The early life social environment and DNA methylation

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/j.1399-0004.2012.01843.x

Clinical Genetics

Abstract

DNA methylation is a chemical modification of DNA that confers upon identical sequences different identities that are reflected in different gene expression programming. DNA methylation has a well established role in cellular differentiation by providing a mechanism for one genome to express multiple phenotypes in a multicellular organism. Recent data points however to the possibility that in addition to the innate process of cellular differentiation, DNA methylation can serve as a genome adaptation mechanism; adapting genome function to changing environmental contexts including social environments. A critical time point for this process is early life when cues from the social and physical environments define life-long trajectories of physical and mental health. DNA methylation and additional epigenetic modifications could therefore serve as molecular links between "Nurture" and "Nature". Data that are consistent with this new role for DNA methylation as a mechanism for conferring an "environment" specific identity to DNA will be discussed.

DNA methylation

Many but not all organisms bear chemically modified bases in their genomes. 5-methyl cytosine is modified by an enzymatic transfer by DNA methyltransferase (DNMT) (1) of a methyl moiety to the 5‘ position in the cytosine ring from the methyl donor S-adenosyl-methionine (SAM) (1, 2). A commonly methylated sequence in vertebrate genome is the dinucleotide CG which is highly enriched in human genomes at promoters (3). Interestingly CG sites around transcription start sites are mostly unmethylated which is consistent with a role for DNA methylation in silencing gene expression (4). The majority of DNA methylation occurs in CG dinucleotides that are either sparsely distributed or in promoters that are silenced by DNA methylation during differentiation or as a consequence of abnormal or pathological processes (5). For example, tumor suppressor genes are abnormally methylated in cancer resulting in silencing of gene expression and tumorigenesis (6). CG methylation is in a palindrome that could be faithfully copied during DNA replication from the template strand to the nascent daughter strand by the semiconservative DNMT1. DNMT1 preferentially recognizes a newly synthesized CG dinucleotide when the CG on the template sequence is methylated (7). Thus, CG methylation could be faithfully methylated through multiple cell divisions offering a mechanism for epigenetic inheritance. However, a certain faction of DNA methylation in the genome occurs in non-CG methylation (8).

Non-CG methylation cannot be automatically inherited from a template strand since there is no C methylation on a template across either a CC (the complementary sequence is GG), CT (AG) or CA (TG) sequences. This suggests that there must be mechanisms other than template-strand for maintaining DNA methylation states (9). Although these non-CG methylation sites were discovered mainly in stem cells (8), it is still possible that non-CG methylation is present to a certain extent in mature cells as well (10) It is known that the two de novo DNMTs DNMT3a and DNMT3b (11) do not require a methylated C on the template and might exhibit more lax dinucleotide sequence specificity(1). Non-CG methylation supports the notion that there is some plasticity built in the DNA methylation pattern (9).

5-methyl-cytosine could be further modified by hydroxylation (12) in a reaction catalyzed by TET enzymes(13), which could then be further carboxylated(14). There is no known mechanism for inheritance of 5-hydroxymethyl cytosine.

DNA methylation patterns and cellular identify of DNA

DNA methylation is part of the DNA molecule chemistry. It is thus clearly differentiated from other epigenetic mechanisms chromatin modification and noncoding RNA. Cell specific DNA methylation patterns that are formed during cellular differentiation by innate developmental programs were described almost two decades ago(15, 16) and were recently confirmed by whole genome methylome mapping(8) (Fig. 1). Thus, the DNA molecule has two identities, the ancestral identity encoded in the sequence and the cell specific identity encoded in the pattern of DNA methylation.

DNA methylation in critical regulatory regions is involved in regulating gene expression. There is an overall inverse correlation between DNA methylation in regulatory regions of genes and gene expression, which was discovered in the early eighties(2, 15) and was confirmed by whole genome approaches(17).

An inverse situation exists in gene-bodies. Increased methylation was observed in the gene bodies of active genes (8, 17) (18) (19).

The role of modified 5-methyl cytosine in controlling gene expression is unclear. Several studies suggest that modified methylcytosines are intermediates in active demethylation pathways(14, 20, 21) but data suggests that, at least in some tissues, 5-hydroxymethylcytosine (5hmC) is maintained stably in the genome and might play a functional role in regulating genome function(12, 22). Genome-wide mapping of 5hmC in the brain suggests that 5hmC is mostly found in genes, is enriched at promoters and in intragenic regions (gene bodies). Presence of 5hmC peaks at transcription start sites did not correlate with gene expression levels, but its presence in gene bodies was positively correlated with gene expression(23). Genome wide mapping of 5-hmC and TET1, showed association with both active or inactive chromatin marks(24, 25). There is good reason to suspect that modified methylated cytosine further refines the gene silencing signal of 5-methylcytosine.

The most established role of DNA methylation is in regulation of promoter activity(26). At least two mechanisms are well established for inhibition of gene activity by DNA methylation. A methyl group positioned in a recognition element for a transcriptional factor can block binding of the transcription factor to the promoter(27, 28). Alternatively,

methylated DNA attracts methylated DNA binding proteins (MBD) such as the Rett syndrome protein Methyl CpG binding protein 2 (MeCP2), which in turn precipitate an inactive gene-silencing chromatin configuration through recruitment of chromatin silencing proteins(29) (Fig. 2).

