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Chapter Title	*Entamoeba histolytica*: Bridging the Gap Between Environmental Stress and Epigenetic Regulation	
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Keywords (separated by "-")	Dnmt2 - *Entamoeba* - Environmental stress - Epigenetic - Protozoan parasite - Retrotransposons - tRNA methylation	

Chapter 11
Entamoeba histolytica: Bridging the Gap Between Environmental Stress and Epigenetic Regulation

Kirschenbaum Michael and Ankri Serge

Abstract Increasing evidence indicates that parasites display unique and diverse mechanisms of epigenetic regulation. In this chapter we present the current state of knowledge about the *Entamoeba histolytica* DNA/tRNA methyltransferase (Dnmt2) machinery and the related EhMLBP, a protein involved in the recognition of methylated DNA targets. The regulation of these epigenetic components by environmental challenges relevant to the biology of the parasite (including heat shock, glucose starvation, oxidative and nitrosative stresses) is also discussed.

11.1 *Entamoeba histolytica*: Life Cycle and Environmental Challenges

Entamoeba histolytica, the protozoan parasite responsible for amebiasis, is a dimorphic organism whose life cycle consists of two stages: the infective cyst and the invasive trophozoite. During its development the parasite moves through a series of different localized microenvironments and biological niches to which it must adapt. Initial infection begins with ingestion of nascent cysts as obtained from contaminated water supplies or food. Upon passage through the upper gastrointestinal tract, the parasites excyst in the terminal ileum, whereupon they migrate to and colonize the large intestine. Here, the parasite's life cycle takes a series of divergent paths depending on the ultimate pathophysiology of the disease. Ninety percent of *E. histolytica* infections are asymptomatic and the parasite remains a commensal organism feeding on the various flora and microbiota of the colon [1]. The trophozoites multiply and divide through binary fission, encyst, and pass through the stools, perpetuating the life cycle. However, in the other 10 % of cases in which symptomatic infection occurs, the trophozoites invade the mucosal lining of the colon, burrowing and coalescing into flask-shaped ulcers, with resultant colitis or dysentery of the host. Disease progression may end here, resolving with the

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T. Nozaki, A. Bhattacharya (eds.), *Amebiasis*,
DOI 10.1007/978-4-431-55200-0_11

infection; or it may continue, with final emergence occurring in distal organs, generally the liver. Other more rare manifestations include pulmonary, cardiologic, and brain involvement [2].

Among the various environmental challenges encountered by *E. histolytica* are drastic changes in pH, pO_2, glucose concentration, biofilm substrate, the surrounding biome, nutrient availability, and the numerous assaults of the host immune system; including oxidative stress, heat shock, complement activation, and phagocytosis ([1, 3–5]). The initial environment, the colonic lumen, is host to a diverse array of resident microflora comprising more than 50 genera and 400 species [3]. Oxygen content is low, pH fluctuates markedly, glucose levels are low (but variant in accordance with the nutritional status of the host), and the metabolic environment consists mostly of acetogenic sugars and short-chain fatty acids [3]. The mucus overlaying the colonic epithelium is a complex gel of glycolipids, glycoproteins, and sugar residues including *N*-acetylglucosamine, *N*-acetylgalactosamine, D-GALACTOSE, fucose, and sialic acids [3]. As mentioned previously, *E. histolytica* begins as a commensal, feeding off this rich diversity of microorganisms in the large intestine. Indeed, it is a voracious predator, and the trophozoites are capable of consuming up to 1,000 bacteria per hour, individually [6]. Regarding the host immune system, it, too, is initially tolerogenic, utilizing both T regulatory cell activation and secretory immunoglobulin A to suppress inflammatory responses and prevent parasitic contact with the colonic mucus, respectively [1]. When the parasite does invade the colonic mucosa, however, it is henceforth subjugated to radically different environments (depending on the bodily compartment/reservoir), most notably characterized as being oxygenated, composed of an extracellular matrix (collagen, elastin, laminin, and fibrinogen) [7], and hostile, resulting from the activated inflammatory immune response.

As such, the amoeba must be capable of adapting to the demands of its surrounding environment. Numerous questions abound, and we find ourselves questioning the precise mechanisms controlling these transitions; which enable the parasite to so perfectly adapt to such a broad range of different situations. Of particular concern is determination of the triggers that change the ultimate pathophysiology of the organism, as it abandons its role as a commensal and becomes an agonist pathogen.

