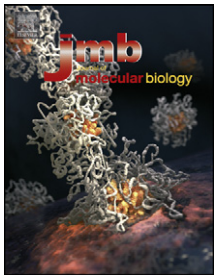

Unexpected Distinct Roles of the Related Histone H3 Lysine 9 Methyltransferases G9a and G9a-Like Protein in Myoblasts

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Abstract

Lysine methyltransferases G9a and GLP (G9a-like protein) are highly homologous and form functional heterodimeric complexes that establish mono- and dimethylation on histone H3 lysine 9 (H3K9me1, H3K9me2) in euchromatin. Here, we describe unexpected distinct roles for G9a and GLP in skeletal muscle terminal differentiation. Indeed, gain- or loss-of-function assays in myoblasts showed, in agreement with previous reports, that G9a inhibits terminal differentiation. While GLP plays a more intricate role in muscle differentiation,in one hand, both GLP gain and loss of function inhibit late steps of differentiation; on the other hand, in contrast to G9a, GLP overexpression promotes abnormal precocious expression of muscle differentiation-specific genes already in proliferating myoblasts. In agreement, transcriptomic analysis indicates that G9a and GLP regulate different sets of genes. Thus, GLP, but not G9a, inhibits proteasome subunit-encoding genes expression, resulting in an inhibition of the proteasome activities. Subsequently, GLP, but not G9a, overexpression stabilizes MyoD that is likely to be responsible for muscle markers expression in proliferating myoblasts.

Introduction

Cellular differentiation involves the coordinated activation and repression of specific subsets of genes, concomitant to significant changes in cellular metabolism. This is established by a network of transcription factors and epigenetic regulators, such as DNA methyltransferases, histone acetyltransferases, histone deacetylases, and lysine methyltransferases (KMTs). The latter are responsible for tri-, di-, or monomethylation of histone and non-histone lysine residues. Depending on the specific histone residue that is methylated, the degree of methylation can either positively or negatively regulate gene expression [1]. Thus, KMTs play key roles in transcriptional regulation during development and are also emerging as crucial players in the control of cellular differentiation, including myogenesis [2–6]. Among more than 50 different KMTs in mammals, histone 3 lysine 9 (H3K9)-specific KMTs of Suv39h family, G9a, G9a-like protein (GLP), Setdb1, and Suv39h are involved in the induction or maintenance of gene repression and heterochromatin formation (as reviewed in Ref [7]).

G9a (also known as KMT1C, EHMT2) and GLP (known as KMT1D, EHMT1) are two highly homologous H3K9 KMTs, bearing a catalytic SET domain and ankyrin repeats involved in protein–protein interactions [8,9] and methyl-lysine binding [10]. Although very similar, while the ankyrin repeat domain of G9a preferentially associates with H3K9me2, the ankyrin repeat domain of GLP preferentially associates with H3K9me1 [10]. Knockout of *G9a* or *GLP* genes in mice induces similar phenotypes, including embryonic lethality, and revealed that G9a and GLP are mainly responsible for mono- and dimethylation of H3K9 in euchromatin [11,12]. Although G9a and GLP can independently

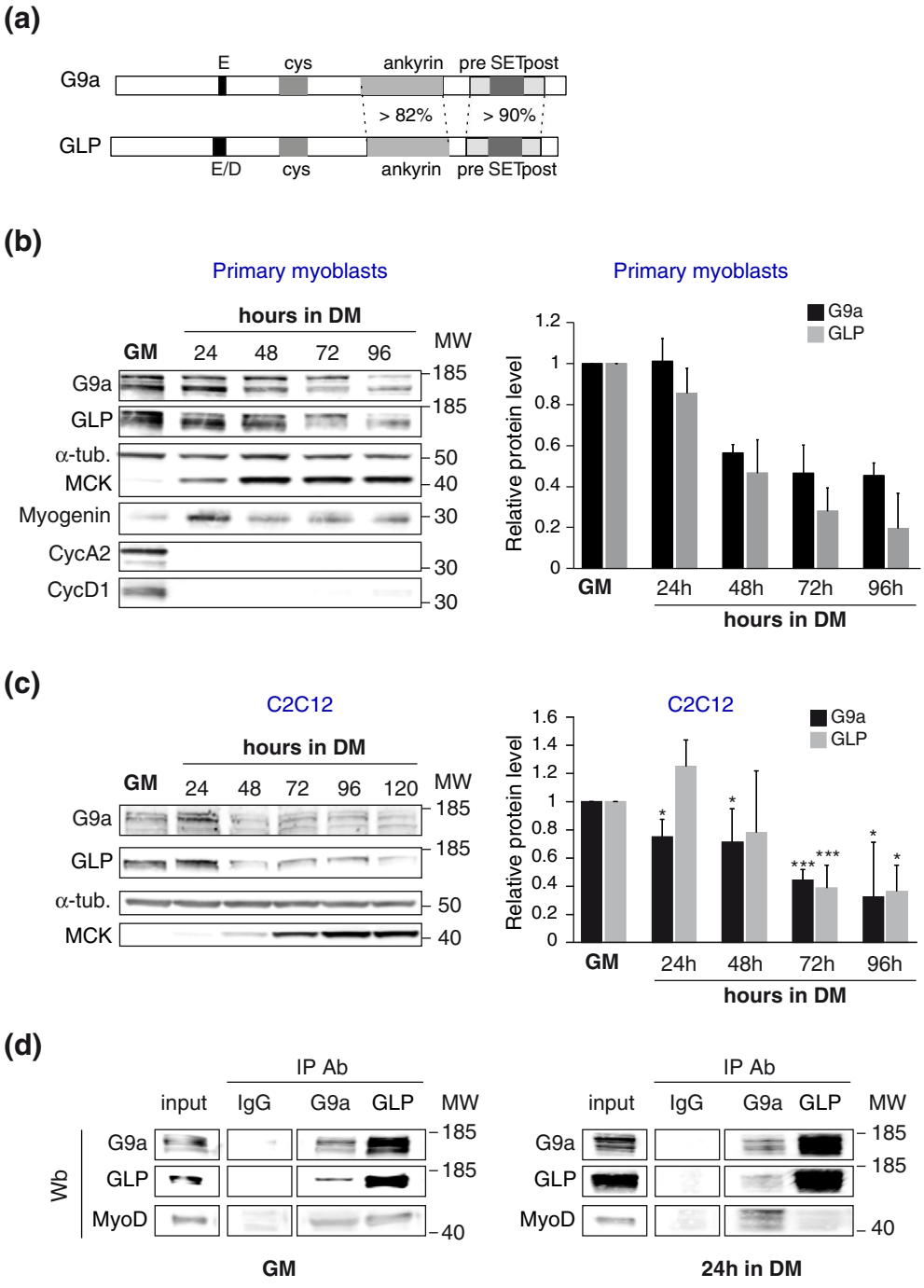


Fig. 1. The highly homologous G9a and GLP are similarly regulated during skeletal muscle terminal differentiation. (a) Schematic representation of G9a and GLP proteins. E: Glu-rich region; E/D: Glu/Asp-rich region; Cys: Cystein-rich region; Ankyrin: Ankyrin repeats; Pre: Pre-SET domain; SET: SET domain; Post: Post-SET domain. In the middle: % of homology for the most conserved domains. (b) Left: Representative WB analysis with the indicated antibodies on protein extracts from proliferating (Growth Media: GM) or differentiating (Differentiation Media: DM, for 24 h, 48 h, 72 h, or 96 h) primary myoblasts. Right: Quantification of WB signals using ChemiSmart system (Vilber Lourmat). GLP or G9a signals were normalized first to α-tubulin and then to protein level in proliferation. n = 2. (c) Left: representative WB as in B, but with protein extracts from C2C12 myoblasts. Right: protein quantification as in B with n > 3. * for p-value <0.05, *** for p-value <0.001. (d) Protein extracts from proliferating (GM) or differentiating (24 h in differentiation medium or DM) C2C12 myoblasts were subjected to IP using G9a, GLP, or irrelevant antibodies (IgG) and then analyzed by WB with the indicated antibodies. Molecular Weight is in kDa.

exert their catalytic activity and form homodimeric and heterodimeric complexes *via* their SET domains, the heterodimeric complex G9a/GLP has been described as the main functional H3K9 mono- and dimethyltransferase, at least in mouse embryonic stem cells [11,12]. However, G9a and GLP are not functionally redundant. Indeed, levels of H3K9me1 and H3K9me2 in euchromatic regions are severely reduced by either G9a and/or GLP knockout [11,13]. In addition, G9a and GLP display different tissue-specific expression profiles [14]. Finally, mutant mice, bearing mutations in GLP ankyrin domain, displayed phenotypic differences from the one bearing ankyrin-mutated G9a [15]. Despite the fact that G9a and GLP have been primarily described to methylate H3K9, they also methylate other histone and non-histone substrates, including a series of transcription factors, chromatin modifiers, including G9a and GLP themselves (for review, see Ref. [7]).

