ASPA Expression is Required to Increase UPC1 Upon Overexpression of Nat8-L in Brown Adipocytes

The Austrian Marshall Plan Foundation 2015

Jenny Paredes, M.S.
Student
The City University of New York

Dr. Juliane Bogner-Strauss
Professor
Graz University of Technology

September 13th of 2015

ASPA Expression is Required to Increase UPC1 Upon Overexpression of Nat8-L in Brown Adipocytes

Paredes Jenny E., Pelzmann Helmut J., and Bogner-Strauss Juliane G.

Molecular Biology Department, The City University of New York, NY, USA; 2 Institute of Biochemistry, Graz University of Technology, Graz, Austria. September, 2015.

Abstract

Brown adipocytes play a primary function in body heat generation in mammals as they contain multiple lipid droplets and high numbers of mitochondria, making them a promising target for possible obesity intervention. Previous studies described the function of the enzyme N-Acetyltransferase 8-like (Nat8l) in brown adipocytes (1) and showed that overexpression of this gene resulted in increased brown phenotype and in a massive increase of the brown marker gene Uncoupling Protein 1 (UCP-1). In this part of the project, we aimed to elucidate the role of the enzyme aspartoacylase (Aspa), which converts NAA into aspartate and acetate, in immortalized brown adipocytes (iBACs) overexpressing Nat8l. Nat8l uses acetate and aspartate to produce N-acetylaspartate (NAA), the substrate for Aspa activity in the NAA pathway. For this assessment, the protein production and gene expression UCP-1 was chosen as the main parameter of evaluation. In this study, we demonstrated that the increased expression of UPC-1 in Nat8-L over-expressing cells is Aspa-dependent. We hypothesize that increased expression of UCP-1 in brown adipocytes through the NAA pathway requires the full or enhanced enzymatic activity Aspa additionally to the overexpression of Nat8-L.

Introduction

Understanding the biological and metabolic mechanisms involved in brown adipogenic differentiation could open different strategies for induction of glucose and Free Fatty Acid (FFA) degradation in obese humans. Brown adipocytes can be activated upon physiological stimuli such as cold for heat generation, by dissipating energy in form of heat, and excess fat diet. It has also been shown that there is a negative correlation between weight gain, obesity and brown fat content. Interestingly, Nat8l overexpression leads to the increase of UCP-1 expression in brown adipocytes and consequently it increases the brown adipogenic phenotype, enhances the lipid turnover and the oxidative potential brown adipocytes.

NAT8L utilizes acetyl-CoA as a substrate to produce NAA, which is then hydrolyzed by the aspartoacylase (Aspa) to yield acetate, which can in turn be used for FFA synthesis. Thus, these FFA could lead to the activation of UCP1 expression.

Previous findings include:

A. B. C.

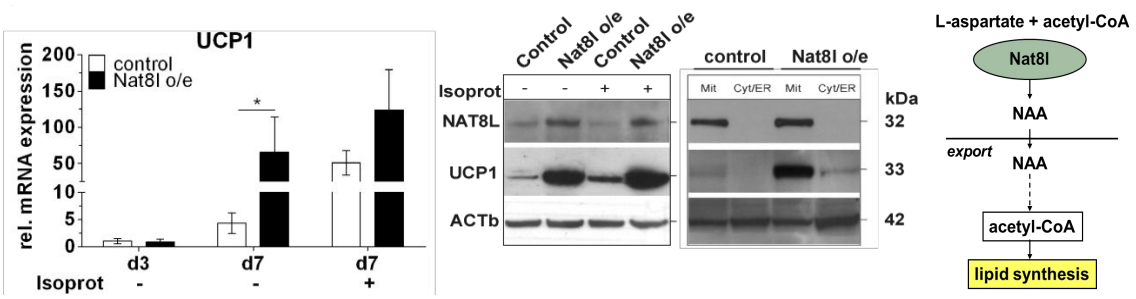


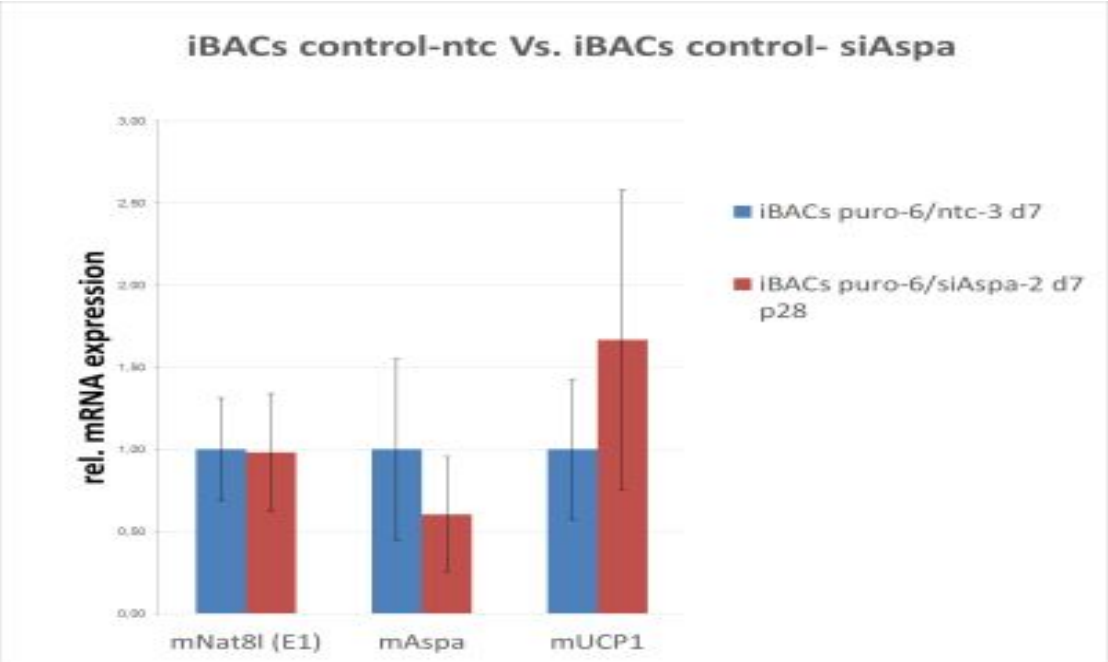
Figure 1.

A. UCP-1 expression is significantly increased in differentiated cells by day 7 and this expression is enhanced in cells over expressing Nat8l.
B. UCP-1 and NAT8L are expressed in the mitochondria of brown adipocytes.
C. Model proposing the NAA pathway as an alternative acetate source for cytosolic acetyl-CoA production.

Results

1. Replicate 1: In order to investigate the role of Aspa silencing in brown adipocytes over expressing Nat8l in terms of UCP-1 expression; relative mRNA expression of Nat8l, UCP-1 and Aspa were measured by qPCR and protein production of UCP-1 and ASPA by western blots.

A.



B.

