**Growth attenuation is associated with histone deacetylase 10-induced autophagy
in the liver**

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Abstract (241)

Our previous data suggested that the histone deacetylase (HDAC) SIRT1 is involved in mediating the effect of nutrition on growth. The aim of the present research was to study the mechanism by which additional HDACs may be involved in nutrition-induced linear growth. The *in vivo* studies were performed in young male Sprague Dawley rats that were either fed ad libitum (AL) or subjected to 10 days of 40% food restriction (RES) and then re-fed (CU). For *in vitro* studies, Huh7 hepatoma cells were used. Food restriction led to significant reduction in liver weight, concomitant with increased autophagy (i.e. a decrease in the level of P62 and an increase in the expression level of Ambra1 and Atg16L2 genes in the RES group). At the same time we found that the level of HDAC10 was significantly increased. Over expression of HDAC10 in Huh7 hepatoma cells led to reduced cell viability and increased autophagy as shown by increased conversion of LC3-I to LC3-II. An increase in the level of HDAC10 was also obtained when mTOR was inhibited by Rapamycin. siRNA directed against HDAC10 abolished the effect of Rapamycin on cell viability and Ambra1 and Atg16L2 increased expression. These results suggest that increased levels of HDAC10 may mediate the effect of malnutrition on growth attenuation and autophagy. Deciphering the role of epigenetic regulation in the nutrition–growth connection may pave the way for the development of new forms of treatment for children with growth disorders.

Key words: HDAC10, catch up growth, autophagy, food restriction, liver

1. Introduction

Children's growth is regulated by both genetic and environmental factors. The most effective environmental factor known today is nutrition; however, the mechanisms connecting nutrition and longitudinal growth are still not fully understood. Several studies have shown a connection between linear growth and epigenetic mechanisms during chondrogenic differentiation [1-3]. Epigenetics is defined as changes in gene function caused by mechanisms other than changes in the genomic DNA sequence, such as methylation of the DNA as well as post-translational modification of the histone proteins [4]. These may include phosphorylation, methylation, ubiquitinylation, sumoylation and acetylation [5, 6]. The most extensively studied epigenetic modification of histones is acetylation. Histone acetylation by histone acetyltransferases (HATs) occurs at the ϵ -amino group of lysine residue within the peptide chain. The already bulky lysine side chain becomes even bulkier, the positive charge is neutralized, and the histone-DNA interaction is weakened. Acetylation is cleared by histone deacetylases (HDACs). As a general rule, histone acetylation by HATs is usually associated with increased transcription activity because of the "loose" chromatin structure. Deacetylation by HDACs leads to condensation and suppression of transcription [7]. HATs and HDACs also use non-histone protein substrates, including transcriptional regulators, chromatin components, and signaling factors, adding another level of regulation [8-11].

There are 18 HDACs in the mammalian genome, divided into four groups according to their homology to yeast HDACs, their size, cellular localization, catalytic domain and mechanism of action. There are 11 zinc-dependent HDACs: class I (HDAC1, -2, -3, and -8); class II, subdivided into class IIa (HDAC4, -5, -7, and 9) and class IIb (HDAC6 and -10); and class IV (HDAC11)[12, 13]- and 7 nicotinamide

adenine dinucleotide (NAD⁺)- dependent HDACs, which comprise class III (sirtuin, SIRT1 to -7). Class I HDACs are generally located in the nucleus and are relatively small in size; class II HDACs are present both in the nucleus and cytoplasm and are generally larger. Several HDACs were shown to be stimulated by food restriction; others were shown to be affected by a high fat diet [14], indicating their sensitivity to the metabolic status and suggesting a possible role in the nutrition-growth connection. The most extensively studied HDACs in this context are the sirtuins, which deacetylate a number of histone and non-histone substrates [15-17].

To study how malnutrition affects linear growth we used a protocol of 40% food restriction for 10 days followed by re-feeding with the same chow for one day, in young, fast-growing male Sprague Dawley (SD) rats [18]. We previously found that SIRT1 of the HDAC III family is significantly increased in the epiphyseal growth plate (EGP) by food restriction, probably as a result of reduction in the level of miR-140 and miR-22, which target it [17]. Furthermore, we found that the effect of food restriction on the level of these miRNAs is mediated by leptin and insulin like growth factor (IGF)-1 as well as other as yet unidentified systemic factors [19]. These findings prompted us to investigate whether there might be parallel effects in other tissues and indeed we found that the weight of the liver was rapidly affected by nutritional manipulation, similar to the EGP. This led to the present study to determine if HDACs could also be involved in food restriction-induced growth attenuation in the liver.

