Paper #1:
Regulation of skeletal myogenesis by association of the MEF2 transcription factor with Class II histone deacetylases,

Lu, J., McKinsey, T.A., Zhang, C., and Olson, E.N.,

Molecular Cell, 6:233-244, 2000.

(233 citations & Impact Factor 14)

Final exam on my section: Things you need to study for papers #1-4

1. You should be able to explain any figure in these papers that is shown to you, after being told the method used:

- What was done (no details in the method but should state basic experimental design and background info relevant to the experiment)
- What it means
- Was it done correctly? Was something left out?
- What could you do to improve the experiment?

Final exam on my section: Things you need to study for papers #1-4

2. You should be able to describe the overall model for each paper:
   - Draw the model and explain it
   - Explain which experiments supported this model (include the outcome of the experiment so it is clear how this experiment supported the model)
   - Suggest other approaches or next step experiments
3. You should understand what each technique is used for and extrapolate this into new situations.

Final exam for my section: Things you need to study for papers #1-4

Please note:

Marks will not be deducted for wrong answers. Please be as complete as possible and assume the broadest interpretation of the question, not the narrowest. You can use diagrams to aid your explanation but you must also have written words to back up the diagram.

You will not be directly tested on:

The general introduction (lecture #1)

but this introduction is relevant to the papers that we cover.

For stem cell therapy lecture (lecture #6):

Understand the different types of stem cells

- Where are they from?
- What advantage or disadvantage do they have?
- What experimental evidence is there that they work?
- Define terms

Eric Olson

- Leader in the field of skeletal and cardiac myogenesis (cloned myogenin, MEF2C, showed synergy)
- Total of 557 papers with 45,685 citations
- H index = 106 (106 papers published with over 106 citations since 1995)

(From Scopus: http://www.scopus.com)

Eric Olson

- Company Myogen sold for $2.5 billion in 2006.
- Development of medicine for the treatment of heart disease
- Also sings in a rock band!

Questions

- Do Class II HDACs regulate myogenesis?
- By what mechanism?
- How is the inhibition regulated?

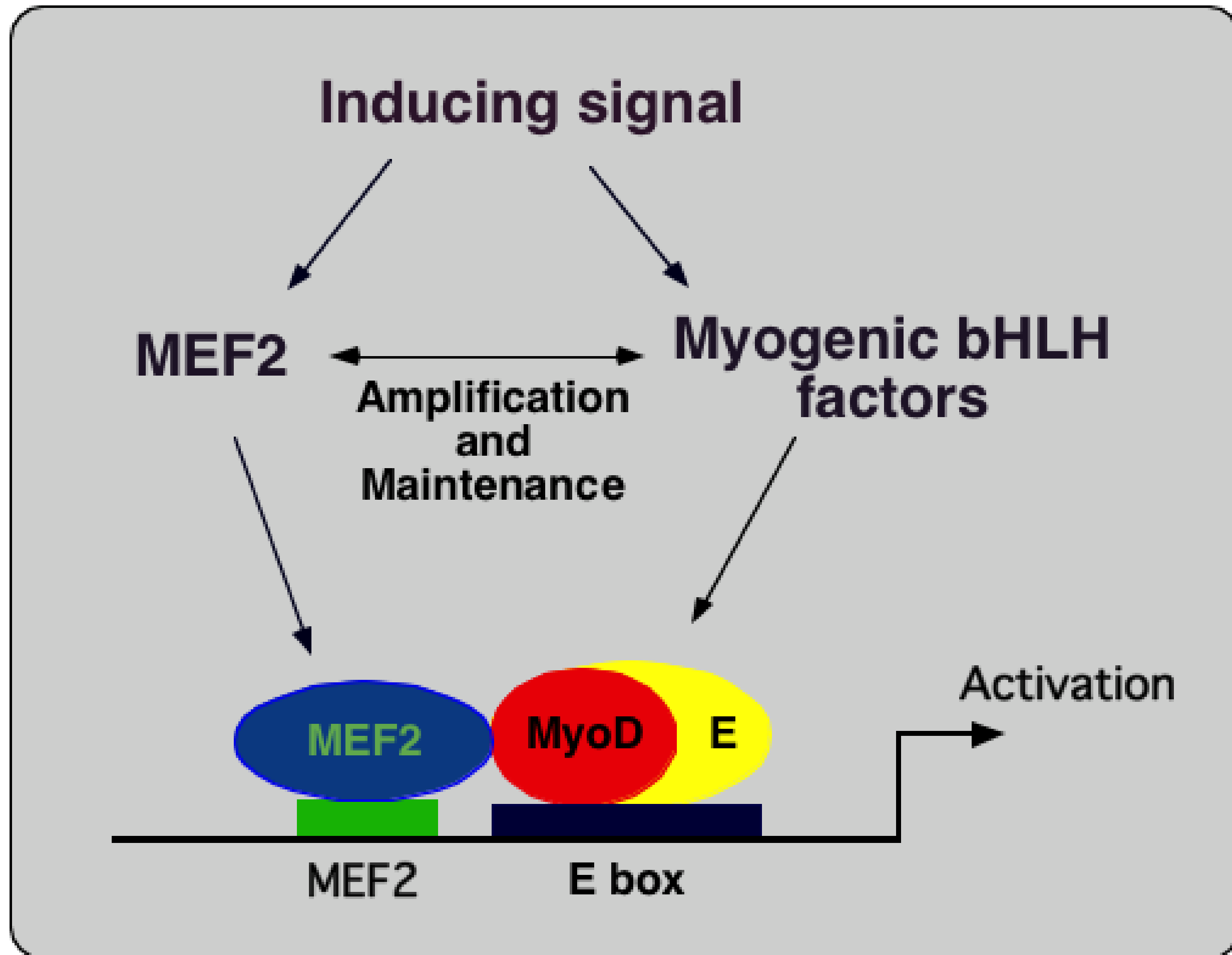
Why is it important to understand these questions?

1. We need to understand how chromosome structure affects the regulation of transcription.
2. Understanding how chromatin structure affects transcription during muscle development may enable new approaches for increasing the amount or regulating the amount of muscle obtained for cell therapy.

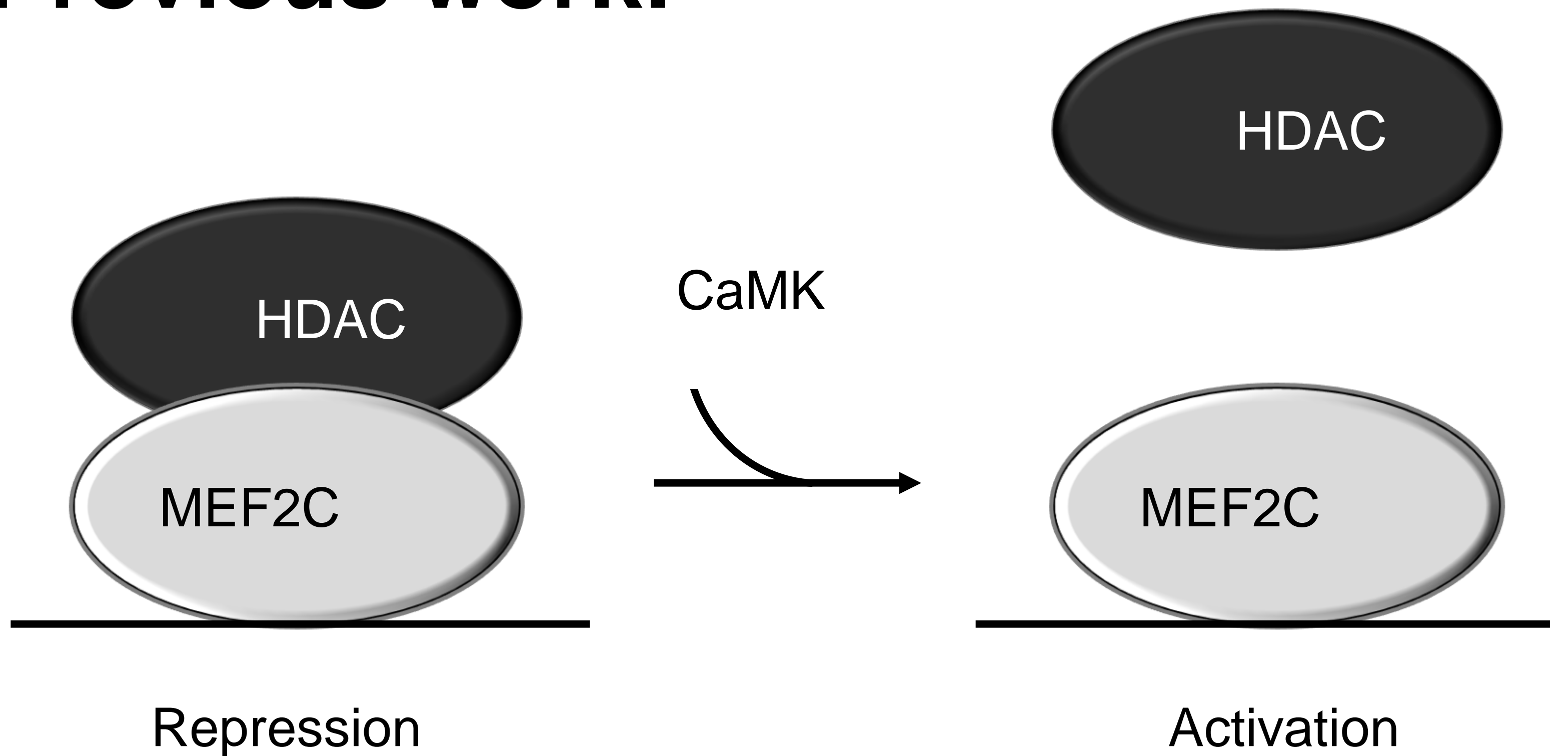
Why understand the details of papers rather than just memorizing the model at the end?

- Being able to understand and critically read papers is essential for one's future ability to do research - whether it is basic science, dental, or medical research
- Critical thinking skills are valuable for everyone

MEF2 and MRFs Act Cooperatively to Activate Muscle-Specific Genes



Previous work:



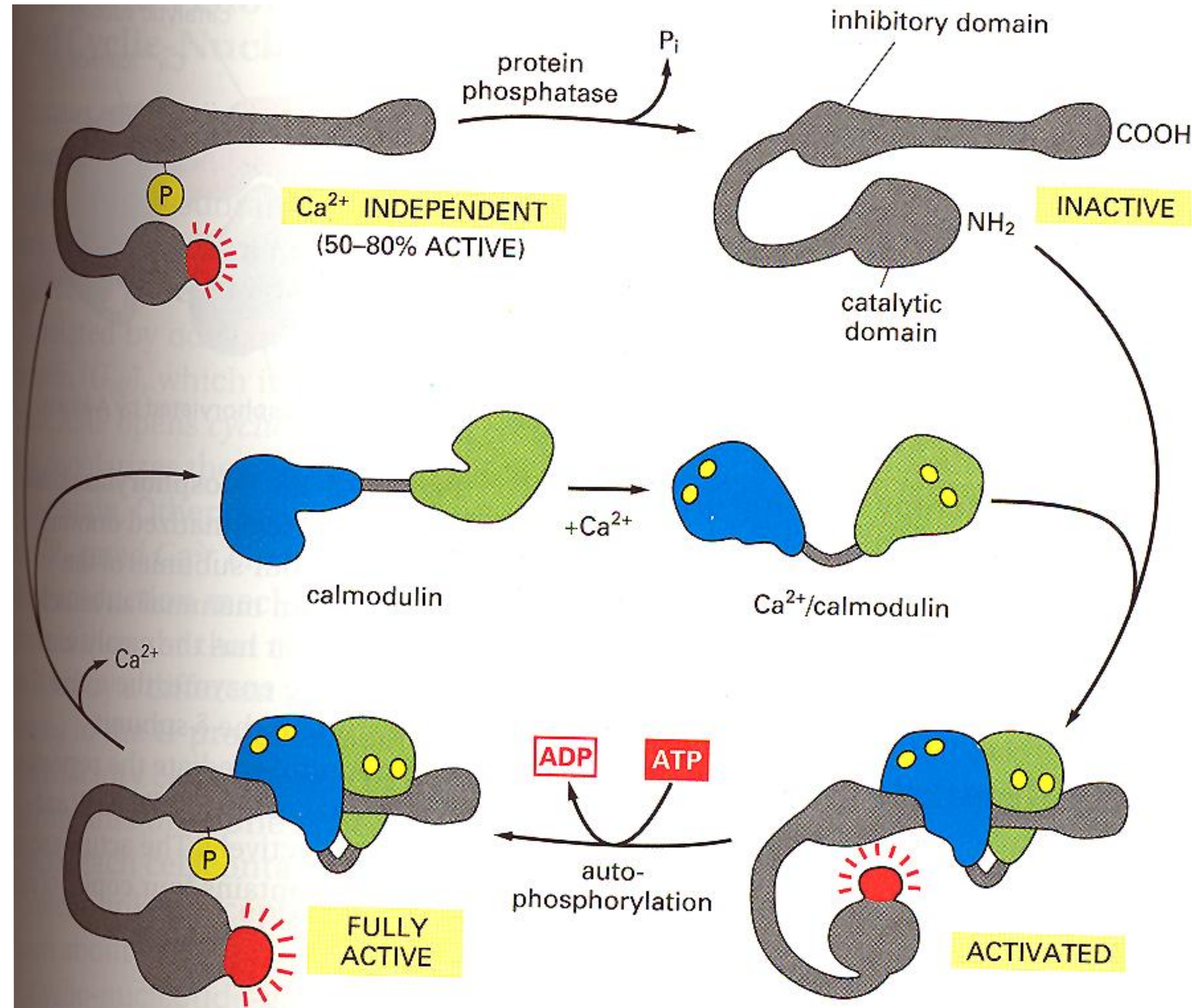
MEF2C:

- Is the first MEF2 to be expressed in developing skeletal muscle
- Binds to HDAC by its MADS/MEF domain
- Binding of HDAC causes repression of MEF2C activity
- The repression can be relieved by CaMK

CaMK (Ca^{2+} /calmodulin-dependent protein kinases):

Calmodulin:

- A ubiquitous Ca^{2+} binding protein
- Activates the serine/threonine kinase activity of CaMK



Hypothesis:

Class II HDACs will inhibit myogenesis by binding to MEF2C, in a CaMK-dependent fashion

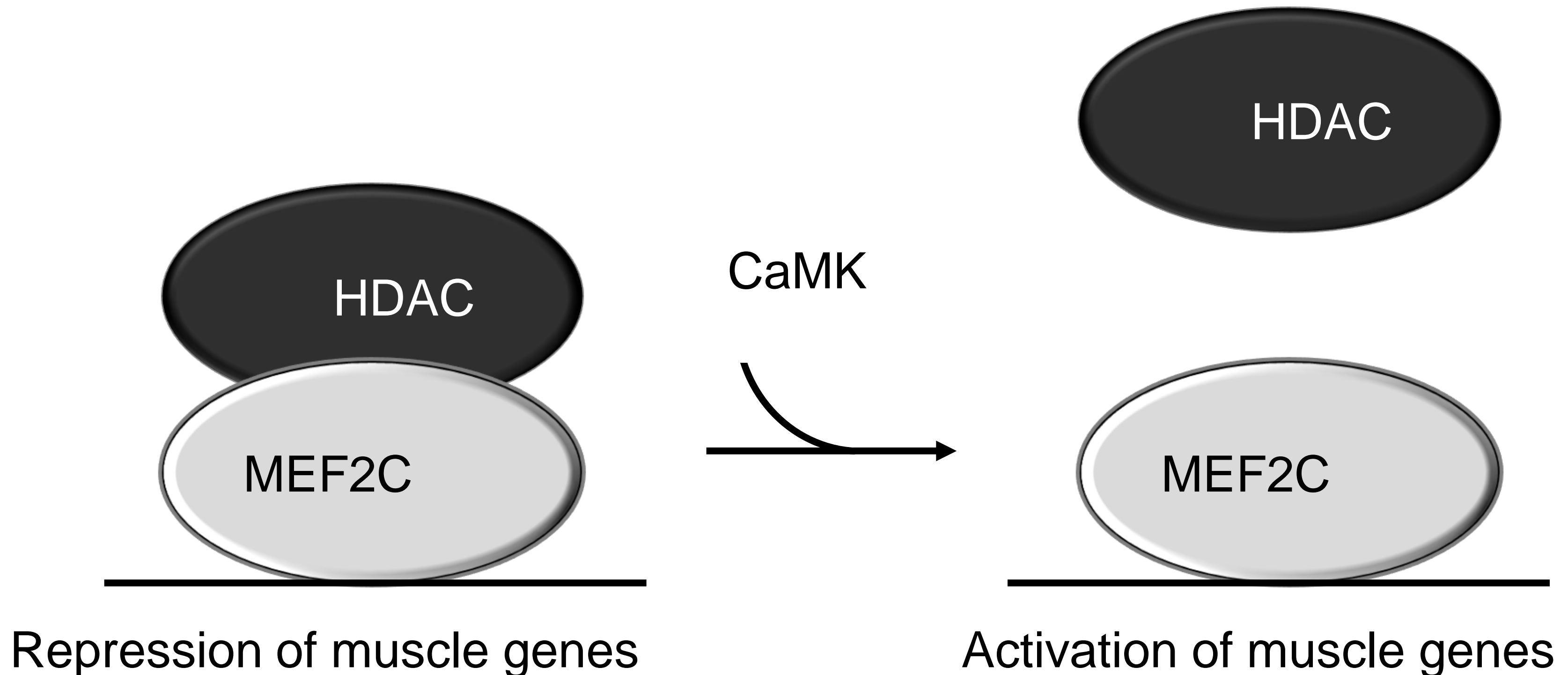
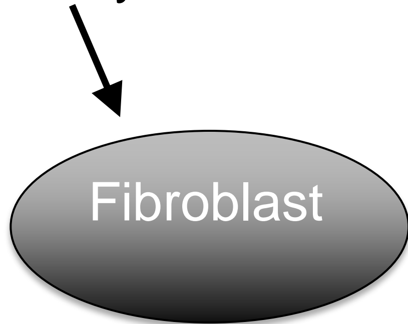
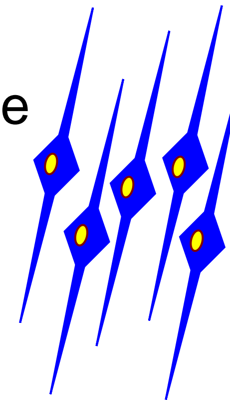


Figure 1: HDACs 4 and 5 Inhibit Conversion of 10T1/2 Cells to Skeletal Muscle by MyoD

Transfect MyoD +/- HDACs



Perform Immunofluorescence



Muscle

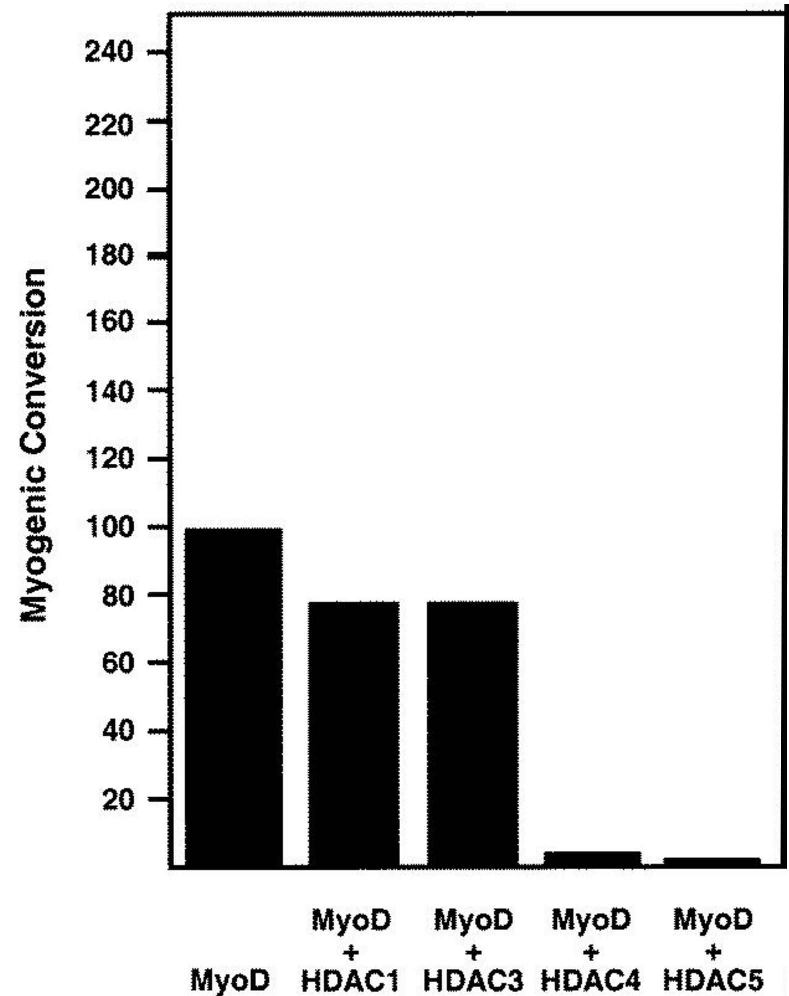
Can HDACs inhibit MyoD function?

