Nupr1 role in adipogenesis

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This certifies that the Master's Thesis of
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ABSTRACT

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The increase of obesity rate in developed countries has been shown to be a serious problem, due to its correlation with the increase of incidence of other metabolic diseases, such as diabetes type 2, and cardiovascular diseases. Recently, adipogenesis is a topic that became widely researched due to its correlation with metabolic diseases, such as Obesity. Thus, studying the biological networks controlling adipogenesis may facilitate greater understanding of the pathophysiology of obesity.

Adipogenesis is characterized by early and late adipogenesis. In early adipogenesis mitotic clonal expansion happens. Whereas preadipocyte cells undergo mitosis, and C/EBPβ gains DNA binding activity. C/EBPβ, consequently, activates C/EBPα and PPARγ, the main drivers of adipogenesis, which stops mitotic clonal expansion, due to their antimitotic nature, and late differentiation phase starts. Consequently, during late adipogenesis, adipocytes

gain insulin sensitivity, adipocyte cells gain lipid droplets that enlarge, and lipogenic enzymes and fatty acid-binding enzymes levels increase. Finally, adipocytes become mature and capable of performing their function as an energy reservoir.

This study data shows that Nupr1 is in fact present in the white adipose tissue and changes its expression levels during adipogenesis. Nupr1 is a ubiquitous nuclear and cytoplasmic stress-activated protein, with an 8kda, known to protect cells from elevated ER stress. Nupr1's function lays in DNA repair, cell regulation, apoptosis, cell migration and invasion. In addition, Nupr1 expression is essential for mitochondria protection and normal function by controlling redox homeostasis, ferroptosis, and energy metabolism[1]. Nupr1 has been extensively studied in cancer, however, its role in metabolism and adipogenesis still has to be greatly explored.

Therefore, in this study, I induced differentiation of mouse 3T3-L1 preadipocytes into mature adipocytes and verified Nupr1 expression and activity during adipogenesis.

I demonstrate that Nupr1 is expressed on day 0 and, is expressed in increased levels in the late stages, days 3 to 8 of adipogenic differentiation. In addition, when Nupr1 is knocked down, via siRNA, in 3T3-L1 cells, adipogenesis is shown to be impaired.

This study also demonstrates that together with Nupr1 depletion, CHOP increases its levels at day 0 of early adipogenesis, which might constitute a cause for the adipogenesis impairment seen before, due to C/EBPβ activity inhibition.

In the same way, after treatment of 3T3-L1 cells with the Nupr1 inhibitor, ZZW-115, during late adipogenesis, cells showed to become impaired and, cell death was also verified. ZZW-115 treated cells showed to have mitochondria superoxide increased presence in relation to the control, and it was correlated with apoptosis, evidenced by proapoptotic BAX and PUMA increased expression in these cells. This happening was accompanied by mitochondrial fragmentation. Similar results were verified when Nupr1 was knocked down via siNupr1 2 days after induction of differentiation of 3T3-L1 cells. Moreover, this study demonstrates that with the depletion of Nupr1, ROS production increases, followed by an increase in mitochondrial fragmentation, impairment and ultimately cell death.

Taken together, my study demonstrates that Nupr1 might have a role in protecting 3T3-L1 cells from CHOP in early adipogenesis and ROS excessive production in late adipogenesis. Consequently, permitting normal occurrence of adipogenesis. Therefore, this protein might constitute a possible target for the treatment of Obesity.

Key words: White adipose tissue, adipogenesis, Nupr1, Obesity, 3T3-L1

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I. INTRODUCTION

Recently, adipogenesis is a topic that became widely researched due to its correlation with metabolic diseases such as obesity and diabetes type 2. The adipose tissue has been shown to be very important for fat/triglyceride's storage and heat production. Moreover, the adipose tissue is divided in white adipose tissue, brown adipose tissue, and beige adipose tissue[2].

The white adipose tissue is composed by white adipocyte cells, and it is mainly present in the subcutaneous and visceral regions of the body. Furthermore, the white adipose tissue is rather important for the body's energy homeostasis, whereas it storages lipids in triglyceride's form, which impedes ectopic deposition of fat that in excess is correlated with various metabolic diseases[3].

Adipocyte cells before maturation are morphologically similar to fibroblasts and when mature, they become spherical, and capable of accumulate lipid droplets. This process of development of adipocyte cells is denominated adipogenesis[4].

Adipogenesis is divided by early differentiation and late or terminal differentiations[5]. During maturation, the CEBP family of transcription factors appears to work as a cascade. Whereas on early differentiation, C/EBP-β and C/EBP-δ appear in higher levels and in late differentiation C/EBP-α, appears in high levels. Differentiation of mouse 3T3-L1 preadipocytes into mature adipocytes, is done using the hormonal cocktail: dexamethasone, Insulin and 3-isobutyl-1-methylxanthine.

Adipogenesis first step consists in the mitotic clonal expansion, in which pre adipocytes enter in the S phase and C/EBPβ and δ combine with centromeres. Consequently, after C/EBP-β and δ gain DNA binding activity by phosphorylation, they transcriptionally activate C/EBP-α and PPARγ, the main drivers of adipogenic differentiation. As soon as C/EBPα is activated, due to its anti-mitotic function, mitosis stops.

Furthermore, during terminal differentiation, adipocytes gain insulin sensitivity, cells gain lipid droplets that enlarge, and lipogenic enzymes and fatty acid binding enzymes levels increase[6]. However, when dysregulations in adipogenesis happen, adipocytes do not mature correctly. These dysregulations can happen due to various factors, as the example of stress[7] or dysfunction/ of adipogenic target gene's expression during differentiation.

This study's microarray data has shown that Nupr1 is present in the white adipose tissue and that increases its expression throughout adipogenesis. Nupr1 is a ubiquitous nuclear and cytoplasm stress-activated protein, with an 8kda. This protein was first found in high levels during acute pancreatitis[8], when in

occurrence of cellular injury. Previous studies have shown that Nupr1 binds to DNA and controls the expression of target proteins, this protein was shown to be a helix–loop–helix protein that has DNA-binding ability. Nupr1's function lays on DNA repair, cell regulation, apoptosis, cell migration and invasion. In addition, this protein has been found to protect cells from high ER stress levels[9] . Moreover, in the last few years, Nupr1 has been found to be correlated with insulin resistance and its deletion correlated with protection against high fat diet obesity[10]. Nupr1 deficiency was, also, found to decrease visceral fat deposition[11].

Furthermore, Nupr1 is a protein that has been extensively studied in cancer, and even though this protein is known to be a stress protein, its role in adipogenesis and metabolism is still left unknown. Therefore, in this study to verify if Nupr1 is present and has a role in adipogenesis, various experiments were performed.

After analyzing public data , I found that Nupr1 is present during adipogenesis, in early adipogenesis, and increases it's levels in late adipogenesis. These findings were in concordance with my data's experiments with 3T3-L1 cells.

And therefore, I performed knockdown experiments via siRNA, followed by induction of differentiation of 3T3-L1 cells and verified that with the depletion of Nupr1, adipogenesis was shown to be impaired. In correlation with these previous findings, this study demonstrates that, together with Nupr1 knockdown, CHOP shows increased levels on day 0, after induction of differentiation of 3T3-L1 cells, on the subsequent 0h and 4 hours. Moreover, supporting my data results, previous studies have correlated CHOP, a ER stress protein, elevated expression

levels with early adipogenesis impairment due to inhibition of C/EBPβ activity and therefore, impairment of early adipogenesis[12].

Additionally, Nupr1 high expression levels during late adipogenesis might point to a role during late adipogenesis. Furthermore, previous studies, performed in cancer cells, have used ZZW-115, a Nupr1 inhibitor, to block Nupr1 activity. ZZW-115, a trifluoperazine compound, binds to the THR68 of the Nupr1's nuclear localization signal and prevents the interaction of the same with importins and consequently, translocation to the nucleus, inhibiting Nupr1 activity.

In the same way, previous studies have correlated ZZW-115 treatment and Nupr1 siRNA treatment with ATP reduction related with ROS excessive production and, ultimately feroptosis[13]. Consequently, in this study, the treatment of 3T3-L1 cells with this compound, on day 4 of adipogenesis, showed to cause cell impairment and cell death. Demonstrating, that Nupr1 might protect late adipogenesis normal occurrence.

Therefore, I hypothesized that Nupr1 might have a role in protecting and permitting normal occurrence of adipogenesis, and therefore Nupr1 might constitute a possible target for the treatment of Obesity.

For these reasons, this study experiment's final intention, and the main focus is to find the role of Nupr1 during adipogenesis.

II. Materials and methods

1. Cell culture

A. 3T3-L1 cell culture and in vitro differentiation

3T3-L1 preadipocytes were cultured and differentiated into mature adipocytes via hormonal induction.

3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium (Gibco 11965-118 –ThermoFisher Scientific) supplemented with 100 µg/ml of Streptomycin, 62.5 µg/ml of Penicillin and 8 µg/ml of biotin, and with 10% of Bovine calf serum (Gibco 16170-078- ThermoFisher Scientific) (DMEM/CS/ABT). The cells were maintained at 37 °C and at 10% CO_2.

3T3-L1 cells were subcultured every Monday, at a density of $4x10^4$, and Friday, at a density of $5x10^4$ into 10cm plates. In addition, media was changed every 2 days, with DMEM/CS/ABT media.

