Potential gene regulatory role for cyclin D3 in muscle cells

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Cyclin D3 is important for muscle development and regeneration, and is involved in post-mitotic arrest of muscle cells. Cyclin D3 also has cell-cycle-independent functions such as regulation of specific genes in other tissues. Ectopic expression of cyclin D3 in myoblasts, where it is normally undetectable, promotes muscle gene expression and faster differentiation kinetics upon serum depletion. In the present study, we investigated the mechanistic role of cyclin D3 in muscle gene regulation. We initially showed by mutational analysis that a stable and functional cyclin D3 was required for promoting muscle differentiation. Using chromatin immunoprecipitation assays, we demonstrated that expression of cyclin D3 in undifferentiated myoblasts altered histone epigenetic marks at promoters of muscle-specific genes like MyoD, Pax7, myogenin and muscle creatine kinase but not non-muscle genes. Cyclin D3 expression also reduced the mRNA levels of certain epigenetic modifier genes. Our data suggest that epigenetic modulation of muscle-specific genes in cyclin-D3-expressing myoblasts may be responsible for faster differentiation kinetics upon serum depletion. Our results have implications for a regulatory role for cyclin D3 in muscle-specific gene activation.

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1. Introduction

The process of muscle differentiation involves expression of early differentiation markers, withdrawal from cell cycle, expression of muscle-specific structural genes and fusion to form multinucleated myotubes (Andres and Walsh 1996). These events are regulated by the paired-box homeodomain proteins (Pax3 and Pax7), muscle regulatory factors (MRFs) – MyoD, Myf5, myogenin and MRF4, and the myocyte enhancer factor 2 (MEF2) proteins (MEF2A-D) (Arnold and Braun 1996). These muscle-specific transcription factors work along with their specific co-activators/co-repressors and epigenetic regulators to control the myogenic gene expression programme (Palacios and Puri 2006; Perdiguero *et al.* 2009). Histone modifications especially the well-characterized histone 3 lysine modifications such as H3K4/27/9 mono/di/trimethylations and H3K9/14/27 acetylations have been shown to be involved in regulating myogenic gene loci, and many epigenetic regulators catalysing these modifications are known to interact with muscle transcription factors (Puri *et al.* 1997; Mal *et al.* 2001; Mal 2006; Asp *et al.* 2011; Tao *et al.* 2011; Ling *et al.* 2012).

Cyclin D3 is an atypical D-type cyclin expressed chiefly in differentiated tissues (Bartkova *et al.* 1998; Jin *et al.* 2009). Cyclin $D3^{-/-}$ mice show retarded growth, significant loss of muscle mass and impaired muscle regeneration upon injury, suggesting important roles for cyclin D3 during myogenesis (Sicinska *et al.* 2003; De Luca *et al.* 2013). Cyclin D3 is expressed at low levels in myoblasts and is up-regulated and stabilized in differentiating muscle cells. Knockdown of cyclin D3 impairs ability of myoblasts to differentiate, resulting in the formation of smaller myofibres and inhibiting MyoD-mediated transdifferentiation of fibroblasts (De Santa *et al.* 2007). In post-mitotic differentiated myotubes, cyclin D3 is tethered at the nucleoskeleton in an insoluble complex with hypophosphorylated retinoblastoma protein (pRb), cyclin-dependent kinase 4 (cdk4) and the cell cycle inhibitor p21 (Cenciarelli *et al.* 1999). In association with pRb, cyclin D3 is involved in reorganization of the nuclear lamina during initial stages of muscle differentiation

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(Mariappan and Parnaik 2005; Mariappan *et al.* 2007). Differentiation is impaired in muscle cells expressing disease-causing mutations in lamin A (Parnaik and Manju 2006), and reduced binding of cyclin D3 to lamin A has been reported to block muscle differentiation in laminopathic cells (Favreau *et al.* 2008). Independent of its cell cycle effects, cyclin D3 acts as a co-activator for peroxisome proliferator-activated receptor γ (PPARγ) and is found to associate along with it at chromatin of PPARγ target genes in differentiated adipose tissue (Sarruf *et al.* 2005). Cyclin D3 also acts as a co-regulator for various steroid hormone receptors and transcription factors (Despouy *et al.* 2003; Liu *et al.* 2004). In B-cells, a subnuclear fraction of cyclin D3 has been shown to regulate transcription of more than 200 genes including the genes coding for the variable segments of immunoglobulin κ (Powers *et al.* 2012). Cytoplasmic cyclin D3–cdk4 complexes in differentiated myotubes phosphorylate and regulate the activity of CUG-binding protein 1 (CUGBP1), an RNA binding protein implicated in the pathology of myotonic dystrophy type 1 and 2 (DM1 and DM2). CUGBP1–eukaryotic initiation factor 2 (eIF2) complexes regulate translation of proteins involved in muscle development and differentiation. Reduced levels of cyclin D3, and therefore CUGBP1-eIF2 complexes, cause a differentiation defect in DM1 cells, which can be alleviated by ectopic expression of cyclin D3 in DM1 myoblasts or restoration of cyclin D3 levels in dystrophic mice (Timchenko *et al.* 2001; Salisbury *et al.* 2008; Jones *et al.* 2012). We have earlier observed that expression of cyclin D3 in C2C12 myoblasts increases levels of muscle-specific genes such as MyoD and myogenin upon serum depletion and enhances the differentiation kinetics of muscle cells but does not initiate precocious differentiation (Gurung and Parnaik 2011). However, the mechanisms involved in the promotion of muscle differentiation by cyclin D3 are not known.

In this study we investigated if cyclin D3 can prime muscle-specific genes for enhanced expression epigenetically, by altering the histone marks at their regulatory regions in C2C12 myoblasts, in the absence of differentiation cues. The C2C12 mouse myoblast cell line has been widely used to study muscle differentiation, which is induced by lowering serum levels in the culture medium. We report that the expression of functional cyclin D3 is essential for enhanced muscle differentiation, and causes remodelling of histone modification marks H3K4me3 (active mark), H3K9ac (active mark) and H3K27me3 (repressive mark) at the regulatory regions of muscle genes such as MyoD, Pax7, myogenin and muscle creatine kinase but not non-muscle genes in undifferentiated myoblasts. These chromatin changes are accompanied by changes in transcript levels of few epigenetic modifier genes. This remodelling of histone marks at muscle-specific gene promoters is likely to contribute to the faster differentiation kinetics observed in cyclin-D3-expressing cells on induction of differentiation.

2. Materials and methods

2.1 *Cell culture, DNA constructs and ectopic expression of cyclin D3*

C2C12 mouse myoblasts were maintained as sub-confluent cultures in DMEM supplemented with 20% FBS (growth medium, GM) (Yaffe and Saxel 1977). For induction of differentiation, 80–90% confluent cultures were transferred to DMEM containing 2% horse serum (differentiation medium, DM). The mammalian expression vector encoding the cyclin D3 gene tagged to HA was a kind gift from P Hinds, Harvard Medical School, MA. Cyclin D3 mutants D3-K112E-HA, D3-mutLXCXE-HA and D3-NΔ75-HA were generated by PCR based mutagenesis as described earlier (Mariappan and Parnaik 2005; Mariappan et al. 2007). DNA transfections were carried out with Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were grown for 24 h before transfection. Lipid and DNA complexes were added to cells in serum-free DMEM. After 3 h, the medium was replaced with GM and cells were harvested 24 h later or differentiated in DM for specified additional time before harvest. For uniform and high expression of ectopic wild-type cyclin D3 the adeno-viral system was used (He *et al.* 1998). Adeno-control and adeno-cyclin D3-HA viral supernatants were generated as previously described (Gurung and Parnaik 2011). Briefly C2C12 myoblasts were seeded 18–24 h before infection and incubated with viral supernatants for 2–3 h following which the supernatants were replaced with fresh GM. Cells were harvested after 24 h of infection and used directly or differentiated in DM for specified additional time before harvest.

