SUPPLEMENTARY data

MYOD REGULATES P57^{KIP2} EXPRESSION BY INTERACTING WITH A DISTANT CIS-ELEMENT AND MODIFYING A HIGHER-ORDER CHROMATIN STRUCTURE

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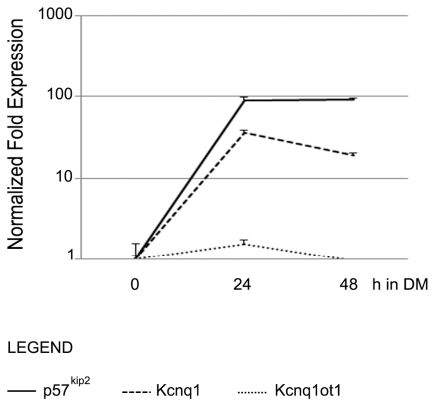
This section contains:

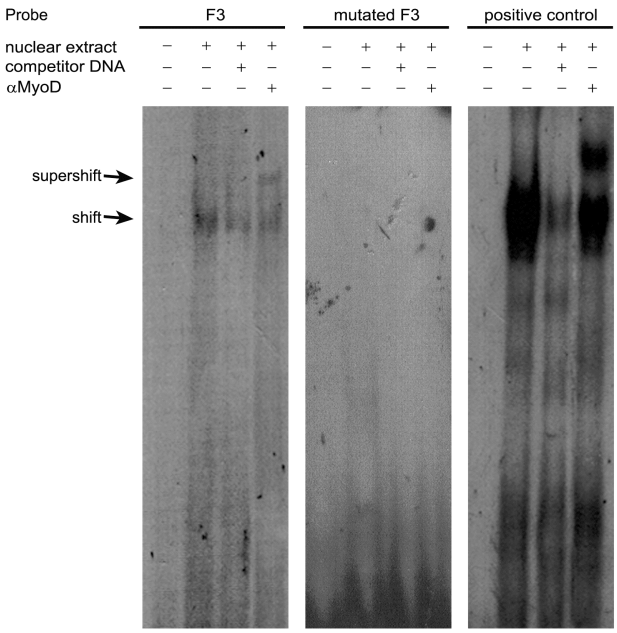
SUPPLEMENTARY FIGURES



Supplementary Figure 1

Hybrid MEFs behave as responsive cells.

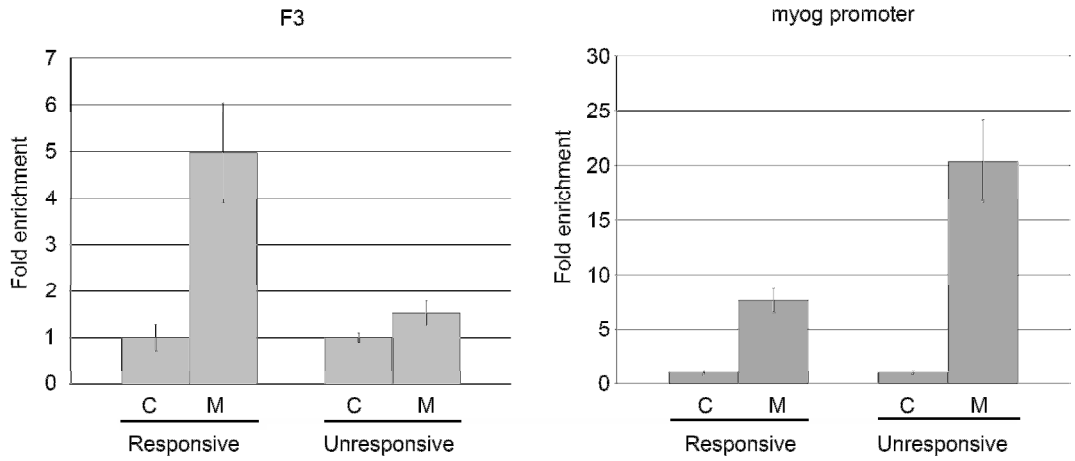
MyoD-expressing C57BL/6 x SD7 F1 hybrid mouse embryo fibroblasts were analyzed by q-RT-PCR at different times after the shift to differentiation medium (hours in DM). The expression levels are relative to the T0 value for each transcript. The results shown are representative of three independent experiments. Error bars indicate s.e.m. for each sample analyzed in triplicate



Supplementary Figure 2

MyoD directly binds to F3 fragment through the E-box-like element

EMSA analysis of MyoD binding to F3 fragment was performed with ^{32}P labelled oligonucleotides designed on fragment 3 and containing either wild type (F3) or mutated (mutated F3) E-box-like sequences. Nuclear extract prepared from differentiated C2 cells were analyzed for the binding to wild type or mutated sequences. Wild type or mutated competitor probes were added in a 100-fold molar excess. The specificity of binding was tested by means of anti-MyoD antibody. Arrows indicate the mobility of protein-DNA complexes (shift) and that of antibody-protein-DNA complexes (supershift). An oligonucleotide designed on MCK enhancer and containing a canonical E-box was used as positive control.



