The Protein Arginine Methyltransferases 1 and 5 affect Myc properties in glioblastoma stem cells

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Protein Arginine (R) methylation is the most common post-translational methylation in mammalian cells. Protein Arginine Methyltransferases (PRMT) 1 and 5 dimethylate their substrates on R residues, asymmetrically and symmetrically, respectively. They are ubiquitously expressed and play fundamental roles in tumour malignancies, including glioblastoma multiforme (GBM) which presents largely deregulated Myc activity. Previously, we demonstrated that PRMT5 associates with Myc in GBM cells, modulating, at least in part, its transcriptional properties. Here we show that Myc/PRMT5 protein complex includes PRMT1, in both HEK293T and glioblastoma stem cells (GSCs). We demonstrate that Myc is both asymmetrically and symmetrically dimethylated by PRMT1 and PRMT5, respectively, and that these modifications differentially regulate its stability. Moreover, we show that the ratio between symmetrically and asymmetrically dimethylated Myc changes in GSCs grown in stem versus differentiating conditions. Finally, both PRMT1 and PRMT5 activity modulate Myc binding at its specific target promoters. To our knowledge, this is the first work reporting R asymmetrical and symmetrical dimethylation as novel Myc post-translational modifications, with different functional properties. This opens a completely unexplored field of investigation in Myc biology and suggests symmetrically dimethylated Myc species as novel diagnostic and prognostic markers and druggable therapeutic targets for GBM.

The Myc (c-myc) oncogene is a transcription factor required for pivotal cell functions. It regulates cell proliferation, metabolism, stemness maintenance and binds to 10% of genomic loci, usually amplifying transcription from already active genes. Myc expression and function are deregulated in a wide variety of cancers, including glioblastoma multiforme (GBM). Despite more than a decade of advances in surgery and chemo-/radiotherapy, GBM treatment options are disconcertingly very poor and patients’ life expectancy is very limited (14.6 to 18 months from diagnosis). GBM poor prognosis and deadly outcome seem to rely on the presence of tumour initiating cells, which share many molecular and biological features with embryonic and adult stem cells and thus are called glioblastoma stem cells (GSCs). They are difficult to eradicate by surgery and are resistant to conventional radio- and chemotherapy, allowing tumour to recur overtime. They also present the so-called “Myc signature”, emphasizing the essential role Myc plays in maintaining GSC properties.

Myc is regulated by a series of post-translational modifications: phosphorylation, acetylation, sumoylation, ubiquitination and consequent degradation by the proteasome machinery. These modifications have a precise role in modulating Myc activity and have been suggested as potential therapeutic targets.

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Figure 1. Myc/PRMT5/PRMT1 complex. (a) Western blot. HEK293T cells were transfected with an empty or a FlagMyc expression vector. After 48 hrs, proteins were resolved onto a 12% polyacrylamide gel. β-actin was used as loading control. Uncropped images are shown in Supplementary Fig. S1a. (b) Western blot. Both HEK293T cells and GSCs were infected with a doxycycline inducible lentivirus carrying a shRNA against Myc (shMyc). After 48 hrs from doxycycline treatment, cells were lysed and proteins resolved onto a 12% polyacrylamide gel. Uncropped images are shown in Supplementary Fig. S1b. (c,d) Immunoprecipitations. FlagMyc/HEK293T cells and GSCs underwent reciprocal immunoprecipitation by using anti-Flag, anti-Myc, anti-PRMT1 and anti-PRMT5 antibodies (and control IgGs). Uncropped images are shown in Supplementary Fig. S1c,d. (e) Western blot. HEK293T cells were transfected with a scrambled siRNA or a pool of siRNAs against PRMT5 or PRMT1. Uncropped images are shown in Supplementary Fig. S1e. (f) Immunoprecipitation. HEK293T cells were transfected with a scrambled siRNA or a siRNAs pool against PRMT5. The day after, cells were transfected again with the FlagMyc expression vector. After further 48 hrs cells were immunoprecipitated with anti-PRMT5, anti-PRMT1 or anti-Flag antibodies (or control IgGs). Input is shown in the middle panel. The cartoon on the right panel outlines immunoprecipitation results. Uncropped images are shown in Supplementary Fig. S1f. (g) Immunoprecipitation experiments as in (f) in cells partially depleted of PRMT1 (see input, middle panel). The right panel outlines immunoprecipitation results. Uncropped images are shown in Supplementary Fig. S1g.
Methylation of arginine (R) residues is the most common methylation event in mammalian cells, which modifies protein interacting properties, ruling a large amount of signaling pathways. Three distinct types of methylated Rs occur in mammalians. Asymmetrically dimethylated arginine (ADMA) is the most frequent, followed by symmetrically dimethylated (SDMA) and monomethylated arginine (MMA). The enzymes responsible for R methylation are the Protein Arginine Methyltransferases (PRMTs). Six proteins have been reported to undoubtedly possess the R methyltransferase catalytic domain (PRMT1, 3, 4, 5, 6 and 8). PRMT2, 7 and 9 have sequence similarity with the other PRMTs and are supposed to possess an equivalent enzymatic activity. Depending on the position of the methyl groups on R terminal guanidino nitrogens, PRMTs are, basically, classified into two types: type I PRMTs (PRMT1, 3, 4, 6 and 8) are responsible for MMA or ADMA whereas the type II PRMT5 the position of the methyl groups on R terminal guanidino nitrogens, PRMTs are, basically, classified into two types: type I PRMTs (PRMT1, 3, 4, 6 and 8) are responsible for MMA or ADMA whereas the type II PRMT5 is associated with transcriptionally active chromatin. PRMT1 is deregulated in a wide variety of cancer types, e.g. pancreatic adenocarcinoma, gastric and lung cancer. It controls the epithelial-mesenchymal transition (EMT) in cancer cells, while playing a major role in muscle and B cell differentiation. PRMT5 has received more attention and has been suggested as a putative druggable target in a wide variety of tumours, including GBM. Like PRMT1, it has multiple nuclear and cytoplasmic targets and catalyzes the symmetrical (S) dimethylation of R3 on histone H4, usually repressing transcription, and of R2, activating transcription. PRMT5 is involved in a variety of cellular processes, such as neurogenesis, myogenesis, somatic cell reprogramming and GSCs self-renewal. Interestingly, PRMT1 and PRMT5 have been shown to interact in GBM cells, being PRMT1 the major responsible for the activation of cancer-related genes. They also catalyze the S- and AS-dimethylation of NMyc, and, very recently, it has been shown that PRMT1 is required for p300 recruitment to Myc-regulated gene promoters.

