

Title of Thesis

**The effect of arachidonic acid on the early and late
stages of adipocyte differentiation**

Evanthia Nikolopoulou

A thesis submitted in fulfilment of the requirements for the
degree of Doctor of Philosophy

Institute of Reproductive and Developmental Biology
Department of Surgery and Cancer
Faculty of Medicine
Imperial College London

Statement of Originality

All experiments included in this thesis were performed by myself unless
otherwise stated in the text

Acknowledgments

I would like to thank my supervisor Dr Mark Christian for giving me the opportunity to work in his lab and being the first ever PhD student working with him. I would also like to thank Professor Malcolm Parker for being a continuous source of knowledge for me and training me to think as a real scientist all these years. Dr Aylin Hanyaloglu has been a great mentor in the cell signalling field and without her advice and guidance this thesis would not have been possible. I would especially like to thank Dr Georgia Papacleovoulou not only for our long scientific conversations but also for being a great friend and always being there for me. Dr Frederique Jean-Alphonse has also been extremely helpful and supportive and I would like to thank him for always offering me advice. I would also like to thank my lab mate Kunal Shah for all these years working together as a real team. Finally, I would like to thank my friends and fellow lab mates Giulia, Alex, Ariel, Asmaà, Txell and David for their help, advice and fun we had together.

I am extremely grateful to my parents who have supported me all the way and have made this possible. Special thanks to my brother Vasilis who has been my best friend since I was born.

Abstract

Dietary fat has been correlated with obesity since it induces the proliferation and differentiation of pre-adipocytes. However it has become clear that the effect of fat on human health depends on the composition and the nature of fatty acids. Arachidonic acid (AA) is a major omega-6 polyunsaturated fatty acid. Its role in adipocyte differentiation is controversial as published data have shown opposing roles of AA in pre-adipocyte differentiation.

We investigated the effect of a brief exposure of AA on pre-adipocyte differentiation. After a 24h of AA treatment during pre-adipocyte differentiation, the expression of aP2, a differentiation marker, and Fra-1, an AP-1 transcription factor, was induced. We showed that this effect was PPAR γ -dependent since AA treatment was unable to up-regulate aP2 and Fra-1 in PPAR γ knockdown cells. Furthermore, we identified that both of these genes were regulated by the AA metabolite, PGF_{2 α} , through binding to its cognate GPCR, the FP receptor, initiating a cascade of signalling pathways involving PKC and ERK activation.

We also showed that short-term treatment of pre-adipocytes with AA at the beginning of differentiation induced events with a long-lasting inhibitory effect on adipogenesis. Treatment of pre-adipocytes with AA for only 24h, blocked adipogenesis as revealed by significantly reduced expression of adipocyte markers and lipid accumulation after 10 days of differentiation. We demonstrated that Fra-1 mediated the inhibitory effect of AA, as Fra-1 depletion rescued the inhibition of differentiation by AA.

We suggest that short-term exposure of pre-adipocytes to AA at the early stages of differentiation has a dual effect. In the early stages, the presence of AA causes a rapid up-regulation in two target genes, the aP2 and Fra-1, in a PPAR γ -dependent way. In the later stages of differentiation, when AA is withdrawn, the early up-regulation of Fra-1 results in inhibition of the differentiation program.

Table of Contents	Page
Title of Thesis	i
Statement of Originality	ii
Acknowledgments	iii
Abstract	iv
Table of Contents	v
List of Figures and Tables	ix
Abbreviations	xii
 <u>Chapter 1</u>	
Introduction	1
1.1 Obesity	2
1.2 Adipose Tissue (AT)	3
1.2.1 White Adipose Tissue (WAT)	3
1.2.2 Brown Adipose Tissue (BAT)	5
1.3 Adipocyte differentiation (Adipogenesis)	8
1.3.1 Determination	12
1.3.2 Terminal differentiation	14
1.3.2.1 Fos-related antigen 1 or Fra-1	14
1.3.2.2 C/EBPs	15
1.3.2.3 PPAR γ	17
1.3.2.4 Adipocyte Fatty acid Binding Protein (or FABP4 or aP2)	24
1.4 Fatty acid metabolism	30
1.4.1 Fatty acids and fatty acid trafficking	30
1.4.2 Triglyceride (TAG) synthesis	34
1.4.3 Lipolysis	35
1.5 Arachidonic acid metabolism	38
1.6 Arachidonic acid and adipogenesis	45
1.7 Hypothesis and Aims	51
 <u>Chapter 2</u>	
Materials and Methods	52
2.1 Materials	53
2.1.1 Antibodies	53
2.1.1.1 Primary Antibodies	53
2.1.1.2 Secondary Antibodies	54
2.1.2 Plasmids	54
2.1.3 siRNA	55
2.1.4 Chemicals and Reagents	55
2.1.5 Kits	58
2.1.6 Cell culture Materials	58
2.1.7 Cell lines	59
2.1.8 Buffers and solutions	59
2.1.8.1 ChIP buffers	59
2.1.8.2 New England Biolabs Restriction Endonuclease buffers (10x)	60

2.1.8.3 Qiagen buffers	61
2.1.8.4 Western blotting buffers	62
2.1.8.5 Miscellaneous buffers	63
2.1.9 Bacterial strains and Media	65
2.1.10 SDS gels	66
2.1.11 Miscellaneous	66
2.2 Methods	67
2.2.1 Cell culture	67
2.2.2 Preparation of Albumin-bound Fatty Acid	68
2.2.3 Oil Red O staining	68
2.2.4 RNA extraction	69
2.2.5 cDNA synthesis	70
2.2.6 Quantitative Real-Time PCR	70
2.2.7 PCR array	72
2.2.8 Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	72
2.2.9 Western Blotting	73
2.2.10 Bacterial propagation of plasmids	74
2.2.10.1 Transformation of chemically competent DH5 α E.Coli by heat shock	74
2.2.10.2 Transformation of electrocompetent DH5 α E.Coli by electroporation	74
2.2.10.3 Small-scale preparation of plasmid DNA (Miniprep)	75
2.2.10.4 Large-scale plasmid DNA preparation (Maxiprep)	75
2.2.11 Molecular Cloning	76
2.2.11.1 Restriction digests	76
2.2.11.2 PCR for cloning	76
2.2.11.3 DNA agarose gel electrophoresis	77
2.2.11.4 Gel extraction of DNA	77
2.2.11.5 Ligations	78
2.2.11.6 DNA sequencing	78
2.2.12 Transient transfections	78
2.2.12.1 Transfection of HEK293 cells using the calcium phosphate method	78
2.2.12.2 Transfection of 3T3-L1 and NIH-3T3 cells using Lipofectamine 2000	79
2.2.12.3 Dharmafect transfection of siRNA	79
2.2.13 Generation of Lentiviruses Containing ATG7 shRNA	80
2.2.14 Reporter Gene assays	80
2.2.15 Chromatin Immunoprecipitation	81
2.2.16 Calcium mobilisation assay	82
2.2.17 PGF _{2α} ELIZA	82
2.2.18 cAMP assay	83
2.2.19 Fluorescence-activated cell sorting (FACS)	83
2.2.20 MTT assay	83
2.2.21 LDH assay	84
2.2.22 AdipoRed assay	84

Chapter 3

Results: Arachidonic Acid effects on the early stages of adipocyte differentiation	85
3.1 Introduction	86
3.2 Results	87
3.2.1 The effect of AA on aP2 expression	87
3.2.1.1 The effect of AA on the adipocyte differentiation markers	87
3.2.1.2 Kinetics of the effect of AA on the adipocyte differentiation markers	92
3.2.1.3 The effect of AA on aP2 expression is metabolite-, time- and condition-specific	95
3.2.2 The effect of the AA metabolites on aP2 expression	97
3.2.2.1 COXs mediate the effect of AA on aP2 expression	97
3.2.2.2 PGs mediate the effect of AA on aP2 expression	100
3.2.3. The signalling pathway mediating the effect of AA on aP2 expression	103
3.2.3.1. ERK MAPK mediates the effect of AA on aP2 expression	103
3.2.3.2. ERK is activated immediately after AA stimulation	105
3.2.3.3. ERK is activated immediately after PGF _{2α} and PGE ₂ stimulation	107
3.2.3.4. The FP receptor mediates AA-induced ERK activation	109
3.2.3.5. The FP receptor mediates the effect of AA on aP2 expression	111
3.2.3.6. PGF _{2α} induces aP2 expression	113
3.2.3.7. AA increases intracellular cAMP levels	115
3.2.3.8 AA increases intracellular calcium levels	117
3.2.3.9. Calcium mediates the effect of AA on aP2 expression and ERK activation	119
3.2.3.10 PKC mediates the effect of AA on aP2 expression and ERK activation	121
3.2.4 The effect of AA on aP2 expression is PPARγ-mediated	123
3.2.4.1 PPARs and aP2 expression	123
3.2.4.2 PPARγ absence prevents AA-dependent induction of aP2	125
3.2.4.3 PPARγ expression enhances the AA-dependent induction of aP2 expression	128
3.2.4.4 PPARγ LBD mediates the effect of AA on aP2 expression	130
3.2.5 The function of aP2 increase after AA treatment	134
3.2.5.1 The role of aP2 increase in FA metabolism	134
3.2.5.2 The role of aP2 increase in ER stress	137
3.2.5.3 The role of aP2 increase in autophagy	139
3.2.6 The effect of AA on Fra-1 expression	144
3.2.6.1 AA increases Fra-1 expression	144
3.2.6.2 The effect of AA on Fra-1 expression is PG-mediated	146
3.2.6.3 The effect of AA on Fra-1 expression is FP receptor-mediated	149
3.2.6.4. The signalling pathway mediating the effect of AA on Fra-1 expression	150
3.2.6.5 The effect of AA on Fra-1 expression is PPARγ-mediated	152
3.2.7 Regulation of aP2 expression by Fra-1	155
3.3 Discussion	160
3.3.1 The effect of AA on adipocyte differentiation markers	160

3.3.2 The AA effect on aP2 expression is PG-mediated	161
3.3.3 The signalling pathway mediating the effect of AA on aP2 expression	163
3.3.4 The effect of AA on aP2 expression is PPAR γ -mediated	167
3.3.5 The function of increased aP2 expression upon AA treatment	170
3.3.6 The effect of AA on Fra-1 expression	172
3.3.7 The regulation of aP2 expression by Fra-1 upon AA treatment	175

Chapter 4

Results: Arachidonic Acid effects on the late stages of adipocyte differentiation

4.1 Introduction	178
4.2. Results	179
4.2.1 Inhibition of adipocyte differentiation after long exposure to AA	179
4.2.2 Inhibition of adipocyte differentiation after short exposure to AA	182
4.2.2.1 Kinetics of the inhibition of differentiation after short exposure to AA	185
4.2.2.2 Differentiating pre-adipocytes are sensitive to the inhibitory effect of AA during the first 24h of differentiation	189
4.2.2.3 A PPAR γ ligand or prolonged exposure to MDI cannot rescue AA-induced inhibition of differentiation	191
4.2.2.4 The inhibition of differentiation after a short exposure to AA is not due to alternations in cell cycle, cell viability or induced cytotoxicity	193
4.2.2.5 Inhibition of adipocyte differentiation after short exposure to AA is mediated by PGs	196
4.2.2.6 Inhibition of adipocyte differentiation after short exposure to PGF $_{2\alpha}$	198
4.2.2.7 Kinetics of the inhibition of differentiation after short exposure to PGF $_{2\alpha}$	203
4.2.2.8 The signalling pathway mediating AA-dependent inhibition of adipocyte differentiation	206
4.2.2.9 Fra-1 mediates AA-induced inhibition of differentiation	210
4.2.2.10 Fra-1 regulates PPAR γ phosphorylation	213
4.2.2.11 Short exposure to AA at the start of differentiation keeps the cells into a pre-adipocyte state	215
4.3 Discussion	218

Chapter 5:

Discussion	225
-------------------	-----

References	231
-------------------	-----

Appendix	266
-----------------	-----

List of Figures and Tables	Page
<u>Chapter 1</u>	
Figure 1.1: Differences between white and brown adipocytes	7
Figure 1.2: Adipocyte differentiation (adipogenesis).	11
Figure 1.3: Gene and protein organization of the mouse and human PPAR γ	20
Figure 1.4: aP2 cellular functions	26
Table 1.1: Nomenclature, structure and level of saturation of different unbranched FAs	31
Figure 1.5: Adipocyte lipogenesis and lipolysis	37
Figure 1.6: AA incorporation in phospholipids	41
Figure 1.7: The three pathways for AA metabolism into eicosanoids	44
Figure 1.8: Opposing actions of AA metabolites on adipocyte differentiation	50
<u>Chapter 3</u>	
Figure 3.1: 3T3-L1 pre-adipocytes showed enhanced LD formation after 24h of AA treatment.	88
Figure 3.2: The effect of AA on the expression of late adipocyte differentiation markers in 3T3-L1 pre-adipocytes	90
Figure 3.3: AA did not affect the expression of early differentiation markers in 3T3-L1 pre-adipocytes.	91
Figure 3.4: The effect of AA on aP2 expression occurred after 24h of treatment in 3T3-L1 pre-adipocytes	93
Figure 3.5: AA does not affect the kinetics of the early differentiation markers in 3T3-L1 pre-adipocytes	94
Figure 3.6: AA up-regulated aP2 expression in 3T3-L1 pre-adipocytes after two days of post-confluency in an IBMX-dependent manner	96
Figure 3.7: The effect of AA on aP2 expression was inhibited by indomethacin	98
Figure 3.8: COXs expression is increased upon AA treatment in 3T3-L1 pre-adipocytes	99
Figure 3.9: The effect of PGs on aP2 expression	100
Figure 3.10: PG receptor expression after 24h treatment with AA	102
Figure 3.11: MAPKs expression after 24h of treatment with AA	103
Figure 3.12: ERK mediates the effect of AA on aP2 expression	104
Figure 3.13: AA-induced ERK activation in 3T3-L1 pre-adipocytes	106
Figure 3.14: PGF $_{2\alpha}$ and PGE $_2$ activate ERK in 3T3-L1 pre-adipocytes	108
Figure 3.15: The FP receptor mediates the AA-induced ERK activation in 3T3-L1 pre-adipocytes	110
Figure 3.16: The FP receptor mediates the effect of AA on aP2 expression	112
Figure 3.17: PGF $_{2\alpha}$ up-regulates aP2 expression	114
Figure 3.18: AA increases intracellular cAMP levels via the EP4 receptor in 3T3-L1 pre-adipocytes	116
Figure 3.19: AA increases intracellular calcium levels via the FP receptor in 3T3-L1 pre-adipocytes.	118

Figure 3.20: Calcium mediates the effect of AA on aP2 expression and ERK activation	120
Figure 3.21: PKC mediates the effect of AA on aP2 expression and ERK activation	121
Figure 3.22: PPARs and aP2 expression	124
Figure 3.23: PPAR γ mediates the effect of AA on aP2 expression	126
Figure 3.24: PPAR γ expression enhances the effect of AA on aP2 expression	129
Figure 3.25: PPAR γ -LBD mediates the effect of AA on aP2 expression	132
Figure 3.26: The effect of AA on lipid metabolism	135
Figure 3.27: The effect of AA on <i>Acs14</i> and <i>CPT1a</i> gene expression	136
Figure 3.28: The effect of AA effect on ER stress	138
Figure 3.29: Inhibition of autophagy enhances the effect of AA on aP2 expression	140
Figure 3.30 aP2 is required for autophagy	142
Figure 3.31 AA treatment does not affect autophagy	143
Figure 3.32 AA increases Fra-1 expression	145
Figure 3.33: The effect of AA on Fra-1 expression is PG-mediated	147
Figure 3.34 PGF $_{2\alpha}$ increases Fra-1 expression	148
Figure 3.35: The FP receptor mediates the effect of AA on Fra-1 expression	149
Figure 3.36: PKA, PKC and ERK mediate the effect of AA on Fra-1 expression	151
Figure 3.37: PPAR γ mediates the effect of AA on Fra-1 expression	153
Figure 3.38 PPAR γ mediates the effect of AA on Fra-1 expression through sequences found upstream the TSS of <i>Fos11</i> gene	154
Figure 3.39 Regulation of aP2 expression by Fra-1	156
Figure 3.40: Expression of the Jun proteins after AA treatment in the early stages of differentiation	158
Figure 3.41: Expression of the Fos proteins after AA treatment and after Fra-1 depletion	159
Figure 3.42: Schematic representation of some of the signalling pathways activated by GPCRs	164
Figure 3.43: Schematic representation of the proposed mechanism triggered by AA to regulate aP2 expression in the early phase of adipogenesis	166
Figure 3.44: Schematic representation of the proposed mechanism triggered by AA to regulate Fra-1 expression in the early phase of adipogenesis	175
Chapter 4	
Figure 4.1: Experimental procedure for long exposure to AA	179
Figure 4.2: AA-dependent inhibition of adipocyte differentiation after long exposure to AA - lipid accumulation	180
Figure 4.3: AA-dependent inhibition of adipocyte differentiation after long exposure to AA – gene expression of adipocyte markers	181
Figure 4.4: Experimental procedure for short exposure to AA	182
Figure 4.5: Inhibition of differentiation after a short exposure to AA – lipid accumulation	183

Figure 4.6: Inhibition of differentiation after a short exposure to AA – gene expression of adipocyte markers	185
Figure 4.7: Kinetics of the inhibition of differentiation after a short exposure to AA – lipid accumulation	186
Figure 4.8: Kinetics of the inhibition of differentiation after a short exposure to AA – gene expression of adipocyte markers	188
Figure 4.9: AA short exposure inhibits the expression of aP2 and PPAR γ	189
Figure 4.10: AA inhibitory effect is maximal when present during the first 24h of differentiation	190
Figure 4.11: Rosiglitazone or prolonged MDI treated does not rescue the inhibition of differentiation by AA	192
Figure 4.12: AA treatment does not affect the cell cycle	194
Figure 4.13: AA treatment does not affect cell viability and does not cause cytolysis	195
Figure 4.14: Indomethacin blocked AA-induced inhibition of differentiation	197
Figure 4.15: PGF $_{2\alpha}$ inhibits lipid accumulation	198
Figure 4.16: AKR1B3, FP receptor expression and PGF $_{2\alpha}$ production after short treatment with AA	200
Figure 4.17: Inhibition of differentiation after a short exposure to PGF $_{2\alpha}$ – gene expression of adipocyte markers	201
Figure 4.18: Inhibition of differentiation after a short exposure to PGF $_{2\alpha}$ – lipid accumulation	202
Figure 4.19: Kinetics of the inhibition of differentiation after a short exposure to PGF $_{2\alpha}$ – gene expression of adipocyte markers	204
Figure 4.20: PGF $_{2\alpha}$ is a potent inhibitor of adipocyte differentiation	205
Figure 4.21: The ERK pathway mediates the inhibition of differentiation by AA	207
Figure 4.22: Depletion of the FP receptor prevents the inhibition of differentiation by AA – gene expression of adipocyte markers	208
Figure 4.23: Depletion of the FP receptor restores PPAR γ and aP2 protein levels upon AA treatment	209
Figure 4.24: Fra-1 protein expression after a short treatment with AA at the early stages of differentiation	211
Figure 4.25 AA-induced inhibition of differentiation requires Fra-1–lipid accumulation	211
Figure 4.26: AA-dependent inhibition of differentiation requires the presence of Fra-1 – gene expression of adipocyte markers	212
Figure 4.27: The phosphorylation of PPAR γ at S112 is Fra-1-dependent	214
Figure 4.28: AA or PGF $_{2\alpha}$ treatments at the beginning of the differentiation procedure retain cells in the pre-adipocyte state	216
Figure 4.29: The FP receptor and Fra-1 are required for the AA-dependent up-regulation of pre-adipocyte marker genes	217
Figure 4.30: Schematic illustration of the proposed mechanism triggered by AA to inhibit pre-adipocyte differentiation	224

Chapter 5

Figure 5.1: Schematic illustration of the proposed mechanism triggered by AA to regulate the early and late stages of adipocyte differentiation	228
---	-----

List of Abbreviations

AA	Arachidonic acid
aa	Amino acids
AC	Adenylyl cyclase
ACBP	Acyl-CoA binding protein
ACC	Acetyl-CoA carboxylase
ACS	Acyl-CoA synthetase
ACSL	Long-chain acyl-CoA synthetase
AGPAT	1-acylglycerol-3-phosphate acyltransferase
AKR	Aldo-keto reductase
ALA	α -linolenic acid
AMPK	Monophosphate- activated protein kinase
ApoE	Apolipoprotein E
APS	Ammonium persulphate
AP-1	Activator protein 1
aP2	Adipocyte protein 2
AT	Adipose Tissue
Atg7	Autophagy-related 7
ATGL	Adipose triglyceride lipase
ATP	Adenosine-5'-triphosphate
BAT	Brown Adipose Tissue
BMI	Body Mass Index
BMPs	Bone Morphogenetic Proteins
BSA	Bovine Serum Albumin
bZip	Basic-leucine zipper
cAMP	cyclic 3'5' Adenosine Monophosphate
CBP	CREB-binding protein
Cdk5	Cyclin-dependent kinase 5
C/EBP- α - β - δ	CCAAT/enhancer binding protein - α - β - δ
CGI-58	Comparative gene identification-58
ChIP	Chromatin immunoprecipitation
CoA-IT	CoA-independent transacylase
CPT1	Carnitine palmitoyltransferase-1

cPGI ₂	Carbaprostacyclin
COX	Cyclooxygenase
CREB	cAMP Response Element-Binding protein
DAG	Diacylglycerol
DBD	DNA binding domain
DEPC	Diethylpyrocarbonate
DEX	Dexamethasone
DGAT	Diacylglycerol acyltransferase
DHA	Docosahexaenoic acid
DHAP	Dihydroxyacetone phosphate
DHET	Dihydroxyeicosatrienoic acid
DIO2	Type 2 iodothyronine deiodinase
DLK-1	Drosophila Homolog-like 1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
dH ₂ O	Distilled water
ECL	Enhanced chemiluminescence
EDTA	Diaminoethanetetraacetic acid
EET	Epoxyeicosatrienoic acid
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-Regulated Kinase/MAPK
ESCs	Embryonic Stem Cells
FA	Fatty acid
FACS	Fluorescence-activated cell sorting
FAT	Fatty acid translocase
FATP	Fatty-acid transport protein
FABPpm	Plasma membrane fatty acid binding protein
FABP4	Fatty acid binding protein 4
FAS	Fatty acid synthase
FBS	Fetal Bovine Serum

FFAs	Free Fatty Acids
Fra-1	Fos-like antigen 1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GATA- 2 -3	GATA-binding protein -2 - 3
Glut4	Glucose transporter type 4
GPAT	Glycerol-3-phosphate acyltransferase
GPCR	G-Protein Coupled Receptor
GR	Glucocorticoid receptor
GyK	Glycerol kinase
G3P	Glycerol-3-phosphate
HDAC	Histone deacetylase
HETE	Hydroxyeicosatetraenoic acid
HFD	High – fat diet
HPETE	Hydroperoxyeicosatetraenoic acid
HSL	Hormone sensitive lipase
H3K4me3	H3 Lys 4 trimethylation
H3K4me2	H3 Lys 4 dimethylation
H3K9ac	H3 Lys 9 acetylation
Ig	Immunoglobulin
IBMX	Isobutylmethylxanthine
IP3	inositol 1,4,5 triphosphate
IP-R	Prostacyclin receptor
IRS-1	Insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
KLF	Krüppel-like factor
LA	Linoleic acid
LAP	Liver-enriched activator protein
LBD	Ligand- binding domain
LCFA	Long-chain fatty acid
LD	Lipid droplet
LDH	Lactate dehydrogenase
LIP	Liver-enriched inhibitory protein
LOX	Lipoxygenase

LPL	Lipoprotein lipase
LTA4,B4	Leukotriene A4, B4
LysoPC	Choline lysophospholipids
MAPK	Mitogen activated protein kinase
MEK	MAPK Kinase/ERK kinase
MG	Monocylglycerol
MGL	Monocylglycerol lipase
MOX	Cytochrome P450 monooxygenases
Myf5	Myogenic factor 5
MSCs	Mesenchymal stem cells
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide
mTOR	Mammalian target of rapamycin
NCoR	Nuclear receptor co-repressor
NEFAs	Non-esterified Fatty Acids
NMR	Nuclear magnetic resonance
NCS	Newborn calf serum
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphatase
PAGE	Polyacrylamide Gel Electrophoresis
PBA	4-phenyl butyric acid
PBS	Phosphate Buffered Saline
PC	Choline glycerophospholipid
PCR	Polymerase chain reaction
PE	Ethanolamine glycerophospholipid
PEPCK	Phosphoenolpyruvate carboxykinase
PET	Positron emission tomography
PFA	Paraformaldehyde
PG	Prostaglandin
PGC-1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGD ₂	Prostaglandin D ₂
PGE ₂	Prostaglandin E ₂
PGF _{2α}	Prostaglandin F _{2α}

PGG ₂	Prostaglandin G ₂
PGHS -1,-2	Prostaglandin endoperoxide synthases -1,-2
PGI ₂	Prostacyclin
PGJ ₂	Prostaglandin J ₂
PI	Phosphatidylinositol
PIP2	PI 4,5-bisphosphate
PKA	Protein Kinase A
PKC	Protein kinase C
PLA2	Phospholipase A2
PMSF	Phenylmethylsulfonyl fluoride
PPAR α - β - γ	Peroxisome proliferator-activated receptor- α - β - γ
PPRE	PPAR Response Element
PRDM16	PR domain containing 16
Pref-1	Preadipocyte factor -1
PUFAs	Polyunsaturated fatty acids
PVDF	Polyvinylidene difluoride
RT-PCR	Real Time PCR
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SFA	Saturated fatty acid
siRNA	Small Interfering RNA
SMRT	Silencing mediator for retinoid or thyroid-hormone receptors
SNS	Sympathetic nervous system
SRC	Steroid receptor coactivator
SREBP	Sterol Regulatory Element-Binding Protein
STAT5A	Signal transducer and activator of transcription 5A
SVF	Stromal vascular fraction
TAGs	Triglycerides
TBS	Tris Buffered Saline
TBST	TBS plus Tween 20
TDF7L1	TCF7-like1
TEMED	Tetramethylethylenediamine

TGF β	Transforming growth factor- β
Tris	Tris (hydroxymethyl) aminomethane
Tween 20	Polyoxyethylene sorbitan monolaurate
TXA ₂	Thromboxane A ₂
TZDs	Thiazolidinediones
UCP1	Uncoupling Protein 1
UFA	Unsaturated fatty acid
VLDL	Very low density lipoproteins
WAT	White Adipose Tissue
3MA	3-methyladenine
15d- $\Delta^{12,14}$ PGJ ₂	15- deoxy- $\Delta^{12,14}$ –Prostaglandin J ₂
17-ODYA	17-octadecynoic acid
¹⁸ F-FDG	[¹⁸ F]-2-fluoro-D-2-deoxy-D-glucose

Chapter 1

Introduction

1.1. Obesity

Obesity has been described as the new epidemic as by 2008, 1.46 billion adults worldwide were overweight (body-mass index [BMI] >25 kg/m²) and 502 million adults were obese (BMI >30 kg/m²) (Swinburn et al., 2011). In western countries the prevalence of obesity is dramatically high e.g. in 2009-2010, in USA the incidence of obesity was 35.5% among adult men and 35.8% among adult women (Flegal et al., 2012). In Great Britain the equivalent was 23% and 25% for adult males and females, respectively in 2002 (Rennie and Jebb, 2005).

Obesity is associated with a series of disorders, such as type 2 diabetes, dyslipidaemia, fatty liver disease hypertension, atherosclerosis, gallbladder disease, degenerative disorders including dementia and some forms of cancer (Bray and Bellange, 2006; Hotamisligil, 2006). Obesity together with insulin resistance, dyslipidaemia and hypertension are the four aspects of the metabolic syndrome which is described as a pre-diabetic state (Grundy, 2012). Even though obesity by itself is unlikely to be the driving factor to metabolic syndrome since lean individuals can become insulin resistant whereas some very obese individuals do not, obesity is still an important risk factor (Virtue and Vidal-Puig, 2010).

Obesity has also programming effects as pregnant women being obese before and during pregnancy, or gaining weight in pregnancy have a higher risk of delivering overweight babies (Desai and Ross, 2011). In the USA 25% of children are now overweight or obese and obesity in childhood or adolescence makes an individual more susceptible to being overweight or obese at 35 years of age with a higher risk of developing diabetes (Guo et al, 2002).

Obesity has been classically termed as the result of an imbalance in energy homeostasis where the energy intake exceeds the energy expenditure. Today this idea is considered to be oversimplistic since obesity is a multi-factorial and complex condition involving the interplay between nutritional, hormonal,

neuronal and psychological factors on a variable genetic background (Sabin et al., 2011; Rosen and Spiegelman, 2006).

Different genes have been associated with the regulation of body weight. Monogenetic mutations such as in leptin and leptin receptor, melanocortin 4 receptor or the FTO gene have been correlated with the onset of obesity (Sabin et al., 2011). Although the genetic background defines the individual's predisposition to obesity, the genotype is susceptible to social trends. Nowadays there is a more "obesogenic" environment characterised by decreased physical activity and increased intake of high caloric food and drink but also other factors such as the ingestion of novel medications, the presence of pollutants, the reduction of the variability in seasonal temperature due to the presence of almost ubiquitous air conditioning (Tam and Ravussin, 2012).

To develop new therapies for the current obesity epidemic, it is essential to elucidate the cellular and molecular mechanisms underlying the regulation of energy balance. Adipose tissue (AT) is a central player of energy metabolism and knowledge of adipose biology is crucial to understand the mechanism by which environmental factors (e.g. diet) affect obesity (Flier, 2004; Ailhaud et al., 2006; Rosen and Spiegelman, 2006).

1.2 Adipose tissue (AT)

AT is a complex organ that plays a central role in energy and metabolic homeostasis. It is composed of different cell types: adipocytes, pre-adipocytes, macrophages, stromal cells, fibroblasts and monocytes. (Frigolet Vazquez-Vela et al., 2008). There are two different types of adipose tissue: the white adipose tissue (WAT) and the brown adipose tissue (BAT).

1.2.1. White Adipose Tissue (WAT)

Most mammals have distinct WAT depots. Some depots have a structural role such as the fat pads of the heels, fingers and toes, and the eye orbital fat. The

two WAT depots that have a role in metabolism are the subcutaneous fat which is associated with the skin and the visceral fat found in the abdominal cavity and is further subdivided into the perigonadal (epididymal in males and periovarian in females), the perirenal, the omental, and the mesenteric fat. The subcutaneous and the visceral fat depots display different patterns of gene expression (including developmental genes), different rates of lipogenesis and lipolysis and different abilities of the pre-adipocytes to differentiate into mature adipocytes *in vitro* (Gesta et al., 2006). Thus, these fat depots are intrinsically different showing distinct developmental characteristics and responses to hormones indicating that these depots are composed of different types of white adipocytes. Fat distribution has a strong hereditary component e.g. Hottentot women have excessive fat accumulation in the buttocks, a condition known as steatopygia (Gesta et al., 2006). Individuals with increased visceral fat are at high risk of type 2 diabetes and cardiovascular disease, whereas body mass index-matched individuals with increased subcutaneous fat are at little or no risk of metabolic disease since it is thought to offer glucose tolerance (Ibrahim, 2010; Yamamoto et al, 2010; Rosen and Spiegelman, 2006)

WAT is the primary site of free-fatty acid (FFAs) or non-esterified fatty acids (NEFAs) storage in the form of triglycerides (TAGs) during energy consumption and the release of FFAs during energy expenditure. White adipocytes also release a repertoire of secreted proteins involved in glucose metabolism (e.g. adiponectin), inflammation (e.g. adiponin), blood pressure (e.g. angiotensinogen) and feeding behaviour (e.g. leptin). Therefore, WAT is not only a storage tissue but also a central regulator of the metabolic program regulating diverse activities, such as insulin sensitivity and lipid metabolism (Hajer et al., 2008). Morphologically, white adipocytes are characterised by a large lipid droplet (LD) (unilocular) occupying 90% of the cell volume. The rest of the cytoplasm contains the nucleus, an under-developed Golgi apparatus, an endoplasmic reticulum (ER), few lysosomes, and few elongated mitochondria with short, randomly arranged cristae (Cinti, 2011).

In rodents, WAT develops mainly after birth, first in the peri-gonadal and subcutaneous depots and only later in the omental depot. The WAT grows in the young animals as a result of both increased adipocyte number (hyperplasia) and adipocyte size (hyperotrophy). However, by the 15th week of life the WAT size is increased solely by an increase in the size of the adipocytes rather than in the cell number (Hirsch and Han, 1969; Billon et al, 2007). In humans, WAT develops from the second trimester, more extensively the third trimester and early in postnatal life. It expands very rapidly after birth as a result of increased cell size but also of increased cell number (Gregoire et al., 1998; Ailhaud et al., 2006). The proliferation and differentiation ability of adipocytes and precursor cells decrease with age. Hence, it was believed that hypertrophy rather than hyperplasia was the main reason for the expansion of the WAT in adults. However, there is now evidence supporting a 10% turnover rate of adipocytes every year in all adult ages confirming that adipogenesis remains active to maintain the adipose compartment (Spalding et al., 2008). Therefore, there must be a pool of precursor cells that are able to differentiate in the adults since mature adipocytes are post-mitotic.

Disrupting the formation of WAT might sound as a quite efficient way to manage obesity but this would result to the storage of lipids in ectopic tissues such as liver and muscle and lead to a syndrome called lipodystrophy. Lipodystrophies are characterised by general (e.g. congenital generalised lipodystrophy) or partial (e.g. familial partial lipodystrophy) absence of the WAT and the symptoms are insulin resistance, fatty liver and dyslipidaemia which are very similar to obesity-associated disorders (Huang-Doran et al., 2010).

1.2.2. Brown Adipose Tissue (BAT)

BAT is found in the interscapular region in rodents whereas in humans it is present in fetuses and newborns in axillary, cervical, perirenal, and periadrenal regions (Cannon and Nedergaard, 2004). Although it was thought to disappear rapidly postnatally, positron emission tomography (PET) using radiotracers such as [^{18}F]-2-fluoro-D-2-deoxy-D-glucose (^{18}F -FDG) revealed

that adults have active brown depots in the neck and chest region, as well as in the kidney area (Nedergaard et al 2007; Nedergaard et al, 2010). Dedicated studies revealed that 30%-100% of adults have BAT, depending on the cohort, with no difference between the genders (Nedergaard et al, 2011).

The main role of the brown adipocytes is the dissipation of energy through the process of adaptive thermogenesis. They express the uncoupling protein 1 (UCP1) which is localised in the inner mitochondrial membrane and its role is to uncouple Adenosine-5'-triphosphate (ATP) from the classical electron transport chain to produce heat (Hansen and Kristiansen, 2006). Other molecular markers of BAT are high-levels of expression of type 2 iodothyronine deiodinase (DIO2), the transcription coregulators PR domain containing 16 (PRDM16), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), and the regulator of lipolysis Cidea (Cypess and Kahn, 2010). Morphologically, brown adipocytes have multiple small LDs (multilocular) and numerous spherical mitochondria rich in lamellar cristae. They are much smaller in diameter, 15–60nm, than white adipocytes which are 25–200 nm in diameter (they are able to increase their size several times). BAT is highly vascularised and innervated by the sympathetic nervous system (SNS) (Cinti, 2011). When there is a need for release of stored energy or for producing heat, then a signal is transmitted from the SNS to individual brown adipocytes. The neurotransmitter norepinephrine via β 3-adrenergic receptors initiate a cascade of events involving the rise of the intracellular cyclic 3'5' Adenosine Monophosphate (cAMP) levels and protein kinase A (PKA) activation that lead to the release of FFA activating UCP1 (Cannon and Nedergaard, 2004).

The human adult BAT is less than 100g and expresses all the BAT gene markers (e.g UCP1, PGC-1 α). If BAT is activated then humans are able to develop non-shivering thermogenesis *i.e.* the ability to produce heat without shivering (which is the acute and immediate heat-producing mechanism) that has the capacity to burn an amount of energy equivalent to approximately 4kg of WAT in a year, making BAT a significant target tissue for medical intervention to manage obesity (Virtanen et al., 2009).

In conclusion, BAT and WAT are anatomically, morphologically and functionally distinct. The different colour between the BAT and the WAT is due to the different number of mitochondria and the difference in the vascularisation (Hansen and Kristiansen, 2006). BAT is mainly a thermogenic tissue whereas WAT is a storing tissue. WAT does not express UCP1 and BAT does not produce leptin. However, under certain conditions e.g. cold temperature, exposure to β 3-adrenergic agonists or exercise, brown-like adipocytes can be found in WAT depots expressing UCP1 representing a different adipocyte type called brite or beige (Cinti, 2011; Bostrom et al, 2011). Beige adipocytes are shown to have a different lineage origin from brown adipocytes (they do not express Myf5, see below) and have a distinct gene expression pattern from either white or brown fat (Wu et al., 2012). There is a debate as to whether these cells are the result of a direct transdifferentiation of mature white adipocytes to brown or they come from totally distinct progenitors.

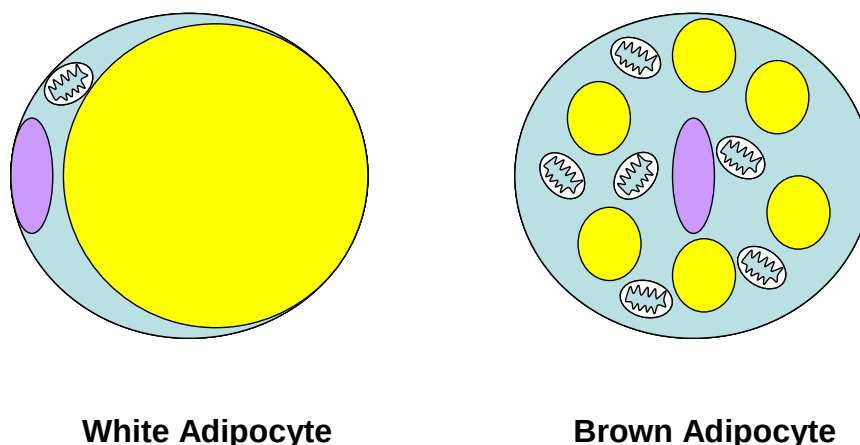


Figure 1.1: Differences between white and brown adipocytes. White adipocytes are characterised by a large LD (yellow) and a few mitochondria. They are specialised to store energy, and they express low levels of UCP1. In contrast, brown adipocytes have many smaller LDs and numerous mitochondria whereas they are specialised to catabolise energy, and they express high levels of UCP1. LD: lipid droplet, UCP1: uncoupling protein 1.

1.3. Adipocyte differentiation (Adipogenesis)

Little is known about the identity, localisation and the biological characteristics of the adipocyte progenitors or pre-adipocytes *in vivo*. The stromal vascular fraction (SVF) of the adipose tissue contains adipose precursor cells which are morphologically identical to other non-adipogenic fibroblast cells making their identification difficult. However, recently, Tang et al (2008) identified a population of adipocyte progenitor cells residing in the mural cell compartment of the adipose vasculature that show specific molecular markers. These cells are found attached to the walls of blood vessels and are often bigger than the endothelial cells with a characteristic basal membrane and glycogen granules. As the pre-adipocytes develop they detach from the capillaries and progressively lose the glycogen vacuoles and accumulate lipid. Thus, the vasculature seems to provide the right local microenvironment for the development of adipocytes (Tang et al, 2008; Cinti, 2011). Brown pre-adipocytes have been also found in the proximity of vessels in the BAT and they have the characteristic basal membrane and glycogen vesicles as the white adipocytes but they also have pretypical mitochondria contrary to the white (Cinti, 2011).

Friedman and colleagues identified a cell population in the adult WAT SVF expressing specific cell-surface markers ($\text{Lin}^- \text{CD29}^+ \text{CD34}^+ \text{Sca-1}^+ \text{CD24}^+$) that was able to differentiate to mature adipocytes *in vitro*. Furthermore, when transplanted into a lipodystrophic mouse models the cells were able to reconstitute a normal size fat pad and correct the diabetic phenotype of these mice (Rodeheffer et al., 2008).

Despite the limited information about adipogenesis *in vivo*, adipocyte differentiation is well characterised, mainly from studies using *in vitro* models. To some extent, pre-adipocyte primary culture has been used. However, the use of primary culture has a number of disadvantages. During the isolation procedure, large amounts of fat tissue are required since pre-adipocytes comprise only a small part of the total fat tissue, but also the result is a heterogeneous population since pre-adipocytes cannot be distinguished from

other fibroblast-like cells. Finally, primary cultures have a limited life span in culture (Ntambi and Young-Cheul, 2000). Thus, adipogenesis has been studied primarily using *in vitro* cell lines. There are two main advantages using these models, the first one that they are clonal populations and hence homogeneous synchronized cells being all at the same stage of differentiation and the second is that they can be kept in culture indefinitely (Ntambi and Young-Cheul, 2000).

From the study of these cell lines and for practical reasons adipogenesis is considered to be a two-phased procedure. The first phase is known as determination. During this phase mesenchymal stem cells (MSCs) become committed to the adipocyte lineage losing their ability to differentiate into other lineages such as osteoblasts, chondrocytes, myoblasts and connective tissue, and essentially becoming pre-adipocytes. In this stage pre-adipocytes have a fibroblast-like morphology and are undistinguishable from their precursors. In the second phase, which is known as terminal differentiation, pre-adipocytes develop into mature adipocytes, acquiring LDs, the ability to respond to insulin and secrete adipocyte specific proteins such as adiponectin (Figure 1.2) (Gesta et al., 2007). The transcriptional regulation of the terminal differentiation phase has been well characterised using *in vitro* pre-adipocyte cell lines that have passed through the commitment step and when appropriately induced, differentiate into cells having the biochemical and morphological phenotype of adipocytes. The most extensively characterised preadipocyte cell lines are the 3T3-L1, 3T3-F442A, and Ob1771 lines. The 3T3-L1 and 3T3-F442A cell lines were derived from disaggregated mouse embryo cells and were selected for their ability to accumulate TAGs (Green and Kehinde, 1975; Green and Kehinde, 1976). When injected into mice, 3T3-L1 pre-adipocytes differentiate and form fat pads that are indistinguishable from normal AT (Green and Kehinde, 1979). Ob17 cells were derived from the stroma of epididymal fat pads from adult ob/ob genetically obese mice, and several sublines, notably the Ob1771 line, were selected (MacDougald and Lane, 1995). The factors that have been identified over the years from the study of these immortalised cell lines have proved to be crucial for adipogenesis *in vivo* either in mouse models or even humans (Gupta et al.,

2012). Based on these *in vitro* models the terminal differentiation can be divided into four stages: growth arrest, clonal expansion, early differentiation and late differentiation (Gesta et al., 2007). First, following confluence, the cells enter growth arrest due to cell-cell contact inhibition. After the exposure of confluent cells to insulin, dexamethasone (DEX) and isobutylmethylxanthine (IBMX) in the presence of fetal bovine serum (FBS), they re-enter the cell cycle for one or two divisions (clonal expansion). However, the clonal expansion step in other cell systems is not always required (e.g. human pre-adipocytes). During the early terminal differentiation, the cells express a cascade of transcription factors including a rapid induction of CCAAT/enhancer binding protein (C/EBP β) β and C/EBP δ . These transcription factors promote the expression of the two most important regulators of terminal differentiation peroxisome proliferator-activated receptor γ (PPAR γ) and C/EBP α leading to a second but permanent growth arrest and the induction of many target genes involved in lipogenesis and adipogenesis (see Section 1.3.2) (Farmer, 2005; Farmer, 2006).

Brown and white adipocytes share many common features in their differentiation despite their functional, morphological and locational differences. However, lineage-tracing studies revealed that brown adipocytes are developmentally closer to skeletal muscle cells than white adipocytes. Both muscle and brown adipocyte progenitors express the muscle marker myogenic factor 5 (Myf5) whereas white adipocytes do not (Seale et al., 2008). Furthermore, knockdown of PRDM16 (a transcriptional co-regulator) in brown preadipocytes leads to a more myogenic phenotype whereas overexpression of PRDM16 in myoblasts leads their differentiation to a brown adipocyte phenotype (Seale et al., 2008).

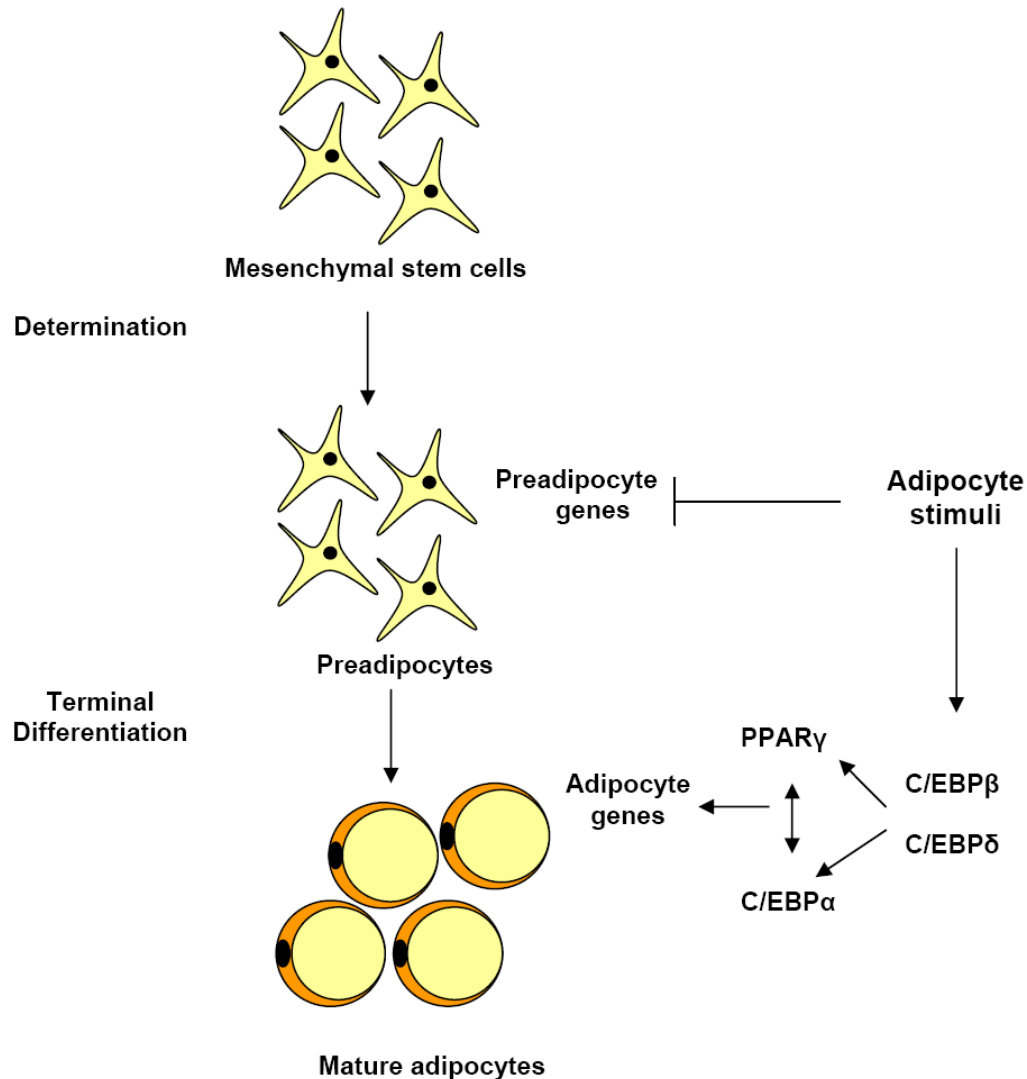


Figure 1.2: Adipocyte differentiation (adipogenesis). Adipocytes arise from multipotent MSCs which become pre-adipocytes when they lose their ability to differentiate into other mesenchymal lineages and become 'committed' to the adipocyte lineage (commitment). During terminal differentiation pre-adipocytes take on the characteristics of mature adipocytes, acquiring LDs and the ability to respond to hormones such as insulin. Terminal differentiation consists of a cascade of transcriptional events. The first wave involves the induction of C/EBP β and C/EBP δ , which, in turn, directly induces expression of C/EBP α and PPAR γ . C/EBP α and PPAR γ subsequently feed back to induce their own expression, in addition to activation of many downstream target genes whose expression defines the adipocyte. MSCs: mesenchymal stem cells, LD: lipid droplet, C/EBP β - δ CCAAT/enhancer binding protein β - δ , PPAR γ : peroxisome proliferator-activated receptor γ (modified from Christodoulides et al., 2009).

1.3.1 Determination

The full repertoire of factors that cause the conversion of an MSC into an adipocyte is largely unknown. Some knowledge about MSC fate decisions has been derived from studies using the immortalised mouse stromal cell line, C3H10T1/2, or mesenchymal precursor populations isolated from adult or fetal tissues (e.g. bone marrow) and embryonic stem cells (ESCs) that have been successfully differentiated to adipocytes *in vitro* (Dani et al, 1997; Billon et al., 2008). In contrast to terminal differentiation the transcriptional regulation of the determination step is not well defined. However, recently Gupta et al., identified Zfp423 transcription factor which is expressed in adipose precursors, as sufficient and necessary for adipogenesis (Gupta et al., 2010). Isolation of GFP⁺ cells from the SVFs of Zfp423^{GFP} transgenic mice differentiated more efficiently in culture compared to their GFP⁻ counterparts proving that Zfp423 expressing cells are *bona fide* committed pre-adipocytes (Gupta et al, 2012). Moreover, Christancho et al., showed that TCF7-like1 (TDF7L1) transcription factor is essential for the pre-adipocyte as it represses cell structure-related genes highlighting the importance of cell structure organisation to the differentiation of pre-adipocytes (Christancho and Lazar, 2011).

A number of factors have been identified that function as molecular switches in controlling the fate of the progenitors. These factors could drive the MSC differentiation into alternative cell fates and have to be inhibited from pro-adipogenic factors for the adipogenesis to proceed.

Two Wnt signaling pathways - the canonical Wnt- β catenin pathway and the non-canonical Wnt pathway - are described to favour the conversion of the MSCs towards the osteoblastic lineage. Wnt10b and Wnt5a are the two main canonical and non-canonical Wnt ligands respectively that are expressed in pre-adipocytes and inhibit adipogenesis by negatively regulating the expression of the two main adipogenic proteins PPAR γ and C/EBP α (Christodoulides et al., 2009; Takada et al, 2009). However, adipogenesis has been found to proceed by the inhibition of Wnt ligand expression in pre-

adipocytes by cAMP signalling which is essential for the first steps of adipogenesis (Fox et al., 2008).

Transforming growth factor- β (TGF β) superfamily members such as TGF β , bone morphogenetic proteins (BMPs) and myostatin have all been described to have roles in adipogenesis. TGF β has been shown to have contradictory effects *in vivo* and *in vitro* promoting and inhibiting adipogenesis, respectively. BMP4 is shown to promote the differentiation of C3H10T1/2 cells to white adipocytes, whereas BMP7 induces brown adipogenesis. Finally ablation of the myostatin gene in mice results in a decrease in AT and an increase in muscle tissue (Zamani and Brown, 2011).

There are also many factors that are specifically expressed in committed pre-adipocytes and their down-regulation sets the stage for terminal differentiation. One of these factors is the preadipocyte factor -1 (Pref-1 or DLK-1: Drosophila Homolog-like 1) which is widely accepted as a pre-adipocyte marker. Pref-1 is synthesised as a transmembrane protein and its extracellular domain is cleaved generating the active 50kDa soluble form that inhibits adipocyte differentiation (Wang et al, 2006). It is highly expressed in pre-adipocyte cell lines (e.g. 3T3-L1 cells) but its expression decreases upon differentiation (Wang et al, 2006). Overexpression of Pref-1 in pre-adipocytes decreases their differentiation to mature adipocytes whereas depleting Pref-1 increases the ability of the cells to undergo differentiation (Wang et al, 2006). Pref-1^{-/-} animals show increased adiposity (Moon et al., 2002) whereas transgenic animals show decreased fat mass, reduced expression of adipocyte markers and lower levels of adipocyte secreted factors (Lee et al., 2003).

Finally, transcription factors such as GATA-binding protein 2 (GATA 2) and 3, members of the Krüppel-like factor (KLF) family and CHOP (a dominant negative partner of C/EBP β) act as inhibitors of differentiation that eventually lead to the repression of PPAR γ expression (Tang and Lane, 2000; Tong et al., 2000; Banerjee et al., 2003).

1.3.2 Terminal Differentiation

As referred to Section 1.3, a number of transcription factors are involved in terminal differentiation. This stage is further divided into the early and late terminal differentiation.

1.3.2.1 Fos-related antigen 1 or Fra-1

Fra-1 or Fos-related antigen 1 is a member of the transcription factor activator protein 1 (AP-1) family. This family consists of the Jun, Fos and activating transcription factor protein families. Fra-1 is one of the Fos proteins together with c-Fos, FosB and Fra-2 and can only heterodimerise with members of the Jun family, whereas the Jun family members (Jun, JunB and JunD) form homodimers and heterodimers with Fos members (Zenz et al., 2008). The different dimer combinations recognise a common AP-1 binding site (TGACTCA) in the promoters and enhancers of target genes. Fra-1 ablation is embryonic lethal around embryonic day 10 due to placental defects. However, specific deletion of Fra-1 only in the mouse, resulted in viable and fertile Fra-1^{-/-} mice which developed osteopenia, indicating a role of Fra-1 on the regulation of bone mass (Eferl et al, 2004).

A role for AP-1 factors in adipogenesis has been implicated and one of the earliest reports in this area identified the binding of c-Fos to the aP2 promoter (Distel et al, 1987). *In vivo* data indicate that AP-1 factors are important in adipogenesis. Transgenic mice expressing a dominant-negative protein preventing the DNA binding of both the C/EBP and Jun families specifically in AT were generated. These mice had no WAT throughout life (Moitra et al, 1998). In 3T3-L1 cells, c-Jun, c-Fos, Jun-B, JunD, Fos-B, Fra-1 and Fra-2 were found to be induced immediately after the induction of adipocyte differentiation (Stephens et al, 1992). Finally, the recently generated Fra-1 transgenic mice present with severe lipodystrophy. Furthermore, Fra-1 is found to suppress adipogenesis by inhibiting C/EBP α expression (Luther et al., 2011).

1.3.2.2 C/EBPs

C/EBPs are members of a family of highly conserved basic-leucine zipper transcription factors (bZip) comprised of six members. All members have a C-terminal leucine zipper domain for dimerisation and a basic domain for DNA binding. These proteins can both homodimerise and heterodimerise with each other and bind to the same C/EBP consensus sequences. Three of the C/EBP family members (C/EBP α , - β and - δ) play an important role in adipogenesis. In 3T3-L1 cells, C/EBP β and C/EBP δ mRNA and protein levels rise very early (2-3h) after the induction of differentiation; they remain constant for the next 36h and then begin to decline (Tang et al., 2004). It has been shown that IBMX (included in the differentiation cocktail), a phosphodiesterase inhibitor causes the increase of cAMP which in turn increases C/EBP β mRNA levels (Cao et al., 1991). Consequently, cAMP response element-binding protein (CREB) binds to the dual CRE-like elements in C/EBP β promoter inducing its expression immediately after the induction of differentiation in 3T3-L1 cells (Zhang et al., 2004). In addition, induction of C/EBP δ is facilitated by C/EBP β and also glucocorticoids which are also included in the differentiation cocktail (Cao et al, 1991). C/EBP β and C/EBP δ single knockouts show a minor decrease in adipose mass whereas in double knockouts the size of the AT is substantially reduced (Tanaka et al, 1997). These data indicate that there is a degree of redundancy between these two transcription factors.

After their induction early in differentiation C/EBP β and C/EBP δ bind directly to C/EBP binding sites on both PPAR γ and C/EBP α promoters but also on promoters of several genes expressed later in the differentiation process such as phosphoenolpyruvate carboxykinase (PEPCK), fatty acid binding protein 4 (FABP4) and glucose transporter type 4 (Glut4) (Wu et al, 1999; Salma et al, 2006; Tang et al., 2004). C/EBP β ectopic expression in NIH-3T3 fibroblasts alone or in combination with C/EBP δ induces PPAR γ expression and adipogenesis, whereas C/EBP δ possesses minimal adipogenic activity (Wu et al., 1995; Farmer, 2006).

C/EBP α is expressed later in the differentiation program and is considered to be an important regulator of white adipocyte differentiation since C/EBP $\alpha^{-/-}$ mice lack WAT but they show normal BAT (Linhart et al., 2001). C/EBP α replaces C/EBP β and C/EBP δ binding to each of the various C/EBP binding sites at the promoters of adipogenic genes (Christy et al., 1989; Salma et al., 2006). Overexpression of C/EBP α in 3T3-L1 cells induces their differentiation into mature adipocytes (Lin and Lane, 1994), whereas constitutive expression of an anti-sense C/EBP α RNA in the same cell model, blocks the expression of adipocyte specific genes and triglyceride accumulation (Lin and Lane, 1992). Moreover, C/EBP $\alpha^{-/-}$ fibroblasts have reduced adipogenic potential, but their capacity to accumulate lipids and activate markers of adipogenesis is restored when PPAR γ is exogenously expressed. It seems that C/EBP α maintains PPAR γ levels as it was found that C/EBP α binds directly to the PPAR γ promoter (Elberg et al., 2000). Finally, C/EBP α is important for establishing insulin sensitivity since it was found to bind and regulate the expression of many genes involved in the insulin signalling such as the insulin receptor and insulin receptor substrate (IRS-1) (Wu et al. 1999).

Despite the important role of C/EBPs in adipogenesis the presence of PPAR γ is pivotal for the function of these transcription factors. For example, C/EBP β cannot induce expression of C/EBP α in the absence of PPAR γ (Zuo et al., 2006) and the ectopic expression of C/EBP α cannot rescue adipogenesis in PPAR $\gamma^{-/-}$ fibroblasts (Rosen et al., 2002).

1.3.2.3 PPAR γ

PPAR γ together with PPAR α and PPAR β constitute a subfamily of the nuclear receptor superfamily which includes receptors for steroids, thyroid hormone, vitamin D, and retinoic acid (Mangelsdorf et al, 1995). All the receptors of this subfamily form obligate heterodimers with another family of nuclear receptors the retinoid X receptors (RXRs). The heterodimers bind to DNA response elements called PPAR response elements (PPREs), the consensus being repeats of the sequence AGGTCA separated by one or two nucleotides (known as DR-1 or DR-2 response elements, respectively). PPARs bind to the 5' repeat unit of PPRE and RXR binds to the 3' repeat (Daynes and Jones, 2002). Using chromatin immunoprecipitation sequencing (ChIP-seq) the genome-wide binding profile of PPAR γ and RXR confirmed the high degree of overlap between the binding sites of the two factors, indicating that PPAR γ and RXR dimerisation is obligatory for DNA binding. This study also revealed that the majority of PPAR γ :RXR binding sites are located far away from the transcription start sites suggesting that looping between enhancers and promoters is a common mechanism for PPAR γ to regulate its target genes (Lefterova et al., 2008; Nielsen et al., 2008).

In humans, the *PPAR γ* gene has nine exons and exists in four mRNA subtypes (γ 1, γ 2, γ 3, and γ 4) whereas in mice there are two mRNA subtypes (PPAR- γ 1 and γ 2) resulting from alternative promoter usage and alternative splicing at the 5' end of the gene. Despite the different mRNA subtypes, PPAR- γ exists in two protein isoforms PPAR- γ 1 and γ 2 in both humans and mice. hPPAR- γ 1, γ 3, and γ 4 mRNAs and mPPAR- γ 1 mRNA, give rise to the protein product, PPAR- γ 1 (477 amino acids (aa) for hPPAR- γ 1 and 475 aa for mPPAR- γ 1). hPPAR- γ 2 and mPPAR- γ 2 mRNA encodes for the PPAR- γ 2 protein with 28 and 30 extra N-terminal aa (505 aa) for human and mouse respectively, encoded by the B exon (Figure 1.3) (Zhu et al, 1995; Fajas et al., 1997; Fajas et al., 1998; Sundvold and Lien, 2001). AT, large intestine and haematopoietic cells express high levels of PPAR γ , kidney, liver and small intestine express intermediate levels whereas muscle barely express PPAR γ . The expression levels of PPAR γ 2 are low in all tissues

compared to PPAR γ 1 with the only exception AT where PPAR γ 2 is expressed in more pronounced amounts (Auwerx, 1999).

PPAR γ protein has the same structure found in most nuclear receptors characterised by 6 regions (A-F). The C-terminal E domain is the ligand-binding domain (LBD). This region is comprised of 12 α helices (H1-H12) and it also contains the ligand-dependent activation domain (AF-2) which depends on the highly conserved helix 12 in the C-terminal end of the LBD. In the unliganded state, the PPAR γ LBD creates a hydrophobic pocket buried among the α -helices. In this state PPAR γ is able to bind DNA and associates with co-repressors such as the silencing mediator for retinoid or thyroid-hormone receptors (SMRT) and the nuclear receptor co-repressor (NCoR) which are part of multiprotein complexes containing histone deacetylase (HDAC) activity that repress target gene transcription. Upon ligand binding, co-repressor proteins are dissociated from PPAR γ and its helix 12 changes conformation creating the appropriate interface for the recruitment of co-activators such as the steroid receptor coactivator (SRC) and p300/CREB-binding protein (CBP). These co-activators have either intrinsic chromatin remodelling activities (such as histone acetyl transferase activity) or recruit other complexes that decondense the chromatin making a transcriptionally permissive environment at the promoter or enhancer (Zieleniak et al., 2008).

Polyunsaturated fatty acids (PUFAs) (such as linoleic acid and arachidonic acid) and their derivatives such as prostaglandins (e.g. 15d-PGJ2) and oxidized FAs [e.g. 9-hydroxyoctadecadienoic (9-HODE) and 13-hydroxyoctadecadienoic (13-HODE)] have been identified to be the endogenous low affinity ligands for PPAR γ binding the receptor at micromolar levels (Forman et al., 1995; Kliewer et al., 1995; Nagy et al., 1998). It has been demonstrated that endogenous PPAR γ ligands are produced during the early stages of adipogenesis in response to cAMP (Tzameli et al., 2004). There are also synthetic, highly specific, ligands for PPAR γ called thiazolidinediones (TZDs). TZDs exhibit high affinities for the receptor and they are used clinically as anti-diabetic agents enhancing insulin sensitivity. However, TZDs have several side effects including osteoporosis, increased

adiposity, fluid retention and heart failure presumably due to the activation of PPAR γ in osteoclasts, collecting duct cells and cardiomyocytes (Sugii and Evans, 2011). However, how TZDs function as potent insulin sensitizers is still unclear since there is not a known PPAR γ deficiency in obesity or insulin resistance cases (Choi et al., 2010). Hence, it is puzzling how activation of this receptor is an efficient anti-diabetic strategy as PPAR γ selective activation in adipocytes in insulin resistant states leads to whole body insulin sensitisation (Sugii et al., 2009)

The N-terminal A/B region is non-conserved between nuclear receptors and it contains another transcription/activation domain the AF-1 which acts in a ligand-independent manner. In PPAR γ 2, residue S112 in the AF-1 domain is subjected to phosphorylation by the mitogen activated protein kinase (MAPK) pathway. This phosphorylation event can disrupt the interdomain interaction between the LBD and the AF-1 domain reducing the ligand binding affinity resulting in negative regulation of the transcriptional and biological functions of PPAR γ (Shao et al., 1998). Furthermore, cyclin-dependent kinase 5 (Cdk5) is activated in obesity and phosphorylates PPAR γ at the S273 residue without affecting its transcriptional activity but it causes dysregulation of its target genes such as adiponectin and adipisin (Choi et al., 2010). Weak PPAR γ agonists (e.g. MRL24) have been shown to inhibit this Cdk5-phosphorylation event making possible the development of improved anti-diabetic medication avoiding the side effects of the full agonists such as TZDs (Choi et al., 2010). The C domain is the DNA binding domain (DBD) and it is the most conserved among nuclear receptors. It contains two zinc-finger-like motifs that mediate DNA binding but it also takes part in the dimerisation with RXR (Zieleniak et al., 2008). The adipogenic activity of PPAR γ requires a functional DNA-binding domain, as DBD mutants were not able to activate target genes (Tontonoz et al., 1994).

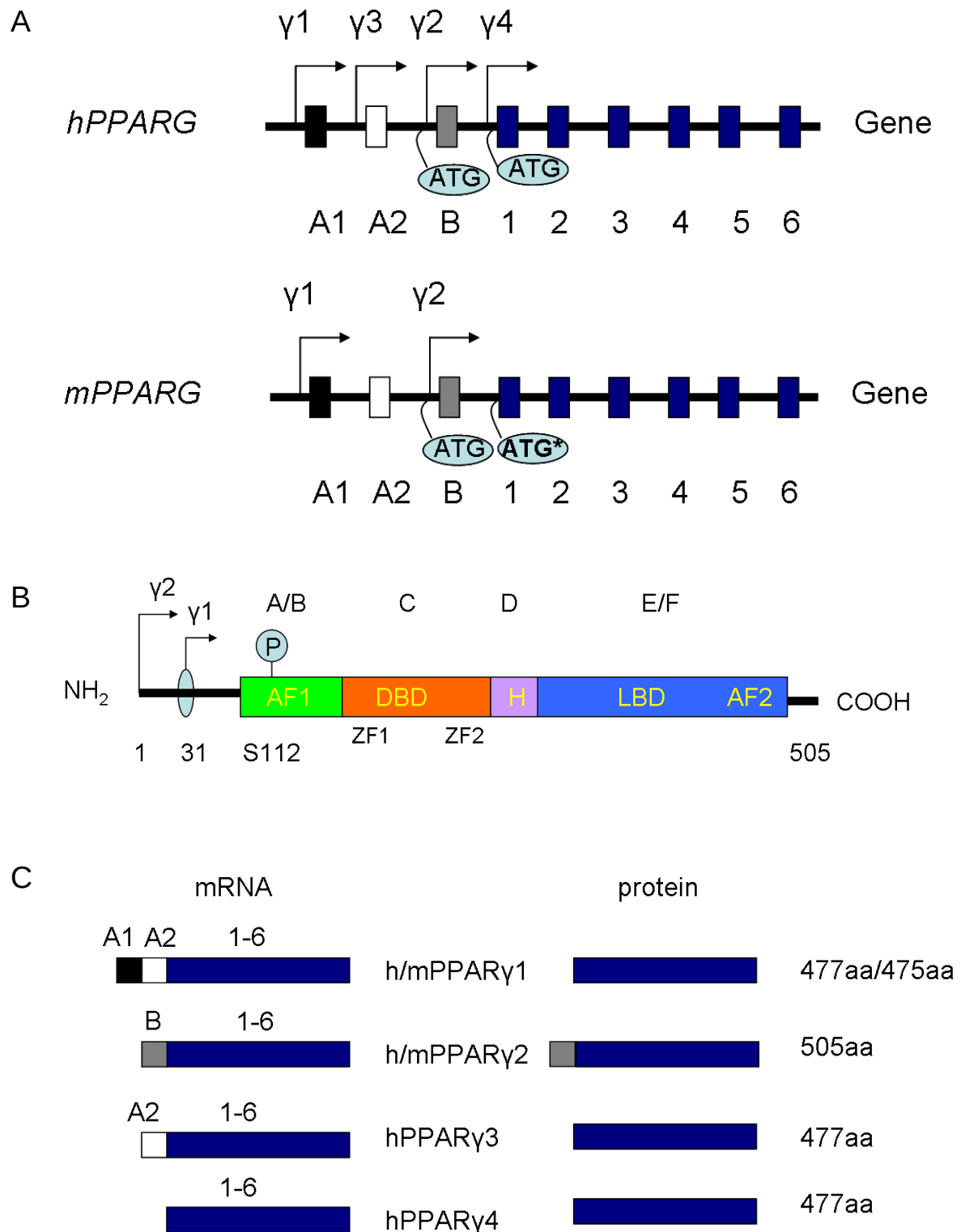


Figure 1.3: Gene and protein organization of the mouse and human PPAR γ . A) PPAR γ gene structure. The genes are shown in 5' to 3' orientation. Exons are denoted by rectangles and introns by a solid line. γ 1, γ 2, γ 3, and γ 4 represent the promoters of PPAR- γ 1, - γ 2 - γ 3, and - γ 4 mRNA, respectively. The location of the ATG start codon is indicated. The asterisk indicates the different ATG used in mPPAR γ 1

(hPPAR γ 1 contains two additional aa relative to mPPAR γ 1). B) PPAR γ protein structure. The hPPAR γ 2 protein contains an additional 28aa at the N terminus compared to hPPAR γ 1, whereas the mPPAR γ 2 30aa. Position of the S112 phosphorylation site, DNA-binding domain (DBD), hinge domain, ligand-binding domain (LBD), and activation function 2 (AF2) are shown. Numbers correspond to aa position. C) The human four subtypes of mRNA give rise to two different PPAR γ proteins. Transcription on the promoters γ 1, γ 3, and γ 4 results in the same protein hPPAR γ 1 477 aa and transcription on the promoter γ 1 results in mPPAR γ 1 475aa. The hPPAR γ 2 and mPPAR γ 2 protein of 505aa is produced by transcription from the promoter γ 2. Abbreviations: γ 1 (PPAR γ 1) and γ 2 (PPAR γ 2); P, phosphorylation site; (adapted from Fajas et al., 1997; Zieleniak et al., 2008; Tontonoz and Spiegelman, 2008).

The D domain is called the hinge domain that can act as a docking site for co-activators. It also contains a nuclear localisation signal which allows the translocation of the nuclear receptor into the nucleus. Finally, it can affect the activities of nearby domains such as the DBD (Zieleniak et al., 2008).

In elucidate PPAR γ transcriptional regulation, DNase I hypersensitivity site analysis combined with deep sequencing was performed. This analysis revealed changes in chromatin structure near the promoter and distal sites of the *PPAR γ* locus within a few hours after the induction of adipocyte differentiation. At the same time a set of early transcription factors (including C/EBP β , C/EBP δ , STAT5A, GR and RXR) bind in a cooperative manner early in the differentiation program forming transcriptional ‘hotspots’. These hotspots are regions of active chromatin remodelling and are found near different PPAR γ target genes but also at the *PPAR γ* locus itself (Siersbæk et al., 2011). Together with the chromatin remodelling, the *PPAR γ* locus displayed increased levels for H3 Lys 4 trimethylation (H3K4me3) and dimethylation (H3K4me2) and H3K9 acetylation (H3K9ac). High levels of H3K4me3 are correlated with transcription start sites whereas high levels of H3K4me2 and H3K9ac are correlated with the presence of enhancers. *Ppar γ 2* transcription start site contains a peak for H3K4me3 on day 1 after the induction of differentiation and is strongly marked by day 10. This is consistent with the fact that PPAR γ 2 is induced 24h after the initiation of differentiation,

and significantly up-regulated in mature adipocytes. A notable area -10kb upstream *Pparγ2* locus is rich in H3K4me2 and H3K9ac levels that increase from day 0 to day 1 and then fall back to day 0 levels at day 10. Finally, this region was found to bind glucocorticoid receptor (GR) and C/EBPβ indicating that enhancer regions can regulate the transition from a pre-adipocyte to a mature adipocyte (Steger et al., 2010).

PPARγ is highly expressed in both WAT and BAT tissue and is considered to be the 'master regulator' of adipogenesis since it is both necessary (Rosen et al., 2002) and sufficient for adipogenesis (Tontonoz et al., 1994). However, PPARγ not only stimulate differentiation to adipocytes of resident AT pre-adipocytes but also promotes the mobilisation of bone marrow-derived circulating progenitor cells to WAT and their subsequent differentiation into adipocytes (Crossno et al., 2006).

In humans, when PPARγ function is reduced, they become lipodystrophic and insulin resistant (Barosso et al., 1999). Homozygous PPARγ^{-/-} mice are not viable due to the role that PPARγ plays in trophoblast formation (Kubota et al., 1999). However, different strategies have been employed to achieve adipose-specific PPARγ knockdown that establish PPARγ essential for adipogenesis (Barak et al., 1999; Rosen et al., 1999; Koutnikova et al., 2003). For example, tetraploid rescue of PPARγ null mutants and chimeric mice made from PPARγ^{-/-} and PPARγ^{+/+} blastocysts show that no adipocytes develop from PPARγ null cells (Barak et al., 1999). On the other hand, in humans, one gain-of-function mutation of PPARγ has been identified (PPARγP115Q). This mutation prevents the adjacent residue being phosphorylated (S114) and PPARγ activity is increased. All patients carrying this mutation were severely obese, supporting the idea of positive correlation between PPARγ activity and adiposity (Hamer et al., 2002). Moreover, gain-of-function experiments have demonstrated that PPARγ alone is sufficient to initiate the entire adipogenic program in non-adipogenic mouse fibroblasts (Tontonoz et al., 1994). PPARγ can induce differentiation in C/EBPα^{-/-} MEFs demonstrating that even though C/EBPα is important for adipogenesis, PPARγ is the dominant factor (Wu et al., 1999). Furthermore, PPARγ^{-/-} cells express reduced levels of C/EBPα

(Rosen et al., 1999) and C/EBP α ^{-/-} cells express only low amounts of PPAR γ (Wu et al., 1999). Hence it seems that there is a positive feedback loop between C/EBP α and PPAR γ expression at the final stage of adipogenesis, which ensures the maintenance of the phenotype of mature adipocytes (Wu et al., 1999).

Genome-wide studies have revealed that 91% of the genomic regions to which PPAR γ is bound contain at least one C/EBP motif (Lefterova et al., 2008). C/EBP α binds nearby to >60% of the locations bound by PPAR γ and also >60% of the up-regulated genes bound both PPAR γ and C/EBP α while only 3% of the genes were bound by PPAR γ alone (Lefterova et al., 2008). Moreover, when PPAR γ , C/EBP α and C/EBP β are all simultaneously depleted the expression of many classical adipogenic genes is remarkably downregulated, suggesting that these factors have synergistic roles in activating transcription (Lefterova et al., 2008).

Finally, besides the role of PPAR γ in adipogenesis, it is also a very important factor for the survival of the mature adipocytes, since selective PPAR γ knockdown in white and brown mature adipocytes leads to the death of these cells (Imai et al., 2004).

1.3.2.4 Adipocyte Fatty acid Binding Protein (or FABP4 or aP2)

As FAs are hydrophobic molecules, they have to bind to albumin, lipocalins and fatty acid-binding proteins (FABPs) to increase their aqueous solubility and facilitate their transport. FABPs comprise a family of intracellular lipid chaperons that consist of nine members having molecular masses around 14-16kDa. FABPs are highly conserved among all species, from *Drosophila melanogaster* and *Caenorhabditis elegans* to mice and humans. In mammals, they are expressed in almost all tissues and they were named after the tissue they were first discovered. The family contains liver (L-FABP/FABP1), intestinal (I-FABP/FABP2), heart (H-FABP/FABP3), adipocyte (A-FABP/FABP4/aP2), epidermal (E-FABP/FABP5/mal1), ileal (Il-FABP/FABP6), brain (B-FABP/FABP7), myelin (M-FABP/FABP8), and testis (T-FABP/FABP9) isoforms. However, this is quite misleading since more than one form is expressed in one tissue (Furuhashi and Hotamisligil, 2008).

The FABP members show a moderate amino acid sequence homology varying from 20-70%. However, crystallography and nuclear magnetic resonance (NMR) analyses revealed that all FABPs share a common tertiary structure with 10 anti-parallel β strands folding as an elliptical β barrel, which is capped by two short α -helices arranged as a helix-turn-helix segment. The binding pocket is located inside the β -barrel whereas the helix-turn-helix motif is thought to act as a portal that regulates FA entry and exit from the internal binding cavity (Furuhashi and Hotamisligil 2008; Storch and Corsiso, 2008).

The β barrel cavity is two to three times larger than the volume of the FA and the structures reveal that on one side of the cavity there are ordered water molecules hydrogen bonded to internal polar residues, whereas a single FA with the carboxylate group oriented inward is on the other side. Most of the FABPs bind a single FA, with the exception of liver-FABP (or FABP1) which has a considerably larger binding pocket allowing the binding of two FAs (Furuhashi and Hotamisligil 2008; Storch and Corsiso, 2008).

All FABPs bind saturated and unsaturated long-chain (≥ 14 -C) FAs (LCFAs) without showing any particular selectivity. Affinities tend to increase with increasing ligand hydrophobicity. Most of the FABPs examined (e.g. intestinal type, adipose-type, heart-type, keratinocyte-type, and myelin-type FABPs) collect and deliver their ligands by contact with a membrane. From there FABPs as lipid chaperones may facilitate the transport of lipids to specific compartments of the cell, such as to the LD for storage; to the ER for signalling, trafficking and membrane synthesis; to the mitochondria or peroxisome for oxidation; to cytosolic or other enzymes to regulate their activity; to the nucleus for lipid-mediated transcriptional regulation; or even outside the cell to signal in an autocrine or paracrine manner (Figure 1.4) (Furuhashi and Hotamisligil, 2008).

PPAR γ and C/EBP α at the final stage of adipogenesis regulate the expression of a large group of genes, including adipocyte FABP, which is highly expressed in mature adipocytes and is considered to be one of the classical adipocyte differentiation markers. Adipocyte FABP (AFABP) is also referred to as aP2, due to its sequence similarity to the so-called myelin P2 protein, and as ALBP, adipocyte lipid-binding protein. aP2, as all FABPs, reversibly binds hydrophobic ligands, such as LCFAs, leukotrienes, and prostaglandins and plays an important role in their cellular uptake and targeting (Furuhashi and Hotamisligil, 2008).

To investigate the *in vivo* role of aP2, Hotamisligil and colleagues generated aP2-deficient mice (aP2 $^{-/-}$) by targeted disruption of the aP2 gene. These animals were phenotypically, developmentally and metabolically normal when maintained on low-fat (4% fat) diets (Hotamisligil et al, 1996). The effects on adipocyte development were minor considering that aP2 makes up 5% of all the soluble cytosolic proteins of an adipocyte (Makowski and Hotamisligil, 2004). This prompted the investigation of other FABPs expressed in adipocytes. Keratinocyte-FABP (K-FABP; FABP5; mal1) is normally expressed in AT at low, but detectable levels (approximately 100-fold lower than that of aP2) and has similar binding properties as aP2 as well as a 52% aa similarity. In adipocytes of aP2 $^{-/-}$ animals 20–40-fold up-regulation of mal1

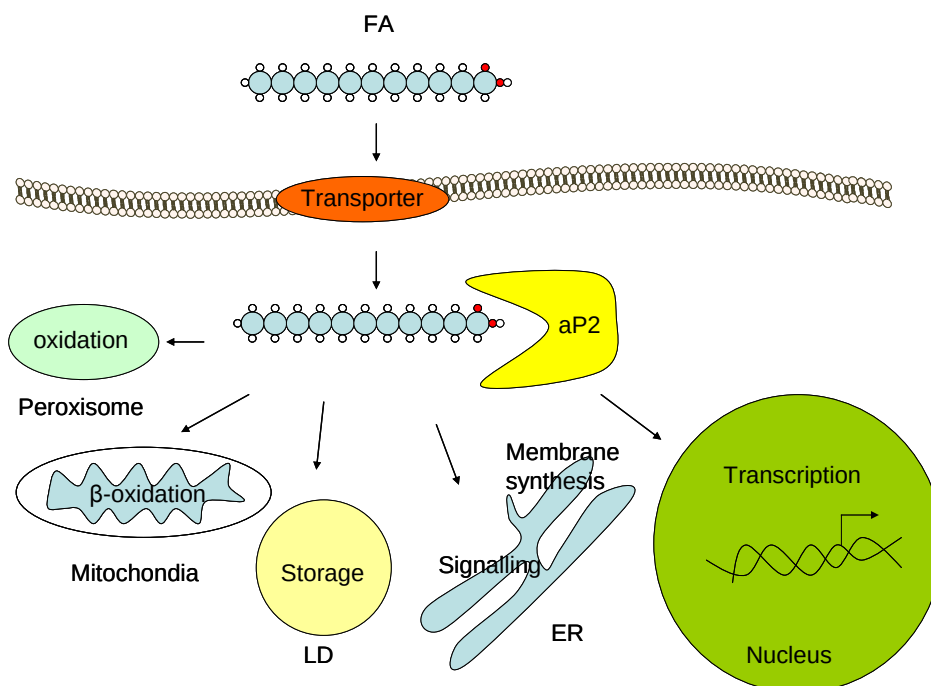


Figure 1.4: aP2 cellular functions. aP2 has been proposed to play a role in the transport of lipids to specific cell compartments: to LD for storage; to the ER for signalling, trafficking and membrane synthesis; to the mitochondria or peroxisomes for oxidation; to the nucleus for the control of lipid-mediated transcriptional programs via nuclear receptors or other transcription factors that respond to lipids. ER: endoplasmic reticulum, LD: lipid droplet (adapted from Furuhashi and Hotamisligil, 2008).

mRNA was detected, with the protein being increased 13-fold potentially compensating for the absence of aP2. However, mal1 was still expressed at a much lower level than that of aP2 in normal AT. Finally, mal1 was not up-regulated to the same extent in macrophages of aP2^{-/-} mice, where both aP2 and mal1 are also expressed (Hotamisligil et al, 1996). Furthermore, in mal1^{-/-} animals an equivalent up-regulation in aP2 expression was not observed in WAT or BAT. Mal1^{-/-} animals as aP2^{-/-} on a normal chow diet showed only modest differences when compared to their wild-type (wt) littermates (Maeda et al., 2003).

When aP2^{-/-} animals were challenged with a high-fat diet (HFD) (40% of calories from fat), they became slightly more obese than wild-type controls but did not develop insulin resistance or diabetes in contrast to their wt and

heterozygotic littermates (Hotamisligil et al, 1996). The same effects were observed when aP2 was disrupted in leptin-deficient (*ob/ob*) mice which are a model of severe genetic obesity and insulin resistance. *ob/ob*-aP2^{-/-} animals gained 15% more weight compared with their *ob/ob* controls, but they were more insulin sensitive, as demonstrated by significantly lower plasma glucose and insulin levels and also had lower plasma TAG and cholesterol levels. Thus, a single disruption in aP2 created a genetic model to uncouple obesity and insulin resistance (Uysal et al., 2000).

