 Marks the important slides

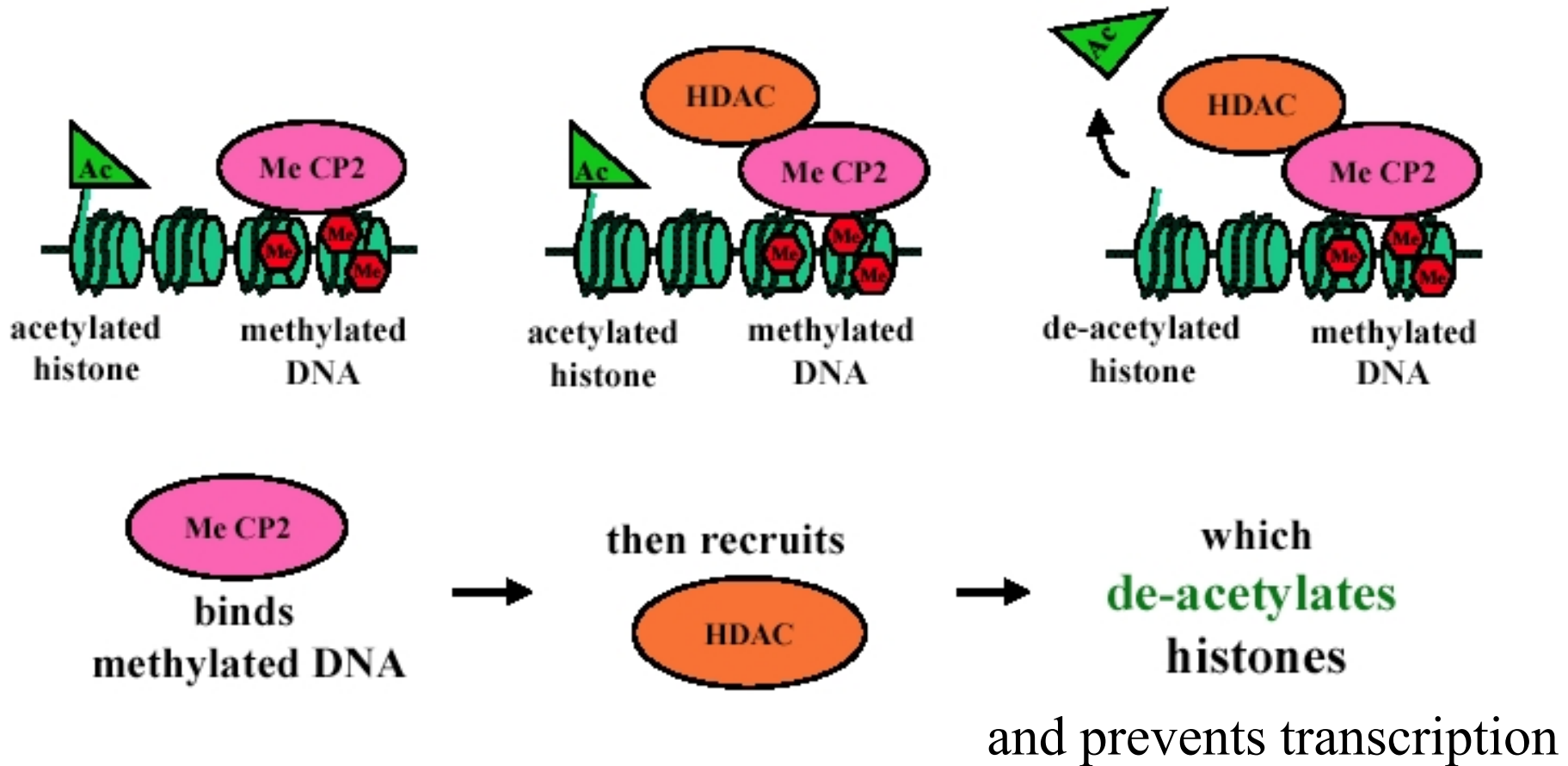
Paper #4: Pax7 activates myogenic genes by recruitment of a histone methyltransferase complex

I.W. McKinnel, J. Ishibashi, F. Le Grand, V.G. J. Punch, G.C. Addicks, J.F. Greenblatt, F.J. Dillworth, and M.A. Rudnicki,

Nature Cell Biology (2008) 10, 77-84.
(IF=19.5, 45 citations)

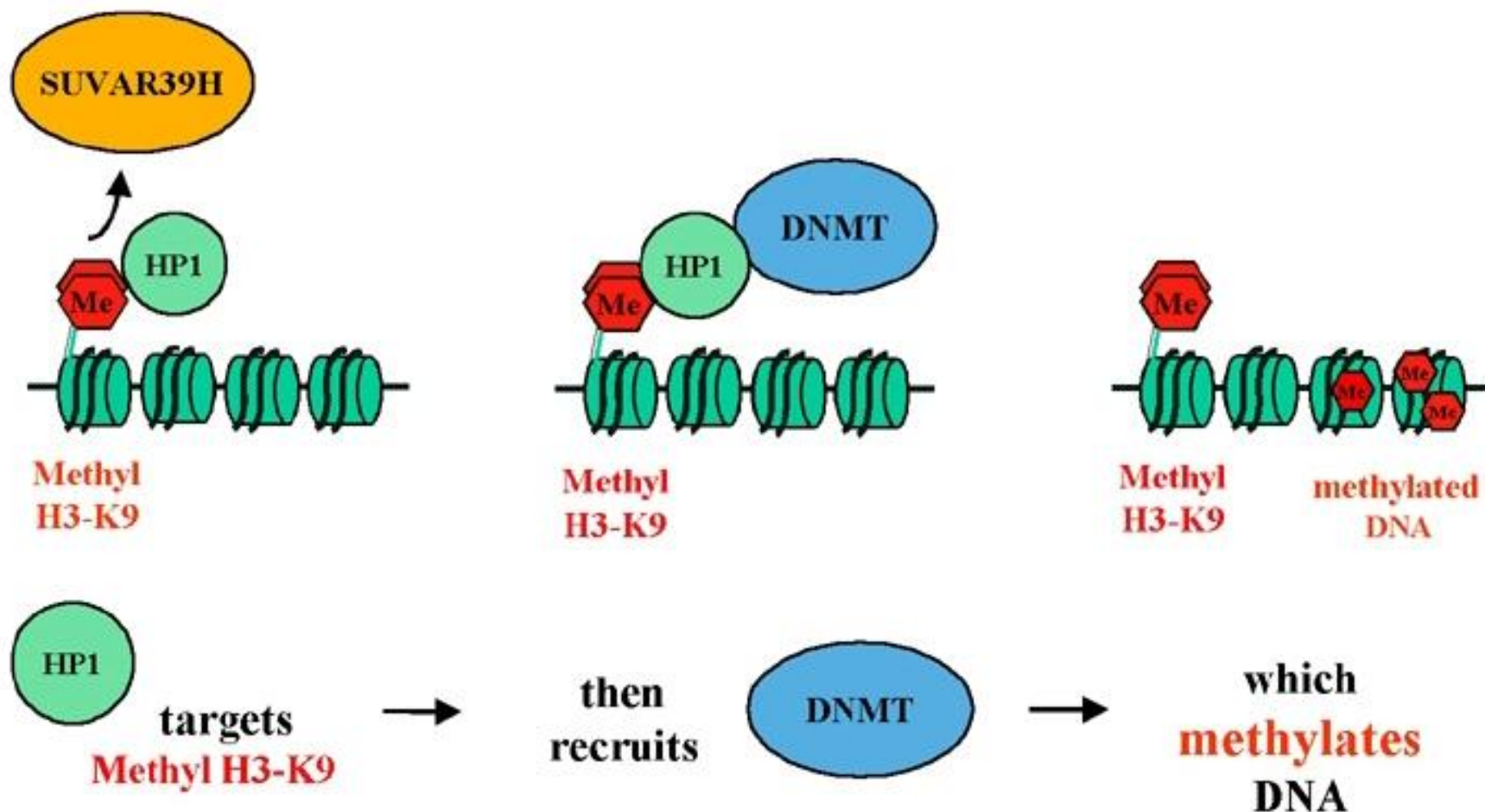
Where does methylation fit into the regulation of transcription?

DNA methylation induces histone de-acetylation



(Ac= Acetyl; Me= Methyl; MeCP2= Methyl-CpG binding Protein 2; HDAC= Histone De-Acetylase)

Histone H3-K9 methylation induces DNA methylation

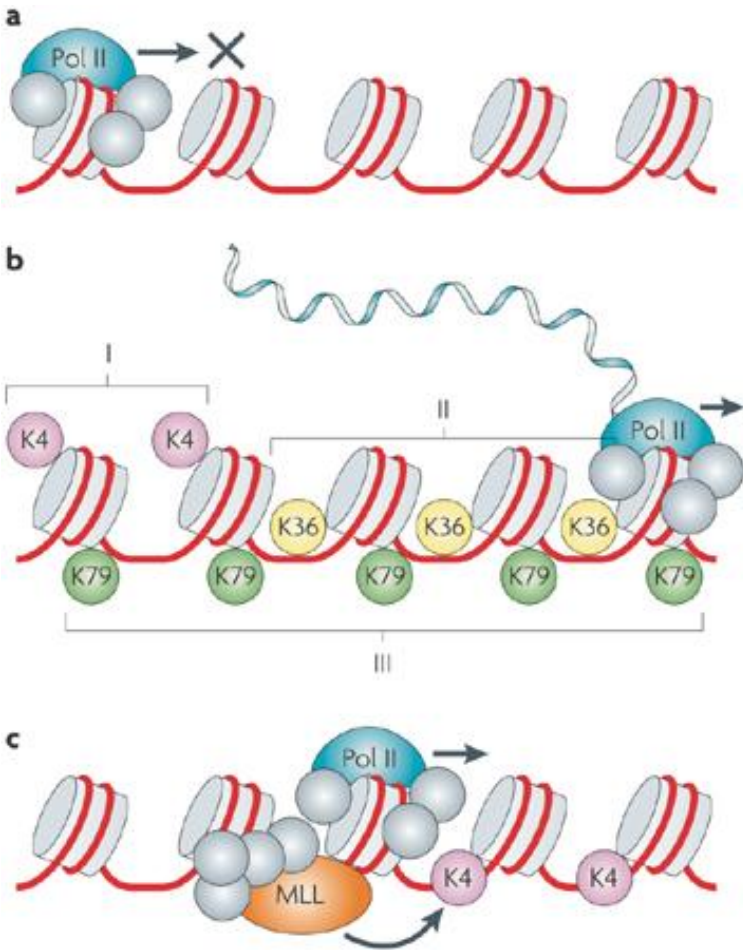


SUVAR39H is a methyltransferase which methylates the Lysine 9 of histone H3, creating a binding site for the Heterochromatin Protein HP1, which recruits a DNA methyltransferase, which methylates CpG in DNA.

(Me= Methyl; Methyl H3-K9= Methyl on Lysine 9 of Histone H3; HP1=Heterochromatin Protein 1; DNMT=DNA Methyltransferase).

Does methylation always
mean inhibition?

Emerging roles for histone methylation

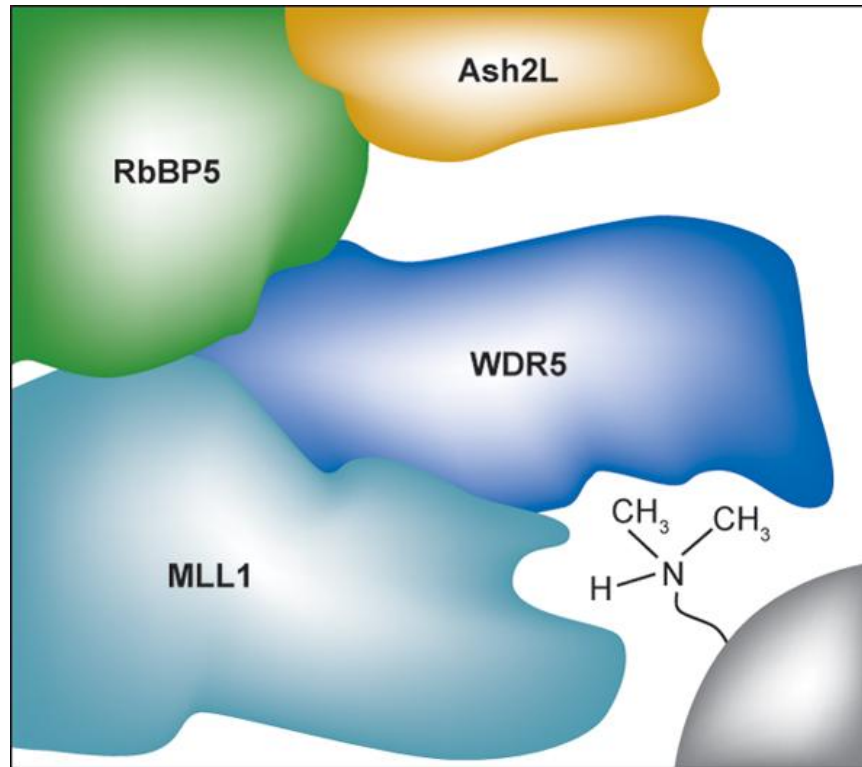


a) RNA polymerase II (Pol II) binds the promoter region of the gene but cannot proceed without specific methylation marks on histone.

b) RNA transcription may be initiated when a promoter region (I) carries histone H3 lysine 4 (H3K4) methylation, and extend when an open-reading frame region (II) carries H3K36 methylation. H3K79 methylation has a broad distribution across promoter and open-reading frame regions (III).

c) Mixed lineage leukaemia (MLL) is a member of a multiprotein complex that mediates methylation of H3K4 within the promoter region of genes occupied by RNA polymerase II.