2.2 *Antibodies*

Antibodies to cyclin D3 were obtained from Cell Signalling Technology (Beverly, MA, USA) and to HA epitope from Roche Applied Sciences (Indianapolis, IN, USA). ChIP-grade antibodies to H3K4me3, H3K27me3 and H3K9ac were from Millipore Corporation (Temecula, CA, USA). Normal IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antibody to lamin B1 was from Abcam (Cambridge, MA, USA). Mouse monoclonal antibody to myogenin (clone F5D) was from Developmental Studies Hybridoma Bank, University of Iowa, IA, USA. Mouse monoclonal antibody to MyoD was from DAKO, Agilent Technologies, Glostrup, Denmark.

2.3 *Western blot analysis*

Whole cell lysates from adeno infected myoblasts prepared in 1X Laemmli's sample buffer were boiled, electrophoresed and electroblotted onto PVDF membranes. Blots were

blocked with 5% Blotto in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST). For immunoblotting, blots were incubated with primary antibody in TBST at appropriate dilution for 1–2 h. Blots were then washed and incubated with peroxidase conjugated secondary antibody for an hour. Bound antibody was visualised using a chemiluminescence kit from Roche Applied Sciences.

2.4 *Immunofluorescence microscopy*

Cells grown on coverslips were washed with phosphate buffered saline (PBS) and fixed with 3.5% formaldehyde in PBS for 10 min at room temperature. Following washing, cells were permeabilised with 0.5% Triton-X 100 (vol/vol) for 6 min at room temperature and blocked with 0.5% gelatin in PBS for about an hour. Cells were then incubated with primary antibody diluted in PBS for 1–2 h followed by species/subtype specific Cy3/FITC-conjugated secondary antibody in PBS for an hour. For double-labelling experiments, fixed cells were incubated with first set of primary antibody and its species/subtype specific fluorescent secondary antibody conjugate followed by the second set of primary antibody and its species/subtype specific fluorescent secondary antibody conjugate. Coverslips were then mounted on glass slides in Vectashield (Vector laboratories, Burlingame, CA, USA) containing 1 μg/mL DAPI to visualise the nucleus. Slides were scanned on a LSM510 META/NLD confocal microscope (Carl Zeiss, Jena, Germany). Images were processed and analysed using the LSM510 META software and assembled using Adobe Photoshop. For quantification of labelled cells, more than n=150 cells were counted per sample in at least three independent experiments. There was no cross-reactivity of fluorescent secondary antibodies in control experiments in which the primary antibody was omitted.

2.5 *Quantitative real time PCR assays*

Total RNA was isolated from adeno-control and adeno-cyclin D3-HA cells using Trizol method. As per manufacturer's instructions, 1–5 μg of DNase treated RNA was reverse transcribed using Superscript II reverse transcriptase kit (Invitrogen). qRT-PCR was performed on ABI 7900HT cycler (Applied Biosystems, Foster City, CA, USA). Primers were designed using Primer3 Input (version 0.4.0) and Primer Blast (NCBI) or obtained from previous reports. Dissociation curves were analysed for each amplicon in every run. $2^{-\Delta\Delta Ct}$ method was used for quantification and each gene was analysed in triplicates (technical replicates) with RPLP0 (ribosome protein large, P0) as internal control. RPLP0 was reported to be one of the most suitable internal controls in a study with human myoblast cultures (Stern-Straeter *et al.* 2009). Final fold changes were derived from average of three independent biological replicates. Statistical significance was calculated using the Student's *t*-test. List of primers used is provided in table 1.

2.6 *Chromatin immunoprecipitation analysis*

Briefly 10^6 cells were cross-linked with 1% formaldehyde for 10 min at 37°C followed by quenching with 0.125 M glycine solution. Cells were washed twice with cold PBS and lysed in SDS lysis buffer (200 μL/10^6 cells) (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1) containing protease inhibitors. Lysate was sonicated to shear chromatin into fragments less than 500 bp in size using Bioruptor (Diagenode). Sheared chromatin was then diluted with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1 and 167 mM NaCl) containing protease inhibitors. Before addition of the antibody, 20 μL of sample (1%) was removed as input. After preclearing, diluted chromatin was incubated overnight with ChIP antibody (2–5 μg) against specific histone marks or normal IgG at 4°C. Following this the antigen-antibody complexes were captured by incubation with pre-blocked protein A agarose for 30 min. The agarose-antigen-antibody complexes were washed sequentially with low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl) and LiCl wash buffers (0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1mM EDTA, 10 mM Tris, pH 8.1) followed by TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0). Immunoprecipitated DNA was obtained by elution of antigen-antibody complexes from agarose using 1 M NaHCO3 and 1% SDS. The input samples and the eluates were reverse cross-linked and treated with proteinase. DNA was isolated and purified using the phenol-chloroform-isoamyl alcohol method. After sodium acetate-ethanol precipitation, DNA was dissolved in 30 μL of water and used for qRT-PCR using 7900HT thermal cycler (Applied Biosystems). Primers specific to different regions of gene promoters were designed or obtained from previous reports. Each primer (promoter region) in individual ChIP experiments was analysed in triplicate qRT-PCR reactions and three independent ChIP experiments were performed. Levels of histone modification marks were expressed as average of percentage of inputs from three independent ChIP experiments and statistical significance was calculated using Student's *t*-test. Figures show percentage of input values obtained for both IgG and specific antibody at each locus in both control and cyclin D3-HA myoblasts. Specific antibody enrichment ≥ 3-fold over IgG was considered to be significant. Enrichment with IgG was negligible (≤0.05%) at most loci hence percentage of input values of IP for control

Table 1. Primers used for transcript analysis by RT-qPCR

No.	Primer name	Forward sequence	Reverse sequence
1	Suv39H1	CGCATCGCATTCTTTGCC	AAGCCGTTGTCCCACATTTG
2	G9a	TCGGGCAATCAGTCAGACAG	TGAGGAACCCACACCATTCAC
3	GLP	TCGCTACGACTGTGTTGTCC	CTGCGAACTGAGACTTGCAC
4	Ezh2	TTACTGCTGGCACCGTCTGATGTG	TGTCTGCTTCATCCTGAGAAATAATC
5	Suz12	ACTATTGCTGTTAAGGAGACGCTGA	GCAGGTCGTCTCTGGCTTCT
6	YY1	GTGGCAAAGCGTTCGTTGAG	ACATAGGGCCTGTCTCCG
7	Ezh1	TCAGACCCCAATGCACTTCC	GGCGTACTCCTTTGCTCCTT
8	Bmi1	ATGAGTCACCAGAGGGATGG	TCGAGGTCTACTGGCAAAGG
9	Ring1	TGCCTGCATAGGTTCTGCTC	GAGATCAGGGCGTCGAAGTT
10	Jarid2	CACTCTTCTGCACGCTATGG	CGGCAAGACTTCTGCTTCTC
11	Set7	CGAATCGAGGCGTTAGAGAC	GCTTCCCTGACATCGTGACT
12	UTX	GCAATGCACCACCTCCAGTA	CGTACCTGTGCAACTCCTGT
13	Jmjd3	GACGTTCTTCACCTCGTTCC	CCTGGAGACCTTGTGTGGAT
14	Jmjd2a	TTCTTCACACTCGGCTCTCC	GGCTCCACTTCATCTCAGGA
15	Jmjd2b	ACTGCGCTGAGTCCACTAAC	GCTGCAGGATGCGTACAAAC
16	Jmjd2c	CCGCAAGCGTATGAAGAGAG	ACCACATAGGGCCAGTCATC
17	Jhdm1a	TGGACCATTTCTAAGGCGGC	GTGTGTGGTCCACTCCATGT
18	Jhdm1d	TGTGCGTAGTTACCCTCAGC	GCCAGCTGCTTTGGAAATGG
19	HDAC1	CTGTGAACTACCCACTGCGA	TTCGTAAGTCCAGCAGCGAG
20	GCN5	ACATCAAGGTGCCCAAGAGC	AAGCAGCTGAGTCCAGGGTA
21	PCAF	GAAGATCCTGATGTGGCTCG	CATCAGGTGGGTTCCATAGC

and cyclin D3-HA samples were compared directly and *p*-values were calculated. Efficacy of ChIP assays was assessed using promoters of GAPDH (constitutively active, marked by active histone marks, absence of repressive marks) and HoxB8 and IgH enhancer region (constitutively repressed, marked by repressive marks, absence of active marks). List of primers used is provided in table 2.

