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Probing p300/CBP Associated Factor (PCAF)-Dependent Pathways with a Small Molecule Inhibitor

Rahul Modak,[†] Jeelan Basha,[†] Narendra Bharathy,[‡] Koustav Maity,[§] Pushpak Mizar,[†] Akshay V. Bhat,[†] Madavan Vasudevan,[∥] Vinay Kumar Rao,[‡] Wai Kay Kok,[‡] Nagashayana Natesh,[⊥] Reshma Taneja,[‡] and Tapas K. Kundu*[,†]

[†]Transcription and Disease Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India 560064

[‡]Department of Physiology, Yong Loo Lin School of Medicine, Block MD9, 2 Medical Drive, National University of Singapore, Singapore 117597

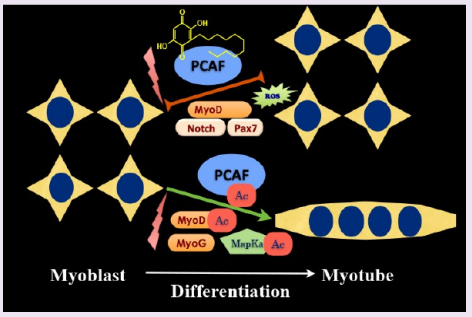
[§]Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India 560012

[∥]Bionivid Technology [P] Ltd, 401 - 4 AB Cross, 1st Main, Kasturi Nagar, East of NGEF, Bangalore, India 560043

[⊥]Central Government Health Scheme Dispensary Number 3, Basavanagudi, Bangalore, India

Supporting Information

ABSTRACT: PCAF (KAT2B) belongs to the GNAT family of lysine acetyltransferases (KAT) and specifically acetylates the histone H3K9 residue and several nonhistone proteins. PCAF is also a transcriptional coactivator. Due to the lack of a PCAF KAT-specific small molecule inhibitor, the exclusive role of the acetyltransferase activity of PCAF is not well understood. Here, we report that a natural compound of the hydroxybenzoquinone class, embelin, specifically inhibits H3Lys9 acetylation in mice and inhibits recombinant PCAF-mediated acetylation with near complete specificity *in vitro*. Furthermore, using embelin, we have identified the gene networks that are regulated by PCAF during muscle differentiation, further highlighting the broader regulatory functions of PCAF in muscle differentiation in addition to the regulation via MyoD acetylation.



PCAF (p300/CBP associated factor, KAT 2B), one of the most important members of the GNAT family of nuclear lysine acetyltransferase (KATs), is a transcriptional coactivator with various biological functions. PCAF is often a part of large multiprotein complexes, which include another major KAT, p300 (KAT 3B). Both PCAF and p300/CBP acetylate histone H3K9 under diverse physiological conditions, making it difficult to distinguish the role of PCAF-mediated acetylation in transcriptional activation. Thus, the coactivator function of PCAF has been better characterized than its KAT activity. The acetylation of nonhistone proteins (e.g., MyoD, p53, and Tat)[1−3] by KATs regulates their biological functions. Significantly, PCAF and p300 have distinct acetylation sites on nonhistone proteins, which have immensely facilitated the identification of their individual functions. Yamauchi et al.[4] have shown that PCAF and its homologue GCN5 play overlapping but distinct roles in mouse embryonic development. PCAF plays a critical role during muscle differentiation. It is part of a large multiprotein complex that includes p300, the chromatin remodeler SWI/SNF, and multiple protein lysine and arginine methyltransferases.[5] PCAF-mediated acetylation of MyoD is essential for MyoD activation and the induction of transcription of its downstream targets, such as myogenin. PCAF and its homologue GCN5 play critical roles in glucose metabolism.[6] A recent report[7] has shown that the KAT activity of PCAF is essential for the activation of p21 transcription and is independent of PCAF-mediated p53 acetylation. These studies underscore the necessity of specific cell-permeable small molecule inhibitors of the PCAF KAT activity to study the hitherto unexplored functions of PCAF.

Several natural, synthetic, and semisynthetic small molecule modulators of KATs have been identified over the past two decades. Natural compounds, such as garcinol and anacardic acid, inhibit both p300 and PCAF. The internal cyclization of garcinol yielded the nonspecific KAT inhibitor isogarcinol, which was further derivatized to synthesize 3 major compounds, including LTK-14, a nontoxic p300-specific inhibitor. Curcumin is a p300-specific HAT inhibitor but has several pleiotropic effects *in vivo* [ref 8 and references therein]. The water-soluble derivative of curcumin CTK7A, however, can efficiently inhibit the KAT activity of p300/CBP and PCAF.[9] Another natural compound, plumbagin, is a p300-specific KAT inhibitor.[10] The first reported synthetic KAT inhibitors lysyl-CoA and H3-CoA-20 are specific toward p300 and PCAF, respectively. Both molecules are relatively large and have been tagged with Tat-peptide to increase their cell permeability and

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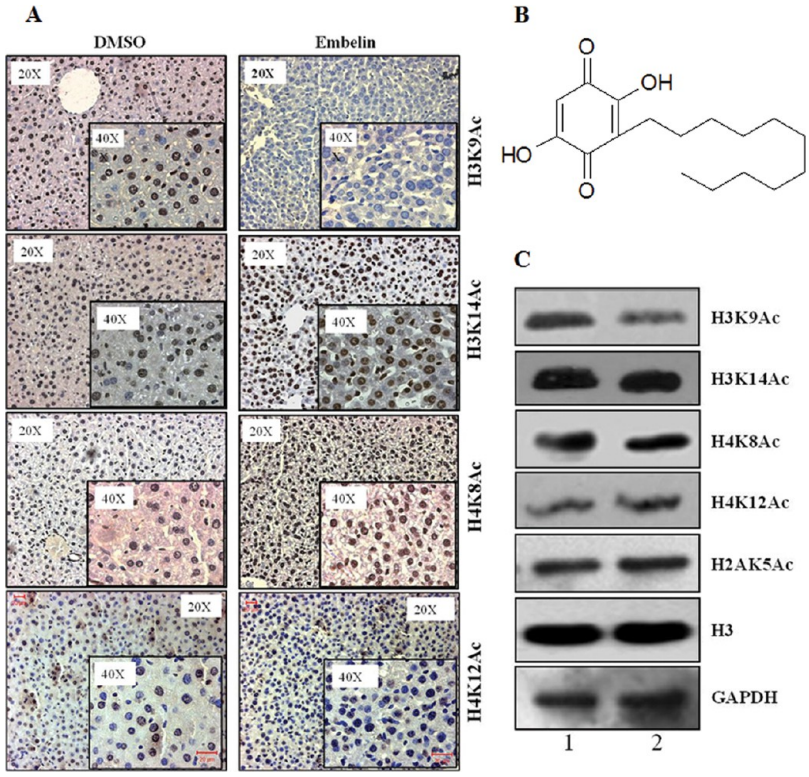

Figure 1. Embelin specifically inhibits H3K9 acetylation in mice. (A) Immunohistochemical analysis of embelin- and control (DMSO)-treated mouse liver tissue indicated a significant downregulation of H3K9 acetylation upon treatment, whereas the H3K14, H4K8, and H4K12 acetylation levels remained unchanged. (B) Structure of embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone). (C) The histone acetylation levels in the embelin-treated mouse liver tissues were analyzed by Western blots using antibodies specific to acetylated H3K9, H3K14, H4K8, H4K12, and H2AK5. The levels of histone H3, H4 and GAPDH were used as loading controls. Lane 1, DMSO-treated and Lane 2, embelin-treated mouse liver tissue.

in vivo efficacy [ref 11 and references therein]. Extensive *in silico* screening of large chemical libraries coupled with biochemical and *in vivo* studies led to identification of two synthetic cell-permeable p300/CBP-specific inhibitors (C646 and C107) with varying potency.[12,13] Recently, the glycosaminoglycan class of compounds has been shown to possess PCAF KAT inhibitor activity at a very high IC_{50}.[14]

From a large-scale screen for KAT modulators, we have identified embelin (Figure 1B), which is a cell-permeable small molecule with various biological activities. The pro-apoptotic, antitumorogenic, and anticancer activities of embelin have been attributed to its inhibitory effect on X-linked Inhibitor of Apoptosis Protein (XIAP).[15] The anti-inflammatory and pro-apoptotic activities of embelin are also mediated through the inhibition of TLR2-mediated activation of NFκB.[16] Embelin treatment leads to the downregulation of genes involved in cell proliferation (COX-2, cyclin-D1, c-Myc) and tumor metastasis (VEGF, ICAM-1, MMP-9) as well as XIAP.[17] Embelin has also been shown to have antiviral,[18] antibacterial,[19] and anthelmintic[20] activities, but the molecular mechanisms underlying these activities are poorly understood. The antifertility activity of embelin has been well studied, but the actual molecular target(s) have not yet been identified.[21] Upon embelin treatment, postcoital female mice delivered smaller and fewer litters, indicating defective embryonic development.[22,23] These studies indicate hitherto unidentified cellular target(s) of embelin, which could be any of the major nuclear KATs, possibly PCAF and/or GCN5.