In the present study we investigated the role of the HDACs in the liver and its effect on growth and autophagy during food restriction. We found that food restriction significantly increased the protein levels of several HDACs in the liver. Our results

suggest that HDAC10 responds to the nutritional status through mTOR and that it may be involved in autophagy induced by food restriction.

1. Materials and Methods

1.1. Antibodies

Anti-HDAC1, 6 and 10 were obtained from Abcam (Cambridge, MA, USA); anti-HDAC10 was obtained also from Biovision (Milpitas, CA, USA); anti-HDAC4 and anti- microtubule associated protein light chain 3 (LC3) were from Cell signaling (Danvers, MA, USA); anti-HDAC 5,7, 8 and 11, anti-SQSTM1/P62 and anti-HSP70/HSC70 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-HDAC2 and anti-HDAC3 were from SAB Signalway Antibody (Baltimore, MD, USA); anti-HDAC9 was from Epitomics (Burlingame, CA , USA); anti- β -actin was from Millipore (Billerica, MA, USA); anti-FLAG were from Sigma – Aldrich (Rehovot, Israel); secondary fluorescent antibodies were obtained from LICOR Biosciences(Lincoln, NE, USA).

1.2. Animals

Male Sprague–Dawley (SD) rats, 24 days old, were purchased from Harlan (Jerusalem, Israel) and housed individually at the animal care facility of the Felsenstein Medical Research Center. The rats were divided into two groups: one was given an unlimited amount of food (complete diet for rats and mice, 3.4 kcal/g, provided by Teklad) (ad libitum group – AL, n=6), and the other was given 60% of the same chow (food-restricted group- RES). All animals had unlimited access to water. The 40% restriction was calculated on the basis of a previous study wherein animals were housed individually and the amount of food consumed each day was

measured, together with the animal's weight and weight gain [18]. The food restriction was maintained for 10 days. At that point, the RES group was further divided into two groups: one was kept restricted (RES, n=6), and the other was given normal chow ad libitum (that induced catch up growth, hence this group was named CU group; n=6). After 1 day of re-feeding, animals from all three groups were sacrificed by CO₂ inhalation; serum was collected and stored at -80°C until further use, internal organs (kidney, lungs, liver and heart) were removed, weighed and immediately frozen in liquid nitrogen until analyzed. Part of each liver was stored in 4% formaldehyde and was later used for paraffin sections. Throughout the study, animals were observed daily, and all remained bright, alert and active, with no evidence of any disorder. The Tel Aviv University Animal Care Committee approved all procedures.

1.3. Huh7 cells

Huh7 cells were cultured in Dulbecco's Modified Eagles' Medium (DMEM) supplemented with 10% fetal calf serum (FCS); 2mM glutamine and penicillin/streptomycin (all cell culture materials were from Biological Industries, Beit Haemek, Israel). Cells were grown in a humidified incubator at 37°C with 95% air/5% CO₂, and the medium was changed every other day.

1.4. Protein extraction and determination

Huh7 human hepatoma cells (washed with PBS) and rat livers were homogenized in a radio-immuno-precipitation assay (RIPA) buffer (20mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.25% Na-deoxycholate), that was supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland) in a 1:12 ratio. Protein

concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, IL, USA) according to the manufacturer's recommendations, and 50µg proteins were analyzed per each sample by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by western immunoblotting. Proteins were transferred to nitrocellulose membranes (GE Healthcare, NJ, USA) by the wet blotting system (Bio-Rad, Hercules, CA, USA). Nitrocellulose membranes were then incubated in TBS-T (10mM Tris-HCl, 150mM NaCl, 0.1% Tween 20) with 5% skim milk solution for one hour to block nonspecific binding, and then incubated with the primary antibody overnight at 4°C. Membranes were washed with TBS-T, incubated with a secondary fluorescent antibody (LI-COR Biosciences, Lincoln, NE, USA) decorated with IRDye® for one hour and washed again. β-actin served as the reference. Quantification was performed using the Odyssey (2.1.) application Software (LI-COR Biosciences, Lincoln, NE, USA).

1.5. *RT² profiler Rat Autophagy PCR Array*

Total RNA was extracted from liver tissues of AL, RES and CU groups with miReasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The quantity and quality of the RNA were evaluated using a Nanodrop spectrophotometer (Nanodrop Ltd, USA) with values of: A260/A280 > 2.0 and A260/A230 > 1.7. Equal amounts of RNA from all groups were used for analysis by the Rat Autophagy RT² Profiler™ PCR Array (SaBiosciences, USA), which profiles the expression of 84 genes related to autophagy. cDNA was synthesized with RT² First Strand Kit (SasBiosciences, USA), using 600 ng of total RNA, preceded by Dnase I treatment. Using real-time PCR, the expression of a focused panel of genes

was measured according to the manufacturer's recommendations, using the ABI Prism 7000 Sequence Detection System (Applied Biosystems (ABI), Foster City, CA).

