High-Throughput Genomic Screen of MEF2 Association During Muscle Differentiation

Mark Takahashi

Associate Scientist

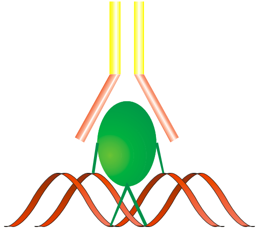
UHN Microarray Centre, Clinical Genomics Centre,
University Health Network, Toronto, Canada.

UHN Microarray Centre

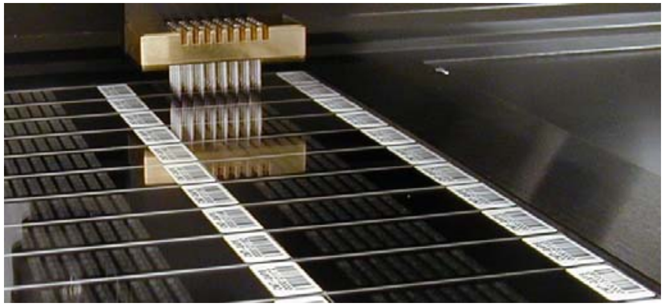
www.microarrays.ca

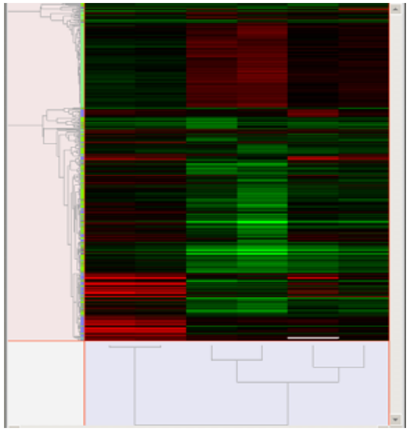
Overview

Chromatin Immunoprecipitation (ChIP)

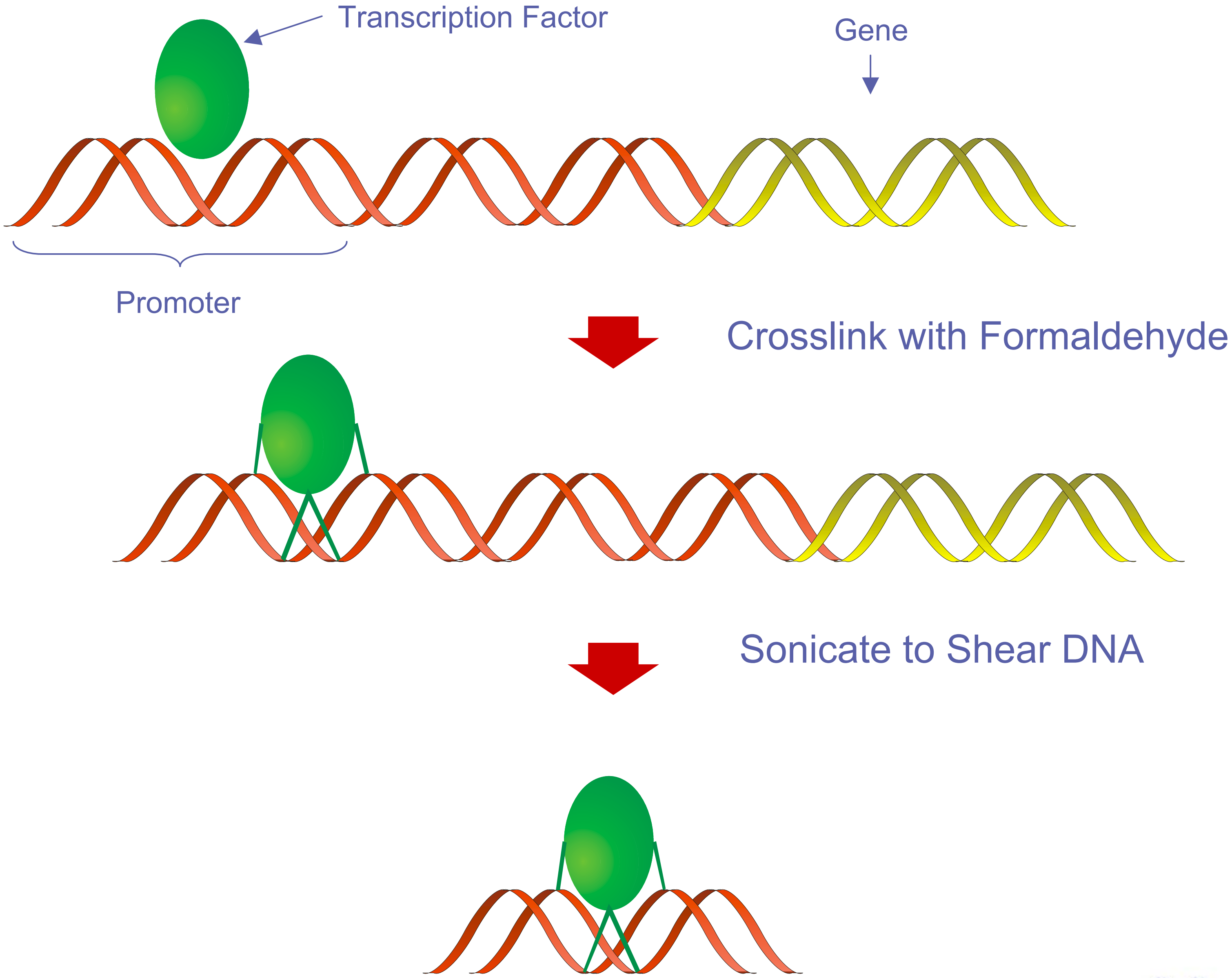


ChIP on Microarray





Novel gene discovery



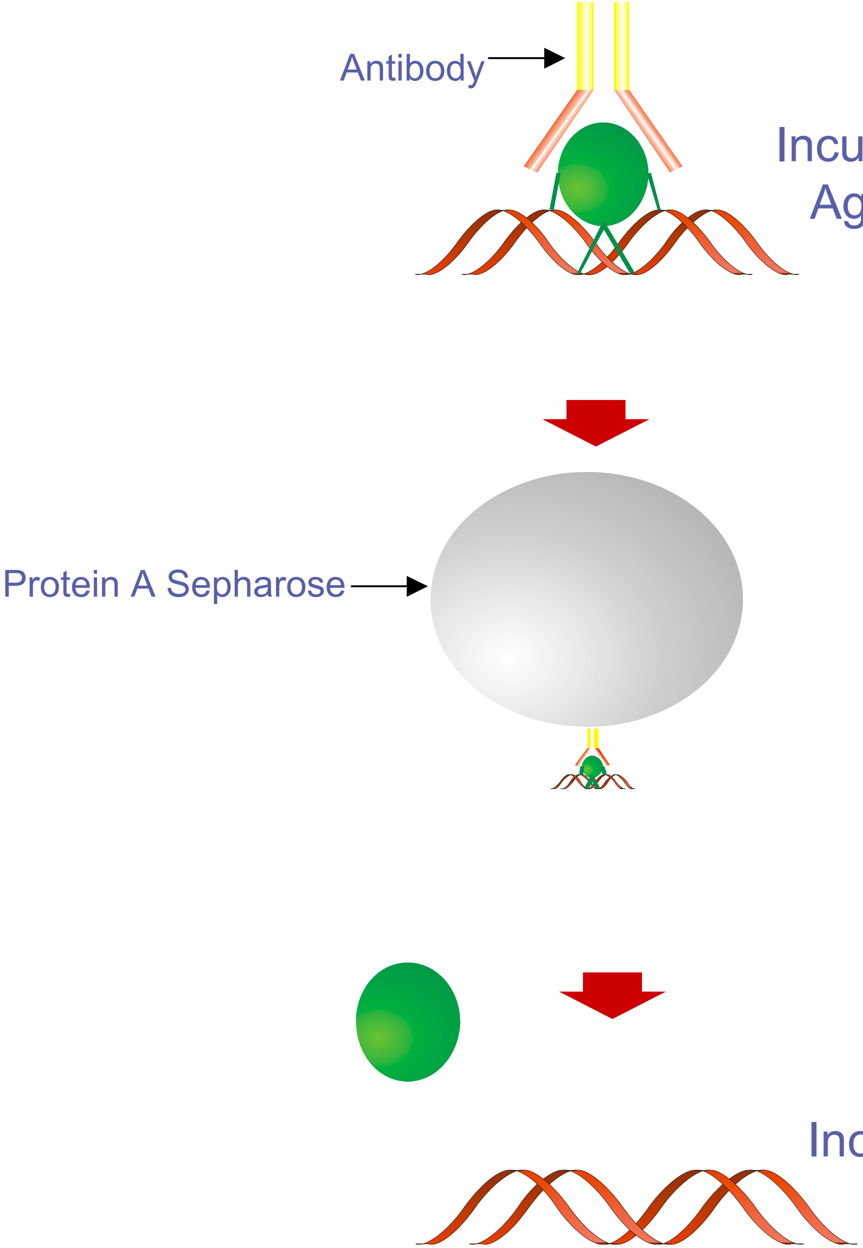
Transcription Factor

Gene

Promoter

Crosslink with Formaldehyde

Sonicate to Shear DNA



Incubate With Antibody Raised Against Transcription Factor

Precipitate complex

Reverse Crosslink by Incubating at High Temperature and High Salt

How can we now apply this to a high throughput screening method?

Ideally, we need a means of profiling all of the regulatory sites/promoter regions of all genes.