In contrast to aP2^{-/-} mice, *mal1*^{-/-} mice when fed HFD or placed on a severe genetic obesity and insulin resistant background (*ob/ob*) they were only moderately more insulin sensitive when compared with matched controls. On the other hand, *mal1* overexpression in WAT had the opposite effect i.e. *mal1*-transgenic mice showed a reduction in insulin sensitivity (Maeda et al., 2003). From these studies, we can conclude that the phenotype of the aP2^{-/-} mice is not accounted for by a compensatory up-regulation of *mal1*, because *mal1* deficiency also improved components of the metabolic syndrome.

Finally, in humans carrying a loss-of-function polymorphism in the *aP2* locus resulted in decreased AT aP2 expression due to alteration of the C/EBP binding and reduced transcriptional activity of the aP2 promoter. In a cohort of 7,899 participants, the individuals carrying this polymorphism had lower serum TAG levels and significantly reduced risk for coronary heart disease and type 2 diabetes compared with subjects homozygous for the wild type allele (Tuncman, 2006).

Studies in aP2^{-/-} primary adipocyte cultures revealed that aP2 disruption does not alter the rate of FA influx or esterification, but revealed a role for aP2 in lipolysis since basal and isoproterenol-stimulated lipolysis was 40% reduced in adipocytes from knockout animals (Coe et al., 1999). This is in agreement with the blunted glycerol blood levels after β -adrenergic stimulation in aP2 null animals (Scheja et al, 1999) and also with the finding that aP2 physically interacts with hormone sensitive lipase (HSL) (Shen et al., 1999). Therefore, aP2–HSL interaction could result in the facilitation of FA efflux from the cell

and in the absence of aP2 FAs are not efficiently released from the adipocyte and accumulate intracellularly (Coe et al., 1999; Shen et al., 1999).

As aP2 is also expressed in macrophages its role in atherosclerosis which is linked with lipid metabolism dysregulation and aberrant inflammatory responses was investigated. Makowski and colleagues addressed this question by crossing Apolipoprotein E deficient mice (ApoE^{-/-}) mice with aP2^{-/-} mice and also performed bone-marrow transplantation experiments in which aP2^{-/-}ApoE^{-/-} mice were donors to recipient aP2^{+/+}ApoE^{-/-}. ApoE-deficient mice exhibited severe hypercholesterolemia and spontaneous atherosclerotic lesions whereas either aP2 total deficiency (ApoE^{-/-}aP2^{-/-} mice) or macrophage specific deficiency (transplantation experiments) resulted in mice with significant less atherosclerosis in the absence of significant changes in serum lipid, glucose or insulin levels. Thus, aP2 seems to have an important role in the development of the metabolic syndrome both through its expression in adipocytes and macrophages regulating lipid homeostasis and inflammatory responses, respectively (Makowski et al, 2001).

Finally, double knockout mice for aP2 and mal1 showed a strong protection from diet-induced obesity, insulin resistance, type 2 diabetes and fatty liver disease, highlighting the central role of these FABPs in the development of key pathologies associated with metabolic syndrome (Maeda et al. 2005).

One common feature of all FABP knockout models, aP2^{-/-}, mal1^{-/-} and double aP2^{-/-}-mal1^{-/-} mice, is that they all show a minor but significant increase in serum FFA levels. Elevated FFAs are positively correlated with the development of obesity and insulin resistance, but adipocyte/macrophage FABP-deficient mouse models were insulin sensitive. This observation challenges the current dogma and indicates that individual FAs or metabolite action at the intracellular level rather than the absolute amounts may be more critical in pathological conditions. Thus, more detailed lipid profiling showed increased shorter-chain (C14) FAs and decreased longer-chain (C18 or C20) FAs in the muscle and adipose tissues of aP2^{-/-}-mal1^{-/-} mice. These changes favoured enhanced insulin receptor signalling, insulin-stimulated glucose

uptake, adenosine monophosphate-activated protein kinase (AMPK) activity, and FA oxidation (Maeda et al, 2005).

One very important question is what the true functional role of aP2 is since its expression promotes a dysfunction of metabolic and inflammatory nature when mice are challenged with stress stimuli (e.g. HFD). One explanation might lie in the role of aP2 on transcriptional regulation. It has been shown that aP2 is able to translocate to the nucleus in response to ligand binding (Tan et al., 2002) and to physically interact with PPAR γ , transferring its ligand to the receptor enhancing its transcriptional activity (Tan et al., 2002; Wolfrum, 2007). Thus, aP2 might be necessary to fine-tune the balance of the available of lipid resources. On the one hand, lipid-ligand activated PPAR γ induces the expression of aP2. On the other hand, aP2 can act as a ligand carrier for PPAR γ but it can also act with a negative mechanism to sequester lipids from PPAR γ . When aP2 is absent PPAR γ is always activated since the signal is not attenuated resulting in a hyperactivated PPAR γ . This model is further supported by the fact that aP2 knockout mice have a similar phenotype to mice or humans treated with TZDs, including improved glucose and lipid metabolism, and decreased insulin resistance, atherosclerosis, and inflammatory response (Makowski and Hotamisligil, 2004).

Finally, Chen et al. demonstrated that aP2 is the major determinant of the FA source for adipocytes. In the presence of aP2, the main source of FAs supplying adipocytes is the diet whereas its absence *de novo* lipogenesis is the major source. As a consequence, the adipocyte FA composition changes and palmitoleate (an unsaturated FA) becomes the major FA produced in adipocytes. In this study, it was clearly demonstrated that the FA composition in AT determines the activity in other metabolic tissues such as liver and muscle. Thus, in specific AT aP2 deficient mice fed a HFD, palmitoleate suppresses hepatosteatosis and stimulates glucose transport in skeletal muscle regardless of the increased total FAs (Chen et al 2008). These observations were confirmed in human studies where palmitoleate levels were strongly associated with insulin sensitivity (Stefan et al., 2010).

In conclusion, it is thought that aP2 is one of the genes involved in the “thrifty” phenotype since on a standard diet aP2^{-/-} mice do not show any significant phenotype whereas they are more insulin sensitive under HFD. Thus, aP2 is essential when there is a limited amount of resources to survive famine whereas, it seems to be responsible for the development of obesity, diabetes, dyslipidaemia and atherosclerosis under caloric excess, making aP2 an ideal candidate for pharmacological intervention (Furuhashi et al., 2011).

1.4 Fatty acid Metabolism

1.4.1 Fatty acids and fatty acid trafficking

Lipids are defined as biological substances that are generally hydrophobic in nature and soluble in organic solvents. Biological lipids usually refer to a broad group of naturally occurring molecules which includes FAs, waxes, eicosanoids, glycerolipids (mono-, di- and triglycerides), phospholipids, sphingolipids, sterols, terpenes, prenols, fat-soluble vitamins (such as vitamins A, D, E and K) and others (Fahy et al., 2005).

FAs represent the major lipid building block of complex lipids and they are one of the most fundamental categories of biological lipids. FAs are either saturated or unsaturated carboxylic acids with carbon chains varying between 2 and 36 carbon atoms (Table 1.1). In animal and plant tissues, the most abundant FAs are those with 16 and 18 carbon atoms, *i.e.* palmitic, stearic, oleic and linoleic. FAs in mammalian organisms reach a chain-length of 12-24 carbon atoms, with 0-6 double bonds whereas FAs with chain lengths shorter than 14 and longer than 22 carbon atoms are present only in minor concentrations (Tvrzicka et al., 2011).

FAs have many different functions in an organism. They are a principal source of energy and have a central role in energy storage. They contain twice as much of energy value compared to proteins and carbohydrates and their storage in AT requires less water as they can be stored anhydrously as TAGs. They are the most important element for the formation of complex lipids such as phospholipids which are structural and functional components of cell

membranes. Finally, they are the precursors for the formation of hormones and signalling molecules that can influence signal transduction pathways and gene transcription (Fahy et al., 2005; Kremmyda et al., 2011).

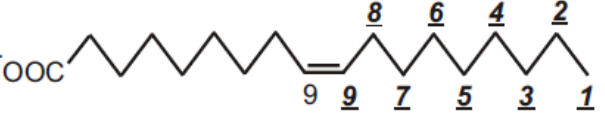
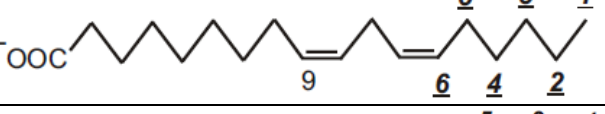
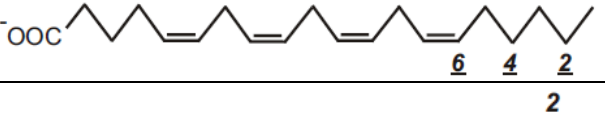
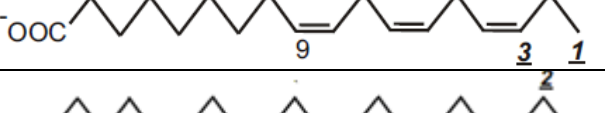
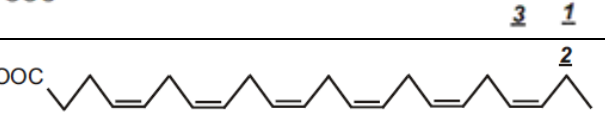
Fatty acids	Structure		Saturation
	Carboxyl end	Methyl end (ω)	
Stearic acid 18:0			Saturated
Oleic acid 18:1, ω -9			Monounsaturated
LA 18:2 ω -6			Polyunsaturated
AA 20:4 ω -6			Polyunsaturated
α -LA 18:3 ω -3			Polyunsaturated
EPA 20:5 ω -3			Polyunsaturated
DHA 22:6 ω -3			Polyunsaturated

Table 1.1: Nomenclature, structure and level of saturation of different unbranched FAs.

FAs are carbon chains with a carboxyl group at one end and a methyl group (designated omega, ω) at the other end. Saturated fatty acids have no double bonds whereas unsaturated have either one (monounsaturated) or more than one (polyunsaturated) double bonds. Unsaturated fatty acids are often expressed as a schematic formula CN:p n-x, where CN (carbon number) represents total number of carbon atoms, p – number of double bonds, x – position of the first double bond from the methyl terminus (ω). FAs: fatty acids, LA: linoleic acid, AA: arachidonic acid, α -LA: α -linolenic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid (adapted from Tvrzicka et al., 2011).

FAs can be obtained from the diet, but can also be released from adipocytes, or synthesized in the liver. The average western diet of a healthy adult person contains approximately 56-78g fat per day for a standard diet of 2000kcal/day. However, the fat intake can be as high as 40-45% of the total energy intake

depending on food resources and habitual consumption. Recommendations for the total fat intake have been set by various bodies worldwide and recently the European Food Safety Authority (EFSA) recommended that total fat should be between 20-35% of total energy intake by healthy adults (EFSA Panel on Dietetic Products, Nutrition and Allergens (NDA); 2010).

Exogenous FAs are supplied to the body mainly as dietary TAGs and are digested into FAs and monoacylglycerols in the small intestine by pancreatic lipases. Then the digestion products are transferred through the luminal membrane of the intestinal epithelial cells, and reesterified into TAGs that are packaged into large lipoproteins called chylomicrons. Chylomicrons are relatively short-lived and are nearly absent in the fasting state. Once formed, they are secreted via the thoracic lymph duct into the blood for delivery to the other tissues in need of FAs. This is called the exogenous lipid transport pathway since the lipids originate from outside the body (Montgomery, 1996).

Under resting conditions, the majority of chylomicrons go to WAT. FFAs are released by the enzymatic hydrolysis of TAGs contained in neutral lipid core of the chylomicrons by the lipoprotein lipase (LPL) which is a 448-amino acid glycoprotein synthesised by the adipocyte and transferred to the luminal pole of microvascular endothelial cells. After FAs enter the adipocyte, reesterification is necessary for lipid storage in TAGs until signals reach the adipocyte to hydrolyse part of their intracellular neutral lipids (Lafontan, 2008).

In the case of excess circulating FAs, they are taken up by the liver where they are esterified into TAGs and packaged in very low density lipoproteins (VLDLs) and released to the blood. VLDLs subsequently transfer the esterified FAs to WAT and muscle (endogenous lipid transport pathway) (Montgomery, 1996).

Energy requirements are constantly changing and cells must match substrate supply with demand, hence FAs are in constant flux and need to enter and leave cells rapidly. In blood, FAs are solubilised and transported in millimolar concentrations (0.1-1) by binding to plasma albumin. The unbound FA (free

FA in equilibrium with albumin), is the species important for cellular uptake. Its concentration is determined by the molar ratio of FA to albumin and is computed based on FA:albumin binding constants. Because albumin binds FA with high affinity, cells should possess a high affinity mechanism allowing recruitment of FA from albumin avoiding its efflux back into the albumin-containing medium (Abumrad et al., 1999; Thompson et al., 2010).

In general, the uptake of FAs from the circulation into cells includes the sequence of three steps. The first step is called adsorption and involves the binding of FAs to the outer surface of the plasma membrane. First, FFAs are generated locally through hydrolysis of TAG-rich lipoproteins by lipases inside the endothelial lumen followed by their binding to albumin. FAs dissociate from albumin followed by binding to plasma membrane proteins or integration into the lipid bilayer. The second step involves the FA transport across the plasma membranes. This step is the most controversial as whether the mechanism of FA translocation is diffusion-mediated or protein-mediated. FAs can traverse the phospholipid bilayer by a rapid passive transport across the lipid bilayer (flip-flop) followed by a slower dissociation of the lipid from the membrane into the intracellular aqueous phase. However, protein-mediated uptake of FAs is also involved especially in cells with high FA demand. Several FA transporters have been identified with potential role in FA uptake such as FA translocase (FAT/CD36), plasma membrane fatty acid binding protein (FABPpm), caveolin-1 and fatty acyl-CoA synthetases (FATP and ACSL). FA transport in adipocytes is enhanced due to the rapid rates of lipid metabolism and incorporation into the TAG droplet which may represent a balance between diffusion-mediated and protein-mediated processes. Finally, the third step is called desorption and involves FAs leaving the cytosolic leaflet of the plasma membrane and interacting with intracellular FABPs and acyl-CoA binding proteins (ACBPs) (Hammilton, 1998; Doege and Stahl, 2006; Thomson et al, 2010).

1.4.2 Triglycerol (TAG) synthesis

In adipocytes, FAs produced by *de novo* lipogenesis (synthesis of FAs from non-lipid substrates), along with those transported into the cell, are used mainly for the synthesis of TAG. The two central enzymes of *de novo* lipogenesis, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) use acetyl-CoA and malonyl-CoA to generate palmitate. Palmitate synthesised by *de novo* lipogenesis as well as dietary lipids, can be modified by endogenous elongase and desaturase enzymes to produce multiple lipid species (Sul and Wang, 1998).

The first commitment step of TAG synthesis is the esterification of fatty acyl-CoA with glycerol-3-phosphate (G3P) at the sn-1 position to form 1-acylglycerol-3-phosphate (lysophosphatidic acid), a reaction catalysed by glycerol-3-phosphate acyltransferase (GPAT) (Brasaemle and Wolins, 2012).

In adipocytes, G3P was classically thought to be generated by insulin dependent uptake of glucose, its conversion to dihydroxyacetone phosphate (DHAP) in the glycolytic pathway and finally generation of G3P by glycerophosphate dehydrogenase (Salway, 2004). However, it has been shown that after TZD treatment adipocytes express glycerol kinase (GyK) that phosphorylates directly glycerol to produce G3P (Guan et al, 2002). Finally, glyceroneogenesis is another pathway that also supplies G3P and involves the carboxylation of pyruvate to oxaloacetate, decarboxylation of oxaloacetate to phosphoenolpyruvate by cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C), and, subsequently, the production of G3P through a partial reversal of glycolysis (Chaves et al., 2006). PEPCK had also been shown (as GyK) to be strongly induced by TZDs revealing another mechanism of the anti-diabetic action of TZDs allowing the adipocyte to use the glycerol and FFA generated from lipolysis for re-esterification, lowering serum FFAs and eliminating their accumulation in ectopic tissues (Tordjman et al, 2003).

Lysophosphatidic acid is further esterified and converted into phosphatidic acid (PA) in the reaction catalysed by 1-acylglycerol-3-phosphate acyltransferase (AGPAT). PA is converted to the intermediate 1,2-diacylglycerol (1,2-DAG) by the phosphatidic acid phosphatase (PAP) reaction. Diacylglycerol acyltransferase (DGAT) catalyzes the acylation of 1,2-DAG to form TAG (Figure 1.5) (Sul and Wang, 1998).

All the enzymes involved in the TAG synthesis are located in the ER but the TAGs are eventually stored in LDs which are monophospholipid layered vesicles. The most widely accepted model of LD formation is that lipids accumulate between the cytosolic and the luminal leaflets of the phospholipid bilayer of the ER, followed by association of LD specific proteins and eventual separation of the growing LD from the ER (Hapala et al., 2011).

1.4.3 Lipolysis

During times of increased energetic demand, WAT has the ability to switch from being a FA storing tissue to a FA provider to the other organs. Lipolysis is the essential mechanism that releases FFAs and glycerol by the hydrolysis of TAGs stored in adipocytes (Duncan et al, 2007). Despite the essential role of FAs, a FA oversupply can cause different detrimental effects all described as lipotoxicity (Unger et al, 2010). Thus, lipolysis and lipogenesis are highly regulated processes releasing or re-esterify FA in a futile cycle in order to avoid non-physiological increases in FA concentrations (Duncan et al, 2007).

Upon β -adrenergic stimulation PKA phosphorylates a protein localised on LDs, perilipin-1, which in the non-stimulated state interacts with comparative gene identification-58 (CGI-58). CGI-58 then dissociates from perilipin-1 and interacts with adipose triglyceride lipase (ATGL) which is the main TAG hydrolase. CGI-58 acts as a coactivator for ATGL which gains full activity and cleaves the first esterbond in a TAG to yield a DAG and a non-esterified FA. HSL is also phosphorylated by PKA and interacts with perilipin-1 to gain access into the LD. Then, HSL hydrolyses DAGs into monacylglycerols (MGs). HSL-phosphorylation and translocation coupled with ATGL activation

by CGI-58 increases the TAG hydrolysis more than 100-fold. Finally, the last step of lipolysis is the hydrolysis of MGs by MG lipase (MGL) (Zechner et al, 2012). The FFAs liberated by lipolysis either exit the adipocyte to be oxidised in other tissues such as liver and muscle, or can be used for the synthesis of TAGs.

Recently macroautophagy of LDs (or macrolipophagy) has been described as an alternative way to produce FFAs apart from lipolysis in long fasting periods (Singh et al, 2009). Macroautophagy is a non-selective lysosomal pathway for the degradation of cytosolic organelles and proteins. These cytoplasmic components are trapped inside double-membrane vesicles termed autophagosomes and fuse with lysosomes where their contents are degraded (Singh et al., 2009). In the case of LDs their lipids are degraded by lysosomal lipases found in lysosomes.

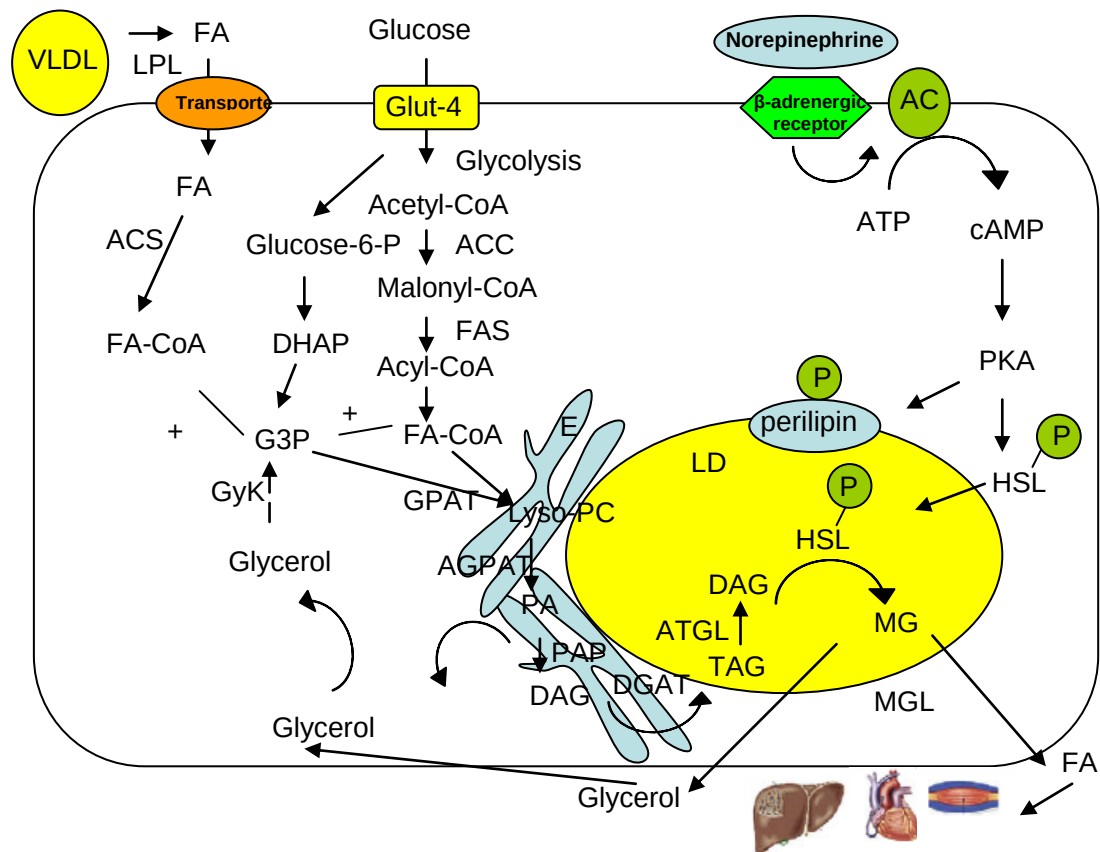


Figure 1.5: Adipocyte lipogenesis and lipolysis. Excess glucose is oxidized via glycolysis to acetyl-CoA in the adipocyte and then converted into acyl-CoA, which is esterified in the ER to TAGs. These are then translocated into the LD. FA obtained from lipoproteins are also esterified into TAG and stored. Under fasting conditions, lipolysis is activated by GPCRs resulting in an increase in cAMP and PKA activation that phosphorylates perilipin located in the membrane of the LD. PKA also phosphorylates HSL that translocates from the cytoplasm to the LD and induces the hydrolysis of DAGs produced by the ATGL to form MGs. MGs are then released to non-adipose tissues, mainly for energy purposes. Some glycolysis and glyceroneogenesis steps are also illustrated for the generation of G3P. ER: endoplasmic reticulum, TAG: triglyceride, LD: lipid droplet, GPCR: G-protein coupled receptor, PKA: protein kinase A, DAG: diacylglycerol, ATGL: adipose triglyceride lipase, MG: monocylglycerol, G3P: glycerol-3-phosphate (adapted from Vázquez-Vela et al., 2008)

1.5 Arachidonic acid metabolism

Polyunsaturated fatty acids (PUFAs) are long chain FA (C16 and above) containing two or more double bonds. PUFAs can be classified in two categories, omega-6 (ω -6 or n-6) and omega-3 (ω -3 or n-3) based on the location of the last double bond relative to the terminal methyl end of the molecule. The precursors of the two classes, linoleic acid (LA; 18:2n-6) and α -linolenic acid (ALA; 18:3n-3) cannot be synthesised endogenously. FAs can only be desaturated endogenously up to the Δ 9 position due to lack of certain enzymes in humans (Δ 12- and Δ 15-desaturases). For this reason LA and ALA must be provided from the diet and are called essential FAs. Further elongation and desaturation of these FAs to produce long-chain PUFA, including eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (AA; 20:4n-6), is possible (with the two FA families competing for the same enzymes) but not very efficient in humans. Thus, these FAs (EPA, DHA and AA) may be characterised as conditionally essential depending on essential FA availability (Schimitz et al., 2008; Tvrzicka et al., 2011). Deficiency of PUFAs leads to different number of disorders such as growth retardation, infertility, skin lesions and dysopsia (Kremmyda et al, 2011).

AA (5,8,11,14-eicosatetraenoic acid), can be synthesised from dietary LA or can be obtained directly from the diet as poultry (eggs and meat) and fish products are high in quantities of preformed AA. In westerns diets, it is calculated that the daily intake of AA is 0.2–0.3g whereas the intake of LA is 10–20g per day, indicating that the formation of AA from LA exceeds the dietary supply of AA (Zhou and Nilsson, 2001). The recommended LA intake by EFSA is 4% of the total energy intake whereas there is not a set recommended dietary reference value for AA (ESFA, 2010).

After consuming n-6 PUFAS, intestinal phospholipids containing AA and LA are taken up by the liver in the form of chylomicron remnants. In the liver, LA is converted to AA by successive actions of Δ 6 desaturase, elongase, and Δ 5 desaturase. Once AA is obtained (from diet) or formed (in liver), it is

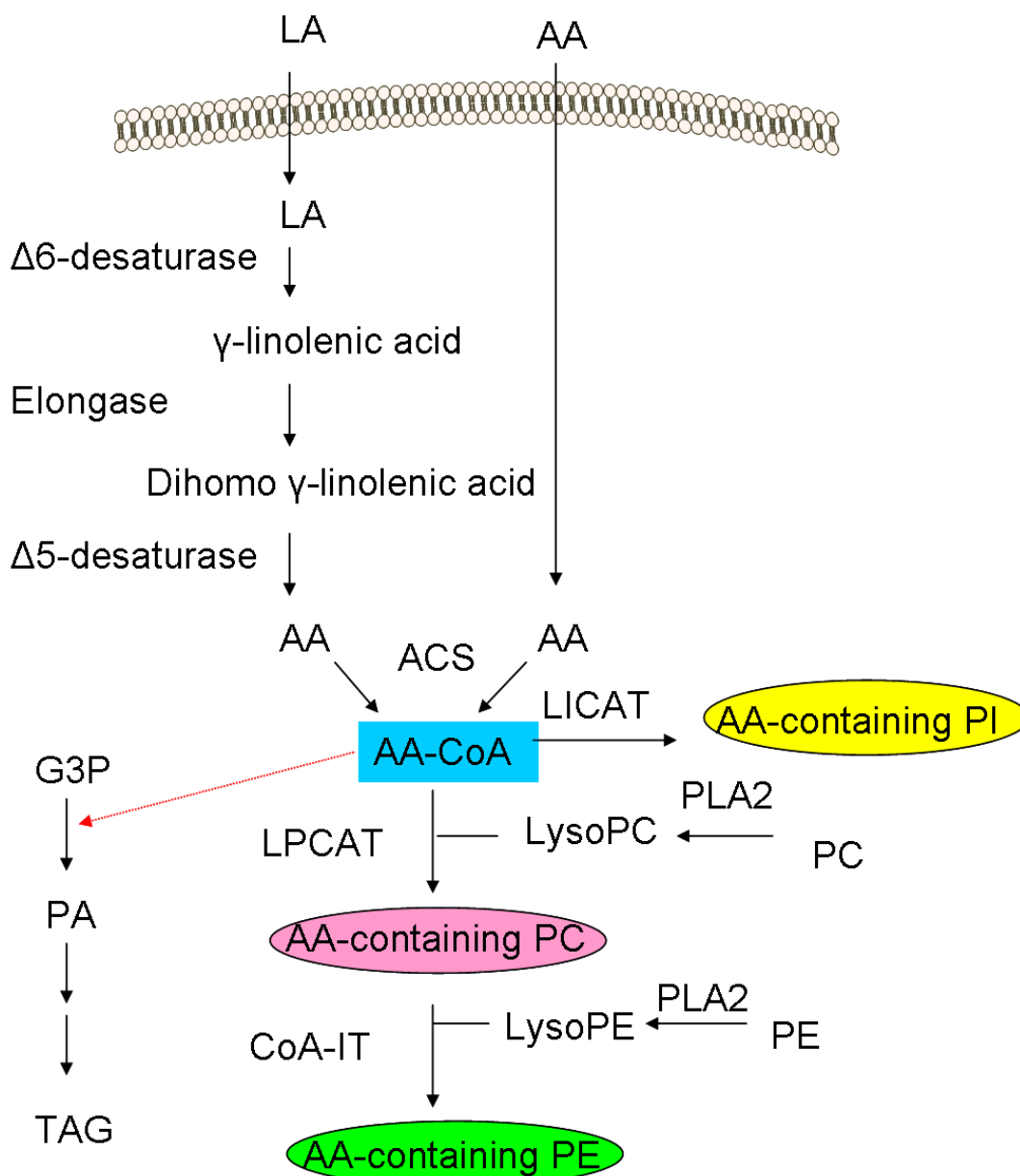
distributed in serum as FFA (complexed to albumin) or as part of complex lipids (phospholipid, TAGs and cholesterol esters) in lipoprotein vesicles. AA is then taken up by cells by a mechanism that is not entirely clear and may involve passive diffusion or facilitated protein-mediated transport (Chilton et al., 1996; Leslie 2004).

There are high levels (500 nmol/ml) of AA (either FFA or complex lipid) in normal human serum (Chilton et al, 1996). Additionally, AA is also found in abundance incorporated into the cell membrane phospholipids particularly choline phospholipids (PCs) ethanolamine phospholipids (PEs), and phosphatidylinositol (PI) which accounts for as much as 25% of all phospholipid FAs. Phospholipids contain FAs esterified to the glycerol backbone at the sn-1 and sn-2 positions. In general, the sn-1 fatty acyl group is saturated whereas the sn-2 fatty acyl group is monounsaturated or polyunsaturated such as AA. The double bond geometries of AA are *cis*, which introduces significant distortion into the membrane bilayer and contributes to its fluidity (Brown and Marnett, 2011).

There are two pathways regulating the incorporation of AA into phospholipids. The first one is a high-affinity pathway that occurs in the presence of low AA concentrations. The first enzymatic step for the incorporation of AA into phospholipids is catalyzed by the enzyme acyl-CoA synthetase (ACS), which activates the carboxyl group of AA by thioesterification with CoA moiety, as a result AA to be converted to arachidonoyl-CoA. The long-chain acyl-CoA synthetases ACSL3, 4 and 6 show marked preference to AA. Arachidonoyl-CoA then is incorporated primarily into choline lysophospholipids (lysoPC) via a reaction catalysed by lysophospholipid CoA dependent acyltransferases resulting in the formation of PCs (Lands pathway). Once AA has been incorporated into phospholipids, a reaction carried out by CoA-independent transacylase (CoA-IT) transfers AA from PCs to PEs. Thus, despite PC being the preferred acceptor for exogenous AA, under equilibrium conditions, AA is more abundant in PE than in PC. This transacylation reaction seems to be important for membrane homeostasis but also for the appropriate cell response during physiological and pathophysiological activation (Astudillo et

al, 2012). The second pathway is a “high-capacity, low affinity” pathway that operates after the high affinity deacylation/reacylation pathway has been saturated due to the high AA concentration and leads via the *de novo* route for phospholipid biosynthesis to accumulation of AA into TAGs (Kennedy pathway) (Figure 1.6) (Chilton et al., 1996; Pérez-Chacón et al., 2009). The incorporation of AA into the TAG pool is postulated to have two roles, one is the utilisation of AA for the production of eicosanoids since prostaglandin synthetases have been found at LDs and the second is a way to store excess AA in order to protect the cells from the toxic effects of high AA concentrations (Chilton et al., 1996).

The production of free AA is a highly regulated process that represents a balance between two competing reactions. On the one hand, phospholipases deacylate phospholipids yielding free AA and on the other hand CoA-dependent acyltransferase re-acylates and re-incorporates AA into phospholipids. Depending on the state of the cell one kind of reaction will dominate over the other. In resting cells, reacylation dominates and the majority of cellular AA is found in the esterified form, keeping free AA at low levels. However, under stimulated conditions (e.g. cytokines, growth factors, mechanical trauma etc) phospholipases are activated and catalyse the hydrolysis of phospholipids generating free AA. Three enzymes can catalyse the deacylation reaction: phospholipase A₂ (PLA₂), phospholipase C (PLC), and phospholipase D (PLD) which have different sites of action on the phospholipid backbone. PLA₂ catalyses the hydrolysis of phospholipids at the sn-2 position releasing free AA in a single-step reaction. By contrast, PLC and PLD generate lipid products containing AA (DAG and PA, respectively), which are released subsequently by DAG- and MG-lipases. Thus, in stimulated cells, the dominant reaction is the phospholipase-mediated deacylation, which results in the release of free AA. However, under activation conditions AA reacylation is still very significant, as only a minor fraction of the AA is released by phospholipases and the remainder is incorporated back into phospholipids (Pérez-Chacón et al., 2009).



Kennedy Pathway Lands Pathway

Figure 1.6: AA incorporation in phospholipids. AA is obtained directly from diet or synthesized from LA. AA is incorporated into the sn-2 position of phospholipids PCs and PIs and later subjected to remodeling processes via CoA-IT reactions transferring AA from PCs to PEs. This pathway is a high affinity pathway called Lands pathway whereas the Kennedy pathway is a low affinity pathway leading to the incorporation of AA to TAGs. AA: arachidonic acid, LA: linoleic acid, PI: phosphatidylinositol, CoA-IT: CoA-independent transacylase, PC: choline glycerophospholipid, PE: ethanolamine glycerophospholipid (adapted from Pérez-Chacón et al., 2009; Astudillo et al, 2012).

The release of AA from phospholipids is the first step and the overall rate-limiting step, for the biosynthesis of AA metabolites or eicosanoids (from the Greek eicosa = twenty; for twenty carbon fatty acid derivatives). Despite the fact that other phospholipases can catalyse the reaction for the release of AA from phospholipids, type IV cytosolic PLA2 α (cPLA2 α) phospholipase shows specificity for phospholipid substrates containing AA. Thus, cPLA2 α is crucial for eicosanoid production and cells lacking cPLA2 α are generally devoid of eicosanoid synthesis (Funk, 2001). Moreover, mice lacking cPLA2 α exhibit a number of striking phenotypes that can be explained by defects in eicosanoid mediated pathways (e.g. reduced airway inflammation (asthma, ARDS, and fibrosis), impaired implantation and parturition in female reproduction, increased intestinal ulcer, delayed blood coagulation, renal dysfunction after aging, protection from Alzheimer's disease, protection from metabolic syndrome (atherosclerosis, obesity and hepatic steatosis) (Murakami et al., 2011).

After its release from phospholipids, the non-esterified AA is rapidly oxygenated in a cell-specific manner by cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450 monooxygenases (MOXs) and give rise to different eicosanoids which have a short half-life (ranging from seconds to minutes) (Figure 1.7) (Funk, 2001).

There are two COX isoforms, COX-1 and -2, which are also known as prostaglandin endoperoxide synthases -1 and -2 (PGHS-1 and -2). COX-1 is constitutively expressed in most tissues and it is responsible for the basal activity, whereas COX-2 is the inducible isoform. COXs catalyse the rate-limiting step in the biosynthesis of prostanoids *i.e.* prostaglandins (PGs) and thromboxane A₂ which is the incorporation of molecular oxygen to AA forming prostaglandin G₂ (PGG₂). PGG₂ is subsequently reduced to PGH₂ by two electrons by the action of the peroxidase active site. PGH₂ is the common precursor of all prostanoids and does not accumulate in cells but rather is converted rapidly to downstream products by the action of specific PGs and thromboxane synthases. These synthases give rise to different 2-series PGs such as PGE₂, PGD₂, prostacyclin (PGI₂), PGF₂ α ("2" stands for the number

of carbon-carbon double bonds in the product) and thromboxane A₂ (TXA₂). PGs are not stored intracellularly but are synthesized *de novo* from phospholipid-released AA every time cells are activated by various stimuli. PGs, once formed, act either by an autocrine and/or paracrine manner by binding to specific surface GPCRs or by directly binding to nuclear receptors (Smith et al., 2000; Smith et al, 2011).

5, 12 and 15 LOX enzymes catalyze the oxygenation of AA (the numbers correspond to the carbon position of AA oxygenation) each forming a distinct hydroperoxy-eicosatetraenoic acid (HPETE). HPETEs are unstable and can be reduced by peroxidases to the corresponding hydroxyeicosatetraenoic acids (HETEs). 5-LOX is the initial enzyme in the synthesis of leukotrienes and catalyses two enzymatic reactions. The first one involves the addition of molecular oxygen to carbon-5 of AA to form 5-HPETE. The second reaction involves the conversion of 5-HPETE into leukotriene A₄ (LTA₄). LTA₄ is quite unstable and becomes a substrate for two discrete enzymes, LTA₄ hydrolase which converts LTA₄ into leukotriene B₄ (LTB₄), and leukotriene C₄ synthase which converts LTA₄ into leukotrienes C₄, D₄, and E₄ (Zimmer et al., 2004).

Finally, MOXs oxygenate AA to form four epoxyeicosatrienoic acids (EETs) which are converted to dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolases. EETs have been associated with anti-inflammatory properties in cardiovascular diseases which function primarily as autocrine and paracrine effectors in the cardiovascular system and kidney (Astudillo et al., 2012).

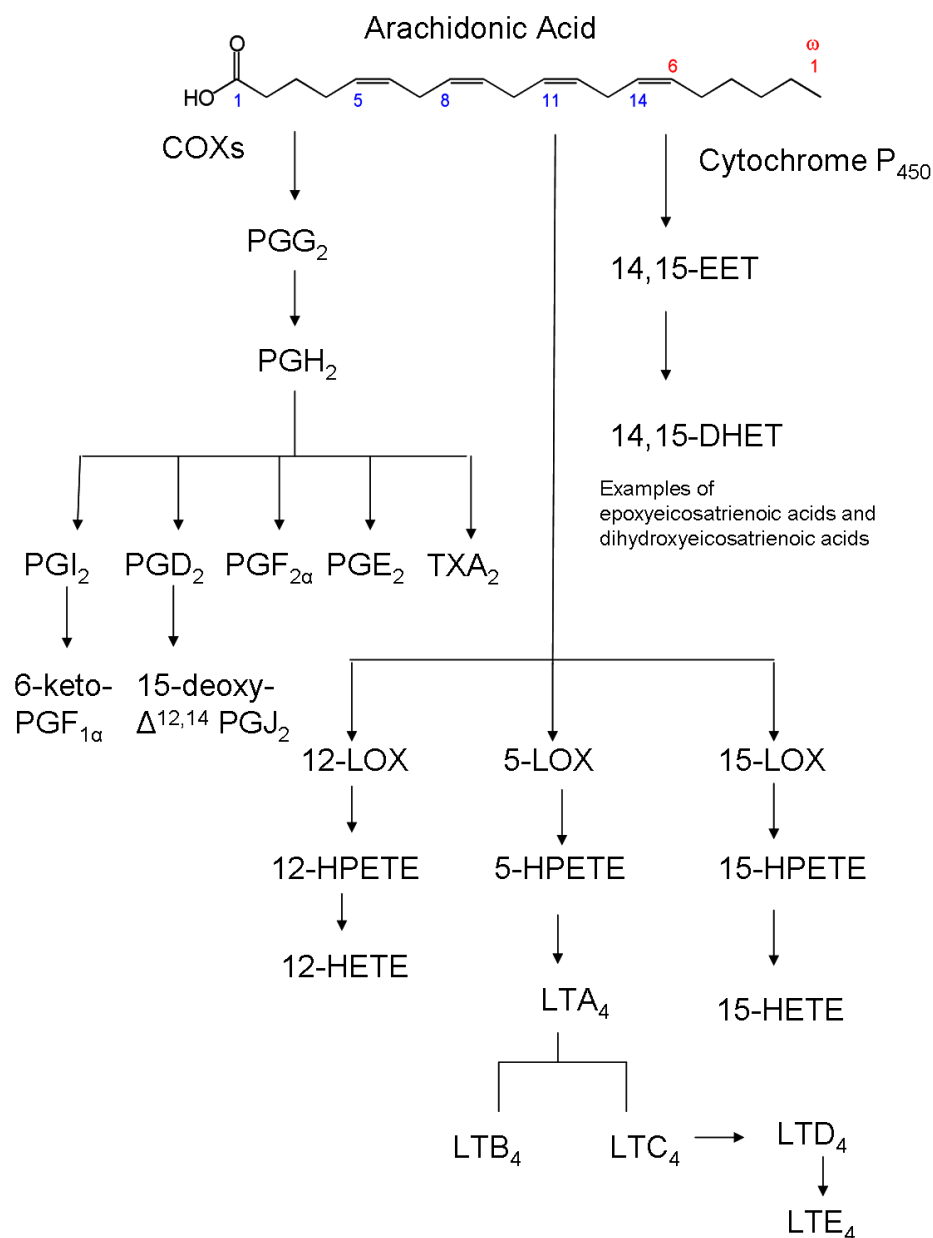


Figure 1.7: The three pathways for AA metabolism into eicosanoids. AA serves as a substrate for cyclooxygenases (COX-1 and COX-2), lipoxygenases (LOX-5, LOX-12 and LOX-15), or P450 monooxygenases (adapted from Astudillo et al., 2012).

1.6 Arachidonic acid and adipogenesis

Dietary fat has always been associated with obesity since it has been shown that HFDs can increase both adipocyte number (hyperplasia) and adipocyte size (hyperotrophy) (Faust et al., 1978). Adipocyte size can increase a lot in response to HFD but has a defined limit of expansion for any given individual. According to the AT expandability hypothesis, when an individual gains weight there is a point that adipocytes cannot expand to store more fat and thus AT can no longer act as a lipid buffering tissue. Once AT storage capacity is exceeded then the levels of circulating NEFAs increase and are deposited ectopically in myocytes, hepatocytes and β -cells where they have lipotoxic effects such as insulin resistance and apoptosis (Virtue and Vidal-Puig, 2010).

However, it is now clear that the effect of dietary fat depends on the nature of FAs *i.e.* chain length, position and number of the double bonds. In general, it seems that saturated FAs (SFAs) are more cytotoxic in comparison to unsaturated FAs (UFAs) and EFSA recommendation for SFAs is that the consumption should be as low as possible (EFSA, 2010). Evidence coming from β -cells revealed that treatment of these cells with SFAs is more lipotoxic than when treated with unsaturated FAs and also unsaturated FAs could counteract SFA toxicity *in vitro* (Diakogiannaki, et al, 2007). Dyslipidaemia, inflammation, oxidative stress and other metabolic abnormalities such as metabolic syndrome and type 2 diabetes, are often accompanied with changes in FA composition in plasma and particularly decreased content of PUFAs and increased content of SFAs (Kremmyda et al., 2011).

PUFAs seem to have different effects on obesity. *In vivo* data demonstrate that dietary intake of n-3 PUFA may reduce body fat accumulation, when exposed to a high fat diet (Hainault et al, 1993), and reduce body weight in adult animals that are already obese (Perez–Matute et al., 2007). On the other hand, animal studies using mice fed diets rich in n-6 PUFA, yielded conflicting results with some studies claiming that n-6 PUFA decrease adipose mass (Matsuo et al., 2002), whereas others associate n-6 PUFA with an increased propensity for obesity (Massiera et al, 2003). Finally, it seems

that the ratio between n-3 and n-6 PUFAs is crucial for the effect of total fat on obesity. Over the last two decades the total amount of ingested fat consumption has not changed but the consistency of the fat has changed dramatically. For example, in France, from 1960 to 2000, an increase in the consumption of LA (2.5-fold) and AA (2.3-fold) has been estimated, whereas the consumption of ALA has decreased by 40%, leading to a 2.9-fold and a 4.2-fold increase in the ratios of n-6/ n-3 PUFAs and LA/LNA, respectively (Ailhaud et al., 2006a). This was also associated with the change in the PUFAs ratio in breast milk which was correlated with increased childhood obesity (Ailhaud et al., 2006b). This notion was confirmed in animal studies where mothers fed either LA or LA and ALA showed that the pups coming from mothers fed LA were more obese in adult life in comparison with the LA/ALA counterparts (Massiera et al., 2003).

Apart from hypertrophy, HFD also increases the adipocyte number (hyperplasia). Adipocyte number is controlled by the rate of adipogenesis. Adipogenesis as already described is a complex process which includes changes in gene expression in response to different stimuli including lipid mediators. Feeding mice and rats with a HFD for 52 weeks increased the proliferation of pre-adipocytes, thereby increasing adipocyte number. The same results were confirmed in Pima Indians fed for 2 weeks with HFD which doubled the population of the small, adipocytes that appear to be recruited from a pool of pre-adipocytes. These observations suggest that hypertrophy and hyperplasia, take place in response to a HFD both in adult rodents and humans (Ailhaud et al., 2008).

Pre-adipocytes and adipocytes have all the machinery for metabolising AA. Several classes of prostanoids are synthesised in pre-adipocytes acting as local hormones at different stages of adipogenesis in response to external signalling molecules or nutritional status, which are involved in regulating differentiation. Pre-adipocytes express COXs which catalyse the first rate-limiting step for AA metabolism. COX-1 is constitutively expressed in pre-adipocytes, as in most cells but COX-2 which is the inducible form is transiently activated in the early stages of adipogenesis (Fujimori et al, 2010).

In vitro studies of the effect of COX-2 on adipogenesis gave contradictory results. Chu et al., showed that expressing COX-2 antisense mRNA resulted in enhanced adipogenesis whereas Fajas et al, showed that COX-2 inhibitors impaired adipogenesis through an inhibition of the clonal expansion step (Fajas et al., 2003; Chu et al., 2010). Finally, COX-2 knockout mice show attenuated adipocyte differentiation (Ghoshal et al, 2011).

AA itself has been described to be a ligand for PPAR γ (Kliewer et al., 1997) making it a good candidate regulating adipogenesis. However, different *in vitro* models gave different results e.g. Gaillard et al. using Ob1771 cells have shown that AA is able to promote their differentiation (Gaillard et al., 1989) whereas Madsen et al. using 3T3-L1 cells showed that AA prevents their differentiation (Madsen et al., 2005). This diversity observed in studies for COX-2 and AA indicate that AA metabolism during adipogenesis is a process governed at multiple levels suggesting a complex role for PGs during fat cell development.

Indeed, it has been shown that PGs have opposing effects on adipogenesis some of them promoting and some of them inhibiting adipogenesis (Figure 1.8). PGI $_2$ stimulates terminal differentiation of Ob1771 adipocytes using the stable analogue of PGI $_2$, carbaprostacyclin (Vassaux et al, 1992). After secretion from pre-adipocytes, prostacyclin acts externally via the prostacyclin receptor (IP-R) on the cell surface. IP-R seems to be coupled to both Gs and Gq resulting in increased cAMP formation and calcium signalling, respectively (Vassaux et al., 1992). IP-R is transiently expressed at the pre-adipocyte stage and prostacyclin synthesis ceases in mature adipocytes (Ailhaud et al., 2008). In order to address the effect of PGI $_2$ in adipose tissue development, IP-R $^{-/-}$ mice were fed with HFD rich in LA during the gestation and suckling period. The pups from IP-R $^{-/-}$ mice did not gain any body weight regardless of the diet indicating that PGI $_2$ is responsible for the enhanced fat mass development of the wild type pups (Massiera et al., 2003). PGI $_2$ has been shown to be pro-adipogenic through two different pathways. PGI $_2$ can up-regulate the expression C/EBP β and C/EBP δ via the PKA pathway (Belmonte

et al., 2001) or can act as a ligand for PPAR β/δ which, in turn, upregulates the expression of PPAR γ (Figure 1.8) (Matsusue et al, 2004).

PGJ₂ derivatives are the products of the non-enzymatic dehydration of PGD₂ to generate 15- deoxy- $\Delta^{12,14}$ -PGJ₂ (15d-PGJ₂) and Δ^{12} -PGJ₂ through PGJ₂ as an intermediate (Kliwer, et a., 1995). 15-deoxy- $\Delta^{12,14}$ -PGJ₂ has been shown to act as a direct ligand for PPAR γ and as expected it is a very potent inducer of adipocyte differentiation *in vitro*, since exogenous addition to the culture converts fibroblasts into adipocytes (Forman et al., 1995; Kliwer, et al., 1995). There have been conflicting reports as to the significance of endogenous production of 15d-PGJ₂ during adipocyte differentiation *in vitro*. Bell-Parikh et al. and Xie et al. show that the levels of 15d-PGJ₂ detected in the medium of 3T3-L1 cultures do not change during the differentiation and that the absolute concentrations are not sufficient to activate PPAR γ making it less possible to act as a natural ligand (Bell-Parikh et al., 2003; Xie et al., 2006). In contrast, Mazid et al. examining the same cell type show an increase in 15d-PGJ₂ production during differentiation which contributes to the stimulation of adipogenesis during the maturation phase of 3T3-L1 cells (Mazid et al., 2006).

PGE₂ acts by binding to four different GPCRs designated subtypes EP₁, EP₂, EP₃, and EP₄. There are four human EP₃ splice variants (EP_{3A}, EP_{3B}, EP_{3C} and EP_{3D}) generated by alternative splicing of the C-terminal tail. The mouse equivalents are designated as EP3 α , EP3 β and EP3 γ (Sugimoto and Narumiya, 2007). EPs show poor sequence homology and trigger different signalling pathways. EP₁ mediates PGE₂ action triggering an increase in intracellular calcium levels through a yet unidentified protein. EP₂ and EP₄ receptors are coupled to Gs and mediate increases in cAMP concentration whereas the major signalling pathway of the EP₃ receptor is inhibition of adenylate cyclase (AC) via Gi (Sugimoto and Narumiya, 2007). However, the splice variants of the EP3 receptor are coupled to different signalling pathways for example, EP_{3A} receptor is coupled to Gi and induces the inhibition of AC, whereas the EP_{3B} and EP_{3C} receptors are coupled to Gs and act to increase levels of cAMP. The EP_{3D} receptor is coupled to Gq, in

addition to Gi and Gs (Sugimoto and Narumiya, 2007). PGE₂ has been shown to inhibit adipogenesis via EP4 receptor which is the only EP receptor isoform to be expressed in pre-adipocytes in a cAMP-dependent pathway (Sugimoto et al., 2004; Tsuboi et al., 2004). However, *in vivo* data from EP4^{-/-} mice are lacking as these mice die shortly after birth and the few that survive do not show any adipose tissue phenotype (Sakuma et al., 2000).

PGF_{2α} is shown to inhibit adipogenesis of primary adipocyte precursor cultures (Serrero et al, 1992a), 1246 cells (Serrero et al, 1992b) and 3T3-L1 cells (Liu and Clipstone, 2007). This result was replicated by using PGF_{2α} analogs, fluprostenol and cloprostenol (Serrero and Lepak, 1997) which act as potent agonists for PGF_{2α} GPCR receptor called FP receptor. PGF_{2α} binds to its membrane FP receptor which is coupled to Gq leading to elevated intracellular calcium levels. The best characterised downstream effector of Gq is PLCβ, which catalyses the hydrolysis of PI 4,5-bisphosphate (PIP2) releasing inositol 1,4,5 triphosphate (IP3) and DAG. In consequence, IP3 causes an increase of intracellular calcium concentration from the ER. The combination of DAG and increased levels of calcium lead to the activation of protein kinase C (PKC). PKC activates the Raf/MEK/MAP kinase signalling pathway affecting various cellular responses (Rhee, 2001). In the context of adipogenesis this activation of MAPK has been shown to phosphorylate PPARγ leading to the inhibition of its activity and as a result the inhibition of the terminal differentiation (Reginato et al., 1998).

Finally, AA eicosanoids derived from the LOX pathway have been shown to affect adipogenesis as inhibition of the LOX pathway, prevents adipocyte differentiation of 3T3-L1 cells (Madsen et al., 2003). Leukotriene B4 (LTB4), 8(S) hydroxyeicosatetraenoic acid [8(S)- HETE] have been shown to bind PPARα, indicating a possible role in FA metabolism (Kliwer et al., 1997; Krey et al., 1997),

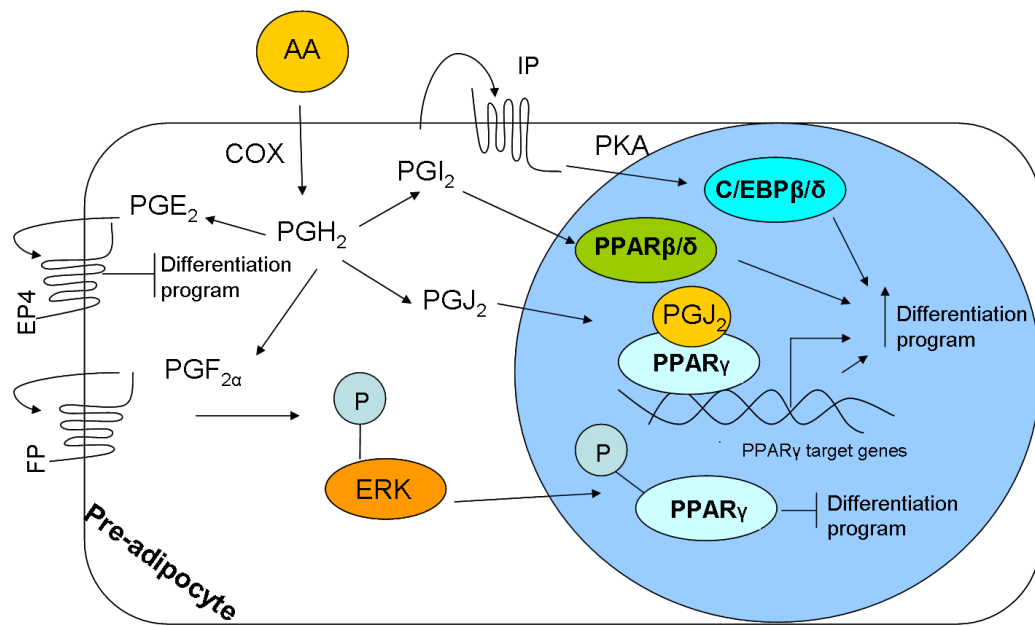


Figure 1.8: Opposing actions of AA metabolites on adipocyte differentiation.

PGI₂ and PGJ₂ activate PPARγ and promote adipocyte differentiation. PGF_{2α} and PGE₂ inhibit adipocyte differentiation with the former by inhibiting PPARγ action and the latter by a yet unknown mechanism. PGI₂: Prostacyclin, PGJ₂: prostaglandin J₂, PGF_{2α}: prostaglandin F_{2α}, PGE₂: prostaglandin E₂, PPARγ: peroxisome proliferator-activated receptor γ.

1.7 Hypothesis and Aims

AT has been recognised to play a central role in energy balance regulation. Besides the genotype, environmental factors such as the diet have been implicated in the development of obesity which is considered to be the modern global epidemic. Obesity is a major risk factor for diet-related diseases, and its health consequences range from increased risk of premature death to chronic conditions such as type 2 diabetes and cardiovascular diseases.

AA is a major n-6 PUFA which is abundant in the western diet. AA itself has been found to be a direct ligand of nuclear receptors but also its metabolites are significant signalling molecules regulating gene expression. However, the role of AA in adiposity and consequently adipocyte differentiation has been ambiguous. Therefore, it was hypothesised that AA can regulate the differentiation potential of pre-adipocytes.

To address this hypothesis the specific aims of this Thesis are:

- To identify the signalling pathways and molecular mechanisms leading to the regulation of AA responsive genes at the early stages of adipocyte differentiation
- To investigate whether the changes triggered by AA at the early stages of differentiation can affect the differentiation potential of pre-adipocytes

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Antibodies

2.1.1.1 *Primary antibodies*

α -tubulin (Sigma T5168)
aP2 (Cell Signalling 3544)
ATG7 (Cell Signalling 2631)
BiP (Cell Signalling C50B12)
C/EBP β (Santa Cruz sc-150)
C/EBP δ (Santa Cruz sc-636)
C-Jun (Cell Signalling 9165)
COX-2 (Santa Cruz sc-1745)
ERK (Cell signalling 9102)
Fra-1 (Santa Cruz sc-605)
GAPDH (Millipore MAB374)
IgG (Santa Cruz sc-2027)
IRE1 α (Cell Signalling 14C10)
JNK (Cell Signalling 9252)
JunB (Santa Cruz sc-46)
JunD (Santa Cruz sc-271938)
LC3 (Novus Biologicals NB100-2220)
mTOR (Cell Signalling 7C10)
PERK (Cell Signalling D11A8)
phospho-c-Jun (Cell Signalling 9164)
phospho-ERK (Cell Signalling 9101)
phospho-JNK (Cell signalling 4671)
phospho-mTOR Ser2448 (Cell signalling D9C2)
phospho-p38 (Cell Signalling 9215)
phospho- PPAR γ S112 (Millipore MAB3632)
PPAR γ (Santa Cruz sc-7196)
p38 (Cell Signalling 9212)

SREBP1 (Novus Biologicals NB100-2215)

2.1.1.2 Secondary antibodies

HRP conjugated Goat Anti-rabbit IgG (Invitrogen 656120)

HRP conjugated Rabbit Anti-goat IgG (Dako P0449)

HRP conjugated Rabbit Anti-mouse IgG (Invitrogen 626520)

2.1.2 Plasmids

pcDNA3.1 (+) (Invitrogen)

pGL3 basic (Promega)

pGL3 basic- ap2 promoter and enhancer (generated during PhD)

pGL3 basic-fra-1-peak2 (generated during PhD)

pGL3-tk-fra-1-peak1 (generated during PhD)

pcDNA3.1 (+) c-jun (generated during PhD)

pcDNA3.1 (+) Fra-1 (generated during PhD)

pcDNA3.1 (+) JunB (generated during PhD)

pcDNA3.1 (+) JunD (a kind gift from Agarwal SK)

pcDNA-GAL4DBD- PPAR γ LBD

pcDNA-PPAR γ (a kind gift from Spiegelman B.)

pcDNA- PPAR γ -S112A (a kind gift from Spiegelman B.)

pCMV-D8.91 (a kind gift from Chandrashekr A.)

PGK (pCCL.sin.PPT.hPGK.GFPWpre) (a kind gift from Cuervo A.)

pMDG2-vsvg (a kind gift from Chandrashekr A.)

pMSCV - FLAG-HA- PPAR γ (a kind gift from Spiegelman B.)

pMSCV - FLAG-HA- PPAR γ Q286P (a kind gift from Spiegelman B.)

pMSCV - FLAG-HA- PPAR γ E499Q (a kind gift from Spiegelman B.)

pCMV- Renilla (generated in the lab)

PSG5- PPAR γ (generated in the lab)

siATG7 (shATG7- pCCL.sin.PPT.hPGK.GFPWpre) (a kind gift from Cuervo A.)

UASx4-TK-luc reporter (generated in the lab)

3xPPRE-tk-Luc reporter (generated in the lab)

2.1.3 siRNA

ON-TARGETplus SMARTpool, Mouse FOSL1 (Dharmacon L-040704-00-0005)

ON-TARGETplus SMARTpool, Mouse PTGFR (Dharmacon L-060399-00-0005)

ON-TARGETplus SMARTpool, Mouse PTGER4 (Dharmacon L-048700-00-0005)

Negative control siRNA (Qiagen)

2.1.4 Chemicals and Reagents

15-deoxy- $\Delta^{12,14}$ PGJ₂ (Sigma)

10mM dNTPs (Invitrogen)

3-Methyadenine (3-MA) (Sigma)

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma)

10x MMLV buffer (Sigma)

17-ODYA (Cayman Chemical)

100x ROX reference dye (Sigma)

5X Reporter lysis buffer (Promega)

AA (Cayman Chemical)

AdipoRed (Cambrex)

Agarose, molecular biology grade (Sigma)

AL 8810 (Cayman Chemical)

Ammonium persulphate (APS) (Sigma)

Ampicillin (Sigma)

Baicalein (Cayman Chemical)

BAPTA-AM (Tocris Bioscience)

Beta-mercaptoethanol (Sigma)

Bovine serum albumin (BSA) and BSA free-fatty acid (Sigma)

Bromophenol blue (Sigma)

Carbaprostacyclin (Cayman Chemicals)

CAY-10441 (Cayman Chemical)

Chloroform (Sigma)

Coelenterazine (LUX)
Complete EDTA-free protease inhibitors tablets (Roche)
COX-1 inhibitor (SC-560) (Sigma)
COX-2 inhibitor (SC-236)(Sigma)
Dexamethasone (Sigma)
DHA (Sigma)
Dharmafect 3 (Dharmacon)
Diethylpyrocarbonate (DEPC) - treated water (Sigma)
Dimethyl sulfoxide (DMSO) (Sigma)
Dithiothreitol (DTT) (Sigma)
DNase I, amplification grade (Invitrogen)
Dynabeads Protein G for Immunoprecipitation (Invitrogen)
EPA (Sigma)
Ethanol (BDH)
Ethylenediaminetetraacetic acid (EDTA)
Formaldehyde 16% (w/v) (Thermo Scientific)
Forskolin (Enzo Life Sciences)
Glycine (Sigma)
H89 (Tocris Bioscience)
IBMX (Sigma)
Imidazole (Sigma)
Indomethacin (Sigma)
Insulin (Sigma)
Jumpstart SYBR Green I Mix (Sigma)
L-161,982 (Cayman Chemical)
LA (Sigma)
Lipofectamine 2000 (Invitrogen)
Luciferase Assay Reagent (Promega)
Methanol (Sigma)
MLLV Reverse Transcriptase (Sigma)
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
(Invitrogen)
Non-fat milk powder (Sigma)

Nonidet P-40 (NP-40) (BDH)
Paraformaldehyde (PFA) (Sigma)
PBA (Sigma)
PD 98059 (Enzo Life Sciences)
PGE₂ (Sigma)
PGF_{2α} (Sigma)
Phenylmethylsulfonyl fluoride (PMSF) (Sigma)
Phosphatase inhibitors cocktail 2 and 3 (Sigma)
PKC inhibitor (GF109203X) (Enzo Life Sciences)
Polybrene (Sigma)
Potassium Chloride (Sigma)
PPARα antagonist (GW-7647) (Sigma)
PPARγ antagonist (GW-9662) (Sigma)
PPARδ agonist (GW-501516) (Sigma)
PPARδ antagonist (GSK-0660) (Sigma)
Propan-2-ol (BDH)
propidium iodide (PI) (Sigma)
Proteinase K (Sigma)
Protogel 30% acrylamide (37.5:1 acrylamide to bisacrylamide) (National
Diagnostics)
Puromycin (Sigma)
Random hexamers (Invitrogen)
Rapamycin (Cayman Chemical)
Restriction endonucleases and buffers (New England Biolabs)
RNase A (Sigma)
RNase OUT Ribonuclease inhibitor (Invitrogen)
Rosiglitazone (Enzo Life Sciences)
SC-51089 (Tocris Bioscience)
Sodium azide (Sigma)
Sodium chloride (Sigma)
Sodium deoxycholate (BDH)
Sodium dodecyl sulphate (SDS) (BDH)
SYBR safe DNA gel stain (Invitrogen)
Tetramethylethylenediamine (TEMED) (Sigma)

TRI reagent (Sigma)
tris(hydroxymethyl)aminomethane (Tris) (Sigma)
Triton X-100 (Sigma)
Tween-20 (Sigma)
U0126 (Enzo Life Sciences)
WY-14643 (Sigma)
 α -tocopherol (Sigma)

2.1.5 Kits

Amplification grade DNase I kit (Sigma)
BCA kit (Thermo Scientific)
cAMP EIA kit (Enzo Life Sciences)
Fluo-4 Direct™ Calcium Assay Kit (Invitrogen)
GenElute DNA Gel extraction kit (Sigma)
HiSpeed Maxiprep kit (Qiagen)
KOD DNA polymerase (Novagen)
PCR array (SaBiosciences)
PGF_{2 α} EIA kit (Enzo Life Sciences)
Profection Mammalian cell Transfection kit (Promega)
QIAprep Spin Miniprep kit (Qiagen)
QIAquick PCR Purification Kit. (Qiagen)
T4 DNA ligase (Invitrogen)

2.1.6 Cell culture Materials

Dulbeco's Modified Eagle Medium containing L-glutamine (DMEM) (Invitrogen)
Heat Inactivated Fetal Bovine Serum (FBS) (Invitrogen)
Heat Inactivated Newborn Calf Serum (NCS) (Invitrogen)
100x Penicillin (100 U/mL)/ Streptomycin (50 μ g/ml) (Invitrogen)
Tissue Culture Plasticware and cell culture plates/dishes (Corning)
0.05% Trypsin-EDTA (Invitrogen)
Optimem reduced serum medium (Invitrogen)

2.1.7 Cell lines

3T3-L1 Mouse embryo fibroblast cell line

aP2^{-/-} 3T3-L1 cells (a kind gift from Bernlohr D.)

PPAR γ ^{-/-} 3T3-L1 cells (a kind gift from Virtue S.)

NIH-3T3 mouse cells

HEK293 human embryonic kidney cells

293FT packaging cells (Invitrogen)

2.1.8 Buffers and solutions

2.1.8.1 ChIP buffers

Elution Buffer

1% SDS

100 mM NaHCO₃

High Salt Buffer

20 mM Tris-HCl pH 8.1

500 mM NaCl

2 mM EDTA

0.1% SDS

1% Triton-X100

IP Buffer

16.7 mM Tris-HCl pH 8.1

16.7 mM NaCl

1.2 mM EDTA

0.01% SDS

1.1% Triton-X100

Lithium Chloride Buffer

10 mM Tris-HCl pH 8.1

250mM LiCl

1 mM EDTA

1% Nonidet p40

1% Deoxycholate

Low Salt Buffer

20 mM Tris-HCl pH 8.1

150 mM NaCl

2 mM EDTA

0.1% SDS

1% Triton-X100

SDS Lysis Buffer

0.5 M Tris-HCl pH 8.1

10 mM EDTA

1% SDS

1% Triton-X100

0.5% Deoxycholate

Swelling Buffer

25 mM HEPES pH 7.9

1.5 mM MgCl₂

10 mM KCl

0.1% Nonidet

Tris-EDTA (TE) buffer

10 mM Tris, pH 8.0

1 mM EDTA, pH 8.0

2.1.8.2 New England Biolabs Restriction Endonuclease Buffers (10x)

NEBuffer 1

10mM Tris Propane-HCl pH 7.0, 10mM MgCl₂, 1mM DTT

NEBuffer 2

10mM Tris-HCl pH 7.9, 10mM MgCl₂, 50mM NaCl, 1mM DTT

NEBuffer 3

50mM Tris-HCl pH 7.9, 10mM MgCl₂, 100mM NaCl, 1mM DTT

NEBuffer 4

20mM Tris-Acetate pH 7.9, 10mM MgCl₂, 50mM potassium acetate, 1mM DTT

2.1.8.3 Qiagen Buffers

Plasmid prep suspension buffer P1

50 mM Tris-HCl, pH 8.0

10 mM EDTA

100 mg/ml RNase A

Plasmid prep lysis buffer P2

200 mM NaOH

1 % w/v SDS

Plasmid prep neutralization buffer P3

3 M KAc pH 5.5

Plasmid prep equilibration buffer QBT

750 mM NaCl

50 mM MOPS, pH 7.0

15 % v/v isopropanol

Plasmid prep wash buffer QC

1 M NaCl

50 mM MOPS, pH 7.0

15 % v/v isopropanol

Plasmid prep elution buffer QF

1.25 M NaCl

50 mM Tris-HCl, pH 8.5

15 % v/v isopropanol

Plasmid prep elution buffer EB

10 mM Tris-HCl, pH 8.5

Tris-EDTA (TE) buffer

10 mM Tris, pH 8.0

1 mM EDTA, pH 8.0

2.1.8.4 Western Blotting Buffers

Blocking Solution

5% (w/v) non-fat milk powder in TBS-T or 5% (w/v) BSA in TBS-T

NuPAGE® Tris-Acetate SDS Running Buffer

Tris base 50 mM pH 8.24

Tricine 50 mM

SDS 0.1%

SDS Running buffer

25mM Tris

190mM glycine

0.1% (w/v) SDS

Transfer Buffer

48 mM Tris-base

39 mM Glycine

0.037% (w/v) SDS

20 % (v/v) methanol

Tris Buffered Saline (TBS)

130mM NaCl,

20mM Tris, pH 7.6

Tris Buffered Saline- Tween 20 (TBS-T)

130mM NaCl,

20mM Tris, pH 7.6,

0.1% Tween

2.1.8.5 Miscellaneous Buffers

10x DNA loading buffer

0.2% (w/v) Bromophenol blue

40% (v/v) Glycerol

100mM EDTA pH 8.0

10x DNAase I Reaction Buffer (Sigma)

200 mM Tris-HCl, pH 8.3

20 mM MgCl₂

10x M-MLV Reverse Transcriptase Buffer (Sigma)

500 mM Tris-HCl, pH 8.3

500 mM KCl,

30 mM MgCl₂

50 mM DTT

2x Laemmli loading buffer

50 mM Tris-HCl, pH 6.8

50 mM Imidazole, pH 6.8

1% (w/v) SDS

10% (v/v) Glycerol

2% (v/v) 2-Mercaptoethanol

0.002% (w/v) Bromophenol blue

5x T4 Ligase Buffer (Invitrogen)

250mM Tris HCl Ph7.6

50mM MgCl₂

5mM ATP

5mM DTT

25% (w/v) polyethylene glycol-8000

Antibody Dilution buffers

5% non-fat milk in TBS-T with 0.1% (v/v) NaN₃

5% BSA in TBS-T with 0.1% (v/v) NaN₃

DNase Stop solution

50 mM EDTA

Oil Red O Stock solution

0.7g Oil Red O

200ml isopropanol

Oil Red O Working solution

6 Parts of Oil Red O Stock and 4 of dH₂O

Phosphate Buffered Saline (PBS)

140mM NaCl

2.5mM KCl

1.5mM KH₂PO₄ pH7.2

10mM Na₂HPO₄ pH7.2

Protein lysis buffer

1% Triton-X

Tris pH 7.0

150mM NaCl

1mM PMSF

1x protease inhibitor cocktail

1x phosphatase inhibitor cocktail 2 and 3

Renilla Buffer

0.5M HEPES pH 7.8

40mM EDTA

Stripping Buffer (Protein)

100mM beta- Mercaptoethanol

2% (w/v) SDS,

62.5mM Tris-HCl pH 6.7

Tris Borate EDTA (TBE)

90mM Tris Borate

2mM EDTA pH 8.0

2.1.9 Bacterial strains and Media

L-agar

1% (w/v) Bactotryptone,

0.5% (w/v) Yeast Extract,

0.5% (w/v) NaCl,

0.1% (w/v) Glucose,

1.5% Bactoagar

LB-broth

1% (w/v) Bactotryptone,

0.5% (w/v) Yeast Extract,

0.5% (w/v) NaCl,

0.1% (w/v) Glucose

S.O.C Medium (Invitrogen)

2% Bactotryptone

0.5% Yeast Extract

10 mM NaCl

2.5 mM KCl

10 mM MgCl₂

10 mM MgSO₄

20 mM glucose

DH5α chemically competent and electrocompetent

F-Φ80*lacZΔM15* Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (rK-,
mK+) *phoA supE44 λ- thi-1 gyrA96 relA1*

2.1.10 SDS gels

Resolving gels

375 mM Tris-HCl, pH 8.9

0.1 % w/v SDS

10 %, 12% or 14% v/v acrylamide/bis-acrylamide (37.5:1)

0.03 % w/v APS

0.06% v/v TEMED

Stacking gels

80 mM Tris-HCl, pH 6.8

80 mM Imidazole pH 6.8

0.1 % w/v SDS

4.1 % v/v acrylamide/bis-acrylamide (37.5:1)

0.09 % v/v APS

0.09 % v/v TEMED

2.1.11 Miscellaneous

Amicon 10,000 NMWL centrifugal filter units (Millipore)

ECL Plus Western Blotting Detection System (Thermo Scientific)

Electroporation Cuvettes (Cell projects)

Hyperladder I and IV DNA ladders (Bioline)

Immobilon Polyvinylidene difluoride (PVDF) membrane (Millipore)

Oil Red O (Sigma)

Oligonucleotides (Invitrogen)

Optically clear adhesive seal sheets (Appleton Woods)

PageRuler pre-stained protein ladder (Fermentas)
RNase ZAP (Ambion)
Super RX autoradiography film (Fujifilm)
384-well plates for qRT-PCR (Applied Biosystems)
NuPAGE® Novex 7% Tris-Acetate Gel 1.5 mm, 10 Well

2.2 Methods

2.2.1 Cell culture

Wild type, PPAR γ ^{-/-} and aP2^{-/-} 3T3-L1 pre-adipocytes were maintained in subconfluent cultures in DMEM, containing 10% NCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. For PPAR γ ^{-/-} and aP2^{-/-} 3T3-L1 cells 2 μ g/ml puromycin was also added to maintain the selection. NIH-3T3, HEK293 and 293FT cells were maintained in DMEM, containing 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin. All cells were grown on 175cm² tissue culture flasks at 37°C in a humid atmosphere maintained at 5% (v/v) CO₂. Cell cultures were divided twice or three times a week depending on growth rate. The medium was removed and the cells were washed once with 10ml PBS before 2 ml of pre-warmed Trypsin/EDTA was applied. The cells were incubated at 37°C for 1 minute. The flask was gently tapped and 10ml pre-warmed DMEM was added (10% (v/v) FBS/NCS) to neutralise the trypsin. The cell suspension was transferred to a sterile 50ml tube and centrifuged at 1000rpm for 5 minutes. The pellet was gently re-suspended in prewarmed medium and cultured at a suitable dilution.

Cells were also prepared for storage in liquid nitrogen. Cells were trypsinized and pelleted as described above. The pellets were resuspended in media containing 10%v/v DMSO. 1 ml aliquots of cells were placed in cryogenic vials and then placed in Mr. Frosty, which is an isopropanol-filled freezing container, to ensure a constant cooling rate of 1 °C/min at -80 °C. The next day the vials were transferred into liquid nitrogen for long term storage. Cells recovered from liquid nitrogen were thawed in a 37 °C water bath and added

to 15 ml of prewarmed 10% (v/v) FBS/NCS DMEM in a 75cm² flask. The medium was changed the next day.

For the differentiation, cells were cultured until confluence and two days post-confluence (Day 0), the cells were stimulated to differentiate in DMEM supplemented with 10% FBS in the presence of 170nM insulin, 0.5 mM IBMX, and 250nM DEX for 2 days (D2). Cells were then treated with DMEM, 10% FBS and 170nM insulin until the end of the differentiation program. The medium was replaced every 2 days with fresh medium.

2.2.2 Preparation of Albumin-bound Fatty Acid

10 mM stock solution of AA was prepared by diluting the free fatty acid in ethanol and precipitating it with the addition of NaOH (final concentration of 0.25 M). The precipitated sodium salt was then evaporated under nitrogen gas, reconstituted with 0.9% (w/v) NaCl, and stirred at room temperature for 10 min with defatted BSA in 0.15 M NaCl. The solution was stored in multiple aliquots at -20 °C protected from light in tubes evacuated under nitrogen gas. Control solution containing NaOH and fatty acid-free BSA was similarly prepared. The FFA/BSA molar ratio was 4:1 (Ou et. al., 2001). All AA treatments (and BSA control) contained 0.22mM α -tocopherol to prevent lipid peroxidation (Kim et. al., 2006). The same procedure was followed for EPA, DHA and LA conjugation.

2.2.3 Oil Red O staining

Differentiated and undifferentiated 3T3-L1 cells were stained with Oil Red O. The culture medium was removed and the cells were washed with PBS. Cells were then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The fixative was discarded and the cells were washed with PBS. Oil Red O working solution (made up with isopropanol) was applied for 1h at room temperature. The cells were then rinsed with 60% isopropanol and PBS. Stained lipid droplets were visualized under light microscope. For Oil Red O

quantification, the dye was extracted in 100% isopropanol, transferred to a white 96-well plate and the absorbance was measured at 510nm.

2.2.4 RNA extraction

Total RNA was isolated from cells using TRI Reagent. Samples were processed according to the manufacturer's protocol. TRI Reagent is a monophasic solution of phenol and guanidine thiocyanate that maintains the integrity of RNA during cell lysis, whilst at the same time disrupting cells and dissolving cellular components. To avoid ribonuclease (RNAase) contamination RNAase-free tips, eppendorfs and DEPC-treated water were used. Moreover, disposable gloves were worn at all times and the surfaces and pipettes were cleaned with RNAase Away.

1ml TRI Reagent was added per well of a 6-well plate to lyse the cells and the lysate was transferred to a 1.5ml microcentrifuge tube. When cells were cultured in smaller formats the volume of TRI Reagent was adjusted accordingly. Samples were incubated at room temperature for 5 minutes to allow dissociation of protein complexes with nucleic acid. 0.2ml chloroform (kept at -20°C) per ml of TRI Reagent was added to each sample, which were then shaken vigorously by hand for 15 seconds before incubating for 2-15 minutes at room temperature. Samples were then centrifuged at 13,000rpm for 15min at 4°C. Following centrifugation, the mixture separated into a lower organic red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase while DNA and proteins remained in the interphase and in the organic phase respectively. Thus, the total RNA isolated by TRI Reagent is free of protein and DNA contamination. The aqueous phase was then transferred to new 1.5ml tubes and total RNA was precipitated by the addition of 0.5 ml of isopropyl alcohol (stored at -20°C), and mix. The samples were left at room temperature for 10 minutes, and then centrifuged 13,000 rpm for 15 minutes at 4°C. The supernatant solution was discarded and the precipitated RNA pellets were washed with at least 1 ml of 75% ethanol (stored at -20%) and centrifuged at 13,000 rpm for 5 minutes at 4°C. Purified RNA was briefly air-

dried and re-dissolved in RNase-free water. The RNA concentration and purity were determined by Nanodrop ND1000 spectrophotometer and samples were stored at -80°C.

2.2.5 cDNA synthesis

Synthesis of cDNA was performed by using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) following the manufacturer's instructions. Before the cDNA synthesis RNA was treated with DNase I to remove any genomic DNA which would be amplified along with the target mRNA. Briefly, 1 µg of total RNA was digested using 0.5U DNase I for 15 minutes at room temperature. The reaction was then inactivated by adding 0.5µl of 25mM EDTA pH 8.0 and the samples were then incubated at 65°C for 10 minutes. 50ng of random primers and 1µl of 10mM dNTP mix were added to the samples and the volume was adjusted to 12µl with DEPC-treated water. The samples were incubated at 65°C for 5 minutes and then placed on ice for 2 minutes. 4µl of 5x first strand RT buffer, 2µl of 0.1M DTT and 40U of RNaseOUT™ Recombinant Ribonuclease Inhibitor were added to the samples which were then incubated for 2 minutes at 37°C. 1 µl (200 units) of M-MLV RT was added to each sample and incubated for 10 min at room temperature followed by incubation for 50 min at 37°C. Finally, the reaction was inactivated by heating at 70°C for 15 min. The synthesized cDNA was then used for quantitative real-time PCR (qRT-PCR) or stored at -20°C.

2.2.6 Quantitive Real-Time PCR

QRT-PCR was carried out using 7900HT Fast Real-Time PCR System (Applied Biosystems). SYBR® Green JumpStart™ Taq ReadyMix™ for Quantitative PCR was used as a fluorescent dye which has undetectable fluorescence in its free form but emits fluorescence once incorporated into double stranded DNA. Therefore as the PCR progresses and more double stranded DNA product accumulates, the more fluorescent signal is produced. The fluorescent signal is detected in each sample following each cycle of the PCR reaction. The data is analysed by the setting of threshold within the

linear phase of the reaction, termed Ct value, and determining the number of cycles required for the fluorescent signal to reach the threshold. The Ct value is seen to decrease with increasing target cDNA.

The reactions were prepared in 384-well plate format. Each reaction contained 4 µl of 2x SYBR Green master mix, the appropriate ratio of primers, 0.3 µl of cDNA made up to a total volume of 8 µl with DEPC-treated water. Negative controls of reaction mix without cDNA template were included. All reactions were carried out in duplicate with the following thermocycler conditions: 94°C for 2 minutes, 94°C for 15 seconds, 60°C for 1 minute and 72°C for 1 minute for a total of 40 cycles. Data were analysed with the SDS 2.3 software (Applied Biosystems).

The method used for quantification was the comparative Ct method which calculates the relative expression of a gene compared to that of a housekeeping gene (L19) and of a calibrator sample. Briefly, the mean Ct value was calculated from the duplicates of the qRT-PCR reactions. Then the ΔCt value was calculated by subtracting the mean Ct for L19 from the mean Ct of the target gene [$\Delta Ct = Ct \text{ (target gene)} - Ct \text{ (L-19)}$]. Then a cDNA sample was selected to act as a calibrator sample in order to relate all other samples to. The $\Delta\Delta Ct$ value was calculated by subtracting the ΔCt of the calibrator sample from the ΔCt values of all the other samples [$\Delta\Delta Ct = \Delta Ct \text{ (sample)} - \Delta Ct \text{ (calibrator)}$]. $\Delta\Delta Ct$ values were then converted to fold differences using the function $2^{-\Delta\Delta Ct}$.

Primers were designed to give a product of approximately 100bps crossing intron exon boundaries to eliminate the amplification of genomic DNA. Sequences were obtained from Ensembl mouse genome database and applied to the software Primer Design or Primer 3. Primers were dissolved in DEPC-treated water to a final concentration of 100pmol/µl, this is then diluted again to a working concentration of 20pmol/µl. The primers used for qRT-PCR are listed in the Appendix.

2.2.7 PCR array

Two days post-confluent 3T3-L1 cells were treated with AA 1mM for 24h and the total RNA was isolated with TRI Reagent as described above. Then 1µg RNA was DNase treated on column using the Qiagen RNeasy Mini kit. cDNA synthesis was conducted according to the manufacturer's guidelines using the RT² First Strand kit. cDNA was loaded into the a 96-well containing the 96-primer set panel together with the qPCR master mix provided. QRT-PCR was conducted using the Step-One ABI PCR cyclers using a two-step cycling program: 95°C for 10min and 40 cycles of 95°C for 15sec and 1 min of 60°C.

2.2.8 Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The BioRad MiniProtean system was used to cast gels. Resolving gels were poured by combining the components mentioned above. The gel solution was added between the glass plates and overlaying with ethanol and allowed to set for at least 20min. Stacking gel was then poured over the set resolving gel and a comb was inserted and allowed to set for 20min.