To induce 3T3-L1 differentiation, first, 3T3-L1 cells were subcultured into 3cm, $2x10^5$/plate, or into 6cm plates at a density of $4x10^5$/plate. 3 days after cell subculture, the cells became post confluent (D2). Furthermore, the cells were supplied with Dulbecco's modified Eagle's medium, supplemented with 100 µg/ml of Streptomycin, 62.5 µg/ml of Penicillin and 8 µg/ml of biotin, and 10% of fetal bovine serum (FBS) (Gibco- 26140-079- ThermoFisher Scientific) (DMEM/FBS/ABT) together with 1uM of dexamethasone, 1 µg/ml of Insulin and 115 µg/ml of 3-isobutyl-1-methylxanthine (Sigma). The cells were incubated at 37 °C and at 10% CO2.

The cells were cultured for another 2 days with DMEM/FBS/ABT and 1 µg/ml of insulin. And consequently, the media was changed every two days.

Oil red O staining was performed on day 6, to verify if the cells accumulated lipid droplets.

2. Oil red O staining (ORO)

6 days after induction of differentiation, the 3T3-L1 cells were washed with 1X PBS. Consequently, 3.7%formaldehyde or 10% neutral buffered formalin (Biosesang) was added to the cells, to crosslink for 20 minutes. The cells were washed 3 times with distilled water (DW). Oil red O staining working solution (ORO) was made with Oil Red O stock and DW in a 6:4 ratio. Consequently, the solution was filtered with a 0.45um syringe filter. Oil red O staining solution was added to the cells and the same were incubated for 1 hour.

Consequently, the cells were washed again 3 times with DW, and were verified on the light microscope to check the presence of lipid droplets. The cells were left to dry, and the plates were scanned.

Oil red O staining stock solution was made with 0.5% Oil Red O powder (Sigma-Aldrich) in isopropanol.

3. siRNA transfection via lipofectamine 2000 and lipofectamine RNAIMAX

3T3-L1 cells were transfected with Lipofectamine™ 2000 Transfection Reagent (Invitrogen) and Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen), following the protocols provided by Invitrogen. The siRNA was purchased from Genolution. The siRNAs used were, respectively : **Genolution negative control**: Sense- CCUCGUGCCGUUCCAUCAGGUAGUU; anti-sense-CUACCUGAUGGAACGGCACGAGGUU.**Nupr1#4**:sense:GAGAAAUUAG GAGUUGUAAUU;antisense:UUACAACUCCUAAUUUCUCUU. 3T3-L1 cells were plated in 3cm plates, at a density of $2x10^5$/plate, or/and 6cm plates, at a density of $4x10^5$/plate. Consequently, after 24hours, 100nM of negative control, and 100nM of Nupr1#4 were transfected to the correspondent plates.

Prior to transfection the media was changed to DMEM supplemented with 10% of Bovine calf serum, because antibiotic presence might inhibit transfection. Consequently, after 24hours media was changed to (DMEM/CS/ABT). Finally on the next day, differentiation was induced as explained before.

In the same way, further transfection experiments, were made. 3T3-L1 cells were plated in 3cm plates, at a density of $1.5x10^5$/plate, or/and 6cm plates, at a density of $4x10^5$/plate. Consequently, differentiation was induced after 3 days, when the cells were in post-confluence state. The cells were maintained in DMEM/FBS/ABT and insulin media until 2 days after differentiation induction. Furthermore, on day 2, the cells were transfected with 250pmole of, respectively,

siRNA negative control, and of siNupr1#4. 48hours after, the media was changed to DMEM/FBS/ABT.

4. Western Blot and antibodies

To verify protein levels, 3T3-L1 cells were washed with 1x PBS and lysed with western blotting lysis buffer, containing 1% Sodium dodecyl sulfate (SDS) and 1M Tris-HCL 60nM, 6.8 pH, and with PRO-PREP™ (iNtRON Biotechnology). The lysis phase was always performed on ice.

Protein concentration was measured via BCA, using the Pierce™ BCA Protein Assay Kit, and the protein samples were made in equal quantities. Furthermore, protein samples were loaded in SDS-PAGE gels and separated in the loading phase of western blot. Consequently, they were transferred to nitrocellulose membranes in the transfer phase of western blot. The membranes were blocked for 1 hour, to prevent unspecific binding.

Immunoblotting analysis was done using the following antibodies: P8 polyclonal antibody (Invitrogen); CHOP (L63F7) mouse mAb (cell signaling technology); C/EBPα (Santa Cruz); PPARγ (Santa Cruz); GAPDH (cell signaling technology); C/EBPβ (Santa Cruz); M2 Anti-Flag monoclonal Mouse (sigma -F3165); DRP1 total (cell signaling technology); pDPR1-S616 (cell signaling technology); pDPR1-S637 (cell signaling technology); MFN2 (Santa Cruz Biotechnology); OPA1 (Santa Cruz Biotechnology); LC3B (Sigma-Aldrich); BAX (Recombinant

Anti-Bax antibody (E63) (Abcam); PUMA (Anti-PUMA antibody (ab9643) (Abcam).

5. Gene expression analysis

RNA was isolated from cells using TRIzol™ Reagent (Invitrogen™), following Invitrogen's protocol. RNA was prepared/extracted for RT/PCR or/and RT/qPCR, cDNA was synthesized from 3μg of total RNA via random hexamer primer and superscript reverse transcriptase II (Invitrogen). Real-time quantitative PCR (RT-qPCR) was performed using the Applied Biosystems QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific). And the mRNA relative expression levels were normalized to L32 levels. Moreover, the following primers were used: **CHOP**5'CTGCCTTTCACCTTGGAGAC3',5'CGTTTCCTGGGGATGAGAT A3';**C/EBPβ**5'AACTCTCTGCTTCTCCCTCTG3';**mNupr1(126)**5'TTCCCA GCAACCTCTAAACC3',5'GCAGCAGCTTCTCTCTTGGT3'**mNupr1(p8)**5' GAAGCTGCTGCCAATACCAACC3',5'TAGCTCTGCCCGTCTACCCTC3'; **RPL32(RT)**5'GCCTCTGGTGAAGCCCAAGATCG3',5'CTCTGGGTTTGG GCCAGTTTCGC3';**PPARγ2**5'CTCTGGGAGATTCTCCTGTTGA3',5'GGT GGGCCAGAATGGCATCT3';**C/EBPα** 5'TGGACAAGAACAGCAACGAG3 ',5'TCACTGGTCAACTCCAGCAC3';**aP2**5'TCTCCAGTGAAAACTTCGAT 3',5'TTACGCTGATGATCATGTTG3';**FAS**5'AAGCCGTTGGGAGTGAAA GT3',5'CAATCTGGATGGCAGTGAGG3';**GAPDH**5**'**ACCACAGTCCATGC CATCAC3',5'TCCACCACCCTGTTGCTGTA3'**.Tfam**5'CAAGTCAGCTG

ATGGGTATGG3',5'TTTCCCTGAGCCGAATCATCC3'**Tfb1m**5'AAT TTCCTCCTGGACTTGAGG3';AGAGAGCATCTGTAACCCTGG; **NRF1:**5**'**GACAAGATCATCAACCTGCCTGTAG3'5'GCTCACTTCCT CCGGTCCTTTG3'

6. Cell counting assay

3T3-L1 cells were plated into 3cm plates in a density of $1.5x10^5$, and 50nM of siRNAs (negative control, , and Nupr1 #4) were transfected after 24 hours. One day after, the cell media was changed to DMEM/CS/ABT media. The cells were trypsinized, and each plate was counted separately, using the cell counter, ADAM (NanoEnTek), on day 0, where the cells were post-confluent and on day 2 after induction of differentiation.

7. Gene expression analysis- Public data

RNA sequencing data set [14] of mouse embryonic fibroblast cells (GSE152750) were downloaded from the Gene Expression Omnibus, National Center of biotechnology information. The cells were incubated with basal growth media supplemented with 3-isobutyl-1-methylxanthine, Insulin, dexamethasone, and rosiglitazone (PPARγ agonist). The RNA sequencing data was made using HPC Cluster Dalma and were normalized using the quantile normalization method in the R language environment, RStudio.

8. ZZW- 115 treatment - 3T3-L1 cells

3T3-L1 cells differentiation was induced. After 4 days cells were treated with ZZW-115 (medchemexpress), dissolved in DMSO, at a concentration of, respectively, 5μM, 10μM. Cell media was changed after 2 days to DMEM/FBS/ABT together with the same concentration of ZZW-115 treatment.

9. Oxygen consumption rate

Seahorse XPs Analyzer (Agilent Technologies) was used to measure OCR. The cells were platted in an Agilent seahorse XPs cell culture Miniplate at a $1.3x10^4$ density. Differentiation was induced and, consequently, ZZW-115 treatment was done on day 4. Before performing OCR measurement media was changed to XF base media, with 1M glucose and 4mMGlutamine at a 7,4 pH.

OCR was measured using Oligomycin (2.6μM) for port A, FFCP (0.5μM) for port B, ROT/AA (1μM) for port C and, Hoechst (20μM) for port D.

10. MitoSOX™ Red mitochondrial superoxide indicator

3T3-L1 cells were platted at a $5x10^4$ density in SPL life Sciences 8 well (30108) plates. 3 days after, differentiation was induced. On the 4^{th} day, ZZW-115 treatment was performed. The cells were washed with warm PBS and incubated

with Mitosox (5ul of 5 μM MitoSOX™ reagent working solution), protected from light. The samples were analyzed by confocal microscopy.

11. Nuclear and cytoplasmic extraction

Nuclear and cytoplasmic extraction was performed using NERPER™ Nuclear and Cytoplasmic extraction reagents (Thermo Scientific). 3T3-L1 cells were treated with ZZW-115, and the cell's nucleus and cytoplasm were extracted following the protocol given by the manufacturer. The extracts were analyzed via western blotting.