In this study, we show that embelin is a novel PCAF-specific non-competitive inhibitor that inhibits the KAT activity of PCAF both *in vitro* and *in vivo*. We have identified the biochemical roles of various functional groups of embelin that are responsible for the PCAF-specific KAT inhibition. The inhibition of the PCAF KAT activity by embelin in turn inhibits the acetylation of MyoD, which blocks the differentiation of myoblasts into myotubes. Microarray analysis of embelin-treated differentiating C2C12 cells identified several genes with altered expression levels. These data are useful in appreciating the wider role of PCAF KAT activity in the process of muscle differentiation.

RESULTS AND DISCUSSION

Embelin Is a Lysine Acetyltransferase (KAT) Inhibitor. Embelin (Figure 1B) was isolated from powdered *Embelia ribes* berries through a series of chromatographic techniques. The purity of the embelin was confirmed by reverse-phase chromatography, mass spectrometry, and NMR. Female Swiss albino mice were intraperitoneally injected with embelin (5 mg/kg body weight) for 3 days. As reported previously,[22] the embelin-treated mice exhibited significantly reduced locomotor activity within 15 min of the treatment, whereas DMSO-treated mice did not show any such symptoms. The embelin-treated mice regained their normal physiological activities 2−3 h after the injection. No further physiological or clinical symptoms were observed at the end of 3 days of embelin treatment. Immunohistochemical analysis of the murine liver tissues showed significant downregulation of histone H3Lys9

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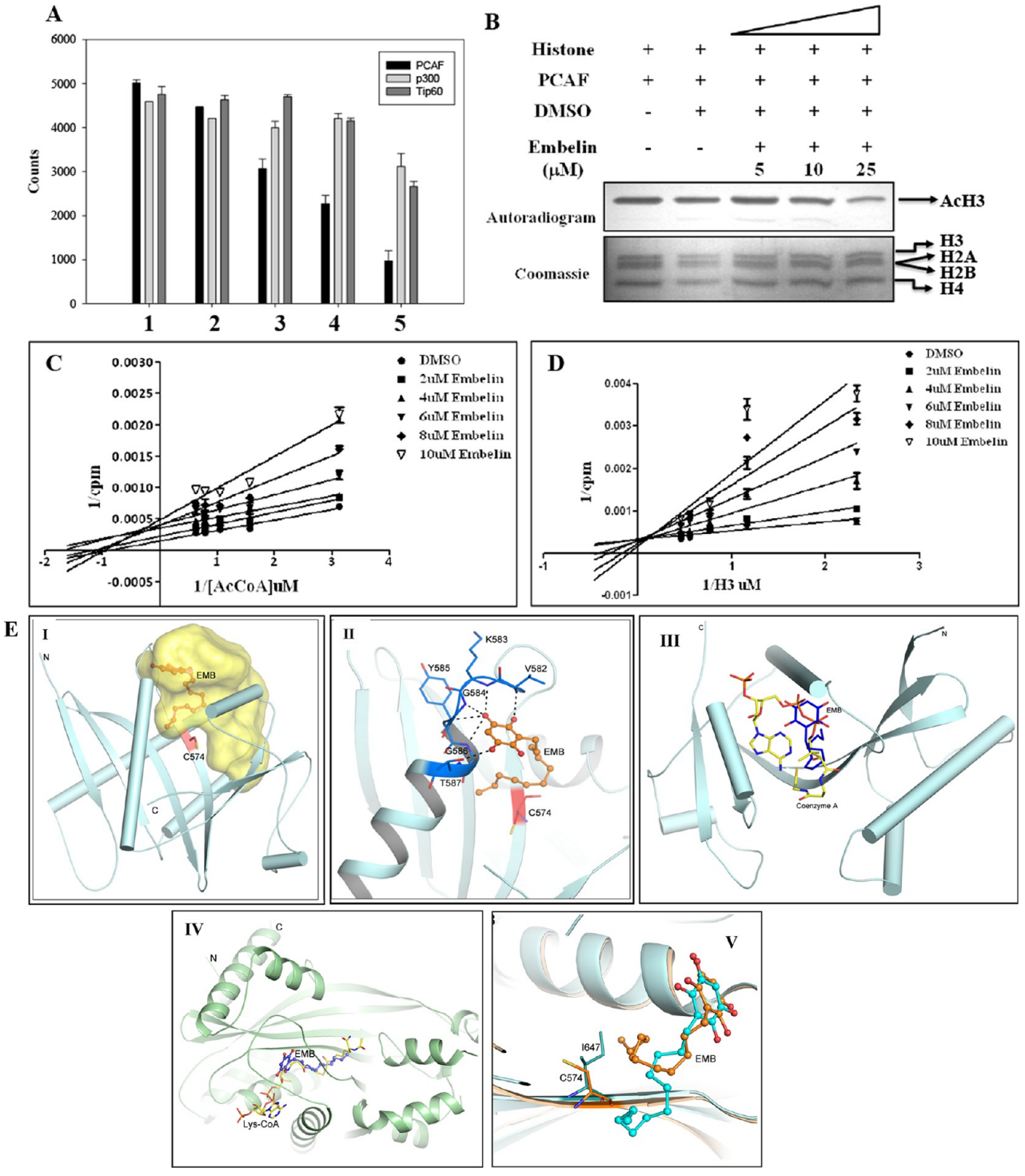

Figure 2. Embelin preferentially inhibits PCAF HAT activity *in vitro*. (A) Filter binding assays for the inhibition of the KAT activity of PCAF, p300, and Tip60 by embelin. The filter binding assays were performed using purified recombinant KATs and highly purified HeLa core histones as the substrate. Set 1, enzyme alone; Set 2, enzyme + DMSO; Sets 3−5, enzyme with 5, 10, and 25 μM embelin. The error bars represent the mean ± SD of triplicate reactions. (B) The dose-dependent inhibition of the KAT activity of PCAF by embelin was confirmed with a gel assay. (C, D) Kinetic analysis of embelin-mediated inhibition of PCAF KAT activity. Lineweaver−Burk plot showing the effect of DMSO and different concentrations (2, 4, 6, 8, and 10 μM) of embelin on the PCAF KAT activity at a fixed concentration of histone H3 and increasing concentrations of ^{3}H-acetyl CoA (C) and at a fixed concentration of ^{3}H-acetyl CoA and increasing concentrations of histone H3 (D). (E) Mechanism of the PCAF-embelin interaction. (I) Molecular modeling studies show that embelin (EMB) is bound to the active site tunnel near the catalytic residue C574. The yellow cloud shows the binding site of CoA on the PCAF KAT domain. (II) Molecular modeling studies showing the hydrogen bonding interactions of the hydroxybenzoquinone moiety of embelin with PCAF. (III) Embelin (blue) binds to PCAF at the CoA (shown in tick line) binding pocket, although in different orientation. (IV) Embelin binds at the KAT domain of p300 but distal from the catalytic residues. (V) Comparison of the binding of embelin with Tip60 (blue) and PCAF (brown). The embelin is docked on Tip60, and the I647 residue of Tip60 is shown in cyan. Embelin docked on PCAF and the catalytic C574 residue of PCAF is indicated in brown.