Whole protein extracts were extracted by lysing the cells with protein lysis buffer containing 1x protease inhibitor cocktail, 1mM PMSF and 1x phosphatase inhibitors. Cells were scraped with a cell scraper on ice and transferred to 1.5 ml microcentrifuge tubes. Lysates were rotated for 10min at 4°C and centrifuged at 13,000rpm for 5min at 4°C. Supernatants were transferred to new eppendorf tubes and either snap frozen for storage at -80°C or the protein concentration was measured. Quantification of total protein was performed using the BCATM Protein Assay Kit. The concentrations of the unknown protein samples were determined based on a standard curve generated from a series of dilutions of known concentrations of Bovine Serum Albumin (BSA; 2mg/ml).

Protein extracts were mixed 1:1 with 2x Laemmli buffer and heated for 5 min at 95°C. At the same time the gels were assembled onto the electrophoresis

equipment in a tank with SDS running buffer. Samples were briefly centrifuged and loaded into the gel wells using gel loading pipette tips together with 5 μ l of the rainbow ladder. Gels were run at 70V for around 10min and at 100-120V until the marker was sufficiently separated.

For PPAR γ protein analysis 70 μ g of lysate was loaded in a 7% Tris-Acetate Novex pre-cast gel. The gel was run in 1x NuPAGE® Tris-Acetate SDS running buffer at 150V until the dye front had migrated to the bottom of the gel

2.2.9 Western Blotting

Following SDS-PAGE, protein samples were transferred to a PVDF membrane using the wet blotting method. Gels were removed from the glass plates and PVDF membrane was activated by soaking in methanol for 10sec. A 'sandwich' was assembled which consisted of a pad, one Whatman filter paper, the gel, the activated PVDF membrane, another piece of Whatman filter paper and another pad. The paper, pad and PVDF had all been pre-equilibrated in western transfer buffer, and the 'sandwich' is put together carefully under the buffer to make sure no air bubbles were present. The 'sandwich' was then placed in a blotting cassette and inserted in the blotting tank immersed in cold western transfer buffer with the PVDF towards the anode and the gel towards the cathode. Transfer was performed for approximately 2h at 4°C at 100V at 4°C. After the transfer, PVDF membranes were removed, allowed to air-dry, re-soaked to methanol and put in blocking buffer for 1h at room temperature with gentle shaking.

After blocking, membranes were incubated with primary antibodies in 50ml tubes at 4°C with constant rolling. Typically, the anti-fungal agent sodium azide was added to allow the storage of diluted antibody at 4°C for subsequent experiments. Consequently, membranes were washed in 1x TBS-T three times for 10 minutes and then incubated for one hour at room temperature with the appropriate secondary antibodies horseradish peroxidase – conjugated diluted at 1:5,000. The membranes were washed in

1x TBS-T three times for 10 minutes. Finally, the signal was detected using enhanced chemiluminescence substrates following manufacture's instructions and the light emission was captured and visualized by exposing the blot to film. Densitometry was carried out by analysing Western blot images with ImageJ software. Densitometry was performed only for the western blot images of p-ERK and total-ERK, to confirm that the stripping of the membrane was efficient, as these two proteins have the same sizes. Densitometry for all other proteins was not performed, as different film exposure times and often saturation, make densitometry an unreliable protein quantification method.

2.2.10 Bacterial propagation of plasmids

2.2.10.1 Transformation of chemically competent DH5α E.Coli by heat shock

For the preparation of large plasmid DNA quantities, chemically competent DH5α cells were thawed on ice (typically 50µl). In an eppendorf tube bacteria were mixed with the relevant plasmid (less than 1ng DNA). After 30 minutes on ice, cells were heat shocked in a 42°C water bath for 30 seconds and then placed on ice for a further two minutes. Then 200 µl of SOC medium was added to the reaction and the cells incubated at 37°C for 1 hour. The cells were then spread on L-agar plates containing 100µg/ml ampicillin to select for transformants. The plates were inverted and incubated at 37°C overnight and the next day colonies were picked.

2.2.10.2 Transformation of electrocompetent DH5α E.Coli by electroporation

Electrocompetent DH5α cells were thawed on ice. 50µl of bacteria was mixed with the ligation in a pre-chilled 1mm electroporation cuvette and electroporated using a Bio-Rad gene pulser, at 1.67kV, giving a time constant of approximately 4 milliseconds. Following electroporation, 200µl SOC medium was added and the cells were incubated at 37°C for 1h. The cells

were then spread on L-agar plates containing 100µg/ml ampicillin, inverted and incubated at 37°C overnight and the next day colonies were picked.

2.2.10.3 Small-scale preparation of plasmid DNA (Miniprep)

Individual bacterial colonies of possible recombinant bacteria were used to inoculate 5 ml of LB containing 50µg/ml ampicillin and allowing the bacteria to grow in a shaking incubator at 37°C overnight. Bacteria were then pelleted by centrifugation for 15 min, at 3900 rpm at 4°C. Plasmid DNA was purified by using QIAprep Spin MiniPrep Kit. Cells were resuspended in 250 µl buffer P1 and lysed under alkaline conditions by the addition of 250 µl buffer P2. The lysates were subsequently neutralised and adjusted to high-salt binding conditions by the addition of 350 µl of buffer N3. Samples were centrifuged and the supernatants were passed through a silica membrane. The membrane allowed selective adsorption of plasmid DNA under the high-salt conditions, while RNA, cellular proteins, and metabolites flow through. Endonucleases were removed by a wash step with buffer PB to ensure plasmid DNA was not degraded. The membrane was then desalted by the addition of 750 µl of buffer PE. Plasmid DNA was eluted with 50 µl elution buffer. Eluted DNA was suitable for diagnostic digestion or sequencing.

2.2.10.4 Large-scale plasmid DNA preparation (Maxiprep)

Higher amounts of correct recombinant plasmids were prepared by using the Qiagen HiSpeed Maxi Kit and were suitable for mammalian cell transfection. As described above, a single colony was picked and 5 ml of LB broth containing 50µg/ml ampicillin was inoculated and grown at 37°C for approximately 8 h with continuous shaking. This was then diluted into 200 ml of LB broth with 50µg/ml ampicillin, which was incubated overnight at 37°C with constant shaking. Bacteria were recovered by centrifugation at 3900 rpm for 15 min at 4°C. Supernatants were removed and pellets were processed according to the manufacturers protocol. The protocol is based on a modified alkaline lysis procedure in which bacterial cells (suspended in buffer P1) were lysed in sodium hydroxide/SDS in the presence of RNase A (buffer P2). SDS

solubilised the phospholipid and protein components of the cell membrane, leading to cell lysis. Sodium hydroxide denatured chromosomal DNA, plasmid DNA and proteins, while RNase A digested the liberated RNA. The lysate was neutralized by the addition of acidic potassium acetate (buffer P3). The high salt condition caused SDS to precipitate with the denatured proteins, chromosomal DNA and cellular debris, trapped in salt-detergent complexes. The smaller, covalently closed plasmid DNA re-natured and remained in solution. The lysates were passed through a pre-equilibrated (buffer QBT) anion-exchange resin operating by gravity flow under low salt and low pH conditions to trap the plasmid DNA, RNA, protein, dyes and low-molecular weight impurities were removed by a medium salt wash (buffer QC). Plasmid DNA was eluted in a high salt buffer (buffer QF) and then concentrated and desalted by isopropanol precipitation. The purified DNA was washed with 70 % v/v ethanol, air-dried and redissolved in TE buffer.

2.2.11 Molecular Cloning

2.2.11.1 Restriction digests

Restriction enzyme digests were performed using enzymes from New England Biolabs at 37°C in the buffers recommended by the manufacturer supplemented with 100x BSA where required. DNA was digested with excess of enzyme. For analysis of digestion products, reactions were terminated by the addition of 10x DNA loading buffer and analysed by gel electrophoresis. For cloning, bands were cut from the gel and purified a DNA gel extraction kit.

2.2.11.2 PCR for cloning

The KOD Hot Start DNA polymerase was used to amplify the fragments needed to be cloned. KOD polymerase is a high-fidelity, proofreading and has 3' to 5' exonuclease activity, so PCR products are of high fidelity. In 0.2ml PCR tubes 1 ng plasmid DNA or 200 ng genomic DNA or 2 µl of a 20-µl reverse transcriptase reaction mix was mixed with 5 µl of 10X PCR Buffer for

KOD Hot Start DNA Polymerase, 3µl of each forward and reverse primers (at final concentration of 0.3µM), 5 µl dNTPs (final concentration 0.2 mM), 2 µl MgSO₄ (final concentration 1 mM), 1µl KOD Hot Start DNA Polymerase (1 U/µl) and PCR grade water to a final volume of 50µl. At the same time non-template control reactions were set up. PCR were run with the following conditions: 95°C for 2min, followed by 35 cycles of 95°C for 20 sec, 55°C for 10 sec and 70°C for 10 sec/15sec/20sec/25sec/kb according to the size of the amplified fragment.

2.2.11.3 DNA agarose gel electrophoresis

Molecular biology agarose was dissolved in 1x TBE in a canonical flask to give the appropriate percentage gels around 1-2 % (w/v). The suspension was heated in a microwave oven until boiling. The solution was allowed to cool, 0.5µg/ml ethidium bromide was added, and poured into appropriate trays with combs. Once set, the gel was submerged in 1x TBE buffer in a gel tank. DNA samples were mixed with 10x DNA loading buffer and loaded into the wells along with the appropriate DNA ladder. Electrophoresis was carried out at 100V. DNA was visualised by illumination on a long wave UV light box and photographed.

2.2.11.4 Gel extraction of DNA

Restriction fragments were purified from TBE agarose gels using GenElute DNA Gel Extraction kit according to manufacturer's protocol. DNA bands of the desired molecular weight were cut from the gel with a clean scalpel under UV light. The gel sliced was weighted and 3 volumes of gel solubilisation solution were added. The gel mixture was incubated at 50°C for 10min with occasional mixing until the gel slice is completely dissolved. At the same time, the binding column was prepared by adding 500µl of column preparation solution and centrifuging at 13,000rpm for 1min and discarding the flow-through. 1 gel volume of 100% isopropanol was added and mixed. The solubilized gel solution was loaded to the prepared binding column and centrifuged at 13,000rpm for 1min. DNA binds to silica membrane and was

washed with 700µl of wash solution. Residual ethanol was removed by centrifugation for 1min. DNA was eluted by adding 30-50µl of elution buffer to the membrane, allowing to stand for 1min and centrifuging at 13,000rpm for 1min.

2.2.11.5 Ligations

Ligation of insert and vector was carried out with T4 DNA ligase. Before the ligation digested vector and insert were electrophoresed on an agarose gels to assess their concentrations. Ligations were performed using a 1:1 -1:6 molar ratio of vector to insert DNA. All ligation reactions were performed in a final volume of 20µl which contained 1x T4 DNA ligase buffer and 1µl T4 DNA ligase. All reactions were incubated overnight at 16°C.

2.2.11.6 DNA sequencing

Plasmid DNA was mixed with 3.2 pmol of sequencing primer to a total volume of 10µl. The Core Genomics Facility performed the DNA sequencing and the chromatographs were analysed with the 4Peaks software.

2.2.12 Transient transfections

2.2.12.1 Transfection of HEK293 cells using the calcium phosphate method

30,000 cells were seeded in 96-well plates in 100µl 10%FBS DMEM. The following day, the transfection mixes were prepared as follows: in a tube the required amount of reporter constructs and expression vectors was mixed with nuclease-free water and 2M calcium chloride whereas in a separate tube an equal volume of 2x HEPES-buffered saline was added. The calcium phosphate mix was added dropwise to 2x HEPES-buffered saline while vortexing. The mix was incubated at room temperature for 20 min to allow formation of calcium phosphate precipitate and then was added to HEK293

cells and incubated with the precipitate for 24h. The following day, cells were treated and harvested 48h post-transfection.

2.2.12.2 Transfection of 3T3-L1 and NIH-3T3 cells using Lipofectamine 2000

3T3-L1 or NIH-3T3 cells were transiently transfected in 24 or 12-well plates using the Lipofectamine 2000 reagent. 80%90 % confluent monolayers were seeded in 10 % FBS/DMEM without antibiotics. The following day, the transfection mixes were prepared as follows: in an appropriate size tube DNA was diluted in 50µl/100µl Opti-MEM® I reduced serum medium and mixed gently, whereas in a separate tube the appropriate volume of lipofectamine 2000 reagent was diluted in Opti-MEM® I reduced serum medium, mixed and incubated at room temperature for 5 min. The diluted DNA and diluted lipofectamine were combined, gently mixed and incubated for 20 minutes to allow DNA-lipofectamine complexes to form. The transfection mix was then added to each well. After 24h the cells were treated and harvested 48h post-transfection.

2.2.12.3 Dharmafect transfection of siRNA

3T3-L1 cells in 12-well /6-well plates were transfected with 100nM siRNA using Dharmafect3. 80%90 % confluent monolayers were seeded in 10 % FBS/DMEM. The following day, the transfection mixes were prepared as follows: in an appropriate size tube siRNA was diluted in Opti-MEM® I reduced serum medium and mixed gently, whereas in a separate tube the appropriate volume of Dharmafect3 reagent was diluted in Opti-MEM® I reduced serum medium, mixed and incubated at room temperature for 5 min. The siRNA mix and the diluted Dharmafect3 were combined, gently mixed and incubated for 20 minutes to allow DNA-lipofectamine complexes to form. The transfection mix was then added to each well containing cells and medium. The cells were treated on the third day after transfection and either harvested after 24h or re-transfected and harvested at the end of the differentiation program.

2.2.13 Generation of Lentiviruses Containing ATG7 shRNA

Lentiviral particles containing PGK vectors were packaged in 293FT cells. The cells were seeded in 10cm dishes so as the next day to be 80% confluent in 10%FBS/DMEM. The following day the cells were transfected with 15µg PGK/siATG7 vector, 10µg pCMV-Δ8.91 and 5µg pMDG2-vsvg per plate using the calcium phosphate method described above. The following day, the transfection mix was exchanged for 7ml per plate. Supernatants were harvested at 24 h and 48 h after the first media change and stored at -80°C. Supernatants from both time points were thawed, pooled and centrifuged at 1000rpm for 5 min to pellet any cells. Viral supernatants were next concentrated to a final volume of 200µl/plate utilizing Amicon 10,000 NMWL centrifugal filter units, and aliquoted for storage at -80°C. Viral titer was calculated using serial dilutions of PGK-GFP-expressing plasmid to infect 3T3-L1 cells in 12-well plates in the presence of 8µg/ml polybrene. GFP was analyzed 72 h later by fluorescence activated cell sorting (FACS), and the minimum volume of virus required to give 90-100% GFP-positive 3T3-L1 cells then used in subsequent experiments.

For experiments, 50,000 3T3-L1 cells were seeded in 12-well plates. After 4-5h of seeding, the cells were infected in the presence of polybrene (8µg/ml). Cells were treated on the third day after infection.

2.2.14 Reporter Gene assays

Transfected cells in 96-well plates or 24-well plates were washed with PBS and lysed in 25 µl or 50 µl 1x Reporter lysis buffer, respectively. The plates were then placed on a shaker for 5min and then placed at -80°C for storage or for a freeze-thaw step which is required for the appropriate lysis of the cells. Plates were then thawed and 10 µl of each lysate was transferred to a white 96-well plate. 50 µl of the luciferase assay reagent was then added to each well and immediately the luciferase activity was measured by reading luminescence on Victor2 plate reader (Perkin Elmer). The remaining lysates were used to measure Renilla luciferase activity to normalise firefly luciferase

activity measurements. Thus, 10 μ l of each lysate was transferred to a white 96-well plate together with 50 μ l of Colenterazine in Renilla buffer. Plates were incubated in dark at room temperature for 20 minutes and then read as described above. Each firefly luciferase activity was divided by renilla activity to correct for variations in the transfection efficiency.

2. 2.15 Chromatin Immunoprecipitation

3T3-L1 cells were grown in 175cm² culture dishes, fixed with 1% formaldehyde and incubated for 10 minutes at 37°C. The fixation was stopped with 125 mM glycine. Then, 1 ml of swelling buffer was added per dish and the cells were scraped and homogenized with a dounce homogenizer in order to isolate the nuclei. The lysates were then rotated at 4°C for 10 minutes. The samples were centrifuged for 3 minutes at 13,000rpm at 4°C and the nuclei, collected in a pellet. The pellets were resuspended in 500 μ l SDS lysis buffer and sonicated for 5 minutes (with 30 second cycles) at 4°C on high power on the Diagenode Bioruptor sonicator. The resulting suspension was centrifuged for 20 minutes at 13,000rpm at 4°C and the supernatant contained the sonicated chromatin. The suspension containing the chromatin was diluted ten times in IP buffer and then precleared at 4°C for 3 hours with 50 μ l of Protein G Dynabeads. At the same time, 5 μ g of antibody was mixed with 50 μ l of Protein G Dynabeads in IP buffer and incubated at 4°C for 3 hrs. 10% of the precleared chromatin was transferred into a clean microfuge tube and frozen at -80°C, which will then be used as the input. The remaining chromatin was then mixed overnight at 4°C with the antibody bound to the Dynabeads. The beads antibody-chromatin complex was then washed with the following buffers: Low Salt Buffer, High Salt Buffer, Lithium Chloride Buffer, TE buffer before eluting the chromatin with 250 μ l Elution buffer and incubated at room temperature for 15 minutes. To reverse crosslink the proteins and the DNA 200 mM NaCl were added to the samples and to the input. The samples and the input were incubated overnight at 65°C and 10 mM EDTA, 40 mM Tris-HCl pH 8 and 40 μ g/ml Protease K were added and incubated for a further hour at 55°C. DNA was then purified using QIAquick PCR Purification Kit. The purified DNA was amplified by RT-qPCR and the data normalized to the input.

2.2.16 Calcium mobilisation assay

3T3-L1 cells were seeded into 35mm tissue culture treated dishes maintained in medium containing 10% NCS/DMEM in an incubator (5% CO₂ at 37°C). Cells were assayed when become two days post-confluent for the calcium mobilization assay. On the day of the assay the dye was prepared according to the manufacturer's instructions. 2x calcium sensitive Fluo-4 Direct buffer was mixed with the same volume of differentiation cocktail (MDI) and cells were incubated for 30min in an incubator (5% CO₂ at 37°C). The cells were removed from the incubator and incubated for another 30min at room temperature. Plates were then placed for live cell imaging on the SP5 confocal microscope (Leica) using the LAS - AF program. Dishes were left for 1 min to gain a basal value before being applied with AA 10μM for approximately 15 min. The data exported from the program as values based on the fluorescence omitted by the experiment. These values were exported and the basal average intensity from the first 1min was subtracted.

2.2.17 PGF_{2α} ELISA

Two days post-confluent 3T3-L1 cells were treated with 100μM, 500μM or 1mM AA and 24h in the presence of MDI. Supernatants were collected and immediately frozen for PGF_{2α} ELISA. PGF_{2α} ELISA kit was used according to manufacturer's instructions. This assay is based on the competition between the PGF_{2α} included in the samples and a conjugated PGF_{2α} with acetylcholinesterase (PGF_{2α} tracer). Because the amount of the tracer is constant whereas the PGF_{2α} amount in our samples varies the amount of the tracer that will bind to the PGF_{2α} -specific rabbit antiserum will be inversely proportional to the concentration of the PGF_{2α} level in the sample. The plate was washed and the substrate for the acetylcholinesterase was added. The product of this reaction had a yellow color and absorbed at 412nm. Thus, the amount measured spectrophotometrically was direct proportional to PGF_{2α} tracer concentration and inversely proportional to the free PGF_{2α} in each well. The PGF_{2α} in our samples were then calculated according to the instructions based on a standard curve of known PGF_{2α} standards.

2.2.18 cAMP assay

3T3-L1 cells were seeded onto 24 well plates. After two days of post-confluency, cells were pre-treated with IBMX, a phosphodiesterase inhibitor, at 500 μ M for 5 min. Cells were then treated with 10 μ M, 100 μ M or 1mM AA for 30min. The experiment was terminated with the addition of 0.1 % HCl-Triton X-100. cAMP was measured using a competitive enzyme immunoassay according to the manufacturer's instructions and normalised to protein concentration.

2.2.19 Fluorescence-activated cell sorting (FACS)

Two days post-confluent 3T3-L1 cells were treated with 100 μ M, 500 μ M or 1mM AA for 24 h in the presence of MDI. Cells were then trypsinized and centrifuged at 1000 rpm for 5min. The pellet was resuspended in medium centrifuged at 1000 rpm for 5min. The pellet was resuspended in PBS, centrifuged and fixed with 5 ml of ice-cold 70% ethanol for at least 1 h at -20°C. The fixed cells were then centrifuged and washed twice in 2 ml of cold PBS. The cells were centrifuged and resuspended in 500 μ l of PBS solution containing RNase (0.2mg/ml) and PI (50mg/ml). After incubation at room temperature for 30min, the stained cells were analysed in a fluorescence-activated cell sorter (FACSCalibur, BD Biosciences, San Jose, CA, USA) with a 488-nm laser line for excitation. Red fluorescence (4600 nm) and side scatter were measured and at least 10000 events were collected. Debris and residuals of necrotic cells was recognized and gated-off.

2.2.20 MTT assay

3T3-L1 cells were seeded on 96-well plates. After two days of post confluency, cells were treated with 100 μ M, 1mM AA or 1% Triton X-100 for 24 h in the presence of MDI. MTT was dissolved in PBS (5 mg/ ml) and 20 μ l was added per well. The plate was then placed on a shaker to thoroughly mix the MTT into the media. The cells were then incubated for 1h at 37°C. The medium was then removed until dry and 200 μ l DMSO was added per well.

MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals and DMSO solubilises the crystals. Samples were read directly in the wells using a spectrophotometer at 570 nm.

2.2.21 LDH assay

Two days post-confluent 3T3-L1 cells were treated with 100 μ M, 500 μ M AA or 2% Triton X-100 for 24 h in the presence of MDI. 100 μ l of supernatants were added into 96-well plate and 100 μ l of the reaction mix was added in each well according to the manufacturer's instructions and incubated for 30min at room temperature. Then the lactate dehydrogenase (LDH) activity was measured with a spectrophotometer at 490nm. LDH activity is directly proportional to cell death and cell lysis, since LDH is released from the cytosol of damaged cells into the supernatant.

2.2.22 AdipoRed assay

AdipoRed is a solution of the hydrophobic stain Nile Red. Nile Red when partitioned in a hydrophobic environment becomes fluorescent. AdipoRed enables the quantification of intracellular LDs in a high-throughput manner. Thus, cells were seeded in a 96-well plate and cultured until ready to be assayed. The plate was then removed from the incubator and allowed to cool to room temperature. The cells were washed once with PBS and then filled with 200 μ l of PBS per well. With a multichannel pipette 5 μ l of the AdipoRed dye was distributed in each well and immediately mixed. After 10minutes the fluorescence was measured using Victor2 plate reader.

Chapter 3

Results: Arachidonic Acid effects on the early stages of adipocyte differentiation

3.1 Introduction

It is apparent that not only the quantity but also the nature of FAs plays an important role on the effect of dietary fat on human health. In evolutionary terms, our diet has changed dramatically at disproportionate rates in comparison with our genes which have changed around 0.005% the past 10,000 years (DNA mutation rate being 0.5% per million years) (Simopoulos, 2002). Several studies suggest that humans evolved on a diet with a ratio of ω -6 to ω -3 FAs of around 1. However, the ω -6/ ω -3 ratio in Western diets is 15/1–16.7/1 (Simopoulos, 2002). It is calculated that 85% of the total dietary PUFA intake in Western diets is ω -6 PUFAs and excessive amounts of ω -6 PUFA has been correlated with the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases (Simopoulos, 2002). Furthermore, studies in rodents link ω -6 PUFA deficiencies with skin conditions, growth retardation and infertility (Kremmyda et al., 2011). In humans PUFA or AA deficiency has not been reported since LA and AA are found in most of the food sources and a completely PUFA free diet must be synthetic or largely so.

Excess fat has been shown to increase both hyperplasia and hypertrophy in AT (Faust et al., 1978). However, even if AA is accepted to be a very important element of our diets, its role in AT development is controversial. Animal studies have yielded conflicting results, with some studies demonstrating that a diet rich in ω -6 PUFAs decreases AT mass (Matsuo et al., 2002), whereas others have associated intake of ω -6 PUFAs with an increased propensity for obesity (Massiera et al., 2003). Very little is known about the effects of AA and its derivatives at the early stages of differentiation and how the early events can affect the pre-adipocyte differentiation potential. In this chapter, experiments were designed to identify AA gene targets during the early stages of differentiation and the signalling and molecular events AA triggers to regulate their expression. To address this question 3T3-L1 cells were used as a pre-adipocyte cell system as it is a well characterised cell line that has been successfully used to identify the key factors for adipocyte differentiation.

3.2 Results

3.2.1 The effect of AA effect on aP2 expression

3.2.1.1 *The effect of AA on the adipocyte differentiation markers*

To address the effect of AA on the early stages of adipocyte differentiation, 3T3-L1 cells were used. 3T3-L1 cells were maintained pre-confluent under growing conditions in medium containing 10% NCS. The differentiation protocol for these cells involves two-day post-confluent cells (D0) treated with 0.5 mM IBMX, 250nM DEX and 170nM insulin (or MDI) for the first 48h (D2) in the presence of 10% FBS. Cells were then treated with 170nM insulin until the end of the differentiation program day 10 (D10) (Figure 3.1 A). In this chapter, cells will be assessed after the initial 24h after the induction of differentiation (D1), unless otherwise stated.

During the growth phase 3T3-L1 cells had a fibroblast-like morphology (Figure 3.1 B). After 24h of the addition of MDI their morphology changed as the cells became spindle-shaped (Figure 3.1 C). AA was added to D0 cells and during the first 24h of differentiation (D1) in the presence of MDI using three doses: 10 μ M, 100 μ M and 1mM. FAs are typically found in fasting or fed mice at plasma levels in a range between 0.1 and 1.2 mM (Distel et al, 1992). Thus, the AA concentrations used were in the physiological range. Cells were stained with Oil Red O which is a dye that stains lipids. LDs could be visualised with increasing doses of AA; no LDs were present using the lowest concentration, 10 μ M AA (Figure 3.1 E), whereas appearance of LDs was observed with 100 μ M AA (Figure 3.1 F) and more LDs could be seen with the highest dose (1mM) (Figure 3.1 G).

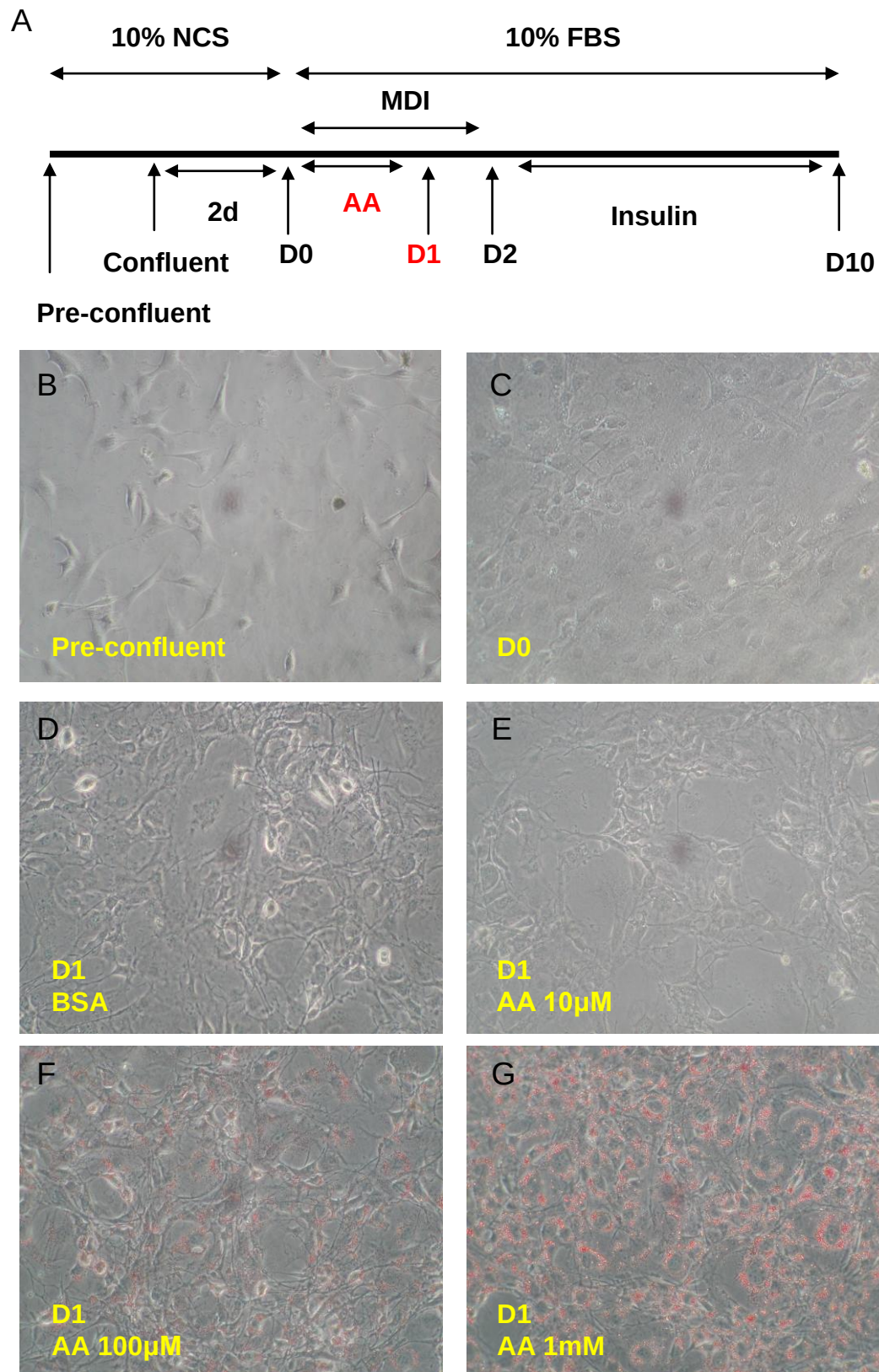


Figure 3.1: 3T3-L1 pre-adipocytes showed enhanced LD formation after 24h of AA treatment. A) For the differentiation protocol of 3T3-L1 pre-adipocytes, cells were kept pre-confluent in medium containing 10% NCS in the growing phase. The differentiation protocol

started with two-day post-confluent cells (D0) treated with 0.5 mM IBMX, 250nM DEX and 170nM insulin (or MDI) for the first 48h (D2) in the presence of 10% FBS. MDI was then withdrawn and the cells were maintained in 10% FBS medium containing 170nM insulin until they were differentiated after 8-10 days. B-G) Oil Red O staining of 3T3-L1 cells at different stages of the differentiation and upon AA treatment for 24h. Cells were captured under a light microscope using 20x magnification. B) pre-confluent cells, C) two-day post-confluent cells (D0), D) treated cells with MDI for 24h and FA-free BSA (vehicle for AA) (D1), E) treated cells with 10 μ M AA in MDI (D1), F) treated cells with 100 μ M AA in MDI (D1), G) treated cells with 1mM AA in MDI (D1).

As discussed in Chapter 1, LD formation has been classically used as an adipocyte differentiation marker as it is an essential readout for adipocyte maturation. To assess if AA promotes the differentiation program of pre-adipocytes after 24h of treatment, the expression of different mature adipocyte gene markers such as aP2, PPAR γ 2/ γ 1, C/EBP α , LPL, FAS, PEPCK, adiponectin and Glut4 was studied (Figure 3.2).

There was not a significant change for FAS, LPL and C/EBP α expression in response to any AA concentrations used in comparison with the vehicle control (Figure 3.2 D, E, F). However, PPAR γ 2 and PPAR γ 1 expression was down-regulated only with the higher AA dose (1mM) (Figure 3.2 B, C). The AA effect was dramatic on aP2 expression. aP2 mRNA levels were up-regulated around 2-fold with 10 μ M, around 80-fold with 100 μ M and around 150-fold with 1mM of AA relative to the vehicle control (Figure 3.2 A). aP2 protein levels also increased in response to AA treatment (Figure 3.2 A). PEPCK, adiponectin and Glut4 expression was not detectable at this early stage of differentiation (data not shown).

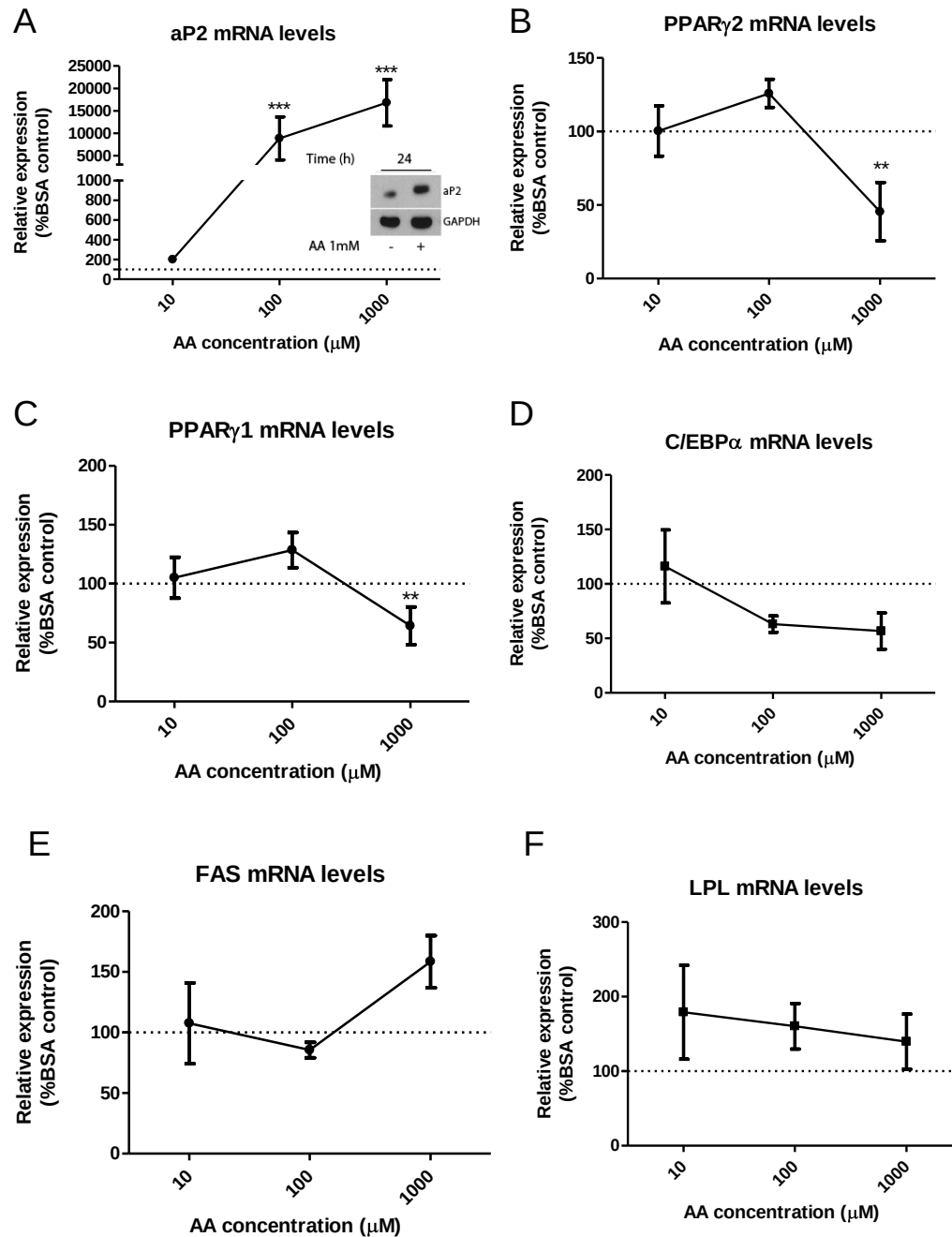


Figure 3.2: The effect of AA on the expression of late adipocyte differentiation markers in 3T3-L1 pre-adipocytes. A) aP2 mRNA and protein, B) PPAR γ 2, C) PPAR γ 1, D) C/EBP α , E) FAS and F) LPL expression profile was monitored in two-day post-confluent 3T3-L1 cells (D0). The pre-adipocytes were incubated for 24h with fatty acid-free BSA vehicle (set as 100%) or AA (10 μM , 100 μM or 1mM) in the presence of MDI. Total RNA was harvested and qRT-PCR was performed according to the Materials & Methods. Protein was also harvested at this time point, to monitor aP2 protein levels. Values shown are mean percentages of three independent experiments (n=3) and error bars represent $\pm\text{SEM}$. **P < 0.01, ***P<0.001. The western blot is a representative of at least three independent experiments.

Moreover, the expression of C/EBP β and C/EBP δ was assessed (Figure 3.3). These transcription factors are known to be expressed early in the differentiation program in response to the MDI induction and their expression declines during the adipocyte differentiation. AA treatment did not significantly change their expression with any dose used during the first 24h of the induction (Figure 3.3)

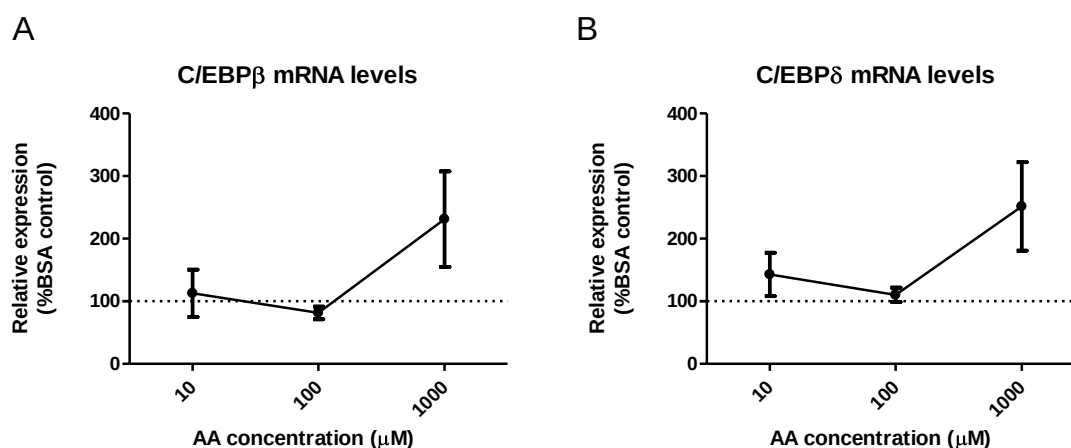


Figure 3.3: AA did not affect the expression of early differentiation markers in 3T3-L1 pre-adipocytes. 3T3-L1 cells (D0) were incubated for 24h with vehicle or AA (10 μ M, 100 μ M or 1mM) in the presence of MDI. qRT-PCR was performed for A) C/EBP β and B) C/EBP δ mRNA transcripts. Values shown are mean percentages of the respective vehicle controls (set as 100%) from three independent experiments (n=3). Error bars represent $\pm$ SEM.

3.2.1.2 Kinetics of the effect of AA on the adipocyte differentiation markers

To further elucidate the effect of AA on the early stages of the differentiation program, time course experiments were performed including time points earlier than 24h using 100 μ M AA. The expression of adipocyte differentiation markers was then assessed including SREBP1, a transcription factor with a role in stimulating the production of ligands for PPAR γ (Kim et al., 1998). PPAR γ 2, C/EBP α , FAS, LPL and SREBP1 levels were not affected by the AA treatment during the early differentiation procedure (Figure 3.4 B, C, D, E, F). However, aP2 was the only late adipocyte differentiation marker which was affected by AA treatment. The aP2 mRNA expression was dramatically up-regulated after 24h of treatment with AA, relative to the vehicle control, and not affected at earlier time points (Figure 3.4 A).

The same time courses were conducted to assess the expression of the early differentiation markers C/EBP β and δ . C/EBP β is expressed in three different protein isoforms: full-length C/EBP β (38-kDa) or liver-enriched activator protein* (LAP*), LAP (34kDa) and liver-enriched inhibitory protein (LIP, 21kDa) which lacks most of the transactivation domain and acts as a dominant negative isoform. C/EBP β protein levels increased after 4h of MDI treatment and continued to be expressed after 24h. In contrast, C/EBP δ was induced after 2h of the MDI treatment and the levels quickly declined after 4h of treatment (Figure 3.5 B, D). AA treatment did not alter C/EBP β and δ RNA or protein expression level relative to the vehicle control at most of the time points. One exception is the 12h time point where AA increased LAP and LIP protein levels that returned to basal levels after 24h of treatment (Figure 3.5).

In conclusion, 24h of AA treatment caused the up-regulation of aP2 mRNA levels which was a gene-specific event that did not affect the differentiation program.

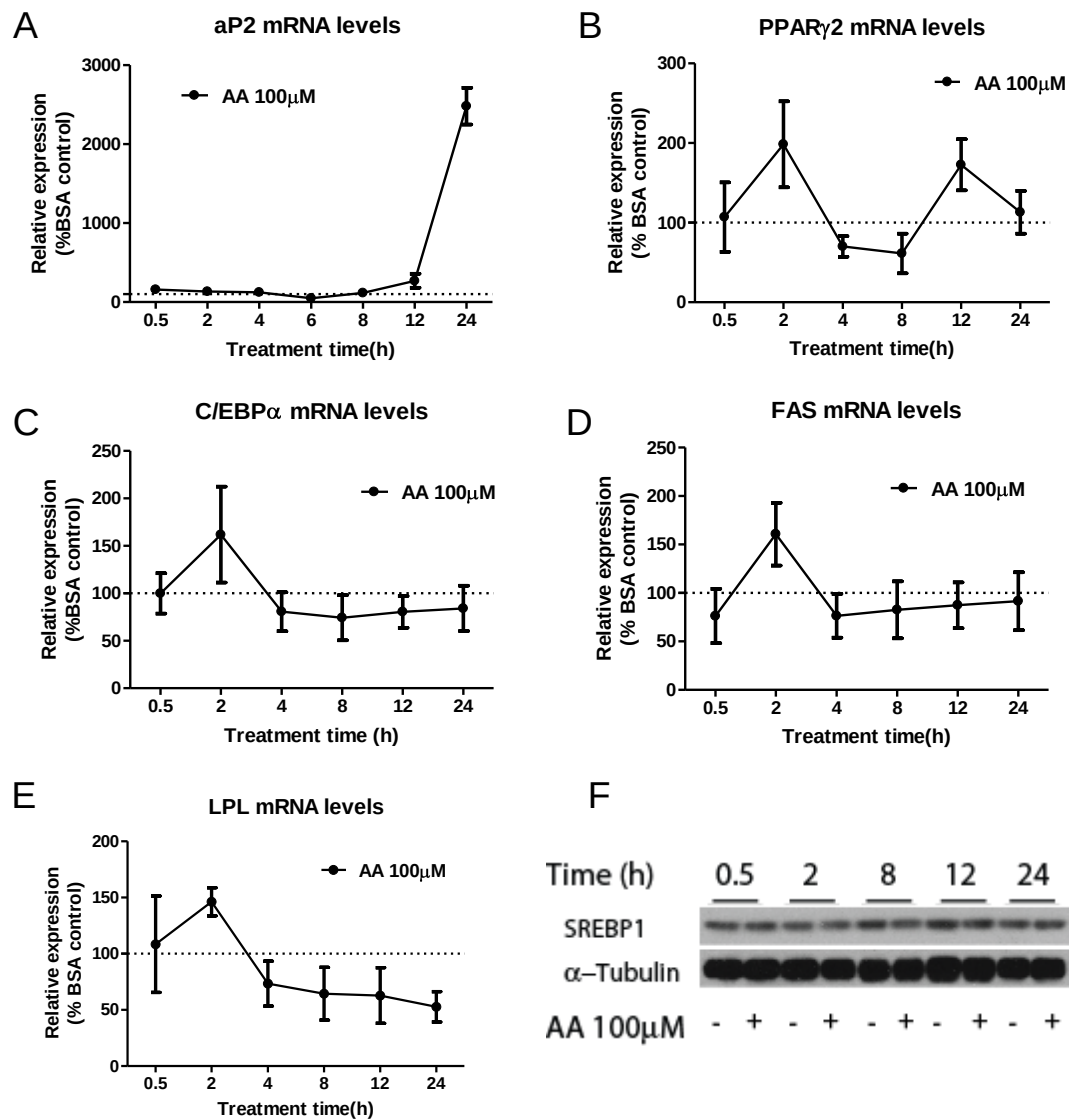


Figure 3.4: The effect of AA on aP2 expression occurred after 24h of treatment in 3T3-L1 pre-adipocytes. 3T3-L1 cells (D0) were treated with 100 μ M AA and total RNA or whole cell lysates were prepared at the indicated time points. A) aP2, B) PPAR γ 2, C) C/EBP α , D) FAS and E) LPL mRNA. F) Western blotting was performed using an anti-SREBP1 antibody. Values are all relative to the vehicle control treated cells (set as 100%). Data are presented as mean $\pm$ SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-test was performed for the 24h time point between the vehicle-treated and the AA-treated.

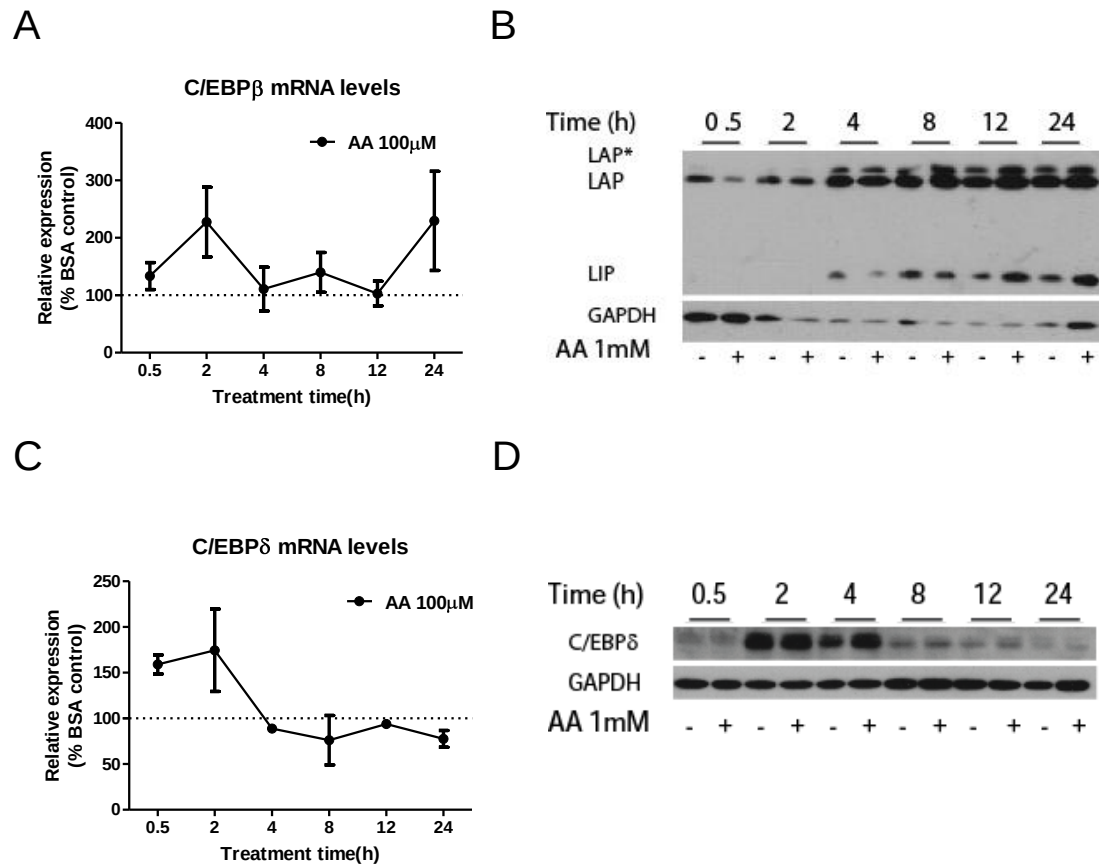


Figure 3.5: AA does not affect the kinetics of the early differentiation markers in 3T3-L1 pre-adipocytes. 3T3-L1 cells (D0) were treated with AA (100 μ M or 1mM) and total RNA or whole cell lysates were prepared at the indicated time points. A) C/EBP β mRNA levels and B) protein levels and C) C/EBP δ mRNA levels and D) protein levels. The graphs and the blots are representatives of two independent experiments. The error bars represent $\pm$ SD.

3.2.1.3 The effect of AA on aP2 expression is metabolite-, time- and condition-specific

To determine if the effect of AA on aP2 expression is specific to AA, 3T3-L1 cells (D0) were treated with EPA, DHA and LA PUFAs for 24h in the presence of MDI. EPA and DHA are both ω -3 PUFAs whereas LA is the precursor of AA and a major ω -6 PUFA. EPA and DHA are both implicated in adipocyte differentiation as they can act as PPAR γ ligands (Krey et al., 1997). All PUFAs were prepared using the same protocol described in Materials and Methods (Section 2.2.2). All the PUFAs tested were able to induce aP2 expression in comparison to the vehicle control. However, AA was the most potent PUFA inducing the expression of aP2 around 300-fold relative to the vehicle-control (set as 100). These data indicate that AA itself or an AA metabolite mediates this effect (Figure 3.6 A).

To further, characterise the time frame of the up-regulation of aP2 expression, 3T3-L1 cells were treated at earlier stages of the growth phase. Thus, cells were treated when pre-confluent, on day 0 of confluency, on day 1 of confluency or on day 2 of confluency with AA (1mM) for 24h in the presence of MDI (Figure 3.6 B). At all time points, AA up-regulated aP2 expression but was more potent when the treatment was initiated at two days post-confluency.

The role of factors included in the serum or in the differentiation cocktail on the up-regulation of aP2 expression by AA was investigated. A time course experiment was conducted with D0 cells without serum and in the presence of the differentiation cocktail (Figure 3.6 C). AA was able to induce aP2 expression even in the absence of serum. Next, 3T3-L1 cells (D0), in serum, were treated with AA in combination with the different components of the differentiation cocktail and aP2 expression was determined after 24h of treatment (Figure 3.6 D). IBMX appeared to be important for the effect of AA on aP2 expression as AA in the presence of serum and IBMX alone could up-regulate aP2 even higher than the MDI. In conclusion, increased cAMP levels,

achieved by the nonselective phosphodiesterase inhibitor (IBMX), might be important in mediating AA effect on aP2 expression.

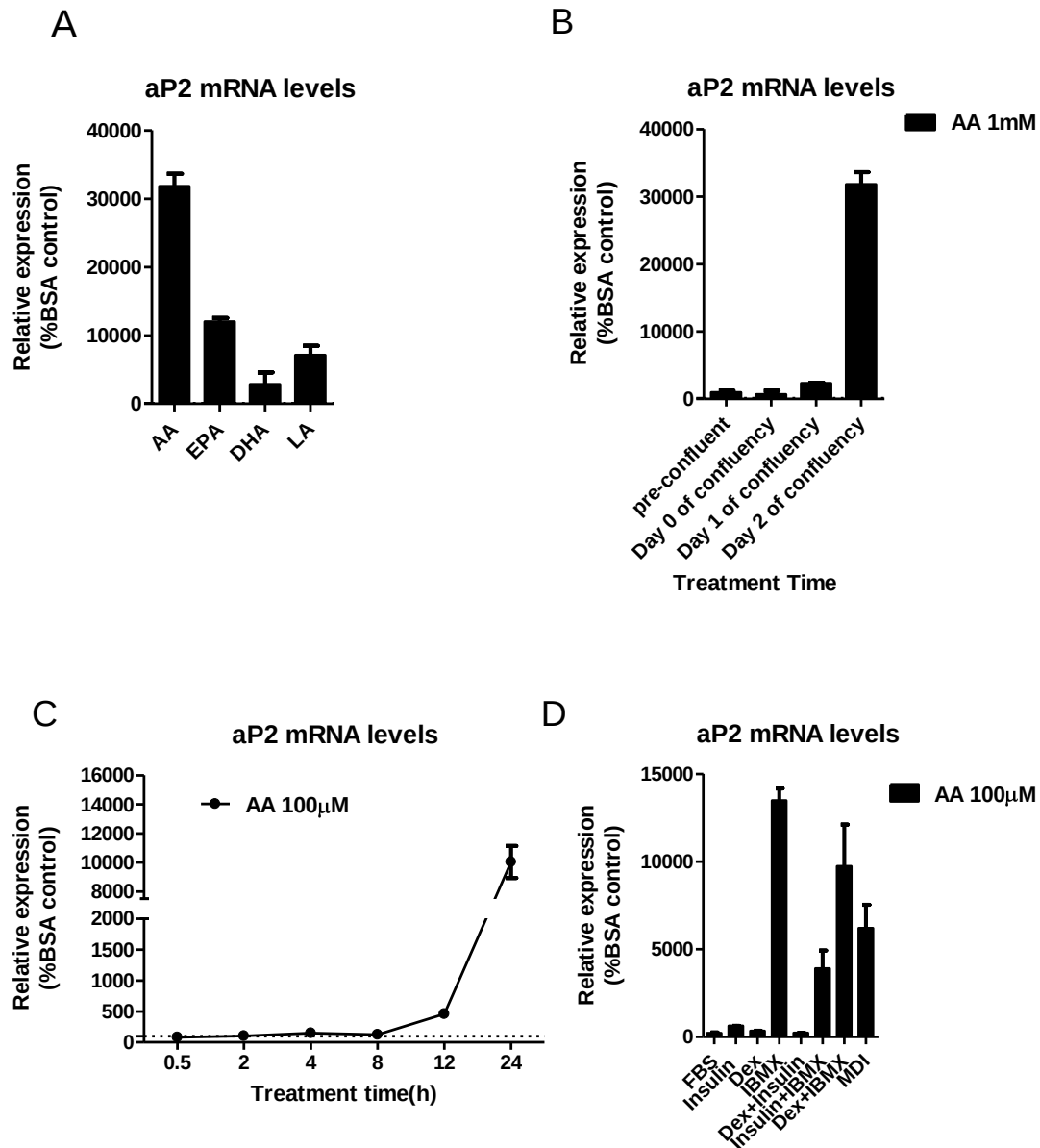


Figure 3.6: AA up-regulated aP2 expression in 3T3-L1 pre-adipocytes after two days of post-confluency in an IBMX-dependent manner. A) 3T3-L1 cells (D0) were treated with AA, EPA, DHA and LA (1mM) for 24h in the presence of MDI. All PUFAs used were conjugated with BSA as described in materials and methods. B) Pre-confluent, day 0 confluent, day 1 confluent and day 2 confluent 3T3-L1 cells were treated with AA (1mM) for 24h. C) 3T3-L1 cells (D0) were treated with AA (100 μ M) in the presence of MDI and in the absence of serum for 24h. D) 3T3-L1 cells (D0) were treated with AA (100 μ M) together with the different components of the MDI or combinations. Total RNA was isolated and qRT-PCR was performed. All relative aP2 mRNA levels are presented as percentages of the respective

vehicle controls (set as 100%). The graphs are representatives of two independent experiments. The error bars represent $\pm$ SD.

3.2.2 The effect of AA metabolites on aP2 expression

3.2.2.1 COXs mediate the effect of AA on aP2 expression

To examine if AA metabolites have a role in aP2 expression or this effect is due to AA itself, 3T3-L1 cells were treated with inhibitors targeting the three enzymes that can metabolise AA. Thus, 3T3-L1 cells were treated with indomethacin which is a general COX inhibitor, baicalein which is a 12/15 LOX inhibitor and 17-octadecynoic acid (17-ODYA) which is a cytochrome P450 epoxygenase inhibitor (Figure 3.7). The effect of AA on aP2 expression was significantly blocked by indomethacin but also from the selective COX-2 (SC-236) and COX-1 inhibitor (SC-560) (Figure 3.7 A, B). However, the AA effect was not blocked by the LOX or MOX inhibitors, indicating that PGs mediate AA on aP2 expression (Figure 3.7 C, D).

COX-2, COX-1 and eLOX3 expression was determined by qRT-PCR in the presence of increasing doses of AA for 24h (Figure 3.8). The expression of both COX isoforms was increased upon AA treatment in a dose-dependent manner (Figure 3.8 A, B). In contrast, eLOX3 expression, which is the only LOX expressed in 3T3-L1s, (Madsen et al., 2003) was not affected by any AA dose (Figure 3.8 C). These data together with the fact that baicalein did not inhibit AA effect, indicates that the metabolites generated through the LOX pathway are not involved in the regulation of aP2.

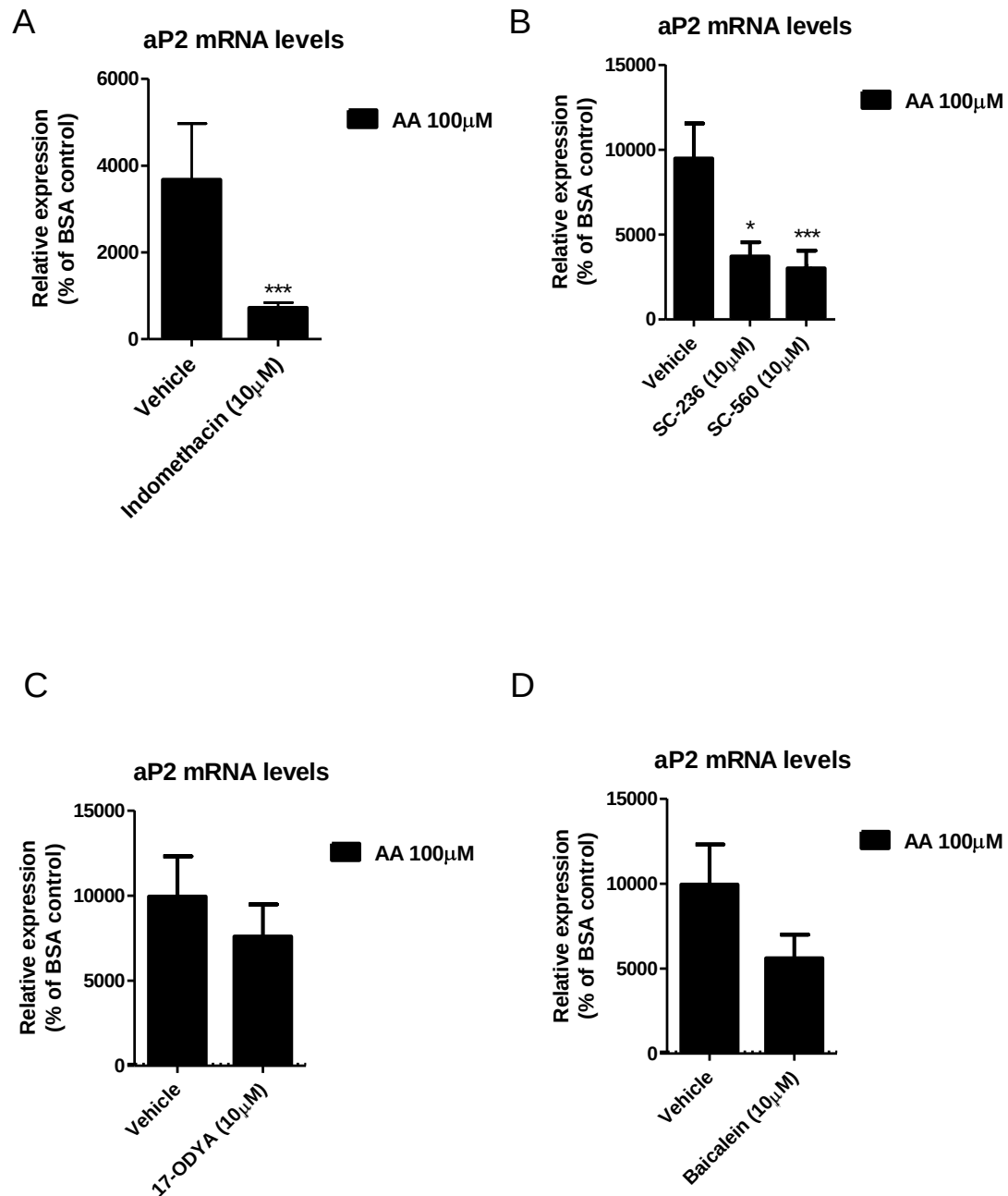


Figure 3.7: The effect of AA on aP2 expression was inhibited by indomethacin. A) 3T3-L1 cells (D0) were pre-treated with A) indomethacin (10µM), B) a selective COX-2 (SC-236, 10µM) and a selective COX-1 inhibitor (SC-560, 10µM), C) 17-ODYA (10 µM) or D) baicalein (10 µM) for 30 min prior to AA (100 µM) treatment for 24h. Total RNA was isolated and qRT-PCR was performed. All relative aP2 mRNA levels are presented as mean percentages of the respective vehicle controls (set as 100%) from three independent experiment (n=3). Error bars represent $\pm$ SEM. **P< 0.01, ***P<0.001.

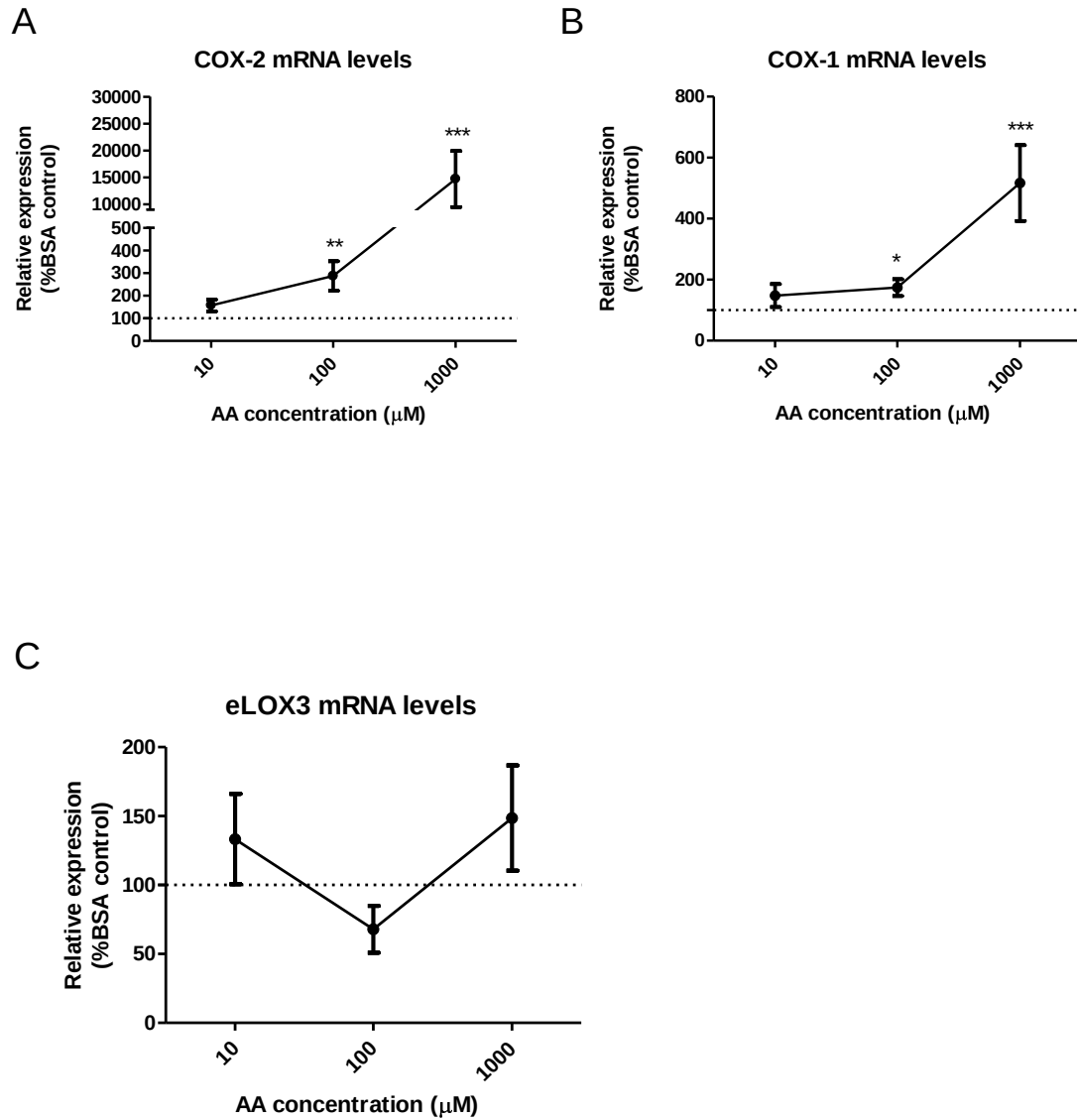


Figure 3.8: COXs expression is increased upon AA treatment in 3T3-L1 pre-adipocytes. 3T3-L1 cells (D0) were treated with 10μM, 100μM or 1mM of AA for 24h. Total RNA was isolated and qRT-PCR was performed for A) COX-2, B) COX-1 and C) eLOX3 mRNA transcripts. All relative mRNA levels are presented as mean percentages of the respective vehicle controls (set as 100%) from three independent experiment (n=3). Error bars represent $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

3.2.2.2 PGs mediate the effect of AA on aP2 expression

To further examine the role of PGs on aP2 expression, 3T3-L1 cells were treated with four different PGs for the first 24h of differentiation. A dose response experiment was carried out using the analogue of PGI₂, carbaprostacyclin (cPGI₂), PGF_{2α}, PGE₂ and 15-deoxy-Δ^{12,14} PGJ₂. From all the PGs tested, PGF_{2α} seems to have a similar effect to AA on aP2 expression (Figure 3.9, red line). Even at the lowest concentration (1nM) tested, PGF_{2α} was able to up-regulate aP2 almost 100-fold. PGE₂ had an effect after using 100nM (30-fold), cPGI₂ had an effect after using 1μM (40-fold) and at 10μM (150-fold) its effect was higher than PGF_{2α} at this dose. Finally, 15-deoxy-Δ^{12,14} PGJ₂ which is a direct PPARγ ligand, did not have any significant effect at all doses tested (Figure 3.9).

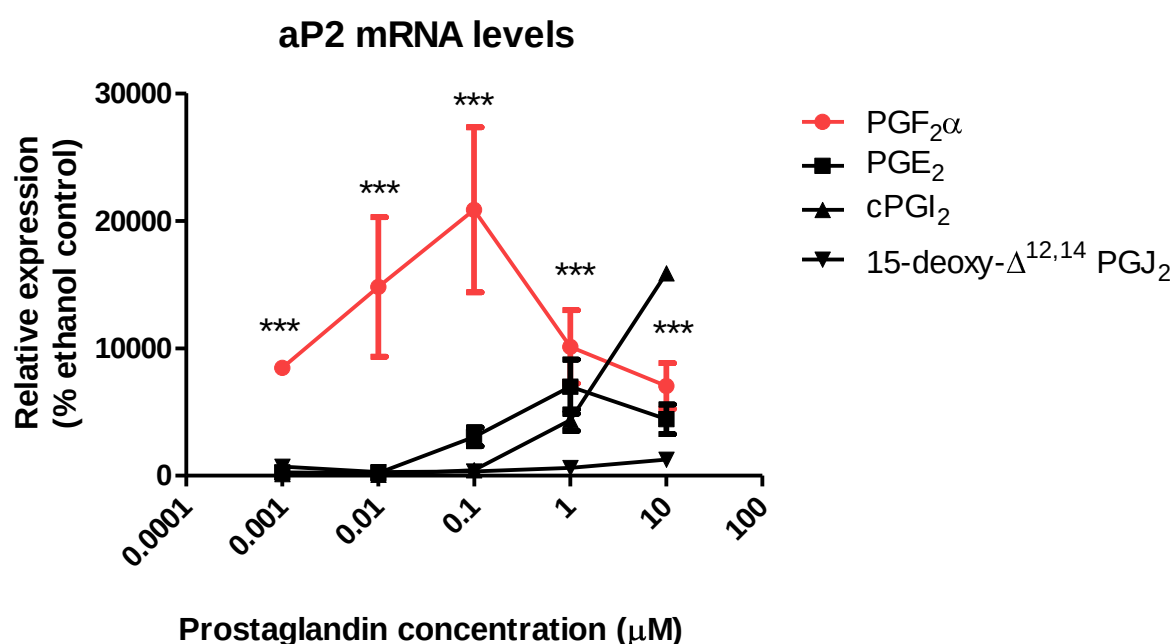
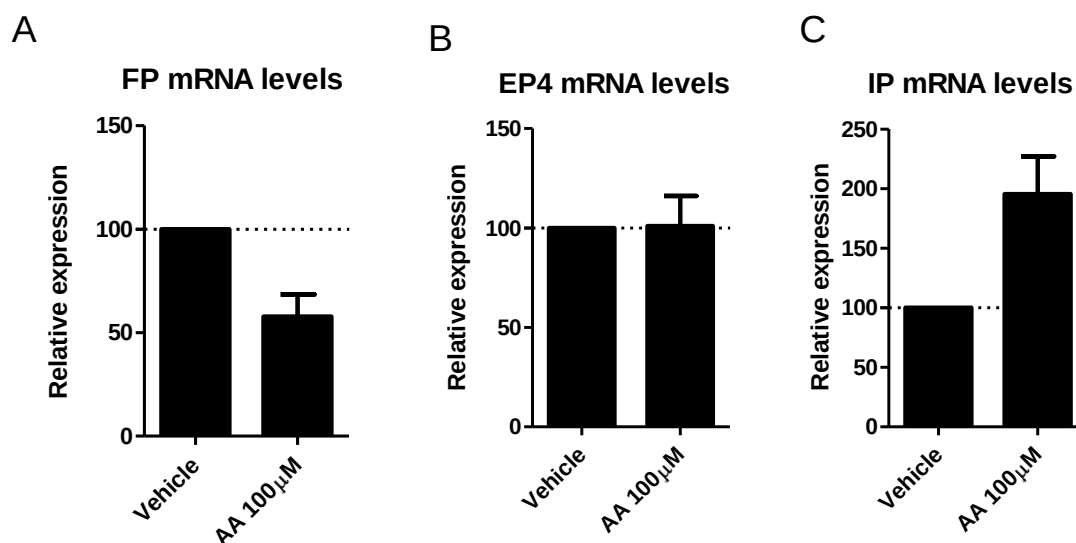


Figure 3.9: The effect of PGs on aP2 expression. 3T3-L1 cells (D0) were treated with 1nM, 10nM, 100μM, 1μM or 10μM of PGF_{2α}, PGE₂, cPGI₂ and 15-deoxy-Δ^{12,14} PGJ₂ for 24h in the presence of MDI. Total RNA was isolated and qRT-PCR was performed for aP2 mRNA transcripts. All relative mRNA levels are presented as mean percentages of the respective vehicle controls (set as 100%) from three independent experiment (n=3). Error bars represent ±SEM. ***P<0.001. The red line is the PGF_{2α} treatment.

Three of the four PGs tested exert their effects through binding to membrane GPCRs. In order to confirm that these GPCRs were expressed in pre-adipocytes and whether AA had any effect on their expression, qRT-PCR was conducted after a 24h of treatment with 100 μ M AA. The PGF_{2 α} receptor called FP receptor, one of the PGE₂ receptors the EP4 receptor and the PGI₂ called IP receptor were all expressed at this stage (Figure 3.10 A-C). AA did not have any significant effect on the expression of the receptors. However, there was a trend to up-regulate the expression of the IP receptor and down-regulate the expression of the FP receptor. The PGE₂ receptor EP1 was also expressed but the levels were low (Ct ~ 35) (Figure 3.10 D). The other PGE₂ receptors, EP2 and EP3- α - β - γ were not detected (Figure 3.10 F-H).



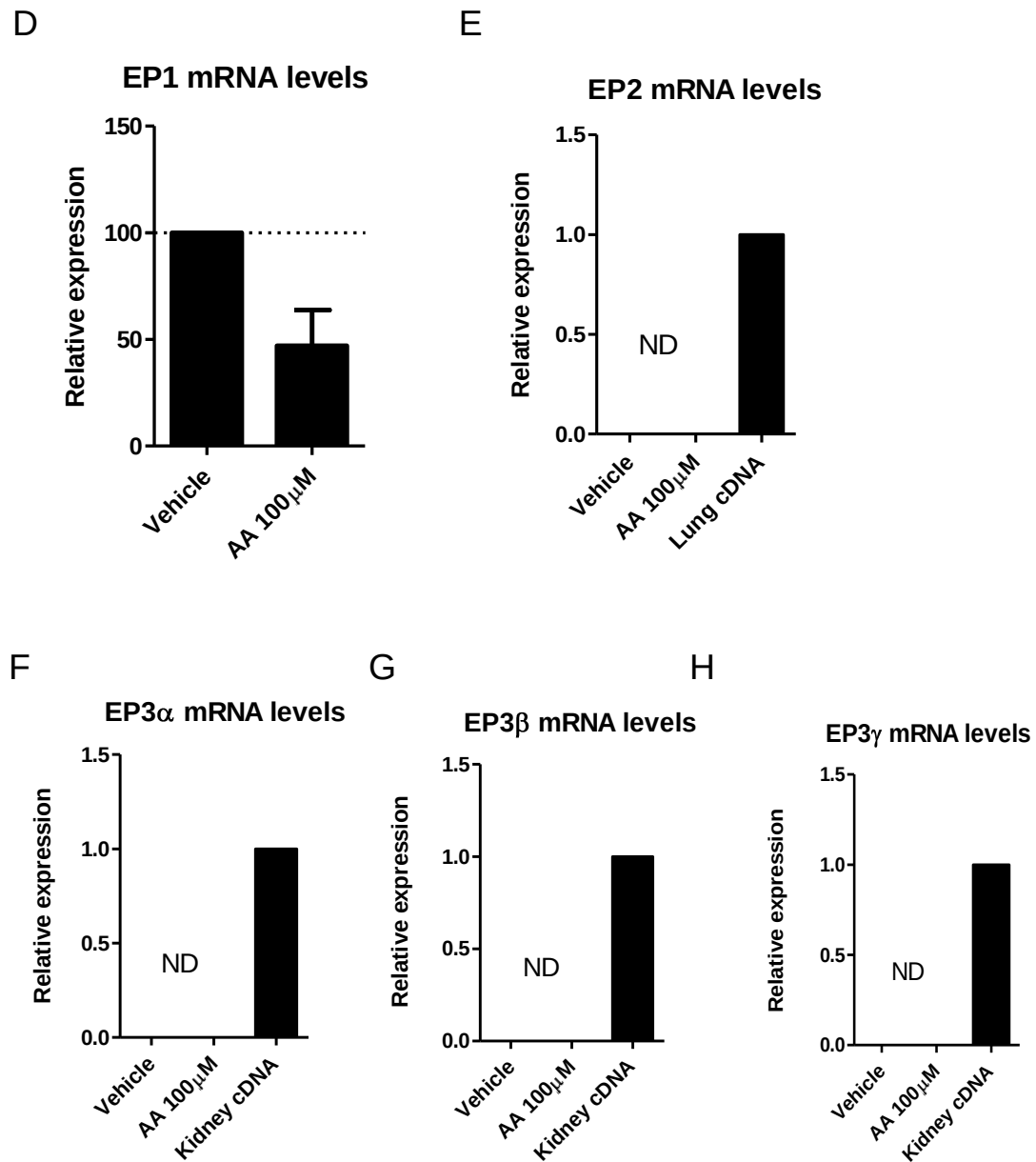


Figure 3.10: PG receptor expression after 24h treatment with AA. 3T3-L1 cells (D0) were treated with 100 μ M AA for 24h in the presence of MDI. Total RNA was extracted and subjected to RT-PCR analysis for the A) FP, B) EP4, C) IP, D) EP1, E) EP2, F) EP3 α , G) EP3 β and H) EP3 γ mRNA transcripts. The relative expression for the FP, EP4, IP and EP1 receptor is shown relative to the vehicle control (set as 100%). Mouse lung RNA was used as a positive control for EP2 and mouse kidney RNA was used for the three EP3 isoforms. Graphs are representatives of two independent experiments (n=2) and error bars represent $\pm$ SD. ND: Not detected.

3.2.3. The signalling pathway mediating the effect of AA on aP2 expression

3.2.3.1. ERK MAPK mediates the effect of AA on aP2 expression

The next question addressed was which signalling pathway AA triggers in order to regulate aP2 expression. For this reason a number of MAP kinases that could be activated by AA were assessed. 3T3-L1s cells were treated with AA over the first 24h of differentiation in the presence of MDI and samples were taken at different time points. p- c-Jun N-terminal kinase (p-JNK) and p-p38 MAPK were not present at high levels in pre-adipocytes and were not regulated by AA at this stage (Figure 3.11 A, B). Together with the 3T3-L1 lysates, a positive control was examined for p-JNK in order to identify the correct bands. As shown in figure 3.11 A, two bands appeared for the 3T3-L1 lysates, which were identical to the extracellular signal-regulated kinase (p-ERK) instead of p-JNK, whereas the correct bands appeared for the positive control. This indicates that this antibody might recognise p-ERK instead of p-JNK in this cell type. However, p-ERK was still expressed after 24h only in the presence of AA using either 100 μ M or 1mM AA (Figure 3.11 C, D).

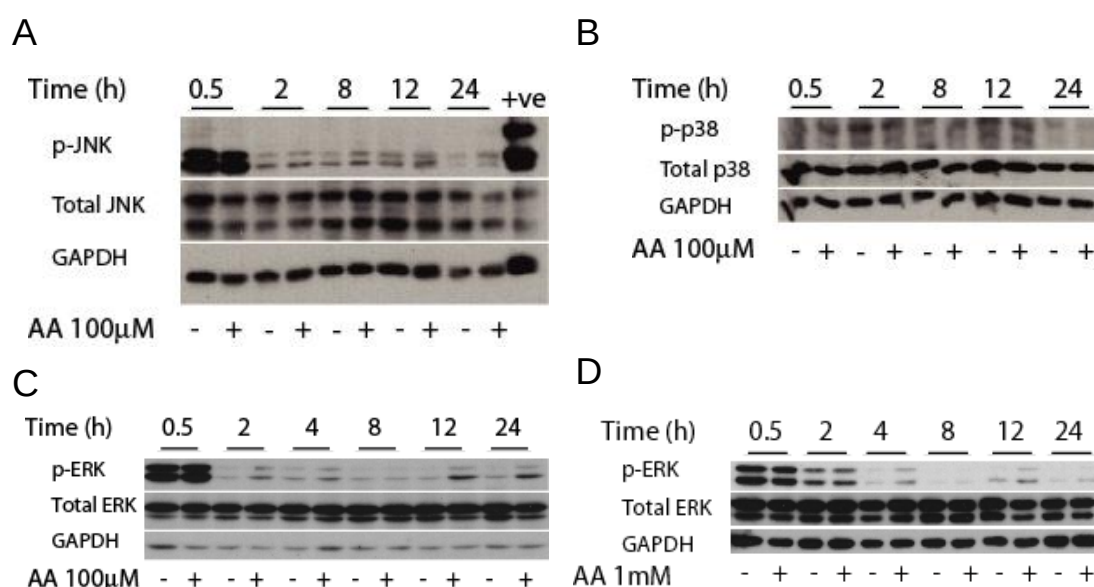


Figure 3.11: MAPKs expression after 24h of treatment with AA. 3T3-L1 cells (D0) were treated with 100 μ M AA for 24h in the presence of MDI. Whole cell lysates were extracted after 0.5, 2, 8, 12 and 24h of AA treatment and analysed by Western blotting with antibodies

against A) p-JNK, total JNK and GAPDH, B) p-p38, total p38 and GAPDH. Similarly, 3T3-L1 cells were treated with either 100 μ M AA (C) or 1mM AA (D) for 24h in the presence of MDI and whole cell protein extracts were analysed by Western blotting with antibodies against p-ERK, total- ERK and GAPDH. +ve: positive control.

To determine if this sustained activation of p-ERK has a role in the up-regulation of aP2 expression after 24h of AA treatment; 3T3-L1 cells were pre-treated with PD98059 which is a potent and selective inhibitor of MAP kinase kinase (also known as MAPK/ERK kinase or MEK kinase). It mediates its inhibitory properties by binding to the ERK-specific MAP kinase MEK, therefore preventing phosphorylation of ERK1/2 (p44/p42 MAPK) by MEK1/2. PD98059 significantly blocked AA effect on aP2 mRNA and protein levels, indicating that ERK activation was occurring after AA treatment and was mediating aP2 up-regulation (Figure 3.12 A, B).

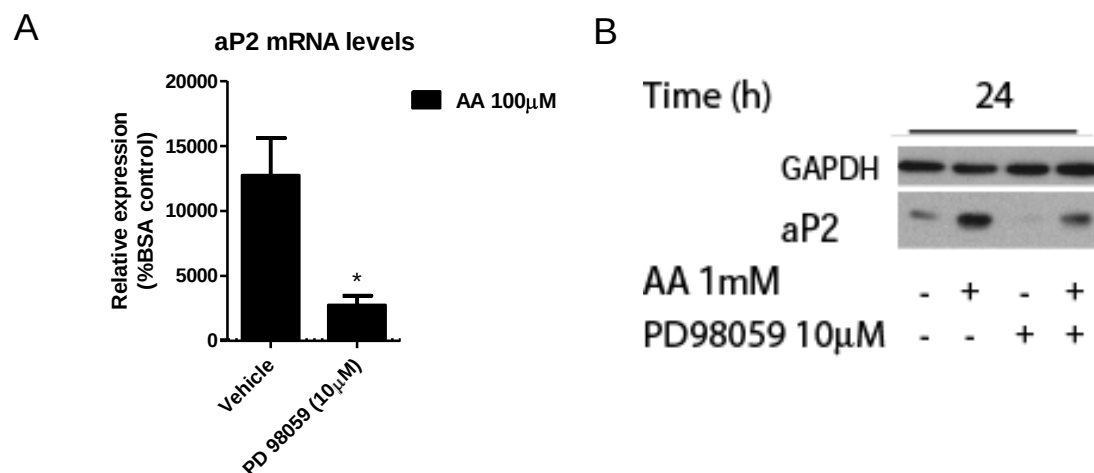


Figure 3.12: ERK mediates the effect of AA on aP2 expression. 3T3-L1 cells (D0) were pre-treated with PD98059 (10 μ M) for 30 min prior to AA (100 μ M or 1mM) treatment for 24h. Total RNA was extracted and subjected to qRT-PCR for aP2 mRNA transcript (A) or whole cell lysates were extracted and subjected to Western blotting using antibodies against aP2 and GAPDH (B). aP2 mRNA levels are presented as mean percentages of the respective vehicle controls (set as 100%) from three independent experiment (n=3). Error bars represent $\pm$ SEM. *P<0.05.