3. Results

3.1 *Analysis of differentiation potential of myoblasts expressing cyclin D3*

We initially investigated the requirement for different functional regions of cyclin D3 for promotion of muscle differentiation in C2C12 mouse myoblasts. The C2C12 mouse myoblast cell line has been widely used to study muscle differentiation, which is induced by lowering serum levels in the culture medium. In a control experiment, C2C12 myoblasts were transfected with a plasmid encoding wild-type cyclin D3-HA or a control vector expressing only GFP. Cells were transferred to differentiation medium after 24 h of transfection and collected at 4, 8, 12 and 20 h of differentiation. Cells were fixed and analysed by immunostaining for the early differentiation marker myogenin. Control vector expressing myoblasts behaved like normal myoblasts and expressed myogenin during the differentiation time course as expected. At all time points tested, differentiating myoblasts expressing cyclin D3-HA showed higher percentage of myogenin-positive cells when compared to control GFP vector, indicating that cyclin D3 expression enhances the differentiation kinetics of myoblasts (figure 1a). Proliferating control and cyclin D3-HA myoblasts (0 h DM) did not show significant number of myogenin positive cells (2–3%), indicating that cyclin D3 expression itself does not induce precocious differentiation. This result is consistent with our previous findings (Gurung and Parnaik 2011). We used three mutants of cyclin D3, namely D3-K112E-HA, D3-mutLXCXE-HA and D3-NΔ75-HA, and analysed their differentiation potential. The point mutation K112E, which causes a lysine to glutamic acid substitution, renders cyclin D3 unable to bind to and activate cdk4, thus impairing differentiation by affecting cell cycle arrest (Baker *et al.* 2005; Hinds *et al.* 1994). D3-mutLXCXE is mutated in the canonical N-terminal LXCXE pRb binding motif, but this mutation only partially impairs pRb binding since pRb has also been shown to interact with cyclin D3 through a stretch of 20 amino acids at the C-terminus (De Santa *et al.* 2007). Interactions of cyclin D3 with pRb are necessary for its stabilization against degradation by glycogen synthase kinase 3β during the differentiation process (De Santa *et al.*

Table 2. Primers used for ChIP-qPCR analysis

No.	Primer name	Forward sequence	Reverse sequence
1	pPax7 (−0.8 kb)	GCGACCCCCTGAGGAAAA	CGAAAAGAAGTCTCCAACGAGTATT
2	pMyoD (−0.39 kb)	CTATGCTTTGCCTGGTCTCC	GGAAGGAGGGCAGAGAGACT
3	pMyoD-DRR (−5.3 kb)	GCCCGCAGTAGCAAAGTAAG	GAAACCGGATCCAACTAGCA
4	pMyogenin (−0.19 kb)	GAATCACATGTAATCCACTGGA	ACGCCAACTGCTGGGTGCCA
5	pMyogenin (−1 kb)	GCCCAGGACAGACAAATGATGCAA	AATGCCTTCTGGCACTAGAACCGT
6	MCK enhancer (−4.2 kb)	AGGGATGAGAGCAGCCACTA	CAGCCACATGTCTGGGTTAAT
7	pCyclin E1 (−0.16 kb)	CTGTGGTCCGTCGAGTCTCT	CTGAGTTCCAAGCCCAAGTC
8	pCyclin D1 (−0.35 kb)	TCCCTCCTAGCTGTCCTCCT	CGGACTGCTTCTCTCCAAAC
9	pGAPDH (−0.25 kb)	TCCCTAGACCCGTACAGTGC	CCGCATCTTCTTGTGCAGT
10	pHoxb8 (−0.09 kb)	CAACAACAGACTCCGGCTTT	GAGGGAATTGGCTGGGTAAC
11	pOct3/4 (−1 kb)	CAGAGCATGGTGTAGGAGCA	GCTGGCGGAAAGACACTAAG
12	pSox2 (−1 kb)	CTTGTGTCAGGGTTGGGAGT	GAGTCCTCTGCCCATGTAGC

2007). As shown in figure 1b, mutants D3-K112E-HA and D3-mutLXCXE-HA localized to the nucleus in the majority of transfected myoblasts. The D3-NΔ75-HA mutant containing an N-terminal deletion of 75 amino acids was mostly localized in the cytoplasm and was highly unstable, as very few cells expressing this mutant survived in either growth or differentiation medium. Analysis of myogenin expression in differentiating cells showed that cells expressing mutant D3-K112E-HA had lower percentage of myogenin expression when compared to control cells. Importantly, D3-mutLXCXE-HA, the partial pRb binding mutant did not show enhanced myogenin levels as wild-type cyclin D3, indicating that a fully functional cyclin D3 is required for promotion of differentiation (figure 1c). The muscle regulatory factor MyoD was also up-regulated upon cyclin D3 expression but this increase was not observed in the presence of cyclin D3 mutants (figure 1c).

Since efficiency of lipofectamine-mediated transfection in myoblasts was only ~30% or less, for further experiments adenoviruses carrying the cyclin D3-HA gene (Ad-ccnD3) or an empty vector construct (Ad-con) both of which co-express GFP were used to infect C2C12 myoblasts and were analysed 24 h after infection. Expression of cyclin D3-HA was confirmed by both Western blotting and immunofluorescence. Immunostaining with anti-HA antibody revealed 90–95% HA positive cells in these myoblasts (figure 1d). Approximately three-fold higher expression of cyclin D3 was obtained in myoblasts infected with cyclin D3-HA adenoviruses when compared to adeno-control infected myoblasts (figure 1e). To validate the adenoviral system and corroborate our findings with cyclin D3-HA plasmid construct we assessed the ability of adeno-infected myoblasts to differentiate. Cells were transferred to differentiation medium 24 h after infection and immunostained for myogenin. Cyclin D3-HA cells showed higher percentage of myogenin positive cells compared to control cells, as observed with transiently transfected cells (data not shown).

3.2 *Epigenetic effects of cyclin D3 expression on promoters of muscle-specific genes*

Chromatin was prepared from myoblasts infected with cyclin D3-HA and control adenoviruses 24 h post adenoviral infection. As cyclin D3 mutants could alter myogenesis through pathways not directly related to gene activation by cyclin D3, absence of cyclin D3 expression (empty vector control) was considered to be a more appropriate control for gene activation by cyclin D3 in the following experiments. ChIP assays were performed with antibodies directed against two transcriptional activating marks (H3K4me3, H3K9ac), a repressive mark (H3K27me3) and normal IgG (negative antibody control), and immunoprecipitated DNA was analysed by qRT-PCR using gene promoter specific primers. Enrichment of ≥ 3-fold over IgG was considered to be significant. As we could not detect any significant enrichment for H3K9me3 mark on most loci that we tested, we did not analyse this mark further (data not shown).

