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COMMENTARY

PROTEIN CARBOXYMETHYLATION: ROLE IN THE REGULATION OF CELL FUNCTIONS

ROBERT F. O'DEA,* O. HUMBERTO VIVEROS†‡ and EMANUEL J. DILIBERTO, JR.†

*Division of Clinical Pharmacology, Departments of Pediatrics and Pharmacology, University of Minnesota, Minneapolis, MN 55455; and †Department of Medicinal Biochemistry, Wellcome Research Laboratories, Research Triangle Park, NC 27709, U.S.A.

In recent years, protein carboxymethylation has received considerable attention as a potential modulator of several basic cellular functions. Ironically, only fifteen years ago it was felt that the role of this enzyme was to hydrolyze *S*-adenosyl-L-methionine (AdoMet), forming methanol. In a study of the distribution of phenylethanolamine-*N*-methyltransferase in various tissues, Axelrod and Daly [1] observed the formation of a volatile product following incubation of AdoMet and pituitary extracts. This product was identified as methanol and, thus, the enzyme was called the "methanol-forming" enzyme. Subsequently, Liss *et al.* [2] and Kim and Paik [3] isolated a protein methylase that transferred labile methyl groups to proteins with AdoMet as the methyl donor. Within eight years of the initial observations by Axelrod and Daly [1], Morin and Liss [4] and Kim [5] established that the "methanol-forming" enzyme was a protein carboxymethylase (PCM; *S*-adenosyl-methionine: protein-carboxyl methyltransferase, EC 2.1.1.24). PCM methylates (esterifies) free carboxyl groups of aspartyl and glutamyl residues of protein. At physiological pH, this reaction involves the neutralization of negative charges of protein (Ref. 6, Fig. 1). This enzyme has since been implicated as an important enzymatic regulator of a number of cellular events including neurosecretion and chemotaxis [7–10]. This commentary attempts to highlight current studies on PCM and to provide a basis for further investigation. For additional information, the reader is encouraged to consult other recent reviews on this subject [11, 12].

‡ Author to whom correspondence should be addressed.

Protein carboxymethylation: The "on" reaction

PCM has been partially purified in a number of laboratories [2, 5, 13, 14] and has been shown to be a cytosolic enzyme in all tissues examined [6, 7]. The enzyme has a molecular weight of approximately 25,000 daltons and migrates as a single polypeptide band on sodium dodecylsulfate (SDS)-polyacrylamide gels [14–16]. Its pH optimum varies with the protein substrate, with a range of 5.5 to 7.0. PCM has a ubiquitous tissue distribution with secretory cells and neural tissue containing the highest specific activities [6]. This distribution of PCM, its presence in synaptosomes, and the evidence for its axonal transport suggested a role for this enzyme in synaptic and secretory function. Since charge neutralization of the cytosolic surfaces of vesicle and plasma membranes may play a major role in exocytotic secretion of exportable products, Diliberto *et al.* [7] suggested the possibility of transient esterification of carboxyl groups of adrenomedullary chromaffin vesicle mem-