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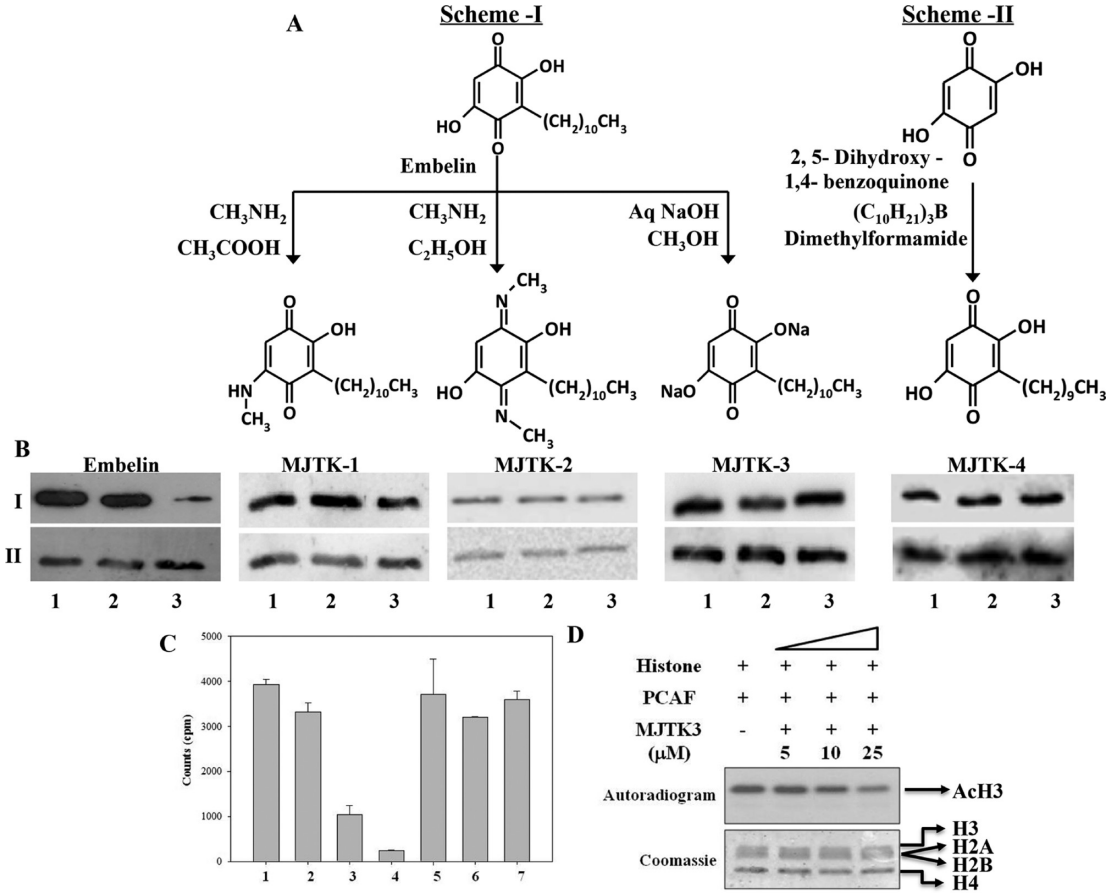

Figure 3. Role of different functional groups of embelin in PCAF inhibition. (A) Schemes for the synthesis of embelin derivatives and their structures. Scheme I: Chemical modification of the hydroxybenzoquinone moiety of embelin to decipher its biological functions. Scheme II: Synthesis of hydroxybenzoquinone derivatives with varying alkyl side chains. (B) Western blots showing the effects of treatments with embelin derivatives on H3K9 acetylation in HEK 293T cells, as indicated on the top of the panel. The cells were treated with (Lane 1) DMSO or (Lane 2) 20 μM or (Lane 3) 40 μM compound. Panel I, H3K9 acetylation and panel II, H3 loading control. (C) Inhibition of PCAF by embelin derivatives. Filter binding assays were performed using purified recombinant PCAF and highly purified HeLa core histones as the substrate. All derivatives were used at the concentration of 25 μM. Sample 1, untreated; sample 2, DMSO; sample 3, embelin; sample 4, MJTK-3; sample 5, MJTK-1; sample 6, MJTK-2; and sample 7, MJTK-4 treated reactions. The error bars represent the mean ± SD of triplicate reactions. (D) Gel assay showing the inhibition of the KAT activity of PCAF by the water-soluble embelin derivative MJTK3 *in vitro*.

acetylation levels compared to the solvent (DMSO) controls (Figure 1A). No significant alterations in the levels of histone H3Lys14, H4Lys8, H4Lys12, and H2ALys5 acetylation were observed (Figure 1A,C). Although several reports have suggested that histone H3Lys9 acetylation is mediated by both PCAF/GCN5 and p300/CBP [ref 24 and references therein], Jin et al.[25] have recently quite convincingly shown that histone H3Lys9 acetylation is predominantly mediated by PCAF/GCN5 *in vivo* under certain cellular conditions. These data thus indicate that embelin could be a potential specific inhibitor of PCAF.

To identify the bona fide target of embelin, filter binding acetyltransferase assays were performed using recombinant, baculovirus-expressed, full-length PCAF and p300 and bacterially expressed Tip60 KAT domain (Figure 2A). As expected, embelin could inhibit KAT activity of PCAF in a dose-dependent manner with an IC_{50} of approximately 7.2 ± 1.5 μM. However, in this *in vitro* assay system, it could also partially inhibit the KAT activity of p300 and Tip60 at higher concentrations. At 25 μM embelin, both p300 and Tip60 retained approximately75% of their original activities, whereas the KAT activity of PCAF exhibited almost 90% inhibition (Figure 2A). The inhibition of the KAT activity of PCAF was further confirmed by a gel assay (Figure 2B), which revealed a greater than 60% reduction in the band intensity in the presence of 10 μM embelin. The gel assay with Tip 60 and p300 did not indicate a marked decrease in histone H4 and H3 acetylation (Supplementary Figure S1), even at concentrations up to 25 μM. To characterise the mechanism of embelin-mediated inhibition of PCAF, bacterially expressed recombinant H3 and [H^3]-acetyl CoA were used. The inhibition pattern obtained upon the varying concentration of [H^3]-acetyl CoA with a fixed concentration of histone H3 suggests that embelin is a non-competitive inhibitor of PCAF KAT activity (Figure 2C). However, when the inhibition assays were performed using a fixed concentration of [H^3]-acetyl CoA and different concentrations of histone H3 (Figure 2D), the enzymatic activity curves resembled competitive inhibition of PCAF. Taken together, these data establish embelin as a non-competitive inhibitor of PCAF that presumably targets acetyl-CoA-PCAF interactions.