MyoD promoter: MyoD is the master regulator of myogenesis known to bind to and regulate several loci in myogenic cells (Cao *et al.* 2010). We previously observed an up-regulation of MyoD transcript levels on induction of differentiation in cyclin-D3-expressing myoblasts (Gurung and Parnaik 2011). To check if the histone mark profile of MyoD regulatory regions is altered in undifferentiated myoblasts expressing cyclin D3, the proximal promoter region and the well characterised DRR (distal regulatory region) of MyoD were analysed. At the proximal promoter region (−0.3

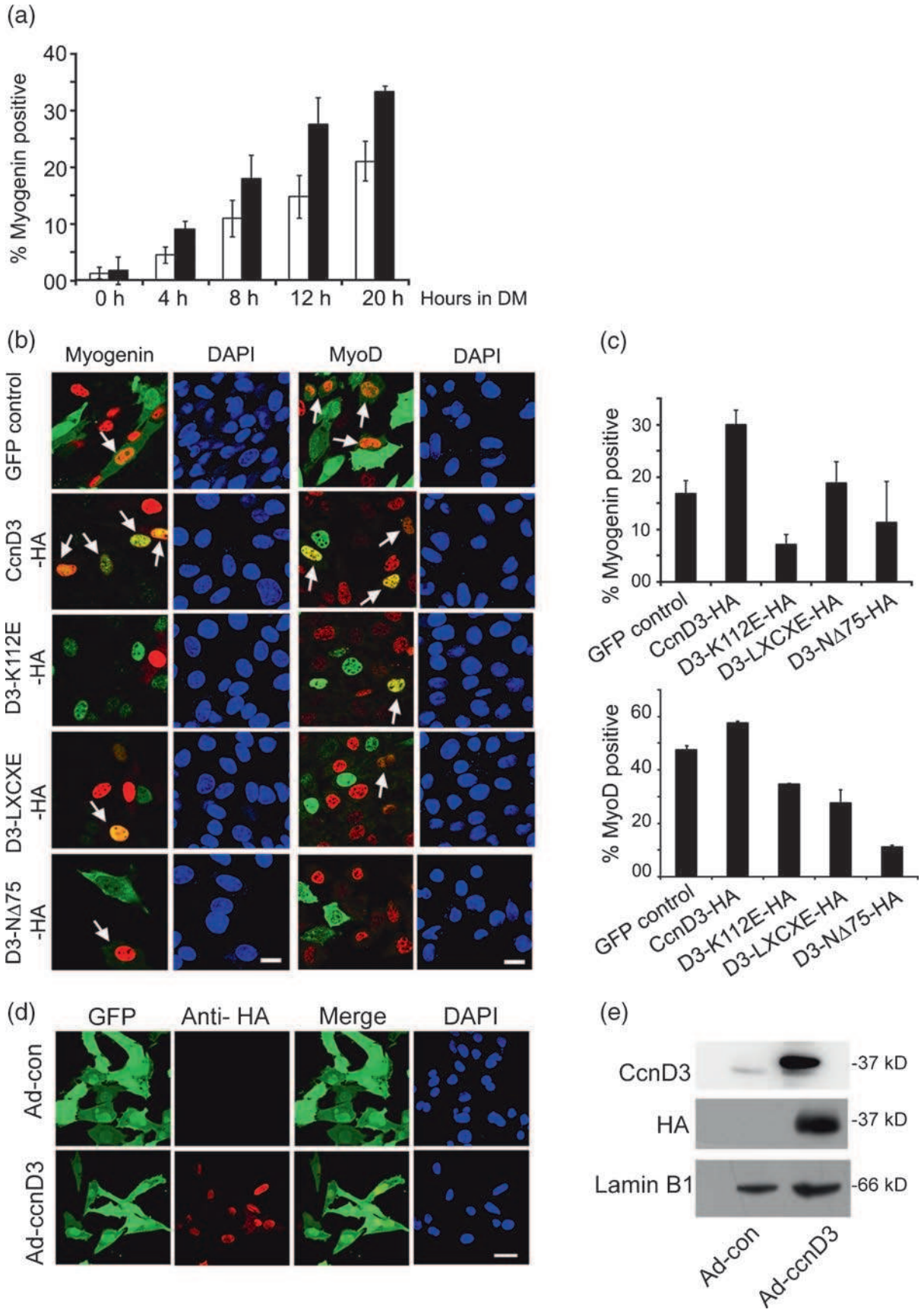
Figure 1. Ectopic expression of cyclin D3 and its mutants. (**a**) C2C12 myoblasts were transfected with cyclin D3-HA (black bars) or GFP vector (white bars), and the number of cells expressing myogenin was studied over a differentiation time course. (**b**) Mutants of cyclin D3 along with wild-type and GFP control were transfected in myoblasts, induced to differentiate, and expression of myogenin and MyoD (red) was analysed in transfected cells by co-immunostaining with antibody to HA (green). Arrows indicate cells positive for both HA and myogenin or MyoD. Bar, 10 μm. (**c**) Quantification of immunostaining of myogenin (12 h in DM) and MyoD (8 h in DM). (**d**) Adenoviral-mediated expression of cyclin D3 in C2C12 myoblasts. C2C12 myoblasts infected with control (Ad-con) and cyclin D3-HA (Ad-ccnD3) adenoviruses were analysed 24 h post infection, and immunostained with anti-HA antibody to reveal HA positive cells in myoblasts expressing cyclin D3-HA; GFP was expressed in both control and cyclin D3-HA myoblasts. Bar, 20 μm. (**e**) Western blotting of whole cell lysates of Ad-con and Ad-ccnD3 myoblasts was performed with anti-HA and anti-cyclin D3 antibodies. Lamin B1 was the loading control. ▶

kb) all three histone marks tested showed higher enrichment over their respective IgG controls in both control and cyclin D3-HA myoblasts. A two-fold increase in the level of H3K4me3 was observed near the TSS (−0.3 kb) in cyclin D3-HA myoblasts when compared to control myoblasts (figure 2a). Levels of H3K27me3 at the proximal promoter were reduced in cyclin D3-HA cells but the reduction was not found to be statistically significant. The DRR of MyoD has serum response factor (SRF) and MEF2 binding sites and is known to regulate expression of MyoD during post-natal myogenesis and muscle regeneration in response to injury in adults (L'Honore *et al.* 2003). Only two-fold enrichment for H3K4me3 over IgG was obtained in both control and cyclin D3-HA myoblasts at the DRR, suggesting the absence of H3K4me3 mark at this locus, an observation typical of enhancer regions. On the other hand higher levels of H3K9ac were observed in cyclin D3-HA myoblasts when compared to control myoblasts at the DRR of MyoD. Levels of H3K27me3 did not vary at this region in both samples (figure 2b). Overall, the MyoD promoter showed a transcriptionally active chromatin structure with increased transcriptional activation marks at its proximal promoter and increased histone acetylation at its enhancer region in cyclin D3-HA myoblasts.

Pax7 promoter: Pax7 is indispensable for satellite cell survival, maintenance and self renewal, and is down-regulated when satellite cells commit to differentiation (Sambasivan *et al.* 2011). In activated muscle satellite cells the Pax7 promoter is reported to be bivalently marked and the bivalency is resolved in response to signalling cues like TNFα (Palacios *et al.* 2010). Cyclin $D3^{-/-}$ mice show significant reduction in $Pax7^{+}$ satellite cells in adult myofibres (De Luca *et al.* 2013). An up-regulation of Pax7 transcript levels in undifferentiated cyclin D3 overexpressing cells was observed in our previous study (Gurung and Parnaik 2011). We analysed a documented regulatory region in the proximal promoter at −0.8 kb (Palacios *et al.* 2010). Consistent with the bivalency of Pax7 promoter, enrichments in specific antibody over IgG were obtained for both active and repressive histone marks at the proximal promoter region in both control and cyclin D3-HA myoblasts. A significant increase in the levels of H3K4me3 and H3K9ac was obtained in cyclin D3-HA myoblasts when compared to control. Levels of H3K27me3 at this region did not vary between control and cyclin D3-HA myoblasts (figure 2c). Thus, the Pax7 promoter showed a transcriptionally permissive chromatin structure with increased active histone marks at its proximal promoter region in cyclin D3-HA myoblasts.