G9a/GLP are master regulators of cell fate changes and lineage-specific gene expression, including during myogenesis. During skeletal muscle differentiation, expression of muscle-specific genes requires and is preceded by the permanent withdrawal of myoblasts from the cell cycle [2]. This cell cycle exit is achieved by silencing of the proliferation genes in H3K9 methylation-dependent manner [5]. Skeletal muscle terminal differentiation program is mainly controlled by a family of specific basic helix–loop-helix transcription factors (including MyoD and Myogenin) that cooperate with members of the MEF2 protein family in the activation of muscle genes [2]. MyoD is considered as the major player in triggering muscle terminal differentiation [16], due to its capacity to induce a myogenic transdifferentiation program in many fully differentiated non-muscle systems [17]. In proliferating myoblasts, MyoD is expressed but is unable to activate its target genes even when it binds to their promoters [4,18,19]. Notably, MyoD activity and stability are also tightly regulated by post-translational modifications [39], including lysine methylation [20].

The role of G9a in skeletal muscle differentiation has been extensively studied using an *in vitro* murine model (C2C12 myoblast cells). The overexpression of G9a inhibited muscle terminal differentiation and its catalytic activity is required for this inhibition [4]; the transcription factor Msx1 recruits G9a to repress its target genes like the *MyoD* gene in myoblasts [21], and G9a interacts with Sharp-1, a basic helix–loop-helix transcription factor, enhancing its ability to repress transcription and to inhibit MyoD activity [22]. Finally, G9a methylates MyoD and MEF2D and inhibits their transcriptional activities [4,23], but it also modulates exogenous MyoD stability in competition with lysine demethylase (KDM) Jmjd2C [20].

In contrast, the role of GLP in regulation of skeletal muscle differentiation is still elusive. Most of the studies on the role of the G9a/GLP complexes have been performed through loss or gain of function of G9a and analyzed through the assumption that GLP would behave as G9a, since they mainly form heterodimers. Here, we have compared the individual roles of G9a and GLP in skeletal muscle differentiation. Through loss- and gain-of-function assays, we found that G9a and GLP have different effects on skeletal muscle terminal differentiation. We show that, unexpectedly and in contrast to G9a, GLP promotes abnormal expression of muscle-specific genes already in proliferating myoblasts when overexpressed. To understand the mechanism behind this apparent discrepancy, we compared the list of genes deregulated upon G9a or GLP acute knockdown in proliferating myoblasts. Our data showed that GLP, but not G9a, specifically regulates expression of subunits and the activity of the proteasome. In turn, GLP, but not G9a, regulates endogenous MyoD stability; GLP overexpression stabilizes MyoD that is likely to be responsible for muscle markers aberrant expression in proliferating myoblasts.

Results

GLP and G9a levels are similarly regulated during terminal skeletal muscle differentiation

G9a and GLP cellular roles have been often associated since they are closely related and form heterodimer [12]. Indeed, G9a and GLP share 73% overall homology, 58% identity, and are structurally similar; they have ankyrin repeats domain with 82% of homology and the catalytic Pre-SET/SET/Post-SET domains where the homology raises to 90% with 80% of identity (Fig. 1A).

In order to compare the roles of G9a and GLP in terminal skeletal muscle differentiation, we first analyzed their expression profiles during skeletal muscle differentiation in primary murine myoblasts and the murine myoblasts cell line C2C12. These myoblasts proliferate in high serum media (GM) and are induced to differentiate in low serum conditions (DM). Differentiation was monitored through the induction of muscle-specific markers expression, namely Myogenin and Muscle Creatine Kinase (MCK), and through the repression of cell cycle-associated cyclins, namely Cyclin D1 and Cyclin A2 (Fig. 1B). Our data show a similar downregulation of G9a and GLP protein levels after differentiation induction in primary (Fig. 1B) and C2C12 (Fig. 1C) myoblasts. In C2C12 myoblasts, G9a and GLP proteins are detected in proliferating myoblasts, peaked early in differentiating myoblasts (24 h of differentiation), dropped again from 48 h of differentiation (Fig. 1C). GLP and G9a have been described to act mainly as heterodimers [12]. Consistently, we

could co-immunoprecipitate GLP and G9a in proliferating and also early differentiating myoblasts (Fig. 1D). Moreover, it has been previously shown that G9a interacts with and methylates MyoD to repress its transcriptional activity in proliferating myoblasts [4,24]. In agreement, we found the