Results were confirmed by real- time PCR reactions performed with specific RT² quantitative PCR primer assay-SYBR Green Rat for AMBRA1 and ATG16L2 genes (SaBiosciences, USA); RPLP1 (ribosomal protein large, P1) and HPRT1 (hypoxanthine phosphoribosyltransferase 1) served as the internal controls. The following thermal cycling conditions were used: one cycle at 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Relative expression was determined using the $2^{-\Delta\Delta C_t}$ method [20]. Each sample was examined in duplicates, 3 biological samples were tested per each point.

1.6. *Transient Transfection*

FLAG-HDAC10 Plasmid was kindly provided by Dr. Hung-Ying Kao from the Case Western Reserve University School of Medicine, Cleveland, Ohio, USA [21]. As a negative control and for transfection efficiency we used the pEGFP-C1 vector expressing a green fluorescent protein (GFP) [17]. Successful transfection was validated by following GFP expression under a fluorescent microscope. Cells were transfected using the TransIT®-LT1 (Mirus, Madison, WI, USA) according to the manufacturer's recommendations.

For the Knockout experiment we used a set of four siRNAs specifically designed to silence HDAC10: siRNA #1 (ID 2150743), #2 (ID 2150744), #4 (ID 2150745), #5 (ID 2150746); (all designed and provided by Qiagen, Valencia, CA, USA). AllStars Neg. siRNA AF 488 (Qiagen) was used as a negative control (NC). This time cells were transfected using the HiPerFect (Qiagen) as the transfection

reagent. All transfection protocols were performed on Huh7 cells grown in 24- and 96-well plate format according to the manufacturer's instructions.

1.7. Assessment of Huh7 cell viability

Huh7 cells were plated in 96-well plates (cell culture clusters, polystyrene, Corning Tewksbury, MA, USA); cell viability was determined by adding 10% Alamar Blue, a metabolic indicator dye (AlamarBlue® assay; AbD Serotec, Raleigh, NC, USA), to each well together with the culture medium and incubating the plates at 37°C for 4 hours. Medium absorbance was measured with a fluorimetric reader (Synergy HT, BioTek Instruments, Winooski, VT, USA), with excitation at 544 nm and emission at 590 nm. The fluorescence in the study groups is expressed as a percentage of the fluorescence in the control group.

1.8. PAS (Periodic Acid Schiff) Staining Protocol for glycogen detection

The PAS staining kit was obtained from Merck (Whitehouse Station, NJ, USA) and was used on paraffin sections of rat liver from all experimental groups (n=3 per each group) according to the manufacturer's protocol.

1.9. Folch method for lipid content determination

Chloroform/methanol in a relative volume of 2:1 was added to homogenized 100 mg of liver tissue. After dispersion, the whole mixture was agitated during 15-20 min on an orbital shaker at room temperature. The homogenate tissue was centrifuged to recover the liquid phase, and then the solvent was transferred to a clean tube, washed with 200 µl water and vortexed for a few seconds to obtain two separate phases. The lower phase was transferred to a new tube and air dried. The amount of

lipid extract that was dried in the tube was weighed and calculated/100 mg of tissue [22].

1.10. Immuno-precipitation

Total cell lysates were incubated over night with anti-Hsp70/Hsc70 antibody following 30 min with protein A/G microbeads. The immune-complexes were washed and eluted using MultiMACSTM Protein A/G Kit according to the manufacturer's protocol (MACS Miltenyi Biotec, BG, Germany).

1.11. Fluorescent Microscopy

For immune-staining, cells were fixed onto glass slides with 4% paraformaldehyde in PBS and then permeabilized and blocked with 0.5% (vol/vol) Triton X-100, 1% BSA and 5% goat serum in PBS for 1h. Cells were incubated with the primary antibody anti-LC3 (Cell signaling, Danvers, MA, USA) overnight followed by PBS washing and 1hr incubation with a secondary antibody (Alexa Fluor® 488 Goat Anti-Rabbit; Thermo Fisher Scientific Inc. Waltham, MA, USA) at room temperature. Immunostained cells were viewed using the Immunofluorescence microscope (Olympus BX52TF, USA).

1.12. Flow cytometry cell cycle analysis

Cells were trypsinized and washed, fixed in 70% Ethanol (diluted in PBS) and kept at -20°C. One day before analysis the cells were washed with PBS and re-suspended in a buffer containing 0.1% Triton X-100 and 1mg/ml RNase A dissolved in PBS for overnight. 1 mg/ml Propidium Iodide (PI) (Sigma-Aldrich, Rehovot,

Israel) was used to label DNA. For analysis we used Cellquest software and the Fluorescent activated cell sorter (FACS) caliber (BD Biosciences, NJ, USA).