DNA methylation and cellular differentiation and the impact of the early environment

Faithful epigenetic inheritance is critical for DNA methylation to play a role in cellular differentiation as maintenance of the differentiated state requires accurate copying of the DNA methylation pattern. This might imply that changes in DNA methylation should not occur outside the context of rigid and innate program of generating cell type identity. If indeed DNA methylation is solely defined by innate programs, there is still room for external processes to affect it accidentally and stochastically through activating or inhibiting the enzymes that either add or remove DNA methylation patterns during gestation.

For example, the impact of diet, or stress during gestation on DNA methylation could be caused by inhibiting or activating DNA methylation/demethylation enzymes at the time when the DNA methylation pattern is laid down. The Jirtle lab demonstrated an effect of maternal diet during gestation on the agouti color phenotype in viable yellow agouti (A(vy)) mice, which was mediated through methylation of a transposable element in the A(vy) transposable element.(30). The impact of methyl-rich diets during gestation or the impact of other chemicals such as bisphenol B during gestation that inhibit DNA methylation (31) could be explained just as a stochastic chemical interference in enzymatic DNA methylation reactions that are actively laying down the DNA methylation pattern during embryogenesis.

Hypothesis: DNA methylation is a genome adaptation mechanism that confers environmental-exposure specific identity to DNA

DNA methylation is a mechanism for diversification of DNA identity by providing within the same chemical entity two layer of information; the ancestral identity encoded in the sequence and the cellular identity encoded in the DNA methylation pattern. It is hypothesized here that similar to the alterations in DNA methylation that occur in response to innate signals during development, external signals triggered by the environment can modulate the DNA methylation pattern to generate differential “environmental-history” DNA methylation identities. This process could occur at different time points in life and act at different time

scales ranging from proximal physiological time scale to life-long as well trans-generational time scales if DNA methylation is reversible after birth (32).

The impact of early life experience on life-long health and behavior trajectories

The social and physical environment influence human development after birth and during different life cycle stations. For example, social adversity early in life has a profound impact on life-long physical health and behavior (33-35). Maternal behavior plays a cardinal role in the behavioral development of mammals. Models of maternal deprivation in primates and rodents and natural variation in maternal care in rodents have demonstrated the significant impact of maternal care on a panel of phenotypes in the offspring that last into adulthood (36, 37).

Hippocampal Glucocorticoid receptor (GR) controls the negative feedback of the HPA axis by glucocorticoids. In the rat, the adult offspring of mothers that exhibit increased levels of pup licking/grooming (i.e., High LG mothers) over the first week of life show increased hippocampal (GR) expression, enhanced glucocorticoid feedback sensitivity, decreased hypothalamic corticotrophin releasing factor (CRF) expression and more modest HPA stress responses compared to animals reared by Low LG mothers (38, 39). These effects could be triggered also by cross-fostering of a Low LG offspring with High LG mother, suggesting that they are not germ line transmitted supporting a nonDNA sequence mediated mechanism. Similarly in non human primates maternal deprivation early in life results in profound phenotypic effects later in life (40-43). These studies provide strong evidence that early life experience could shape life-long phenotypes. We proposed that DNA methylation mediated this environmental -responsive phenotypic variation by conferring environmental-exposure identities to similar DNAs?

A dynamic DNA methylation pattern; reversibility of DNA methylation in postmitotic tissue

The main question is whether DNA methylation is reversible after birth (44)? Is the pattern of DNA methylation that is fashioned by innate developmental processes a final state or is it in a dynamic state, which is responsive to external signals?

Although there was a resistance in the field to accept the possibility of a reversible DNA methylation in postmitotic tissues there is significant evidence for this hypothesis (45-49). It has been shown that brain extracts are capable of demethylating “naked” DNA substrate *in*

vitro (10, 50, 51). The strongest evidence for dynamic methylation-demethylation comes from several studies showing active demethylation in postmitotic neurons (52-55). Conditional knock out of DNMT1 in postmitotic neurons results in DNA demethylation suggesting the presence of demethylation activity in nondividing neurons which is critical for a dynamic methylation pattern in the brain (56).

The main issue in the field remains however whether DNA methylation is truly a reversible reaction that involves removal of the methyl moiety and its release (44, 57) or whether DNA demethylation requires excision of the methylated base and its replacement by an unmethylated cytosine through a process of DNA repair (58, 59). First, DNMTs were proposed to deaminate the methyl cytosine to thymidine creating a C/T mismatch, which is then corrected by a mismatch-repair mechanism (60). Second, Growth arrest and DNA-damage-inducible, alpha (GADD45A), a DNA repair protein was proposed to participate in catalysis of active DNA demethylation by an unknown DNA repair based mechanism (61). However, this was disputed (62). Other studies have suggested involvement of GADD45B in demethylation in the brain (63). Third, a complex sequence of coupled enzymatic reactions of deamination and mismatch repair were shown to be involved in demethylation in zebrafish: activation-induced cytidine deaminase (AID, which converts 5-meC to thymine), a G:T mismatch-specific thymine glycosylase methyl-CpG binding domain protein 4 (MBD4) and repair promoted by GADD45A (64). AID has been implicated in the global demethylation in mouse primordial germ cells as well (65). An open question is the role of the newly discovered modification 5-hydroxymethylcytosine as a potential intermediate in the DNA demethylation reaction (12). Recent data suggest that TET1 the enzyme that catalyzes the hydroxylation of 5-methylcytosine is present and required for stem cell maintenance of inner cell mass specification (66) and for activity driven demethylation in neurons(20). 5-hydroxymethylation catalyzed by TET1 is followed by deamination of the 5-hydroxymethylated base by AID/APOBEC (apolipoprotein B mRNA-editing enzyme complex) family of cytidine deaminases and base excision repair enzymes replace the deaminated base with an unmethylated cytosine (BER)(20). More recently it has been proposed that 5-hydoxymethylcytosine is further carboxylated and this serves as a substrate for yet unknown decarboxylases that release the entire modified methyl moiety (14) (Fig. 3 for model).