★ Class II HDACs inhibit MyoD function

% Myogenic conversion where 100% = 50 cells/coverslip

Found:

- HDAC4 and HDAC5 inhibited the ability of MyoD to convert fibroblasts into muscle, but not HDAC1 or HDAC3

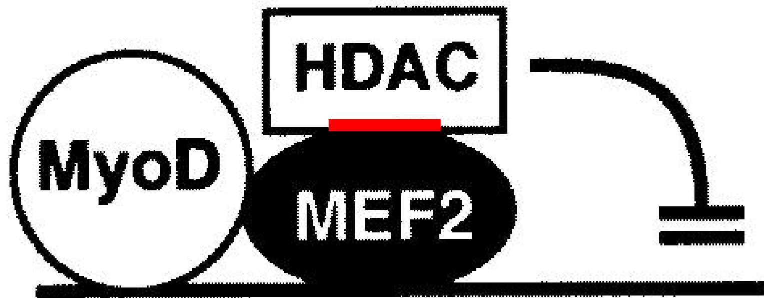


What domains of HDAC4 and HDAC5 are important for inhibiting MyoD?

HDAC-4 & -5: N-terminal mutants

MEF2 binding

(repressor)



If the MEF2 binding region of HDAC is mutated, HDAC cannot bind to MEF2.

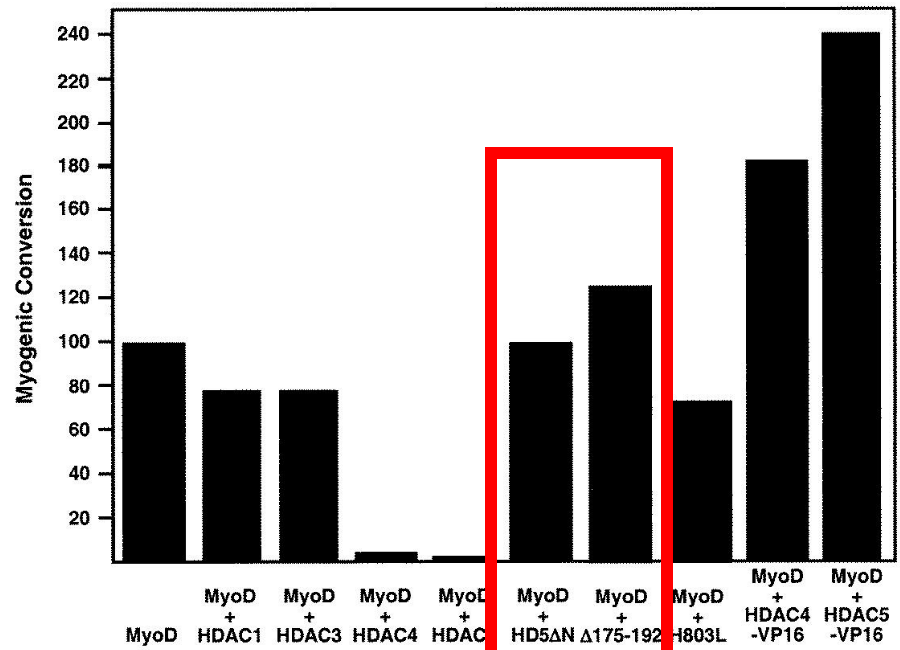
Why do they hypothesize that MEF2 is involved when MEF2 wasn't added in the experiment?

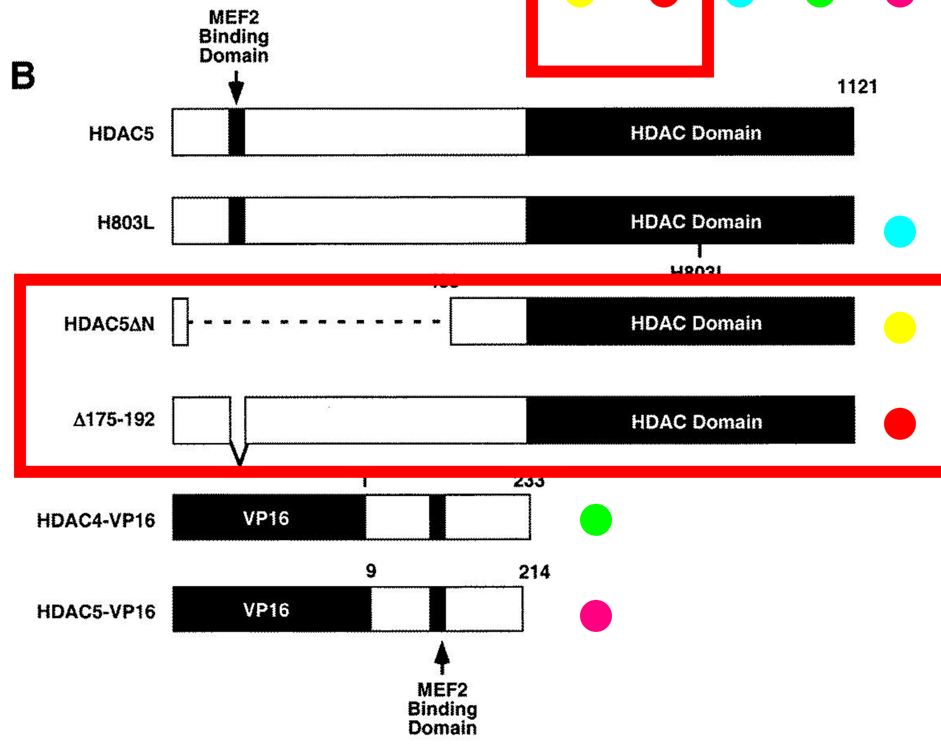
★ Class II HDACs inhibit MyoD function

% Myogenic conversion where 100% = 50 cells/coverslip

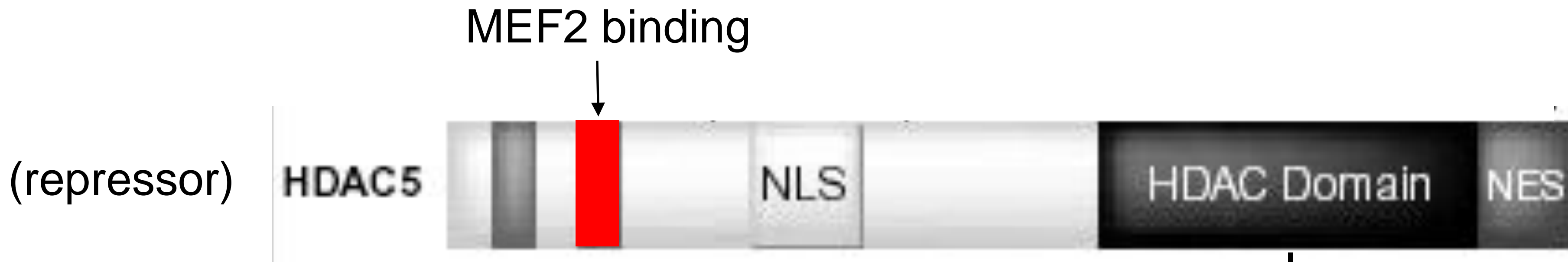
Found:

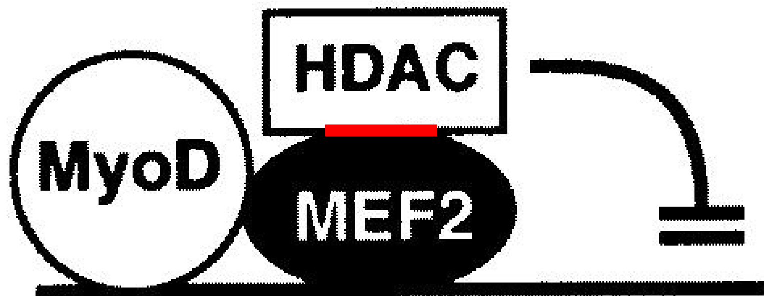
- HDAC4 and HDAC5 inhibited the ability of MyoD to convert fibroblasts into muscle, but not HDAC1 or HDAC3
- Inhibition required the MEF2 binding domain





HDAC-5: Catalytic mutant





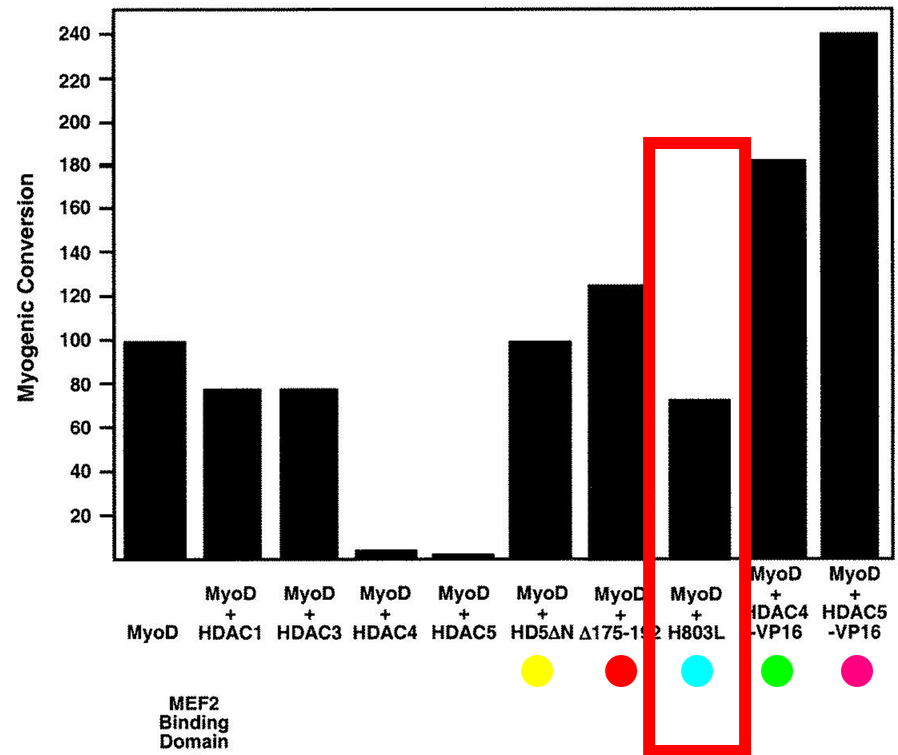
If there is no catalytic activity, HDAC cannot deactylate the histones and is not able to repress transcription

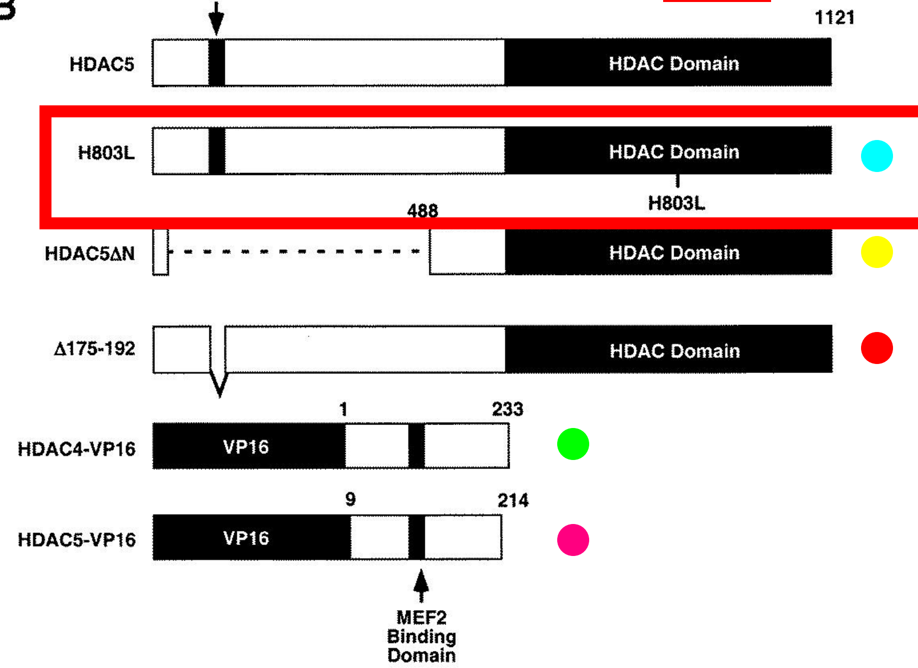
★ Class II HDACs inhibit MyoD function

% Myogenic conversion where 100% = 50 cells/coverslip

Found:

- HDAC4 and HDAC5 inhibited the ability of MyoD to convert fibroblasts into muscle, but not HDAC1 or HDAC3
- Inhibition required the MEF2 binding domain and a functional catalytic domain



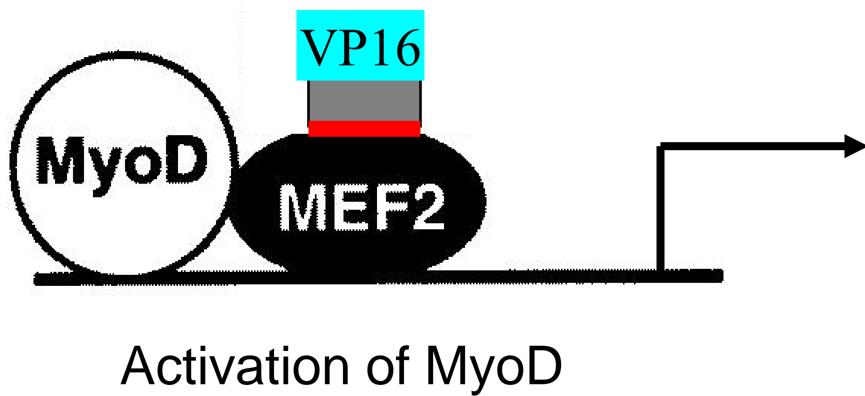


Class IIa = HDAC-4 & -5

HDAC
-VP16

(activator)

HDAC-VP16: The fusion of the MEF2 binding domain with VP16. VP16 is a powerful transcriptional activation domain from a viral transcription factor. It can activate the function of many transcription factors.



Activation of MyoD

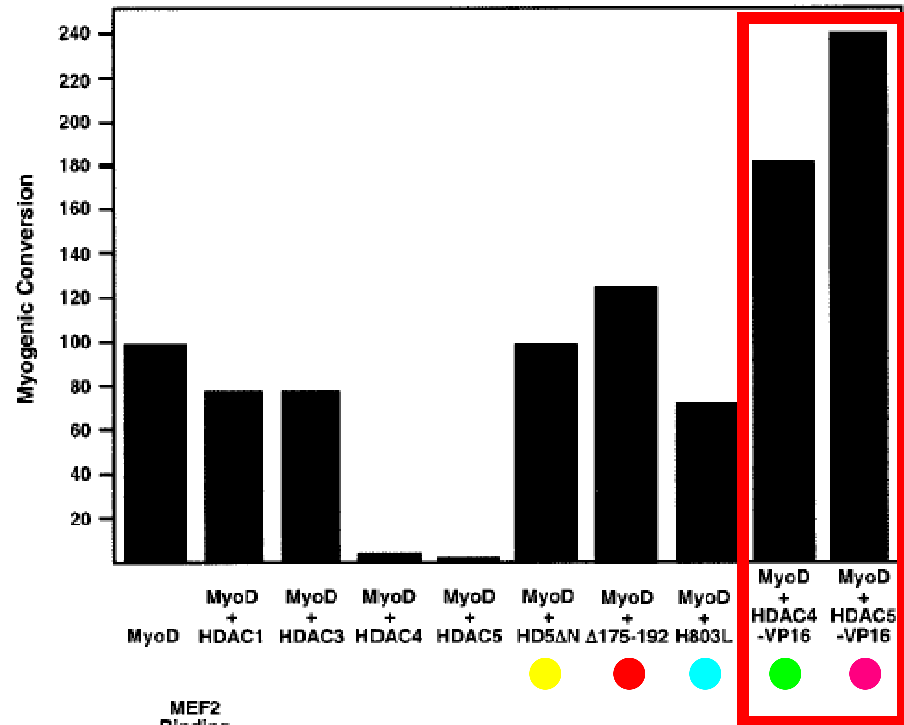
If the transcriptional activator, VP16 can be targeted to MEF2 by the MEF2 binding site of HDAC, then we would expect transcriptional activation to occur

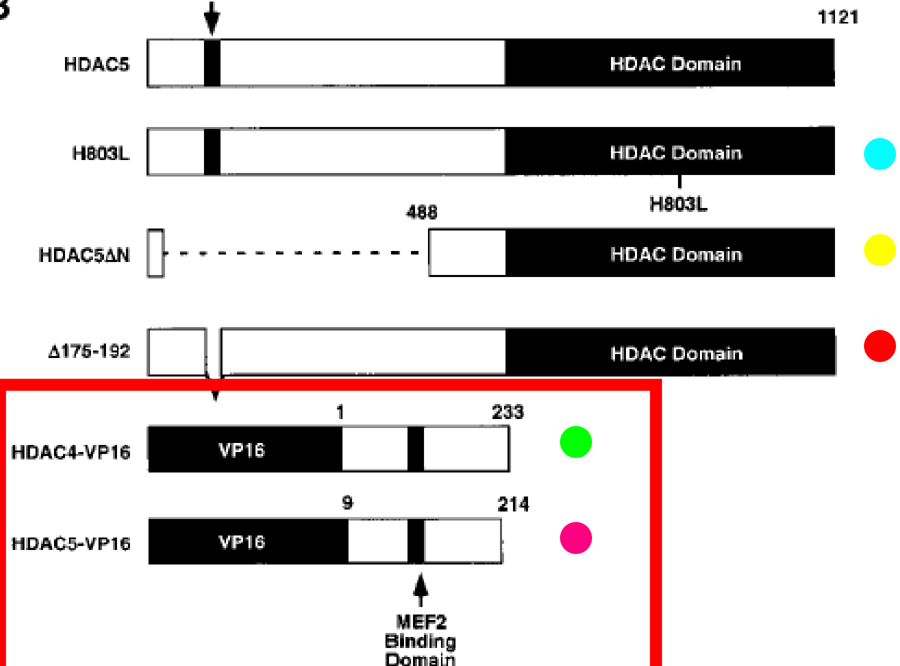
★ Class II HDACs inhibit MyoD function

% Myogenic conversion where
100% = 50 cells/coverslip

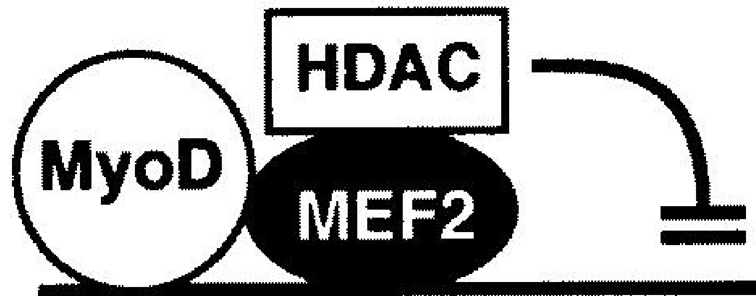
Found:

- HDAC4 and HDAC5 inhibited the ability of MyoD to convert fibroblasts into muscle, but not HDAC1 or HDAC3
- Inhibition required the MEF2 binding domain and a functional catalytic domain
- Replacing the HDAC catalytic domain with VP16 resulted in an enhancement



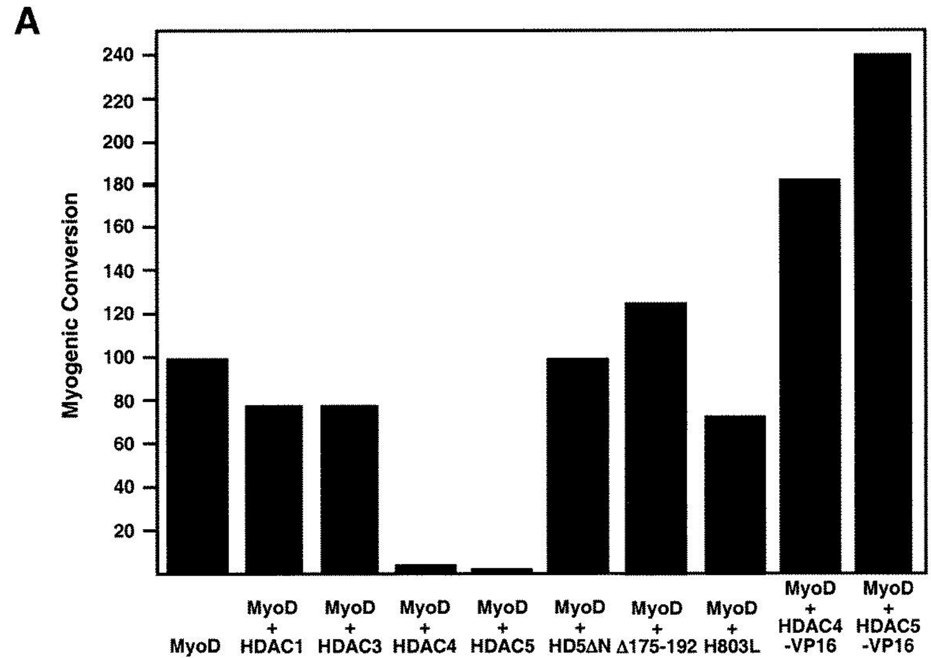


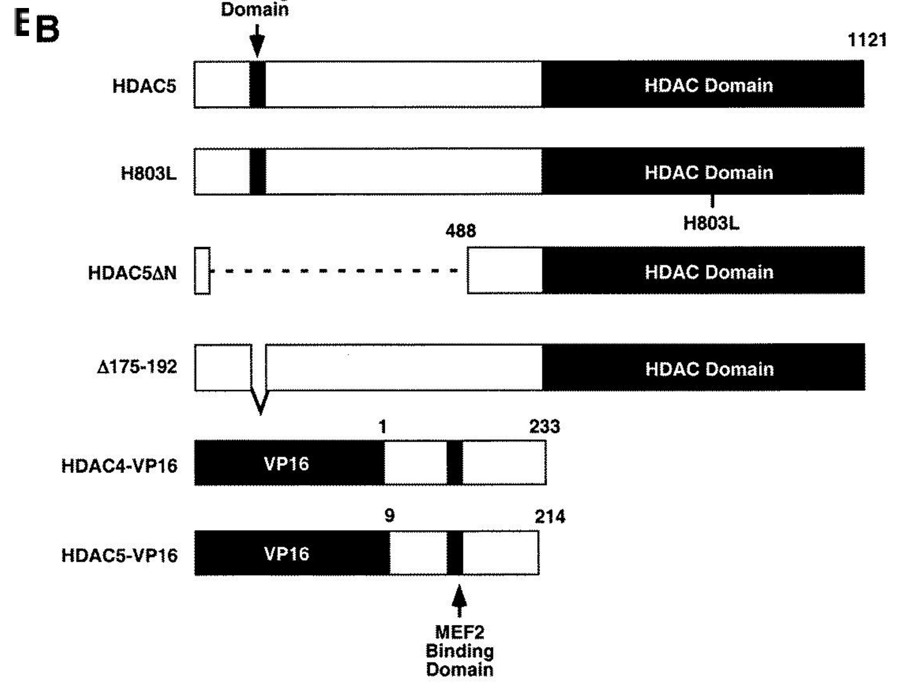
The data is consistent with a model in which HDAC inhibits myogenesis by binding to MEF2, which binds to MyoD



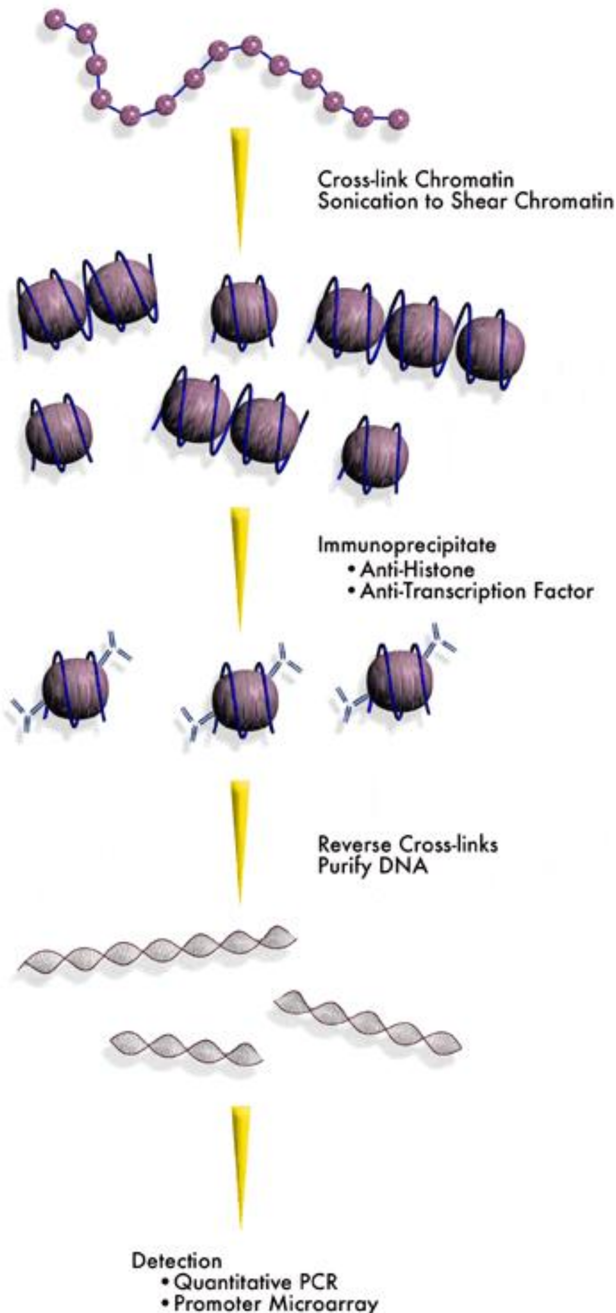
★ Class II HDACs inhibit MyoD function

What is missing from this figure?

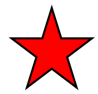




Do HDACs change the acetylation status of muscle-specific genes during myogenesis?



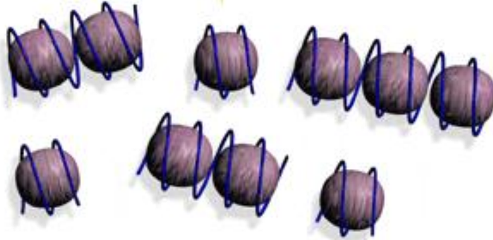
Chromatin Immunoprecipitation Assay (CHIP): A method to determine the location of transcription factor binding sites or chromatin modifications



Chromatin Immunoprecipitation Assay (CHIP)

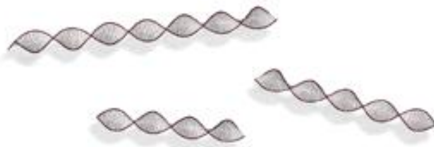
-Isolate chromatin from cells of interest

-Cross-link chromatin and sonicate to shear



-Immunoprecipitate with antibodies to the transcription factor of interest or to a modified histone (acetylated histone or methylated histone)

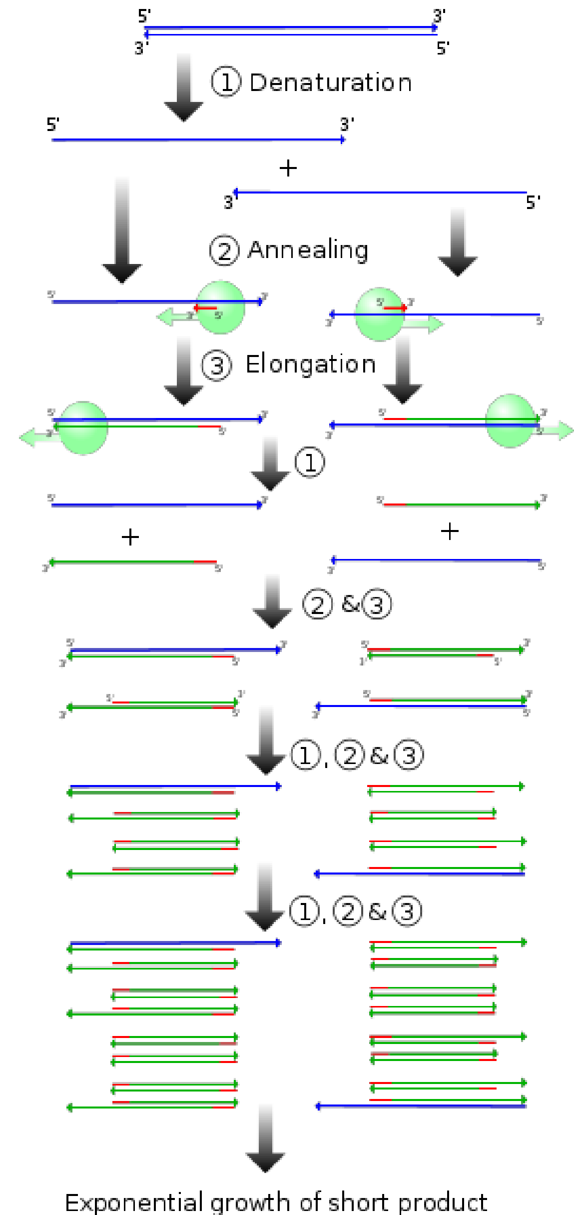
-Reverse the cross-links , remove the protein, and purify the DNA



-Analyze DNA by PCR or quantitative PCR, by microarray, or by high throughput sequencing

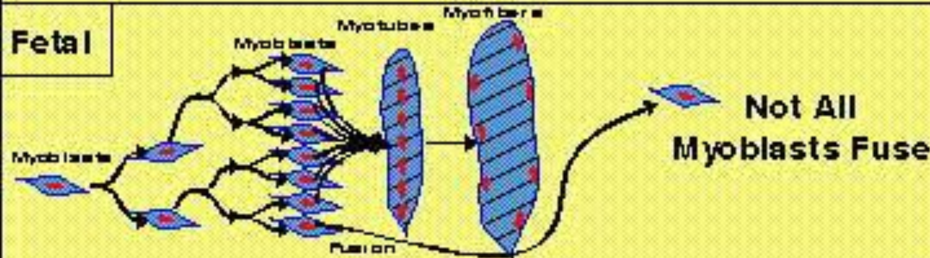
Polymerase Chain Reaction (PCR)

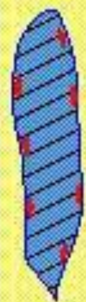
- Denature DNA
- Anneal primers to specific genes
- Elongate DNA with DNA polymerase
- Repeat the cycle to create exponential growth of a short product

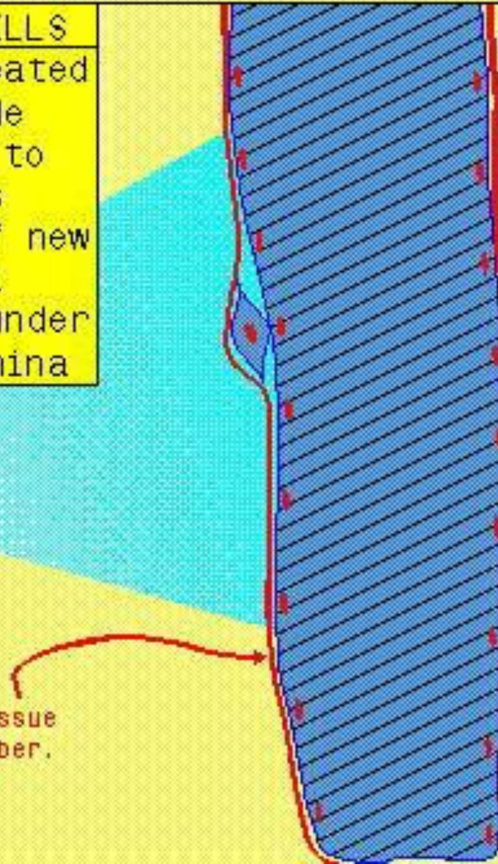


Satellite cells

Where do myonuclei come from?







- Present in adult muscle
- Can be activated to proliferate and differentiate into skeletal muscle by injury
- Are the source of cells for various skeletal myoblast cell lines:

C2 myoblasts

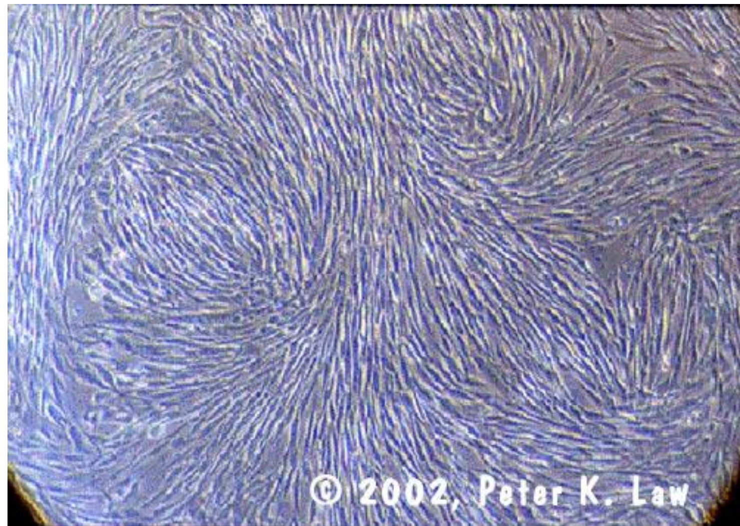
C2 myoblasts differentiate into skeletal myotubes

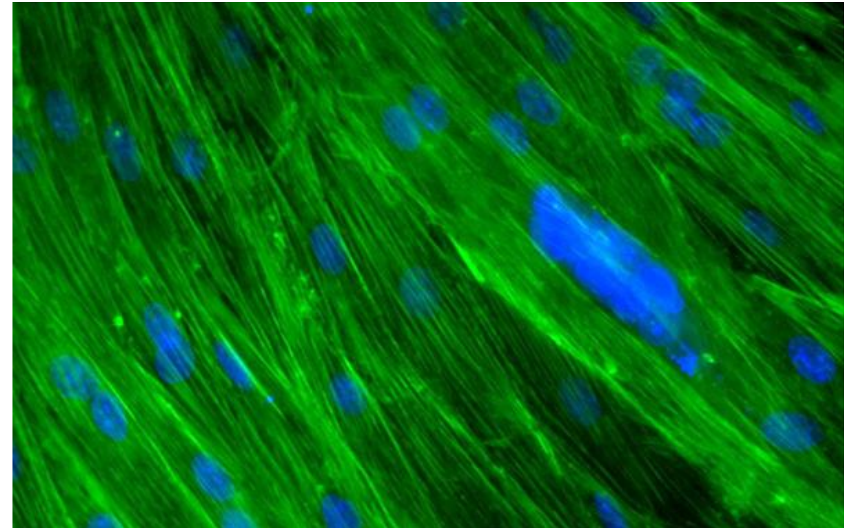
GM

Growth media (High serum)

DM

Differentiation media (Low serum)





Phase contrast cells under light (Day 0)

Immunofluorescence labeling with Myosin heavy chain (green) and Hoechst dye for nuclei (day 5)

MyoD

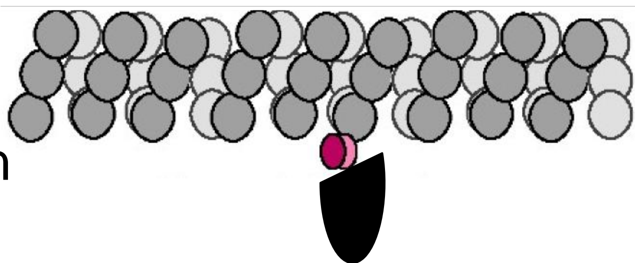
Myogenin*, MEF2C, actin, myosin, muscle creatine kinase (MCK)*

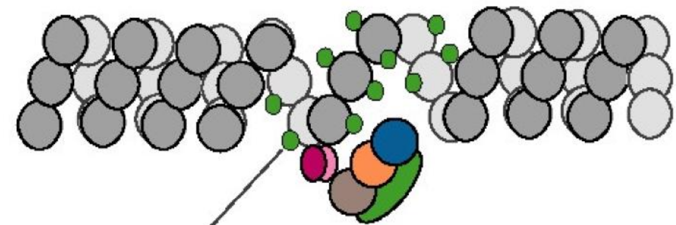
Hypothesis:
Acetylation of histones will occur on genes regulated by MEF2 during differentiation in C2 myoblasts

Growth Media (GM)

Differentiation Media (DM)

MCK
or
myogenin





MEF2 bound to HDAC

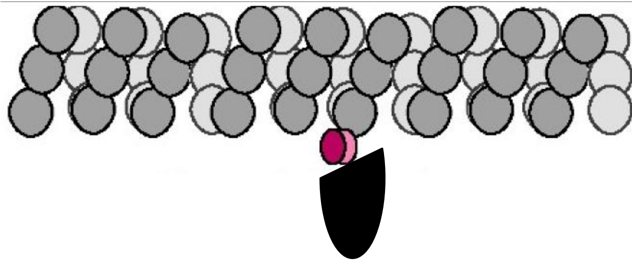
MEF2 bound to HAT

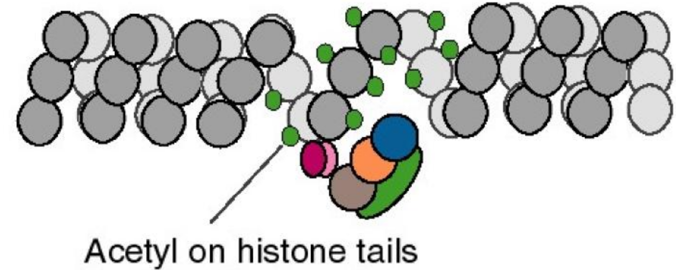
Chromatin around MEF2 sites in MCK or myogenin promoters is closed (unacetylated) in growing C2 myoblasts and open during differentiation of myoblasts into muscle.