12. Transmission electron microscopy

The samples are fixed in 2% Glutaraldehyde-2% Paraformaldehyde in 0.1M phosphate buffer(pH 7.4), 12hours. Consequently, the specimens are washed 2 hours with 0.1M phosphate buffer and, post fixed for 2 hours with 1% OsO4 in 0.1M phosphate buffer. After, the specimen is dehydrated with ethanol (ascending ethanol series) for 10 minutes/each and infiltrated with propylene oxide for 10min. Specimens are embedded with Poly/Bed 812 kit (Polysciences), and consequently polymerized in an electron microscope oven (TD-700, DOSAKA, Japan) for 12hours (at 65℃). The block, with a diamond knife in the Ultramichrome, is cut into a 200 ㎚ semi–thin section and stained with toluidine blue. After observation in the optical microscope, the region needed is cut into a 80 ㎚ section via the ultramicrotome and is double stained with 3% uranyl acetate,

30 minutes and 3% Lead citrate for a total of 7 minutes. The specimen is imaged with a transmission electron microscopy (JEM-1011, JEOL, Tokyo, Japan) at 80Kv via Megaview III CCD camera (Soft imaging system-Germany)

13. Statistical analysis

The data are represented as means ± standard error of mean (S.E.M). Data sets were analyzed for statistical significance via non-parametric Mann-Whitney tests. All p-values<0.05, are significant.

III. RESULTS

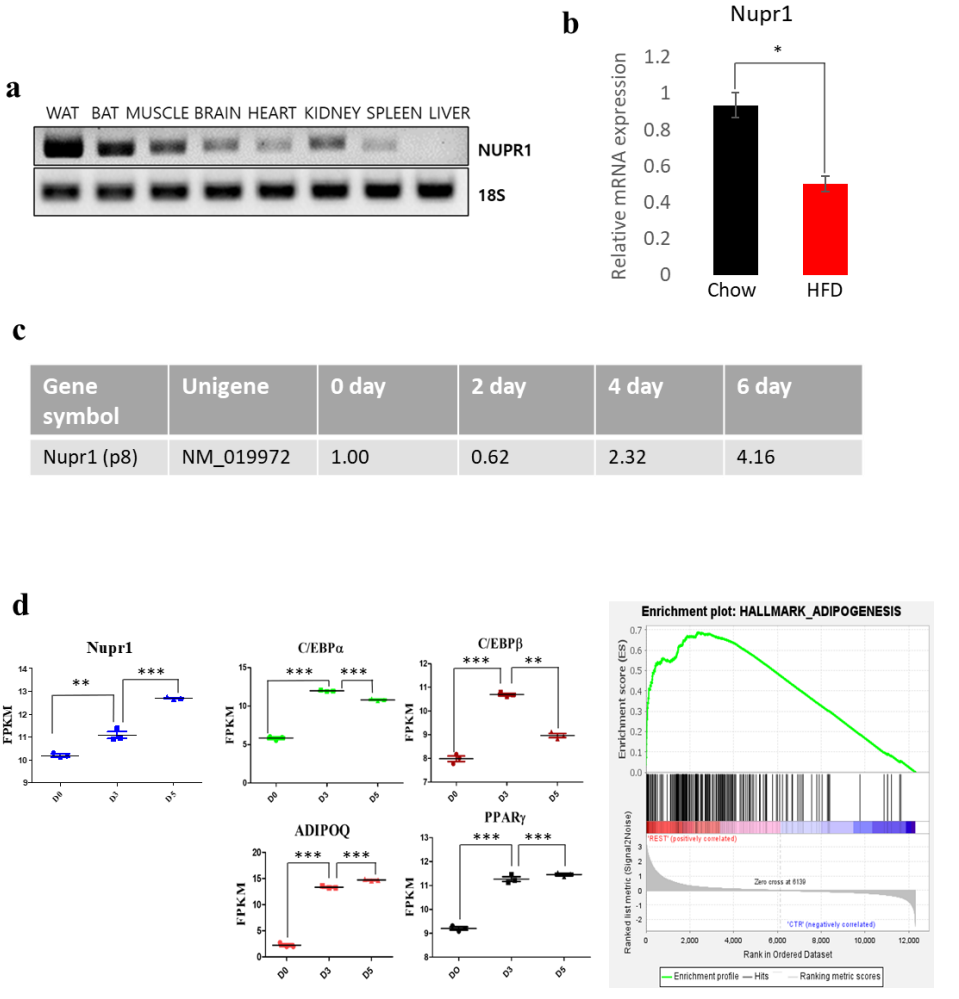
1. Nupr1 is mainly present in the white adipose tissue

To verify the expression of Nupr1 in different tissues I checked its RNA relative expression levels in various tissues. I verified that Nupr1 is highly expressed on the white adipose tissue (WAT) (Fig.1 a). The fact that Nupr1 is highly expressed in the WAT might indicate a role in adipogenesis and lipid accumulation. In addition, Nupr1 shows to be less expressed in the adipose tissue of high fat diet fed mice relative to mice that were fed with chow diet, as shown in the figure 1b.

And therefore, following the last findings, I verified microarray data of 3T3-L1 cells from our team, and I found Nupr1 to increase its RNA levels from day 0 to day 7 of adipogenesis and to be present in the adipose tissue (Fig.1 c).

Consequently, I analyzed a RNA sequencing file from Mouse Embryonic Fibroblasts (GSE152750). This data was downloaded from the Gene Expression Omnibus, National Center of Biotechnology Information[14]. To obtain this data, RNA was extracted on days 0, 3, and 5. Mouse Embryonic Fibroblast cells were incubated with basal growth media supplemented with 3-isobutyl-1-methylxanthine, Insulin, dexamethasone, and rosiglitazone (PPARγ agonist). And therefore, analysis of this data showed that while adipogenic genes increase their levels, in the same way, Nupr1 increases its levels too. This information is supported by the enrichment plot: Hallmark. Adipogenesis that shows enrichment of various genes during adipogenesis (Fig.1 d).

Together these data demonstrated that Nupr1 is present in the white adipose tissue during adipogenesis and changes its expression throughout it.



Gene symbol	Unigene	0 day	2 day	4 day	6 day
Nupr1 (p8)	NM_019972	1.00	0.62	2.32	4.16

Figure 1. Nupr1 is mainly present in the white adipose tissue (a) Expression of Nupr1 in mouse Tissues. The relative mRNA expression levels of Nupr1 and internal control (18s) were measured, by RT-PCR. Mouse tissues of the white adipose tissue (WAT), brown adipose tissue (BAT), muscle, brain, heart, kidney, spleen, and liver were analyzed. (b) qPCR analysis of Nupr1 (left) in chow diet fed mouse adipose tissue and high fat diet fed mice adipose tissue.

(n=3). Error bars represent mean ±S.E.M, * P<0.05. (c) microarray data of 3T3-L1 cells supplied by our team, Jae-woo Kim laboratory. (d) Public data, Mouse Embryonic Fibroblasts (MEFs), Adipogenic Potential: A Comprehensive Transcriptome Analysis - GSE152750. Whereas RNA was extracted on days 0,3 and 5. Incubation was made with basal growth media with 10 μg/mL insulin, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 10 μM rosiglitazone. (n=3). Error bars represent mean ±S.E.M, * P<0.05- samples compared to D0.

2. Nupr1 is expressed and increases its expression levels on days 0 and 3,4 and 8 of adipogenesis in 3T3-L1 cells.

Consequently, adding to the previous information, to check when Nupr1 is prevalent in adipogenesis, I verified the expression of Nupr1 and adipogenic target genes on 3T3-L1 adipocyte cells during adipogenesis. Nupr1 expression is apparent on day 0 and very apparent on days 4 and 8 at the end of adipogenesis (Fig 2. a and b). In the same way, I verified adipogenic target genes and Nupr1 protein levels via western blotting and I found that Nupr1 protein levels increased on days 0, 4, and 8, supporting the mRNA data. (Fig 2. C). These findings might indicate a role of Nupr1 in early and late adipogenesis.

Therefore, knockdown experiments were performed , via Nupr1 specific siRNA, to check if depletion of this protein will affect in any way adipocyte's differentiation. As a consequence, depletion of Nupr1 showed to impair adipogenesis. Oil red O staining showed, that in fact, there is less lipid droplet accumulation in the siNupr1 sample in relation to the control sample (Fig 2 d).

In summary, all together, these data demonstrate that Nupr1 is in fact present during adipogenesis and changes its levels throughout it, showing an increase of expression at the end of adipogenesis. In the same way, when Nupr1 is knocked down from 3T3-L1 cells, adipogenesis shows to become impaired. These findings evidence that Nupr1 presence and function are necessary for the normal occurrence of adipogenesis.