TET3 was recently shown to be required for the programmed demethylation of the paternal

genome after fertilization including the demethylation of the paternal *oct4* and *nanog* genes (67). However it is becoming clear that TET3 is not involved in an enzymatic process of demethylation during programmed DNA demethylation during early embryogenesis, but rather in marking certain sequences for escaping DNMT catalyzed DNA methylation during cell division (68), possibly since 5-hydroxymethylcytosine is a poor template for DNMT1 (69). It is also possible that TET3 acts by a 5-hydroxymethylcytosine independent mechanism to "mark" certain sequences either directly or "indirectly" through recruitment of other chromatin modification enzymes. The global programmed "demethylation" of the paternal epigenome few hours following fertilization has been the best-known example of global "active-demethylation" during embryogenesis (70). However, the experimental evidence for this "active global demethylation" was based on reduction in immunostaining with an antibody directed against 5-methylcytosine (70). This antibody stains 5-methylcytosine selectively but not 5-hydroxymethylcytosine. The global "demethylation" appears to be just a further modification of the methylated cytosine by hydroxylation rather than loss of the methyl moiety (71). Interestingly, 5-hydroxymethylcytosine is maintained for a considerable time during early embryogenesis (71) and is gradually diluted through synthesis of DNA that is not methylated by the maintenance DNMT(68). These data shed serious doubt on the possibility that TET enzymes participate in an active process of enzymatic DNA demethylation during early embryogenesis. The role of TET enzymes and 5-hydroxymethylcytosine might be mainly as a variation on the methyl-cytosine mark. This could also lead to loss in DNA methylation in mitotic cells in absence of a mechanism to copy modified methyl moieties during cell division.

Interestingly, *TET2* catalytic mutations are frequently observed in myeloid cancers such as myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), MDS/MPN overlap syndromes including chronic myelomonocytic leukemia (CMML), acute myeloid leukemias (AML) and secondary AML (sAML) (72-74). If enzymatic conversion of 5-methylcytosine to 5-hydroxymethylcytosine by TET enzymes were required for active enzymatic DNA demethylation, one would expect that a catalytic mutant in *TET2* would cause loss of 5-hydroxymethylcytosine as well as DNA hypermethylation. Interestingly, although samples from patients with *TET2* mutations displayed as expected low levels of 5hmC in genomic DNA compared to bone marrow samples from healthy controls, the samples with low 5hmC were hypomethylated and not hypermethylated relative to controls

(74). This data is again inconsistent with the hypothesis that 5-hyxroxymethylation catalyzed by TET enzymes serves as an intermediate in active DNA demethylation.

Notwithstanding the precise biochemistry, the fact that DNA methylation is reversible even in postmitotic tissue provides justification for examining the possibility that DNA methylation patterns are adapted to environmental signals including social signals in early life (Fig. 4).

DNA methylation association with early life social experience; lessons from candidate genes

The first line of data that showed association of early life experience with long-term changes in DNA methylation came from a candidate gene approach. Weaver et al. showed that variations in maternal care result in differences in DNA methylation and histone acetylation in the *GR*/*NR3C1* gene encoding the glucocorticoid receptor (GR exon 1_7 promoter) that emerge early in life and remain stable into adulthood (52). Cross fostering experiments showed a causal relationship between maternal care and the DNA methylation differences and reversal of the phenotypes with epigenetic drug treatments supported a causal relationship between DNA methylation differences and phenotypic variation (75, 76). Exposure of infant rats to stressed caretakers that displayed abusive behaviour produced persisting changes in methylation of *bdnf* gene promoter in the adult prefrontal cortex (77). Similarly, early-life stress (ELS) in mice caused sustained DNA hypomethylation of an important regulatory region of the arginine vasopressin (AVP) gene (78).

Although it is impossible to provide causal evidence for early life experience altering DNA methylation states in humans as it is ethically unfeasible to randomize in humans early life abuse, it is possible to associate DNA variations with differences in early life experience. The state of methylation of *rRNA* gene promoters and *NR3C1* promoter in the hippocampus were examined in a cohort of suicide victims in Quebec who were abused as children and their control group. Ribosomal RNA (rRNA) forms the skeleton of the ribosome, the protein synthesis machinery. We have previously demonstrated a critical role for DNA methylation in regulating expression of rRNA genes (79). Our results showed that the suicide victims who experienced childhood abuse had higher overall methylation in their rRNA genes and expressed less rRNA in a brain region specific manner (80). We also examined in this cohort the same promoter of *NR3C1* gene that was affected by maternal care in rats. Site-specific

differences in DNA methylation in the *NR3C1* exon 1f promoter and its expression were detected between suicide completers who had reported social adversity early in life and suicide completers who did not experience social adversity early in life (81).