In silico docking studies (Figure 2E) were performed to understand the molecular mechanism of the PCAF−embelin interaction. Embelin was docked on the active site of PCAF

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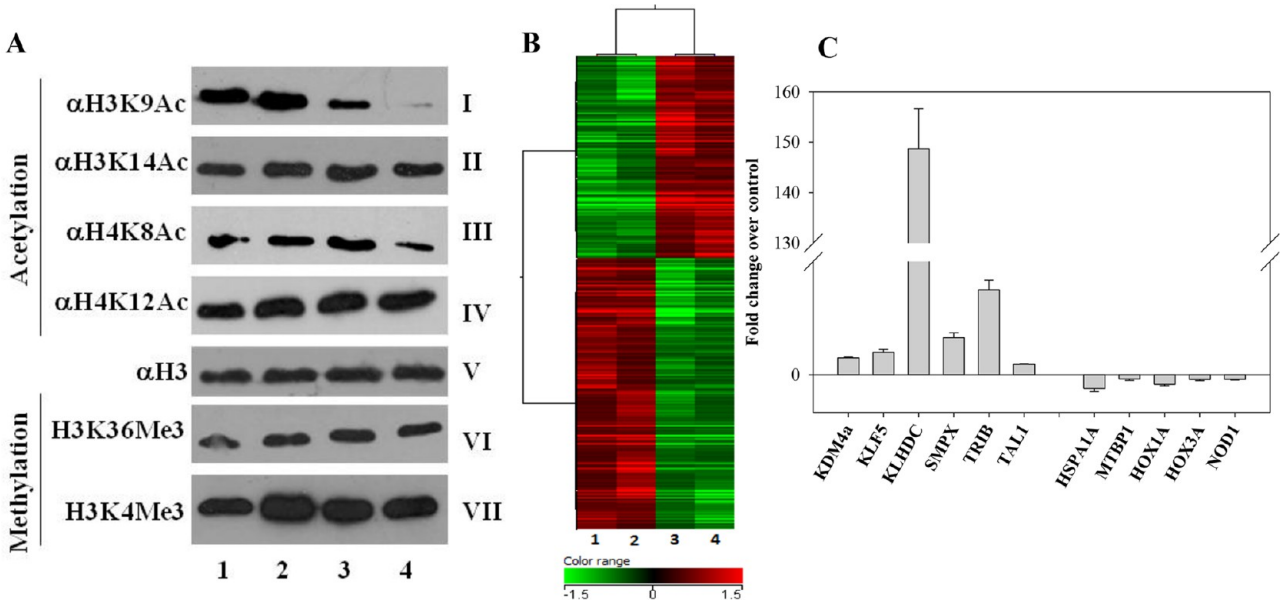

Figure 4. Embelin inhibits H3K9 acetylation *in vivo*. (A) Embelin-treated HEK 293T cells were analyzed by Western blot. Lane 1, untreated; Lane 2, DMSO-treated; Lane 3, 20 μM embelin-treated; and Lane 4, 40 μM embelin-treated cells. The histone modification-specific antibodies used in the analysis are indicated on the left side of the panels. (B) Microarray analysis of embelin-treated cells. Embelin-mediated downregulation of histone H3K9 acetylation alters the expression of a select set of genes in HEK 293T cells. Lanes 1 and 2, 20 μM embelin-treated cells; lanes 3 and 4, DMSO-treated cells. (C) The expression of specific genes in the embelin-treated HEK 293T cells was analyzed by real time quantitative RT-PCR. The fold overexpression of the genes in embelin-treated cells over the DMSO control-treated cells was calculated and plotted as the average of three biological replicates.

(Figure 2E-III) with a theoretical binding energy of −6.78 kcal/mol. The estimated inhibition constant (K_i) from docking was 10.64 μM. The docked conformation of embelin indicated that the hydrophobic tail of the inhibitor was situated near the catalytic residue (C574) (Figure 2E-I). The hydroxybenzoquinone headgroup of embelin forms several hydrogen bonds with the main chain and side chains of the residues Val582, Lys583, Gly584, Tyr585, Gly586 and Thr567 (Figure 2E-II). A comparison of coenzyme A (CoA) and embelin binding demonstrated that the headgroup of embelin mimics the pyrophosphate of the CoA (Figure 2E-III); therefore, embelin is strongly bound to PCAF through specific interactions. However, the tail group of embelin and the β-mercaptoethylamine arm of the CoA follow different paths in the CoA-binding tunnel (Figure 2E-I). Both the *in vitro* and *in vivo* data suggest that embelin inhibits H3K9 acetylation by inhibiting the PCAF KAT activity with high specificity. To gain insight into this specificity, embelin was further docked on the p300 and Tip60 HAT/KAT domain. A comparison between Lysyl-CoA, a p300-specific synthetic inhibitor,[26] and embelin binding on p300 (Figure 2E-IV) showed a significant difference in the mode of binding. The hydroxybenzoquinone group of embelin does not mimic the position of pyrophosphate compared to CoA binding to p300, and embelin was found to be situated far from the catalytic residue. This finding is in sharp contrast with PCAF-embelin binding, in which embelin was positioned at the entrance to the active site. A structural superposition of the Tip60-embelin and PCAF-embelin binding conformations indicated the presence of a bulky residue (Ile647) in Tip60 (Figure 2E-V) that prevents the tail group of embelin from reaching the catalytic residue, which might renderTip60 insensitive to embelin. Collectively, these data indicate that embelin is a lysine acetyltransferase inhibitor with a significant specificity toward PCAF.

Role of Various Functional Groups of Embelin in Small Molecule−PCAF Interactions. Molecular docking studies have suggested that the carbonyl oxygen and the hydroxyl group of hydroxybenzoquinone headgroup of embelin make extensive contacts with the peptide backbone and side chains of various residues. The conversion of one hydroxyl group to methylamine (MJTK-1, Scheme I, Figure 3A) completely abrogated the PCAF inhibition, both *in vitro* (sample 5, Figure 3C) and *in vivo* (Figure 3B). Similarly, the conversion of both the carbonyl groups to methylimine (MJTK-2) also resulted in a loss of activity (sample 6, Figure 3B and C).

Molecular docking studies have shown that an 11-carbon length of the alkyl is optimal for the closest approach to the catalytic C-574 residue of PCAF. This alkyl chain of embelin (Figure 2E-I, orange) approaches this residue through a distinct short hydrophobic tunnel present near the tunnel accommodating the β-mercaptoethylamine arm of CoA (Figure 2D-I, shown in yellow space filled). Closer observation of the embelin binding pocket revealed that there is a small bend at the end of the tunnel, which firmly holds the alkyl chain close to the C574. We have synthesized a new molecule (MJTK-4) with a 10-carbon long alkyl chain from 1,4-dihydroxy benzoquinone (Scheme II, Figure 3A). MJTK-4 has also lost the PCAF inhibitory activity both *in vitro* (sample 7, Figure 3C) and *in vivo* (Figure 3B).

The water-soluble derivative of embelin (MJTK-3) exhibited improved inhibition of PCAF *in vitro* both by a filter binding assay (sample 4, Figure 3C) and gel assay (Figure 3D). However, MJTK-3 did not show any effect *in vivo*, either on HEK 293T cells (Figure 3B) or treated mice (data not shown). Taken together, these data identify the specific molecular entities of embelin that are responsible for the inhibition of KAT activity.

