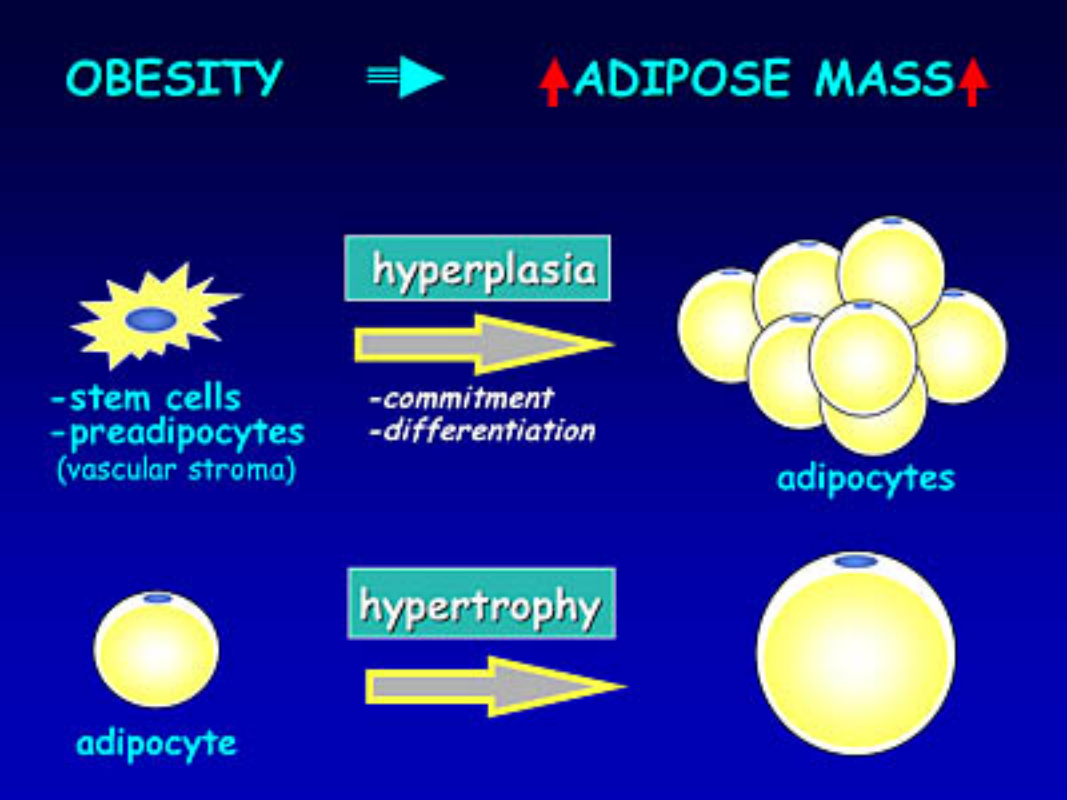
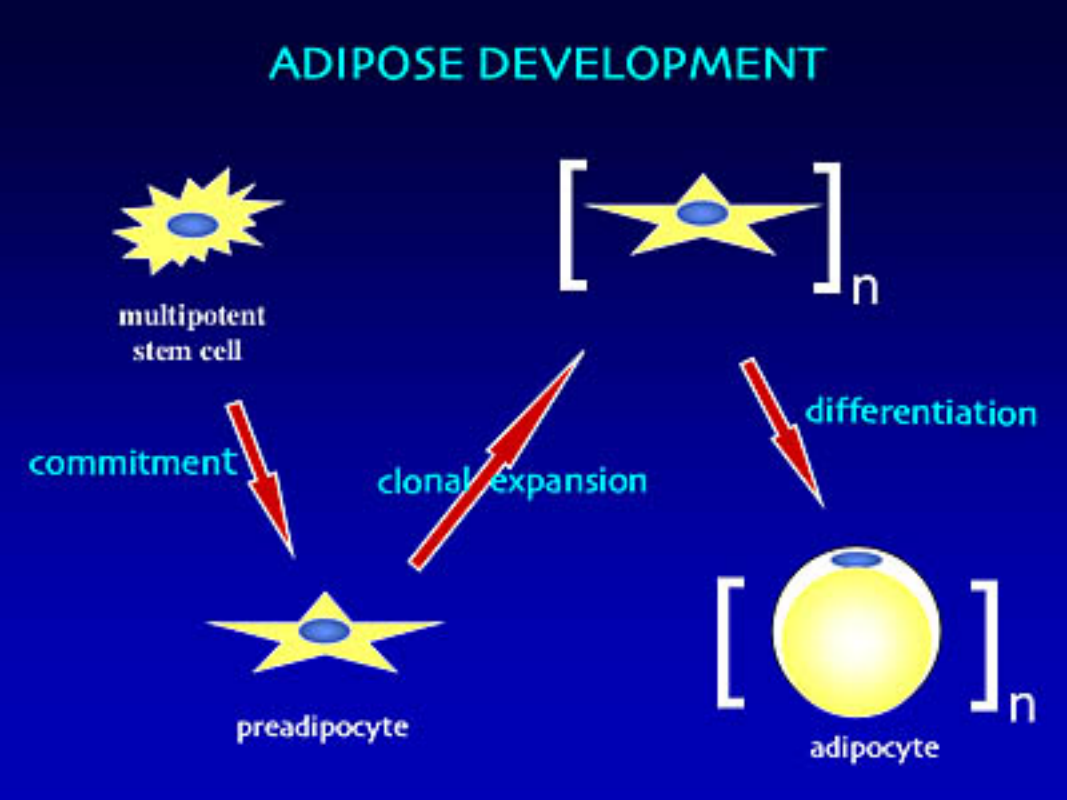
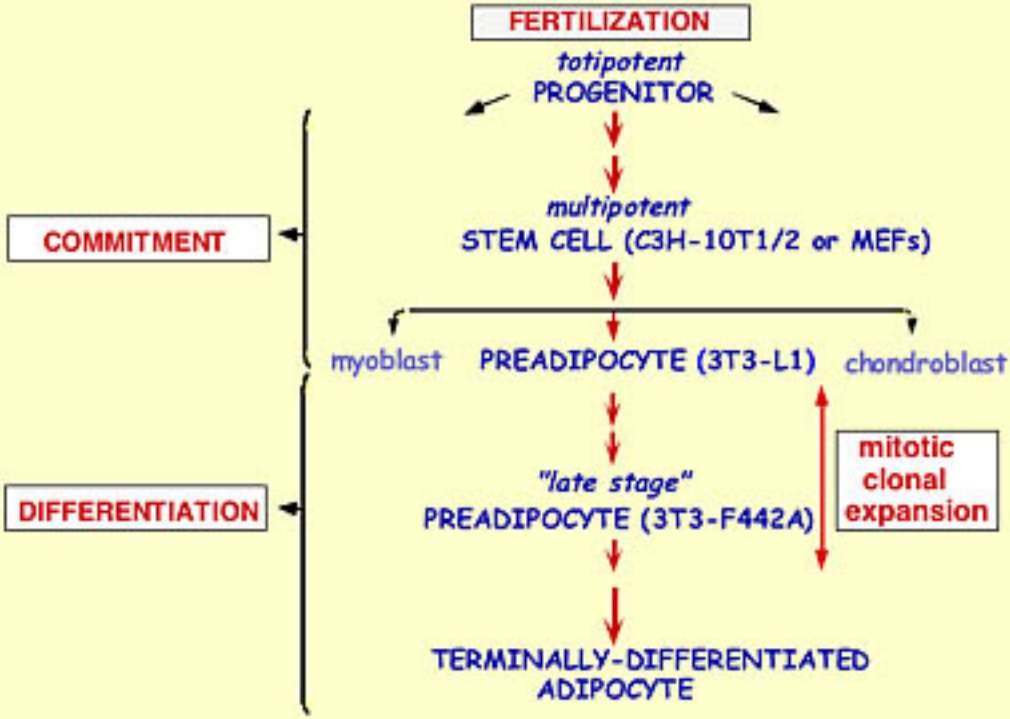

OBESITY
ADIPOSE MASS
hyperplasia
-stem cells
-preadipocytes
(vascular stroma)
-commitment
-differentiation
adipocytes
hypertrophy
adipocyte

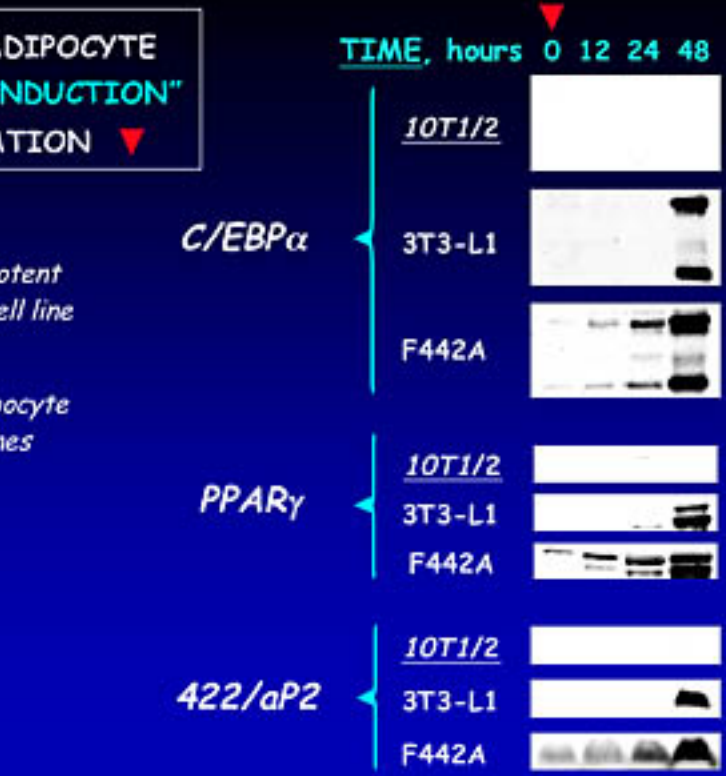

ADIPOSE DEVELOPMENT
multipotent
stem cell
n
commitment
clonal expansion
differentiation
preadipocyte
n
adipocyte

