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p38 MAPK signaling regulates recruitment of Ash2L-containing methyltransferase complexes to specific genes during differentiation

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Cell-specific patterns of gene expression are established through the antagonistic functions of trithorax (TrxG) and polycomb (PcG) group proteins. Several muscle-specific genes have previously been shown to be epigenetically marked for repression by PcG proteins in muscle progenitor cells. Here we demonstrate that these developmentally regulated genes become epigenetically marked for gene expression (H3K4me3) during muscle differentiation through a specific recruitment of Ash2L containing methyltransferase complexes. Targeting of Ash2L to specific genes is mediated by the transcriptional regulator Mef2d. Furthermore, this interaction is modulated during differentiation through activation of the p38 MAPK signaling pathway via phosphorylation of Mef2d. Thus, we provide the first evidence that signaling pathways regulate the targeting of TrxG mediated epigenetic modifications at specific promoters during cellular differentiation.

Cell specific gene expression programs are established during embryogenesis through transient environmental signals. These programs are then stably maintained and passed to daughter cells through a process of cellular memory. Experiments in *Drosophila* have identified Polycomb (PcG) and Trithorax (TrxG) group proteins as the mediators of this memory[1]. The list of genes that fall into PcG and TrxG protein groups is extensive[1,2], but those of known function are modifiers of chromatin structure: histone methyltransferases, ubiquitin E3 ligases, and ATP-dependent chromatin remodeling factors. This suggests that epigenetic modification of chromatin plays an important role in establishing cellular memory.

PcG and TrxG proteins act antagonistically to establish and maintain tissue specific patterns of gene expression, the former marking genes for repression while the later marks genes for expression. This is accomplished, in part through trimethylation of histone H3 at lysine 9 (H3K9me3) and/or lysine 27 (H3K27me3) for repressed genes, or histone H3 at lysine 4 (H3K4me3) for active genes[1,3]. Indeed, ChIP-Chip studies have suggested that developmentally regulated genes which are actively expressed are enriched for the H3K4me3 epigenetic mark in the 5'-end of their coding sequence[4]. The H3K27me3 and H3K9me3 modifications are enriched in facultative, and constitutive heterochromatin respectively[5]. The repressive transcriptional effects of PcG proteins is thought to be the default state since these proteins can be found at both active and inactive genes, while TrxG proteins appear to be specifically

targeted to active genes[6]. In agreement with this, loss of TrxG function prevents the expression of developmentally regulated genes[7]. In contrast, double mutations where both a PcG protein (Ez) and a TrxG (Ash1) protein were mutated resulted in ectopic expression of the developmentally regulated Hox genes[8]. This suggests that TrxG proteins act as anti-repressors in a tissue specific manner to ensure PcG proteins do not repress expression of developmentally important genes.

During myogenesis, the Mef2 family of MADS-box transcription factors and the muscle specific transactivator MyoD target specific promoters[9] to establish a precise gene expression program, ultimately resulting in the formation of multinucleated myotubes. This myogenic gene expression program is temporally ordered[10], and is proposed to be mediated through a feed-forward mechanism[11]. Among the cofactors “feeding” into the myogenic program is the p38 mitogen activated protein kinase (MAPK)[11] which plays a critical role in establishing the muscle specific gene expression program[10-13].

While several important factors responsible for establishing muscle specific gene expression have been identified, the mechanism by which transcription is activated at muscle specific loci had remained elusive. Some insight has come from studies which demonstrate that several muscle specific genes are epigenetically marked for repression (H3K27me3, or H3K9me3) in growing myoblasts[14,15]. In the case of H3K27me3 marked genes, the PcG protein Ezh2 was shown to be targeted to the promoter by the transcriptional regulator

YY1[14]. The fact that muscle specific genes are targeted for repression by PcG proteins suggests that activation of these genes should require the anti-repressive function of TrxG proteins. Here, we set out to determine whether muscle specific genes are epigenetically marked for gene expression in differentiating mouse C2C12 myoblasts, and if yes, how are TrxG proteins targeted to these promoters.

Results

Muscle-specific genes are marked for expression during myogenesis

Genes poised for transcription are proposed to be marked by H3K4me2, while those actively transcribing are marked by H3K4me3[4,16]. Here we set out to examine whether genes that are expressed during myogenesis may be marked by the epigenetic modification H3K4me3 during the transactivation process. The mouse C2C12 myoblast cell line was used as a model for these studies since it can be induced to undergo myogenesis under conditions of serum withdrawal[17]. To avoid potential epitope masking by proteins that bind modified histone tails, we used a native ChIP protocol to immunoprecipitate nucleosomes with an anti-H3K4me3 antibody. Using Taqman probes that recognized the 5' end of the genes, we found that both the Myog and Ckm genes became enriched in nucleosomes containing H3K4me3 during C2C12 cell differentiation (Figure 1A). This enrichment coincides temporally with the increased expression of these two genes during myogenesis (Figure 1B,C). Consistent with previous results

suggesting that not all active genes are enriched for the H3K4me3 mark[18], we did not observe extensive enrichment of this mark within the transcribed Acta1 gene (Figure 1A,B). Similarly, no enrichment of H3K4me3 was observed at the Cdkn1a gene that is transcribed before differentiation, and whose expression is further upregulated during myogenesis; or at the transcriptionally silent Igh locus (Figure 1A,B). Expanding our analysis to examine the distribution of H3K4me2 and H3K4me3 epigenetic marks across the Myog gene during C2C12 differentiation, we observed that the promoter region is enriched for the H3K4me2 while the H3K4me3 mark peaks within the transcribed region of the gene (Supplementary Figure 1).

TrxG protein Ash2L associates with the transcriptional activator Mef2d

Recent studies have suggested that the Ash2L protein is required for H3K4me3 to occur at specific loci[19,20]. As Ash2L is expressed in both skeletal muscle[21], and C2C12 cells (Supplementary Figure 2A), we examined whether Ash2L-containing complexes may be recruited to muscle-specific genes by developmentally regulated transcriptional activators. To identify candidate transcriptional activators that mediate recruitment of TrxG proteins, we turned to the Myog promoter. In this case, DNA binding elements responsible for establishing cellular memory should lie in a fragment of DNA containing the region between -184 and +18 relative to Myog transcription start site since this region is sufficient to ensure muscle specific expression of the gene[22,23]. As Myog is a well characterized promoter[22-26], we looked for interactions between Ash2L

and transcriptional activators known to bind this region. Western blot analysis of proteins co-immunoprecipitating with Ash2L indicated the presence of Mef2, but not the muscle-specific transcriptional activators MyoD, or Myogenin (Figure 2A). In a reciprocal experiment, immunoprecipitation of Mef2 using a pan-Mef2 antibody confirms the interaction between the transcriptional activator and the Ash2L complex subunits Ash2L, RbBP5, Menin, along with the methyltransferase MLL2 (Figure 2B). Furthermore, methyltransferases assays performed with Mef2 immunoprecipitates confirmed that the transcriptional activator associates with an H3K4 methyltransferase activity in differentiating C2C12 cell extracts (Supplementary Figure 2C,D). Interestingly, the association between Ash2L and Mef2 was not observed in undifferentiated C2C12 cells, or at the early stages of differentiation (data not shown), suggesting that the interaction between these proteins may be developmentally regulated. As Ash2L is present in at least 5 known methyltransferase complexes[27], we examined whether the interaction between Ash2L and Mef2 was limited to MLL2 containing complexes. Using antibodies directed at different H3K4 methyltransferases, we were able to immunoprecipitate Mef2 with antibodies directed at MLL2, Set1, and MLL3, but not MLL or MLL4 (Supplementary Figure 2B). Thus, Mef2 can interact with multiple Ash2L complexes, but preferentially interacts with MLL2 containing complexes.

To determine whether Mef2 may be recruiting the Ash2L complex to mediate H3K4 trimethylation on muscle specific genes, we used ChIP analysis to

examine whether these two factors bind to the Myog and Ckm promoters during C2C12 differentiation. Consistent with an association between Mef2 and the Ash2L complex, we observed a good correlation between their recruitment to the Ckm promoter (Figure 2C). In contrast, recruitment of Ash2L did not coincide temporally with the recruitment of Mef2 at the Myog gene. Instead, Mef2 was recruited to the Myog promoter within 6 h after serum withdrawal while recruitment of Ash2L, marking of nucleosomes by H3K4me3, and gene activation are maximal at later stages (48 h) in the differentiation process (see Figure 2C, 1A, 1B, and 1C). Thus, recruitment of Ash2L is temporally distinct from that of Mef2 at the Myog promoter.

Phosphorylation of Mef2d by p38 permits its association with Ash2L

As our antibody recognizes all four Mef2 isotypes (Mef2a, Mef2b, Mef2c, and Mef2d), one possibility is that Ash2L interacts with a specific Mef2 isotype that is differentially regulated during differentiation. To test this possibility, we performed interaction studies between Ash2L and the three Mef2 isotypes that are known to be expressed in muscle. Baculovirus encoding C-terminal Flag-tagged Mef2a (FL-Mef2a), Mef2c (FL-Mef2c), and Mef2d (FL-Mef2d) were engineered. Recombinant Mef2 proteins were then purified from baculovirus infected Sf9 cell extracts using M2-Flag agarose beads. As phosphorylation has been proposed to activate Mef2 gene products[28], the different purified Mef2 proteins were incubated in the presence (or absence) of active p38α MAPK[29] prior to incubation with an extract prepared from differentiating (48 h) C2C12

cells. While Ash2L does not bind to FL-Mef2a, we consistently observed an interaction with FL-Mef2d (Figure 3A). Interestingly, this interaction is enhanced when FL-Mef2d is phosphorylated by p38α. A weaker interaction between p38α-treated FL-Mef2c and Ash2L could also be observed. Thus, Ash2L appears to selectively associate with the phosphorylated Mef2d (and to a lesser extent Mef2c) isotype. It is not clear why Mef2a does not also interact with Ash2L. However, it is not a case of differential β-exon usage since all three constructs contained the previously described isoform-specific acidic transactivation domain[30]. We did observe some interaction between Ash2L and Mef2d in the absence of exogenous p38α. This is likely due to the presence of endogenous p38 activity present in the C2C12 cell extracts. To examine the importance of Mef2d phosphorylation by p38α in mediating its interaction with Ash2L, we generated a Mef2d construct with mutated consensus p38 phosphorylation sites (T308A/T315A). These mutations greatly reduced *in vitro* phosphorylation of the Mef2d protein by p38α (Figure 3B), while completely blocking its interaction with Ash2L (Figure 3C). Furthermore, this protein behaved as a dominant negative mutant leading to decreased expression of the endogenous Myog gene in transfected C2C12 cells (Supplementary Figure 3). To further confirm that p38 dependent phosphorylation of Mef2d regulates its interaction with Ash2L, we performed Mef2 IPs using extracts prepared from C2C12 cells that had been differentiated in the presence or absence of the p38 MAPK inhibitor SB203580. Use of this p38α/β specific kinase inhibitor has previously been demonstrated to

block phosphorylation of Mef2 proteins in differentiating L8 myoblasts[13]. As expected, treatment of C2C12 cells with SB203580 markedly reduced the amount of Ash2L that coimmunoprecipitated with Mef2 (Figure 3D). These results strongly suggest that Ash2L interacts with phosphorylated Mef2d.

