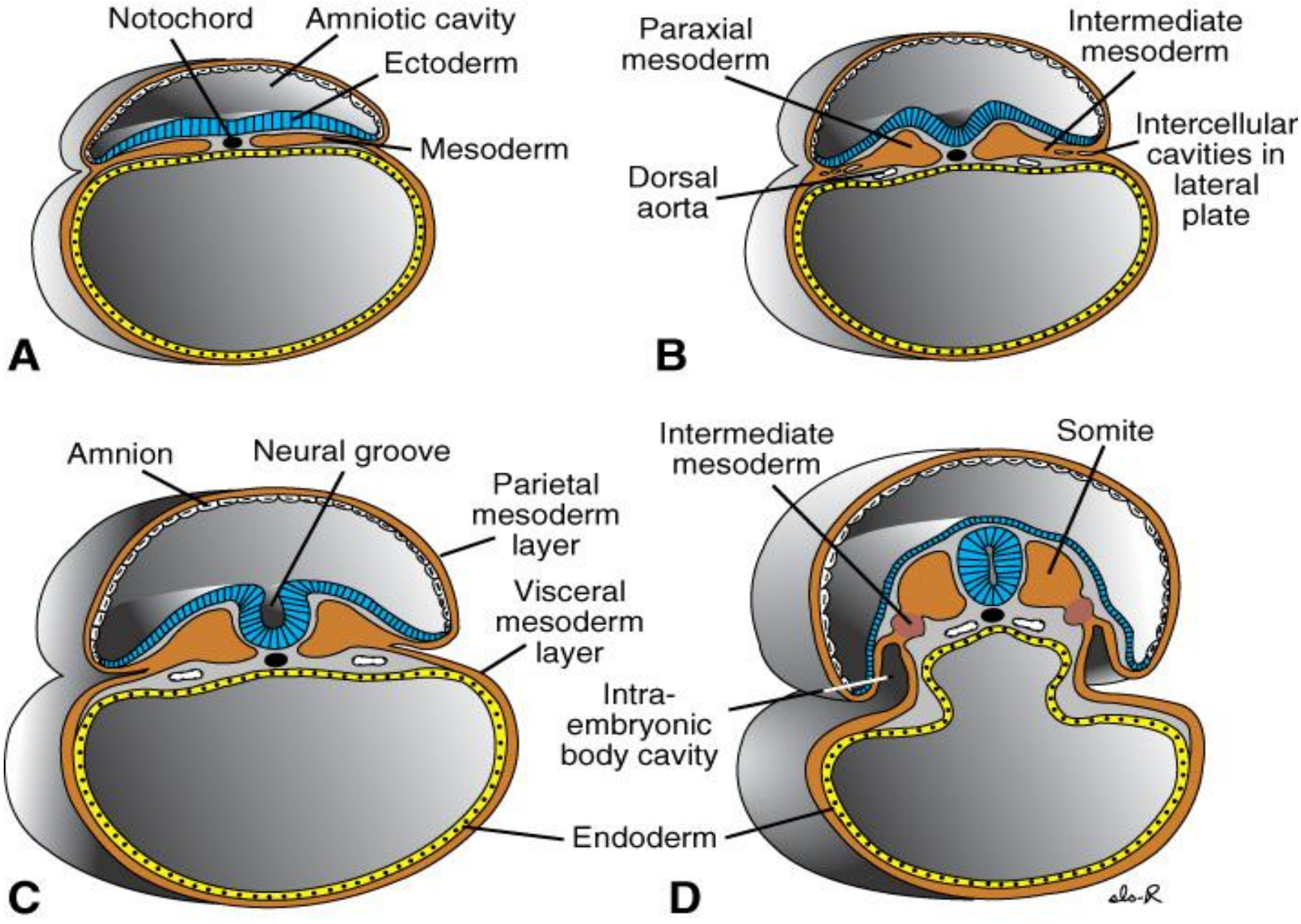
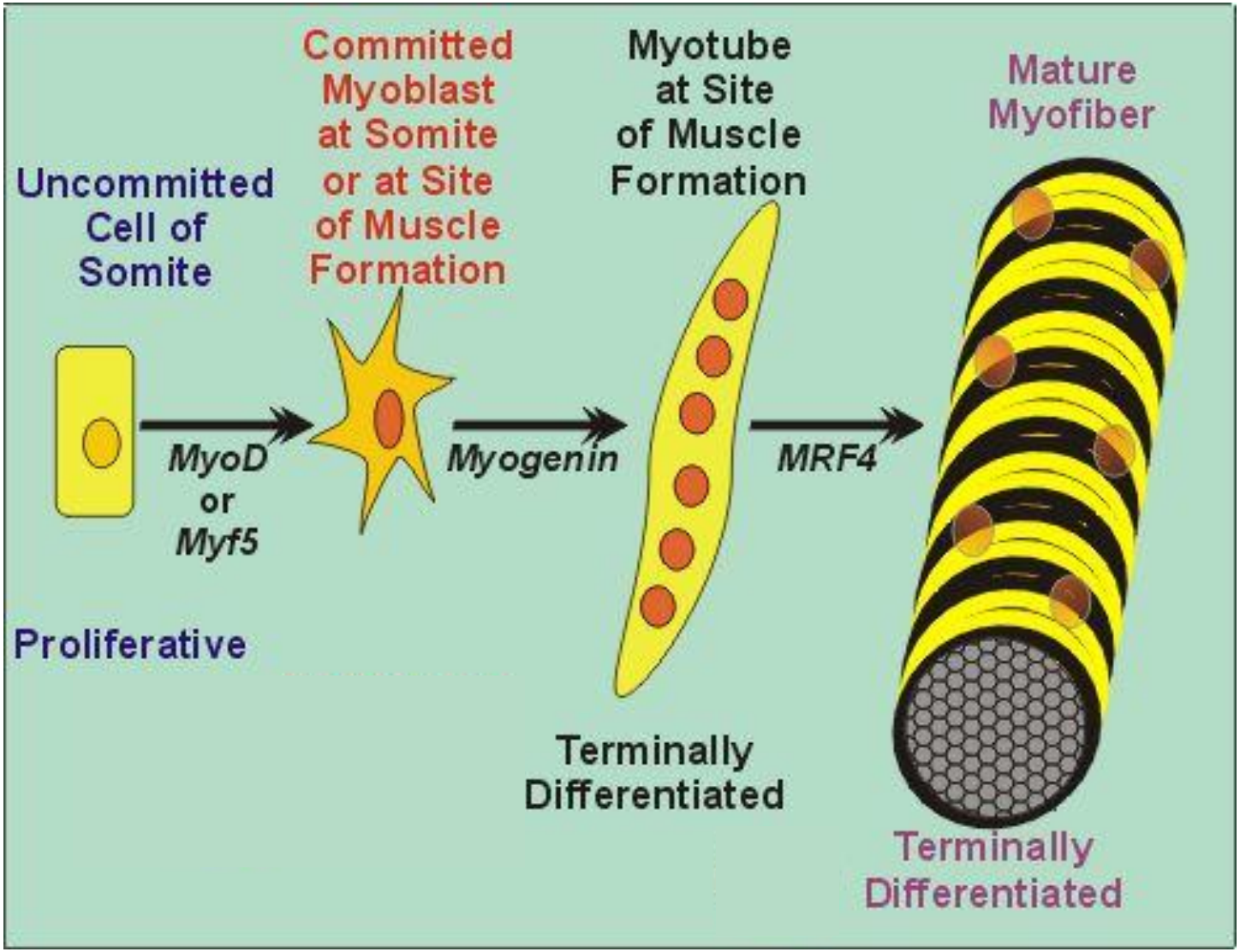
Bch 4122
Paper # 3: Skeletal Muscle Specification by myogenin and Mef2D via the SWI/SNF ATPase Brg1

Ohkawa Y., Marfella C., Imbalzano, A.
(2006). *EMBO Journal* **25**: 490-501.

(Impact Factor 8.3; 36 citations)


Notochord
Amniotic cavity
Ectoderm
Mesoderm
A
Paraxial mesoderm
Intermediate mesoderm
Intercellular cavities in lateral plate
Dorsal aorta
B
Amnion
Neural groove
Parietal mesoderm layer
Visceral mesoderm layer
Endoderm
C
Intermediate mesoderm
Somite
Intra-embryonic body cavity
D

Myogenic Regulatory Factors Control Myogenesis



MyoD and Myf-5

Knockout studies suggest functional redundancy among MyoD and Myf5:

Mice lacking MyoD = normal skeletal muscle

Mice lacking Myf5 = normal skeletal muscle

Mice lacking MyoD and Myf5 = no skeletal muscle

Therefore MyoD and Myf5 thought to be involved in the early events of myogenesis

(Simplified summary)

Method to create knockout mice

QuickTime™ and a
decompressor
are needed to see this picture.

Myogenin

Mice lacking Myogenin display normal myoblast formation but are unable to complete the later stages of skeletal muscle development

What is Myogenin’s role in differentiation?

MEF2D

Mef2D has a skeletal muscle specific isoform (Mef2D1b) and can accelerate muscle development in synergy with MyoD.

Mef2 can interact with HDACs and the components SWI/SNF chromatin remodeling enzyme.

The Question

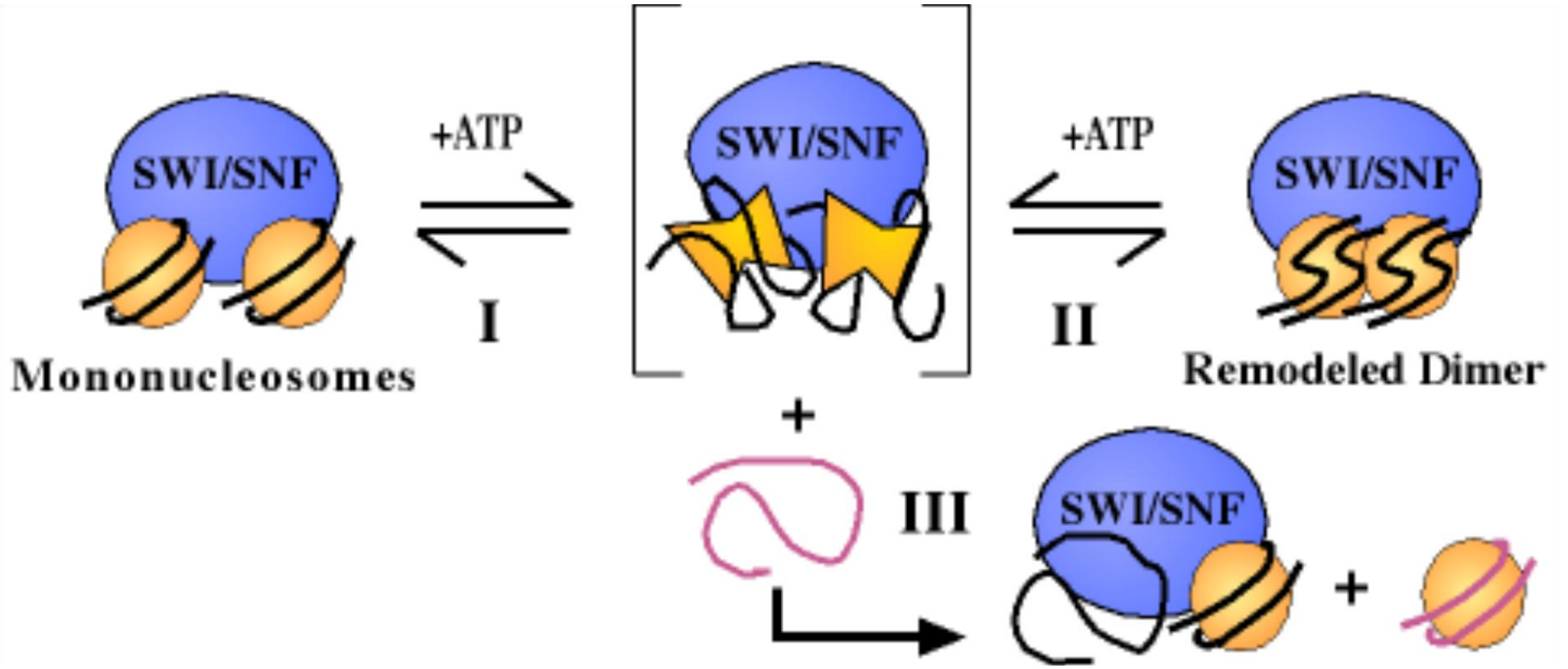
What is the relationship between muscle specific transcription factors (specifically Mef2D and myogenin) and chromatin remodeling-enzymes (SWI/SNF) in the activation of late myogenic genes?

The Hypothesis:

Brg1 function is essential for skeletal myogenesis to occur.

Chromatin remodeling ATPases catalyze stable alteration of the nucleosome

Brg1 = SWI/SNF in this paper



II: form a **stably remodeled** **dimer**, **altered DNAse digestion pattern**

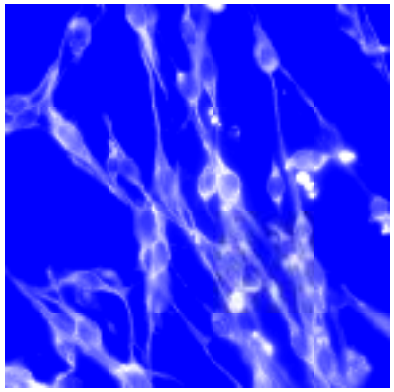
III: transfer a histone octamer to a different DNA fragment

Early versus late myogenesis in the mouse embryo

E=Embryonic Day

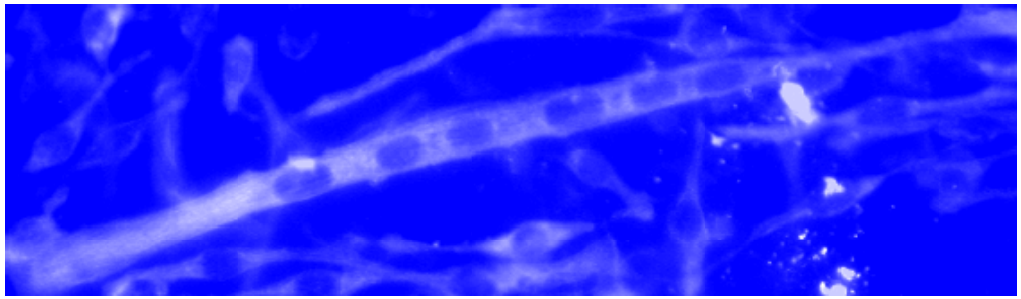
E 8.5-early

First myotomal muscle (myocytes) bipolar,mononucleate

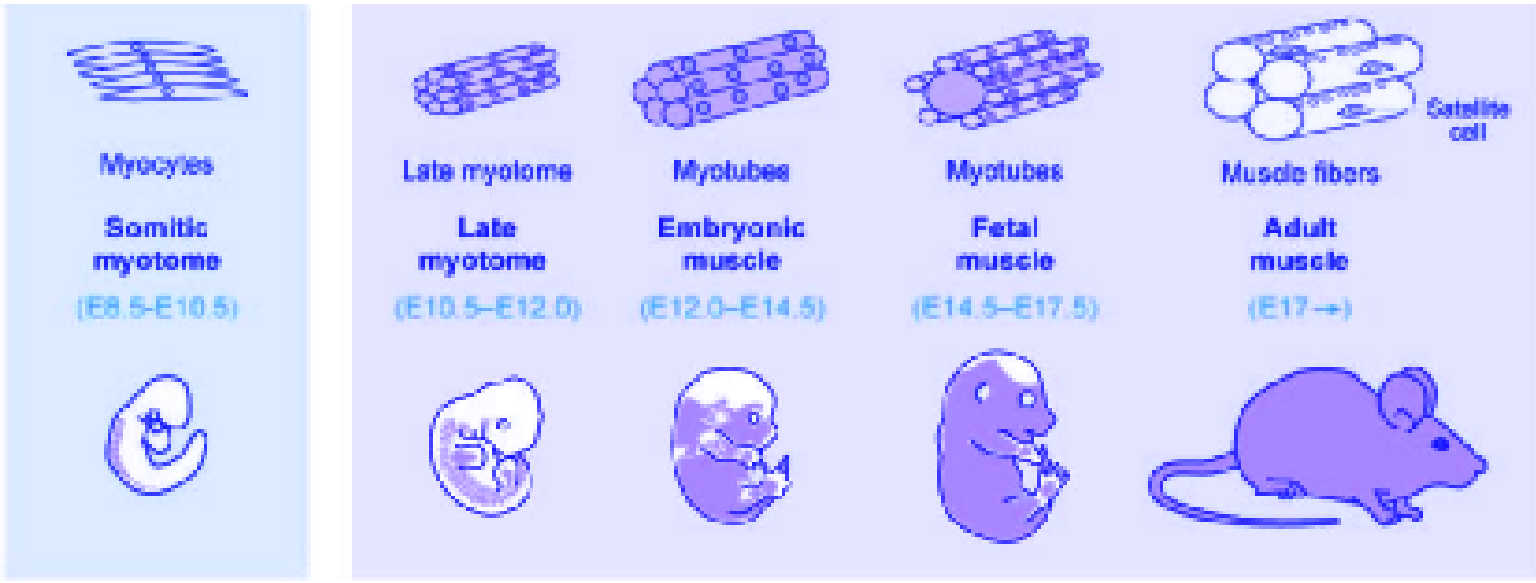


E 13-15 late

Primary muscle formation (myotubes/myofibres) multinucleated elongated cells



Murine Embryogenesis



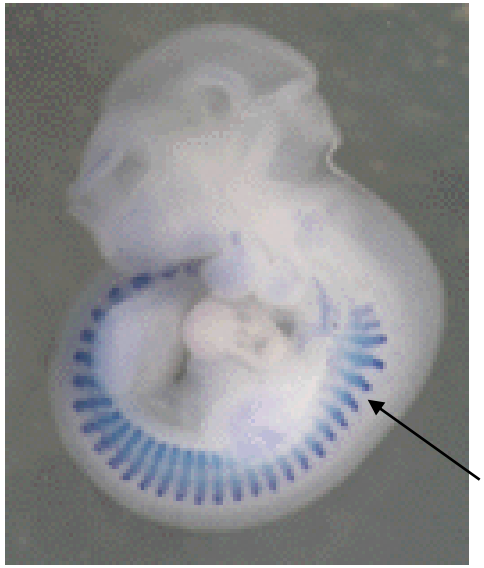
Times used in paper:

10.5 12.5 14.5

Is there a correlation with late myogenesis and increases in muscle structural gene expression?