We previously demonstrated that PRMT5 interacts with Myc in glioblastoma cells, including GSCs, and that PRMT5 participates, at least in part, in the activation of Myc target genes. Here we show that PRMT1 associates with Myc/PRMT5 in both HEK293T cells and GSCs and that Myc is both S- and AS-dimethylated by PRMT5 and PRMT1, respectively, both in vitro and in living cells. At the functional level, S-dimethylation protects Myc from degradation, while AS-dimethylation ensure Myc proper turnover. Finally, the inhibition of either PRMT1 or PRMT5 activity affects Myc recruitment at promoters and has a profound effect on GSCs biological functions, such as neurospheres formation and differentiation capacity. These findings represent the first demonstration in GSCs of the presence of differentially dimethylated Myc species, with distinct properties, opening a completely new field of investigation in Myc-dependent GBM biology. Further, they support the hypothesis that acting on S-Myc post-translational modification may represent a possible route to control its function.

Results

Myc interacts with PRMT1 and PRMT5. We have previously shown that Myc induces S-dimethylation of R3 on histone H4 (H4R3me2s, Fig. 1a, left and ref.) and associates with PRMT5 in both HEK293T and glioblastoma cells. Since PRMT5 and PRMT1 were found associated in GBM cells, we sought to determine whether Myc was able to promote also AS-dimethylation of R3 on histone H4 (H4R3me2as). To this aim, HEK293T cells were transfected with either a Flag-tagged Myc construct (FlagMyc/HEK293T) or an empty vector and the level of H4R3me2as was detected by western blot. Figure 1a, right, shows H4R3me2as induction in the presence of FlagMyc construct. We reasoned that these histone modifications should decrease by Myc interference. However, in both HEK293T and mesenchymal GSCs transduced with a lentiviral, doxycycline inducible shRNA against Myc (shMyc), the level of H4R3me2s were reduced, while H4R3me2as increased (Fig. 1b), suggesting that impairing Myc-dependent PRMT5 activity is still sufficient to make H4R3 available for PRMT1 activity. Based on these data, we asked whether PRMT1, PRMT5 and Myc may interact. A series of reciprocal immunoprecipitation experiments, performed in FlagMyc/HEK293T cells, showed that FlagMyc associates with both PRMT5 and PRMT1 (Fig. 1c). No interactions were observed by transfecting the CBs-Flag vector alone, as expected (not shown). Consistently, the same result was obtained, at the endogenous level in GSCs (Fig. 1d). Overall, these data validate PRMT5/Myc interaction and indicate PRMT1 as a novel partner in this protein complex.

PRMT5 is required for the formation of Myc/PRMT5/PRMT1 protein complex. We next wondered which protein member was necessary for complex assembly. Therefore, PRMT5 and PRMT1 expression was blunted by specific siRNAs in HEK293T cells (Fig. 1e). In siPRMT5/HEK293T cells, PRMT5 depletion was associated with a decrease in H4R3me2s levels, as expected, and with an increase in H4R3me2as, underlying the competition between PRMT5 and PRMT1 for the same histone substrate. Intriguingly, Myc protein also decreased. No effect on PRMT1 expression was observed. In siPRMT1/HEK293T cells, PRMT1 decreased together with H4R3me2s levels, as expected, while H4R3me2as increased. Myc protein slightly increased, while no effect on PRMT1 expression was detected. Although PRMT5 depletion was incomplete, Fig. 1f shows that the anti-Flag antibody did not co-immunoprecipitate PRMT1; accordingly, the anti-PRMT1 antibody did not co-immunoprecipitate the FlagMyc protein, as outlined in the model shown in the right panel. In cells partially depleted of PRMT1 (Fig. 1g) we were still able to observe a residual amount of PRMT5/PRMT1/FlagMyc complex, when an anti-PRMT1 antibody was used for immunoprecipitation. However, in anti-Flag and anti-PRMT5 immunoprecipitates, PRMT1 was undetectable, whereas PRMT5 and FlagMyc still interacted (see the scheme in the right panel). Overall, these data suggest that Myc/PRMT1 binding is mediated by PRMT5 in FlagMyc/HEK293T cells.