As the interaction between Ash2L and Mef2d was not observed in growing myoblasts, we examined whether expression of the transcriptional activator might be upregulated during C2C12 cell differentiation. However, both semi-quantitative RT-PCR and western blot analysis demonstrated that Mef2d levels remain relatively constant throughout C2C12 differentiation (Supplementary Figure 4). Thus, we next examined whether p38 MAP kinase activity was modified during C2C12 differentiation. In accordance with previous reports[12], we found that total levels of p38 remained constant throughout differentiation. In contrast, active (phosphorylated) p38 is markedly increased during differentiation, attaining its maximal levels 48h after serum withdrawal (Supplementary Figure 4B). Thus, our results suggest that Mef2d recruits Ash2L to muscle specific promoters in a p38 dependent manner.

p38 regulates recruitment of Ash2L to muscle-specific promoters

To examine this possibility *in vivo*, we performed ChIP experiments in C2C12 cells that have been differentiated in the presence of SB203580. Treatment of C2C12 cells with SB203580 did not affect total p38 levels in the cell (Figure 4A). As previously observed[31], expression of both Ckm and Myog in differentiating C2C12 is inhibited by SB203580 treatment (Figure 4B). However,

this is not a global effect on transcription since expression of Acta1, Mef2c, and Mef2d is not affected. Furthermore, ChIP experiments demonstrated that the block in transcription at the Myog and Ckm genes was not due to reduced recruitment of MyoD, Mef2, p300, or Pol II to these loci (Figure 4C, and Supplementary Figure 5). Similarly, acetylation of histone H4 was not affected by SB203580 treatment suggesting that the promoters are poised for transcription. Treatment of differentiating C2C12 cells with SB203580 did however result in a substantial decrease in Ash2L recruitment, and H3K4me3 at both of these muscle specific promoters (Figure 4C and Supplementary Figure 5). This shows that p38 kinase activity is required *in vivo* for efficient recruitment of the Ash2L complex and H3K4 trimethylation at the Myog and Ckm promoters.

To confirm that Ash2L is recruited to the muscle specific promoters through a Mef2 dependent mechanism, we used siRNA mediated knock-down of Mef2d and Mef2c in C2C12 cells. While knock-downs did not appreciably effect Acta1 expression, both Myog and Ckm genes were markedly downregulated in response to Mef2d, or Mef2c knock-downs (Figure 5A and 5B). Since both Mef2c and Mef2d are required for maximal transactivation of the Ckm and Myog genes (Figure 5B), we decided to knock down both Mef2 isotypes in the same population of C2C12 cells for ChIP studies (Figure 5C,D). Analysis of promoter occupancy at both the Ckm and Myog genes demonstrated an 80% reduction in recruitment of Mef2 during differentiation after siRNA treatment, while MyoD levels remained constant (Figure 5D, and Supplementary Figure 6). Loss of Mef2

binding at these promoters resulted in decreased H3K4me3 within the gene, and a concomitant increase in H3K4 dimethylation within these regions (Figure 5C, Supplementary Figure 6). Similarly, knock-down of Mef2d resulted in a marked decrease in both Ash2L and MLL2 recruitment to the Myog and Ckm promoters (Figure 5D, Supplementary Figure 6). Our observation that Ash2L binding is only reduced by 50% when Mef2 binding is reduced by 80% is likely explained by the fact that the TrxG complex can interact with multiple elements within the promoter region, including CBP/p300[32], non-methylated CpG dinucleotides[33], and/or H3K4me2[34]). Thus, while phosphorylated Mef2d is likely required for initial recruitment of Ash2L, the increased affinity accorded by multiple interactions at the promoter might further stabilize the binding of the Ash2L complex.

Lastly, to confirm the importance of the Ash2L complex in regulating gene expression at muscle specific loci, we examined the effect of siRNA knock-down of Ash2L in C2C12 cells. Indeed, reducing levels of Ash2L in C2C12 cells (Figure 6A) lead to decreased expression of both the Myog and Ckm genes while having no effect on Acta1 RNA levels during muscle differentiation (Figure 6B). This decreased expression could be correlated to reduced recruitment of Ash2L and lower H3K4me3 at the Myog (Figure 6C), and Ckm genes (data not shown). Thus, our studies have revealed that establishment of the H3K4me3 mark at the Myog and Ckm promoters during muscle differentiation occurs via a series of ordered events involving Mef2d phosphorylation by p38 MAPK and subsequent recruitment of the Ash2L methyltransferase complex.

Discussion

In this study, we show that Ash2L is recruited to muscle specific promoters through its association with the transcriptional activator Mef2d. Furthermore, we demonstrate that Ash2L recruitment is regulated via phosphorylation of Mef2d by the p38 MAPK. Indeed, we observed Mef2 recruitment to the Myog promoter early in differentiation, whereas Ash2L was not recruited until p38 activity becomes upregulated during later stages of differentiation. Interestingly, Ash2L demonstrated a differential ability to associate with the various members of the Mef2 family of transcriptional activators. Combined with the fact that continued expression of Mef2a in Mef2d/Mef2c knock-down cells does not allow expression of the Myog and Ckm genes, our results demonstrate for the first time that the Mef2 isotypes are playing functionally different roles during myogenesis. In particular, we show that Mef2d integrates the p38 MAPK signaling pathway to establish the epigenetic H3K4me3 mark at muscle specific promoters.

The essential role for the p38 MAPK signaling pathway in myogenesis has been well documented[35]. Treatment of myoblasts with SB203580 inhibits the formation of myotubes, and blocks expression of a subset of muscle specific genes[10-13]. Many reports have demonstrated that factors involved in transcriptional regulation in muscle are targets for phosphorylation by p38, including Mef2a/Mef2c[28], and Mef2d[11]. Furthermore, phosphorylation of the Mef2 activation domains by p38 has been shown to markedly increase its ability to

activate transcription[28]. Finally, it has been demonstrated that precocious activation of p38 in the presence of Mef2d leads to altered kinetics of transcription during myogenesis where late expressing genes are expressed during the early stages of differentiation[11]. While these previous studies suggested that p38-dependent phosphorylation of Mef2 proteins could be involved in establishing muscle specific patterns of gene expression, the functional role for phosphorylation of the Mef2 activation domain had not previously been defined. Our study shows that the Ash2L methyltransferase complex is selectively recruited to muscle specific promoters through p38-dependent phosphorylation of Mef2d providing the first insight into how MAPK signaling acts to regulate muscle specific transcription. Based on the results obtained in this study, we propose a model for Mef2d-dependent activation of muscle specific genes during myogenesis (Figure 7). During differentiation, MyoD and Mef2 bind to muscle specific promoters leading to the recruitment of coactivators (including p300), and the basal transcriptional machinery to establish a transcriptionally poised promoter. Consistent with previous observations[31], we detected the acetyltransferase p300 and Pol II at the Myog and Ckm promoters in the absence of p38 activity. This results in a promoter which contains acetylated histone H4. Once poised for transcription, expression of muscle specific genes is then initiated through activation of the p38 MAPK pathway. Recent studies have suggested that prolonged activation of the p38 MAPK pathway could be mediated through activation of the membrane bound

receptor Cdo as a result of cell-cell contact[36]. Once activated, p38 can then phosporylate Mef2d thereby permitting the recruitment of the Ash2L complex to muscle specific promoters, ultimately leading to trimethylation of histone H3 at lysine 4. Combined with the p38-dependent remodeling of nucleosomes within the promoter region by SWI/SNF[31], the H3K4me3 modification would permit high level expression of developmentally regulated genes. Interestingly, our siRNA studies demonstrate that a decrease in H3K4me3 is matched by an increase in H3K4me2 (Figure 5C, and Supplementary Figure 6) suggesting that these two modifications exist in a dynamic equilibrium that may allow the rapid conversion from a poised promoter to an actively transcribing gene.

The p38 MAPK pathway has previously been shown to be essential for the recruitment of the ATP-dependent chromatin remodeling complex SWI/SNF to the Myog and Ckm promoters[31]. This is particularly interesting, since the catalytic subunits of these complexes (BRM/BRG1) falls into the same TrxG family of proteins as Ash2L[1,2]. Furthermore, in the case of the Ckm gene, it has been shown that Mef2d cooperates with Myog to recruit SWI/SNF to its promoter region[37]. In contrast, several studies have suggested that SWI/SNF is recruited to the Myog promoter through an interaction with MyoD[31,38] in the absence of Mef2[31]. While SWI/SNF appears to be recruited to the Myog and Ckm promoters through different mechanisms, we clearly observe a Mef2d/p38 dependent recruitment of Ash2L to the same two loci. Thus, there appears to be some difference in the mechanism by which Ash2L and SWI/SNF are targeted to

developmentally regulated genes. However, we highlight the fact that p38 regulates the recruitment of these two TrxG complexes to specific genes. Furthermore, we propose that one of the mechanisms by which p38 promotes myogenesis is through targeting of TrxG proteins to muscle specific genes to establish tissue specific expression.

In conclusion, we demonstrate that muscle specific genes do become epigenetically marked for gene expression during myogenesis. Targeting of the H3K4me3 mark to specific promoters is mediated by the Ash2L methyltransferase complex which is recruited by the DNA-bound transcriptional activator Mef2d. Furthermore, we found that this recruitment is regulated through the p38 MAPK mediated phosphorylation of Mef2d to enhance the association between the transactivator and the methyltransferase complex. Thus, our study demonstrate the first mechanistic link between the p38 MAPK signaling pathway and the activation of muscle specific genes during myogenesis.

Materials and Methods

Antibodies*.* Histone H3K4me3 (Abcam ab8580), Histone H3K4me2 (Upstate 07-030), Acetyl-Histone H4 (Upstate 06-866), WDR5 (Abcam ab22512), RbBP5 (Bethyl BL766), Mef2 (Santa Cruz sc-17785; sc-13917), Mef2d (BD Biosciences 610774), Set1 (Bethyl A300-289A) MLL (Bethyl A300-087A) MLL2/Trx2 (Bethyl A300-113A), Menin (Bethyl A300-105A), Pol II (Santa Cruz sc-17798; sc-9001; sc-5943), p300 (Santa Cruz sc-584), p38 (Santa Cruz sc-7972),

Phosphorylated(Thr180/Tyr182)-p38 (Cell Signaling 9211S), Myog (Developmental Studies Hybridoma Bank - F5D), Flag (Sigma F-3165). Antibodies against MLL3 and MLL4/ALR have previously been described[27]. Antibodies against MyoD, Ash2L, DPY30 were raised in rabbits using purified full-length proteins.

Cell Culture. The mouse myoblast cell line C2C12[17] was maintained at less than 80% confluency in DMEM containing 10%(v/v) FCS, L-glutamine, penicillin, and streptomycin. For differentiation studies, cells were washed with PBS when they attained 80% confluence, and then incubated in differentiation media (DMEM containing 2%(v/v) horse serum, insulin (10μg mL^{-1}), transferrin (10μg mL^{-1}), L-glutamine, penicillin, and streptomycin) for 48 h (unless otherwise noted). Mef2c, Mef2d, Ash2L or control siRNAs (Ambion) were transfected at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen) and transferred to differentiation media immediately. For p38 MAPK inhibition studies, SB203580 was added directly to the differentiation media at a final concentration of 10 μM.

Native ChIPs. Approximately 1x10^6 C2C12 cells obtained at various stages of differentiation were lysed in buffer A (15 mM Tris pH 7.5, 15 mM NaCl, 60 mM KCl, 250 mM sucrose, 5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 mM DTT, and 1 mM PMSF). Nuclei were recovered by centrifugation, and quantitated by measuring total ribonucleic acid content. Chromatin (15 μg) was digested with 1 U of MNase

(Sigma) for 10 min at 37°C to give maximum visible fragment size of 550 bp (3 nucleosomes). NaCl was then added to a final concentration of 600 mM, and the nuclei were incubated with 10 mg of hydroxyapatite resin to generate a slurry. After extensive washing, nucleosomes were eluted from the resin using a buffer containing 300 mM $NaPO_4$ pH 7.2. Eluted nucleosomes were then subjected to immunoprecipitation with either an anti-H3K4me3, anti-H3K4me2 or control rabbit IgG as described in the figure legend. Immunoprecipitated DNA was purified, and subjected to Real-Time PCR analysis using Taqman probes (see Supplementary Table 1 for primer/probe sequences). To determine relative enrichment, the signal observed in the control IP was subtracted from the signal observed using the specific antibody. This value was then divided by the signal observed from 1/50th of the ChIP input material.