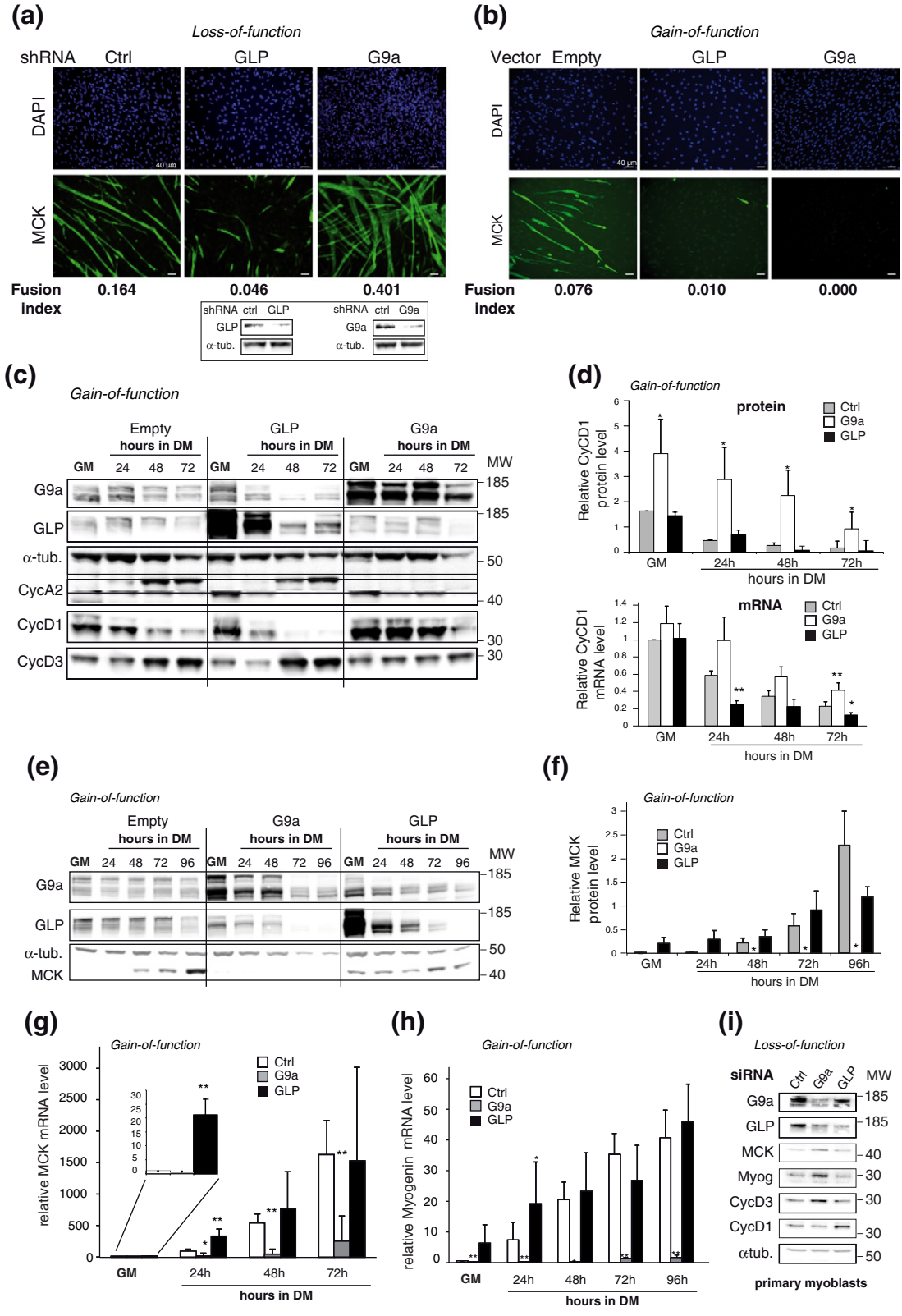


Fig. 2 *(legend on next page)*

interaction between G9a and MyoD in proliferating C2C12 myoblasts (Fig. 1d). We found that GLP also interacts with MyoD in proliferating C2C12 myoblasts (Fig. 1d, top panel). However, we were able to detect the interaction with MyoD in differentiating C2C12 myoblasts exclusively for G9a but not GLP (Fig. 1d, right panel), suggesting different functional links between MyoD and these related enzymes. Taken together, these results suggest that although G9a and GLP are similarly regulated and form a heteromeric complex in proliferating and in differentiating myoblasts, they might have different functions in differentiating myoblasts.

Modulation of GLP and G9a levels have distinct effects in proliferating myoblasts and on terminal skeletal muscle differentiation

Previous studies have shown that G9a negatively regulates skeletal muscle terminal differentiation [4,22]. Considering the similar regulation of G9a and GLP and their interaction during myogenic differentiation (Fig. 1), we sought to investigate whether GLP is also involved in this process. To this end, we performed individual loss- and gain-of-function assay for G9a or GLP in C2C12 and in mouse primary myoblasts. We confirmed that G9a knockdown favors muscle differentiation, as we could observe that the myotubes were higher in number and markedly thicker when compared to the control, and the relative fusion index was 2.4 times higher when G9a was downregulated (Fig. 2a). Surprisingly, GLP knockdown inhibited muscle differentiation, resulting in formation of only a few short myotubes that nevertheless express MCK (Fig. 2a). Consistently, the relative fusion index was 3.5 times lower when GLP is downregulated (Fig. 2a). These results show that GLP and G9a downregulation has opposite effects on terminal muscle differentiation.

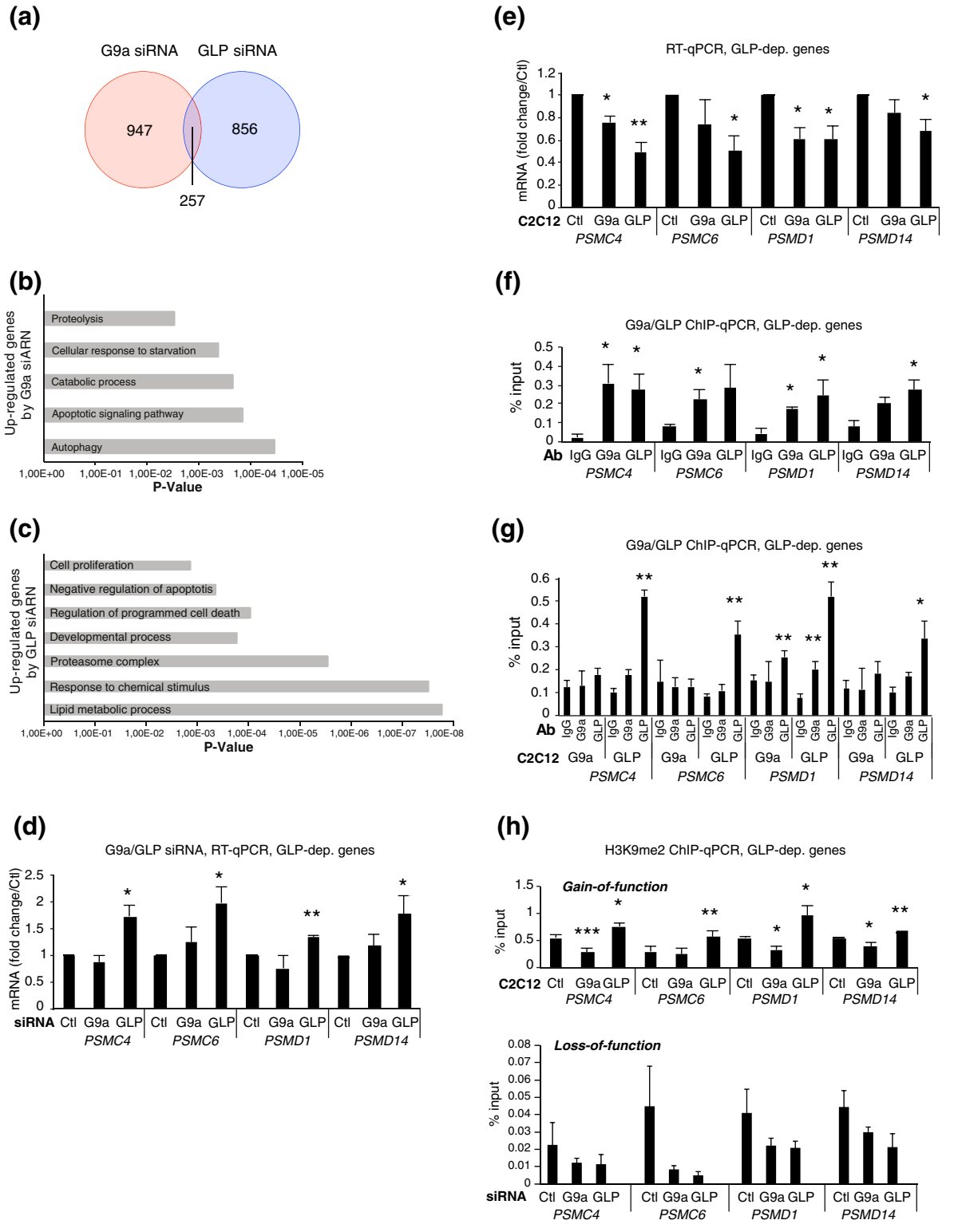
We next performed the mirror experiment by establishing polyclonal C2C12 myoblasts stably overexpressing GLP or G9a. As expected, G9a stable overexpression totally inhibited C2C12 terminal differentiation (Fig. 2b). Overexpression of GLP also inhibited myotubes formation (relative fusion index was 7.6 times lower than in control cells) even if few cells expressed MCK (Fig. 2b). These results confirmed G9a function as a strong negative regulator for skeletal muscle terminal differentiation. Importantly, these data suggest that GLP and G9a have different functions during terminal muscle differentiation. Moreover, GLP has a more subtle role since both its downregulation and its overexpression have an inhibitory effect on C2C12 differentiation, suggesting that GLP balance is particularly important. Such subtle effect is likely to be achieved through the regulation of different sets of genes with antagonistic effects.

Interestingly, overexpression of exogenous Flag-HA-tagged GLP or G9a is clearly visible in proliferating myoblasts, but these exogenous proteins are downregulated during differentiation similarly to the endogenous ones (Fig. 2c and e), suggesting that the downregulation of these KMTs during differentiation is regulated on protein level rather than on transcriptional level. Notably, while in mouse embryonic stem cells, G9a is stabilized by GLP [12,25]; in myoblasts, we detect only a slight increase in G9a upon GLP overexpression and no stabilization of GLP upon G9a overexpression (Fig. 2c and e).

To get a better insight on the distinct effects on terminal skeletal muscle differentiation observed upon overexpression of G9a or GLP, we analyzed expression of cell cycle and muscle-specific markers by western blot (WB). First, we analyzed expression of cell cycle markers in cells overexpressing G9a or GLP in differentiation conditions, since muscle terminal differentiation is correlated to an irreversible cell cycle exit. We observed that G9a overexpression results in a clear delay in Cyclin D1 downregulation and absence of Cyclin D3 accumulation (Fig. 2c and d), which is usually observed during irreversible cell cycle exit prior to differentiation [26].