Epigenetic modulation of other candidate genes was implicated in suicide; the *Gamma-aminobutyric acid A receptor alpha 1* subunit (*GABRA1*) promoter (82) within the frontopolar cortex (83) and *Tropomyosin-related kinase B* (*TRKB*) in the frontal cortex of suicide completers (84). It is unknown yet whether these changes in DNA are also associated with early life adversity.

DNA methylation association with early life social experience; involvement of broad genomic regions in a clustered and organized response

Genes don't act independently but through functional gene circuitries. In addition, the phenotypic response to early life adversity involves multiple phenotypes suggesting a system wide response. If indeed the response of DNA methylation states to early life adversity is an adaptation rather than a stochastic disruption, it should involve an organized change in DNA methylation across the genome (Fig. 5). We tested this hypothesis in several studies. All studies point to the conclusion that the impact of early life adversity on the epigenome is broad and that it involves multiple systems and is not limited to the brain. This has diagnostic and mechanistic implications. It supports the idea that it might be worthwhile to study behavioral epigenetics in peripheral tissues. We have documented several examples that support this hypothesis.

First, natural variations in maternal care in the rat are associated with coordinate changes in DNA methylation, chromatin, and gene expression spanning over a hundred kilobase pairs. Interestingly, a chromosomal region containing a cluster of the PROTOCADHERIN α, -β, and -γ (Pcdh) gene families implicated in synaptogenesis show the highest differential response to maternal care. The entire cluster reveals epigenetic and transcriptional changes in response to maternal care (85). Second, we showed that a similar pattern of response to childhood abuse is associated with DNA methylation differences throughout the genomic region spanning the six and a half million base-pair region centered at the *NR3C1* gene in the hippocampus of adult humans suggesting evolutionary conservation of this adaptation (Suderman et al., submitted 2011). Third, similar to the rat and human, the changes in DNA methylation associated with differences in rearing in rhesus monkeys are widespread in the genome, that they are not limited to the brain and occur in T cells as well (Provencal,

Tremblay, Suomi et al., submitted 2011). Fourth, we have initiated a study of the impact of socioeconomic positioning on DNA methylation that examined blood DNA from the British birth cohort of 1958. This study detected a signature of DNA methylation that is associated with early life adversity (86) supporting the hypothesis that social environment DNA methylation signatures are found system wide and could be examined in peripheral blood cells.

Three other studies have demonstrated that epigenetic effects associated with behavioral adversity could be detected in blood cells. First, the *NR3C1* promoter was more methylated in lymphocytes in newborns exposed prenatally to maternal depression than control newborns (87). Second, Pituitary adenylate cyclase-activating polypeptide (PACAP), a protein known to be involved in stress response in the pituitary was found to be differentially methylated in peripheral blood cells in humans with post traumatic stress syndrome (88). Third, telomere lengths differences were identified between orphans in the Bucharest Early Intervention Project who were placed under high quality foster care compared with those subjected to continued care in institutions(89). As discussed above, a long line of data have established that the physiological response to early life socio-economic adversity is not limited to the brain (34, 90, 91). There is no reason therefore to believe that DNA methylation changes in response to adversity should not occur in the periphery as well as the brain.

Summary

The scope of involvement of DNA methylation in long-lasting regulation of genome function is wider than has originally been thought. DNA methylation acts as a mechanism for providing differential identities to similar DNA sequences. Originally, it was believed that such a mechanism is exclusive for cellular differentiation when an identical genome acquires different identities expressing different phenotypes. We propose that DNA methylation can also act as a mechanism for adaptation of the genome to different environments. There is data in both animals and humans that supports this hypothesis. However, the mechanisms that mediate between external social signals and DNA methylation changes that seem to cluster across the genome are unknown. Future studies are required to unravel these mechanisms.

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Acknowledgements
Work in MS lab was funded by the Canadian Institute for Health Research, the National Institute of Canada, The National Institute of Child Health (USA), the Sackler program in psychobiology and epigenetics at McGill University and the Candian Institute for Advanced Research.

Figure legends

Figure 1**. DNA methylation patterns.** The DNA methylation pattern is sculpted during gestation by de novo methylation of sites that were not previously methylated ,demethylation and maintenance methylation which is accurate copying of the DNA methylation pattern during mitosis. CH_3 -methyl moiety; DNMT-DNA methyltransferase.

Figure 2. **Mechanisms of silencing of gene expression by DNA methylation**. Active genes are characterized by association with acetylated histones and lack of methylation in promoters. A methylation event can either disrupt binding of transcription factor to their recognition sequence (upper right panel) or recruit methyalted binding proteins which in turn recruit chromatin modifying enzymes resulting in changes to chromatin modification and silencing of gene expression.

