Brown adipose tissue
BMI	Body mass index
C/EBPs	CCAAT/enhancer binding proteins
cAMP	Cyclic adenosine monophosphate
CDK4	Cyclin-dependent kinase 4 or cell division protein kinase 4
CRD	Cytokine receptor domain
DIO	Diet induced obesity
DMEM	Dulbecco's Modified Eagle Medium
DXM	Dexamethasone
e-WAT	Epididymal WAT
E2F	Elongation factor
EBs	Embryoid bodies
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FFAs	Free fatty acids
FOXO1	Forkhead box O1
G6Pase	Glucose 6-phosphatase
HDAC	Histone deacetylase
HFD	High Fat Diet
hMADS	Human multipotent adipose-derived stem
HPA	Hypothalamic-pituitary-adrenal axis
IBMX	3-isobutyl-1-methylxanthine
Ig	Immunoglobulin domain
Igfpb2	Insulin-like groWTh factor-binding protein 2
IKK	IκB kinase
IFN-γ	Interferon γ
IL	Interleukin
IR	Insulin Receptor
IRS	Insulin receptor substrate
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KO	Knock Out
Lep	Leptin
LepR	Leptin receptor
Lipo2000®	Lipofectamine 2000.
MAPK	Mitogen-activated protein kinase
MEKK	MEK kinase
MKK	MAPK kinases
MEFs	Mice Embryonic Fibroblasts
mESCs	Mouse embryonic stem cells
MSCs	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin

NC	Neural crest
NCD	Noncommunicable diseases
p21/WAF1/CIP	Cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1
p53	Protein 53 or tumor protein 53
PGC-1	Peroxisome proliferator-activated receptor coactivator 1
PI3K	Phosphatidylinositol 3-kinases
PPARs	Peroxisome proliferator-activated receptors
pRb	Retinoblastoma protein
ROS	Reactive oxygen species
S6K	Ribosomal s6 kinase
SAPK	Stress Activated Protein Kinase
SC	Sub cutaneous WAT
SD	Standard diet
SREBPc1	Sterol regulatory element binding transcription factor 1
STAT3	Signal transducer and activator of transcription 3
STZ	Streptozotocin
SV	Simian virus
SVF	Stromal vascular fraction cells
TNF-α	Tumour necrosis factor alpha
TZD	Thiazolidinediones
UCP1	Uncoupling protein 1
uDM	Uncontrolled, insulin-deficient diabetes
UPR	Unfolded protein response
WAT	White adipose tissue
WHO	World Health Organization