Fig. 2. Distinct roles of G9a and GLP during skeletal muscle terminal differentiation. (a) Loss-of-function assays. C2C12 myoblasts were transfected with a vector expressing control shRNA (Ctrl) or shRNA directed against GLP or G9a. The knockdown efficiency, as assessed by WB, is presented below the IF panels. After 72 h in DM, transfected C2C12 myoblasts were subjected to IF against muscle creatine kinase (MCK) (Green) and DAPI stained (blue). Relative fusion indexes (number of nuclei in myotubes/total number of nuclei) are indicated under images. (b) Gain-of-function assays. C2C12 myoblasts were stably transfected with an empty vector (empty), G9a or GLP expression vectors. Myoblasts were then treated as in (A). Relative fusion indexes [as in (A)] are indicated under images. (c) Representative WB analysis of cell cycle markers with the indicated antibodies on protein extracts from proliferating (GM) or differentiating (in DM for 24 h, 48 h, or 72 h) C2C12 myoblasts described in (b). (d) Quantification of Cyclin D1 levels. Upper panel: quantification on WB of Cyclin D1 protein normalized to α-tubulin ($n > 3$). Lower panel: Cyclin D1 mRNA quantification by RT-qPCR normalized to cyclophiline A mRNA ($n > 3$). Error bars indicate mean ± SD, * for p-value <0.05, ** for p-value <0.01. (e) Similar experiment as in (C) to measure the muscle marker MCK expression. (F) Quantification on WB of MCK protein normalized to α-tubulin ($n > 3$). Error bars indicate mean ± SD, * for p-value <0.05. (G) MCK mRNA quantification by RT-qPCR normalized to cyclophiline A mRNA ($n > 3$). Error bars indicate mean ± SD, * for p-value <0.05, ** for p-value <0.01. (h) As in (g) but for Myogenin mRNA. Error bars indicate mean ± SD, * for p-value <0.05, ** for p-value <0.01. (i) Loss-of-function assay. Proliferating primary myoblasts were transfected with siRNA control (Ctrl) or directed against G9a or GLP. Representative WB analysis with the indicated antibodies is shown.

We found five- to eightfold (depending on the differentiation time point) increase in Cyclin D1 in G9a-overexpressing C2C12 (Fig. 2d). On the opposite, overexpression of GLP results in a faster downregulation of Cyclin D1 and Cyclin A2 without alteration in Cyclin D3 accumulation (Fig. 2c and d). For Cyclin D1, we showed by RT-qPCR that G9a and GLP act at the transcriptional level (Fig. 2d).



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Next, we analyzed the expression of muscle-specific markers in the G9a- or GLP-overexpressing C2C12 myoblasts (Fig. 2e–h). As expected, overexpression of G9a totally abolishes MCK expression induction during differentiation. In contrast, GLP overexpression induces a premature expression of MCK since the protein is clearly visible in proliferating myoblasts when it is normally undetectable (Fig. 2e). Quantification on multiple WBs of MCK protein levels upon GLP overexpression showed a 17-fold increase at 24 h of differentiation (Fig. 2f), but its levels are not significantly different later in differentiation (Fig. 2f). Moreover, MCK and Myogenin mRNA are already detectable in proliferating GLP-overexpressing C2C12 myoblasts and are increased more than in the control in early differentiation (24 h of differentiation) (Fig. 2g and h). On the contrary, MCK and Myogenin mRNA increase were drastically impaired during differentiation of G9a-overexpressing C2C12 myoblasts (Fig. 2g and h). Therefore we concluded that the overexpression of GLP or G9a has distinct effects on muscle markers expression. Suggestively, overexpression of GLP in proliferating myoblasts promotes myogenic genetic program when it should be "poised". Importantly, we did not notice myotube formation in GLP-overexpressing myoblasts in proliferation conditions (not shown), indicating that GLP overexpression *per se* is not sufficient to induce terminal differentiation. This is consistent with our results that showed that the late differentiation is also disrupted by the overexpression of GLP, and myotubes do not form properly (Fig. 2b). Premature expression of some myogenic markers might not directly lead to terminal differentiation, since KMTs might act directly on one set of genes and/or indirectly on other genes expression, leading to deregulation of the other steps of the differentiation process. Finally, consistent with the suggested distinct roles of GLP and G9a in skeletal muscle differentiation, we found that in proliferating primary murine myoblasts, knockdown of G9a increases the expression of Myogenin and MCK proteins, while knockdown of GLP increases Cyclin D1 expression (Fig. 2i).

Collectively, these results indicated that G9a and GLP do not have the same functions during skeletal muscle terminal differentiation. G9a represses differentiation by promoting proliferation, while GLP activates muscle terminal differentiation program in proliferating myoblasts.

GLP and G9a regulate distinct sets of genes and GLP specifically represses proteasome regulatory subunits

To shed light on how G9a and GLP exert opposite effects in proliferating myoblasts despite their very high homology, we performed a transcriptomic analysis on proliferating C2C12 myoblasts after acute G9a or GLP knockdown. A Venn diagram representing the overlap between G9a and GLP deregulated genes showed that the downregulation of G9a or GLP do not affect the same sets of genes (Fig. 3a). In fact, on the 2061 genes detected from the transcriptomic study, only 257 (12.5%) are commonly regulated by both G9a and GLP, while 87.5% of the genes are exclusively regulated by G9a or GLP. Thus, only 23% of the GLP-dependent genes are also regulated by G9a (Fig. 3a).

We next performed gene ontology analyses and found that G9a and GLP negatively regulate different cellular pathways, further showing that they have different roles in proliferating myoblasts. G9a knockdown affects autophagy and apoptosis signaling pathways, consistent with previous studies (Fig. 3b) [27–31]. GLP is rather involved in lipid metabolic processes, response to chemical stimuli and proteasome (Fig. 3c). Concerning the proteasome, the majority of the genes dependent on GLP encode for 26S proteasome subunits.

We focused our study on the unexpected and less-documented GLP-specific functions. In particular,

Fig. 3. G9a and GLP regulate different sets of genes, and GLP negatively regulates proteasome subunit-encoding genes. (aA) Venn diagram showing overlap between deregulated genes (upregulated or downregulared) in proliferating C2C12 myoblasts transfected either by G9a or GLP siRNA. (b) Gene ontology, using Genomatix software, for upregulated genes in proliferating C2C12 myoblasts transfected with G9a compared to scrambled siRNA. (c) As in (b) but transfected with GLP siRNA. (D) mRNA quantification by RT-qPCR of the indicated GLP regulated genes in proliferating C2C12 myoblasts transfected either with G9a, GLP siRNA or scrambled siRNA (Ctl). Histograms represent the mRNA quantification normalized first to cyclophiline A mRNA then to the control. Error bars indicate mean ± SD, $n > 3$, * for p-value <0.05, ** for p-value <0.01. (e) mRNA quantification as in (d) but proliferating C2C12 were transfected with expression vectors for GLP or G9a or empty vector (Ctl). Error bars indicate mean ± SD, $n > 3$, * for p-value <0.05, *** for p-value <0.001. (F) ChIP-qPCR from C2C12 proliferating myoblasts with control IgG, G9a, or GLP antibodies and primers specific of the promoter of the indicated GLP regulated genes. Results are expressed as a % of immunoprecipitated DNA compared to the input DNA (% input). Error bars indicate mean ± SD, $n > 3$, * for p-value <0.05. (g) ChIP-qPCR as in F but from proliferating C2C12 transfected with expression vector for GLP or G9a. Results are expressed as in (f). Error bars indicate mean ± SD, $n > 3$, * for p-value <0.05, ** for p-value <0.01. (H) ChIP-qPCR as in (F) but with H3K9me2 antibody and from proliferating C2C12 myoblasts transfected with expression vector for GLP or G9a (upper) or upon G9a or GLP loss of function (lower). Results are expressed as in (f). Error bars indicate mean ± SD, $n > 3$, * for p-value <0.05, ** for p-value <0.01, *** for p-value <0.001.