CpG islands

-CpG islands are unmethylated C-G rich regions of the genome

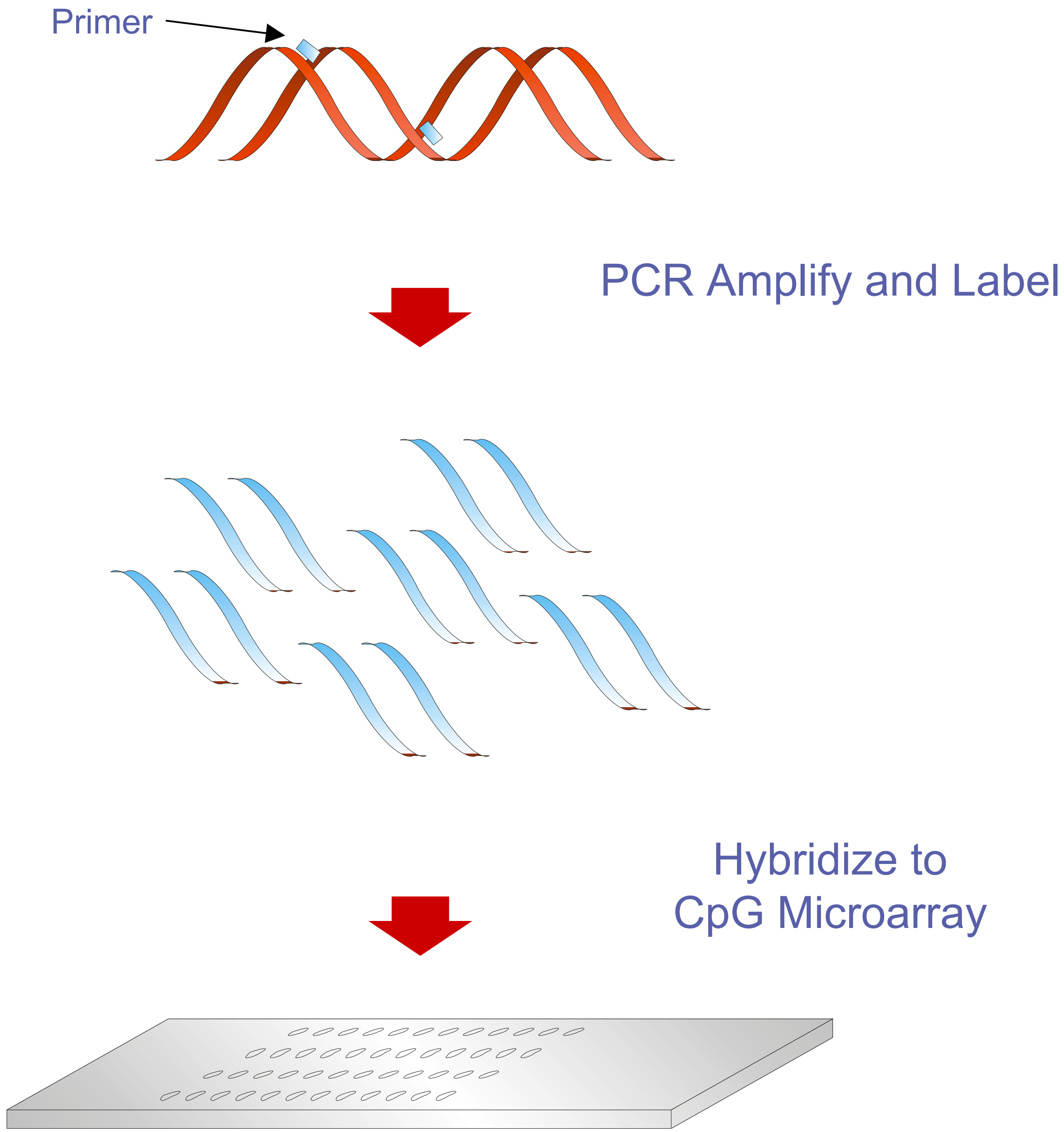
-account for approximately 2% of the genome

-associated with the 5' ends of all house-keeping genes and a large number of regulated genes

-About 60% of human genes and 47% of mouse genes are associated with CpG islands

-about 80% of CpG islands are common between human and mouse

Based upon methodologies established in the P.O. Brown lab



Primer

PCR Amplify and Label

Hybridize to
CpG Microarray

Cross-link protein to DNA

DNA Shearing

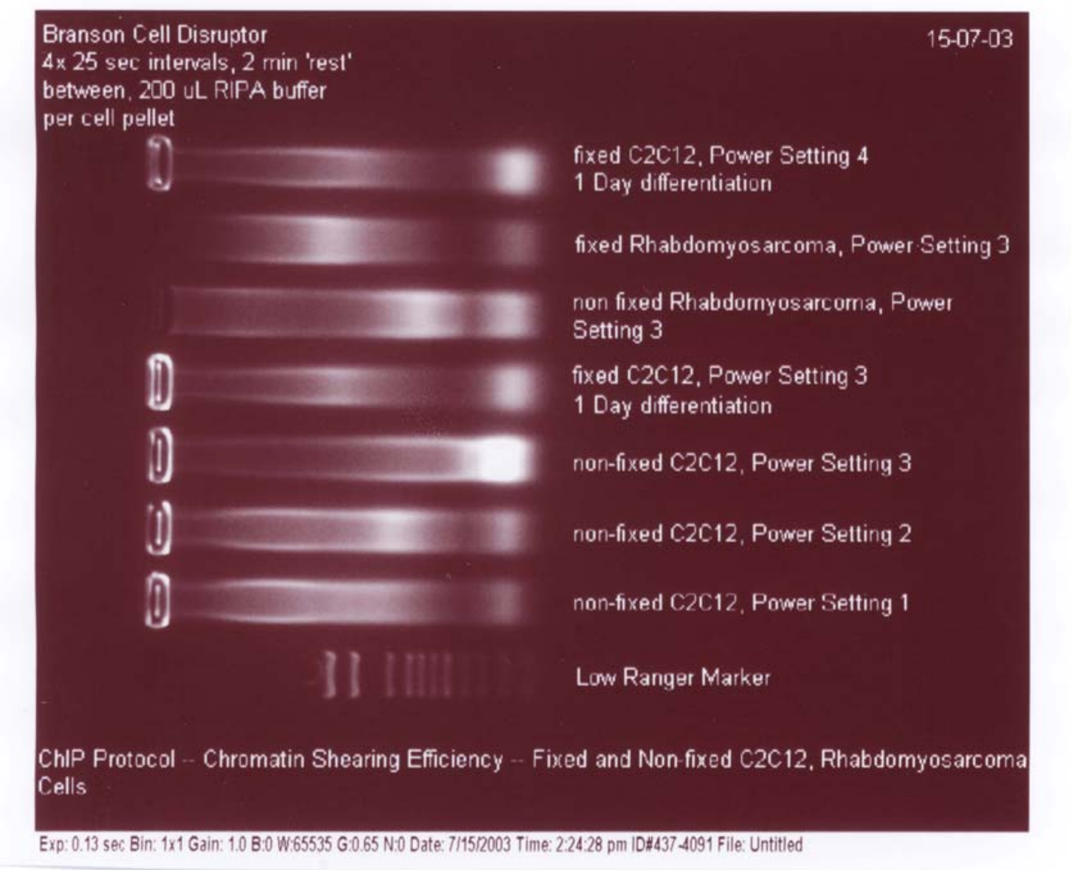
Immunoprecipitation

DNA recovery

Amplification

Microarray analysis

DNA Shearing



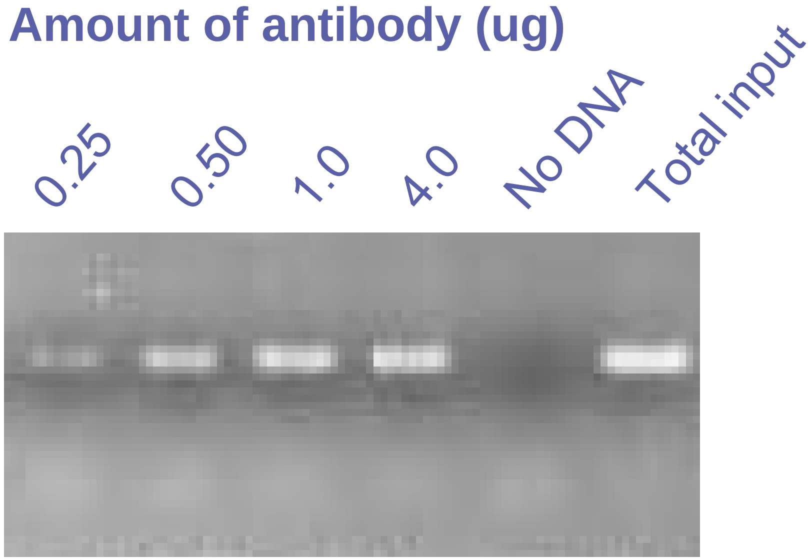
Branson Cell Disruptor
4x 25 sec intervals, 2 min 'rest'
between, 200 uL RIPA buffer
per cell pellet

15-07-03

ChIP Protocol -- Chromatin Shearing Efficiency -- Fixed and Non-fixed C2C12, Rhabdomyosarcoma Cells

Exp: 0.13 sec Bin: 1x1 Gain: 1.0 B:0 W:65535 G:0.65 N:0 Date: 7/15/2003 Time: 2:24:28 pm ID#437-4091 File: Untitled