Supplementary Figure 3

MyoD induces H3 acetylation at F3 in responsive cells

q-ChIP assays were performed to analyze H3 acetylation at F3 and myogenin (myog) promoter in responsive (C57BL) and unresponsive (C3H10T1/2) mouse embryo fibroblasts, infected with either a retroviral empty vector as a control (C) or with a retroviral vector expressing MyoD (M) and analyzed after 24 hours of differentiation.

qPCR values, normalized relative to the input chromatin and to the Pancreatic amylase 2 (*Amy2*) signal, used as a baseline control, were expressed as fold increase respect to control cells.

The values reported in the graphs are representative of three independent experiments. The error bars indicate the standard deviation for each sample analyzed in triplicate.

SUPPLEMENTARY METHODS

Primer sequences

OLIGONUCLEOTIDES USED FOR qRT-PCR		
Gene	**Primers**	**Primers Sequences 5'-3'**
18S	Forward	ACGACCCATTCGAACGTCTG
	Reverse	GCACGGCGACTACCATCG
HPRT	Forward	TCCGGAGCGGTAGCACCTCC
	Reverse	GACTGCGGGTCGGCATGACG
p57	Reverse	AACTTCCAGCAGGATGTGCC
	Reverse	CATCCACTGCAGACGACCAG
Kcnq1ot1	Forward	TTCTGGAGGCGATTGAGGC
	Reverse	AGCAACCAGAACCAGGTGAGAG
Kcnq1	Forward	TGAGAAAGATGCGGTGAACG
	Reverse	GCGTAGCTGCCAAACTCGAT

OLIGONUCLEOTIDES USED FOR RT-PCR AND ALLELIC EXPRESSION ANALYSES					
Gene	**Primers**	**Primers Sequences 5'-3'**	**Article reference**	**Polymorphism**	**Enzyme**
p57	Forward	TTCAGATCTGACCTCAGACCC	(Caspary et al., 1998)	RFLP	AvaI
	Reverse	AGTTCTCTTGCGCTTGGC			
Kcnq1	Forward	GATCACCACCCTGTACATTGG	(Caspary et al., 1998)	RFLP	PvuII
	Reverse	CCAGGACTCATCCCATTATCC			
HPRT	Forward	GCCGACCCGCAGTCCCAGCGTCG	(Figliola et al., 2008)	-	-
	Reverse	GGCTGTACTGCTTAACCAGGGAAAG			

OLIGONUCLEOTIDES USED FOR ChIP				
Region	**Primers**	**Primers Sequences 5'-3'**	**Article reference**	**Region of amplification**
p57 promoter	Forward	CGTCGCGGTGTCACGTTA	(Figliola et al., 2008)	From 579 to 999 in AF160190
	Reverse	CTTGTCCTGTCCAGCTTGG		
F1	Forward	GGCTGCCACGTCACCAA	(Fitzpatrick et al., 2007)	From 1855 to 1992 in AF119385
	Reverse	CCTGACTGGACCAAAATGCA		
F2	Forward	TTTTTCACGGTGAGGTCATATCAGC	(Fitzpatrick et al., 2007)	From 2151 to 2252 in AF119385
	Reverse	GAGGTGTAGTGCTCAAGTGATCCGA		
F3	Forward	TTTTTCTCGGCATGGTTCTC	This work Primer-BLAST	From 2586 to 2802 in AF119385
	Reverse	AAACGAATACGGAGCCACTG		

OLIGONUCLEOTIDES USED FOR ChIP qPCR.				
Region	**Primers**	**Primers Sequences 5'-3'**	**Article reference**	**Region of amplification**
F3	Forward	GCACAAGTCGCAAGTCCGCG	This work Primer-BLAST	From 2655 to 2770 in AF119385
	Reverse	ATGGAGCCCAGCCGCGAAAG		
myog promoter	Forward	TGGCAGGAACAAGCCTTTTGCGA	This work Primer-BLAST	From 26 to 166 in NC_000067
	Reverse	AGTCCGCTCATAGCCCGGGG		
amy2 promoter	Forward	TCAGATGGGAGGACTGCTATTGT	(Cao et al., 2006)	From 219 to 288 in NC_000069.6
	Reverse	GCTCACATTCCTTGGCAATATCA		