The MLL complex



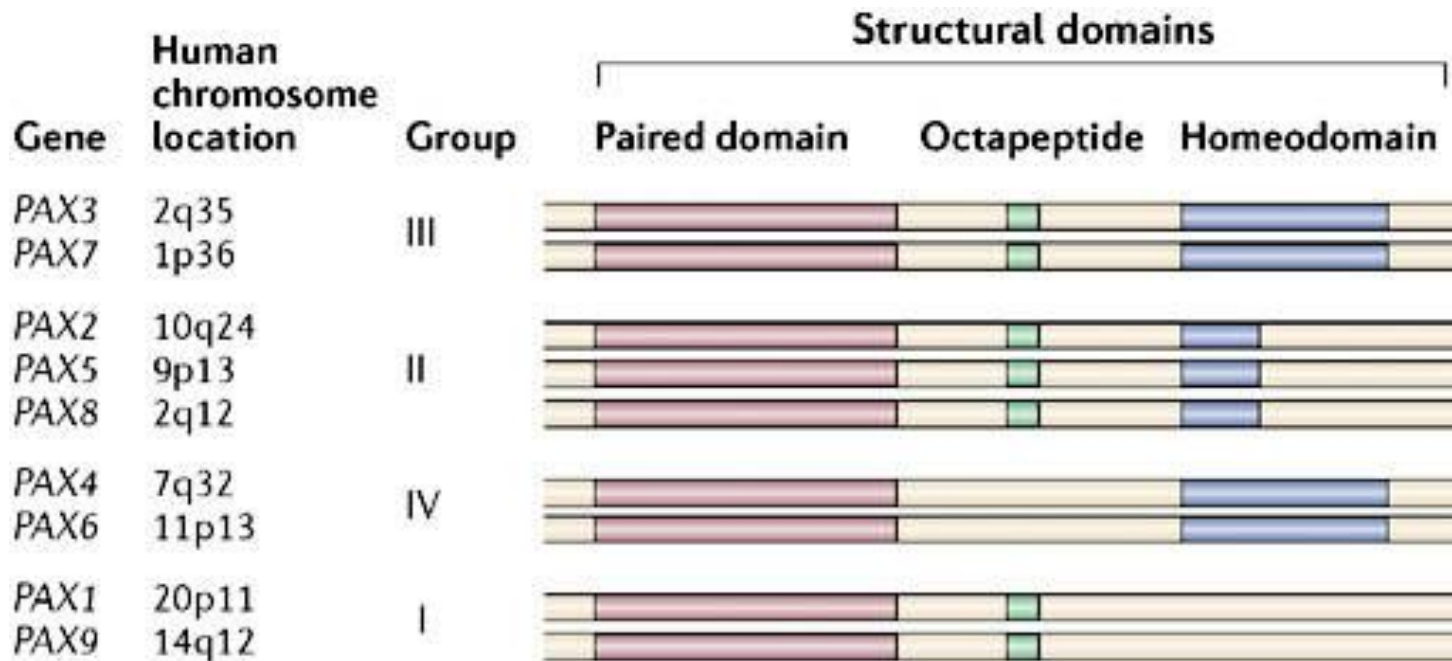
Nomenclature:
H3K4
H3K4-me
H3K4-2me
H3K4-3me

WDR5 interacts with methylated H3K4, presenting it for further methylation by linking the catalytic MT (methyltransferase) domain of MLL1 with its substrate. This interaction is necessary for H3K4 trimethylation and subsequent transcription of MLL1 target genes.

What targets the MLL complex
to specific genes?

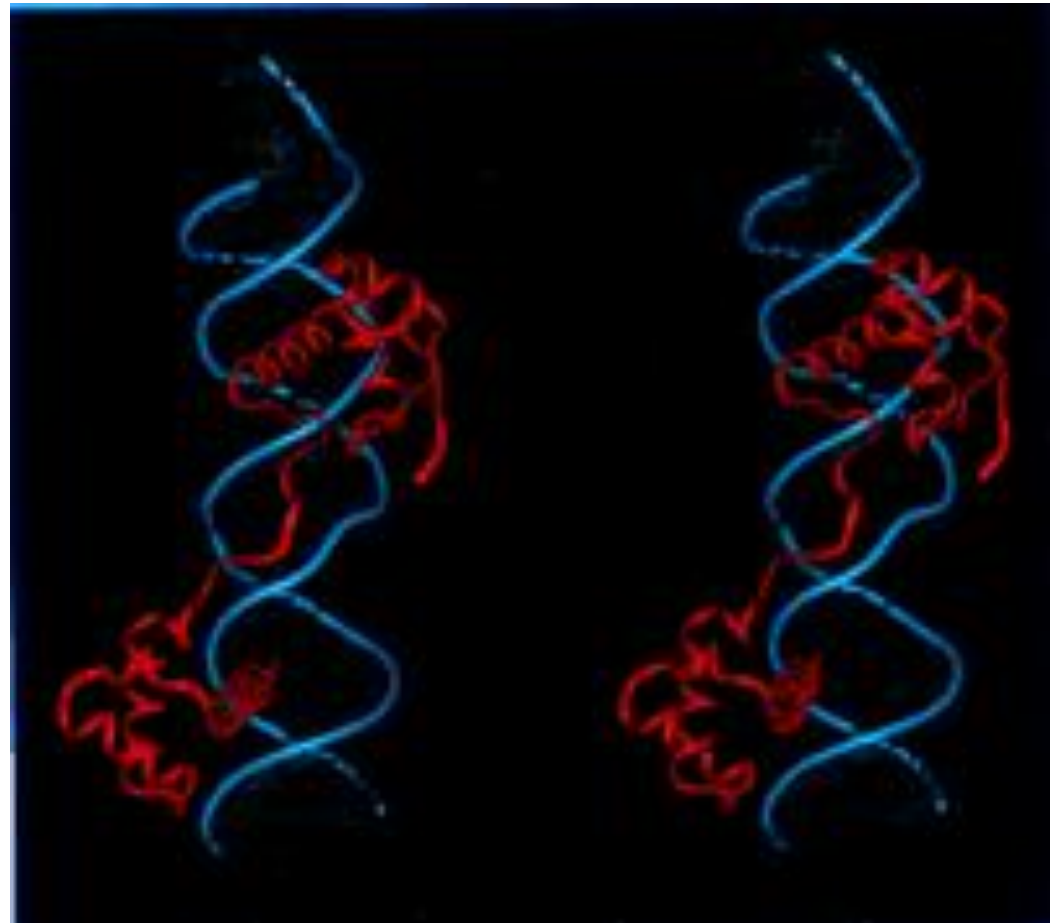
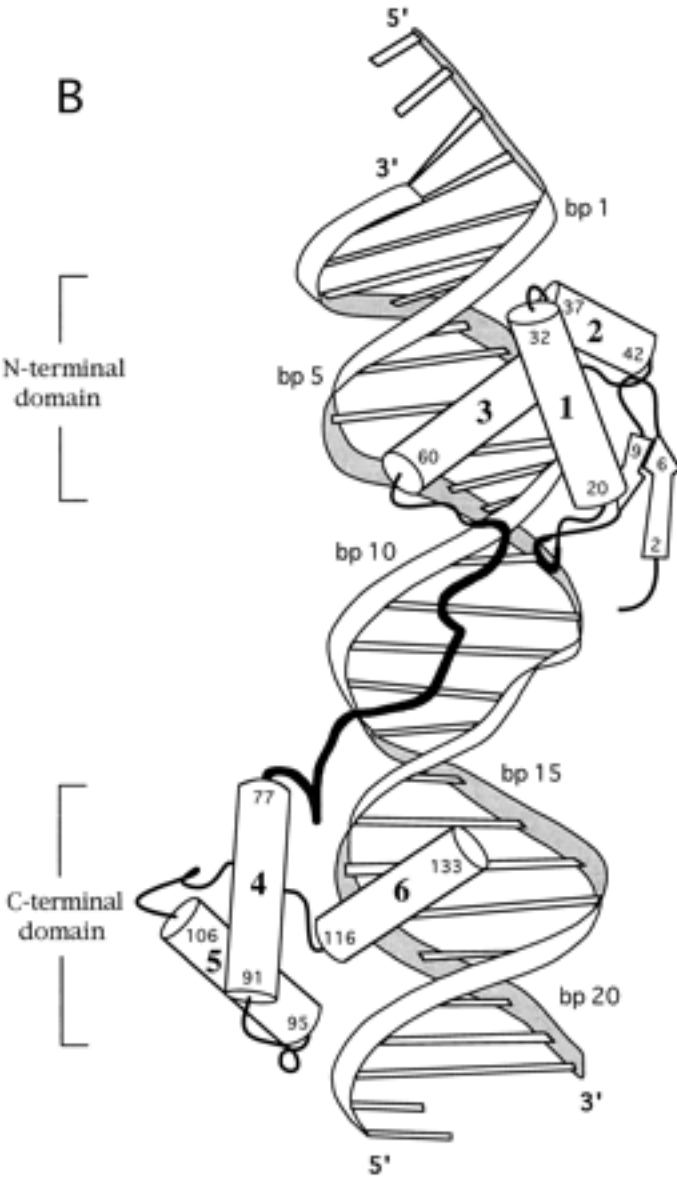
What is the role of Pax7
transcription factors?

The structure of Pax family members



Robson EJD *et al.* (2006) A PANorama of PAX genes in cancer and development
Nat. Rev. Cancer. **6**: 52–62 doi:10.1038/nrc1778

Structure of the Pax6 paired domain with DNA



What is the role of Pax3/7 in myogenesis?

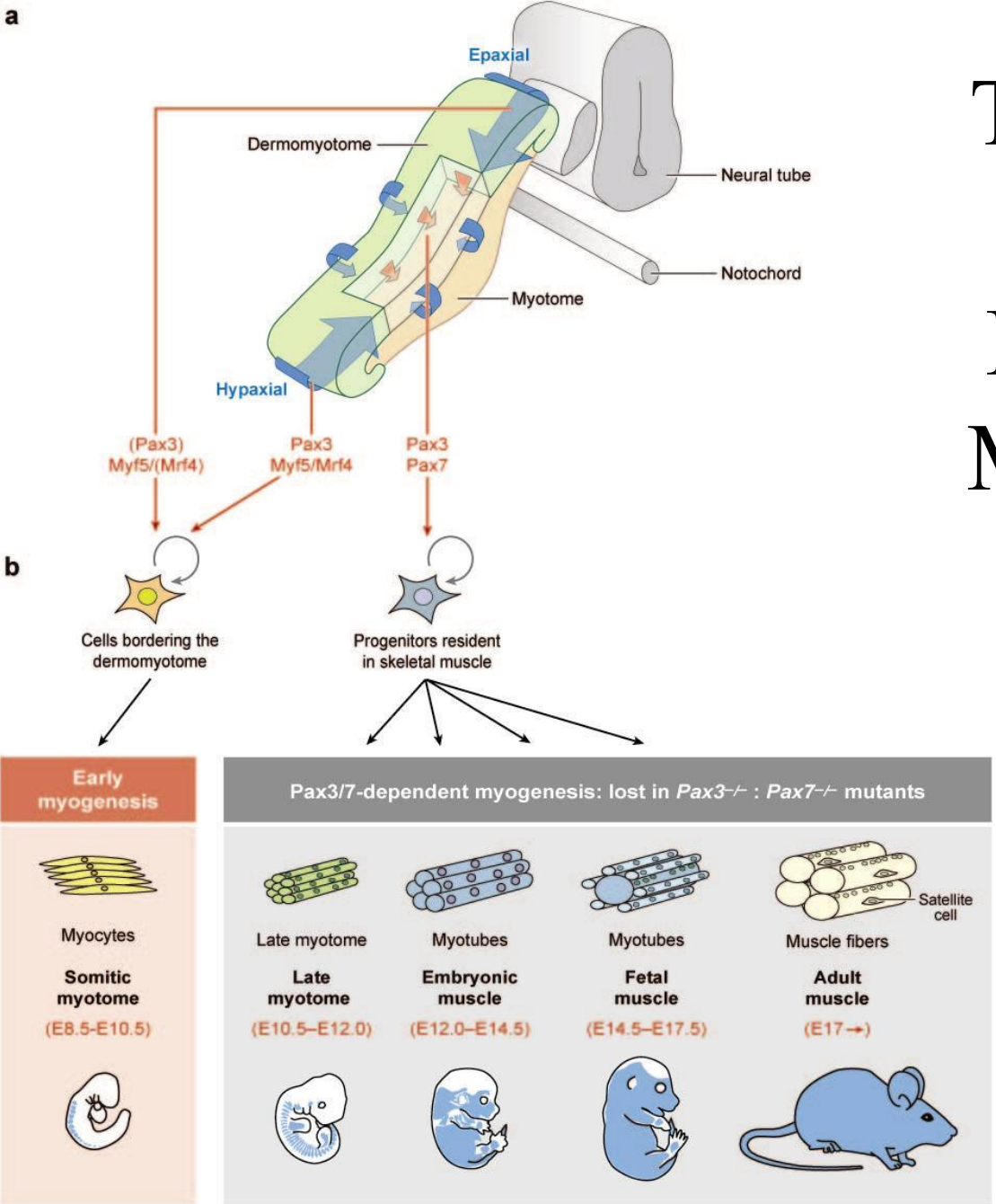
Mice lacking Pax3/7 have defects in muscle development

Pax3 $-/-$ mice: Do not form limb muscle

Pax7 $-/-$ mice: Do not form satellite cells

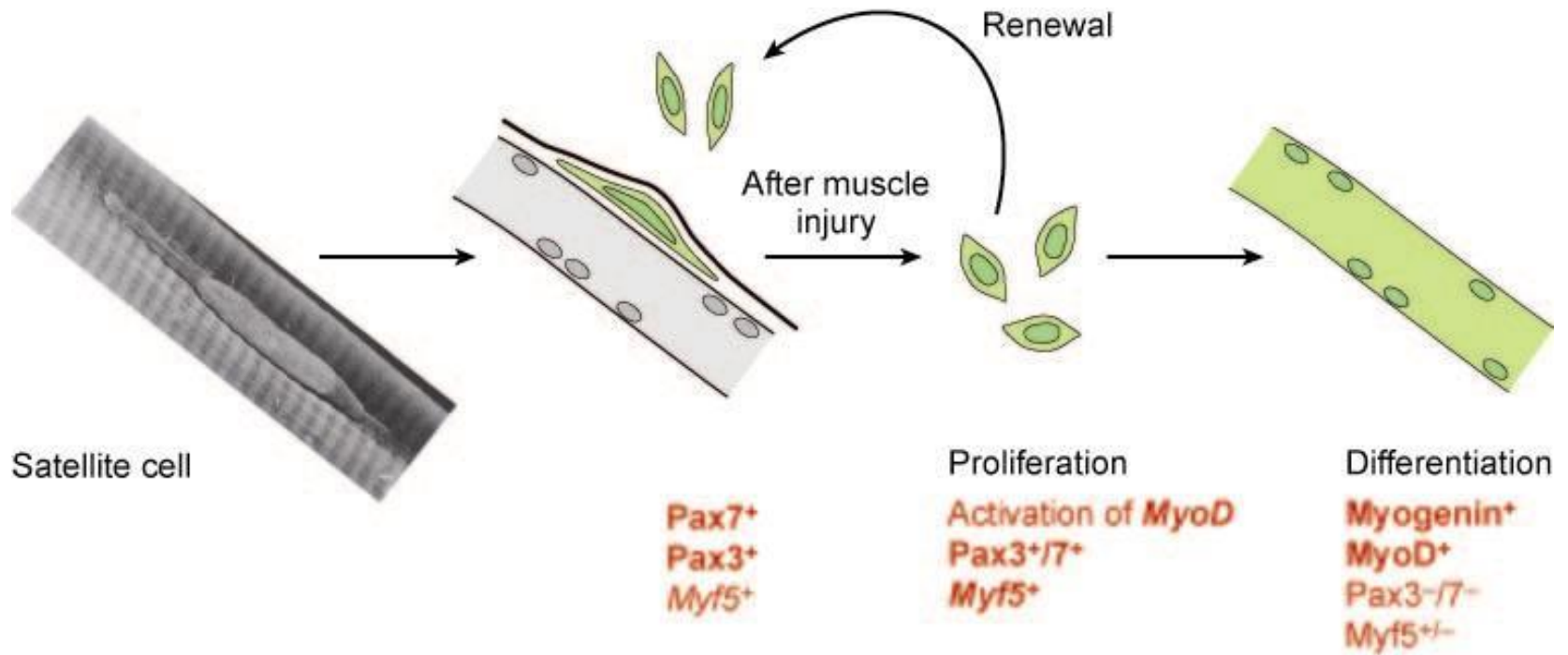
Pax3 $-/-$, Pax7 $-/-$ mice: Do not form most muscle or satellite cells

The Role of Pax3/7 in Embryonic Myogenesis



Annual Reviews

The Role of Pax3/7 in Satellite Cell Repair of muscle



AR Buckingham M, Relaix F. 2007.
Annu. Rev. Cell Dev. Biol. 23:645–73

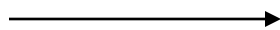
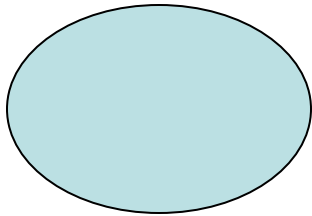
Hypothesis:

Pax7 regulates muscle development
by recruiting methyltransferases to
the Myf5 locus

(Remember: Myf5 is one of the 4
MRFs)

What genes are regulated by Pax7?