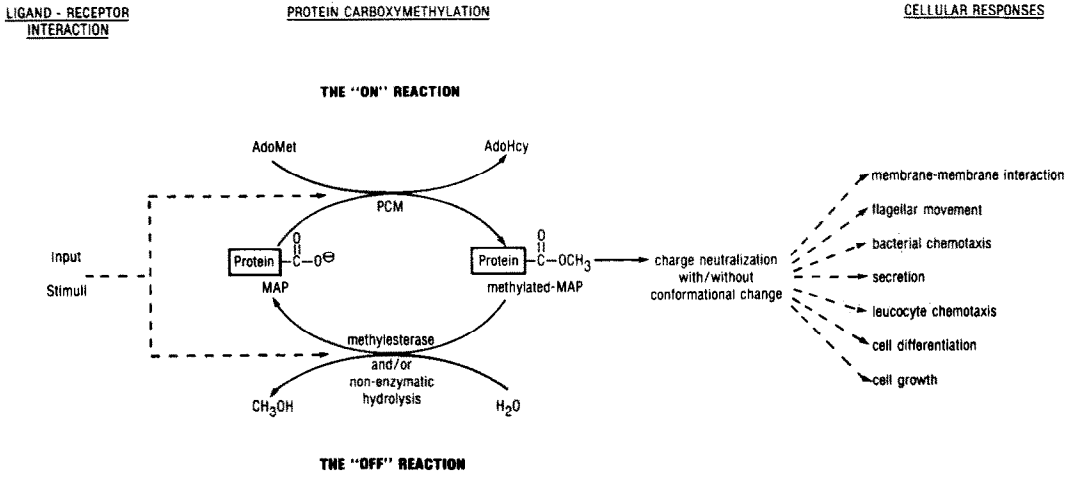

Fig. 1. A scheme representing the role of protein carboxymethylation and demethylation in the transduction of ligand–receptor interaction to cellular responses. Abbreviations: PCM, protein carboxymethylase; MAP, methyl-acceptor protein; AdoMet, *S*-adenosyl-L-methionine; and AdoHcy, *S*-adenosyl-L-homocysteine.

brane proteins by PCM as a step in excitation–secretion coupling. This led to a search for the localization of methyl-acceptor proteins (MAPs) in the cytosol and various membrane functions [6, 7, 17–19]. One attractive feature of the product of mammalian PCM is its instability. At physiological pH and temperature, protein-methyl esters undergo rapid non-enzymatic hydrolysis to liberate methanol [6]; thus, a readily reversible system appears to be available for charge neutralization of proteins (Fig. 1). This property of mammalian protein methylesters, however, has hampered investigation of specific MAP(s) involved in the various cellular functions. In attempting to correlate protein carboxymethylation with various cellular functions, one should be aware of the fact that the levels of carboxymethylated protein(s) result from the balance between the rates of carboxymethylation and demethylation. Thus, either the rate of esterification or the steady-state levels of methylated proteins may be involved in transduction of the cellular response. Shortly after our investigations on the role of PCM in the secretory process, other laboratories provided evidence of the role of protein carboxymethylation in bacterial chemotaxis [8, 9]. These studies prompted O'Dea *et al.* [10] to examine a similar role for PCM in eukaryotic cells. One major difference between the PCM systems of prokaryotic and eukaryotic cells is the stability of the protein-methyl esters. The bacterial system appears to generate a product with a stability similar to authentic glutamic acid 5-methyl ester [20, 21]. The mammalian enzyme, as mentioned earlier, yields a product with much greater instability. Thus, until recently, the de-esterification of carboxymethylated proteins was felt to be strictly non-enzymatic in eukaryotes [6].

The methylesterase: the "off" reaction

Consideration of a regulatory role of PCM in bacterial chemotaxis has evolved from the elegant work recently performed in the laboratories of Koshland [8, 20] and Adler [9, 22] who demonstrated that PCM participates in the modulation of bacterial motility. Exposure of the mobile bacterium to a chemoattractant results in an increase in methylation of glutamyl residues on a specific group of membrane proteins of approximately 65,000 daltons [23]. Through the use of bacterial mutants, these investigators have shown that PCM is essential for chemotaxis and that in non-chemotactic mutants PCM or its methyl-accepting chemotaxis proteins (MCPs) are lacking. Motile bacteria also contain a methylesterase that catalyzes the de-esterification of the MCP, thus influencing the steady-state levels of carboxymethylated proteins (Ref. 23 and 24, Fig. 1). In response to attractants, there is an increase in the methylation of the MCPs, whereas dilution of the attractant causes a decrease in the level of methylated MCP. Repellents produce the opposite effect [9]. These changes in carboxymethylation are rapid and result in a new plateau level of methylated MCP. The changes in the levels of methylated MCPs are coincident with the behavioral response, i.e. an altered frequency of tumbling, whereas during the plateau the bacteria return to the unstimulated frequency of tumbling. This latter phenomenon is called adaptation [22]. This switching mechanism implicates PCM as an important biological transducer in this stimulus–response cycle.

