Epigenetic silencing of myogenic gene program by Myb-binding protein 1a suppresses myogenesis

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Skeletal myogenesis involves highly coordinated steps that integrate developmental cues at the chromatin of muscle progenitors. Here, we identify Myb-binding protein 1a (Mybbp1a) as a novel negative regulator of muscle-specific gene expression and myoblast differentiation. The mode of action of Mybbp1a was linked to promoter regulation as illustrated by its interaction with MyoD at the genomic regions of silent muscle-specific genes as well as its negative effect on MyoD-mediated transcriptional activity. We propose that Mybbp1a exerts its repressive role by inducing a less permissible chromatin structure following recruitment of negative epigenetic modifiers such as HDAC1/2 and Suv39h1. At the onset of differentiation, Mybbp1a undergoes a promoter disengagement that may be due to the differentiation-responsive, miR-546-mediated downregulation of Mybbp1a expression. Moreover, such alteration gave rise to promoter enrichment of activators and histone acetylation, an epigenetic status amenable to gene activation. Together, these findings unveil a hitherto unrecognized transcriptional co-repressor role of Mybbp1a in proliferating muscle progenitor cells, and highlight an epigenetic mechanism by which Mybbp1a and miR-546 interplay to control myoblast differentiation transition.

The EMBO Journal (2012) **31**, 1739–1751. doi:10.1038/emboj.2012.24; Published online 14 February 2012

Subject Categories: chromatin & transcription; differentiation & death

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Received: 17 August 2011; accepted: 23 January 2012; published online: 14 February 2012

Keywords: epigenetics; miR-546; Mybbp1a; MyoD; myogenesis

Introduction

Myb-binding protein 1a (Mybbp1a) was originally identified as an interacting partner of *c-myb* protooncogene product (c-Myb) (Favier and Gonda, 1994; Tavner *et al.*, 1998). It interacts with the negative regulatory domain (NRD) of c-Myb and suppresses its transactivation activity. Mybbp1a has also been shown to bind a number of other transcription factors, including PGC-1 α , RelA/p65, Prep1, Aire, and CRY1, and similarly exert inhibitory effect on their transactivation activity through yet unresolved mechanism (Fan *et al.*, 2004; Diaz *et al.*, 2007; Owen *et al.*, 2007; Oriente *et al.*, 2008; Hara *et al.*, 2009; Abramson *et al.*, 2010). These findings are consistent with a context-dependent co-repressor function of Mybbp1a. In further support of this notion, Mybbp1a was recently identified as a component of several co-repressor and ATP-dependent chromatin remodelling complexes, including Ret-CoR and esBAF complex (Takezawa *et al.*, 2007; Ho *et al.*, 2009). These enzymatic activities are intimately associated with the processes of differentiation and stem cell physiology, and mostly contain common constituents such as HDACs. While the roles of Mybbp1a in these repressor complexes remain unclear, it may likely serve similar epigenetic and cellular functions. Mybbp1a is also known to preferentially interact with dimethylated histone H3K9, a marker of transcriptional repression (Hara *et al.*, 2009). Taken together, these observations strongly implicate Mybbp1a in the epigenetic regulation of gene expression.

Due to the lack of further information on the cellular and transcriptional functions of Mybbp1a, particularly concerning its downstream target genes, we set out to first address this issue by performing microarray-based gene expression profiling on Mybbp1a-knockdown C2C12 myoblast cells. Subsequent analysis revealed an enrichment of differentially expressed genes (DEGs) implicated in muscle differentiation and development process.

Differentiation of skeletal muscle cells, or myogenesis, involves highly coordinated processes that progress from myogenic determination of pluripotent mesodermal precursor, withdrawal from the cell cycle, subsequent expression of myotube-specific genes, and to the formation of multinucleated myotube. At the molecular level, this process entails tight integration of extracellular and intracellular cues at the chromatin of muscle progenitors (Guasconi and Puri, 2009; Perdiguero *et al.*, 2009; Saccone and Puri, 2010). During myogenesis, a group of basic helix-loop-helix (bHLH) family of transcription factors—myogenic differentiation 1 (MyoD), myogenic factor-5 (Myf5), myogenin (MyoG), and myogenic regulatory factor 4 (MRF4), collectively termed the myogenic regulatory factors (MRFs)—has been shown to establish the

myogenic lineage during embryogenesis and regulate the myogenic program (Pownall *et al*, 2002; Tapscott, 2005). Muscle-specific genes that are essential for the differentiation process are transcriptionally silenced in the undifferentiated cells. Such repressed status is maintained by a multicomponent epigenetic system that encompasses a few key players, such as HDAC1, HDAC2, Ezh2, HP1, and Suv39h1 (Zhang *et al*, 2002; Mal and Harter, 2003; Caretti *et al*, 2004; Guasconi and Puri, 2009). Consequently, Suv39h1-mediated methylation of H3 lysine 9 and Polycomb-mediated trimethylation of H3 lysine 27 are critical epigenetic modifications that restrict the temporal expression of muscle genes in myoblasts. At the onset of differentiation, the epigenetic repressors disengage from the promoter, thereby allowing productive interactions with positive co-activators such as PCAF, p300, and SWI/SNF. These complexes then create a permissive chromatin environment for RNA Pol II binding and function.

Results from our present work are consistent with the scenario that Mybbp1a is an integral constituent of the myogenesis-associated epigenetic regulation. Mybbp1a acts as a suppressor of muscle differentiation by interacting with MyoD and inhibiting associated transcriptional activation of downstream genes. We demonstrated that its function in this regard is mediated through associating with HDAC1/2 and further stabilizing the co-repressor complex to the regulatory region of muscle-specific genes, thereby maintaining the local histone hypermethylation and gene silencing. In response to differentiation cues, expression of Mybbp1a was downregulated by both miR-546 and reduced transcription, leading to its disengagement from the promoters. Such downregulation destabilized promoter association of co-repressor complexes and was in turn accompanied by binding of co-activators, enrichment of histone H3 acetylation, and subsequent gene activation. These findings collectively establish a regulatory circuitry comprising Mybbp1a and miR-546 that contributes to scheduled gene program transitions during myogenesis.

Results

Identification of putative Mybbp1a target genes by global gene expression profiling

To characterize the cellular role of Mybbp1a, we first established clones of myoblast C2C12 cells stably expressing Mybbp1a-targeting shRNA (Figure 1A). Microarray analysis of RNA levels revealed that, under growth condition (GM or growth medium), about 500 genes were upregulated ($P < 0.05$, ≥ 2 -fold change; Supplementary Table S1) and 70 genes downregulated ($P < 0.05$, ≤ 0.5 -fold change; Supplementary Table S2) significantly in these cells relative to the control. Given its putative repressor role, these upregulated genes are potential targets of Mybbp1a-mediated repression. By analysing the functional and cellular attributes of the upregulated genes with the bioinformatics tool MetaCore (GeneGo), we found an enrichment of genes implicated in skeletal muscle development and contraction processes (Figure 1B).

