cAMP Signaling Regulates DNA Demethylation by Augmenting the Intracellular Labile Ferrous Iron Pool

Vladimir Camarena\(^1\), David W. Sant\(^1\), Tyler C. Huff\(^1\), Sushmita Mustafi\(^1\), Ryan K. Muir\(^2\), Adam R. Renslo\(^2\), Paula Monje\(^3\), Gaofeng Wang\(^{1,4*}\)

\(^1\) John P. Hussman Institute for Human Genomics, Dr. John T. Macdonald Foundation Department of Human Genetics, University of Miami Miller School of Medicine, Miami, Florida, USA.

\(^2\) Department of Pharmaceutical Chemistry, University of California San Francisco, San Francisco, California, USA.

\(^3\) The Miami Project to Cure Paralysis and Department of Neurological Surgery, University of Miami Miller School of Medicine, Miami, Florida, USA.

\(^4\) Dr. Nasser Ibrahim Al-Rashid Orbital Vision Research Center, Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, Florida, USA.

*Correspondence to: Gaofeng Wang, Ph.D., Biomedical Research Building 608, 1501 NW 10th Ave, University of Miami Miller School of Medicine, Miami, FL 33136, USA. E-mail: gwang@med.miami.edu.
Abstract

It is widely accepted that cAMP regulates gene transcription principally by activating the protein kinase A (PKA)-targeted transcription factors. Here, we show that cAMP enhances the generation of 5-hydroxymethylcytosine (5hmC) in multiple cell types. 5hmC is converted from 5-methylcytosine (5mC) by Tet methylcytosine dioxygenases, for which Fe(II) is an essential cofactor. The promotion of 5hmC was mediated by a prompt increase of the intracellular labile Fe(II) pool (LIP). cAMP enhanced the acidification of endosomes for Fe(II) release to the LIP likely through RapGEF2. The effect of cAMP on Fe(II) and 5hmC was confirmed by adenylate cyclase activators, phosphodiesterase inhibitors, and most notably by stimulation of G protein-coupled receptors (GPCR). The transcriptomic changes caused by cAMP occurred in concert with 5hmC elevation in differentially transcribed genes. Collectively, these data show a previously unrecognized regulation of gene transcription by GPCR-cAMP signaling through augmentation of the intracellular labile Fe(II) pool and DNA demethylation.

Introduction

Cyclic AMP (cAMP), the second messenger, converts the binding of ligands with many different G-protein coupled receptors (GPCRs, also known as seven-transmembrane domain receptors) into various biological activities (Sutherland, 1970). Principally, cAMP exerts its function through three major targets – cAMP-dependent protein kinase A (PKA), cyclic nucleotide-gated ion channels (CNGCs), and exchange protein directly activated by cAMP (Epac). It is well known that cAMP has an impact on gene transcription, which is currently considered to be mediated by the PKA-targeted transcription factors (Montminy, 1997). Three transcription factors, including cAMP response element-binding protein (CREB), cAMP response element modulator (CREM), and activating transcription factor 1 (ATF1), can be phosphorylated by PKA and subsequently
bind to cAMP response elements (CRE) in gene promoters, generally to activate gene transcription (Sands and Palmer, 2008).

Schwann cells form the myelin sheath of axons within the peripheral nervous system. cAMP is a known instructive signal for Schwann cell differentiation into a myelinating phenotype (Jessen et al., 1991). cAMP directly induces cell cycle arrest along with the expression of a wide variety of myelination-associated genes such as Egr2/Krox20, a transcription factor that is considered a master regulator of the myelin program, and myelin protein zero, the main peripheral nerve myelin protein (Bacallao and Monje, 2015). However, the mechanism by which cAMP regulates transcription and promotes the differentiation of Schwann cells is not fully understood.

DNA methylation is one major epigenetic mark that regulates gene transcription. Active DNA demethylation is catalyzed by ten-eleven translocation (Tet) methylcytosine dioxygenases, which oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) (Tahiliani et al., 2009; Kriaucionis and Heintz 2009), and further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (He et al., 2011; Ito et al., 2011). 5fC and 5caC are ultimately replaced by unmodified cytosine (5C) to complete cytosine demethylation (Pastor et al., 2013). It is noteworthy that 5hmC is also a unique epigenetic mark with regulatory capacities in addition to being a DNA demethylation intermediate (Shen et al., 2014). Tet belongs to the iron and 2-oxoglutarate (2OG, alternatively known as α-ketoglutarate)-dependent dioxygenase family which utilizes labile Fe(II) as a cofactor and 2OG as a co-substrate. We and others have shown that ascorbate, which has the capacity to reduce the redox-inactive Fe(III)/Fe(IV) to Fe(II), is another cofactor for Tet (Minor et al., 2013; Yin et al., 2013; Blaschke et al., 2013; Dickson et al., 2013; Chen et al., 2013). Thus, ascorbate has an impact on DNA demethylation by promoting the availability of redox-active Fe(II) to Tet. 5mC and 5hmC are major epigenetic marks that govern both cell identity and cellular phenotype transformation. Thus, it is plausible that such marks are involved in the transition of Schwann cells from the immature to myelinating phenotype.
Similar to cAMP, ascorbate was identified as another essential factor for Schwann cells to initiate and promote myelin formation (Bunge et al., 1986). Intracellular ascorbate deficiency can cause hypomyelination in modeled rodents (Gess et al., 2011). It is plausible that, by regulating DNA demethylation, ascorbate alters the cellular phenotype of Schwann cells towards a myelinating state. Based on this shared function in enhancing Schwann cell myelination, we reasoned that cAMP, like ascorbate, might also play a role in Tet-mediated DNA demethylation. Here, we investigated the potential role of cAMP in DNA demethylation. We identified that cAMP enhanced the generation of 5hmC in cultured Schwann cells. The effect of cAMP on 5hmC was not limited to Schwann cells but is likely a general effect, as it has been verified in other cell types. cAMP promptly increased the intracellular labile Fe(II) pool (LIP) independently of major cAMP targets but likely through RapGEF2 to enhance the acidification of endosomes and subsequently elevate the LIP. The increase of labile Fe(II) could mediate the effect of cAMP on 5hmC. The effect of cAMP on Fe(II) and 5hmC was mimicked by the addition of AC activators and phosphodiesterase (PDE) inhibitors such as caffeine. Stimulation of Schwann cells with isoproteenol or calcitonin gene-related peptide (CGRP), two ligands known to increase intracellular cAMP through Gs-coupled receptors (Cheng et al., 1995), elevated levels of intracellular labile Fe(II) and 5hmC. Furthermore, stimulation with cAMP caused a genome-wide shift in 5hmC profile, which correlated with a majority of the differentially expressed transcripts in Schwann cells.

Results

cAMP promotes 5hmC generation.

In the absence of cAMP treatment, 5hmC was barely detectable by dot blot in primary cultured rat Schwann cells. However, after treatment with membrane-permeable cAMP (100 µM), 5hmC
signal emerged at comparable levels to cells treated with ascorbate (50 µM, Figure 1A and 1B).

Encouraged by this initial observation, we investigated the role of cAMP on 5hmC by immunofluorescence (IF). The addition of cAMP (0 – 250 µM) dose-dependently increased 5hmC generation in Schwann cells (Figure 1C and 1D). The IF signal for 5hmC was sustained for 1 – 7 days upon continuous cAMP (100 µM) treatment (Figure 1E and 1F), suggesting that continuous cAMP treatment largely maintained 5hmC. An enhanced generation of 5hmC was also verified in other cell types examined, such as HEK-293 cells, mouse embryonic fibroblasts (MEF) and neuroblastoma SH-SY5Y cells, indicating that the effect of cAMP on 5hmC is not limited to Schwann cells and is likely to be a general effect (Figure 1G).

