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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 group (TrxG) and Polycomb group (PcG) 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 (trimethylated on histone H3 Lys4, H3K4me3) during muscle differentiation through 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 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 melanogaster* have identified PcG and TrxG 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 is important 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 whereas the later marks genes for expression. This is accomplished in part through trimethylation of histone H3 at Lys9 (H3K9me3) and/or Lys27 (H3K27me3) for repressed genes, and through the H3K4me3 modification for active genes[1,3]. Indeed, studies based on chromatin immunoprecipitation (ChIP) coupled to DNA microarray analysis (ChIP-chip experiments) have suggested that developmentally regulated genes that are actively expressed are enriched for the H3K4me3 epigenetic mark in the 5′ ends of their coding sequences[4]. The H3K27me3 and H3K9me3 modifications are enriched in facultative and constitutive heterochromatin, respectively[5]. Repression of transcription by PcG proteins is thought to be the default state, as these proteins can be found at both active and inactive genes, whereas TrxG proteins seem 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 protein (Ash1) are mutated, result in ectopic expression of the developmentally regulated *Hox* genes[8]. This suggests that TrxG proteins act as anti-repressors to ensure that PcG proteins do not repress expression of developmentally important genes in specific tissues.

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 co-factors feeding into the myogenic program is the p38 mitogen-activated protein kinase (MAPK)[11], which is crucial in establishing the muscle-specific gene expression program[10–13].

Although several important factors responsible for establishing muscle-specific gene expression have been identified, the mechanism by which transcription is activated at muscle-specific loci has remained elusive. Some insight has come from studies demonstrating that several muscle-specific genes are epigenetically marked for repression (by H3K27me3 or H3K9me3) in growing myoblasts[14,15]. In the case of H3K27me3-marked genes, it has been shown that the transcriptional regulator YY1 targets the PcG protein Ezh2 to promoters[14].

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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. We set out to determine whether muscle-specific genes are epigenetically marked for gene expression in differentiating mouse C2C12 myoblasts, and if so, how TrxG proteins are targeted to these promoters.

RESULTS

Specific genes are marked for expression during myogenesis

It has been proposed that genes poised for transcription are marked by H3K4me2, whereas those actively transcribing are marked by H3K4me3 (refs. 4,16). Here we set out to examine whether genes that are expressed during myogenesis are marked by H3K4me3 during the transactivation process. The mouse C2C12 myoblast cell line was used as a model for these studies, as 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 H3K4me3-specific antibody. Using hydrolysis probes for quantitative PCR (qPCR) that recognized the 5′ ends of the genes, we found that both the *Myog* and *Ckm* genes became enriched in nucleosomes containing H3K4me3 during C2C12 cell differentiation (**Fig. 1a**). This enrichment coincided temporally with the increased expression of these two genes during myogenesis (**Fig. 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 in the transcribed *Acta1* gene (**Fig. 1a**,**b**). Similarly, no enrichment of H3K4me3 was observed at the *Cdkn1a* gene, which is transcribed before differentiation and whose expression is further upregulated during myogenesis, nor was there enrichment at the transcriptionally silent *Igh* locus (**Fig. 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 mark, whereas the H3K4me3 mark peaks within the transcribed region of the gene (**Supplementary Fig. 1** online).

Ash2L associates with 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 Fig. 2a** online), 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 promoter, DNA binding elements responsible for establishing cellular memory should lie in a fragment of DNA containing the region between positions –184 and +18 relative to the *Myog* transcription start site, as this region is sufficient to ensure muscle-specific expression of the gene[22,23]. As *Myog* has a well-characterized promoter[22–26], we looked for interactions between Ash2L and transcriptional activators known to bind this region. Western blotting analysis of proteins co-immunoprecipitating with Ash2L indicated the presence of Mef2 but not the muscle-specific transcriptional activators MyoD or myogenin (**Fig. 2a**). In a reciprocal experiment, immunoprecipitation of Mef2 with a pan-Mef2 antibody confirmed the interaction between the transcriptional activator and the Ash2L complex subunits Ash2L, RbBP5 and menin, along with the methyltransferase MLL2 (**Fig. 2b**). Furthermore, methyltransferase assays performed with Mef2 immunoprecipitates confirmed that the transcriptional activator associates with an H3K4 methyltransferase activity in differentiating C2C12 cell extracts (**Supplementary Fig. 2c**,**d**). Notably, 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 five known methyltransferase complexes[27], we examined whether the interaction between Ash2L and Mef2 is limited to MLL2-containing complexes. Using antibodies directed against various H3K4 methyltransferases, we were able to immunoprecipitate Mef2 with antibodies to MLL2, Set1 and MLL3, but not MLL or MLL4 (**Supplementary Fig. 2b**). Thus, Mef2 can interact with several Ash2L complexes, but it preferentially interacts with MLL2-containing complexes.