11.2 Epigenetics as a Tool for Adaptation

Epigenetic regulation of protein expression has long been recognized to be a key component in the cellular development, adaptability, and physiology of all living things, ranging from the simple prokaryotes and Archaebacteria to plants, animals, and human beings. Epigenetics specifically refers to chemical or structural modifications to DNA that preserve the genetic code but ultimately result in altered RNA transcription and protein expression. This trait may, in fact, be heritable, resulting in altered phenotype/differentiation of all descendant cells, despite the fact that they all share the same genotype and overall genetic code. Numerous epigenetic signals have already been elucidated, most prominently featuring DNA methylation and covalent modifications of histone proteins (e.g., acetylation, phosphorylation).

These modifications result in overall changes in chromatin structure and accessibility to transcription factors [8] and other nuclear proteins, such as methyl binding domain proteins [9].

In recent years, epigenetic regulation of gene expression has emerged as a crucial aspect of parasite biology. Indeed, this genomic plasticity has been demonstrated as a key factor in the virulence, differentiation, and lifecycles of protozoa as varied as *Toxoplasma gondii*, *Plasmodium falciparum*, and *Trypanosoma brucei* [10–13]. Alternative transcriptomes have also been obtained for the virulent HM1:IMSS *E. histolytica* strain versus the avirulent Rahman strain, with differential protein expression profiles for key virulence genes including the cysteine proteases, Gal/GalNAc lectins, and the protective peroxiredoxin [14]. Although many of the fundamental principles of epigenetic gene regulation are similar to those in mammalian cells and model systems, protozoan parasites also display unique and diverse mechanisms of epigenetic gene regulation [15–17]. This chapter presents our current state of knowledge about Dnmt2-mediated methylation in the parasite *E. histolytica* and its regulation by the environment.

11.3 Evidence for 5-Methylcytosine in the Genome of *E. histolytica*

DNA methylation is associated with gene silencing and transposon control [18, 19]. In mammals, 3 % to 8 % of cytosine residues are methylated, generally in a CpG context [20]. Typically, DNA methylation leads to recruitment of methylated CpG binding domain (MBD) proteins, which themselves interact with histone deacetylase to alter chromatin structure; condensing it, and silence gene expression [21]. The first clue about the presence of m5C in *E. histolytica* came 13 years ago when transfected *E. coli* activated their *mrr* methylation-restricting systems in response to exogenous *E. histolytica* transfectant plasmids (unpublished results). Direct evidence of the presence of methylated cytosine in the parasite's genome was then achieved via immunoblotting with m5C-specific antibody [22]. Recently, high pressure liquid chromatography (HPLC) coupled to mass spectrometry revealed low amounts of m5C in *E. histolytica* DNA (about 0.05 %) but definitely more than the detection level of the method (unpublished data). This presence of m5C in the genome of the parasite raises questions regarding its formation, the cellular/signaling events regulating this phenomenon, and its role or roles in the parasite life cycle and virulence.

11.4 *E. histolytica* Dnmt2 (Ehmeth) is a DNA Methyltransferase

The formation of m5C is catalyzed by 5-cytosine methyltransferase (m5C-MTase) with *S*-adenosylmethionine as a cofactor. The mammalian DNA machinery consists of three active DNA MTases: Dnmt1, Dnmt3a, and Dnmt3b. Dnmt1 has a high

preference for hemi-methylated DNA [23, 24] as a substrate, functioning as a "maintenance" DNA MTase, which preserves epigenetic differentiation throughout descendent cell lines during mitotic events. Dnmt3a and Dnmt3b, however, are de novo DNA MTases acting on nonmethylated DNA (for review, see Jeltsch [20]) and initiate active epigenetic regulation. A fourth enzyme, Dnmt2, is the most conserved of all DNA MTases; belonging to a large family of proteins conserved in all species from *Schizosaccharomyces pombe* to humans [25]. It is also the most enigmatic, however. The enzyme has very weak methylation activity on DNA [26–28]. More recently, methylation of $tRNA^{Asp}$ could be attributed to Dnmt2 [29]. Although this could indicate a biological function of the enzyme, the phenotype of knockout (KO) mutants is usually very mild or not detectable [29, 30]. Remarkably, the tRNA methylation activity follows a DNA methylation motif (utilizing cysteine79 present in motif IV of the catalytic site) and not the one employed by the structurally similar tRNA methyltransferases (which use an alternative cysteine to stabilize the Michael addition of a methyl group) [31]. Indeed, what distinguishes Dnmt2 from the other DNA MTases is its comparatively shorter N-terminal regulatory domain, which may play a role in its highly discriminate DNA-binding activity. The catalytic C-terminal domain is shared by all DNA methyltransferases; and structural analysis of human Dnmt2 showed a high similarity to the M.*Hha*I methyltransferase from *Haemophilus haemolyticus* [32].