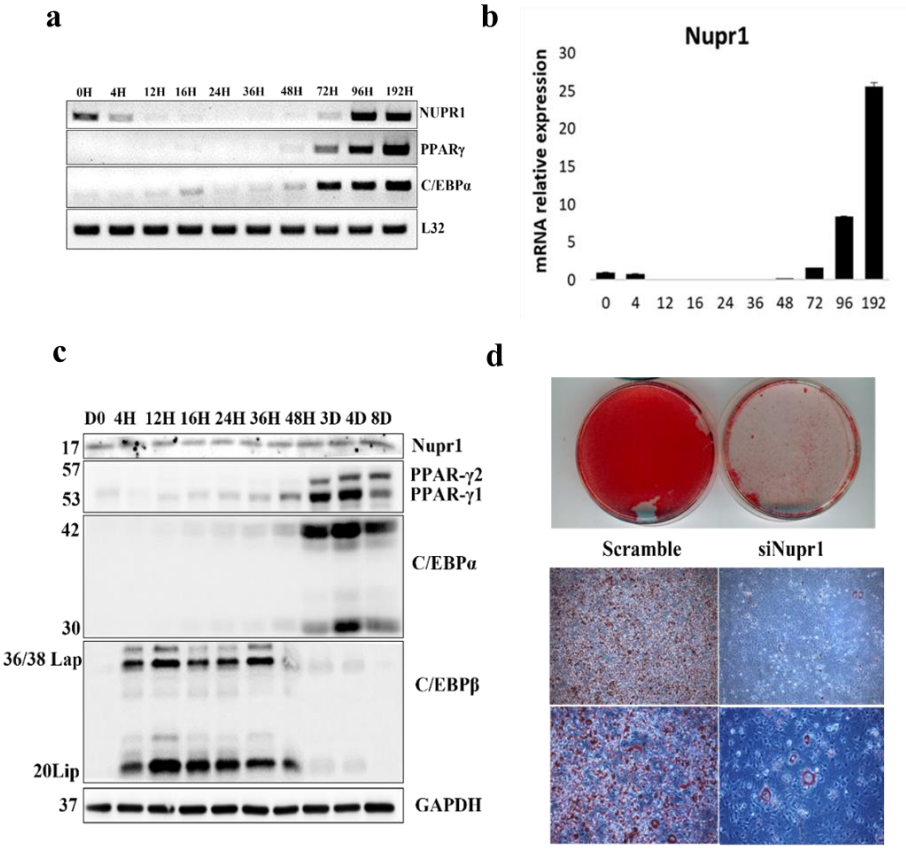


Figure 2. Nupr1 is expressed and increases its expression levels on days 0 and 3,4 and 8 of adipogenesis in 3T3-L1 cells. (a) Nupr1 expression in 3T3-L1 after differentiation's induction, 3T3-L1 cells were differentiated, and Nupr1, PPARγ, C/EBPα levels were measured at 0H, 4H, 12H, 16H, 24H, 36H, 48H, 72H, 96H and 192H using RT-PCR. L32 was used as a housekeeping gene for control (above) (b) Nupr1 levels were also measured via RT-qPCR for the same samples (left). (c) Western blotting analysis of Nupr1- PPARγ, C/EBPα, C/EBPβ of 3T3-L1 cells, after

differentiation induction, at 0H, 4H, 12H, 16H, 24H, 36H, 48H, 72H, 96H and 192H. GAPDH was used as control (bottom figure). (d) light microscope analysis of lipid accumulation in Nupr1 knocked down 3T3-L1 cells, at day 6 of adipogenic differentiation (bellow). Oil red O staining of control (left) and knocked down Nupr1 3T3-L1 cells (right), on day 6 of differentiation

3. Nupr1 absence causes impairment of early adipogenesis

Following the last findings, the analysis of the mRNA expression of adipogenic and lipogenesis target genes was done.

C/EBPα and adipocyte protein 2 levels decreased when Nupr1 was knocked down. In the same way, fatty acid synthetase (Fasn) showed to decrease its levels substantially on day 6. PPARγ, one of the main drivers of adipogenesis, showed to decrease its levels too (fig 3a). These data demonstrate that, in fact, the depletion of Nupr1 impairs adipogenesis due to the decreased expression of adipogenic target genes.

Following, CHOP expression levels were also verified, when in absence of Nupr1, during adipogenesis, in 3T3-L1 cells. CHOP showed to have increased expression on day 0 and decreased expression on day 6 of adipogenesis. And due to the fact that Nupr1 is expressed on days 0 and 6 of adipogenesis, these data might suggest that Nupr1 might protect adipogenesis from CHOP. CHOP is known to be very important in early differentiation, whereas its expression decrease, after induction of adipogenesis, is very important and necessary for the normal occurrence of adipogenesis.

Moreover, to check if early adipogenesis is impaired with the absence of Nupr1 further knockdown experiments were performed. Consequently, cell counting experiments were performed. As seen in figure 3b, the cell number for Nupr1 knocked down cells didn't change significantly from D0 to D2. Furthermore, for normal adipogenesis to happen, during the mitotic clonal expansion phase, on the first 2 days of differentiation, the cell number has to increase substantially, as

seen in the control sample. Whereas, 3T3-L1 cells reenter the cell cycle and undergo mitosis[15]. And therefore, the fact that the cell number didn't change for Nupr1 depleted cells points to a possible impairment in the mitotic clonal expansion phase in early adipogenesis.

Consequently, with the depletion of Nupr1, CHOP mRNA relative expression shows to be increased during early adipogenesis at OH and 4H relative to the control (Fig 4 c and d). These data points to dysfunctions in early adipogenesis due to high expression of CHOP.

And therefore, these findings, all together, point to an essential function of Nupr1 in protecting the normal occurrence of early adipogenesis and without it early adipogenesis shows to be inhibited.

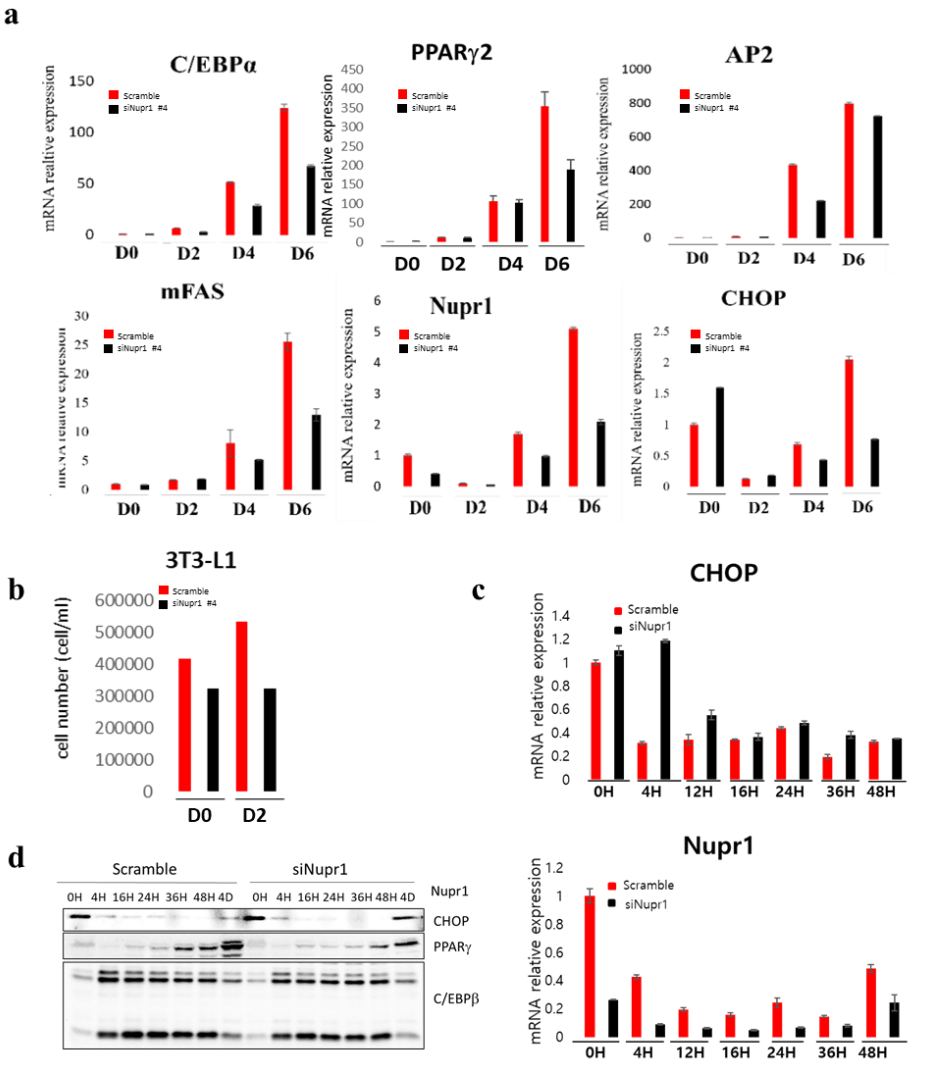


Figure 3. Nupr1 absence causes impairment of early adipogenesis (a) relative mRNA levels of C/EBPα, PPARγ, AP2, CHOP, FAS and Nupr1 of 3T3-L1 cells during adipogenesis. L32 was used as a housekeeping gene. The mRNA levels were measured on days: 0,2,4,6, after induction of differentiation. (b) 3T3-L1 cell

counting after Nupr1 Knockdown- siNupr1 and Scramble siRNA were transfected in to 3T3-L1 cells, and the cell number was counted on days 0 and 2. (c) Nupr1 knockdown, via siRNA, RT-qPCR - Nupr1 levels were measured. Harvest was done at 0H, 4H, 12H, 16H, 24H, 36H and 48H. L32 was used as a housekeeping gene. CHOP mRNA relative expression was also verified with the same samples. (d) Nupr1 knockdown, via siRNA, western blotting. Harvest was done at 0H, 4H, 16H, 24H, 36H, 48H and D4.

4. Nupr1 protects mature adipocytes from ROS production

Nupr1 is not only expressed in preadipocytes, but also at the end of adipogenesis and in mature adipocytes. Therefore, to verify the function of this protein in late adipogenesis, 3T3-L1 cells were treated with ZZW-115, 4 days after induction of differentiation.

ZZW-115, an organic synthetic compound, is known to be a Nupr1 inhibitor. Whereas it binds to Nupr1 and inhibits its translocation to the nucleus. This compound is highly used in studies with Nupr1 in cancer.

As seen in figure 4a, when 3T3-L1 cells were treated with ZZW-115, in relation to the control, their state showed to deteriorate with time. The cell's shape changed, the shape of lipid droplets changed, and lipid content decreased, ultimately, cell death was seen, as demonstrated in the light microscope pictures (bellow). In the same way, oil red o staining supports the last data findings. There was less lipid content in cells treated with 5 and 10μM of ZZW-115. This lipid content decrease rate showed to be dependent on the treatment concentration given. Therefore, these data altogether, demonstrates that the treatment of 3T3-L1 cells with ZZW-115, causes cell impairment and cell death.