To determine whether Mef2 recruits the Ash2L complex to mediate H3K4 trimethylation on muscle-specific genes, we used ChIP analysis to measure the binding of these two factors to the *Myog* and *Ckm* promoters during C2C12 differentiation. Consistent with an association between Mef2 and the Ash2L complex, we observed a good correlation of their recruitment to the *Ckm* promoter (**Fig. 2c**). In contrast, the recruitment of Ash2L did not coincide temporally

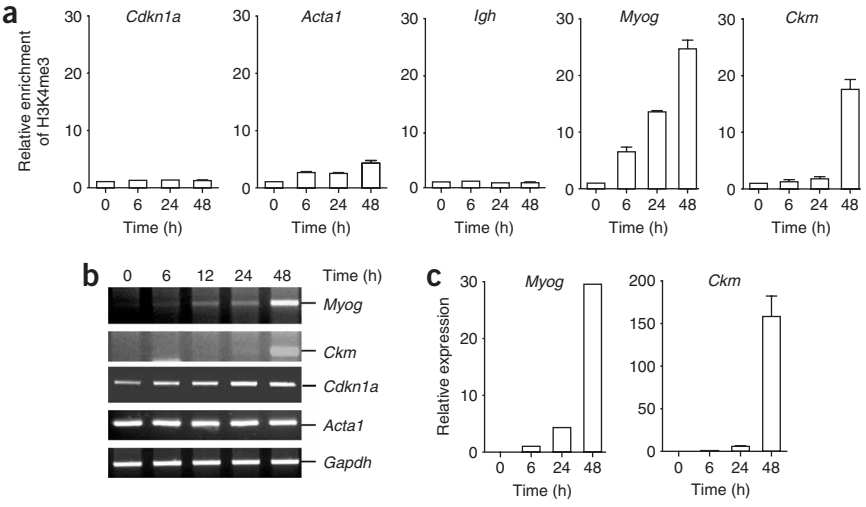

Figure 1 Muscle-specific genes are trimethylated at H3K4 during myogenesis. (**a**) Growing (0 h) or differentiating (6, 24 or 48 h) C2C12 cells were subjected to native ChIP analysis with antibodies to H3K4me3 or control rabbit IgG. Immunopurified nucleosomes were then deproteinated and subjected to qPCR analysis with primers recognizing the genes indicated. Average values of duplicate qPCR reactions are shown; error bars represent s.d. Each experiment was performed at least twice with independent chromatin samples and yielded similar results both times. (**b**) RNA was extracted from growing or differentiating C2C12 cells, reverse-transcribed and subjected to semiquantitative PCR analysis of gene expression with primers specific for the transcripts indicated. (**c**) cDNA was prepared as in **b** and subjected to qPCR analysis of gene expression with primers specific for either the *Myog* or *Ckm* transcripts and with primers specific for 18S RNA. Expression is reported relative to the control 18S RNA signal. Average values of triplicate qRT-PCR reactions are shown; error bars represent s.d. Each experiment was performed at least twice with independently isolated RNA.