E. histolytica belongs to the so-called Dnmt2 only organisms and does not contain any of the canonical DNA methyltransferases (Dnmt1 and Dnmt3). Substantial evidence supports *E. histolytica* Dnmt2 (called Ehmeth), as a genuine DNA MT. First, a number of DNA sequences have been identified via methylated DNA immunoprecipitation (MedIP) using the 5mC antibodies. These sequences include ribosomal DNA (*rDNA*), heat-shock genes (HSP70 and HSP 100), and retrotransposons [22, 33, 34]. Further analysis of these sequences utilizing bisulfite sequencing indicated that, in contrast to mammals, where cytosine is methylated predominantly within the CpG dinucleotides, the DNA methylation pattern in *E. histolytica* is not restricted to a CpG context, but can also occur at non-CpG sites [22]. Interestingly, non-CpG-methylation in mammals is primarily found in viral or stably integrated plasmid sequences [35], as well as in the endogenous long interspersed nuclear element, LINE-1 [36]. In higher plants, DNA methylation is commonly found not only in the symmetrical motifs, CpG and CpNpG, but also in some asymmetrical contexts, such as CpN, and is needed for normal development [37]. Therefore, it has been proposed that non-CpG methylation may reflect the substrate specificity of Dnmt2.

The role of the Dnmt2 protein family is still under investigation. Conventionally, DNA methylation in higher eukaryotes is linked with the silencing of gene expression. A correlation between DNA methylation and gene expression has been reported for the HSP100 gene of *E. histolytica* [34]. This apparently is not its most important function in *E. histolytica* because treatment with 5-azacytidine (a potent inhibitor of DNA methyltransferase) has a limited effect on gene expression in the parasite [38]. Remarkably, however, the ability of 5-azacytidine (23 μM)-treated *E. histolytica* trophozoites to form liver abscesses in infected hamsters is significantly reduced [22], suggesting that Ehmeth activity [39] regulates *E. histolytica* virulence.

One of the other functions of DNA methylation in higher eukaryotes is to provide protection from selfish DNAs that include retroelements [40]. The non-long-terminal repeat (non-LTR) retrotransposons encode a reverse transcriptase (RT) and other proteins that are needed for transposition. Non-LTR retrotransposons consist of short interspersed nuclear elements (SINEs) or long interspersed nuclear elements (LINEs), which are also transposed by reverse transcription of mRNA directly into the site of integration [41]. The sequencing of the *E. histolytica* genome revealed multiple LINE families and SINE elements that are also abundantly transcribed [42, 43]. Nevertheless, most of the LINEs have lost their transposition ability, probably because of mutations in some of their essential genes, such as reverse transcriptase [44]. It has been proposed that these mutations are the result of the accelerated deamination that occurs to the methylated cytosines that are present in the LINEs [45]. Newly emergent biotechniques may enable us to explore this phenomenon. Recent work has established a retrotransposition-competent cell line in *E. histolytica*, that is, reconstructed ORF2 (reverse transcriptase and accompanying endonuclease) serving as an activated LINE element, coupled with a secondary vector consisting of marked SINE linked to a targeted hotspot of integration [46]. Double transfectants displayed retrotransposition capability, mobilizing the marked SINEs and inserting them into the neighboring hotspot. It will be interesting to examine the relationship between Ehmeth expression and the frequency of retrotransposition, the stability of these activated SINEs in both their respective mosaics and tendency to accumulate polymorphisms (as correlated to Ehmeth expression), and, finally, the methylation status of the newly mobilized SINEs. This control of retrotransposons via Dnmt2-mediated DNA methylation has been demonstrated in other Dnmt2 only organisms including *Dictyostelium* and *Drosophila* spp. [28, 47], and the control of repetitive DNA elements by the trematode *Schistosoma mansoni* Dnmt2 has also been recently proposed [48].