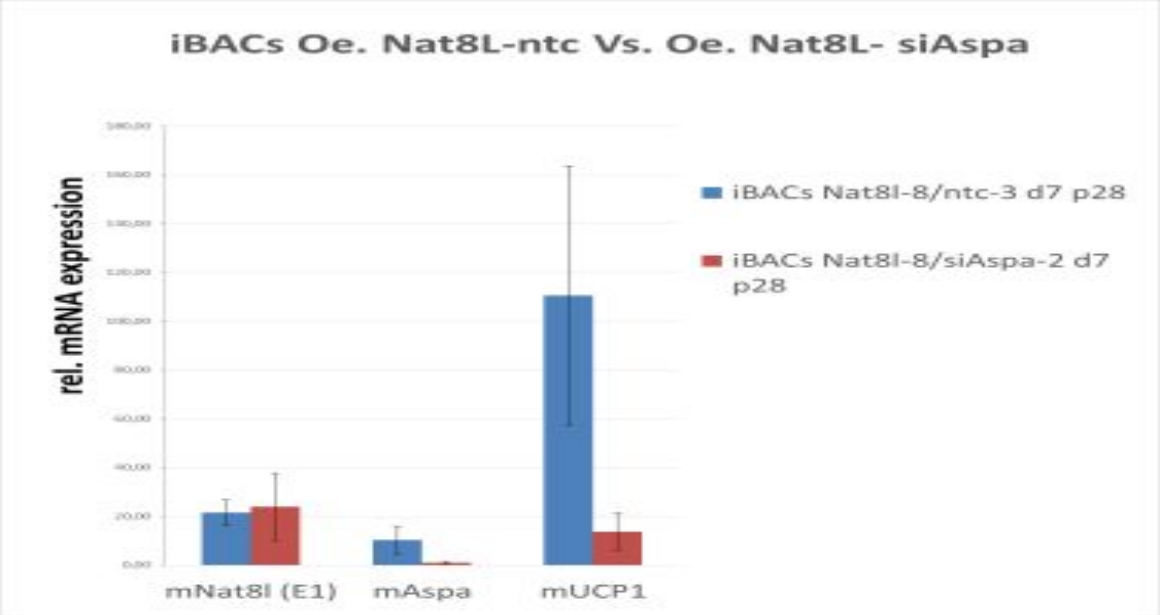
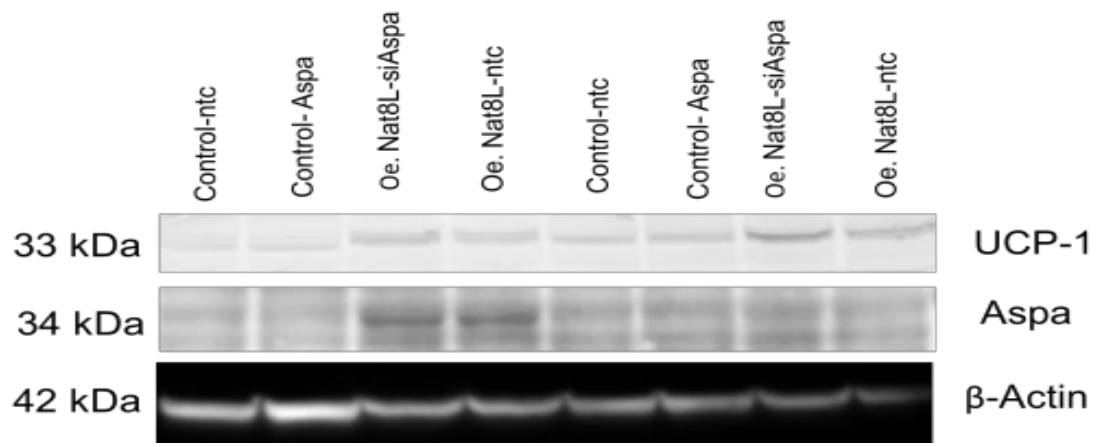


Figure 2.

A. Aspa silencing was successful in control cells.

B. Aspa is silenced in Nat8l o/e iBACs and the increased UCP-1 upregulation is blunted when Aspa is silenced. However, the standard variation between the strains replicates suggest that new biological replicates are necessary to confirm this findings.

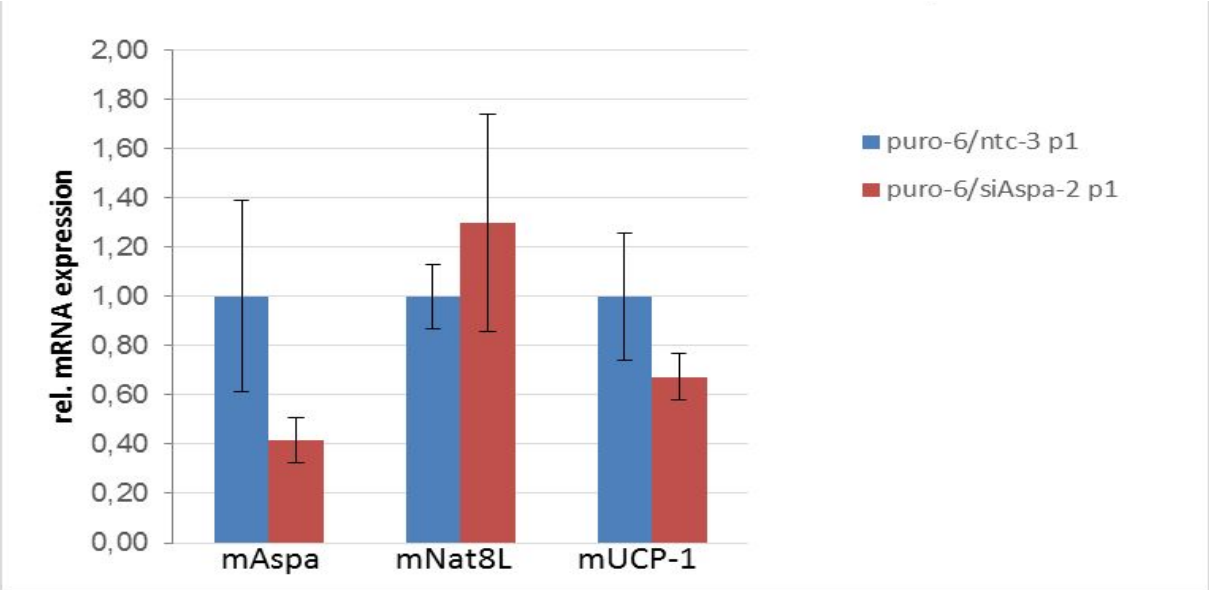
C.



C. The UCP-1 and Aspa protein expression in the used cell lines (all differentiated to brown adipocytes) was inconclusive. The western blots results cannot be taken into consideration as protein expression across the cell lines and duplicates are inconsistent. Beta-Actin was used as a loading control and it can be concluded that the protein profiles must be repeated.

2. Replicate 2: The overall objective of Replicate 2 was to corroborate the results obtained from Replicate 1 and to reduce the standard variation across samples. This experiment followed the same procedures, described in the methods section, as Replicate 1 for iBACs adipocytes.