1.13. Statistical analysis

Results are expressed as mean $\pm$ SD. A one-way analysis of variance (ANOVA) was used to test for significant differences in variables across the groups. Where differences were found, Schaffé and Tukey's post-hoc tests were used to ascertain the location of the difference. Significance level was set at $p < 0.05$. SPSS v15 software (SPSS Inc) was used for statistical analysis of the data. The data of the realtime PCR analysis was checked using the SPSS statistical software using One sample Kolmogorov-Smirnov Test. Student's T Test was used when only two groups were compared.

2. Results

2.1. Effect of food restriction on tissue weight

We have previously shown that after a period of food restriction of 40% for 10 days the weight and bone length of the food- restricted rats (RES) was significantly lower than those of the control, unrestricted group fed ad libitum (AL) and that one day of re-feeding (enabling catch up growth; CU) led to a significant increase in body weight [18]. To determine if additional organs were similarly affected and that the rapid effect on weight was not due solely to the food load in the gut we determined the weight of additional internal organs. Fig. 1 shows that the weight of the internal organs from the RES rats was significantly less than that of the controls (AL). Already after one day of re-feeding there was a significant increase in the weight of the liver, kidneys and heart.

269

270 2.2. *Effect of re-feeding on the liver*

271 To investigate the basis for the rapid increase in liver weight, fat and glycogen
272 levels were measured. There were no significant differences in fat accumulation
273 between the groups (Fig. 2A); however, a significant decrease in glycogen content
274 was noted in liver sections taken from the food-restricted animals. Glycogen levels
275 very rapidly reverted to normal levels: after one day of re-feeding there was no
276 significant difference between the CU and AL groups (Fig. 2B).

277 Our measurement of serum levels of IGF-1 showed that food restriction
278 significantly reduced serum IGF-1 levels and that already after one day of re-feeding
279 levels had returned to normal [19], indicating proper liver function.

280

281 2.3. *Effect of food restriction on liver autophagy*

282 Autophagy is a highly regulated cellular process that mediates the degradation
283 of intracellular components, including macromolecules and organelles within the
284 lysosomes. It is activated in response to diverse forms of stress and physiological
285 conditions, including food deprivation, hyperthermia and hypoxia [23-25]. To
286 determine if the food restriction protocol of our study led to autophagy in the liver we
287 used the Rat Autophagy RT² Profiler™ PCR Array, which includes probes for 84
288 genes involved in autophagy.

289 The expression level of 24 genes was greater in the food restriction group than
290 in the controls (RES vs. AL). It is of note that of these, 15 were reduced back to
291 normal levels already after one day of re-feeding (CU) (Table 1 and supplementary
292 tables 1 and 2). Results of the array were further verified using realtime PCR for the
293 two genes which expression was mostly affected: Activating Molecule in Beclin-1-

Regulated Autophagy (Ambra1) and Autophagy Related 16-Like 2 (Atg16L2), which are directly related to autophagy regulation. The expression levels of Atg16L2 and Ambra1 in the liver were significantly greater in the RES group compared to the AL and CU groups (Fig 3A &B; for Ambra1 only a tendency was observed, $p=0.06$).

P62/SQSTM1 protein is involved in autophagy-lysosome degradation: by binding directly to Atg8/microtubule associated protein light chain 3 (LC3) it facilitates degradation of ubiquitinated protein aggregates and is degraded during autophagy induction [26]. Thus a reduction in its level may be another indication of autophagy. Indeed, the P62 protein level was significantly lower in the liver of the RES animals compared with the levels of the control group (AL), with no significant change after one day of re-feeding (CU) (Fig. 3C). These results suggest that food restriction stimulates the autophagy process in the liver and that this process is partially reversible already after one day of re-feeding.

2.4. Effect of nutrition on the level of HDAC proteins in liver cells

We have previously found that the levels of SIRT1 [17] is increased in the EGP of RES animals and rapidly reduced by re-feeding. These results are in line with our previous observation of global changes in gene expression within the EGP [18], suggesting epigenetic regulation by HDAC. To determine if other HDACs are also affected by food restriction the profile of the HDACs protein level in liver tissue from the three groups (AL, RES and CU) was tested. As shown in Fig. 4, all eleven zinc binding HDACs were detected in the liver. After 11 days of food restriction the protein levels of HDAC1, 3, 6, 7 and 10 were increased in the liver (RES). It is of note that one day of re-feeding with an unlimited amount of the same chow (CU) led to a reduction of the level of HDAC1, 6, 7 and 10.