Myc is both symmetrically and asymmetrically dimethylated. We wondered whether Myc was dimethylated by both PRMT5 and PRMT1. To ask this question, in vitro methylation assays were performed...
Figure 2. Symmetrical and asymmetrical Myc dimethylation. (a) Top, left. Western blot showing the efficacy of a siPRMT5 in inhibiting the expression of PRMT5-6myc (PRMT5-myc in the figure). Top, right. *In vitro* methylation assay showing S-dimethylation of a recombinant Myc protein in the presence of an overexpressed PRMT5. Bottom, left. Western blot showing the efficacy of a siPRMT1 in inhibiting the expression of PRMT1-myc-Dkk (PRMT1-myc in the figure). Bottom, right. *In vitro* methylation assay showing AS-dimethylation of a recombinant Myc protein in the presence of an overexpressed PRMT1. Uncropped images are shown in Supplementary Fig. S2a. (b) Left. Western blot analysis showing PRMT1 and PRMT5 expression levels upon FlagMyc transfection in HEK293T cells. Middle. FlagMyc/HEK293T cells underwent immunoprecipitation with control IgG or ASYM24, SYM10 and anti-Flag antibodies. Western blot were performed by using anti-Flag antibody for ASYM24 and SYM10 immunoprecipitations and ASYM10 or SYM10 antibodies for anti-Flag immunoprecipitation. Right. Western blot showing the input of the immunoprecipitations. Uncropped images are shown in Supplementary Fig. S2b. (c) Left. FlagMyc/HEK293T cells were treated for 24 hrs with EPZ015666 or 9i. After additional 24 hrs, immunoprecipitations were performed by using either SYM10 or ASYM24 antibody. Uncropped images are shown in Supplementary Fig. S2c. (d) The model depicts the competition between PRMT1 and PRMT5 for Myc. Inhibiting PRMT5 activity with EPZ015666 restrained FlagMyc S-dimethylation, while enhancing AS-dimethylation (left). The opposite was obtained by inhibiting PRMT1.
in HEK293T cells, overexpressing either myc-tagged PRMT5 or PRMT1, in the presence or absence of specific siRNAs (Fig. 2a). S- or AS-dimethylation of a recombinant human Myc protein was detected by immunoprecipitation experiments using antibodies recognizing either S- (SYM10 antibody) or AS- (ASYM24 antibody) dimethylated R residues. The recombinant human Myc was S-dimethylated in the presence of overexpressed PRMT5. S-dimethylation was restrained in the presence of a pool of PRMT5-specific siRNAs, as expected (Fig. 2a, top). Recombinant Myc AS-dimethylation was detected in the presence of a PRMT1-myc-Dkk overexpressed plasmid. No signal was observed in the presence of a specific siPRMT1 pool (Fig. 2a, bottom).

Moreover, protein extracts from HEK293T/FlagMyc cells were reciprocally immunoprecipitated by using SYM10 or ASYM24 or anti Flag antibodies. Western blots were performed with anti-Flag or SYM10 and ASYM24 antibodies. Figure 2b, middle panel, shows the presence of both S and AS-dimethylated FlagMyc in HEK293T cells. HEK293T cells transfected with pcBSFlag vector alone represented the negative control (not shown). To confirm that S- and AS-Myc dimethylation were PRMT3 and PRMT1-dependent, respectively, HEK293T/FlagMyc cells were treated for 24 hours with either 5 μM EPZ015666 – a commercially available PRMT5 specific inhibitor – or 3 μM 1,5-bis(3,5-Br2-4-hydroxyphenyl)-1,4-pentadien-3-one, hereafter named inhibitor 9 (9i), which specifically inhibits PRMT1 at this concentration30. Therefore, immunoprecipitation experiments were performed with SYM10 and ASYM24 antibodies. Figure 2c, left, shows that PRMT5 inhibition impairs FlagMyc S-dimethylation, as expected. Conversely, PRMT5 inhibition increases the level of AS-dimethylated FlagMyc. These results were mirrored by similar experiments performed on cells treated with 9i. Indeed, PRMT1 inhibition reduced the level of AS-FlagMyc, while enhancing S-FlagMyc (Fig. 2c, left and scheme in d). In siPRMT1 transfected HEK293T/FlagMyc cells, S-FlagMyc was found at low levels, as expected. Interestingly, also AS-FlagMyc decreased (Fig. 2e). This is not surprising, as FlagMyc/PRMT1 interaction requires the presence of PRMT5 in HEK293T cells (Fig. 1f). In siPRMT1 transfected HEK293T/FlagMyc cells (Fig. 2e) we detected higher levels of S-FlagMyc, whereas AS-FlagMyc was severely impaired, confirming the binding of PRMT5 and Myc in the absence of PRMT1, as previously shown in Fig. 1g.

PRMT1 and PRMT5 modulate Myc stability. It has been shown that both PRMT1 and PRMT5 stabilize NMyc protein30,31. We observed that upon PRMT5 interference Myc expression decreased, while in PRMT1-depleted cells, Myc levels slightly increased (Fig. 1e). The same results were obtained inhibiting PRMT5 or PRMT1 activity, respectively (Fig. 2c, right). Therefore, two sets of experiments were performed, in order to investigate whether Myc stability was affected by either PRMT5 or PRMT1 catalytic inhibition in HEK293T/FlagMyc cells and in GSCs. To this aim, cells were treated with 5 μM EPZ015666 and 3 μM 9i for 24 hrs. Thereafter, cycloheximide (CHX, Fig. 3a,c) or the proteasome inhibitor MG132 (Fig. 3b,d) were added to the culture medium, along a time course between 0 and 100 minutes (CHX) or 0 and 360 minutes (MG132). In HEK293T/FlagMyc cells, we found that PRMT5 inhibition produced a decrease in FlagMyc protein levels upon CHX administration (Fig. 3a, middle), when compared to the control vehicle. This effect was also confirmed, and more evident, on endogenous Myc in GSCs (Fig. 3c, middle). Importantly, the enhanced degradation of Myc protein in the presence of EPZ015666 was not responsible for the decrease in Myc S-dimethylation (Fig. 2c). Indeed, immunoprecipitation experiments revealed that MG132 treatment did not recover S-Myc protein levels but only total FlagMyc (Supplementary Fig. S6), indicating that the decrease in Myc S-dimethylation was due to PRMT5 catalytic inhibition, although a combined effect with the proteasome degradation may also be hypothesized. Concretely, PRMT1 inhibition stabilized both FlagMyc and endogenous Myc upon CHX treatment (Fig. 3a,c, right). Proteasome inhibition by MG132 produced a similar, slight, but statistically significant, increase of FlagMyc protein levels in EPZ015666 and 9i HEK293T/FlagMyc treated cells (Fig. 3b). In GSCs, control vehicle and EPZ015666 produced similar effects upon MG132 exposure (Fig. 3d, left and middle, respectively). Conversely, 9i dramatically enhanced endogenous GSCs Myc protein content upon MG132 administration (Fig. 3d, right). These experiments suggest that in both HEK293T cells and GSCs, PRMT5 have a protective effect on Myc protein, while PRMT1 may allow its proper turnover or promote its degradation. However, these effects are particularly evident in GSCs.