ChIPs. ChIPs were performed as previously described[39], except that DNA was sonicated using a bioruptor to obtain fragments of approximately 400 bp in length. Immunoprecipitated DNA was reverse-crosslinked, purified, and subjected to Real-Time PCR analysis using Taqman probes (see Supplementary Table 1 for primer/probe sequences). Relative enrichment was calculated as described for native ChIP studies.

Protein Expression. N-terminal Flag-tagged MyoD (FL-MyoD); and C-terminal Flag-tagged Mef2aα1,β (FL-Mef2a), Mef2cα1,β,γ (FL-Mef2c), and

Mef2dα1,β (FL-MEF2D) were expressed using baculovirus in Sf9 cells, and purified using anti-Flag M2-agarose beads (Sigma). The phosphorylation deficient mutant of Mef2d (FL-Mef2d(Mut)) was generated by mutating both threonine 308 and 315 to alanine using the QuikChange system (Stratagene). Activated p38α was purified from a BL-21 strain stably co-expressing His-p38α, MEK4, and MEKK-C[29] using Ni-NTA resin (Qiagen).

Immunoprecipitations and Flag pull down assays*.* Cell extracts were prepared from 48 h differentiating C2C12 cells in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1%(v/v) NP-40, 0.1%(w/v) SDS, 0.5%(v/v) Na-deoxycholate, and Complete protease inhibitors(GE Healthcare)). Either protein A or protein G Dynabeads were coated with antibodies as described before[39]. Approximately 400 μg of protein lysate was subjected to immunoprecipitation using various antibodies (as indicated in figure legends). Beads were then washed three times with buffer containing 300 mM KCl and 0.1%(v/v) NP40 and proteins were eluted in SDS loading dye. Eluted proteins were then subjected to western blotting.

For Flag pull down assays, extracts prepared from baculovirus-infected Sf9 cells expressing recombinant proteins of interest were incubated with Flag M2-Agarose beads for 1 hr. The beads were then washed using EX-100 buffer[40] to remove unbound proteins. For phosphorylation experiments protein bound beads were incubated in the presence of 1 *μ*M rATP with (or without) p38α kinase for 30 min at 30° C. After washing to remove ATP and p38α, beads were

incubated with either 48 h differentiated C2C12 cell extract. Interacting proteins were eluted in SDS loading dye and subjected to western blotting using various antibodies as indicated.

RT-PCR assays. Total RNA (3μg) was reverse transcribed using MuMLV reverse transcriptase. Random primed cDNA obtained was subjected to semi-quantitative RT-PCR using gene specific primers for Myog, Ckm, Cdkn1a, Acta1, Mef2a, Mef2c, Mef2d, and β-Actin (see Supplementary Table 1 for primer sequences). For qRT-PCRs, duplex reactions were performed using 5'FAM/3'BHQ labeled gene specific primers (see Supplementary Table 1 for primer sequences) and 5'YY/3'EDQ labeled 18S rRNA primers (Eurogentec).

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Author Contributions

S.R. and F.J.D. conceived and designed the experiments. S.R. was responsible for all ChIP, immunoprecipitation, RT-PCR, and interaction studies. F.J.D. and

M.B. generated the polyclonal antibody against full-length Ash2L. L.L. generated the polyclonal antibodies against full-length DPY and MyoD. E.M. generated the Mef2D mutant. S.R. and E.M. performed the transfections studies with the Mef2 mutants. K.G. provided unpublished antibodies against MLL3 and MLL4. S.R. M.B., S.J.T. and F.J.D. provided scientific direction to the project. F.J.D. wrote the paper. S.R., M.B., and F.J.D. discussed and commented on the manuscript.

Competing Interests Statement

The authors declare that they have no competing financial interests.

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Figure Legends

Figure 1. Muscle specific genes are trimethylated at H3K4 during myogenesis. A) Growing (0 h) or differentiating (6 h, 24 h, or 48 h) C2C12 cells were subjected to native ChIP analysis using antibodies directed against H3K4me3 or control rabbit IgG. Immunopurified nucleosomes were then deproteinated, and subjected to qPCR analysis using primers recognizing either the Myog, Ckm, Cdkn1a, Acta1, or Igh genes. Average values of duplicate qPCR reactions are displayed with error bars corresponding to ± s.d. Each experiment was performed at least twice using independent chromatin samples, and yielded similar results. B) RNA was extracted from growing (0 h) or differentiating (6 h, 12 h, 24 h, or 48 h) C2C12 cells, reverse transcribed, and subjected to semi-quantitative PCR analysis of gene expression using primers specific for either the Myog, Ckm, Cdkn1a, Acta1, or Gapdh transcripts. C) cDNA was prepared as in B) and subjected to duplex qPCR analysis of gene expression using primers specific for either the Myog or Ckm transcripts along with 18S RNA. Expression of Myog and Ckm is reported relative to the control 18S RNA signal. Average values of triplicate RT-qPCR reactions are displayed with error bars corresponding to ± s.d. Each experiment was performed at least twice using independently isolated RNA.

Figure 2. Mef2 interacts with the Ash2L methyltransferase complex. A) Mef2 co-immunoprecipitates with Ash2L in C2C12 cells. Nuclear extracts

prepared from differentiating C2C12 cells (48 h) were subjected to immunoprecipitation using an anti-Ash2L or control IgG antibody (Mock). Immunopreciptated proteins were analyzed by western blot using indicated antibodies. B) Cell extracts prepared from differentiating C2C12 cells (48 h) were subjected to immunoprecipitation using an anti-Mef2 or control IgG antibody. Immunopreciptated proteins were analyzed by western blot using indicated antibodies. C) Relative recruitment of Mef2 or Ash2L was measured at the Myog promoter or Ckm enhancer at various time points during differentiation using ChIP. After deproteination, immunopurified DNA was quantitated by Real-Time qPCR using Taqman probes. Average values of duplicate qPCR reactions are displayed with error bars corresponding to ± s.d. Each experiment was performed at least twice using independent chromatin samples, and yielded similar results.

Figure 3. Ash2L interacts preferentially with phosphorylated Mef2d and Mef2c. A) Flag-tagged Mef2a, Mef2c, or Mef2d treated with (or without) activated p38α were incubated with an extract prepared from differentiating (48 h) C2C12 cells. After washing, Mef2-associated proteins were subjected to western blot analysis using anti-Ash2L or anti-Flag antibodies. B) Flag-tagged Mef2d(Wt), or Mef2d(Mut) were incubated with activated p38α and [^{32}P]-ATP. Reactions were separated on a 10% SDS-PAGE gel, dried and exposed to autoradiographic film. C) Interaction studies using Flag-tagged Mef2d(Wt) or Mef2d(Mutant) were performed as outlined in A). D) Cell extracts prepared from differentiating C2C12

cells (48 h) that had been incubated in the presence (+SB) or absence(-SB) of the p38 MAPK inhibitor SB203580 were subjected to immunoprecipitation using an anti-Mef2 or control IgG antibody. Immunopreciptated proteins were analyzed by western blot using indicated antibodies.

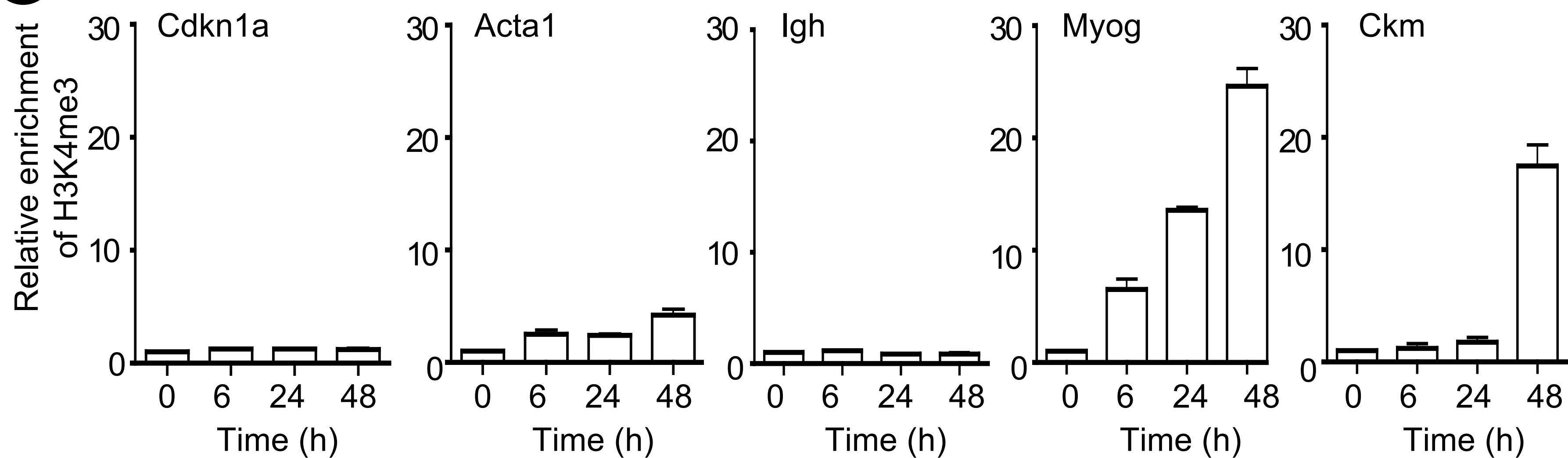
Figure 4. Inhibition of p38 MAPK activity prevents recruitment of Ash2L to the Myog promoter. A) Western blot analysis of protein extracts prepared from C2C12 cells treated in the presence (+SB) or absence (-SB) of SB203580 under either growth (0 h) or differentiation (48 h) conditions using indicated antibodies. B) Inhibition of the p38 kinase activity blocks transcription of several muscle specific genes in C2C12 cells. RNA was isolated from C2C12 cells treated in the presence (+SB) or absence (-SB) of SB203580 under either growth (0 h) or differentiation (48 h) conditions. After reverse transcription, random primed cDNA was subjected to semi-quantitative PCR analysis using primers specific for the genes indicated. C) Inhibition of the p38 kinase activity blocks recruitment of Ash2L and prevents H3K4me3 at the Myog promoter. ChIP was used to measure relative enrichment of the indicated proteins to the Myog promoter in C2C12 cells differentiated (48 h) in the presence or absence of SB203580. After deproteination, immunopurified DNA was quantitated by Real-Time qPCR using Taqman probes. Average values of duplicate qPCR reactions are displayed with error bars corresponding to ± s.d. Each experiment was performed at least twice using independent chromatin samples, and yielded similar results.

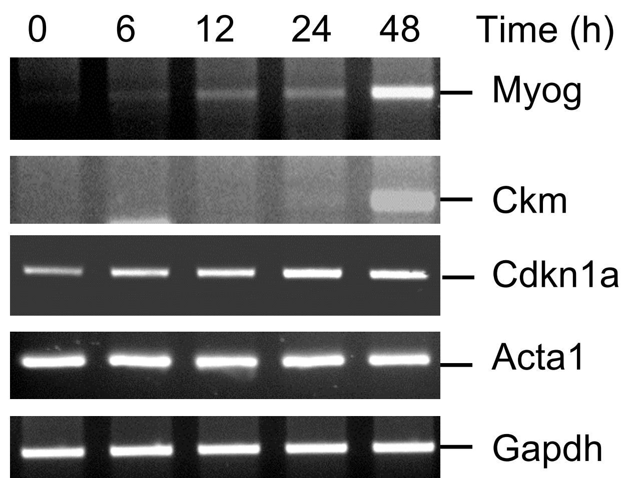
Figure 5. Knock-down of Mef2d and Mef2c in C2C12 cells leads to reduced levels of Ash2L at the Myog and Ckm promoters. A) RNA was isolated from differentiating C2C12 cells (48h) that were untransfected or had been transfected with siRNA targeting Mef2d, Mef2c, or control (untargeted). RNA was reverse transcribed, and subjected to duplex qPCR analysis of gene expression using primers specific for Myog, Ckm, or Acta1 transcripts along with 18S RNA. Expression of Myog, Acta1, and Ckm is reported relative to the control 18S RNA signal. Average values of triplicate RT-qPCR reactions are displayed with error bars corresponding to ± s.d. Each experiment was performed at least twice using independently isolated RNA. B) cDNA was prepared as described in A), and analyzed by semi-quantitative PCR using primers specific to the genes indicated. C) C2C12 cells were transfected with siRNA targeting both Mef2c and Mef2d (such that both family members would be knocked down), or an untargeted control siRNA. Cells were then differentiated for 48 h, and analyzed by ChIP for enrichment of H3K4 methylation at the Myog promoter. Immunopurified DNA was quantitated by Real-Time qPCR using Taqman probes.x D) Experiments were performed as described in C) except that relative recruitment of transcriptional activators, and coactivators was examined. For C) and D) average values of duplicate qPCR reactions are displayed with error bars corresponding to ± s.d. Each experiment was performed at least twice using independent chromatin samples, and yielded similar results.