319

320 **2.5. *Effect of HDAC10 on cell viability in vitro***

321 In our further studies we focused on HDAC10. Hepatoma cell line (Huh7)
322 were subjected to serum depletion to simulate food restriction and obtain growth
323 inhibition. Fluorescence-activated cell sorting (FACS) analysis (Fig. 5A) shows that
324 following 48h hours of serum depletion there was a 10% increase in accumulation of
325 cells in the G0/G1 phase ($P<0.05$), compared to cells grown in the presence of 10%
326 FCS. Under these conditions the level of HDAC10 protein was increased, similar to
327 the increase observed in the livers of the RES animals (Fig. 5B).

328 To further study the connection between HDAC10 and growth inhibition, a
329 plasmid expressing HDAC10 [21] was inserted into the Huh7 cells. Fig.6A shows the
330 increase in HDAC10 protein level following transfection. Cell viability measured
331 using the Alamar Blue metabolic dye demonstrated a decrease in viability (10%;
332 $p<0.05$) in cells transfected with the HDAC10 plasmid (Fig. 6B), suggesting a role for
333 HDAC10 in regulating cell viability.

334

335 **2.6. *Over-expression of HDAC10 increases autophagy in Huh7 cells***

336 Autophagy was monitored by measuring the conversion of LC3-I to LC3-II
337 (LC3-phosphatidylethanolamine conjugate, localized to the autophagosome
338 membrane) and by P62 protein level. Indeed, over-expression of HDAC10 in Huh7
339 cells significantly increased the ratio of LC3-II/LC3-I and decreased P62 protein level
340 (Fig.6C, D&E respectively). Immunofluorescent staining of LC3 antibody show an
341 increase of autophagosomes formation after 48h of serum starvation and 72h of
342 HDAC10 overexpression (Fig.6F).

343

2.7. *Inhibition of mTOR promotes autophagy by induction of HDAC10*

mTOR (mammalian target of Rapamycin) is an evolutionarily conserved serine/threonine protein kinase that promotes anabolic programs such as mRNA translation and protein synthesis and represses catabolic activities such as autophagy [27]. Under conditions of nutrient starvation, mTOR is inactivated, protein synthesis is inhibited, cell growth is arrested and autophagic protein degradation takes place [28-30]. To determine whether inhibition of mTOR mediates the effect of nutrient depletion (food restriction *in vivo* or serum starvation *in vitro*) on the level of HDAC10, cells were treated with the known mTOR inhibitor Rapamycin for 72 hours (10nM) [31]. Indeed an increase in the level of the HDAC10 protein was found, suggesting an association between mTOR-HDAC10 and autophagy (Fig. 7A). HDAC10 levels were suppressed by using siRNA (Fig.7B&C). Assessment of the effect of Rapamycin on cell viability under these conditions showed that reduction of HDAC10 protein levels partially prevented Rapamycin-induced growth attenuation (Fig.7D). Furthermore, reduction in HDAC10 level, resulted in a partial decrease in the Rapamycin induced autophagy as shown in the ratio level of LC3-II/ LC3-I (Fig.7E &F) and inhibited the induction of Atg16L2 and Ambra1 mRNA expression level (Fig.8).

2.8. *HDAC10 directly interacts and deacetylates heat shock protein 70 (Hsp70)*

The major non- histone target proteins of HDAC10 are the Hsp70 protein family. Hsc70, a constitutively expressed, cognate protein of the Hsp70 family, is known to have regulatory role in the autophagy process [32]. We hypothesized that HDAC10 may exert its activity of controlling autophagy by interacting and de-

acetylating the Hsc70 protein. We therefore transfected Huh7 cells with the FLAG-HDAC10 plasmid [21] and by using immuno-precipitation and immune-fluorescence we showed that HDAC10 interacts with Hsp70 proteins (Fig.9A&C). Using immunohistochemistry staining we also found that both proteins co-localize in the cytoplasm and perinuclear regions of the cells (Fig.9C), suggesting a direct interaction between HDAC10 and Hsp70/Hsc70 protein. In addition, we demonstrate deacetylation of Hsp70/Hsc70 in extracts obtained from cells overexpressing HDAC10, showing indeed that Hsp70/Hsc70 is a target for HDAC10 deacetylase activity (Fig.9B).