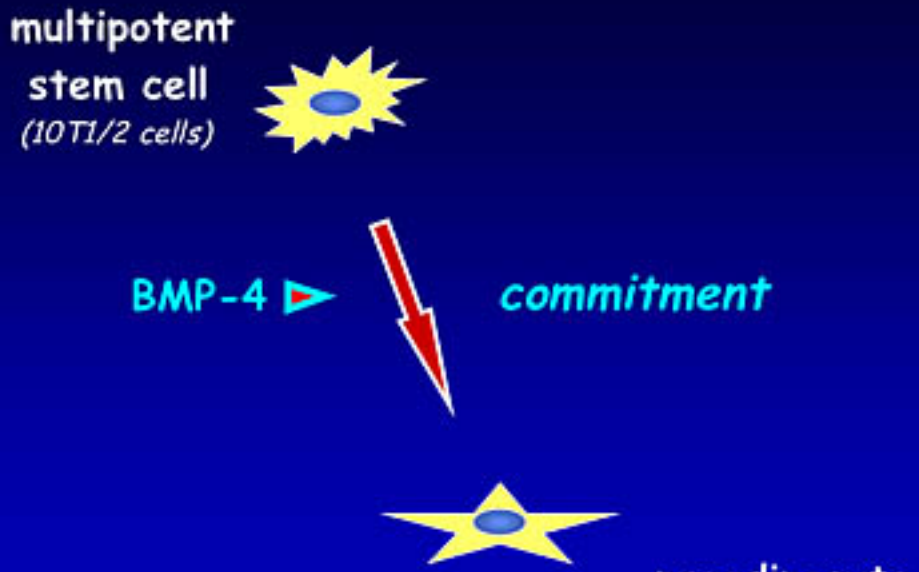

FERTILIZATION
totipotent
PROGENITOR
multipotent
STEM CELL (C3H-10T1/2 or MEFs)
COMMITMENT
myoblast
PREADIPOCYTE (3T3-L1)
chondroblast
"late stage"
PREADIPOCYTE (3T3-F442A)
mitotic clonal expansion
DIFFERENTIATION
TERMINALLY-DIFFERENTIATED ADIPOCYTE

EXPRESSION OF ADIPOCYTE MARKERS UPON "INDUCTION" OF DIFFERENTIATION

10T1/2 = multipotent stem cell line

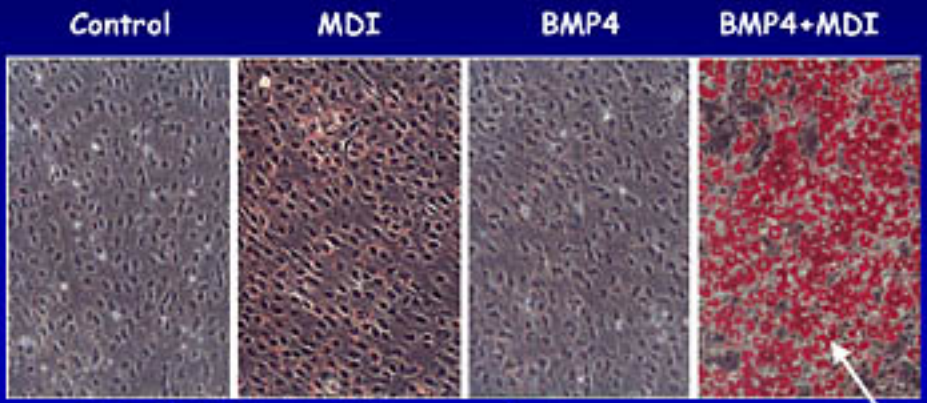
3T3-L1, 3T3-F442A = preadipocyte cell lines




multipotent
stem cell
(10T1/2 cells)
BMP-4
commitment
preadipocyte

BMP-4 COMMITS 10T1/2 STEM CELLS TO THE ADIPOCYTE LINEAGE

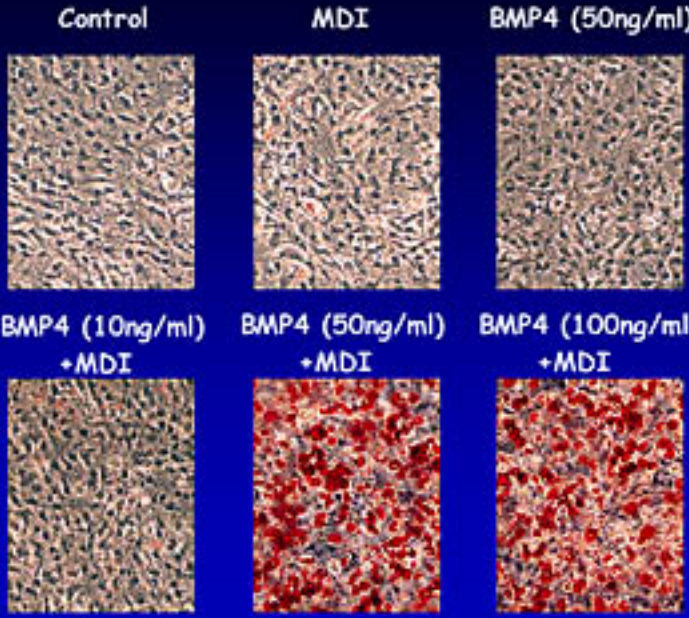
- *medium from 293 cells transfected with BMP-4 expression vector*
- *MDI = differentiation inducers*



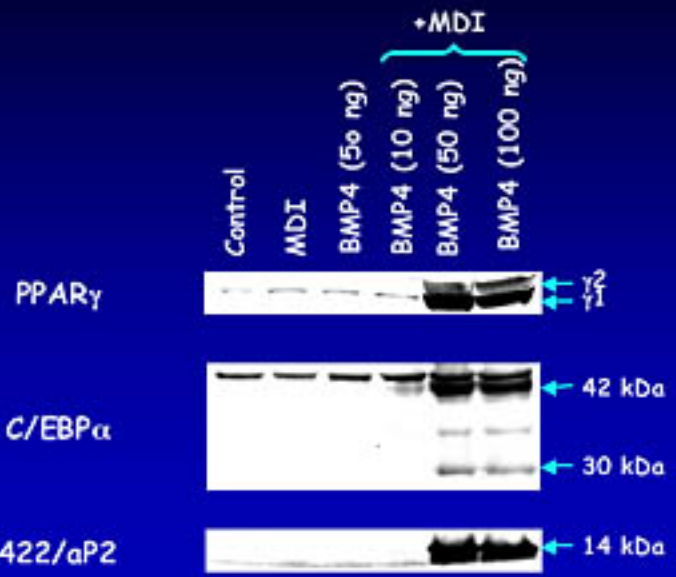
Only BMP4+MDI-treated cells express adipocyte marker proteins (C/EBPα, PPARγ and 422/aP2)

Oil Red-O stained fat

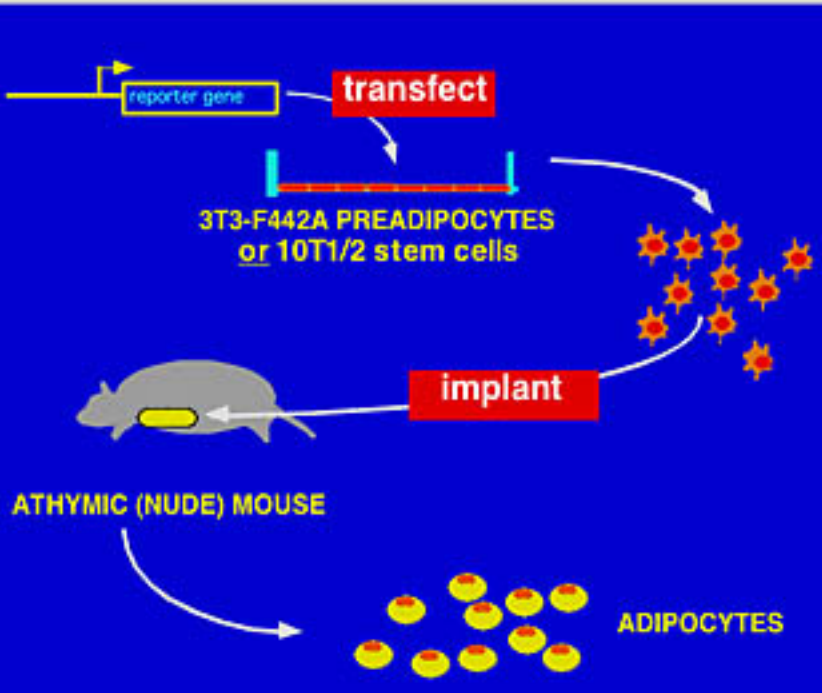
EFFECT OF rBMP4 ON COMMITMENT



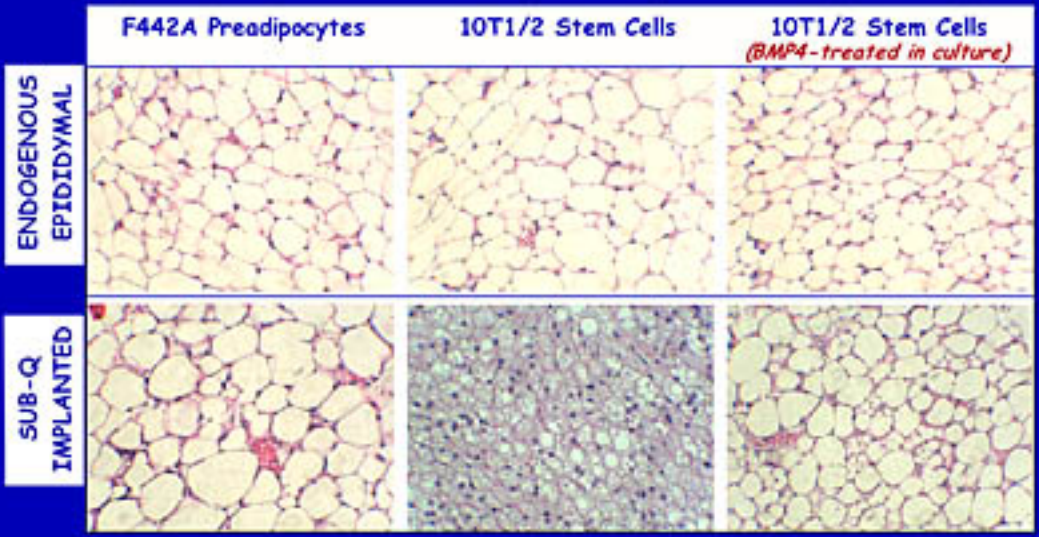
EXPRESSION of ADIPOCYTE MARKERS AFTER COMMITMENT of 10T1/2 STEM CELLS WITH rBMP4 & INDUCTION OF DIFFERENTIATION