In parallel, we sought to determine whether the differentially expressed gene set in Mybbp1a-knockdown cells was enriched in genes normally regulated during myogenic differentiation. Towards this end, we used the previously published gene expression profiles of wild-type C2C12 cells undergoing differentiation (growth condition versus 6 days

post differentiation) (Chen *et al*, 2006a). We first found that, of the genes upregulated (≥ 2 -fold) in the course of differentiation, there was a considerable overlap (153 genes) with the upregulated genes (≥ 1.5 -fold change, $P < 0.05$; $n = 995$) in our data set (Supplementary Table S3). We then performed gene set enrichment analysis (GSEA; Subramanian *et al*, 2005) to explore correlation in a more statistically significant manner (Materials and methods). Such interrogation subsequently revealed highly significant enrichments (GSEA $P < 0.001$), in which genes that were altered in the Mybbp1a-knockdown experiment corresponded to genes that were differentially regulated upon myogenic differentiation (Figure 1C; Supplementary Table S4). Moreover, overlapping genes were enriched in processes such as cell-cycle regulation (downregulation) and muscle differentiation and development (upregulation) (Figure 1C and D; Supplementary Table S5). Taken together, these observations strongly imply that Mybbp1a may be involved in controlling the expression of muscle differentiation-associated genes. Moreover, based on the gene profiles (Figure 1C), Mybbp1a-depleted myoblasts may enter the phase of early differentiation, even in the presence of mitogens. Interestingly, a recent report of large-scale whole-mount *in-situ* hybridization studies also pinpointed Mybbp1a expression temporally (E9.5–E11.5) and spatially (limb bud) to early embryonic myogenesis (Yokoyama *et al*, 2009).

Mybbp1a represses muscle genes expression and contrasts myogenic differentiation

To begin elucidating Mybbp1a function in skeletal muscle, we initially assessed the effect of Mybbp1a knockdown on muscle differentiation by examining multinucleated myotubes formation and accumulation of skeletal muscle-specific marker proteins. In the knockdown culture undergoing differentiation, we observed more extensive myotube formation, a morphology that is consistent with a greater extent of differentiation (Figure 2A). Moreover, in the absence of Mybbp1a, there was a further upregulation of myogenic markers MyoG and MHC during differentiation, as revealed by our western blot and immunofluorescence analyses (Figure 2B and C). Quantitative determination of the extent of MyoG and MHC expression (Figure 2C) are respectively shown in Figure 2D and E. In line with these observations, and consistent with our microarray data, some of the muscle-specific genes were upregulated in the knockdown cells even when cells were in the growth condition (GM), and underwent further augmentation when differentiation was induced (differentiation medium (DM)) (Figure 2F). Conversely, overexpression of Mybbp1a had a negative effect on most of these muscle-specific genes (Figure 2G). As controls, two genes that did not exhibit expression changes in the microarray analysis, *Amy1* and *Morf4l2*, were not altered by the knockdown or overexpression of Mybbp1a (Supplementary Figure S1). Similar phenotypes on muscle differentiation were also observed when we performed transient RNAi experiments with synthetic siRNAs targeting different regions of the *Mybbp1a* transcript (Supplementary Figure S2). Therefore, these results together imply that Mybbp1a is a negative regulator of muscle gene expression and may be important for maintaining the undifferentiated state of myoblast.

Figure 1 Identification of Mybbp1a target genes in proliferating C2C12 cells by microarray analysis. **(A)** Expression of Mybbp1a in the mixed clones of C2C12 cell line that stably harbour control and Mybbp1a-targeting shRNA. **(B)** Preponderance of differentially expressed genes in distinct cellular processes/networks, as revealed by bioinformatics analysis using MetaCore (P -value < 0.05 and False Discovery Rate < 0.05). **(C)** GSEA comparing the gene expression profile on Mybbp1a depletion (in growth medium) and those upregulated (left, GSE4694_UP) or downregulated (right, GSE4694_DOWN) during 6-day myogenic differentiation (GSE4694). The curves correspond to the observed enrichment score profiles in the Mybbp1a gene set to the GSE4694 gene sets. The bar-code plot below enrichment score plot denote the rank of each member in the Mybbp1a-knockdown gene list; the schematic colour bar represents extent of correlation, with positive in red and negative in blue. The genes in yellow shade are the components of leading edges (see Supplementary Table S4 for full lists). **(D)** Expression level of genes co-regulated by myogenic differentiation and Mybbp1a knockdown (leading-edge genes shown in C), as represented by the heat map. The expression values were displayed in shades of red or blue (Log2 scale) relative to the means of all corresponding values within individual experimental groups. Clusters of genes, indicated by bar codes of different colours, are denoted by their experimental types/conditions. Hierarchical clustering was performed on the samples by Euclidian distance and average linkage method.

Mybbp1a interacts with MyoD at the chromatin regulatory region of transcriptionally inactive muscle genes and inhibits MyoD-mediated transactivation

We further characterized the functional involvement of Mybbp1a in transcriptional regulation by examining the candidate target genes. Interestingly, results from the literature search (Ishibashi *et al*, 2005) indicated a clear enrichment of these preferentially regulated genes in the MyoD regulatory network (Supplementary Table S6), thus potentially linking Mybbp1a to this MRF. Next, to elucidate how Mybbp1a could be functionally associated with MyoD at the transcription level, we performed promoter reporter assay. To this end, we used several muscle (MyoD)-specific reporter constructs, including *MyoG*-Luc, *MCK*-Luc, and 4RE-tk-Luc (pure MyoD binding reporter construct), all of which were responsive to MyoD expression as shown by the dramatic upregulation (Figure 3A–C). In contrast, overexpression of Mybbp1a markedly repressed the MyoD responsive promoter activation (Figure 3A–C). The degree of repression in some

cases was even more pronounced than that exerted by Ezh2, which has been regarded as a myogenesis repressor (Carette *et al*, 2004). Additionally, as a more direct means to probe its possible promoter regulation, we performed chromatin immunoprecipitation (ChIP) assay and verified Mybbp1a's physical occupancy of the promoter regions of MyoD-regulated muscle genes (Figure 3D). As a control, sequence of the *GAPDH* promoter was not significantly enriched in the immunoprecipitates (Figure 3D). Further, consistent with Mybbp1a's repressor function, there was a differentiation-responsive decrease in its promoter binding (Figure 3D), which may likely be attributed to its downregulated expression in this context (Figure 2B, compare lanes 1–3).