To verify if Nupr1 entrance in the nucleus is inhibited by ZZW-115 in adipocyte cells, 3T3-L1 cells were treated with ZZW-115 and their nucleus and cytoplasm were, consequently extracted. Furthermore, western blotting data, fig.4b, shows that Nupr1 is not present in the nucleus in ZZW-115 treated cells relative to control, and is mainly present in the cytoplasm. These data shows that Nupr1 might have a role in the nucleus in 3T3-L1 cells.

Moreover, figure2c shows that pro apoptotic genes BAX and PUMA are in increased levels 72H after treatment with ZZW-115, demonstrating that 3T3-L1 cells undergo apoptosis when Nupr1 is inhibited (Fig 4c). In the same way, the autophagy target gene LC3b shows to be in increased levels 24H, 48H and 72H after the treatment. pDRP1 S616 shows to be in increased levels in ZZW-115 treated cells in relation to the control, demonstrating increased mitochondrial fission. In concordance, Mfn2 and opa1, fusion controlling genes, were down regulated in relation to the control, showing that with the treatment of 3T3-L1 cells with ZZW-115, and inhibition of Nupr1, the balance between fission and fusion that maintains the mitochondria integrity is disrupted causing dysfunction of the same (Fig2.d).

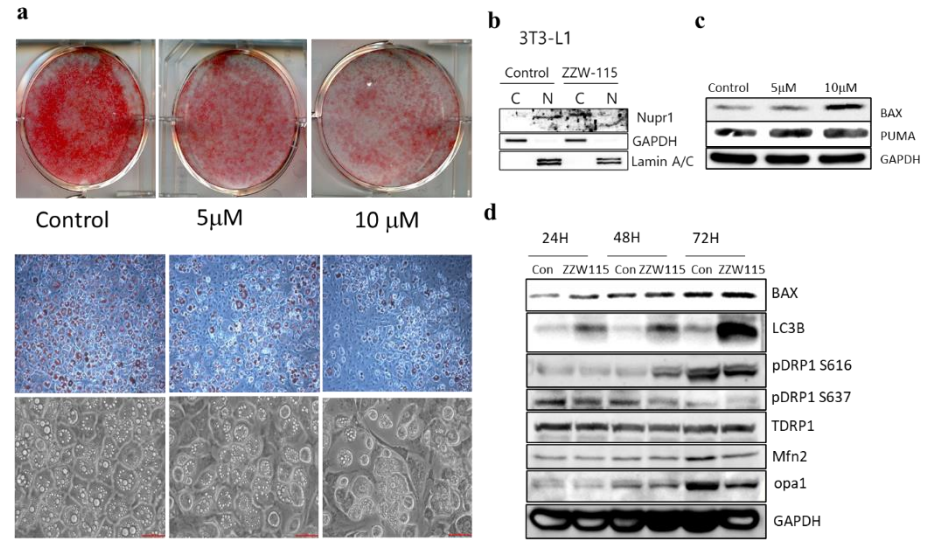
Confocal microscopy of MitoSOX Red mitochondrial superoxide indicator stained ZZW-115 treated cells and control cells, showed that ZZW-115 treated cells present more superoxide relative to the control (Fig.2e). Superoxide is an oxygen free radical belonging to the reactive oxygen species (ROS), that in excessive levels causes hydroxyl-radical formation and DNA damage[16].

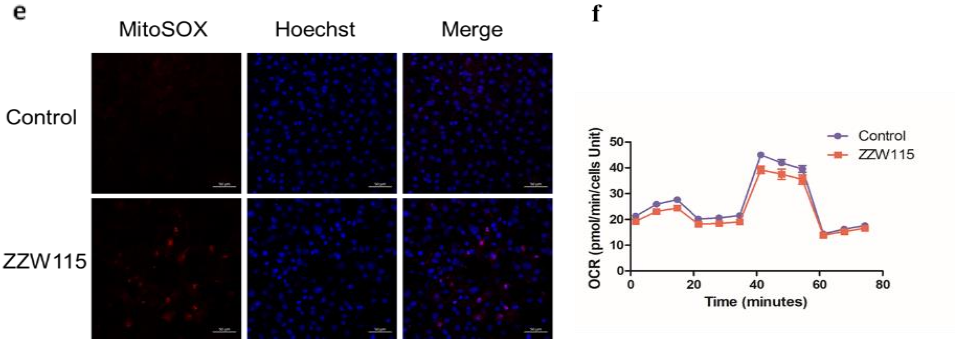
In the same way, oxygen consumption rate (OCR) was verified, in control and ZZW-115 treated 3T3-L1 cells. ZZW-115 treated 3T3-L1 cells showed a lower OCR relative to control (Fig 4f).

Moreover, transmission electron microscopy experiments evidence that ZZW-115 treated cells, at 24h, show a large number of autolysosomes, in the autophagy degradation phase, in comparison to control cells, that don't show any, and abnormal mitochondria are found in low quantities (Fig 4. g). After 48H, ZZW-

115 treated 3T3-L1 cells show a large number of abnormal mitochondria, with abnormal cristae fold, and similarly to what was seen at 24h, autolysosomes are largely present in these cells in relation to the control. (Fig. 4h) .These data show that, with the treatment of ZZW-115, autophagy is seen after 24h, and mitochondrial dysfunction occurs more slowly and is largely seen 48H after ZZW-115 treatment.

These data demonstrate that Nupr1 is important in late adipogenesis and with the depletion of this protein, differentiating adipocytes show to have increased production of ROS and consequently, mitochondrial dysfunction, autophagy occurrence, and ultimately cell death.





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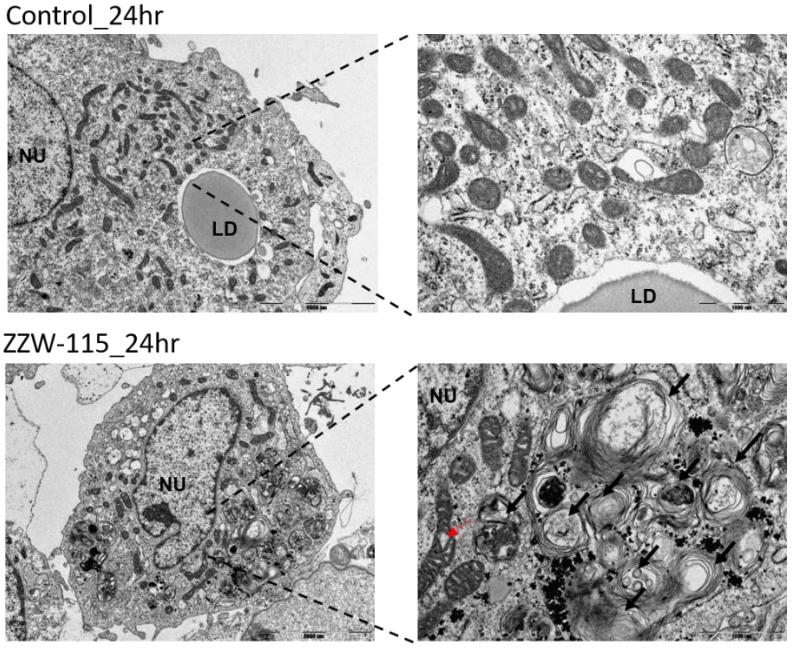

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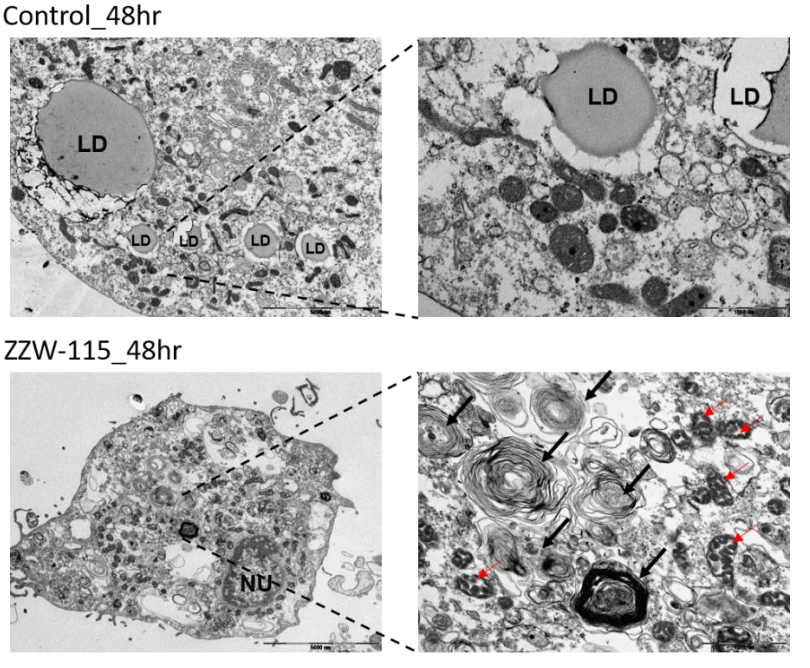

Figure 4. Nupr1 protects mature adipocytes from ROS production (a) 3T3-L1 cells were treated with ZZW-115, 4 days after induction of differentiation. The cells were treated, respectively, with 5μM, and 10μM. Oil red O staining was performed on day 7 of differentiation, and pictures were taken (light microscope analysis). (b) ZZW-115 treatment was done at a concentration of 10μM, consequently on the next day, nucleus and cytoplasm were extracted and Nupr1 localization was checked via western blotting. (c) 3T3-L1 cells were treated with ZZW-115 at a concentration of 5μM, and 10μM for 72 Hours, then the cells were harvest and western blotting was performed. (d) Differentiation of 3T3-L1 cells was induced, and ZZW-115 treatment was done at a concentration of 10μM. The samples were harvested at 24H, 48H and 72H after the treatment. (e) Confocal microscopy image of MitoSOX™ Red mitochondrial superoxide indicator staining. Differentiating 3T3-L1 cells were treated with ZZW-115 at a concentration of, 10μM. A control was used for comparation. MitoSOX stained cells (left), Hoechst-stained cells (right), and merged pictures (right). (f) Oxygen consumption rate of 3T3-L1 cells control and ZZW-115 treated (10μM) cells. The treatment was done 4 days after differentiation induction.