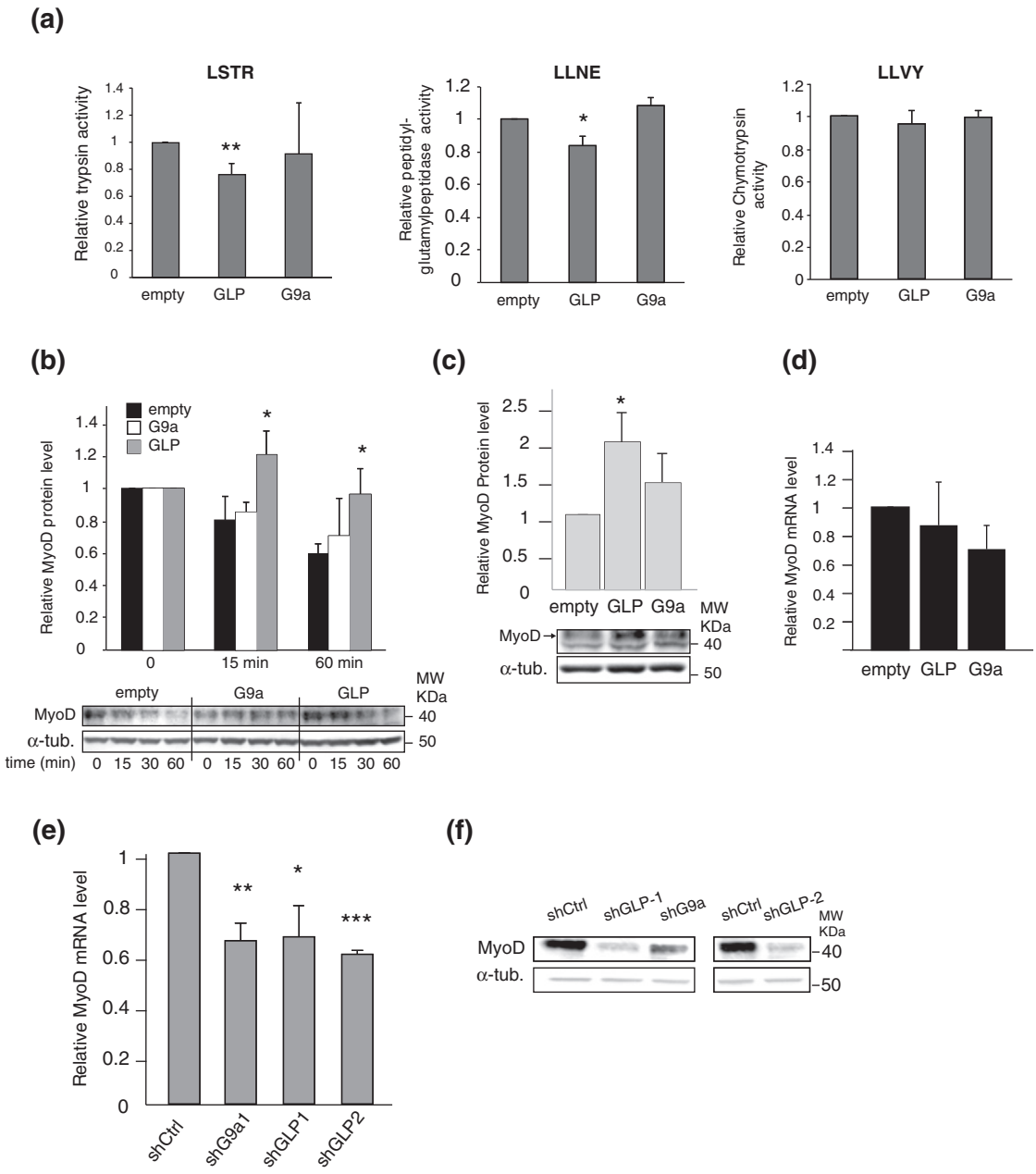

Fig. 4. Overexpression of GLP, but not G9a, decreases proteasome activity and stabilizes MyoD. (a) C2C12 proliferating myoblasts were transfected with expression vectors for GLP or G9a or an empty vector. Total lysates were assayed for proteasome activity using fluorescent peptides LSTR, LLNE, LLVY to measure activity of trypsin, peptidyl-glutamylpeptidase, and chymotrypsin, respectively. Histograms represent activities normalized to the control (empty). Error bars indicate mean ± SD, $n > 3$, *$p < 0.05$, **$P < 0.01$. (b) C2C12 proliferating myoblasts were transfected with expression vectors for GLP or G9a or an empty vector. MyoD protein was quantified from WB at 0, 15, or 60 min after addition of cycloheximide. Histograms represent protein levels normalized to time 0. Error bars indicate mean ± SD, $n > 3$, *$p < 0.05$. A typical WB result is shown below. (c) MyoD WB signal quantification in extracts from proliferating C2C12 myoblasts transfected with expression vectors for GLP or G9a or an empty vector. Histograms represent the quantification normalized to α-tubulin. Error bars indicate mean ± SD, $n > 3$, *$p < 0.05$. A typical WB result is shown below. (d) As in (c), MyoD mRNA quantification by RT-qPCR. Histograms represent the quantification normalized to cyclophilineA mRNA. $n = 2$. (E) MyoD mRNA quantification by RT-qPCR from proliferating C2C12 transfected with scrambled shRNA (shCtrl) or shRNAs directed against GLP or G9a. Histograms represent the quantification normalized to cyclophiline A and to the control. Error bars indicate mean ± SD, $n > 3$, *$p < 0.05$, **$P < 0.01$, ***$P < 0.001$. (f) WB analysis with the indicated antibodies of protein extracts from proliferating C2C12 transfected with scrambled shRNA (shCtrl) or shRNAs directed against GLP or G9a.

we further addressed GLP role in the regulation of proteasome subunits expression. Indeed, the proteasomal protein degradation plays pivotal roles in cell fate changes, particularly in skeletal muscle terminal differentiation [32–34]. Interestingly, we found that GLP regulates, to different extent, many genes encoding subunits of the proteasome, such as *Psma1 [Proteasome* (*Prosome*, *Macropain*) *Subunit*, *Alpha Type*, *1]*, *Psma4*, *Psmc4*, *Psmc6*, *Psmd1*, *Psmd3*, *Psmd5,* and *Psmd14*.

We focused our studies on the regulation of the 26S proteasome subunit genes *Psmc4*, *Psmc6*, *Psmd1,* and *Psmd14*, which encode for subunits of the 19S regulatory base of the proteasome. First, we confirmed by RT-qPCR that these four subunits of the proteasome are specifically and significantly upregulated upon GLP, but not G9a, knockdown (Fig. 3d). Consistently, the four genes were downregulated upon overexpression of GLP, while overexpression of G9a has no effect on *Psmd14* and *Psmc6* and downregulates also *Psmc4* and *Psmd1* (Fig. 3e).

GLP and G9a are known to repress transcription *via* methylation of H3K9 at the regulatory regions of their target genes. In an attempt to understand the mechanism of the regulation of proteasome subunit genes by GLP, we performed ChIP-qPCR to analyze the presence of endogenous GLP and G9a on the promoters of the GLP-regulated genes *Psmd1*, *Psmc4*, *Psmc6,* and *Psmd14*. We detected both endogenous G9a and GLP at the promoter region of the tested proteasome subunit genes in proliferating C2C12 myoblasts (Fig. 3f). We also performed ChIP-qPCR in proliferating G9a- or GLP-overexpressing C2C12 myoblasts or in C2C12 myoblasts upon G9a or GLP knockdown (Fig. 3). In comparing results obtained with C2C12 overexpressing either G9a or GLP, we did not find any increase in G9a recruitment on the promoter region of these GLP target genes (Fig. 3g). However, GLP overexpression induced an increase in its binding ranging between 1.8- and 2.9-fold on the promoter of its target genes in C2C12 overexpressing this KMT (Fig. 3g). Finally, we analyzed H3K9 dimethylation for these GLP target genes and detected a significant increase of this repressive mark when GLP is overexpressed, while, surprisingly, overexpression of G9a results in a decrease in this mark (Fig. 3h, upper panel), while both GLP and G9a knockdown lowered H3K9 dimethylation at these genes (Fig. 3h, lower panel).