To interrogate whether a physical association with MyoD underlies Mybbp1a's negative role on MyoD transcriptional activity, we next performed *in-vitro* glutathione S-transferase (GST) pull-down assay using GST-MyoD fusion protein and Myc-Mybbp1a proteins synthesized either by the *in-vitro* transcription/translation system or by ectopic expression in

Figure 2 Mybbp1a suppresses muscle gene expression and myogenic differentiation. (A) Representative Mybbp1a-knockdown (si-Mybbp1a) and control (ctrl) C2C12 lines were cultured in differentiation medium (DM) for 0, 24, or 48 h, and myotube formation was examined by phase contrast microscopy. Bar, 80 μ m. (B) Control (ctrl) and Mybbp1a-knockdown C2C12 lines were cultured in DM for 0, 24, or 48 h prior to western blot analysis for Mybbp1a, Ezh2, MyoG, and MHC expression. GAPDH serves as the internal control. (C) Morphological characterization of myotube formation in response to Mybbp1a knockdown. Myotubes (DM 48 h) were visualized by indirect immunofluorescence with antibody against MHC (top, red) or MyoG (bottom, red) and nuclear counterstaining with DAPI (blue). Bar, 40 μ m for MHC and 80 μ m for MyoG. (D, E) Quantitative analysis of the indicated myotube morphological attributes based on the immunofluorescence assays ($n = 500$). * $P < 0.05$. (F) Expression analysis of putative Mybbp1a target genes in GM (growth medium) versus DM (48 h) cell culture. RNA was isolated from these cells and subjected to real-time RT-PCR with primers specific to the indicated genes. The bar graph shows normalized values with the control knockdown-GM cells represented as 1. Note the log scale of y axis. (G) Effect of Mybbp1a overexpression in C2C12 cells on muscle genes, as analysed by changes in target mRNA level in DM (48 h) culture. Expression analysis was done as in (F).

C2C12 cells (Figure 3E). Mybbp1a from both sources was efficiently and specifically pulled down by GST-MyoD but not by the GST protein (Figure 3E, right). Co-immunoprecipitation experiment was also carried out to assess the Mybbp1a-MyoD interaction in the cells. To this end, Mybbp1a was found in the immunocomplexes pulled down by the anti-MyoD antibody under the growth (GM) but not the differentiation (DM) condition (Figure 3F), further supporting their interaction in the proliferating myoblasts. Although MyoD is generally considered as a transcription activator, its presence on the muscle-specific silenced gene promoters prior to skeletal myogenesis has been reported previously (Mal and Harter, 2003). The functional connection between Mybbp1a and MyoD in this context was further corroborated by the sequential ChIP assay, which demonstrated a specific and concurrent association of these two proteins with the same chromatin regions only under growth

condition (Figure 3G). Collectively, these data strengthen the negative regulatory role of Mybbp1a on muscle-specific genes in the myoblasts, a function likely mediated through antagonizing the transcriptional activity of MyoD.

Histone deacetylase 1/2 contribute to Mybbp1a-mediated transcriptional repression

We sought to further dissect the molecular basis of Mybbp1a's inhibitory role in muscle gene transcription. Despite its binding and negative effect on MyoD transactivation activity, knockdown of Mybbp1a did not alter the promoter chromatin binding of MyoD itself (Figure 3H). Given the requirement of HDACs in maintaining an inactive state of MyoD-responsive promoters (Bailey *et al*, 1999; Mal and Harter, 2003), and the reported association of Mybbp1a with several HDAC1/2-containing co-repressor complexes (Takezawa *et al*, 2007), we next aimed to assess whether

Figure 3 Mybbp1a is present at the chromatin regulatory region of transcriptionally inactive muscle genes and inhibits MyoD-mediated transactivation. (A–C) Promoter reporter assay was conducted on C2C12 myoblast cells in growth medium. Cells were transfected with the *MyoG-Luc* (A), *MCK-Luc* (B) or pure MyoD binding (4RE-TK-Luc) reporter construct, together with expression vectors for MyoD, Ezh2, Mybbp1a, or the indicated combinations. ** $P < 0.01$; *** $P < 0.001$. (D) ChIP analysis of Mybbp1a binding on chromatin isolated from C2C12 under GM (0 h) versus DM (24 and 48 h) culture, using control (IgG), or Mybbp1a antibody. The precipitated DNA fragments were quantitatively analysed by real-time PCR using primers for the *MyoG* promoter, *MCK* enhancer, *MHCIIb* promoter, and *GAPDH* promoter (non-binding control), and normalized to the values of IgG. Statistical significance of the indicated comparisons: * $P < 0.05$; *** $P < 0.001$; n.s., not significant. (E) *In-vitro* pull-down assay was performed using bacterially expressed GST or GST-MyoD as the bait (left). Non-labelled Mybbp1a generated from *in-vitro* transcription/translation reaction ('Mybbp1a: IVT', top right) or lysates of Mybbp1-overexpressing C2C12 ('Mybbp1a: C2C12', bottom right) were subjected to the pull-down assay. (F) Co-immunoprecipitation of Mybbp1a with MyoD. Proliferating (GM) and differentiating (DM 48 h) C2C12 cells were harvested for immunoprecipitation (IP) with antibody against MyoD. Immunoprecipitates and lysate input (Input, 1/60 of IP) were probed for Mybbp1a and MyoD, as indicated. (G) Sequential ChIP assay was performed to assess the co-occupancy of *MyoG* promoter by both MyoD and Mybbp1a in proliferating (GM) and differentiating (DM 48 h) C2C12 cells. Chromatin was first immunoprecipitated by the control IgG and Mybbp1a antibody ('ChIP 1'). A second round of ChIP ('ChIP 2') was carried out using control IgG and MyoD antibody. Levels of precipitated DNA were normalized to the values of IgG. (H) MyoD binding to *MyoG* promoter in the Mybbp1a-knockdown versus control C2C12 cells, in both GM and DM (48 h) conditions, was examined by ChIP. For (G, H), the precipitated DNA fragments were quantified by real-time PCR using primers for the *MyoG* and *GAPDH* promoters. *GAPDH* promoter serves as non-binding control. n.s., not significant.

HDAC1/2 contribute to Mybbp1a-mediated gene silencing in the myoblasts. Towards this end, we first performed a co-immunoprecipitation assay and showed that there was a co-precipitation of HDAC1 and HDAC2 with the exogenous (Myc-Mybbp1a) or endogenous Mybbp1a (Figure 4A). Notably, knockdown of Mybbp1a led to reduced promoter binding of the HDAC enzymes under both GM and DM conditions, implicating Mybbp1a in stabilizing their promoter association (Figure 4B and C).