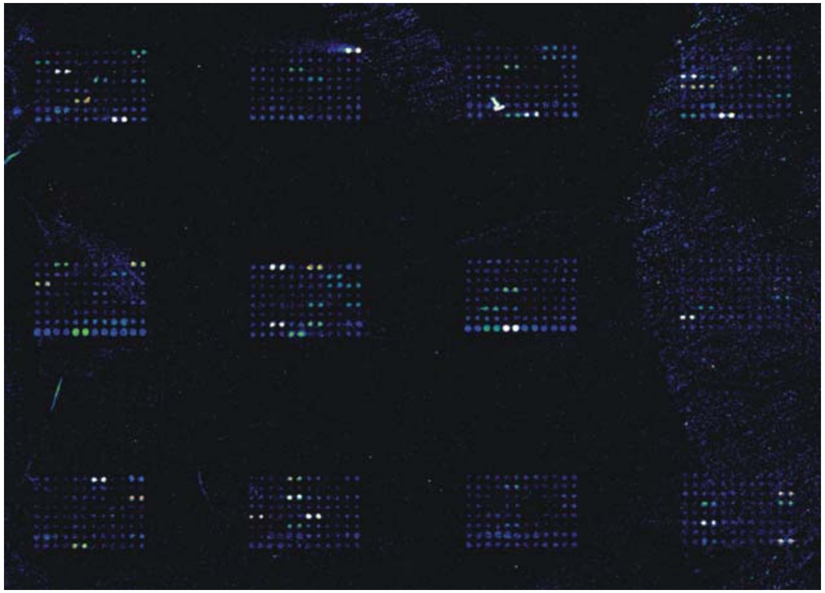
Immunoprecipitation

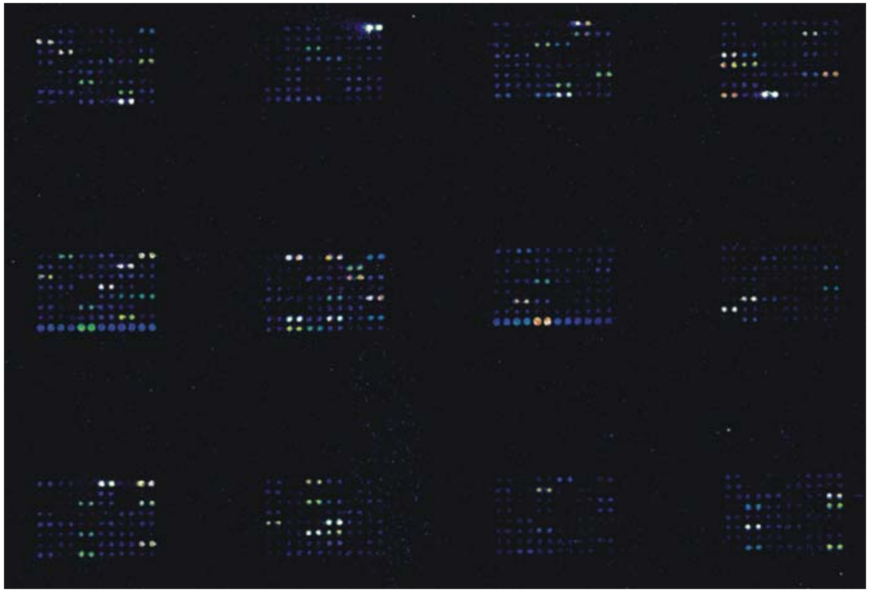


DNA recovery

Protein A beads

Staph A cells





Amplification

10 cycles

20 cycles

30 cycles

Microarray analysis

Microarray Data Filtering

-Establish minimum intensity cutoff value

-Threshold for determining true protein associated DNA

Genomic Database Search

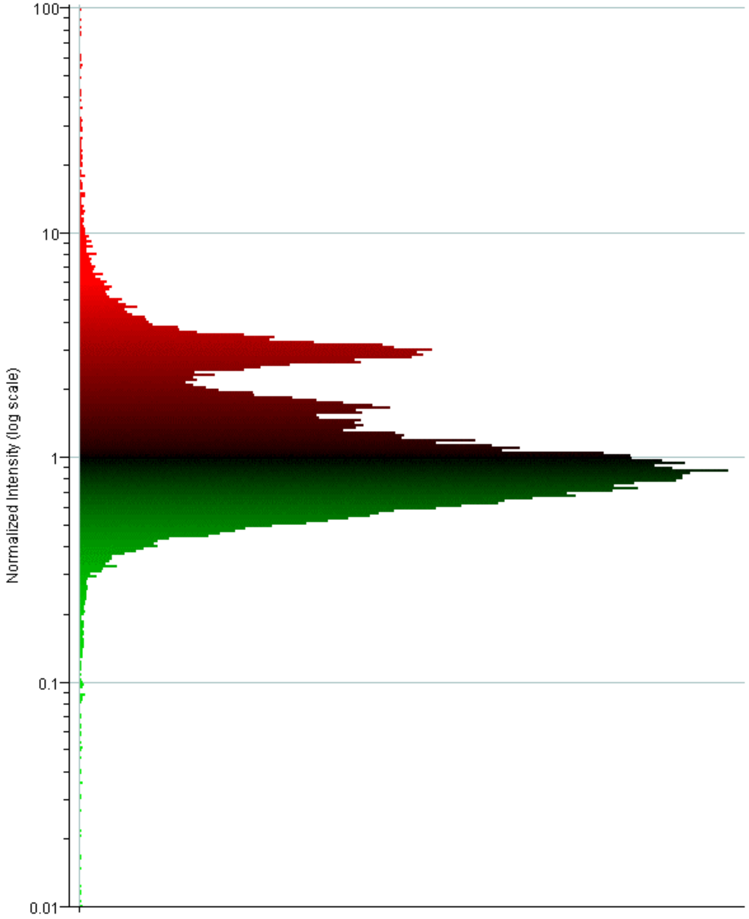
-Place clone sequence in genomic DNA sequence

-Search for potential gene targets within location of target sequence

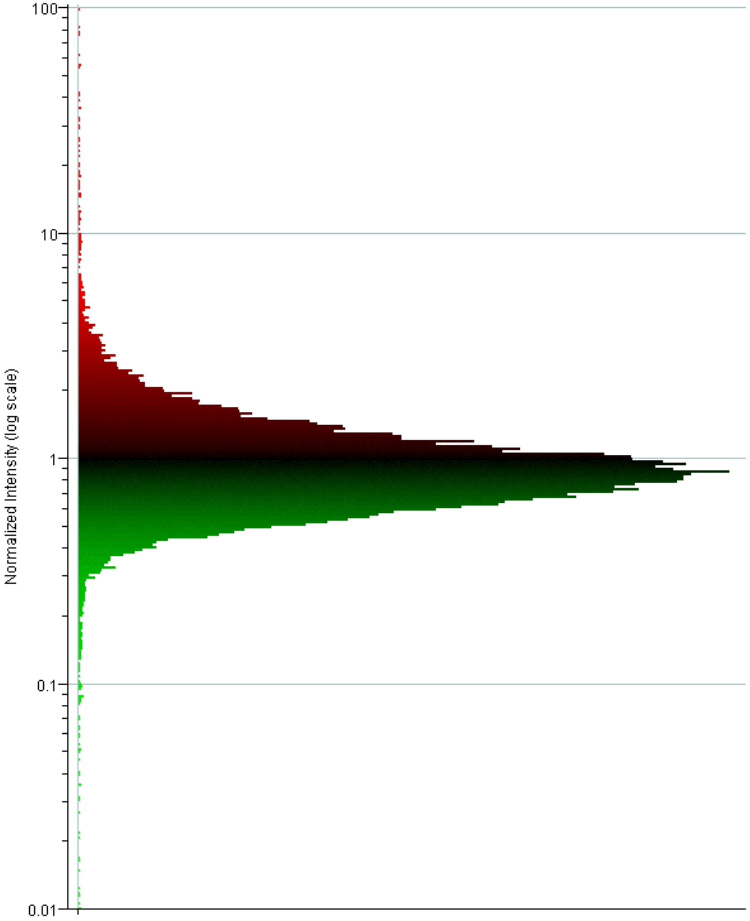
Verification

-Carry out ChIP using primers specifically associated with the identified genes

Microarray Data Filtering



All Intensities



Threshold filtered

Genomic Database Search

- UCSC Genome Bioinformatics
  http://genome.ucsc.edu/

- UHN CpG Microarray Database
  mouse; http://data.microarrays.ca/cpgmouse/
  human; http://data.microarrays.ca/cpg/

Verification

Requires identification of potential transcription factor binding sites;