Method: Isolated RNA from these time points and locations:

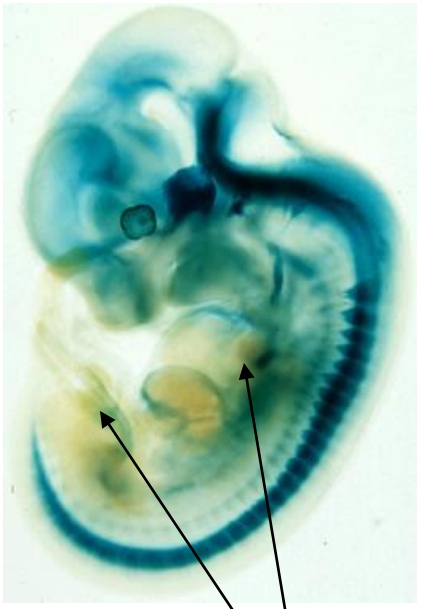
E10.5



myogenin

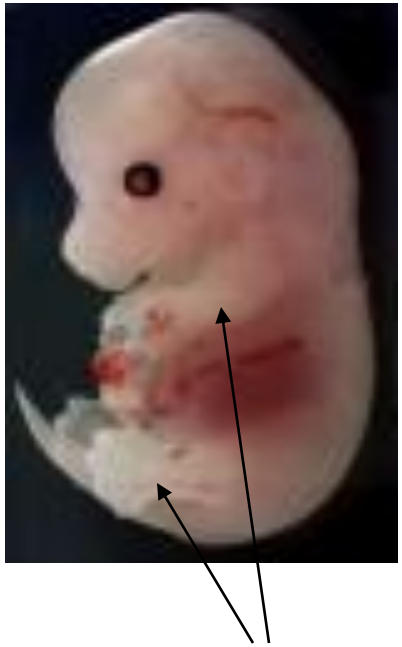
Took: The body
(no head or organs)

E12.5



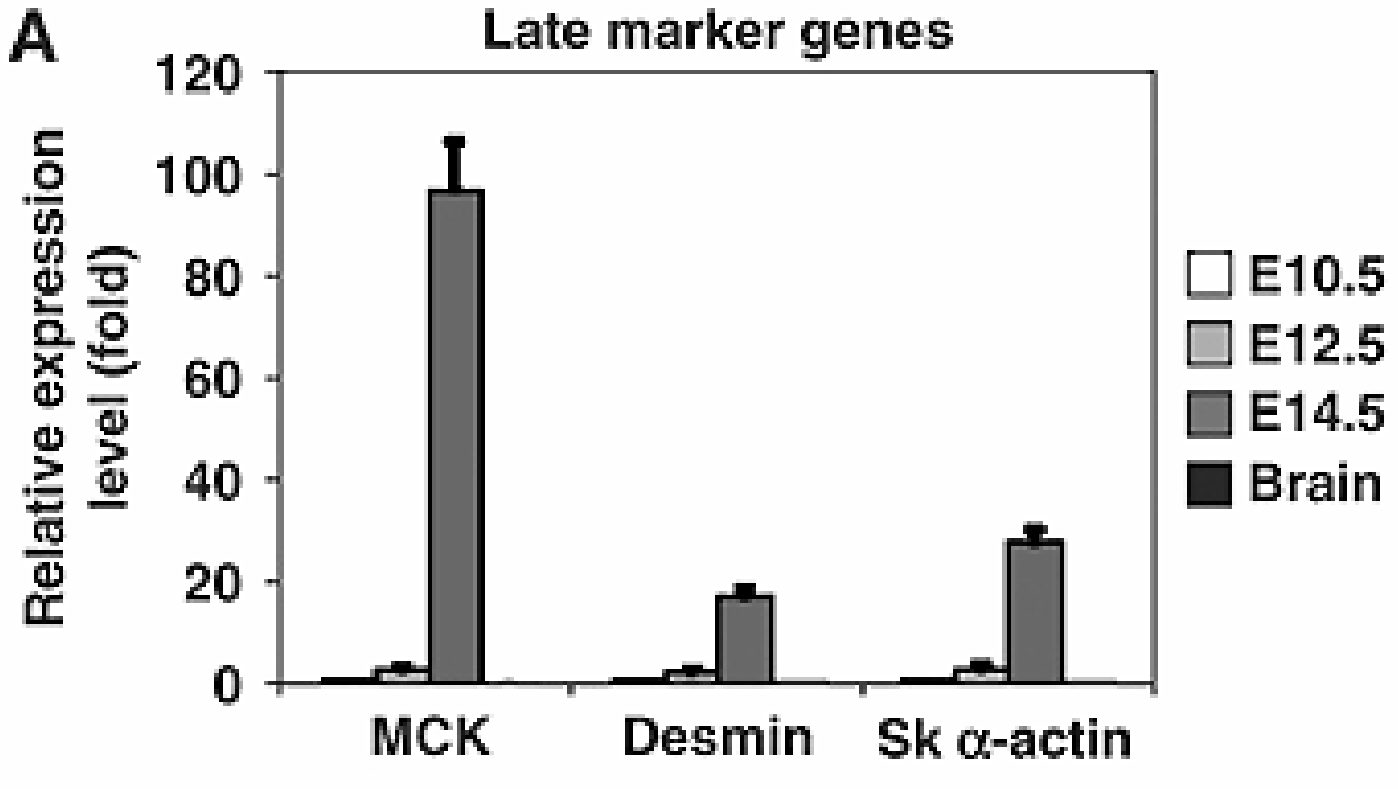
The limb buds

E 14.5



The limbs

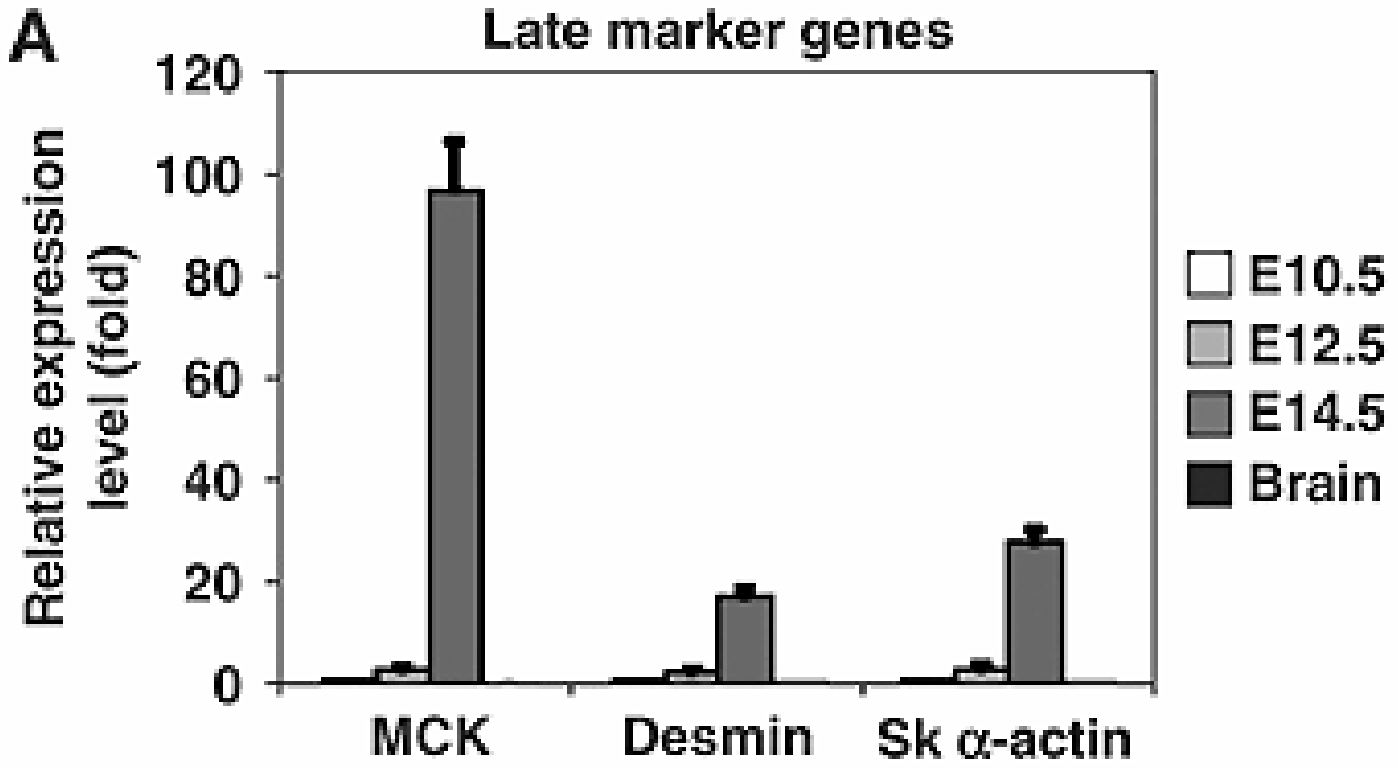
Fig. 1A Examining the Expression of Muscle-specific genes in the developing mouse embryo by RT-PCR



MCK = Muscle creatine kinase

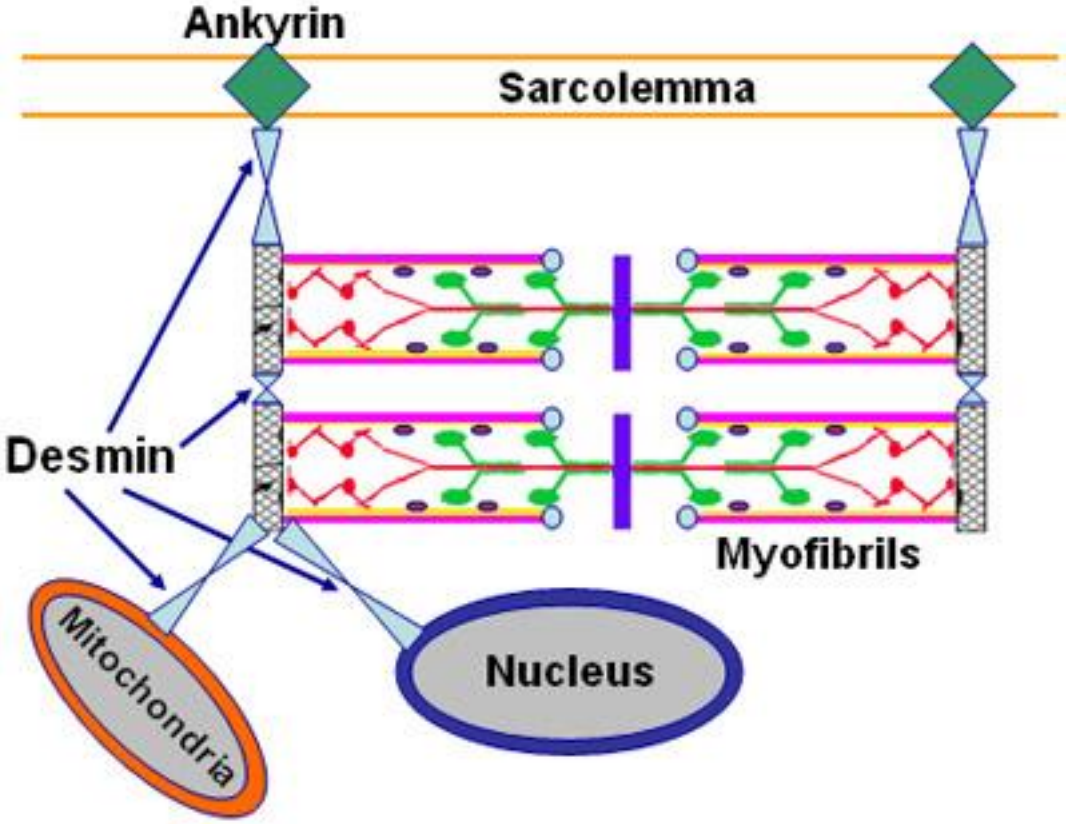
Found: The greatest increase in muscle-specific genes occurs from E12.5 to E14.5

Fig. 1A Examining the Expression of Muscle-specific genes in the developing mouse embryo by RT-PCR



Does this mean there is no MCK, Desmin, or sk α-actin in E10.5 embryos?

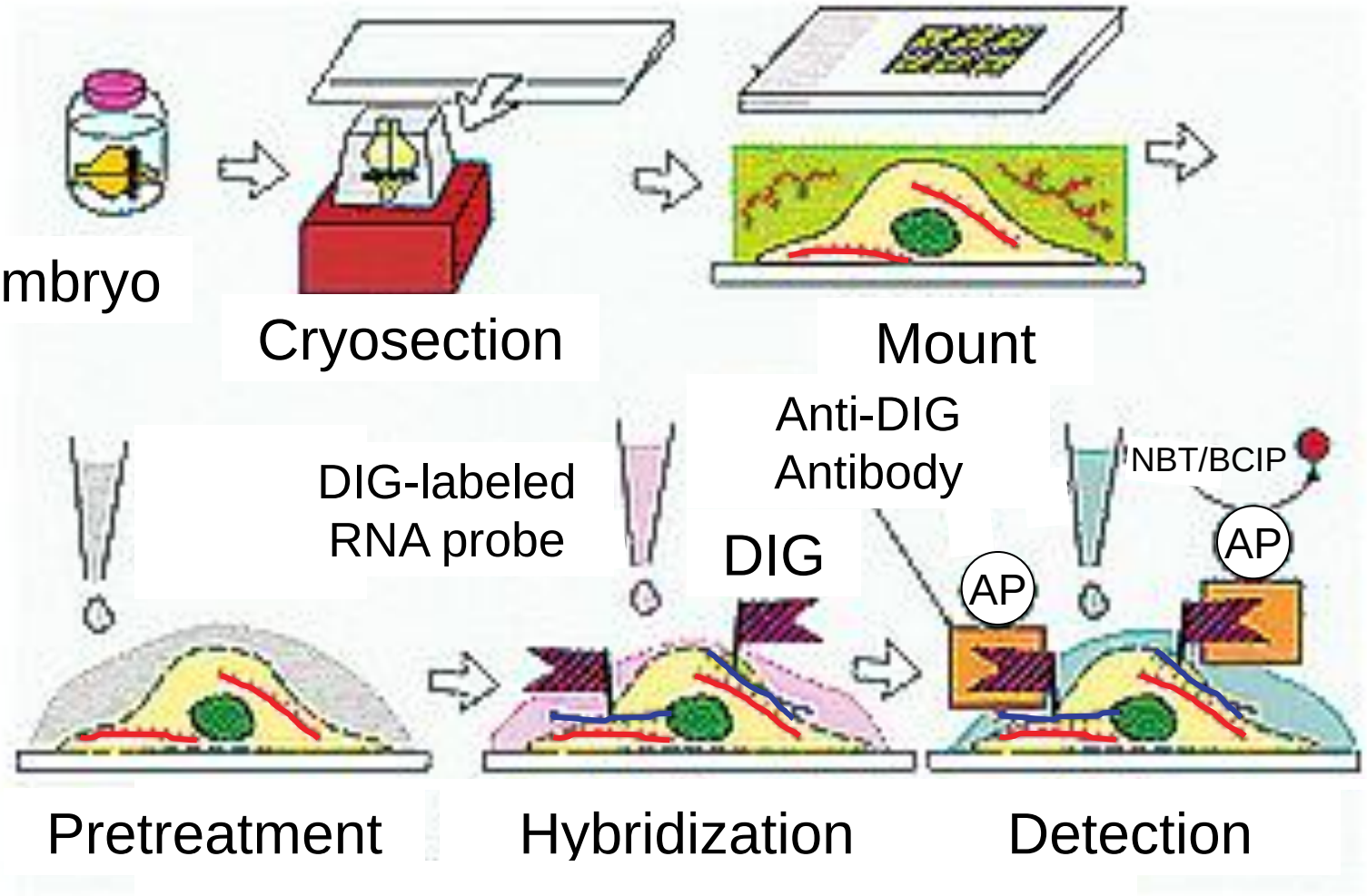
What is desmin?



Desmin is a Type III intermediate filament found in muscle

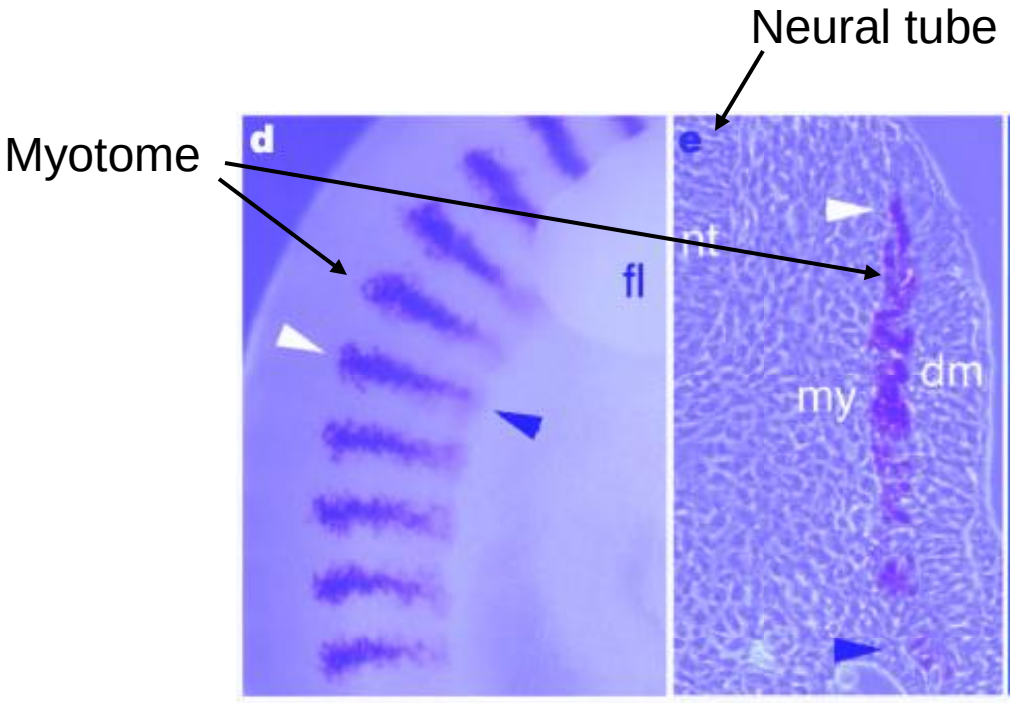
How early is desmin expressed in embryogenesis?

Method of In Situ Hybridization



Red line= endogenous RNA; Blue line with flag = DIG-labeled RNA probe
Orange box = Anti-DIG antibody; White circle = alkaline phosphatase

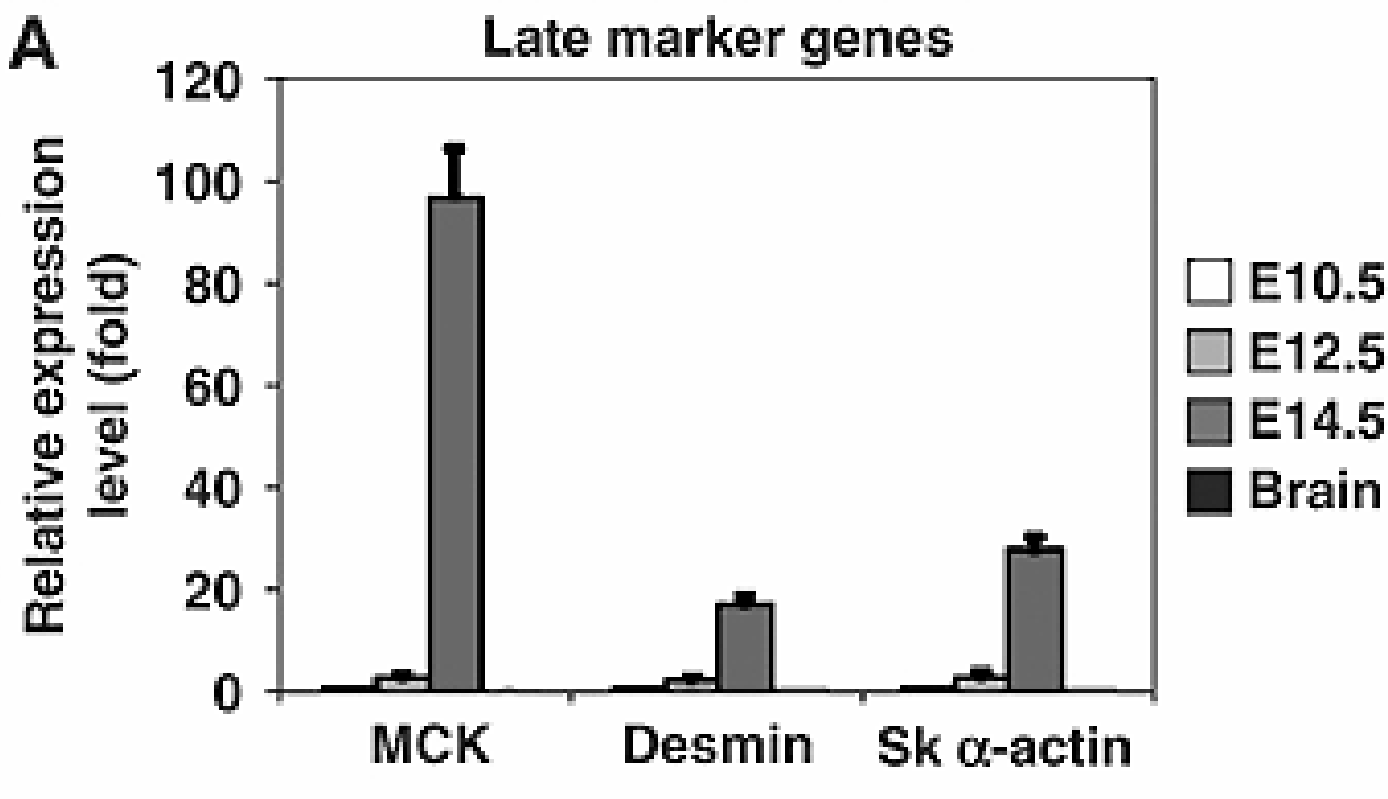
In situ hybridization of desmin in E10.5 embryos