3.2.3.2. *ERK is activated immediately after AA stimulation*

To further elucidate the signalling pathway triggered by AA in pre-adipocytes to regulate aP2 expression, we studied the events taking place immediately after the addition of AA to the cells. To test the effect of MDI and/or serum on ERK activation, an experiment was conducted with the normal differentiation cocktail and samples were taken over a period of one hour. As expected, under these conditions the effect of AA was masked (Figure 3.13 A). Therefore, 3T3-L1 cells were serum starved overnight and then treated with AA (Figure 3.13 B). However, with serum-starved cells we reduced the AA concentration to 10 μ M as with 100 μ M AA the BSA used as the vehicle-control activated ERK after 10 or 15 min (Figure 3.13 B). Using this protocol, AA (10 μ M) significantly activated ERK after 5 minutes of treatment and its activation declined thereafter (Figure 3.13 C, D). Finally, a lower AA concentration was also tested (1 μ M) but the activation of ERK was very modest (Figure 3.13 E).

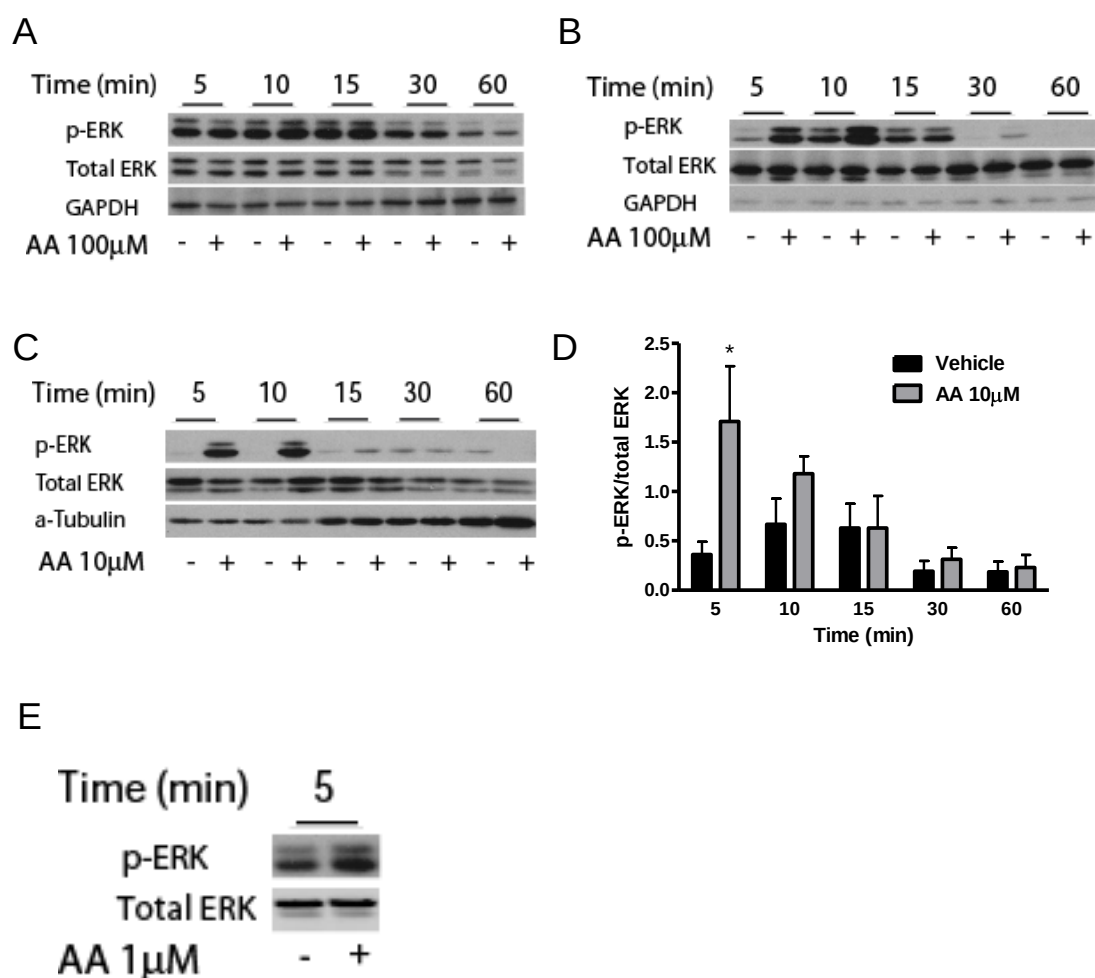


Figure 3.13: AA-induced ERK activation in 3T3-L1 pre-adipocytes. A) 3T3-L1 cells (D0) were treated with 100μM AA for 1h in the presence of MDI. Whole cell lysates were extracted after 5, 10, 15, 10 and 60min and analysed by Western blotting with antibodies against p-ERK, total- ERK and GAPDH. B) 3T3-L1 cells (D0) were serum starved overnight and treated with 100μM AA for 1h. Whole cell lysates were extracted after 5, 10, 15, 10 and 60min and analysed by Western blotting with antibodies against p-ERK, total- ERK and GAPDH. C) 3T3-L1 cells (D0) were serum starved overnight and lysates were extracted as described in B but were treated with 10μM AA for 1h. D) Densitometry results of three independent experiments using the protocol described in C. Densitometry was performed on scanned immunoblot images using the ImageJ software. Relative intensity for each sample was calculated by normalizing its p-ERK to the corresponding control total-ERK. *P< 0.05. E) The same protocol was used as described in C but cells were treated with 1μM AA for 5min.

3.2.3.3. ERK is activated immediately after $\text{PGF}_{2\alpha}$ and PGE_2 stimulation

As it was shown in section 3.2.4, the effect of AA on aP2 expression was blocked with either indomethacin or selective COX inhibitors indicating a role for PG production. Therefore, to address whether the effect of AA on ERK activation is PGs mediated, 3T3-L1s cells were serum starved overnight, pre-treated with indomethacin for 30min and stimulated for 5 min with AA (Figure 3.14 A). Indomethacin completely blocked ERK activation by AA, indicating that this signalling pathway is PGs-dependent. To determine which PGs activate ERK, serum-starved 3T3-L1s cells were treated with $\text{PGF}_{2\alpha}$, PGE_2 and cPGI_2 . These PGs were chosen as they can signal through membrane GPCRs and could potentially activate ERK. From the three PGs tested, $\text{PGF}_{2\alpha}$ and PGE_2 activated ERK after 5 min of treatment (Figure 3.14 B-D).

A full time course was conducted over an hour using $\text{PGF}_{2\alpha}$ and PGE_2 (Figure 3.14 E, F). Both PGs activated ERK after 10 min of treatment and the activation declined after one hour of treatment. However, p-ERK levels were above the basal levels even after one hour of $\text{PGF}_{2\alpha}$ treatment. It should be noted that the PG concentration used and able to activate ERK was 1000 times lower than the AA concentration that achieved the same activation. The high concentration of AA required to activate ERK suggests that an AA metabolite rather than AA itself, exerted this effect. In conclusion, $\text{PGF}_{2\alpha}$ and PGE_2 seem to be the PGs that activate ERK when cells are challenged with AA.

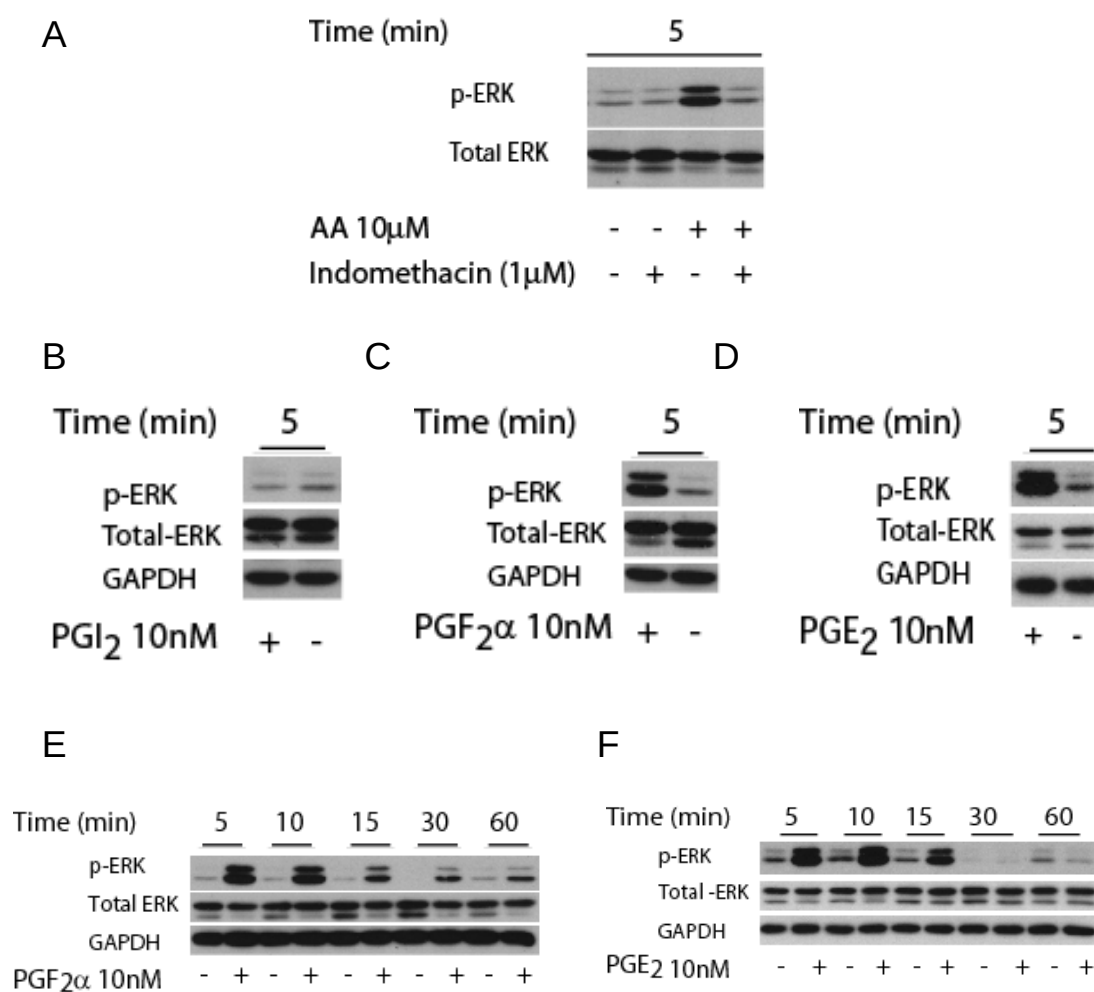


Figure 3.14: PGF₂ α and PGE₂ activate ERK in 3T3-L1 pre-adipocytes. A) Serum-starved 3T3-L1 cells (D0) were pre-treated with indomethacin (1 μ M) for 30min prior to 10 μ M AA stimulation for 5min. Whole cell lysates were extracted and probed with antibodies against p-ERK and total-ERK. B), C), D) Serum-starved cells were treated with PGI₂ (10nM), PGF₂ α (10nM) and PGE₂ (10nM) for 5min. Whole cell lysates were extracted and probed with antibodies against p-ERK, total-ERK and GAPDH. E) and F) Serum-starved cells were treated with PGF₂ α (10nM) and PGE₂ (10nM) for 1h. Whole cell lysates were extracted after 5, 10, 15, 30 and 60min of stimulation and probed with antibodies against p-ERK, total-ERK and GAPDH.

3.2.3.4. The FP receptor mediates AA-induced ERK activation

To determine which PG GPCRs are activated upon AA treatment and lead to activation of ERK, 3T3-L1 cells were pre-treated with selective antagonists against the PGs receptors expressed during the early stages of differentiation (see figure 3.10 A-C). Serum-starved cells were pre-treated for 30 min with AL 8810, a selective antagonist for FP receptor (PGF_{2α} receptor), L-161,982, selective for EP4 receptor (PGE₂ receptor), SC-51089, for selective EP1 receptor (PGE₂ receptor) and CAY- 10441, selective for IP receptor (PGI₂ receptor). Then the cells were treated with AA or the corresponding receptor agonists (as a positive control) for 5 min and the phosphorylation of ERK was assessed (Figure 3.15). Only the FP receptor antagonist (AL 8810) could significantly block the phosphorylation of ERK by AA, indicating that PGF_{2α} is the PG that mediates AA effect on the phosphorylation of ERK.

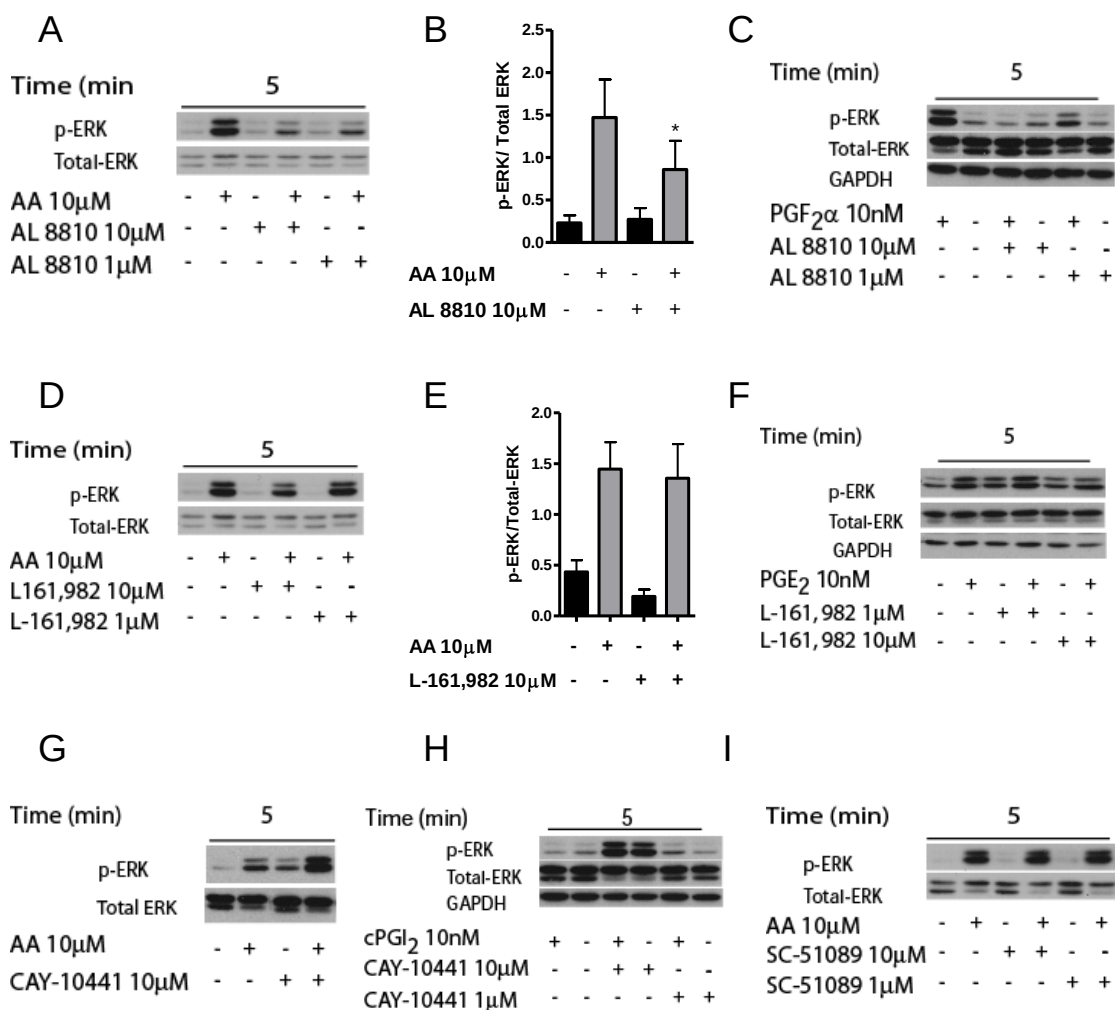


Figure 3.15: The FP receptor mediates the AA-induced ERK activation in 3T3-L1 pre-adipocytes. A), D), G) and I) 3T3-L1 cells (D0) were serum-starved overnight pre-treated with AL 8810 (10μM, 1μM), L-161,982 (10μM, 1μM), CAY-10441 (10μM) or SC-51089 (10μM, 1μM) for 30min prior to AA (10μM) 5min stimulation. Whole cell lysates were extracted and probed with antibodies against p-ERK and total-ERK. B) and E) Densitometry results of three independent experiments. p-ERK relative to total-ERK were calculated from experiments pre-treated with AL 8810 and L-161,982 respectively prior to the AA stimulation. C) 3T3-L1 cells (D0) were serum starved overnight pre-treated with AL 8810 (10μM, 1μM) for 30min prior to PGF_{2α} (10nM) 5min stimulation and p-ERK and total-ERK expression was determined. F) 3T3-L1 cells (D0) were serum starved overnight pre-treated with L-161,982 (10μM, 1μM) for 30min prior to PGE₂ (10nM) 5min stimulation and p-ERK and total-ERK expression was determined. H) 3T3-L1 cells (D0) were serum starved overnight pre-treated with CAY-10441 (10μM) for 30min prior to cPGI₂ (10nM) 5min stimulation and p-ERK and total-ERK expression was determined. *P<0.05. Error bars represent ±SEM.

3.2.3.5. The FP receptor mediates the effect of AA on aP2 expression

To address if there is any correlation between PG-induced ERK activation and aP2 expression at 24h, two days post-confluent cells were pre-treated with the antagonists against the FP receptor, the EP4 receptor and the IP receptor for 30 min prior to 24h AA treatment and aP2 mRNA levels were assessed. Only the FP receptor antagonist, AL 8810 could significantly block the ability of AA to increase aP2 mRNA levels whereas L-161,982 and CAY- 10441 had no effect on AA-dependent induction of aP2 (Figure 3.16 A-C).

To investigate the role of the FP receptor on aP2 expression, the FP receptor was depleted by a targeting siRNA (siFP). After 24h of treatment with AA, the up-regulation of aP2 expression was blocked in the presence of siFP (Figure 3.16 D). At the same time FP receptor mRNA levels were assessed and its expression was reduced around 80% (Figure 3.16 E). In addition, ERK activation was reduced in the absence of the FP receptor when AA was added for 24h (Figure 3.16 F). As a negative control, cells were transfected with the siRNA targeting the EP4 receptor (siEP4). Knocking down EP4 receptor (around 50%) did not block the ability of AA to induce aP2 expression but on the contrary enhanced aP2 expression around 2-fold, indicating that PGE₂ does not mediate the effect of AA (Figure 3.16 G, H). In conclusion, the AA-dependent induction of aP2 expression is mediated by PGF_{2α} which acts through its membrane GPCR, the FP receptor.

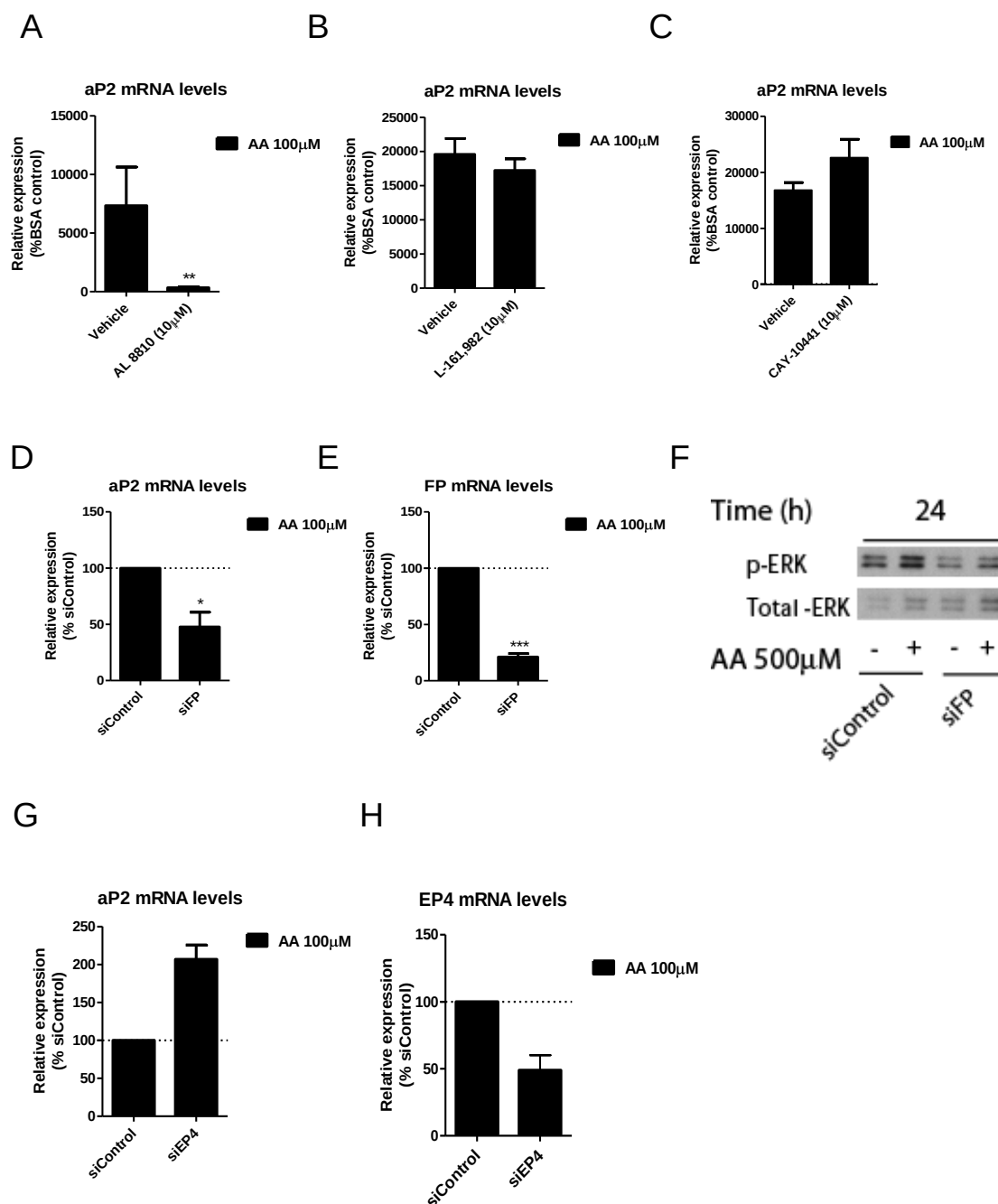


Figure 3.16: The FP receptor mediates the effect of AA on aP2 expression. A), B) and C) 3T3-L1 cells (D0) were pre-treated with AL 8810 (10 μ M), L-161,982 (10 μ M) or CAY-10441 (10 μ M) for 30 min prior to 100 μ M AA treatment for 24h. Total RNA was extracted and subjected to qRT-PCR for aP2 mRNA transcript. D) and E) 80% confluent 3T3-L1 cells were transfected with a non targeting control (siControl) siRNA or an siRNA targeting the FP receptor (siFP). On the third day after transfection, cells were treated with 100 μ M AA for 24h in the presence of MDI. Cells were harvested and total RNA was isolated and qRT-PCR was conducted for aP2 mRNA and FP receptor transcripts. F) Cells were transfected with siFP as described in D and E and treated with 500 μ M AA for 24h in the presence of MDI. Whole lysates were harvested and probed for p-ERK and total-ERK. G) and H) Cells were

transfected with an siRNA targeting EP4 receptor (siEP4) as described in D and E and aP2 and EP4 receptor transcripts were measured. All mRNA levels are presented as mean percentages of the respective vehicle controls from three independent experiment (n=3). Error bars represent $\pm$ SEM. *P<0.05, ***P<0.001.

3.2.3.6. $\text{PGF}_{2\alpha}$ induces aP2 expression

To confirm that $\text{PGF}_{2\alpha}$ is produced after AA treatment, 3T3-L1 cells were treated with increasing doses of AA in the presence of MDI and supernatants were collected and assayed for $\text{PGF}_{2\alpha}$ levels. $\text{PGF}_{2\alpha}$ was produced after AA treatment in a dose-dependent manner (Figure 3.17 A). A time-course experiment was performed using $\text{PGF}_{2\alpha}$ over a course of 24h and aP2 mRNA levels were measured (Figure 3.17 B). $\text{PGF}_{2\alpha}$ could increase aP2 mRNA expression in a time-dependent manner and the up-regulation was significant at the 12h and 24h time points. This correlated with the dose-dependent increase of the $\text{PGF}_{2\alpha}$ synthase in 3T3-L1 cells, aldoketo reductase (AKR)1B3 (Fujimori et al., 2010), when cells were challenged with AA (Figure 3.17 C). However, the FP receptor mRNA levels were not significantly affected by AA treatment (Figure 3.17 D).

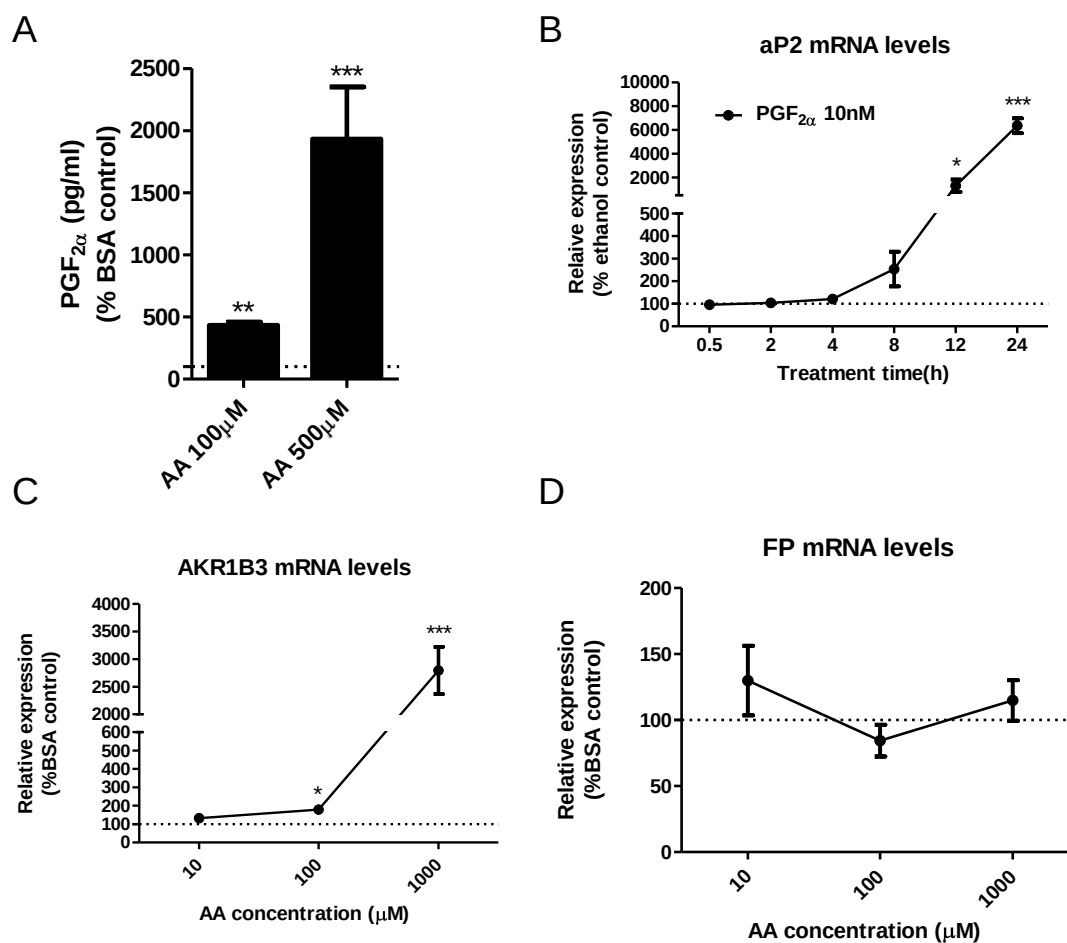


Figure 3.17: PGF_{2α} up-regulates aP2 expression. A) 3T3-L1 cells (D0) were treated with AA (100μM or 500μM) in the presence of MDI for 24h. Supernatants were collected and assessed for PGF_{2α} levels with EIA. B) 3T3-L1 cells (D0) were treated with PGF_{2α} (10nM) in the presence of MDI for 24h. Total RNA was isolated and aP2 transcript was measured. C) and D) 3T3-L1 cells (D0) were treated with increasing doses of AA for 24h in the presence of MDI. Total RNA was isolated and qRT-PCR was conducted for AKR1B3 and FP receptor transcripts. All relative mRNA levels are presented as mean percentages of the respective vehicle controls (set as 100%) from three independent experiment (n=3). Error bars represent ±SEM. *P< 0.05, ***P<0.001.

3.2.3.7. AA increases intracellular cAMP levels

The next step in elucidating the signalling pathway was to investigate which is the second messenger that the FP receptor triggers after AA treatment. The two most abundant second messengers are cAMP and Ca^{2+} . Thus, cAMP levels were measured using two days post-confluent 3T3-L1 cells, serum starved overnight and pre-treated with a phosphodiesterase inhibitor (IBMX) for 5 min prior to treatment with increasing doses of AA for 30min (Figure 3.18 A). AA stimulated an increase in cAMP levels with all doses tested, therefore the lowest of 10 μM was used for the following experiments. To determine if the rise of cAMP levels was PG-mediated, cells were pre-treated with indomethacin the general COX inhibitor (Figure 3.18 B). Indomethacin completely blocked the effect of AA on cAMP levels. Finally, to identify the PG GPCR mediating the increase in cAMP levels by AA, cells were pre-treated with the FP, EP4 and IP receptor selective antagonists prior to the AA stimulus. Only the EP4 receptor antagonist (L-161,982) could block the effect of AA on cAMP levels (Figure 3.18 C).

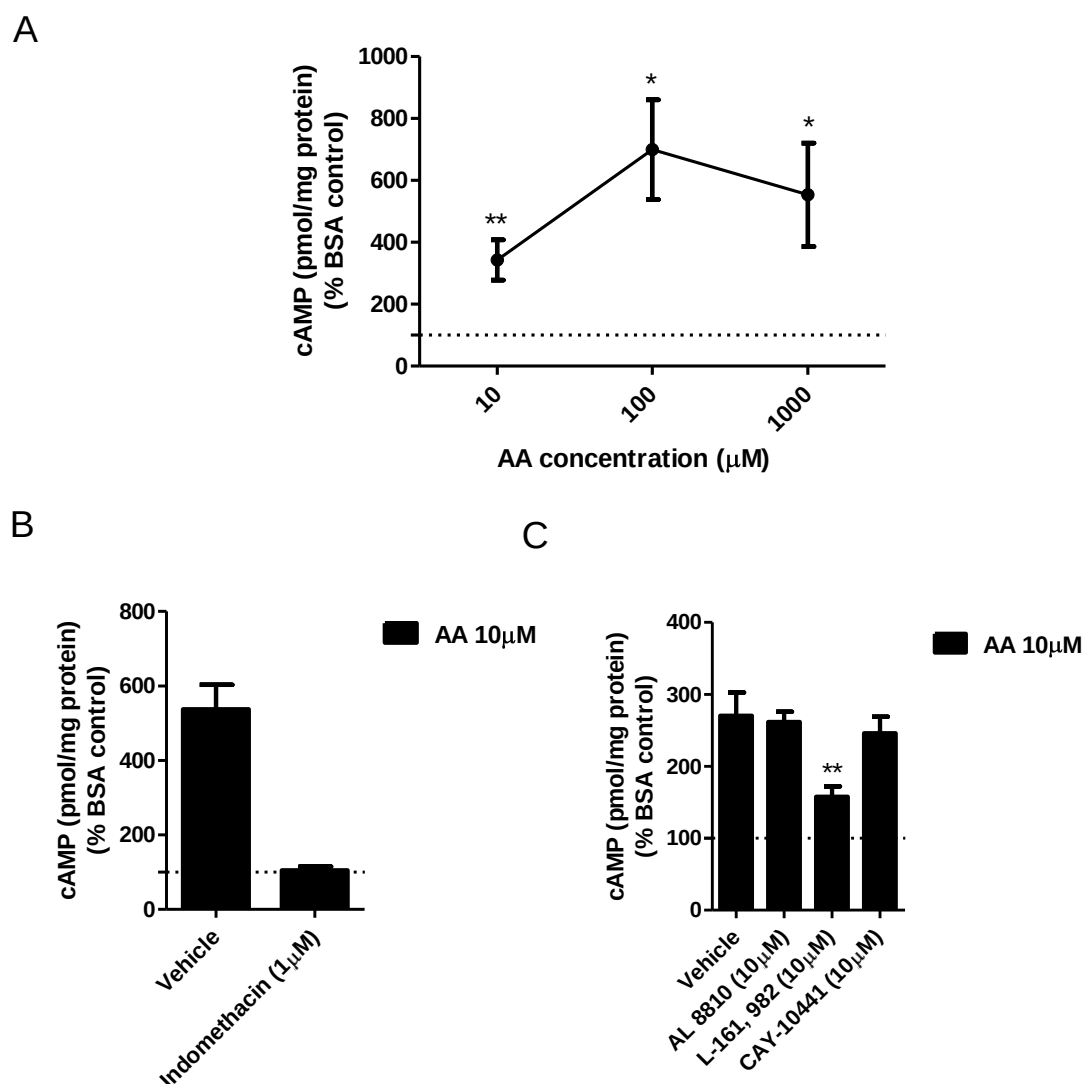


Figure 3.18: AA increases intracellular cAMP levels via the EP4 receptor in 3T3-L1 pre-adipocytes. A) 3T3-L1 cells (D0) were serum starved overnight, pre-treated, with IBMX for 5 min and incubated with AA (10μM, 100μM or 1mM) for 30 min. Cells lysed with 0.1 % HCl / Triton X- 100. cAMP levels were measured using an EIA system. All relative cAMP levels are presented as mean percentages of the respective vehicle controls (set as 100%) from three independent experiment (n=3). Error bars represent ±SEM. B) 3T3-L1 cells (D0) were serum starved overnight, pre-treated with indomethacin for 60 min before stimulation with 10μM AA for 30 min. Cells were lysed and cAMP was measured as described above. The graph is representative of one of the two experiments performed. C) 3T3-L1 cells were serum starved overnight and pre-treated with AL 8810 (10μM), L-161,982 (10μM) and CAY-10441 (10μM) for 60 min before stimulation with 10μM AA for 30 min. Cells were lysed and cAMP was measured as described above. All relative cAMP levels are presented as mean percentages of the respective vehicle controls (set as 100%) from three independent experiment (n=3). Error bars represent ±SEM. *P<0.05, **P<0.01.

3.2.3.8 AA increases intracellular calcium levels

To assess if AA triggers an increase of intracellular calcium levels, calcium mobilisation studies were performed. Two days post-confluent 3T3-L1 cells were loaded with the calcium dye Fluo-4 and treated with 10 μ M AA during live cell imaging. Addition of AA induced a rapid and robust increase in calcium in cultured 3T3-L1 cells (Figure 3.19 A). This effect was PG-mediated as pre-treatment with indomethacin completely blocked the mobilization of calcium stores (Figure 3.19 B). The FP receptor antagonist (AL 8810) was able to block the effect of AA on the intracellular calcium levels (Figure 3.19 C). This was consistent with previous reports illustrating that the FP receptor is coupled to G α q leading to an increase in intracellular calcium concentrations (Liu and Clipstone, 2007).

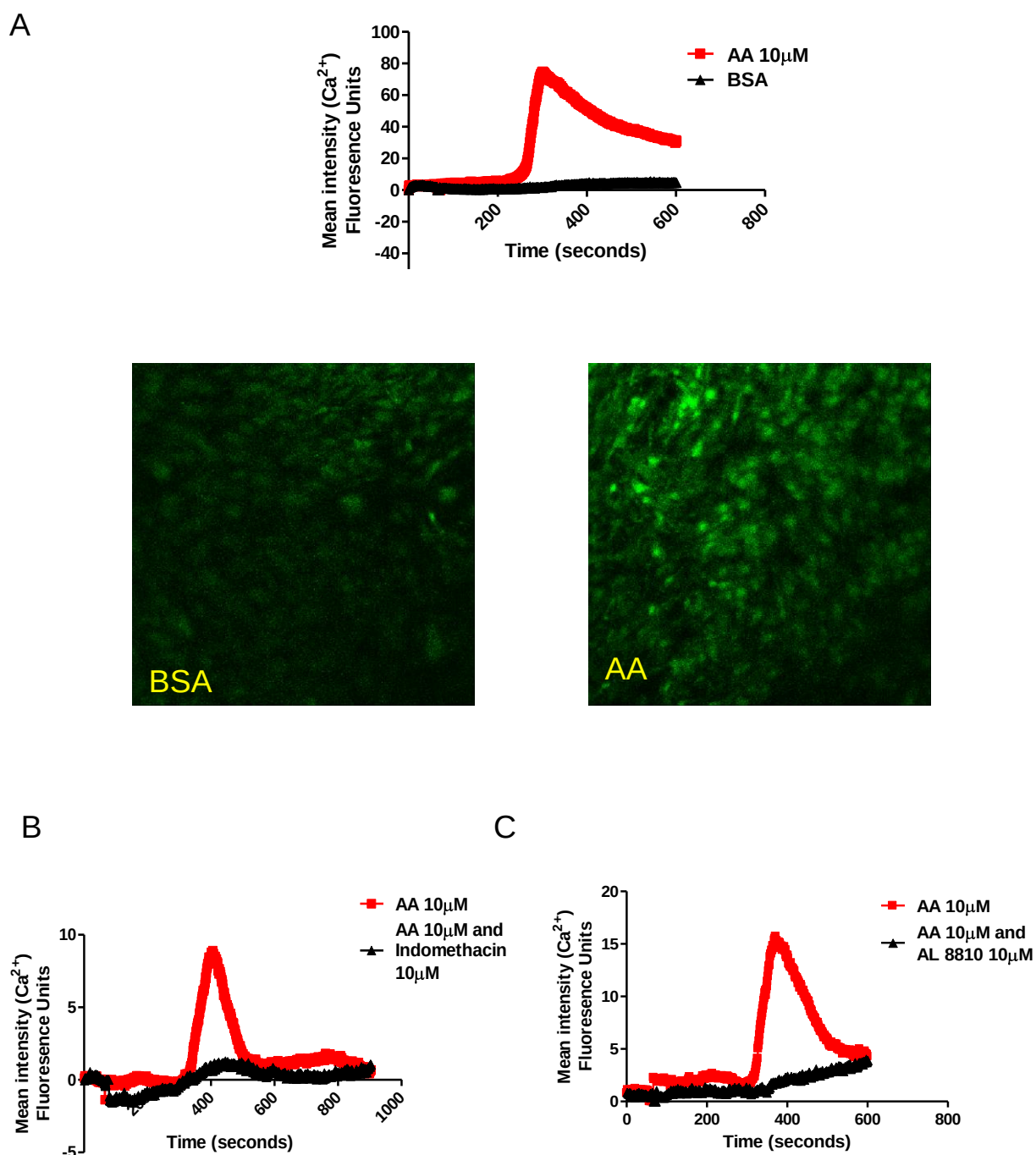


Figure 3.19: AA increases intracellular calcium levels via the FP receptor in 3T3-L1 pre-adipocytes. A) 3T3-L1 cells (D0) were loaded with calcium sensitive Fluo-4 before stimulation with 10 μM AA or BSA as vehicle control and calcium mobilisation was determined by confocal microscopy. Single image of calcium loaded 3T3-L1 cells after a vehicle or AA (10 μM) treatment. (B) Cells were pre-treated with 10 μM indomethacin for 30min before the stimulation with 10 μM AA. (C) Cells were pre-treated with 10 μM AL 8810 for 30min prior 10 μM AA stimulation. A representative experiment is shown ($n = 3$).

3.2.3.9 Calcium mediates the effect of AA on aP2 expression and ERK activation

To correlate the calcium response with the regulation of aP2 expression at 24h of treatment, cells were pre-treated with a membrane-permeant calcium chelator BAPTA-AM to establish an intracellular zero free calcium level. BAPTA-AM significantly blocked the up-regulation of aP2 expression by AA but also the activation of ERK (Figure 3.20 A, B, C). Finally, to determine if the calcium signalling influences the cAMP production, cells were pre-treated with BAPTA-AM prior to the AA stimulation and the cAMP intracellular levels were assessed (Figure 3.20 D). BAPTA-AM did not have any effect on cAMP production, indicating that these signalling pathways are independent. From these data can be concluded that calcium is the second messenger that mediates the effect of FP receptor leading to the increase in aP2 expression.

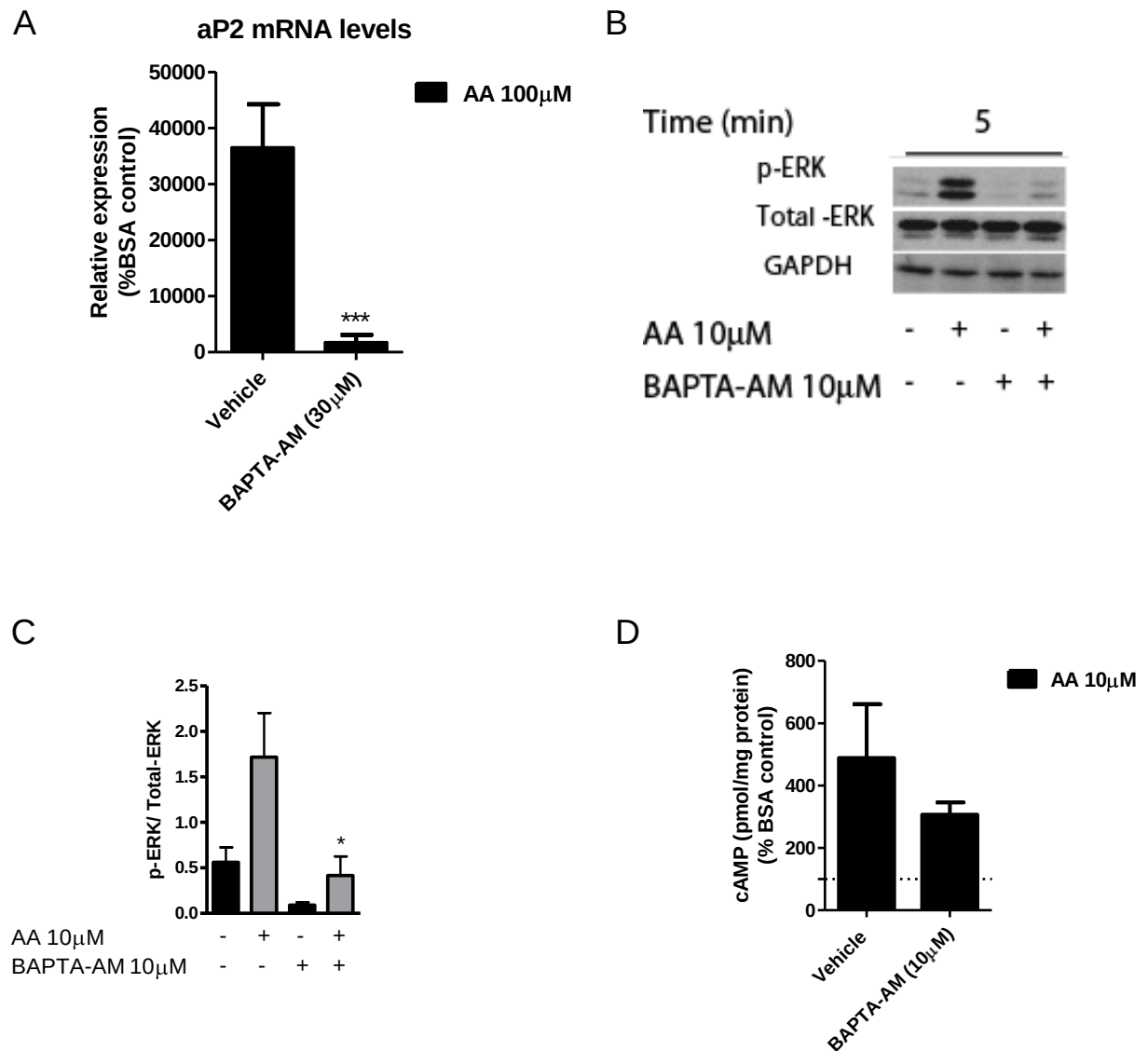


Figure 3.20: Calcium mediates the effect of AA on aP2 expression and ERK activation.

A) 3T3-L1 cells (D0) were pre-treated with BAPTA-AM (30 μ M), for 30 min prior to 100 μ M AA treatment for 24h. Total RNA was extracted and subjected to qRT-PCR for aP2 mRNA transcript. mRNA levels are presented as mean percentages of the respective vehicle controls (set as 100%) from three independent experiment (n=3). Error bars represent $\pm$ SEM. ***P<0.001. B) Serum-starved 3T3-L1 cells (D0) were pre-treated with BAPTA-AM (10 μ M) for 30min prior to AA (10 μ M) 5min stimulation. Whole cell lysates were extracted and analysed by Western blotting with antibodies against p-ERK, total-ERK and GAPDH. C) Densitometry results of three independent experiments performed as described in B. p-ERK relative to total-ERK were calculated and plotted. Error bars represent $\pm$ SEM. *P<0.05. D) Serum-starved 3T3-L1 cells (D0) were pre-treated BAPTA-AM (10 μ M) for 60 min before stimulation with 10 μ M AA for 30 min. The experiment was terminated and lysed with 0.1 % HCl / Triton X-100. cAMP was measured using an EIA system. All relative cAMP levels are presented as mean percentages of the respective vehicle controls (set as 100%).

3.2.3.10 PKC mediates the effect of AA on aP2 expression and ERK activation

Finally, it was addressed which protein kinase is important for the regulation of aP2 expression. Thus, 3T3-L1 cells were pre-treated with a PKA inhibitor (H89) for 30min prior to the 100 μ M AA treatment for 24h. H89 did not significantly block the effect of AA on aP2 expression (Figure 3.21 A). In addition, a PKC inhibitor (GF 109203X) was used to pre-treat the cells. The induction of aP2 and the ERK activation was completely blocked by pre-incubation with the PKC inhibitor prior to AA treatment. These data indicate that PKC is activated downstream of the FP receptor and the calcium release (Figure 3.21 C, D).

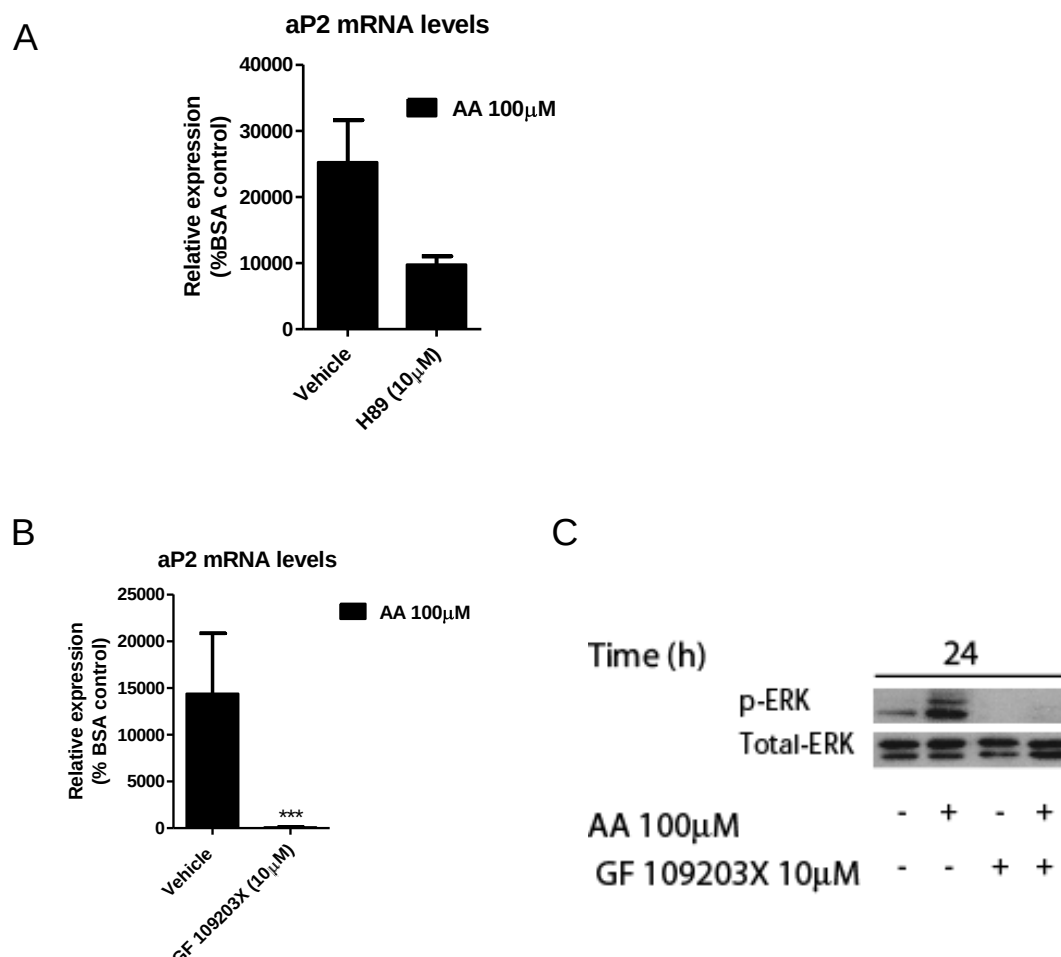


Figure 3.21. PKC mediates the effect of AA on aP2 expression and ERK activation. A) 3T3-L1 cells were pre-treated with H89 (10 μ M) for 30 min prior to 100 μ M AA treatment for 24h. Total RNA was extracted and subjected to qRT-PCR for aP2 mRNA transcript. mRNA

levels are presented as mean percentages of the respective vehicle controls (set as 100%) from three independent experiment (n=3). Error bars represent $\pm$ SEM. B) Cells were treated as described in A but they were pre-treated with GF109203X (10 μ M) for 30 min prior to 100 μ M AA treatment for 24h. mRNA levels are presented as mean percentages of the respective vehicle controls (set as 100%) from three independent experiment (n=3). Error bars represent $\pm$ SEM. ***P< 0.001. C) Cells were treated as described in B but whole cell lysates were subjected to western blotting and probed for antibodies against p-ERK and total-ERK. The blot shown is a representative of two independent experiments.

3.2.4 The effect of AA on aP2 expression is PPAR γ -mediated

3.2.4.1 PPARs and aP2 expression

aP2 is one of the best characterised gene targets of PPAR γ as it was the first PPAR γ target gene identified (Tontonoz et al, 1994a). aP2 has a 518bp enhancer element from -5.4kb to -4.9kb relative to the transcription start site, that drives gene expression in AT (Ross et. al., 1990). In this enhancer region two PPRE elements are present (Graves et. al., 1992), where the heterodimer PPAR γ 2/RXR α binds and drives adipose-specific aP2 expression (Tontonoz et. al., 1994a). For this reason it was hypothesised that PPAR γ could be the transcription factor mediating the effect of AA. However, PPAR δ and PPAR α are also expressed in 3T3-L1 cells and could also bind to PPRE elements present at the aP2 enhancer. To elucidate the role of PPARs on AA-dependent aP2 transcription, two days post-confluent 3T3-L1s were treated with rosiglitazone, GW7647 and GW501516 which are synthetic agonists selective for PPAR γ , PPAR α and PPAR δ , respectively. Rosiglitazone had the strongest effect of all the PPAR agonists, up-regulating aP2 expression around 20-fold. PPAR δ agonist also had a small effect around 2-fold and the PPAR α agonist did not have any effect (Figure 3.22 A, B, C).

To further elucidate the role of PPARs in mediating the effect of AA on aP2 expression, HEK293 cells were transfected with a reporter construct containing three copies of a functional PPRE or the aP2 reporter construct containing both promoter and enhancer sequences. The cells were then treated with AA (1mM) in the presence or absence of PPARs. The respective agonist for each PPAR was also included as a positive control. AA treatment induced the PPRE promoter activity in the presence of all PPARs (Figure 3.22 D, E, F). However, the effect of AA on the aP2 promoter construct was less pronounced than observed for the PPRE construct. AA enhanced reporter gene activity from both reporter constructs in the presence of PPAR γ (Figure 3.22 D, G), whereas only for the PPRE construct could AA increase its activity in the presence of PPAR α or PPAR δ (Figure 3.22 E, F).

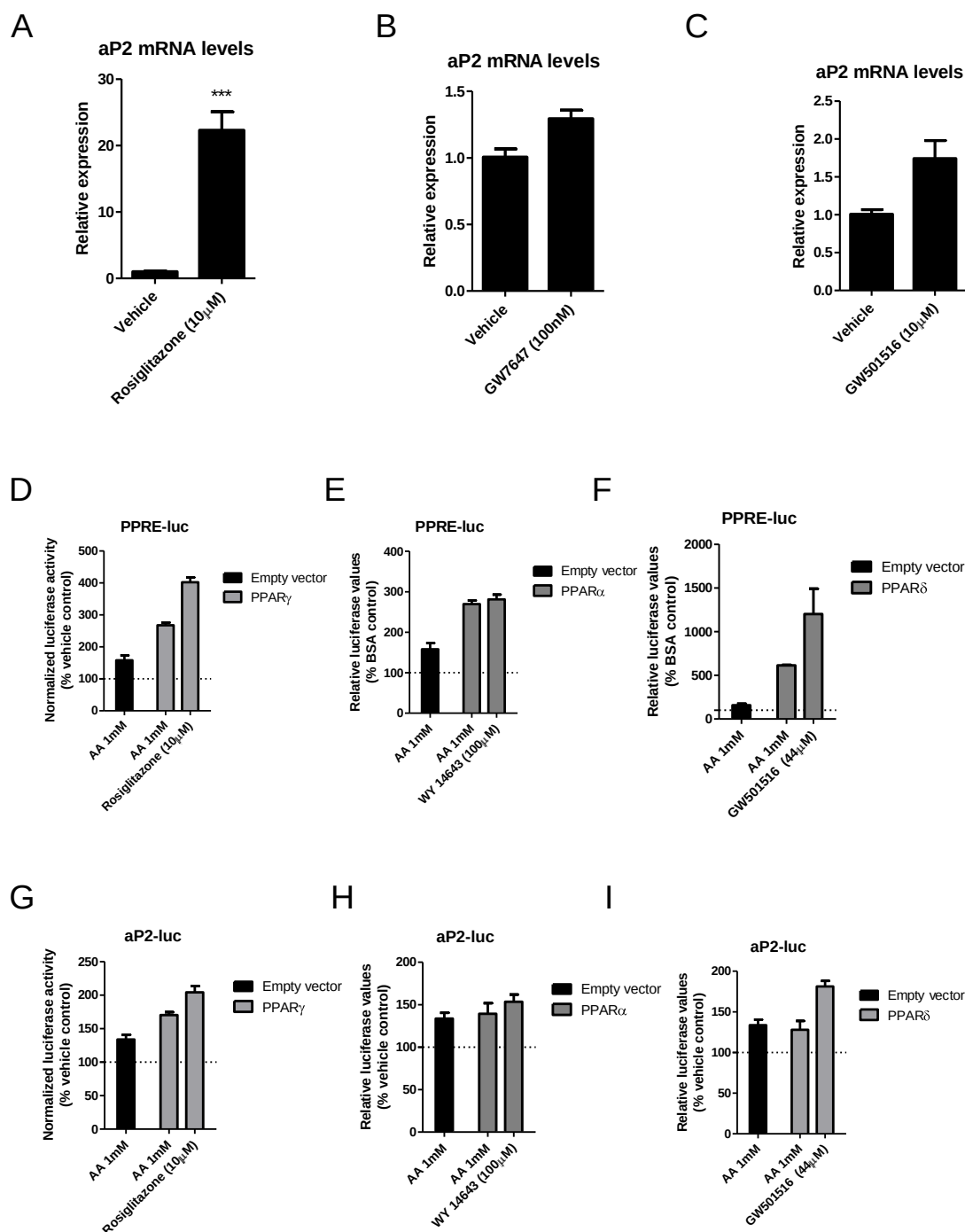


Figure 3.22: PPARs and aP2 expression. A) Two-day post-confluent 3T3-L1 cells were treated with a PPAR γ agonist (rosiglitazone, 10 μ M) for 24h in the presence of MDI. Total RNA was extracted and subjected to qRT-PCR. The plot represent the mean values of three independent experiment (n=3). Error bars represent $\pm$ SEM. ***P< 0.001. B) and C) 3T3-L1 cells were treated with a PPAR α agonist (GW 7647, 100nM) or a PPAR δ agonist (GW 501516, 10 μ M), respectively for 24h in the presence of MDI. Total RNA was extracted and subjected to qRT-PCR. The plots are representative of two independent experiments (n=2), Error bars represent $\pm$ SD. D), E) F) HEK293 cells were transfected using the calcium phosphate method with the 3x-PPRE luciferase reporter construct (PPRE-luc) and a renilla

construct together with the expression vector for PPAR γ , PPAR α and PPAR δ respectively or an empty vector. The next day, cells were treated with AA (1mM) or the respective PPAR agonist i.e a PPAR γ agonist (rosiglitazone, 10 μ M), a PPAR α agonist (WY14643, 100 μ M) and a PPAR δ agonist (GW 501516, 44 μ M) for 24h. The cells were harvested and the luciferase activity was measured. Activation of the luciferase reporter is expressed relative to the vehicle control (set as 100) after normalisation for the renilla luciferase activity. The plots are representative of two experiments (n=2). Error bars represent $\pm$ SD. G), H) and I) HEK293 cells were transfected and treated as described above but this time with the aP2-luciferase (aP2-luc) was transfected. The plots are representative of two experiments (n=2). Error bars represent $\pm$ SD.

3.2.4.2 PPAR γ absence prevents AA-dependent induction of aP2

Since the selective agonists for PPAR γ and PPAR δ were able to up-regulate aP2 expression and since they were the PPARs that enhanced the effect of AA on both promoter constructs, it was hypothesised that these PPARs most likely mediate AA effect. To test this hypothesis, 3T3-L1s were pre-treated with a PPAR γ (GW 9662) or a PPAR δ selective antagonist (GSK 0660) for 30min before AA (100 μ M) treatment for 24h (Figure 3.23 A, B). Only the PPAR γ antagonist could significantly block the ability of AA to induce aP2 expression (Figure 3.23 A), indicating that PPAR γ is the nuclear receptor mediating the effect of AA.

To further confirm this observation, PPAR γ was stably silenced in 3T3-L1 cells. A cell line expressing shPPAR γ displayed a reduction of PPAR γ expression more than 90% compared to cells expressing a non-specific shRNA (shScrambled) (Figure 3.23 E). Two days post-confluent shPPAR γ and shScrambled 3T3-L1 cells were treated with AA (100 μ M) for 24h and the aP2 mRNA and protein levels were assessed. The effect of AA on aP2 expression was significantly blocked in the absence of PPAR γ , indicating that PPAR γ is essential for this up-regulation (Figure 3.23 C, D). As a positive control the same experiment was repeated but the cells were treated with the PPAR γ selective agonist, rosiglitazone. The ability of rosiglitazone to induce aP2 levels was also prevented in the absence of PPAR γ (Figure 3.23 E). Finally, shPPAR γ and shScrambled expressing cell lines were treated with

PGF_{2α} (10nM) (Figure 3.23 F). The effect of PGF_{2α} on aP2 levels also prevented by the absence of PPAR_γ.

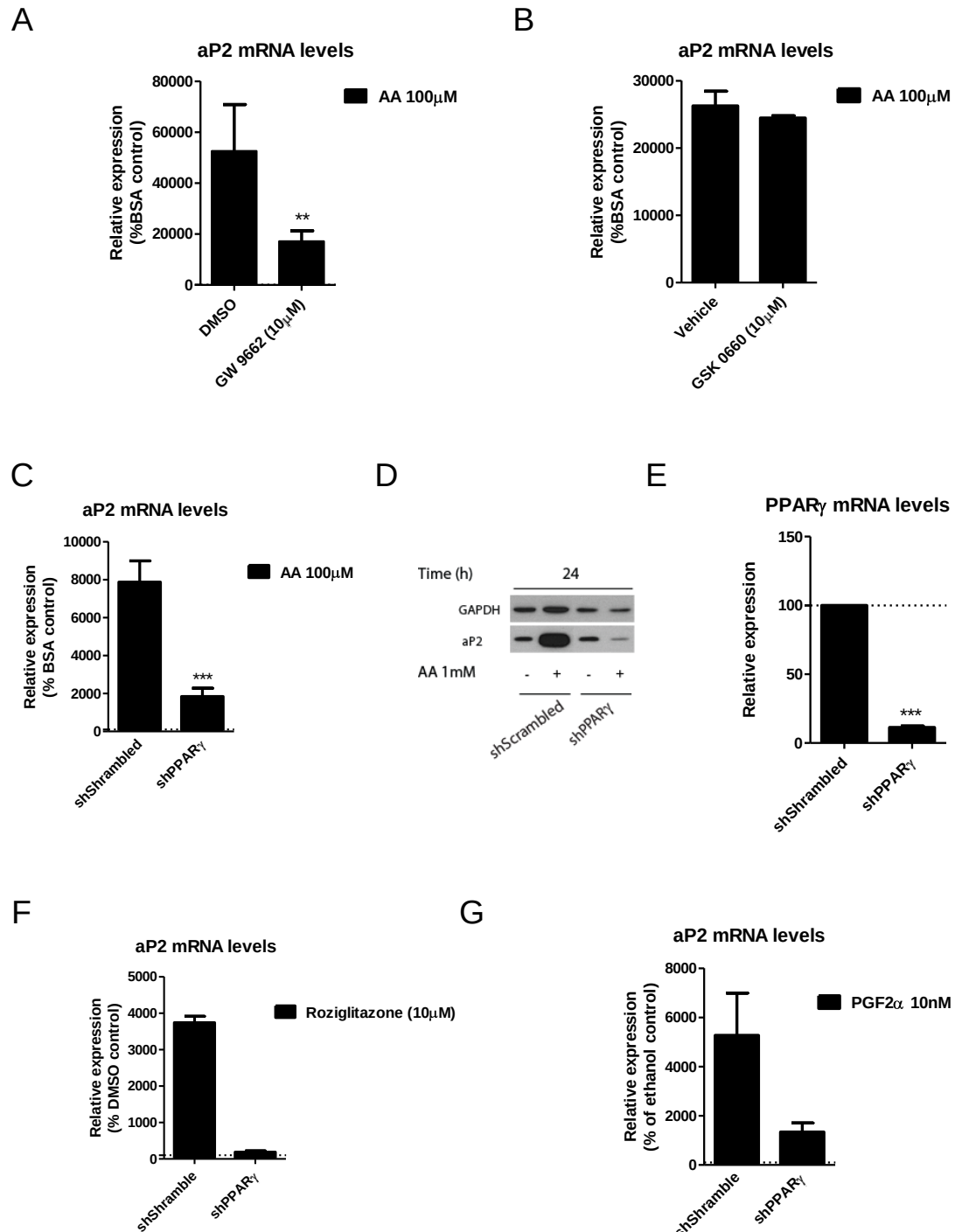


Figure 3.23: PPAR_γ mediates the effect of AA on aP2 expression. A) 3T3-L1 cells (D0) were pre-treated with a selective PPAR_γ antagonist (GW 9662, 10_μM) for 30min before the AA (100_μM) treatment in the presence of MDI for 24h. Total RNA was isolated and analyzed using qRT-PCR. Relative aP2 mRNA levels were expressed as percentage to the respective negative control stimulated with vehicle only (set as 100%). The plot represent the mean values of three independent experiment (n=3). Error bars represent $\pm$ SEM. *** P< 0.001. B)

As in A but cells pre-treated with a selective PPAR δ antagonist (GSK 0660, 10 μ M). The plot is a representative of two independent experiments (n=2). Error bars represent $\pm$ SD C) Two days post-confluent PPAR γ knockdown 3T3-L1 cells (shPPAR γ) together with the negative control cells (shScrambled), were treated with 100 μ M AA for 24h in the presence of MDI. The total RNA of the cells was isolated and analyzed using qRT-PCR. Relative aP2 mRNA levels were expressed as percentage to the respective negative control stimulated with vehicle only (set as 100%). The plot represents the mean values of three independent experiment (n=3). Error bars represent $\pm$ SEM. *** P< 0.001. D) As in C but whole cell extracts were isolated and subjected to western blotting using antibodies against aP2 and GAPDH. E) As in C. Relative PPAR γ mRNA levels in the basal conditions of the shPPAR γ cells were expressed as percentage to the respective negative control (shScrambled). The plot represent the mean values of three independent experiments (n=3). Error bars represent $\pm$ SEM. *** P< 0.001. F) and G) As in C but cells were treated with rosiglitazone (10 μ M) or PGF2 α (10nM) respectively, for 24h in the presence of MDI. Relative aP2 mRNA levels were expressed as percentage to the respective negative control stimulated with vehicle only. The plots are representatives of two independent experiments (n=2). Error bars represent $\pm$ SD.

3.2.4.3 PPAR γ expression enhances the AA-dependent induction of aP2 expression

An alternative approach was used to address the role of PPAR γ in the AA-mediated aP2 induction. PPAR γ was overexpressed in wild type 3T3-L1 cells and endogenous aP2 expression was measured after 24h of AA treatment. PPAR γ was expressed around 15-fold more than the empty vector control in basal conditions (Figure 3.24 A). aP2 mRNA levels were up-regulated in the presence of PPAR γ and there was a pronounced increase in the presence of AA (around 4-fold) (Figure 3.24 B).

These data indicate that PPAR γ is the PPAR that mediates the effect of AA on aP2 expression. The next step was to determine if this regulation is direct or indirect. For this reason ChIP experiments were conducted using a PPAR γ antibody (data not shown) and primers designed to amplify the PPRES on aP2 enhancer. None of the experiments performed yielded any enrichment of the target sequences with the PPAR γ antibody and the reason might lie in the decrease of PPAR γ protein levels upon AA treatment (Figure 3.24 C).

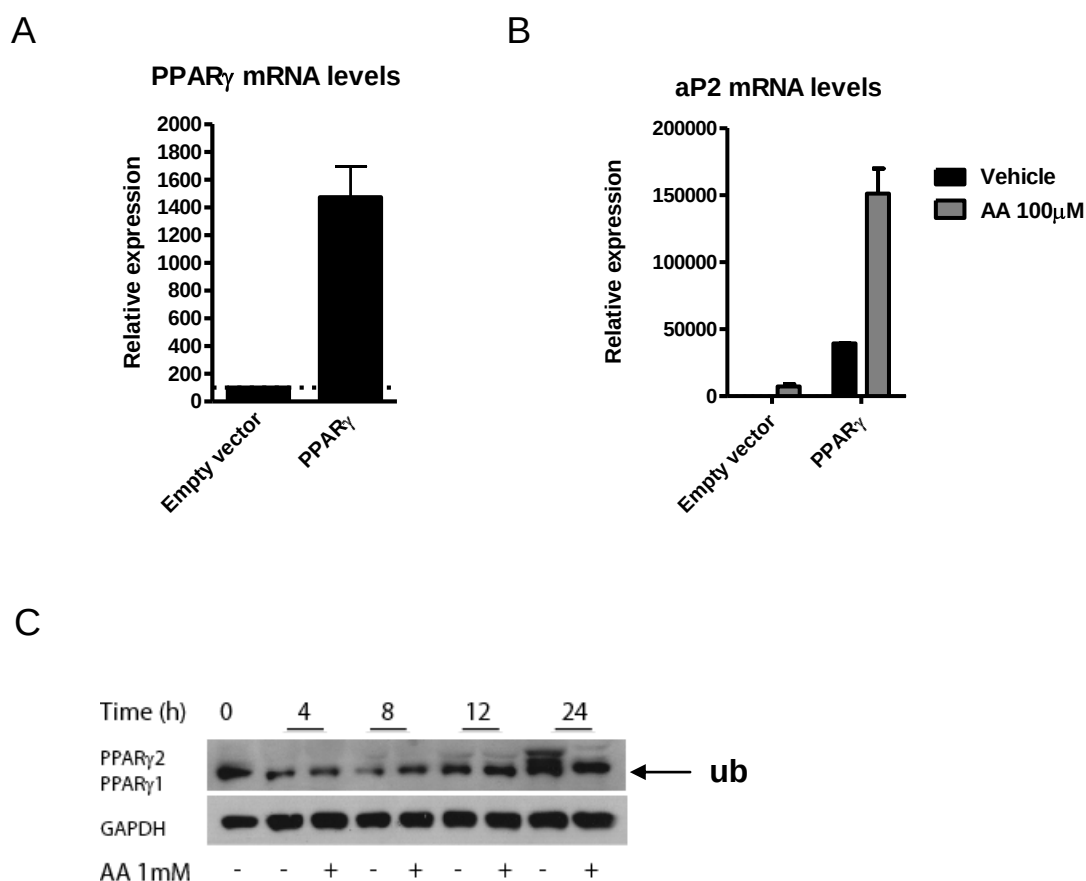


Figure 3.24: PPAR γ expression enhances the effect of AA on aP2 expression. A) and B) 80% confluent 3T3-L1 cells were transfected with a PPAR γ expression vector (pSG5-PPAR γ) or an empty vector (pcDNA) as a negative control and the next day were treated with AA (100 μ M) in the presence of MDI for 24h. The total RNA of the cells was isolated and analyzed using qRT-PCR for PPAR γ and aP2 transcripts, respectively. These are representative graphs of two independent experiments (n=2). All expression levels are relative to the empty vector vehicle control set as 100. Error bars represent $\pm$ SD. C) 3T3-L1 cells were treated with 1mM AA for 24h. Whole cell extracts were isolated and subjected to western blotting using antibodies against PPAR γ and GAPDH. PPAR γ migrates as two closely spaced bands on an SDS–polyacrylamide gel, the upper band is PPAR γ 2 and the lower band is PPAR γ 1. This antibody also recognises one unspecific band (ub) lower than PPAR γ 1.

3.2.4.4 PPAR γ LBD mediates the effect of AA on aP2 expression

Finally, the last question that was addressed was which domain of PPAR γ was involved in the signalling of AA. These experiments were carried out in NIH-3T3 cells as they have been used previously to investigate the role of PPAR γ in adipogenesis (Hu et al., 1996). Cells were transfected with wild type and different mutant versions of PPAR γ expression vectors, treated for 24h with AA (1mM) and the endogenous aP2 expression was assessed (Figure 3.25).

The PPAR γ 2-S112A mutant expresses a PPAR γ 2 form that cannot be phosphorylated at the serine 112, the PPAR γ 2-E499Q mutant express a PPAR γ 2 form with a disrupted α -helix as a result a disrupted ligand-dependent conformational change of the AF2 region and/or docking of cofactors (Hauser et al., 2000; Walkey and Spiegelman, 2008). Finally, the mutation of the PPAR γ 2-Q286P is in the middle of α -helix 3 of the LBD, which forms the backbone of the ligand-binding pocket. This mutant is unable to bind or be activated by all known agonists (Walkey and Spiegelman, 2008).

As can be seen in figure 3.25 B and C, AA enhanced endogenous aP2 expression with the empty vector control in comparison with the vehicle control and its effect was further increased when the cells were transfected with the wild type PPAR γ 2. When cells were transfected with the AF-2 mutant (PPAR γ 2-E499Q) the effect of AA on aP2 was lower in comparison with the wild type PPAR γ 2, whereas when transfected with the LBD mutant (PPAR γ 2-Q286P) the effect of AA was similar to the basal levels. In addition, when cells were transfected with the phosphorylation site mutant (PPAR γ 2-S112A), the ability of AA to induce aP2 expression was not affected. These data indicate that the effect of AA on aP2 is mediated thorough PPAR γ 2 in a LBD-dependent manner.

Finally, to confirm the importance of the LBD, pre-confluent 3T3-L1 cells were transfected with a UASx4-TK-luc reporter plasmid, a Gal4-DBD-PPAR γ -LBD expression plasmid and a renilla normalisation vector. The cells were

subsequently grown to confluence and induced to differentiate using the MDI protocol in the presence or absence of 100 μ M AA. Cells were harvested at 4, 8, 12 and 24h time points and luciferase activity was measured and normalised to renilla activity. Normalised luciferase activity increased approximately 2-fold during the first 24h of the differentiation process in the presence of AA. These data indicate that AA could enhance the production of endogenous PPAR γ ligands during this period (Figure 3.25 D).

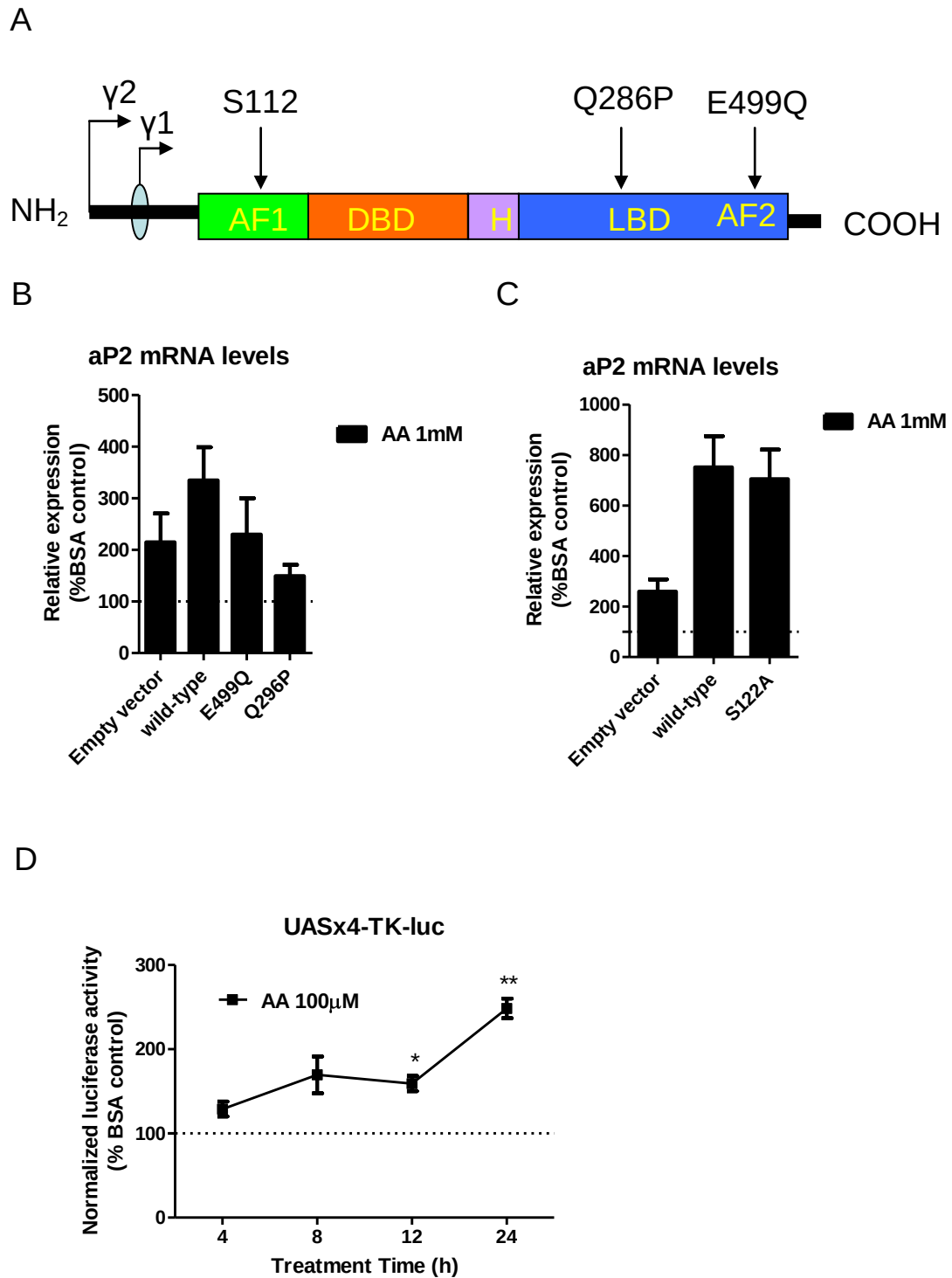


Figure 3.25: PPAR γ -LBD mediates the effect of AA on aP2 expression. A) Schematic representation of PPAR γ with indications of the sites of the point mutations. B) and C) pre-confluent NIH-3T3 were transfected with expression plasmids for PPAR γ wild-type or PPAR γ mutants. 24h after transfection, the cells were treated for 24h with or without 1mM AA. Total RNA was isolated and subjected to qRT-PCR for endogenous aP2. The graphs are representative of two independent experiments. Error bars represent $\pm$ SD. D) Pre-confluent 3T3-L1 cells were transfected with a UASx4-TK-luc reporter plasmid, a Gal4-DBD- PPAR γ - LBD expression plasmid and renilla normalization vector, re-plated and subsequently induced

to differentiate in the presence or absence of 100 μ M AA. Cells were harvested at the indicated time points and analysed for luciferase and renilla activities. At each time point, luciferase activity was normalized to renilla activity. The plot represent the mean values of three independent experiment (n=3). Error bars represent $\pm$ SEM. * P< 0.05, **P<0.01.

3.2.5 The function of aP2 increase after AA treatment

3.2.5.1 *The role of aP2 increase in FA metabolism*

aP2 has been suggested to have role on the distribution of lipids between the different compartments of the cell, such as to the LD for storage; to the ER for signalling, trafficking and membrane synthesis; to the mitochondria or peroxisome for oxidation; to cytosolic or other enzymes to regulate their activity; to the nucleus for lipid-mediated transcriptional regulation; or even outside the cell to signal in an autocrine or paracrine manner. Thus, it was hypothesised that the role of the aP2 increase at the early stages of differentiation after AA treatment could be related to changes in lipid metabolism. To investigate this, a PCR array was conducted on two days post-confluent 3T3-L1 cells after treatment with AA (1mM) for 24h. By using this PCR array we could monitor any changes in gene expression in 84 genes involved in FA catabolism, FA transport, FA biosynthesis regulation, ketogenesis and TAG catabolism (Figure 3.26). In figure 3.26 the fold increase of the genes after AA treatment in comparison with the BSA control used as a vehicle is shown. For significant changes we considered that a gene would have to change more than 2 fold (dotted line). In addition to aP2 that showed around a 200-fold increase in comparison with the vehicle control, two other genes were found up-regulated by AA, changing around 3-fold, *Acsl4* and carnitine palmitoyltransferase 1A (*CPT1a*). To confirm the results taken from the PCR array, qRT-PCR was conducted in two days post-confluent cells treated with increasing doses of AA. *Acsl4* and *CPT1a* mRNA levels were increased but the up-regulation was significant only after 24h of treatment with the higher dose of AA. This result indicates that AA overload may regulate β -oxidation (Figure 3.27).

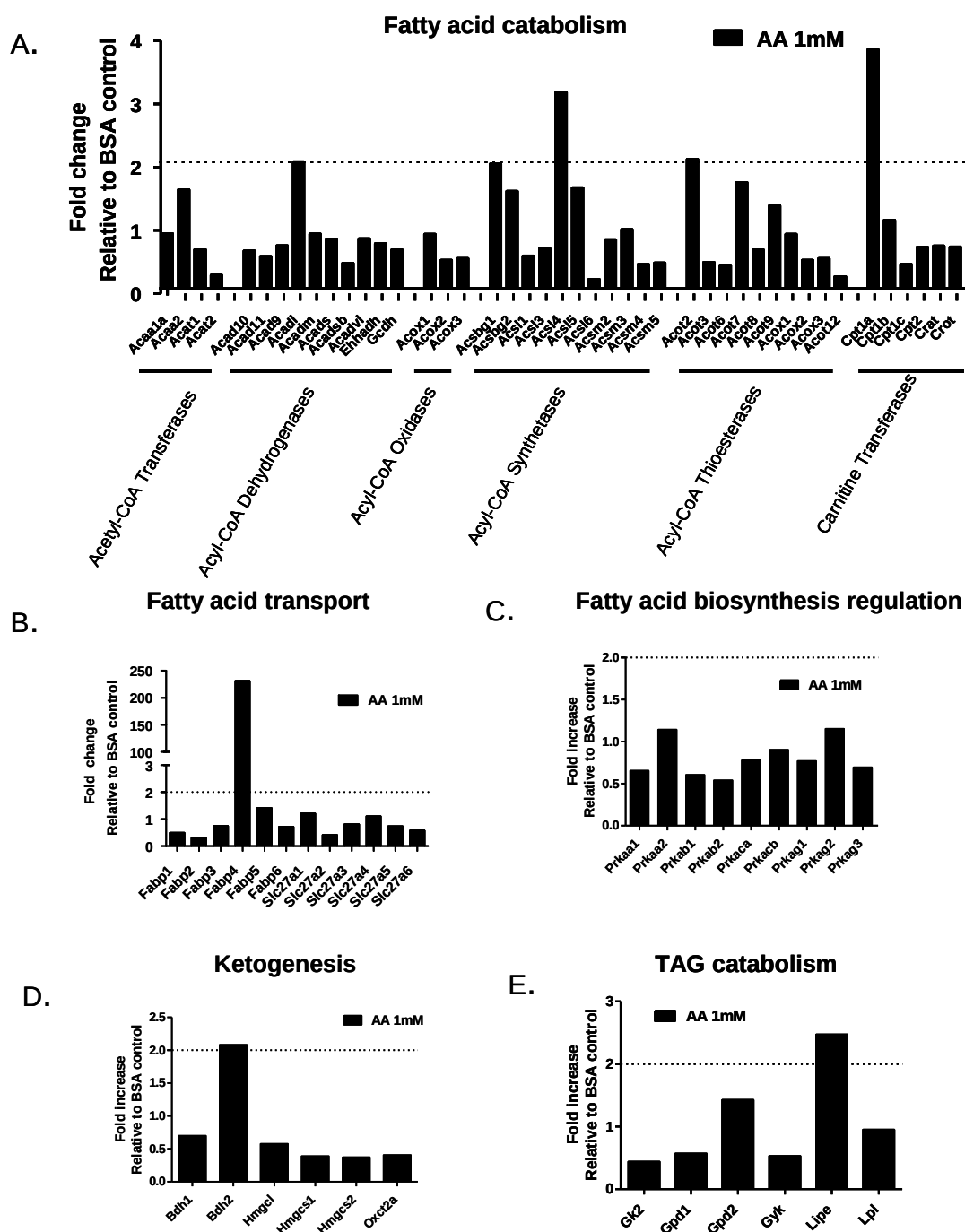


Figure 3.26: The effect of AA on lipid metabolism. A) PCR array was conducted in two days post-confluent 3T3-L1 cells treated with 1mM AA for 24h in the presence of MDI. RNA and cDNA was prepared according to the manufacturer's guidelines. The expression of genes involved in different aspects of lipid metabolism was assessed A) Relative expression of genes involved in FA catabolism, B) in FA transport, C) in FA biosynthesis regulation, D) in ketogenesis and E) in TAG catabolism. The graphs represent the mean values of duplicate samples.