Figure 3. **DNA methylation reactions**. DNA is methylated by a transfer of a methyl moiety from the methyl donor S-adenosyl-L-methionine (AdoMet) to the 5' position on a cytosine ring by DNA methyltransferases (DNMT) releasing S-adenosyl-homocysteine (AdoHcy). Several demethylation reactions were suggested. Direct demethylation by a demethylase enzyme (dMTase) (MBD2 is a putative candidate) could release a methyl moiety (CH3) in the form of either methanol or formaldehyde. Alternatively, the methyl cytosine ring could be modified by either deamination catalyzed for example by AID or hydroxylation of the methyl moiety catalyzed by TET1. The modified base is then excised by glycosylases and repaired. Alternatively, the bond between the sugar and the base is cleaved (by glycosylases such as MBD4 or 5-methylcytosine glycosylase 5-MCDG) followed by repair. Repair proteins shown to be associated with demethylation were GADD45(a and b).

Figure 4. **The dynamic relationship between DNA methylation and chromatin structure, a model**. The DNA methylation and chromatin modification equilibrium is laid down during embryogenesis. However, the DNA methylation state is not final. A balance of DNA methylation and demethylation activities as well as chromatin activating modifications are dynamically maintained. The chromatin modification states and DNA methylation states are interrelated. Signals from the environment can tilt the DNA methylation balance to either increased or decreased methylation.

Figure 5. **DNA methylation a system wide genome adaptation mechanism**. Signals from the social, bio-environment and physical environment act on signaling pathways to trigger changes in DNA methylation in multiple tissues and across many regions in the genome to adapt the genome and the phenotype to the anticipated life-long environment. A misfit between the environment and programmed DNA methylation could result in disease.