Pax7-flag retrovirus



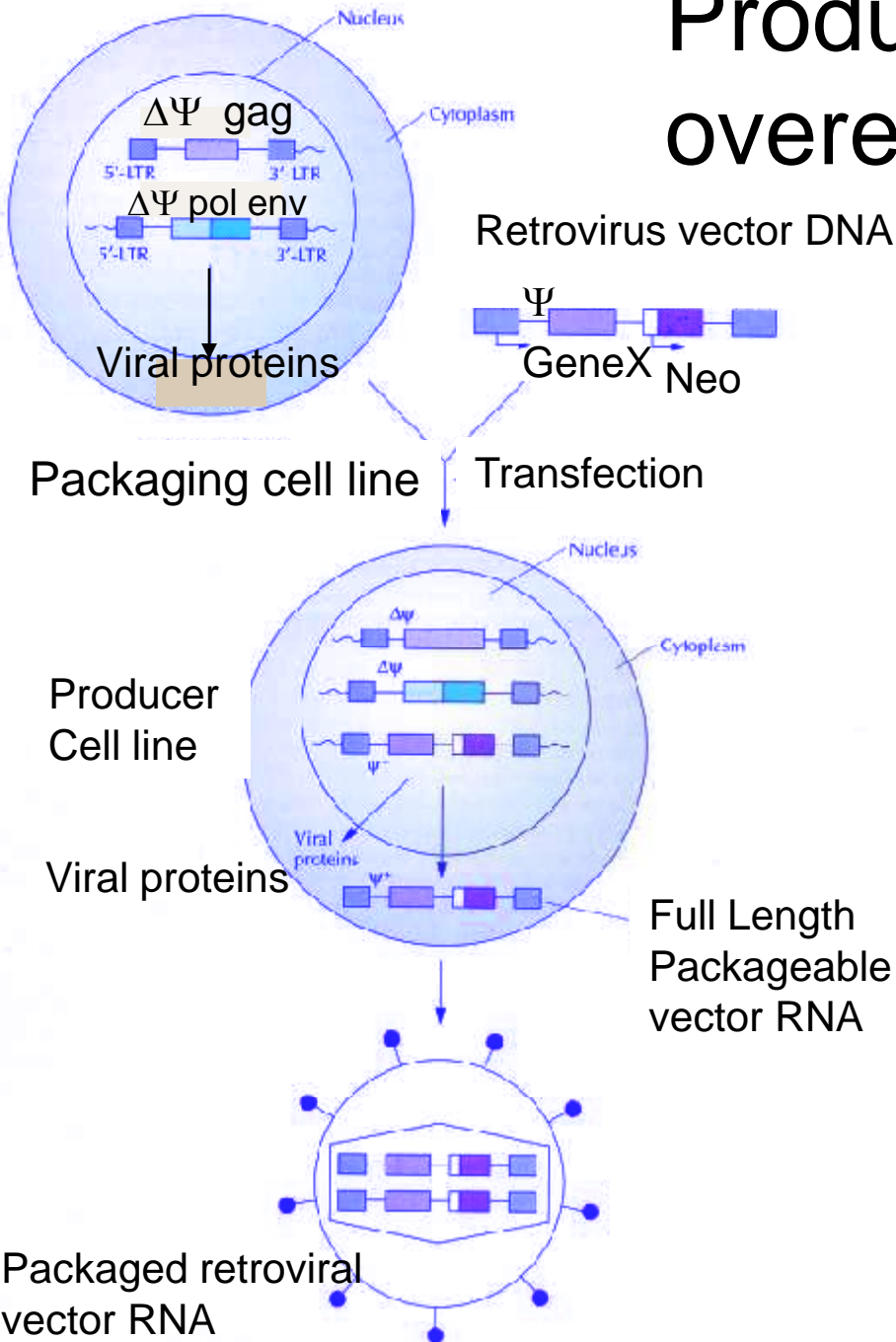
Microarray analysis



C2C12 myoblasts

Validation of genes

Production of retrovirus for overexpression of Gene X



Ψ =encapsidation sequence

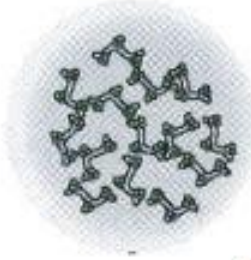
Transfection of the retrovirus vector into the packaging cell line results in packaged retroviral vector RNA, which can infect cells, causing Gene X to be expressed, but can't create more virus particles.

gag: Internal capsid structural proteins.
pol: reverse transcriptase and integrase.
env: envelope protein.
LTR: long terminal repeat

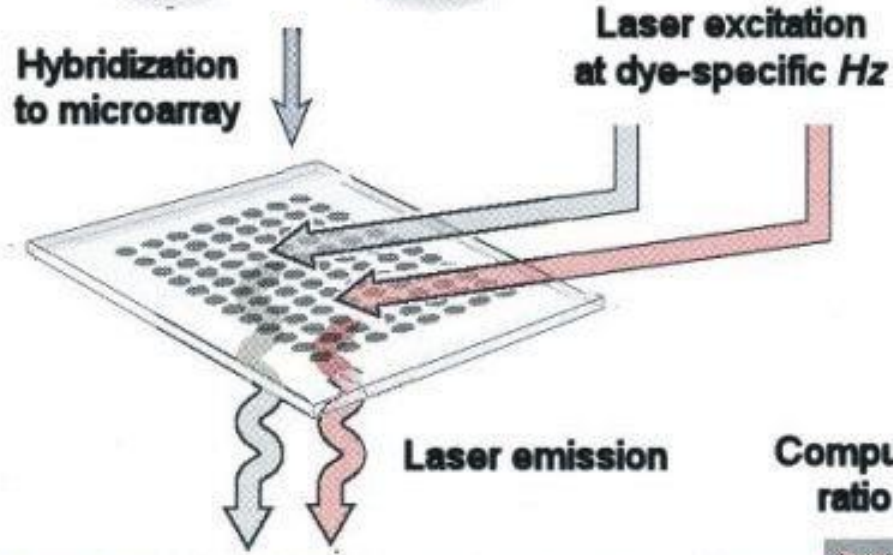
Make cDNA reverse transcript
Label cDNAs with fluorescent dyes

Control

Experimental



Principle of cDNA microarray
assay for gene expression
(after Gibson & Muse 2002)



Red = "up-regulation"
Green = "down-regulation"
Black = constitutive expression

Computer calculates
ratio of intensity

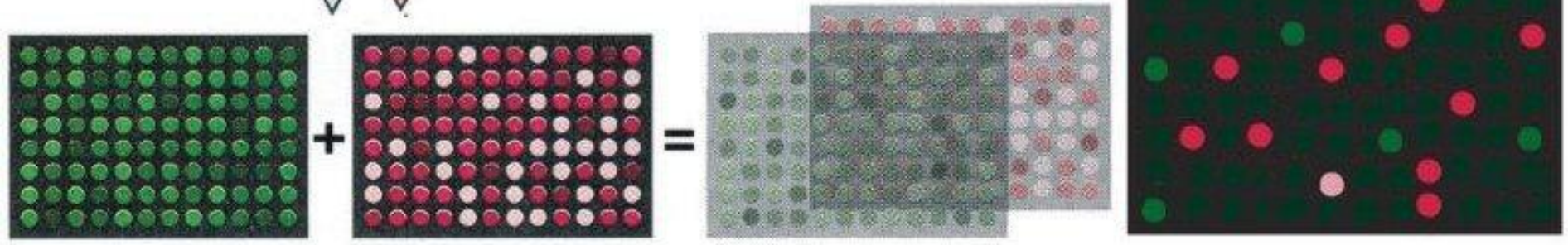
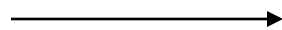
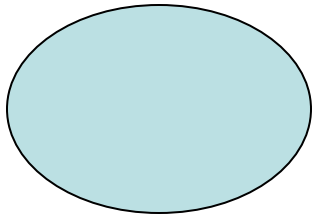


Table 1. Pax7-induced increases in expression in C2C12 myoblasts

Symbol	GID	Fold	Name	RLT-PCR
Plagl1	NM_009538	385	Pleiomorphic adenoma gene-like 1	135.8
Lix1	NM_025681	12	Limb expression 1 homolog (chicken)	34.8
Syne2	BF582734	11	Synaptic nuclear envelope 2	2.2
Cipar1	AK008716	8.1	Castration-induced prostatic apoptosis-related 1	166.6
Trim54	NM_021447	7.2	Tripartite motif-containing 54	4.1
Il13ra1	NM_133990	5.4	Interleukin 13 receptor, alpha 1	-
Mest	NM_008590	5	Mesoderm specific transcript	17.9
Peg3	NM_008817	4.6	Paternally expressed 3	-
3110001A13Rik	NM_025626	4.6	RIKEN cDNA 3110001A13 gene	-
Ahr	NM_013464	4.4	Aryl-hydrocarbon receptor	-
Npnt	NM_033525	4.1	Nephronectin	-
Ltb4dh	NM_025968	4.1	Leukotriene B4 12-hydroxydehydrogenase	-
Msln	NM_018857	4.1	mesothelin	-
C1qtnf3	NM_030888	3.8	C1q and tumour necrosis factor related protein 3	-
-	BB369191	3.7	Sim. to mouse pentylenetetrazol-related mRNA	-
Pparg	NM_011146	3.4	Peroxisome proliferator activated receptor gamma	-
Cd24a	NM_009846	3.1	CD24a antigen	-
Pkia	NM_008862	3	Protein kinase inhibitor, alpha	-
Nqo1	NM_008706	2.9	NAD(P)H dehydrogenase, quinone 1	-
Marcks	NM_008538	2.6	Myristoylated alanine-rich protein kinase C substrate	-
Gch1	NM_008102	2.6	GTP cyclohydrolase 1	-
Cdh11	NM_009866	2.6	Cadherin 11	-
Myo1d	NM_177390	2.5	Myosin ID	-
Cxcr4	NM_009911	2.5	Chemokine (C-X-C motif) receptor 4	-
Crlf1	NM_018827	2.5	Cytokine receptor-like factor 1	-
Sema3e	NM_011348	2.4	Semaphorin 3E	-
Mdfic	NM_175088	2.4	MyoD family inhibitor domain-containing	-
Ass1	NM_007494	2.4	Argininosuccinate synthetase 1	-
Id3	NM_008321	2.4	Inhibitor of DNA binding 3	-
Ppap2a2	NM_008903	2.3	Phosphatidic acid phosphatase 2a isoform 2	-
Myf5	NM_008656	2.2	Myogenic factor 5	3

What genes are regulated by Pax7?

Pax7-flag retrovirus



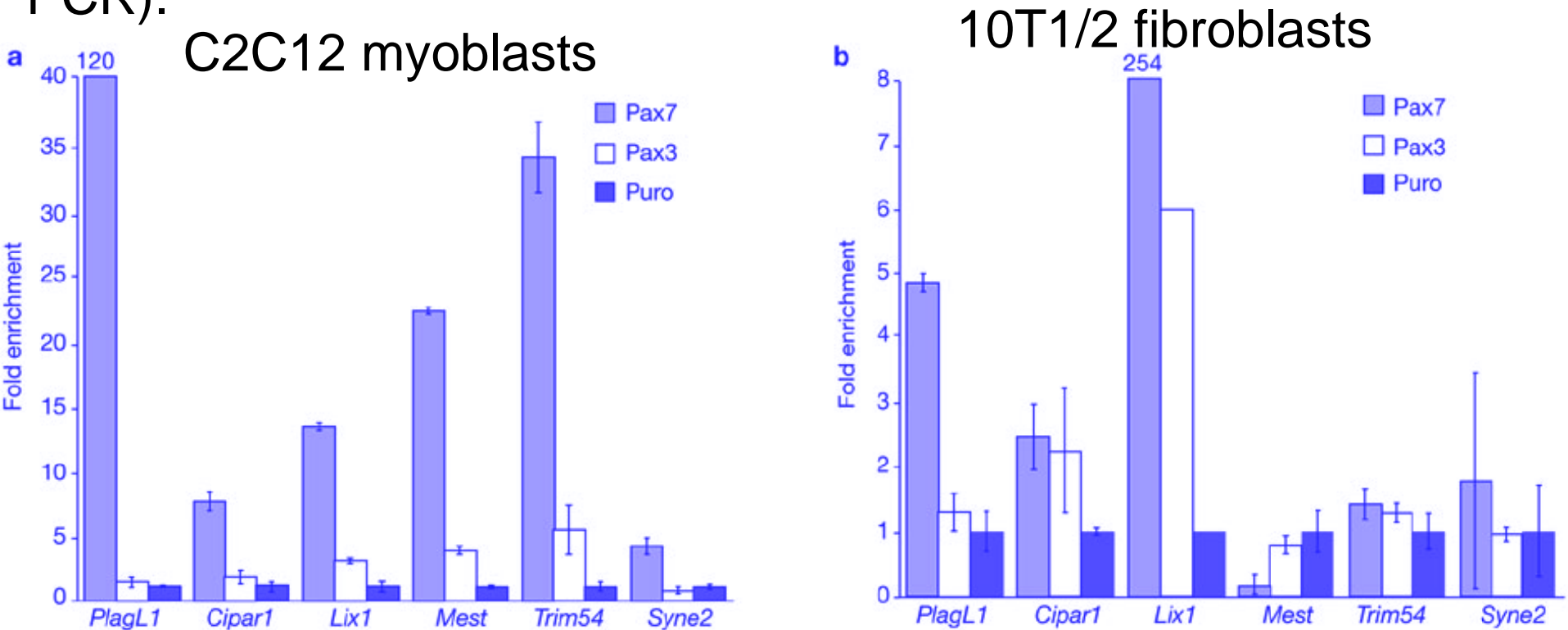
Microarray analysis



C2C12 myoblasts

Validation of genes

Figure 1. Target genes are specifically activated by Pax7 in C2C12 myoblasts and only weakly responsive to Pax3 (Q-PCR).