Found: Staining of desmin throughout the myotome as early as E10.5

From:
Jay L. Vivian, Eric N. Olson, and William H. Klein, Developmental Biology **224,** 29–41 (2000)

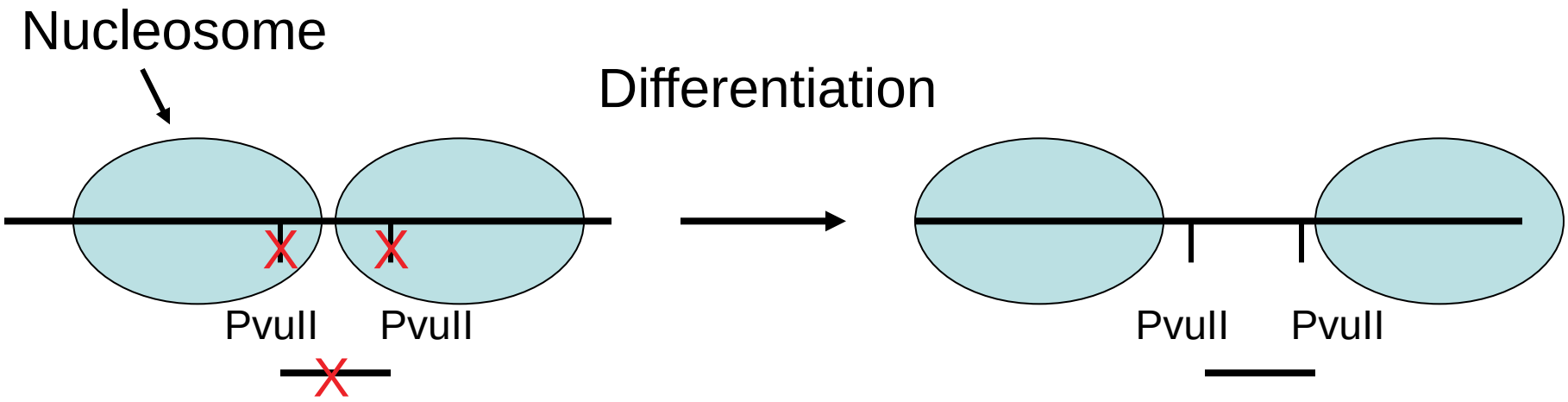
* Fig. 1A Examining the Expression of Muscle-specific genes in the developing mouse embryo by RT-PCR



What does the previous in situ hybridization of desmin expression show with respect to the interpretation of these RT-PCR results?

Is there a correlation with muscle gene expression and changes in the chromatin structure?

* Fig. 1B Restriction Enzyme Accessibility Assay (chromatin accessibility)



Myoblast

- No product

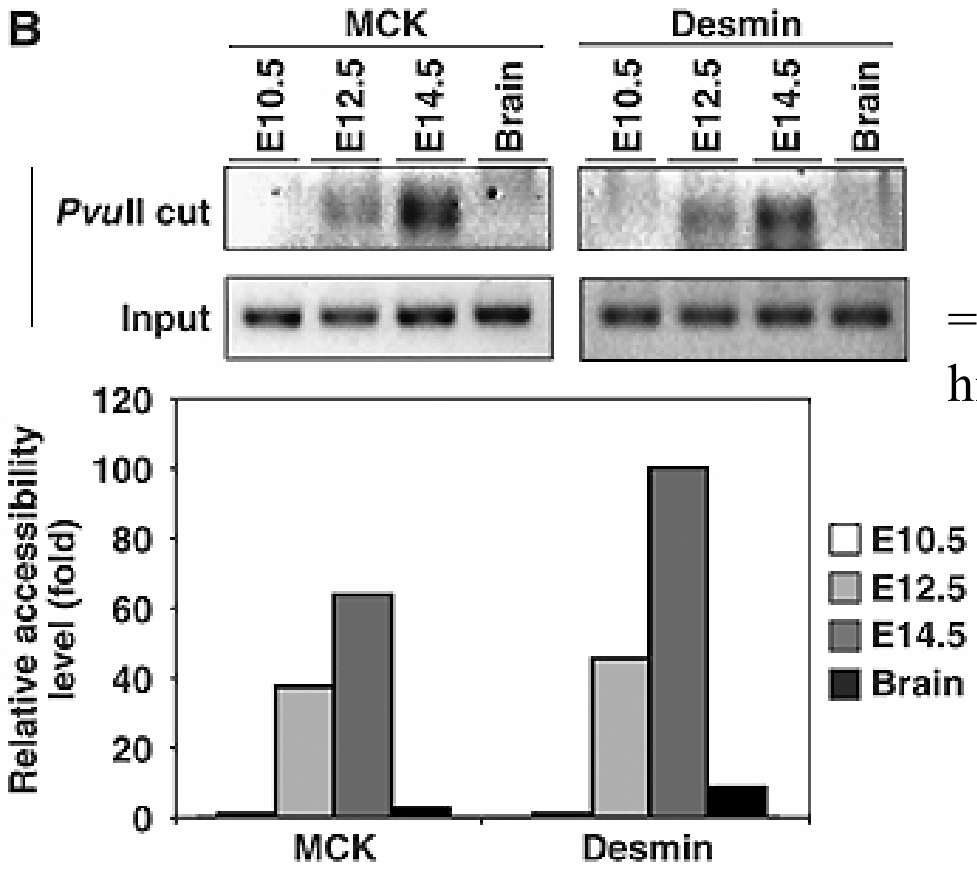
Myotube

-Defined band identified by PCR

(DNA product subcloned and PCR amplified)

Fig. 1B Quantitative Restriction enzyme Accessibility Assay

PCR products

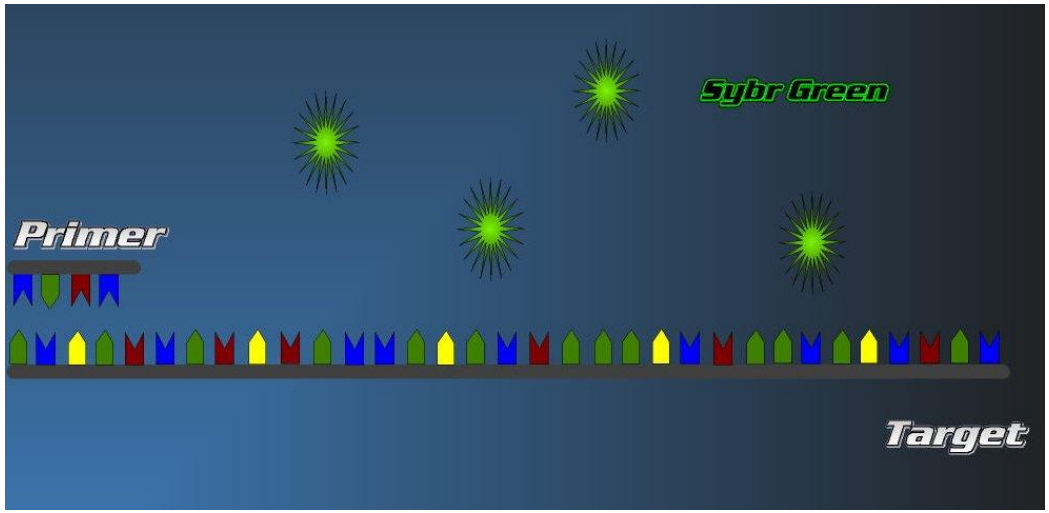


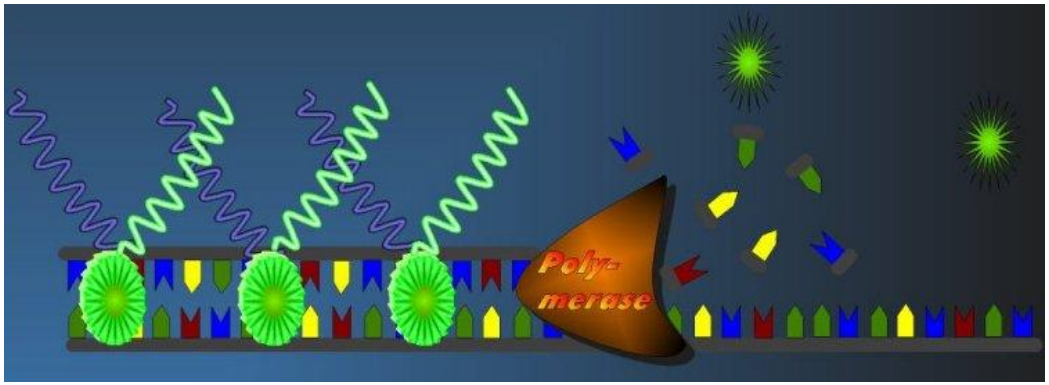
=genomic DNA with histones removed

Increased chromatin accessibility is coincident with increased mRNA levels in embryonic tissue.

Is there a correlation between expression levels of muscle-specific transcription factors or chromatin remodeling factors with late myogenesis?

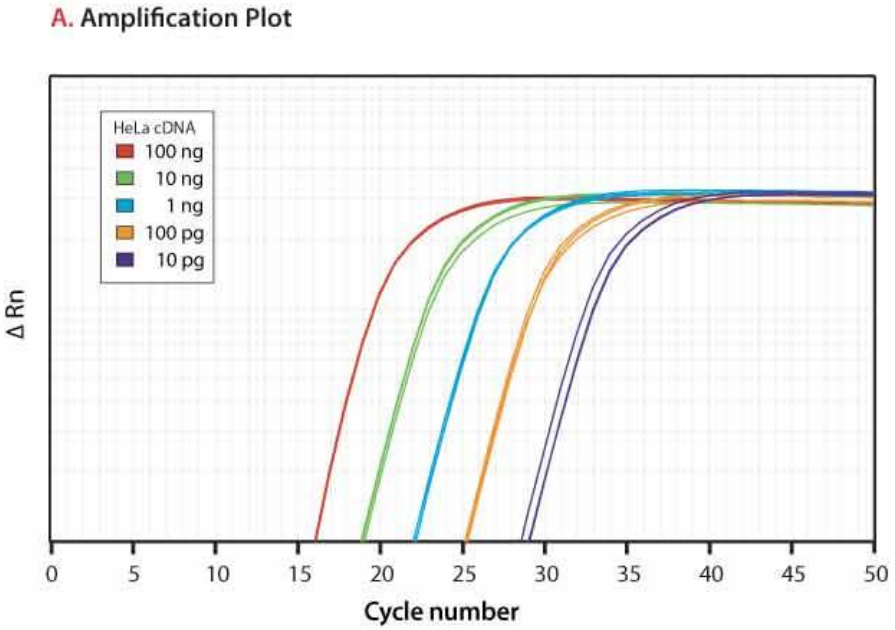
Real-Time or Quantitative PCR

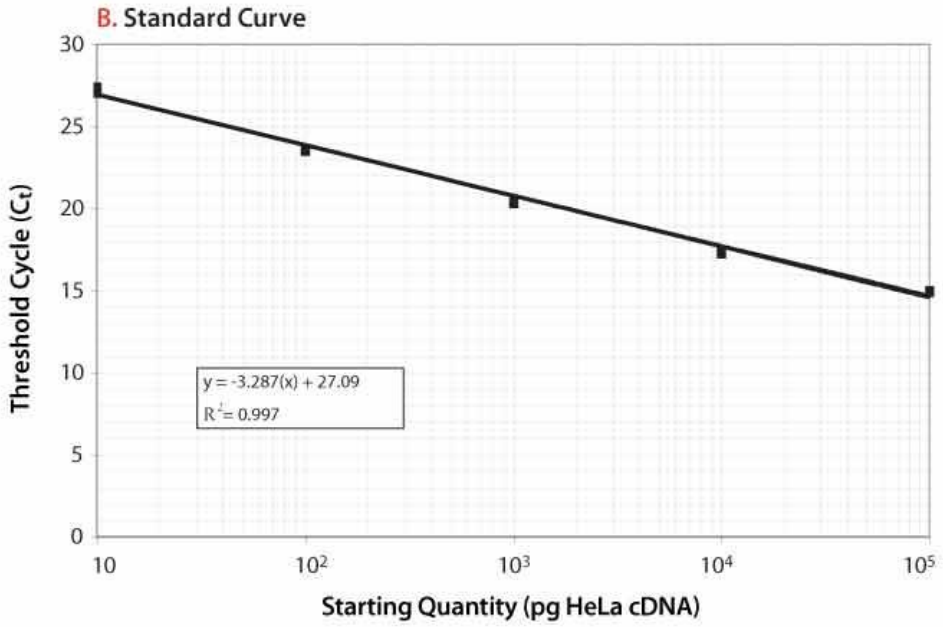




- Sybr Green = fluorescent dye that binds to dsDNA and emits light
- Real Time PCR machine follows the change in fluorescence for each cycle

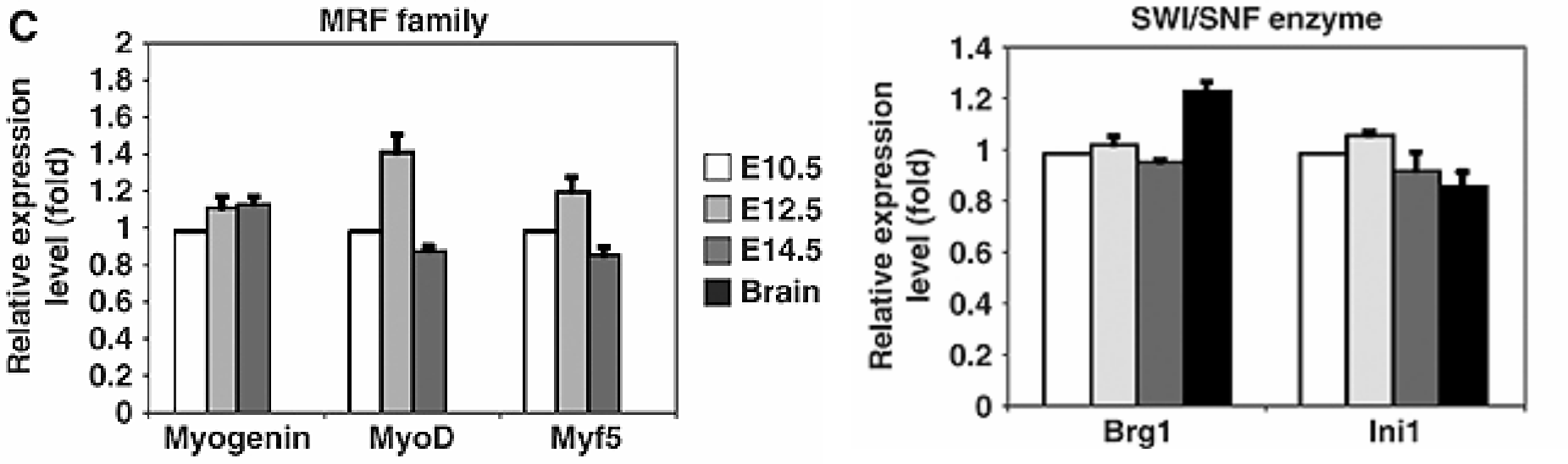
Real Time PCR is Quantitative





*

Fig. 1C Quantitative RT-PCR

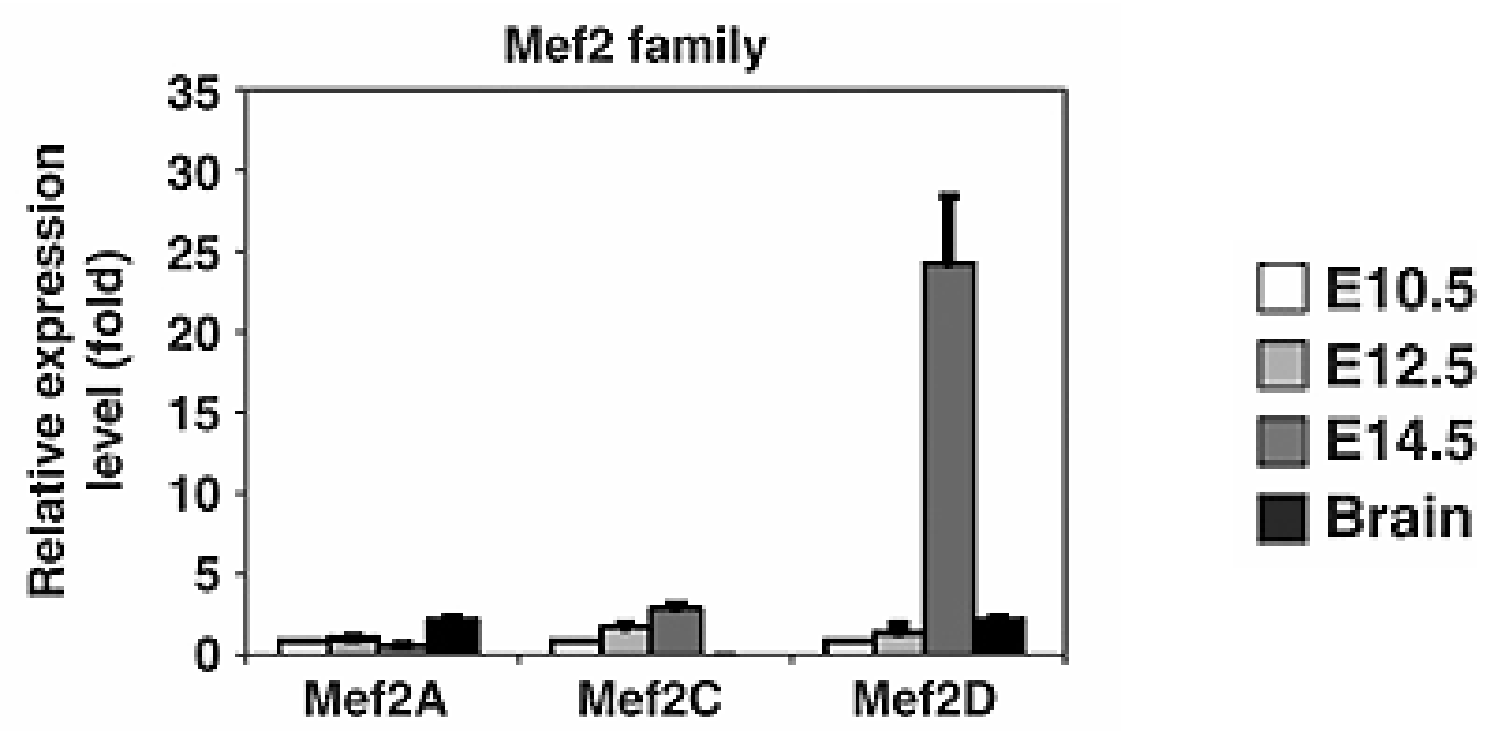


Normalized to EF1α

Expression levels of MRFs and SWI/SNF components do not change significantly through E14.5