11.5 Ehmeth is a tRNA MT

The observation that Dnmt2 methylates tRNA was first reported by Goll et al. [29]. In this seminal paper, the authors showed that Dnmt2 has a strong methylation activity at C38 of $tRNA^{Asp}$ in mice, *Drosophila melanogaster*, and *Arabidopsis thaliana* [29]. In addition to $tRNA^{Asp}$, $tRNA^{Val}$, $tRNA^{Gly}$ and $tRNA^{Glu}$ are also methylated by Dnmt2 [49, 50]. Interestingly, Dnmt2 modifies these tRNAs at cytosine 38 following the reaction mechanism established for 5-cytosine DNA methyltransferases [29, 49]. This observation extends to *E. histolytica* as well. Recombinant Ehmeth prepared from *E. coli* was able to methylate synthetic $tRNA^{Asp}$. Concurrently, global $tRNA^{Asp}$ methylation in *E. histolytica* was measured via incorporation of radioactive methyl group into the tRNA of the parasite, utilizing hDnmt2. In this assay, the amount of SAM incorporated is proportional to the amount of unmethylated tRNA [39]. Recently, bisulfite sequencing of tRNA has been developed as well. This method offers direct detection of cytosine methylation in tRNA, accurately

localizing the methylated cytosines within the sequence. In applying this method to *E. histolytica*, we showed that Ehmeth, as do other Dnmt2 proteins [29], methylates $tRNA^{asp}$ at C38 (manuscript in preparation).

Therefore, in contrast to human Dnmt2, which apparently has a strong substrate preference for tRNA, Ehmeth can use both DNA and tRNA as substrates. This dual specificity for DNA and tRNA has also been proposed for the Dnmt2 homologue in *Drosophila melanogaster* [31]. The exact biological role of Dnmt2-mediated tRNA methylation is not yet known in *E. histolytica*, however. The position next to the anticodon loop suggests a role in the basic transcriptional process, but influence on tRNA folding and stability is also possible. A recent work points to the role of *Drosophila* Dnmt2 in the regulation of tRNA degradation. In this work, stress-induced cleavage of tRNAs was Dnmt2 dependent, and Dnmt2-mediated methylation protected tRNAs against ribonuclease cleavage [49]. Additionally, Dnmt2 has been implicated as an agent in the *Drosophila* innate immune response; intercepting exogenous viral RNA and labeling it for disposal utilizing the Dicer/Argonaute RNAi machinery [51]. In *Saccharomyces cerevisiae*, Trm9-mediated tRNA methylation is linked to the translation enhancement of genes related to stress response, DNA damage, and other cellular functions. These results together with previously published data support a role of tRNA methylation in the control of tRNA stability and consequently protein synthesis [52]. Recently, it has been shown that disrupting both the Dnmt2 and the NSun2 tRNA methyltransferases in mice led to the complete loss of tRNA methylation, reduced protein synthesis, and lethality [30].

11.6 Regulation of Ehmeth Activity and the Role of the Environment

It is well documented that long-term culture of pathogens, particularly parasites, leads to virulence attenuation [53, 54]. This observation also applies to *E. histolytica* for which regular passage through hamster liver is necessary to keep functional the ability of the parasite to form a liver abscess [55]. Similarly, we observed that continuous culture of *E. histolytica* in *TYI*-S-33 media progressively lowers the expression of Ehmeth to a barely detectable level (Fig. 11.1). This observation raises new and interesting questions about the regulation of Ehmeth expression and the impact of environment on this regulation. An analogous observation about the effect of growth conditions on the expression of Dnmt2 in *Saccharomyces pombe* was reported stating that nutrition (peptone) regulates *Schizosaccharomyces pombe* Dnmt2-dependent tRNA methylation [50].