MSCAN

http://mscan.cgb.ki.se/cgi-bin/MSCAN

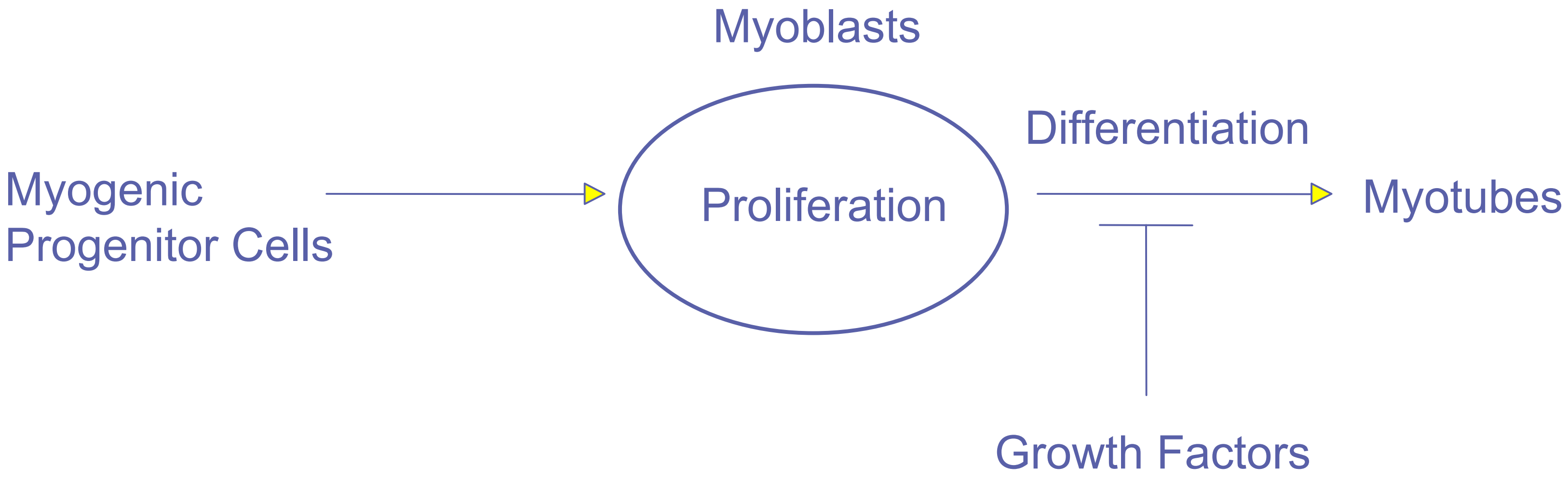
Transfac

http://www.gene-regulation.com/pub/databases.html#transfac

MOTIF search

http://motif.genome.jp/

Regulation of Skeletal Muscle Differentiation



Myoblasts

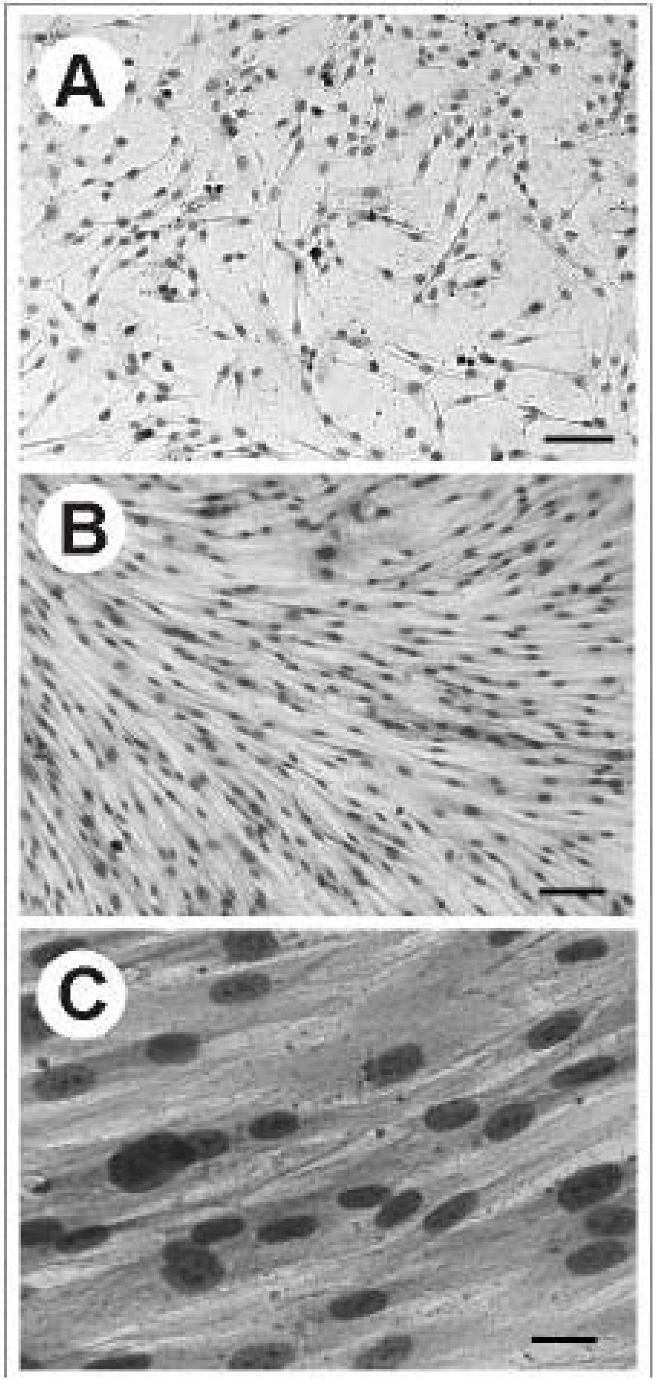
Myogenic
Progenitor Cells

Proliferation

Differentiation

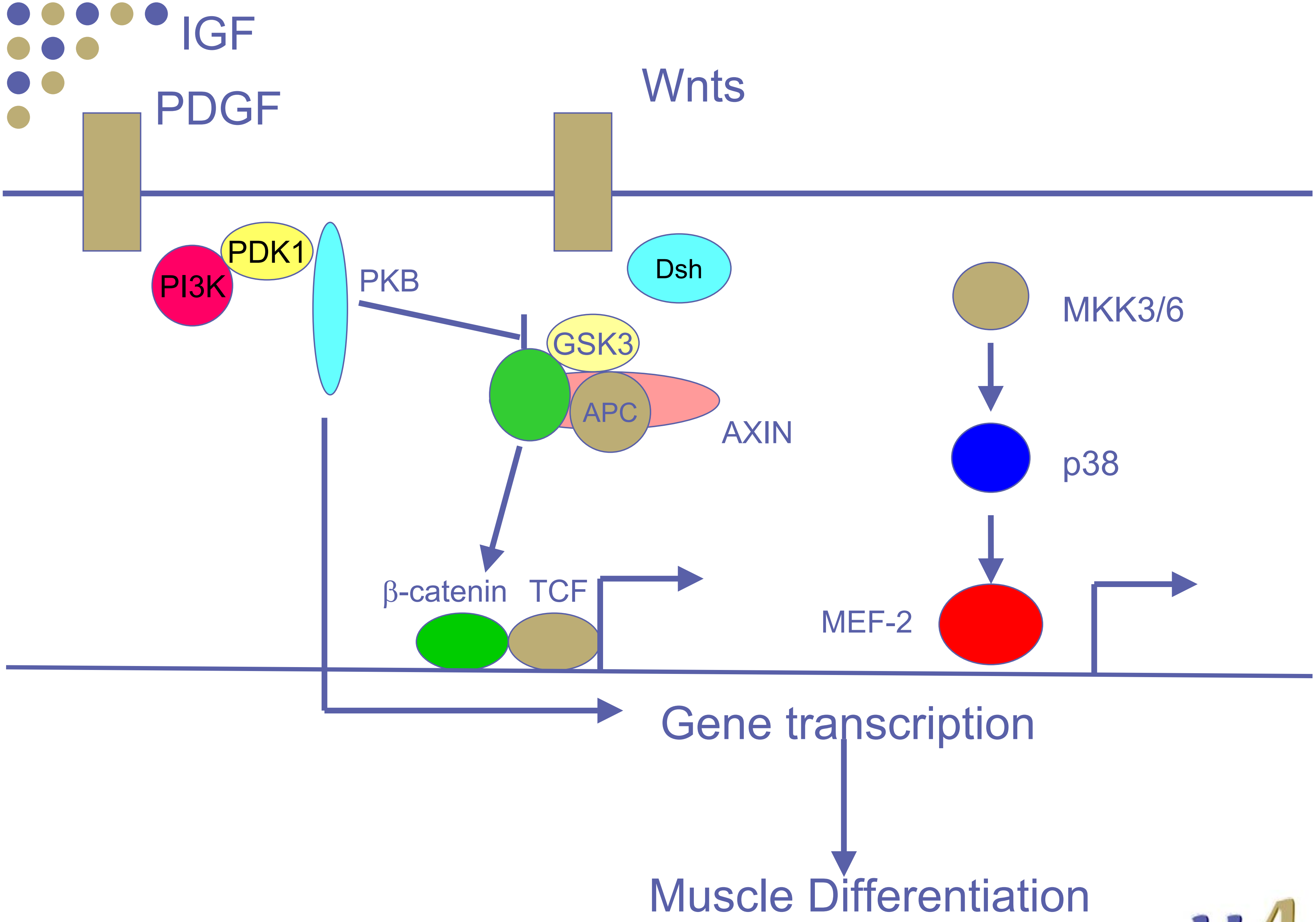
Myotubes

Growth Factors



Undifferentiated myoblasts

Differentiated myotubes



IGF

PDGF

Wnts

PDK1

PI3K

PKB

Dsh

MKK3/6

GSK3

APC

AXIN

p38

β-catenin TCF

MEF-2

Gene transcription

Muscle Differentiation

A number of studies have investigated gene expression in skeletal muscle cells undergoing differentiation.

However, these studies often do not distinguish primary changes in gene expression from secondary or tertiary ones.

How can we narrow the focus of genes involved during the process of differentiation?