*

Fig. 1C Quantitative RT-PCR



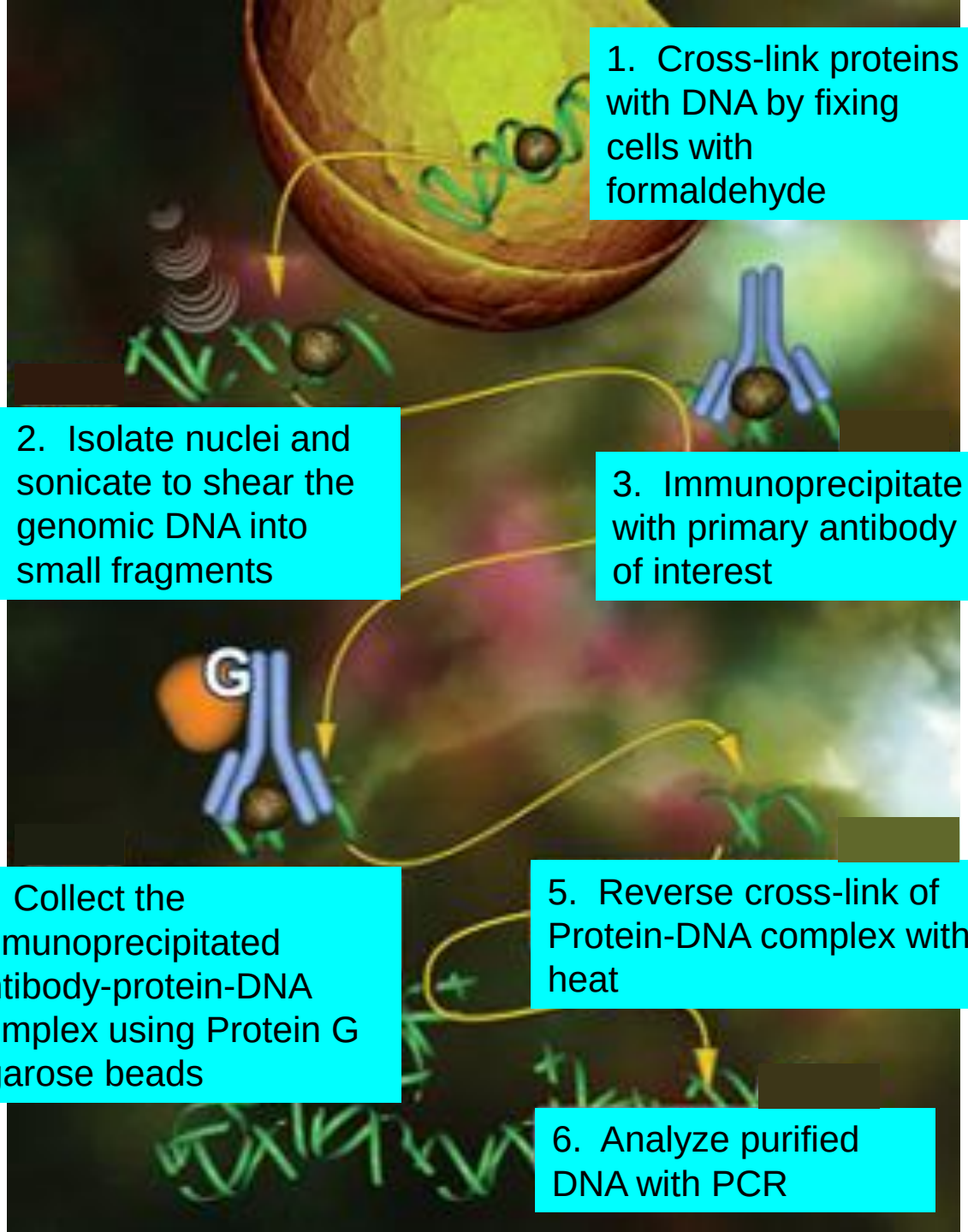
Upregulation of Mef2D is coincident with increased chromatin accessibility of Desmin and MCK

Summary:

Fig. 1: Increased chromatin accessibility correlates with increased MEF2D, MCK, and desmin mRNA levels in the embryo.

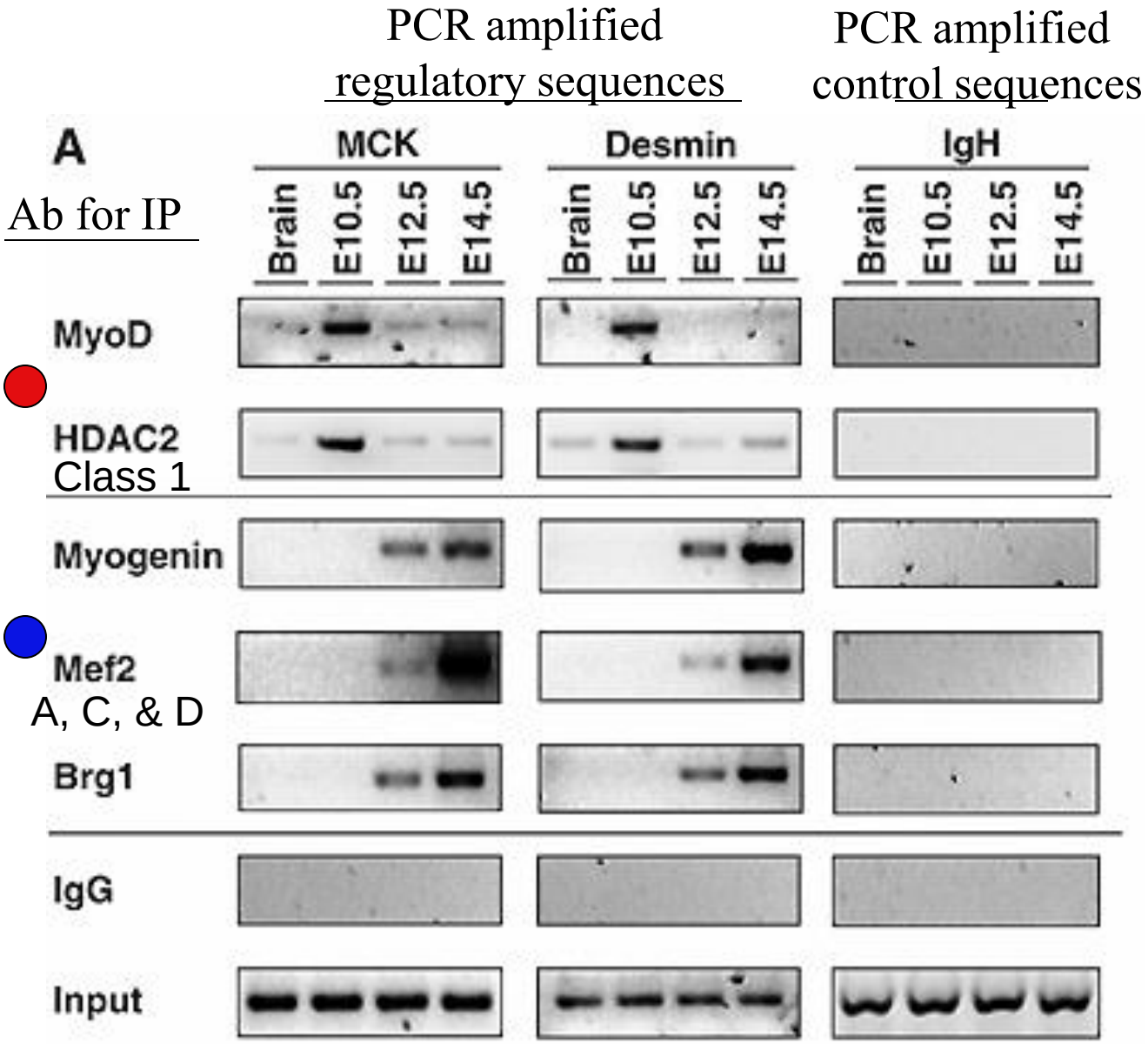
Who is bound to the MCK and Desmin promoters during early and late myogenesis?

Chromatin Immunoprecipitation Assay



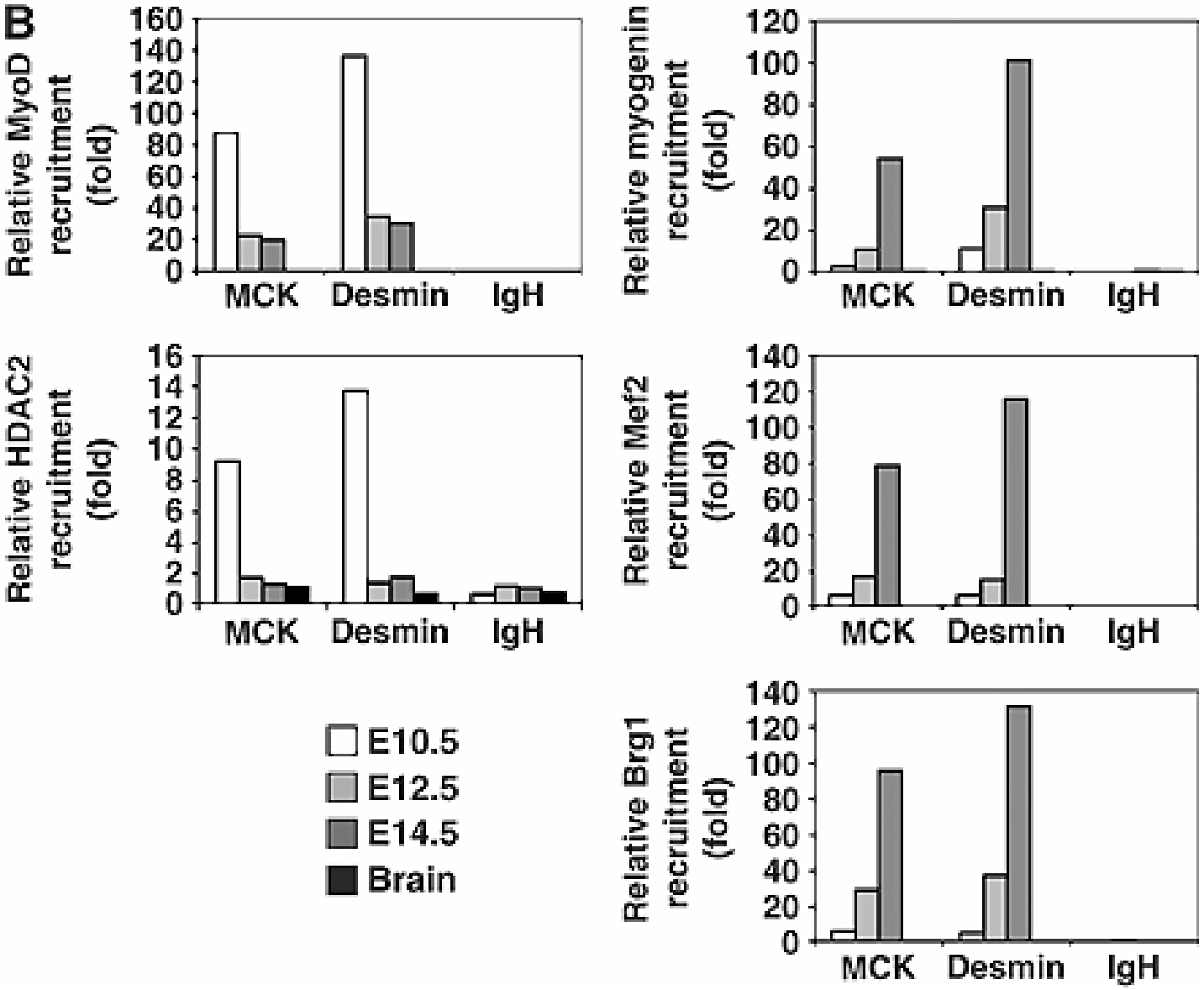
Imgenex.com

Fig. 2A ChIP assays



- MyoD and HDAC2 are bound to the regulatory sequences of MCK and Desmin at early time points.
- They are replaced by myogenin, Mef2 and Brg1 at later time points, coincident with increased mRNA levels.

Fig. 2B Quantification of ChIP assays

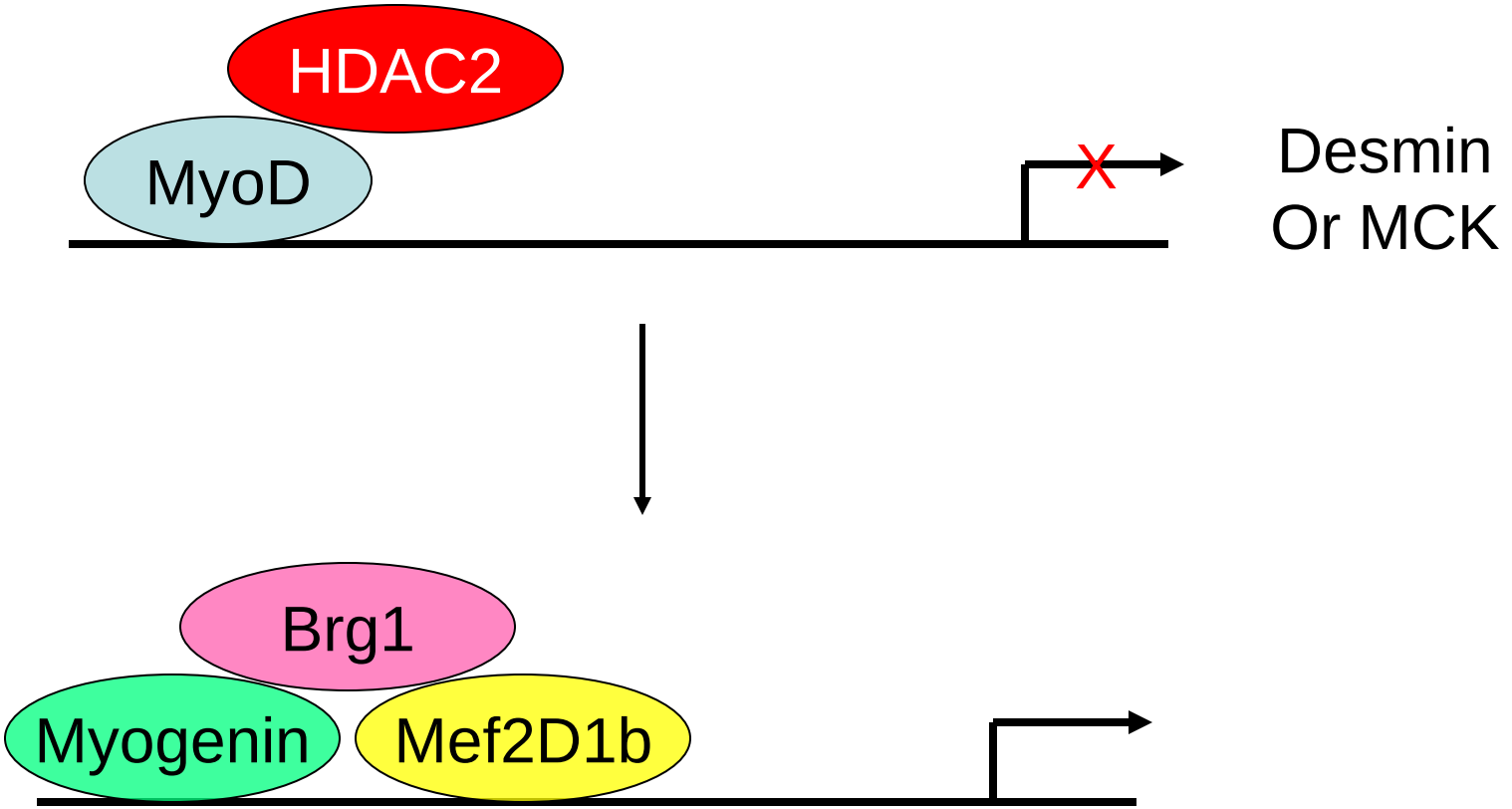


Summary:

Fig. 1: Increased chromatin accessibility correlates with increased MEF2D, MCK, and desmin mRNA levels in the embryo.

Fig. 2: MyoD and HDAC2 are bound to the MCK/desmin regulatory sequences early in the embryo, and myogenin, MEF2D1B, and Brg1 are bound late.

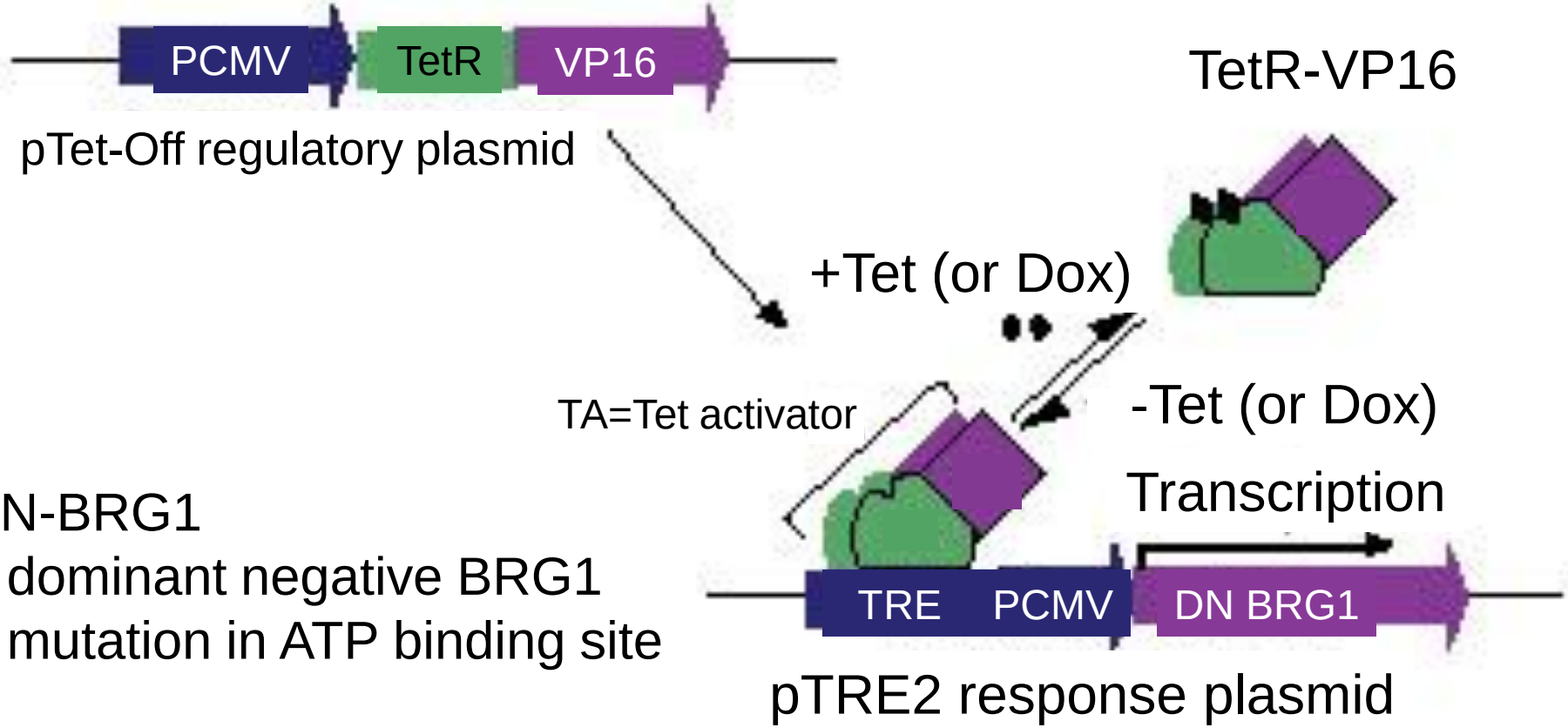
* Model consistent with ChIP data from embryos



Does this also happen in cell culture?