Myogenin promoter: Expression of myogenin signifies commitment to differentiation (Andres and Walsh 1996). Epigenetic regulation of myogenin promoter regions −0.196 kb and −1 kb has been extensively reported (Palacios and Puri 2006; Perdiguero *et al.* 2009). Cyclin-D3-overexpressing cells showed an early activation of myogenin expression on induction of differentiation (Gurung and Parnaik 2011). Two regions upstream of myogenin TSS were analysed (−0.196 kb and −1 kb). These regions were previously shown to undergo dynamic epigenetic changes in response to induction of differentiation (Seenundun *et al.* 2010). At the TSS (−0.196 kb) levels of active histone marks were similar in cyclin D3-HA and control myoblasts. Level of H3K27me3 mark at this region was higher in cyclin D3-HA myoblasts when compared to control myoblasts (figure 3a). Upstream of TSS, at region −1 kb, levels of H3K4me3 did not vary but a significant decrease in levels of both H3K27me3 and H3K9ac mark was obtained in cyclin D3-HA myoblasts when compared to control myoblasts (figure 3b). The reduction in levels of H3K27me3 and H3K9ac mark was specific to this region and was not observed at a region tested downstream of TSS (+1 kb) in cyclin D3-HA myoblasts (data not shown). The reduction in H3K27me3 levels may contribute to early activation of myogenin gene expression in cyclin D3-HA myoblasts

Muscle creatine kinase (MCK) promoter: Numerous histone modification marks at the MCK enhancer region (−4.1 kb) have been reported and dynamic changes in these marks has been shown to be important for MCK gene regulation. In proliferating myoblasts MCK enhancer is marked by repressive histone marks which are replaced by active histone marks on induction of differentiation (Caretti *et al.* 2004; Seenundun *et al.* 2010). The enhancer region of MCK was


(a)
% Myogenin positive
40
30
20
10
00
0 h
4 h
8 h
12 h
20 h
Hours in DM
(b)
Myogenin
DAPI
MyoD
DAPI
GFP control
CcnD3 -HA
D3-K112E -HA
D3-LXCXE -HA
D3-NΔ75 -HA
(c)
% Myogenin positive
30
20
10
00
GFP control
CcnD3-HA
D3-K112E-HA
D3-LXCXE-HA
D3-NΔ75-HA
% MyoD positive
60
40
20
00
GFP control
CcnD3-HA
D3-K112E-HA
D3-LXCXE-HA
D3-NΔ75-HA
(d)
GFP
Anti- HA
Merge
DAPI
Ad-con
Ad-ccnD3
(e)
CcnD3
-37 kD
HA
-37 kD
Lamin B1
-66 kD
Ad-con
Ad-ccnD3

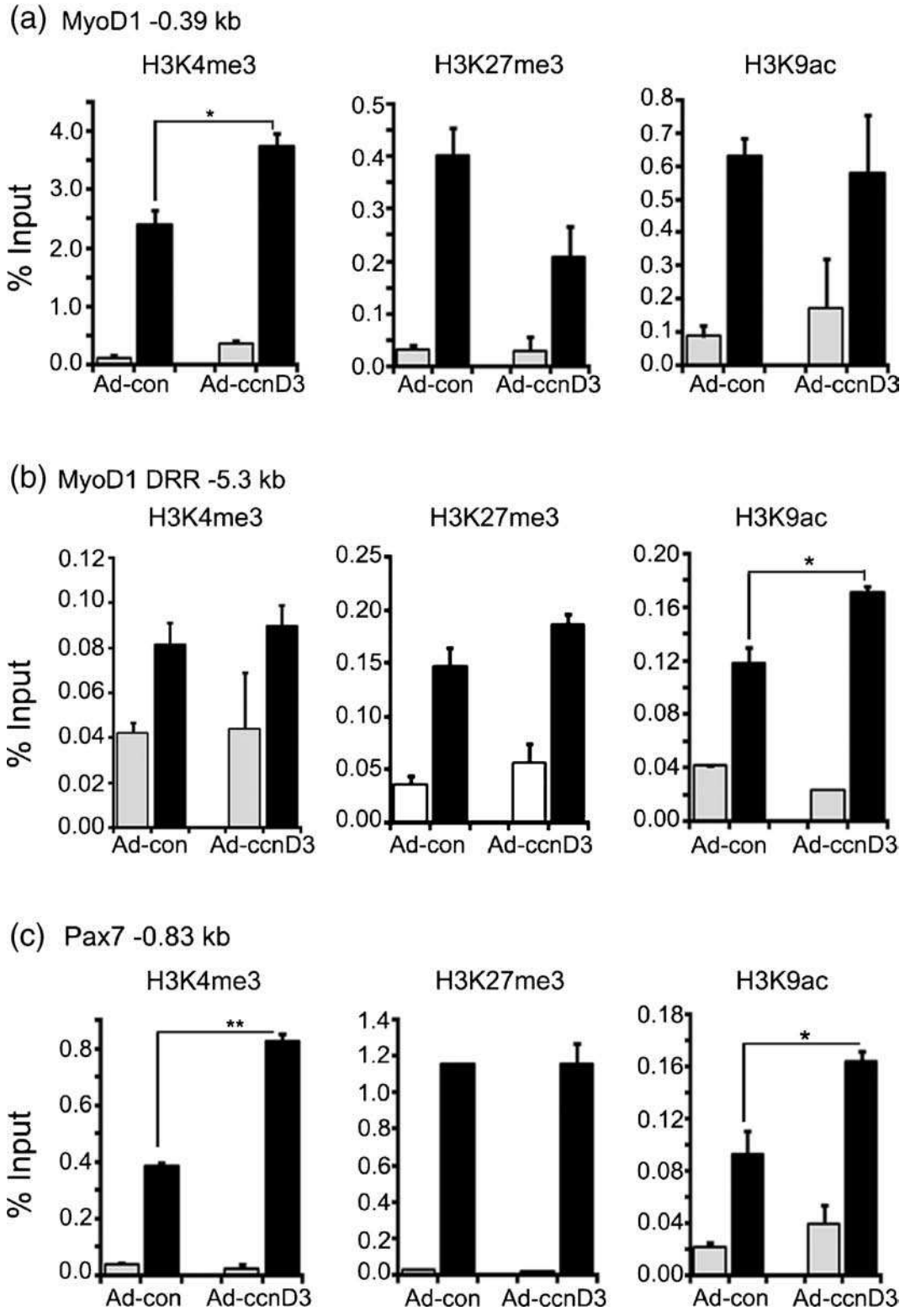


Figure 2. ChIP-qPCR analysis of MyoD and Pax7 promoters. Enrichments at MyoD promoter regions (**a**) −0.39 kb and (**b**) DRR, and (**c**) Pax7 promoter, −0.83 kb, are shown for both control and cyclin D3-HA myoblasts. ChIP-qPCR with indicated histone modification mark antibodies and IgG was performed with chromatin from adeno-control (Ad-con) and adeno-cyclin D3-HA (Ad-ccnD3) myoblasts. Mean of percent inputs ±S.E. (from three independent biological replicates) for IgG (grey bars) and specific antibody (black bars) are represented (*p<0.05, **p<0.01).