To further clarify whether HDAC1/2 underlie Mybbp1a-mediated gene repression, we next assessed expression of muscle genes in the context of unbalanced Mybbp1a and HDAC1/2 expression. Although overexpression of Mybbp1a downregulated *MyoG* and *MHC* genes in differentiating C2C12 cells (Figures 2G and 4D), mimicking a more repressed promoter state, simultaneous knockdown of HDAC1/2 by siRNAs effectively abolished such gene suppression (Figure 4E; Supplementary Figure S3A). In contrast, in the absence of Mybbp1a, overexpression of HDAC1/2 did not counteract the upregulation of muscle gene expression (Figure 4E; Supplementary Figure S3B). This is in line with the above observations (Figure 4B and C) that implicated the presence of Mybbp1a in the promoter binding of these repressive enzymes. Considered together, these data suggest that interaction with and recruitment of histone deacetylases may be a key step through which Mybbp1a facilitates the silencing of myogenic promoters.

RNAi depletion of Mybbp1a alters epigenetic status of muscle gene promoter

Silencing of the *MyoD*-responsive genes in the cycling myoblasts is enforced by additional epigenetic repressors (Guasconi and Puri, 2009; Perdiguero *et al*, 2009; Saccone and Puri, 2010). We thus conducted additional ChIP assays to assess Mybbp1a's role in the promoter binding of other known resident co-repressors. The results showed that the *MyoG* promoter association of repressive factors, such as HP1 and Suv39h1, also declined in the Mybbp1a-knockdown cells under both GM and DM conditions (Figure 5A and B), while their protein expression remained unaltered (Supplementary Figure S4). Conversely, knockdown of Mybbp1a augmented the recruitment of activators (such as p300, PCAF, and Brg1) to the *MyoG* promoter upon differentiation induction (Figure 5C–E). Concomitantly with the switch of occupying epigenetic factors, abrogation of Mybbp1a also triggered reciprocal changes in the levels of promoter-associated histone modifications—there was a rise in H3K9Ac, which is an activating mark (Figure 5F), but a loss of H3K9Me2, a repressive mark (Figure 5G). Finally, loss of Mybbp1a did not affect DNMT binding (Figure 5H), or the methylation status of the muscle gene promoters (data not shown), indicating that gene regulatory function of Mybbp1a in muscle differentiation is independent of DNA methylation. Collectively, these observations strongly suggest that Mybbp1a is indispensable for the convergence of epigenetic repressive signals at the myogenic gene promoters and consequently the maintenance of their silenced state in the undifferentiated cells.

miR-546 targets Mybbp1a in differentiating myoblast and enhances muscle differentiation

Based on our data, downregulation of Mybbp1a protein and its disengagement from promoter is a critical step of muscle

Figure 4 Histone deacetylase 1/2 contributes to Mybbp1a-mediated transcriptional repression. (A) Co-immunoprecipitation of HDAC1 and HDAC2 with endogenous Mybbp1a (–) or Myc-Mybbp1a (+) in proliferating C2C12 cells. Immunoprecipitation (IP) was done with antibodies against the indicated proteins. Immunoprecipitates and lysate input (Input, 1/40 of IP) were probed for the specified proteins. (B, C) HDAC1 and HDAC2 binding to the *MyoG* promoter in the Mybbp1a-knockdown versus control C2C12 were examined. Chromatin fragments were prepared from these cells and subjected to ChIP with control (IgG), HDAC1 (B), or HDAC2 (C) antibody. Quantitative determination of the bound DNA, carried out with real-time PCR, is shown by the bar graphs. Data presented are normalized to the IgG values. n.s., not significant; ** $P < 0.01$; *** $P < 0.001$. (D) Control (ctrl) or Mybbp1a-overexpressing (Myc-Mybbp1a) C2C12 myoblasts were transfected with control (si-ctrl) or HDAC1/2-targeting synthetic siRNAs (si-HDAC1/2). After 1 day, cells were cultured in differentiation medium (DM) for additional 48 h and subsequently harvested for mRNA expression analysis of the differentiation marker genes *MyoG* and *MHC*. (E) Control (ctrl) or Mybbp1a-knockdown (si-Mybbp1a) C2C12 myoblasts were transfected with empty (over-ctrl) or both HDAC1- and HDAC2-encoding plasmids (over-HDAC1/2). After transfection, cells in DM (48 h) were harvested for mRNA expression analysis of myogenic markers *MyoG* and *MHC*. Real-time PCR was done to assess the relative RNA levels. Data presented are normalized to the control (ctrl; grey bars) of each transfection group. n.s., not significant; *** $P < 0.001$.

differentiation. To further interrogate the mechanism underlying such control, we turned our attention to miRNA-mediated post-transcriptional regulation owing to its reported link to myogenesis (Ge and Chen, 2011). To this end, sequence analysis of the Mybbp1a 3'UTR using TargetScan revealed the presence of putative target sites for three miRNAs—miR-546, miR-703, and miR-199a-5p (Figure 6A). The expression of miR-199a-5p was previously found to elevate during myogenesis (Juan *et al*, 2009). The relatively unknown miR-546 shares a host gene, *Ctdsp2*, with miR-26a,

Figure 5 RNAi depletion of Mybbp1a alters epigenetic status of muscle gene promoter. Control and knockdown cell lines were cultured in GM or DM (48 h) and subsequently subjected to ChIP for analysing *MyoG* promoter binding of various epigenetic factors/marks (see Materials and methods): HP1 (A), Suv39h1 (B), p300 (C), PCAF (D), Brg1 (a SWI/SNF complex component) (E), H3K9Ac (F), H3K9Me2 (G), and DNMT (H). ChIP was carried out with control (IgG) or the specific antibodies, as denoted. Quantitative determination of the bound DNA, carried out with real-time PCR, is depicted by the bar graphs. Data presented are normalized to IgG values, with the mean $\pm$ s.d. values from at least three experiments also shown (n.s., not significant; * P < 0.05; ** P < 0.01; *** P < 0.001).

another myogenesis-associated miRNA implicated in targeting *Ezh2* (Wong and Tellam, 2008). Interestingly, expression of the *Ctdsp2* transcript underwent a significant increase during myoblast differentiation (Supplementary Figure S5). Taken together, we thus suspected a possible involvement of these miRNAs in the negative regulation of Mybbp1a protein expression. Expression profiling revealed that miR-546 and miR-199a-5p were clearly induced when C2C12 (Figure 6B) and primary mouse satellite (Supplementary Methods; Supplementary Figure S6A–C) cells underwent differentiation. Such accumulation in differentiating cells coincided with initial Mybbp1a protein reduction (Figure 2B, compare lanes 1–3) as well as the upregulation of two known myogenesis-associated miRNAs, miR-26a and miR-26b (Figure 6B). Conversely, levels of the other candidate miRNA, miR-703, remained relatively constant throughout the early differentiation course (Figure 6B).