* B22 cells - Created by expressing inducible DN BRG1 in Fibroblasts

Tet-Off



DN-BRG1
= dominant negative BRG1
= mutation in ATP binding site

Inducible means: DNBRG1 turned on in the absence of Tet (tetracycline)

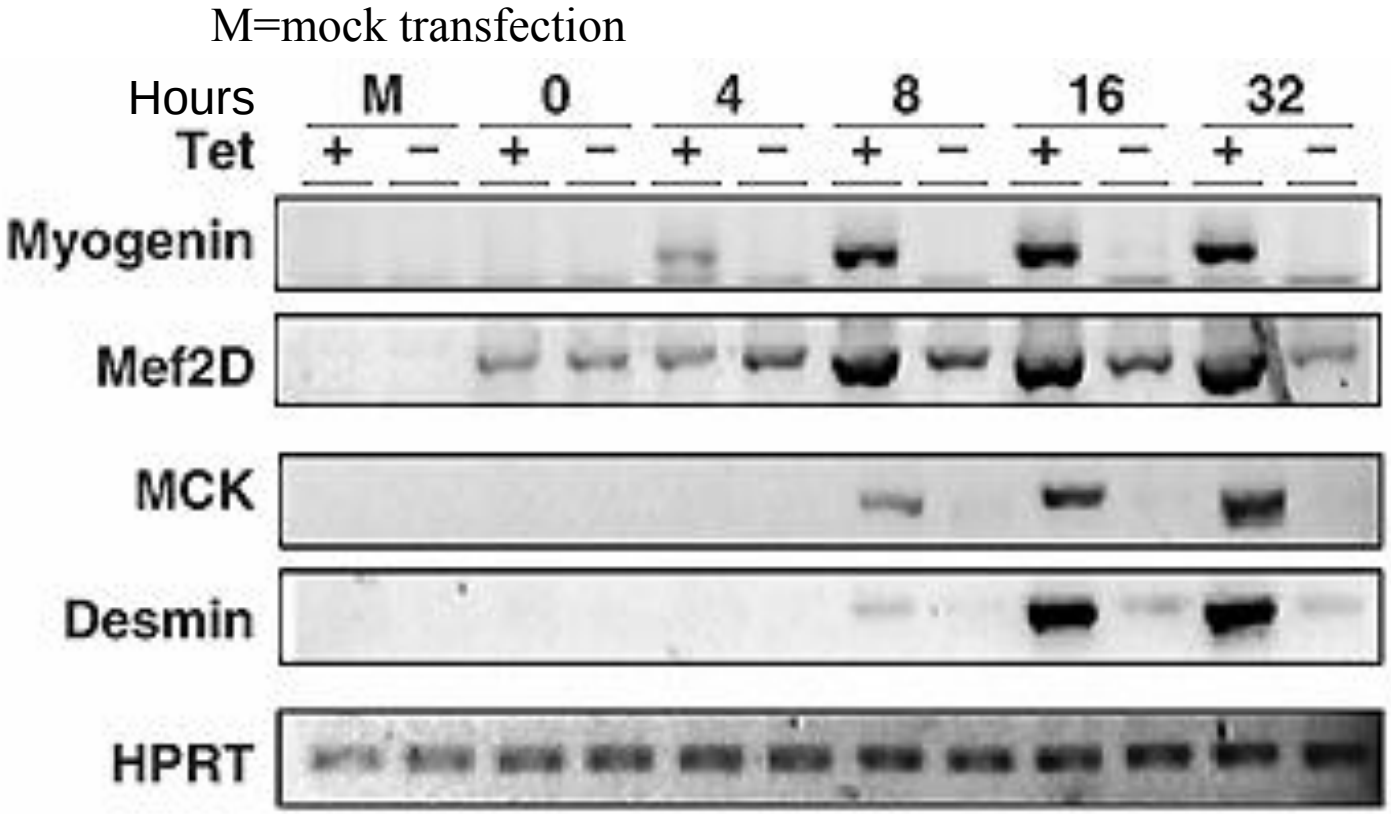
Tetracycline repressor protein class D TetR (Escherichia coli)

QuickTime™ and a
decompressor
are needed to see this picture.

1. TetR is a homodimer that binds DNA when bound to tetracyclin
2. TetR family of transcription factors repress a variety of pathways, including efflux pumps, that mediate the antibiotic effect.

Note: In the tet-off system, TetR-VP16 binds DNA in the absence of Tetracycline

* Fig. 3 A Time course of MyoD directed differentiation in B22 cells (RT-PCR)

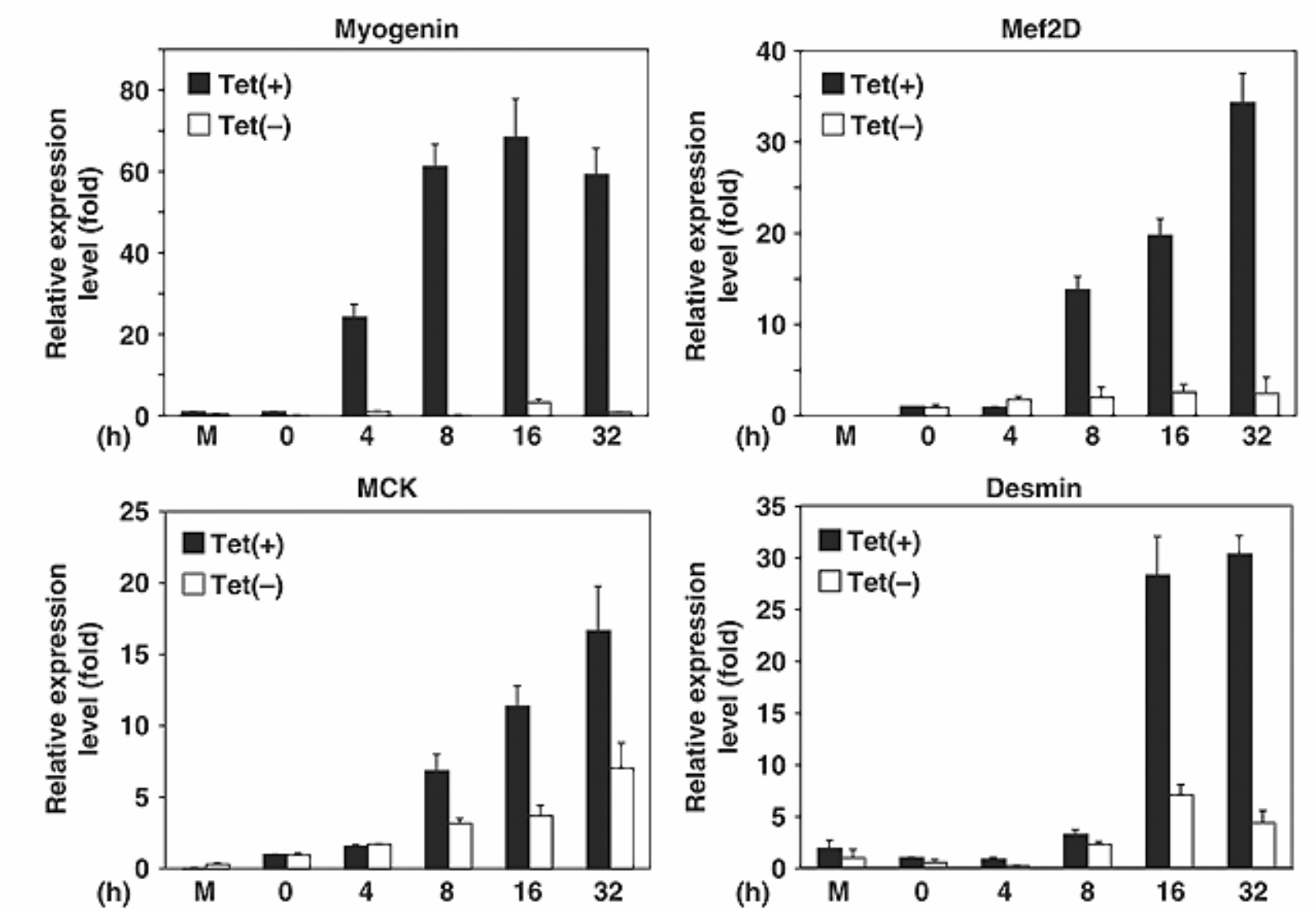


MyoD-directed differentiation was inhibited by the expression of dominant negative BRG1

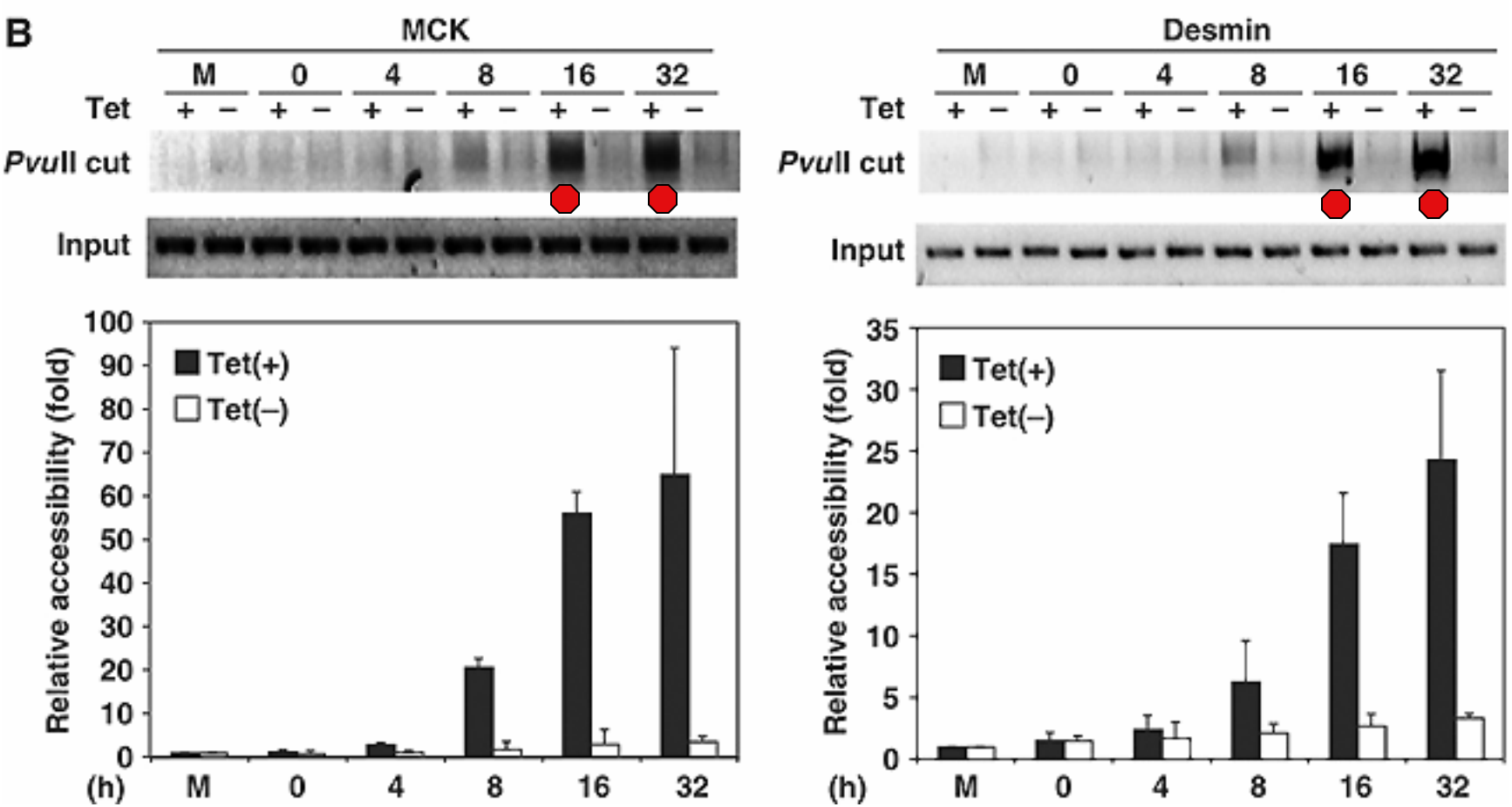
What is missing?

*

Fig. 3A Quantification



* Fig. 3B Restriction enzyme accessibility assays



Found: Accessibility increases ● at endogenous late marker loci during differentiation and requires functional Brg1

Summary:

Fig. 1: Increased chromatin accessibility correlates with increased MEF2D, MCK, and desmin mRNA levels in the embryo.

Fig. 2: MyoD and HDAC2 are bound to the MCK/desmin regulatory sequences early in the embryo, and myogenin, MEF2D1B, and Brg1 are bound late (ChIP assay).

Fig. 3: MyoD-directed differentiation and opening of chromatin were inhibited by the expression of dominant negative BRG1 in fibroblasts (accessibility assay).

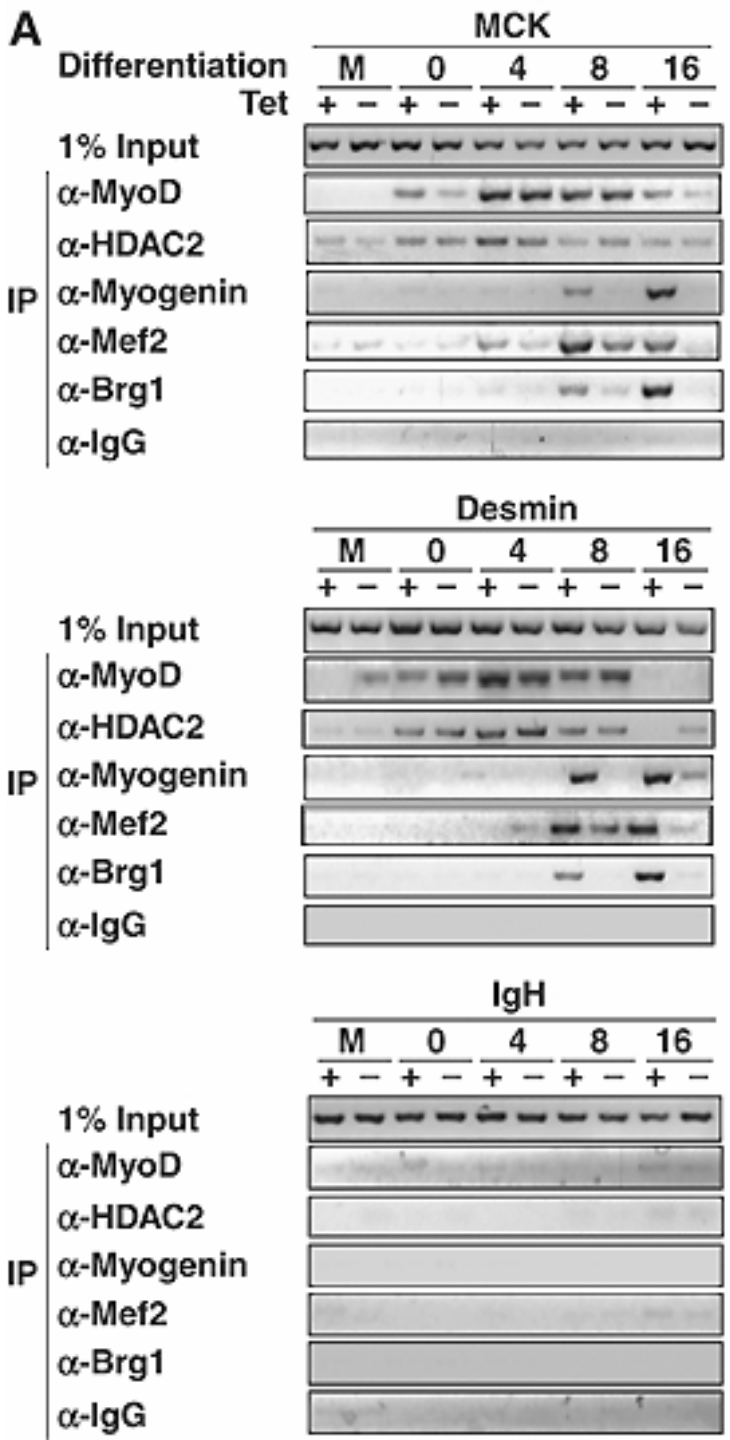
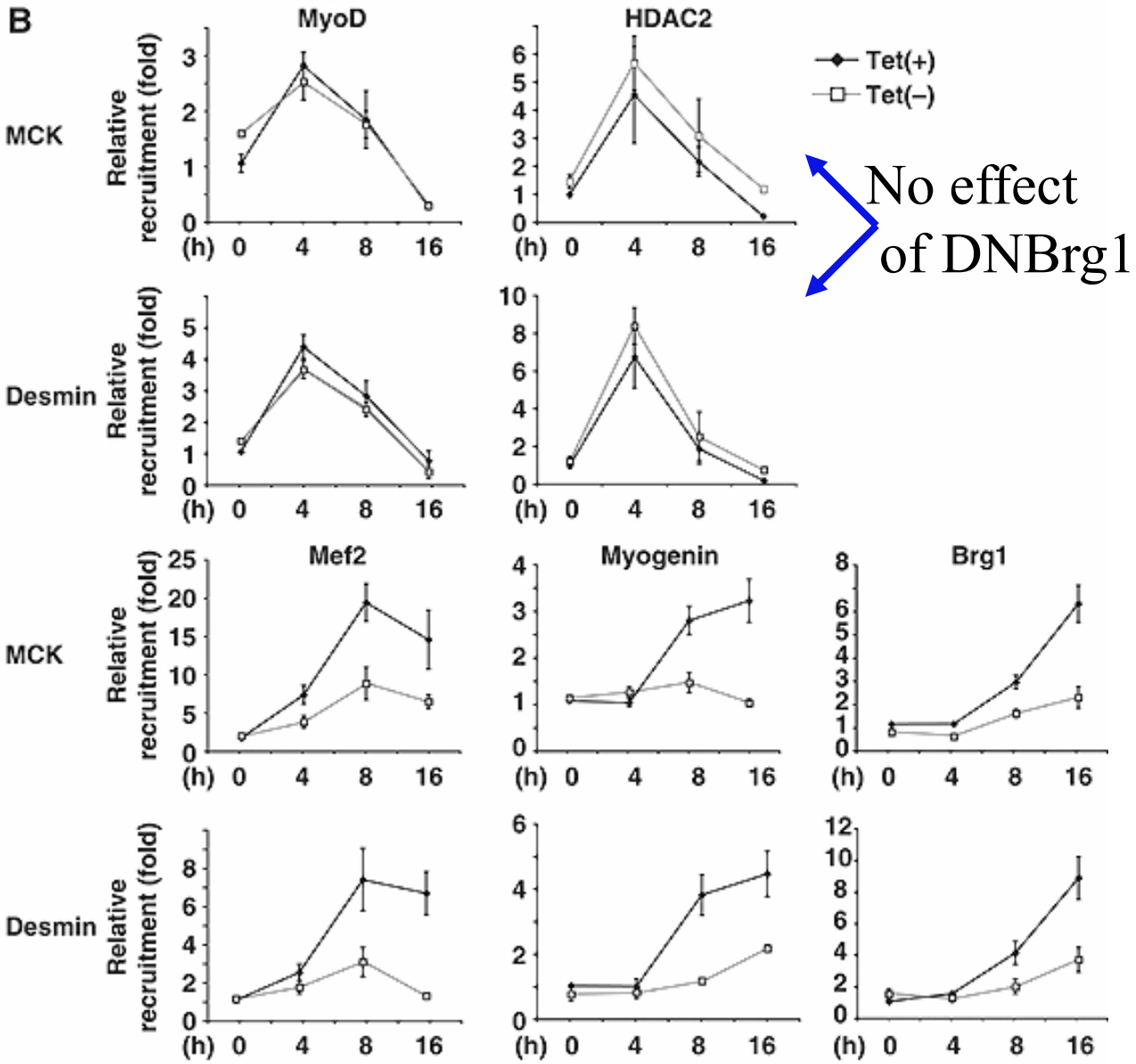


Fig. 4A ChIP assays were performed on mock (M) or MyoD differentiated cells (0-16 hours).

Found:

- Results mimic those seen in embryonic muscle tissue, with MyoD and HDAC2 being replaced by Myogenin, Mef2, and Brg1.
- Inability of dominant-negative BRG1 to inhibit MyoD binding.
- DNBRG1 inhibits myogenin, MEF2, and wtBrg1 binding.

* Fig. 4B Quantification of ChIP assays



No effect of DNBrg1

Binding inhibited by DNBrg1

Relative recruitment of MyoD and HDAC2 decreases with time while relative recruitment of myogenin, Mef2 and Brg1 increases with time.