A. Control cells vs. siAspa



B. Oe. Nat8l-Control vs. Oe. Nat8l-siAspa

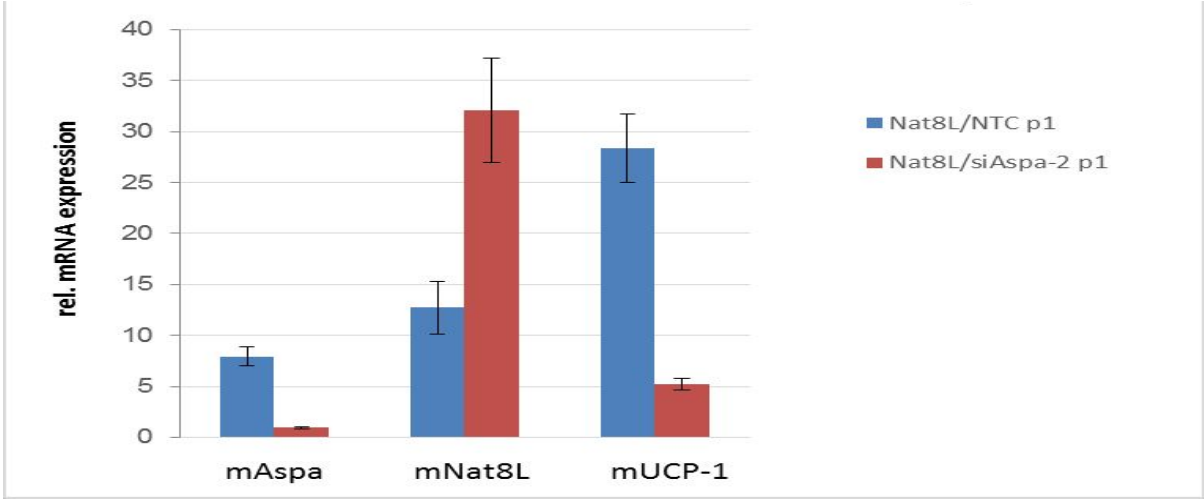


Figure 3.

A. Aspa silencing resulted in a reduction of UCP-1 mRNA expression in comparison to control adipocytes.

B. Aspa is silenced in Nat8l o/e iBACs and the increased UCP-1 upregulation is blunted when Aspa is silenced. It can be concluded that in order to increase the gene expression levels of UCP-1 the overexpression of Nat8l must be accompanied by Aspa expression.

A.

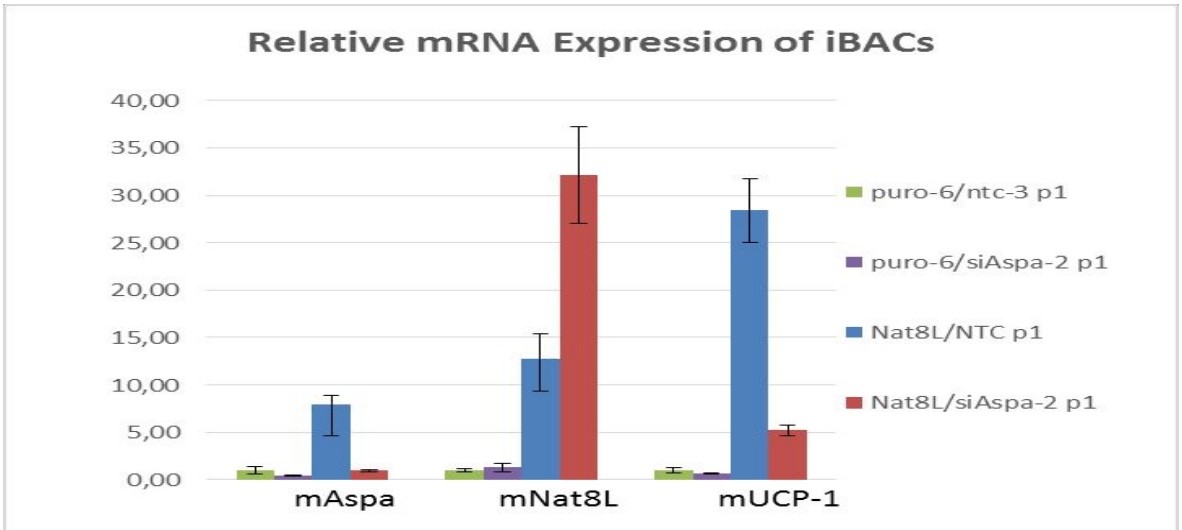
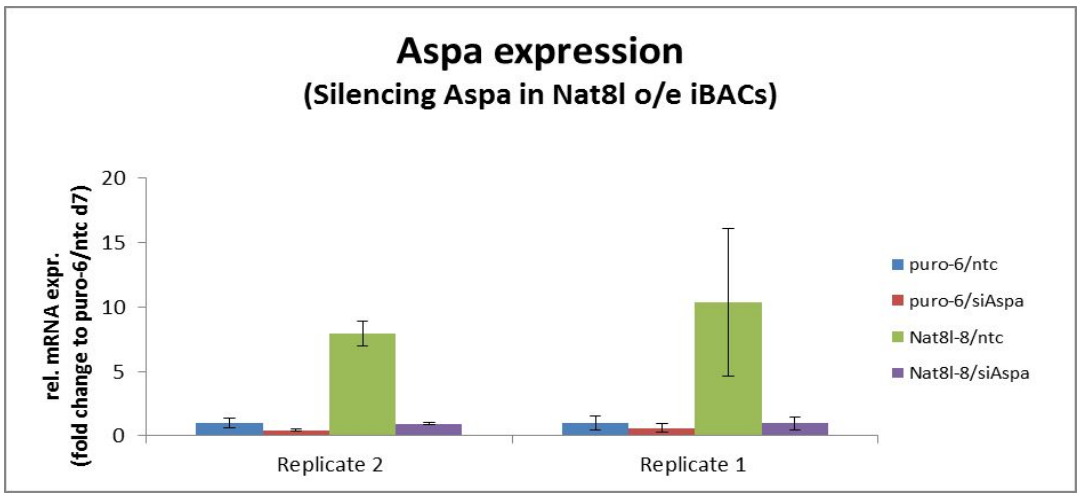


Figure 3.

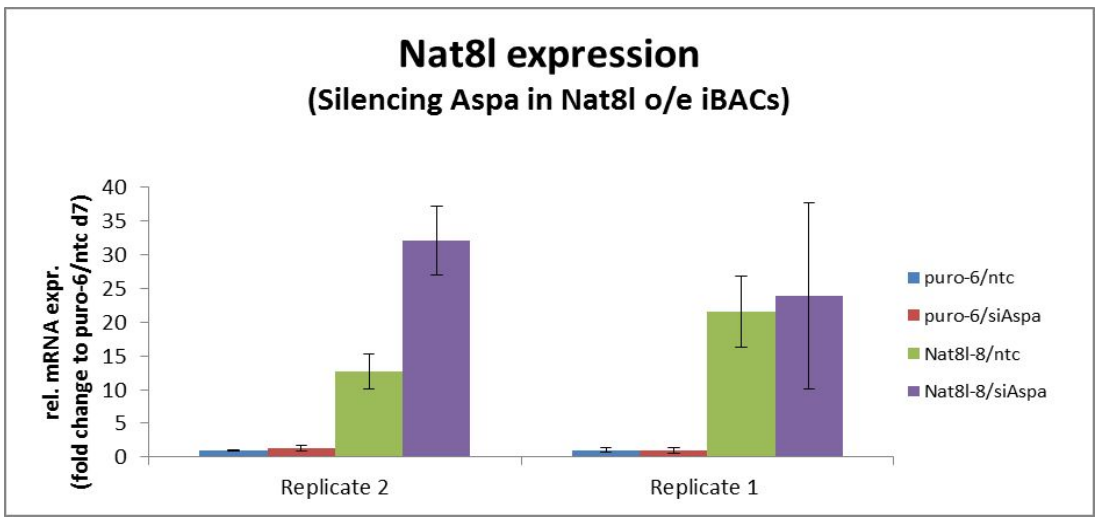
A. The relative mRNA expression of Aspa, Nat8l and UCP-1 can be observed across strains in this graph. It can be concluded that gene expression of the 3 targeted genes is enhanced in the overexpressing Nat8l cell lines.

