Obesity, whose incidence has increased dramatically in recent decades, is a major risk factor for other chronic diseases such as insulin resistance and type 2 diabetes. Because of the serious social problem posed, it is necessary to increase our knowledge of these diseases and to develop new and more effective therapeutic approaches. To design new effective therapies, in addition to the identification of new genes involved in the pathogenesis of this disease, a better knowledge of the molecular regulation of diabetogenic genes is necessary. Gene regulation at transcriptional level has been described to be directly correlated, among other factors, with alterations in chromatin structure by covalent modifications of NH2-terminal end of histones, mainly through acetylation. This MIRG Project has allowed us to start and set up our research, focused on the study of the pathophysiological causes of obesity, insulin resistance and type 2 diabetes. Specifically, we are centered in determining how epigenetic alterations (especially changes in chromatin structure) in glucose metabolism key tissues (such as liver and/or adipose tissue) can lead to the development of these diseases. To do that, we have developed two approaches:

1. Identification of genes regulated by the histone deacetylase (HDAC) SIRT1 in the pathogenesis of obesity, insulin resistance and type 2 diabetes:

In this first part, which constitutes the main objective of the project, we proceeded to study the effect of chromatin deacetylation by members of the family of Sirtuins (mainly SIRT1) in the pathogenesis of obesity and insulin resistance. As we already described in the previous report of this project, we have also developed the ChIPSeq technology (chromatin immunoprecipitation (ChIP) associated with massive sequencing (Seq)) in liver samples from animals that have developed an obese phenotype and insulin resistance. Specifically, we carried out ChIP experiments by using specific antibodies against SIRT1 in liver lysates from SIRT1 knockout mice and control mice and transgenic overexpressing SIRT1 which were provided with a high lipid content diet (HFD) as a model of obesity and type 2 diabetes. With the resulting immunoprecipitated DNA fragments, we performed massive sequencing and alignment with the entire mouse genome. This allowed us to identify hotspots that will indicate specific SIRT1 reaction with endogenous genes, suggesting potential target genes for this histone deacetylase.

Due to its intrinsic HDAC activity, SIRT1 is involved in promoting the formation of heterochromatin and, therefore, increasing the presence of changes in repression/gene silencing chromatin markers in its target genes (such as histone 3 trimethylation in lysines 9 and 27 (H3K9me3 and H3K27me3, respectively)). In contrast, in active gene regions we will observe a decrease in SIRT1 and increased euchromatin/gene activation markers (such as histone 3 trimethylation in lysine 4 (H3K4me3) and, in particular, SIRT1 has been found to specifically deacetylate the histone 4 lysine 16 residue when it is acetylated (H4K16Ac)). Thus, during this project, we conducted ChIPSeq experiments using antibodies against chromatin modifications, allowing us to confirm the specificity of candidate target genes obtained after the SIRT1ChIPSeq. Therefore, in addition to the ChIPSeq of SIRT1, we also have performed ChIPSeq experiments with antibodies against H3K9me3, H3K27me3, H3K4me3 and H4K16Ac in liver samples from SIRT1 knockout mice and control mice and transgenic overexpressing SIRT1, which were provided with a HFD as a model of obesity and type 2 diabetes. Those genes showing a high signal SIRT1, enrichment in H3K9me3 and H3K27me3 signal and a marked decrease in H4K16Ac and H3K4me3, are the ones we focused to validate biologically. All these results are allowing us to determine the effect of chromatin remodeling mediated by SIRT1 in the development of obesity, insulin resistance and type 2 diabetes. Thus, SIRT1 transgenic mice fed a HFD showed alterations in genes involved in regulation of oxidative stress, drug metabolism, arachidonic acid metabolism, immune response and PPAR signaling pathway, among others. By contrast, control animals in a HFD showed marked alterations in genes regulating biosynthetic process of macromolecules, cell motility and transcription factor activity. Therefore, the (dys) regulation in the control of these metabolic pathways may be involved in the onset and development of obesity and type 2 diabetes.

To complement the results of the ChIPSeq of SIRT1, we have also carried out a transcriptomic approach. We have determined the expression of the 7 sirtuins members in the liver of mouse models of obesity, such as HFD mice and *ob/ob*. Our results indicated that amongst the Sirtuin family, the hepatic expression of SIRT1 was markedly decreased as a result of obesity and insulin resistance. In addition, we also performed a gene expression microarray study in liver samples from animals detailed in the previous paragraph. These results allowed us to complement the previous ChIPSeq experiment (target genes obtained in the ChIPSeq should match the differentially expressed genes on the microarray). Thus, we have observed a clear correlation between the two technologies (Figure 1).