IMPLANTED 10T1/2 STEM CELLS or PREADIPOCYTES → ADIPOSE TISSUE *IN VIVO*



COMMITMENT/DIFFERENTIATION OF 10T1/2 STEM CELLS IN ATHYMIC MICE (@ 4 weeks)

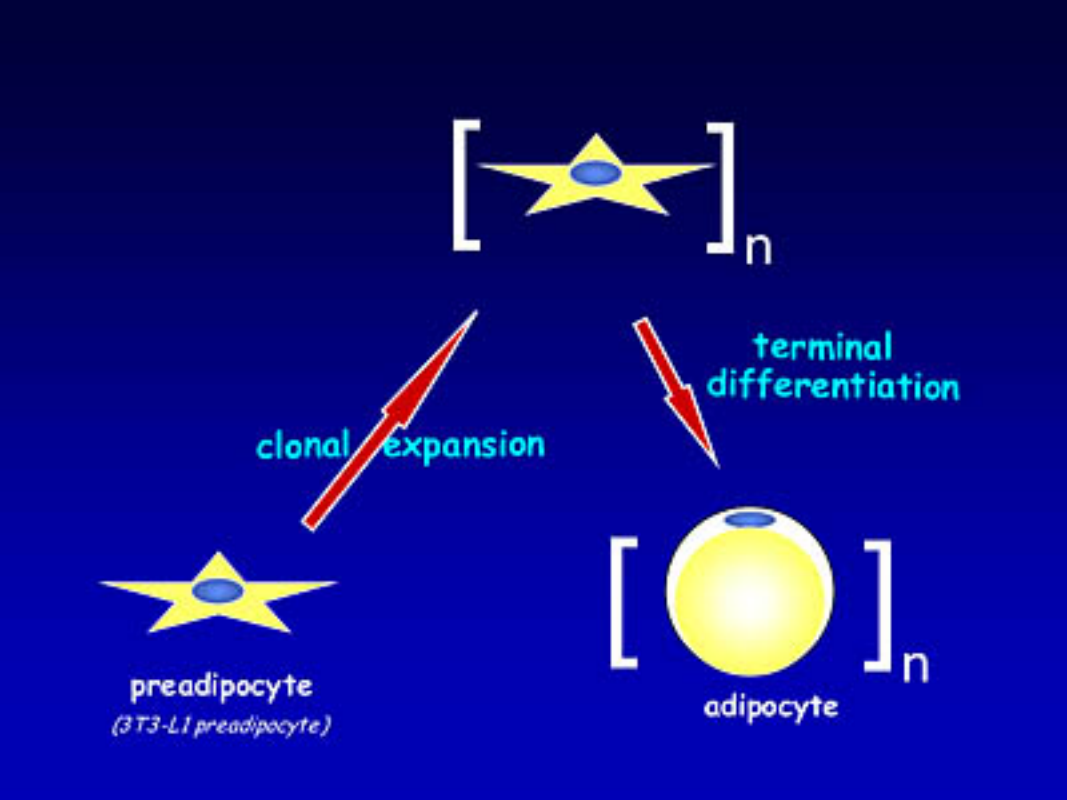


- We now have systems to study stem cell commitment to the adipocyte lineage both in cell culture and in an "in vivo" context

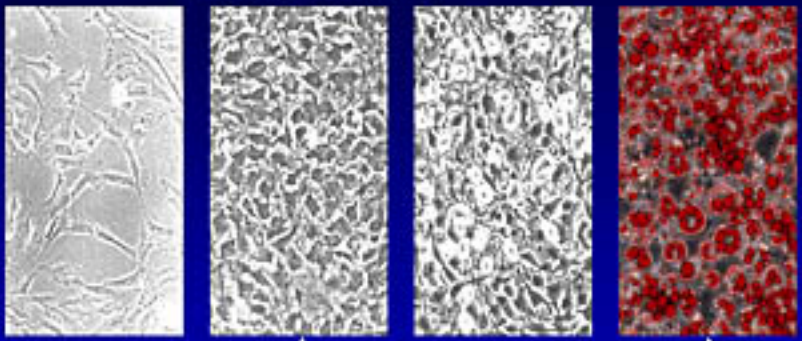
GOALS:

- identify the gene(s) or protein modification(s) that triggers stem cell commitment

- verify the role(s) of the gene or protein modification in an *in vivo* context


n
terminal
differentiation
clonal expansion
preadipocyte
(3T3-L1 preadipocyte)
n
adipocyte

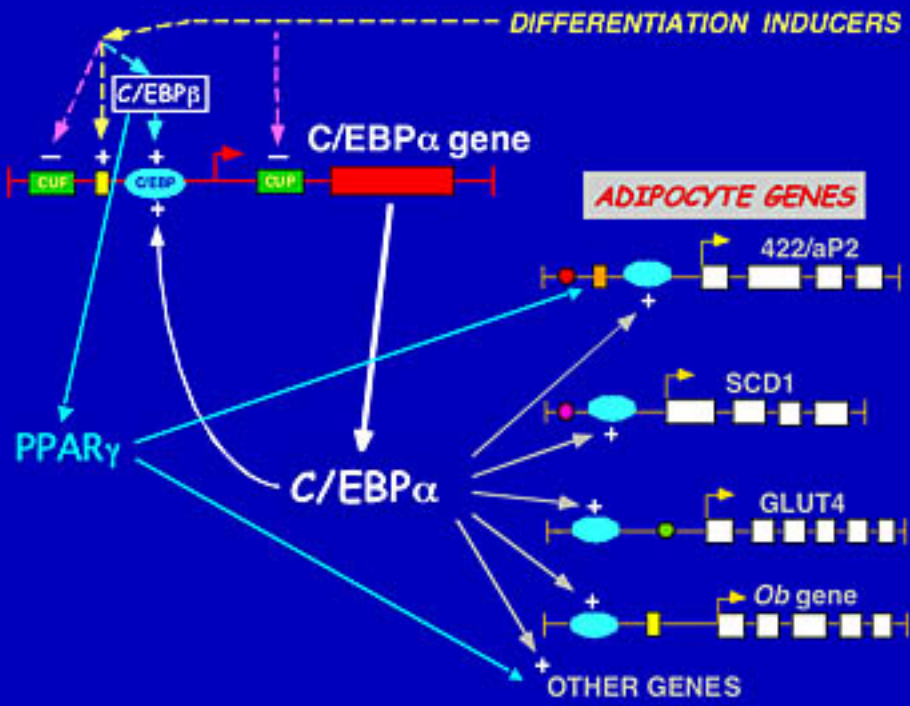
Differentiation of 3T3-L1 preadipocytes



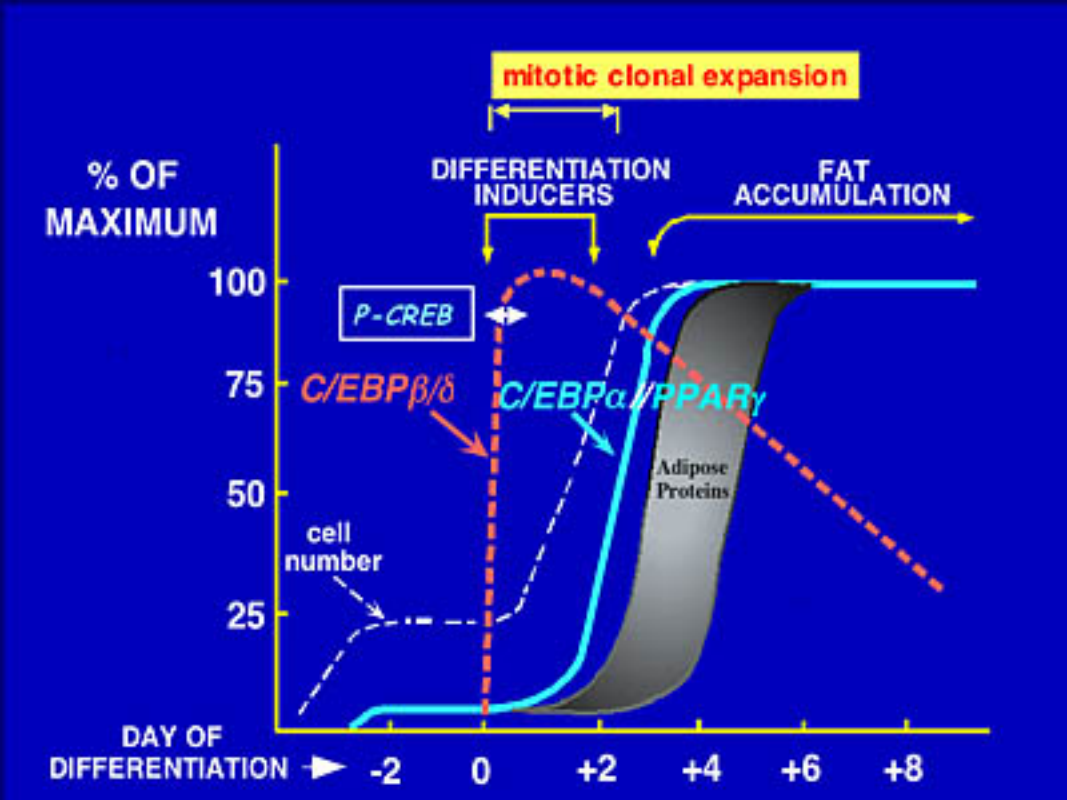
MIX (methylisobutylxanthine), a cAMP-phosphodiesterase inhibitor