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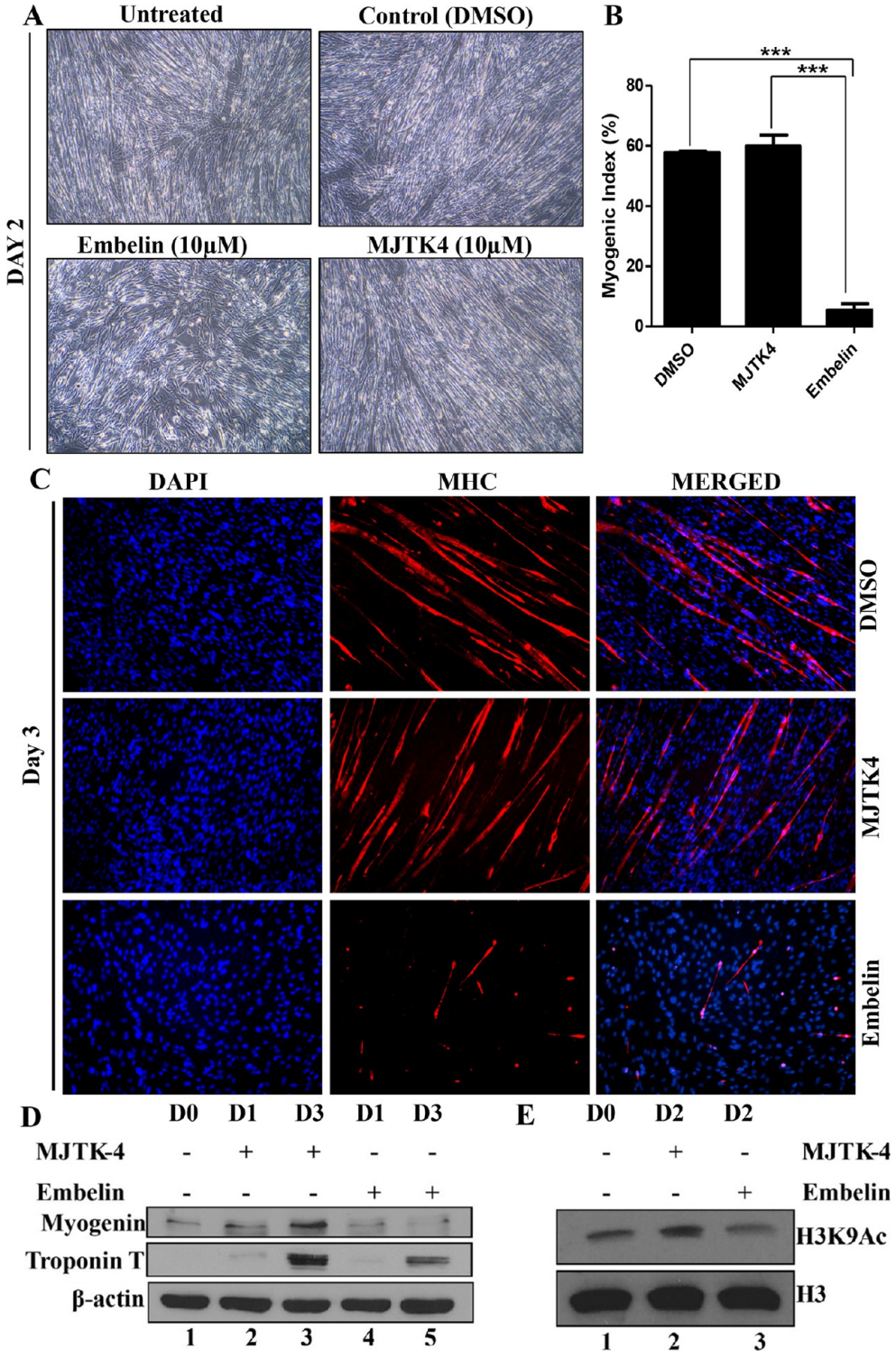

Figure 5. Embelin inhibits skeletal muscle differentiation. (A) C2C12 cells were treated with 10 μM embelin or MJTK-4 for 48 h in differentiation medium and analyzed morphologically for myotube formation. Untreated and vehicle (DMSO)-treated cells were used as controls. Impaired myotube formation was evident in the embelin-treated cells compared to the controls and MJTK-4-treated cells. (B) Quantification of the myogenic index revealed a significant downregulation in the embelin-treated cells. The error bars indicate the mean ± SD. (C) C2C12 cells were treated with vehicle (DMSO), 10 μM MJTK-4 or embelin. Three days after differentiation, the cells were immunostained with α-MHC antibody (red), and the nuclei were stained with DAPI. A clear reduction in MHC^+ cells was observed in the embelin-treated cells compared to the DMSO- and MJTK-4-treated controls. (D) C2C12 cells were left untreated (Day 0) or treated with 10 μM embelin or MJTK-4 for 24 h (D1) or 72 h (D3) in differentiation medium. Myogenin and Troponin T expression was determined by Western blot, and β-actin was used as a loading control. (E) C2C12 cells were left untreated (Day 0) or treated with 10 μM embelin or MJTK-4 for 48 h (D2) in differentiation medium. Total histone H3K9 acetylation was determined by Western blot, and H3 was used as a loading control.

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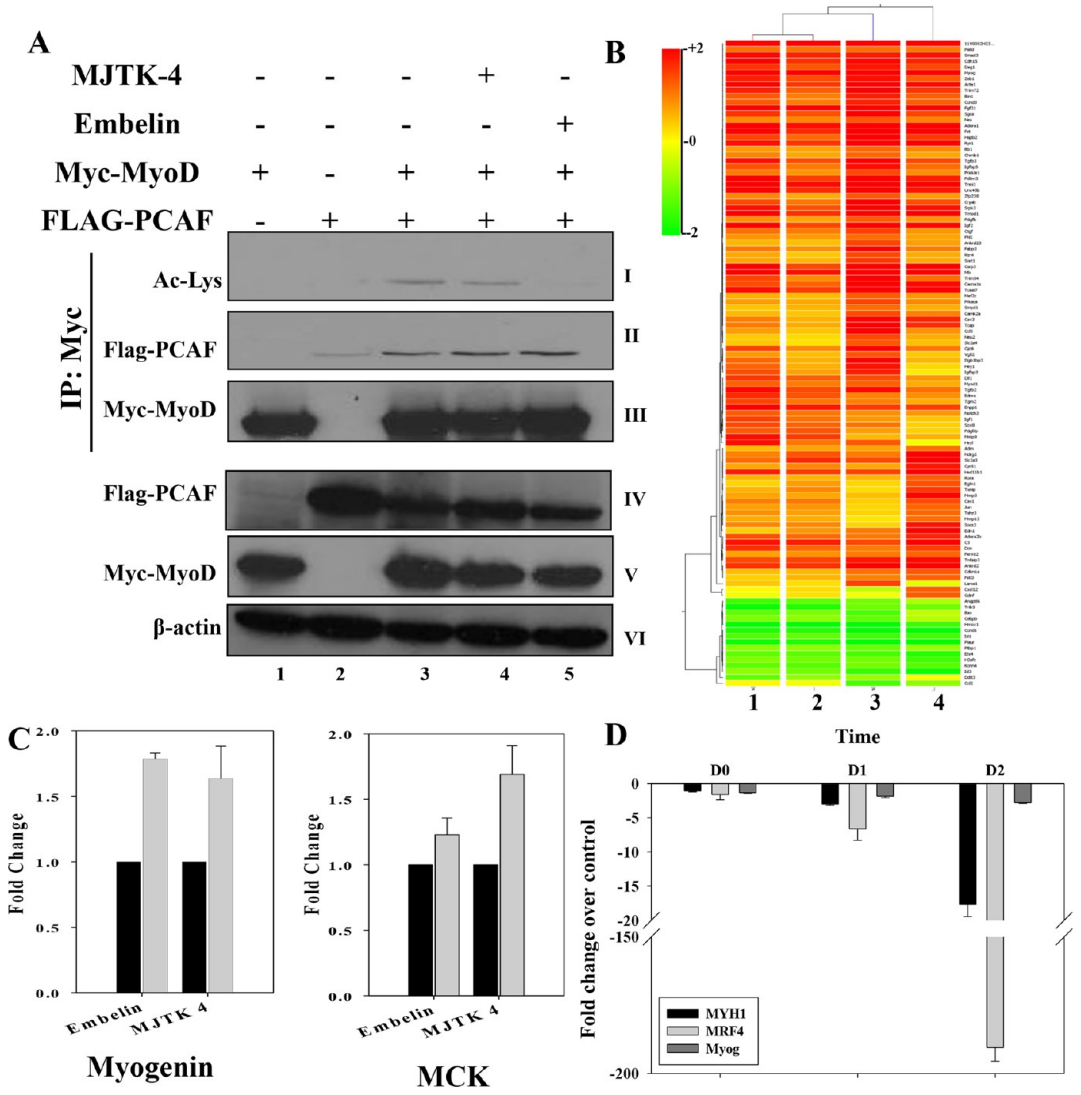

Figure 6. Embelin specifically inhibits PCAF-mediated MyoD acetylation *in vivo*. (A) HEK 293T cells were transfected with FLAG-PCAF (panel IV) and Myc-MyoD (panel V) and treated with 15 μM embelin or MJTK-4. MyoD was immunoprecipitated (panel III) and probed with α-FLAG (panel II) and α-acetylated Lys (panel I) antibodies. Panel VI: β-actin loading control. (B) Treatment with 10 μM embelin for 48 h alters the expression of a set of genes in C2C12 cells in DM. The differential expression of various genes after 24 and 48 h of treatment with DMSO (lane 1 and lane 3, respectively) and embelin (lane 2 and lane 4, respectively) upon the induction of differentiation is presented. (C) H3K9 acetylation of the myogenin and M-type creatine kinase (MCK) promoters were detected by ChIP assays. C2C12 cells were untreated (D0) or treated with embelin or MJTK-4 for 2 days during differentiation (D2). The relative enrichment of H3K9ac on D0 (black bars) and D2 cells (gray bars) was determined. Primers flanking the MyoD regulatory site were used for Q-PCR. β-actin was used as a control. (D) Expression of certain muscle differentiation lineage-specific genes in the embelin-treated C2C12 cells was analyzed by real time quantitative RT-PCR. The fold expression of the genes in the embelin-treated cells over the DMSO control-treated cells was calculated. These data show a significant correlation with the fold change observed in the genome-wide expression analysis.