Moreover, thanks to our collaboration with the group of Dr. Abián (CSIC, UAB), we have begun to perform a proteomic approach to identify proteins associated with SIRT1. Specifically, we are using liver samples from the above mice. After homogenization, we have conducted an immunoprecipitation against SIRT1, allowing us to precipitate the target proteins likely associated with SIRT1. Subsequently, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed to the protein targets for identifying possible candidates. Once identified the potential candidates, we will proceed to validate them by co-immunoprecipitation, chromatin immunoprecipitation and Western blot techniques in these samples. These studies will be mostly prolonged after the finalization of this grant with the funding of other Agencies.

The identification and characterization of target genes that are upstream/downstream in the regulatory pathways and target protein(s) associated with SIRT1 will provide more precise information on how we could exploit these pathways therapeutically to treat these diseases. Because of the novelty of these experiments and results, we believe that we can publish at least one manuscript in a journal of high impact. At the moment, we have presented our results in the prestigious Chromatin and Structure Function Meeting and we are already proceeding to the writing of a manuscript with the ChIPSeq results described above.

2. - To study the role of adipogenesis in the pathogenesis of insulin resistance and type 2 diabetes:

Obesity is a complex metabolic disorder in which dysfunction of adipocyte and adipose tissue has been described as one of the main causes of its etiology. Therefore, understanding the mechanisms regulating adipogenesis is of crucial interest to develop better future therapies for obesity. Epigenetic mechanisms (such as remodeling of chromatin structure) are involved in adipogenesis. Members of the "architectural" family of proteins High Mobility Group A1 (HMGA1) regulate the function and chromatin structure. During this MIRG project we have generated and determined the effects of the overexpression of HMGA1 in adipose tissue of transgenic mice:

A. - Throughout the life of transgenic animals. We have performed histological, gene expression and metabolic analysis in control and transgenic animals from fetuses to 14 months. In all time points we have observed the same phenotype: lower fat mass, reduction of metabolites related to lipid metabolism, no changes in glucose metabolism, and alterations in the expression of genes related to mitochondrial energy metabolism in brown adipose tissue (BAT) without major changes observed in the expression of genes involved in metabolism of white adipose tissue (WAT).

B. - In response to exposure to low temperatures: Because these animals showed a marked alteration in the BAT, we have conducted similar determinations as before in transgenic and control animals subjected to a temperature of 4-6 ° C for 8 to 72 hours. As in the previous section, in these transgenic animals we observed a reduction in fat mass and a reduction in the expression of genes involved in thermogenesis and BAT metabolism (PPARγ, UCP1, etc.). At this point it is noteworthy that we are counting on the collaboration of Dr. Francesc Villarroya (in the Department of Biochemistry, UB), which has extensive experience in the metabolic and functional analysis of BAT.

C. - In terms of obesity and insulin resistance: In particular, we are studying the effect of HMGA1 overexpression during long-term exposure (4 months) to a HFD as a model of obesity and insulin resistance. Preliminary results indicate that overexpression of HMGA1 in adipose tissue of transgenic animals leads to increased protection against obesity and insulin resistance produced by the HFD. Thus, transgenic animals showed less weight gain than control mice, which was associated with decreased fat mass and reduced WAT adipocyte size. Moreover, these transgenic animals showed higher sensitivity to glucose and insulin together with an improvement in serum parameters and hormones related to glucose and lipid metabolism. However, transgenic animals show hepatic steatosis, probably due to a shortfall in adipogenesis, which might lead to decreased ability of accumulating lipids in WAT and/or burning lipids in BAT. To analyze the potential changes at the transcriptional level in BAT and WAT of these transgenic animals, we performed gene expression analysis in 384-well plate in the adipose tissues of these animals. In addition, we are currently analyzing gene expression microarrays to further identify HMGA1 target genes in WAT and BAT. So far, we have observed large differences at the expression of genes involved in, among others, adipogenesis, inflammation, cytokines and apoptosis in both standard and HFD diet in BAT. It is important to note that in the context of obesity and type 2 diabetes, the role of HMGA1 in adipose tissue remains to be elucidated.