To further assess the importance of miR-546 in Mybbp1a 3'UTR regulation, we generated Mybbp1a 3'UTR reporter construct (WT) by grafting the 3'UTR to a luciferase reporter. This construct was introduced into C2C12 cells co-expressing ectopic miR-546, miR-199a-5p, or both. Only cells with

ectopic miR-546 suppressed the luciferase activity (Figure 6C), whereas miR-199a-5p had no significant effect on the reporter. Conversely, reporter construct with modified miR-546 presumptive site in the 3'UTR (Mut) lost its responsiveness to overexpressed miR-546 (Figure 6D). In further support to the miR-546-Mybbp1a link, overexpression of miR-546 in proliferating C2C12 cells led to a marked downregulation of Mybbp1a protein (Figure 6E, top) and upregulation of muscle-specific genes (Figure 6F). On the contrary, in the presence of anti-miR-546 oligomers, level of Mybbp1a protein underwent an increase (Figure 6E, bottom) and the anticipated muscle-specific gene expression was repressed (Figure 6G). The physiological significance of miR-546-Mybbp1a network was further corroborated by the effects of altering Mybbp1a (Supplementary Figure S6D) or miR-546 (Supplementary Figure S6E) on primary satellite cells differentiation. Overall, these results support the notion that miR-546 directly targets Mybbp1a's 3'UTR in C2C12 cells with a functional implication in skeletal muscle differentiation.

Furthermore, we noted that the ectopic expression of miR-546 did not have discernable effect on the RNA levels of Mybbp1a (Figure 7A). However, Mybbp1a mRNA expression

Figure 6 miR-546 is upregulated and targets the *Mybbp1a* 3'UTR in differentiating myoblast. (A) Putative miRNA target sites within the *Mybbp1a* 3'UTR (top). The alignment of the indicated *Mybbp1a* 3'UTR/miRNA complements (bottom). Numbers denote sequences relative to the stop codon (0). (B) Relative expression levels of miR-546, miR-199a-5p, miR-26a, miR-26b, and miR-703 in differentiating C2C12 (0, 24, and 48 h post DM culture), as determined by real-time RT-PCR. (C, D) Luciferase reporter construct that contains wild-type 3'UTR sequence of *Mybbp1a* (pGL3-Mybbp1a 3'UTR, or 'WT'; C) or 3'UTR with mutated miR-546 target sites ('Mut'; D) was transfected into C2C12 myoblasts. In addition, synthetic miR-546, miR-199a-5p, or both were co-transfected, as indicated. Luciferase activity was determined 48 h after transfection, with controls being represented as 1. (E) Immunoblots of Mybbp1a in C2C12 myoblasts transfected with plasmids encoding LacZ control or miR-546 (top), and in those transfected with the anti-miR-546 oligomers and corresponding control (bottom). (F) C2C12 myoblasts were ectopically expressed with LacZ control or miR-546 as in (E) and harvested under GM condition for mRNA expression analysis of the differentiation marker genes *MyoG* and *MHC*. (G) C2C12 myoblasts were transfected with control or anti-miR-546 oligomers. Forty-eight hours after transfection, cells were induced to differentiate for 24 h before being harvested for mRNA expression analysis. For (F, G), real-time PCR was done to assess the relative RNA levels. (For statistical analyses shown in this figure: n.s., not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.)

was reduced when cells underwent differentiation (Figure 7B; Supplementary Figure S1A), suggesting that a transcriptional regulation may also be in effect. To resolve this issue, we carried out ChIP assay to examine the extent of RNA Pol II binding to the *Mybbp1a* promoter. Our data demonstrated a decline in the levels of proximal (Figure 7C) and distal (Figure 7D) promoter-bound RNA Pol II coinciding with C2C12 differentiation, contrary to the expectedly enhanced RNA Pol II occupancy of the *MyoG* promoter (Figure 7E). Therefore, these findings collectively imply a combined action of miR-546 and reduced transcription in effectively restricting *Mybbp1a*'s expression and functions during myogenesis. Similar mode of regulation is also evident in the case of *Ezh2*, which is repressed in differentiating myoblasts at both transcriptional and post-transcriptional (miR-214) levels (Juan *et al*, 2009), hinting at a common

mechanism for the regulation of myogenesis-associated transcriptional co-repressors.

Discussion

During cell lineage commitment and differentiation, both co-repressor and co-activator proteins constitute an important regulatory mechanism contributing to the proper and timely expression of lineage-specific genes. Compared with the extensive knowledge of various enzyme-associated regulatory complexes involved in the muscle differentiation program, much less is understood about the mode of their promoter association and disengagement. In this study, we identified a novel myogenesis-associated co-repressor protein, Mybbp1a, whose binding to MyoD and presence on the promoters of muscle-specific genes were implicated in

Figure 7 Differentiation-responsive downregulation of *Mybbp1a* gene transcription. (A) C2C12 myoblasts were ectopically expressed with control or miR-546 oligomers, and harvested under GM condition for real-time RT-PCR analysis of *Mybbp1a* mRNA expression. (B) C2C12 cells were cultured under DM (0, 24, and 48 h) conditions. RNA was isolated from these cells and subjected to real-time RT-PCR with primers specific to *Mybbp1a*. The bar graph shows the normalized values with the DM/0 h cells represented as 1, and with the mean $\pm$ s.d. values from three independent experiments. (C–E) Chromatin was prepared from C2C12 cells cultured under DM condition for 0, 24, and 48 h, as shown. ChIP analysis was carried out using control antibody (IgG) or antibody against RNA Pol II. The graph represents the quantitative determination of bound DNA corresponding to the *Mybbp1a* proximal (C) or distal (D) promoter, or the *MyoG* promoter (E) in the immunoprecipitates, as determined by quantitative RT-PCR. Data presented are normalized to the values of IgG. (For statistical analyses shown in this figure: n.s., not significant; *** $P < 0.001$).

maintaining the stability of co-repressor promoter association prior to myoblast differentiation. Consequently, *Mybbp1a* serves a critical role in conferring a repressive state of these genes that antagonizes *MyoD*-mediated transcriptional activation. Despite previous reports on the potential role of *Mybbp1a* in gene regulation, demonstration of its cell biological significance has been largely elusive. A recent study showed that *Mybbp1a*, as a novel Aurora B kinase substrate, plays an essential role in the normal progression of mitosis (Perrera *et al*, 2010). In addition, *Mybbp1a* signals a response to nucleolar stress by facilitating the p300-dependent acetylation and activation of p53 (Kuroda *et al*, 2011). Interestingly, previous profiling of the survival of motor neurons protein (SMN) interactome identified *Mybbp1a* as a novel binding partner (Fuller *et al*, 2010). Furthermore, the expression of *Mybbp1a* is significantly reduced in the cells of type I spinal muscular atrophy (SMA). In addition, *Mybbp1a* has been linked, via PGC-1 α and Prep1, to proper mitochondrial respiration and insulin-mediated glucose uptake in muscle

(Fan *et al*, 2004; Oriente *et al*, 2008), thus strengthening a relevant role of *Mybbp1a* in muscle physiology. To our knowledge, our study is the first report of a hitherto unrecognized link of the *Mybbp1a* protein to muscle development and possibly differentiation in general. Our findings also uncovered a new component of the epigenetic regulatory network that underlies myogenesis.