Experimental design:

Growth Media (GM)

Differentiation Media (DM)





MEF2 bound to HDAC

MEF2 bound to HAT

Isolate chromatin/X-link/sonicate

Immunoprecipitate with Acetylated H4-specific AB or non-immune serum

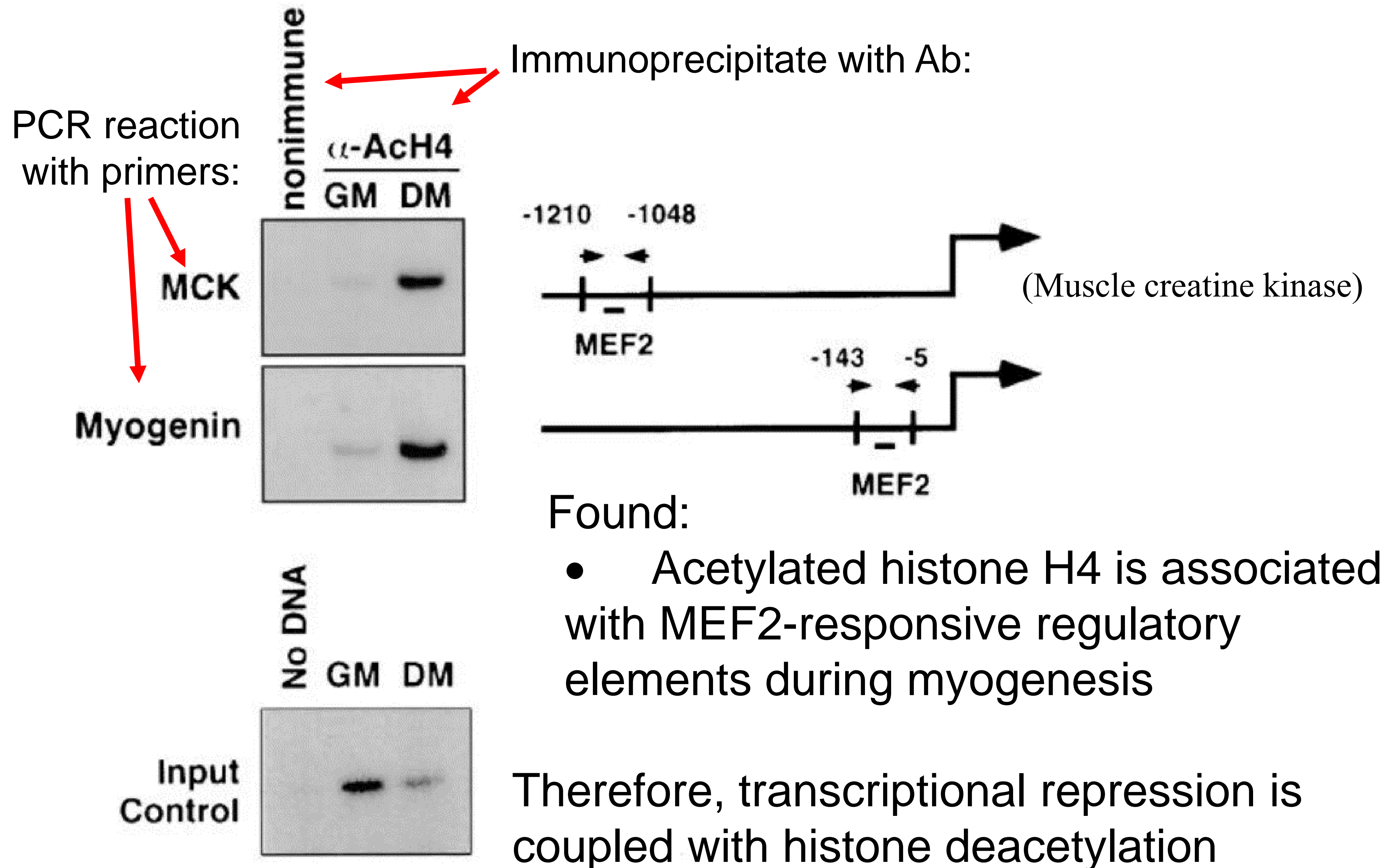
Input control

Reverse cross-link DNA and PCR amplify with primers to either MCK or myogenin promoters

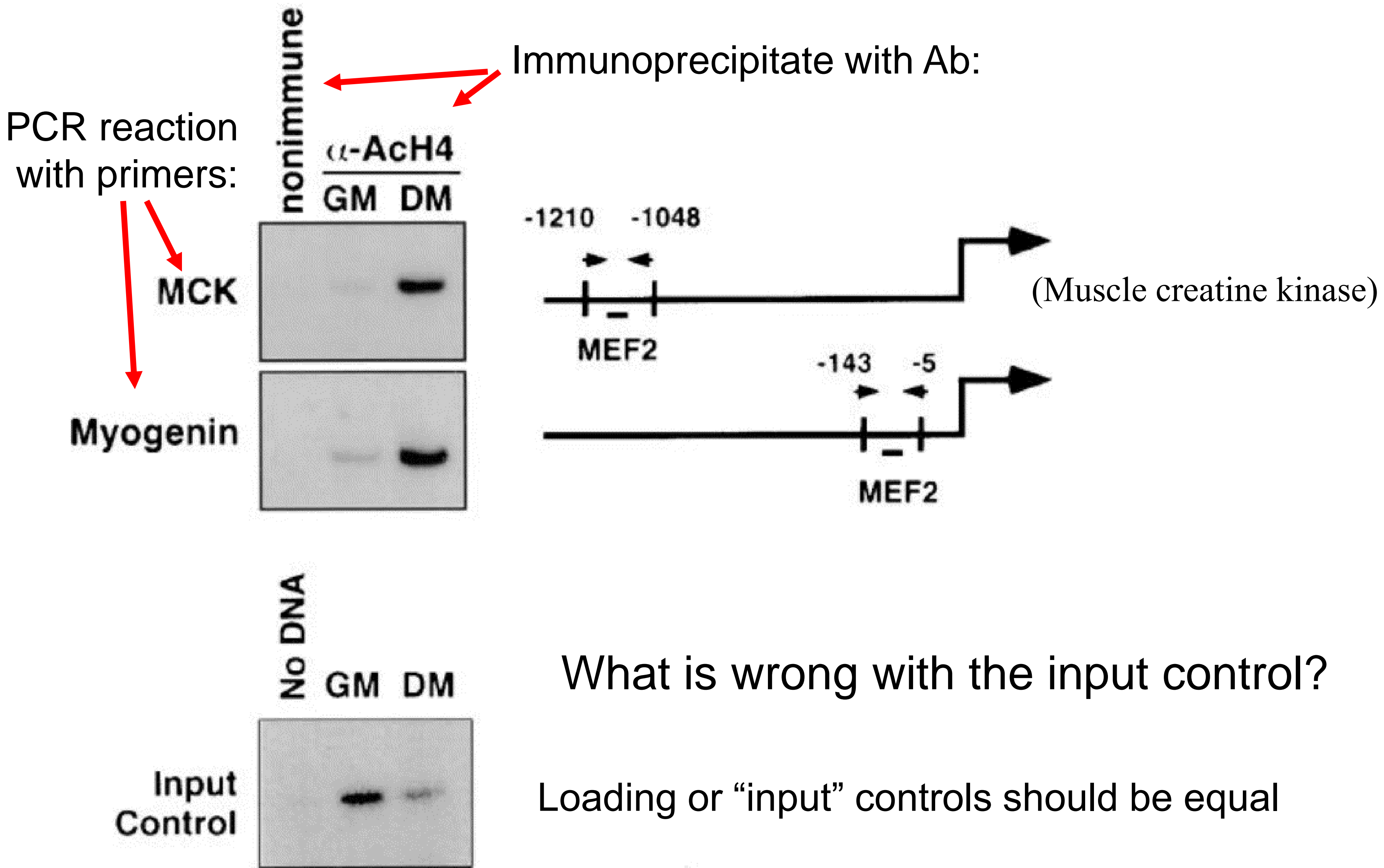
How to make antibodies:

1. Take blood from a rabbit before immunization. This is called pre-immune or nonimmune serum.
2. Inject the antigen into the rabbit and wait a few weeks. Inject again and wait.
3. Test the rabbit blood for antibodies against the antigen. Repeat injection if necessary.

Figure 2A: Histone H4 is acetylated at MEF2 target sites during skeletal myogenesis

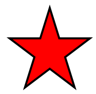


★Figure 2A: Histone H4 is acetylated at MEF2 target sites during skeletal myogenesis

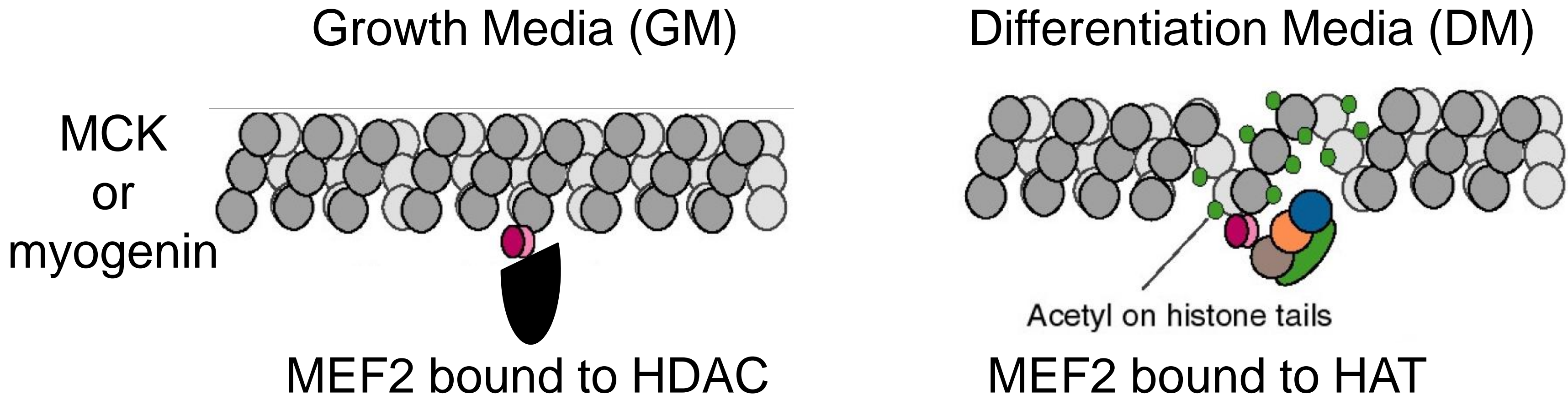


What is wrong with the input control?

Loading or “input” controls should be equal



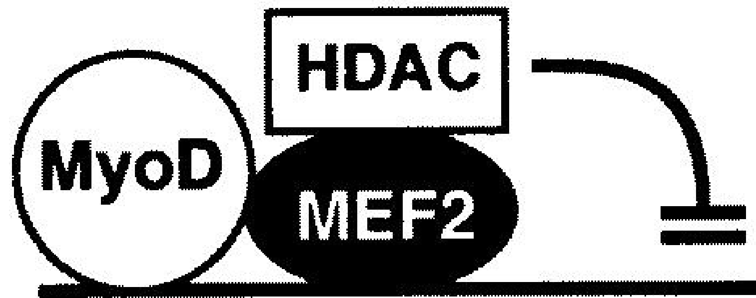
The data is consistent with the following model:

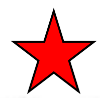


Chromatin around MEF2 sites in MCK or myogenin promoters is closed (unacetylated) in growing C2 myoblasts and open during differentiation of myoblasts into muscle.

Can HDAC4/5 overexpression inhibit myogenesis in myoblasts?

Make stable cell lines overexpressing HDAC: Use mouse C2 myoblasts and human HDAC cDNA





wild-type | HDAC4 (#2) | HDAC5 (#6)

Days in DM: 0 1 3 5 0 1 3 5 0 1 3 5

α-skeletal actin

MCK

Myogenin

MyoD

MEF2C

L7

HDAC4

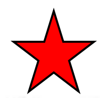
HDAC5

FIG. 2B RT-PCR:Overexpress HDAC4 and HDAC5 in C2 myoblast cells.

Found:

- Late differentiation markers, Muscle Creatine Kinase and Myosin Heavy Chain (data not shown), missing
- Early markers, myogenin and α-skeletal actin, down-regulated
- MEF2C, a late differentiation marker, was lost
- MyoD still expressed
- Endogenous HDAC4 and 5 expression is constant

Therefore, HDAC4 and 5 overexpression inhibits myogenesis



	wild-type				HDAC4 (#2)				HDAC5 (#6)			
Days in DM:	0	1	3	5	0	1	3	5	0	1	3	5

α-skeletal actin

MCK

Myogenin

MyoD

MEF2C

L7

HDAC4

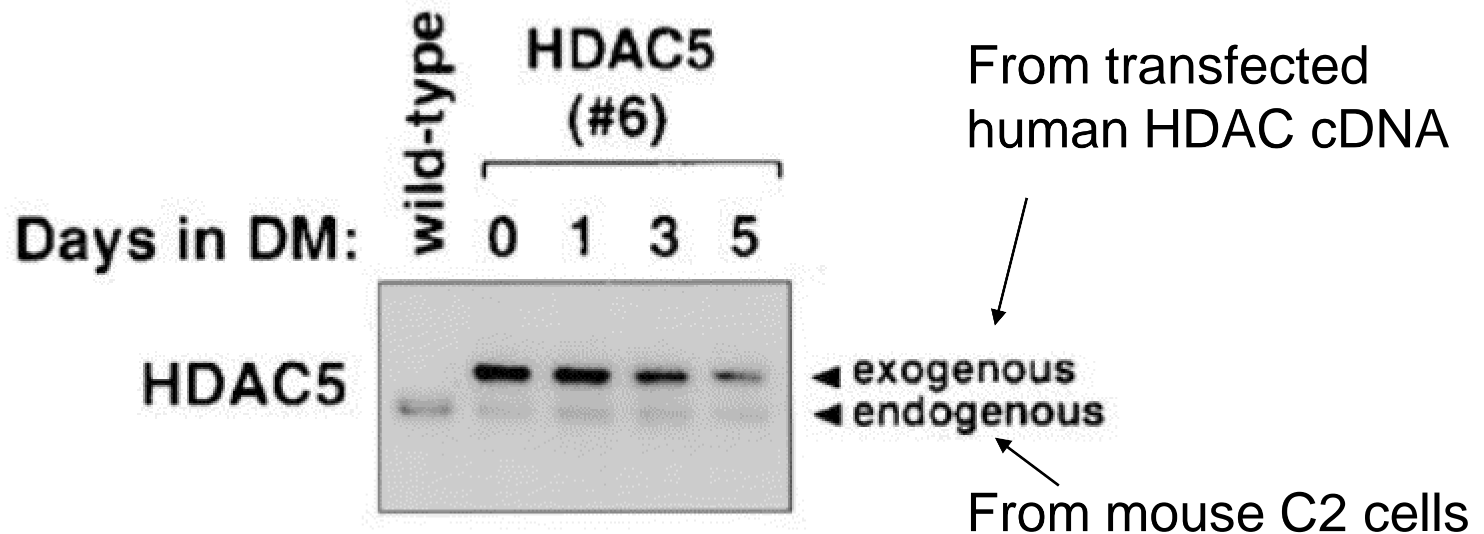
HDAC5

FIG. 2B RT-PCR:Overexpress HDAC4 and HDAC5 in C2 myoblast cells.

What is missing from this figure?

FIG. 2C Semi-quantitation of the levels of exogenous HDAC5

Used RT-PCR to distinguish between the endogenous (mouse) and exogenous (human) HDAC5 transcripts.

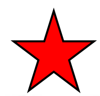


Found:
Exogenous levels were 4 times higher than endogenous levels

Is this enough of a difference to cause the inhibition of myogenesis?

Problems with overexpression:

High levels of exogenous protein may bind to protein/DNA that the endogenous protein would not bind to. This would lead to an "artifact" and not a true representation of the endogenous protein function.



wild-type HDAC4 (#2) HDAC5 (#6)

Days in DM: 0 1 3 5 0 1 3 5 0 1 3 5

α-skeletal actin

MCK

Myogenin

MyoD

MEF2C

L7

HDAC4

HDAC5

FIG. 2B RT-PCR:Overexpress HDAC4 and HDAC5 in C2 myoblast cells.

What is confusing about these results?

How can MEF2C regulate MyoD function before it is expressed?

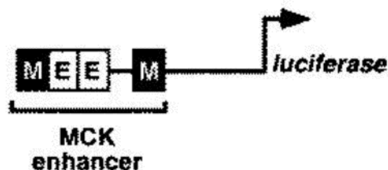
1. Low levels of MEF2C are present
2. Another MEF2 factor is present and inhibits MyoD function

Can HDAC inhibit MyoD and/or MEF2 activity in a promoter assay?