Effect of Embelin on Histone Acetylation and Global Gene Expression. Previously, it was observed that embelin-induced cytotoxicity is highly dependent on cell type, with an IC_{50} ranging from 3.7 to 70 μM.[15,17] The cancerous cells expressing XIAP protein are more susceptible to embelin treatment compared to normal immortalized cells. However, we have found that HEK 293T cells treated with 20 and 40 μM embelin did not show any cytotoxicity and cell cycle arrest as revealed by FACS analysis (Supplementary Figure S1C) and MTT assay (data not shown). Western blotting analysis with various histone acetylation and methylation specific antibodies showed that in embelin-treated cells the histone H3K9 acetylation is dramatically reduced in a dose-dependent manner (Figure 4A, panel I). In agreement with the inhibition pattern observed in mice, embelin treatment does not alter the acetylation levels of H3K14, H4K8, and H4K12 residues significantly. In addition to acetylation, we have also investigated the status of histone methylation upon embelin treatment, and no change in the trimethylation of the H3K36 and H3K4 residues could be observed (Figure 4A). These data argue for establishing embelin as a specific inhibitor of lysine acetyltransferase that is preferentially targeted toward PCAF.

PCAF is a well-known transcriptional coactivator with intrinsic lysine acetyltransferase activity, but the KAT activity may or may not be involved in the transcriptional coactivator capacity. The absence of a specific PCAF inhibitor presented a major obstacle for the identification of genes regulated by the KAT activity of PCAF. To address this long awaited question,

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HEK 293T cells were treated with 20 μM embelin or DMSO in 3 biological replicates. Total RNA was isolated, and the RNA quality was verified with a bioanalyzer (Agilent). RNA from two biological replicates of each set were used to analyze global gene expression, as summarized in Figure 4B. The same batch of RNA in triplicate was used to validate the microarray data by quantitative real-time PCR (Figure 4C). The embelin treatment significantly (>2-fold) downregulated the expression of 457 genes and simultaneously upregulated the expression of 294 genes (Supplementary Figure S3A). On the basis of gene ontology, these differentially regulated genes could be further grouped into major cellular pathways, among which genes involved in transcriptional regulation formed the largest group, with 23 candidates (Supplementary Figure S3B, Supplementary Table S3). We observed the downregulation of all of the histones, heat shock proteins, such as HSPA1A and NOD1, and homeobox proteins, including HOXA1, HOXA3, and PHOX2A. The most upregulated gene upon embelin treatment was kelch domain containing 7B (KLHDC7B), which has been reported as a novel biomarker for breast cancer.[27] Other upregulated genes, such as TRIB3, SMPX, TAL1, and KLFs, play diverse roles in the cell.

Embelin Inhibits Muscle Cell Differentiation. Previous studies have demonstrated a key role for chromatin modifying and remodelling enzymes in mediating the dynamic modifications required for reprogramming during the transition from undifferentiated to differentiated muscle cells.[28] In particular, PCAF plays an essential role in the activation of MyoD by mediating its acetylation at lysine residues 99, 102, and 104, whereas the role of p300 is to mediate the acetylation of H3 and H4, which allows for enhanced MyoD transcriptional activity.[2,29,30] Given embelin appears to be a specific PCAF inhibitor, we determined its impact on skeletal muscle differentiation. C2C12 cells were treated with embelin or its inactive analogue MJTK4 for various time points, and the differentiation was examined morphologically. As shown in Figure 5A, treatment of myoblasts with 10 μM embelin, but not the inactive compound MJTK-4, resulted in significantly impaired myotube formation over time. To validate these findings, we examined myogenic differentiation by immunostaining with anti-myosin heavy chain (MHC) antibody, a marker of terminally differentiated cells. In contrast to the DMSO-treated controls and cells treated with MJTK-4, the embelin treatment resulted in a reduction in the MHC^+ cells (Figure 5C) and in the myogenic index (Figure 5B). To further investigate the mechanisms underlying the inhibition of myogenic differentiation, we analyzed the expression of myogenin and Troponin T, which are MyoD target genes that depend on the transcriptional activity of MyoD. In MJTK-4-treated cells, the expression levels of myogenin and Troponin T were upregulated at day 1 (D1) and day 3 (D3) after the induction of differentiation (Figure 5D, lanes 2 and 3). However, the embelin-treated cells manifested an apparent reduction in myogenin and Troponin T levels, confirming of the inhibition of myogenesis (Figure 5D, lanes 4 and 5).We have further examined the total histone H3K9 acetylation levels in the C2C12 cells upon induction of differentiation. In presence of 10 μM embelin the global level of H3K9ac remained at the level of undifferentiated cells (D0) (Figure 5E, lanes 1 and 3), whereas the level of H3K9ac was enhanced normally in MJTK-4-treated cells in differentiation medium (Figure 5E, lane 2).

Embelin Specifically Inhibits PCAF-Mediated Acetylation of MyoD. Given the expression of MyoD target genes is dependent on its transcriptional activity, which in turn depends on acetylation by PCAF, we examined whether the treatment of cells with the PCAF inhibitor resulted in altered MyoD acetylation. 293T cells were transfected with MyoD and PCAF in the presence of either 15 μM embelin or MJTK-4. MyoD was immunoprecipitated, and the extent of acetylation was determined using an anti-acetyl lysine antibody (Figure 6A). As expected, MyoD was acetylated when expressed along with PCAF. Interestingly, embelin treatment reduced the MyoD acetylation, while MJTK-4 had no effect. To examine whether embelin inhibits H3K9ac on muscle promoters, we performed chromatin immunoprecipitation assays. C2C12 cells were cultured in the presence of MJTK-4 or embelin for 2 days, and the levels of H3K9ac at the myogenin and M-type creatine kinase (MCK) promoters were analyzed. In both the MJTK-4- and embelin-treated cells, the levels of H3K9ac were increased to a similar extent during differentiation (Figure 6C) at myogenin gene promoter. At MCK promoter, there is partial reduction of H3K9 acetylation. Together, these studies confirm that embelin treatment had a selective impact on MyoD acetylation and no impact H3K9ac on muscle promoters, validating its specificity as a PCAF inhibitor.

Embelin Alters Muscle-Specific Gene Expression during Differentiation. There was no significant difference in global gene expression in the undifferentiated C2C12 cells upon embelin treatment (Supplementary Figure S3C). To identify genes involved in muscle differentiation, the gene expression patterns upon the induction of differentiation in the presence and absence of embelin on D1 and D2 were first compared with the corresponding D0 values. Overall, DMSO-treated C2C12 cells exhibited similar changes in gene expression patterns during differentiation as reported in the literature.[31] On D1, the majority of the genes had comparable expression patterns in the presence and absence of embelin (Figure 6B, lanes 1 and 2), although the absolute changes in the expression levels from D0 were marginally lower in the presence of embelin. Significant changes in gene expression upon embelin treatment were observed on D2 (Figure 6B, lanes 3 and 4). The gene expression pattern in the DMSO-treated cells on D2 exhibited similar trends as on D1 (Figure 6B, lane 3 vs lane 1), although the fold changes in gene expression were much higher. After 48 h of embelin treatment, we observed a drastic reduction in myoblast differentiation. There was also a drastic reduction in the expression of MyoD-responsive genes, such as Myog, MYH1, and MRF4, which were validated by Q-PCR (Figure 6D). Overall, the embelin treatment leads to altered expression of muscle differentiation-specific genes, selective transcription factors, and histone modifiers, among others, which collectively inhibit myoblast differentiation.