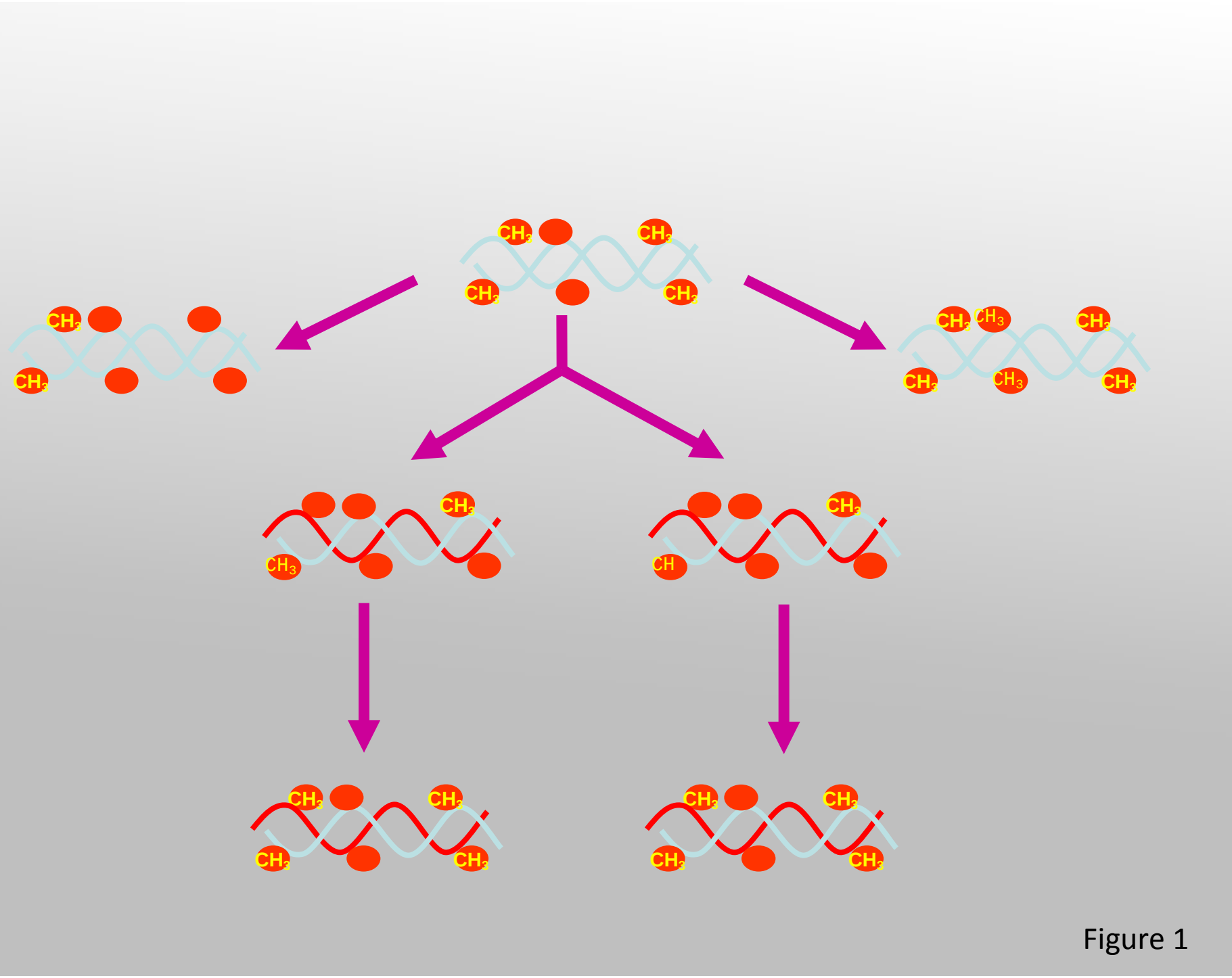
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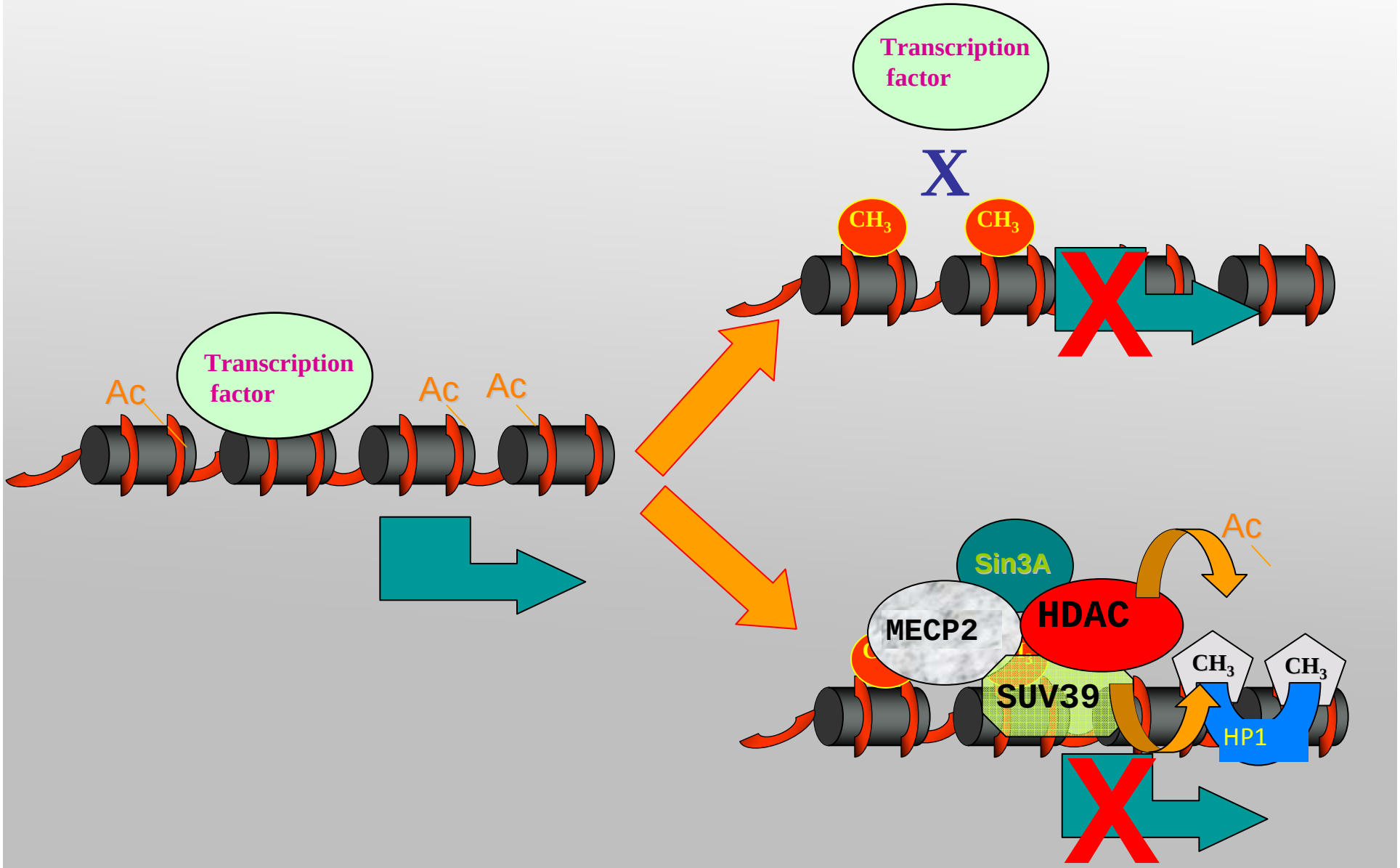
Figure 1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CH3
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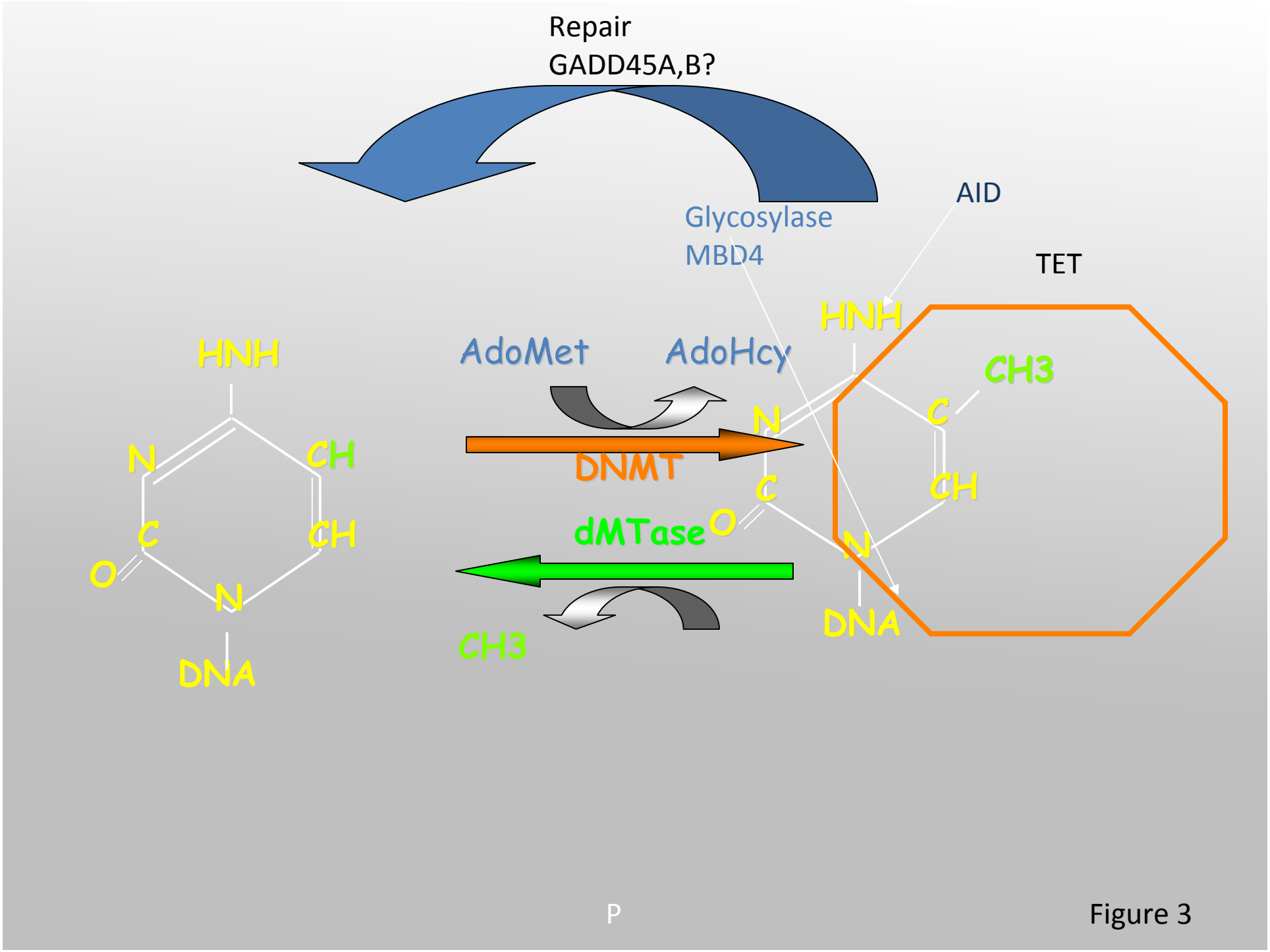
SUV39
HP1
X