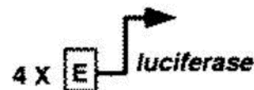
Promoter Assay

- Transiently transfect the reporter plasmid, with and without MyoD, MEF2C, or HDAC4/5
- Look for luciferase activity

1. MCK enhancer has both MEF2 and E-Box sites:



2. 4XE promoter has only E-box sites:

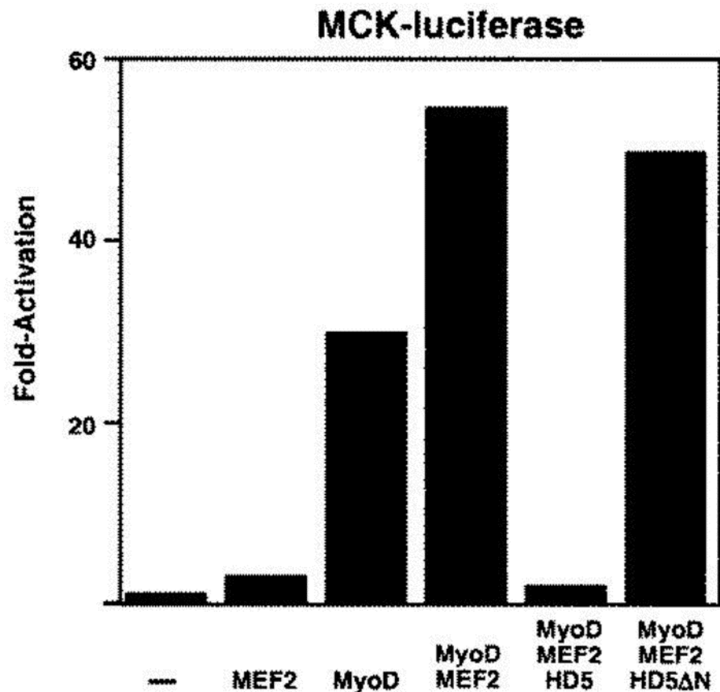


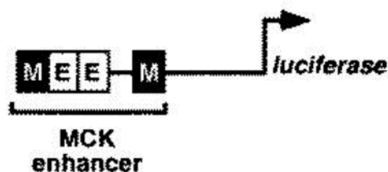
3. 3XMEF2 promoter has only MEF2 sites:

3XMEF2-luciferase

FIG. 3 HDAC5 Selectively Inhibits MyoD-Dependent Promoters that Contain MEF2 sites

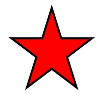
Promoter analysis in 10T1/2 fibroblast cells



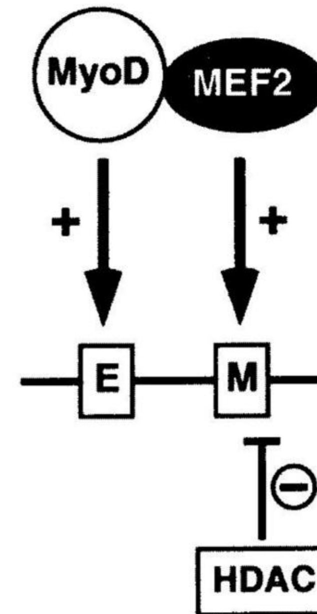


Found:

- HDACs 4 and 5 completely inhibited activation of the MCK enhancer by MyoD
- This inhibition requires the N-terminus of HDAC5 (the MEF2 binding site)



This data is consistent with the following model:



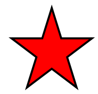
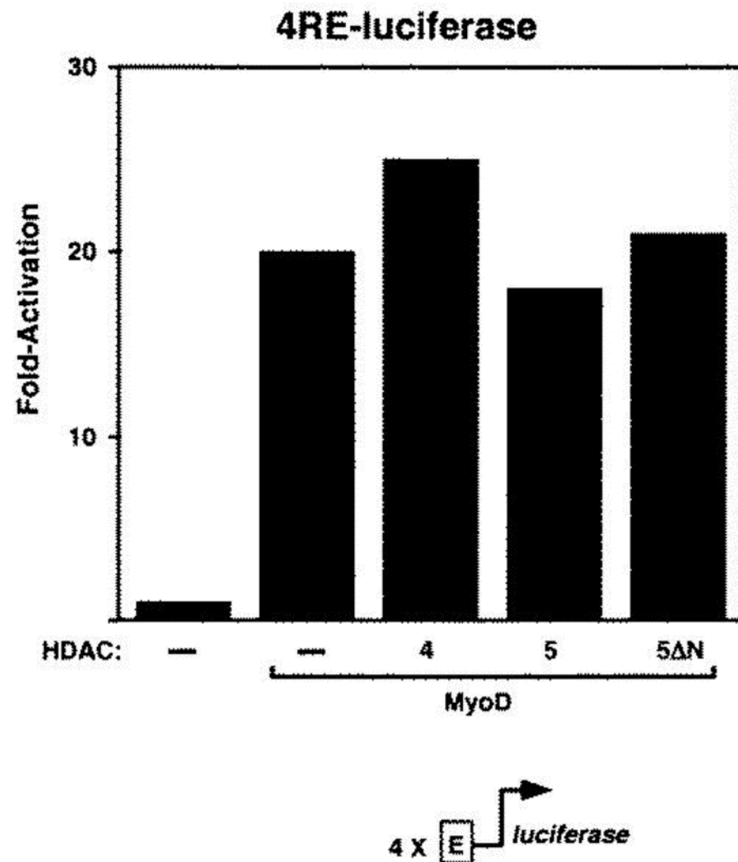


FIG. 3 HDAC5 Selectively Inhibits MyoD-Dependent Promoters that Contain MEF2 sites

Promoter analysis in 10T1/2 fibroblast cells

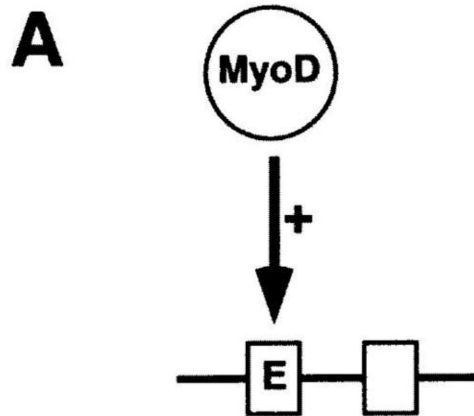


Found:

- HDACs 4 and 5 were unable to inhibit the ability of MyoD to activate a reporter construct in the absence of MEF2 sites.

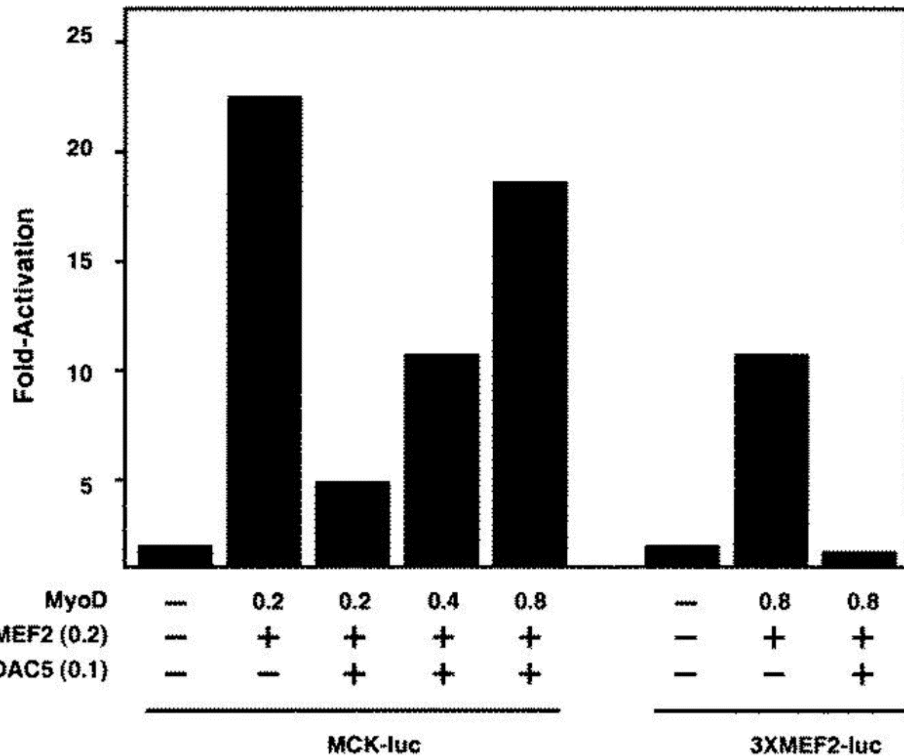
Therefore, the repression of MyoD-dependent transcription requires MEF2 sites, that recruit a MEF2-HDAC complex.

This data is consistent with the following model:



MyoD-dependent
MEF2-independent
HDAC-insensitive

Fig. 3C Excess MyoD can relieve the inhibitory activity of HDAC5 on the MCK enhancer



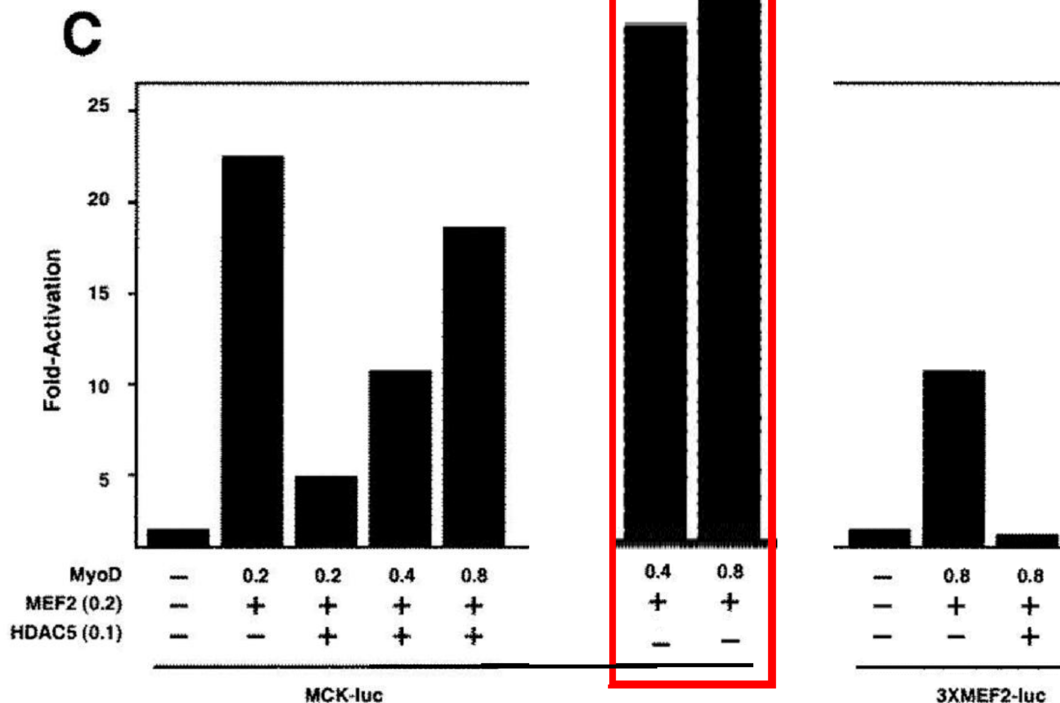
Found:

- High levels of MyoD could bypass the inhibition of HDAC5 on the MCK enhancer.
- High levels of MyoD could not bypass the inhibition of HDAC5 on the 3XMEF2-luc, containing 3 tandem MEF2 sites.

Therefore, MyoD can selectively overcome the inhibitory effects of HDACs on promoters containing both MyoD and MEF2 binding sites, but not on promoters containing only MEF2 sites.

Fig. 3C Excess MyoD can relieve the inhibitory activity of HDAC5 on the MCK enhancer

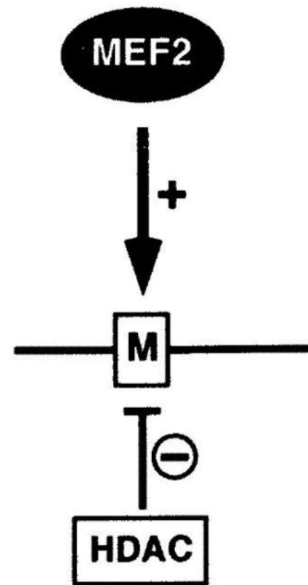
What is missing?



What if they would have included the proper controls? Would this be the data they found?

Therefore this paper does not provide convincing evidence that excess MyoD can relieve HDAC inhibition.

This data is consistent with the following model:



Can HDAC inhibit in transient promoter assays?

Yes: Histones are still present but the nucleosomes are unorganized

What part of MEF2 interacts with HDAC 4/5?

Fig. 4 Mapping residues in the MADS/MEF2 domains of MEF2C required for binding HDAC

(Note: exact amino acids are confusing between text and figure)

A

MADS Box

MEF2 Domain

Data from:

this paper

earlier paper

10 20 30 40 50 60 70 80

MADS Box

MEF2 Domain

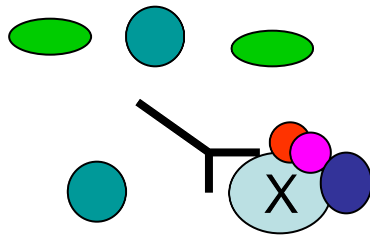
MEF2C MGRKKIQITRIMDERNRQVTFTKRKFGLMKKAYELSVLCDCEIALIIFNSTNKLFQYASTDMDKVLLKYTEYNEPHESRTNSDIVE

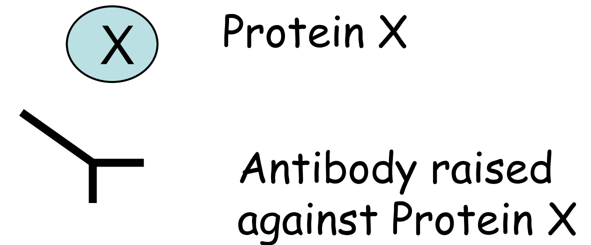
	HDAC-4 Binding	DNA Binding	Dimerization
MEF2C	+	+	+
K4S	+	+	+
RKK3-5TNQ	+	-	+
R10S	+	+	+
E14T	+	+	+
R15L	+	+	+
R17V	+	+	+
K25N	+	+	+
K30H	+	-	+
K31L	+	-	+
CDC39-41SDD	-	-	-
LI45, 46RN	-	-	-
IF47, 48DS	-	-	-
STDMD59-63RAVMH	-	-	+
VLL65-67ASR	-	+	+
KYTEY68-72ECNDN	-	-	+
NEPH73-76IAPL	+	+	+
ESRT77-80VNQA	+	+	+
Δ40-57	-	-	-
Δ58-87	-	-	-
Δ70-85	-	-	-

HDAC Binding Region

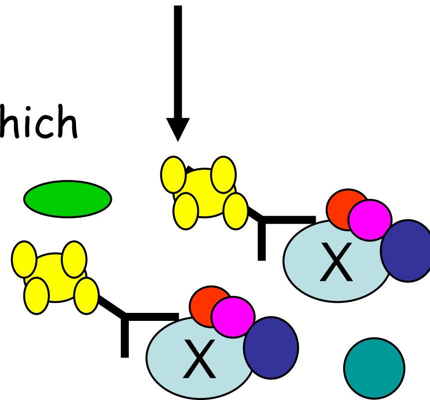
Detection of protein-protein interactions by co-immuno precipitation assay

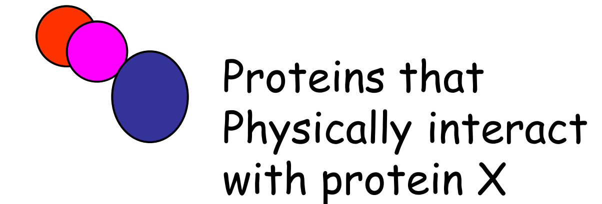
Incubate whole cell extract with antibody to protein X

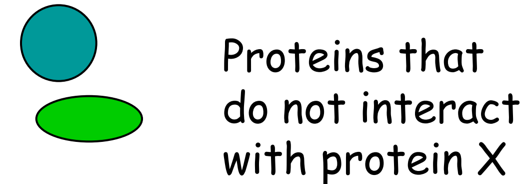




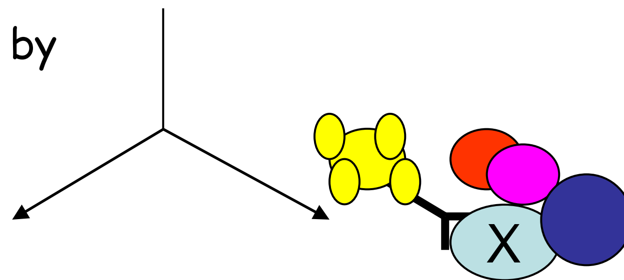
Incubate extract with Protein-A-Sepharose, which binds antibody







Collect Sepharose beads by centrifugation

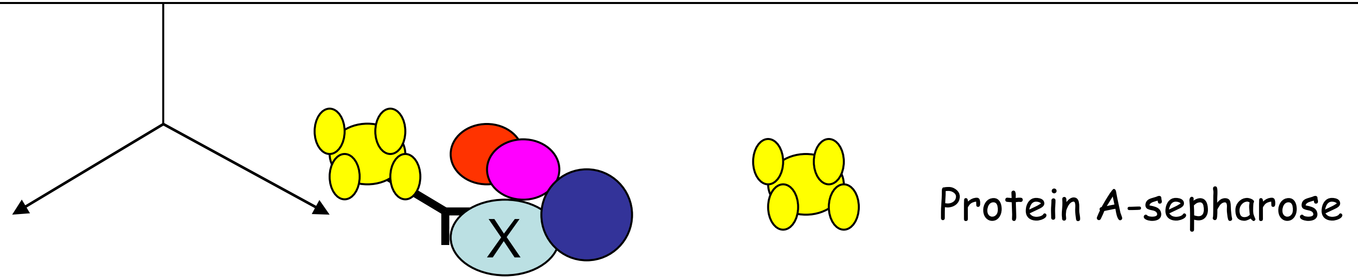


Discard supernatant

Wash pellet several times
Dissociate proteins from Protein-A Sepharose
Separate proteins by SDS Page

http://www.mcb.uct.ac.za/UG%20courses/mcb300a/lect8-pres.ppt#280,4,Slide 4

Detection of protein-protein interactions by co-immuno precipitation assay



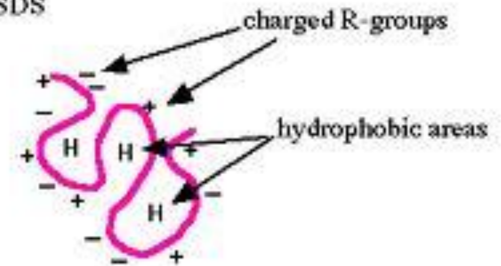
Discard supernatant

Wash pellet several times
Dissociate proteins from Protein-A Sepharose
Separate proteins by SDS Page

How does SDS-PAGE separate proteins?