We next tested whether the elevation of endogenous cAMP imparts a similar effect as exogenously applied cAMP. Treating cells with forskolin, which directly activates transmembrane adenylate cyclase (AC) to produce endogenous cAMP, promoted 5hmC generation in Schwann cells as did bicarbonate, an activator of soluble AC (Figure 1H). The production of 5hmC was also observed when cells were treated with phosphodiesterase (PDE) inhibitors caffeine or IBMX, both of which prevent cAMP degradation (Figure 1H). Conversely, no 5hmC signal was observed after cells were treated with AMP (100 µM) (Figure 1H). Collectively, these observations suggest that endogenous cAMP is indeed involved in 5hmC generation.

**cAMP increases the intracellular labile Fe(II) pool to generate 5hmC.**

To understand how cAMP enhances 5hmC generation, we first examined the transcription of Tet. mRNA levels of Tet2 and Tet3 were decreased, whereas Tet1 mRNA remained unchanged after treatment with cAMP (100 µM) for 1 day (Figure 1 – figure supplement 1 and table supplement 1), a time point at which cAMP clearly promoted 5hmC generation (Figure 1E and 1F). Thus, the increased level of 5hmC does not appear to be mediated by an effect of cAMP on
the expression of Tet. In healthy cells such as primary cultured Schwann cells, the most plausible rate-limiting factor for Tet’s enzymatic activity is the replenishment of the Fe(II) cofactor rather than 2OG, a relatively abundant intermediate of the Krebs cycle. We thus hypothesized that cAMP may increase access to bioavailable Fe(II), as does ascorbate. To test this hypothesis, we used Trx-Puro (Spangler et al., 2016), a recently developed reactivity-based probe that is highly specific for redox–active labile Fe(II) within the cell. Reaction of Trx-Puro with labile Fe(II) unmasks the aminonucleoside puromycin through a Fenton-type reaction. Released puromycin is incorporated in cellular proteomes, which can be visualized using a convenient IF assay (Spangler et al., 2016). Treatment with cAMP at a dose as low as 1 µM increased intracellular labile Fe(II), which reached even higher levels after treatment with 10 – 100 µM cAMP (Figure 2A and 2B). Incubation of the cells with Diox-Puro (Spangler et al., 2016), a dioxolane control conjugate that is non-peroxidic, showed no IF signal after cAMP treatment indicating the Fe(II) dependence of the signal detected by Trx-Puro (Figure 2 – figure supplement 1). The action of cAMP on intracellular labile Fe(II) was rapid. Fe(II) signal appeared within 2 hours (the minimal time for simultaneous incubation of cAMP and Trx-Puro) and peaked at 4 hours after cAMP treatment (Figure 2C and 2D). Similar to 5hmC, the effect of cAMP on intracellular labile Fe(II) was also validated in other types of cells including HEK-293, MEF, and SH-SY5Y (Figure 2E), suggesting that it is likely a general effect.

Treatment with AMP had no observable effect on labile Fe(II) (Figure 3A). Elevating endogenous cAMP also augmented intracellular labile Fe(II), as demonstrated by the treatment of Schwann cells with AC activators (forskolin, bicarbonate) and PDE inhibitors (caffeine, IBMX) (Figure 3A). Ascorbate at physiological levels has been shown to have no obvious influence on intracellular labile Fe(II) (Spangler et al., 2016). Indeed, no obvious change in the intracellular labile Fe(II) pool was observed in Schwann cells after treatment with ascorbate at a
physiological concentration (50 µM) (Figure 3 – figure supplement 1), suggesting that ascorbate may only increase the bioavailability of Fe(II) to Tet and other enzymes locally.

To determine whether cAMP promotes 5hmC generation via labile Fe(II), Schwann cells were treated with two different iron chelators, 2,2-bipyridyl (20 µM) and deferoxamine (20 µM) prior to cAMP stimulation. These iron chelators drastically decreased the level of labile Fe(II), and further abolished the effect of cAMP on 5hmC in Schwann cells (Figure 3B). The abolishing effect of the iron chelators on cAMP-induced 5hmC generation was also verified in other cells such as human melanoma A2058 cells (Figure 3 – figure supplement 2). These results suggest that cAMP upregulates 5hmC by elevating the intracellular labile Fe(II).

cAMP increases the intracellular labile Fe(II) by enhancing endosome acidification.

The molecular mechanism by which cAMP alters labile iron in principle could be related to iron uptake and storage in the cell. Cellular uptake of iron is a complicated cascade involving the internalization of transferrin-transferrin receptor complex and Fe(III) discharge from transferrin after the acidification of endosomes, via vacuolar H⁺-ATPase (V-ATPase, the H⁺ pump). Subsequently, Fe(III) is converted to Fe(II) by Steap3 and leaves the endosome via divalent metal transporter 1 (DMT1) to enter the LIP (De Domenico et al., 2008). One key step in the iron uptake is endosome acidification. cAMP treatment consistently decreased the pH in endocytotic vesicles as measured using vesicle pH indicator that emits increasing levels of fluorescence as pH decreases from 8 to 4 (Figure 4A). These results suggest that cAMP enhances the acidification of endosome, which could underlie the elevated LIP.

We then tested whether elevated labile Fe(II) and 5hmC by cAMP signaling is mediated by enhanced endosome acidification. Cells were pretreated with V-ATPase inhibitor Bafilomycin A1 (200 nM). Indeed, Bafilomycin A1 largely abolished the effect of cAMP on labile Fe(II) as well as 5hmC in the cells (Figure 4B). Furthermore, knocking down the expression of ferritin did not
block the induction of labile Fe(II) elevation by cAMP indicating that iron storage may not be a major target of cAMP signaling (Figure 4 – figure supplement 1). Overall, these results suggest that cAMP signaling upregulates the LIP, and subsequently 5hmC generation by acidifying the endosome.

In an attempt to understand how cAMP signaling causes endosome acidification, we examined the major known targets of cAMP, which include PKA, CNGCs, and Epac. PKA phosphorylates substrate proteins and Epac acts as a guanine nucleotide exchange factor for the small G protein Rap20. CNGCs are nonselective cation channels for Na\(^+\), Ca\(^{++}\), and other cations, but have no known role in transporting iron. To determine whether labile Fe(II) depends on the major known signaling targets of cAMP, we pretreated the cells with PKA inhibitors (KT5720, H89), an Epac inhibitor (ESI09) or a CNGC channel blocker (LCD). Neither the inhibitors nor the channel blocker prevented the observable alterations in labile Fe(II) in cAMP-treated Schwann cells (Figure 5A). Using an in vitro assay, we confirmed that H89 (20 \(\mu\)M) and KT5720 (2 \(\mu\)M) indeed abolished the cAMP-induced phosphorylation of Peptag peptide which is catalyzed by PKA, thus confirming the potency of the two PKA inhibitors (Figure 5 – figure supplement 1). Furthermore, the transcripts of CNGC and Epac (RapGEF3, RapGEF4) were expressed at either null or extremely low levels in control and cAMP-treated Schwann cells (Figure 5 – table supplement 1), suggesting that the impact of CNGC and Epac on intracellular labile Fe(II) is likely to be negligible. These results suggest that the upregulation of intracellular labile Fe(II) by cAMP is likely independent of these known pathways.