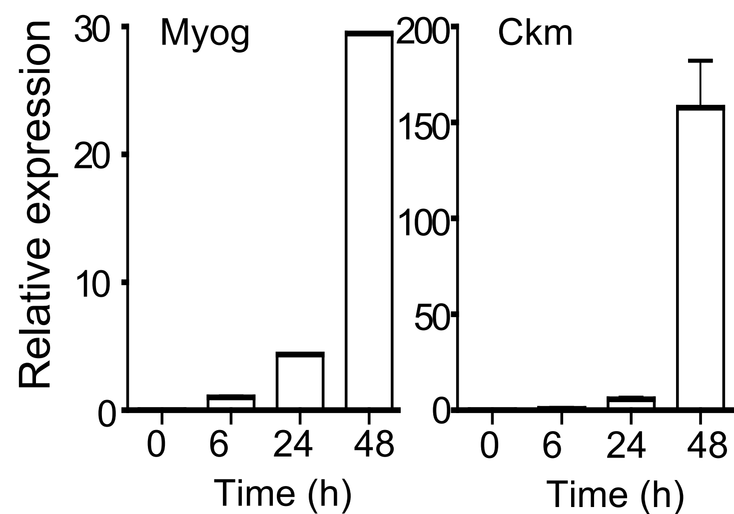
Figure 6. Knock-down of Ash2L in C2C12 cells leads to reduced transcription of the Myog and Ckm genes. A) Whole cell extracts prepared from differentiating C2C12 cells after different siRNA treatments were subjected to western blot analysis using anti-Ash2L or anti-β-actin antibodies. B) Knock-down of Ash2L leads to decreased expression of muscle specific genes. RNA was isolated from differentiating C2C12 cells (48 h) that were transfected with siRNA targeting Ash2L, or control (untargeted). RNA was extracted, reverse transcribed, and subjected to duplex qPCR analysis. Expression of Myog, Acta1, and Ckm is reported relative to the control 18S RNA signal. Average values of triplicate RT-qPCR reactions are displayed with error bars corresponding to ± s.d. Each experiment was performed at least twice using independently isolated RNA. C) C2C12 cells were transfected with siRNA targeting Ash2L, or an untargeted control siRNA. Transfected cells were then differentiated for 48 h, and analyzed by ChIP for enrichment of Ash2L, H3K4me3, and Mef2 at the Myog promoter. After deproteination, immunopurified DNA was quantitated by Real-Time qPCR using Taqman probes. Average values of duplicate qPCR reactions are displayed with error bars corresponding to ± s.d. Each experiment was performed at least twice using independent chromatin samples, and yielded similar results.

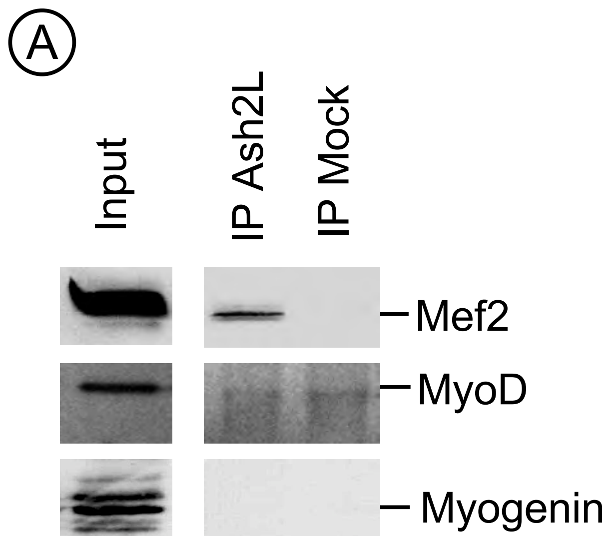
Figure 7. Model for the integration of p38 MAPK signaling pathway with Mef2 dependent recruitment of the Ash2L complex to muscle specific

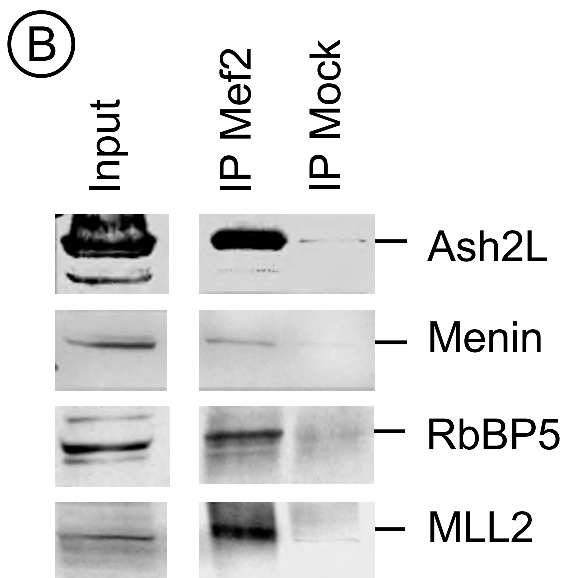
promoters. Prior to activation of p38, MyoD/E47(MD/E) cooperate with Mef2d dimers (homo- or heterodimers) to establish a transcriptionally poised promoter through recruitment of the acetyltransferase p300 and RNA Polymerase II (Pol II). This leads to acetylation of nucleosomes on histone H4 within the promoter. As differentiation proceeds, cell-cell contact activates the membrane bound receptor Cdo leading to p38 MAPK activation via the scaffold protein JLP, and MKK6 kinase[36]. Once activated, p38 MAPK phosphorylates Mef2d leading to the targeting of the Ash2L methyltransferase complex to muscle specific promoters. The methyltransferase complex then establishes the epigenetic H3K4me3 mark required for high-level expression of developmentally regulated genes.

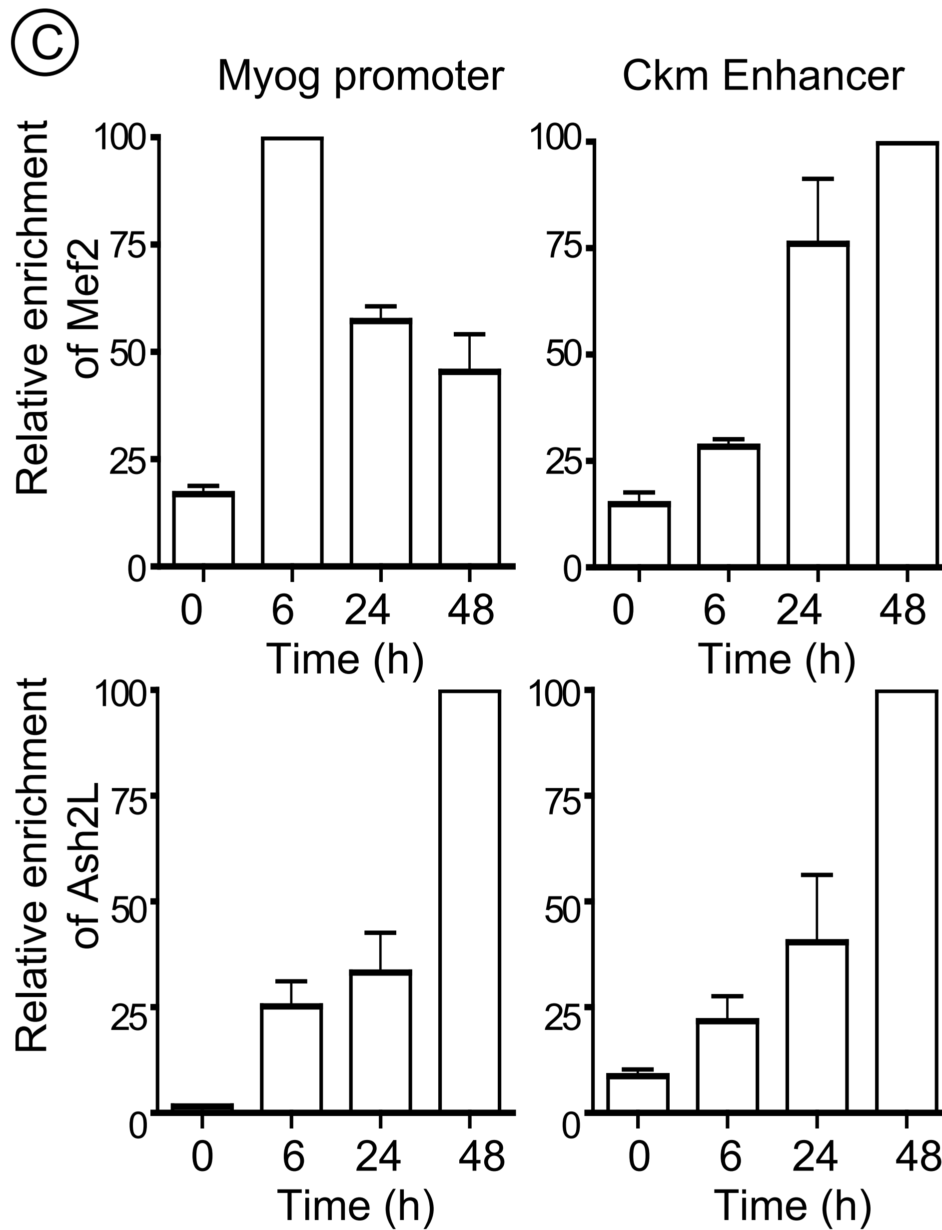

A
Relative enrichment of H3K4me3
Cdkn1a
Acta1
Igh
Myog
Ckm
30
20
10
0
0 6 24 48
Time (h)