Taken together, our results show that GLP and G9a mainly regulate different sets of genes in myoblasts. GLP specifically negatively regulates the transcription of genes encoding subunits of the proteasome; GLP is located at the promoters of these target genes and its levels correlate with the dimethylation of H3K9 (Fig. 3f–h), suggesting the implication of its catalytic activity. Most likely, GLP establishes this repressive histone modification independently of G9a, since overexpression of G9a does not modulate its level on these GLP target gene promoters. These results indicate that GLP, but not G9a, could, *via* its H3K9 methylation activity, negatively regulate these four studied proteasome subunit genes.

GLP inhibits proteasome activity resulting in an increase in MyoD protein level

We found that GLP regulates several genes encoding subunits of the proteasome and showed that four subunits of the regulatory proteasome base are negatively regulated by GLP but not by G9a. We thus asked whether this could, in turn, regulate the proteasome activity. To test this, we studied three different protease activities associated with proteasome functions. We monitored these proteasome activities in total protein extracts from control, G9a- or GLP-overexpressing C2C12 myoblasts using synthetic peptides whose cleavage will reveal specifically trypsin, chymotrypsin, and peptidyl-glutamylpeptidase activities. G9a overexpression did not modify any of these three protease activities (Fig. 4a). GLP overexpression significantly decreased trypsin activity and the peptidyl-glutamyl-peptidase activities (Fig. 4a).

Previous studies have shown that proteasome-dependent degradation pathway is involved in the regulation of MyoD during cell cycle and myogenic differentiation [32–34]. MyoD increases during G1 phase, reaching its highest level of expression. If external stimuli are favorable, cells undergo terminal differentiation, and if not, MyoD is degraded *via* the proteasome pathway and cell stay proliferating [33]. Therefore, we asked whether the abnormal expression of the differentiation markers in proliferating myoblasts that we observed with GLP overexpression (Fig. 2e–h) could be at least partially attributed to the changes in MyoD degradation dynamics due to GLP-dependent regulation of proteasome activities. Using cycloheximide to inhibit protein synthesis, we studied MyoD stability in control, G9a- or GLP-overexpressing myoblasts and observed a stabilization of MyoD protein upon GLP, but not G9a, overexpression (Fig. 4b). Consistently, we found an increase in MyoD protein levels in proliferation conditions (Fig. 4c). Notably, overexpression of GLP did not significantly affect MyoD mRNA level, but rather decreased it (Fig. 4d), clearly indicating that GLP-induced increase of MyoD protein level is associated with higher stability of the protein.

Downregulation of G9a or GLP by shRNA resulted in decrease of MyoD mRNA level (Fig 4e), as described for G9a [4]. Importantly, at the protein level, the MyoD decrease was clearly more pronounced upon downregulation of GLP than G9a (Fig. 4f).

Notably, we did not observe changes in MyoD stability following cycloheximide treatment upon GLP downregulation (not shown), even if it was sufficient to induce overexpression of four tested proteasome subunits (Fig. 3d). We speculate that the overexpression of only part of the proteasome subunits is not enough to increase proteasome activity, while their downregulation makes them the limiting factors and therefore affects protease activity.

Together, these results suggested that GLP could control MyoD protein levels by regulation of at least two protease activities of the proteasome, most likely through the expression regulation of the 19S proteasome regulatory subunit.

In summary, we found that on one hand, overexpression of GLP in proliferating myoblasts increases MyoD protein level and we observed a premature expression of myogenic specific genes; on the other hand, downregulation of GLP reduces MyoD levels consistently with the observed alterations in myogenic differentiation.

Discussion

Skeletal muscle terminal differentiation requires the selective activation and repression of specific genetic programs, which involves transcription factors and epigenetic mechanisms. Transcription factors from the MRF family, such as Myf5, MyoD, and Myogenin, regulate negatively or positively genes at the proliferation-to-differentiation switch [1]. This gene expression regulation involves mainly epigenetic mechanisms that are established through recruitment of chromatin-modifying enzymes, such as histone acetyltransferases, deacetylases, KMTs, and lysine demethylases KDMs. Methylation/demethylation of histone lysines and non-histone proteins plays a central role in epigenetic regulation of gene expression by recruiting effectors bearing methyl-binding modules with distinct activities. In turn, KMTs and KDMs are highly specific for the targeted residue in the histone tail and so far, around 50 KMTs and 30 KDMs have been described [7]. In agreement with a role of histone lysine methylation in regulating specific genetic programs, we and others have shown that many KMTs and KDMs are involved in the regulation of muscle-specific genes, and particularly *myogenin* during the process of muscle differentiation [4,5,18,35]. Moreover, some master proteins controlling the balance between proliferation and differentiation, such as MyoD and MEF2, are known to be methylated on lysines themselves [4,23] and are thus substrates of KMTs and KDMs.

Here, we have dissected the role of two related H3K9 KMTs, G9a and GLP. These enzymes are highly homologous, have similar enzymatic activities, and are considered to act mainly as heterodimers [12]. Despite all these similarities, our data showed that they play different roles in skeletal muscle terminal differentiation, even if they share similar expression patterns during myoblasts–myotubes transition. Gain- and loss-of-function assays in myoblasts showed that G9a negatively regulates myogenic differentiation, since its overexpression totally abolished myotubes formation and increased Cyclin D1 proliferation marker expression in myoblasts, while its downregulation enhances myotube formation. Surprisingly, both knockdown and overexpression of GLP abolished C2C12 differentiation, suggesting that GLP could play a subtle role at the proliferation–differentiation transition. Terminal muscle differentiation is the result of a complex network of both silencing and activating of different sets of genes. Indeed, myoblasts must first exit cell cycle and then enter terminal differentiation. Modulation of KMT levels could affect (directly or indirectly) antagonist sets of genes and therefore result in an impaired differentiation upon their either upregulation or downregulation. Thus, GLP dosage is crucial for both normal cell cycle exit and terminal differentiation entry, similar to the other H3K9 KMT Suv39h1 [5,36], which is also involved in both cell cycle exit and entry in terminal differentiation. Unexpectedly, GLP involvement in muscle differentiation is different to G9a. Consistently, with this different mode of involvement, we have also shown that the interaction of G9a, GLP, and MyoD evolved differently since GLP, but not G9a, lost its association with MyoD upon differentiation.

Interestingly, overexpression of GLP in proliferating myoblasts is associated with abnormal expression of the MCK myogenic marker and Myogenin, when they should be totally silenced. Such aberrant expression is clearly observed during proliferation and early differentiation when the level of exogenous GLP is the highest. Notably, overexpression of G9a has no detectable effect on MCK expression in proliferating myoblast and totally inhibits its activation upon differentiation. Thus, our results showed that these two highly homologous KMTs, GLP and G9a, have different roles in proliferating myoblasts.

In order to uncover how GLP and G9a could behave in opposite ways in proliferating myoblasts, we have performed transcriptomic analyses in myoblasts upon loss of function of each of these two KMTs. Unexpectedly, we found that only 12.5% of the identified genes are commonly regulated by GLP and G9a. Gene ontology analyses indicated that the genes upregulated upon G9a knockdown are mainly involved in survival control (autophagy, apoptosis, starvation). This result is in concordance with the increasing number of studies involving G9a in the protection from cell death [28,37,38]. Differently, genes upregulated upon GLP knockdown are involved in metabolic and chemical processes in the

Table 1. List of primer, siRNA and shRNA.