Glucose starvation (GS), one of the most studied metabolic stresses, has been investigated in the malaria parasite *Plasmodium falciparum*. Interestingly, the PfEMP (*var*) genes, key components in malaria pathogenesis, are among the genes upregulated by GS [56]. Accordingly, it has been proposed that ambient glucose concentration is a good indicator of the environmental changes to which the parasite is exposed during its life cycle. This regulatory role of glucose is particularly

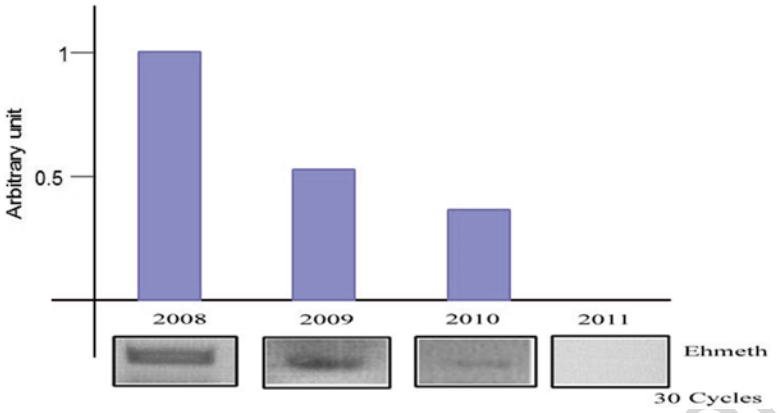

Fig. 11.1 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of Ehmeth expression in an HMI:MSS strain that has been kept in continuous culture for more than 4 years (2008–2011). The HMI strain (a gift of Prof. Mirelman, Weizmann Institute) used in this study was originally isolated from a hamster that developed a liver abscess following injection of trophozoites in its liver. The strain was kept under continuous culture in TYI media without further passage in hamster liver. Semiquantitative *RT-PCR* was used for measuring Ehmeth expression

relevant to *E. histolytica* because, as already mentioned, it lives in the colon, a niche where the amount of available glucose for fermentation is usually small (about 0.2 g/kg tissue) because of the high absorptive capacity of the glucose transporters in the small intestine [57–59]. On rare occasions, it has been reported that *E. histolytica* trophozoites leave the colon and migrate to the liver. In this organ, the concentration of glucose was estimated to be twice that of perfusing blood (about 2.0 g/kg tissue) [60–62]. We recently reported that *E. histolytica* is capable of responding to changes in its surrounding glucose concentration: short-term glucose starvation (12 h) led to the accumulation of enolase, a glycolytic enzyme, and the inhibition of the Ehmeth activity in its nucleus (Tovy et al.). Extending the condition of glucose starvation beyond 12 h led to the progressive death of most of the parasite population. Surprisingly, some individual clones survived and adapted to this absence of glucose in the media. Adaptations included a number of metabolic changes. Specifically, the increased expression of various catabolic enzymes involved in amino acid regulation; in particular, methionine gamma lyase, aspartate ammonia lyase, and dihydropyrimidine dehydrogenase (DPD), an important effector of the pyrimidine catabolism pathway. Indeed, DPD is crucial for parasite growth when the availability of glucose is limited [5]. Undergoing experiments also point toward increased $tRNA^{asp}$ methylation levels in these glucose-starved parasites (unpublished data). This result raises many intriguing questions about the role of tRNA methylation in the adaptive mechanism to glucose starvation.

[AU1]

Until recently, no interacting partner had been identified for Dnmt2. We have identified that enolase interacts with the catalytic site of Ehmeth, subsequently inhibiting both its DNA and tRNA methyltransferase activity [39]. Additionally, short-term glucose starvation (12 h) triggers the accumulation of enolase from the

cytoplasm to the nucleus, resulting in activated Ehmeth inhibiton [39]. We recently analyzed the crystal structures of both *E. histolytica* enolase and of Ehmeth [63, 64], but the molecular details on the Ehmeth–enolase hybrid remained elusive. Hence, the three-dimensional structure of the Ehmeth–enolase complex still needs to be elucidated.