Summary:

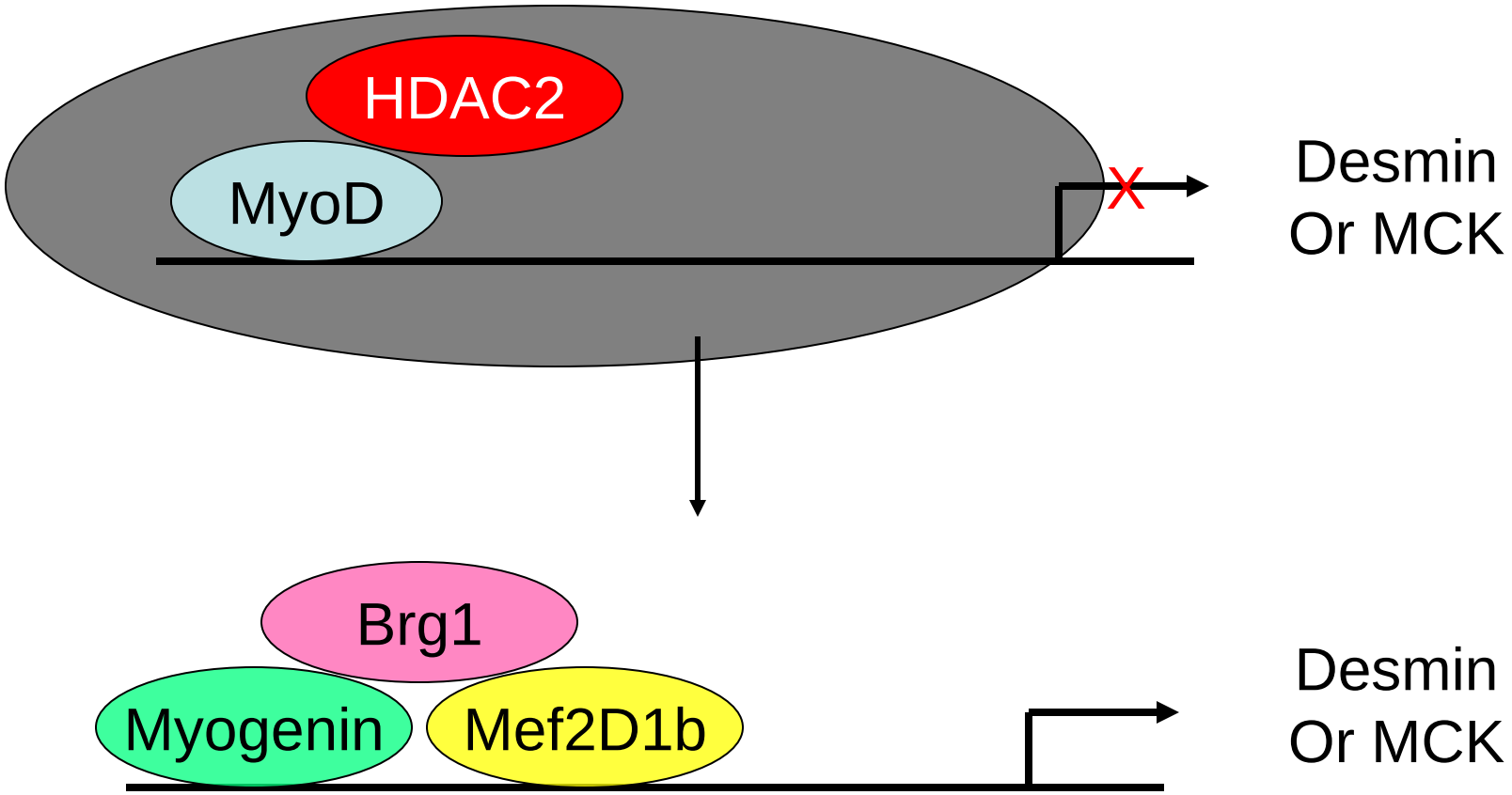
Fig. 1: Increased chromatin accessibility correlates with increased MEF2D, MCK, and desmin mRNA levels in the embryo.

Fig. 2: MyoD and HDAC2 are bound to the MCK/desmin regulatory sequences early in the embryo, and myogenin, MEF2D1B, and Brg1 are bound late. (ChIP assay)

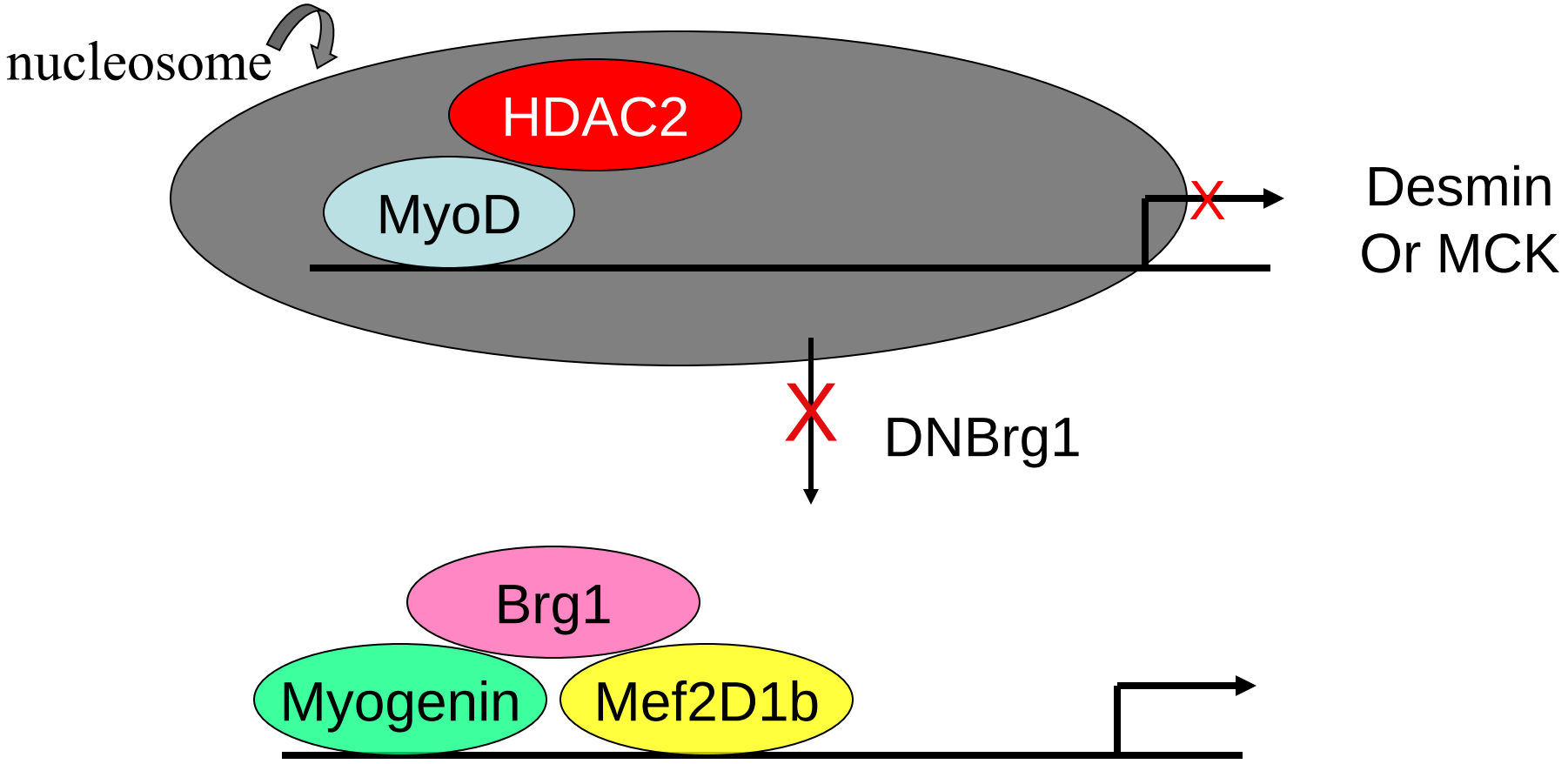
Fig. 3: MyoD-directed differentiation (mRNA) and opening of chromatin were inhibited by the expression of dominant negative BRG1 in fibroblasts (accessibility assay)

Fig. 4: Binding of early and late factors in MyoD-directed fibroblast myogenesis, by ChIP assay, is the same as in the embryo (Fig. 2) and late binding is inhibited by BRG1.

* The data in MyoD-directed fibroblasts is consistent with the following model:



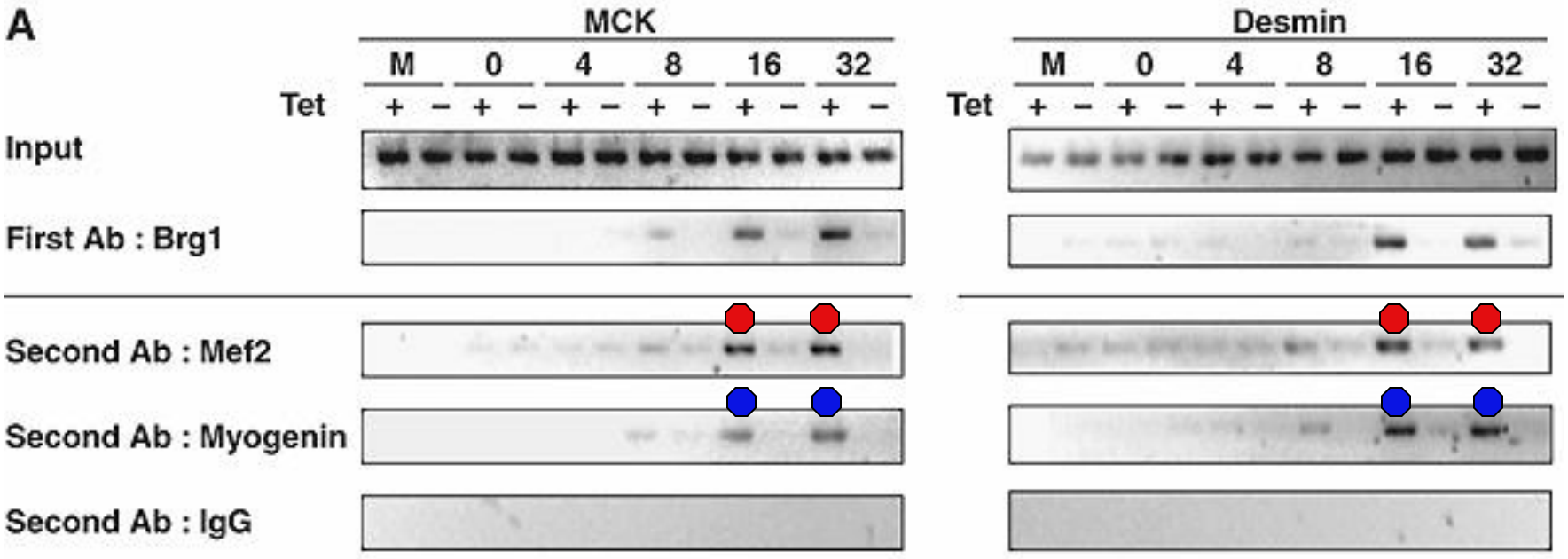
* The data in MyoD-directed fibroblasts is consistent with the following model in the presence of a dominant negative Brg1 mutant:

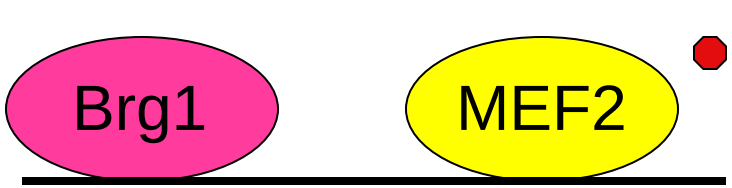


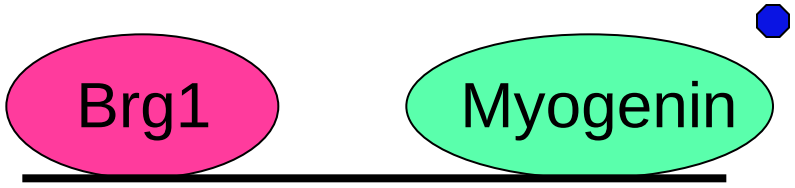
Do Brg1, MEF2, and Myogenin bind to the same DNA fragments?

*

Fig. 5A Re-ChIP assays show co-recruitment of Brg1, myogenin and Mef2 at MCK and Desmin promoters



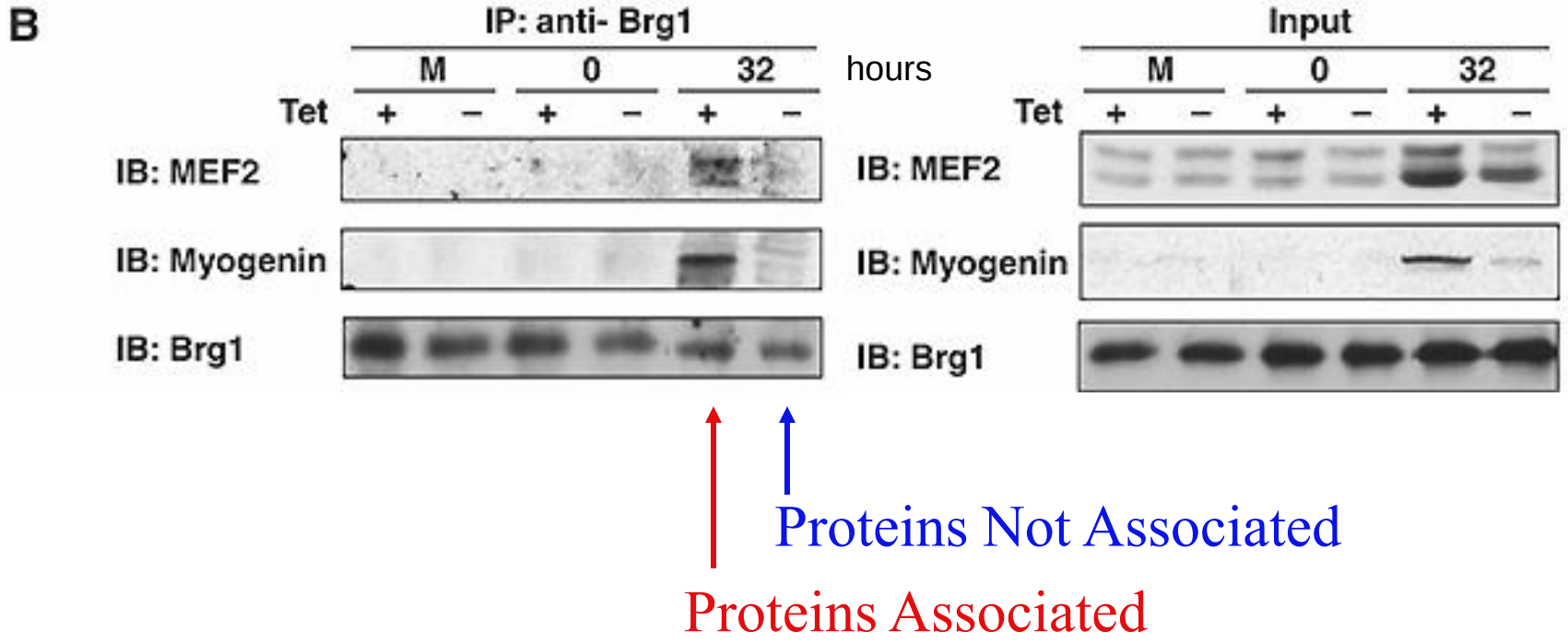




Does Brg1 bind directly to MEF2 and myogenin?

*

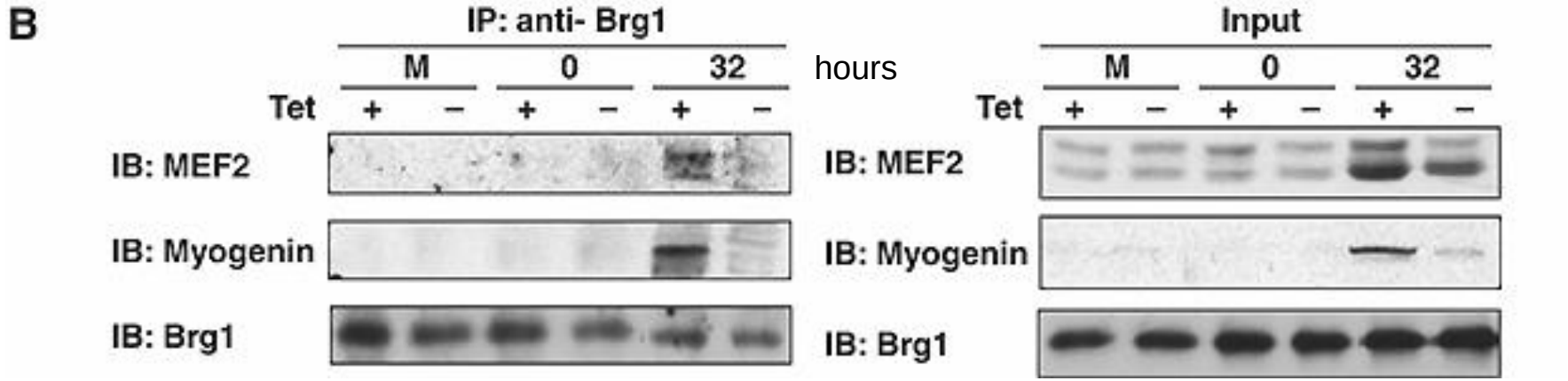
Fig. 5B Co-IP shows that endogenous Brg1 interacts with endogenous Mef2 and myogenin



Suggests that Brg1 is targeted to late genes by Mef2 and myogenin

*

Fig. 5B Co-IP shows that endogenous Brg1 interacts with endogenous Mef2 and myogenin



What controls are missing?

1. A control immunoprecipitation in the absence of primary Ab (beads alone).
2. The reciprocal immunoprecipitations (IP with MEF2 or myogenin and western blot with the other factors).

Summary:

Fig. 1: Increased chromatin accessibility correlates with increased MEF2D, MCK, and desmin mRNA levels in the embryo.