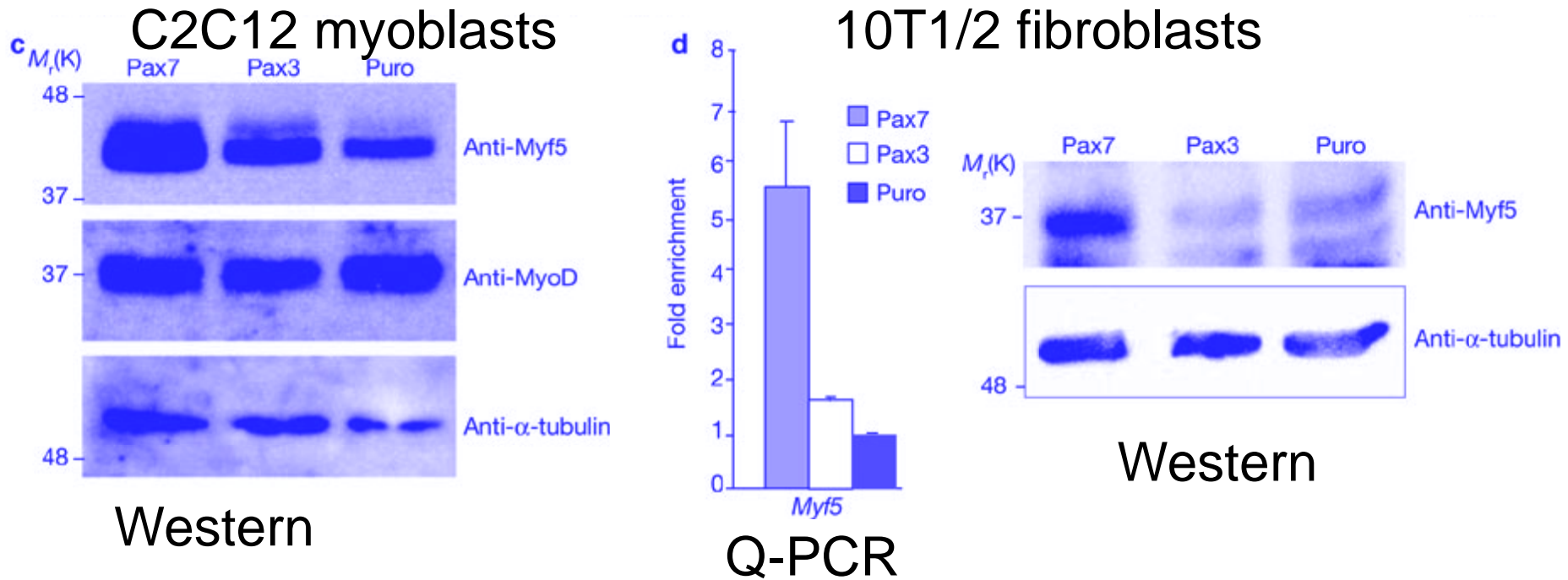


Found:

- Series of genes specifically upregulated by Pax7 and not Pax3 in C2C12 myoblasts, indicating differences between Pax3 and Pax7
- Many of these genes were not upregulated in 10T1/2 fibroblasts, suggesting other factors in myoblasts help Pax7 function.



Fig. 1 Pax7 activates Myf-5 expression



Found:

- Myf-5, but not MyoD, was upregulated by Pax7, and to a lesser extent Pax3, in C2C12 myoblasts and 10T1/2 fibroblasts.

What proteins work with Pax7?

TAP=Tandem Affinity Purification

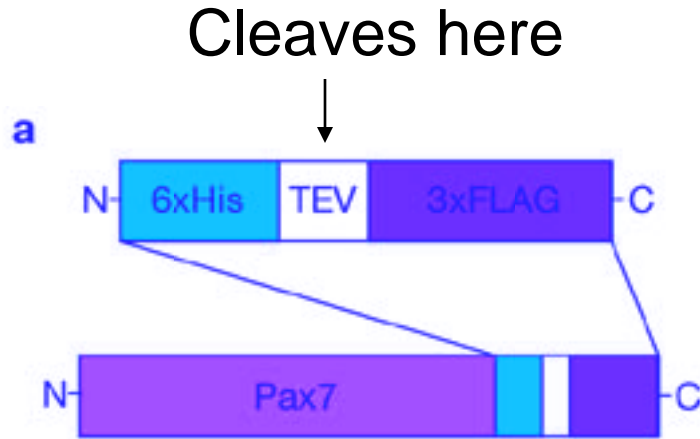
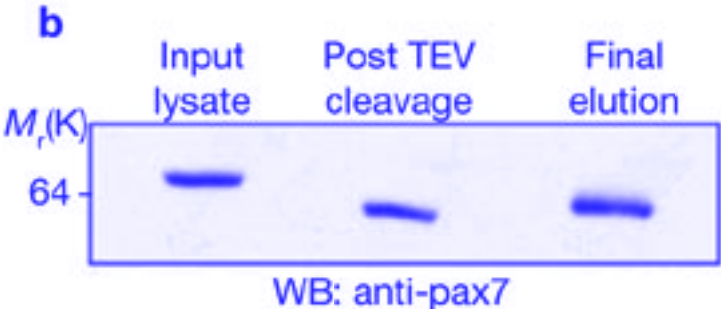


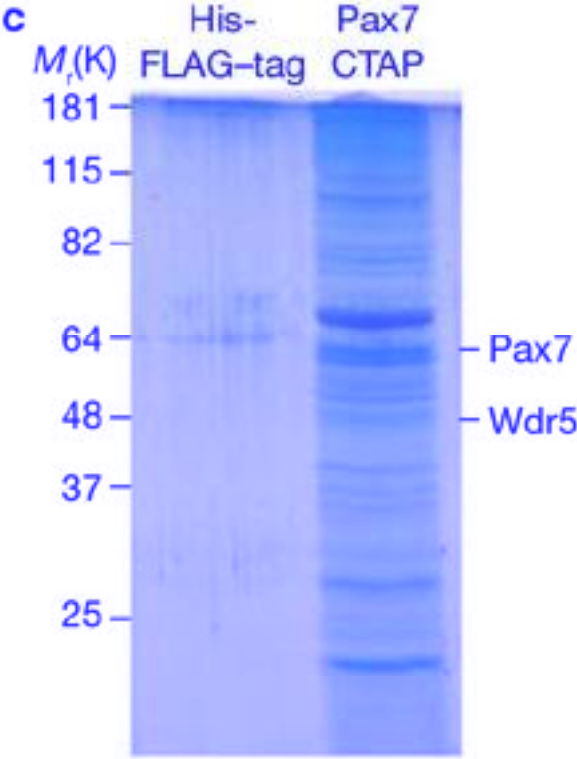
Fig. 2a. A TAP-tag method was used to purify Pax7

1. Bind Pax7-TAG to an anti-Flag column and wash
2. Cleave off Pax7-6xHis with TEV enzyme (tobacco etch virus)
3. Bind to a Ni⁺ column and wash
4. Elute with imidazole

Fig. 2b&c Pax7-CTAP was purified from C2C12 cells and associated proteins identified by Mass Spectrometry



Silver stained gel



Found:

- Pax7 was purified by the TAP method
- Many more proteins were associated with Pax7-CTAP than the CTAP alone
- Wdr5 was identified associated with Pax7-CTAP and not CTAP alone.

Different cell types:



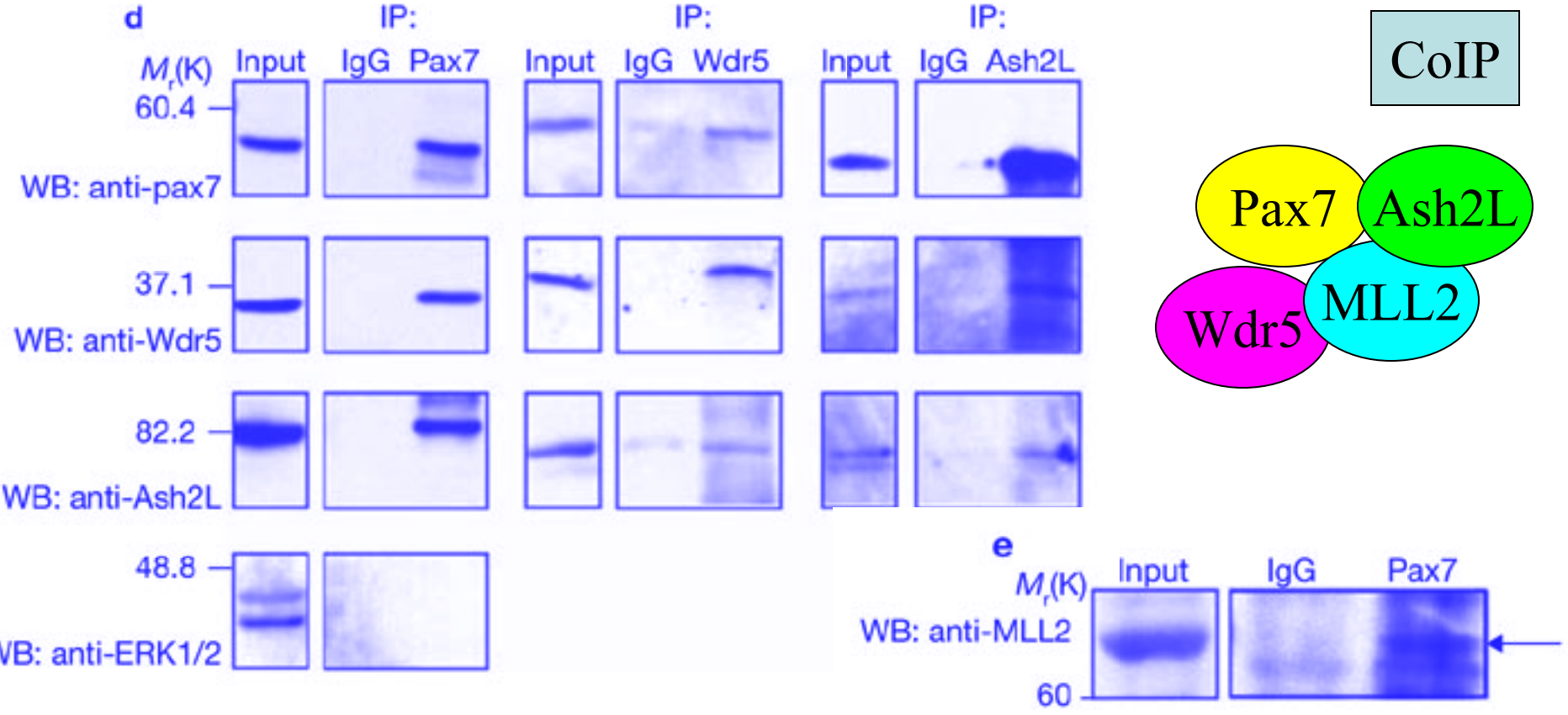
Primary myoblasts:

As soon as quiescent satellite cells are removed from the muscle, they activate MRFs and start to proliferate, called primary myoblasts.

C2C12 cells:

Primary myoblasts that have been immortalized to form a stable cell line.