ChIp (Chromatin Immunoprecipitation)

Provides us with the ability to identify genomic DNA associated with specific proteins (transcription factors)

Potentially have the capability to identify gene targets associated with specific transcription factors

Example: MEF2 (Myocyte Enhancing Factor)

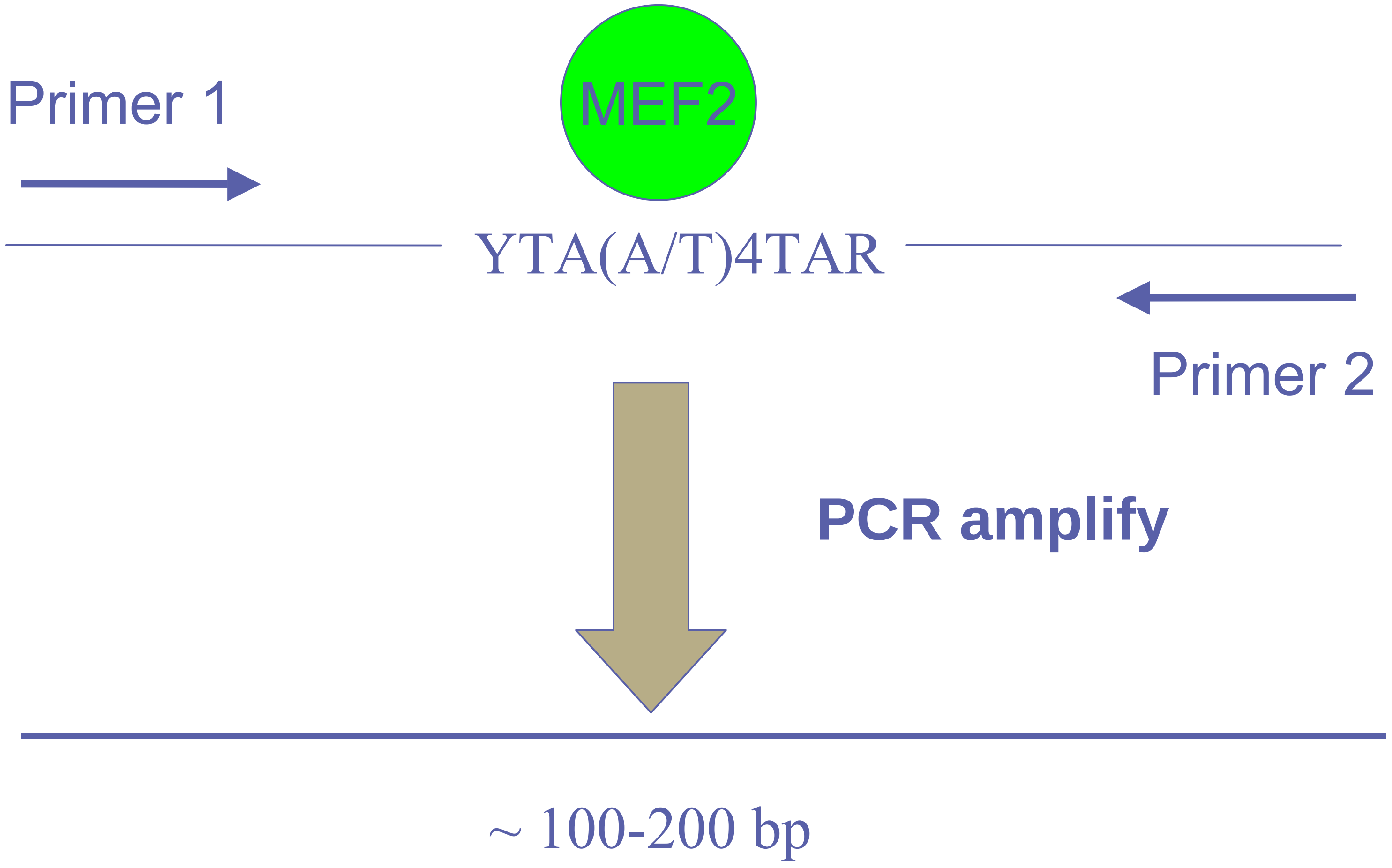
Muscle transcription factor known to be a key player in the regulation of muscle differentiation

Expression of a number of genes are known to be regulated by MEF2 e.g. muscle creatine kinase, skeletal α-actin, myosin light chain, and myoglobin