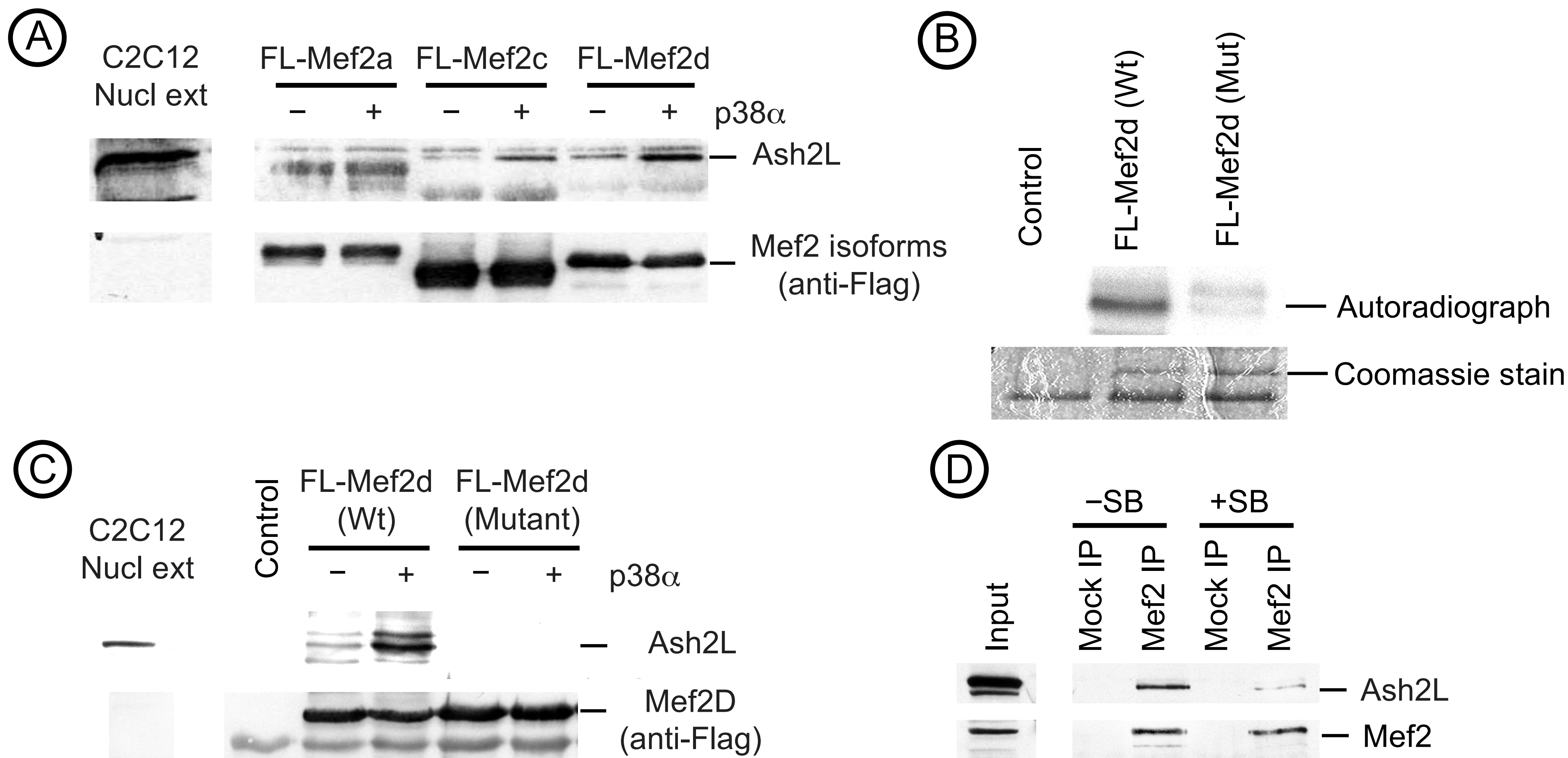

B
0 6 12 24 48 Time (h)
Myog
Ckm
Cdkn1a
Acta1
Gapdh


C
Relative expression
Myog
Ckm
30
20
10
0
200
150
100
50
0 6 24 48
Time (h)

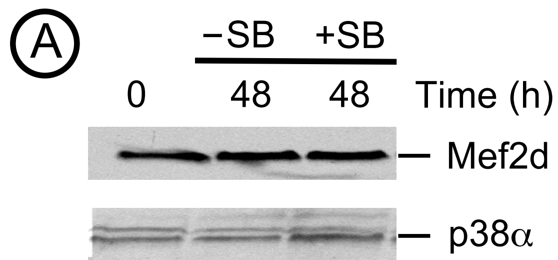

A
Input
IP Ash2L
IP Mock
Mef2
MyoD
Myogenin


B
Input
IP Mef2
IP Mock
Ash2L
Menin
RbBP5
MLL2

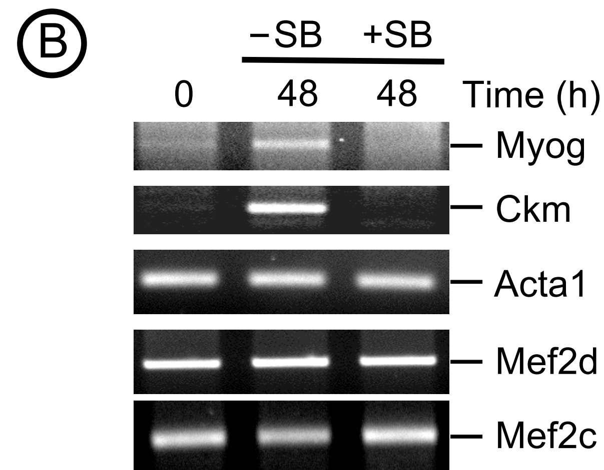

C
Myog promoter
Ckm Enhancer
Relative enrichment of Mef2
Relative enrichment of Ash2L
0
25
50
75
100
0
6
24
48
Time (h)



A
C2C12
Nucl ext
FL-Mef2a
FL-Mef2c
FL-Mef2d
−
+
−
+
−
+
p38α
Ash2L
Mef2 isoforms
(anti-Flag)
B
Control
FL-Mef2d (Wt)
FL-Mef2d (Mut)
Autoradiograph
Coomassie stain
C
C2C12
Nucl ext
Control
FL-Mef2d
(Wt)
FL-Mef2d
(Mutant)
−
+
−
+
p38α
Ash2L
Mef2D
(anti-Flag)
D
−SB
+SB
Input
Mock IP
Mef2 IP
Mock IP
Mef2 IP
Ash2L
Mef2

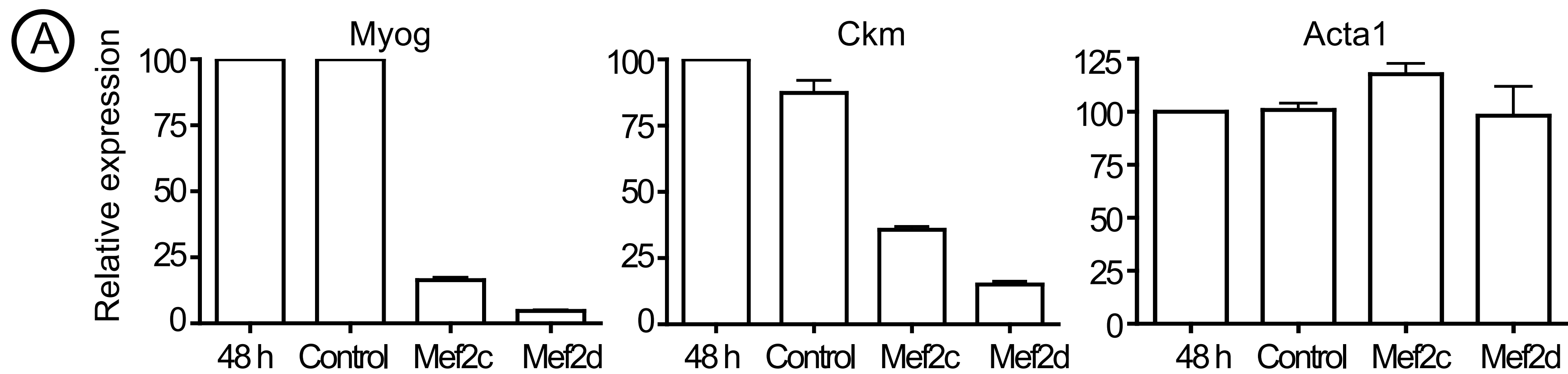


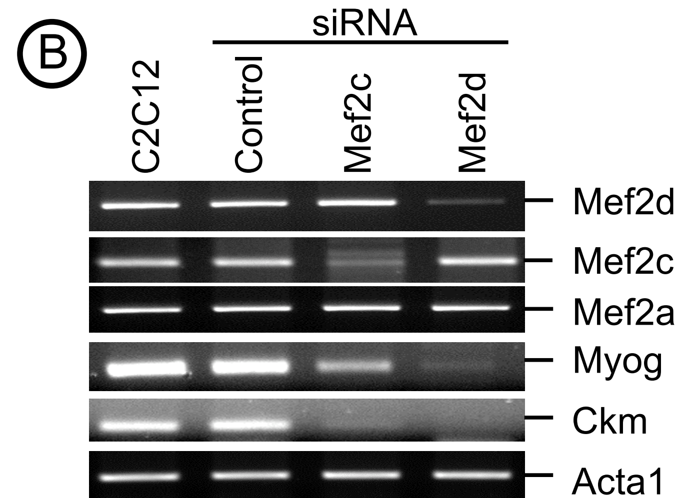
A
−SB
+SB
0
48
48
Time (h)
Mef2d
p38α

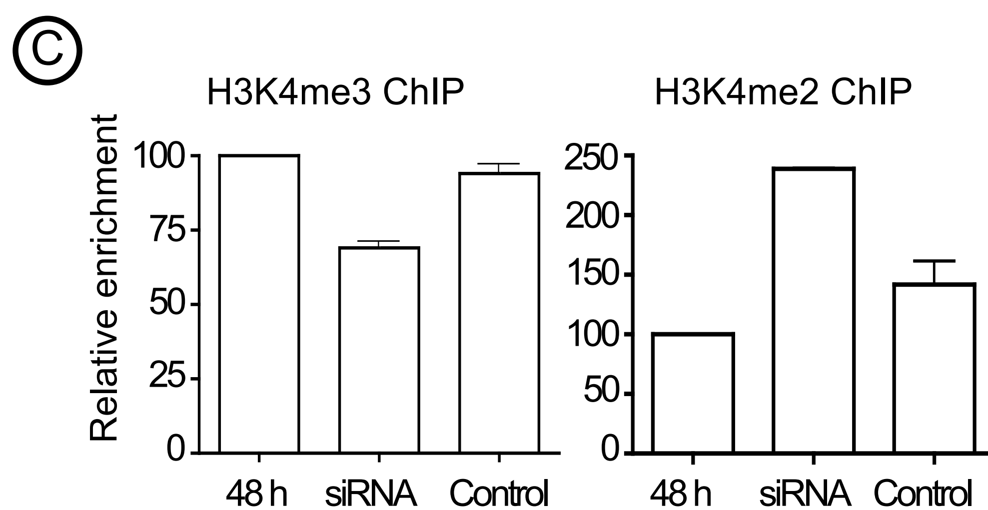


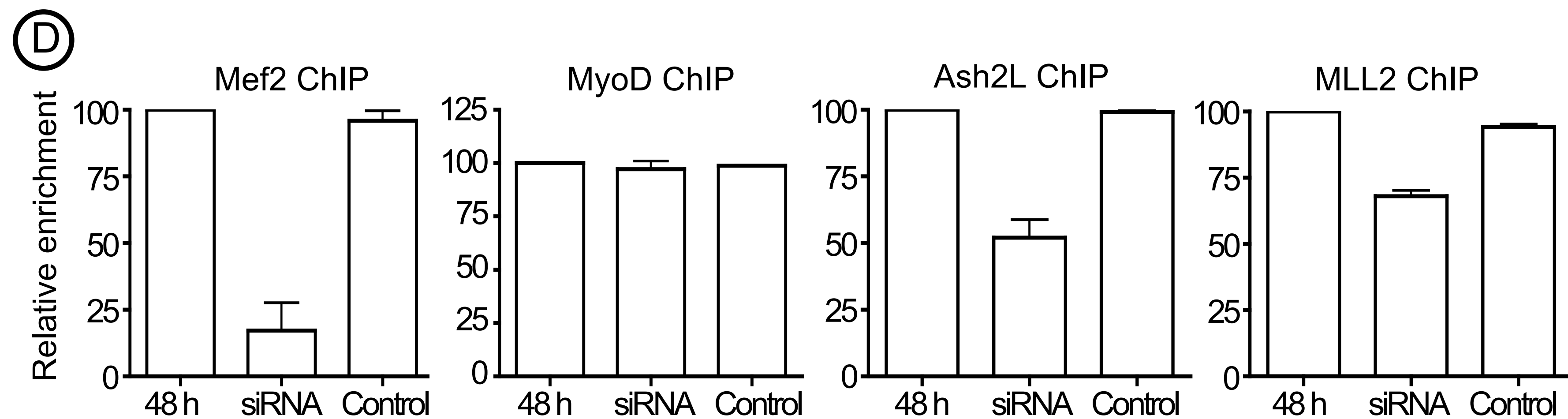
B
−SB
+SB
0
48
48
Time (h)
Myog
Ckm
Acta1
Mef2d
Mef2c