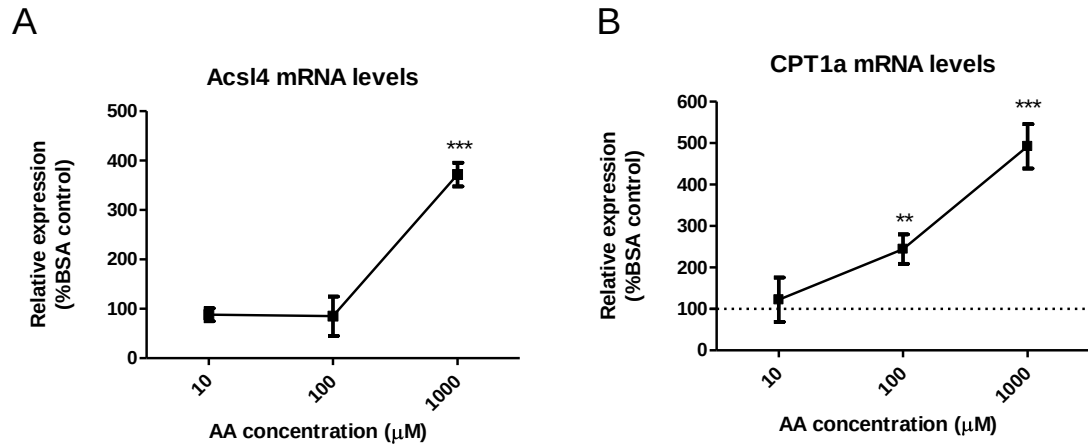


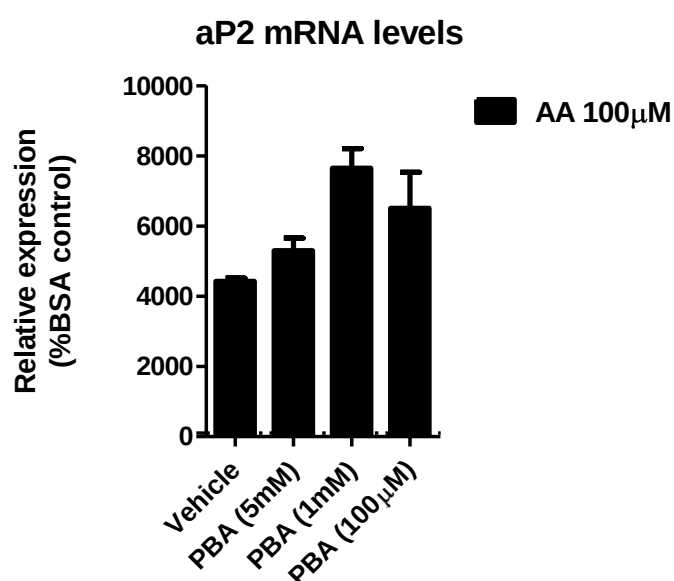
Figure 3.27: The effect of AA on Acs14 and CPT1a gene expression. A) and B) Two days post-confluent 3T3-L1 cells were treated with increasing doses of AA for 24h in the presence of MDI. Total RNA was isolated and subjected to qRT-PCR for Acs14 and CPT1a, respectively. The graphs represent the mean normalized to vehicle control values of three independent experiments (n=3). Error bars represent $\pm$ SEM. **P< 0.01, ***P<0.001.

3.2.5.2 The role of aP2 increase in ER stress

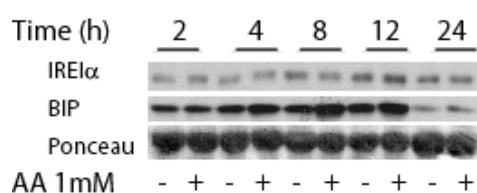
It was shown that palmitate can induce ER stress and also aP2 expression in macrophages. Both of these effects could be rescued by treating the cells with the chemical chaperone 4-phenyl butyric acid (PBA) which alleviates ER stress, showing that ER stress is mediated by aP2 in these cells (Erbay et al., 2009). In addition, palmitate does not induce ER stress in aP2^{-/-} macrophages which do not have any defects in FA uptake or response to other ER inducers. The same results were confirmed in vivo (Erbay et al., 2009).

We hypothesised that the increase in aP2 expression stimulated by AA in pre-adipocytes could have the same role as in macrophages by mediating ER stress. For this reason, two day post-confluent 3T3-L1 cells were treated with increasing doses of PBA together with 100µM AA for 24h. PBA, as mentioned before, can alleviate ER stress and, hence, provides an experimental opportunity to approach the role of aP2 up-regulation by AA mediating ER stress in pre-adipocytes. In contrast to macrophages, PBA could not block the AA-induced up-regulation of aP2 levels, indicating that in pre-adipocytes the role of aP2 is not connected with ER stress (Figure 3.28 A). Finally, to investigate if AA was inducer of ER stress, the expression of three ER markers were monitored after AA treatment (Figure 3.28 B, C). IRE1α, BiP and PERK expression was not regulated by any dose of AA, confirming that the aP2 up-regulation by AA is not correlated with ER stress.

A



B



C

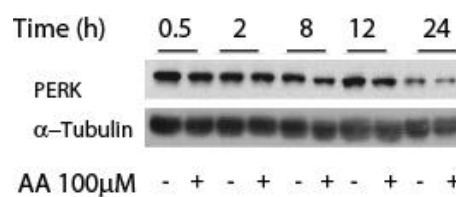


Figure 3.28: The effect of AA effect on ER stress. A) Two-day post-confluent 3T3-L1 cells were treated with increasing doses of PBA (100µM, 5mM and 10mM) together with 100µM AA for 24h in the presence of MDI. Total RNA was isolated and subjected to qRT-PCR for aP2. B) and C) Two-day post-confluent 3T3-L1 cells were treated with 1mM or 100µM AA and whole cell lysates were subjected to western blotting with antibodies against IREα, BIP and PERK.

3.2.5.3 The role of aP2 increase in autophagy

Macroautophagy is a lysosomal pathway for the degradation of cytosolic organelles and proteins. Macroautophagy can either be non-selective where a cytosol cargo with any content present is sequestered or it can be selective (mitophagy, ribophagy, lipophagy, aggregophagy, etc.). Thus any cargo (whole organelles or portions of cytosol) is engulfed by double-membrane-bound structures termed autophagosomes that translocate to lysosomes for fusion to form autolysosomes and sequestered intra-autophagosomal components are degraded by lysosomal hydrolases (Singh and Cuervo, 2011). Macroautophagy has been implicated in adipocyte differentiation. Knocking down a critical macroautophagy gene autophagy-related 7 (Atg7) in 3T3-L1 cells resulted in inhibition of differentiation. The same observation was confirmed in adipocyte-selective *Atg7*^{-/-} knockout mice which displayed reduced WAT mass (Singh et al., 2009a). Moreover, in hepatocytes inhibition of autophagy increased lipid accumulation, confirming that autophagy and lipid metabolism are interrelated (Singh et al., 2009b). We hypothesised that since the intracellular FA content is increasing with AA treatment then the increase in aP2 expression is a homeostatic mechanism for the transport of excess FA load to the autophagosomes for degradation.

To investigate if AA-induced aP2 expression is related to the regulation of autophagy, 3T3-L1 cells were treated either with an inhibitor of autophagosome formation (3-methyladenine, 3MA) or an inducer of autophagy (rapamycin) and aP2 expression was monitored. 3MA enhanced the AA-dependent induction of aP2 expression whereas rapamycin inhibited AA effect on aP2 expression (Figure 3.29 A, B). To further confirm the pharmacological inhibition of autophagy on aP2 mRNA levels, 3T3-L1 cells were stably infected with an empty lentiviral vector (PGK) or with a vector expressing an shRNA to target the essential autophagy gene *Atg7* (siATG7). Levels of *Atg7* were markedly decreased in siATG7 cells after 24h of the differentiation induction (Figure 3. 29 D). PGK or siATG7 3T3-L1 cells were treated with AA (100µM) or PGF_{2α} (10nM) for 24h in the presence of MDI and

the aP2 expression was assessed. In both AA and PGF_{2α} treatments aP2 expression was induced when autophagy was prevented (Figure 3.29 E).

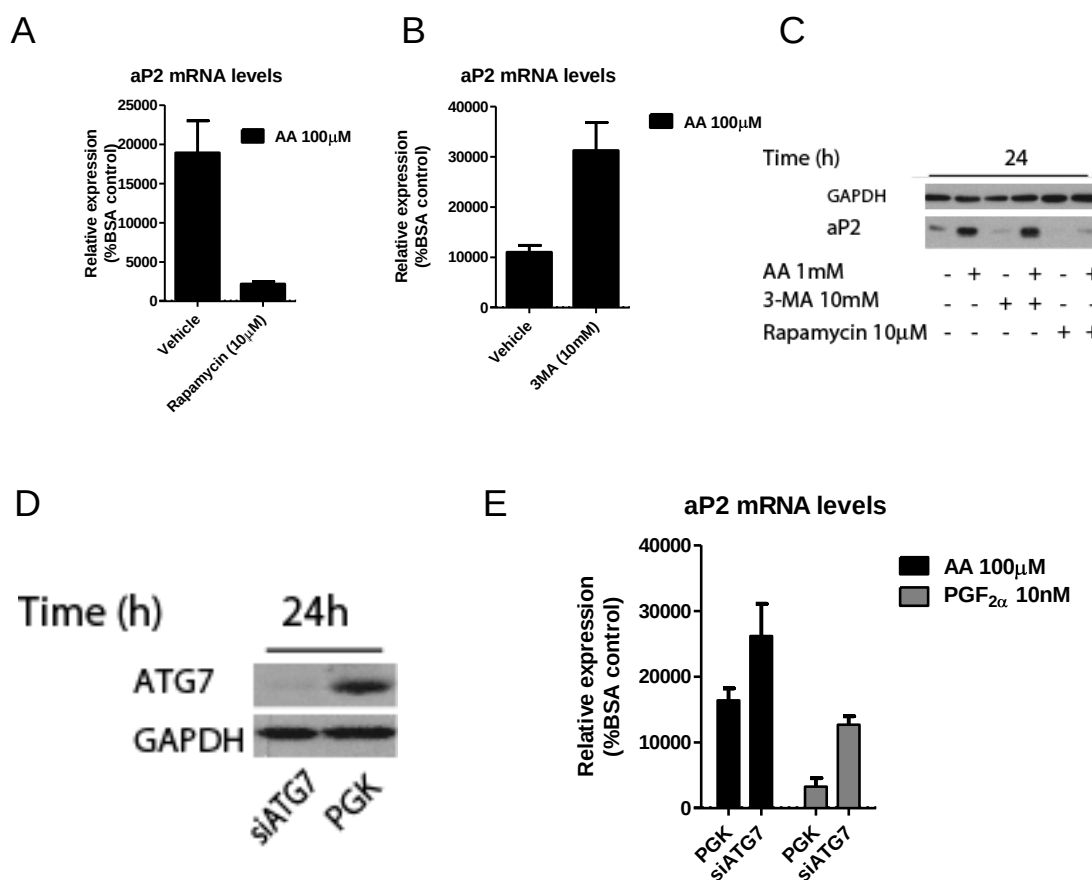


Figure 3.29: Inhibition of autophagy enhances the effect of AA on aP2 expression. A) 3T3-L1 cells pre-treated with rapamycin (10μM) for 30min before the treatment with 100μM AA for 24h in the presence of MDI. Total RNA was isolated and subjected to qRT-PCR for aP2 transcripts. B) Cells were pre-treated with 3MA (10mM) prior to the AA (100μM) treatment. C) 3T3-L1 cells were treated as described in A and B and whole cell lysates were subjected to western blotting using antibodies against aP2 and GAPDH. D) 80% confluent 3T3-L1 cells were infected with empty lentiviral vector (PGK) or with vector expressing an shRNA targeting Atg7 (siATG7). Total protein was isolated from PGK and siATG7 cells and immunoblotted with antibodies for Atg7 and GAPDH. E) On the third day after the infection, PGK and siATG7 3T3-L1 cells were treated with 100μM AA or 10nM PGF_{2α} for 24h in the presence of MDI. Total RNA was isolated and subjected to qRT-PCR for aP2. All graphs are representatives from two independent experiments (n=2). Error bars represent ±SD.

To investigate whether aP2 has a role in autophagy we investigated responses in 3T3-L1 cells depleted of aP2 with a targeting shRNA. aP2 was increased upon AA treatment in the shScrambled cells whereas aP2 levels markedly decreased in shaP2 3T3-L1 cells with or without AA treatment (Figure 3.30 A). ShScrambled and shaP2 cells were treated with 100 μ M AA for 24h in the presence of MDI and the expression of microtubule-associated protein light chain 3 (LC3) was determined. LC3 is a reliable marker of autophagosomes. Upon induction of autophagy, cytosolic LC3 I is conjugated to PE through two consecutive ubiquitylation-like reactions conducted by ATG7 and ATG3 to form LC3 II which is targeted to autophagic membranes. During the fusion of autophagosomes with the lysosomes LC3 II is also degraded by the lysosomal proteases. Thus, the amount of LC3-II correlates well with the number of autophagosomes and this characteristic conversion of LC3 can be used to monitor autophagic activity (Tanida et al., 2008).

As it can be seen in figure 3.30 B, LC3 II levels were reduced in the absence of aP2 indicating that aP2 is required for autophagy. This effect does not seem to be AA related as treatment with AA does not influence autophagy. To further confirm that observation a time course experiment was conducted in 2-days post-confluent wild-type 3T3-L1 cells treated with 100 μ M AA for 24h and LC3 and ATG7 levels were assessed (Figure 3.31 A, B). Autophagy as it can be assessed by LC3 II levels or ATG7 levels was declining over the period of 24h and was not regulated by the presence of AA. Finally, the activity of the mammalian target of rapamycin (mTOR) which inhibits autophagy was not altered as determined by the phosphorylation status (Figure 3.31 C).

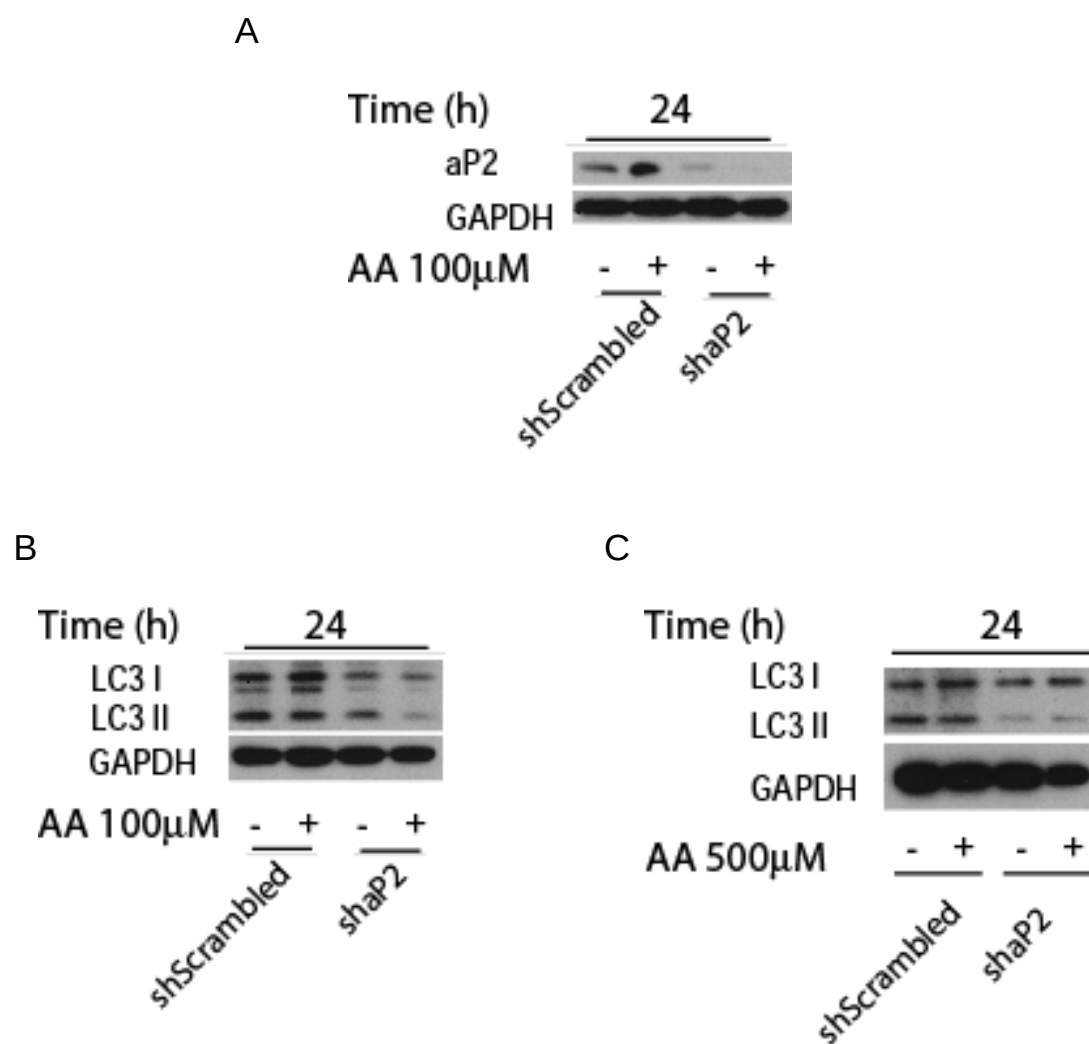


Figure 3.30 aP2 is required for autophagy. Two days post-confluent shaP2 and shScrambled 3T3-L1 cells were treated with 100μM or 500μM AA for 24h in the presence of MDI. Whole lysates were extracted and subjected to western blot using antibodies against A) aP2 and GAPDH. B) and C) LC3 and GAPDH.

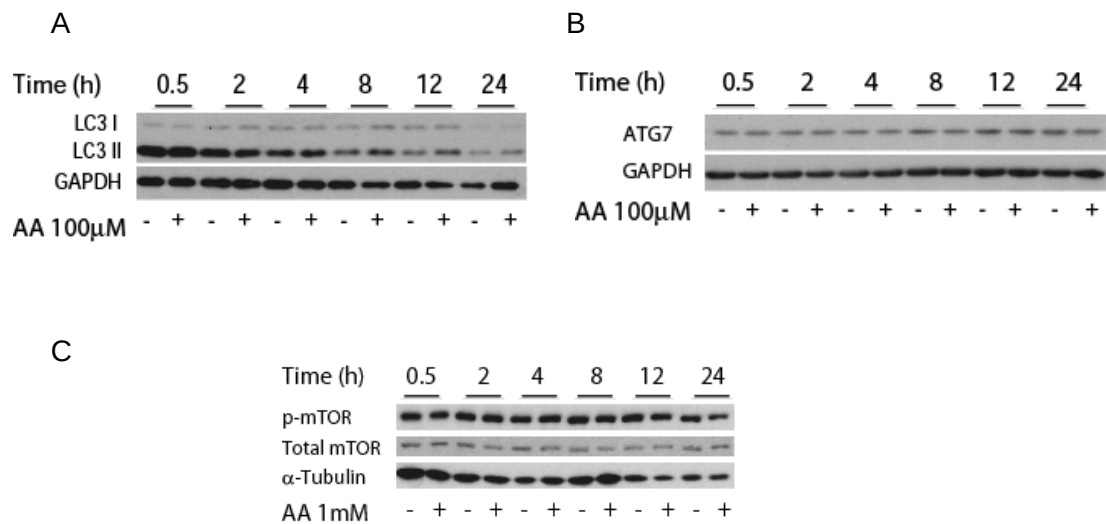


Figure 3.31 AA treatment does not affect autophagy. 3T3-L1 cells were treated with 100μM or 1mM AA for 24h in the presence of MDI. Whole cell lysates were subjected to western blotting with antibodies against A) LC3 and α-tubulin B) ATG7 and GAPDH, C) p-mTOR, total-mTOR and GAPDH.

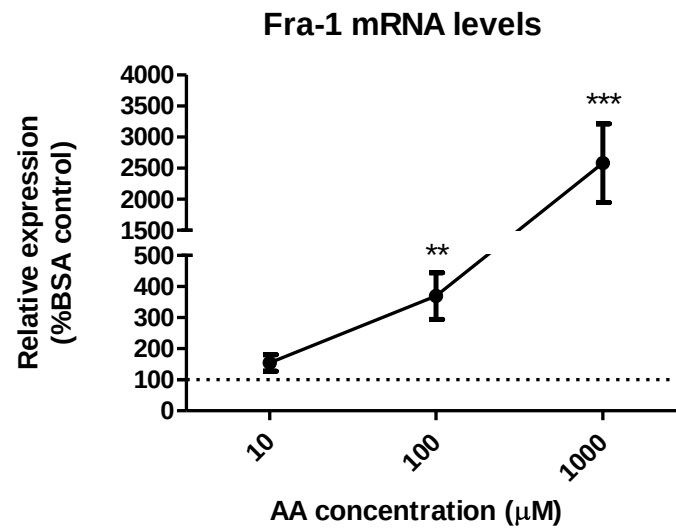
3.2.6 The effect of AA on Fra-1 expression

3.2.6.1 AA increases *Fra-1* expression

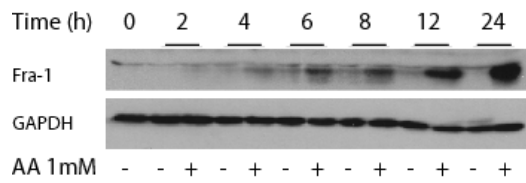
Fra-1 is an AP-1 transcription factor that is expressed early in adipocyte differentiation (Stephens et al., 1992), and Fra-1 transgenic mice show lipodystrophy. It has been shown to be induced by FAs and in particular oleate at the early stages of differentiation through transcriptional regulation (Distel et al, 1992).

To determine if AA had any effect on Fra-1 expression during the early stages of adipocyte differentiation, 3T3-L1 cells were treated with increasing doses of AA in the presence of MDI and harvested after 24h of treatment. Fra-1 mRNA levels were increased with increasing doses of AA (Figure 3.32 A). Similarly, Fra-1 protein levels were increased by AA at 1mM and 100µM doses in the first 24h of the differentiation procedure. The effect of AA on Fra-1 protein was detectable after only 4h of treatment which was more evident after 8h (Figure 3.32 B, C).

A



B



C

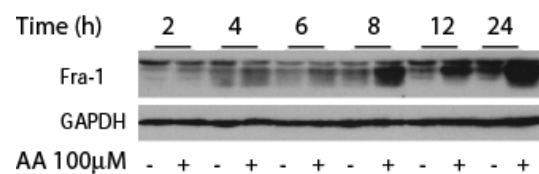


Figure 3.32 AA increases Fra-1 expression. A) 3T3-L1 cells were treated with increasing doses of 10μM, 100μM or 1mM AA for 24h in the presence of MDI. Total RNA was isolated after 24h of treatment and qRT-PCR was performed. All relative Fra-1 mRNA levels are presented as mean percentages of the respective BSA (set as 100%) controls from three independent experiment (n=3). Error bars represent $\pm$ SEM. **P< 0.01, ***P<0.001. B) Cells were treated as described in A and whole lysates were harvested and probed with antibodies against Fra-1 and GAPDH. The blots are representative of two independent experiments.

3.2.6.2 The effect of AA on Fra-1 expression is PG-mediated

To determine if this effect is due to AA itself or its metabolites 3T3-L1 cells were pre-treated with indomethacin (a general COX inhibitor) or selective inhibitors for COX-2 (SC-236) and COX-1 (SC-560) for 30min and then treated with AA 100 μ M for 24h. Similarly, cells were pre-treated with a 12/15 LOX inhibitor (baicalein) and a general P450 monooxygenase inhibitor (17-ODYA). 17-ODYA did not have any effect on Fra-1 expression whereas baicalein increased levels of Fra-1, indicating unspecific effects (Figure 3.33 C, D). The effect of AA on Fra-1 expression was substantially reduced by COX inhibitors indicating that PGs mediate the effect of AA on Fra-1 expression (Figure 3.33 A, B).

To determine which PG is important for Fra-1 up-regulation, a dose–response experiment was conducted treating pre-adipocytes with increasing doses of cPGI₂, PGF_{2 α} , PGE₂ and 15-deoxy- $\Delta^{12,14}$ PGJ₂ and Fra-1 expression was assessed. Only PGF_{2 α} increased significantly Fra-1 levels using the 10nM concentration (Figure 3.34 A). A time course experiment was conducted using 10nM PGF_{2 α} and RNA and protein were harvested at different time points over a period of 24h. Fra-1 mRNA levels were increased with AA over time peaking at 24h (Figure 3.34 B). However, the kinetics for Fra-1 protein levels were different from the RNA data showing an increase at 8h, then declining at 12h and then rise again at 24h (Figure 3.34 C).

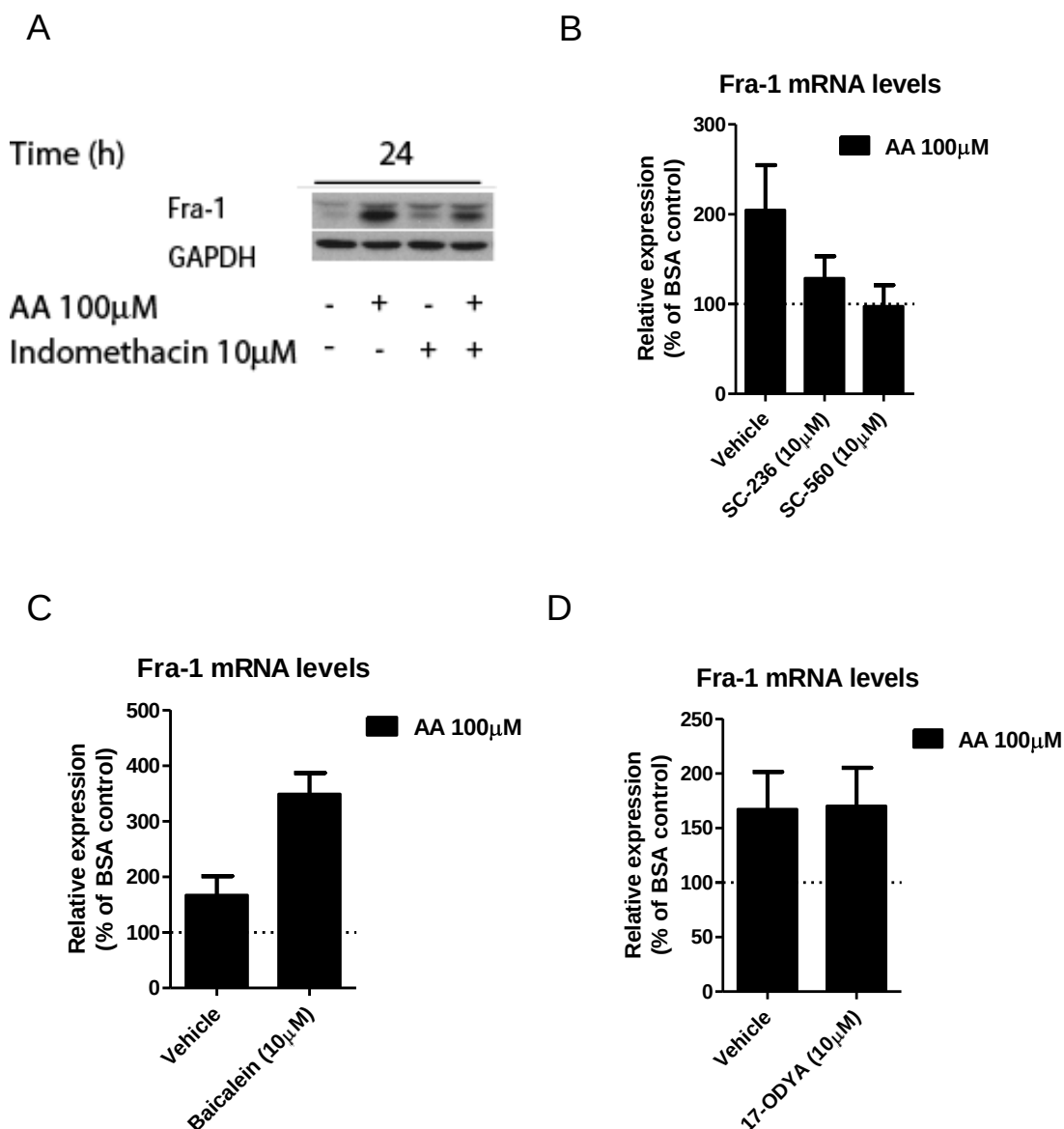
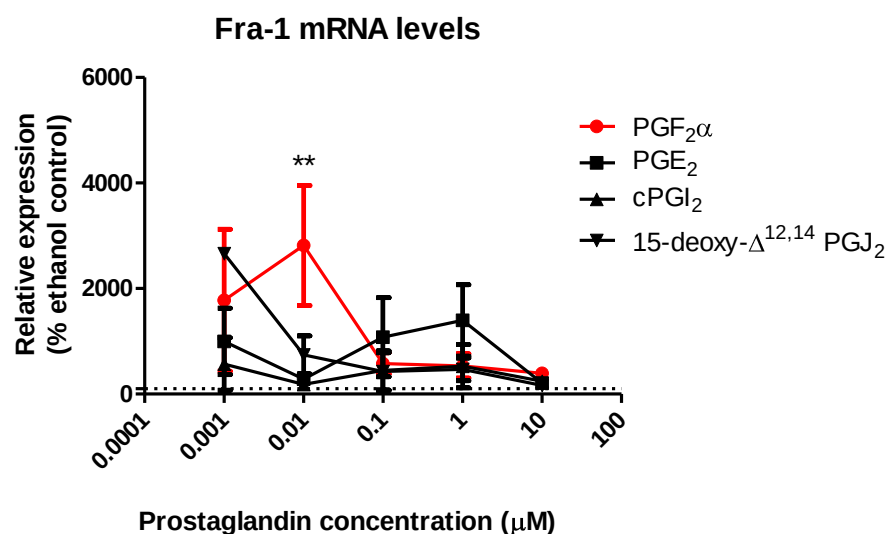
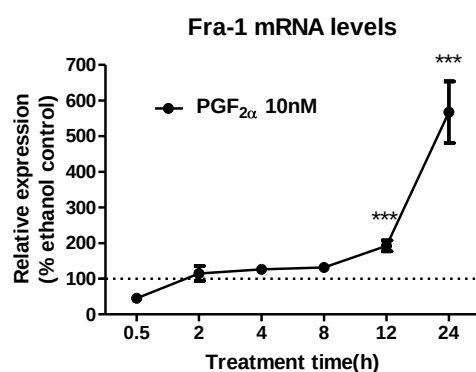


Figure 3.33: The effect of AA on Fra-1 expression is PG-mediated. A) 3T3-L1 cells were pre-treated with A) indomethacin (10 μ M), B) a selective COX-2 (SC-236, 10 μ M) and a selective COX-1 inhibitor (SC-560, 10 μ M), C) baicalein (10 μ M) or D) 17-ODYA (10 μ M) for 30 min prior to AA (100 μ M) treatment for 24h. Total RNA or whole cell lysates were isolated and qRT-PCR or western blot was performed. All relative Fra-1 mRNA levels are presented as mean percentages of the respective vehicle controls (set as 100%) from two independent experiment (n=2). Error bars represent $\pm$ SD. The antibodies used were against Fra-1 and GAPDH. The blot is representative of two independent experiments.

A



B



C

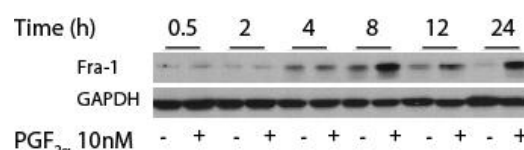


Figure 3.34 PGF_{2α} increases Fra-1 expression. A) Two days post-confluent 3T3-L1 cells were treated with 1nM, 10nM, 100μM, 1μM or 10μM PGF_{2α}, PGE₂, cPGI₂ and 15d-PGJ₂ for 24h in the presence of MDI. The total RNA was isolated after 24h of treatment and qRT-PCR was performed for Fra-1 mRNA transcripts. All relative mRNA levels are presented as mean percentages of the respective vehicle controls from three independent experiments (n=3). Error bars represent ±SEM. **P<0.01. The red line is the PGF_{2α} treatment. B) Two days post-confluent 3T3-L1 cells were treated for 24h with 10nM PGF_{2α} and B) RNA and C) protein were harvested at the indicated time points and subjected to qRT-PCR and western blot respectively. All relative mRNA levels are presented as mean percentages of the respective vehicle controls from three independent experiments (n=3). Error bars represent ±SEM. ***P<0.001.

3.2.6.3 The effect of AA on Fra-1 expression is FP receptor-mediated

To further confirm that $\text{PGF}_{2\alpha}$ regulates Fra-1, cells were pre-treated with an FP receptor antagonist (AL 8810), an EP4 receptor antagonist (L-161,982) and an IP receptor antagonist (CAY-10441) before the AA treatment for 24h (Figure 3.35). Only the FP antagonist could significantly block the effect of AA on Fra-1 expression (Figure 3.35 A). In addition, Fra-1 up-regulation by AA was blocked completely when cells were depleted of the FP receptor using a targeting siRNA (siFP) when compared with siControl (Figure 3.35 B).

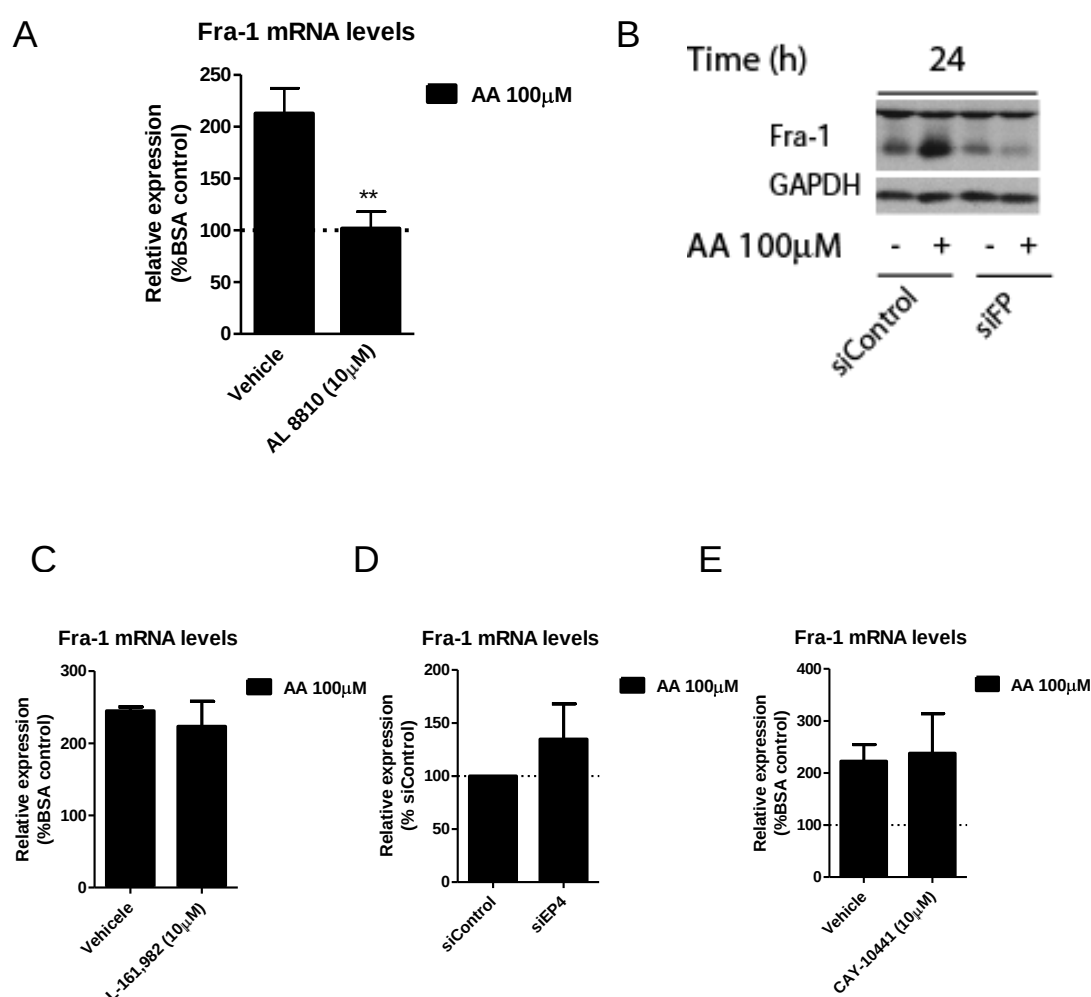


Figure 3.35: The FP receptor mediates the effect of AA on Fra-1 expression. A), C) and E) 3T3-L1 cells were pre-treated with AL 8810 (10 μ M), L-161,982 (10 μ M) or CAY-10441 (10 μ M) for 30 min prior to 100 μ M AA treatment for 24h. Total RNA was extracted and subjected to qRT-PCR for Fra-1 mRNA transcript. All relative mRNA levels are presented as mean percentages of the respective vehicle controls from three independent experiments

(n=3). Error bars represent $\pm$ SEM. **P<0.01. B) 80% confluent 3T3-L1 cells were transfected with a non targeting control siRNA (siControl), an siRNA targeting the FP receptor (siFP) On the third day after transfection, cells were treated with 100 μ M AA for 24h in the presence of MDI. Whole lysates were isolated and subjected to western blotting with antibodies against Fra-1 and GAPDH. The blot is a representative of three independent experiments. D) Cells were treated as described in B but cells were transfected with an siRNA targeting the EP4 receptor (siEP4). Total RNA was extracted and subjected to qRT-PCR for Fra-1 mRNA transcript. Graph is a representative from two independent experiments. Error bars represent $\pm$ SD.

3.2.6.4 The signalling pathway mediating the effect of AA on Fra-1 expression

To elucidate the signalling pathway by which AA regulates Fra-1 expression, 3T3-L1 cells were pre-treated with a PKA inhibitor (H89) or a PKC inhibitor (GF 109203X) prior to AA treatment for 24h (Figure 3.36 A-E). H89 substantially blocked the ability of AA to induce Fra-1 expression. As a positive control, cells were pre-treated with H89 and then treated with forskolin for 24h (Figure 3.36 B, C). Forskolin activates adenylyl cyclase (AC) and causes the increase of the intracellular levels of cAMP. Forskolin was able to increase Fra-1 expression which was blocked with H89, indicating that cAMP increase is important in Fra-1 regulation. In addition, 3T3-L1 cells were pre-treated with a PKC inhibitor (GF 109203X) prior to AA treatment (100 μ M) for 24h. The PKC inhibitor blocked significantly the effect of AA on the increase in Fra-1 protein and mRNA levels (Figure 3. 36 D, E). In conclusion, both PKC and PKA inhibitors blocked the effect of AA on Fra-1 expression.

Finally, Fra-1 is reported to be regulated by the ERK pathway both transcriptionally and post-translationally (Casalino et al., 2003). Therefore, to investigate if ERK mediates the effect of AA on Fra-1 expression, 3T3-L1 cells were pre-treated with a MEK1 inhibitor (PD98059) or a MEK 1/2 inhibitor (U0126), before AA stimulation for 24h. PD98059 or U0126 blocked the ability of AA to induce Fra-1 expression, suggesting a role of ERK in Fra-1 up-regulation by AA (Figure 3.36 F, G).

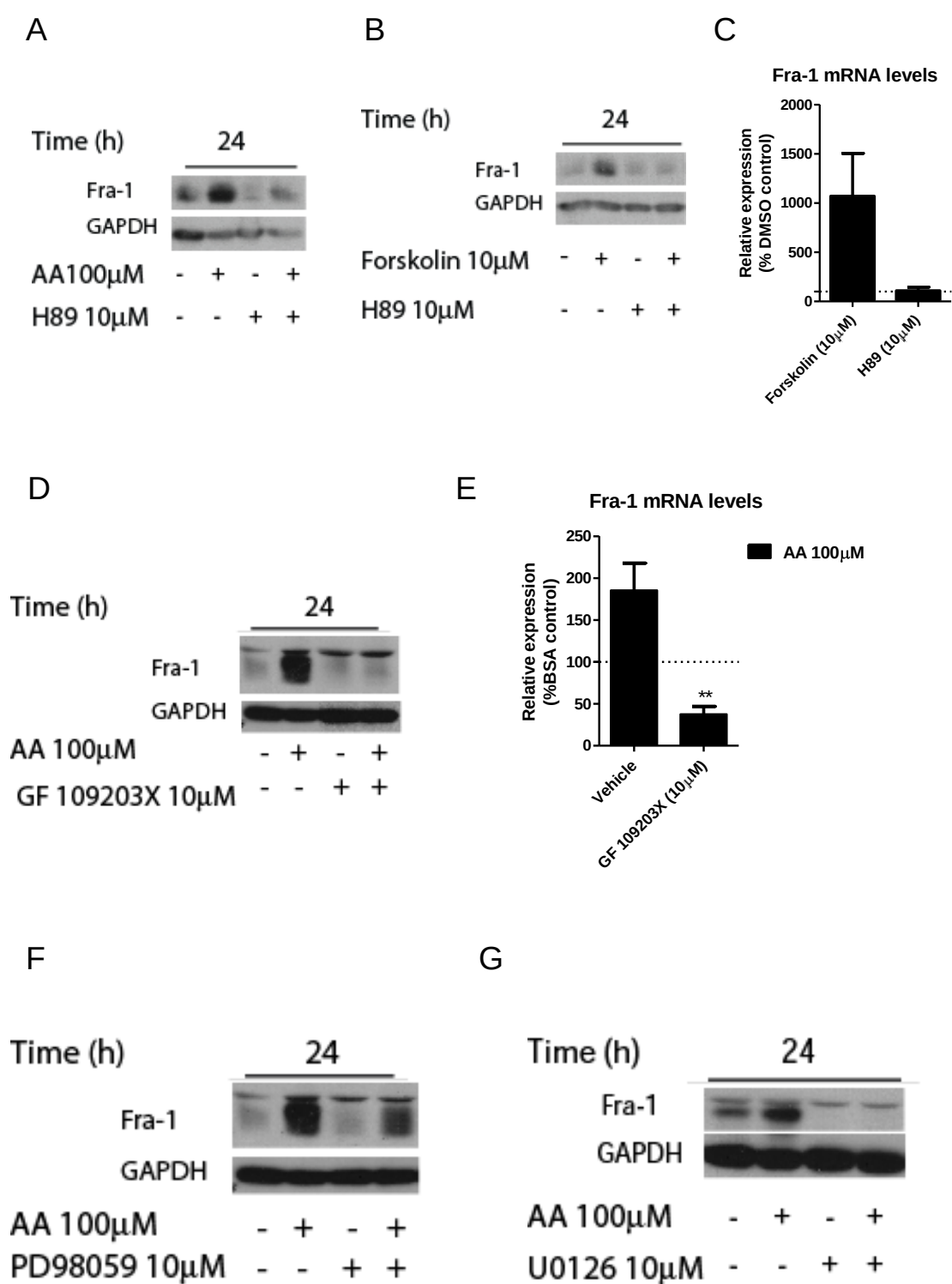


Figure 3.36: PKA, PKC and ERK mediate the effect of AA on Fra-1 expression. A) 3T3-L1 cells were pre-treated with H89 (10μM) for 30 min prior to 100μM AA treatment for 24h. Whole cell lysates were isolated and subjected to Western blotting using antibodies against Fra-1 and GAPDH. The blot is a representative of two independent experiments. 3T3-L1 cells were pre-treated with H89 (10μM) for 30 min prior to Forskolin (10μM) treatment for

24h. Whole cell lysates (B) or total RNA (C) was isolated and subjected to Western blotting or qRT-PCR, respectively for Fra-1 expression. D) As described above but cells pre-treated with a PKC inhibitor (GF109203X) prior to AA (100 μ M) stimulation for 24h. F) and G) as described above but cells pre-treated with PD98059 (10 μ M) or a U0126 (10 μ M), respectively.

3.2.6.5 The effect of AA on Fra-1 expression is PPAR γ -mediated

It has been suggested that Fra-1 might be regulated by PPAR γ since its expression is elevated after rosiglitazone treatment in 3T3-L1 cells (Albrektsen and Fleckner, 2001). To investigate the role of PPAR γ mediating the effect of AA on Fra-1 expression, 3T3-L1 PPAR γ knockdown cells (shPPAR γ) were treated with AA (100 μ M) or PGF $_{2\alpha}$ (10nM) for 24h and Fra-1 expression was assessed (Figure 3.37 A, B). In the absence of PPAR γ , AA- or PGF $_{2\alpha}$ -dependent induction of Fra-1 mRNA levels was significantly reduced, indicating that PPAR γ is crucial for the regulation of Fra-1 by AA. We also examined Fra-1 expression in wild-type 3T3-L1 cells where PPAR γ was overexpressed. PPAR γ mRNA levels were increased around 15-fold (Figure 3.24 A). Fra-1 mRNA levels were increased after AA treatment in the empty vector negative control whereas Fra-1 levels were enhanced in the presence of exogenous PPAR γ and further increased with AA treatment (Figure 3.37 C).

To further characterise the regulation of Fra-1 by AA through PPAR γ , two sequences of Fra-1 promoter were cloned upstream the luciferase gene. These sequences were chosen from a PPAR γ ChIP sequencing experiment during the differentiation of 3T3-L1 that identified binding of PPAR γ on the Fra-1 promoter at these sites (Nielsen R. personal communication) (Figure 3.38 A). These sequences are upstream the transcription start site and likely to be regulatory elements. Peak 1 is designated the 736bps element found from -14,728 to -13,992bp and peak 2 is the 849bps element found -3,277 to -2,488bp upstream Fra-1 transcription start site. 3T3-L1 cells were transfected with Fra-1 peak 1 and peak 2 luciferase constructs together with PPAR γ and treated with AA (1mM) for 24h in the presence of MDI. Both reporter constructs responded to some extent to AA but Fra-1-peak 1 displayed a greater response to AA that was further enhanced by the presence of PPAR γ

(Figure 3.38 B, C), indicating that this sequence might be important for the regulation of Fra-1 expression by AA via PPAR γ .

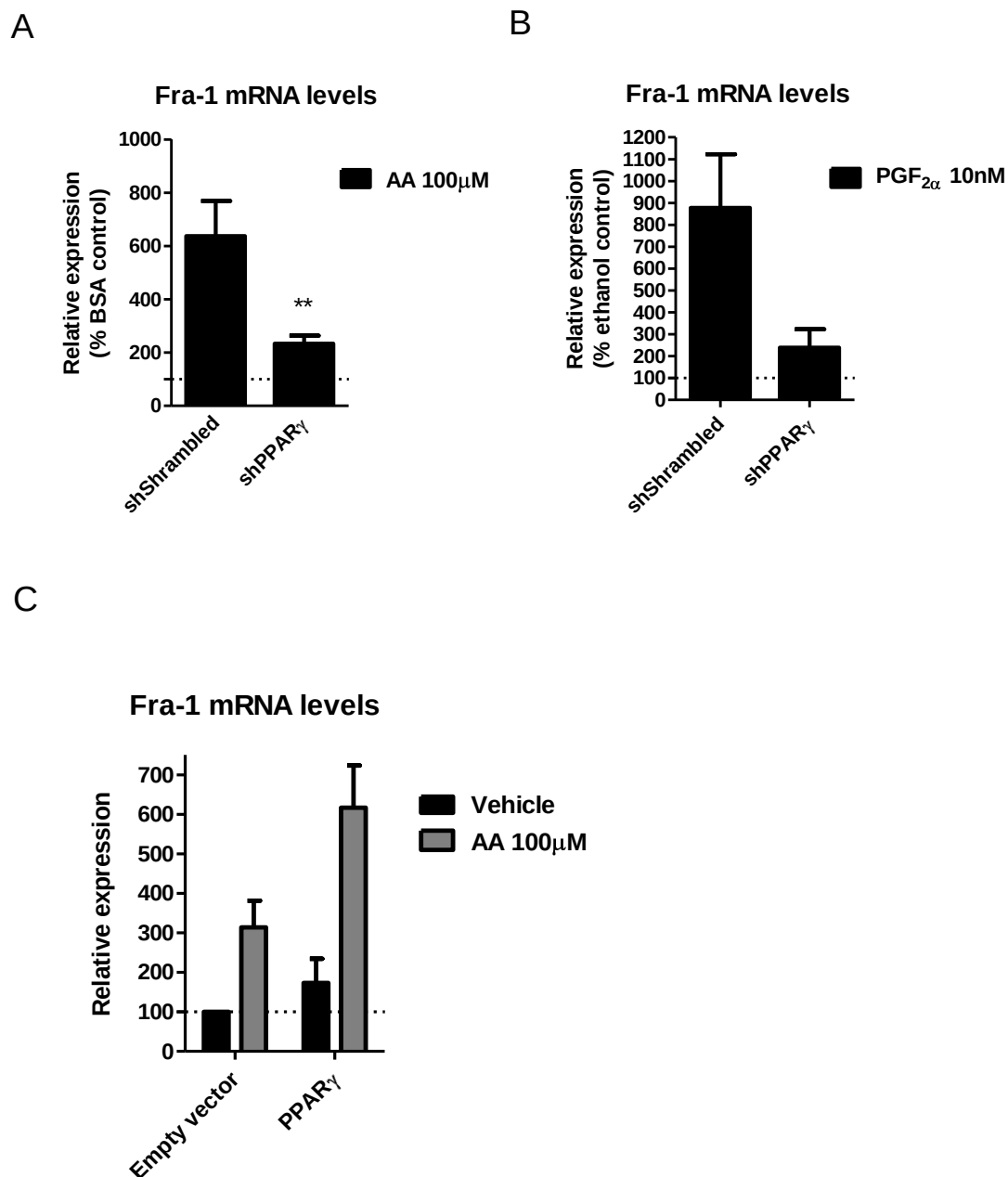
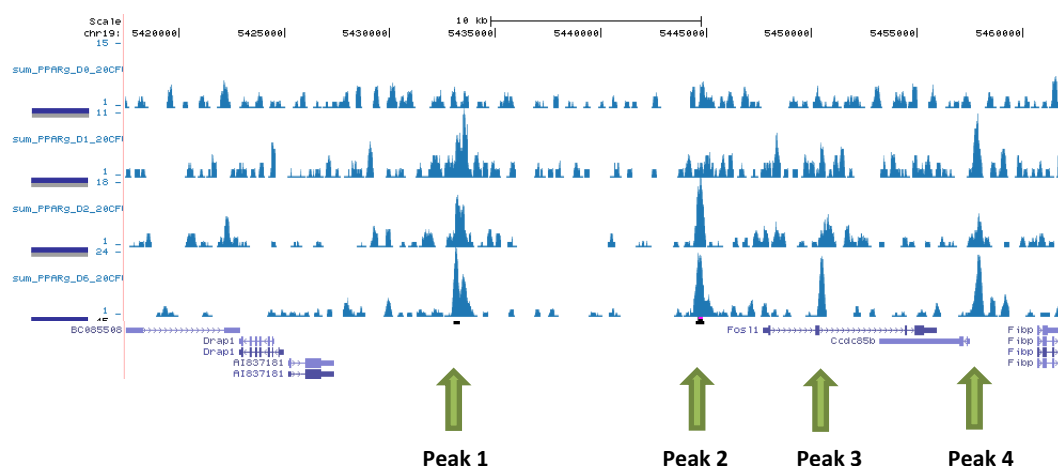


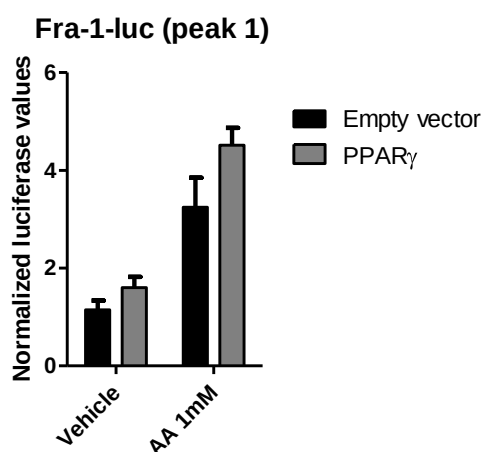
Figure 3.37: PPAR γ mediates the effect of AA on Fra-1 expression. A) shPPAR γ and shScrambled 3T3-L1 cells were treated with AA 100 μ M for 24h in the presence of MDI. The total RNA was isolated and analyzed using qRT-PCR. Relative Fra-1 mRNA levels were expressed as percentage of the respective negative control stimulated with vehicle only. The plot represent the mean values of three independent experiment (n=3). Error bars represent $\pm$ SEM. **P< 0.01. B) As in A, but cells were treated with PGF $_{2\alpha}$ 10nM respectively, for 24h in the presence of MDI. Relative Fra-1 mRNA levels were expressed as percentage to the respective negative control stimulated with vehicle only. The plots are representatives of two

independent experiments (n=2). Error bars represent $\pm$ SD. C) 80% confluent wild-type 3T3-L1 cells were transfected with a PPAR γ expression vector (pSG5- PPAR γ) or an empty vector (pcDNA) as a negative control and the next day were treated with 100 μ M AA in the presence of MDI for 24h. The total RNA of the cells was isolated and analyzed using qRT-PCR for Fra-1 transcripts. The graph is a representative of two independent experiments (n=2). All expression levels are relative to the empty vector vehicle control set as 100. Error bars represent $\pm$ SD.

A.



B



C

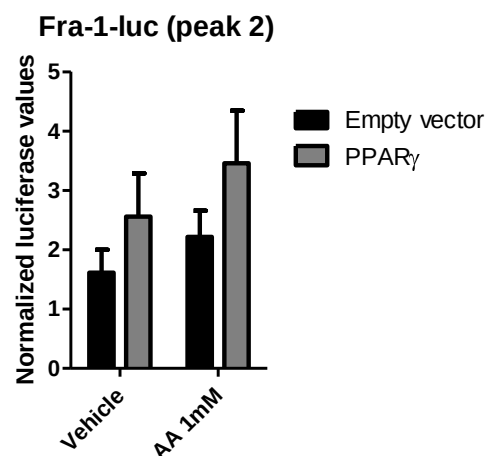


Figure 3.38 PPAR γ mediates the effect of AA on Fra-1 expression through sequences found upstream the transcription start site of *Fos/1* gene. A) ChIP-seq data during 3T3-L1 adipocyte differentiation showing PPAR γ sites for *Fos/1* locus. Base positions are indicated above. Green arrows indicate PPAR γ binding (adapted from Nielsen R., personal communication). 80% confluent 3T3-L1 cells were transfected with Fra-1-peak 1 (B) and Fra-1 peak 2 (C) luciferase constructs together with PPAR γ expression vector and a renilla

construct. The next day cells were treated with 1mM AA in the presence of MDI. After 24h the cells were harvested and the luciferase activity was measured. Activation of the luciferase reporter is expressed after normalisation for the renilla luciferase activity. The plots are representative of two experiments (n=2). Error bars represent $\pm$ SD.

3.2.7 Regulation of aP2 expression by Fra-1

aP2 and Fra-1 genes are both regulated from AA at the early stages of differentiation. The aP2 promoter has an AP-1 site where c-fos binds (Distel et al, 1987). Therefore, we hypothesised that in addition to PPAR γ , Fra-1 might also regulate aP2 expression. ChIP experiments were conducted using two days post-confluent 3T3-L1 and treated with AA (1mM) for 24h and chromatin was immunoprecipitated with a Fra-1 antibody. The data show that Fra-1 is bound to the aP2 promoter and binding is further enhanced by treatment with AA (Figure 3.39 A).

To further investigate the functional significance of the Fra-1 binding on aP2 promoter, reporter assays were conducted using the aP2 promoter/enhancer construct, co-transfecting increasing doses of Fra-1 expression vector and treating with AA for 24h in the presence of MDI. aP2 promoter/enhancer activity was not enhanced by Fra-1 (Figure 3.39 B). However, Fra-1 binds on promoter/enhancer sequences as a heterodimer with a member of the Jun protein family. Thus, 3T3-L1 cells were transfected with Fra-1 in combination with different Jun proteins, treated with AA in the presence of MDI and endogenous aP2 mRNA levels were assessed. Only the Fra-1 and JunD combination was able to enhance the effect of AA on aP2 expression (Figure 3.39 C).

Finally, to confirm the role of Fra-1 on aP2 expression, Fra-1 was depleted by an siRNA targeting endogenous Fra-1, the cells were then treated with AA for 24h and aP2 mRNA levels were assessed. Depleting cells from Fra-1 (around 50%) did not have any effect on aP2 expression (Figure 3.39 D, E).

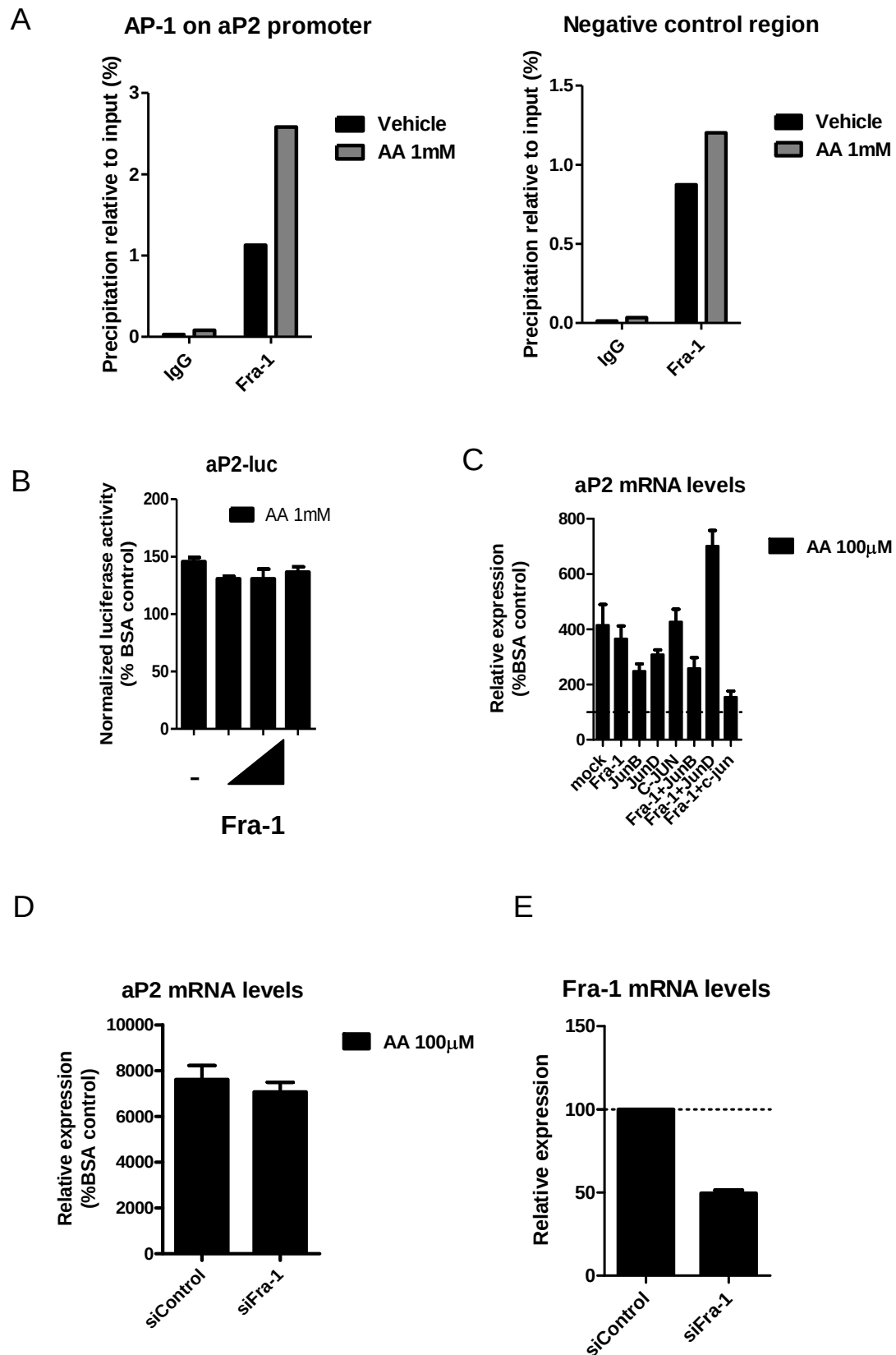


Figure 3.39 Regulation of aP2 expression by Fra-1. A) ChIP experiments were performed with cross-linked chromatin from two days post-confluent 3T3-L1 cells after treatment with 1mM AA in the presence of MDI. Antibodies used were against Fra-1 and IgG as a negative

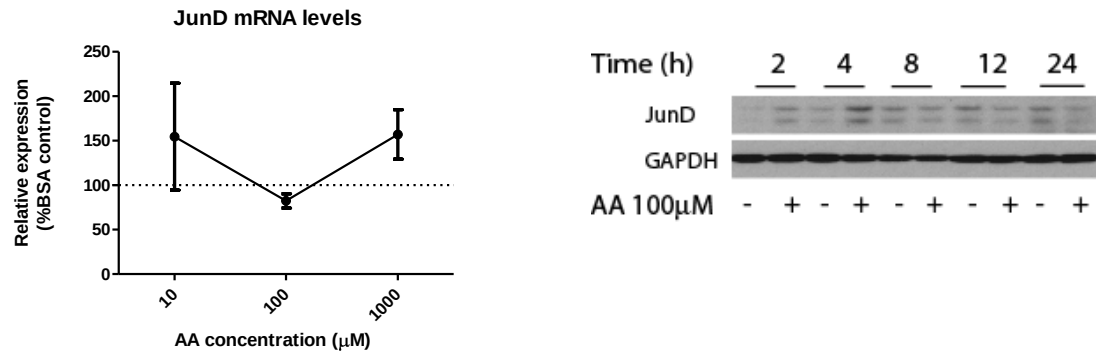
control. Precipitated DNA fragments were analysed by qRT-PCR and normalized to the input. The graph is a representative of three independent experiments. B) 80% confluent 3T3-L1 cells were transfected with aP2 promoter/enhancer luciferase construct together with a Fra-1 expression vector and a renilla construct. The next day cells were treated with 1mM AA in the presence of MDI. After 24h the cells were harvested and the luciferase activity was measured. Activation of the luciferase reporter is expressed after normalisation for the renilla luciferase activity. The plots are representative of two experiments (n=2). Error bars represent $\pm$ SD. C) 80% confluent 3T3-L1 cells were transfected with Fra-1, JunB, JunD and c-jun expression vectors. The next day cells were treated with 100 μ M AA in the presence of MDI. After 24h the cells total RNA was isolated and subjected to qRT-PCR for aP2 expression. The graph is a representative of two independent experiment (n=2). Error bars represent $\pm$ SD. D) and E) 80% confluent 3T3-L1 cells were transfected with a non targeting control (siControl) siRNA or an siRNA targeting Fra-1 (siFra-1). On the third day after transfection, cells were treated with 100 μ M AA for 24h in the presence of MDI. Cells were harvested and total RNA was isolated and qRT-PCR was conducted for aP2 mRNA and Fra-1 transcripts. The graphs are representative of two independent experiments (n=2). Error bars represent $\pm$ SD.

This observation indicates that maybe JunD is not expressed in sufficient concentrations upon AA treatment at this early time point of differentiation in order to interact with Fra-1 and regulate aP2. Based on this, the expression of different Jun proteins was examined by qRT-PCR and western blot (Figure 3.40). JunB and c-Jun RNA levels were up-regulated with a high dose of AA (1mM) but JunD expression was not affected by any dose of AA. The protein levels of JunB and c-Jun were increased after 30min of the MDI induction in an AA-independent way and then declined in the 24h time course. In contrast, JunD protein levels were increased with AA treatment in the first 4h and then declined. In conclusion, the protein levels of all Jun proteins are very low after 24h of induction of differentiation, and are not affected by AA treatment, making difficult to define the partner of Fra-1.

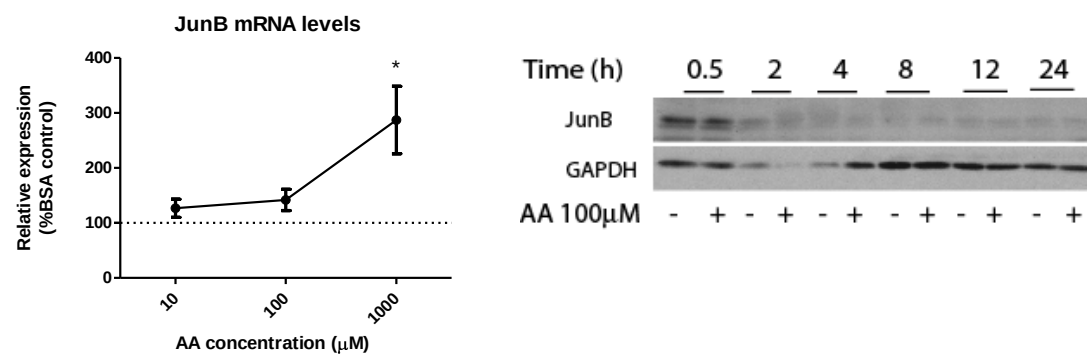
Another explanation for the lack of any effect of Fra-1 absence on aP2 expression could be a compensatory increase of another fos protein. For this reason the expression of c-Fos, Fra-2 and FosB was examined with increasing doses of AA or in the absence of Fra-1 (Figure 3.41). Only Fra-2 expression was up-regulated with a high dose of AA whereas the other two Fos proteins were not affected by any AA dose. In the absence of Fra-1 none of the Fos proteins expression tested was affected.

In conclusion, Fra-1 is present on aP2 promoter after AA treatment and could regulate aP2 expression but further experiments need to confirm if Fra-1 has an effect on aP2 expression

A



B



C

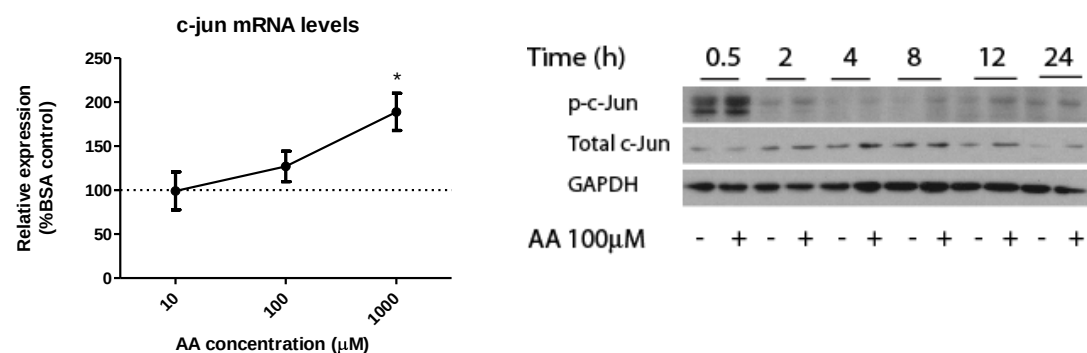


Figure 3.40: Expression of the Jun proteins after AA treatment in the early stages of differentiation. 3T3-L1 cells were treated either with increasing doses of AA and RNA was harvested after 24h of treatment or treated with 100μM AA and harvested at the indicated time points and whole cell lysates were harvested. qRT-PCR or western blotting was conducted for A) c-Jun, B) JunB and C) JunD. The values are mean percentages relative to

vehicle control (set as 100) of three independent experiments (n=3). Error bars indicate $\pm$ SEM.

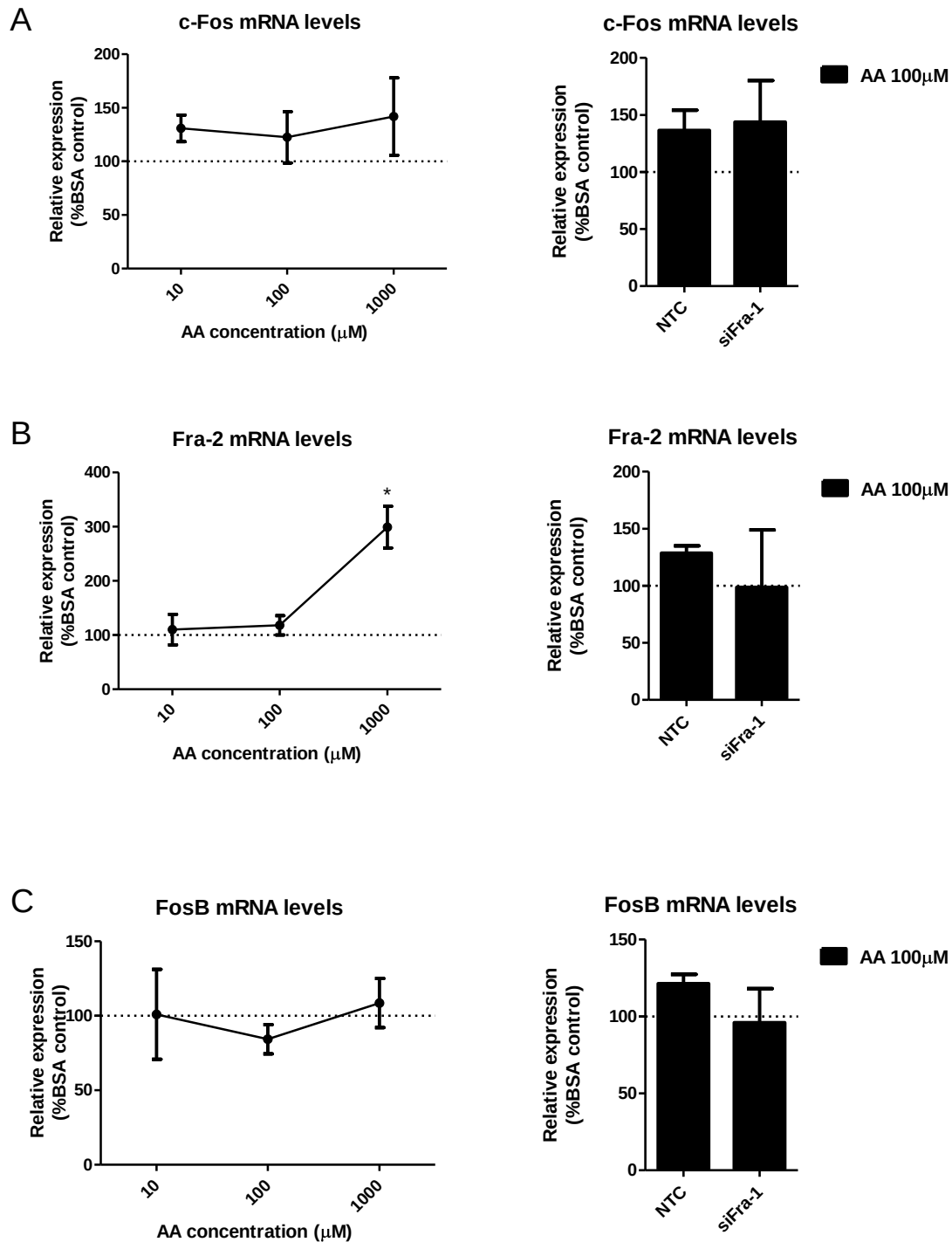


Figure 3.41: Expression of the Fos proteins after AA treatment and after Fra-1 depletion. 3T3-L1 cells were treated either with increasing doses of AA and RNA was harvested after 24h of treatment or transfected with siFra-1 and treated with 100 μ M AA for 24h. qRT-PCR was conducted for A) c-Fos, B) Fra-2 and C) FosB. The values are mean percentages relative to vehicle control (set as 100) of three independent experiments (n=3). Error bars indicate $\pm$ SEM.

3.3 Discussion

Excess fat has been correlated with hyperplasia and hypertrophy of AT. AA is one of the major PUFAs that is found in abundance in western diets. However, the role of AA in adipogenesis is controversial. Pre-adipocytes are exposed to changing levels of FAs under various normal and pathological metabolic states. It is not currently known how AA affects gene expression in pre-adipocytes and how this effect can alter their function. In the following sections, it will be discussed how AA regulates gene expression in the early stages of differentiation.

3.3.1 The effect of AA on adipocyte differentiation markers

The first observation made following treatment of pre-adipocytes with AA was a dose-dependent LD formation. LDs are predominantly found at the later stages of adipocyte differentiation when adipocytes are mature enough to store TAGs. However, LDs exist in virtually all types of cells when faced with lipid overload. In non-adipocytes, LDs are believed to function as lipid storage sites for later β -oxidation, membrane biogenesis, hormone synthesis, and other cellular functions. To delineate a potential enhancement of the differentiation program, the expression and the kinetics of a number of early and late adipocyte markers was assessed. The genes examined have a well-established role in adipocytes and some of them are also implicated in lipid metabolism.

It was found that AA altered the expression of only one late differentiation marker, aP2. The effect of AA on aP2 expression was dose- and also time-dependent. This observation is in agreement with previous reports where the expression of aP2 in adipocytes has been shown to be transcriptionally controlled by FAs (Amri et al, 1991). In particular, it has been shown that aP2 expression was increased by oleate treatment in pre-adipocyte lines (Distel et al, 1992).

Pre-adipocytes were treated with other PUFAs, such as EPA, DHA and LA to test the specificity of AA on aP2 expression. *In vivo* studies have shown that ω -3 PUFAs prevent HFD induced obesity in contrast to *in vitro* studies where EPA and DHA did not inhibit the differentiation program (Madsen et al., 2005; Manickam et al., 2010). However, there are no data describing the effect of these PUFAs on aP2 expression during the early stages of differentiation. All the PUFAs tested enhanced aP2 expression but AA seemed to be the most potent one. It should be noted that LA did not have a similar effect to AA on aP2 expression even though it is the precursor of AA. This might be because either the desaturases and elongases required to convert LA to AA are not highly expressed at this time point or these additional steps are delaying the potential effect of LA on aP2 expression.

Finally, the effect of AA on adipocyte differentiation in combination with the different components of the differentiation cocktail has been reported previously. In this context, AA was a weak inducer of the differentiation in the presence of DEX, in the presence of insulin and DEX, AA was a promoter of differentiation, whereas in the presence of the full MDI, AA inhibited differentiation (Madsen et al, 2005). These results indicate that elevated cAMP levels (with the inclusion of IBMX) are central for the inhibitory effect of AA on differentiation. In the context of aP2 regulation by AA elevated cAMP levels seem to be also crucial.

In conclusion, regulation of aP2 expression by AA is a gene-specific effect and does not alter the differentiation program. AA is a very potent PUFA enhancing aP2 expression and elevated cAMP levels might be important for this regulation.

3.3.2 The AA effect on aP2 expression is PG-mediated

Since AA can be metabolised by a number of enzymes to give rise to different eicosanoids, pre-adipocytes were treated with inhibitors of the three main enzymes using AA as a substrate. It has been shown before that these inhibitors can affect the adipocyte differentiation. Indomethacin is known to be

a direct ligand for PPAR γ and is included in the differentiation cocktail for various pre-adipocyte lines (Lehmann et al, 1997) and as expected it does not inhibit adipocyte differentiation (Madsen et al, 2003). A concentration of baicalein between 10 and 20 μ M has been shown to inhibit the differentiation of 3T3-L1 cells (Madsen et al, 2003). Finally, 17-ODYA does not have any effect on adipocyte differentiation (Madsen et al., 2003). From, these inhibitors only indomethacin blocked the induction of aP2 by AA, indicating that PGs mediate this effect. This observation is in agreement with previous reports, where PGs mediated the inhibitory effect of AA on adipocyte differentiation (Casimir et al., 1996).

Further support for this observation is the fact that both COX-2 and COX-1 mRNA expression was enhanced upon AA treatment. According to Pettersen et al., COX-2 and COX-1 are both expressed at the early stages of differentiation and they both peak during the first hours after the induction of differentiation and by 24h their expression decline. However, in the presence of AA, their expression is maintained after 24h of treatment (Pettersen et al., 2003). Thus our data together with the previous reports indicate that products from both COX isoforms are necessary for mediating the effect of AA on aP2 expression.

PGs have been implicated in adipocyte differentiation and can have opposing effects. PGI $_2$ and 15-deoxy- $\Delta^{12,14}$ PGJ $_2$ have a promoting effect whereas PGF $_{2\alpha}$ and PGE $_2$ have an inhibiting effect on adipocyte differentiation (Vassaux et. al, 1992; Forman et. al., 1995; Kliewer, et. al., 1995; Sugimoto et. al., 2004; Tsuboi et. al., 2004; Liu and Clipstone, 2007). The expression of different PG GPCRs was assessed and it was found that the FP, the EP4, the IP and the EP1 receptors are expressed at this stage of the differentiation. Our results are in agreement with previous publications where the expression of different PG receptor expression was tested during the differentiation of 3T3-L1 cells (Tsuboi et al, 2004). However, the authors could not detect the EP1 expression early in the differentiation. This might be due to the fact that they used semi-quantitative PCR instead of qRT-PCR in combination with the low levels of the receptor.

3.3.3 The signalling pathway mediating the effect of AA on aP2 expression

To begin delineating the signalling pathway triggered by AA that leads to aP2 expression regulation, the expression of different MAPKs was assessed. Using a p-JNK antibody we found that the bands detected are most likely attributable to ERK MAPK. This antibody is widely used and it was not possible to confirm with another one. In addition, AA did not show any effect on p-p38 MAPK activation. Although AA has been demonstrated to activate MAPKs in various cell types (Alexander et al, 2001; Talukdar et al, 2005), including p38 and JNK, there is relatively little reported about these pathways in the early stages of differentiation in adipocytes. Whereas the JNK pathway does not seem to be involved directly in adipocyte differentiation, p38 MAPK role remains unclear. There are conflicting results showing that p38 MAPK is essential for 3T3-L1 differentiation, whereas loss of p38 MAPK promotes adipogenesis in MEFs (Engelman et al., 1998; Aouadi et al., 2006). ERK MAPK signalling has been shown to be important for the early steps of the adipocyte differentiation program, whereas sustained activation of this signalling inhibits adipocyte differentiation (Prusty et al., 2002). Furthermore, it has been demonstrated that AA can activate ERK during the early stages of adipocyte differentiation (Pettersen et al, 2003). This is in agreement with our observations, where ERK was activated very early after the addition of MDI in the presence or absence of AA but its expression was above the basal levels when treated with AA. Finally, treating with a MEK inhibitor the effect of AA on aP2 expression was substantially blocked, showing that the ERK pathway is involved in the pathway triggered by AA to regulate aP2.

The next step to define the signalling pathway activated by AA stimulation, leading to aP2 up-regulation was to determine the PG GPCR that mediates the effect of AA. GPCRs interact with G proteins composed of α , β and γ subunits. Agonist binding triggers a conformational change in the receptor, which catalyses the dissociation of $G\alpha$ from $G\beta\gamma$ subunits. The α subunits of G proteins are divided into three main subfamilies: $G_{\alpha s}$, $G_{\alpha i}$ and $G_{\alpha q}$. A single GPCR can couple to either one or more families of $G\alpha$ proteins.

Typically $G_{\alpha s}$ stimulates adenylyl cyclase (AC) and increases levels of cAMP, $G_{\alpha i}$ inhibits AC and lowers cAMP levels, and $G_{\alpha q}$ bind to and activate PLC, which cleaves PIP_2 into DAG and IP₃. IP₃ triggers the release of calcium from intracellular stores, and DAG in conjunction with calcium ions recruits PKC to the membrane and activates it (Neves et al., 2002) (Figure 3.42).

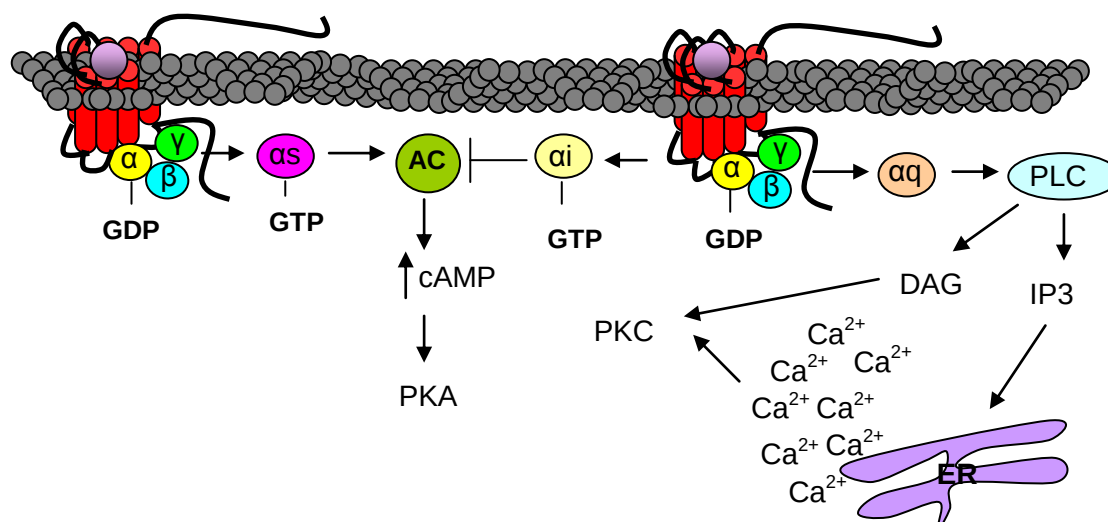


Figure 3.42: Schematic representation of some of the signalling pathways activated by GPCRs. GPCRs interact with G proteins subunits α , β and γ which are bound to GDP under resting conditions. Agonist binding results in dissociation of the GDP from the G_{α} subunit and binding to GTP whereas at the same time causes the dissociation of the G_{α} and $G_{\beta\gamma}$ subunits from each other and from the GPCR. The $G_{\beta\gamma}$ subunits can regulate certain downstream effectors, such as ion channels and PLC. The G_{α} subunits are divided into three subfamilies: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$. Typically the $G_{\alpha s}$ subunit activates AC leading to increases in intracellular cAMP, while $G_{\alpha i}$ subunit inhibits AC activity, decreasing cAMP levels. The $G_{\alpha q}$ subtype activates PLC, resulting in the production of IP₃ and DAG. IP₃, via its receptor, releases calcium from intracellular stores. These second messengers (cAMP, DAG and IP₃) can modulate further downstream effectors, such as PKA and PKC (adapted from Ritter and Hall, 2009).