3. Discussion

The present study shows that HDAC10 in the liver is influenced by the nutritional status. It is significantly increased by 11 days of food restriction of 40% and rapidly reduced by re-feeding. Furthermore, in an *in vitro* system we have shown by manipulating HDAC10 protein level that HDAC10 is involved in autophagy process by deacetylating Hsp70 protein family (a model is described in Fig.10). To the best of our knowledge, this is the first study showing that the HDAC10 protein level is increased by food restriction and is involved in nutrition- induced autophagy in both animal and *in vitro* models of food restriction.

In the present study we found a significant increase in the expression level of several autophagy-related genes in the livers of the RES animals, followed by a reduction in the expression of the same genes once nutrition was replenished. Among them, we found Atg16L2 and Atg5 which are known to be associated with autophagosomes formation [33, 34] and Ambra1 and Bak1 which are involved in the crosstalk between autophagy and apoptosis [35]. Stress stimulus can often activate

autophagy and apoptosis, in such cases, autophagy usually takes place first and inhibits the induction of apoptosis by engulfing pro-apoptotic proteins, such as caspase8 and Bak1, and damaged mitochondria to prevent the release of cytochrome C [36]. When the intensity or duration of stress persists beyond the cell's tolerance, suicidal cell death programs are activated. Apoptosis then inhibits the autophagy machinery by degrading Ambra1, Beclin 1, Atg3 and Atg5 through caspases and calpains [37-39]. It seems that autophagy is used by the cells when a minor insult is detected, but when the insult (for example, food restriction) is enhanced, cell death may occur [40]. In our study, probably because the restriction was partial, and as re-feeding had such a rapid and robust effect on liver weight and function, we believe that autophagy did not lead to apoptosis. It is possible that under these conditions, autophagy provided the cells with required building blocks and energy, and protected the cells from death fate.

A role for histone modification in autophagy was not obvious at first, as cells without a nucleus were shown to undergo autophagy as well [41]. However, in recent years several studies have shown that treatment of cells with HDAC inhibitors increased the level of cellular acetylation as well as autophagy [42], providing compelling evidence that the nucleus is also a regulator of autophagy. Many promoters contain ATGs that are protected from a global hypo-acetylation and therefore remain accessible, allowing their selective transcription under conditions of food shortage (reviewed by [43]). In the present study we have shown that several HDACs were increased by food restriction in the liver: HDAC 1, 3, 6, 7 and 10. Upon review of the scientific literature we found publications showing that HDAC1 is an inhibitor of autophagy, as inhibition, genetic knock down or disruption of HDAC1 all induced significant levels of autophagic vacuole formation and conversion of LC3-I to

LC3-II [44]. Studies in HDAC3 Knockout mice showed that mTOR was repressed in chondrocytes lacking HDAC3, demonstrating that it is required for normal mTOR function [45]. No further information on the role of HDAC7 in autophagy was found.

The two HDACs that could have been associated with induction of autophagy are HDAC6 and 10. HDAC6 and HDAC10 are structurally similar and may be functionally associated through their effect on Hsp. The main non-histone target proteins of HDAC6 and HDAC10 are Hsp90 and Hsp70 respectively [10]. Hsp90 is a co-chaperon of Hsp70 [46]; Hsc70, a constitutively expressed, cognate protein of the Hsp70 family, has a regulatory role in autophagy [32]. Furthermore, HDAC6 has been defined as a basal component of the autophagic machinery [47], and HDAC10 was recently shown to promote autophagy-mediated survival of neuro-blastoma cells [48]. Knockdown of HDAC10 effectively disrupted autophagy and induced accumulation of autophagosomes as well as P62/sequestosome1 protein level in neuroblastoma cells [48]. In this study, we have shown that by changing the level of HDAC10 we can affect autophagy significantly.

HDAC10 was first described by several groups in 2002 [21, 49-51]. It was characterized as the second member of the HDAC IIb subfamily, and was found to be similar in structure to HDAC6. HDAC10 is widely expressed and can be found in both nuclear and cytoplasmic cellular fractions. Its function was unknown until 2011, when several studies found that HDAC10 is deregulated in several cancer types, including CLL [52, 53], neuroblastoma [48], and cervical cancer [54].

HDAC10 was shown to have multiple targets, including histone H3 and H4. By binding to the metalloproteinase (MMP)-2 and -9 promoter regions, and deacetylating H3 and H4 histones in this region, it inhibits the binding of RNA polymerase II and leads to inhibition of MMP- 2 and 9 expression [54]. In other

studies it was found to interact with the transcriptional regulators, paired box protein 3 (Pax3) and KRAB-associated protein 1 (KAP1) [55] while in neuroblastoma, as well as in the current study, it was found to deacetylate the chaperone protein Hsc70 [48].