It is not surprising, therefore, that eukaryotes might conserve the PCM system as an efficient biochemical regulator of chemotaxis. In 1978, O'Dea *et al.* [18] examined the role of protein carboxymethylation in chemotactically stimulated rabbit neutrophils. It was observed that PCM activity and its protein substrates were distributed in cytoplasmic and membranous fractions of the cell, respectively, and that formylated peptides rapidly and transiently stimulated protein carboxymethylation in intact neutrophils. Both the behavioral response and the increase in carboxymethylation could be prevented by the prior addition of an attractant antagonist [18, 25]. Subsequently, Chiang *et al.* [26] and Pike *et al.* [27] showed that inhibitors of carboxymethylation simultaneously suppressed methylation and the chemotactic behavioral response in leukocytes. These increases in carboxymethylated protein(s), however, have not been observed upon exposure of guinea pig macrophages to chemoattractant peptides [28]. Additional support for PCM as a modulator of motile cells has been presented by Gagnon *et al.* [29], who observed a high level of PCM and MAPs in normal spermatids and, in contrast, a deficient level of PCM activity in sperm of necrospermic, infertile male rats [30].

Previously, it was shown that de-esterification of enzymatically carboxymethylated mammalian proteins is a rapid non-enzymatic process at physiologic pH [6]. This is in sharp contrast to the stability of the *N*-methylated lysyl, arginyl or histidyl residues in proteins [31]. Although preliminary experiments suggested that *in vivo* demethylation was more rapid than the spontaneous hydrolysis rate [32], it was not until recently that this process was also shown to be enzymatic in eukaryotes. The presence of such an enzyme in mammalian tissue was first reported by Gagnon [33] in a variety of organs, with the highest specific activity present in the kidney. Venkatasubramanian *et al.* [34] examined whether a similar carboxymethylesterase was present within the chemotactically stimulated neutrophil. In the neutrophil, the methylesterase was primarily cytosolic in distribution. In intact cells, the presence of a chemoattractant evoked a rapid stimulation of enzymatic activity with half-maximal stimulation induced by a 3 nM concentration of f-met-leu-phe, a concentration of peptide quite similar to that (1 nM) which produced a half-maximal chemotactic response. Furthermore, the presence of specific chemotactic antagonists inhibited the stimulated methylesterase response in neutrophils. The rapid, transient nature of these phenomena is temporally related to the earliest events in the leukotactic response, suggesting that turnover of protein-methyl esters function in the transduction of the chemotactic signal into directed migration of the mammalian cell.

PCM as a neurosecretory regulator

Protein carboxymethylation may perform a crucial role in the modulation of neurosecretion. This hypothesis is supported by the observation that neural and endocrine tissues have the highest levels

of PCM and MAPs [6, 14]. The stimulated release of secretory products from the adrenal, pituitary, and parotid glands has been shown to induce a concomitant increase in protein carboxymethylation [7, 13, 32, 35, 36].

The first studies in this area were performed by Diliberto *et al.* [7], using an adrenal medullary preparation. In this tissue, PCM was found to be cytoplasmic, whereas the MAPs were associated with both membranes and the soluble contents of the chromaffin vesicles. Stimulation of catecholamine secretion from the adrenal medulla secondary to insulin-induced hypoglycemia promoted a rapid increase in the carboxymethylation of membrane protein(s) and a concurrent release of membrane protein(s) from the chromaffin vesicle fraction [32, 36]. These findings prompted the authors [7, 32, 36] to suggest that the biologic function of PCM was to neutralize the negative charges of anionic proteins on membrane surfaces, resulting in a decrease in the electrostatic barrier and promoting vesicle–membrane fusion and, thus, stimulus–secretion coupling.

Employing an isolated rat parotid gland preparation, Strittmatter *et al.* [35] described a rapid and reversible *in vitro* and *in vivo* stimulation of PCM and an increase in MAP capacity following exposure of the gland to the beta-adrenergic agonist isoproterenol. This effect paralleled amylase secretion and appeared to be restricted to the methylation of two parotid proteins with molecular weights of less than 25,000 daltons.