Our results indicate that the expression of *Mybbp1a* in proliferating myoblasts is immediately downregulated upon differentiation induction. While *Mybbp1a* is still present to a certain extent in the differentiated myotubes (Figure 2B), such downregulation implies a stage-dependent function. Although depletion of *Mybbp1a* led to partial de-repression of muscle-specific genes during proliferating phase (Figure 2F), such alteration was not sufficient for the myoblasts to attain terminal differentiation. In line with this finding, while *Mybbp1a*-knockdown destabilized repressor complex binding to the promoters, replacement by the activator complex did not fully take place unless cells were cultured in DM (Figure 5). The observed phenotypes are also in accordance with those previously reported for other myogenesis-associated epigenetic or structural factors. For instance, while *MyoD* is bound by PcG proteins and associated histone marks in the ES cells, the mere absence of Suz12 or Eed (Boyer *et al*, 2006; Lee *et al*, 2006) is not sufficient to transcriptionally activate the derepressed promoter. Given that myogenesis is characterized by stepwise cellular changes, these results collectively suggest the significance of *Mybbp1a* in the maintenance of undifferentiated state and/or the early stages of the muscle differentiation process.

While a co-repressor function on muscle-specific genes has been ascribed to *Mybbp1a* by our results, how such a role is manifested remains an unanswered question. *Mybbp1a* has been known to associate with diverse transcriptional regulators, acting largely as a repressor. Sequence characterization reveals the presence of several domains, such as leucine zipper-like, basic, and acidic motifs, that are characteristic of transcriptional regulators, but no enzymatic activity has been described for this protein. Intriguingly, a previous report demonstrated the binding of *Mybbp1a* with histone H3 amino terminus that is dimethylated on the Lys9 residue (Hara *et al*, 2009). This finding preliminarily implies that *Mybbp1a* may be capable of recognizing distinct chromatin domains, particularly the silenced regions, and consequently exerting its regulatory functions. On the basis of this attribute as well as the observations in this study, we propose that *Mybbp1a* may serve as a structural adaptor for the co-repressor complexes, or an enhancer for the promoter binding and activity of the various components.

A small number of muscle-specific miRNAs have been recently identified and shown to impact myogenesis and/or cardiac functions (Wong and Tellam, 2008; Juan *et al*, 2009; Chen *et al*, 2010; Sarkar *et al*, 2010; Dey *et al*, 2011). Our findings provide evidence that miR-546 may be a new important addition to this functional group of myomiRs. Intriguingly, several of these myomiRs—miR-1/206, miR-26a, and miR-214—are implicated in downregulating histone-modifying factors such as Ezh2 and HDAC4 (Chen *et al*, 2006b; Wong and Tellam, 2008; Juan *et al*, 2009), thus suggesting a regulatory cross-talk between different epigenetic mechanisms in the context of myogenesis. Our

observation of the connection between miR-546 and Mybbp1a lends further support to such paradigm, which may serve to increase the robustness of the transcriptomic transition during muscle differentiation process. Notably, miR-546 is part of a gene cluster associated with miR-26a that resides in the *Ctdsp2* gene, whose expression was also upregulated upon myoblast differentiation (Supplementary Figure S5). The muscle-specific miR-1 family of myomiRs, composed of six miRNA genes distributed in three paralogous clusters, is subject to tissue-specific regulation and exhibits similar expression alteration. Thus, this parallelism in miRNA gene organization and expression signifies the biological relevance of the miR26a/546 gene cluster in myogenesis. Characterization of downstream targets of this gene cluster may further elucidate such functional role and possible link to other differentiation programs.

While the present study substantiated the involvement of Mybbp1a in the differentiation of myoblast, several lines of evidence have also hinted at a more widespread role of Mybbp1a in cell differentiation in general. In this capacity, Mybbp1a was also found to be critical for the neuronal differentiation of the human amniotic fluid mesenchymal stem cells (AF-MSCs), although the underlying mechanism was not fully delineated (data not shown). Further expression analysis by our group revealed that the levels of Mybbp1a were likewise diminished when the myelogenous leukaemic K562 cell line was induced to enter either the monocyte or erythroid lineage (data not shown). Reduced expression was also observed during neuronal differentiation of P19 mouse embryonal carcinoma cells (Watkins *et al*, 2008). Such differentiation-responsive expression alteration may be indicative of a function of Mybbp1a in regulating cell differentiation state that is similar to what was observed in the myogenesis. Importantly, Mybbp1a was recently identified as a component of two protein complexes with essential functions in the maintenance of embryonic stem cell self-renewal and pluripotency: esBAF (Ho *et al*, 2009) and Nanog (Wang *et al*, 2006). Owing to the intimate link of these two factors to the transcriptomic network underlying stem cell physiology, it is a formal possibility that Mybbp1a, through such physical and functional association, may be involved in determining cell pluripotency versus differentiation. Further understanding of the mode of action and cellular role of this protein may shed new light on developmental pathways and perhaps regenerative medicine.

Materials and methods

Cell culture and differentiation

Mouse C2C12 myoblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum (FBS; Invitrogen) and 100 U/ml penicillin and streptomycin solution (Invitrogen) (GM), at subconfluent densities. Cells were maintained in a 5% CO₂ humidified incubator at 37°C. To induce C2C12 myotube differentiation, the medium was replaced by DMEM supplemented with 2% heat-inactivated horse serum (DM) at subconfluent culture and maintained for the time indicated in the corresponding experiments.