Although the transcription of Epac1 (RapGEF3) and Epac2 (RapGEF4) is very low, other RapGEFs, especially RapGEF2 transcripts are expressed at a much higher level (> 330 fold of Epac1 or Epac2) in Schwann cells. RapGEF2 appears to be also a target of cAMP, which could be further involved in the activation of other small GTPases (Pak et al., 2002). To explore whether RapGEF2 mediates the elevation of labile Fe(II) via endosome acidification, we used
siRNA to knockdown its expression. Due to the low transfection efficiency of primary cultured Schwann cells, we alternatively used HEK-293 cells, which also showed a robust elevation of labile Fe(II) and 5hmC in response to cAMP treatment. In scramble siRNA treated cells, cAMP treatment decreased the pH value in endocytic vesicles and elevated the intracellular labile Fe(II). However, after the expression of RapGEF2 was reduced by siRNA, the effect of cAMP on endosome acidification and labile Fe(II) was largely abolished (Figure 5B). The knockdown effect of siRNA on RapGEF2 was confirmed by qRT-PCR (Figure 5 – figure supplement 2). These results suggest that cAMP signaling, via RapGEF2, acidifies the endosome to augment the LIP.

**Activation of Gs-coupled receptors increases intracellular labile Fe(II) and 5hmC.**

Signaling of many GPCRs either up- or downregulates intracellular cAMP, depending on the coupled Gs or Gi. It was shown that isoproterenol and calcitonin gene-related peptide (CGRP) elevate cAMP in Schwann cells by binding to β-adrenergic receptors and CGRP receptors respectively (Cheng et al., 1995), both of which are coupled with Gs. We therefore used these two GPCRs as models to test whether their signaling can change DNA demethylation. Indeed, stimulation with isoproterenol or CGRP increased 5hmC in Schwann cells (Figure 6A), which correlated with an elevated level of intracellular labile Fe(II) (Figure 6B). Furthermore, AC inhibitor SQ22536 largely abolished the effect of isoproterenol and CGRP on labile Fe(II) (Figure 6B). Overall, these results indicate that GPCR signaling regulates DNA demethylation by augmenting the intracellular Fe(II) pool via activated ACs and elevated cAMP.

**cAMP changes the 5hmC profile in differentially expressed genes.**

It has been previously shown that cAMP shifts the transcriptome (Montminy, 1997). Indeed, 7,020 transcripts were differentially transcribed in Schwann cells in response to elevated cAMP.
as shown by RNA-seq (Figure 7A and 7B). Of the differential transcripts, 54% were upregulated and 46% downregulated, which is concordant with the bi-directional transcriptional regulation of 5hmC (Wu et al., 2011). Furthermore, genome-wide 5hmC profiles were also altered by cAMP treatment as revealed by hMeDIP-seq (Figure 7C). In total, 66,963 5hmC peaks were upregulated and 10,026 peaks were downregulated by cAMP treatment. By integrating RNA-seq and hMeDIP-seq, we found that 4,071 differential transcripts (58% of total differential transcripts) correlate with altered 5hmC peaks located within promoter regions or gene bodies (Figure 8A). Overall, cAMP increased 5hmC level mainly in gene bodies of these differential transcripts, suggesting that changes in 5hmC could be responsible for the differential transcription or involved in its regulation.

Currently, it is thought that PKA-dependent phosphorylation of three transcription factors including cAMP response element-binding protein (CREB), cAMP response element modulator (CREM), and activating transcription factor 1 (ATF1) are responsible for the transcriptional changes caused by cAMP (Sands and Palmer, 2008). However, 3,965 transcripts (56.5% of total differential transcripts) might be targeted by the three transcription factors after reviewing ChIP data in ENCODE and ChEA databases (Yip et al., 2012; Lachmann et al., 2010). Of the 3,965 transcripts, 2,372 transcripts were also accompanied with differential 5hmC peaks, suggesting that these genes might be dually regulated by 5hmC and the transcription factors (Figure 8B). Additionally, 24.2% of differentially transcribed genes have differential 5hmC peaks but have not been previously shown to be targeted by CREB, CREM, or ATF1. For example, cAMP dramatically induced the transcription of Egr2 (also known as Krox-20), which plays a key role in Schwann cell myelination (Arthur-Farraj et al., 2011). After cAMP treatment, there was an obvious increase of 5hmC peaks in the gene body of Egr2 (Figure 8C), which is also thought to be targeted by CREB (Hossain et al., 2012). On the other hand Pmp2, which is predominantly expressed in myelinating Schwann cells (Zenker et al., 2014), has no obvious binding motif for the PKA-dependent transcription factors. cAMP treatment elevated the 5hmC level in the gene
body and upregulated its transcription dramatically, suggesting this gene could be regulated by 5hmC and not the PKA-dependent transcription factors.

Discussion

It is known that epigenetic changes, such as DNA methylation, reflect the interface of a dynamic extracellular microenvironment and the genome. However, the specific signaling molecules that serve as mediators between environmental variation and epigenomic changes remains largely elusive. On the other hand, GPCRs, the largest and most diverse group of membrane receptors, sense extracellular changes by binding with specific ligands. Signaling of cAMP, one major second messenger of the GPCRs, and its targets has been thoroughly studied and was thought to be well established. Our results show a previously unknown pathway of GPCR-cAMP signaling in promoting the intracellular labile Fe(II) pool, enhancing DNA demethylation and consequently changing gene transcription.

The convergence of 5hmC generation and labile Fe(II) concentrations suggests a previously unrecognized role for labile Fe(II) in propagating a signal initiated by cAMP and mediated by the Fe(II)-dependent oxidase activity of Tet. This new role for iron is a plausible one given that cellular iron uptake and the shuttling of iron from storage in ferritin as Fe(III) to its entry into the cytosolic labile pool of Fe(II) is a fundamental aspect of cellular iron metabolism. While this process is clearly involved in maintaining iron homeostasis and the biosynthesis of Fe(II) cofactors, it appears also to have been appropriated for cellular signaling in the particular case of enzymes that employ labile Fe(II) as an essential co-factor, such as the iron and 2OG-dependent Tet and Jumonji C domain-containing histone demethylases. The changes to labile cytosolic Fe(II) measured by Trx-Puro likely also reflect the nuclear Fe(II) pool, since there is a rapid equilibrium between these two Fe(II) pools, presumably via nuclear pores (Ma et al., 2015).
Thus, the results described above suggest that cAMP increases the accessibility of Fe(II) to Tet by augmenting labile Fe(II) in the cytosol and nucleus.

An increase in labile Fe(II) can be induced by cAMP in as little as 2 hours, indicating that no new protein synthesis is required. The molecular mechanism by which cAMP alters labile Fe(II) appears to be related to iron uptake rather than storage. Cellular iron uptake is a multistep process including internalization of the transferrin-transferrin receptor complex, vesicle formation, endosome acidification, Fe(III) discharge, conversion of Fe(III) to Fe(II), and release of Fe(II) from the endosome to the LIP. cAMP could potentially be involved in each step of iron uptake. Our results suggest that cAMP signaling increases the acidification of endocytotic vesicles, which is essential for Fe(III) discharge from the internalized transferrin. This is consistent with earlier studies showing that cholera and pertussis toxins, by increasing intracellular cAMP, enhance endosome acidification (Van Dyke. 1997). It could be that cAMP signaling increased the number and/or activity of V-ATPase to pump H⁺ into the endosome to release Fe(II) to the LIP, which subsequently promotes 5hmC generation. Indeed, we showed that inhibitors of V-ATPase largely abolish the effect of cAMP on labile Fe(II) and 5hmC.

The elevation of labile Fe(II) by cAMP signaling appears independent of the major targets of cAMP including PKA, Epac, and CNGC. We examined the effect of RapGEF2 initially based on its higher expression level in Schwann cells. cAMP failed to induce labile Fe(II) and 5hmC once the expression of RapGEF2 was reduced, suggesting that RapGEF2 could be at least one major mediator of cAMP to regulate labile Fe(II) and 5hmC. Thus, it is plausible that RapGEF2, directly or indirectly, affects the number and/or activity of V-ATPase in the endosome. Future studies are warranted to investigate how RapGEF2 regulates the function of V-ATPase.