Fig. 2: MyoD and HDAC2 are bound to the MCK/desmin regulatory sequences early in the embryo, and myogenin, MEF2D1B, and Brg1 are bound late. (ChIP assay)

Fig. 3: MyoD-directed differentiation and opening of chromatin were inhibited by the expression of dominant negative BRG1 in fibroblasts (accessibility assay)

Fig. 4: Binding of early and late factors in MyoD-directed fibroblast myogenesis is the same as in the embryo (Fig. 2) and inhibited by BRG1. (ChIP assay)

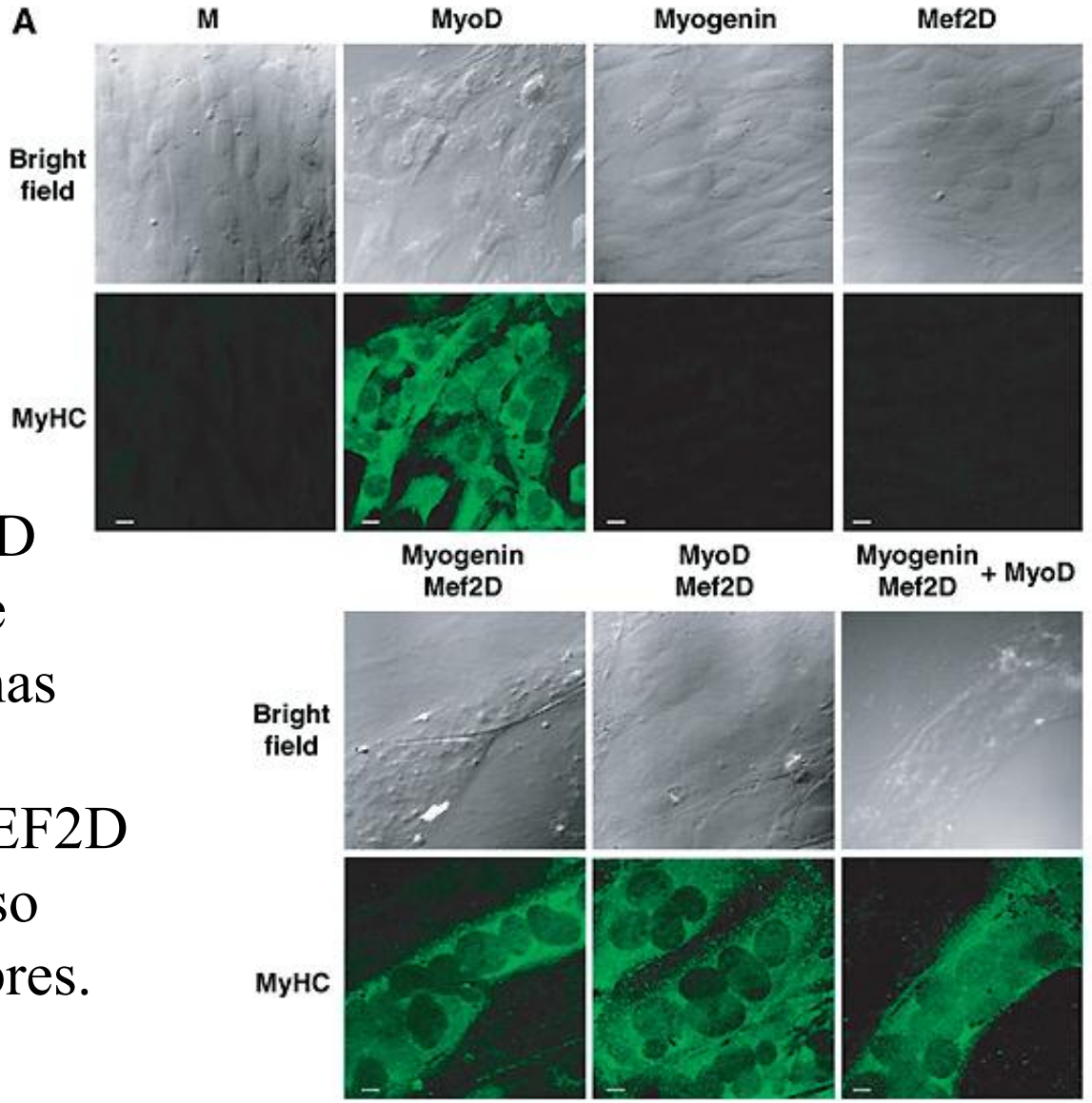
Fig. 5: BRG1, myogenin, and MEF2 form a protein complex during MyoD-directed myogenesis in fibroblasts. (Re-ChIP and IP)

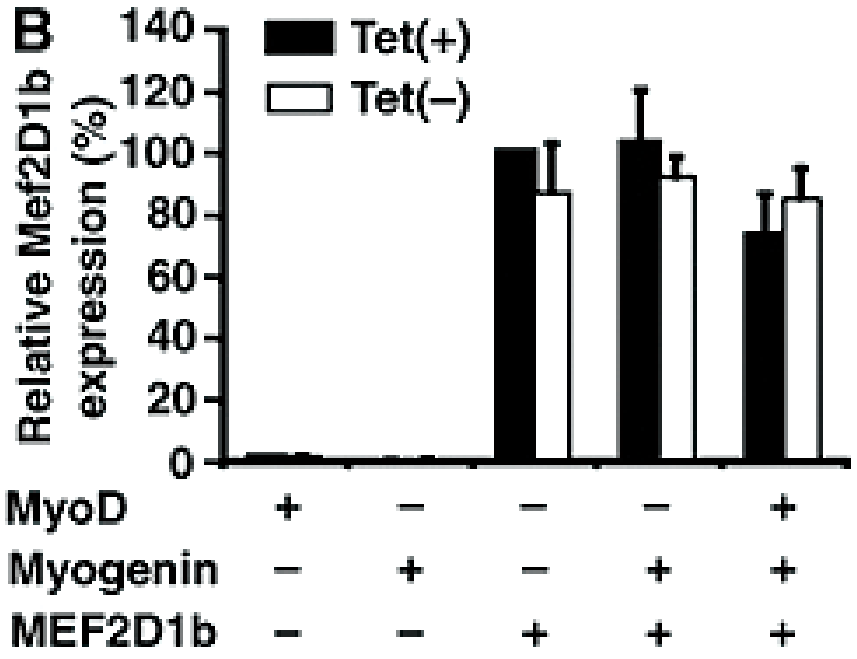
Does the combination of these genes affect the ability to make skeletal muscle?

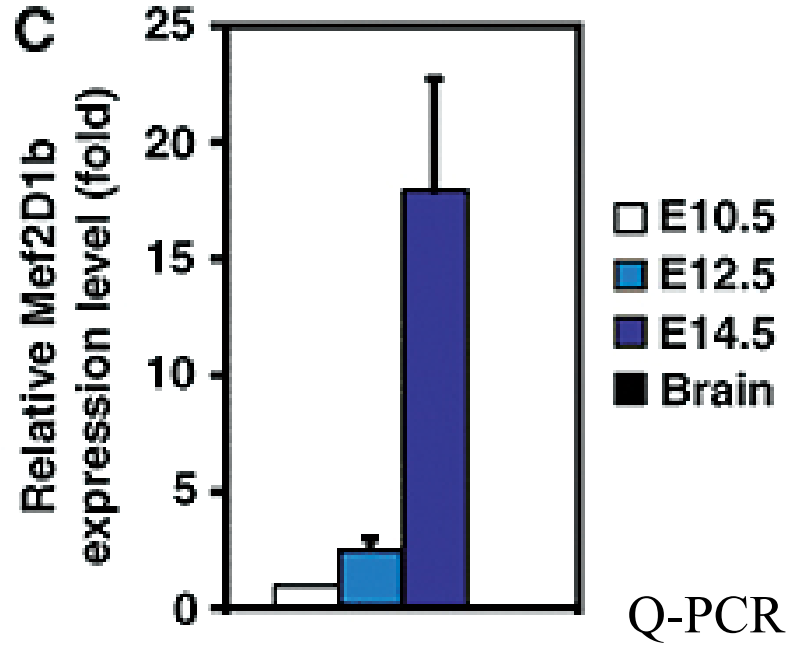
Fig. 6A MyoD alone or Mef2D with myogenin can induce skeletal myogenesis in B22 cells

Found:
Top Panel: Only MyoD alone can make muscle.

Bottom Panel: Myogenin with MEF2D together can also make muscle. This muscle has more nuclei/cell and is larger. MyoD with MEF2D or all three together also make larger muscle fibres.



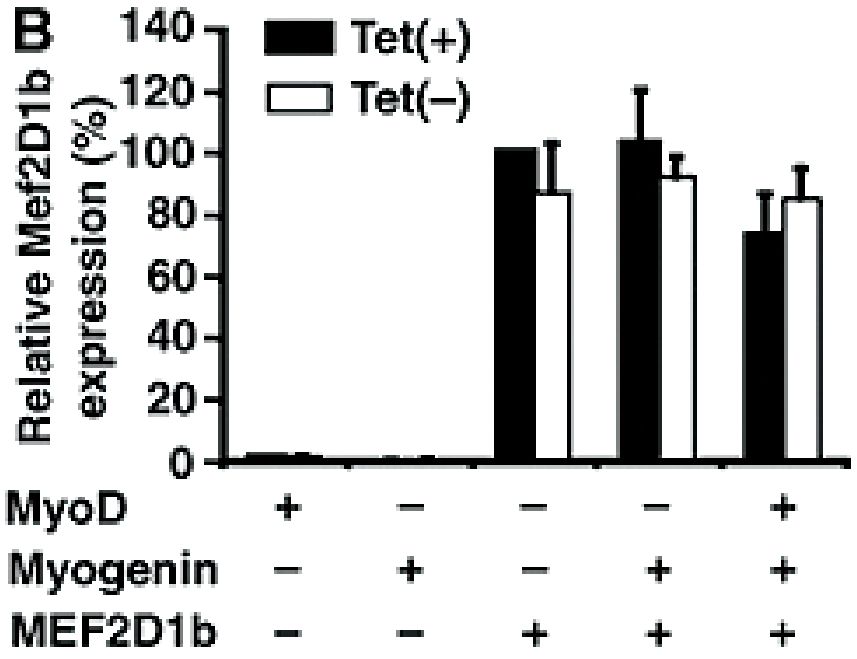




Q-PCR analysis

Fig. 6B & C Neither MyoD nor myogenin could induce Mef2D1b expression in fibroblasts

- Implication is that mature myotubes are only formed in the presence of Mef2D1b



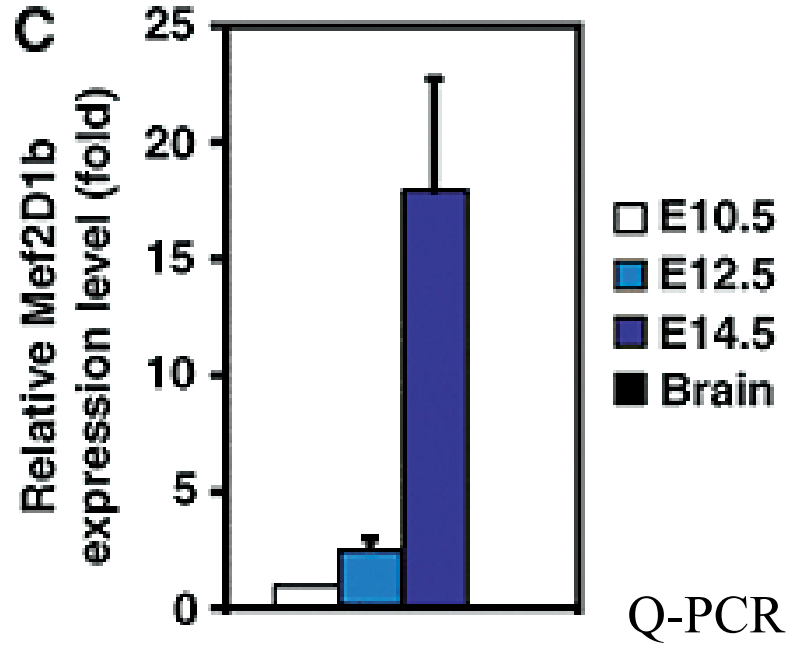


Fig. 6B & C Neither MyoD nor myogenin could induce Mef2D1b expression in fibroblasts

How meaningful is it to compare Mef2D1b expression with MyoD alone vs transfected Mef2D1b?

Q-PCR analysis

Summary:

Fig. 1: Increased chromatin accessibility correlates with increased MEF2D, MCK, and desmin mRNA levels in the embryo.

Fig. 2: MyoD and HDAC2 are bound to the MCK/desmin regulatory sequences early in the embryo, and myogenin, MEF2D1B, and Brg1 are bound late (ChIP)

Fig. 3: MyoD-directed differentiation and opening of chromatin were inhibited by the expression of dominant negative BRG1 in fibroblasts (accessibility assay)

Fig. 4: Binding of early and late factors in MyoD-directed fibroblast myogenesis is the same as in the embryo (Fig. 2) and inhibited by BRG1 (ChIP)

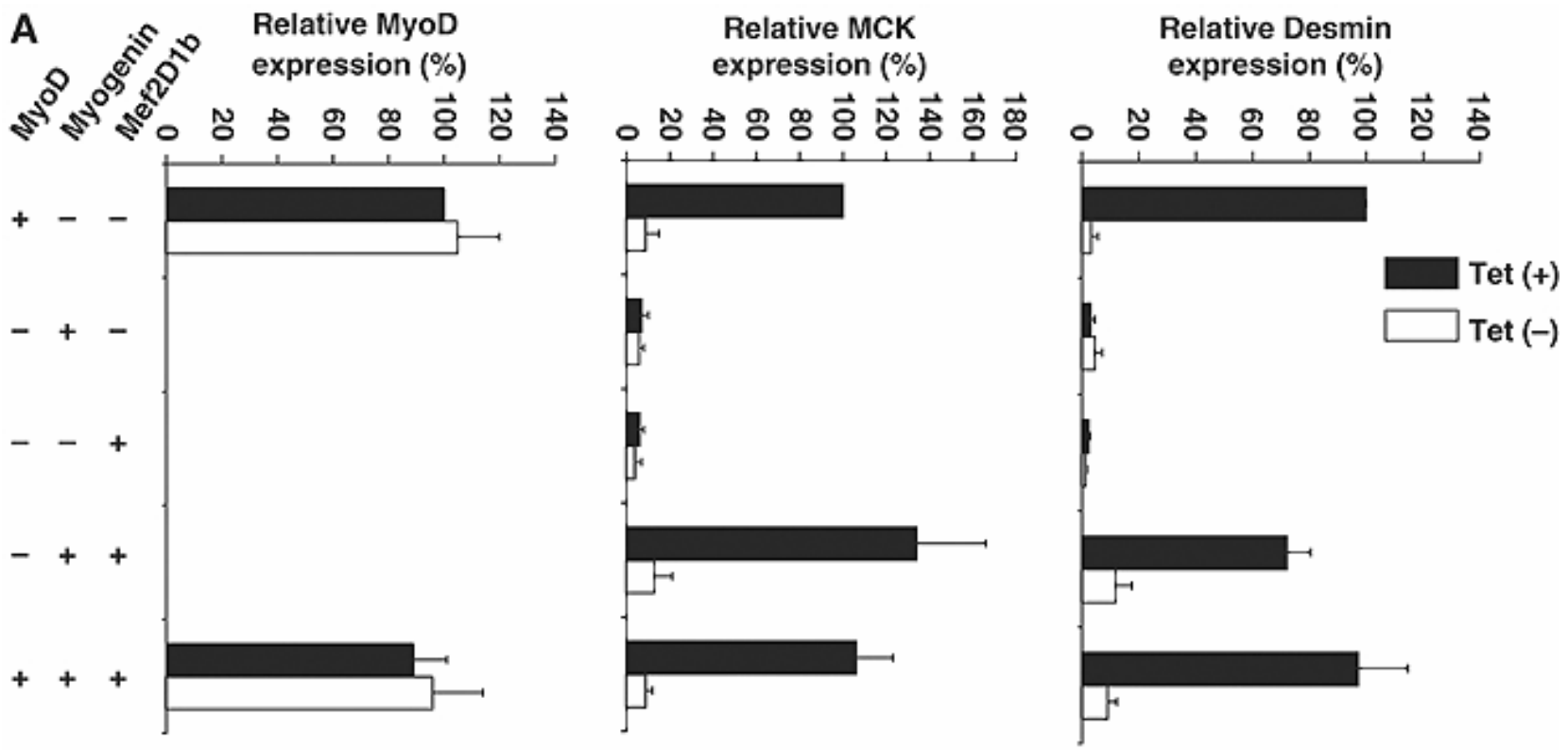
Fig. 5: BRG1, myogenin, and MEF2 form a protein complex during MyoD-directed myogenesis in fibroblasts (Re-ChIP & IP)

Fig. 6: MyoD alone or Mef2D with myogenin can induce skeletal myogenesis in fibroblasts (IF & Q-PCR)

Do Myogenin and Mef2D1b require Brg1 to induce myogenesis?

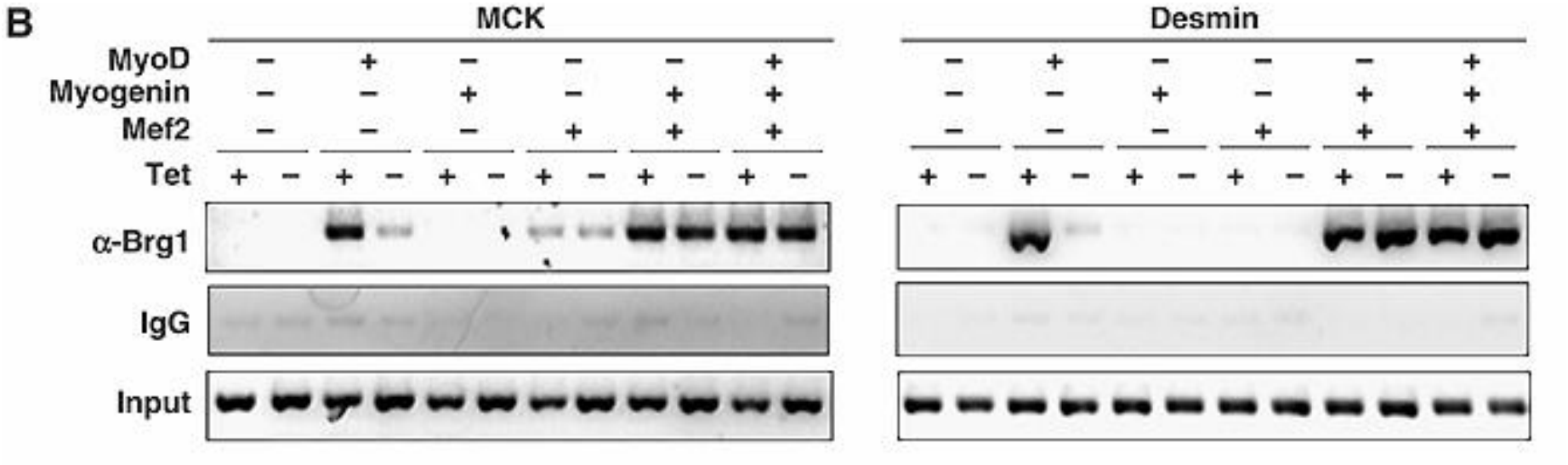
Figure 7A Myogenin and Mef2D induce myogenesis in B22 fibroblasts

Q-PCR analysis



Found: Myogenin and MEF2D1b together can induce the expression of desmin and MCK in fibroblasts, in a BRG-1-dependent manner.

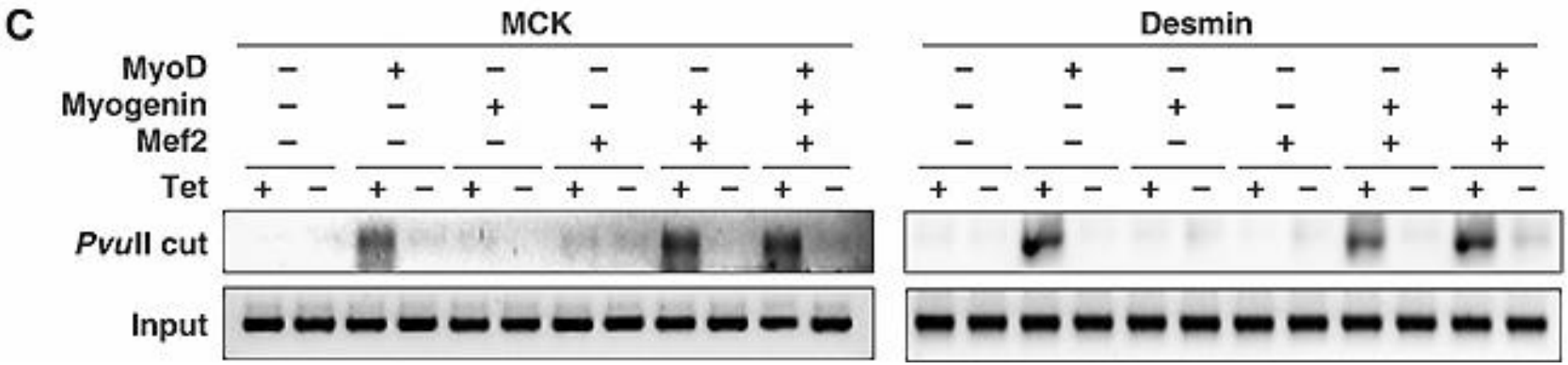
Fig. 7B Myogenin and Mef2D recruit Brg-1 to Desmin and MCK promoters (ChIP)



Found: ChIP assays show that coexpression of Myogenin and Mef2D can promote the targeting of Brg-1 to the regulatory sequences of Desmin and MCK.

Note: It is not really clear why Myogenin & MEF2 can recruit DNBrg-1, but MyoD cannot.

Fig. 7C Myogenin and Mef2D induce myogenesis by promoting chromatin remodeling by Brg-1



Coexpression of Mef2D1b and myogenin increases chromatin accessibility in a Brg-1 dependent manner.