	Target	Forward primer	Reverse primer
RT-qPCR primers	Cyclophilin A	GTCAACCCCACCGTGTTCTT	GTCAACCCCACCGTGTTCTT
	MCK	CACCATGCCGTTCGGCAACA	GGTTGTCCACCCCAGTCT
	MyoD	GGCGACTCAGATGCATCCA	GCTGTAATCCATCATGCCATCA
	Myogénine	GAATCACATGTAATCCACGGA	ACGCCAACTGCTGGGTGCCA
	Psmc4	GGATCAGAACACAGCCATCGT	GGTACTCAGGATGCGCACATAA
	Psmc6	CCTCCAAAAGGCTGTTTGCT	CTCGTGCCAAGAGTGTTTTCC
	Psmd1	GGAAAAGCCTGATTTCATCAATG	CACTCACAGCCTGAGGATCATC
	Psmd14	TGGACACAGCAGAACAAGTTTATATCT	CCAGCACGACCATGTTTTAACA
ChIP-qPCR	Psmc4	GGGCAAAAGCCTACGGAAA	TATGGTGCTTCTACTTGTTTTAAATGG
	Psmc6	GGGAATCATGTGGTTTTTTCTGA	ATGTGTGTGCAGCACGTGTGT
	Psmd1	TTCCTGCCTTTTCCTCTGCTT	GCTGGGTGTTGTGGTTCACA
	Psmd14	CCAACACCCAAGTCAGAAAGC	GGCATCAGATCATGCGTAGCT
		Sequence	
siRNA	Scrambled	ACUUAACCGGCAUACCGGCTT	
	G9a 1	UAACAAGGAUGGCGAGGUUUU	
	G9a 2	CAGGACAGGUGGACGUCAAUU	
	GLP 1	GCACCUUUGUCUGCGAAUAUU	
	GLP 2	GGAUCAAACCUGCUCGGAAUU	
shRNA	Scrambled	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	
	G9a 1	CCGGAATAACAAGGATGGCGAGGTTCTCGAGAACCTCGCCATCCTTGTTATTTTTTG	
	G9a 2	CCGGAACAGGACAGGTGGACGTCAACTCGAGTTGACGTCCACCTGTCCTGTTTTTTG	
	GLP 1	CCGGAAGCACCTTTGTCTGCGAATACTCGAGTATTCGCAGACAAAGGTGCTTTTTTG	
	GLP 2	CCGGAAGGATCAAACCTGCTCGGAACTCGAGTTCCGAGCAGGTTTGATCCTTTTTTG	

proteasome complex, developmental processes, and regulation of programmed cells death, further supporting the different functions of G9a and GLP in myoblasts.

Our transcriptomic analyses revealed that at least eight subunits of proteasome complex are specifically upregulated upon GLP but not G9a knockdown. It was particularly interesting since one of the main non-epigenetic mechanisms to regulate myoblasts fate is to modulate the amount of MRF transcription factors, Myf5 and MyoD, at the protein level through ubiquitin/proteasome-dependent degradation [33]. By using GLP or G9a gain- and loss-of-function assays, we confirmed that at least four of these proteasome subunits (namely, *PSMC4*, *PSMC6*, *PSMD1,* and *PSMD14*) are specifically regulated by GLP but not by G9a. Endogenous G9a and GLP are located to the promoter of these genes, but only the overexpression of GLP leads to an increase in the specific H3K9me2 repressive epigenetic mark. Furthermore, this epigenetic mark decreases upon downregulation of either GLP or G9a. Taking together with the fact that both endogenous G9a and GLP are enriched at the promoters of these genes, we speculate that G9a participates in targeting or/and stabilization of GLP at these sites, while GLP carries out the enzymatic function. Therefore, loss of any of these proteins will be limiting for the deposition of H3K9me2. We further analyzed the functional outcome of GLP overexpression on proteasome activity and we could show that the trypsin and peptidyl-glutamylpeptidase proteasome enzymatic activities are decreased. In parallel, we showed that the GLP overexpression increases MyoD stability and induces a twofold increase at protein levels, which is important for this key transcription factor; driving myogenic differentiation and, on the opposite, GLP knockdown drastically decreased MyoD protein levels. These effects on MyoD levels could explain the biological effects we have observed upon loss and gain of function for GLP. In one hand, GLP overexpression in proliferating myoblasts increases MyoD protein levels, resulting in the transcriptional activation of myogenic marker genes such as MCK. On the other hand, GLP downregulation decreases MyoD levels impairing muscle terminal differentiation. Notably, MyoD protein stability is regulated by a complex crosstalk between post-translational modifications including phosphorylation, methylation, and ubiquitinylation [20,39]. MyoD stability is controlled during cell cycle of myoblasts [40,41], so that any changes in its stability could modify the outcome of the balance between proliferation and differentiation. It has been shown that G9a, known to deposit the repressive H3K9 or H3K27 methylation, also methylates non-histone substrates like MyoD itself, resulting in its stabilization on chromatin and inhibition of its transcriptional activity [4]. On the opposite, the demethylase Jmjd2C, known to demethylate H3K9, also demethylates MyoD [20]. Notably, we were able to detect changes neither in endogenous MyoD ubiquitination nor methylation upon modulation of GLP levels (not shown), which could be explained by the differences in experimental approaches, since all published reports were using overexpression of

MyoD. Nevertheless, we have demonstrated that GLP, which is able to methylate H3K9, also influences MyoD stability but by a new mechanism that passes through regulation of proteasome activity.

Most of the previous studies on G9a/GLP KMTs have focused their experimental approaches on G9a, assuming that the highly homologous GLP would behave the same way. However, previous studies suggested that in some tissues, for example, in brown adipose tissue, GLP could function by itself, since G9a is poorly expressed [14,42]. Our data point out for the first time that during early steps of skeletal muscle differentiation, these two highly homologous KMTs have opposite biological functions. We demonstrate that they transcriptionally regulate mainly different sets of genes, GLP being specifically involved in proteasome complex regulation. It is thus important to consider these differences when these key enzymes are targeted by chemical inhibitors, especially in therapeutical considerations.

Materials and methods

Cell culture and transient transfections

C2C12 cells were grown in DMEM media supplemented with 15% of fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin, and sodium pyruvate (PAA, Les Mureaux, France). Primary myoblasts were grown in DMEM media supplemented with 20% of FBS, 2.5 ng/ml of bFGF (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin, and sodium pyruvate (PAA, Les Mureaux, France). C2C12 cells and mouse primary myoblasts were differentiated, as described in Yahi H, *et al*., 2008). siRNAs were purchased from Sigma and transfected using Hi-Perfect reagent (Qiagen) (Table 1). We transfected 0.2 µmol of siRNA per 100 mm cell culture dish. Cycloheximide and MG132 (Sigma) were used at 100 µg/ml and 20 µM, respectively.

Stable cell lines

Polyclonal C2C12 cell lines stably expressing Flag-HA-tagged G9a and GLP were established by retroviral transduction of full-length transgenes followed by a selection of infected cells using magnetic beads covered by anti-CD25 antibody, as described in Ref. [23]. A cell line transduced with the empty vector has been used as control.

Production of lentivirus and infections

Lentiviral particles were produced in HEK-293 T cells. HEK-293 T cells were grown in DMEM media supplemented with 10% of FBS, penicillin/streptomycin, and sodium pyruvate (PAA, Les Mureaux, France). Cells were transfected with pLKO.1 vectors containing shRNA targeting G9a, GLP, or scramble control (Table 1), psPax2 lentiviral packaging plasmid, and pMD2.G VSV-G envelope expressing plasmid using Calcium Phosphate transfection method. Supernatant from 293 T cells were collected 48 h after transfection, filtered through a 0.45 µm filter, and concentered by 2 h of centrifugation at 25,000 rpm at 4 °C. C2C12 cells were infected with 10 viral particle per cell.

Antibodies

Antibodies against Cyclin A2 (sc-596), Cyclin D1 (sc-753 and sc-20044), Cyclin D3 (sc-182), and Myogenin (sc-576) from Santa Cruz Biotechnologies; G9a (07–551) from Upstate or from Cell Signaling Technology (3306) and GLP (PP-B0422–00) from R&D Systems; and MCK that were a kind gift from Dr. H. Ito (Aichi Human Service Center, Japan) were used for the WB. Antibodies against G9a (3306) from Cell Signaling Technology and GLP (PP-B0422–00) from R&D Systems; H3K9me2 (ab1220) from Abcam; mouse normal IgG (sc-2025) and rabbit normal IgG (sc-2027) from Santa Cruz Biotechnologies were used for ChIP and/or immunoprecipitation (IP).

Western blot

Purified protein complexes or whole-cell extracts were resolved on pre-cast NuPage 4–12% acrylamide gradient SDS-PAGE gel (Life Technologies) and transferred into nitrocellulose membrane in phosphate transfer buffer. Membrane was blocked in 10% milk and incubated overnight at 4 °C with the primary antibodies. Membranes were incubated with the appropriate secondary antibodies coupled to horseradish peroxydase, and revealed using West Dura kit (Pierce, Rockford, USA) and ChemiSmart 5000 system (Vilber Lourmat).