Both cAMP and ascorbate appear to enhance the activity of Tet to generate 5hmC. However, the mechanisms of cAMP and ascorbate in promoting the availability of Fe(II) to Tet are likely different. Ascorbate at physiological concentrations has no obvious influence on intracellular labile Fe(II) shown here and before (Spangler et al., 2016), but is thought to maintain local iron
in the active form of Fe(II) for Tet as it does for other hydroxylases (Mandl et al., 2009). In contrast, cAMP elevates the global intracellular labile Fe(II) pool.

For the first time, GPCRs via cAMP are directly linked to epigenetic regulation. The first hint of a possible role for cAMP in DNA demethylation came from studies on Schwann cells. Using Schwann cells as a model, we showed that intracellular cAMP elevation increased the intracellular labile Fe(II) pool, changed genome-wide 5hmC profile, and altered the transcription of many genes. More importantly, activation of different Gs-coupled receptors demonstrated increases in intracellular labile Fe(II) and 5hmC similarly to treatments that elevate intracellular cAMP including membrane permeable cAMP, AC activators, or PDE inhibitors. Coupled with Gs, Gi, or both, GPCRs can raise or decrease the intracellular cAMP, which will further change DNA methylation-demethylation dynamics. Thus, GPCRs via cAMP transduce microenvironmental cues to epigenetic alterations.

One recent study showed that cAMP has an impact on DNA methylation by mainly increasing the expression of Tet or DNA methyltransferases (Fang et al., 2015). This may not be a general effect but more likely a cell specific effect since we observed a decreased expression of Tet2 and Tet3 in Schwann cells after cAMP treatment.

In this study, most experiments were conducted in Schwann cells. However, the effect of cAMP on 5hmC is not limited to Schwann cells and is likely a general effect, as it has been verified in other cell types. Studies have shown that 5hmC is not only a demethylation intermediate but also a unique epigenetic mark that regulates transcription by recruiting a different set of binding proteins compared to 5mC (Spruijt et al., 2013). Indeed, altered 5hmC profiles caused by cAMP correlated with a majority of differentially transcribed genes. Thus, cAMP regulates the transcriptome by enhancing DNA demethylation, in addition to the known pathway of PKA-targeted transcription factors. In silico analysis suggests that some differential transcripts correlate with either 5hmC or the three transcription factors, while other transcripts correlate with both or none of them. The focus of this study is to link the dots from GPCR to
cAMP, Fe(II), 5hmC, and ultimately transcription. Future studies can dissect the impact of cAMP on the transcriptome and the contribution of 5hmC and/or the three transcription factors in different cell types.

In conclusion, our results show a novel function of cAMP signaling in regulating DNA demethylation by increasing the intracellular labile Fe(II) pool likely via a non-canonical pathway, which consequently promotes the oxidation of 5mC to 5hmC by Fe(II)-dependent Tet. Dynamic intracellular cAMP, regulated by Gs-/Gi-coupled receptors and factors affecting AC/PDE directly, therefore could have a profound impact on the epigenome.

Materials and Methods

Materials

cAMP [8-(4-Chlorophenylthio) adenosine-3',5'-cyclic monophosphate] and AMP (8-Bromoadenosine-5-O-monophosphate) were purchased from Biolog (US distributor, Axxora LLC, San Diego, CA). Recombinant herregulin-β1 (herein referred to as neuregulin) was purchased from Peprotech (Rocky Hill, NJ). Forskolin, poly-L-lysine, bicarbonate, sodium ascorbate, bafilomycin A1, 2,2'-bipyridyl, deferoxamine and laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane were purchased from Sigma-Aldrich (St. Louis, MO). Caffeine was bought from Enzo Life Sciences (Farmingdale, NY). SQ 22536, IBMX, ESI-09, CGRP (Calcitonin Gene-Related Peptide (rat)) and Isoproterenol hydrochloride were purchased from Tocris Bioscience (Bristol, UK). KT5720 and H-89 Dihydrochloride were purchased from Millipore (Billerica, MA). LCD (L-cis-Diltiazem) was purchased from Abcam (Cambridge, UK). Dulbecco’s modified eagles’s medium (DMEM) that contain reduced level of sodium bicarbonate (1.5 g/L) (cat#30-2002) was purchased from American Type Culture Collection (ATCC) (Manassas, VA).
Cell culture and treatments

Primary cultured Schwann cells were isolated from the sciatic nerves of 3 month-old Fisher rats as described previously (Bacallao and Monje, 2015). Briefly, sciatic nerves were cut into small segments and allowed to degenerate in vitro by incubation for 10 days in DMEM medium containing 10% heat inactivated FBS. Degenerated nerve explants were dissociated with a mixture of 0.25% dispase and 0.05% collagenase and the resulting cell suspension was plated on poly-L-lysine (PLL)-coated dishes. The purified Schwann cells were expanded up to passage one in DMEM supplemented with 2 \( \mu \)M forskolin and 10 nM neuregulin. Experiments were performed on Schwann cells between 3 to 5 rounds of expansion plated on PLL-laminin coated glasses in 24-well dishes. The media used were DMEM supplemented with 10% FBS and without forskolin and neuregulin. Treatment of Schwann cells with cAMP was carried out as reported previously (Bacallao and Monje, 2015). HEK-293, A2058, and SH-SY5Y cell lines were obtained from ATCC (Manassas, VA). Mouse embryonic fibroblasts were a gift from Dr. Katherine Walz from the University of Miami. All these cells were cultured in DMEM containing 10% heat-inactivated FBS. Each treatment group contains three wells and major experiments were replicated at least three times.

5hmC dot-blot assay

Genomic DNA was extracted from cultured Schwann cells using QIAamp DNA mini kits (Qiagen, Hilden, Germany). A Qubit Fluorometer (Life Technologies, Carlsbad, CA) was used to quantify the concentration of DNA. DNA samples were diluted with 2N NaOH and 10 mM Tris·Cl, pH 8.5, then loaded on a Hybond N+ nylon membrane (GE Healthcare, Little Chalfont, UK) using a 96-well dot-blot apparatus (Bio-Rad Laboratories, Hercules, CA). After hybridizing at 80°C for 30 min and blocking with 5% non-fat milk for 1 hour at room temperature, the membrane was incubated in polyclonal anti-5hmC antibody (Active Motif #39769, 1:10,000,
Carlsbad, CA) at 4°C overnight. 5hmC was visualized by chemiluminescence. The densities of the dots on membrane were captured and quantified by ImageJ. To ensure equal loading, the membrane was stained with methylene blue post-immunoblotting. Statistical significance of differences in 5hmC content between treatments was assessed by Student t test, at α = 0.05.

**Immunofluorescence assay for 5hmC**

Cells were seeded in 24-well plates with coverslips at 10,000–50,000 cells per well. After treatments, cells were fixed with 4% paraformaldehyde and then incubated with 2N HCl at 37°C for 20 min and neutralized with 100 μM Tris-HCl for 10 min. After washing with PBS and blocking with 0.4% Triton X-100 with 10% FBS in PBS for 1 hour, cells were incubated with anti-5-hmC antibody (Active Motif #39769, 1:1,000) at 4°C overnight. Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:500) was used as an immunofluorescent secondary antibody. Cells were then counterstained with DAPI.