Binding sequence: $\mathrm{YTA(A/T)_4TAR}$

Identify potential upstream regulatory sites of genes containing this sequence

Design primers to flank this region



Primer 1

MEF2

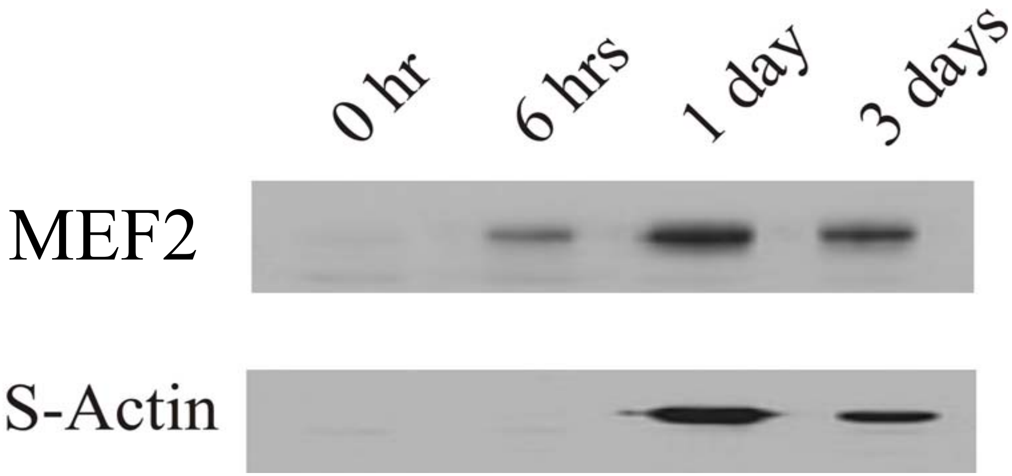
YTA(A/T)4TAR

Primer 2

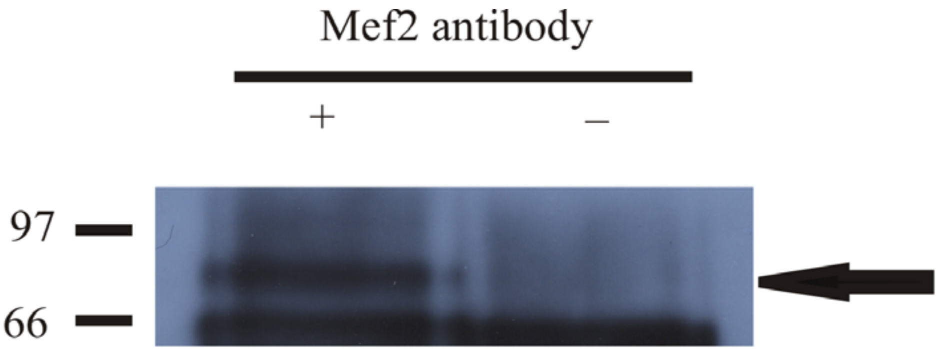
PCR amplify

~ 100-200 bp

MEF2 Expression During Muscle Differentiation

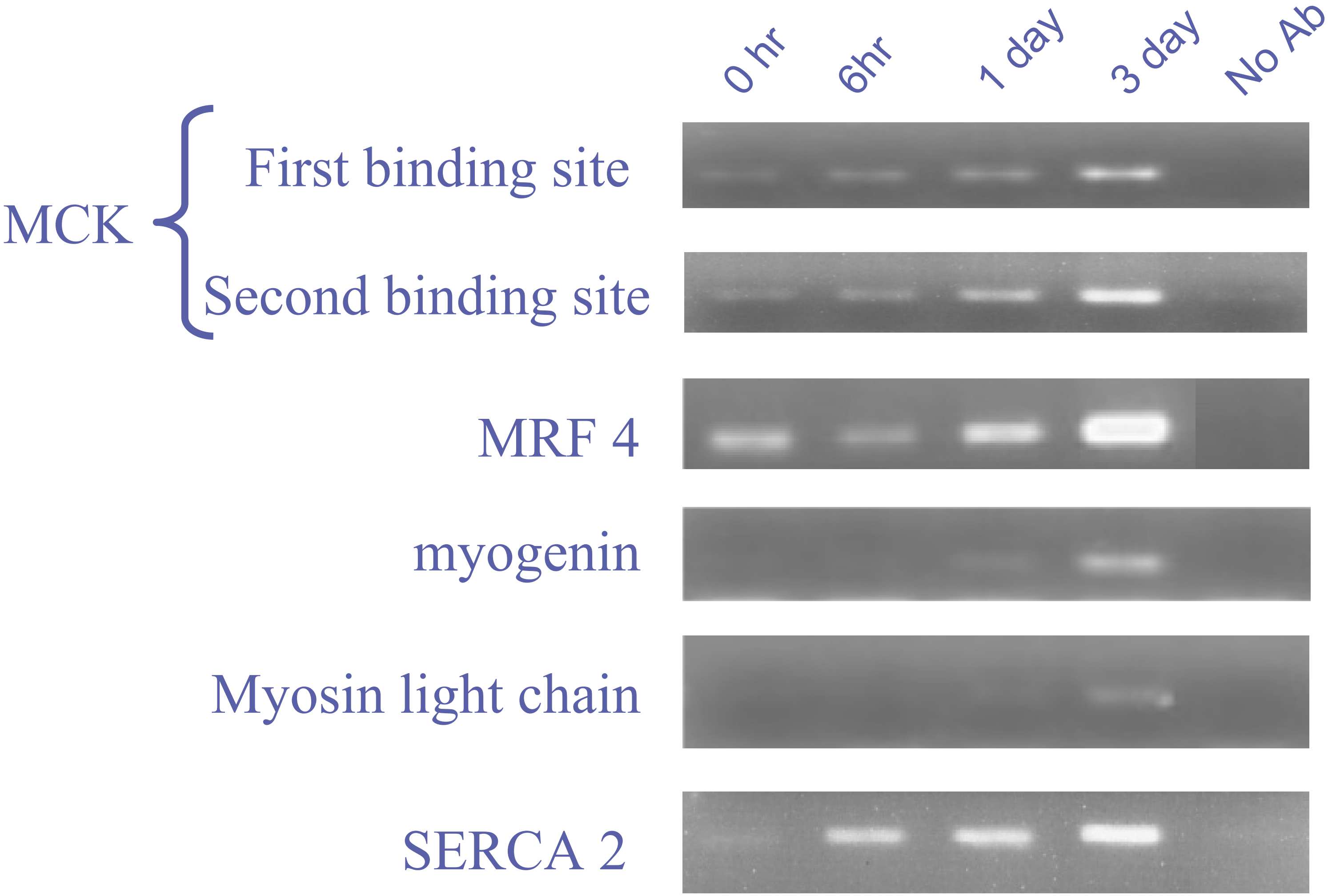


MEF2 Immunoprecipitation



3 day differentiated muscle

ChIP Analysis of Known MEF2 Associated Genes



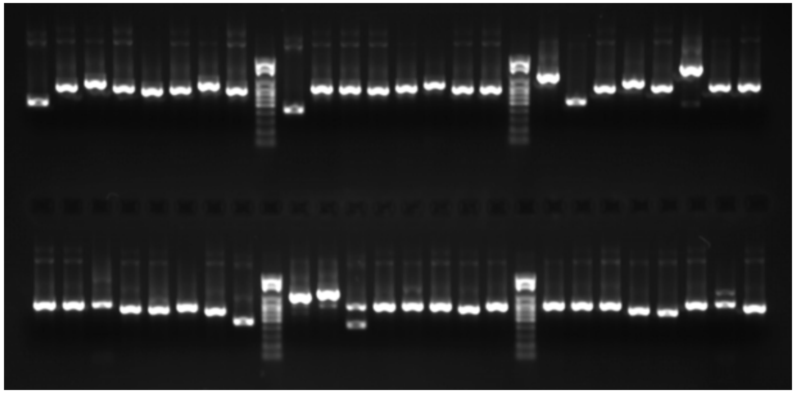
Construction of the 7K mouse CpG island array

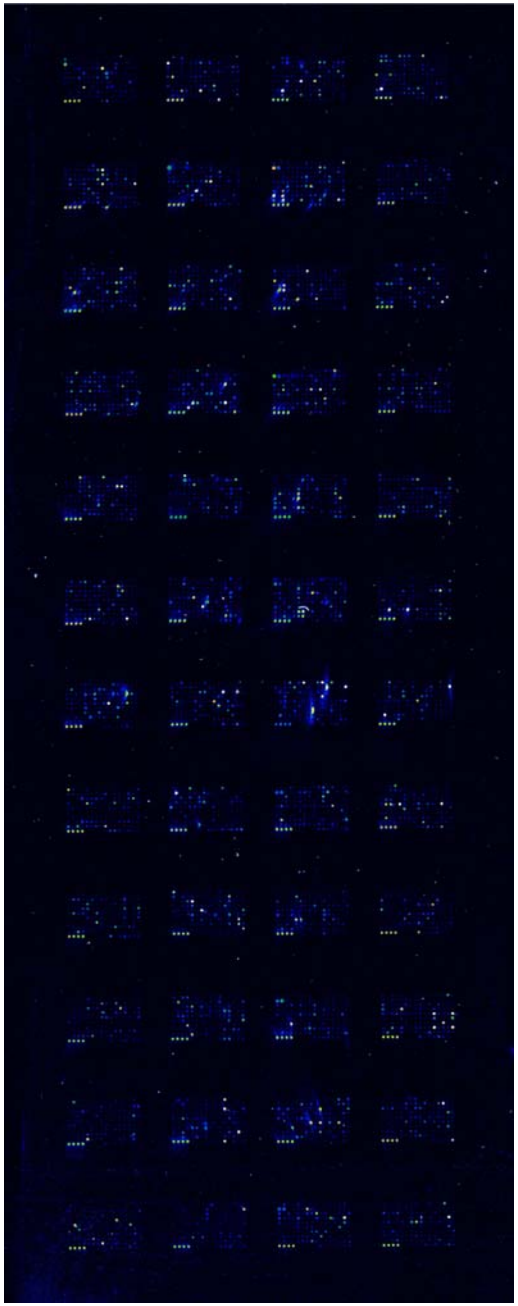
-Obtained mouse CGI library from the Sanger Institute UK

-all CpG islands were cloned into pGEM-5Zf vectors and plated

-used colony picker (Genetix Q-pix2)

-amplified inserts using T7/SP6 primers





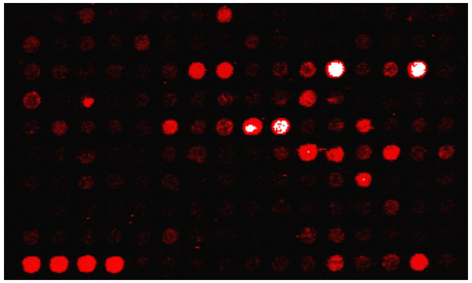
Sample mouse CGI array v.1

Total number of spots: 7680

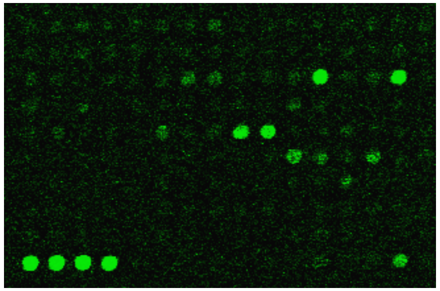
Methods

- C2C12 mouse skeletal muscle myoblast cell line
  - Grown to 80% confluence
  - Differentiation initiated by serum withdrawal and addition of 10 µg/ml IGF-1
  - Harvest cells and isolate total RNA
- Time course: 0, 6 hr, 1, 2, and 3 days

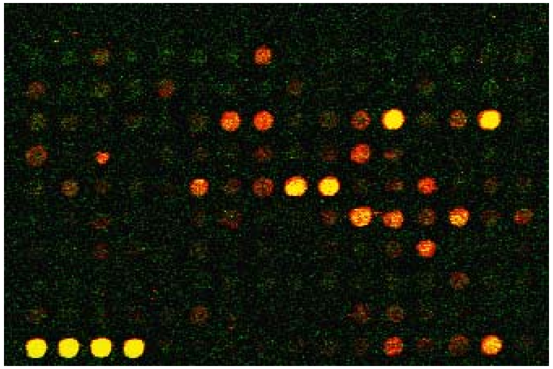
Antibody

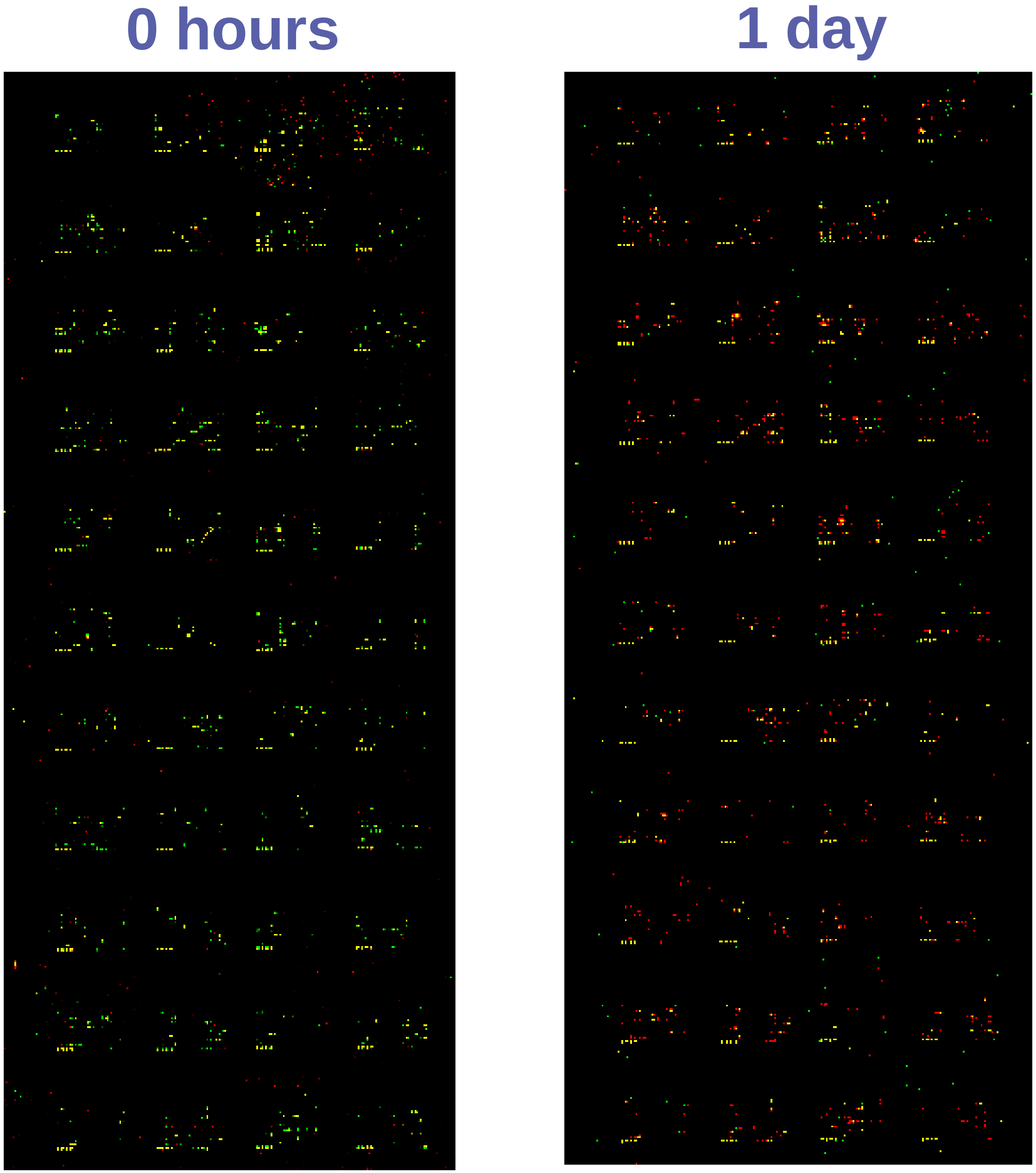


No Antibody



Composite




0 hours
1 day

Informatics Pipeline

Hits from microarrays

(>75% quartile intensities, >2-fold) → 291 clones

↓

Sequence clones → 247

↓

Remove redundancy → 79 remaining

↓

Scan for sequences with repetitive elements → 60 remaining

↓

Submit sequences to BLAT (UCSC)