3. Replicates 1 & 2 mRNA expression: The gene expression profiles of the 2 biological and technical replicates were graphed together for comparison.

A.



B.



C.

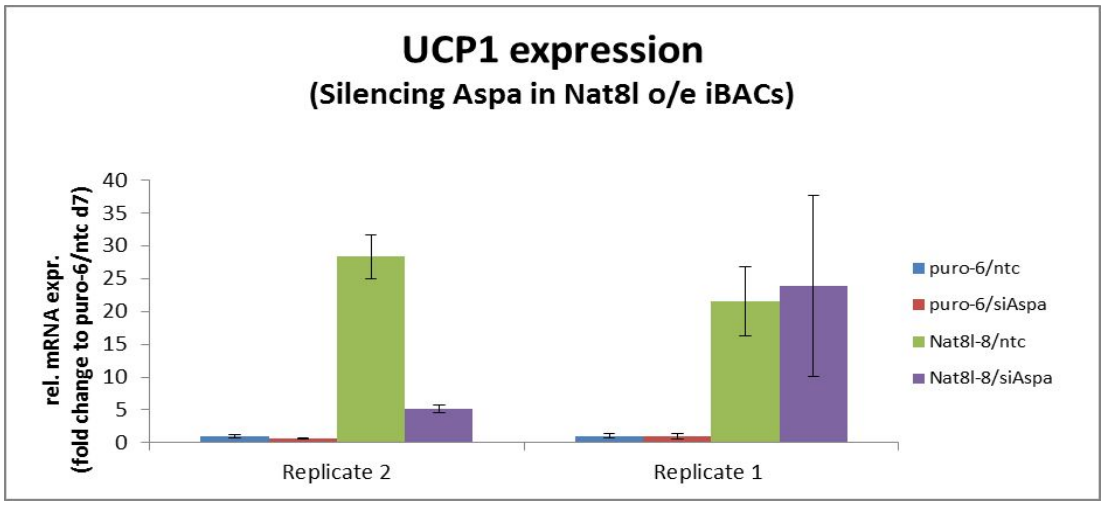
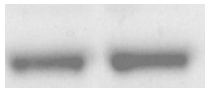
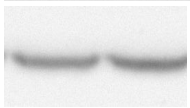
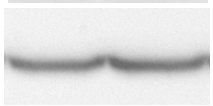
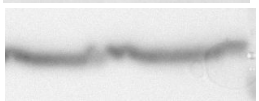


Figure 4.

A,B,C. Replicates 1 & 2 showed a similar gene expression pattern of Aspa, Nat8l and UCP-1. Nevertheless, it can be observed that the standard variation in Replicate 1 is much higher than Replicate 2 and therefore unreliable. It could be concluded that the results obtained from Replicate 2 are significant and follow the initial hypothesis of this project.

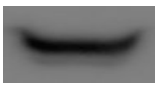
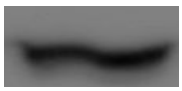
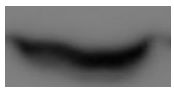
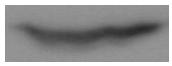
4. Protein expression of UCP-1 and Aspa in iBACs overexpressing Nat8l: The protein production was detected by the western blot technique. Table 1 shows protein profiles for passages 1 and 2, and Table 2 demonstrates passage 3 for each of the iBACs strains evaluated in this project. 70ng of protein were loaded onto 12% gels and runned in MOPS buffer. The imaging process was done through chemiluminescence.

Table 1.

Protein	Control	Cntl-siAspa	Oe.Nat8l	Oe. Nat8l-siAspa	kDa
UCP-1					33
B-Actin					42
ASPA					34

UCP-1 expression is increased in brown adipocytes that have overexpression of Nat8l as well as Aspa activity. In the absence of Aspa, UCP-1 protein levels are notoriously reduced. Beta-Actin detection included as loading control. On the contrary, expression of Aspa is inconclusive; the protein levels differ even among strains' duplicates. Replicates of Aspa expression are suggested for further experiments.

Table 2. Replicate 3: UCP-1 and ASPA protein expression of Passage 3 of iBACs strains. B-Actin loading controls are included for each target. As observed in Table 2, UCP-1 protein levels are increased in overexpressing Nat8l cells that also have Aspa activity. In conjuction, this replicate also confirmed that the inconclusive protein profile of Aspa in the strains observed in previous samples persisted in replicate 3. Levels of ASPA protein don't seem to be reduced in the strains with the silencing of the Aspa gene.

Protein	Control	Cntl-siAspa	Oe.Nat8l	Oe. Nat8l-siAspa	kDa
UCP-1					33
B-Actin					42
ASPA					34
B-Actin					42

Conclusions:

- Overexpression of Nat8l in iBACs leads to an increase in gene expression of the brown marker UCP1.
- We observed that the increase of UCP1 expression in overexpressing Nat8l cells is dependent on Aspa activity.
- The NAA pathway could serve as an additional acetyl-CoA-metabolizing mechanism in brown adipocytes. Overexpressing Nat8l and expression of Aspa in brown adipocytes could result in increased acetyl-CoA flux via the NAA pathway and higher cytoplasmic FFA anabolism, resulting in elevated triglycerides' synthesis. A parallel increase in lipolysis followed by an activation of β-oxidation can then restore acetyl-CoA back to the mitochondria, inducing

lipid turnover and the oxidative potential of the brown fat cell and thereby boosting the brown adipogenic phenotype.

- The results from the protein production do not correlate with the gene expression patterns observed in this project. Further replicates of Aspa protein expression in similar experimental conditions are recommended.

Methods:

An immortalized brown adipocyte cell line (iBACs), was used to test over-expression of Nat8-L accompanied by silencing of ASPA in terms of UCP-1 gene expression and protein levels. Cells were induced to differentiate at the day of confluence with 0,5 mM 3-isobutyl-1-methylxanthine, 0,05nM dexamethasone, 20nM insulin, 1 nM triidthyronine and 0.125 nM indomethacin. Two days after induction, medium was changed to maintenance medium containing 20 nM insulin and 1 nM triiodothyronine and cells were maintained in this medium till harvesting.

Quantification of protein production was assessed by Western Blot Analysis with the following antibodies: anti NAT8-L anti-ASPA and anti-UCP-1 and anti-β-Actin. Gene expression was measured by Real Time PCR; mRNA was assessed as described in (Bogner-Strauss J.G., 2010) and gene expression was normalized to the housekeeping gene TFIIβ.

Acknowledgements:

I thank Ariane R. Pessentheiner, PhD for her mentoring and the technical assistance from Thomas Schreiner and Wolfgang Krispel.

Grant Support from: The Austrian Marshall Plan Foundation, The LSAMP-CUNY Program and The Austrian science fund FWF: Doktoratskolleg DK-MCD W1226 and NAT8L P27108.

References:

(1) Pessentheiner AR & Pelzmann HJ et al., J Biol Chem 2013.