(g) Transmission electron microscopic pictures of ZZW-115 treated 3T3-L1 cells and control 3T3-L1 cells. ZZW-115 treatment was done on the 4th day of differentiation and the samples were prepared 24h after. Red arrow (impaired mitochondria), black arrow (autolysosome) (h) electron microscopic pictures of ZZW-115 treated 3T3-L1 cells and control 3T3-L1 cells. ZZW-115 treatment was done on the 4th day of differentiation and the samples were prepared 48h after.

5. Nupr1 depletion causes cell death in late adipogenesis

Following the last data findings, to verify if Nupr1's function is inhibited via ZZW-115 and causes ultimately cell death, further knock-down experiments were performed.

Differentiation of 3T3-L1 cells was induced and, 2 days after, siNupr1 was transfected to the cells. Furthermore, Oil red O staining was performed on day 7 of differentiation.

As seen in figure 5a the siNupr1 transfected sample shows less lipid accumulation in relation to the control sample. In the same way, light microscope pictures evidence loss of cell integrity, change of cell's shape, change in the shape of lipid droplets, and lipid content decrease, ultimately, cell death was seen (Fig.5a).

Similar to the previous figure 4 data, Nupr1 knocked down cells showed an increase in pro-apoptotic BAX expression, demonstrating that the cells are undergoing apoptosis. In the same way, phosphorylated DRP1 S616 shows to increase its levels in relation to the control demonstrating an increase of mitochondria fission. LC3B shows an increase in its levels on day 6 (Fig 5b).

Additionally, Nupr1 mRNA expression showed to be decreased in relation with the control sample, evidencing successful knockdown of Nupr1. In the same way, mitochondrial NRF1 and its target genes TFAM and TFB1M show to have a lower mRNA relative expression in relation to the control. NRF1, a transcription factor, activates the expression of genes that control cellular growth, nuclear genes required for respiration, mitochondrial DNA transcription, and replication and activates the following genes mtTFB1 and TFAM. These genes are very

important for DNA transcription in mitochondria. Therefore, the decreased expression of these genes points to a dysfunction of mitochondria.

Putting all together, Nupr1 knockdown shows a similar effect to ZZW-115 treatment of 3T3-L1 cells, evidencing that ZZW-115 might specifically inhibit this protein and that Nupr1 is vital for the maintenance of 3T3-L1 mature adipocytes, and the depletion of the same causes cell death.

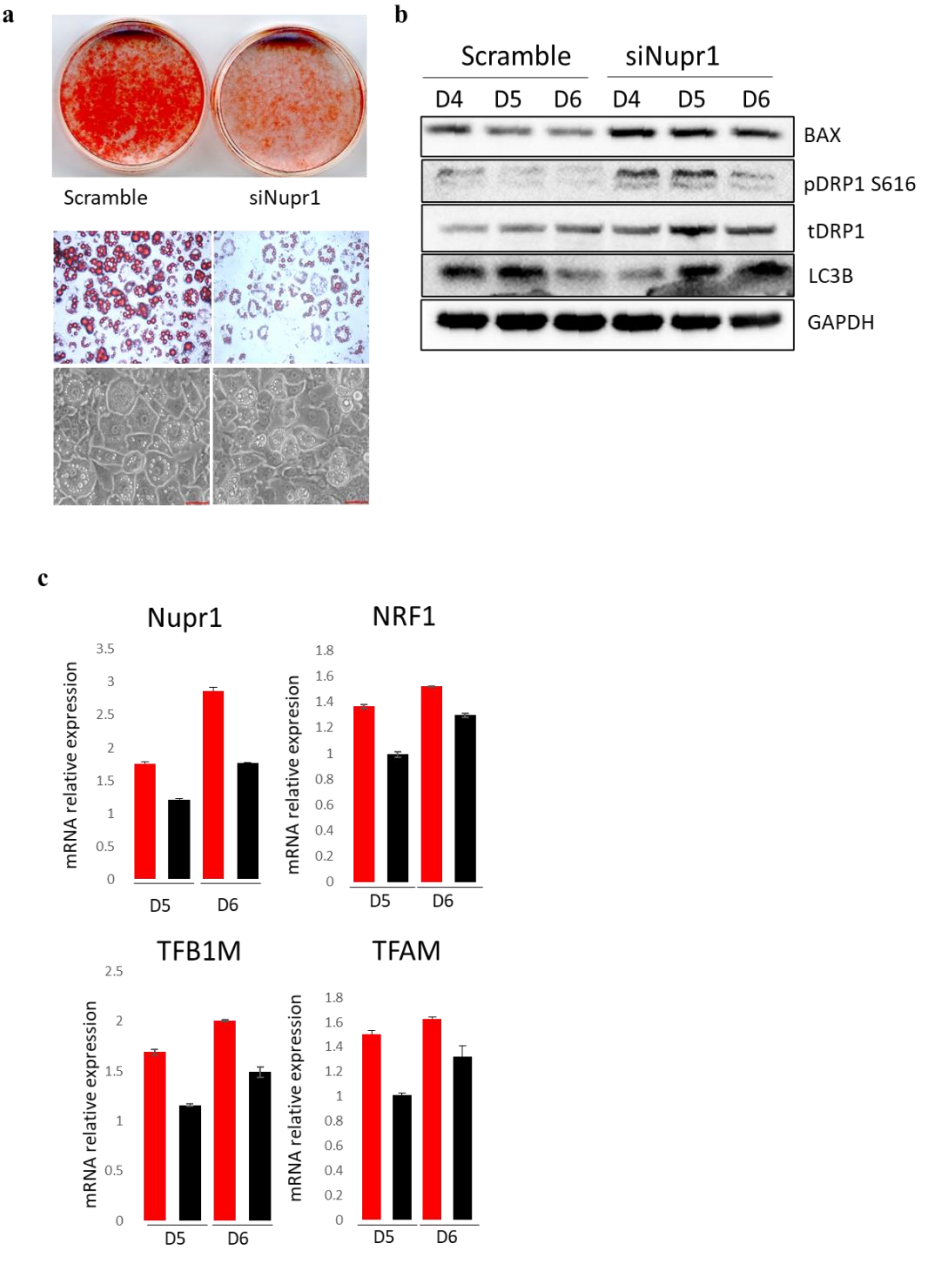

Figure 5. Nupr1 depletion causes cell death in late adipogenesis (a) Nupr1 knockdown via siRNA. Differentiation of 3T3-L1 cells was induced and 2 days after siNupr1 was transfected via lipofectamine 2000. Insulin was also introduced into FBS/ DMEM media (without antibiotics). Oil red O staining was performed on day 7 and light microscope pictures were taken on the same day. (b) The same samples were harvested on days 4, 5 and 6, and western blotting was performed to verify, BAX, phosphorylated DRP1-S161, Total DRP1 and LC3B. (c) siNupr1 treated 3T3-L1 cells and control were harvested on days 5 and 6 and Nupr1, NRF1, TFAM and TFB1M mRNA relative expressions were verified.

IV. DISCUSSION

The white adipose tissue is rather important for the storage of energy in the form of triglycerides, and therefore, dysfunctions in adipogenesis are correlated with various metabolic diseases, such as obesity.

Nupr1 has been shown to have a role in cancer, for example, cell migration and cancer cell growth, however, its role in metabolism is not well established.

This study data demonstrate that Nupr1 is present in the white adipose tissue during adipogenesis and that it increases its levels throughout the end of it (Fig 1 and 2). In concordance, this study shows that Nupr1 is important for the normal function of the white adipose tissue. First, Nupr1 is shown to be present in the white adipose tissue, during adipogenesis, on day 0 but even more expressed on days 3,4 and 8 (Fig 2), that is, Nupr1 is expressed at the beginning of adipogenesis, but it is even more expressed at the end of adipogenesis.

Therefore, to check Nupr1 function during adipogenesis, Nupr1 specific siRNA was transfected before induction of differentiation of 3T3-L1 cells, and accordingly, Nupr1 depletion showed to cause adipogenesis impairment. Correlated with the decrease of Nupr1 expression levels the same happened with the adipogenic target genes, C/EBPα, PPARγ, adipocyte protein 2 (AP2), and lipogenesis target gene, fatty acid synthetase (Fasn). AP2 and FAS are only expressed at the end of adipogenesis (Fig 3). Therefore, Nupr1 might protect adipogenesis permitting its normal occurrence.

In addition, in this study CHOP levels also showed to be in increased levels on day 0 when Nupr1 is depleted, relative to control. And therefore, there might be a possibility that Nupr1 protects adipogenesis from CHOP high levels during ER stress in early adipogenesis. This statement is supported by previous studies that have shown that CHOP heterodimerizes C/EBPβ and therefore, inhibits DNA binding activity gain. In normal circumstances CHOP levels decrease in the S phase in preadipocytes, C/EBPβ gains DNA binding activity and consequently activates C/EBPα and PPARγ allowing adipogenesis to happen correctly. Therefore, CHOP overexpression inhibits C/EBPβ activity causing, consequently, early adipogenesis impairment[17,18]. In the same way, CHOP overexpression was found to be correlated with cell cycle arrest and apoptosis induction[19]. These studies might support my data, from figure 3, whereas with the depletion of Nupr1, CHOP levels increase at 0H and 4H after 3T3-L1 differentiation's induction.