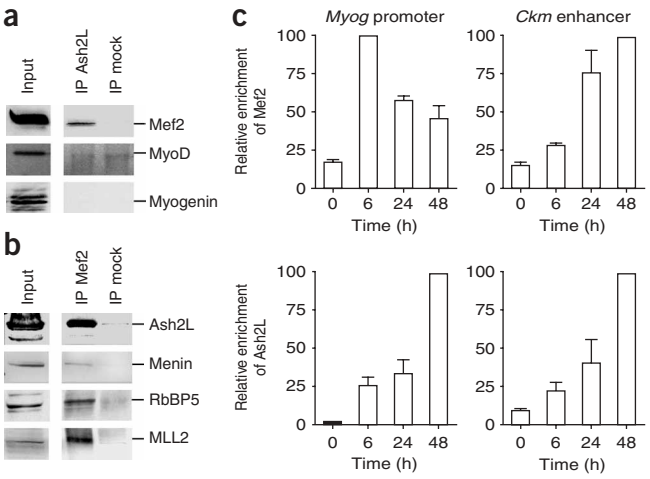

Figure 2 Mef2 interacts with the Ash2L methyltransferase complex. (**a**) Mef2 co-immunoprecipitates with Ash2L in C2C12 cells. Nuclear extracts prepared from differentiating C2C12 cells (48 h) were subjected to immunoprecipitation (IP) with anti-Ash2L or control IgG (mock). Immunoprecipitated proteins were analyzed by western blotting with indicated antibodies. (**b**) Cell extracts prepared from differentiating C2C12 cells (48 h) were subjected to immunoprecipitation with anti-Mef2 or control IgG antibody. Immunoprecipitated proteins were analyzed by western blotting with indicated antibodies. (**c**) Relative recruitment of Mef2 or Ash2L was measured by ChIP at the *Myog* promoter or *Ckm* enhancer at indicated time points during differentiation. After deproteination, immunopurified DNA was quantified by qRT-PCR with hydrolysis probes. Average values of duplicate qRT-PCR reactions are shown; error bars represent s.d. Each experiment was performed at least twice with independent chromatin samples and yielded similar results both times.

with the recruitment of Mef2 at the *Myog* gene. Instead, Mef2 was recruited to the *Myog* promoter within 6 h after serum withdrawal, whereas recruitment of Ash2L, marking of nucleosomes by H3K4me3 and gene activation were maximal at later stages (48 h) in the differentiation process (**Fig. 1** and **Fig. 2c**). Thus, recruitment of Ash2L is temporally distinct from that of Mef2 at the *Myog* promoter.

Phosphorylation of Mef2d permits 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 whose expression or activity changes during differentiation. To test this possibility, we studied interactions between Ash2L and the three Mef2 isotypes that are known to be expressed in muscle. Baculoviruses encoding C-terminally 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] before incubation with an extract prepared from differentiating (48 h) C2C12 cells. Although Ash2L did not bind FL-Mef2a, we consistently observed an interaction with FL-Mef2d (**Fig. 3a**). Notably, this interaction was enhanced when FL-Mef2d was phosphorylated by p38α. A weaker interaction between p38α-treated FL-Mef2c and Ash2L could also be seen. Thus, Ash2L seems to associate selectively with the phosphorylated Mef2d isotype (and, to a lesser extent, with phosphorylated Mef2c). It is not clear why Mef2a does not also interact with Ash2L. However, it is not a case of differential β-exon usage, as 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 probably 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 (**Fig. 3b**) and completely blocked its interaction with Ash2L (**Fig. 3c**). Furthermore, this protein behaved as a dominant-negative mutant, leading to decreased expression of the endogenous *Myog* gene in transfected C2C12 cells (**Supplementary Fig. 3** online). To further confirm that p38-dependent phosphorylation of Mef2d regulates its interaction with Ash2L, we immunoprecipitated Mef2 from extracts of 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 co-immunoprecipitated with Mef2 (**Fig. 3d**). These results 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 is upregulated during C2C12 cell differentiation. However, both semiquantitative reverse-transcription (RT)-PCR and western blotting analysis showed that Mef2d levels remained relatively constant throughout C2C12 differentiation (**Supplementary Fig. 4** online). Therefore, we next examined whether p38 MAPK activity varies during C2C12 differentiation. In accordance with previous reports[12], we found that levels of total p38 remained constant throughout differentiation. In contrast, there was markedly more active (phosphorylated) p38 during differentiation, with the maximal