Figure 2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GADD45A,B?
Glycosylase
MBD4
AID
TET
HNH
AdoMet
AdoHcy
CH3
DNMT
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Figure 3

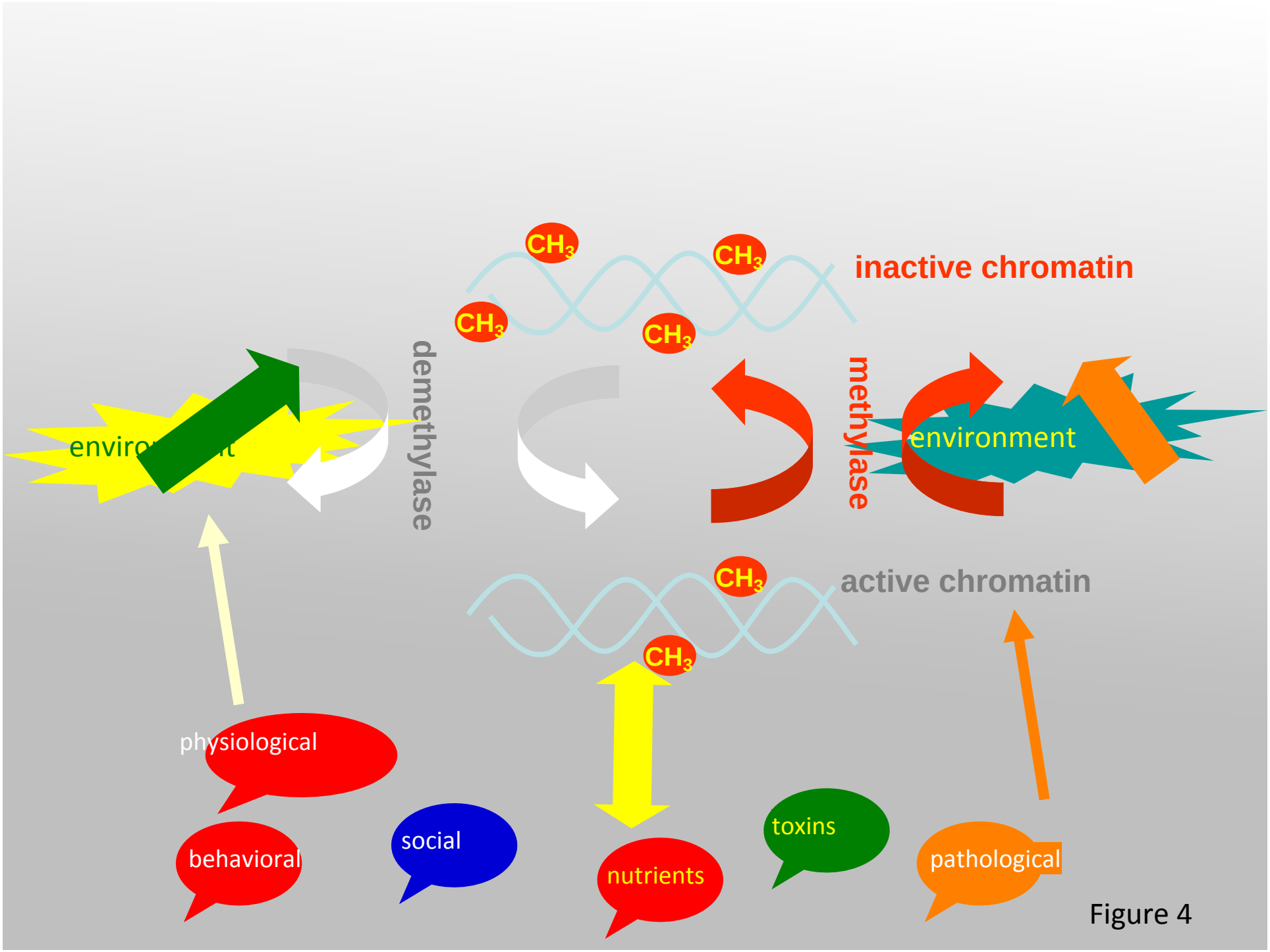
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Figure 4