Among neuroendocrine structures, the pituitary gland has been shown to have high levels of both PCM and MAPs [6, 13]. The endogenous substrates for PCM in the pituitary have been associated primarily with neurosecretory storage granule lysates [37]. These MAPs have molecular weights of less than 25,000, suggesting that these proteins are polypeptide hormones and/or neurophysins. A number of anterior pituitary hormones including LH, FSH, ACTH, GH, TSH and prolactin, and the posterior pituitary carrier proteins, neurophysins, were observed to be good substrates for PCM, whereas oxytocin and vasopressin were poor substrates for PCM [13, 38]. Furthermore, Gagnon *et al.* [39] have demonstrated that neurophysin itself constitutes over 80 per cent of neurohypophyseal lysate MAPs. These workers also observed that prolonged stimulation of the rat neurohypophyseal axis by salt loading, a technique that depletes secretory products, also promoted a decrease in posterior pituitary MAPs. These experiments and those mentioned earlier suggest that PCM may have a unique regulatory role in either the packaging or release of secretory products. It has not been demonstrated, however, that activators or inhibitors of the PCM system can interfere with these secretory events. Recent studies performed in hypothalamic tissue by Eiden *et al.* [40] have revealed that increased neurotransmitter release by elevated K^+ and veratridine caused a decrease in protein carboxymethylation in a catecholamine-secreting synaptosomal preparation. Again, the rate of esterification rather than the steady-state levels of protein-methyl esters may be important in neurotransmitter secretion. Further work is necessary to establish the relation between protein carboxymethylation and synaptic function.

The methyl-acceptor proteins

The lack of known regulation of PCM has prompted investigators to direct their energies toward the identification of those proteins that may be physiological substrates for this enzyme. Unlike the bacterial system, where specific proteins with molecular weights of 56,000–65,000 daltons have been identified as being methyl-acceptors [23], the substrate specificity of mammalian PCM is quite heterogeneous when lyzed cell preparations are analyzed. MAP specificity, however, may be different in the intact cell or *in vivo* situation. In the chromaffin vesicle membrane, MAPs have been identified as two minor proteins having molecular weights of 32,000 and 55,000 daltons [19]. Chromogranin A, a soluble protein, is the primary MAP in the vesicle lysate, although it is doubtful that this protein is ever exposed to the enzyme under physiological conditions. Previous investigations have shown that the erythrocyte is unique among tissues in that the putative MAPs are associated exclusively with the membrane [18] while PCM, as in other cells, is cytosolic [17, 18]. Galletti *et al.* [41] demonstrated that methylesterification of erythrocyte membrane proteins occurred selectively in proteins with apparent molecular weights of 97,000, 75,000 and 48,000 daltons. Recently, this group has partially characterized these proteins as being glycophorin and other unidentified proteins [42]. Although the biological functions of these erythrocyte methylations are unknown, it is possible that a methylation and demethylation cycle exists in this cell as well. Changes in the anionic charge concentration on the cell membrane may be important in the transfer of charged molecules across the red cell membrane and/or in the plasticity of the membrane.

The role of calcium as a mediator in the coupling of stimulus to secretion in the adrenal medulla and other secretory cells is well established [43]. Although neither calcium nor magnesium alters the rate of carboxymethylation of adrenomedullary membrane fractions *in vitro*, the chemotactic peptide-induced increase in leukocyte protein carboxymethylation *in vivo* appeared to be calcium dependent [25]. Cox *et al.* [44] recently examined the question of whether a ubiquitous anionic calcium-binding protein, calmodulin, might be a substrate for protein carboxymethylase. Calmodulin has been shown to be a critical regulator of calcium-dependent enzymatic processes in the cell, including such events as cyclic nucleotide degradation, calcium-dependent ATPase, phosphorylation of myosin light chain kinase, and reassembly of cytoskeletal units [45]. In this study, calmodulin was shown to be an excellent substrate for PCM. The K_m for calmodulin as a substrate was approximately 7×10^{-5} M, a value similar to the actual concentration of calmodulin in the brain [46] and testis [47]. Furthermore, alkaline hydrolysis of native calmodulin resulted in a 10-fold increase in the incorporation of methyl groups into the calmodulin molecule, presumably via de-esterification of preformed methyl esters. Nevertheless, later studies did not demonstrate that carboxymethylation of

calmodulin altered its ability to activate cyclic GMP phosphodiesterase or calcium-dependent ATPase (D. E. Cox, R. F. O'Dea, M. Y. Hurwitz and R. D. Edstrom, unpublished observations). Recently, Gagnon *et al.* [48] reported a 60 per cent methyl esterification of the calmodulin molecule by using a highly purified PCM preparation which resulted in a 60 per cent decrease in calcium-stimulated cyclic GMP phosphodiesterase activity. This apparent discrepancy was probably due to the availability of a high concentration of purified PCM which enabled a degree of carboxymethylation greater than that obtained by Cox *et al.* The above observations represent a new lead and suggest a role for PCM as a modifier of calcium-dependent activities in the cell. This concept is particularly interesting in view of the hypothesis of Naccache *et al.* [49] that the role of PCM in the neutrophil is to modulate the rapid, chemoattractant-stimulated changes in calcium ion movement occurring in that cell. These observations represent a new objective for PCM studies which should be explored intensively in the future.