DEX (dexamethasone), a synthetic glucocorticoid agonist

INS, insulin @ high concentration binds to the IGF-1 R

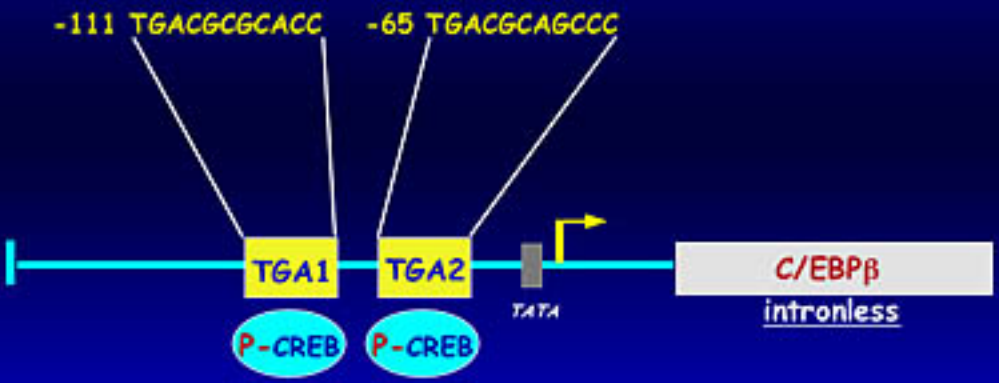

DIFFERENTIATION INDUCERS
C/EBPβ
C/EBPα gene
CUP
CEBP
CUP
ADIPOCYTE GENES
422/aP2
SCD1
GLUT4
Ob gene
PPARγ
C/EBPα
OTHER GENES

Sequence of events from preadipocyte to adipocyte


mitotic clonal expansion
% OF MAXIMUM
DIFFERENTIATION INDUCERS
FAT ACCUMULATION
100
75
50
25
P-CREB
C/EBPβ/δ
C/EBPα/PPARγ
Adipose Proteins
cell number
DAY OF DIFFERENTIATION
-2
0
+2
+4
+6
+8

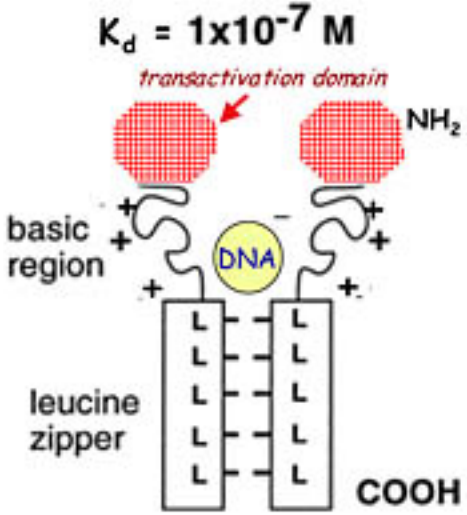
P-CREB activates transcription of the C/EBPβ gene

cAMP regulatory elements in the C/EBPβ gene at which P-CREB activates transcription

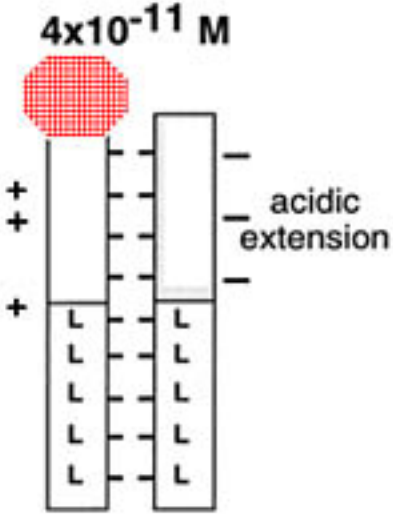


(mutation of TGA1 and 2 abolishes activity)

Structure of dominant-negative CREB (A-CREB)

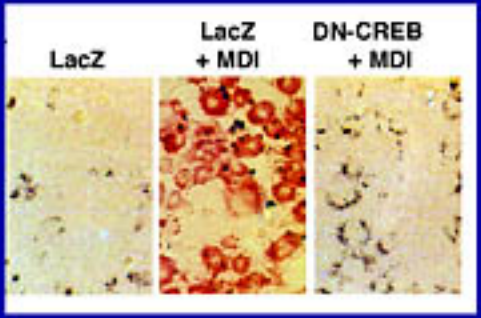


CREB homodimer



CREB : A-CREB heterodimer

Effect of dominant-negative CREB on differentiation of 3T3-L1 preadipocytes

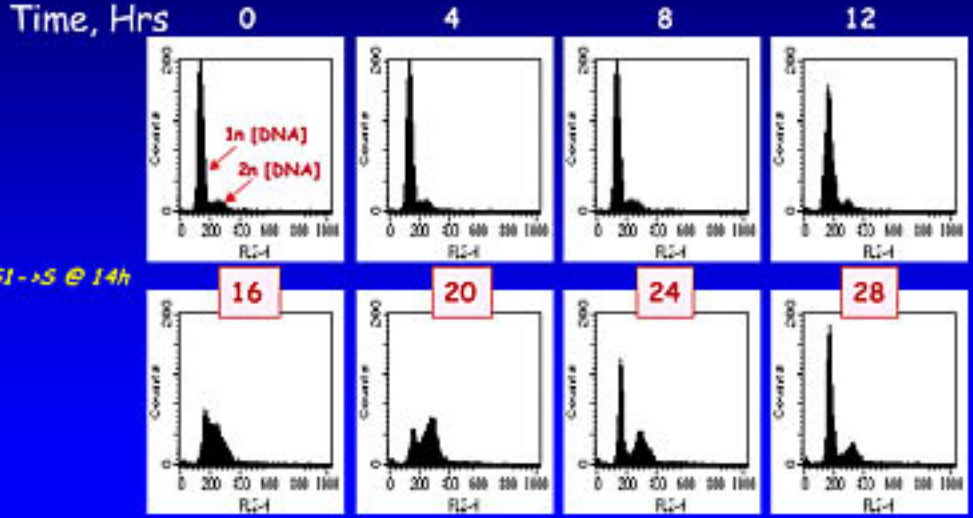


MDI = differentiation inducers

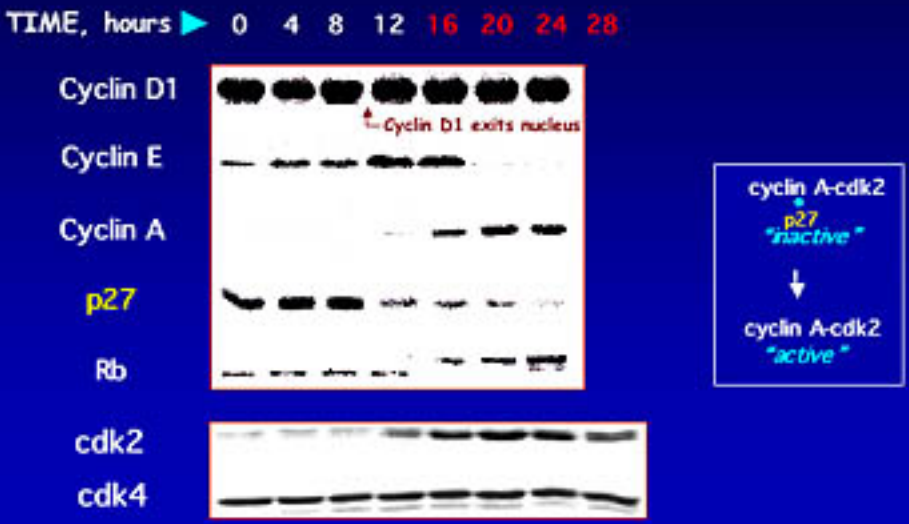
Is mitotic clonal expansion required for adipogenesis ??