Immunoprecipitation

C2C12 cell pellets were lysed in hypotonic buffer (20 mM HEPES pH 7, 0.15 mM EDTA, 10 mM KCl, 0.15 mM EGTA) with freshly added spermine, spermidine (0.15 mM, 0.5 mM, respectively) and protease inhibitors (Sigma) by 20 strokes in Dounce homogenizer. Cell lysates were centrifuged at 2000 g for 7 min. The nuclei pellets were suspended in sucrose buffer (20 mM Tris pH 7.65; 60 mM NaCl; 15 mM KCl; 0.34 M Sucrose) and then high salt buffer was added (20 mM Tris-HCl pH 7.65; 0.2 mM EDTA; 25% glycerol; 900 mM NaCl; 1.5 mM $MgCl_2$) to a final NaCl concentration of 300 mM. The nuclear extracts were treated with Micrococcal nuclease (0.0025 U/ml) with 1 mM $CaCl_2$ at 37 °C during 10 min, EDTA was added to 4 mM final concentration and finally sonication for 10 min (15 sec ON, 45 sec OFF) at high frequency. The lysates were ultracentrifuged at 40000 rpm for 30 min and pre-cleared with protein G-agarose beads (Sigma) during 2 h at 4 °C. Immunoprecipitations were carried out overnight at 4 °C using 5 µg of each antibody. Ultralink beads (Perbio) were blocked overnight at 4 °C with 0,3 % BSA and 0,5 µg/µl ssDNA and then incubated with the immunocomplexes for 4 h at 4 °C. The immunocomplexes are washed four times in wash buffer (50 mM Tris-HCl, pH 7.65, 150 mM NaCl, Triton X-100 0.5%) and

the proteins were eluted in NuPAGE® LDS Sample Buffer (Life Technologies) at 96 °C during 5 min. Finally, the immunoprecipitates were examined by western blot.

RNA, microarray, and quantitative RT-PCR

Total RNA was extracted using RNeasy mini-kit (Qiagen) following manufacturer's procedures. With High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), 0.5 µg of total RNA was reverse transcribed. Real-time quantitative PCR was performed to analyze relative gene expression levels using SYBR Green Master mix (Applied Biosystems) following manufacturer indications. Relative expression values were normalized to the housekeeping gene mRNA Cyclophillin A. Primer sequences are listed in Table 1. For Microarray, raw data were normalized using the Robust Multichip Algorithm in Bioconductor R. Then, all quality controls and statistics were performed using Partek GS®. We first made a hierarchical clustering (Pearson's dissimilarity and average linkage) and Principal Component Analysis to control the data. To find differentially expressed genes, we applied a classical analysis of variance for each gene and made pairwise Tukey's post hoc tests between groups. Then, we used p-values and fold changes to filter and select differentially expressed genes. Genomatix software was used for Gene Ontology analyses.

Chromatin Immunoprecipitation (ChIP)

Formaldehyde (Sigma) was added to the culture medium to a final concentration of 1%. Cross-linking was allowed to proceed for 10 min at room temperature and stopped by addition of glycine at a final concentration of 0.125 M. For G9a and GLP ChIP, we introduced another step of cross-linking before formaldehyde by adding DSG (Di-Succinimidyl Glutarate; Santa Cruz) at a final concentration of 2 mM for 45 min at room temperature, as described in Ref. [24]. Fixed cells were washed and harvested with PBS. Chromatin was prepared by two subsequent extraction steps (10 min at 4 °C) with Buffer 1 [50 mM Hepes/KOH (pH 7.5); 140 mM NaCl; 1 mM EDTA; 10% Glycerol; 0.5% NP-40; 0.25% Triton X-100] and Buffer 2 [200 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 10 mM Tris (pH 8.0)]. Nuclei were then pelleted by centrifugation, resuspended in Buffer 3 [50 mM Tris (pH 8.0); 0.1% SDS; 1% NP-40; 0.1% Na-Deoxycholate; 10 mM EDTA; 150 mM NaCl], and sonicated with Bioruptor Power-up (Diagenode), yielding genomic DNA fragments with a bulk size of 150–300 bp. Chromatin was precleared with Protein A/G ultralink beads (Pierce) for 2 h at 4 °C and immunoprecipitation with the specific antibodies carried out overnight at 4 °C. Immune complexes were recovered by adding pre-blocked protein A/G ultralink beads and incubated for 2 h at room temperature. Beads were washed twice with Low salt buffer [0.1% SDS; 1% Triton; 2 mM EDTA; 20 mM Tris (pH 8.0); 150 mM NaCl], twice with High salt buffer [0.1% SDS; 1% Triton; 2 mM EDTA; 20 mM Tris (pH 8.0); 500 mM NaCl], once with LiCl wash buffer [10 mM Tris (pH 8.0); 1% Na-deoxycholate; 1% NP-40, 250 mM LiCl; 1 mM EDTA], and twice with TE supplemented with 50 mM NaCl. Beads were eluted in TE with addition of 1% SDS at 65 °C, and cross-link was reversed overnight at 65 °C. The eluted material was phenol/chloroform extracted and ethanol precipitated. DNA was resuspended in water, and q-PCR performed using PowerSYBR Green PCR Master mix (Applied Biosystems) and analyzed on a 7300 PCR System (Applied Biosystems). ChIP-qPCR results were represented as percentage (%) of IP/input signal (% input). Primer sequences are listed in Table 1.

Immunofluorescence (IF)

Cells were cultured in Lab-Tek chamber slides (Nunc) and fixed for 10 min with 4% formaldehyde in PBS. Residual formaldehyde was neutralized with 0.1 M Glycine (pH 8.0) and washed with PBS. Cells were permeabilized and blocked using 1% BSA, 1% goat serum, 0.3% Triton-X100 in PBS. Primary (anti-MCK, from Dr. H. Ito) and secondary [Anti-rabbit IgG Alexa 488, A-11008, (Life technologies)] antibodies were diluted in the permeabilizing/blocking solution and were washed with 0.3% Triton-X-100 in PBS. Nuclei are stained with DAPI, and the glass lid is fixed using an anti-fading polymerizing media from DakoCytomation (Dako).

In vitro proteasome assay

Cells were collected in 25 mM Tris (pH 7.5) buffer and lysed by passing 10 times through a needle (21G). Lysate was centrifuged at 12,000 g for 30 min at 4 °C. Then, 25 µg of total protein of cell lysates were transferred to a 96-well microtiter plate (BD Falcon), and the fluorogenic substrate was then added to lysates. We measure trypsin-like, the chymotrypsin-like, and the postglutamylpeptide hydrolase activities of the protease using, respectively, Leu-Ser-Thr-Arg -AMC (LSTR-AMC), Leu-Leu-Val-Tyr-AMC (LLVY-AMC) both at 50 mM and the Leu-Leu-Glu-NA (LLE-NA) peptides at 400 mM. Reactions were monitored on a FlexStation 3 every 5 min for 1 h at 37 °C. For the peptides-AMC (aminomethylcoumarine), we used 360 nm excitation, 460 nm emission setting, and for the peptide-NA (Naphtylamide) 320 nm excitation, 420 nm emission. Cells treated with MG132 were used as negative control and the activity = sample activity minus negative control activity.

Statistical analysis

All values are presented as mean +/– standard deviation (SD). For statistical significance, a unilateral t-test was applied. P-values less than 0.05 (*) were considered significant. P-values less than 0.01 are marked as **, and less than 0.001 as ***.

Accession numbers

Microarray Raw data have been submitted on GEO Omnibus site: GSE72626.

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Conflict of interests

The authors declare that they have no conflict of interest with the content of this article.

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Keywords:
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Abbreviations used:
GLP, G9a-like protein; H3K9, histone H3 lysine 9; KMT, lysine methyltransferase; KDM, lysine demethylase; MCK, muscle creatine kinase; FBS, fetal bovine serum; ChIP, chromatin immunoprecipitation; IP, immunoprecipitation; SD, standard deviation; WB, western blot.

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