PCM: A regulator of cell growth

In previous studies it has been suggested that PCM is a modulator of basic cellular functions, including motility and secretion. For this reason PCM was examined as a possible effector of cellular growth and differentiation. Recently, S. H. Zuckerman, R. F. O'Dea, J. M. Olson and S. D. Douglas (unpublished observations) have shown that the cultured human monocyte, a cell that undergoes spontaneous, time-dependent differentiation into a macrophage-like cell [50], displays a dramatic increase in both cellular PCM and endogenous MAP activity concomitant with the profound morphologic changes of cellular differentiation. In contrast, cultured human pulmonary alveolar macrophages, a totally differentiated cell, do not display any increases in PCM activity with culture. A large increase in the endogenous MAPs, however, was observed. The identity of these protein substrates is unknown. It is interesting to speculate that changes in cell growth and the timing of differentiative events may be dependent upon the availability of specific MAPs.

Pharmacological probes and PCM

A number of criteria should be met to support the claim that a given stimulus elicits a biological response in a target organ by stimulating protein carboxymethylation. One of these is that agents capable of blocking PCM activity should also be able to suppress the stimulated biological event. Unfortunately, no substance currently available has been demonstrated to specifically inhibit PCM; however, considerable data have been obtained using compounds that inhibit methyltransferase reactions in general. Although mammalian tissues abound in assorted small and large molecule methyltransferases (e.g. COMT, PNMT, histamine-*N*-methyltransferase, PCM, protein-*N*-methyltransferases, and phospholipid and tRNA methylases), all of these enzymes share a strict requirement for AdoMet as the methyl donor and exhibit a sensitivity to inhibition by the transmethylation product, *S*-adenosyl-L-homocysteine (AdoHcy) [51]. As reviewed by Borchardt [12], attempts to alter cellular methyltransferase reactions have proceeded along four investigative lines with various degrees of success. These include the effects of: (1) analogues of the methyl-acceptor substrate; (2) inhibitors of AdoMet synthesis, such as cycloleucine [52]; (3) analogues of AdoHcy, including the antifungal antibiotic, sinefungin [53]; and (4) alterations in the feedback regulatory mechanism of AdoHcy, employing inhibitors of the hydrolytic enzyme AdoHcy hydrolase (EC 3.3.1.1). This latter enzyme, which catalyzes the hydrolysis of AdoHcy to homocysteine and adenosine [54], constitutes an interesting target for pharmacologic manipulation. Inhibition of AdoHcy hydrolase by compounds such as 3-deazaadenosine [55] and 5′-deoxy-5′-(isobutylthio)-3-deazaadenosine [56] has been shown to elevate AdoHcy levels in rat liver [57], rat spleen [58], isolated hepatocytes [56], leukocytes [59, 60], and lymphocytes [60].

PCM was shown initially by Diliberto and Axelrod [13] to be quite sensitive to the inhibiting effects of AdoHcy with a K_i approximately one order of magnitude less than inhibitor constants obtained with most "small molecule" methyltransferases [51]. Recently, Fuller and Nagarajan [53] reported that an antifungal antibiotic, sinefungin, was an effective inhibitor of the methyltransferases responsible for the metabolism of catechols, histamine, and norepinephrine. This compound differs from AdoHcy only in having the sulfur replaced with an amino-substituted methylene unit. Borchardt *et al.* [61] reported than sinefungin and a related metabolite A-9145C (Lilly) were potent competitive inhibitors of calf thymus and bovine adrenal PCM with K_i values for both enzymes substantially lower than that seen with AdoHcy. Employing a 20,000 *g* soluble fraction from the murine C-1300 neuroblastoma, O'Dea has observed that both sinefungin and AdoHcy are potent inhibitors ($K_i = 0.3$ to $0.7\ \mu M$) of PCM obtained from this neoplasm (R. F. O'Dea, G. P. Pons and B. L. Mirkin, unpublished observations). Currently, work is underway to examine whether these analogues can modify growth and/or differentiation of this tumor.