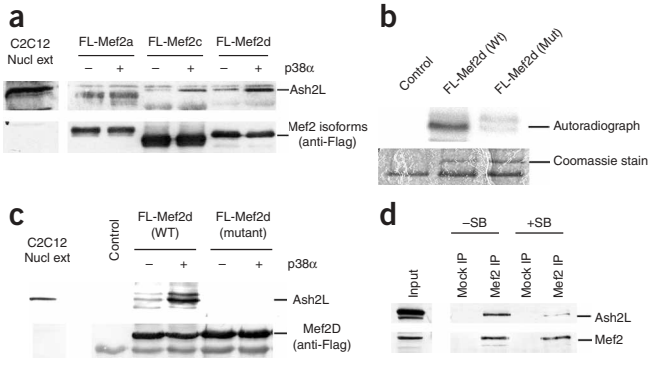

Figure 3 Ash2L interacts preferentially with phosphorylated Mef2d and Mef2c. (**a**) Flag-tagged Mef2a, Mef2c or Mef2d treated with activated p38α (or untreated) were incubated with extract prepared from differentiating (48 h) C2C12 cells. After washing, Mef2-associated proteins were subjected to western blotting analysis with anti-Ash2L or anti-Flag. (**b**) Flag-tagged wild-type (WT) or mutant Mef2d were incubated with activated p38α and [^{32}P]ATP. Reactions were separated by SDS-PAGE. (**c**) Interaction studies with Flag-tagged WT and mutant Mef2d as 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 with anti-Mef2 or control IgG. Immunoprecipitated proteins were analyzed by western blotting with indicated antibodies.

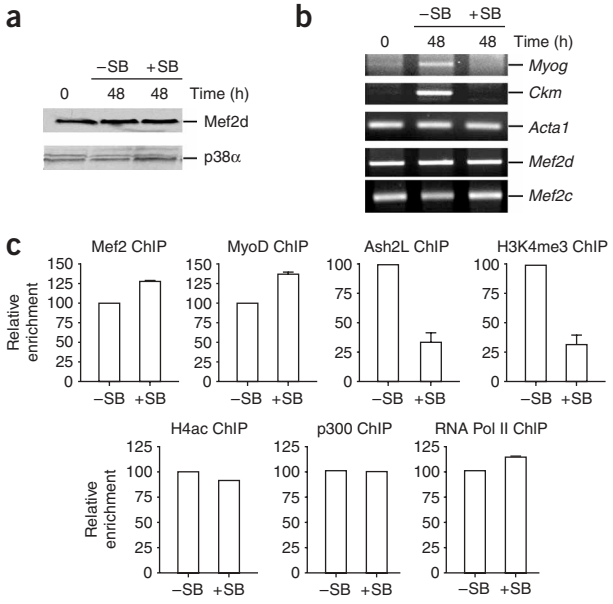

Figure 4 Inhibition of p38 MAPK activity prevents recruitment of Ash2L to the *Myog* promoter. (**a**) Protein extracts prepared from C2C12 cells treated with SB203580 (+SB) or untreated (–SB) under either growth (0 h) or differentiation (48 h) conditions were analyzed by western blotting with indicated antibodies. (**b**) Inhibition of p38 kinase activity blocks transcription of several muscle-specific genes in C2C12 cells. RNA was isolated from +SB or –SB C2C12 cells under either growth or differentiation conditions. After reverse transcription, random primed cDNA was subjected to semiquantitative PCR analysis with primers specific for the genes indicated. (**c**) Inhibition of p38 kinase activity blocks recruitment of Ash2L and prevents H3K4me3 modification at the *Myog* promoter. ChIP was used to measure relative enrichment of the indicated proteins at the *Myog* promoter in C2C12 cells differentiated (48 h) in the presence or absence of SB203580. After deproteination, immunopurified DNA was quantified by qRT-PCR with hydrolysis probes. Average values of duplicate qRT-PCR reactions are shown; error bars represent s.d. Each experiment was performed at least twice with independent chromatin samples and yielded similar results both times.