Discussion. Reversible acetylation by KATs and lysine deacetylases (KDACs) plays a key role in the regulation of gene expression and thereby in the process of muscle differentiation. The coactivator functions of PCAF and GCN5 have been studied in detail, but the contributions of their KAT activities have yet to be understood. We found that embelin specifically inhibits histone H3K9 acetylation in mice and blocks *in vitro* the KAT activity of recombinant PCAF. Microarray analyses of embelin-treated HEK 293T and C2C12 cells demonstrated that indeed embelin alters expression of an array of genes, known to be regulated by PCAF. PCAF-mediated acetylation of MyoD is

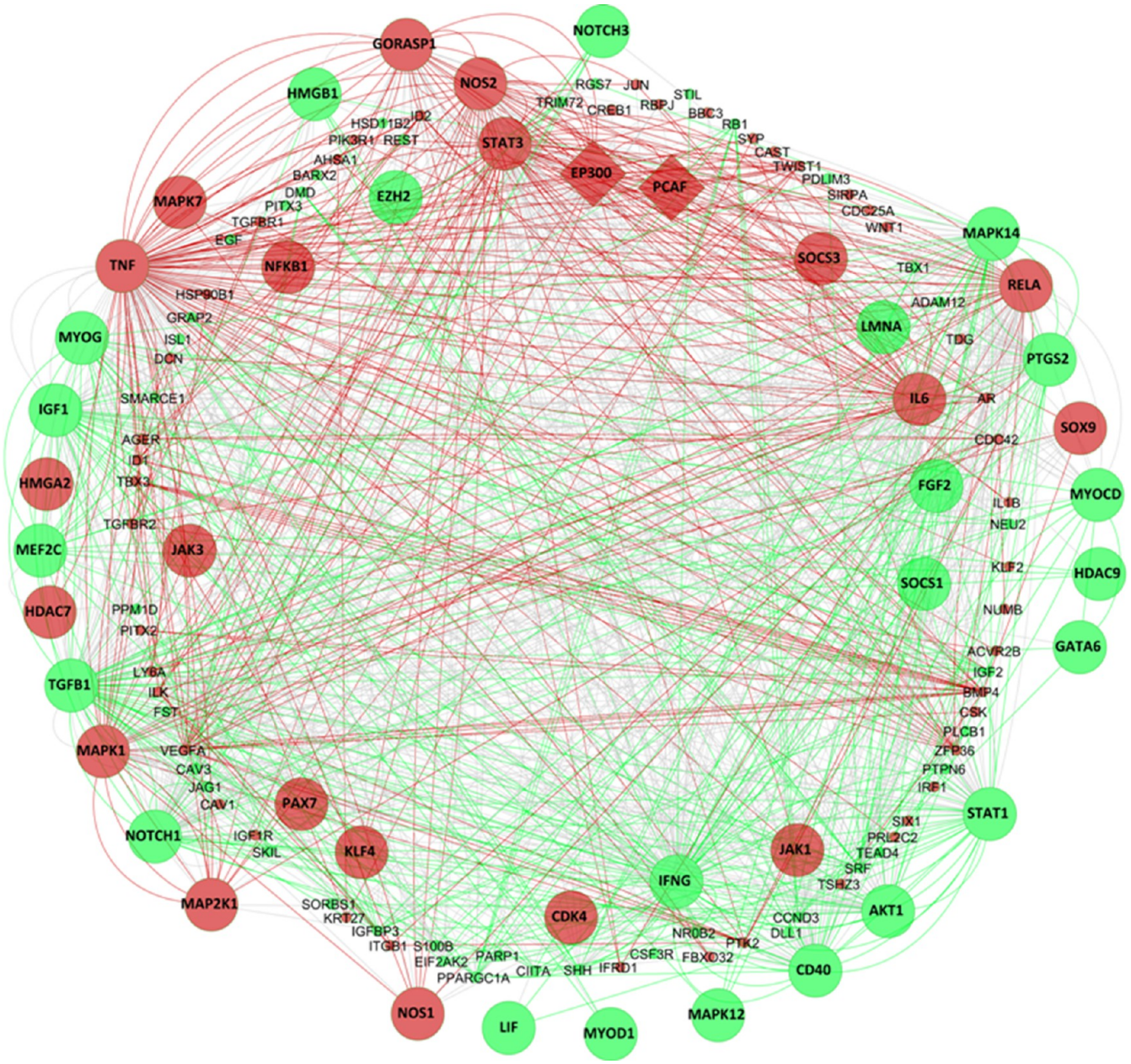

Figure 7. Inhibition of PCAF KAT activity alters the expression of several genes involved in the regulation of muscle differentiation. Biological network analysis showing the differential expression of various genes involved in muscle differentiation in C2C12 cells cultured in DM for 48 h in the presence embelin. Genes that are overexpressed compared to DMSO on D2 are shown in red, and repressed genes are marked in green. Genes known to play a critical role in muscle cell differentiation and proliferation of progenitor cells are shown in a bigger size. The lines connecting overexpressed nodes are shown in red and down-regulated nodes are shown in green.

essential for muscle differentiation, and embelin treatment specifically inhibits MyoD acetylation, but not PCAF−MyoD interactions (Figure 6A), which led to inhibition of muscle cell differentiation. The C2C12 microarray data further identified several genes, yet to be established as important regulators of differentiation processes.

In vitro biochemical studies have shown that embelin preferentially inhibits PCAF at low micromolar concentrations (Figure 2A and B, Supplementary Figure S1A and B). Embelin is a non-competitive inhibitor of PCAF (Figure 2C) and binds to the CoA binding pocket although with a slightly different orientation from CoA (Figure 2E). It has been reported earlier that H3 can bind to PCAF/GCN5 only after acetyl coA is bound to PCAF.[32,33] It is possible that embelin binding somehow alters the PCAF-acetyl CoA intermediate structure in a manner that disfavors H3 binding, thus showing competitive inhibition (Figure 2D).[34] H3-PCAF and embelin-PCAF cocrystal structures and site directed mutagenesis studies will unravel the exact molecular mechanism of inhibition. Molecular docking studies predicted the roles of various functional groups in the PCAF-embelin interaction. The conversion of the hydroxyl and carbonyl groups of embelin to amines (MJTK-1) and imines (MJTK-2), respectively, led to reduced hydrogen bonding interactions and thus the abolition of the inhibition of the KAT activity of PCAF. Docking studies also postulated that an 11-carbon-long alkyl chain in embelin is optimal for binding to PCAF, and removing one methyl group from the alkyl chain led to complete loss of the activity. None of these derivatives

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were active *in vitro* or *in vivo*, and thus MJTK-4 served as an effective negative control for further studies (Figure 3A−C). The disodium salt of embelin (MJTK-3) presented a very interesting scenario. MJTK-3 was completely water-soluble at physiological pH and exhibited comparable PCAF inhibitory activity *in vitro* (Figure 3C and D). HEK 293T cells treated with MJTK-3 did not show any change in H3Lys9 acetylation (Figure 3B), and MJTK-3 was completely inactive when injected into mice (data not shown). This loss of *in vivo* activity can be attributed to the cellular impermeability of MJTK-3. MJTK-3 has accumulated 2 negative charges over embelin, which induce electrostatic repulsion when it approaches a cell membrane, and in turn, passive diffusion through the cell membrane is inhibited.