11.7 Ehmeth Protects *E. histolytica* from Oxidative and Nitrosative Stresses

Although the overall biological function of Dnmt2/Ehmeth is not yet completely understood, recent work has enabled us to view their expression, pleiomorphically, in a broader context; particularly in terms of survival, longevity, and adaptability to oxidative stresses. Dnmt2 expression has been implicated as a necessary component to maintaining the normal lifespan in *D. melanogaster*; and, indeed, overexpression induces longevity in fruit flies [65]. It has been proposed that the underlying mechanism behind this observation is an increased resistance to oxidative damage; which has a well-established association with both degenerative diseases and aging [66]. Dnmt2 overexpression induces small heat-shock protein (Hsp) expression in *Drosophila melanogaster* [65], which facilitates the stabilization/sequestration of damaged or misfolded proteins [67]. Similarly, our group has demonstrated Hsp 70 upregulation in Ehmeth overexpressing *E. histolytica* transfectant [68]. Moreover, these Ehmeth-overexpressing trophozoites exhibit significantly greater resistance/survivability to H_2O_2 exposure. H_2O_2 is one of the principal convergent intermediate metabolites in oxidative stress. Activated resistance to oxidative damage is not surprising when considered in the context of *E. histolytica* virulence. Passage from the anoxic luminal colon into the tissues or bloodstream of the human host necessitates a dramatic change in environmental pO_2. Moreover, the parasite must now withstand the assaults of the human immune system, including oxidative bursts of superoxide anion and nitric oxide. Hsp induction, coupled with the upregulation of other protective antioxidant proteins (e.g., peroxiredoxin, iron containing superoxide dismutase), is thus seen in virulent [14] and even in laboratory-made drug-resistant strains of *E. histolytica* [69]. Puzzlingly, however, Ehmeth expression does not seem to directly induce Hsp 70 expression via methylation of its promoter, implying that there are other agents or mediators involved in the process [68].

Nitric oxide (NO) is the major cytotoxic molecule released by activated macrophages for defense against *E. histolytica* [70]. It is synthesized from L-arginine utilizing the calmodulin dependent iNOS dimer and has been implicated as a major effector for immunomediated antimicrobial defense. *E. histolytica* actually responds to NO and initiates fragmentation/mobilization of its proto-endoplasmic reticulum-like mitosomes, in addition to upregulating numerous genes involved in oxidative control and glycolysis [71]. *S*-Nitrosylation is an emerging redox-based posttranslational modification. *S*-Nitrosylation of crucial virulence factors and metabolic enzymes has been reported [72, 73]. There is increasing evidence to

support NO as a regulator of key epigenetic events. NO can have direct or indirect effects on the nucleosome assembly and chromatin structure by inhibiting or activating transcription factors, histone deacetylases, histones, and nuclear receptors. In addition, NO can disrupt the binding of transcription factors with their interacting proteins and can inhibit their nuclear localization (for a recent review, see Illi et al. [74]). The regulation of DNA methylation pattern by "stress" in some specific loci in plants, basal chordates, and mammals, including humans, has been well documented. However, the mechanisms that control this regulation are not well understood [75]. Thus, nothing is known about the effect of NO on Dnmt activity in general and on Dnmt2 in particular. Indeed, we do not know if the same protective effect of Ehmeth against oxidative stress applies to nitrosative stress [68]. Our ongoing research to address these issues indicates that Ehmeth protects the parasite from nitrosative stress, although the mechanism behind this protective effect is still under study.

11.8 Recognition of Methylated Cytosine by EhMLBP

Conventional methyl-CpG-binding proteins contain the conserved DNA-binding motif methyl-cytosine binding domain (MBD), which preferentially binds to methylated CpG dinucleotides. These proteins serve as transcriptional repressors, mediating gene silencing via DNA cytosine methylation (for a recent review, see Clouaire and Stancheva [76]). Information about methylated DNA-binding proteins in protozoa, however, was nonexistent. Indeed, bioinformatics analysis of the *E. histolytica* genome revealed an absence of MBD homologues, raising the very important question of how *E. histolytica* senses the aforementioned methylated regions in its DNA. Research initiated 3 years ago has established that a protein named *E. histolytica* methylated LINE binding protein (EhMLBP) [77] is involved in DNA methylation recognition. Specifically, it has a tendency to interact with those portions of the genome known already to be methylated (e.g., RT LINE DNA, rDNA) but competitive DNA probe binding assays have shown it to be a strong sensor of DNA methylation in a variety of genes including dihydrouridine synthetases, RAP GTPase-activating protein, serine/threonine protein kinase, and leucine-rich repeat containing protein. The common ground is that EhMLBP binds with a much higher affinity to methylated DNA over its nonmethylated counterpart. Further characterization of EhMLBP revealed that its C-terminal DNA-binding region has strong homology with histone H1 of *Xanthomonas oryzae* and *Trypanosoma brucei gambiense*; however, it shares no homology with the *E. histolytica* histone H1, or any of the other "classical MBDs" in mammals, plants, or insects. Thus, an in-depth analysis of EhMLBP localization, cognate protein partners, and DNA targets was carried out. The results revealed EhMLBP to be a perinuclear protein with strong preference for "kinked" DNA containing adenine stretches as present in LINE and SINE retrotransposons at their 3′-ends, and a consensus motif shared by the aforementioned genes [77, 78].