SYNCHRONOUS ENTRY OF MITOTIC CLONAL EXPANSION

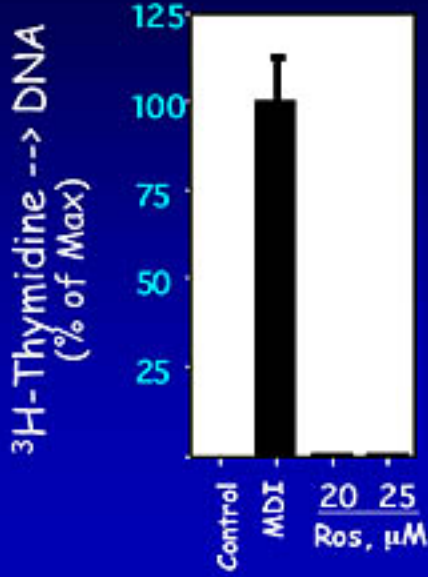
(DNA CONTENT: FAX analysis after fixation & Propidium I staining)

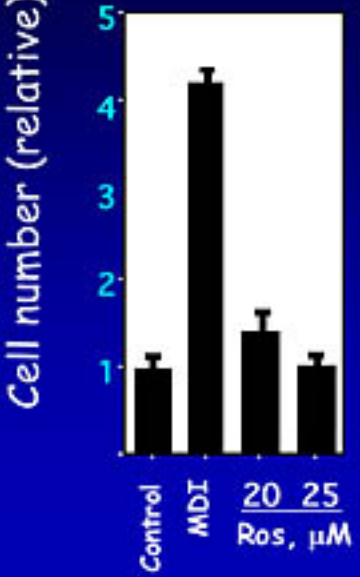


Expression of cell cycle proteins during mitotic clonal expansion

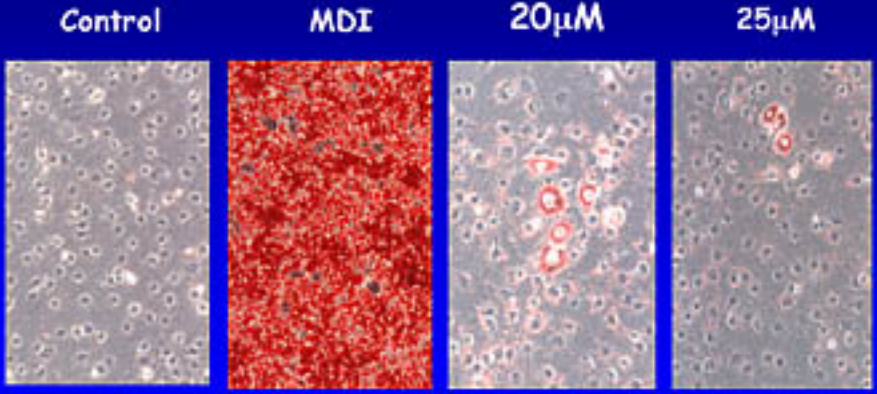


BLOCKADE OF MITOTIC CLONAL EXPANSION WITH A cdk2 INHIBITOR (ROSCOVITINE)

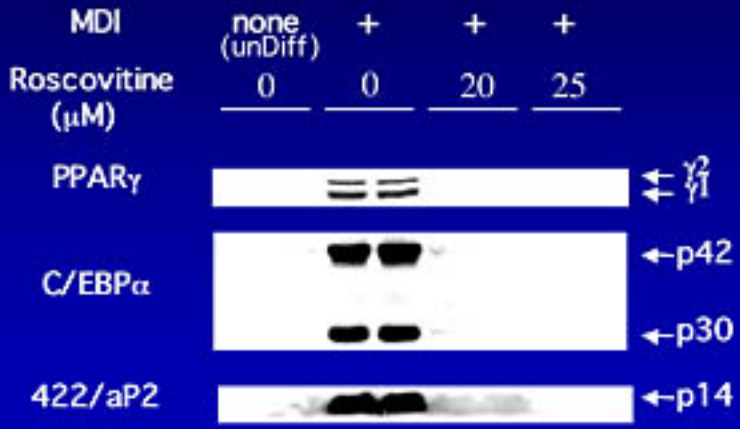




BLOCKADE OF DIFFERENTIATION WITH THE CDK2 INHIBITOR, ROSCOVITINE



EFFECT OF THE cdk2 INHIBITOR (ROSCOVITINE) ON THE EXPRESSION OF ADIPOCYTE MARKERS



OTHER EVIDENCE:

Blocking down-regulation of p27 (a requirement for S-phase entry) prevents MCE and differentiation

Inhibitors of MEK (a requirement for cell cycle progression) prevents MCE and differentiation

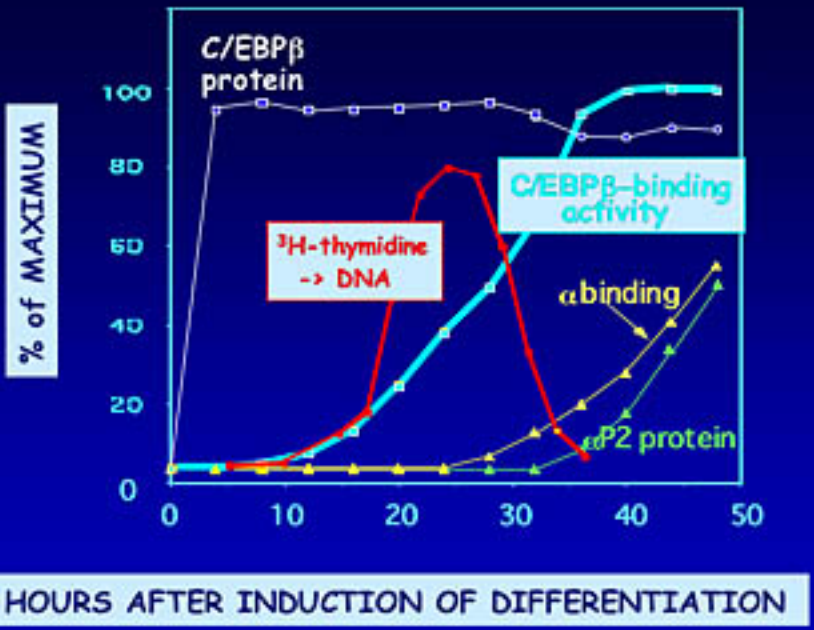
C/EBPβ (-/-) MEFs neither undergo MCE nor differentiate

Dominant-negatuve C/EBP blocks MCE and differentiation

CONCLUSION:

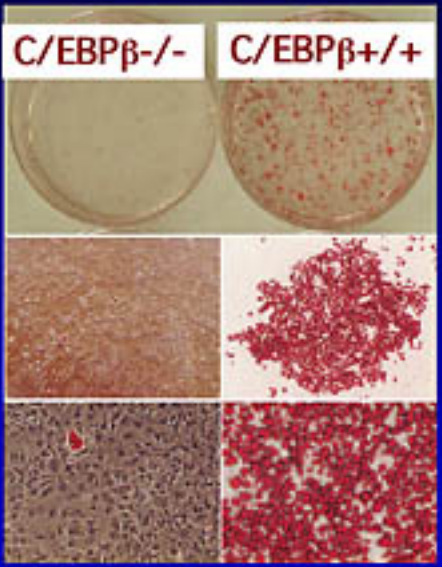
Mitotic clonal expansion is required for differentiation

Is C/EBPβ required for mitotic clonal expansion during adipogenesis ??


C/EBPβ protein
% of MAXIMUM
100
80
60
40
20
0
0
10
20
30
40
50
C/EBPβ–binding activity
³H-thymidine -> DNA
αbinding
αP2 protein
HOURS AFTER INDUCTION OF DIFFERENTIATION

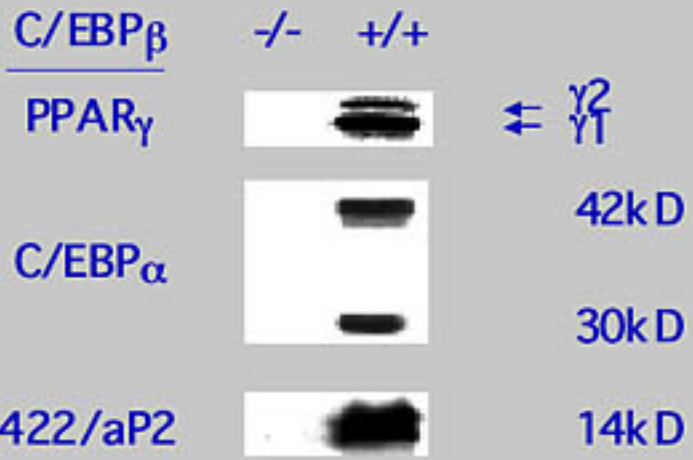
C/EBPβ(+/+) MEFs undergo mitotic clonal expansion & differentiate; C/EBPβ(-/-)* do not

(induced to differentiate with MDI)



*from Peter Johnson (NCI/NIH)

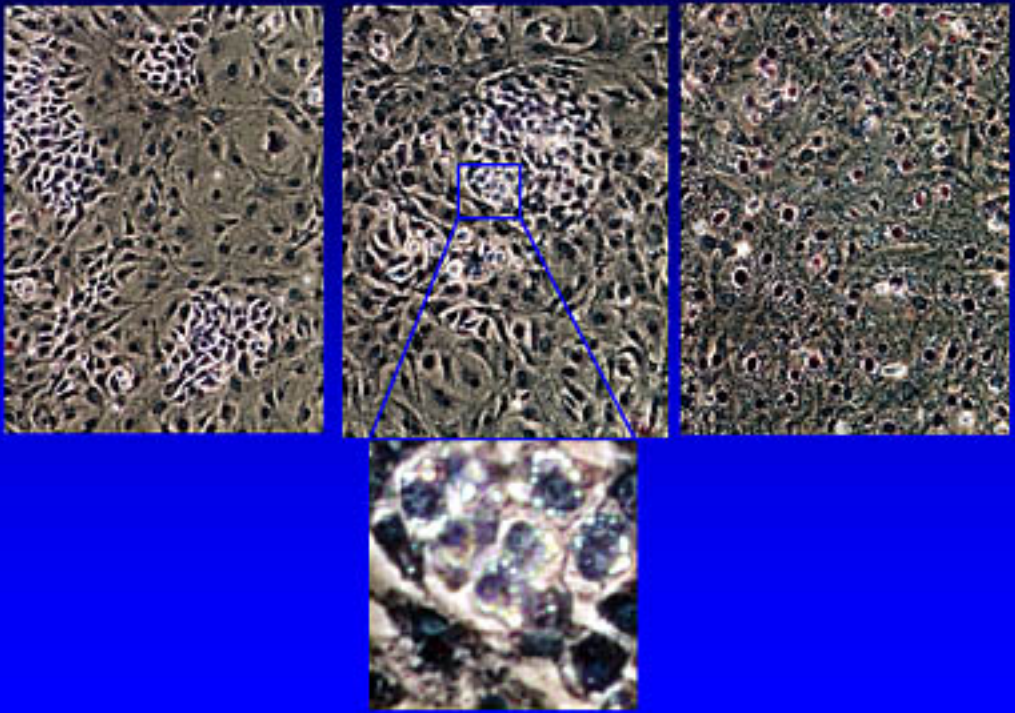
Role of C/EBPβ in adipocyte differentiation