Myc is preferentially S-dimethylated in GSCs grown as neurospheres. To evaluate endogenous Myc dimethylation in GSCs, neurospheres were treated for 24 hrs with either 5 μM EPZ015666 or 3 μM 9i. Cell extracts were immunoprecipitated with either SYM10 or ASYM24 antibody. An anti-Myc antibody was used to detect endogenous Myc. As shown in Fig. 4a, EPZ015666 treatment inhibited and 9i enhanced S-Myc, which represented the major Myc dimethylated species in GSCs. Consistently, 9i treatment decreased and EPZ015666 increased AS-Myc levels, which were, conversely, barely detectable in vehicle-treated GSCs. The presence of almost exclusively S-Myc species in GSCs grown as neurospheres prompted us to investigate whether Myc dimethylation pattern could change exposing GSCs to different stimuli. Indeed, we found that in GSCs grown in 2% FBS-containing medium, which promotes their astrocytic differentiation30, S-Myc levels were progressively reduced along a time course between 4 hours and 7 days, while AS-Myc remained quite stable (Fig. 4b, left). Consistently, almost total disappearance of PRMT5 and a scarce effect on PRMT1 expression upon FBS culture were observed, together with a reduction in Myc levels (Fig. 4b, middle). This also paralleled with immunoprecipitation experiments showing that Myc and PRMT1 could still interact in differentiating GSCs, while PRMT5 was
only barely detected (Fig. 4b, right and model in panel c). In summary, these data suggest that also in GSCs Myc is both S- and AS-dimethylated and that PRMT5 and PRMT1 are competitors for Myc as a substrate. Furthermore, S-dimethylated Myc is the prevalent Myc species in GSCs grown as neurospheres whereas in differentiating GSCs its levels strongly decrease in a time-dependent manner. Differently, PRMT1 protein level and activity were weakly reduced during GSCs differentiation.

Figure 3. PRMT1 and PRMT5 control Myc protein stability. FlagMyc/HEK293T cells (a) and GSCs (c) were treated with either DMSO, EPZ015666 or 9i for 24 hrs. Thereafter, cycloheximide was added along a time course between 0 and 100 minutes and western blot analysis was performed. FlagMyc/HEK293T cells (b) and GSCs (d) were treated with either EPZ01566 or 9i for 24 hrs. Thereafter, MG132 was added along a time course between 0 and 360 minutes and western blot analysis was performed. *p < 0.05 vs DMSO. Uncropped images are shown in Supplementary Fig. S3a–d.
PRMT1 and PRMT5 inhibition differentially modulates Myc recruitment at its target promoters in GSCs. To evaluate the effect of PRMT1 and PRMT5 on Myc binding at its specific chromatin loci in GSCs, ChIP experiments were performed, either in stem or differentiation conditions, in the presence or absence of EPZ015666 or 9i or control vehicle (Fig. 5). Two well known Myc promoter targets - cyclin D1 (CCND1) and the Epithelial Growth Factor Receptor variant III (EGFRvIII)\textsuperscript{37} - the stem gene promoters SRY-box 2 (SOX2), bound by Myc in GSCs\textsuperscript{34} together with the Glial Fibrillary Astrocytic Protein (GFAP) promoter\textsuperscript{38}, were tested. In stem conditions, either PRMT5 or PRMT1 inhibition did not affect Myc binding on CCND1 and EGFRvIII promoters. Conversely, SOX2 promoter was sensitive only to PRMT1 inhibition, whereas, either PRMT5 or PRMT1 inhibition decreased Myc binding on GFAP promoter. Interestingly, Myc detached from all these promoters in differentiating control cells. PRMT5 inhibition had no effect on Myc recruitment in these experimental conditions, whereas 9i treatment restored Myc binding above control levels only on CCND1 and EGRvIII promoters. Altogether, these data address a role to both PRMT5 and PRMT1 catalytic activity in regulating Myc loading at promoters.

PRMT5 affects GSCs cell cycle progression and neurospheres formation. To evaluate in depth the effect of either PRMT1 or PRMT5 on GSCs biological properties, cell cycle, neurospheres formation, viability and apoptosis were evaluated. Cell cycle analysis (Fig. 6a, left) shows that a low, but statistically significant, number
of GSCs treated for 7 days with EPZ015666 were arrested in S-phase, whereas an accumulation of cells in the subG1 phase was mostly observed upon 9i administration. Consistent with the role of PRMT5 in sustaining GSCs self-renewal28, neurospheres formation was extremely compromised after 10 days of EPZ015666 treatment, while 9i had no effect, when compared to control solvent (Fig. 6a, middle). This was paralleled by a decrease of SOX2 protein upon EPZ015666 exposure (Fig. 6a, right). Nevertheless, no differences in cell viability nor in apoptosis rate were observed in the presence of the two drugs, when compared to controls (not shown).