amount attained 48 h after serum withdrawal (**Supplementary Fig. 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 with C2C12 cells that had been differentiated in the presence of SB203580. Treatment of C2C12 cells with SB203580 did not affect total p38 levels in the cell (**Fig. 4a**). As previously observed[31], expression of both *Ckm* and *Myog* in differentiating C2C12 was inhibited by SB203580 treatment (**Fig. 4b**). This is not a global effect on transcription, as expression of *Acta1*, *Mef2c* and *Mef2d* was 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 RNA polymerase II (Pol II) to these loci (**Fig. 4c** and **Supplementary Fig. 5** online). Similarly, acetylation of histone H4 (H4ac) was not affected by SB203580 treatment, suggesting that the promoters are poised for transcription. Treatment of differentiating C2C12 cells with SB203580 did, however, substantially decrease Ash2L recruitment and H3K4me3 at both of these muscle-specific promoters (**Fig. 4c** and **Supplementary Fig. 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 an Mef2-dependent mechanism, we used short interfering RNA (siRNA)-mediated knockdown of Mef2d and Mef2c in C2C12 cells. Whereas the knockdowns did not appreciably affect *Acta1* expression, both *Myog* and *Ckm* genes were markedly downregulated

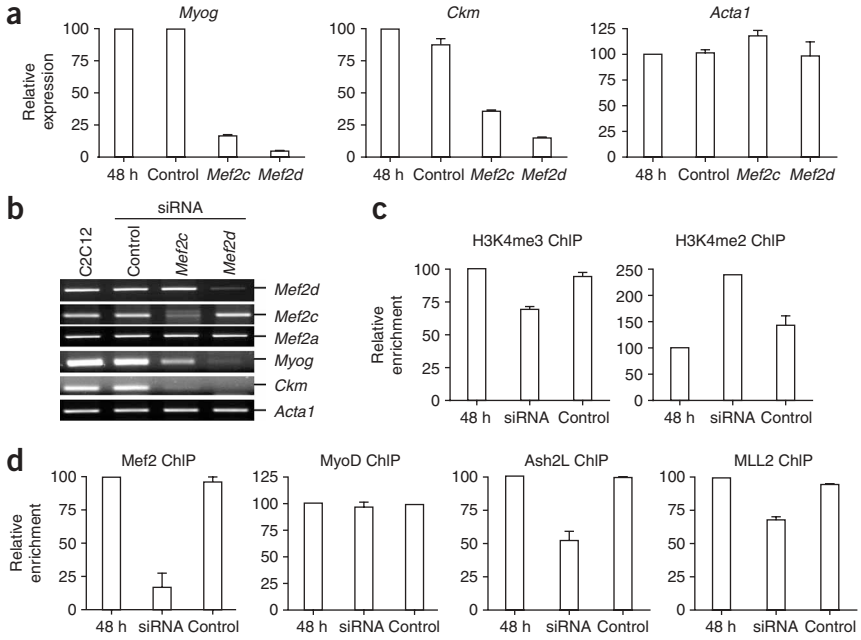

Figure 5 Knockdown of Mef2d and Mef2c in C2C12 cells decreases Ash2L at the *Myog* and *Ckm* promoters. (**a**) RNA was isolated from differentiating C2C12 cells (48 h) that were untransfected or transfected with siRNA targeting *Mef2d* or *Mef2c* or with untargeted control siRNA. RNA was reverse-transcribed and subjected to qPCR analysis of gene expression with primers specific for *Myog*, *Ckm* or *Acta1* transcripts along with primers specific for 18S RNA. Expression is reported relative to the control 18S RNA signal. Average values of triplicate qRT-PCR reactions are shown; error bars represent s.d. Each experiment was performed at least twice with independently isolated RNA. (**b**) cDNA was prepared as in **a** and analyzed by semiquantitative PCR with primers specific to the genes indicated. (**c**,**d**) C2C12 cells were transfected with siRNA targeting both *Mef2c* and *Mef2d* (such that both family members would be knocked down) or with an untargeted control siRNA. Cells were then differentiated for 48 h and analyzed by ChIP to measure enrichment of H3K4 methylation at the *Myog* promoter (**c**) or relative recruitment of transcriptional activators and coactivators (**d**). Immunopurified DNA was quantified by qRT-PCR with hydrolysis probes. Average values of duplicate qRT-PCR reactions are shown; error bars represent s.d. Each experiment was performed at least twice with independent chromatin samples and yielded similar results both times.