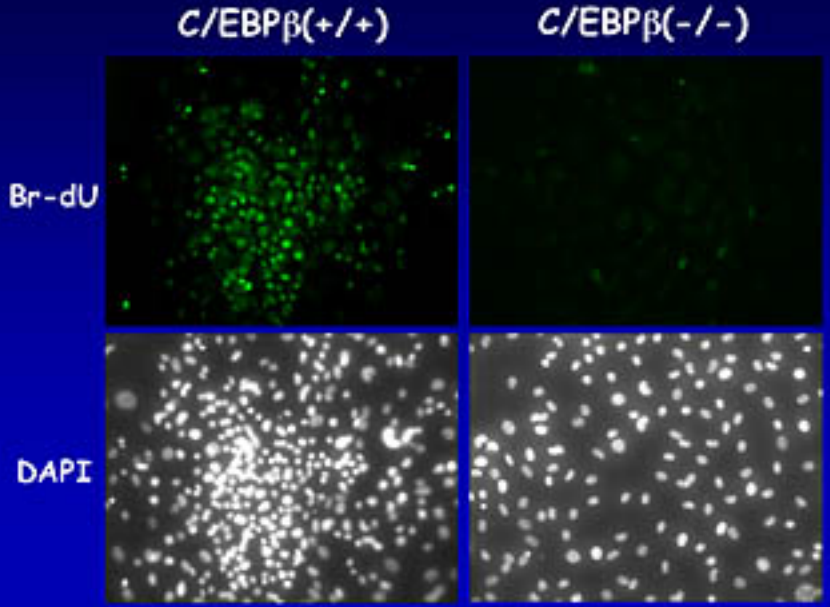
*cell # increases

C/EBPβ+/+

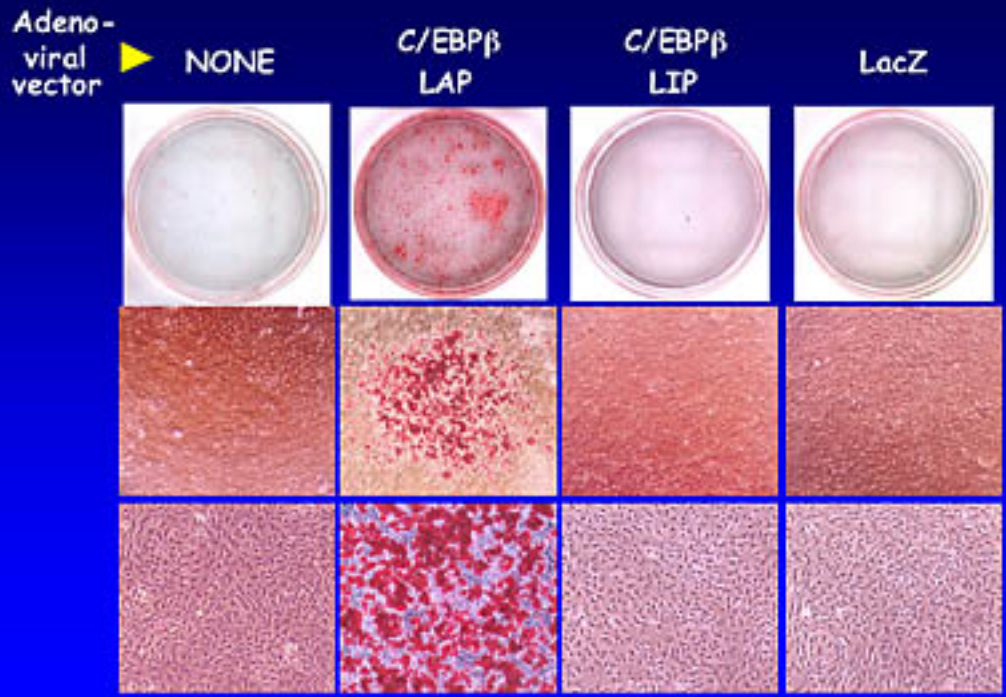
C/EBPβ-/-

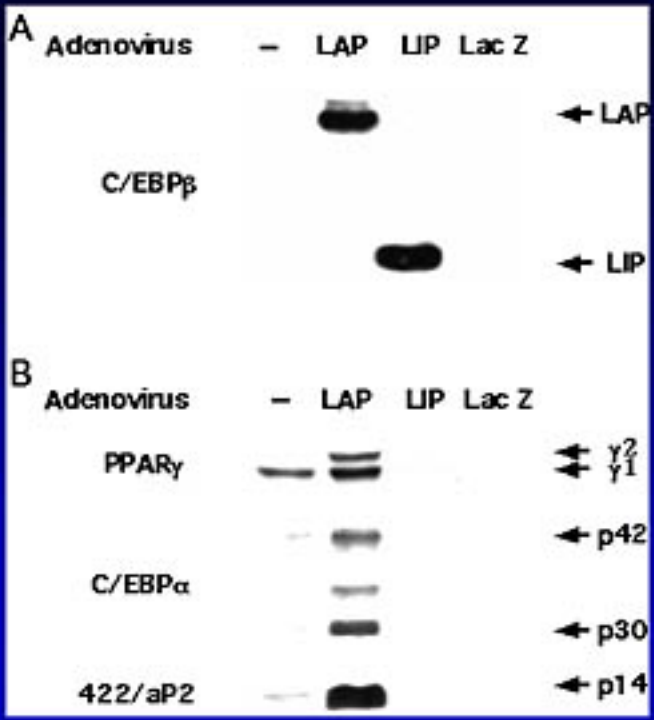


Br-dU LABELING OF DNA OCCURS ONLY IN FOCI THAT UNDERGO DIFFERENTIATION



"Rescue" of C/EBP(-/-) MEFs by expressing C/EBPβ LAP



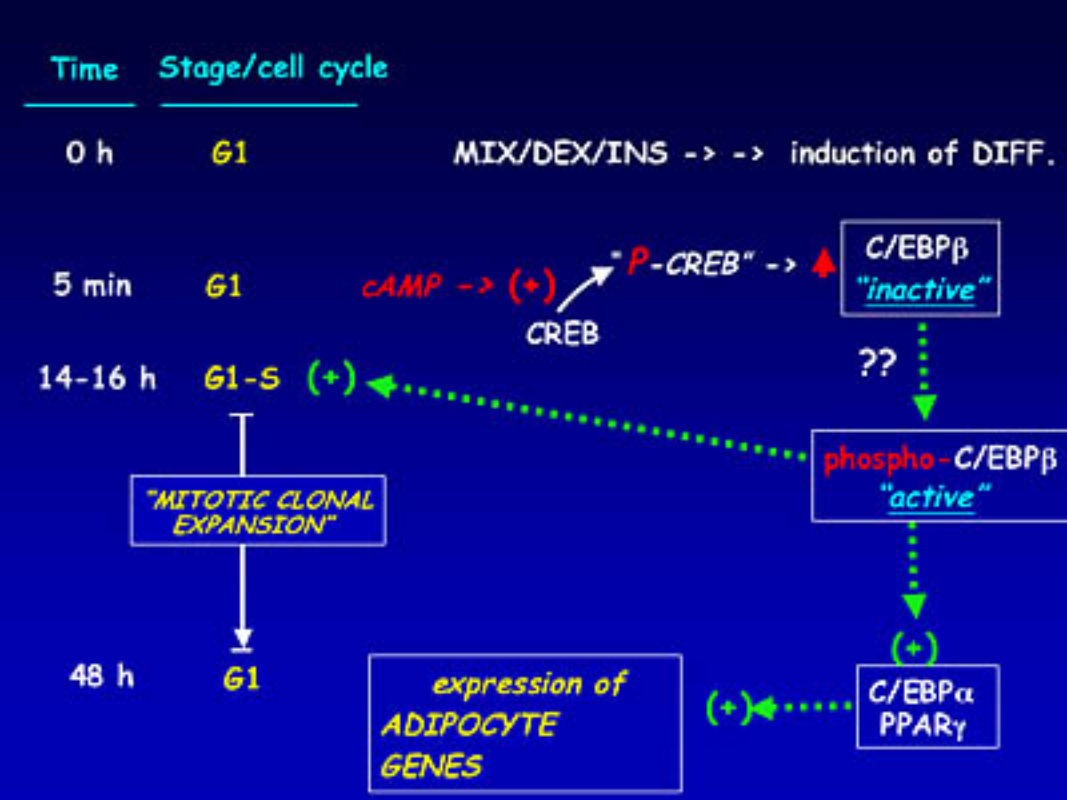

A
Adenovirus
–
LAP
LIP
Lac Z
LAP
C/EBPβ
LIP
B
Adenovirus
–
LAP
LIP
Lac Z
PPARγ
γ2
γ1
p42
C/EBPα
p30
422/aP2
p14

CONCLUSION:

C/EBPβ is required for mitotic clonal expansion and differentiation

WHY IS MITOTIC CLONAL EXPANSION NECESSARY ??