SDS-PAGE: Separates proteins by size because the proteins are denatured by SDS = sodium dodecyl sulfate

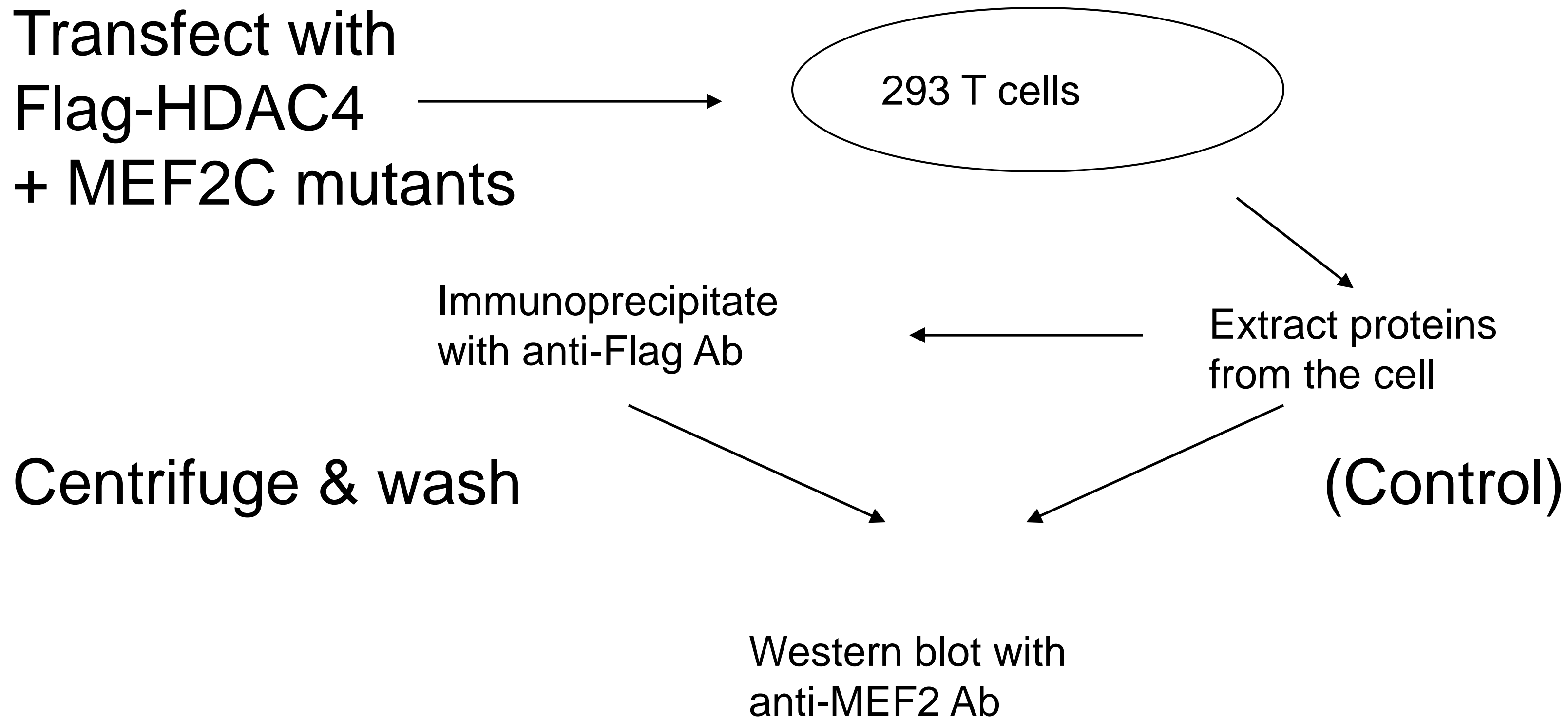
SDS has what charge? Negative!



http://www.mcb.uct.ac.za/UG%20courses/mcb300a/lect8-pres.ppt#280,4,Slide 4

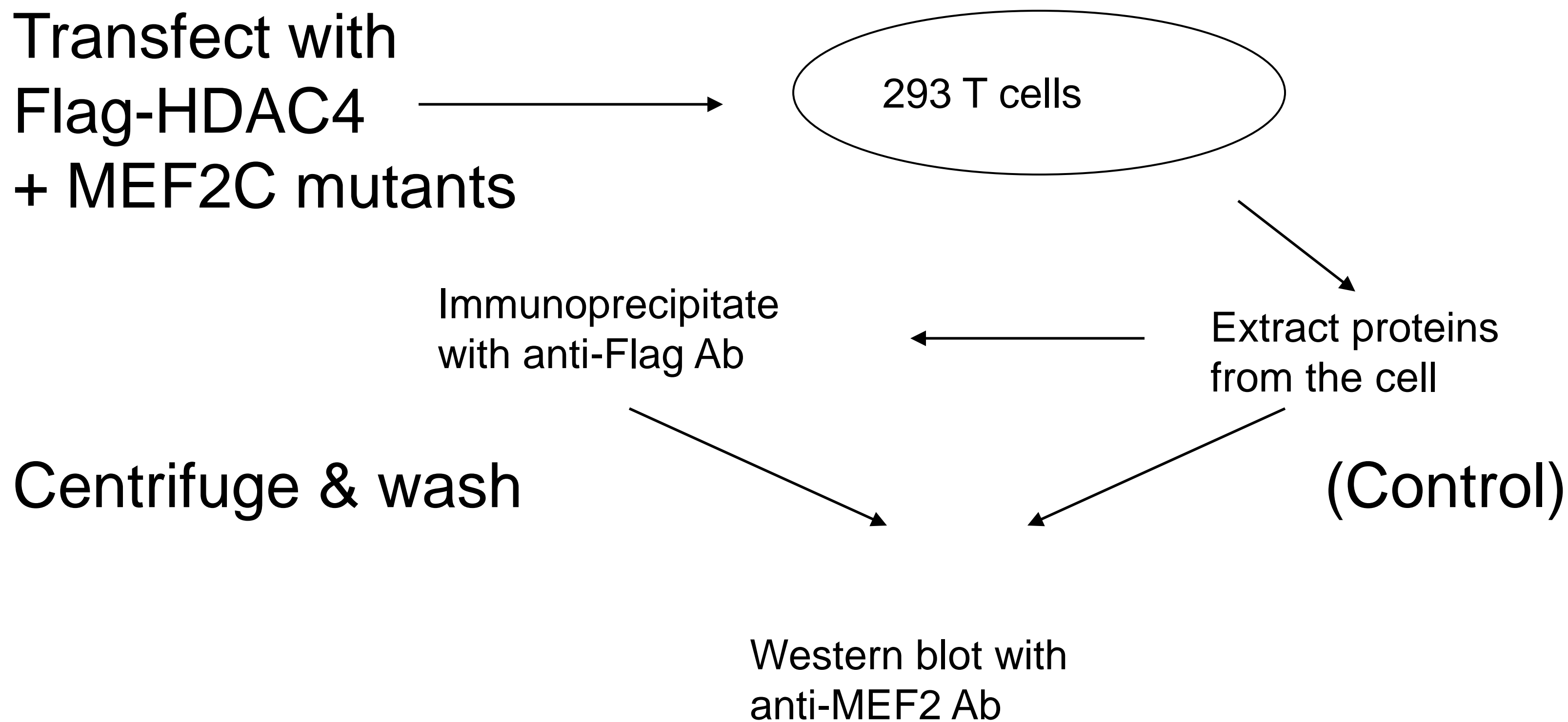
Fig. 4B Co-immunoprecipitation of HDAC4 and MEF2C mutants

HEK293T cells: Transformed Human Embryonic Kidney cells



Which control is missing?

Fig. 4B Co-immunoprecipitation of HDAC4 and MEF2C mutants

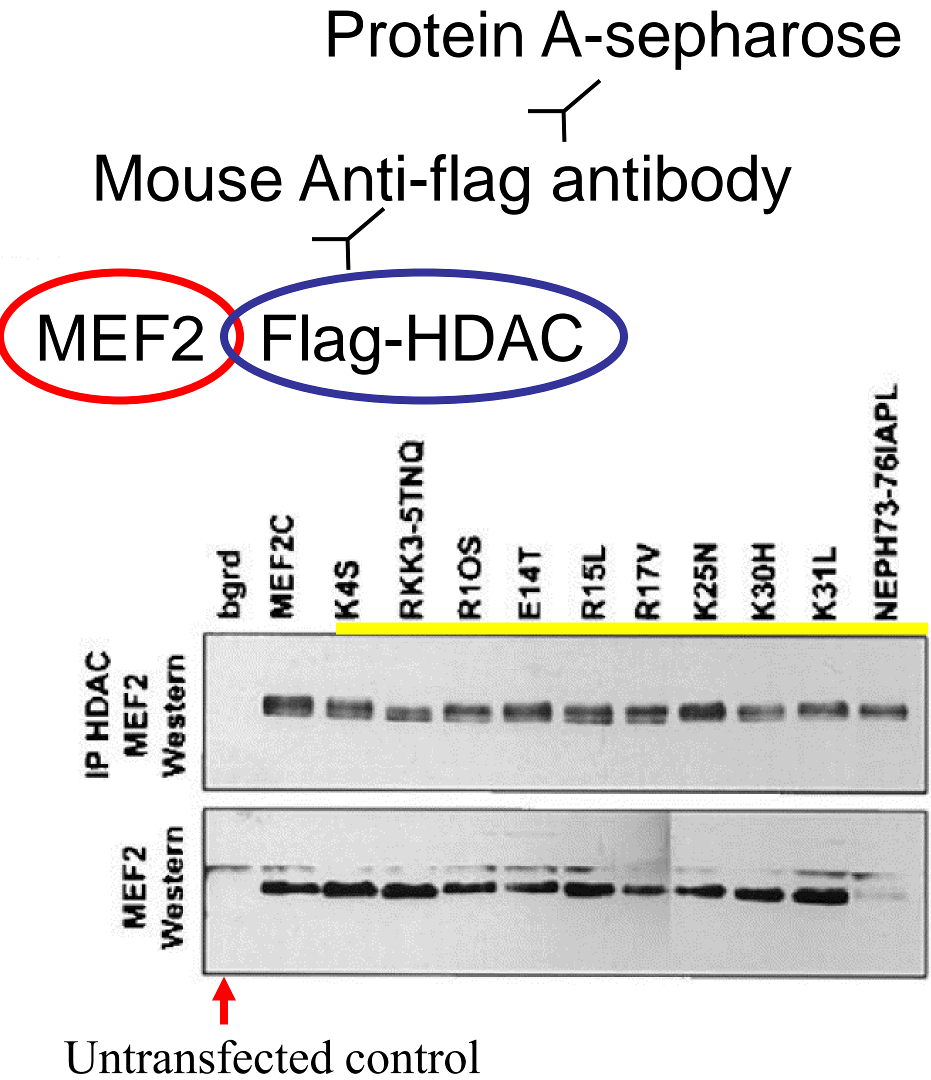


Which control is missing?

Either: 1) Cells transfected with MEF2C and not Flag-HDAC4

2) Immunoprecipitate with sepharose beads alone

★ Fig. 4B. Co-immunoprecipitation of HDAC4 and MEF2C mutants.



Found:

- Mutation in the first 31 amino acids of MEF2C had no effect on HDAC4 binding (not 25 as in text)
- Therefore, the HDAC binding region is between 31 and 86 aa.
- Switched to an in vitro system because other mutants couldn't be detected after transfection in cell culture.

How to find protein-protein interactions: *GST Affinity Chromatography*

Genes of interest can be cloned in frame with glutathione-S-transferase (GST) to create a GST:fusion protein

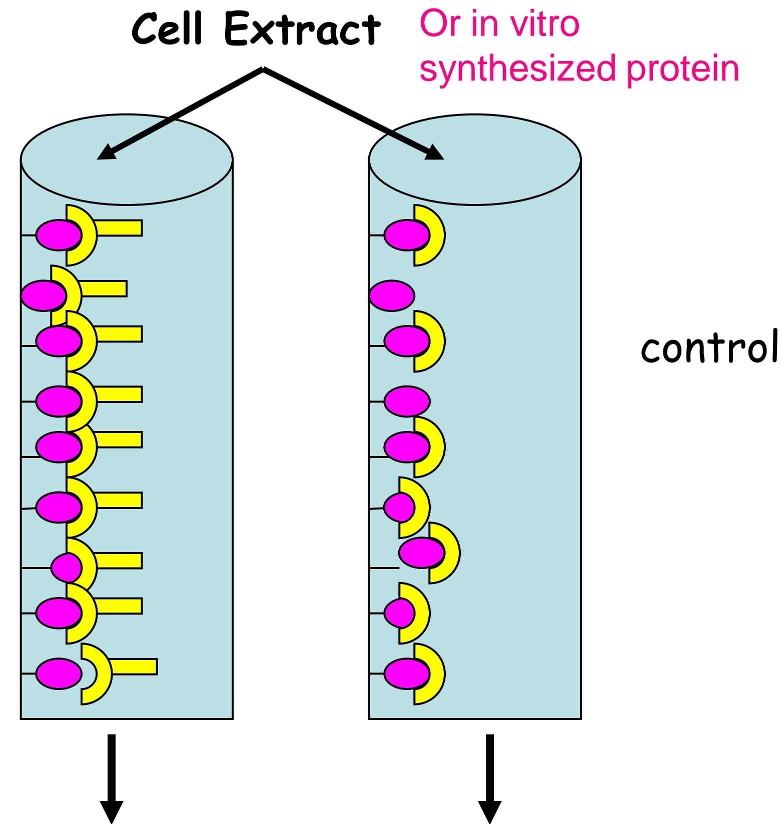
GST binds to glutathione with high affinity

Agarose bead with attached glutathione

GST:fusion protein (Made in bacteria)

GST (Made in bacteria)

1) Flowthrough
2) Elution with bound protein using salt
3) Analysis of eluted protein

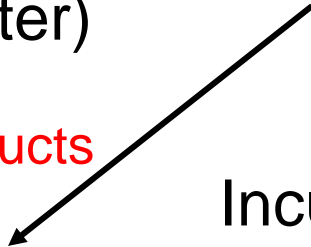


GST fusion protein can be eluted from agarose beads by incubation with excess reduced glutathione.

Fig. 4C. Binding of MEF2C mutants to GST-HDAC4 *in vitro*

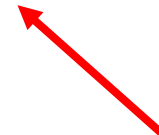
In vitro transcription of MEF2C and mutants (using strong viral promoter)

In vitro translation with ^{35}S-met (using reticulocyte lysate)



Input control or translation products

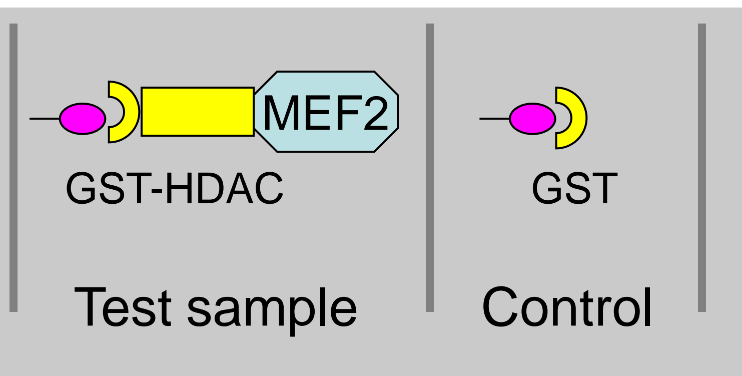
Incubate with unlabelled GST-HDAC4 OR GST alone bound to glutathione-agarose beads (GST = Glutathione S-transferase)



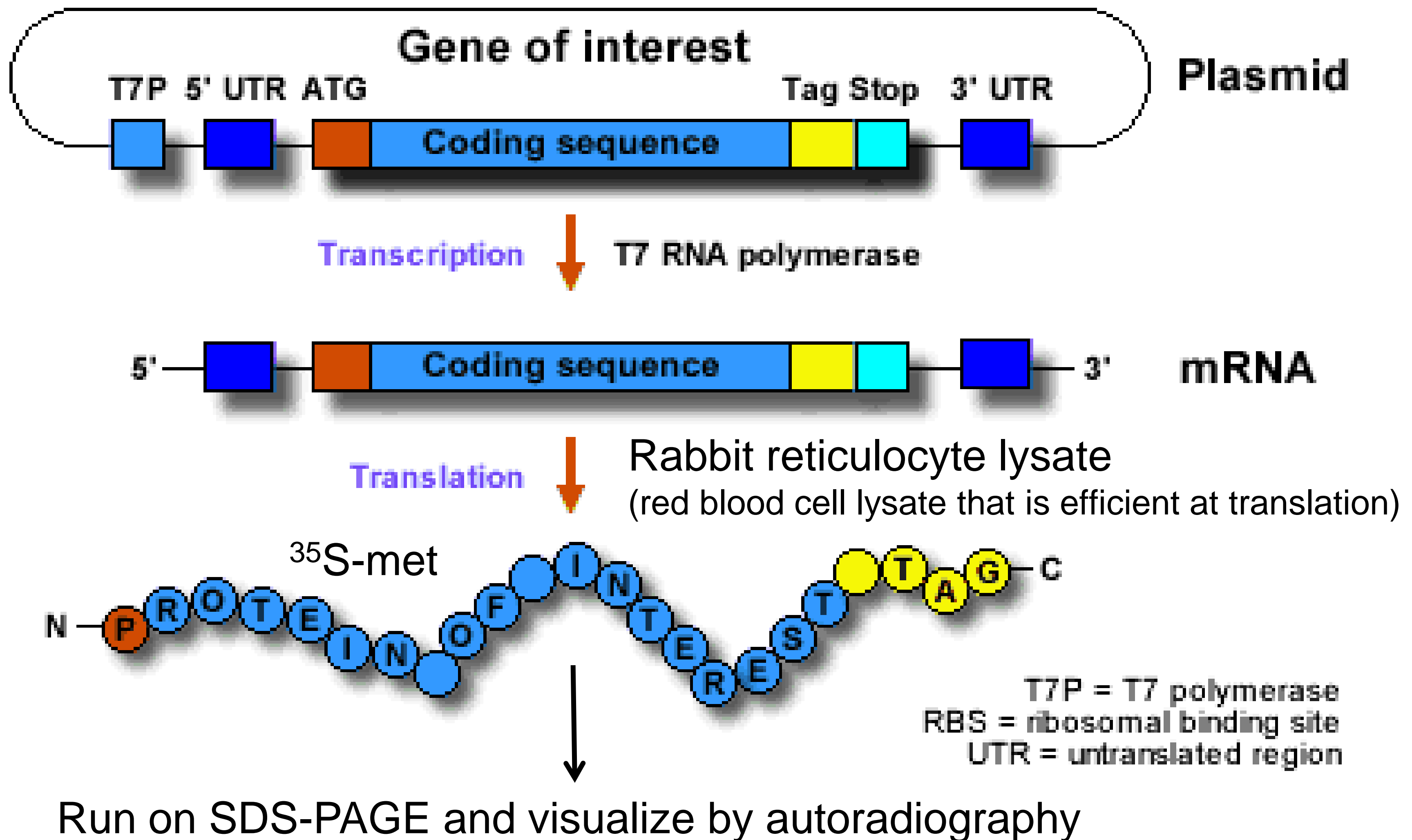
Usually from bacterial purification

Wash and separate by SDS-PAGE

Visualize by autoradiography



In vitro Transcription/Translation



Run on SDS-PAGE and visualize by autoradiography

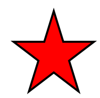


Fig. 4C – Deletion of residues 39-67 of MEF2C prevented interaction with GST-HDAC4

SRF=MADS box factor (negative control)

MEF2C
1-117
Δ40-57
Δ58-87
Δ70-85
D-MEF2
SRF
CDC39-41SDD
LI45, 46RN
IF47, 48DS
STDMD59-63RAVMH
VLL65-67ASR
KYTEY68-72ECNDH
NEPH73-76IAPL
ESRT77-80VNQA
HDAC4

+GST-HDAC4
+GST
Translation Products

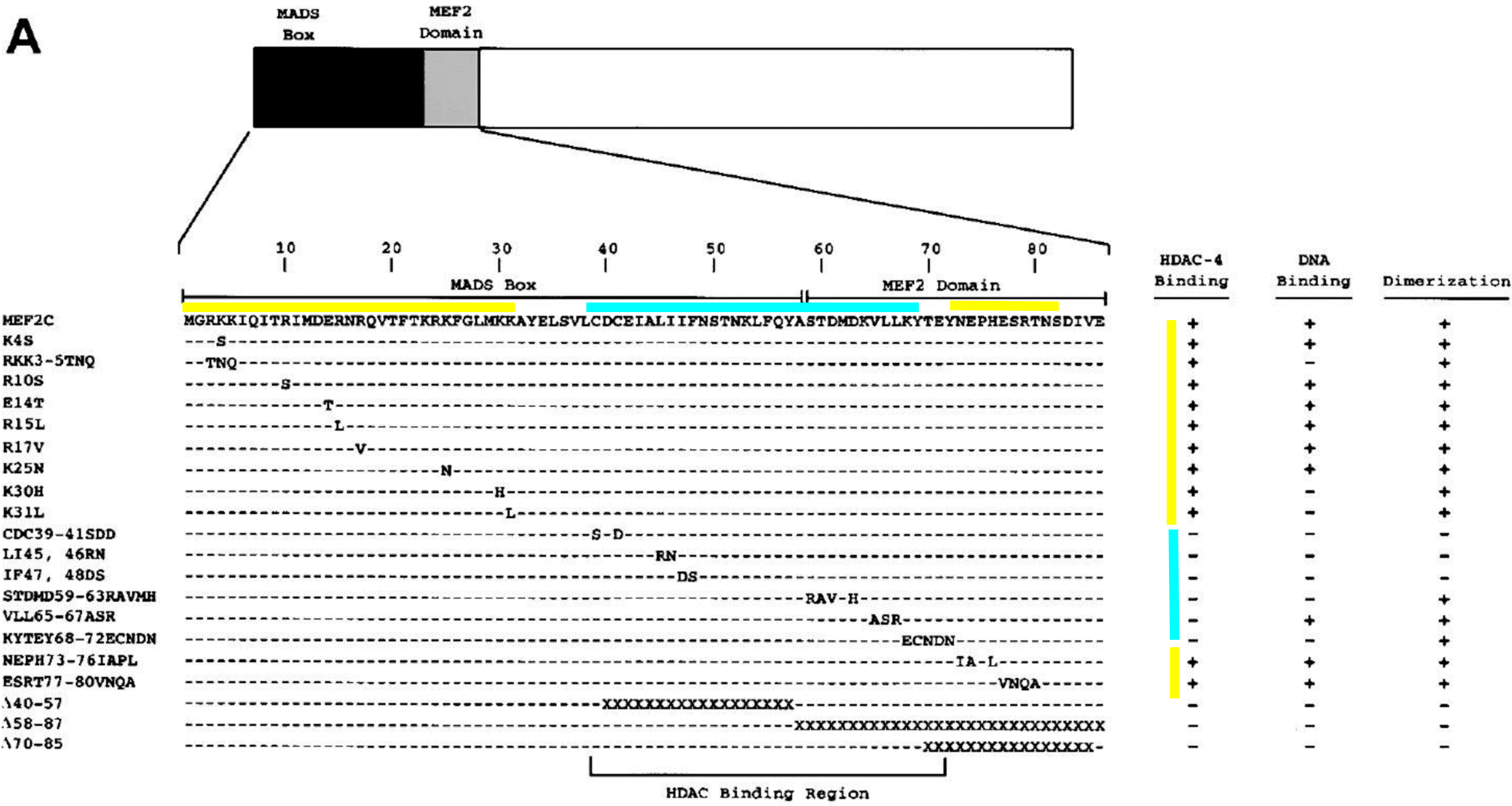
+GST-MEF2C(1-86)
+GST
Translation Products