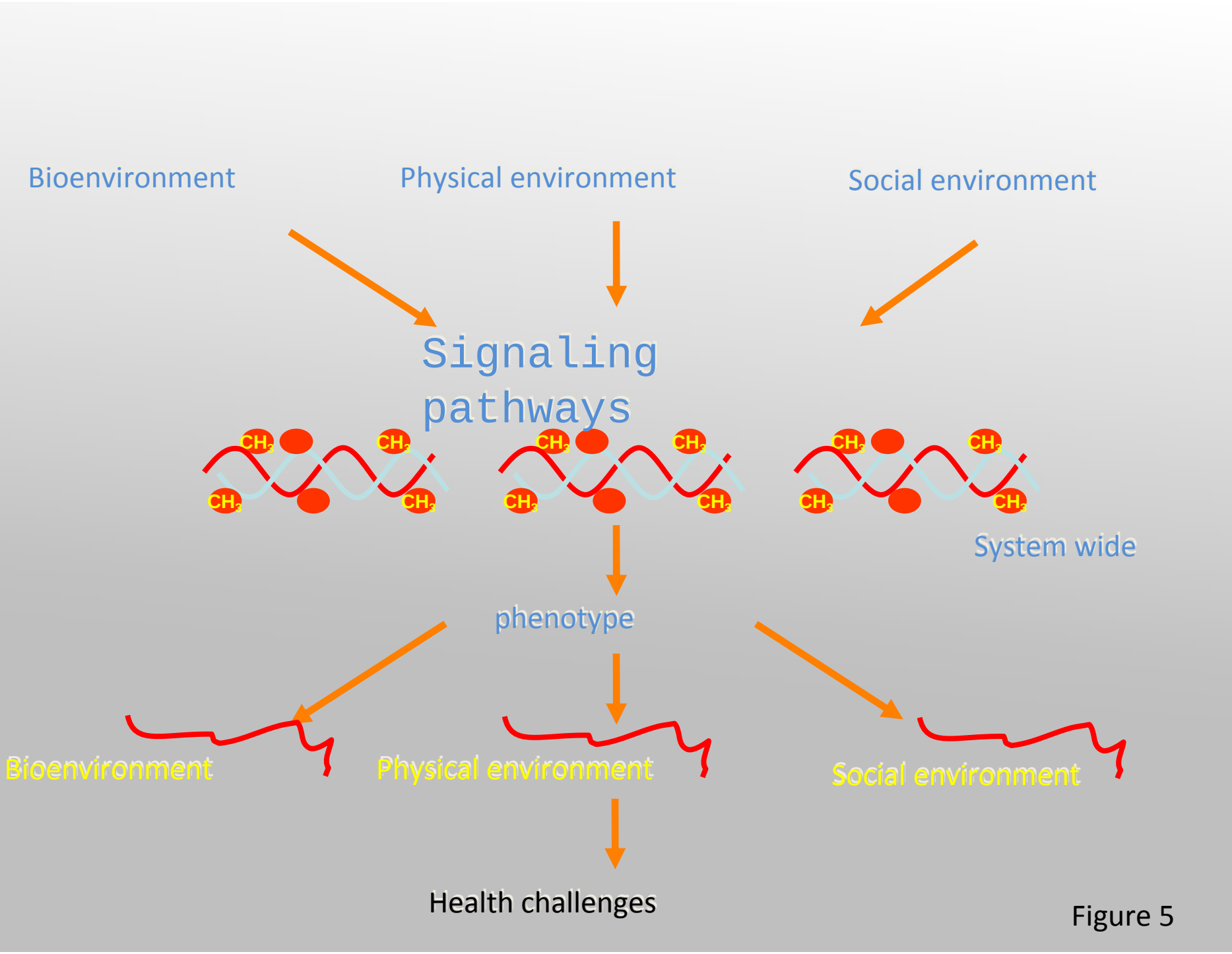
Bioenvironment
Physical environment
Social environment
Signaling pathways
CH3
System wide
phenotype
Bioenvironment
Physical environment
Social environment
Health challenges

Figure 5