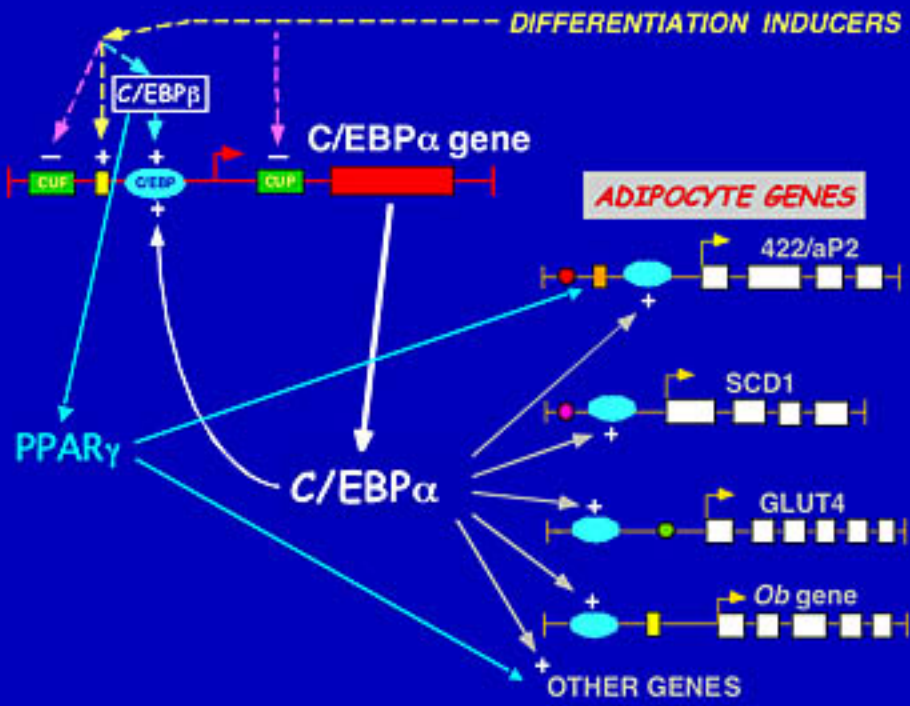
- perhaps to allow access to transcription and other factors to adipocyte genes during DNA replication


Time
Stage/cell cycle
0 h
G1
MIX/DEX/INS -> -> induction of DIFF.
5 min
G1
cAMP -> (+)
CREB
"P-CREB" ->
C/EBPβ
"inactive"
??
14-16 h
G1-S
(+)
phospho-C/EBPβ
"active"
"MITOTIC CLONAL EXPANSION"
48 h
G1
(+)
C/EBPα
PPARγ
(+)
expression of
ADIPOCYTE
GENES

The C/EBPα and PPARγ Gene Promoters

* Both the C/EBPα and PPARγ genes possess "functional" C/EBP regulatory elements

* C/EBPβ transactivates both genes

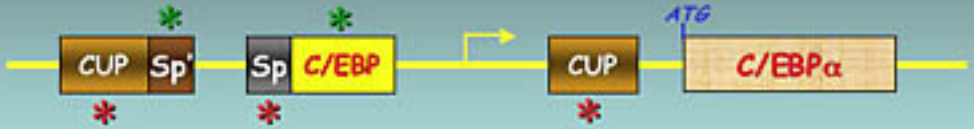

DIFFERENTIATION INDUCERS
C/EBPβ
C/EBPα gene
CUP
CEBP
CUP
ADIPOCYTE GENES
422/aP2
SCD1
GLUT4
Ob gene
PPARγ
C/EBPα
OTHER GENES

END

COLLABORATORS

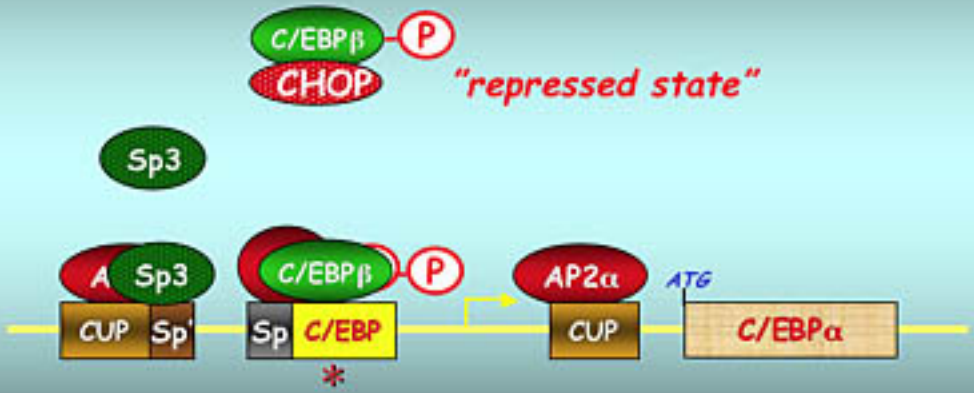
Qi-Qun Tang	*Biological Chemistry/Johns Hopkins*
Jiangwen Zhang	*Biological Chem.stry/Johns Hopkins*
Tammy Otto	*Biological Chemistry/Johns Hopkins*
Chuck Vinson	*NCI/NIH*
Dwight Klemm	*University of Colorado Med. Cntr.*

THE C/EBPα GENE PROMOTER



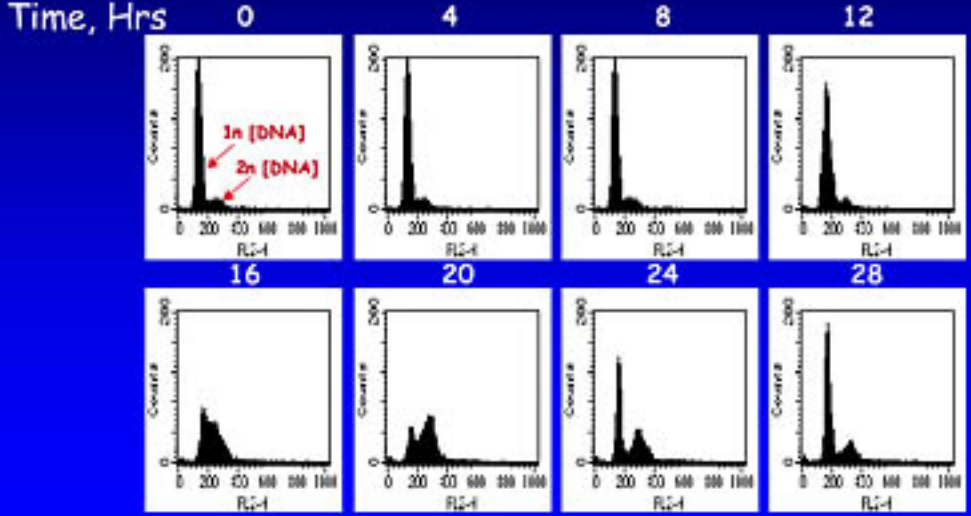
*activator binding site
*repressor binding site

REPRESSION-DEREPRESSION OF THE C/EBPα gene (2h after MDI)

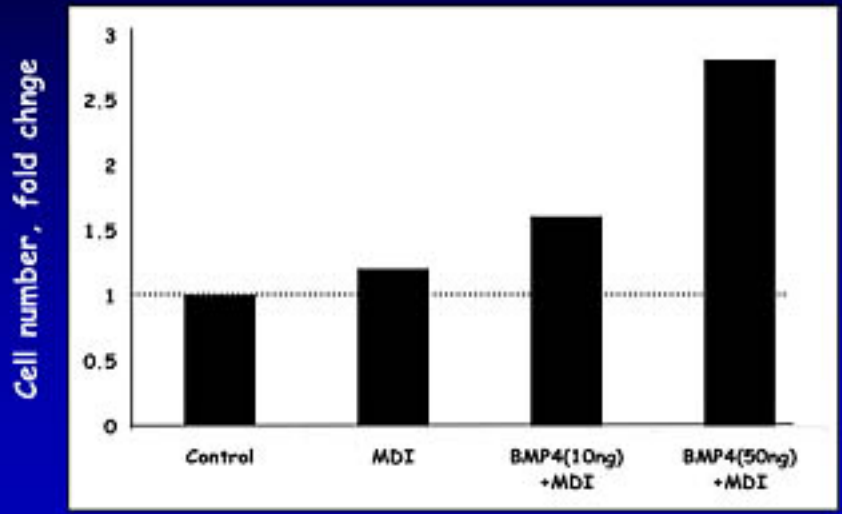


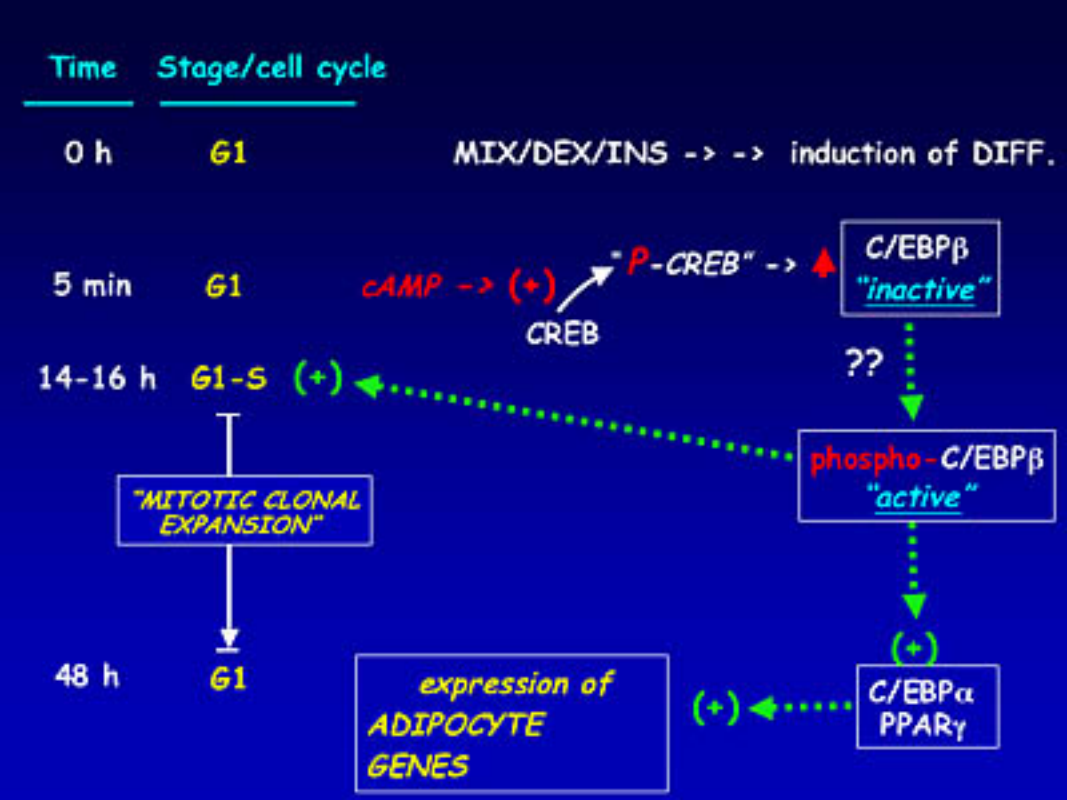
SYNCHRONOUS ENTRY OF MITOTIC CLONAL EXPANSION