We have found that HDAC10 responds to the nutritional status in normal animals. Interestingly, we have found that the level of HDAC10 was increased by food restriction, while Funato et al [14] showed that after 16h fasting, HDAC10 mRNA levels decreased in medial hypothalamus of mice. These differences may be due to different protocols of the food restriction, the species or the organ studied.

Cells have a complex sensing system to ensure that they do not grow in the absence of the nutrients that supply the energy and building blocks to support that growth, leading to protein synthesis inhibition. In spite of this global reduction in protein synthesis, we found a marked increase in the level of several proteins of the HDAC family, including SIRT1 and SIRT6 ([17] and Pando R, unpublished data), as well as HDAC 1, 3, 6, 7 and 10. The increase in SIRT 1 and 6 under conditions of food restriction was also reported by others [15, 16, 56] and was shown to be associated with increased longevity. However, the increase in HDAC1,3,6,7 and 10 was not previously reported.

The cell's translational machinery for the synthesis of proteins is activated by mTOR, an evolutionarily conserved serine/threonine protein kinase [27]. mTOR is regulated by insulin and nutrients, including glucose and amino acids, particularly leucine, as well as a variety of cellular stresses. In some cell types, it is activated by amino acids alone; in others, amino acids collaborate with growth factors such as insulin [52, 57]. When nutrient levels or growth factors are reduced, mTOR is inactivated and autophagic protein degradation takes place. Mutations in one of the mTOR machinery components, Lamtor 2, was reported to lead to an immune disease

that is associated with a significant growth deficit [58] supporting a role for the mTOR complex in growth regulation.

Autophagy is a catabolic process that results in autophagosome-dependent lysosomal degradation of bulk cytoplasmic content, abnormal protein aggregates, and excess of damaged organelles. It occurs continuously at basal levels during the homeostatic turn-over of cytoplasmic components required to meet metabolic demands. It is induced mostly by stress stimuli, especially under energy-restricted environmental conditions, and is inhibited by nutrient sufficiency. Autophagy involves dynamic membrane rearrangement, controlled by a set of ATG proteins. Cells degrade the cytosolic content by the formation of a double-walled vesicular structure that eventually fuses with lysosomes. In this manner, energy and building blocks can be generated from the cell's own protein and lipid stores. Nutrient-stimulated activation of mTOR leads to the phosphorylation and inactivation of components of the autophagy pathway [59].

We have shown that a number of HDACs are increased by food restriction and GH deficiency, and that HDAC10 is involved in autophagy. Deciphering the role of epigenetic regulation in the epigenetic-growth connection may open a new era of research and pave the way for the development of new forms of treatment for children with growth disorders. It will be fascinating to follow this research in the coming years and to translate the knowledge gained to clinical benefits.

Disclosure of Conflicts of Interest

All authors state that they have no conflict of interest.

Acknowledgment

The authors are grateful to Ms. Ruth Fradkin for editing the paper. This work was performed in partial fulfillment of the requirements for a Ph.D. degree by Galit Pinto for the Sackler Faculty of Medicine, Tel Aviv University, Israel.

Funding:

This study was partially supported by the Bill and Melinda Gates Foundation (Grand Challenge Exploration round 8).

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Legends to figures and tables

Fig. 1. Effect of nutrition on the weights of internal tissues at sacrifice; * $p < 0.05$ compared with AL, ** $p < 0.05$ compared with RES. **Numbers indicate the average weight.**

Fig. 2. Effect of re-feeding on metabolic parameters in the liver. **(A)** Fat content extracted from rat's liver from all experimental groups (presented as mg fat/100 mg liver tissue). **(B)** PAS staining for glycogen detection.

Fig. 3. Autophagy induction in the liver of food restricted animals. Real-time PCR for autophagy-related genes: **(A)** Atg16L2 **(B)** Ambra1 (RPLP1 served as control). **(C)** Protein level of p62 in animals' livers; * $p < 0.05$ compared with AL; \$ $p = 0.06$ compared with RES.

Fig. 4. Effect of food restriction and re-feeding on the level of HDACs proteins extracted from the liver of all experimental groups; * $p < 0.05$ compared with AL, ** $p < 0.05$ compared with RES.

Fig. 5. Effect of serum depletion on Huh7 cells. Huh7 cells were subjected to 48hrs serum starvation. **(A)** Cell cycle as monitored by FACS analysis. **(B)**- HDAC10 level was monitored by Western blot (beta actin served as loading control).