In the same way, cell counting experiments showed that Nupr1 depleted cell's number was reduced relative to the control and didn't increase from day 0 to day 2 of differentiation, pointing to a possible dysfunction of adipogenesis during the mitotic clonal expansion phase, when Nupr1 is knocked down. This phase is very important for the normal occurrence of adipogenesis and any disruption that happens during the mitotic clonal expansion phase inhibits adipogenesis due to, also, the decreased expression levels of the late adipogenic target genes PPARγ and C/EBPα[20].

Furthermore, Nupr1 high expression in late adipogenesis might show a function of this protein in this phase of differentiation and in mature adipocyte

maintenance. Previous studies have stated that mitochondria function is very important for the normal differentiation of 3T3-L1 adipocyte cells. Moreover, mitochondrial target genes are shown to increase 20-fold during adipogenesis, and consequently, dysfunction in mitochondria causes impairment of adipogenesis[21]. Mitochondrial biogenesis, also, increases throughout adipogenesis, ATP levels demand increases, and there's also an increase in oxygen consumption rate. Mitochondrial oxidation is also very important, whereas oxidative phosphorylation levels increase throughout adipogenesis[22].

Moreover, in addition, previous studies have also correlated Nupr1 expression with mitochondria protection and normal function by controlling redox homeostasis, ferroptosis, and energy metabolism. Nupr1 deficiency, also, causes elevated production of ROS, loss of membrane potential, mitochondria relocation, and, as a consequence, mitochondrial failure. And, in the same way, Nupr1 depletion shows to cause a decrease in ATP production and necrosis due to elevated ER stress[9.]

Moreover, these late statements are correlated with the figure 4 data results. ZZW-115 treatment did in fact cause adipogenesis impairment by cell dysfunction, cell morphology change, change in lipid droplet's shape, decrease in lipid content, and cell death. In the same way, my data demonstrate that mitochondria become dysfunctional by an excess of ROS presence and therefore, leading to cell death. Cell death was evidenced by proapoptotic genes BAX and PUMA increased expression in ZZW-115 treated cells relative to controls.

In the same way, as seen in the transmission electron microscopy images, on figures 4 g and h, Nupr1 absence/inhibition causes autophagy and mitochondrial dysfunction which, points to a protective role of this protein in the maintenance of mitochondrial function and normal metabolism in 3T3-L1 cells.

Previous studies have shown that treatment with ZZW-115 inhibited Nupr1 translocation to the nucleus, by binding to the Thr68, located in the nuclear location signal region of Nupr1, competing with importin-α3[23]. Nupr1 was also shown to interact with DNA repair proteins, and to localize DNA damaged sites, however after treatment with ZZW-115 DNA damage was seen, followed by cell death[24]. This inhibition of Nupr1 translocation to nucleus was also seen in this study in 3T3-L1 cells.

Nupr1 nuclear presence might point to a role as a transcription factor. Previous studies have shown that Nupr1 induces the expression of PDGFA, to promote angiogenesis in hepatocellular carcinoma. Nupr1 has been demonstrated to be similar to high motility group-like proteins and binds to A/T rich sequences. Moreover, Nupr1 was shown to bind to the -3090/-2128 promoter region of PDGFA, evidenced by an increase in luciferase transcriptional activity. CHIP assay experiments also demonstrated that Nupr1 is recruited to the promoter of PDGFA at the -2231/-2027 region and binds to the same[25]. In the same way, Nupr1 was previously found to bind to promoter regions of genes involved in drug resistance and autophagy, such as BECN1, RAB31 and, CYP1B1, and control their transcriptional activity, maintaining cancer cells resistance to tamoxifen[26]. These statements evidence that Nupr1 might control the expression

of other proteins. Moreover, a previous study from 2004, has shown that human Nupr1 is constituted by a basic helix-loop-helix domain and a nuclear localization signal domain[27], which verifies the transcription factor role of Nupr1. However, Nupr1 transcriptional regulating activity is unknown in the white adipose tissue, during adipogenesis.

In addition, Nupr1 further knockdown experiments, as shown in figure 5, have evidenced that with the depletion of Nupr1, 3T3-L1 cells show similar but less drastic changes in cell shape and lipid content in relation to control. BAX also is shown to be more expressed in Nupr1 knocked down samples, showing that these cells are undergoing cell death. Mitochondrial fission/fusion imbalance increase is also evidenced in these samples. These findings, show that with inhibition of Nupr1, via ZZW-115 and Nupr1 knockdown, mitochondria become impaired, and cell death happens, showing a crucial role of Nupr1 in maintaining 3T3-L1 cells metabolism and mitochondria normal function, while protecting cells from high stress.

This study's data mainly evidence that depletion of Nupr1 might cause mitochondria dysfunction and cell death in late adipogenic differentiation, due to the increase of sensitivity of 3T3-L1 cells to stress caused by excessive ROS production.

Altogether, this study data shows that Nupr1 depletion during adipogenesis might happen due to CHOP overexpression that inhibits C/EBPβ in early adipogenesis or by excessive presence of ROS that causes differentiating adipocyte's cell mitochondria dysfunction and ultimately cell death.

In summary, when Nupr1 is blunted adipogenesis doesn't happen correctly. Therefore, Nupr1 might constitute a therapeutical target for Obesity, due to its possible protective role that allows adipogenesis to proceed correctly and maintenance of mature 3T3-L1 cells.

V. CONCLUSION

1. Nupr1 is present in the white adipose tissue

2. Nupr1 is present in the white adipose tissue at the beginning and the end of adipogenesis.

3. Nupr1 deficiency causes adipogenesis impairment

4. Nupr1 deficiency causes CHOP increased expression in early differentiation and consequently early adipogenesis impairment

5. ZZW-115, Nupr1 inhibitor, treatment causes differentiating adipocyte cell's mitochondria impairment, autophagosome formation and, ultimately, cell death.

6. Nupr1 knockdown, similar to ZZW-115, causes mitochondria impairment and cell death.

REFERENCES

1. Huang, C., Santofimia-Castaño, P. and Iovanna, J., 2021. NUPR1: A Critical Regulator of the Antioxidant System. Cancers, 13(15), p.3670.
2. Choe, S., Huh, J., Hwang, I., Kim, J. and Kim, J., 2016. Adipose Tissue Remodeling: Its Role in Energy Metabolism and Metabolic Disorders. Frontiers in Endocrinology, 7.
3. Kwok, K., Lam, K. and Xu, A., 2016. Heterogeneity of white adipose tissue: molecular basis and clinical implications. Experimental & Molecular Medicine, 48(3), pp.e215-e215.
4. Ghaben, A. and Scherer, P., 2019. Adipogenesis and metabolic health. Nature Reviews Molecular Cell Biology, 20(4), pp.242-258.
5. GREGOIRE, F., SMAS, C. and SUL, H., 1998. Understanding Adipocyte Differentiation. Physiological Reviews, 78(3), pp.783-809
6. Lane, M., Tang, Q. and Jiang, M., 1999. Role of the CCAAT Enhancer Binding Proteins (C/EBPs) in Adipocyte Differentiation. Biochemical and Biophysical Research Communications, 266(3), pp.677-683.
7. Muir, L., Neeley, C., Meyer, K., Baker, N., Brosius, A., Washabaugh, A., Varban, O., Finks, J., Zamarron, B., Flesher, C., Chang, J., DelProposto, J., Geletka, L., Martinez-Santibanez, G., Kaciroti, N., Lumeng, C. and O'Rourke, R., 2016. Adipose tissue fibrosis,

hypertrophy, and hyperplasia: Correlations with diabetes in human obesity. Obesity, 24(3), pp.597-605.

8. Mallo, G., Fiedler, F., Calvo, E., Ortiz, E., Vasseur, S., Keim, V., Morisset, J. and Iovanna, J., 1997. Cloning and Expression of the Rat p8 cDNA, a New Gene Activated in Pancreas during the Acute Phase of Pancreatitis, Pancreatic Development, and Regeneration, and Which Promotes Cellular Growth. Journal of Biological Chemistry, 272(51), pp.32360-32369.
9. Santofimia-Castaño, P., Lan, W., Bintz, J., Gayet, O., Carrier, A., Lomberk, G., Neira, J., González, A., Urrutia, R., Soubeyran, P. and Iovanna, J., 2018. Inactivation of NUPR1 promotes cell death by coupling ER-stress responses with necrosis. Scientific Reports, 8(1).
10. Barbosa-Sampaio, H., Liu, B., Drynda, R., Rodriguez de Ledesma, A., King, A., Bowe, J., Malicet, C., Iovanna, J., Jones, P., Persaud, S. and Muller, D., 2013. Nupr1 deletion protects against glucose intolerance by increasing beta cell mass. Diabetologia, 56(11), pp.2477-2486.
11. Barbosa-Sampaio, H., Drynda, R., Liu, B., Rodriguez De Ledesma, A., Malicet, C., Iovanna, J., Jones, P., Muller, D. and Persaud, S., 2015. Reduced nuclear protein 1 expression improves insulin sensitivity and protects against diet-induced glucose intolerance through up-

regulation of heat shock protein 70. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1852(5), pp.962-969.