We are currently deepening into the study of the mechanisms through which HMGA1 exerts its action. 1) We are performing plasmid co-transfections of HMGA1 together with promoters of genes involved in adipogenesis (Pref-1, PPARγ, UCP1, etc.) in BAT-like cells HIB-1b. 2) As described before, we are also conducting a proteomic approach to identify proteins associated with HMGA1. In this case, we will use WAT and BAT samples of transgenic animals. The results of this study may help to a better understanding of the mechanisms for adipogenesis gene regulation both at transcriptional and at epigenetic level. At the moment, we have presented our results in several National and International Meetings and we have already sent a manuscript to PLoS One journal with the results described above.

Therefore, taken all the results of this MIRG Project, from the standpoint of public health, the identification of potential SIRT1 and HMGA1 target genes will be very useful for future understanding and treatment of these diseases, which could lead to possible new treatments for obesity, resistance to insulin and type 2 diabetes. In addition, together with the Patents' Office of our University, we are studying carefully whether the results obtained in this Project may lead to patents that could be exploited commercially.

Finally, we would stress out that due to the results obtained in this MIRG project, we have been awarded with funds from a very competitive call from the Spanish Government (SAF2011-23649). The results in terms of present and future publications and dissemination are detailed in the relevant sections of this report.

Figure 1

ANALYSIS OF SIRT1-MEDIATED HISTONE MODIFICATIONS IN THE LIVER OF DIET-INDUCED OBESITY MICE

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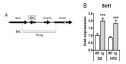
Abstract

Obesity, whose incidence has increased dramatically in recent decades, is a major risk factor for other chronic diseases such as insulin resistance and type 2 diabetes. Because of the serious social problem posed, it is necessary to increase our knowledge of these diseases and to develop new and more effective therapeutic approaches. In addition to the identification of new genes involved in the pathogenesis of obesity, a better knowledge of the molecular regulation of obesogenic genes is necessary. Gene regulation at transcriptional level has been described to be correlated with epigenetic modifications (i.e. DNA methylation, histone modifications, etc.). However, epigenetic changes associated with obesity are still poorly understood. It has been suggested that histone deacetylation might play an important role in the pathogenesis of obesity. Sirtuin 1 (SIRT1), a histone deacetylase class III, links chromatin epigenetic changes and transcriptional regulation with energy metabolism. It has been reported that hepatic overexpression of SIRT1 partially protects against the metabolic consequences of chronic exposure to a high-fat diet, via protein-protein interaction. Therefore, understanding the mechanisms regulated by SIRT1 is of crucial interest to develop better future therapies for obesity. Nevertheless, the genetic network/s that SIRT1 regulates at the level of histone deacetylation in metabolic tissues are unknown. In this work, we have performed genome-wide binding of SIRT1 and several histone modifications by using chromatin immunoprecipitation combined with sequencing (ChIP-Seq), together with gene expression microarrays. These analysis were carried out in liver homogenates from standard and high fat diet (HFD) fed wild type, SIRT1 transgenic and SIRT1 knock-out mice. Preliminary results indicate a direct correlation between gene expression levels and SIRT1 genome-wide binding in the liver of mice fed a high-fat diet. Moreover, in the liver of HFD fed SIRT1 transgenic mice we have identified specific histone H4 lysine 16 acetylation (H4K16Ac) binding in genes involved in oxidation reduction, drug metabolism and PPAR signalling pathway. In contrast, enrichment of H4K16Ac in genes involved in transcription factor activity and RNA metabolic processing has been observed in HFD wild type. The results of this study may contribute to a better understanding of the mechanism of SIRT1 regulation both at a transcriptional and at an epigenetic level, which could lead us to the development of new therapies for obesity related diseases.

1) Experimental design:

1) Mice:

- **Livers from KO, WT and Tg mice overexpressing Sirt1 ubiquously in STD and in a HFD**, as a model of obesity, IR and T2Db (**Refs.**: Sirt1 protects against high-fat diet-induced metabolic damage: Pfluger et al; PNAS (2008) and Sirt1 improves healthy ageing and protects from metabolic syndrome-associated cancer (Herranz et al.: Nat. Commun. (2010).