↓

↓

Find only those with exact matches (no gaps)

↓

Look for any genes (predicted or known) in region

↓

Use MSCAN to search for potential MEF2 binding consensus sequence

↓

Extract regulatory region

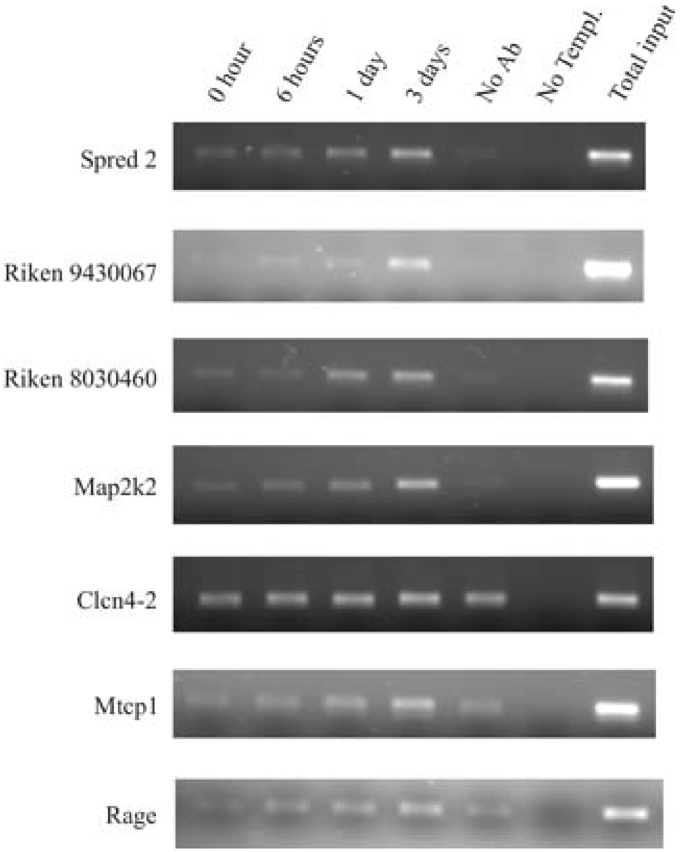
↓

Design primers to this region

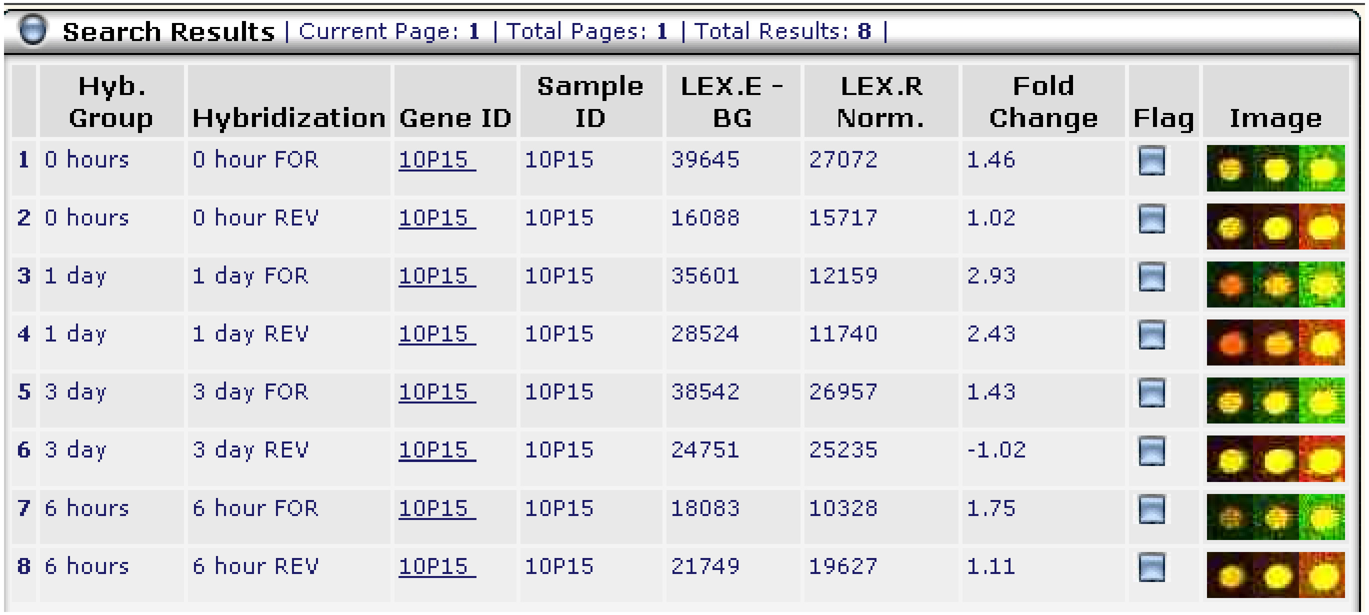
↓

Run ChIP with these specific primers and confirm microarray result

ChIP Reconfirmation of MEF2 ChIP on Chip Targets



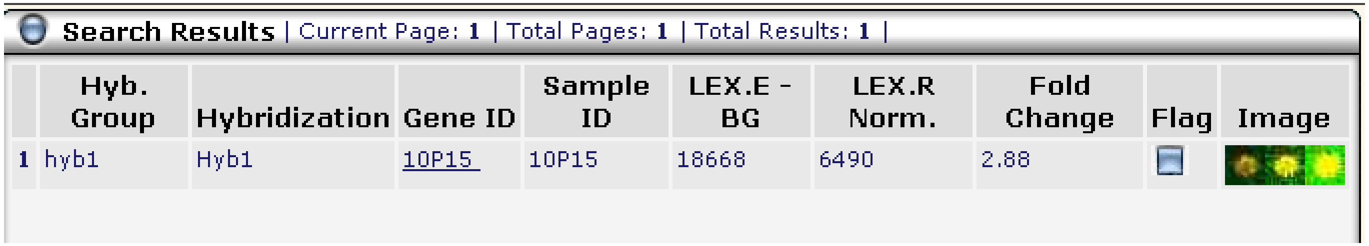
Amplified DNA



Search Results | Current Page: **1** | Total Pages: **1** | Total Results: **8** |

	Hyb. Group	Hybridization	Gene ID	Sample ID	LEX.E - BG	LEX.R Norm.	Fold Change	Flag	Image
1	0 hours	0 hour FOR	10P15	10P15	39645	27072	1.46		
2	0 hours	0 hour REV	10P15	10P15	16088	15717	1.02		
3	1 day	1 day FOR	10P15	10P15	35601	12159	2.93		
4	1 day	1 day REV	10P15	10P15	28524	11740	2.43		
5	3 day	3 day FOR	10P15	10P15	38542	26957	1.43		
6	3 day	3 day REV	10P15	10P15	24751	25235	-1.02		
7	6 hours	6 hour FOR	10P15	10P15	18083	10328	1.75		
8	6 hours	6 hour REV	10P15	10P15	21749	19627	1.11		