12. Batchvarova, N., Wang, X. and Ron, D., 1995. Inhibition of adipogenesis by the stress-induced protein CHOP (Gadd153). The EMBO Journal, 14(19), pp.4654-4661.
13. Huang, C., Santofimia-Castaño, P., Liu, X., Xia, Y., Peng, L., Gotorbe, C., Neira, J., Tang, D., Pouyssegur, J. and Iovanna, J., 2021. NUPR1 inhibitor ZZW-115 induces ferroptosis in a mitochondria-dependent manner. Cell Death Discovery, 7(1).
14. Al-Sayegh, M., Ali, H., H Jamal, M., ElGindi, M., Chanyong, T., Al-Awadi, K. and Abu-Farha, M., 2021. Mouse Embryonic Fibroblast Adipogenic Potential: A Comprehensive Transcriptome Analysis. Adipocyte, 10(1), pp.:1-20.
15. Patel, Y. and Lane, M., 2000. Mitotic Clonal Expansion during Preadipocyte Differentiation: Calpain-mediated Turnover of p27. Journal of Biological Chemistry, 275(23), pp.17653-17660.
16. Keyer, K. and Imlay, J., 1996. Superoxide accelerates DNA damage by elevating free-iron levels. Proceedings of the National Academy of Sciences, 93(24), pp.13635-13640.
17. Tang, Q. and Lane, M., 2000. Role of C/EBP homologous protein (CHOP-10) in the programmed activation of CCAAT/enhancer-

binding protein-beta during adipogenesis. Proceedings of the National Academy of Sciences, 97(23), pp.12446-12450.

18. Liu, D., Lin, Y., Kang, T., Huang, B., Xu, W., Garcia-Barrio, M., Olatinwo, M., Matthews, R., Chen, Y. and Thompson, W., 2012. Mitochondrial Dysfunction and Adipogenic Reduction by Prohibitin Silencing in 3T3-L1 Cells. PLoS ONE, 7(3), p.e34315.
19. Oyadomari, S. and Mori, M., 2003. Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death & Differentiation, 11(4), pp.381-389.
20. Tang, Q., Otto, T. and Lane, M., 2002. Mitotic clonal expansion: A synchronous process required for adipogenesis. Proceedings of the National Academy of Sciences, 100(1), pp.44-49.
21. Chen, Q., Hao, W., Xiao, C., Wang, R., Xu, X., Lu, H., Chen, W. and Deng, C., 2017. SIRT6 Is Essential for Adipocyte Differentiation by Regulating Mitotic Clonal Expansion. Cell Reports, 18(13), pp.3155-3166.
22. Zhang, Y., Marsboom, G., Toth, P. and Rehman, J., 2013. Mitochondrial Respiration Regulates Adipogenic Differentiation of Human Mesenchymal Stem Cells. PLoS ONE, 8(10), p.e77077.
23. Santofimia-Castaño, Xia, Peng, Velázquez-Campoy, Abián, Lan, Lomberk, Urrutia, Rizzuti, Soubeyran, Neira and Iovanna, 2019.

Targeting the Stress-Induced Protein NUPR1 to Treat Pancreatic Adenocarcinoma. Cells, 8(11), p.1453.

24. Lan, W., Santofimia-Castaño, P., Swayden, M., Xia, Y., Zhou, Z., Audebert, S., Camoin, L., Huang, C., Peng, L., Jiménez-Alesanco, A., Velázquez-Campoy, A., Abián, O., Lomberk, G., Urrutia, R., Rizzuti, B., Geli, V., Soubeyran, P., Neira, J. and Iovanna, J., 2020. ZZW-115–dependent inhibition of NUPR1 nuclear translocation sensitizes cancer cells to genotoxic agents. JCI Insight, 5(18).
25. Chen, C., Wu, S., Lin, Y., Chi, H., Lin, S., Yeh, C., Chuang, W. and Lin, K., 2019. Induction of nuclear protein-1 by thyroid hormone enhances platelet-derived growth factor A mediated angiogenesis in liver cancer. Theranostics, 9(8), pp.2361-2379.
26. Wang, L., Sun, J., Yin, Y., Sun, Y., Ma, J., Zhou, R., Chang, X., Li, D., Yao, Z., Tian, S., Zhang, K., Liu, Z. and Ma, Z., 2021. Transcriptional coregualtor NUPR1 maintains tamoxifen resistance in breast cancer cells. Cell Death & Disease, 12(2).
27. Goruppi, S. and Kyriakis, J., 2004. The Pro-hypertrophic Basic Helix-Loop-Helix Protein p8 Is Degraded by the Ubiquitin/Proteasome System in a Protein Kinase B/Akt- and Glycogen Synthase Kinase-3-dependent Manner, whereas Endothelin Induction of p8 mRNA and

Renal Mesangial Cell Hypertrophy Require NFAT4. Journal of Biological Chemistry, 279(20), pp.20950-20958.

ABSTRACT (IN KOREAN)

Adipogenesis 에서 Nupr1 의 역할

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선진국의 비만율 증가는 제 2 형 당뇨병, 심혈관 질환 등과 같은 대사 질환의 발생 증가와 상관관계가 있기 때문에 심각한 문제로 대두되고 있다. 지방세포분화 조절 메커니즘은 비만과 같은 신진대사 질환과의 상관관계 때문에 널리 연구되는 주제이다. 그러므로, 지방세포분화를 조절하는 생물학적 네트워크를 연구하는 것은 비만의 병리생리학에 대한 더 큰 이해를 가져올 수 있다. 지방세포 분화는 초기 지방 분화와 후기 지방 분화과정을 거친다. 초기 지방분화에서 지방세포의 유사 분열이 일어나고 C/EBPβ는 DNA 결합 활성을 얻는다. 활성화된 C/EBPβ는 지방 분화의 결정적 역할을 하는 C/EBPα와 PPARγ을 활성화하고, 증가된 C/EBPα와 PPARγ에 의해 유사 분열을 멈추게 한 후, 후기 지방 분화 단계가 시작된다.

결과적으로, 후기 지방 분화 동안, 지방세포는 인슐린 민감도를 얻고, 지방세포는 확대되는 지질 방울을 얻으며, 지방 생성 효소와 지방산 결합 효소 발현을 증가시킨다. 마지막으로, 지방세포는 성숙해지고 에너지 저장고로서의 기능을 수행할 수 있게 된다.

이번 연구에서 Nupr1 이 백색 지방 조직에 존재하고 지방 분화 동안 Nupr1 의 발현이 변화하는 것을 확인했다. Nupr1 은 증가된 소포체 스트레스로부터 세포를 보호하는 것으로 알려진 8kd 의 핵 및 세포질 스트레스 활성화 단백질이다.

Nupr1 은 DNA 복구, 세포 조절, 세포 사멸, 세포 이동 및 침윤을 조절한다고 알려져 있다. 또한, Nupr1 발현은 산화환원 항상성, 페롭토시스, 에너지 대사를 조절하여 미토콘드리아를 보호하고 정상기능을 유지시키는 단백질로 알려져 있다. Nupr1 은 암에서 광범위하게 연구되어 왔지만, 신진대사와 지방 분화에 있어서 Nupr1 의 역할은 알려져 있지 않아 연구가 필요하다.

따라서 본 연구에서는 마우스 3T3-L1 지방선구세포를 성숙한 지방세포로의 분화를 유도하고 지방분화과정 중 Nupr1 발현과 역할을 확인하였다. Nupr1 은 지방 분화 Day 0 에 발현되고, 지방 분화의 Day 3 일에서 Day 8 일째인 말기에 증가된 수준으로 발현된다는 것을 확인했다. 또한, 3T3-L1 세포에서 siRNA 를 통해

Nupr1 발현이 억제시키면, 지방분화 생성이 억제되는 것을 확인했다. Nupr1 발현이 억제되면 초기 지방형성 Day0 에 CHOP 의 발현이 증가되는데, 이는 C/EBPβ 활성 억제 및 그에 따른 유사분열의 억제로 인해 지방세포분화 손상의 원인이 될 수 있음을 보여줍니다.

또한, 지방 세포 분화 과정에서 Nupr1 은 후기에도 발현이 되는데, 이는 Nupr1 이 후기 지방 생성 동안 역할을 할 가능성이 있다. 지방 분화 후기에 Nupr1 의 억제제인 ZZW-115 를 처리하여 지방 생성이 잘 이루어지지 않으며 세포사멸이 일어나는 것을 확인했다. pro-apoptotic 유전자인 BAX 와 PUMA 는 ZZW-115 처리된 세포에서 autophagy 표적 유전자 LC3b 와 함께 그 수준을 증가시키는 것으로 나타났습니다. 같은 방식으로 처리된 세포에서 미토콘드리아 융합 관련 유전자 opa1 및 MFN2 의 하향 조절에 의해 융합과 관련하여 미토콘드리아 분열이 증가하는 것으로 나타났다. 추가 데이터는 ZZW-115 처리된 세포의 미토콘드리아가 대조군과 관련하여 초과산화물 존재를 증가시켰고 산소 소비율이 대조군 샘플보다 상대적으로 낮아 Nupr1 이 고갈될 때 미토콘드리아의 기능장애를 나타내는 것을 보여줍니다. 3T3-L1 세포의 분화 유도 2 일 후 siRNA 를 통해 Nupr1 을 녹다운 시킨 경우에도 유사한 결과가 확인되었다.

이러한 결과는 Nupr1 의 스트레스 보호 기능을 통해 지방 분화 및 지방생성의 정상적인 발생을 유지시킨 다는 것을 예측할 수 있다.

본 연구결과를 통해, Nupr1 이 지방 생성의 정상적인 발생을 보호하고 허용하는 역할을 할 수 있으며 결과적으로 이 단백질이 비만 치료의 가능한 표적이 될 수 있음을 확인했다.

백색 지방 조직, 지방세포분화, 비만, Nupr1, 3T3-L1