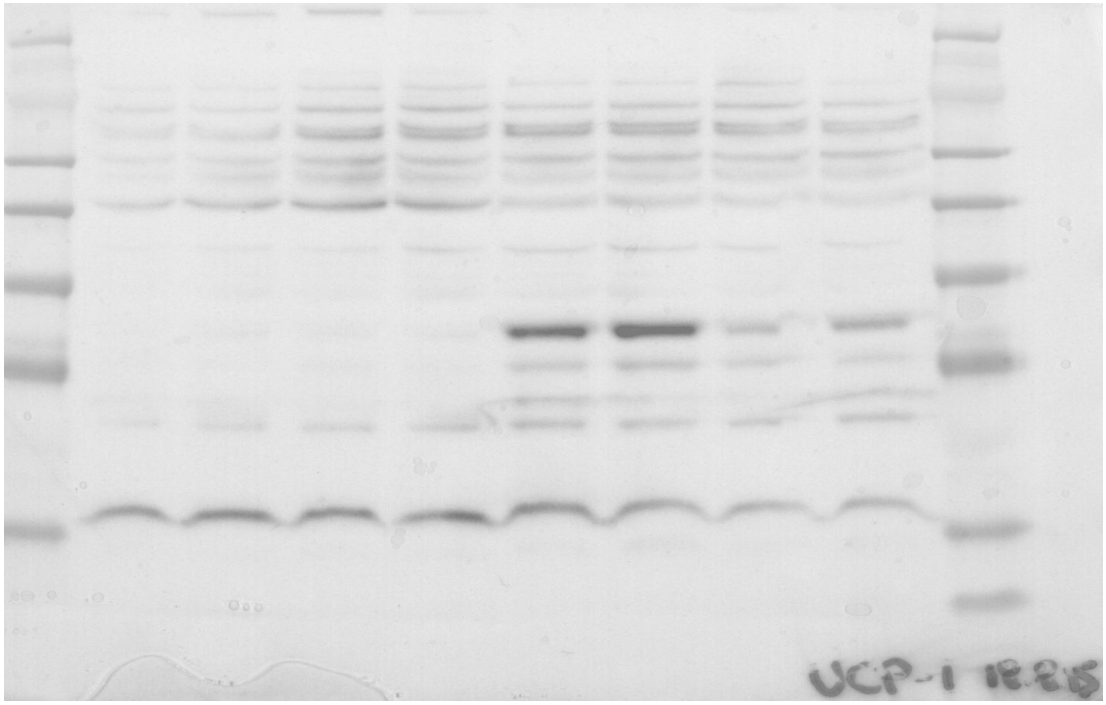
Annexes:

1. Raw data from qPCR for Replicates 2 and 3. Three passages of each of the iBACs strains (control, control-siAspa, Oe.Nat8l and Oe.Nat8l-siAspa) were assessed for relative mRNA expression.

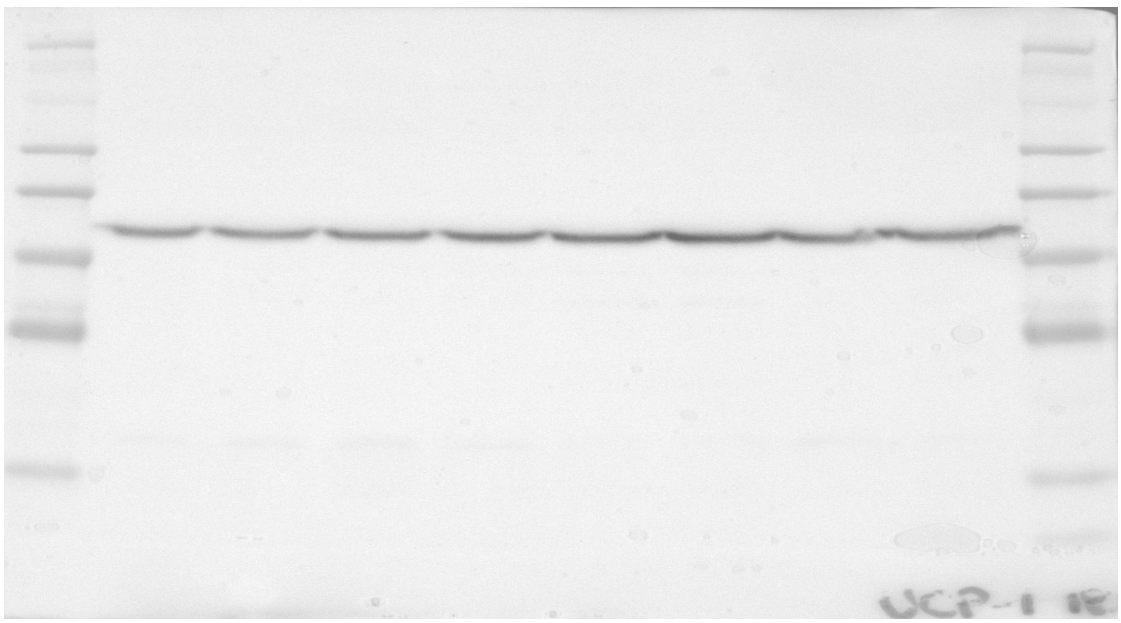
Replicate	Strain	Target	Norm	Average	SD	Replicate 1	2^(-ΔΔCт)/Ave	Average	SD
Replicate 2	**puro-6/ntc-3 p1**	**Aspa**	**0,77450111**	**1**	**0,38907086**	**iBACs puro-6/ntc-3 d7**	**0,256895101**	**1**	**0,55075936**
Replicate 2	puro-6/ntc-3 p2	Aspa	1,44925921			iBACs puro-6/ntc-3 d7 p29	1,169438284		
Replicate 2	puro-6/ntc-3 p3	Aspa	0,77623968			iBACs puro-6/ntc-3 d7 p30	1,573666616		
Replicate 2	puro-6/siAspa-2 p1	Aspa	0,49757301	0,41604215	0,09349839	iBACs puro-6/siAspa-2 d7 p28	0,114229749	0,60526408	0,34918615
Replicate 2	puro-6/siAspa-2 p2	Aspa	0,31398653			iBACs puro-6/siAspa-2 d7 p29	0,896173071		
Replicate 2	puro-6/siAspa-2 p3	Aspa	0,43656691			iBACs puro-6/siAspa-2 d7 p30	0,805369405		
Replicate 2	Nat8L/NTC p1	Aspa	7,00662082	7,94577925	0,94441109	iBACs Nat8l-8/ntc-3 d7 p28	2,21917936	10,340369	5,742588
Replicate 2	Nat8L/NTC p2	Aspa	7,93536013			iBACs Nat8l-8/ntc-3 d7 p29	14,42713303		
Replicate 2	Nat8L/NTC p3	Aspa	8,89535679			iBACs Nat8l-8/ntc-3 d7 p30	14,37479452		
Replicate 2	Nat8L/siAspa-2 p1	Aspa	0,90018085	0,94424742	0,1157972	iBACs Nat8l-8/siAspa-2 d7 p28	0,299516588	0,95256795	0,51605189
Replicate 2	Nat8L/siAspa-2 p2	Aspa	0,85695279			iBACs Nat8l-8/siAspa-2 d7 p29	0,996946024		
Replicate 2	Nat8L/siAspa-2 p3	Aspa	1,07560862			iBACs Nat8l-8/siAspa-2 d7 p30	1,561241236		
Replicate 2	**puro-6/ntc-3 p1**	**NAT8L (E1)**	**1,02405625**	**1**	**0,12992362**	**iBACs puro-6/ntc-3 d7 p28**	**1,376749254**	**1**	**0,31463945**
Replicate 2	puro-6/ntc-3 p2	NAT8L (E1)	1,1162143			iBACs puro-6/ntc-3 d7 p29	1,01666677		
Replicate 2	puro-6/ntc-3 p3	NAT8L (E1)	0,85972945			iBACs puro-6/ntc-3 d7 p30	0,606583976		
Replicate 2	puro-6/siAspa-2 p1	NAT8L (E1)	0,88914755	1,29708937	0,44164719	iBACs puro-6/siAspa-2 d7 p28	1,459823343	0,98093003	0,35738062