2) Techniques:

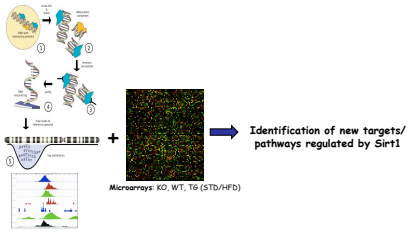


Figure 1. Experimental design. We have performed microarray analysis and ChIPSeq techniques in liver extracts of KO, WT STD, TG STD, WT HFD and TG HFD mice. For the RNA microarray experiments, the Gene Chip Affimetrix platform® was used, following manufacturer's protocol. ChIP was carried out by using Diagenode's High Cell Number ChIP kit and all protocols for Solexa/ Illumina ChIP-Seq analysis (sample preparation and sequencing) were carried out following the manufacturer's protocol.

2) Expression microarrays from liver:

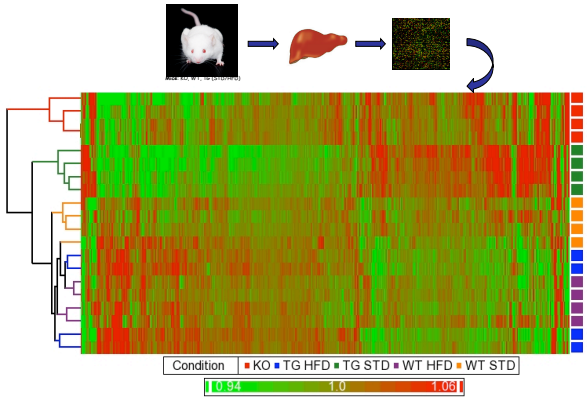


Figure 2. Heat map representation of differential gene expression pattern by the genotype and the HFD. Total RNA was extracted from the liver of KO, WTSTD, TGSTD, WTHFD and TGHFD mice (4 mice in ach group), column purified, converted to cRNA and subjected to a Gene Chip Affymetrix microarray. Gene sequences with $P \leq 0.05$ are shown (green: down-regulated genes and in red: up-regulated genes).

	Kegg Scheme	# Genes	p value	Fold enrichment
A	Spliceosome	36	3.4E-4	1,8
	Insulin signaling pathway	38	8,9E-4	1,7
	PPAR signaling pathway	21	1,0E-2	1,8
	Lysosome	30	1,2E-2	1,6
	Fructose and mannose metabolism	12	3,5E-2	1,9
	mTOR signaling pathway	15	4,2E-2	1,7
	Pancreatic cancer	19	4,6E-2	1,6
B	Pathways in cancer	10	3,7E-2	2,2
	Acute myeloid leukemia	4	4,6E-2	4,9
C	Ribosome	31	8,3E-12	4,2
	Steroid biosynthesis	7	1,8E-3	4,9
	Lysosome	21	1,8E-3	2,1
	Fatty acid elongation in mitochondria	5	2,6E-3	7,5
	Retinol metabolism	14	3,6E-3	2,5
	Citrate cycle	8	1,2E-2	3,1
	Pancreatic cancer	12	3,6E-2	2,0
	Drug metabolism-p450	12	4,6E-2	1,9

Figure 3. Enrichment of KEGG pathways in genes changed in different experimental conditions. List of KEGG pathways significantly enriched ($p < 0.05$) in (A) KO vs. WT STD; (B) WTHFD vs. TG HFD and (C) WT STD vs. WT HFD. The inset figure (on the *right*) corresponds to the heat map representation of the differential gene expression pattern observed with the 38 genes modified in the insulin signaling pathway.

3) Analysis of ChIPSeq histone modifications in liver:

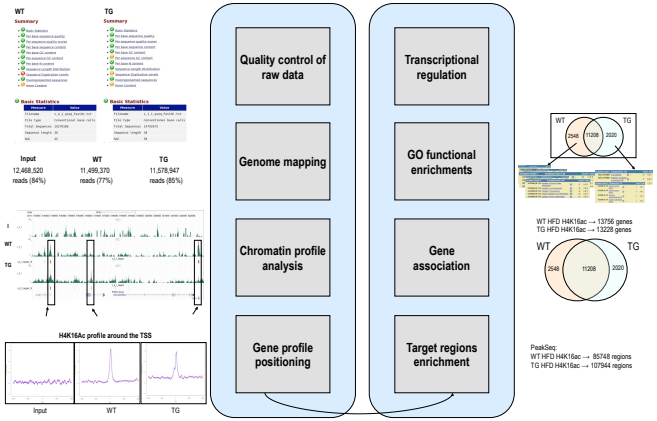


Figure 4. ChIPSeq experimental pipeline followed in liver extracts from KO, WTSTD, TGSTD, WTHFD and TGHFD mice. We have performed ChIPSeq experiments by using H4K16Ac (a direct target of SIRT1) and H3K4me3 antibodies. Schematic results shown from H4K16Ac ChIPSeq experiment using liver extracts from WTHFD, TGHFD and the input sample.