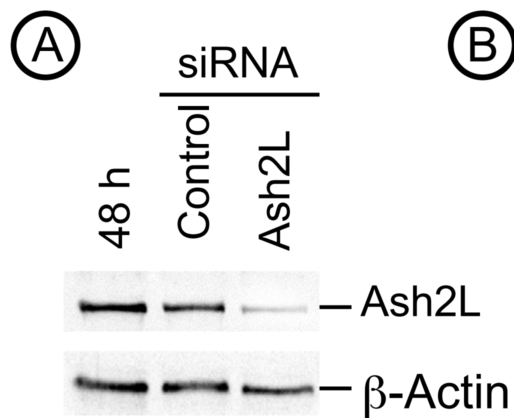
C
Mef2 ChIP
MyoD ChIP
Ash2L ChIP
H3K4me3 ChIP
Relative enrichment
−SB
+SB
H4ac ChIP
p300 ChIP
RNA Pol II ChIP
Relative enrichment
−SB
+SB

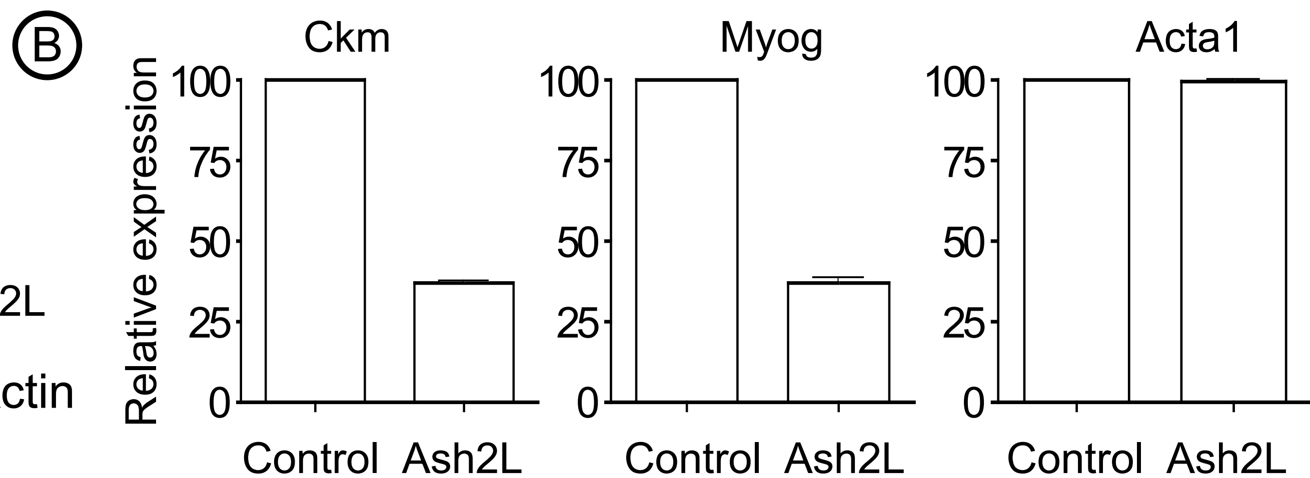

A
Myog
Ckm
Acta1
Relative expression
100
75
50
25
0
125
48 h
Control
Mef2c
Mef2d

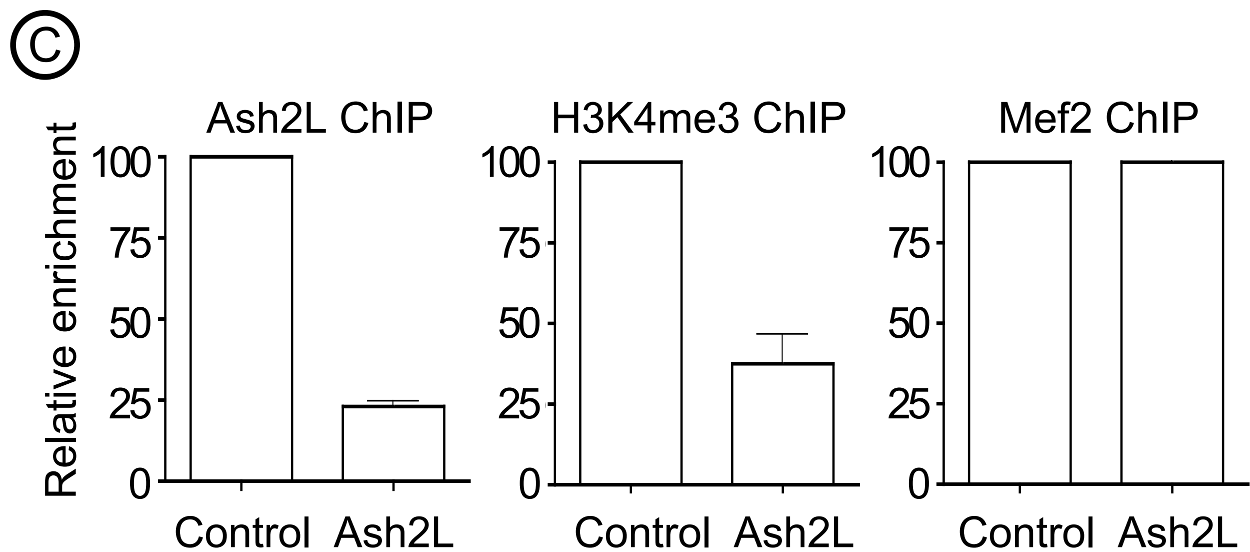

B
siRNA
C2C12
Control
Mef2c
Mef2d
Mef2d
Mef2c
Mef2a
Myog
Ckm
Acta1

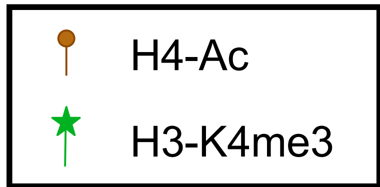

C
H3K4me3 ChIP
H3K4me2 ChIP
Relative enrichment
100
75
50
25
0
250
200
150
48 h
siRNA
Control

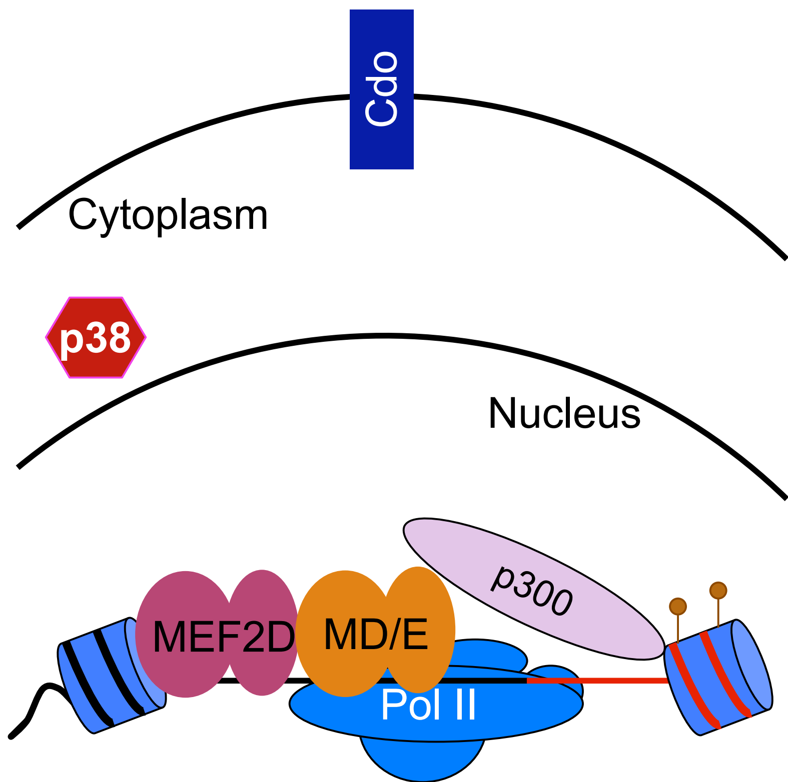

D
Mef2 ChIP
MyoD ChIP
Ash2L ChIP
MLL2 ChIP
Relative enrichment
125
100
75
50
25
0
48 h
siRNA
Control

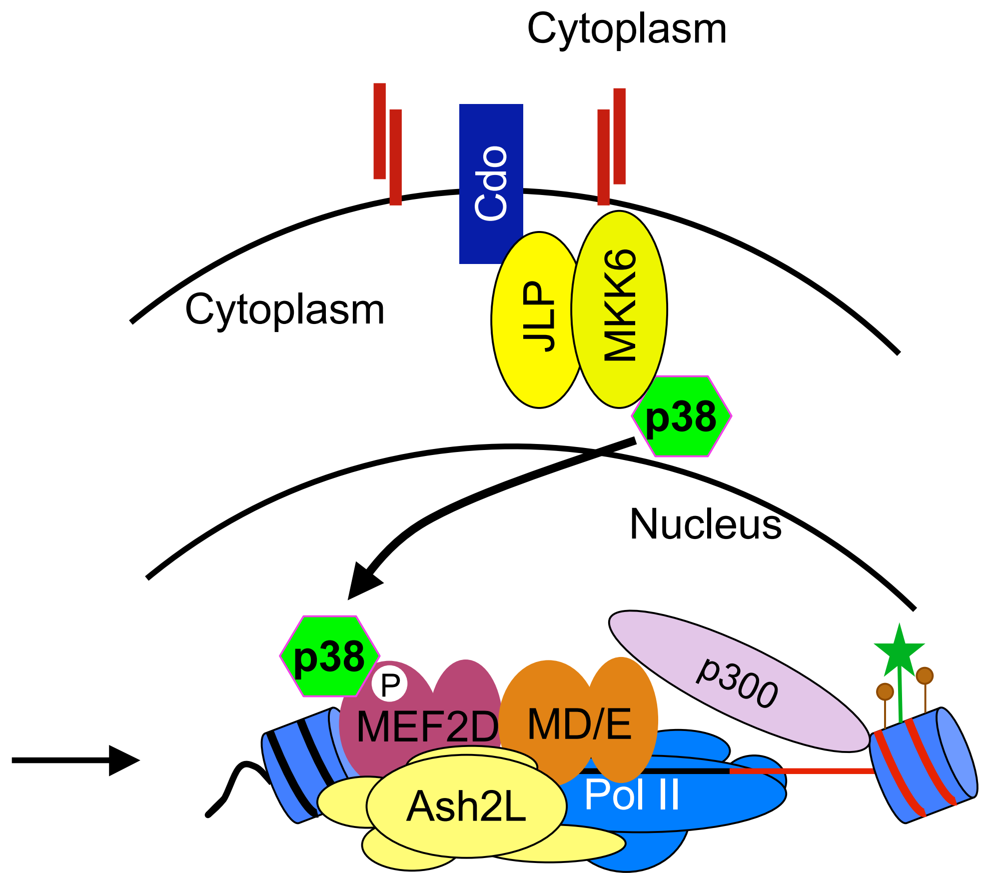

A
siRNA
48 h
Control
Ash2L
Ash2L
β-Actin


B
Ckm
Myog
Acta1
Relative expression
100
75
50
25
0
Control
Ash2L


C
Ash2L ChIP
H3K4me3 ChIP
Mef2 ChIP
Relative enrichment
100
75
50
25
0
Control
Ash2L