RNAi and establishment of stable cell lines expressing the Mybbp1a-targeting shRNA constructs

Plasmid-based shRNA-expressing constructs (pSuper) targeting Mybbp1a (5'-GAGACCAAGAAGCGAAAGA-3' and 5'-CTGAGTGGGA GCAGCTGAT-3') and luciferase (control) were transfected into C2C12 cells using Lipofectamine 2000 reagent (Invitrogen) to

obtain stable lines. Stable clones overexpressing Mybbp1a were established using pcDNA3.1 vector encoding Myc-Mybbp1a (Fan *et al*, 2004). These cells were plated at low density, subjected to drug selection with 850 µg/ml G418 (Invitrogen), and clonal colonies were isolated. Several drug-resistant C2C12 cell lines were established for both constructs and pooled for further characterization. Their levels of Mybbp1a protein were determined by immunoblotting. For transient knockdown of Mybbp1a in C2C12, cells were transfected with control or a pool of two Mybbp1a-specific siRNAs with Lipofectamine RNAiMAX (Invitrogen). Twenty-five nucleotide siRNA duplexes (Stealth, Invitrogen) were designed targeting different regions of the mRNA: +1718 to +1743 (5'-AGATGATGAGTACTCTGAAGGAATT-3') and +3135 to +3160 (5'-CCTGATGCTCCAGAAGACTCTGTCT-3') for Mybbp1a; +982 to +1006 (5'-CAGAGAUCCCUAAUGAGCUGCCCUA-3') and +521 to +545 (5'-GAGGUGUCUCUAUUAUGACAUUGAU-3') for HDAC1; +1497 to +1521 (5'-GAGGUCGUAGGAAUGUUGCUGAUCA-3') and +1042 to +1066 (5'-UGUCAAAAGUCAUGCUAAAUGUGUA-3') for HDAC2.

Microarray experiments and statistical analysis

For global gene expression profiling, total RNA was isolated from the indicated cell lines under growth condition (GM) using TRIzol (Invitrogen), reverse transcribed and labelled, and subsequently hybridized to Agilent Mouse Whole Genome Oligo 4 × 44 K. LOWESS normalization was applied to the intensity derived from individual experiments. We performed Log2 transformation on the expression values and then integrated two batches of experiment based on the 37 308 co-expressed probes (21 309 unique genes). ANOVA statistics was used to evaluate and correct the effect caused by experiment batch. In addition, we retrieved from GEO database a public microarray data (GSE4694) composed of 3-paired chips for the comparison of 6-day-old differentiated versus undifferentiated murine myoblast C2C12 cells. The expression values were exacted from series matrix files that were normalized by GCRMA algorithm in the original paper. Student's *T*-test was performed to identify DEGs as gene signatures associated with muscle development. We used stringent criteria ($P < 0.005$ and three-fold changes) to identify 913 DEGs, including 473 upregulated and 440 downregulated genes. For GSEA, these two gene signatures were combined with 214 canonical KEGG pathways in murine, as customized gene sets. All the microarray data preprocessing and statistical analyses described above were performed with Partek Genome Suite 6.5. The data have been submitted to the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) under accession no. GSE31319.

Gene set enrichment analysis

To identify the significantly altered pathways representative to both the myogenic differentiation-associated (GSE4694) and Mybbp1a-knockdown cells (in-house data) gene expression profiles, GSEA, a pathway-based enrichment analysis (Subramanian *et al*, 2005), was performed. The offline GSEA software was downloaded from Broad Institute website (<http://www.broad.mit.edu/gsea/>). A normalized Kolmogorov-Smirnov statistic was used in GSEA to estimate the significance level of specific gene sets. Because the gene sets in the Molecular Signatures Database (MSigDB) v3.0 are for human only, we created a bundle of gene sets consisting of the 214 mouse KEGG canonical pathways and two manually curated myogenic-differentiation signatures described above. The numbers of members for individual gene sets were limited between 10 and 500. Entrez IDs were used to identify the components in individual gene set. For the microarray data, the expression values of genes with multiprobes were collapsed to single gene level using median values. We then generated a ranked gene list by signal-to-noise algorithm. GSEA identified the most representative gene sets, whose *P*-values were evaluated using 1000 gene set permutation.

Sequence alignments and microRNA target prediction

The potential microRNA target sites within the 3'UTR of Mybbp1a were identified using web-based bioinformatics database TargetScan (Grimson *et al*, 2007; <http://www.targetscan.org>).

Synthetic microRNA mimics and hairpin inhibitors

For ectopic expression of miRNA mimics and antago-miRNA hairpins, C2C12 myoblasts were transiently transfected at 50% confluence with the indicated synthetic RNA oligonucleotides and corresponding controls (all purchased from Thermo Fisher Scien-

tific) using RNAiMAX (Invitrogen). Overexpression of miR-546 was also achieved by a plasmid-based system (pcDNA6.2) that harbours coding sequence for either miR-546 or the LacZ control (Invitrogen) and subsequent transfection using Lipofectamine 2000 (Invitrogen).

Reagents and antibodies

All chemicals were purchased from Sigma (St. Louis, MO), except where otherwise indicated. Mybbp1a-specific antibody (Cell Signaling Technology, Danvers, MA, USA) was raised in rabbit using a recombinant protein corresponding to amino acids 1092–1214 of mouse Mybbp1a, followed by antigen-specific purification. Anti-Myc-tag and anti-Ezh2 monoclonal antibodies were from Cell Signaling Technology. Rabbit polyclonal antibodies against MyoG, MyoD, p300, Brg1, RNA Pol II, and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-MHC mouse monoclonal antibody was purchased from Sigma (Sigma-Aldrich, MO, USA). Polyclonal antibodies against HDAC1, HDAC2, and HP1 were from Millipore (Temecula, CA, USA). α H3K9Ac, α H3K9Me2, α Suv39h1, and α PCAF rabbit polyclonal antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-DNMT antibody was obtained from Bethyl Laboratories (Montgomery, TX, USA). Secondary antibodies used in the western blot assays were from Vector Laboratories (Burlingame, CA, USA), whereas those used in immunofluorescence analysis were obtained from Invitrogen.

RNA isolation and reverse transcription (RT)–PCR

Total RNA from cells was isolated using the TRIzol reagent (Invitrogen) according to manufacturer's instructions. Genomic DNA was removed by digestion with 2 U of DNase I (Ambion, Foster City, CA, USA). cDNA and microRNA cDNA were synthesized by MMLV reverse transcriptase (Invitrogen) using random hexamers and microRNA RT-loop primers, respectively. Sequences for the microRNA RT-loop primers and primers for real-time PCR assays are listed in Supplementary Tables S7 and S8, respectively. Quantitative determination of the cDNA levels was done by real-time PCR using the Bio-Rad iQ5 Gradient Real Time SYBR-Green PCR system. Levels of cDNA were normalized to the GAPDH values of the respective samples, and microRNA cDNA was normalized to U6 control. All results represent the mean $\pm$ s.d. of at least three independent experiments.