Summary:

Fig. 1: Increased chromatin accessibility correlates with increased MEF2D, MCK, and desmin mRNA levels in the embryo (accessibility & Q-PCR)

Fig. 2: MyoD and HDAC2 are bound to the MCK/desmin regulatory sequences early in the embryo, and myogenin, MEF2D1B, and Brg1 are bound late (ChIP)

Fig. 3: MyoD-directed differentiation and opening of chromatin were inhibited by the expression of dominant negative BRG1 in fibroblasts (accessibility)

Fig. 4: Binding of early and late factors in MyoD-directed fibroblast myogenesis is the same as in the embryo (Fig. 2) and inhibited by BRG1 (ChIP)

Fig. 5: BRG1, myogenin, and MEF2 form a protein complex during MyoD-directed myogenesis in fibroblasts (Re-ChIP & IP)

Fig. 6: MyoD alone or Mef2D with myogenin can induce skeletal myogenesis in fibroblasts (IF & Q-PCR)

Fig. 7: Myogenin and MEF2D induce myogenesis by promoting Brg-1-induced chromatin changes, shown by approaches from Fig. 3 & 4.

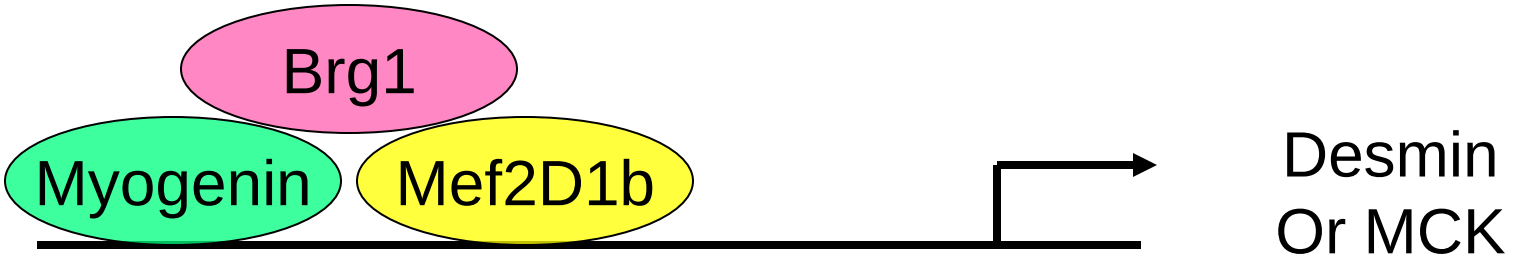
Summary of Experiments in Paper #3

System/Assay	Expression (Q/RT-PCR)	Chromatin accessibility	ChIP	Re-ChIP	IP	IF
Embryo	Fig.1	Fig. 1	Fig. 2	-		-
MyoD-B22	Fig.3 and 6	Fig. 3	Fig.4	Fig. 5	Fig. 5	Fig. 6
Myogenin/Mef-B22	Fig. 6	Fig. 7	Fig. 7	-	-	Fig. 6

Need to know Figs. 1-5 for the exam

*

The data from myogenin and Mef2D1b-directed myogenesis in fibroblasts is consistent with the following model:

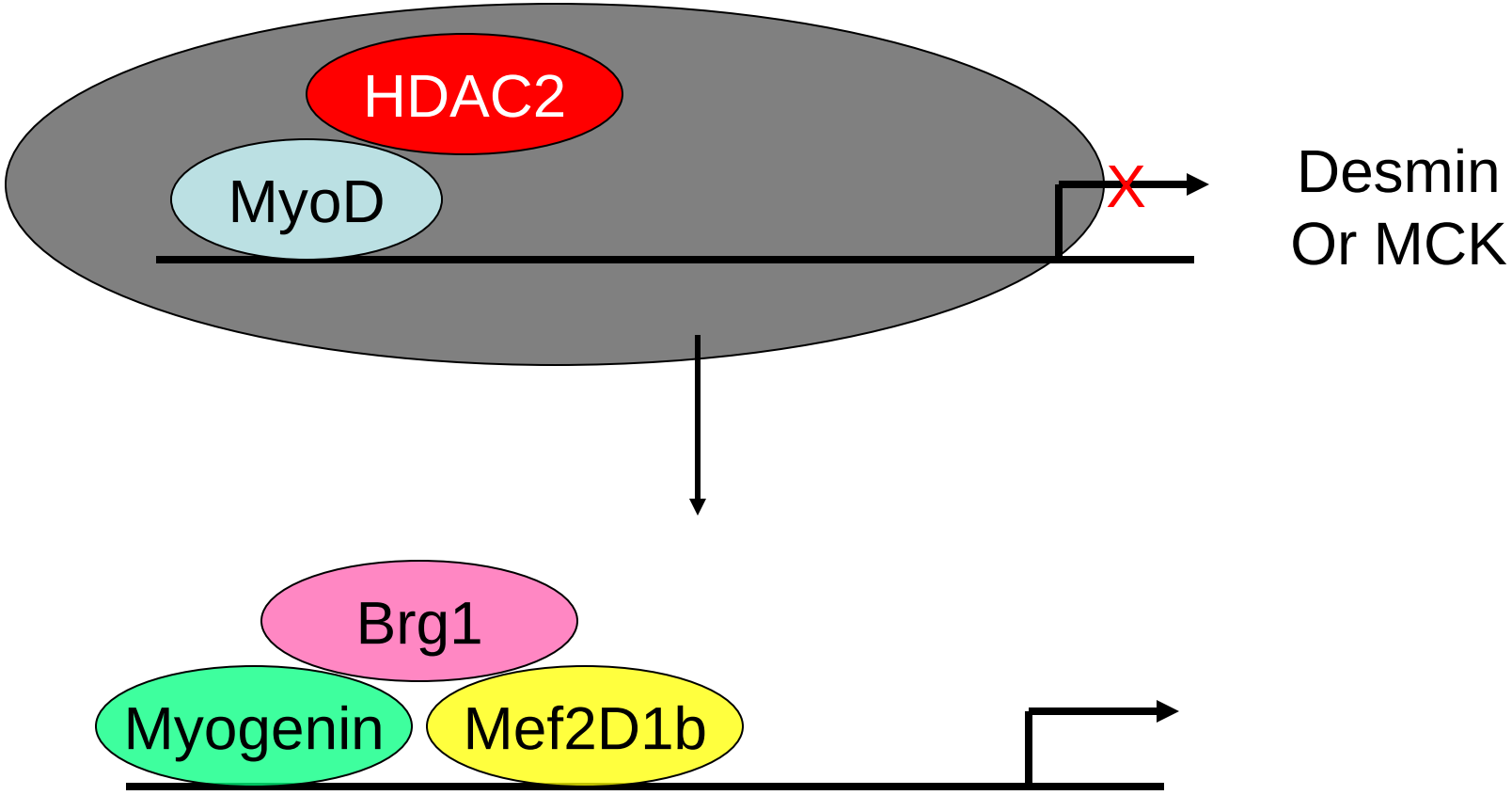


Conclusions

- Myogenin and Mef2D are sufficient to induce skeletal myogenesis, in a Brg1-dependent manner, in the absence of MyoD.
- Myogenin and Mef2D cooperate to promote chromatin remodeling by SWI/SNF at myogenic late gene regulatory sequences.
- MyoD can bind the promoters of late myogenic genes but the induction of these same genes is coincident with the replacement of MyoD and HDAC2 with myogenin, Mef2D and Brg1.

*

Model



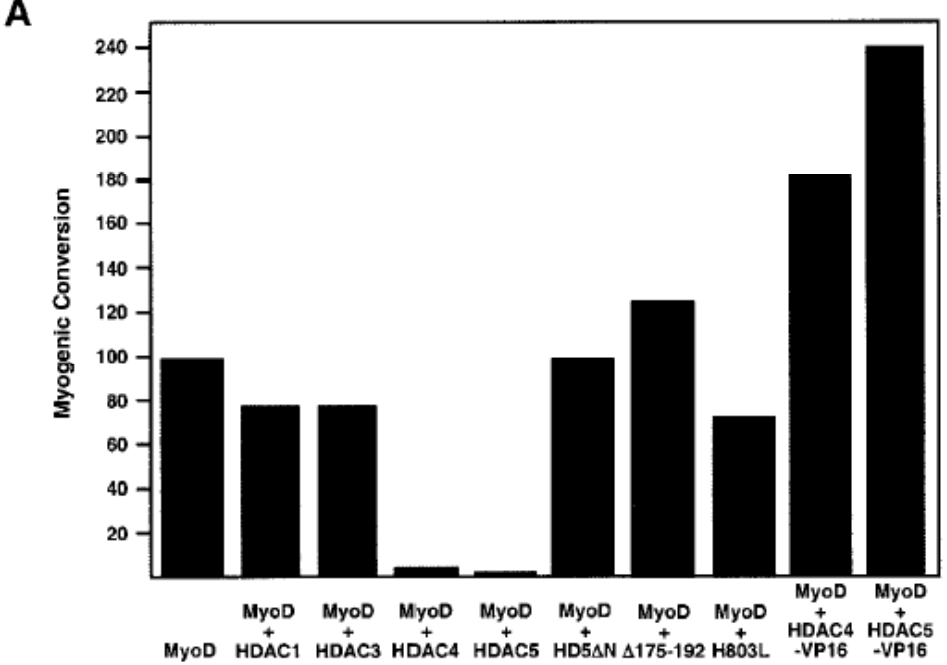
Does this contradict the first 2 papers?

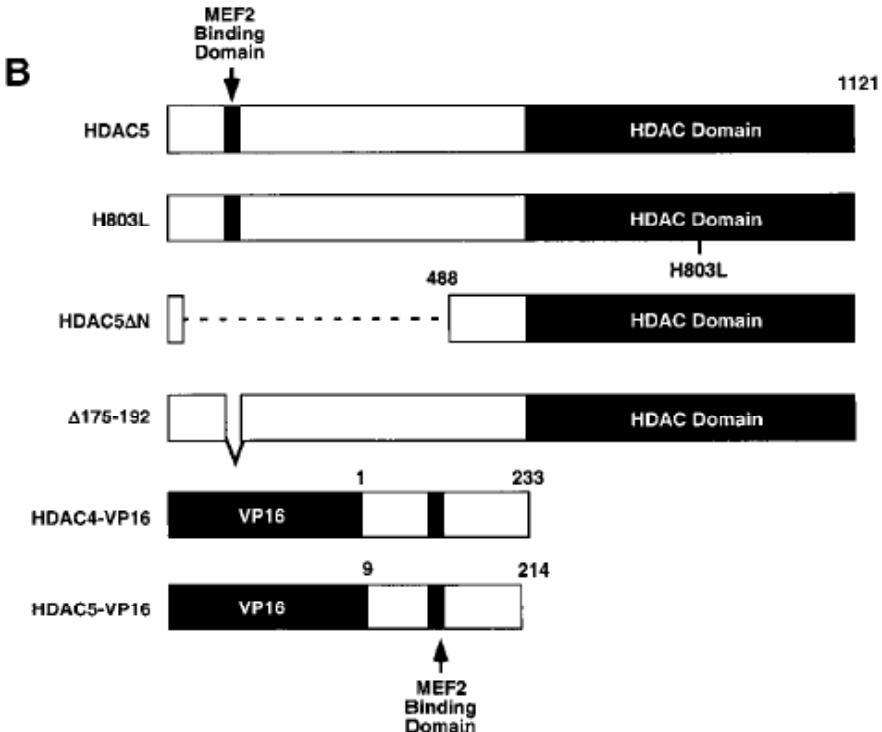
* Fig. 1 Paper #1 Class II HDACs inhibit MyoD function

% Myogenic conversion where 100% = 50 cells/coverslip

They didn't look at HDAC2!

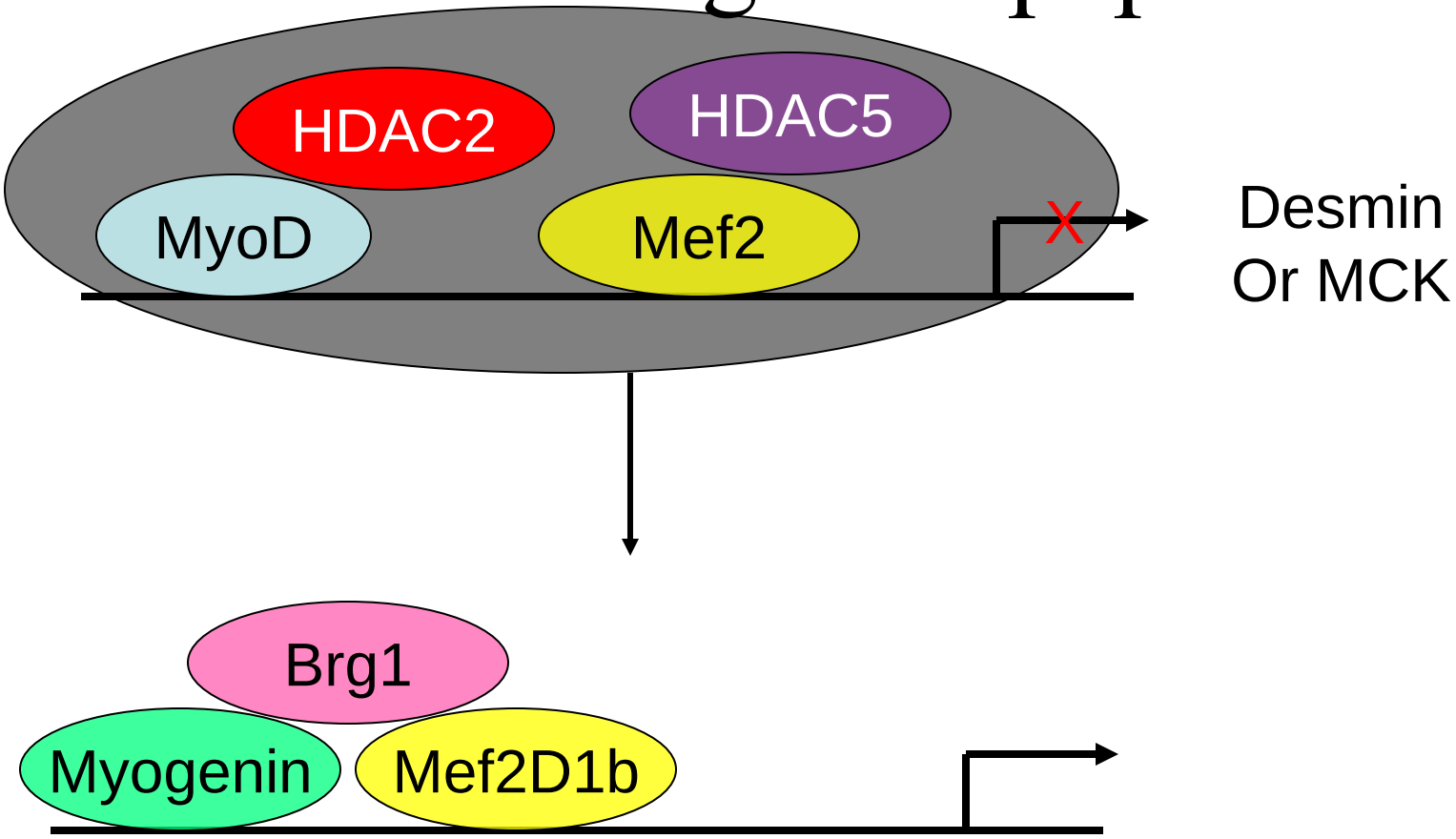
They didn't do ChIP for myogenin





*

Model combining all 3 papers



- Fig 4A shows MEF2 bound to MCK promoter at early times
- The EMBO J paper never looked at class II HDACs

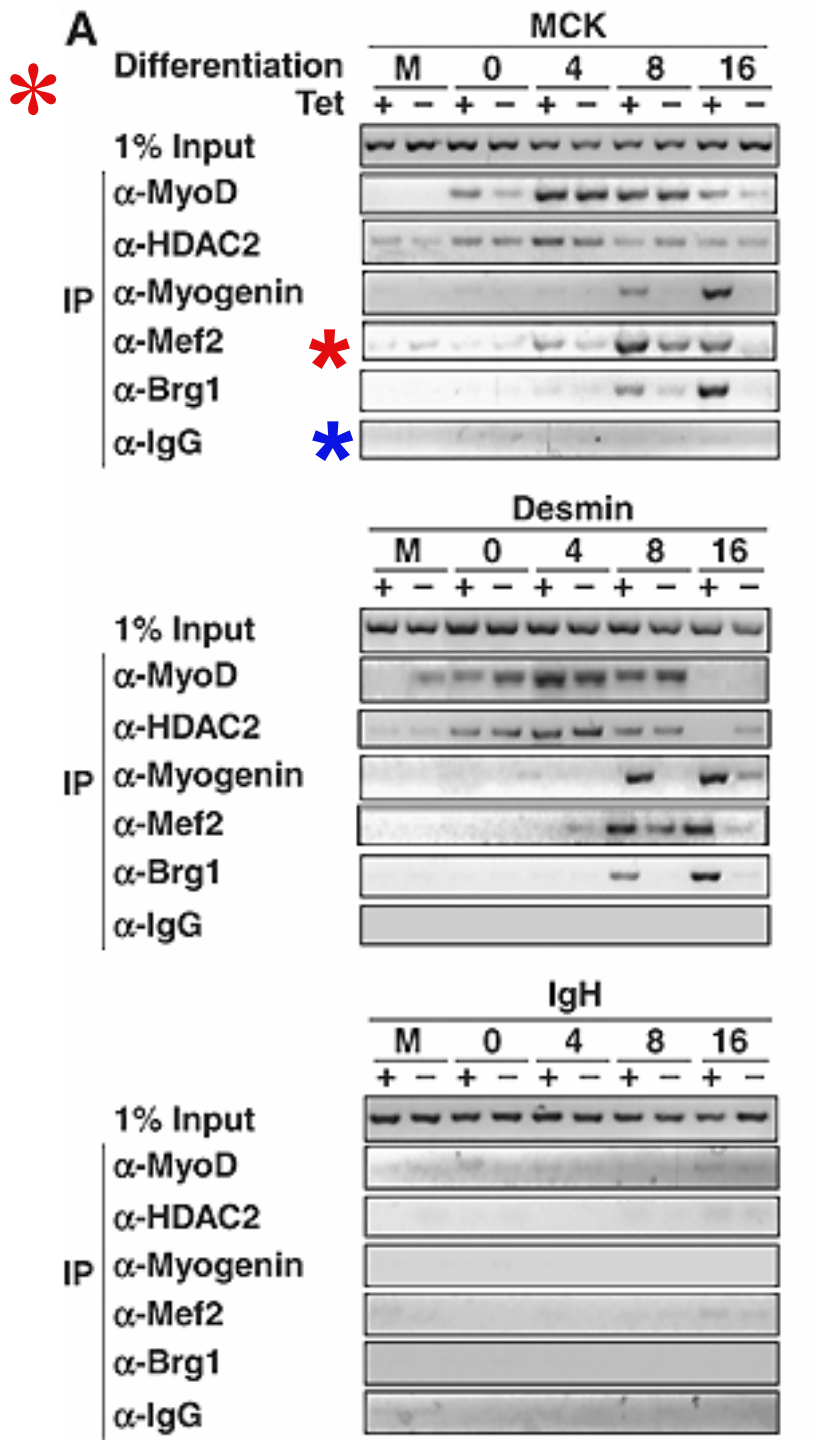


Fig. 4A ChIP assays were performed on mock (M) or MyoD differentiated cells (0-16 hours).

Found:

•Low levels of MEF2 are in fact present on the MCK promoter * over the IgG background * in the mock transfected and day 0 transfected cells.

Is the data convincing that MEF2D1b specifies myotubes?

- Showed that MEF2D1b, when transfected with myogenin, made myotubes (Fig. 6A)
- Missing the negative controls - would the other MEF2 isoforms give the same answer?
- Embryo data confusing because desmin and MCK are expressed on E10.5! Likely, their results are due to the relative amount of muscle present at early times and the way they collected and compared RNA

Why is this paper important?

- It provided the first evidence that Brg1 was essential for skeletal myogenesis and that myogenin and MEF2D can cooperate to induce myogenesis