Replicate 2	puro-6/siAspa-2 p2	NAT8L (E1)	1,23603225			iBACs puro-6/siAspa-2 d7 p29	0,881402143		
Replicate 2	puro-6/siAspa-2 p3	NAT8L (E1)	1,76608829			iBACs puro-6/siAspa-2 d7 p30	0,601564612		
Replicate 2	Nat8L/NTC p1	NAT8L (E1)	11,8522257	12,7325358	2,60839126	iBACs Nat8l-8/ntc-3 d7 p28	28,88035781	21,5360529	5,24496727
Replicate 2	Nat8L/NTC p2	NAT8L (E1)	15,6671839			iBACs Nat8l-8/ntc-3 d7 p29	18,76412863		
Replicate 2	Nat8L/NTC p3	NAT8L (E1)	10,6781979			iBACs Nat8l-8/ntc-3 d7 p30	16,96367211		
Replicate 2	Nat8L/siAspa-2 p1	NAT8L (E1)	37,6303946	32,085129	5,10335699	iBACs Nat8l-8/siAspa-2 d7 p28	42,6434303	23,9297423	13,861617
Replicate 2	Nat8L/siAspa-2 p2	NAT8L (E1)	27,5857146			iBACs Nat8l-8/siAspa-2 d7 p29	9,516709246		
Replicate 2	Nat8L/siAspa-2 p3	NAT8L (E1)	31,0392779			iBACs Nat8l-8/siAspa-2 d7 p30	19,62908725		
Replicate 2	**puro-6/ntc-3 p1**	**UCP1**	**0,70892468**	**1**	**0,25910385**	**iBACs puro-6/ntc-3 d7 p28**	**1,376749254**	**1**	**0,31463945**
Replicate 2	puro-6/ntc-3 p2	UCP1	1,20546408			iBACs puro-6/ntc-3 d7 p29	1,01666677		
Replicate 2	puro-6/ntc-3 p3	UCP1	1,08561125			iBACs puro-6/ntc-3 d7 p30	0,606583976		
Replicate 2	puro-6/siAspa-2 p1	UCP1	0,62483316	0,67312269	0,09530727	iBACs puro-6/siAspa-2 d7 p28	1,459823343	0,98093003	0,35738062
Replicate 2	puro-6/siAspa-2 p2	UCP1	0,61162538			iBACs puro-6/siAspa-2 d7 p29	0,881402143		
Replicate 2	puro-6/siAspa-2 p3	UCP1	0,78290954			iBACs puro-6/siAspa-2 d7 p30	0,601564612		
Replicate 2	Nat8L/NTC p1	UCP1	26,4687721	28,4015543	3,34204104	iBACs Nat8l-8/ntc-3 d7 p28	28,88035781	21,5360529	5,24496727
Replicate 2	Nat8L/NTC p2	UCP1	26,4752817			iBACs Nat8l-8/ntc-3 d7 p29	18,76412863		
Replicate 2	Nat8L/NTC p3	UCP1	32,260609			iBACs Nat8l-8/ntc-3 d7 p30	16,96367211		
Replicate 2	Nat8L/siAspa-2 p1	UCP1	5,80438773	5,20191	0,56742105	iBACs Nat8l-8/siAspa-2 d7 p28	42,6434303	23,9297423	13,861617
Replicate 2	Nat8L/siAspa-2 p2	UCP1	5,12367806			iBACs Nat8l-8/siAspa-2 d7 p29	9,516709246		
Replicate 2	Nat8L/siAspa-2 p3	UCP1	4,67766422			iBACs Nat8l-8/siAspa-2 d7 p30	19,62908725		