(autoradiography after SDS-PAGE)

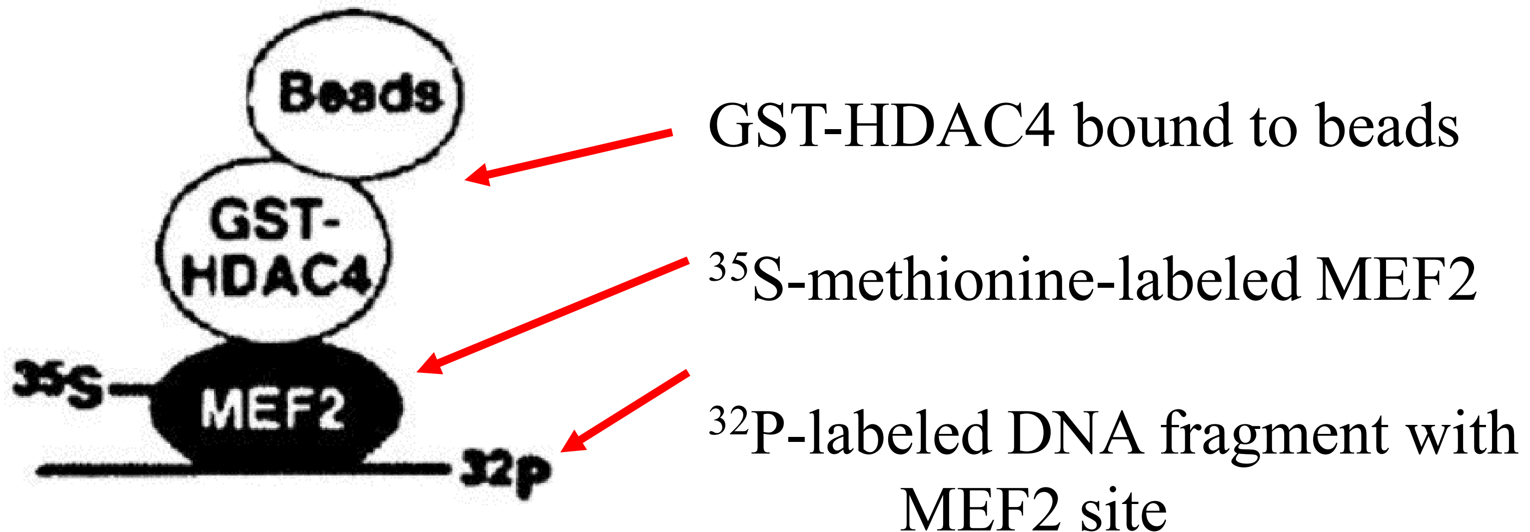
Found:

- Residues 39-67 of MEF2C are required for the interaction with HDAC4 but residues 68-80 are not.
- SRF does not interact with HDAC4 and does not contain this conserved region

Fig. 4 Mapping residues in the MADS/MEF2 domains of MEF2C required for binding HDAC



Can MEF2 bind HDACs and DNA at the same time?



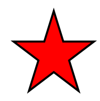


Figure 5 Simultaneous binding of MEF2 to DNA and HDACs

A

^{35}S (cpm x 10^3)

50 100 150 200

^{35}S

^{32}P

+GST-HDAC4

SRF

L145I, 46RN

RKK3-5TNQ

MEF2C

+GST

MEF2C

200 400 600 800 1000 1200

^{32}P (cpm)

Beads

GST-HDAC4

^{35}S

MEF2

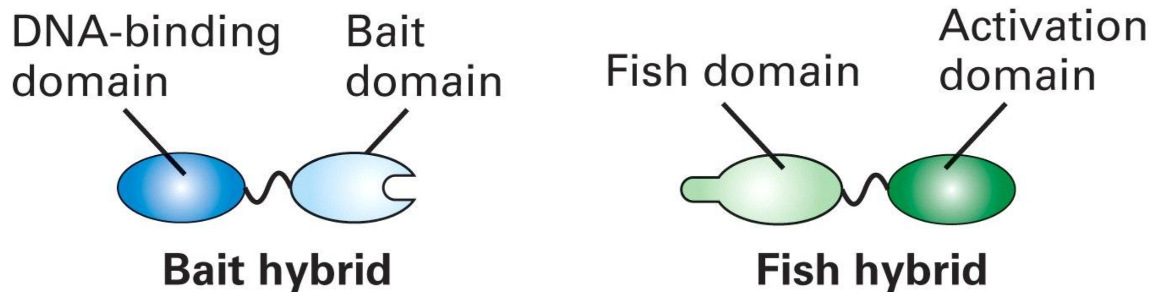
^{32}P

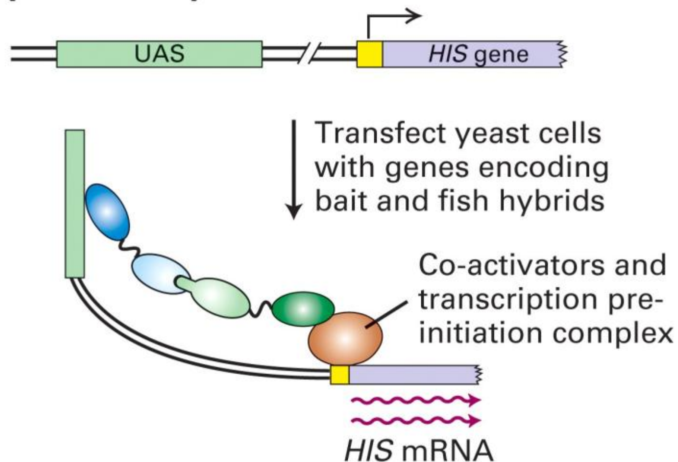
Found:

- GST-HDAC4 interacted with [^{35}S]-labeled MEF2C bound to its [^{32}P]-labeled target site.
- RKK3-5TNQ binds HDAC in vivo but not DNA (control expt.)
- LI45,46RN and SRF can't interact with GST-HDAC (control expt.)

How to find interactions:

Transcription factors have 2 essential domains: the DNA-binding domain and the activation domain.





The DNA-binding domain and the activation domain can activate transcription from two separate proteins that can bind to each other. This property is important for identifying protein-protein interactions

Fig 11.39 Lodish et al. Molecular Cell Biology

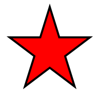
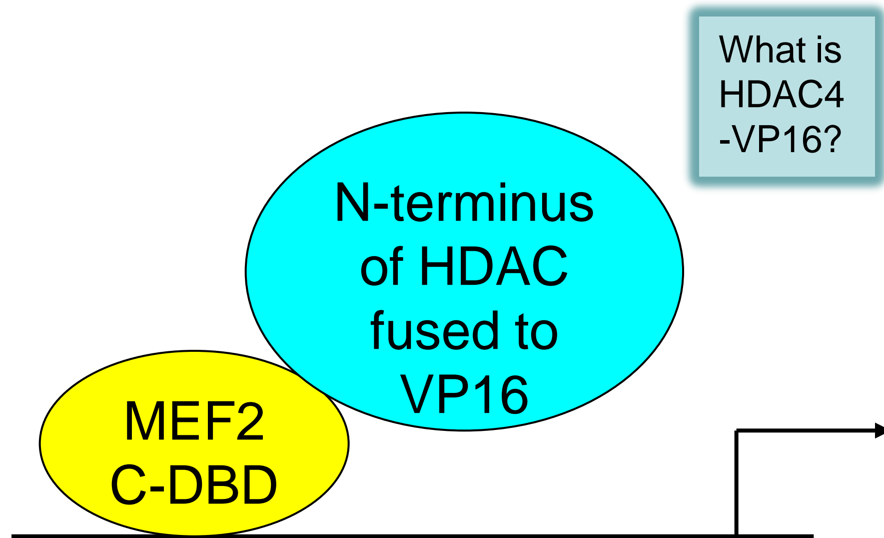
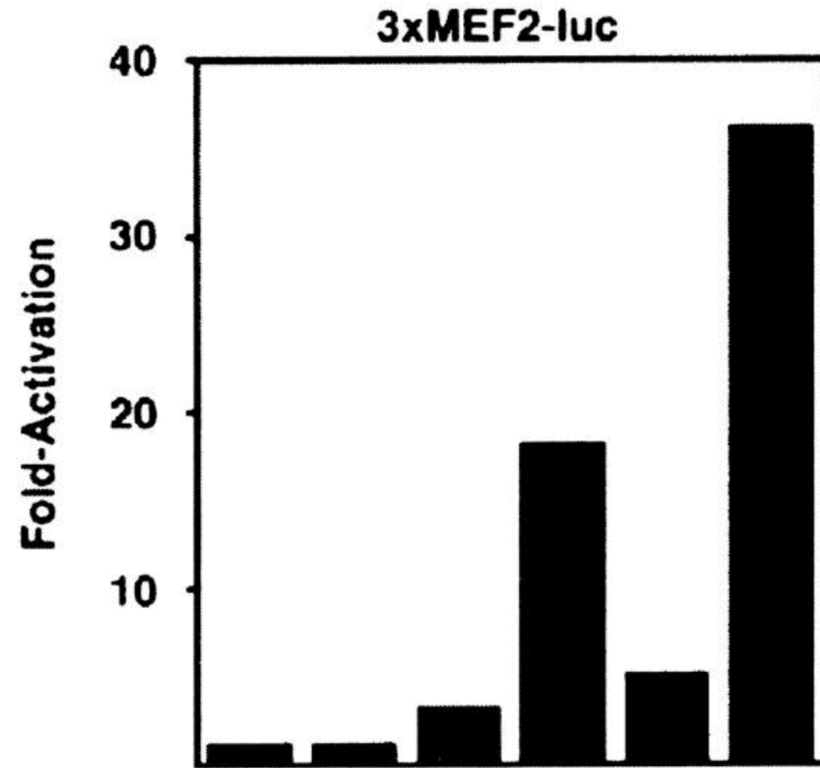


FIG. 5B. Modified one-hybrid assay

Reporter assay in 10T1/2 cells





Therefore, interaction of MEF2 with HDAC4 or 5 does not interfere with binding of MEF2 to DNA.

Summary of paper #1 so far:

1. Showed MyoD function was inhibited by HDAC on:
- endogenous promoters (C2 myoblasts and MyoD-transfected fibroblasts)
- exogenous promoters (reporter assays)

2. Showed MEF2 bound HDAC by MADS/MEF domain using:
- co-IP
- GST pull down,
- radioactive pull down
- yeast one-hybrid

Can CamK signaling relieve HDAC repression?

Hypothesis:

Class II HDACs will inhibit myogenesis by binding to MEF2C, in a CaMK-dependent fashion

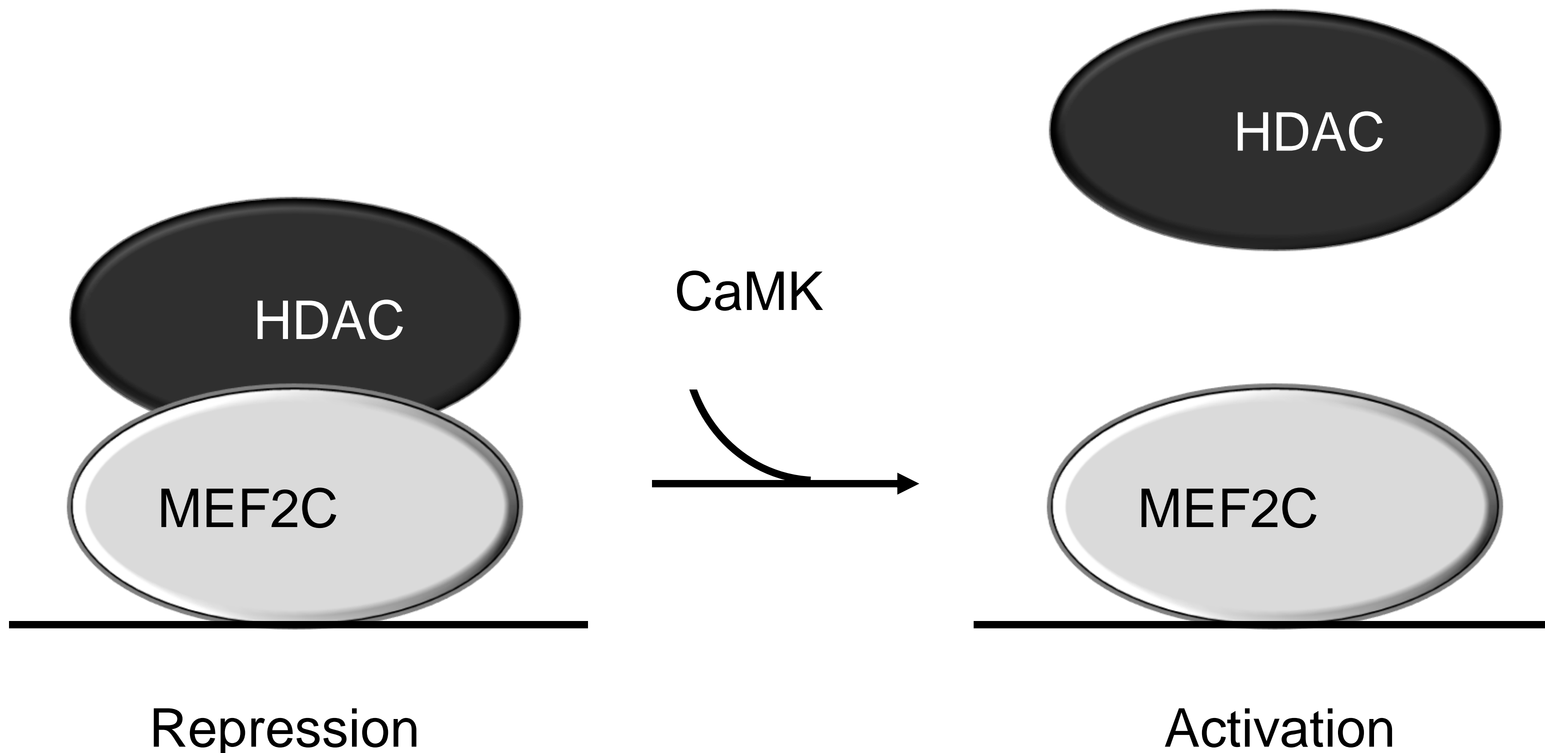
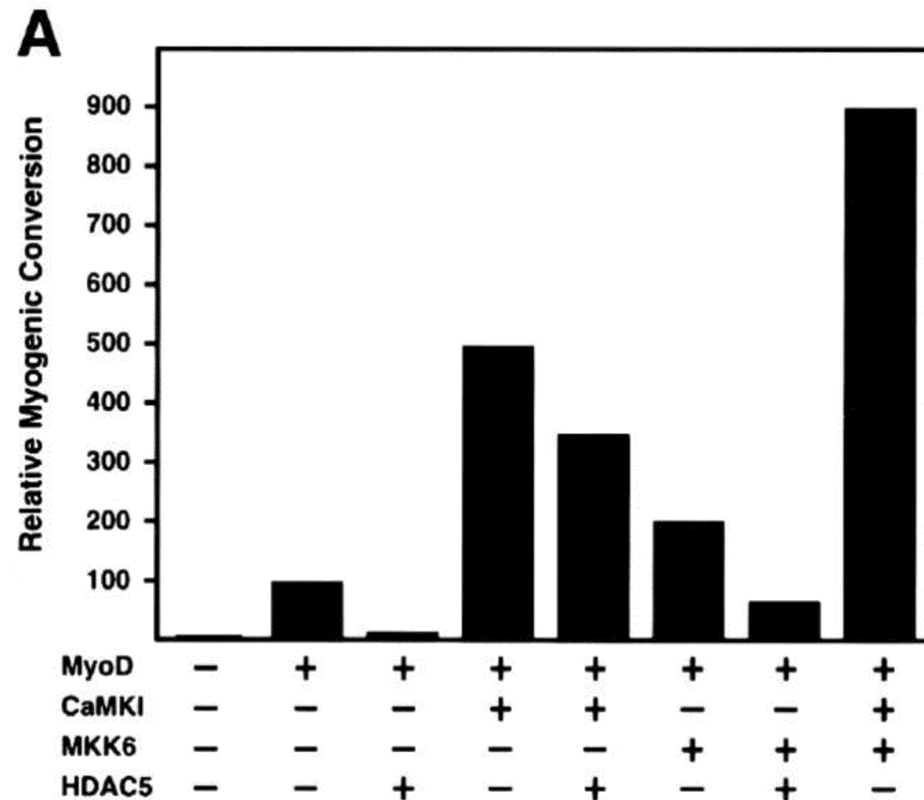


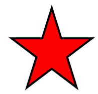
Fig. 6A CaMK signaling overcomes HDAC-Mediated repression of MyoD activity

Myogenic conversion assay with CaMKI and MKK6 (which activates p38 kinase, which phosphorylates and activates MEF2C)



Found:

- MyoD was able to convert fibroblasts to muscle in the presence of activated CaMKI and HDAC
- MyoD was not able to efficiently convert fibroblasts to muscle in the presence of activated MKK6 and HDAC
- The ability of MyoD to induce myogenesis was synergistically activated by CaMKI and MKK6



This data is consistent with the following model:

Myoblasts

HDAC

MyoD

MEF2

Repression of Muscle Genes

CaMK

HDAC

Myotubes

MAPK (p38)

P

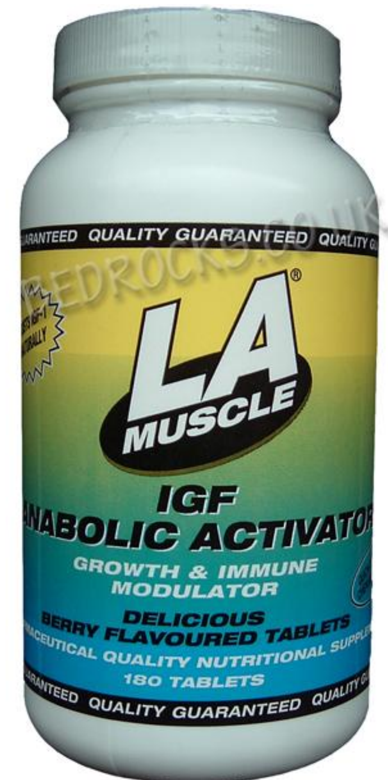
MyoD

MEF2

Activation of Muscle Genes

Are Insulin-like growth factors (IGFs) involved?