OLIGONUCLEOTIDES USED FOR ALLELE-SPECIFIC ChIP					
Region	**Primers**	**Primers Sequences 5'-3'**	**Article reference**	**Polym.**	**SNP**
F3	Forward	TTTTTCTCGGCATGGTTCTC	This work Primer-BLAST	SSCP	Substitution C57/BL6 (T) SD7 (C) position 2622, Deletion SD7 (G) position 2634, Insertion SD7 (T) position 2743 in AF119385
	Reverse	AAACGAATACGGAGCCACTG			

Electrophoretic Mobility Shift Assay (EMSA)

Differentiated C2 cells were scraped into 1.5 ml of cold phosphate buffered saline (PBS), pelleted and lysed in 0.5% NP-40 and centrifuged to pellet the nuclei. Nuclear proteins were then extracted in 20 mM HEPES (pH 7.6), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.05% NP40, 0.5 mM dithiothreitol, plus a standard protease-inhibitor cocktail. Protein concentration was determined by Bradford assay. The upper strands of the double-stranded oligonucleotides used as probes in EMSA experiments correspond to the following sequences: 5'-CGCAAGTCCGCGTGGCCGCGCTCAT-3', containing the wild type F3 E-box-like sequence (F3), 5'-CGCAAGTCCGCGGGCCGCGCTCATC-3', containing the mutated F3 E-box-like sequence (mutant F3), 5'- GATCCCCCCAACACCTGCTGCCTGA-3', containing the MCK enhancer E-box consensus (Weintraub et al., 1990), used as a positive control.

EMSAs were carried out by incubating 10 mg of each extract in a final volume of 15 µl of 20 mM HEPES (pH 7.9), 0.1 mM EDTA, 1.5 mM DTT containing 2 µg of polydI-polydC for 10 min on ice and for 20 min at room temperature. For supershift experiments, anti-MyoD antibody ([sc-760] from Santa Cruz biotechnology) was added and incubated for 10 minutes on ice and 20 min at room temperature. For competition experiments 100 fold molar excess of unlabeled oligonucleotides carrying F3, mutant F3 or the positive control was used. ^{32}P-labeled double stranded oligonucleotides ($4x10^4$ cpm) were added and incubated for 15 min at room temperature. DNA-protein complexes were separated by electrophoresis on a 6% polyacrylamide gel in 0.25x TBE buffer (25 mM Tris, 25 mM boric acid, 0.6 mM EDTA) and visualised by autoradiography.

Plasmids

Plasmids used in the enhancer-blocking assays, pneo, Epneo and 11-22 [3'-5'], containing the 11-22 fragment from KvDMR1 cloned into Epneo vector between the Eδ enhancer and the Vδ promoter using SalI and ClaI sites (Fitzpatrick et al., 2007 and Figure 3) were kindly provided by Professor M. J. Higgins (*Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, New York*). For the realization of the control plasmid for 3C, a KvDMR1 fragment spanning from 2101 to 2463 of AF119385 and including the NcoI restriction site used in 3C assay was amplified and cloned into pCR2.1 vector. A p57 promoter region spanning from 587 to 1245 of AF160190 was amplified and the PCR product was digested with SacI and NcoI and cloned into SacI-NcoI sites of the recombinant plasmid in order to join p57 promoter and KvDMR1 sequences.

H3Ac chromatin immunoprecipitation

ChiP assays were carried out with anti-H3Ac antibody [Upstate 06-599] according to the manufacturer's recommended protocol. The amplification reactions were performed in triplicate as described in Materials and Methods using 2.5 ng of DNA.

qPCR values for F3 and myogenin promoter, normalized relative to the input chromatin and to the Pancreatic amylase 2 (*Amy2*) signal, used as a baseline control, were expressed as fold increase respect to control cells.

SUPPLEMENTARY REFERENCES

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Weintraub, H., Davis, R., Lockshon, D., and Lassar, A. (1990). MyoD binds cooperatively to two sites in a target enhancer sequence: occupancy of two sites is required for activation. Proceedings of the National Academy of Sciences of the United States of America *87*, 5623-5627.