**PRMT1 affects GSCs differentiation.** As known, mesenchymal GSCs differentiate into astrocytes when cultured in the presence of foetal bovine serum (FBS)36 and we found that, in this condition, Myc is preferentially AS-dimethylated (Fig. 4b). Therefore we asked whether the inhibition of either PRMT1 or PRMT5 catalytic activity could modulate GSCs differentiation properties. To this aim, GSCs were pre-treated for 24 hrs with either EPZ015666 or 9i (or control vehicle); thereafter cells were shifted to FBS-containing medium and allowed to differentiate for 7 days. Drugs were refreshed every 48 hrs. Immunofluorescence analysis showed that control cells expressed GFAP but were negative for the stemness markers SOX2, Oct4, Nanog and Olig239 (Fig. 7a). Also TuJ1, a neuronal marker, was undetectable, as expected. Comparable data were obtained with EPZ015666 (Fig. 7b,d), whereas different results were observed upon PRMT1 inhibition. Differentiating GSCs exposed to 9i still did not express Olig2, TuJ1, SOX2, Oct4 and Nanog proteins, and dramatically lost GFAP (Fig. 7c,d), as also
confirmed by a decrease in its mRNA level, as shown in panel e. mRNAs encoding for the other protein markers analyzed were not modulated, upon neither PRMT5 nor PRMT1 inhibition (not shown). Furthermore, since PRMT1 activity is associated with astrocytic differentiation40, we monitored H4R3me2as and H4R3me2s levels. A prevalence of H4R3me2as was observed in control and EPZ015666 treated cells. Consistently, 9i blocked H4R3 AS-dimethylation, whereas a slight increase in H4R3me2s was observed (Fig. 7d). In summary, PRMT1 appears to support GSCs astrocytic differentiation, since its inhibition led to the loss of FBS-induced GFAP-positive cells. On the contrary, PRMT5 inhibition did not have any impact on GSCs commitment into the astrocytic lineage.

Discussion

GBM still represents a great challenge for oncologists. Its high genetic, epigenetic and phenotypic heterogeneity have made impossible to identify valuable therapeutic routes, so far. PRMT1, PRMT5 and Myc have been all indicated as important players in GBM progression5,41. Specifically, Myc and PRMT5 have been proved as master regulators of GSC pluripotency8,28, while PRMT1 loss does not affect typical pluripotency markers42. Myc interacts with PRMT543 and PRMT1 is required for p300 recruitment at Myc-regulated gene promoters43. Further, PRMT1 and PRMT5 interact in GBM29. However, no direct association between Myc, PRMT1 and PRMT5 has been reported. Here we show, for the first time, that Myc, PRMT5 and PRMT1 form a ternary complex, both in recipient HEK293T cells and GSCs (Fig. 1c,d). More importantly, we show that Myc is dimethylated by both

Figure 7. PRMT1 inhibition impairs GSCs astrocytic differentiation. (a–d) GSCs were pre-conditioned for 24 hrs in the presence of EPZ015666, 9i or control vehicle. The day after, cells were dissociated and plated in DMEM/F12 plus 2% FBS for 7 days. Drugs were refreshed every 48 hrs. Thereafter, cells were fixed and processed in immunofluorescence analyses. (e) Real time PCR for GFAP. *p < 0.05 vs DMSO.
PRMT1 and PRMT5 (Figs 2 and 4), being the presence of PRMT5 required for Myc/PRMT1 binding, at least in HEK293T cells (Figs 1f and 2e). Therefore, PRMT5 seems to function not only as a catalytic enzyme but also as a fundamental platform of the complex (Fig. 1f), at least in this cell system. Indeed, PRMT5 inhibition impaired also Myc AS-dimethylation (Fig. 2e). The inhibition of the catalytic activity of either PRMT1 or PRMT5 enhances Myc S- or AS-dimethylation, respectively (Figs 2b,c and 4a), without disrupting the complex and indicating that, as occurs for H4R3, they compete for Myc as a substrate. Myc dimethylation is consistent with the S- and AS-dimethylation of other transcription factors. Indeed, PRMT5 dimethylates and activates the Nuclear Factor kB (NF-kB) p65 subunit43, while AS-dimethylation by PRMT1 results in an impairment of the NF-kB RelA

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In conclusion, our work reports, for the first time, that Myc associates with both PRMT1 and PRMT5 being differentially dimethylated. S-dimethylation protects Myc from degradation, while AS-dimethylation allows Myc proper turnover. We hypothesize S-Myc as typical of aggressive GSCs, as S-Myc/AS-Myc ratio decreases in favour of AS-Myc. Consistently, we still detected Myc/PRMT1 binding after 7 days of differentiation (Fig. 4b, middle), together with a residual amount of PRMT5 (Fig. 4b, middle). However, it has to be still determined whether Myc/PRMT1 interaction is direct or indirect and we cannot exclude the presence of other interactors to mediate Myc/PRMT1 association (see model in Fig. 4c).

Medium/F12 mixture (1:1), 1% Glutamine, 1% Penicillin/Streptomycin and 2% Foetal Bovine Serum (FBS, Merck, Darmstadt, Germany).

We are aware that our observations lack mechanistic insights. In fact, the modifications in Myc binding ability at promoters (Fig. 5), GSCs cell cycle profile, failure of neurospheres formation (Fig. 6) and changes in the differentiation capability (Fig. 7) cannot be definitely addressed to S- or AS-Myc species. The identification of Myc dimethylated arginine(s), the production of specific, either unmethylatable or methyl-mimicking, Myc mutants, followed by depletion/reconstitution experiments to substitute the endogenous wild type Myc, is still missing and will be object of our future investigations.