(DNA CONTENT: FAX analysis after fixation & propidium I staining)



"COMMITTED" 10T1/2 CELLS UNDERGO MITOTIC CLONAL EXPANSION WHEN INDUCED TO DIFFERENTIATE




Time
Stage/cell cycle
0 h
G1
MIX/DEX/INS -> -> induction of DIFF.
5 min
G1
cAMP -> (+)
CREB
"P-CREB" ->
C/EBPβ
"inactive"
??
14-16 h
G1-S
(+)
phospho-C/EBPβ
"active"
"MITOTIC CLONAL EXPANSION"
48 h
G1
(+)
C/EBPα
PPARγ
(+)
expression of
ADIPOCYTE
GENES

Effect of dominant-negative CREB on C/EBPβ promoter-reporter gene

Co-transfection of C/EBPβ promoter-luciferase

Expression vectors:

CREB-VP16 (CREB DNA binding domain constitutively active)

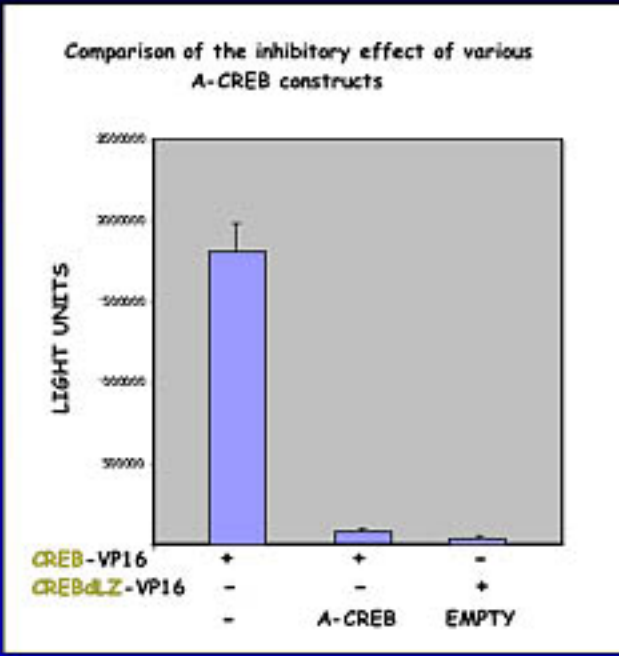
or

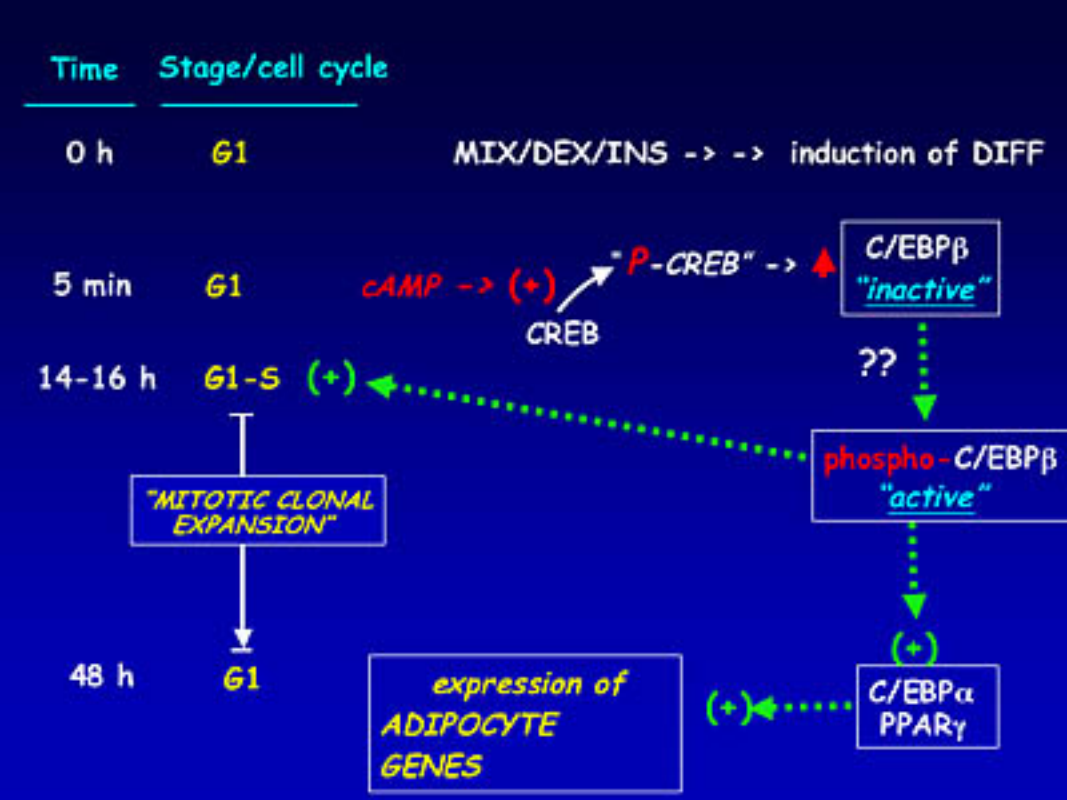
CREBdLZ-VP16 (lacks functional DNA binding domain)

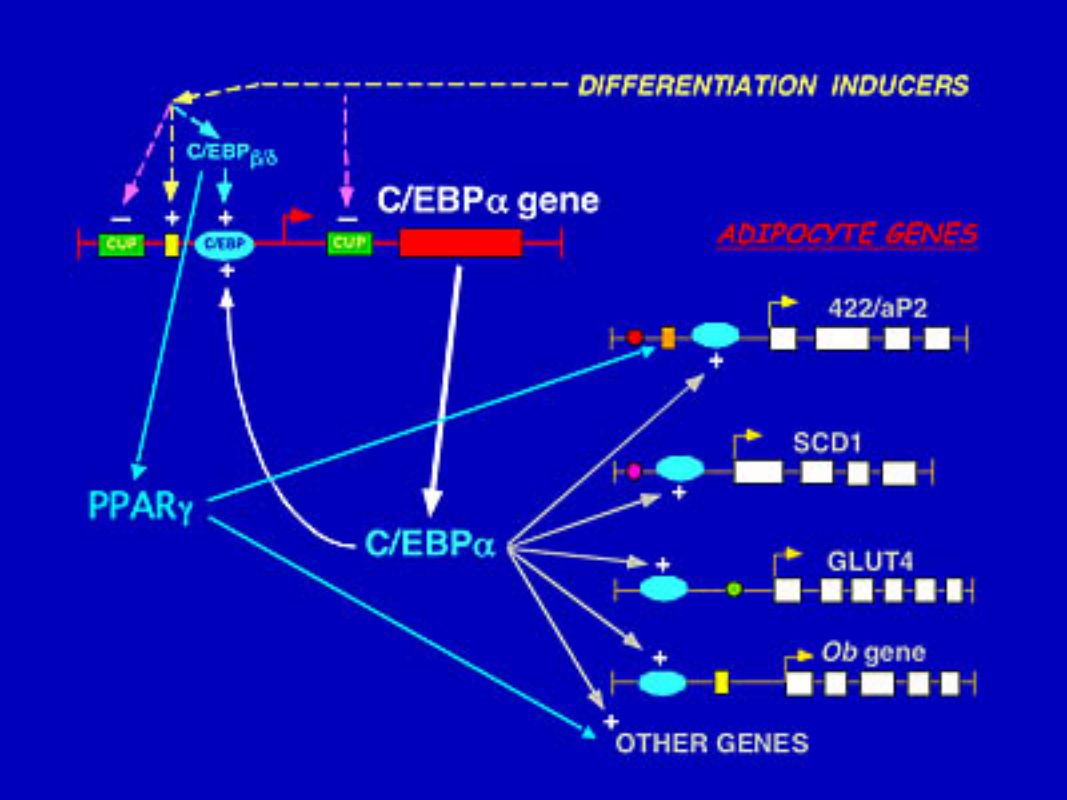
A-CREB (dominant negative)

or

Empty vector




Time
Stage/cell cycle
0 h
G1
MIX/DEX/INS -> -> induction of DIFF
5 min
G1
cAMP -> (+)
CREB
"P-CREB" ->
C/EBPβ
"inactive"
??
14-16 h
G1-S
(+)
phospho-C/EBPβ
"active"
"MITOTIC CLONAL EXPANSION"
48 h
G1
(+)
C/EBPα
PPARγ
(+)
expression of
ADIPOCYTE
GENES


DIFFERENTIATION INDUCERS
C/EBPβ/δ
C/EBPα gene
CUP
C/EBP
CUP
ADIPOCYTE GENES
422/aP2
SCD1
GLUT4
Ob gene
PPARγ
C/EBPα
OTHER GENES