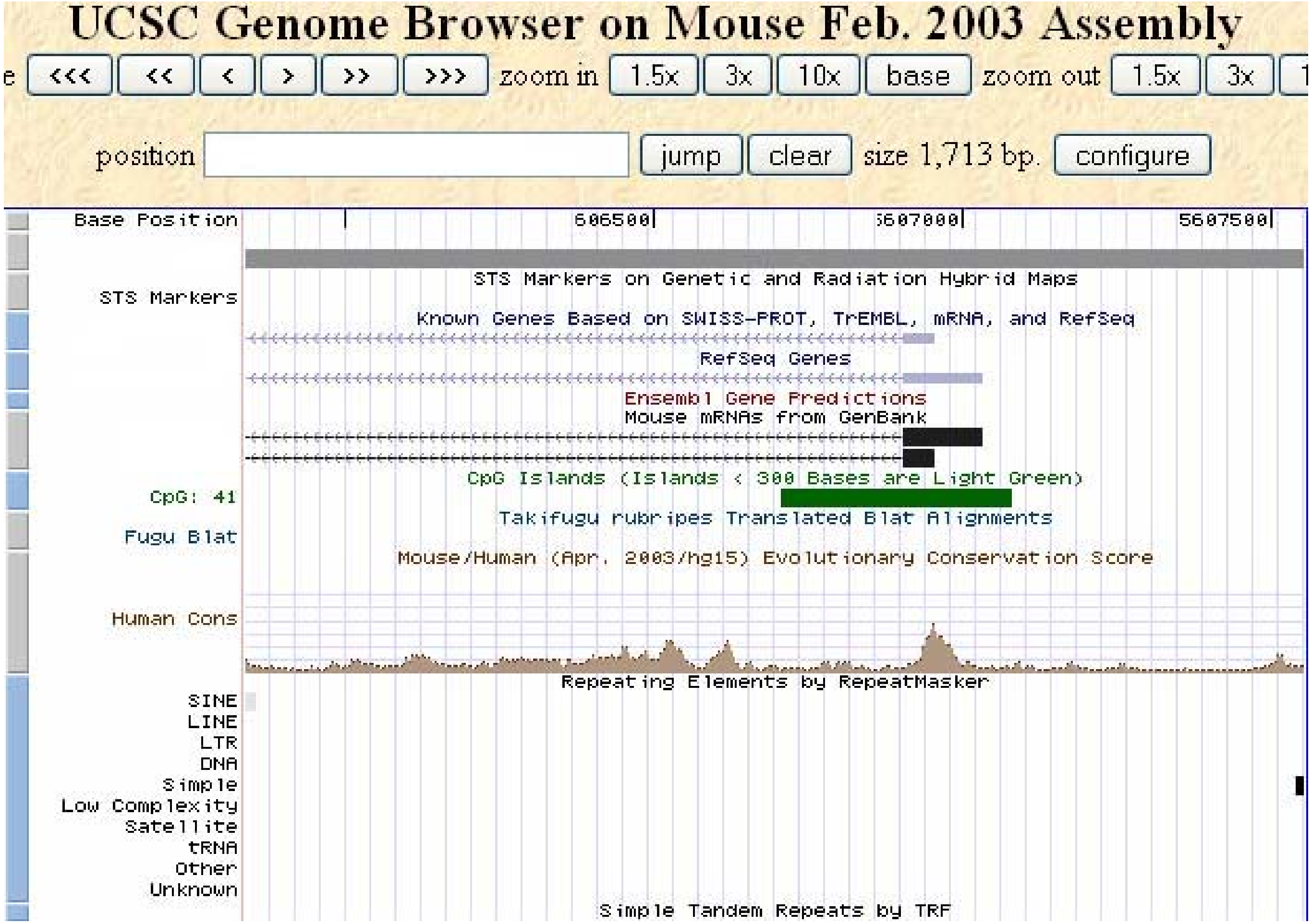
Non-amplified DNA



Search Results | Current Page: **1** | Total Pages: **1** | Total Results: **1** |

	Hyb. Group	Hybridization	Gene ID	Sample ID	LEX.E - BG	LEX.R Norm.	Fold Change	Flag	Image
1	hyb1	Hyb1	10P15	10P15	18668	6490	2.88		

Identification of a Novel Gene Target

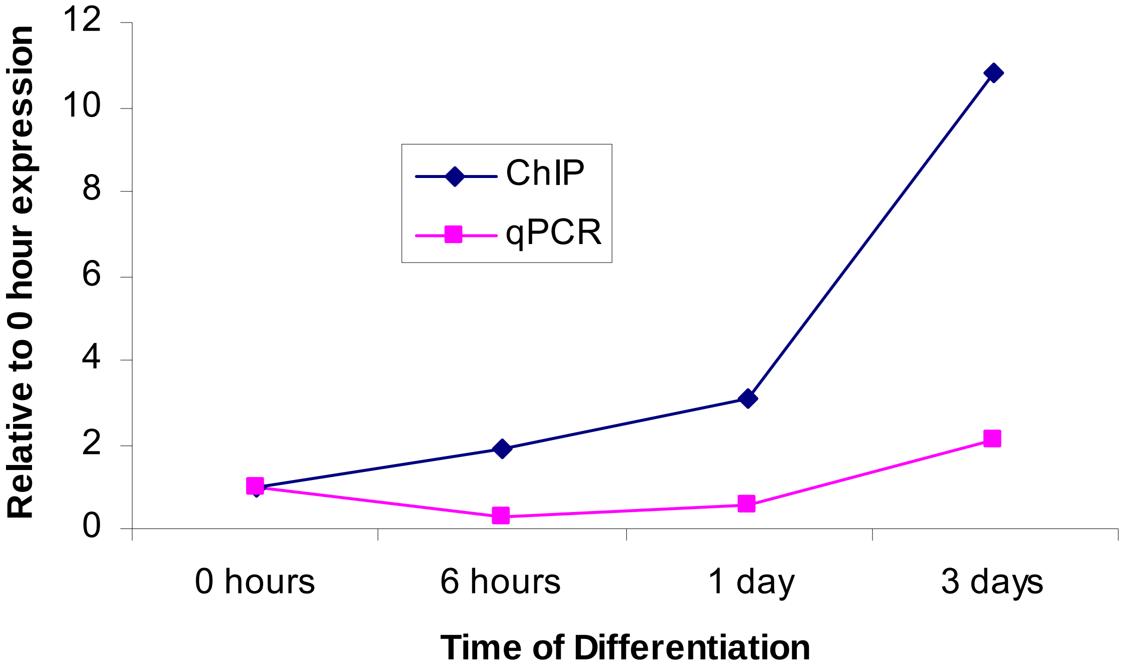


UCSC Genome Browser on Mouse Feb. 2003 Assembly

position

size 1,713 bp.

MEF2 Binding and Gene Expression

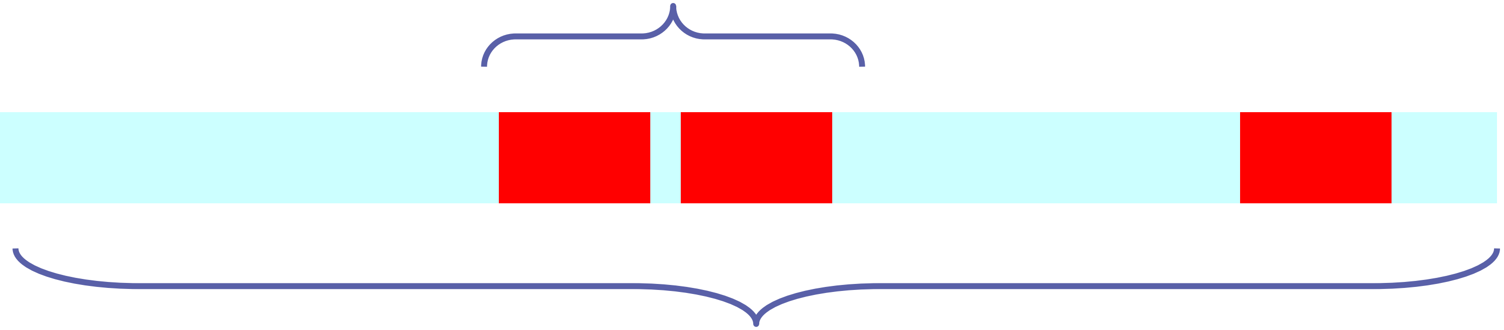


Transcript

mRNA = 4297 bp

ORF = 2285 bp

Protein

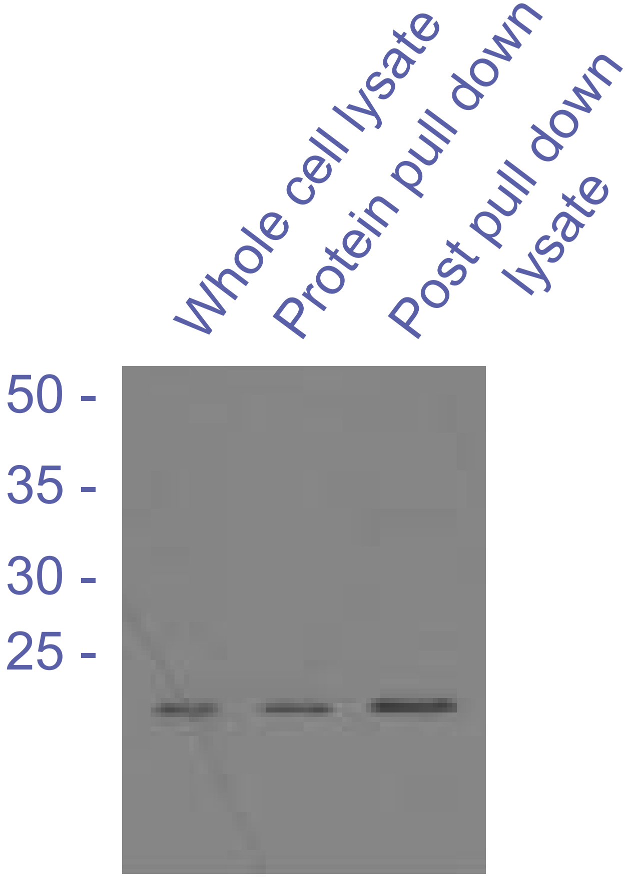


PH domains

Ras binding domain

761 aa/86.6 kDa

GST Fusion Protein Pull Down



Anti-ras WB

Future Work

Biological function

- Expression studies; in vitro and in vivo
- Subcellular localization
- Protein-protein associations
- Deletion mutants

Summary

- ChIP allows for the identification of primary gene targets directly associated with a specific transcription factor
- Coupled with microarrays ChIP provides a high throughput means to identify potential gene targets

UHN Microarray Centre

James Paris
Carl Virtanen
Xhibin Liu

Thank you

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