2. **UCP-1 and Aspa protein expression Replicate 2:** 12% gels, MOPS buffer and 30uL per well were used for western blots assays. Passages 1 & 2 were evaluated.

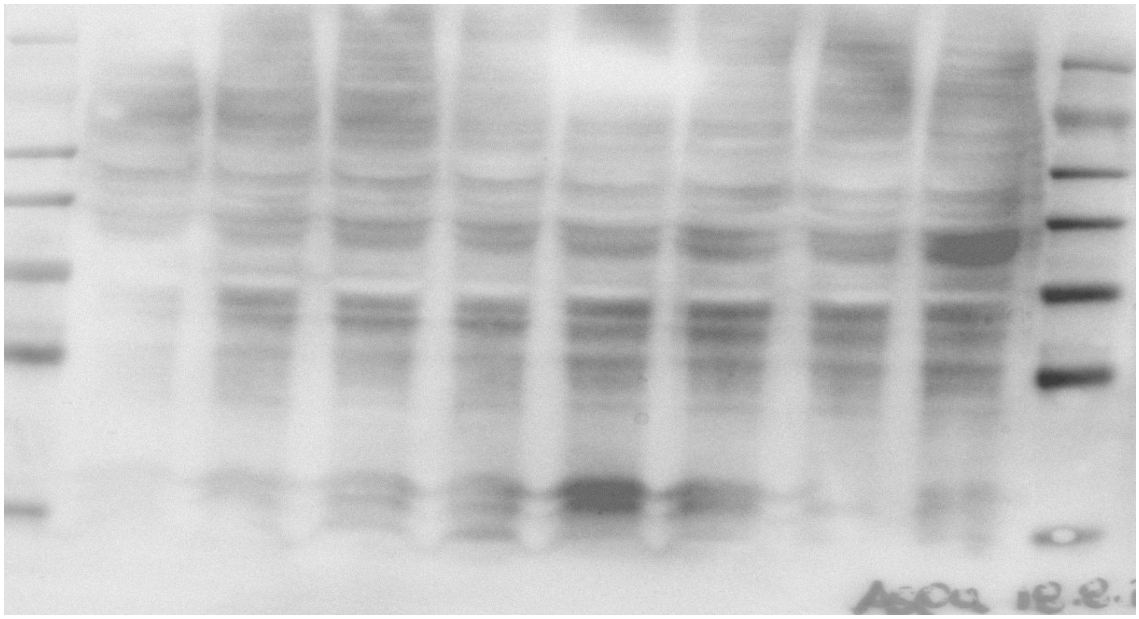
A. UCP-1 expression



B. B-Actin control of UCP-1 Expression

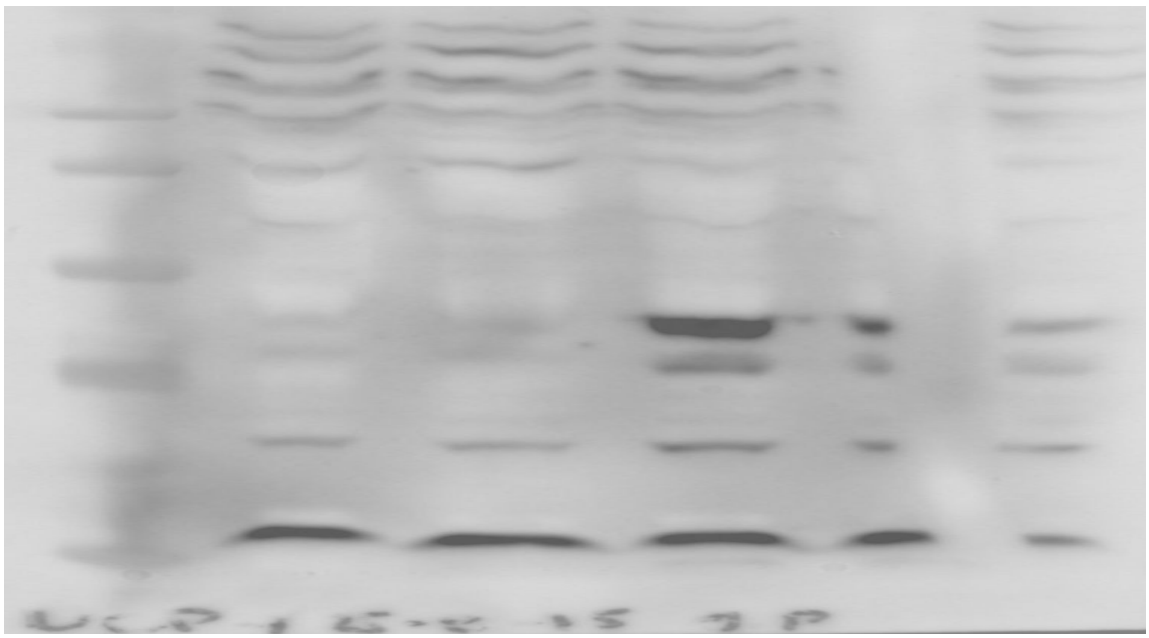


C. Aspa Expression

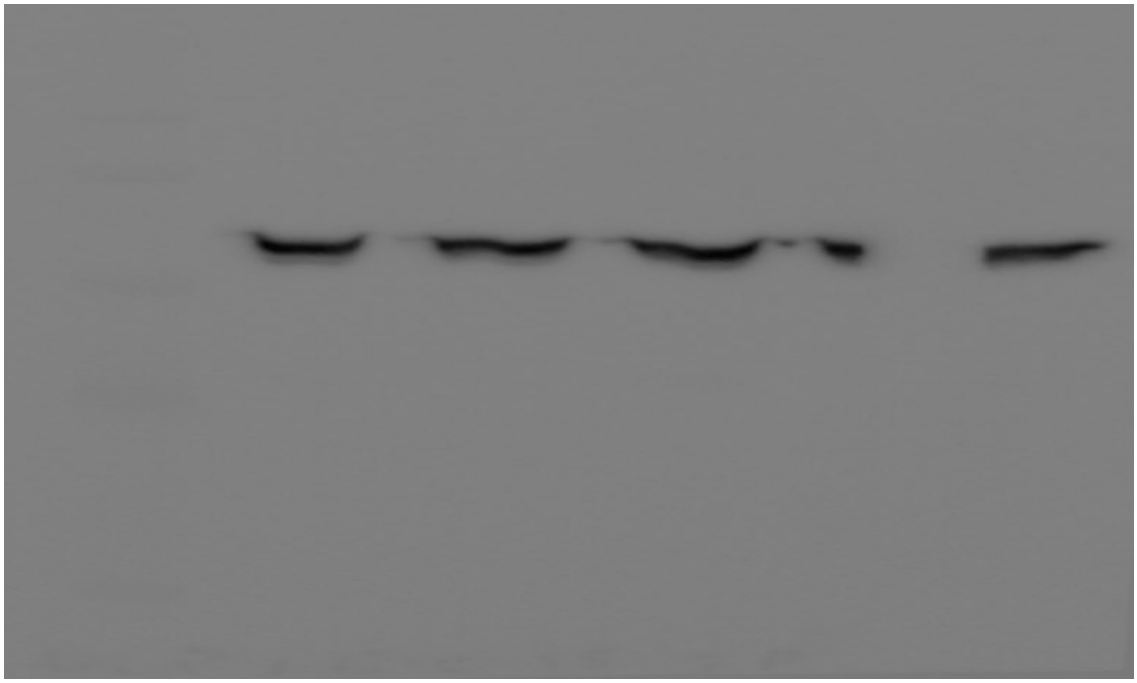


3. UCP-1 and Aspa protein expression Replicate 3: 12% gels, MOPS buffer and 30uL per well were used for western blots assays. Passages #3 of each strain were loaded.