Fig. 2d & e. Pax7 co-immunoprecipitated with Wdr5, Ash2L, and MLL2 in primary myoblasts

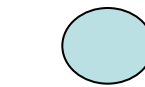
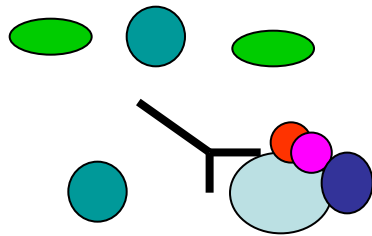


Found:

- The Pax7/Wdr5/Ash2L complex, but not ERK1/2, could be immunoprecipitated with Ab to any component of the complex
- Antibodies to Pax7 co-immunoprecipitate MLL2

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

Incubate whole cell extract with antibody to protein X

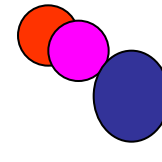
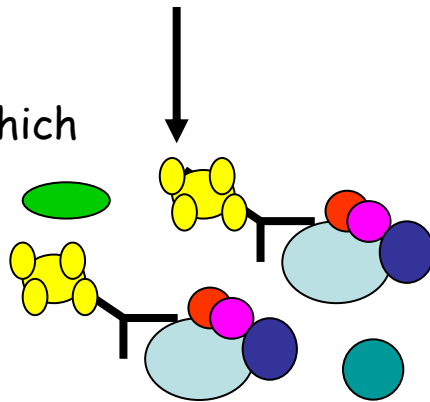


Protein X



Antibody raised against Protein X

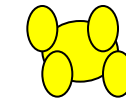
Incubate extract with Protein-A-Sepharose, which binds antibody



Proteins that Physically interact with protein X



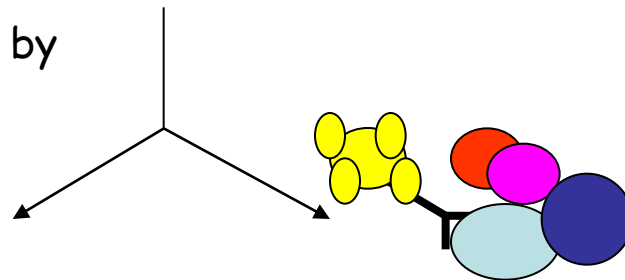
Proteins that do not interact with protein X



Protein A-sepharose

Collect Sepharose beads by centrifugation

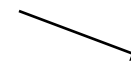
Discard supernatant



Wash pellet several times

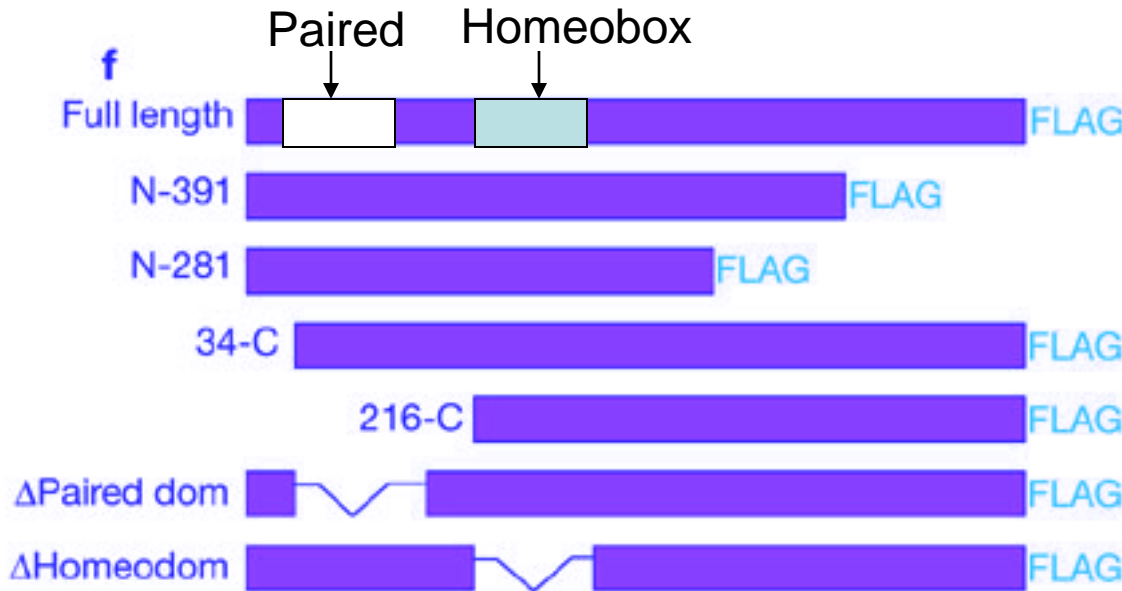
Dissociate proteins from Protein-A Sepharose

Separate proteins by SDS Page

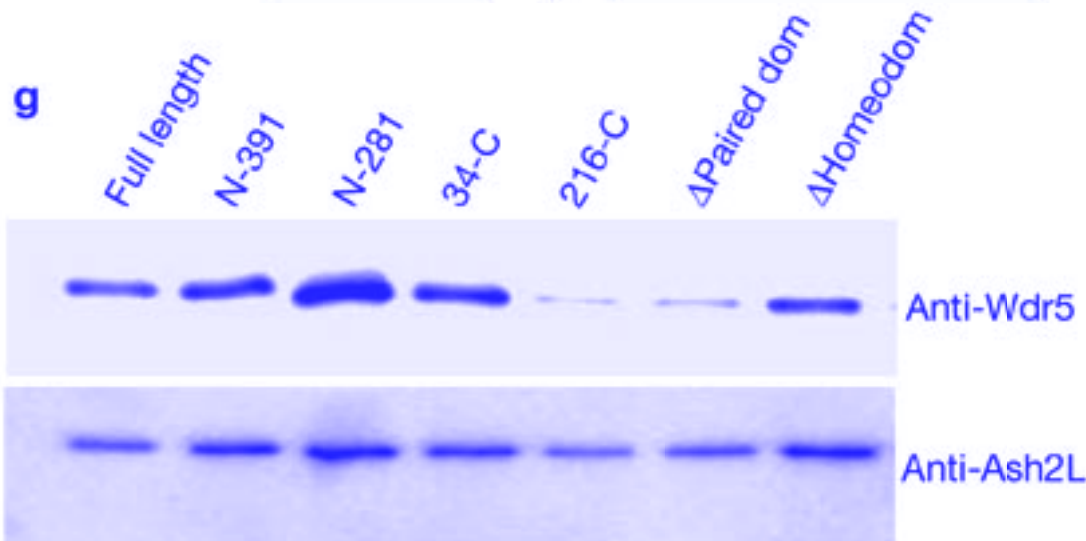


Western blot analysis

Fig. 2. Loss of the Paired domain abolished binding between Pax7 and Wdr5



Mutants of Pax7 were created and co-immunoprecipitated. Western blots were probed with Ab to Wdr5 and Ash2L.

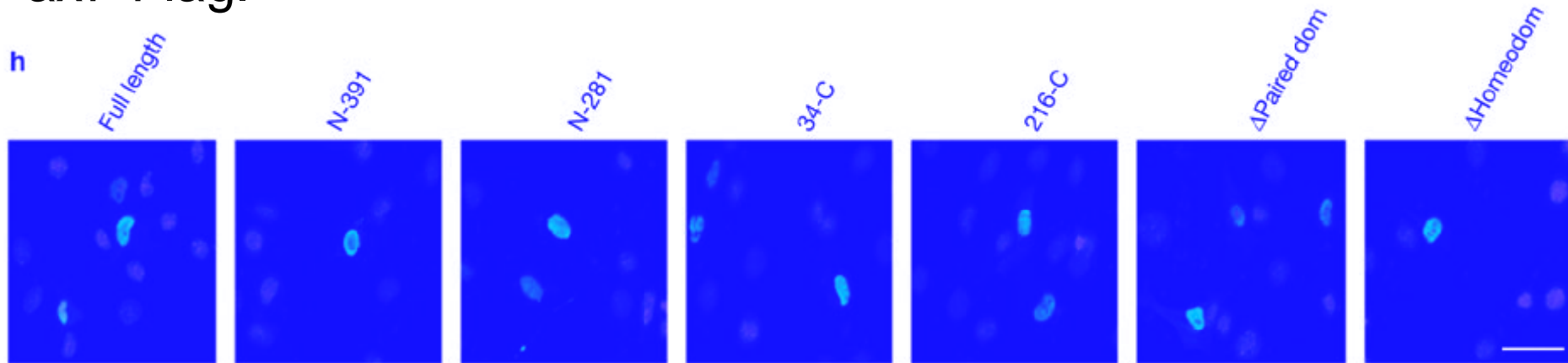


Found:

- Wdr5 interaction with Pax7 was lost when the Paired domain was deleted.

Fig. 2 Pax7-Flag mutations were found in the nucleus

Immunofluorescence was performed with Flag Ab to visualize Pax7-Flag.

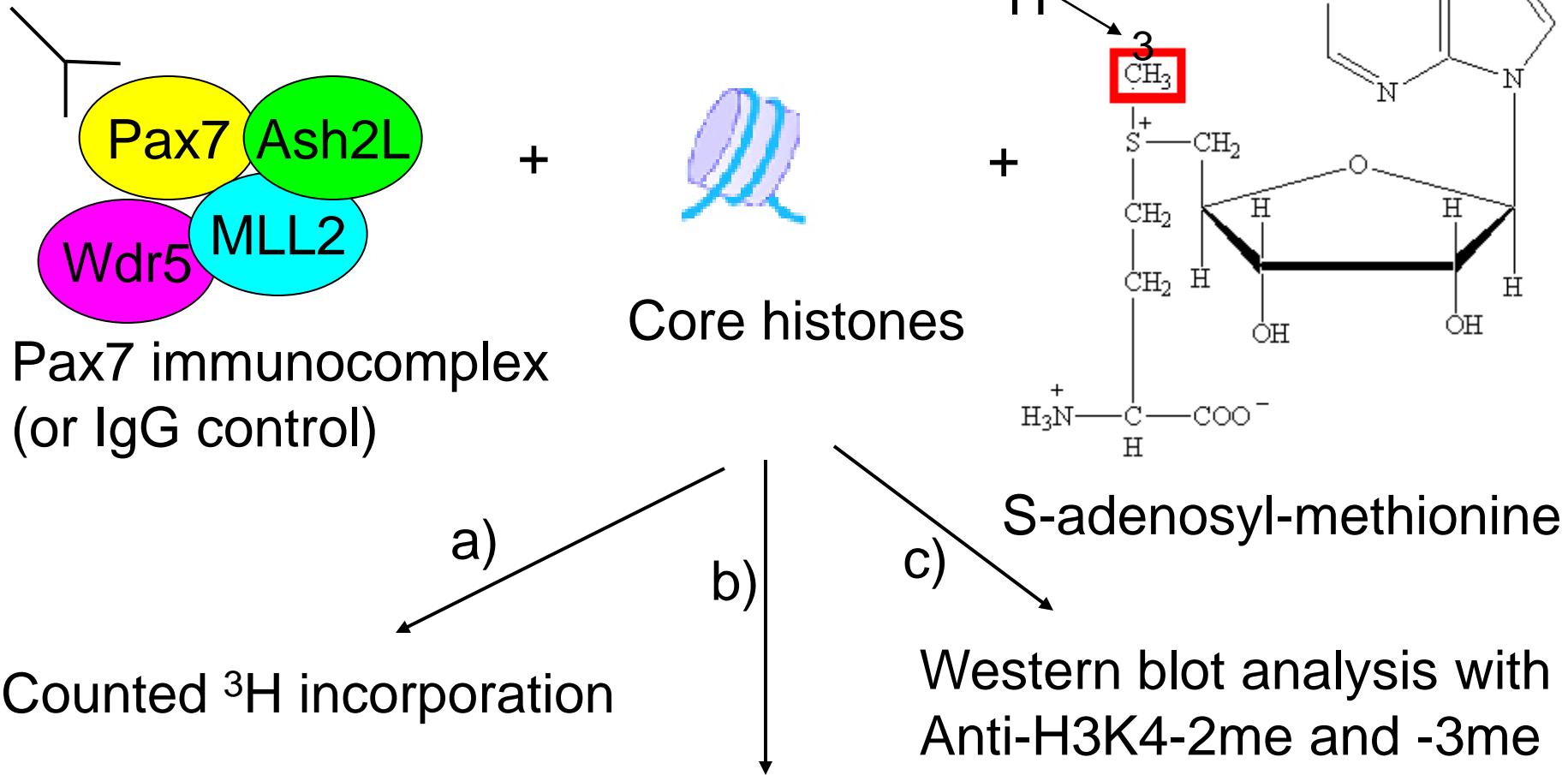


Found:

All Pax7-Flag deletion mutants were present in the nucleus. Therefore, the loss of WDR5 interaction with the paired domain mutants is not due to the lack of Pax7-flag mutant expression.

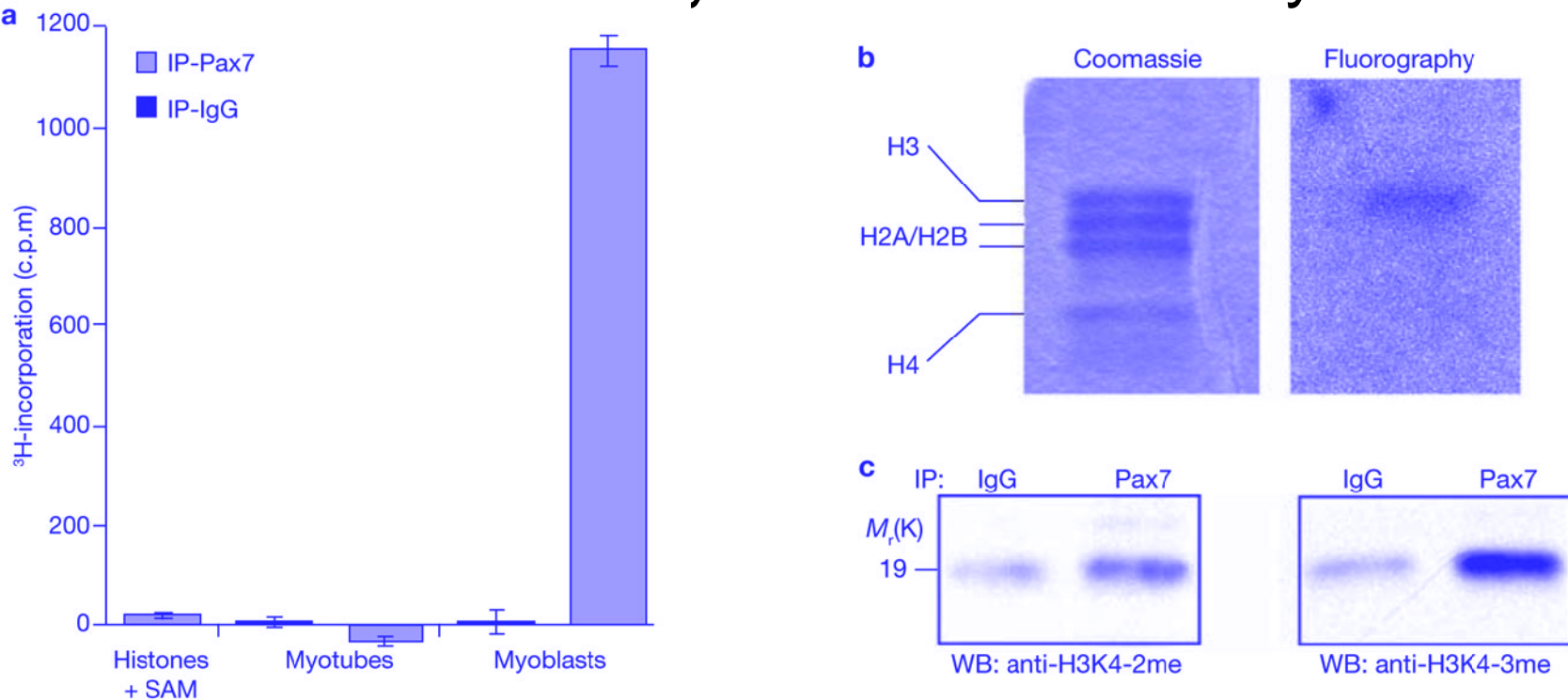
Do Pax7 complexes contain methyltransferase activity?