**Intracellular labile Fe(II) detection**

Labile Fe(II) was detected using the reported method (Spangler et al., 2016). First, cells were treated with puromycin conjugates TRX-Puro, Diox-Puro (a dioxolane conjugate as non-peroxidic control), or puromycin (1 μM) for 2 hours (Spangler et al., 2016). After washing with PBS, cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were then permeabilized with 0.4% Triton X-100 with 10% FBS and 0.25% fish skin gelatin in PBS for 30 min. Cells were incubated with anti-puromycin antibody (1:500) (Kerafast, Boston, MA) overnight at 4°C. After washing, cells were incubated with anti-mouse Alexa Fluor 488 secondary antibody (1:500) for 1 hour. Cells were then counterstained with DAPI.

**Endocytotic vesicle pH detection**
The acidification of endocytotic vesicles was measured by pHrodo Red Dextran (Thermo Scientific, Waltham, MA) according to the manufacturer’s instructions. Briefly, after plating and cAMP treatments, cells were washed 3 times with PBS and incubated with pHrodo Red Dextran at a final concentration of 20 µg/ml for 20 min in the incubator. Cells were then washed again with PBS and fixed with 4% PFA for 10 min. After fixation, cells were counterstained with Hoechst 33342. Images were captured by a Zeiss LSM 710 confocal microscopy.

**In vitro PKA assay**

To confirm the activity of PKA inhibitors, a non-radioactive PepTag PKA assay (Promega, Madison, WI) was used to measure PKA activity. A2058 cells were plated at 10 cm dishes and and Schwann cell plated in 24 well plates, which were incubated with or without PKA inhibitors (H-89 and KT5720) for 30 min. After treatment with cAMP (100 µM) for 30 min at 37°C, cells were harvested for lysates and for intracellular labile (FeII) detection. The lysate was incubated with the PepTag peptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), which is a substrate of PKA for phosphorylation. The phosphorylated peptides have a negative charge that was then separated from the non-phosphorylated peptide by agarose gel electrophoresis. The bands were excised and the phosphorylated peptide was quantified by reading the absorbance at 570 nm using a Synergy 2 Biotek spectrophotometer.

**Gene silencing by siRNA**

Small short interfering RNA (siRNA) was used to transiently silence Ferritin heavy chain 1 (FTH1) and RAPGEF2. To decrease the expression of Ferritin, a pool of three siRNA duplexes (cat# SR301663, Origene Technologies, Rockville, MD) against FTH1 and scramble siRNA (cat# SR30004) were used. To reduce the expression of RapGEF2, a pool of four siRNA duplexes (cat# L-009742-00-0005, Dharmacon, Lafayette, CO) was applied. siRNA were
delivered to cells by SiTran Transfection reagent (Origene Technologies). After transfection for 72 hours, cells were treated with cAMP for intracellular labile Fe(II) measurement. Cells in a subset of wells were harvested for RNA and protein extraction and subsequent qRT-PCR and immunoblot assays.

**Immunoblot**

Cells were washed twice with PBS and then lysed with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40) in the presence of protease and phosphatase inhibitors. Prior to SDS-PAGE, cell lysates were re-suspended in SDS sample buffer (60 mM Tris–HCl, 1% SDS, 10% glycerol, 0.05% bromophenol blue, pH 6.8, with 2% β-mercaptoethanol). Samples were subjected to 10% SDS-PAGE (Bio-Rad, Hercules, CA) and transferred to PVDF membranes (Bio-Rad) for immunoblot. Transfer efficiency was determined by Ponceau S staining (Sigma-Aldrich, St. Louis, MO). PVDF membranes were incubated with blocking solution (TBS containing 0.1% Tween 20 and 5% BSA) and were probed with specific antibodies including anti-Ferritin heavy chain (B-12) (sc-376594, Santa Cruz Biotechnology, Dallas, TX) and anti-β-Actin (13E5) (cat# 4970, Cell Signaling Technology, Danvers, MA). Protein bands were detected using a chemiluminescence kit (Millipore, Billerica, MA).

**Image acquisition and analysis**

Cell fluorescence images were acquired into a 512 × 512 frame size by averaging 16 times at a bit depth of 8 using a Zeiss LSM 710 confocal microscope (Oberkochen, Germany). Fluorescence intensity was quantified using Fiji (ImageJ) (Schindelin et al., 2012). Average intensity values were measured from every cell within the image field from a minimum of five 20× images per condition (Around 450 cells per condition). The intensity values from individual
cells were plotted and statistically analyzed by one-way ANOVA with Tukey post hoc test using GraphPad Prism 7 from GraphPad Software (La Jolla, CA).

Quantitative real-time RT-PCR
RNA was extracted from cultured Schwann cells using RNeasy kits from Qiagen (Hilden, Germany). A nanodrop 8000 photospectrometer (Thermo Scientific, Waltham, MA) was used to quantify RNA. The qScript Flex cDNA kit from Quanta Biosciences (Beverly, MA) was used for reverse transcription (RT) according to the manufacturer’s instructions. Quantitative real-time RT-PCR (qRT-PCR) was performed in triplicate on a QuantStudio 12K Flex using the PowerUp Sybr Green Master Mix from Life Technologies (Carlsbad, CA). Primers were designed to span introns (Figure 1—table supplement 1). The transcript amplification results were analyzed with the QuantStudio 12K Flex software, and all values were normalized to the levels of the Sdha using the $2^{\Delta\Delta Ct}$ method. Statistical significance of differences in expression levels was assessed by Student $t$ test, at $\alpha = 0.05$.

RNA-seq
Total RNA was extracted from cells using the RNEasy Mini Kit from Qiagen (Hilden, Germany). A Bioanalyzer 2000 was used to measure the quality of RNA. All samples’ RNA integrity numbers (RIN) were above 9. Whole transcriptome sequencing (also known as RNA-seq) was carried out at the Sequencing Core of John P. Hussman Institute of Human Genomics at the University of Miami using the TruSeq Stranded Total RNA Library Prep Kit from Illumina (San Diego, CA). RNA-seq was performed with 3 replicates per group to give ~89% power to detect a 1.5-fold change between and a standard deviation of 14.5% of the mean. This standard deviation was calculated from actual data used for analysis. Briefly, after ribosomal RNA (rRNA) was depleted, sequencing libraries were ligated with standard Illumina adaptors and
subsequently sequenced on a HiSeq2000 sequencing system (125 bp paired-end reads, 4 samples per lane; Illumina, San Diego, CA, USA). All samples after sequencing had between 39,094,152 and 54,392,564 reads. Raw read data was first run through quality control metrics using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were trimmed with trim_galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to remove low quality bases from reads (scores <20 in Phred + 33 format) and Illumina adapters. After quality control was checked and trimming performed, sequence reads were aligned to the rat transcriptome (Rnor_6.0, Ensembl.org), quantified using the STAR aligner (Dobin et al., 2013), and normalized using CuffQuant and CuffNorm, which are part of the Tuxedo Suite (Trapnell et al., 2012). All samples had between 37,714,718 and 52,162,956 aligned reads. Statistical significance was determined using 3 alternative differential expression calculators: edgeR, DESeq2, and CuffDiff (Robinson et al., 2010; Love et al., 2014; Trapnell et al., 2012). To reduce false positives, differentially expressed features were determined by cutoff adjusted P values of 0.05 across all 3 methods (false discovery rate, FDR). DESeq2 called 8,589 differential transcripts, edgeR called 8,029 differential transcripts, and CuffDiff called 8,076 differential transcripts with a total of 7,020 transcripts called as differential by all three programs. Of the 7,020 transcripts, 3,809 transcripts increase in expression and 3,211 transcripts decrease after treatment with cAMP. Differential transcripts were visualized using z-scores per transcript where blue represents lower expression and red represents higher expression in the form of a heatmap generated using the heatmap.2 function of gplots (https://cran.r-project.org/web/packages/gplots/index.html).