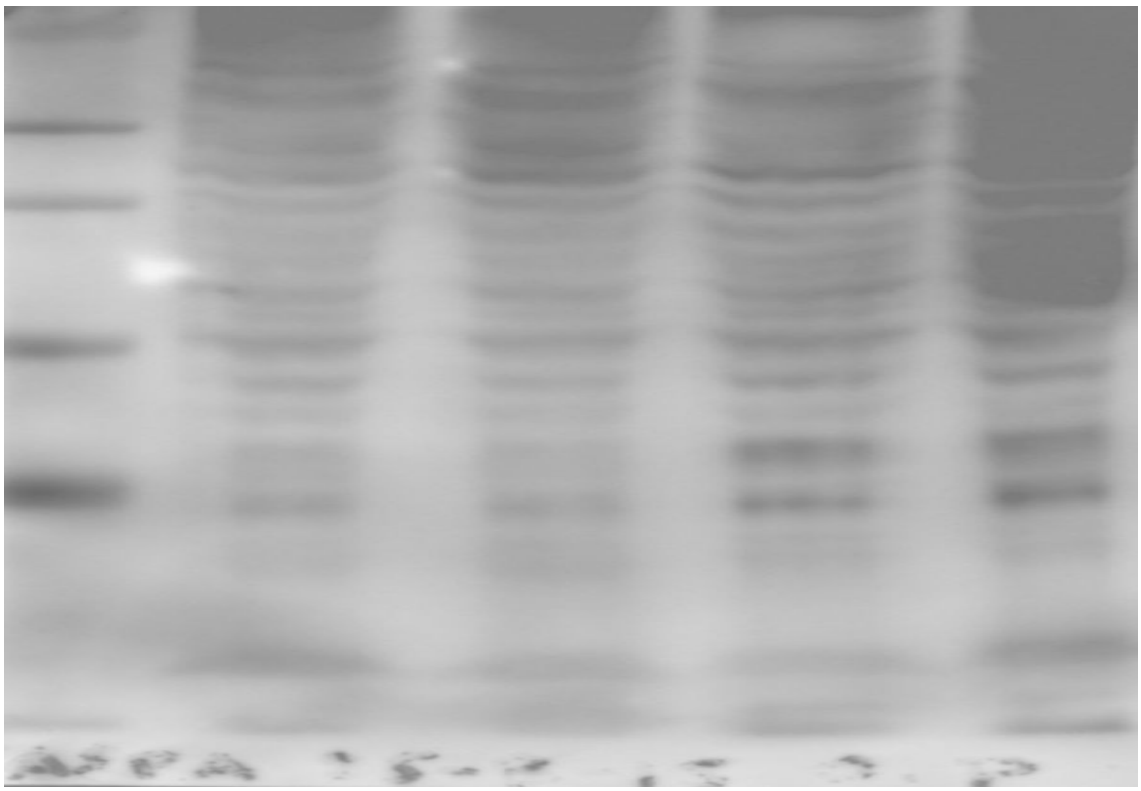
A. UCP-1 expression



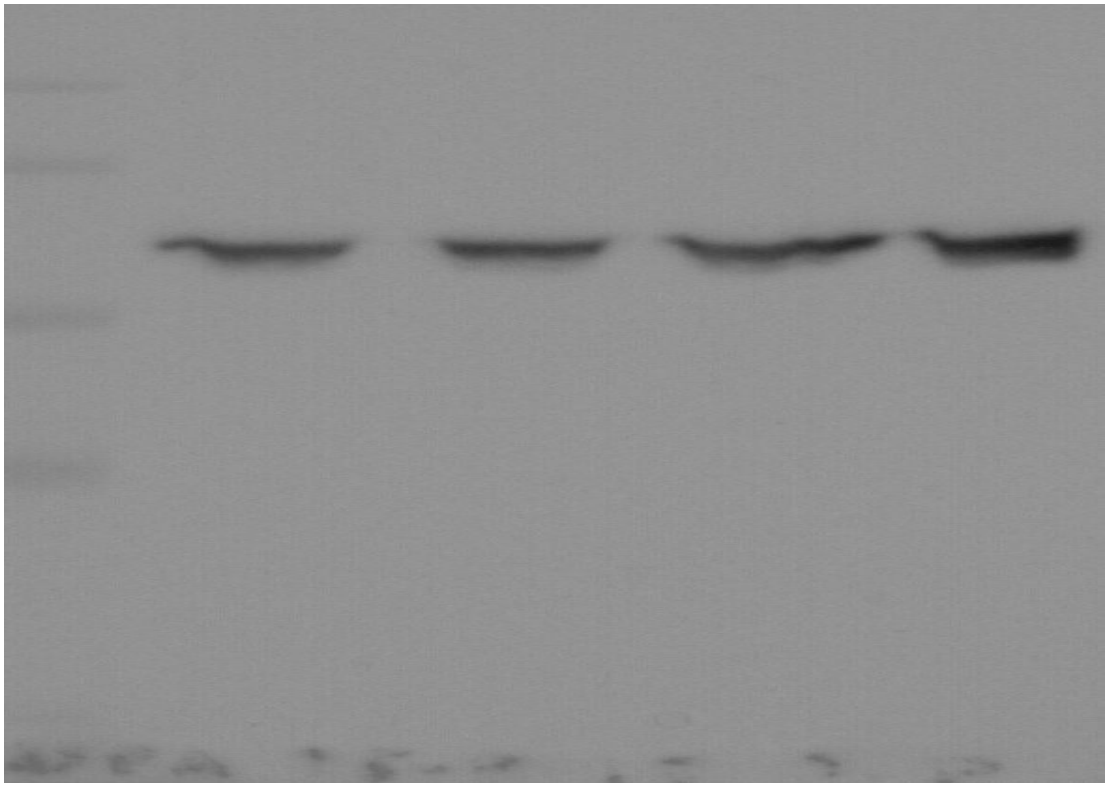
B. B-Actin control of UCP-1 expression



C. Aspa expression



D. B-Actin control of Aspa expression

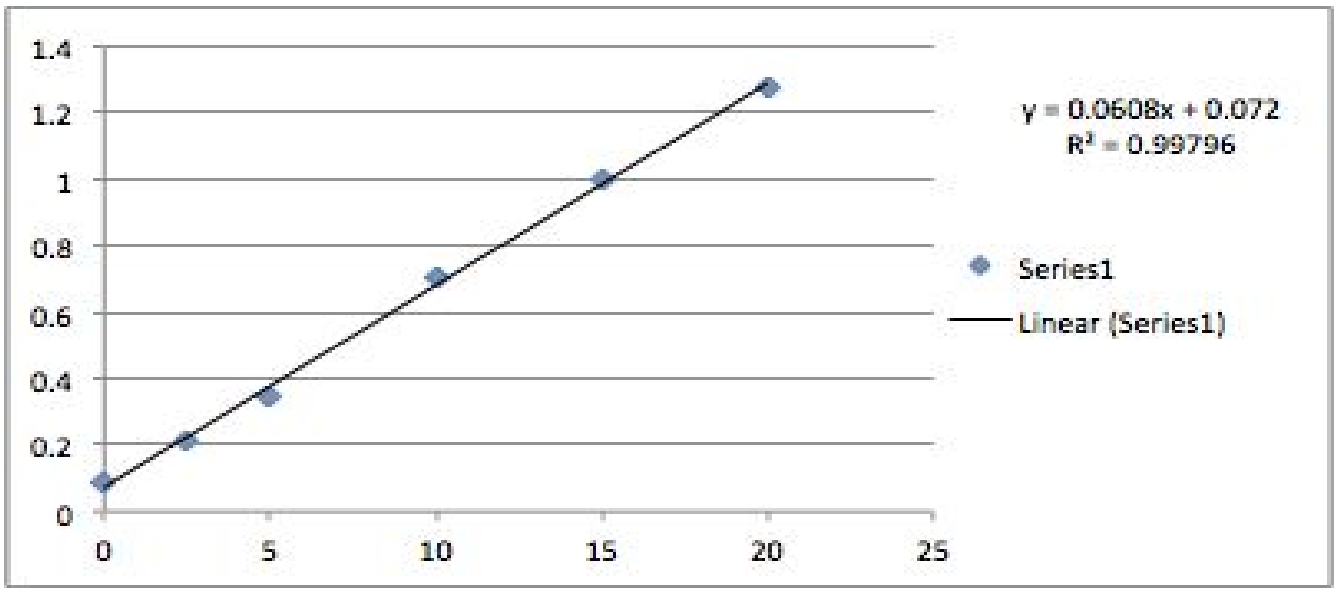


Protein Raw data Replicate 1: Example of Western Blot

Raw Data Wavelength: 562.0 Protein Concentration												
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.276	0.995	0.703	0.344	0.214	0.09	0.045	0.046	0.046	0.046	0.048	0.045
B	0.425	0.457	0.559	0.74	0.711	0.689	0.543	0.818	0.045	0.046	0.047	0.046
C	0.493	0.422	0.568	0.56	0.732	0.765	0.807	0.686	0.045	0.047	0.047	0.046
D	0.045	0.046	0.049	0.046	0.046	0.046	0.045	0.046	0.046	0.046	0.047	0.046
E	0.046	0.045	0.046	0.046	0.046	0.045	0.045	0.045	0.047	0.047	0.045	0.045
F	0.045	0.046	0.046	0.046	0.046	0.046	0.046	0.045	0.045	0.046	0.046	0.046
G	0.046	0.046	0.046	0.046	0.046	0.046	0.046	0.046	0.047	0.047	0.045	0.046
H	0.048	0.047	0.046	0.046	0.046	0.045	0.046	0.046	0.045	0.046	0.047	0.045

Standard Curve BCA vs. Absorbance	
BCA [µg]	Absorbance
0	0.09
2.5	0.214
5	0.344
10	0.703
15	0.995
20	1.276

Linear Regression for Protein Replicate 1



UCP-1 Detection ASPA Detection Membranes 1 and 2:

Saturation of Membrane: 5% BSA / PBST

1. AB: Anti-UCP1: 1:750 in 5% BSA / PBST over night 1.

AB: Anti-Aspa: 1:10,000 in 5% BSA over night

2. AB: Anti-rabbit 1:2,000 in PBST 2. AB: Anti-Goat 1:3,000 in PBST

Imaging UCP-1: MW ca. 33kDa

Imaging ASPA: MW ca. 34kDa

Beta-Actin Detection:

Saturation of Membrane: 5% BSA / PBST

1. AB: Anti-Beta-Actin: 1:25,000 in 1% Milk / PBST over night

2. AB: Anti-Mouse 1:3,000 in PBST

Imaging: MW ca. 44kDa