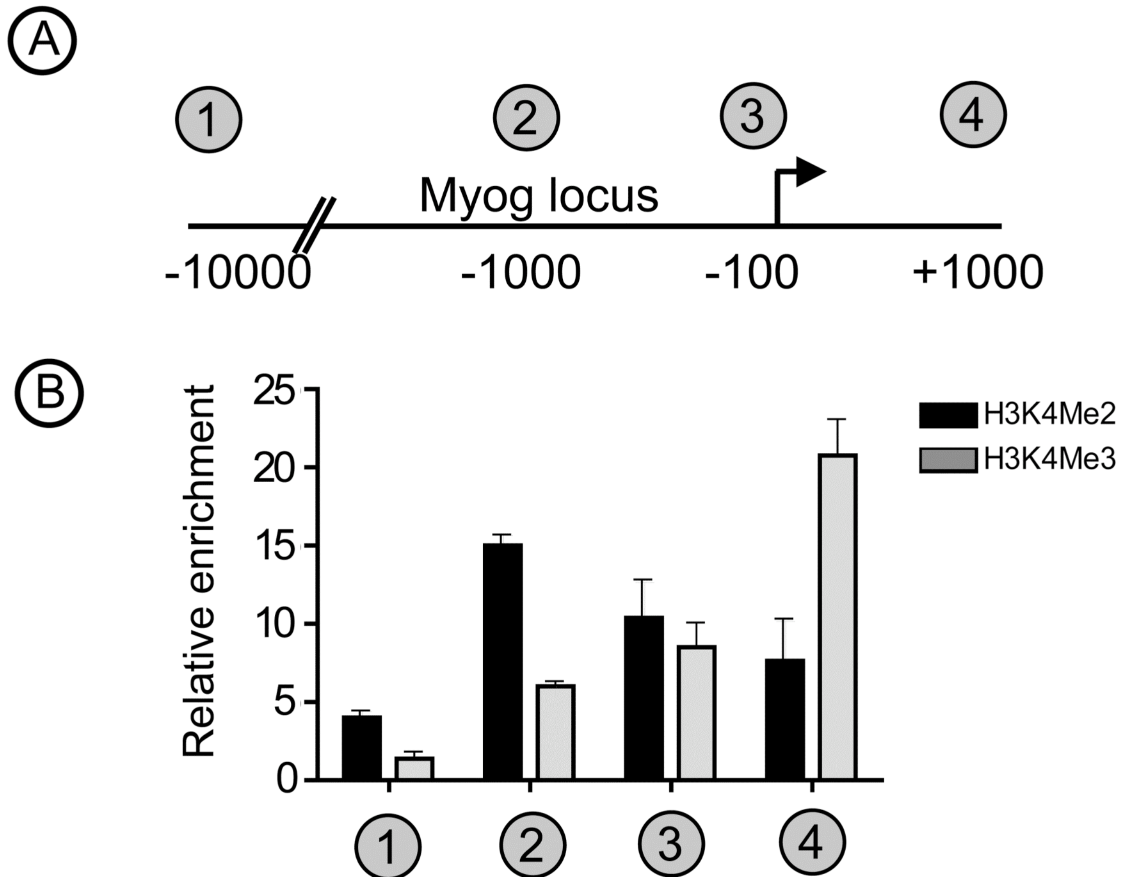

H4-Ac
H3-K4me3


Cdo
Cytoplasm
p38
Nucleus
MEF2D
MD/E
p300
Pol II

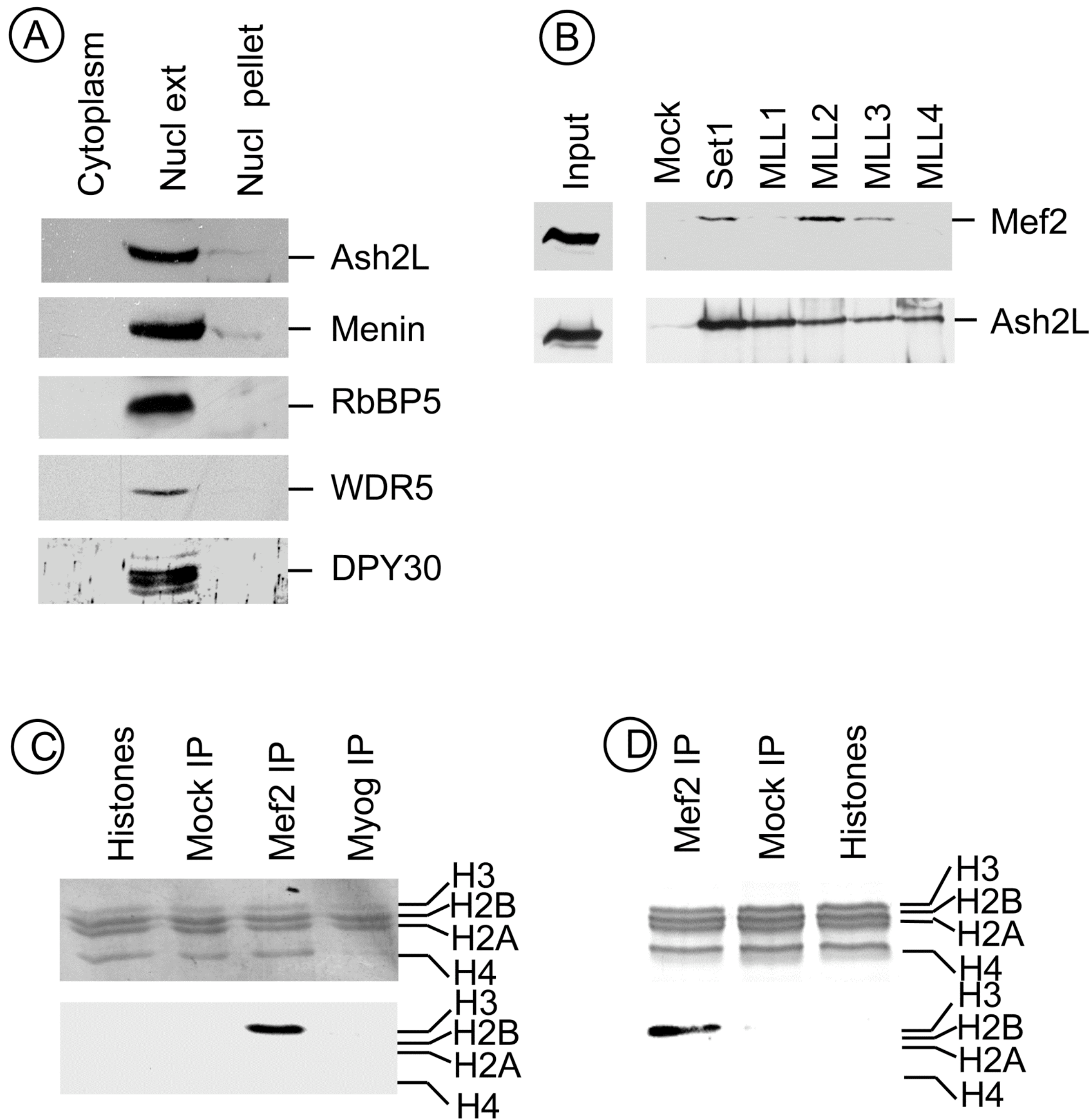

Cytoplasm
Cdo
JLP
MKK6
Cytoplasm
p38
Nucleus
p38
P
MEF2D
MD/E
p300
Ash2L
Pol II

p38 MAPK signaling regulates recruitment of Ash2L-containing methyltransferase complexes to specific genes during differentiation

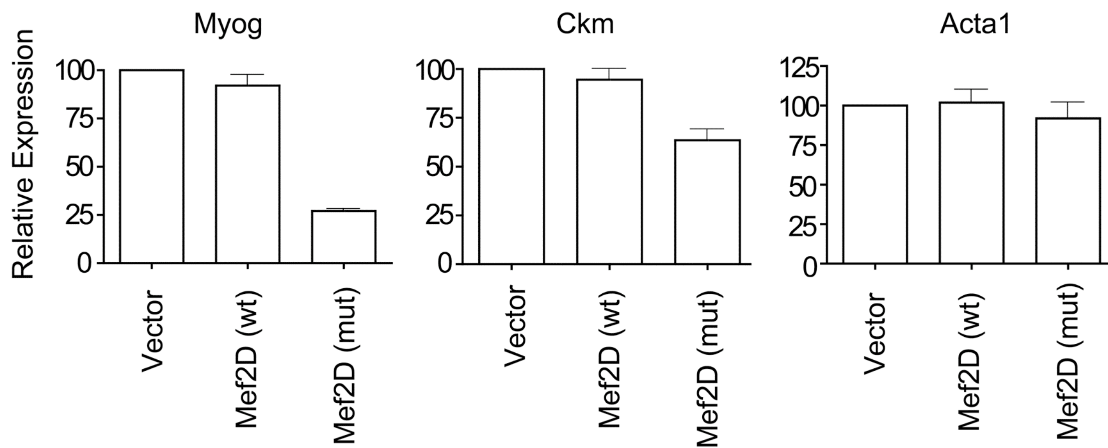
Shravanti Rampalli, LiFang Li, Esther Mak, Kai Ge, Marjorie Brand, Stephen J. Tapscott, and F. Jeffrey Dilworth



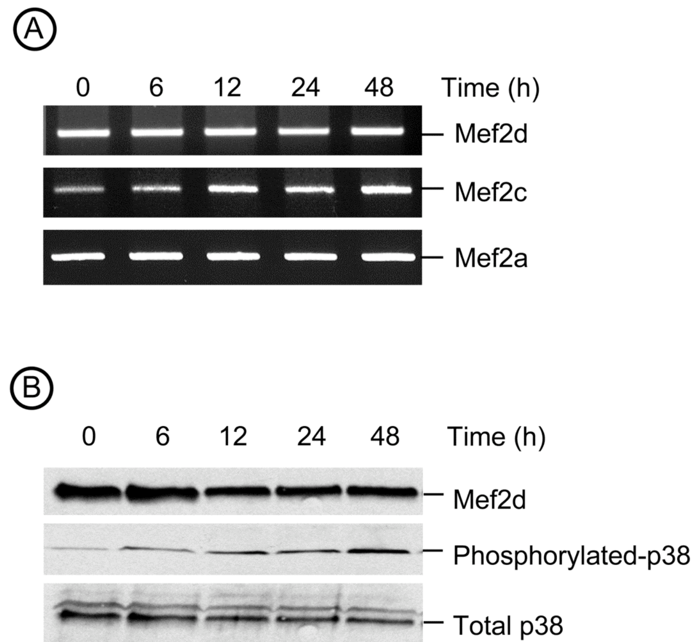
Supplemental Figure 1. Distribution of H3K4me2 and H3K4me3 across the Myog locus. A) Schematic representation of the Myog gene locus. The numbers 1 through 4 represent the position of primer sets used in the ChIP studies. B) Native ChIP analysis was used to measure relative enrichment of H3K4me2 and H3K4me3 at various locations along the Myog locus in differentiating (48 h) C2C12 cells as described above. Resolution of the native ChIP was ~550 bp after micrococcal nuclease digestion. Relative enrichment is expressed as the signal observed on the Myog gene with respect to that observed at the inactive Igh gene. Average values of duplicate qPCR reactions are displayed with error bars corresponding to ± s.d. Each experiment was performed at least twice independently.