Fig. 3 a-c. In vitro methytransferase assay:



Separated protein on SDS-PAGE and visualized by coomassie or fluorography

Fig. 3 a-c. Pax7 immunocomplex has histone methyltransferase activity



Found:

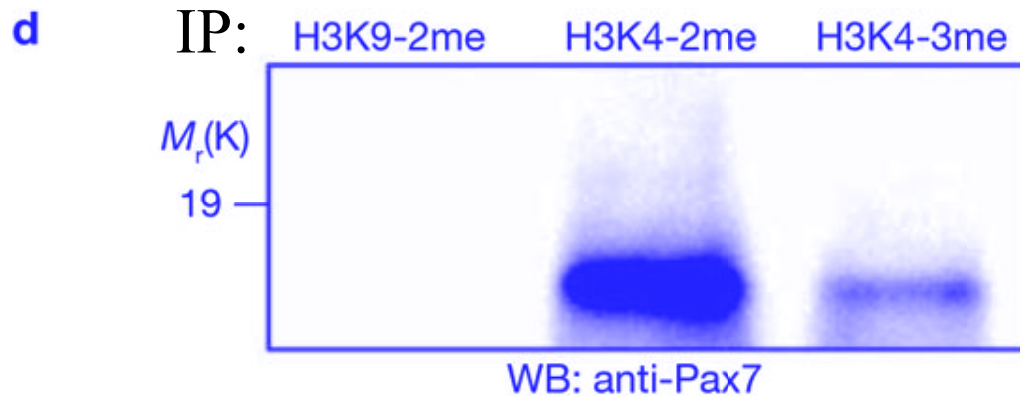
- 3H incorporation in histones incubated with Pax7 immunocomplexes from myoblasts (which express Pax7) but not myotubes (no Pax7)
- Methylation occurred on H3 and not H2a/b or H4
- Methylation occurred primarily as H3K4-3me, and some as -2me

Therefore, Pax7 recruits a protein complex capable of methylating H3K4

Fig. 3d Pax 7 associates with sites of H3K4 methylation on chromatin



IP-western Method: Immunoprecipitate with methylation-specific antibodies from primary myoblasts and look for Pax7 association by western blot



Found:

- Pax7 associated strongly with chromatin containing H3K4-2me
- Pax7 associated weakly with chromatin containing H3K4-3me
- Pax7 didn't associate with H3K9-2me

Therefore, Pax7 provides the missing function that targets histone methyltransferases to specific genes

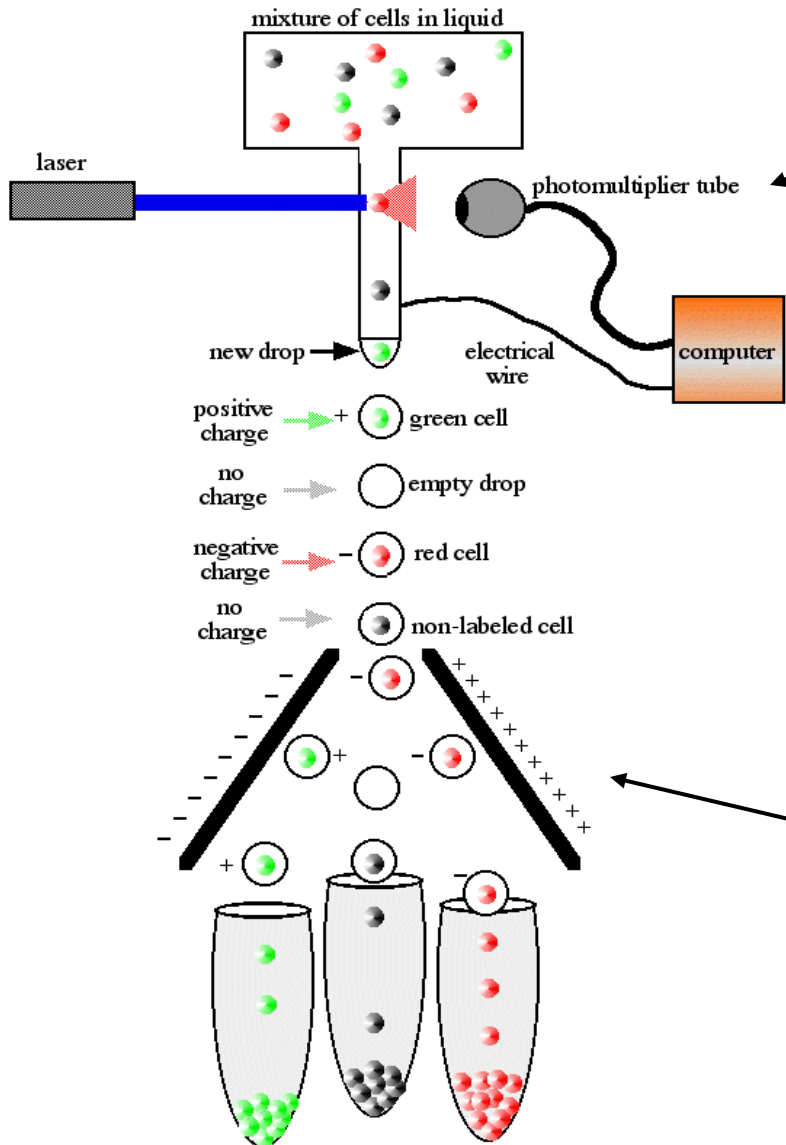
Is Pax7 important for Myf-5 expression in satellite cells?

Approach

Examine Myf5 expression in satellite cells (satellite cells are isolated directly from muscle by FACS): ■

1. Overexpressing Pax7 (Fig. 4a)
2. With reduced Pax7 levels by siRNA (Fig. 4b)
3. Genetically lacking Pax7 (Fig. 4c-e)

Fluorescent activated cell sorting

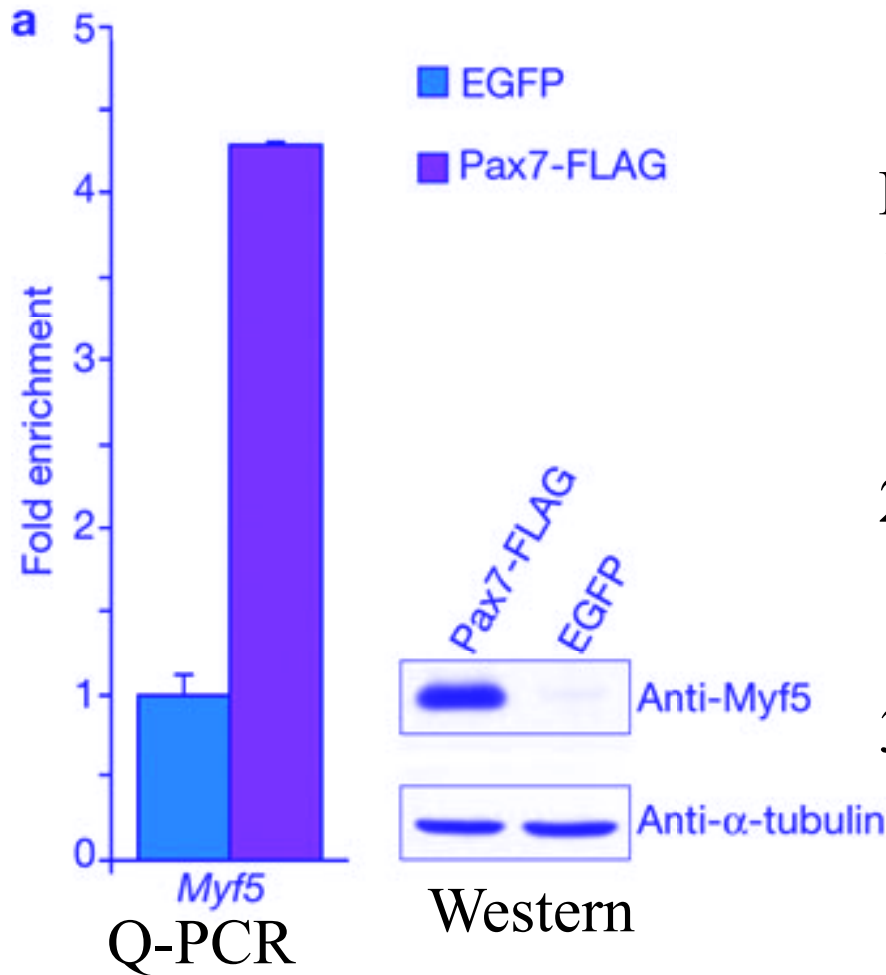


The photomultiplier tube reads the fluorescence information and conveys this to a computer

Charge is applied to the drop by the computer, based on information from the photomultiplier tube

Cells are separated based on their charge

Fig. 4a. Pax7 regulates Myf5 expression in satellite cell-derived myoblasts (Q-PCR & western) 



Method:

1. Satellite cell-derived myoblasts were infected with Pax7-Flag or EGFP
2. mRNA was harvested and analyzed for Myf5 mRNA expression
3. Protein was harvested and analyzed for Myf-5 protein expression


Found:

- Overexpression of Pax7 upregulates Myf5 mRNA and protein

Is Pax7 important for Myf-5 expression in satellite cells?

Approach

Examine Myf5 expression in satellite cells:

1. Overexpressing Pax7 (Fig. 4a)
2. With reduced Pax7 levels by siRNA (Fig. 4b) 
3. Genetically lacking Pax7 (Fig. 4c-e)

siRNA-mediated Gene Suppression

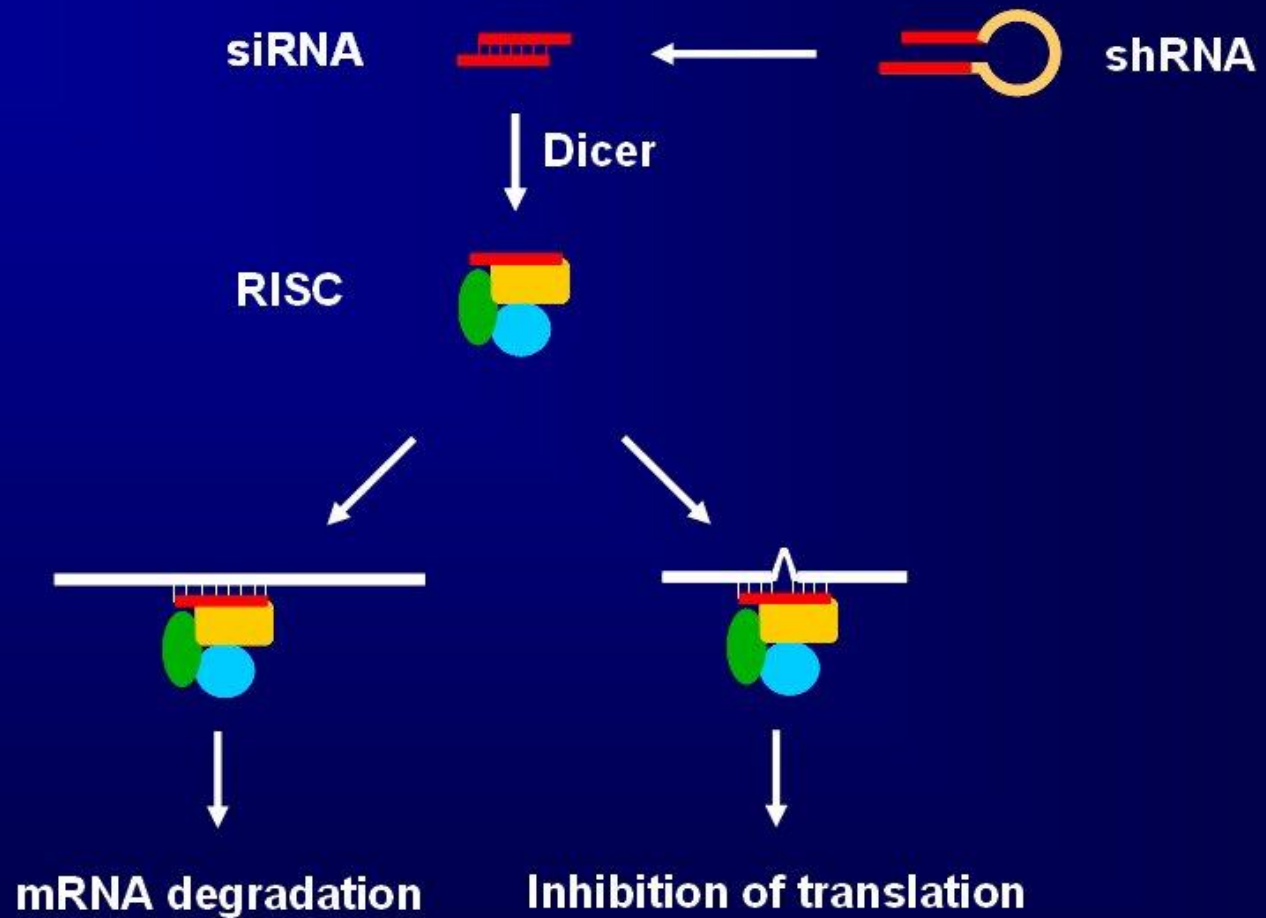
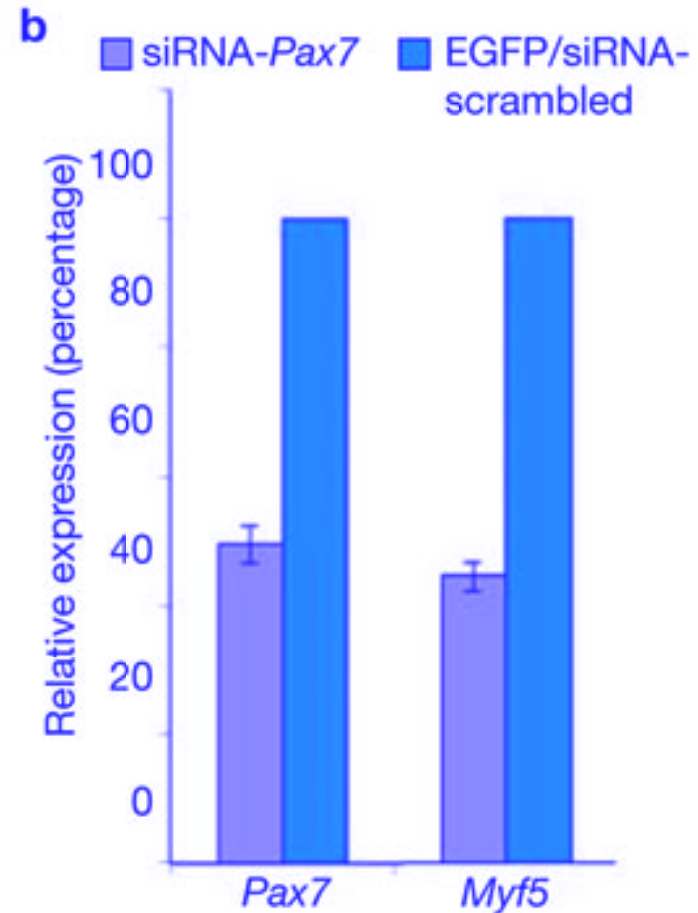