Fig. 6. Growth inhibition and induction of autophagy in response to HDAC10 over-expression. Huh7 cells were transfected with a plasmid expressing HDAC10. **(A)**

Western blot showing HDAC10 in control and transfected cells (β -actin was used as a loading control). **(B)** Viability measured 72h after cell transfection; $*p<0.05$ compared to control. **(C)** Western blot showing P62 protein level and the two conformations of LC3 upon HDAC10 overexpression. **(D)** Quantification of P62 protein level; $*p<0.05$. **(E)** Quantification of the ratio of LC3-II/LC3-I relative to actin; $*p<0.05$. **(F)** Autophagosome formation was visualized in cells after serum starvation or HDAC10 over-expression. Punctate staining is indicative for the redistribution of LC3 to autophagosomes.

Fig. 7. mTOR mediates the effect of nutrient depletion on HDAC10 level. **(A)** Huh7 cells were treated with 10nM Rapamycin for 72 hours and the level of HDAC10 was measured by western blot, $*p<0.05$. **(B)** Western blot of whole-cell lysates of Huh7 cells transfected with siRNAs#2 targeting HDAC10, showing HDAC10 protein level after 48h of Rapamycin treatment and 72h of transfection. **(C)** Quantification of relative protein level of HDAC10 compared to actin **from three different experiments; $*p<0.05$** . **(D)** Viability assay performed after 48h of Rapamycin treatment and 72h of siRNA-HDAC10 #2 transfection; $*p<0.01$ $**p<0.05$ compared to control. **(E)** Representative results of the effect of Rapamycin treatment in cells transfected with siRNA on the ratio of LC3-II to LC3-I. **(F).** Quantification of the ratio of LC3-II/LC3-I; **from three different experiments $*p<0.05$** .

Fig. 8: The effect of Rapamycin on Atg16L2 and Ambra1 is partially inhibited by siRNA-HDAC10. Real-time PCR for autophagy-related genes: **(A)** Atg16L2 **(B)** Ambra1 (HPRT1 served as control). $*p<0.05$ compared to Rapamycin +NC (siRNA negative control)

708

709 **Fig. 9:** HDAC10 physically interacts with Hsp70/Hsc70 and de-acetylates it. **(A)** Co-
710 immuno-precipitation of HDAC10 with Hsp70/Hsc70: Whole-cell extracts of Huh7
711 cells transfected with FLAG- HDAC10 plasmid, were immuno-precipitated with an
712 antibody directed against Hsp70/Hsc70. On western blot, HDAC10 was detected
713 using an antibody against the FLAG tag; Hsp70/Hsc70 were detected using the same
714 antibody used for IP. **(B)** HDAC10 over expression leads to reduced lysine acetylation
715 (Ac-Lys) of Hsp70/Hsc70. **(C)** Immunofluorescent detection of HDAC10 and
716 Hsp70/Hsc70: Cells transfected with FLAG-tagged HDAC10 were immune-stained
717 with anti-FLAG tag antibody (red) and anti Hsp70/Hsc70 antibody (green).
718 Cytoplasmic co-localization is shown by overlay in yellow.

719

720 **Fig. 10:** Model suggested: food restriction inhibits mTOR activity (↓), inducing
721 HDAC10 protein level (↑) which leads to an increase in the autophagy process (↑)
722 and growth arrest.

723

724 **Table 1.** Autophagy gene expression following food restriction and re-feeding (Rat
725 Autophagy RT² Profiler™ PCR Array).

726

727

Table 1

Genes which level was increased in the livers of the RES group compared with AL and CU.		
Gene Symbol	Description	Function
AMBRA1	Autophagy/beclin 1 regulator 1	Co-Regulators of Autophagy and Apoptosis
ATG16L2	ATG16 autophagy related 16-like 2	Responsible for protein transport
ATG5	ATG5 autophagy related 5 homolog	Involved in autophagic vacuole formation
BAK1	BCL2-antagonist/killer 1	Co-Regulators of Autophagy and Apoptosis
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Co-Regulators of Autophagy and the Cell Cycle
ESR1	Estrogen receptor 1	Autophagy in Response to Other Intracellular Signals
FAS	Fas (TNF receptor superfamily, member 6)	Co-Regulators of Autophagy and Apoptosis
GABARAPL1	GABA(A) receptor-associated protein like	Responsible for protein transport
HTT	Huntingtin	Co-Regulators of Autophagy and Apoptosis
LAMP1	Lysosomal-associated membrane protein 1	Autophagy Induction by Intracellular Pathogens
PIK3CG	Phosphoinositide-3-kinase, catalytic,	Autophagy in Response to Other Intracellular Signals
RAB24	RAB24, member RAS oncogene family	Responsible for protein transport
UVRAG	UV radiation resistance associated gene	Activates the Beclin1-PI(3)KC3 complex
WIP1	WD repeat domain, phosphoinositide interacting 1	Involved in autophagic vacuole formation

A **B**

Food Restriction

mTOR

HDAC10

Autophagy

Growth Arrest