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Methods

Cell culture and treatments. HEK293T and mesenchymal Brain Tumour 168 Neural Stem Cells33,36 were cultured as described35. For differentiation experiments, GSCs were cultured in Dulbecco’s Modified Eagle Medium/F12 mixture (1:1), 1% Glutamine, 1% Penicillin/Streptomycin and 2% Foetal Bovine Serum (FBS, Merck, Darmstadt, Germany).

EPZ015666 (Merck) was administered at a concentration of 5 μM and the PRMT1 inhibitor 1,5-bis(3,5-Br2-4-hydroxyphenyl)-1,4-pentadien-3-one (9i) at 3 μM45. Control solvent was represented by dimethylsulfoxide
Western blot analyses. Cells were directly lysed in Laemmli buffer and western blots were performed as described33. Anti-H4R3me2s (cat. #ab5823; dilution 1:1000), anti-H4R3me2as (cat. #ab194683; dilution 1:1000), anti-PRMT5 (cat. #ab109451; dilution 1:1000) and anti-PRMT1 (cat. #ab37678; dilution 1:400) antibodies were from Abcam (Cambridge, UK). Anti-Myc (clone 9E10, cat. #sc-40; dilution 1:500) was from Santa Cruz Biotechnology (Dallas, TX, U.S.A.). Anti-actinHRP (cat. #A3854; dilution 1:5000) was from Merck. Anti-SOX2 (cat. #MAB4343; dilution 1:500), SYM10 (cat. #07-412; dilution 1:1000) and ASYM24 (cat. #07-414; dilution 1:1000) antibodies were from Millipore (Burlington, MA, U.S.A.). Anti-Flag antibody (cat. #F1804; dilution 1:1000) was from Merck. Secondary antibodies were donkey anti-rabbit IgG Alexa Fluor 555 (cat. #A5040, dilution 1:5000) and anti-mouse IgG (cat. #A9044; dilution 1:2000) were from Merck. In most cases, filters were directly cut after the blocking procedure, according to the molecular weight of the proteins of interest. For immunofluorescence experiments: cells were captured with a ChemiDoc XRS+ Gel Imaging System (Bio-Rad, Hercules, CA, U.S.A.) and the band intensity of western blot images was slightly adjusted with the Power Point software.

Immunoprecipitation. For immunoprecipitation experiments, cells were lysed in 140 mM NaCl, 10 mM Tris pH 7.6, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA, protease and phosphatase inhibitors. Cells were collected on ice for 30′, then centrifuged for 20′ at 4 °C, at 13000 rpm. Thereafter, supernatant (1–2 mg) underwent immunoprecipitation as described33, by using 4 μg of each antibody or control IgGs (mouse IgGs were from Santa Cruz, cat. #sc-2025; rabbit IgGs were from Millipore, cat. #sc-18140). To avoid IgGs detection, either ImmunoCruz IP/WB Optima B, C, F, E (Santa Cruz Biotechnology; cat. #sc-45039, cat. #sc-45040, cat. #sc-45043, cat. #sc-45042, respectively) or the VeriBlot reagent (Abcam; cat. #ab131366 and #ab131368) were used in western blot, after the immunoprecipitation procedure, according to the manufacturer’s instructions.

Plasmid transfection and lentiviral transduction. Plasmid transfections were performed by using Lipofectamine 2000 (ThermoFisher Scientific, Waltham, MA, U.S.A.) according to the manufacturer’s instructions. Plasmids were: pCBSFlagMyc33, pCMVPRMT5-6myc (courtesy of Dr. Levi A.) and pCMV-V6PRMT1-myc-Dkk (Origene, Rockville, MD, U.S.A.), together with the corresponding empty vectors. The shMyc insert was subcloned from the KRAB lentiviral vector into the Tet-on pSLIK lentivirus33. Lentiviral production and infection were performed as described33.

siRNA transfection. siPRMT5, siPRMT1 (ON-Target Plus Human PRMT5 and PRMT1 siRNA, Dharmacon, Lafayette, CO, U.S.A.) and scrambled siRNAs (sc-44230, Santa Cruz Biotechnology) were transfected by using the DHARMAfect reagent (Dharmacon), according to the manufacturer’s instructions.

In vitro methylation assay. For in vitro methylation assays, HEK293T cells (3,5 × 105) were transfected with 50 nM siRNAs (or scrambled siRNA) and the day after with either PRMT5-6myc or PRMT1-myc-Dkk plasmids or control vectors (10 μg). 48 hrs later, cells were lysed in 20 mM Hepes pH 7.9, 137 mM NaCl, 20% Glycerol, 1% Nonidet P40, 2 mM EDTA, protease and phosphatase inhibitors. Cell extracts were kept in rotation for 30′, at 4 °C and then centrifuged at 12000 rpm for 20′ at 4 °C. Cell extracts were pre-cleared with 5 μg of control IgGs, for 1 hr on ice; thereafter, 20 μl of equilibrated protein A/G Plus agarose beads (Santa Cruz Biotechnology; cat. #sc-2003) were added and the mixture was kept in rotation for 30′, at +4 °C, and then centrifuged at 10000 rpm, at 4 °C. The supernatant was incubated with either anti-Myc antibody (Santa Cruz Biotechnology), or control IgG, for an overnight, at 4 °C (to purify either overexpressed PRMT5-6myc or PRMT1-myc-Dkk). The day after, 40 μl of equilibrated protein A/G Plus agarose beads were added and the mixture was incubated in rotation for 4 hrs, at 4 °C. Beads were extensively washed with lysis buffer by centrifuging at 3000 rpm, at 4 °C. Beads and bound PRMT5-6myc or PRMT1-myc-Dkk were resuspended in assay buffer (20 mM Tris HCl pH 7.5, 20% glycerol, 100 mM KCl, 1 mM DTT, 0.2 mM EDTA, protease and phosphatase inhibitors) and 500 ng of human recombinant c-Myc protein (Abcam; cat. #ab84132) plus 1 mM of S-adenosylmethionine (Merck) were added. The reaction mixture (100 μl) was incubated for 1 hr, at 35 °C. Thereafter, 400 μl of assay buffer were added, and dimethylated Myc was immunoprecipitated by incubating the reaction mixture with 4 μg of either SYM10 or ASYM24 antibody (Millipore), for an overnight, at 4 °C. The day after, 40 μl of equilibrated protein A/G Plus agarose beads were added and the mixture incubated for 4 hrs, in rotation, at 4 °C. After three washes in assay buffer, 20 μl of Laemmli buffer 2X were added and the immunocomplexes boiled for 5′. Dimethylated Myc was revealed by western blot.