hMeDIP-seq

Genomic DNA was extracted from Schwann cells using QIAamp DNA mini kits from Qiagen (Hilden, Germany) according to the manufacturer’s instructions. A Qubit Fluorometer from Life Technologies (Carlsbad, CA, USA) was used to quantify the concentration of DNA. A
Bioanalyzer 2000 was used to measure the quality of DNA. DNA was submitted for hydroxymethylated DNA immunoprecipitation sequencing (hMeDIP-seq) at the Epigenomics Core at the University of Michigan (Mohn et al., 2009). hMeDIP-seq was performed with 3 replicates per group to give ~98% power to detect a 2-fold change between groups and a standard deviation of 19% of the mean. This standard deviation was calculated from actual data used for analysis. Briefly, DNA is sonicated to approximately 100 bp and then ligated with Illumina adaptors. A portion of the DNA was set aside for sequencing as unprecipitated input DNA and the remaining DNA was incubated overnight at 4°C with an antibody against 5hmC from Active Motif (Carlsbad, CA). The antibodies, along with immunoprecipitated DNA, were then pulled out of solution using Protein G magnetic beads from Invitrogen, (Carlsbad, CA). Magnetic beads were then washed with immunoprecipitation buffer (10 mM sodium phosphate pH 7.0 with 140 mM NaCl and 0.05% triton x-100). The beads were then resuspended in proteinase K buffer and incubated for 3 hours at 55°C to remove the antibodies from the DNA. Unprecipitated input DNA was incubated with proteinase K buffer alongside the precipitated DNA. After proteinase K treatment, DNA was purified using AMPure beads from Beckman Coulter (Brea, California). Efficiency of immunoprecipitation was evaluated using the 5hmC, 5mC, cytosine DNA standard pack from Diagenode (Searing, Belgium). DNA was then sequenced on a HiSeq4000 sequencing system (50 bp single-end reads, 3 samples per lane; Illumina, San Diego, CA). Immunoprecipitated samples all had between 123,192,512 and 143,992,231 reads and unprecipitated input samples all had between 84,030,758 and 142,427,775 reads. Reads were trimmed with trim_galore to remove low quality bases from reads (scores <20 in Phred+33 format), and Illumina adapters. After quality control was checked sequence reads were aligned to the rat genome (Rnor_6.0, Ensembl.org) using BWA (Li et al., 2009a). All immunoprecipitated samples had between 117,762,176 and 137,740,430 aligned reads and unprecipitated input samples had between 80,822,191 and 138,629,799 aligned reads.
Multimapped reads were removed using Samtools ([Li et al., 2009b](Li et al., 2009b)) and duplicate reads were removed using PicardTools (https://broadinstitute.github.io/picard/). All precipitated samples had between 63,471,316 and 78,618,595 uniquely aligned reads and all unprecipitated input samples had between 44,195,649 and 80,394,782. To identify regions of the genome with substantial levels of 5hmC, peaks were called and filtered using the Irreproducible Discovery Rate (IDR) method developed for the ChIP-seq portion of the ENCODE project ([Li et al., 2011](Li et al., 2011)).

Briefly, peak calling was performed with MACS2 using the narrow peak mode and a relaxed threshold of 0.001 ([Zhang et al., 2008](Zhang et al., 2008)). The number of peaks called for the control samples were 364,776, 346,424, and 331,841. The number of peaks called for the cAMP samples were 367,389, 356,223, and 351,249. Peaks were ranked from strongest to weakest peaks in each individual sample, and the rank order of common peaks among samples was compared to filter for only peaks with similar strength across all samples using the R-scripts provided for the IDR pipeline with a threshold of 0.02. After filtering for peaks in common, 106,807 peaks remained for the cAMP samples and 120,057 peaks remained for the control samples. The lower number of filtered peaks among the cAMP samples is likely a result of treatment causing higher variability in 5hmC levels in samples. When peaks across sample types were merged, a total of 192,143 peaks remained to be investigated in all samples. Reads within peak regions were quantified using HT-Seq-count ([Anders et al., 2015](Anders et al., 2015)). Statistical significance was determined using edgeR as was used for RNA-seq, except without using the calcNormFactors function so as to normalize to total read counts rather than counts within peaks. This normalization was necessary to account for a global change in 5hmC levels. Differential expression was also calculated using DESeq2. To minimize false positives, we considered only peaks with a minimum of 2X fold change and below an adjusted $P$-value (false discovery rate, FDR) below 0.05 by both edgeR and DESeq2. By this method, 66,963 peaks were upregulated, and 10,026 peaks were downregulated after treatment with cAMP, and 115,154 peaks remained unchanged.
**Sequencing reads Visualization**

For each individual sample, coverage at each base in the genome was calculated and normalized to total read count using bedtools genomecov (http://bedtools.readthedocs.io/en/latest/content/tools/genomecov.html). Coverage files were converted to bigwig files using the wigToBigWig program from UCSC genome browser (http://hgdownload.cse.ucsc.edu/admin/exe/linux.x86_64.v287/). Read density for peaks in each sample was visualized using version 2 of DeepTools (*Ramirez et al.*, 2016) and individual bigwig files. Visualization of read density across differential genes was also performed using DeepTools and the merged bigwig files.

**Integration of hMeDIP-seq and RNA-seq**

5hmC peaks were assigned to regions of the genome using Region_analysis in the diffReps package (*Shen et al.*, 2013). Peaks whose center was within 3,000 bp of the transcription start site (TSS) of a given gene were considered promoter regions for that gene, and peaks whose center lay in the remainder of the gene or up to 1,000 bp following the transcription end site (TES) were considered gene body for that gene. Peaks outside of gene body regions and gene promoter regions were classified as intergenic. Differential transcripts, with differential peaks assigned to either the promoter region or gene body of that gene, were considered to be potentially regulated by 5hmC. Of the 7,020 differential transcripts, 4,071 (58%) were found to be potentially regulated by 5hmC. Further, visualization of both RNA-seq and hMeDIP-seq data was done using UCSC genome browser (https://genome.ucsc.edu/) and bigwig files showing loci of differential transcripts.

It is known that elevation of cAMP regulates the activity of three different transcription factors: CREB1, CREM and ATF1. The Ma’ayan laboratory has posted analyzed data online from both the ChEA and ENCODE projects (http://amp.pharm.mssm.edu/X2K/#downloads). To
find genes potentially regulated by these three transcription factors, we used the ENCODE 2015 and ChEA 2015 databases (Yip et al., 2012; Lachmann et al., 2010). Experiments using rat cells are uncommon in these databases, so we combined the lists of genes found regulated by all experiments of a given transcription factor from human, mouse, or rat experiments. ATF1 was found to regulate 2,000 genes across a variety of human cells in the ENCODE projects, of which 1,996 genes were found to have a rat homolog in the Jackson Labs Complete Homology Class report (http://www.informatics.jax.org/homology.shtml). CREM was found to regulate 5,776 genes in mouse testicular cells (GC1-SPG) in the ChEA project, of which 5,773 were found to have a rat homolog in the Jackson Labs Complete Homology Class report. CREB1 was found to regulate 4,040 genes across human leukemia (K562) and adenocarcinoma (A549) cells in the ENCODE project, as well as 957 genes in human embryonic kidney (HEK293) cells in the ChEA project. CREB1 was also found to regulate 2,393 genes in rat hippocampus and 3,057 genes in mouse testicular cells (GC1-SPG) in the ChEA project. Combining data from all CREB1 experiments in both ChEA and ENCODE gave 6,997 rat genes potentially regulated by CREB1. Combining results from all experiments of all three transcription factors, 9,767 genes have been found to potentially be regulated by at least one of these three transcription factors.