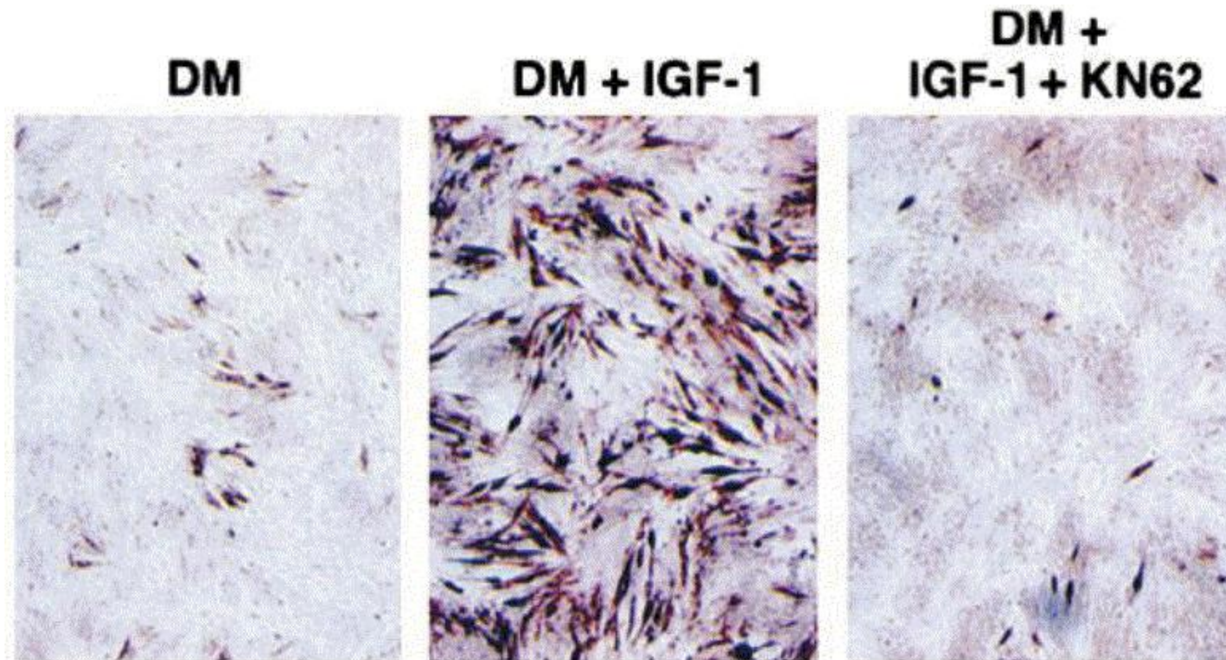
IGF-1 (Insulin-like growth factor) stimulates skeletal muscle growth (hypertrophy) and activates intracellular Ca^{2+} signaling.



★ Fig. 6B: IGF-1 signaling overcomes HDAC-mediated repression of MyoD activity

Is CaMK needed for IGF-1 to enhance myogenesis? Used a CamK inhibitor, KN62.

Rat L6 myoblasts stained with immunoperoxidase system/anti-MHC antibody



Found:
IGF-1 dramatically stimulated differentiation of L6 myoblasts – this was lost in the presence of a CaMK inhibitor, KN62.

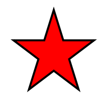
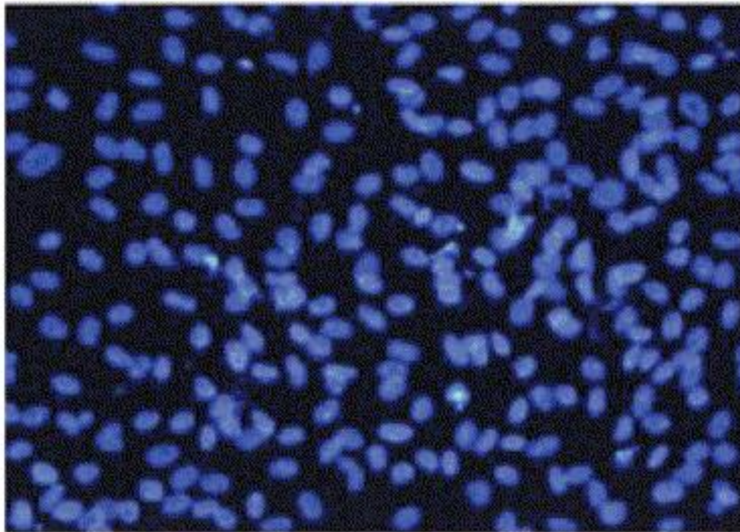
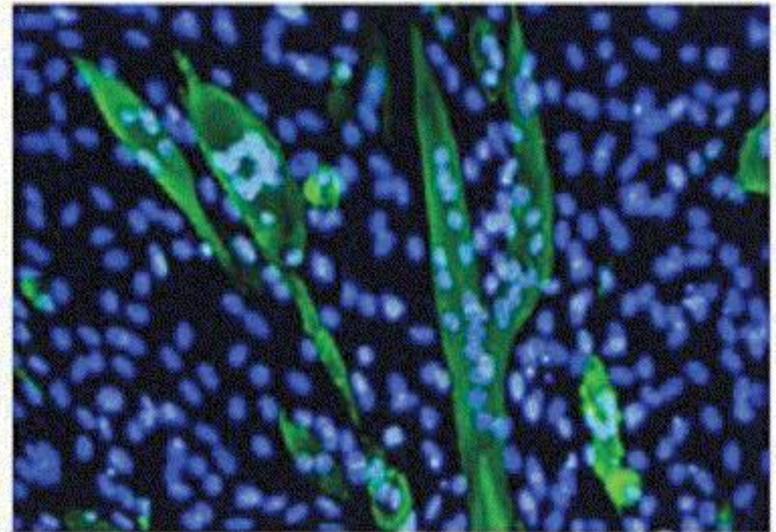


Fig. 6C: Can IGF-1 override the HDAC inhibition?

HDAC-4 transfected C2 myoblasts:
- Immunofluorescence with MHC (green) and nuclear stain(blue)

DM | DM + IGF-1





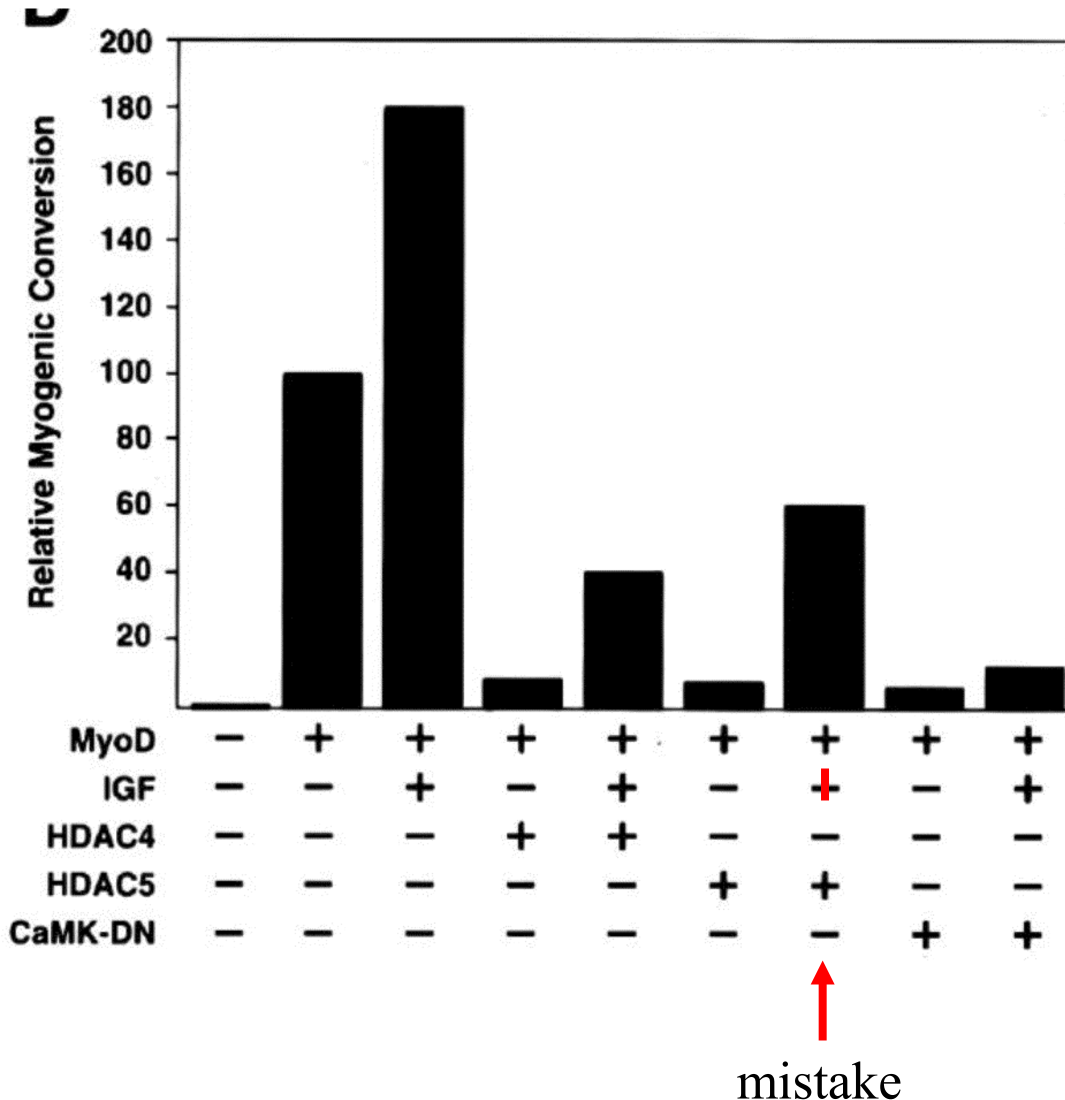
Found:

- IGF-1 could reverse the inhibition by HDAC-4 overexpression

Can a dominant negative CamK inhibit the stimulation of myogenesis by IGFs?

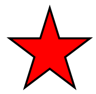
DN-CamK = catalytically inactive kinase, binds Ca^{2+} and calmodulin

Fig. 6D. A dominant negative CaMKIV can interfere with the stimulatory effect of IGF-1 on MyoD activity



Found:

- IGF-1 enhanced the ability of MyoD to activate myogenesis
- MyoD lost all myogenic activity in the presence of a dominant negative CaMK mutant
- IGF-1 could overcome the inhibition of MyoD by HDAC
- IGF-1 could not overcome the inhibition by CaMK-DN



The data is consistent with the following model:

Myoblasts

IGF

Myotubes

MAPK

HDAC

MyoD

MEF2

CaMK

P

MyoD

MEF2

Repression of Muscle Genes

Activation of Muscle Genes

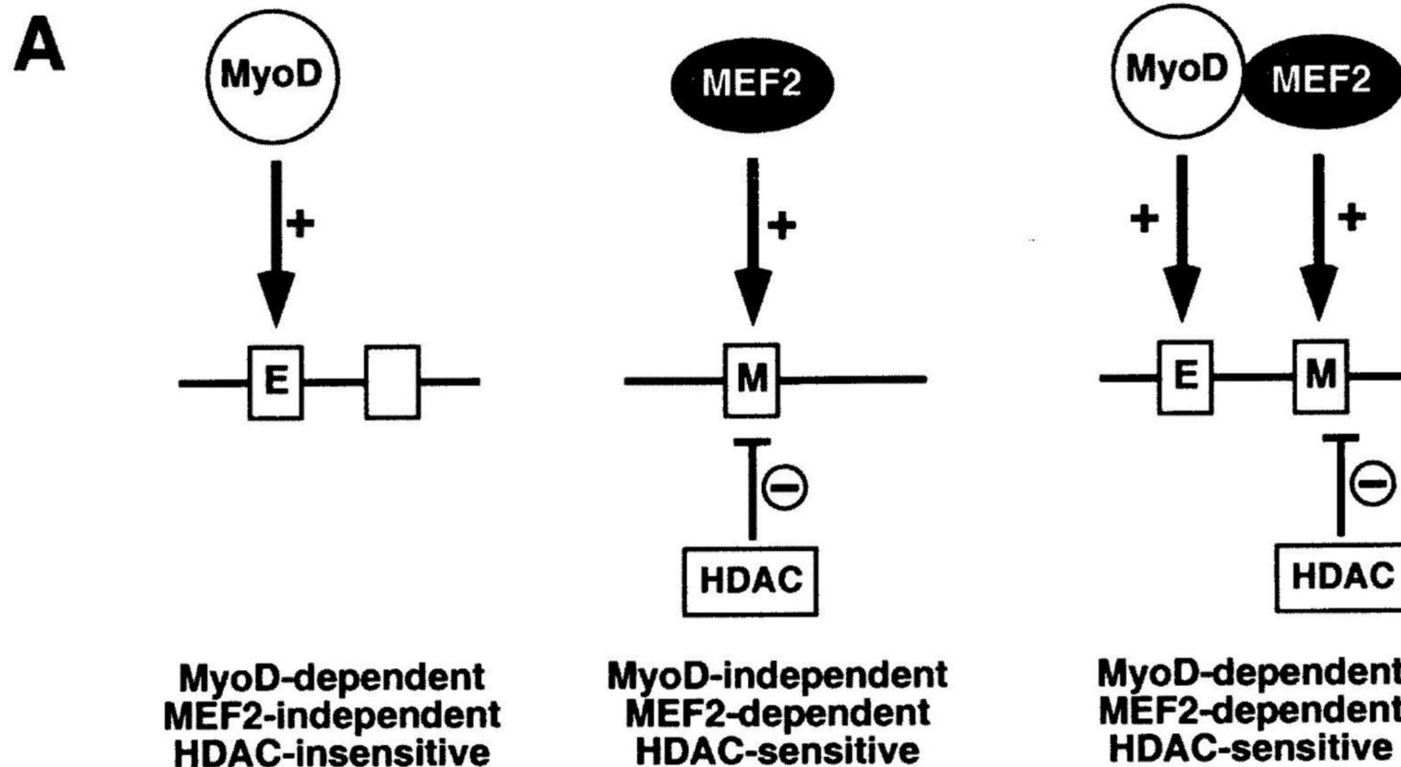
HDAC

IGF can relieve HDAC inhibition in:

1. L6 myoblasts
2. C2 myoblasts
3. A myogenic conversion assay

Fig. 6 Summary:
Therefore, CaMK activity is required for MyoD-mediated myogenesis and for IGF-mediated stimulation of myogenesis.

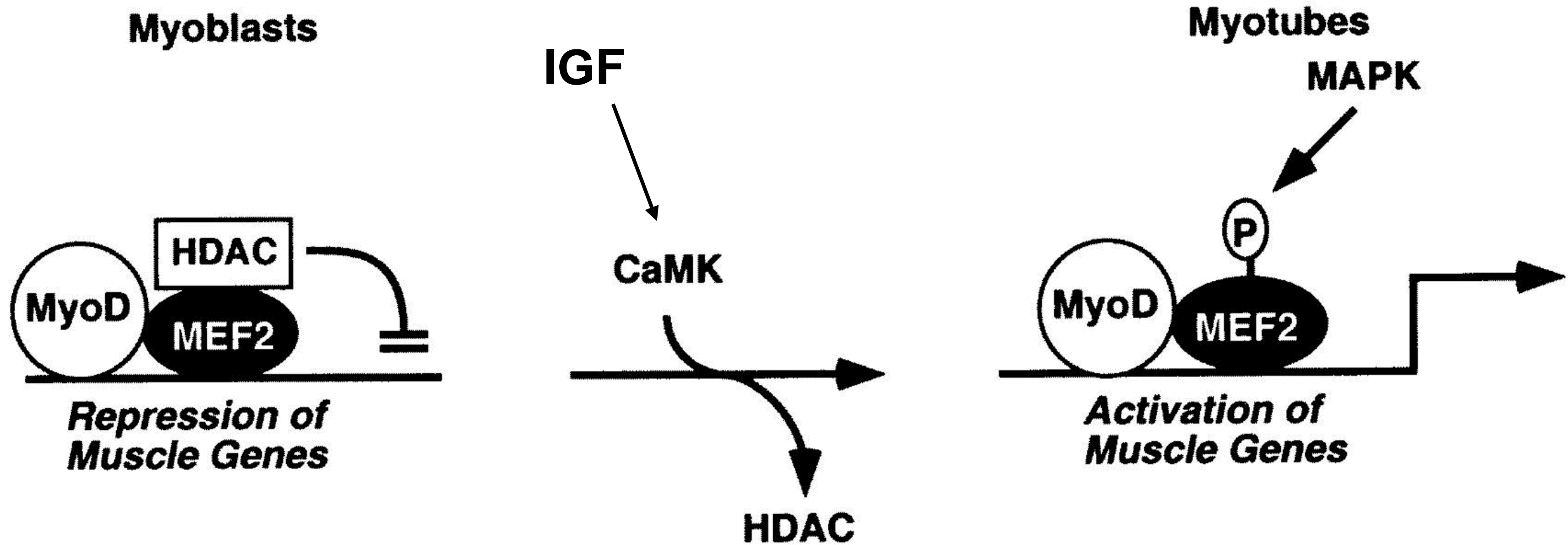
Fig. 7A: A Model for the Role of HDACs in Skeletal Myogenesis



Hypothesis:

A. There are three types of muscle-specific target genes, distinguished by their responsiveness to MyoD, MEF2, and HDAC.

Fig. 7B: A Model for the Role of HDACs in Skeletal Myogenesis



B. In myoblasts, HDAC binds MEF2 and represses muscle gene expression. CaMK signaling and MAPK (MKK6) signaling stimulate myogenesis by enhancing MEF2 activity (IGF is involved in activating CaMK).

Why is this paper important?

- It was the first observation that Class II HDACs could inhibit myogenesis
- It provided a mechanism by which this occurs: Direct binding to MEF2, which could be relieved by CamKinase or IGF signaling
- Understanding the mechanism of inhibition could lead to small molecule screens to reverse the inhibition and to use for therapies.

Did the approach of the paper convince you?

1. Showed MyoD function was inhibited by HDAC on:

- endogenous promoters (C2 myoblasts and MyoD-transfected fibroblasts)
- exogenous promoters (reporter assays)

2. Showed MEF2 bound HDAC by MADS/MEF domain using:

- co-IP
- GST pull down,
- radioactive pull down
- yeast one-hybrid

Did the approach of the paper convince you?

3. Showed CamK:

- Can override HDAC inhibition in a myogenic conversion assay,
- Inhibition can reverse IGF enhancement of L6 differentiation and myogenic conversion assay

4. Showed IGF can relieve HDAC inhibition in:

- L6 myoblasts,
- C2 myoblasts,
- A myogenic conversion assay

What were the problems with this paper?

- General lack of error bars and statistical analysis
- Some critical controls were missing