Immunofluorescence analyses. Immunofluorescence analyses were performed as described33. Anti-Olig2 (cat. #sc-293163; dilution 1:50), anti-Tuj1 (cat. #sc-51670; dilution 1:50), anti-Oct4 (cat. #sc-101534; dilution 1:50) antibodies were from Santa Cruz Biotechnology; anti-SOX2 (cat. #MAB4343; dilution 1:50) and anti-Nanog (clone 7F1.1, cat. #MAB2D4; dilution 1:50) were from Millipore. Anti-GFAP (cat. #ab7260; dilution 1:100), anti-H4R3me2s (cat. #ab5823; dilution 1:100) and anti-H4R3me2as (cat. #ab194683; dilution 1:100) were from Abcam. Secondary antibodies were donkey anti-rabbit IgG Alexa Fluor 555 (cat. #A-31572; dilution 1:100) and goat anti-mouse Alexa Fluor 488 (cat. #A-11029; dilution 1:100), both from Thermo Fisher Scientific.

Cell cycle analysis. Cells were dissociated, counted, resuspended in PBS and then fixed in 70% ethanol for a minimum of 24 hrs. After three washes in PBS, cells were incubated with 50 μg/ml Propidium Iodide (Merck) plus 0.1 U/L RNase (Merck) for 3 hrs at room temperature before FACS analysis by Coulter Epics XL flow cytometer.
(Beckman Coulter, Brea, CA, U.S.A.). Cells were gated using both area vs peak to eliminate doublets and forward vs side scatter to exclude debris. The percentage of cells in the different cycle phases and in subG1 (composed of dead cells and debris) was calculated using FACS Express Flow Research Edition software.

**Neurospheres formation.** 3 × 10^5 GSCs were plated in duplicate into a six-well multi-plate. Cells were allowed to grow for 10 days, in the presence or absence of EPZ015666 or 9i or control vehicle. Neurospheres formation was monitored daily by a phase contrast microscope. Images were captured with the ZOE Fluorescent Cell Imager (Bio-Rad).

**Real time PCR.** Real time PCR were performed as described.

**Chromatin Immunoprecipitation.** Chromatin immunoprecipitations were performed as described.

**Oligonucleotides.** ChiPs oligonucleotides were:

- hEGFr3prom_F GGCTGTTTTGTCAGCAGCTTTA
- hEGFr3prom_R TTGAAGCCAAATGTGTGAAGCA
- hSOX2prom_F AACGGACGTGCCTGACATT
- hSOX2prom_R TCCCATTTGTCGAGCTGA AAA
- hGFAProm_F TGGGAATGAGGCCTAGTAGGAA
- hGFAProm_R GGGAAATTGCTGGCGAAGAAAGA
- hCCND1prom_F GCTTTCATCCATGAGGTGTGT
- hCCND1prom_R CTACCTTGACCGACTGGTCTT
- GFAP oligonucleotides used in real time PCR experiments were:
  - GFAP_F ACCAGGACCTGCTCAGTG
  - GFAP_R ATCTCACCAGTGTCCTACAC

**Quantification and statistical analyses.** All data are expressed as mean ± SEM, and significance (p value) was calculated using two-tailed paired Student’s t test; p values < 0.05 were considered statistically significant.