Either through pharmacological inhibition, by using a selective FP antagonist, or by depleting the FP receptor using siRNA, the effect of AA on ERK activation and aP2 up-regulation was blocked. Therefore, as ERK mediates the effect of AA on aP2 expression and since the FP receptor is essential for the ERK activation, then the FP receptor upon AA, signals to ERK which regulates aP2. This is in agreement with previous reports, showing that upon activation the FP receptor is triggering the MEK/ERK pathway to regulate the

expression of the COX-2 gene at the early stages of differentiation in 3T3-L1 cells (Ueno and Fujimori, 2011). This report together with our data indicate that there is a positive feedback loop upon AA treatment COX-2 is induced which then elevates PG levels which in turn allow a further induction of COX-2 and aP2 expression.

In addition, AA causes the generation of two second messengers cAMP and Ca^{2+} . We showed that the cAMP generation is through the EP4 receptor. This observation is in agreement with previous reports describing the EP4 to be G α s coupled, activating AC to raise cAMP intracellular levels and through this pathway EP4 inhibits adipocyte differentiation (Tsuboi et al., 2004). We also show that the calcium generation is mediated through the FP receptor, as predicted for GPCRs coupled to G α q. This observation is in accordance with reports that PGF $_{2\alpha}$ inhibits adipocyte differentiation via a calcium-dependent signalling pathway (Liu and Clipstone, 2007). Therefore, AA treatment seems to activate both EP4 and FP receptors triggering different signalling pathways. These pathways do not appear to interact as a calcium chelator did not affect AA-mediated cAMP production. However, it cannot be excluded that cAMP could affect calcium signalling. Nevertheless, the calcium chelator blocked both ERK activation and aP2 up-regulation triggered by AA showing the importance of calcium as a second messenger for mediating responses to AA.

As discussed in the previous section IBMX was the component of the differentiation cocktail necessary for the AA-dependent induction of aP2 expression. This observation in combination with the fact that AA increases cAMP production, suggested that AA could act through a PKA-dependent pathway. However, when cells were treated with a PKA inhibitor, AA-induced aP2 up-regulation was not significantly blocked. On the other hand, both AA effects on aP2 and ERK were completely blocked when cells were treated with a PKC inhibitor. However, other targets of cAMP cannot be excluded.

In conclusion, AA after its conversion to PGs by COXs, PGF $_{2\alpha}$ is generated and binds to its membrane GPCR, the FP receptor. This binding causes the increase of intracellular calcium levels, which activate PKC and in turn

activates ERK. ERK signals through undefined steps to increase aP2 transcription (Figure 3. 43).

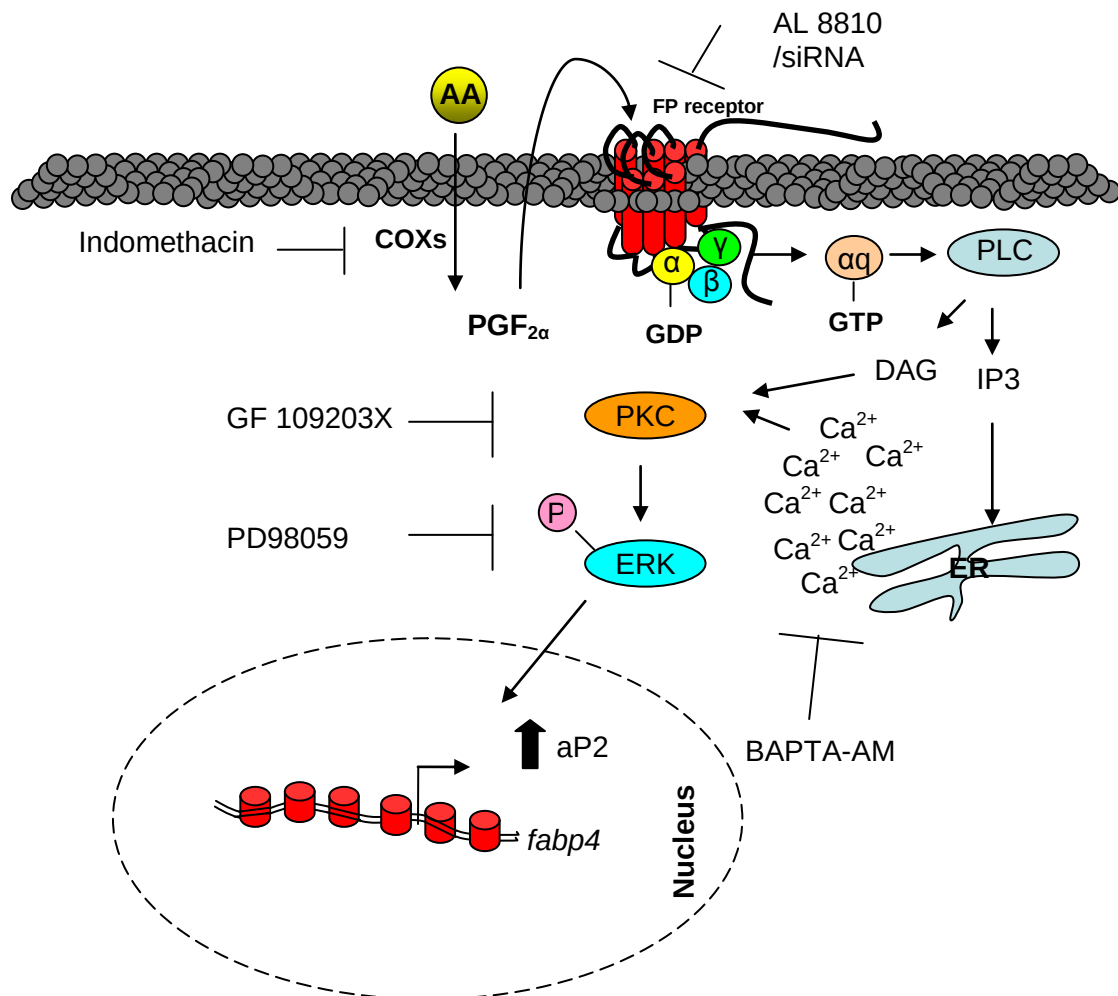


Figure 3.43: Schematic representation of the proposed mechanism triggered by AA to regulate aP2 expression in the early phase of adipogenesis AA is metabolised through COXs to PGs. PGF_{2α} is generated and binds to the FP receptor which is coupled to Gαq triggering a calcium response. PKC is then activated and phosphorylates ERK which induces (through an unknown mechanism) aP2 transcription. It is also illustrated the pharmacological tools used to elucidate the signalling pathway. Indomethacin: COX inhibitor, GF109203X: PKC inhibitor, PD98059: ERK inhibitor, AL8810: FP antagonist, BAPTA-AM: calcium chelator.

3.3.4 The effect of AA on aP2 expression is PPAR γ -mediated

The importance of PPAR γ in adipocyte differentiation is well recognised. Exogenous FAs are required for adipocyte differentiation since pre-adipocytes cannot differentiate in delipidated serum (Chawla and Lazar, 1994). Tzameli and colleagues have shown that PPAR γ ligand(s) is produced early and transiently during adipocyte differentiation in a cAMP-dependent manner (Tzameli et al., 2004). However, the identity of the endogenous PPAR γ ligand(s) promoting the early stages of adipocyte differentiation has so far not been identified. Studies showed that PGs can act as potential endogenous agonists promoting adipocyte differentiation. The naturally occurring 15d-PGJ₂ has been shown to activate PPAR γ and promote adipocyte differentiation (Forman et al., 1995). However, there is evidence arguing that 15d-PGJ₂ is not a *bona fide* endogenous agonist during adipocyte differentiation as the absolute levels detected in adipocyte cultures were not sufficient to activate PPAR γ (Bell-Parikh et al., 2003; Xie et al., 2006).

aP2 transcriptional regulation has been thoroughly described as both the aP2 proximal promoter and enhancer have been well characterised. The aP2 proximal promoter region contains binding sites for AP-1 at -120bp and C/EBPs at -140bp relative to the transcription start site (Christy et al, 1989). However, this proximal promoter is not sufficient or necessary to direct adipose-specific gene expression. Further upstream there is a 518bp enhancer element from -5.4kb to -4.9kb that drives gene expression to AT (Ross et al., 1990). In this enhancer region two PPRE elements were found (Graves et al., 1992), where the heterodimer PPAR γ 2/RXR α binds and drives adipose-specific aP2 expression (Tontonoz et al., 1994a).

Here we show that PPAR γ mediates the effect of AA on aP2 expression in the early stages of adipocyte differentiation. The effect of AA on aP2 expression was blocked by both a pharmacological inhibition (using a selective PPAR γ antagonist) and stable depletion of PPAR γ . However, it was not possible to determine if the effect of PPAR γ on aP2 expression was direct or indirect *i.e.* by binding directly to the PPRES of aP2 enhancer, as PPAR γ protein levels

are greatly reduced upon AA treatment. This observation is in agreement with reports that ligands for PPAR γ can decrease the amount of PPAR γ protein (Hauser et al., 2000). However, according to the literature PPAR γ requires a functional DBD to activate its target genes (Tontonoz et al., 1994b), indicating that it must bind on the aP2 enhancer. In the presence of reduced PPAR γ levels ChIP might not be a sensitive enough technique to detect this binding.

Regulation of aP2 expression by FAs has been reported before in response to oleate in pre-adipocytes. However, the authors dismissed the idea of this event being PPAR γ -mediated as none of other PPAR γ gene targets were affected (Distel et al., 1992). Nevertheless, there are reports identifying PPAR γ 2 to be activated very early in the differentiation procedure when the ligands are not abundant, suggesting a key role for PPAR γ 2 in driving the initiation of adipogenesis (Saladin et al., 1999). Moreover, PPAR γ has been shown to have a larger ligand binding cavity than observed in other nuclear receptors. This is likely to allow PPAR γ to be more sensitive to the presence of a low concentration of ligands as it could be able to sense not only a single ligand but a pool of related ligands, making PPAR γ a sensor of FAs (Itoh et al., 2008). Thus, as PPAR γ 2 is expressed early in the differentiation process the possibility is raised that it can be activated upon AA treatment during these early stages. AA could cause the generation of ligands for PPAR γ which would activate it in order to regulate aP2, which unlike other PPAR γ gene targets, is constitutively associated with co-activators (Guan et al., 2005).

To test if AA is acting through the LBD of PPAR γ , we used different mutant versions of PPAR γ 2, transfected into NIH-3T3 cells, treated with AA and assessed endogenous aP2 expression. These PPAR γ mutants have different effects in the differentiation procedure. The PPAR γ 2-S112A mutant which express a PPAR γ 2 form that cannot be phosphorylated drives the differentiation program even more efficiently than the wild-type receptor (Hu et al., 1996). The PPAR γ 2-E499Q mutant has a disrupted α -helix as a result a perturbed ligand-dependent conformational change of the AF2 region and/or docking of cofactors. This mutated form can bind ligands but cannot drive the differentiation program as the mutated receptor is inactive (Hauser et al.,

2000; Walkey and Spiegelman, 2008). Finally, the mutation of the PPAR γ 2-Q286P occurs in the middle of α -helix 3 of the LBD, which forms the backbone of the ligand-binding pocket. This mutant is unable to bind or be activated by virtually all known agonists but can drive the differentiation program, indicating that a functional LBD is not necessary for adipogenesis (Walkey and Spiegelman, 2008). In the induction of aP2 the PPAR γ 2-Q286P mutant seems to block the action of AA, indicating that AA acts through the LBD. To confirm this observation, we presented evidence that the activation of a PPAR γ LBD reporter during adipogenesis was sensitive to AA.

We showed that PGF $_{2\alpha}$ is the PG that mediates the effect of AA on aP2 expression. However, PGF $_{2\alpha}$ has been shown not to be a ligand for PPAR γ (Yu et al., 1995; Forman et al., 1995), indicating that PGF $_{2\alpha}$ is not the ligand produced by AA to mediate both effects on aP2 expression and PPAR γ activation. PGF $_{2\alpha}$ has been reported before (but also shown here) to activate ERK (Ueno and Fujimori, 2011). This ERK activation leads to the phosphorylation of PPAR γ 2 at the S112 residue (equivalent to S82 of PPAR γ 1). The phosphorylation of that residue has been shown to decrease PPAR γ transcriptional activity (Camp and Tafuri, 1997; Hu et al., 1996). The same phosphorylation event has been shown to be caused by insulin that is an adipogenic agent included in the differentiation cocktail, showing that the ability to balance cell growth and differentiation is critical (Hu et al., 1996).

There are reports of studies that elucidate the mechanism by which this phosphorylation event inhibits PPAR γ activity. Adams et al. showed that phosphorylation of S112 affected both ligand-independent and ligand-dependent transcription. The authors speculated that this phosphorylation event could influence transcription by altering the preinitiation complex formation or stability or might influence PPAR γ interaction with specific coactivators or corepressors (Adams et al., 1997). Moreover, Shao et al. showed that there is an interdomain communication between the AF-1 domain (where the phosphorylation event occurs) with the LBD, indicating that the phosphorylation is affecting the ligand binding (Shao et al., 1998).

Taking together all our data, the $\text{PGF}_{2\alpha}$ -FP receptor-calcium-PKC-ERK pathway seem to lead to the phosphorylation of PPAR γ . However, AA increases also the intracellular cAMP levels which are connected with the production of PPAR γ ligands at the early stages of differentiation (Tzameli et al., 2004) but also with the inhibition of ERK activity in adipocytes (Sevetson et al., 1993). Thus, it seems there is parallel pathway that is opposing the $\text{PGF}_{2\alpha}$ pathway producing ligands for PPAR γ . In conclusion, during the normal adipocyte differentiation program the endogenous PPAR γ is in equilibrium between the phosphorylated and non-phosphorylated state. In the early stages of differentiation and upon AA treatment, PPAR γ is activated by the AA-produced ligands which lead to aP2 activation whereas on the other hand, it gets phosphorylated and inactivated.

3.3.5 The function of increased aP2 expression upon AA treatment

Since aP2 up-regulation was established the next question addressed was the function of this increased expression. To answer that question, a PCR array was performed including 84 genes involved in FA metabolism. Following this approach we hypothesised that the pathways being affected by the increase of aP2 expression could be identified. For this reason pre-adipocytes were treated with AA and the genes *Acsl4* and *CPT-1a* (in addition to aP2) were found to be up-regulated more than 2-fold.

Acsl4 is a long chain acyl-CoA synthetase which preferentially uses AA as a substrate (Cao et al, 1998). *Acsl4* converts AA into AA-CoA esters reducing intracellular free AA for both synthesis of cellular lipids, and degradation via β -oxidation. Thus, it was logical for *Acsl4* to be up-regulated upon AA treatment since AA has to be esterified in order to be used. The other gene up-regulated was *CPT1a* which is the key enzyme in FA oxidation. *CPT1a* is found in the outer membrane of mitochondria and catalyses the formation of long chain acyl-carnitine, which is enabled to traverse the inner mitochondrial membrane and thus committed to β -oxidation in the mitochondria (Gao et al., 2011). Overall this data suggested that the increase in the aP2 levels could mean that aP2 escorts the oversupply of AA to be degraded through the β -oxidation

process. However, preliminary data measuring β -oxidation (data not shown) indicated that β -oxidation was actually lower in the AA-treated cells in comparison with the untreated cells. Increased CPT1a mRNA along with increased β -oxidation has been reported before in epididymal fat of mice fed a rich EPA/DHA diet (Flachs et al., 2005). Moreover, increased β -oxidation but not increased CPT1a mRNA levels were observed in 3T3-L1 adipocytes when treated with EPA (Guo et al., 2005). There are currently no reports assessing the effect of AA treatment on β -oxidation in pre-adipocytes. In conclusion, more experiments are needed to define whether AA treatment promotes β -oxidation, mitochondrial biogenesis or the effect on CPT1a mRNA levels is a gene-specific event.

In parallel, genes that were down-regulated upon AA treatment were assessed. Similarly, genes that were down-regulated more than 2-fold considered to be significantly down-regulated. Only one gene appeared to be more than 2-fold down-regulated and also the vehicle control Ct value to be less than 30. This gene was the *Acat2* or acyl CoA:cholesterol acyltransferase. *Acat2* together with *Acat1* are the enzymes that catalyse the cholesterol ester synthesis from cholesterol and long-chain fatty acyl-CoA (Tomoda and Omura, 2007). Down-regulation of this enzyme may indicate means that the main lipid found in LDs are TAGs and to a lesser extent cholesterol esters in pre-adipocytes upon AA treatment.

A possible function of the increased aP2 expression could be to transfer AA for degradation through the macroautophagy pathway. Macroautophagy has been implicated in adipocyte differentiation. Knocking down *Atg7* in 3T3-L1 cells inhibited differentiation (Singh et al., 2009a). Inhibiting autophagy either by a pharmacological inhibitor or by a stable depletion of *Atg7* further enhanced aP2 expression upon AA treatment. These data indicate that by blocking this degradative process, aP2 expression is enhanced due to increases in cellular lipid levels. Thus, to avoid the cellular lipotoxic effects, aP2 is induced to bind the excess lipid. On the other hand, when aP2 was depleted autophagy decreased but this time this effect was not AA-related.

These data show that aP2 expression is essential for the autophagy procedure to occur in pre-adipocytes, indicating that aP2 is mediating the transfer of lipids to autophagosomes or LDs which later could be sequestered in autophagosomes.

3.3.6 The effect of AA on Fra-1 expression

Fra-1 is a basic-leucine zipper (bZIP) AP-1 transcription factor that has been shown to be induced early in the adipocyte differentiation program (Stephens et al., 1992). In addition, Fra-1 expression is enhanced by PPAR γ agonists (Albrechtsen and Fleckner, 2001), FAs (Distel et al., 1992), mitogens (Adiseshaiah et al., 2003) and insulin (Hurd et al., 2002). We showed that Fra-1 mRNA and protein levels were enhanced upon AA treatment at the early stages of differentiation. This effect was COX-mediated and PGF $_{2\alpha}$ was the PG that could mimic the effect of AA. Furthermore, this observation was further confirmed by inhibiting the FP receptor either by pharmacological inhibition (FP antagonist) or by depleting the FP receptor. However, it should be noted that the effect of PGF $_{2\alpha}$ on Fra-1 expression was not dose-dependent as the higher doses of PGF $_{2\alpha}$ failed to increase Fra-1 expression. One explanation is the homologous desensitisation *i.e.* a process occurring in response to high doses of an agonist to GPCRs which are then “turned off” or desensitized (Hosey, 1999). Thus, the FP receptor in response to high doses of PGF $_{2\alpha}$ is inactivated unable to mediate the PGF $_{2\alpha}$ effect on Fra-1 expression. In conclusion, the pathway AA-COX-PGF $_{2\alpha}$ -FP regulates Fra-1 expression at the early stages of adipocyte differentiation. This is the first time reported that Fra-1 is regulated by an AA metabolite.

Since AA induces cAMP and calcium intracellular levels, PKA and PKC kinases could potentially be activated and regulate Fra-1 expression. Using selective inhibitors we found that both pathways have the potential to regulate, to some degree, the expression of Fra-1. However, the PKC inhibitor more efficiently blocked the effect of AA on Fra-1 mRNA and protein levels. This observation is in agreement with the FP receptor activation and its coupling with G α_q . Although there is no literature supporting these observations in

adipocytes, others have observed increased Fra-1 expression after treatment with isoproterenol, a cAMP-increasing and PKA-activating compound in chondrocytes (Mitchell et al., 2011). In astrocytes increased levels of Fra-1 expression are reported after treatment with a phorbol-12-myristate-13-acetate (PMA), a PKC-activating compound (Won and Suh, 2000). In conclusion, both PKA and PKC pathways could activate Fra-1 expression upon AA treatment.

In addition, it was tested if the ERK activation by AA was involved in Fra-1 induction. AA-induced Fra-1 up-regulation could be blocked by the addition of one of two different MEK inhibitors. This is in agreement with previous reports that have identified ERK as an important regulator of Fra-1 expression but not JNK1/2 or p38 (Adiseshaiah et al., 2008). The ERK pathway regulates Fra-1 expression at two levels, transcriptionally but also post-translationally. It has been shown that ERK activates the transcription factors binding to regulatory elements found upstream the transcription start site of Fra-1 gene (Adiseshaiah et al., 2003; Adiseshaiah et al., 2005). ERK also phosphorylates Fra-1 protein in two serines, S252 and S265 stabilising the Fra-1 protein (Basbous et al., 2007). In addition, different thresholds of ERK activity control Fra-1 transcription and stability *i.e.* moderate ERK activation is enough to induce Fra-1 transcription whereas strong ERK activation leads to Fra-1 phosphorylation preventing its degradation by the proteasome (Vial and Marshall, 2003). Finally, the ERK pathway has been shown to affect Fra-1 function beyond regulating its transcription or protein stability but also regulating its transactivation activity as phosphorylation of Thr-231 by ERK is required for Fra-1 activation of its transactivation domain (Young et al., 2002). We show that AA increases cAMP intracellular levels through the EP4 receptor that could lead to a pathway activating PKA. We also showed that PGE₂ is activating ERK. Thus, it is possible even though the EP4 receptor antagonist does not have any effect on Fra-1 mRNA levels that there are two parallel pathways triggered by AA. One would be the AA-COX-PGF_{2α}-FP-PKC-ERK and the other one AA-COX-PGE₂-EP₄-PKA-ERK. In conclusion, it seems that the PGF_{2α} pathway is the dominant pathway in Fra-1 induction as

the EP4 antagonist or the EP4 knockdown does not affect Fra-1 mRNA levels but still may play a role as its induction is PKA-dependent (Figure 3.44).

Finally the role of nuclear receptor in driving Fra-1 transcription was investigated. Fra-1 has been shown to be transcriptionally regulated by c-Fos in rat fibroblasts by interacting with an AP-1 site on the first intron of Fra-1 (Bergers et al., 1995). The same site was also shown to bind Fra-1 itself, revealing an autoregulatory mechanism sustaining Fra-1 expression (Casalino et al., 2003). In addition, three transcription factor binding elements, a 12-O-tetradecanoylphorbol-13-acetate (TPA) response element (TRE/AP-1), an activating transcription factor (ATF/CREB) element and a serum response element (SRE) have been characterised in an enhancer element upstream Fra-1 transcription start site (Adiseshaiah et al., 2003; Adiseshaiah et al., 2005). Rosiglitazone enhances Fra-1 expression in 3T3-L1 cells, indicating that PPAR γ could play a role (Albrektsen and Fleckner, 2001). A PPAR γ ChIP-sequencing experiment revealed that PPAR γ binds to four different sequences upstream and downstream of the Fra-1 gene but also to a sequence found in the second intron, during 3T3-L1 differentiation (Nielsen R. personal communication). Thus, using two different approaches of depleting or overexpressing PPAR γ , we showed that PPAR γ can regulate Fra-1 expression upon AA treatment. To further characterise this regulation, two sequences of the four detected from the PPAR γ ChIP-sequencing experiment, were cloned upstream the luciferase gene. Peak 1 is the potential PPAR γ binding element found approximately -14,000bps upstream the transcription start site. Based on the ChIP-sequencing analysis, PPAR γ binds to that region as early as the first day of differentiation and the binding appears to get stronger during the course of the differentiation. The luciferase reporter construct harbouring peak 1 sequence was activated upon AA treatment and this activation was potentiated in the presence of PPAR γ . These data indicate that this sequence could mediate PPAR γ action on Fra-1 expression. However, further experiments need to be done in order to characterise this sequence as a novel PPAR γ binding site on Fra-1 promoter.

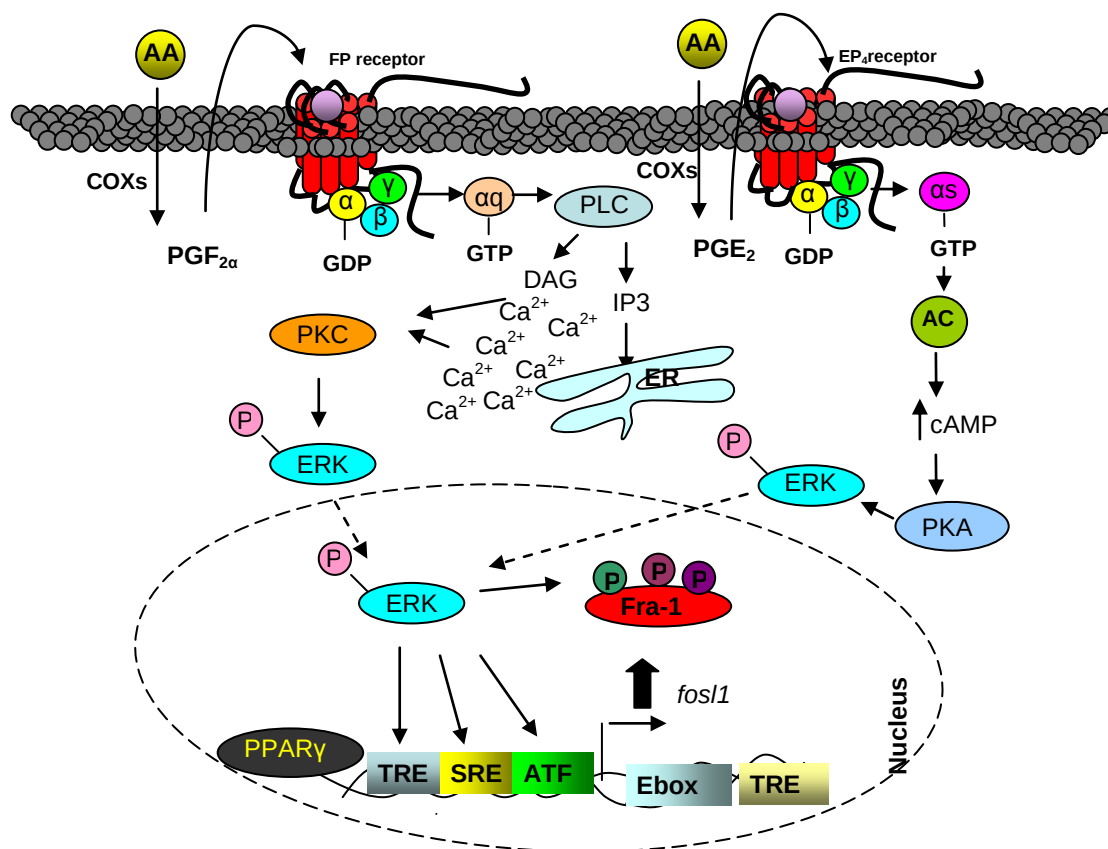


Figure 3.44: Schematic representation of the proposed mechanism triggered by AA to regulate Fra-1 expression in the early phase of adipogenesis. AA is metabolised through COXs to PGs. PGF_{2α} and PGE₂ generated bind to their respective GPCRs, FP and EP₄ receptor respectively. FP is coupled to Gαq and EP₄ to Gαs triggering calcium and cAMP generation, respectively. Both of these pathways activate ERK but the FP-mediated pathway is the dominant one (thick arrow). ERK induce Fra-1 transcription, stabilise and activate Fra-1 protein. PPARγ is also regulating Fra-1 expression probably by a direct binding upstream the transcription start site of *fosl1* gene.

3.3.7 The regulation of aP2 expression by Fra-1 upon AA treatment

It was investigated whether the up-regulation seen in both aP2 and Fra-1 genes upon AA treatment was connected. There is only one report by Distel et al., correlating an AP-1 transcription factor with aP2. The authors identified binding of c-Fos on aP2 promoter characterising c-Fos binding as a negative regulator of aP2 gene expression (Distel et al., 1987). It is possible that Fra-1 replaces c-Fos on the AP-1 site on aP2 promoter as Fra-1 has been shown to substitute for c-Fos (Fleischmann et al., 2000). Thus, Fra-1 binding on the aP2 promoter was investigated using primers flanking the AP1 site found at -

120bps upstream the transcription start site of the aP2 gene (Christy et al., 1989). Fra-1 was found to be recruited to the aP2 promoter in the vehicle-treated cells indicating that the differentiation cocktail might drive this event due to Fra-1 being an early induced gene during the differentiation of 3T3-L1 cells (Stephens et al., 1992). However, upon AA treatment a greater enrichment of Fra-1 on aP2 promoter was observed, indicating that AA drives Fra-1 recruitment to the aP2 promoter. It has been shown that AP-1 factors can act as either positive or negative regulators of transcription depending on their composition, the target gene, the cell context, or the signals received from the environment (Chinenov and Kerppola, 2001). To further investigate if Fra-1 binding has a positive or a negative impact on aP2 expression, different approaches were undertaken. Fra-1 overexpression and Fra-1 depletion did not affect on aP2 expression. However, Fra-1 depletion was around 50% which could mean that the remaining Fra-1 is enough to drive aP2 expression. Fos and Jun family proteins have both unique roles that cannot be compensated for by other family members but also overlapping functions. Thus, different Fos members could compensate for the lack of Fra-1. However, more experiments need to be undertaken to assess this question as the gene expression profile of the other Fos proteins did not indicate any up-regulation to compensate for Fra-1 depletion.

To further investigate the impact of Fra-1 on aP2 expression, Fra-1 was expressed in combination with different Jun proteins. AP1 factors through dimerization can form 18 different homo and heterodimers (Chinenov and Kerppola, 2001). The only combination that appeared to have a positive effect on aP2 expression greater than the empty vector control was the Fra-1 and JunD combination. The Fra-1 and JunD interaction has been shown before in differentiated 3T3-L1 cells upon rosiglitazone treatment (Albrechtsen and Fleckner, 2001). However, further experiments need to be undertaken to characterise if this is a *bona fide* interaction upon AA treatment. Although, it should be noted that Fra-1 can interact with other bZIP transcription factors such as ATFs (such as ATF4) (Hai and Curran, 1991) but also with basic-helix-loop-helix-leucine zipper (bHLHZip) transcription factors (such as USF1) (Pognonec et al., 1997).

Chapter 4

Results: Arachidonic acid effects on the late stages of adipocyte differentiation

4.1 Introduction

Adipocyte differentiation is a complex procedure involving the coordinated action of many different factors in a temporal sequence. In humans, AT develops in the second trimester of pregnancy and more extensively in the third trimester and after birth. However, adipogenesis occurs throughout the adult life as approximately 10% of adipocytes turn over in human AT every year (Spalding et al., 2008). Very little is known about the number or location of pre-adipocytes *in vivo*. Even less is known about how different stimuli at the early events of differentiation can affect the differentiation potential of pre-adipocytes. Dietary fat has been correlated with hyperplasia of AT, thereby affecting the number of adipocytes (Faust et. al., 1978). Therefore, it was hypothesised that exposure of pre-adipocytes to AA can affect the early stages of AT development.

In this chapter, experiments were designed to address if AA stimulus at the early stage of adipocyte differentiation can affect the differentiation potential of pre-adipocytes. The mechanism connecting the effect of AA at early events with the late events of differentiation is also addressed.

4.2. Results

4.2.1 Inhibition of adipocyte differentiation after long exposure to AA

To elucidate the effect of AA on the differentiation of 3T3-L1 cells, two days post-confluent cells (designated as Day 0 or D0) were treated with 1 mM, 100 μ M or 10 μ M AA in the presence of the differentiation cocktail (MDI) for 10 days (D10) (Figure 4.1). As a first indicator of differentiation, lipid accumulation was assessed by staining the cells with Oil Red O on D10 after the induction of differentiation. Control vehicle-treated cells became spherical and accumulated LDs. Treatment with 10 μ M AA did not have any effect on lipid accumulation, whereas treating with 100 μ M AA resulted in less lipid accumulation compared to the vehicle-control. Finally, in the presence of 1mM AA there was even less lipid accumulation (Figure 4.2).

To further confirm the effect of AA on the adipocyte differentiation process, the expression of a number of differentiation markers was assessed. Treatment with 1mM AA resulted in the down-regulation of adipocyte marker genes such as aP2, PPAR γ , C/EBP α , FAS, GLUT-4 and LPL. Down-regulation of these genes was also observed with 100 μ M but not with the lower dose 10 μ M (Figure 4.3).

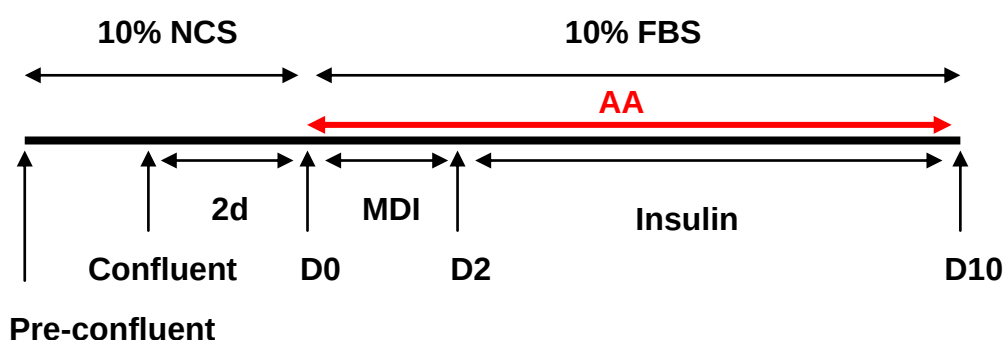


Figure 4.1: Experimental procedure for long exposure to AA. 3T3-L1 cells were induced to differentiate at two days post-confluence (D0) with DMEM containing 10% (v/v) FBS, 250nM DEX, 0.5 mM IBMX and 170nM insulin. After 48 h and for the rest of the differentiation, the cells were replenished with DMEM containing 10% (v/v) FBS and 170nM insulin and the medium was changed every second day. AA conjugated with BSA was added when differentiation was induced on D0 and was present throughout the differentiation period.

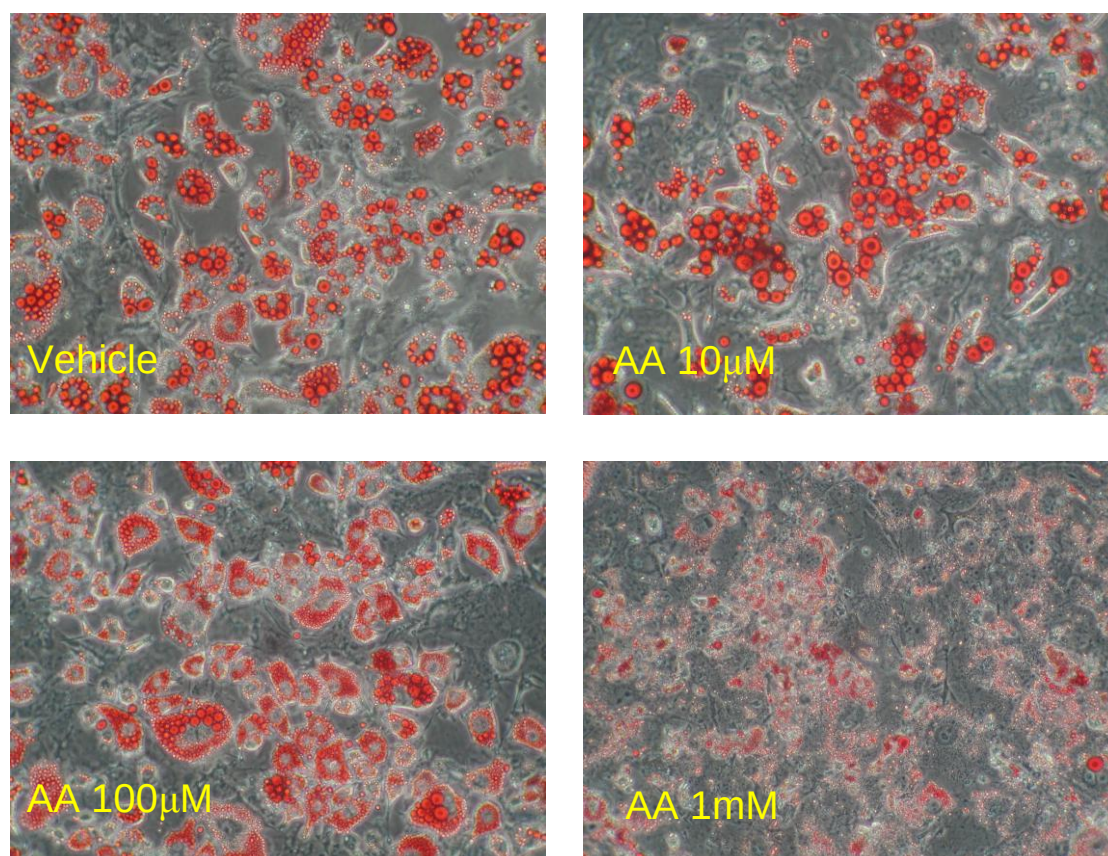


Figure 4.2: AA-dependent inhibition of adipocyte differentiation after long exposure to AA-lipid accumulation. Two-day-post-confluent 3T3-L1 pre-adipocytes were induced to differentiate in the absence or presence of AA (10μM, 100μM or 1mM) with the standard differentiation cocktail. Cells were fixed and stained with Oil Red O on D10 of the differentiation program (20x magnification).

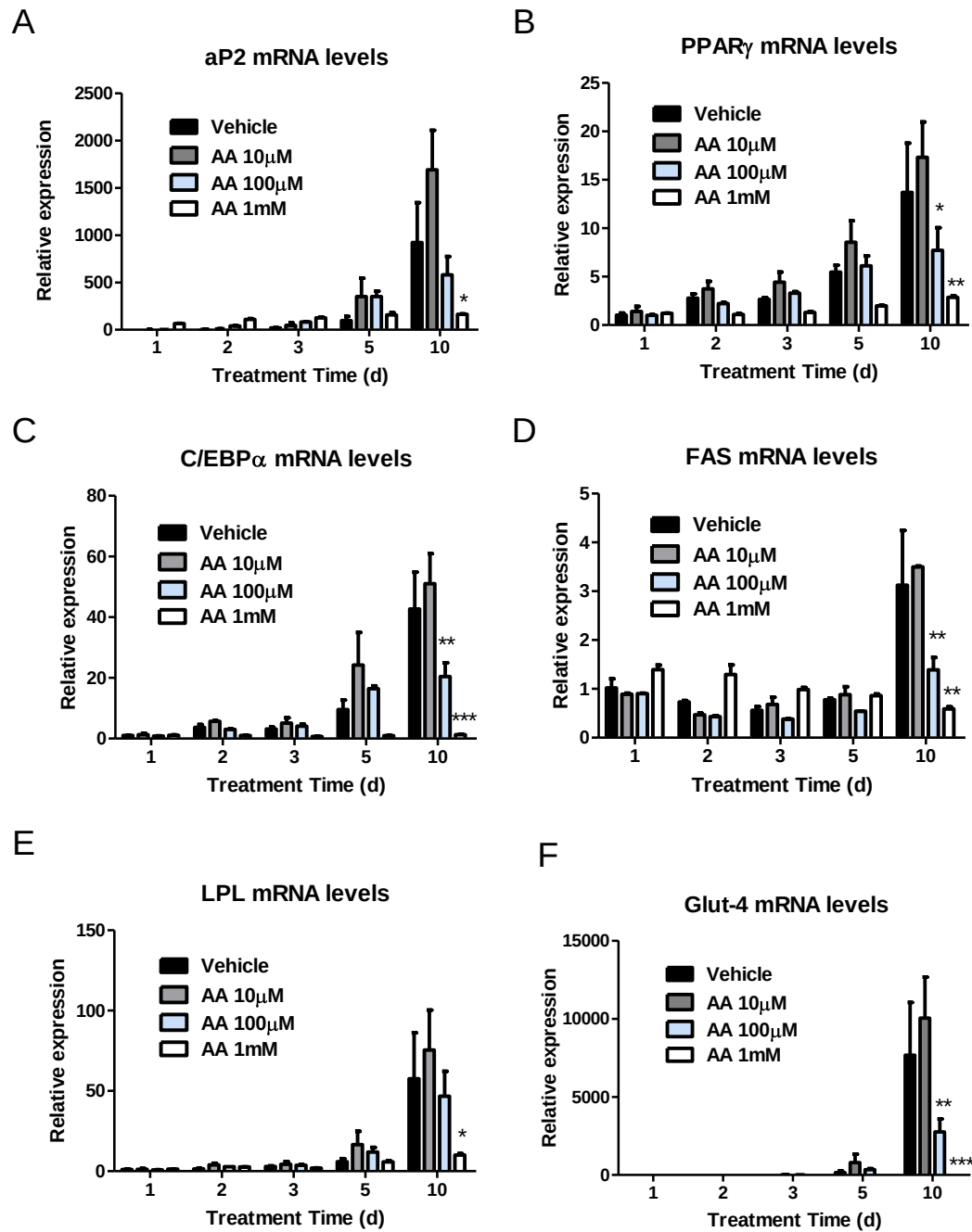


Figure 4.3: AA-dependent inhibition of adipocyte differentiation after long exposure to AA – gene expression of adipocyte markers. Cells were treated as described above. Briefly D0 3T3-L1 pre-adipocytes were induced to differentiate in the presence of increasing doses of AA throughout the differentiation program. Total RNA was harvested at the indicated time points and the expression of adipocyte markers A) aP2, B) PPAR γ , C) C/EBP α , D) FAS, E) LPL and F) Glut-4 was analysed by qRT-PCR. All the time points indicate days post induction of differentiation. Data are presented as mean $\pm$ SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-test was performed for the D10 time point between the vehicle-treated and the AA-treated. *P<0.05, **P<0.01, ***P<0.001.

4.2.2 Inhibition of adipocyte differentiation after short exposure to AA

The next question addressed was the effect of a short treatment with AA at the early stages of differentiation on the differentiation potential of the pre-adipocytes. Thus, two days post-confluent 3T3-L1 cells were treated with different doses of AA for the first 24h of differentiation, then AA was withdrawn and the standard differentiation protocol was followed until D10 after the induction (Figure 4.4). The lipid accumulation was assessed by staining the cells with Oil Red O dye. Increasing doses of AA were inversely correlated with lipid accumulation (Figure 4.5 A). Furthermore, the ability of AA to inhibit lipid accumulation was confirmed by quantification of the intracellular TAG (Figure 4.5 B). To further investigate this observation the expression of some adipocyte differentiation markers was assessed by qRT-PCR. The expression of all the differentiation markers tested was much lower in the treated cells compared with the vehicle control-treated cells, for all the doses used (Figure 4.6).

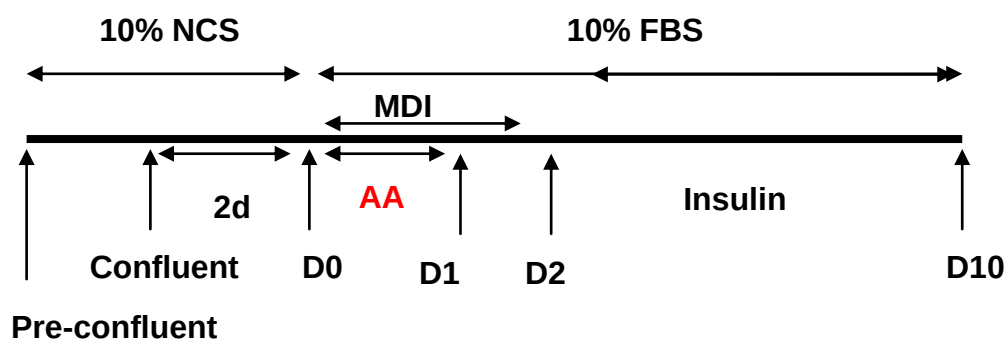
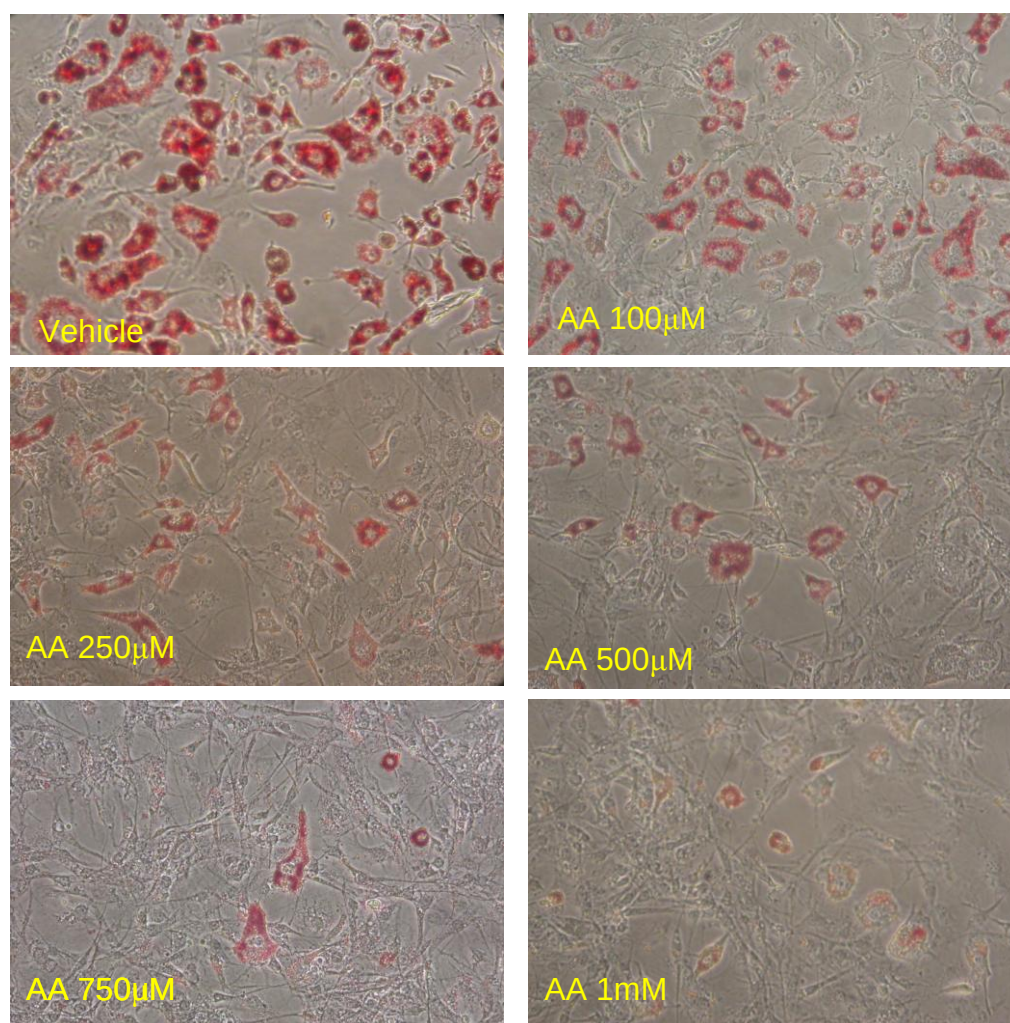


Figure 4.4: Experimental procedure for short exposure to AA. D0 3T3-L1 cells were treated with AA for 24h in the presence of MDI. After 24h, AA was withdrawn and cells were kept in MDI for another 24h. The next day cells were kept in medium with insulin until the end of the differentiation D10.

A



B

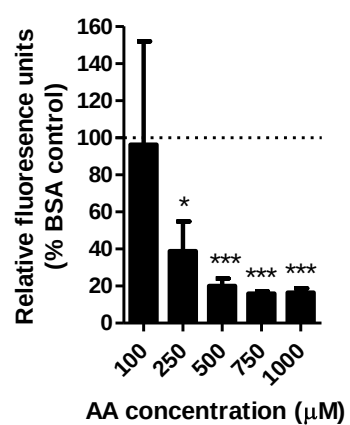
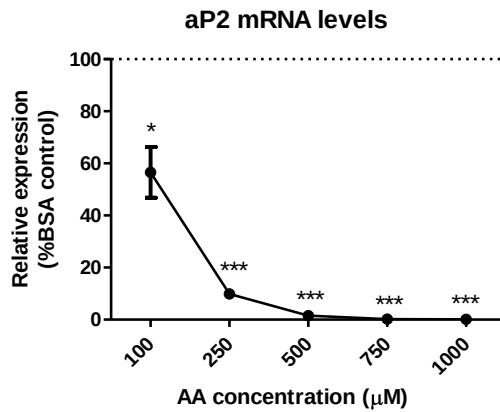


Figure 4.5 Inhibition of differentiation after a short exposure to AA – lipid accumulation.

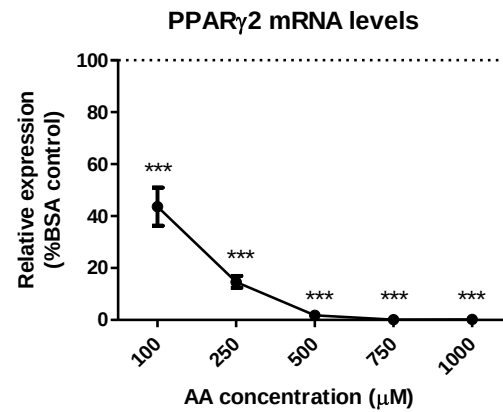
A) Cells were treated as described in Fig 4.4. Briefly cells exposed to 100 μ M, 250 μ M, 500 μ M, 750 μ M or 1mM AA for the first 24h of differentiation. Cells were fixed and stained with Oil Red O on D10 of the differentiation program (20x magnification). B) Cells were treated as described in A and lipids were quantified using AdipoRed stain and fluorescence measured

on D10 after the induction. Values shown are mean percentages of the respective vehicle controls (set as 100%). Data are presented as mean $\pm$ SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-test was conducted between the vehicle-treated and the AA-treated. * $P < 0.05$, *** $P < 0.001$.

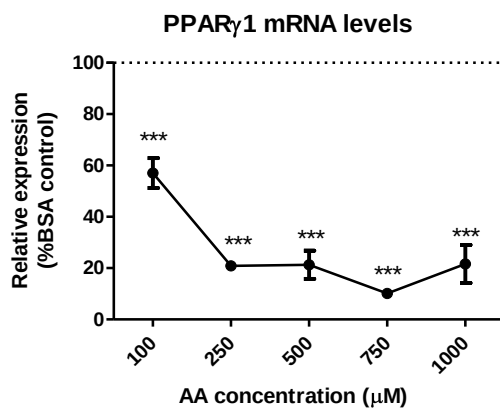
A



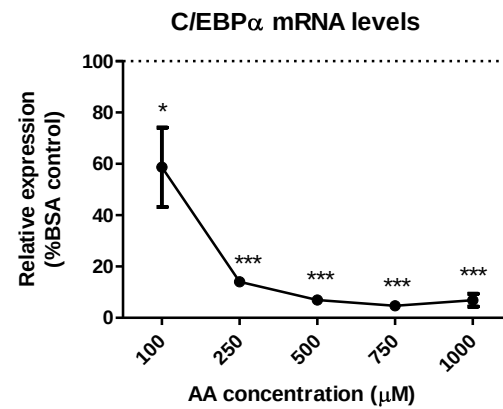
B



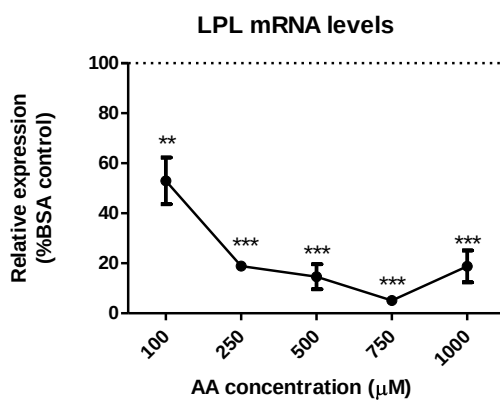
C



D



E



F

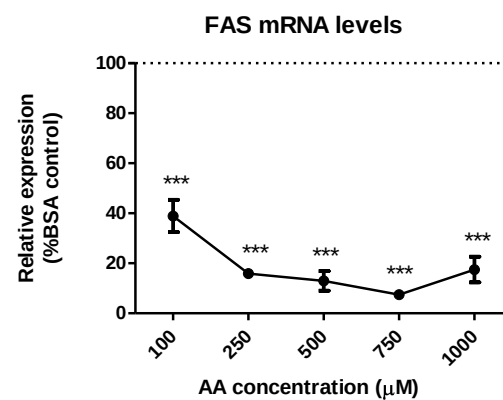


Figure 4.6: Inhibition of differentiation after a short exposure to AA – gene expression of adipocyte markers. 3T3-L1 cells were treated as described in Fig 4.4. Briefly, cells were treated with increasing doses of AA for 24h in the presence of MDI. After the 24h, AA was withdrawn and total RNA was isolated on D10 and subjected to qRT-PCR for A) aP2, B) PPAR γ 2, C) PPAR γ 1, D) C/EBP α , E) FAS and F) LPL. Values shown are mean percentages of the respective vehicle controls (set as 100%). Data are presented as mean $\pm$ SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-tests were conducted between the vehicle-treated and the AA-treated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.2.2.1 Kinetics of the inhibition of differentiation after short exposure to AA

To investigate the kinetics of the inhibition, cells were treated for 24h with 500 μ M AA in the presence of MDI, then withdrawn and harvested at different time points during the eight days of differentiation. On the eighth day of differentiation cells were stained with Oil Red O and quantified (Figure 4.7). LDs appeared after 24h AA of treatment in contrast to the vehicle control. After the withdrawal of AA and on the second day of differentiation, fewer LDs were present compared to the 24h time-point,, whereas there were no visible LDs in the vehicle-treated cells. On the fourth day of differentiation the vehicle-treated cells appeared to change their morphology, becoming more rounded and accumulating some lipid in contrast to the AA-treated which lost almost all their LDs. On the eighth day of differentiation the vehicle-treated cells had mature LDs whereas in the AA-treated cells, only few of them had LDs. Lipid content quantification confirmed the lipid accumulation in the AA-treated cells for the first two days whereas the levels declined after the fourth day and appeared to be lower in comparison to the vehicle control on the eighth day (Figure 4.7 B).

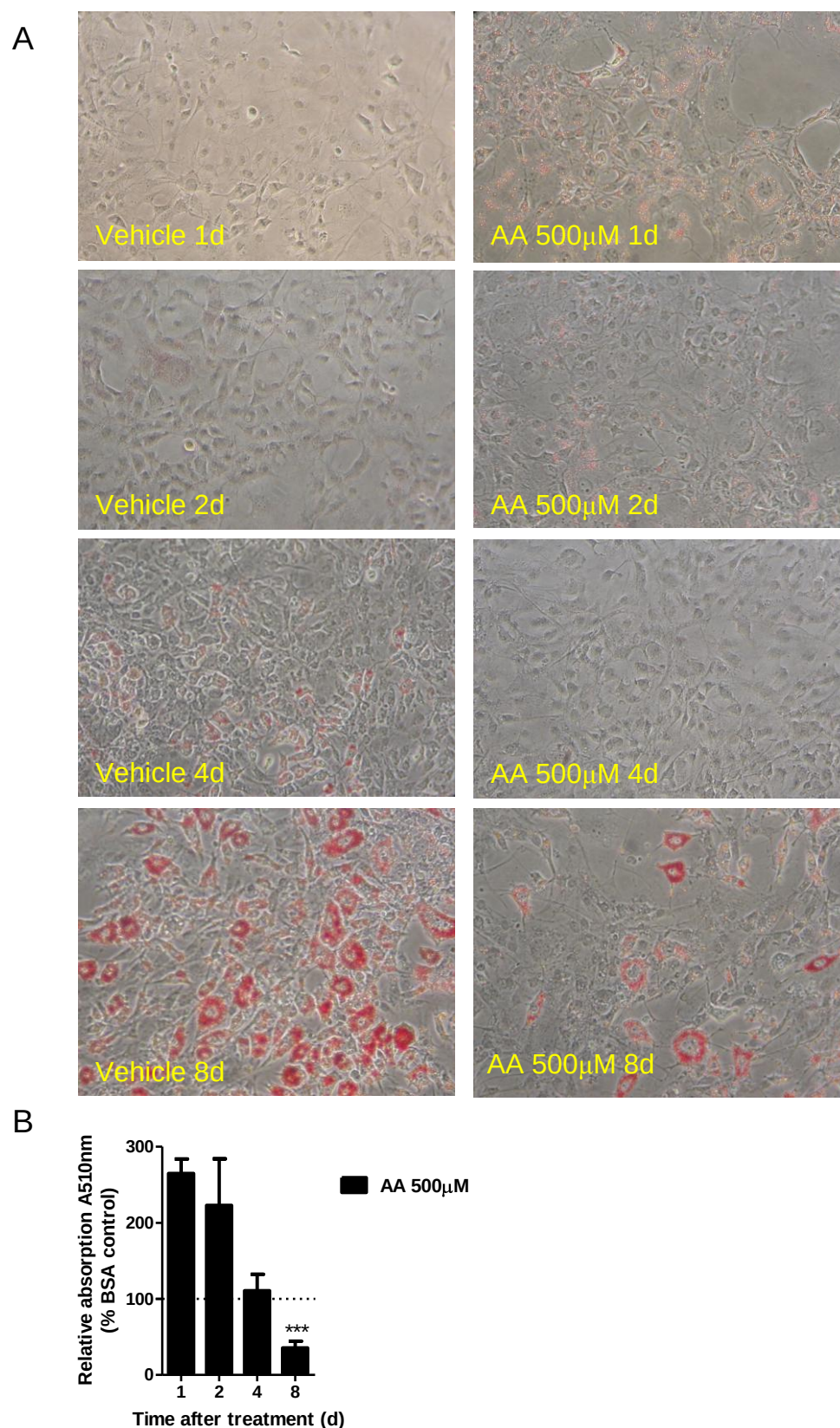


Figure 4.7: Kinetics of the inhibition of differentiation after a short exposure to AA – lipid accumulation. A) Cells were treated as described in Fig 4.4. Briefly cells exposed to 500µM AA for the first 24h of differentiation. Cells were fixed and stained with Oil Red O at the indicated time points (20x magnification) B) Cells were treated as described for A) and Oil

Red O was extracted in 100% isopropanol and quantified at the indicated time points. Values shown are mean percentages of the respective vehicle controls (set as 100%). Data are presented as mean $\pm$ SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-test was conducted between the vehicle-treated and the AA-treated. ***P<0.001.

Total RNA and protein were isolated and the expression of some differentiation markers was assessed. The expression of all differentiation markers tested was lower in the cells exposed to AA for 24h at all points examined, with the exception of aP2. Its levels were much higher after 24h of treatment (in agreement with the previous chapter) but its levels fell below the vehicle-treated cells at the later time points (Figure 4.8). These observations were confirmed at the protein level. aP2 protein expression was increased after 24h of AA treatment and was above the basal levels after 48h. From the fourth day onwards the vehicle-treated cells appeared expressed more aP2 in comparison to AA-treated cells. The expression of PPAR γ in AA-treated cells was below the basal levels in all time points tested (Figure 4.9).

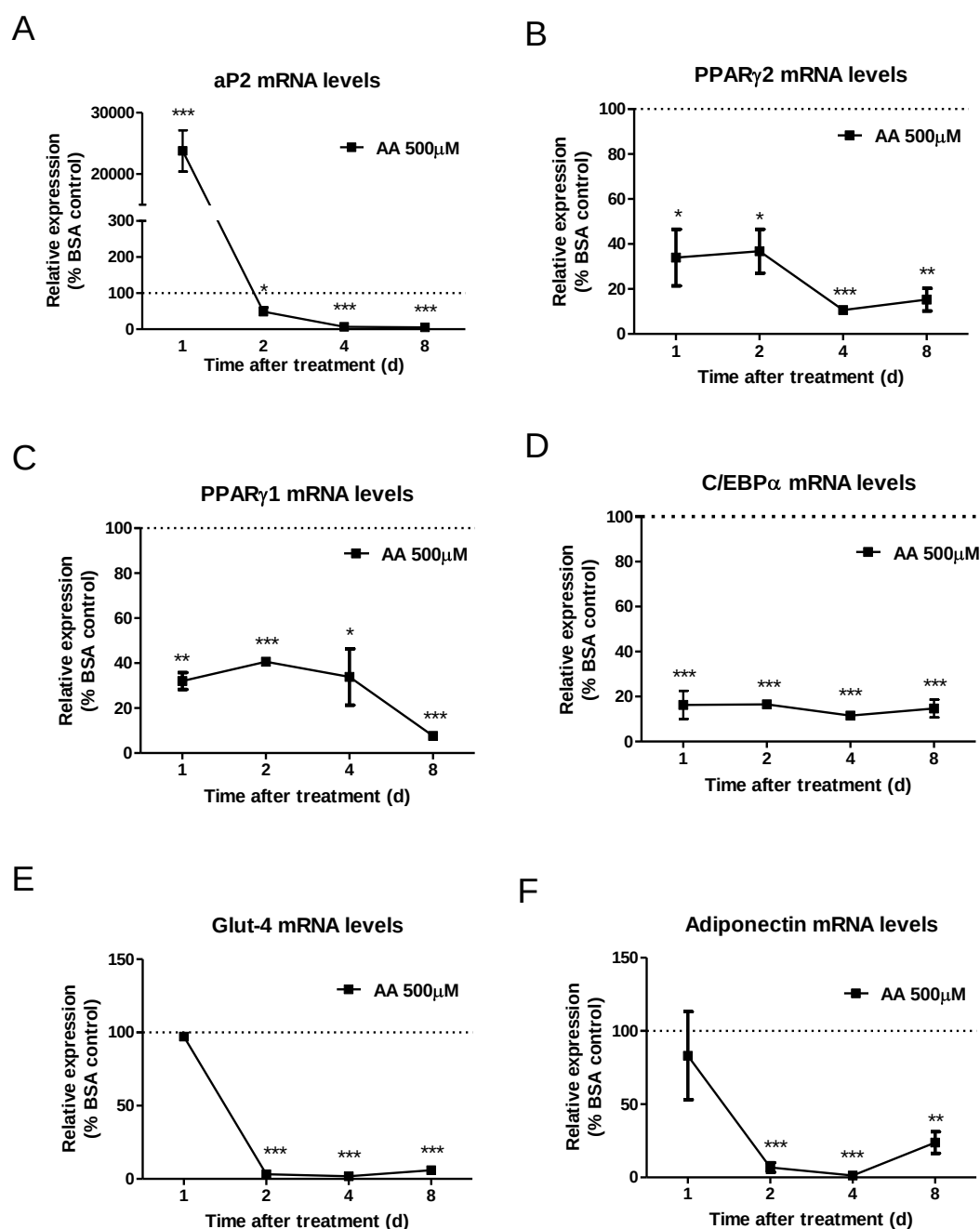
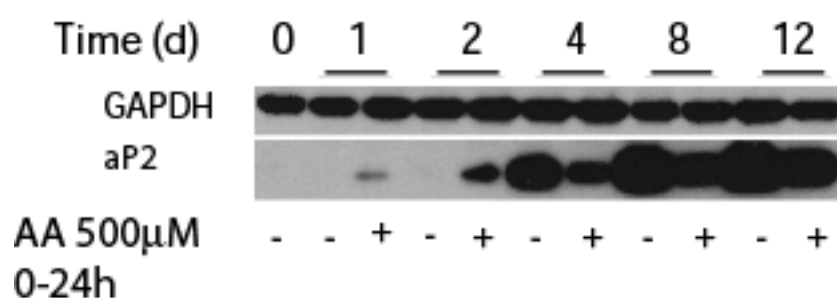


Figure 4.8: Kinetics of the inhibition of differentiation after a short exposure to AA – gene expression of adipocyte markers. Cells were treated as described in Fig 4.4. Briefly, cells were treated with 500 μ M AA for 24h in the presence of MDI. After the 24h, AA was withdrawn and total RNA was extracted at the indicated time points. qRT-PCR was conducted for A) aP2, B) PPAR γ 2, C) PPAR γ 1, D) C/EBP α , E) Glut-4 and F) adiponectin. Values shown are mean percentages of the respective vehicle controls (set as 100%). Data are presented as mean $\pm$ SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-tests were conducted between the vehicle-treated and the AA-treated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

A



B

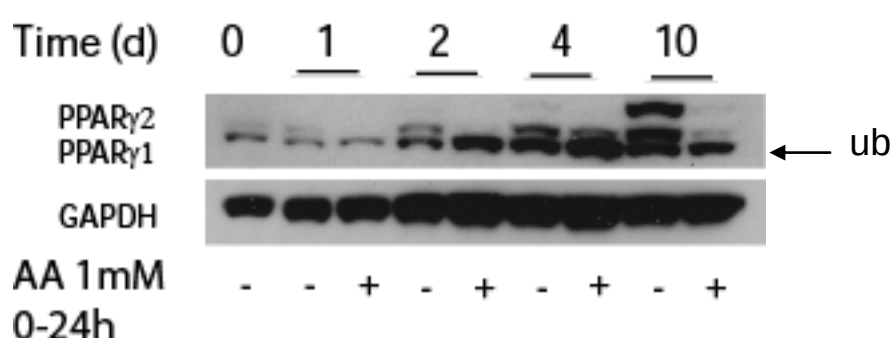


Figure 4.9 AA short exposure inhibits the expression of aP2 and PPAR γ . After AA treatment for the first 24h of differentiation whole cell lysates were harvested at the end of the differentiation procedure day 10-12. Western blotting was performed using antibodies against A) aP2 and B) PPAR γ . The blots are representatives of two independent experiments. Ub: unspecific band.

4.2.2.2 Differentiating pre-adipocytes are sensitive to the inhibitory effect of AA during the first 24h of differentiation.

To define the time frame at which pre-adipocytes are sensitive to the inhibitory effect of AA, cells were treated for different time intervals during the differentiation procedure. The expression of adipocyte markers was assessed at the end of the differentiation procedure (Figure 4.10). The inhibitory effect of AA was maximal when present during the first 24h of differentiation. When AA was present between day 1 and 2 it still had an inhibitory effect on differentiation but was less in comparison to the 24h treatment. When AA was present after the second day of differentiation it did not affect the

differentiation. In conclusion, the presence of AA at the beginning of the differentiation procedure is crucial in order to exert its inhibitory effect.

To further elucidate the timing of AA treatment to inhibit differentiation, cells were treated for shorter periods of time at the start of differentiation and the latter was assessed on D10 (Figure 4.10). There was no effect after treating the cells with AA for 2h or 4h at the beginning of the differentiation procedure. A small inhibitory effect was detectable after 8h of treatment. After 12h of AA treatment the effect was more evident but AA exert its maximum inhibitory effect on differentiation only after 24h.

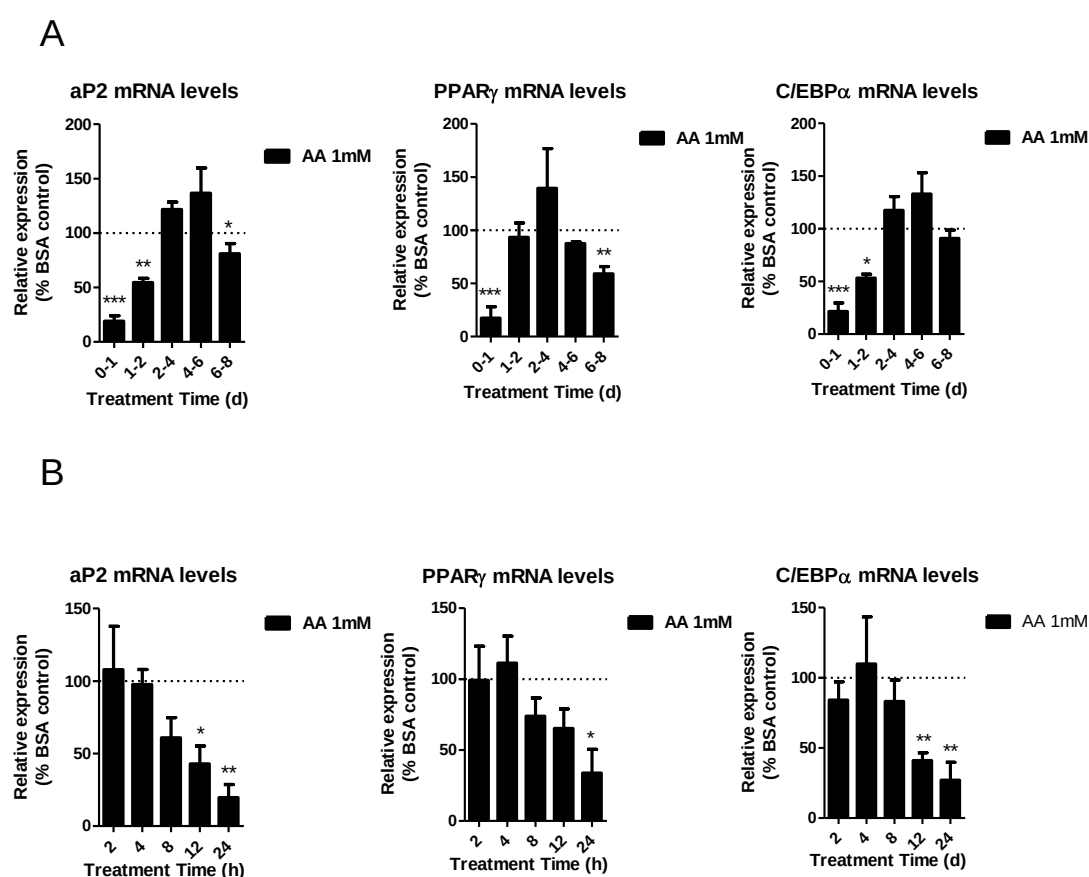


Figure 4.10: AA inhibitory effect is maximal when present during the first 24h of differentiation. A) Cells were treated with 1mM AA for the first 24h, between 24h and 2 days, between 2d and 4d, between 4d and 6d and between 6d and 8d. B) Cells were treated with 1mM AA for the first 2, 4, 8, 12, or 24h of differentiation. Total RNA was extracted on D10 and qRT-PCR was performed for aP2, PPAR γ and c/EBP α . Values shown are mean percentages of the respective vehicle controls (set as 100%). Data are presented as mean $\pm$ SEM based on triplicate determinations from a representative experiment of two independent experiments.

Student's t-test was conducted between the vehicle-treated and the AA-treated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.2.2.3 A PPAR γ ligand or prolonged exposure to MDI cannot rescue AA-induced inhibition of differentiation

Since PPAR γ is the nuclear receptor regulating adipocyte differentiation, it was hypothesised that a high affinity PPAR γ ligand would rescue cells from the inhibitory action of AA. To test this hypothesis, cells were treated with the PPAR γ agonist, rosiglitazone in the presence or absence of AA for different time intervals at the beginning of differentiation and then aP2 and PPAR γ mRNA levels were assessed on D10. As shown above, AA exerts its inhibitory effect after 24h of treatment. In contrast, rosiglitazone enhanced differentiation after 24h of treatment but lost this promoting activity when AA was present (Figure 4.11 A, B).

In addition, it was hypothesised that AA instead of inhibiting it was rather causing a delay in the differentiation. To test this hypothesis, cells were treated with MDI for longer periods after the initial 24h AA treatment and the differentiation was assessed on D10. Thus, after AA withdrawal, MDI was added for one, two or three more days. The ability of AA to inhibit differentiation could not be rescued with the prolonged MDI treatment as shown by the expression of the differentiation markers aP2, PPAR γ and C/EBP α , which was below the basal levels (Figure 4.11 C-E).

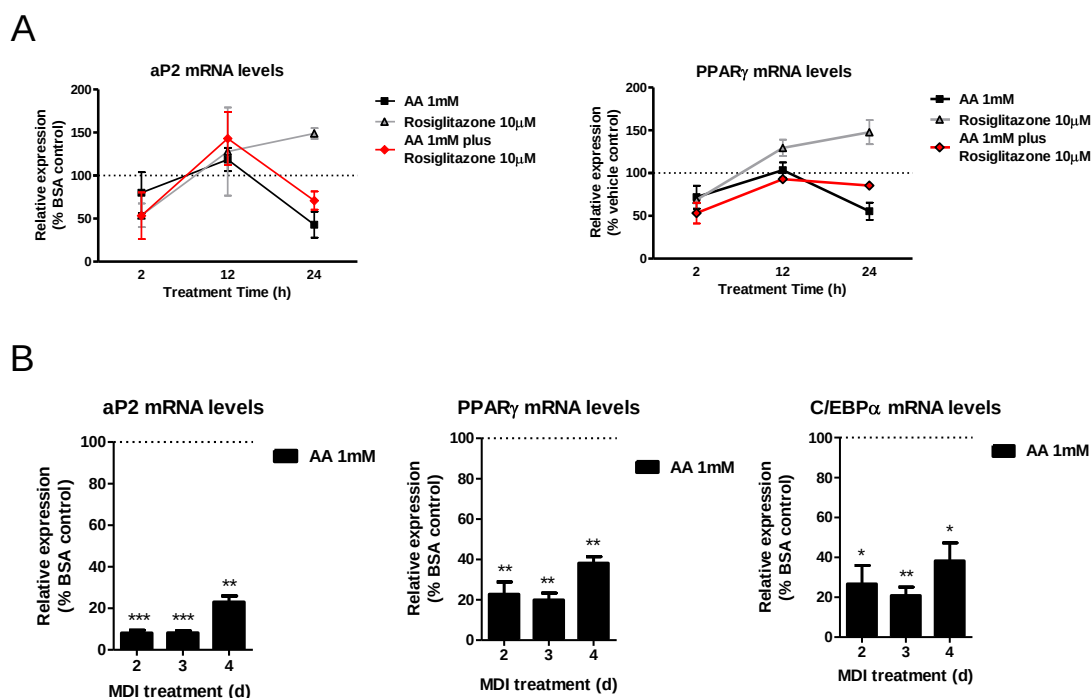


Figure 4.11: Rosiglitazone or prolonged MDI treated does not rescue the inhibition of differentiation by AA. A) 3T3-L1 cells were treated with 1mM AA, 10 μ M rosiglitazone or both for 2, 12 and 24h at the beginning of differentiation, after which the treatments were withdrawn and total RNA was isolated on D10. qRT-PCR was performed for aP2 and PPAR γ . Graphs are representative of two independent experiments. Error bars indicate $\pm$ SD. B) Cells were treated with 1mM AA for the first 24h of differentiation in the presence of MDI. The next day AA was withdrawn and the cells kept in MDI for 1, 2 or 3 extra days (*i.e.* for a total of 2, 3 or 4 days, respectively). RNA was isolated on D10 and qRT-PCR was performed for B) aP2, PPAR γ and C/EBP α mRNA. Values shown are mean percentages of the respective vehicle controls (set as 100%). Data are presented as mean $\pm$ SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-test was conducted between the vehicle-treated and the AA-treated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.2.2.4 The inhibition of differentiation after a short exposure to AA is not due to alternations in cell cycle, cell viability or induced cytotoxicity.

To exclude any other possible causes for the inhibition of differentiation by AA, cell cycle, cell viability and cytotoxicity were tested after 24h of AA treatment.

When post-confluent, growth-arrested 3T3-L1 pre-adipocytes are induced to differentiate with MDI the cells undergo two sequential rounds of mitosis over the next two days which is referred to as mitotic clonal expansion. The first round of mitosis is completed 24 to 36 h after induction, and the second round is completed 48 to 60h after induction. Therefore, since the treatment with AA is initiated during the period of the first mitosis, flow cytometry was employed to study the cell cycle by propidium iodide staining of cellular DNA. Cells were treated with a high dose of AA (1mM) for 24h. A total of 10,000 events were counted and the number of cells at each stage of the cell cycle was similar for control and AA-treated cells (Figure 4.12).

In addition, D0 cells were treated with a high (1mM) and a low dose (100 μ M) of AA for 24h and assessed for cell viability using the MTT assay. An overnight treatment with 1% Triton X-100 was used as a positive control per supplier's instructions. None of the doses of AA used affected cell viability relative to the vehicle control (Figure 4.13 A).

Finally, the supernatants from cells following a 24h treatment with AA in two doses (100 μ M and 500 μ M) were assessed for the release of lactate dehydrogenase (LDH) activity. LDH is released from the cytosol of damaged cells into the supernatant. None of the two doses examined caused an increase in the LDH activity relative to the vehicle control (Figure 4.13 B).

In conclusion, the inhibition of the adipocyte differentiation by a short exposure to AA, is not due to effects on cell cycle, cell viability or cytotoxicity caused by the doses of AA.

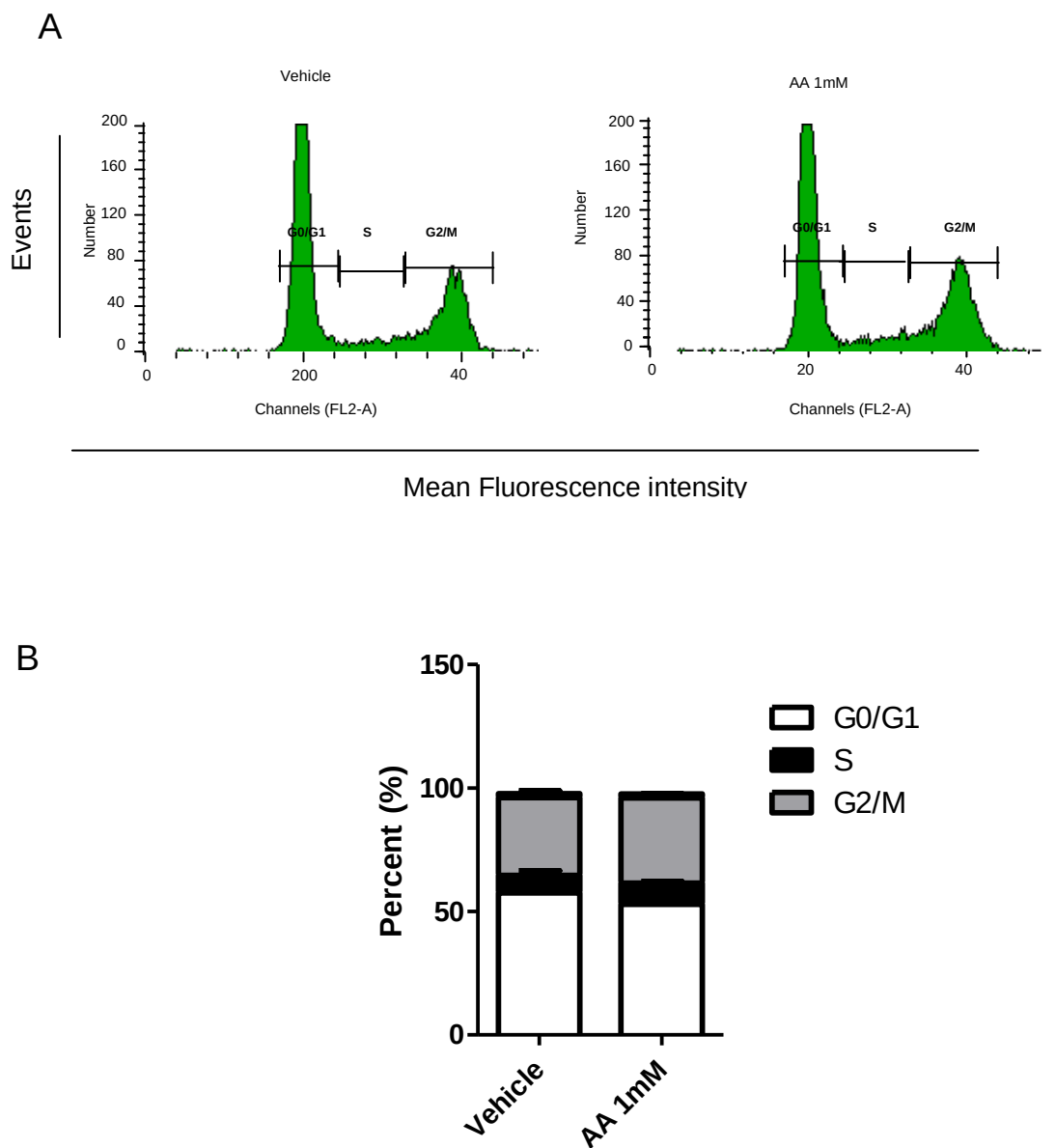


Figure 4.12: AA treatment does not affect the cell cycle. A) D0 3T3–L1 pre-adipocytes were cultured in the differentiation medium containing 1mM AA for 24 h, stained with a propidium iodide solution, and analysed by fluorescence-activated cell sorting. B) The percentage of cell population at each stage of the cell cycle was determined. The values represent mean percentages of three independent experiments (n=3).