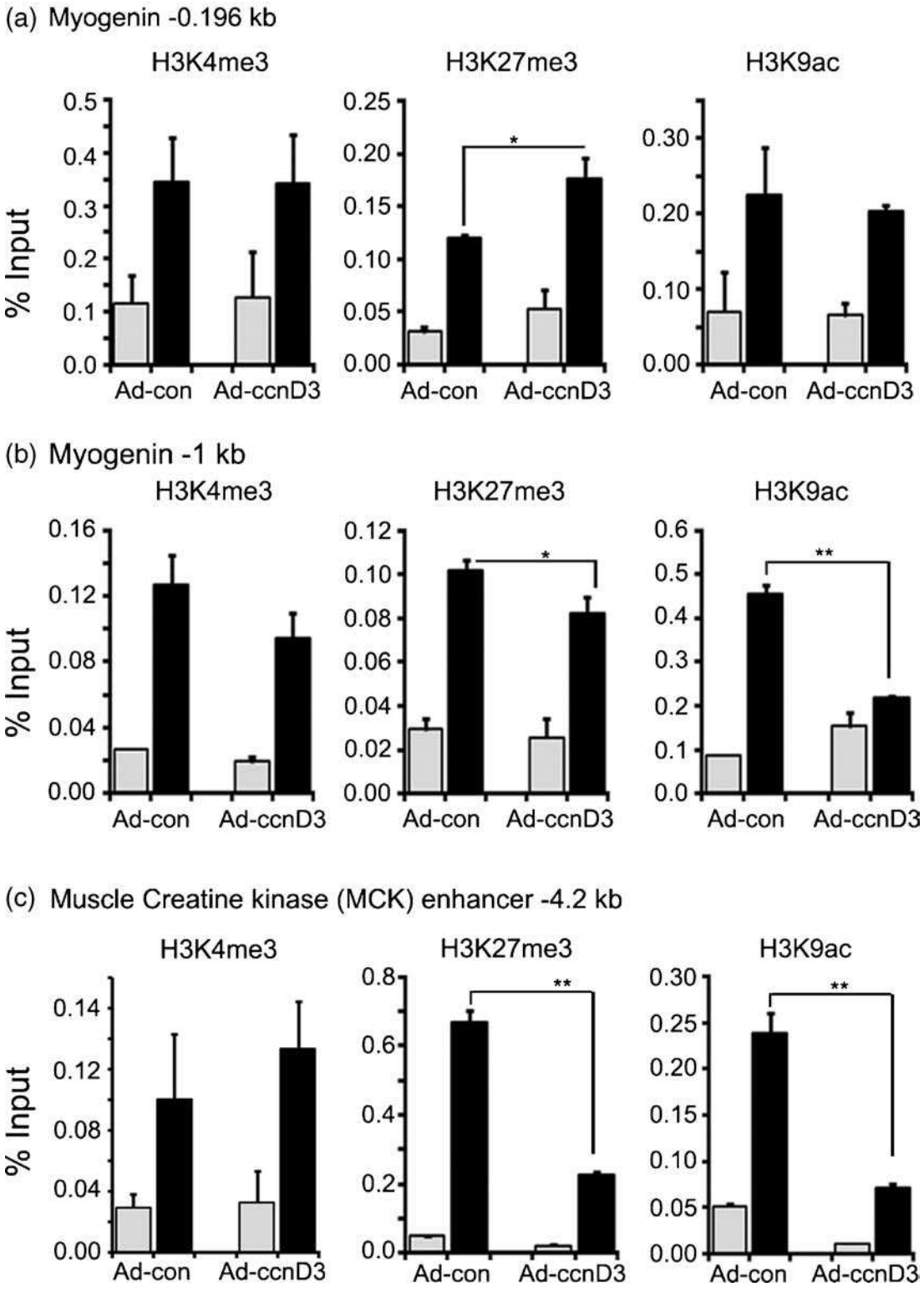


Figure 3. ChIP-qPCR analysis of myogenin promoter and MCK enhancer. Enrichments at myogenin promoter regions (**a**) −0.196 kb and (**b**) −1 kb, and (**c**) MCK enhancer are shown. ChIP-qPCR with indicated histone modification mark antibodies and IgG was performed with chromatin from adeno-control (Ad-con) and adeno-cyclin D3-HA (Ad-ccnD3) myoblasts. Mean of percent inputs ±S.E. (from three independent biological replicates) for IgG (grey bars) and specific antibody (black bars) are represented. (*p<0.05, **p<0.01).

marked with both H3K27me3 and H3K9ac as evidenced by their higher enrichment levels over IgG in both control and cyclin D3-HA myoblasts (figure 3c). On the other hand, levels of H3K4me3 at this region were negligible in both control and cyclin D3-HA myoblasts suggesting that the enhancer region was not marked by H3K4me3. Similar to the finding at upstream region of myogenin promoter, significantly lower levels of both H3K27me3 and H3K9ac were observed at the MCK enhancer region in cyclin D3-HA myoblasts.

3.3 *Epigenetic effects of cyclin D3 expression on promoters of non-muscle genes*

Promoters of cell cycle genes: Cyclin D1 and cyclin E1 are expressed in proliferating cells and are involved in phosphorylation and inactivation of pRb, leading to progression of G1-S phase of the cell cycle. Expression of both these genes is down-regulated on induction of differentiation. Overexpression of cyclin D3-HA in myoblasts does not significantly affect cell proliferation or expression levels of cyclin D1 and cyclin E1 (Gurung and Parnaik 2011). In accordance with this observation, robust enrichment of transcriptional activation marks, both H3K4me3 and H3K9ac over IgG was obtained at promoters of cyclin D1 and cyclin E1 in both control and cyclin D3-HA myoblasts (figure 4a, b). Differences in levels of the active histone marks on these promoters were not observed between control and cyclin D3-HA myoblasts. Enrichment for repressive mark, H3K27me3 over IgG was not obtained in both cyclin D3-HA and control myoblasts at these promoters consistent with earlier reports (Blais *et al.* 2007; Mal 2006).

Promoters of GAPDH, HoxB8, Oct3/4 and Sox2: In accordance with its role as a housekeeping gene, robust enrichment for active histone marks, H3K4me3 and H3K9ac over IgG, was obtained at GAPDH promoter in both cyclin D3-HA and control myoblasts (figure 5a). The H3K27me3 mark was not associated with the GAPDH promoter in both these samples. HoxB8 of the HoxB gene cluster is constitutively repressed in C2C12 myoblasts by deposition of H3K27me3 by the polycomb complexes (Cao and Zhang 2004; Asp *et al.* 2011). Enrichment of H3K27me3 over IgG was observed at the HoxB8 promoter while no enrichments of active marks over IgG were detected at this locus in both cyclin D3-HA and control myoblasts (figure 5b). The levels of H3K27me3 at the HoxB8 and H3K4me3 at the GAPDH promoters did not vary between control and cyclin D3-HA myoblasts.

We also determined H3K27me3 enrichments at promoters of pluripotency genes known to be silenced by deposition of H3K27me3 in C2C12 myoblasts. Pluripotency genes in general are highly expressed in embryonic stem cells but are down-regulated in lineage committed and differentiated cells (Barrand and Collas 2009). Significant enrichments for H3K27me3 over IgG at promoters (−1 kb) of Oct4 and Sox2 were obtained but levels of H3K27me3 did not vary between cyclin D3-HA and control myoblasts (figure 5c). This experiment further supports the specificity of the altered levels of H3K27me3 obtained in cyclin D3 myoblasts for myogenin and MCK promoters.

3.4 *Transcriptional changes associated with epigenetic modifier genes in cyclin-D3-expressing cells*

In order to investigate whether the alterations in histone marks at myogenic genes observed in cyclin D3-HA myoblasts were accompanied by changes in expression of histone modifier genes, transcript levels of histone modifier genes along with few positive and negative regulators of muscle differentiation were analysed. The results are summarized in table 3 and significant changes are discussed below. Transcript levels of components of polycomb repressor complexes PRC1 and PRC2 (H3K27me3-catalysing) in cyclin D3-HA myoblasts were found to be same as that in control myoblasts except for enhancer of zeste homolog 1 (Ezh1) (Margueron *et al.* 2008; Juan *et al.* 2011; Stojic *et al.* 2011). More than two-fold reduction in the transcript levels of Ezh1 was observed in cyclin D3-HA myoblasts when compared to control myoblasts. A two-fold reduction in two H3K9me3 demethylases, Jmjd2B/KDM4B and Jmjd2C/KDM4C was observed in cyclin D3-HA myoblasts when compared to control myoblasts (Loh *et al.* 2007; Das *et al.* 2013). Changes in transcript levels of H3K27me3 demethylases, UTX and JMJD3 or deacetylase, HDAC1 or acetylases, PCAF and GCN5 were not observed in cyclin D3-HA myoblasts.