Of the 7,020 differential transcripts, 3,965 (56.5%) were included in the list of genes potentially regulated by one of the three transcription factors that is activated by cAMP/PKA. Differences in transcription can be possibly attributed to both transcription factors and changes in 5hmC for 2,372 (33.8%) of the 7,020 transcripts and 1,356 (19.3%) are not likely regulated by either transcription factor or 5hmC.

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**Competing interests**

The authors declare that no competing interests exist.

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Figure 1

A

Control  Ascorbate  cAMP

Methyl blue

0.8 μg
0.4 μg

B

ShmC (fold)

Control  Ascorbate  cAMP

0
1
2
3
4
5

C

cAMP (μM)  0  1  10  100  250

ShmC

ShmC/DAPI

D

Fluorescence intensity

0  1  2  3  4  5  6  7

Fluorescence intensity

0  10  20  30  40  50  60  70

E

Time (days)  0  1  3  5  7

ShmC

ShmC/DAPI

F

Time (days)

0  1  3  5  7

G

HEK-293  MEF  SH-SY5Y

cAMP (μM)  0  10  0  10  0  10

ShmC

ShmC/DAPI

H

Control  AMP  Forskolin  Bicarbonate  Caffeine  IBMX

ShmC

ShmC/DAPI
Figure 1. cAMP induces 5hmC in cells. (A) Dot-blot shows that treatment with ascorbate (50 µM) for 3 days or cAMP (100 µM) for 7 days induced 5hmC in Schwann cells. (B) Semi-quantification of the dot-blot shows that both cAMP and ascorbate enhanced 5hmC generation in the Schwann cells. (C) cAMP (10 – 250 µM) increased 5hmC in Schwann cells after 7 days treatment shown by IF. (D) IF quantification (percent normalized to control) indicates the dose-dependent effect of cAMP on 5hmC in Schwann cells. (E) cAMP (100 µM) treatment for 1 – 7 days increased 5hmC in Schwann cells. (F) IF quantification (percent normalized to control) shows that the effects of cAMP treatment from 1 to 7 days induces comparable effects on 5hmC in Schwann cells. (G) cAMP (10 µM) also induced 5hmC in HEK-293 cells, MEF, and SH-SY5Y cells after treatment for 8 hours. (H) Endogenous cAMP promotes 5hmC in Schwann cells. AMP (100 µM) treatment for 2 days did not induce 5hmC while AC activators (Forskolin (100 µM), Bicarbonate (50 mM)), and PDE inhibitors (Caffeine (100 µM), IBMX (100 µM)) enhanced 5hmC generation. * P < 0.0005, Scale bar = 20 µm (n = 3 independent experiments with 3 biological replicates in each group, error bars denote standard deviation).
Figure 1 – Figure supplement 1. Treatment with cAMP does not increase Tet transcripts. qRT-PCR shows that Tet1 mRNA remained at a similar level ($P = 0.478$) after treatment of Schwann cells with cAMP (100 µM) for 1 day. In contrast, levels of Tet2 mRNA ($P = 0.001$) and Tet3 mRNA ($P = 0.0001$) were decreased after treatment of rat Schwann cells with cAMP (100 µM) for 1 day ($n = 2$ independent experiments with 3 biological replicates, error bars denote standard error).
**Figure 1 – Table supplement 1.** Primers used for quantitative RT-PCR.

| Gene   | Forward (5'→3')                | Reverse (5'→3')               |
|--------|--------------------------------|--------------------------------|
| Tet1   | CATGGAGACTAGGTATGGCCAGAAGG     | CTTCTTCTAATCACCACCTTGGGCAC    |
| Tet2   | GAACAAATCAGGAAGAAGGTGGAGAG    | GTACTTGACCTCCGATACCCATTAG     |
| Tet3   | GGGAACCTCAGGAGATCGGTATGGAG    | GTGTGTGCTTTGGGATCCACCTTTAG    |
| Rapgef2| TAGAGGCTAGTCCCATCG             | TCTGCAAGGAGTGAGTTTC           |
| Sdha   | GAAACGAATCCGCTAAAGTTCAGAAGCCG | GCAGCATGACCTCCCTGGCTG         |

**Note:** *Sdha* was used as a loading control.
Figure 2
**Figure 2.** cAMP increases the intracellular labile Fe(II) pool in cells. (A) cAMP (1 – 100 µM) treatment for 4 hours increased the intracellular labile Fe(II) pool detected by Trx-Puro ferrous iron probes. (B) IF quantification (percent normalized to control) shows the dose-dependent effect of cAMP on labile Fe(II). (C) cAMP (100 µM) treatment for 2 – 24 hours increased labile Fe(II). (D) IF quantification (percent normalized to control) shows the peak effect of cAMP on labile Fe(II) was at 4 hours treatment. (E) cAMP (10 µM) also enhanced the intracellular labile Fe(II) pool in HEK-293, MEF and SH-SY5Y cells after treatment for 4 hours. * P < 0.0005, Scale bar = 20 µm (n = 3 independent experiments with 3 biological replicates in each group, error bars denote standard deviation).
Figure 2 – *figure supplement 1*. Negative control for puromycin incorporation with labile Fe(II) probe TRX-puro in Schwann cells. (A) No immunofluorescence (IF) signal was observed after incubation with negative control dioxolane compound, Diox-Puromycin, (1 $\mu$M) for 2 hours, indicating no puromycin incorporation. Strong IF signal showed after incubating cells with unconjugated puromycin in rat Schwann cells. Scale bar = 20 $\mu$m. (B) The chemical structure of TRX-puromycin labile Fe(II) probe. (C) The chemical structure of nonperoxidic dioxolane compound, Diox-Puromycin. The $\alpha$-amine of puromycin is carbamoylated in both compounds. Consequently, these two conjugated compounds cannot be incorporated into nascent polypeptides, unlike unconjugated puromycin. Labile Fe(II) can react with the 1,2,4-trioxolane ring of TRX-Puromycin causing release of the puromycin and its incorporation into nascent polypeptides, which can then be detected by anti-puromycin antibody. Labile Fe(II) does not react with the bioisosteric nonperoxidic dioxolane compound, Diox-Puromycin, which was used as negative control for all labile Fe(II) IF experiments (n = 6 independent experiments with 3 biological replicates).
Figure 3. Induction of labile Fe(II) by endogenous cAMP and the dependency of 5hmC generation on labile Fe(II). (A) AMP (100 µM) treatment for 4 hours did not induce labile Fe(II) while AC activators (Forskolin (100 µM), Bicarbonate (50 mM)) and PDE inhibitors (Caffeine (100 µM), IBMX (100 µM)) increased labile Fe(II). (B) Pretreatment with iron chelators 2,2, bipyridyl (20 µM) and deferoxamine (20 µM) for 20 min blocked the elevation of labile Fe(II) and 5hmC in Schwann cells by cAMP (100 µM) treatment. Scale bar = 20 µm (n = 3 independent experiments with 3 biological replicates in each group).
Ascorbate does not increase the intracellular labile Fe(II) pool in Schwann cells. Treatment of Schwann cells with 50 µM sodium ascorbate for 4 hours caused no obvious change in labile Fe(II). In comparison, immunofluorescence shows an increase of labile Fe(II) in cells after treatment with cAMP (100 µM) for 4 hours. Scale bar = 20 µm (n = 2 independent experiments with 3 biological replicates each).
Figure 3 – figure supplement 2

Pretreatment with iron chelators 2,2', bipyridyl (20 μM) and deferoxamine (20 μM) for 20 min blocked the upregulation of 5hmC by cAMP (100 μM) treatment in A2058 cells. Scale bar = 20 μm (n = 2 independent experiments with 3 biological replicates each).