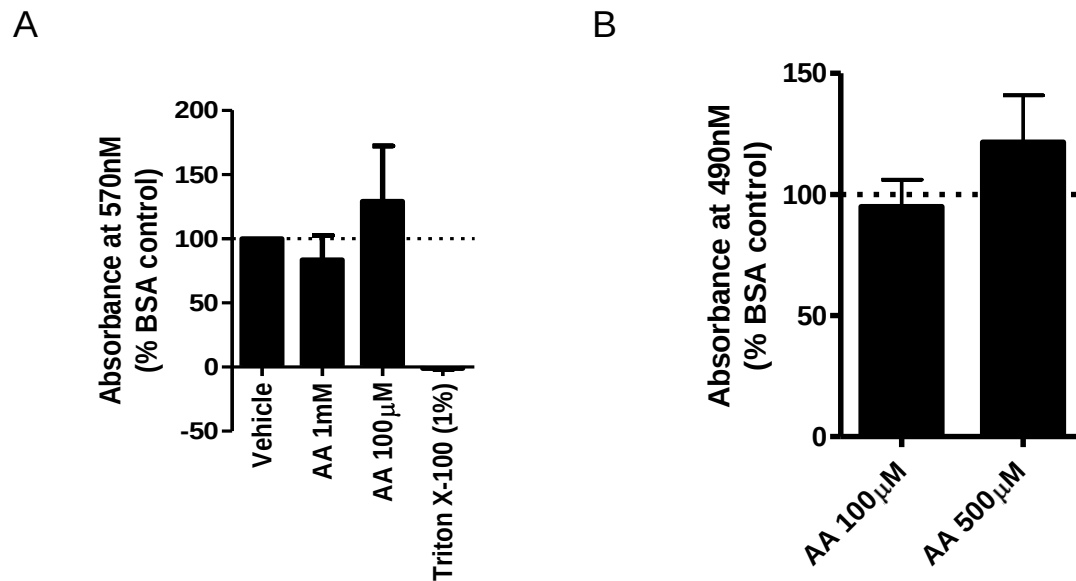


Figure 4.13: AA treatment does not affect cell viability and does not cause cytolysis. A) 3T3-L1 cell viability was assessed after AA treatment (1mM or 100 μ M) for 24h by MTT assay, graph of absorbance at 570 nm. 1% Triton X-100 was used as a positive control B) 3T3-L1 supernatants were assessed after 24h of AA treatment for lactate dehydrogenase (LDH) activity, graph of absorbance at 490 nm. Values shown are mean percentages to vehicle-treated (set as 100%) of three independent experiments (n=3). Error bars represent $\pm$ SEM.

4.2.2.5 Inhibition of adipocyte differentiation after short exposure to AA is mediated by PGs

As it was discussed in Chapter 3 (Results 3.2.4 and 3.3.2), the effect of AA treatment in the first 24h of differentiation on aP2 and Fra-1 induction is mediated by PGs. To test if PGs also have a role in mediating the effect of AA on the inhibition of differentiation, cells were pre-treated with indomethacin, a general COX-inhibitor, prior to a 24h treatment with AA. Total RNA was isolated at the end of the differentiation procedure (D10) (Figure 4. 14 A, B). Indomethacin appeared to block the inhibition of differentiation by AA, as the levels of aP2 and PPAR γ increased to levels comparable to the vehicle-treated cells. Furthermore, COX-2 protein and RNA levels were assessed and both increased after a 24h of AA treatment in comparison with the vehicle control (Figure 4.14 C, D). RNA levels started declining on the second day of differentiation in contrast with the protein levels but both were still above the basal levels. At the later stages of differentiation COX-2 protein remained elevated in the AA-treated cells compared to the vehicle control levels, although RNA expression levels were similar between treated and untreated cells.

Finally, cells were treated with individual PGs for the first 24h of differentiation, withdrawn and stained with Oil Red O on D10 of differentiation (Figure 4.15). Cells treated for the first 24h with PGF $_{2\alpha}$ appeared to have reduced lipid accumulation in comparison with the other PGs and control.

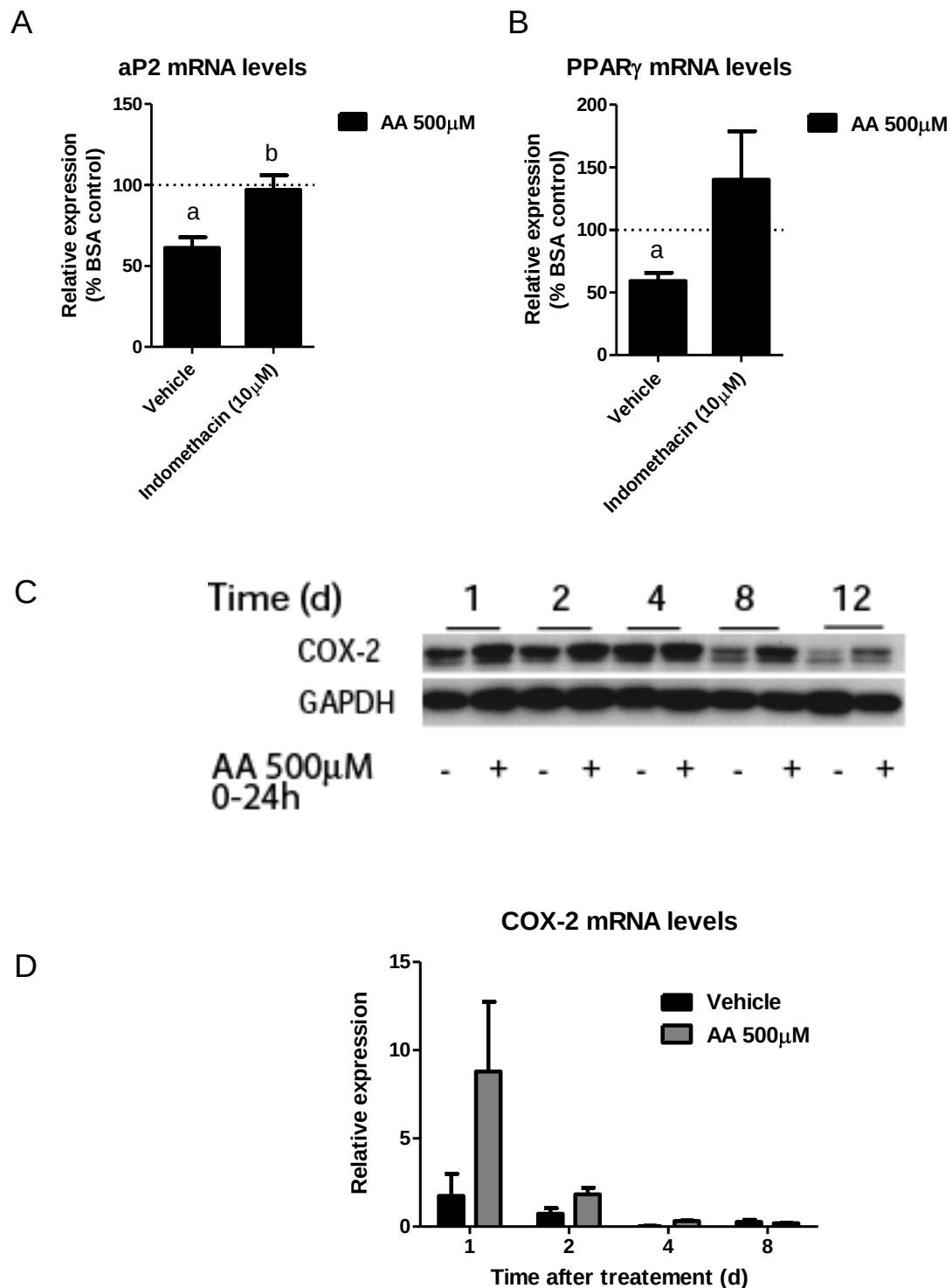


Figure 4.14: Indomethacin blocked AA-induced inhibition of differentiation. Cells were pre-treated with indomethacin (10µM) for 30min before AA treatment (500µM) for the first 24h of differentiation in the presence of MDI. After 24h AA was withdrawn and the cells were treated according to the standard differentiation protocol until D10. Total RNA was isolated and qRT-PCR was performed for the differentiation markers A) aP2, B) PPAR γ . The values represent means of three independent experiments (n=3) and they are expressed as percentages relative to the vehicle-control (set as 100%). Error bars indicate $\pm$ SEM. Student's

t-tests were conducted and a indicates $p < 0.05$ between AA-treated and BSA-treated cells and b indicates $p < 0.05$ between AA-treated cells in the presence or absence of indomethacin. C), D) Cells were treated as described above and RNA or whole cell lysates were harvested at the indicated time points. Western blotting was performed using antibodies against COX-2 and GAPDH and qRT-PCR was performed for COX-2 transcripts. The blot and the graph are representatives of two independent experiments. Error bars represent $\pm$ SD.

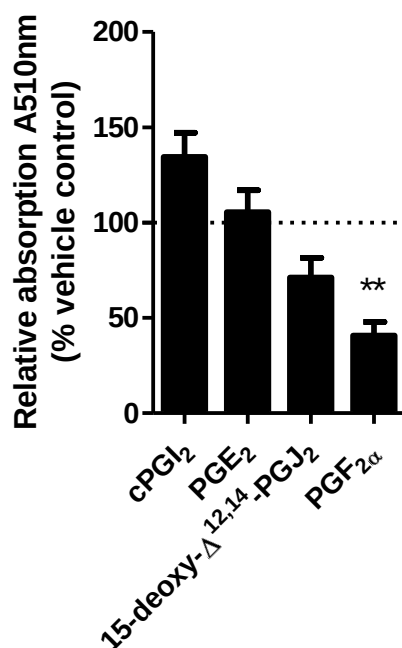


Figure 4.15: PGF_{2α} inhibits lipid accumulation. D0 cells were treated with 10μM cPGI₂, PGE₂, 15d-Δ^{12,14}-PGJ₂ and PGF_{2α} for the first 24h of differentiation. After 24h PGs were withdrawn and stained with Oil Red O at the D10 of differentiation. Oil Red O was extracted in 100% isopropanol and quantified. Data are presented as mean $\pm$ SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-test was conducted between the vehicle-treated and the PGF_{2α}-treated. **P < 0.01.

4.2.2.6 Inhibition of adipocyte differentiation after short exposure to PGF_{2α}

It was hypothesised that AA-dependent inhibition of differentiation was mediated by PGF_{2α} given that PGF_{2α} has been reported to inhibit adipocyte differentiation when present throughout the differentiation period (Liu and Clipstone, 2007). In addition, we had found that PGF_{2α} mediated the effect of AA at the early stages of differentiation. To further investigate this hypothesis, cells were treated with AA for the first 24h of differentiation and the expression

of the $\text{PGF}_{2\alpha}$ synthase, AKR1B3 was examined during the whole course of differentiation (Figure 4.16 A). AKR1B3 mRNA expression in 3T3-L1 cells has been reported to be induced early in the differentiation procedure showing its peak at 24h and quickly decreased on the next day and maintained its low level during their differentiation into adipocytes (Fujimori et al., 2010). Upon AA treatment, AKR1B3 levels were increased after the 24h of differentiation, remained elevated after day two and declined to basal levels after day four of differentiation. The same trend was seen in the production of $\text{PGF}_{2\alpha}$ but the levels were still above the basal levels on day eight (Figure 4.16 C). In addition, the expression levels of the $\text{PGF}_{2\alpha}$ receptor, the FP receptor were increased upon AA treatment compared to the vehicle-treated cells on the second and fourth of differentiation (Figure 4.16 B).

To test if the effect of a short treatment with $\text{PGF}_{2\alpha}$ is capable of inhibiting differentiation in a similar manner to AA, cells were treated with serial doses of $\text{PGF}_{2\alpha}$ for the first 24h of the differentiation, then withdrawn and either total RNA was isolated or the cells were stained with Oil Red O on D10 (Figure 4.17). $\text{PGF}_{2\alpha}$ at 10 μM or 1 μM inhibited the expression of all the differentiation markers tested. An inhibitory effect was observed with 100nM $\text{PGF}_{2\alpha}$ but it was much weaker than the higher doses and finally 10nM did not affect the expression of the differentiation markers tested. In accord with this observation, staining the cells with Oil Red O, 10 μM or 1 μM $\text{PGF}_{2\alpha}$ strongly inhibited lipid accumulation whereas the lower concentration (100nM) had a weaker effect (Figure 4.18). In conclusion, $\text{PGF}_{2\alpha}$ reproduced the inhibition of differentiation by AA after a short treatment of pre-adipocytes.

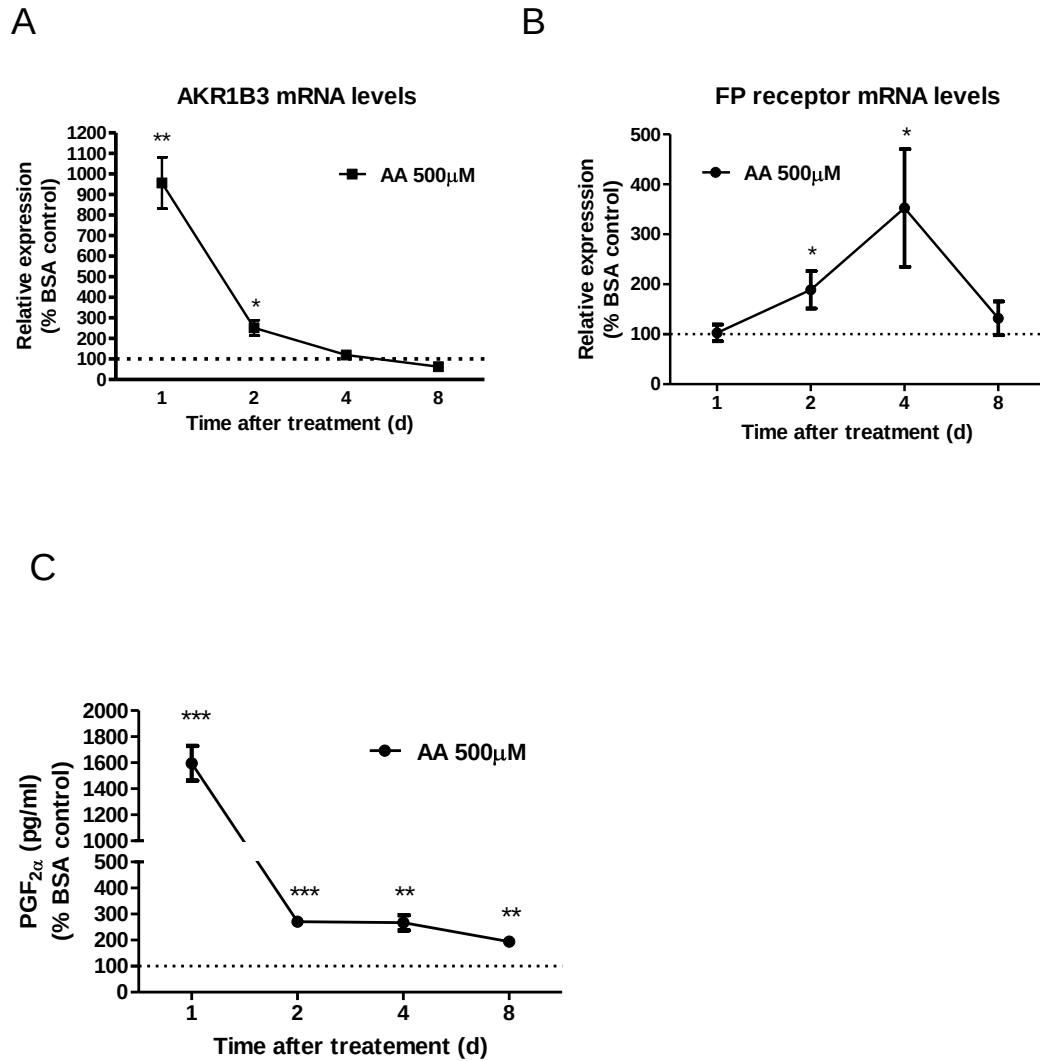


Figure 4.16: AKR1B3, FP receptor expression and PGF_{2α} production after short treatment with AA. D0 cells were treated with AA (500µM) for the first 24h of differentiation. RNA and supernatants were isolated at the indicated time points until the eighth day of differentiation. qRT-PCR was performed for A) AKR1B3 and the B) FP receptor and C) PGF_{2α} levels were measured using an EIA kit. Values shown as relative to the respective vehicle control (set as 100%). Data are presented as mean±SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-test was conducted between the vehicle-treated and the AA -treated. * P < 0.05, **P<0.01, ***P<0.001.

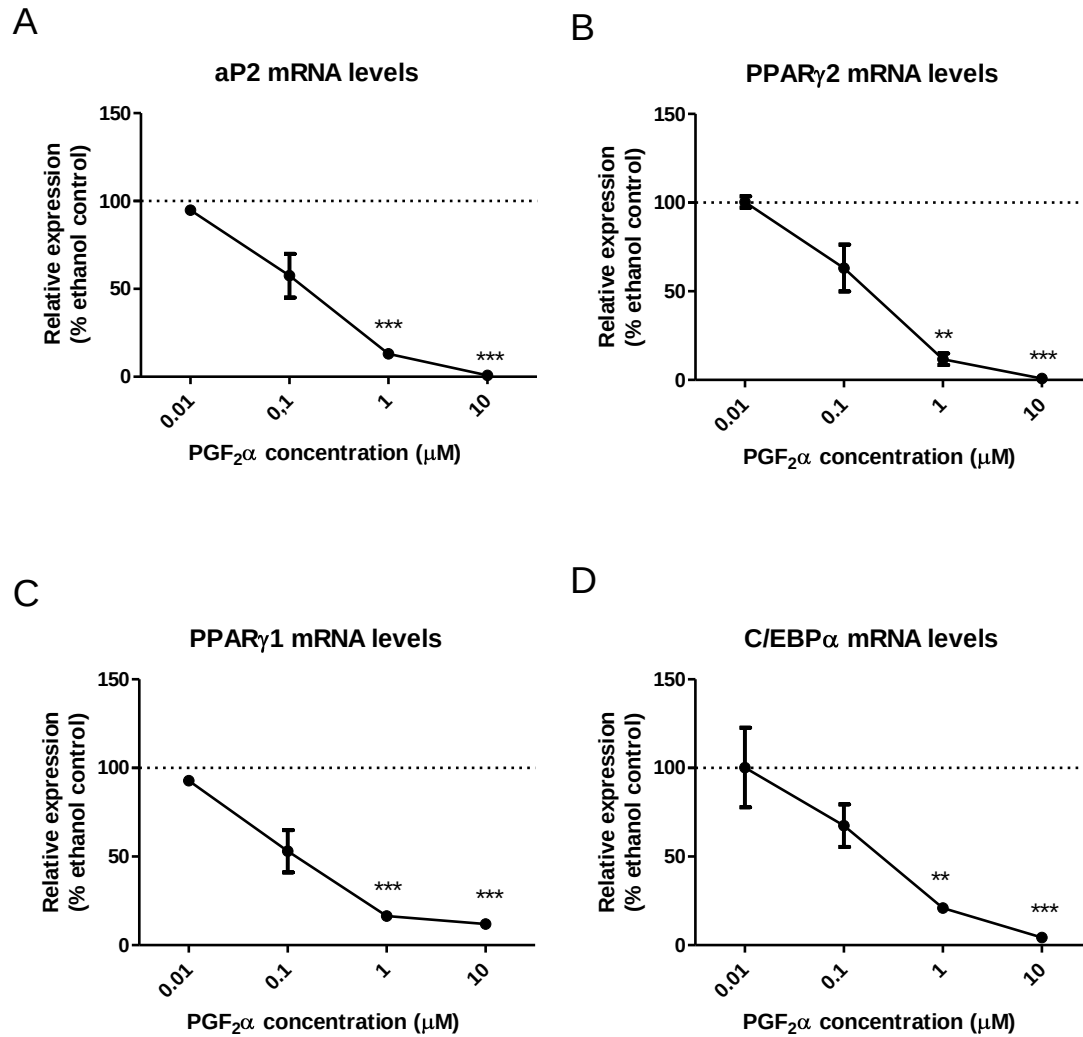


Figure 4.17: Inhibition of differentiation after a short exposure to PGF_{2α} –gene expression of adipocyte markers. D0 3T3-L1 cells were treated with 10nM, 100nM, 1μM or 10μM of PGF_{2α} for 24h in the presence of MDI. After 24h PGF_{2α} was withdrawn and the cells were maintained in the differentiation cocktail. Total RNA was isolated on D10 and the qRT-PCR was performed for A) aP2, B) PPAR_γ2, C) PPAR_γ1, D) C/EBP_α, E) LPL and F) FAS mRNA. Relative expression was expressed as a percentage of the vehicle control (set as 100%). Data are presented as mean±SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-test was conducted between the vehicle-treated and the PGF_{2α}-treated. *P<0.05, **P<0.01, ***P<0.001.

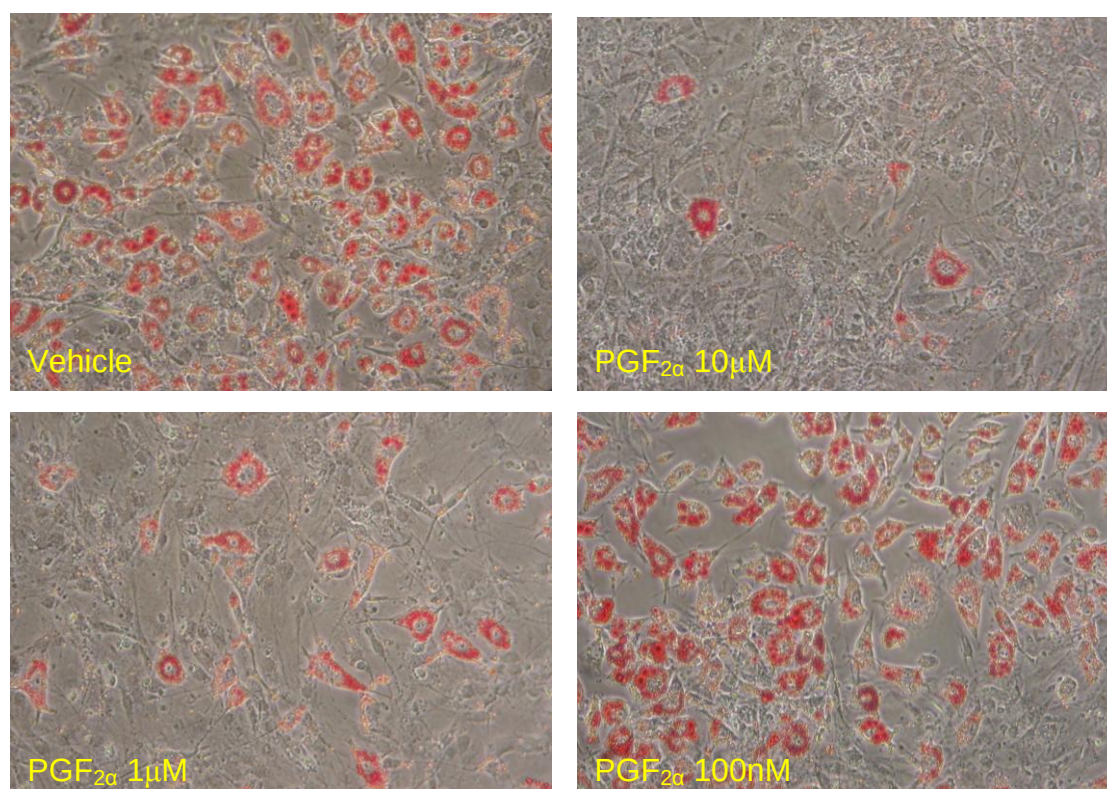


Figure 4.18: Inhibition of differentiation after a short exposure to PGF_{2α} – lipid accumulation. D0 cells were treated with 10μM, 1μM or 100nM PGF_{2α} for the first 24h of differentiation. Cells were fixed and stained with Oil Red O on D10 of differentiation (20x magnification).

4.2.2.7 Kinetics of the inhibition of differentiation after short exposure to $\text{PGF}_{2\alpha}$

To determine the kinetics of the inhibition of differentiation by $\text{PGF}_{2\alpha}$, cells were treated with $1\mu\text{M}$ $\text{PGF}_{2\alpha}$ for the first day of differentiation, then withdrawn and RNA isolated at different time points during the course of differentiation (Figure 4.19). aP2 expression was increased after 24h $\text{PGF}_{2\alpha}$ treatment in agreement with findings shown in Chapter 3. On the second day of differentiation and after $\text{PGF}_{2\alpha}$ withdrawal, aP2 expression remained above the basal levels but from the fourth day onwards aP2 levels were lower than measured in the control cells. PPAR γ and adiponectin expression levels were lower than control cells from the second day after treatment whereas C/EBP α expression was below the control levels after the first 24h of treatment (Figure 4.19).

To define more precisely the time at which $\text{PGF}_{2\alpha}$ exerts its inhibitory effect, cells were treated for different time intervals during the differentiation procedure and the expression of aP2, PPAR γ or C/EBP α was assessed at the end of the differentiation procedure. The inhibitory effect of $\text{PGF}_{2\alpha}$ was dramatic when present either at the early time points between day 0 and day 1 or between days 1 and 2 or present at the later stages of differentiation between day 4 and 8 (Figure 4.20 A). Finally, $\text{PGF}_{2\alpha}$ had a strong inhibitory effect on the differentiation even when it was present only the first 2h of the differentiation (Figure 4.20 B). In conclusion, $\text{PGF}_{2\alpha}$ strongly abrogated differentiation when added at the start or at the later stages of differentiation.

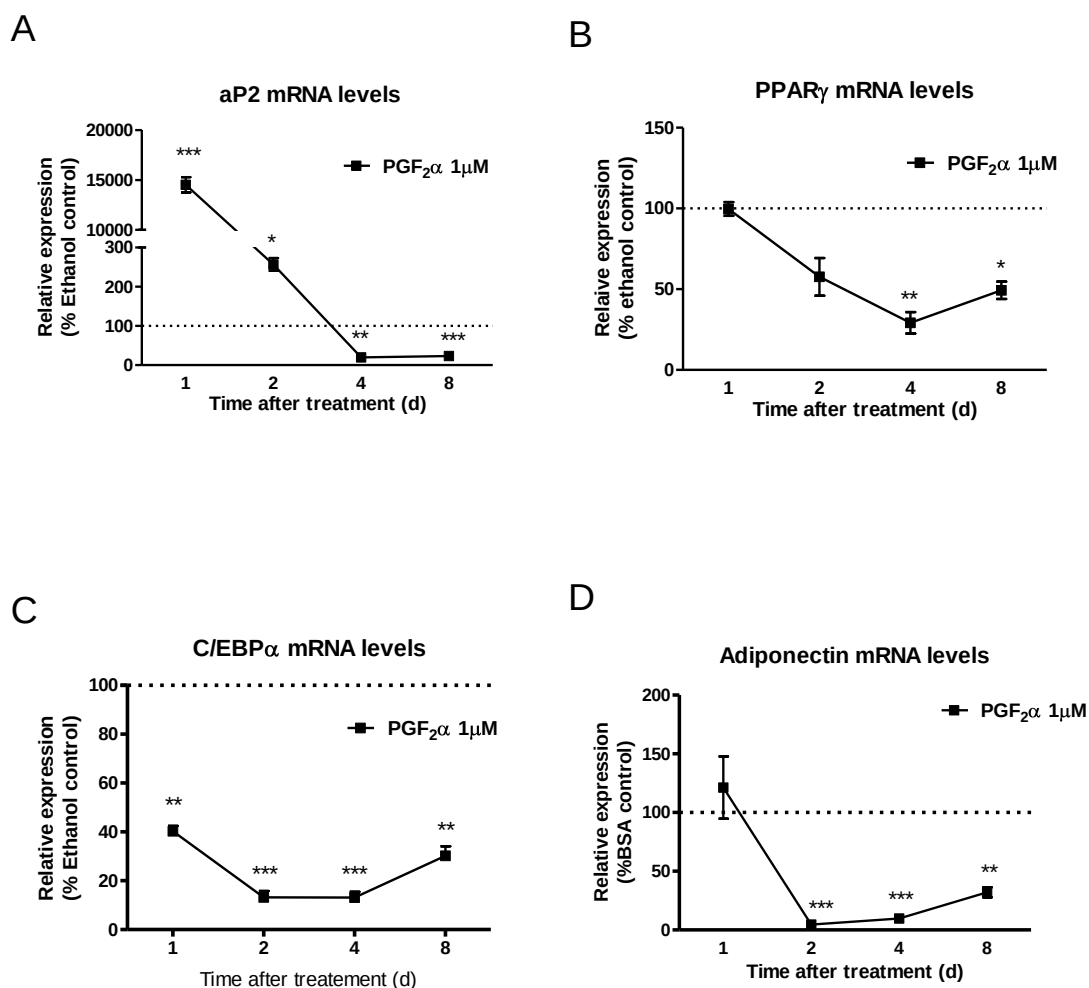
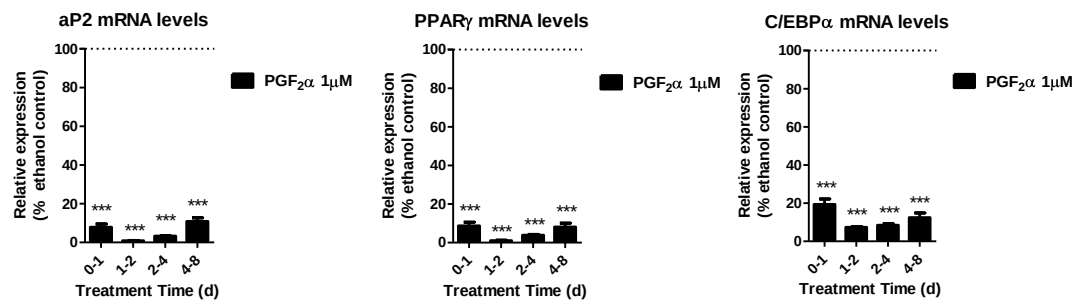


Figure 4.19: Kinetics of the inhibition of differentiation after a short exposure to PGF $_{2\alpha}$ – gene expression of adipocyte markers. Cells were treated as described above. Briefly cells were treated with PGF $_{2\alpha}$ (1 μ M) and withdrawn after the first 24h of differentiation. Total RNA was purified at the indicated time points. qRT-PCR was conducted for A) aP2, B) PPAR γ , C) C/EBP α and D) adiponectin mRNA. Relative expression was expressed as a percentage of the vehicle control (set as 100%). Data are presented as mean $\pm$ SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-test was performed for the vehicle-treated and the PGF $_{2\alpha}$ -treated cells. *P<0.05, **P<0.01, ***P<0.001.

A



B

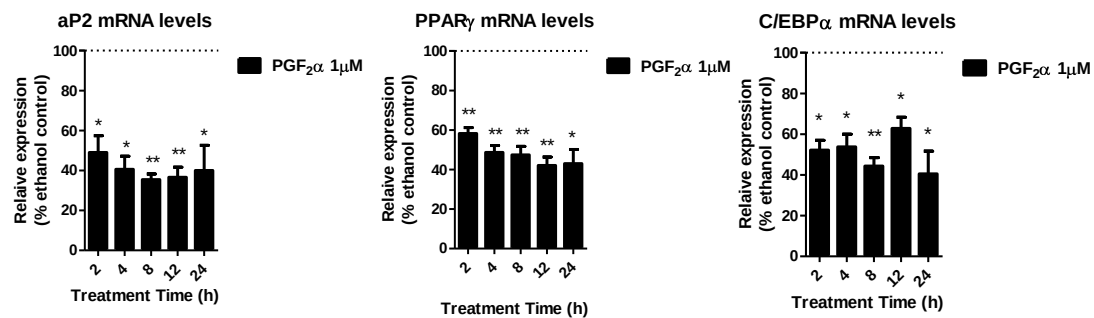


Figure 4.20: PGF $_{2\alpha}$ is a potent inhibitor of adipocyte differentiation. A) D0 cells were treated with PGF $_{2\alpha}$ 1 μ M for the first 24h, between 24h and 2 days, between 2d and 4d, between 4d and 6d and between 6d and 8d. B) Cells were treated with PGF $_{2\alpha}$ 1 μ M for the first 2, 4, 8, 12, or 24h of differentiation. Total RNA was isolated on D10 and qRT-PCR was performed for aP2, PPAR γ and C/EBP α mRNA. The values are presented relative to the corresponding vehicle-treated control (set as 100%). Data are presented as mean $\pm$ SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-test was performed for the vehicle-treated and the PGF $_{2\alpha}$ -treated. *P<0.05, **P<0.01, ***P<0.001.

4.2.2.8 The signalling pathway mediating AA-dependent inhibition of adipocyte differentiation

As demonstrated in section 3.2.5, $\text{PGF}_{2\alpha}$ activates ERK through its membrane GPCR, the FP receptor. Therefore whether the same pathway mediates the inhibition of differentiation by AA was examined. Cells were treated with AA (500 μM) for the first 24h of differentiation only, and cells were harvested at different time points until D12 of differentiation. ERK was activated early in the differentiation procedure and p-ERK levels started to decline after the fourth day. However, in the presence of AA p-ERK levels were increased in comparison with the vehicle control on day 12 (Figure 4.21 A). These data indicated that ERK can play a role in the inhibition of differentiation by AA. Thus, cells were pre-treated with the MEK inhibitor PD9809 (10 μM) before the AA 24h treatment and RNA was extracted on D10 of the differentiation (Figure 4.21 B). The reason that this MEK inhibitor was used was that it has been shown not to interfere with the clonal expansion or the differentiation of pre-adipocytes (Tang et al., 2003). PD9809 could rescue the inhibition of differentiation by AA, since the expression levels of aP2, PPAR γ and C/EBP α were similar to the vehicle-control-treated cells. These data confirmed that ERK pathway mediates the effect of AA on the inhibition of differentiation.

Finally, to determine if the inhibition of differentiation by AA is regulated by the FP receptor, an siRNA targeting the receptor (siFP) was used. The cells were maintained with the siRNA until the end of the differentiation (D10) and RNA or protein samples were prepared. siFP blocked the ability of AA to inhibit differentiation of 3T3-L1 cells, as judged by expression of aP2, PPAR γ 2/ γ 1, C/EBP α , LPL and FAS which were almost restored to the vehicle-treated cells (Figure 4.22). The protein expression of two differentiation markers aP2 and PPAR γ was also determined. 24h of AA treatment at the beginning of differentiation inhibited the expression of aP2 and PPAR γ protein in cells transfected with siControl, whereas in the presence of the siFP the levels of these markers were restored to levels similar to the vehicle-treated cells (Figure 4.23).

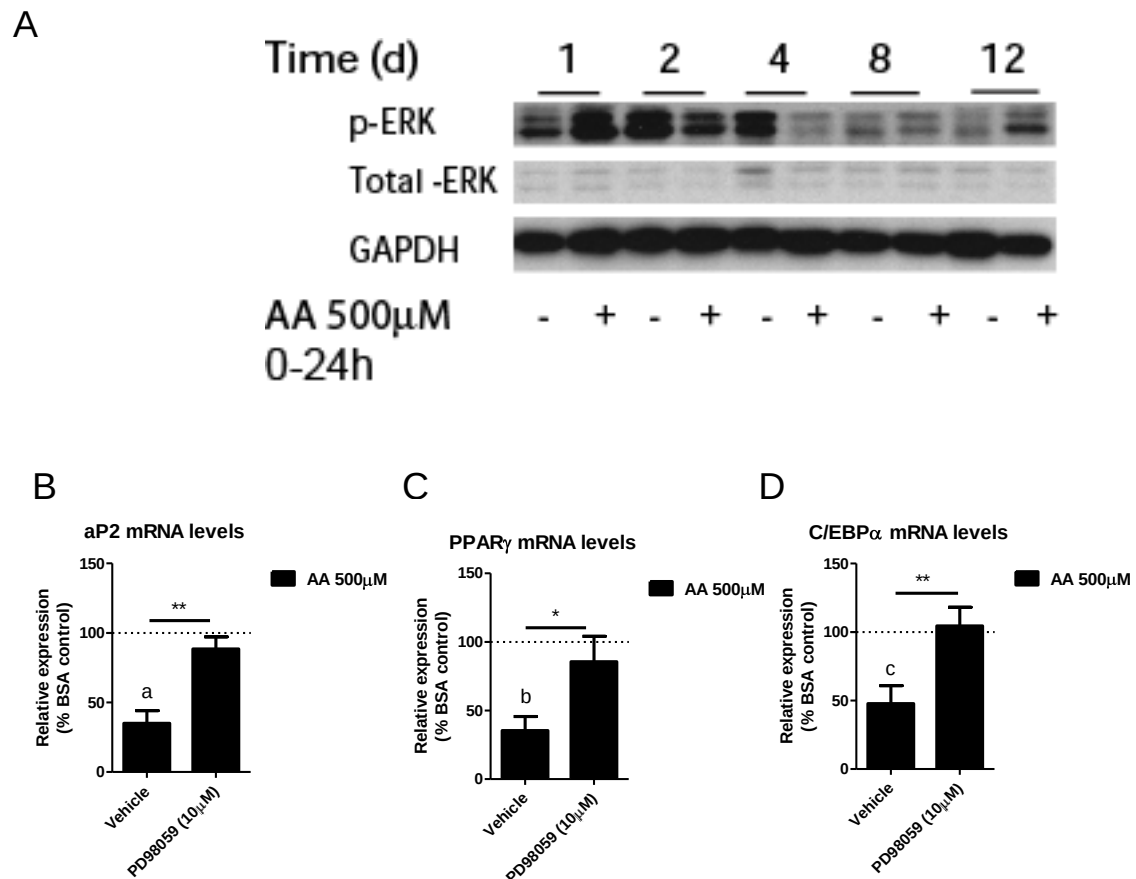


Figure 4.21: The ERK pathway mediates the inhibition of differentiation by AA. A) 3T3-L1 cells were treated with AA (500μM) for the first 24h of differentiation and whole cell lysates were harvested at the indicated time points. Western blot was performed using antibodies for p-ERK, total-ERK and GAPDH. B) Cells were pre-treated with PD98059 (10μM) for 30min prior to 24h AA treatment. Total RNA was isolated at D10 and qRT-PCR was performed for B) aP2, C) PPAR γ and D) C/EBP α . The values are presented relative to the corresponding vehicle-treated control (set as 100%). Data are presented as mean $\pm$ SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-tests were performed for AA-treated cells in the presence or absence of PD98059. *P<0.05, **P<0.01. Student's t-tests were performed for BSA and AA-treated cells in the absence of PD98059. a: ***P < 0.001, b: **P < 0.01, c: *P<0.05.

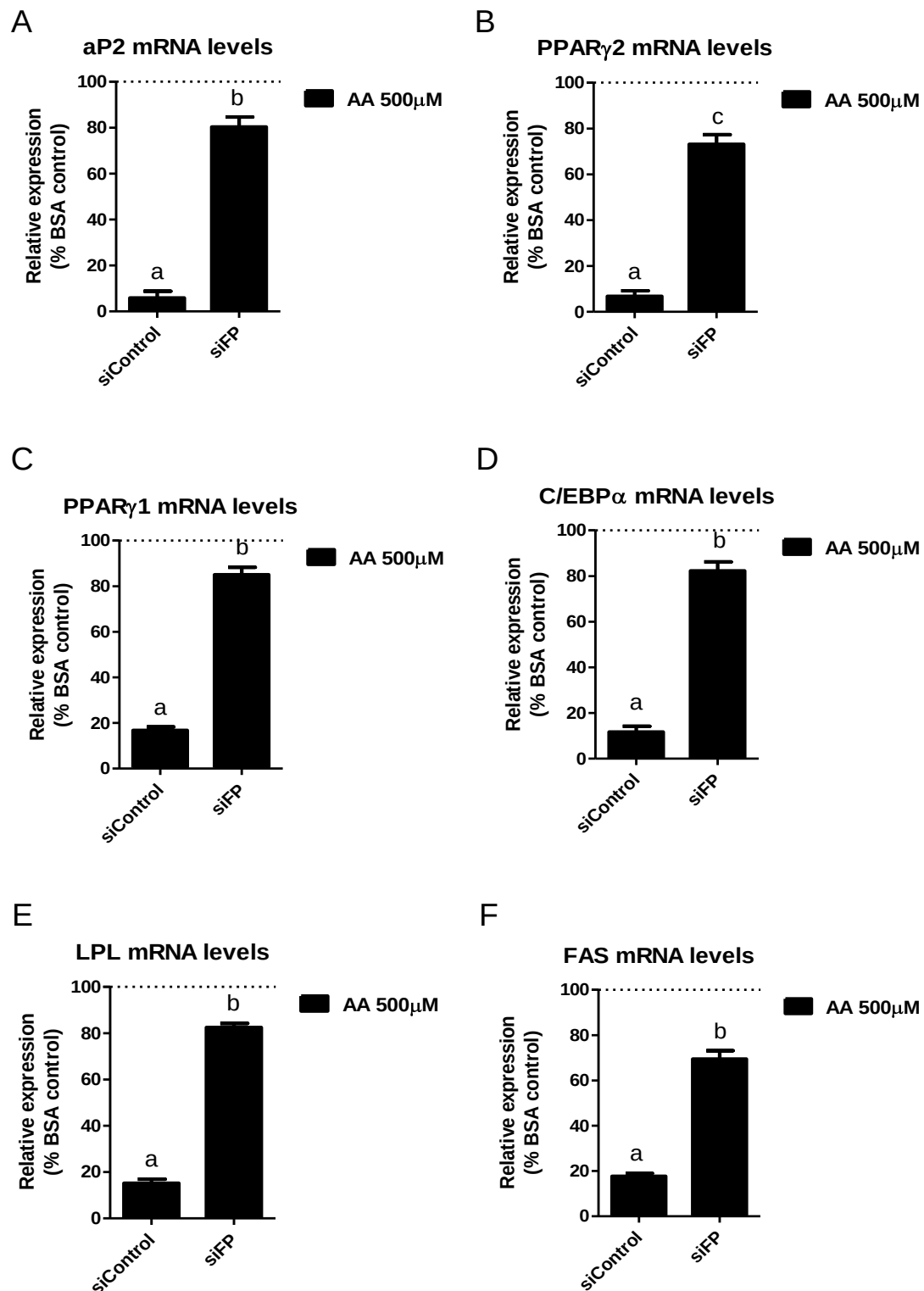


Figure 4.22: Depletion of the FP receptor prevents the inhibition of differentiation by AA—gene expression of adipocyte markers. 80% confluent cells were transfected with a non-targeting control (siControl) or an siRNA targeting the FP receptor (siFP). Three days after transfection the cells were treated with AA in the presence of MDI for 24h when AA was withdrawn and the transfection was repeated. The differentiation protocol was followed until D10. Total RNA was isolated and qRT-PCR was performed for A) aP2, B) PPAR γ 2, C)

PPAR γ 1, D) C/EBP α , E) LPL and F) FAS mRNA. The values are presented relative to the corresponding vehicle-treated control (set as 100%). Data are presented as mean $\pm$ SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-tests were performed for BSA and AA-treated siControl. a: ***P < 0.001. Student's t-tests were performed for AA-treated siControl and siFP. b: **P < 0.01, c: ***P<0.001.

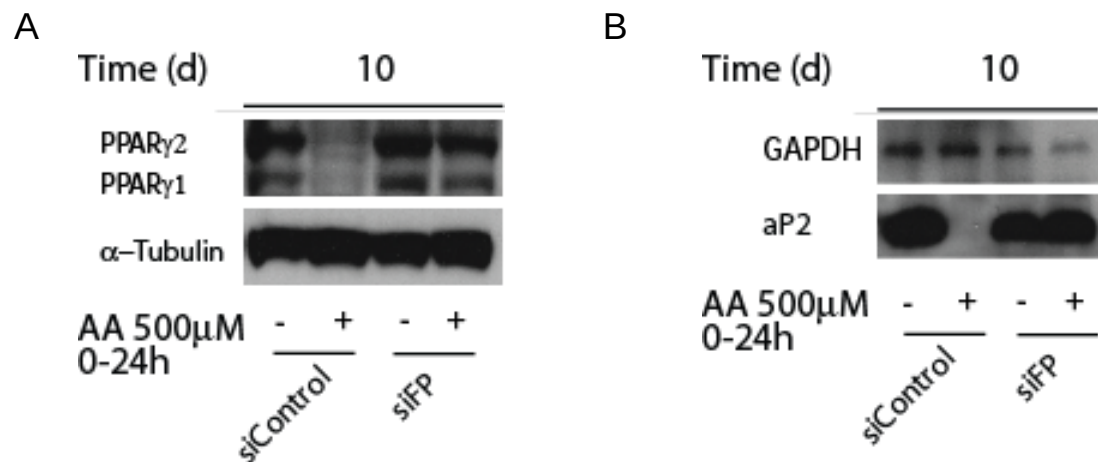


Figure 4.23: Depletion of the FP receptor restores PPAR γ and aP2 protein levels upon AA treatment. 3T3-L1 cells were transfected and treated as described in Fig 4.22. Briefly, cells were transfected with siControl or siFP and treated for 24h with AA (500 μ M). Whole lysates were extracted on D10 and subjected to Western blotting using antibodies for A) PPAR γ and α -tubulin or B) aP2 and GAPDH.

4.2.2.9 Fra-1 mediates AA-induced inhibition of differentiation

We found that Fra-1 expression is increased upon AA treatment at the first stages of differentiation (see section 3.3.1). Thus, it was hypothesised that Fra-1 could play a role in mediating the AA-dependent inhibition of differentiation. First, to test this hypothesis, Fra-1 expression levels were examined using the protocol of the short AA treatment for the first 24h of differentiation and cell lysates were harvested at different time points until the end of the differentiation procedure (Figure 4.24). Fra-1 protein levels increased after 24h AA treatment and started declining from the second day onwards. On the second day, the expression of Fra-1 in AA-treated cells was still above the basal levels. From the fourth day both vehicle-control and AA treated cells showed minimal levels of Fra-1, confirming that Fra-1 is expressed only during the early stages of differentiation (Figure 4.24).

To elucidate a potential role of Fra-1 in the inhibition of differentiation by AA, cells were transfected with an siRNA targeting Fra-1, then treated with AA for the first 24h and the effect on the differentiation was evaluated on D10 either by an Oil Red O stain (Figure 4.25), or by qRT-PCR for the main differentiation markers (Figure 4.26). Depletion of Fra-1 was able to block the ability of AA to inhibit differentiation as the expression of all the differentiation markers tested in the presence of siFra-1 was similar to the vehicle-treated cells. This observation was further confirmed by Oil Red O staining where cells treated with AA and transfected with an siControl did not accumulate any lipid. Cells that were AA-treated and transfected with an siFra-1 they accumulated lipid at similar levels to the vehicle-control levels.

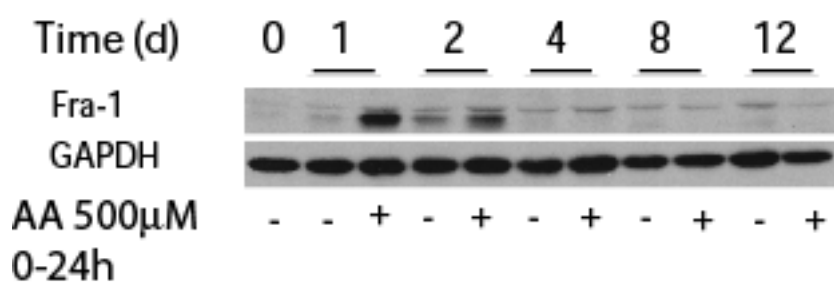


Figure 4.24: Fra-1 protein expression after a short treatment with AA at the early stages of differentiation. 3T3-L1 cells were treated with 500µM AA for the first 24h and whole cell lysates were isolated at the indicated time points during the differentiation procedure. Western blotting was performed using antibodies against Fra-1 and GAPDH.

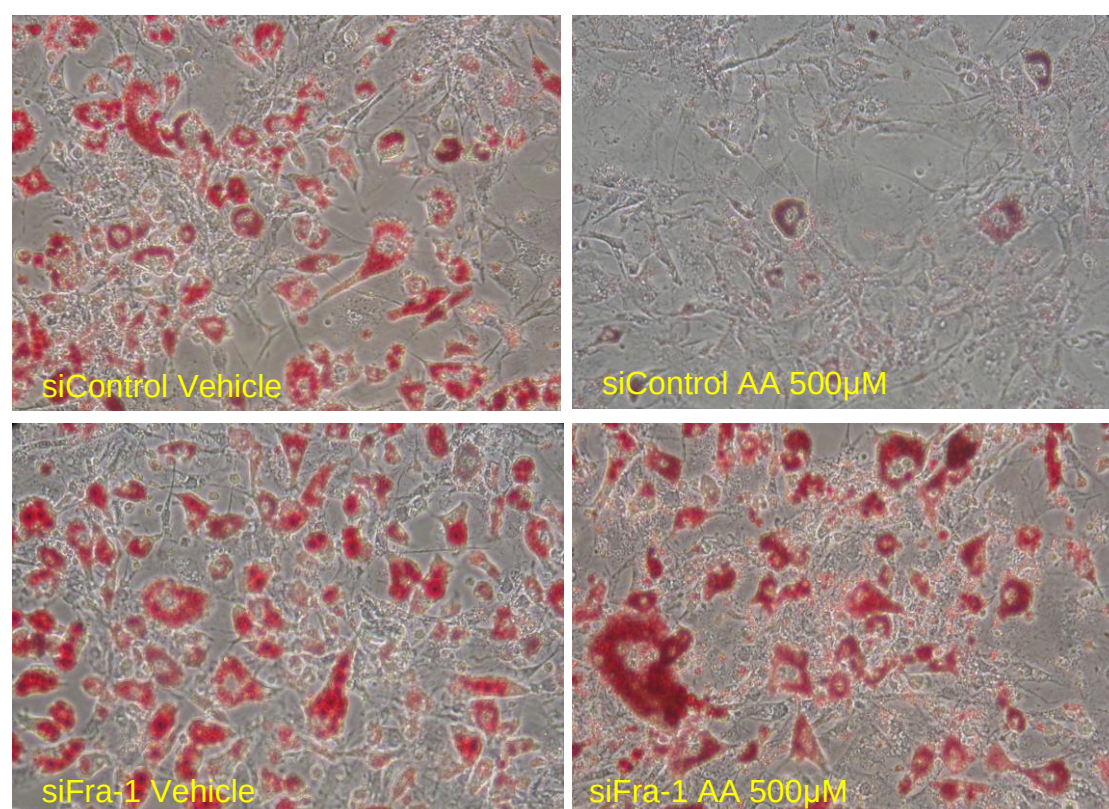


Figure 4.25 AA-induced inhibition of differentiation requires Fra-1–lipid accumulation. 80% confluent cells were transfected with either siFra-1 or a non-targeting control (siControl). On the third day of transfection cells were treated with 500µM AA for 24h. The next day, AA was withdrawn and the differentiation protocol was carried out until D10. Cells were fixed and stained with Oil Red O and pictures were captured under a light microscope (20x magnification).

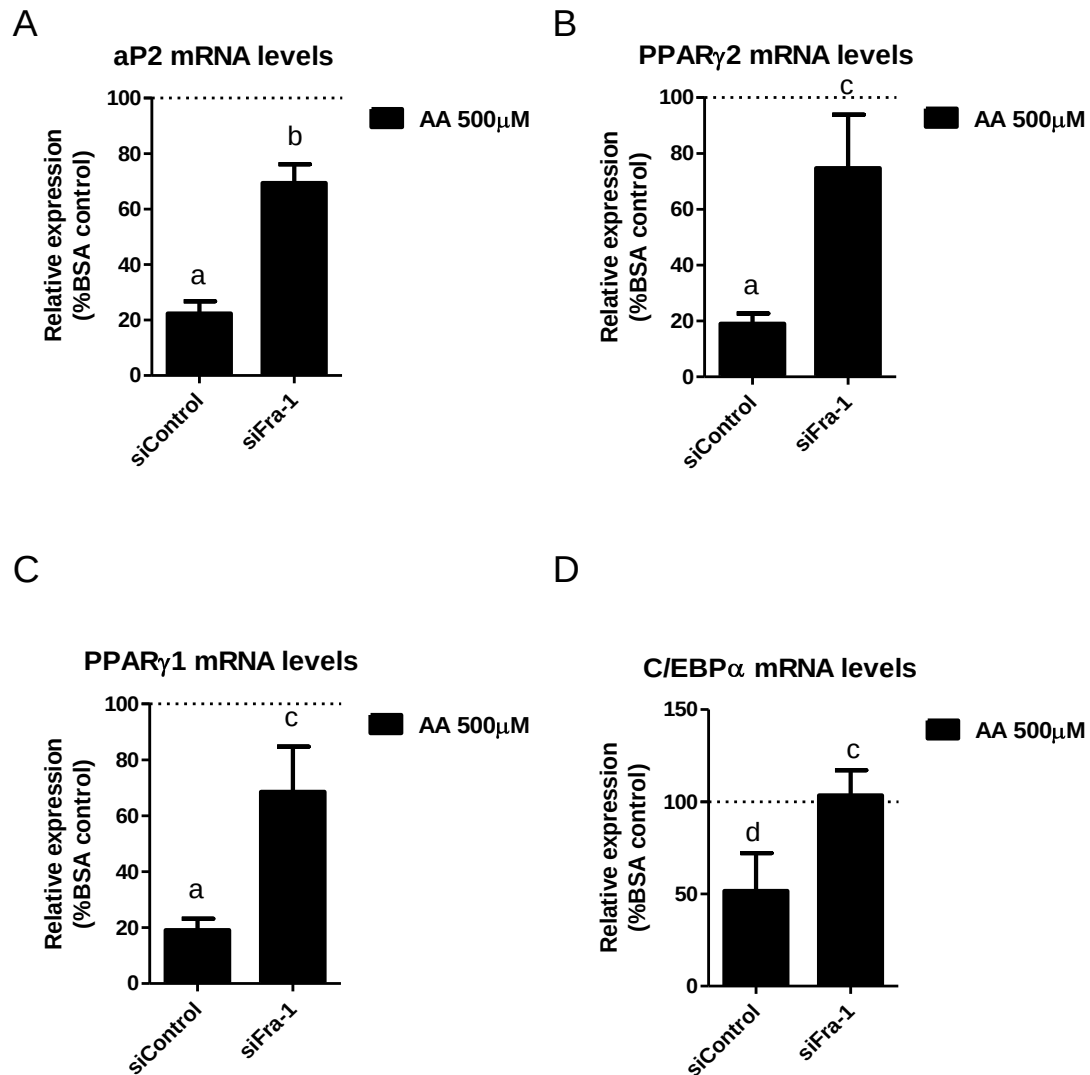


Figure 4.26: AA-dependent inhibition of differentiation requires the presence of Fra-1 – gene expression of adipocyte markers. 3T3-L1 cells were transfected with either siFra-1 or a non-targeting control (siControl), treated with 500 μ M AA for 24h and total RNA was isolated on D10. qRT-PCR was performed for A) aP2, B) PPAR γ 2, C) PPAR γ 1 and D) C/EBP α mRNA. Values shown are mean percentages of the respective vehicle control (set as 100%) of three independent experiments (n=3). Error bars represent $\pm$ SEM. Student's t-tests were performed for BSA and AA-treated siControl. a:***P < 0.001, d:*P<0.05. Student's t-tests were performed for AA-treated siControl and siFra-1. b: ***P < 0.001, c: *P<0.05.

4.2.2.10 Fra-1 regulates PPAR γ phosphorylation

PPAR γ is the key nuclear receptor of adipogenesis. When PPAR γ is not expressed or non-functional in pre-adipocytes then the cells lose their differentiation potential. Any agent that inhibits adipocyte differentiation is likely to affect either directly or indirectly the expression and/or function of PPAR γ . Thus, it was hypothesised that AA treatment at the start of the differentiation could dysregulate PPAR γ action resulting in the inhibition of differentiation. PGF_{2 α} has been shown before to result in increase phosphorylation of PPAR γ at S112 leading to decreased transcriptional activity (Reginato et al., 1998). Thus, cells were treated for the first 24h of the differentiation with AA and whole cell lysates were harvested at the indicated time points (Figure 4.27 A). Total PPAR γ levels were less in AA-treated compared to the vehicle-treated cells from the first 24h of treatment. The same trend was observed until the last of differentiation (day 8) which is consistent with the AA-induced inhibition of differentiation. In contrast, the levels of PPAR γ phosphorylation at S112 appear to be elevated after AA treatment after the first 24h of treatment compared to the vehicle-treated cells and increases at the subsequent time points. In the untreated cells the phosphorylation status of PPAR γ appears to be biphasic with an increase on the second day and then an increase on day 8. However, at both time points the phosphorylation was increased in the AA-treated cells (Figure 4.27 A).

As showed in section 4.12, the depletion of Fra-1 could prevent the ability of AA to inhibit differentiation. Thus, it was investigated if the absence of Fra-1 would affect the phosphorylation status of PPAR γ at S112. Cells were transfected with the siControl and siFra-1 siRNAs, treated with AA and whole cell lysates were prepared at the end of the differentiation procedure. In siControl cells, AA treatment caused the increase in the phosphorylation status of PPAR γ in comparison with the vehicle-treated cells. However in siFra-1 cells the phosphorylation status of PPAR γ in AA-treated cells was similar with the untreated cells. In addition, the levels of total PPAR γ appeared to be restored in the siFra-1 transfected cells treated with AA. (Figure 4.27 B).

In conclusion, it appears that Fra-1 is mediating the phosphorylation of PPAR γ at S112 by an unknown mechanism.

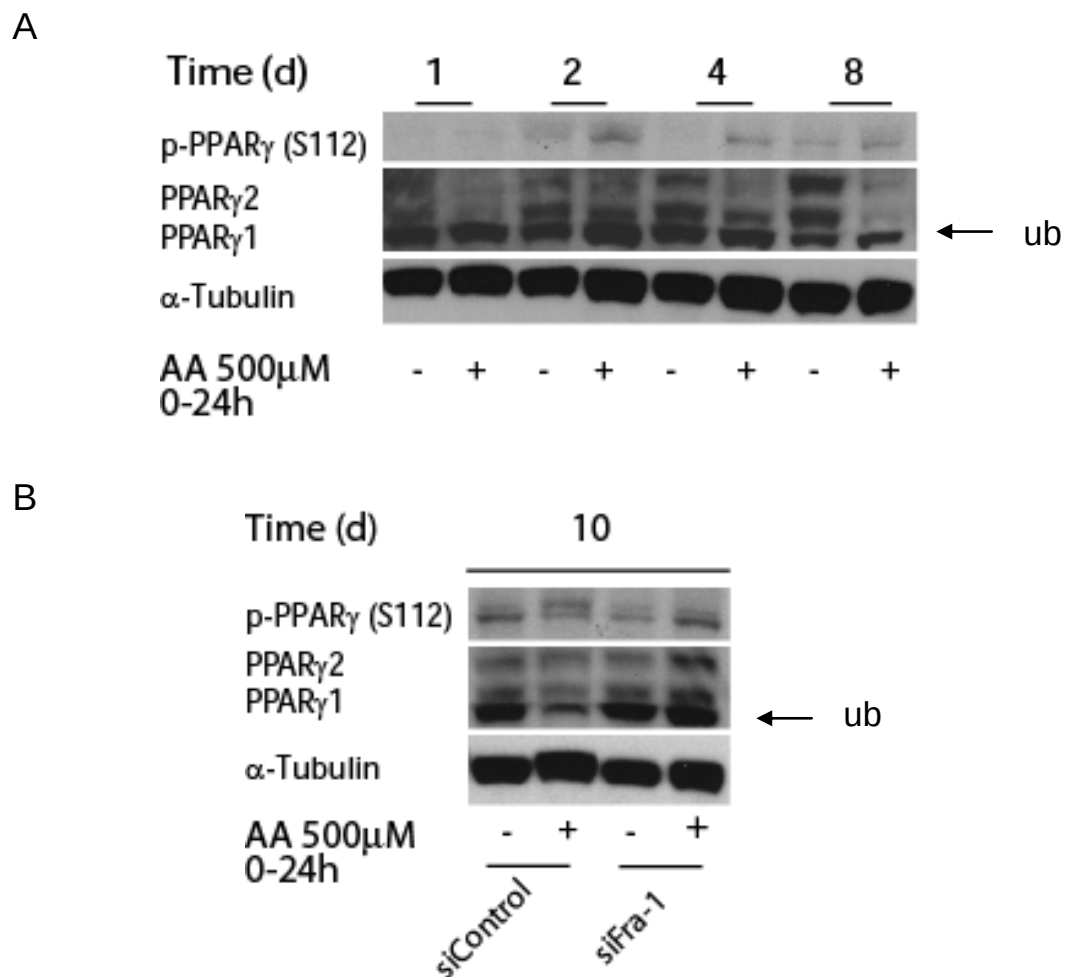


Figure 4.27: The phosphorylation of PPAR γ at S112 is Fra-1-dependent. A) 3T3-L1 cells were treated with 500 μ M AA for the first 24h and whole cell lysates were isolated at the indicated time points during the differentiation procedure. Western blotting was performed using antibodies against for p-PPAR γ (S112), total PPAR γ and α -tubulin. B) 3T3-L1 cells were transfected with siControl or siFra-1 and treated for 24h with AA (500 μ M). Whole lysates were extracted on D10 and subjected to Western blotting using antibodies for p-PPAR γ (S112), total PPAR γ and α -tubulin.

4.2.2.11 Short exposure to AA at the start of differentiation keeps the cells into a pre-adipocyte state

A short exposure to AA at the beginning of the differentiation procedure inhibits differentiation. However, the effects on cellular identity following the treatment could either be to promote the phenotype of non-functional adipocytes or cells restricted to the pre-adipocyte state. To assess this, the expression of Pref-1, GATA-2 and KLF-2 was examined. These genes are highly expressed in pre-adipocytes but decrease during differentiation and thus they are used as pre-adipocyte markers. Pref-1 expression increased dramatically after eight days of differentiation in the AA or PGF_{2α}-treated cells in comparison with the vehicle-treated cells (Figure 4.28 A, B). GATA-2 expression increased after the fourth day in the AA-treated cells whereas in the PGF_{2α}-treated cells the expression was above the basal levels at all time points (Figure 4.28 C, D). Finally, KLF-2 expression was always above the basal levels at all time points in either AA- or PGF_{2α} -treated cells (Figure 4.28 E, F). In conclusion, short-term exposure of pre-adipocytes to AA or PGF_{2α} promotes the expression phenotype of a pre-adipocyte.

Since both the depletion of the FP receptor and Fra-1 rescued the inhibition of differentiation by AA, the expression of the pre-adipocyte markers was also assessed in cells under these conditions (Figure 4.29). Both siFP and siFra-1 were able to prevent the increased levels of the pre-adipocyte markers due to AA treatment, indicating that both have a role in the sustained pre-adipocyte state caused by AA.

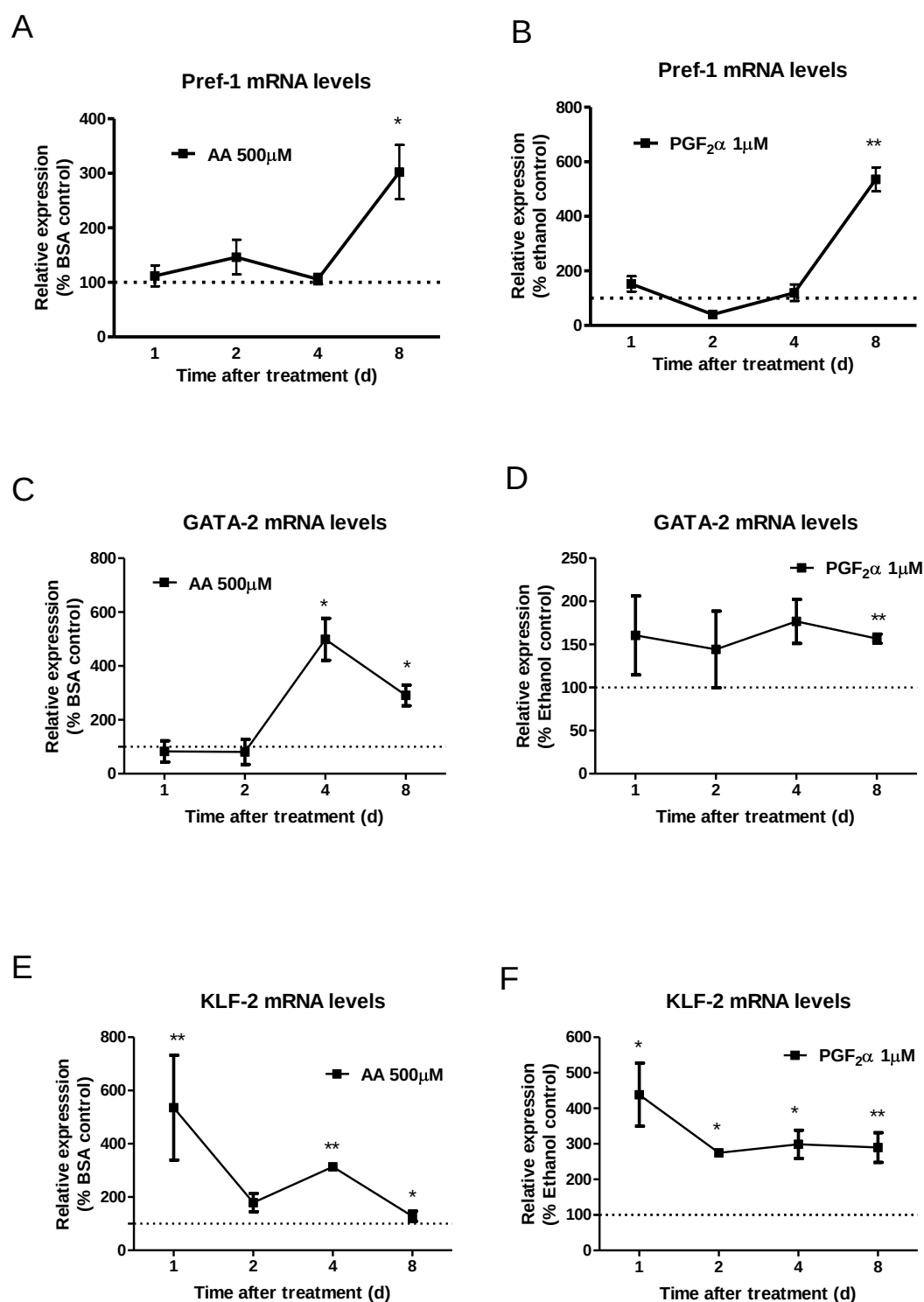
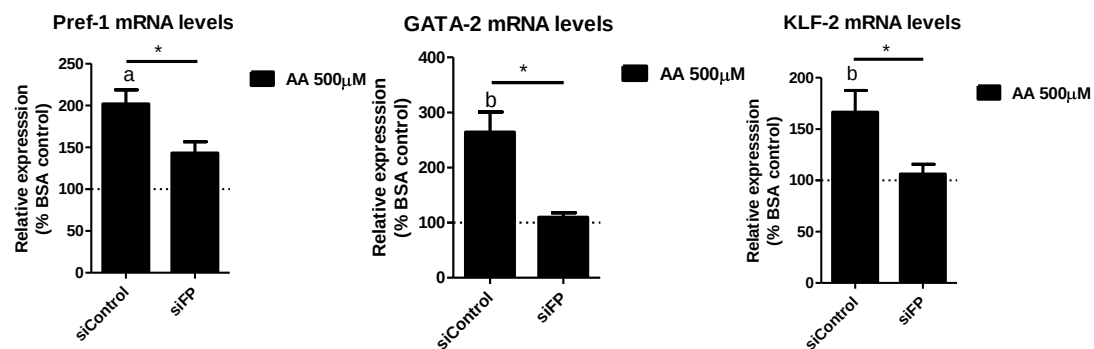


Figure 4.28: AA or PGF₂α treatments at the beginning of the differentiation procedure retain cells in the pre-adipocyte state. 3T3-L1 cells were treated for the first 24h of differentiation with either AA (500 μM) or PGF₂α (1 μM), then withdrawn and RNA was isolated at the indicated time points. qRT-PCR was conducted for A) and B) Pref-1, C) and D) GATA-2 and E) and F) KLF-2 mRNA. Data are presented as mean±SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's

t-test was conducted for the vehicle-treated and the AA- or PGF_{2α}-treated cells. *P<0.05, **P<0.01.

A



B

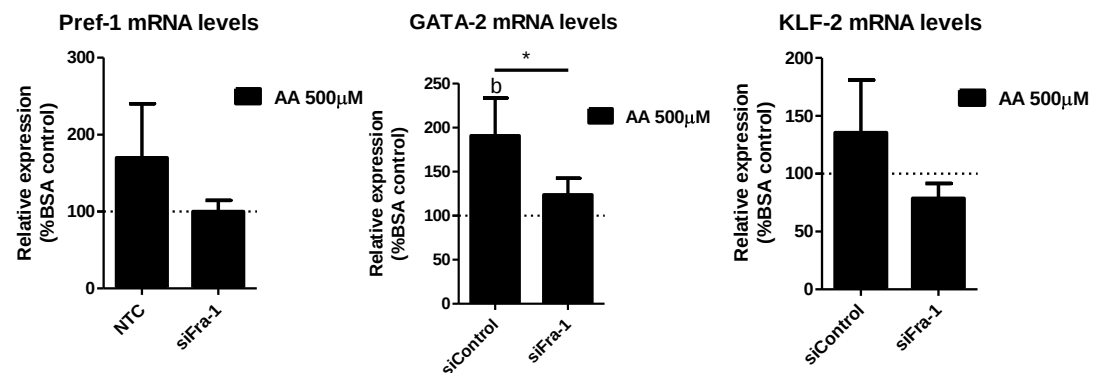


Figure 4.29: The FP receptor and Fra-1 are required for the AA-dependent up-regulation of pre-adipocyte marker genes. 3T3-L1 cells were transfected with siFP, siFra-1 or siControl, treated with 500 μM AA for 24h and total RNA was isolated on D10. The expression of Pref-1, GATA-2 and KLF-2 was assessed after the depletion of the A) FP receptor or B) Fra-1. For A, data are presented mean±SEM based on triplicate determinations from a representative experiment of two independent experiments. Student's t-tests were performed for AA-treated siControl and siFP *P<0.05 or for BSA and AA-treated siControl. a:**P < 0.01, b:*P<0.05. For B, the graphs represent mean values from three independent experiments and error bars represent ±SEM Student's t-tests were performed for AA-treated siControl and siFra-1 *P<0.05 or for BSA and AA-treated siControl b:*P<0.05.

4. 3 Discussion

To determine whether AA affects adipocyte differentiation, 3T3-L1 cells were treated with increasing doses of AA for the entire differentiation program. AA inhibited adipocyte differentiation in a dose-dependent manner. This observation is supported by previous reports showing that AA can inhibit the differentiation of 3T3-L1 cells (Casimir et al., 1996; Madsen et al., 2005) but also primary pre-adipocytes (Serrero and Lepak, 1997) and 1246 cells (Serrero et al., 1992).

As reported in chapter 3, AA treatment for the first 24h of differentiation triggered a number of changes in cell signalling and gene expression. To investigate whether a short stimulation with AA at the beginning of the differentiation program modulates the differentiation potential of adipocytes, pre-adipocytes were treated during the first 24h and the differentiation was evaluated. AA abrogated the differentiation even when it was present only at the early stages of differentiation as the majority of the cells retained the morphological characteristics of fibroblasts and did not accumulate LDs. This was a dose-dependent effect as increasing doses of AA had an inverse effect on differentiation.

The negative effect on the differentiation became apparent around the fourth day of the differentiation program when the AA-treated cells lost their initial LDs, whereas at the same time point the vehicle-treated cells became rounded and started accumulating lipid. Around the same time vehicle-treated cells started to express the mature differentiation markers whereas AA-treated cells failed to do so. In addition, AA lost its inhibitory effect when treatment started after the second day of differentiation or when present less than 24h. These data indicated that there is a window at the early stages of differentiation for AA to exert its inhibitory effect. This effect was not reversible either by the addition of a PPAR γ ligand or a prolonged MDI treatment, showing that AA interferes with a critical early event during pre-adipocyte differentiation.

During the first 24h of differentiation, growth-arrested 3T3-L1 pre-adipocytes re-enter the cell cycle synchronously, undergoing mitotic clonal expansion, which is essential for the differentiation to occur (Tang et al, 2003). For this reason, cell cycle progression was tested and AA did not affect the cell number going through mitosis. Previous observations showed that $\text{PGF}_{2\alpha}$, a major AA metabolite does not interfere with the mitotic clonal expansion phase of adipogenesis (Liu and Clipstone, 2007). In addition, AA did not affect cell viability nor did it cause cytotoxicity indicating that there is another mechanism by which AA inhibits differentiation.

PG synthesis was required for the action of AA as when cells were pre-treated with indomethacin, a general cyclooxygenase inhibitor, the expression of the differentiation markers of the AA-treated cells were at similar levels to the vehicle-control cells. In addition, elevated COX-2 expression was detected in both protein and RNA levels following AA treatment. In particular, COX-2 protein expression was increased in the presence of AA above the basal levels until the end of the differentiation program. Elevated COX-2 expression by AA has been observed before. It has been also reported that indomethacin reduces COX-2 expression in AA-treated cells, indicating that PGs are necessary for maintaining COX-2 expression (Petersen et al., 2003). PG levels, in general, were shown to be lower in differentiated 3T3-L1 adipocytes compared to confluent or dividing pre-adipocytes (Hyman et al., 1982), supporting the observation that the inhibitory effect of AA on differentiation is likely to be an early event.

The present study showed that $\text{PGF}_{2\alpha}$ mimics the effect of AA on differentiation by inhibiting lipid accumulation when cells were treated for the first 24h of differentiation. $\text{PGF}_{2\alpha}$ -dependent inhibition of differentiation was also a dose-dependent event as shown for AA. In addition, the kinetics of $\text{PGF}_{2\alpha}$ inhibition was similar to AA as the inhibitory effect was apparent after the fourth day of differentiation. However, $\text{PGF}_{2\alpha}$ proved to be a more potent inhibitory factor than AA, since $\text{PGF}_{2\alpha}$ could exert its inhibitory effect during any time period tested and also as little as a 2h treatment was enough to inhibit differentiation. This finding is supported by previous reports showing

that fluprostenol, a selective FP receptor agonist could inhibit differentiation more than 60% when was present for only one hour at the beginning of the differentiation (Casimir et al., 1996).

PGF_{2α} has been shown to inhibit the differentiation of a number of adipocyte cell systems such as primary adipocytes (Serrero and Lepak, 1997), 1246 cells (Serrero et al., 1992) and 3T3-L1 cells (Casimir et al., 1996; Miller et al., 1996). PGF_{2α} concentrations decreased during 3T3-L1 differentiation, an event that may allow cells to progress into terminal differentiation as maintaining preadipocyte-like levels of PGF_{2α} prevents cells from differentiating (Casimir et al., 1996). This report was in agreement with our observations as in AA-treated cells, PGF_{2α} levels were elevated above the basal levels until the end of the differentiation program, indicating the generation of a continuous inhibitory signal for differentiation. Furthermore, PGF_{2α} synthase (AKRB13) expression had the same kinetics as COX-2 as it was increased after 24h of treatment and then reduced to basal levels by the end of differentiation. In addition, the FP receptor expression has been shown to be similar between pre-adipocytes and adipocytes (Miller et al., 1996) and the current data showed that in AA-treated cells the FP receptor expression was higher in comparison to the vehicle-treated cells. All these data indicate that the machinery for PGF_{2α} production and function is expressed until the late stages of differentiation, at lower levels compared to the early stages, but still there to mediate the inhibitory effect of PGF_{2α}.

AA and PGF_{2α} have been shown to enhance the activation of ERK1/2 (Reginato et al., 1998; Petersen et al., 2003). This observation was replicated herein as ERK activation was sustained after 12 days of differentiation in AA-treated cells. ERK activation has been shown to promote the clonal expansion at the early stages of differentiation (Prusty et al., 2002) but sustained expression has been linked with inhibition of differentiation (Petersen et al., 2003). The prevention of ERK activation has little or no effect on adipogenesis (Petersen et al., 2003) and for this reason a MEK-inhibitor (PD98059) was used to assess whether the AA-induced inhibition of differentiation was ERK-mediated. PD98059 could restore full adipogenesis in the presence of AA as

the expression levels of all the differentiation markers tested were similar to the vehicle-treated cells. Thus, ERK-activity was necessary for AA-mediated inhibition of adipocyte differentiation.

As demonstrated in Chapter 3, ERK activation is mediated by the FP receptor, thus we hypothesised that $\text{PGF}_{2\alpha}$ secreted after AA treatment, binds to FP receptor, activates ERK and inhibits differentiation. Depletion of the FP receptor could rescue the blockage of differentiation by AA, as cells in the absence of FP could express the differentiation markers at similar levels to vehicle-treated cells. However, further experiments need to be undertaken to directly link the FP receptor with ERK activation at the late stage of differentiation in the presence of AA. For example, assessing ERK phosphorylation after depletion of the FP receptor would establish that ERK activation is mediated by the FP receptor. However, this approach is limited as it is likely that ERK activation could be mediated by a variety of GPCR receptors expressed at the late stages of differentiation.

It has been shown that the inhibitory effect of AA on differentiation is dependent upon the addition of cAMP elevating agents (Madsen et al., 2008) suggesting that the effect of AA is mediated by a PKA-sensitive pathway. However, although a PKA inhibitor was able to prevent the inhibition of differentiation by AA it could not inhibit ERK activation (Petersen et al., 2003). As revealed in Chapter 3, AA could enhance the generation of a calcium response. It has been established before that Ca^{2+} -mobilising agents could inhibit adipogenesis (Ntambi and Takova, 1996). Furthermore, it has been suggested that the $\text{PGF}_{2\alpha}$ -mediated inhibition of differentiation is through FP receptor-mediated increase in intracellular calcium increase (Miller et al., 1996). However, a PKC inhibitor was not able to block the inhibition of differentiation by $\text{PGF}_{2\alpha}$ (Miller et al., 1996). In conclusion, it is apparent that the regulation of adipocyte differentiation involves both PKA and PKC-mediated signalling pathways.

As described in Chapter 3, Fra-1 expression was increased by AA after a 24h treatment through a PKA- and a PKC-mediated pathway. Fra-1 was

expressed transiently, early in the differentiation procedure, but upon AA treatment its expression was increased above the basal levels until the second day of differentiation. Thus, we predicted that Fra-1 could play a role in the inhibitory effect of AA on adipocyte differentiation. Indeed, depleting Fra-1 expression could prevent the inhibitory effect of AA on the adipocyte differentiation. The role of Fra-1 in adipogenesis is largely unknown but it has been demonstrated recently that Fra-1 transgenic mice are lipodystrophic (Luther et al., 2011). This observation is in agreement with our data, implicating Fra-1 as an internal break in the differentiation procedure. It was suggested that Fra-1-dependent lipodystrophy was mediated by negatively regulating C/EBP α (Luther et al., 2011). However in our hands, although C/EBP α expression was reduced upon AA treatment, Fra-1 was not recruited on the C/EBP α promoter upon AA treatment (data not shown).

As PPAR γ is a crucial transcription factor regulating adipogenesis, it was hypothesised that, AA-induced inhibition of differentiation would have an impact on PPAR γ expression and/or activity. Indeed, AA-treated cells showed lower expression of total PPAR γ and more of the PPAR γ phosphorylated form until the end of the differentiation program. It has been demonstrated that both ERK activation and PGF $_{2\alpha}$ treatment lead to the phosphorylation of PPAR γ at the S112 residue decreasing PPAR γ transcriptional activity (Camp and Tafuri, 1997; Reginato et al., 1998). Therefore, the AA-enhanced ERK activation in combination with the fact that PGF $_{2\alpha}$ mediates the inhibitory effect of AA raises the possibility that the AA-PGF $_{2\alpha}$ -FP receptor-ERK pathway leads to PPAR γ phosphorylation and inhibition of differentiation. Furthermore, the role of Fra-1 on PPAR γ expression was assessed as depletion of Fra-1 was enough to restore the normal differentiation upon AA treatment. Furthermore, depletion of Fra-1 was able to restore the normal levels of both total and phosphorylated PPAR γ in AA-treated cells. In conclusion, Fra-1 appears to mediate PPAR γ phosphorylation either directly or indirectly. Further studies need to be undertaken to elucidate whether Fra-1 regulates PPAR γ expression and/or phosphorylation directly or to identify Fra-1 target genes that could lead to PPAR γ phosphorylation. In conclusion, Fra-1 appears to be

a key mediator of AA-dependent inhibition of differentiation, highlighting the importance of early events in pre-adipocyte differentiation potential.

A role for aP2 early up-regulation, upon AA treatment, in the inhibition of differentiation was investigated. Using aP2-depleted 3T3-L1 cells and the control shScramble cells, it was not possible to draw a conclusion as AA-induced inhibition of differentiation was not observed in the shScramble cells. Moreover, a cell-permeable selective aP2 inhibitor targeting the fatty acid binding pocket did not block the inhibitory effect of AA, indicating that aP2 early up-regulation was not related to the inhibitory effect of AA (data not shown).

As the levels of PGs are higher in pre-adipocytes compared to differentiated 3T3-L1 adipocytes, this indicates that the presence of PGs may maintain cells in the pre-adipocyte state (Hyman et al., 1982). Therefore, to determine the cellular identity of cells at the late stage of differentiation after the early short exposure to AA, the expression of Pref-1, GATA-2 and KLF-2 pre-adipocyte markers was assessed. The expression of all three pre-adipocyte markers was above the basal levels at the late stages of differentiation in AA-exposed cells. Pref-1 is classically used as a pre-adipocyte marker as it is specifically expressed in pre-adipocytes but not in mature adipocytes (Wang et al., 2006). GATA-2 transcription factor has been shown to inhibit adipogenesis by direct binding to the C/EBP β protein and to the GATA binding site on the PPAR γ 2 promoter (Tong et al., 2000; Tong et al., 2005). Constitutive expression of GATA-2 suppressed adipocyte differentiation and maintained cells at the pre-adipocyte stage (Tong et al., 2000). Finally, KLF-2 is expressed in pre-adipocytes but not in mature adipocytes and acts as a negative regulator of adipogenesis via direct interaction with the PPAR γ promoter and inhibiting its expression (Banerjee et al., 2003). These data indicated that the presence of AA at the early stages of differentiation restricts the cells to the pre-adipocyte stage. Moreover, this reveal the importance of the FP receptor and Fra-1 in maintaining the pre-adipocyte state and their down-regulation required for adipogenesis to proceed.

In conclusion, this chapter demonstrated that AA-induced events at the beginning of the differentiation process affect the differentiation potential of pre-adipocytes. We suggest that AA after being metabolised to $\text{PGF}_{2\alpha}$, which binds to the FP receptor, activates ERK which regulates Fra-1 expression and in turn inhibits the differentiation possibly through deactivating PPAR γ (Figure 4.30).