Fig. 4b. Pax7 regulates Myf5 expression in satellite cell-derived myoblasts (Q-PCR)



Method:

1. Satellite cell-derived myoblasts were transfected with siRNA against Pax7 or a scrambled control
2. mRNA was harvested and analyzed for Pax7 and Myf5 expression



Found:

- Reduction in Pax7 expression results in a loss of Myf5 mRNA

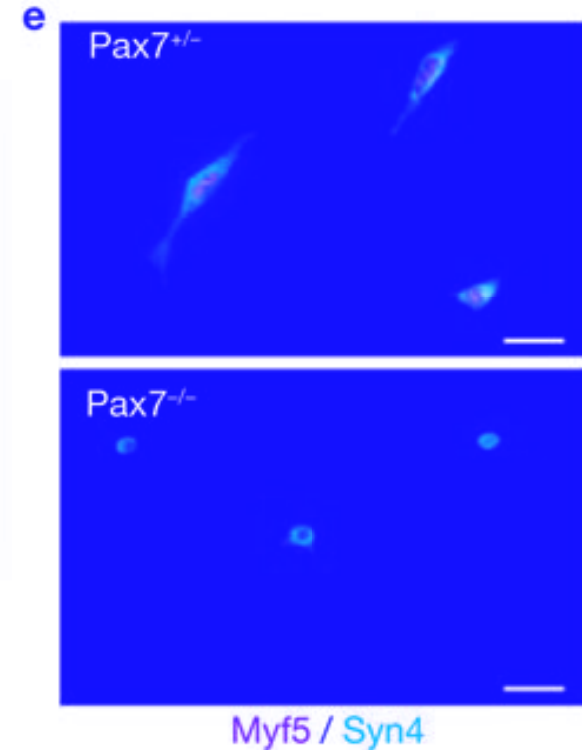
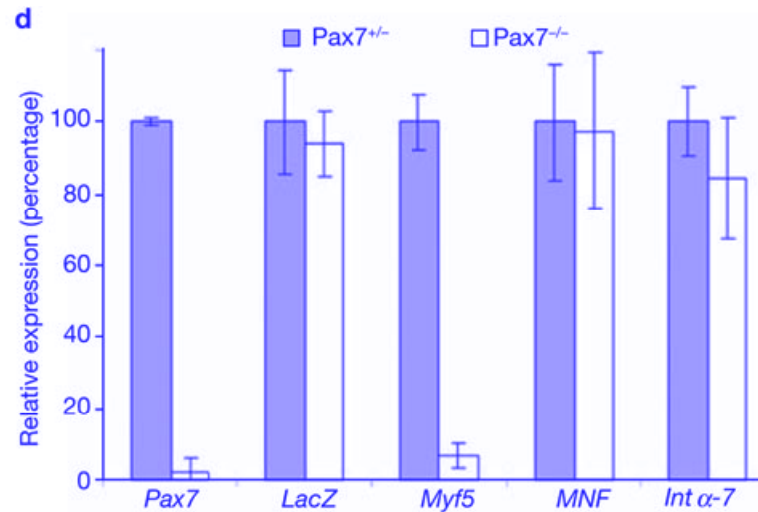
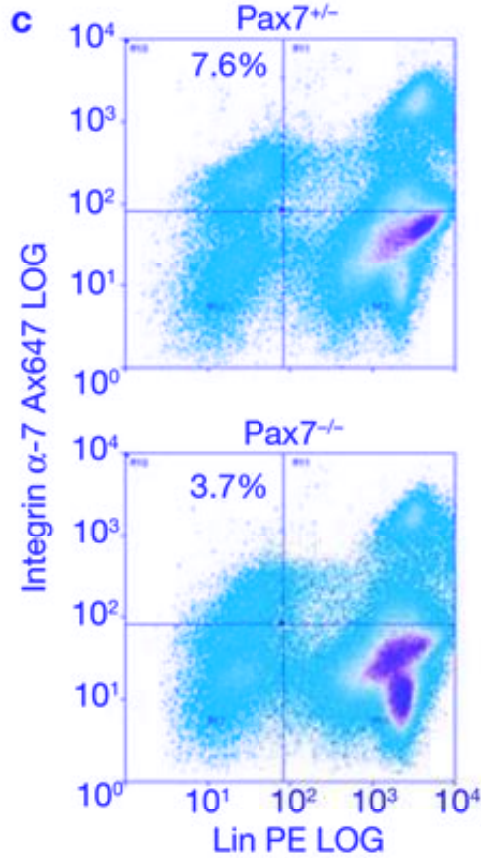
Is Pax7 important for Myf-5 expression in satellite cells?

Approach

Examine Myf5 expression in satellite cells:

1. Overexpressing Pax7 (Fig. 4a)
2. With reduced Pax7 levels by siRNA (Fig. 4b)
3. Genetically lacking Pax7 (Fig. 4c-e)

Fig. 4 c-e. Satellite cells lacking Pax7 also lack Myf5 (Syn4 and Integrin α -7 are satellite cell markers)



Found:

- Pax7^{-/-} mice have half the number of satellite cells
- These satellite cells don't express Myf-5

Therefore, Pax7 is required for the expression of Myf5 and the commitment of satellite cells to the myogenic lineage.

Summary of Experiments (Fig. 1-4e):

1. Pax7 regulates Myf5 expression
 - Overexpression of Pax7 enhances Myf5 in C2C12 myoblasts, 10T1/2 fibroblasts, satellite cells
 - Loss of Pax7 reduces Myf5 expression in satellite cells, by siRNA and Pax7 ^{-/-} mice
2. Pax7 binds a complex containing Wdr5, Ash2L, and MLL2
 - Mass spectrometry analysis of Pax7-CTAP purification
 - Co-immunoprecipitation in satellite cell myoblasts
3. The Pax7/Wdr5/Ash2L/MLL2 complex has Histone Methytransferase activity
 - In vitro methylation assay with 3H-SAM and western blot analysis

Does Pax7 bind Myf5 directly? *

Approach - Perform chromatin immunoprecipitation in pax7-FLAG and EGFP control satellite cell-derived myoblasts with:

1. Flag Ab (PCR amplifying a known Pax3 binding site at -57.5 Kb, and a negative control at -315 bp)
2. Ash2L Ab (PCR amplifying as in 1.)
3. H3K4-3me Ab (PCR amplifying the promoter/coding regions)

The Myf5 gene:

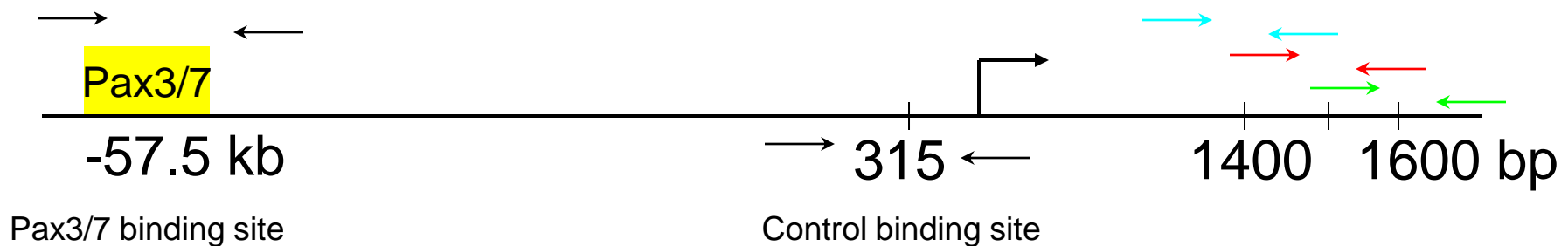
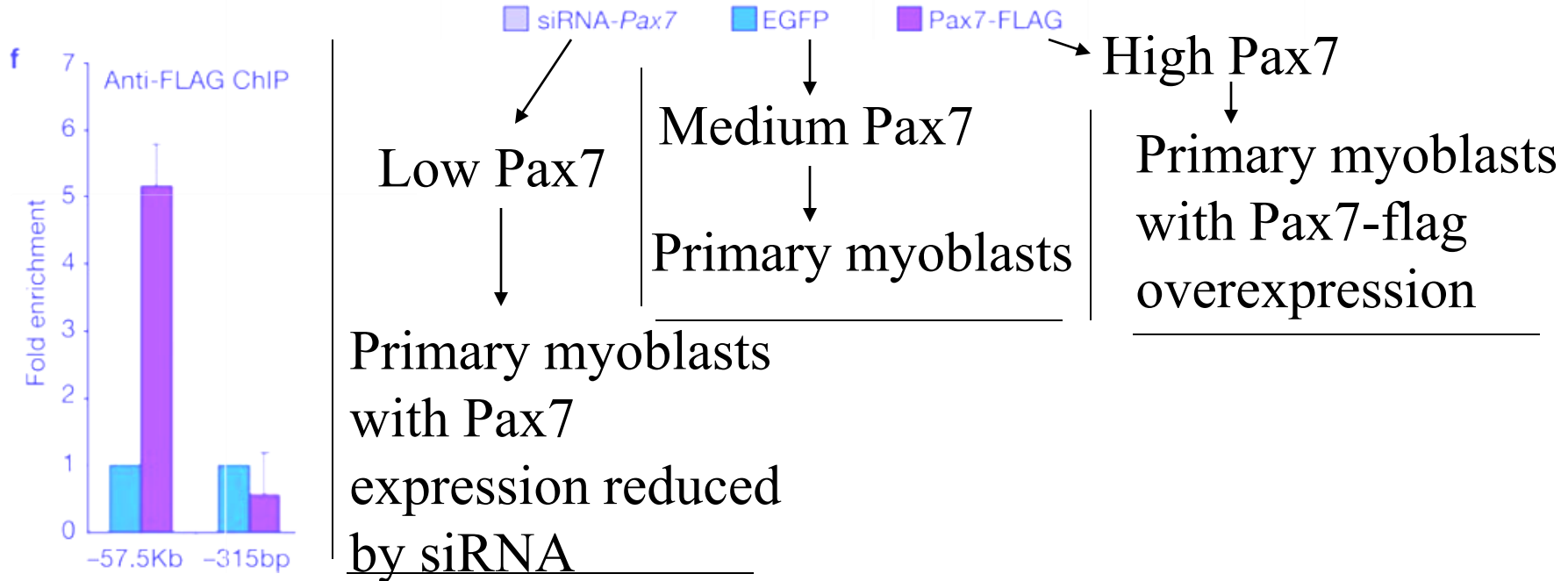


Fig. 4 Flag-Pax7 binds directly to the -57.5* Kb region of Myf5



Found:

1. Pax7 immunocomplexes were enriched for the -57.5 kb Pax7 binding region but not the control -315 bp region

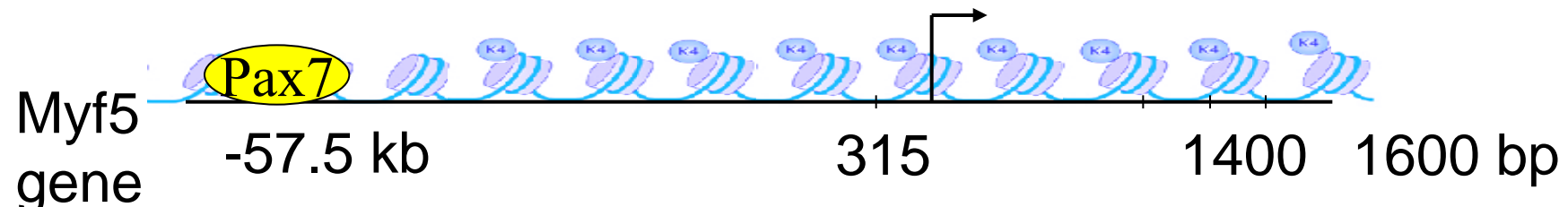
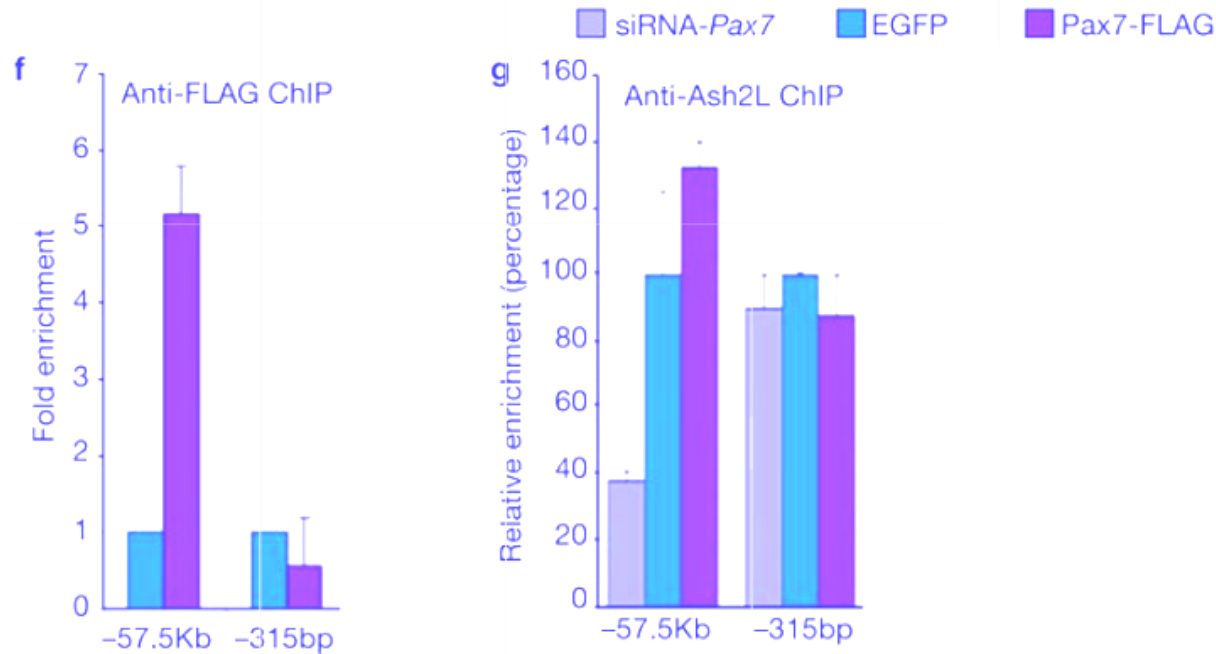