|                   | cAMP (μM) |       |       |
|-------------------|-----------|-------|-------|
|                   | 0         | 100   |       |
| No pretreatment   |           |       |       |
| 2,2' Bipyridyl (20 μM) | 0         | 100   |       |
| Deferoxamine (20 μM) | 0         | 100   |       |

**Figure 3 – figure supplement 2.** Pretreatment with iron chelators 2,2', bipyridyl (20 μM) and deferoxamine (20 μM) for 20 min blocked the upregulation of 5hmC by cAMP (100 μM) treatment in A2058 cells. Scale bar = 20 μm (n = 2 independent experiments with 3 biological replicates each).
Figure 4. cAMP increases labile Fe(II) and 5hmC by enhancing the acidification of endosomes. (A) cAMP (100 µM) treatment decreases the pH in intracellular vesicles. Red fluorescence increases when the pH decreases from 8 to 4. (B) V-ATPase inhibitor Bafilomycin A1 (200 nM) pretreatment for 30 min blocks the effect of cAMP on both labile Fe(II) and 5hmC in Schwann cells. Scale bar = 20 µm (n = 3 independent experiments with 3 biological replicates each).
**Figure 4 – figure supplement 1.** Deceasing the expression of Ferritin does not block the induction of labile Fe(II) by cAMP. (A) cAMP (100 µM) treatment induces labile Fe(II) at a comparable level in Ferritin heavy chain knockdown cells compared to the cells treated with scramble siRNA or with no pretreatment. Scale bar = 20 µm. (B) Immunoblot of Ferritin shows that the level of Ferritin heavy chain and Ferritin complex are decreased by siRNA compared to cells transfected by scramble siRNA (n = 2 independent experiments with 3 biological replicates for IF and 2 biological replicates for immunoblot).
Figure 5. The regulation of labile Fe(II) by cAMP likely via RapGEF2. (A) Pretreatment with PKA inhibitors (KT5720 (2 \( \mu \)M), H89 (10 \( \mu \)M)), Epac inhibitor ESI09 (10 \( \mu \)M) or CNGC blocker LCD (10 \( \mu \)M) for 20 min prior to cAMP addition showed no effect on labile Fe(II) induced by cAMP (100 \( \mu \)M) treatment in Schwann cells. (B) Knocking down the expression of RapGEF2 in HEK-293 cells blocked the acidification of vesicles and labile Fe(II) elevation after cAMP (100 \( \mu \)M) treatment compared to the scramble siRNA group. Scale bar = 20 \( \mu \)m (n = 3 independent experiments with 3 biological replicates in each group).
**Figure 5 – figure supplement 1.** Abolishment of PKA activity by inhibitors H89 and KT5720. (A) Pretreatment with H-89 (20 µM) and KT5720 (2 µM) decreased the band of phosphorylated Peptag peptide induced by cAMP (100 µM) treatment. (B) Quantification of the assay showing that 20 µM of H89 and 2 µM of KT5720 were sufficient to block the activity of PKA (n = 2 independent experiments with 1 biological replicates each).
Figure 5 – *Figure supplement 2*. qRT-PCR shows that the mRNA level of RapGEF2 is lower in the siRNA group compared to the scramble siRNA group ($P = 0.042$) ($n = 3$ independent experiments with 3 biological replicates each, error bars denote standard error).
**Figure 5 – table supplement 1.** Fragments per kilobase per million (FPKM) of CNG and Rapgef genes in Schwann cells.

| Gene Name | Control-1 | Control-2 | Control-3 | Control Average | cAMP-1 | cAMP-2 | cAMP-3 | cAMP Average |
|-----------|-----------|-----------|-----------|-----------------|--------|--------|--------|--------------|
| Cnga1     | 0.00      | 0.02      | 0.00      | 0.01            | 0.02   | 0.00   | 0.00   | 0.01         |
| Cnga2     | 0.00      | 0.00      | 0.00      | 0.00            | 0.00   | 0.00   | 0.00   | 0.00         |
| Cnga3     | 0.00      | 0.00      | 0.00      | 0.00            | 0.00   | 0.00   | 0.00   | 0.00         |
| Cnga4     | 0.14      | 0.10      | 0.09      | 0.11            | 0.01   | 0.02   | 0.00   | 0.01         |
| Cngb1     | 0.24      | 0.23      | 0.11      | 0.19            | 0.17   | 0.24   | 0.21   | 0.20         |
| Cngb3     | 0.00      | 0.00      | 0.00      | 0.00            | 0.00   | 0.00   | 0.00   | 0.00         |
| Rapgef1   | 8.86      | 9.77      | 7.18      | 8.60            | 5.26   | 5.13   | 5.31   | 5.23         |
| Rapgef2   | 27.82     | 28.13     | 26.69     | 27.55           | 21.33  | 18.11  | 20.35  | 19.93        |
| Rapgef3   | 0.00      | 0.00      | 0.00      | 0.00            | 0.02   | 0.01   | 0.03   | 0.02         |
| Rapgef4   | 0.14      | 0.04      | 0.09      | 0.09            | 0.08   | 0.05   | 0.05   | 0.06         |
| Rapgef5   | 0.01      | 0.01      | 0.04      | 0.02            | 0.01   | 0.00   | 0.00   | 0.00         |
| Rapgef6   | 8.01      | 9.00      | 7.61      | 8.21            | 11.28  | 11.49  | 11.16  | 11.31        |

**Note:** The RNA-seq data shows that the expression of CNG genes, Rapgef3 and Rapgef4 are extremely low in both control and cAMP-treated Schwann cells.
Figure 6. GPCR stimulation induces labile Fe(II) and 5hmC generation in Schwann cells. (A) Treatment with Gs-coupled receptor ligands isoproterenol (10 µM) and CGRP (100 nM) for 2 days induced 5hmC, an effect comparable to treatment with cAMP (100 µM) as shown by IF. (B) Treatment with isoproterenol (10 µM) and CGRP (100 nM) for 4 hours enhanced intracellular labile Fe(II), which is comparable to treatment with cAMP (100 µM). The induction of labile Fe(II) by isoproterenol and CGRP was decreased after pretreatment with SQ22536 (100 µM) for 20 minutes prior to isoproterenol and CGRP stimulation. Scale bar = 20 µm (n = 3 independent experiments with 3 biological replicates in each group).
Figure 7. cAMP shifts the transcriptome and hydroxymethylome of Schwann cells. (A) cAMP (100 µM) treatment for 7 days changes genome-wide transcription as shown by the heatmap of the relative abundance of reads for differential transcripts. (B) Venn Diagram of the RNA-seq results from the comparison of Schwann cells treated with or without cAMP (100 µM) for 7 days. The numbers within the circles represent the number of differential transcripts called by DESeq2 (yellow), edgeR (green), and CuffDiff (Blue) respectively. (C) cAMP (100 µM) treatment upregulated 5hmC peaks genome-wide as detected by hMeDIP-seq (n = 1 experiment with 3 biological replicates).
Figure 8. 5hmC profile changes correlate with gene transcription. (A) cAMP (100 µM) upregulated 5hmC at promoter and gene body regions of differential transcripts. (B) 33.8% of differential transcripts were associated with both 5hmC peaks and PKA-dependent transcription factors (TF). 24.2% of differential transcripts were associated with 5hmC peaks only and 22.7% of differential transcripts were associated with TF only. The rest of the differential transcripts (19.3%) was not associated with either 5hmC peaks or TF. (C) cAMP (100 µM) increased transcription and 5hmC, mainly in the gene bodies, of Egr2 and Pmp2 shown by UCSC Genome Browser views of hMeDIP-seq and RNA-seq reads (n = 1 experiment with 3 biological replicates).