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**References**

1. Eilers, M. & Eisenman, R. N. Myc's broad reach. Genes Dev. 22, 2755–2766 (2008).
2. Niu, Z. et al. c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. Cell. 151, 68–79 (2012).
3. Lin, C. Y. et al. Transcriptional amplification in tumor cells with elevated c-Myc. Cell. 151, 56–67 (2012).
4. Orian, J. M. et al. UCP2 and PRMT1 are key prognostic markers for lung carcinoma patients. Cancer. 76, 7049–7058 (2016).
5. Liu, W. et al. Nuclear PRMT1 expression is associated with poor prognosis and chemosensitivity in gastric cancer patients. Gastric Cancer. 19, 789–797 (2016).
6. Madreiter-Sokolowski, C. T. et al. UCP2 and PRMT1 are key prognostic markers for lung carcinoma patients. Oncotarget. 8, 80278–80285 (2017).
7. Zhang, Y. et al. Protein arginine methyltransferase 1 coordinates the epithelial-mesenchymal transition/proliferation dichotomy in gastric cancer cells. Exp Cell Res. 362, 43–50 (2017).
8. Banares-Moratalla, A. et al. Arginine methyltransferase 1 promotes tumorigenicity in colorectal cancer cells. Mol Cell Biol. 37, https://doi.org/10.1128/MCB.00457-16 (2017).
9. Infantino, S. et al. Arginine methyltransferase catalyzed by PRMT1 is required for B cell activation and differentiation. Nat Commun. 8, 891 (2017).
10. Lacroix, M. et al. The histone-binding protein COPR3 is required for nuclear functions of the protein arginine methyltransferase PRMT5. EMBO Rep. 9, 452–458 (2013).
11. Migliorini, V. et al. Symmetric dimethylation of H3R2 is a newly identified histone mark that supports euchromatin maintenance. Nat Struct Mol Biol. 19, 136–44 (2012).
25. Chittka, A., Nitaroska, J., Grazini, U. & Richardson, W. D. Transcription factor positive regulatory domain 4 (PRDM4) recruits protein arginine methyltransferase 5 (PRMT5) to mediate histone arginine methylation and control neural stem cell proliferation and differentiation. J Biol Chem. 287, 42995–43006 (2012).
26. Dacwag, C. S., Ohkawa, Y., Pal, S., Sif, S. & Imbalzano, A. N. The protein arginine methyltransferase Prmt5 is required for myogenesis because it facilitates ATP-dependent chromatin remodeling. Mol Cell Biol. 27, 384–394 (2007).
27. Nagamatsu, G. et al. A germ cell–specific gene, Prmt5, works in somatic cell reprogramming. J Biol Chem. 286, 10641–10648 (2011).
28. Banavaswadi-Siddeswada, Y. K. et al. PRMT5–PTEN molecular pathway regulates senescence and self-renewal of primary glioblastoma neurosphere cells. Oncogene. 36, 263–274 (2017).
29. Takai, H. et al. 5-Hydroxymethylcytosine plays a critical role in glioblastomagenesis by recruiting the CHTOP-methylosome complex. Cell Rep. 9, 48–60 (2014).
30. Park, J. H. et al. Protein arginine methyltransferase 5 is a key regulator of the MYCN oncoprotein in neuroblastoma cells. Mol Oncol. 9, 617–627 (2015).
31. Eberhardt, A. et al. Protein arginine methyltransferase 1 is a novel regulator of MYCN in neuroblastoma. Oncotarget. 7, 63629–63639 (2016).
32. Tikhanovich, I. et al. Arginine methylation regulates c-Myc-dependent transcription by altering promoter recruitment of the acetyltransferase p300. J Biol Chem. 292, 13333–13344 (2017).
33. Mongiardi, M. P. et al. Myc and Omomyc functionally associate with the Protein Arginine Methyltransferase 5 (PRMT5) in glioblastoma cells. Sci Rep. 5, 15494 (2015).
34. Galardi, S. et al. Resettling cancer stem cell regulatory nodes upon MYC inhibition. EMBO Rep. 17, 1872–1889 (2016).
35. Mai, A. et al. Synthesis and biological validation of novel synthetic histone/protein methyltransferase inhibitors. Chem Med Chem. 2, 987–991 (2007).
36. De Bacco, E. et al. The MET oncoprotein is a functional marker of a glioblastoma stem cell subtype. Cancer Res. 72, 4537–4550 (2012).
37. Zhao, K. et al. EGFR/c-myc axis regulates TGFβ3/HiPpo/Notch pathway via epigenetic silencing miR-524 in gliomas. Cancer Lett. 406, 12–21 (2017).
38. Panicker, S. P. et al. p300- and Myc-mediated regulation of glioblastoma multiforme cell differentiation. Oncotarget. 1, 289–303 (2010).
39. Kupp, R. et al. Lineage-Restricted OLG2-RTK Signaling Governs the Molecular Subtype of Glioma Stem-like Cells. Cell Rep. 16, 2836–2845 (2016).
40. Honda, M., Nakashima, K. & Katada, S. PRMT1 regulates astrocytic differentiation of embryonic neural stem precursor cells. J Neurochem. 142, 901–907 (2017).
41. Han, X. et al. Expression of PRMT5 correlates with malignant grade in gliomas and plays a pivotal role in tumor growth in vitro. J Neurooncol. 118, 61–72 (2014).
42. Wu, Q. et al. CARM1 is required in embryonic stem cells to maintain pluripotency and resist differentiation. Stem Cells. 27, 2637–2645 (2009).
43. Wei, H. et al. PRMT5 dimethylates R30 of the p65 subunit to activate NF-κB. Proc Natl Acad Sci USA. 110, 13516–13521 (2013).
44. Reintjes, A. et al. Asymmetric arginine dimethylation of RelA provides a repressive mark to modulate TNFα/NF-κB response. Proc Natl Acad Sci USA. 113, 4326–4331 (2016).
45. Zheng, S. et al. Arginine methylation-dependent reader-writer interplay governs growth control by E2F-1. Mol Cell. 52, 37–51 (2013).
46. Nanni, S. et al. Endothelial NOS, estrogen receptor beta, and HIFs cooperate in the activation of a prognostic transcriptional pattern in aggressive human prostate cancer. J Clin Invest. 119, 1093–1108 (2009).

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Author contributions
Favía A. took care of cell cultures, performed transfections, including siRNAs transfections, western blots, immunoprecipitations, FACS analysis, neurospheres formation, apoptosis, viability assays and real time PCR experiments; Salvatori L. supervised the experiments and reviewed the manuscript; Nanni S. performed ChIPs; Iwamoto-Stohl L.K. performed western blots; Valente S. and Mai A. prepared and supplied the PRMT1 inhibitor 9i; Fontanella R. and Scagnoli F. took care of cell cultures and performed western blots; Totta P. contributed to the revised manuscript; Nasi S. reviewed the manuscript; Illi B. designed all the experiments, performed in vitro experiments; Salvatori L. supervised the experiments and reviewed the manuscript; Nanni S. performed ChIPs; Iwamoto-Stohl L.K. performed western blots; Valente S. and Mai A. prepared and supplied the PRMT1 inhibitor 9i; Fontanella R. and Scagnoli F. took care of cell cultures and performed western blots; Totta P. contributed to the revised manuscript; Nasi S. reviewed the manuscript; Illi B. designed all the experiments, performed in vitro experiments, lentiviral infections, immunofluorescences, analyzed results and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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