In addition to the direct interference with PCM activity by AdoHcy analogues, alteration in intracellular AdoHcy/AdoMet ratios has been demonstrated to alter both PCM and cellular responses. 3-Deazaadenosine has been shown to be a potent inhibitor of leukocyte chemotaxis [59], resulting in a 3-fold increase in AdoHcy levels in the cells, with no change in AdoMet. This agent also significantly inhibited both protein carboxymethylation [25] and phospholipid methylation in neutrophils; however, the concentration of 3-deazaadenosine used was ten times greater than that necessary to achieve a 100 per cent inhibition of chemotaxis [62]. A similar disparity in dose–response relationships was reported by Zimmerman *et al.* [60] who observed a potent suppression of murine lymphocyte-mediated cytolysis and cellular PCM by 3-deazaadenosine. The IC_{50} for 3-deazaadenosine as an inhibitor of PCM was 20-fold lower than that seen for the cytolytic response. This may indicate that nearly complete inhibition of PCM is required for observable changes

in the cytolytic response. Taken together, these data suggest that PCM, and probably other methyltransferase reactions, may play an integral part in cellular stimulus–response coupling. Further investigation will be needed, however, to establish the regulatory role and identification of endogenous methyl-acceptor substrates.

The use of pharmacological probes for the elucidation of biochemical mechanisms underlying cellular responses has had a history of successful applications. Misinterpretation of their use, however, is also well known. With regard to inhibitors of transmethylation, we should consider recent data by Zimmerman *et al.* [63]. Prior loading of lymphocytes with AdoHcy and *S*-3-deazaadenosyl-L-homocysteine induced a marked enhancement in cyclic AMP levels in response to agonists. Although the mechanism for this effect is not well understood, it is obvious that the effects observed after these apparently pure transmethylation inhibitors may be the consequence of their modification of other unrelated cellular reactions.

Final comments

We have presented a brief review of the functional evidence that leads to the conclusion that carboxymethylation of membrane protein(s) plays an important role in the regulation of such apparently different functions as bacterial and leucocyte chemotaxis, cell secretion, flagellar movement, and cell differentiation (Fig. 1). From a physicochemical standpoint, protein carboxymethylation is a unique post-translational modification of proteins allowing for rapid and reversible neutralization of negatively charged groups. It is easy to envision how a change in the charge on a single aspartyl or glutamyl residue adjacent to, or part of, the active site of an enzyme may modify its kinetics. More extensive carboxymethylation of exposed negative charges may similarly affect the conformation and function of a regulatory protein. Furthermore, because PCM seems to have a special affinity for proteins in membranes, charge modification by methylation may alter the properties of ionic channels and transporter proteins, the degree of exposure of receptor proteins in equilibrium with the bulk phase of the membrane, or membrane–membrane interactions. Therefore, it is tempting to speculate that a common mechanism such as charge modification, may underlie the regulation of such varied and diverse cellular functions as were noted above. Although our current knowledge on the role of protein carboxymethylation in cell function has been derived from physiological and biochemical studies, an area which remains unexplored is the biophysical changes resulting from the carboxymethylation of endogenous soluble and membrane-bound substrate proteins.

As frequently happens with the development of new areas of interest in biological research, chance played an important role in the discovery of PCM, the "methanol-forming" enzyme. While Diliberto and Viveros were studying if PCM was regulated by divalent cations, serendipity was propitious again: neither Ca^{2+} nor Mg^{2+} modified the rate of carboxymethylation but Mg^{2+} produced a large increase of methyl group incorporation into membrane lipids. This observation resulted in the identification of a previously unknown Mg^{2+}-dependent enzyme, phosphatidylethanolamine-*N*-methyltransferase, in membrane fractions from various tissues [64]. This phospholipid methylation also has the ability to participate in regulation of a large variety of cell functions through modification of the lipid phase of membranes. Within 2 years of the first report of this phospholipid methyltransferase, great research interest was focused on this enzyme (for a review of its possible physiological role see Ref. 65). While another dimension of the role of *S*-adenosyl-L-methionine-dependent transmethylation reactions in cell function may be uncovering, it is important to point out that there are real difficulties that may arise in attempting to relate the specific role of protein carboxymethylation and lipid methylation to a particular function. A search for specific inhibitors of these two methyltransferases may provide important tools to this end.

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