4. Discussion

We have previously shown that overexpression of cyclin D3 causes early activation of muscle-specific genes, which in turn enhances the muscle differentiation programme (Gurung and Parnaik 2011). Our present study suggests that a stable and functional cyclin D3 is required for this process as mutants of cyclin D3 were found to be deficient for myogenin expression when compared to wild-type cyclin D3. Importantly cyclin D3 overexpression caused alterations in histone modifications marks on regulatory regions of muscle-specific genes MyoD, Pax7, myogenin, and MCK in undifferentiated myoblasts. Significant changes in transcript levels of certain histone modifier genes in these cells

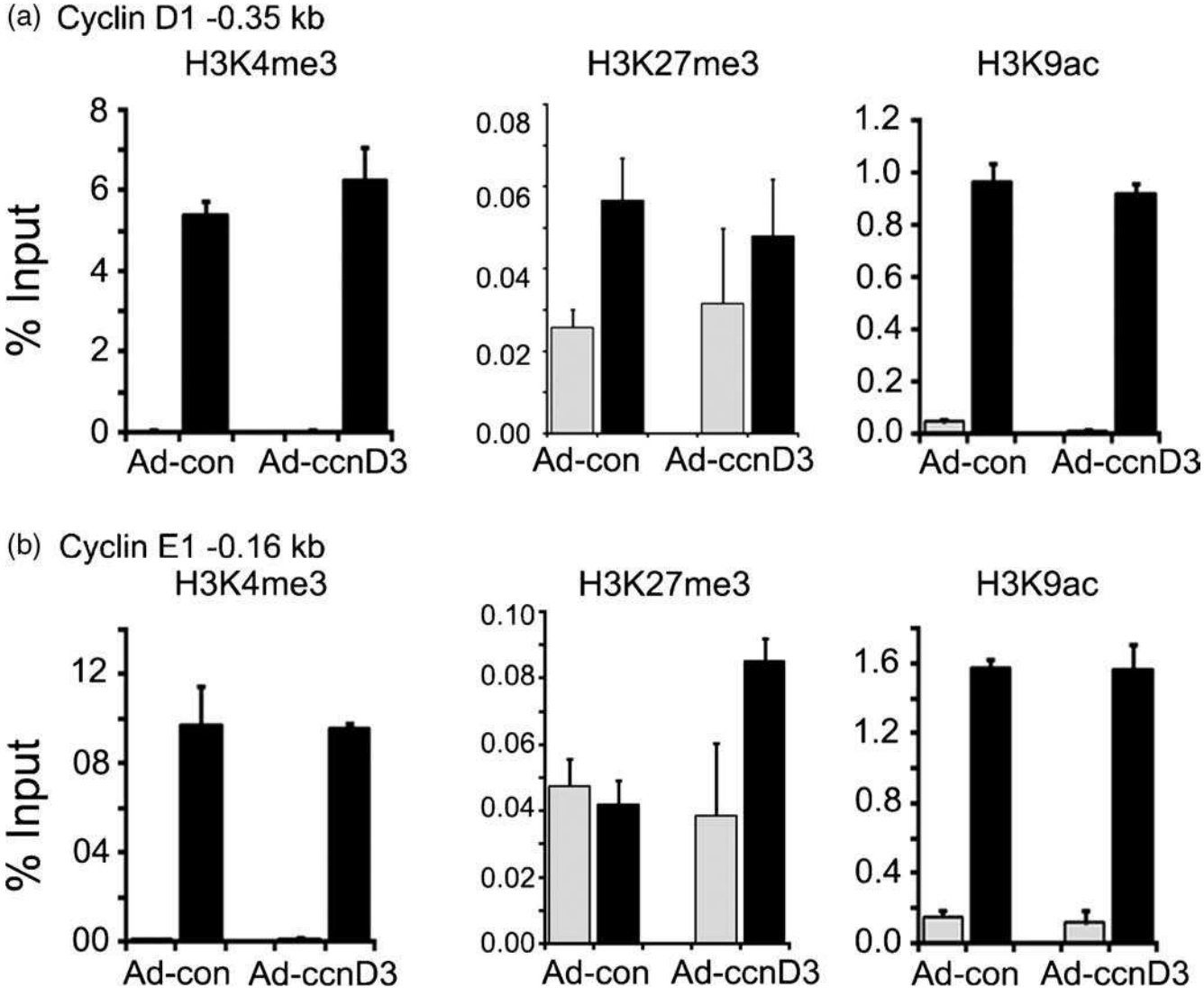


Figure 4. ChIP-qPCR analysis of cell cycle gene promoters. Enrichments at proximal promoter regions of (**a**) cyclin D1 and (**b**) cyclin E1 are shown. ChIP-qPCR with indicated histone modification mark antibodies and IgG was performed with chromatin from adeno-control (Ad-con) and adeno-cyclin D3-HA (Ad-ccnD3) myoblasts. Mean of percent input ±S.E. (from three independent biological replicates) for IgG (grey bars) and specific antibody (black bars) are represented.

were also observed. The alterations in the histone marks were specific to the regulatory regions of muscle genes and not the non-muscle genes tested and may be responsible for the accelerated kinetics of differentiation observed in cyclin-D3-expressing myoblasts.

A transcriptional regulatory role for cyclin D3 has been reported in several tissues. Cyclin D3 is shown to associate with and function as a co-regulator for steroid hormone receptors like retinoic acid receptor (Despouy *et al.* 2003), androgen receptor (Knudsen *et al.* 1999), estrogen receptor-PCAF complex (McMahon *et al.* 1999), vitamin D receptor (Jian *et al.* 2005), and transcription factors like hATF5 (human activating transcription factor 5) and DMP1 (cyclin-D-binding myb-like protein 1) (Inoue and Sherr 1998; Liu *et al.* 2004). In differentiated adipose tissue, it has been shown that along with its cdk partner, cyclin D3 phosphorylates PPAR γ and also associates with it at its target promoters. In B-cells, a subnuclear fraction of cyclin D3 has been shown to regulate transcription of more than 200 genes (Sarruf *et al.* 2005; Powers *et al.* 2012). We have previously reported an up-regulation of myogenic genes upon serum depletion in cyclin-D3-overexpressing myoblasts (Gurung and Parnaik 2011). Our present findings further support a transcriptional regulatory role for cyclin D3 in muscle cells