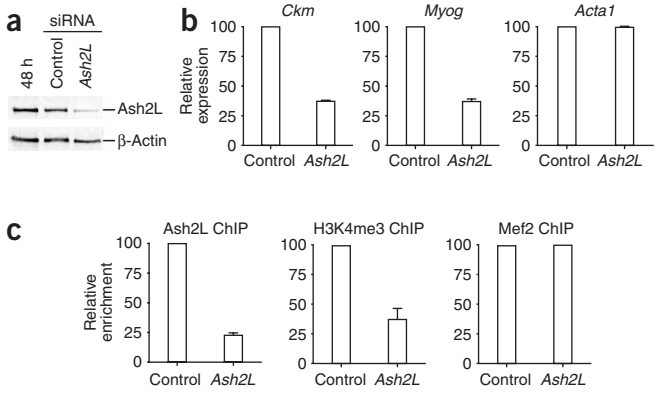

Figure 6 Knockdown of Ash2L in C2C12 cells leads to reduced transcription of the *Myog* and *Ckm* genes. (**a**) Whole-cell extracts prepared from differentiating C2C12 cells after indicated siRNA treatments were subjected to western blotting analysis with anti-Ash2L or anti–β-actin. (**b**) Knockdown of Ash2L leads to decreased expression of muscle-specific genes. RNA was isolated from differentiating C2C12 cells (48 h) transfected with siRNA targeting *Ash2L* or with untargeted control siRNA. RNA was extracted, reverse-transcribed and subjected to qPCR analysis with primers recognizing the genes indicated. Expression is reported relative to the control 18S RNA signal. Average values of triplicate qRT-PCR reactions are shown; error bars represent s.d. Each experiment was performed at least twice with independently isolated RNA. (**c**) C2C12 cells were transfected with siRNA targeting *Ash2L* or with 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 quantified by qRT-PCR with hydrolysis probes. Average values of duplicate qPCR reactions are shown; error bars represent s.d. Each experiment was performed at least twice with independent chromatin samples and yielded similar results both times.

in response to knockdown of Mef2d or Mef2c (**Fig. 5a**,**b**). Because both Mef2c and Mef2d are required for maximal transactivation of the *Ckm* and *Myog* genes (**Fig. 5b**), we decided to knock down both Mef2 isotypes in the same population of C2C12 cells for ChIP studies (**Fig. 5c**,**d**). Analysis of promoter occupancy at both the *Ckm* and *Myog* genes revealed that the siRNA treatment reduced recruitment of Mef2 during differentiation by 80%, whereas MyoD levels remained constant (**Fig. 5d** and **Supplementary Fig. 6** online). Loss of Mef2 binding at these promoters coincided with decreased H3K4me3 and a concomitant increase in H3K4 dimethylation (**Fig. 5c** and **Supplementary Fig. 6**). Similarly, knockdown of Mef2d markedly decreased recruitment of both Ash2L and MLL2 to the *Myog* and *Ckm* promoters (**Fig. 5d** and **Supplementary Fig. 6**). Our observation that Ash2L binding was reduced by only 50% when Mef2 binding was reduced by 80% is probably explained by the ability of the TrxG complex to interact with multiple elements in the promoter region, including CBP/p300 (ref. 32), unmethylated CpG dinucleotides[33] and H3K4me2 (ref. 34). Thus, although phosphorylated Mef2d is probably 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 knockdown of Ash2L in C2C12 cells. Indeed, reduced levels of Ash2L in C2C12 cells (**Fig. 6a**) led to decreased expression of both the *Myog* and *Ckm* genes while having no effect on *Acta1* RNA levels during muscle differentiation (**Fig. 6b**). This decreased expression can be correlated with reduced recruitment of Ash2L and less H3K4me3 at the *Myog* gene (**Fig. 6c**) and *Ckm* gene (data not shown). Thus, our studies reveal that establishment of the H3K4me3 mark at the *Myog* and *Ckm* promoters during muscle differentiation occurs through 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 became upregulated during later stages of differentiation. Notably, Ash2L showed different affinities for the various members of the Mef2 family of transcriptional activators. Combined with our observation that continued expression of Mef2a in Mef2d- and Mef2c-knockdown cells did not allow expression of the *Myog* and *Ckm* genes, these results demonstrate that the Mef2 isotypes have different functions 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 studies have demonstrated that factors involved in transcriptional regulation in muscle are targets for phosphorylation by p38, including Mef2a and Mef2c[28] as well as 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