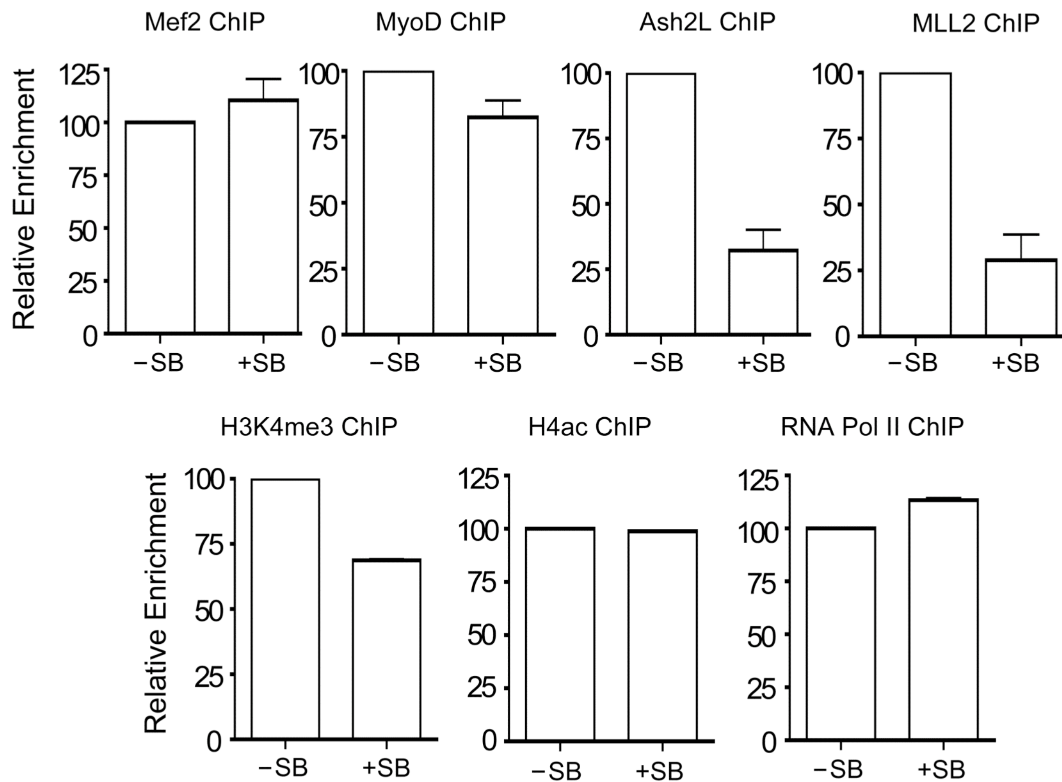
Supplemental Figure 2. Mef2 interacts with multiple Ash2L-containing methyltransferase complexes. A) The Ash2L complex is expressed in C2C12 cells. Extracts were prepared from C2C12 cells corresponding to the cytoplasm, nucleus, or nuclear pellet. Extracts were analyzed by western blot using indicated antibodies. B) Mef2 interacts with the MLL2, MLL3, and Set1 containing Ash2L-complexes. Nuclear extracts prepared from differentiating C2C12 cells (48 h) were subjected to immunoprecipitation using anti-MLL, -MLL2, -MLL3, -MLL4, -Set1 or control Rabbit IgG antibodies. Immunopreciptated proteins were analyzed by western blot using either Mef2 or Ash2L antibodies. C) Mef2 associates with an H3K4 methyltransferase activity in C2C12 cells. Mef2, Myog, and mock immunoprecipitates from differentiating (48 h) C2C12 cells were incubated with *S*-[methyl-^{3}H]-adenosyl-*L*-methionine in the presence of purified core histones. Reactions were split into 2 fractions, separated by SDS-PAGE, and either stained with coommassie blue, or transferred to PVDF membrane and exposed to autoradiographic film. D) Mef2 and mock immunoprecipitates from differentiating (48 h) C2C12 cells were incubated with 10 μM *S*-adenosyl-*L*-methionine in the presence of purified core histones. Reactions were split into 2 fractions, separated by SDS-PAGE, and either stained with coommassie blue, or analyzed by western blotting using antibodies directed against H3K4me3.



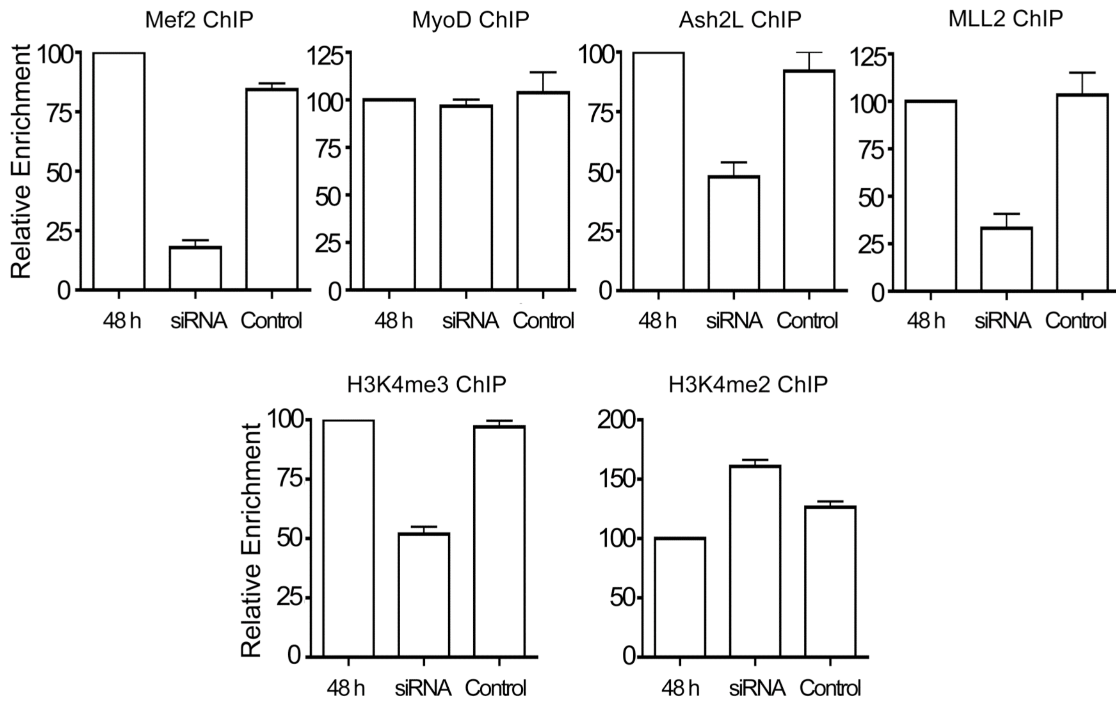
Supplementary Figure 3. A mutant Mef2d that is not phosphorylated by p38 acts in a dominant negative manner to inhibit Myog and Ckm expression. C2C12 cells were transfected with pBABE (Vector), pBABE-Mef2d(wt), or pBABE-Mef2d(mut), and allowed to differentiate. After 48 h, RNA was extracted and subjected to reverse transcription and duplex qPCR analysis using Taqman probes. Expression of Myog, Acta1, and Ckm is reported relative to the control 18S RNA signal. Average values of triplicate RT-qPCR reactions are displayed with error bars corresponding to ± s.d. Each experiment was performed at least twice independently.



Supplementary Figure 4. Phosphorylation of p38 is increased during differentiation. A) RNA isolated from C2C12 cells at different stages of differentiation was reverse transcribed and subjected to semi-quantitative PCR analysis using primers specific for the genes indicated. B) Whole cell proteins extracts prepared from C2C12 cells at different stages of differentiation were analyzed by western blot using indicated antibodies



Supplementary Figure 5. Inhibition of p38 activity prevents recruitment of Ash2L and prevents H3K4me3 at the Ckm promoter. ChIP was used to measure relative enrichment of the proteins indicated to the Ckm promoter in C2C12 cells differentiated (48 h) in the presence or absence of SB203580. After deproteination, immunopurified DNA was quantitated by Real-Time PCR using Taqman probes. Average values of duplicate qPCR reactions are displayed with error bars corresponding to ± s.d. Each experiment was performed at least twice independently.



Supplementary Figure 6. Knock-down of Mef2d and Mef2c in C2C12 cells leads to reduced recruitment of the Ash2L complex to the Ckm gene promoter. Prior to differentiation, C2C12 cells were transfected with siRNA targeting both Mef2c and Mef2d (such that both family members would be knocked down), or an untargeted control siRNA. Both transfected and untransfected cells were then differentiated for 48 h, and further analyzed by ChIP for enrichment of H3K4 methylation at the Ckm promoter. After deproteination, immunopurified DNA was quantitated by Real-Time qPCR using Taqman probes. Average values of duplicate qPCR reactions are displayed with error bars corresponding to ± s.d. Each experiment was performed at least twice independently.

Supplementary Table 1. Primers Used for PCR analysis.

Myog Coding (+1000)	
Probe	5'FAM-ACCCTGGTAACATGGCTCAAATCCCT-3'BHQ1
Forward	5'-TCCCAACCCAGGAGGTAAGTGAAT-3'
Reverse	5'-AAGGGTGATTGGGACAGGGCTTAT-3'
Myog Start Site (-145)	
Probe	5'FAM-CTTGATGTGCAGCAAC-3'BHQ1
Forward	5'-TCACATGTAATCCACTGGAAACG-3'
Reverse	5'-CCTGAGCCCCCCTCTAAGC-3'
Myog Promoter (-1000)	
Probe	5'FAM-TCGACCCTTCTACAGAAAGGAAAGAGTCA-3'BHQ1
Forward	5'-GCCCAGGACAGACAAATGATGCAA-3'
Reverse	5'-AATGCCTTCTGGCACTAGAACCGT-3'
Myog Upstream (-10000)	
Probe	5'FAM-TCCTTTGGCTCTCACCGTGTCTACAT-3'BHQ1
Forward	5'-CTCTCTTCTGAATGGCGTTTGCGT-3'
Reverse	5'-TTTGAAAGAGCTCCAGCTTTGGGC-3'
Ckm Enhancer (-1340)	
Probe	5'FAM-TCTAGGCTGCCCATGTAAGGAGGCAA-3'BHQ1
Forward	5'-GCTCCTGTCATATTGTGTCCTGCT-3'
Reverse	5'-TTATAACCAGGCATCTCGGGTGTC-3'
Ckm Intron 1 (+915)	
Probe	5'FAM-AGCAAGGAGGGAGGACAGGT-3'BHQ1
Forward	5'-CAATGCTGACTTAGTGCAAGGCGA-3'
Reverse	5'-AGACAGAGACCCAAAGCCCTTGAA-3'
Acta1 Promoter (-317)	
Probe	5'FAM-CCTTTGGCCCAGCACAGCCCT-3'BHQ1
Forward	5'-ACCAGCGGTCAAAGCAGTG-3'
Reverse	5'-CTCCCAACTGGCTCCAAGG-3'
Cdkn1a Promoter (-297)	
Probe	5'FAM-ATAGATGTATGTGGCTCTGCTGGTGC-3'BHQ1
Forward	5'-AGTTGGTCAGGGACAGACCCATAA-3'
Reverse	5'-ACACCTGGGCTATTCTCTTGTCAC-3'
Igh Enhancer (+4367)	
Probe	5'FAM-CGCCTCTGAGCCTGGGCAAGG-3'BHQ1
Forward	5'-GCCTGGGCTTGCTTTGTCT-3'
Reverse	5'-TGACCCCTTTCGCTCATTG-3'
Myog cDNA	
Probe	5'FAM-ATGGTGCCCAGTGAATGCAACTCCCA-3'BHQ1
Forward	5'-CATCCAGTACATTGAGCGCCTACA-3'
Reverse	5'-AGCAAATGATCTCCTGGGTTGGGA-3'
Ckm cDNA	
Probe	5'FAM-TCAACCACGAGAACCTCAAGGGTGGA-3'BHQ1
Forward	5'-ACCCACAGACAAGCATAAGACCGA-3'
Reverse	5'-AGGCAGAGTGTAACCCTTGATGCT-3'
Acta1 cDNA	
Probe	5' FAM-TATGTGGCCCTGGACTTCGAGAATGA-3'BHQ1
Forward	5'-TTGTGCGCGACATCAAAGAGAAGC-3'
Reverse	5'-GAAACGCTCATTGCCGATGGTGAT-3'

Mef2a cDNA	
Forward	5’-ATTCTCCAATTGTGCTTGGCCGAC-3’
Reverse	5’-AGGAAGTGCCAGACTGGTCTGTTT-3’
Mef2c cDNA	
Forward	5’-TCAACAGCACCAACAAGCTGTTCC-3’
Reverse	5’-ACCTGTTATGGCTGGACACTGGGA-3’
Mef2d cDNA	
Forward	5’-TTCAGGCGCTATGGGTCATCTGTT-3’
Reverse	5’-AGGCTCCATTAGCACTGTTGAGGT-3’
Cdkn1a cDNA	
Forward	5’-TGTCCAATCCTGGTGATGTCC-3’
Reverse	5’-TCAGACACCAGAGTGCAAGAC-3’
Gapdh cDNA	
Forward	5’-AGCCACATCGCTCAGACACC-3’
Reverse	5’-GTACTCAGCGGCCAGCATCG-3’