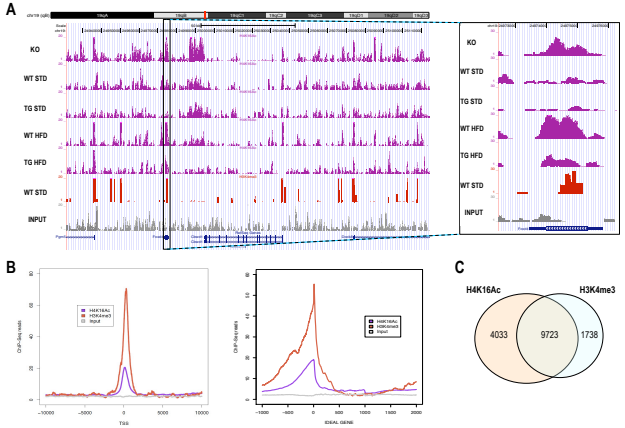


Figure 5. Landscape of histone modifications profiles in liver extracts of the different experimental conditions. (A and inset) UCSC Genome Browser overview of the ChIP-Seq reads across a region of chromosome 19: from the top, H4K16Ac (lilac) of KO, WTSTD, TGSTD, WTHFD and TGHFD; H3K4me3 (red) of WTSTD; the input (grey) sample and RefSeq genes (blue). The height of the peaks represents the number of reads obtained for each mark in each region by ChIP-Seq. (B) Projection of H4K16Ac and H3K4me3 over the TSS and the idealized gene. (C) Venn diagrams showing the intersection H4K16Ac.

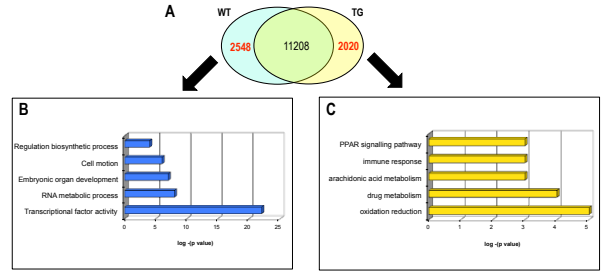


Figure 6. H4K16Ac direct targets in liver extracts of WT HFD and TG HFD. (A) Venn diagrams showing the intersection between target genes identified in the liver of WT HFD and TG HFD. (B and C) Gene Ontology (GO) term enrichment of H4K16Ac target genes (2548) uniquely identified in WT HFD (B) and in C the ones uniquely identified in TG HFD (2020).

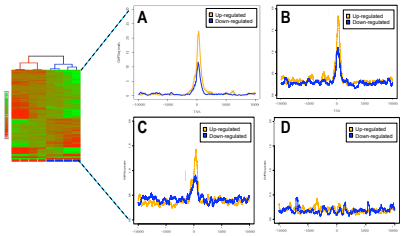


Figure 7. Correlation between gene expression levels and histone marks (H3K4me3 and H4K16Ac). Genes on the Affymetrix array (left, Figure 2) are classified as up-regulated/ expressed (orange) or down-regulated/silenced (blue). Distribution of ChIP-Seq reads of genes belonging to each expression category in the Affymetrix array across the TSS for WT H3K4me3 (A); WT H4K16Ac (B); TG H4K16Ac (C) and Input (D).

Summary

We have identified, in the liver of HFD fed SIRT1 transgenic mice, specific histone H4 lysine 16 acetylation (H4K16Ac) binding in genes involved in oxidation reduction, drug metabolism and PPAR signalling pathway, that correlated with the array data. H4K16Ac modifications showed a similar profile than the ones observed for H3K4me3, suggesting a likely role of SIRT1 in promoter activation/inactivation. However, the metabolic implications of SIRT1-mediated liver dysfunction during obesity/ T2Db at chromatin level must be further determined. The results of this study may contribute to a better understanding of the mechanism of SIRT1 regulation both at a transcriptional and at an epigenetic level.

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