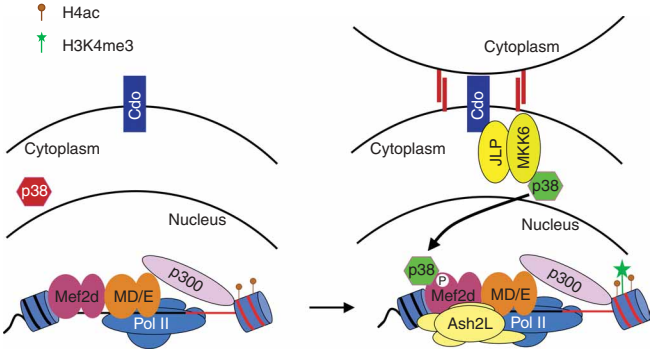

Figure 7 Model for the integration of the p38 MAPK signaling pathway with Mef2-dependent recruitment of the Ash2L complex to muscle-specific promoters. Before activation of p38, MyoD and E47 (MD/E) cooperate with Mef2d dimers (homo- or heterodimers) to establish a transcriptionally poised promoter through recruitment of the acetyltransferase p300 and 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 the MKK6 kinase[36]. Once activated, p38 MAPK phosphorylates Mef2d, leading to targeting of the Ash2L methyltransferase complex to muscle-specific promoters. The methyltransferase complex then establishes the epigenetic H3K4me3 mark required for abundant expression of developmentally regulated genes.

kinetics of transcription during myogenesis such that ordinarily late-expressing genes are expressed during the early stages of differentiation[11]. Although these observations have suggested that p38-dependent phosphorylation of Mef2 proteins might be involved in establishing muscle-specific patterns of gene expression, the function of phosphorylation of the Mef2 activation domain has 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 insight into the regulation of muscle-specific transcription by MAPK signaling. On the basis of the results obtained in this study, we propose a model for Mef2d-dependent activation of muscle-specific genes during myogenesis (**Fig. 7**). During differentiation, MyoD and Mef2 bind 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 Pol II and the acetyltransferase p300 at the *Myog* and *Ckm* promoters in the absence of p38 activity. This results in a promoter that contains H4ac. Once the muscle-specific genes are poised for transcription, their expression is 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 phosphorylate Mef2d, thereby permitting the recruitment of the Ash2L complex to muscle-specific promoters, which ultimately would lead to trimethylation of H3K4. Combined with the p38-dependent remodeling of nucleosomes in the promoter region by the ATP-dependent chromatin-remodeling complex SWI/SNF[31], the H3K4me3 modification would permit abundant expression of developmentally regulated genes. Notably, our siRNA experiments demonstrate that a decrease in H3K4me3 is matched by an increase in H3K4me2 (**Fig. 5c** and **Supplementary Fig. 6**), suggesting that these two modifications exist in a dynamic equilibrium that may allow 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 SWI/SNF to the *Myog* and *Ckm* promoters[31]. This is particularly noteworthy because the catalytic subunits of SWI/SNF (BRM/BRG1) are in the same TrxG family of proteins as Ash2L[1,2]. Furthermore, it has been shown that Mef2d cooperates with myogenin to recruit SWI/SNF to the promoter region of the *Ckm* gene[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 (ref. 31). Whereas SWI/SNF seems to be recruited to the *Myog* and *Ckm* promoters through different mechanisms, we observed Mef2d- and p38-dependent recruitment of Ash2L to both *Myog* and *Ckm*. Thus, there seems to be some difference in the mechanism by which Ash2L and SWI/SNF are targeted to developmentally regulated genes. However, p38 regulates the recruitment of both these TrxG complexes to specific genes (**Fig. 4c** and ref. 31). 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 show here 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 find that this recruitment is regulated through the p38 MAPK–mediated phosphorylation of Mef2d, which enhances the association between the transactivator and the methyltransferase complex. Thus, our study mechanistically links the p38 MAPK signaling pathway with the activation of muscle-specific genes during myogenesis.