In-vitro GST pull-down assay

GST and GST–MyoD proteins were expressed in *Escherichia coli* strain BL21 and purified by affinity chromatography using glutathione-agarose (GE Healthcare) beads. The GST and GST–MyoD proteins were analysed by SDS/PAGE (10% gel) to examine their integrity and to normalize the protein levels. There were two sources of the prey protein: Mybbp1a protein (Myc tagged) was either expressed using an *in-vitro* transcription and translation system with cold Methionine (TnT-coupled system; Promega), or ectopically expressed in C2C12 by transient transfection. In all, 10 μ l of the rabbit reticulocyte lysates or 800 μ g of nuclear extracts from transiently transfected C2C12 was used in the *in-vitro* binding assays with 1 μ g GST or fusion proteins pre-adsorbed on the agarose beads, in 500 μ l WCE buffer (20 mM HEPES, pH 7.4, 0.2 M NaCl, 0.5% Triton X-100, 5% glycerol, 1 mM EDTA, 1 mM EGTA, 10 mM β -glycerophosphate, 2 mM Na₃VO₄, 1 mM NaF, 1 mM DTT, cocktail protease inhibitor). The reaction was allowed to proceed for 4 h at 4°C, with gentle rocking. The affinity beads were collected by centrifugation at 500 g for 5 min and washed six times with 1 ml WCE buffer each, and subsequently boiled in 2 $\times$ sample buffer dye for subsequent PAGE and immunoblotting analysis as described above.

Promoter and 3' UTR reporter constructs, mutagenesis, and luciferase reporter assay

Luciferase reporter construct containing a 202-bp DNA fragment corresponding to the MyoG regulatory region (–184 to +18) was kindly provided by Dr Stavnezer. The 338-bp DNA fragment containing the MCK enhancer region (–1217 to –879) and a 4RE-TK promoter construct (containing four E-boxes placed upstream to the TK (thymidine kinase) promoter) were also used. To generate 3'UTR reporter, PCR fragment corresponding to the mouse Mybbp1a 3'UTR was subcloned into the luciferase vector pGL3-control (Promega) (WT). Site-directed mutagenesis was employed to generate a mutant version that harbours altered miR-546

target site (predicted seed complement GCCACCAA mutated to GCCAAAA; Mut).

For promoter reporter assay, C2C12 cells were seeded in six-well plates before being co-transfected (at 50% confluence) with the indicated reporter constructs and expression vectors for MyoD, Mybbp1a or both using Lipofectamine 2000 (Invitrogen). For 3'UTR reporter assay, co-transfections were done with the reporter constructs and the indicated synthetic microRNA using RNAiMAX (Invitrogen). After 2-day incubation, cells were lysed in Reporter Lysis 5 $\times$ Buffer (Promega) with two rounds of freeze thaw, followed by incubation at 4°C. Cell debris was removed by centrifugation, and luciferase activity in the supernatant was measured by a Dual-Luciferase Reporter Assay System (Promega). The relative light units were firefly luciferase units normalized to absorption units of the co-expressed β -gal.

ChIP and real-time PCR analysis

ChIP assay was performed as described previously (Hsieh *et al*, 2011). Crosslinked, sonicated chromatin was precleared before being incubated with 2.5 μ g of the indicated antibodies and rotated at 4°C overnight. Normal mouse or rabbit IgG (Millipore) was used for the mock immunoprecipitation. After extensive washes, immunocomplexes were treated with Proteinase K and decrosslinked. Bound DNA in the precipitates, as well as input DNA (1/10 fragmented chromatin), was extracted, purified, and subjected to real-time PCR analysis using primers corresponding to the mouse MyoG regulatory region regions (–169 to +39) (Forward, 5'-CCCTGCCCCACAGGGGCTGTG-3'; Reverse, 5'-ACGCCACAGAAACCTGAGC CC-3'), MHCIIb promoter (GenBank M92099) (Forward: 5'-CACC CAAGCCGGGAGAAACAGCC-3'; Reverse: 5'-GAGGAAGGACAGGA CAGAGGCACC-3'), MCK enhancer (GenBank M21390) (Forward: 5'-AGGGATGAGAGCAGCCACTA-3'; Reverse: 5'-CAGCCAC ATGTCT GGGTTAAT-3'), and GAPDH promoter (Forward: 5'-AGAGAGGGAG GAGGGGAAATG-3'; Reverse: 5'-AACAGGGAGGACAGAGAGCA C-3'). For determining RNA Pol II binding to Mybbp1a promoter, primer pairs corresponding to a proximal region (–110 to –12: Forward, 5'-AAGCCACTTCCAACCTGCTA-3'; Reverse, 5'-ACGTGGG CTCAGAAGACAGT-3') and a distal site within the gene region (+1085 to +1156: Forward, 5'-ACAAGCCATGAACAAGTGCGT G-3'; Reverse, 5'-GGGTCCCTCCCCATAGCTGTTC-3') were used. For the sequential ChIP assay, anti-Mybbp1a immunocomplexes from the first round were recovered by an elution solution (0.1% SDS, 0.5 M NaCl, and 25 mM DTT in TE buffer) and diluted 10 $\times$ by binding buffer, prior to a second round of ChIP using the MyoD antibody. Real-time PCRs were conducted on the Bio-Rad iQ5 Gradient Real-Time PCR system, using the 2 $\times$ SYBR-Green Master mix (Bio-Rad, USA). Results were corrected for non-specific binding to IgG (whose values were considered as 1 and not shown in the bar graphs). Triplicate PCRs for each sample were carried out.

Statistical analysis

Data are presented as mean with error bars indicating the standard deviation (s.d.). Student's *t*-test or two-way ANOVA with Bonferroni's correction (post tests) was used to determine the statistical significance of quantitative comparisons done in the gene expression, ChIP, and reporter assays. Degrees of statistical significance (n.s., not significant; *P < 0.05; **P < 0.01; ***P < 0.001) are indicated in the respective figure legends.

Descriptions for isolation and culture of mouse primary myoblasts, indirect immunofluorescence and confocal microscopy, cell lysate preparation, western blot analysis and immunoprecipitation are provided in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Ed Stavnezer for the MyoG-Luc promoter reporter construct. We are grateful to members of the BC-MT laboratory for critical reading of the article and important discussions. This work was supported by grants from the Hong Kong Polytechnic University (#1-BD03, #G-U702, and #G-U915 to BY-MY), the National Science Council of Taiwan (NSC100-2320-B-182-022 and NSC99-2632-B-182-001-MY3 to BC-MT and NSC98-2312-B-182-

001-MY3 to HL), Chang Gung Memorial Hospital (CMRPD170303 to BC-MT and CMRPD160364 to BY-MY), National Health Research Institute of Taiwan (NHRI-EX100-9923SC to BC-MT), and the Ministry of Education, Taiwan.

Author contributions: C-CY, HL, BY-MY, and BC-MT conceived and designed the study; YH, S-JC, and H-CC performed data analysis and informatics; C-CY, HL, and C-LH designed and

executed experiments; T-HW, SLC, H-CC, and BY-MY provided reagents; C-CY, HL, and BC-MT wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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