The acetylation of MyoD by PCAF and histones H3 and H4 at specific residues by p300 are essential for the differentiation of C2C12 cells from myoblasts into myotubes.[29,35] Treatment with 10 μM embelin completely inhibited myotube formation (Figure 5A) and PCAF-mediated acetylation of H3K9 residue globally (Figure 5E). Biochemical analyses have shown that embelin specifically inhibits the PCAF-mediated acetylation of MyoD (Figure 6A). An immuno-affinity pull-down of Myc-MyoD protein also copurified PCAF, indicating stable MyoD-PCAF complex formation even in the presence of embelin. In the case of XIAP inhibition, embelin binds to the baculovirus inhibitor of apoptosis protein (IAP) domain-3 (BIR-3) and thus inhibits its interaction with caspases[15] and possibly with surviving,[36] rendering the cells susceptible to apoptosis. These data clearly argue that in contrast to the case of XIAP, embelin inhibits the KAT activity of PCAF but does not interfere with the interaction between PCAF and MyoD. Our results also suggest that embelin is a competitive inhibitor of PCAF that inhibits the binding to acetyl CoA. ChIP assays have shown that there is no change in the level of histone H3K9 acetylation on the myogenin and creatine kinase M-type (MCK) promoter upon embelin treatment. This observation is highly significant. First, these data suggest that, consistent with a previous report, histone acetylation by PCAF is not involved in MyoD-responsive gene expression (p300 is responsible for H3K9ac on the myogenic and MCK promoter).[30] Second, these results further establish the specificity of embelin toward PCAF, as only MyoD acetylation was found to be affected upon embelin treatment.

Biological network analysis from the C2C12 microarray data (Figure 7 and Supplementary Figure S4) allowed for the prediction of broader regulatory networks associated with PCAF during muscle differentiation. Embelin treatment induced the expression of CXCL12, which activates the chemokine receptor CXCR4 and induces intracellular calcium flux. It also binds to CXCR7 and activates the β-arrestin pathway. CXCL12 plays important role in β-cell lymphopoiesis, myelopoiesis, and heart ventricular septum formation during embryonic development.[37] Embelin treatment resulted in the downregulation of laminin-α1, which is involved in the attachment, migration, and organization of cells into tissues during embryonic development.[38] Laminin-α1 is also essential for mouse cerebellar development. The overexpression of GDNF was observed in the embelin-treated C2C12 cells, which would promote cell survival in the absence of a functional Myf5-MyoD complex.[39] The embelin-treated cells had higher levels of TNFα and Bax, which would inhibit myogenic differentiation by inducing the Bax-caspase9 pathway.[40,41] Embelin treatment altered the MAPK pathway. MAPK-$\alpha/\beta/\gamma$, which activate MyoD through the E47 pathway and regulate MEF2 function, were drastically downregulated. PCAF-mediated acetylation of MAPKα is essential for the activation of MAPKα in cardiomyocytes;[42] thus, embelin-mediated inhibition would also be affected in a MyoD-independent manner. Embelin treatment led to induction of expression of STAT3 and repression of STAT1. STAT3 interacts with MyoD[43] and also sequesters p300/CBP and PCAF from interacting with MyoD,[44] resulting in the inhibition of differentiation. STAT3/STAT1/JAK1 signaling is necessary for myoblast proliferation[45] and at the early stage of differentiation. This dual function is regulated by the SOCS1/SOCS3/PIAS1 complex, which is present at higher levels in embelin-treated cells. SOCS1 binds to JAK1 and PIAS1 to STAT1 to prevent STAT1 from binding DNA, and thus they inhibit differentiation.[46] The presence of high levels of JAK3[47] and KLF4[48] and low quantities of GATA6[49] in embelin-treated cells would also contribute to the decrease in the MHC, myogenin, MyoD, MEF2, and myocardin levels and thus lead to the inhibition of differentiation. The higher levels of Sox9 in embelin-treated cells would lead to reduced expression of α-sarcoglycan during the early stages of differentiation through a MyoD-independent pathway.[50] Higher levels of HMGA2 would promote the proliferation of embelin-treated cells.[51] Embelin-treated cells have higher levels of Pax7, which is essential for the proliferation of satellite cells and the inhibition of myogenic differentiation.[52] Higher levels of Pax7 are maintained by sustained notch expression[53] and reduced levels of the TNF/PRC2/EZH2 repressive complex,[54] which has been observed upon embelin treatment. A decrease in the levels of HDAC7 and HDAC9 was also observed in embelin-treated cells, which would lead to sustained cyclin-D1 levels and myoblast survival.[55] Embelin treatment also induced the expression of NOS1, NOS2, and NFκB, which are essential for myoblast survival and for the inhibition of differentiation.[56,57] Presumably, PCAF regulates muscle cell survival and differentiation through various MyoD-dependent and -independent pathways.

METHODS

Protein Purification and Enzyme Assays. Recombinant baculovirus expressed full length FLAG-tagged PCAF, CARM1, and CBP were purified by immunoaffinity chromatography using anti-FLAG M2 agarose beads (Sigma). Hexa-histidine tagged full length baculovirus expressed p300 and G9a as well as bacterially expressed KAT domain of Tip60 were purified by Ni-NTA agarose affinity chromatography (Merck). Detailed protocols for purifications have been reported earlier.[58] All four core histones from HeLa nuclear pellet and rat liver were purified as described earlier.[59] *In vitro* histone acetylation and methylation assays were performed as reported earlier.[60] Kinetic analysis of the bisubstrate KAT reaction was carried out in the presence of varying concentrations of embelin.

Chromatin Immunoprecipitation (ChIP). C2C12 cells were cultured in growth medium for 24 h (day 0) and differentiation medium for 48 h (day 2), in the presence of 10 μM embelin and MJTK-4. Acetylated H3K9 levels on the myogenin promoter were examined by ChIP assays using H3K9ac (Abcam) antibodies described earlier.[61] The following primers were used for PCR. Myogenin promoter: 5′-TGGCTATATTTATCTCTGGGTTCATG-3′ and 5′-GCTCCCGCAGCCCCT-3′; β-actin: 5′-GCTTCTTTGCAGCTC-CTTCGTTG-3′ and 5′-TTTGCACATGCCGGAGCCGTTGT-3; MCK promoter: 5′CGCCAGCTAGACTCAGCACT-3′ and 5′-CCCTGAGAGCAGATGAGCTT-3′.

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Statistical Analysis. Statistical analysis was ascertained by Student's *t* test, and *p*-values of <0.05 were considered significant. Data are mean ± standard deviation (SD).

Microarray on HEK293T Cells. Whole genome gene-expression profiling of the HEK 293T cells was performed by single-color labeling of the total RNA and hybridization to an Agilent 8 × 60k human microarray. The array was scanned with an Agilent Microarray Scanner, and the raw data were extracted using the Agilent Feature Extraction software (iLife Discoveries, New Delhi, India).

Microarray on C2C12 Cells. C2C12 cells were grown in growth medium for 24 h (D0) in the presence of 10 μM embelin or DMSO and shifted to differentiation medium in the presence of embelin or DMSO respectively for 24 h (D1) and 48 h (D2). Total RNA was extracted from proliferating and differentiated C2C12 cells using TRIzol (Invitrogen) reagent. The experiment included two biological replicates of the DMSO- and embelin-treated cells. RNA was purified using the RNeasy MinElute Cleanup Kit (Qiagen) and reverse transcribed, labeled, and subsequently hybridized to the Illumina mouse WG-6 v2.0 array. The gene expression data from both sets were analyzed with Genespring GX 12.0 (Agilent) by shifting the intensities to the 75th percentile, and baseline transformation was performed to the medians of all samples (treated and control) (Bionivid Technologies, Bangalore, India). For detailed protocols for the RNA extraction, quantitative real-time PCR, and microarray data analysis, please refer to the Supporting Information.

For detailed protocols for all other experiments, please refer to the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

Accession Codes

Accession code for microarray data: GEO reference GSE39827 and GSE39828.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: tapas@jncasr.ac.in.

Author Contributions

T.K.K. and R.M. were responsible for overall conceptualization of the study and writing manuscript. R.M. initiated the study and performed all *in vitro*, human cell line, and mice experiments. J.B. purified embelin from plant source and prepared MJTK-1, MJTK-2, and MJTK-3 derivatives. P.M. synthesized MJTK-4 derivative. A.B. was associated with IHC analysis. K.M. and R.M. were responsible for *in silico* analysis. N.B., V.K., W.K.K., and R.T. performed all muscle differentiation experiments. R.M. and M.V. analyzed all the microarray data. N.N. collected the authentic plant material.

Notes

The authors declare no competing financial interest.

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