Regarding downregulation of EhMLBP, antisense technology, peptide targeting, and the lexotropic agent distamycin A (shown to be a potent inhibitor of EhMLBP) [79] all resulted in trophozoites with impaired growth and virulence; this finding identified EhMLBP as an essential constituent of the parasite *E. histolytica* and a possible target for anti-amebic chemotherapy. Interestingly, functional analysis revealed that EhMLBP also contains heat-shock domains, heat-shock transcriptional elements, and an N-terminal fibrinogen α-chain. What it lacks, however, is the conserved α-crystallin domain shared by Hsps in all three domains of life: Archea, Bacteria, and Eukarya. This lack indicates convergent evolution and a possible link between environmental heat stress and epigenetic control of transcription. Indeed, heat shock has been shown to induce EhMLBP expression both in vitro and in vivo, and the heat-shock element promoter (shared with the other Hsps) is induced by the same transcription factor [80]. Moreover, heat shock also induces pan-nuclear mobilization of EhMLBP along with its appearance in cytoplasmic vesicles that appear as putative stress granules. Not surprisingly, EhMLBP overexpression has been shown to protect heat-shocked trophozoites and even reduces overall protein aggregation in both control and heat-shocked trophozoites [80].

The fundamental question confronting us is whether EhMLBP is a sensor of DNA methylation initiating an adaptive response to methylated portions of the genome or whether it may, in fact, induce DNA methylation via recruitment of proteins such as Ehmeth. A study in EhMLBP overexpression revealed increased transcription of RT LINE DNA [80]. It would be interesting to investigate further the methylation status of this DNA, as well as concurrent overexpression/underexpression of Ehmeth. Conversely, what happens to Ehmeth/EhMLBP expression under overexpression of the cognate DNA targets? Finally, further research may reveal details about a putative protein scaffold, interactions with *S*-MARs, and/or cytoplasmic interactions with proteins and their expression/degradation.

11.9 Concluding Remarks

During the past few years, we have improved our knowledge on the biochemistry of Ehmeth, its mode of action, its targets, and the effects of their respective interactions. The data we have obtained thus far imply Ehmeth activity is induced under conditions threatening the genomic integrity of the parasite (i.e., external challenges such as stress, nutrients, and foreign genetic material). That Ehmeth expression seems less vital or pervasive under laboratory conditions suggests that this artificial atmosphere (in vitro) favors the emergence of strains with more lethargic phenotypes. Alternative, demanding environments may reveal more about Ehmeth expression, activity, and virulence. The identification of these conditions constitutes an important challenge for the coming years. The adage “Tell me who your friends are and I’ll tell you who you are” has been shown to be true when we identified enolase as the first Dnmt2-interacting protein implicated in both epigenetic regulation and metabolism in *E. histolytica*. More of these interacting proteins must be identified

in the future if we want to understand the full mechanism of Ehmeth expression as it relates to ultimate proteome expression.

Although most of our research is fundamental and tends to focus on the characterization of the epigenetic components in the parasite, we cannot ignore that several epigenetic drugs are being tested in clinical trials or even already being used (e.g., anticancer or antiepileptic drugs). It may thus be possible to test epigenetic targets as putative drugs for the treatment of amebiasis. Indeed, we may extend this philosophy toward treatment of other parasitic infections as well. From a clinical perspective, this possibility is very attractive because of the lack of homology between parasitic proteins such as EhMLBP (which has no mammalian counterpart) and human epigenetics. This possibility is particularly relevant because of emergent reports of amebiasis refractive to pharmaceutical treatment [81, 82] and various laboratory strains with existing metronidazole and even multidrug resistance [83, 84]. Furthermore, a host of adverse effects is associated with some of the conventional treatments. Possible side effects for metronidazole, for example, include nausea, diarrhea, thrombophlebitis, and even CNS toxicity [85]. Our previous work on EhMLBP has shown that it is possible to find an inhibitory peptide that blocks specifically the activity of this protein, which highlights the idea that epigenetics may be exploited for the development of alternative pharmaceutical agents that will serve as novel drugs, targeting a parasite's unique metabolism or reproductive niche that is not manifested in human physiology.

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