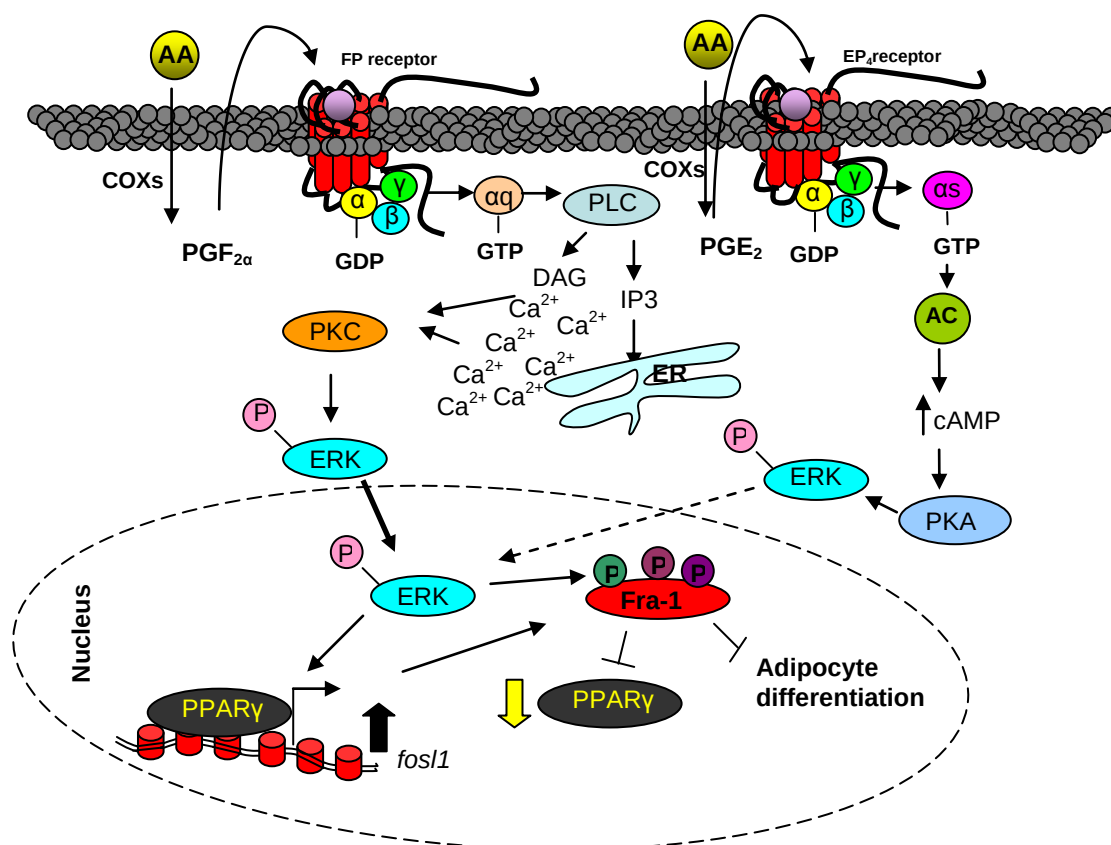


Figure 4.30: Schematic illustration of the proposed mechanism triggered by AA to inhibit pre-adipocyte differentiation. Upon treatment of pre-adipocytes with AA, COXs mediate its conversion to PGs particularly $\text{PGF}_{2\alpha}$ and PGE_2 . These PGs bind to their membrane GPCRs, the FP and the EP_4 receptor, respectively and trigger two different pathways. Both pathways lead to ERK activation but the FP mediated pathway is the dominant one. ERK then up-regulates Fra-1 transcription but also phosphorylates Fra-1, stabilising Fra-1 protein. PPAR γ also up-regulates Fra-1 expression. Increased levels of Fra-1 inhibit terminal differentiation, keeping the cells at the pre-adipocyte stage, probably by mediating PPAR γ deactivation.

Chapter 5

Discussion

This thesis explored the mechanisms by which a major omega-6 PUFA, AA affects adipocyte differentiation. It is important to understand how adipogenesis is modulated as the formation of new adipocytes prevents the appearance of insulin resistant adipocytes, as well as the lipid deposition in peripheral organs such as muscle, liver, and heart. Classically, AA has been correlated with increased inflammation and obesity as excessive caloric intake (especially consumption of food rich in fat) relative to expenditure promotes both hyperplasia and hypertrophy of adipocytes. However, the role of AA on adipocyte differentiation has been controversial with some studies showing it is pro-adipogenic and some anti-adipogenic (Massiera et al., 2003; Madsen et al., 2005). Besides adipogenesis, there is increasing evidence from human studies correlating higher plasma levels of AA with decreased levels of serum proinflammatory markers and reduced risk of coronary heart disease (Harris et al., 2009). Thus, the role of AA in whole body metabolism appears to be more complex than originally thought.

It is also becoming apparent that the differentiation of pre-adipocytes into mature adipocytes is a complex process involving both positive and negative signalling pathways which contribute to the adipogenic gene program. Indeed, our data showed that regulation of adipogenesis by AA had a dual role in adipogenesis with effects observed at the early and late stages of differentiation. In the early stages of differentiation, AA was found to positively regulate the expression of aP2 and Fra-1, whereas at the late stages AA negatively impacted adipocyte differentiation. The main focus of this thesis was to elucidate the signalling pathways and the molecular mechanisms underpinning these two events.

Upon treatment of pre-adipocytes with AA, COXs convert AA to several PGs. We observed that this event occurred within the first few minutes after the addition of AA to pre-adipocyte cell monolayers. This is in agreement with previous reports showing that PGs are generated most effectively in 10-15 minutes after treatment with AA in skeletal muscle (Berlin et al., 1979). Subsequently, PGs mediate the effect of AA to increase the levels of the two

key second messengers, cAMP and calcium. We showed that the AA-induced cAMP increase was mediated by EP4 receptor which is a PGE₂ GPCR, whereas calcium increase was downstream of the FP receptor which is a PGF_{2α} receptor. However, the EP4 antagonist did not block completely the cAMP production, indicating that other GPCRs could contribute to the increase in cAMP levels upon AA treatment. Alternatively, in addition to coupling to Gαq, it is conceivable that the FP receptor could be coupled to Gαs, mediating the cAMP increase, as it is not uncommon for GPCRs to be coupled with multiple α subunits.

Investigating downstream effects of AA-dependent signalling we showed that AA, through PGs, activated ERK. ERK activation was mediated predominantly by the FP receptor but it cannot be excluded that other parallel pathways, triggered by AA, could also affect ERK activation. ERK has been shown to have a diverse range of substrates as it can translocate to the nucleus and phosphorylate transcription factors or it can regulate the activity of other cytoplasmic protein kinases (Davis, 1993). ERK has also been reported to phosphorylate cPLA₂ enhancing its activity (Sa et al., 1995), a mechanism that could also result in sustained signal after AA treatment.

We showed that AA signalling in the early stages of differentiation had two main downstream targets. One of them was the AA-mediated early and profound up-regulation of aP2. This up-regulation was mediated by the PGF_{2α}-FP receptor-calcium-PKC-ERK pathway (Figure 5.1). Moreover, we showed that aP2 expression was enhanced by PPARγ upon AA treatment. aP2 is a classical gene target of PPARγ in the later stages of differentiation but it appears that in the early stages upon AA treatment, PPARγ ligands were generated to enhance its activity. However, it appears that at the same time AA treatment produced signals to cause the phosphorylation of PPARγ and its deactivation. One such metabolite has been reported to be PGF_{2α} (Reginato et al., 1998). This would explain the obvious discrepancy between the decreased PPARγ levels and the increased levels of aP2. In conclusion, it appears to be a balance between positive and negative signals upon AA treatment that regulate PPARγ activity.

Furthermore, this is the first study associating a FABP with autophagy. We suggest that upon excess lipid treatment, aP2 mediates the transport of lipids to autophagosomes to avoid lipotoxic effects. On the other hand, when aP2 is not expressed autophagy cannot function, indicating that aP2 is critical for the autophagy to occur. However, other possible functions of aP2 cannot be excluded. For example, aP2 can translocate to the nucleus in response to specific ligands and interact directly with PPAR γ enhancing its activity (Tan et al., 2002). This up-regulation of aP2 could also result in a positive feedback loop sustaining its expression by providing ligands to PPAR γ

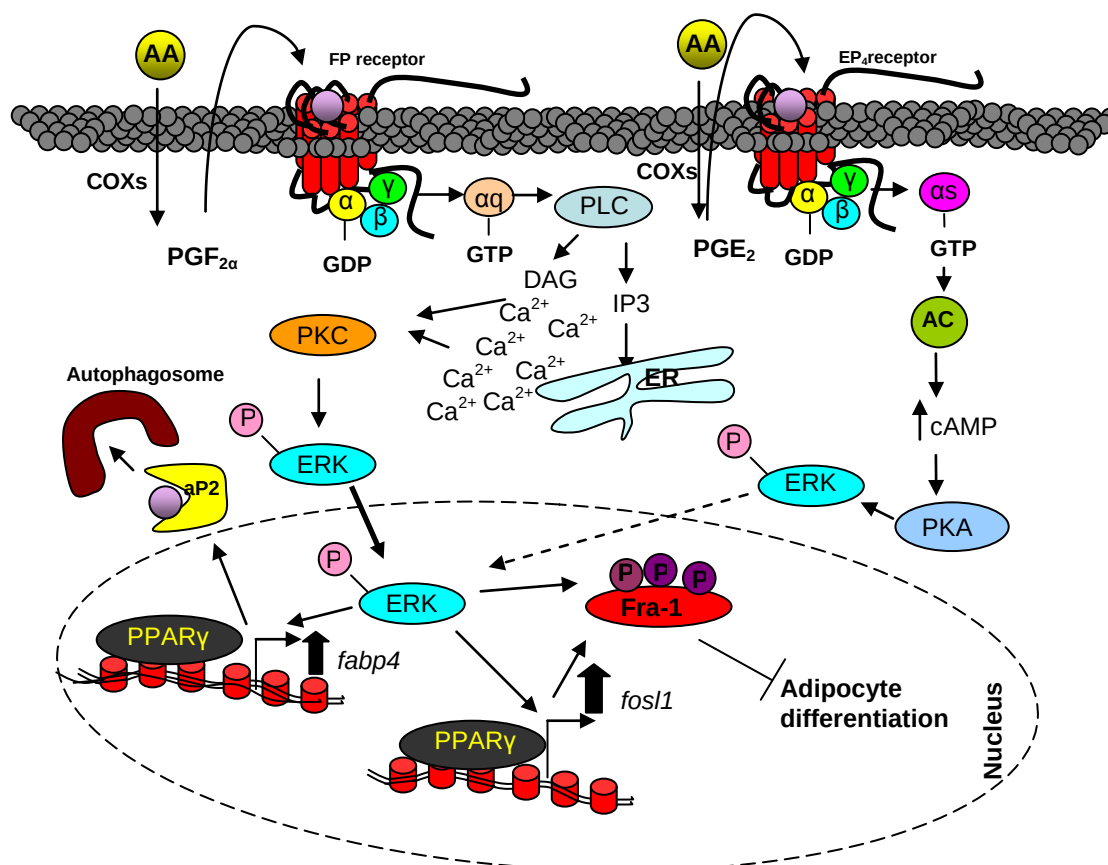


Figure 5.1: Schematic illustration of the proposed mechanism triggered by AA to regulate the early and late stages of adipocyte differentiation. Upon treatment of pre-adipocytes with AA, COXs mediate its conversion to PGs particularly PGF_{2α} and PGE₂. These PGs bind to their membrane GPCRs, the FP and the EP4 receptor, respectively and trigger two different pathways. Both pathways lead to ERK activation but the FP mediated pathway is the dominant one. ERK then mediates the up-regulation of aP2 and *fra-1* gene transcription increasing the expression of aP2 and Fra-1 mRNA, respectively. ERK also phosphorylates

Fra-1, stabilising Fra-1 protein. PPAR γ also up-regulates Fra-1 and aP2 expression. Increased levels of Fra-1 inhibit terminal differentiation, keeping the cells at the pre-adipocyte stage, probably by mediating PPAR γ deactivation.

From this study we showed for the first time that Fra-1 is a new factor regulating adipocyte differentiation upon AA treatment. We showed that the main receptor mediating Fra-1 up-regulation was the FP receptor which classically signals to PKC activation. Even though we showed that PKC was important for Fra-1 up-regulation, PKA signalling also appeared to be critical (Figure 5.1). This observation might explain the different effects of AA on adipogenesis in the presence of different cAMP levels. Madsen et al demonstrated that when cAMP levels are high AA inhibits differentiation whereas when cAMP levels are low, AA stimulates adipogenesis (Madsen et al., 2008). This could be mediated mechanistically by different levels of Fra-1 protein. Thus, it is plausible that in the presence of high cAMP levels, Fra-1 is up-regulated, stabilised and able to inhibit differentiation, whereas in the presence of low cAMP levels, Fra-1 is expressed at levels too low to inhibit differentiation. However, it appears that there is a cross-talk between PKA and PKC pathways as it has been shown that signalling through AC/cAMP does not necessarily involve PKA but also PKC (Parada et al., 2005). Furthermore, the activation of PKC by PKA cannot be excluded (Gomes and Soares-da-silva, 2002). In addition, calcium-activated ACs have been identified to mediate the activation of the cAMP/PKA pathway from calcium influx (Dunn et al., 2009). Finally, there is also a cross-talk between GPCRs. One such mechanism is the heterologous desensitisation. This involves the activation of one GPCR to generate a signal that causes inhibition of signalling by a second, heterologous GPCR mediated via phosphorylation by G protein coupled receptor kinases or PKA and PKC (Hosey, 1999). In conclusion, further studies need to be undertaken to fully elucidate the mechanism Fra-1 is regulated by AA.

Fra-1, in common with all AP-1 transcription factors, converts extracellular signals into changes in the expression of target genes, by binding to AP-1 binding sites found in the promoter/enhancer regions of a large number of

genes. Furthermore, the activity of Fra-1 is controlled at multiple levels, at gene transcription, mRNA turnover, protein stability by interaction with other transcription factors, and by phosphorylation of its transactivation domains by MAPKs. Thus, the complex control of Fra-1 makes it difficult to assess its impact on gene expression and its relevance to human disease.

Little is known about Fra-1 functions or about Fra-1 target genes. However, it has been reported that Fra-1 has a transactivation domain and can positively regulate gene expression (Young et al., 2002). In addition, Fra-1 can interact with HDAC1, indicating that it can negatively regulate gene expression by altering the chromatin landscape (Hoffmann et al., 2005). Thus, it is possible for Fra-1 to regulate both negatively and positively the expression of its target genes in order to drive the inhibition of differentiation. Further studies need to be performed to identify the target genes of Fra-1 after AA treatment leading to the inhibition of differentiation. However, one possible mechanism might be that Fra-1 regulates PPAR γ activity. We showed that depletion of Fra-1 could restore normal PPAR γ levels placing Fra-1 upstream PPAR γ in the pathway regulating differentiation. Further experiments need to be performed to elucidate the mechanism by which PPAR γ is regulated by Fra-1. In addition, our finding that Fra-1 is a negative regulator of adipogenesis was further supported by the generation of Fra-1 transgenic mice, which became lipodystrophic (Luther et al., 2011).

In conclusion, this study established that AA can trigger different signalling pathways affecting the function and the differentiation potential of pre-adipocytes. Thus, the regulation of adipogenesis by FAs appears to be more complicated than originally thought. It is now well-recognised that FAs can regulate adipogenesis in both a positive and a negative way, indicating the existence of a homeostatic mechanism that regulates the balance of positive and negative transcriptional regulators, promoting and inhibiting adipocyte differentiation upon high fat challenge.

References

Abumrad N., Coburn C., Ibrahimi A. (1999). Membrane proteins implicated in long-chain fatty acid uptake by mammalian cells: CD36, FATP and FABPm. *Biochim Biophys Acta*. 1441(1):4-13.

Adams M., Reginato M.J., Shao D., Lazar M.A., Chatterjee V.K.(1997) Transcriptional activation by peroxisome proliferator-activated Receptor γ is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. *J Biol Chem*. 272(8):5128-32.

Adiseshaiah P, Papaiahgari SR, Vuong H, Kalvakolanu DV, Reddy SP. (2003) Multiple cis-elements mediate the transcriptional activation of human fra-1 by 12-o-tetradecanoylphorbol-13-acetate in bronchial epithelial cells. *J Biol Chem*. 278(48):47423-33.

Adiseshaiah P, Peddakama S, Zhang Q, Kalvakolanu DV, Reddy SP. (2005) Mitogen regulated induction of FRA-1 proto-oncogene is controlled by the transcription factors binding to both serum and TPA response elements. *Oncogene*. 24(26):4193-205.

Adiseshaiah P, Li J, Vaz M, Kalvakolanu DV, Reddy SP.(2008) ERK signaling regulates tumor promoter induced c-Jun recruitment at the Fra-1 promoter. *Biochem Biophys Res Commun*. 371(2):304-8.

Ailhaud G. (2006a). n-6 Fatty acids and adipogenesis. *Scandinavian Journal of Food and Nutrition*. 50 (S2): 17 -20.

Ailhaud G., Massiera F., Weill P., Legrand P., Alessandri J.-M, Guesnet. P. (2006b). Temporal changes in dietary fats: Role of n-6 polyunsaturated fatty acids in excessive adipose tissue development and relationship to obesity. *Progress in Lipid Research*. 45; 203–236.

Ailhaud G., Guesnet P., Cunnane S.C. (2008). An emerging risk factor for obesity: does disequilibrium of polyunsaturated fatty acid metabolism

contribute to excessive adipose tissue development? *Br J Nutr.* 100(3):461-70.

Albrektsen T. and Fleckner J. (2001) The transcription factor Fos-related antigen 1 is induced by thiazolidinediones during differentiation of 3T3-L1 cells. *Mol Pharmacol.* 59(3):567-75.

Alexander LD, Cui XL, Falck JR, Douglas JG. (2001) Arachidonic acid directly activates members of the mitogen-activated protein kinase superfamily in rabbit proximal tubule cells. *Kidney Int.* 59(6):2039-53.

Amri E.Z., Bertrand B., Ailhaud G., Grimaldi P. (1991) Regulation of adipose cell differentiation. 1. Fatty acids are inducers of the aP2 gene expression. *J Lipid Res.* 1991 Sep;32(9):1449-56.

Aouadi M, Laurent K, Prot M, Le Marchand-Brustel Y, Binétruy B, Bost F.(2006) Inhibition of p38MAPK increases adipogenesis from embryonic to adult stages. *Diabetes.* 55(2):281-9.

Astudillo A.M., Balgoma D., Balboa M.A., Balsinde J. (2012). Dynamics of arachidonic acid mobilization by inflammatory cells. *Biochim Biophys Acta.* 1821(2):249-56.

Auwerx J. (1999). PPAR γ , the ultimate thrifty gene. *Diabetologia.* 42(9):1033-49.

Banerjee S.S., Feinberg M.W., Watanabe M., Gray S., Haspel R.L., Denkinger D.J., Kawahara R., Hauner H., Jain M.K. (2003) The kruppel-like factor KLF2 inhibits peroxisome proliferator activated receptor- γ expression and adipogenesis. *J Biol Chem.* 278(4):2581-4.

Barak Y., Nelson M.C., Ong E.S., Jones Y.Z., Ruiz-Lozano P., Chien K.R., Koder A., Evans R.M. (1999). PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol Cell.* 4, 585-595.

Barroso I., Gurnell M., Crowley V.E., Agostini M., Schwabe J.W., Soos M.A., Maslen G.L., Williams T.D., Lewis H., Schafer A.J., Chatterjee V.K., O'Rahilly S. (1999). Dominant negative mutations in human PPARgamma associated with severe insulin resistance, diabetes mellitus and hypertension. *Nature* . 402880–883.883.

Basbous J, Chalbos D, Hipskind R, Jariel-Encontre I, Piechaczyk M. (2007) Ubiquitin-independent proteasomal degradation of Fra-1 is antagonized by Erk1/2 pathway-mediated phosphorylation of a unique C-terminal destabilizer. *Mol Cell Biol*. 27(11):3936-50.

Bell-Parikh L.C., Ide T., Lawson J.A., McNamara P., Reilly M., FitzGerald G.A. (2003). Biosynthesis of 15-deoxy- $\Delta^{12,14}$ -PGJ2 and the ligation of PPAR γ . *J Clin Invest*. 112(6):945-55.

Belmonte N., Phillips B.W., Massiera F., Villageois P., Wdziekonski B., Saint-Marc P., Nichols J., Aubert J., Saeki K., Yuo A., Narumiya S., Ailhaud G., Dani C. (2001). Activation of extracellular signal-regulated kinases and CREB/ATF-1 mediate the expression of CCAAT/enhancer binding proteins beta and – delta in preadipocytes. *Mol Endocrinol*. 15(11):2037-49.

Bergers G, Graninger P, Braselmann S, Wrighton C, Busslinger M. (1995) Transcriptional activation of the fra-1 gene by AP-1 is mediated by regulatory sequences in the first intron. *Mol Cell Biol*. 15(7):3748-58.

Berlin T., Cronstrand R., Nowak J., Sonnenfeld T., Wennmalm A. (1979) Conversion of arachidonic acid to prostaglandins in homogenates of human skeletal muscle and kidney. *Acta Physiol Scand*. 106(4):441-5.

Billon N., Iannarelli P., Monteiro M.C., Glavieux-Pardanaud C., Richardson W.D., Kessar N., Dani C., Dupin E. (2007). The generation of adipocytes by the neural crest. *Development*. 134(12):2283-92.

- Billon N., Monteiro M.C., Dani C. (2008). Developmental origin of adipocytes: new insights into a pending question. *Biol Cell*. 100(10):563-75.
- Boström P., Wu J., Jedrychowski M.P., Korde A., Ye L., Lo J.C., Rasbach K.A., Boström E.A., Choi J.H., Long J.Z., Kajimura S., Zingaretti M.C., Vind B.F., Tu H., Cinti S., Højlund K., Gygi S.P., Spiegelman B.M. (2011). A PGC1- α -dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature*.11;481(7382):463-8.
- Brasaemle D.L. and Wolins N.E. (2012). Packaging of fat: An evolving model of lipid droplet assembly and expansion. *J Biol Chem*. 287(4):2273-9.
- Bray G. A. and Bellanger T. (2006) Epidemiology, Trends, and Morbidities of Obesity and the Metabolic Syndrome. *Endocrine*, 29(1): 109–117.
- Brown H.A. and Marnett L.J. (2011). Introduction to lipid biochemistry, metabolism, and signaling. *Chem Rev*. 111(10):5817-20.
- Camp H.S. and Tafuri S.R. (1997) Regulation of peroxisome proliferator-activated receptor γ activity by mitogen-activated protein kinase. *J Biol Chem*. 272(16):10811-6.
- Cannon B., Nedergaard J. (2004). Brown adipose tissue: function and physiological significance. *Physiol Rev*. 84(1):277-359.
- Cao Z., Umek R.M., McKnight S.L. (1991). Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev*. 5,1538–1552.
- Cao Y, Traer E, Zimmerman GA, McIntyre TM, Prescott SM. (1998) Cloning, expression, and chromosomal localization of human long-chain fatty acid-coa ligase 4 (FACL4). *Genomics*. 49(2):327-30.

- Cao H., Gerhold K., Mayers J.R., Wiest M.M., Watkins S.M., Hotamisligil G.S. (2008). Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. *Cell*. 134(6):933-44.
- Casalino L, De Cesare D, Verde P.(2003) Accumulation of Fra-1 in ras-transformed cells depends on both transcriptional autoregulation and MEK-dependent posttranslational stabilization. *Mol Cell Biol*. 23(12):4401-15.
- Casimir D.A., Miller C.W., Ntambi J.M. (1996) Preadipocyte differentiation blocked by prostaglandin stimulation of prostanoid FP² receptor in murine 3T3-L1 cells. *Differentiation*. 60(4):203-10.
- Coe N.R., Simpson M.A., Bernlohr D.A. (1999). Targeted disruption of the adipocyte lipid-binding protein (aP2 protein) gene impairs fat cell lipolysis and increases cellular fatty acid levels. *J Lipid Res*. 40(5):967-72.
- Cristancho A.G. and Lazar M.A. (2011). Forming functional fat: a growing understanding of adipocyte differentiation. *Nat Rev Mol Cell Biol*. 28;12(11):722-34.
- Chaves V.E., Frasson D., Martins-Santos M.E., Boschini R.P., Garófalo M.A., Festuccia W.T., Kettelhut I.C, Migliorini R.H. (2006). Glyceroneogenesis is reduced and glucose uptake is increased in adipose tissue from cafeteria diet-fed rats independently of tissue sympathetic innervation. *J Nutr*. 136(10):2475-80.
- Chilton F.H., Fonteh A.N., Surette M.E., Triggiani M., Winkler J.D. (1996). Control of arachidonate levels within inflammatory cells. *Biochim Biophys Acta*. 1299(1):1-15.
- Chinenov Y. and Kerppola TK. (2001) Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene*. 20(19):2438-52.

Choi J.H., Banks A.S., Estall J.L., Kajimura S., Boström P., Laznik D., Ruas J.L., Chalmers M.J., Kamenecka T.M., Blüher M., Griffin P.R., Spiegelman B.M. (2010). Anti-diabetic drugs inhibit obesity-linked phosphorylation of PPAR γ by Cdk5. *Nature*. 466(7305):451-6.

Christodoulides C., Lagathu C., Sethi J. K., Vidal-Puig A. (2009). Adipogenesis and WNT signalling. *Trends Endocrinol Metab*. 20,16-24.

Christy R.J., Yang V.W., Ntambi J.M., Geiman D.E, Landschulz W.H., Friedman A.D., Nakabeppu Y., Kelly T.J., Lane M.D. (1989). Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes Dev*. 3,1323-1335.

Chu X., Nishimura K., Jisaka M., Nagaya T., Shono F., Yokota K. (2010). Up-regulation of adipogenesis in adipocytes expressing stably cyclooxygenase-2 in the antisense direction. *Prostaglandins Other Lipid Mediat*. 91(1-2):1-9.

Cinti S. (2011). Between brown and white: Novel aspects of adipocyte differentiation. *Ann Med*. 43(2):104-15.

Claesson H.E. (2009) On the biosynthesis and biological role of eoxins and 15-lipoxygenase-1 in airway inflammation and Hodgkin lymphoma. *Prostaglandins Other Lipid Mediat*. 89(3-4):120-5.

Cook J.S., Lucas J.J., Sibley E., Bolanowski M.A., Christy R.J., Kelly T.J., Lane M.D. (1988) Expression of the differentiation-induced gene for fatty acid-binding protein is activated by glucocorticoid and cAMP. *Proc Natl Acad Sci U S A*. 85(9):2949-53.

Crossno J.T. Jr., Majka S.M., Grazia T., Gill R.G., Klemm D.J. (2006) Rosiglitazone promotes development of a novel adipocyte population from bone marrow–derived circulating progenitor cells. *J Clin Invest*. 116(12):3220-8.

Cypess A.M. and Kahn C.R. (2010). Brown fat as a therapy for obesity and diabetes. *Curr Opin Endocrinol Diabetes Obes.* 17(2):143-9.

Dani C. Smith A.G., Dessolin S., Leroy P., Staccini L., Villageois P., Darimont C., Ailhaud G. (1997). Differentiation of embryonic stem cells into adipocytes in vitro. *J Cell Sci.* 110 (Pt 11):1279-85.

Daynes R.A and Jones D.C. (2002). Emerging roles of ppars in inflammation and immunity. *Nat Rev Immunol.* 2(10):748-59.

Davis R.J. (1993) The mitogen-activated protein kinase signal transduction pathway. *J Biol Chem.* 268(20):14553-6.

Desai M. and Ross MG. (2011). Fetal programming of adipose tissue: Effects of intrauterine growth restriction and maternal obesity/high-fat diet. *Semin Reprod Med.* 29(3):237-45.

Diakogiannaki E., Dhayal S., Childs C.E., Calder PC., Welters H.J., Morgan N.G. (2007). Mechanisms involved in the cytotoxic and cytoprotective actions of saturated versus monounsaturated long-chain fatty acids in pancreatic b-cells. *J Endocrinol.* 194(2):283-91.

Distel R.J., Ro H.S., Rosen B.S., Groves D.L., Spiegelman B.M. (1987) Nucleoprotein complexes that regulate gene expression in adipocyte differentiation: direct participation of c-fos. *Cell.* 49(6):835-44.

Distel R.J., Robinson G.S., Spiegelman B.M. (1992) fatty acid regulation of gene expression. Transcriptional and post-transcriptional mechanisms. *J Biol Chem.* 267(9):5937-41.

Doege H. and Stahl A. (2006). Protein-mediated fatty acid uptake: Novel insights from in vivo models. *Physiology (Bethesda).* 21:259-68.

Duncan R.E., Ahmadian M., Jaworski K., Sarkadi-Nagy E., Sul H.S. (2007). Regulation of lipolysis in adipocytes. *Annu Rev Nutr.* 27:79-101.

Dunn TA, Storm DR, Feller MB. (2009) Calcium-dependent increases in protein kinase-A activity in mouse retinal ganglion cells are mediated by multiple adenylate cyclases. *PLoS One.* 4(11):e7877.

Eferl R., Hoebertz A., Schilling A.F., Rath M., Karreth F., Kenner L., Amling M., Wagner E.F.(2004) The Fos-related antigen Fra-1 is an activator of bone matrix formation. *EMBO J.* 23(14):2789-99.

Erbay E, Babaev VR, Mayers JR, Makowski L, Charles KN, Snitow ME, Fazio S, Wiest MM, Watkins SM, Linton MF, Hotamisligil GS. (2009) Reducing endoplasmic reticulum stress through a macrophage lipid chaperone alleviates atherosclerosis. *Nat Med.* 15(12):1383-91.

EFSA Panel on Dietetic Products, Nutrition and Allergens (NDA).(2010). Scientific Opinion on Dietary Reference Values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. *EFSA Journal.* 8:1461.

Elberg G., Gimble J.M., Tsai S.Y. (2000). Modulation of the murine peroxisome proliferator-activated receptor γ 2 promoter activity by CCAAT/enhancer-binding proteins. *J Biol Chem.* 275(36):27815-22.

Engelman JA, Lisanti MP, Scherer PE. (1998) Specific Inhibitors of p38 mitogen-activated protein kinase block 3T3-L1 adipogenesis. *J Biol Chem.* 273(48):32111-20.

Fahy E., Subramaniam S., Brown H.A., Glass C.K., Merrill A.H. Jr, Murphy R.C., Raetz C.R., Russell D.W., Seyama Y., Shaw W., Shimizu T., Spener F., van Meer G., VanNieuwenhze M.S., White S.H., Witztum J.L., Dennis E.A. (2005). A comprehensive classification system for lipids. *J Lipid Res.* 46(5):839-61.

Fajas L., Auboeuf D., Raspé E., Schoonjans K., Lefebvre A.M., Saladin R., Najib J., Laville M., Fruchart J.C., Deeb S., Vidal-Puig A., Flier J., Briggs M.R., Staels B., Vidal H., Auwerx J. (1997). The organization, promoter analysis, and expression of the human PPAR γ gene. *J Biol Chem.* 272(30):18779-89.

Fajas L., Fruchart J.C., Auwerx J. (1998). PPAR γ 3 mRNA: a distinct PPAR γ mRNA subtype transcribed from an independent promoter. *FEBS Lett.* 438(1-2):55-60.

Fajas L., Miard S., Briggs M.R., Auwerx J. (2003). Selective cyclo-oxygenase-2 inhibitors impair adipocyte differentiation through inhibition of the clonal expansion phase. *J Lipid Res.* 44(9):1652-9.

Farmer S.R. (2005). Regulation of PPAR α activity during adipogenesis. *International Journal of Obesity.* 29, S13–S16.

Farmer S. R. (2006). Transcriptional control of adipocyte formation. *Cell Metab.* 4, 263–273.

Faust I.M., Johnson R., Stern J.S., Hirsch J (1978). Diet-induced adipocyte number increase in adult rats: a new model of obesity. *Am J Physiol.* 235, E279–E286.

Feltenmark S., Gautam N., Brunnström A., Griffiths W., Backman L., Edenius C., Lindbom L., Björkholm M., Claesson H.E. (2008). Eoxins are proinflammatory arachidonic acid metabolites produced via the 15-lipoxygenase-1 pathway in human eosinophils and mast cells. *Proc Natl Acad Sci U S A.* 105(2):680-5.

Flachs P, Horakova O, Brauner P, Rossmeisl M, Pecina P, Franssen-van Hal N, Ruzickova J, Sponarova J, Drahota Z, Vlcek C, Keijer J, Houstek J, Kopecky J. (2005) Polyunsaturated fatty acids of marine origin upregulate

mitochondrial biogenesis and induce β -oxidation in white fat. *Diabetologia*. 48(11):2365-75.

Fleischmann A, Hafezi F, Elliott C, Remé CE, Rüther U, Wagner EF. (2000) Fra-1 replaces c-Fos-dependent functions in mice. *Genes Dev*. 14(21):2695-700.

Flegal K.M., Carroll M.D., Kit B.K., Ogden C.L. (2012) Prevalence and trends in obesity among US adults, 1999-2010. *JAMA*. 1;307(5):491-7.

Flier J. S. (2004) Obesity wars: molecular progress. Confronts an expanding epidemic. *Cell*. 116, 337–350.

Forman B. M., Tontonoz P., Chen J., Brun R. P., Spiegelman B. M., Evans R. M. (1995). 15 Deoxy- $\Delta^{12,14}$ -Prostaglandin J_2 is a ligand for the adipocyte determination factor PPAR γ . *Cell*. 83, 803-812.

Fox K.E., Colton L.A., Erickson P.F., Friedman J.E., Cha H.C., Keller P., MacDougald O.A., Klemm D.J. (2008). Regulation of Cyclin D1 and wnt10b gene expression by cAMP-responsive element-binding protein during early adipogenesis involves differential promoter methylation. *J Biol Chem*. 283(50):35096-105.

Frigolet Vazquez-Vela M. E., Torres N., Tovar A. R. (2008). White adipose tissue as endocrine organ and its role in obesity. *Archives of Medical Research*. 39, 715-728.

Fujimori K., Ueno T., Nagata N., Kashiwagi K., Aritake K., Amano F., Urade Y. (2010). Suppression of adipocyte differentiation by aldo-keto reductase 1B3 acting as prostaglandin $F_{2\alpha}$ synthase. *J Biol Chem*. 285(12):8880-6.

Funk C.D. (2001). Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science*. 294(5548):1871-5.

Furuhashi M. and Hotamisligil G. S. (2008). Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. *Nat Rev Drug Discov.* 7,489-503.

Furuhashi M., Ishimura S., Ota H., Miura T. (2011). Lipid chaperones and metabolic inflammation. *Int J Inflam.* 2011:642612.

Gaillard D., Negrel R., Lagarde M., Ailhaud G. (1989). Requirement and role of arachidonic acid in the differentiation of pre-adipose cells. *Biochem. J.* 257 389– 397.

Gao X, Li K, Hui X, Kong X, Sweeney G, Wang Y, Xu A, Teng M, Liu P, Wu D. (2011) Carnitine palmitoyltransferase 1A prevents fatty acid-induced adipocyte dysfunction through suppression of c-Jun N-terminal kinase. *Biochem J.* 435(3):723-32.

Gesta S, Blüher M, Yamamoto Y, Norris AW, Berndt J, Kralisch S, Boucher J, Lewis C, Kahn CR. (2006). Evidence for a role of developmental genes in the origin of obesity and body fat distribution. *Proc Natl Acad Sci U S A.* 25;103(17):6676-81.

Gesta S., Tseng Y.-H., Kahn C. R. (2007). Developmental origin of fat: tracking obesity to its source. *Cell.* 131, 242 – 256.

Ghoshal S., Trivedi D.B., Graf G.A., Loftin C.D. (2011). Cyclooxygenase-2 deficiency attenuates adipose tissue differentiation and inflammation in mice. *J Biol Chem.* 286(1):889-98.

Gomes P. and Soares-da-Silva P. (2002) Role of cAMP-PKA-PLC signaling cascade on dopamine-induced PKC-mediated inhibition of renal Na⁺-K⁺-ATPase activity. *Am J Physiol Renal Physiol.* 282(6):F1084-96.

- Graves R. A., Tontonoz P., Spiegelman B. M. (1992). Analysis of a tissue specific enhancer: ARF6 regulates adipogenic gene expression. *Molecular and Cellular Biology*. 12, 1202-1208.
- Green H. and Kehinde O. (1975). An established preadipose cell line and its differentiation in culture. II Factors affecting the adipose conversion. *Cell*. 5,19–27.
- Green H. and Kehinde O. (1976). Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell*. 7, 105–113.
- Green H. and Kehinde O. (1979). Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *J Cell Physiol*. 101(1):169-71.
- Gregoire F. M., Smas C. M., Sul H. S. (1998). Understanding adipocyte differentiation. *Physiological Reviews*. 78, 783 - 809.
- Grundy SM. (2012). Pre-Diabetes, Metabolic Syndrome, and Cardiovascular Risk. *J Am Coll Cardiol*. 14;59(7):635-43.
- Guan H.P., Li Y., Jensen M.V., Newgard C.B., Stepan C.M., Lazar M.A. (2002). A futile metabolic cycle activated in adipocytes by antidiabetic agents. *Nat Med*. 8(10):1122-8.
- Guo S.S., Wu W., Chumlea W.C., Roche A.F. (2002). Predicting overweight and obesity in adulthood from body mass index values in childhood and adolescence. *Am J Clin Nutr*. 76(3):653-8.
- Guo W, Xie W, Lei T, Hamilton JA. (2005) Eicosapentaenoic acid, but not oleic acid, stimulates β -oxidation in adipocytes. *Lipids*. 40(8):815-21.

Gupta R.K., Arany Z., Seale P., Mepani R.J., Ye L., Conroe H.M., Roby Y.A., Kulaga H., Reed R.R., Spiegelman B.M. (2010). Transcriptional control of preadipocyte determination by Zfp423. *Nature*. 25;464(7288):619-23.

Gupta R.K., Mepani R.J., Kleiner S., Lo J.C., Khandekar M.J., Cohen P. Frontini A., Bhowmick D.C., Ye L., Cinti S., Spiegelman B.M. (2012). Zfp423 expression identifies committed preadipocytes and localizes to adipose endothelial and perivascular cells. *Cell Metab*. 8;15(2):230-9.

Hai T. and Curran T.(1991) Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Natl Acad Sci U S A*. 88(9):3720-4.

Hainault I., Carlotti M., Hajduch E., Guichard C., Lavau M. (1993). Fish oil in a high lard diet prevents obesity, hyperlipidemia, and adipocyte insulin resistance in rats. *Ann N Y Acad Sci*. 683, 98–101.

Hajer G. R., Van Haeften T. W., Visseren F. L.J. (2008). Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *European Heart Journal*. 29, 2959–2971.

Hamer O.W., Forstner D., Ottinger I., Ristow M., Bollheimer L.C., Schölmerich J., Palitzsch K.D. (2002) The Pro115Gln polymorphism within the PPAR γ 2 gene has no epidemiological impact on morbid obesity. *Exp Clin Endocrinol Diabetes*.110(5):230-4.

Hamilton J.A. (1998). Fatty acid transport: difficult or easy? *J Lipid Res*. 39(3):467-81.

Hansen J.B. and Kristiansen K. (2006). Regulatory circuits controlling white versus brown adipocyte differentiation. *Biochem J*. 2006 Sep 1;398(2):153-68.

Hapala I., Marza E., Ferreira T. (2011). Is fat so bad? Modulation of endoplasmic reticulum stress by lipid droplet formation. *Biol Cell*. 103(6):271-85.

Harris WS, Mozaffarian D, Rimm E, Kris-Etherton P, Rudel LL, Appel LJ, Engler MM, Engler MB, Sacks F. (2009) Omega-6 fatty acids and risk for cardiovascular disease a science advisory from the american heart association nutrition subcommittee of the council on nutrition, physical activity, and metabolism; council on cardiovascular nursing; and council on epidemiology and prevention. *Circulation*. 119(6):902-7.

Hauser S, Adelmant G, Sarraf P, Wright HM, Mueller E, Spiegelman BM. (2000) Degradation of the peroxisome proliferator-activated receptor γ is linked to ligand-dependent activation. *J Biol Chem*. 275(24):18527-33.

Hirsch J. and Han P.W. (1969) Cellularity of rat adipose tissue: effects of growth, starvation, and obesity. *J Lipid Res*. 10(1):77-82.

Hoffmann E, Thiefes A, Buhrow D, Dittrich-Breiholz O, Schneider H, Resch K, Kracht M. (2005) MEK1-dependent delayed expression of Fos-related antigen-1 counteracts c-Fos and p65 NF- κ B-mediated interleukin-8 transcription in response to cytokines or growth factors. *J Biol Chem*. 280(10):9706-18.

Hosey MM (1999) What molecular events underlie heterologous desensitization? Focus on "Receptor phosphorylation does not mediate cross talk between muscarinic M3 and bradykinin B2 receptors. *Am J Physiol*. 277(5 Pt 1):C856-8.

Hotamisligil G.S., Johnson R.S., Distel R.J., Ellis R., Papaioannou V.E., Spiegelman B.M. (1996). Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science*. 274(5291):1377-9.

Hotamisligil G. S. (2006). Inflammation and metabolic disorders. *Nature*. 14, 860-7.

Hu E, Kim JB, Sarraf P, Spiegelman BM.(1996) Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPAR γ . *Science*. 274(5295):2100-3.

Huang-Doran I., Sleight A., Rochford J.J., O'Rahilly S., Savage D.B. (2010). Lipodystrophy: metabolic insights from a rare disorder. *J Endocrinol*. 207(3):245-55.

Hurd TW, Culbert AA, Webster KJ, Tavaré JM. (2002) Dual role for mitogen-activated protein kinase (Erk) in insulin-dependent regulation of Fra-1 (fos-related antigen-1) transcription and phosphorylation. *Biochem J*. 368(Pt 2):573-80.

Hyman B.T., Stoll L.L., Spector A.A. (1982) Prostaglandin production by 3T3-L1 cells in culture. *Biochim Biophys Acta*. 713(2):375-85.

Ibrahim M.M. (2010). Subcutaneous and visceral adipose tissue: structural and functional differences. *Obes Rev*. 11(1):11-823 11-18.

Imai T., Takakuwa R., Marchand S., Dentz E., Bornert J.M., Messaddeq N., Wendling O., Mark M., Desvergne B., Wahli W., Chambon P., Metzger D. (2004). Peroxisome proliferator-activated receptor γ is required in mature white and brown adipocytes for their survival in the mouse. *Proc Natl Acad Sci U S A*. 101(13):4543-7.

Kim J.B., Wright H.M., Wright M., Spiegelman B.M. (1998) ADD1/SREBP1 activates PPAR γ through the production of endogenous ligand. *Proc Natl Acad Sci U S A*. 95(8):4333-7.

Kletzien R.F., Foellmi L.A., Harris P.K., Wyse B.M., Clarke S.D. (1992) Adipocyte fatty acid-binding protein: regulation of gene expression in vivo and in vitro by an insulin-sensitizing agent. *Mol Pharmacol*. 42(4):558-62.

Kliwer S.A., Lenhard J.M., Willson T.M., Patel I., Morris D.C., Lehmann J.M. (1995). A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell*. 1;83(5):813-9.

Kliwer S.A., Sundseth S.S., Jones S.A., Brown P.J., Wisely G.B., Koble C.S., Devchand P., Wahli W., Willson T.M., Lenhard J.M., Lehmann J.M. (1997). Fatty acids and eicosanoids regulate gene expression through direct interactions with *Proc Natl Acad Sci U S A* peroxisome proliferator-activated receptors alpha and gamma.. 29;94(9):4318-23.

Koutnikova H, Cock TA, Watanabe M, Houten SM, Champy MF, Dierich A, Auwerx J. (2003). Compensation by the muscle limits the metabolic consequences of lipodystrophy in PPAR gamma hypomorphic mice. *Proc Natl Acad Sci USA*.100,14457–14462.

Kremmyda L.S., Tvrzicka E., Stankova B., Zak A. (2011) Fatty acids as biocompounds: Their role in human metabolism, health and disease – a review. Part 2: Fatty acid physiological roles and applications in human health and disease. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*, 155(3):195-218.

Krey G., Braissant O., L'Horset F., Kalkhoven E., Perroud M., Parker M.G., Wahli W. (1997). Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator dependent receptor ligand assay. *Mol Endocrinol*. 11(6):779-91.

Kubota N., Terauchi Y., Miki H., Tamemoto H., Yamauchi T., Komeda K., Satoh S., Nakano R., Ishii C., Sugiyama T., Eto K., Tsubamoto Y., Okuno A., Murakami K., Sekihara H., Hasegawa G., Naito M., Toyoshima Y., Tanaka S., Shiota K., Kitamura T., Fujita T., Ezaki O., Aizawa S., Kadowaki T., (1999). PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol Cell*. 4,597-609.

Lafontan M. (2008). Advances in adipose tissue metabolism. *Int J Obes (Lond)*. 32 Suppl 7:S39-51.

Lee K., Villena J. A., Moon Y. S., Kim K.-H., Lee S., Kang C., Sul H. S. (2003). Inhibition of adipogenesis and development of glucose intolerance by soluble preadipocyte factor-1 (Pref-1). *J. Clin. Invest*. 111, 453–461.

Lefterova M.I., Zhang Y., Steger D.J., Schupp M., Schug J., Cristancho A., Feng D., Zhuo D., Stoeckert C.J. Jr, Liu X.S., Lazar M.A. (2008). PPAR γ and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. *Genes Dev*. 22(21):2941-52.

Lehmann JM, Lenhard JM, Oliver BB, Ringold GM, Kliewer SA. (1997) Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J Biol Chem*. 272(6):3406-10.

Leslie C.C. (2004). Regulation of arachidonic acid availability for eicosanoid production. *Biochem Cell Biol*. 82(1):1-17.

Lin F.-T. and Lane M. D. (1992). Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. *Genes & Development*. 6,533-544.

Lin F.-T. and Lane M. D. (1994). CCAAT/enhancer binding protein α is sufficient to initiate the 3T3-L1 adipocyte differentiation program. *Proc. Natl. Acad. Sci. USA*. 91,8757-8761.

Linhart H.G., Ishimura-Oka K., DeMayo F., Kibe T., Repka D., Poindexter B., Bick R.J., Darlington G.J. (2001). C/EBP α is required for differentiation of white, but not brown, adipose tissue. *Proc Natl Acad Sci U S A*. 23;98(22):12532-7.

Liu L. and Clipstone N.A. (2007). Prostaglandin F2 α inhibits adipocyte differentiation via a gaq-calcium-calcineurin-dependent signaling pathway. *J Cell Biochem*. 100(1):161-73.

Luther J., Driessler F., Megges M., Hess A., Herbort B., Mandic V., Zaiss M.M., Reichardt A., Zech C., Tuckermann J.P., Calkhoven C.F., Wagner E.F., Schett G., David J.P. (2011) Elevated Fra-1 expression causes severe lipodystrophy. *J Cell Sci*. 124(Pt 9):1465-76.

MacDougald O.A. and Lane M.D. (1995). Transcriptional regulation of gene expression during adipocyte differentiation. *Annu Rev Biochem*. 64:345-73.

Madsen L., Petersen R.K., Sørensen M.B., Jørgensen C., Hallenborg P., Pridal L., Fleckner J., Amri E.Z., Krieg P., Furstenberger G., Berge R.K., Kristiansen K. (2003). Adipocyte differentiation of 3T3-L1 preadipocytes is dependent on lipoxygenase activity during the initial stages of the differentiation process. *Biochem J*. 375, 539-49.

Madsen L.T, Petersen R. K., Kristiansen K. (2005). Regulation of adipocyte differentiation and function by polyunsaturated fatty acids. *Biochimica et Biophysica Acta*. 1740, 266– 286.

Madsen L., Pedersen L.M., Liaset B., Ma T., Petersen R.K., van den Berg S., Pan J., Müller-Decker K., Dülsner E.D., Kleemann R., Kooistra T., Døskeland S.O., Kristiansen K. (2008) cAMP-dependent signaling

regulates the adipogenic effect of n-6 polyunsaturated fatty acids. *J Biol Chem.* 283(11):7196-205.

Maeda K., Uysal K.T., Makowski L., Görgün C.Z., Atsumi G., Parker R.A., Brüning J., Hertzel A.V., Bernlohr D.A., Hotamisligil G.S. (2003). Role of the fatty acid binding protein mal1 in obesity and insulin resistance. *Diabetes.* 52(2):300-7.

Maeda K., Cao H., Kono K., Gorgun C.Z., Furuhashi M., Uysal K.T., Cao Q., Atsumi G., Malone H., Krishnan B., Minokoshi Y., Kahn B.B., Parker R.A., Hotamisligil G.S. (2005). Adipocyte/macrophage fatty acid binding proteins control integrated metabolic responses in obesity and diabetes. *Cell Metab.* 1(2):107-19.

Makowski L., Boord J.B., Maeda K., Babaev V.R., Uysal K.T., Morgan M.A., Parker R.A., Suttles J., Fazio S., Hotamisligil G.S, Linton M.F. (2001). Lack of macrophage fatty-acid-binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis. *Nat Med.* 7(6):699-705.

Makowski L. and Hotamisligil G.S. (2004). Fatty acid binding proteins—the evolutionary crossroads of inflammatory and metabolic responses. *J Nutr.* 134(9):2464S-2468S.

Mangelsdorf D.J., Thummel C., Beato M., Herrlich P., Schütz G., Umesono K., Blumberg B., Kastner P., Mark M., Chambon P., Evans R.M. (1995). The nuclear receptor superfamily: The second decade. *Cell.* 83(6):835-9.

Manickam E, Sinclair AJ, Cameron-Smith D. (2010) Suppressive actions of eicosapentaenoic acid on lipid droplet formation in 3T3-L1 adipocytes. *Lipids Health Dis.*9:57.

Massiera F., Saint-Marc P., Seydoux J., Murata T., Kobayashi T., Narumiya S., Guesnet P., Amri E.Z., Negrel R., Ailhaud G. (2003). Arachidonic acid and

prostacyclin signaling promote adipose tissue development: a human health concern? *J Lipid Res.* 44(2):271-9.

Matsuo T., Takeuchi, H., Suzuki, H., Suzuki, M. (2002). Body fat accumulation is greater in rats fed a beef tallow diet than in rats fed a safflower or soybean oil diet *Asia Pac. J. Clin. Nutr.* 11, 302–308.

Matsusue K., Peters J.M., Gonzalez F. (2004). PPARbeta/delta potentiates PPARgamma-stimulated adipocyte differentiation. *FASEB J.* 18(12):1477-9.

Mazid M.A., Chowdhury A.A., Nagao K., Nishimura K., Jisaka M., Nagaya T., Yokota K. (2006). Endogenous 15-deoxy-D12,14-prostaglandin J2 synthesized by adipocytes during maturation phase contributes to upregulation of fat storage. *FEBS Lett.* 580(30):6885-90.

Melki S.A. and Abumrad N.A. (1993) Expression of the adipocyte fatty acid-binding protein in streptozotocin-diabetes: effects of insulin deficiency and supplementation. *J Lipid Res.* 34(9):1527-34.

Miller C.W., Casimir D.A., Ntambi J.M. (1996) The mechanism of inhibition of 3T3-L1 preadipocyte differentiation by prostaglandin F2 α . *Endocrinology.* 137(12):5641-50.

Mitchell J, Lai LP, Peralta F, Xu Y, Sugamori K. (2011) β 2-Adrenergic receptors inhibit the expression of collagen type II in growth plate chondrocytes by stimulating the AP-1 factor Jun-B. *Am J Physiol Endocrinol Metab.* 300(4):E633-9.

Montgomery R., Conway T.W., Spector A.A., Chappell D. (1996). *Biochemistry: A case-oriented approach.* Sixth Edition. Mosby.

Moitra, J., Mason, M. M., Olive, M., Krylov, D., Gavrilova, O., Marcus-Samuels, B., Feigenbaum, L., Lee, E., Aoyama, T., Eckhaus, M. et al. (1998). Life without white fat: a transgenic mouse. *Genes Dev.* 12, 3168-3181.

- Moon Y. S., Smas C. M., Lee K., Villena J. A., Kim K.-H., Yun E. J., Sul H. S. (2002). Mice lacking paternally expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity. *Molecular and Cellular Biology*. 22, 5585–5592.
- Murakami M., Taketomi Y., Miki Y., Sato H., Hirabayashi T., Yamamoto K. (2011). Recent progress in phospholipase A2 research: From cells to animals to humans. *Prog Lipid Res*. 50(2):152-92.
- Nagy L., Tontonoz P., Alvarez J.G., Chen H., Evans R.M. (1998). Oxidized LDL regulates macrophage gene expression through ligand activation of PPAR γ . *Cell* 93: 229-240.
- Nedergaard J, Bengtsson T, Cannon B. (2007). Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab*. 293(2):E444-52.
- Nedergaard J., Cannon B. (2010). The changed metabolic world with human brown adipose tissue: Therapeutic visions. *Cell Metab*. 7;11(4):268-72.
- Nedergaard J, Bengtsson T, Cannon B. (2011). Three years with adult human brown adipose tissue. *Ann N Y Acad Sci*. 1212:E20-36.
- Neves SR, Ram PT, Iyengar R. (2002) G Protein pathways. *Science*. 296(5573):1636-9.
- Nielsen R., Pedersen T.A., Hagenbeek D., Moulos P., Siersbaek R., Megens E., Denissov S., Børgesen M., Francoijs K.J., Mandrup S., Stunnenberg H.G. (2008). Genome-wide profiling of PPAR γ :RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. *Genes Dev*. 22(21):2953-67.

Ntambi J.M. and Takova T. (1996) Role of Ca^{2+} in the early stages of murine adipocyte differentiation as evidenced by calcium mobilizing agents. *Differentiation*. 60(3):151-8.

Ntambi J.M. and Young-Cheul K. (2000). Adipocyte differentiation and gene expression. *J Nutr*. 130(12):3122S-3126S.

Parada CA, Reichling DB, Levine JD (2005) Chronic hyperalgesic priming in the rat involves a novel interaction between cAMP and PKC ϵ second messenger pathways. *Pain* 113:185–190.

Pérez-Chacón G., Astudillo A.M., Balgoma D., Balboa M.A., Balsinde J. (2009). Control of free arachidonic acid levels by phospholipases A2 and lysophospholipid acyltransferases. *Biochim Biophys Acta*. 1791(12):1103-13.

Pérez-Matute P, Pérez-Echarri N, Martínez JA, Martí A, Moreno-Aliaga MJ. (2007). Eicosapentaenoic acid actions on adiposity and insulin resistance in control and high-fat-fed rats: role of apoptosis, adiponectin and tumour necrosis factor- α . *Br J Nutr*. 97, 389–398.

Petersen R.K., Jørgensen C., Rustan A.C., Frøyland L., Muller-Decker K., Furstenberger G., Berge R.K., Kristiansen K., Madsen L. (2003) Arachidonic acid-dependent inhibition of adipocyte differentiation requires PKA activity and is associated with sustained expression of cyclooxygenases. *J Lipid Res*. 44(12):2320-30.

Pognonec P, Boulukos KE, Aperlo C, Fujimoto M, Ariga H, Nomoto A, Kato H. (1997) Cross-family interaction between the bHLHZip USF and bZip Fra1 proteins results in down-regulation of AP1 activity. *Oncogene*. 14(17):2091-8.

Prusty D., Park B.H., Davis K.E., Farmer S.R. (2002) Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor γ (PPAR γ) and C/EBP α Gene expression during the differentiation of 3T3-L1 preadipocytes. *J Biol Chem*. 277(48):46226-32.

- Reginato M.J., Krakow S.L., Bailey S.T., Lazar M.A. (1998). Prostaglandins promote and block adipogenesis through opposing effects on peroxisome proliferator-activated receptor gamma. *J Biol Chem.* 23;273(4):1855-8.
- Rennie K.L. and Jebb S.A. (2005) Prevalence of obesity in Great Britain. *Obes Rev.*6(1):11-2.
- Rhee S.G. (2001). Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem.* 70:281-312.
- Ritter S.L. and Hall R.A. (2009) Fine-tuning of GPCR activity by receptor-interacting proteins. *Nat Rev Mol Cell Biol.* 10(12):819-30.
- Rodeheffer M.S., Birsoy K., Friedman J.M. (2008). Identification of white adipocyte progenitor cells in vivo. *Cell.* 2008 135(2):240-9.
- Rosen E.D., Sarraf P., Troy A.E., Bradwin G., Moore K., Milstone D.S., Spiegelman B.M., Mortensen R.M. (1999). PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol Cell.*4, 611-617.
- Rosen, E. D., Hsu CH., Wang X., Sakai S., Freeman M. W., Gonzalez J. F., Spiegelman B.M. (2002). C/EBP α induces adipogenesis through PPAR γ : a unified pathway. *Genes Dev.* 16, 22–26.
- Rosen E. D. and Spiegelman B. M. (2006). Adipocytes as regulators of energy balance and glucose homeostasis. *Nature.* 444, 847-853.
- Ross S.R., Graves R.A., Greenstein A., Platt K.A., Shyu H.L., Mellovitz B., Spiegelman B.M.(1990) A fat-specific enhancer is the primary determinant of gene expression for adipocyte P2 in vivo. *Proc Natl Acad Sci U S A.* 87(24):9590-4.
- Sa G, Murugesan G, Jaye M, Ivashchenko Y, Fox PL. (1995) Activation of cytosolic phospholipase A2 by basic fibroblast growth factor via a p42

mitogen-activated protein kinase-dependent phosphorylation pathway in endothelial cells. *J Biol Chem.* 270(5):2360-6.

Sabin M.A., Werther G.A., Kiess W. (2011). Genetics of obesity and overgrowth syndromes. *Best Practice & Research Clinical Endocrinology & Metabolism.* 25(1):207-20.

Sakuma Y., Tanaka K., Suda M., Komatsu Y., Yasoda A., Miura M., Ozasa A., Narumiya S., Sugimoto Y., Ichikawa A., Ushikubi F., Nakao K. (2000). Impaired bone resorption by lipopolysaccharide in vivo in mice deficient in the prostaglandin E receptor EP4 subtype. *Infect Immun.* 68(12):6819-25.

Salma N., Xiao H., Imbalzano A.N. (2006). Temporal recruitment of CCAAT/enhancer-binding proteins to early and late adipogenic promoters in vivo. *J Mol Endocrinol.* 36(1):139-51.

Salway, J.G. (1994) *Metabolism at a Glance*. Third edition. Blackwell Scientific Publishing Ltd, Oxford.

Scheja L., Makowski L., Uysal K.T., Wiesbrock S.M., Shimshek D.R., Meyers D.S., Morgan M., Parker R.A., Hotamisligil G.S. (1999). Altered insulin secretion associated with reduced lipolytic efficiency in $\alpha P2^{-/-}$ mice. *Diabetes.* 48(10):1987-94.

Schmitz G. and Ecker J. (2008). The opposing effects of n-3 and n-6 fatty acids. *Prog Lipid Res.* 47(2):147-55.

Seale P., Bjork B., Yang W., Kajimura S., Chin S., Kuang S., Scimè A., Devarakonda S., Conroe H.M., Erdjument-Bromage H., Tempst P., Rudnicki M.A., Beier D.R., Spiegelman B.M. (2008). PRDM16 controls a brown fat/skeletal muscle switch. *Nature.* 454(7207):961-7.

Shao D., Rangwala S.M., Bailey S.T., Krakow S.L., Reginato M.J., Lazar M.A. (1998). Interdomain communication regulating ligand binding by PPAR- γ . *Nature*. 396(6709):377-80.

Serrero, G., Lepak, N. M., Goodrich, S. P. (1992). Paracrine regulation of adipose differentiation by arachidonate metabolites: prostaglandin F₂ α inhibits early and late markers of differentiation in the adipogenic cell line 1246. *Endocrinology*. 131, 2545–2551.

Serrero G. and Lepak N.M. (1997). Prostaglandin F_{2a} receptor (FP receptor) agonists are potent adipose differentiation inhibitors for primary culture of adipocyte precursors in defined medium. *Biochem Biophys Res Commun*. 233(1):200-2.

Sevetson BR, Kong X, Lawrence JC Jr. (1993) Increasing cAMP attenuates activation of mitogen-activated protein kinase. *Proc Natl Acad Sci U S A*. 90(21):10305-9.

Shen W.J., Sridhar K., Bernlohr D.A., Kraemer F.B. (1999). Interaction of rat hormone-sensitive lipase with adipocyte lipid-binding protein. *Proc Natl Acad Sci U S A*. 96(10):5528-32.

Siersbæk R., Nielsen R., John S., Sung M.H., Baek S., Loft A., Hager G.L., Mandrup S. (2011). Extensive chromatin remodelling and establishment of transcription factor ‘hotspots’ during early adipogenesis. *EMBO J*. 30(8):1459-72.

Simopoulos A.P. (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother*, 56(8):365-79.

Singh R., Xiang Y., Wang Y., Baikati K., Cuervo A.M., Luu Y.K., Tang Y., Pessin J.E., Schwartz G.J., Czaja M.J. (2009a). Autophagy regulates adipose mass and differentiation in mice. *J Clin Invest*. 119(11):3329-39.

Singh R., Kaushik S., Wang Y., Xiang Y., Novak I., Komatsu M, Tanaka K, Cuervo AM, Czaja MJ. (2009b) Autophagy regulates lipid metabolism. *Nature*. 458(7242):1131-5.

Singh R and Cuervo AM. (2011) Autophagy in the cellular energetic balance. *Cell Metab*. 13(5):495-504.

Smith W.L., DeWitt D.L., Garavito R.M. (2000). Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem*. 69:145-82.

Smith W.L., Urade Y., Jakobsson P.J. (2011). Enzymes of the cyclooxygenase pathways of prostanoid biosynthesis. *Chem Rev*. 111(10):5821-65.

Spalding K.L., Arner E., Westermark P.O., Bernard S., Buchholz B.A., Bergmann O., Blomqvist L., Hoffstedt J., Näslund E., Britton T., Concha H., Hassan M., Rydén M., Frisén J., Arner P. (2008). Dynamics of fat cell turnover in humans. *Nature*. 453(7196):783-7.

Stefan N., Kantartzis K., Celebi N., Staiger H., Machann J., Schick F., Cegan A., Elcnerova M., Schleicher E., Fritsche A., Häring H.U. (2010). Circulating palmitoleate strongly and independently predicts insulin sensitivity in humans. *Diabetes Care*. 33(2):405-7.

Steger D.J., Grant G.R., Schupp M., Tomaru T., Lefterova M.I., Schug J., Manduchi E., Stoeckert C.J. Jr, Lazar M.A. (2010). Propagation of adipogenic signals through an epigenomic transition state. *Genes Dev*. 24(10):1035-44.

Stephens J.M., Butts M.D., Pekala P.H.(1992). Regulation of transcription factor mRNA accumulation during 3T3-L1 preadipocyte differentiation by tumour necrosis factor- α . *J Mol Endocrinol*. 9(1):61-72.

Storch J. and Corsico B. (2008). The emerging functions and mechanisms of mammalian fatty acid-binding proteins. *Annu Rev Nutr.* 2008;28:73-95.

Sugii S., Olson P., Sears D.D., Saberi M., Atkins A.R., Barish G.D., Hong S.H., Castro G.L., Yin Y.Q., Nelson M.C., Hsiao G., Greaves D.R., Downes M., Yu R.T., Olefsky J.M., Evans R.M. (2009). PPAR γ activation in adipocytes is sufficient for systemic insulin sensitization. *Proc Natl Acad Sci U S A.* 106(52):22504-9.

Sugii S. and Evans R.M. (2011). Epigenetic codes of PPAR γ in metabolic disease. *FEBS Lett.* 585(13):2121-8.

Sugimoto Y., Tsuboi H., Okuno Y., Tamba S., Tsuchiya S., Tsujimoto G., Ichikawa A. (2004). Microarray evaluation of EP4 receptor-mediated prostaglandin E2 suppression of 3T3-L1 adipocyte differentiation. *Biochem Biophys Res Commun.* 322(3):911-7.

Sugimoto Y. and Narumiya S. (2007). Prostaglandin E Receptors. *J Biol Chem.* 282(16):11613-7.

Sul H.S. and Wang D. (1998). Nutritional and hormonal regulation of enzymes in fat synthesis: Studies of fatty acid synthase and mitochondrial glycerol-3-phosphate acyltransferase gene transcription. *Annu Rev Nutr.* 18:331-51.

Sundvold H. and Lien S. (2001). Identification of a novel peroxisome proliferator-activated receptor (PPAR) γ promoter in man and transactivation by the nuclear receptor ROR α 1. *Biochem Biophys Res Commun.* 287(2):383-90.

Takada I., Kouzmenko A.P., Kato S. (2009). Wnt and PPAR γ signaling in osteoblastogenesis and adipogenesis. *Nat Rev Rheumatol.* 5(8):442-7.

- Talukdar I, Szeszel-Fedorowicz W, Salati LM. (2005) Arachidonic Acid inhibits the insulin induction of glucose-6-phosphate dehydrogenase via p38 MAP Kinase. *J Biol Chem.* 280(49):40660-7.
- Tam C.S., Ravussin E. (2012). Energy balance: an overview with emphasis on children. *Pediatr Blood Cancer.* 58(1):154-8.
- Tan N.S., Shaw N.S., Vinckenbosch N., Liu P., Yasmin R., Desvergne B., Wahli W., Noy N. (2002). Selective cooperation between fatty acid binding proteins and peroxisome proliferator-activated receptors in regulating transcription. *Mol Cell Biol.* 22, 5114-5127.
- Tanaka T., Yoshida N., Kishimoto T., Akira S. (1997). Defective adipocyte differentiation in mice lacking the C/EBP β and/or C/EBP δ gene. *EMBO J.* 16(24):7432-43.
- Tang Q.Q. and Lane M.D. (2000) Role of C/EBP homologous protein (CHOP-10) in the programmed activation of CCAAT/enhancer-binding protein-beta during adipogenesis. *Proc Natl Acad Sci U S A.* 97(23):12446-50.
- Tang Q.Q., Otto T.C., Lane M.D. (2003). Mitotic clonal expansion: A synchronous process required for adipogenesis. *EMBO J.* 16 (24):7432-43.
- Tang Q.Q., Zhang J.W., Daniel Lane M. (2004). Sequential gene promoter interactions by C/EBP β , C/EBP α , and PPAR γ during adipogenesis. *Biochem Biophys Res Commun.* 2004 May 21;318(1):213-8.
- Tang W., Zeve D., Suh J.M., Bosnakovski D., Kyba M., Hammer R.E., Tallquist M.D., Graff J.M. (2008). White fat progenitor cells reside in the adipose vasculature. *Science.* 24;322(5901):583-6.

- Tanida I., Ueno T., Kominami E. (2008). LC3 and Autophagy. *Methods in Molecular Biology, Autophagosome and Phagosome*, Volume 445, 77-88.
- Thompson B.R., Lobo S., Bernlohr D.A. (2010). Fatty acid flux in adipocytes: The in's and out's of fat cell lipid trafficking. *Mol Cell Endocrinol.* 318(1-2):24-33.
- Tomoda H. and Omura S. (2007) Potential therapeutics for obesity and atherosclerosis: Inhibitors of neutral lipid metabolism from microorganisms. *Pharmacol Ther.* 115(3):375-89.
- Tong Q., Dalgin G., Xu H., Ting C.N., Leiden J.M., Hotamisligil G.S. (2000). Function of GATA transcription factors in preadipocyte- adipocyte transition. *Science.* 6;290(5489):134-8.
- Tong Q., Tsai J., Tan G., Dalgin G., Hotamisligil G.S. (2005) Interaction between GATA and the C/EBP family of transcription factors is critical in GATA-mediated suppression of adipocyte differentiation. *Mol Cell Biol.* 25(2):706-15.
- Tontonoz P, Graves RA, Budavari AI, Erdjument-Bromage H, Lui M, Hu E, Tempst P, Spiegelman BM. (1994a) Adipocyte-specific transcription factor ARF6 is a heterodimeric complex of two nuclear hormone receptors, PPAR γ and RXR α . *Nucleic Acids Res.* 22(25):5628-34.
- Tontonoz, P., Hu, E. and Spiegelman, B. M. (1994b). Stimulation of adipogenesis in fibroblasts by PPAR γ 2, a lipid activated transcription factor. *Cell.* 79, 1147–1156.
- Tordjman J., Chauvet G., Quette J., Beale E.G., Forest C., Antoine B. (2003). Thiazolidinediones block fatty acid release by inducing glyceroneogenesis in fat cells. *J Biol Chem.* 278(21):18785-90.

Tsuboi H., Sugimoto Y., Kainoh T., Ichikawa A. (2004). Prostanoid EP4 receptor is involved in suppression of 3T3-L1 adipocyte differentiation. *Biochem Biophys Res Commun.* 322(3):1066-72.

Tuncman G., Erbay E., Hom X., De Vivo I., Campos H., Rimm E.B., Hotamisligil G.S. (2006). A genetic variant at the fatty acid-binding protein *aP2* locus reduces the risk for hypertriglyceridemia, type 2 diabetes, and cardiovascular disease. *Proc Natl Acad Sci U S A.* 103(18):6970-5.

Tvrzicka E., Kremmyda L.S., Stankova B., Zak A. (2011). Fatty acids as biocompounds: their role in human metabolism, health and disease – a review. Part 1: classification, dietary sources and biological functions. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub.* 155(2):117-30.

Tzamelis I., Fang H., Ollero M., Shi H., Hamm J.K., Kievit P., Hollenberg A.N., Flier J.S. (2004). Regulated production of a peroxisome proliferator-activated receptor- γ ligand during an early phase of adipocyte differentiation in 3T3-L1 adipocytes. *J Biol Chem.* 279(34):36093-102.

Ueno T. and Fujimori K. (2011) Novel suppression mechanism operating in early phase of adipogenesis by positive feedback loop for enhancement of cyclooxygenase-2 expression through prostaglandin F2 α receptor mediated activation of MEK/ERK-CREB cascade. *FEBS J.* 278(16):2901-12.

Unger R.H., Clark G.O., Scherer P.E., Orci L. (2010). Lipid homeostasis, lipotoxicity and the metabolic syndrome. *Biochim Biophys Acta.* 1801(3):209-14.

Uysal K.T., Scheja L., Wiesbrock S.M., Bonner-Weir S., Hotamisligil G.S. (2000). Improved glucose and lipid metabolism in genetically obese mice lacking *aP2*. *Endocrinology.* 141(9):3388-96.

Vassaux G., Gaillard D., Ailhaud G., Négrel R. (1992). Prostacyclin is a specific effector of adipose cell differentiation its dual role as a cAMP- and Ca^{+2} -elevating agent. *J Biol Chem.* 1992 Jun 5;267(16):11092-7.

Vázquez-Vela M.E., Torres N., Tovar A.R. (2008). White adipose tissue as endocrine organ and its role in obesity. *Arch Med Res.* 39(8):715-28.

Vial E. and Marshall C.J.(2003) Elevated ERK-MAP kinase activity protects the FOS family member FRA-1 against proteasomal degradation in colon carcinoma cells. *J Cell Sci.* 116(Pt 24):4957-63.

Virtanen K.A., Lidell M.E., Orava J., Heglind M., Westergren R., Niemi T., Taittonen M., Laine J., Savisto N.J., Enerbäck S., Nuutila P. (2009). Functional brown adipose tissue in healthy adults. *N Engl J Med.* 9;360(15):1518-25.

Virtue S. and Vidal-Puig A. (2010). Adipose tissue expandability, lipotoxicity and the metabolic syndrome — An allostatic perspective. *Biochim Biophys Acta.* 1801(3):338-49.

Walkey CJ and Spiegelman BM.(2008) A functional peroxisome proliferator-activated receptor- γ ligand-binding domain is not required for adipogenesis. *J Biol Chem.* 283(36):24290-4.

Wang Y., Kim K-A., Kim J.-H., Sul H. S. (2006). Pref-1, a preadipocyte secreted factor that inhibits adipogenesis. *J. Nutr.* 136, 2953–2956.

Wolfrum C. (2007). Cytoplasmic fatty acid binding protein sensing fatty acids for peroxisome proliferator activated receptor activation. *Cell. Mol. Life Sci.* 64, 2465 – 2476.

Won JS and Suh HW. (2000) The differential molecular mechanisms underlying proenkephalin mRNA expression induced by forskolin and phorbol-

12-myristic-13-acetate in primary cultured astrocytes. *Brain Res Mol Brain Res*.84(1-2):41-51.

Wu Z., Xie Y., Bucher N.L., Farmer S.R. (1995). Conditional ectopic expression of C/EBP β in NIH-3T3 cells induces PPAR γ and stimulates adipogenesis. *Genes Dev*. 1;9(19):2350-63.

Wu Z., Rosen E.D., Brun R., Hauser S., Adelmant G., Troy A.E., McKeon C., Darlington G.J., Spiegelman B.M. (1999). Cross-Regulation of C/EBP α and PPAR γ controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Mol Cell*. 3(2):151-8.

Wu J., Boström P., Sparks L.M., Ye L., Choi J.H., Giang A.H., Khandekar M., Virtanen K.A., Nuutila P., Schaart G., Huang K., Tu H., van Marken Lichtenbelt W.D., Hoeks J., Enerbäck S., Schrauwen P., Spiegelman B.M. (2012) Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell*.150(2):366-76.

Xie Y., Kang X., Ackerman W.E., Belury M.A., Koster C., Rovin B.H., Landon M.B., Kniss D.A. (2006). Differentiation-dependent regulation of the cyclooxygenase cascade during adipogenesis suggests a complex role for prostaglandins. *Diabetes Obes Metab*. 8(1):83-93.

Yamamoto Y., Gesta S., Lee K.Y., Tran T.T., Saadatirad P., Kahn C.R. (2010). Adipose depots possess unique developmental gene signatures. *Obesity (Silver Spring)*.18(5):872-8.

Young MR, Nair R, Bucheimer N, Tulsian P, Brown N, Chapp C, Hsu TC, Colburn NH. (2002) Transactivation of Fra-1 and consequent activation of AP-1 occur extracellular signal-regulated kinase dependently. *Mol Cell Biol*. 22(2):587-98.

Yu K, Bayona W, Kallen CB, Harding HP, Ravera CP, McMahon G, Brown M, Lazar MA. (1995) Differential activation of peroxisome proliferator-activated receptors by eicosanoids. *J Biol Chem.* 270(41):23975-83.

Zamani N. and Brown C.W. (2011). Emerging roles for the transforming growth factor- β superfamily in regulating adiposity and energy expenditure. *Endocr Rev.* 32(3):387-403.

Zechner R., Zimmermann R., Eichmann T.O., Kohlwein S.D., Haemmerle G., Lass A., Madeo F. (2012). Fat signals - Lipases and lipolysis in lipid metabolism and signaling. *Cell Metab.* 15(3):279-91.

Zenz R., Eferl R., Scheinecker C., Redlich K., Smolen J., Schonhaler H.B., Kenner L., Tschachler E., Wagner E.F.(2008) Activator protein 1 (Fos/Jun) functions in inflammatory bone and skin disease. *Arthritis Res Ther.*10(1):201.

Zhang J.W., Klemm D.J., Vinson C., Lane M.D. (2004). Role of CREB in transcriptional regulation of CCAAT/ enhancer-binding protein beta gene during adipogenesis. *J Biol Chem.* 279, 4471–4478.

Zhou L.and Nilsson A. (2001). Sources of eicosanoid precursor fatty acid pools in tissues. *J Lipid Res.* 42(10):1521-42.

Zhu Y., Qi C., Korenberg J.R., Chen X.N., Noya D., Rao M.S., Reddy J.K. (1995). Structural organization of mouse peroxisome proliferator activated receptor γ (mPPAR γ) gene: Alternative promoter use and different splicing yield two mPPAR γ isoforms. *Proc Natl Acad Sci U S A.* 92(17):7921-5.

Zieleniak A., Wójcik M., Woźniak L. A. (2008). Structure and physiological functions of the human peroxisome proliferator-activated receptor γ . *Immunol. Ther. Exp.* 56, 331–345.

Zimmer J.S., Dyckes D.F., Bernlohr D.A., Murphy R.C. (2004). Fatty acid binding proteins stabilize leukotriene A₄ competition with arachidonic acid but not other lipoxygenase products. *J Lipid Res.* 45(11):2138-44.

Zuo, Y., Qiang, L. & Farmer, S. R. (2006). Activation of CCAAT/enhancerbinding protein (C/EBP) α expression by C/EBP β during adipogenesis requires a peroxisome proliferator-activated receptor- γ -associated repression of HDAC1 at the C/EBP α gene promoter. *J. Biol. Chem.* 281, 7960–7967.

Appendix

List of qRT-PCR primers		
Gene		Sequence 5' to 3'
Acs14	F	GCTTCCTATCTGATTACTAGTGTTGA
	R	ATCCACATAAATGATATGTTTAACACAATT
Adiponectin	F	GAGATGCAGGTCTTCTTGGTCCTA
	R	CCCCGTGGCCCTTCAG
aP2	F	ACACCGAGATTTCTTCAAAGTG
	R	CCATCTAGGGTTATGATGCTCTTCA
AKR1B3	F	TCGTGGTGACAGCATATAGTCC
	R	CCAGGAGAGACGGATCTTCA
C/EBP α	F	TGGAGTTGACCAGTGACAATGAC
	R	CAGTTCACGGCTCAGCTGTTC
C/EBP β	F	GAGCGACGAGTACAAGATGCGG
	R	TTGTGCTGCGTCTCCAGGTTG
C/EBP δ	F	CTGAACGACCTATACCTCAGACC
	R	AGCTTCTCTCGCAGTCCAGT
C-FOS	F	CCTGCCCCTTCTCAACGAC
	R	GCTCCACGTTGCTGATGCT
C-Jun	F	CGGACCGTTCTATGACTGCAA
	R	GGAGGAACGAGGCGTTGA
COX-1	F	TGCCACCTTCATCCGAGAA
	R	TAAGGTTGGACCGCACTGTG
COX 2	F	TCATAAGCGAGGACCTGGGT
	R	TTCACCATAAATGTGATTTAAG
Cpt1 α	F	TTAACAGCAACTACTACGCC
	R	CCAGAAGACGAATAGGTTTGA
eLOX3	F	CCGCTCTGTCATCCAGTCTACA
	R	TCTTGCGATGGTGTTCACTTG
EP1	F	GGTTCACGCACACGATGTG
	R	GCTGCAGGGAGTTAGAGTTCCA
EP2	F	CACCTCATTCTCCTGGCCATT
	R	TTCATCCATGTAGGCAAAGATTGT
EP3 α	F	GCTTCCAGCTCCACCTCCTT
	R	CATCATCTTTCCAGCTGGTCACT
EP3 β	F	GGAAGTTCTGCCAGATGATGAA
	R	ATTCTCAGACCCAGGGAAACAG
EP3 γ	F	AGTTCTGCCAGGTAGCAAACG
	R	GCCTGCCCTTTCTGTCCAT
FAS	F	TGCGGAAACTTCAGGAAATGT
	R	AGAGACGTGTCACTCCTGGACTT
FosB	F	CAACCACCAGTGGACCTGTGT
	R	CGACGGTTCCTGCACTTAGC

FP	F	TTCAGCTCCTGGCCATAATGT
	R	AAAAAGTGTCGTTTCACAGGTCCT
Fra-1	F	CGCAAGCTCAGGCACAGA
	R	AATGAGGCTGCACCATCCA
Fra-2	F	ATCCGGAGGGAGAGAAACAAG
	R	GCCCAGACTTCTCCTCTTCCA
GATA-2	F	GGAGACGATTGTGCTGAGTCAA
	R	TTAGGGTGCTCTGCAGCAACT
Glut-4	F	CTATTCAACCAGCATCTTCGAG
	R	CTACTAAGAGCACCGAGACC
IP	F	GTGTGCTCCCTGCCTCTCAT
	R	TCCCCCATCTCTCTGCTGTCT
Jun B	F	CTACAACTCCTGAAACCCACCTT
	R	GCCCCAGGACCCTTGAGA
JunD	F	GGAGCTGGAGAGGCTGATCAT
	R	CGGGTAGAGGAACTGCGTACTG
KLF-2	F	ACCAAGAGCTCGCACCTAAA
	R	TCCTTCCCAGTTGCAATGAT
LPL	F	CCAGGATGCAACATTGGAGA
	R	CAACTCAGGCAGAGCCCTTT
L19	F	GGAAAAAGAAGGTCTGGTTGGA
	R	TGATCTGCTGACGGGAGTTG
PPAR γ	F	CGAGTCTGTGGGGATAAAGC
	R	GGATCCGGCAGTTAAGATCA
PPAR γ 1	F	AGAAGCGGTGAACCACTGATATTC
	R	AGAGGTCCACAGAGCTGATTCC
PPAR γ 2	F	TGGGTGAACTCTGGGAGATTC
	R	AGAGGTCCACAGAGCTGATTCC
PREF-1	F	CTCTTGCTCCTGCTGGCTTT
	R	ATTGCTAGCCTCGCAGAATC

ChIP primers		
Promoter		Sequence 5' to 3'
aP2-AP1	F	CCAGGGAGAACCAAAGTTGAG
	R	CCTTCATGACCAGACCCTGT

Cloning primers		
Construct		Sequence 5' to 3'
aP2-PGL3	F	GAATTCCCAGCAGGAATCA
	R	GGCTGCAGCACAGGAGGGT
c-Jun-pcDNA	F	ATGACTGCAAAGATGGAAA
	R	AAACGTTTGCAACTGCTGCGTT
JunB-pcDNA	F	ATGTGCACGAAAATGGAACA
	R	GAAGGCGTGTCCCTTGA
Fra-1 peak1-PGL3	F	AAGTCTGATTGGTTTCTACCATTG
	R	GGTGCCAACCTCCTGCTCTG
Fra-1 peak2-PGL3	F	ACCAATCCAGGCACTTATGG
	R	GCCTCTCGGAGTCTGGTCTT
Fra-1-pcDNA	F	ATGTACCGAGACTACGGGGAAC
	R	CAAAGCCAGGAGTGTAGGA