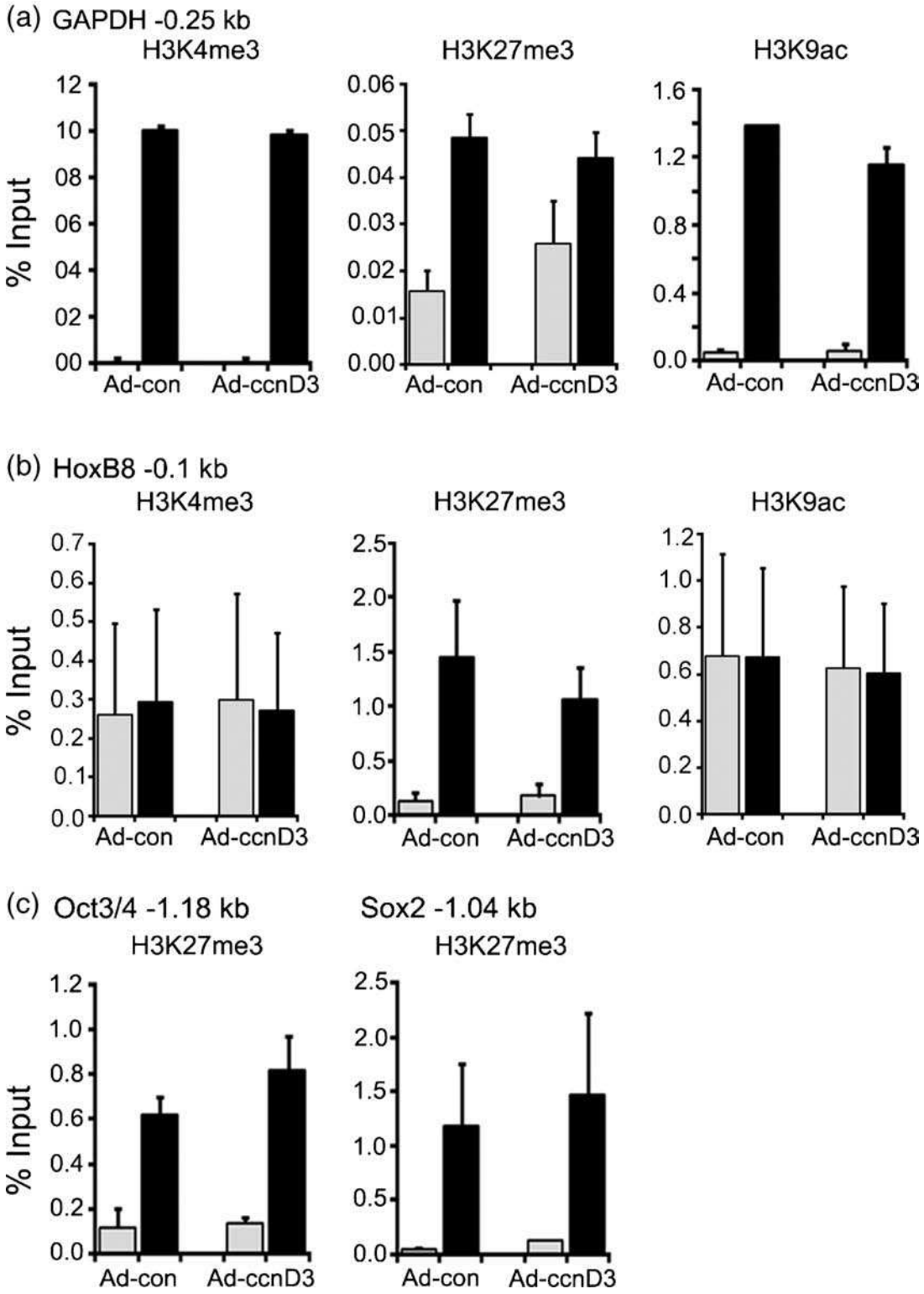


Figure 5. ChIP-qPCR analysis of non-myogenic gene promoters. Enrichments at promoter regions of (**a**) GAPDH, (**b**) HoxB8 and (**c**) H3K27me3 levels at the promoters of pluripotency genes are shown. ChIP-qPCR with indicated histone modification mark antibodies was performed with chromatin from adeno-control (Ad-con) and adeno-cyclin D3-HA (Ad-ccnD3) myoblasts. Mean of percent inputs ± S.E. (from three independent biological replicates) for IgG (grey bars) and specific antibody (black bars) are represented.

Table 3. Real time analysis of epigenetic modifier genes in cyclin-D3-expressing cells

	Fold change ± S.E.
Gene	Ad-ccnD3 Myoblasts
Histone methyl transferases (HMT) and components of HMT complexes	
Ezh2	−1.31±0.09
Suz12	1.51±0.12
YY1	1.51±0.12
Jarid2	−1.48±0.10
Ezh1	−2.41±0.13**
Carm1	−1.10±0.06
Bmi	−1.10±0.06
Ring1	−1.64±0.12
G9a	−1.79±0.10
GLP	−1.30±0.23
Suv39H1	−1.85±0.14*
Set7	−1.20±0.05
Histone acetyl transferases and deacetylases	
HDAC1	−1.27±0.11
GCN5	−1.22±0.06
PCAF	−1.26±0.04
Histone demethylases	
UTX	−1.40±0.16
Jmjd3	1.03±0.13
Jmjd2a	−1.11±0.05
Jmjd2b	−2.23±0.18**
Jmjd2c	−2.20±0.12**
Jhdm1a	1.14±0.07
Jhdm1d	1.09±0.11

Transcript levels of epigenetic modifier genes were determined in undifferentiated cyclin D3-HA expressing cells by qRT-PCR. Fold changes ± S.E. normalized to their respective controls are expressed (*p<0.05, **p<0.01).

and this may be important in the context of its implication in the pathology of myotonic dystrophy and laminopathies (Favreau *et al.* 2008; Salisbury *et al.* 2008).

Our ChIP-qPCR analysis showed that the promoters of myogenic lineage determinant genes like Pax7 and MyoD showed increased levels of active histone marks, H3K4me3 and H3K9ac, at their proximal promoter regions in cyclin D3-HA myoblasts. Hyperacetylation of H3K9 at DRR of the MyoD promoter was also observed, which may explain the enhanced activation of MyoD transcription on induction of differentiation in cyclin-D3-expressing myoblasts. Regulatory regions of myogenin promoter and MCK enhancer showed significant decrease in levels of H3K27me3 repressive mark in cyclin-D3-expressing myoblasts. Reduction in levels of H3K27me3 repressive mark may be instrumental in priming of these myogenic loci for enhanced activation on differentiation. A similar reduction of H3K27me3 levels was not found at the upstream regions of promoters of pluripotency genes (constitutively repressed in C2C12), suggesting that this effect was specific to myogenin and MCK promoters. The reduced levels of H3K27me3 may be attributed to impaired/decreased methylation or increased demethylation of these genomic loci. A down-regulation in the transcript levels of Ezh1, the catalytic component of the PRC1/2 histone methylating complex, was observed in cyclin-D3-expressing myoblasts when compared to control myoblasts. This down-regulation of Ezh1 transcripts may be related to the reduction in H3K27me3 levels at the regulatory regions of myogenin and MCK genes. However, we cannot rule out the possibility that the observed reduction of expression of these histone methylases or demethlases is an unrelated consequence of cyclin D3 overexpression. It would be interesting to determine whether cyclin D3 plays a direct or indirect role in the recruitment of specific histone modifiers to muscle genes.

We also observed reduction in the levels of the active mark H3K9ac at the upstream region of myogenin promoter and at the enhancer region of MCK gene in cyclin-D3-expressing myoblasts compared to control myoblasts. Myogenin and MCK transcripts are normally not expressed in proliferating myoblasts, but only in differentiating cells that have undergone cell cycle arrest; hence, their possible untimely expression needs to be blocked. Moreover, it has been reported earlier that up-regulation of cyclin D3 does not induce expression of differentiation markers like myogenin in myoblasts due to sustained phosphorylation of pRb and expression of cyclin D1 in these myoblasts, which continue to proliferate (De Santa *et al.* 2007; Gurung and Parnaik 2011). Hence, it is likely that proliferative signals induce the hypoacetylation of H3K9 in the myogenin and MCK genes to prevent their expression in myoblasts to counter the down-regulation of repressive histone marks by cyclin D3. This was not observed in the case of MyoD and Pax7, which are already expressed in proliferating myoblasts. The H3K9 hypoacetylated regions were not methylated as significant enrichment for H3K9me3 mark was not detected on these or most other loci that were tested (data not shown). Furthermore, ectopic cyclin D3 did not influence enrichment levels of histone modification marks on cell cycle gene promoters. This is consistent with earlier reports that cyclin D3 does not alter cell proliferation (Gurung and Parnaik 2011). Our findings suggest that cyclin D3 can mediate alteration of chromatin marks on differentiation-specific genes to prime them for enhanced transcriptional activation even in the absence of differentiation cues. Although we could not detect an association of cyclin D3 itself with the chromatin of muscle genes in our ChIP experiments (data not shown), our mutant analysis suggests that the stability, kinase activating function and

interaction with pRb may all be important for the enhanced differentiation capacity of cyclin-D3-expressing cells.

Reduced number and size of adult myofibres, impaired proliferation and precocious differentiation of satellite cells in cyclin D3$^{-/-}$ mice argue for a role of cyclin D3 in post-natal myogenesis and adult muscle regeneration (De Luca *et al.* 2013). Indeed, physiologically, cyclin D3 is maximally up-regulated during the post-natal period when myogenesis is at its peak levels and also during muscle repair and regeneration subsequent to injury (Bartkova *et al.* 1998; De Luca *et al.* 2013). Cyclin D3 has also been implicated in the pathology of myotonic dystrophy 1 (DM1). Analysis of DM1 patient cells showed that on initiation of differentiation, these cells fail to up-regulate cyclin D3 levels, resulting in impaired myoblast fusion and a differentiation defect, which could be alleviated by expression of cyclin D3 in cells or restoration of cyclin D3 levels in dystrophic mice (Timchenko *et al.* 2001; Salisbury *et al.* 2008; Jones *et al.* 2012).

In conclusion, we propose a novel transcriptional regulatory function for cyclin D3 in muscle differentiation mediated by the alteration of the histone modification marks on myogenic genes. This transcriptional regulatory function of cyclin D3 may have implications in the process of muscle regeneration and in the pathology of muscular dystrophies.

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