Fig. 4 Pax7 binds directly to the -57.5 Kb region of Myf5 in complex with Ash2L



Found:

2. In the presence of high Pax7, Ash2L levels at the -57.5 Kb region were regulated by Pax7 levels

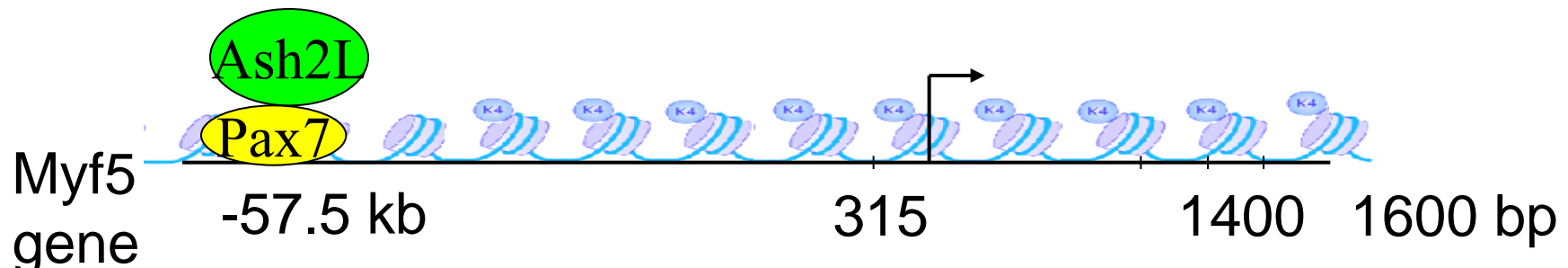
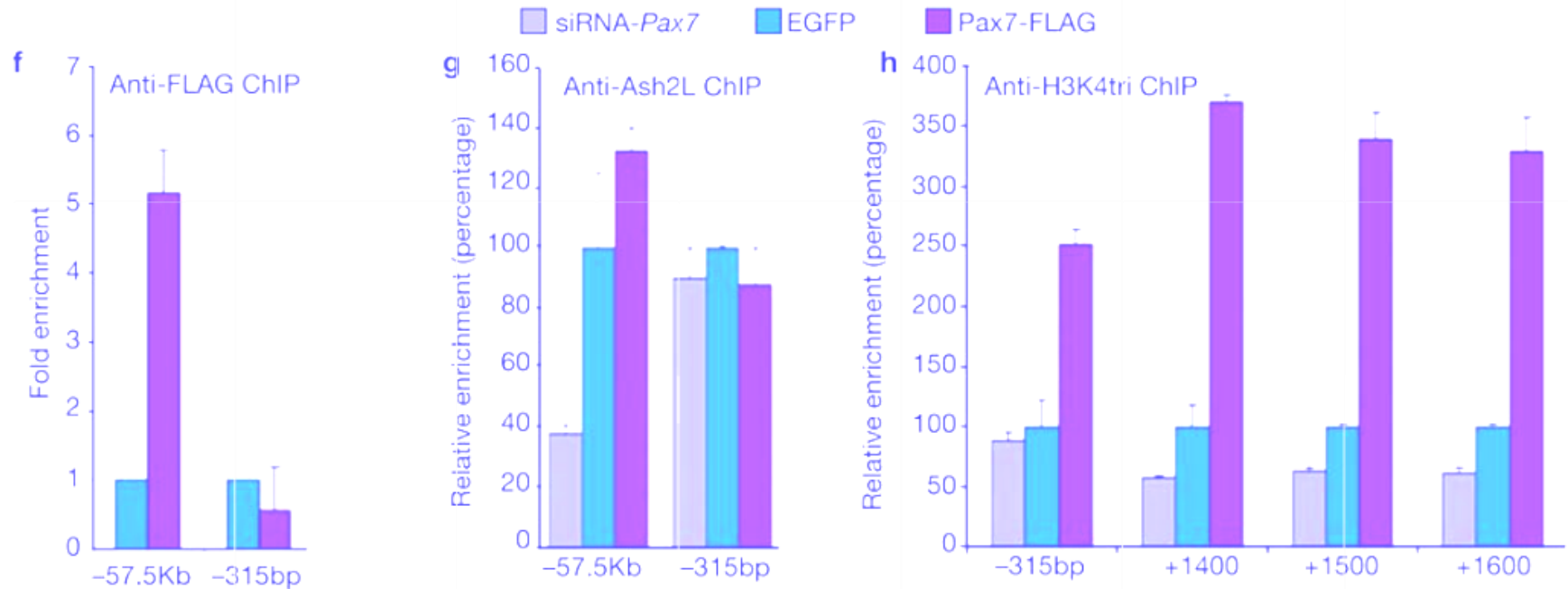


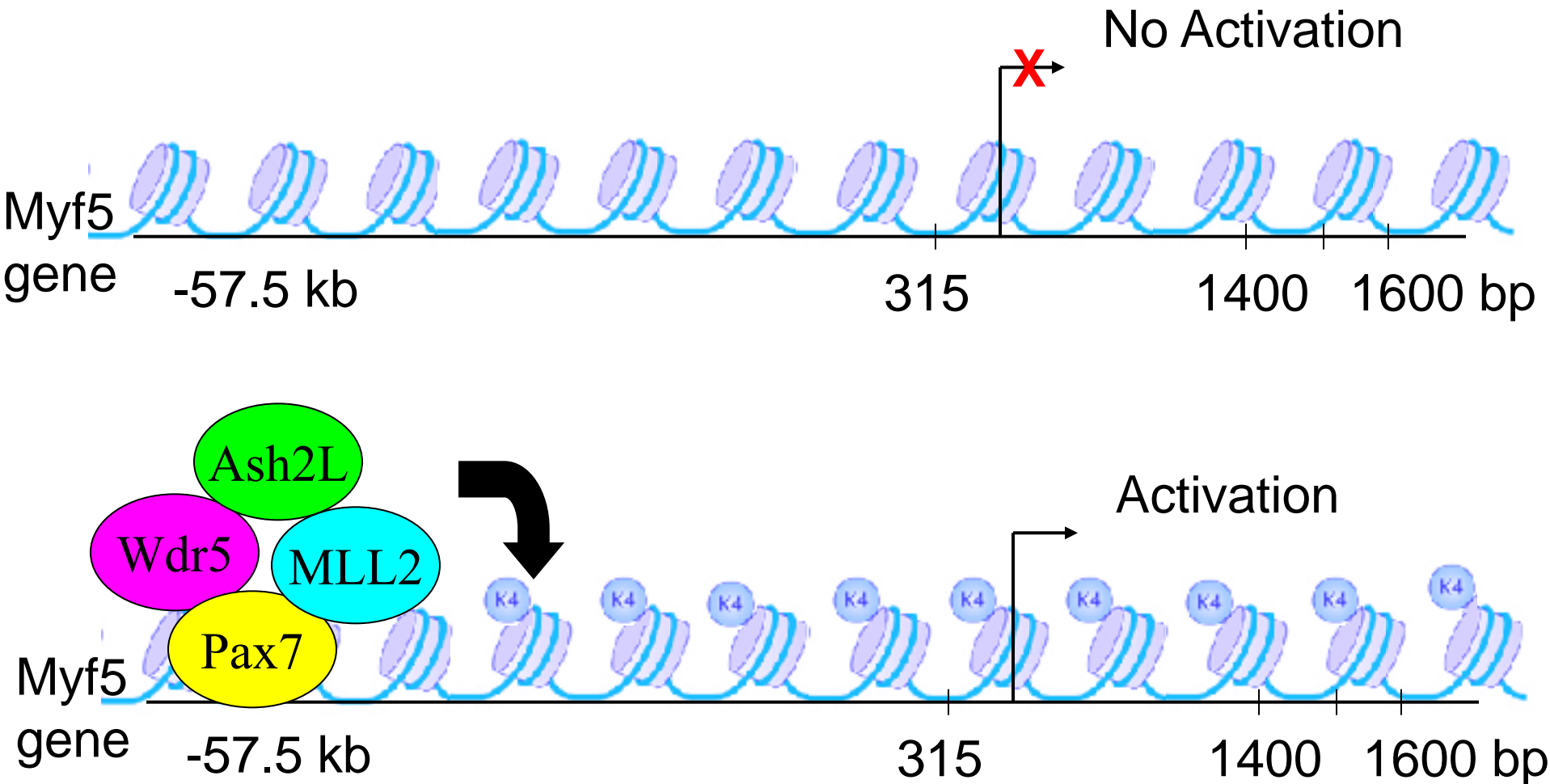
Fig. 4 Pax7 binds directly to the -57.5 Kb region of Myf5 in complex with Ash2l



Found:

1. Pax7 immunocomplexes were enriched for the -57.5 kb Pax7 binding region but not the control -315 bp region
2. In the presence of high Pax7, Ash2L levels at the -57.5 Kb region were regulated by Pax7 levels
3. H3K4 trimethylation of the 5' and coding regions was regulated by the levels of Pax7

Model proposed for the regulation of transcription of Myf5



Summary of Experiments:

1. Pax7 regulates Myf5 expression
 - Overexpression of Pax7 enhances Myf5 in C2C12 myoblasts, 10T1/2 fibroblasts, satellite cells
 - Loss of Pax7 reduces Myf5 expression in satellite cells, by siRNA and Pax7 $-/-$ mice
2. Pax7 binds a complex containing Wdr5, Ash2L, and MLL2
 - Mass spectrometry analysis of Pax7-CTAP purification
 - Co-immunoprecipitation of satellite cell myoblasts
3. The Pax7/Wdr5/Ash2L/MLL2 complex has Histone Methyltransferase activity
 - In vitro methylation assay with 3H-SAM and western blot analysis
4. Pax7 binds directly to the Myf5 promoter and is associated with enhanced levels of Ash2L and H3K4 trimethylation (ChIP)

This paper is important because:

1. It shows the first direct link between Pax7 and Histone Methyltransferase activity
2. It shows the first direct link between Pax7 and Myf5
3. Since Pax-family genes are essential for the specification of diverse tissues, Pax recruitment of Histone Methyltransferase activity could be a conserved mechanism regulating developmental programs.

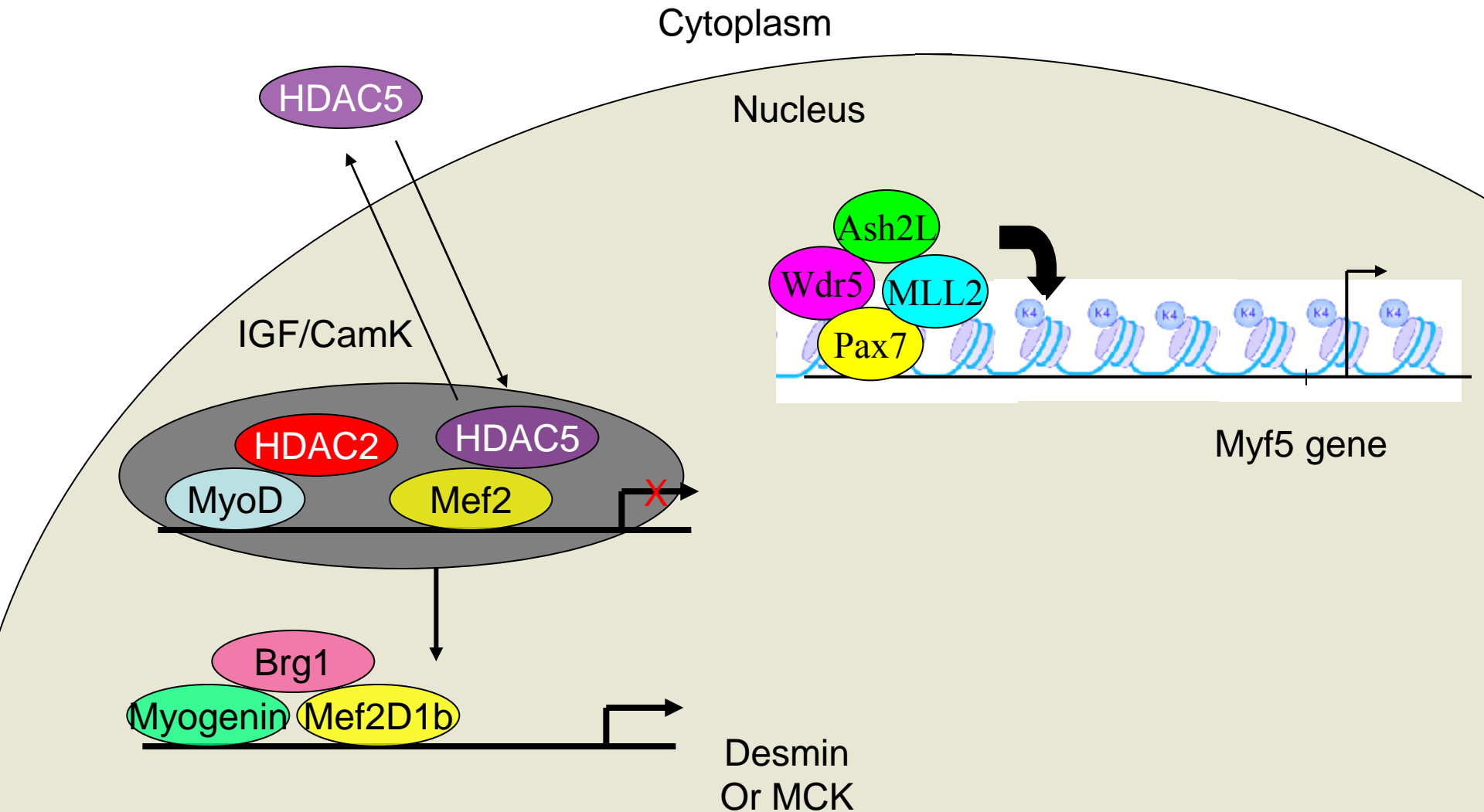
This paper is also important
because:

It marks the beginning of “high through-put”
approaches to study transcription

Future:

Combination of datasets from microarray, mass spectrometry of interacting proteins, and Chip-Sequencing. We will need bioinformatics and mathematical modeling to analyze and understand the incredible complexity!

Summary of models from the 4 papers: *



Therefore, we've seen 3 ways that chromatin can regulate transcription:

1. Histone deacetylation and closure of the chromatin structure by HDACs
2. Nucleosome remodelling by SWI/SNF ATPases
3. Methylation of H3K4 by the MLL complex

What questions remain?

- Are all of the genes regulated by these three mechanisms?
- Are there other mechanisms?
- How does Myf5 fit into the model?