METHODS

Antibodies. We used antibodies specific to H3K4me3 (Abcam ab8580), H3K4me2 (Upstate 07-030), H4ac (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), p38 phosphorylated on Thr180 and Tyr182 (Cell Signaling 9211S), myogenin (Developmental Studies Hybridoma Bank F5D) and Flag (Sigma F-3165). Antibodies to MLL3 and MLL4/ALR have 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 (ref. 17) was maintained at less than 80% confluency in DMEM containing 10% (v/v) FBS, L-glutamine, penicillin and streptomycin. For differentiation studies, cells were washed with PBS when they attained 80% confluence and then incubated in differentiation medium (DMEM containing 2% (v/v) horse serum, 10 μg ml^{-1} insulin, 10 μg ml^{-1} transferrin, L-glutamine, penicillin and streptomycin) for 48 h (unless otherwise noted). Control siRNAs or siRNAs targeting *Mef2c*, *Mef2d* or *Ash2l* (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 chromatin immunoprecipitation. Approximately 1×10^7 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 quantified by measuring total ribonucleic acid content. Chromatin (15 μg) was digested with 1 U MNase (Sigma) for 10 min at 37 °C to give a maximum visible fragment size of 550 base pairs (three 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 with 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 legends. Immunoprecipitated DNA was purified and subjected to qPCR analysis with hydrolysis probes (see **Supplementary Table 1** online for primer and probe sequences). To calculate relative enrichment, we subtracted the signal observed in the control immunoprecipitation experiment from that observed with the specific antibody, then divided the resulting difference by the signal observed from one-fiftieth of the ChIP input material.

Chromatin immunoprecipitation. ChIP assays were done as described[39], except that DNA was sonicated using a bioruptor to obtain fragments approximately 400 base pairs in length. Immunoprecipitated DNA was reverse–cross-linked, purified and subjected to qPCR analysis with hydrolysis probes (see **Supplementary Table 1** for primer and probe sequences). Relative enrichment was calculated as described for native ChIP studies.

Protein expression. N-terminally 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 with anti-Flag M2-agarose beads (Sigma). The phosphorylation-deficient mutant of Mef2d was generated by mutation of both Thr308 and Thr315 to alanine using the QuikChange system (Stratagene). Activated p38α was purified from a BL-21 strain stably coexpressing His-p38α, MEK4 and MEKK-C[29] with nickel–nitrilotriacetic acid (Ni-NTA) resin (Qiagen).

Immunoprecipitation and Flag pull-down assays. Cell extracts were prepared in lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.1% (w/v) SDS, 0.5% (v/v) sodium deoxycholate and Complete protease inhibitors (GE Healthcare)) from differentiating C2C12 cells at the 48 h time

point. Either protein A or protein G Dynabeads were coated with antibodies as described[39]. Approximately 400 μg of protein lysate were subjected to immunoprecipitation with various antibodies (as indicated in figure legends). Beads were then washed three times with buffer containing 300 mM KCl and 0.1% (v/v) Nonidet P-40, and proteins were eluted in SDS loading dye and 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 h. The beads were then washed with EX-100 buffer[40] to remove unbound proteins. For phosphorylation experiments, protein-bound beads were incubated in the presence of 1 μM ATP with or without p38α kinase for 30 min at 30 °C. After washing to remove ATP and p38α, beads were incubated with 48 h–differentiated C2C12 cell extract. Interacting proteins were eluted in SDS loading dye and subjected to western blotting with indicated antibodies.

Reverse-transcription PCR assays. Total RNA (3 μg) was reverse-transcribed with MuMLV reverse transcriptase. The resulting random primed complementary DNA was subjected to semiquantitative RT-PCR with gene-specific primers (see **Supplementary Table 1** for primer sequences) for *Myog*, *Ckm*, *Cdkn1a*, *Acta1*, *Mef2a*, *Mef2c*, *Mef2d* and *Actb* (encoding β-actin). For quantitative RT-PCR (qRT-PCR), duplex reactions were performed using 6-fluorescein phosphoramidite (FAM)-labeled and 3′ black hole quencher-1 (BHQ)-labeled gene-specific primers (see **Supplementary Table 1** for primer sequences) and 5′ yakima yellow (YY)-labeled and 3′ eclipse dark quencher (EDQ)-labeled primers specific for 18S ribosomal RNA (Eurogentec).

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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

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